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Περίληψη

Η σήψη είναι μια δυνητικά θανατηφόρα κατάσταση και οι καρδιαγγειακές επιπλοκές συχνά την περιπλεκουν, αυξάνοντας το ποσοστό θνησιμότητας από 20% σε 90%. Πολλαπλοί μηχανισμοί έχουν ενεπλακεί στη παθοφυσιολογία της σηπτικής καρδιομυοπάθειας όπως η αυξημένη φλεγμονή, το οξειδωτικό στρες, διαταραχές του β-αδρενεργικού συστήματος και του μεταβολισμού και μειωμένη παραγωγή ενέργειας από τα καρδιομυοκύτταρα. Οι υπεύθυνοι μηχανισμοί ωστόσο, δεν είναι, πλήρως μελετημένοι, και αυτό αποτελεί την αιτία για την οποία δεν υπάρχει αποτελεσματική θεραπεία για τη σηπτική καρδιομυοπάθεια. Στη πρώτη μας μελέτη, δείχνουμε ότι αναστέλλοντας τη δράση της NOX2 χρησιμοποιόντας το φάρμακο apocynin, τα ποντίκια προστατεύονται από συστολική καρδιακή δυσλειτουργία που εμφάνιζουν λόγω σήψης. Δείξαμε ότι η αναστολή της δράση της NOX2 διατήρησε τη καρδιακή λετουργία, μείωσε το οξειδωτικό στρες, μείωσε την ενεργοποιήση του JNK και την αρνητική ρύθμιση μεταβολικών γονιδίων, χωρίς να επηρεάζει τη φλεγμονή. Αναδεικνύοντας έτσι, ένα μοναδικό ρόλο της NOX2 στη παθοφυσιολογία της ασθένειας, να δρα μέσω του καρδιακού μεταβολισμού κατά ένα τρόπο που σε μεγάλο ποσοστό είναι ανεξάρτητος της συστηματικής φλεγμονής. Στη δεύτερη έρευνα που διεξάγαμε, χρησιμοποιήσαμε το φάρμακο LGM2605, χημική σύνθεση του SDG, και δείξαμε ότι το φάρμακο απέτρεψε επιτυχώς την εμφάνιση σηπτικής καρδιακής δυσλειτουργίας. Το LGM2605 μείωσε τη παραγωγή του ανιόντος του σουπεροξειδίου από τα μιτοχόνδρια που προκλήθηκε λόγω σήψης *in vitro*, αποκατάστησε τη διαταραχή στον αριθμό των μιτοχονδρίων που προκλήθηκε από τη σήψη, αύξησε την έκφραση του MCU και αύξησε τα επίπεδα μιτοχονδριακής αναπνοής όπως παρατηρήσαμε από την ανάλυση seahorse. Και στις δύο μελέτες μας δείξαμε τη σπουδαιότητα της αντιοξειδωτικής και μεταβολικής θεραπείας στην αντιμετώπιση της σηπτικής καρδιομυοπάθειας. Περαιτέρω μελέτες χρειάζονται προκειμένου να υποστηρίξουν την υπόθεση μας, για στόχευση του οξειδωτικού στρες και της μιτοχονδριακής δυσλειτουργίας ως ένα μέσο για τη θεραπεία της σηπτικής καρδιομυοπάθειας.

Role of oxidative stress and mitochondrial dysfunction in septic cardiomyopathy

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Abstract

Sepsis is a life-threatening condition and cardiovascular dysfunction frequently complicates sepsis, as its presence increases mortality rate from 20% to 90%. Various mechanisms have been implicated in the pathophysiology of septic cardiac dysfunction such as increased inflammation, oxidative stress, impaired β-adrenergic signaling, impaired metabolism and reduced ATP synthesis in cardiomyocytes. The underlying mechanisms, though, are not fully elucidated, which constitutes the reason for the lack of an effective treatment for septic cardiomyopathy. In our first study, we showed that *in vivo* inhibition of NOX2 by apocynin protects mice from sepsis-induced systolic dysfunction using both the LPS-injection model and the CLP model of polymicrobial sepsis. We showed that NOX2 inhibition preserved cardiac function, alleviated oxidative stress, reduced JNK activation and downregulation of metabolic genes without resolution of the inflammatory component of the disease, identifying a potentially novel role for NOX2 in the pathophysiology of the disease, acting through cardiac metabolism in a way that is largely independent of systemic inflammation. In the second study we performed, we used LGM2605, which is chemically synthesized SDG and we showed that the drug could successfully prevent the sepsis-induced cardiac dysfunction using the CLP model of polymicrobial sepsis. LGM2605 suppressed the sepsis-induced mitochondrial superoxide generation *in vitro*, restored the sepsis-induced impairment on mitochondrial number, increased the expression of MCU and increased the mitochondrial respiration levels as we observed by seahorse analysis. In both studies we showed the importance of anti-oxidant and metabolic therapies for treating septic cardiac dysfunction. Further studies are needed to support our hypothesis for targeting oxidative stress and mitochondrial dysfunction as a way to treat septic cardiomyopathy.

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List of acronyms

ROS	Reactive oxygen species
ICU	Intensive care unit
ATP	Adenosine triphosphate
TNFa	Tumor necrosis factor
IL-1,6,8,12	Interleukin 1,6,8,12
IFN-γ	Interferon γ
O_2	Superoxide anion
H_2O_2	Hydrogen peroxide
HO	Hydroxyl radical
bARs	Beta adrenergic receptors
Gs protein	Stimulatory G protein
AC	Adenynyl cyclase
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
NO	Nitric oxide
CD36	cluster differentiation
FATP	Fatty-Acid Transport Protein
TCA	tricarboxylic acid
NADH	Nicotinamide adenine dinucleotide
FADH2	Flavin adenine dinucleotide
ADP	Adenosine diphosphate
GLUTs	Glucose transporters
G6P	glucose 6-phosphate
PDH	pyruvate dehydrogenase
PPARα	Peroxisome proliferator activated receptor α
RxRs	retinoid-X receptors
TRs	thyroid receptors
PGC-1	PPARγ- coactivator-1
NOX	NADPH oxidases
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
PKC	protein kinase C
JNK	c-Jun N-terminal kinase
PMA	Para- Methoxyamphetamine
LPS	Lipopolysaccharide
CLP	Cecal Ligation and Puncture
EF	Ejection fraction
FS	Fractional shortening
BNP	Brain natriuretic peptide
PLB	Phospholampan
IL1a, IL1b	Interleukin 1a or 1b

LVIDs	Left ventricular internal diameter end systole
CPT1b	Carnitine palmitoyltransferase 1b
UCP	Uncoupling protein
SDG	Secoisolariciresinol diglucoside
CVD	Cardiovascular disease
ARDS	Acute Respiratory Distress Syndrome
HO-1	Heme oxygenase-1
MFN 1, MFN2	Mitofusin 1 and 2
OPA1	Optic atrophy protein 1
PINK1	Phosphatase and tensin homolog–induced putative kinase protein 1
LC3-II	Light chain 3 II
MCU	Mitochondria calcium uniporter
MICU	Mitochondrial calcium uptake
DRP1	Dynamin-related protein-1
FIS1	Fission 1
NRF-1 and NRF-2	Nuclear respiratory factor 1 and 2
TFAM	Transcription factor A mitochondrial
TFBM	Transcription factor B mitochondrial
ARE	Antioxidant response element
GSTM1	Glutathione S-Transferase Mu1
NQO1	NAD(P)H Quinone Dehydrogenase 1
KEAP1	Kelch Like ECH Associated Protein 1
LCAD	Long-chain acyl-CoA dehydrogenase
VLCAD	Very long-chain acyl-CoA dehydrogenase
MCAD	Medium-chain acyl-CoA dehydrogenase
PDK4	Pyruvate dehydrogenase kinase 4
IKBa	NFKB inhibitor alpha
NFKB	Nuclear Factor Kappa B

CHAPTER I: Introduction

Sepsis is a life-threatening condition that is caused by infection and dysregulated response of the host. Symptoms of sepsis include shivering, fever, extreme pain or general discomfort, pale or discolored skin, sleepiness, confusion, feeling of upcoming death and shortness of breath. If sepsis is not treated properly, it can progress to septic shock which is a combination of low blood pressure, ischemia and multiple organ failure that is a lethal condition [1].

Sepsis is characterized by two hemodynamic phases. The early phase, called hyperdynamic phase, is a hyper-inflammatory response characterized by massive production of proinflammatory cytokines and ROS by the neutrophils and macrophages, a situation known as respiratory burst. Immune cells such as neutrophis and mononuclear phagocytes can rapidly produce superoxide and hydrogen peroxide, due to the increased oxygen uptake that occurs when they are exposed to certain stimuli such as different bacteria[2]. In this phase death can occur with acute organ dysfunction due to the cytokine storm. The late phase, called hypodynamic phase is a hypo-inflammatory response. Due to the existing immunosuppression, death in this phase can occur either due to the primary or secondary infection [3].

Cardiovascular dysfunction frequently complicates sepsis. One hospital-based study found that 43% of patients with bacteremia had increased serum troponin, which indicates myocardial damage [4]. Other clinical studies have shown that the presence of cardiovascular dysfunction in sepsis is associated with significantly increased mortality rate of 70% to 90% compared with 20% in septic patients without cardiovascular impairment [5]. Sepsis can also cause abnormal cardiac electrophysiology, as indicated by the fact that septic patients often have abnormal electrocardiograms and/or arrhythmias [6,7]. Atrial fibrillation in patients admitted to the ICU with sepsis

compared to other diagnoses is six times more common [8]. Septic cardiac dysfunction is characterized by impaired contractility, diastolic dysfunction, as well as reduced cardiac index and ejection fraction (EF) [9,10]. Cardiac index stands for the amount of blood ejected by the heart in a unit of time divided by the body surface area and ejection fraction stands for the percentage of blood that is pumped out of a filled ventricle with each heartbeat. Currently, there is no therapy for sepsis-induced cardiomyopathy.

Various mechanisms have been implicated in the pathophysiology of septic cardiac dysfunction such as increased inflammation, oxidative stress, impaired β-adrenergic signaling, impaired metabolism and reduced ATP synthesis in cardiomyocytes [11,12,13].

Inflammation in sepsis

The inflammatory component of the pathophysiology of sepsis is complex. Innate immune responses lead to activation of the cytokine system, which has plenty of effects on a variety of organs as well as the vascular system, that leads to changes in vascular permeability, the function of the endothelium, and activation of mediators such as bradykinin, the complement, and the coagulation system. Cytokines and particularly pro-inflammatory mediators such as TNFα, IL-1, IL-6, IL-8, IL-12, and IFN-γ play an important role in the pathophysiology of sepsis[14,15].

Oxidative stress in sepsis

. The formation of chemically reactive species known as reactive oxygen species (ROS) is the result of univalent reduction of molecular oxygen[16]. Low concentration of ROS is necessary for adequate cell physiology. When ROS though is excessively produced or when antioxidants that tightly control ROS production are depleted, there is a high intracellular ROS concentration that leads to oxidative stress and that ultimately results in cellular damage [17].

The most common ROS in biological systems are:

1. Superoxide anion (O_2^-) is a byproduct of respiration that is mainly produced by NADPH oxidases. The fact that O_2^- is instable and cannot diffuse through membranes because of its negative charge makes this ROS a poor molecule for signaling.
2. Hydrogen peroxide (H_2O_2). The toxicity of H_2O_2 is essentially the consequence of its reduction to HO. H_2O_2 is relatively stable and its selective reactivity and ability to diffuse makes it fit for signaling
3. Hydroxyl radical (HO). The highly toxic HO has high reactivity, which limits its diffusion only to sites of production [16].

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β-adrenergic signaling in sepsis

Impaired β-adrenergic signaling has also been implicated in the pathophysiology of septic cardiomyopathy. The main subtypes of cardiac β adrenergic receptors (βARs) are the β1- and β2-adrenergic receptors. Approximately 80% of the cardiac βARs are β1 and 20% are β2 [18,19]. Adrenergic receptors are a class of G protein-coupled receptors. The β1- and β2-adrenergic receptors are coupled to the stimulatory G protein, named Gs. Stimulation of the receptor protein by a βAR agonist such as norepinephrine or epinephrine causes a conformational change that leads to the dissociation of the G protein into its components Gαs and βγ. That results in stimulation of adenylyl cyclase (AC) which is necessary for the conversion of ATP to cAMP. The last one increases the activity of the protein kinase A (PKA) which phosphorylates threonine and serine residues on plenty of proteins. Some of the downstream targets of the PKA that can affect the contractility of the myocardium are the β-ARs, the sarcoplasmic reticular Ca++/ATPase inhibitory protein and the phospholamban[20]. In the early stages of sepsis, the catecholamine levels are elevated, which most likely acts as a compensatory adrenergic response that aims to increase cardiac contractility and heart rate [21,22]. However, this adaptive response finally becomes maladaptive since this excessive stimulation of the cardiac βARs cause damage of the myocardium

mostly due to the overload of intracellular calcium and to the induction of cell death**.** In ill patients this sympathetic overstimulation in long term is harmful for the heart as it can lead to tachycardia and tachyarrhythmia, myocardial ischemia, apoptosis and necrosis. As the septic cardiac dysfunction progresses, βAR density is reduced, and there is significant cardiac desentization and downregulation of bARs potentially caused by cytokines [23,24]. So, the sympathetic system appears incapable of stimulating the heart any further and the myocardium becomes unresponsive to catecholamines. This lack of βAR responsiveness during sepsis has been attributed to either auto-oxidation by reactive oxygen species (ROS)[25] or to elevated Nitric Oxide (NO) levels[26].

Cardiac metabolism in sepsis

Cardiac metabolism and ATP synthesis are compromised in sepsis. Cardiac ATP synthesis mostly (70%) depends on fatty acid oxidation, while 20% depends on glucose oxidation and 10% on lactate and ketone bodies [27]. Fatty acids are taken up by the cardiomyocytes via cluster differentiation (CD36)/ Fatty-Acid Transport Protein (FATP) and are directly converted to fatty acyl-CoA in the cytosol. Fatty acyl-CoA are stored in triglycerides and then used for ATP synthesis [28]. In order to transported into the mitochondria, CoA is replaced by carnitine. In the mitochondria carnitine is replaced again with CoA. Fatty acyl-Co-A then enters the beta oxidation cycle (shortening of the fatty acyl-CoA) that divides the long carbon chains into short acetyl-CoA molecules that are further oxidized in the tricarboxylic acid (TCA) cycle. The TCA cycle generates NADH and FADH2, which enter the electron transport chain (oxidative phosphorylation) that leads to proton transfer in the intramitochondrial space resulting in a proton gradient across the inner mitochondrial membrane. This proton gradient drives ATP Synthase that converts ADP to ATP.

Glucose is transported into the cytosol by glucose transporters (GLUTs), specifically GLUT1 and GLUT4. Cytosolic free glucose is rapidly phosphorylated to glucose 6-phosphate (G6P), which then enters many metabolic pathways, such as pentose phosphate pathway, the glycogen pathway and the hexosamine pathway. Glycolysis

represents the major pathway in glucose catabolism. Glycolysis generates pyruvate, NADH, and 2 ATP molecules. In the cytosol, pyruvate can either be converted to lactate under anaerobic conditions such as strenuous exercise, or during resting and steady state conditions it can be transported into the mitochondrial matrix, where pyruvate dehydrogenase (PDH) oxidizes pyruvate to acetyl-CoA. Pyruvate can also undergo carboxylation to form oxaloacetate or malate [29].

In heart failure, fatty acid oxidation is reduced, and glucose oxidation is increased to compensate for the energy production[30]. However, this doesn't happen in sepsis that insulin resistance occurs[31]. Thus, both cardiac fatty acid oxidation and glucose catabolism are suppressed. More specifically, during sepsis, the expression of fatty acid metabolism genes and transcriptional factors, important for their regulation such as peroxisome proliferator activated receptor α (PPARα), retinoid-X receptors (RxRs), thyroid receptors (TRs), PPARγ-coactivator-1 (PGC-1), is downregulated [13,32,33,34]. CD36, lipoprotein lipase, and lipoprotein remnant receptors are also downregulated, leading to increased fatty acid and triglyceride-carrying lipoprotein content in the circulation. In addition, cardiac β-oxidation is inhibited, and mitochondrial number is reduced via mitophagy, resulting in intracellular accumulation of the unused fatty acids in triglycerides [15].

It has been suggested that the mitochondrial dysfunction is triggered by increased production of ROS that occurs in septic hearts[35]. Downregulation of complex II and IV levels of the oxidative phosphorylation machinery and of the enzymatic activity of mitochondrial enzymes such as NADH cytochrome c reductase, succinate cytochrome c reductase and cytochrome c oxidase are some of the changes in the mitochondrial metabolism that occur during septic cardiomyopathy [36].

So, the basic mechanisms that have been implicated in the pathophysiology of septic cardiac dysfunction are increased inflammation, oxidative stress, impaired energetics and attenuated adrenergic signaling in the heart, indicating the complexity of this disease. Most of the treatments that have been tested so far, target one of these mechanisms leading to some or partial restoration of the cardiac function. Since this disease is so complex, it is

possible, by identifying a drug that can target more than one of these pathways, to gain full restoration or even prevention of the cardiac dysfunction during sepsis. So, in our studies we were interested in testing drugs that potentially can target multiple pathways or central mediators through distinct properties. Apocynin which was the drug we tested on our first study was known to inhibit NOX2 which produces ROS and particularly superoxide, so we hypothesized that by blocking NOX2 we could prevent oxidative stress, impaired energetics and therefore the development of cardiac dysfunction. LGM2605 that was the drug we tested on our second study was shown to have anti-inflammatory and antioxidant properties, but it was never tested on sepsis, and since inflammation and oxidative stress are two main causes of septic cardiomyopathy we hypothesized that by using this drug we could potentially alleviate the cardiac dysfunction.

CHAPTER II: Inhibition of NAPDH oxidase 2 (NOX2) prevents sepsis-induced cardiomyopathy

Relevant publication: Inhibition of NADPH oxidase 2 (NOX2) prevents sepsis-induced cardiomyopathy by improving calcium handling and mitochondrial function. Joseph LC, Kokkinaki D, Valenti MC, Kim GJ, Barca E, Tomar D, Hoffman NE, Subramanyam P, Colecraft HM, Hirano M, Ratner AJ, Madesh M, Drosatos K, Morrow JP. JCI Insight. 2017 Sep 7;2(17). pii: 94248. doi: 10.1172/jci.insight.94248. [Epub ahead of print] PMID: 28878116

Introduction

NADPH oxidases (NOX) are a family of enzyme complexes that produce ROS in mammalian cells, and particularly O_2^- and H_2O_2, by transferring electrons from NADPH inside the cell across the membrane and coupling them to molecular oxygen. To date, the known NOX enzymes are NOX1, NOX2, NOX3, NOX4 and NOX5. NOX1 is expressed in smooth muscle cells and other vascular cells, is important in host defense and cell growth and leads to generation of O_2^-. Nox2 is expressed in endothelial cells, phagocytes, but also in vascular, cardiac, renal, and neural cells, is important for blood pressure regulation and inflammation and generates O_2^-. Nox3 is expressed in fetal tissue, in the brain and in the adult inner ear and is involved in processes such as hearing and balance. Nox4 is mainly expressed in the kidney, in vascular smooth muscle and endothelial cells, is important in differentiation and is responsible for the basal production of H_2O_2. Lastly, Nox5 is a Ca^{2+}-dependent homolog, which is mostly expressed in testis and in immature lymphoid tissue and leads to the generation of H_2O_2. [37,38]. It is suggested that the NADPH oxidases which are expressed in cardiovascular cells, are involved in many physiological and/or pathological processes such as regulation of blood pressure and vascular tone, cell growth, cell migration, apoptosis and hypertrophy [39].Data showing the implication of NOX2 in the development of cardiac hypertrophy, even more support this involvement [40].

Multiple stress-related kinases are activated in the septic heart, including protein kinase C (PKC) and c-Jun N-terminal kinase (JNK) [13,41]. A recent study [42] proposed that NOX2 can be activated by PKC isoforms in cardiomyocytes. Measurement of NOX2 activity with a lucigenin assay showed significant increase following 2 hours of palmitate treatment. The increase in NOX2 activity was prevented by either inhibition of mitochondrial

uptake of fatty acids, using etomoxir, a carnitine palmitoyl transferase inhibitor, or by the PKC inhibitors Go6983 and LY333531. Furthermore, they measured the production of total ROS and mitochondrial ROS and they showed that pharmacological inhibition of NOX2 was almost as effective in preventing the increase in ROS production as PKC inhibition, demonstrating that PKC activation increases total cellular ROS predominantly via NOX2 in cardiomyocytes.

Oxidative stress and impaired energetics have been implicated in the pathophysiology of septic cardiomyopathy, but the mechanisms responsible for these cardiac abnormalities are not clearly identified. In our study, we hypothesized that NOX2 activation accounts for sepsis-induced oxidative stress and impaired energetics. Thus, NOX2 inhibition would prevent septic cardiomyopathy.

Experimental models of sepsis

There are three models of sepsis that are being used for experimental studies:

1. Bacterial infusion
2. Intraperitoneal (i.p.) injection of *E. coli* lipopolysaccharide (LPS)
3. Cecal ligation and puncture (CLP)

Previous studies of our laboratory, using the LPS model of sepsis, have shown early impairment of cardiac function (6 hours after LPS i.p injection) accompanied by decreased fatty acid and glucose metabolism and reduced ATP synthesis from the cardiomyocytes [43]. In our study, we chose to work with a peritonitis model, which is induced with cecal ligation and puncture (CLP). The CLP model includes ligation and perforation of the cecum allowing the release of fecal material into the peritoneal cavity to generate polymicrobial infection that elicits an exacerbated immune response. This model mimics human sepsis condition and therefore it is considered more clinically relevant. As in humans, mice that undergo CLP

with fluid resuscitation show the first (early) hyperdynamic phase that in time progresses to the second (late) hypodynamic phase [44].

