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The Emerging Role of Neutrophils as Modifiers of Recovery After Traumatic Injury to the Developing Brain

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Abstract

The innate immune response plays a critical role in traumatic brain injury (TBI), contributing to ongoing pathogenesis and worsening long-term outcomes. Here we focus on neutrophils, one of the “first responders” to TBI. These leukocytes are recruited to the injured brain where they release a host of toxic molecules including free radicals, proteases, and pro-inflammatory cytokines, all of which promote secondary tissue damage. There is mounting evidence that the developing brain is more vulnerable to injury that the adult brain. This vulnerability to greater damage from TBI is, in part, attributed to relatively low antioxidant reserves coupled with an early robust immune response. The latter is reflected in enhanced sensitivity to cytokines and a prolonged recruitment of neutrophils into both cortical and subcortical regions. This review considers the contribution of neutrophils to early secondary pathogenesis in the injured developing brain and raises the distinct possibility that these leukocytes, which exhibit phenotypic plasticity, may also be poised to support wound healing. We provide a basic review of the development, life cycle, and granular contents of neutrophils and evaluate their potential as therapeutic targets for early neuroprotection and functional recovery after injury at early age. While neutrophils have been broadly studied in neurotrauma, we are only beginning to appreciate their diverse roles in the developing brain and the extent to which their acute manipulation may result in enduring neurological recovery when TBI is superimposed upon brain development.

Keywords

Granules; Neutropenia; Neutrophil Elastase; Oxidative Stress; Polarization; Recruitment

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Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in children, with approximately 640,000 TBI-related visits to the emergency room in children under 14 in the United States in 2013 (Faul et al., 2010; Taylor et al., 2017). Infants and young children (aged 0-4) are at greatest risk of sustaining a TBI (Corrigan et al., 2013; Faul and Coronado, 2015; Faul et al., 2010), and compared to other age groups, young children show poorer functional recovery as measured by IQ and related cognitive performance tests, which may persist for years after injury (Anderson et al., 2000; Anderson et al., 2005; Catroppa et al., 2008). As processes such as myelination, synaptogenesis and pruning continue years beyond birth in humans (Koizumi, 2004), there is substantial risk that an early age injury will disrupt these developmental processes, and result in long-term behavioral consequences including anxiety, attention deficit hyperactivity disorder, psychosocial problems and increased risk-taking behavior (Corrigan et al., 2013).

This review will consider neutrophils, the most abundant leukocytes, which are recruited to the injured young brain, as mediators of early secondary pathogenesis and determinants of long-term neurological recovery. Infiltrated neutrophils are a common denominator of the human brain after a TBI (Gahm et al., 2002; Hausmann et al., 1999; Rhind et al., 2010) and rodent models of TBI, designed to interrogate their contribution to early secondary tissue damage (Clark et al., 1994; Claus et al., 2010). The latter have provided an opportunity to not only address the contribution of neutrophils to early secondary pathogenesis but the neurological consequences at adulthood after exposure of the developing brain to these leukocytes.

The Basics of Neutrophils

In order to understand the potential role(s) of neutrophils in early-age TBI, we first provide an overview of their general biology including their development, lifecycle, and granular contents.

Development and lifecycle of neutrophils

Neutrophils, basophils, and eosinophils are collectively known as granulocytes. Like monocytes, neutrophils descend from granulocyte-monocyte progenitor cells in the bone marrow. During the roughly 14 day process (in humans) of granulopoiesis, neutrophils mature, proliferate, differentiate, and develop their characteristic granules (Bainton et al., 1971; Mortaz et al., 2018).

The systemic response of neutrophils to brain injury is centered in the liver, which produces a host of cytokines in response to inflammation in the central nervous system (CNS), which drive the maturation and granulation of neutrophils in bone marrow (Anthony et al., 2012; Campbell et al., 2008; Furze and Rankin, 2008). Chief among the many cytokines capable of regulating granulopoiesis is granulocyte-colony stimulating factor (G-CSF), which is regulated by the interleukin (IL)-23/IL-17 axis (Aggarwal et al., 2003; Li et al., 2017; Schwarzenberger et al., 2000), and is elevated acutely after TBI in both clinical and preclinical studies (Banks et al., 2016). The factors that regulate neutrophil death are similar

to those that regulate granulopoiesis, notably G-CSF (Colotta et al., 1992). In fact, there is a homeostatic feedback loop linking neutrophil birth and death via the IL-23/IL-17/G-CSF axis; phagocytosis of apoptotic neutrophils by macrophages and dendritic cells leads to upregulation of granulopoiesis via IL-17 and G-CSF (Stark et al., 2005).

Under basal conditions, neutrophils live only a few hours after being released, dying by apoptosis and being removed by macrophages in the liver and spleen (Kolaczkowska and Kubes, 2013; Stark et al., 2005), However, the lifespan and death of neutrophils can be altered by the cellular environment. Inflammatory conditions such as congestive heart failure and hypoxia increase neutrophil lifespan (Hannah et al., 1995; Tracchi et al., 2009), and under circumstances of extensive tissue damage, like TBI, clearance of circulating neutrophils by the liver or spleen is delayed (Kolaczkowska and Kubes, 2013).

Fully mature neutrophils are released from the bone marrow and transmigrate across the epithelium and into the blood stream. Under normal conditions, there are 5-6 times as many mature neutrophils in the bone marrow as in circulation (Cartwright et al., 1964; Cowland and Borregaard, 2016; Tak et al., 2013). The chemokine receptor (CXCR) type 4, a G-protein coupled receptor expressed at low levels on the cell surface of mature neutrophils (Martin et al., 2003), binds with its ligand stromal derived factor (SDF)-1 to retain neutrophils in the bone marrow (Eash et al., 2009; Summers et al., 2010). Downregulation of CXCR4 and SDF-1 by G-CSF allows mobilization of neutrophils from bone marrow (Kim et al., 2006; Semerad et al., 2005).

Neutrophil recruitment and migration

Mature neutrophils move through the body via the circulatory system, resulting in a distribution that can be subdivided into two subpopulations: circulating neutrophils, which flow freely in the vasculature, and marginated neutrophils, which travel more slowly, as they transit through organs (Summers et al., 2010). Both circulating and nearby marginated neutrophils are acutely recruited to the site of injury (de Oliveira et al., 2016). Notably, there is recent preclinical evidence that when stimulated by stroke or aseptic meningitis, the majority of responding neutrophils in the brain arise from the marrow of the skull rather than that of long bones such as the femur or tibia, and that some of these neutrophils pass directly from the bone marrow to the cerebrospinal fluid of the meningeal compartment, ostensibly bypassing the blood-brain barrier (Herisson et al., 2018). In the case of TBI, the number of circulating neutrophils does not seem to be a rate-limiting factor for neutrophil recruitment, as treatment with G-CSF prior to controlled cortical impact (CCI) causes dramatic increases in circulating neutrophils with no effect on neutrophils recruited to the injured cortex (Whalen et al., 2000).

