ACTIVATED MACROPHAGES DECREASE RAT CARDIAC MYOCYTE CONTRACTILITY: IMPORTANCE OF ICAM-1 DEPENDENT ADHESION

By

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ABSTRACT

Macrophages are found in the heart as part of the inflammatory response. Our goal was to determine whether macrophages could contribute to myocardial dysfunction. Rat ventricular myocytes were isolated using a collagenase perfusion system and cultured for 24 hours. Then myocytes were co-cultured with elicited peritoneal macrophages in media alone or in media containing TNFα, IL-1β, or endotoxin for 4 hours. Myocytes were electrically stimulated and fractional shortening was determined using videomicroscopy. When myocytes alone were challenged with TNFα, or LPS, fractional shortening did not decrease. When macrophages were prevented from adhering to myocytes by well inserts, TNFα and LPS did not have an effect. Fractional shortening of myocytes decreased from 20.3 ± 1.1% in unchallenged macrophage-myocyte co-cultures, without inserts, to 15.3 ± 1.1% and 15.1 ± 1.8% when challenged for 4 hours with TNFα or endotoxin respectively ($p<0.05$). After 4 hours, the challenged myocytes had a mean adherence of 4.2 ± 0.2 macrophages compared to 2.6 ± 0.3 for controls ($p<0.05$). The number of adherent macrophages was associated with the decrease in fractional shortening. ELISA showed dose dependent ICAM-1 expression on myocytes, while VCAM-1 did not induce measurable expression. Antibody to ICAM-1 reduced macrophage adherence and prevented the decrease in fractional shortening. The decrease in fractional shortening was also prevented by anti-TNFα, desferoxamine, SOD, and L-NAME. These results suggest that activated macrophages adhere to cardiac myocytes via ICAM-1 and adherent macrophages decrease cardiac myocyte contractile function via TNFα, oxygen free radicals, and nitric oxide.

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LIST OF ABBREVIATIONS

ABC   Avidin-Biotin Complex

BSA   Bovine Serum Albumin

ELISA   Enzyme-Linked Immunosorbent Assay

ICAM-1   Intercellular adhesion molecule-1

IL-1β Interleukin-1 beta

LFA-1   Lymphocyte function-associated antigen-1

L-NAME   N-nitro-L-arginine methyl ester

LPS   Lipopolysaccharide

MCP-1   Monocyte Chemotactic Protein-1

MEM   Minimal Essential Media

NO   Nitric Oxide

OPD   1,2-Phenylenediamine dihydrochloride

PBS   Phosphate-Buffered Saline

rTNFα recombinant tumor necrosis factor-alpha

SIRS   Systemic Inflammatory Response Syndrome

SOD   Superoxide dismutase

TNFα Tumor necrosis factor-alpha

VCAM-1   Vascular cell adhesion molecule-1

ACKNOWLEDGEMENTS

CHAPTER 1: INTRODUCTION

1.1 History of Septic Shock

Sepsis is the presence of various pathogenic organisms and their toxins, in the blood or tissues while septic shock is defined as the system's response to an invasive infection caused by these organisms. The term "sepsis" comes from an old Greek term referring to putrefaction (1). Early physicians recognized the syndrome as a putrefactive process whereby a rotting or decaying odor was detected along with its systemic response (2). To the general population, sepsis was formerly known by its other more recognized name, "blood poisoning".

Septic shock is due to the spread of infection from an initial site to the systemic circulation. Some of the common causes of septic shock are:

1) Pneumonia and subsequent spread of a gram positive or gram negative infection.

2) Peritonitis, caused by the leak from the gut from an intestinal disease or wounds.

3) Infection spreading into the blood from the kidney or urinary tract, commonly caused by gram negative bacilli.

4) Post surgical wound infections.

5) Cutaneous or deeper gangrenous infection resulting from gram positive or anaerobic organisms spreading first through the tissues, then to the blood.

Due to the multiple causes of septic shock, it is often difficult to categorize this condition. Common features are: high fever, high cardiac output, marked systemic vasodilatation, sludging of the blood, and development of microclots. Many studies on sepsis and septic shock have formulated a set of criteria for enrolling patients (Table 1). Patients are selected for sepsis trials if they are known or even suspected of having a gram-negative infection in addition to either fever or hypothermia, tachycardia or tachypnea. Patients must also have at least one of the signs of organ dysfunction such as hypoxemia, increased serum lactate concentration, decreased urine output, altered mental mentation and unexplained coagulation (3).

Table 1: Inclusion criteria for patients with sepsis.

I)   Known or suspected gram-negative infection
 	
Plus at least one of these signs:
 	
    a) Fever or hypothermia: >38.2°C or < 36.5° C
 	
    b) Tachycardia: > 90 beats/min
 	
    c) Tachypnea: >20 breaths/min

II)   Plus at least one of these signs of organ dysfunction:
 	
    a) Hypoxemia: $PaO_2/FiO_2 \leq 280$
 	
    b) Increased serum lactate concentration: above normal value
 	
    c) Oliguria: < 0.5mL/kg of body weight/hr
 	
    d) Altered mentation: Glasgow Coma Scale <15, or decrease ≥ 1
 	
    e) New coagulopathy: Unexplained

1.2 Statistics of the Septic Shock Syndrome

Septic shock syndrome, which is the most frequently recognized cause of morbidity in the postoperative period (4), has been estimated to occur in 1% of all hospitalized patients, affecting more than 40,000 people in Canada every year (5). There has been a steady increase in these numbers, which has been attributed to the recent advances in surgical and medical treatments such as aggressive chemotherapy, the use of immunosuppressive drugs for organ transplantation, the increased survivability of patients, and the use of invasive medical procedures (6). Mortality rates in patients suffering from this septic shock range from 20-30%. Treatment of patients with septic shock usually consists of aggressive antibiotic therapy and surgical intervention. Unfortunately, in many cases these treatments are not enough (7). Many times, physicians treating individuals have been puzzled over the mechanism by which shock and tissue injury, resulting from complications due to sepsis, may fatally progress in some cases, even after the patient's infection has been appropriately treated (12).

In 40% of cases, sepsis is complicated by cardiovascular dysfunction, causing a dramatic increase in the mortality rate, anywhere from 40-70% (7). Ejection fraction, which is a measure of contractility, is decreased in septic humans and recovers to normal values over approximately ten days (9,38). Similar reductions in ejection fraction (39) and in load-insensitive indexes of ventricular contractility (26,40) have been observed in animal models of sepsis. One important and often observed characteristic of this cardiovascular dysfunction is the decrease in left ventricular contractility (8). Unfortunately, the cause of

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decreased contractility is not fully understood. One popular theory is that a circulating myocardial depressant factor is released during sepsis since plasma from septic humans and pro-inflammatory cytokines can cause decreased myocyte shortening (8,9,12). Endotoxin or products from bacterial cells trigger the inflammatory cascade by activating monocytes and macrophages. These cells then elaborate a number of cytokines including TNFα (tumor necrosis factor-alpha) and IL-1β (interleukin-1 beta). Several studies have implicated TNFα as a systemic soluble mediator that is important in sepsis and septic shock (10,11). These cytokines then activate cells which are involved in the inflammatory process such as neutrophils and endothelial cells. Oxygen free radicals, which have been documented to be released from macrophages and neutrophils, have been shown to represent another pathway for tissue damage. This inflammatory cascade during sepsis can cause significant damage to the vascular and capillary bed structure and function, tissue damage, edema, and cell death by hypoxia (12).

1.3   Previous Research

Although the term "sepsis" is related to the bacterial infection, recent studies have proposed that the problems associated with sepsis may not be caused by the invading organism. They propose that the development of sepsis is due to the body's immunological response to the event, which occurs when mediators such as TNFα and IL-1β are released (1). It has been proposed that a more general term, called Systemic Inflammatory Response Syndrome (SIRS), be employed in order to describe the clinical manifestation of systemic inflammation (13).

Macrophages have been shown to play an essential part of the immune response to sepsis by presenting antigens to lymphocytes during the development of specific immunity, ingesting and destroying invading micro-organisms, serving as accessory cells to lymphocytes, and as the source of several soluble factors involved in host defense (14). The transformation from resident bone marrow-derived monocytes to tissue macrophages is an important step in its maturation process. In responding to an invading micro-organism, tissue macrophages undergo a conversion from a normal resting macrophage to an activated macrophage. These activated cells are usually larger, have an increased capacity for adherence, are more metabolically active, and more able to release soluble substances and oxidative metabolites. Macrophages, when exposed to endotoxin (lipopolysaccharide; LPS), release cytokines such as TNFα, which can activate other macrophages (23-26,43). This raises the possibility that during sepsis, LPS-exposed macrophages, located at sites of infection, may self-activate (14). LPS has been shown on many occasions to induce septic shock by the stimulation of these macrophages (11,15)

Macrophages have been discovered in the heart after ischemia-reperfusion (16,17); in inflammatory cardiomyopathies (18-20), after orthotopic heart transplantation (21); and in animal models of sepsis after injection of endotoxin (57,58). In a related inflammatory state in patients who underwent orthotopic heart transplantation, a significant increase in macrophage infiltration of the heart was observed along with a corresponding increase in TNFα production (28). Leukocytes also have been shown to contribute to decreased myocardial contractility after endotoxin infusion (22) but whether the macrophage subset

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contributes to the inflammatory process is not known. Activated leukocytes have been shown to decrease left ventricular function (43). In animal models of sepsis, leukocytes are slowed and retained in myocardial capillaries (51). This is associated with capillary endothelial and cardiac myocyte damage, myocardial edema (41), and decreased contractility (22). Similarly, leukocytes decrease contractility in animal models of ischemia-reperfusion (52).

