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## Complement and Sepsis-induced Heart Dysfunction

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### Abstract

It is well known that cardiac dysfunction develops during sepsis in both humans and in rodents (rats, mice). These defects appear to be reversible, since after “recovery” from sepsis, cardiac dysfunction disappears and the heart returns to its function that was present before the onset of sepsis. Our studies, using in vivo and in vitro models, have demonstrated that C5a and its receptors (C5aR1 and C5aR2) play key roles in cardiac dysfunction developing during sepsis. Use of a neutralizing antibody to C5a largely attenuates cardiac dysfunction and other adverse events developing during sepsis. The molecular basis for cardiac dysfunctions is linked to generation of C5a and its interaction with C5a receptors present on surfaces of cardiomyocytes (CMs). It is established that C5a interactions with C5a receptors leads to significant reductions involving faulty contractility and relaxation in CMs. In addition, C5a interactions with C5a receptors or CMs results in reductions in  $\text{Na}^+/\text{K}^+$ -ATPase in CMs. This ATPase is essential for intact action potentials in CMs. The enzymatic activity and protein for this ATPase were strikingly reduced in CMs during sepsis by unknown mechanisms. In addition, C5a interactions with C5aRs also caused reductions in CM homeostatic proteins that regulate cytosolic  $[\text{Ca}^{2+}]_i$  in CMs: sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2) and  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX). In the absence of C5a receptors, defects in SERCA2 and NCX in CMs after sepsis are strikingly attenuated. These observations suggest new strategies to protect the heart from dysfunction developing during sepsis.

### Keywords

Complement C5a; C5aR1; C5aR2 (GPR77);  $\text{Na}^+/\text{K}^+$ -ATPase; sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2);  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX)

### 1. Introduction

It has long been known in humans with sepsis that, as the situation progresses to septic shock, this may result in high lethality ( 70%) (Russell, 2006). Rather surprisingly, if patients survive septic shock, the post-sepsis situation usually involves hearts that resume their function that existed before the onset of sepsis (Kan and Finkel, 2003; Parker et al., 1984). In other words, the events that lead to septic shock and heart dysfunction may be

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completely reversible. Many decades ago, it was postulated that “warm shock” in sepsis progressed to “cold shock” linked to low cardiac output (MacKenzie, 2001; Rabuel and Mebazaa, 2006). It was postulated that early in shock there was a “hyperdynamic phase” followed by a “hypodynamic phase”. However, recent studies suggest that aggressive fluid resuscitation of septic patients results in abolition of the early “hyperdynamic” phase. The hypodynamic phase was thought to be associated with reduced cardiac output and elevated systemic vascular resistance (Clowes et al., 1966; MacKenzie, 2001).

However, over the past several decades, the use of sophisticated physiological monitoring of septic humans has described normal or elevated cardiac output and decreased systemic vascular resistance in septic patients aggressively treated with fluid resuscitation (Gunnar et al., 1973; Marik and Varon, 1998; Packman and Rackow, 1983; Sharma and Dellinger, 2006; Winslow et al., 1973). It is important to note that direct measurements of coronary artery blood flow during sepsis have revealed increased blood flow. Furthermore, myocardial necrosis or apoptosis had not generally been found in hearts from patients who have died during sepsis (Hotchkiss et al., 1999; Lanone et al., 2000; Rabuel and Mebazaa, 2006). Together, it appears that global ischemia of the heart during sepsis does not occur during sepsis.

There has been evidence that septic shock was associated with suppressive factors in plasma negatively affecting cardiac function (Alhamdi et al., 2015; Kalbitz et al., 2015; Parrillo et al., 1985). Circulating cardio-suppressive factors altered  $[Ca^{2+}]_i$  flux in cardiomyocytes (CMs). These factors have been identified with TNF, IL-1 and C5a (Atefi et al., 2011; Finkel et al., 1992; Kumar et al., 1996; Niederbichler et al., 2006; Parrillo et al., 1985). While these cytokines impair heart function *ex vivo*, there is no consensus that such mediators play a major factor in septic shock. All in all, our understanding of events that lead to septic shock and cardiac dysfunction have changed dramatically as sensitive and reliable technologies have been developed to measure physiological parameters during development of septic shock. Such developments have dramatically changed our understanding of molecular pathways responsible for development of septic shock.

