Clinical/Basic Science Research Article

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Compartment syndrome-induced muscle injury is diminished by the neutralization of pro-inflammatory cytokines

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Abstract

Objectives: Compartment syndrome (CS) is one of the most devastating consequences of musculoskeletal trauma. The pathophysiology of CS includes elevation of intracompartmental pressure (ICP), causing damage to the microcirculation, decreased oxygen delivery, tissue anoxia, and cell death. CS is a combined ischemic and inflammatory condition that induces the systemic inflammatory cascade. In complete ischemia, within the first hour of reperfusion, a peak in the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) has been previously reported. The purpose of this study was to examine the suspected systemic inflammatory cytokine/chemokine release in response to CS, and to evaluate the microvascular dysfunction, tissue injury, and inflammatory response following the neutralization of pro-inflammatory cytokines TNF-α and/or interleukin-1 beta (IL-1β).

Methods: Twenty-eight male Wistar rats were randomly assigned into 5 groups: Sham (no CS), CS (with isotype control), CS+TNF-α neutralizing antibody (NA), CS+IL-1β NA, CS+Combo (both TNF-α and IL-1β NA). CS was induced by elevation of ICP above 30 mm Hg through an infusion of isotonic saline into the anterior compartment of the hind limb for 2 hours; NA were administered just prior to fasciotomy. Microvascular perfusion, cellular tissue injury, and inflammatory response within the extensor digitorum longus muscle were assessed using intravital video microscopy for 45 minutes after fasciotomy. Systemic levels of 24 different cytokines/chemokines were also measured, using the xMAP Luminex technology.

Results: Of the 24 cytokines/chemokines sampled, 6 were significantly elevated from their baseline levels, and included the pro-inflammatory cytokines TNF-α, IL-1β, growth-related oncogene/keratinocyte chemoattractant (GRO/KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), and the anti-inflammatory cytokine IL-10. CS resulted in a significant decrease in microvascular perfusion, from 75 ± 2% continuously perfused capillaries in the sham to 31 ± 4% in CS ($P < .001$), a significant increase in tissue injury (0.33 ± 0.4 versus 0.04 ± 0.01 in sham) and leukocyte activation (14 ± 2 adherent leukocytes/1000 μm^2 versus 2 ± 1 adherent leukocytes/100 μm^2 in sham, $P < .001$). CS-associated tissue injury was significantly decreased with TNF-α neutralization ($P < .05$), both when administered alone or in combination with IL-1β ($P < .05$). Additionally, TNF-α neutralization blocked CS-associated leukocyte activation ($P < .05$); IL-1β neutralization also diminished leukocyte adhesion ($P < .05$). Perfusion remained virtually unchanged in CS animals treated with NA (36 ± 4%, 32 ± 3% and 30 ± 2% in CS+TNF-α, CS+IL-1β and CS+Combo groups, respectively).

Conclusion: The results of this study indicate that CS induces a systemic inflammation, as evidenced by upregulation of inflammatory cytokines/chemokines in circulation. Neutralization of TNF-α led to a significant reduction in tissue injury; however, it had no effect on the CS-induced microvascular dysfunction. This suggests a distinct role of TNF-α in the pathophysiology of muscle injury in CS.

Abbreviations: BB = bisbenzimide, CPC = continuously-perfused capillaries, CS = compartment syndrome, EB = ethidium bromide, EDL = extensor digitorum longus, ELISA = enzyme-linked immunosorbent assay, GRO/KC = growth-related oncogene/keratinocyte chemoattractant, I/R = ischemia-reperfusion, ICP = intracompartmental pressure, IL-1β = interleukin-1 beta, IPC = intermittently-perfused capillaries, IVVM = intravital video microscopy, MCP-1 = monocyte chemoattractant protein 1, MIP-1a = macrophage inflammatory protein 1 alpha, NA = neutralizing antibodies, NPC = non-perfused capillaries, ROS = reactive oxygen species, TNF-α = tumor necrosis factor alpha.

Keywords: compartment syndrome, cytokines/chemokines, inflammation, tissue injury, tissue perfusion, TNF-α, IL-1β, cytokine neutralization

The study was supported by 2015–2016 Orthopaedic Trauma Association Resident Research grant, awarded to Dr Donohoe, and Lawson Health Research Institute Internal Research Fund (IRF-19-14), awarded to Dr Lawendy.

This work was presented at the 2016 Orthopaedic Trauma Association Annual Meeting in Washington, DC.

The authors have no conflicts of interest to disclose.

