Cellular Physiology and Biochemistry

Cell Physiol Biochem 2019;52:565-579
DOI: 10.33594/000000040
Published online: 22 March 2019
Accepted: 18 March 2019

Original Paper

Amitriptyline Reduces Inflammation and Mortality in a Murine Model of Sepsis

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Key Words
Sepsis • Amitriptyline • Macrophage • IL-10 • p38

Abstract
Background/Aims: During sepsis, an unchecked pro-inflammatory response can be detrimental to the host. We investigated the potential protective effect of amitriptyline (AT). ***Methods:*** We used two murine models of sepsis: Cecal ligation and puncture and endotoxemia following LPS challenge. Aural temperatures were taken and cytokines quantified by cytometric bead assay. Lung injury was determined histologically and by protein determination in bronchoalveolar lavage fluid. Cell accumulation in the peritoneum was analyzed by flow cytometry, as well as cytokine production and p38-phosphorylation. Neutrophil chemotaxis was evaluated using an *in vitro* transwell assay. ***Results:*** Our findings demonstrate that AT-treated septic mice have improved survival and are protected from pulmonary edema. Treatment with AT significantly decreased serum levels of KC and monocyte chemoattractant protein-1, as well as the accumulation of neutrophils and monocytes in the peritoneum of septic mice. Peritoneal IL-10 levels in septic mice were increased upon AT treatment. Direct treatment of septic mice with IL-10 recapitulated the effects of AT. Endotoxemic mice also exhibited enhanced IL-10 production upon AT-administration and peritoneal macrophages were identified as the AT-influenced producers of IL-10. Treatment of these cells with AT *in vitro* resulted in increased p38-phosphorylation and IL-10 generation, whereas ceramide and p38 inhibition had the opposite effect. ***Conclusion:*** Altogether, AT treatment improved survival, increased IL-10 levels, and mitigated a pro-inflammatory response during sepsis. We conclude that AT is a promising therapeutic to temper inflammation during septic shock.

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Introduction

Among hospitalized patients diagnosed with sepsis or septic shock, the mortality secondary to sepsis-related complications approaches 25% [1]. Timely initiation of critical care, including early antibiotic administration, has been shown to significantly decrease mortality rates [2, 3]. However, despite these interventions, sepsis deaths have increased within the past decade [4].

Macrophages are among the first responders to the septic insult and can produce a number of cytokines, including pro-inflammatory tumor necrosis factor alpha (TNFα) but also anti-inflammatory IL-10. The balance between pro- and anti-inflammatory cytokines released after insult influences the extent of inflammation during sepsis and the outcome. Increased IL-6:IL-10 ratios are seen in non-survivors 48 hours after onset of sepsis [5]. When the initial host response causes exaggerated inflammation, this can result in tissue injury and may progress further to single- or multi-organ dysfunction [6], even if the infectious source is controlled with antibiotics. Thus, the hyper-inflammatory response may still give way to the lethal sequelae of tissue injury, multi-organ dysfunction and death.

Previous work from our group demonstrates that AT has anti-inflammatory effects in scald-injured mice [7] and mitigates sepsis-induced TNFα expression and coagulation in a mouse model of sepsis [8]. Furthermore, Peng et al. have demonstrated that AT mitigates superoxide formation, which normally results in the breakdown of tissue tight junction proteins during *Staphylococcus aureus* (*S. aureus*) sepsis [9]. Regarding cytokines, AT-administration for 4 weeks has been reported to increase splenic IL-10 levels in mice [10] and in *in vitro* studies, AT mitigated IL-1ß and TNFα production by macrophages in response to *S. aureus* α-toxin [11]. Taken together, these data highlight a potential therapeutic benefit of AT treatment in sepsis.

Mechanistically, AT has been reported to upregulate microglia p38 mitogen-activated protein kinase activity and IL-10 expression in chronically morphine-infused rats [12]. However, the impact of AT-treatment in the context of sepsis is relatively unexplored. Altogether, we postulated that AT-treatment during sepsis may also result in increased IL-10 generation, potentially ameliorating the early pro-inflammatory response associated with sepsis.

Materials and Methods

Ethical statement

All murine experiments were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati (protocol number: 08-09-19-01).

Mouse housing

Male CF-1 outbred mice aged 6-8 weeks (Charles River Laboratories, Wilmington, MA) were used for experiments. Mice were acclimated at least one week prior to experimentation and were housed in standard environmental conditions with corn-cob bedding in groups of four. A standard pellet diet and water *ab libitum* was provided. All murine experiments were performed between 8 AM and 1 PM.

Polymicrobial sepsis and endotoxemia models

Mice were subjected to cecal ligation and puncture (CLP) to induce polymicrobial sepsis, as previously described [7, 13]. During surgeries, mice were anesthetized with 2% isoflurane in oxygen, shaved over the abdominal area, disinfected with povidone-iodine, and placed supine on a heating pad. Following a 1-2 cm midline laparotomy incision, 66% of the cecum was ligated with a 4-0 silk tie (Syneture, Norwalk, CT). A through-and-through puncture was made on the anti-mesenteric side with a 22-gauge needle and a small amount of feces was extruded through the puncture holes to ensure perforation. The cecum was replaced in its original location, and the midline incision was closed in two layers with 4-0 silk. Following CLP, mice were resuscitated with 1 mL sterile normal saline (NS), (Hospira, Lake Forest, IL, USA) injected subdermally,

and placed on a heating pad for one hour. Sham mice underwent the above process except for ligation and puncture.

Endotoxemia was induced as previously described [8] through a 1 mg intraperitoneal (IP) injection of lipopolysaccharide (LPS), which was derived from Escherichia coli 0111:B4 strain (Sigma-Aldrich, St. Louis, MO) and solubilized in NS (Hospira).

Amitriptyline treatment

At the time of CLP or LPS challenge, mice were randomized to IP delivery of 0.9% NS (Hospira, Lake Forest, IL) or 16 mg/kg AT (Sigma-Aldrich) solubilized in NS, as previously described by Peng et al. [9].

IL-10 treatment

To evaluate the role of IL-10 during the early phase of sepsis, mice were randomized to intraperitoneal delivery of NS or 500 ng recombinant murine IL-10 (BioLegend, San Diego, CA) solubilized in NS at the time of CLP.

Cytokine analysis

Whole blood was harvested by cardiac puncture, collected in serum separator tubes (BD Biosciences, San Jose, CA), and centrifuged at 10, 000 x g for 10 minutes at 20°C. Peritoneal fluid was harvested by peritoneal lavage and centrifuged at 450 x g for 10 min at 9°C. The serum and cell-free peritoneal fluid were stored in sterile tubes and frozen at -80°C until cytokine analysis. The IL-6, IL-10, KC and MCP-1 concentrations in the samples were analyzed by cytometric bead array (BD Biosciences), as previously described [14].