Results

In order to standardize the CLP method, we had to determine the following parameters:

- Ligation site.
- Size of the needle used to perforate the cecum.
- Number of punctures
- Duration between application of the CLP and analysis of cardiac function. The length of ligated cecum defined as the distance from the distal end of cecum to ligation point will determine the degree of severity. A distance of >1cm produces high grade sepsis while a distance of ⩽1 cm produces mid-to-low grade sepsis. After a majority of experiments, we performed we concluded to a procedure that produces mid-to-low grade sepsis (ligation site:1cm), using a 19-gauge needle to perforate the cecum twice and follow up the mice at a timepoint of 12 hours post-surgery. In order to define this suitable timepoint for our experiments we evaluated when significant cardiac dysfunction occurs. Male C57BL/6 mice demonstrated significant cardiac dysfunction 9h post-CLP, which deteriorated further 12h post-CLP as shown by Fig 1B-D. On the other hand, female mice had only a slight decline in cardiac function even at 12 h post-CLP (Fig 1A).

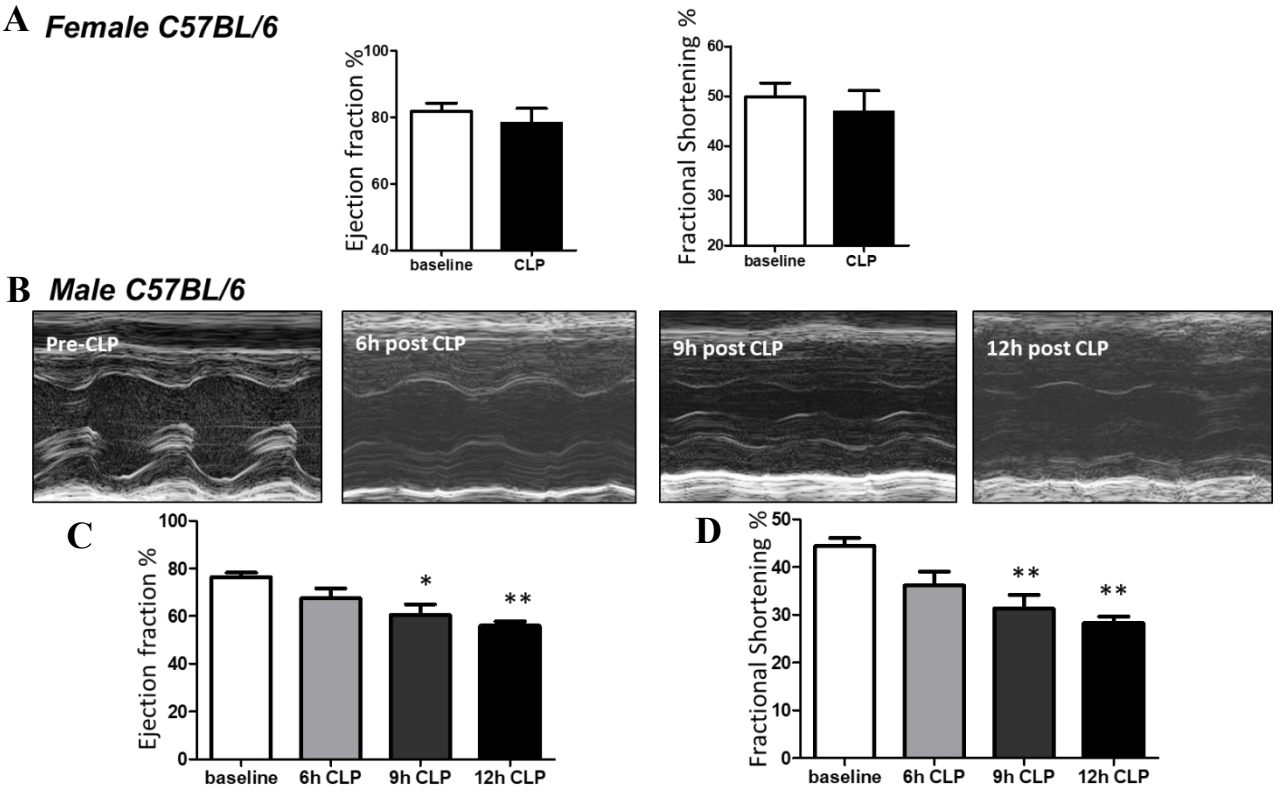

Figure 1: Cardiac function was impaired in male but not in female C57BL/6 mice – (A) Graph of ejection fraction (EF) and fractional shortening (FS) **(B)** Time-course of representative M-mode echocardiograms after CLP surgery. **(C)** Graph of ejection fraction (EF). **(D)** Graph of fractional shortening (FS), N=5 mice, One-way ANOVA analysis *p:<0.05 vs baseline. **p:<0.01 vs baseline. Data are presented as mean with SEM

NOX2 inhibition is cardioprotective in a mouse model of sepsis.

Previous studies in our group, performed by Dr. Christine Pol and Ms. Mesele-Christina Valenti, assessed the significance of NOX2 activation in septic cardiac dysfunction *in vivo*, using the LPS mouse model of sepsis. Wild-type (WT) mice were injected with LPS, that is known to decrease cardiac contractility [43] and then cardiac function was assessed by 2D-echocardiography. Inhibition of NOX activity with apocynin had a protective effect on systolic function after LPS injection (Fig 2A-C). Apocynin also prevented the increase in ventricular brain

natriuretic peptide (BNP) expression, a marker of heart failure (Fig 2D). To assess whether apocynin was protective in a more clinically relevant model of sepsis, we used an established model of polymicrobial sepsis, CLP. C57BL/6 mice that underwent CLP were treated with apocynin, during CLP surgery and 6 hours post-CLP and assessed cardiac function with 2D-echocardiography 12h post-CLP. This analysis showed that apocynin prevented cardiac systolic dysfunction (Fig 3A). To evaluate the therapeutic potential of apocynin we performed CLP in another group of mice and we treated them with apocynin 9 hours post-CLP, when consistent reduction in ejection fraction occurs. We showed that apocynin given after the onset of sepsis showed only a trend of improvement of cardiac function, as shown by ejection fraction and fractional shortening (Fig 3B). We also evaluated cardiac function 24 hours after CLP surgery. For this experiment, we administered the first dose of apocynin during CLP and the second dose 12 hours post-CLP. We evaluated cardiac function with 2D-echocardiography 24 hours post-CLP. Apocynin preserved the ejection fraction at 24 hours (Fig 3C). Collectively, these data suggest that inhibition of NOX2 in the early stage of sepsis improves cardiac systolic function.

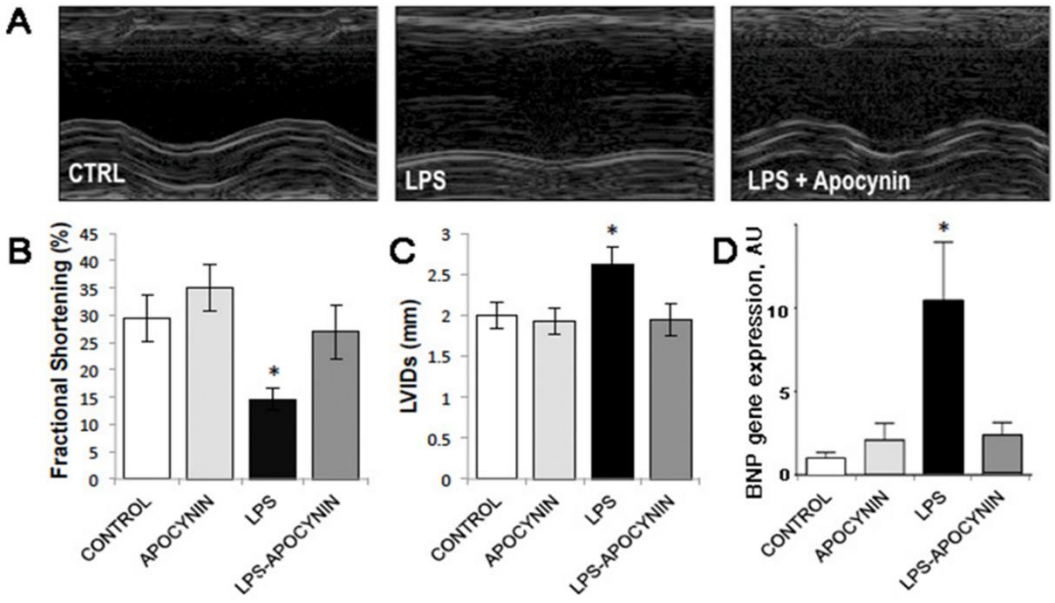

Figure 2. NOX2 inhibition prevents cardiomyopathy *in vivo* in the LPS model of sepsis.
(**A**) Representative M-mode echocardiograms. (**B**) Graph of fractional shortening (FS), $n = 4–5$ mice. (**C**) Graph of left ventricular internal dimensions during systole (LVIDs), $n = 4–5$ mice. (**D**) Graph of ventricular BNP mRNA levels, $n = 4–5$ mice. All panels show data from C57BL/6 mice treated with saline (control), apocynin, LPS, or combination of LPS and apocynin. *$P < 0.05$, compared with control by ANOVA with post-hoc testing. (Courtesy of Dr. Konstantinos Drosatos-Data produced by Mesele-Christina Valenti,).

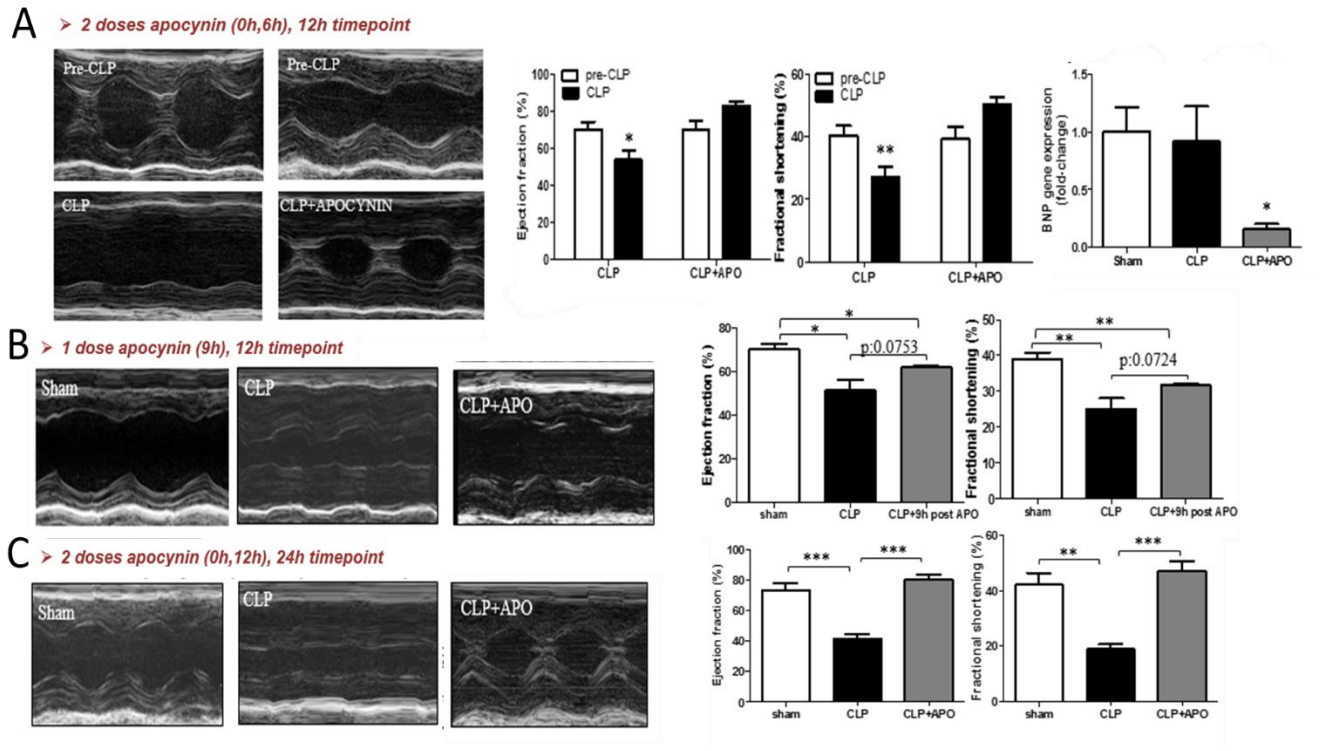

Figure 3. NOX2 inhibition prevents cardiomyopathy *in vivo* in the CLP model of sepsis
-(**A**) Representative M-mode echocardiograms from C57BL/6 mice treated with vehicle control, or apocynin, at the time of cecal ligation and puncture (CLP) surgery and 6hrs post-CLP. Graph of echo data before and after CLP surgery. Graph of ventricular BNP mRNA levels. (**B**) Representative echocardiograms after sham, CLP surgery, or CLP with apocynin given 9 hours after CLP surgery. Graphs of the echo parameters. N= 4 mice/group. **(C)** Representative echocardiograms after sham, CLP surgery or CLP with apocynin given at the time of surgery and 12 hours after the CLP surgery. Sham: n=4, CLP: n=4, CLP+apocynin: n=5 Graphs of echo parameters. The means are different by ANOVA; *p<0.05, **p<0.01, ***p:<0.001.

NOX2 inhibition reduces superoxide in cardiac ventricular tissue

With the help of the lab of Madesh Muniswamy (Dr. Nicholas Hoffman and Dr. Dhanendra Tomar) we performed dihydroethidium staining of ventricular tissue obtained from control mice, mice treated with apocynin, mice treated with LPS and mice treated with both LPS and apocynin, to assess ROS formation and particularly superoxide. This analysis showed that LPS caused a significant increase in oxidative stress, which was prevented by apocynin. This analysis also showed that LPS increased cardiac NADPH levels, which was

not prevented by apocynin. Since NOX2 activation would tend to consume NADPH and increase levels of NADP+, there must be compensatory mechanisms to increase NADPH after exposure to LPS. For example, NADPH is used in many anabolic reactions, so a decrease in anabolic metabolism could increase tissue levels. Collectively, these data suggest that NOX2 activation is the major mechanism causing oxidative stress in the early stages of sepsis.

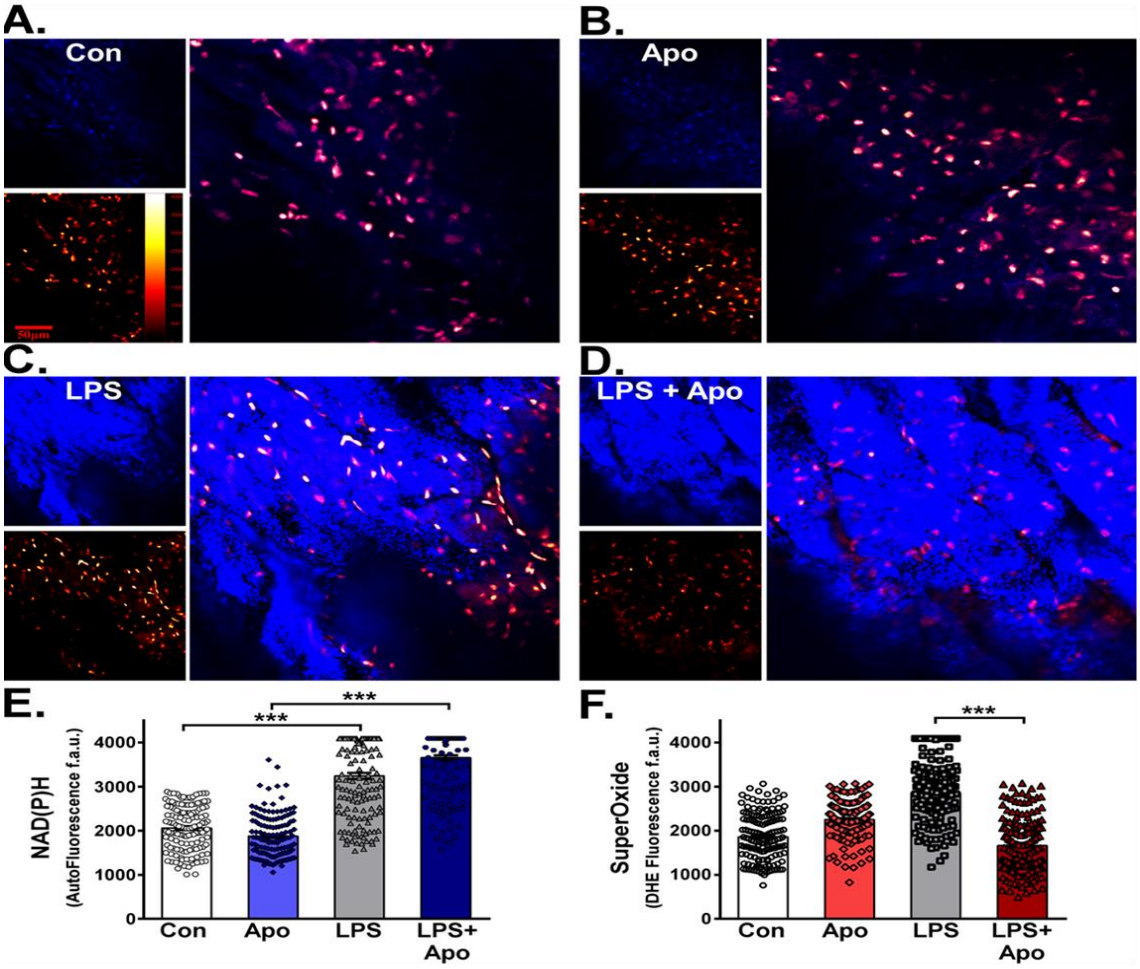

Figure 4. Apocynin reduces superoxide in cardiac tissue (left ventricle). (**A**) Representative confocal images of superoxide detection by dihydroethidium (DHE) and NADPH autofluorescence (blue color) in left ventricular tissue slices from control mice. DHE signal is indicated by the red to white range indicator. Scale bars: 50 μm, with high magnification (×2 zoom of merged images) inset indicated by dashed square. (**B**) Representative confocal image from apocynin-treated tissue for superoxide and NADPH. Same scale as **A**. (**C**) Representative confocal image from LPS-treated tissue for superoxide and NADPH. (**D**) LPS+apocynin–treated representative confocal image. (**E**) Quantification of NADPH signal from control, apocynin, LPS, and LPS+apocynin heart samples. (**F**) Quantification of superoxide signal from control, apocynin, LPS, and LPS+apocynin heart samples. n = 3–6 mice/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA

with post-hoc testing. (Courtesy of Dr. Madesh Munsiwamy – Data produced by Dhanendra Tomar and Nicholas Hoffman).

NOX2 inhibition improves cardiac fatty acid metabolism-related gene expression in LPS-treated mice

NOX inhibition prevented activation of cardiac JNK in LPS-treated mice (Fig. 5A). We have previously shown that the JNK pathway is activated in the heart during sepsis and is responsible for downregulation of genes required for normal mitochondrial respiration [13]. Consistent with this prior work, NOX inhibition prevented LPS-induced -alterations in FAO-related gene expression. LPS reduced cardiac mRNA levels of several genes that are critical for metabolism and mitochondrial function such as Pparα, Pgc1α, and Cpt1β (Fig 5B). Apocynin prevented downregulation of these genes (Fig. 5B). Not all of the genes that are critical for metabolism and mitochondrial function were downregulated by LPS. We found that LPS increased cardiac mRNA levels of uncoupling protein (Ucp) 2 and Ucp3, and these levels were normalized by apocynin (Fig. 5C). In contrast, NOX2 inhibition did not improve cardiac fatty acid metabolism in the CLP model of sepsis (Fig. 5D).

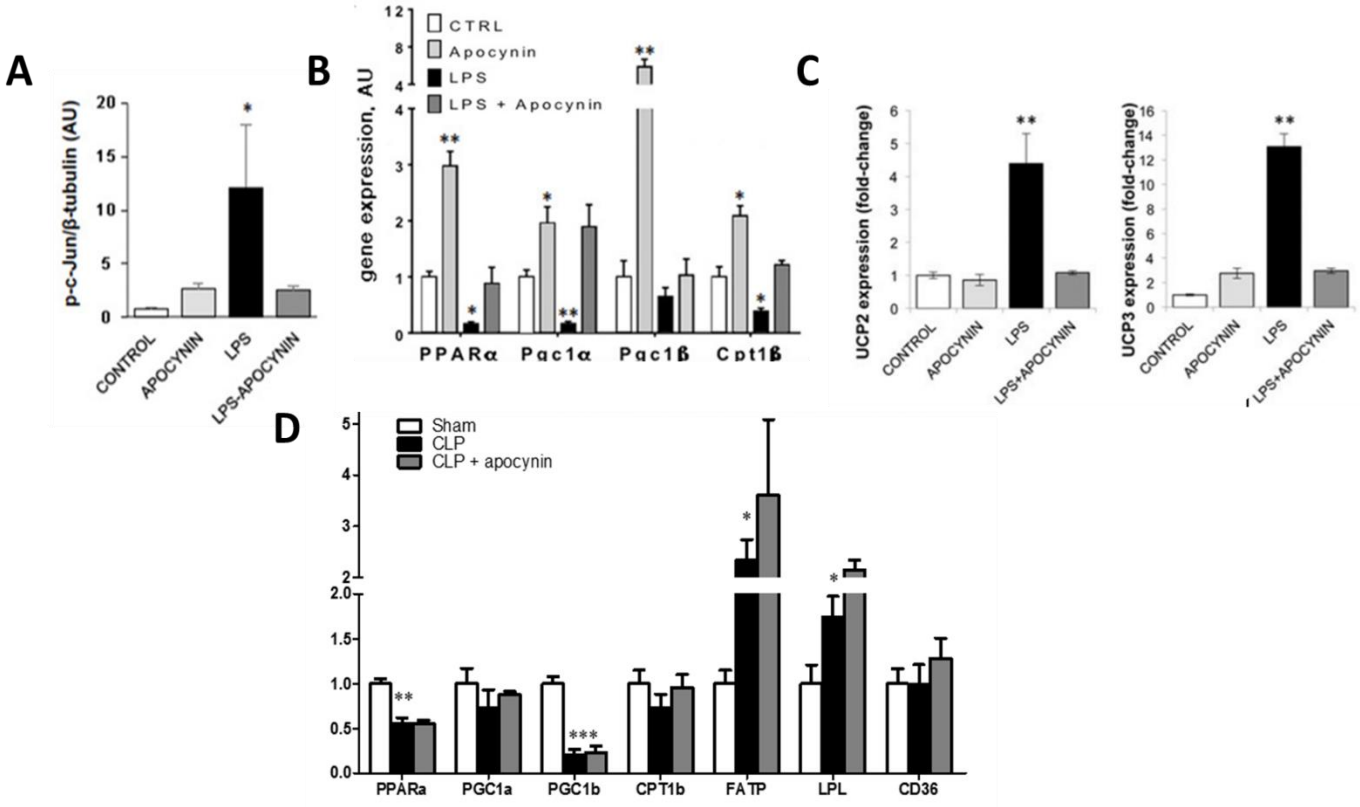

Figure 5. NOX2 inhibition improves cardiac fatty acid metabolism in LPS-treated mice. (**A**) Graph of multiplex protein analysis for p-JNK adjusted relative to β-tubulin. *n* = 4–5 mice. (**B**) Graph of metabolic gene expression from ventricular tissue. *n* = 6–8 heart samples. **(C)** Graph of cardiac Ucp2 and Ucp3 mRNA expression. All panels at (A), (B), (C) show data from C57BL/6 mice treated with saline (control), apocynin, LPS, or combination of LPS and apocynin. Courtesy of Dr. Konstantinos Drosatos -data produced by Mesele-Christina Valenti. –(**D**) Graph of metabolic gene expression from ventricular tissue, *n* = 4-5 mice per group. Data from C57BL/6 mice that underwent sham, CLP surgery or CLP and were treated with apocynin at the time of the CLP and 6hrs after the CLP, **P* < 0.05, ***P* < 0.01 compared with control by ANOVA with post-hoc testing.

