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Sirt3 Deletion Increases Inflammation and Mortality in Polymicrobial Sepsis

Hanna E. Labiner,[1] Kelli M. Sas,[1] Joseph A. Baur,[2] and Carrie A. Sims[1]

Abstract

Background: Sirtuin 3 (SIRT3) is a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase that confers resilience to cellular stress by promoting mitochondrial activity. Mitochondrial dysfunction is a major driver of inflammation during sepsis. We hypothesize that *Sirt3* expression improves survival in polymicrobial sepsis by mitigating the inflammatory response.
Materials and Methods: *Sirt3* knockout (S3KO) and wild-type (WT) mice underwent cecal ligation and puncture (CLP) or sham surgery. mRNA expression was quantified using quantitative polymerase chain reaction (qPCR) and protein expression was quantified using enzyme-linked immunosorbent assay (ELISA). Spectrophotometric assays were used to quantify serum markers of organ dysfunction. For in vitro studies, bone marrow-derived macrophages (BMDMs) were harvested from S3KO and WT mice and treated with lipopolysaccharide (LPS).
Results: After CLP, hepatic *Sirt3* levels decreased from baseline by nine hours and remained depressed at 24 hours. Peak serum interleukin-6 (IL-6) protein levels were higher in S3KO mice. In LPS-treated BMDMs, *IL-6* mRNA levels peaked earlier in S3KO cells, although peak levels were comparable to WT. Although S3KO mice had decreased median survival after CLP compared with WT, there was no difference in five-day survival or organ dysfunction.
Conclusions: Although S3KO mice initially had increased inflammation and mortality, this difference abated with time, and overall survival was comparable between the groups. This pattern is consistent with the timeline of sepsis-induced *Sirt3* downregulation in WT mice, and suggests that *Sirt3* downregulation occurring in sepsis is at least partially responsible for the initial hyperinflammatory response and subsequent mortality. Our data support upregulation of *Sirt3* as a promising therapeutic strategy for further research in sepsis.

Keywords: anti-inflammatory; dysregulated host response; mitochondrial activity; sirtuin

Despite international campaigns to improve the early identification and supportive care of sepsis, there are approximately 11 million sepsis-related deaths worldwide every year.[1] The morbidity and mortality associated with sepsis is not, however, due solely to the underlying infectious agent. Instead, it is a product of the dysregulated host response that causes inflammation, oxidative stress, mitochondrial dysfunction, and cellular apoptosis.[2–6] Therefore, to improve outcomes in septic shock, it is imperative that we discover new strategies to modulate the inflammatory response.

One group of proteins that may mitigate the dysfunctional host response to sepsis are sirtuins. Sirtuins are a highly conserved group of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases that confer resilience to cellular stress.[7–9] Sirtuin 1 has been studied extensively in

[1]Division of Trauma, Critical Care, and Burn at The Ohio State University Wexner Medical Center, The Ohio State University, Columbus, Ohio, USA.
[2]Institute for Diabetes, Obesity and Metabolism and Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA.
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sepsis, but the importance of sirtuin 3 (*Sirt3*) has only recently begun to be elucidated.[10–12] *Sirt3* is a mitochondrial sirtuin the expression of which in sepsis is known to improve mitochondrial function[13,14] and reduce tissue oxidative stress.[15,16] Enhancing *Sirt3* can therefore prevent inflammation[17,18] and cellular apoptosis.[19] Indeed, increased *Sirt3* expression may decrease sepsis-induced organ dysfunction in multiple organs including kidney,[19,20] heart,[21,22] and brain.[14]

Although many benefits have been postulated for *Sirt3* expression in a polymicrobial sepsis model, the overall effects of *Sirt3* on morbidity and mortality in vivo remain unknown. In this study, we demonstrate that *Sirt3* expression is necessary to mitigate the inflammatory response to sepsis and improve early survival. Furthermore, we provide evidence that the anti-inflammatory effects of *Sirt3* may be mediated via its expression in macrophages.

Materials and Methods

Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania and The Ohio State University. *Sirt3* knockout (S3KO) mice were originally generated by Lombard et al.[23] and were backcrossed on a C57BL/6J background for 10 generations prior to use. Studies were performed on S3KO mice and their wild-type (WT) littermates.

We performed cecal ligation and puncture (CLP) on eight to 12-week-old mice as described by Rittirsch et al.[24] Briefly, we anesthetized the animals using 2% isoflurane prior to making a midline abdominal incision. We ligated the cecum with 4-0 silk suture just below the ileocecal valve and punctured the cecum twice with a 23-gauge needle. The abdominal incision was closed in two layers with 4-0 vicryl suture. Control animals underwent sham surgery, which consisted of midline abdominal incision under anesthesia with manipulation of the cecum without ligation or puncture, followed by closure of the abdominal incision with 4-0 vicryl suture.

