An essential role for complement C5a in the pathogenesis of septic cardiac dysfunction

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Defective cardiac function during sepsis has been referred to as “cardiomyopathy of sepsis.” It is known that sepsis leads to intensive activation of the complement system. In the current study, cardiac function and cardiomyocyte contractility have been evaluated in rats after cecal ligation and puncture (CLP). Significant reductions in left ventricular pressures occurred in vivo and in cardiomyocyte contractility in vitro. These defects were prevented in CLP rats given blocking antibody to C5a. Both mRNA and protein for the C5a receptor (C5aR) were constitutively expressed on cardiomyocytes; both increased as a function of time after CLP. In vitro addition of recombinant rat C5a induced dramatic contractile dysfunction in both sham and CLP cardiomyocytes, but to a consistently greater degree in cells from CLP animals. These data suggest that CLP induces C5aR on cardiomyocytes and that in vivo generation of C5a causes C5a–C5aR interaction, causing dysfunction of cardiomyocytes, resulting in compromise of cardiac performance.

Sepsis and septic shock are complex and therapeutically challenging disorders of the immune system. Organ dysfunction during sepsis is a life-threatening and extremely cost-intensive clinical problem. Organ dysfunction during sepsis is a life-threatening and extremely cost-intensive clinical problem affecting ~600,000 patients annually in the United States, with an associated mortality rate ranging from 20 to 60% (1–3). Despite tremendous research efforts over the last 20 yr, sepsis remains the leading cause of death in intensive care units. Most importantly, myocardial dysfunction puts septic patients at high risk to develop multi-organ failure, which is associated with a high mortality. Multi-organ failure results from a “vicious cycle” initiated by impaired cardiac function, decreased cardiac output leading to compromised tissue/organ perfusion, decreased oxygen and nutrient supply, ischemia, organ dysfunction, and a hypo- or hyper-reactive immune system (6). Therefore, cardiac dysfunction plays a pivotal role and is often decisive in determining survival or death.

In light of the multifactorial pathogenesis of sepsis and septic shock, extensive work has been done to characterize the numerous agents and mediators that could cause myocardial dysfunction (7–13). A “myocardial depressant substance” in the serum of septic patients has been proposed to account for the cardiac dysfunction observed during the hypodynamic phase of sepsis (7). Activation of the complement system...
system is a hallmark of sepsis that leads to robust generation of potent proinflammatory complement factors. Among those factors, C5a is one of the most potent inflammatory peptides (14–17). There is accumulating evidence that C5a–C5a receptor (C5aR) signaling plays an essential role in septic shock (18). In earlier work, we demonstrated that blockade of either C5a or C5aR greatly improves survival in sepsis after cecal ligation and puncture (CLP) in rodents (19, 20). Furthermore, we have demonstrated that anti-C5a treatment resulted in decreased levels of bacteremia, preservation of innate immune functions of blood neutrophils, greatly reduced thymocyte apoptosis, and improvement in the overall survival in the rat model of CLP-induced sepsis (19–22).

The contribution of the complement system to septic cardiomyopathy has not been studied. Recently, we showed that C5aR expression is significantly elevated in whole heart homogenates (based on in vivo binding studies of anti-C5aR IgG and immunostaining), perhaps setting the stage for C5a-induced organ dysfunction (23). However, the expression of C5aR on the cell surface of cardiomyocytes has not been determined. The present study was designed to evaluate the impact of C5a and C5a blockade on septic cardiac dysfunction both in vivo and in vitro. To investigate the effects of anti-C5a on preventing cardiomyocyte contractility deficits, we used the rat sepsis model (CLP) to examine in vivo left ventricular function and in vitro single cardiomyocyte sarcomere contractile performance.

RESULTS
Expression of C5aR on cardiomyocytes
Both mRNA and protein for C5aR were measured in cardiomyocytes from sham rats and CLP rats as a function of time after surgery. As shown in Fig. 1, mRNA for C5aR in extracts of cardiomyocytes from CLP rats showed progressive increases 12, 24, and 48 h after CLP, whereas any changes in C5aR mRNA in sham cardiomyocytes were much less evident. When homogenates from cardiomyocytes were evaluated by Western blot analysis, there were progressive increases 6–48 h after CLP (Fig. 2). Cardiomyocytes from sham surgery rats showed no increases in C5aR expression (unpublished data).