Improvement in cardiac function by NOX2 inhibition is not associated with alleviation of systemic or cardiac inflammation

Experiments performed by Dr. Christine Pol and Ms. Mesele-Christina Valenti showed that treatment of C57BL/6 mice with LPS induced plasma IL-6 (fold change) and TNF-α (fold change) levels (Fig. 6A). Treatment of endotoxemic mice with apocynin did not reduce plasma IL-6 and TNF-α levels (Fig. 6A), indicating lack of improvement in inflammation by NOX2 inhibition.

In accordance with the LPS sepsis model, NOX2 inhibition did not have a significant anti-inflammatory effect in the CLP model based on cardiac tissue inflammatory gene expression (Fig 6B). We also drew blood 24 h post-CLP and measured white blood cell (WBC) number, which constitutes another measure of immune system activation. Both septic mice that were treated with vehicle or apocynin had lower WBC count compared with the sham mice (Fig 6C).

We also wanted to evaluate if apocynin could improve mortality in septic mice. So, we treated septic mice with apocynin at the time of the CLP surgery and then every 12 hours and we monitored the survival rate compared to the septic and sham mice. Both septic mice that were treated with vehicle (DMSO) or apocynin had significantly lower survival rate compared to the sham mice, and apocynin could not improve the mortality of the septic mice (Fig 6D).

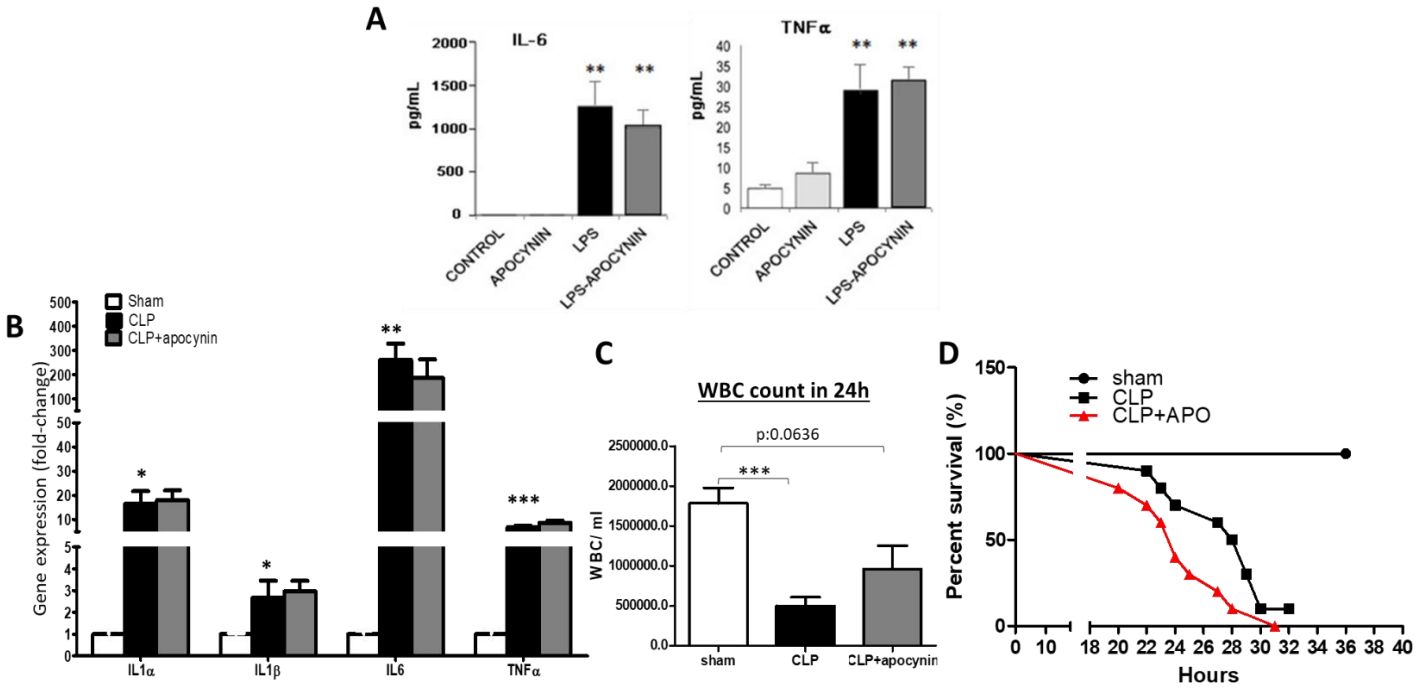

Figure 6. The benefit of NOX2 inhibition is not mediated by reduction of systemic or cardiac inflammation. (**A**) Plasma levels of IL-6 and TNF-α, of mice treated with saline (control), apocynin, LPS, or combination of LPS and apocynin $n = 4–5$ mice. Courtesy of Dr. Konstantinos Drosatos -data produced by Mesele-Christina Valenti (**B**) Inflammatory markers (IL1a, IL1b, IL6, TNFa) gene expression in ventricular tissue of mice that underwent sham, CLP surgery or CLP and were treated with apocynin at the time of the CLP and 6hrs after the CLP $n = 4–5$ mice per group. (**C**) Measurement of WBC count from blood collected at 24 hrs timepoint from mice that underwent sham, CLP surgery or CLP and were treated with apocynin at the time of the CLP and 12hrs after the CLP (**D**) Survival curves of mice that underwent sham, CLP surgery or CLP and were treated with apocynin at the time of the CLP and every 12hrs after the CLP. N=4 mice in the sham group, N=10 mice in the CLP group, N=10 mice in the CLP+apocynin group. The sham and CLP mice received DMSO (vehicle control) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by ANOVA with post-hoc testing.

LPS and CLP activate PKCδ and PKCβ

Since we suspected that PKC activation could be upstream of NOX2 activation in isolated cardiomyocytes [42], we evaluated PKC isoforms in membrane preparation lysates from ventricular tissue of mice injected with LPS or vehicle control. This analysis showed that membrane: cytosolic PKCα ratio showed a nonsignificant trend of increase. PKCβ and PKCδ were significantly activated in the hearts of mice treated with LPS. There was also a corresponding decrease in membrane-bound PKCε (Fig 7).

We also confirmed that PKC isoforms were activated in the hearts of mice after CLP, particularly PKCβ and PKCε were significantly activated (Fig 8). Notably, PKCβ was activated in both the LPS and the CLP sepsis models.

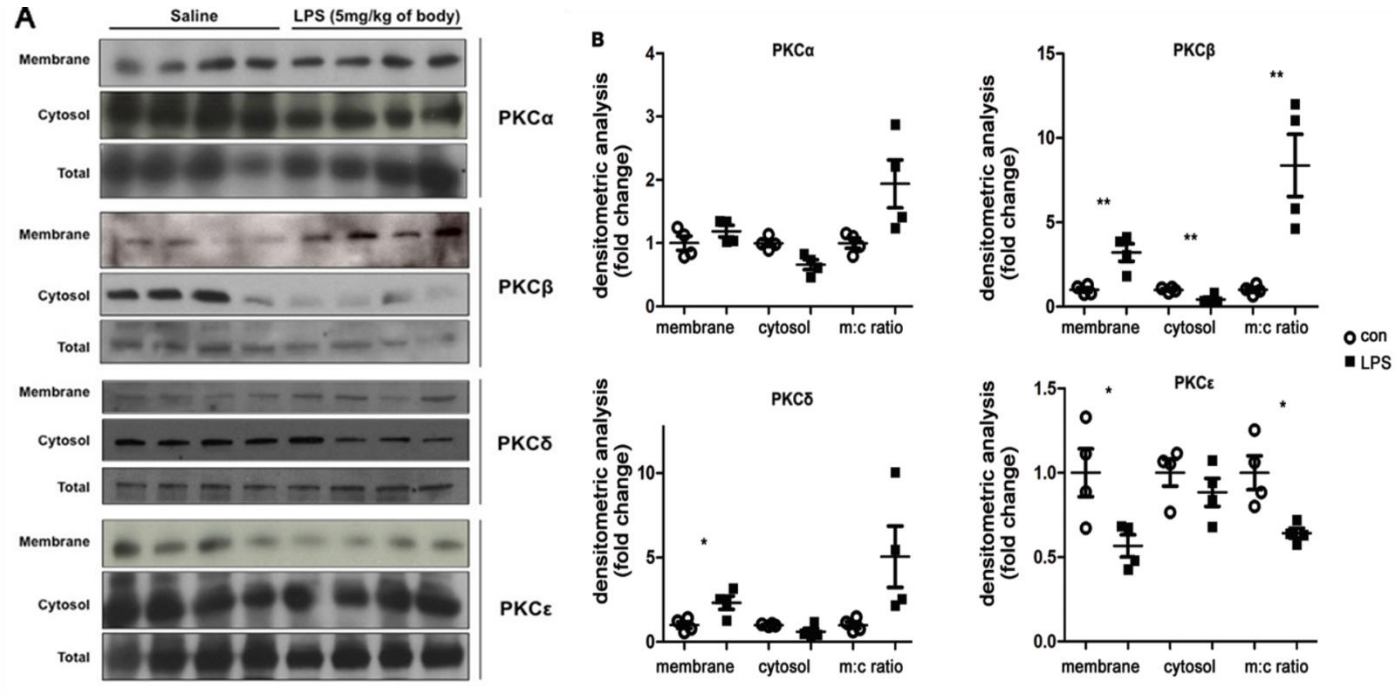

Figure 7. LPS activates PKCβ and PKCδ (**A**) Western blots of PKC isoforms, membrane and cytosolic fraction, from ventricular tissue of mice treated with LPS or vehicle control. (**B**) Densitometric analysis of PKC Western blots, in relative units. **P* < 0.05, ***P* < 0.01 by unpaired *t* test (control vs. LPS for each fraction). Open circles are control, squares are LPS treatment. (Data produced by Dr. Konstantinos Drosatos).

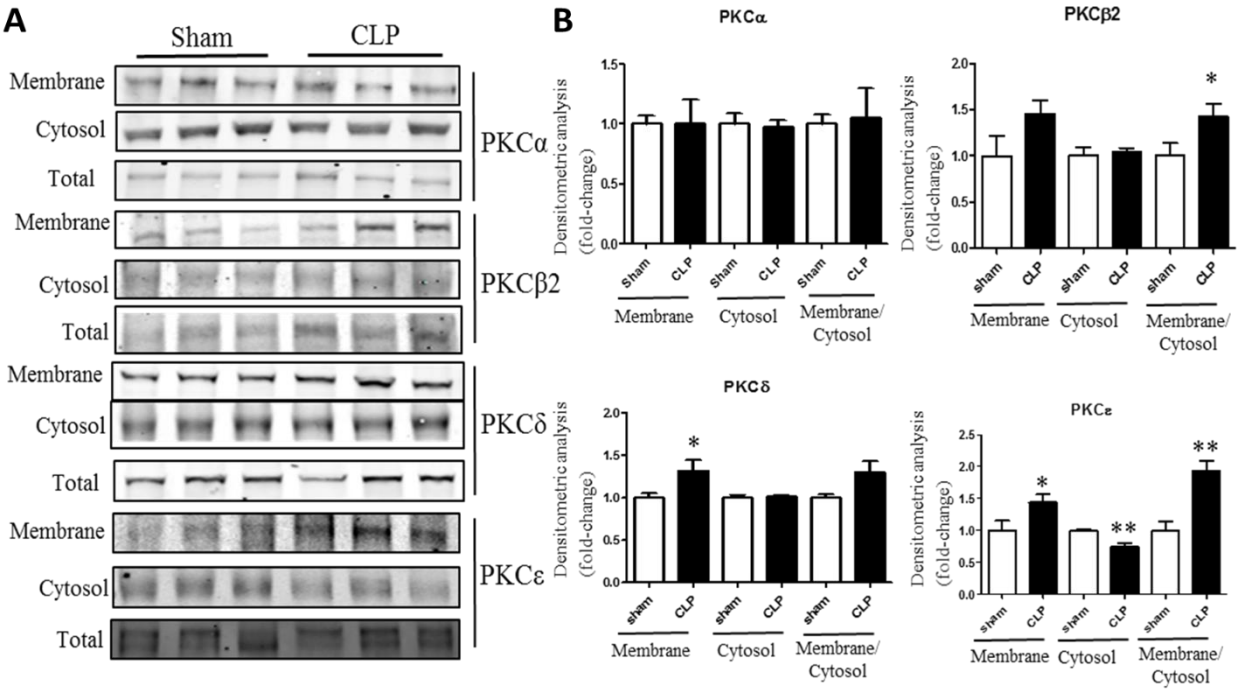

Figure 8: Cardiac PKCβand PKCε are activated in C57BL/6 mice that underwent CLP
PKCα, PKCβ, PKCδ and PKCε western blots (A) and densitometric analysis (relative units) (B), of total, membrane and cytosolic protein lysates obtained from ventricular tissue of C57BL/6 mice that underwent sham surgery or CLP. Unpaired t-test *p<0.05, **p<0.01

PKC inhibition is cardioprotective in a mouse model of sepsis.

We evaluated the *in vivo* benefit of PKC inhibition using the LPS and CLP animal models of sepsis. Previous studies performed by Dr. Konstantinos Drosatos, where C57BL/6 mice were treated with saline, LPS and/or the PKC inhibitor, showed that the broad-spectrum PKC inhibitor Ro-31-8220 prevented cardiac systolic dysfunction in LPS-treated mice (Fig. 9A). PKC inhibition also prevented the increase in ventricular brain natriuretic peptide (BNP) expression (Fig 9B).

Consistent with the LPS model, PKC inhibition had a protective effect on cardiac systolic function that was assessed in the CLP model. C57BL/6 mice underwent CLP and were treated with the PKC inhibitor during CLP and 6 h post-CLP. The cardiac function of the mice that underwent CLP and were treated with the PKC

inhibitor was normal 12h post-CLP (Fig. 10A). Metabolic gene expression in the heart was improved by the PKC inhibitor in the CLP model as shown by the improved mRNA levels of PPARa, PGC1a and PGC1b (Fig 10B). Furthermore, in both the LPS (Fig. 9C) and CLP model (Fig 10C). PKC inhibition did not have a significant anti-inflammatory effect, as shown by plasma cytokine levels (Fig. 9C) and cardiac inflammatory gene expression (Fig 10C), respectively.

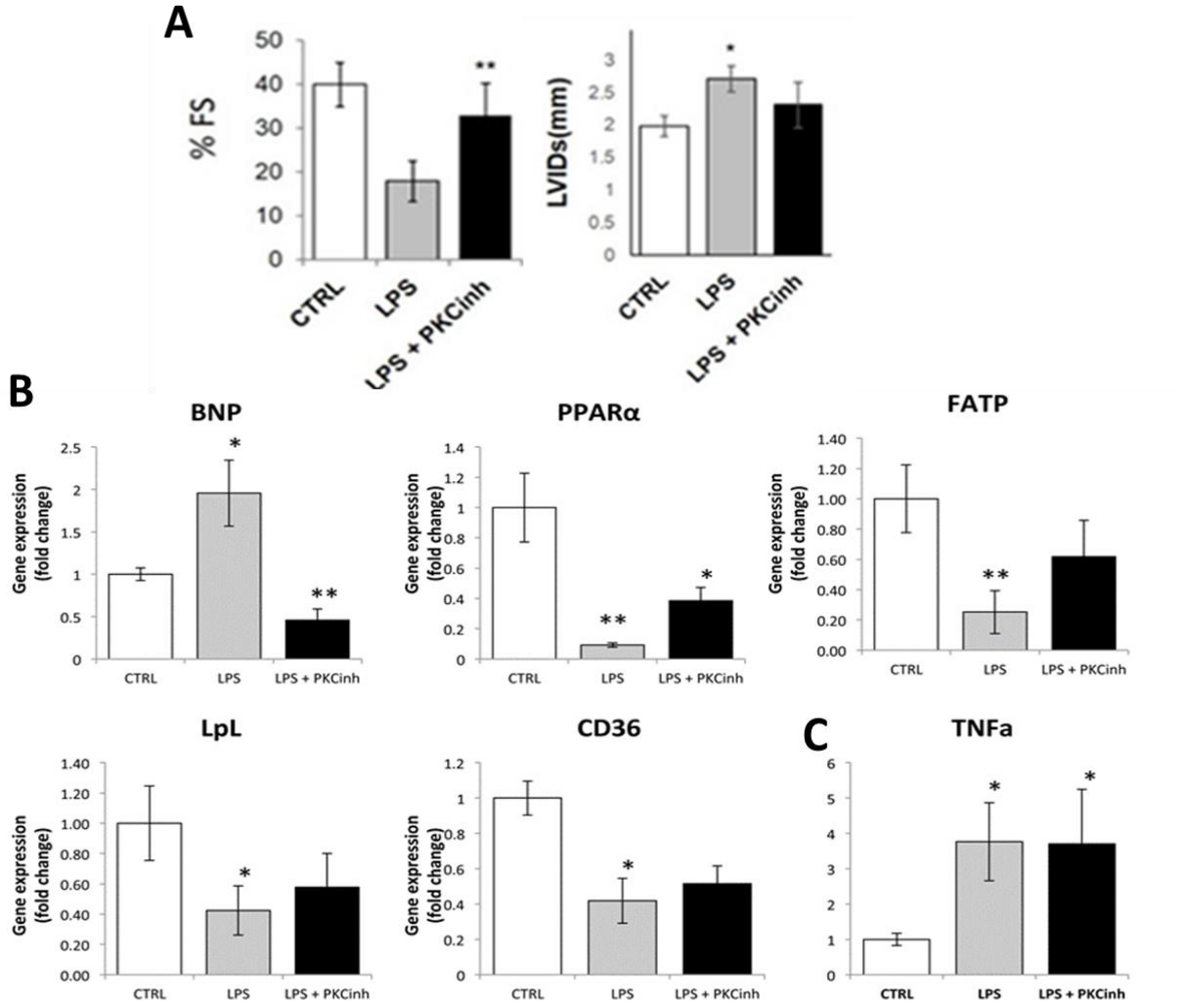

Figure 9: PKC inhibition improves cardiac function in the LPS model of sepsis (**A**) Fractional shortening (FS) and left ventricular internal diameter during systole (LVIDs). (**B**) Cardiac mRNA levels of BNP, PPARa, FATP, LPL and CD36 (**C**) Plasma levels of TNF-α, **P* < 0.05, ***P* < 0.01 by ANOVA with post-hoc testing. All data are from C57BL/6 mice treated with saline, LPS, and/or PKC inhibitor Ro-31-8220, *n* = 4 (Courtesy of Dr.Konstantinos Drosatos -data produced by Mesele-Christina Valenti).

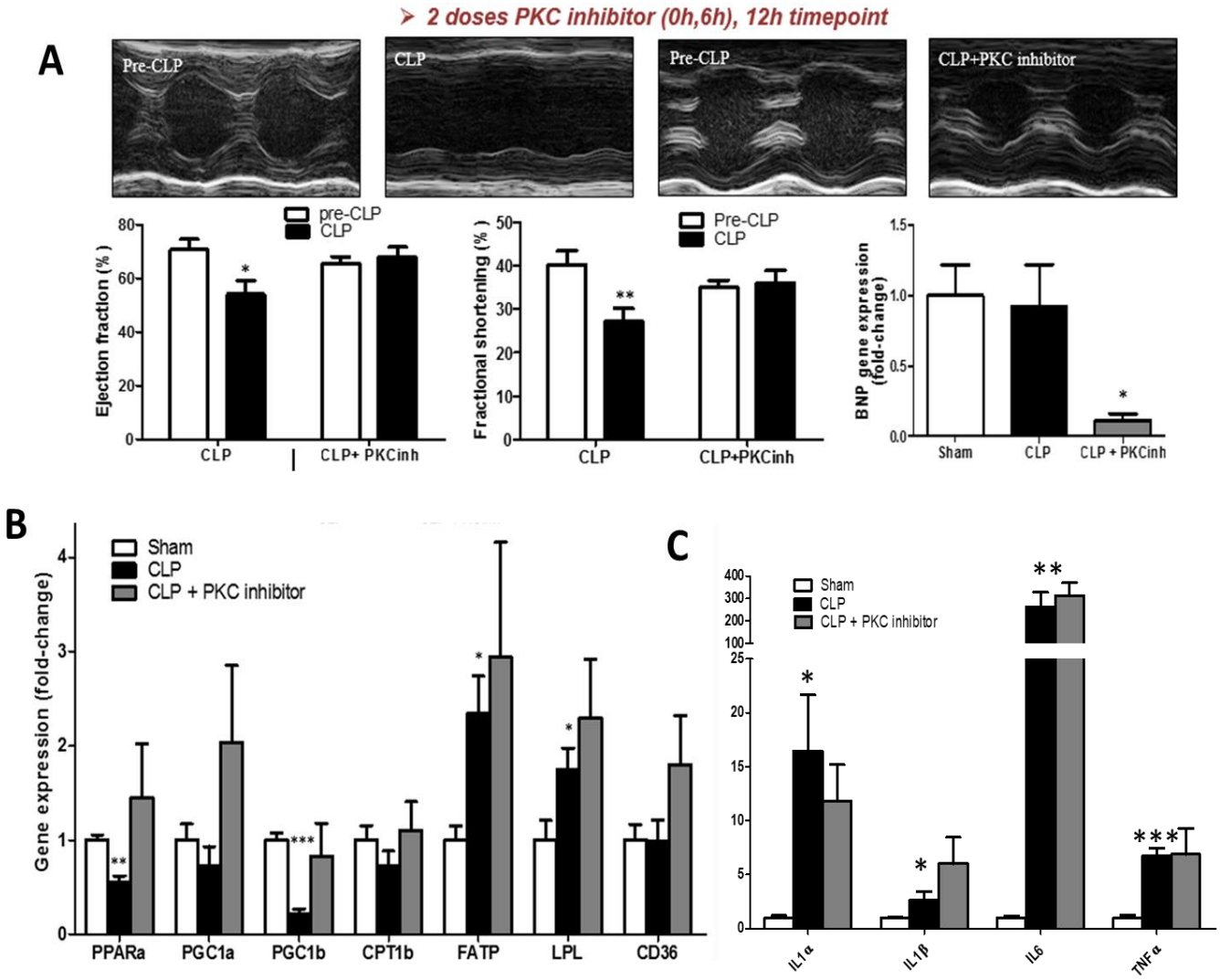

Figure 10: PKC inhibition improves cardiac function in the CLP model of sepsis (**A**) Representative M-mode echocardiograms from C57BL/6 mice treated with vehicle control or PKC inhibitor Ro-31- 8220, at the time of cecal ligation and puncture (CLP) surgery and 6 hours after CLP, ejection fraction before and after CLP surgery, fractional shortening before and after CLP surgery, cardiac BNP mRNA levels (**B**) Cardiac mRNA levels of PPARα, PGC1α, PGC1b, CPT1b, FATP, LPL and CD36 from mice that underwent sham, CLP surgery and mice that underwent CLP surgery and were treated with the PKC inhibitor at the time of CLP and 6hrs post-CLP, n = 4–5 mice per group. (**C**) Inflammatory markers (IL1a, IL1b, IL6, TNFa) gene expression from ventricular tissue of mice that underwent sham, CLP surgery and mice that underwent CLP surgery and were treated with the PKC inhibitor at the time of CLP and 6hrs post-CLP, n = 4-5 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with post-hoc testing.