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OTA (2018) e011

Received: 13 February 2018 / Received in final form: 21 August 2018 / Accepted: 28 August 2018

http://dx.doi.org/10.1097/OI9.0000000000000011

Introduction

CS remains one of the most devastating and challenging sequelae of musculoskeletal trauma. CS develops secondary to increased pressure within a closed osseofascial compartment, most commonly following a tibial diaphyseal fracture.[1–8] Elevated ICP compromises the microcirculation, leading to decreased oxygen and nutrient delivery, tissue anoxia and, ultimately, myonecrosis.[4,9,10]

The pathophysiology of CS-associated tissue injury is only partially understood. While ischemia-reperfusion (I/R) injury appears to be the main driving force, there is a critical distinction between CS and complete I/R: unlike complete I/R, tissue necrosis and ischemic injury in CS ensue despite a patent macrocirculatory system and palpable distal pulses.[11] Previous studies have demonstrated that CS-induced tissue injury is more extensive than that of a complete ischemic insult of the same duration.[12]

Normal microvascular perfusion is comprised of predominantly continuously perfused capillaries (CPC). Elevated ICP results in a shift of perfusion towards IPC and NPC,[13,14] leading to a "low flow" ischemic state. As a result, the metabolic demands of the tissue cannot be met,[13] resulting in the production of reactive oxygen species (ROS). ROS, generated following the re-introduction of oxygen into previously ischemic tissue are known to trigger the acute inflammatory cascade and eventual cellular death via apoptosis or necroptosis.[15,16] Unlike a complete I/R (as observed following organ transplantation and re-vascularization procedures[17,18]), the defined phases of injury (ischemia, reperfusion) cannot be clearly delineated in a "low-flow" ischemia, initiating an early and ongoing reperfusion injury concurrent with ischemic period. The reperfusion injury not only persists throughout the duration of the CS, but also it is further intensified by the restoration of normal blood flow into the capillary bed after fasciotomy.

Activated leukocytes appear to play a major role in CS pathophysiology.[14] While leukopenia does not restore or maintain perfusion, it appears to provide significant protection against muscle injury.[14] The pro-inflammatory cytokine TNF-α has been shown to be acutely upregulated following reperfusion in multiple I/R studies.[19–21]

The purpose of this study was to examine the specific cytokines/chemokines released during CS in a rat model, as well as to evaluate the microvascular dysfunction, tissue injury, and leukocyte activation following the neutralization of pro-inflammatory cytokines TNF-α and IL-1β at the time of fasciotomy. We hypothesized that CS is associated with the initiation of the systemic inflammatory cascade, and that neutralization of ischemia-reperfusion injury-linked cytokines would diminish the microvascular dysfunction and tissue injury associated with CS.

Materials and methods

Animal preparation

The experimental protocol was approved by the Canadian Council on Animal Care and the Animal Use Subcommittee at The University of Western Ontario. Male Wistar rats (body weight 180–250 g) were used for all experiments.

Animals were anesthetized with isoflurane (5% induction, 2% maintenance) in a 1:1 O_2:N_2 mixture. The left carotid artery was cannulated for the continuous monitoring of blood pressure, fluid replacement, and blood sampling; core body temperature was maintained at 37°C by the means of a heat lamp. A normotensive and normothermic model of CS was used.

CS was induced by an infusion of isotonic saline into the anterior compartment of the right-hind limb via a 24-gauge angiocatheter to raise the ICP, as previously described.[13] Compartment pressures were measured by an electronic compartmental pressure monitoring system (Synthes USA, Paoli, Pennsylvania), inserted into the posterior compartment via a 14-gauge angiocatheter. Elevated ICP was maintained at 30 to 40 mm Hg for 2 hours, followed by fasciotomy and 45 minutes of reperfusion. The skeletal muscle was then imaged by intravital video microscopy (IVVM).

Experimental groups

Animals were randomly assigned into 1 of 5 groups: Sham (n = 8), CS (with isotype control) (n = 8), CS with TNF-α neutralization (CS+TNF-α) (n = 4), CS with IL-1β neutralization (CS+IL-1β) (n = 4), and CS with neutralization of both TNF-α and IL-1β (CS +Combo) (n = 4). Sham animals underwent all the procedures as the CS groups, but they did not receive saline infusion into the anterior compartment of the hind limb and the ICP was maintained at the baseline level of 0 mm Hg.

Serum cytokines/chemokines

All blood samples were drawn from the indwelling carotid line at 7 different time points: baseline (just prior to the elevation of ICP); at 1 hour CS; at 2 hours CS, just prior to fasciotomy; at 10 minutes reperfusion, at 20 minutes reperfusion, at 30 minutes reperfusion; at 45 minutes reperfusion, just prior to IVVM. At each time point, 0.1 mL of blood was withdrawn and replaced with 0.1 mL of normal saline.

Serum levels of cytokines/chemokines were assessed by xMAP Luminex assay (EMD Millipore, Mississauga, Ontario), as per manufacturer's instructions, simultaneously testing 24 different cytokines/chemokines: TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IL-18, IFN-γ, Eotaxin, IP-10, MIP-1α, Leptin, MCP-1, GRO/KC, RANTES, GM-CSF, G-CSF, and VEGF. The samples were analyzed using Luminex 200 instrument (EMD Millipore, Mississauga, Ontario).

Neutralization of serum TNF-α and IL-1β

NA to TNF-α and/or IL-1β (eBioscience, San Diego, CA) was administered to CS animals just before fasciotomy at a dose of 3.5×10^4 neutralizing units/100 g body weight (0.3 mL IV). Syrian hamster IgG (eBioscience, San Diego, California) was used as an isotype control. Neutralization of serum TNF-α and IL-1β was confirmed by enzyme-linked immunosorbent assay (ELISA, Pierce Biotechnology, c/o Thermo Scientific, Rockford, Illinois) as per manufacturer's instructions. ELISAs were sensitive to <5 pg/mL.

