Published in final edited form as:

Brain Behav Immun. 2020 February ; 84: 115–131. doi:10.1016/j.bbi.2019.11.016.

Loss of tissue-nonspecific alkaline phosphatase (TNAP) enzyme activity in cerebral microvessels is coupled to persistent neuroinflammation and behavioral deficits in late sepsis

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Abstract

Sepsis is a host response to systemic inflammation and infection that may lead to multi-organ dysfunction and eventual death. While acute brain dysfunction is common among all sepsis patients, chronic neurological impairment is prevalent among sepsis survivors. The brain microvasculature has emerged as a major determinant of sepsis-associated brain dysfunction, yet the mechanisms that underlie its associated neuroimmune perturbations and behavioral deficits are not well understood. An emerging body of data suggests that inhibition of tissue-nonspecific alkaline phosphatase (TNAP) enzyme activity in cerebral microvessels may be associated with changes in endothelial cell barrier integrity. The objective of this study was to elucidate the connection between alterations in cerebrovascular TNAP enzyme activity and brain microvascular dysfunction in late sepsis. We hypothesized that the disruption of TNAP enzymatic activity in cerebral microvessels would be coupled to the sustained loss of brain microvascular integrity,

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⁵. Author Contributions

D.C.N., D.D., and C.M.B. designed the studies. D.C.N. and A.L.B. performed CLP surgeries and monitored animals after sepsis procedure. D.C.N., C.A.G., and E.B.E.-C. performed behavioral tests and analyzed behavioral data. D.C.N. and S.A.B. performed immunohistochemistry and image analysis. D.C.N., S.C., W.W., and S.J. performed and analyzed flow cytometric data. D.C.N., D.D., C.M.B. wrote the manuscript. J.L.M. and A.B.P. provided the SBI-425 reagent. All authors read and revised the final manuscript.

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Conflicts of Interest: A.B.P. and J.L.M. are inventors on a patent application covering SBI-425 (PCT WO 2013126608). All other authors declare no competing interests.

elevated neuroinflammatory responses, and behavioral deficits. Male mice were subjected to cecal ligation and puncture (CLP), a model of experimental sepsis, and assessed up to seven days post-sepsis. All mice were observed daily for sickness behavior and underwent behavioral testing. Our results showed a significant decrease in brain microvascular TNAP enzyme activity in the somatosensory cortex and spinal cord of septic mice but not in the CA1 and CA3 hippocampal regions. Furthermore, we showed that loss of cerebrovascular TNAP enzyme activity was coupled to a loss of claudin-5 and increased perivascular IgG infiltration in the somatosensory cortex. Analyses of whole brain myeloid and T-lymphoid cell populations also revealed a persistent elevation of infiltrating leukocytes, which included both neutrophil and monocyte myeloid derived suppressor cells (MDSCs). Regional analyses of the somatosensory cortex, hippocampus, and spinal cord revealed significant astrogliosis and microgliosis in the cortex and spinal cord of septic mice that was accompanied by significant microgliosis in the CA1 and CA3 hippocampal regions. Assessment of behavioral deficits revealed no changes in learning and memory or evoked locomotion. However, the hot plate test uncovered a novel anti-nociceptive phenotype in our septic mice, and we speculate that this phenotype may be a consequence of sustained GFAP astrogliosis and loss of TNAP activity in the somatosensory cortex and spinal cord of septic mice. Taken together, these results demonstrate that the loss of TNAP enzyme activity in cerebral microvessels during late sepsis is coupled to sustained neuroimmune dysfunction which may underlie, in part, the chronic neurological impairments observed in sepsis survivors.

Keywords

sepsis; cecal ligation and puncture; tissue non-specific alkaline phosphatase; blood-brain barrier; nociception; astrogliosis; microgliosis; neuroimmune

1. Introduction

Sepsis is a potentially fatal clinical syndrome that results from an excessive systemic inflammatory response to an infection (Stare et al., 2015; Varatharaj & Galea, 2017; Zaghoul et al., 2017). This syndrome accounts for 30 million cases annually, and about 6 million deaths worldwide (Fleischmann et al., 2016). A unique feature of sepsis is that it is often comorbid or may precipitate from other disease conditions such as stroke, cancer, or diabetes (Boehme, Ranawat, Luna, Kamel, & Elkind, 2017; Nwafor et al., 2019; H. E. Wang et al., 2012). It is anticipated that the rise in these chronic diseases will increase the global prevalence of sepsis (Ovbiagele et al., 2013; Stoller et al., 2016). Yet, therapeutic strategies to treat septic patients remain limited, as there are no FDA-approved drugs to treat sepsis, and the primary treatments are largely limited to anti-microbial drugs, supportive fluids, and vasopressors (Fink & Warren, 2014). In addition, the mechanisms underlying the long-term consequences of sepsis on the brain and periphery remain elusive (Iskander et al., 2013; Iwashyna, Ely, Smith, & Langa, 2010).

Sepsis survivors are burdened with an increased risk of infections, sensorimotor abnormalities, and cognitive decline (Iwashyna et al., 2010; Nwafor et al., 2019; Zauner et al., 2002). The central nervous system (CNS), particularly the brain, is severely affected in sepsis and sepsis-associated cognitive impairment is an independent predictor of mortality

(Gofton & Young, 2012). Persistent peripheral inflammation in sepsis contributes to a dysfunctional brain microvasculature and facilitates the trafficking of peripheral immune cell populations, cytokines and neurotoxins into the brain which, collectively, promote neuroinflammation and alter brain function (Andonegui et al., 2018; Singer et al., 2016). Short-term brain microvascular dysfunction in murine models of experimental sepsis has been demonstrated by increased barrier permeability and diminished tight junction (TJ) proteins 24 h post-sepsis (Towner et al., 2018; P. Wang, Hu, Yao, & Li, 2018). Thus, a better understanding of the cellular and molecular mechanisms that contribute to brain microvascular dysfunction in sepsis may provide insights to mitigate and improve long term neurological outcomes in sepsis survivors.

We and others have shown that the ectoenzyme tissue-nonspecific alkaline phosphatase (TNAP) may play a critical role in brain microvascular function and dysfunction (Deracinois, Lenfant, Dehouck, & Flahaut, 2015; Nwafor et al., 2019). TNAP, also known as bone/liver/kidney alkaline phosphatase, is one of four alkaline phosphatase genes in the mammalian genome (Millan, 2006). Peripheral TNAP enzymatic activity, most likely generated within neutrophils, liver, and kidneys, has been shown to play an important role in attenuating inflammation and improving survival in sepsis rodent models (Bender et al., 2015; Verweij et al., 2004). TNAP enzyme activity is also highly elevated in cerebral microvessels and has been used as a histological marker of the brain microvasculature for nearly a century (Betz, Firth, & Goldstein, 1980; Friede, 1966; Vorbrodt, Lossinsky, & Wisniewski, 1986). While a clearly delineated physiological role for TNAP at the brain microvascular interface remains less understood, recent studies suggest a role for TNAP in the maintenance of brain endothelial cell integrity. An *in vitro* study demonstrated that inhibition of TNAP activity on brain endothelial cells by levamisole, a pan-AP enzyme inhibitor, worsened barrier function and increased cellular permeability (Deracinois et al., 2015). In a recent *in vivo* study, we reported that brain TNAP enzyme activity is decreased 24 h post-sepsis compared to sham-injured mice (Nwafor et al., 2019). Taken together, both findings suggest a plausible role for TNAP in maintaining brain microvascular barrier integrity during inflammation. However, it remains unclear whether the observed decrease in TNAP's enzyme activity persists beyond 24 hours and whether the decrease in TNAP enzyme activity is coupled to brain microvascular dysfunction *in vivo*. The delineation between early (< 24 h) and late sepsis (> 36 h) is particularly important considering that most sepsis-related deaths occur in the late/hypo-inflammatory phase of sepsis (Otto et al., 2011).