## 2. Non-invasive methods to assess heart function in sepsis

There are several non-invasive methods like functional and electrical measurements to assess heart function after onset of sepsis including:

### 2.1. Echocardiography

Echocardiography is one of the noninvasive and accurate tools used in the intensive care unit (ICU) to assess cardiac function and monitor hemodynamics in patients with sepsis (Guerin and Vieillard-Baron, 2016; Haileselassie et al., 2016). During severe sepsis or septic shock, several mechanisms can lead to hemodynamic failure which is important to be diagnosed quickly to apply appropriate hemodynamic therapy. Echocardiography can provide physicians with this diagnostic possibility, whether or not there is fluid responsiveness, cardiac dysfunction, or persistent vasoplegia (Guerin and Vieillard-Baron, 2016). In the clinical setting of critically ill patients like sepsis echocardiography is used to identify the presence of left ventricular (LV)/right ventricular (RV) failure, optimize fluid replacement,

and guide therapies used to alter pulmonary and systemic vascular resistance (Price et al., 2006). Using echocardiography, it is possible to measure cardiac output, intravascular pressures and volumes, systolic and diastolic function of both ventricles, and preload responsiveness of patients suffering from sepsis (De Backer, 2014). There is slower LV filling and aberrant LV relaxation time in humans with sepsis (Jafri et al., 1990; Munt et al., 1998), suggest that impaired compliance may contribute to myocardial depression during sepsis. Myocardial depression during sepsis can perhaps be best described as a global (systolic and diastolic) dysfunction of both the left and right sides of the heart (Antonucci et al., 2014). The main echocardiographic views used to assess hemodynamic status in patients with septic shock was extensively described in the past showing LV and RV dysfunction (Vieillard-Baron et al., 2003). Cardiac dysfunction occurs in up to 80% of patients with septic shock (Beraud et al., 2014). Echocardiography study on 76 patients admitted to the ICU with septic shock within 72 hours of their admission showed cardiac dysfunction and heart failure. Ejection fraction was abnormal in 35%, wall motion abnormalities were present in 17%, right ventricular was impaired in 31%, diastolic function was abnormal in 53% of patients, and the left atrium was dilated in 42% of patients with diastolic dysfunction (Beraud et al., 2014). These data show the potential usefulness of echocardiography to assess myocardial function in patients with septic shock and to assist their management.

Echocardiography can also be used in murine septic models to study their cardiovascular performance (Alhamdi et al., 2015; Boluyt et al., 2004; Hollenberg et al., 2001; Hoover et al., 2015; Kalbitz et al., 2016; Zanotti-Cavazzoni et al., 2009). We evaluated the cardiac function in our CLP (cecal ligation and puncture) mice model using this technique. All echocardiograms were performed by a registered echocardiographer who was blinded to mouse genotype. Imaging was performed using a Vevo 770 High-Resolution In Vivo Imaging System (Visualsonics Inc., Toronto, ON, Canada) equipped with a RMV-707 30 MHz RMV (Real-Time Visualization) (up to 45MHz) scanhead. LV volumes were measured from the parasternal long axis view at the level of the tips of the leaflets of the mitral valve at end systole (VolS) and end diastole (VolD) and used to calculate stroke volume ( $SV = VolD - VolS$ ) and ejection fraction ( $EF \% = \text{endocardial } SV / \text{endocardial } VolD \times 100$ ). Cardiac output was calculated from stroke volume and heart rate ( $CO = SV \times \text{heart rate}$ ). Mitral valve E and A wave inflow velocities were sampled at the tips of the leaflets of the mitral valve from the apical four chamber view. Doppler tissue imaging was performed with acquisition of peak E' velocity from the lateral (E'la) and septal annulus (E'sa) of the mitral valve imaged from the apical four chamber view. Isovolumic relaxation time (IVRT), from the closure of the aortic valve to the opening of the mitral valve, was measured from the apical five chamber view using Doppler flow imaging. For all experiments heart functions, diastolic and systolic parameters in septic mice were compared with those before inducing the CLP (Kalbitz et al., 2016).

## 2.2. Electrocardiogram (ECG)

ECG is another non-invasive tool to study heart function after sepsis. Two-thirds of the patients with sepsis and myocardial depression showed ST-elevation myocardial infarction ECG changes. Septic patients showed to develop significant attenuation of QRS complexes as well as increases in QRS duration may be due to altered cardiac excitability, with or

without bundle branch block (Madias and Bazaz, 2003; Rich et al., 2002). These ECG changes returned to normal following recovery from septic shock, showing reversible reductions of QRS amplitudes (Rich et al., 2002). Another ECG complication during sepsis is QT prolongation (Tisdale et al., 2013; Varriale and Ramaprasad, 1995). Prolongation of the QT interval was a negative predictive factor in septic patients (Tisdale et al., 2013). ECG from human with sepsis also reported myocardial depression and takotsubo syndrome (TS), which the latter may be the cause of the majority of cases of sepsis-induced myocardial depression (Y-Hassan et al., 2014). Takotsubo, which is a Japanese term, is a pot with a round base and narrow neck used in Japan for trapping octopuses (Tako octopus and Tsubo pot). With increasing recognition of takotsubo syndrome (TS), cases of sepsis-triggered TS have been reported (Y-Hassan et al., 2014).