Intravital video microscopy (IVVM)

Following fasciotomy, the extensor digitorum longus (EDL) muscle was prepared for IVVM, as previously described.[13,22–24] Briefly, following isolation of EDL, the animal was transferred onto the stage of an inverted microscope (Nikon, Nikon Instruments, Inc., Mississauga, Ontario, Canada); the EDL muscle was detached from its bony insertion and reflected into a saline bath containing 5 μg/mL each of the fluorescent vital dyes bisbenzimide (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 cells with damaged cell membranes. The EB/BB ratio, therefore, was used as a measure of tissue injury.[13,22]

Microvascular perfusion and leukocyte activation within the post capillary venules were recorded by translumination with 20× and 40× objectives, respectively, in 5 adjacent fields of view, and captured into a computer for offline video analysis. Fluorescence microscopy was used to visualize the BB- and EB- labeled cells from the same fields of view. At the conclusion of the experiment, the animals were euthanized by an overdose of anesthetic agent.

Offline video analysis

Capillary perfusion was evaluated by counting the number of CPCs, IPCs, and NPCs capillaries crossing 3 parallel lines drawn perpendicular to the capillary axis on the computer monitor, and was expressed as a percentage (%) of total capillaries.[13,22]

Tissue injury was evaluated by counting the number of EB- and BB-labeled nuclei, and expressed as EB/BB ratio.[13,22]

Leukocyte activation (inflammation) was assessed by counting the number of rolling and adherent leukocytes in the postcapillary venules, and conveyed as cells per unit area (i.e., 1000 μm^2). Venular area was measured using ImageJ software (NIH, Bethesda, Maryland). A leukocyte was deemed adherent if it remained immobile for at least 30 seconds and rolling if it remained in contact with the wall of the vessel but remained in motion.[13,22]

Statistical analysis

All data were expressed as mean ± SEM (standard error of the mean). Serum levels of cytokines/chemokines were analyzed by repeated measures analysis of variance (ANOVA) (GraphPad Prism v. 5.0c for Mac, San Diego, California); each sample within the time course was compared to its baseline levels. One-way ANOVA was used to compare the degree of perfusion, tissue injury, and leukocyte activation (adhesion, rolling). Newman—Keuls multiple comparison post-hoc test was used where appropriate. A sample size calculation was performed (StatMate, GraphPad Inc, San Diego, California), with power set at 85%. Statistical significance was defined as $P < .05$.

Results

Systemic cytokines/chemokines

Sixteen of the 24 cytokines/chemokines sampled were detectable using xMAP Luminex technology. These included TNF-α, IL-1α, IL-1β, IL-10, IL-12p70, IL-13, IL-18, IFN-γ, Eotaxin, MIP-1α, Leptin, MCP-1, GRO/KC, IP-10, RANTES, and IL-6. Six of the detectable cytokines/chemokines were significantly elevated from their baseline levels at 2-hour CS (Table 1). These included pro-inflammatory cytokines TNF-α and IL-1β, anti-inflammatory cytokine IL-10, and chemokines GRO/KC, MCP-1, and MIP-1α.

Pro-inflammatory cytokine neutralization

Systemic TNF-α increased from 18.1 ± 3.4 pg/mL at baseline to 707.1 ± 181.9, 1495.0 ± 152.7, 1573.0 ± 188.0, 1460.4 ± 283.3, 1624.3 ± 546.9, and 2303.7 ± 394.9 pg/mL at 1-hour CS, 2-hour CS, and 10 minutes, 20 minutes, 30 minutes, and 45 minutes post-fasciotomy, respectively ($P < .05$). Administration of TNF-α NA at fasciotomy resulted in a complete inhibition of TNF-α to a nondetectable level, where it remained for the rest of the experiment ($P < .05$) (Fig. 1A).

Serum IL-1β was elevated from the baseline of 40.0 ± 26.7 pg/mL to 97.6 ± 32.1, 141.3 ± 13.1, 102.2 ± 22.1, 119.9 ± 17.6, 46.0 ± 46.0, and 61.2 ± 31.7 pg/mL at 1-hour CS, 2-hour CS, and 10 minutes, 20 minutes, 30 minutes, and 45 minutes post-fasciotomy, respectively ($P < .05$). Administration of IL-1β NA at fasciotomy resulted in a complete inhibition of IL-1β, to a nondetectable level, for the rest of the experiment ($P < .05$) (Fig. 1B).

Serum TNF-α and IL-1β levels in the sham group showed no significant changes from the baseline levels of 12.1 ± 6.9 pg/mL

Table 1

Serum levels of cytokines/chemokines detected in a rat model of CS.