Neutrophils are one of the first myeloid-derived cells to infiltrate the injured brain, peaking at approximately 24 hours post injury (See review; Jassam et al., 2017; Kochanek et al., 2017). Circulating neutrophils are recruited to the acutely injured brain through a series of steps along the endothelium that involve tethering and rolling and their subsequent migration into the parenchyma (Yilmaz and Granger, 2010). The first step of this process involves attachment of neutrophils to endothelial cells (Abbassi et al., 1993; Schmidt et al., 2011). Neutrophils tether to and roll along the endothelial cell layer through the action of a class of

cell adhesion molecules, of which the P-selectin glycoprotein ligand is the primary ligand (Eriksson et al., 2001). These adhesion molecules are known as P, E and L-selectins and are each expressed in different cells with unique actions and kinetics (Kansas, 1996). L-selectin is expressed on the surface of neutrophils and other leukocytes, while endothelial cells present P- and E-selectin on their luminal-facing surface (Kansas, 1996). To enter the injured brain, rolling neutrophils must overcome the shear force of blood flow to slow down to a crawl and then fully arrest, adhering firmly to the endothelial layer (Yilmaz and Granger, 2010). This crawling and subsequent arrest is achieved through the action of integrins, transmembrane receptors which connect the extracellular matrix to the cytoskeleton of the cell and promote cell-to-cell binding (Kourtzelis et al., 2017). Endothelial intercellular cell adhesion molecule −1 and 2 facilitate the extravasation of neutrophils from blood vessels through the barrier via their strong transmembrane binding action (Gorina et al., 2014).

Once neutrophils have arrived at the site of injury and adhere to the endothelial cell layer, they traverse the endothelium in a process termed transmigration, which can occur by either paracellular or transcellular routes (Wittchen, 2009; Zen and Parkos, 2003). In paracellular transmigration, cells penetrate the barrier by traversing adjacent endothelial cells through tight junctions (Muller, 2015). In contrast, transcellular migration involves a neutrophil passing through the endothelial cell proper, emerging on the parenchymal side after full engulfment into the cytoplasmic compartment (Hyun and Hong, 2017). It is generally thought that most transmigration occurs via the paracellular route; however, the transcellular route may be preferred when endothelial cells express high levels of intercellular adhesion molecule-1 (Yang et al., 2005). *In vitro* studies that model the inflamed blood-brain barrier have demonstrated a neutrophilic preference for transcellular routes, with cells passing fully through the endothelial cell proper rather than the paracellular route (Gorina et al., 2014; von Wedel-Parlow et al., 2011).

Neutrophilic granules

Neutrophils influence their surroundings primarily through their granular contents, which are released either extracellularly or into phagosomes (Papayannopoulos, 2018). The immune response from TBI creates an environment where neutrophils readily degranulate, their granule contents acting in a variety of potentially harmful and helpful ways (Hager et al., 2010).

There are three general classifications of granules (Lacy, 2006): primary, which include myeloperoxidase, neutrophil elastase, and cathepsin G; secondary, which include lactoferrin; and tertiary granules, which include matrix metalloproteinases (MMPs). The primary functions of myeloperoxidase and lactoferrin are antimicrobial; the former generates cytotoxic hypochlorous acid, whereas the latter sequesters iron and degrades nucleic acid. MMP-9, a member of the family of matrix metalloproteinases, may facilitate paracellular migration of neutrophils as zonulae occludins-1 (a key component of the tight junctional complexes) is a known substrate of this protease (Turner and Sharp, 2016). Each type of granule also contains many other molecular contents, and the content of each granule seems to be determined by when in granulopoiesis it formed (Cowland and Borregaard, 2016). Classification of neutrophil granules (primary, secondary, tertiary) stems mainly from rodent

models, but the nature of human neutrophil granules may be somewhat different, with more heterogeneity and overlap between granule contents (Bainton et al., 1971; Cowland and Borregaard, 2016; Kjeldsen et al., 1993).

Polarization of neutrophils and their phenotypic diversity

Emerging evidence supports the heterogeneity of neutrophils. Early studies suggested an N1 (pro-inflammatory)/N2 (anti-inflammatory) phenotypic polarization (Fridlender et al., 2009) modeled after the M1/M2 polarization identified in macrophages (Xu et al., 2017). Recently though, the concept of a binary M1/M2 classification has been replaced by the idea that a broader spectrum of macrophage phenotypic diversity exists (Morganti et al., 2016; Ransohoff, 2016). Similarly, neutrophils may not be so easily classified into two binary categories (Deniset and Kubes, 2016). One alternative scheme categorizes neutrophils by degrees of activation; namely, 1) naïve (circulating neutrophils), 2) mildly activated (wound- or tumor associated), 3) activated (acute infection) and 4) highly activated (cytotoxic; Houghton, 2010). Although neutrophil polarization has not been studied in great detail nor has the phenotypic spectrum been fully delineated, these differentially activated subsets of neutrophils have been reported in both humans and in animal models in different types of disease and infection (Silvestre-Roig et al., 2016; Tsuda et al., 2004).

Specific to inflammation in the brain, there is some evidence that neutrophils may be more likely to assume an anti-inflammatory phenotype, crucial to the resolution of inflammation and neuroprotection (Cuartero et al., 2013). For example, in a model of intracerebral hemorrhage, the anti-inflammatory cytokine IL-27 alters the phenotype of maturing neutrophils, decreasing their production of cytotoxic granular contents and increasing production of the beneficial iron-scavenging protein, lactoferrin (Zhao et al., 2017). No studies to date have considered the possibility that neutrophils may exhibit phenotypic diversity in the traumatically injured brain. However, it is clear that investigation of neutrophil polarization is crucial to understanding how these leukocytes behave in an evolving lesion where they may exhibit temporally distinct roles.

