Chapter

Peripheral Immune Response Following Traumatic Brain Injury

Amanda Hazy, Elizabeth Kowalski, Nathalie Groot and Michelle Theus

Abstract

Traumatic brain injury (TBI) represents a leading contributor to long-term neurological damage. Though TBI is a leading cause of death and neurological damage worldwide, there exists no therapeutic treatments to alleviate deleterious secondary injury due to neuroinflammation. The continuum of pro- and anti-inflammatory response elicited by TBI is suggested to play a key role in the outcome of TBI; however, the underlying mechanisms remain poorly defined. This chapter explores rodent models of injury used to study the disease pathology of TBI, as well as the major contributions of the peripheral immune response following injury. Further, this chapter discusses the influence of individual immune cell types on neuroinflammation following TBI, focusing on peripheral monocyte/macrophages, their polarization state, and the current literature surrounding their behavior within the TBI milieu. Finally, cell-to-cell contact regulators that effect peripheral-induced neuroinflammation and may serve as novel targets for therapeutics will be highlighted.

Keywords: inflammation, monocytes, traumatic brain injury, blood-brain barrier

1. Introduction

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality in the United States and worldwide [1–4]. TBI results from an injury to the brain following exposure to external physical forces including falls, car accidents, explosive blasts, and assault [5, 6]. These injuries often have long-term consequences to the health of injured individuals, and few effective treatments are currently available [6]. The pathophysiology is characterized by damage to the neuronal and glial cells of the brain as well as the associated vasculature [6], and the role of inflammation as a causative agent of tissue injury has emerged as a focus of TBI research [7]. Preclinical research focusing on the mechanisms underlying secondary inflammation and treatment of TBI employs various animal models [8]. This review will discuss TBI as a public health problem, the pathology of TBI and the significance of the peripheral immune response in the outcome of TBI in human and animal models.

2. Prevalence of TBI

Traumatic brain injury is a major cause of death and disability in the United States and worldwide [1–4]. An estimated 69 million people sustain a TBI each year

around the world [9]. In the United States, incidence of TBI has risen steadily over recent years. An average of 1.7 million TBIs occurred per year from 2002 to 2006 [1], but an estimated 2.8 million TBIs occurred in 2013 [4]. There is a gender disparity in groups most affected by TBI—in the United States, males are more commonly affected than females. Age group differences are also evident in TBI prevalence, with young children, young adults, and the elderly most frequently suffering from TBI. The specific age groups that most commonly sustain TBIs are ages 0–4 years, 15–24 years, and 75 years and older [4]. Traumatic brain injuries arise from a variety of causes including traffic accidents, falls, abuse, sports injuries, and traumatic impact with an object [4, 5]. The most prevalent causes of injury vary predictably with patient age. Injuries in younger patients are most commonly associated with sports activities or high-risk behaviors such as distracted driving, while injury in the older population of patients is more frequently associated with falls [5]. These events cause injuries of a range of clinical severities including mild, moderate, and severe TBI. In the clinical setting, these injuries are most frequently classified using the Glasgow Coma Score (GCS) [10]. The GCS assesses overall consciousness of the patient and classifies injury severity based on eye, motor, and verbal responses to stimuli [5, 10]. Scores range from 3 to 15. Higher scores correlate with decreased injury severity—for clinical classification purposes, a GCS range of 13–15 has been used to demarcate mild injury, 9–12 for moderate injury, and 8 or less to indicate severe TBI [5]. Imaging modalities including CT and MRI are also used to further assess the severity of TBI and inform prognosis [10].

3. Pathology of TBI

A traumatic brain injury may be defined as an injury to brain tissue caused by direct external force [10]. The physical impact of TBI initiates a plethora of downstream processes with deleterious effects on neuronal and glial tissue. Overall, the pathophysiology of a TBI can be divided into primary and secondary phases of injury [5, 10–12]. The primary phase of injury includes the cellular damage caused at the instant of injury by the direct mechanical impact of trauma. Primary injury can manifest as cell death, hemorrhage, and/or diffuse axonal injury. First, neurons and supporting vasculature can be directly torn by the shear forces of injury. This damage to the neurovascular network results in intracranial hemorrhage, which can lead to increased intracranial pressure as blood builds up inside the skull. Intracranial bleeding can also generate hematomas. Both increased intracranial pressure and hematoma formation have negative impacts on neural recovery [11, 13]. Primary injury can also encompass diffuse axonal injury. Diffuse axonal injury is damage to neurons going beyond the initial lesion area, caused by dynamic forces spreading through the brain from the primary impact [11]. These physical forces resulting from traumatic brain injury can be either linear accelerational forces or rotational forces. Since neural tissue is elastic and does not have a strong internal structure, the brain has little tolerance for this disruption and is very susceptible to injury from these forces [12]. Primary injury also disturbs autoregulation of cerebral blood flow and cellular metabolism. Normal control mechanisms for blood flow and metabolism fail due to the cellular damage of TBI, resulting in cellular effects similar to those seen in ischemic stroke. As the massive damage overwhelms cellular metabolism, ATP production cannot match demand, and neuronal and glial supplies of ATP become inadequate to fuel cellular ion pumps. The resulting dysregulation of ion flow initiates various downstream pathways leading to necrosis, apoptosis, and oxidative damage [14]. Additional mechanisms of secondary injury have been described and

include the long-term changes resulting from the physiological processes triggered by the primary phase of injury [12].

3.1 Neuroinflammation

Neuroinflammation plays a major role in the secondary phase of injury. While all resident brain cells are involved in some way in the response to TBI, the role of microglia, the resident immune cells of the brain, in neuroinflammation has been particularly well-studied. When brain injury occurs, cells damaged in the primary phase of injury release cell signals known as damage-associated molecular patterns (DAMPs). In the early stages of injury, resident microglial cells are activated by these DAMPs and migrate to the injury site [7, 15]. These cells have a profound effect on both acute and chronic injury processes as they secrete both pro- and anti-inflammatory cytokines and can remain activated for up to 18 years after TBI [7, 16]. Cytokines released by microglia have a plethora of effects including alteration of local blood flow and modification of the blood-brain barrier (BBB) [15]. Microglia also assist in walling off the injured area in a protective effort to prevent the spread of bleeding and cellular damage. However, these cells can also generate additional reactive oxygen species (ROS) with damaging effects on cells [17]. While glial cell activation is a key part of the secondary phase of TBI, there is also an important role for the peripheral immune system in TBI recovery. The central nervous system is typically viewed as an immune-privileged site, with few or no peripheral-derived immune cells present. However, following TBI, the blood-brain barrier is damaged, allowing infiltration of peripheral-derived circulating immune cells including neutrophils, macrophages, and lymphocytes [17]. Glutamate excitotoxicity, oxidative stress, and neuroinflammation all contribute to the cellular damage observed in the secondary phase of injury, and the long-term damage resulting from these processes can be extensive. This secondary phase of injury is the primary target for TBI therapeutics—while efforts can be made to reduce TBI incidence, once a TBI has occurred nothing can be done to treat primary injury. Therefore, potential TBI treatments are aimed at reducing damage from the secondary phase of injury [14].

