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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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**SYSTEMIC CYTOKINES/CHEMOKINES CONTRIBUTE TO MICROVASCULAR
DYSFUNCTION AND TISSUE INJURY IN COMPARTMENT SYNDROME**

(Thesis format: Integrated-Article)

by

Erin Siobhan Donohoe

Graduate Program in Surgery

**A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science in Surgery**

The School of Graduate and Postdoctoral Studies

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London, Ontario, Canada

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ABSTRACT

There are few more devastating complications of musculoskeletal trauma than compartment syndrome (CS). It occurs secondary to elevated pressure within a closed osseofascial compartment, leading to microvascular dysfunction, hypoperfusion of the tissues, cellular anoxia and ultimately cell death. The aim of this thesis was to prove that CS leads to a systemic inflammatory response, and to examine the specific cytokines/chemokines associated with CS. Twenty-four cytokines/chemokines were measured in a rat model of CS. Additionally, microvascular dysfunction, tissue injury and inflammatory response following the neutralization of pro-inflammatory cytokine, TNF- α , at the time of fasciotomy were assessed using intravital video microscopy (IVVM). The results of our studies were the first to confirm that CS is associated with an acute inflammatory response, and that neutralization of TNF- α at the time of fasciotomy provides some protection against tissue injury due to CS.

Keywords: *compartment syndrome, microvascular dysfunction, systemic cytokine/chemokine release, ischemia-reperfusion, tissue injury, inflammation, TNF- α .*

CO-AUTHORSHIP

Each of the co-authors listed below provided invaluable contributions to this work. I performed the experiments, data collection and analysis. I have written the manuscripts presented in this thesis with consultation, support and critical review by the co-authors.

Abdel-Rahman Lawendy, MD, FRCSC, PhD and **David Sanders, MD, FRCSC**, in their role as joint supervisors, provided guidance and direction on this thesis.

Aurelia Bihari, MSc taught me all of the experimental techniques used in this project, assisted with the animal protocols and setup, data collection, analysis, technical support, and manuscript editing.

DEDICATION

I would like to dedicate this work to my parents who have supported me unconditionally. They immigrated to Canada so that my sister and I would be unbound from social status, and free to pursue our dreams. You have both taught me the true meaning of hard work and support, and I am forever grateful.

To my sister, who is wise beyond her years. Your insight and guidance have meant more to me than I can ever express.

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LIST OF ABBREVIATIONS

ANOVA, analysis of variance

BB, bisbenzimidazole

CPC, continuously-perfused capillaries

CS, compartment syndrome

EB, ethidium bromide

EDL, extensor digitorum longus

ELISA, enzyme-linked immunosorbent assay

E-selectin, endothelial selectin

FADD, *Fas*-associated death domain

G-CSF, granulocyte-colony stimulating factor

GM-CSF, granulocyte macrophage-colony stimulating factor

GRO/KC, growth regulated oncogene/keratinocyte-derived chemokine

ICAM-1, intercellular adhesion molecule-1

ICP, intra-compartmental pressure

IFN- γ , Interferon Gamma

IL-1, interleukin-1

IL-1 α , interleukin -1 alpha

IL-1 β , interleukin-1 beta

IL-2, interleukin-2

IL-4, interleukin-4

IL-5, interleukin-5

IL-6, interleukin-6

IL-9, interleukin-9

IL-10, interleukin-10

IL-12p70, interleukin-12p70

IL-13, interleukin-13

IL-17, interleukin-17

IL-18, interleukin-18

IP, Interferon Gamma-Induced Protein

IPC, intermittently-perfused capillaries

I/R, ischemia-reperfusion

IVVM, intravital video microscopy

KC, keratinocyte chemoattractant

L-selectin, leukocyte selectin

MCP-1, monocyte chemotactic protein-1

MIP-1 α , macrophage inflammatory protein-1 alpha

NIR, near infra-red spectroscopy

NPC, non-perfused capillaries

P-selectin, platelet selectin

PPS, pain on passive muscle stretch

RANTES, regulated on activation, normal T cell expressed and secreted

RIP1, receptor-interacting protein 1

RIP3, receptor-interacting protein 3

ROS, reactive oxygen species

TNF- α , tumor necrosis factor alpha

TNFR1, tumor necrosis factor receptor 1

TNFR2, tumor necrosis factor receptor 2

TNF- α NA, tumor necrosis factor alpha neutralizing antibody

VCAM-1, vascular adhesion molecule-1

VEGF, vascular endothelial growth factor

Chapter 1

Introduction

CHAPTER 1: INTRODUCTION

1.1 COMPARTMENT SYNDROME

Compartment syndrome (CS) results from increased pressure within a closed osseofascial compartment; microvascular compromise, cellular anoxia and ultimately cell death ensues (Whitesides, Haney et al. 1975, Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen, Winkquist et al. 1980, Hartsock, O'Farrell et al. 1998). CS is a surgical emergency, whereby fasciotomy is the only effective treatment (Tzioupi, Cox et al. 2009).

The etiology of CS is varied and commonly includes fractures, burns, crush injuries, ischemia-reperfusion injury, exertion, and prolonged limb compression. Less frequent causes of CS include bleeding disorders (Hope and McQueen 2004), lithotomy position (Mathews, Perry et al. 2001), hypothyroidism (Hsu, Thadhani et al. 1995), and snake bites (Vigasio, Battiston et al. 1991).

1.2 DIAGNOSIS

The diagnosis of CS is currently rooted in clinical signs and symptoms. Prompt diagnosis is critical in order to minimize long-term complications. A high suspicion must be maintained at all times; knowledge of the early clinical signs and symptoms, as well as a recognition of high-risk patients are vital to its timely diagnosis (Matsen, Winkquist et al. 1980, McQueen, Gaston et al. 2000). Risk factors include male gender, young age, tibial fracture, high-energy forearm

fracture, femoral diaphyseal fractures, bleeding diathesis, and anticoagulation (McQueen, Gaston et al. 2000).

Two of the hallmark clinical signs of early CS include pain out of proportion to the injury and pain on passive stretch (PPS) of the affected compartment (Whitesides and Heckman 1996). In children, or in patients whose level of consciousness is altered, progressively increasing analgesia requirements may be an early sign of developing CS (Bae, Kadiyala et al. 2001). Pain out of proportion to the injury and PPS are often the only findings that precede neurologic dysfunction of the affected compartment. Unfortunately the sensitivity of these clinical signs in combination is alarmingly low (13 - 19%), while the specificity is high (97%) (Whitesides and Heckman 1996, Ulmer 2002). The absence of pain and PPS is, perhaps, more useful in ruling out CS (Ulmer 2002) than diagnosing it when they are present.

A palpably tense compartment, resulting from increased intra-compartmental pressure (ICP), is recognized as an early physical sign of CS (Mubarak, Owen et al. 1978). Any patient being evaluated, or who is at high risk for CS, should have their dressings or cast removed for thorough assessment. Serial examination is also paramount, as the onset of CS is unpredictable.

It is important to note that the absence of a distal pulse and/or pallor of the affected limb are not features of CS. Likewise, paresis and/or paralysis of the muscles of the involved compartment are late signs of CS (Matsen and Clawson 1975, Ulmer 2002). These are important distinctions to draw, as waiting for all

clinical signs and symptoms to be present for diagnosis would lead to poor functional outcomes, and increased morbidity and mortality.

As an adjunct to the physical examination, intra-compartmental pressure monitoring is available. In patients who are unable to communicate verbally, for example pediatric or obtunded patients, invasive compartment pressure monitoring can provide valuable objective information in diagnosing acute CS (Gelberman, Garfin et al. 1981, Hargens, Akeson et al. 1989). While there are multiple methods of measuring compartment pressures, the use of electronic transducer-tipped systems are the most accurate and are not dependent on limb position or height of the transducer (McDermott, Marble et al. 1984, Willy, Gerngross et al. 1999).

While ICP monitoring can serve as a valuable adjunct in the diagnosis of acute CS, there remains no consensus regarding the threshold at which fasciotomy is necessary. Multiple absolute pressure thresholds have been suggested, and include 30 mmHg (Mubarak, Owen et al. 1978), 40 mmHg (Schwartz, Brumback et al. 1989), and 45 mmHg (Matsen, Winquist et al. 1980). A differential pressure threshold has been popularized, which theorizes that tissue ischemia begins when the difference between the ICP and the diastolic blood pressure is 20 mmHg (Whitesides, Haney et al. 1975). This differential pressure threshold has since been expanded to 30 mmHg based on retrospective observation that this value led to no missed cases of CS (McQueen and Court-Brown 1996). The differential pressure threshold is useful clinically in

hypotensive trauma patients, and has a lower overall rate of fasciotomy when compared to the absolute pressure threshold (McQueen and Court-Brown 1996).

The diagnosis of CS requires a thorough evaluation of patients, maintenance of a high suspicion, awareness of risk factors, and if any doubt exists, frequent serial examinations with the use of invasive ICP monitoring. To date there is no reliable objective diagnostic test for CS; therefore clinical judgment remains at the forefront of diagnosis in order to prevent the catastrophic complications of a late diagnosis or a misdiagnosis.

1.3 TREATMENT

CS is a surgical emergency, as it is both a limb- and life-threatening condition. Treatment involves emergent surgical decompression of the affected limb by fasciotomy, regardless of the underlying cause of CS. CS, however, occurs most frequently following fracture of the tibia; approximately 6% of patients under the age of 35 with a tibial diaphyseal fracture develop compartment syndrome (McQueen, Gaston et al. 2000).

There are multiple described techniques for fasciotomy of the lower leg, with the two-incision technique being the preferred method (Appendix C). In brief, it involves incising and releasing the fascial layer that surrounds the affected compartment. This facilitates tissue swelling, allowing the compartments to expand and therefore decreasing the ICP. All non-viable tissue is debrided, wounds are left open, and a repeat debridement is performed 48-72 hours later.

At the second operation, wounds are either closed primarily, with skin grafting, or occasionally with more advanced reconstructive techniques.

1.3.1 Anatomy of the Lower Leg

The lower leg has four distinct osseofascial compartments: anterior, lateral, deep posterior, and superficial posterior (Figure 1.1). The anterior compartment includes the extensor muscles of the foot and ankle. The lateral compartment contains the peroneal muscles, which evert the foot. The posterior compartments, both deep and superficial, include the flexor muscles of the foot and ankle.

1.4 COMPLICATIONS OF COMPARTMENT SYNDROME

CS is both a limb and life-threatening condition, and despite appropriate and timely treatment, devastating complications can ensue. In a systematic review of 1920 cases of CS, overall 5.5% of patients required an amputation, and there was a 3.3% CS-associated mortality rate (Hayakawa, Aldington et al. 2009). Timing of fasciotomy is critical: when comparing fasciotomy within 6 hours to delayed fasciotomy beyond 12 hours, there was a higher rate of amputation (14% vs. 3.2%) and death (4.3% vs. 2.0%) (Hayakawa, Aldington et al. 2009). A retrospective review of 336 combat casualties in Iraq and Afghanistan over a 20-month period found that delayed fasciotomy resulted in double the rate of amputations, and increased the mortality rate threefold, as compared with patients who had early fasciotomy (Ritenour, Dorlac et al. 2008).

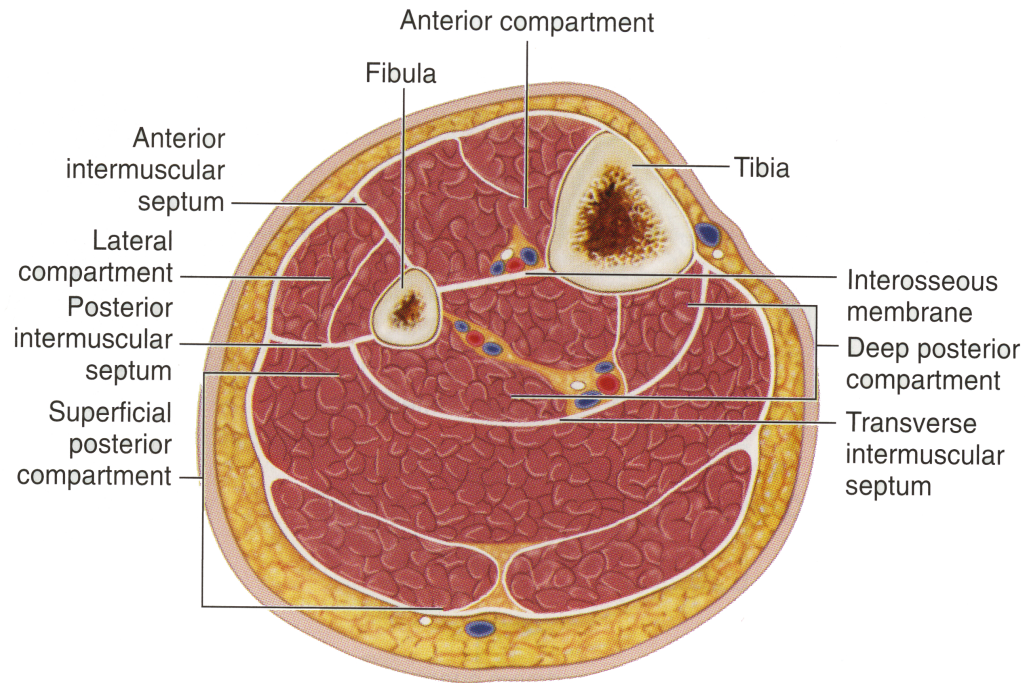


Figure 1.1. Cross-section of the lower leg. The lower leg contains two bones (tibia and fibula) and is divided into four distinct osseofascial compartments: (1) anterior compartment (containing tibialis anterior, extensor hallucis longus, extensor digitorum longus, peroneus tertius, the deep peroneal nerve and the anterior tibial vessels); (2) lateral compartment (containing peroneus longus, peroneus brevis, superficial peroneal nerve); (3) deep posterior compartment (contains tibialis posterior, flexor digitorum longus, and flexor hallucis longus, tibial nerve and posterior tibial vessels); (4) superficial posterior compartment (contains gastrocnemius, soleus, and plantaris, and the sural nerve).