After surgery, all animals received 0.1 mg/kg subcutaneous buprenorphine, followed by a lower subcutaneous dose (0.05 mg/kg) every 12 hours for the first 48 hours postoperatively. Subcutaneous normal saline (75 mcL/g) was administered every 12 hours starting immediately after CLP. Antibiotic agents (ceftriaxone, 25 mg/kg; metronidazole, 12.5 mg/kg) were administered 12 hours after CLP and every 12 hours subsequently for the duration of the experiment. After surgery, animals were monitored at 12 hours and every six hours thereafter. Glucose was measured via AlphaTRAK rodent glucometer (Abbott Laboratories, Abbott Park, IL). Animals were weighed daily. A cohort of animals was euthanized at 36 hours or five days, and serum was collected for quantification of interleukin-6 (IL-6) and markers of organ dysfunction. Kidney and liver tissue were also harvested at these time points and either snap-frozen or used immediately for mitochondrial function testing. Five-day survival studies were carried out on a separate cohort of mice. To minimize animal discomfort, mice were euthanized when they reached predetermined end points of euthanasia (Supplementary Table S1). After five days all surviving animals were euthanized.

Bone marrow-derived macrophages

We harvested bone marrow from eight to 12-week-old S3KO and WT mice. After euthanasia, the bilateral lower extremities were prepped and sterilized with alcohol. Both femurs and tibias were harvested using sterile technique. Marrow was extracted with RPMI +1% P/S, filtered through a 100 mcm filter, and centrifuged at 300*g* for 10 minutes at 4°C. The cells were resuspended in growth media (400 mL RPMI, 1% P/S, 20% fetal bovine serum [FBS], 10 mcg macrophage colony-stimulating factor [MCSF]), plated, and incubated at 37°C for seven days. Media was changed on day 4. Cells were then washed with phosphate buffered saline (PBS), incubated for five minutes with 1 mL 0.25% trypsin, and resuspended in growth media without MCSF. Cells were centrifuged at 300*g* for 10 minutes at 4°C and then resuspended in growth media and counted. In a six-well plate, $0.5 \times 10\text{^}6$ cells in 1 mL growth media were added per well and allowed to incubate overnight at 37°C. Lipopolysaccharide (LPS) was added for a concentration of 1 mcg/mL and allowed to incubate for either four or eight hours. Cells were then collected for RNA extraction.

mRNA expression

Sirt3, *IL6*, and *SOD2* mRNA levels were measured using quantitative polymerase chain reaction (qPCR). Liver and kidney RNA was extracted from frozen tissue and bone marrow-derived macrophages (BMDMs). RNA was extracted from fresh cells with ethanol precipitation. One microgram of RNA was used to create cDNA using a High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Waltham, MA) according to manufacturer's recommendations. Quantitative PCR was performed with SYBR green using an Applied Biosystems 7900HT system. Cycle threshold (CT) was normalized to β-actin to calculate ΔCT, and individual sample ΔCTs were compared with the average sham ΔCT at each timepoint (for in vivo) or untreated ΔCT (for in vitro) to calculate ΔΔCT. Relative mRNA levels were expressed as fold change, calculated as $2^{-\Delta\Delta CT}$ for each sample. Primers used: β-*actin* (F: GGCTGTATTCCCCTCCATCG, R: CCAGTTGGTAACAA TGCCATGT), *IL6* (F: TAGTCCTTCCTACCCCAATTTCC, R: TTGGTCCTTAGCCACTCCTTC), *SOD2* (F: GCCTGC ACTGAAGTTCAATG, R: ATCTGTAAGCGACCTTGCTC), *Sirt3* (F: GCTGCTTCTGCGGCTCTATAC, R: GAAGGA CCTTCGACAGACCGT).

Protein expression

Serum blood urea nitrogen (BUN) was quantified using point of care veterinary cartridges (i-STAT, Abbott Point of Care, Inc., Princeton, NJ). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified spectrophotometrically using commercially available kits (Pointe Scientific kit A7561150 and A7526150, Canton, MI). Serum IL-6 was quantified using enzyme-linked immunosorbent assay (ELISA; Invitrogen BMS603HS, Thermo Fisher Scientific, Waltham, MA). Each sample was run in duplicate with known standards and according to the manufacturer's guidelines.

Mitochondrial content

Citrate synthase was used to quantify mitochondrial content. Kidney or liver tissue (100 mcg) was suspended in assay

buffer (0.1 mM 5,5-dithio-bis-(2-nitrobenzoic acid) [DTNB] in 1 M Tris buffer (pH 8.0), 0.3 mM acetyl coenzyme A, and 0.05% Triton X-100), and 1 mM oxaloacetate was added. Citrate synthase activity was then determined spectrophotometrically at 37°C with absorbance at 412 nm.[25]

Mitochondrial isolation

Liver and kidney tissue was harvested at 36 hours and five days post-surgery for mitochondrial isolation. Immediately after the mice were euthanized, 4–6 g of liver and kidney tissue were immersed and homogenized separately in ice-cold mitochondrial isolation buffer (MIB; 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.5% fatty acid-free bovine serum albumin [BSA], potassium hydroxide [KOH] to pH 7.2). Differential centrifugation was performed as previously described.[26] The mitochondrial pellet was then resuspended in MIB.

