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Microglial polarization in post-traumatic epilepsy – potential mechanism and treatment opportunity.

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Highlights

- The biological mechanisms which underpin the development of post-traumatic epilepsy (PTE) are not understood
- Neuroinflammatory processes are intimately linked to acquired epilepsies, and are triggered by traumatic brain injury (TBI)
- Microglial activation after TBI can be heterogeneous, resulting in pro- and anti-inflammatory environments, which may be relevant for PTE
- Driving M2 microglial polarisation is emerging as a potential disease-modifying, and/or disease-preventing strategy for PTE

Abstract

Owing to the complexity of the pathophysiological mechanisms driving epileptogenesis following traumatic brain injury (TBI), effective preventive treatment approaches are not yet available for post-traumatic epilepsy (PTE). Neuroinflammation appears to play a critical role in the pathogenesis of the acquired epilepsies, including post-traumatic epilepsy, but despite a large preclinical literature demonstrating the ability of anti-inflammatory treatments to suppress epileptogenesis and chronic seizures, no anti-inflammatory treatment approaches have been clinically proven to date. TBI triggers robust inflammatory cascades, suggesting that they may be relevant for the pathogenesis of PTE. A major cell type involved in such cascades are the microglial cells – brain-resident immune cells which become activated after brain injury. When activated, these cells can oscillate between different phenotypes, and such polarization states are associated with the release of various pro- and anti-inflammatory mediators which may influence brain repair processes, and also differentially contribute to the development of PTE. As the molecular mechanisms and key signalling molecules associated with microglial polarization in brain are being discovered, strategies are now emerging which can modulate this polarization, promoting this as a potential therapeutic strategy for PTE. In this review, we discuss the relevant literature regarding the polarization of brain-resident immune cells following TBI and attempt to put into perspective a role in epilepsy pathogenesis. Finally, we explore potential strategies that could polarize microglia/macrophage towards a neuroprotective phenotype to mitigate PTE development.

Keywords: microglial polarisation; post-traumatic epilepsy; neuroinflammation; disease-modification; traumatic brain injury

Introduction

Epilepsy is a common group of chronic neurological diseases that is characterized by the occurance of recurrent unprovoked seizures. Post-traumatic epilepsy (PTE) is a common, long-term and debilitating consequence of traumatic brain injury (TBI), and is a significant cause of related death and disability in patients and an economic burden to society [1; 2]. It accounts for up to 20% of all acquired epilepsies in the general population [3]. Following the initial traumatic event, it can take months and even years before PTE is diagnosed. Though it provides an opportunity for potential preventive therapies, the extended duration of the pathogenesis makes it challenging to separate causal mechanisms critically involved in the development of PTE from non-specific neuropathologies related to TBI [3]. A growing body of evidence suggests involvement of neuroinflammation in the pathophysiology of acquired epilepsies, although a proven preventive treatment for PTE or for other acquired forms of epilepsy is yet to reach the clinic to benefit patients **[4-6].**

Neuroinflammation is a heterogeneous process which evolves following a brain insult, incorporating a variety of infiltrating and brain resident immune cells [7]**, such as neurons, glial cells and cerebral vasculature.** Microglia are brain-resident immune cells and are emerging as central players in regulating pathways of CNS inflammation [8]. As initially shown in monocytes from *in vitro* studies, microglia can undergo acute classical activation, the M1-like phenotype, that leads to the release of pro-inflammatory cytokines and regulates the clearance of pathogenic stimuli, or cell debris in the case of a brain insult such as TBI [9]. **While performing this phagocytic role, mediators of the neuroinflammatory response may induce collateral damage, often leading to a feed-forward loop resulting in enduring neuroinflammation, and contributing to chronic brain diseases.** Microglia can also undergo an alternate activation, the M2-like phenotype, and release anti-inflammatory mediators that participate in the resolution of the inflammatory

processes and promote repair mechanisms [9]. Such differences in the activation states are mainly characterised to date by the expression of specific immunogenic markers, but are not identified by any distinct morphological traits. M1-like activated microglia, or more precisely the mediators of M1 signalling, including IL-1β, TNFα as well as HMGB-1, have been implicated in epileptogenesis [10]. In contrast, M2 inflammation mediators including anti-inflammatory cytokines act to downregulate the responses of M1-like microglia, thereby potentially limiting epileptogenesis. In addition, these anti-inflammatory molecules are also directly associated with some of the pathological outcomes **thought to be** relevant to epileptogenesis, but generally work in opposition to the effects of M1 phenotype activation. **These processes - discussed in detail in next sections - include increased neurogenesis, reducing neuronal death, improving white matter integrity, extracellular matrix remodelling, oxidative stress and synaptic remodelling [11-16], although it should be noted that these may not necessarily be causative of epilepsy independently, but may team up to enhance epileptogenesis**. Therefore, modulation of microglial activation towards a M2-like phenotype could be a novel therapeutic approach to prevent the development of PTE [17].

