RESEARCH ARTICLE

Sirtuin-2 Regulates Sepsis Inflammation in ob/ob Mice

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Abstract

Objective

Obesity increases morbidity and resource utilization in sepsis patients. Sepsis transitions from early/hyper-inflammatory to late/hypo-inflammatory phase. Majority of sepsis-mortality occurs during the late sepsis; no therapies exist to treat late sepsis. In lean mice, we have shown that sirtuins (SIRTs) modulate this transition. Here, we investigated the role of sirtuins, especially the adipose-tissue abundant SIRT-2 on transition from early to late sepsis in obese with sepsis.

Methods

Sepsis was induced using cecal ligation and puncture (CLP) in ob/ob mice. We measured microvascular inflammation in response to lipopolysaccharide/normal saline re-stimulation as a “second-hit” (marker of immune function) at different time points to track phases of sepsis in ob/ob mice. We determined SIRT-2 expression during different phases of sepsis. We studied the effect of SIRT-2 inhibition during the hypo-inflammatory phase on immune function and 7-day survival. We used a RAW264.7 (RAW) cell model of sepsis for mechanistic studies. We confirmed key findings in diet induced obese (DIO) mice with sepsis.

Results

We observed that the ob/ob-septic mice showed an enhanced early inflammation and a persistent and prolonged hypo-inflammatory phase when compared to WT mice. Unlike WT mice that showed increased SIRT1 expression, we found that SIRT2 levels were increased in ob/ob mice during hypo-inflammation. SIRT-2 inhibition in ob/ob mice during the hypo-inflammatory phase of sepsis reversed the repressed microvascular inflammation in vivo via activation of endothelial cells and circulating leukocytes and significantly improved survival. We confirmed the key finding of the role of SIRT2 during hypo-inflammatory phase of sepsis in this project in DIO-sepsis mice. Mechanistically, in the sepsis cell model, SIRT-2 expression modulated inflammatory response by deacetylation of NFκBp65.
Conclusion

SIRT-2 regulates microvascular inflammation in obese mice with sepsis and may provide a novel treatment target for obesity with sepsis.

Introduction

Sepsis and septic shock are the leading causes of death in non-coronary intensive care units. In the United States alone, they kill more than 200,000 patients each year, with an annual cost of treatment $16 billion[1]. The acute systemic inflammatory response of sepsis quickly transitions from an early/hyper-inflammatory phase to a late/hypo-inflammatory phase with persistent multi-organ dysfunction [2,3]. Evidence suggests that the hypo-inflammatory phase of sepsis, the host is unable to clear the pre-existing infection or superadded infections effectively and succumb to sepsis[4]. Immune-repressor hypo-inflammatory sepsis phenotype switches from glycolysis to fatty acid metabolism as a primary source for ATP production [5]. While some patients die early during sepsis, most sepsis-related deaths occur during the late/hypo-inflammatory phase of sepsis [6]. Over 30 different treatment modalities, all targeting early sepsis have failed to improve survival [7]. Evidence supports a new approach of treating sepsis by enhancing the repressed immune response[4].

The repressive stage of sepsis is characterized by the phenomenon of “endotoxin tolerance”, first described by Beeson as a decreased response to subsequent endotoxin stimulation after the first typhoid vaccine in rabbits [8]. Repressed immunity/endotoxin tolerance occurs after sepsis initiation, thereby providing a biomarker of immune disruption; endotoxin tolerance can be used as a test for “immune function” in mouse. Using endotoxin (E. coli lipopolysaccharide: LPS) as a “second hit” to study the response to further inflammatory stimuli, we can test whether or not a cell/organism immunity is intact (response to second-hit preserved) vs. repressed (response to second-hit impaired). We have shown that in lean WT mice with sepsis, the initial hyper-inflammatory (endotoxin responsive) phase transitions to hypo-inflammatory/repressed immunity (endotoxin tolerant) phase[2].

Obesity, a disease of rising prevalence, affects more than a third of the adult USA population [9]. Healthcare-related expenditure in obese individuals is projected increase by $549 billion by the year 2030[10] if the obesity trend continues. Although the association between sepsis-mortality and obesity is debated, literature, unequivocally, shows increased morbidity and resource utilization in obese with critical illness [11,12].

Mounting evidence supports a role for the sirtuin family of highly conserved NAD+ dependent deacetylases in directing the course of sepsis [5,13,14], but whether and how this concept applies to obesity is unknown. First identified in yeast, the seven-member mammalian sirtuins (SIRT-1-7) are now recognized as regulators of metabolic homeostasis and guardians of metabolism, immunity and bioenergetics[13] [15]. We discovered that in cell and lean mouse models of sepsis that nuclear SIRT-1 couples with nuclear NFκB p65/RelB, nuclear SIRT-6 and mitochondrial SIRT-3 as homeostasis checkpoints during acute sepsis-inflammation [5,14]. Sepsis shifts metabolism between the early activated and subsequent repressive stage by switching glycolysis as an energy source to mitochondrial oxidation of fatty acids as primary energy. How sirtuins affect obesity with inflammation is not clear, but obesity alone can decrease sirtuin expression [16].

