Investigating the Roles of Microglia and Astrocytes in Multiple Sclerosis: Allies or Adversaries?

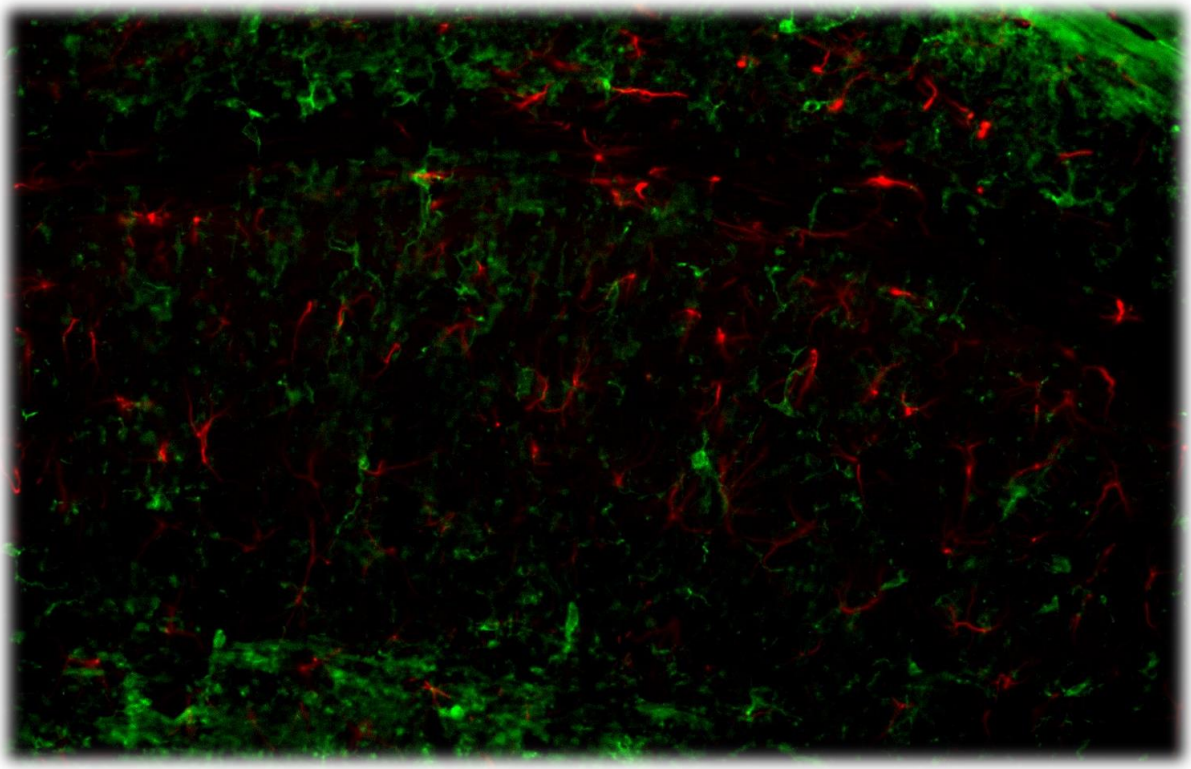

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Illustration: Composite of IBA-1 (green) and GFAP (red) in the CA1 hippocampal region of EAE mice.

1 FOREWORD

The picture on the cover page is an original creation of mine, designed to visually represent the main themes of this thesis. This picture was created by making a composite of an IBA-1 staining and GFAP staining, both in the CA1 region of the hippocampus. The stainings are from two distinct EAE mouse brains.

2 SUMMARY

Multiple sclerosis (MS) is an immune-mediated multifactorial neurodegenerative disease predominantly diagnosed in young adults. Microglia and astrocytes are glial cells that have been associated with MS disease progression. However, other studies suggest these cells have been associated with amelioration of MS. The aim of this literature study is to investigate how microglia and astrocytes behave in MS lesions and how their interactions with other glial cells affect MS disease progression. Microglia seem to exert more protective effects in and around MS lesions compared to astrocytes. However, in certain circumstances, microglial activity in MS lesions can be fatal to oligodendrocytes. In addition, microglia can induce a pro-inflammatory phenotype in astrocytes, amplifying the pro-inflammatory response. Astrocytes have predominantly been associated with detrimental behaviors in MS. Some studies have found that astrocyte-oligodendrocyte communication via gap junctions can be impaired in MS, harming oligodendrocytes and possibly impairing remyelination. On the other hand, astrocytes provide OPCs with essential differentiation factors in MS. Finally, astrocytes can recruit microglia to MS lesions, which can contribute to disease progression as well as disease amelioration. In conclusion, the roles of microglia and astrocytes and their interactions in MS remain complex, which limits the ability of making a general statement on their roles in MS. Future research should focus on investigating the environmental triggers that create harmful phenotypes of microglia and astrocytes in MS to develop novel strategies to combat adversary roles in these glial allies.

