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Resveratrol Attenuates Microvascular Inflammation in Sepsis via SIRT-1-induced Modulation of adhesion molecules in *ob/ob* mice

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Abstract

Objective—Obesity, a Sirtuin-1 (SIRT-1) deficient state, increases morbidity and resource utilization in critically ill patients. SIRT-1 deficiency increases microvascular inflammation and mortality in early sepsis. Our objective was to study the effect of resveratrol (RSV), a SIRT-1 activator, on microvascular inflammation in obese septic mice.

Methods and procedures—*ob/ob* and C57Bl/6 (WT) mice were pre-treated with RSV vs. DMSO (Vehicle) prior to cecal ligation and puncture (Sepsis). We studied 1) Leukocyte/Platelet adhesion 2) E-selectin, ICAM-1 and SIRT-1 expression in small intestines 3) 7-day survival. A group of RSV treated mice received SIRT-1 inhibitor (EX-527) with sepsis-induction and leukocyte/platelet adhesion and E-selectin/ICAM-1 expression were studied. We treated endothelial (HUVEC) cells with RSV to study E-selectin/ICAM-1 and p65-acetylation (AC-p65) in response to LPS.

Results—RSV treatment decreased leukocyte/platelet adhesion and E-selectin/ICAM-1 expression with increased SIRT1 expression in septic *ob/ob* and WT mice; decreased E-Selectin/ICAM-1 expression via increased SIRT-1 expression and decreased AC-p65 expression in HUVEC. EX-527 abolished RSV-induced attenuation of microvascular inflammation in *ob/ob* septic mice. Finally, *ob/ob* mice in Sepsis+RSV group had significantly increased 7-day survival vs. Sepsis+Vehicle group.

Conclusion—RSV increases SIRT-1 expression in *ob/ob*-septic mice to reduce microvascular inflammation and improves survival.

Keywords

Sepsis; Obesity; SIRT-1; Resveratrol; Adhesion Molecule; Leukocyte adhesion

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INTRODUCTION

Severe sepsis and septic shock are the leading causes of death in critically ill patients in non-coronary intensive care units worldwide^{1,2}. In the USA alone they cause over 200,000 deaths per year and costs \$16 billion annually³. High mortality is generated both by the inciting infection and by host-derived inflammatory injury to multiple organs.

Microvasculature with its strategic place between systemic circulation and local tissue environment plays a central role in sepsis-induced inflammation with multiple organ failure. Circulating cell-endothelial cell interactions in the microcirculation are the rate determining factors in inflammatory response in various tissues in response to variety of stimuli⁴ including sepsis. Others and our previous work demonstrated increased leukocyte adhesion in post-capillary venules after induction of sepsis⁵⁻⁷.

Obesity, now recognized as a disease, affects more than one third of the adults in the USA. Estimates project obesity-related healthcare cost to increase by \$48-66 billion/year in USA by year 2030⁸. While controversy exists regarding the effect of obesity and overweight on mortality in critically ill patients⁹, literature suggests that morbid obesity is associated with increased resource utilization and cost of care in already expensive care of critically ill patients in the intensive care units^{10,11}. We have used a mouse model to better understand the interaction of obesity and sepsis and found increased mortality and exaggerated microvascular inflammation during early sepsis⁶ but the mechanisms responsible are unclear.

Previously, we reported that NAD⁺ dependent activation of class III histone deacetylase sirtuin 1 (SIRT-1) coordinates the acute inflammatory response in sepsis. We found that SIRT-1 inhibition during early sepsis significantly exaggerates leukocyte adhesion and increases mortality¹². Growing body of evidence supports that obesity with chronic inflammation is associated with low levels of NAD⁺ and SIRT-1¹³. In contrast to the state of over nutrition of obesity, calorie restriction¹⁴ and weight loss lead to increased SIRT-1 expression¹³ and reduce inflammation and imbalanced metabolism. The effects of low SIRT-1 expression during obesity on microvascular inflammation associated with sepsis are not well defined, nor is it known whether SIRT-1 activation might reduce microvascular inflammation and limit systemic injury due to sepsis.

Resveratrol (3, 5, 4'-trans-trihydroxystilbene; RSV), a potent activator of SIRT-1^{15,16} present in grapes, berries, peanuts and red wine, is known for its anti-inflammatory and anti-cancer properties^{14,17-19}. The anti-inflammatory effects of RSV, which also link to activation of AMP-activated protein kinase (AMPK), attenuate lung and kidney injury and decrease mortality in rodent sepsis^{18,20,21}. While beneficial in lean mice with sepsis, the effect of RSV on sepsis with obesity, a state of reduced SIRT-1 expression, are unclear. Also, it is unknown whether the circulating cell-endothelial cell interactions in septic mice are modulated by RSV treatment. Here, we report the effect of increased SIRT-1 expression using RSV on circulating cell-endothelial cell interactions and survival in obese mice with sepsis.

MATERIAL AND METHODS

Animals

This study was approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine and was performed according to the NIH guidelines. The wild type (WT: C57Bl/6) and *ob/ob* (B6.Cg-Lepob/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Mice were injected with RSV (RSV) 30mg/kg (4ml/kg) or equivalent volume of DMSO (Vehicle) (4ml/kg) intraperitoneally 18 hours pre-sepsis. This dose of RSV in mice was as per documented literature²¹. In one group of mice, RSV pre-treated mice received EX-527 (10 mg/kg intraperitoneally; 4ml/kg, Vehicle: DMSO) within 10 minutes of cecal ligation and puncture.

Cecal ligation and puncture (CLP)

Sepsis was induced using CLP as described in the literature^{12,22}. Briefly, laparotomy incision (1-1.5 cm) was performed; cecum was identified, ligated and perforated two times with a 22-gauge needle. Contents were returned to abdominal cavity and incision was closed in two layers (peritoneum and skin), followed by fluid resuscitation (normal saline 1ml subcutaneously) given to each mouse. The Sham-operated mice underwent laparotomy and fluid resuscitation as described above, without cecal ligation or puncture. Mice with CLP (Sepsis) and Sham surgeries were used for tissue harvest (small intestinal tissue) or intravital fluorescent video microscopy 6 hours post-surgery.

Intravital fluorescent video microscopy (IVM)

Six hours after CLP/Sham surgery, the mice underwent carotid artery and jugular venous cannulations. The laparotomy incision was opened, small intestine (ileum) was exteriorized and care was taken to keep small intestine moist through the entire procedure. After allowing 30 minutes to stand, the small intestinal microcirculation was studied (n= 4-6 mice/group) using Nikon Eclipse FN1 microscope as described previously^{5,12}. Mice were injected with Rhodamine G(Sigma-Aldrich; St Louis, MO, USA: 0.02% in 100 μ L of PBS) for *in vivo* leukocyte visualization (labeled red) while platelets were labeled green *ex vivo* with carboxyfluorescein diacetate succinimidyl ester (CFSE: Sigma-Aldrich; St Louis, MO, USA; 90 μ M). This allowed simultaneous monitoring of leukocytes and platelets in each venule.

The details of the platelet isolation technique are as outlined previously⁶. Leukocyte contamination of the platelet suspension was evaluated from a 25 μ l sample to ensure that the leukocyte count of the infusate did not exceed 0.01%. The platelets (n=100 \times 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 platelets²³.