4. Rodent models of TBI

Multiple rodent models have been used to study the role of inflammation in TBI. Due to the variety of injury causes and individual patient health effects, human TBI exhibits multifaceted disease processes, and different animal models are used to recapitulate different aspects of human injury. Here, we discuss three common mouse models of TBI: weight drop, fluid percussion injury (FPI), and controlled cortical impact (CCI). All three of these models generate TBI by direct impact, either applied directly to the brain through a craniectomy or applied to the intact skull. While each of these models replicate certain features of human TBI, no one model fully expresses the varied picture of clinical TBI.

- Weight drop and fluid percussion injury are both used to produce diffuse injury in rodent models of TBI. Weight drop injury relies on gravity-driven fall of the weight to generate injury. Injury severity can be controlled by adjusting both the height of the drop and the mass of the weight used. Modification of injury severity allows this model to reproduce features of mild, moderate, or severe TBI. Weight drop injury results in cortical cell death, cerebral edema, neuroinflammation, and blood-brain barrier compromise, and this method of

injury is relatively time-efficient to perform [18, 19]. In addition, weight drop injury results in demonstrable cognitive deficits, which may reproduce features of human TBI [20].

- FPI can also reproduce certain histological features of human TBI and can be modified to generate different severities of injury. The FPI method is one of the most commonly used models of experimental TBI and can be adjusted to generate mild, moderate, or severe TBI [21]. Fluid percussion injury is performed using the injection of fluid into the cranial cavity, generating injury as a pressure wave spreads through the fluid applied to the brain [8, 22]. This model results in cortical contusion, hemorrhage, inflammation, diffuse axonal injury, and gliosis, with accompanying memory and motor deficits [21, 23, 24]. Application of FPI causes both focal and diffuse damage to the brain and has been used to assess multiple prospective TBI therapeutics [25].

- CCI generates injury by application of a mechanical focal impact to the brain using a controlled piston. This technique was initially developed to replicate features of injuries caused by automobile accidents but is now commonly used to study multiple aspects of focal TBI pathology [26]. Controlled cortical impact machines allow modification of the depth, velocity, dwell time, and angle of the impact, as well as variation of the size and shape of the impactor tip. These highly reproducible features make the CCI model especially well-suited to induce a wide range of injury severities, and the tight control of injury parameters is an important advantage of this model [26, 27]. The CCI method of experimental TBI typically includes a craniotomy before impact to the intact dura mater, although this method can also be used to produce closed-head injury [27]. Injury induced by CCI replicates many histopathological changes seen in human TBI, including cortical contusion, blood-brain barrier compromise, inflammation, and oxidative stress [26]. Corresponding to the histological features observed in this model, CCI results in functional deficits, including memory, learning, and motor deficits similar to those observed in human TBI patients [26]. These deficits are observed in both the acute and chronic periods, while other models including FPI less frequently report the chronic persistence of cognitive deficits [27]. The CCI model also has an overall higher survival rate compared to the fluid percussion model [26]. The reproducibility, tight control of experimental parameters, persistence of cognitive deficits, and high survival rate induced by CCI make it an excellent model for TBI research. For these reasons, the work outlined in this dissertation takes advantage of the CCI model.

5. Peripheral-derived immune cell response to TBI

The peripheral-derived immune cell response is a key feature of the physiologic response to traumatic brain injury, which can have both positive and negative effects. The central nervous system is typically regarded as an immune-privileged site due to the action of the blood-brain barrier (BBB), which prevents peripheral immune cells from readily entering CNS tissue [7, 15]. However, following TBI, the integrity of the blood-brain barrier is compromised by a variety of mechanisms, allowing infiltration of peripheral-derived immune cells into brain parenchyma [28]. Various immune cells including neutrophils, macrophages, and lymphocytes have been shown to infiltrate the lesion area following injury, releasing cytokines that influence recovery [17]. These peripheral immune cells have a profound effect on injury recovery—impact of these infiltrating cells can be either beneficial or

deleterious to recovery depending on the specific cells and mechanisms involved. While all of these cell types may affect TBI recovery, macrophages in particular have been a focus of TBI research [29]. This review will discuss the general mechanisms of blood-brain barrier compromise after TBI and survey the effects of peripheral immune cell infiltration, with a focus on macrophages.

5.1 Blood-brain barrier compromise and immune cell infiltration in TBI

The blood-brain barrier (BBB) forms a protective layer separating the CNS from the surrounding environment, including circulating peripheral immune cells. The brain is typically regarded as an immune-privileged site due to the operation of the BBB—under normal physiologic conditions, peripheral immune cells in the vasculature cannot enter CNS tissue [7, 15]. The healthy brain exists in a tightly regulated system, and proper operation of the BBB is critical in maintenance of the correct microenvironment for healthy neural function [30]. Multiple cell types including brain endothelial cells, astrocytes, and pericytes compose the BBB [30]. Traumatic brain injury compromises the BBB by direct damage to the cells composing this barrier. The direct damage to cerebral vasculature and disruption of endothelial tight junctions allows entry of immune cells and proteins from the vasculature into cerebral tissue [28, 31]. Rising calcium concentrations activate caspases in endothelial cells, initiating apoptosis of brain endothelial cells and resulting in additional damage to the BBB [28]. The glutamate excitotoxicity observed in TBI also has been shown to increase production of reactive oxygen and nitrogen species (known as oxidative stress), causing further apoptosis of brain endothelial cells [31]. Reactive oxygen species can also increase migration of peripheral monocytes through up-regulation of cellular adhesion molecules [31]. The physical damage to brain endothelial and glial cells combined with the activation of apoptotic and stress-related pathways in the endothelium that disrupt tight junctions can increase BBB permeability, allowing circulating peripheral immune cells to enter the brain. Massive influx of peripheral immune cells, induced by brain-derived cytokine release (IL-6, TNF, IL-1β, etc.) at the lesion area over time, further contributes to BBB damage. Additional cytokine, matrix metallopeptidase (MMP), and reactive oxygen species (ROS) released by activated neutrophils and monocyte/macrophages further disrupt the BBB via down-regulation of tight junction proteins as well as through recruitment of additional inflammatory cells [28, 31–34]. An overview of the major peripheral immune cell response is depicted in **Figure 1**.