Microvasculature, with its highly strategic interface between systemic circulation and local tissue environment, plays a critical role in modulating the course of sepsis inflammation and organ function [2]. Interactions between circulating immune cells and endothelial cells in the
microcirculation are rate-determining factors for inflammation [17]. This critical area of inflammation/immune regulation is often missing from the studies of inflammatory and immune diseases, but when performed in vivo offers an insight not available from ex vivo or in vitro cell or tissue research. We have adapted this tool to study microvascular inflammation of sepsis temporally and to assess immune function by studying leukocyte adhesion in vivo, in response to endotoxin as a second-hit at different time points post-sepsis.

In this project, we determined in ob/ob mice: 1) the effect of obesity on the repressed inflammation phenotype (hypo-inflammation) and its duration; 2) whether sirtuins contribute to the nature and duration of sepsis reprogramming of inflammation and immunity; 3) if modifying sirtuin function alters inflammation and immune sepsis reprogramming; and 4) the effect of blockage of sirtuins on duration of these phases and sepsis-survival. Unlike WT mice, we show that in ob/ob mice with sepsis, the initial phase of hyper-inflammation is exaggerated and shortened and the repressed/hypo-inflammatory response is prolonged. In a distinct contrast our reports in lean septic mice [2], we implicate SIRT-2 deacetylase and not SIRT-1 as a regulator of microvascular function and survival in ob/ob mice. In our sepsis cell model, we show that SIRT-2 deacetylates master inflammatory and immune transcription factor NFkB p65, which is known to repress transcription of inflammation and immune activator genes, like TNF-α, IL-6 etc. [18]. Lastly, we examined the key findings in our manuscript in mice with diet induced obesity (DIO) with sepsis.

Methods

Animals: Study was approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine and experiments performed according to the NIH guidelines. The wild type (WT; C57Bl/6; 6–8 week old) and ob/ob (B6.Cg-Lepob/J; 6–8 week old) and diet induced obesity (DIO; 13–15 week old) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Diet induced obesity was induced in C57Bl/6 mice who received 60% fat diet (Research Diets Inc. cat no: D12492) for 7–9 weeks, starting as 6 weeks of age. A group of mice were injected with a single dose of either EX-527 (10mg/kg) or AK-7 40mg/kg (4ml/kg) or equivalent volume of DMSO (Vehicle) (4ml/kg) intraperitoneally, 18 hours post-sepsis. These doses of EX-527 and AK-7 in mice were per literature [2] [19].

Cecal ligation and puncture (CLP): Mice were anesthetized using isoflurane anesthesia (1–3% Isoflurane/ O2 mixture via nose cone). CLP was used to induce sepsis as described in the literature [2]. Laparotomy was performed; cecum identified, ligated, perforated two times with a 22-guage or a 25-guage needle (see below), contents returned, abdomen closed in two layers (peritoneum and skin) and animal received 1 ml normal saline fluid resuscitation subcutaneously. Sham-operated mice underwent laparotomy and fluid resuscitation without cecal ligation and puncture.

Sepsis “dose titration in ob/ob” mice: We started out studying model of CLP (sepsis) used in previous publications [2,20] and observed that the ob/ob mice had significantly decreased survival compared to WT lean mice. As shown in S1A Fig, we observed that using 22-guage needle and two punctures model of sepsis (CLP 22.2), there was 0% 7-day survival in ob/ob mice vs. 40% in WT mice; all ob/ob mice died within 48 hours. As shown in S1B Fig, with CLP using 25-guage needle and two punctures model of sepsis (CLP 25.2) we were able to achieve a comparable (30% survival) in ob/ob mice with our previous publications [2,20]) and decided that CLP25.2 was the optimal dose of sepsis in ob/ob mice to study early and late sepsis. However, the survival in WT mice with CLP25.2 was 100% (S1B Fig). Mice with CLP25.2 (Sepsis)/ Sham surgeries were used for tissue harvest or intravital fluorescent video microscopy at specified time points post-surgery as indicated.
Endotoxin tolerance in vivo: We used in vivo endotoxin tolerance to test immune function in sepsis mice at different time points as indicated. To do this, mice received either E. coli lipopolysaccharide O111:B4 (LPS: 5 μg/mouse intraperitoneally; 4 hours of stimulation) or normal saline (NS) as a "second hit" after CLP. We used CLP25.2 in WT and obese mice.

Pain and distress: All mice were monitored at least twice per day. Pain and distress were relieved using buprenorphine analgesia (0.05mg/kg body weight; intraperitoneally). Pain scoring system, indications for analgesia and humane end points for euthanasia are described in detail in Table 1. We used isoflurane overdose followed by cervical dislocation (secondary method) for euthanasia. Despite rigorous monitoring, the rate of unexpected deaths was 15–20% and cause of death was found to be due to intra-abdominal abscess formation.

Intravital fluorescent video microscopy (IVM): Mice were anesthetized using ketamine (150mg/kg) + Xylazine (7.5 mg/kg) intramuscularly. Intravital microscopy procedures were described previously [2]. The mice underwent carotid artery (to measure invasive blood pressure, MAP monitoring) and jugular venous cannulations (to inject platelets/Rhodamine G intravenously), laparotomy incision opened, small intestine exteriorized and the small intestinal microcirculation was studied (n = 4–6 mice/group) [2]. In vivo visualization of leukocyte achieved by injecting mice with Rhodamine G (labeled red) while platelets were labeled green ex vivo with carboxyfluorescein diacetate succinimidyl ester (CFSE: Sigma-Aldrich; St Louis, MO, USA; 90 μM) to allow simultaneous monitoring of leukocytes and platelets. The details of the platelet isolation technique are as outlined previously [20,21]. The platelets (n = 100 x 10⁶) were infused intravenously over 5 min (yielding <5% of the total platelet count) and allowed to circulate for a period of 5 min before recording on a DVD. Literature suggests that these platelet isolation procedures have no significant effect on the activity or viability of isolated platelets [22].

