C5a-Blockade Improves Burn-Induced Cardiac Dysfunction


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C5a-Blockade Improves Burn-Induced Cardiac Dysfunction

Laszlo M. Hoesel,1,2* Andreas D. Niederbichler,2† Julia Schaefer,* Kyros R. Ipaktchi,† Hongwei Gao,§ Daniel Rittirsch,* Matthew J. Pianko,* Peter M. Vogt,‡ J. Vidya Sarma,* Grace L. Su,‡ Saman Arbabi,† Margaret V. Westfall,† Stewart C. Wang,† Mark R. Hemmila,† and Peter A. Ward3*  

We previously reported that generation of the anaphylatoxin C5a is linked to the development of cardiac dysfunction in sepsis due to C5a interaction with its receptor (C5aR) on cardiomyocytes. Burn injury involves inflammatory mechanisms that can lead to C5a generation as well. In this study, we investigated the effects of C5a blockade on burn-induced cardiac dysfunction. Using a standardized rat model of full thickness scald injury, left ventricular pressures were recorded in vivo followed by in vitro assessment of sarcomere contraction of single cardiomyocytes. Left ventricular pressures in vivo and cardiomyocyte sarcomere contractility in vitro were significantly reduced following burn injury. In the presence of anti-C5a Ab, these defects were greatly attenuated 1, 6, and 12 h after burn injury and completely abolished 24 h after burn. In vitro incubation of cardiomyocytes with bacterial LPS accentuated the impaired contractility, which was partially prevented in cardiomyocytes from burned rats that had received an anti-C5a Ab. Based on Western blot analyses, real-time PCR, and immunostaining of left ventricular heart tissue, there was a significant increase in cardiomyocyte expression of C5aR after burn injury. In conclusion, an in vivo blockade of C5a attenuates burn-induced cardiac dysfunction. Further deterioration of contractility due to the exposure of cardiomyocytes to LPS was partially prevented by C5a-blockade. These results suggest a linkage between C5a and burn-induced cardiac dysfunction and a possible contribution of LPS to these events. The Journal of Immunology, 2007, 178: 7902–7910.

S evere burn injury induces pathologic alterations in cellular signaling pathways that can lead to life-threatening end-organ dysfunctions. Among the latter, cardiac dysfunction plays a crucial role in the outcome of the burned patient. Compromised cardiac function results in organ hypoperfusion leading to impaired peripheral microcirculation, burn zone extension, and reduced resistance to bacterial infection at the wound site. Burn-associated cardiac dysfunction has been shown previously to occur independently of intravascular volume loss (1–3). Assuming that circulating “myocardial depressant factors” trigger burn-induced cardiac contractility deficits, numerous experimental studies have been done to identify a causative factor (4–11), but a single agent responsible for burn-induced myocardial dysfunction has not been found.

The complement system represents a powerful component of innate immune defense, becoming activated during a variety of inflammatory and immunological diseases (12). Excessive production of the complement anaphylatoxin C5a has been implicated in the pathogenesis of various immunoinflammatory diseases (13). Until recently, there has been little evidence for direct effects of C5a on parenchymal cells resulting in organ dysfunction. We have previously shown that C5a plays a central role in the induction of cardiomyocyte (CM) contractility deficits during sepsis (14). To date, the complement system has not been implicated in the pathogenesis of burn-induced myocardial dysfunction. Previous reports showed elevated C3a and C5a levels in burned human subjects, suggesting a possible linkage between the complement system and the induction of organ dysfunction after burn injury (11).

Secondary infections represent one of the major complications of burn care, putting burn patients at additional risk. Recently, we reported that LPS, a bacterial cell wall component, directly inhibits cardiac contractility in a time-dependent manner (15). LPS binding and signaling occurs through the TLR4/CD14/MD2 complex, which represents another important component of the innate immune system, in addition to the complement system (16). Local or systemic infections lead to the activation of both systems. After ligand binding, both TLRs and complement receptors activate a cascade of intracellular downstream mediators such as MAPK and the transcription factor NF-κB, leading to the release of proinflammatory and regulatory cytokines (IL-1β, TNF-α, IL-6, and IL-12) and chemokines (17, 18). However, the possible effects of blocking either the C5a/C5aR or LPS/TLR4 pathway on the outcome after burn injury are not known. Based on the fact that both C5a and LPS trigger CM dysfunction, we have investigated the role of...
C5a in the pathogenesis of burn-induced cardiac dysfunction and a possible contribution of LPS to these effects.

