

# Microglia at the Crossroads of Pathogen-Induced Neuroinflammation

Ana María Rodríguez, Julia Rodríguez,  
and Guillermo Hernán Giambartolomei 

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## Abstract

Microglia are the resident tissue macrophages of the central nervous system (CNS). Recent findings point out that in the steady state the major role of microglia, is to instruct and regulate the correct function of the neuronal networks and different components of the neurovascular unit in the adult CNS, while providing immune surveillance. Paradoxically, during CNS infection immune activation of microglia generates an inflammatory milieu that contributes to the clearance of the pathogen but can, in the process, harm nearby cells of CNS. Most of the knowledge about the harmful effects of activated microglia on CNS has arisen from studies on neurodegenerative diseases. In this review we will focus on the beneficial role and detrimental functions of microglial cells on the neighboring cells of the CNS upon infection.

## Keywords

microglia, CNS infection, neuroinflammation, immunopathology

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## Introduction

Microglia are the resident tissue macrophages of the CNS and are cells that belong to the innate immune system. Since its discovery, the immunological role of macrophages have focused on their phagocytic activity (Tauber, 2003) which is central not only for host defense but also for various house-keeping functions, such as the removal of apoptotic cells and the remodeling of the extracellular matrix (ECM). Ongoing research has started to point out a much more general role of macrophages in vertebrate biology, including their roles in cold adaptation, systemic metabolism, tissue homeostasis and development (Gordon et al., 2014; Wynn et al., 2013). The normal development and function of some tissues and organs is critically dependent on macrophages that reside in these organs (Gordon et al., 2014; Wynn et al., 2013), microglial cells in CNS. As with any other tissue macrophage, microglia were considered to be in a resting state in the healthy CNS, while CNS infection and inflammation caused activation of these cells to a professional phagocyte that either resolves these situations, or potentially induces a pathological process. Here, we will briefly discuss the homeostatic functions of microglia and the impact that microglia have in the well-being of the different CNS cells. Moreover, we will summarize the current state of art of the

never resting microglia, focusing on how microglia immune activation upon infection creates an inflammatory milieu that while defending CNS from invading pathogens can damage surrounding tissues.

## The Surveying Never Resting Microglia Keeping the Homeostatic State of the CNS

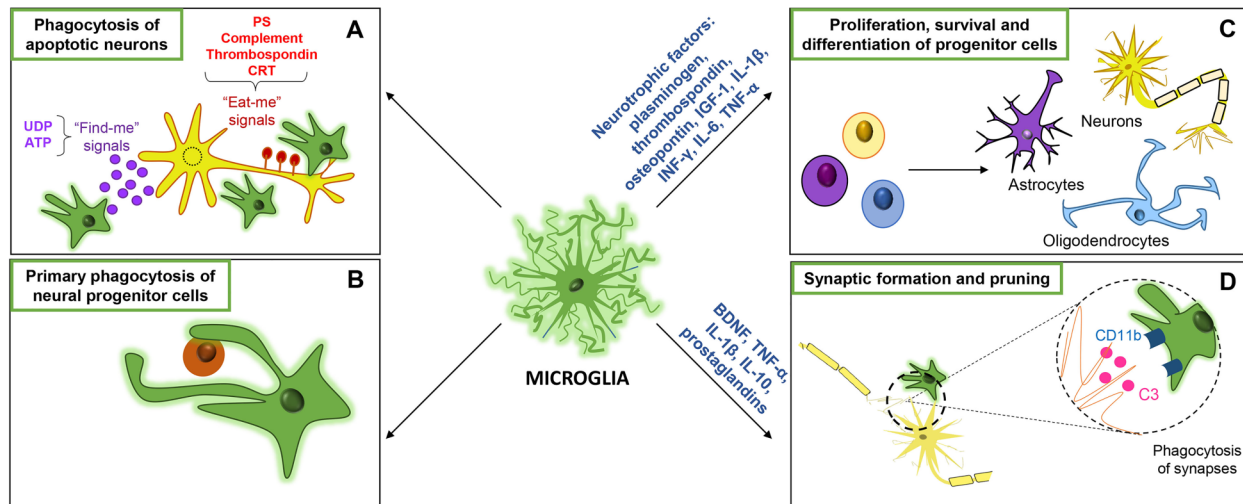
Microglia have important homeostatic functions in the adult CNS (Hanisch & Kettenmann, 2007; Tremblay et al., 2011). These cells participate in CNS development and homeostasis by regulating neural cell numbers, migration of interneurons, as well as promoting connectivity, synapse formation, and pruning (Thion et al., 2018) (Figure 1). Also, microglia are considered the first line of immune defense in the brain by

Instituto de Inmunología, Genética y Metabolismo (INIGEM). CONICET. Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

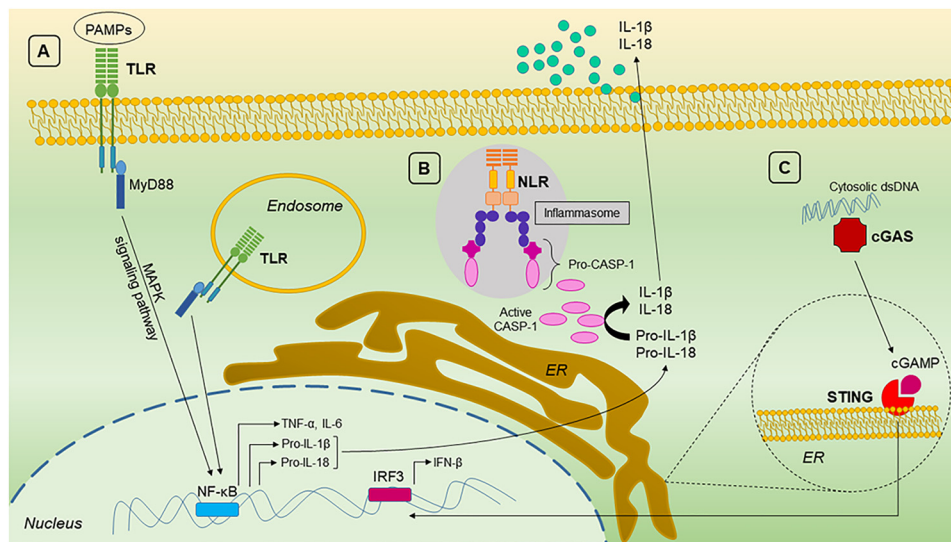
### Corresponding Author:

Guillermo Hernán Giambartolomei, Instituto de Inmunología, Genética y Metabolismo (INIGEM). CONICET. Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Av. Córdoba 2351, C1120AAR, Buenos Aires, Argentina.  
Email: ggiambart@ffyb.uba.ar





**Figure 1.** Physiological functions of microglia. (A) Phagocytosis of apoptotic neurons. Apoptotic neurons release “find-me” signals which attract microglia and expose in their surface “eat-me” signals stimulating microglial phagocytosis. (B) Phagocytosis of viable neural progenitor cells. Microglia phagocytoses viable neural progenitor cells in a direct manner, without inducing apoptosis. (C) Trophic support of proliferation, survival and differentiation of neural and other glial progenitor cells. Microglia secrete trophic factors that promote neurogenesis, astrogenesis and oligodendrogenesis. (D) Refinement of synaptic formation and pruning. Microglia regulate the extension of synaptic networks through phagocytosis during CNS development, as well as in the adult CNS.