Cytokine/Chemokine	Baseline, pg/mL	At 2-hour CS, pg/mL	Fold change	*P*-value
TNF-α	18.1 ± 3.5	1495.1 ± 152.7*	82	.0004
IL-1β	40.0 ± 20.0	141.3 ± 18.6*	3	.0384
IFN-γ	326.6 ± 98.6	360.4 ± 159.3	0.1	.8463
IL-6	2566 ± 1758	2351 ± 1494	−0.1	.9084
MIP-1α	0.0 ± 0.0	84.8 ± 41.0*	8	.0077
MCP-1	313.4 ± 31.4	698.8 ± 30.6*	1	.0172
GRO/KC	990.2 ± 213.3	2984 ± 1310*	2	.0001
IL-1α	473.9 ± 291.7	434.7 ± 179.3	−0.1	.8887
Leptin	9596 ± 1038	8606 ± 1353	−0.1	.5344
Eotaxin	228.0 ± 161.3	175.7 ± 144.3	−0.2	.4431
IL-13	225.3 ± 60.0	254.9 ± 106.9	0.1	.7161
IL-12p70	196.1 ± 68.1	199.2 ± 83.5	0	.9718
IL-18	86.6 ± 7.3	76.4 ± 6.1	−0.1	.1621
IP-10	145.4 ± 108.6	155.7 ± 108.9	0.1	.9424
RANTES	1673 ± 250	1680 ± 193	0	.3560
IL-10	68.4 ± 68.4	1138.3 ± 372.3*	16	.0012

Twenty-four different cytokines and chemokines were tested; those with detectable values are shown here.
* $P < .05$.

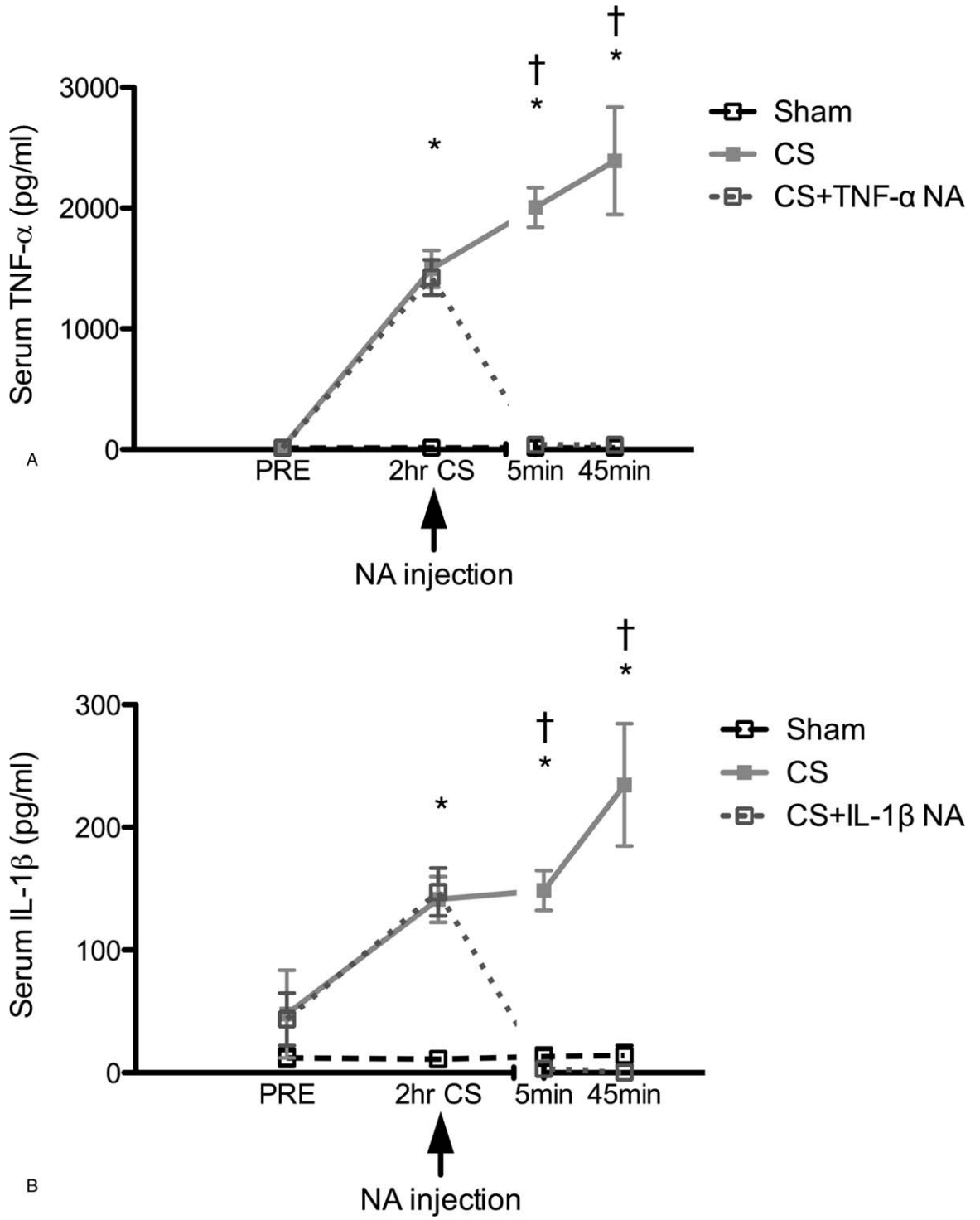

Figure 1. Serum levels of pro-inflammatory cytokines in a rat model of CS. Level of TNF-α is shown in panel A; level of IL-1β is shown in panel B. Elevated ICP resulted in a significant increase in systemic TNF-α and IL-1β, both of which continued to rise during the 45 minutes reperfusion period (solid line). The administration of NA at fasciotomy resulted in a complete inhibition of TNF-α or IL-1β levels (dotted line). Systemic TNF-α or IL-1β levels remained constant in sham animals (dashed line) ($^{*}P < .05$ from baseline, †$P < .05$ from CS+TNF-α or CS+IL-1β group; repeated measures two-way ANOVA). *PRE*, baseline levels.