Pathobiology of the Injured Developing Brain

Long-term neurological deficits, reported in brain-injured children, are likewise paralleled in preclinical models. In response to CCI, rodents at either post-natal day (PND) 17 or 21, ages roughly equivalent to the toddler-aged child (Semple et al., 2016), exhibit a variety of abnormal behavioral phenotypes at adulthood, findings that speak to the long-term consequences of early age injury. These include deficits in spatial learning and memory (Adelson et al., 2013; Ajao et al., 2012; Kamper et al., 2013; Pop et al., 2013) and sensorimotor function (Kamper et al., 2013; Pullela et al., 2006). Hyperactivity is evident as early as adolescence, persisting into adulthood (Pullela et al., 2006). In contrast, deficits in sociosexual function are not detected at adolescence but rather present at adulthood (Semple et al., 2014).

Preclinical models of TBI at an early age have elucidated the pathobiology of secondary injury. In rodents exposed to a CCI at PND 21, neuronal cell loss is evident within the first several days of injury and coincides with activated microglia/macrophages in both the ipsi-

and contralateral cortices, the ipsilateral hippocampus and the thalamus (Igarashi et al., 2007; Tong et al., 2002). Ongoing pathogenesis may persist for months after injury at either PND 17 or 21, culminating in reduced cortical thickness and numbers of neurons in the hippocampus as well as atrophy of the hippocampus and the corpus callosum (Ajao et al., 2012; Kamper et al., 2013; Tsuru-Aoyagi et al., 2009).

The brain at PND 21 is more vulnerable to damage as a result of TBI compared to that of the adult. This is due, in part, to inadequate antioxidant reserves (Tsuru-Aoyagi et al., 2009) coupled with prolonged recruitment of neutrophils that exceeds what is seen in adult injured brain (Claus et al., 2010; Potts et al., 2006). The injured brain is exposed to a high degree of oxidative stress resulting from reactive oxygen and nitrogen species, released by dying and damaged cells, infiltrating leukocytes, and free iron liberated by degradation of heme subsequent to hemorrhage (Potts et al., 2006). The young brain lacks adequate antioxidant reserves necessary to combat TBI-related oxidative damage (Bayir et al., 2002; Tsuru-Aoyagi et al., 2009) and the capacity to fully engage antioxidant pathways. For example, the antioxidant glutathione peroxidase is produced in the injured brains of rodents at PND 21 at levels similar to that seen in adults, but is not upregulated in response to injury as it is in adults (Fan et al., 2003). Overexpression of this enzyme in transgenic rodents results in improved long-term behavioral outcomes after TBI at PND 21, demonstrating the importance of this antioxidant in reducing pathogenesis (Tsuru-Aoyagi et al., 2009).

There is a growing interest in neutrophils in TBI, implicating their involvement in secondary injury (Dickens et al., 2017; Makinde et al., 2017; Roth et al., 2014; Russo et al., 2018; Zhao et al., 2017). Their recruitment to the injured brain (Biagas et al., 1992; Clark et al., 1994; Hartl et al., 1997; Schoettle et al., 1990; Soares et al., 1995) coincides with disruption of the blood-brain-barrier (Anthony et al., 1998; Anthony et al., 1997; Kaczorowski et al., 1995; Soares et al., 1995) and the release of free radicals, proteases, and pro-inflammatory cytokines, all of which promote tissue damage (Jickling et al., 2015; Kawabata et al., 2002; Keeling et al., 2000; Lee and Downey, 2001; Owen and Campbell, 1999).

The injured developing brain exhibits a unique inflammatory signature, characterized by prolonged recruitment of neutrophils into the damaged cortex. In a rodent model of CCI, neutrophil recruitment into the injured adult brain occurs over a period of about 3 days. In contrast, TBI at PND 21 results in recruitment of neutrophils that persists up to 14 days post injury (DPI) (Figure 1; Claus et al., 2010). These findings raise questions about the mechanism(s) underlying these differences in recruitment timecourse. An interesting series of studies, focused on cortical delivery of cytokines, have shown a dramatic age-dependency in neutrophil recruitment, cementing the idea of a developmental “window of susceptibility” in the response of neutrophils to neuroinflammation. In line with this age-dependent response of neutrophils to TBI (Claus et al., 2010), intraparenchymal delivery of IL-1β into the naive rodent brain at PND 21 results in far more recruitment of neutrophils and disruption of the blood-brain barrier compared to the adult rodent brain exposed to similar conditions, highlighting the enhanced sensitivity in the young brain to cytokines (Figure 2; Anthony et al., 1997). A recent study examining the response to intraparenchymal administration of IL-1β at PND 7, 14, 21, and 60 (young adult) has revealed an exquisite sensitivity to IL-1β at early developmental stages (Sa-Pereira et al., 2018). These results

show elevated numbers of neutrophils in the brain with almost a 5-fold increase at PND 14 compared to both PND 7 and 21, with minimal to no detection at PND 60, indicating an age-dependent response by neutrophils specific to development. Lastly, intraparenchymal administration of the downstream cytokine-induced neutrophil chemoattractant 1 (CINC-1, also known as GRO in rat; homologue to human IL-8) produces a more rapid recruitment of neutrophils in rodents at PND 21 compared to adult (Anthony et al., 1998). These collective findings suggest that the time-course for neutrophil recruitment in response to neuroinflammation is directly impacted by age at time of injury.

Neutrophils as Modifiers of Pathogenesis

A variety of approaches have been used to assess the pathogenicity of neutrophils in the injured brain. These include pharmacologic and immunologic depletion strategies, and the use of genetically modified animals to reduce the number of circulating neutrophils, target receptors that facilitate their transmigration, or address the consequences of selective deletion of their granular content. Surprisingly, very few of these studies in the injured adult brain have employed quantitative methods to address changes in recruitment and only one study has been conducted in the injured developing brain (Table 1). Importantly, some of these strategies may lack specificity to neutrophils, confounding interpretation of the results.

Strategies to induce neutropenia

One of the earliest studies induced neutropenia (an abnormally low neutrophil cell count) via a relatively non-specific approach; namely, intraperitoneal administration of the antimitotic vinblastine sulfate prior to a weight drop injury in adult rodents (Uhl et al., 1994). The resulting neutropenia, confirmed by approximately a 90% depletion in circulating neutrophils, was associated with a reduction in cerebral blood flow in the absence of any changes in edema, as measured by brain water content, or lesion size at one day post injury. These findings suggest possible interactions between neutrophils and improved blood flow-findings that have been more recently linked to the protein cathepsin G, a serine protease that is stored in neutrophilic primary granules (Faraday et al., 2013). While this was one of the earliest studies to attempt to understand the role of neutrophils in the injured brain through their systemic depletion, the interpretation of these early findings are confounded by a number of factors, namely the lack of specificity of vinblastine and its potential toxicity and unanticipated findings including reduced cerebral blood flow in the sham group and the absence of any evidence of neutrophils in the injured brain.