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4 ABBREVIATIONS

Blood-brain barrier	BBB
Central Nervous System	CNS
Glial fibrillary acidic protein	GFAP
Interferon-1 gamma	IFN-γ
Ionized calcium binding adapter molecule 1	IBA-1
Multiple Sclerosis	MS
Myelin oligodendrocyte protein	MOG
Oligodendrocyte progenitor cell	OPC
Pertussis toxin	PT
Primary progressive Multiple Sclerosis	PPMS
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Relapsing-remitting Multiple Sclerosis	RRMS
Secondary progressive Multiple Sclerosis	SPMS
Tumor necrosis factor alpha	TNF-α
Tumor necrosis factor alpha receptor 1	TNFR1
Tumor necrosis factor alpha receptor 2	TNFR2

5 INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated multifactorial neurodegenerative disease that affects over 2 million people worldwide (Kingwell et al., 2013; Walton et al., 2020). MS is usually diagnosed between the ages of 30 and 40, making it one of the most commonly diagnosed neurological diseases in young adults (Kingwell et al., 2013; WHO & Rompani, 2008). A sex bias has been observed in MS, with women being diagnosed with the disease more often compared to men (Constantinescu et al., 2011; Dobson & Giovannoni, 2019). Early stages of MS often present as acute episodes of neurological dysfunction, often accommodated with partial or complete remission afterwards (Confavreux et al., 2000). This neurological dysfunction can manifest in different ways, such as optic neuritis, diplopia, transverse myelitis and ataxia (Reich et al., 2018). Although the life expectancy of most MS patients is like that of healthy adults, around 60% of MS patients are disabled 20 years after diagnosis (WHO & Rompani, 2008).

The risk of developing MS can be increased by both genetic as well as environmental factors. More than 150 single-nucleotide polymorphisms have been associated with an elevated risk for developing MS (Dobson & Giovannoni, 2019). Multiple genes have been associated with an increased risk for MS as well (Hollenbach & Oksenberg, 2015). Some environmental factors that increase the risk of MS are smoking, obesity, Epstein-Barr virus infection and head injury (Stephenson et al., 2018). Furthermore, a sex bias in MS with women being more susceptible compared to men has been thoroughly described in literature (Constantinescu et al., 2011; Dobson & Giovannoni, 2019; International Multiple Sclerosis Genetics Consortium, 2019; Murphy et al., 2020; Stephenson et al., 2018).

Classical hallmarks of MS are persistent perivenular neuroinflammation and lesions in the central nervous system (CNS) (Doshi & Chataway, 2016). MS can be categorized into different types. Most MS patients experience a relapsing-remitting type (RRMS), where demyelinated lesions improve and can be remyelinated with the help of oligodendrocytes, although this is not always the case. After a while, RRMS can develop into a secondary progressive type (SPMS) where demyelinated axons deteriorate, resulting in neurodegeneration. A smaller percentage of patients exhibit a primary progressive development of the disease (PPMS) (Lassmann et al., 2012; Yong, 2022). Recently, an appreciation of the continuum within and between these three MS subtypes has been found (Yong, 2022).

The etiopathology of this disease is still actively debated. Two popular models for the etiopathogenesis of MS are the 'inside out' model and the 'outside in model'. The 'outside in' model proposes that peripheral inflammation occurs first, after which pro-inflammatory B and T cells trigger an inflammatory response in the CNS. The 'inside out' model proposes that an autoimmune response in the CNS occurs first, after which the peripheral immune system gets activated (Sen et al., 2020; Titus et al., 2020). Although a definitive answer to this debate remains elusive, significant progress has been made in exploring other aspects of the disease, particularly the role of glial cells in MS.

Glial cells are residents of the CNS. They are, in one way or another, responsible for neuron homeostasis and health. Oligodendrocytes are the glial cells responsible for myelination in the CNS. During development, oligodendrocytes create thick myelin sheaths around the axons. When demyelination occurs, these cells get activated to remyelinate the unmyelinated axons (Peferoen et al., 2014). Microglia, which are CNS-resident macrophages, could protect neurons from pathogens

and toxins (Yong, 2022). Astrocytes, which can be considered the 'housekeepers' of the CNS, could mediate blood flow and metabolism for optimal neuronal activity (Verkhratsky et al., 2021). In MS, these protectors of the CNS sometimes tend to behave differently. This different behavior can either be beneficial or detrimental to the surrounding neurons (Brambilla, 2019; Muzio et al., 2007; Sofroniew & Vinters, 2010; Takeuchi et al., 2006). Studying the exact roles microglia and astrocytes play in MS is crucial to understand their contributions to disease progression as well as to develop insights into novel therapeutics for MS. The aim of this literary study is to elucidate how microglial and astrocytic behavior in MS lesions and their interactions with other glial cells affect MS disease progression. To study this, the focus will lie on how well techniques to study MS represent the disease in humans, as well as how microglia and astrocytes are affected by the peripheral immune system and vice versa.

6 ANIMAL MODELS OF MS

6.1 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

EAE is an MS model most often induced in mice and rats to mimic MS (Brambilla, 2019; Murphy et al., 2020). EAE shares key symptoms of MS such as focal lesions in the CNS, demyelination and axonal damage (Murphy et al., 2020). As will be discussed in the following chapters, EAE also mimics the increased levels of microglia and astrocytes found in MS lesions (Bradl & Linington, 1996; Brambilla, 2019; Kuhlmann et al., 2008; Kuhlman et al., 2017).