and 12.3 ± 7.1 pg/mL, respectively. Levels of TNF-α at 2 hours and 45 minutes reperfusion time-matched points were 14.8 ± 3.8 pg/mL, and 12.6 ± 5.9 pg/mL, respectively, while those of IL-1β were 11.0 ± 6.0 pg/mL and 14.0 ± 8.0 pg/mL, respectively (Fig. 1).

Microvascular perfusion

Elevation of ICP resulted in significant changes to microvascular perfusion, as shown in Figure 2. The number of CPC decreased from 75 ± 2% in the sham to 31 ± 3%, 36 ± 4%, 31 ± 3%, and

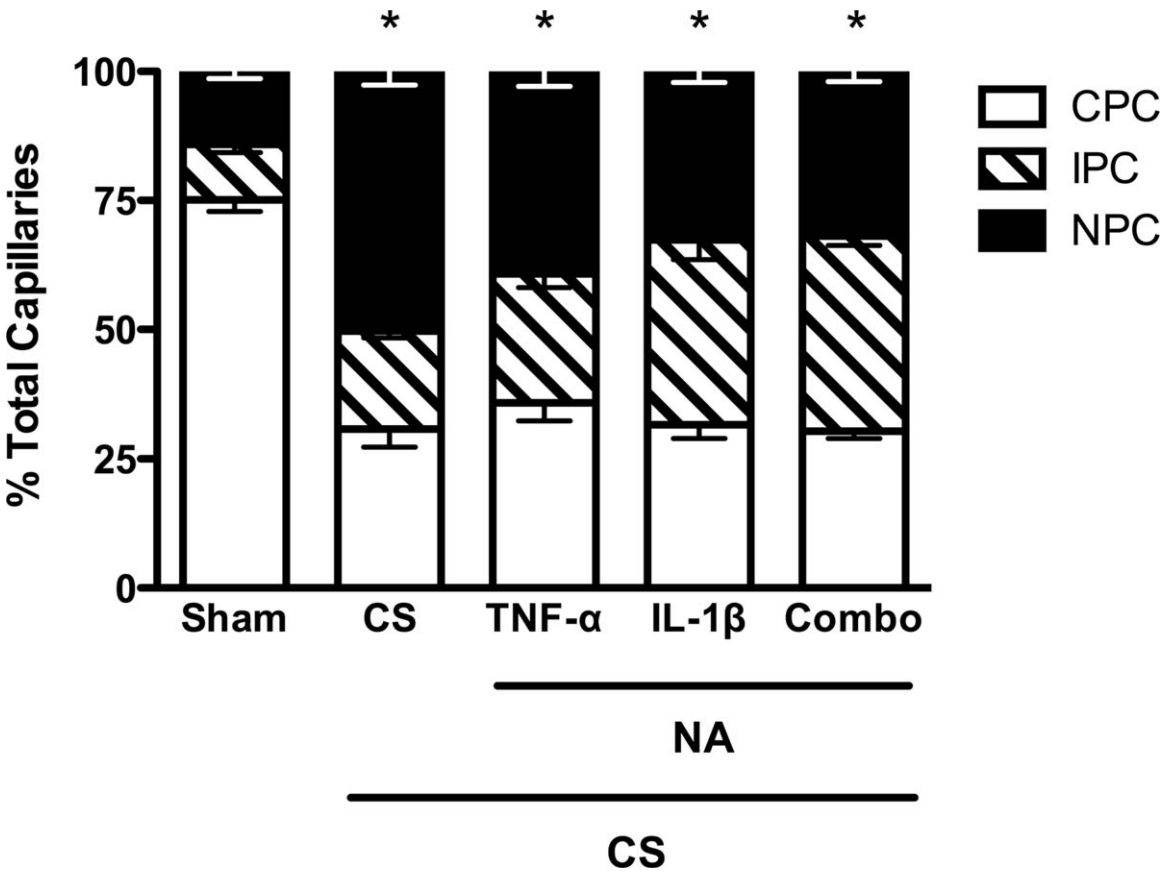

Figure 2. The effect of pro-inflammatory cytokine neutralization on the severity of microvascular perfusion deficit in a rat model of CS. Elevated ICP resulted in a severe microvascular perfusion deficit; neutralization of TNF-α and/or IL-1β at fasciotomy did not restore continuous perfusion ($^{*}P < .05$ from sham; one-way ANOVA).