TNFα has been implicated as a systemic soluble mediator which is important in sepsis and septic shock (10,11). TNFα administered alone in whole animal models (24,26) or as one of the mediators released after endotoxin infusion (44), results in a decrease in ventricular contractility within 4-6 hours (24,26,44). In rats, injections of recombinant TNFα led to severe hypotension, lactic acidosis and shock within minutes (11). These effects were blocked when animals were pre-injected with antibodies to TNFα (30). TNFα has also been shown to induce the release of IL-1β from endothelial cells and macrophages while IL-1β subsequently stimulates the biosynthesis of other cytokines (10). TNFα may also act synergistically with IL-1β (43) and appears to decrease myocardial contractility by activation of other mediator pathways (23,26). This would indicate that TNFα and IL-1β both play early roles in the body's inflammatory response to infection, since the fever associated with the early stages of sepsis, has been found to be, at least, partly induced by IL-1β. IL-1β, which is produced by blood monocytes and tissue macrophages, has a particularly potent effect on the inflammatory response (31). In the response of the host

to sepsis, IL-1β is a non-specific mediator of the acute phase response.

A number of investigators have shown that part of the effect of pro-inflammatory cytokines in vitro is due to stimulation of nitric oxide production in cardiac myocytes (23,27,45,46). The release of TNFα and nitric oxide from macrophages is known to contribute to myocardial dysfunction (23-29). Similarly, in isolated working hearts, TNFα and IL-1β contribute to decreased contractile function via nitric oxide and peroxynitrite (47). Thus, cytokine mediators released from macrophages may contribute to myocardial dysfunction in sepsis. Therefore, a role for intramyocardial macrophages in myocardial depression is plausible since macrophages could potentially contribute to both mediator and leukocyte-induced myocardial depression, although very little is known about their interaction with cardiac myocytes.

Antibody to TNFα decreases the functional impairment induced by ischemia-reperfusion (48). Ischemia-reperfusion upregulates expression of mRNA for the pro-inflammatory cytokines TNFα and IL-1β in the heart (49). In isolated rat hearts, myocardium releases significant amounts of TNFα after ischemia-reperfusion (17). TNFα release in ischemia-reperfusion is associated with the accumulation and activation of macrophages (42). TNFα may contribute to further leukocyte accumulation by stimulation of chemokine production (34) and upregulation of leukocyte adhesion molecules on cardiac myocytes (50).

Intercellular adhesion molecule-1 (ICAM-1) is a cell-surface antigen for lymphocyte function-associated antigen-1 (LFA-1) (Figure 1). Cytokine induction of this adhesion molecule on cardiac myocytes may be a critical step in myocarditis (32). Smith et al. have shown that canine cardiac myocytes synthesize and express ICAM-1 on their membrane and that this receptor allows neutrophils to adhere. When exposed to IL-1β, TNFα or LPS, neutrophil adhesion was significantly increased and that this adherence can be blocked by anti-ICAM-1 (33).
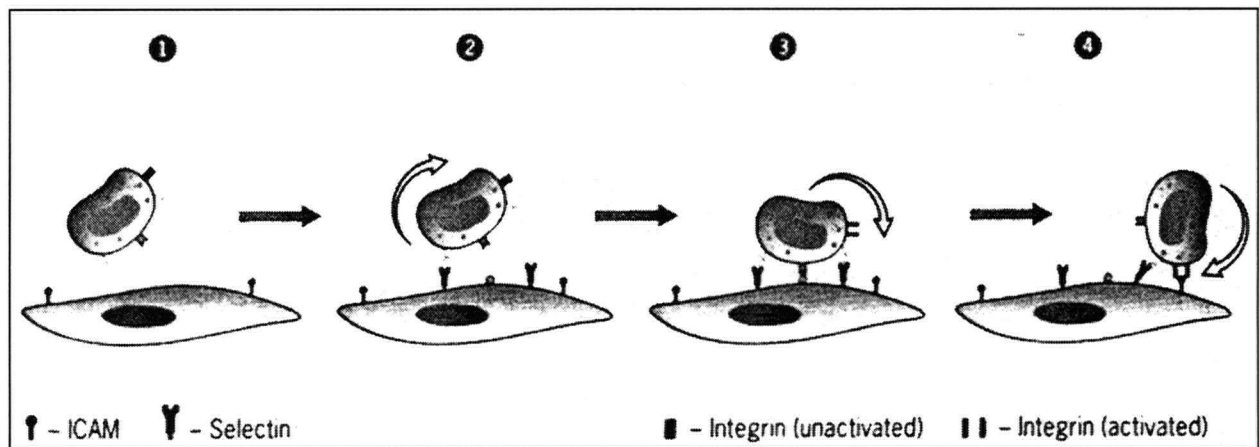
Figure 1: Steps in the adhesion of leukocytes via ICAM. (1) expression of ICAM receptors (2) leukocyte trapping, (3) leukocyte activation of integrin receptors, 4) leukocyte adhesion. Adapted from Gerald Karp: Cell and molecular biology: concepts and experiments, John Wiley & Sons, Inc., 1996.

By co-incubating isolated ventricular myocytes with a variety of cytokines, one is in a better position to observe the accumulation and adhesion of leukocytes to cardiac myocytes, as opposed to whole heart experiments. Massey et al. have shown that cardiac myocytes directly recruit activated leukocytes into areas of injury (34). These cell culture studies have shown that myocytes play an important role in the pathogenesis of inflammation-mediated myocardial injury such as ischemia/reperfusion. Their unique morphology (rod shaped, striated) allows them to not only be easily differentiated from non-myocytes, but their functional properties can also be easily measured. Other studies have shown that by studying leukocyte migrations in isolated cultures, it may aid in understanding the mechanisms whereby these cells are brought in close proximity to myocytes where secretory products would have enhanced injurious effects, particularly if leukocyte adherence to myocytes occurs (35).

Our goal was to determine whether macrophages could alter cardiac myocyte function. To address this issue, we first co-cultured rat ventricular cardiac myocytes and rat peritoneal macrophages with various inflammatory mediators and measured cardiac myocyte contractile function. Interestingly, we noted that decreased myocyte contractile function was associated with adhesion of macrophages to myocytes. This led to the examination of macrophage adhesion to myocytes and the role of receptor-mediated adhesion in myocyte contractile dysfunction. Finally we investigated the potential mechanisms whereby adherent macrophages could mediate myocyte contractile dysfunction, including $TNF\alpha$, reactive oxygen intermediates, and nitric oxide production.

CHAPTER 2: METHODS

This study was approved by the University of British Columbia Animal Care Committee and adheres to Canadian and NIH guidelines for animal experimentation.

2.1   Isolation of Rat Ventricular Myocytes

Male Sprague-Dawley rats (250-300 g) were anesthetized with 3% halothane by inhalation and 1000 units of heparin (Organon Teknika, Canada) were injected intraperitoneally in order to prevent the blood from clotting. Ten minutes later a midline thoracotomy was performed and the heart was excised and placed in oxygenated (95% O_2, 5% CO_2) HEPES-Joklik modified calcium-free MEM buffer (minimal essential media, Gibco BRL, Grand Island, NY) at 0°C. Within two minutes, the heart was mounted, via the aorta, on a modified Langendorff apparatus and perfused with oxygenated MEM, at 37°C for 2 minutes. After blood in the coronary circulation was cleared, the perfusate was changed to 30 mL recirculating calcium free MEM containing 1mg/mL of 236 U/mL collagenase (type 2, Worthington Biochemical, Freehold, NJ). At 15 and 20 minutes, the calcium concentration was increased, stepwise to 25 and 75 µmol/L by adding 75 µL and 150 µL of 10mM $CaCl_2$ respectively to the recirculating buffer. After a 30-40 minute period of digestion the ventricles were removed from the perfusion system and gently teased apart. The tissue, in oxygenated 75 µmol/L calcium perfusion buffer, was then agitated for 2-3 minutes at 37°C in a water bath. The tissue and dispersed cells in solution were then filtered through a 200 µm nylon mesh. The filtrate was then centrifuged at 500 rpm for 2

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minutes and the supernatant was replaced with fresh perfusion buffer. The cells were then washed three times at 37°C in MEM containing increasing Ca^{2+} concentrations (200 μM, 500 μM and 1 mM), allowing the cells to settle by gravity for 10 minutes between each wash. Cells were resuspended in 37°C HEPES-modified M199 buffer (pH 7.4) (Gibco BRL) with 1% BSA (bovine serum albumin, (98% purity) Sigma Chemicals, St. Louis, MO) and viable cell concentration was determined using a hemocytometer. The cell concentration was adjusted to 50,000 cells/mL. 5000 cells were loaded into each well of a laminin-coated 96 well plate (VWR Canlab, Canada) and incubated at 37°C with 95% O_2 and 5% CO_2. At 90 minutes, the media was changed to fresh M199 with BSA and incubated for 24 hours. Cells were considered viable if they demonstrated a characteristic rod shape without cytoplasmic blebbing. This morphometric assessment of viability was confirmed in a subset of experiments with trypan blue exclusion. We found that the fraction of viable myocytes was always greater than 85%.

2.2   Isolation of Rat Peritoneal Macrophages

Ten mL of sodium caseinate was injected intraperitoneally in rats anesthetized using 3% halothane by inhalation. (59) The rats were allowed to recover and 72 hours later were again anesthetized using 3% halothane by inhalation. Peritoneal lavage was performed using 3 aliquots of 30 mL normal saline containing 1 U/mL heparin at 37°C. Peritoneal lavage fluid was then filtered through sterile gauze into 50 mL tubes. Cells were then centrifuged at 1700 rpm for 6 min. The pellet was resuspended in 4 times volume of 0.144 mol/L NH_3Cl solution to lyse any red blood cells. The macrophages were centrifuged again,

and then resuspended in M199 buffer containing 1% BSA. After a final centrifugation, the pellet was resuspended in M199 buffer with BSA and the concentration of macrophages was determined using a hemocytometer. The concentration of macrophages was then adjusted to 500,000 cells/mL. The percentage of macrophages was found to be 90-95% and these cells were viable as indicated by trypan blue exclusion.

2.3 Co-culture of Macrophages and Cardiac Myocytes

After 24 hours of cardiac myocyte incubation, 50,000 macrophages were added to each well of cardiac myocytes (ratio of 10 macrophages per cardiac myocyte (33) in the 96 well laminin-coated plates for co-culture experiments. These co-cultures were incubated at 37°C in 95% O_2, 5% CO_2 for 4 hours. After the 4 hour incubation, fractional shortening of electrically stimulated myocytes and the number of adhering macrophages per myocyte was determined.