In septic mice prolongation of the PR, QRS complex (Bustamante et al., 2002; Hoover et al., 2015) and prolongation of QTc intervals was reported in the ECG (Hoover et al., 2015). Details of ECG recordings method in mice was described in some reports (Hoover et al., 2015).

### 2.3. Evaluating biomarkers in plasma

There are hundreds of biomarkers including C-reactive protein (CRP), procalcitonin (PCT), lipopolysaccharide binding protein (LBP), various cytokines, and cell surface markers which could be potentially used for diagnosis and prognosis in septic patients (Cho and Choi, 2014; Pierrakos and Vincent, 2010; Prucha et al., 2015). Only some of them are used in routine clinical practice because many lack sufficient sensitivity or specificity (Cho and Choi, 2014; Prucha et al., 2015). Among them PCT and CRP protein have been most widely used however neither PCT nor CRP fulfills the role of an ideal biomarker in the diagnostics of sepsis (Pierrakos and Vincent, 2010; Prucha et al., 2015), having limited abilities to distinguish sepsis from other inflammatory conditions or to predict outcome (Henriquez-Camacho and Losa, 2014; Pierrakos and Vincent, 2010). In neonatal sepsis, the diagnostic utility of the following biomarkers seems to be most practical in the early (IL-6, IL-8, TNF, neutrophil CD64), mid (PCT) and late (CRP) phases (Bhandari, 2014).

Recently, usefulness of biomarker-guided antibiotic supervisions was reported. However, the other side of these numerous biomarkers is that no novel single laboratory marker can diagnose, predict, and track the treatment of sepsis (Cho and Choi, 2014).

Some novel markers were introduced during last decade in the setting of sepsis including presepsin (sCD14-ST) (Endo et al., 2012; Masson et al., 2014; Novelli et al., 2013; Shozushima et al., 2011; Ulla et al., 2013; Yaegashi et al., 2005) and soluble triggering receptor expressed on myeloid cells (sTREM-1) (Adib-Conquy et al., 2007; Ferat-Osorio et al., 2008), soluble urokinase plasminogen activator receptor (suPAR) (Eugen-Olsen et al., 2002; Koch et al., 2011), (proadrenomedullin (proADM) (Suberviola et al., 2012) and CD73 (Bellingan et al., 2014; Hasko et al., 2011).

Cardiac Troponin (cTn) is another biomarker which can be detectable in the plasma of critically ill patients like patients with sepsis. The septic patients can have an elevated cTn levels in the plasma in up to 60% of cases (Hamilton et al., 2012) in which its elevated levels

is associated with increased mortality risk (Fromm, 2007; Gualandro et al., 2014). Septic patients with elevated cTn levels can have a 2–5 fold increased risk of death, even in the absence of known cardiovascular disease (Mantzouris et al., 2013). Another serum cardiac marker used in sepsis disease is Creatine Kinase (CK) which its high level is associated with higher mortality rate (Oliveira et al., 2008; Zhang et al., 2012). This biochemical parameter improved after different treatment approaches in murine (He et al., 2014; Smith et al., 2016; Zhang et al., 2015) and human (Hua et al., 2012; Zhang et al., 2012) sepsis.

Systemic levels of Pentraxin 3 (PTX3) is also a prognostic value in patients with systemic inflammatory response syndrome (SIRS) or sepsis (Liu et al., 2014).

One of the novel biomarkers newly introduced are extracellular histones which currently draw much attention and are detected in sepsis and some disorders like ischemic reperfusion injuries and trauma. Histones act as damage-associated molecular pattern molecules (DAMP) when they are released into the extracellular space (Chen et al., 2014). Extracellular histones can lead to excessive and overwhelming cell damage and death due to the high cytotoxic and proinflammatory effects, thus contributing to the pathogenesis of sepsis (Xu et al., 2015). Release of histones into the extracellular compartment has been postulated to be a major cause of death during sepsis (Chaput and Zychlinsky, 2009; Ekaney et al., 2014; Nakahara et al., 2013; Xu et al., 2009). More than 2 decades ago there was overwhelming evidence that myocardial performance was depressed in sepsis and in endotoxin shock, the possible role of circulating toxins was suggested to be associated with depressed myocardial performance (Abel, 1989). Circulating histones were reported as major mediators of cardiac injury in patients with sepsis (Alhamdi et al., 2015). We recently showed that extracellular histones appearing in septic plasma play an important role in the cardiomyopathy of CLP mice (Kalbitz et al., 2015). Both C5a receptors (C5aR1 and C5aR2) were required for histone presence (Bosmann et al., 2013; Grailer et al., 2015; Kalbitz et al., 2015) perhaps being released from NETs (neutrophil extracellular traps) (Fattahi et al., 2015). Presence of histones after CLP is linked to disturbances in functional responses of hearts as defined by ECHO/Doppler parameters and this cardiac dysfunction is preserved using antibody against histones (Kalbitz et al., 2015). Moreover, in ECG tracings on perfused mouse hearts with histones or isolated cardiomyocytes incubated with histones we showed harmful effect of histones (Kalbitz et al., 2015). In line, other studies showed antihistone-based treatments (e.g., neutralizing antibodies, activated protein C, and heparin) to have protective effects and have significantly improved the outcomes of mice with sepsis (Xu et al., 2015). Neutralization of Cit H3 also showed survival improvement in septic mice (Li et al., 2014). This data together indicates extracellular histones could serve as potential biomarkers and novel therapeutic targets in sepsis.

