Role of Interleukin-6 in Cardiac Inflammation and Dysfunction after Burn Complicated by Sepsis

Hongchao Zhang¹, Huan-You Wang², Rhonda Bassel-Duby³, David L. Maass⁴, William E. Johnston¹, Jureta W. Horton⁴, and Wei Ke Tao¹

Departments of Anesthesiology and Pain Management¹, Pathology², Molecular Biology³, and Surgery⁴, the University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390-9068

Address Correspondence to: Wei Ke Tao, Department of Anesthesiology and Pain Management, the University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines, Dallas, TX 75390-9068. Phone: 214-648-4840; Fax: 214-648-2229; email: wei ke.tao@utsouthwestern.edu

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ABSTRACT
To examine the role of myocardial interleukin-6 (IL-6) in myocardial inflammation and dysfunction after burn complicated by sepsis, we performed 40% total body surface area contact burn followed by late (7 days) *S. pneumoniae* pneumonia sepsis in wild type (WT) mice, IL-6 knockout (IL-6 KO) mice, and transgenic mice over-expressing IL-6 in the myocardium (TG). Twenty-four hrs after sepsis was induced, isolated cardiomyocytes were harvested and cultured in vitro, and supernatant concentrations of IL-6 and tumor necrosis factor (TNF)-α were measured. Cardiomyocyte intracellular calcium ($[Ca^{2+}]_i$) and sodium ($[Na^+]_i$) concentrations were also determined. Separate mice in each group underwent in vivo global hemodynamic and cardiac function assessment by cannulation of the carotid artery and insertion of a left ventricular pressure volume conductance catheter. Hearts from these mice were collected for histopathologic assessment of inflammatory response, fibrosis, and apoptosis. In the WT group, there was an increase in cardiomyocyte TNF-α, $[Ca^{2+}]_i$, and $[Na^+]_i$, after burn plus sepsis, along with cardiac contractile dysfunction, inflammation and apoptosis. These changes were attenuated in the IL-6 KO group but accentuated in the TG group. We conclude myocardial IL-6 mediates cardiac inflammation and contractile dysfunction after burn plus sepsis.
**Introduction:**

Pro-inflammatory cytokines have been implicated in cardiac dysfunction after acute injuries, such as burn (13, 25) and sepsis (4). Our laboratory sequentially evaluated the effect of TNF-α, IL-1β, and IL-6 on cardiac contractile dysfunction, and showed a complementary role of IL-6 to TNF-α and IL-1β: IL-6 mainly accentuated the myocardial depressive effects of TNF-α and IL-1β but lacked an independent effect (24). As part of the acute phase reaction, IL-6 is acutely upregulated after burn and sepsis. IL-6 is also involved in a wide range of physiologic and disease processes, including aging (18). Whether and how such acute or chronic changes in IL-6 regulations mediate myocardial depression after acute injuries remain largely unknown. Currently, there is controversy regarding the exact role of IL-6 in the inflammatory response. While many studies suggest an active role of this cytokine in acute inflammation (32), others indicate its role as a marker (31), or even as an anti-inflammatory mediator (44). The complex roles of IL-6 in mediating essential cell function and the inflammatory response after burn and sepsis prompted the present study examining the effects of IL-6 on cardiac inflammation and function using our clinically relevant model of burn complicated by sepsis (burn plus sepsis) (38, 43).

Based on our previous findings (24), we hypothesize that IL-6 mediates myocardial dysfunction after burn plus sepsis. We used loss-of-function and gain-of-function approaches to evaluate the role of IL-6 in cardiac inflammation and dysfunction using mice deficient in IL-6 (IL-6 knockout mice, KO) and transgenic mice over-expressing IL-6 specifically in the heart (α-MHC-IL-6, TG). TNF-β and IL-6 production were measured in the supernatant of isolated cardiomyocytes. Intracellular calcium and sodium overload was assessed in isolated cardiomyocytes as an indicator of myocardial injury (41, 43). In addition, we measured in vivo hemodynamic and cardiac contractile function, followed by histopathologic assessment of cardiac inflammation, fibrosis, and apoptosis after burn plus sepsis.