High-resolution respirometry

Freshly isolated mitochondria (0.15 mg) were suspended in respiration medium B (RM-B; 110 mM mannitol, 0.5 mM ethyleneglycoltetraacetic acid [EGTA], 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 60 mM K lactobionate, 0.3 mM dithiothreitol [DTT], 1% fatty acid-free bovine serum albumin [BSA], adjusted to pH 7.1 with KOH). Oxygen consumption was measured using high resolution respirometry at 37°C (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria). After stabilization, real-time oxygen concentration and flux data were continuously collected (DatLab software 4.3, Oroboros Instruments, Innsbruck, Austria). A standard substrate/inhibitor titration (SUIT) protocol was used to analyze respiratory function as a factor of rate of oxygen consumption. Basal respiration was recorded. For rates of fatty acid oxidation (FAO), 0.04 mM palmitoyl carnitine and 0.1 mM malate were added, followed by 1 mM adenosine diphosphate (ADP). For activity of individual complexes, complex I (CI)-dependent respiration was induced by adding 10 mM glutamate, 5 mM malate, and 1 mM ADP. Complex II (CII)-dependent respiration was then determined by adding 0.5 mcM rotenone (a CI inhibitor) and CII substrate succinate (10 mM). Complex III (CIII) inhibitor antimycin A (5 mcM) was then added and complex IV (CIV) substrates tetramethyl-para-phenylenediamine (TMPD; 0.5 mM) and ascorbate (2 mM) were added to measure CIV-dependent respiration.

Statistical analysis

Results are expressed as mean ± standard error. Comparisons between groups were performed using Student t-test, Mann-Whitney test, analysis of variance (ANOVA), or Kruskal-Wallis test. Mean survival was analyzed using a Kaplan-Meier curve with a Gehan-Breslow-Wilcoxon test, and 48 hour and five-day survival were analyzed using Fisher exact test. Statistical significance was designated as $p \leq 0.05$. All statistical analysis was performed using Prism 7 (GraphPad Software, Inc).

Results

Sepsis induces a functional knockdown of Sirt3 in WT mice

We first evaluated the timeline of *Sirt3* expression changes during sepsis in WT mice. Compared with sham mice, hepatic *Sirt3* gene expression was downregulated by nine hours in mice subjected to CLP (77% decrease, $p < 0.01$), and remained decreased at 24 hours (75% decrease, $p < 0.01$; Fig. 1A). In contrast, there was no significant difference in *Sirt3* expression in the kidney at three, nine, or 24 hours after CLP induction (Fig. 1B). Levels of *Sirt3* mRNA were consistently downregulated in S3KO mouse tissue (data not shown).

Deleting Sirt3 is proinflammatory in polymicrobial sepsis

Serum IL-6 protein levels increased after CLP and peaked around six hours. Peak serum IL-6 levels were higher in S3KO compared with WT mice (169.5 ± 30.1 ng/mL vs. 84.0 ± 16.6 ng/mL; $p = 0.03$) and remained elevated in S3KO mice at 12 hours (101.7 ± 21.8 ng/mL vs. 54.9 ± 5.5 ng/mL; $p = 0.05$). By 36 hours, there was no difference between S3KO and WT mice as serum IL-6 levels returned to baseline (Fig. 1C).

Sirt3 may increase hepatic mitochondrial activity in polymicrobial sepsis

We next aimed to determine whether the increased inflammation in septic S3KO mice could be related to changes in mitochondrial function. Sepsis caused a general decrease in hepatic and renal mitochondrial function in WT mice at 36 hours and five days after CLP. In the S3KO mice, the respiratory activity of complexes I and IV in liver tissue were increased at 36 hours post-CLP compared with WT (180.6 ± 12.5 vs. 140.2 ± 8.6 nmol O_2/mg/min; $p = 0.02$ and 1741.1 ± 60.1 vs. 1,496.5 ± 96.4 nmol O_2/mg/min; $p = 0.05$, respectively; Fig. 2A). By day 5, however, this difference had dissipated, and hepatic mitochondrial complex function was comparable between the septic WT and S3KO groups (Fig. 2B). There was no difference in renal mitochondrial complex activity between the S3KO and WT mice at 36 hours or five days (Fig. 2C and 2D). *Sirt3* deletion had no effect on renal or hepatic fatty acid oxidation (Fig. 2A–2D) or mitochondrial content (Fig. 2E and 2F) at 36 hours or five days post-CLP.

