A novel role for tissue-nonspecific alkaline phosphatase at the blood-brain barrier during sepsis

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Sepsis and the blood-brain barrier (BBB): Sepsis is a life threatening systemic inflammatory condition involving multi-organ dysfunction. The World Health Organization estimates that about 30 million people are affected by sepsis every year, and at least 6 million people die from sepsis each year. Out of approximately 24 million sepsis survivors, it is estimated that 70% of these individuals will experience some form of long-term neurological impairment (Iwashyna et al., 2010). Thus, when combined with prevention, diagnosis, and therapeutic management strategies, a functional understanding of the mechanisms that promote sepsis-associated neurological impairment is necessary to address the clinical challenges and economic burdens faced when treating sepsis.

An emerging area in basic and translational sepsis research is the role of the BBB in sepsis pathophysiology. The BBB is an important dynamic interface between the brain and periphery. Its stringent structure regulates the transport of molecules from the brain to the periphery and *vice versa* (Varatharaj and Galea, 2017). However, in many neuroinflammatory and neurodegenerative diseases, e.g., Alzheimer's disease, stroke, sepsis, the function and structure of the BBB are disrupted – a process which may result in acute and/or chronic perturbations in neurological function (Varatharaj and Galea, 2017). Studies have suggested that approaches to mitigate the loss of BBB integrity seen in neuroinflammatory conditions like sepsis must incorporate a functional understanding of proteins localized to brain microvascular endothelial cells (BMECs) in order to elucidate their role(s) in preserving BBB integrity (Varatharaj and Galea, 2017; Nwafor et al., 2019). This concept is exemplified in a recent study which showed that the sphingosine 1-phosphate receptor localized to BMECs is required for small-molecule-selective opening of the BBB (Yanagida et al., 2017). Hence, it is imperative that researchers assess canonical as well as noncanonical functions of proteins localized to the BMECs to elucidate how they preserve or impair BBB integrity.

Tissue-nonspecific alkaline phosphatase in physiology-from bone to brain: Alkaline phosphatases (APs) were first discovered in 1923 and are well known for their roles in bone and teeth mineralization. There are four human AP isoenzymes that include; tissue-nonspecific alkaline phosphatase (TNAP), germ cell alkaline phosphatase, intestinal alkaline phosphatase, and placental alkaline phosphatase. The latter three isoenzymes are expressed in tissues which they are aptly named while TNAP is expressed in multiple tissues types such as bone, liver, intestine, kidney, and brain. TNAP as well as other AP isoforms are ectophosphatases anchored to the cell plasma membrane by glycosylphosphatidylinositiol protein (Buchet et al., 2013). Although there is an extensive literature on the important role played by TNAP in skeletal mineralization, a paucity of data exists on the mechanisms underlying TNAP's function in other tissues, particularly the brain (Nwafor et al., 2019).

In human, non-human primate, and rodent brains, quantification of TNAP activity has been employed to study cerebral microvessel architecture and to quantify changes in the cerebral microvessel density during aging or injury (Brown et al., 2007; Fonta et al., 2015). Intriguingly, although TNAP activity has been employed as a tool to assess quantitative and qualitative changes in cerebral microvessels for nearly a century, the physiological role for TNAP in cerebral microvessels has remained elusive. Deracinois et al. (2015) were the first to demonstrate a functional role for TNAP in bovine capillary endothelial cells using a pan-AP inhibitor (levamisole). Results from this study demonstrated that bovine capillary endothelial cell barrier integrity was decreased via cytoskeleton remodeling following levamisole treatment. Although the Deracinois et al study uncovered a significant role for TNAP in endothelial cell barrier integrity, the results raised several additional questions for further study: 1) Could these *in vitro* observations be replicated *in vivo*? 2) What is the physiological role for TNAP in BMECs? 3) Is inhibition of TNAP activity observed in specific disease states? 4) What are the repercussions of diminished brain endothelial cell TNAP activity on other brain cell populations? 5) Is the inhibition or loss of TNAP activity associated with behavioral deficits? These questions have guided the direction of our current research and three recent publications from our group have attempted to address them (Brichacek et al., 2019; Nwafor et al., 2020). These novel findings are addressed in the next section.