The studies were extended by the rise of immunostaining of cardiomyocytes for C5aR protein (Fig. 3). Confocal immunostaining revealed intense cortical staining in cardiomyocytes 12 h after CLP (Fig. 3 A), whereas cells obtained from sham animals show evidence of constitutive expression (Fig. 3 B) that was much less intense. Surface immunostaining of individual cardiomyocytes for C5aR proteins revealed intense staining of cardiomyocytes obtained 12 h after CLP (Fig. 3 C) and much less staining of sham cardiomyocytes (Fig. 3 D), consistent with the confocal staining data. When irrelevant rabbit IgG was used, there was very little staining of either CLP or sham (Fig. 3, E and F) cardiomyocytes, respectively.

Reduced left ventricular pressure (LVP) in septic animals and functional preservation by in vivo C5a blockade
LVP was recorded in CLP and sham-operated rats treated with either anti–C5a or an equivalent amount of nonspecific IgG. The results are shown in Fig. 4. Rats subjected to CLP developed significantly lower mean LVPs than their sham-treated counterparts (P < 0.001, analysis of variance [ANOVA]) (Fig. 4 A). Antibody-induced in vivo blockade of C5aR, which was given intravenously immediately after CLP, resulted in greatly improved mean, maximum, and minimum LVPs (LVP\textsubscript{mean}, LVP\textsubscript{max}, LVP\textsubscript{min}) when compared with septic rats treated with nonspecific IgG (P < 0.001, ANOVA). Injection of either anti–C5a or preimmune IgG into sham rats had no effect on LVP values. No differences were found in measured heart rates between the various groups (unpublished data). These data indicate that in vivo myocardial dysfunction is abolished by blockade of C5aR in septic animals when compared with sham animals. Whether the mean LVP, the maximum LVP, or the minimum LVP values were used, the results were the same in all cases.

Evidence for C5a-dependent cardiomyocyte dysfunction after CLP
We next investigated whether polymicrobial sepsis (CLP) influences cardiomyocyte sarcomere contractility. Baseline sarcomere length values did not differ significantly among all
cardiomyocytes analyzed. Cardiomyocytes from CLP-injured animals demonstrated an ~50% decrease in relative peak sarcomere shortening compared with sham animals (peak height CLP + nonspecific IgG 0.099 ± 0.005 μm vs. sham 0.201 ± 0.008 μm, P < 0.001, ANOVA, Fig. 5 A).

Analysis of single cell cardiomyocyte sarcomere shortening revealed a substantial improvement in peak shortening of cells isolated from CLP animals treated with anti-C5a compared with CLP treated with nonspecific IgG (peak height CLP + anti-C5a IgG, 0.168 ± 0.007 μm vs. CLP + nonspecific IgG, 0.086 ± 0.005 μm, P < 0.001, ANOVA, Fig. 5 A). To assess whether the effects of anti-C5a were injury dependent, we also included a group of rats that underwent sham operation and received the blocking antibody intravenously. Relative peak sarcomere shortening values were not different after administration of IgG or anti-C5a in sham animals (Fig. 5 A). These results demonstrate that the presence of C5a, which is generated during sepsis, is linked to cardiomyocyte dysfunction of CLP.

In addition to relative peak sarcomere shortening, normalized maximum contraction and relaxation velocities were determined. Interestingly, infusion of anti-C5a greatly improved the maximum velocities of contraction (−dL/dt) and relaxation (+dL/dt) comparable to those measured in sham animals (Fig. 5, B and C). Cardiomyocytes from CLP + anti-C5a animals contracted significantly faster than animals treated with nonspecific IgG (CLP + anti-C5a 27.98 ± 0.92 s⁻¹ vs. CLP + nonspecific IgG 22.68 ± 0.41 s⁻¹, P < 0.001, ANOVA) (Fig. 5 B). Similar results were obtained for values of relaxation velocities (+dL/dt) (Fig. 5 C). Cardiomyocytes derived from CLP + anti-C5a animals exhibited significantly faster relaxation velocity than cells isolated from CLP + IgG animals (CLP + anti-C5a 24.35 ± 0.91 s⁻¹ vs. CLP + nonspecific IgG 18.58 ± 0.42 s⁻¹, P < 0.001, ANOVA). Furthermore, maximum contraction and relaxation velocities occurred significantly earlier in CLP animals treated with anti-C5a than in rats infused with nonspecific IgG (Fig. 5, D and E). These data indicate that CLP induces C5a-dependent dysfunction in cardiomyocytes and that blockade of C5a reverses these changes.