### 3. Roles of complement C5a in septic cardiomyopathy

Complement C5a which is one of the powerful anaphylatoxins generated from the complement system shown to be associated with septic cardiomyopathy (Hoesel et al., 2007b; Kalbitz et al., 2015; Niederbichler et al., 2006). An excessive C5a generation occurs during sepsis (Hoesel et al., 2007b; Niederbichler et al., 2006). In below we explain some of the effects of C5a on cardiomyocytes contributing in cardiac dysfunction.

### 3.1. C5a causes increased ROS and $[Ca^{2+}]_i$ in CMs

Intracellular calcium  $[Ca^{2+}]_i$  is one of the most important factors for corresponding and efficient contractility in which CM dysfunction after sepsis was linked to the altered calcium transient properties (Ren et al., 2002). In fact, impaired calcium handling in CMs was suggested as one of the underlying mechanisms in septic cardiomyopathy (Maitra et al., 1999).

Reactive oxygen species (ROS) are also increased in the failing heart, has been shown to play an important role in the pathophysiology of cardiac remodeling and reduced LV function. ROS influence contractile function by modifying proteins involved in excitation-contraction link including ryanodine receptor (RyR) to enhance its open probability, the suppression of L-type calcium channel, and oxidative interaction with  $Ca^{2+}$ -ATPase in the sarcoplasmic reticulum (SR) to inhibit  $Ca^{2+}$  uptake (Tsutsui et al., 2011; Zima and Blatter, 2006).

We recently showed the effects of C5a on  $[Ca^{2+}]_i$  homeostasis and electrophysiological functions in single CMs (Kalbitz et al., 2016). We also found ability of rat recombinant C5a to cause increased ROS in isolated rat CMs (Kalbitz et al., 2016). We also found increased  $[Ca^{2+}]_i$  influx in rat CMs after treating them with C5a in vitro (unpublished data). In line with in vitro data, inducing CLP in wild type (Wt) mice resulted in  $[Ca^{2+}]_i$  release (unpublished data) and increased ROS (Kalbitz et al., 2016) in mouse CMs. Collectively, the data provide direct evidence of the effects of C5a on the loss of homeostatic control of ROS and  $[Ca^{2+}]_i$  in CMs during sepsis.

Interestingly, both  $[Ca^{2+}]_i$  and ROS release require C5a receptors (C5aR1 and C5aR2) (Kalbitz et al., 2016) as both responses were significantly attenuated in the lack of either C5aR1 or C5aR2 especially in C5aR1<sup>-/-</sup> mice.

### 3.2. C5a induces defects in contractility and relaxation in CMs

We showed in the past that C5aR1 is expressed in whole heart homogenates of septic rats, perhaps setting the stage for C5a-induced impairment of cardiac function (Riedemann et al., 2002). By evaluating contractility parameters of single cardiomyocytes on the sarcomere level, we were able to find association of the complement system and septic cardiomyopathy (Niederbichler et al., 2006). There was significant reduction in LV pressures in rats after CLP which was in line with earlier reports (Kumar et al., 2001). There was also dramatically impaired contractility in rat cardiomyocytes in vitro after their incubation with 10.0 nM C5a but to a consistently greater degree in cells from CLP rats (Niederbichler et al., 2006). 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 (Nakae et al., 1994).

C5a can also result in defective action potentials in CMs especially in septic CMs. Using patch-clamp techniques, CMs from rats showed early after depolarizations (EADs) and widened and prolonged action potentials 24 hr after CLP. Addition of C5a (45ng/ml) accentuated this abnormalities and EAD profile. Our very recent data showed exposure of CMs from septic hearts to C5a results in dramatically altered action potentials, with defective values for resting membrane potential, maximal upstroke velocities, prolongation



of action potential duration and the appearance of EADs indicating profound prolongation in action potentials as a result of sepsis together with presence of C5a (Kalbitz et al., 2016).