Sirt3 deletion does not affect morbidity, but does decrease median survival

After CLP, 48-hour survival in S3KO mice was lower than WT (55.0% vs. 94.1%; $p < 0.01$). Five-day survival trended lower in S3KO mice (50.0% vs. 64.7%; $p = 0.0952$), however, this did not reach statistical significance. Median survival in S3KOs was 84 hours, whereas median survival for WT mice was not reached (Fig. 3A). Body temperature and glucose both decreased after CLP and nadired at 18 hours, however, there was no difference in either between the WT and S3KO mice (Fig. 3B and 3C). The S3KO mice weighed less at baseline compared with WT (25.6 ± 0.5 g vs. 27.0 ± 0.4 g; $p = 0.04$), but by day 5 post-CLP, both S3KO and WT mice

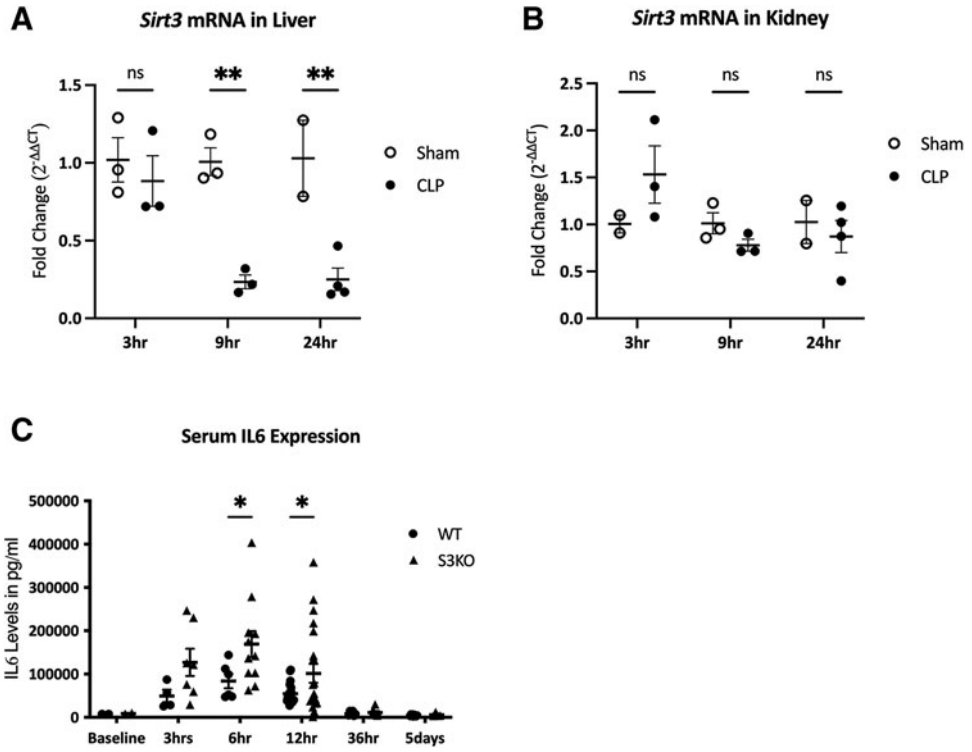

FIG. 1. *Sirt3* and IL-6 expression over time in wild-type (WT) mice after cecal ligation and puncture (CLP). Fold change is expressed as change from average sham normalized cycle threshold (ΔCT) values at each time point. **A:** Changes in liver *Sirt3* mRNA over time in WT sham versus CLP mice. **B:** Changes in kidney *Sirt3* mRNA over time in WT sham versus CLP mice. **C:** Serum interleukin-6 (IL-6) protein levels in pg/mL in WT mice and *Sirt3* knockout (S3KO) mice before CLP and three hours, six hours, 12 hours, 36 hours, and five days after CLP. *$p < 0.05$; **$p < 0.01$.

had similar weights (23.1 ± 0.6 g vs. 23.1 ± 0.9 g; $p = 0.98$; Fig. 3D). *Sirt3* deletion also did not affect serum markers of kidney or liver dysfunction at 36 hours or five days after CLP (Fig. 4A–4D).

Sirt3 deletion promotes inflammation in LPS-stimulated BMDMs

Bone marrow-derived macrophages are known to play a vital role in the immune response to sepsis, and our previous work suggests that the inflammatory response to sepsis may be due in large part to expression of sirtuin proteins within myeloid cells.[27] We therefore questioned whether *Sirt3* deletion within BMDMs was sufficient to promote the BMDM hyperinflammatory response. Compared with baseline levels in untreated BMDMs, *Sirt3* mRNA expression in LPS-treated BMDMs decreased by 66% in the first four hours ($p = 0.019$) but returned to baseline by eight hours (Fig. 5A). Although there was no difference between WT and S3KO cells in terms of the maximum *IL6* mRNA expression observed, the *IL6* expression peaked earlier in S3KO cells compared with WT (4 vs. 8 hours; Fig. 5B). LPS-treated S3KO BMDMs had an increase in *SOD2* mRNA compared with WT at four hours (12.4-fold increase vs. 5.8-fold increase; $p < 0.01$). *SOD2* expression in both WT and S3KO continued to increase at 8 hours but was not different between the groups (Fig. 5C).

