

# Targeting the eCIRP/TREM-1 Interaction with a Small Molecule Inhibitor Improves Cardiac Dysfunction in Neonatal Sepsis

**Naomi-Liza Denning**

1Center for Immunology and Inflammation, The Feinstein Institutes for Medical Research, Manhasset, NY 2Elmezzi Graduate School of Molecular Medicine, Manhasset, NY 3Department of Surgery, Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, H

**Monowar Aziz**

1Center for Immunology and Inflammation, The Feinstein Institutes for Medical Research, Manhasset, NY 2Elmezzi Graduate School of Molecular Medicine, Manhasset, NY

**Li Diao**

1Center for Immunology and Inflammation, The Feinstein Institutes for Medical Research, Manhasset, NY

**Jose M Prince**

1Center for Immunology and Inflammation, The Feinstein Institutes for Medical Research, Manhasset, NY 2Elmezzi Graduate School of Molecular Medicine, Manhasset, NY 3Department of Surgery, Donald and Barbara Zucker School of Medicine at Hofstra/Northwell,

**Ping Wang** (✉ [pwang@northwell.edu](mailto:pwang@northwell.edu))

Northwell Health Feinstein Institutes for Medical Research

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## Research

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# Abstract

**Background:** Neonatal sepsis and the associated myocardial dysfunction remain a leading cause of infant mortality. Extracellular cold-inducible RNA-binding protein (eCIRP) acts as a ligand of triggering receptor expressed on myeloid cells-1 (TREM-1). M3 is a small CIRP-derived peptide that inhibits the eCIRP/TREM-1 interaction. We hypothesize that the eCIRP/TREM-1 interaction in cardiomyocytes contributes to sepsis-induced cardiac dysfunction in neonatal sepsis, while M3 is cardioprotective.

**Methods:** Serum was collected from neonates in the Neonatal Intensive Care Unit (NICU). 5-7-day old C57BL/6 mouse pups were used in this study. Primary murine neonatal cardiomyocytes were stimulated with recombinant murine (rm) CIRP with M3. TREM-1 mRNA and supernatant cytokine levels were assayed. Mitochondrial oxidative stress, ROS, and membrane potential were assayed. Neonatal mice were injected with rmCIRP and speckle-tracking echocardiography was conducted to measure cardiac strain. Sepsis was induced by *i.p.* cecal slurry. Mouse pups were treated with M3 or vehicle. After 16 h, echocardiography was performed followed by euthanasia for tissue analysis. A 7-day survival study was conducted.

**Results:** Serum CIRP levels were elevated in septic human neonates. rmCIRP stimulation of cardiomyocytes increased TREM-1 gene expression. Stimulation of cardiomyocytes with rmCIRP upregulated TNF- $\alpha$  and IL-6 in the supernatants, while this upregulation was inhibited by M3. Stimulation of cardiomyocytes with rmCIRP resulted in a reduction in mitochondrial membrane potential (MMP) while M3 treatment returned MMP to near baseline. rmCIRP caused mitochondrial calcium overload; this was inhibited by M3. rmCIRP injection impaired longitudinal and radial cardiac strain. Sepsis resulted in cardiac dysfunction with a reduction in cardiac output and left ventricular end diastolic diameter. Both were improved by M3 treatment. Treatment with M3 attenuated serum, cardiac, and pulmonary levels of pro-inflammatory cytokines compared to vehicle-treated septic neonates. M3 dramatically increased sepsis survival.

**Conclusions:** Inhibition of eCIRP/TREM-1 interaction with M3 is cardioprotective, decreases inflammation, and improves survival in neonatal sepsis.

**Trial registration:** Retrospectively registered.

## Background

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.[1] Neonates are particularly vulnerable to sepsis.[2] Estimates of the global burden of neonatal sepsis vary considerably, but neonatal sepsis is thought to account for approximately 1.4 million neonatal deaths per year.[3, 4] Sepsis-associated cardiac dysfunction in neonates is not as well studied as in older patients. Among adults with sepsis, a recent literature review found that myocardial dysfunction is common with a reported incidence ranging from 10–70%.[5] This large range is likely due, in part, to the lack of a clinical consensus definition of sepsis-associated cardiac dysfunction.[5]

Myocardial dysfunction in sepsis considerably worsens outcomes.[5–7] Although there is a relative paucity of data in the neonatal population, pediatric studies have recapitulated these findings.[8–12] In fact, cardiac dysfunction appears to occur more frequently in pediatric and neonatal septic patients as compared to their adult counterparts.[13]

Damage-associated molecular patterns (DAMPs) propagate inflammation in sepsis[14, 15] and some have been implicated in increasing sepsis-associated cardiac dysfunction.[6, 16, 17] Cold-inducible RNA binding-protein (CIRP) is an 18-kDa nuclear RNA chaperone protein that is released extracellularly both passively after necrotic cell death and in response to sepsis, hemorrhage or ischemia-reperfusion injury. [18, 19] We have previously demonstrated that extracellular CIRP (eCIRP) is a DAMP, promoting activation of several cell types including macrophages, lymphocytes, and neutrophils, potentiating cytokine and chemokine production, and propagating formation of neutrophil extracellular traps (NETs).[19, 20] eCIRP causes endoplasmic reticulum (ER) stress and Nlrp3 inflammasome activation.[21] Conversely, CIRP knockout mice are protected from sepsis and acute lung injury.[21, 22] In adult human patients, elevated plasma levels of eCIRP have been correlated with a poor prognosis in patients with sepsis and with lung dysfunction after cardiac surgery.[19, 23, 24] However, to our knowledge, eCIRP levels have never been assessed in neonates. Furthermore, the impact of eCIRP on cardiac tissue or cardiac function has never been studied.

Triggering receptor expressed on myeloid cells-1 (TREM-1) is an innate immune pattern recognition receptor expressed primarily on neutrophils and macrophages.[25] We have recently shown that eCIRP is a ligand of TREM-1.[26] TREM-1 is an activating, proinflammatory receptor, propagating inflammation independently[27], as well as synergistically with the toll-like receptor 4 (TLR4) pathway.[27, 28] Both eCIRP and TREM-1 are upregulated in sepsis to serve as mediators of inflammation.[19, 26] We recently developed a small eCIRP-derived peptide (RGFFRGG), named M3, that is an antagonist of the eCIRP/TREM-1 interaction. We successfully implemented M3 as a therapeutic agent in adult murine models of sepsis.[26]

However, the neonatal immune response is dramatically different than its adult counterpart.[29] Neonates rely almost exclusively on innate immunity yet have a relatively downregulated NF- $\kappa$ B pathway compared to adults. Additionally, they have a diminished upregulation of TNF- $\alpha$  related genes, diminished pattern recognition signaling, and impaired neutrophil function.[29, 30] Furthermore, despite some evidence that soluble TREM-1 may be a biomarker for neonatal sepsis,[31, 32] in neonates with sepsis, TREM-1 upregulation is decreased compared to older infants, children, and adults.[2] Given the differences between the adult and neonatal immune response in sepsis, an investigation into the efficacy of M3 in neonatal sepsis is warranted.

In this study using both *in vitro* studies and echocardiogram of neonatal mice, we establish the link between eCIRP and cardiac dysfunction in sepsis for the first time. We demonstrate the significance of this finding by reporting elevated levels of eCIRP in septic neonates in the neonatal intensive care unit. We determine the impact of the eCIRP/TREM-1 interaction on systemic inflammation, cardiac function, and

survival in a mouse model of neonatal sepsis. Finally, we demonstrate the therapeutic potential of M3 as an inhibitor of eCIRP/TREM-1's interaction in neonatal sepsis.