Bacterial counts

Whole blood and peritoneal fluid were harvested as previously described [15, 16] using cardiac puncture and peritoneal lavage, respectively. Aerobic samples were serially diluted in sterile PBS and cultured on Tryptic Soy Agar plates (BD Biosciences). The plates were incubated at 37°C for 24 hours, after which colony counts were performed.

Lung histology

Mice were sacrificed sixteen hours after CLP. Lungs were inflated with 1 mL 10 % formalin, harvested and fixed in 10 % formalin for 48 hours, dehydrated in 70 % ethanol for 72 hours and embedded in paraffin. 5-10 µm thick sections were prepared and stained with hematoxylin and eosin. Samples were viewed and graded with a 20 x light microscopy magnification by a blinded investigator. A pathology grading system with a numerical score was adopted from previous definitions and evaluated alveolar edema (0-3) and leukocyte infiltration (0-4) [17].

Bronchoalveolar lavage protein assay

Bronchoalveolar lavage (BAL) of NS and AT-treated mice were obtained by instillation of NS through the trachea with a 20-gauge catheter, followed by removal of 2 ml fluid. Protein concentration was determined using the BCA Protein Assay kit (Thermo Scientific, Waltham, MA). Absorbance was read at 562 nm using the ELx800 Microplate Reader (BioTek Instruments, Winooski, VT). A standard curve of BSA dilutions with known concentrations was used to calculate the protein concentrations of the samples.

Flow cytometry and PhosFlow analysis

Peritoneal cells were harvested by peritoneal lavage and centrifuged at 450 x g for 10 min. The pelleted cells enumerated with a cell counter (Beckman Coulter, Brea, CA), and analyses of cell surface antigen and intracellular cytokine expression were performed.

To analyze intracellular IL-10 expression *in vivo*, mice were pre-treated with protein transport inhibitor brefeldin A (BFA, BioLegend) as previously described [18]. 250 µg of BFA were administered IP 30 min prior to challenge with LPS and administration of NS or 16 mg/kg AT. Peritoneal macrophages were isolated 90 min after LPS injection by peritoneal lavage and intracellular staining was performed as previously described [19].

To evaluate macrophage IL-10 generation *in vitro*, peritoneal cells were obtained by lavage from healthy mice. 100 ng / mL LPS was administered along with 2 μM protein transport inhibitor monensin (MilliporeSigma, Darmstadt, Germany) and 100 μM AT or 10 μM ceramide (Avanti Polar Lipids, Alabaster, AL) or 20 μM p38 kinase inhibitor SB239063 as indicated. Intracellular IL-10 levels were assessed 3 hours later as previously described [19].

To determine phosphorylated p38, macrophages were incubated with 100 ng / mL LPS for 30 minutes and subsequently fixed by addition of 90 % methanol.

The following antibodies were used for flow cytometry analysis: Ly6G (Clone: 1A8, BD Biosciences), Ly6C (Clone: AL-21, BD Biosciences), CD11b (Clone: M1/70, BD Biosciences), F4/80 (Clone: T45-2342, BD Biosciences), IL-10 (Clone: JES5-16E3, BioLegend) and phospho-p38 (Clone: pT180/pY182, BD Biosciences). Flow cytometry acquisition and analysis were performed on an Attune Flow Cytometer (Life Technologies, Foster City, CA).

Chemotaxis assay

Bone marrow cells were harvested from femur and tibia in HBSS (ThermoFisher Scientific, Waltham, MA), centrifuged at 450 x g and counted on a cell counter (Beckman Coulter, Brea, CA). 2 million cells were seeded on a transwell insert with 3 μm pores (ThermoFisher Scientific). 100 ng KC were added to the bottom well and cells were incubated for 3 h at 37°C. Non-migrated cells from the transwell insert and migrated cells from the bottom well were recovered and analyzed by flow cytometry.

Statistics

GraphPad Prism 6.0 (Graphpad Software, La Jolla, CA) was used to perform statistical comparisons. A two-tailed Student's t-test was used for two groups, or one-way ANOVA with Tukey *post-hoc* analysis were used for more than two groups. Kaplan-Meier method was used to calculate survival distributions, which were then compared using log-rank test.

Results

Amitriptyline treated septic mice demonstrate improved survival and decreased occurrence of hypothermia

Administration of AT at the time of CLP increased survival of septic mice compared to control mice (Fig. 1A, p = 0.03). Endotoxemic mice show rapid mortality within 24h after the LPS challenge, which was also significantly ameliorated by AT administration (data not shown). Systemic IL-6 levels did not differ between treated and control mice six hours after CLP (Fig. 1B). Survival differences first became apparent sixteen hours after CLP. At this time, AT-treated septic mice were less hypothermic than control mice (Fig. 1C, -2.06 ± 0.46 °C CLP+NS vs -0.88 ± 0.43 °C CLP+AT, p = 0.03). There were no differences in local and systemic bacterial burden at this time (Fig. 1D), nor at an earlier time-point four hours after CLP (data not shown).

Amitriptyline treatment mitigates pulmonary injury during sepsis

AT treatment has previously been reported to ameliorate lung injury during *S. aureus* infection [9]. Here, we assessed lung pathology in our polymicrobial sepsis model. Tissues were harvested 16 h after CLP. Lung edema and polymorphonuclear leukocyte infiltration was ameliorated in AT-treated septic mice, compared to sham and NS-treated septic mice (Fig. 2A+B). In line with these observations, AT-treated septic mice also had reduced protein concentrations in BAL 3 h after CLP (Fig. 2C, 0.79 ± 0.24 mg/mL CLP+NS vs 0.19 ± 0.02 mg/mL CLP+AT, p = 0.03).