Materials and Methods

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich.

Experimental animals

Adult male Sprague-Dawley rats (Harlan) weighing 300–350 g were used in all experiments. Before use, the animals were housed in a specific pathogen-free environment and allowed to acclimate to their surroundings for 1 wk. Standard rat chow and water were available to the animals ad libitum. All experiments were performed in accordance with the guidelines set forth by the National Institutes of Health for the care and use of animals. The experimental protocol was approved by the University Committee on Use and Care of Animals at the University of Michigan (Ann Arbor, MI).

Burn procedure

Under isoflurane anesthesia dorsal hair was closely clipped and the remainder removed using Nair depilatory cream (Church & Dwight). The rats were then placed in a prefabricated mold device with a rectangular opening that exposed the dorsal skin surface while protecting the remaining skin from burn exposure. The exposed skin surface was immersed in 60°C water for 40 s, producing a full-thickness dermal burn over 30% of the total body surface area. Sham animals underwent an identical procedure, except that they were immersed in room temperature water (24°C). The rats were immediately dried and resuscitated with lactated Ringer’s solution (4 ml/kg/percentage of total body surface area burn). One-half of the calculated resuscitation volume was given i.p. and the remaining volume was given as divided dose by s.c. injections immediately postburn. The animals also received buprenorphine at 0.1 mg/kg by s.c. injection every 8 h for the first 24 h following burn injury. Sham animals received the same resuscitation and analgesia treatment. Where indicated, animals received 500 µg of anti-C5a IgG Ab in 500 µl of sterile Dulbecco’s PBS solution by i.v. injection immediately after burn or sham injury. Control animals received a similar dose of preimmune IgG Ab. Rats were sacrificed at 1, 6, 12, 24, or 48 h after burn injury for in vivo and in vitro experiments.

Production of anti-C5a

The C-terminal end (aa residues 58–77) of the rat C5a molecule was coupled to keyhole limpet hemocyanin and used to immunize goats for the production of anti-sera. The polyclonal anti-C5a specific Ab was affinity purified and cross-reactivity with recombinant rat C5a was confirmed in Western blots.

In vivo measurement of left ventricular pressure (LVP)

Under deep isoflurane anesthesia the right carotid artery was exposed and a Mikro-Tip 2.5 French catheter (Millar Instruments) was advanced into the left ventricle. Correct positioning in the left ventricle was verified by fluoroscopy and the appearance of the characteristic pressure curve. Mean (LVPmean), maximum (LVPmax), and minimum (LVPmin) left ventricular pressure as well as heart rate (beats per minute) were recorded for 5 min using a signal transduction and amplification system connected to a standard Microsoft Windows operating system personal computer equipped with the appropriate recording and analysis software (PowerLab 8SP Base, Bridge Amp, Chart 5 Software; ADInstruments).

Cardiomyocyte isolation

Rats were anaesthetized i.p. with 1000 U of heparin sodium (Abbott Laboratories) and deeply anaesthetized with isoflurane anesthesia at the indicated time points. A bilateral thoracotomy flap was created and the heart was rapidly excised and rinsed in ice-cold Krebs-Henseleit buffer supplemented with 5 mM taurine. The heart perfusion and the CM isolation procedures were performed as described previously (14). Briefly, the distal aorta and pulmonary arteries were cannulated and mounted on a Langendorff perfusion system (ADInstruments) connected to a computer-based signal transduction/amplification system (PowerLab; ADInstruments). Retrograde coronary artery perfusion was performed using Krebs-Henseleit buffer. Enzyme digestion was then performed by using a calcium-free Krebs-Henseleit perfusion solution containing collagenase type II at 0.5 mg/ml (Worthington Biochemical) and hyaluronidase at 0.2 mg/ml (Sigma-Aldrich). After a gradual increase of calcium concentration the heart was removed from the cannula, gently minced, and the resulting CM pellet was resuspended in DMEM (Invitrogen Life Technologies). Cardiomyocyte concentration was determined using a hemacytometer and cell viability was assessed by trypan blue dye exclusion and cell morphology. Myocytes with a rod-like shape, clearly defined edges, and sharp striations were counted as viable cells, whereas cells with membrane bleeding, loss of striation pattern, and rounded cells were classified as nonviable. Cell suspensions with a viability of >75% were used for all subsequent experiments. The purity of the CM suspension and the possible contamination with leukocytes (neutrophil granulocytes, lymphocytes, and macrophages) was assessed with the FACScan flow cytometry system (BD Biosciences), revealing a purity of >99% CMs.