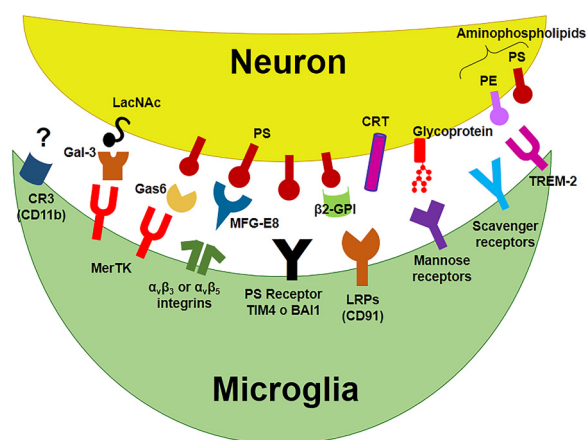
**Figure 2.** Microglial immune receptors. Microglia recognize, through different pattern recognition receptors (PRRs), microorganisms that are capable of invading the CNS parenchyma. (A) Toll-like receptors (TLR) sense PAMPs expressed in microorganisms. TLR activation leads to the enrollment of the adaptor MyD88 and the consequent activation of MAPK that triggers the translocation to the nucleus of the transcription factor NF- $\kappa$ B, promoting the expression of many inflammatory cytokines and the precursor forms of IL-1 $\beta$  and IL-18. (B) Nod-like receptors (NLR) are cytosolic receptors that oligomerize, and together with adaptor proteins form the inflammasome. This multi-subunit complex activates caspase 1 (CASP-1) enzyme, which produce the proteolytic cleavage of pro-IL-1 $\beta$  and pro-IL-18 to their mature forms that are then released to the extracellular milieu. (C) GMP-AMP synthase (cGAS) is a cytosolic receptor that recognizes double-stranded DNA. The activation of cGAS produces the second messenger cGAMP, which binds to STING in the ER and promotes downstream IFN- $\beta$  expression via IRF-3.

monitoring the brain parenchyma under homeostatic conditions and resolving cerebral insults (Aloisi, 2001). This multifunctional task is accomplished by performing four major

defined functions: i) phagocytosis of apoptotic neurons, ii) trophic support of developing neurons and other cells glial cells, iii) guidance of the developing vasculature of the

CNS, and iv) support and refinement of developing neural circuits (Figure 1). The main mechanisms for these functions are phagocytosis and cell-to-cell communication through direct intercellular contacts or via soluble mediators.

Microglia contributes substantially to adult neurogenesis at several areas of the healthy CNS (Norris & Kipnis, 2019; Sato, 2015; Sierra et al., 2014). Neurogenesis is influenced by microglia by exerting suppressive (Figure 1 A, B) or supportive functions (Figure 1 C, D). Microglia can also contribute to neural repair and regeneration through phagocytosis and the production of immune-regulatory mediators such as IL-1 $\beta$ , IL-6, TNF- $\alpha$  and interferon (IFN)- $\gamma$ , as well as neuronal growth factors such as insulin-like growth factor 1 (Figure 1 C-D) (Lannes et al., 2017). In addition, microglial cells stimulate the formation of new spines in the cortex by secreting brain-derived neurotrophic factor (Parkhurst et al., 2013) (Figure 1 D). Interestingly, the commitment of microglial specific pathways could also drive the modulation of synaptic activity. For instance, fractalkine signaling pathway regulates excitatory synaptic transmission and plasticity (Rogers et al., 2011) but also Toll-like receptor (TLR) 4 signaling controls glutamate release of presynaptic fibers through the astrocyte mobilization (Pascual et al., 2012). These findings have led to the contention that microglial cells scrutiny but also control synaptic and neuronal activity in the healthy adult brain (Tremblay et al., 2011). These observations also suggest that the profound changes in microglia phenotype that take place upon the occurrence of an infection or a pathological condition will have two consequences: i) the loss of the constitutive influence of surveying microglia on homeostasis and ii) the emergence of new functions related to the immune nature of these cells.



**Figure 3.** Microglial phagocytic receptors. Microglia express several membrane phagocytic receptors that recognize different target molecules (“eat-me” signals) on apoptotic or live damaged neurons. The recognition can be direct or mediate by the interaction with bridge molecules. LacNAc, N-acetyl-lactosamine; PE, phosphatidylethanolamine.

## Immune Functions of Microglia

### Immune Receptors of Microglia

Microglia recognize a wide array of pathogens that can invade the CNS as bacteria, viruses, parasites and fungi. They are armed with a vast repertoire of pattern recognition receptors (PRRs) that include mainly TLRs and Nod-like receptors (NLRs); along with a vast array of phagocytic receptors, which function together to sense and eliminate microbes that invade the CNS parenchyma. PRRs sense pathogen-associated molecular patterns (PAMPs) which are highly conserved microbial motifs, such as lipopolysaccharide (LPS) or viral nucleotides, as well as danger-associated molecular patterns (DAMPs) such as amyloid (A $\beta$ ) or cytosolic proteins released from necrotized cells; and as a result, they elicit the production of inflammatory mediators from microglia (Hanamsagar et al., 2012). TLRs detect PAMPs expressed on a wide variety of microbial pathogens (Kawai & Akira, 2010). Most of TLR engagement leads to the recruitment of MyD88 and subsequent NF- $\kappa$ B and MAPK-mediated transcriptional activation of inflammatory mediators (Brown et al., 2011) (Figure 2A). NOD-like receptors (NLRs), particularly NLRP1, NLRP3, and NLRC4 in microglia, are cytoplasmic receptors that oligomerize to form a platform known as inflammasome, a multi-protein complex that finally cleaves pro-IL-1 $\beta$  and pro-IL-18 into their mature forms via caspase-1 (CASP-1) action. Among them, the most abundant inflammasome present in the CNS is NLRP3 (Katuri et al., 2019). Both, TLRs and inflammasomes receptors act in concert in a two-signal model. Ligand binding to TLR induces the expression of pro-IL-1 $\beta$  and pro-IL-18, after which NLR-dependent activation of CASP-1 regulates their proteolytic processing and release (Franchi et al., 2009) (Figure 2B). Cytosolic double-stranded DNA can also activate an innate immune response. It is recognized by GMP-AMP synthase (cGAS) (Paludan & Bowie, 2013), which produces cyclic-GMP-AMP (cGAMP), a second messenger, which in turn activates downstream STING. This signaling pathway ultimately leads to IFN- $\beta$  production via IRF-3 (Sun et al., 2013) (Figure 2C).