#### 4. ECHO/Doppler defects in heart after onset of sepsis preserved in septic mice lacking C5a receptors

We recently showed sepsis-induced heart dysfunction is preserved in septic mice lacking C5a receptors (C5aR1<sup>-/-</sup> and C5aR2<sup>-/-</sup>) (Kalbitz et al., 2016). We studied the cardiovascular performance by echocardiography in septic mice 8 hr after CLP to see if there is any protective effect in septic mice lacking C5a receptors (Kalbitz et al., 2016). Echocardiograms were performed as previously described (Boluyt et al., 2004) according to the recommendations of the American Society of Echocardiography.

Our results showed diastolic function (as measured by IVRT and Doppler tissue imaging) were prolonged 8 hr after CLP induction in Wt but not in C5aR1<sup>-/-</sup> and C5aR2<sup>-/-</sup> mice. Tissue Doppler Imaging demonstrated a reduction in annular velocities of the mitral valve at the lateral (E'la) and septal annulus (E'sa) suggesting reduced diastolic function after CLP that is at least partially reversed in the setting of C5aR1 or C5aR2 K.O. Correcting E wave velocities for diastolic function (E/E' ratio) revealed that CLP resulted in a reduction of LV filling pressures, likely as the result of reduced preload in Wt animals. In C5aR1<sup>-/-</sup> and C5aR2<sup>-/-</sup> mice, the E/E' ratios were similar to sham operated Wt controls, suggesting normalization of ventricular filling pressures which was supported by the assessment of ventricular volumes. Similarly, the significantly decreased end diastolic volumes noted after CLP in Wt mice were less pronounced in the C5aR1<sup>-/-</sup> and C5aR2<sup>-/-</sup> mice. These data suggest that CLP mice have reduced cardiac performance that was muted in mice lacking either C5a receptor.

Generally our echo data in Wt CLP mice is in line with earlier reports (Hollenberg et al., 2001; Zanotti-Cavazzoni et al., 2009), but no other echocardiography studies is present looking at the protective effect of lacking C5a receptors. This highlights the importance of the C5a receptors as a potential target for therapeutic purposes in the clinical setting of sepsis. We showed by immunofluorescence staining both C5a receptors are present in human hearts. Fig. 1 shows confocal immunofluorescence using frozen sections of two different human LV samples, confirming the presence of both C5aR1 and C5aR2 proteins in human ventricular wall. As shown in Fig. 1, human myocardium from normal hearts revealed the presence of cytosolic troponin T and both C5a receptors in CMs. Heart-specific troponin T staining (red) was present in CMs and C5a receptors (green staining) were also found in CMs. In the PAS frame, there was clear definition of CMs. The presence of both C5a receptors in human CMs suggests that C5a may interact with the corresponding receptors on CMs during human sepsis. These observations may have therapeutic implications for treatment of humans developing cardiomyopathy during sepsis.

## 5. Proteins studies in heart homogenates from CLP mice and protective effect of lacking C5a receptors

### 5.1. Reduced SERCA2 and NCX protein levels in CLP murine

As stated in section 3.1, one of the mechanisms suggested for sepsis-induced cardiomyopathy is impaired calcium handling in cardiomyocytes.  $[Ca^{2+}]_i$  is a requirement for contraction and relaxation. During diastole, the  $[Ca^{2+}]_i$  is maintained at sufficiently low levels to prevent activation of contractile proteins. With each heartbeat,  $Ca^{2+}$  influx via the L-type  $Ca^{2+}$  channel triggers release of  $Ca^{2+}$  from the SR which functions as the storage area for calcium during myocyte relaxation. These two sources combine to elevate  $[Ca^{2+}]_i$ , which promotes  $Ca^{2+}$  binding to troponin and activation of the contractile process. Contraction is terminated as  $Ca^{2+}$  is pumped back into the SR by the SR  $Ca^{2+}$ -ATPase (SERCA) and out of the cell via the sarcolemmal  $Na^+$ - $Ca^{2+}$  exchanger (NCX). Modulation of the calcium regulating proteins may have important functional consequences during heart failure, arrhythmias and sepsis. The depressed contractility of the failing heart is thought to involve alterations in myocyte  $Ca^{2+}$  regulation (Piacentino et al., 2003). Impairment of calcium transport mechanisms in the myocardium occurs during sepsis (Dong et al., 2001) SR  $Ca^{2+}$  handling dysfunction also shown to be an early event during endotoxemia (Hassoun et al., 2008). Several studies on rats demonstrated reduced activity of SERCA after CLP (He et al., 2007; Ren et al., 2002; Wu et al., 2004; Wu et al., 2016; Wu et al., 2001; Zhu et al., 2005). In accordance with these findings, SERCA inhibition has been demonstrated in endotoxemia in rats (Comini et al., 2005; Hassoun et al., 2008) and mice (Hobai et al., 2013; Ichinose et al., 2007; Turdi et al., 2012). This SERCA inhibition in endotoxemia was LPS dose dependent (Hobai et al., 2013), further confirming a direct contributing relation. It has also been reported that ATP-dependent calcium uptake by SERCAs is impaired during later stages of endotoxemia and sepsis (Wu et al., 2001; Wu and Liu, 1991). NCX is also shown to be decreased in sepsis (Hobai et al., 2015a; Hobai et al., 2015b).