30 ± 2% in the CS, TNF-α, IL-1β and Combo neutralizing groups, respectively ($P < .001$). There were no significant differences in CPC between the CS and any of the neutralizing groups.

The number of IPC increased from 11 ± 2% in sham to 19 ± 1%, 25 ± 3%, 36 ± 4% and 38 ± 2% in the CS and TNF-α, IL-1β and Combo neutralizing groups, respectively ($P < .001$). The number of IPC in all neutralizing groups was significantly higher than that in CS group ($P < .05$).

The number of NPC increased from 14 ± 1% in sham to 50 ± 3%, 39 ± 3% 33 ± 2% and 32 ± 2% in the CS and TNF-α, IL-1β and Combo neutralizing groups, respectively ($P < .001$). The number of NPC in the CS+TNF-α neutralizing group was significantly lower as compared to the CS group ($P < .05$).

Tissue injury

Elevation of ICP resulted in a significant increase in tissue injury, from 0.04 ± 0.02 in sham to 0.33 ± 0.04 in CS group ($P < .001$) (Fig. 3). Administration of TNF-α NA resulted in a decrease to 0.19 ± 0.04 ($P < .001$) and 0.13 ± 0.02 ($P < .001$) in CS+TNF+α and CS + Combo groups, respectively, while neutralization of IL-1β had no effect (0.29 ± 0.02 in CS+IL-1β group, not significant).

Inflammation

Elevation of ICP resulted in an increase in leukocyte rolling, from 2 ± 1 leukocytes/30 s/1000 μm^2 in sham to 10 ± 3 leukocytes/30 s/1000 μm^2 in CS group ($P < .001$). Administration of TNF-α NA resulted in a significant decrease to 2 ± 1 and 3 ± 1 rolling leukocytes/30 s/1000 μm^2 in CS+TNF+α and CS+Combo groups, respectively ($P < .01$) (Fig. 4A). Administration of IL-1β following CS had no effect (15 ± 3 rolling leukocytes/30 s/1000 μm^2, not significant).

There was a significant increase in leukocyte adherence following elevation of ICP, from 2 ± 1 to 14 ± 2 leukocytes/30 s/1000 μm^2 in CS group ($P < .001$) (Fig. 4B). Administration of TNF-α NA produced a significant decrease to 1 ± 0 adherent leukocytes/30 s/1000 μm^2 in both CS+TNF+α and CS+Combo groups ($P < .001$). Administration of IL-1β also significantly decreased leukocyte adherence (6 ± 1 adherent leukocytes/30 s/1000 μm^2, $P < .05$).

Discussion

In spite of the fact that CS has been a recognized complication of musculoskeletal trauma since Richard von Volkmann's description of ischemic contracture in 1881, the pathophysiology of CS remains largely unknown.[25] The use of IVVM imaging has been instrumental in illustrating the derangement of microvascular perfusion following a CS insult. Under live conditions, we were able to observe the presence of CPC, IPC, and NPC within the same capillary bed, validating CS as a "low-flow" ischemic environment.[13]

Inflammation has been shown to be a major contributor to the microvascular dysfunction associated with I/R (TNF-α has been