Discussion

Sepsis is a very common and complicated disease. Cardiac complications are associated with worse prognosis. Currently, the underlying mechanisms are not fully elucidated, which constitutes the reason for the lack of an effective treatment for septic cardiomyopathy. In our study, we showed that *in vivo* inhibition of NOX2 protects mice from sepsis-induced systolic dysfunction. The protective effect of NOX2 inhibition was observed in both the LPS-injection and the CLP model of sepsis.

This is the first time to our knowledge that pharmacologic NOX2 inhibition has been shown to prevent sepsis-induced contractile dysfunction *in vivo*. There was a prior report, which showed that NOX2-KO hearts were protected from the harmful effects of LPS emphasized though in the role of inflammation [45]. In our study, NOX2 inhibition preserved cardiac function, alleviated oxidative stress, reduced JNK activation and downregulation of metabolic genes without resolution of the inflammatory component of the disease based on both serum cytokines and cardiac tissue inflammatory gene expression, although apocynin may have an effect on WBC function. Thus, our study identifies a potentially novel role for NOX2 in the pathophysiology of the disease, acting through cardiac metabolism in a way that is largely independent of systemic inflammation.

Since it is suggested that PKC activation could be upstream of NOX2 activation in isolated cardiomyocytes [42] and since in our study we found activation of PKCβ in both models of sepsis (LPS and CLP) we think that this is probably the activator of NOX2. Although we show that NOX inhibition is effective at preserving contractility *in vivo*, it is also possible that activation of PKC isoforms by LPS has harmful effects that are not mediated by NOX2. For example, the gene expression data from the CLP model indicate that PKC inhibition is more effective at normalizing metabolic gene expression than apocynin, the NOX inhibitor.

Although immune system activation may contribute to cardiac dysfunction in later stages of sepsis [11,46], our experiments show that the NOX2 inhibitor apocynin prevents systolic dysfunction *in vivo* without reducing

circulating proinflammatory cytokines or cardiac inflammatory gene expression. This demonstrates that cardiac contractility and systemic inflammation can be dissociated. This finding is consistent with our previous findings showing that induction of cardiac fatty acid oxidation and energy production improved cardiac function prior to alleviation of inflammation [13,43]. These new findings, in combination with our prior work, suggest a crucial role for restoration of cardiac energetics as a way to maintain cardiac function in sepsis, independently of systemic inflammation, at least for the early stage of the disease. The fact that NOX activation during sepsis decreases expression of genes that are required for metabolism and mitochondrial function such as PPARa, PGC1a and CPT1b is a potentially novel finding. We have previously shown that LPS-mediated JNK activation is responsible for the decrease in expression of *PPARa* and other genes required for fatty acid oxidation by mitochondria [13]. NOX2 inhibition prevented activation of JNK, which improved the expression of these genes.

We also found that cardiac expression of UCP2 and UCP3 was upregulated in the mouse model of sepsis. UCPs are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis. Superoxide activates UCPs [47], and UCP activation may decrease mitochondrial superoxide production [48]. Although this may be an adaptive response to increased oxidative stress, increased UCP activity and/or expression could compromise cardiac efficiency by decreasing the mitochondrial inner membrane potential.

The NOX inhibitor apocynin did not improve mortality in the rodent CLP model. A reason for that may be that NOX2 inhibition may inhibit respiratory burst in immune cells and thus compromise the removal of the pathogen. For that reason, maybe the LPS model might be more appropriate to test the effect of apocynin on the survival. It is possible though that with intravenous fluid resuscitation and broad-spectrum antibiotics, NOX inhibitors could have additional benefit during septic shock complicated by cardiomyopathy.

In conclusion, our results indicate that NOX2 activation is a critical factor in the pathophysiology of sepsis-induced cardiomyopathy, acting at least in part independently of systemic inflammation. NOX2 activation

increases cardiac superoxide production, causes mitochondrial dysfunction, and decreases contractility. We used a small-molecule inhibitor of NOX2 in 2 different models of sepsis to demonstrate the potential for translational application of these findings. Although NOX2 inhibition could potentially reduce bactericidal functions of WBCs, it is possible that selective, partial inhibition of NOX2 in combination with antibiotics would be beneficial during sepsis.

Materials/Methods

Animal care, sepsis models - C57BL/6 mice were purchased from The Jackson Laboratory and were 8–12 weeks old at the time of experiments. LPS injections were performed as previously described and animals were sacrificed 6–9 hours after LPS injections. Briefly, 5 mg/kg of LPS (Sigma-Aldrich), which is a sublethal dose, was administered by intraperitoneal injection. Control mice were treated with an equal volume of saline. Mice received intraperitoneal injection of 6 mg/kg Ro-31-8220 for PKC inhibition or 50 mg/kg apocynin for NOX inhibition. Both pharmacologic agents were administered 30 minutes prior to LPS injection or as stated in the "Results" section. For the CLP surgery, a 1- to 2-cm midline laparotomy was created to expose the cecum with the adjoining intestine. The cecum was ligated 1 cm from the distal end of the cecum. The cecum was punctured twice with a 19-gauge needle and gently squeezed so that a small amount of feces was extruded. Mice were resuscitated by injecting 1 ml of 0.9% saline solution subcutaneously. Mice were injected with inhibitor compounds at the time of surgery and again 6 hours later. Control mice received vehicle (DMSO) at both time points as a control.

2D-echocardiography - Echocardiograms were performed at 6 hours after LPS injection and at 12 hours after CLP or as stated in the "Results" section. Two-dimensional echocardiography was performed on anesthetized

mice (inhaled isoflurane) using a VisualSonics Vevo 2100 machine. Echocardiographic images were recorded in a digital format. A single observer blinded to the respective treatments of mice then analyzed images off-line.

Histology - Freshly isolated ventricular tissue sections were loaded with dihydroethidium (150 μm) in serum-free media for 30 minutes at room temperature with shaking at 30 rpm, to visualize ROS. NADPH autofluorescence was acquired using a 2-photon laser at 720 nm excitation and emission at 380–550 nm. Images were acquired using a Zeiss 710 Microscope equipped with 10× objective and a Coherent Chameleon 2-photon laser.

RNA purification and gene expression analysis - Total RNA was purified from cells or hearts using TRIzol reagent according to the manufacturer's instructions (Invitrogen). DNase-treated RNA was used for cDNA synthesis using the ProtoScript II First-Strand cDNA Synthesis Kit (New England Biolabs). Quantitative real-time PCR was performed with the SYBR Select Master Mix (Applied Biosystems). Incorporation of the SYBR green dye into the PCR products was monitored in real time with an Mx3000 sequence detection system (Stratagene) or with the Applied Biosystems StepOnePlus Real-Time PCR System. Samples were normalized against 18S ribosomal RNA or 36B4 RNA. The sequences of the primers have been described previously.

Protein purification and analysis - Isolated heart tissues or cells were homogenized in radioimmune precipitation assay (RIPA) buffer containing protease inhibitors (1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM ethylene glycol tetraacetic acid, and 2 mM ethylene diamine tetraacetic acid; Sigma-Aldrich) as well as 1 mM dithiothreitol and phosphatase inhibitors (Halt phosphatase inhibitor mixture, Thermo Fisher Scientific). Total protein extract was applied (25 μg) to SDS-PAGE and transferred onto nitrocellulose membranes. Antibodies were obtained from Millipore (PKCα, 05-154), Santa Cruz Biotechnology (β-actin, C4sc47778; JNK, D2sc7345; PKCβ, E3sc8049; PKCδ,

C17sc213; and PKCε, E5sc1681), and Cell Signaling Technology (phospho-PKCδ, 9374S; phospho-JNK, 2993S; phospho-JNK-Ser-63, 9261S; phospho-JNK-Ser-73, 9164S; and phospho-ERK, 9101).

Multiplex cytokine and phosphoprotein analysis - Cytokine concentrations in mouse serum were determined using the mouse cytokine/chemokine magnetic bead panel (Millipore) according to the manufacturer's instructions. The final data were obtained and analyzed via the Bioplex Magpix multiplex reader system (Bio-Rad). Loading for the protein panels was normalized via determination of total protein concentration (BCA Protein Assay kit, Pierce).

Measurement of WBC count - We used ACK buffer-erythrocyte lysis buffer to isolate WBCs from total blood, incubated for 5–10 minutes on a rocker. This was followed by addition of equal volume of cold phosphate-buffered saline (PBS), centrifugation at 300 *g* for 5 minutes (2°C–8°C), and removal of the supernatant. The pellet was resuspended with ACK lysis buffer and the procedure continued until the pellet became white. We measured the WBCs using the TC20 Automated Cell Counter from Bio-Rad.

Statistics - Results are presented as the mean ± SEM. The unpaired *t* test was used for comparisons of 2means; a 2-tailed *P* value of less than 0.05 was considered statistically significant. For groups of 2 or more, ANOVA was used with Bonferroni as a post-hoc testing (Prism v5, GraphPad Software).

Study approval.

Animal protocols were reviewed and approved by Temple University Institutional Animal Care and Use Committees (Philadelphia, PA,) and were carried out in accordance with the NIH guidelines for the care and use of laboratory animals.

CHAPTER III: Chemically synthesized Secoisolariciresinol diglucoside prevents sepsis-induced cardiomyopathy

Introduction

Flaxseed, which is the richest known source of secoisolariciresinol diglucoside (SDG), the mammalian lignan precursor, is a non-toxic whole grain that consists of high concentrations of omega-3 fatty acids and lignans. Flaxseed and SDG are potent antioxidants with anti-inflammatory and anti-fibrotic properties [49, 50,51]. It has been proposed that its antioxidant activities are accounted for by its hydroxyl radical scavenging activity [52].

SDG has shown potential health benefits in many diseases, such as hypercholesterolemia, diabetes, postmenopausal symptoms, CVD, metabolic syndrome, and bone health, ARDS, Ischemia-Reperfusion Injury, radiation pneumonopathy and hyperoxia [50]. As we mentioned in the beginning of the thesis, during sepsis a lot of abnormalities occur in which potentially SDG may play a role through its distinct properties. For instance, during sepsis both CPT-1 and GLUT4 are downregulated, and there are previous studies that have shown that SDG increases CPT-1 in skeletal muscle and GLUT4 during obesity [53]. Furthermore, triglycerides, free fatty acids, oxidative stress and inflammation are found to be increased during sepsis and SDG has been shown to decrease or normalize them in CVD and metabolic syndrome models [54]. In a model of radiation-induced lung injury, SDG has also shown to prevent DNA damage, enhance the antioxidant capacity of normal lung cells and decrease the oxidative tissue damage as well as the nitrosative stress by increasing the gene and protein levels of the antioxidants HO-1, GSTM1 and NQO1, though activation of the Keap1/Nrf2/ARE pathway that regulates these antioxidants and cytoprotective genes [55]. All the above are possible mechanisms through which SDG may act and prevent septic cardiomyopathy.

In our study, we used the LGM2605 which is chemically synthesized SDG [56]. LGM2605 is a free radical scavenger and antioxidant with DNA-protective activity similarly to the natural SDG extracted from whole

grain flaxseed [57]. LGM2605 has also been shown to have potent cell-protective and antioxidant properties, while being capable of inducing phase II antioxidant, cell-protective enzymes that are transcriptionally regulated by the Nrf2-ARE signaling pathway [55].

Inflammation and oxidative stress are major complications that are associated with cardiac dysfunction in sepsis. Based on the antioxidant and anti-inflammatory properties of LGM2605, we hypothesized that it would alleviate septic cardiac dysfunction. Cardiac mitochondrial function and ATP synthesis are also compromised in sepsis [13]. As LGM2605 has antioxidant properties, we hypothesized, that it would restore cardiac energetics and cardiac function in sepsis. Since increased production of ROS has been shown to impair cardiac β-adrenergic signaling [25] which is impaired in sepsis [12], we anticipated that the anti-oxidant effect of LGM2605 would improve β-adrenergic receptor signaling, increase cardiac contractility, heart rate and therefore reverse cardiac dysfunction triggered by sepsis and increase the survival rate in the septic mice. Because oxidative stress and inflammation have been described in various organs during sepsis, we hypothesized that treatment of septic animals with LGM2605 would likely improve the function of other organs and increase survival rate. Thus, we assessed whether LGM2605 can prevent septic cardiac dysfunction and mortality in mice and investigated the mechanisms that may mediate this beneficial effect.

Results

LGM2605 prevents septic cardiac dysfunction

To assess the effect of LGM2605 administration in septic cardiac dysfunction *in vivo* we performed cecal ligation and puncture (CLP), an established model of polymicrobial sepsis. We administered intraperitoneally LGM2605 at a dose of 100 mg/kg body weight either 2hours prior of CLP or 6 hours after CLP. At 12 hours after CLP, we assessed cardiac function by two-dimensional echocardiography. The mice that underwent CLP showed significant impairment of their systolic cardiac function in 12 hours accompanied by decreased ejection fraction

and fractional shortening (Fig. 11). On the other hand, LGM2605 treatment prevented cardiac dysfunction both in mice that received the drug 2 hours prior to CLP or 6 hours after CLP (Fig. 11) . So, the chemically synthetized SDG prevented septic cardiac dysfunction.

In order then to assess the mechanisms that mediate the beneficial effects of LGM2605 in septic cardiac dysfunction, we evaluated each one of the mechanisms that have been associated with the pathophysiology of septic cardiomyopathy: inflammation, impaired β-adrenergic signaling, oxidative stress and impaired metabolism and reduced ATP synthesis.

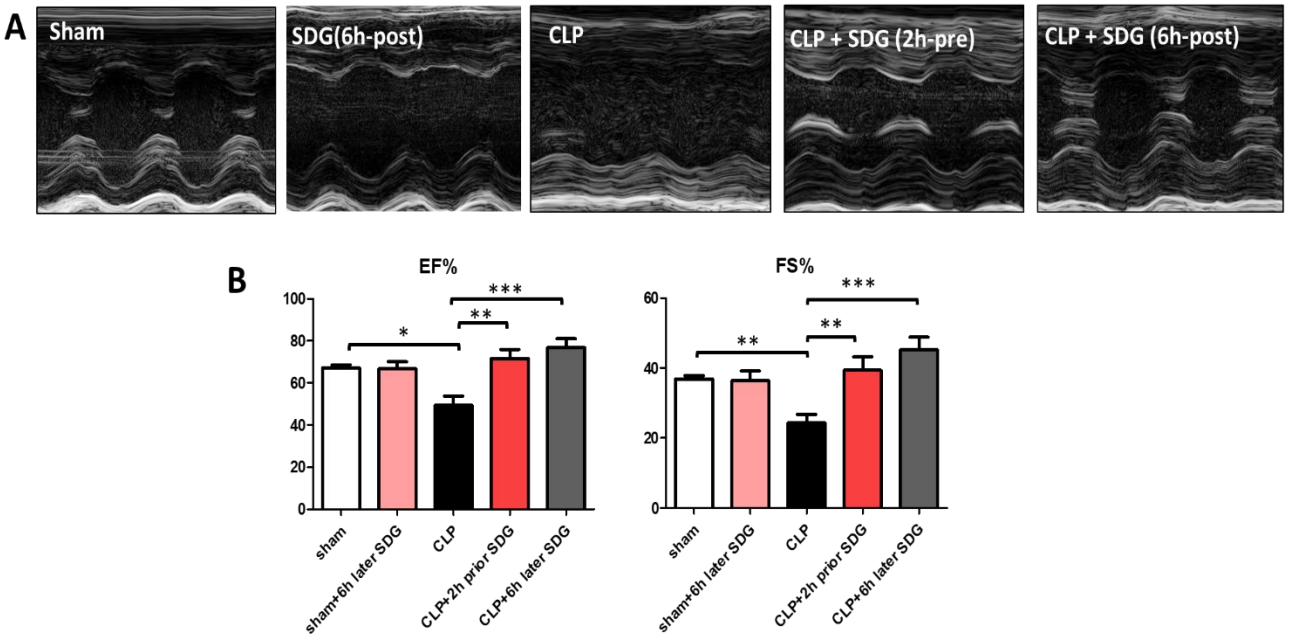

Figure 11. LGM2605 prevents septic cardiac dysfunction *in vivo* in C57BL/6 mice that underwent CLP - (**A-B**) Representative M-mode echocardiograms (A), ejection fraction (EF) and fractional shortening (FS) (B) of C57BL/6 mice treated with LGM2605 2hrs prior to CLP or 6hrs post-CLP and monitored for 12hrs after the surgery. Sham: n=4, Sham+6hrs SDG: n=4, CLP: n=7, CLP+2hrs prior SDG: n=4, CLP+6hrs SDG: n=8. Unpaired t-test comparison; *p<0.05, **p<0.01, ***p:<0.001.

LGM2605 influences NF-kB activation but not cardiac gene expression of inflammatory cytokines

NF-κB is a major component of inflammation [58]. NF-κB protein levels were, as expected, significantly increased (fold change) in AC16 cells treated with LPS 1 μg/ml for 12 hours (Fig.12A). Combined treatment with LPS and LGM2605 at a dose of 50mM tended to restore NF-κB expression (p=0.06). We then tested if LGM2605

alleviates the expression of cardiac inflammatory markers in the hearts of septic C57BL/6 mice. Specifically, in collaboration with the group of Dr. Anastasios Lymperopoulos, we assessed phosphorylation of IκBα, which is the inhibitor of NF-κB. Higher phosphorylation of IκBα and lower levels of total IκBα are associated with NF-κB activation, since IκBα phosphorylation leads to degradation. Mice with CLP showed a trend for increased phosphorylation of IκBα, and lower levels of total IκBα at 12 hours post-CLP (Fig. 12B), which suggests increased NF-κB activation. Administration of LGM2605 in these mice tended to restore the phosphorylated IκBα/total IκBα ratio.

In contrast, the anti-inflammatory effect of the drug did not reflect to the cardiac mRNA levels of inflammatory markers that we assessed in septic C57BL/6 mice (Fig. 12C-D). Cardiac mRNA levels of inflammatory markers such as IL1α, IL1β, IL6, and TNFa in septic mice were significantly increased at both 6 hours (Fig 12C) and 12 hours after CLP (Fig.12D). However, LGM2605 did not seem to restore the expression to normal levels (Fig. 12C-D).