Post-capillary venules (three to five in number; 4-6 mice per group) in each mouse intestine were recorded to quantify leukocyte and platelet adhesion. Recordings were analyzed for one minute. A cell (leukocyte/platelet) was considered adherent if it remained stationary for at least 30 consecutive seconds of the one minute recording. The mean of the average values of leukocyte (LA) and platelet adhesion (PA) determined in each mouse were then used to generate a group mean.

Immunohistochemistry of small intestinal tissue

Small intestinal tissue was harvested and frozen using OTC medium. Acetone fixed frozen sections of tissue were stained using SIRT-1 (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). CyTM3-conjugated labeled secondary antibodies for SIRT-1 and E-selectin and FITC conjugated secondary antibody for VWF were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA).

Image acquisition—Virtual images were captured with an Olympus VS110 (20x/0.75 objective). Static images were captured utilizing OlyVIA (Olympus), at the designated magnification. We used Olympus XM10 camera for image acquisition.

Cell Culture

Human umbilical vein/vascular endothelium (HUVEC) cell line was obtained from American Type Culture Collection (CRL-1730) and maintained in F-12K medium containing 0.1mg/mL of heparin, 0.03-0.05mg/mL endothelial cell growth supplement, 100 units/ml penicillin, 100mg/ml streptomycin and 10% of heat inactivated FBS at 37 °C and 5% CO₂. Early passage (2-4) cultures were used.

RNA extraction and RT-PCR

HUVEC were pre-treated with Vehicle (DMSO) or 10uM of RSV for 24 h prior to further stimulation with LPS (100ng/mL) for 4 h. RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). The mRNA expression levels of target genes were quantified by quantitative real-time PCR with SensiFASTTM Probe Lo-ROX One-Step Kit (Bioline, BIO-78005) as described before¹². All results were normalized to GAPDH RNA levels. Relative quantification was calculated using the comparative threshold formula. All samples were run in quadruplicates to calculate average and SE value. TaqMan primer/probes for GAPDH, E-Selectin and ICAM-1 were purchased from Invitrogen (Grand Island, NY, USA).

Western blot

HUVEC were seeded in 6-well plate (3×10⁵ cells per well) the before the treatment and stimulation as described above. Total cell lysate was collected in lysis buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% glycerol, plus Complete mini Protease Inhibitor Cocktail from Roche) followed by the sonication for 10min. Cell debris were removed, protein concentration determined with Bio-Rad DC protein assay and adjusted into the same value

and resolved by SDS-PAGE, Protein levels were measured by immunoblotting, using GAPDH as loading control. The membrane was blocked and then incubated with antibodies against SIRT-1 (sc-15404, Santa Cruz Biotechnology, USA), Ac-p65 (Abcam, ab19870, 1:300), total p65 (Santa Cruz Biotechnology, sc-372, 1:1000) and GAPDH (AM4300, 1:15000; Invitrogen, Carlsbad, CA, USA) overnight. The blots were incubated with Alexa Fluor 680-conjugated secondary antibodies (827-10080 and 827-10081, 1:10,000; LI-COR Biosciences, Lincoln, NE, USA) on day 2. The signal was developed and quantified with a Li-COR Odyssey Infrared Imager system (Li-COR Biosciences).

Statistics

All data were 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

Mean arterial blood pressure, weights and leukocyte counts of mice in different groups

The mean arterial blood pressure (MAP) and weights of different groups of mice are shown in Table 1. As expected, weights of all *ob/ob* mice were significantly higher than the corresponding WT cohort. We did not find significant differences in the mean arterial blood pressure (MAP) between *ob/ob* or WT mice experimental groups.

We show that the total leukocyte counts of *ob/ob* Sham +Vehicle mice were significantly lower compared to WT counterpart, while no such differences were found in between Sham +RSV, Sepsis+Vehicle and Sepsis+RSV groups. Similarly, there were no statistically significant differences found in polymorphonuclear neutrophil counts (PMN) between different groups of mice.

Resveratrol decreases leukocyte and platelet adhesion in intestinal microcirculation in obese mice with sepsis

We have shown previously that leukocyte and platelet adhesion is increased in WT mice with polymicrobial sepsis; we further observed that *ob/ob* mice⁶ and SIRT-1 deficient WT mice sepsis had further increase in leukocyte adhesion¹². These observations supported that SIRT-1 deficiency participated in an exaggerated adhesion response and suggested that SIRT-1 activation may attenuate leukocyte adhesion during sepsis. We first tested this concept in WT sepsis and found that pre-treatment with RSV decreased leukocyte and platelet adhesion in early sepsis. We then tested the effect of RSV in *ob/ob* mice with and without sepsis. As depicted in **Figure 1**, we show that leukocyte and platelet adhesion in vehicle treated *ob/ob* mice were significantly higher compared to WT mice with sepsis, a finding consistent with our previous report⁶. More importantly, mice pre-treated with RSV showed significantly decreased leukocyte and platelet adhesion compared to vehicle treated mice in *ob/ob* septic mice. Leukocyte and platelet adhesion in intestinal microcirculation in *ob/ob* and WT mice were significantly increased compared to respective Sham controls and there were no significant differences leukocyte/platelet adhesions between different sham

groups. These results demonstrate that RSV limits leukocyte and platelet adhesion in *ob/ob* and WT mice with sepsis.

Resveratrol decreases E-selectin and ICAM-1 expression in intestinal tissue in obese mice with sepsis

We and others^{4,12,24} have reported that the E-selectin and ICAM-1 expression modulate leukocyte adhesion in models of inflammation and sepsis^{12,24}. Recently, we showed that SIRT-1 inhibition increases E-selectin/ICAM-1 expression during sepsis¹² and together with results in Figure 1, suggest an inverse relationship between SIRT-1 and E-selectin/ICAM-1 expression. To test this inverse relationship, we used immunohistochemistry (IHC) to observe the effect of RSV on E-selectin and ICAM-1 expression in small intestinal tissue in WT and *ob/ob* mice with sepsis. As we had previously found, E-selectin and ICAM-1 expression increased in small intestinal tissue of WT and *ob/ob* mice with sepsis (**Figure 2 A/B and C/D**, E-selectin and ICAM-1 respectively). We observed better co-localization between ICAM-1-vWF compared to E-selectin-vWF, probably due to non-specific staining pattern of either vWF or E-selectin. To resolve the issue of effect of RSV on endothelial-specific E-selectin expression, (see below).

Consistent with an inverse relationship hypothesis however, we found decreased E-selectin and ICAM-1 expression in RSV treated mice. Taken together, these results suggest that E-selectin and ICAM-1 expression correlate with leukocyte adhesion during sepsis. Moreover, we observed an inverse relationship between E-selectin/ICAM-1 and SIRT-1.

Resveratrol decreases E-selectin and ICAM-1 expression via increased SIRT-1 in endothelial cells

To examine the effect of RSV on endothelial cell SIRT-1 and adhesion molecule expression, we treated HUVEC with RSV overnight and stimulated with LPS. First we tested whether RSV treatment increased SIRT-1 expression in HUVEC. As shown in **Figure 3 A and B** we show that there was significantly increased expression of SIRT-1 in RSV treated HUVEC.