The objective of this study was to determine the neuroimmune and behavioral changes which paralleled the loss of TNAP in cerebral microvessels in late sepsis, i.e. up to seven days post-sepsis. We incorporated an established a seven day model of late sepsis, cecal ligation and puncture (CLP) (Crowell, Phillips, Kelleher, Soybel, & Lang, 2017; Vachharajani et al., 2014; X. Wang et al., 2016). We assessed novel behavioral and neuroinflammatory outcomes in late sepsis and coupled these outcomes to distinct alterations in the brain microvasculature and integrity. Our results revealed a loss of TNAP enzymatic activity in cerebral microvessels, diminished tight junctions and increased permeability, indicating a loss of barrier integrity. Enhanced cortical astrogliosis and microgliosis was accompanied by sustained leukocyte infiltration, and we also identified a

novel cell population infiltrate with phenotypic characteristics of myeloid-derived suppressor cells (MDSCs). Assessment of behavioral deficits in late sepsis uncovered a novel anti-nociceptive phenotype in the absence of an impairment in learning and memory. Taken together, these results demonstrate that late sepsis is characterized by a novel neuroimmune phenotype that embodies loss of sustained loss of cerebral microvascular TNAP activity and persistent neuroinflammation.

2. Materials and Methods

2.1. Animals

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at West Virginia University. Male wild-type (WT; *C57BL/6J*) mice were bred in West Virginia University Health Sciences Center vivarium facilities and used for experiments at 3-5 months old. All mice were generated from *C57BL/6J* breeding pairs obtained from Jackson Labs (Bar Harbor, ME, Catalog # 000664). Mice were group housed in environmentally controlled conditions with a reverse light cycle (12:12 h light/dark cycle at $21 \pm 1^\circ\text{C}$) and provided food and water *ad libitum*.

2.2. SBI-425 preparation

5-((5-chloro-2-methoxyphenyl)sulfonamido) nicotinamide (SBI-425) was synthesized as previously described (Pinkerton et al., 2018). SBI-425 powder was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milwaukee, WI), heated, sonicated, and stored at -80°C . The stock solution was diluted to 1 mg/mL in a vehicle solution comprised of: 10% DMSO, 10% Tween-80 (Sigma-Aldrich, Milwaukee, WI), and 80% water.

2.3. Brain endothelial cell barrier function assays

The human cerebral microvascular endothelial cell line (hCMEC/D3) is an immortalized line that retains BBB characteristics *in vitro* (Weksler et al., 2005). hCMEC/D3 cells were seeded onto 3 independent collagen-coated 16-well E-Plate PET arrays (ACEA Biosciences, San Diego, CA) at a concentration of 20,000 cells/well and loaded onto an xCelligence RTCA DP system (ACEA Biosciences) enclosed in a tissue culture incubator. Once cells reached confluence ~ 24 h after seeding, duplicate wells in each array were treated with 200 μL of the following: vehicle (DMSO, 0.3%), tissue-nonspecific alkaline phosphatase inhibitor (TNAPi 100 μM ; Millipore, Temecula, CA), lipopolysaccharide (LPS 100 ng/mL; Sigma-Aldrich, Milwaukee, WI), or LPS (100 ng/mL) and TNAPi (100 μM). Cell index (CI) was recorded continuously for 24 h and analyzed with RTCA Software 2.0 (ACEA Biosciences). CI for all wells was normalized to a single timepoint, i.e. ~ 1 h after treatment and reported as normalized CI.

2.4. Cecal ligation and puncture (CLP)

The cecal ligation and puncture (CLP) model of moderate polymicrobial sepsis was employed as previously described (Vachharajani et al., 2014). Briefly, mice were anesthetized by the inhalation of 1-2% isoflurane. Abdominal access was obtained via a midline incision following topical application of 2% lidocaine (Hospira, Lake Forest, IL).

The cecum was isolated and ligated with a 4-0 silk ligature below the ileocecal valve and then punctured twice with a 22 G needle through and through. Fecal matter (approximately 1 mm) was extruded from the hole, and the cecum was placed back into the abdominal cavity. The abdominal muscle layer was closed with 6-0 sutures (Ethilon, Cornelia, GA), while the abdominal skin layer was closed with 5-0 sutures (Ethilon, Cornelia, GA). Sham-operated animals had their cecum isolated and then returned to the peritoneal cavity without being ligated or punctured. One mL of sterile 0.9% saline was administered subcutaneously after sepsis procedure for fluid resuscitation in sham- and CLP-operated mice. All mice were housed according to treatment group and placed on heating pads for three days following sepsis procedure. Mice were monitored for survival and sepsis-associated clinical signs twice daily for six days and underwent a series of behavioral assessments. To assess the role of TNAP in maintaining brain endothelial cell barrier integrity *in vivo*, a third cohort of septic mice was used for SBI-425 studies. Briefly, mice were injected intraperitoneally with 25 mg/kg SBI-425 or vehicle 1 h following surgery and once daily for the next six days; this dosage was derived from a previously published study (Sheen et al., 2015). Mice used for all experiments were euthanized at day seven post-sepsis.

2.5. Modified murine sepsis score (MMSS) and average weight loss score

We modified a validated murine sepsis severity score (MSS) (Shrum et al., 2014) to examine eight sickness behavior components: appearance, weight loss, activity, response to stimulus, eyes, posture, diarrhea, and respiratory rate, henceforth referred to as the modified murine sepsis severity score (MMSS). Each component is scored from 0 (normal/no change) to 4 (severe change), and the MMSS is a grand total of all eight aforementioned sickness behavior components (i.e. 32 total points) collected each day post-sepsis over a 6-day period for each animal. The average percent weight loss score was derived from the MMSS and defined on a scale from 0 to 4 (i.e. a score of 0=0-5%, 1=5.1-10%, 2=10.1-15%, 3=15.1-20%, and 4=>20.1% average percent weight loss). A score of 24 or greater on the MMSS represents a humane endpoint for all animals used in our studies. Animals with a MMSS greater than 24 were euthanized and excluded from behavioral assessments.

2.6. Tissue collecting and processing

Mice were deeply anesthetized with isoflurane and perfused intracardially with a perfusion pump (Masterflex 7524-10, Cole-Parmer, Vernon Hills, IL) set to 5.0 mL/min. Blood was removed with 25 mL of 0.9% saline followed by perfusion and fixation with 50 mL 4% chilled paraformaldehyde (PFA, Fisher Scientific, Pittsburgh, PA). Perfused brains and spinal cords were removed from the skull and spinal canal, respectively, and post-fixed in 4% PFA overnight at 4°C. On the following day, brains and spinal cords were rinsed in 0.01 M phosphate buffered saline (PBS) and incubated sequentially in 10%, 20%, and 30% sucrose in PBS for 24 h each. Following successive incubations in sucrose, brains and spinal cords were co-embedded in a 15% gelatin matrix in groups of twelve per matrix for simultaneous sectioning. The gelatin block was processed sequentially through 4% PFA for 24 h, 15% sucrose for 24 h, and 30% sucrose for 48 h. The block was trimmed and placed in a -80°C freezer for 30 min. Sectioning was performed in the coronal plane at 30 µm on a sliding microtome (HM 450, ThermoFisher Scientific, Waltham, MA) equipped with a 3×3 freezing stage (BFS-40MPA, Physitemp, Clifton, NJ) at -20°C. Sections were collected into

a series of six cups filled with PBS/0.06% sodium azide. Adjacent cups were used for sequential histological staining or immunostaining.

2.7. Immunohistochemistry

Following sequential cuts of the gelatin blocks on the microtome, sections were immunostained using standard free-floating immunohistochemistry techniques as described (Bachman, 2013). Briefly, all sections were blocked with 80 mL PBS, 10 mL of methanol (Fisher Scientific, Pittsburgh, PA), and 10 mL of 30% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) and incubated on a shaker (Model 55D, Reliable Scientific, Hernando, MS) for 15 min. Sections were then washed three times and permeabilized for 30 min on a shaker with 1.83% lysine (Fisher Scientific, Pittsburgh, PA) in 1% Triton (Sigma-Aldrich, St. Louis, MO), and 4% heat-inactivated horse serum (Sigma-Aldrich, St. Louis, MO). Sections were then incubated for 24 h with primary antibodies at room temperature, followed by a 2 h incubation with the appropriate secondary antibody at room temperature. The following primary antibodies were used with working dilutions and antibody identification indicated in parentheses: Iba-1 (Invitrogen (1:1000), [AB 2544912](#), Carlsbad, CA), GFAP (Agilent (1:10,000), [AB 2811722](#), Santa Clara, CA), Claudin-5 (GeneTex (1:500), [AB 2811720](#), Irvine, CA), TMEM 119 (Abcam (1:1000), [AB 2800343](#), Cambridge, MA), NeuN (Cell Signaling Technologies (1:500), [AB 2651140](#), Danvers, MA), and choline acetyltransferase (ChAT) antibody (Abcam (1:1000), [AB 2721842](#), Cambridge, MA).