The post-capillary venules (n = 3–5/mouse; 4–6 mice per group) were recorded (1 min 20 seconds each) and leukocyte/platelet adhesion quantified. Cell (leukocyte/platelet) was considered adherent if stationary for at least 30 consecutive seconds of the one minute recording. The mean of the average values of leukocyte adhesion determined in each mouse were then used to generate a group mean.

Immunohistochemistry of small intestinal tissue: Small intestinal tissue was harvested and fixed frozen sections of tissue were stained using antibodies against SIRT-2(Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), E-selectin and ICAM-1 (BD biosciences, San Jose, CA, USA) and Von Willebrand factor (VWF, Abcam, Inc. Cambridge, MA, USA). Cy-3-conjugated

Table 1. Monitoring pain and distress.

| Pain score | Observation                                                                                     | Recommendation                                      |
|-----------|-------------------------------------------------------------------------------------------------|----------------------------------------------------|
| 0         | Asleep or awake. Normal appearance and behavior. Respiratory rate normal                         | No analgesic indicated                              |
| 1         | Mild agitation. Depressed and uninterested in surrounding, frequent position change of reluctant to move. Mild changes in appearance, eyes partially closed, decreased interaction. Respiratory rate up to 30% above normal. | Buprenorphine 0.05mg/kg intraperitoneally. Frequent (at least every four hours) monitoring. Euthanasia if persistent score 1 for four hours or progression to score 2. |
| 2         | Moderate agitation, restless and uncomfortable. Moderate changes in eyes, sunken or glazed, unthrifty. Moderate changes in behavior, less mobile, less alert, unaware of surroundings. (Reluctant to move, but will if coaxed. Respiratory rate 30–45% above normal. | Buprenorphine 0.05mg/kg intraperitoneally. Monitoring at least 2 hours post-analgesia. If unchanged, euthanasia. |
| 3         | Extremely agitated thrashing. Severe changes in appearance. Eyes pale, enlarged pupils. Guarding, hunched in appearance, legs in abnormal position, teeth grinding. Respiratory rate more than 45% above normal. | Euthanasia.                                        |

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labeled secondary antibodies for SIRT-2, E-selectin, ICAM-1 and FITC conjugated secondary antibody for VWF were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA) [2,20]. Virtual images were captured as described previously[2] and immunofluorescence quantification were performed using NIH Image J software [23].

Cell Culture: RAW264.7 (ATCC® TIB-71™: RAW) and HEK 293 (ATCC® CRL- 1573™: HEK) cells were obtained from ATCC. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (iFBS), 100units/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5%CO2. Early passage (2–10) cultures were used in all experiments.

SIRT2 overexpression and knockdown: To overexpress SIRT2 protein, SIRT-2 (or control plasmid pcDNA3) plasmids were co-transfected with p65 or/and CBP as indicated based on reported in literature [24]. Plasmids were diluted in Opti-MEM medium, followed by the addition of SuperFect (QIAGEN, #301305). The transfection complex was added to HEK 293 cells and the medium was replaced with DMEM containing 10% iFBS and whole cell lysates were collected for western blotting 48 hours post-transfection. HEK cells were used for overexpression experiment due to low transfection efficiency for SIRT2 plasmid in RAW cells.

For knock-down of SIRT-2, we co-transfected RAW cells with p65 and CBP plasmids together with either SIRT2 siRNA (si-SIRT2) (Dharmacon Cat #: L-061727-02-0005) or scrambled control (si-Ctrl) (Dharmacon Cat #: D-001810-10-05) with RNAiMax reagent and incubated and RAW cells were detached and suspended DMEM containing 10% iFBS. The whole cell lysates were collected for western blotting.

We obtained pcDNA3-β-FLAG-CBP-HA (Addgene plasmid # 32908), SIRT2 Flag (Addgene plasmid # 13813) and pCMV4 p65 (Addgene plasmid # 21966) from Addgene.

RNA extraction and RT-PCR: RNA extraction method and RT-PCR were completed as described previously [2,20]. The mRNA expression was quantified by quantitative real-time PCR with SensiFAST Probe Lo-ROX One-Step Kit (Bioline, BIO-78005). GAPDH was used to normalize the gene expression data. Relative quantification was calculated using the ΔΔ comparative threshold formula. All samples were run in quadruplicates to calculate average and SE value. TaqMan primer/probes were purchased from Invitrogen (Grand Island, NY).

Whole blood leukocytes: Blood was collected via carotid arterial cannulation in anesthetized mice, peripheral leukocytes were stained with anti-CD45 (eBioscience, Inc., San Diego, CA, USA) and anti-PSGL-1 (BD Biosciences, San Jose, CA, USA) antibodies. The samples were assayed by flow cytometry using a BD Acuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) as described before[2]. We gated for CD45+ cells (total leukocytes). PSGL-1 expression was determined by MFI analysis of leukocytes (PSGL-1+/CD45+ cells). MFI values were normalized to control.