5.2 Immune cell-specific contribution to TBI

Neutrophils: Neutrophils arrive at the lesion area in the early stages of injury—these cells migrate to the area of injury and infiltrate damaged brain tissue within the first 24 hours postinjury [33]. These cells are recruited by the release of IL-8, a chemoattractant cytokine known to be generated in the early stage of TBI [35]. Numbers of circulating neutrophils rise significantly in the acute phase of TBI. One study found that neutrophils present following TBI appear to be less susceptible to apoptosis than neutrophils in uninjured patients, which may contribute to the increased numbers observed [36]. In contrast to the few studies implicating a positive role for neutrophils in TBI recovery [33, 37], numerous show deleterious effects. One study, using the CCI model, found that neutrophil depletion improved tissue recovery. Neutrophil-depleted mice in this study showed decreased cell death and tissue loss following TBI [38]. Another study assessed the effects of decreased immune cell infiltration following TBI via administration of anti-intercellular adhesion molecule 1 (ICAM1) antibody in a fluid percussion model of rat TBI. Rats given anti-ICAM1

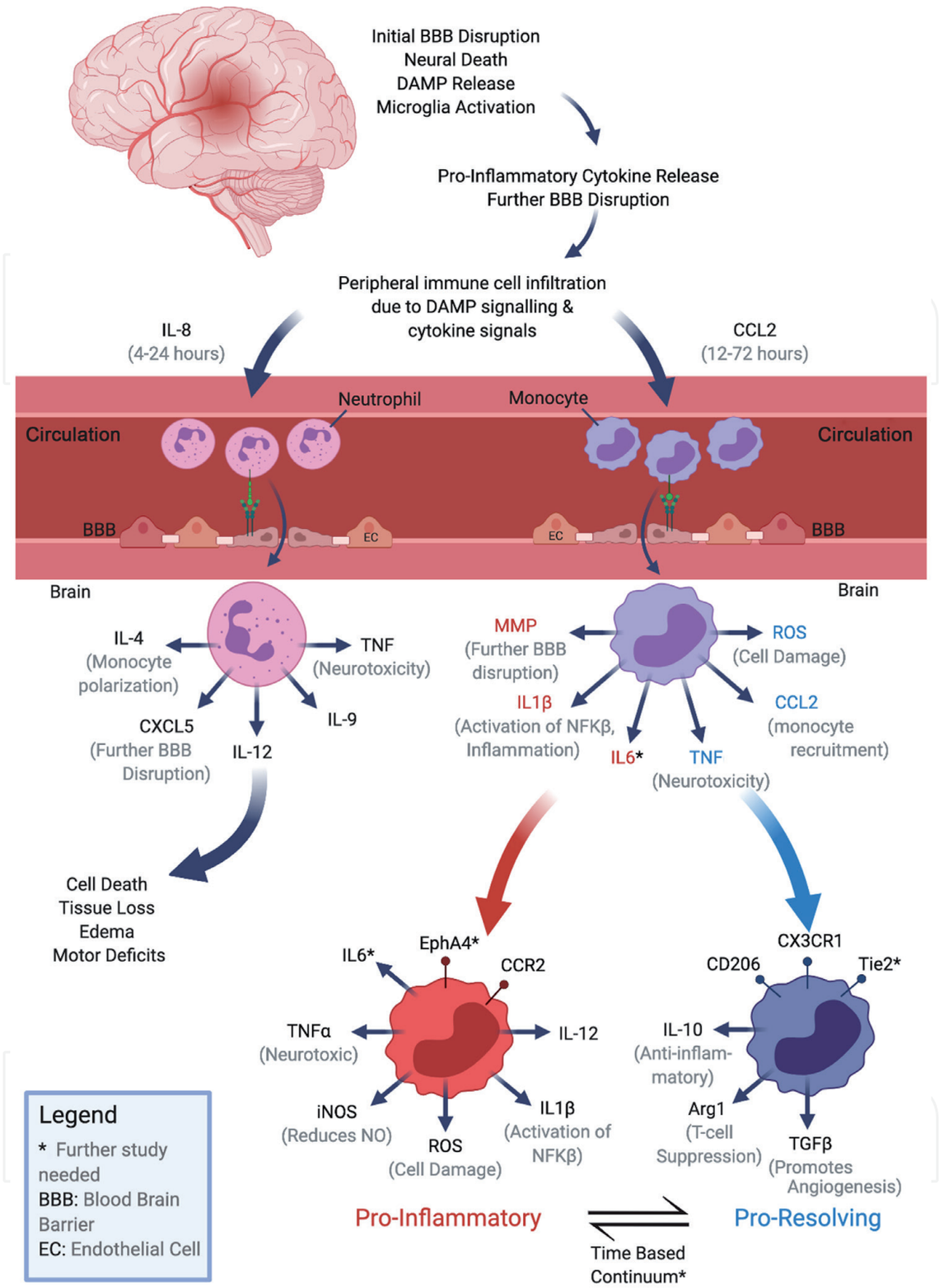

Figure 1.
Overview of major peripheral immune cell response to TBI.

showed decreased neutrophil infiltration following injury 26 hours following TBI, which correlated with increased motor recovery [39]. Several mechanisms have been suggested to explain these negative effects. Some studies have indicated that neutrophils bind endothelial cells and platelets after TBI, decreasing blood flow and promoting ischemia [33]. As previously mentioned, neutrophils can also damage the BBB through release of MMPs and reactive oxygen and nitrogen species [33]. In addition, many of the cytokines generated by neutrophils following TBI have been

shown to have negative effects on neural recovery. These cytokines include IL-9, IL-12, CXCL5, and TNFα. IL-9 can increase the damage caused by excitotoxicity following TBI, and high levels of IL-12 have been correlated with poor postinjury outcome (**Figure 1**). CXCL5 contributes to BBB compromise, and TNFα plays a role in neurotoxicity [33]. However, other studies have found that infiltration of peripheral cells in the acute stage of injury has little effect on recovery, suggesting instead that infiltration of peripheral-derived monocytes in the later stages of injury (greater than 48 hours after injury) has the greatest influence on injury progression [7].