Reproduced with permission from Lawendy and Sanders (2010).

Infection is a common complication following fasciotomy, with high rates of 7.3% even when treated early, versus 28% if fasciotomy is delayed greater than 12 hours (Williams, Luchette et al. 1997). A retrospective study of 60 patients found that 70% of patients had long-term neurologic dysfunction following fasciotomy (62% weakness, 66% paresthesia, 71% dysaesthesia) (Dover, Memon et al. 2012). Fitzgerald et al performed a retrospective review of patient outcomes over an 8-year period, and reported that 77% of their patient population had residual sensory deficits around their wounds, and 10% had chronic pain (Fitzgerald, Gaston et al. 2000). In another study 15.4% of all patients have residual pain at rest, and 26.9% have pain with exertion following CS (Frink, Klaus et al. 2007).

1.5 PATHOPHYSIOLOGY

The pathophysiological mechanism of the tissue injury seen in compartment syndrome remains only partially understood. The literature supports raised ICP as the initial insult, which then leads to microvascular dysfunction. Damage to the microcirculatory system results in hypo-perfusion, causing cellular anoxia and muscle death (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Rorabeck and Clarke 1978, Matsen, Winkquist et al. 1980, Harvey, Sanders et al. 2012). In contrast with complete ischemia, CS causes tissue necrosis despite a patent macrocirculatory system: patients with CS almost always have a palpable distal pulse (Seddon 1966, Lawendy, Bihari et al. 2015).

Live *in vivo* imaging using intravital video microscopy (IVVM) of the microcirculatory system in an animal model of CS, has demonstrated that raised ICP causes significant impairment in capillary perfusion. Under normal physiological conditions there is continuous flow through the capillary beds. Raised ICP, however, causes a shift in perfusion toward non-perfused capillaries (NPC), and intermittently perfused capillaries (IPC) (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). Decreased tissue perfusion leads to inefficient nutrient and gas exchange for the metabolic demands of the tissue. NPCs have no ability for nutrient or gas exchange, and represent a state of ischemia. The presence of some continuously perfused (CPC), IPC, and NPC in the same muscle bed therefore suggests that CS is a low-flow ischemic state.

IVVM has also been able to demonstrate that the microvascular dysfunction seen secondary to raised ICP is associated with rapid activation of leukocytes in post-capillary venules, suggesting the inflammatory pathway may play a role in the tissue injury seen in CS (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). We know from complete ischemia and reperfusion (I/R) literature that the re-introduction of oxygenated blood into an ischemic muscle bed causes a significant activation of leukocytes (Gute, Ishida et al. 1998, Lakshminarayanan, Lewallen et al. 2001). It has been well documented in complete I/R that increasing ischemia time causes increasing accumulation of activated leukocytes in the post-capillary venules. Leukocyte activation leads to increased vascular permeability to plasma protein leakage, tissue edema, and increased interstitial pressure. This increased interstitial pressure is believed to

physically compress capillaries, leading to failure of capillaries to reperfuse upon restitution of blood flow to ischemic tissue. The microvascular dysfunction associated with reperfusion injury is known as no-reflow phenomenon, whereby despite successful resolution of ischemia there is impaired nutrient and gas exchange to meet the metabolic demands of the tissue (Sexton, Korthuis et al. 1990, Kurose, Anderson et al. 1994, Gute, Ishida et al. 1998). A strong correlation has been noted between the number of leukocytes in the capillaries of post ischemic tissue and the percentage of capillaries exhibiting no-reflow (Engler, Dahlgren et al. 1986, Barroso-Aranda, Schmid-Schonbein et al. 1988, Del Zoppo, Schmid-Schonbein et al. 1991, Gute, Ishida et al. 1998).

Further support that activated leukocytes are involved in no-reflow phenomenon is observed in leukopenic models of complete I/R. The microvascular dysfunction of no-reflow in re-perfused myocardium, brain, and skeletal muscle tissue has been abolished in neutrophil-deplete models of complete I/R (Schmid-Schonbein, Shih et al. 1975, Barroso-Aranda, Schmid-Schonbein et al. 1988, Jerome, Smith et al. 1993). Leukopenia does not, however, appear to be protective in restoring or maintaining perfusion following a CS insult (Lawendy, Bihari et al. 2015). Additionally, a greater impairment to the metabolic state and structural integrity of skeletal muscles has been shown in canines subjected to 3 hours of hind limb raised ICP, followed by 2 hours of reperfusion, compared to animals undergoing 3 hours of complete ischemia, followed by 2 hours of reperfusion (Heppenstall, Scott et al. 1986). These findings suggest that while compartment syndrome falls within the spectrum of

ischemia – reperfusion injury, the pathophysiology of tissue injury is distinct from that of complete I/R.

1.6 LEUKOCYTE ACTIVATION

Live *in vivo* studies have demonstrated that the microvascular dysfunction associated with CS occurs in conjunction with leukocyte accumulation and activation in the post capillary venules (Lawendy, Bihari et al. 2015). Leukocyte activation occurs via a series of events, which ultimately results in extravasation of the leukocyte from the blood stream to the extravascular tissue. With the reintroduction of oxygenated blood after an ischemic insult, reactive oxygen species (ROS) are generated, which promote a pro-inflammatory stimulus. Additionally, ROS modify the expression of adhesion molecules on the surface of leukocytes and endothelial cells, resulting in their activation (Gute, Ishida et al. 1998). In the initial phase of leukocyte activation, leukocytes come into contact with the endothelial cell surface and begin to roll along the endothelium of the post-capillary venule following tissue injury or inflammation (Atherton and Born 1973, Carlos and Harlan 1994). Leukocytes are considered rolling if they remain in contact with the vessel wall but do not remain static. A family of three proteins, known as selectins, mediates rolling; they include E- (endothelial), P- (platelet), and L- (leukocyte) selectin, where E- and P-selectin are expressed by stimulated endothelial cells, and L-selectin is expressed by leukocytes (Bevilacqua and Nelson 1993, Carlos and Harlan 1994).

In the second phase of leukocyte activation, leukocytes become firmly adherent to the endothelium. A leukocyte is considered adherent if it remains fixed to the vessel wall. Adhesion is modulated via leukocyte integrins; the transmembrane glycoprotein family CD11/CD18 has been identified as the most likely integrins to modulate neutrophilic adhesion (Dana, Fathallah et al. 1991, Gute, Ishida et al. 1998). Binding of chemokines, cytokines, or chemoattractants can increase the affinity of the integrins for endothelial adhesion proteins (Carlos and Harlan 1994). The counter-receptor for CD11/CD18 expressed by endothelial cells is the intercellular adhesion molecule-1 (ICAM-1) (Horgan, Ge et al. 1991, Ma, Lefer et al. 1992).

Once firmly adherent to the endothelium, activated leukocytes extravasate into the subendothelial matrix towards sites of inflammation or injury, where they are thought to be responsible for parenchymal injury via release of oxidants and hydrolytic enzymes. Additionally, extravasated leukocytes disrupt the microvascular barrier, increasing the permeability of intravascular proteins into the extravascular space and causing tissue edema (Gute, Ishida et al. 1998).

1.7 SYSTEMIC CYTOKINES/CHEMOKINES

Cytokines comprise a group of cell-derived secreted proteins that effect the interactions and communications between cells (Feghali and Wright 1997, Zhang and An 2007). Cytokine is a general term, and includes cytokines produced by lymphocytes (lymphokines), and monocytes (monokines). Chemokines are a subset of cytokines with chemotactic properties. Interleukins

are cytokines that are produced by leukocytes, and exert their effects on other leukocytes. Cytokines can exert their effects on the cell which secretes them (autocrine action), on nearby cells (paracrine action), or at distant sites (endocrine action) (Zhang and An 2007).

Cytokines are responsible for the make-up of the cellular infiltrate, cellular activation, and the initiation of the systemic response to inflammation. Most cytokines are multifunctional and have redundant activities. They may act synergistically or antagonistically, and can exhibit both negative and positive regulatory effects on their target cells. Cytokines bind with high affinity to cell surface receptors on their target cells, initiating a series of intracellular signal transduction pathways (Feghali and Wright 1997).

Pro-inflammatory cytokines are predominantly produced by activated macrophages, and result in the upregulation of inflammatory reactions (Zhang and An 2007). A number of cytokines have been implicated in mediating acute inflammatory reactions. Interleukin 1 (IL-1), and tumor necrosis factor alpha (TNF- α) are two of the primary cytokines that mediate acute inflammation, and have pro-inflammatory properties (Feghali and Wright 1997).

1.8 TUMOR NECROSIS FACTOR ALPHA

TNF- α is a potent pro-inflammatory cytokine released by macrophages (innate immune cells) following trauma, inflammation, or infectious stimulation (Stein and Gordon 1991). TNF- α plays a role in the regulation of cell proliferation, survival, differentiation, and apoptosis (Parameswaran and Patial 2010). TNF- α

has been found in multiple I/R studies to be acutely upregulated following the initiation of reperfusion (Coty, Guice et al. 1990, Brock, Lawlor et al. 1999, Krishnadasan, Naidu et al. 2003). TNF- α exerts its effects through two transmembrane receptors: TNF receptor 1 (TNFR1), and TNF receptor 2 (TNFR2). TNFR1 is ubiquitously expressed in most tissues, where TNFR2 is found mainly on cells of the immune system (Banner, D'Arcy et al. 1993). Activation of TNFR1 by TNF- α results in a complex series of signalling pathways, ultimately leading to altered gene transcription in the activated cell (Parameswaran and Patial 2010). TNFR1 can couple with the adaptor protein *Fas*-associated death domain (FADD), which causes progression toward apoptosis, i.e. programmed cell death (Wallach 1997, Jiang, Wang et al. 2009). In the context of acute limb ischemia, signals in the TNF- α /TNFR1 pathway have been found to be upregulated, however it was unknown whether this would induce apoptosis. In 2009, Jiang et al. induced hind limb ischemia in TNFR1 knockout mice; they found that blocking the TNF- α /TNFR1 pathway prevented the activation of death-related proteins downstream to TNF- α , and significantly decreased cell death (Jiang, Wang et al. 2009).

The role of TNFR2 is less clear; however, it has been reported to play a role in the proliferation of lymphoid cells and it appears to contribute to TNF- α -induced E-selectin, vascular adhesion molecule 1 (VCAM-1), and ICAM-1 expression (Chandrasekharan, Siemionow et al. 2007).

1.9 CELL DEATH

The activation of TNFR1 is able to signal cell death via its cytoplasmic death domain in a multitude to cell types (Wajant, Pfizenmaier et al. 2003). TNF- α has been shown to initiate cell death by either apoptosis or necroptosis (Wu, Tan et al. 2011). Apoptosis is a form of genetically programmed cell death, whereby cell death occurs without the initiation of the inflammatory pathway. It plays an essential role in embryonic development, tissue homeostasis, immune function, tumor suppression, infection resistance, and eliminating damaged cells (Reed 2000, Walsh 2014).

Briefly, apoptosis involves a highly regulated series of events which involves chromatin condensation, nuclear fragmentation, plasma blebbing, and cell shrinkage. Cellular fragments are then removed via phagocytosis without triggering the inflammatory response (Reed 2000). Caspases are a family of intracellular cysteine proteases critical to triggering apoptosis. Caspases are present in virtually all cells in their inactive forms, and must be triggered to assume their active state (Reed 2000, Walsh 2014).

Apoptosis can be triggered by activation of either the extrinsic or intrinsic pathways. The intrinsic pathway involves the release of cytochrome C from the mitochondria into the cytoplasm, which then activates a variety of caspases (Walsh 2014). The extrinsic pathway is initiated by TNF- α binding to TNFR1, whereby the cytoplasmic death domain recruits other death domain associated proteins, ultimately inducing caspase activity and apoptosis (Wang, Du et al. 2008, Wu, Tan et al. 2011).

An additional mechanism of programmed cell death is inducible via activation of TNFR1 by TNF- α . It has been termed programmed necrosis, or necroptosis, and occurs independent of caspase activity (Linkermann and Green 2014, Walsh 2014). Necroptosis ensues when there is pan-caspase inhibition, and the cell is, therefore, unable to undergo apoptosis (Linkermann and Green 2014). It is typically associated with pathological conditions including infection, ischemia-reperfusion injury, stroke, myocardial infarction, pancreatitis, Alzheimer's, and inflammatory bowel disease, (Vandenabeele, Galluzzi et al. 2010, Linkermann and Green 2014). The initiation of necroptosis by death receptors associated with TNFR1 requires the kinase activity of receptor-interacting protein 1 (RIP1), and RIP3. In contrast to apoptosis, necroptosis involves the disintegration of mitochondrial, lysosomal and plasma membranes which are then engulfed by macrophages, and elicits an immune response (Vandenabeele, Galluzzi et al. 2010). Necroptotic cell death has been associated with the tissue injury that results following I/R. We can deduce from these findings that this pathway of programmed cell death is also responsible for the tissue injury associated with CS.

1.10 AIM OF THESIS

The pathophysiology underlying the tissue injury seen in CS remains largely unknown. It has been well documented in complete I/R literature that activation of the systemic inflammatory system plays a significant role in the parenchymal damage associated with reperfusion injury. CS appears to be a low-

flow ischemic insult; therefore, we have extrapolated from this that the inflammatory response may contribute to the tissue injury associated with CS. Leukopenic animal models of CS have demonstrated a significant reduction in leukocyte activation and tissue injury associated with raised ICP (Lawendy, Bihari et al. 2015).