## Methods

### Determination of blood CIRP levels in human neonatal samples

After obtaining Institutional Review Board approval (Northwell Health IRB # 19-0511), serum samples were obtained from patients in a neonatal intensive care unit at a tertiary care children's hospital. Serum samples were collected initially for patient care and the remaining volume in a sample after use for diagnostic testing was used for this study. The medical record was reviewed for demographics and clinical and diagnostic information. Serum was stored at -80°C until analysis. eCIRP levels in the serum were measured using a human CIRP ELISA kit (American Research Products, Waltham, MA) according to manufacturer's instructions.

### Experimental animals

House-bred male and female C57BL/6 mice were kept in a 12 h light/dark, temperature-controlled room and fed standard Purina rodent diet. Females were closely observed to ensure an accurate date of birth for all litters. Five to seven-day old neonatal mice were used for all *in vivo* experiments. Sex determination via external characteristics is difficult to reliably determine at this age and weight (three to four grams). As such, pups were not identified as either male or female and both genders were therefore used in all experiments. Pups remained with their mothers throughout all experimental timeframes and could breastfeed ad libitum. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of the Feinstein Institutes for Medical Research.

### Murine model of neonatal sepsis

Neonatal sepsis was induced with cecal slurry (CS) as originally developed by Wynn et al[33] and modified as previously described by us.[34, 35] Sepsis was induced in neonatal mice via by administration of 0.525 mg/g body weight (BW) intraperitoneal CS. Pups were removed and returned to their cage with their mothers as a group. Sham mice received an intraperitoneal injection of an equivalent volume of 5% dextrose. At 16 hours after CS injection, pups were anesthetized using 2.5% isoflurane anesthesia, underwent echocardiogram as described below, and were subsequently euthanized by cardiac puncture. Blood, heart, and lungs were collected. Heart and lungs were immediately flash-frozen in liquid nitrogen. Blood was centrifuged at 1,000 rotations per minute (rpm) for 10 minutes and serum was collected. Serum, heart, and lungs were then stored at -80°C until analysis. For the survival study, pups received a diluted dose of CS (0.175 mg/g BW) and were monitored for seven days for survival.

### *In vivo* administration of mCIRP and M3

Recombinant murine CIRP (rmCIRP) was produced as previously described.[19] rmCIRP at a dose of 10 mg/kg BW or an equivalent volume of normal saline was administered intraperitoneally. M3 (10 mg/kg BW) or vehicle (normal saline) was given *i.p.* at the time of cecal slurry injection.[26] For the survival study, an additional group of mice received M3 2 h after CS injection.

### **Isolation of primary murine neonatal cardiomyocytes**

Neonatal cardiomyocytes were isolated from 0 to 2-day old neonatal C57BL/6 mice using the Pierce Primary Cardiomyocyte Isolation kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Cells were cultured in DMEM from the kit supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### **Measurements of reactive oxygen species, mitochondrial depolarization, and mitochondrial calcium levels**

Cardiomyocyte and mitochondrial reactive oxygen species (ROS), mitochondrial depolarization, and mitochondrial calcium levels were assayed as described previously by Joseph et al.[36, 37] Cardiomyocytes were plated in 96-well plates. Plated cells were pre-treated with 10 µg/mL M3 peptide or equivalent volume additional media for 20 minutes. Cells were then stimulated with either PBS as a control or rmCIRP at various doses for 4 h to assess ROS and mitochondrial depolarization or 1.5 h to determine calcium levels. To measure total ROS, cardiomyocytes were loaded with 25 µM 2',7'-dichlorofluorescein diacetate (DCF) (Sigma-Aldrich, St Louis, MO) for 30 minutes in the dark. Excess DCF was removed by washing and DCF fluorescence was recorded at excitation/emission wavelengths of 490/530 nm. To measure mitochondrial ROS, MitoSOX Red, a mitochondrial superoxide indicator, (5 µM, ThermoFisher Scientific) was added to cardiomyocytes and incubated in the dark for 30 minutes. Excess MitoSOX Red was removed, and fluorescence was recorded at excitation/emission wavelengths of 525/620 nm. Tetramethylrhodamine methyl (TMRM) ester (1 nM, ThermoFisher Scientific) was used to assess changes in mitochondrial membrane potential. Cardiomyocytes were stained with TMRM ester for 30 minutes and fluorescence was recorded at excitation/emission 540 nm/590nm. Due to its internal positive charge, TMRM preferentially accumulates inside mitochondria. As the mitochondria depolarizes, less TMRM is trapped. Therefore, the TMRM signal is proportional to the inner membrane potential of the mitochondria.[36] Finally, changes in mitochondrial calcium were determined using a 30 minute incubation with 10 µM Rhod-2-AM (ThermoFisher Scientific) followed by a 1 hour washout- and cytosolic quenching with 0.5 mM manganese (Trevigen, Gaithersburg, MD). Fluorescence was measured at 552 nm (excitation)/581 nm (emission).

### **Enzyme-linked immunosorbent assay (ELISA)**

Cardiomyocyte supernatant was analyzed by ELISA kits for interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Cardiac and lung tissue was crushed in liquid nitrogen, and equal weights of powdered tissues (~50 mg) were dissolved in 500 µl of lysis buffer (10 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1% Triton X-100, and 2 mM each of

EDTA and EGTA), and subjected to sonication on ice. Protein concentration was determined by the BioRad protein assay reagent (Hercules, CA). Equal amounts of proteins (250-500 µg) were loaded into respective ELISA wells for the assessment of IL-6 and IL-1β (Invitrogen, Carlsbad, CA). Serum was analyzed using a Bio-Plex Pro Mouse Cytokine Th17 Panel A -6-Plex kit (BioRad).

### **Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

To examine sepsis-associated lung and cardiac inflammation, the lung and heart mRNA expression of IL-6 and IL-1 β were measured. TREM-1 expression in neonatal murine cardiomyocytes was assessed after 24 hours of rmCIRP stimulation. Total RNA was extracted from cells and tissue using Trizol reagent (Invitrogen). cDNA was synthesized using MLV reverse transcriptase (Applied Biosystems, Foster City, CA). PCR reactions were carried out in 20 µl of a final volume of 0.08 µM of each forward and reverse primer, cDNA, water, and SYBR Green PCR master mix (Applied Biosystems). Amplification was performed in a Step One Plus real-time PCR machine (Applied Biosystems). Mouse β-actin or GAPDH mRNA was used as an internal control for amplification for lung and cardiac tissue, respectively, and relative gene expression levels were calculated using the DDCT method. Relative expression of mRNA was expressed as fold change in comparison with sham tissues or PBS treated cells.

### **Echocardiogram**

Two hours after injection of rmCIRP or 16 hours after sepsis induction with CS, cardiac function in neonatal mice was assessed by transthoracic echocardiography. Echocardiography was conducted using a 40 MHz center frequency transducer coupled to a Vevo®3100 Imaging System (Fujifilm VisualSonics, Toronto, ON, Canada). Sedation was induced with 2.5% isoflurane and maintained with 0.5-1% isoflurane for the duration of the echocardiogram. Mice were maintained on a heated table during this time. Parasternal long axis views were taken in B and M modes. VevoLab (Fujifilm VisualSonics) software was used to determine cardiac parameters. VevoStrain (Fujifilm VisualSonics) software was used to measure myocardial strain and strain rate using speckle-tracking echocardiography. [38]

### **Statistical analysis**

Data represented in the figures are expressed as mean ± SE. All data has been tested for normality using the Kolmogorov-Smirnov Test of Normality. Normally distributed data was analyzed using the two-tailed Student's *t* test for two-group comparisons and One-way ANOVA for comparison among multiple groups with the significance between individual groups determined using the Tukey method. Nonparametric data was analyzed using one-way comparison among multiple groups with the Kruskal-Wallis test with a Dunn's multiple comparison test. Significance was considered for  $p \leq 0.05$  between study groups. Data analyses were carried out using GraphPad Prism graphing and statistical software (GraphPad Software, San Diego, CA).