Alternative, immunologic-based approaches have been used to induce neutropenia prior to TBI (Kenne et al., 2012; Whalen et al., 1999). Continuous intraventricular infusion of the RP-3 monoclonal antibody generated against neutrophils (Sekiya et al., 1989) before and after CCI induced neutropenia and resulted in an almost complete lack of neutrophil accumulation in the injured cortex but no change in blood-brain barrier permeability at 4 hours post injury, indicating that barrier leakage at early time-points is not mediated by neutrophils (Whalen et al., 1999). Systemic administration of an anti-GR-1 monoclonal antibody (RB6-8C5) 12 hours prior to and 12 hours following CCI to the adult brain resulted in decreased GR-1+ labeled neutrophils in the injured cortex and a reduction in the apoptotic

marker cleaved-caspase 3, brain edema, and microglia/macrophage activation within the first week post injury that corresponded to an attenuation in lesion volume at 7 and 14 DPI (Kenne et al., 2012). This suggests an adverse role of neutrophils in early secondary pathogenesis. However, this interpretation is confounded by the use of the RB6-8C5 monoclonal antibody which reacts strongly with mouse Ly6G, a marker of neutrophils, but also weakly with mouse Ly6C, which is found on subsets of monocytes and lymphocytes (Daley et al., 2008). Thus, it remains unclear if these findings are solely due to neutropenia.

Purinergic signaling

Two-photon laser scanning microscopy, coupled with a mild closed head compression injury (CHI), has offered unique insights into the early dynamic roles of microglia and neutrophils in the meningeal and parenchymal compartments (Roth et al., 2014). The very acute signatures of this injury speak to the vulnerability of the meningeal compartment, where cell death precedes that in the parenchyma. Within 30 minutes post injury there is vascular leakage in the meninges, the emergence of reactive oxygen species, and the subsequent breach of the glial limitans. Microglia respond to the compromised glial limitans by forming a transient adjacent phagocytic cell layer. This early pathogenesis coincides with the recruitment of neutrophils within the meningeal compartment (and not the parenchyma) where they show marked motility and close interactions with dead cells. Subsequent experiments implicated purinergic (P2) receptors, which respond to ATP released by dying cells, in recruitment of these neutrophils. Antagonism of P2X7, a receptor involved in recruitment of these leukocytes in a model of sterile inflammation in the liver (McDonald et al., 2010), resulted in a near absence of LysM $^{gfp/+}$ neutrophils in the meningeal space. Importantly, this resulted in increased cell death in the meningeal space with no impact on cell death in the parenchyma, suggesting that neutrophils may play a beneficial role in this acute scenario after a mild CHI.

Targeting the CXCR2 receptor

Neutrophil migration is regulated by chemokine signaling through CXCR2, which is elevated after TBI and has been associated with the progression of secondary damage (Kobayashi, 2008; Morganti-Kossmann et al., 2018). Performing CHI on wildtype and transgenic rodents deficient in CXCR2 showed that neutrophil recruitment in the injured hemisphere was greatly reduced in CXCR2 null rodents compared to wildtype at both 12 hours and 7 DPI (Semple et al., 2010). CXCR2 null rodents also had reduced tissue damage, neuronal loss, and cell death at 7 DPI, suggesting that neutrophil recruitment contributes to secondary tissue damage after TBI. It is noteworthy, however, that CXCR2 deficient rodents may demonstrate a compensatory increase in CXC neutrophil chemokines and G-CSF at 12 and 24 hours post injury, but no changes in other chemokines, indicating that the acute cytokine response may not be mediated primarily by neutrophils in CHI.

Another recent study showed that in two models of peripheral inflammatory response, CXCR2 deficient rodents displayed a transient exaggerated acute inflammatory response, including increased pro-inflammatory markers, reduced inflammatory resolution markers, and increased macrophage accumulation (Dyer et al., 2017). The marked contrast between the response of CXCR2 null animals to peripheral inflammation versus CHI highlights the

complexity of the role neutrophils play in the immune response to injury. Though neutrophils may not directly mediate acute cytokine response, in certain circumstances, their recruitment may be vital to the early inflammatory response through their interaction with other immune cells, such as macrophages.

Monocyte/neutrophil interactions

Monocytes infiltrate the injured adult brain through the C-C-chemokine receptor 2 (CCR2) and have been shown to adversely influence neuronal survival and neurological recovery (Hsieh et al., 2014). Recent studies have revealed that monocytes also modulate the recruitment of neutrophils into the injured adult brain. Systemic depletion of monocytes prior to injury, using liposome-entrapped clodronate, resulted in a reduction in neutrophils in the injured brain at 1 DPI, in the absence of any change in circulating neutrophils (Makinde et al., 2017). Clodronate-treated animals also showed reduced vasogenic edema and improved motor coordination and working memory, further supporting their adverse role in the injured brain. In rodents, monocytes are subdivided into classical monocytes ($CD115^{+}$, $Ly6C^{hi}$, $CD62^{+}$, $CCR2^{hi}$) and nonclassical monocytes ($CD115^{+}$, $Ly6C^{b}$, $CD62^{-}$, $CCR2^{lo}$), which also have high levels of the CX3CR1 fractalkine receptor, involved in leukocyte adhesion and migration (Geissmann et al., 2003; Ziegler-Heitbrock et al., 2010). Immunological depletion of classical monocytes with an anti-CCR2 antibody that recognizes $Ly6C^{hi}$ monocytes (Shi and Pamer, 2011) did not impact the recruitment of neutrophils. Conversely, a transgenic rodent model lacking CX3CR1 with reduced nonclassical monocytes showed a decrease in neutrophil recruitment after CCI. Taken together, these results suggest that neutrophil recruitment may be specifically regulated by nonclassical monocytes (Makinde et al., 2017).