Statistics: All data were analyzed using Graph Pad Prism 6.0 (Graph Pad Software, La Jolla, CA, USA). Analyses with more than three groups were analyzed using one-way ANOVA or
two-way ANOVA with Tukey’s post hoc comparisons as appropriate. A p < 0.05 was designated as significant. Leukocyte adhesion was analyzed using Tukey-Kramer post-hoc analysis (Statview, SAS; Cary, NC, USA). A p < 0.05 was designated as significant. Log rank test was used to compare survival between the groups in the Kaplan-Meier survival curves and a p < 0.05 was designated as significant.

Results
Ob/ob mice rapidly enter a prolonged immune-repressor hypo-inflammatory phase of sepsis

We defined temporal changes in leukocyte adhesion, using endotoxin stimulation as a “second hit” to study microvascular response to inflammatory stimulus at different time points after sepsis-induction. Increased leukocyte adhesion in response to endotoxin (endotoxin-responsiveness) indicates immune-competence while endotoxin tolerance serves as a marker for putative immune-repression in vivo. To achieve this, we challenged WT and ob/ob- septic mice with either normal saline (NS) or lipopolysaccharide (LPS: endotoxin) as a second-hit at different time points after sepsis and studied small intestinal microcirculation for leukocyte adhesion. The leukocyte adhesion in Sepsis +NS in ob/ob mice peaks at 12h post-sepsis and decreases thereafter (Fig 1A). The leukocyte adhesion in WT mice with CLP25.2 peaked at 6 hours and decreased thereafter (supportive information S1 Fig). The leukocyte adhesion in Sepsis+ NS groups remained significantly higher in ob/ob vs. WT mice in 6, 12, 24 and 72 hours post-CLP (data not shown).

In ob/ob mice, when challenged with endotoxin (Sepsis +LPS), further accentuation of the leukocyte adhesion (vs. Sepsis +NS) occurred only in the first 6h after sepsis to be followed by an endotoxin-tolerant phase; the endotoxin tolerance (repressor/hypo-inflammatory phase) persists for up to 7 days in septic ob/ob mice (Fig 1A). This suggests a prolonged hypo-inflammation/ delayed sepsis resolution in ob/ob mice. As shown in Table 2, there were no significant differences in body weight and carotid arterial invasive mean arterial blood pressure (MAP) between the groups. In WT counterparts with CLP 25.2, Sepsis+ LPS groups had significantly increased leukocyte adhesion vs. Sepsis+NS at all the time points studied, i.e. 6, 12, 24 and 72 hours post-sepsis. This suggests that microvasculature remained endotoxin-responsive (S2A Fig). Thus, we show while WT mice remain endotoxin responsive with 100% survival, the ob/ob mice show prolonged endotoxin tolerance with this model of cecal ligation and puncture (CLP 25.2).

SIRT-2 expression in ob/ob septic mice increases during immune repressor hypo-inflammatory phase

We first studied whether there was a difference in SIRT-1 expression in small intestinal tissue during hyper-inflammatory phase (6 hours) vs. hypo-inflammatory phase (24 hours) of sepsis. We showed that in ob/ob mice, SIRT-1 expression during hypo-inflammatory phase of sepsis decreased vs. hyper-inflammatory phase as shown in Fig 1B. Next, we investigated whether there was change in SIRT-2 expression and found that SIRT-2 expression in the small intestinal tissue of ob/ob mice increased during the hypo-inflammatory phase vs. hyper-inflammatory phase as shown in Fig 1C. We found similar results, i.e. decreased SIRT-1 and increased SIRT2 expression during the hypo-inflammatory phase in the liver tissue of ob/ob mice with sepsis (not shown). We also studied the SIRT2 expression in WT mice with sepsis during corresponding time points (6 and 24 hours post-sepsis) and show in supportive information S2B Fig that in WT mice, the SIRT2 expression remained unchanged at 6 vs. 24 hours post-sepsis. Taken
Fig 1. Ob/ob mice rapidly and persistently enter the sepsis repressor hypo-inflammatory phase. A: Ob/ob mice show a prolonged repressor/hypo-inflammatory phase of sepsis: In ob/ob mice with sepsis, leukocyte adhesion in small intestinal microcirculation (mice n = 5/group) peaked at 12h in Vehicle (normal saline) group and decreased in after 18 h in Sepsis (CLP) group. LPS challenge in sepsis mice significantly increased leukocyte further (vs. Vehicle) only in 6h Sepsis group (hyper-inflammatory) while it did not in 12, 24, 72h and 7 day Sepsis groups, indicating endotoxin tolerance (immune repression/hypo-
together, we show that the \textit{ob/ob} mice show prolonged endotoxin tolerance with increased SIRT2 expression. Thus, SIRT-2 and not SIRT-1 expression was increased during hypo-inflammatory phase of \textit{ob/ob}-sepsis mice. 

**SIRT-2 and not SIRT-1 inhibition during sepsis repressor/ hypo-inflammatory phase improves sepsis survival in \textit{ob/ob} mice**

To further investigate the potential differences in \textit{ob/ob} vs. WT sepsis, we tested whether treatment with SIRT1-specific inhibitor EX-527 improved 7-day survival in \textit{ob/ob} mice. In contrast to our findings in lean/WT sepsis, where EX-527 improved survival, we found that in \textit{ob/ob} mice with sepsis EX-527 significantly decreased survival in \textit{ob/ob} mice with sepsis. (Fig 2). Since SIRT-2 expression appears to be increased in \textit{ob/ob} mice with CLP, we tested the effects of SIRT-2 specific inhibitor AK-7 on 7-day sepsis survival and observed there was a significant increase in survival of \textit{ob/ob} septic mice treated with AK-7 (Fig 2). Thus, the data suggest that SIRT-2 and not SIRT-1 plays a crucial role in \textit{ob/ob} mice with sepsis.