The aim of this thesis was, therefore, to investigate the suspected inflammatory cytokine/chemokine release in response to CS. It has been previously hypothesized that TNF- α may play a role in the microvascular dysfunction associated with CS. Therefore, we undertook the evaluation of the microvascular dysfunction, tissue injury, and inflammatory response following neutralization of TNF- α at the time of fasciotomy.

We hypothesized that a CS insult is associated with a systemic activation of the inflammatory response, and that neutralization of TNF- α would decrease the microvascular dysfunction associated with CS.

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Chapter 2

Compartment Syndrome as an Inflammatory Process

CHAPTER 2: COMPARTMENT SYNDROME AS AN INFLAMMATORY PROCESS

2.1 INTRODUCTION

Compartment syndrome (CS), one of the most devastating consequences of musculoskeletal trauma, is defined as elevated pressure within a closed osseofascial compartment (McQueen, Gaston et al. 2000). CS commonly occurs following fracture of the lower leg; approximately 6% of patients under the age of 35 with a tibial diaphyseal fracture will develop CS (McQueen, Gaston et al. 2000).

CS is a surgical emergency, and the gold standard treatment involves fasciotomy of the affected limb in order to relieve the raised intra-compartmental pressure (ICP) (Harvey, Sanders et al. 2012). Despite appropriate and early fasciotomy, complications of CS include neurologic injury, decreased function of the extremity, ischemic contractures, infection, loss of limb, and death (Giannoudis, Nicolopoulos et al. 2002, Dover, Memon et al. 2012, Fry, Wade et al. 2013).

The pathophysiology of CS is only partly understood. Current teachings propose that raised ICP compromises the microcirculation, which ultimately leads to decreased oxygen delivery, tissue anoxia, and cell death (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Rorabeck and Clarke 1978). In contrast to complete ischemia, however, CS induces myonecrosis and nerve injury despite patent blood vessels and a palpable distal pulse (Seddon 1966). CS is, therefore, considered a 'low flow' ischemic state, which results in microvascular hypo-perfusion that is insufficient to meet the metabolic demands of the tissues (Lawendy, Sanders et al. 2011). By comparison, complete ischemia, results in macro-vascular

dysfunction, with a loss of distal pulses. Complete ischemia has both a defined ischemic time and onset of reperfusion once blood flow is restored, while in 'low flow' ischemia there is no cessation of blood flow. Therefore, the true onsets of the ischemic insult, as well as the onset of reperfusion, are unknown.

The initiation of the inflammatory response has been well delineated in complete ischemia-reperfusion injury, as seen primarily in organ transplant and revascularization procedures (Forbes, Harris et al. 1996, Harris and Skalak 1996). CS falls within the spectrum of ischemia-reperfusion injury. Therefore, we have postulated that the ongoing ischemia-reperfusion injury, associated with CS, will also cause an early and concurrent initiation of the systemic inflammatory cascade.

A previous study by our group had assessed CS-induced microvascular dysfunction, tissue injury, and leukocyte activation in a leukopenic rat model (Lawendy et al. 2015). We demonstrated that leukopenia provided significant protection against CS-induced muscle injury, concurrent with a significant reduction in leukocyte activation. It did not, however, provide any protection against the microvascular dysfunction associated with CS.

We hypothesized that the injury seen as a result of CS is secondary to both ischemia and the early initiation of the inflammatory cascade, with ongoing and simultaneous reperfusion, thereby inducing the systemic inflammatory response. The purpose of our study was to examine the suspected inflammatory cytokine/chemokine release in response to CS.

2.2 METHODS

2.2.1 Animal Handling and Care

Male Wistar rats were utilized for these experiments. Animals had access to food and water *ad libitum*. Animal housing, care, and associated protocols were conducted in agreement with the Canadian Council on Animal Care. The Animal Use Subcommittee at The University of Western Ontario approved the animal protocol for this study.

2.2.2 Experimental Protocol

Five rats (body weight 180 - 250g) were anesthetized with inhalational isoflurane (5% induction, 2% maintenance, titrated as needed to maintain an adequate level of general anesthesia) in a 1:1 O₂:N₂ mixture. The left carotid artery was cannulated for continuous blood pressure monitoring, blood sampling, and fluid replacement. A rectal probe was inserted to monitor core body temperature, which was adjusted using a heat lamp, as needed. We used a normotensive and normothermic model of CS, where the mean arterial blood pressure was maintained at 100mmHg, and core body temperature at 37°C.

2.2.3 Compartment Syndrome

Isotonic saline was infused into the anterior compartment of the right hind limb via a 24-gauge catheter in order to elevate the ICP, as previously described (Lawendy, Sanders et al. 2011). The ICP was measured by an electronic compartmental pressure monitoring system (Synthes USA, Paoli, PA), which was

inserted into the posterior compartment through a 14-gauge angiocatheter (Figure 2.1). The ICP was maintained at 30-40mmHg for 120 minutes; this was followed by fasciotomy and then 45 minutes of reperfusion.

2.2.4 Blood Sampling

Blood samples were collected from the indwelling carotid line. At each sampling time, 0.3ml of blood was extracted and replaced with an equal volume of normal saline. Blood sampling was done as follows: (1) at the time of carotid artery cannulation, for baseline levels; (2) after 1hr of CS; (3) 2hrs of CS; following (4) 10-; (5) 20-; (6) 30-; and (7) 45-minutes of reperfusion. All blood samples were stored on ice during the surgical procedure and then centrifuged at 1200xg for 15min at room temperature. Serum was collected and placed into clean micro-centrifuge tubes and stored at -80°C.

2.2.5 Cytokines/Chemokines Sampled

Serum samples were diluted at 1:5 dilution, as per the manufacturer's instructions, and run on a 24-plex xMAP Luminex assay (Millipore, Billerica MA) for simultaneous testing of the following pro-inflammatory cytokines/chemokines: Tumor Necrosis Factor (TNF)- α , Interleukin (IL)-1 α , IL-1 β , IL-2, IL-5, IL-12p70, IL-13, IL-17, IL-18, Interferon Gamma (IFN- γ), Eotaxin, Interferon Gamma-Induced Protein (IP)-10, Macrophage Inflammatory Protein (MIP)-1 α , Leptin, Monocyte Chemotactic Protein (MCP)-1, Growth Regulated Oncogene/Keratinocyte-Derived Chemokine (GRO/KC), RANTES.

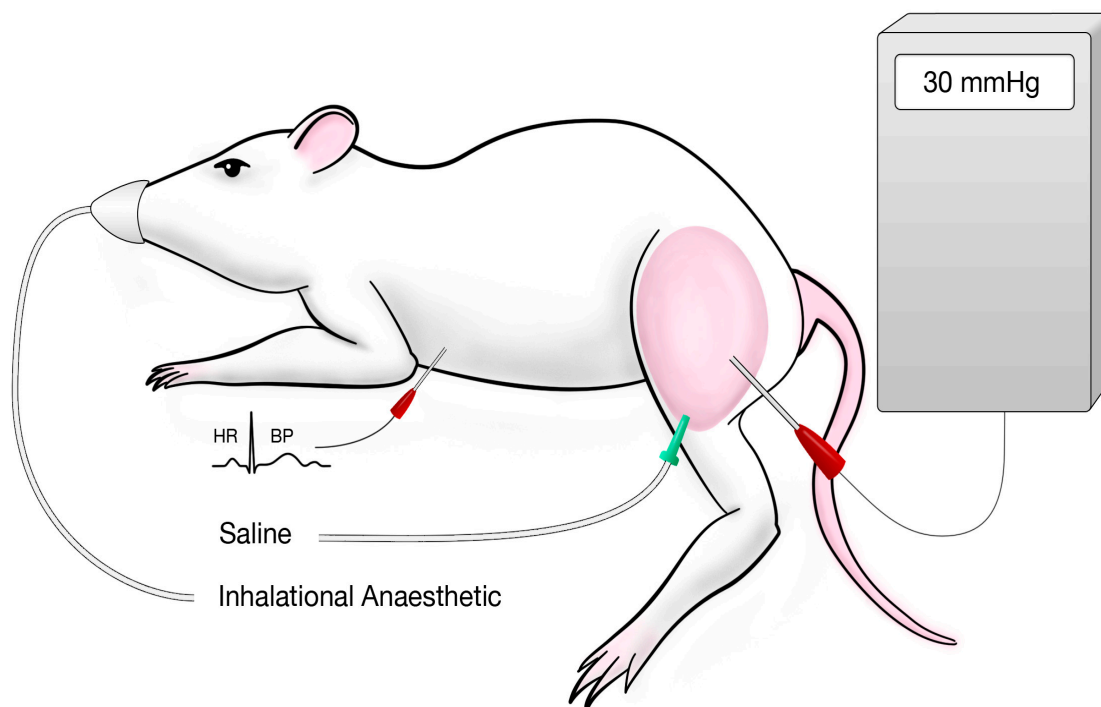


Fig 2.1. Experimental Set-Up for Rat Model of CS. Rat was anesthetized with isoflurane, and left carotid artery cannulated for blood pressure monitoring. Isotonic saline was infused into the anterior compartment to raise ICP, with continuous ICP monitoring via 14G catheter in the posterior compartment.

White blood cell growth factors Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Granulocyte-Colony Stimulating Factor (G-CSF) were tested, along with the angiogenic cytokine Vascular Endothelial Growth Factor (VEGF). Additionally, a number of anti-inflammatory cytokines were tested and included IL-4, IL-9, IL-10, as well as IL-6, which can be both pro- and anti-inflammatory.

2.2.6 Statistical Analysis

The numbers were expressed as means \pm SEM. Repeated measures one-way analysis of variance testing (ANOVA) was used to compare each sample within the time course to its baseline levels, with Newman-Keuls multiple comparison post hoc test where appropriate (GraphPad Prism V5.0, San Diego, CA). Sample size calculation was performed using StatMate (GraphPad Software Inc., San Diego, CA), with power set at 85%. Statistical significance was defined as $p < 0.05$.

2.3 RESULTS

2.3.1 Detectable Cytokines/Chemokines

Of the 24 cytokines/chemokines sampled, 14 were detectable using xMAP Luminex technology. These included TNF- α , IL-1 α , IL-1 β , IL-12p70, IL-13, IL-18, IFN- γ , Eotaxin, MIP-1 α , Leptin, MCP-1, GRO/KC, IL-10, and IL -6.

2.3.2 Significantly Elevated Cytokines/Chemokines

Six of the 14 cytokines/chemokines that were detectable were significantly elevated from their baseline levels: TNF- α significantly increased across all time points, from 32.1 ± 2.6 pg/ml at baseline to 226.3 ± 31.0 pg/ml, 335.7 ± 64.7 pg/ml, 317.7 ± 68.5 pg/ml, 338.4 ± 85.5 pg/ml, 403.3 ± 131.5 pg/ml, and 798.7 ± 370.7 pg/ml at 1hr of CS, 2hrs of CS, 10min, 20min, 30min, and 45min of reperfusion, respectively ($p<0.05$) (Figure 2.2).

IL-1 β levels were significantly elevated from the baseline of 26.7 ± 26.7 pg/ml to 97.7 ± 32.1 pg/ml, 141.3 ± 13.1 pg/ml, 102.2 ± 22.1 pg/ml, and 119.9 ± 17.6 pg/ml at 1hr of CS, 2hrs of CS, 10, and 20 minutes of reperfusion, respectively ($p<0.05$). By 30 and 45minutes of reperfusion, systemic IL-1 β levels returned to the baseline levels of 46.0 ± 46.0 pg/ml and 61.2 ± 31.7 pg/ml, respectively (Figure 2.3).

GRO/KC levels were significantly elevated from 990.2 ± 190.8 pg/ml at baseline, to 27217.0 ± 6939.0 pg/ml, 29073.0 ± 7169.0 pg/ml, 31115.0 ± 7961.0 pg/ml, 32494.0 ± 5763.0 pg/ml, and 35529.0 ± 2624.0 pg/ml at 2hrs of CS, 10, 20, 30, and 45 minutes of reperfusion, respectively ($p<0.05$). After 1hr of CS, GRO/KC level was 2985.0 ± 1172.0 pg/ml (n.s.) (Figure 2.4).

MCP-1 levels were significantly elevated from the baseline level of 313.4 ± 31.4 pg/ml to 698.8 ± 300.6 pg/ml, 803.4 ± 374.6 pg/ml, 926.0 ± 549.6 pg/ml, 849.7 ± 395.3 pg/ml, and 778.2 ± 289.4 pg/ml after 2hrs of CS, 10, 20, 30, and 45 minutes of reperfusion, respectively ($p<0.05$). After 1hr of CS, MCP-1 level was 305.0 ± 34.0 pg/ml (n.s.) (Figure 2.5).

MIP-1 α levels were significantly elevated across all time points, from 0.0 pg/ml at baseline, to 41.9 \pm 34.3pg/ml, 84.8 \pm 36.6pg/ml, 68.9 \pm 14.5pg/ml, 81.4 \pm 21.3pg/ml, 103.3 \pm 24.9pg/ml, and 131.1 \pm 29.3pg/ml after 1hr of CS, 2hrs of CS, 10, 20, 30, and 45 minutes of reperfusion, respectively (p <0.05) (Figure 2.6).

IL-10 levels were significantly elevated across all time points, from the baseline of 68.4 \pm 68.4pg/ml to 676.9 \pm 358.7pg/ml, 1138.0 \pm 372.3pg/ml, 898.4 \pm 225.8pg/ml, 591.7 \pm 225.2pg/ml, 727.0 \pm 180.5pg/ml, and 1023.0 \pm 97.3pg/ml after 1hr of CS, 2hrs of CS, 10, 20, 30, and 45 minutes of reperfusion, respectively (p <0.05) (Fig 2.7).