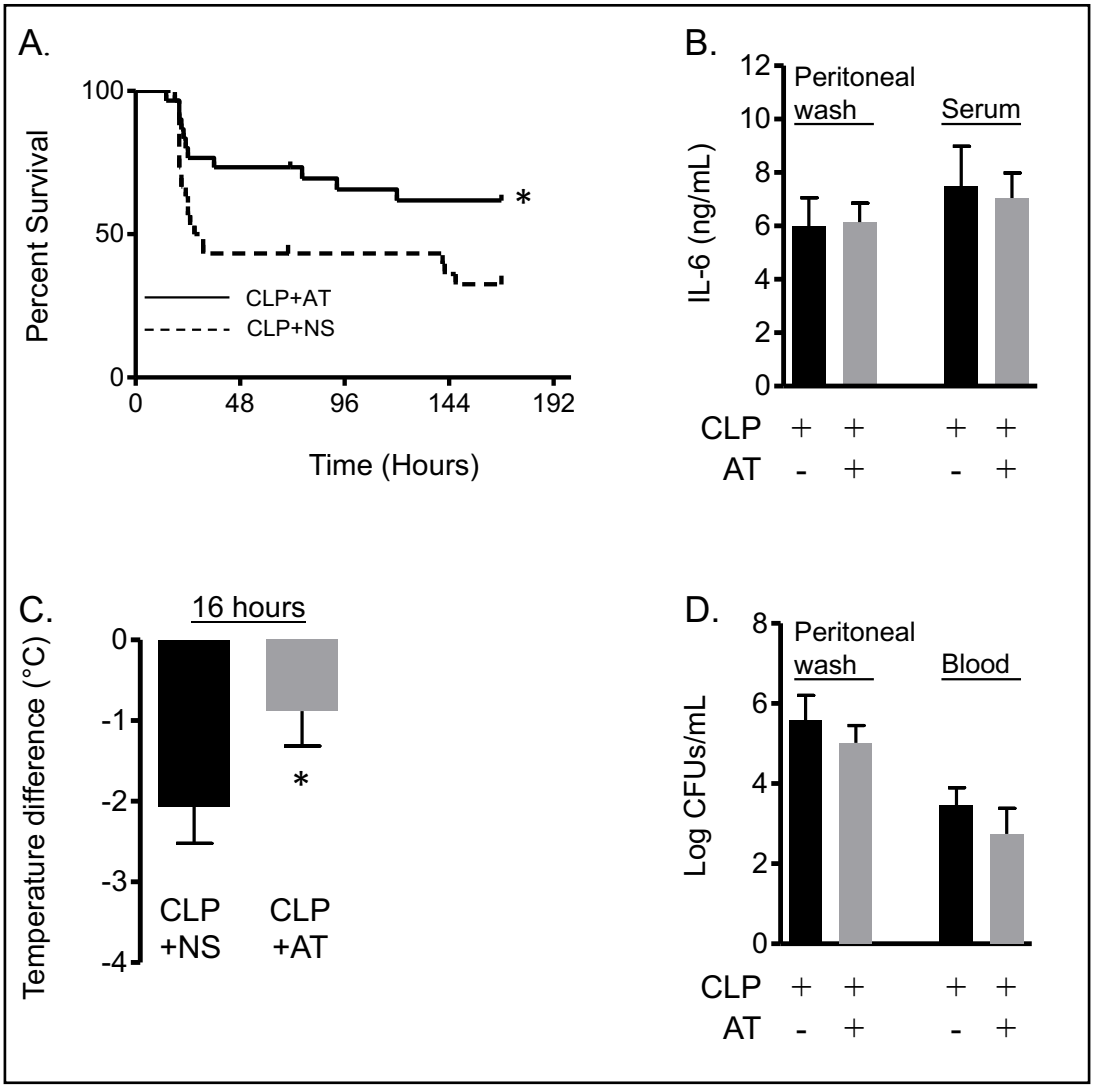

Fig. 1. Amitriptyline treated septic mice demonstrate improved survival and decreased hypothermia, despite no differences in local and systemic IL-6 levels and bacterial burden. Mice underwent CLP. A) Control and AT-treated mice were monitored for survival. * $p<0.05$ vs CLP+NS; n = 30 mice/group. B) Peritoneal wash and blood samples were collected 6 hours after CLP for cytokine analysis, and IL-6 was quantified for NS- and AT-treated septic mice. n = 10 mice/group. C) Aural temperatures of septic mice treated with NS or AT are shown 16 h after CLP. * $p<0.05$ vs CLP+NS; n = 25 mice/group. D) Peritoneal wash and blood were collected 16 hours after CLP and samples were cultured to determine bacterial burden. n = 6 mice/group.

Leukocyte chemoattractant levels and accumulation are decreased with AT treatment

The observed differences in leukocyte recruitment to the lung prompted us to also examine the recruitment of neutrophils and monocytes to the site of the septic insult – the peritoneal cavity. We analyzed leukocyte recruitment to the peritoneum 3 h after CLP and observed a 1.7-fold decrease in peritoneal neutrophil accumulation in AT-treated septic mice compared to NS saline treated mice (Fig. 3A, 2.26 ± 0.48 x 10^6 cells/mL CLP+NS vs 1.31 ± 0.23 x 10^6 cells/mL CLP+AT, p = 0.04). This coincided with a 1.4-fold decrease in serum KC in the AT-treated group (Fig. 3B, 1.90 ± 0.13 ng/mL CLP+NS vs 1.37 ± 0.17 ng/mL CLP+AT, p = 0.02). In an *in vitro* assay, we also tested whether AT influences chemotaxis of neutrophils directly. AT treatment decreased neutrophil chemotaxis towards KC 2.4-fold (Fig. 3C, 55.38 ± 12.45 % CLP+NS vs. 22.98 ± 13.59 % CLP+AT, p = 0.0005). Similarly, a 1.9-fold decrease in peritoneal monocyte accumulation (Fig. 3D, 1.00 ± 0.11 x 10^6 cells/mL CLP+NS vs 0.52 ±