**Time-dependent changes in cardiomyocytes contractility after CLP**

Cardiomyocytes were obtained as a function of time after sham surgery or after CLP, as described in Fig. 6. As shown in Fig. 6 A, contractility values in the sham cardiomyocytes not otherwise treated were essentially stable as a function of time (0–48 h after sham surgery). In striking contrast, as shown in Fig. 6 B, there was a progressive decrease in sarcomere shortening 6, 12, and 24 h after CLP, with a slight improvement at 48 h. When sham cardiomyocytes were exposed to either 5.0 or 10.0 nM recombinant rat (rr)C5a, there was a progressive lost of contractility. With an ~30% reduction in contractility after incubation with 5.0 nM C5a and a 53% reduction after exposure to 10.0 nM C5a. In the case of CLP cardiomyocytes, as shown in Fig. 6 B, there was a very substantial loss of contractility which was progressive with time up to 24 h after CLP. The addition of 5.0 nM of...
C5a caused further reductions and greater reductions occurred with exposure to 10.0 nM C5a. Thus, it appears that both sham cardiomyocytes as well as CLP cardiomyocytes show adverse effects after exposure to C5a, but with greater decrements in function in CLP cardiomyocytes.

Effects of duration of in vitro exposure of cardiomyocytes to C5a

For these studies, cardiomyocytes were isolated from sham rats as well as CLP rats 24 h after sham surgery or CLP. Thereafter, they were exposed in vitro to 10.0 nM C5a for 0–60 min and the changes on contractility were measured immediately after the indicated exposure time. As shown in Fig. 6 C, with no exposure to C5a, the expected significant differences in contractility between sham and CLP cardiomyocytes were verified. In general, contractility measurements diminished as a function of time of exposure to C5a, especially up to 30 min, seeming to approach a plateau thereafter. Thus, the effects of C5a on induction of contractility defects in cardiomyocytes seem to require no more than an exposure time of 30 min.

In companion studies, sham or CLP cardiomyocytes were exposed to C5a for either 15 min or 3 h, followed by washing, and contractility measurements were performed. Similar to the data in Fig. 6, there were decrements in cardiomyocyte contractility after exposure to C5a. There was no difference in the reductions in contractility in cardiomyocytes...
exposed for only 15 min versus 3 h (unpublished data). These data suggest that the interaction of C5a with cardiomyocytes occurs rapidly and that removal of the C5a soon thereafter will not prevent the progressive loss in contractility.

**DISCUSSION**

**The complement system and septic cardiomyopathy**

During sepsis and septic shock, cardiac function is often impaired, placing the patient at risk for multi-organ dysfunction and eventual death. The complement system is activated during sepsis, resulting in generation of potent proinflammatory factors, which may contribute to impaired function of the immune system and vital organs. To what extent complement activation products can be linked to cardiac dysfunction during sepsis is not known. In an endotoxin shock model, C3 deficiency was associated with a decreased left ventricular ejection fraction (24). The present study investigated possible protective effects of blocking the potent complement activation product, C5a, on cardiac performance during experimental sepsis. Our results demonstrate for the first time that the C5aR is expressed on the surface of single cardiomyocytes obtained from septic and sham animals. C5aR content was higher on CLP cardiomyocytes. Furthermore, we provide evidence that in vivo interception of C5a (with anti-C5a) given at the time of onset of CLP significantly improved cardiac performance both in vivo (Fig. 4), as reflected by restored levels of LVP (LVP<sub>mean</sub>, LVP<sub>max</sub>, LVP<sub>min</sub>), and in vitro as reflected by significantly restored peak sarcomere shortening levels and improved contractile velocities (±dL/dt, Fig. 5).