Delineating TNAP's role in BMECs: Elucidating the mechanisms underlying TNAP biology outside of teeth and bone has been challenging due to its presence in numerous tissues, e.g., liver, kidney, spleen, lung, heart, and its presence in numerous cell types in the brain, e.g., BMECs, neurons, astrocytes, and microglia. Mice with a global knockout of TNAP, known as $Alpl^{-/-}$ or $Akp2^{-/-}$ mice, die a few weeks after birth from hypophosphatemia and seizures (Buchet et al., 2013; Fonta et al., 2015). Thus, TNAP's redundancy in numerous tissues makes the global knockout model an insufficient tool to delineate tissue-specific TNAP functions. The main objective of our study was to address our research questions by establishing an *in vivo* role for TNAP in BMECs. We employed a sepsis disease model since we aimed to understand how brain endothelial dysfunction affects brain function and we knew that sepsis targets the microcirculation (Nwafor et al., 2019). Our study utilized the gold standard sepsis model of cecal ligation and puncture to study the effects of both early sepsis (24 hours post-sepsis) and late sepsis (7 days post-sepsis). Our results showed that TNAP activity was diminished at 24 hours and 7 days post-sepsis in the cortex, striatum, and spinal cord. Interestingly, the sustained decrease in TNAP activity at 7 days did not extent to the hippocampus (Nwafor et al., 2020). These findings are supported by earlier studies which have shown that different brain regions have specific TNAP expression patterns. For example, human and rodent cortical regions express the highest level of TNAP activity compared to other brain regions (Fonta et al., 2015). Therefore, we speculated that the cortex, striatum, and spinal cord are affected at 24 hours and 7 days post-sepsis because of their high TNAP activity levels compared to other brain regions like the hippocampus which show lower levels of TNAP activity. It is also likely that hippocampal alterations in TNAP activity are more evident at post-sepsis timepoints (> 14 days) when learning and memory deficits are present in septic mice (Andonegui et al., 2018). An important part of our study was to demonstrate that the decrease in TNAP activity in cerebral microvessels was not due to the loss of brain endothelial cells, as earlier studies reported that a decrease in TNAP activity was caused by the loss of cerebral microvessels (Brown et al., 2007; Fonta et al., 2015). We employed CD31 immunostaining and showed that cerebral microvessel density remained unchanged in the face of diminished TNAP activity on those same vessels. Furthermore, it was apparent from mere histological observation that the loss of TNAP activity on CD31 positive vessels was complete in some vessel segments and incomplete in others (Nwafor et al., 2020).

To further elucidate a functional role for TNAP on BMECs, we carried out a series of complementary *in vitro* and *in vivo* experiments. We showed that pharmacological inhibition of TNAP with two highly specific inhibitors, TNAP inhibitor and SBI-425, significantly diminished barrier function in a human cerebral microvascular endothelial cell line (hCMEC/D3 cells) and primary murine BMECs, respectively (Brichacek et al., 2019; Nwafor et al., 2020). Our *in vitro* experiments carefully demonstrated that TNAP inhibition during inflammation results in a greater loss of barrier integrity than TNAP inhibition alone under basal conditions. We extrapolated our *in vitro* findings to the experimental design of our *in vivo* septic studies and found that cortical areas with diminished TNAP activity showed increased permeability to IgG with a concomitant loss of the tight junctional protein claudin-5. Likewise, when septic mice were treated with *in vivo* TNAP inhibitor, SBI-425, we observed a decrease in the expression of claudin-5 in the cortex of septic mice that received SBI-425 compared to septic mice that received vehicle (Nwafor et al., 2020). Our *in vitro* and *in vivo* results complement each other and demonstrate a descriptive yet functional role for TNAP in BMECs through the maintenance of brain endothelial cell integrity. An additional set of experiments addressed whether the observed decrease in TNAP activity was coupled to canonical neuroinflammatory pathology such as microgliosis and astrogliosis seen in systemic inflammation (Varatharaj and Galea, 2017). We found that these two indices of neuroinflammation, i.e., microgliosis and astrogliosis, were present and elevated in a pattern consistent with observed spatial and temporal changes in TNAP activity and increased cerebral microvessel permeability (Nwafor et al., 2020).

Our final experiments explored a critical functional outcome in neuroscience research – behavior. We hypothesized that sensorimotor function would be impaired since the cortex and spinal cord both showed a loss of TNAP activity. Conversely, we also hypothesized that our mice would not exhibit any deficits in learning and memory since the hippocampus showed no alterations in TNAP activity at 7 days post-sepsis. As expected, our results revealed no deficits in learning and memory at 7 days post-sepsis; however, septic mice did exhibit decreased spontaneous movement, diminished exploratory behavior in a novel environment, and hypoanalgesia compared to controls. Interestingly, evoked locomotion and coordination were preserved as early as 3 days post-sepsis (Nwafor et al., 2020), a time period when mice typically exhibit the most severe sickness behavior. Collectively, the behavioral deficits we observed closely mimic clinical features observed in human sepsis patients. The presence of spontaneous locomotor deficits and the absence of evoked motor deficits suggests that septic mice exhibit altered mood and motivational behaviors previously described in humans

(Barichello et al., 2019).