EAE is induced by an external compound called an adjuvant. Adjuvants often contain a bacterial element. There are a handful of adjuvants available to induce EAE, contributing to the diversity of EAE animal models (Constantinescu et al., 2011; Lassmann & Bradl, 2017). Out of all adjuvants, myelin oligodendrocyte glycoprotein (MOG) is the most commonly used one, as well as one that induces one of the most aggressive forms of EAE. Interestingly, anti-MOG proteins are also found in some MS patients. Some adjuvants require co-administration with the bacterial pertussis toxin (PT) (Bittner et al., 2014; Brambilla, 2019; Lassmann & Bradl, 2017). PT is a compound which increases the inflammatory response and the permeability of the blood-brain barrier (BBB) (Murphy et al., 2020). In EAE, T cells infiltrate the CNS (Fletcher et al., 2010), which is possibly mediated by the leaky BBB.

Besides the administration of different adjuvants and toxins called active EAE, EAE can also be induced by isolating CD4+ T cells from EAE animals that received direct immunization (i.e. were injected with an adjuvant and possibly co-administration of PT) and transferring these T cells to naïve recipient animals. This is called passive EAE (Brambilla, 2019; Lassmann & Bradl, 2017; Wekerle et al., 1994).

In summary, EAE seems to be a valuable model for MS due to its ability to mimic MS in terms of disease progression, lymphocyte infiltration, increased activation of glial cells and to some extent the mechanisms of MS. However, the model has some limitations. One of these limitations is how EAE is induced. Although a lot is still unknown, MS seems to be induced spontaneously, unlike EAE. Transgenic EAE mouse models have been developed to overcome this limitation (Brambilla, 2019). Another limitation found is that EAE induced with PT can mask the sexual dimorphic symptoms of MS (Murphy et al., 2020). Moreover, the different adjuvants in EAE add heterogeneity. On the one hand, this might be beneficial because of the wide range of effects that can be induced. For example, the different subtypes of MS (RRMS, PPMS, SPMS) can be modeled with different adjuvants (Libbey & Fujinami, 2011). On the other hand, heterogeneity in EAE models adds a lot of variability in literature. This can make it more difficult to accurately compare different studies with each other. The commonly used adjuvant MOG has some serious limitations as well; Lassmann & Bradl (2017) reported that in EAE mice induced with MOG, axonal degeneration occurred before demyelination did, which is very different from MS, where demyelination leads to axonal degeneration. Furthermore, while MS lesions are predominantly found in the brain, EAE lesions are more commonly found in the spinal cord (Fujinami, 2001; Lassmann & Bradl, 2017). Furthermore, some therapeutics that ameliorated symptoms in EAE mice had no effect or worsened symptoms in MS patients (Libbey & Fujinami, 2011). Finally, because of the way EAE is induced, it always follows an ‘outside in’ model for MS. This is a possible limitation of the animal model, as it is not clear yet whether MS follows an ‘outside in’ or ‘inside out’ trajectory or a combination of both (Sen et al.,

2020; Titus et al., 2020). These limitations could hinder the accuracy of the MS animal model when translating the findings to MS in humans.

6.2 CUPRIZONE MICE

Cuprizone is a molecule that induces demyelination by killing oligodendrocytes. Although the exact mechanisms of cuprizone toxicity are not clear, it is hypothesized that cuprizone dysregulates the metabolism of mature oligodendrocytes, resulting in apoptosis of mature oligodendrocytes. This might result in demyelination of axons (Sen et al., 2022; Torkildsen et al., 2008).

Although cuprizone mice seem to develop demyelinated lesions in a similar manner to MS, it still mimics more of a toxin-induced lesion than an autoimmunity-induced lesion (Torkildsen et al., 2008). Cuprizone mice respond to lesions in a similar manner as is observed in MS. Like MS lesions, an increase in activation of astrocytes and microglia is reported when mice are fed cuprizone (Leo & Kipp, 2022). McMahon et al. (2002) reported an increase in microglia but mostly macrophages in the CNS in cuprizone-treated mice. Furthermore, cuprizone-fed mice show a disease pattern that is more like early MS, as the symptoms of these mice are more similar to what MS patients might experience in their early stages (Sen et al. (2022). However, differences between the cuprizone treatment and MS in humans are still observed. In cuprizone lesions, T cells do not invade the CNS as has been observed in MS lesions (Torkildsen et al., 2008).

It has been found that MS patients have an elevated risk of developing schizophrenia (Meier et al., 2020). Recent studies indicate that a dysregulated immune response may play a role in schizophrenia like it does in MS (Herring & Konradi, 2011; Meier et al., 2020; Pape et al., 2019). Therefore, cuprizone mice are also used as a model for schizophrenia (Herring & Konradi, 2011). For this reason, cuprizone mice could serve as a bridge to investigate the link between schizophrenia and MS.

6.3 COMPARISON OF EAE AND CUPRIZONE MODELS IN MS

Differences between EAE and cuprizone treatment have also been observed. An overview of the two MS models can be seen in figure 1.