The current literature provides evidence for complement activation in humans and animals with septic disorders based on elevated plasma levels of C3a, C4a, and C5a (14–17). Excessive generation of complement anaphylatoxin, C5a, and its interaction with C5aR induces a plethora of cellular stress response mechanisms such as the modulation of cytokine expression, apoptosis, and activation of the coagulation pathway (22, 25–28). Previously, our laboratory showed that C5a generation during sepsis plays a key role in the regulation of various pathophysiologic changes that can be reversed by blockade of C5a (19, 20, 22). Anti-C5a treatment and complement depletion with cobra venom factor led to an increased oxygen extraction ratio and oxygen consumption in a porcine model of sepsis (29). Septic cardiomyopathy is a key pathophysiologic event during the course of sepsis and it frequently determines whether the patient survives or dies. Given the immense socioeconomic impact of sepsis in terms of morbidity, mortality, and cost to the health system (estimated $16.7 billion per year), the development of novel therapeutic strategies for septic cardiomyopathy is clearly warranted (30).

**Constitutive and up-regulated expression of C5aR**

Western blotting (for C5aR protein) and real-time PCR (C5aR mRNA content) of cardiomyocyte lysates revealed characteristic bands indicating expression of C5aR on rat cardiomyocytes. Constitutive expression of C5aR on cardio-

**myocytes was found in both CLP and sham animals, but higher levels were seen in cardiomyocytes obtained from CLP rats. This is in contrast with our findings in neutrophils where surface C5aR expression on PMNs was down-regulated in septic animals compared with shams (20). These contrasting findings underline the polyfunctional properties of the C5a–C5aR system. The differences in the C5aR status in blood neutrophils and cardiomyocytes after CLP may simply reflect a higher concentration of C5a in blood than in the extravascular compartment. The central role of the neutrophil for innate immune function necessitates the presence and activation of neutrophils in the face of invading microorganisms. Interference in this system because of excessive C5a production has been shown to compromise host defenses (20). In contrast, constitutive expression of C5aR on the cardiomyocyte surface ensures the rapid initiation of cellular stress response mechanisms (alteration of intracellular calcium, etc.). When excessive C5a generation occurs as in sepsis, these potentially protective mechanisms may turn into detrimental derangements both at the functional and cellular level. Additional support for this hypothesis was obtained by in vitro incubation of cardiomyocytes with C5a. The dose of 10.0 nM dramatically impaired contractility parameters in cardiomyocytes after sham surgery (Fig. 6 A). These data correlate with the serum levels of C5a found in septic humans, which have been shown to range between 1.0 and 15.0 nM (14, 15). Similarly, 10.0 nM C5a abolished the already impaired contraction of cardiomyocytes from septic animals (unpublished data).

In summary, we provide evidence for constitutive and enhanced expression of C5aR on rat cardiomyocytes, which underscores the functional importance of the C5a–C5aR system. This may explain the early onset of septic myocardial dysfunction and suggests potential therapeutic options based on systemic interception of C5a or C5aR. Constitutive C5aR expression does not appear to be harmful under baseline physiological conditions.

**Effect of C5a blockade on cardiac function in vivo**

CLP reproduces many of the characteristics of polymicrobial sepsis found in humans (31). We investigated alterations in cardiac function during the hypodynamic phase of sepsis, which usually occurs 20–24 h after CLP (32), when hemodynamic parameters are decreased (33). In animal models, sepsis-induced deficits in cardiac contractility have been controversial. Decreased parameters of contractility independent of preload and changes in the myocardial structure have been reported in ex vivo studies on Langendorff-perfused hearts (34). In contrast, no changes of cardiac contractility parameters or myocardial ultrastructure were reported during the hypodynamic phase of sepsis (35). Using a rat CLP model, a reduction in cardiac contractility parameters such as LVP and ±dP/dt has been described previously (36). Our results demonstrate significantly lower LVP values in CLP animals compared with shams, suggesting severely impaired cardiac contractility in sepsis. We provide evidence that infusion of
anti-C5a prevented impairment of LVP in vivo in CLP animals. Our cellular studies show that the interaction of C5a–C5aR on cardiomyocytes is a major contributor to the reduced levels of LVP observed in vivo during sepsis. C5a and C3a have previously been shown to exert profound vasodilatative effects on the systemic and coronary vasculature, which might exacerbate the myocardial contractile deficits in sepsis and partially explain the low systemic blood pressure values seen in septic humans and animal models (37, 38).