## **Results**

## **eCIRP levels are increased in neonates with sepsis**

Blood samples were obtained from human neonates in the neonatal intensive care unit (NICU) of a tertiary care children's hospital. Extracellular CIRP levels in the serum were measured by ELISA. Several comparisons were made between groups. Infants were designated as septic versus non-septic according to review of the electronic medical record (EMR) and documentation on the day of specimen collection. First, eCIRP levels in non-septic infants versus septic infants were compared. Extracellular CIRP levels were significantly elevated in septic neonates (**Fig 1A**). Second, because sepsis rule-out is so common in neonates, we included infants in that category in the comparison. Infants that had been clinically classified as "sepsis rule-out" in the EMR on the day of serum collection were divided into two groups. Infants ultimately receiving only 48 hours of antibiotics, and thus clinically determined to not have sepsis, were included in the no sepsis group. Infants who received more than 48 hours of antibiotics were included in the sepsis group. There was a dramatic difference between the two groups (**Fig 1B**). Next, infants with sepsis were compared to a group of infants in the well-baby nursery or with full term infants in the NICU with a short (less than 3 days) length of stay and a non-infectious reason for NICU admission, rendering them likely to be a fairly homogenous, well-baby population (**Fig 1C**). Finally, given the large difference in corrected gestational age of infants in the NICU, we compared septic vs non-septic infants with a gestational age at birth of under 32 weeks and again saw large disparities in serum CIRP levels (**Fig 1D**). The significant elevations in serum CIRP levels of septic neonates highlights the clinical relevance of the findings in this study.

## **M3 attenuates systemic inflammation in murine neonatal sepsis**

To evaluate the impact of the eCIRP/TREM-1 interaction in neonatal sepsis, we treated pups with the M3 peptide or normal saline vehicle after sepsis induction with CS. Sixteen h after CS, as a surrogate of systemic inflammation, we measured the serum levels of IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  (**Fig 2**). Compared with sham pups, septic pups had significant elevations in serum levels of all four cytokines; IL-6 (**Fig 2A**) increased over 200-fold, from an average of 0.17 ng/mL in sham pups to 35.8 ng/mL in vehicle-treated pups. Treatment with M3 dampened this inflammation: M3-treated septic pups had a 36.6% reduction in IL-6 levels to an average of 22.7 ng/mL (**Fig 2A**). Similarly, IL-1 $\beta$  was elevated in septic vehicle-treated pups to an average of 666.0 pg/mL compared to shams' average level of 8.7 pg/mL. M3 improved this inflammation by 42.7%, resulting in an average level of 381.3 pg/mL (**Fig 2B**). Sepsis induction resulted in analogous increases in TNF- $\alpha$  and IFN- $\gamma$  with increases of 38.7-fold and 22.4-fold, in vehicle-treated septic pups compared to sham pups, respectively. M3 treatment attenuated these levels, by 41.2% and 36.0%, respectively (**Figs 2C-D**). These data clearly suggest that M3 plays an anti-inflammatory role by decreasing systemic cytokine levels in neonatal sepsis.

## **Treatment with M3 pulmonary and cardiac inflammation in neonatal sepsis**

Sepsis-associated acute lung injury is responsible for a large percentage of the morbidity and mortality of intra-abdominal sepsis.[39] We assessed both mRNA expression and protein levels of neonatal lung tissue 16 hours after sepsis induction with cecal slurry. In the lungs, both mRNA and protein levels of the

pro-inflammatory cytokines IL-6 and IL-1 $\beta$  were significantly increased in vehicle treated septic pups compared to sham neonates (**Figs 3A-D**). M3 treatment significantly decreased the expression of IL-6 and IL-1 $\beta$  at mRNA level by 50.7% and 24%, respectively, and at protein level by 41.3% and 24.8%, respectively compared to vehicle-treated samples (**Figs 3A-D**). Cytokine production from the heart has been shown to propagate inflammation and worsen cardiac dysfunction.[40, 41] Analogously, in the cardiac tissues, both mRNA and protein levels of the pro-inflammatory cytokines IL-6 and IL-1 $\beta$  were significantly increased in vehicle treated septic pups compared to sham neonates (**Fig 3E-H**). M3 treatment significantly decreased the expression of IL-6 and IL-1 $\beta$  at mRNA level by 77.5% and 61.7%, and at protein level by 37.3% and 46.3%, respectively, compared to vehicle-treated samples (**Fig 3E-H**). Thus, M3 decreases proinflammatory cytokine levels in the lungs and cardiac tissues in neonatal septic mice.

### **Treatment with M3 improves cardiac function in neonatal sepsis**

To determine if M3 improved cardiac function in neonatal sepsis, we performed echocardiogram 16 hours after sepsis induction (**Fig 4A, Supplemental Video**). Cardiac output (CO) was dramatically decreased 16 hours after sepsis induction. M3 improved CO by 42% (**Fig 4B**). Diastolic function was also impaired by sepsis. Left ventricular end diastolic diameter was decreased in vehicle treated mice and improved by 20% in M3 treated pups (**Fig 4C**).

### **M3 improves survival in neonatal sepsis**

To verify that the reduction in inflammatory markers and improvement in cardiac function resulted in improved outcomes, we performed a survival study on septic neonates. Pups were injected with a reduced concentration of CS and either M3 or vehicle. Neonatal pups were monitored for seven days. Survival improved from 8% in the vehicle group to 54% in the group of pups who received simultaneous M3 treatment (**Fig 5**). To increase the clinical applicability of the model, we also tested the survival in a group of pups exposed to M3 two hours after sepsis induction with CS. This group also had improved survival at 58% (**Fig 5**). There was no statistically significant difference in survival between the simultaneous and delayed administration treatment groups. These findings confirm that inhibiting the eCIRP/TREM-1 inhibition improves survival in murine neonatal sepsis.

### **Treatment with M3 inhibits rmCIRP-mediated inflammation in neonatal cardiomyocytes**

Given that M3 was successful in improving cardiac function in neonatal sepsis, we aimed to further identify if this impact was, in part, due to the eCIRP/TREM-1 interaction in cardiomyocytes or if it was merely a reflection of reduced sepsis severity seen with eCIRP inhibition in previous preclinical sepsis models.[19, 34] We first examined the response of neonatal murine cardiomyocytes to rmCIRP. Primary murine neonatal cardiomyocytes were isolated from 1 to 2-day old neonatal mice and plated in 96-well plates. After 24 hours of rmCIRP stimulation, cells were lysed and assessed for TREM-1 mRNA expression. rmCIRP increased TREM-1 gene expression by approximately 100-fold compared to PBS-treated cardiomyocytes (**Fig 6A**).

To demonstrate that rmCIRP results in cardiac inflammation, primary murine neonatal cardiomyocytes were again isolated and exposed to rmCIRP. rmCIRP stimulation significantly increased levels of IL-6 (**Fig 6B**) and TNF- $\alpha$  (**Fig 6C**) in the supernatant by 3.1 and 10.3-fold, respectively. However, treatment with rmCIRP after 20 minutes of pre-treatment with M3 reduced IL-6 and TNF- $\alpha$  levels to near-baseline, by 70.9% and 84.8%, respectively (**Figs 6B, C**). Cumulatively, this data indicates that TREM-1 expression increases during inflammatory conditions in cardiac cells and that inhibition of the eCIRP/TREM-1 interaction is anti-inflammatory.