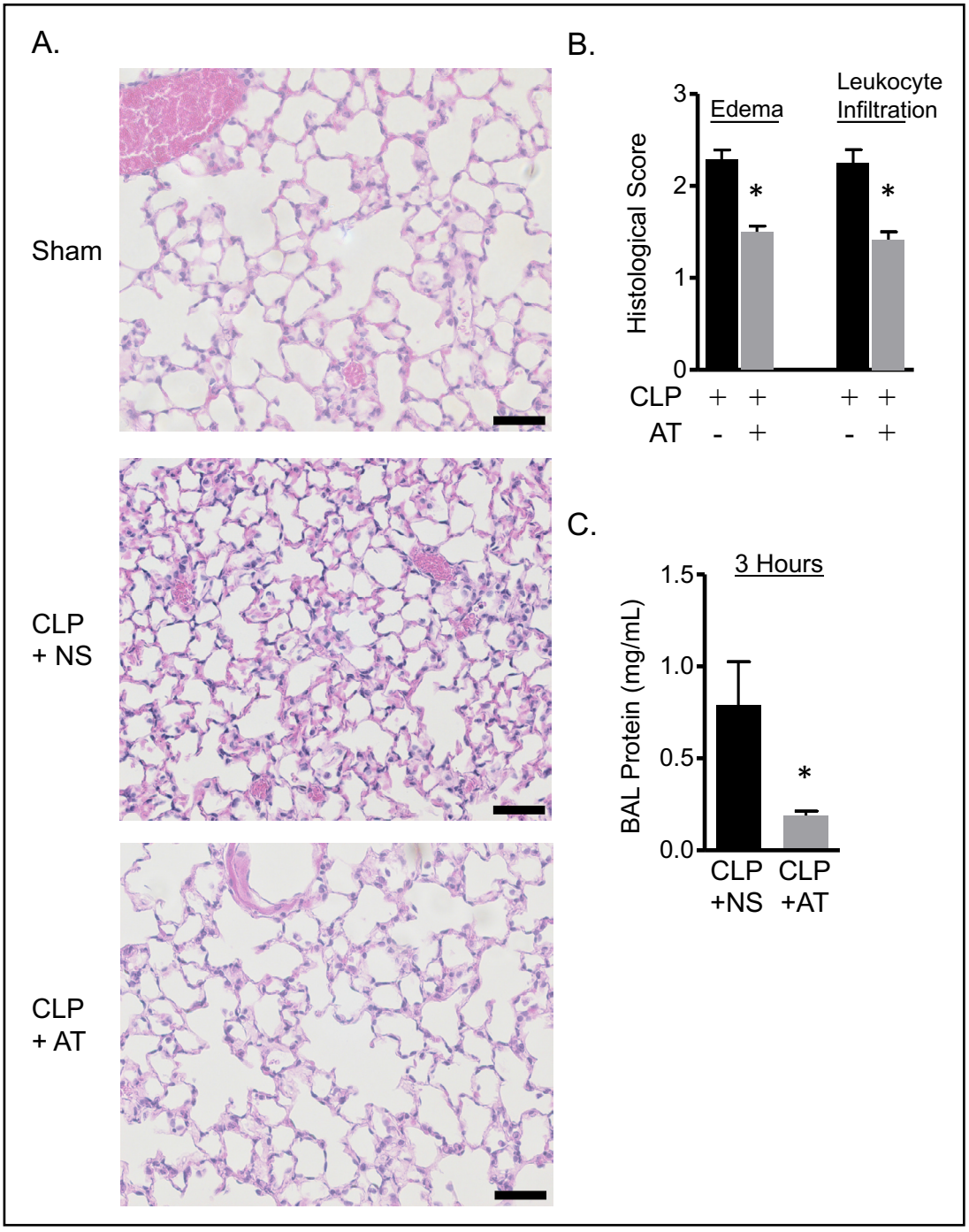

Fig. 2. Amitriptyline treatment mitigates pulmonary injury during sepsis. Lungs from sham and septic mice were collected 16 hours after CLP. A) Samples were embedded in paraffin, stained with hematoxylin and eosin, and sectioned. B) Edema and leukocyte infiltration histology scores were compared between septic control and AT-treated mice. * $p<0.05$ vs CLP+NS; n = 6 mice/group. C) BAL fluid was collected and protein concentration was determined. * $p<0.05$ vs CLP+NS; n = 6 mice/group.

0.11 x 10^6 cells/mL CLP+AT, $p < 0.01$) and a 3-fold decrease in serum MCP-1 levels (Fig. 3E, 4.13 ± 0.97 ng/mL CLP+NS vs 1.32 ± 0.37 ng/mL CLP+AT, $p = 0.02$) was observed with AT-treatment. Serum KC and MCP-1 levels in sham mice were below the detection limit (data not shown).