Plating of CMs and stimulation with LPS

Cardiomyocytes were plated onto sterile 22 × 22-mm glass coverslips precoated with 40 µg/ml natural mouse laminin (Invitrogen Life Technologies) at a density of 5 × 10^5 cells/coverslip/well and incubated in 6-well tissue culture plates (37°C at 5% CO2) for 3 h to allow for cell attachment. DMEM was then carefully pipetted off and replaced with 2 ml/well serum-free M199 medium supplemented with 10 mM glutathione, 0.2 mg/ml BSA (Invitrogen Life Technologies), 15 mM HEPES, and 26 mM NaHCO3. Bacterial endotoxin (LPS derived from Escherichia coli 0111:B4) was prepared by dissolving 25 mg of the lyophilized powder in 25 ml of M199 medium. This suspension was then sonicated and boiled for 10 min, and 10 µg of LPS per milliliter of M199 medium was added where indicated. The procedures involving single cell suspensions of CMs with or without LPS were placed in an incubator (37°C with 5% CO2) for an additional 3 h. All media and other reagents used for the CM isolation were certified endotoxin-free by the manufacturers.

Single-cell sarcomere contraction and relaxation analysis

Plated CMs that had been incubated for a total of 6 h underwent single-cell sarcomere contraction and relaxation analysis using a variable rate charge-coupled device video camera system (MyoCam; IonOptix) equipped with sarcomere length detection software (IonWizard; IonOptix). Microscopic imaging was performed using an inverted microscope system (Eclipse TE-2000S; Nikon) connected to the charge-coupled device video camera system. A coverslip with the plated CMs was placed in a prefabricated chamber that was filled with warm (37°C) M199 medium and mounted on the microscope system. The chamber system was connected to a Grass stimulator system. Electrical pacing stimulation was performed using a 100-mV stimulus of 4-ms duration and a frequency of 1 Hz. The measurement of sarcomere contraction at the different experimental conditions and the selection of the CMs from each coverslip were performed in a randomized fashion. For each measured CM, a rectangle-shaped region of interest was defined and sarcomeres within the focused region were selected for analysis; typically 15–20 sarcomeres. Sarcomere contractions were recorded for 75 s. The parameters measured using the IonWizard (IonOptix) software included peak sarcomere shortening, contraction velocity, and relaxation velocity.

Western blot analysis

Protein from CM lysates (50 µg; harvested at the indicated time points after burn injury) was separated by electrophoresis in a denaturing 10% polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. Equal loading was facilitated by protein estimation (BCA protein assay; Pierce) and confirmed by the detection of β-actin as the housekeeping protein. Nonspecific binding sites were blocked with TBST containing 5% nonfat dry milk for 1 h at room temperature. The membrane was then incubated with a rabbit anti-rat C5aR Ab at 1/500 dilution overnight. After washing, the membrane was incubated in a 1/5,000 dilution of HRP-conjugated donkey anti-rabbit IgG as the secondary Ab (Amersham Biosciences). The membrane was developed by the ECL technique according to the manufacturer’s protocol (Amersham Biosciences). The results were quantified using digital pixel density and Kodak ID image analysis software (Scientific Imaging Systems), normalized to β-actin, and expressed as a ratio of C5aR/β-actin pixel densities.

RNA isolation and detection of C5aR mRNA in CMs by real-time quantitative PCR analysis

Rat CMs were isolated as described at the indicated time points after burn injury. Total RNA was isolated using the TRizol method (Invitrogen Life Technologies) according to the manufacturer’s instructions. Digestion of any contaminating DNA was achieved by the treatment of samples with RQ1 RNase-free DNase at 0.2 U/µl (Promega). Reverse transcription was performed with 5 µg of RNA by using the SuperScript II RNase H reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s protocol. Real-time PCR was then performed with the following primers for...
Anti-C5a treatment after thermal injury preserves left ventricular function