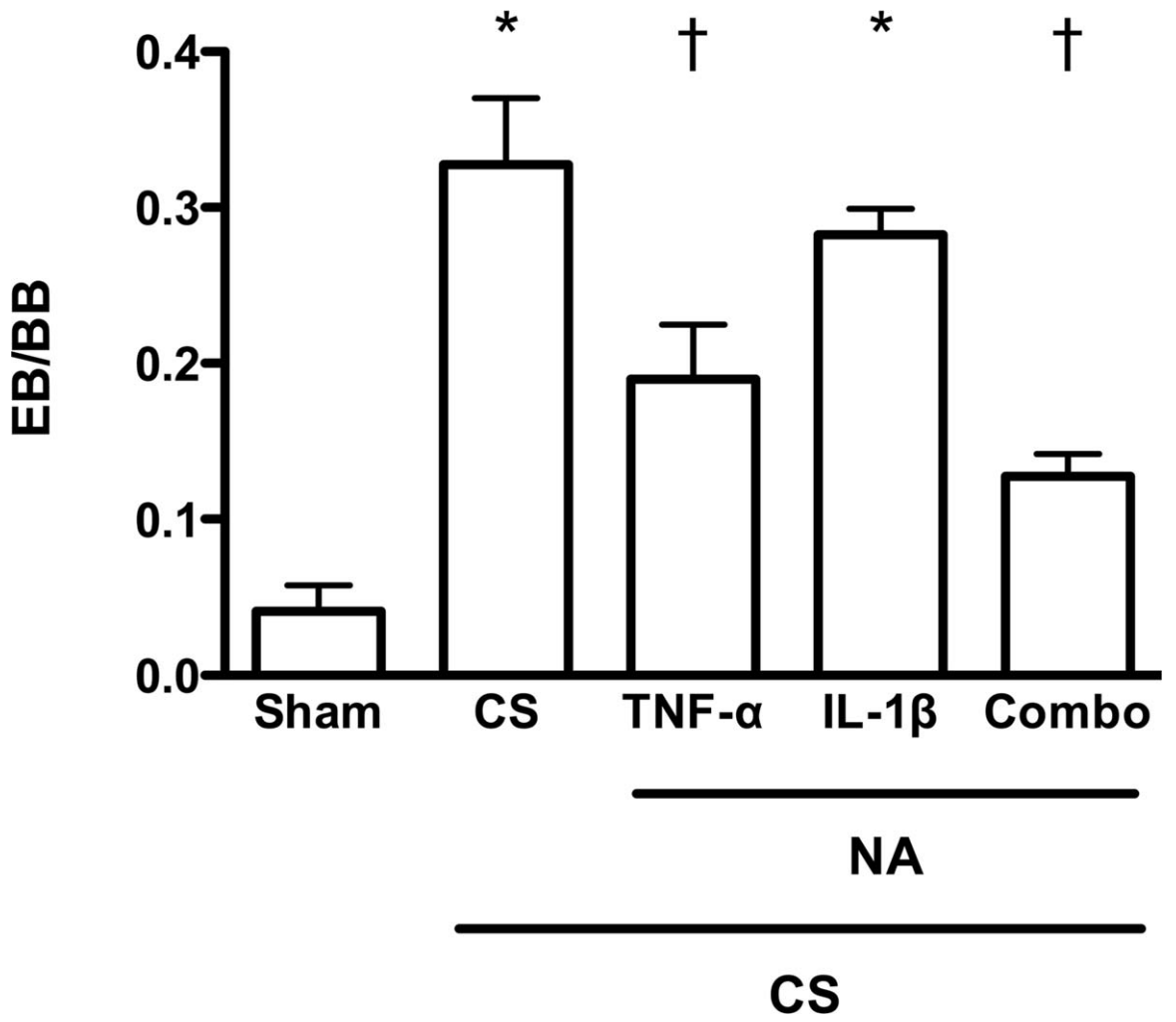

Figure 3. The effect of pro-inflammatory cytokine neutralization on skeletal muscle injury in a rat model of CS. Administration of TNF-α or a combination of TNF-α/IL-1β NA at fasciotomy significantly diminished CS-induced tissue injury; neutralization of IL-1β alone had no effect (*$P < .001$ from sham, †$P < .01$ from CS; one-way ANOVA).

shown to be elevated following a complete I/R injury[19,20]), and hence CS. The purpose of this study was to identify the mediators (i.e., cytokines/chemokines) involved, and to observe the effect of neutralizing 2 major pro-inflammatory cytokines, TNF-α, and IL-1β, on microvascular perfusion, tissue injury, and leukocyte activation in an animal model of CS.

This study confirms that CS induces a systemic response, as evidenced by the upregulation of pro-inflammatory cytokines/chemokines in circulation. Of the 14 cytokines/chemokines detected in the serum of rats challenged with CS, 6 were significantly elevated from their baseline levels after 2 hours of ICP elevation ($P < .05$): TNF-α, IL-1β, GRO/KC, MCP-1, MIP-1α, and IL-10. With the exception of IL-10, (which is an anti-inflammatory cytokine),[26] all detected cytokines/chemokines are pro-inflammatory.[27–35]

TNF-α is a powerful pro-inflammatory cytokine released following trauma, inflammation, or infection.[36] TNF-α acts as a chemoattractant for neutrophils, causes up-regulation of cytokine/chemokine production, and activates cell death via apoptosis and necroptosis.[27–30,37] Similarly, IL-1β is another pro-inflammatory cytokine that plays an important role in sterile inflammation.[28] IL-1β is produced by activated macrophages, monocytes, endothelial cells, fibroblasts, and appears to be involved in cell proliferation, differentiation and apoptosis, mediating an inflammatory sequence similar to that of TNF-α.[28] As both TNF-α and IL-1β have been demonstrated to play a significant role in I/R,[29,38] our current study focused on assessing the contribution of these 2 cytokines to the pathophysiology of CS.

CS resulted in a significant rise in systemic TNF-α and IL-1β, in conjunction with a marked increase in activated leukocytes. Modulating the inflammatory response significantly decreased leukocyte activation, illustrated by a decrease in both rolling and adherent leukocytes within the post-capillary venules ($P < .05$ and $P < .0001$, respectively).

Tempering the inflammatory response through neutralization of TNF-α (alone or in combination with IL-1β) also led to a significant relative reduction of approximately 40% in tissue injury following 2 hours of elevated ICP. Neutralization of IL-1β by itself had no appreciable effect; however, when given in combination with TNF-α, it augmented the reduction in parenchymal injury to levels previously found in a leukopenic model of CS.[14] One possible explanation as to why neutralization of IL-1β alone failed to offer any protection may be due to the existence of temporal relationship between TNF-α and IL-1β release.[38] In I/R injury, TNF-α release appears to precede that of