2.4 DISCUSSION

CS, a complication of muscle trauma, poses a particular challenge. The pathophysiological mechanisms of CS are complex and not fully understood. The purpose of this study was to determine if the low-flow ischemia associated with CS would result in the systemic activation of the inflammatory response.

2.4.1 Systemic Inflammatory Response

Acute inflammation is characterized by the activation of circulating leukocytes, whereby activation results in leukocyte rolling and adhesion. Activated leukocytes produce reactive oxygen species, and release cytokines and chemokines, as a consequence of the reintroduction of oxygen into ischemic tissue (Harkin, Barros D'sa A et al. 2001). The generation of reactive oxygen species ultimately triggers cellular apoptosis and necrosis (Schlag, Harris et al. 2001).

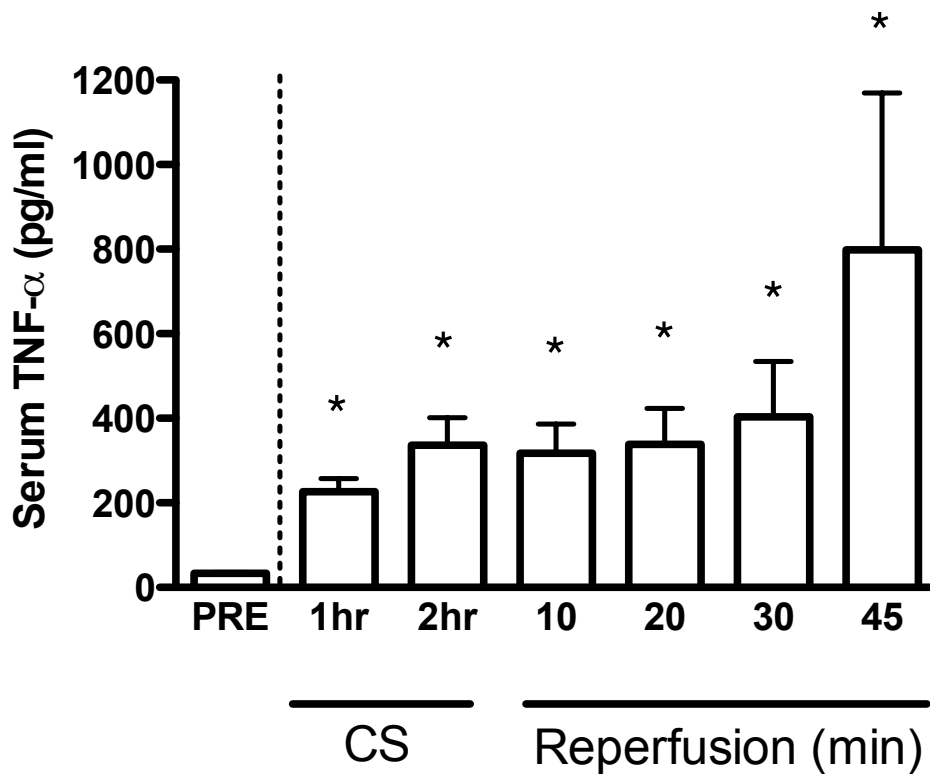


Fig 2.2. Time Course of TNF- α Expression in Rat Model of CS. CS induced a significant increase in systemic TNF- α after 1hr and 2hrs of raised ICP, and at each time point measured during reperfusion. Peak TNF- α levels were seen after 45-minutes of reperfusion with a 25-fold increase from baseline (* p <0.05 from baseline, repeated measures one-way ANOVA).

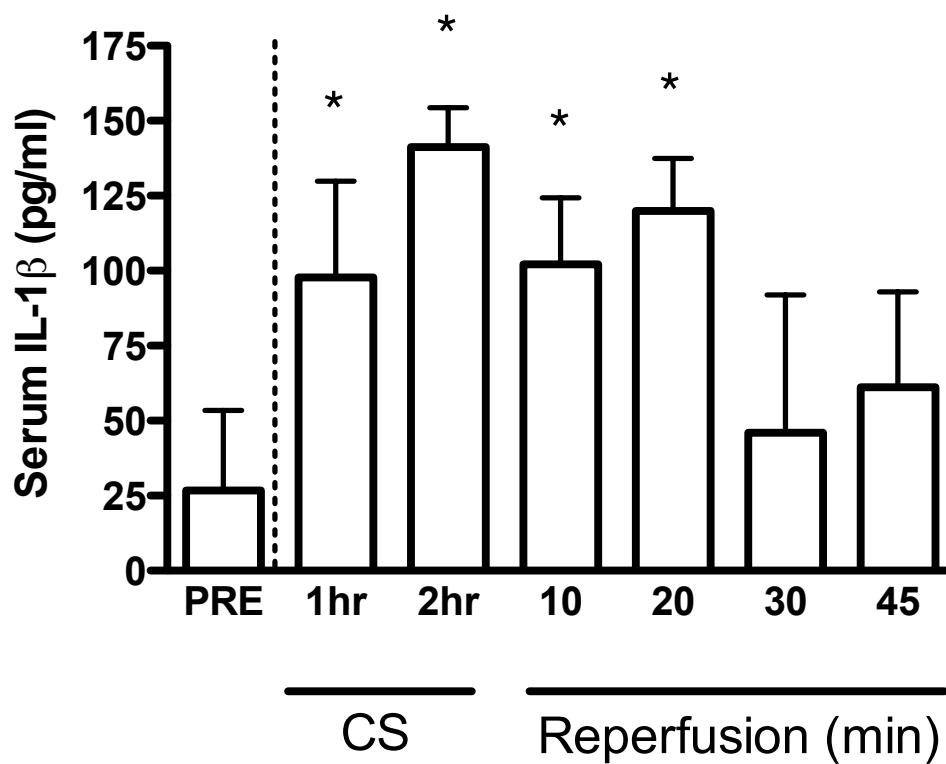


Fig 2.3. Time Course of IL-1 β Expression in Rat Model of CS. CS induced a significant increase in systemic IL-1 β after 1hr and 2hrs of raised ICP, and through 20 minutes of reperfusion. Levels were no longer significantly elevated from baseline after 30 or 45 minutes of reperfusion (* p <0.05, repeated measures one-way ANOVA).

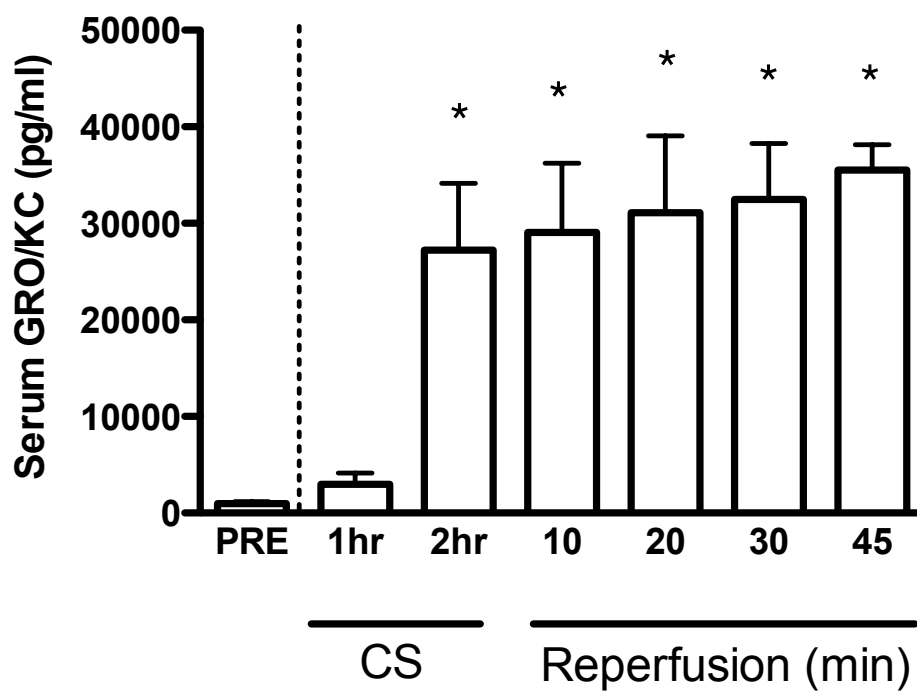


Fig 2.4. Time Course of GRO/KC Expression in Rat Model of CS. CS induced a significant increase in systemic GRO/KC after 2hrs of raised ICP, and at each time point measured throughout reperfusion (* $p < 0.05$, repeated measures one-way ANOVA).

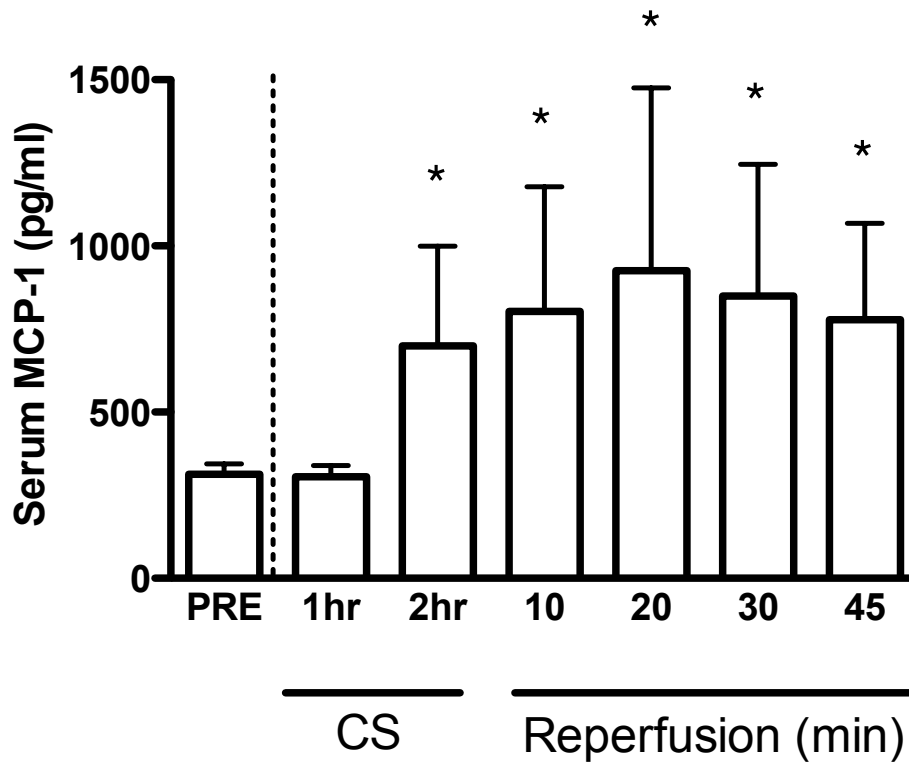


Fig 2.5. Time Course of MCP-1 Expression in Rat Model of CS. CS induced a significant increase in systemic MCP-1 after 2hrs of raised ICP, and at each time point measured throughout the reperfusion period (* $p < 0.05$, repeated measures one-way ANOVA).

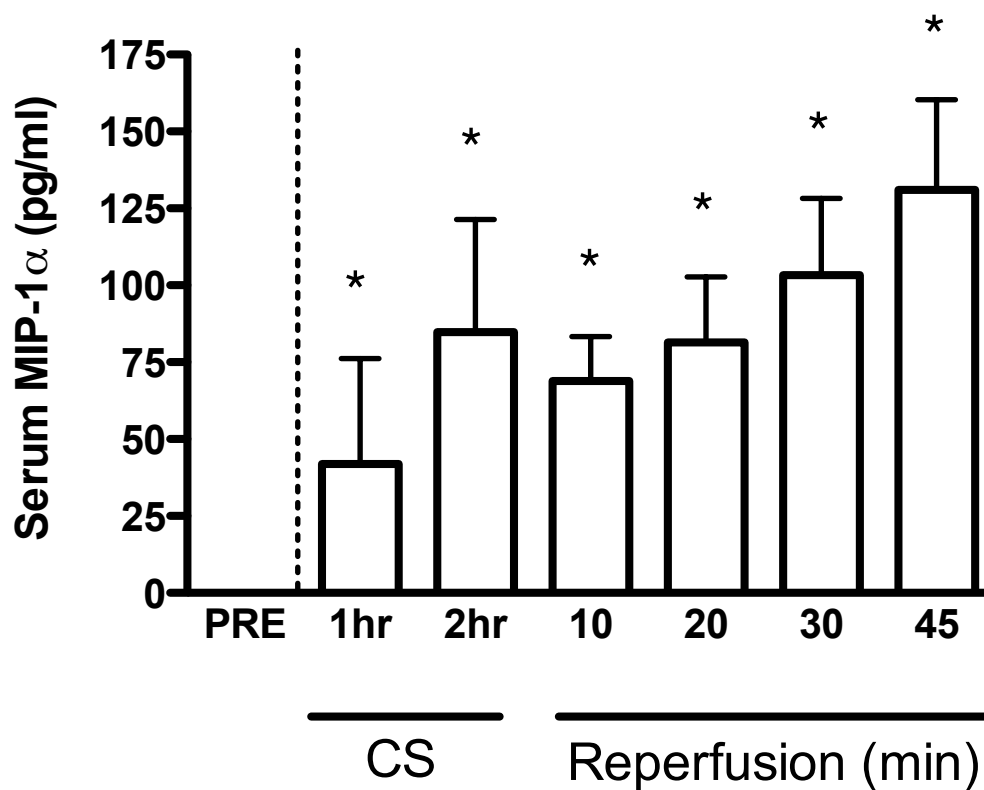


Fig 2.6. Time Course of MIP-1 α Expression in Rat Model of CS. CS induced a significant increase in systemic MIP-1 α after 1hr of raised ICP, and at each time point measured throughout the 45 minutes reperfusion period (* $p < 0.05$, repeated measures one-way ANOVA).