Using a functional analysis approach, we found that blockade of C5a–C5aR interaction led to a significant improvement of cardiac function, as reflected in restored mean LVP and max/min LVP values comparable to sham animals. These results support the importance of C5a–C5aR interaction at the cardiomyocyte level in septic cardiomyopathy.

Effect of C5a blockade on cardiomyocyte function in vitro
A major finding in the present study is that cardiomyocytes isolated from CLP animals exhibited significantly decreased sarcomere contractility parameters (peak sarcomere shortening, ±dL/dt) compared with sham animals. At the sarcomere level, our study showed myocellular contractile deficits during CLP-induced sepsis. In our analysis, we used a standardized area that included at least 15–20 sarcomeres and acquired real-time sarcomere-shortening values. This technique is sarcomere specific and independent of cell size compared with the cellular edge detection shortening measurement used in a prior study (39).

We sought to determine the role of C5a toward inhibition of sarcomere shortening in sepsis. Confirming our in vivo data, anti-C5a treatment of septic rats completely reversed the sepsis-induced reduction in sarcomere contraction and resulted in cardiomyocyte function comparable to that found in sham animals. Coupled with our finding of constitutive expression of C5aR on cardiomyocytes and the detrimental effect of C5a incubation on mice on contractility parameters, these data strongly suggest that specific C5a–C5aR interaction occurs in septic rat cardiomyocytes, leading to impaired cardiac performance. In agreement with our already demonstrated beneficial effects of anti-C5a on survival after CLP (19), improved cardiomyocyte contractility resulting in improved end-organ perfusion and oxygen supply may be the mechanistic cornerstone leading to improved survival rates.

Further studies are needed to evaluate intracellular signaling pathways involved after C5a–C5aR interaction in impaired myocardial function. Various mediators such as reactive oxygen intermediates and proinflammatory cytokines (TNF-α, IL-1β, IL-6) have been proposed to be myocardial depressant substances underlining that upstream modulation, such as interception of the C5a–C5aR interaction, might be useful to improve cardiac dysfunction during sepsis (8, 11, 40, 41). Because intracellular calcium is one of the most important factors for coordinated and efficient contractility, various studies have focused on alterations in calcium fluxes in cardiomyocytes. It has been suggested that septic cardiomyocyte dysfunction may be linked to altered calcium transient properties (39). Recently, an abnormal Ca²⁺ leakage from the sarcoplasmic reticulum has been found and may contribute significantly to the depressed cardiomyocyte shortening in sepsis (42). The interaction between phospholamban and sarcoplasmic reticulum Ca²⁺-ATPase2a (SERCA2a), two major modulators of calcium-dependent contractility, has been shown to play a central role in the pathogenesis of congestive heart failure and septic myocardial dysfunction (43, 44). Therefore, it is intriguing to hypothesize that C5a–C5aR interaction may affect intracellular calcium handling, possibly via modulation of SERCA2a activity leading to functional deficits. Our in vitro results support this theory. The maximum contraction and relaxation velocities are significantly higher in cardiomyocytes from CLP rats treated with anti-C5a when compared with infusion of nonspecific IgG. The improved contraction and relaxation velocities and peak shortening levels may indicate improved intracellular calcium handling.

There are suggestions that TNFα may contribute to cardiac dysfunction after sepsis or endotoxemic shock (41). After injection of LPS, circulating levels of TNFα reached 10 ng/ml, resulting in evidence of cardiac dysfunction (45). Our own studies of CLP in rats suggest extremely low levels of TNFα in the serum (<5 pg/ml) (46). Accordingly, on the basis of the CLP model used in the current studies it seems unlikely that TNFα is responsible for the cardiomyocyte defects.