2.8. Tissue-nonspecific alkaline phosphatase (TNAP) enzyme histology

Brains and spinal cords were evaluated for alkaline phosphatase enzyme activity with the BCIP/NBT substrate kit (SK-5400, Vector Laboratories, Burlingame, CA) as previously described (Nwafor et al., 2019). Sections were rinsed in PBS twice for 5 min each, once in 0.1M Tris-HCl (pH = 9.5) for 5 min and incubated in the staining solution for 4 h at room temperature. Following three rinses in 0.01 M PBS, the sections were mounted onto microscope slides (Unifrost+, Azer Scientific, Morgantown, PA), air-dried overnight, dehydrated through a standard dehydration series, and cover-slipped with Permount (Fisher Scientific, Pittsburgh, PA).

2.9. Image analysis

Sections were viewed on a Leica DM6B microscope (Leica Camera, Allendale, NJ) and images were captured using Leica LASX software (Leica Microsystems, Buffalo Grove, IL). All brain regions of interest i.e. the striatum, CA1, CA3, somatosensory cortex, and basal forebrain (diagonal band of Broca and medial septum) were identified by referring to the Allen Institute Brain Atlas (<http://mouse.brain-map.org>). Analysis of Iba-1 and TMEM 119 was conducted in the somatosensory cortex (20X (TMEM119), and 20X and 40X (Iba-1) magnifications), CA1 (40X (TMEM119 and Iba-1) magnification), and CA3 brain regions (40X (TMEM119 and Iba-1) magnification). Analysis for GFAP was conducted in the somatosensory cortex (20X magnification), CA1 (40X magnification), and CA3 (40X magnification). Cell counting analysis for NeuN was conducted in the CA1 and CA3 hippocampal regions at 40X magnification. Cholinergic (ChAT-positive) neurons were analyzed in the basal forebrain region at 40X magnification. Analysis for TNAP enzyme activity was conducted in the somatosensory cortex at 20X magnification. Three random

images were collected per animal per brain region of interest. Spinal cords were analyzed for GFAP, Iba-1, and TNAP. All collected images were converted to an 8-bit image and quantified in FIJI/Image J version 2.0 software.

2.10. Flow cytometric sample preparation, gating strategy and analysis

Single cell suspensions were obtained as previously described using the Miltenyi adult brain dissociation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions (Garcia-Bonilla et al., 2018; Vachharajani et al., 2014). Briefly, mice were deeply anesthetized with isoflurane and were perfused intracardially with a perfusion pump (Masterflex 7524-10, Cole-Parmer, Vernon Hills, IL) set to 5.0 ml/min. Blood was removed with 25 mL 0.9% saline. Perfused whole brains were removed from the skull and processed with the Adult Brain Dissociation Kit (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions. Briefly, brains were minced followed by mechanical homogenization in a gentleMACS C tube with the gentleMACS Octo Dissociator (Miltenyi Biotec). Following centrifugation, cells were further dissociated using a 70 μ m strainer to obtain a single cell suspension. Myelin and other debris were removed, followed by red blood cell lysis with ACK Lysis Buffer (Lonza, Walkersville, MD). CD45⁺ cells within the suspension were isolated with mouse CD45 microbeads (Miltenyi Biotec) and magnetic separation with an autoMACS separator (Miltenyi Biotec). Viability and total cell yield were determined by trypan blue exclusion and cells were re-suspended to a final concentration of $2.5\text{--}3 \times 10^6$ cells/ml with FACS buffer (0.01 M PBS, 5mM EDTA, 2% FBS). Cells were washed twice in cold 0.01 M PBS and stained with fixable viability dye eFluor780 (eBioscience, San Diego, USA) for 30 min at 4°C in the dark. Cells were then briefly washed with FACS buffer and blocked with Ultra-Leaf purified anti-mouse CD16/32 (BioLegend, AB 2783156, San Diego, CA) for 20 min. Following non-specific blocking, cells were stained with monoclonal antibodies for CD45-PE (AB 2727965), CD11b-VioBlu (AB 2726322), CD11c-PerCp-Vio700 (AB 2660163), Ly6G-APC (AB 2660046), Ly6C-PE-Vio770 (AB 2660034) (Miltenyi Biotec, Auburn, CA) or CD4-FITC (AB 464896), CD25-PE (AB 465607), CD19-PerCp-Cy5.5 (AB 1106999) and CD8a-eFluor450 (AB 1272198) (eBioscience, San Diego, USA) for 10 min at 4°C. Appropriate single stained controls were prepared for fluorophore compensation using compensation beads (Invitrogen, Carlsbad, CA). For intracellular FOXP3 detection cells were fixed/permeabilized using a mouse regulatory T cell staining kit (eBioscience) after staining the surface markers (CD4 or CD25, Invitrogen, Carlsbad, CA) and stained with anti-FOXP3-PE-Cy5 (eBioscience, AB 468806, San Diego, USA). Fluorescence was measured using a BD LSR Fortessa with FACS Diva software (BD Biosciences, San Jose, CA). All data were compensated and spectral overlap was minimized using automatic compensation method of BD FACS diva software (BD Biosciences, San Jose, CA).

Further analyses were performed in FCS Express 6.0 (De Novo Software, Glendale, CA). Single cells were identified by forward scatter and side scatter, and viable cells were gated for further analysis of positive cell populations. Viable cells were gated for CD45⁺ populations and then divided into T-lymphoid cells, which included cytotoxic T-cells (CD45^{hi}, CD3⁺, CD8⁺), helper T-cells (CD45^{hi}, CD3⁺, CD4⁺), and T-regulatory cells (CD4⁺, CD25⁺, Foxp3⁺); myeloid cells, which included neutrophils (CD45^{hi}, CD11b⁺, Ly6C

⁻, Ly6G⁺), monocytes (CD45^{hi}, CD11c⁺, Ly6C⁺, Ly6G⁻), polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) (CD45^{hi}, CD11b⁺, Ly6C^{low}, Ly6G⁺), and monocytic myeloid-derived suppressor cells (M-MDSCs) (CD45^{hi}, CD11b⁺, Ly6C^{hi}, Ly6G⁻). The quantified results represent the percentage of positive cells from the total CD45⁺ live cells.

2.11. Behavioral testing

2.11.1. Open field (OF)—The open field testing paradigm was used to evaluate passive locomotor activity as previously described (Doll et al., 2015) and was administered on day two post-sepsis. A white plastic box (60 cm × 60 cm × 15 cm) was placed upon a table accompanied by five 60-watt lamps that provided indirect illumination. At the start of the trial, each mouse was placed at the center of the box and received a 1 h session recorded over 5 min intervals. Locomotor activity was recorded using a 4×8 photobeam activity system by San Diego Instruments (San Diego, CA). The dependent variables are the total horizontal movements (x, y direction) and rearing movements (z direction) over a 60 min period.

2.11.2. Accelerating rotarod (AR)—The accelerating rotarod test was used to evaluate evoked locomotor performance and coordination as previously described (Doll et al., 2015) and was administered on day three post-sepsis. A textured plastic horizontal rod (3 cm in diameter) was mounted 14.5 cm above a pressure-sensitive base (Ugo Basile, Gemonio, Italy). For each trial, the mouse was placed on a moving rod that accelerates from 4 rpm to 44 rpm when the operator hits the start button. Acceleration is continued until the mouse fell onto the padded base or until 300 s elapsed. Latency to fall (s) onto the padded base was the dependent variable. All mice received 4 trials on the rotarod.

2.11.3. Passive avoidance (PA)—The passive avoidance test is used to assess non-spatial aversive learning and memory (Barichello et al., 2005) and was administered days four and five post-sepsis. The test is performed using a two-compartment (illuminated and darkened) chamber connected by an automated door (Med Associates, VT). All animals undergo 3 trials (120 s acquisition, 15 min immediate retention, and 24 h retention). During the 120 s acquisition, each animal is placed in the illuminated compartment and receives a 0.3mA shock when it enters the darkened compartment. For the 300 s retention trials, the animal is placed in the illuminated chamber; however, no shock is initiated upon entry into darkened compartment. The dependent variable is the latency to enter the darkened compartment (s).