Monocytes: The role of monocyte/macrophages has been particularly well-studied in regard to the effects of infiltrating peripheral-derived immune cells after TBI. Although a minority in terms of numbers of circulating immune cells, composing only 5–10% of the peripheral immune cell population, monocytes play an important role in TBI recovery [36]. Monocytes are the primary infiltrating immune cells observed at 3–5 days following injury [29]. While some studies have even argued that peripheral monocytes are the most prominent infiltrating immune cell at 24 hours postinjury as well [40]. Circulating monocytes can display pro- or anti-inflammatory properties. When monocytes migrate into affected tissue, they mature into macrophages with pro- or anti-inflammatory characteristics [36]. These cells can have a neuroprotective effect via phagocytosis of dead cell debris, release of growth factors, and production of anti-inflammatory cytokines. Monocyte/macrophages also release granulocyte-macrophage colony-stimulating factor (GM-CSF), which may have a neuroprotective effect through promotion of stem cell differentiation and suppression of apoptotic pathways [41]. However, monocyte/macrophages may have differing effects on TBI recovery depending on their inflammatory profile.

While monocyte/macrophages may be beneficial in some aspects of TBI recovery, other studies have found that these cells may also negatively affect neural recovery through different mechanisms. One study assessed the influence of macrophages on TBI recovery using a chemokine CC ligand-2 (CCL2) knockout mouse model. This study found increased levels of CCL2 following TBI in both human patients and in a murine weight drop injury model. CCL2 knockout mice showed decreased macrophage accumulation and smaller lesion volumes at 2 and 4 weeks after injury [42]. One study showed that depletion of monocytes using clodronate liposomes decreased neutrophil infiltration and edema and resulted in improved neurobehavioral recovery [43]. Several mechanisms have been suggested by which macrophages could exert neurotoxic effects. Infiltrating macrophages may release reactive oxygen and nitrogen species, increase additional recruitment of neutrophils and monocytes, and generate multiple pro-inflammatory cytokines including TNF, IL-1β, and IL-6 (**Figure 1**) [41]. The apparent discrepancy between the neurodegenerative and pro-resolving effects of macrophages following TBI is most likely due to the release of both pro- and anti-inflammatory signals from these cells, with corresponding positive or negative effects [29]. As previously mentioned, monocytes are capable of maturing into macrophages with either pro- or anti-inflammatory characteristics [36]. These two populations are traditionally defined as M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. Although the overall balance between these phenotypes is driven by injury processes [44], their differential characteristics and the mechanisms underlying their fate choice remain under investigation.

6. The M1/M2 continuum in TBI

Monocyte/macrophages display different phenotypes depending on the cellular microenvironment. Classical macrophages, called M1 macrophages, specialize in promoting inflammation and phagocytosing pathogens. The second class of

macrophages, called M2 macrophages, serves to promote tissue recovery [45]. Macrophages are a critical part of the tissue repair process following injury, but these cells can be either helpful or damaging depending on M1/2 status. Following TBI, macrophage polarization toward the M1 phenotype has been associated with neurodegeneration, while polarization toward the M2 phenotype has been shown to reduce oxidative stress [46]. However, these classes are not absolute—macrophages respond to their cellular environment to become more or less M1/2, existing on a continuum with M1 and M2 subcharacteristics at either end [45]. The varied expression of M1 pro-inflammatory vs. M2 pro-recovery traits can be a critical factor in recovery during the peripheral-derived inflammatory response to TBI.

6.1 Classical role of macrophages as pro-inflammatory cells in TBI (M1 phenotype): time course

The classically activated or M1 phenotype macrophages are known to function as pro-inflammatory cells. Early studies indicated that these cells become activated by a combination of IFNγ signaling and either direct TNF signaling or Toll-like receptor-induced production of TNF, usually triggered by lipopolysaccharide (LPS) [47]. In the typical response to wound healing outside the CNS, these cells are important in protection against bacterial infection. M1-polarized macrophages generate reactive oxygen species and also activate inducible nitric oxide synthase (iNOS) to generate nitric oxide as well as an array of pro-inflammatory cytokines including IL-12, TNFα, IL-6, IL-1β, and nitric oxide [47, 48]. Identification of M1 macrophages is typically done by measuring gene expression of characteristic markers including IL-12, IL-1β, iNOS, TNFα, and IL-6 [46]. High levels of CCR2 with low CX3CR1 expression have also been used as an indicator of pro-inflammatory status in macrophages [49]. Bystander tissue damage from M1 macrophages can be catastrophic in the normally immune-privileged setting of the CNS.

Peripheral-derived macrophages have been shown to rapidly infiltrate the injured brain within the first 1–3 days postinjury [46]. Although both M1 and M2 macrophages are likely present at this stage, early studies of macrophage polarization following TBI indicated that the M1 phenotype predominates in the *initial response* to brain trauma [48]. CCI-induced increase in expression of pro-inflammatory markers has been demonstrated as early as 6 hours following injury, suggesting that macrophages expressing M1 traits are a key part of the acute response to TBI [46]. One study found that increases in the number of IL-12-expressing macrophages/microglia were evident by 24 hours following CCI injury, and the number remained increased compared to sham controls out to 7 days postinjury [46]. Other work demonstrated that either macrophages polarized toward the M1 phenotype or a transitional phenotype between M1/2 (to be discussed later) become predominant over M2 phenotype by 7 days following CCI injury. This phenomenon correlates with neurodegeneration [46].