In summary, we have shown that rat cardiomyocytes constitutively express C5aR. We show in vivo and in vitro evidence of the beneficial effects of anti-C5a treatment, leading to reversal of septic cardiomyopathy. These data indicate a possible novel and promising approach for the treatment of patients with septic cardiomyopathy, using interventions directed either at systemic C5a interception or blockade of C5aR.

MATERIALS AND METHODS
Reagents. Unless otherwise indicated, all reagents were purchased from Sigma Aldrich.

Experimental animals. Adult male Sprague-Dawley rats (Harlan Inc.) weighing 300–350 g were used in all experiments. 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 during the course of the experiment ad libitum. All experiments were performed in accordance with the guidelines set forth by the National Institutes of Health for Care and Use of Animals. Approval for the experimental protocol was obtained from the University Committee on Use and Care of Animals at the University of Michigan.

Experimental sepsis. Sepsis was induced by the CLP procedure as described previously (31). In brief, rats were anesthetized with isoflurane (2–3%, oxygen flow 3L O2/min). After a midline incision, the cecum was exposed and ligated ∼2/3 of the distance from the distal pole. The ligated cecum was punctured through and through with an 18-gauge needle and a small portion of feces was expressed. The abdomen was closed in layers using 4–0 sutures (Ethicon Inc.) and metallic clips. Sham animals underwent the same procedure except for ligation and puncture of the cecum. Where indicated, animals received 500 mg/kg anti-C5a antibody in 500 μl sterile DPBS by intravenous injection immediately after CLP. Control animals received a similar dose of nonspecific IgG antibody. Rats were killed at 6, 12, 24, or 48 h after CLP for in vivo and in vitro experiments.
Production of anti-C5a. The COOH-terminal end (amino residues 58–77) of the rat C5a molecule was coupled to keyhole limpet hemocyanin and was used to immunize goats for the production of anti-sera (47). The polyclonal anti-C5a specific antibody was affinity purified, and cross-reactivity with rC5a was confirmed in Western blots.

In vivo measurement of LVP. Under isoflurane anesthesia, the left carotid artery was exposed, the left vagus nerve was dissected and freed from the carotid artery, and microclips were positioned to gain proximal and distal vascular control. A 2.5 French microtip catheter (Millar Instruments) was inserted into the left carotid artery and 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) LVP 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 PC equipped with the appropriate recording and analysis software (PowerLab 8SP Base, Bridge Amp, Chart 5 Software, ADInstruments).

Isolated perfused hearts (Langendorff system) and cardiomyocyte isolation. CLP or sham-operated rats were anaesthetized using heparin sodium (100 U i.p.) (Abbott Laboratories) and deeply anesthetized using isoflurane (3%). At 12 or 24 h after CLP, the heart was rapidly excised and rinsed in ice-cold, sterile Krebs-Henseleit buffer (containing [in mM] 118 NaCl, 4.7 KCl, 21 NaHCO3, 1.8 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 11 glucose, pH 7.4) supplemented with 5 mM 2-aminoethanesulfonic acid (taurine). The aortic stump was cannulated and the heart was mounted on a Langendorff perfusion system (ADInstruments Inc.). Cannulation and initiation of heart perfusion took place within 60 s of removal of the organ from the animal. Retrograde perfusion was initiated with calcium-containing (1.2 mM) Krebs-Henseleit buffer for 5 min to stabilize the heart on pump. Flow rate was set to 7.5 ml/min, temperature was kept at 38°C (±0.5°C), and perfusion pressure was continuously monitored. Perfusion with calcium-free Krebs-Henseleit buffer was performed for 3 min, and the flow rate increased to 9.0 ml/min. Collagenase type II (Worthington Biochemical Corp.) and hyaluronidase type II were added and the hearts were perfused as described previously (48). Cardiomyocytes were counted using a hemocytometer, cell viability was assessed by trypan blue dye exclusion, and cell morphology. Myocytes with a rod-like shape, clearly defined edges and sharp strations were counted as viable cells, whereas cells with membrane bleeding, loss of stration pattern and rounded cells were classified as nonviable. Cell suspensions with a viability of >75% were used for all subsequent experiments.