2.4 Measurement of Cardiac Myocyte Fractional Shortening

Fifteen minutes before the 4 hour time point, 2 μL of trypsin (0.5% w/v) was added to each well in order to cleave the myocytes off the laminin-coated bottom. The plates were then returned to the incubator for the final fifteen minutes. Preliminary experiments demonstrated that this concentration of trypsin did not alter cell viability and cleaved more than 95% of the adherent myocytes from their attachments to the bottom of the well. At the 4 hour time point, specially designed platinum electrodes were lowered into each well in the 96 well plate and the cardiac myocytes were electrically stimulated (Grass S48

stimulator, W. Warwick, RI, 45 volts, 2.2 ms duration, 25Ω resistance) while being recorded by videomicroscopy (Sony SLV-760HF) (Figure 2). This electrical stimulus was chosen from preliminary threshold experiments as twice the minimum electrical stimulus required to maximally contract the cardiac myocytes. Still frames of systolic and diastolic myocytes were captured for computer analysis from the video recording and myocyte fractional shortening was then measured using Scion ImagePC (Scion Corporation, Frederick, MD).

2.5   Measurement of the Number of Macrophages Adherent to Myocytes

In similar experiments, myocytes and macrophages were co-incubated for 4 hours and the number of adherent macrophages was counted for all myocytes in 12 wells at five random microscope fields per well.

2.6   ELISA for ICAM-1 and VCAM-1 Expression

The cardiac myocyte culture without added macrophages was challenged with human recombinant tumor necrosis factor-alpha (rTNFα, R&D Systems, Minneapolis, MN) in concentrations of 0-50 ng/mL for 4 hours. ICAM-1 and VCAM-1 expression was then quantified by ELISA. Briefly, after the 4 hour incubation cells were fixed using 4% paraformaldehyde (100 µl/well) for 10 minutes at room temperature and then washed twice with 1x PBS and stored at 4°C until the ELISA. Plates were then washed twice with 1x PBS and blocked by adding 250 µL/well of 5% normal goat serum (Dako Diagnostics Canada, Mississauga, ON) in PBS for one hour at 37°C. Then, 100 µl/well of rabbit anti-rat ICAM-1 or rabbit anti-rat VCAM-1 (gift from Dr. P. Ward, University of Michigan), diluted

to 1:5000 in PBS was added, and incubated for one hour at 37°C, following a PBS wash. Cells were washed and 100 µL/well of a biotinylated goat anti-rabbit (Dako Diagnostics Canada, Mississauga, ON), diluted 1:5000 in PBS, was added and incubated at 37°C for 30 minutes. Then ABC complex (Dako Diagnostics Canada, Mississauga, ON) was added followed by chromogen substrate (OPD, Dako). The colorimetric reaction was quenched by the addition of 0.5 mol/L H_2SO_4, according to the manufacturer's instructions. Plates were then read at 490 nm using a microplate reader (Rainbow Reader, SLT Lab Instruments, Salzburg, Austria).

2.7   Experimental Protocols

2.7.1 Fractional shortening of myocytes.

We studied 1) cultures of cardiac myocytes alone, 2) cultures of cardiac myocytes with macrophages added into a culture well insert (0.45 µm pores so that myocytes and macrophages were cultured in the same well but the cells were kept separated), and 3) co-cultures of cardiac myocytes and macrophages where cell interaction was not prevented. TNFα (2µL), LPS (lipopolysaccharide, 2µL), or control saline (2µL) was added to each well at the start of the cardiac myocyte-macrophage co-culture. The final concentration of TNFα and endotoxin in the wells was 20 ng/mL and 10 µg/mL respectively. At the end of a 4 hour incubation, one microscope field was randomly selected in each well and was electrically stimulated once. Previous experiments showed that more than one stimulation of the myocytes would render them inactive. Generally, 1 to 4 myocytes were present in each field. Diastolic length and fractional shortening were determined for each myocyte.

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2.7.2 Macrophage adherence to myocytes.

Co-cultured myocytes and macrophages were challenged using TNFα, IL-1β, LPS, or vehicle. TNFα and IL-1β were added at a concentration of 20 ng/mL (28) while LPS was added at 10 µg/mL at the start of the 4 hour co-culture. Then the number of adherent macrophages to myocytes was counted by morphological assessment as well as observing their adhesion during myocyte contraction. In additional experiments to determine the role of adhesion molecules expressed by cardiac myocytes, antibodies to ICAM-1 and VCAM-1 were added at 200 pg/mL immediately prior to stimulation with TNFα. The number of macrophages adherent to myocytes after 4 hours was counted.

2.7.3 Role of macrophage adhesion on myocyte function.

We repeated the co-culture experiments using pre-treatment with antibody to ICAM-1. TNFα (20 ng/mL), IL-1β (20 ng/mL), LPS (10 µg/mL) (28), or control saline was immediately added and incubated for the 4 hour period. Fractional shortening of cardiac myocytes and the number of adherent macrophages were then measured.

2.7.4 The role of TNFα, oxygen free radicals, NO, and peroxynitrite.

In order to assess the role of TNFα, oxygen free radicals, NO, and peroxynitrite we used the same co-culture as previously described. Anti-TNFα, oxygen free radical scavengers desferoxamine (0.6568 mg/mL (36))and superoxide dismutase (SOD, 23.3 µg/mL (36)), the NO synthase inhibitor L-NAME (10^{-4} mol/L (23)), and the peroxynitrite inhibitor urate

(100 μg/mL (37)) were added to co-cultures just prior to challenging with TNFα (20 ng/mL) or saline. Urate was used since peroxynitrite being part of the nitric oxide synthase cascade. At 4 hours of co-culture fractional shortening was measured along with the number of adherent macrophages per cardiac myocyte.

2.8   Data Analysis

We tested for differences in fractional shortening and number of adherent macrophages using an analysis of variance choosing $p<0.05$ as significant. When a significant difference was found we identified specific differences between groups using a sequentially rejective Bonferroni procedure. Data are expressed as mean ± standard error throughout.
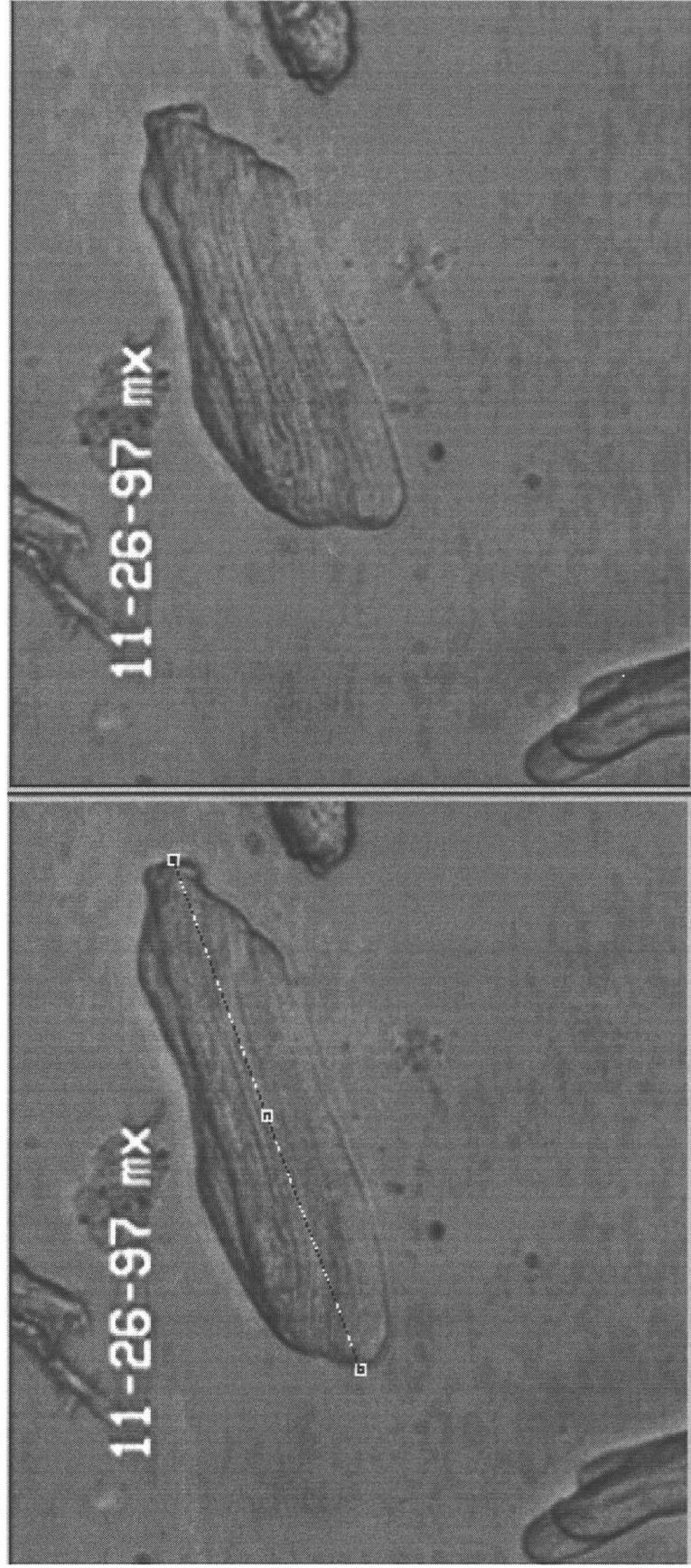
FIGURE 2-Diastolic and systolic videomicroscopic images of a typical cardiac myocyte are shown. Fractional shortening is determined as the difference between diastolic length and systolic length, divided by diastolic length

CHAPTER 3: RESULTS

3.1　Fractional Shortening

Fractional shortening of myocytes alone exposed to TNFα or LPS was no different from unchallenged myocytes (Figure 3). Similarly, when myocytes were cultured with macrophages but physical contact was prevented by placing macrophages in culture well inserts, neither TNFα nor LPS had a statistically significant effect on fraction shortening (Figure 3). Fractional shortening of myocytes in co-culture with macrophages (no well inserts) without activation was not statistically different from unchallenged myocytes alone (Figure 3). However, TNFα and LPS added to macrophage-myocyte co-cultures decreased myocyte fractional shortening by 24 ± 8% ($p<0.05$) and 26 ± 9% ($p<0.05$) respectively (Figure 3). The diastolic length of myocytes in co-cultures was not significantly altered by TNFα or LPS indicating that the decrease in fractional shortening was due to a change in systolic contractility and not due to varying myocyte lengths. In these experiments, the number of adherent macrophages was significantly greater in co-cultures challenged with TNFα and LPS (4.2 ± 0.2) than in unchallenged co-cultures (2.6 ± 0.3) ($p<0.05$) and, as the number of adherent macrophages increased from 2 to 6 per cardiac myocyte, fractional shortening of the cardiac myocyte decreased by 0.7% per adherent macrophage ($p=0.07$). Thus, macrophage adhesion appeared to be important in decreasing myocyte function.