Based on these reports, macrophages seem to be skewed toward the M1 phenotype for an extended period following CNS injury, with corresponding negative effects on recovery. This contrasts with the typical immune response outside neural tissue, where an early increase in M1 macrophages gives way to pro-recovery M2 macrophages [46]. The neurotoxic effect of M1 macrophages is most likely mediated by pro-inflammatory cytokines. Levels of M1-associated pro-inflammatory cytokines transiently increase in brain tissue during the acute response to injury. Specifically, IL-1β, IL-6, and TNFα levels have been shown to significantly increase in brain tissue by 12 hours postinjury in a mouse model. These cytokines return to sham levels by 7 days postinjury [50]. Support for the importance of these cytokines as mediators of M1-induced secondary neural damage following TBI is provided by a study targeting

these cytokines as a potential TBI therapeutic. For example, treatment with Minozac, an inhibitor of pro-inflammatory cytokines, ameliorated the TBI-induced increase of pro-inflammatory cytokines in cortex and hippocampus and resulted in decreased neuronal damage and improved neurocognitive function following TBI [50].

6.2 Pro-resolving macrophages in TBI (M2 phenotype): time course

The alternatively activated or M2 phenotypic macrophages are known to serve as pro-recovery or anti-inflammatory cells. These cells are activated by IL4 and serve an immunoregulatory function, in contrast to the microbe-killing function of their M1 counterparts [47]. Like M1 macrophages, identification of M2 macrophages employs gene expression levels of a wide array of characteristic markers. Markers commonly used for this purpose include CD206, Fizz1, Ym1, IL1-RN, Arg1, TGFβ, SOCS3, and IL4-RA [46]. Low levels of CCR2 with high CX3CR1 expression have also been used as a marker for pro-repair macrophages [49]. Studies using these markers have demonstrated an important role for M2-polarized macrophages at multiple time points following TBI. Increases in M2 markers have been shown as early as 6 hours following CCI injury [46]. The reported timeline of M2 influence varies depending on the specific markers assessed. For example, the number of TGFβ-expressing macrophages/microglia has been demonstrated to increase by 24 hours post-CCI injury and remain elevated compared to sham out to 7 days following injury [46]. Increase in expression of Arg1 has also been demonstrated in macrophages/microglia following CCI. Interestingly, the increase in Arg1 expression in macrophages/microglia, which first becomes evident at 24 hours post-CCI, continues to rise out to 7 days postinjury rather than decreasing back toward normal levels as was observed for TGFβ [46]. Expression of CD163, another marker of the pro-resolving M2 phenotype, has also been investigated following TBI. One study showed increased expression of $CD163^+$ macrophages following weight-drop TBI in a rat model. This may have anti-inflammatory effects following TBI through suppression of the pro-inflammatory macrophage phenotype [51]. While timing of expression of specific markers can vary, these studies indicate that macrophages expressing M2 phenotypic traits are a significant factor in TBI recovery.

Macrophage polarization toward the alternatively activated or M2 phenotype has beneficial effects on recovery following TBI through a variety of mechanisms. M2-polarized macrophages are characterized by expression of multiple markers including arginase 1 (Arg1), CD206, CD301, resistin-like α, and PDL2 [48]. Alternatively activated macrophages have been shown to decrease T-cell proliferation, promote angiogenesis, assist in generation of extracellular matrix components, and benefit wound healing and tissue repair [47]. In addition, the anti-inflammatory cytokines TGFβ and IL10 secreted by alternatively activated macrophages help to decrease activation of classical macrophages, reducing bystander tissue damage [47]. One study demonstrated that experimentally altering macrophage/microglia phenotype to favor M2 polarization by inhibition of NOX2 results in decreased oxidative damage [46]. In another report, inhibition of high-mobility group box 1 (HMGB1) decreased M1 and increased M2 polarization of macrophages/microglia, which correlated with decreased lesion volume and improved recovery [52]. Moreover, activation of the cannabinoid receptor CB2R decreased M1 and promoted M2 macrophage polarization, accompanied by decreased edema and improved blood flow and behavioral recovery [53]. These studies employed different methods to influence macrophages toward the M2 phenotype with similar results—*increased expression of M2 traits has a positive impact on TBI recovery.* Additional studies are needed to confirm this beneficial role of M2 macrophages in TBI.

7. Continuum of expression between M1/2 and influence on TBI

As previously mentioned, macrophages *in vivo* do not always show a sharply demarcated M1 or M2 phenotype. Several studies have shown expression of both M1 and M2 traits in macrophages following TBI, while others have demonstrated that macrophages can switch between phenotypes [54, 55]. One study using the CCI model demonstrated co-expression of iNOS, a classical M1 marker, with Arg1, an M2 marker, in perilesional macrophages/microglia following injury [46]. Another study assessed expression of a wide array of pro-inflammatory (associated with M1) and anti-inflammatory (associated with M2) genes in mouse cortical tissue following CCI injury and found that both sets of genes are co-expressed at 1, 2, and 7 days postinjury. This study also showed that perilesional microglia/macrophages co-labeled with both M1 and M2 markers at all three time points [56]. A different study using flow cytometry to sort Arg1-positive and Arg1-negative brain macrophages following TBI demonstrated that neither $Arg1^+$ or $Arg1^-$ cells displayed gene expression profiles consistent with the M1 or M2 patterns defined by *in vitro* studies, although two distinct populations of macrophages did seem to exist in this context [48]. These findings suggest that the classic M1 and M2 traits may actually coexist in the same macrophages following TBI. To confirm this at the level of the individual macrophage, one study employed single-cell RNA sequencing to assess the expression of classical and alternative markers in individual macrophages 1 day following TBI. This work demonstrated that traditional M1/2 markers are frequently co-expressed at high levels in the same cell [55]. This study also demonstrated that high expression of well-known M1 or M2 markers did not seem to down-regulate expression of markers of the opposite class. Some macrophages with high expression of Arg1, an established M2 marker, also displayed high expression of TNF and/or IL-1β, known M1 markers [55]. This type of M1/2 combination profile was displayed in a variety of genes, demonstrating that macrophage polarization *in vivo* can widely differ from the traditional M1/2 paradigm established primarily by *in vitro* studies [55]. Surprisingly, this study actually failed to find any macrophages that fit entirely within the M1 or M2 category, suggesting that all macrophages responding to TBI respond to injury stimuli along a continuum of expression [55]. Intermediate macrophage phenotypes with traits of both M1 and M2 have also been found in studies of spinal cord injury and Alzheimer's disease [46]. The results of these studies indicate the existence of a continuum between M1 and M2 macrophages in the setting of brain injury and disease.