Purity of the cardiomyocyte suspension and possible contamination with leukocytes (neutrophil granulocytes, lymphocytes, macrophages) was assessed with FACSscan Flow Cytometry System (Becton Dickinson). Using the size of a known reference population of leukocytes for comparison, cardiomyocytes were gated by size with the typical forward and side light scatter profiles revealing a purity of >99% cardiomyocytes.

Cells were plated onto laminin-coated (Invitrogen), sterile 22 × 22 mm glass coverslips at a density of 5 × 104 cells/coverslip/well. Subsequently, coverslips were cultured in six-well culture plates (37°C, 5% CO2) in DMEM containing 50 U/ml penicillin, 50 µg/ml streptomycin (pen/strep), and 5% fetal calf serum to allow for cell attachment and adhesion to the laminin matrix. Media was then replaced with serum-free M199 media supplemented with 10 mM glutathione, 0.2 mg/ml BSA (GIBCO BRL), 15 mM Hepes (Sigma-Aldrich), and 26 mM NaHCO3 and placed in an incubator (37°C, 5% CO2) for 3 h. All media and other reagents used for the cardiomyocyte isolation were certified endotoxin-free by the manufacturers.

Single cell sarcomere contraction and relaxation analysis. Plated cardiomyocytes that had been incubated for 3 h underwent single cell sarcomere contraction and relaxation analysis using a variable rate CCD video camera system (MyoCam, IonOptix Corp.) equipped with sarcomere length detection software (IonWizard, IonOptix Corp.). A coverslip with the plated cardiomyocytes was placed in a prefabricated chamber, which was filled with warm (37°C) M199 and mounted on the microscope stage (Eclipse; Nikon Corp.) connected to the CCD video camera system. The chamber system was connected to a stimulator system (Grass Inc.) and electrical pacing stimulation was initiated (100 mL, 4 ms, 1 Hz). A total number of 1,247 cardiomyocytes were analyzed. Measurement of cardiomyocyte function, by groups, was performed in a randomized fashion if different experimental groups were present. A rectangle-shaped region of interest within each randomly chosen cardiomyocyte was then defined and sarcomeres within the focused region were selected for analysis. Typically, this region included ~15–20 sarcomeres. Cardiomyocyte contractile parameters (relative peak sarcomere shortening, normalized maximum contraction velocity –dL/dt, normalized maximum relaxation velocity +dL/dt, time of –dL/dt and +dL/dt) were recorded for 75 s.

Western blot analysis. Total protein from cardiomyocyte lysates (50 µg, harvested 0, 6, 12, 24, or 48 h after CLP or sham operation) 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 Chemical Co.) and confirmed by detection of β-actin as housekeeping protein. Non-specific binding sites were blocked with TBST (40 mM Tris, pH 7.6; 300 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at 4°C. The membrane was incubated with rabbit anti–rat C5aR antibody at a 1:1,000 dilution overnight. After five washes in TBST, the membrane was incubated in a 1:5,000 dilution of horseradish peroxidase–conjugated donkey anti–rabbit IgG as the secondary antibody (GE Healthcare). The membrane was developed by enhanced chemiluminescence technique according to the manufacturer’s protocol (Fig. 1). The results were quantified using digital pixel density and image analysis software Kodak ID (Scientific Imaging Systems). The results were normalized to β-actin and expressed as a ratio of C5aR/β-actin pixel densities (Fig. 1).