2.11.4. Hot plate (HP)—The hot plate test is used to assess nociception (Vachon et al., 2013) and was administered on day five post-sepsis. Mice were individually placed on a preheated 55°C hot plate (IITC Life Science, CA), inside an open-ended cylindrical Plexiglas tube with a diameter of 30 cm. The latency (s) to respond to thermal stimuli and the total number of nociceptive behaviors (jumps as well as hind limb-lick, flick or flexion) exhibited over a 30 s time frame were measured. To prevent tissue damage, mice were removed from the hot plate after 30 s regardless of their response.

2.11.5. Two-day radial arm water maze (2D-RAWM)—The 2D-RAWM was used as previously described (Colton et al., 2014; Kan et al., 2015) with minor modifications. Briefly, a 6-arm maze is submerged in a pool of opaque water, and an invisible platform placed at the end of 1 arm below the surface of the water. Each mouse received 15 trials per day for two days and the start arm was changed on each trial, however, the goal arm remained the same for all animals. Using static visual cues, the mouse learned the position of the platform. The number of errors (incorrect arm entries) is counted across 1 min trials. If a mouse failed to locate the platform in 1 min, the mouse was guided to the platform by the investigator and allowed to stay on the platform for 15 s before returning the mouse to a heated cage. The number of errors is averaged over two trials per block, resulting in five blocks (15 trials) per day per animal, and a total of 10 blocks (30 trials) for the two-day period. The latency to get to the platform (s) and the number of errors are the dependent variables measured. The 2D-RAWM was performed on days five (acquisition) and six (retention) post-sepsis. It is important to note that mice that participated in the hot plate test and the passive avoidance test did not participate in the 2D-RAWM and vice versa due to the stressful impact of both tests on the mice. To avoid any potential influence of aversive testing experience, a separate cohort of mice was used for PA/HP testing than those that underwent 2D-RAWM.

2.12. Experimental design and statistical analysis

All experiments were executed to enhance rigor and avoid experimenter bias according to ARRIVE guidelines (Kilkenny et al., 2010). Only male mice were used for all experiments because males are more susceptible to sepsis than females as previously published (Angele, Pratschke, Hubbard, & Chaudry, 2014). The use of male mice also allows for comparisons with a number of published studies which address similar outcomes (Andonegui et al., 2018; Denstaedt et al., 2018; Zaghloul et al., 2017). Behavioral assays were conducted from the least aversive (i.e. open field) to most aversive (i.e. 2D-RAWM). The investigator was blinded to the treatment groups for all behavior tests and image analysis. Animals that died 24 h after a behavior test was conducted were excluded from the study. See Figure 1 for an experimental timeline and outline of behavioral assays performed. All statistical analyses were conducted in GraphPad Prism 8.1 (GraphPad Software, La Jolla, CA). Results are expressed as mean $\pm$ SEM and *p*-values ≤ 0.05 were considered significant. Survival was analyzed using a log-rank test. All other results were analyzed using a two-tailed unpaired Student's *t*-test, repeated two-way analysis of variance (ANOVA), or two-way analysis of variance followed by Tukey's multiple comparisons *post hoc* test. Datasets that did not display a Gaussian distribution were subjected to a comparable non-parametric analysis as indicated in the text and figure legends. All *p*-values and *n* values are indicated in the text and figure legends.

3. Results

3.1. Survival analysis, sickness score, and average weight loss

The objective of our first set of experiments was to characterize our sepsis model by investigating the detrimental effects of sepsis on mortality and sickness behavior. Septic mice had a significantly increased mortality rate compared to sham mice (***) $p = 0.001$,

Log-rank) (Fig. 2A). Thereafter, we examined sickness score and average weight loss using the modified murine sepsis score (MMSS). Our results showed a significantly increased sickness score in septic mice compared to sham mice ($F(1, 26) = 22.55$, **** $p < 0.0001$, repeated measures two-way ANOVA) (Fig. 2B). Furthermore, septic mice had a significantly higher average weight loss score compared to sham mice ($U(104, 302) = 26$, *** $p = 0.0005$, Mann-Whitney test) (Fig. 2C). Due to the downward trend in the sickness score and an improvement in overall physical appearance of septic animals after day five, we examined blood serum levels of inflammatory cytokines interleukin 6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) chemokine on day seven in a terminal blood sample. Our results show that septic mice exhibit a persistent peripheral inflammatory phenotype denoted by the significant increase in the levels of inflammatory cytokines IL-6 ($U(16, 62) = 1$, ** $p = 0.005$, Mann-Whitney test) and MCP-1 ($U(11, 55) = 1$, * $p = 0.01$, Mann-Whitney test) (Fig. 2D).

3.2. TNAP's activity in brain microvascular endothelial cells is altered in late sepsis

We next hypothesized that elevated levels of the serum pro-inflammatory cytokines, IL-6 and MCP-1 (Fig. 2D), in late sepsis would support a sustained loss of TNAP enzyme activity in cerebral microvessels. To investigate this, we co-immunolabelled cerebral microvessels with the endothelial cell marker CD31 and subsequently quantified *in vivo* AP activity in the cerebral microvessels within the same tissue. It is important to note that TNAP is the only AP isozyme found in microvessels of the brain and spinal cord (Brun-Heath et al., 2011; Street et al., 2013). Therefore, assessment of total AP activity is adequate to assess total TNAP activity in cerebral microvessels. Our *in vivo* results demonstrated a significant decrease in TNAP enzyme activity in the somatosensory cortex ($t = 2.8$, * $p = 0.02$, unpaired t-test) (Fig. 3A, B) of septic mice. Parallel AP histology of the spinal cord also showed decreased TNAP enzyme activity in the spinal cords of septic mice (Supplementary Fig. 1). Co-labeling of TNAP enzyme activity (purple) with cerebral microvessels (CD31, brown) provides further support that the observed decrease in TNAP's enzyme activity is not the result of a loss of cerebral microvessels, and thus most likely demonstrates that TNAP enzyme activity is reduced in cortical cerebral microvessels during late sepsis (Fig. 3C). In contrast, we did not observe any significant differences in TNAP enzyme activity in the hippocampal CA1 or CA3 region of septic and sham mice on day seven (data not shown). Collectively, our findings reveal a sustained loss of cortical and spinal TNAP enzyme activity in late sepsis.

3.3. TNAP is critical for maintaining cerebrovascular barrier integrity

Past studies by Deracinois *et al.* revealed that inhibition of TNAP enzyme activity with a pan-AP inhibitor (levamisole) decreased barrier integrity in bovine capillary endothelial cells (Deracinois et al., 2015). However, levamisole is not a specific inhibitor of TNAP as it is capable of decreasing the activity of other phosphatases (Debray et al., 2013; Nowak, Rosay, Czege, & Fonta, 2015; Pinkerton et al., 2018). Likewise, it is not clear whether TNAP inhibition during inflammation further exacerbates loss of barrier integrity. Therefore, we utilized a specific *in vitro* inhibitor of TNAP (TNAPi) to examine the effect of TNAP inhibition in hCMEC/D3 cells under basal conditions and during inflammation. Our results showed that treatment of hCMEC/D3 with TNAPi (* $p = 0.026$, Tukey's multiple