In the following section, we describe inflammatory processes occurring after TBI, mainly focusing on microglia polarization, and attempt to delineate processes that might be pro- or anti-epileptogenic. **For this narrative review, we searched the PubMed database using combinations of the following search criteria: microglial polarization, M2 polarization, M2 phenotype activation, epilepsy, TBI, pharmacological intervention, anti-inflammatory intervention. We included all original studies that assessed microglia/macrophage polarization in TBI or epilepsy models. We excluded all non-original articles such as reviews, letters, books and conference abstracts.** We hope that linking this evidence will provide greater insights in understanding disease mechanisms of

epileptogenesis, and identify new therapeutic targets to prevent PTE development based on manipulation of neuroinflammation.

Neuroinflammation

The brain traditionally has been considered an immune-privileged site, but it is now widely accepted that the brain responds to insults or infection with an immune response. Following a primary brain insult, such as a traumatic brain injury, a secondary phase of injury results from a range of etiologies such as edema, inflammation, ischemia, and excitotoxicity. In experimental models, inflammation is regarded as a major contributing factor to secondary brain injury processes.

A variety of different cell types are involved in mediating the neuroinflammatory response including brain resident glial cells such as microglia and astrocytes, and neurons themselves contribute by mediating homeostasis through their interaction with glia. **Other cellular players involved in this response including infiltrating leukocytes, as well as cerebral vasculature.** Microglia are the primary innate immune effector cells of the CNS and are considered to be derived from yolk sac progenitors [18]. In physiological conditions, microglia assist in homeostatic survellience, retaining a state of ramified morphology with long processes protruding from the cytoplasm. **These functions are in turn regulated by neurons.** Healthy neurons secrete factors such as TREM2 and fractalkine, and these signal by activating receptors present on microglia [18], maintaining the survellience state of microglia. **Following a brain insult, microglia are exposed to factors released due to cellular injury that may include nuclear or cytosolic proteins such as HMGB1, ATP and heat shock proteins, or may include proteins released due to damaged extracellular matrix [19]. In turn, microglia then attain an activated morphological state with retraction and hyperatrophied cellular processes, and release neuroinflammatory cytokines with an**

aim to clear cellular debris after brain injury [20]. The released cytokines and chemokines promote mobilization of additional immune cells to the site of injury, leading to their proliferation and activation [21; 22]. However, this probably oversimplifies the situation, with microglia responding in different ways to different stimuli after brain injury, and these differing responses lead to a variety of functions and functional outcomes. If these could be better understood and harnessed, it could lead to new therapeutic targets.

While the focus of this review is to highlight the role of microglia, other cellular components also contribute to the neuroinflammatory response, and could also potentially be targeted for therapeutic intervention. Astrocytes are another abundant glial cell in brain and are commonly described to be dysregulated in epilepsy [23]. In addition to their function of providing biochemical support to the endothelial cells in maintaining blood brain barrier, they can also regulate synaptic transmission and neuronal excitability. They perform this by virtue of their surface expression of ion channels, neurotransmitter receptors and transporters, and aquaporin channels that regulate the electrolyte homeostasis and regulate neurotrasmitter levels in extracellular space [23; 24]. In animal models, as well as in resected tissues from epilepsy patients, these homeostatic mechanisms are impaired suggesting a critical role in the pathophysiological alterations observed in epilepsy (see ref[25] for a detailed review). Interestingly, different polarization states of astrocytes have also been described - based on their transcriptomic profiles, these states are referred to as A1 or A2 in analogy to microglial M1 and M2 phenotypes[26].

Peripheral cells such as monocytes, neutrophils and T lymphocytes can migrate to the brain and also contribute to neuroinflammation in epilepsy [27]. Migration of these cells is induced by direct injury to components of blood-brain barrier such as pericytes and astrocytes [28], and also by the release of proinflammatory cytokines which can

compromise blood-brain barrier [29]. Similar to microglia, macrophages (of monocyte lineage) can also polarise once they have infiltrated into brain parenchyma. Despite having very different endogenous roles, separating out neuroinflammatory effects of microglia and macrophages is quite challenging, since there are no clear markers to indistinguish them. One strategy which has been used to achieve this is transgenic co-labelling of CX3CR1 cells (brain resident) and CCR2 cells (monocytes). Using this approach, it appears as though these cell types may respond differently to interleukin-13, an M2-inducing cytokine [30]: in a model of spinal cord injury, IL-13 secretion from mesenchymal stem cells increased alternatively activated macrophages but reduced overall number of microglia [30]. This suggests that not only are they difficult to distinguish, macrophages and microglia may also differentially influence the neuroinflammatory environment. As such, delineation of exclusive roles of these two immune cells in pathology have not been clearly defined to date, and also the mechanisms and therapeutic potential of cell polarization described later may be related to a combination effects of microglia and macrophages. Nevertheless, any outcomes on brain pathology would not be mediated only by microglia/macrophages but would be a combined effect of a response from other cellular mediators as well.