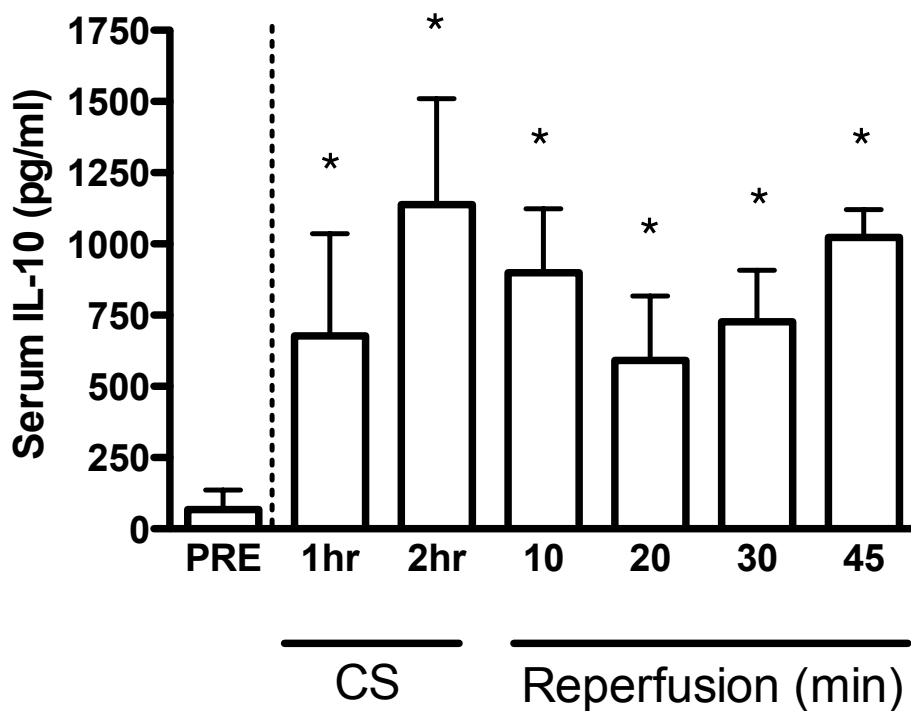


Fig 2.7. Time Course of IL-10 Expression in Rat Model of CS. CS induced a significant increase in systemic IL-10 after 1hr of CS, and at each time point measured throughout the 45-minute reperfusion period (* $p < 0.05$, repeated measures one-way ANOVA).

It has been well documented that revascularization following complete ischemia results in the initiation of acute inflammation (Gute, Ishida et al. 1998, Lum and Roebuck 2001, Schlag, Harris et al. 2001). Using a model of complete hind limb ischemia, animals rendered neutropenic prior to the onset of ischemia had a delay in the onset of muscle injury, and a decrease in the magnitude of injury seen (Forbes, Harris et al. 1996). Additionally, an earlier and more rapid onset of leukocyte activation was shown in a rodent model of partial ischemia when compared to complete ischemia (Heppenstall, Scott et al. 1986, Forbes, Harris et al. 1996, Lakshminarayanan, Lewallen et al. 2001).

2.4.2 Systemic Cytokine/Chemokine Release

Cytokines are intercellular signaling peptides released from stimulated leukocytes, which induce the production of other cytokines and cell surface receptors through their effects on gene transcription (Cannon 2000, Grivennikov, Kuprash et al. 2006).

Chemokines are a family of chemotactic cytokines, which recruit monocytes, neutrophils, and lymphocytes to sites of injury and inflammation. Chemotaxis is induced through the activation of G-protein-coupled receptors, and the receptors that a given leukocyte expresses usually determines the chemokines to which it will respond (Charo and Taubman 2004). Chemokines are secreted by a variety of cell types, including leukocytes, fibroblasts, and endothelial cells (Roebuck, Carpenter et al. 1999).

While an increasing amount of literature exists to suggest that the initiation of the systemic inflammatory response plays a role in the myonecrosis associated with CS, no study to date has directly measured the cytokine/chemokine release induced by CS. A normothermic and normotensive model of CS was used in order to remove the possibility of a systemic inflammatory response secondary to hypoperfusion and shock.

Our study has confirmed that CS induces a systemic cytokine/chemokine release. Of the 24 cytokines/chemokines measured, 14 were detectable. Of the 14 detected, 12 were pro-inflammatory, 1 was anti-inflammatory, and 1 had both a pro- and an anti-inflammatory effect.

Six of the 14 cytokines/chemokines that were detectable were significantly elevated from their baseline levels after 2hrs of CS. These include TNF- α , IL-1 β , GRO/KC, MCP-1, MIP-1 α , and IL-10.

TNF- α is a pro-inflammatory cytokine, and one of the first cytokines released with the initiation of inflammation. It serves as a chemoattractant for neutrophils, up-regulation of downstream cytokine/chemokine production, and it promotes the expression of adhesion molecules (Ascer, Gennaro et al. 1992, Yi and Ulich 1992, Seekamp, Warren et al. 1993, Krishnadasan, Naidu et al. 2003, Zhang, Hu et al. 2005).

IL-1 β is a pro-inflammatory cytokine, and it is produced by activated macrophages. It is involved in multiple cellular functions including cell proliferation, differentiation, and apoptosis (Gao, Madi et al. 2014).

GRO/KC is a pro-inflammatory cytokine, and it is produced by macrophages and neutrophils. It serves to induce neutrophil chemotaxis. It is also seen in high levels in chronic inflammatory states such as chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (Bechara, Chai et al. 2007).

MCP-1 is a pro-inflammatory cytokine whose primary role is to recruit monocytes/macrophages to sites of injury (Shireman, Contreras-Shannon et al. 2007). MCP-1 is secreted by macrophages that have been induced by pro-inflammatory cytokines including TNF- α and IL-1 β , and has been shown to play a role in angiogenesis following an ischemic stress (Lakshminarayanan, Lewallen et al. 2001). Additionally, MCP-1 exerts its effects by binding to leukocytes, and targeting them for activation (Yadav, Saini et al. 2010).

MIP-1 α is a pro-inflammatory cytokine and is produced by macrophages, lymphocytes, and dendritic cells. It is chemotactic for cells of the monocyte lineage as well as for lymphocytes. Additionally it appears to inhibit the proliferation of hematopoietic stem cells (Cook 1996).

IL-10 is an anti-inflammatory cytokine, and is produced by monocytes, macrophages, dendritic cells, and lymphocytes. It is an inhibitor of antigen presentation, and prevents the differentiation of monocyte precursors, and the maturation of dendritic cells (Mosser and Zhang 2008).

The results of our study have confirmed that CS induces a substantial, and predominantly pro-inflammatory response. The initiation of the systemic inflammatory response should, therefore, be considered in the pathophysiology of CS. The systemic inflammatory response is complex and redundant therefore, the

exact mechanism of initiation and the interplay between cytokines and chemokines in CS needs to be further delineated.

To our knowledge, this is the first study that directly confirms CS as an inflammatory process. The results are the beginning step, in order to extrapolate the mechanisms by which injury occurs. The identification of specific mediators could potentially serve as pharmacologic targets in future studies in an attempt to decrease the devastating consequences of CS.

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Chapter 3

Systemic TNF- α Release Contributes to Microvascular Dysfunction and Tissue Injury in Compartment Syndrome

CHAPTER 3: SYSTEMIC TNF- α RELEASE CONTRIBUTES TO
MICROVASCULAR DYSFUNCTION AND TISSUE INJURY IN
COMPARTMENT SYNDROME

3.1 INTRODUCTION

There are few more devastating complications of musculoskeletal trauma than compartment syndrome (CS). CS is caused by increased pressure within a closed osseofascial compartment, the most common etiology being fracture of the lower extremity (Matsen 1975, Whitesides, Haney et al. 1975, Whitesides, Haney et al. 1975, Mubarak, Owen et al. 1978, Rorabeck and Clarke 1978, Matsen, Winkquist et al. 1980, Rorabeck 1984, Tornetta and Templeman 1997). Increased intra-compartmental pressure (ICP) leads to microvascular dysfunction, which results in limited oxygen and nutrient delivery to the tissues. Cellular anoxia eventually leads to myonecrosis, the sequelae of which include permanent functional impairment, loss of limb, and even death (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Heckman, Whitesides et al. 1994, Fry, Wade et al. 2013).

In contrast to complete ischemia, CS causes myonecrosis despite vessels being patent: it has been demonstrated that ischemic changes occur even in the presence of a distal pulse (Seddon 1966). This clinical paradox suggests that the pathophysiology of CS is more complex than previously thought.

The use of intravital video microscopy (IVVM) in animal models of complete limb ischemia and reperfusion (I/R) have shown that activated leukocytes directly impair capillary perfusion, and contribute to the parenchymal injury occurring with the onset of reperfusion (Forbes, Carson et al. 1995, Forbes, Harris et al. 1996, Harris and Skalak 1996). Previous studies by our group have shown a dramatic increase in leukocyte activation in the post-capillary venules of skeletal muscle following a CS insult (Lawendy, Sanders et al. 2011). It has been extrapolated from this data that CS appears to induce a low-flow ischemic state, and due to the patent macro-circulatory system there is, therefore, an ongoing low-flow ischemia and reperfusion injury occurring.

The initiation of the inflammatory pathway has been well described in the I/R literature, whereby activated leukocytes impair capillary perfusion and also result in parenchymal damage following reperfusion (Forbes, Carson et al. 1995, Forbes, Harris et al. 1996, Harris and Skalak 1996). Within the first hour of reperfusion, a peak in the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF- α) has been reported, and it is believed to be a driving force of inflammation following a complete ischemic insult (Caty, Guice et al. 1990, Brock, Lawlor et al. 1999, Krishnadasan, Naidu et al. 2003). TNF- α appears to act as a chemoattractant for neutrophils, causes up-regulation of downstream cytokine/chemokine production, and promotes the expression of adhesion molecules (Ascer, Gennaro et al. 1992, Yi and Ulich 1992, Seekamp, Warren et al. 1993, Krishnadasan, Naidu et al. 2003, Zhang, Hu et al. 2005).

It has been hypothesized that TNF- α may act as an important driving force of CS pathophysiology. Therefore, the purpose of this study was to evaluate the microvascular dysfunction, tissue injury, and inflammatory response following the neutralization of TNF- α at the time of fasciotomy, in an animal model of CS.

3.2 METHODS

3.2.1 Animal Handling and Care

Male Wistar rats were utilized for these experiments. Animals had access to food and water *ad libitum*. Animal housing, care, and associated protocols were conducted in agreement with the Canadian Council on Animal Care. The animal protocol for this study was approved by the Animal Use Subcommittee at The University of Western Ontario.

3.2.2 Experimental Protocol

A total of twelve male Wistar rats (body weight 180 - 250g) were randomly assigned into three groups: (1) sham (n= 4), (2) CS (with isotype control) (n=4), and (3) CS with TNF- α neutralization (CS+TNF- α) (n=4). Animals were anesthetized with inhalational isoflurane (5% induction, 2% maintenance, titrated as needed to maintain an adequate level of general anesthesia) in a 1:1 O₂:N₂ mixture. The left carotid artery was cannulated for continuous blood pressure monitoring, blood sampling, and fluid replacement. A rectal probe was inserted to monitor core body temperature, which was adjusted using a heat lamp, as needed. A normotensive and normothermic model of CS was used, where the

mean arterial blood pressure was maintained at 100mmHg, and core body temperature at 37°C.

3.2.3 Compartment Syndrome

Isotonic saline was infused into the anterior compartment of the right hind limb via a 24-gauge catheter in order to elevate the ICP, as previously described (Lawendy, Sanders et al. 2011). The ICP was measured by an electronic compartmental pressure monitoring system (Synthes USA, Paoli, PA), which was inserted through a 14-gauge angiocatheter into the posterior compartment (Figure 2.1). The ICP was maintained at 30-40mmHg for 2 hours; this was followed by fasciotomy and 45 minutes of reperfusion.

Animals that underwent CS followed by isotype control for TNF- α were administered 0.3ml Armenian hamster IgG (3.5 μ g of IgG per 200g body weight, IV, eBioscience, SanDiego, CA) at the time of fasciotomy, while those in the CS+TNF- α neutralization group were administered 0.3ml TNF- α neutralizing antibody (3.5 μ g TNF- α neutralizing antibody per 200g body weight, IV, eBioscience, SanDiego, CA) at the time of fasciotomy.

Sham animals underwent the same procedure as those in the CS groups; however the ICP was kept at the baseline level of 0mm Hg for 2 hours, followed by fasciotomy and 45 minutes of reperfusion.

Intravital video microscopy (IVVM) was then performed on all animals to assess microvascular dysfunction, leukocyte activation and the parenchymal injury to the extensor digitorum longus (EDL) muscle.

3.2.4 Blood Sampling

Blood samples were collected via an indwelling carotid line. At each sampling time, approximately 0.3ml of blood was extracted and replaced with an equal volume of normal saline. Blood sampling was done as follows: (1) at the time of carotid artery cannulation, for baseline levels; (2) after 2 hours of CS, just prior to fasciotomy/antibody injection; and (3) following 45-minutes of reperfusion. All blood samples were allowed to coagulate on ice during the surgical procedure and then centrifuged at 1200xg for 15min at room temperature. Serum was collected and placed into clean micro-centrifuge tubes and stored at -80°C.