**The role of SIRT-2 in the endotoxin tolerant RAW cells**

We next sought to elucidate the mechanism role of SIRT-2 in endotoxin tolerance as it relates to SIRT-2 expression, using mouse macrophage cell line, the RAW cells. Endotoxin stimulation and subsequent tolerance is described in RAW cells has been studied as a cell culture sepsis model in the literature. We primed RAW cells with LPS 100 ng/ml for indicated times (0h, 4h, 6h, 8h, and 24h) and then re-stimulated with LPS/vehicle (normal saline: NS) as a second-hit for additional 4h and then assessed TNF-α mRNA expression and show that the second dose of LPS is unable to further increase TNF-α mRNA expression, confirming previous studies and further showing endotoxin tolerance in RAW cells as early as 4h after the first dose of LPS (Fig 3A). We next examined SIRT-2 expression in endotoxin tolerant RAW cells. Similar to \textit{ob/ob} tissue (Fig 1), we show increased SIRT-2 expression in endotoxin tolerant RAW cells (Fig 3B).

Sirtuins directly deacetylate and inactivate NFκB p65. Next, we studied whether SIRT-2 deacetylates NFκBp65. Due to very low plasmid transfection efficiency in RAW cells, we studied p65 deacetylation as a proof of principle study, first in HEK cells. We studied p65 deacetylation as a proof of principle study, first in HEK cells. We studied acetylated

| Table 2. Weight in grams and mean arterial blood pressure (MAP) in different groups. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Body Weight (gms) | Mean ± SEM | MAP (mmHg) | Mean ± SEM |
|                                 | NS               | LPS          | NS           | LPS           |
| 6 h sepsis                      | 41.8±3.9         | 40.97±4.3    | 55.6±1.8     | 61.20±3.2     |
| 12 h sepsis                     | 36.76±0.53       | 37.85±1.4    | 56.0±4.6     | 64.75±5.6     |
| 24 h sepsis                     | 35.56±0.33       | 36.92±1.5    | 60.66±0.33   | 58.33±8.6     |
| 72 h sepsis                     | 35.02±1.3        | 35.27±1.05   | 62±2.9       | 68.20±6.61    |
| 7 days                          | 37.01±1.1        | 35.57±0.80   | 69±5.3       | 72.4±5.8      |

Body weight and carotid arterial mean arterial blood pressure in different groups of \textit{ob/ob} mice with and without LPS second-hit studied show no significant differences from each other. Similarly, the mean arterial blood pressures (MAP) between different groups were not significantly different from each other.
(AC-p65) expression in cells transfected with SIRT-2 vs. empty (Ctrl) plasmid with p65+CBP (to increase baseline p65 acetylation) co-transfection. We then studied AC-p65 using western blot analysis. As shown in Fig 3C, AC-p65 expression decreased significantly in cells transfected with p65+CBP+SIRT-2 compared to those with Ctrl+p65+CBP. Taken together, our data suggest that in a cell model that SIRT-2 participates in endotoxin tolerance by deacetylating and inactivating NFkB p65.

SIRT-2 inhibition reverses endotoxin tolerance in RAW cells

First we studied the effect of SIRT-2 inhibitor AK-7 pre-treatment on hyper-inflammatory phase in the RAW cells. We show that there was a significant increase in TNF-α mRNA expression in endotoxin sensitive RAW cells pre-treated with AK-7 vs. Vehicle (Fig 4A). Next, we determined whether SIRT-2 specific inhibitor AK-7 could reverse endotoxin tolerance. We treated cells at 4 and 6h post-LPS (when cells are transitioning to the repressed state) with either vehicle or AK-7 and then re-stimulated with LPS. We show that AK-7 treated cells significantly increase TNF-α, IL-6 and IL1-1β mRNA levels (Fig 4B) compared to Vehicle treated cells, suggesting that SIRT-2 inhibition can reverse endotoxin tolerance in RAW cells.

To further study the effect of SIRT-2 deficiency on AC-p65 expression, we treated RAW cells with SIRT2 siRNA (si-SIRT2) vs. scramble siRNA (si-Ctrl) co-transfected with p65+CBP. We then studied AC-p65 expression in these cells using western blot analysis shown in Fig 4C.
AC-p65 increased further in RAW cells treated with si-SIRT-2 +p65+CBP compared to si-Ctrl +p65+CBP. Together, our data suggests that SIRT2 inhibition or deficiency is associated with increased ACp65 expression.

SIRT-2 inhibition in ob/ob septic mice reverses microvascular hypo-inflammation in vivo

Data in Fig 1A suggests that ob/ob mice show a prolonged hypo-inflammatory phase; AK-7 treatment during the hypo-inflammatory phase of sepsis significantly improves survival. So
next, we sought to study whether this beneficial effect on survival is via reversal of hypo-inflammatory phase of sepsis in ob/ob mice in vivo. Specifically, using endotoxin tolerance in vivo as a tool for tracking repressed immunity in the microvasculature, we tested the effect AK-7 in ob/ob mice. As shown in Table 3, there were no significant differences in body weight and MAP between different groups studied. We then treated ob/ob mice with AK-7/ Vehicle after hypo-inflammatory phase onset (18h post-CLP) and assessed microvascular leukocyte adhesion in
response to second-hit LPS. As shown in Fig. 5A, vehicle treated mice remain endotoxin tolerant, with no significant difference between Sepsis+NS vs. Sepsis+LPS. However, in AK-7 treated mice, we observed significant increases in leukocyte adhesion in Sepsis +LPS vs. Sepsis +NS groups, indicating reversal of the hypo-inflammatory phase; in contrast with prolonged hypo-inflammation in untreated ob/ob mice shown in Fig 1A. Thus, SIRT-2 inhibition with AK-7 reverses hypo-inflammation and restores the microvascular function in ob/ob-sepsis mice.