A large difference between EAE and cuprizone as an animal model for MS is how stopping cuprizone treatment results in remyelination of axons. This could mimic the way MS lesions in RRMS get remyelinated after demyelination. Another benefit of the remyelination in cuprizone mice is that it allows for studying the factors that contribute or inhibit remyelination (Torkildsen et al., 2008). An additional difference between the EAE and cuprizone models is that cuprizone does not compromise the BBB as severely as the EAE model does, especially in combination with PT (Bradl & Linington, 1996; Sen et al., 2022; Vankriekelsvenne et al., 2022).

Whereas EAE lesions are predominantly found in the spinal cord, cuprizone lesions rarely present themselves in that area. Instead, cuprizone lesions are more commonly found in the corpus callosum and the somatosensory cortex region (Fujinami, 2001; Zhan et al., 2020). The difference in lesion sites between the models should be considered when comparing results between these two MS models.

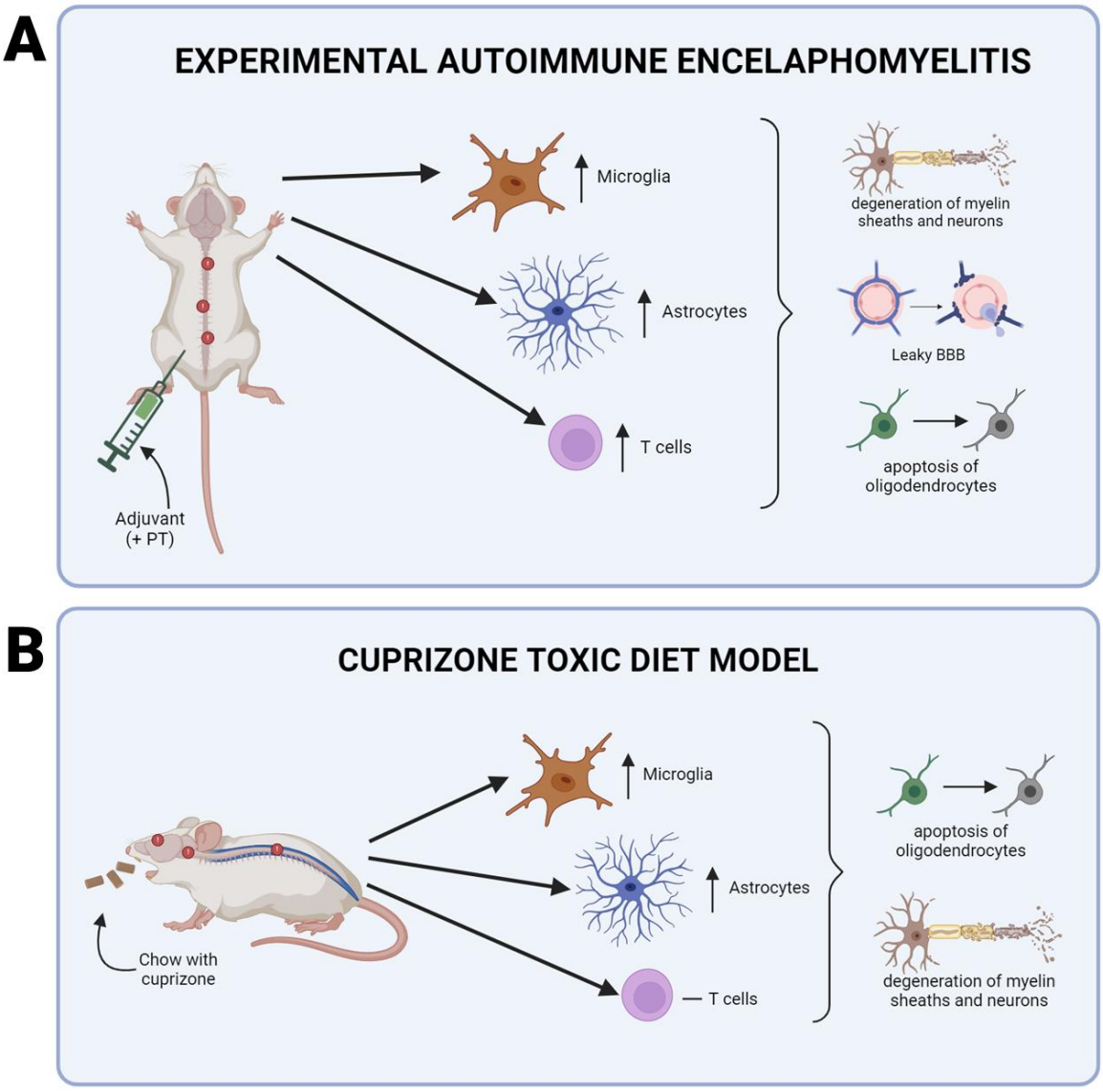

Figure 1. Overview of the animal models experimental autoimmune encephalomyelitis (EAE) and cuprizone. (A) Overview of EAE. (B) Overview of cuprizone. Figure created by using BioRender.com.