Next we tested whether the RSV treatment decreased adhesion molecule expression in HUVEC. As shown in **Figure 4 A and B**, there was a significant decrease in E-selectin and ICAM-1 mRNA expression in RSV treated HUVEC vs. Vehicle when stimulated with LPS.

SIRT-1 is known to deacetylate p65²⁵. So we further tested whether RSV treatment in HUVEC modulates acetylated p65 expression. As shown in **Figure 4C**, there was a dose dependent decrease in Ac-p65 in HUVEC treated with RSV.

Resveratrol increases SIRT-1 expression in intestinal tissue in obese mice with sepsis

The beneficial effects of RSV are attributed to its ability to increase SIRT-1 expression in SIRT-1 deficient states such as aging and obesity^{14,25-27}. The next experiments are designed to confirm (1) *ob/ob* as a SIRT-1 depleted state in mouse intestine and (2) the inverse relationship between SIRT-1 and E-selectin/ICAM-1 expression. We confirmed decreased SIRT-1 expression in the intestine of *ob/ob* mice compared to WT Sham and observed that SIRT-1 decreases with sepsis in both, WT (**Figure 5A**) and *ob/ob* mice (**Figure 5B**).

Consistent with our results in HUVEC, we also found that RSV treated mice had increased SIRT-1 expression in the small intestine in both, *ob/ob* and WT mice with sepsis.

Resveratrol decreases microvascular inflammation in obese septic mice via SIRT-1 modulation

To confirm that decreased leukocyte adhesion and E-selection expression in *ob/ob* mice is dependent on increased SIRT-1 expression we treated *ob/ob* mice with both, RSV and a SIRT-1 specific inhibitor EX-527. As shown in **Figure 6A and B**, the attenuation of leukocyte and platelet adhesion seen in RSV treated *ob/ob* mice with sepsis was completely abolished by the addition of EX-527. This supports that RSV acts to decrease cell adhesion in *ob/ob* septic mice through increased SIRT-1 expression.

We then determined the contribution of SIRT-1 dependent adhesion molecule expression to this increase in leukocyte/platelet adhesion in Sepsis + RSV + EX-527 compared to Sepsis + RSV. We studied E-selectin and ICAM-1 expression in the small intestinal tissue. As shown in **Figure 6 C and D**, we found that in *ob/ob* mice, the E-selectin and ICAM-1 expression in Sepsis + RSV + EX-527 were increased compared to Sepsis + RSV although not to the same extent as Sepsis + Vehicle groups. The data from Figure 6 suggest that RSV attenuates microvascular inflammation via modulation of SIRT-1.

Taken together, our data confirm and extend an observation that obesity is a SIRT-1 deficient state. We found that *ob/ob* mice have decreased intestinal SIRT-1 and increased microvascular inflammation compared to WT mice with sepsis. Conversely, increased SIRT-1 using RSV treatment attenuated microvascular inflammation via E-selection/ICAM-1 expression *ob/ob*-septic mice.

RSV improves survival in obese septic mice

Given decreased adhesion/adhesion molecule expression and microvascular inflammation with RSV treatment, we then determined whether RSV pre-treatment would affect 7-day survival in *ob/ob* mice with sepsis. As shown in **Figure 7**, in *ob/ob* mice treated with RSV had 100% survival compared to 12.5% survival in vehicle treated mice.

DISCUSSION

This study improves our understanding of the role of NAD⁺ sensor SIRT-1 in sepsis-induced inflammation, with focus on the microvasculature. The major findings include: 1) RSV acts by inducing SIRT-1 to limit the degree of leukocyte-platelet adhesion in the small intestine of septic *ob/ob* and WT mice without significant differences in the circulating leukocyte/PMN counts, 2) This microvascular response is associated with decreased expression of E-selectin and ICAM-1 via decreased Ac-p65 expression in endothelial cells 3) The effect of RSV in sepsis, at least in part, is by inducing SIRT-1 activity and 4) RSV pre-treatment markedly improves survival in *ob/ob* mice with sepsis.

Sepsis is the 11th leading cause of death in US with high cost of care in the non-coronary intensive care units^{28,29}. Microvasculature with its strategic place at the interface of systemic circulation and local tissue environment is highly sensitive to changes in both,

local and systemic inflammation. Leukocyte adhesion is a rate determining factor in inflammatory responses including sepsis. Not surprisingly, endothelial cell activation markers, especially adhesion molecule expression biomarkers, like E-selectin, directly correlate with mortality in sepsis³⁰. We have studied leukocyte and platelet adhesion in early and late phases of sepsis and shown that increased leukocyte adhesion in early sepsis is followed by endotoxin tolerance in the microcirculation in late sepsis¹². We have also shown that the leukocyte and platelet adhesion are exaggerated in obese compared to lean mice with early sepsis⁶. The results presented here suggest that leukocyte adhesion in the microvascular circulation can be modulated to improve sepsis outcomes.

Obesity increases resource utilization and cost of care in critically ill patients^{10,11}. Obesity is a SIRT-1 deficient state¹⁴. Recently, we discovered that SIRT-1 genetic deficiency or pharmacologic inhibition SIRT-1 during early sepsis exaggerates leukocyte adhesion via increased E-selectin and ICAM-1 expression and decreases sepsis survival in mice¹². We also have shown previously that the E-selectin and ICAM-1 antibody administration abolishes leukocyte adhesion in microcirculation in our previous work^{6,24}. While RSV is shown to reduce chronic inflammatory phenotype in obesity¹⁷, the effect of RSV in acute inflammation in obese subjects is not very well known. Moreover, the effect of RSV on microvascular dysfunction in sepsis is also not very well studied. To begin to address this knowledge gap, we studied the effect of RSV induced increase in SIRT-1 expression in SIRT-1 deficient *ob/ob* mice with acute inflammation of sepsis. We show that RSV pre-treatment decreases circulating cell-endothelial cell interaction in the microcirculation via modulation of E-selectin and ICAM-1 expression.

To further examine the effect of RSV on endothelial cells specifically, we treated HUVEC with RSV and studied E-selectin, ICAM-1 and SIRT-1 expression in response to LPS stimulus. Similar to intestinal tissue, we show inverse relationship between RSV induced SIRT-1 and adhesion molecule expression in HUVEC.

The anti-inflammatory action of RSV *in vivo* may be due to either increased expression of SIRT-1 or AMPK. To further study the contribution of increased SIRT-1 in decreasing cell adhesion and adhesion molecule expression we inhibited SIRT-1 with EX-527 in RSV pre-treated *ob/ob* septic mice. EX-527 is known to be a selective sirtuin inhibitor³¹. We show that the attenuation of leukocyte and platelet adhesion seen in RSV treated *ob/ob* mice with sepsis were completely abolished by the addition of EX-527 via modulation of E-selectin and ICAM-1 expression (**Figure 6**). Together these data support the inverse relationship between SIRT-1 and E-selectin expression in *ob/ob* septic mice.