Before the isolation of rat CMs the left cardiac ventricle was catheterized with a 2.5-F microcatheter and LVP values were recorded. Animals were injected i.v. with either 500 μg of anti-C5a IgG or preimmune IgG at time 0. LVP and heart rate values were recorded 1 and 24 h following burn or sham injury. At either time point the LVPmean, LVPmax, and LVPmin values were all significantly reduced in burn plus IgG compared with the sham-injured animals. Injection of an anti-C5a Ab at the time of the burn injury resulted in restoration of LVP similar to the levels of sham animals. Because similar results were obtained at both time points (1 and 24 h after burn injury), data for 24 h only are shown in Fig. 1. Interestingly, while LVP values were reduced in burn plus IgG compared with sham animals, anti-C5a IgG injection improved the burn-induced LVPmean as early as 1 h after injury and even normalized the pressure values back to baseline sham levels 24 h after burn (Fig. 1, A–C). It is important to note that anti-C5a treatment of sham animals had no effect on LVPmean, LVPmax, or LVPmin values at either time point, suggesting that the generation of C5a is necessary for the cardiac dysfunction to develop after burn injury. Heart rates did not differ significantly in any of the groups at either time point (Fig. 1D for the 24-h time point).

C5aR: 5'-TATAGTCTGCGCTCCTTCT-3' (5' primer) and 5'-TCAC CACCCACGCCGTCTGG-3' (3' primer). The primers were designed for 409-bp cDNA amplification in the middle region of the rat C5aR cDNA (positions 373–781). The primers for the “housekeeping” gene GAPDH were 5'-GGCTTCGCTCATAAGCAGATG-3' (5' primer) and 5'-CAG TAGACTCCAGCACATAC-3' (3' primer). Reactions were prepared in duplicate as 50-μl reactions using the iQ SYBR Green Supermix reagent (Bio-Rad). An amplification plot was generated using 2-fold dilutions of the cDNA generated from a known amount (1 μg) of mRNA. The cycle threshold (Ct) was set above the baseline fluorescence. Plotting the log of the dilutions vs the Ct values then generated a standard curve. Quantitation of C5aR and GAPDH in the samples was determined using the standard curves. Purity of the products was assessed by generating melting curves. Further, the PCR products were run out on a 1.2% agarose gel to confirm that the amplicons generated were single bands at the expected size. The C5aR to GAPDH ratios were then plotted for the various time points in burn and sham rats. Real-time PCR was performed using the Smart Cycler base (Cepheid). Ct values, standard curves, and melting curves were generated using the software provided by the manufacturer.
Anti-C5a preserves burn-induced peak sarcomere shortening after burn injury

We investigated the effects of burn injury and anti-C5a treatment (at the time of injury) on peak sarcomere shortening 1, 6, 12, and 24 h after burn or sham injury. The data demonstrate that the interception of C5a at the time of injury abolishes the burn-induced depression of peak sarcomere shortening. As early as 1 h following burn injury in burn plus IgG-treated rats, peak sarcomere shortening was markedly reduced (by \( \frac{1}{1022} 40\% \)) with a further decline at 6 and 12 h and a slight restoration 24 h after burn (Fig. 2A). Peak sarcomere shortening values for CMs isolated from burn plus anti-C5a animals were markedly improved compared with the values of burn plus IgG animals at all time points (Fig. 2A). Anti-C5a treatment resulted in normal sarcomere function by 24 h after burn injury.

Impaired CM contraction and relaxation velocities are restored by anti-C5a treatment

In addition to an evaluation of peak sarcomere shortening (Fig. 2A), we analyzed sarcomere contraction velocity (Fig. 2B) and relaxation velocity (Fig. 2C) values at 1, 6, 12 and 24 h after burn or sham injury. Both velocity parameters were significantly slower in CMs isolated from burn animals compared with cells derived from sham animals at all time points. In contrast to the in vivo findings described above, anti-C5a treatment of burn rats did not improve contractile velocities 1 h after injury (Fig. 2, B and C). Significantly slower contraction and relaxation velocities occurred in CMs isolated from burn plus IgG animals compared with their sham counterparts at 6, 12, and 24 h after burn (Fig. 2, B and C). However, at 6 and 12 h after burn injury anti-C5a treatment (at the time of injury) improved the contraction velocity as well as the relaxation velocity. By 24 h after burn, CMs from rats treated with anti-C5a had sarcomere shortening velocities comparable to levels recorded in CMs isolated from sham animals (Fig. 2, B and C). To rule out an intrinsic effect of anti-C5a on velocities, sham rats injected with anti-C5a were compared with sham rats injected with preimmune IgG; there was no difference in sarcomere shortening velocities between these two treatment groups.