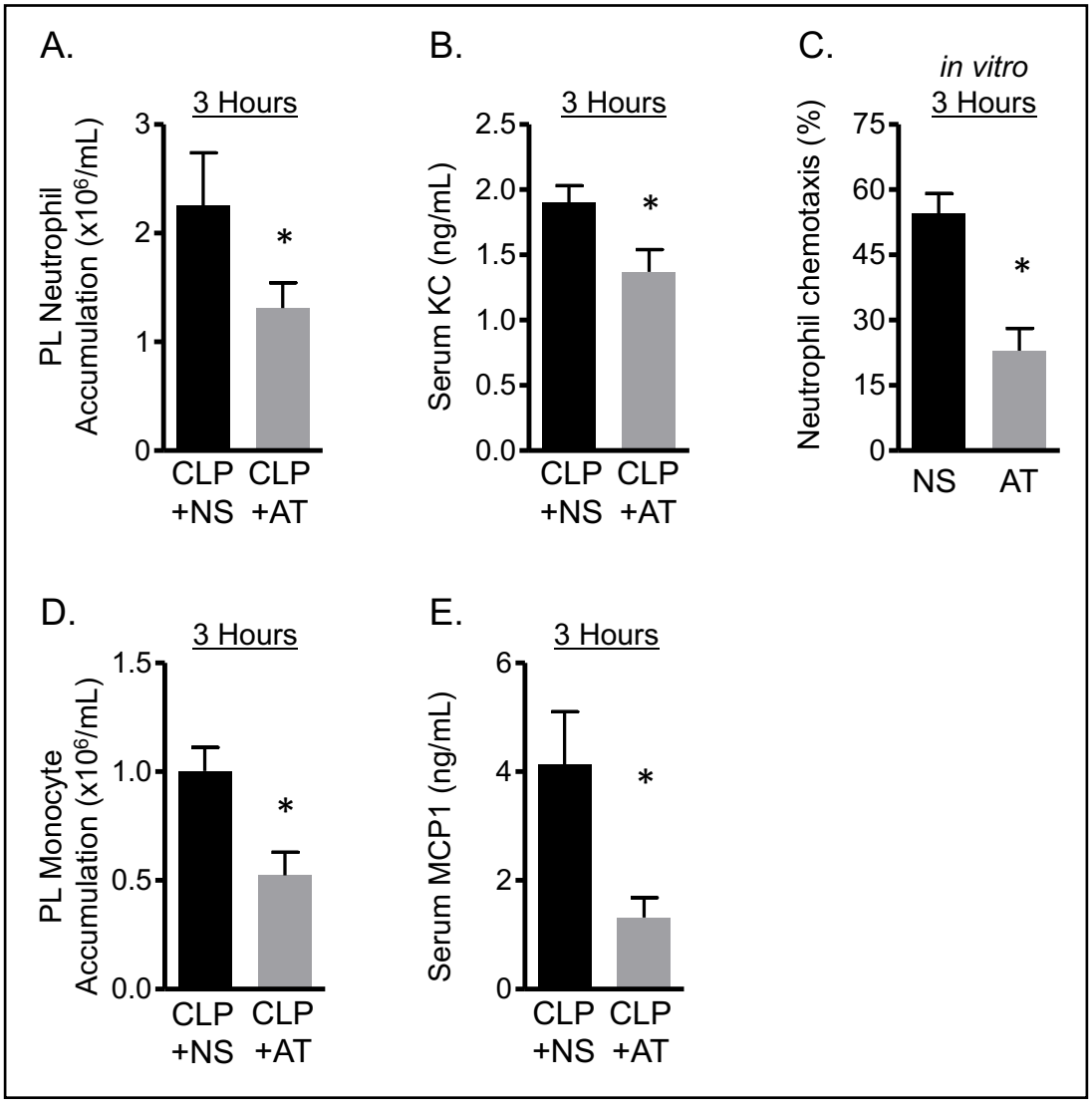

Fig. 3. Leukocyte chemoattractant levels and accumulation are decreased with AT treatment. Peritoneal lavage (PL) and blood were collected for cellular and cytokine analysis, respectively. A) Neutrophil accumulation and B) serum KC levels were determined for septic control and AT-treated mice three hours after CLP. * $p<0.05$ vs CLP+NS; n = 8 mice/group. C) Neutrophil chemotaxis toward KC was determined in an in vitro transwell assay and analyzed by flow cytometry. * $p<0.05$ vs NS; n = 8 replicates in 2 independent experiments. D) Monocyte accumulation and E) serum MCP-1 levels were determined for septic control and AT-treated mice three hours after CLP. * $p<0.05$ vs CLP+NS; n = 8 mice/group.

IL-10 production is increased in septic AT-treated mice and exogenous IL-10 administration reiterates the amelioration of the host response seen with AT treatment

Due to suppressed leukocyte accumulation and unchanged IL-6 levels in AT-treated septic mice compared to septic control mice, we investigated the role of anti-inflammatory IL-10. AT-treatment caused a 2.7-fold increase in peritoneal IL-10 concentration in septic mice 6 hours after CLP (Fig. 4A, 26.50 ± 4.92 pg/mL CLP+NS vs 72.20 ± 21.16 pg/mL CLP+AT, p = 0.04). To establish the role of IL-10 in the response of AT-treated mice to sepsis, mice were treated with exogenous IL-10 at the time of CLP. Similar to AT-treatment, administration of IL-10 reduced hypothermia in septic mice (Fig. 4B, -4.63 ± 0.81°C CLP+NS vs -1.69 ± 0.52°C CLP+IL-10, p = 0.01). Treatment with IL-10 also decreased peritoneal KC levels (Fig. 4C, 2.75 ± 0.20 ng/mL CLP+NS vs 1.99 ± 0.20 ng/mL CLP+IL-10, p=0.02) and neutrophil accumulation

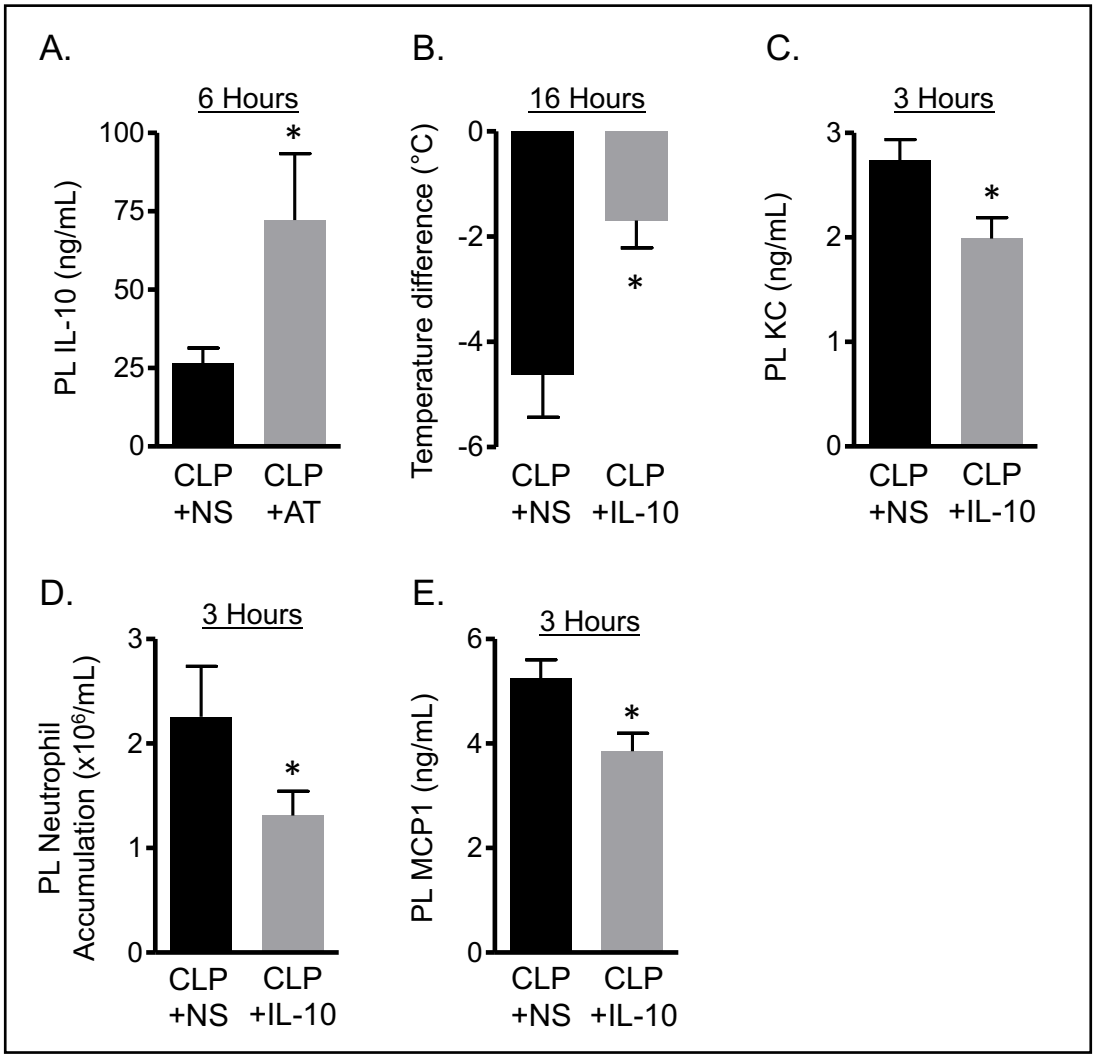

Fig. 4. IL-10 production is increased in septic AT-treated mice and exogenous IL-10 administration reiterates the amelioration of septic shock and leukocyte response seen with AT treatment. A) IL-10 levels were quantified from peritoneal lavage (PL) six hours after CLP; * $p<0.05$ vs CLP+NS; n = 10 mice/group. B) Mice were randomized to intraperitoneal delivery of NS or 500 ng recombinant murine IL-10 at the time of CLP. Aural temperature was determined 16 hours post-CLP. * $p<0.05$ vs CLP+NS; n = 8 mice/group. PL and blood were collected for cellular and cytokine analysis, respectively. C) Serum KC levels and D) local neutrophil accumulation were determined for septic control and IL-10-treated mice three hours after CLP. * $p<0.05$ vs CLP+NS; n = 16 mice/group. E) Serum MCP-1 levels were determined for septic control and IL-10-treated mice three hours after CLP. * $p<0.05$ vs CLP+NS; n = 16 mice/group.