In line with these data, our recent studies using mRNA expression, Western blot and ELISA methods show decreased levels of these calcium regulatory proteins (SERCA2 and NCX) in LV homogenized mice after CLP (Fig. 2) (Kalbitz et al., 2016). These reductions were dramatically attenuated in the mice lacking either C5aR1 or C5aR2 (Kalbitz et al., 2016), indicating that CLP-induced reductions in these calcium regulatory proteins are C5a receptor-dependent.

### 5.2. Reduced enzyme activity and protein levels of $Na^+/K^+$ -ATPase in CLP mice

In addition to the calcium regulatory proteins we recently measured enzymatic activity of  $Na^+/K^+$ -ATPase in LV homogenates from mice after CLP and found profound reductions in this enzyme activity. In the absence of C5aR1, there was almost no reduction in  $Na^+/K^+$ -ATPase activity after CLP. In C5aR2<sup>-/-</sup> mice, there appeared to be less protection against the CLP-induced fall in  $Na^+/K^+$  ATPase, whereas in C5aR1<sup>-/-</sup> mice the sepsis-induced fall was virtually abolished.

Our Western blot data for measuring  $Na^+/K^+$ -ATPase activity in LV homogenates was consistent with enzymatic activity results showing reductions in  $Na^+/K^+$ -ATPase. We found



profound reductions in intensity of LV blots for Na<sup>+</sup>/K<sup>+</sup>-ATPase occurred 8 hr after CLP. When we further determined whether C5aR deficiency prevented loss of Na<sup>+</sup>/K<sup>+</sup>-ATPase proteins levels in heart homogenates by ELISA, we found significant reduction in heart homogenate proteins in Wt for Na<sup>+</sup>/K<sup>+</sup>-ATPase 16 hr after CLP (Fig. 2) while this reduction was attenuated in the absence of C5aR1 or C5aR2 (Kalbitz et al., 2016).

## 6. Discussion

Figure 3 summarizes our current data, linking C5a and C5a receptors to events that compromise the functional integrity of heart (CMs or LV frozen sections). Several years ago we described that polymicrobial sepsis caused substantial increases in C5aR1 on surfaces of CMs. We further showed that addition of C5a to sham CMs or CMs from septic mice dramatically caused CM dysfunction, involving both contractility and relaxation (Niederbichler et al., 2006). We also demonstrated that blocking antibodies to C5a or blockade or absence of C5aR2 greatly improved survival of septic mice (Huber-Lang et al., 2001b) and reduced evidence of cardiac dysfunction during sepsis based on ECHO/Doppler technology (Kalbitz et al., 2015). We have recently found that sepsis caused both enzymatic and protein reductions in Na<sup>+</sup>/K<sup>+</sup>-ATPase in CMs, leading to interference with repolarization of CMs that had undergone depolarization (Kalbitz et al., 2016). In addition, there were reductions in SERCA2 and NCX, involving mRNAs and protein content in CMs from hearts after onset of sepsis. Collectively, changes in these three proteins in CM interfered with the ability to repolarize the cells after CM activation, resulting in defective contractility and relaxation. Reductions in SERCA2 and NCX interfered with clearance of diastolic [Ca<sup>2+</sup>]<sub>i</sub> in CMs following their activation (electrical) or during spontaneous systole. During diastole, impairment of SERCA2 or NCX resulted in the inability of CMs to clear diastolic [Ca<sup>2+</sup>]<sub>i</sub>, resulting in serious defects in contractility and relaxation (Lompre et al., 1994; Misquitta et al., 1999). During sepsis, there may also be excessive activation of RyR receptors, which would further accentuate the buildup of cytosolic [Ca<sup>2+</sup>]<sub>i</sub> during diastole. In the setting of sepsis, the strategy to reduce cardiac dysfunction could include infusion of a neutralizing antibody to C5a or use of one of the new blocking compounds to prevent C5a activation or the functional activity of C5a (Czermak et al., 1999; Flierl et al., 2009; Guo et al., 2000; Guo et al., 2006; Hoesel et al., 2007a; Huber-Lang et al., 2001a; Huber-Lang et al., 2001b; Laudes et al., 2002; Riedemann et al., 2004; Sprong et al., 2003). Since the neutralizing mAb to C5a is safe in normal humans (Hammerschmidt et al., 1980; Stevens et al., 1986) this antibody might become a candidate to intervene in septic individuals entering the phase of septic shock.