**Materials and Methods**
**IL-6 knockout and transgenic mice**

Male, 8-10 week-old, wild type (WT, C57BL/6J) and IL-6 knockout (IL-6 KO, C57BL/6J-II6tm1Kopf) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The IL-6 KO mice have a targeted mutation in the IL-6 gene and do not produce IL-6 mRNA or protein. The colony has been maintained by the Jackson Laboratory for many years and used in numerous studies. The low level IL-6 activity detected in our immunoassay in the cultured cardiomyocyte supernatant (see Results below) probably represents cross-reactivity with other structurally or functionally similar cytokines in the IL-6 family. To generate transgenic mice over-expressing myocardial IL-6 (TG mice), a regulatory region derived from the alpha-myosin heavy chain (α-MHC) promoter is used to drive IL-6 expression (37). A Flag-tagged form of mouse IL-6 cDNA (gift from Dr. Jian Zhang, University of Michigan) was placed downstream of the alpha-MHC promoter (11), followed by a 0.6 kb fragment of the human growth hormone intronic and polyA signal sequences. The UT Southwestern Transgenic Core Facility generated three founder α-MHC-IL-6 transgenic mouse lines. Genotyping was performed by PCR using DNA extracted from a tail biopsy with the following primers (forward primer: 5'-GACGAATTCGCCTTCCCTACTTCACAA-3' and reverse primer: 5'-AATGCGGCCGCCTAGGTTTGCCGAGTAGA-3'). Forward and reverse primers were designed to target corresponding IL-6 and human growth hormone DNA sequence, respectively (Figure 1). Positive offspring was crossed with C57BL/6J mice for at least nine generations. Mice were handled in accordance with institutional and National Institutes of Health guidelines. A total of 14-19 mice were used in each group, and at least eight mice were assigned to in vivo hemodynamic and cardiac functional assessment. The rest of the mice were used for in vitro Western blot analysis of myocardial IL-6, cardiomyocyte supernatant cytokine secretion, and intracellular calcium \([\text{Ca}^{2+}]_i\) and sodium \([\text{Na}^+]_i\) concentrations. The entire experimental procedure is illustrated in Figure 2.

**Burn plus sepsis injury model**

We used our previously established model of 40% total body surface area (TBSA)
contact burn followed by *S. Pneumoniae* sepsis induced at seven days after the initial burn (38, 43). We used this model since our institutional data showed most infectious complications occurred between 4-7 days after burn (9), and our experimental data showed a maximum myocardial depression after this injury model of burn complicated by sepsis (38, 43). The total body weight (TBW) of each mouse was obtained before the injury. The mice were anesthetized by placement in a plastic chamber supplied with 2.5% isoflurane in oxygen. Brass probes preheated to 100°C were applied to the torso back of mice to induce the 40% TBSA third-degree burn. The mice were given 4 mL/kg/% TBSA burn intraperitoneal lactated Ringer's solution for resuscitation, and 0.05 mg/kg buprenorphine was administered every 12 hrs for pain control. Mice were allowed to recover in cages placed on warming blankets with free access to food and water. Seven days after burn, mice were anesthetized again with isoflurane, and 1 x 10^5 colony forming units of *S. pneumoniae* was instilled into the trachea. Sepsis was confirmed by positive blood cultures 24 hrs after bacterial instillation (38).

*Western blot analysis of myocardial IL-6*

Western blot analysis of IL-6 was adopted from our previous methods in rats (2). Briefly, heart tissues from sham WT, KO, and TG mice were harvested and stored at -80°C. Frozen hearts were homogenized in ice-cold lysis buffer (0.5 g tissue/mL) containing 10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, and one Mini Complete Protease Cocktail Inhibitor tablet per 10 mL of compete buffer (Roche Biochemicals, Mannheim, Germany). The homogenized samples were incubated on ice for 30 min and centrifuged at 10,000 g for 10 min at 4°C. Protein concentration was determined by the Bradford assay, using BSA for the standard curve (Bio-Rad Protein Assay Reagents, Hercules, CA). The protein samples were separated on a 4-20% SDS-polyacrylamide gel. The protein was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked for 1 hr in 5% nonfat milk and probed for 1 hr with goat polyclonal mouse IL-6 antibody (Cat. no. SC-1265, 1:1000, Santa Cruz Biotechnology,
Santa, Cruz, CA). After the blot was washed, donkey anti-goat IgG-HRP antibody was added (Cat. no. SC-2033, Santa Cruz Biotechnology). After three washes (20 mM Tris, 135 mM NaCl, 0.1% Tween, pH 7.6), the bound antibody was visualized by enhanced chemiluminescence (ECL system, Amersham, Biosciences, Piscataway, NJ).