The translational implications of our findings are summarized in the model shown in **Figure 1**. We demonstrated that TNAP's enzymatic activity is novel modulator of BBB integrity in sepsis. Under healthy conditions (**Figure 1A**), TNAP activity in BMECs is maintained at basal levels and this activity is important for maintaining barrier integrity and cerebral homeostasis. However, during sepsis, key brain regions (**Figure 1B**), undergo a loss of TNAP activity earlier than others. This loss of TNAP activity occurs as early as 24 hours and is sustained up to 7 days post-sepsis. Following a loss of TNAP activity in BMECs, the disruption of junctional proteins enhances the infiltration of leukocytes which release proinflammatory cytokines into the parenchyma. This process, in turn, increases numbers of activated microglia and reactive astrocytes that release additional pro-inflammatory cytokines in the brain parenchyma. Thus, an acute systemic inflammatory disorder such as sepsis may sustain a chronic state of neuroinflammation that eventually leads to neurological impairment and increased mortality and morbidity in sepsis survivors (**Figure 1C**).

Concluding remarks: Our results demonstrate a novel role for TNAP in maintaining BBB integrity and adds to the only existing scientific literature which had previously examined TNAP's role in maintaining barrier function (Deracinois et al., 2015). Furthermore, the contributions from our study highlight the emerging importance of TNAP in other neuroinflammatory and neurodegenerative diseases. Most importantly, our study lends support for TNAP as a therapeutic target to mitigate the long-term cognitive impairment symptoms seen in septic patients via the maintenance of BBB integrity. Conversely, TNAP activity could be manipulated to allow pharmacological therapies to penetrate the brain and target cancerous cells. Nevertheless, we must stress that much research is needed to fully elucidate the mechanisms and signaling pathways through which TNAP is able to maintain BBB integrity. Additional studies are also needed to understand how other TNAP expressing cell types communicate with brain endothelial cells to preserve BBB integrity in the face of injury.

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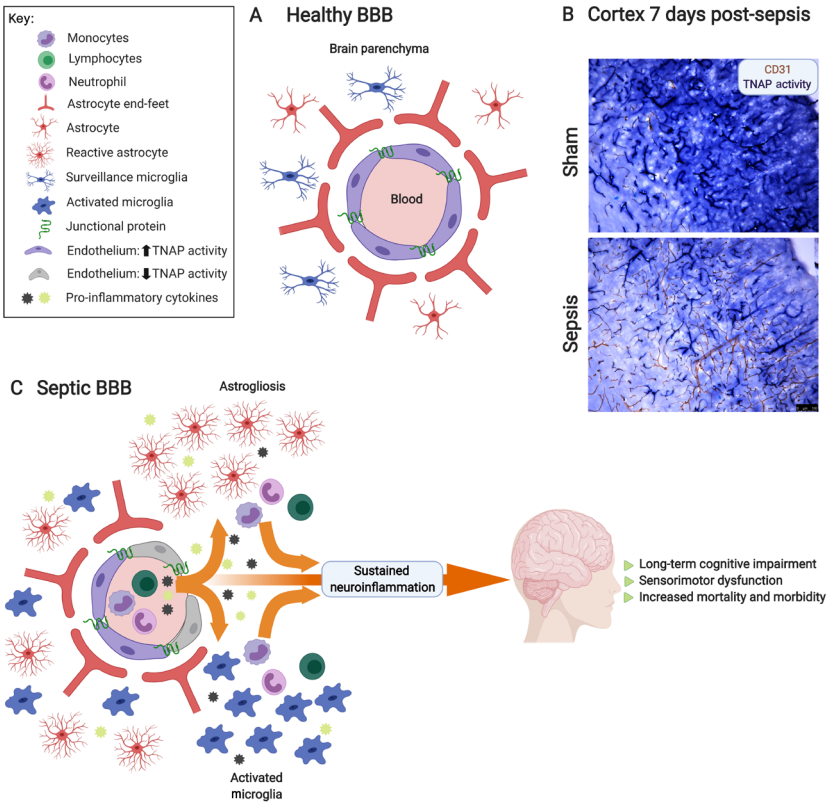

Figure 1 | **Schema for TNAP's role at the BBB during acute systemic inflammation.**
(A) Under physiologic conditions, the BMEC is enriched with TNAP activity (shown in purple), and this activity is required to maintain precise cerebral homeostasis. (B) Key brain regions (cortex depicted) become susceptible to loss of TNAP activity (i.e. purple stain) in septic compared to sham mice. The loss of TNAP activity on $CD31^{+}$ (brown) cerebral microvessels is sustained up to 7 days post-sepsis. (C) Following a loss of TNAP activity in BMECs, junctional protein disruption occurs and promotes the infiltration of peripheral pro-inflammatory cytokines and leukocytes into the brain parenchyma. Infiltrating cytokines increase the number of activated microglia and reactive astrocytes. Activated microglia and reactive astrocytes contribute to a state of sustained neuroinflammation which promotes long-term cognitive impairment, sensorimotor dysfunction, and increased mortality and morbidity. Images were taken at 10× magnification, scale bar: 100 µm. BBB: Blood-brain barrier; BMECs: brain microvascular endothelial cells; TNAP: tissue-nonspecific alkaline phosphatase.

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