The specific stimuli and mechanisms involved in the continuum of M1/2 expression are currently areas of active research. Some authors have suggested that dual expression of M1 and M2 characteristics is a necessary part of the macrophage response to TBI, as these cells must respond to both pro- and anti-inflammatory environmental signals simultaneously in the setting of brain trauma [54]. This concept is supported by the results of the previously mentioned study demonstrating concurrent expression of both pro- and anti-inflammatory gene signatures [56]. The function of infiltrating monocyte/macrophages, therefore, appears to depend more on the specific gene expression and cytokine profile than on overall classification as M1 or M2. These findings underscore the importance of improving our understanding of the pathways involved in regulation of expression on the M1/2 continuum. Data from multiple studies have indicated that the Tie2/Angiopoietin pathway is an important factor in the continuum of expression between M1 and M2 macrophages. In addition, data from our project, to be discussed in the following chapters, have specifically implicated this pathway in the context of M1/2 polarization after TBI.

8. Tie2/Angiopoietin signaling in immune cells

The Tie2/Angiopoietin signaling axis was first identified for its key role in the regulation of angiogenic pathways, but this receptor complex is also gaining increasing recognition for its importance in peripheral immune cells. The receptor tyrosine kinase Tie2 (also known as Tek) interacts with its ligands, the angiopoietin family of proteins, to influence vascular development [57]. Studies in endothelial cells have shown that Tie2 is differentially regulated by its ligands Angiopoietin 1 (Angpt1) and Angiopoietin 2 (Angpt2). Angpt1 typically acts as an agonist for Tie2, while Angpt2 serves as an antagonist with several exceptions [58, 59]. Although Tie2/Angiopoietin signaling has been most studied for its role in regulation of vascular function, Tie2 is also expressed in a subpopulation of monocyte/macrophages called Tie2-expressing monocytes (TEMs) implicated in tumor formation and inflammation [60]. This review will discuss the mechanisms involved in the Tie2/Angiopoietin signaling axis and investigate the function of TEMs in various cellular contexts.

8.1 Overview of the Tie2/Angiopoietin axis

Tie2 is a receptor tyrosine kinase first identified on vascular endothelial cells [61]. There are multiple components to the Tie2 signaling pathway where the angiopoietin ligands serve as binding partners [59]. In addition to its expression on endothelial cells, Tie2 is expressed in TEMs, hematopoietic stem cells, neutrophils, eosinophils, and some muscle satellite cells [59, 62]. Angiopoietin 1 (Angpt1) is primarily expressed in platelets and perivascular cells, while Angiopoietin 2 (Angpt2) is expressed in endothelial cells [63]. Expression of both Angiopoietins has also been demonstrated in hematopoietic stem cells and some immune cell types including monocyte/macrophages [64, 65]. Angpt1 serves as a Tie2 agonist, activating this receptor and increasing endothelial vessel stability [59]. However, the function of Angiopoietin 2 (Angpt2) is more variable. Some studies have shown that Angpt2 can act as either an agonist or antagonist of Tie2 depending on cellular context, and increased expression of Angpt2 has been demonstrated in multiple disease states [59]. Angpt2 has been found to act as a Tie2 agonist in the context of decreased Angpt1 signaling, absence of Tie1/Tie2 heterocomplexes, or inhibition of vascular endothelial protein tyrosine phosphatase (VE-PTP) in the endothelium [59, 66, 67]. However, the dominant role of Angpt2 and/or these co-complexes in TBI has not been established. This ligand has repeatedly been shown to act as an antagonist in the setting of inflammation [68]. Although less studied than its counterpart, Tie1 has also been found to interact with Tie2 to promote Tie2/Angiopoietin interactions in vascular remodeling [59, 69]. The interactions between Tie2, Tie1, Angpt1, and Angpt2 have a profound influence on cell survival and vascular permeability [59, 61].

The downstream cellular effects of Tie2 binding with an Angiopoietin ligand can vary widely with cellular context. This is partially due to the differing effects of Angpt1 vs. Angpt2—Angpt1 binding has been shown to oppose the effects of inflammatory cytokines and decrease vascular permeability, while Angpt2 has been found to increase vascular permeability in a number of inflammatory models [59]. Binding patterns of these two ligands with Tie2 are distinct from each other, which may contribute to their differing effects. The fibrinogen-like domain of Angpt1 binds an immunoglobulin domain of Tie2, which may help Angpt1 increase cluster formation and cross-phosphorylation of Tie2 upon binding [68]. In contrast, Angpt2 has a slightly different amino acid sequence in the fibrinogen-like domain

and is also more likely to form dimers than oligomers. These structural differences may contribute to the different effects of the two ligands [68]. The central importance of clustering in Tie2 activation is confirmed by the results of one study that used an anti-Angpt2 antibody to cluster Angpt2. The clustering of Angpt2 caused it to act as an agonist to Tie2 rather than an antagonist, resulting in decreased vascular permeability and increased organ protection in the setting of sepsis [70]. Once Tie2 is activated, multiple downstream signaling pathways could be involved as effectors. Specifically, the Akt/PI3K (phosphatidylinositol 3 kinase) pathway has been implicated as a downstream effector of Tie2. This pathway is critical for cell survival and M2 macrophage polarization [61, 71, 72]. In the context of inflammation, Tie2 activation is decreased by a variety of mechanisms, (1) Angpt2 can be released from endothelial cells and competitively inhibits Angpt1/Tie2 binding, (2) overall expression of Tie2 and Angpt1 may be decreased, or (3) the extracellular domain of Tie2 can be cleaved [68]. The decrease in Tie2-Akt/PI3K signaling up-regulates Angpt2. This creates a feedback loop that further decreases Tie2 signaling [68]. The overall effect of the increasing endothelial-derived Angpt2 signaling is an increase in vascular permeability and amplification of inflammatory processes; however, these effects in the brain have not been established following TBI-induced neuroinflammation [63].