Microglia polarization between M1/M2-like phenotypes

Depending on the stimuli, activated microglia can be polarized into two major phenotypes - proinflammatory (M1-like) and anti-inflammatory (M2-like) phenotypes [9]. The M1-like phenotype is associated with the expression of proinflammatory cytokines and chemokines that promote chronic neuroinflammation, phagocytosis, oxidative stress, neurodegeneration, and inhibit regeneration. The mechanical damage to neurons after an injury leads to release of factors such as purine metabolites, High-mobility group box 1 (HMGB1) and heat shock

proteins that regulate release of pro-inflammatory cytokines and activation of Toll-like receptors to induce a M1-like phenotype. These activated microglia then release high levels of pro-inflammatory mediators, including cytokines (IL-1β, IL-12, TNF-α), chemokines (CCL2, CXCL9, CXCL10), and reactive oxygen species (ROS), that play roles in host defence [31; 32]. In many instances, microglia with a M1-like phenotype could be viewed as providing neuroprotection as they clear dead/damaged cells, and their expression then is downregulated once the stimuli have been removed. However, in certain cases, prolonged chronic upregulation of M1-like activation in the brain can lead to neurotoxicity, eventually triggering **persistent** neurodegeneration [33].

In contrast, M2-like microglia downregulate the M1 signals by releasing anti-inflammatory cytokines, such as IL-10, IL-4 and IL-13, as well as growth factors such as transforming growth factor-β (TGFβ) and Insulin-like growth factor-1 (IGF-1) [32]. The cytokines aid in resolving inflammation, whilst growth factors can promote regeneration. Within this activation state, there are three described M2 polarization subtypes, M2a, M2b, and M2c, each with unique functionality [34]. M2a involves upregulation of markers, such as Arginase 1, CD206, Ym1, Fizz 1, and leads to inhibition of NF-κB signalling and this downregulation of the M1 activation mediating pro-inflammatory cytokine expression. The M2b phenotype has either a pro-inflammatory or anti-inflammatory functionality and in the peripheral system is associated with immunological memory. It has characteristics of both M1 (can express markers such as CD 86 and MHCII) and M2 (including IL-10) cells [35]. The third M2 sub-phenotype described for microglia involves the M2c phenotype that is attained in response to IL-10, glucocorticoids, or uptake of apoptotic cells- this phenotype is generally associated with tissue remodelling and matrix deposition following downregulation of inflammation. M2c-polarized cells exhibit upregulation of CD163, CD206, TGFβ and Sphk-1 [32; 34; 36]. Despite this description of the heterogeneity of M2 polarization subtypes – to date

mainly observed in cell culture - the exclusive identification of any particular sub-type within the complex in vivo environments, including post-TBI, are not reported. Indeed, mixed M2a and M2c markers have been reported 72h following TBI in mice [21], reinforcing the concept that discrete phenotypes of M1/M2 cells are rare. Indeed, single cell RNA sequencing studies have suggested that microglia can display signature genes of both M1 and M2 activation simultaneously and a balance between these two gene subtypes regulates its phenotypic transition [37]. Therefore, depending on the severity and time after brain insult, the microglial cells can display both M1-like and M2-like phenotypes, as well as expression patterns of microglia in a transition mode. This is further corroborated by a recent study using M1 or M2 polarized peripheral macrophages - when they selectively underwent gene knockdown of their specific regulators, the cells did not fully polarize from one subset to the other, but rather resulted in macrophages with both characteristics [38]. For this reason, researchers have acknowledged in recent years that microglia polarization within the CNS is likely to be a spectrum between M1 and M2 phenotypes, with cells 'oscillating' between the two extremes [9].

Heterogeneity of microglial phenotypes following TBI

Following brain injury, microglia can adopt different phenotypes. This has been suggested to be an innate attempt to resolve brain damage, and aid in the return to a healthy homeostatic environment. As discussed earlier, this can range from neurotoxic (M1-like phenotype activation) to a neuroprotective (M2-like phenotype activation) state.

As our understanding of microglial polarization has expanded, it has been increasingly considered that such polarization states in a local population of microglia are not exclusive to one particular phenotype and overlap is observed in many cases [39]. Indeed, the presence of mixed microglial phenotypic activation is commonly observed in the early acute

time points (6-72h) following TBI in animal studies with markers of both M1-like and M2-like phenotypes being expressed simultaneously [21; 40; 41]. The increased expression of M2 markers appears only transiently, returning to sham levels in the sub-chronic periods after TBI [42]. To add to the complexity of microglia polarization after TBI, others reported emergence of Mtrans form of activated microglia [21], defined by the expression of specific markers such as Arginase1 (M2 marker) and iNOS (M1 marker). At chronic timepoints, the injury site is dominated by microglia of the M1 phenotype [40; 42; 43]. Published studies report some variability in the extent and temporal evolution of these phases. For example, one study compared M2-like responses in young and aged mice after controlled cortical impact (CCI) [44]. The early M2-like response after CCI was diminished in aged mice when compared to young mice, and this was associated with a larger lesion size in the aged animals [44], suggesting that a prolonged M2 activation state may limit neurodegeneration. Similarly, in a projectile concussive impact model in rats, despite the predominance of M1 markers among the mixed state of polarization, a single impact resulted in faster resolution of the M1-like phenotype whereas multiple impacts resulted in a persistent M1-like activation [45]. **In the chronic phase after TBI, microglia of predominantly a M1-like phenotype with limited capacity for tissue repair are common, leading to enduring neuroinflammation which coincides with subsequent behavioural consequences** [21]. As discussed in detail in following sections, mixed microglia phenotypes are also observed in epilepsy models, further supporting the strategy that tilting the balance between M1/M2-like activation phenotypes might alter disease progression, rather than specifically express one phenotype or the other.