Sir2 gene was first linked to replicative lifespan of budding yeast as a nuclear regulator of heterochromatin, and it acts by sensing nicotinamide adenine dinucleotide (NAD⁺) and deacetylating nearby histones³². There are seven mammalian homologues of Sir2 proteins. Of these, SIRT-1 is studied most extensively. SIRT-1 is a critically important director of the innate immune response, as well as the metabolic shifts associated with acute and chronic inflammation³³⁻³⁵. As a chromatin modifier, SIRT-1 directs many cell and organism processes. One clearly established effect on inflammation is its connection with the NFκB, where it controls RelA transactivation properties by deacetylating lysine 310, thereby

deactivating and degrading this critically important gene activator. Among the many genes whose transcription is supported by RelA and its control by SIRT-1 are proinflammatory cytokines like TNF- α and IL-1 β , as well as adhesion regulators, including E-selectin^{12,36}

The SIRT-1 concept also applies to human inflammation^{37,38}. RSV, a known anti-inflammatory and anti-cancer agent increases SIRT-1 expression, which limits the transactivation of RelA (p65)^{26,27,32}. One plausible contributing mechanism for our observation that RSV protects microvascular proinflammatory adhesion responses in septic mice is by limiting RelA activity and reducing expression of E-selectin/ ICAM-1. Moreover, E-selectin/ ICAM-1 induction would be amplified in *ob/ob* mice with its association with low SIRT-1 activity and further decreases in early sepsis as noted in this study. We examined the mechanism responsible for the “anti-inflammatory” effect of RSV in endothelial (HUVEC) in this project. Specifically, we studied acetylated p65 expression in HUVEC in response to LPS and showed that RSV, in a dose-dependent manner, decreased Ac-p65 and adhesion molecule expression in HUVEC stimulated with LPS. Other mechanisms likely contribute, such as limiting the systemic inflammation induced by amplified expression of proinflammatory cytokines, like TNF-alpha-induced acetylation of NF-kappa B p65²⁵ and increased AMPK activity^{15,39}. Although p65 acetylation has an established role in acute inflammation, additional effects of RSV on histone and/or chromatin modification need further study.

Accumulating evidence supports SIRT-1 as a critically important regulator of immune and metabolic homeostasis⁴⁰. In contrast to the effects of low SIRT-1 on early inflammation, as demonstrated by this study, late sepsis is associated with persistent elevations in NAD⁺ and SIRT-1^{12,38}. Sustained SIRT-1 activity due to unknown mechanisms contributes to the high mortality of late sepsis, as demonstrated by our recent report that inhibiting SIRT-1 by EX-527 reverses immune suppression and rescues septic mice from death¹². More studies are needed to better understand how SIRT-1 activity can be targeted to treat chronic and acute inflammatory diseases. In the case of sepsis, this is a critical need, since no such treatments are available and deaths are rising. This study supports a preventive approach to improving sepsis outcomes, especially in at risk obese or elderly patients, by modifying inflammation at the microvascular interface.

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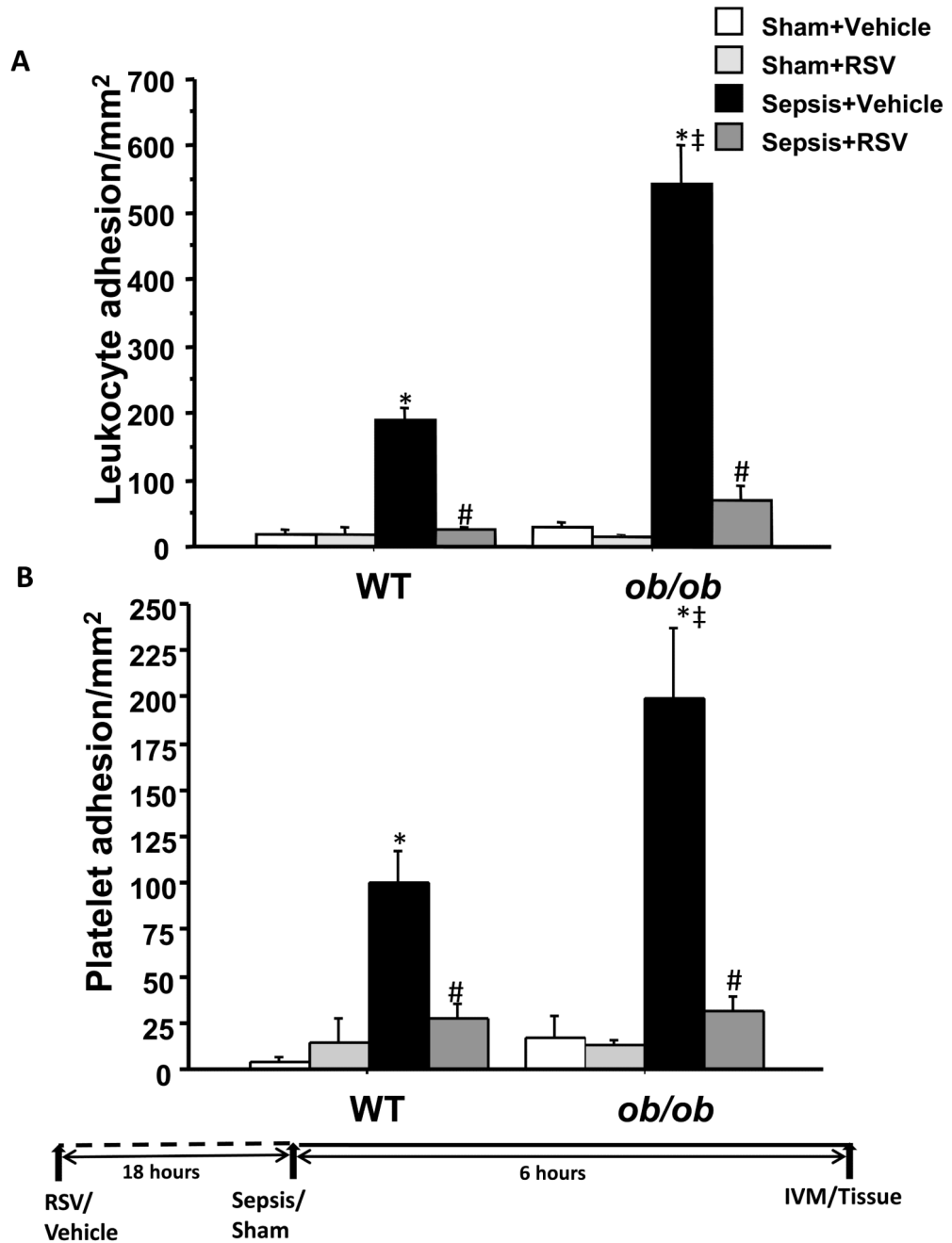


Figure 1. RSV attenuates leukocyte and platelet adhesion in small intestinal microcirculation Leukocyte (**Figure 1A**) and platelet (**Figure 1B**) adhesion in small intestinal microcirculation (mice n=4-6/group) (mean \pm SEM) were measured using intravital microscopy (IVM). Leukocyte and platelet adhesion in Sepsis + Vehicle groups (black bars) were significantly increased vs. Sham + Vehicle (white bars) groups in both, WT and *ob/ob* strains. Sepsis + Vehicle treated *ob/ob* mice had significantly higher leukocyte and platelet adhesion than WT mice. Sepsis + RSV (dark gray) groups had significantly attenuated leukocyte and platelet adhesion compared to Sepsis + Vehicle in both WT and *ob/ob* strains;

n=4-6/group * p<0.05 vs. respective Sham + Vehicle group; # p<0.05 vs. respective Sepsis + Vehicle groups' ‡ p<0.05 vs. WT Sepsis + Vehicle group using Tukey's post-hoc analysis; error bars: s.e.m.