Discussion

Sepsis remains a highly morbid and fatal disease process, in part because of the dysregulated host response that induces inflammation, oxidative damage, and mitochondrial dysfunction.[2–6] Although the use of sepsis protocols and antibiotic agents have improved outcomes, further advancements will require finding ways to modulate this dysregulated host response.[1] *Sirt3* is generally thought to promote mitochondrial function and inhibit oxidative stress[13–16]; therefore, we evaluated its role on inflammation and survival in polymicrobial sepsis.

Although it has been shown previously that *Sirt3* deletion upregulates proinflammatory cytokines in myeloid and nonmyeloid cells during sepsis,[18,19] we are the first to demonstrate a temporal link, with *IL6* expression peaking earlier in S3KO myeloid cells compared with WT cells. This is consistent with our finding that WT cells become functional SIRT3 knockdown cells a few hours after a septic insult. Together these data support that a natural downregulation of SIRT3 contributes to the lethal hyperinflammatory response known to occur early during sepsis.[28]

Oxidative stress is another marker of cellular stress and is closely linked with inflammation.[15,18] Superoxide has been shown to activate the inflammatory mediator nuclear factor kappa B (NFkB), which stimulates production of *SOD2* as a compensatory mechanism.[18,29–32] We found that LPS treatment was associated with increased expression of the antioxidant *SOD2* in BMDMs. Similar findings in a murine model of sepsis by Bloise et al.[33] confirmed that this upregulation of *SOD2* correlated with an increase in superoxide production. Therefore, our results likely represent an overall increase in cellular oxidative stress with LPS treatment. The earlier increase in *SOD2* expression in S3KO cells compared