comparison test, two-way ANOVA) or LPS ($*p = 0.01$, Tukey's multiple comparison test, two-way ANOVA) significantly decreased barrier integrity to similar levels compared to DMSO treated cells. Furthermore, combination treatment of LPS and TNAPi significantly decreased barrier integrity when compared to TNAPi only ($****p < 0.0001$, Tukey's multiple comparison test, two-way ANOVA), LPS only ($****p < 0.0001$, Tukey's multiple comparison test, two-way ANOVA), and DMSO ($****p < 0.0001$, Tukey's multiple comparison test, two-way ANOVA) treated hCMEC/D3 cells (Fig. 4A). As reported previously, the expression of the tight junction (TJ) protein claudin-5 is decreased at 24 h following either CLP or LPS injection (Banks et al., 2015; Yang et al., 2015). We hypothesized that the sustained loss of TNAP enzyme activity in the somatosensory cortex (Fig. 2D) would be coupled to a persistent loss of brain microvascular integrity in late sepsis. To investigate this, we co-immunolabelled for the endothelial cell marker CD31 (brown) and claudin-5 (red) in the somatosensory cortex. Our results showed that claudin-5 expression around CD31-labelled microvasculature was decreased in the somatosensory cortex of septic mice compared to sham mice (Fig. 4B). However, we did not observe any differences in hippocampal claudin-5 expression between septic and sham mice (data not shown). Since the TNAPi can only be used *in vitro*, we next used a highly specific *in vivo* TNAP inhibitor (SBI-425) to investigate whether further inhibition of TNAP enzymatic activity exacerbates additional loss of cortical claudin-5 in septic mice. Our results showed that SBI-425 treated septic mice exhibited an increased loss of cortical claudin-5 compared to vehicle-treated septic and naïve mice (Fig. 4C). Furthermore, co-immunolabelling for immunoglobulin G (IgG, brain permeability marker) and TNAP enzyme activity in the somatosensory cortex revealed increased vascular (cyan arrow) and perivascular IgG permeability (red arrow) in the brains of septic mice compared to sham mice (Fig. 4D). Our results also showed that septic cortical microvascular brain regions with moderate TNAP enzyme activity exhibited decreased IgG brain parenchymal infiltration (green arrow) compared to cortical microvascular brains regions devoid of TNAP enzyme activity (red arrow) (Fig. 4D). Together, our results demonstrate that modulation of TNAP enzyme activity during inflammation may be critical for barrier maintenance. Thus, both *in vitro* and *in vivo* findings support TNAP's critical role in maintaining endothelial barrier integrity in normal physiology and during inflammation.

3.4. Dysfunction of the neuroimmune axis post-sepsis

3.4.1. Sustained peripheral myeloid immune cell trafficking into brain parenchyma of septic mice

—To investigate whether alterations in TNAP activity within cerebral microvessels (Fig. 3A, B) are coupled to trafficking of peripheral immune cells into the brain parenchyma at day seven post-sepsis, we next characterized the percentage of myeloid cell populations in the brains of septic and sham mice using flow cytometry. Myeloid cells are gated using the strategy shown in Supplementary Figure 2. FCS express analyses of inflammatory myeloid cell populations showed that neutrophils ($CD45^{hi}$, $CD11b^{+}$, $Ly6C^{-}$, $Ly6G^{+}$) (Fig. 5A, B) and monocytes ($CD45^{hi}$, $CD11c^{+}$, $Ly6C^{+}$, $Ly6G^{-}$) (Fig. 5C, D) are significantly increased ($U(15, 51) = 0$, $**p = 0.004$, Mann-Whitney test) in the brains of septic compared to sham mice. Myeloid derived suppressor cells (MDSCs) i.e. polymorphonuclear-MDSCs/PMN-MDCs ($CD45^{hi}$, $CD11b^{+}$, $Ly6C^{lo}$, $Ly6G^{+}$) (Fig. 5E, F; $U(15, 51) = 0$, $**p = 0.004$, Mann-Whitney test) and monocytic-MDSCs/M-MDSCs ($CD45^{hi}$,

CD11b⁺, Ly6C^{hi}, Ly6G⁻) (Fig. 5E, G; $U(15, 51) = 0$, $**p = 0.004$, Mann-Whitney test) were also significantly increased in the brains of septic compared to sham mice.

3.4.2. Sustained peripheral T-lymphoid immune cell trafficking into brain parenchyma of septic mice

Next, we explored T-lymphoid immune cell trafficking post-sepsis using the gating strategy for CD45^{hi} live cells shown in Supplementary Figure 3. FCS express analyses of CD8⁺ (CD45^{hi}, CD3⁺, CD8⁺) (Fig. 6A, B; $U(15, 51) = 0$, $**p = 0.004$, Mann-Whitney test) and CD4⁺ T-cells (CD45^{hi}, CD3⁺, CD4⁺) (Fig. 6C, D; $t = 2.3$, $*p = 0.046$, unpaired t-test) revealed increased populations of these cells in the brains of septic compared to sham mice. We also assessed whether T-regulatory (Tregs) cells from the T-lymphoid lineage were similarly increased in the brains of septic mice. To do this, CD4⁺ T-cells were gated further for Foxp3 and CD25 markers. Our results showed that Treg cells (CD4⁺, CD25⁺, Foxp3⁺) (Fig. 6E, F) are significantly increased ($t = 4.7$, $**p = 0.0011$, unpaired t-test) in the brains of septic mice compared to sham mice. Together both findings from Figures 5A–G and 6A–F demonstrate a persistent dysfunction in the neuroimmune axis in septic mice.

3.5. Regional analyses of brain residential microglia populations in late sepsis

Our next step was to determine the effects of sepsis associated brain microvascular dysfunction on residential microglia cell populations. For our analyses, we performed immunohistochemistry and assessed microglia in three different brain regions (i.e. somatosensory cortex, CA1, and CA3) for the microglia/macrophage ionized calcium binding adaptor molecule 1 (Iba-1). Iba-1 immunoreactive cells were significantly increased in the somatosensory cortex ($U(15, 51) = 0$, $**p = 0.004$, Mann-Whitney test), and CA1 ($t = 3.5$, $**p = 0.007$, unpaired t-test) and CA3 ($t = 3.9$, $**p = 0.004$, unpaired t-test) hippocampal regions of the brain (Fig. 7). Further analysis for Iba-1 positive cells in the spinal cords of septic mice were increased compared to sham mice (Supplementary Figure 4A). To confirm whether the stained Iba-1 positive cells were microglia and not macrophages, we stained for microglia using a specific marker, TMEM119, which is not found on macrophages (Bennett et al., 2016). Contrary to the observed increase in Iba-1 immunoreactive cells shown in Figures 7 A–F, there were no significant differences in TMEM119 immunoreactive cells in the somatosensory cortex ($t = 0.22$, $p = 0.13$, unpaired t-test), and the CA1 ($t = 0.07$, $p = 0.94$, unpaired t-test) and CA3 ($t = 0.42$, $p = 0.68$, unpaired t-test) hippocampal regions (Supplementary Table 1). This suggests that these cells are infiltrating monocytes rather than resident microglia.

3.6. Sustained astrogliosis in the somatosensory cortex of septic mice

Given the alterations in brain microglial/monocyte activation, we also assessed changes in astrocyte proliferation and morphology post-sepsis. We performed glial fibrillary acidic protein (GFAP) immunolabeling and quantified the proliferation of reactive astrocytes in the somatosensory cortex, in hippocampal CA1 and CA3 and in the spinal cord. Our results demonstrate a significant increase in astrocyte proliferation in the somatosensory cortex ($t = 3.5$, $**p = 0.006$, unpaired t-test) of septic compared to sham mice (Fig. 8A, B). However, we did not observe any significant differences in astrocyte proliferation in the CA1 ($t = 0.5$, $p = 0.61$, unpaired t-test) and CA3 ($t = 0.60$, $p = 0.56$, unpaired t-test) hippocampal regions

of the brain (Fig. 8C–F). Parallel to the results observed in the somatosensory cortex of the brain, we observed an increase in proliferative astrocytes in the spinal cords of septic mice compared to sham mice (Supplementary Figure 4B). These results demonstrate that the somatosensory cortex and the spinal cord exhibit sustained astrogliosis post-sepsis.

3.7. Septic mice displayed an impairment in spontaneous locomotion and exhibited a novel anti-nociception phenotype

Septic and critically ill patients often experience long-term sensorimotor impairment (Iwashyna et al., 2010; Khan, Harrison, Rich, & Moss, 2006; Zauner et al., 2002). Therefore, we implemented a series of behavioral assays on days two (open field testing, OFT) and three (rotarod) to assess motor dysfunction and on day five (hot plate) to assess sensory dysfunction in septic mice. The OFT was used to evaluate spontaneous motor dysfunction while the rotarod was utilized to assess evoked motor dysfunction. Our results showed that spontaneous locomotion in the horizontal ($F(1, 24) = 20.51$, $***p < 0.0001$, repeated measures two-way ANOVA) and vertical axes ($F(1, 24) = 48.4$, $***p < 0.0001$, repeated measures two-way ANOVA) were significantly decreased in septic compared to sham mice on day two over a 60 min trial (Fig. 9A, B). However, we did not observe any differences in evoked locomotion on the rotarod ($F(1, 13) = 1.6$, $p = 0.23$, repeated measures two-way ANOVA) between septic and sham mice over four 300 s trials (Fig. 9C). Next, we utilized the hot plate test to assess sensory dysfunction post-sepsis. Our results show that septic mice have an increased latency to respond to thermal stimuli ($t = 2.8$, $**p = 0.01$, unpaired t-test) and exhibit a decreased total number of nociceptive behaviors (hind limb-lick, flick, and jump) ($t = 2.9$, $**p = 0.0096$, unpaired t-test) compared to sham mice (Fig. 9D, E). From these findings, we report, for the first time, a discrepancy between two measurements of locomotion, OFT and rotarod, in septic mice, and reveal a novel anti-nociceptive phenotype exhibited by septic mice.