AK-7 treatment restores adhesion molecule expression

Having found reactivation of NFkB p65 function after AK-7 treatment in our cells, we tested the effect of SIRT-2 inhibition on p65-dependent adhesion molecules. Rolling and adhesion of circulating leukocytes in microcirculation are facilitated via selectins and ICAM-1, respectively [21,27]. We reported in septic mice that SIRT-1 represses leukocyte adhesion in vivo microcirculation, via E-selectin and ICAM-1 expression in lean mice [2,20]. Here, we examined the effect of AK-7 on E-selectin and ICAM-1 expression in the small intestinal microcirculation using immunohistochemistry. As shown in Fig 5B and 5C, E-selectin and ICAM-1 expression increased in AK-7 vs. vehicle treated mice, supporting a role for SIRT-2 in repressing inflammatory reactions in microvasculature during obesity with sepsis.

AK-7 treatment activates circulating leukocytes: Next, we examined the effect of AK-7 treatment on circulating cells. The circulating leukocytes interact with the selectins expressed on the endothelial cells via P-selectin glycoprotein ligand (PSGL-1), a ligand for E-selectin. We co-stained leukocytes with anti-CD45 antibody and PSGL-1 antibody and gated for CD45 positive cells. As shown in Fig 6, PSGL-1 expression increased in leukocytes of mice treated with AK-7 compared to Vehicle treatment. Fig 6A shows quantification of mean fluorescence intensity CD45+PSGL-1+ cells expression in three groups of mice: Control, Sepsis+ Vehicle and Sepsis+AK-7 treatment. Fig 6B shows representative dot-plots and corresponding histograms for all CD-45+ cells. Together, these data indicate that AK-7 acts both on circulating leukocytes and endothelium during sepsis in ob/ob mice.

Diet induced obese mice with sepsis and SIRT2 expression

Finally, we confirmed our key findings in ob/ob mice in DIO mice with sepsis. The ob/ob mice are leptin deficient and the effect of leptin on innate immunity is controversial [28,29]. We have shown previously that the microvascular inflammation in early sepsis in leptin deficient ob/ob, leptin resistant db/db and melanocortin 4 receptor knock out mice associated obese

| Table 3. Body weight and mean arterial blood pressure |
|---------------------------------|-----------------|
|                                 | Body Weight (gms) Mean± SEM | MAP (mmHg) Mean± SEM |
|                                 | NS               | LPS             | NS               | LPS             |
| Ob/ob sepsis+ Vehicle           | 37.03±3.9        | 39.25±1.47      | 59.0±2.6         | 60.0±3.08       |
| Ob/ob sepsis+ AK-7              | 36.34±1.13       | 39.39±0.41      | 57.80±5.03       | 60.40±4.40      |
| DIO sepsis+ Vehicle             | 33.89±1.03       | 35.54±2.46      | 68.6±2.1         | 58.20±0.73      |
| DIO sepsis+ AK-7                | 33.89±0.98       | 38.04±1.13      | 60.0±4.02        | 50±5.2          |

Body weight and mean arterial pressure in ob/ob and DIO mice with sepsis with vehicle vs. AK-7 treatment:

Body weight and carotid artery mean arterial blood pressure were measured in ob/ob and DIO mice treated with vehicle vs. AK-7 and re-stimulated with normal saline (NS) and LPS. Different groups studied show no significant differences from each other. Similarly, the mean arterial blood pressures (MAP) between different groups were not significantly different from each other.

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Fig 5. SIRT-2 inhibition in ob/ob septic mice restores repressed microvascular leukocyte adhesion. A: SIRT-2 inhibition during hypo-inflammation enhances leukocyte adhesion in ob/ob: mice with sepsis: ob/ob-septic mice were treated with Vehicle or AK-7 during hypo-inflammatory phase of sepsis, challenged with either normal saline (NS) or LPS and studied leukocyte adhesion 4h later. While Vehicle treated mice showed no further increase in leukocyte adhesion in response to LPS, AK-7 treated mice showed significant increase in leukocyte adhesion in small intestinal microcirculation. *p<0.05 vs. Vehicle.
phenotypes are similar[21,30]. We confirmed the key findings of this project in another model of obesity, namely the diet induced obesity (DIO). First we studied whether the hypo-inflammatory phase of sepsis in DIO mice with sepsis is associated with increased SIRT2 expression. As depicted in Fig 7A, there was a marked increase in the SIRT-2 expression small intestinal tissue in DIO mice with sepsis during the hypo-inflammatory phase of sepsis. We also found similar results in liver tissue (data not shown). We then studied microvascular endotoxin tolerance using leukocyte adhesion in the small intestinal microcirculation with and without AK-7 treatment in DIO mice. As shown in Table 3 there were no significant differences in body weight and MAP between different groups. Fig 7B shows that while the vehicle treated DIO sepsis mice remained endotoxin tolerant during the hypo-inflammatory phase, the AK-7 treated mice showed endotoxin responsiveness similar to ob/ob mice. Thus, our data suggest that the hypo-inflammatory phase of sepsis is associated with increased SIRT2 expression in DIO mice as well. Moreover, SIRT-2 inhibition during the hypo-inflammatory phase in DIO mice with sepsis reverses the endotoxin tolerance similar to ob/ob mice.