### **rmCIRP increases oxidative stress in cardiomyocytes and causes mitochondrial dysfunction**

To better identify how eCIRP results in cardiac dysfunction in sepsis, we examined cardiac oxidative stress and mitochondrial dysfunction *in vitro*. 2',7'-dichlorofluorescein diacetate [DCF] fluorescence was used to quantify total cellular ROS in cardiomyocytes after rmCIRP exposure (**Fig 7A**). There was a dose dependent increase in ROS after rmCIRP stimulation. M3 was able to significantly inhibit this rmCIRP-mediated increase in ROS (**Fig 7A**). To look preferentially at mitochondrial ROS, we used MitoSox Red. We again found a dose dependent increase in ROS after rmCIRP stimulation that was ameliorated by M3 treatment (**Fig 7B**). We next quantified the mitochondrial inner membrane potential using tetramethylrhodamine methyl ester (TMRM). rmCIRP resulted in a significant reduction in mitochondrial membrane potential in cardiomyocytes which was partially restored by M3 (**Fig 7C**). In a homeostatic cell, the mitochondrial inner membrane has a voltage gradient – the interior is negative as compared to the cytosol. rmCIRP caused partial depolarization of the mitochondrial inner membrane potential, an indication of cardiomyocyte mitochondrial dysfunction. The inner membrane potential of a mitochondria can be depolarized by a surplus of mitochondrial calcium uptake.[37] As such, we sought to evaluate mitochondrial calcium levels after rmCIRP stimulation. Using Rhod2-AM fluorescence, we found that rmCIRP caused mitochondrial calcium overload, in a dose-dependent manner, which was again mitigated by M3 treatment (**Fig 7D**). We conclude from this data that rmCIRP increases oxidative stress in cardiomyocytes and contributes to mitochondrial impairment.

### **Global longitudinal and radial strain declines following exposure to rmCIRP**

In order to determine if eCIRP was detrimental to cardiac function *in vivo*, 5-7 day old neonatal mouse pups were injected i.p. with rmCIRP. Two hours later, using VevoStrain, we assessed radial and longitudinal strain. Myocardial strain allows for quantification of myocardial deformation and has been demonstrated to be an effective assessment of cardiac dysfunction in sepsis.[38] Two hours after rmCIRP injection, pups demonstrated impairment in both longitudinal and radial strain and strain rate (**Figs 8A-D**) highlighting the impact of eCIRP on cardiac dysfunction.

## **Discussion**

The initial response of the innate immune system in neonatal sepsis includes an unregulated, disproportionate release of proinflammatory cytokines in response to PAMPs and DAMPs. This “cytokine storm” can lead to organ dysfunction and death.[29, 42, 43] Furthermore, excessive inflammation in the

neonatal period has been associated with neurodegenerative effects and cognitive deficiencies.[44, 45] Excessive inflammation in the lungs predispose infants to an increased risk of bronchopulmonary dysplasia, while cardiac inflammation has been associated with the development of ventricular failure. [46, 47] In the present study of a neonatal murine model of polymicrobial abdominal sepsis, we demonstrated that sepsis treatment with the eCIRP-derived TREM-1 inhibitor M3 reduced systemic, pulmonary, and cardiac inflammation. M3 treatment also improved survival after neonatal sepsis. Although several TREM-1 antagonists are available, most of them were developed and designed from the extracellular TREM-1 sequence and serve as TREM-1 decoy receptors.[48] M3 was developed from eCIRP's sequence to specifically abrogate the eCIRP/TREM-1 ligand/receptor interaction[26], while allowing other molecules that regulate inflammation to be un-interrupted.

The presence of cardiac dysfunction in sepsis can double mortality.[49] In our study, all neonatal mice who were subjected to sepsis induction via CS demonstrated some degree of cardiac functional impairment as seen on echocardiogram. Unlike adults, sepsis-associated cardiac dysfunction in children is typically a nonhyperdynamic state, with reduced cardiac output and increased systemic vascular resistance.[50] Although neonates can have a more variable response to severe sepsis,[50] we found the anticipated reduction in CO in the neonatal mice. Treatment of septic mouse pups with M3 resulted in an improvement in CO. Maintenance of cardiac output is a key predictor of sepsis survival in infants and children and an important therapeutic target.[51–53] Additionally, we found that M3 was able to increase left ventricular end diastolic diameter, a characteristic that has also been associated with increased survival.[54, 55] As such, we postulated that the myocardial dysfunction in sepsis was due, in part, to eCIRP.

Although there have been years of research without the identification of a central mechanism for sepsis-associated cardiac dysfunction, several factors have been identified as being contributory. These include upregulation of innate immune receptors in the heart itself, circulating DAMPs and cytokines, altered production of nitric oxide, lack of calcium homeostasis, and oxidative stress.[6, 56, 57] We have previously published ample evidence that eCIRP is released into the circulation in sepsis and results in systemic inflammation.[20] In this study, we have demonstrated that circulating eCIRP in sepsis plays a direct role in many of the aforementioned mechanisms of sepsis-associated cardiac dysfunction in neonates.

To demonstrate the direct impact of the eCIRP/TREM-1 interaction on cardiac tissue, primary neonatal cardiomyocytes were stimulated with rmCIRP. rmCIRP was able to upregulate TREM-1 expression in cardiomyocytes. Additionally, stimulated cells increased secretion of both TNF- $\alpha$  and IL-6. Cardiomyocytes are known to develop NF- $\kappa$ B-dependent inflammation and activation of the transcription of genes involved in producing pro-inflammatory cytokines, including IL-6, TNF- $\alpha$ , and IL- $\beta$ , during sepsis, which leads to contractile dysfunction in the myocardium.[41, 56, 58–60] This inflammation was originally thought to be entirely TLR4 dependent, however evidence is growing that TREM-1 is important in sepsis-associated cardiac inflammation.[61–63] In addition to promoting inflammation independently[27], TREM-1 is known to be synergistic with TLR4 and amplify TLR4-mediated

inflammation[28, 64, 65] TREM-1 is also known to proceed through the NF- $\kappa$ B pathway.[66, 67] Thus the protective effects of M3 on cardiac inflammation are likely multifaceted, preventing TREM-1 mediated inflammation and downregulating TREM-1 amplification of TLR-4 signaling.

Mitochondrial dysfunction is also thought to be a key component of sepsis-associated cardiac impairment.[57, 68] Sepsis results in partial depolarization of mitochondria and mitochondrial calcium leak, as well as the upregulation of mitochondrial reactive oxygen species, all of which lead to decreased cardiac contractility and dysfunction.[37] Oxidative stress as a cause of mitochondrial dysfunction appears to be particularly prominent in neonates.[69] In this study, we have demonstrated that eCIRP is sufficient to increase cardiomyocyte ROS and mitochondrial superoxide, increase calcium influx into mitochondria, and promote depolarization of the mitochondria. Furthermore, we have shown that inhibition of the eCIRP/TREM-1 interaction with the M3 peptide ameliorated these effects. In an observational study of pediatric patients with sepsis, persistent mitochondrial dysfunction was linked to prolonged organ dysfunction,[70] therefore the ability to improve mitochondrial function with the M3 peptide is advantageous.

Finally, to verify that eCIRP directly impacts cardiac function *in vivo* and not just in cell culture, we injected rmCIRP into neonatal mice and measured longitudinal and radial cardiac strain using speckle tracking echocardiography. Cardiac strain is more sensitive than conventional echocardiography for detecting early cardiac dysfunction in sepsis.[38, 71] Myocardial strain can identify sepsis-associated cardiac dysfunction even in the presence of a normal ejection fraction, which can occur with decreased cardiac work and does not accurately reflect cardiac dysfunction.[72] In a meta-analysis of adult patients with sepsis, increased (i.e., less negative) global longitudinal strain values were associated with mortality; this finding was not seen with ejection fraction – the most commonly used parameter to evaluate adult cardiac function in sepsis.[73] Additionally, changes in myocardial strain have been shown to correlate with the upregulation of inflammatory cytokines and mitochondrial dysfunction in the form of an increased in mitochondrial ROS.[72] In our study, eCIRP worsened both longitudinal and radial cardiac strain, providing direct evidence that eCIRP causes cardiac dysfunction in neonatal sepsis.