7 THE ROLES OF GLIAL CELLS IN MS

7.1 MICROGLIA

Microglia are present in a wide variety of species (Geirsdottir et al., 2019). The conservation of microglia across this wide variety of species indicates the importance of these CNS-resident immune cells. The most important function of microglia is to protect the CNS from pathogens, toxins and other forms of injury. Microglia have long branches extending from their cell body, allowing them to scavenge a large surface around them. They are also able to rapidly move to injured sites (Davalos et al., 2005). At the injured site, microglia clear up debris and phagocytose dead or dying cells (Hickman et al., 2018). Another function of microglia is regulation of synaptic homeostasis (Lui et al., 2016).

Microglia are derived from the yolk sac during development, and they move into the CNS before the BBB forms. This distinguishes them from peripheral macrophages, as these originate from hematopoietic stem cells (Guerrero & Sicotte, 2020; Yong, 2022). Microglia also differ from macrophages in their ability to self-regenerate (Prinz & Priller, 2014; Yona et al., 2013).

Traditionally, microglia were categorized into two subtypes: M1 microglia, like M1 macrophages, are considered pro-inflammatory, whereas M2 microglia are considered anti-inflammatory. However, multiple studies have reported that this dichotomy is overlooking the versatility in microglia and therefore inaccurate (Bachiller et al., 2018; Guerrero & Sicotte, 2020; Kwon & Koh, 2020; Yong, 2022). Therefore, in this literature review these terms will not be used.

The heterogeneity within microglia can also be found in different lesion types. In active lesions, microglia/macrophages with pro-inflammatory markers are frequently present. In contrast, inactive lesions are more frequently visited by microglia/macrophages with anti-inflammatory markers (Guerrero & Sicotte, 2020).

Multiple markers have been used for visualizing microglia. Due to the phenotypic differences within microglia depending on their active state, differentiating microglia from macrophages can be difficult (Yong, 2022). Ionized calcium binding adapter molecule 1 (IBA-1) is a commonly used marker which is not able to distinguish microglia from macrophages (Guerrero & Sicotte, 2020; Yong, 2022). New markers which should be able to distinguish microglia from macrophages, such as transmembrane protein 119 have been developed (Bennett et al., 2016). While the development of new markers has been appreciated by some (Satoh et al., 2016), the new markers have also been criticized for being less effective at binding to activated microglia (Schwabenland et al., 2021), being downregulated proteins in MS (Lloyd & Miron, 2020) or not being specific to microglia only (Vankriekelsvenne et al., 2022). In this essay, when the distinction between microglia and macrophages has not been made, it will be referred to as 'microglia/macrophages'.

Because the presence of microglia/macrophages in and around lesions has been clinically associated with MS (Kuhlmann et al., 2008; Kuhlman et al., 2017), these cells are critical to study in MS. In MS lesions the inflammatory response overrides the tissue regeneration phase, resulting in significant tissue damage (Muzio et al., 2007). Microglia, able to showcase both pro-inflammatory and anti-inflammatory phenotypes, are seen as one of the key regulators of initiating and terminating inflammation (Muzio et al., 2007). Furthermore, a genomic map of MS has shown that

microglia carry a surplus of genes that are associated with an increased risk of MS (International Multiple Sclerosis Genetics Consortium, 2019). Investigating the complex roles of microglia in the context of MS is not only essential to gain a better understanding of the pathophysiology of MS, but also for the development of novel therapies. First, we will go over the beneficial effects. Then, the detrimental effects will be discussed.

7.1.1 Beneficial effects of microglia in MS

Even though the M1/M2 polarization has been disputed, an important takeaway from this dichotomy is that microglia can be pro-inflammatory and anti-inflammatory. Muzio et al. (2007) reported that microglia can secrete anti-inflammatory cytokines. They also reported that the pro-inflammatory cytokine interferon gamma (IFN-γ) can promote neuroprotective effects in low doses (Muzio et al., 2007). In addition to secretion of anti-inflammatory cytokines, microglia/macrophages can also phagocytose pro-inflammatory leukocytes. Wasser et al. (2020) reported that microglia/macrophages can engulf Th-17 cells in EAE mice, both *in vivo* as well as *ex vivo*, after which the Th-17 cells either escape or undergo cell death. This engulfment mechanism suppresses the immune response in EAE mice (Wasser et al., 2020). Moreover, microglia also exhibit a self-limiting mechanism to restrict the damage they cause while exerting a pro-inflammatory phenotype. Takeuchi et al. (2006) found that microglia can undergo apoptosis by upregulating pro-apoptotic genes when exposed to IFN-γ to limit the damage they can cause.

Microglia/macrophages can also phagocytose other potentially harmful molecules, such as oxidized phospholipids (Dong & Yong, 2022). Oxidation of phospholipids occurs under oxidative stress. During demyelination, myelin sheaths are destroyed, leaving myelin debris at the lesion site. Myelin debris can be oxidized due to the oxidative stress present in these lesions. The detrimental effects of oxidative stress will also be highlighted in the following sections. Oxidized phospholipids kill neurons and oligodendrocytes *in vitro*, which drives neurodegeneration and demyelination, exacerbating MS symptoms. When oxidized phospholipids were injected *in vivo*, microglia were shown to phagocytose the phospholipids, ameliorating the disease (Dong et al., 2021). In addition to phagocytosing oxidized phospholipids, microglia can also clean up myelin debris. Phagocytosis of myelin debris is essential, as this debris inhibits remyelination and the differentiation of oligodendrocyte progenitor cells (OPCs) (Kotter et al., 2006; Loyd & Miron, 2019; Miron et al., 2013). This highlights the importance of not always associating pro-inflammatory microglia - which are able to phagocytose myelin debris - with harmful microglia. Furthermore, microglia can secrete factors which stimulate the recruitment and differentiation of OPCs, directly stimulating remyelination (Lloyd & Miron, 2019; Miron et al., 2013).