## 7. Conclusions

The early initiating events in sepsis are complement activation with C5a generation which acts directly by engaging its both receptors (C5aR1 and C5aR2) that involve in septic cardiomyopathy. So development of defective cardiac function can be linked to C5a signaling via C5a receptors (summarized in Fig. 3). This figure describes events occurring in the heart after polymicrobial sepsis, resulting in defective action potentials and loss of homeostatic proteins that are crucial for preventing buildup of [Ca<sup>2+</sup>]<sub>i</sub> in CMs during diastole. C5a interacts with CM receptors (C5aR1, 2), which results in defective action

potentials (associated with faulty repolarization in CMs) as well as the cytosolic buildup of  $[Ca^{2+}]_i$  in CMs during diastole. These changes cause faulty contractility and relaxation in CMs. Molecular mechanisms linked to these outcomes appear to be related to reduced levels of SERCA2 and NCX and reduced levels of  $Na^+/K^+$ -ATPase. This triad of defects may be key to development of cardiac dysfunction after sepsis.

A neutralizing mAb to C5a might represent an effective strategy for treatment of patients with sepsis. Moreover, presence of extracellular histones occurring after CLP can be linked to appearance of defects in cardiac function during sepsis. Neutralizing histone antibodies or drugs that block histone interactions with CMs, besides neutralizing mAb to C5a, may be potential therapeutic strategies to prevent or ameliorate septic cardiomyopathy.

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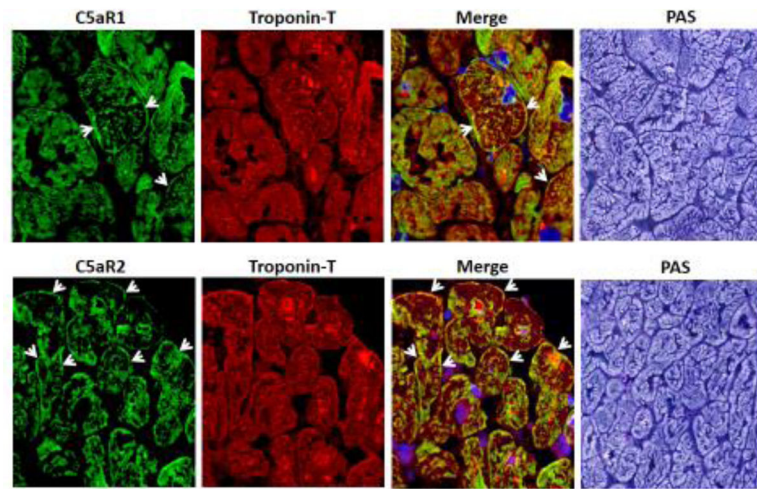


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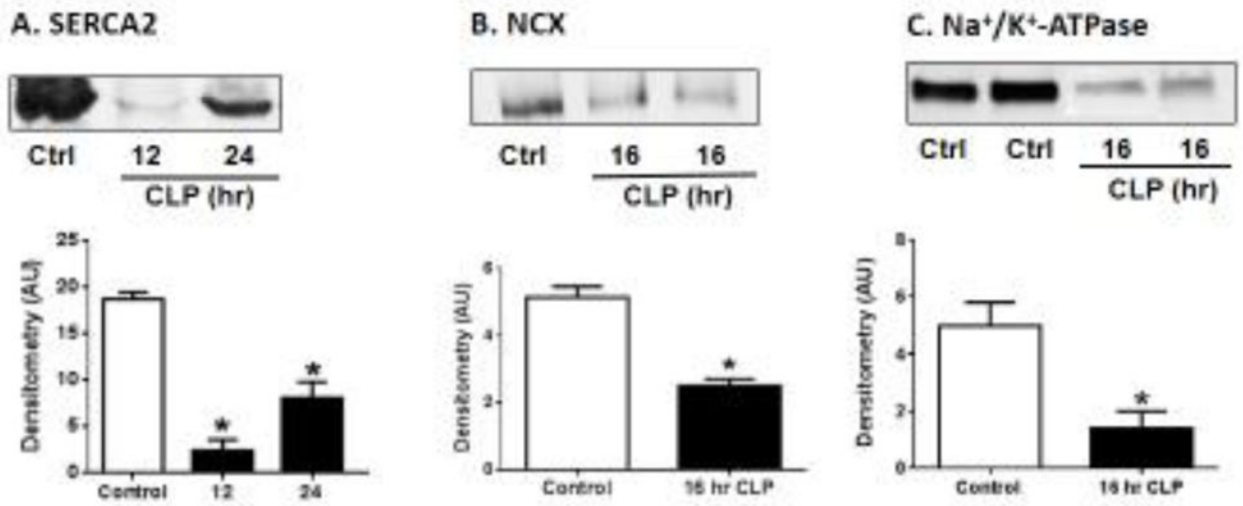
### Highlights