In an attempt to verify the clinical applicability of these findings, we included in our study an analysis of serum eCIRP levels in human neonates. Serum levels of eCIRP have been shown to correlate with severity of illness and survival in adult patients with sepsis.[23] However, plasma levels of DAMPs and cytokines are known to vary with age, and a level that is prognostic in one age group does not necessarily extrapolate to another.[74, 75] In our study, higher serum eCIRP levels were found in infants with sepsis than compared to non-septic neonates in the NICU. Recognizing that NICU infants are a heterogeneous group, many of whom may have chronic inflammation due to noninfectious comorbidities, we further compared septic neonates with a small group of well infants. Differences between these two groups were even more apparent.

Our study is limited by the lack of invasive cardiac monitoring in the neonatal mice, a limitation due to technical difficulty given their small size. Additionally, we demonstrated the improvement in inflammation

and cardiac function when M3 was given concurrently with CS injection. In the clinical setting, it can be difficult to detect the onset of neonatal sepsis. We attempted to mitigate this limitation by providing a survival curve with a delayed M3 administration.

## Conclusion

In conclusion, in this study, we used both *in vitro* and *in vivo* data to demonstrate that eCIRP is responsible for some of the cardiac dysfunction seen in septic shock. We further demonstrate that M3, an eCIRP-derived TREM-1 inhibitor, decreases systemic, pulmonary, and cardiac inflammation, improves cardiac dysfunction, and increases survival in a murine model of neonatal sepsis. Pharmacologic inhibition of the eCIRP/TREM-1 interaction is a new potential therapeutic strategy in the treatment of neonatal sepsis.

## Abbreviations

eCIRP	extracellular cold-inducible RNA-binding protein
CIRP	cold-inducible RNA-binding protein
rmCIRP	recombinant murine cold-inducible RNA-binding protein
TREM-1	triggering receptor expressed on myeloid cells-1
MMP	mitochondrial membrane potential
DAMP	damage-associated molecular pattern
NETs	neutrophil extracellular traps
ER	endoplasmic reticulum
CS	cecal slurry
BW	body weight
DCF	dichlorofluorescein diacetate
TMRM	tetramethylrhodamine methyl
NICU	neonatal intensive care unit
CO	cardiac output
LVED Diameter	left ventricular end diastolic diameter
EMR	electronic medical record
i.p.	intraperitoneal

## Declarations

**Ethics approval and consent to participate:** Human samples were studied under the approval of Institutional Review Board (Northwell Health IRB # 19-0511). All animal studies were performed under the approval of the Institutional Animal Care and Use Committee of the Feinstein Institutes for Medical Research.

**Consent for publication:** Yes.

**Availability of data and materials:** All data were presented in the manuscript and in the supplemental section and these are readily available to the readers.

**Competing interests:** One of the authors (PW) is an inventor of patent applications (WO/2010/120726 and 61/881.798) covering the fundamental concept of targeting CIRP for the treatment of inflammatory

diseases, licensed by TheraSource LLC. PW is a co-founder of TheraSource LLC. Other authors declared that they have no competing interests.

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**Authors' contributions:** NL Denning, M Aziz, and P Wang designed the experiments. NL Denning and L Diao performed all animal experiments. NL Denning, M Aziz, and JM Prince analyzed the data. NL Denning prepared the figures and wrote the manuscript. M Aziz, L Diao, JM Prince, and P Wang critically reviewed and edited the manuscript. P Wang supervised the project.

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## Figures

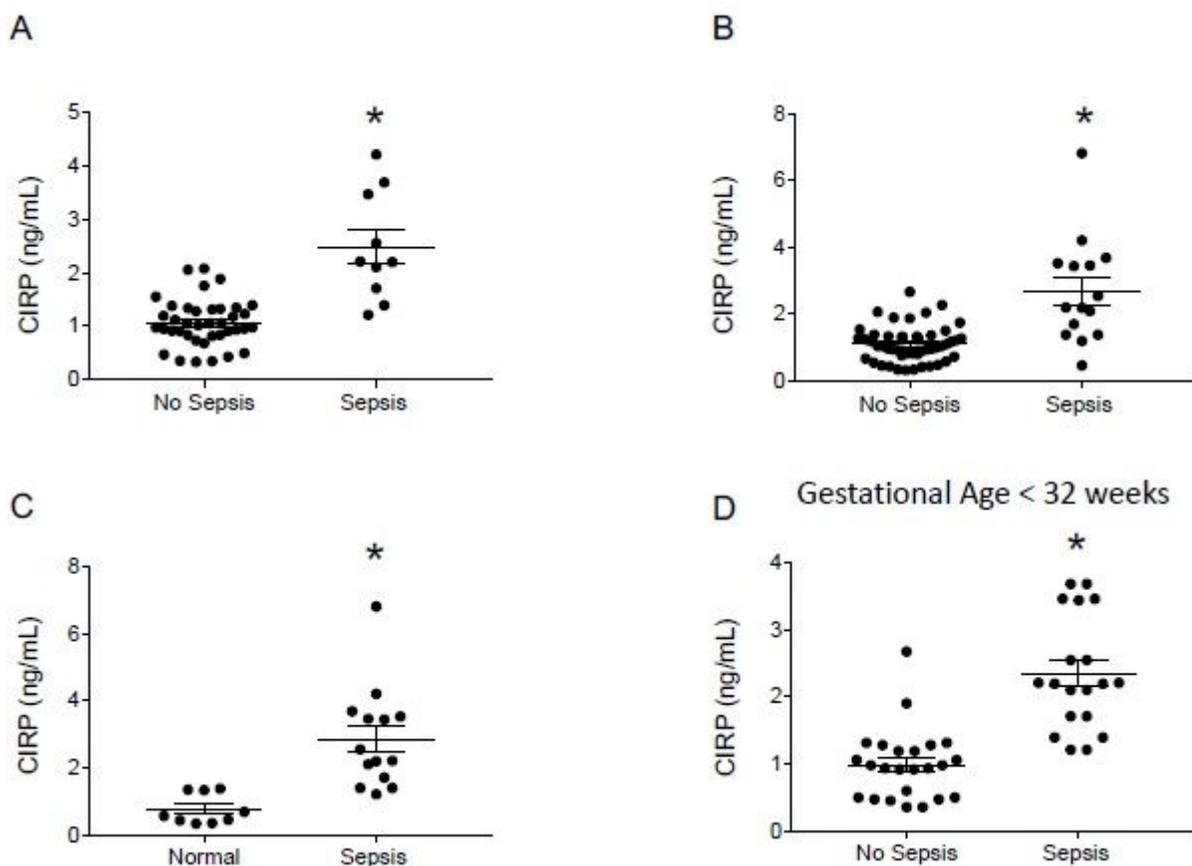
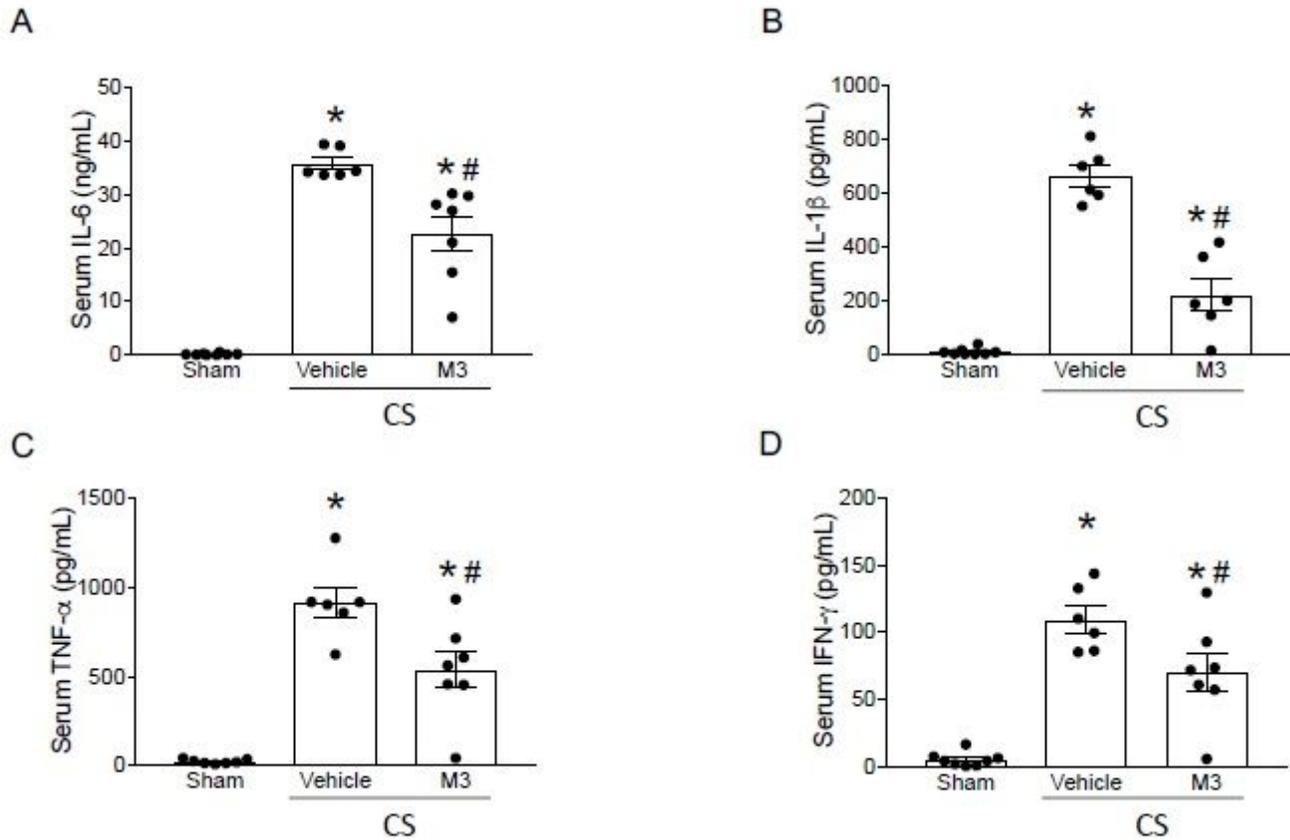