Glutamate is an excitatory neurotransmitter which is abundant in the CNS. If its homeostasis is disrupted, glutamate can be very toxic. In MS, glutamate levels are significantly elevated in the CSF, indicating glutamate toxicity (Stover et al., 1997). The role of microglia/macrophages regarding glutamate has been debated for some time (Schwartz et al., 2003). Although microglia/macrophages are often activated when glutamate toxicity occurs, Schwartz et al. (2003) argue that this is not necessarily because microglia/macrophages are expressing this glutamate. Instead, they argue that it could be that microglia/macrophages are actually providing beneficial effects in presence of glutamate, but these are not strong enough to overcome glutamate toxicity.

7.1.2 Detrimental effects of microglia in MS

While microglia play pivotal roles in providing neuroprotection as mentioned above, their activation in MS can also lead to detrimental effects that exacerbate disease progression.

Gerritse et al. (1996) found that inhibition of CD40, a protein which is highly expressed in microglia/macrophages, inhibits EAE development *in vivo*, suggesting that microglia/macrophages are involved in the disease onset of EAE. Additionally, Heppner et al. (2005) found that microglial paralysis slowed down MS symptom onset and decreased symptom severity, indicating that microglia are contributing to the development and severity of EAE symptoms. These findings further demonstrate the harmful effects microglia can pose on EAE.

Microglia/macrophages can generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as NADPH oxidase, an enzyme that generates ROS. These free radicals are harmful to other cells, like oligodendrocytes (Fischer et al., 2012; Haider et al., 2011; Hickman et al., 2018; Ohl et al., 2015). Additionally, ROS can oxidize phospholipids in the CNS, which are abundantly present in membranes and myelin sheaths (Dong & Yong, 2022). Microglia/macrophages can also sustain the inflammatory response in MS lesions by releasing pro-inflammatory cytokines and chemokines, which attract leukocytes from the periphery (Heppner et al., 2005). These recruited leukocytes can induce more damage to neurons and oligodendrocytes (Hickman et al., 2018; Muzio et al., 2007). The pro-inflammatory cytokine IFN-γ activates microglia/macrophages into a detrimental phenotype that releases cytotoxic agents, such as nitric oxide and glutamate (Takeuchi et al., 2006). Another pro-inflammatory cytokine called tumor necrosis factor alpha (TNF-α) has been shown to be elevated in microglia, astrocytes, monocytes and T cells present in MS lesions, possibly exacerbating the inflammatory response (Klinkert et al., 1997). By secreting pro-inflammatory cytokines such as TNF-α, microglia induce a pro-inflammatory phenotype of astrocytes in MS (Liddelow et al., 2017). As will be covered in the next chapter, astrogliosis is detrimental to MS as it induces a lot of damage (Brambilla, 2019; Healy et al., 2022; Sofroniew & Vinters, 2010).

One could question how microglia are able to generate such harmful molecules without killing themselves in the process. Mitrovic et al. (1994) found that microglia, when compared to astrocytes and oligodendrocytes, are resistant to nitric oxide (NO), one of the RNS found in MS (Lan et al., 2018). They hypothesized that this is due to NO targeting Fe-S complexes, which microglial enzymes do not possess in their active centers (Mitrovic et al., 1994).