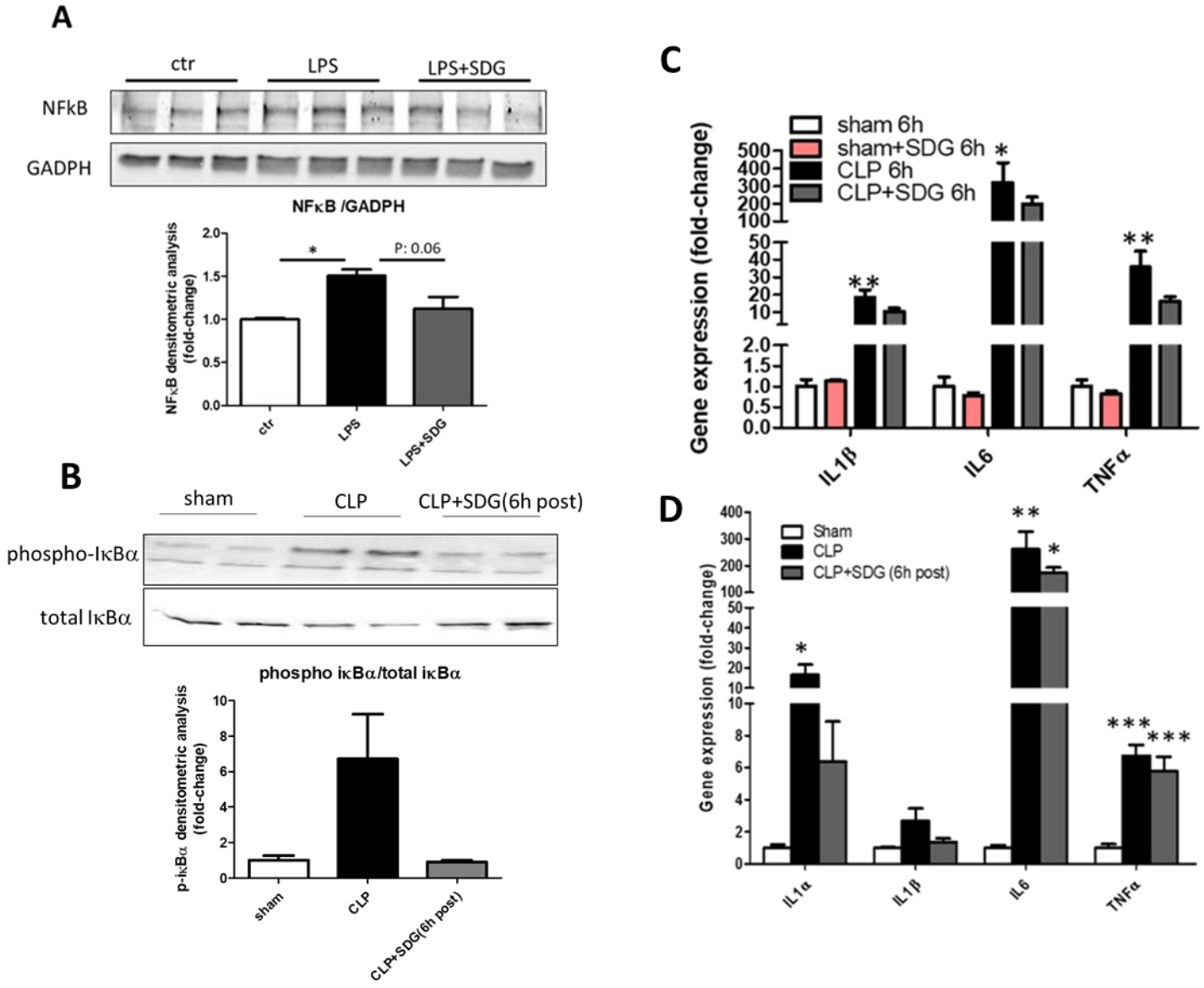

Figure 12: LGM2605 influences NF-kB activation but not cardiac gene expression of inflammatory cytokines (**A**) Immunoblotting and densitometric analysis of NF-κB in cell lysates obtained from AC16 cells either untreated or treated with LPS or LPS+LGM2605 for 12 h. (**B**) Immunoblotting and densitometric analysis of phosphorylated and total IkBα from ventricular tissue of mice 12hours post- control sham surgery, CLP or CLP followed by treatment with LGM2605 (Courtesy of Dr. Anastasios Lymperopoulos) (**C**) Cardiac IL-1β, IL-6, and TNFα mRNA levels of mice 6hours post-sham surgery, CLP or CLP+LGM2605, *n* = 3-4 mice per group. (**D**) Cardiac IL-1α, IL-1β, IL-6, and TNFα mRNA levels of mice 12hours post-sham surgery, CLP or CLP followed by treatment of LGM2605, *n* = 4-5 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by ANOVA with Bonferroni post-test.

LGM2605 stimulates components of β-AR signaling but not during sepsis

It has been shown that sepsis affects cardiac contractility [10] and cAMP levels, which are controlled by β-adrenergic receptor signaling. In order to assess whether the improved cardiac function of septic mice that were treated with LGM2605 may be attributed to improved β-AR signaling, we first assessed cAMP levels in AC-16 cells treated with LPS and LGM2605. All the different groups of cells that we used for the experiment, control AC16 cells, AC16 cells treated with LGM2605 alone, and LPS-treated cells that were incubated with or without LGM2605 had increased cAMP levels following stimulation with isoproterenol (Fig 13C). LGM2605 did not have a separate effect in cAMP levels in cells treated with LPS for 12h and either not stimulated or stimulated with forskolin or isoproterenol. In contrast, treatment of AC16 cells with LGM2605 alone increased significantly cAMP levels 12 hours in non-stimulating conditions compared to the control cells (Fig 13C). This increase in cAMP levels in LGM2605-treated cells did not have an additive effect in cells that were stimulated with forskolin or isoproterenol (Fig. 13C). So, it seems that LGM2605 has an effect on cAMP levels in non-stimulating conditions, which though is not pronounced in stimulating conditions or in the disease state.

Based on the increased cAMP levels in cells treated with LGM2605 alone, we sought to explore potential role of LGM2605 in β-adrenergic signaling and cardiac contractility *in vivo*. Therefore, we performed *in vivo* hemodynamic measurements aiming to identify if the mice that underwent CLP and received LGM2605, had improved responsiveness to increasing doses of isoproterenol compared to mice that underwent CLP. Both CLP and CLP+LGM2605 mice showed significant lower baseline $LVdP/dt_{max}$ (index of cardiac contractility) and higher baseline $LVdP/dt_{min}$ (index of myocardial relaxation) levels compared to sham mice 12 hours after the surgery (Fig. 13H). Surprisingly we didn't observe significant responsiveness to increasing doses of isoproterenol in control mice that underwent sham surgery (Fig. 13H). On the other hand, we observed significant responsiveness to 5ng of isoproterenol in mice that underwent CLP and to 5ng and 10ng isoproterenol in mice that underwent CLP and received LGM2605 compared to their baseline measurements (Fig 13H). Similarly, we

didn't observe significant relaxation (LVdP/dtmin) following increasing doses of isoproterenol neither in the control mice nor in the septic mice but we observed significant responsiveness compared to baseline measurements in the septic mice that received LGM2605 and were stimulated with 0,5ng, 1ng, 5ng and 10ng of isoproterenol (Fig 13H).

As basal levels of $LVdP/dt_{max}$ and $LVdP/dt_{min}$ were lower in mice with CLP, in collaboration with the group of Dr. Anastasios Lymperopoulos (Nova Southeastern University), we performed radioligand binding assay to assess the density of cardiac β-adrenergic receptors in hearts of mice 12 hours after sham surgery, CLP surgery and CLP surgery followed by treatment with LGM2605 6 hours post CLP. With this assay we observed significant downregulation of β-adrenergic receptors in the hearts of mice that underwent CLP but the treatment with LGM2605 6 hours after CLP could not reverse this downregulation (Fig 13A).

Since the drug did not affect β-adrenergic receptor density, but we had observed increased cAMP levels in cells treated with LGM2605 alone, we assessed the expression of adenylate cyclase (AC) both *in vivo* and *in vitro*. AC is responsible for the conversion of ATP to cAMP. LGM2605 did not have any effect on AC protein expression in AC16 cells treated with LPS for 12 or 24 hours (Fig 13B). In contrast, in mice that underwent CLP we observed a trend for reduced cardiac AC protein levels 12 hours post-CLP and this reduction tended to be restored following administration of LGM2605 6 hours post-CLP.

Based on our observations showing the effect of LGM2605 on cAMP levels at basal conditions, we assessed if this is accompanied by activation of protein kinase A (PKA), which follows cAMP formation. Treatment of AC16 cells with LGM2605 alone for 12 hours augmented the PKA activation after isoproterenol stimulation but not in the absence of isoproterenol (Fig 13D). The positive effect of LGM2605 in isoproterenol-stimulated PKA activation was blunted when cells were treated with LPS (Fig. 13D). In accordance with this observation, treatment of AC16 cells with LGM2605 alone for 6 hours didn't alter the protein levels of PKA (Fig. 13E). Similar to the *in vitro* data, treatment of septic mice with LGM2605 did not have a significant effect neither

in the PKA activity nor in the protein levels of PKA in the heart tissue at 12 hours (Fig 13F-G). We then assessed phosphorylation of phospholampan (PLB) at Serine 16, which is a main protein target of PKA activation. We found that phosphorylation of PLB tended to be lower in the hearts of mice that underwent CLP and this decrease tended to reverse with the administration of LGM2605. These data suggest that LGM2605 treatment has an effect on the PKA activation after isoproterenol stimulation in vitro, but this effect is not pronounced in the absence of isoproterenol or in the disease state.

Based on these results LGM2605 may play a role in β-adrenergic signaling as indicative by the increase in cAMP levels we observed in basal conditions and the increase in PKA activity after isoproterenol stimulation.

We conclude that although we didn't observe significant improvement in responsiveness to the increasing doses of isoproterenol in the control mice, we did observe such improvement in the septic mice that received LGM2605. However, septic mice had significantly lower basal levels of LVDP/dtmax and LVDP/dtmin. To conclude on the role of LGM2605 in cardiac β-adrenergic signaling in sepsis, it seems that the beneficial effect is not accounted for by improvements in the number of β-adrenergic receptors or AC protein expression. The improvement in cAMP levels occurs in the absence of inotropic stimulation but not in the disease state. This is also not in accordance with the increased PKA activity levels, which are observed only upon stimulation with isoproterenol but again not in the disease state, *in vitro*.

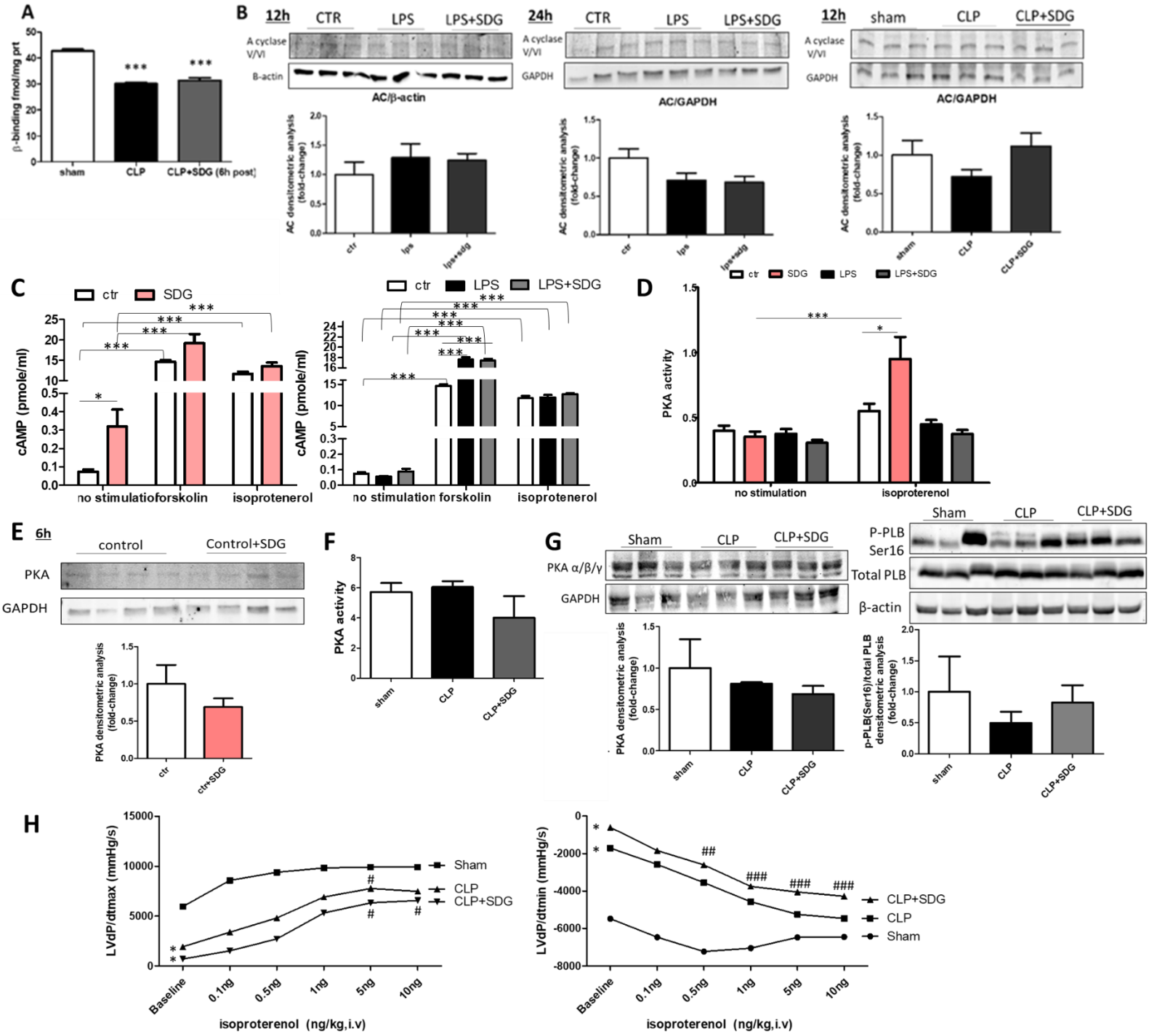

Figure 13: LGM2605 stimulates components of β-AR signaling but not during sepsis (**A**)Density of β adrenergic receptors using radio ligand binding assay, n=4-5 mice per group. ****P* < 0.001 by ANOVA with Bonferroni post-test. (**B**) Immunoblot and densitometric analysis of adenylate cyclase (AC) and GAPDH, in relative units, from AC16 cells treated with and without LPS or LPS+LGM2605 for 12hrs and 24hrs (2 left graphs), and from ventricular tissue of mice 12 h after control sham surgery, CLP or CLP+LGM2605 at 6hrs post CLP (right). (**C**) cAMP levels in AC16 cells either untreated or treated with LGM2605 for 12 hours (left) and control cells, LPS-treated and LPS+LGM2605 -treated cells (right). **P* < 0.05, ****P* < 0.001; unpaired t-test for the left graph and ANOVA with Bonferroni post-test for the right

panel. (**D**) PKA activity in AC16 cells either untreated or treated with LGM2605, or LPS or LPS+LGM2605 for 12hrs. *P < 0.05, ***P < 0.001 by ANOVA with Bonferroni post-test. (**E**) Immunoblot and densitometric analysis of protein kinase A (PKA), in relative units, in AC16 cells untreated or treated with LGM2605 for 6hrs. (**F**) PKA activity from sham, CLP and CLP+LGM2605 mice at 12hrs timepoint, n = 4-5 mice per group. (**G**) Immunoblot and densitometric analysis of the PKA, in relative units (left) and phosphor-phospholamban at Serine 16 (right) from sham, CLP and CLP+LGM2605 mice at 12hrs timepoint. (**H**)LVdP/dtmax (left) as an index of cardiac contractility and LVdP/dtmin (right) as an index of myocardial relaxation to increasing doses of isoproterenol in mice that underwent sham surgery, CLP and combined CLP and LGM2605 treatment (6 h post-CLP), at 12 hrs timepoint. n=2-4 mice per group, *P < 0.05 versus sham, *P < 0.05 versus baseline, **P < 0.01 versus baseline, ***P < 0.001 versus baseline by ANOVA with Bonferroni post-test.

LGM2605 reduces LPS-mediated increase in mitochondrial superoxide *in vitro*, without having a significant effect in the expression of antioxidant genes *in vivo*.

In order to assess the role of LGM2605 in the sepsis-induced oxidative stress, we measured superoxide generation, using Mitosox Red staining, in AC16 cells treated with LPS. Treatment of AC16 cells with LPS for 12 hours increased (fold-change) mitochondrial superoxide levels. This LPS-mediated increase was abrogated when the cells were treated with LGM2605 (Fig 14A). So, LGM2605 suppresses the LPS-mediated increase in mitochondrial superoxide generation.

In mice LGM2605 did not prevent the CLP-mediated changes in the expression of antioxidant genes such as NRF2, HO1, GSTM1, NQO1 in the heart tissue at both 6 and 12 hours timepoint (Fig 14B).

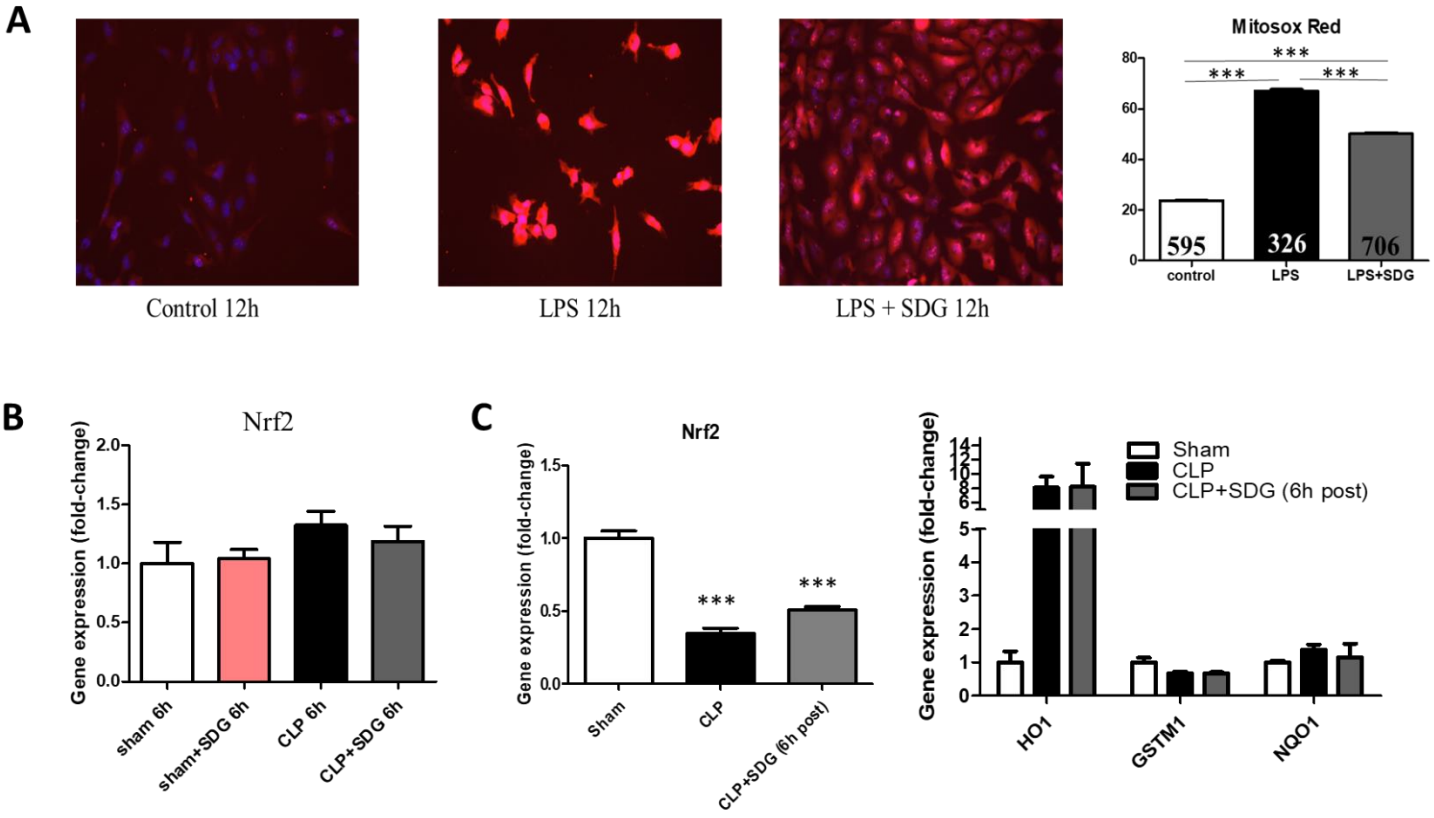

Figure 14: LGM2605 reduces LPS-mediated increase in mitochondrial superoxide *in vitro*, without having a significant effect in the expression of antioxidant genes *in vivo*. (**A**) Representative fluorescence microscopy images and quantification of superoxide levels based on Mitosox Red staining (red color) and DAPI nuclear staining (blue color) in AC16 cells treated with or without (control) LPS or with combination of LPS and LGM2605 for 12 hours. (B) NRF2 mRNA levels in ventricular tissue of mice 6hrs post sham surgery, CLP and CLP +LGM2605, $n = 4$ mice per group (left graph), NRF2, HO1, GSTM1, NQO1 gene expression from ventricular tissue of mice 12hrs post sham surgery, CLP and CLP followed by treatment with LGM2605 at 6hrs post CLP, $n = 4$-5 mice per group (right graph). ***$P < 0.001$ by ANOVA with Bonferroni post-test.

LGM2605 doesn't have a significant effect on fatty acid or glucose metabolism, but has a significant effect on mitochondrial oxidative metabolism.

In order to assess further the mechanisms that may mediate the beneficial effect of LGM2605 in alleviating septic cardiomyopathy, we examined separately potential effects of LGM2605 in the expression of proteins that are associated with glucose metabolism, fatty acid metabolism, as well as mitochondrial biology.

LGM2605 doesn't have a significant effect on glucose metabolism

We evaluated the expression of the main glucose uptake and catabolism markers such as GLUT1, GLUT4 and PDK4 in the hearts of mice that underwent CLP surgery. GLUT 1 and GLUT4 cardiac mRNA levels didn't seem to change significantly in the septic mice, but we observed a significant increase in the mRNA levels of cardiac PDK4 at 12 hours in the mice that underwent CLP (Fig 15A), consistent with the downregulation of the glucose metabolism that is known to occur during sepsis. This increase was not alleviated by administration of LGM2605. So, LGM2605 couldn't improve the CLP-mediated changes in cardiac glucose uptake and catabolism markers. Furthermore, we measured plasma glucose levels in septic mice. We observed at both 6 and 12 hours post-CLP a significant hypoglycemia in the mice that underwent CLP surgery that was not alleviated by the administration of LGM2605 (Fig 15B). Collectively, these data suggest that the beneficial effect of LGM2605 doesn't involve alterations in glucose metabolism of septic mice.