3.2   Adherence of Macrophages

Anti-ICAM-1 prevented increased adherence of macrophages to cardiac myocytes induced by TNFα, IL-1β, and LPS (Figure 4). Anti-VCAM-1 did not prevent increased adherence due to TNFα, IL-1β, or LPS in macrophage-myocyte co-culture (data not shown). ELISA for ICAM-1 showed that when myocytes were challenged with human recombinant TNFα, the expression of ICAM-1 on cardiac myocytes was dose dependent, reaching a plateau at a dose of 20 ng/mL of TNFα (Figure 5). TNFα did not induce measurable VCAM-1 expression on cardiac myocytes (data not shown).

3.3   Role of Macrophage Adhesion on Myocyte Function

To determine if ICAM-1 mediated adhesion played a role in causing the decrease in cardiac myocyte function induced by activated macrophages, we added anti-ICAM-1 to co-cultures. Anti-ICAM-1 prevented the increase in adherence due to TNFα, IL-1β, and LPS challenge (Figure 4) and also the decrease in myocyte fractional shortening (Figure 6).

3.4   The Role of TNFα, Oxygen Free Radicals, NO, and Peroxynitrite

To further understand how adherent macrophages decrease cardiac myocyte fractional shortening we first examined the role of TNFα. Anti-TNFα prevented the decrease in cardiac myocyte fractional shortening (Figure 6) induced by TNFα or LPS challenge which might be due to the macrophages which produce TNFα when exposed to LPS. Interestingly, anti-TNFα also decreased adherence of macrophages to myocytes (Figure