Blockade of C5a partially abolishes burn-induced impairment of CM contractility after exposure to LPS in vitro

To investigate the effects of LPS on contractility parameters after burn injury, we exposed isolated CMs to 10 \( \mu \)g of E. coli-derived LPS per milliliter of medium in vitro. Exposure of CMs to LPS in vitro resulted in substantial deterioration of peak sarcomere shortening at all time points (1, 6, 12, and 24 h) after burn (Fig. 3) or sham injury (Table I) when compared with CMs not exposed to LPS (shown in Fig. 2). In the case of CMs isolated from burned animals, LPS led to an additional decrease in contractility beyond that caused by the burn injury itself. However, even after CM exposure to LPS, peak sarcomere contraction of CMs isolated from burn animals treated with anti-C5a was significantly improved compared with CMs isolated from burned animals treated with IgG.
at either time point (Fig. 3A). With regard to contraction and relaxation velocities, a blockade of C5a had no protective effect on CMs obtained 1, 6, or 12 h after burn injury and subsequent in vitro challenge with LPS (Fig. 3, B and C). In contrast, the infusion of anti-C5a improved velocity levels in CMs isolated 24 h after burn injury. The restoration of contractility parameters by treatment with anti-C5a was only partial and never reached the levels of the CMs that had not been exposed to LPS. Anti-C5a Ab administered to sham animals had no effect on peak sarcomere shortening of CMs stimulated with LPS (Table I).

To highlight and summarize the effects of burn injury, C5a blockade, and the additional exposure of CMs to LPS on contractility parameters, the results obtained at 24 h after burn injury are combined in Fig. 4. Without LPS (black bars), blockade of C5a abolished burn-induced impairment of CM contractility parameters (Fig. 4). Whereas LPS itself further impaired contractility (Fig. 4, open bars vs filled bars), a blockade of C5a significantly improves contractility even in the presence of LPS (open bars).

Expression of C5aR on CMs following burn injury
Both mRNA and protein for C5aR were measured in CMs from sham and burn animals as a function of time (Fig. 5). The mRNA for C5aR in extracts of isolated CMs from burned rats showed an increase starting 1 h after burn injury and continued to progressively increase up to 48 h after burn when compared with CMs

### Table I. Cardiomyocyte dysfunction after sham burn injury

<table>
<thead>
<tr>
<th>Group</th>
<th>In Vitro Addition</th>
<th>Peak Sarcomere Shortening (µm ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Sham plus IgG</td>
<td>None</td>
<td>0.159 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>0.117 ± 0.002 (-26%)b</td>
</tr>
<tr>
<td>Sham plus anti-C5a</td>
<td>None</td>
<td>0.153 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>0.114 ± 0.013 (-28%)b</td>
</tr>
</tbody>
</table>

a Data presented at hours 1, 6, 12, and 24 after sham injury are expressed as mean ± SEM.

b Statistical significance of LPS vs. no LPS, p < 0.05. Percentage values in parentheses are relative to animals without the addition of LPS. Similar results were obtained for parameters contraction velocity and relaxation velocity (data not shown).
obtained from sham animals (Fig. 5A).

Similarly, when homogenates from CMs were evaluated for C5aR protein by using Western blot analysis, there was an increase in a time-dependent pattern from 1 to 48 h after burn injury when compared with CMs obtained from sham animals (Fig. 5B).

C5aR expression was further examined by the immunostaining of frozen sections of the left ventricular wall to visualize the presence of C5aR on CMs (Fig. 6). Surface immunostaining for C5aR of heart sections obtained 24 h after burn injury revealed an intense staining of the edges of CMs (B, arrows) and much less staining of sham CMs (A), consistent with our Western blot and PCR data. When preimmune rabbit IgG was used as a negative control there was very little staining of either burn injury or sham CMs (not shown).