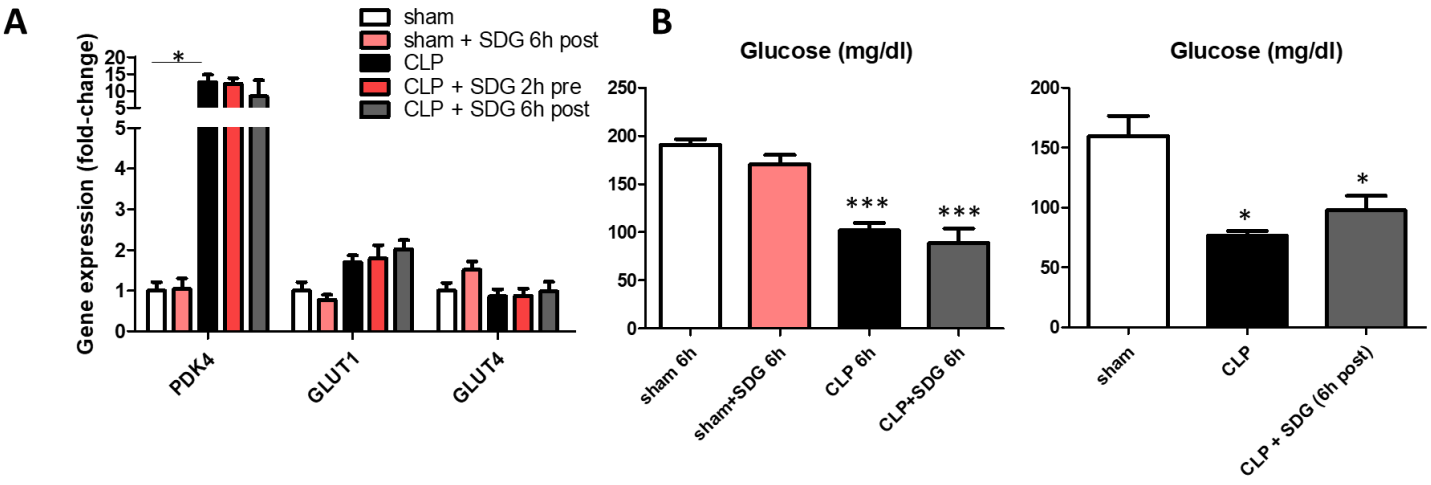

Figure 15: LGM2605 doesn't have a significant effect on glucose metabolism
(**A**) PDK4, GLUT1, GLUT4 gene expression levels from ventricular tissue of mice 12hrs after sham surgery, sham followed by treatment with LGM2605 at 6hrs post-sham operation, CLP surgery, CLP + LGM2605 treatment at 2 hrs prior to CLP and CLP followed by treatment with LGM2605 at 6hrs post-CLP, n=4 mice per group. (**B**) Plasma glucose levels from mice at 6 hrs (left) and 12 hrs timepoint (right), n= 3-5mice per group. *P<0.05, ***P < 0.001 by ANOVA with Bonferroni post-test.

LGM2605 doesn't have a significant effect on fatty acid metabolism

We assessed cardiac expression of several genes associated with fatty acid metabolism that are known to be affected during septic cardiac dysfunction. LGM2605 did not reverse the CLP-mediated changes in the peroxisome proliferator-activated receptors (PPARα, PPARβ, PPARγ) and neither did it for CD36, a marker for fatty acid uptake, fatty acid oxidation genes (CPT1β, MCAD, LCAD, VLCAD), uncoupling proteins (UCP2, UCP3), and PGC1α and PGC1β (Fig 16A). LGM2605 caused only a slight, non-statistically significant reduction, in plasma triglyceride levels which were increased in septic mice at 12 hours timepoint (Fig 16B). Collectively, these data suggest that the beneficial effect of LGM2605 in septic cardiac dysfunction is not associated with improvement in cardiac fatty acid metabolism.

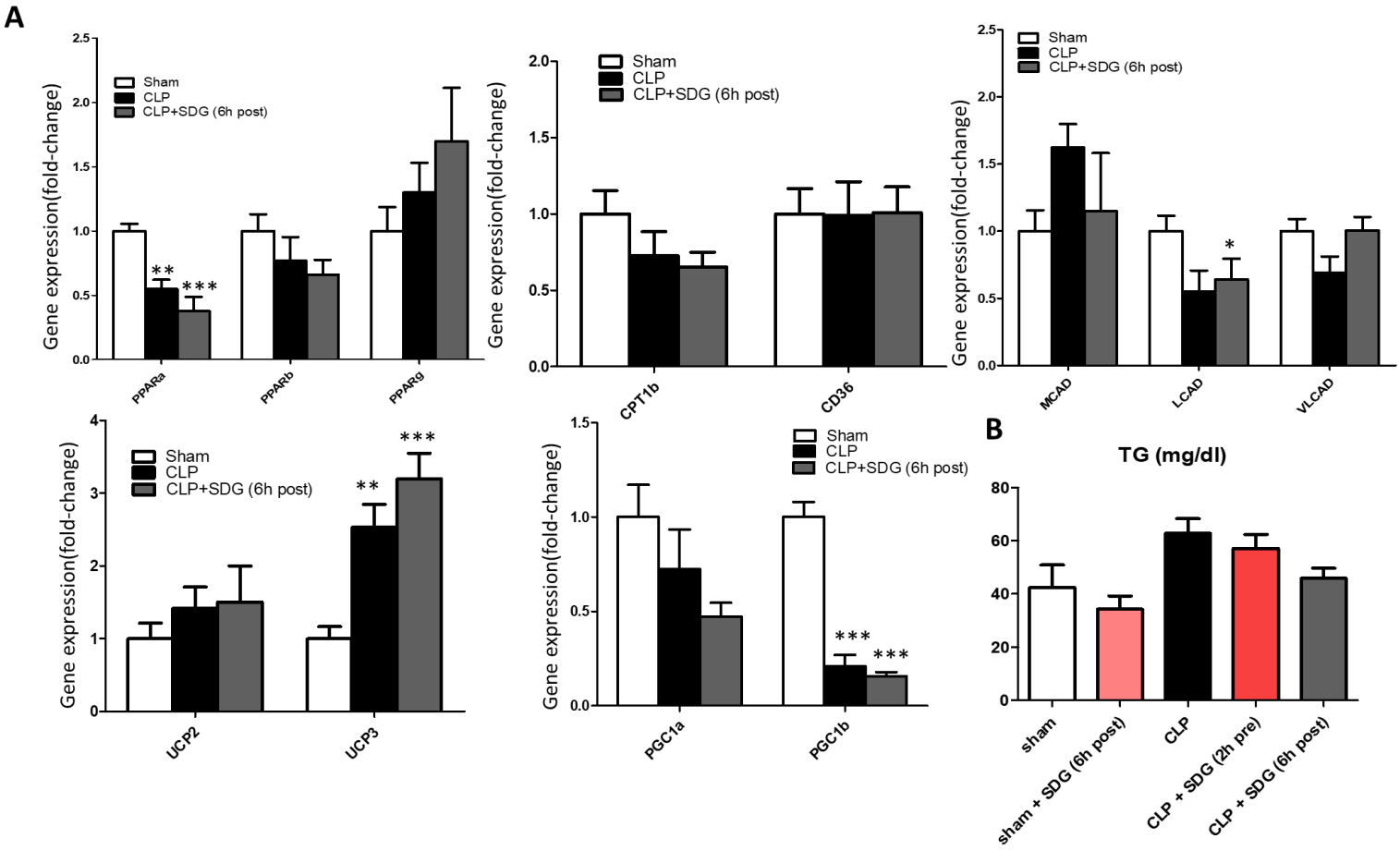

Figure 16: LGM2605 doesn't have a significant effect on fatty acid metabolism (**A**) Gene expression of PPARa, PPARb, PPARg, CPT1b, CD36, MCAD, LCAD, VLCAD, UCP2, UCP3, PGC1a and PGC1b from ventricular tissue of mice 12hrs after control- sham surgery, CLP surgery and CLP followed by treatment with LGM2605 at 6hrs post-CLP n=4-5 mice per group. (**B**) Plasma triglyceride levels from mice at 12hrs after sham surgery, sham followed by treatment with LGM2605 at 6hrs post-sham operation, CLP surgery, CLP + LGM2605 treatment at 2 hrs prior to CLP and CLP followed by treatment with LGM2605 at 6hrs post-CLP, n= 4 mice per group. **P*<0.05, ** *P*<0.01, ****P* < 0.001 by ANOVA with Bonferroni post-test.

The role of SDG in the sepsis-induced impairment of mitochondrial metabolism

The mitochondrial life cycle consists of four major events, biogenesis, fusion, fission and mitophagy. PGC-1α is the main molecule that regulates biogenesis and activates NRFs, TFAM and TFBM. Mitochondria under normal conditions undergo cycles of fusion and fission. Fusion which is mainly mediated by MFN1, MFN2, and OPA1 is important for the formation of elongated mitochondrial networks. Fission is mainly mediated by DRP1 and FIS1 and leads to the formation of smaller individual organelles. Accumulation of damage is possible to happen during the normal life cycle of the mitochondria or during increased oxidative stress. In that case, a mechanism to gather the damaged components that will be eliminated is provided through the fission process. The forth major event of the mitochondrial life cycle is mitophagy. Important proteins that are involved in this process are PINK1, which is retained in the mitochondrial membrane, Parkin which is recruited during mitophagy and targets the mitochondria to autophagosome, beclin-1 and LC3-II which play a role in the assembly of the phagosome [59].

We first assessed whether administration of LGM2605 changes mitochondrial number. So, we treated AC16 cells with 1μg/ml LPS and 50μM LGM2605 and we evaluated the mitochondrial number using the Mitotracker Red staining. LPS treatment for 12 hours decreased mitochondrial number, which was prevented when we treated the cells also with LGM2605 (Fig 17). In order to explain this effect of the drug in the mitochondrial number, we examined each one of the main events of the mitochondrial life cycle such as biogenesis, fusion, fission and mitophagy, separately, to see in which of these events LGM2605 may have an effect that can explain restoration of mitochondrial number.

Firstly, we evaluated gene expression of mitochondrial biogenesis markers both *in vitro* and *in vivo*. We observed a trend of reduction in the mRNA levels of Nrf2 and PGC1α in LPS-treated cells at 6 hours that tended to restore with the administration of the drug (Fig 18A). However, treatment of LPS-treated AC16 cells with LGM2605 for 6, 12 or 24 hours didn't have a significant effect in the expression of mitochondrial biogenesis markers compared to AC16 cells that were treated only with LPS for the same timepoints. Accordingly, our *in vivo* data, did not show any significant effect of the drug in the cardiac gene expression of mitochondrial biogenesis markers at both 6 and 12 hours timepoints (Fig 18B).

Since LGM2605 didn't seem to have a profound effect in the expression of mitochondrial biogenesis markers, we examined whether there is a possible effect in mitochondrial fusion and fission. *In vitro* data, showed that combined treatment of AC16 cells with LPS and LGM2605 tended to increase the mRNA levels of fusion (MFN1, MFN2, OPA1) and fission (DRP1) markers at 6 hours, without any significant effect in the mRNA levels of these genes at 12 or 24 hours of treatment (Fig 19A). In contrast to the *in vitro* data, in the heart tissue of mice that underwent CLP we found that the mRNA levels of MFN1, MFN2 and DRP1 tended to increase and the levels of FIS1 tended to decrease at 12 hours. LGM2605 treatment seemed to restore these CLP-mediated changes with the restoration of MFN2 reaching statistical significance (Fig 19B).

Assessment of autophagy markers showed that LGM2605 did not alter significantly the gene expression of ATG7 and BCN1 in LPS-treated AC16 cells neither at 6 nor at 12 and 24 hours (Fig 20A). In agreement with the *in vitro* data, LGM2605 did not have a significant effect on the gene and protein expression of autophagic markers in the heart tissue of mice that underwent CLP at 12 hours (Fig 20B-D).

Another very important aspect of mitochondrial metabolic pathways is the mitochondrial calcium uptake that affects intracellular calcium signaling, cell metabolism and survival by buffering cytosolic calcium and regulating mitochondrial effectors [60]. Calcium enters the mitochondria primarily through the mitochondria calcium uniporter (MCU) [61]. MICU1 and MICU2 are two other proteins that are in the same complex with MCU.

MICU2 has an inhibitory effect so during resting conditions, MICU1-MICU2 heterodimers act as the gatekeeper for MCU. When the calcium concentration is increased though, the whole dimer changes and that leads to MICU1-mediated increase in the channel activity of MCU [62].

Firstly, we assessed the expression of MCU and MICU1 in AC16 cells treated with LPS and LGM2605 for 6, 12 and 24 hours. At 6 hours of treatment we observed a trend of decrease in the mRNA levels of MCU and MICU1 in the LPS-treated cells, that tended to be reversed with the administration of the drug (Fig 21A). At 12 hours, MCU gene expression tended to be increased in the LPS-treated cells and this increase tended to be restored in the cells that were treated also with LGM2605 (Fig 21A). At the same timepoint MCU protein expression tended to be attenuated in the LPS-treated cells and this decrease seemed to be restored with the administration of LGM2605 (Fig 21B). MICU1 gene and protein expression didn't change at 12 hours of LPS or combined LPS and LGM2605 treatments (Fig 21 A-B). At 24 hours of treatment we didn't observe any difference between our groups in both MCU and MICU1 gene expression (Fig 21 A). Overall, we didn't observe a significant effect of the LGM2605 administration in LPS-treated cells, in vitro.

We also explored whether LGM2605 has any effect in our *in vivo* model. Interestingly, sham mice that were treated with LGM2605 (drug was administrated at the time of the sham operation) had significantly higher mRNA levels and protein levels of MCU in the heart tissue compared to the control sham mice at 6 hours timepoint (Fig 22A). This indicates that the drug influences the expression of MCU *in vivo* in basal conditions. MICU1 gene and protein expression didn't seem to change in the heart tissue of the sham mice that were treated with LGM2605 compared to the control mice at the 6 hours timepoint (Fig 22A). The mice that underwent CLP didn't show any significant change in the gene or protein expression of MCU or MICU1 in the heart tissue at 6 hours after the surgery, but the mice that underwent CLP and received LGM2605 during the CLP surgery showed a trend of elevated mRNA levels of MICU1 and significant increase in the protein levels of cardiac MCU compared to the sham mice at 6 hours after the treatment (Fig 22B). At 12 hours after the CLP surgery, the mRNA

levels of MCU tended to be attenuated in the heart tissue of septic mice, while the protein levels did not seem to change (Fig 22C). Cardiac MICU1 mRNA levels were not altered, although the protein levels showed a trend of increase in septic mice, at 12 hours after the CLP surgery (Fig 22C). Administration of LGM2605 in mice that underwent CLP didn't have a significant effect in the mRNA levels of cardiac MCU and MICU1 at 12 hours, although the protein levels of MCU in the heart tissue of mice that underwent CLP and received LGM2605 at 6 hours post -CLP were significantly increased compared to the mice that underwent CLP at 12 hours (Fig 22C). Cardiac MICU1 protein levels were not altered in mice that underwent CLP and received the drug compared to the mice that underwent CLP and were not treated with the drug (Fig 22C).

Since we saw an effect of the drug in the MCU and MICU1 expression in our *in vivo* model of sepsis, we evaluated further the role of LGM2605 in mitochondrial respiration, performing seahorse analysis. For this experiment we had three groups of C57BL/6 mice. We had the mice that underwent sham surgery as our control mice, the mice that underwent CLP surgery and the mice that underwent CLP surgery and were treated with LGM2605 at 6 hours post CLP. So, we performed the surgeries in C57BL/6 mice and isolated adult cardiomyocytes 12 h post-surgeries that we assessed with Seahorse analysis. The Seahorse analysis showed that LGM2605 increases oxygen consumption rate of cardiomyocytes in septic mice. More particularly, administration of LGM2605 in mice that underwent CLP increased basal respiration levels, maximal respiration levels and oxygen consumption rate associated with ATP synthesis in the cardiomyocytes, compared to both CLP and sham group of mice (Fig 23).

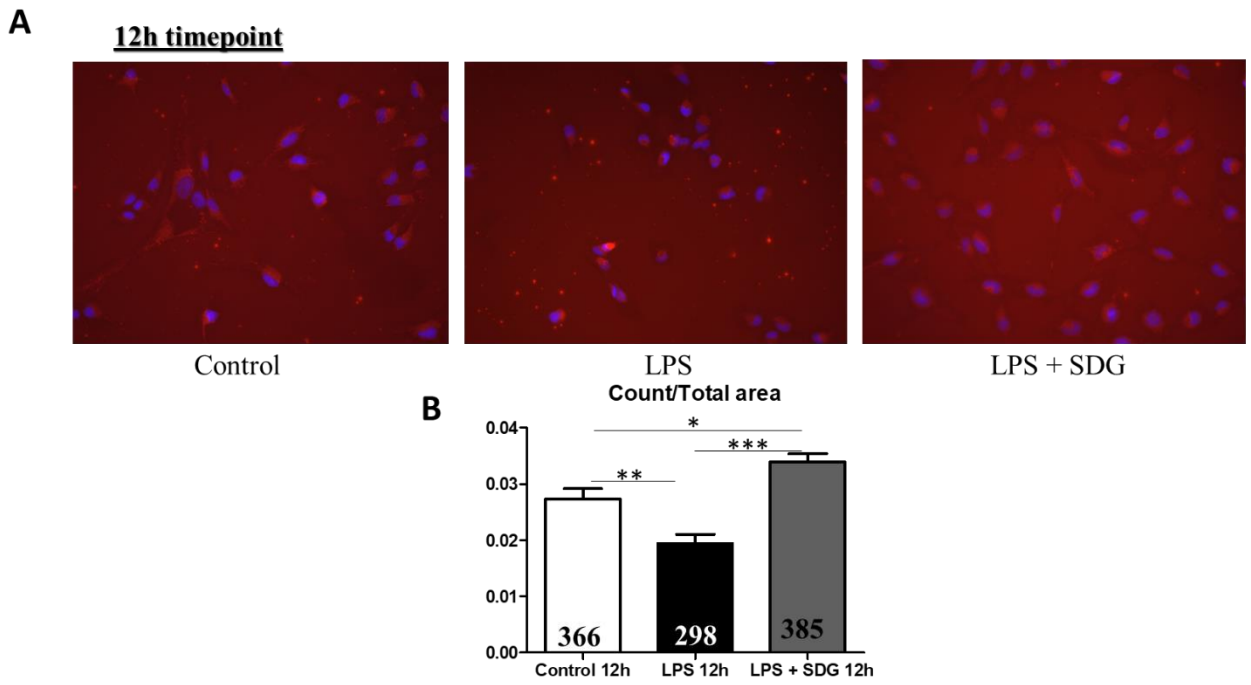

Figure 17: SDG prevents LPS-mediated decrease in mitochondrial number in AC16 cells (**A**) Representative fluorescent images of mitochondria detection by Mitotracker Red (red color) and DAPI nuclear staining (blue color) from AC16 cells either untreated(control) or treated with LPS or with LPS+LGM2605 for 12 hours. (**B**) Quantification of Mitotracker Red signal from control, LPS, and LPS+SDG AC16 cell samples. **P*<0.05, ** *P*<0.01, ****P* < 0.001 by ANOVA with Bonferroni post-test.

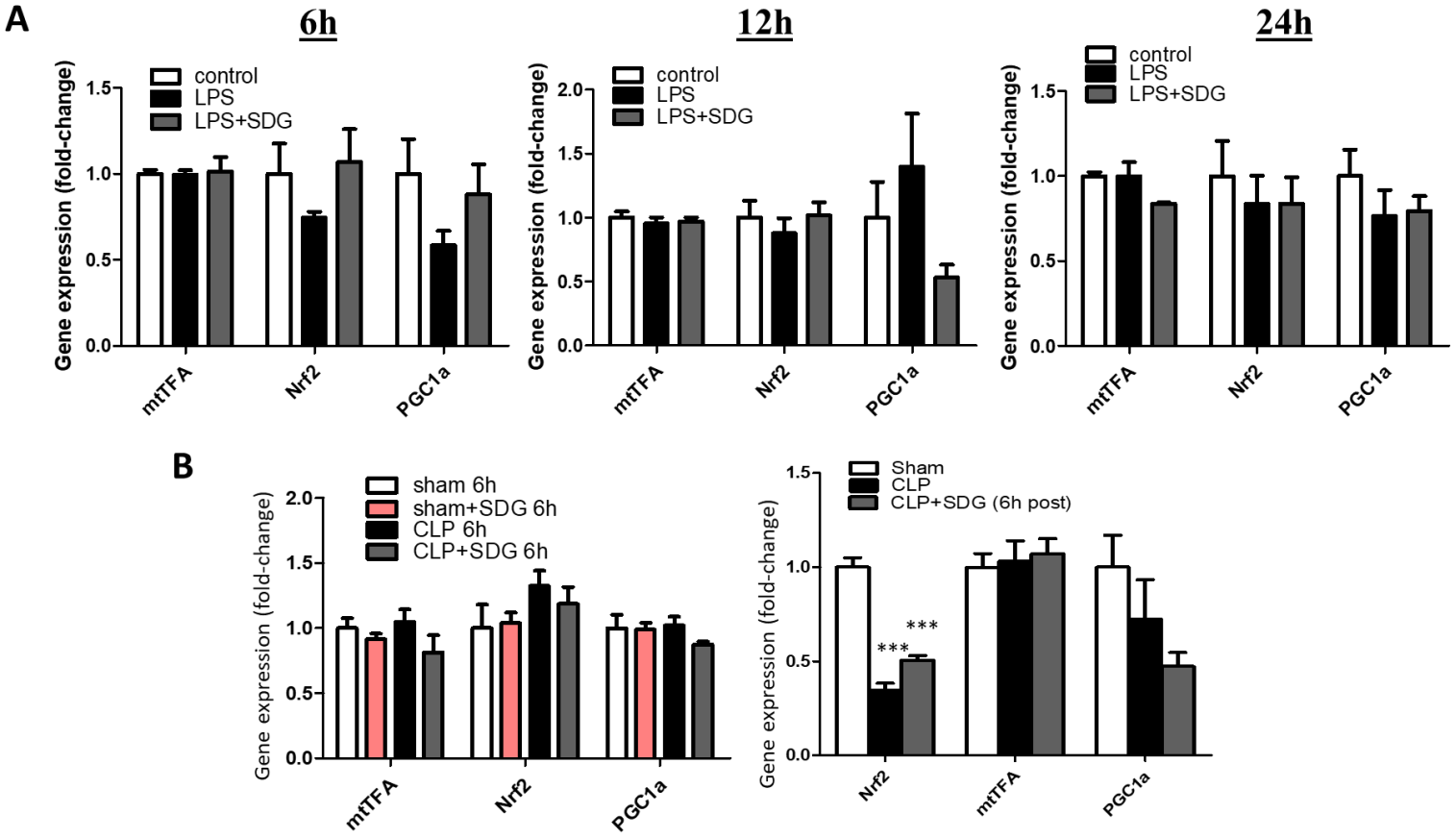

Figure 18: LGM2605 doesn't have a significant effect on mitochondrial biogenesis (**A**) Graphs of mitochondrial biogenesis markers (mtTFA, Nrf2, PGC1a) gene expression from AC16 cells either untreated (control) or treated with LPS or LPS+LGM2605 for 6hrs, 12hrs and 24hrs. (**B**) Graphs of mitochondrial biogenesis markers (mtTFA, Nrf2, PGC1a) gene expression from ventricular tissue of mice 6hrs after sham surgery, sham surgery and administration of LGM2605 during surgery, CLP surgery , and CLP with administration of LGM2605 during surgery (left), *n* =4 mice per group, and from mice 12hrs after sham surgery, CLP and CLP surgery followed by treatment with LGM2605 at 6hrs post-CLP (right), *n* = 4-5 mice per group. ****P* < 0.001 by ANOVA with Bonferroni post-test.