Discussion

The results of this study introduce previously unreported discoveries with potential implications for obesity and sepsis. Specifically, we report for the first time to our knowledge, that SIRT-2 modulates sepsis-related inflammation in obese-septic mice. From the pathophysiologic standpoint, we find that obese septic mice not only increase the magnitude of the initial sepsis inflammatory response in microvasculature, as assessed in vivo, but also more rapidly transition in the repressed state of established sepsis, which correlates with profound immune repression of both innate and adaptive immunity[4,31]. Moreover, we discover that the inflammation and immunity repressor hypo-inflammatory phase persists in ob/ob septic mice at a lower dose (intensity) of sepsis for at least 7 days, which is significantly longer than we found lean septic mice with equivalent dose of sepsis[2]. Mechanistically, we find that in vivo repression of leukocyte adhesion by SIRT-2 is mediated, at least in part, via repression of endothelium (E-selectin, ICAM-1 expression) and circulating cells (PSGL-1 expression); AK-7 treatment reverses both (Figs 5 and 6). We extended mechanistic study in a mouse macrophage cells model in vitro, which simulated our in vivo results with SIRT-2. SIRT-2 deacetylates master immune and inflammation regulator NFkB p65 thus inactivating p65 function. Taken together, this study implicates NAD + dependent SIRT-2 deacetylase function as promoting the immune repressive hypo-inflammatory phase and influencing survival in sepsis with obesity. Ob/ob mice are leptin deficient. Although the evidence regarding the effect of leptin on inflammatory response is controversial, we have confirmed our findings in other models of obesity such as db/db and melanocortin 4 receptor knock out mice[21,30]. In the current project, we confirmed the key findings in clinically significant obesity model, the DIO mice with sepsis. We show that similar to ob/ob mice, the hypo-inflammatory phase of DIO mice with sepsis is also associated with increased SIRT-2 expression and SIRT-2 inhibition during the hypo-inflammatory phase reverses it.

Sepsis is the 11th leading cause of death in the US [32], and an even more dominant cause of death worldwide. Sepsis is considered to be the “most expensive condition” in the US[33].
Fig 6. SIRT-2 inhibition during hypo-inflammatory phase activates circulating leukocytes. We treated ob/ob mice with either Vehicle or AK-7 during hypo-inflammatory phase of sepsis and studied whole blood leukocytes for PSGL-1 and CD45 expression using flow cytometry. Fig 6A shows mean fluorescence intensity from three different groups while Fig 6B and 6C show representative histogram and dot plots respectively. There was a significant increase in PSGL-1 expression in CD45 positive cells in Sepsis+ AK-7 compared to Sepsis +Vehicle group using Tukey’s post-hoc analysis; error bars: s.e.m.

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Fig 7. Diet induced obese mice with sepsis and SIRT2 expression. A: Immunohistochemistry of small intestinal tissue (6B) from DIO mice with sepsis were stained for SIRT-2 (Cy3: red), Von Willebrand factor (VWF, FITC: green), nuclear stain (DAPI: blue) and merged color image show that SIRT-2 expression increased during immune repressor/ hypo-inflammatory phase (24 h) Sepsis compared to hyper-inflammatory phase (6 h) similar to that in ob/ob mice. B: SIRT-2 inhibition during hypo-inflammation enhances leukocyte adhesion in DIO mice with sepsis. DIO mice with sepsis were
Early, hyper-inflammatory phase of sepsis transitions to a muted inflammatory phase within hours. If homeostasis is quickly retrieved, immune competence and general homeostasis returns, and patient survival is high. If repressed immunity persists, both the original and acquired infections, including latent viruses, are a source of high mortality [3]. Additional mortality is associated with sustained failure of sepsis target organs: heart, lung, kidney, brain, and liver. Mortality remains high in late phase sepsis and has not mechanism-specific treatment. Since the incidence of sepsis is rising, a public health crisis has emerged [34]. Added to this is the epidemic of obesity, which in itself has high morbidities and mortality rates. Sepsis impacts obesity morbidity and mortality.

Endotoxin tolerance is described in sepsis and other systemic inflammatory states [35–37]. Majority of the studies regarding endotoxin tolerance were performed in vitro. We adapted this tool to our in vivo system to study sepsis-induced endotoxin tolerance in the microvasculature [2]. In the current project we further applied the tool of endotoxin tolerance to assess immune function in vivo to obese mice.

Potentially important observation in obesity is that sirtuins are essential sensors and regulators of metabolism [13]. SIRT-1, by far the most studied sirtuin, mobilizes fatty acid and increase gluconeogenesis in liver in addition to improving insulin secretion from pancreas [38]. Emerging data indicate that SIRT-2 also regulates the beta oxidation of fatty acids via PGC1-α pathway [39]. Thus, sirtuins likely play potentially important but so far unknown roles in obesity metabolic and inflammatory pathways. For example, it is known that obesity-associated chronic inflammation—a systemic metabolic syndrome—is associated with a "low SIRT state" [38], which may directly contribute to metabolic syndrome and/or prime obese immune cells for amplified inflammation. This "priming or sensitizing" property may prompt excessive inflammation during early sepsis. Supporting this concept is that SIRT-1 and 2 activation attenuates obesity-related inflammation [39,40].