Discussion

Impaired cardiovascular function following thermal injury is a central risk factor for the development of multiorgan dysfunction (19). Initially, this was attributed to the loss of blood, plasma fluid shifts, decreased venous blood return, and reduced cardiac filling. However, the presence in burn patients of a “serum myocardial depressant factor” suggests another possible cause of cardiac dysfunction (20). Myocardial dysfunction following thermal injury is characterized by slowed isovolemic relaxation, impaired contractility, and decreased diastolic compliance of the left ventricle (1, 2). Our own and other studies have revealed that impaired cardiac function is detectable as early as 1 h after burn injury and reaches a nadir at 24–30 h after the insult (15, 21, 22). From a clinical standpoint, impaired cardiac function may pose a grave threat to burn patients, especially to those who are elderly or immunocompromised.
Several mediators have been proposed to be involved in the development of cardiac dysfunction after burn injury. Prolinflammatory cytokines (such as TNF-α, IL-1β, and IL-6) have been described as cardiodepressant agents (21–23). Reactive oxygen intermediates and macrophage migration inhibitory factor (MIF) have been shown to exert cardiodepressant effects following thermal injury (24). To date, the potential role of complement activation products in the setting of cardiac dysfunction following burn injury has not been investigated. It is well known that burn injuries lead to extensive activation of the complement system, producing C5a and other products (11, 25). Recently, we demonstrated the expression of the C5a receptor, C5aR, on isolated rat CMs. We showed that experimental sepsis resulted in the increased expression of C5aR on CMs in a time-dependent manner (14). The interaction of C5a with C5aR resulted in an impaired cardiac function, as measured in vivo and in vitro, that was restored by a blockade of C5a.

In the present study, Western blotting and real-time PCR of isolated rat CMs (as well as immunostaining of the ventricular wall) revealed a clearly up-regulated expression of C5aR on CMs following thermal injury, setting the stage for interaction with its ligand, C5a. Although the intracellular downstream signaling effects after C5a-C5aR interaction are not well understood, it is possible that the increased expression of C5aR after thermal injury affects the performance-on-demand of CMs. However, excessive and unregulated generation of C5a, which impairs contractility as seen in sepsis, results in defective cardiac function (18). Analogous to this would be our studies in sepsis in which low levels of C5a prime neutrophils for enhanced innate immune functions while high levels of C5a cause a loss of MAPK signaling pathways and greatly impaired innate immune function (26).

Focusing on effects of the blockade of C5a, we provide evidence that infusion of anti-C5a improved LVP values in vivo at both 1 and 24 h after burn injury. LVP values at 24 h (and to a lesser extent at 1 h) after burn injury in anti-C5a treated rats were restored to levels comparable to those found in sham rats. This suggests that, despite the rapid activation of the complement system after burn injury, limited beneficial effects of C5a blockade occur on the cellular level as early as 1 h, whereas it clearly takes longer (24 h) to fully engage the protective impact of anti-C5a. Our previous cellular studies have shown that the interaction of C5a-C5aR on CMs is a major contributor to the reduced LVP observed in vivo during sepsis (14). C5a and C3a have previously been shown to exert profound vasodilatative effects on the systemic and coronary vasculatures that might exacerbate the myocardial contractile deficits in sepsis and partially explain the low systemic blood pressure values seen in septic humans and animal models (27, 28). Using a functional analysis approach, we now provide evidence that blockade of C5a-C5aR interaction leads to a significant improvement of cardiac function after burn injury as reflected in preserved LVP mean, LVP max, and LVP min values comparable to those of sham animals, especially 24 h after burn injury. These results point to the prominent role of C5a-C5aR interaction at the CM level following thermal injury.

The protective effects of blocking C5a in vivo after burn injury prompted us to determine whether similar treatment would have beneficial impacts on the in vitro contractility parameters of CMs. We show that CMs isolated from burn animals exhibit significantly decreased peak sarcomere shortening as well as reduced contraction and relaxation velocities when compared with CMs from sham animals. Our analysis is sarcomere specific and independent of cell size and provides results comparable to the method of a cellular edge detection shortening measurement used in a prior study (29). In accord with our in vivo data, anti-C5a treatment of burned rats reversed the burn-induced reduction in sarcomere contractility. Similar to the effects seen in LVP values, complete restoration of peak sarcomere shortening to levels comparable with those of CMs from sham animals was observed at 24 h after burn injury, whereas a blockade of C5a only partially improved contractility at 1, 6, and 12 h after injury. This confirms our paradigm that, despite the early activation of the complement system, other intracellular pathways need to be activated first before the full effect can be seen. Further evidence to support this paradigm was obtained when we analyzed contraction and relaxation velocities. Blockade of C5a had no effect on these parameters early at 1 h after burn injury, while both parameters reached levels comparable to those of sham animals 24 h after injury. In other words, the ultimate deterioration in CM function after burn injury may be directly linked to the effects of C5a on CMs as well as to the enhancing effects of C5a on mediators that will also depress cardiac function (e.g., TNF-α, IL-1β, IL-6, MIF, etc.) (30).