Phagocytosis is a well-controlled process that requires the activation of several non-redundant signaling pathways that recognize different molecules on the target cell surface (Ravichandran, 2011) (Figure 3). These molecules, also called “eat-me” signals, are recognized directly by phagocytic membrane receptors, or by the interaction with bridge proteins, that are then recognized by a phagocyte receptor. Phosphatidylserine (PS) is one of the best characterized “eat-me” signal. It normally resides in the inner leaflet of cell plasma membrane, but it is exposed on the surface of apoptotic or damaged cells (Ravichandran & Lorenz, 2007). This exposed PS binds to its receptors or connective molecules and triggers phagocytosis on macrophages, including microglia. Two receptors that recognize PS have been identified: TIM4

and BAI. In addition to this, various adaptor molecules facilitate ingestion of cells through PS binding: Gas6, MFG-E8,  $\beta_2$ -GPI and annexin V (Sierra et al., 2013). The connective molecules are bridge proteins with two binding domains, the first one binds to PS and the other one binds the phagocyte receptor (Li, 2012). One of the best characterized bridge PS-binding protein is MFG-E8 (lactadherine), which recognizes PS through its C-terminal domain and binds to integrins  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  (vitronectin receptor, for example) through N-terminal RGD motifs (Akakura et al., 2004). Another signaling pathway that initiates phagocytosis involves the recognition of N-acetyl-lactosamine by Galectin 3 (Gal-3), which is secreted by activated phagocytes and binds to MerTK receptor. This important phagocytic receptor belongs to the family of tyrosine kinase receptors TAM that was recently involved in phagocytosis of neurons by microglia (Nomura et al., 2017).

In addition to non-protein molecules, membrane-bound proteins can act as “eat-me” signals. The expression of calreticulin is increased in apoptotic or damaged cells, which leads to its recognition by the LRP receptor (low-density lipoprotein receptor-related protein, CD91), although phagocytosis mediated by this route seems to depend on the exposure of PS in the damaged cell (Fricker et al., 2012a; Gardai et al., 2005). Other receptors that have been reported to be involved in phagocytosis are mannose receptors, scavenger receptors, sialic acid binding immunoglobulin-like lectins (Siglecs) and TREM-2. The last one can bind to LPS, peptidoglycan, lipoteichoic acid (LTA), and other molecules that they have repeated anionic residues (Daws et al., 2003; Wang et al., 2015; Yeh et al., 2016). Recently, it was described that TREM-2-dependent signal transduction in response to apoptotic neurons is mediated by aminophospholipid ligands, PS and phosphatidylethanolamine, which are exposed on apoptotic cells (Shirotani et al., 2019). Activation of TREM-2 led to phagocytosis of apoptotic cell debris (Fu et al., 2014). Other receptors involved in the fine regulation of phagocytosis are the Fc $\gamma$ R and the complement receptors. Fc $\gamma$ R (I, II and III) bind the constant fragment of immunoglobulins. Complement receptors CR1, CR3, and CR4 bind the component C3bi, while C1qR $\beta$  binds C1q (Aloisi, 2001). Activation by ligands of CR1, CR3 and Fc $\gamma$ R trigger phagocytosis of opsonized targets *in vitro*. C1qR $\beta$  activation enhances FcR- and CR1-mediated phagocytosis. Moreover, signaling through CR3 and Fc $\gamma$ R may also enhance microglia pro-inflammatory and cytotoxic functions as secretion of pro-inflammatory cytokines and reactive oxygen species/nitric oxide (ROS/NO) (Aloisi, 2001) (Figure 3).

On the other hand, microglia also express siglecs receptors, which bind to sialylated ligands on neurons or CNS tumor cells (Linnartz-Gerlach et al., 2014), modulating the activation of microglia and thus also phagocytosis activity. Microglial phagocytosis also is modulated by LRP, signal-regulatory protein alpha (SIRP $\alpha$ , CD172a) and TREM-2 (Gitik et al., 2011; Hadas et al., 2012; Yang et al., 2016).

Other kind of receptors, the purinergic receptors, recognize chemotactic signals called “find me” signals. Professional phagocytes, as microglia, are mobile and are constantly patrolling tissues in search of dead or damaged cells. ATP and UDP nucleotides are the best characterized “find me” signals released by damaged cells (Ravichandran, 2011). ATP and UDP released by these cells generate a gradient for microglial chemotaxis that recognizes them through their purinergic receptors. P2Y $_6$ , P2Y $_{12}$ , P2X $_4$  and P2X $_7$  are expressed by microglia. ATP and UDP are agonists of P2Y $_6$ . UDP-stimulated microglia changed their morphology extending microglial processes, filopodia-like protrusions and phagosome-like vacuoles, increasing the phagocytosis activity (Koizumi et al., 2007). P2Y $_{12}$  and P2X $_7$  receptors are responsible of ATP responses. Using KO mice of both receptors it was showed that P2Y $_{12}$  is involved on microglia recruitment *in vivo* (Fekete et al., 2018).

### The Positive side of Microglial Immune Responses

After pathogen ingress through the endothelial barrier, microglia are the first line of defense (Forrester et al., 2018). In response to pathogens infection, activated microglia produce pro-inflammatory mediators, including NO/ROS, cytokines and chemokines; and increase their phagocytic activity. One immune key role of microglia activation during CNS infection is the recruitment and subsequent activation of infiltrating immune peripheral cells, such as granulocytes (neutrophils, eosinophils, and basophils), monocytes and lymphocytes. The recruitment of these inflammatory cells is the most common manifestation of neuroinfectious diseases, which mediate local resistance to viral, bacterial and parasitic organisms within CNS (Klein & Hunter, 2017). Most data on activated microglia is about the immunopathology caused by these cells during activation, but this immune response can be also beneficial to the host, executing the control and clearance of the pathogen.