3.8. Learning and memory is preserved seven days post-sepsis

To determine whether learning and memory deficits are evident during late sepsis, we used the two-day radial arm water maze (2D-RAWM) to assess for spatial learning and memory on days five and six, and the passive avoidance test to assess non-spatial learning and memory on days four and five. Septic mice showed no difference in the number of errors ($F(1, 20) = 0.76$, $p = 0.39$, repeated two-way ANOVA) or the latency to find the hidden platform ($F(1, 20) = 0.87$, $p = 0.36$, repeated two-way ANOVA) on day two of the retention trial compared to sham mice (Fig. 10A, B). Assessment of non-spatial learning and memory with the use of the passive avoidance test showed no difference in latency to enter the darkened compartment ($F(1, 12) = 0.09$, $p = 0.77$, repeated two-way ANOVA) between septic and sham mice at the 15 min and 24 h retention trials (Fig. 10C). Due to the absence of learning and memory impairments at these timepoints, we assessed neuronal loss via NeuN immunolabeling in hippocampal regions CA1 and CA3. No apparent differences in neuronal loss were observed in these regions between sham and septic mice (Supplementary Figure 5). To confirm that other brain regions involved in memory consolidation were not affected by sepsis, we performed immunostaining for choline acetyltransferase (ChAT) in cholinergic neurons of the basal forebrain. Similarly to results observed in the hippocampus, there were no significant differences in the number of ChAT positive neurons in the basal

forebrain of septic mice compared to sham mice (Supplementary Table 2). Taken together, the absence of neuronal loss in the hippocampus and cholinergic neurons of the basal forebrain in septic mice further substantiated the results derived from the 2D-RAWM and passive avoidance behavioral tests (Fig. 10A–C).

4. Discussion

Brain function in humans is significantly impaired in both early and late sepsis and is an independent predictor of mortality (Gofton & Young, 2012). An emerging hypothesis suggests that the pronounced hyper-inflammatory phase seen in early sepsis disrupts brain microvascular function and barrier integrity (Mazeraud et al., 2016; Widmann & Heneka, 2014). The subsequent infiltration of peripheral immune cells and systemic pro-inflammatory molecules into the brain parenchyma that accompanies changes in brain microvascular dysfunction may increase the likelihood of significant short-term and long-term deficits in neurocognitive function (Andonegui et al., 2018; Sonnevile et al., 2013). Therefore, a better understanding of brain microvascular mechanisms that maintain barrier integrity may foster the development of therapeutics to mitigate the neurological impairments seen in sepsis survivors. The results from this study support a mechanism through which loss of TNAP activity is linked to brain microvascular dysfunction. Our results demonstrate that TNAP is critically important for maintenance of brain microvascular integrity in model of experimental sepsis and that perhaps modulation of TNAP's enzymatic activity may foster the development of new therapies that treat long-term sepsis associated cognitive impairment.

We employed the cecal ligation and puncture model of experimental sepsis to identify neuroimmune mechanisms and behavioral deficits that accompanied brain microvascular dysfunction following a loss of TNAP enzyme activity in cerebral microvessels. Results from our sepsis model closely align with previously reported findings in similar models of sepsis (Bi et al., 2019; Zaghloul et al., 2017). However, a unique feature of our study involves our focus on the relationship between suppressed TNAP enzyme activity to brain microvascular dysfunction, leukocyte trafficking, and behavioral deficits in late sepsis. Very little is known about TNAP-associated functions and signaling mechanisms outside of its well-characterized role in bone and mineral metabolism (Buchet, Millan, & Magne, 2013). The physiological importance of suppressed TNAP activity in brain endothelium was suggested by an earlier *in vitro* study which showed that inhibition of TNAP with a nonspecific pan-AP inhibitor (levamisole) worsened brain endothelial cell barrier integrity (Deracinois et al., 2015). However, the experiments in this study established a role for TNAP in basal endothelial cell permeability but did not address the effects of TNAP inhibition during inflammation or injury. Moreover, levamisole is a nonspecific AP inhibitor that induces neurological deficits (Helms et al., 2016; Pinkerton et al., 2018). Thus, we carried out a series of *in vitro* and *in vivo* studies with highly specific TNAP inhibitors to further investigate a putative role for TNAP in brain endothelium. Our findings in the current study expand on previously published data showing that TNAP's enzymatic activity is decreased in septic mice 24 h post-sepsis (Nwafor et al., 2019), i.e. early sepsis, and we demonstrate that suppression of TNAP's enzymatic activity extends into late sepsis.

Results from the current study also reveal a mechanistic role for TNAP as an important regulator of barrier function during inflammation. First, our *in vitro* results in hCMEC/D3 cells demonstrated that inhibition of TNAP activity significantly suppressed barrier function in brain endothelium over time. More importantly, TNAP inhibition combined with LPS treatment synergistically worsened barrier function compared to LPS or TNAPi alone. Second, *in vivo* results showed that a decrease in claudin-5 coupled with increased extravasation IgG in the somatosensory cortex suggests that inhibition of TNAP is coupled to cerebral microvascular dysfunction. Furthermore, *in vivo* inhibition of TNAP on cerebral microvessels of septic mice with SBI-425 decreased claudin 5 immunolabeling in the somatosensory cortex. However, a limitation of our SBI-425 studies is that because TNAP is expressed in numerous brain cell types (Langer et al., 2008), and it is likely that SBI-425 crosses the BBB in sepsis. Therefore, we cannot discern whether the effects SBI-425 on brain endothelial cell cells are direct or indirect due to crosstalk with other brain resident cells that express TNAP, e.g. neurons, astrocytes. Of note, there was no detectable loss of hippocampal TNAP enzyme activity or claudin-5 in this model. These findings suggest that specific brain regions are more susceptible to sepsis-induced brain dysfunction than others in late sepsis. Collectively, our experiments demonstrate that TNAP modulation may be critical in attenuating the brain microvascular dysfunction described in many published preclinical and clinical sepsis studies (Widmann & Heneka, 2014).

Our experiments also addressed whether alterations in brain myeloid and lymphoid cell populations paralleled the loss of brain microvascular integrity. While alterations in peripheral myeloid and T-lymphoid cell populations have been described in numerous murine sepsis studies (Deutschman & Tracey, 2014; Hotchkiss, Coopersmith, McDunn, & Ferguson, 2009), few studies have addressed changes in brain immune cells populations and trafficking peripheral leukocyte populations in late sepsis. Our results are consistent with findings from Singer *et al.* who also showed that monocytes and neutrophils persist up to day 14 in septic rodent brains (Singer et al., 2016). Importantly, our findings showed that elevated numbers of leukocytes are present in the brain parenchyma post-sepsis in spite of a downward trend in sickness score, physical appearance of animal, and cessation of additional mortality past day five. Additionally, we also uncovered two novel findings that support a role for brain immunosuppression in late sepsis. It has been demonstrated that sepsis-associated immunosuppression in peripheral tissues contributes to poor recovery and long-term morbidity post-sepsis (Delano & Ward, 2016; Mira, Brakenridge, Moldawer, & Moore, 2017), but a role of brain immunosuppression in sepsis is less clear. There are no reports, to our knowledge, that have investigated alterations in brain T-regulatory cell or MDSC populations in sepsis. Our observation that brain CD4⁺/CD25⁺/Foxp3⁺ T-regulatory cells (Tregs) were elevated in septic mice suggests that dysregulation of peripheral Tregs may play a role in generating the immunosuppressive phenotype associated with late sepsis (Cao et al., 2018; Molinaro et al., 2015; Tatura et al., 2015); however, further studies are needed to substantiate this finding. While the role of MDSCs in neuroinflammation is not well understood, a recent study in a mouse model of traumatic brain injury showed that circulating PMN-MDSCs and M-MDSCs entered the brain shortly after injury to suppress neuroinflammation; however, PMN-MDSCs declined thereafter while M-MDSCs remained elevated in the brain (Hosomi et al., 2019). In contrast, the results from our study suggest

that both PMN-MDSCs and M-MDSCs remain elevated in late sepsis. While MDSCs have been shown to express an immunosuppressive phenotype, we did not perform a functional analysis of MDSC populations and cannot determine whether this population contributes to sepsis-associated immunosuppression.