Relevance of microglial polarization to post-traumatic epilepsy

Strong evidence links epilepsy to neuroinflammation, and pro-inflammatory cytokines can regulate neuronal excitability and neuropathological outcomes related to epileptogenesis [43; 46;

[47], suggesting bidirectionality. Proinflammatory cytokines are both the markers and effector signals of M1-like microglia polarization. Expression of these molecules is upregulated in the brains of patients and animal models of temporal lobe epilepsy [48]. These cytokines can promote neuronal excitability by upregulation of excitatory neurotramission affecting both ligand gated and voltage gated ion channels as well as reducing the GABAergic inhibitory neurotransmission. For example, IL-1β can promote translocation of NR2B subunits of NMDARs to the membrane and can promote NMDAR mediated Ca^{+} currents [49]. For a discussion of the detailed effects of pro-inflammatory cytokines on neurotransmission, the readers are referred to the review by Vezzani et al [50]. The overall outcome of such effects is an imbalance in the excitatory/inhibitory neurotransmission that could favour seizure generation and promote epileptogenesis. These M1 proinflammatory cytokines have also been implicated in pathological mechanisms associated with epilepsy development and progression, such as neurodegeneration, axonal injury, neuroplasticity, and aberrant hippocampal neurogenesis (reviewed in [43; 50]). Finally, blocking these M1-cytokines or their biosynthesis is known to reduce epileptogenesis in both genetic and acquired models of epilepsy [47; 48; 51]. For instance, treating mice with an IL-1R antagonist for 1 week after pediatric TBI resulted in reduced susceptibility to provoked seizures in adulthood [52]. Similarly, inhibiting pro-inflammatory signalling using HMGB1 and TLR4 antagonists reduced established epileptic seizures in a model of temporal lobe epilepsy [48]. So it would seem that suppressing proinflammatory responses may be beneficial, but caution should be taken since these are also associated with triggering repair mechanisms; therefore, complete suppression of such processes may not be an ideal strategy.

Research describing and characterising the heterogeneity of brain inflammation following injury, specifically relating to M1 and M2-like microglial polarization, is still in its infancy. Furthermore, little is known about its relevance to epileptogenesis. One relevant

study compared microglial polarization in two post-status epilepticus (SE) models of acquired temporal lobe epilepsy in mice which exhibit different severities of epilepsy [31]. Pilocarpine-induced SE was associated with mixed expression of **M1/M2** markers, whereas only M1 upregulation was seen acutely in the kainic acid model. This difference in microglia polarization might play a role in epilepsy development, since more frequent epileptic seizures were observed in the kainic acid model [31], suggesting that the presence of M2-like microglia limited the severity of the resultant epilepsy. Unlike in TBI models, where M1 markers dominate in the chronic phase (see Figure 1), upregulation of both M1 and M2 markers was maintained in the chronic epilepsy phase in the kainic acid-induced SE model. It is not clear whether the presence of M2-activation markers in the chronic phase is related to a more severe epilepsy phenotype, or whether this is a compensatory mechanism to control the epileptic seizures. It is possible that the contribution to disease development of microglial M2 phenotypes is different during epileptogenesis to the period when chronic seizures are observed (established epilepsy). Also, in a genetic mouse model of epilepsy, mixed microglia polarization were observed at 2-week postnatal age when no seizures are observed, whereas the balance is tilted to predominantly M1-like phenotype immediately before the development of epileptic seizures in this model [46]. These findings suggest that microglia polarization can be a critical contributor to epilepsy outcomes, and support the hypothesis that promoting and sustaining the M2-like phenotype after epileptogenic injury may provide anti-epileptogenic effects. However, more insights are needed to prove this concept, perhaps from other models of epilepsy specifically relating to PTE. In the next section, we discuss linkages between inflammatory mediators and epilepsy, focussing on how different epileptogenic pathologies might be differentially regulated by M1 and M2-like microglia phenotypes.

Possible epileptogenic mechanisms influenced by microglial polarization

The above evidence associates M1-effector proinflammatory cytokines with the pathogenesis of epilepsy, raising the possibility that enhancing M2-like polarization might reduce epileptogenesis. Theoretically, this could be achieved via a number of different methods or combination of mechanisms. Here we briefly describe these.

Altering excitation/inhibition balance

M2 cytokines suppress the release of proinflammatory cytokines via inhibition of the NF-κB pathway [53], thereby inhibiting the excitatory effects of pro-inflammatory cytokines, as discussed above. Furthermore, anti-inflammatory cytokines, such as IL-4 and IL-13, secreted from M2-like microglia also modulate neuronal excitability themselves by regulating GABAergic transmission [54]. For example, in neuronal cultures, IL-4 increased inward GABA currents thereby promoting inhibition, which was opposite to the effect of the pro-inflammatory cytokine, IL-2 [54]. Overall, this could restore the excitatory/inhibitory balance generally considered to be associated with epilepsy, and increase seizure threshold.