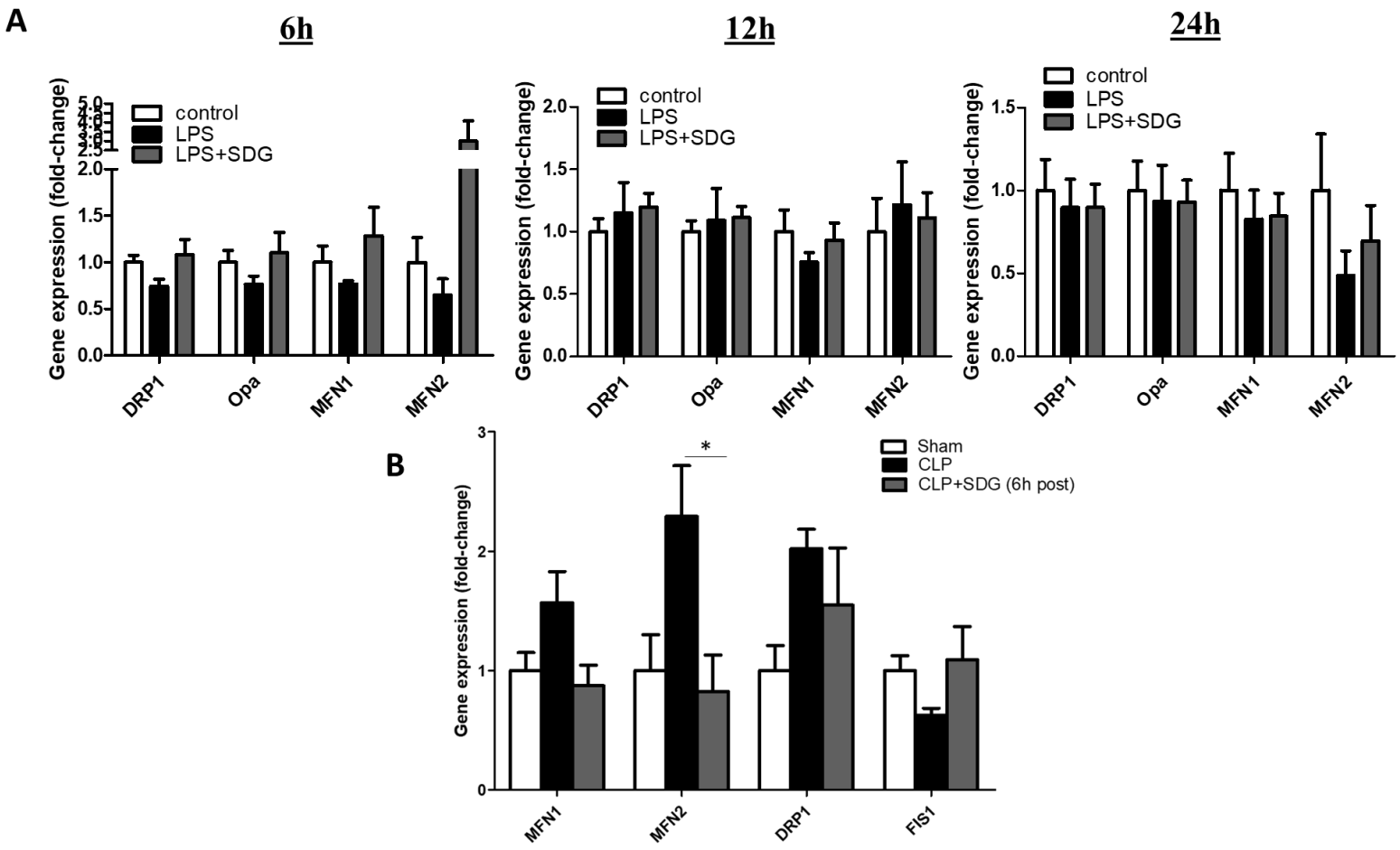

Figure 19: LGM2605 doesn't have a significant effect on mitochondrial fusion and fission (**A**) Fusion (MFN1, MFN2, OPA) and fission (DRP1) gene expression-related markers in AC16 cells either untreated (control) or treated with LPS or LPS+LGM2605 for 6h, 12h and 24h. (**B**) Fusion (MFN1, MFN2) and fission (DRP1, FIS1) gene expression-related markers in ventricular tissue of mice 12hrs after sham surgery, CLP and CLP surgery followed by treatment with LGM2605 at 6hrs post-CLP, n = 4-5 mice per group. *$P < 0.05$ by ANOVA with Bonferroni post-test.

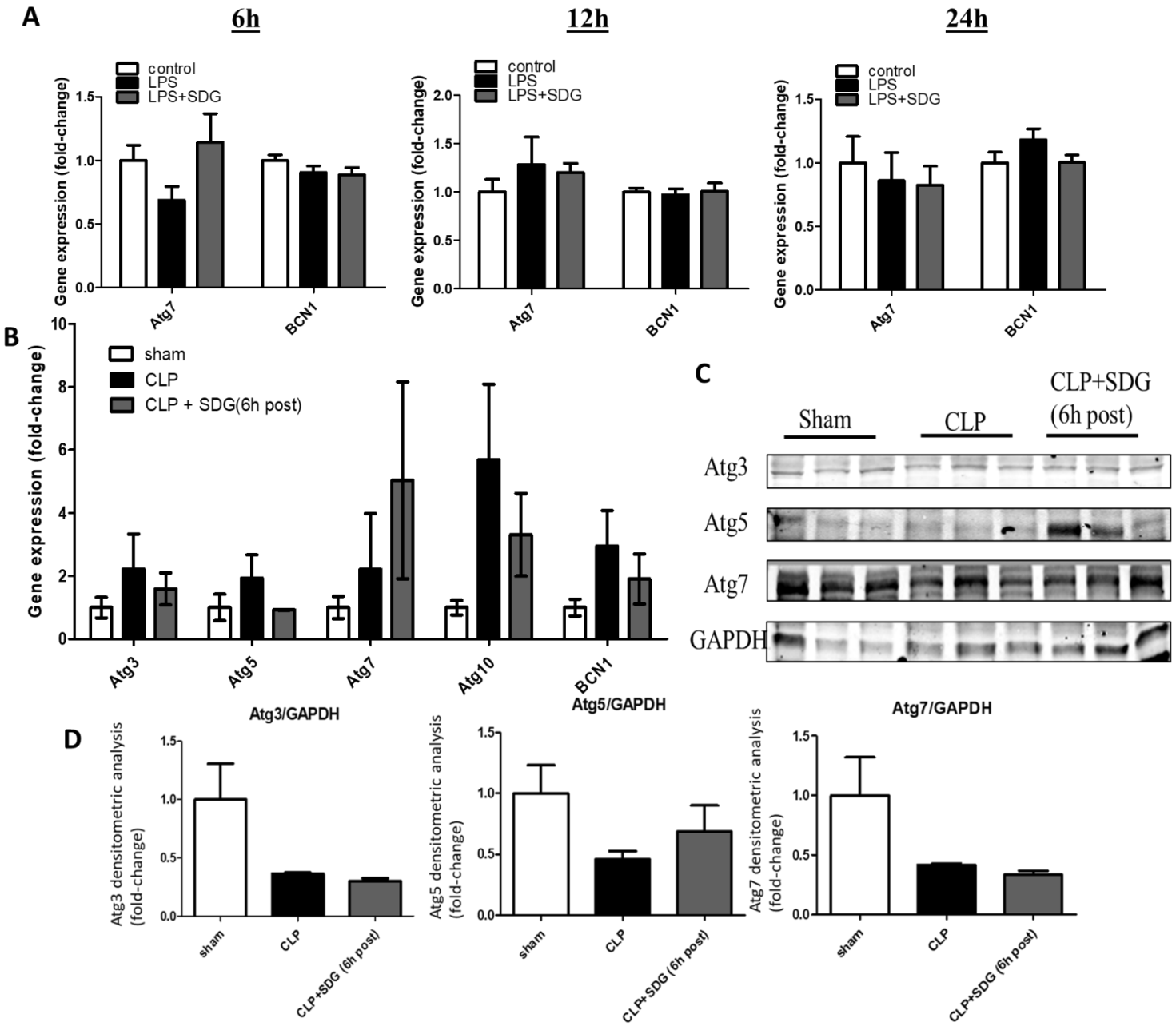

Figure 20: LGM2605 doesn't have a significant effect on autophagy (**A**) Gene expression of autophagy markers (ATG7, BCN1) in mRNA obtained from AC16 cells that were either untreated (control) or treated with LPS or LPS+LGM2605 for 6 h, 12 h and 24 h, as indicated. (**B**) Gene expression of autophagy markers (ATG3, ATG5, ATG7, ATG10, BCN1) in mRNA obtained from ventricular tissue of C57BL/6 mice 12hrs after sham surgery, CLP and CLP surgery followed by treatment with LGM2605 at 6hrs post-CLP, *n* = 4-5 mice per group. (**C, D**) Immunoblots (C) and densitometric analysis (D) of ATG3, ATG5, ATG7, in relative units, in protein lysates obtained from ventricular tissue of C57BL/6 mice 12hrs after sham surgery, CLP and CLP surgery followed by treatment with LGM2605 at 6hrs post-CLP. Means are not significant by ANOVA with Bonferroni post-test (n=4-5 mice per group).

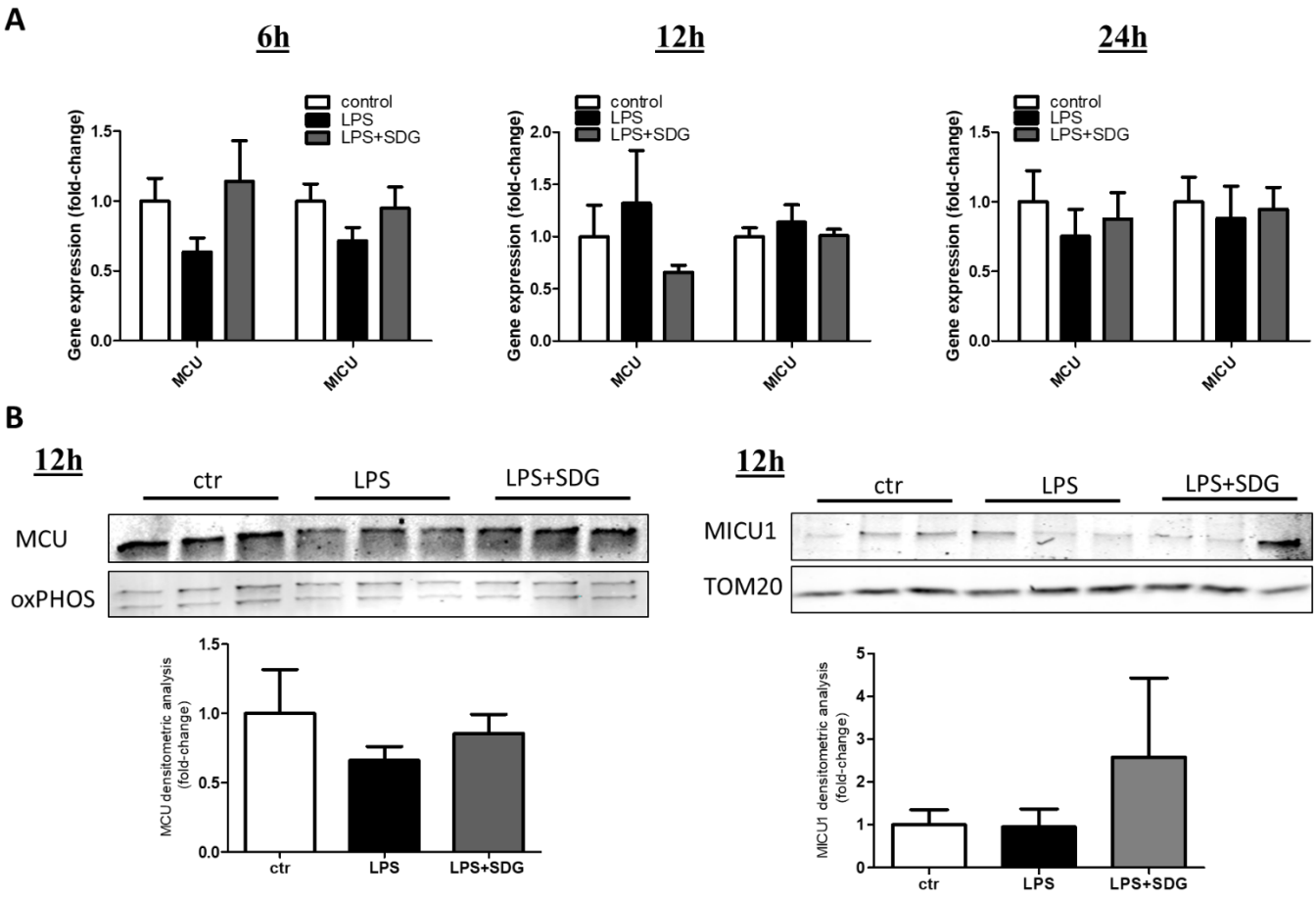

Figure 21: LGM2605 doesn't have a significant effect on MCU, MICU1 expression *in vitro* (**A**) MCU and MICU1 mRNA levels in AC16 cells either untreated (control) or treated with LPS or LPS+LGM2605 for 6 h, 12 h and 24 h. (**B**) Immunoblot and densitometric analysis of MCU and MICU1, in relative units, of protein lysates obtained from AC16 cells treated with LPS or combination of LPS and SDG for 12 h. Means are not significant by ANOVA with Bonferroni post-test.

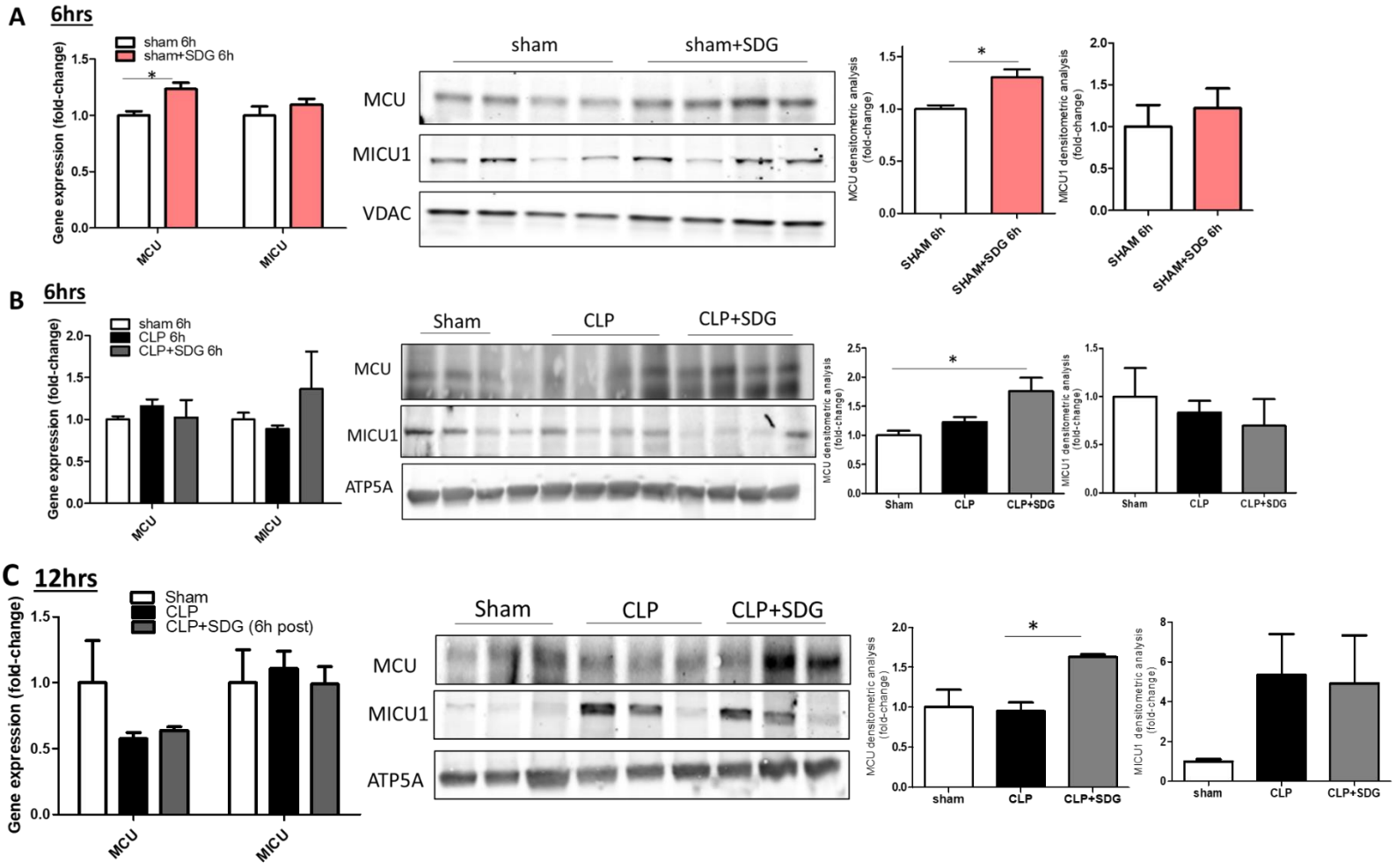

Figure 22: LGM2605 increases MCU expression *in vivo* without having a significant effect on MICU1 expression (**A**) MCU, MICU1 gene expression in mRNA obtained from ventricular tissue of C57BL/6 mice 6hrs after sham surgery and sham surgery with administration of LGM2605 during surgery, n = 4 mice per group. Immunoblot and densitometric analysis of MCU, MICU1, in relative units, from protein lysates obtained from ventricular tissue of C57BL/6 mice 6hrs after sham surgery and sham surgery with administration of LGM2605 during surgery. *$p < 0.05$ by unpaired t-test. (**B**)Graph of MCU, MICU1 gene expression from ventricular tissue of mice 6hrs after sham surgery, CLP and CLP surgery with administration of LGM2605 during surgery, n = 4 mice per group. Densitometry analysis of MCU, MICU1 western blots, in relative units, from ventricular tissue at 6hrs timepoint. *$P < 0.05$ by ANOVA with Bonferroni post-test. (**C**) Graph of MCU, MICU1 gene expression from ventricular tissue of mice 12hrs after sham surgery, CLP and CLP surgery followed by treatment with LGM2605 at 6hrs post-CLP, n = 4-5 mice per group. Densitometry analysis of MCU, MICU1 western blots, in relative units, from ventricular tissue at 12hrs timepoint. *$P < 0.05$ by ANOVA with Bonferroni post-test.

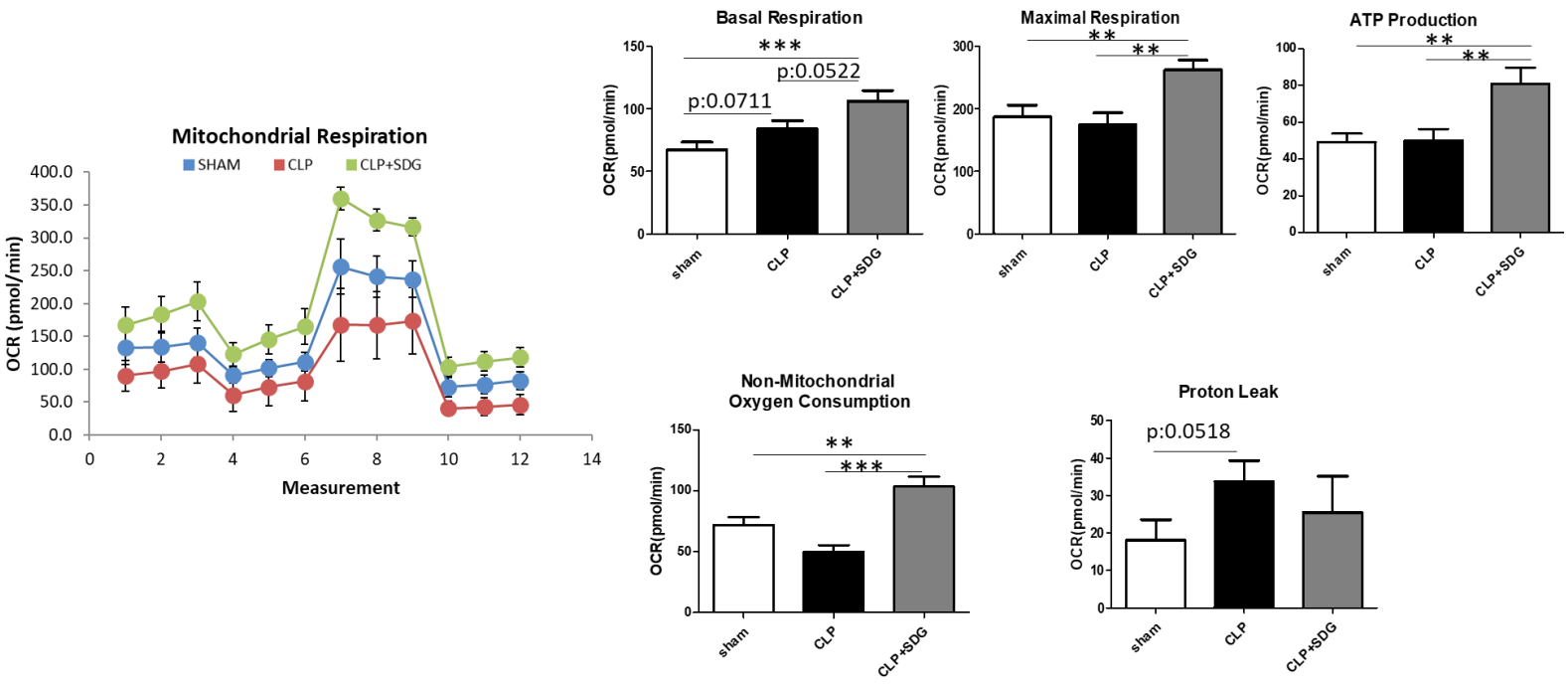

Figure 23: LGM2605 increases the oxygen consumption rate in isolated primary cardiomyocytes in septic mice. Seahorse experiment. For this experiment adult cardiomyocytes were isolated 12hrs after sham surgery, CLP surgery and CLP followed by treatment with LGM2605 at 6hrs post-CLP. Graphs of basal respiration, maximal respiration, oxygen consumption rate used for ATP synthesis, non-mitochondrial oxygen consumption and proton leak. $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA with Bonferroni post-test. Mice used in the seahorse experiment and wells analyzed: 3 sham mice (total 34 wells), 4 CLP mice (total 30 wells), 4 CLP+SDG mice (total 38 wells)

Discussion

In this study, we examined the effect of chemically synthesized SDG, LGM2605, which has potent anti-inflammatory and anti-oxidant properties[55], in cardiac function of a mouse model of polymicrobial sepsis. Various mechanisms have been associated with the pathophysiology of septic cardiomyopathy such as increased inflammation [11], impaired β-adrenergic signaling [12], increased oxidative stress [63], impaired metabolism and reduced ATP synthesis in the cardiomyocytes [13]. We hypothesized that LGM2605 will alleviate oxidative stress and inflammatory pathological mechanisms, prevent cardiac dysfunction, and improve mortality during sepsis.