Microglia can be infected by several virus such as herpes simplex virus (HSV) (Chen et al., 2019), human immunodeficiency virus type 1 (HIV-1) (Chen et al., 2019) and Zika virus (ZIKV) (Lum et al., 2017), among others. Virus-activated microglia have shown to play an important role in immune defense against viruses. Mice infected with HSV show an increase of microglia number at 6 days post-infection. These microglia surround HSV-infected neurons. Stressed infected neurons seem to release ATP since it has been described that there exists a reduction of more than 50% in the numbers of microglia recruited to infected neurons when P2Y $_{12}$  KO mice were employed, indicating that microglia are recruited around the infected neurons via P2Y $_{12}$  signaling. Interestingly, P2Y $_{12}$ -deficient microglia has a decreased amount of CD68 $^+$  phagolysosomes compared with normal mice, which indicates that P2Y $_{12}$  is essential for the phagocytic activity of microglia (Fekete et al., 2018). This phenomenon was also present in human patients. Brain

specimens of patients with HSV encephalitis shows that P2Y<sub>12</sub>-positive microglia processes extend to HSV-positive neurons, and there are many activated microglia around each infected neuron (Fekete et al., 2018). The same study demonstrated *in vivo* that if mice were depleted of microglia, there were an increase in the number of HSV-infected neurons and viral proteins inside neuronal cells were higher than control mice, showing the importance of microglia in the clearance of HSV infection of CNS (Fekete et al., 2018). According with this, viral loads were higher in microglia-depleted mice and they succumb to infection earlier. Besides, rapidly deteriorating neurological symptoms may be associated with significantly increased neuronal infection (Chen et al., 2019). Importantly, it seems that cGAS-STING and TLR3 are the receptors involved in HSV infection control in the CNS (Chen et al., 2019). Moreover, TLR3 signaling pathway is critical in mediating resistance to HSV encephalitis in mice and in humans (Klein & Hunter, 2017). A recent work has confirmed that microglia are the main source of HSV-induced type I IFN, which is induced in a cGAS-STING-dependent manner. Brains of mice lacking cGAS and STING have higher viral load and viral spreading throughout the brain parenchyma (Reinert et al., 2016).

The mouse model of vesicular stomatitis virus (VSV) encephalitis demonstrated that infected microglia produce type I IFN and activate innate immunity which limits the trans-synaptic spread of VSV (Drokhlyansky et al., 2017). Interestingly, the same model has shown that upon intranasal VSV infection activated microglia aggregate in the olfactory bulb. It was shown that microglia accumulating around the olfactory bulb form a natural immune barrier that plays a critical role in limiting the spread of VSV in the CNS and prevents lethal encephalitis. VSV-infected mice have higher viral load in microglia-depleted brain and display higher mortality, indicating that microglia is crucial to limit the spread of VSV (Chhatbar et al., 2018). Also, early depletion of microglia in a model of murine hepatitis virus resulted in increased mortality while later depletion (after six days post-infection) had no influence on survival (Wheeler et al., 2018).

Cytomegalovirus-activated microglia suppresses viral replication in astrocytes by secreting IFN- $\gamma$  and TNF- $\alpha$ , also attract T-cell by CXCL10/IP-10 action (Rock et al., 2004). Interestingly, single-cell analysis of neuro-inflammatory responses following intracranial injection of rabies viruses have revealed distinct states of microglia activation that may serve different functions, which range from surveillance to antigen presentation and cytokine secretion. Among the later, antiviral responses are orchestrated by Type I and Type II IFN signaling from microglia (Huang & Sabatini, 2020). Microglia can also affect the adaptive immune response of the CNS. Depletion of microglia changes the response of CD4<sup>+</sup> T cell to viral infection in the brain, since MHC II is required for re-stimulation and activation of CD4<sup>+</sup> T cells. A decrease in MHC II as a result of microglial depletion has been shown to reduce the response of

virus-specific CD4<sup>+</sup> T cells (Wheeler et al., 2018). Also, during dengue virus infection it has been described a reduced CD8<sup>+</sup> T cell response and increased viral replication as a consequence of pharmacological deletion of microglia, demonstrating a functional role for microglia in modulating this cell population (Tsai et al., 2016).

With respect to bacterial infections, *Streptococcus pneumoniae* activates microglia throughout TLR2. As consequence, microglia secrete pro-inflammatory mediators and recruit neutrophils, monocytes, and T-cells from periphery to the infection site. It has been reported that TLR2 and MyD88 KO mice shown increased bacterial burdens and symptoms of more severe disease (Hanamsagar et al., 2012; Koedel et al., 2004). Moreover, IL-1 $\beta$  appears to be critical to pathogen containment and host survival during bacterial abscess formation in the CNS parenchyma (Kielian et al., 2004). Also, microglia are capable of exerting antimicrobial activity *per se*, for example, by secreting the antimicrobial cathelicidin peptide LL-37, that plays an important role in the innate immune response against CNS pneumococcal pathogens (Brandenburg et al., 2008). During *Staphylococcus aureus* CNS infection microglia recruit leukocytes into the CNS to facilitate bacterial clearance during abscess development (Mariani & Kielian, 2009; Rock et al., 2004). It has been demonstrated in *in vitro* assays that primary microglia, as well as different microglia cell lines, were able to internalize and kill *S. aureus* (Kielian et al., 2002). Gram-negative bacterial meningitis is caused principally by *Neisseria meningitidis* and *Haemophilus influenzae*. The principal compound of gram-negative bacterial wall is LPS, a TLR4 ligand. LPS is a potent stimulus for the microglial production of cytokines, chemokines, prostaglandins and NO (Rock et al., 2004).

Microglia has also been vindicated as an active participant in host defense against parasites that invade CNS. In the murine model of toxoplasmosis microglia are strongly activated, showing up-regulation of MHC class I and II molecules, and other antigens such as CD200, CD11a, CD11b; and secrete cytokines and chemokines (Schluter & Barragan, 2019). Once in the CNS, CD4<sup>+</sup>, CD8<sup>+</sup> T-cells and NK cells, work together with microglia to suppress *Toxoplasma gondii* proliferation, mainly by IFN- $\gamma$  and TNF- $\alpha$  action (Chao et al., 1994). In addition, the anti-parasite effects of activated microglia are due to NO-mediated inhibition of *T. gondii* intracellular replication, as the use of NO inhibitors abrogated the inhibitory effect (Chao et al., 1993). All together, these data suggest that microglia activation is beneficial for *T. gondii* clearance from CNS. Interestingly, experimental placement of *Trypanosoma brucei* into the brain results in rapid death of the parasites. This data is consistent with microglia activation in response to trypanosomes (Figarella et al., 2019). These authors speculated that the latency of human brain infection, that is the appearance of neuronal infection at late *T. brucei* disease stage, could be reflecting the ability of microglia to destroy parasites that occasionally cross the BBB (Figarella et al., 2019). Also

cerebral leishmaniasis as well as *T. cruzi* CNS infections can occur, but are largely controlled by the immunity in the brain, although the actual role of microglia is unknown (Figarella et al., 2019).