3.2.5 Intravital Video Microscopy (IVVM)

Following fasciotomy, the EDL muscle was prepared for IVVM, as previously described (Potter, Dietrich et al. 1993, Forbes, Carson et al. 1995, Manjoo, Sanders et al. 2010, Lawendy, Sanders et al. 2011). The animal was then transferred onto the stage of an inverted microscope (Nikon, Nikon Instruments, Inc., Mississauga, Ontario, Canada) and the EDL muscle was reflected into a saline bath containing 5µg/ml each of the fluorescent vital dyes bisbenzimidazole (BB; excitation wavelength (Ex) 343 nm, emission wavelength (Em) 483 nm) and ethidium bromide (EB; Ex 482 nm, Em 616 nm). BB stains nuclei of all cells while EB stains the nuclei of only those cells with damaged cell membranes. The EB/BB ratio, therefore, provides a measure of tissue injury.

Microvascular perfusion and leukocytes within the post capillary venules were recorded by transillumination with 20x and 40x objectives respectively, in

five adjacent fields of view, and captured into a computer for offline video analysis. Fluorescence microscopy was used to visualize the BB- and EB-labelled cells from these same fields of view. At the conclusion of the experiment, the rats were euthanized by an overdose of anesthetic agent.

3.2.6 Offline Video Analysis

Capillary perfusion was assessed by counting the number of continuously perfused (CPC), intermittently perfused (IPC), and non-perfused (NPC) capillaries crossing three parallel lines drawn perpendicular to the capillary axis on the video monitor, and was expressed as a percentage (%) of total capillaries.

Tissue injury was assessed by counting the number of EB- and BB-labelled nuclei, and expressed as EB/BB ratio.

Leukocyte activation was assessed by counting the number of rolling and adherent leukocytes in post-capillary venules, and expressed as cells per unit area (i.e. $1000 \mu\text{m}^2$). Venular area was measured using ImageJ software (NIH, Bethesda, Maryland). A leukocyte was considered adherent if it remained stationary for at least 30 seconds and rolling if it remained in contact with the wall of the vessel but did not remain stationary.

3.2.7 Measurement of Serum TNF- α

To evaluate the effect of CS on TNF- α production, and to confirm TNF- α neutralisation, serum levels of TNF- α were assessed in all animals using enzyme-linked immunosorbent assay (ELISA) kit (Pierce Biotechnology, c/o

Thermo Scientific, Rockford, IL), as per manufacturer's instructions. The assay was sensitive to less than 5pg/ml.

3.2.8 Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the degree of perfusion, tissue injury and leukocyte activation (both adherence and rolling). Repeated measures two-way ANOVA was used to compare the serum levels of TNF- α . Newman-Keuls multiple comparison post hoc test was used where appropriate (GraphPad Prism V5.0, San Diego, CA). Sample size calculation was performed using StatMate (GraphPad Software Inc., San Diego, CA), with power set at 85%. Statistical significance was defined as a p-value <0.05.

3.3 RESULTS

3.3.1 Microvascular Perfusion

The perfusion profile seen in the sham group demonstrated predominately continuously perfused capillaries. The perfusion profile of both the CS and the CS+TNF- α neutralizing groups demonstrated a shift toward IPC and NPC (Figure 3.1).

The number of CPC decreased from $75.08 \pm 2.3\%$ in the sham group to $30.66 \pm 3.6\%$, and $35.7 \pm 3.5\%$ in the CS and CS+TNF- α neutralizing groups, respectively ($p < 0.0001$). There was no significant difference in CPC between the CS and CS+TNF- α neutralizing groups.

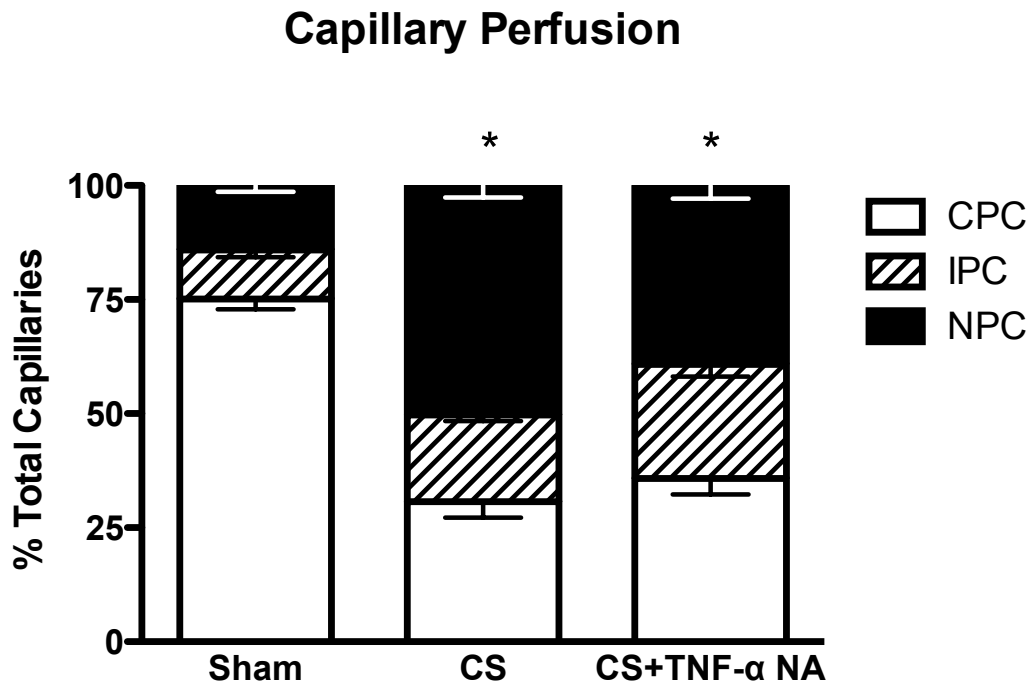


Figure 3.1. The Effect of Elevated ICP on Microvascular Perfusion in Rat Model of CS. CS resulted in a significant microvascular perfusion deficit. Neutralization of TNF- α at fasciotomy did not restore the microvascular perfusion (* $p < 0.05$ from Sham; one-way ANOVA). *CPC*, continuously-perfused capillaries; *IPC*, intermittently-perfused capillaries; *NPC*, non-perfused capillaries.

The number of IPC increased from $10.76 \pm 1.5\%$ in sham to $19.08 \pm 1.3\%$, and $25.09 \pm 2.7\%$ in the CS and CS+TNF- α neutralizing groups, respectively ($p < 0.001$). The number of IPC in the CS+TNF- α neutralizing group was significantly higher than that in CS group ($p < 0.05$).

The number of NPC increased from $14.18 \pm 1.4\%$ in sham to $50.27 \pm 2.6\%$, and $39.19 \pm 2.8\%$ in the CS and CS+TNF- α neutralizing groups, respectively ($p < 0.0001$). The number of NPC in the CS+TNF- α neutralizing group was significantly lower as compared to the CS group ($p < 0.05$).

3.3.2 Tissue Injury

Tissue injury significantly increased following elevation of ICP, from 0.04 ± 0.02 in sham to 0.33 ± 0.04 in CS group ($p < 0.001$) (Figure 3.2). Administration of TNF- α neutralizing antibody resulted in a significant decrease when compared to CS group, to 0.21 ± 0.04 ($p < 0.05$).

3.3.3 Leukocyte Activation

Elevation of ICP resulted in a significant increase in leukocyte adherence, from 1.7 ± 0.4 leukocytes/30s/1000 μm^2 to 14.1 ± 1.6 leukocytes/30s/1000 μm^2 in CS group ($p < 0.05$) (Figure 3.3). Administration of TNF- α neutralizing antibody produced a significant decrease to 0.9 ± 0.2 adherent leukocytes/30s/1000 μm^2 ($p < 0.05$).

Elevation of ICP resulted in an increase in leukocyte rolling, from 2.1 ± 0.8 leukocytes/30s/1000 μm^2 in sham to 9.8 ± 3.2 leukocytes/30s/1000 μm^2 in CS

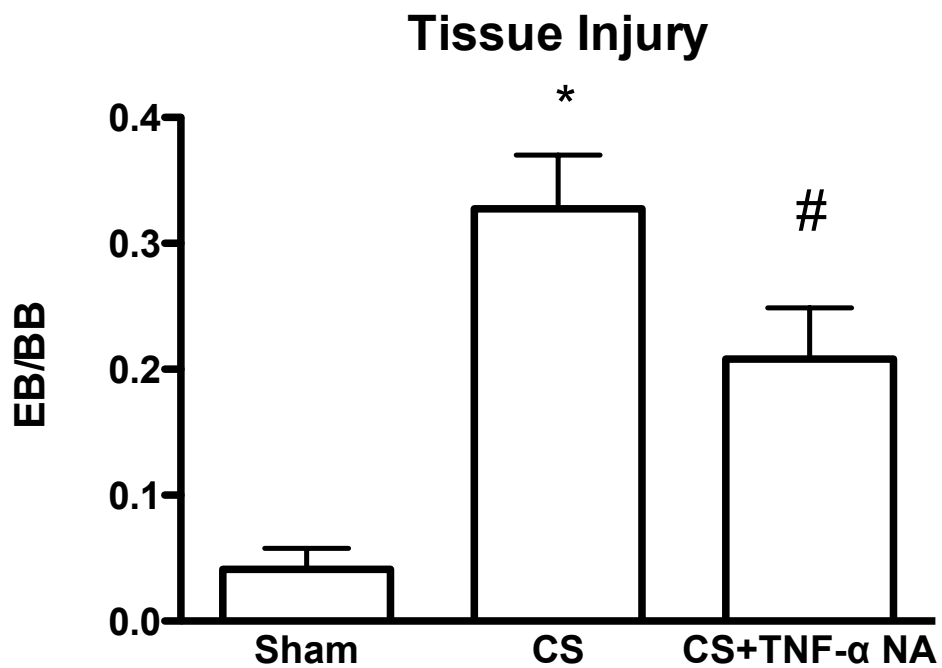


Figure 3.2. Tissue Injury In a Rat Model of CS. Elevation of ICP resulted in a significant increase in tissue injury. Administration of a TNF- α neutralizing antibody at fasciotomy significantly diminished tissue injury (* $p < 0.05$ from Sham, # $p < 0.05$ from CS; one-way ANOVA).

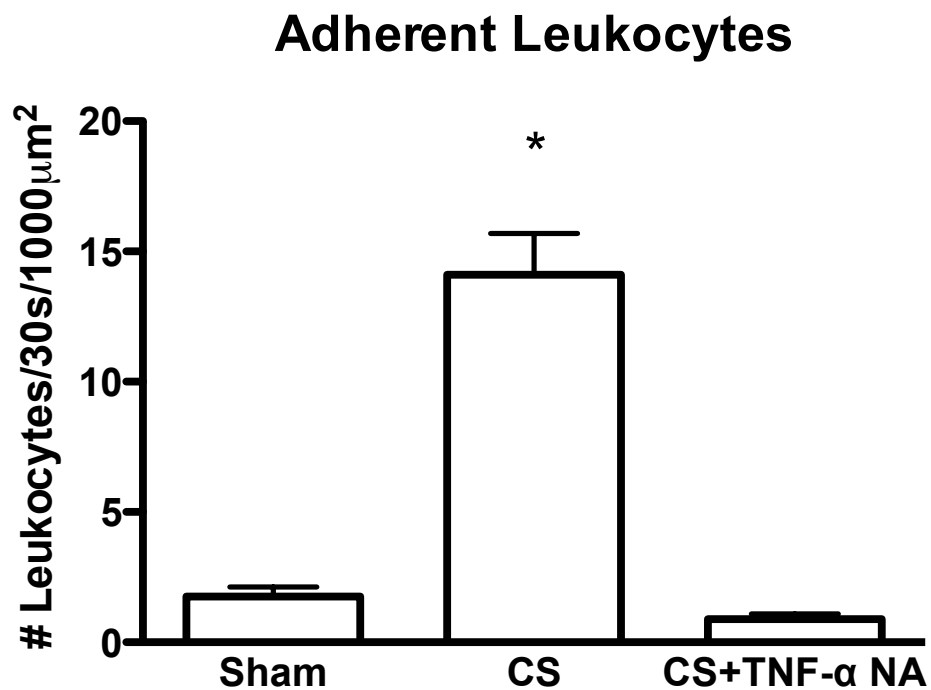


Figure 3.3 Leukocyte Activation (Adhesion) in a Rat Model of CS.

Elevation of ICP resulted in an increase in adherent leukocytes.

Administration of TNF- α neutralizing antibody at fasciotomy blocked

leukocyte adhesion (* $p < 0.0001$ from Sham; one-way ANOVA).

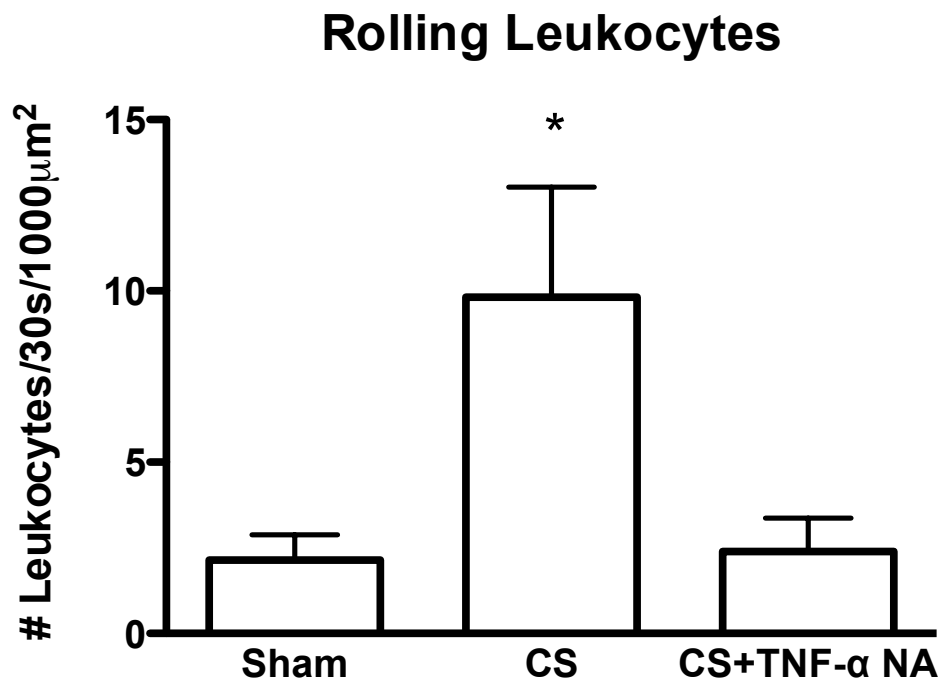


Figure 3.4 Leukocyte Activation (Rolling) in a Rat Model of CS. Elevation of ICP resulted in a significant increase in leukocyte rolling. Administration of TNF- α neutralizing antibody at fasciotomy resulted in leukocyte rolling returning to the baseline level (* p <0.0001 from Sham; one-way ANOVA).

group ($p < 0.05$) (Figure 3.4). Administration of TNF- α neutralizing antibody resulted in a significant decrease to 2.4 ± 1.0 leukocytes/30s/1000 μm^2 ($p < 0.05$).