Extracellular matrix remodelling

Along with the increased expression of anti-inflammatory cytokines, M2-like microglia also promote expression and release of markers such as FIZZ1 and YM1 that are associated with extracellular matrix remodelling, along with expression of growth factors that would further facilitate brain repair after an injury [9; 17; 36]. Extracellular matrix remodelling by these M2 markers is mediated by their involvement in collagen deposition and fibroblast differentiation [13] and their association with heparin sulphate that serves as docking sites for growth factors in the extracellular matrix [15]. Neural extracellular matrix functions have been linked with epilepsy [55]. The expression of urokinase-type plasminogen activator (uPA) and kallikrein-

related peptidase 8 (KLK8), proteins involved in remodeling of the matrix proteins, are reduced in rodent model of temporal lobe epilepsy and this reduced expression related inversely with seizure burden in epileptic animals [56].

Oxidative Stress

Similarly, Arginase 1 is another protein upregulated by M2 microglia that, apart from being involved in collagen deposition, negatively regulates the Nitric Oxide Synthase pathway and the production of nitric oxide that would, in turn, limit the collateral damage to neurons caused by ROS [57]. Oxidative stress has been increasingly considered as an epileptogenic mechanism[12; 14] and thus could be another mechanism negatively regulated by M2-like microglia polarization.

Synaptic remodelling

Synaptic remodelling of neural networks is another prominent mechanism that can lead to seizure generation. It has been shown that short and long term synaptic plasticity responses may change gradually in the hippocampus and cerebral cortex following epileptic seizures. There is evidence that inflammatory cytokines, such as IL-1β and TNF-α, can modulate neuronal plasticity and adjust the strength of the synapses [50]. In the context of M2 polarization, recent studies have shown a role for M2-like microglia phenotypes to modulate neuroplasticity, and this is associated with increased expression of synaptophysin with improved memory outcomes in a model of chronic cerebral hypoperfusion [58]. However, predicting the consequences of such neuroplastic changes with regards to overall excitability and epilepsy development is challenging with the limited amount of data currently available.

Neuroprotection and white matter regeneration

Other neuropathological outcomes of epileptogenic brain insults, such as traumatic brain injury, include neurodegeneration and white matter disintegration [59]. These are common pathological features observed in both experimental and clinical cases, and may promote epilepsy development [43; 60], **although whether preventing neurodegeneration can actually impede epileptogenesis is a subject of debate** [61]. Neuroinflammatory responses, mainly relating to M1 polarised microglia in general, are recognised contributors to these pathological outcomes [62]. In contrast, studies have shown that M2-polarized microglia provide neuroprotection, and perhaps also contribute to white matter regeneration [11; 63].

Hippocampal neurogenesis

Aberrant hippocampal neurogenesis is another neuropathological characteristic of acquired temporal lobe epilepsy. There is a strong upregulation in neuronal proliferation following an epileptogenic insult [64]. This has been suggested as a potential compensatory mechanism to combat the enhanced excitation, but often these cells aberrantly migrate to the dentate hilus region within the hippocampus to generate epilepsy [65]. However, other interventions, such as environmental enrichment, are recognised to boost neurogenesis [66] and impede epileptogenesis [67; 68], so the role of new neurons generated may be related to the timing of their birth and the associated guidance factors present at the time. Of relevance, therefore, M1-like microglia are known to suppress neurogenesis, whereas M2-like microglia promote it [16]. The complexities associated with the role of neurogenesis in epileptogenesis make it difficult to predict how changing neurogenesis patterns by different microglial polarization might directly influence epileptogenesis.

Overall, this evidence points to the potential contribution of M1/M2-like microglia polarization in epilepsy development, with M1-like microglia contributing to epileptogenesis

through several potential mechanisms, and M2-like microglia acting to combat these processes or initiate antiepileptogenic mechanisms in their own right.

Approaches to induce microglia M2 polarization: potential against PTE

As discussed in the previous section, microglial phenotypes appear to play an important role in neuropathological outcomes which are associated with epilepsy development. Anti-inflammatory drugs, including those that suppress global microglia activation or specific inhibitors of pro-inflammatory cytokine pathways, have been evaluated as therapies following TBI, as well for the early management of post-traumatic seizures [69; 70]. Despite the fact that some of these drugs have shown neuroprotective effects in animal models of brain disease, none have succeeded in clinical trials. Strategies promoting M2 activation of microglia and macrophages have shown improved pathological and functional outcomes in CNS diseases associated with acute or chronic neuroinflammation [71-73]. Different strategies have been adopted to achieve this, including pharmacological treatment, cell delivery systems and gene therapy, strategies which should be explored for the treatment and prevention of post-traumatic epilepsy (Table 1). **The outcomes of the studies discussed below may not be exclusively related to microglia/macrophage polarization but a complex interaction of inflammatory mediators released from these cells with the others cellular mediators of inflammation such as brain resident astrocytes as well as the peripheral cells that migrate to the site of injury. Moreover, even if genetic tools such as Cre-recombinase systems were to be utilised to specifically induce microglia/macrophage polarization, as stated earlier the overall outcomes may not be exclusively driven by these cells, but would be the result of overall interaction with other immune cells.**