Infection with *Cryptococcus neoformans* on the CNS led to MHC class II increase in perivascular microglia, showing activation. Importantly, adaptive immune response plays a key role in the defense, especially increasing the antifungal activity of microglia by T-cell-secreted IFN- $\gamma$ . In addition, microglia can ingest and inhibit the growth of *C. neoformans*, opsonized or not, depending on the species of microglia (Rock et al., 2004).

Finally, microglia play a key role in limiting neuroinflammation. Microglia can produce IL-27 in the inflamed CNS. In IL-27 absence, mice infected with different pathogens as *T. gondii* or Coronaviruses show elevated production of IL-17 and GM-CSF in the CNS, which correlate with the development of T cell-mediated immune pathology (Klein & Hunter, 2017). IL-27 can directly limit T cell production of IL-17 and GM-CSF, and also promote T cell production of IL-10, Treg cell responses, and T cell expression of inhibitory receptors (Klein & Hunter, 2017). On the other hand, *T. gondii* infection of microglia induces secretion of transforming growth factor- $\beta$  (TGF- $\beta$ ) which inhibits the NO production by IFN- $\gamma$ -activated microglia, promoting neuronal viability (Rozenfeld et al., 2005). Moreover, as other regulatory mechanisms, it was described an indirect effect of soluble immunoregulatory mediators released by *T. gondii*-infected astrocytes which mediate neuron viability by inhibiting NO production by IFN- $\gamma$ -activated microglia (Rozenfeld et al., 2003).

### When Friends Become Enemies: Harmful Consequences of Pathogen-Activated Microglia on Cells of the CNS

Whatever the pathogen and the receptor involved in its recognition, the activation of the microglia leads, not only to the defense and elimination of the pathogen itself, but also to the damage of brain tissue and cells. Thus, beyond their roles in physiological conditions, microglia contribute to neuroinflammation in response to injury, stroke and infection (Aloisi, 2001; Kugler et al., 2021). They act as primary initiators of the inflammation cascade by increased reactivity and the secretion of factors such as chemokines (Karve et al., 2016).

#### Changes in Permeability and BBB Disruption due to Pathogen Activation of Microglia

BBB integrity is necessary to protect the brain from systemic toxins and germs, as well as to maintain the necessary level of nutrients for normal neuronal function. BBB activation or dysfunction is a significant contributor to the

pathogenesis of a variety of brain pathologies (Almutairi et al., 2016).

Pro-inflammatory cytokines secreted by pathogen-activated microglia, as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, act on the BBB activating endothelial cells (EC), leading to disruption of homeostasis and increasing its permeability. They also cause MMP secretion. MMPs have been implicated in inflammatory tissue destruction in several pathological situations in different tissues, including CNS. These proteins can act directly breaking the junctions between EC. Moreover, MMP-9 can degrade type IV collagen, laminins and fibronectin, which are structural components of the BBB and CNS tissue matrix, increasing BBB permeability as a consequence (Novak & Kaye, 2000). BBB disruption occurs in patients suffering cerebral malaria (CM) leading to severe neurological complications like intracerebral hemorrhage and intracranial edema, which finally resulted in axonal damage, CNS dysfunction and death (Brown et al., 2001; Nishanth & Schluter, 2019). One cause of the BBB damage is the excessive intracerebral inflammation resulting from pro-inflammatory cytokine response. It has been suggested that ECs are directly activated by recognizing *Plasmodium*-infected red blood cells, but microglia also recognize and phagocytize *Plasmodium*-infected red blood cells; and this result on their activation too (Shrivastava et al., 2017). Activated microglia produce pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$  that have been implicated in the pathogenesis of the CM (Nishanth & Schluter, 2019). TNF- $\alpha$  and IL-1 $\beta$  alter the expression of tight-junction proteins claudin-5, ZO-1, occludin, and P-glycoprotein 1 (Landoni et al., 2012; Ravindran et al., 2011). These cytokines also activate EC, which release chemokines, inducing the recruitment of immune cells to the brain. In addition, microglia secrete CXCL10, which also participated on CD8<sup>+</sup> T-cell recruitment which are critical in the BBB disruption. Studies using knock-out mice to several chemokines and their receptors have shown a reduced pathology of experimental CM (Nishanth & Schluter, 2019). MMP, produced by microglia and others CNS cell in response to *P. falciparum*, also has been implicated in BBB disruption and neuronal loss (Mariani & Kielian, 2009).

We have described that microglia and astrocytes release MMP-9 in response to *Brucella* spp. infection (Miraglia et al., 2013). This could contribute to the disruption of BBB in this pathology. Moreover, astrocytes and microglia infected by *B. abortus* secrete IL-1 $\beta$ , among others pro-inflammatory factors, throughout TLR2 activation. In addition, *B. abortus* can be recognized through TLR6, inducing cell activation (de Almeida et al., 2013). This cytokine activate brain EC, increasing the permeability of the BBB (Miraglia et al., 2016). *In vivo* injection of *B. abortus* in the brain of mice lead to an inflammatory infiltrate (Miraglia et al., 2016; Samartino et al., 2010), and this was dependent of microglia and astrocytes IL-1 $\beta$  secretion, since mice deficient in ASC and NLRP3 KO (that are unable to produce IL-1 $\beta$ ) exhibit

less leukocyte infiltrate in the CNS (Miraglia et al., 2016). On the other hand, *B. abortus* traverse the BBB and access to the CNS by the Trojan horse mechanism, within an infected monocyte (Miraglia et al., 2018). Therefore, this evidence would indicate that a breakdown of BBB and chemokine secretion by microglia would lead to monocytes and other infected immune cells to migrate into the CNS, generating a feedback that would perpetuate infection (Rodríguez et al., 2019).