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4) induced by TNFα, IL-1β, or LPS challenge. We then examined the role of oxygen free radicals, NO, and peroxynitrite, since these three are released by macrophages. As in previous experiments, TNFα challenge of macrophage-myocyte co-culture significantly decreased cardiac myocyte fractional shortening (Figure 7). This decrease was completely inhibited by desferoxamine, SOD, and L-NAME. Urate did not prevent the decrease in fractional shortening to a statistically significant degree. The number of adherent macrophages did not decrease to account for improved fractional shortening with desferoxamine, SOD, or L-NAME.
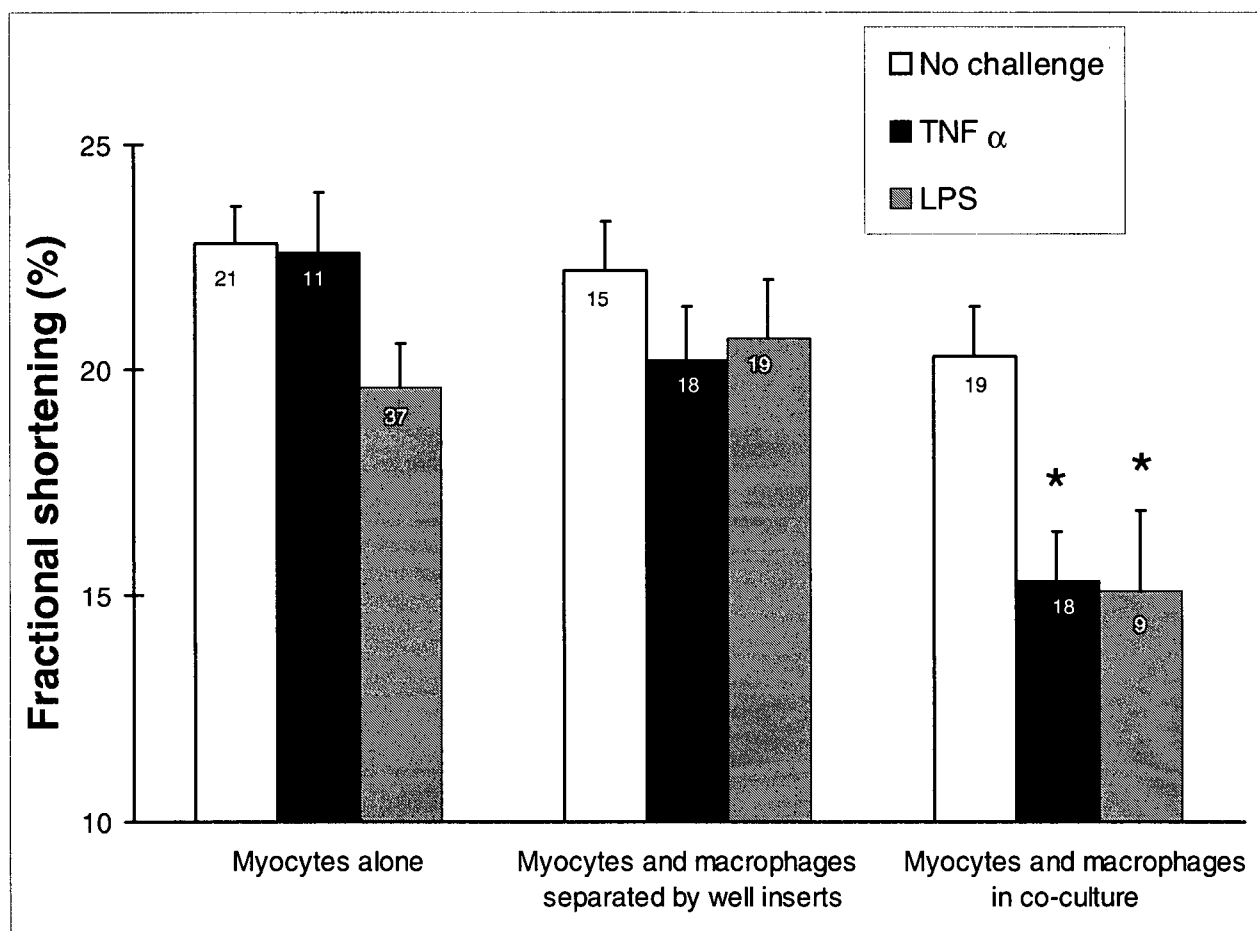
FIGURE 3- Fractional shortening of cardiac myocytes is shown, as determined by diastolic minus systolic, divided by diastolic. (* indicates p<0.05 compared to all other groups, n is indicated on bars)
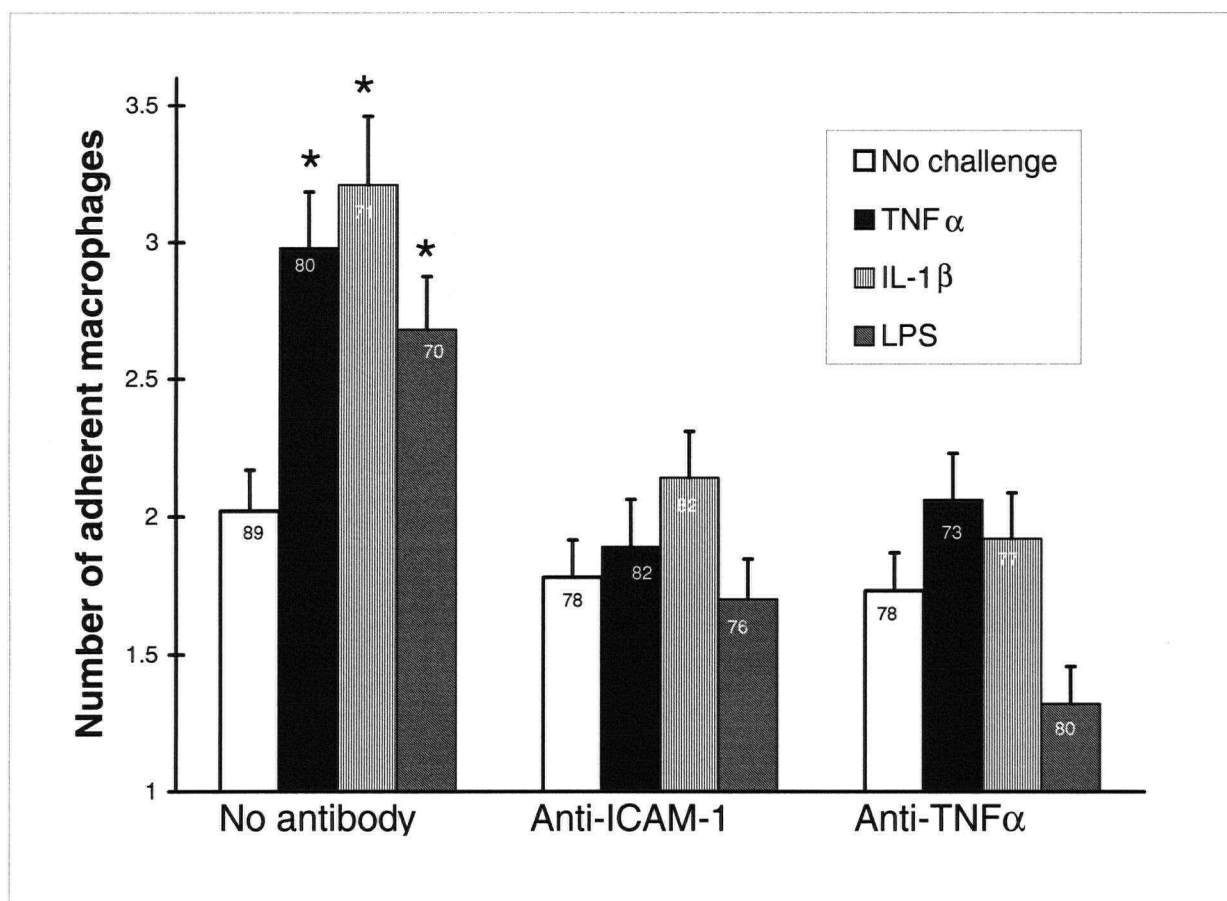
FIGURE 4-The number of macrophages adherent to cardiac myocytes after 4 hours of co-culture is shown.(* indicates p<0.05 compared to other groups, n is indicated on bars)
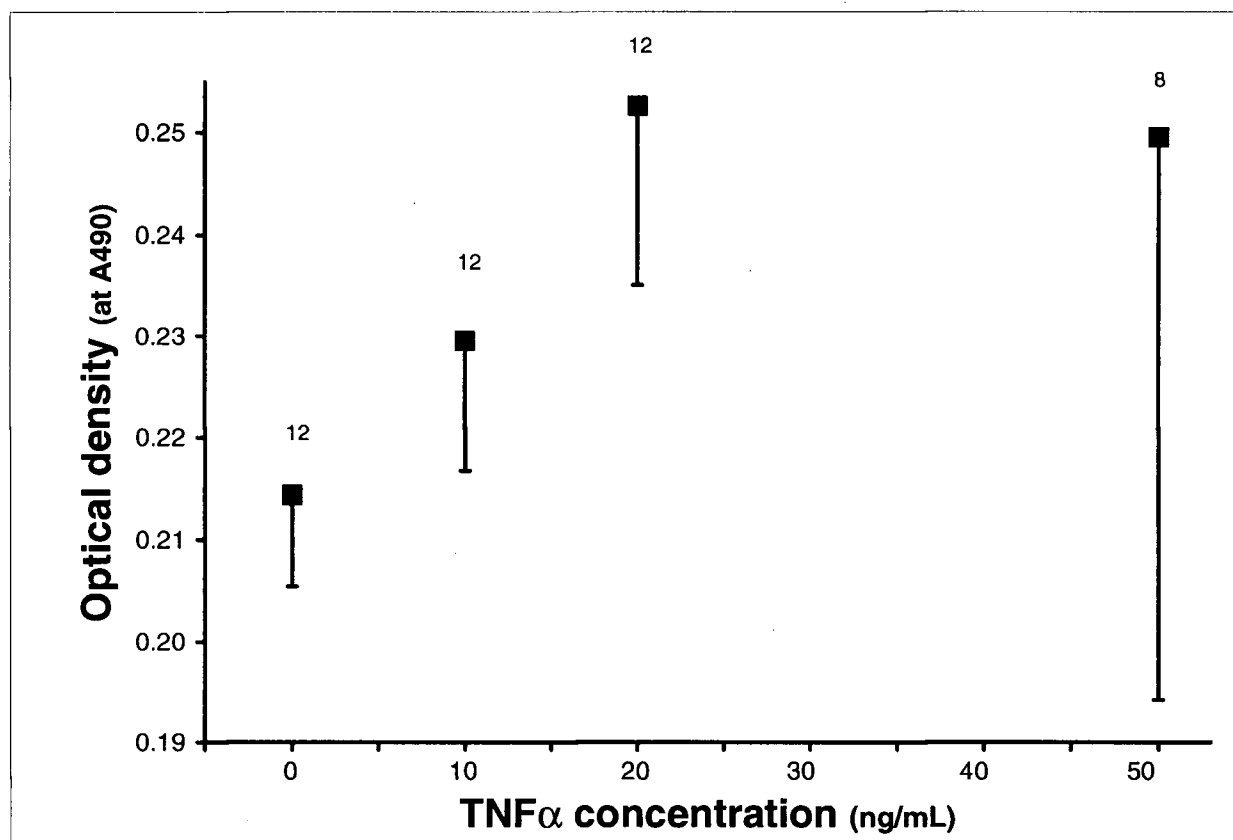
FIGURE 5-Optical density as determined by the ELISA for ICAM-1 expression on rat cardiac myocytes after 4 hours of TNFα challenge is shown. (n is indicated above)
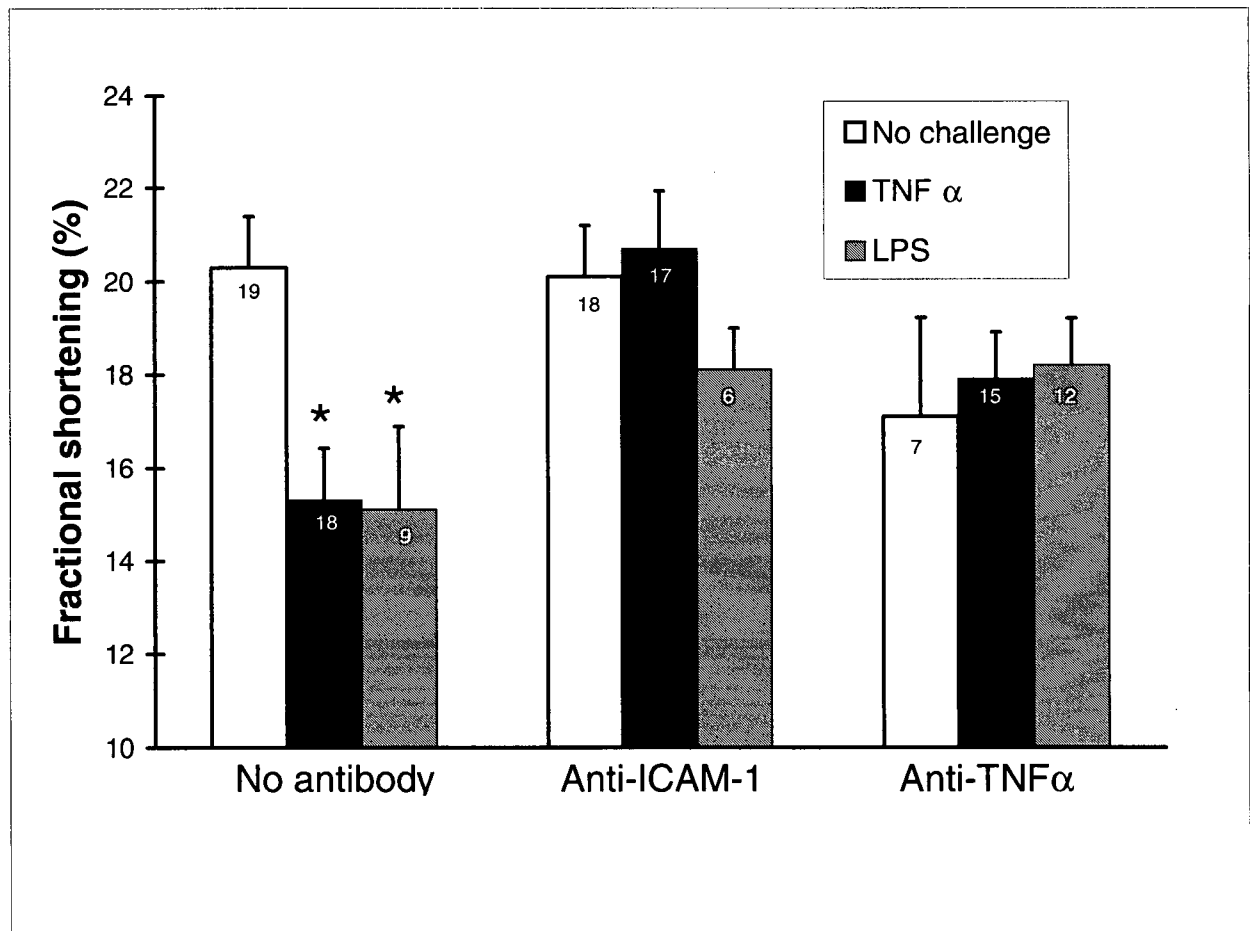
FIGURE 6-Fractional shortening of rat cardiac myocytes co-incubated with rat macrophages after 4 hour exposure to TNFα or LPS. (* indicates p<0.05 compared to control, n is indicated on bars).
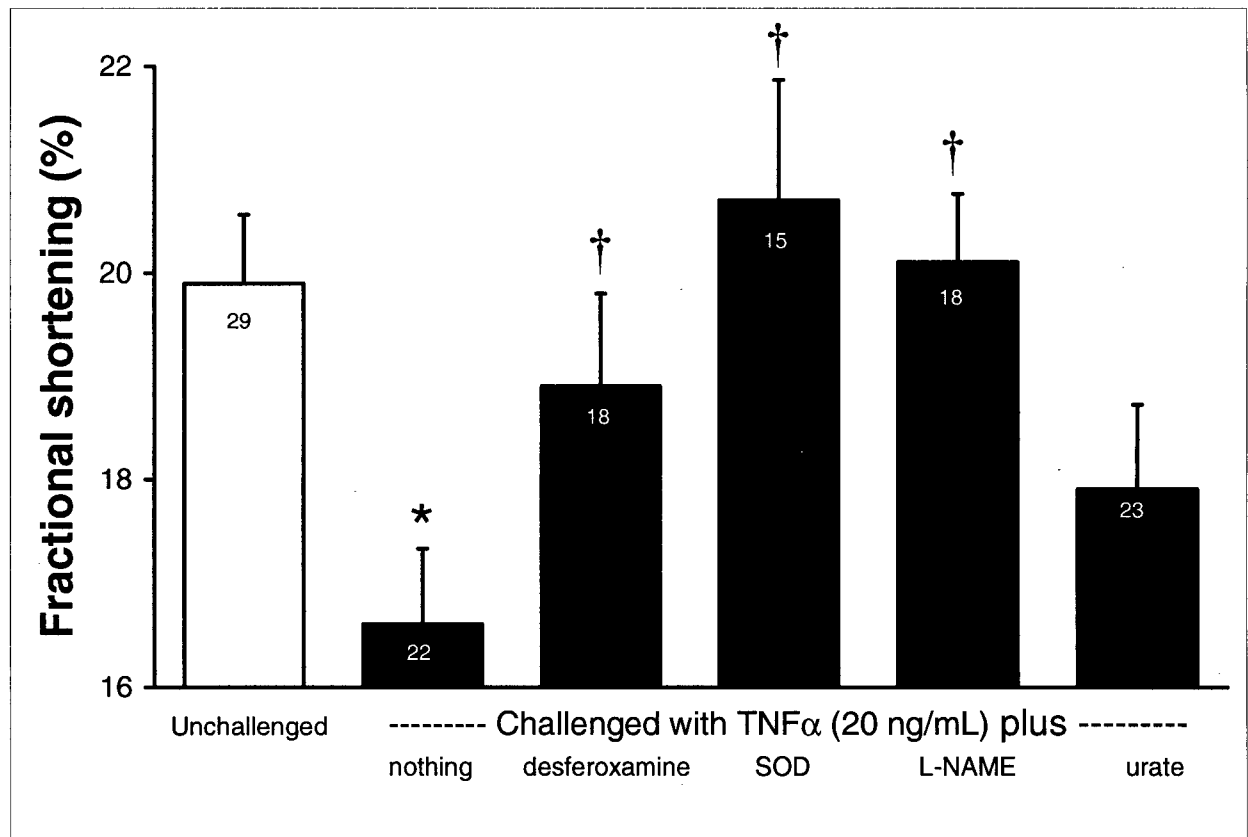
FIGURE 7-Fractional shortening of rat cardiac myocytes co-incubated with rat macrophages after 4 hour exposure to TNFα. (* indicates p < 0.05 compared to control and †, n is indicated on bars)