(Fig. 4D, 2.59 ± 0.35 x 10^6 cells/mL CLP+NS vs. 1.82 ± 0.21 x 10^6 cells/mL CLP+IL-10, p = 0.03) 3 hours after CLP. Peritoneal MCP-1 levels were also reduced in the IL-10 treated group (Fig. 4E, 5.25 ± 0.35 ng/mL CLP+NS vs 3.85 ± 0.34 ng/mL CLP+IL-10, $p<0.01$), but monocyte recruitment was not significantly changed (data not shown).

Peritoneal macrophage IL-10 production is increased in AT-treated septic mice

In order to confirm the increase in IL-10 production seen in CLP mice upon AT treatment in another model of sepsis, we tested the IL-10 response of endotoxemic mice 90 min after the LPS challenge. AT treatment of endotoxemic mice increased serum IL-10 levels 10-fold compared to the control group (Fig. 5A, 0.32 ± 0.06 ng/mL LPS+NS vs 3.14 ± 0.33 ng/mL LPS+AT, $p < 0.01$).

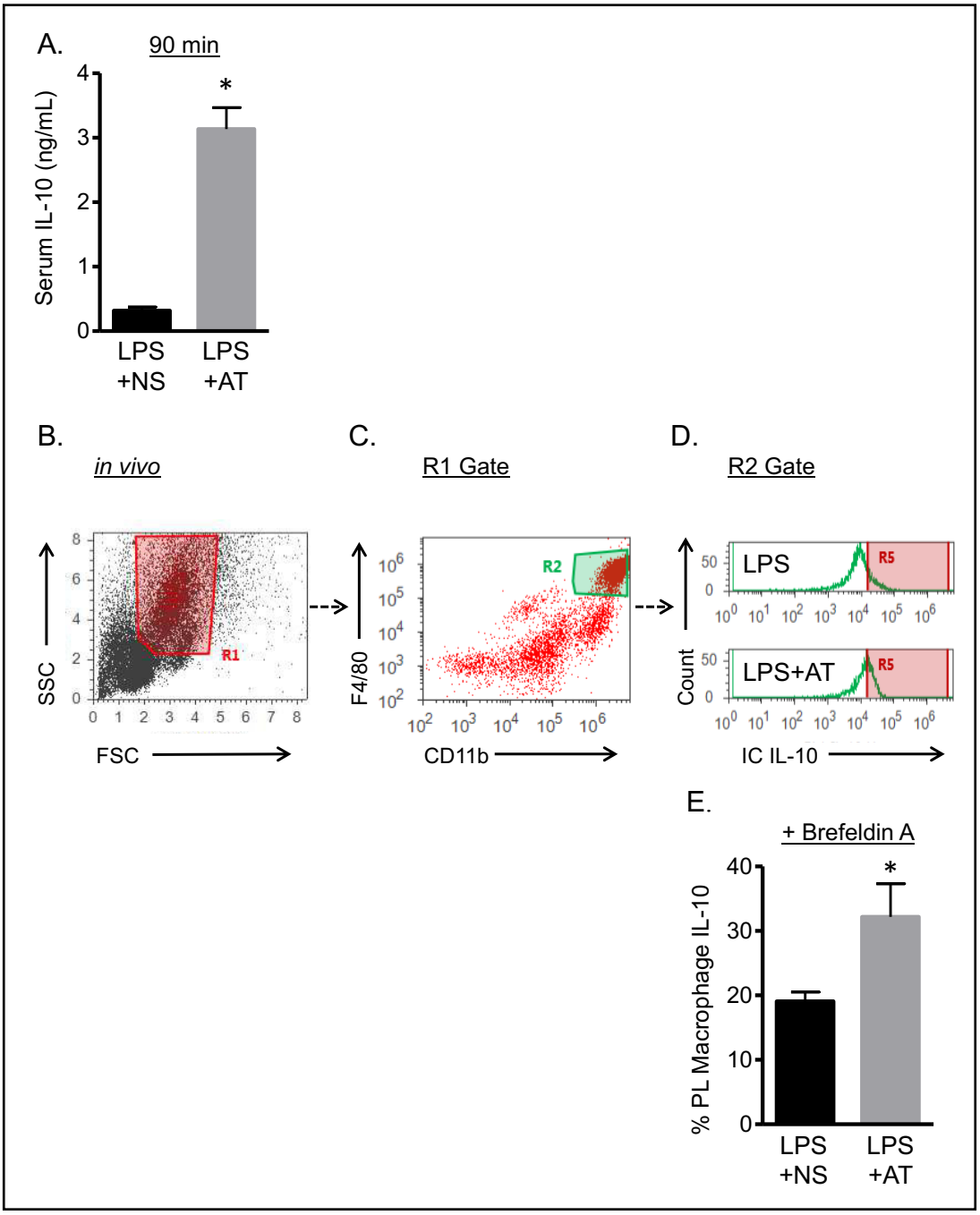

Fig. 5. Macrophages are the predominant source of IL-10 production in AT-treated septic mice. A) IL-10 levels were quantified from blood 90 minutes after LPS challenge; *, p<0.05 vs LPS+NS; n = 8 mice/group. B-E) Mice were treated with protein transport inhibitor Brefeldin A, challenged with LPS and treated with either NS or AT in vivo. Peritoneal leukocytes were isolated and analyzed by flow cytometry for intracellular IL-10 content. Macrophages were gated based on CD11b and F4/80 expression. * p<0.05 vs LPS+NS; n = 4 mice/group.