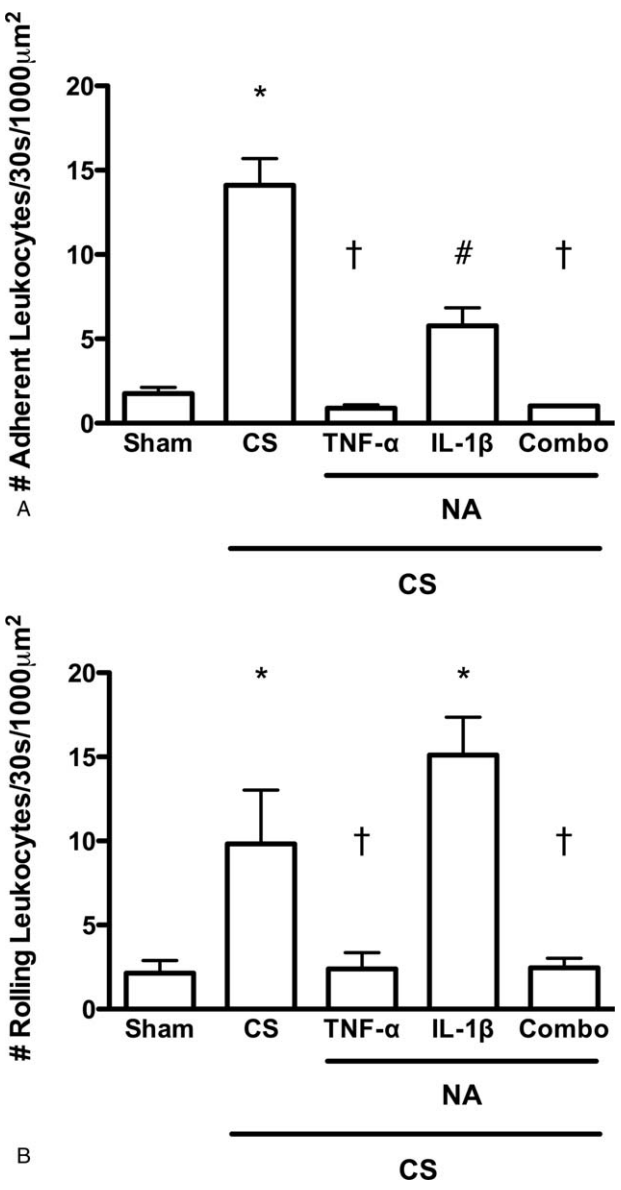

Figure 4. The effect of systemic pro-inflammatory cytokine neutralization on tissue inflammation, as quantified by (A) leukocyte adhesion and (B) leukocyte rolling, in a rat model of CS. CS-associated leukocyte activation (both (A) adhesion and (B) rolling) was significantly diminished by the administration of TNF-α NA (alone or in combination with IL-1β) at fasciotomy, while administration of IL-1β had no effect ($^{*}P < .001$ from sham, †$P < .001$ from CS, #$P < .01$ from CS+TNF-α; one-way ANOVA).

IL-1β; this would indicate that IL-1β probably plays a more significant role at later stages of ischemia. Thus, our findings suggest that, although TNF-α appears to play a significant role in the inflammatory response associated with CS, the contribution of other cytokines/chemokines (e.g., IL-1β) to the pathophysiology is not inconsequential.

Neutralizing TNF-α and/or IL-1β at the time of fasciotomy did not provide any protection to the microvascular perfusion following a CS insult. These results corroborate previous work by our group, whereby leukopenia was not protective in preventing the microvascular dysfunction associated with CS.[14] Again, a distinction from complete I/R must be highlighted: following complete ischemia, microvascular dysfunction, and tissue injury were significantly decreased in a leukopenic I/R animal model.[17] Our current study, therefore, provides further evidence that the pathologic changes in microvascular dysfunction following CS have a distinct pathophysiology compared to complete I/R injury.

This study demonstrates that CS is associated with a significant activation of the systemic inflammatory response, and its initiation should, therefore, be considered in the pathophysiology of CS. In addition to its role as an acute inflammatory mediator, TNF-α has also been implicated in initiating cell death via apoptosis and necroptosis.[39] The necroptotic pathway is also associated with the tissue injury following I/R.[40,41] In contrast to apoptosis, necroptosis generates a systemic inflammatory response. It can be inferred from our findings that this pathway of programmed cell death may contribute to the tissue injury associated with CS. It is plausible that the early and sustained reperfusion injury associated with 'low-flow' ischemia induces an early and sustained stimulus for the necroptotic pathway. This could in part, explain the greater tissue injury associated with CS versus complete I/R.

Surprisingly, neutralization of TNF-α (alone or in combination with IL-1β) did not confer the same type of protection to the microvasculature as that seen following administration of carbon monoxide (CO) donor, CORM-3, in a rat model of CS.[42] CO has been shown to exhibit potent vasodilatory, anti-inflammatory and anti-apoptotic properties in various models of disease.[43,44] As such, the results of our current study confirm that inflammatory response is not the only component of CS pathophysiology, although it plays a major role. Hence, neutralization of pro-inflammatory cytokines does not address other factors that contribute to CS-associated damage (e.g., vasodilation, ROS production, apoptosis) and thus is insufficient in restoring the microcirculation back to normal.

To our knowledge, this is the first study that directly confirms CS as an inflammatory process. The systemic inflammatory cascade is complex and redundant, and these results are merely the beginning steps in an attempt to extrapolate the pathophysiologic mechanism by which CS injury occurs.

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