To assess the effect of LGM2605 administration in septic cardiac dysfunction *in vivo* we used an established model of polymicrobial sepsis, cecal ligation and puncture (CLP). We chose this model of sepsis over the LPS and bacterial infusion models since it can mimic human sepsis conditions and therefore it is generally considered more clinically relevant. Within 12 hours, the mice that underwent CLP showed a significant impairment in their systolic cardiac function. On the other hand, mice that received LGM2605 either 2 hours prior to CLP or 6 hours post-CLP had significant restoration of their cardiac function. So, the chemically synthetized SDG, LGM2605, prevents septic cardiac dysfunction.

We then investigated whether LGM2605 alleviates any of the pathological mechanisms that are known to mediate septic cardiac dysfunction.

Firstly, we investigated the effect that LGM2605 had in sepsis-induced inflammation. Treatment with the drug prevented the increase in cardiac NF-kB levels *in vitro* and decreased phosphorylation of IkBa, suggesting inhibition of NF-κB activation *in vivo*. In contrast, the drug couldn't restore the increased cardiac mRNA levels of inflammatory cytokines. So, it seems that LGM2605 influences NF-kB activation but not cardiac gene expression of inflammatory cytokines. NF-κB activation is the endpoint of various signal transduction events that are initiated by a vast majority of stimuli related to many processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. So, the effect that the drug seems to have on NF-κB activation may be associated with other processes and not only inflammation. Further experiments are needed in order to delineate the signaling mechanisms that may mediate the inhibitory effect of LGM2605 in NFκB activation.

Another mechanism that we evaluated was the sepsis-induced impairment of β-adrenergic signaling. As we mentioned also in the introduction, during the early stages of sepsis, catecholamine levels are elevated and there is increased stimulation of the cardiac βARs [21,22] most possibly as a compensatory mechanism that aims to increase cardiac contractility. As septic cardiac dysfunction though progresses βAR density is reduced and the

myocardium becomes unresponsive to catecholamines [23,24]. Based on our data, LGM2605 does not improve β-AR density and neither does it change AC protein expression. However, we found that LGM2605 increases basal cAMP levels in non-stimulated cells. On the other hand, LGM2605 does not potentiate further responsiveness of β-AR when cells are treated with LPS. LGM2605 also increases PKA activity following isoproterenol stimulation but also not in LPS-treated cells. These data suggest that LGM2605 stimulates components of β-AR signaling but not during sepsis. Thus, this effect does not account for the improvement in cardiac function during sepsis. In order to confirm that the beneficial effect of LGM2605 is not via improved β-AR signaling we performed *in vivo* hemodynamic measurements. During this analysis we experienced technical flaws that surprisingly led us to the observation of lack of significant responsiveness of control (sham surgery) mice to increasing doses of isoproterenol. However, no significant improvement in cardiac contractility and relaxation was observed between mice that underwent CLP and were treated with LGM2605 compared to mice with CLP only. Thus, LGM2605 does not have a beneficial effect in cardiac adrenergic signaling that might explain the benefit in heart function during sepsis.

Then we investigated the effect of LGM2605 in sepsis-induced oxidative stress. The drug reduced LPS-mediated increase in mitochondrial superoxide *in vitro*, but it didn't have a significant effect in the expression of antioxidant genes *in vivo*. Further experiments need to be done in order to address if the effect we saw *in vitro* is also present *in vivo*. Staining of ventricular tissue with dihydroethidium (DHE) for superoxide detection, as we performed in our recent study [64], would help to address if the drug alleviated oxidative stress in septic hearts.

The last cause of septic cardiac dysfunction we evaluated for being alleviated by LGM2605 was impaired cardiomyocyte metabolism and reduced ATP synthesis in the cardiomyocytes. LGM2605 didn't have a significant effect on fatty acid or glucose metabolism-related gene expression. LGM2605 had a more significant effect in mitochondrial oxidative metabolism as shown by Seahorse analysis. This was associated with increased mitochondrial number that cells treated with LPS and LGM2605 had in comparison with cells treated with LPS

only. Further validation is needed with *in vivo* experiments in order to attribute the beneficial effect of LGM2605 in cardiac function in sepsis. The preliminary data we obtained in mice with CLP were inconclusive in what pertains to assessment of gene expression markers for mitochondrial biogenesis, autophagy, and mitochondrial fusion and fission, as we observed only trends and not significant changes.

Mitochondrial calcium uptake is a very important aspect of mitochondrial metabolic pathways as it affects intracellular calcium signaling, cell metabolism and survival by buffering cytosolic calcium and regulating mitochondrial effectors [65] .To better evaluate potential involvement of calcium handling changes in the improvement in cardiac function of septic mice that were treated with LGM2605, we assessed protein MCU and MICU1 levels. We observed trends of restoration of the sepsis-induced changes in the MCU and MICU1 expression *in vitro.* We then evaluated *in vivo* whether LGM2605 affects cardiac MCU and MICU1 in our mouse models of sepsis. Interestingly, sham mice that received LGM2605 had significantly higher gene and protein levels of MCU in the heart tissue compared to the sham mice at 6 hours after LGM2605 administration. That indicates that the drug stimulates basal expression levels of cardiac MCU. In addition, mice that underwent CLP and received LGM2605 had increased cardiac MCU levels compared to sham mice at 6 hours. Mice that underwent CLP and were treated with LGM2605 6hrs post-CLP had increased protein levels of cardiac MCU compared to the mice that underwent CLP at 12 hours. So, these data indicate that the drug has a significant effect on the MCU expression, which may affect mitochondrial calcium uptake. In order to verify that MCU increase may alter mitochondrial calcium uptake and improve mitochondrial metabolism, new calcium uptake experiments need to be performed. However, we evaluated mitochondrial respiration with the Seahorse system. We found that LGM2605 increases oxygen consumption rate of cardiomyocytes in septic mice. More specifically, administration of LGM2605 in mice that underwent CLP increased basal respiration levels, maximal respiration levels and the oxygen consumption rate used for ATP synthesis in cardiomyocytes, compared to both CLP and sham group. The effect of LGM2605 in mitochondrial respiration was very striking. However, although mitochondrial respiration

showed a trend for decrease in cardiomyocytes obtained from mice with CLP, statistical significance was not reached. Thus, analysis of additional mice is needed. It is also possible that the 12 hours timepoint that we chose for our experiments is an early one and we may not see profound impairment of mitochondrial respiration. So, further seahorse experiments should be performed possibly in a later timepoint of sepsis, for instance 24 hours after the surgery in order to address this question.

To conclude, in this study we evaluated the role of LGM2605, which is chemically synthesized SDG with anti-inflammatory and antioxidant properties in septic cardiac dysfunction. We showed that the drug could successfully prevent the cardiac dysfunction at 12 hours using an *in vivo* model of polymicrobial sepsis, the cecal ligation and puncture model. We evaluated the role of LGM2605 in all the possible mechanisms that have been associated with the pathophysiology of septic cardiomyopathy. The more pronounced effects of LGM2605 were regarding oxidative stress and mitochondrial metabolism. Further experiments should be performed though to address the clear mechanism through which the drug acts to prevent the septic cardiomyopathy.

Material and Methods

Animal care, cecal ligation and puncture procedure and echocardiography - Animal protocols were approved by the Temple University Institutional Animal Care and Use Committee and were carried out in accordance with the NIH guidelines for the care and use of laboratory animals. Wild type (WT) C57BL/6 mice were purchased from Jackson labs. Cecal ligation and puncture (CLP) was performed as previously described [44]. Briefly, for the procedure 7-9 weeks old mice were used. Under aseptic conditions, a 1 to 2 cm midline laparotomy was performed and exposure of the cecum with adjoining intestine. The cecum was tightly ligated at its base below the ileo-cecal valve at a distance of 1cm and was punctured twice with a 19-gauge needle. The length of the ligated cecum defined as the distance from the distal end of cecum to ligation point will determine the degree of severity. A distance of >1cm produces high grade sepsis while a distance of ≤ 1cm produced mid-to-low grade sepsis. Then the cecum is squeezed to extrude a small amount of feces and it is returned to the peritoneal cavity. The peritoneum and the skin are closed with sutures. The mice were resuscitated by injecting subcutaneously 1ml of pre-warmed 0,9% saline solution to induce the hyperdynamic phase of sepsis and for post-operative analgesia the mice received subcutaneously buprenorphine (0.05mg.kg body weight). At 12h post CLP, two-dimensional echocardiography was performed on anesthetized mice (inhaled isoflurane) using a VisualSonics Vevo 2100 machine. Echocardiographic images were recorder in a digital format. A single observer blinded to the respective treatments of mice then analyzed images off-line. The mice were sacrificed 12h post-CLP after the echocardiography. Mice received a single intraperitoneal injection of LGM2605 100mg/kg that was administrated either 2h prior to CLP or 6h post CLP for the 12 hours experiments and at the time of CLP for the 6 hours experiments.

Cell Culture - A human ventricular cardiomyocyte-derived cell line, designated AC-16, was used for the in vitro experiments. Cells were maintained in Dulbecco's modified Eagle's medium-nutrient mixture F-12 (DMEM-F-12; Invitrogen,Carlsbad,CA).

Measurement of mitochondrial superoxide and mitochondrial number- AC16 cells were cultured in 2-well plates with cell concentration at 10^4 cells/ml. After the overnight incubation, cells were divided into three groups, in the first group cells were treated with LPS (1μg/ml), in the second one with LPS and SDG (50mM) and the third group were the untreated cells. LPS and SDG were diluted in serum free media and the control group was incubated as well with serum free media. At 12 hours and 24 hours after the treatment the AC16 cells were loaded with MitoSOX Red (5Mm, M36008, Molecular Probes), a mitochondrial superoxide indicator, to analyze mitochondrial superoxide generation within the mitochondria of the AC16 cells, and incubated for 30 minutes in the dark. Excess MitoSOX Red was removed with three washed of PBS solution. MitoSOX Red fluorescence was recorded at 510 (excitation) and 580nm (emission). To assess possible changes in the mitochondrial number, AC16 cells were stained with 200nM Mitotracker Red (M22425, Molecular Probes), and incubated for 30 minutes in the dark. Excess Mitotracker Red was removed with three washed of PBS solution. Mitotracker Red fluorescence was recorded at 581 (excitation) and 644nm (emission).

Radio-ligand binding assay: Number of β-adrenergic receptors -Plasma membranes from excised mouse hearts were prepared, and saturation ligand binding was performed as described previously [66], using ^{125}I-CYP (iodocyanopindolol; PerkinElmer, Waltham, MA) for β-AR density measurement. Data were analyzed by nonlinear regression analysis using GraphPad Prism (GraphPad Software, La Jolla, CA).

Cyclic AMP assay- AC16 cells were cultured (100μl/well) in standard 96-well microtiter plates with cell concentration at 10.000 cells per well. After the overnight incubation, the control, SDG-treated cells, LPS-

treated cells and LPS+SDG-treated cells were washed with Krebs-Ringer Bicarbonate buffer and stimulated with 100nM isoproterenol in PBS. Control treatments were performed with PBS and 20μM forskolin in PBS. The concentration of cyclic AMP (cAMP) was determined with the cAMP Enzyme Immunoassay Kit according to the instructions of the manufacturer (CA201, Sigma-Aldrich, St.Louis, MO).

PKA activity assay- AC16 cells were cultured (100μl/well) in standard 96-well microtiter plates with cell concentration at 10.000 cells per well. After the overnight incubation, control, SDG-treated cells, LPS-treated cells and LPS+SDG-treated cells were washed with Krebs-Ringer Bicarbonate buffer and stimulated with 100nM isoproterenol in PBS. Control treatments were performed either with PBS or 20μM forskolin in PBS. The concentration of PKA kinase was determined with the PKA kinase Activity Kit according to the instructions of the manufacturer (ADI-EKS-390A, ENZO Life Sciences Inc, Farmingdale, NY). Obtained values were normalized with total protein concentration as determined with the Modified Lowry Protein Assay Kit (Thermo Scientific, Fremont,CA).

Adult Mouse Cardiomyocyte Isolation–Adult mouse cardiomyocytes (ACMs) were isolated from ventricles of C57BL/6 mice at 12 hours after they underwent sham surgery, or CLP surgery or CLP and received LGM2605 at 6 hours after the surgery. Hearts from heparinized mice (90 USP; ip) were cannulated through the aorta. Hearts were perfused with perfusion buffer (120.4 mMNaCl, 14.7 mM KCL, 0.6 mM NaH2PO4, 0.6 mM KH2PO4, 1.2 mM MgSO4, 10 mM Hepes, 4.6 mM NaHCO3, 30 mMtaurine, 10 mM BDM, 5.5 mM glucose; pH 7.4) for 3 min followed by digestion with perfusion buffer containing 19250 units Collagenese type II (Worthington), 5-6 mg trypsin and 0.02 mM CaCl2 for 7 min. Ventricles were gently teared in small pieces, perfusion buffer containing 5 mg/ml BSA and 0.125 mM CaCl2 was added and filtered with 100 μm nylon. The filtrate was pelleted by gravity for 5 min, centrifuged for 30 sec at 700 rpm and the

pellet resuspended in perfusion buffer containing 5 mg/ml BSA and 0.225 mM CaCl2. The cells were pelleted by gravity for 10 min, centrifuged for 30 sec at 700 rpm and the pellet resuspended in perfusion buffer containing 5 mg/ml BSA and 0.525 mM CaCl2.

Seahorse Analysis - Isolated primary ACMs were counted with Hematocytometer. Dead cells were detected with Trypan Blue Dye staining. Cells were platted (3000 cells per well) in XF96 Seahorse® plates pre-coated with laminin with 20 µg/ml laminin (Invitrogen, 23017). In order to assess oxygen consumption rates (OCR) for fatty acid oxidation (FAO) recordings, cells were incubated in substrate limited medium (DMEM containing 10mM Glucose, 1.025mM CaCl2, 0.5mM carnitine, pH=7.4) and assayed with fatty acid oxidation medium as per manufacturer's protocol. Before starting the assay, 1mM palmitate conjugated with BSA was added in each well. Drugs used for maximal response during fatty acid oxidation were: Oligomycin (3µM) (Sigma, O4875) which blocks complex V, FCCP (2µM) (Sigma, C2920) that leads to the collapse of the proton gradient, and Rotenone/Antimycin A (0.5µM) (Sigma, A8674)/ (Sigma, R8875) where rotenone blocks complex I and antimycin A blocks complex III. The pre-hydrated with XF assay calibrant, XF cartridges were filled with the drugs and the cartridge was calibrated for 30 minutes in Seahorse Analyzer. All experiments were performed at 37ºC. Calculations were made as described in the Seahorse manual and XF Seahorse Mito Stress Test kit user guide. Briefly, basal respiration was calculated with subtraction of non-mitochondrial respiration rate from the last measurement prior to first injection. Maximal respiration was calculated by subtraction of the non-mitochondrial respiration measurement from maximum measurement after FCCP injection. ATP synthesis-related OCR was obtained indirectly by measuring ATP-linked respiration in the presence of complex V inhibitor (Oligomycin). The decrease of oxygen consumption rate representing the portion of basal respiration that was used to drive ATP synthesis was calculated with subtraction of the minimum measurement after

Oligomycin injection from the last measurement prior to Oligomycin injection. Spare Respiratory Capacity was equal to (maximum respiration)- (basal respiration).

***In vivo* cardiac function measurements -**Mice from the three different groups (sham, CLP, CLP+LGM2605) were anesthetized. The right carotid artery was cannulated with a catheter/pressure transducer, and the catheter was advanced into the left ventricle. Another catheter was inserted into the femoral vein for administration of drugs. After completion of all the surgical procedures, the animal was allowed to stabilize for 5 min, followed by recording of basal hemodynamic parameters. Myocardial responses to increasing doses of isoproterenol (0.1ng, 0.5ng, 1ng, 5ng, 10ng) administered at 1- to 2- min intervals were determined. Data calculation was achieved using the PowerLab software.

RNA Purification and Gene Expression Analysis- Total RNA was purified from AC16 cells or hearts using the TRIzol reagent according to the instructions of the manufacturer (Invitrogen). DNase-treated RNA was used for cDNA synthesis using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs). Quantitative real-time PCR was performed with the Sybr Select Master Mix (Applied Biosystems). Incorporation of the SYBR green dye into the PCR products was monitored with the Applied Biosystems StepOnePlus Real-Time PCR System. Samples were normalized against mouse 36B4 or human rps13 RNA.

Protein Purification and Analysis- Isolated heart tissue or AC16 cells were homogenized in radioimmune precipitation assay buffer containing protease and phosphatase inhibitors (Pierce Protease and Phosphatase Inhibitor Mini Tablets, Thermo Scientific). 30-50 μg of total protein extract was applied to SDS-PAGE and transferred onto nitrocellulose membranes.

Statistical Analysis- Results are presented as mean ±SEM. The unpaired t-test was used for comparisons of two means; a 2-tailed value of $P<0.05$ was considered statistically significant. For groups of 2 or more ANOVA was used with Bonferroni as a post-hoc testing (Prism v5, GraphPad Software)

Chapter IV: Discussion and Future Directions

Sepsis is a common disease with a high mortality rate. Cardiac complications of sepsis are associated with a worse prognosis. Currently, there is no effective treatment for sepsis-induced cardiomyopathy. Studies using human tissue samples have shown that cardiomyocyte cell death is rare during sepsis and does not explain the decrease in systolic function [67]. Despite years of research, the cellular mechanisms of systolic dysfunction during sepsis remain unclear [68]. Multiple abnormalities have been reported, using cardiomyocytes and animal models, without the identification of a central mechanism. There are plenty of mechanisms that have been associated with the pathophysiology of septic cardiac dysfunction such as increased oxidative stress, increased inflammation, impaired β-adrenergic signaling, impaired metabolism and reduced ATP synthesis in the cardiomyocytes, but still remains unclear which one contributes more or is the central mechanism or mediator responsible for the presence of cardiac complications during sepsis.

Our goal in the studies we performed was to identify the contribution of several responsible mechanisms to the presence of septic cardiomyopathy and to identify drugs or molecules that by inhibiting them we can preserve the cardiac function, improve the mortality and contribute to the current knowledge of treating cardiac complications during sepsis.

In the first study we performed, we focused on the inhibition of NOX2 and its role in the pathophysiology of septic cardiac dysfunction. There are other papers though that have shown that other NOXes are also implicated

in septic cardiomyopathy such as NOX1[69] or in the pathophysiology of heart failure such as NOX4, with autophagy being a potential mechanism for this effect [70].

In our study, we showed that *in vivo* inhibition of NOX2 protects mice from sepsis-induced systolic dysfunction using both the LPS-injection model and the CLP model of polymicrobial sepsis. We showed that NOX2 inhibition preserved cardiac function, alleviated oxidative stress, reduced JNK activation and downregulation of metabolic genes without resolution of the inflammatory component of the disease based on both serum cytokines and cardiac tissue inflammatory gene expression. Thus, our study identified a potentially novel role for NOX2 in the pathophysiology of the disease, acting through cardiac metabolism in a way that is largely independent of systemic inflammation. Although immune system activation may contribute to cardiac dysfunction in later stages of sepsis [11,46], our experiments show that cardiac contractility and systemic inflammation can be dissociated. These new findings, suggest a crucial role for alleviation of oxidative stress, and restoration of the mitochondrial function and cardiac energetics as a way to maintain cardiac function in sepsis, independently of systemic inflammation, at least for the early stage of the disease. Although the NOX inhibitor apocynin did not improve mortality by itself in the rodent CLP model, it is possible that with intravenous fluid resuscitation and broad-spectrum antibiotics, NOX inhibitors could have additional benefit during septic shock complicated by cardiomyopathy.

In the second study we performed, we used LGM2605, which is chemically synthesized SDG with similar anti-inflammatory and antioxidant properties with the natural SDG extracted from flaxseed, and we evaluated its role in the septic cardiac dysfunction. We showed that the drug could successfully prevent the cardiac dysfunction at 12 hours using an *in vivo* model of polymicrobial sepsis, the cecal ligation and puncture model. We evaluated the role of LGM2605 in all the mechanisms that have been associated with the pathophysiology of septic cardiomyopathy and the more pronounced effects of LGM2605 were regarding oxidative stress and mitochondrial metabolism. More particularly, we showed that the drug could suppress the sepsis-induced

mitochondrial superoxide generation *in vitro*, restore the sepsis-induced impairment on mitochondrial number, increase the expression of MCU and increase the mitochondrial respiration levels as we observed by seahorse analysis.

The majority of mechanisms that have been implicated in the pathophysiology of septic cardiac dysfunction, indicate the complexity of this disease. Most of the treatments that have been tested so far, target one of the associated mechanisms leading to some or partial restoration of the cardiac function. Since this disease is so complex, and a clear responsible mechanism has not yet been identified, it is possible, that by targeting more than one of these pathways, to gain full restoration or even prevention of the cardiac dysfunction during sepsis. In our studies we showed the importance of anti-oxidant and metabolic therapies for treating septic cardiac dysfunction. It is possible that these two mechanisms account more for the induction of cardiomyopathy during at least the early stages of sepsis since by targeting them we observed significant restoration of the cardiac function in both of our studies.

Future studies are needed to evaluate further and support our data and hypothesis for targeting oxidative stress and mitochondrial dysfunction as a way to treat septic cardiomyopathy.

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