3.3.4 Serum TNF- α

Serum TNF- α levels in the sham animals showed no significant changes from the baseline level of 12.1 ± 6.9 pg/ml: serum levels of TNF- α at 2hr CS and 45min reperfusion were 14.8 ± 3.8 pg/ml, and 12.6 ± 5.9 pg/ml respectively ($p = 0.357$, n.s.) (Figure 3.5).

Elevation of ICP resulted in a significant increase in serum TNF- α , from 16.1 ± 4.8 pg/ml at baseline to 1496.0 ± 152.6 pg/ml just prior to fasciotomy ($p < 0.05$) (Figure 3.5). TNF- α level remained significantly elevated, at 2391.0 ± 445.7 pg/ml after 45 minutes of reperfusion ($p < 0.05$). Administration of TNF- α neutralizing antibody at fasciotomy resulted in a complete inhibition of TNF- α , to 0 pg/ml ($p < 0.05$).

3.4 DISCUSSION

The underlying pathophysiological mechanism of the tissue injury associated with acute compartment syndrome remains largely unknown. Previous work by our group, and others, has begun to recognize the role of the inflammatory pathway in microvascular compromise, and tissue injury, seen in CS (Manjoo, Sanders et al. 2010, Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). The immune system is amenable to modulation, and, therefore,

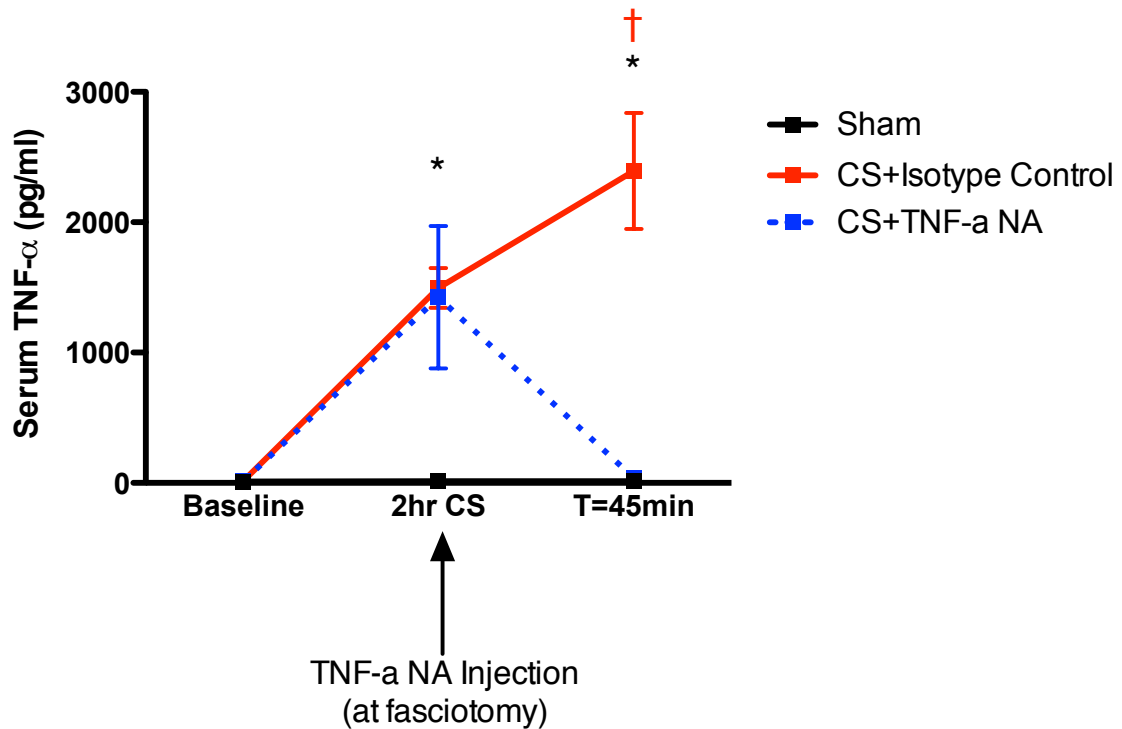


Figure 3.5. Serum TNF- α Levels in a Rat Model of CS. Elevation of ICP resulted in a significant increase in systemic TNF- α , which continued to rise during the 45-minute reperfusion period (red line). Injection of TNF- α neutralizing antibody at fasciotomy resulted in a complete inhibition of serum TNF- α levels (blue dotted line). Systemic TNF- α levels remained constant in sham animals (black line) (*p<0.05 from baseline, †p<0.05 from CS+TNF- α group; repeated measures two-way ANOVA).

potentially represents a therapeutic target in order to decrease the tissue injury associated with CS. We chose to target TNF- α as it has been shown to be acutely elevated in complete I/R injury (Coty, Guice et al. 1990, Brock, Lawlor et al. 1999).

Microvascular perfusion, under normal physiological conditions, is primarily that of continuous blood flow. CS causes a shift in perfusion toward intermittently perfused and non-perfused capillaries. An ischemic environment within the affected capillary bed ensues, resulting in decreased nutrient and gas exchange. There remains, however, continuous perfusion within a small portion of the capillaries, creating a low-flow ischemic environment in contrast to a complete ischemic state.

The neutralization of TNF- α essentially had no effect on the microvascular perfusion profile seen in CS (Figure 3.1). Tempering the inflammatory response did not, therefore, provide protection in restoring microvascular perfusion in the face of elevated compartment pressures. These results are in keeping with the previous work, whereby a leukocyte-deplete animal model was not shown to be protective in restoring the microvascular perfusion following a CS insult (Lawendy, Bihari et al. 2015). A distinction with complete I/R must again be highlighted: microvascular dysfunction was prevented, and parenchymal injury was reduced in a leukocyte deplete I/R model (Forbes, Harris et al. 1996). This data further corroborates that the microvascular dysfunction associated with compartment syndrome has a distinct pathophysiology compared to that of complete ischemia-reperfusion.

The results of our study confirm that CS induces a systemic inflammatory response. Elevation of ICP for 2 hours resulted in a significant increase in serum TNF- α , coupled with the recruitment of activated leukocytes, and arrest of leukocytes, observed under live microscopy (Figure 3.5). Blocking the inflammatory response post-fasciotomy, through the administration of TNF- α neutralizing antibody, significantly decreased leukocyte activation, as evidenced by a decrease in both rolling and adherent leukocytes within the post-capillary venules ($p < 0.05$ and $p < 0.0001$ respectively) (Figure 3.3 and Figure 3.4). Activated leukocytes are known to produce reactive oxygen species and proteolytic enzymes; this results in an increased vascular permeability and tissue edema, cellular injury, and raised interstitial pressure. Combined, the effects may exacerbate perfusion dysfunction in the microvascular bed.

In this study, elevation of ICP resulted in a significant increase in the number of EB-labelled nuclei (i.e. tissue injury). Neutralization of TNF- α led to a significant relative reduction of approximately 36% in tissue injury (Figure 3.2). This data confirms that TNF- α plays at least some role in the inflammatory response observed in a CS insult. In a leukocyte-deplete animal model of CS, tissue injury was reduced by more than 50%, implying that there are also other cytokines/chemokines involved in the pathophysiology of CS (Lawendy, Bihari et al. 2015).

Leukocyte activation within the post capillary venules appears to play a role in producing the tissue injury observed in CS, without directly affecting the perfusion profile of the microvascular beds. In the early phase of CS, at least,

TNF- α plays a role in the tissue injury associated with CS. Neutralizing TNF- α in isolation had less of an effect on reducing tissue injury in comparison to a completely leukocyte deplete animal model, again suggesting a role for also other cytokines/chemokines in the pathophysiology of CS (Lawendy, Bihari et al. 2015).

The study validates immune modulation as a potential therapeutic target in order to diminish the parenchymal injury associated with CS. While TNF- α is only one of many cytokines/chemokines in a complex and redundant system, its neutralization did result in a significant decrease in tissue injury. Continued investigation is still required, as this is only one small piece of a complex pathophysiological mechanism.

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Chapter 4

General Discussion and Conclusions.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1 OVERVIEW OF RESULTS

4.1.1 Pathophysiology of Compartment Syndrome

Compartment syndrome (CS) remains one of the most devastating complications of musculoskeletal trauma. It occurs secondary to elevated pressure within a closed osseofascial compartment, and is both a limb and life-threatening condition (McQueen, Gaston et al. 2000, Hayakawa, Aldington et al. 2009). Despite being a recognized complication of trauma since Richard von Volkmann's description of ischemic contracture in the upper limb of children in 1881, to date the underlying pathophysiological mechanism remains largely unknown (von Volkmann 1881).

Raised intra-compartmental pressure (ICP) is the initial insult seen in CS, which then leads to microvascular dysfunction, hypo-perfusion of the tissues, and ultimately cellular anoxia and cell death (Sheridan and Matsen 1975, Whitesides, Haney et al. 1975, Rorabeck and Clarke 1978, Harvey, Sanders et al. 2012). There are a few important distinctions that must be drawn between CS and complete ischemia-reperfusion (I/R) injury: CS causes tissue necrosis despite a patent macrocirculatory system, where ischemic tissue injury occurs in the face of a palpable distal pulse (Seddon 1966), and a CS insult is more injurious than a complete ischemic insult of the same duration (Heppenstall, Scott et al. 1986). These findings suggest that the pathophysiology of CS involves a low-flow

ischemic state, is more complex than previously understood, and is distinct from that of complete I/R injury.

4.1.2 Compartment Syndrome as *Low-Flow Ischemia*

Under normal conditions, microvascular perfusion exhibits predominantly continuously perfused capillaries (CPC). Raised ICP has been shown to cause a shift in perfusion toward intermittently perfused capillaries (IPC), and non-perfused capillaries (NPC) (Lawendy, Sanders et al. 2011, Lawendy, Bihari et al. 2015). The presence of CPC in the same muscle bed as IPC and NPC suggests that CS induces a low-flow ischemic state, as there is some degree of continuous perfusion still present. Ischemia ensues as a consequence of inefficient nutrient and gas exchange to meet the metabolic demands of the tissue, despite some continuous oxygen delivery (Lawendy, Sanders et al. 2011). We believe that this ongoing perfusion in the face of an essentially ischemic tissue bed is analogous to the reperfusion injury associated with complete I/R injury. The presence of oxygen in ischemic tissue results in the generation of reactive oxygen metabolites, the initiation of an acute inflammatory state, and triggers cellular death via apoptosis and necroptosis (Gute, Ishida et al. 1998, Lum and Roebuck 2001). This pattern of tissue injury is, however, distinct from that of complete I/R, which has defined phases of injury (ischemia, then reperfusion). We believe the low-flow ischemic state associated with CS initiates an early and continuous reperfusion injury, which not only persists for the duration of the raised ICP, but is exacerbated by fasciotomy and the restitution of blood flow to the capillary beds.

Heppenstall *et al* (1986) have demonstrated, in a canine model, that a CS insult is more injurious than a complete ischemic insult of the same duration, further corroborating our theory that CS includes an early and continuous state of reperfusion injury.

The first aim of this thesis was to confirm that CS induces the systemic inflammatory response, and to examine the specific cytokines/chemokines released as a result. A further focus of this thesis was to evaluate the microvascular dysfunction, tissue injury, and inflammatory response following the neutralization of TNF- α at the time of fasciotomy. The background for these studies has been primarily extrapolated from I/R literature and, more recently, from studies by our group; thus the role of inflammation in CS-induced tissue injury is beginning to be recognized.

4.1.3 Compartment Syndrome as an Inflammatory Process

The study was designed to confirm that CS is associated with the initiation of a systemic inflammatory response, and to examine the suspected inflammatory cytokine/chemokine release in response to CS. The initiation of the inflammatory response has been well described following complete I/R injury, however, to our knowledge, had not been studied to-date in CS. The use of IVVM imaging of the capillaries following CS has demonstrated significant microvascular dysfunction, concurrent with a significant increase in leukocyte activation in the post-capillary venules of skeletal muscle (Lawendy, Bihari et al. 2015). These findings suggested that CS induces a pro-inflammatory

environment; however, the specific cytokines/chemokines involved remained unknown. Following a complete I/R injury, activated leukocytes in the post-capillary venules have been shown to directly inhibit capillary perfusion, cause direct parenchymal damage following the onset of reperfusion, increase vascular permeability, and increase tissue edema (Kurose, Anderson et al. 1994, Forbes, Harris et al. 1996, Harris and Skalak 1996).