CHAPTER 4: DISCUSSION

Taken together, these observations demonstrate that activated macrophages can contribute to cardiac myocyte contractile dysfunction. Adherence of activated macrophages to ICAM-1 expressed on activated cardiac myocytes appears to be an important step in mediating the observed contractile dysfunction. TNFα, which has not only been clearly implicated as an important mediator in sepsis (10,11), has been shown to induce the release of IL-1β from macrophages (10). This cytokine plays a role by contributing to macrophage activation and adhesion and, possibly, by mediating part of the effect of adherent macrophages. Both NO and oxygen free radicals seem to contribute to the myocardial depressant effect of adherent macrophages.

4.1   Fractional Shortening

An important characteristic of cardiovascular dysfunction in sepsis is the decrease in left ventricular contractility (8). By using myocyte fractional shortening as representative of contractility, our results extend previous findings by demonstrating that activated macrophages can contribute to myocardial dysfunction. In contrast, TNFα in the absence of macrophages did not decrease fractional shortening of cardiac myocytes. Lack of direct TNFα effects in our study differs from the initial observations of Finkel et al. (23) but agree with the observations of Kumar et al. who also found that TNFα and IL-1β separately do not decrease cardiac myocyte function. TNFα and IL-1β, which are released during the body's immunological response to sepsis, may act synergistically to decrease contractility

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(43). It follows that one potential explanation for our results that macrophages decreased myocyte contractility because they release both TNFα and IL-1β. However, if this were the only mechanism of macrophage effect then physical separation of macrophages and myocytes using culture well inserts should not have prevented the decrease in inflammatory mediator-induced myocyte contractility. Similarly, anti-ICAM-1, which simply prevented macrophage adhesion to cardiac myocytes, should not have prevented decreased myocyte contractility.

4.2   Role of Macrophage Adherence on Myocyte Function

Cytokine induction of ICAM-1 on cardiac myocytes have been observed as being a critical step in myocarditis (32). Macrophage binding to cardiac myocytes has been shown to involve ICAM-1. Our ELISAs showed this ICAM-1 expression on cardiac myocytes after TNFα challenge. This expression on cardiac myocytes has also been observed following TNFα, IL-1β, and monocyte chemotactic protein-1 (MCP-1) challenge (32). Adherence of macrophages to myocytes via ICAM-1 appears to be also important in macrophage-induced myocyte dysfunction. Smith et al. have shown that when exposed to either TNFα, IL-1β, or LPS, canine cardiac myocytes synthesize and express ICAM-1 on their membrane, which allows leukocytes to adhere while their adherence to myocytes can also be blocked by anti-ICAM-1 (33).

Adherence could contribute to decreased contractility in at least two ways. First, adherence may simply keep macrophages in very close proximity to cardiac myocytes so that

mediator concentrations (for example, TNFα and IL-1β, as well as oxygen free radicals and NO) are high in the immediate vicinity of the cardiac myocyte. In other tissues, leukocytes can adhere to cells, enveloping a pocket between the two cells that may contain very high concentrations of mediators. Thus, physical proximity may be an important issue. The second possibility is that cardiac myocyte ICAM-1 may act as a receptor that, by itself or in conjunction with co-stimuli, may result in intracellular signalling leading to decreased contractile function.

4.3 Role of Oxygen Free Radicals and Nitric Oxide on Myocyte Function

In addition to TNFα and other cytokines, oxygen free radicals and nitric oxide also seem to play important roles by mediating the contractile dysfunction induced by activated, adherent macrophages (23-29). This mediation of dysfunction was observed when the decrease in fractional shortening was inhibited following the co-incubation of the co-culture with nitric oxide and NO synthase inhibitors after TNFα challenge (Figure 7). Several studies support these findings by showing that part of the effect of pro-inflammatory cytokines in vitro is due to stimulation of nitric oxide production in cardiac myocytes (23,27,45,46). There is growing evidence, as shown in isolated working hearts, that TNFα and IL-1β release contribute to the production of oxygen free radicals and NO. The production of oxygen free radicals and NO could then mediate part of their effect as components of intracellular signal transduction by combining to form peroxynitrites which could cause damage to cell membranes, thus contributing to decreased contractile function (47). Another possible pathway for NO in mediating contractile function is that production

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of NO stimulates the guanylate cyclase pathway within the cardiac myocytes, reducing intracellular calcium, which could, in turn, decreases cardiac contractility (28,45,53-55).

It is important to recognize that we used macrophages that had emigrated from the blood to the peritoneal cavity. Emigrated leukocytes express different cell adhesion molecules than the circulating blood population (56). While peritoneal macrophages may differ from those recruited to the heart during an inflammatory response we felt that emigrated peritoneal macrophages may better model emigrated intramyocardial macrophages than peripheral blood monocytes.

In summary, activated macrophages adhering to cardiac myocytes via ICAM-1, after challenging with TNFα, could contribute to myocardial contractile dysfunction through a decrease in myocyte fractional shortening. The effects of adherent macrophages are mediated in part by oxygen free radicals and nitric oxide.

4.4 Further Studies

Neutrophils and macrophages have been found to both bind via ICAM-1. Since these two cells are both present during the sepsis, further work could examine which cell binds with greater affinity when both macrophages and neutrophils are co-cultured with myocytes.

IL-1β and TNFα are present in the early stages of the septic inflammatory cascade. Other studies could also examine the cytokines which are triggered later on, such as IL-6.

ICAM-1 has been shown to be very important in macrophage-induced myocyte dysfunction. However, when anti-ICAM-1 was used, the effect of increased macrophage adhesion and decreased myocyte contractility was not completely blocked. By exploring other adhesion molecules, one might be in a better position to understand what other molecules besides ICAM-1 play a role in macrophage adhesion.

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APPENDIX I: EXPERIMENTAL DATA

1a)   Fractional shortening of myocytes co-incubated with antibodies, after no challenge.

| | No macrophages | | No Antibodies | | anti-ICAM-1 | | anti-TNFa | |
	cell	%diff	Cell	%diff	cell	%diff	Cell	%diff
No Challenge	1	30.73	1	19.11	1	23.76	1	13.58
	2	19.81	2	20.57	2	16.97	2	23.08
	3	17.28	3	27.51	3	21.71	3	16.1
	4	23.7	4	28.49	4	14.56	4	15.78
	5	21.39	5	28.21	5	23.16	5	7.98
	6	17.24	6	15.08	6	26.2	6	18.65
	7	18.89	7	15.56	7	26.89	7	24.3
	8	24.29	8	16.49	8	9.25		
	9	21.61	9	14.8	9	20.85		
	10	30.07	10	22.34	10	24.76		
	11	26.19	11	16.46	11	20.02		
	12	21.69	12	20.44	12	14.51		
	13	29.33	13	13.62	13	21.25		
	14	19.44	14	19.15	14	22.04		
	15	21.02	15	22.92	15	18.02		
	16	23.65	16	25.96	16	16.07		
	17	21.79	17	18.61	17	23.73		
	18	21.15	18	23.25	18	17.14		
	19	22.91	19	16.47				
	20	24.32						
	21	21.94						
Mean		22.78		20.27		20.05		17.07
Std Err.		0.82		1.09		1.10		2.12

1b)   Fractional shortening after TNFα challenge.

	no macrophages		no antibodies		Anti-ICAM-1		anti-TNFa	
	cell	%diff	cell	%diff	Cell	%diff	cell	%diff
TNFa	1	29.71	1	17.44	1	21.65	1	14.91
	2	19.71	2	12.79	2	21.91	2	24
	3	28.06	3	14.77	3	18.47	3	11.76
	4	20.76	4	15.54	4	26.22	4	23.36
	5	28.75	5	17.94	5	17.49	5	17.63
	6	20.19	6	15.79	6	15.6	6	14.25
	7	23.65	7	19.37	7	23.08	7	21.34
	8	21.14	8	9.07	8	23.49	8	12.92
	9	16.49	9	2.23	9	10.94	9	21.61
	10	22.07	10	19.12	10	16.82	10	17.56
	11	17.75	11	13.36	11	28.89	11	16
			12	11.97	12	13.67	12	19.16
			13	22.75	13	20.68	13	14.08
			14	15.73	14	26.14	14	20.78
			15	13.13	15	22.05	15	20.38
			16	21.21	16	24.79		
			17	14.81	17			
			18	19.12				
Mean		22.57		15.34		20.74		17.98
Std Err		1.35		1.12		1.23		1.00

1c)   Fractional shortening after LPS challenge.

	no macrophages		no challenge		anti-ICAM-1		anti-TNFa	
	cell	%diff	cell	%diff	cell	%diff	cell	%diff
LPS	1	21.35	1	19.9	1	17.67	1	15.45
	2	14.79	2	13.96	2	14.56	2	15.24
	3	22.79	3	14.66	3	17.45	3	13.88
	4	19.72	4	17.98	4	18.41	4	12.23
	5	17.58	5	23.49	5	21	5	18.4
	6	20.91	6	7.28	6	19.5	6	21.21
	7	6.48	7	7.91			7	17.12
	8	15.08	8	17.85			8	19.82
	9	27.66	9	12.73			9	20.61
	10	25.52					10	22.22
	11	19.13					11	19.36
	12	26.29					12	23.28
	13	18.44						
	14	15.55						
	15	22.62						
	16	18.57						
	17	18.26						
	18	19.38						
	19	18.18						
	20	6.06						
	21	7.83						
	22	24.1						
	23	12.25						
	24	23.53						
	25	23.19						
	26	24.83						
	27	21.21						
	28	20.89						
	29	20.05						
	30	36.55						
	31	24.36						
	32	19.93						
	33	17.87						
	34	16.87						
	35	18.64						
	36	14.3						
	37	22.19						
Mean		19.54		15.08		18.10		18.24
Std Err		0.96		1.78		0.89		1.00

2) Macrophage adherence to cardiac myocytes after 3 and 4 hours of challenge.