While expression of Tie2 has been most studied in endothelial cells, Tie2 has also been shown to be expressed in hematopoietic cell types. The role of Tie2 has been studied in hematopoietic stem cells and a subset of monocytes in addition to vascular and lymphatic endothelial cells [59]. Interestingly, Tie2 expression has also been demonstrated on neutrophils—Angiopoietin 1 has been shown to interact with Tie2 on neutrophils to promote neutrophil migration [73]. The role of Tie2 in macrophages has been increasingly recognized for its importance in tumorigenesis and inflammation. This critical function of Tie2 signaling will be discussed in the following sections of this review.

8.2 Tie2-expressing macrophages

Tie2 has been shown to play an important role in a subset of monocyte/macrophages known as Tie2-expressing monocytes or macrophages (TEMs). TEMs have been most studied in the setting of tumorigenesis and have been found to promote tumor development through a variety of mechanisms. In addition to potentiating overall tumor growth and metastasis, TEMs have been demonstrated to directly promote tumor angiogenesis [74]. Other research has shown that TEMs not only promote tumorigenesis but are necessary for tumor angiogenesis and tumor recurrence following chemotherapy [75]. Several mechanisms have been proposed as effectors of this process. The interaction of Angpt2 with Tie2 in TEMs has been implicated in the pro-angiogenic effect of TEMs as well as in metastasis. One study found that inhibition of Angpt2 blocked the pro-angiogenic function of TEMs in tumors, and another study suggested that inhibition of Angpt2 could help to limit metastasis [59]. Tumor-associated expression of Angpt2 has also been shown to increase expression of pro-angiogenic factors in TEMs [76]. In addition, TEMs in tumors display increased expression of the anti-inflammatory cytokine IL-10. Stimulation of these cells by Angpt2 can work through IL-10 to influence activity of T cells by decreasing T-cell proliferation and increasing regulatory T cells for an overall immunosuppressive effect [77]. Angpt1-Tie2 interaction may also influence tumor development. TEMs are known to express Angpt1 [78], indicating that they may be able to activate Tie2 through autocrine signaling. Angpt1 expression in tumor-infiltrating TEMs has been suggested as a mechanism of increasing tumor angiogenesis through interaction with

endothelial cells [78]. While both Angpt1 and Angpt2 may influence the tumor-promoting activity of TEMs, studies agree that the protumorigenic activity of these cells is under control of Tie2/Angiopoietin signaling. This discovery has established the Tie2/Angiopoietin signaling axis as a target of interest in tumor therapeutic research. Several treatments aimed at blocking Tie2/Angiopoietin signaling are currently in development, with three Tie2/Angiopoietin inhibitors currently in clinical trials as cancer therapeutics [79]. No trials are currently underway for brain injury.

The origin and M1/2 polarization status of TEMS is currently under active investigation. Some studies have found that these cells seem to be polarized toward the M2 phenotype [80]. TEMs have been shown to display increased expression of arginase 1 (Arg1) and scavenger receptors accompanied by decreased expression of pro-inflammatory and anti-angiogenic mediators compared to tumor-associated macrophages that lack Tie2 expression. This expression pattern is consistent with an M2 polarization state [78]. In addition, TEMs exert an anti-inflammatory effect in the context of tumorigenesis. These cells release IL-10 and VEGF, decrease T-cell proliferation, inhibit antigen presentation by dendritic cells, and promote T-cell conversion to regulatory T cells [80]. However, TEMs may also play important roles in a variety of disease settings aside from tumorigenesis. Specifically, many studies have implicated TEMs as key regulators of inflammation.

9. TEMs in inflammation

An influential role of TEMs under inflammatory conditions remains under investigation. In the setting of inflammation, Tie2 expression may influence macrophage phenotype on the M1/2 continuum [45]. While TEMs have been shown to favor the M2 phenotype in the context of tumor infiltration, Tie2 expression has been demonstrated in monocytes polarized to both M1 and M2 phenotypes [60, 78]. Investigations of whether Tie2 expression in inflammatory disease correlates with M1 or M2 phenotype have shown conflicting results. One study showed Tie2 activation in synovial macrophages of human patients with autoimmune rheumatoid arthritis. In this study, Angpt2/Tie2 signaling interacted with TNF to up-regulate IL-6 and macrophage inflammatory protein 1α (MIP-1α), and antagonizing this pathway reduced synovial inflammation in a mouse model of disease [81]. Exogenous Angpt1 application to human monocyte cultures has been shown to up-regulate TNF and possibly regulate their polarization state [45]. Another study found that Angiopoietin binding works synergistically with TNF to drive expression of pro-inflammatory cytokines in human-cultured monocytes under several polarized conditions [60]. In contrast, previous studies showed anti-inflammatory effects of Angpt1 binding in TEMs and found that Angpt1 blocks LPS-induced TEM migration and ameliorates LPS-induced TNF expression via NF-KB [82]. Angpt2 has also been shown to augment immunosuppressive cytokines and T-reg chemokines expressed by TEMS *in vitro* [77]. These conflicting results suggest that Tie2 signaling may serve differential functions depending on acute and chronic conditions and may be dependent upon the activation state of the cells. Furthermore, the role of clustering and oligomerization of angiopoietin molecules on Tie2 binding and activation [83] raises the possibility that Tie2 may be differentially regulated under these conditions, although individual studies failed to confirm p-Tie2 states directly. Therefore, the role of Tie2 activation in the M1/M2 continuum remains unclear. While Tie2 signaling has been implicated in promoting injury-induced and tumor-promoting vascular health in numerous non-CNS models [59, 74, 75], its role

in regulating monocyte/macrophage polarization in CNS inflammation remains unexplored. Furthermore, limited data exist regarding novel pathways that may regulate Tie2 function in TBI-induced peripheral immune response.

10. Cell-to-cell contact in TBI-induced inflammation

Many cell-to-cell interactions become key in the regulation of inflammation following TBI. As previously mentioned, one of the most detrimental results of TBI is the breakdown of the BBB. Adhesion molecules contribute to cell-cell and cell-extracellular matrix (ECM) interactions that mediate inflammation by promoting peripheral leukocyte infiltration across the BBB and aggregation to the site of injury. This represents the initiation of the inflammatory response [84]. After tissue injury, circulating immune cells will recognize signals released from injured tissue, will stop on the luminal surface of blood vessels, transmigrate paracellularly across the endothelial layer, and enter the injured milieu [85, 86]. This process is referred to as the leukocyte adhesion cascade, which involves tethering, rolling, activation, firm adhesion, and transmigration. Numerous preclinical models have determined the detrimental role of leukocyte migration and accumulation during neuroinflammation in TBI [39, 87].