Cardiomyocyte isolation, culture, and supernatant cytokine measurements
Twenty-four hrs after *S. pneumoniae* instillation, mice were heparinized, decerebrated, and the heart removed through a medial sternotomy. The heart was immediately placed in ice-cold calcium-free Tyrode’s solution, followed by cannulation of the aorta and retrograde perfusion with calcium-free Tyrode’s solution equilibrated with 95% O_2 and 5% CO_2. The heart then underwent enzymatic digestion by perfusion with 80 mL of calcium-free Tyrode’s solution containing 20 mg collagenase A (Boehringer Mannheim, Indianapolis, IN) and 2 mg of protease (Polysaccharide XIV, Sigma, St. Louis, MO). The ventricular tissue was gently minced in Tyrode’s solution containing 100 µM calcium. The myocyte suspension was filtered and the cells were allowed to settle. The supernatant was removed and cells resuspended in 50 mL Tyrode’s solution. The rinsing and settling steps were repeated as calcium concentration gradually increased from 100 µM to 1.88 mM. Cell viability was measured with Trypan Blue dye exclusion. Cardiomyocytes were plated 50,000 cells/mL/well and incubated at 37°C in 5% CO_2 for 18 hrs. Microtiter plates were removed from the incubator and the supernatant was harvested to measure secreted TNF-α and IL-6 using ELISA kits (TNF-α, Endogen, Woburn, MA; IL-6, Biosource, Camirillo, CA) (42).

Cardiomyocyte intracellular calcium ([Ca^{2+}]_i) and sodium ([Na^+]_i) measurements
Cardiomyocyte harvested from above were loaded with Fura-2 AM for 45 min or sodium-binding benzofurzan isophthalate (SBFI) for 60 min at room temperature in the dark. They were suspended in Tyrode’s solution containing 1.88 mM calcium and washed to remove extracellular dye. Cardiomyocytes were placed in a perfusion
chamber under an inverted microscope and stimulated (0.5 sec) with platinum electrodes. Fluorescent images at 340 and 380 nm wavelengths in response to cell excitation were captured, with [Ca^{2+}]_i and [Na^+]_i quantified by InCyt Im 2™ Imaging System (Intracellular Imaging, Cincinnati, OH) (42).

**Global hemodynamics and cardiac contractile function**

Twenty-four hrs after *S. pneumoniae* instillation, mice were anesthetized with 1.5% to 2% inhaled isoflurane in oxygen. Tracheostomy was performed to allow endotracheal intubation and positive pressure mechanical ventilation. The left carotid artery was cannulated for mean arterial blood pressure measurements and administration of lactated Ringer’s solution. A clamshell thoracotomy incision was performed to expose the heart. The pericardium was excised and a 6-0 silk tie was placed around the inferior vena cava (IVC). The left ventricular pressure-volume catheter (SPR 839; Millar Instruments, Houston, TX) was inserted through the apex of the heart. Steady-state hemodynamic variables were measured, followed by contractility parameters with preload reduction achieved by transient IVC occlusion while suspending mechanical ventilation. Data were digitally converted and displayed using Chart software (version 4.12; ADInstruments, Castle Hill, Australia). Steady-state hemodynamic variables included heart rate (HR), stroke volume (SV), cardiac output (CO), and systemic vascular resistance (SVR). Variables obtained during IVC occlusion included left ventricular end-systolic pressure-volume relationship (ESPRV), preload-recruitable stroke work (PRSW), and time-varying maximum elastance (E_{max}). All data were analyzed with pressure-volume analysis software (PVAN version 3.1; Millar Instruments) (38).