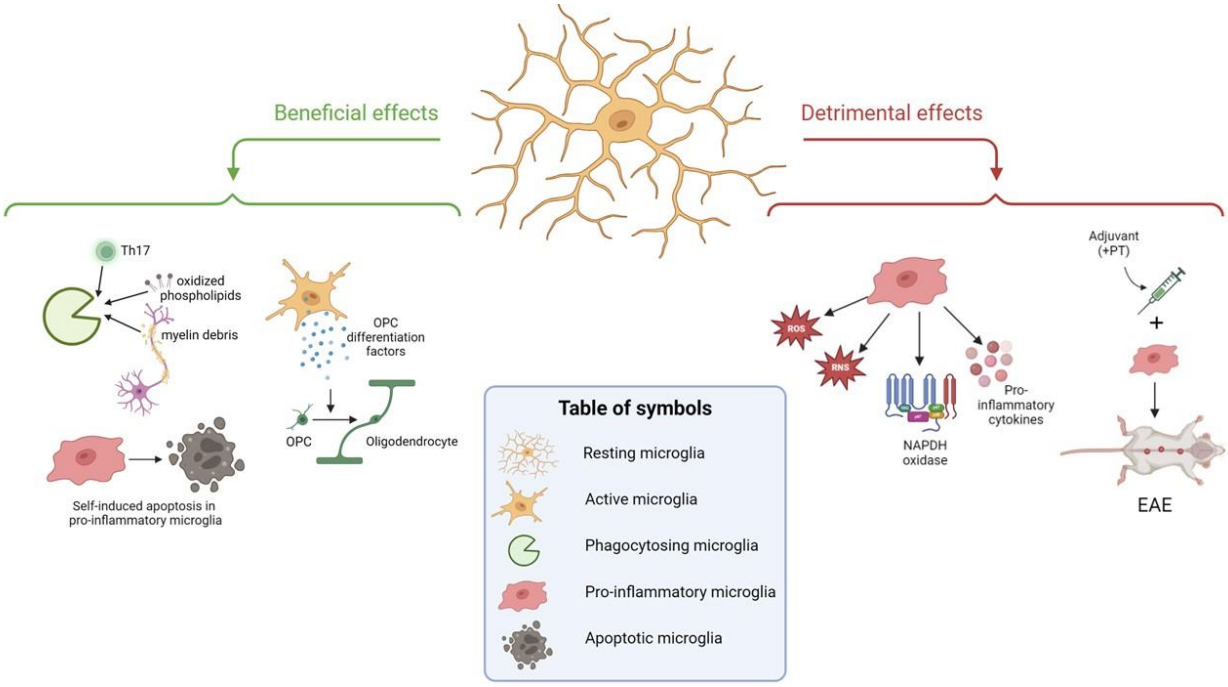

Figure 2. Overview of the roles of microglia in MS. On the left-hand side, the beneficial effects of microglia in MS are shown. These include the phagocytosis of pro-inflammatory cells and neurotoxic compounds, as well as the secretion of OPC differentiation factors and the induction of apoptosis in microglia with a pro-inflammatory phenotype. On the right-hand side, detrimental effects of microglia in MS are shown. These include the release of reactive species and pro-inflammatory cytokines, as well as playing a key role in the induction of EAE in mice. In the table of symbols, an overview of the different symbols used for microglia is shown. Figure created by using BioRender.com.

7.2 Astrocytes

Astrocytes are the most common cells in the CNS. Astrocytes can have a wide variety of functions, although specific populations of astrocytes in the CNS may have distinct roles.

In general, astrocytic function can be described as *multifaceted neuron support* (Ludwin et al., 2016). Astrocytes can support neurons in direct and indirect manners. Astrocytes can support neurons by providing trophic factors, such as glycogen and cholesterol (Brambilla, 2019; Brown & Ransom, 2007; Ludwin et al., 2016). Additionally, astrocytes regulate the permeability of the BBB (Qian et al., 2023). Astrocytes can also release vasodilating and vasoconstricting agents, regulating the blood flow in the CNS (Gordon et al., 2007; Ludwin et al., 2016). Moreover, astrocytes can also play a role in the formation of the extracellular matrix (ECM) (Ludwin et al., 2016). Astrocytes are also a key constituent of the BBB. Finally, astrocytes have immunomodulatory functions, such as the ability to secrete cytokines and chemokines (Brambilla, 2019; Kwon & Koh, 2020).

Unlike microglia, how astrocytes develop is poorly understood. It is thought that astrocytes develop from neural stem cells by transitioning from neurogenesis to gliogenesis. Then, astrocytes migrate across the CNS in poorly understood manners. It is thought that the astrocyte precursors mature further after migration has taken place (Molofsky & Deneen, 2015).

Astrocytes have traditionally also been separated into A1 and A2 astrocytes, with the A1 astrocytes being pro-inflammatory and A2 being anti-inflammatory (Brambila, 2019; Stephenson et

al., 2018). However, like microglia the A1/A2 dichotomy can be insufficient to properly describe the versatility of astrocytes. Therefore, a plea for steering away from a binary system in astrocyte nomenclature should be considered (Escartin et al., 2021).

Two terms that are also often mentioned in literature are 'reactive astrocytes' and 'reactive astrogliosis'. It is important to note that the terms 'reactive astrocytes' and 'reactive astrogliosis' are not unanimously agreed upon in neuroscientific literature (Sofroniew, 2009). In this literary study, the terms will be used to describe "*a sub-type of astrocytes that 'react' to CNS pathology, such as MS, with pro-inflammatory, anti-inflammatory, or mixed responses*" and "*a state of astrocytes which can be pro-inflammatory, anti-inflammatory, or mixed*" respectively.

Glial fibrillary acidic protein (GFAP) is a protein abundant in astrocytes and is therefore widely used as a marker for astrocytes (Escartin et al., 2021; Sofroniew & Vinters, 2010). GFAP is present in the branches of astrocytes, resulting in star-like stained cells (Ludwin et al., 2016). Elevated levels of GFAP are associated with response to injury and are commonly seen in CNS disorders such as MS (Escartin et al., 2021; Ludwin et al., 2016). However, it should be noted that elevated GFAP levels do not always correlate with elevated levels of reactive astrogliosis. For one, this can be due to different regions in the CNS having natural differences in levels of GFAP (Escartin et al., 2021; Sofroniew & Vinters, 2010).