Due to a persistent presence of brain microvascular dysfunction post-sepsis, we also investigated the impact of these changes on two key glial cell populations: microglia and astrocytes. Prior studies that have utilized Iba-1 and CD11b as markers to assess alterations in microglia populations have shown that microglia numbers are increased in the cortex and hippocampus of septic mice (Hoogland, Houbolt, van Westerloo, van Gool, & van de Beek, 2015; Singer et al., 2016). In agreement with prior reports, we found that Iba-1 positive cell populations were increased in cortex and hippocampus. It is important to note that the Iba-1 and CD11b markers label microglia and/or infiltrating monocytes (Hoogland et al., 2015; Prinz, Erny, & Hagemeyer, 2017). Further immunostaining with TMEM119, a marker for residential microglia, did not reveal any differences between microglial populations in sham and septic mice. The observed differences reflect the heterogeneity of the brain microglial/monocyte populations and emphasize their importance in the pathophysiology of sepsis (Colton, 2009; Smolders et al., 2019). These findings further suggest that the resultant increase in Iba-1 positive cells may be attributed to infiltrating monocyte populations that differentiate into macrophages expressing Iba-1.

Clinical studies have shown that elevated serum GFAP concentrations are associated with a poor prognosis and quality of life post-sepsis (Wu et al., 2019). Similarly, preclinical studies have shown that GFAP-mediated astrogliosis is associated with brain microvascular dysfunction in many neuroinflammatory conditions (Varatharaj & Galea, 2017). Therefore, we speculated that the presence of brain microvascular dysfunction in late sepsis would support sustained astrogliosis in the somatosensory cortex and spinal cord but not in the hippocampus because there were no differences in hippocampal TNAP enzyme activity between sham and septic mice. Our results confirmed prominent astrogliosis in the somatosensory cortex and spinal cord. Taken together, our results demonstrate that brain microvascular dysfunction is also coupled to sustained microgliosis and astrogliosis in late sepsis.

Since septic survivors are burdened with neurological impairments (Iwashyna et al., 2010), we investigated whether the neuroinflammatory findings in our sepsis model paralleled sensorimotor, nociceptive, and learning and memory impairments. Importantly, we identified several novel behavioral outcomes that are supported by findings in human sepsis survivors. We speculate that the significant decrease in spontaneous locomotion we observed was due to a lack of motivation to explore a novel environment post-sepsis; however, when septic mice were forced to engage in motor activity using the rotarod test, this motor impairment disappeared. These differences may be due to an effect of sepsis on motivation. Motivation is an affective behavior that is difficult to quantify in mice (Ward, 2016), and sepsis-induced changes in motivation have been observed following lipopolysaccharide injection (Anderson, Commins, Moynagh, & Coogan, 2015). Recent studies have also reported similar neuropsychiatric disturbances in motivation in sepsis survivors (Barichello et al., 2019; Erbs et al., 2019). We also assessed deficits in thermal sensory dysfunction using the

hot plate test since altered pain perception has previously been reported in sepsis patients (Thomas, Stover, Lambert, & Thompson, 2014). Increased sensitivity to deep pain but not cutaneous pain has been reported in studies of human experimental endotoxemia (Karshikoff et al., 2015). Our results uncovered a novel anti-nociceptive phenotype in septic mice. The mechanism through which the observed anti-nociceptive phenotype is unclear, but we speculate that this phenotype may result from loss of TNAP enzyme activity observed in the somatosensory cortex and spinal cord, as these regions are part of critical neural circuits involved pain perception and processing biobehavioral pathways (Garland, 2012).

In contrast to previous studies, we did not observe any alterations in either spatial or non-spatial learning and memory tests between septic and sham mice. The absence of neuronal loss in the basal forebrain or the hippocampus supports these behavioral findings. It is highly likely that the seven day post-sepsis time frame was too early to detect cognitive impairment, as other studies have shown that learning and memory are significantly altered at one month post-sepsis in mice and may be mediated via infiltration of CCR2⁺ monocytes and/or increased levels of high mobility group box 1 protein (HMGB1) secreted by inflammatory cells (Andonegui et al., 2018; Chavan et al., 2012). Conceivably, activation of TNAP enzyme activity may limit the infiltration of CCR2⁺ monocytes and HMGB1 into the brain and prevent the cognitive impairment seen one-month post-sepsis. It is also plausible that TNAP activity may be suppressed in other brain regions, e.g. hippocampus, at later time points such as one-month post-sepsis when learning and memory deficits typically become more apparent. Thus, a limitation of our behavioral studies is that they spanned from day two (early sepsis) to day six (late sepsis).

In summary, this study establishes that diminished TNAP enzyme activity in cerebral microvessels is a consequence of the brain's sustained response to systemic inflammation and infection, i.e. sepsis. We propose a model in which TNAP functions as part of a dynamic complex of barrier integrity proteins on the luminal and abluminal surfaces of cerebral microvessels. Inhibition of TNAP activity disrupts this complex, thereby resulting in the loss of tight junction proteins, e.g. claudin-5, elevated extravasation of circulating proteins, e.g. IgG, and increased leukocyte trafficking into the brain parenchyma. In turn, these events exacerbate neuroinflammation and behavioral deficits in sepsis survivors. A better understanding of these mechanisms will support the development of targeted therapies to mitigate chronic neurological impairments in sepsis survivors. It is highly likely that this novel regulatory mechanism extends beyond sepsis and encompasses a common mechanism of cerebral microvascular dysfunction in both acute and chronic systemic inflammatory disorders that cause neurological impairment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors gratefully thank Jessica Povroznik, M.S. for her technical support and assistance with rodent behavioral training. We acknowledge the technical support from Dr. Kathleen Brundage in the West Virginia University Flow Cytometry and Single Cell Core Facility, which is supported by the National Institutes of Health (NIH) equipment

grant number S10 OD016165 and the Institutional Development Awards (IDeA) from the National General Medical Sciences of the National Institutes of Health under grant numbers P30 GM103488 (Cancer CoBRE) and P20 GM103434 (INBRE). Funding for this work was supported by the NIH T32 AG052375 (D.C.N, A.L.B), K01 NS081014 (C.M.B), West Virginia Clinical and Translational Science Institute (U54 GM104942), and the West Virginia University Stroke CoBRE (P20 GM109098).

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Highlights

- Alkaline phosphatase activity in brain microvessels is diminished in sepsis.
- Loss of alkaline phosphatase activity is coupled to the loss of barrier integrity.
- Brain infiltration of myeloid and T-lymphoid cells persists in late sepsis.
- Septic mice exhibit a novel anti-nociceptive phenotype.
- Cortical astrogliosis and microgliosis persist in late sepsis.

Experimental Timeline

Figure 1. Experimental design and behavioral testing paradigm to study neuroimmune and brain microvascular function in late sepsis.

Animals in cohorts 1 and 2 were randomized and underwent either sham or sepsis (cecal ligation and puncture, CLP) surgery. All animals were subjected to a series of daily behavioral assays from day two to day six. Mice were euthanized on day seven and their brains were harvested for flow cytometry (cohort 1) or immunohistochemistry (IHC), and alkaline phosphatase (AP) histology (cohort 2). Spinal cords were used for IHC and AP histology (cohort 2). Animals in cohort 3 underwent sepsis surgery alone and were injected with SBI-425 or vehicle intraperitoneally 1 h post-CLP and daily for six days post-CLP. Mice in cohort 3 were euthanized on day seven post-sepsis and brains collected for IHC.

Figure 2. Survival is decreased while longitudinal clinical scores and serum proinflammatory cytokines are increased in sepsis.