**Histopathology**

Immediately after cardiac function assessment, the heart was removed and placed in ice-cold lactated Ringer’s solution. The ventricles were isolated at the level of the atrioventricular annulus, rinsed, and weighed. The ventricle was cut transversely at the mid-papillary level. Tissue blocks were fixed in 10% PBS-buffered neutral
formaldehyde overnight, dehydrated with ethanol, and embedded with paraffin. They were sliced into 5 µm thickness sections according to standard protocols. Routine histomorphology was examined with Hematoxlin-Eosin (H&E)-stained slides. A semi-quantitative grading system described by Nyska (26) was employed to evaluate the extent of cardiac inflammation: minimal (grade 1) changes involved 1–10% of the section; mild (grade 2) involved 11–40%; moderate (grade 3) involved 41–80%; and severe (grade 4) involved 81–100%. Programmed cell death (apoptosis) of cardiomyocytes was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP nick end labeling (TUNEL) assay using the Promega Fluorescein Apoptosis detection kit (Promega, Madison, WI). Briefly, tissue sections were deparaffinized, pre-incubated with the equilibration buffer for 5 min, and incubated with TdT in the presence of digoxigenin-conjugated deoxyuridine triphosphate for 1 hr at 37°C. The reaction was terminated by the stopping buffer. The sections were incubated with fluorescein-labeled anti-digoxigenin antibody (green, nuclear stain). An excess (approximately 100 µl) of 1% propidium iodide (red) was added to each slide for nuclear counterstain. The slides were examined using 470 nm (FITC-green) and 535 nm (Cy-3, red) fluorescence microscope. All cell nuclei were stained red, but nuclei of apoptotic cells were stained green, and an overlap of green and red generated an orange image indicating the apoptotic cell. The distinction between apoptotic cardiomyocytes from non-cardiomyocytes was made based on the morphology and location of the nuclei. Ten random high-power fields (800X) representing approximately 4,000 cells were counted to enumerate TUNEL-positive cells expressed as percentage of total cells (17). Normal thymus tissue was used as positive control, and negative controls were conducted in the same fashion as positive controls except no TdT was added.

Cardiac fibrosis was assessed with slides stained with Masson's Trichrome according to protocols described (1). Collagen was stained blue, and myocytes red. A scoring system was used to define the degree of myocardial fibrosis as described by Shimizu at el (34): grade 0, no fibrosis; grade I, focal fibrosis; grade II, moderate fibrosis; grade III, diffuse fibrosis. Four different areas (left, anterior, posterior walls, and
septum) were analyzed from each section, and the results were averaged.

**Statistical analysis**

All values are expressed as mean ± standard error. For each variable, comparisons were made among all groups using the WT group as control by one-way analysis of variance and Dunnett’s multiple comparisons test. In addition, Student's t test was performed for each variable within each group between sham and burn plus sepsis mice. A value of P < 0.05 was considered statistically significant.

**Results**

There were no differences in cardiac structure and function between the commercial WT (C57BL/6J) mice and the wild type littermates of the IL-6 transgenic mice. Therefore, only the WT group was used as the control group.

**Myocardial IL-6 protein by Western blot analysis**

Myocardial IL-6 protein content in WT, IL-6 KO, and TG groups are shown in Figure 3. There was no detectable IL-6 protein in the KO group. Mice in the TG group had an abundance of IL-6 compared to the WT group (Figure 3).

**Cardiomyocyte supernatant TNF-α and IL-6 levels**

In WT mice, both TNF-α and IL-6 increased after burn plus sepsis. In the IL-6 KO group, there was a low level of IL-6 activity at baseline and after burn plus sepsis, but the level was lower than the WT group. There was an increase in TNF-α levels in this group after burn plus sepsis injury. In TG mice, there was an increase in IL-6 and TNF-α levels after sham injury compared to the WT mice. After burn plus sepsis injury, both IL-6 and TNF-α further increased compared to the WT mice (Figure 4).

**Cardiomyocyte [Ca^{2+}] and [Na^{+}]**

Burn plus sepsis caused an increase in cardiomyocyte [Ca^{2+}] and [Na^{+}] in the WT mice. In IL-6 KO mice, baseline [Ca^{2+}] and [Na^{+}] were not different from the WT
group. After burn plus sepsis, \([\text{Ca}^{2+}]\) and \([\text{Na}^+]\), both increased, but the levels were lower than the WT group. The TG group had higher levels of \([\text{Ca}^{2+}]\) and \([\text{Na}^+]\), at baseline, and these levels were higher than the WT group after burn plus sepsis (Figure 5).

Global hemodynamics and cardiac contractile function

In the WT group, there was a decrease in HR and CO after burn plus sepsis; however, MAP was maintained with an increase in SVR during steady state hemodynamic measurements. All variables obtained during IVC occlusion showed decreased contractility after the injury. In IL-6 KO group, the global hemodynamic and cardiac contractile changes were attenuated. In the TG group, hemodynamic changes were accentuated after burn plus sepsis, with a further reduction in CO and an increase in SVR, and all contractility variables showed myocardial depression compared to the WT group (Table 1). Representative pressure-volume tracings during IVC occlusions are shown in Figure 6.