MMP-9 and Neutrophils

Clinical studies have reported an elevation of MMP-9 in cerebrospinal fluid and blood of patients with TBI (Grossetete et al., 2009; Guilfoyle et al., 2015), and preclinical studies demonstrate its presence in the acutely injured brain. Preclinical models have offered insights into the source and contributions of this protease to secondary pathogenesis. Neutrophils represent one potential source of MMP-9; it is stored in tertiary granules and in studies of the ischemic rodent brain, elevation of MMP-9 is attributed to infiltrating neutrophils (Gidday et al., 2005; Justicia et al., 2003). MMP-9 is likewise upregulated in the adult rodent brain after traumatic injury where it is responsible for the degradation of myelin and disruption of the blood-brain barrier (Wang et al., 2000). Such findings are in line with known substrates of MMP-9; namely, myelin basic protein and the tight junction protein zonulae occludens-1 (Hannocks et al., 2017; Liu et al., 2012). However, to the best of our knowledge, no studies have provided definitive evidence that infiltrating neutrophils are a key source of this protease in the brain after a traumatic injury or if its targeted deletion alters the recruitment of neutrophils. Only one study has studied MMP-9 in the injured developing brain; similar to the adult brain, it is upregulated acutely post injury (Semple et al., 2015a). However, its blockade using a gelatinase inhibitor does not alter early cell death nor long-term neurological function. Such findings contrast that of the injured adult brain, where blockade with a gelatinase inhibitor resulted in a smaller cortical lesion volume, reduced dendritic degeneration and microglial and astrocyte activation, and improvements in

motor function (Hadass et al., 2013). Collectively, these findings support the upregulation of MMP-9 in both the injured adult and developing brain, but the extent to which neutrophils utilize this protease for transmigration into the parenchyma remains unclear. Moreover, the pathogenicity of MMP-9 may differ based upon age at time of injury. It is noteworthy that the activity of MMP-9 is more prominent in the acutely injured brain at PND 21 compared to that of the adult brain (Semple et al., 2015a; Wang et al., 2000). Such a distinction may impart greater vulnerability in the young brain to TBI as a consequence of enhanced proteolytic activity.

NeutrophilElastase

Neutrophil elastase is one of the most destructive enzymes in inflammatory mediated pathogenesis (Lee and Downey, 2001; Owen and Campbell, 1999). When neutrophils become activated, it is rapidly released from primary granules into the adjacent extracellular space (Lee and Downey, 2001). If left unchecked neutrophil elastase may not only intensify the host inflammatory response but can degrade a variety of matrix and nonmatrix proteins (i.e. plasma proteins, pro-inflammatory mediators, adhesion receptors) due to its broad specificity.

The destructive nature of neutrophil elastase is revealed in early preclinical models of spinal cord injury and cerebral ischemia. Pharmacologic blockade of this protease stabilized the barrier, resulted in an improvement in neurologic recovery in the injured spinal cord (Taoka et al., 1998; Tonai et al., 2001), reduced vasogenic edema and infarct volume (Shimakura et al., 2000), and protected hippocampal neurons (Matayoshi et al., 2009) in models of brain ischemia. The damaging consequences of neutrophil elastase are further realized in studies employing both a neutrophil elastase inhibitor and knockout models, where infarct volume is reduced in neutrophil elastase deficient animals after transient brain ischemia (Stowe et al., 2009). Such cross validation of genetic and pharmacologic approaches confirms the causal involvement of neutrophils in neurovascular pathology.

Most recently, neutrophil elastase has been studied in a model of CCI to the developing brain at PND 21 using both pharmacologic and genetic strategies (Semple et al., 2015b). A number of key findings have supported its potent pathogenicity and long-term impact on behavioral recovery. Neutrophil elastase activity is elevated in the cortex within the first 24 hours post injury, a finding that is consistent with the peak of infiltration of neutrophils into the injured brain (Claus et al., 2010). Brain-injured neutrophil elastase knockout animals show a reduction in vasogenic edema in the cortex and significant neuroprotection to the hippocampus as evidenced by reduced numbers of TUNEL and caspase+ cells. Upregulation of heme oxygenase, an indicator of oxidative stress, is likewise reduced in these animals. Subsequent behavioral assessments at adulthood reveals a reduction in injury-induced hyperactivity and improvement in spatial learning in the knockout compared to wildtype controls. Similar findings of early neuroprotection are reported in brain-injured wildtype rodents, treated with a neutrophil elastase inhibitor within the first 12 hours post injury (Semple et al., 2015b). These collective findings highlight the damaging consequences of neutrophil elastase. As neutrophils are recruited to the injured developing rodent brain over several weeks post injury (Claus et al., 2010) there is risk that prolonged exposure to this

protease furthers secondary damage and thus contributes to the long-term neurological deficits.

Closing Remarks

The developing rodent brain shows greater vulnerability to damage from a traumatic injury compared to that of the injured adult brain (See review; Potts et al., 2006). This is, in part, attributed to a “window of susceptibility”, characterized by a more robust response to chemokines and a prolonged recruitment of neutrophils, compared to that of the injured adult brain. Activated neutrophils, recruited to the injured brain, have the capacity to support secondary pathogenesis through the generation of reactive oxygen species (superoxide anion radicals, hydrogen peroxide, hypochlorous acid) (Snelgrove et al., 2018) and release of granular content, including neutrophil elastase and MMP-9, that collectively mediate cell death, disruption of the blood-brain barrier and degradation of the extracellular matrix. With a high content of fatty acids that is further compounded by low antioxidant reserves, the developing brain is poorly positioned to respond to these toxic products produced by activated neutrophils (See review; Bayir et al., 2006).

We are only beginning to understand the role of neutrophils in the injured adult brain. Studies involving both systemic depletion of neutrophils and genetic deficiency in CXCR2 argue for their involvement in secondary pathogenesis (Dyer et al., 2017; Semple et al., 2010; Uhl et al., 1994). The caveats to this interpretation include the lack of specificity in methods of depletion which could result in changes in other leukocyte populations (Daley et al., 2008) and the presence of CXCR2 on other myeloid lineage cell types including monocytes, macrophages, and mast cells (Olson and Ley, 2002).

Beyond the traditional viewpoint which considered neutrophils as terminally differentiated leukocytes that served antimicrobial functions, there is a growing body of evidence that speak to their ability to perform specialized functions. This has led to a much broader perspective on neutrophils, as transcriptionally active leukocytes that assume distinct phenotypes; there is evidence for tissue/organ specificity during homeostasis as well as subpopulations that reflect disease states including inflammation and metabolic dysregulation (Rosales, 2018). While much of this work has been done outside of the CNS, early studies in stroke models have identified subpopulations of neutrophils that are defined as either pro- or anti-inflammatory (Cuartero et al., 2013) and may be, in part, related to a “threshold” number of neutrophils with higher numbers more closely associated with an anti-inflammatory phenotype (Easton, 2013).