- Cardiomyopathy is a common complication of sepsis, often with a high mortality rate.
- Cardiomyopathy has requirements for complement C5a and its receptors.
- Molecular defects in cardiac dysfunction of sepsis are complement-dependent and develop during early sepsis, with specific defects:
  - Defective action potentials;
  - Reduced amounts of SERCA2, NCX and Na<sup>+</sup>/K<sup>+</sup>-ATPase;
  - Development in CMs of increased amounts of ROS and [Ca<sup>2+</sup>]<sub>i</sub> which are known to impair contractility and relaxation of cardiomyocytes (CMs).
  - Extracellular histones formed during sepsis which can lead to excessive damage and death.
- Cardiac dysfunction of sepsis disappears after recovery from sepsis, indicating that cardiac dysfunction in sepsis is reversible over time.
- Presence of C5a receptors on surfaces of human CMs implies that CMs may respond to complement products, resulting in adverse consequences.



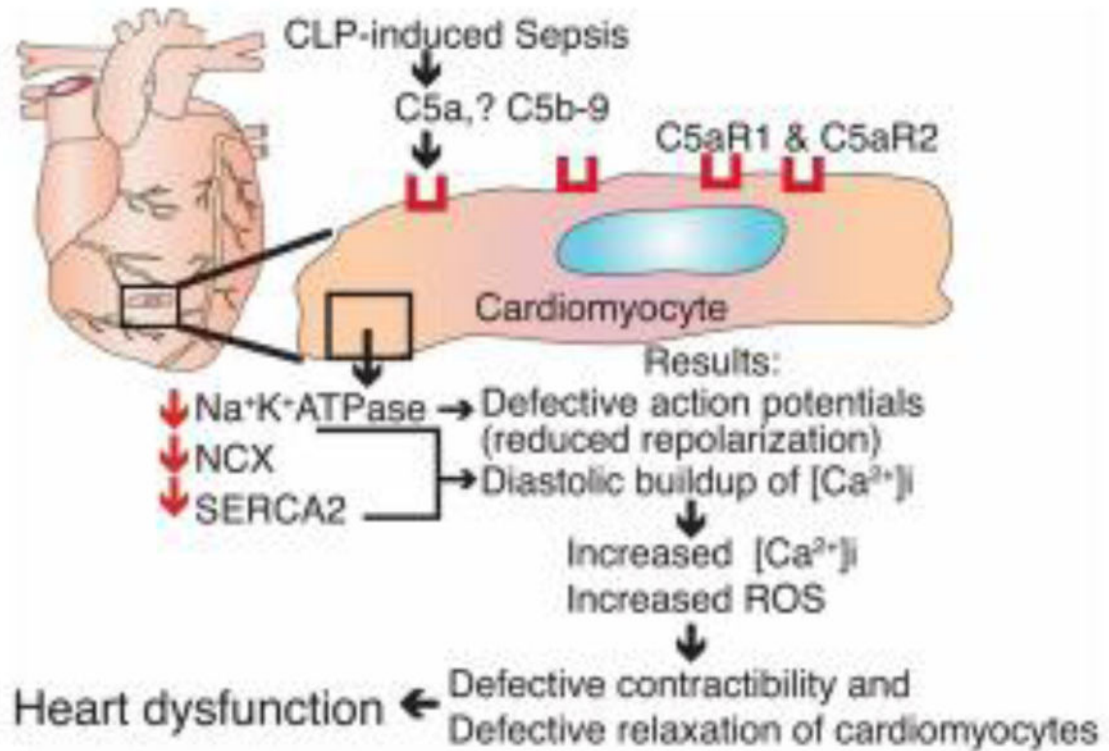
**Figure 1. Expression of C5aRs in Human Heart**

Confocal immunofluorescence for C5aR1 and C5aR2 protein, x63. Double-staining with heart-specific troponin T, and for C5aR1 and C5aR2, both x63. Far right, periodic acid-Schiff (PAS) staining of frozen sections from normal human heart tissue, x63.



**Figure 2. Changes in CM Protein Levels for SERCA2 (A), NCX (B) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (C) after CLP**

Changes based on Western blots of CM proteins, SERCA2 (A), NCX (B) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (C) at indicated time points after CLP. For each bar, n>6 separate samples. \*p<0.05



**Figure 3. Proposed Cascade of Events after CLP Leading to Septic Heart Dysfunction**  
 Reductions in key Ca<sup>2+</sup> regulatory proteins in CMs and reduction in Na<sup>+</sup>/K<sup>+</sup>-ATPase are associated with inability to clear cytosolic Ca<sup>2+</sup> after systole and defective action potentials, ultimately leading to the “cardiomyopathy of sepsis”.