10.1 Adhesion molecules involved in TBI-induced inflammation

Adhesion molecules involved in these processes include three major families: selectins, integrins, and immunoglobulins. Selectins are a group of transmembrane glycoproteins expressed on the surface of leukocytes, which express L-selectin, and endothelial cells, which express P- and E-selectins following activation [88]. These glycoproteins mediate the initial tethering of leukocytes to the vessel wall by binding to counter-receptors and rolling within moments of tissue injury [89]. Integrins are a family of adhesion molecules broken into subclassifications of α and β subunits that are responsible for cellular attachment to the ECM and leukocyte-endothelial cell adhesion and are denominated by the β subunit CD18. These molecules include CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18, and CD11d/CD18 [90, 91]. Immunoglobulins are a superfamily in which some members are glycoprotein adhesion molecules that regulate the adhesion and migration between leukocytes and endothelial cells during the inflammatory process. These molecules include ICAM-1, ICAM-2, VCAM-1, and PECAM-1 [92]. Key adhesion molecules involved in TBI inflammatory response are summarized in **Table 1**. Eph receptors and their ephrin ligands have also been implicated in the migration step of leukocyte infiltration into injured tissue and subsequent inflammation and will be discussed further.

10.2 Overview of membrane-bound Eph receptors and ephrin ligands

Eph receptors tyrosine kinases and their membrane-bound ephrin ligands function as mediators of cell migration and a wide-range of cellular functions across different cell types. Eph receptors are the largest family of receptor tyrosine kinases that are activated following cell-to-cell contact [107]. The Eph receptors are classified as either EphA or EphB receptors based on ligand binding. EphB receptors typically bind to transmembrane ephrin B ligands [107–109], while some Eph receptors, such as EphA4, can bind to both A and B ephrins [110]. Eph receptors play critical roles in axon guidance, synaptogenesis, neuromuscular junctions, and vascular remodeling among other roles [107, 109, 111]. Importantly, multiple

Adhesion molecule family	Molecule	Involvement/association with TBI	Expression	Mediates
Selectin	E-selectin (CD62E, ELAM-1)	Up-regulated 2–24 hrs in percussion model of TBI in rats, activated by IL-1 and TNFα [93].	Activated endothelial cells	Slow leukocyte rolling
	P-selectin (CD62P)	Increased CSF levels in children with severe TBI and associated with poor outcome [94]. Stimulated by TNFα and IL-1 [95].	Secretory granules of platelets and endothelial cells	Leukocyte rolling
Integrins	CD11b	Depletion of CD11b macrophages in diphtheria toxin receptor mice increased inflammatory signaling during TBI [96]. This may be due to critical mechanisms for TBI recovery being impaired.	Macrophages and microglia	Pathogen and DAMP recognition, phagocytosis, and cell survival [97]
	CD18/CD11b (Mac-1)	Blockade attenuates neutrophil accumulation following TBI in rats [98].	Neutrophils, monocytes/ macrophages, and NK cells	Firm adhesion during transmigration of leukocytes
	CD18/CD11d	Blockade reduces lesion volume and macrophage infiltration 3 d post-TBI in rats [99].	Neutrophils and monocyte/ macrophages	Adhesion of leukocytes
Immunoglobulin	ICAM-1	Increased significantly in TBI up to 72 hours postinjury, and blockade reduced leukocyte accumulation and improved neurological function following TBI [100, 101]. Soluble ICAM-1 in CSF was found in patients with severe cerebral injuries and BBB impairment [102]. Stimulated by IL-8, IL-1, and TNFα.	Endothelial cells	Leukocyte passage across vascular endothelial cell layer to injured tissue. Promotes leukocyte adhesion and migration [103]
	VCAM-1	Significantly decreased in children suffering from inflicted TBI [104].	Activated endothelial cells [105]	Promotes leukocyte adhesion through VLA-4 receptor [106]

Table 1.
Adhesion molecules involved in TBI inflammatory response.

Eph receptors and ephrins play a critical role in inflammation [111]. Ephrin A1 in endothelial cells responds to TNF stimulation, and multiple Eph receptors and ephrins respond to LPS [111]. EphA4 has been demonstrated to influence both spinal cord injury and TBI [111, 112].

10.3 Eph signaling in immune cells

Eph/ephrin signaling contributes to immune cell function. For example, EphA4 expression influences multiple different immune cell types including T cells, B cells, platelets, monocyte/macrophages, and dendritic cells [113–115]. Both $CD4^+$ and $CD8^+$ T cells have been shown to express EphA4 [116], and EphA4 expression in $CD4^+$ T cells has been implicated in T-cell migration [117, 118]. EphA4 is also critical in migration of memory T cells in response to ephrin A1 stimulation [116]. EphA4 expression in monocyte/macrophages effects their polarization status by mediating their pro-inflammatory (M1-like) state [115]. Moreover, ephrin A1 stimulation increased monocyte adhesion in a cell culture model through interaction with EphA4 on endothelial cells [119]. While these studies highlight that Eph/ephrin signaling is important in peripheral-derived immune cells, a significant research gap exists concerning the specific mechanisms involved in bi-direction signaling and its role in the function of peripheral immune cells following TBI.

11. Conclusions

Understanding the role of the peripheral-derived immune response to TBI is an important unmet need in TBI research. TBI is a leading cause of death and disability worldwide, and the secondary phase of injury is a critical target for therapeutics. Infiltration of peripheral immune cells through the compromised blood-brain barrier forms a major component of this phase, which can have both beneficial and deleterious effects. Monocyte/macrophages impact the response to TBI by a variety of mechanisms. These cells can cause tissue damage through pro-inflammatory traits or exert pro-recovery effects through anti-inflammatory traits, and the continuum of M1/2 expression is a growing research focus. Tie2 and cell-to-cell contact signaling is gaining attention for its role in peripheral immune cells, which provides additional opportunity for developing novel therapeutic treatments following TBI.

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Amanda Hazy, Elizabeth Kowalski, Nathalie Groot and Michelle Theus*
Virginia Tech, Blacksburg, VA, USA

*Address all correspondence to: mtheus@vt.edu

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