To the best of our knowledge, no studies have yet examined neutrophil subpopulations in the brain after a TBI. As neutrophils show protracted recruitment to the injured developing brain (Claus et al., 2010), it is possible that they will exhibit phenotypic changes, reflecting diverse environments that span acute injury to wound healing. Based upon studies of the neutrophil elastase knockout, it is clear that neutrophils are damaging to the acutely injured brain (Semple et al., 2015b). However, no studies to date have evaluated the properties of these leukocytes during wound healing after a TBI. Neutrophils have the potential to support wound healing in the injured brain. This is exemplified in studies using a model of ischemia

to skeletal muscle (Massena et al., 2015). Neutrophils, recruited to ischemic muscle, displayed a unique phenotype; namely, CD49d^{hi} CXCR4^{hi} VEGFR1, where VEGFR1 is the receptor for vascular endothelial growth factor (VEGF)-A. This study revealed that neutrophil recruitment to VEGF-A was dependent on activation of both VEGFR1 on neutrophils and VEGFR2 on endothelial cells and that this subpopulation of neutrophils was essential for angiogenesis (Massena et al., 2015). Such findings provide an additional perspective on neutrophils beyond their involvement in the inflammatory cascade, where they could be critical players in facilitating angiogenesis in the injured brain.

While most attention has been directed at understanding pathogenicity of activated neutrophils, there is a growing recognition of their potential benefits in the context of both early neuroprotection and tissue repair (See review; Liu et al., 2018; Snelgrove et al., 2018). From the perspective of neuroprotection, this is exemplified in recent studies of the meningeal compartment after a mild CHI to the adult brain where early purinergic-dependent recruitment of neutrophils results in reduced death of meningeal cells (Roth et al., 2014).

Such findings are in sharp contrast to a moderate CCI to the developing cortex, where neutrophil elastase, produced by infiltrating neutrophils, is a determinant of early neuronal death (Semple et al., 2015b). Neutrophils may behave differently in each of these scenarios, based on their known plasticity, where there is opportunity to change phenotypes and functions (Rosales, 2018); neutrophils may assume a more damaging phenotype with increasing injury severity, and/or when exposed to different environments (parenchyma of the brain versus meninges) and/or age at time of injury (young versus adult brain). These major unanswered questions may guide future investigations into heterogeneity of neutrophils in the context of injury (see Figure 3 for summary). Additionally, while data do support sex as a key biological variable in the young injured brain (Fraunberger et al., 2019; Spani et al., 2018), it has to be considered in the context of neutrophil function and warrants further investigation.

There is now substantial evidence to support pleotrophic functions (Snelgrove et al., 2018); beyond their established ability to phagocytose and clear apopotic cells and debris, these leukocytes are immune-regulatory, spanning pro-inflammatory functions, facilitating resolution of inflammation via pro-resolving lipid mediators and production of anti-inflammatory cytokines, and supporting wound healing events including neovascularization. As noted by others (Snelgrove et al., 2018), it is time to shift the perspective of neutrophils as “indiscriminate killers” to a broader viewpoint whereby their function is defined by context, timing, and location. For the injured developing brain, neutrophil recruitment spans acute injury to wound healing. Thus, these leukocytes may change their functionality depending upon the local environment that is responding to injury while simultaneously evolving in accordance with ongoing maturational cues.

Abbreviations

TBI	Traumatic brain injury
PND	postnatal day

CCI	controlled cortical impact
CNS	central nervous system
G-CSF	granulocyte-colony stimulation factor
MMP	matrix metalloproteinase
IL	interleukin

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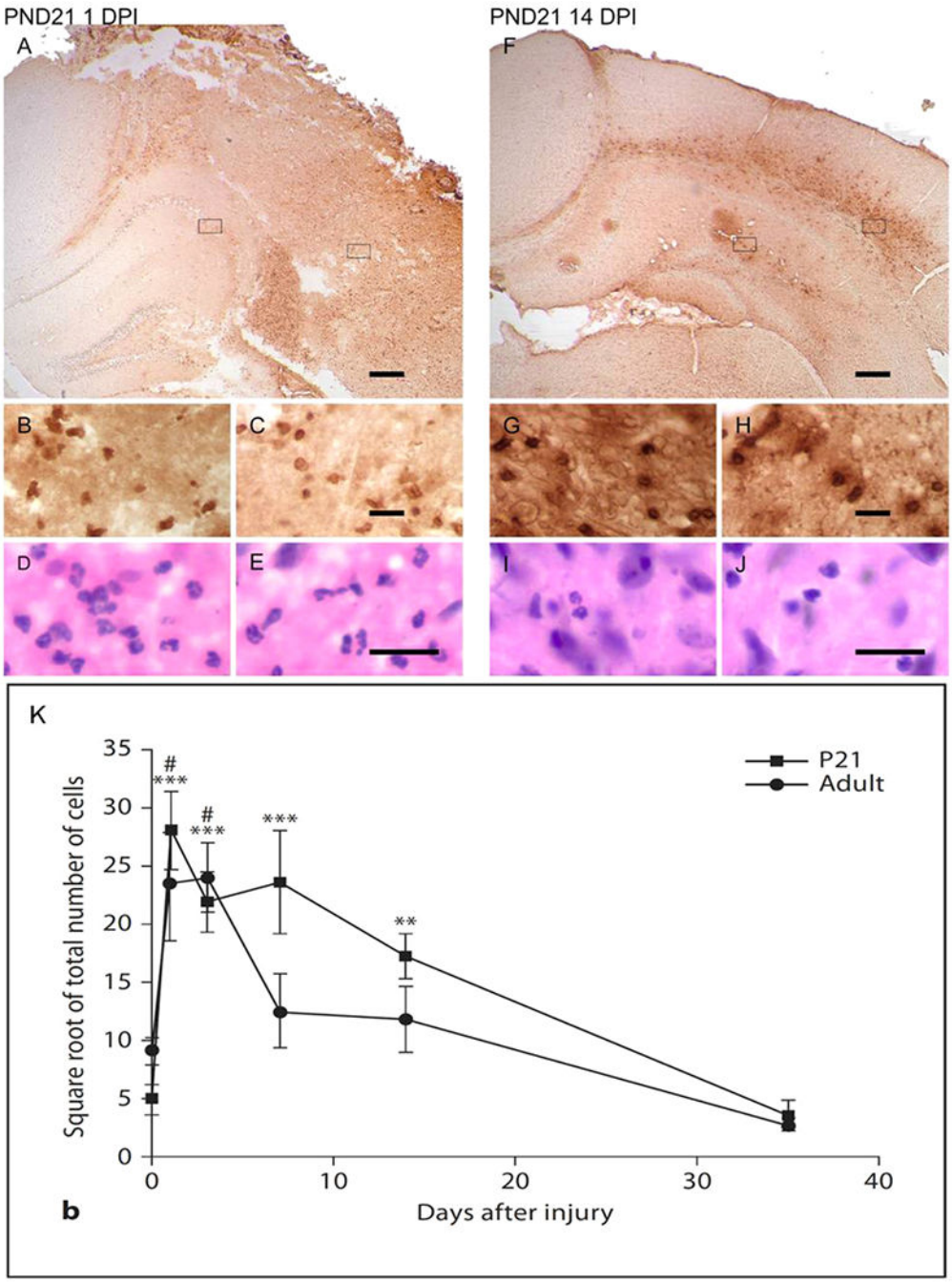

Figure 1.