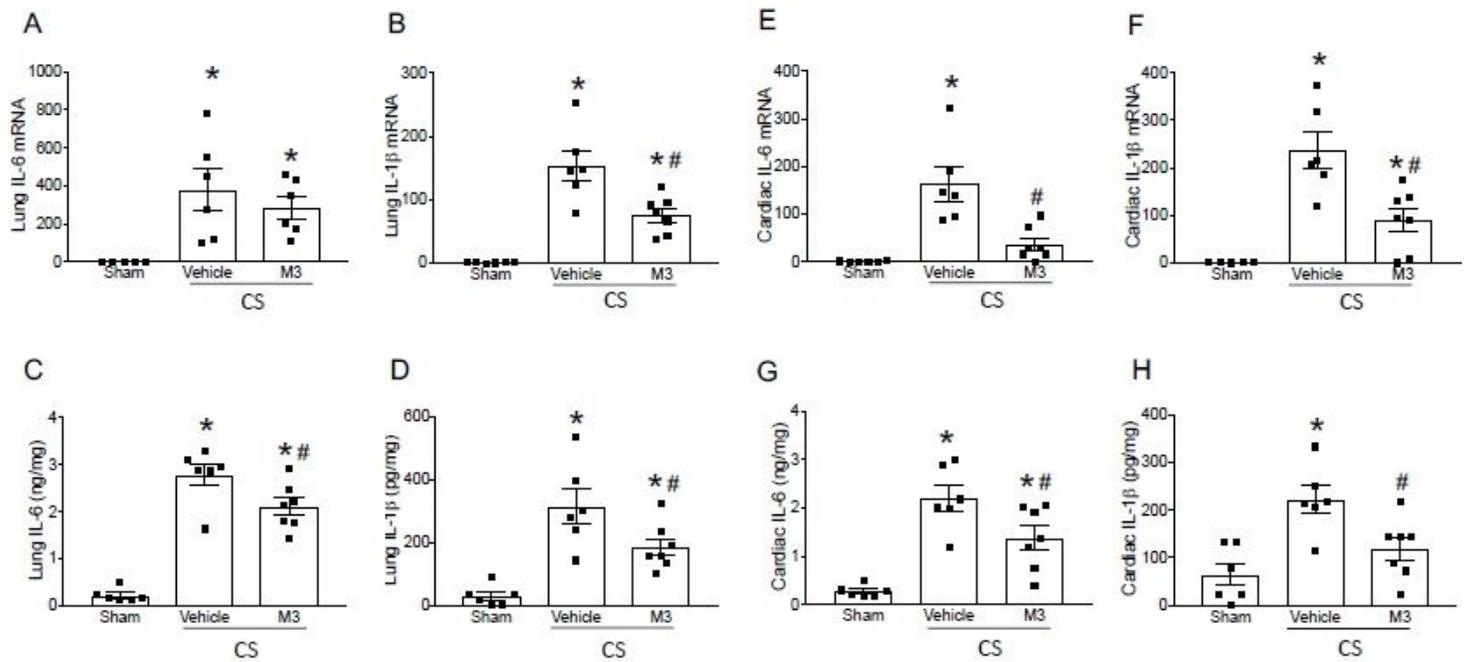
Figure 1

Serum C1RP levels are elevated in septic neonates. Serum levels of C1RP were compared in (A) neonates classified as non-septic versus septic according to the electronic medical record. (B) Infants clinically classified as “sepsis rule-out” were divided into the non-septic vs septic cohorts according to duration of antibiotics therapy. (C) Serum levels of C1RP in full term, well infants were compared to septic neonates. (D) Infants with a gestational age < 32 weeks were compared. Data are expressed as means  $\pm$  SE and compared by two-tailed student’s t test (\*  $p < 0.05$  vs no sepsis).