	3 hours		4 hours	
	unchallenged	TNFα challenge	unchallenged	TNFα challenge
	3	4	3	3
	4	7	2	6
	1	3	3	5
	6	5	4	5
	2	1	2	3
	1	5	1	4
	0	2	1	2
	3	1	5	5
	0	5	4	1
	0	2	4	2
	2	4	1	8
	4	3	4	5
	0	6	0	2
	3	1	2	5
	1	5	1	5
	1	1	3	3
	0	3	1	5
	1	2	6	2
	4	3	4	3
	0	5	3	5
	1	1	4	6
	2	2	1	5
	0	6	3	8
	0	4	2	2
	2	1	0	5
	2	0	3	7
	0	3	2	4
	0	5	5	5
	0	5	0	0
	2	7	3	3
	0	2	4	4
	1	5	2	4
	0	4	2	6
	2	9	5	4
	3	3	5	3
	4	4	2	5
	2	1	1	4
	0	4	2	8
	3	2	4	0

	3 hours (cont'd)		4 hours (cont'd)	
	Unchallenged	TNFα chall	Unchallenged	TNFα challenged
	5	4	0	2
	6	5	3	9
	2	6	2	4
	0	2	1	3
	3	8	2	
	4	3	4	
	3		1	
	0		3	
	0		5	
	5		2	
	1			
mean	1.78	3.64	2.59	4.19
Std Err	0.71	0.55	0.32	0.24

3) ICAM Expression data from ELISA.

Dose of TNF (ng/mL)	0	10	20	50
Optical Density	0.185	0.197	0.185	0.136
	0.187	0.242	0.217	0.085
	0.194	0.260	0.254	0.112
	0.268	0.315	0.312	0.462
	0.209	0.203	0.207	0.492
	0.198	0.226	0.181	0.291
	0.216	0.193	0.252	0.163
	0.240	0.297	0.309	0.255
	0.191	0.186	0.216	
	0.200	0.176	0.272	
	0.208	0.208	0.236	
	0.276	0.251	0.390	
mean	0.214	0.230	0.253	0.250
Standard Error	0.0089	0.013	0.018	0.055

4) Fractional shortening of myocytes co-incubated with oxygen free radical scavengers, NO synthase inhibitors and peroxynitrite inhibitors.

Cell #	No chall	chall	nchall desfer	desferox	Nchall SOD	SOD	nchall L-NAME	L-NAME	nchall urate	urate
1	22.63	15.33	12.93	21.83	20.62	21.91	16.9	16.1	25.41	15.15
2	21.7	16.77	17.56	15.25	18.19	24.62	17.87	19.68	18.77	17.06
3	22.84	16.33	18.49	15.36	21.58	18.8	14.97	16.24	18.4	5.18
4	20.37	16.3	20.38	19.23	18.43	23.37	23.35	20.79	11.57	23.99
5	16.2	15.87	16.03	24.34	21.45	10.44	17.89	22.56	20	16.19
6	16.84	12.21	33.04	25.84	22.87	22.06	12.25	18.28	18.31	19.1
7	18.71	7.79	23.32	14.32	22.24	18.26	13.39	20.76	18.24	20.3
8	19.56	15.53	24.81	16.78	23.14	20.99	15.85	19.57	15.54	16.02
9	17.72	16.22	18.95	21.17	20.02	23.79	19.68	22.71	22.39	21.56
10	23.3	20.46	13.9	18.33	6.53	20.25	15.14	19.03	21.59	20.31
11	13.23	10.43	9.94	20.42	21.56	29.27	19.35	17.72	12.35	15.31
12	19.53	17.49	26.9	22.1	18.15	17.1	17.71	17.01	14.54	15.4
13	21.91	24.35	17.4	20.3	19.96	20.75	13.92	19.02	17.8	14.86
14	21.19	19.37	16.67	10.54	20.94	24.59	17.07	22.26	23.01	20.43
15	15.16	15.21	14.53	16.27	20.31	15	15.48	17.06	16.64	17.62
16	21.61	17.05	15.05	17.02			23.13	23.81	18.68	14.87
17	11.82	18.41	15.91	19.01			16.78	19.06	13.58	24.31
18	19.59	19.97	18.57	22.12			17.85	25.73	20.4	20.1
19	22.6	16.91	23.72				21.2	23.83	22.45	21
20	14.7	17.86	20.87				15.07		16.89	17.02
21	20.92	17.26	19.49				14.35		20.68	17.9
22	19.64	18.54	27.82				20.94		16	20.08
23	18.7		16.23				14.74		16.34	17.71
24	21.5		20.62				17.96		19	
25	28.57						23.87		28.79	
26	24.32									
27	18.68									
28	23.67									
29	19.77									
mean	19.90	16.62	19.30	18.90	19.73	20.75	17.47	20.06	18.69	17.89
St Err	0.66	0.73	1.07	0.90	1.03	1.16	0.63	0.66	0.79	0.82

5)   Mean macrophage adherences after co-incubation with oxygen free radical scavengers, peroxynitrite inhibitors, NO synthase inhibitors.

Cell #	No chall	chall	No chall desferox	desfer ox	No chall SOD	SOD	No chall L-NAME	L-NAME	nchall urate	urate
1	3	1	2	3	3	3	2	2	2	2
2	2	2	4	1	3	2	2	1	1	2
3	2	2	6	1	0	4	3	4	1	4
4	2	2	6	1	2	2	1	2	1	4
5	1	3	2	3	4	2	1	1	1	1
6	3	5	5	1	3	1	2	4	2	2
7	2	2	0	2	1	2	1	5	2	3
8	3	1	3	4	4	2	2	0	0	2
9	3	4	6	3	3	2	2	3	3	3
10	5	3	6	0	4	4	4	1	4	2
11	3	4	2	4	4	6	2	6	4	2
12	3	3	2	3	5	3	2	3	2	2
13	3	0	2	2	5	2	3	2	0	3
14	4	1	1	4	6	3	1	3	5	1
15	5	4	5	3	4	5	2	3	3	0
16	4	3	7	3	5	2	1	1	0	1
17	4	4	3	2	3	5	3	3	4	0
18	5	2	3	4		2	4	2	2	2
19	2	2	2			4	1	2	4	1
20	3	3	3			1	3	1	3	3
21	3	3	4			3	1	3	1	1
22	4	0	1			5	0		3	
23	2		4				2		2	
24	3		3				2		1	
25			5				1			
26			4							
27			5							
28										
29										
mean	3.08	2.45	3.56	2.44	3.47	2.95	1.92	2.48	2.13	1.95
Sd Err	0.22	0.29	0.35	0.29	0.37	0.30	0.20	0.32	0.29	0.24

6) Relationship between adherent macrophages and fractional shortening.

#macrophages	2	3	4	5	6
	17.44	19.11	27.51	16.46	15.56
	12.79	20.57	28.21	20.44	11.97
	15.54	14.77	15.08	25.96	13.13
	17.94	28.49	13.36	16.47	19.12
	15.79	2.23	15.73	13.96	18.61
	9.07	19.12	14.8	12.73	14.66
	19.15	22.75	22.92	13.96	7.91
	17.44	22.34	19.9	12.73	11.97
	12.79	14.77	7.28	20.02	13.13
	17.98	17.85	13.36	16.07	19.12
	23.49	2.23	15.73	10.94	14.66
	15.54	19.12	19.9	20.68	7.91
	17.94	22.75	7.28	18.41	23.76
	15.79	17.85	26.2	19.5	26.89
	9.07	21.71	17.14	21.61	14.56
	17.98	14.56	17.49	17.12	11.76
	23.49	20.85	23.49		13.88
	24.76	21.65	28.89		
	18.47	21.91	17.67		
	26.22	15.6	17.45		
	23.08	13.58	23.08		
	16.82	7.98	18.65		
	22.05	24	23.36		
	24.79	14.25	17.63		
	21	12.92	20.38		
	16.1	17.56	19.36		
	15.78	19.16			
	24.3	18.4			
	21.34	19.82			
	16	20.61			
	20.78	22.22			
	15.24				
	21.21				
	23.28				
Mean	18.54	17.77	18.92	17.32	15.21
Std Error	0.74	1.03	1.10	0.99	1.22

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7) Diastolic lengths of cardiac myocytes after TNFα and LPS challenge.

	no challenge	TNFα challenge	LPS challenge
lengths	77	67	75
	65	61	48
	74	55	79
	69	61	71
	55	65	62
	79	79	84
	63	51	73
	63	55	68
	74	45	59
	74	67	75
	83	56	57
	60	64	63
	68	62	
	71	56	
	63	82	
	69	75	
	72	71	
	66	52	
	58		
	67		
	55		
	62		
	71		
Mean	67.74	62.44	67.87
Std Err	1.54	2.35	2.99

8) Myocytes incubated with macrophages after addition of well inserts.