Recruitment of neutrophils into the injured brain at PND 21. GR-1+ neutrophils are immunolocalized in the cortex and hippocampus at 1 (A-E) and 14 days (F-J) post injury. Boxes in A, F are represented at higher magnification in the cortex (B,G) and hippocampus (C,H). Adjacent sections stained with hematoxylin and eosin show cells with lobulated nuclei in the cortex (D,I) and hippocampus (E,J). Scale bars = 200 μm (A,F) and 20 μm (C,E,H,J). (K) Quantification of neutrophils in sections of the cortex after injury at either PND 21 or at adulthood. GR-1+ neutrophils are significantly higher in the injured young

brains within the first 2 weeks after injury relative to shams. In contrast, GR-1 is significantly elevated at only 1 and 3 days after injury to the adult brain. * * $p < 0.01$, * * * $p < 0.001$, one-way ANOVA followed by Newman-Keuls multiple comparison test (sham vs. PND 21); # $p < 0.05$, one-way ANOVA followed by Newman-Keuls multiple comparison test (sham vs. adult). Figures modified from Claus et al., 2010, with permission from S. Karger AG Publishers, Basel, Switzerland.

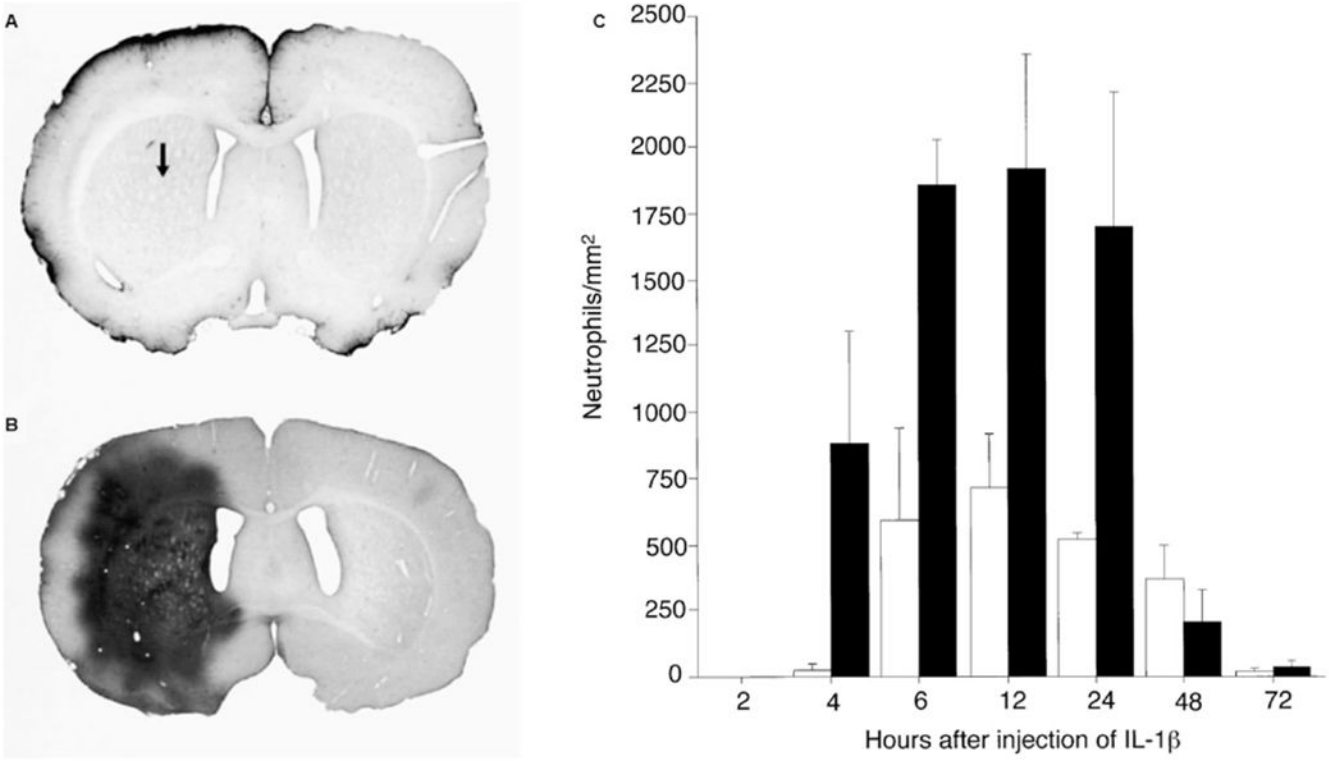

Figure 2.

Breakdown of the blood-brain barrier to the vascular tracer horseradish peroxidase and recruitment of neutrophils after intracortical administration of interleukin-1β. There is more limited leakage to horseradish peroxidase at 4 hours after administration to the adult brain (3 months old) compared to the brain at PND 21 (A-B) (Arrow in A shows the position of the injection site for all sections). Dark staining indicates regions of abnormal vascular permeability to horseradish peroxidase. Intracortical administration of interleukin-1β likewise resulted in a more robust recruitment of neutrophils into the young compared to the adult brain (C). The number of neutrophils was quantified in a region of striatum immediately adjacent to the site of injection. Solid black bars represent mean number of neutrophils present in the PND21 brain, open bars represent mean number of neutrophils present in the adult brain. Values are the mean ± SD. Figure modified from Anthony et al., 1997, with permission from Oxford Press.