Microglia secrete NO in response to different pathogens such as *B. abortus* (Rodríguez et al., 2017), group B *Streptococcus* (GBS) (Lehnardt et al., 2006), as well as when they are activated by PAMPs (Chao et al., 1992; Neher et al., 2011). High levels of NO have a key role in BBB dysfunction. NO acts by different mechanisms. NO form highly active nitrides, as peroxynitrite and nitrate, which result in the production of nitrotyrosine which causes lipid peroxidation and cell damage. On the other hand, NO is a vasodilator and it can increase permeability of BBB, allowing the influx of immune cells to CNS, generating damage in the CNS parenchyma (Liu et al., 2019; Rock et al., 2004). Brain ECs exposed to hypoxic conditions demonstrated an increased paracellular permeability that was significantly attenuated by inhibition of NOS (Mark et al., 2004). In addition, NO diffuses through endothelial cell membranes to activate soluble guanylyl cyclase, a cytoplasmic enzyme that produces cGMP, which regulates cGMP-dependent protein kinase, which is cytoskeleton-associated and indirectly led to changes on BBB functions and disruption by NO (Chen et al., 2003).

Finally, direct LPS treatment of ECs monolayer showed no effect on permeability, however, when the LPS treatment was performed on co-cultures of ECs plus microglia, permeability was increased and this was dependent on the amount of microglial cells in culture (da Fonseca et al., 2014). In support of this, LPS-activated microglial disrupt tight junction proteins ZO-1 and claudin-5, decreasing trans-endothelial electrical resistance of an endothelial monolayer *in vitro* (Sumi et al., 2010) and triggering endothelial cell injury (Kacimi et al., 2011). Furthermore, LPS administration in rodent induces BBB leakage and neutrophil recruitment via TLR4-expressing microglial cells (Zhou et al., 2006) (Table 1).

### **Neuronal Demise Induced by Pathogen-Activated Microglia**

There are different reports describing several inflammatory mechanisms generated by microglia in response to an infectious process that induce neuronal death instead of host defense. First, neuronal damage can be induced by microglia-released factors. It was described neuronal loss as a consequence of microglia release of factors such as NO (Chao et al., 1992; Dawson et al., 1991; Lehnardt et al.,

2006), IL-1 $\beta$ , IFN- $\gamma$  (Hu et al., 1997), TNF- $\alpha$  (Medana et al., 2000; Takeuchi et al., 2006; Venters et al., 2000), MMPs (Thornton et al., 2008) and others. Lehnardt and collaborators have deepened the study, describing neuronal apoptosis generated by microglia-secreted NO by TLR2-recognition of Group B *Streptococcus* antigens (GBS). They observed neuronal apoptosis in co-cultures of neurons and microglia, and this neuronal apoptotic death was abrogated by using aminoguanidine, an NO inhibitor, and using TLR2-deficient mice (Lehnardt et al., 2006). Among cytokines, it has been described the neurotoxic effect of IL-1 $\beta$  and IL-1 $\beta$  + IFN- $\gamma$  combination, that act indirectly by induction of NO release in glia/neurons mixed cultures (Hu et al., 1997). Pathogen-activated microglia secrete TNF- $\alpha$ , a cytokine that induces neurodegeneration by several mechanisms. TNFR1 (p55) is a very well characterized receptor, which has ability to induce signals that led to cellular death by apoptosis (Venters et al., 2000). Other mechanism whereby TNF- $\alpha$  secreted from activated microglia may mediate neuronal death is glutamate secretion. LPS-activated or TNF- $\alpha$ -stimulated microglia secrete glutamate. This glutamate induces neuronal death throughout NMDA receptor, inducing a drop on intraneuronal ATP levels and mitochondrial dysfunction. Neuronal death was inhibited by anti-TNF- $\alpha$ , anti-TNFR1 or anti-NMDA antibodies (Takeuchi et al., 2006). Using a murine model of CM, which exhibits neurological alterations that are similar to human pathology; it has been shown the importance of microglia in malaria CNS pathology. Microglia activation occur 2–3 days post-inoculation of parasites, before symptoms appear. Mice treated with dexamethasone, which inhibits TNF- $\alpha$  secretion by activated-microglia, showed less microglia-activation which correlate with less neurological symptoms (Medana et al., 2000; Rock et al., 2004). Pathogen-activated microglia can secrete MMP-2 and MMP-9. It has been described a neurotoxic effect of MMP-9 in glial–neuronal co-cultures, which was abrogated using a MMP-9 inhibitor (Thornton et al., 2008). Analyzing histopathological features of patients with HIV-associated neurocognitive disorders (HAD), it was observed that the number of activated microglia has a better correlation with HAD degree than the presence and amount of cells infected by HIV in the brain parenchyma, and that activated microglia is a better marker of neuronal damage than productive HIV-1 infection in the CNS (Glass et al., 1995). These data highlight the importance of activated microglia in HAD disease (Rock et al., 2004) (Table 1).

Cell-to-cell contact is another important factor that has been vindicated in the induction of neuronal death by activated microglia (Myers et al., 2009; Neher et al., 2012; Rodríguez et al., 2017). This is the case of neuronal apoptosis mediated by microglia activated by *Borrelia burgdorferi*. Although it was observed that microglia are capable of secreting multiple soluble factors, using transwell inserts to separate microglia from neuron, the authors observed that

**Table 1.** Detrimental Effect of Pathogen-Activated Microglia on the Neurovascular Unit.

<b>Change in permeability and BBB disruption due to pathogen activation of microglia</b>				
<b>IL-6</b> <b>TNF<math>\alpha</math></b> <b>IL-1<math>\beta</math></b>	Induction of MMP9 secretion	Degrade components of ECM of BBB such as type IV collagen, laminins and fibronectin, increasing BBB permeability	LPS <i>B. abortus</i>	Novak & Kaye, 2000 Miraglia et al., 2013
<b>TNF<math>\alpha</math></b> <b>IL-1<math>\beta</math></b>	Disrupt tight junction proteins ZO-1, claudin-5, occludin and P-glycoprotein 1		<i>Plasmodium</i> spp Shiga toxin 1 LPS	Ravindran et al., 2011 Landoni et al., 2012 Sumi et al., 2010
<b>IL-1<math>\beta</math></b>	Activates brain EC, increasing the permeability of the BBB		<i>B. abortus</i>	Miraglia et al., 2016
<b>NO</b>	Forms peroxy nitrite and nitrate, which causes lipid peroxidation and cell damage Vasodilator, increases permeability of BBB, allowing CNS invasion of immune cells, generating damage Diffuses into EC to activate soluble guanylyl cyclase, which regulates cGMP-dependent protein kinase, which is cytoskeleton-associated and indirectly led to changes and disruption on BBB		Herpes simplex	Rock et al., 2004 Liu et al., 2019  Chen et al., 2003
<b>Neuronal demise induced by pathogen-activated microglia</b>				
<b>NO</b> <b>IL-1<math>\beta</math></b> <b>IFN-<math>\gamma</math></b> <b>TNF<math>\alpha</math></b>	Neurotoxic effect -> Inducing apoptosis		LPS / group B <i>Streptococcus</i>	Dawson et al., 1991 Chao et al., 1992 Lehnardt et al., 2006 Hu et al., 1997
<b>MMP-9</b> <b>Cell-to-cell contact</b>	Act indirectly by induction of NO release in glia/ neurons mixed cultures Primary Phagocytosis	(For more detail see Figure 4)	<i>Plasmodium</i> spp	Medana et al., 2000 Rock et al., 2004
	Induces glutamate secretion -> Inducing a drop on intraneuronal ATP levels and mitochondrial dysfunction		LPS	Venters et al., 2000
	Neurotoxic effect			Thornton et al., 2008
			LPS / LTA <i>B. abortus</i>	Neher et al., 2012 Rodriguez et al., 2017
<b>Effect of pathogen-activated microglia on oligodendrocytes</b>				
<b>Glutamate</b> <b>TNF<math>\alpha</math></b> <b>IL-1<math>\beta</math></b> <b>NO</b> <b>TNF<math>\alpha</math></b> <b>IL-1<math>\beta</math></b> <b>IL-1<math>\alpha</math>,</b> <b>TNF-<math>\alpha</math></b> <b>C1q</b> <b>IL-33</b>	Generate damage	Poor quality of myelin	LPS	Peferoen et al., 2014 Pang et al., 2003 Sherwin & Fern, 2005
	Contribute to oligodendrocytes and myelin damage			
	Induce astrocyte activation	Oligodendrocyte damage and death		
	Induces secretion of cytokines by microglia such as IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , and chemokines such as CXCL9 and CXCL10		<i>Plasmodium</i> spp	Reverchon et al., 2017