Heart, total body weight (TBW), and histopathologic evaluation

The heart weight, TBW, and the heart / total body weight ratio (H/TBW) are summarized in Table 2. The IL-6 KO mice had a similar heart weight and TBW compared to the WT mice. In the TG mice, the TBW was similar to the WT mice, but the heart weight was higher. As a result, H/TBW was higher in the TG group than in the WT group. The left ventricular (LV) wall thickness was similar in the WT and IL-6 KO groups, but higher in the TG group.

Cardiac inflammation, apoptosis, and fibrosis are summarized in Table 3. In the WT group, there was no inflammation associated with sham injury, but inflammation increased to grade II after burn plus sepsis injury. In the KO group, the inflammatory changes were attenuated after burn plus sepsis. In the TG group, there was basal inflammation associated with sham injury, and the inflammatory response increased to grade III after burn plus sepsis injury (Table 3, Figure 7). The apoptosis was increased after burn plus sepsis injury in the WT group, but such changes were
attenuated in the KO group. Apoptosis was most pronounced in the TG group (Figure 8). There was no discernible fibrosis in both WT and KO mice, but there is moderate collagen (blue) deposition (grade II) in the TG group (Table 3 and Figure 9).

Discussion

Myocardial depression after acute injuries such as burn or burn plus sepsis is largely considered to be mediated by TNF-α and IL-1β, which initiate a cascade of events, including induced production of nitric oxide and free radicals (21), altered calcium and sodium handling (2, 43), and increased myocardial apoptosis (22). Although IL-6 is increased as part of the acute phase reaction, its role in mediating myocardial depression after acute injuries is not as clearly defined as the roles of TNF-α and IL-1β. We focused on the role of IL-6 in cardiac inflammation and function after burn plus sepsis as a clinically relevant model of injury where our laboratory has repeatedly shown that myocardial depression after this two-hit injury is exaggerated compared to burn alone or sepsis alone (33, 38, 42, 43).

Results in our study support the hypothesis that IL-6 is an active mediator in the inflammatory response of the heart: mice deficient in IL-6 shows attenuated inflammation, reduced apoptosis, and improved contractile function, whereas mice over-expressing myocardial IL-6 showed more pronounced inflammation, increased apoptosis, and depressed contractile function. These results are consistent with previous findings that IL-6 mediates the inflammatory response. For example, intrinsic production of IL-6 by cardiomyocytes was increased after burn plus sepsis (43) or hemorrhagic trauma (45). Treatment with anti-IL-6 antibodies reduced mortality after endotoxin injection alone (36) or after burn followed by endotoxin injection (27, 28). More recently, the IL-6 gene has been shown to be upregulated in patients suffering from meningococcal septic shock, and removal IL-6 from serum of these patients reversed the negative inotropic effects on rat cardiomyocytes in vitro (29).

The fact that IL-6 increases in parallel with other pro-inflammatory cytokines, most notably TNF-α and IL-1β, makes it difficult to study the effect of an increase in IL-6 alone. It is generally believed that IL-6 participates in a wide range of immunologic
actions, such as activation of intracellular adhesion molecule-1 (20), stimulation of antigen-presenting macrophages and dendritic cells, recruitment of leukocytes, all promoting the inflammatory process (16). Whether and how IL-6 independently promotes myocardial inflammatory responses and cardiac dysfunction have not been well studied. In a model of isolated cardiomyotes, Yu et al showed incubation of cardiomyocytes with IL-6 increased inducible nitric oxide synthase activity and decreased cardiomyocyte shortening (46). Finkel et al had previously demonstrated the relationship between IL-6-induced nitric oxide synthase activity and altered myocardial tension in vitro (8). Using isolated heart preparations, our group has shown IL-6 possessed little direct myocardial depressive effect, but its synergism with TNF-α and IL-1β exaggerated their myocardial depressive effects (24). Our present study further demonstrates the role of IL-6 in exacerbating cardiac dysfunction in intact subjects.