Exposure to M2-promoting cytokines

One therapeutic approach to modify the polarization state of microglia is to use viral vector-based gene delivery to upregulate M2-promoting cytokines, or by delivering these cytokines using genetically modified stem cells. The interleukins, IL-13, IL-4 and IL-10, are regarded as essential modulators of neuroinflammation by promoting M2-like microglial polarization [17]. IL-10 possesses anti-inflammatory properties through activation of Jak1/STAT3 signalling that inhibits NF-kB to suppresses pro-inflammatory cytokine expression [74]. This pathway has been implicated in epilepsy: using hippocampal cultures from a picrotoxin-mediated seizure model in mice, IL-10 over-expression was able to suppress IL-1β expression [75]. Using the closed cortical impact model of TBI, M2 polarization was induced by IL-10 secretion from transplanted, genetically modified mesenchymal stem cells (MSCs) [76]. The M2 polarized microglia in the CA1 area of the hippocampus led to improvements in fine motor skills and prevented cell loss [76]. Similar beneficial effects of IL-10 administration have been reported after fluid-percussion traumatic brain injury [77]. IL-10 expressing MSCs can also induce M2 polarization in other brain pathologies that have epileptogenic potential such as stroke and improve functional outcomes [78].

IL-4 and IL-13 are other immunoregulatory cytokines that have great potential in triggering M2 polarization by acting on their common heterodimeric receptor composed of the IL-13 Receptor alpha 1 (IL-13Rα1) and the Interleukin 4 Receptor alpha (IL-4Rα) subunits [79]. This in turn activates downstream signalling cascades including the JAK/STAT6 pathway and insulin receptor substrate (IRS) and phosphatidylinositol 3′-kinase pathways, leading to the transcription of genes relevant to M2-like microglia polarization [79]. Despite their M2-inducing properties, IL-13 or IL-4 mediated M2 polarization strategies have not been investigated in TBI or PTE models. However, in one study, administration of IL-13-producing MSCs modulated infiltrating immune cells to a M2-like state but not the resident cells in the surrounding pathological area caused by Status Epilepticus [80], demonstrating that,

in principle, exogenous IL-13 can influence microglial phenotypes. This aligns with other studies showing that MSCs delivering IL-13 are able to induce a M2 neuroprotective phenotype in a stroke model in mice [72], and that viral vector based delivery of IL-13 provides protection against cuprizone-induced demyelination, perhaps due to the observed polarization of microglia toward a M2-phenotype [73]. Regarding IL-4, a recent study explored the effectiveness of this cytokine in the pilocarpine-induced post-SE model of epilepsy in mice. Delivering IL-4 (with Interferon-γ) via ip injection modified the inflammatory response caused by the SE, increasing M2-like polarization during the acute phase (1-4 d post-SE), as well as reducing the M2-like state (5-9 d after SE), which led to reduced seizures in the chronic period. However, the exact cellular mechanisms regarding these intriguing outcomes are not known yet and more research is needed in this direction [81]. Nevertheless, the modulation of neuroinflammation by delivery of anti-inflammatory cytokines to induce M2-like microglia polarization represents a novel intervention strategy for the management of neuroinflammation following TBI and PTE.

In addition to treatment strategies which aim to directly modulate microglial phenotypes, several alternate strategies, including pharmacological and cell-based therapy, provide associative evidence to support the promise of M2-like microglial polarization as a potential therapy against PTE.

Pharmacological treatments

Some of the pharmacological interventions we focus on, such as glycyrrhizin, rosiglitazone and gp91ds-tat, target pathways that suppress pro-inflammatory cytokine expression after TBI, and therefore could induce expression of M2-like microglia polarization markers. Glycyrrhizin is a bioactive substance that exhibits anti-inflammatory properties by inhibiting HMGB1 phosphorylation [82]. Glycyrrhizin treatment suppressed HMGB1 expression in a

weight drop injury model in rats, subsequently inducing M2-like microglia polarization and improving functional recovery [83]. Accordingly, HMGB1 is also reported to be an important mediator of inflammation in epilepsy models and has potential as a predictive biomarker of epileptic seizures [10; 84]. Rosiglitazone, a potent peroxisome proliferator-activated receptor (PPAR)-γ agonist with anti-inflammatory properties, has been reported to induce M2 polarization in a TBI model in mice, and attenuate axonal injury and improved neurological function [85]. However, improved TBI outcomes by rosiglitazone may also involve other biological mechanisms, such as limiting the mitochondrial dysfunction and oxidative damage, as well as suppressing autophagy [86; 87]. Previous studies also suggest that PPAR-γ agonists provide neuroprotection and suppress development of epileptic seizures in animal models of temporal lobe epilepsy [88; 89]. The selective peptide inhibitor, gp91ds-tat, is an inhibitor of NADPH oxidase (NOX2), an enzyme that generates ROS, leading to activation of microglia and release of M1- proinflammatory cytokines [90]. Administration of this peptide to mice following CCI injury led to a reduction in M1 markers and promotion of M2 markers in the hippocampus, and reduced neurodegeneration with enhanced motor recovery [91]. Subtypes of metabotropic glutamate receptors mGluR are expressed in reactive astrocytes in epilepsy models and may contribute to increased inflammation [92]. Treatment with a positive allosteric modulator of mGluR5 following CCI induced M2-like microglia polarization and significantly reduced lesion size, reduced hippocampal neurodegeneration, and improved sensorimotor performance [93]. Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein that plays an essential role for the downstream cell signalling of proinflammatory cytokines such as IL-1 and IL-18 [94]. A recent study has shown that MyD88 knockout or administration of a MyD88 inhibitory peptide in the pilocarpine SE model in mice is associated with decreased M1 and increased M2-like microglia in the hippocampus that inturn related to reduced hippocampal pyramidal cell death [95]. So, while

these pharmacological tools come from various diverse drug classes with different biological targets, together this evidence acts to strengthen the associations between increased M2 polarization and improved functional and anti-epileptogenic outcomes.