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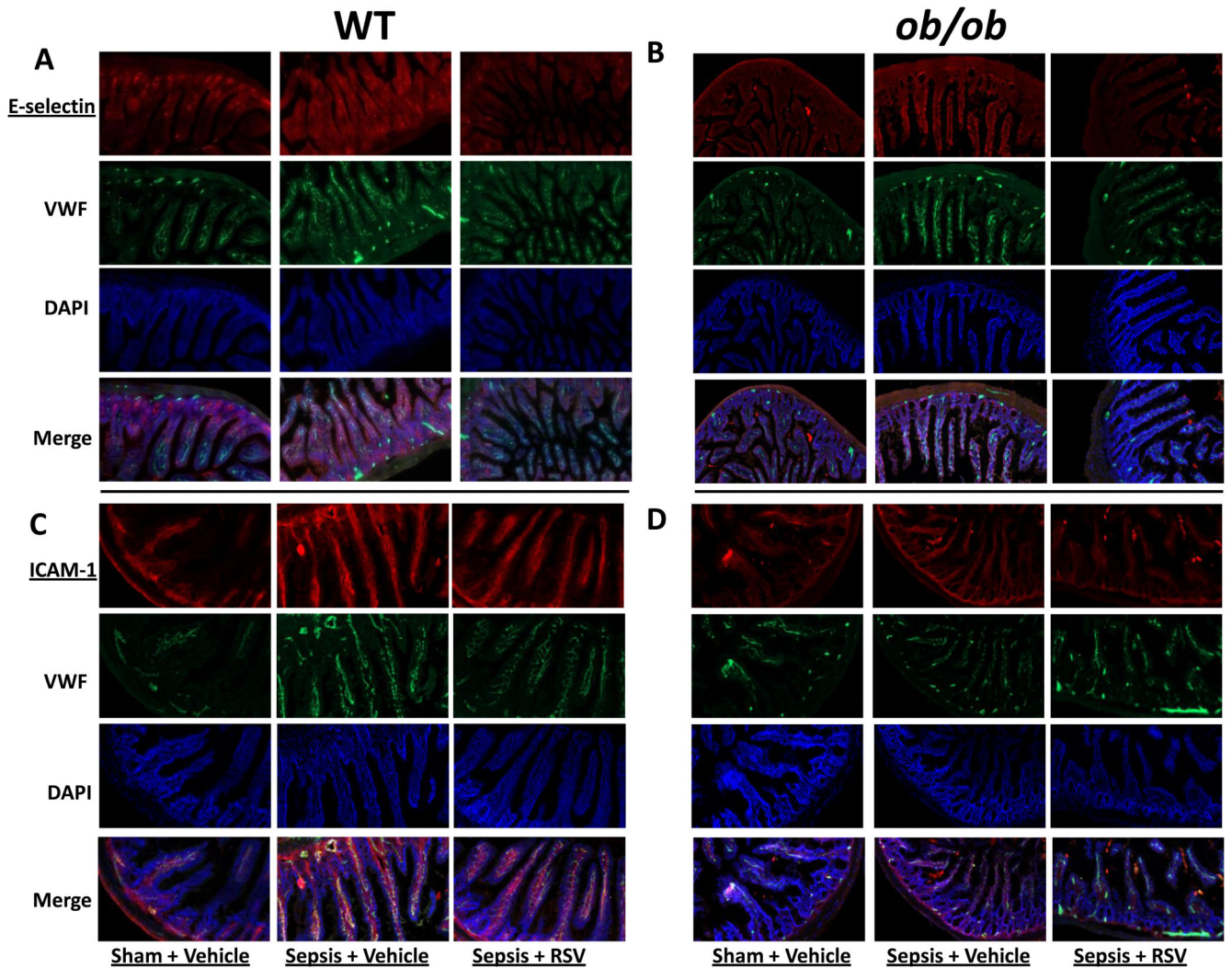


Figure 2. RSV attenuates E-selectin and ICAM-1 expression in small intestinal tissue

We show the E-selectin and ICAM-1 expression in small intestinal tissue sections were studied using immunohistochemistry (20× magnification) in WT and *ob/ob* mice. E-selectin expression in Sepsis + Vehicle group was significantly increased vs. Sham + Vehicle group in both, WT (2A) and *ob/ob* (2B) strains. Sepsis + RSV groups had significantly decreased E-selectin expression compared to Sepsis + Vehicle in both WT and *ob/ob* strains.

We show ICAM-1 expression in small intestinal tissue sections in WT (Figure 2C) and *ob/ob* (Figure 2D) mice. Similar to E-selectin expression, ICAM-1 expression in Sepsis + Vehicle group were significantly increased vs. Sham + Vehicle group in both, WT and *ob/ob* tissue sections. Sepsis + RSV groups had significantly decreased ICAM-1 expression compared to Sepsis + Vehicle in both WT and *ob/ob* strains.