Another objective of this study was to investigate the extent to which the exposure of single CMs from burn animals to LPS would lead to impaired contractility and whether these changes could be attenuated by in vivo blocking of C5a. Although burn wounds are initially aseptic, the burn injury disrupts the normal barrier function of the skin against bacterial invasion. The open and damaged tissues of a burn wound, in conjunction with a naturally laden environment, ensure a high likelihood of bacterial wound invasion and LPS exposure. Furthermore, the severe physiologic derangements following burn injury can increase bacterial translocation from the gastrointestinal tract (31–33). As a result, myocardial function may be worsened by the effects of endotoxin in addition to the burn injury itself. LPS, a glycolipid molecule located within the wall of Gram-negative bacteria, has been shown to induce hemodynamic compromise and shock (34). Several studies have reported significantly impaired myocardial contractility following the infusion of LPS (35, 36). To mimic a Gram-negative bacterial superinfection (e.g., burn wound infection), we incubated isolated CMs with LPS to evaluate the potential additional effects on CM performance after burn injury. We show that LPS significantly decreased contractility parameters in sham animals, confirming earlier studies and the validity of our model. However, when analyzing CMs from burn animals we found that incubation with LPS reduced contractility even further when compared with burn animals without an in vitro exposure to LPS. Further, this LPS-induced effect was not restored completely by the administration of anti-C5a. The dose of 10 μg of LPS per milliliter of medium used in our in vitro CM studies is very high in comparison to the stimulation of other cells (e.g., macrophages, neutrophils) with LPS. However, it is known from our own studies as well as from other groups that high levels of LPS are needed for primary cultures of CMs to see effects on contractility or cytokine production (15, 37, 38).

It is now well established that LPS binding and signaling occurs through the TLR4/CD14/MD2 complex, which is expressed on the surface of CMs (39, 40). Recently, it has been reported that the binding of LPS to TLR4 on rat CMs clearly contributes to myocardial depression (41). We now provide evidence that similar pathways are activated following thermal injury, leading to impaired cardiac contractility. The complement system and the TLRs are two major factors of the innate immune system that are often activated upon contact with most pathogens. There are no published data that definitively support a direct linkage or intersection between the C5aR and TLR4.
pathways (16, 42, 43). However, there are several reports that indicate the ability of C5a to alter the outcomes of LPS-induced activation of phagocytic cells (16, 26, 44, 45). Recently, it has been reported that the induction of the IL-12 family of cytokines in activated macrophages was suppressed in the expression of C5a, potentially setting the stage for the immunosuppressive effects seen in sepsis (18). Collectively, these data indicate that C5a interacting with C5aR can alter LPS-triggered responses in a variety of cell types. The findings from our study suggest that the cellular mechanisms mediating CM response to C5a or endotoxin and leading to depressed contractility are different but may partially overlap. It seems clear that some of the CM dysfunction resulting from burn injury is C5a dependent. When active, this C5a-mediated suppression pathway is enhanced by LPS exposure. However, LPS-induced contractile defects are at least in part independent of the effects of C5a.

In search of other mechanisms to explain cardiac dysfunction, “reversible hibernation”, altered calcium handling, and uptake by the sarcoplasmatic reticulum have been proposed (29, 46–48). Furthermore, MIF, a known cardio-depressant factor, is released during various immunoinflammatory states (3). We have previously reported that C5a plays an important role in enhancing MIF release from neutrophils, which may explain the restored cardiac performance by a blockade of C5a after burn injury (44). Also, we and others have reported that C5a induces the phosphorylation of p38 and other MAPKs as well as the up-regulation of NF-κB (17, 26). Therefore, the cardioprotective effects seen after a blockade of C5a may be partially due to inhibition of these intracellular signaling pathways.

In summary, the expression of C5aR is increased following burn injury and the blockade of C5a in burn animals restores cardiac contractility indices in vivo and in vitro. In the light of increased levels of C5a following burn injury combined with the known detrimental effects of C5a on contractility parameters in vitro, our data support the concept that specific C5a-C5aR interaction occurs in CMs, resulting in impaired cardiac performance (11, 14). C5a-induced, impaired cardiac function resulting in decreased end-organ perfusion and oxygen supply to tissue may be a crucial mechanism in the development of multiorgan dysfunction following burn injury.

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Disclosures
The authors have no financial conflict of interest.

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