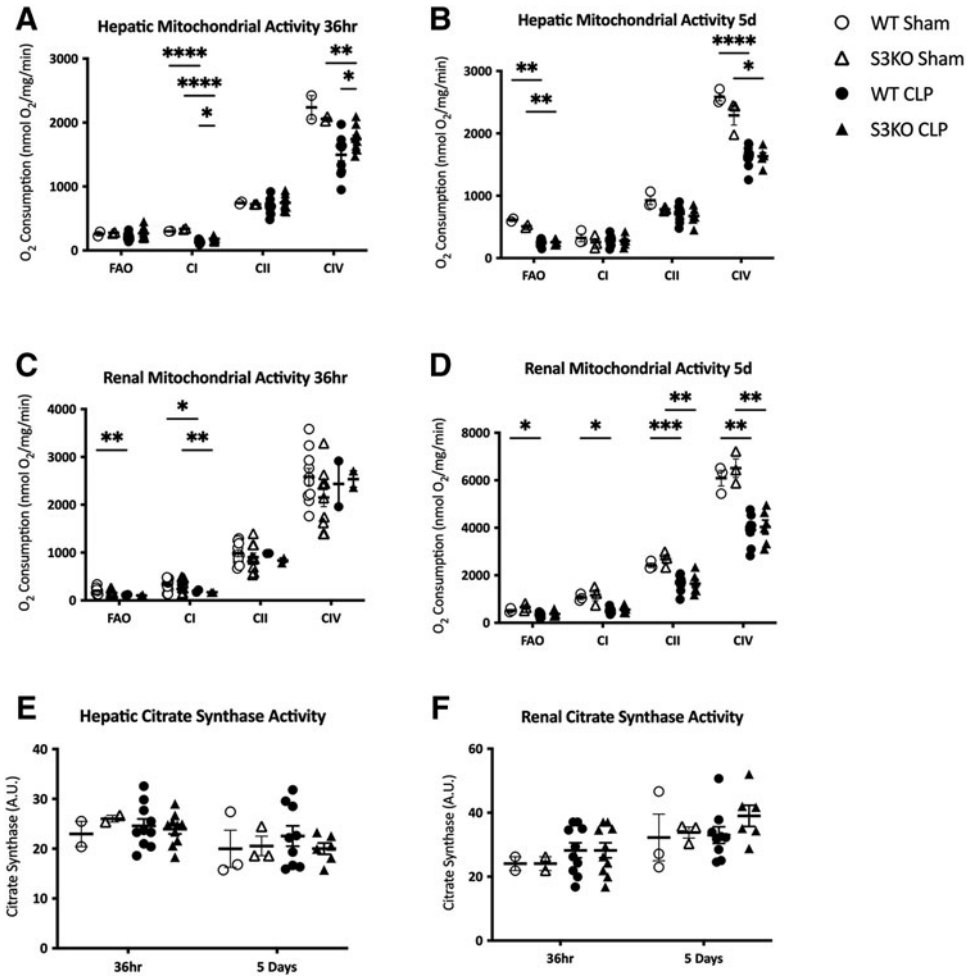

FIG. 2. Hepatic and renal mitochondrial content and function after cecal ligation and puncture (CLP) or sham surgery in wild-type (WT) and *Sirt3* knockout (S3KO) mice. Respiratory function is determined by rate of oxygen consumption (nmol O_2/mg/min). Rate of oxygen consumption was measured for fatty acid oxidation (FAO) and mitochondrial CI (complex I), CII (complex II), and CIV (complex IV). **A:** Oxygen consumption in hepatic tissue at 36 hours after CLP. **B:** Oxygen consumption in hepatic tissue at five days after CLP. **C:** Oxygen consumption in renal tissue at 36 hours after CLP. **D:** Oxygen consumption in renal tissue at five days after CLP. Mitochondrial content is measured by citrate synthase activity at 36 hours and five days in liver (**E**) and kidney (**F**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

with WT that we identified is also consistent with our inflammatory cytokine findings and supports the theory that *Sirt3* deletion contributes to early oxidative stress. Future studies will need to further examine the role of SIRT3 on early oxidative stress by studying lipid peroxidation in the tissues of septic mice with downregulated SIRT3 expression as well as those overexpressing SIRT3.

The S3KO mice had a shorter median survival after CLP compared with WT mice. The increase in early mortality, but lack of difference in five-day mortality, is consistent with our finding that WT animals become functional *Sirt3* knockdown animals several hours after septic insult. We expect that because S3KO animals have an earlier increase in hyperinflammation and oxidative stress, they have increased mortality early on. This early survival advantage among the WT mice appears to be lost between days 3 and 5, however, consistent with the delayed *Sirt3* downregulation in the WT mice and subsequent increases in inflammation and oxidative stress.

There is currently conflicting evidence regarding the effects of sepsis on mitochondrial activity, with some studies showing increased respiration in response to infection.[34,35] This is believed to be due in part to increased mitochondrial uncoupling in response to cellular stress, which promotes reactive oxygen species (ROS) production.[36,37] Whereas *Sirt3* deletion is generally thought to decrease mitochondrial respiration,[18] we found increased hepatic mitochondrial oxygen consumption in S3KO mice. Given our findings of increased *SOD2* in S3KO mice, this increased oxygen consumption most likely reflects an increase in mitochondrial uncoupling and subsequent complex-dependent ROS production, as opposed to improved adenosine triphosphatase (ATP) production.[36,37]

We saw no other significant *Sirt3*-dependent effects on mitochondrial activity or serum markers of organ function in sepsis at 36 hours or five days. Of note, because serum markers of organ dysfunction tend to be a delayed sign of tissue damage, this lack of difference in serum marker of organ dysfunction is likely due to a survival bias.