	No chall co-incub.	No chall (insert)	TNF co-incub.	TNF (insert)	LPS co-incubated	LPS (insert)
	21.53	16.33	13.37	14.09	15.59	14.15
	16.05	24.34	9.35	17.98	12	38.09
	18.83	22.96	16.11	14.57	22.54	17
	15.46	19.14	20.48	28.3	11.81	15.95
	24.59	26.23	18.11	17.82	12.97	18.42
	22	27.26	19.95	20.18	15.96	14
	17.31	21.99	17.76	19.18	18.06	25.01
		19.12	24.8	16.19	16.91	18.2
		28.09	16.74	20.26	22.01	19.61
		29.47	22.41	16.93	11.24	19.24
		19.33	11.25	17.64	14.07	20.94
		16.62	18.43	18.42	20.36	14.14
		21.22	16.22	21.14	22.29	27.1
		24.39	15.67	22.36	15.41	21.56
		17.16		27.64	21.51	22.72
				26.95		25.28
				20.49		21.99
				22.64		19.41
						20.99
Mean	19.74	22.24	17.19	20.15	16.85	20.73
Sd Err	1.28	1.1	1.16	1.00	1.05	1.29

9) Mean macrophage adherences after co-incubation with antibodies and challenge.

	No antibody				Anti-ICAM-1				anti-TNF			
	No Stim	TNF	IL-1	LPS	No Stim	TNF	IL-1	LPS	No Stim	TNF	IL-1	LPS
	3	4	2	3	4	1	7	1	4	1	0	3
	3	1	1	3	0	6	6	3	2	1	2	5
	6	0	1	2	1	0	2	5	3	0	0	0
	3	3	5	5	0	6	1	5	3	2	0	5
	0	1	2	5	2	1	0	3	1	1	0	5
	4	1	8	3	3	1	2	3	1	6	1	2
	3	2	4	5	3	4	1	2	0	5	1	1
	3	4	4	3	1	0	2	1	2	2	0	1
	3	6	1	2	2	3	7	1	0	4	2	1
	3	4	3	5	1	4	2	4	0	1	5	2
	1	6	5	5	2	2	6	2	3	1	3	3
	2	4	5	6	3	4	4	2	1	3	3	3
	3	3	3	4	2	4	3	0	2	2	0	3
	0	7	5	0	4	5	1	2	2	5	5	1
	3	2	9	2	0	5	0	5	1	6	5	4
	1	6	5	1	2	0	3	3	1	4	1	1
	2	3	1	0	3	4	0	4	1	3	3	3
	2	4	10	2	2	4	2	3	1	0	1	2
	2	3	2	4	1	3	1	2	1	5	3	0
	3	3	6	2	2	0	2	2	3	3	6	1
	3	5	4	2	3	2	1	1	3	1	0	0
	1	7	5	5	1	0	2	0	2	1	1	1
	3	1	8	9	1	1	3	1	0	4	1	3
	4	4	11	2	3	4	0	2	4	3	3	0
	2	4	4	1	2	2	4	0	1	5	3	0
	0	3	1	2	3	1	3	1	1	2	1	0
	2	7	3	3	1	0	2	3	3	4	2	0
	1	9	3	2	0	1	3	1	2	2	2	0
	1	4	5	4	0	2	2	2	2	4	2	1
	3	5	3	2	4	4	1	2	2	0	2	1
	2	1	4	2	2	2	2	5	3	3	2	0
	2	5	4	5	3	6	2	3	0	3	2	0
	3	7	3	2	4	4	1	0	3	0	7	2
	1	4	1	5	3	3	2	2	2	2	3	2
	2	6	3	2	1	1	5	1	5	1	2	2
	3	3	4	1	3	1	1	2	2	3	4	1
	3	2	4	4	0	0	2	1	3	1	3	1
	3	5	2	6	4	1	3	0	1	1	2	2
	2	2	3	3	1	0	5	0	1	0	3	1
	3	2	3	1	2	1	1	4	0	1	3	2
	4	5	1	3	2	2	0	2	1	3	3	3
	1	4	5	7	1	3	3	4	5	2	2	3
	2	3	6	5	3	4	5	1	4	2	1	0
	0	4	1	5	3	1	1	3	2	0	4	1
	2	6	2	3	5	4	3	2	1	4	5	3
	2	5	3	2	3	0	3	0	5	3	2	2
	0	3	1	2	1	0	1	3	1	1	4	1
	0	4	4	3	3	3	3	2	2	3	3	1

No antibody (cont'd)				Anti-ICAM-1 (cont'd)				Anti-TNF (cont'd)			
No chall	TNF	IL-1	LPS	No chall	TNF	IL-1	LPS	No chall	TNF	IL-1	LPS
1	4	3	1	1	2	2	1	2	3	2	1
1	2	2	2	2	3	2	2	1	2	4	1
5	3	7	3	3	3	2	3	2	2	2	2
5	3	3	1	0	3	4	1	5	2	1	0
6	2	3	3	0	2	4	1	1	0	2	0
9	0	0	2	1	1	4	1	0	1	0	2
0	0	3	0	2	1	5	0	3	1	3	1
0	1	4	1	3	1	2	0	3	3	1	0
1	3	3	3	1	2	3	2	1	1	0	1
2	2	1	1	2	3	0	2	0	1	1	1
3	3	2	1	2	0	1	1	0	2	0	1
2	2	0	2	4	0	0	1	2	1	0	2
2	2	1	2	2	1	1	1	2	0	2	0
0	4	2	2	3	0	2	0	3	1	1	1
1	1	2	2	3	2	1	1	1	4	1	1
0	1	3	1	0	3	3	3	3	4	3	1
2	1	2	4	2	0	2	0	2	1	1	0
0	2	3	2	1	2	2	0	1	1	2	0
1	3	7	2	1	1	5	1	2	1	1	1
2	1	1	0	1	1	1	0	2	1	1	0
2	0	0	1	0	0	2	1	1	0	4	4
2	2	1	1	1	2	1	2	0	2	1	1
2	2	2	3	1	1	1	0	1	2	0	1
1	4	5	1	0	2	2	1	0	1	3	0
1	5	3	3	1	4	0	2	1	1	1	1
1	3	0	4	0	0	3	1	1	0	0	4
3	3	1	1	0	1	1	2	0	1	0	2
1	1	1	1	3	0	3	0	2	0	1	2
1	0	2	3	1	1	1	4	0	1	2	0
2	4	3	0	4	1	2	2	2	4	0	2
2	3	3	4	1	0	1	0	2	3	3	2
1	2	3	2	0	0	1	0	3	4	1	0
0	0	1		1	0	4	3	2	1	1	0
0	1			1	2	0	2	2	2	1	1
2	1			3	3	0	1	3	3	0	0
2	1			2	2	1	1	0		1	0
3	2			1	1	2	2	2		4	0
2	1			2	4	0	0	1		1	1
3	3			1	5	1		0		2	2
1	1			0	1	1		0			0
1	0				1	2					1
3	2				1	3					0
2					1	1					
0					0	2					
2											
1											
3											
2											
3											
3											
0											

	2											
Mean	2.02	2.98	3.21	2.68	1.78	1.89	2.14	1.70	1.73	2.06	1.92	1.32
Sd Err	0.15	0.20	0.25	0.19	0.13	0.17	0.17	0.15	0.14	0.17	0.17	0.13

APPENDIX II: STATISTICAL ANALYSES

NOTE: n is calculated by examining individual cultured rat ventricular myocytes

1)   Fractional shortening of myocytes co-incubated with antibodies, after challenge.

<u>No macrophages</u>:   ANOVA: $\underline{F}$ (2,66) = 3.34, (not significant)

<u>No antibodies</u>:   ANOVA: $\underline{F}$ (2,43) = 5.90, p < 0.05

                          Post hoc (Bonferroni):   a) No challenge > TNFα challenge

                                                        b) No challenge > LPS challenge

<u>anti-ICAM-1</u>:   ANOVA: $\underline{F}$ (2,37) = 0.749, (not significant)

<u>anti-TNFα</u>:   ANOVA: $\underline{F}$ (2,31) = 0.18, (not significant)

2)   Macrophage adherence to cardiac myocytes:

After 3 hours incubation: ANOVA: $F_{(1,93)} = 22.59$, $p < 0.001$

After 4 hours incubation: ANOVA: $F_{(1,90)} = 18.04$, $p < 0.001$

3)   Fractional shortening of myocytes co-incubated with oxygen free radical scavengers, NO synthase inhibitors and peroxynitrite inhibitors.

After comparing challenged cells with various additives:

ANOVA: $F_{(4,92)} = 3.82$, $p < 0.005$

Post hoc (Bonferroni):   a) challenged < SOD

b) challenged < L-NAME

c) challenged < desferoxamine

4) Mean macrophage adherences after co-incubation with oxygen free radical scavengers, peroxynitrite inhibitors, NO synthase inhibitors.

After comparing challenged cells vs unchallenged cells:

      a) no additives: ANOVA: $F_{(1,44)} = 3.15$, (not significant)

      b) desferoxamine: ANOVA: $F_{(1,40)} = 3.21$, (not significant)

      c) SOD: ANOVA: $F_{(1,44)} = 0.13$, (not significant)

      d) L-NAME: ANOVA: $F_{(1,43)} = 2.57$, (not significant)

      e) urate: ANOVA: $F_{(1,43)} = 0.20$, (not significant)

5) Relationship between adherent macrophages and fractional shortening.

ANOVA: $F_{(4,119)} = 1.65$, $p = 0.07$

Fractional shortening: a) 2 adherent macrophages > 6 macrophages

                                b) 4 adherent macrophages > 6 macrophages

6)   Diastolic lengths of cardiac myocytes.

ANOVA: $\underline{F}$ (1,39) = 3.83, (not significant)

7)   Myocytes incubated with macrophages after addition of well inserts.

ANOVA for co-incubated cells vs inserts:

a) no challenge: $\underline{F}$ (1,20) = 2.36, (not significant)

b) TNFα challenge: $\underline{F}$ (1,30) = 4.01, p < 0.05

c) LPS challenge: $\underline{F}$ (1,32) = 5.01, p < 0.05

8)   Mean macrophage adherences after co-incubation with antibodies and challenge.

a) <u>No antibodies</u>:   ANOVA: $\underline{F}$ (3,347) = 7.21, p < 0.001

Post hoc (Bonferroni):   a) No challenge > TNFα challenge

b) No challenge > LPS challenge

c) No challenge > IL-1β challenge

b) <u>Anti-ICAM-1</u>:   ANOVA: $\underline{F}$ (3,354) = 1.51, (not significant)

c) <u>Anti-TNFα</u>:   ANOVA: $\underline{F}$ (3,344) = 4.50, (not significant)