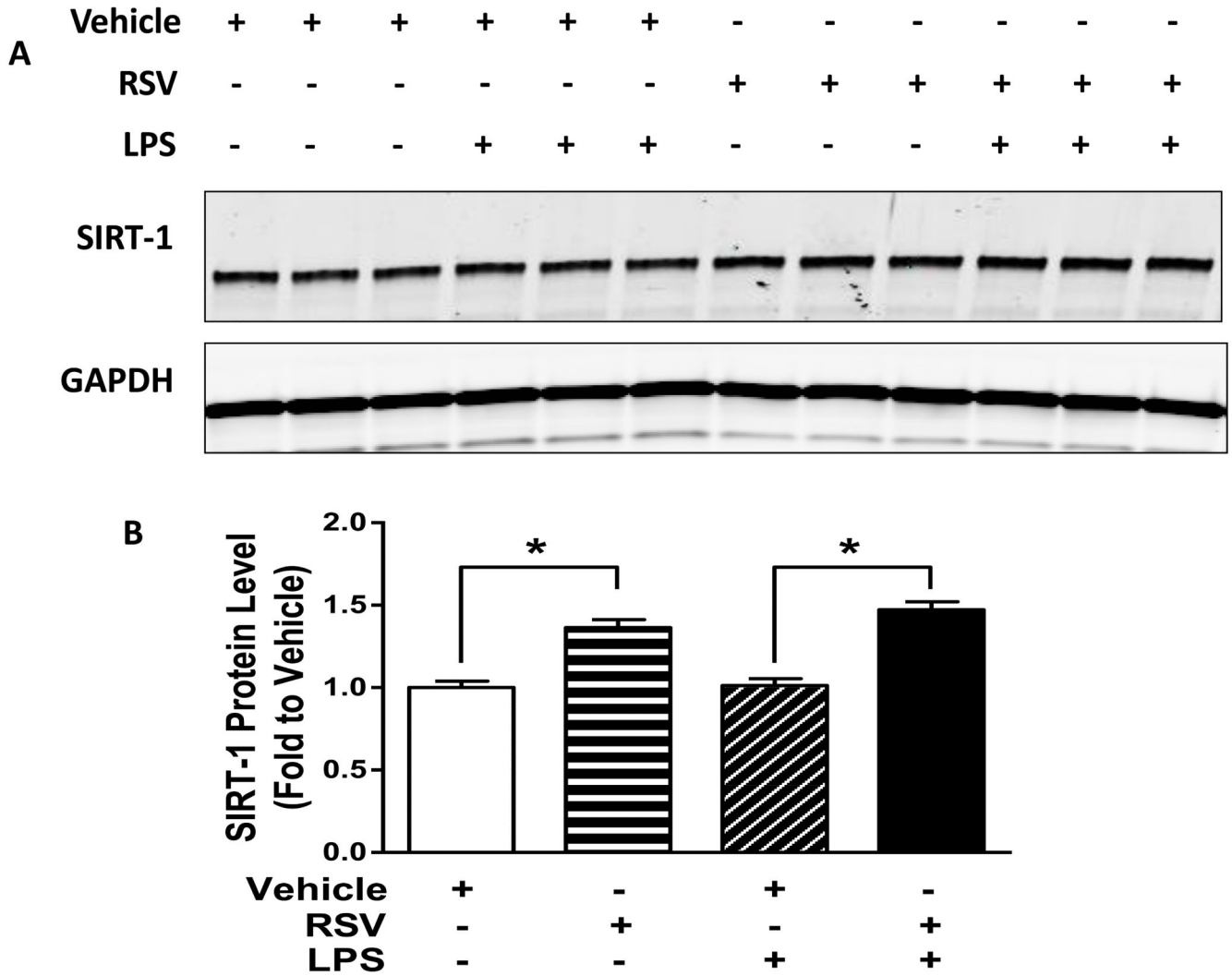


Figure 3. RSV increases SIRT-1 expression in HUVEC

We next studied if RSV treatment increased SIRT-1 protein expression in HUVEC using Western Blot. Cells were pre-treated with RSV and stimulated with LPS as indicated.

Representative WB and quantitative bar graph are shown in **Figures 3A and B** respectively.

RSV treatment increased SIRT-1 protein expression significantly (* $p < 0.05$ vs. Vehicle treatment).

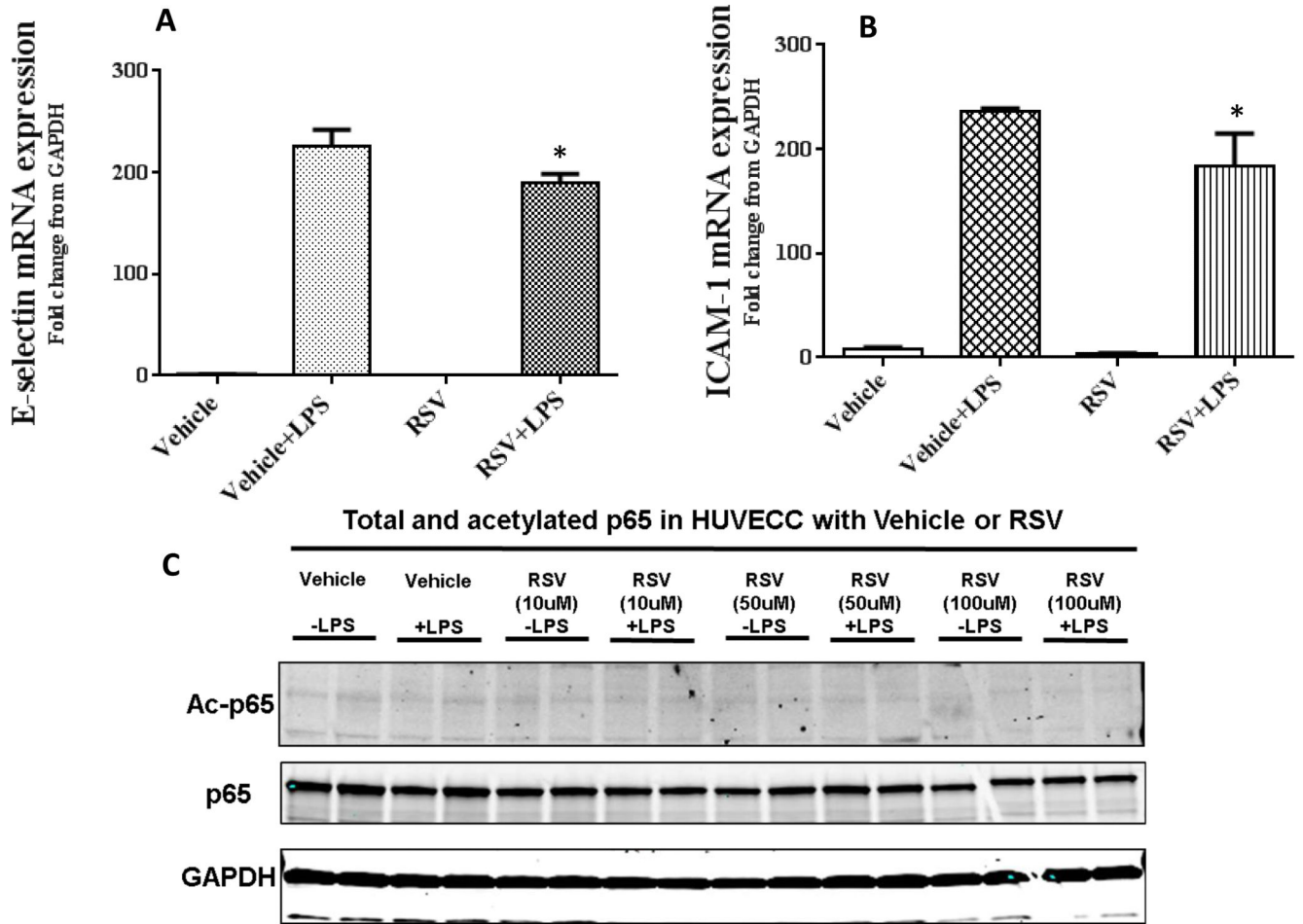


Figure 4. Resveratrol attenuates E-selectin and ICAM-1 expression and decreases p65 acetylation

We next examined the effect of RSV on E-selectin and ICAM-1 expression in human umbilical vein endothelial cells (HUVEC). Cells were pre-treated with RSV/Vehicle, stimulated with LPS and E-selectin and ICAM-1 mRNA (mean \pm SEM) expression were studied. We show that RSV treatment attenuates E-selectin (Figure 4A) and ICAM-1 (Figure 4B) expression with LPS stimulus compared to Vehicle (* $p < 0.05$ vs. Vehicle + LPS). Next we studied the effect of RSV treatment on p65 acetylation in endothelial cells with and without LPS stimulus. As shown in the representative WB blots in **Figure 4C**, there was reduction in AC-p65 expression in HUVEC with RSV in a dose dependent manner.