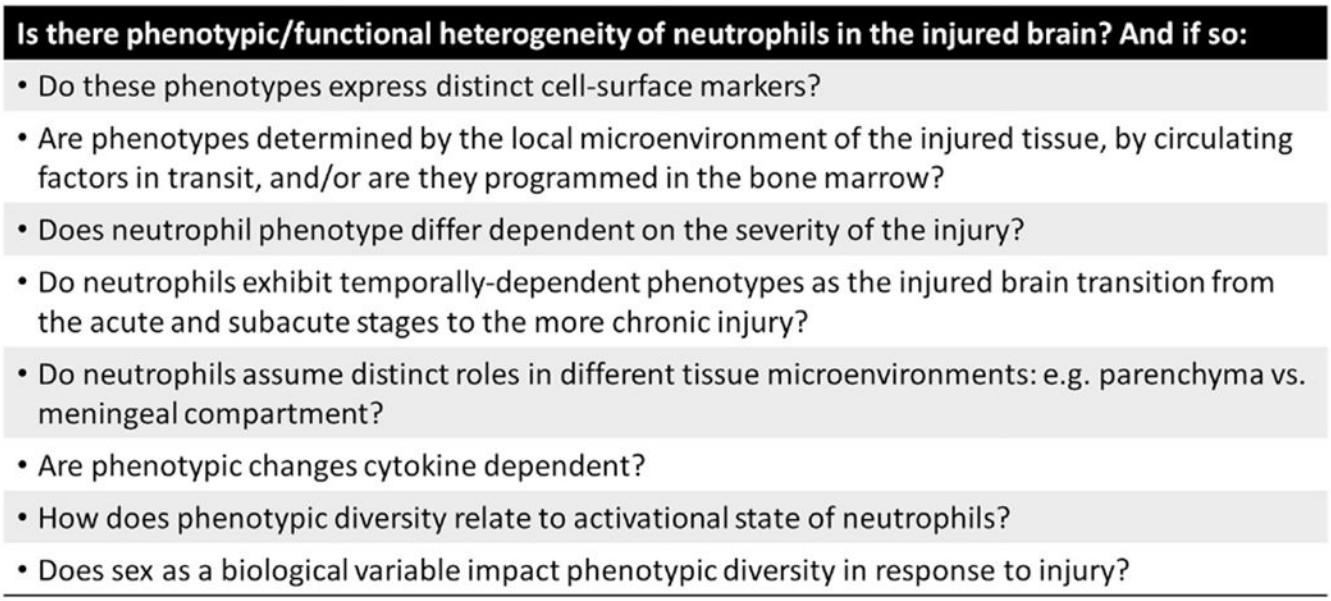

Figure 3.
Key emerging questions regarding neutrophil function.

Table 1.

Summary of Studies Targeting Neutrophil Recruitment after TBI

Summary of strategies that have altered recruitment of neutrophils in pre-clinical rodent models of TBI at adulthood and at postnatal day (PND)21. This table considers only those studies that have modified neutrophil recruitment via either immunological depletion of these leukocytes or pharmacologic methods, or by use of genetically modified animals. Acute, subacute and long-term neurological consequences are summarized in accordance with each study.

Adult Brain						
Reference	**TBI Model**	**Approach**	**Treatment Strategy**	**Neutrophil Numbers**	**Acute Findings**	**Subacute & Chronic Findings**
Uhl et al. 1994	WD	Vinblastine Sulfate	Route: IP (5 d prior to TBI)	Absolute cell count in blood (prior to & 24 hrs PI)	↓ CBF. No change-edema or lesion size (24 hrs PI)	Not Studied
Whalen et al. 1999	CCI	Anti-RP-3 mAb to neutrophils	Route: IV (1 hr prior to TBI & for experimental period)	Absolute cell count in blood and histology: RP-3+ cells (4 hrs PI)	↓ Neutrophil recruitment. No change- BBB permeability (4 hrs PI)	Not Studied
Semple et al. 2010	WD	CXCR2 KO	Not Applicable	Histology: NIMP-R14+ cells (12 hrs & 7d PI)	↑ Neutrophil chemokine production (12 & 24 hrs PI)	↑ tissue damage. No change- BBB permeability or neurologic recovery (7 d PI)
Kenne et al. 2012	CCI	Anti-GR-1 mAb RB6-8C5 to neutrophils	Route: IP (12 hrs prior & PI)	Histology: GR-1+ cells (24 hrs PI)	↓ Edema & cell death (24 hrs PI) ↓edema (48 hrs PI)	↓ lesion volume & microglial activation (7 d PI) ↓ lesion volume (14 d PI)
Roth et al. 2014	CHI	Purinergic Receptor (P2) Antagonist Or Glutathione (GSH)	Route: Topical to skull (30 mins prior to TBI)	Histology: $LysM^{GFP/+}$ cells (1, 3, & 6 hrs PI)	P2: ↓ Neutrophil recruitment (3 & 6 hrs PI) ↑ Meningeal cell death (12 hrs PI) GSH: ↓ Parenchymal cell death, microglial activation & breakdown of glial limitans (>1 hr PI) ↓ Neutrophil recruitment (3 & 6 hrs PI)	Not Studied
Makinde et al. 2017	CCI	Liposomal clodronate Or CX3CR1 KO	Route: IV (24 hrs prior to TBI)	Flow Cytometry: (24 hrs PI)	Clodronate and KO: ↓ Neutrophil recruitment & monocyte infiltration (24 hrs PI)	↓ edema, ↑ motor function & working memory (assessed only in clodronate group; 1 mo post injury)
Developing Brain (Postnatal day 21)						

Adult Brain						
Reference	**TBI Model**	**Approach**	**Treatment Strategy**	**Neutrophil Numbers**	**Acute Findings**	**Subacute & Chronic Findings**
Semple et al. 2015b	CCI	Neutrophil elastase (NE) Inhibitor Or NE KO	Route: IP (2, 6, & 12 hrs PI)	Histology: GR-1+ cells-drug study only (24 hrs PI)	NE KO: ↓ edema, cell death & oxidative stress (24 hrs PI) NE inhibitor: ↓ edema, cell death & oxidative stress (24 hrs PI)	NE KO: ↑ spatial memory, ↓ hyperactivity; no change in anxiety or motor deficits (2 mos PI) NE inhibitor: No change in neurologic recovery (2 mos PI)

Abbreviations/Explanations: BBB (blood-brain barrier), CBF (cerebral blood flow), CCI, (controlled cortical impact), CHI (Closed head injury), CXCR2 (Cytokine receptor 2, mediates neutrophil recruitment), CX3CR1 (C-X3-C motif chemokine receptor 1, impairs recruitment of monocyte-derived macrophages), d (day), hr (hour), IP (intraperitoneal), IV (intravenous), KO (knockout), $LysM^{GFP/+}$ (neutrophils are brightly labeled in this construct), mAb (monoclonal antibody), mo (month), PI (post injury) TBI (traumatic brain injury), WD (weight drop)