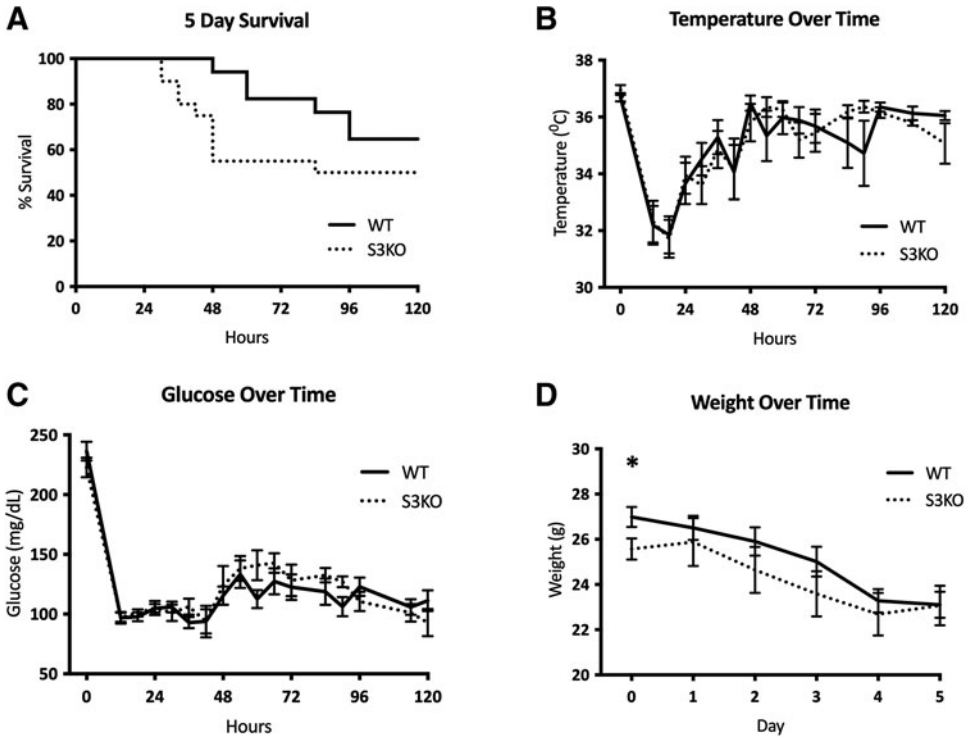

FIG. 3. Physiologic changes over time in wild-type (WT) versus *Sirt3* knockout (S3KO) mice after cecal ligation and puncture (CLP). (**A**) Five-day survival, p value calculated using Gehan-Breslow-Wilcoxon test. (**B**) Temperature (C°) over time in the five days after CLP. (**C**) Glucose (mg/dL) over time in the five days after CLP. **D:** Daily weight (g) after CLP. *$p < 0.05$.

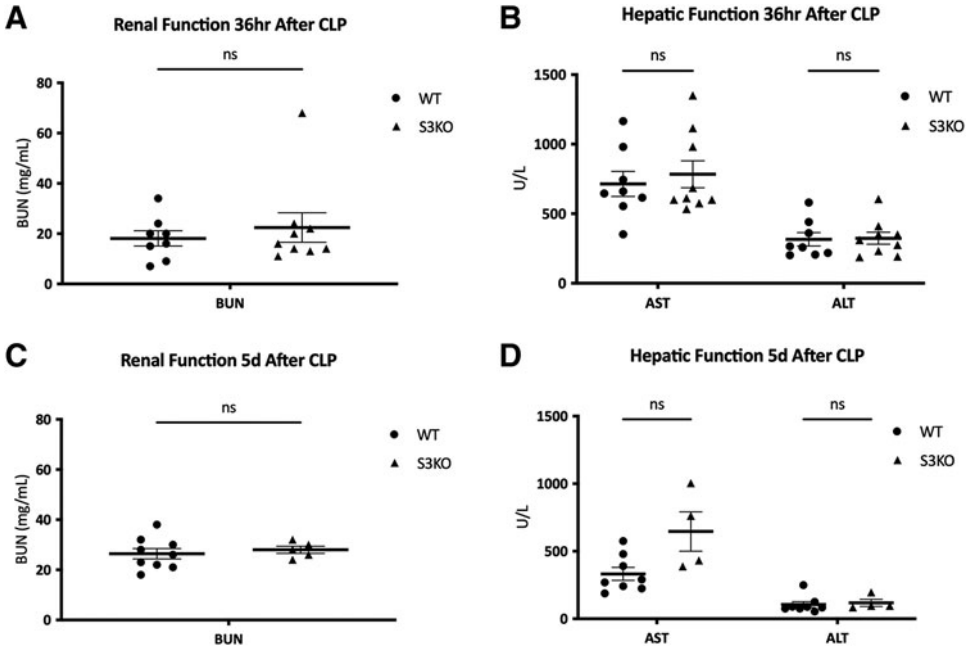

FIG. 4. Markers of organ dysfunction in wild-type (WT) versus *Sirt3* knockout (S3KO) mice after cecal ligation and puncture (CLP). **A:** Serum blood urea nitrogen (BUN; mg/mL) in WT versus S3KO mice at 36 hours after CLP. **B:** Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT; U/L) in WT versus S3KO mice at 36 hours after CLP. **C:** Serum BUN (mg/mL) in WT versus S3KO mice at five days after CLP. **D:** Serum AST and ALT (U/L) in WT versus S3KO mice at five days after CLP.