The different levels of inflammation and apoptosis after burn plus sepsis among the three experimental groups in our study are well illustrated. These findings further support the theory that the heart is not only a mechanical pump, but also an immune active organ (39). Locally produced pro-inflammatory cytokines, including TNF-α and IL-1β, probably mediate a large part of the myocardial dysfunction associated with injury and disease. In the present study, IL-6 likely mediated myocardial dysfunction either through its own action or by promoting the production of and synergizing with TNF-α and IL-1β. Indeed, myocardial IL-6 over-expression was associated with increased TNF-α production even after sham injury, and this increase was even more pronounced after burn plus sepsis.

The inflammatory response after burn plus sepsis was accompanied by intracellular calcium and sodium overload. Such an overload was attenuated in the KO mice, but accentuated in the TG mice. Intracellular calcium and sodium overload has been shown in a variety of disease models, including cardiac hypertrophy and failure (30) and ischemia-reperfusion injury (7). It is considered a major indicator of myocardial cell injury. Our group has shown that intracellular calcium and sodium overload is a contributing factor to myocardial contractile dysfunction after burn or burn plus sepsis.
The mechanisms of increased $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ are not completely understood: burn and/or sepsis may alter sarcoplasmic calcium ATPase expression and function (2), causing an increase in $[\text{Ca}^{2+}]_i$, with a compensatory increase in $[\text{Na}^+]_i$, as a result of accelerated $\text{Ca}^{2+}$-$\text{Na}^+$ exchange, or such injuries induce intracellular acidification which results in increased $\text{Na}^+$-$\text{H}^+$ exchange and, subsequently, $\text{Ca}^{2+}$-$\text{Na}^+$ exchange (35).

In addition to promoting the inflammatory response, IL-6 also appears to be associated with myocardial and non-myocardial cell apoptosis. It has been well established that apoptosis is a major event after myocardial injury such as ischemia/reperfusion (40), and it has been implicated in endotoxin-induced myocardial dysfunction (19). Our coworkers have demonstrated the importance of apoptosis in myocardial dysfunction after burn (5, 23) and endotoxemia (6). Similar to findings by Carlson et al (6), the percentage of cells showing positive TUNEL stains was numerically too low to correlate with the degree of functional deterioration, which could be explained in part by the rapid clearance of apoptotic cells by macrophages. In addition, pro-inflammatory cytokines, especially TNF-$\alpha$, not only participate in triggering apoptosis (6, 19), but also initiate a wide range of other inflammatory processes, including activation of inducible nitric oxide synthase (21). It was not clear whether the different levels of apoptosis in our study was the result of IL-6 itself or different activities of TNF-$\alpha$ and other inflammatory cytokines. The observed apoptosis may serve more as a marker of the inflammatory activity than as a critical component of contractile dysfunction. Nonetheless, it is reasonable to link the existence or over-abundance of IL-6 to the more pronounced apoptotic activities after burn plus sepsis.

All three groups of mice showed similar global hemodynamic and cardiac contractile function after sham injury, although TG mice showed mild inflammation with increased TNF-$\alpha$ production and cardiomyocyte $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$. The reason for the normal function in sham TG mice despite the mild inflammation is not clear. It is possible that the basal inflammatory state that occurred as result of IL-6 over-expression produced numerous compensatory responses in the intact animals,
masking mild or modest cardiac contractile dysfunction. In addition, IL-6 overproduction in the myocardium does not simulate a true pathological insult, such as burn injury, with regards to signaling derangements (3) that contribute to myocardial dysfunction. While a rise in basal IL-6 production is seen in a variety of processes including aging (10), major organ dysfunction predominantly occurs after major insults such as burn or burn plus sepsis (9).

In the present study, the KO mice were deficient in IL-6 in all tissues, whereas TG mice over-expressed IL-6 in the myocardium only. We chose total body IL-6 knockout because IL-6 is produced by many organs and tissues, including liver, gut, macrophages, leukocytes, and Kupffer cells. IL-6 produced in these tissues after burn plus sepsis will most likely circulate to the heart, making it impossible to investigate the effect of the loss of IL-6 on the myocardium. On the other hand, we had to use myocardium-specific IL-6 over-expressing mice since IL-6 exerts a wide range of physiologic effects, including participation of total body immune response, regulation of body mass, and more importantly, stimulation of adrenocorticotrophic hormones, and interaction with estrogen (18). Selective cardiac over-expression of IL-6 enables us to focus on its role on myocardial structure and function without affecting other organ systems. We believe such selection of genetic groups of mice allowed us to better address the role of IL-6 in the myocardium after burn plus sepsis.