We have implicated sirtuins as major contributors to sepsis outcome, both early and late [13,41]. Importantly, sirtuins occupy fundamentally important checkpoints for guarding immuno-metabolic homeostasis. Homeostasis deviation is extreme during sepsis. As a rheostat, low expression of SIRT-1 and other sirtuins amplify early sepsis and high expression sustain repressed immunity [20,42]. Immune cells require activation of glycolysis for energy to generate an effector antimicrobial response and promote anabolic channels for growth and differentiation [43]. During the switch of sepsis from glycolysis dependency for energy of hyper-inflammation, immune cells require fatty acid oxidation to supply ATP in a low energy catastrophic hypo-inflammatory phase that characterizes repressor immune cells and possibly poor retrieval of organ function [44]. This hypo-inflammatory of sepsis is promoted by SIRT expression and its interaction with AMP kinases [45]. These SIRT-regulated immuno-metabolic processes likely impact many obesity interactions with sepsis.

In this study, we show that at two different doses of sepsis, ob/ob mice show decreased survival (S1A and S1B Fig); at lower dose of CLP also show increased immune dysfunction (prolonged hypo-inflammation) compared to lean mice with equivalent dose of CLP. The “survival” observation is in contrast to the clinical observation with controversial data regarding obesity and sepsis-mortality [46]. However, the literature almost unanimously indicates that there is increased morbidity in obese-sepsis vs. lean-sepsis patients [12]. Our data
regarding the prolonged hypo-inflammatory immune repressor phase in \( ob/ob \) mice is consistent with that observation.

Surprisingly, in this study, we found that phenotypic shift from hyper-inflammatory to hypo-inflammatory phase is not controlled by SIRT-1, but rather by SIRT-2; thus, lean and \( ob/ob \) mice differ in that respect too. SIRT-2 is the most abundant of sirtuins in the white adipose tissue where it critically regulates adipocyte growth in obesity; SIRT2 expression is down-regulated in differentiating pre-adipocytes to increase glycolysis and decrease fatty acid oxidation, allowing adipogenesis to occur. [39,47]. Yet SIRT2 is dominant in mature adipose tissue, perhaps as a checkpoint. Whether SIRT-2 participates in obesity related acute inflammation is largely unknown. Research suggests that while predominantly cytosolic, under acute inflammatory stress, SIRT-2 translocates to the nucleus [48]. This supports our observation that SIRT-2 controls NFkB dependent adhesion molecule gene expression and shifts in NFkB p65 acetylation/deacetylation [49].

This study poses major unanswered questions. First, do the changes of \( ob/ob \) mice reflect those in nutritional obesity-associated sepsis? From the SIRT perspective this seems likely, but whether SIRT-2 is specific for obesity is unknown. From our data shown in Fig 7 starts to address this important issue. Here we show that the DIO mice also show increased SIRT-2 expression during the hypo-inflammatory phase and SIRT-2 inhibition during the hypo-inflammatory phase reverses it. Secondly, the exact mechanism of how obesity modulates SIRT-2, especially during the acute inflammatory states, needs to be further elucidated. This highly significant concept urges further research in cell, mouse, and human models of obesity.

In summary, we report for the first time to our knowledge, that septic \( ob/ob \) mice show a prolonged the immune-repressor/ hypo-inflammatory phase that frequently accompanies lethal human sepsis. We implicate SIRT-2 as a novel checkpoint for guarding homeostasis in obesity, but with persistent activity adversely affect survival.

Supporting Information

S1 Fig. Sepsis dose titration for \( ob/ob \) mice. We examined 7-day survival in WT lean vs. \( ob/ob \) mice. As shown in S1A Fig, the 7-day survival in \( ob/ob \) mice with CLP22.2 was significantly decreased (0%) vs. WT (40%) mice. Moreover, all the mice from \( ob/ob \) groups died within 48 hours post-sepsis. As shown in S1B Fig, with CLP25.2, the 7-day survival in \( ob/ob \) mice was 30% vs. WT mice 100%. * \( p<0.05 \) vs. corresponding WT CLP using Log-Rank test.

S2 Fig. phases of sepsis in WT mice with CLP25.2. A: In WT mice with CLP25.2, leukocyte adhesion in small intestinal microcirculation (mice \( n = 5 \)/group) in WT mice with LPS “second-hit” was significantly increased vs. NS groups at 6, 12, 24 and 72 hours post CLP25.2 indicating no hypo-inflammatory phase with CLP25.2 in WT mice. * \( p<0.05 \) vs. respective Sepsis+NS group Tukey’s post-hoc analysis; error bars: s.e.m. B: SIRT2 expression in WT mice with CLP25.2: Small intestinal tissue were stained for SIRT-2 at 6 vs. 24 hours post-CLP25.2. SIRT-2 (Cy3: red), Von Willebrand factor (VWF, FITC: green), nuclear stain (DAPI: blue) and merged color image show that SIRT-2 expression in WT mice at 6 vs. 24 hours post-CLP25.2 remained unchanged.

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Author Contributions
Conceived and designed the experiments: VV.
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Analyzed the data: XW NLB VV JW BY.
Contributed reagents/materials/analysis tools: VV BY CEM.
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Data collection: XW NLB AM JW BY VV CEM.

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