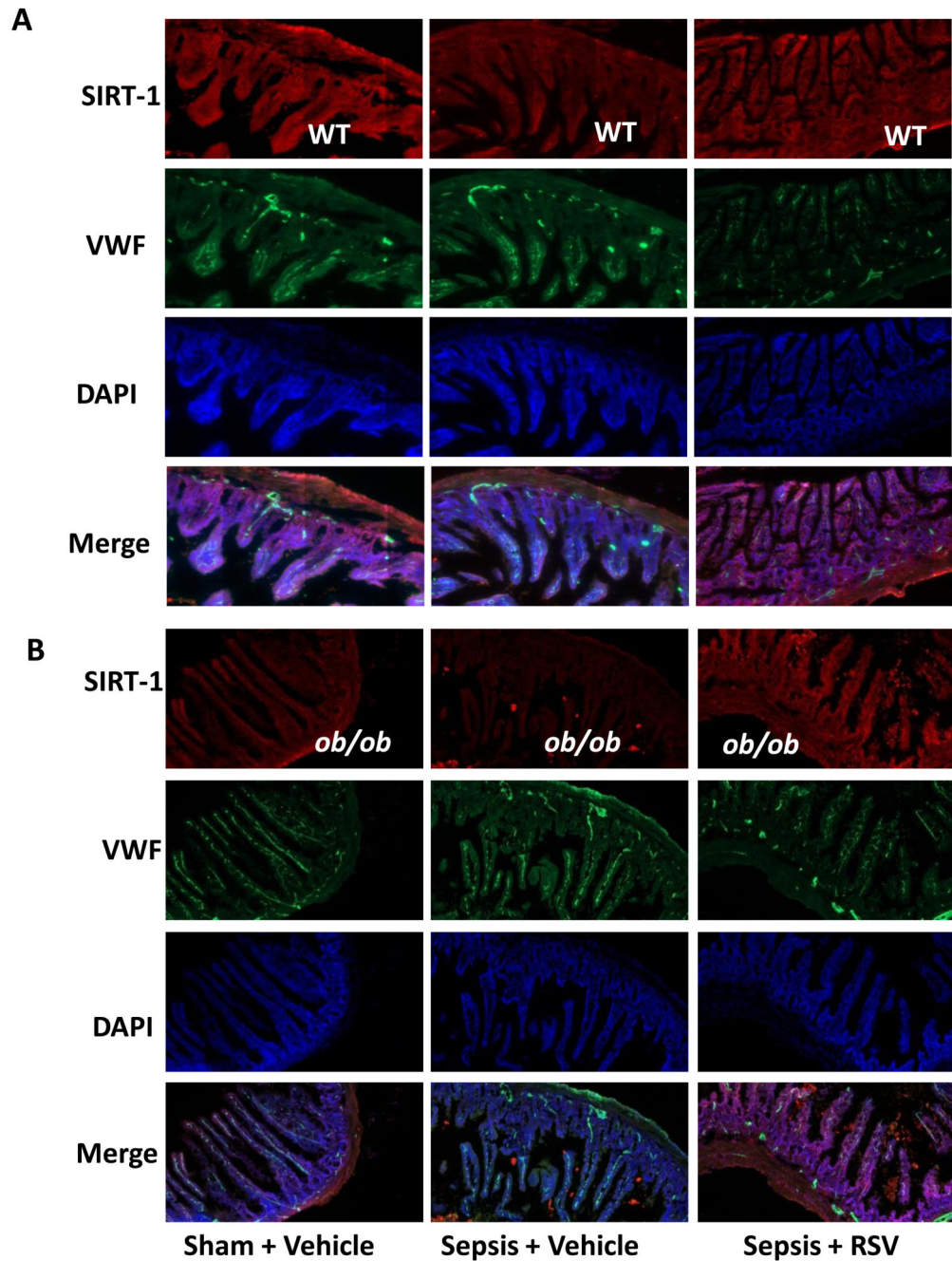


Figure 5. RSV increases SIRT-1 expression in small intestinal tissue

SIRT-1 expression in small intestinal tissue sections was studied using immunohistochemistry (20× magnification) in WT (**Figure 5A**) and *ob/ob* (**Figure 5B**) mice. There was a lower SIRT-1 expression in *ob/ob* vs. WT Sham+Vehicle groups. SIRT-1 expression in Sepsis + Vehicle group was significantly decreased vs. Sham + Vehicle group in both, WT and *ob/ob* strains. Sepsis + RSV groups had significantly increased SIRT-1 expression compared to Sepsis + Vehicle in both WT and *ob/ob* strains.