We next sought to determine which cells produce IL-10 in an AT-mediated manner. In order to stop cytokine secretion and thus enable intracellular detection, we pre-treated mice with the protein transport inhibitor brefeldin A. This was followed by LPS challenge and treatment with NS or AT. Ninety minutes later, peritoneal cells were isolated and analyzed by

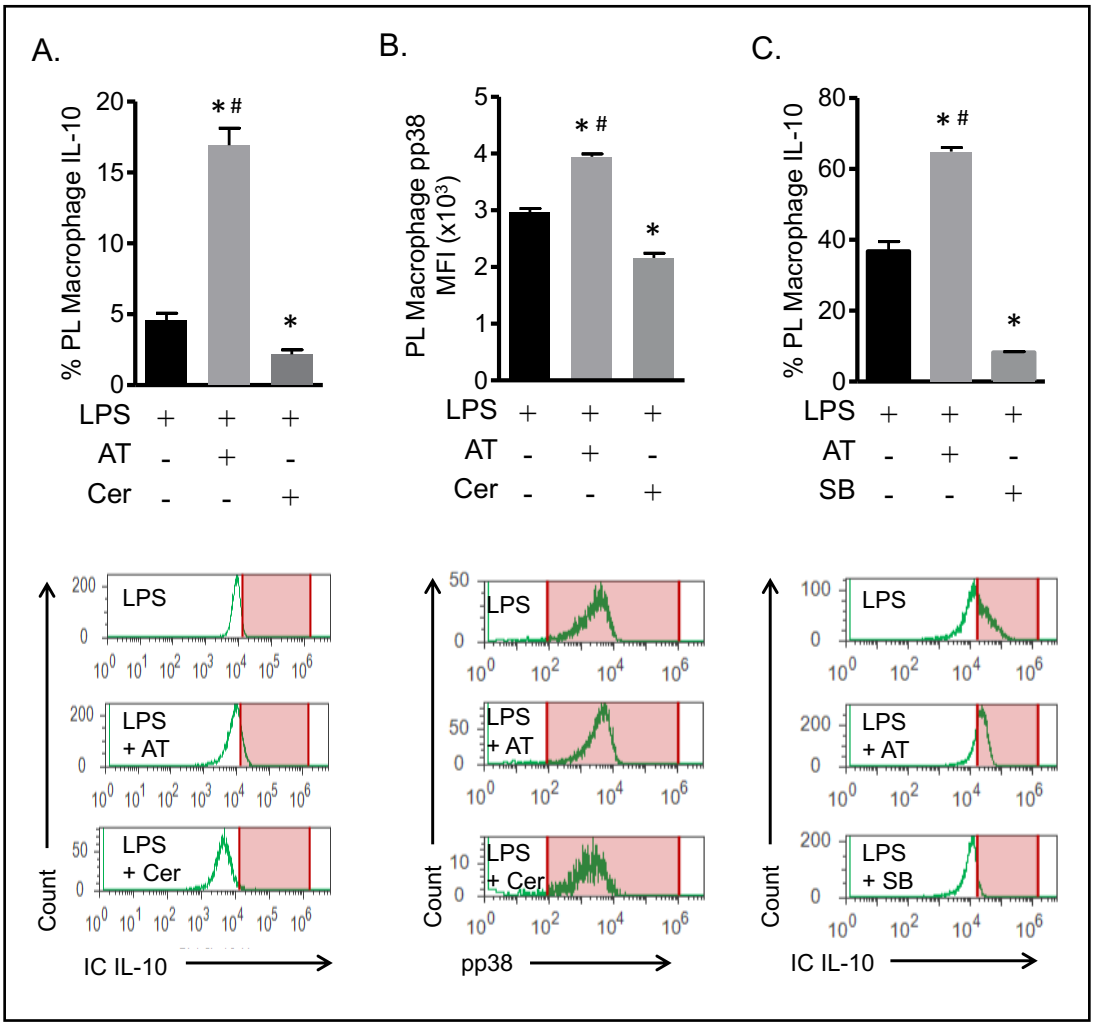

Fig. 6. Ceramide regulates peritoneal macrophage IL-10 production through p38 signaling. Peritoneal lavage (PL) macrophages were pre-treated with either AT or ceramide and stimulated with LPS. A) The percentage of IL-10 producing macrophages was quantified. * p<0.05 vs +LPS; # p<0.05 vs +LPS+Cer; n = 4-8 mice/group, as well as B) phospho-p38 levels in macrophages. * p<0.05 vs +LPS; # p<0.05 vs +LPS+Cer; n = 4 mice/group. C) The percentage of IL-10 producing macrophages was measured and compared when treated with AT compared to LPS alone or LPS and selective p38 inhibitor SB239063. * p<0.05 vs +LPS; # p<0.05 vs +LPS+SB; n = 4 mice/group. The gating strategy is the same as shown in Fig. 5. One representative histogram per group is shown.

flow cytometry. Peritoneal macrophages were found to produce IL-10 in an AT-driven fashion (Fig. 5B-E). Specifically, there was a 1.7-fold increase in intracellular IL-10 expression in macrophages from endotoxemic mice that received AT over endotoxemic mice that received NS (Fig. 5E, 19.08 ± 1.42 % LPS+NS vs 32.20 ± 5.12 % LPS+AT, p <0.01).

Ceramide regulates peritoneal macrophage IL-10 production through p38 signaling

We next investigated potential pathways through which AT could drive macrophage IL-10 production. AT affects a number of different targets, including the ceramide-generating enzyme Asm. We first established that the *in vitro* treatment of peritoneal macrophages with AT increased IL-10 production on a per cell basis. Ceramide, on the other hand, reduced IL-10 production (Fig. 6A, 4.56 ± 0.52% +LPS vs 16.92 ± 1.20 % +LPS+AT vs 2.17 ± 0.33 % +LPS+Ceramide, p < 0.01).

The p38 mitogen-activated kinase (MAPK) has previously been shown to be required for IL-10 production [20, 21]. To determine whether AT exerts its effects on IL-10 production through the p38-MAPK pathway, we analyzed p38 phosphorylation in LPS-challenged macrophages treated with AT or ceramide. Thirty minutes after LPS challenge, p38 phosphorylation was significantly increased in AT-treated cells and significantly decreased in ceramide-treated cells compared to LPS stimulation alone (Fig. 6B, 2.97 ± 0.06 x 10^3 MFI LPS vs 3.94 ± 0.05 x 10^3 MFI LPS+AT vs 2.16 ± 0.08 x 10^3 MFI LPS+Ceramide, $p < 0.01$). Similarly, macrophage IL-10 production in response to LPS was increased upon AT treatment, but decreased upon treatment with the selective p38 inhibitor SB239063 (Fig. 6C, 36.80 ± 2.70 % LPS vs 64.93 ± 1.12 % LPS+AT vs 8.22 ± 0.24 % LPS+SB, $p < 0.01$). Thus, AT treatment increased IL-10 generation and p38 phosphorylation, while inhibition of the p38 pathway decreased IL-10 production. We postulate that this is mediated through inhibition of ceramide generation, however, further studies are necessary to support this.