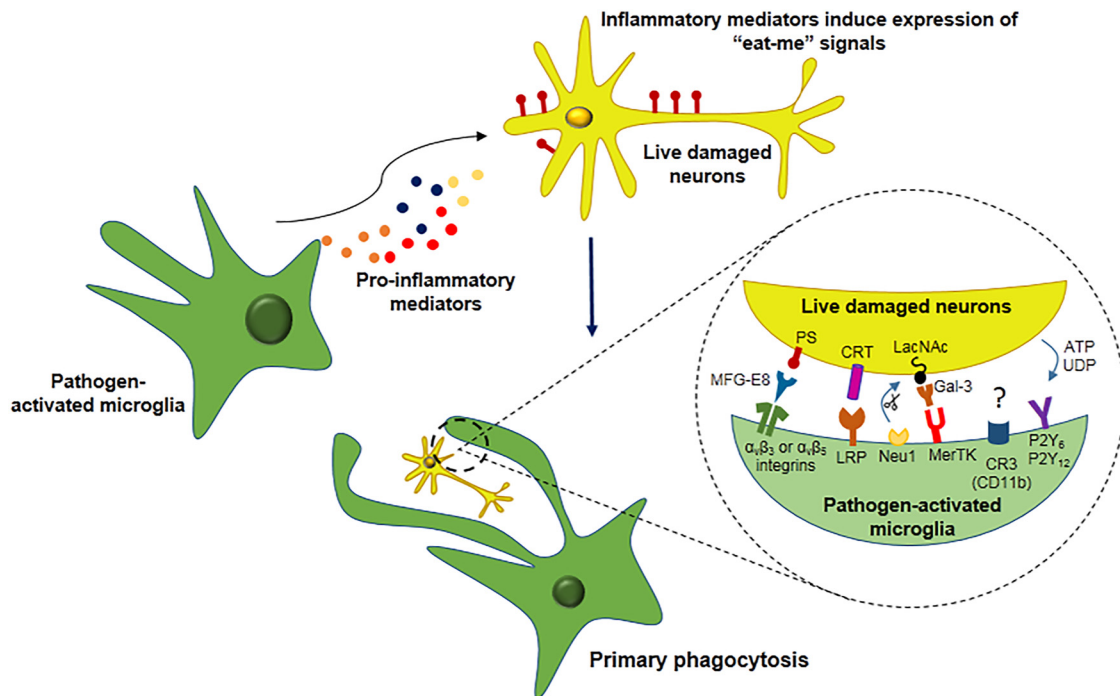
ECM: extracellular matrix.

EC: endothelial cells.

contact between cells is required for infected microglia to kill neurons (Myers et al., 2009). In recent years, a peculiar cell-to-cell contact mechanism implicated in the death of neurons induced by activated microglia during inflammation of the CNS has been described. It is named primary phagocytosis or phagoptosis (Figure 4). This mechanism involves the phagocytosis of live but stressed neurons by activated microglia (Brown & Neher, 2012, 2014), and is opposed to the traditional secondary phagocytosis of neurons that have

been killed by apoptosis and latter are phagocytosed. Activation of microglia by different PAMPs, such as LPS or LTA, lead to neuronal death by primary phagocytosis (Fricker et al., 2012a, 2012b; Neher et al., 2012). In these works, authors elegantly demonstrate that this mechanism require cell-to-cell contact. Neurons expose to low levels of NO and ROS expose in their membrane PS, as mentioned before a well know “eat-me” signal. Microglia recognizes this PS by MFG-E8 protein, which acts as bridge between





**Figure 4.** Microglial primary phagocytosis or phagoptosis. Pathogen-activated microglia increase their phagocytic activity and secrete several pro-inflammatory mediators. These mediators induce the expression of “eat-me” signals on live neurons. The exposure of these signals in viable damaged neurons triggers neuronal death by microglial primary phagocytosis.

PS and the vitronectin receptor located on microglia membrane (Fuller & Van Eldik, 2008). This triggered phagocytosis. When the interaction PS/MFG-E8/vitronectin receptor was inhibited, neuronal death was abolished. Importantly, neurons in the culture did not undergo apoptosis, demonstrating that the phagocytosis was the cause of the death but not a consequence (Neher et al., 2012). Moreover, it has also been described that the expression of calreticulin (CRT) on the surface of neurons, viable or apoptotic, is required for triggering their phagocytosis via LRP receptors. Using antibodies against anti-LRP or CRT to block this interaction, the phagocytosis of neurons was abolished, as well as by using soluble CRT that blocks microglial phagocytosis of neurons (Fricker et al., 2012a). Also, LPS-activated microglia secrete Gal-3 and increase the neuraminidase 1 activity in their cell membrane triggering the desialylation of neighboring neurons to expose N-acetyl-lactosamine residues, which can be recognized by Gal-3. Gal-3 binds to MerTK receptor on the surface of microglia and trigger phagocytosis (Nomura et al., 2017). In addition, recently it has been described that adding desialylated microglia to cultures of neurons, without neuronal desialylation, also caused neuronal death. This was prevented by using CD11b blocking antibody. Phagocytosis of neurons by LPS-activated microglia was also inhibited using CD11b blocking antibody, demonstrating that autocrine desialylation activity is necessary for microglia to phagocytose neurons, stimulating CR3-mediated phagocytosis (Allendorf et al., 2019). Yet,

the mechanism by which CR3 is involved in phagocytosis of neurons is unknown.