Our study confirmed that CS induced a systemic cytokine/chemokine release. Twenty-four cytokines/chemokines were measured, and 14 were detectable. Of the 14 detected, 12 were pro-inflammatory, 1 was anti-inflammatory, and 1 had both a pro- and an anti-inflammatory effect. Six of the 14 cytokines/chemokines that were detectable were significantly elevated from their baseline levels after 2hrs of CS ($p < 0.05$). These included TNF- α (Figure 2.2), IL-1 β (Figure 2.3), GRO/KC (Figure 2.4), MCP-1 (Figure 2.5), MIP-1 α (Figure 2.6), and IL-10 (Figure 2.7). TNF- α , IL-1 β , GRO/KC, MCP-1, and MIP-1 α are pro-inflammatory cytokines/chemokines, while IL-10 is an anti-inflammatory cytokine. These results confirm that CS induces a significant and, predominantly, pro-inflammatory response.

4.1.4 TNF- α Neutralization

The purpose of this study was to evaluate the microvascular dysfunction, inflammatory response, and tissue injury following the neutralization of TNF- α at the time of fasciotomy, in an animal model of CS. TNF- α is a potent pro-inflammatory cytokine released in response to trauma, inflammation, or infectious

stimulation (Stein and Gordon 1991). Previously, multiple I/R studies have demonstrated that TNF- α is up-regulated in as little as 1 hour following reperfusion (Coty, Guice et al. 1990, Brock, Lawlor et al. 1999, Krishnadasan, Naidu et al. 2003). TNF- α acts as a chemoattractant for neutrophils, causes up-regulation of downstream cytokine/chemokine production, promotes the expression of adhesion molecules, and activates cell death via apoptosis and necroptosis (Ascer, Gennaro et al. 1992, Yi and Ulich 1992, Seekamp, Warren et al. 1993, Zhang, Hu et al. 2005, Parameswaran and Patial 2010).

CS causes a shift in perfusion from CPC (under normal physiological conditions) to that of IPC and NPC. The neutralization of TNF- α at the time of fasciotomy essentially had no effect on the microvascular perfusion profile seen in CS (Figure 3.1). Modulation of the inflammatory response did not, therefore, provide protection in restoring microvascular perfusion in the face of raised ICP. These results corroborate previous work by our group, whereby a leukocyte-deplete animal model was not shown to be protective in restoring the microvascular perfusion following a CS insult (Lawendy, Bihari et al. 2015).

The results of this study, again, confirm that CS induces a systemic inflammatory response. Two hours of CS produced a significant increase in serum TNF- α , coupled with the recruitment of activated leukocytes (Figure 3.5). Modifying the inflammatory response post-fasciotomy, through the administration of TNF- α neutralizing antibody, significantly decreased leukocyte activation, as evidenced by a decrease in both rolling and adherent leukocytes within the post-capillary venules ($p < 0.05$ and $p < 0.0001$ respectively) (Figure 3.3 and Figure 3.4).

When compared to control animals, neutralization of TNF- α led to a significant relative reduction of approximately 36% in tissue injury (Figure 3.2) following elevated ICP. This data confirms that TNF- α plays at least some role in the inflammatory response observed in a CS insult. In a leukocyte-deplete animal model of CS, tissue injury was reduced by more than 50%, implying that there are also other cytokines/chemokines involved in the pathophysiology of CS (Lawendy, Bihari et al. 2015).

Our findings confirm that CS is associated with a significant up-regulation of the systemic inflammatory response, and its induction should, therefore, be considered in the pathophysiology of CS. TNF- α plays a role in the tissue injury associated with CS, at least in the acute phase. Neutralizing TNF- α in isolation had less of an effect on reducing tissue injury in comparison to a completely leukocyte deplete animal model, suggesting that other cytokines/chemokines may also contribute to the pathophysiology of CS (Lawendy, Bihari et al. 2015). There are, of course, multiple potential mechanisms of tissue injury, including the generation of reactive oxygen species, increased expression of adhesion molecules on the surface of leukocytes and endothelial cells, and direct parenchymal injury via extravasated leukocytes (Gute, Ishida et al. 1998).

TNF- α has been recognized in initiating cell death via apoptosis and necroptosis (Wu, Tan et al. 2011). Necroptosis, in particular, is associated with pathological conditions including I/R; and, in contrast to apoptosis, it generates an inflammatory response (Vandenabeele, Galluzzi et al. 2010, Linkermann and Green 2014). It is, therefore, plausible that the low-flow ischemia associated with

CS, and consequently the early and sustained reperfusion injury, stimulates an earlier and sustained trigger of the apoptotic and necroptotic pathways. This could in part, account for the greater tissue injury associated with CS versus complete I/R.

4.2 LIMITATIONS AND FUTURE DIRECTIONS

Inflammation has been shown to play an important role in the microvascular dysfunction and tissue injury associated with CS. One of the limitations of these studies is its use of a small animal model. The injury process and clinical significance of the observed decrease in tissue injury remain to be translated to a human model. Future directions would include a large animal model, whose physiology more resembles that of humans, and recovery studies in order to assess functional outcomes.

The study also validates immune modulation as a potential therapeutic target in order to decrease the tissue injury associated with CS. TNF- α is already a mainstay of treatment in chronic pro-inflammatory conditions, such as Crohn's disease, rheumatoid arthritis, and psoriasis (Ford, Sandborn et al. 2011). TNF- α is only one of many cytokines/chemokines that were found to be significantly up-regulated following CS. Targeting different cytokines/chemokines, or a combination of them, could further elucidate the pathophysiological mechanism of CS.

4.3 CONCLUSIONS

The maintenance of microvascular perfusion throughout the CS insult allows oxygenated blood to enter an essentially ischemic compartment, which ultimately leads to the early initiation of the inflammatory cascade, and activation of leukocytes. This, in turn, stimulates the release of predominantly pro-inflammatory cytokines/chemokines, and in particular, the production of TNF- α . Neutralization confirms that TNF- α plays a role in the tissue injury associated with CS, likely through early activation of the apoptotic and necroptotic pathways.

The systemic inflammatory response is a complex and redundant system, and continued investigation is still required, as this study has only begun to elucidate one small piece of a complex pathophysiological mechanism.

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APPENDIX A

Animal Protocol Approval Letter

APPENDIX A: ANIMAL PROTOCOL APPROVAL LETTER**11.01.13**

This is the original approval for this protocol
 A full protocol submission will be required in 2017

Dear Dr. Lawendy:

Your animal use protocol form entitled:

Direct and Remote Organ Injury Following Hind Limb Compartment Syndrome

Funding agency Orthopaedic Trauma Association – Direct and Remote Organ Injury Following Hind Limb Compartment Syndrome – Grant #R4889A04 has been approved by the University Council on Animal Care.

This approval is valid from **11.01.13 to 11.30.17** with yearly renewal required.

The protocol number for this project is **2009-083**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
 If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 YEARS

Species	Strain	Other Detail	Pain Level	Animal # Total for 4 years
Rat	Wistar	150-350 g	C	680
Pig	Yorkshire-Landrace	50-60 kg	B	30

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. R Bihari, T Carter, K Bothwell, P Coakwell

APPENDIX B

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APPENDIX C

Surgical Approach to Leg Compartment Syndrome

APPENDIX C: SURGICAL APPROACH TO LEG COMPARTMENT

SYNDROME

There are multiple surgical techniques for complete fascial release of the lower leg. These include a two-incision fasciotomy, a single-incision perifibular fasciotomy, and fibulectomy. The preferred method is the two-incision technique (described below), as it allows for adequate visualization of all compartments, assessment of muscle viability, and sufficient exposure to safely avoid injury to the neurovascular structures.

C1. Two-Incision Fasciotomy (Mubarak and Hargens 1981)

The patient is positioned supine on the operating room table. A tourniquet is applied above the knee, but not insufflated. The limb is prepped and draped free. A 25 cm incision is made, centered halfway between the fibular shaft and the tibial crest (Figure C1). The subcutaneous tissue is dissected and full thickness skin flaps are elevated in order to achieve wide exposure of the fascial compartments. The intermuscular septum is exposed, and the superficial peroneal nerve is identified distally, lying posterior to the septum. Using dissecting scissors, the anterior compartment is released proximally and distally, in line with the tibialis anterior. The lateral compartment is released proximally and distally, in line with the fibular shaft. Care must be taken at the distal end to avoid damaging the superficial peroneal nerve.

A second longitudinal incision 2 cm posterior to the posterior margin of the tibia is made. The subcutaneous tissue is dissected, and full thickness skin flaps are elevated for exposure. The saphenous vein and nerve are identified and protected. The septum between the deep and superficial posterior compartments is identified. The superficial posterior compartment is released by incising the fascia along the entire length of the gastrocnemius-soleus complex. The deep posterior compartment is released by incising the fascia over the flexor digitorum longus muscle. Proximally, if the soleus bridge extends more than halfway down the tibia, this extended origin must be released. Finally, after release of the posterior compartment the tibialis posterior muscle must be identified. If there is increased tension within this compartment, it is released.

Wounds are left open and packed, and a posterior plaster splint is applied with the foot plantigrade.

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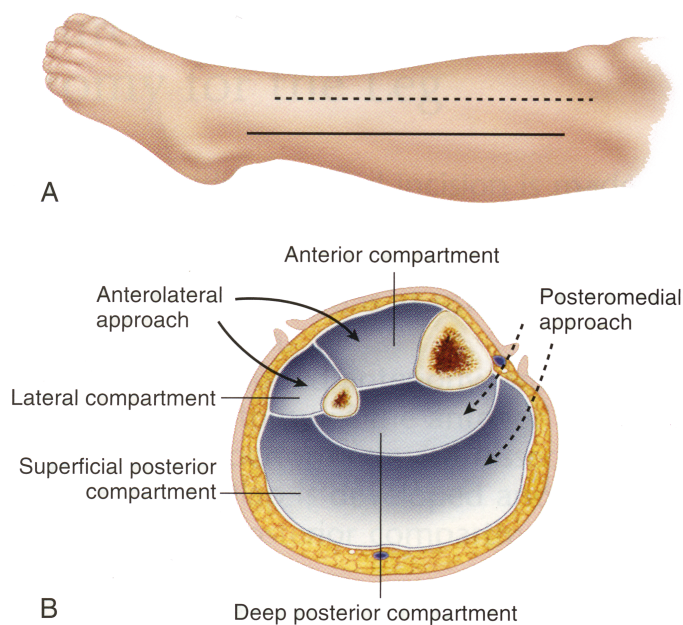


Figure C1. Two-Incision Fasciotomy. An incision is made halfway between the fibular shaft and the crest of the tibia. The lateral intermuscular septum is exposed and the superficial peroneal nerve is identified. The anterior compartment is released by incising the fascia in line with the tibialis anterior. The lateral compartment is released by incising the fascia in line with the fibular shaft. A second incision is made 2 cm posterior to the posterior margin of the tibia. The superficial posterior compartment is released by incising the fascia over the entire length of the gastrocnemius-soleus complex. Finally, the deep posterior compartment is released with a fascial incision overlying the flexor digitorum longus muscle.

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APPENDIX D

Luminex x-MAP Assay Protocol

APPENDIX D: LUMINEX x-MAP ASSAY PROTOCOL

Sera were subjected to a cytokine/chemokines multiplex panel (MILIPLEX® MAP, EMD Millipore, St. Charles, MO), based on Luminex® x-MAP technology, simultaneously quantifying the expression of 24 different cytokines/chemokines: Eotaxin, G-CSF, GM-CSF, IL-1 α , MCP-1, leptin, MIP-1 α , IL-4, IL-1 β , IL-2, IL-6, IL-9, IL-13, IL-10, IL-12p70, IL-5, IFN- γ , IL-17, IL-18, IP-10, GRO/KC, RANTES, TNF- α , VEGF. This immunoassay technology uses fluorescently labelled microsphere beads.

In principle, internally colour-coded microspheres are coated with two fluorescent dyes. Through precise concentrations of these, a hundred distinctly coloured bead sets can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with the reporter conjugate (streptavidin-phycoerythrin) to complete the reaction on the surface of each microsphere. The microspheres are allowed to pass rapidly through a laser, which excites the internal dyes marking the microsphere set. A second laser excites phycoerythrin, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals, comparing it to the standard curve.

All samples were run in duplicate. Standard curve was obtained by performing serial dilutions of rat standard containing all 24 cytokines/chemokines; 20,000pg/ml, 5,000pg/ml, 1,250pg/ml, 312.5pg/ml, 78.13pg/ml, 19.53pg/ml, 4.88pg/ml and 0pg/ml were used to run the standard curve. 25µl of each sample or standard, followed by 25µl of beads were added to the appropriate wells on a 96-well plate, and incubated with agitation overnight at 4°C. Following two washes with the wash buffer, 25µl of detection antibody were added to each well, and incubated for 2 hours at room temperature. This was followed by addition of 25µl of phycoerythrin to each well and 30-minute incubation at room temperature. The plate was washed two times with wash buffer; 150µl of sheath fluid was then added to each well to reconstitute the microbeads. The plate was then read on Luminex200 instrument equipped with MILLIPLEX® Analyst 5.1 software, recording the median fluorescent intensity (MFI). The results were calculated by 4-point logistic curve fitting software against the MFI of the standards.

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Donohoe E, Sanders D, Howard J, Somerville L, Lawendy A. **Rate of Total Knee Arthroplasty after Tibial Plateau Fracture.** Podium Presentation, 70th Canadian Orthopedic Association Annual Meeting, Vancouver, British Columbia, June 2015

Donohoe E, Sanders D, Desai S, Abdo H, Carey T, Cashin M, Bartley D, Lawendy A. **Atypical and Late Diagnosis of Acute Pediatric Compartment Syndrome Leads to Devastating Consequences.** Poster Presentation, 70th Canadian Orthopedic Association Annual Meeting, Vancouver, British Columbia, June 2015