Cellular therapies

Cellular therapies, especially with stem cells of different regenerative capacities, have been trialled in neurological disorders with chronic loss of specific cell types, such as Parkinson's disease and Alzheimer's disease, as well as brain injury associated with ongoing neurodegeneration such as stroke and TBI [96]. Such therapies have also shown promising outcomes in epilepsy models- administering the stem cells programmed to differentiate into GABAergic interneurons in order to counter the excessive network excitability [97; 98]. Genetically modified cells have also been injected into the brain in order to continuously deliver different neuropeptides or other molecules with anti-epileptic potential and have shown positive outcomes [99; 100]. In addition, different types of stem cells, such as mesenchymal stem cells (multipotent cells of stromal origin) and neural stem cells (multipotent cells with capacity to generate into neurons and glial cells), have been observed to interact with the host cells by the exchange of trophic growth factors and modulation of immune response [101]. These interactions between host and grafted cells limit the pathological damage in neurological disorders by triggering the M2-like microglia phenotype [102; 103] and indeed such cellular interventions have shown positive outcomes in an animal model of epilepsy (summarized in [104]. In the context of TBI, stem cells have shown potential in improving the pathological and functional outcomes in rodent models, and these benefits are consistently associated with an ability to induce an M2-like microglial phenotype [41; 105; 106]. For instance, intracerebral transplantation of human neural stem cells after CCI improved neuronal cell survival and reduced axonal injury in mice [106]. Transplanting these cells leads

host microglia towards a M2 polarization state that contributes to the observed neuroprotective effects [106]. The immunomodulatory property (independent of cell replacement effects) of stem cells in a TBI model is also exemplified by a study by Walker et al, [41] which showed that injecting multipotent adult progenitor cells intravenously could preserve blood brain barrier permeablity, in addition to stimulating the release of anti-inflammatory cytokines from these cells. These findings indicate the potential of stem cells not only in providing cell replacement after an injury but inducing the regeneration and indeed provide neuroprotection by their immunomodulatory properties of mainly inducing an M2-like microglia phenotype.

Concluding remarks

Inflammatory mechanisms have been increasingly considered as important contributors to the epileptogenic process, and as a target for preventive therapies. Over the past decades, substantial advances have been made towards understanding the underlying mechanisms associated with neuroinflammation, and the field appreciates the heterogeneity involved in such inflammatory processes. The concept of microglia polarization has also evolved from being distinctly present either as M1 or M2-like subtype to being acknowledged to be a spectrum, where more than one phenotype can be observed simultaneously in a pathological environment. Despite this, the polarization of microglia towards M2-like phenotype or at least tilting the balance in favour of M2 can be of therapeutic value in treating brain insults [17]. M2-like microglia have the potential to remove the cell debris and toxic metabolites via phagocytosis, without excessive collateral damage to the surroundings, in addition to promoting regeneration and improving recovery. These effects have been documented in various neurodegenerative conditions [107] as well as following injury to the CNS, including epileptogenic insults such as TBI. Interventions to promote M2 in PTE models, or even

evaluating the microglia phenotypes in these models, have not been reported in great detail. We provide here a series of compelling arguments to suggest that microglia polarization could be highly relevant to the mechanisms relevant to epileptogenesis including excitation/inhibition balance, white matter and extracellular matrix integrity, synaptic plasticity, as well as neurodegeneration. This evidence warrants more detailed investigations, as well as development of strategies promoting M2-like microglia phenotypes for potential anti-epileptogenesis pre-clinical trials. Most of the evidence describing microglia polarization in epilepsy pathology points to a potential involvement in acquired epilepsies. However, a switch in the M2/M1 ratio is also reported in a genetic seizure model [46] and the fact that reactive astrocytes, along with expression of the M1 marker, IL-1β, is observed in the somatosensory cortex of genetic absence epilepsy rats [47], suggest the possibility of M2 polarization strategies being relevant for genetic forms of epilepsies.