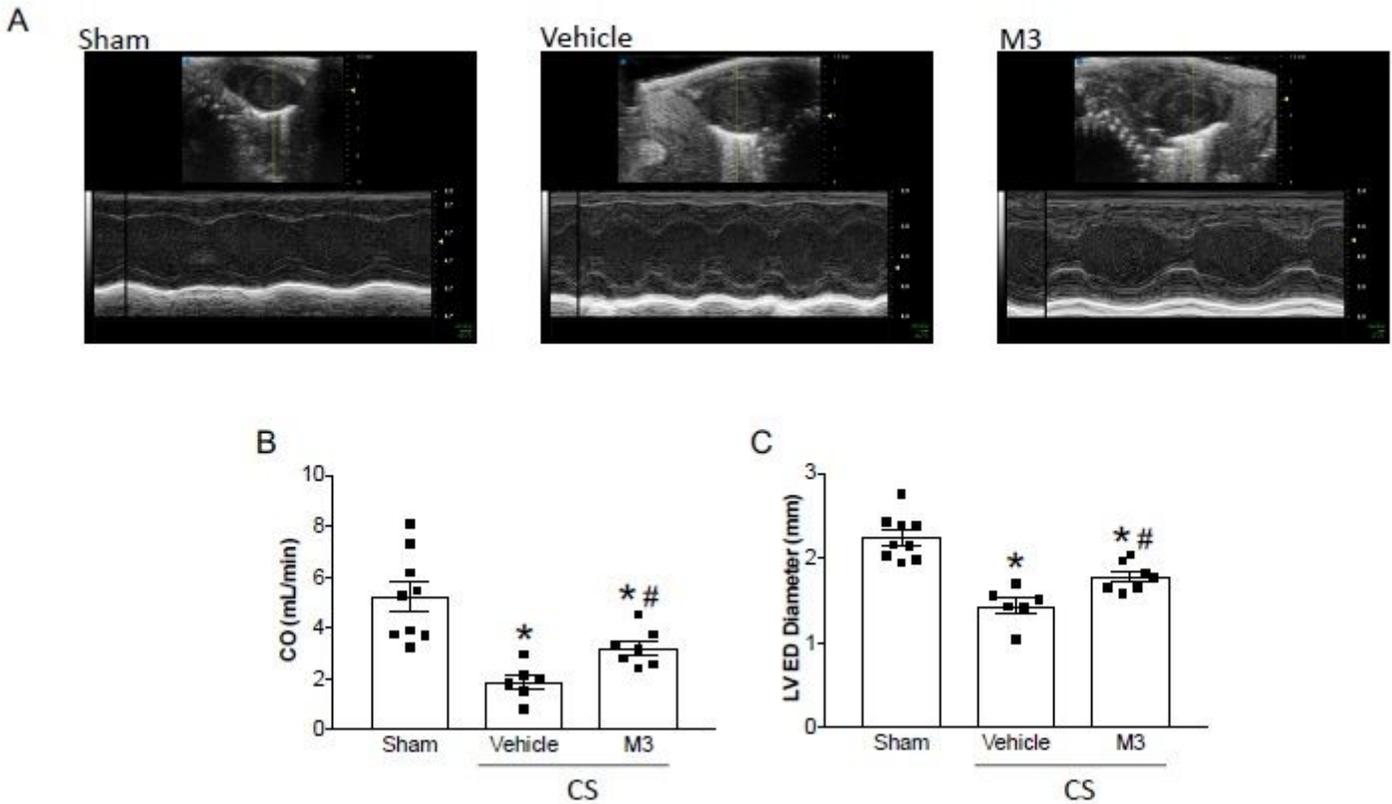
**Figure 2**

Treatment with M3 reduces proinflammatory cytokines in neonatal sepsis. 16 hours after CS injection, serum from sham, vehicle, or M3 treated pups was collected to measure serum cytokines (A) IL-6, (B) IL-1 $\beta$ , (C) TNF- $\alpha$ , and (D) IFN- $\gamma$  by ELISA. Data are expressed as means  $\pm$  SE. Multiple groups were compared by one-way ANOVA and Tukey method (\* $p < 0.05$  vs. sham; # $p < 0.05$  vs. Vehicle).



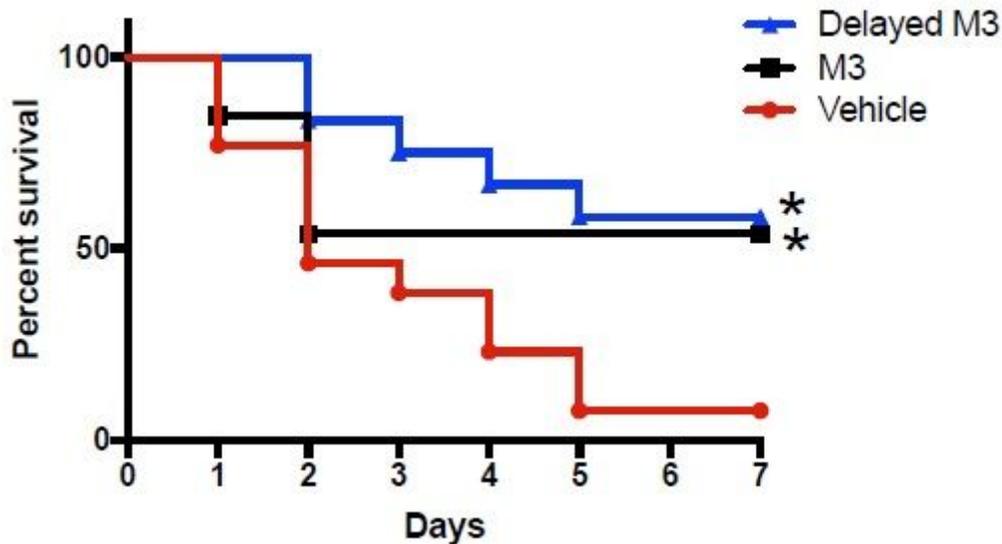
**Figure 3**

M3 improves pulmonary and cardiac inflammation. 16 hours after cecal slurry sepsis induction, mRNA and protein levels of proinflammatory cytokines were measured by RT-PCR and ELISA. Lung levels of IL-6 and IL-1 $\beta$  (A, B) mRNA and (C, D) protein were increased by sepsis and reduced in M3 treated pups. Analogously, cardiac IL-6 and IL-1 $\beta$  (E, F) mRNA and (G, H) protein were elevated in vehicle treated pups as opposed to M3 treated septic neonates. Data are expressed as means  $\pm$  SE. Multiple groups were compared by one-way ANOVA and Tukey method (\* $p < 0.05$  vs. sham; # $p < 0.05$  vs. Vehicle).



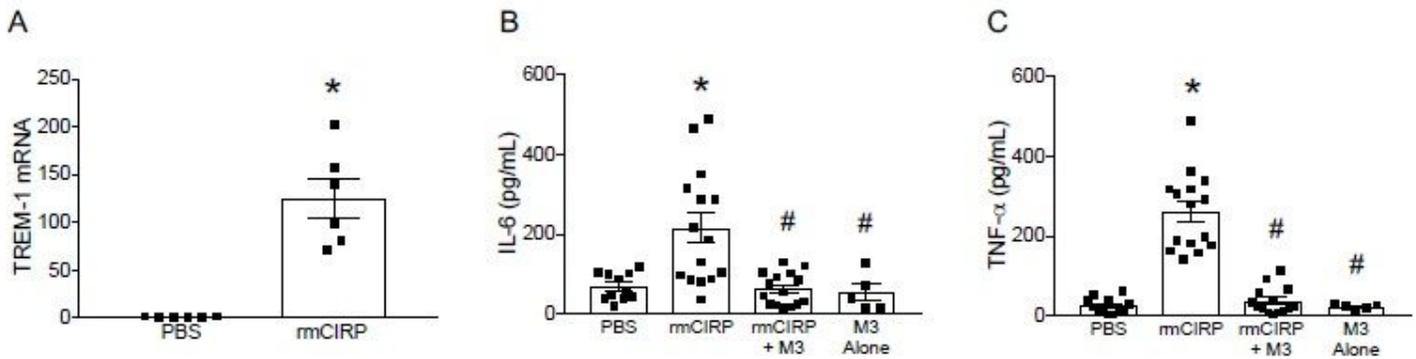
**Figure 4**

M3 improves pulmonary and cardiac inflammation. 16 hours after cecal slurry sepsis induction, mRNA and protein levels of proinflammatory cytokines were measured by RT-PCR and ELISA. Lung levels of IL-6 and IL-1 $\beta$  (A, B) mRNA and (C, D) protein were increased by sepsis and reduced in M3 treated pups. Analogously, cardiac IL-6 and IL-1 $\beta$  (E, F) mRNA and (G, H) protein were elevated in vehicle treated pups as opposed to M3 treated septic neonates. Data are expressed as means  $\pm$  SE. Multiple groups were compared by one-way ANOVA and Tukey method (\* $p$ <0.05 vs. sham; # $p$ <0.05 vs. Vehicle).