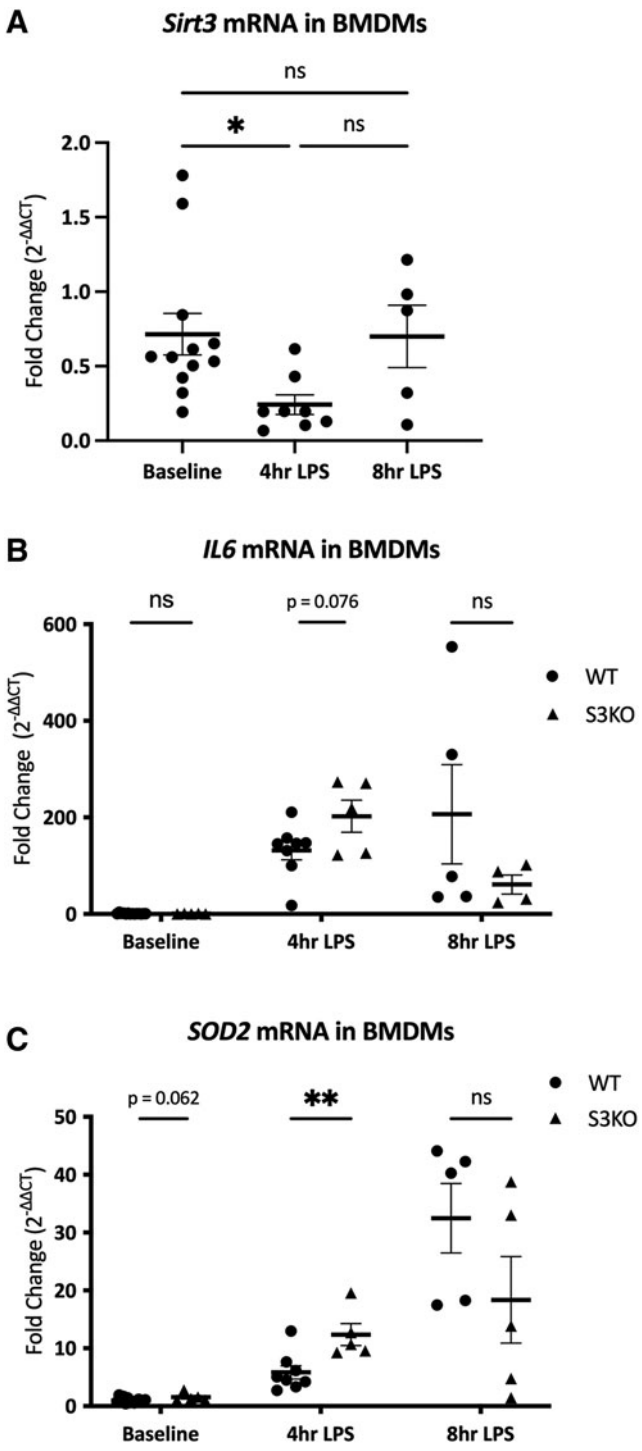

FIG. 5. mRNA Expression over time in lipopolysaccharide (LPS)-treated bone marrow-derived macrophages (BMDMs): fold change is expressed as change from average untreated wild-type (WT) normalized cycle threshold (ΔCT) values at each time point. **A:** Changes in *Sirt3* mRNA over time in untreated versus LPS-treated WT BMDMs. **B:** Changes in *IL6* mRNA in untreated versus LPS-treated WT and S3KO BMDMs over time. **C:** Changes in *SOD2* mRNA in untreated vs. LPS-treated WT and S3KO BMDMs over time. *$p < 0.05$; **$p < 0.01$.

Although 36 hours was chosen as a timepoint to capture the delayed presentation of elevated AST, ALT, and BUN, we found that 25% of the S3KO mice, but none of the WT mice, had died by the 36-hour timepoint. It may therefore be beneficial for future studies on SIRT3 in early sepsis to focus on tissue oxidative stress as a surrogate for morbidity.

Our study had several limitations. We primarily measured the expression of inflammatory cytokines and *Sirt3* using mRNA. Although our mRNA findings were supported by our studies of serum IL-6 protein levels, further experiments are needed to confirm that the changes in mRNA do, in fact, translate to changes in tissue protein expression. Additionally, because sepsis appears to create a functional knockdown of *Sirt3* in WT mice, the results of our study may underestimate the true effects of *Sirt3* during sepsis. Further studies comparing septic responses of S3KO and WT mice to mice overexpressing *Sirt3* will be necessary to better elucidate the role of *Sirt3* in polymicrobial sepsis.

Conclusions

In this study, we demonstrated that *Sirt3* is naturally downregulated in sepsis, and that this downregulation promotes a hyperinflammatory response, contributing to increased mortality. Furthermore, our in vitro work established that *Sirt3* expression in BMDMs influences their inflammatory potential, and therefore BMDMs may be particularly important in modulating the *Sirt3*-dependent response to sepsis. *Sirt3* is a promising therapeutic target that could help ameliorate the harmful inflammatory response in early sepsis and improve survival.

Authors' Contributions

Investigation: Labiner, Sims. *Formal analysis:* Labiner, Sas, Sims. Writing: Labiner, Sas, Sims. *Editing:* Labiner, Sas, Sims. *Conceptualization:* Baur, Sims. *Methodology:* Baur, Sims.

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Author Disclosure Statement

The authors have no conflicts of interest to disclose.

Supplementary Material

Supplementary Table S1

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Address correspondence to:
Dr. Carrie A. Sims
Division of Trauma, Critical Care, and Burn
The Ohio State University Wexner Medical Center
Faculty Office Tower
395 12th Avenue, Room 654
Columbus, OH 43210
USA

E-mail: Carrie.Sims@osumc.edu