In summary, we showed myocardial IL-6 production was increased after burn plus sepsis injury, along with intracellular calcium and sodium overload, myocardial inflammation and apoptosis, and depressed myocardial contractile function. These effects were partially attenuated by deficiency in IL-6 and accentuated by myocardial IL-6 over-expression. Myocardial over-expression of IL-6 was also associated with elevated basal inflammation as evidenced by more prominent inflammatory infiltration and apoptosis, and increased cardiomyocyte TNF-α secretion. Our findings further demonstrate the role of IL-6 in mediating cardiac inflammation and contractile dysfunction.

There are limitations to the present study. Although we documented histopathologic and functional changes associated with IL-6 after burn plus sepsis, there were no
data that directly explain the molecular mechanisms of IL-6 in mediating myocardial
dysfunction. In addition, we have noted changes in heart weight and left ventricular
wall thickness in the TG group, but we have not initiated the detailed analysis of the
nature of such structural changes. Future studies aimed at the mechanisms of IL-6
will help us understand how IL-6 mediates the inflammatory response in the heart and
explore interventions directed at preserving cardiac function after burn plus sepsis.
Figure 1

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Figure 2

STEP 1

Commercial WT and KO mice
TG mice

Genotyping
Heart tissue IL-6 Western Blot

STEP 2

Day 1
1. Total Body Weight
2. Sham or Burn Injury
3. Resuscitation

Days 2-6
1. Recovery
2. Food and water access
3. Pain Control

Day 7
1. Vehicle or S. pneumoniae instillations
2. Resuscitation

Day 8
In vivo Function
Heart weight and pathology

In vitro cardiomyocyte cytokine and [Ca$$^{2+}$$], and [Na$$^+$$],

STEP 3

Commercial WT and KO mice
TG mice

Genotyping
Heart tissue IL-6 Western Blot

In vivo Function
Heart weight and pathology

In vitro cardiomyocyte cytokine and [Ca$$^{2+}$$], and [Na$$^+$$],
Figure 3

Heart tissue IL-6 →

WT    KO    TG
Figure 4

A. Cardiomyocyte TNF-α Production (pg/mL)

B. Cardiomyocyte IL-6 Production (pg/mL)
Figure 5.

A.

B.
Figure 6

WT

KO

TG
Figure 7

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Figure Legends

Figure 1. PCR analysis of DNA isolated from tails of wild type (WT), IL-6 knockout (KO) and α-MHC-IL-6 (TG) mice shows insertion of IL-6 transgene. Control is α-MHC-IL-6 transgene DNA.

Figure 2. Flowchart of the experimental procedure.

Figure 3. Western blot analysis of heart tissues isolated from wild type (WT), IL-6 knockout (KO) and α-MHC-IL-6 (TG) mice using goat anti-mouse IL-6 polyclonal as primary antibody, donkey anti-goat IgG-HRP as secondary antibody and enhanced chemiluminescence to detect IL-6 protein.

Figure 4. Cardiomyocyte supernatant TNF-α (A) and IL-6 (B) levels measured by ELISA. B+S: Burn plus sepsis. * p < 0.05 vs. the WT group, and † p < 0.05 vs. sham.

Figure 5. Cardiomyocyte [Ca^{2+}] (A) and [Na^{+}] (B) levels. B+S: Burn plus sepsis. * p < 0.05 vs. the WT group, and † p < 0.05 vs. sham.

Figure 6. Representative left ventricular pressure-volume relationship tracings during IVC occlusion. The slope of the end-systolic pressures generated in response to changes in preload is end-systolic pressure-volume relationship. Flattening of the slope indicates myocardial contractile dysfunction.

Figure 7. Representative histopathology of heart tissue. After burn plus sepsis (B+S), there was significant inflammatory infiltration, disorganization of cardiomyocytes, with cell dropouts in WT mice. Such inflammatory response was attenuated in the IL-6 KO group. In TG mice, there was inflammation after sham injury, and such inflammation became more pronounced after burn plus sepsis injury (H&E, original magnification 200X).
Figure 8. Representative images of heart tissue apoptosis. After burn plus sepsis (B+S), there was apoptotic activity in the WT, but not in the IL-6 KO group. In the TG group, there was apoptosis after sham, and apoptosis became more significant after B+S (TUNEL, original magnification 800X. All nuclei were stained red, and apoptotic nuclei were stained green. Overlapping the red and green images generated orange nuclei indicating the type and location of cells undergoing apoptosis).