Several strategies have been adopted to induce M2-like microglia phenotypes in TBI models and have relevance to epileptogenesis. Among these, the delivery of M2- promoting cytokines by genetically modified cells and viral vector based gene therapies seem promising and can be easily investigated for their potential in acquired epilepsies. Stem cell-based approaches are a promising new strategy for a range of human conditions, and have been trialled for cell replacement and modulating the levels of neuroprotective agents [96]. Similarly, the viral vector- mediated modulation of inflammation could be used to modulate neuroinflammation after an epileptogenic brain insult, considering that these have been observed to be safe in mediating gene therapy in neurological conditions [108; 109]. However, before contemplating clinical translation, a detailed understanding of microglia polarization in the context of epileptogenesis is needed. This could initially be tested in post-status epilepticus epilepsy models for practical reasons including a shorter follow up period and lower sample size needed for an adequately powered study due to a high rate of epilepsy in

these models. However, this would then need to be followed up in post-TBI models for evaluation of PTE, which would be laborious and expensive due to longer timeline and the need of bigger sample size in such studies as only a proportion of animals develop PTE [110]. This would create additional opportunities to evaluate the long term survival of the transplanted cells, or the resident genetically modified cells induced by viral vectors. Similarly, several pharmacological and cell-based therapies in TBI models, although providing associative evidence, supports the promise of M2-like microglial polarization as a potential therapy against PTE. The field is in its infancy, but is highly promising, and thus needs further exploration.

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Figure legend:

Figure 1: Schematic illustration proposing the timecourse of polarization of brain-resident immune cells following TBI, overlain on a proposed timeline of PTE development. Following TBI, epileptogenesis can be initiated, and persist throughout a latent period of months to years. Within hours following injury, the molecular signals from injured tissue drive phenotypic and functional responses in microglia. Mixed cellular phenotypes are present during the acute phases after injury, transitioning predominantly to M1-like-

phenotype in the chronic phase. The possibility of intervention by upregulating M2-like responses may limit M1 microglial activation, and impede epileptogenesis.

Table Legend:

Table 1: Studies that have evaluated M2 polarizing interventions for experimental TBI and epilepsy

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Treatment	Model	Intervention	Outcomes	Reference
(I) Exposure to M2 promoting cytokines				
IL-10 producing mesenchymal stem cells (MSCs)	CCI model in rat	Implantation of IL-10 MSCs, 36 hours post TBI	Treatment polarized microglia towards M2 phenotype. This activation state reduced expression of pro-inflammatory markers and triggered alternative inflammatory expression markers such as CD163. Improved fine motor skills and reduced tissue loss.	65
IL-13 producing MSCs	Intrahippocampal kainic acid model in mouse	Implantation of IL-13 MSCs, 1 week prior to SE	Treatment led to the polarization of cell graft-infiltrating microglia towards an M2 phenotype. Did not influence the SE mediated neuroinflammation or seizure outcomes	69
IL-4/IFN-γ	Pilocarpine-induced epilepsy model in mouse	Intraperitoneal injection of IL-4 and IFN- γ, 5 hours prior to pilocarpine injection	Treatment induced M2 polarization and suppressed frequency and severity of spontaneous recurrent seizures. Treated mice also showed improved cognitive performance.	70
(II) Pharmacological treatments				
Glycyrrhizin	Weight drop injury model in rat	Intravenous injection of glycyrrhizin, 3 hours post TBI	Treatment suppressed M1 phenotype activation and promoted M2 microglial activation. Also reduced lesion volume.	71
Rosiglitazone	FPI model in mouse	Intraperitoneal injection of rosiglitazone 15 minutes prior to FPI	Treatment induced PPAR-γ activation, and promoted microglia polarization towards M2 phenotype. Downregulated inflammatory response, reduced axonal injury and improved neurological function.	74
NOX2 inhibitor	CCI model in mouse	Intraperitoneal injection of gp91ds-tat, 24 hours post TBI	Treatment promoted M2 phenotype activation, increasing IL-4Rα signalling in infiltrating macrophages. Also, limited neurodegeneration and improved motor recovery.	80
mGluR5 positive allosteric modulators	CCI model in mouse	Intraperitoneal injection of VU0360172, 3 hours post TBI	Treatment reduced CD68 and NOX2 expression in activated microglia, and promoted M2 phenotypes. Also reduced the lesion volume and hippocampal neurodegeneration, and improved motor function recovery.	82
Inhibitor of MyD88	Pilocarpine-induced epilepsy model in mouse	Intrahippocampal injection of MyD88 inhibitory peptide, 20 hours prior pilocarpine injection	MyD88 inhibition decreased M1 microglial phenotype activation with increased M2 phenotype activation. Also prevents apoptosis of pyramidal neurons.	84
(III) Cellular therapies				
Bone marrow mesenchymal stromal cells	CCI model in mouse	Intracerebroventricular infusion of mesenchymal stromal cells, 24 hours post TBI	Treatment modified the local inflammatory response by upregulating Ym1 and Arginase-1 mRNA expression together with decreased phagocytosis which aid in the polarization towards M2 phenotype. Also promoted growth stimulation and tissue repair.	94
Multipotent adult progenitor cell therapy (MAPC)	CCI model in mouse	Intravenous injection of multipotent adult progenitor cells, 24 hours post TBI	Treatment preserved blood brain barrier integrity and increased expression of anti-inflammatory cytokines. Also provided neuroprotection.	26
Human neural stem cell transplantation (hNSC)	CCI model in mouse	Intracerebral infusion of cells 24 hours post TBI	Treatment modulated host microglia towards M2 polarization state. Also associated with replaced neuronal cells and reduced axonal injury.	95

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