(A) A Kaplan-Meier log-rank survival curve analysis of sham ($n = 22$) and septic ($n = 38$) mice from day 0 of surgery to euthanasia at day seven showed that septic mice had a significant decrease in survival (** $p = 0.001$) compared to sham mice. (B, C) Septic mice ($n = 16$) had significantly higher sickness scores (**** $p < 0.0001$, repeated two-way ANOVA) and average weight loss scores (** $p = 0.0005$, Mann-Whitney test) compared to sham mice ($n = 12$). (D) Examination of blood serum inflammatory cytokines at day seven revealed a persistent increase in IL-6 (** $p = 0.005$, Mann-Whitney test) and MCP-1 (* $p = 0.01$, Mann-Whitney test) in septic mice ($n = 7$) compared to sham mice ($n = 4-5$). * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and is considered significant. All data are presented as mean $\pm$ SEM.

Figure 3. Tissue-nonspecific alkaline phosphatase (TNAP) enzyme activity in cerebral microvessels is decreased in late sepsis.

(A) Representative images of TNAP enzyme activity on brain microvessels showed that TNAP's enzyme activity in the somatosensory cortex (20X magnification) of septic mice is decreased compared to sham mice. (B) Quantification of TNAP enzyme activity on sections of the somatosensory cortex showed that TNAP enzyme activity is significantly decreased ($*p = 0.02$, unpaired t-test) in septic ($n = 6$) compared to sham treated mice ($n = 5$). (C) Co-labelling of TNAP enzyme activity (purple) with CD31 cerebral microvessel marker (brown) in the somatosensory cortex (10X and 40X magnification) further demonstrates that loss of TNAP's enzyme activity in brain sections is due to a loss of TNAP enzyme activity in cerebral microvessels rather than a loss of brain microvessels. * indicates $p < 0.05$, and is

considered significant. All data are presented as meant $\pm$ SEM. 10X, 20X, and 40X magnification scale bar = 160 μ m, 80 μ m, and 40 μ m respectively.

Figure 4. Changes in claudin-5 expression and IgG permeability are consistent with diminished tissue-nonspecific alkaline phosphatase (TNAP) enzyme activity in cerebral microvessels. (A) Normalized cell index is an impedance-based measure of barrier integrity. Treatment of hCMEC/D3 cells with TNAPi significantly decreased barrier integrity ($*p = 0.026$, Tukey's multiple comparison test, two-way ANOVA) compared to DMSO treated hCMEC/D3 cells. Combination treatment of LPS and TNAPi significantly decreased barrier integrity compared to TNAPi only ($****p < 0.0001$, Tukey's multiple comparison test, two-way ANOVA), LPS only ($****p < 0.0001$, Tukey's multiple comparison test, two-way ANOVA), and DMSO ($****p < 0.0001$, Tukey's multiple comparison test, two-way ANOVA) treated

hCMEC/D3 cells. (B) Claudin-5 (red) expression surrounding CD31 (brown) labelled brain microvessels is decreased in septic compared to sham mice (20X magnification). (C) Daily injections of SBI-425 up to day six post-sepsis dramatically reduced claudin-5 expression compared to vehicle (Veh) treated septic mice or naive mice. 20X and 40X magnification scale bar = 80 μ m and 40 μ m respectively. (D) Vascular (cyan arrow) and perivascular IgG permeability (red arrow) is increased in septic brains compared to sham brains (white arrow) (40X magnification). Also, note that cortical microvessels with slight TNAP enzyme activity (green arrow) in septic brains showed reduced perivascular IgG permeability compared to areas without TNAP enzyme activity (red arrow) (40X magnification). * indicates $p < 0.05$, *** $p < 0.0001$, and is considered significant. All data are presented as mean $\pm$ SEM. 20X and 40X magnification scale bar = 80 μ m and 40 μ m respectively.

Figure 5. Brain infiltrating myeloid cell populations are elevated in late sepsis.

(A-D) Representative dot plot and quantification of inflammatory myeloid cells showed a significant increase in neutrophil (** $p = 0.004$, Mann-Whitney test) and monocyte (** $p = 0.004$, Mann-Whitney test) cell populations in the brains of septic compared to sham mice. (E-G) Analyses of myeloid derived suppressor cells (MDSCs) showed that septic brains have a significant increase in polymorphonuclear-MDSC/PMN-MDSC (** $p = 0.004$, Mann-Whitney test) and monocytic-MDSCs/M-MDSC (** $p = 0.004$, Mann-Whitney test) cell populations compared to sham mice. Flow cytometric results are expressed as the percentage

of an indicated cell type in total CD45⁺ live cells in septic mice (n = 6) or sham mice (n = 5). Analyzed population is indicated by the encircled gating. ** indicates $p < 0.01$, and is considered significant. All data are presented as mean $\pm$ SEM.

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Figure 6. Multiple T-lymphoid cell populations are elevated in the brain during late sepsis. (A-D) Representative dot plot and quantification of T-lymphoid cells revealed that septic brains have a significant increase in CD8+ (** $p = 0.004$, Mann-Whitney test) and CD4+ T-cell (* $p = 0.046$, unpaired t-test) populations compared to sham mice. (E, F) Further analyses of a specific subset of CD4+ cells (T-regulatory cells, Tregs) using Foxp3 and CD25 markers revealed a significant increase in the percentage of Tregs (** $p = 0.0011$, unpaired t-test) in the brains of septic compared to sham mice. Quantification of flow cytometric results are expressed as the percentage of an indicated cell type in total CD45+

live cells in septic mice (n = 6) or sham mice (n = 5). The analyzed population is indicated by the encircled gating. * indicates $p < 0.05$, ** $p < 0.01$, and is considered significant. All data are presented as mean $\pm$ SEM.

Figure 7. Late sepsis is characterized by increased microglia/infiltrating monocyte populations. (A-F) Representative histological images (A,C,E) and quantification of Iba-1 (B,D,F) showed an increased number of Iba-1 immunoreactive cells in the somatosensory cortex (** $p = 0.004$, Mann-Whitney test), CA1 (** $p = 0.007$, unpaired t-test) and CA3 (** $p = 0.004$, unpaired t-test) brain regions of septic ($n = 6$) compared to sham mice ($n = 5$). ** indicates $p < 0.01$, and is considered significant. All data are presented as mean $\pm$ SEM. 20X and 40X magnification scale bar = 80 μm , and 40 μm respectively.

Figure 9. Spontaneous sensorimotor activity and nociceptive functions are impaired in septic mice.

(A, B) Open field testing of spontaneous locomotion on day two showed that septic mice ($n = 14$) had a significant decrease in horizontal (** $p < 0.0001$, repeated two-way ANOVA) and vertical (** $p < 0.0001$, repeated two-way ANOVA) spontaneous locomotion compared to sham mice ($n = 10$). (C) Rotarod assessment of evoked locomotion on day three showed no difference ($p = 0.23$, repeated two-way ANOVA) between septic mice ($n = 8$) and sham mice ($n = 7$). (D, E) Evaluation of sensory dysfunction with the hot plate test revealed a novel antinociceptive behavior in septic mice i.e. septic mice exhibited a significant increase in the latency to respond to thermal stimuli (** $p = 0.01$, unpaired t-test) and a significant decrease in the total number of nociceptive behaviors (hind limb - lick, flick, and jump) (** $p = 0.0096$, unpaired t-test). ** indicates $p < 0.01$, **** $p < 0.0001$, and is considered significant. All data are presented as mean $\pm$ SEM.

Figure 10. Absence of spatial and non-spatial learning and memory behavioral deficits in late sepsis.

(A, B) Mice underwent two-day radial arm water maze (2D-RAWM) testing to assess spatial learning and memory deficits on days five and six post-sepsis. Septic mice ($n = 12$) showed no difference in the number of errors ($p = 0.39$, repeated two-way ANOVA) or the latency to find the hidden platform ($p = 0.36$, repeated two-way ANOVA) on day two of the retention trial compared to sham mice ($n = 10$). (C) The passive avoidance assessed non-spatial learning and memory on days four and five post-sepsis. Septic mice ($n = 7$) showed no difference in latency to enter the darkened compartment ($p = 0.77$, repeated two-way ANOVA) compared to sham mice ($n = 7$). All data are presented as mean $\pm$ SEM.