RNA isolation and detection of C5aR mRNA in cardiomyocytes by real-time quantitative–PCR analysis. Rat hearts were obtained 0, 6, 12, 24, or 48 h after the CLP or sham procedure and cardiomyocytes were isolated as described in previous paragraphs. Total RNA was isolated using the TRIzol method (Life Technologies Inc.) according to the manufacturer’s instructions. Digestion of any contaminating DNA was achieved by treatment of samples with RNase-Free DNase (Promega Corp.). Reverse transcription was performed with 5 µg RNA using the Superscript II Reverse Transcriptase (GIBCO BRL; Life Technologies Inc.) according to the manufacturer’s protocol. Real-time PCR was then performed with primers for C5aR, 5′ primer, 5′-TATAGTCTGGCGCTCGCTTAC-3′; and 3′ primer, 5′-TCACACTTGTGACCCGTCTTG-3′. 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′ primer, 5′-GCTCTGGTTCATAGAAGATG-3′; and 3′ primer, 5′-CAGTAGACTCCACGACATAC-3′. Reactions were prepared in duplices as 50 µl reactions using the iQ SYBR green Supermix reagent (BioRad Laboratories). After a “hot-start” for 3 min at 94°C, 45 cycles were used for amplification with a melting temperature of 94°C (15 s), an annealing temperature of 60°C (15 s), and an extending temperature of 72°C (30 s), followed by a melting curve generation starting at 60°C with gradual increase in the temperature up to 95°C in 0.2°C steps. An amplification plot was generated using the iCycler software 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 versus 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. Furthermore, the PCR products were run out on a 1.2% gel to confirm that the amplons generated were single bands at the expected size (unpublished data). The C5aR to GAPDH ratios were then plotted for the various time points in CLP and sham rats (Fig. 1). Real-time PCR was performed using a Smart cycler (Cepheid). Ct values,
standard curves, and melting curves were generated using the software provided by the manufacturer (Cepheid).

**Incubation of cardiomyocytes with rC5a.** Cardiomyocyte suspensions (5 × 10^5) isolated 0, 6, 12, 24, or 48 h after CLP or sham procedure were incubated with recombiant, endotoxin-free (<5 pg/ml) rat C5a at various doses for various times. Dose-dependent effects of C5a on cardiomyocyte contractile parameters were then recorded measured and analyzed using the MyoCam system.

**Confocal microscopy and immunohistochemical staining.** Cardiomyocytes isolated 12 h after CLP or sham injury were plated on coverslips and cells were fixed with 4% paraformaldehyde. For confocal microscopy, non-specific binding sites were blocked with 10% gelatin for 20 min and with 4% normal goat serum for 30 min. Cells were incubated with either rabbit anti-rat C5aR antibody (1:50 dilution) or preimmune serum (1:50) for 2 h, washed, and incubated with goat anti-rabbit Alexa-568 Fab2 (1:200) (Invitrogen). After adding SlowFade solution (Invitrogen), cells were visualized using a confocal fluorescence microscope. Both projection view and optical sections were developed electronically and processed digitally. Optical scanning and digital processing of the images were performed to determine the topographic distribution of the Alexa Fluor–conjugated IgG on the surface of cardiomyocytes. For immunohistochemical staining, cardiomyocytes were incubated with anti-C5aR antibody in a dilution of 1:100 in PBS for 2 h. After washing, cells were incubated with a 1:500 diluted peroxidase-conjugated goat anti-rabbit IgG for 30 min (Jackson ImmunoResearch Laboratories). After washing with PBS for 2 min, cells were stained using the AEC vector staining kit (Vector Laboratories Inc.) followed by counterstaining with hematoxylin for 30 s. Tissue sections were fixed and cover slides were mounted with Permount medium (Fisher Scientific Co.). Staining was documented using light microscopy and digital imaging.

**Limulus amebocyte assay.** A chromogenic Limulus amebocyte assay (QLC-1000, Cambrex Corp.) was performed to confirm endotoxin-free cell supernatants and chromogenic substrate reagent over a short incubation period (16 min) and read on a spectrophotometer at a wavelength of 405 nm. The assay has a sensitivity range of 0.1–1.0 EU/ml.

**Statistical analysis.** All statistical analysis was performed using STATA Statistics/Data Analysis 8.0 software (Stata Corporation). Results are expressed as the mean value ± SEM unless otherwise noted. ANOVA followed by Tukey’s post-hoc tests was used to test for differences among the groups. Statistical significance was defined as P ≤ 0.05.

We are very thankful to B. Schumann for secretarial support in preparation of the manuscript. This work is supported by the National Institutes of Health (NIH) grant nos. GM54911 (to S.C. Wang) and GM61656, GM02960, and HL-3193, (to P.A. Ward) and by the American College of Surgeons C. James Carvicco Faculty Research Fellowship for the Study of Trauma and Critical Care (to M.R. Hemmlia). The authors have no conflicting financial interests.

Submitted: 15 June 2005
Accepted: 17 November 2005

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