Recently, we have demonstrated *in vitro* for the first time in a bacterial infection that phagoptosis occurred during *B. abortus* infection. As mentioned before, *B. abortus* access to the brain parenchyma and it can infect and activate microglia and astrocytes (Miraglia et al., 2018; Rodríguez et al., 2019; Samartino et al., 2010). Both types of cells are able to secrete pro-inflammatory factors such as TNF- $\alpha$ , IL-1 $\beta$  and NO in response to bacterial infection (Rodríguez et al., 2017; Samartino et al., 2010). Using primary cultures of murine neurons and microglia, we have shown that *B. abortus*-activated microglia kill neurons by phagoptosis. If the recognition of PS on the neurons by MFG-E8 is blocked, neurons are rescued. Interestingly, there were necessary two features of *B. abortus*-activated microglia to kill neurons by primary phagocytosis: secretion of NO, which induce neurons PS expression on the surface, and the increase of the phagocytic activity by *B. abortus*-activated microglia (Rodríguez et al., 2017).

Finally, it has been demonstrated that phagocytosis of neurons by microglia also requires the release of “find-me” signals such as neuronal UDP/ATP. Both, nucleosides and nucleotides of adenine and uridine, can function as extracellular signals inducing a change in the microglia from migratory to phagocytic phenotype; causing the formation of the so-called phagocytic cup (the membrane invagination around the target cell) and enabling neuronal ingestion

(Koizumi et al., 2007). Their action is mediated by purinergic receptors capable of modulating a wide variety of responses, such as inflammatory response, insulin secretion, vascular tone regulation, among others (Lazarowaki & Schwarzbaum, 2009). It has been shown that cell phagocytosis requires activation of these signaling pathways (Ravichandran, 2011). In particular, it has been described that in the final stage of phagocytosis of neurons by microglia the release of UDP by the target cell is required to change the phenotype of microglia from a migratory profile towards a phagocytic profile (Bernier et al., 2013; Koizumi et al., 2007). UDP released from damaged neurons induces adjacent microglia to phagocyte them through the P2Y<sub>6</sub> receptor. More recently, it has been shown that this signaling pathway is also involved in the recognition and subsequent phagocytosis of live neurons. Microglia activated by LPS or LTA (TLR4 and TLR2 ligand, respectively) phagocytose live neurons and this phenomenon was inhibited by MRS2578, a specific inhibitor of P2Y<sub>6</sub> receptor (Neher et al., 2014).

### ***Oligodendrocytes and Astrocytes Alterations Induced by Pathogen-Activated Microglia***

Oligodendrocytes are susceptible to be damaged by microglia-derived factors as glutamate, TNF- $\alpha$ , IL-1 $\beta$  and NO among others, particularly because of their high metabolic activity and energy demands. As consequence of this damage, they produce a poor quality myelin, which may contribute to the pathology observed in CNS infection diseases (Peferoen et al., 2014). Intracerebral injection of LPS lead to activation of astrocytes and microglia. Both type of cells produce pro-inflammatory cytokines, that lead to hypo-myelination (Pang et al., 2003). *In vitro*, LPS-activated microglia induce arrest of oligodendrocyte progenitor cell proliferation and their death (Sherwin & Fern, 2005). In addition, because of cell damage or death, oligodendrocytes also release ATP/UDP that work as a find-me signals for microglia. TNF- $\alpha$  and IL-1 $\beta$  released by microglia might further contribute to oligodendrocytes and myelin damage (Sherwin & Fern, 2005).

Recently, it has been described the ability of LPS-activated microglia to induce a kind of reactive astrocytes named A1. This phenomenon was induced by microglia-secreted IL-1 $\alpha$ , TNF- $\alpha$  and C1q in concert. These astrocytes lose the ability to promote neuronal survival and, on the contrary, lead to neurons and oligodendrocytes to death (Liddelow et al., 2017). In addition, it was recently reported the involvement of the IL-33 in mice suffering experimental CM. It was demonstrated that astrocytes and oligodendrocytes secrete IL-33 in hippocampus at 7 days post-infection with *P. berghei*. The same study showed that microglia from ST2 (IL-33 receptor) KO mice have problems to be fully activated. Although microglia display the typical activated morphology, i.e.

hypertrophied cell body and thick cytoplasmic extension, together with an increase in IBA1<sup>+</sup> expression and proliferation activity at 7 days post-infection; there was less production of the pro-inflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , as well as the chemokines CXCL9 and CXCL10. IL-33 secreted by oligodendrocytes and astrocytes induce microglial secretion of IL-1 $\beta$  and, conversely, IL-1 $\beta$  induce the secretion of IL-33 by oligodendrocytes, generating an inflammatory loop that contribute to *P. berghei*-induced cognitive disorders (Reverchon et al., 2017) (Table 1).

### **Concluding Remarks**

Most of the information gathered to present has come from the acknowledgment of microglia as being the resident macrophages of the CNS and the recently discovered evidences of the homeostatic functions of tissue macrophages, despite their traditional immune functions (Gordon et al., 2014; Wynn et al., 2013). Considering the skills of microglia to remodel their phenotype according to environmental signals, they can be considered as the most plastic cell type of the CNS, acting as immune “double-edge swords” either bringing benefits and defense to the CNS or causing immunopathology. An important aspect that this review has brought into attention is that the molecular tools (immune receptors, neurotropic factors, inflammatory mediators, etc.) that microglial cells use to perform “good and evil” are practically the same. During development and in the adult CNS the particular phenotype that microglia adopt is related to environmental cues that these cells received from other cells (astrocytes, neurons, ECs, etc.), microbes or spatio-temporal tissue profiles.

Much of the information we have learnt from the harmful function of microglia have aroused from studies on neurodegenerative diseases and stroke but less is known about the damage that microglia inflict to the CNS cells when is activated by a pathogen. It has come to the attention of the field that there exist a functional diversity of microglia (Sankowski et al., 2019) and not a single uniform population of microglial cells in the CNS. Thus, it might be possible that thorough investigations on how different subpopulation of microglia respond to infection allow showing such a diversity.

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## ORCID iD

Guillermo Hernán Giambartolomei  <https://orcid.org/0000-0001-7441-5311>

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