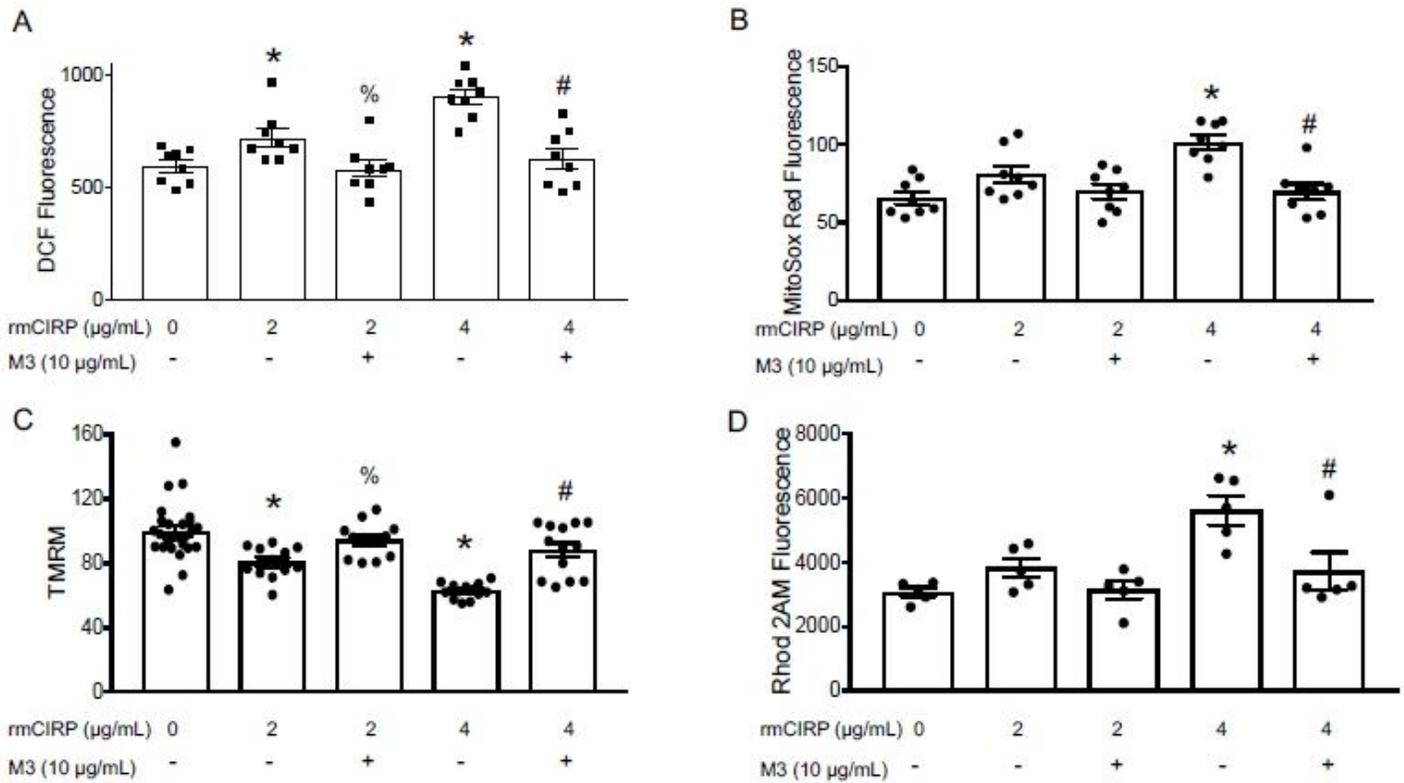
## Figure 5

M3 improves survival in neonatal murine sepsis. Mouse pups were injected with a reduced dose of cecal slurry (0.175 mg/g BW) and treated with either M3 or normal saline vehicle. The square black line represents M3 treatment at the time of cecal slurry injection. The triangle blue line represents M3 treatment 2 hours after sepsis injection. N = 13 mice per group, \* $p < 0.05$  vs. vehicle, determined by the log-rank test.



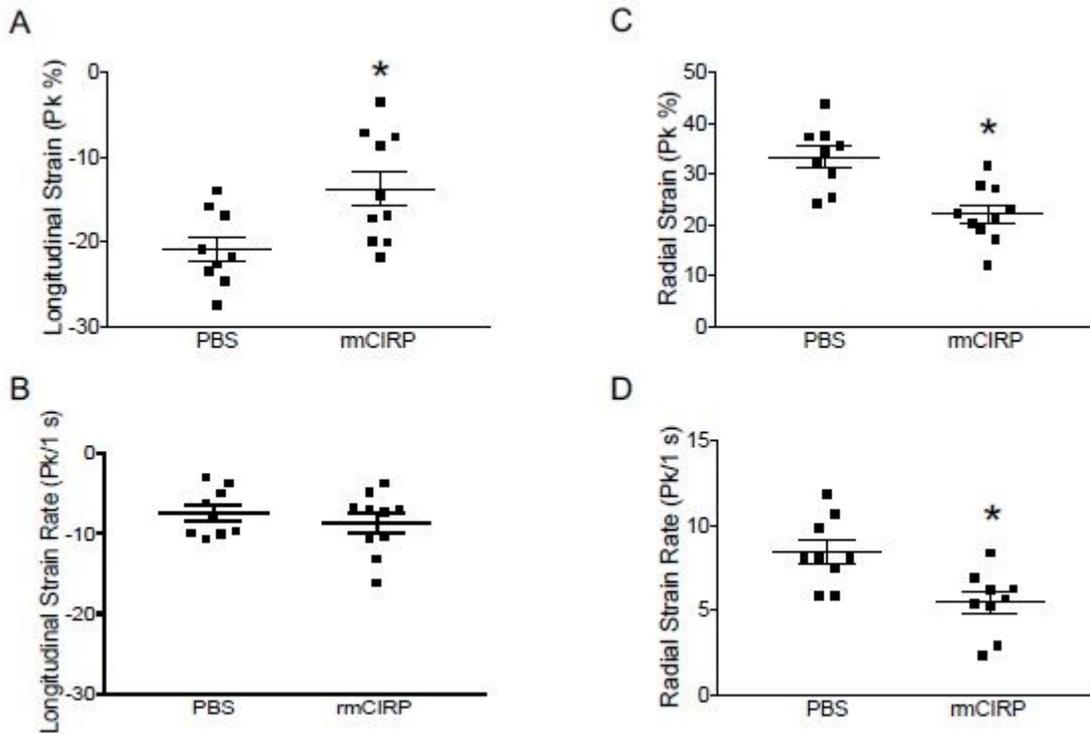
## Figure 6

The eCIRP/TREM-1 interaction increases inflammation in cardiomyocytes. Primary murine neonatal cardiomyocytes were isolated and treated with rmCIRP for 24 hours. (A) TREM-1 expression was assessed by RT-PCR. Cardiomyocytes were treated with rmCIRP for 24 hours with or without 20-minute pretreatment with 10  $\mu\text{g}/\text{mL}$  M3. rmCIRP stimulation increased levels of (B) IL-6 and (C) TNF- $\alpha$  in the supernatant. M3 mitigated this inflammatory response. The last column shows M3 treatment alone as a control. Data are result of 2 independent experiments and expressed as means  $\pm$  SE and compared by Kruskal-Wallis test with Dunn method (\* $p < 0.05$  vs. PBS control and # $p < 0.05$  vs. rmCIRP-treated cells).



**Figure 7**

The eCIRP/TREM-1 interaction increases inflammation in cardiomyocytes. Primary murine neonatal cardiomyocytes were isolated and treated with rmCIRP for 24 hours. (A) TREM-1 expression was assessed by RT-PCR. Cardiomyocytes were treated with rmCIRP for 24 hours with or without 20-minute pretreatment with 10 μg/mL M3. rmCIRP stimulation increased levels of (B) IL-6 and (C) TNF-α in the supernatant. M3 mitigated this inflammatory response. The last column shows M3 treatment alone as a control. Data are result of 2 independent experiments and expressed as means ± SE and compared by Kruskal-Wallis test with Dunn method (\*p<0.05 vs. PBS control and #p<0.05 vs. rmCIRP-treated cells).



**Figure 8**

rmCIRP impairs cardiac strain. In vivo rmCIRP exposures causes cardiac dysfunction as evidenced by changes in global longitudinal and radial strain and strain rate. 2 hours after rmCIRP injection, mouse pups underwent echocardiogram. B modes images were analyzed using VivoStrain software. rmCIRP injection resulted in impaired (A) longitudinal strain and (B) strain rate as well as impaired (C) radial strain and (D) strain rate. Data are expressed as means  $\pm$  SE and compared by two-tailed student's t test (\*  $p < 0.05$  vs PBS control).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [M3NeonatalSepsisJTranslationalVideo.mp4](#)