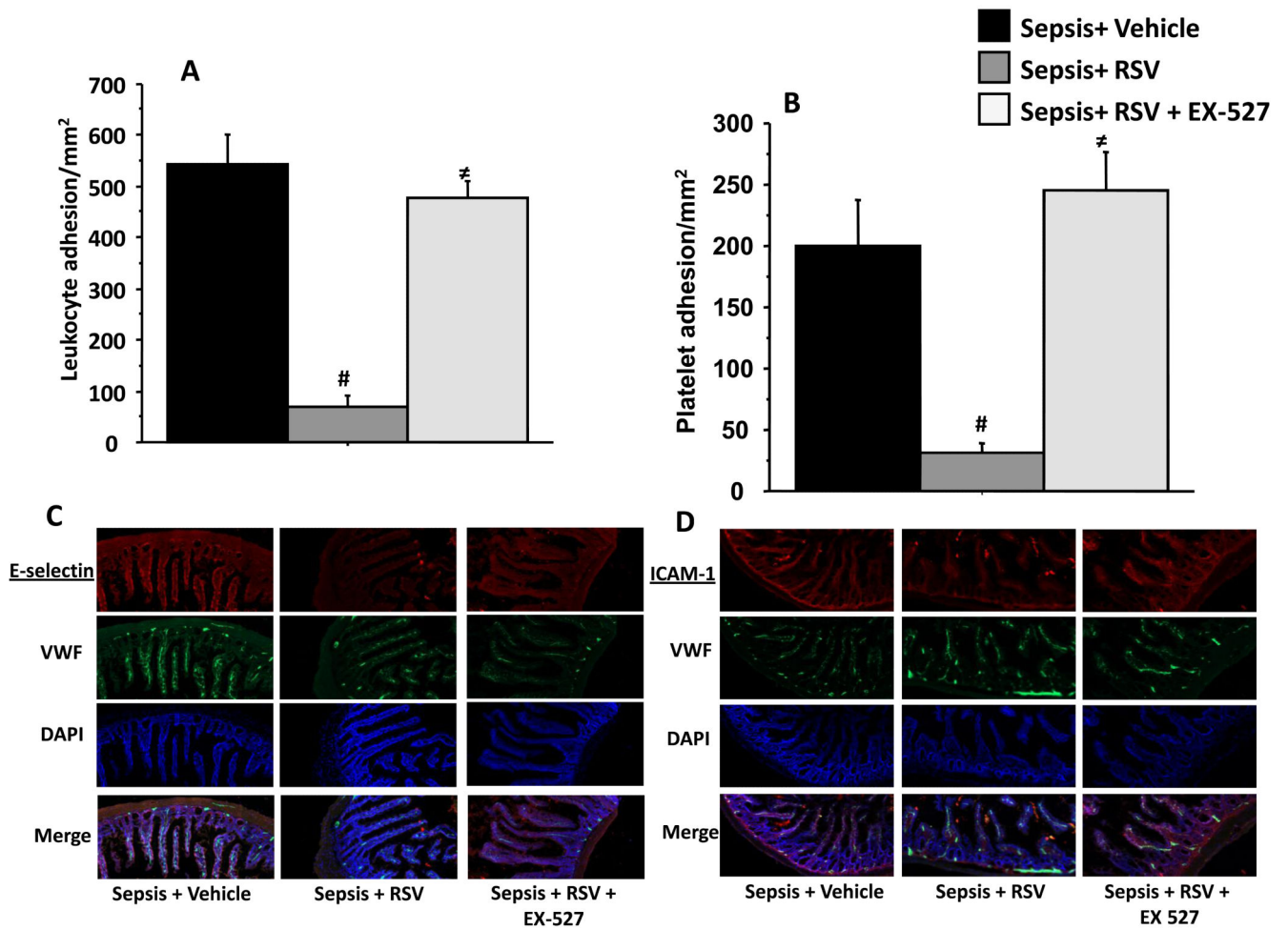


Figure 6. RSV decreases leukocyte adhesion in obese septic mice via SIRT-1 modulation
 RSV treated *ob/ob* mice with sepsis were treated with SIRT-1 specific inhibitor EX-527 and leukocyte and platelet adhesion were studied in small intestinal microcirculation using IVM and compared to Sepsis + Vehicle (black bars) and Sepsis + RSV (dark grey) treated groups. Leukocyte (**Figure 6A**) and platelet adhesion (**Figure 6B**) in Sepsis + RSV + EX-527 (light grey) groups were significantly increased compared to Sepsis + RSV group. There was no statistically significant difference in leukocyte and platelet adhesion between Sepsis + Vehicle vs. Sepsis + RSV + EX-527 group. # $p < 0.05$ vs. Sepsis + Vehicle; $p < 0.05$ vs. Sepsis + RSV. We next studied the E-selectin and ICAM-1 expression using immunohistochemistry (20 \times magnification) in Sepsis + Vehicle, Sepsis + RSV and Sepsis + RSV + EX-527 treated *ob/ob* mice with sepsis. As shown in Figure 6C and D, E-selectin and ICAM-1 expression in Sepsis + RSV + EX-527 treated mice were increased compared to Sepsis + RSV respectively.

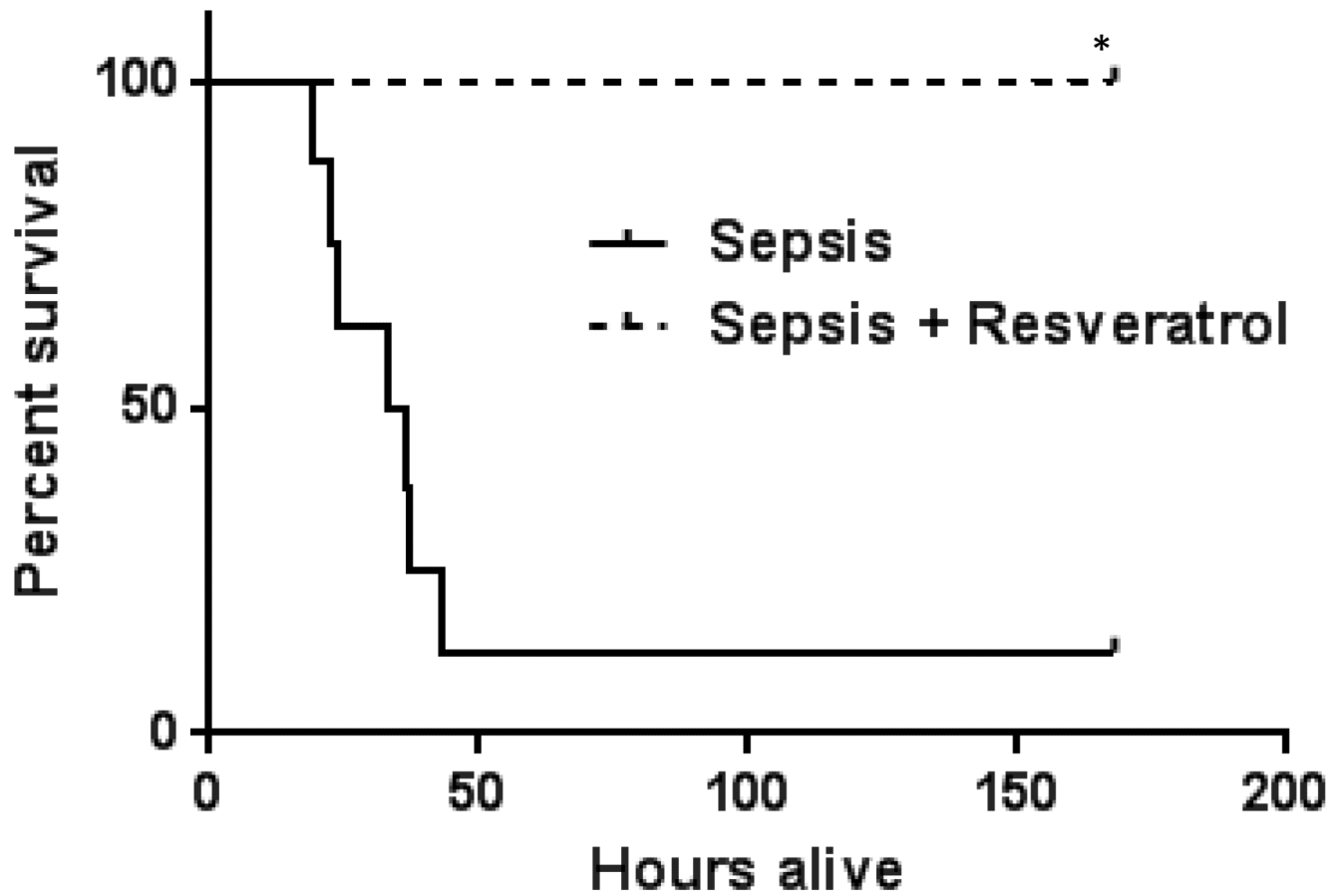


Figure 7. RSV improves survival in obese septic mice

We treated *ob/ob* septic mice with either Vehicle or RSV and studied 7-day survival. As shown in **Figure 7**, in *ob/ob* mice Sepsis+ RSV treated mice had significantly higher survival compared to Sepsis+ Vehicle group. * $p < 0.05$ vs. Sepsis.

Table 1
Mean arterial blood pressure (MAP), peripheral leukocyte/neutrophil count and weight in different groups

	MAP (Mean ± SEM)	Weight (Mean ± SEM)	Total leukocytes/ μ l (Mean ± SEM)	PMN/ μ l (Mean ± SEM)
WT Sham + Vehicle	68±3	24.64±1.34	7687±187	3125±1000
WT Sepsis + Vehicle	66±7	29.03±0.48	3125±544 [#]	1154±624
WT Sham + RSV	77±3	24.13±1.49	6937±187	2625±375
WT Sepsis + RSV	73±3	26.69±0.83	6625±190	1750±144
<i>ob/ob</i> Sham + Vehicle	63±1	42.41±1.95 [*]	5750±250 [*]	2312±187
<i>ob/ob</i> Sepsis + Vehicle	64±2	40.70±3.04 [*]	3458±110	2582±397
<i>ob/ob</i> Sham + RSV	69±4	37.31±3.5 [*]	5750±375	1268±981
<i>ob/ob</i> Sepsis + RSV	73±3	38.30±2.19 [*]	5333±512	2833±480

* p<0.05 vs. respective WT control group

[#] p<0.05 vs. WT Sham+ Vehicle

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