Figure 9. Representative histograms of collagen deposition in the heart. Cardiac fibrosis was more significant in the TG mice (Masson’s Trichrome, original magnification x400).
Table 1. Changes in global hemodynamic (steady state) and cardiac contractility (IVC occlusion)

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<td>MAP</td>
<td>86 ± 3</td>
<td>85 ± 5</td>
<td>88 ± 5</td>
</tr>
<tr>
<td>CO</td>
<td>9.1 ± 0.4</td>
<td>5.0 ± 0.4†</td>
<td>9.2 ± 0.8</td>
</tr>
<tr>
<td>SVR</td>
<td>9.4 ± 0.4</td>
<td>17.5 ± 1.7†</td>
<td>9.6 ± 0.9</td>
</tr>
<tr>
<td><strong>Cardiac Contractility</strong> (IVC Occlusion)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESPVR</td>
<td>19.1 ± 9.0</td>
<td>5.1 ± 1.1†</td>
<td>19.3 ± 4.3</td>
</tr>
<tr>
<td>PRSW</td>
<td>106 ± 6</td>
<td>39 ± 2†</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>E_max</td>
<td>41.5 ± 3.5</td>
<td>16.9 ± 2.9†</td>
<td>43.4 ± 4.3</td>
</tr>
</tbody>
</table>

HR: heart rate (bmp); MAP: mean arterial pressure (mmHg); CO: cardiac output (µL/min); SVR: systemic vascular resistance (mmHg/µL/min); ESPVR: end-systolic pressure-volume relationship (µL/mmHg); PRSW: preload-recruitable stroke work (µL-mmHg); E_max: maximum elastance (µL/mmHg). * p < 0.05 vs. the WT group, and † p < 0.05 vs. sham. B+S: burn plus sepsis.
Table 2. Heart weight, total body weight, heart / total body weight ratio, and left ventricular (LV) wall thickness

<table>
<thead>
<tr>
<th></th>
<th>Heart (g)</th>
<th>TBW (g)</th>
<th>Heart / TBW (%)</th>
<th>LV Wall Thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.119 ± 0.006</td>
<td>26.33 ± 0.75</td>
<td>0.451 ± 0.021</td>
<td>1.500 ± 0.0200</td>
</tr>
<tr>
<td>KO</td>
<td>0.110 ± 0.007</td>
<td>24.69 ± 0.82</td>
<td>0.446 ± 0.015</td>
<td>1.583 ± 0.224</td>
</tr>
<tr>
<td>TG</td>
<td>0.124 ± 0.008*</td>
<td>26.27 ± 1.05</td>
<td>0.472 ± 0.012*</td>
<td>1.887 ± 0.259*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. the WT group. TBW: total body weight. LV: left ventricle.
Table 3. Quantification of Inflammation, Apoptosis, and Fibrosis

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th>KO</th>
<th></th>
<th>TG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>B+S</td>
<td>Sham</td>
<td>B+S</td>
<td>Sham</td>
<td>B+S</td>
</tr>
<tr>
<td>Grade of Inflammation</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Apoptosis - Myocardial (%)</td>
<td>0</td>
<td>0.17</td>
<td>0</td>
<td>&lt; 0.1</td>
<td>0.11</td>
<td>0.40</td>
</tr>
<tr>
<td>Apoptosis - Non-Myocardial (%)</td>
<td>0</td>
<td>0.23</td>
<td>0</td>
<td>&lt; 0.1</td>
<td>0.26</td>
<td>0.43</td>
</tr>
<tr>
<td>Grade of Fibrosis</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>II</td>
</tr>
</tbody>
</table>

B+S: burn plus sepsis
References


19. Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock KL, Glentzokski CC, Quintana PJ, and Sabbadini RA. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of


36. Starnes HF, Jr., Pearce MK, Tewari A, Yim JH, Zou JC, and Abrams JS. Anti-IL-6 monoclonal antibodies protect against lethal Escherichia coli infection and lethal tumor necrosis factor-alpha challenge in