Discussion

We previously observed that AT treatment mitigates sepsis-induced tumor necrosis factor expression and coagulopathy [8]. In this study, we aimed to identify further anti-inflammatory properties or protective effects of AT in sepsis. First, we observed that AT ameliorated septic shock, as demonstrated by improved hypothermia in AT-treated septic mice and increased survival compared to controls. Neither bacterial burden nor systemic IL-6 levels were changed. Secondly, AT-treated septic mice also had decreased pulmonary edema and reduced leukocyte accumulation in the lungs and peritoneum. This can be explained by the observed decrease of myeloid cell chemoattractants upon AT treatment. Finally, AT also directly impaired neutrophil chemotaxis at the cellular level.

The ameliorative effects of AT appear to be driven by IL-10 production. The administration of AT increases IL-10 production in both septic and endotoxemic mice, and the administration of exogenous IL-10 recapitulates the cellular changes seen with AT treatment in septic mice. Previous publications also showed a survival advantage in septic mice treated with human recombinant IL-10 when given 5h or 6h after CLP [22, 23] and extended the therapeutic window when combined with rescue surgery at 30h after CLP [24]. We show that peritoneal macrophages are the predominant source of AT-mediated IL-10 production. Our further *in vitro* studies indicate a p38-dependent mechanism: ceramide inhibits p38 phosphorylation and thus activation, whereas AT, possibly through the functional inhibition of the ceramide-generating enzyme acid sphingomyelinase (Asm), enhances p38 phosphorylation upon LPS stimulation. In line with previous reports, IL-10 production greatly depended on p38 activity, as it was markedly reduced upon addition of a specific p38 inhibitor [20, 21].

Although IL-10 levels were increased in AT-treated septic mice, it is important to note that AT treatment did not cause a state of general immunosuppression, as local and systemic bacterial burden at 4 and 16 hours, and systemic IL-6 levels 6 hours after CLP were similar in AT-treated and control mice. Thus, AT treatment only tempered the innate immune response, but did not lead to broad immunosuppression.

In line with this, the survival advantage of AT-treated mice was evident at 16-20 hours after CLP and their survival curve plateaued shortly after. In contrast, the mortality of septic control mice still increased at late time-points (> 144 hours). These delayed deaths may reflect a "second-hit" due to an unresolved infection in mice that survived the initial hyper-inflammatory response [25]. Previous reports from animal models and patients indicate that the delayed, compensatory increase in anti-inflammatory mediators may be mitigated by blocking the initial inflammatory response [26, 27]. Consistent with this, the amelioration of the initial inflammatory response through administration of AT avoided the second-hit phenomenon in AT-treated septic mice.

Another clinically promising finding is that AT-treatment mitigated pulmonary injury in septic mice, as defined by edema and leukocyte infiltration and BAL protein concentrations. These results are complemented by several prior studies. For example, ceramide accumulation in bronchial epithelial cells has previously been shown to increase pulmonary infection susceptibility in cystic fibrosis patients and murine models [28-30]. Reducing pulmonary ceramide levels through AT treatment or the use of other Asm inhibitors was shown to decrease infection-susceptibility to *P. aeruginosa* [28, 30]. Reduction of Asm activity also decreased pulmonary inflammation in cystic fibrosis mice even prior to infection, consistent with the present finding of an anti-inflammatory effect of AT treatment. Additionally, genetic and pharmacological Asm activity reduction was reported to protect mice from lung edema during *S. aureus* sepsis [9]. Mechanistically, Peng et al. demonstrated that accumulation of ceramide due to increased Asm activity promoted superoxide formation in endothelial cells, which induced degradation of tight junction proteins and resulted in leakiness of the pulmonary endothelial barrier [9]. Peng et al. pre-treated mice with AT for several days before inducing sepsis [9]. Our study shows immunomodulatory effects and protection from septic shock with a single loading dose of AT, which is more relevant clinically. Additionally, the fast onset of effects of a single dose of AT reported here highlight its potential to attenuate the initial hyper-inflammatory response and thus prevent the second-hit phenomenon as described above.

AT can regulate a number of targets, including serotonin transporters, histamine and muscarinic acetylcholine receptors, ion channels and sphingolipid-metabolizing enzymes such as Asm [31]. Asm inhibition with AT has been previously shown to mediate anti-inflammatory effects through reduction of ceramide levels [30]. Reduction of Asm activity has also been previously implicated in the regulation of p38 activation in the central nervous system [12, 32, 33] and in cardiac ischemic-reperfusion injury [34]. We observed that ceramide had opposing effects to AT on IL-10 production and p38 phosphorylation in our *in vitro* studies, suggesting that these effects of AT may be due to the drug's effects on sphingolipids. However, further studies are necessary to support this conclusion.

It is estimated that approximately one-third of trauma patients over the age of 45 years take a neuropsychiatric medication [33]. AT is a common drug with well-established dosing and safety profiles, used to treat mental illnesses such as depression and anxiety. It is also used off-label for migraines, irritable bowel syndrome and fibromyalgia [35-39]. Despite the prevalence of AT in the United States, a national database examining AT usage among trauma/septic patients and its association with hospital outcomes and survival is lacking. This information, however, would help to tailor AT as an appropriate therapy. A healthy eighteen-year-old trauma patient's septic course will not be identical to that of an elderly immunosuppressed patient with multiple comorbidities. To effectively treat a heterogeneous patient population with a timeline sensitive illness and tailor appropriate immune-modulating therapies, we need to more effectively stage sepsis and determine the immune status and functional deficits of the critically ill patient [40].

Conclusion

Altogether, AT treatment improved survival in septic shock and increased peritoneal macrophage IL-10 production through p38 activation, mitigating an initial overwhelming inflammatory burden. This resulted in a checked inflammatory milieu and controlled leukocyte response. Therefore, we propose that AT treatment is a promising therapeutic approach in septic shock. Future focus on staging sepsis to determine patients who would benefit the most from AT treatment is needed.

Acknowledgements

BX, NB, LW, AP: Design of the work, data collection, data analysis and interpretation, drafting the article, critical revision of the article, and final approval of the version to be published. TP, VN, EG and CC: Conception of the work, data analysis and interpretation, drafting the article, critical revision of the article, and final approval of the version to be published. The authors would like to acknowledge H. Goetzman and L. England for excellent veterinarian and flow cytometrical support.

Disclosure Statement

The authors declare no conflicts of interest.

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