Next to microglia, astrocytes have been recognized as a key player in EAE and MS. Because reactive astrocytes and increased levels of GFAP are observed throughout the CNS in MS, studying astrocytes in MS is crucial (Brambilla, 2019). Reactive astrocytes can exert pro-inflammatory as well as anti-inflammatory agents (Brambilla, 2019; Kwon & Koh, 2020; Sofroniew & Vinters, 2010), yielding both positive and negative effects in MS. We will begin by investigating the beneficial effects of astrocytes, followed by an analysis of their detrimental effects. A brief overview of the beneficial and detrimental effects of astrocytes in MS is shown in figure 3.

7.2.1 Beneficial effects of astrocytes in MS

In EAE lesions, reactive astrocytes are colocalized with TNF receptor 2 (TNFR2), a receptor predominantly associated with anti-inflammatory effects, cell survival and tissue regeneration (Brambilla et al., 2011; Dong et al., 2015). This suggests that reactive astrocytes are promoting anti-inflammatory pathways (Brambilla et al., 2011). Additionally, in cuprizone mice, it was found that astrocytes expressed a promyelinating factor responsible for the proliferation and differentiation of OPCs. By knocking out TNFR2 in cuprizone mice, Patel et al. (2012) demonstrated that the expression of this factor was due to activation of TNFR2 in astrocytes. In addition to anti-inflammatory effects mediated via TNFR2, it has also been found that astrocytes upregulate anti-oxidative enzymes in active MS lesions (Lassmann & van Horssen, 2016; van Horssen et al., 2008). Sanmarco et al. (2021a) found that a subset of astrocytes expresses anti-inflammatory factors, inducing apoptosis in infiltrating T cells in the CNS.

While reactive astrocytes are able to attract pro-inflammatory cells to the MS lesion, they can limit the spread of these pro-inflammatory cells to healthy CNS regions (Sofroniew & Vinters, 2010). One of the ways astrocytes limit this spread is by forming a glial scar, which is a severe outcome of reactive astrogliosis. The glial scar consists of not only glial cells but also other types of cells, and it is formed to shield the injured tissue from receiving more damage (Sofroniew, 2009;

Stichel & Müller, 1998). This further illustrates the idea of astrocytes promoting anti-inflammatory and neuroprotective effects in MS.

The role of astrocytes in the BBB in MS is complex. Bush et al. (1999) found that depleting the CNS of astrocytes significantly prevented healing of the BBB. Stimulating this process in MS could have therapeutic potential. With that said, the ability of astrocytes to repair the leaky BBB may depend on certain factors, such as the location in the CNS and the astrocytic phenotype (Sofroniew & Vinters, 2010). Moreover, one of the early signs of MS is BBB disruption as well as other harmful effects, which have been thoroughly covered in literature (Archie et al., 2021; Bush et al., 1999; Sofroniew & Vinters, 2010; Qian et al., 2023; van Doorn et al., 2012). The next section will elaborate further on the destructive effects of the BBB.

7.2.2 Detrimental effects of astrocytes in MS

While the reactions of astrocytes mentioned above seem to positively impact MS, their activation in MS can also lead to detrimental effects that exacerbate disease progression.

Similarly to microglia, reactive astrocytes have the potential to produce ROS and RNS which are neurotoxic molecules (Brambilla, 2019). As mentioned earlier, reactive astrocytes can also produce glutamate, which is neurotoxic when its homeostasis is dysregulated (Sofroniew & Vinters, 2010). A study found that a subpopulation of astrocytes had dysregulated glutamate and potassium ion transporters in postmortem brains of MS patients, which is thought to contribute to neurodegeneration in MS (Colón Ortiz & Eroglu, 2024). Astrocytes can also produce complement proteins, which elicit a strong immune response. This can cause collateral damage to surrounding tissue (Baines & Brodsky, 2017; Healy et al., 2022). Furthermore, reactive astrocytes can inhibit the regeneration of axons (Sofroniew & Vinters, 2010).

In MS lesions, reactive astrocytes have been shown to upregulate TACE/ADAM-17, which is an enzyme responsible for cleaving membrane-bound TNF-α, which has predominantly been associated with anti-inflammatory effects, into soluble TNF-α, which has predominantly been associated with pro-inflammatory effects (Brambilla, 2019). Moreover, it is thought that astrocytes have detrimental impacts on the BBB in CNS diseases such as MS (Archie et al., 2021; Bush et al., 1999; Sofroniew & Vinters, 2010; Qian et al., 2023; van Doorn et al., 2012). Multiple hypotheses surrounding the roles of astrocytes in the BBB have been proposed. One of these hypotheses is that astrocytic endfeet detach from the BBB in MS (Araya et al., 2008; Díaz-Castro et al., 2023). This creates a leaky BBB, resulting in increased inflammation and infiltration in the CNS (Sofroniew & Vinters, 2010). As mentioned earlier, astrocytes can create a glial scar, which is a severe outcome of reactive astrogliosis. Although the glial scar limits infiltration of pro-inflammatory cells from MS lesions into the healthy CNS, in EAE it has been found that the glial scar might contribute to damage more than it averts. In EAE mice, the glial scar is often formed with the help of astrocytes, but it often exacerbates the inflammatory response and worsens the condition of the mice (Sofroniew & Vinters, 2010) by inhibiting infiltration of oligodendrocytes into the lesion site, effectively blocking remyelination (Ludwin et al., 2016).

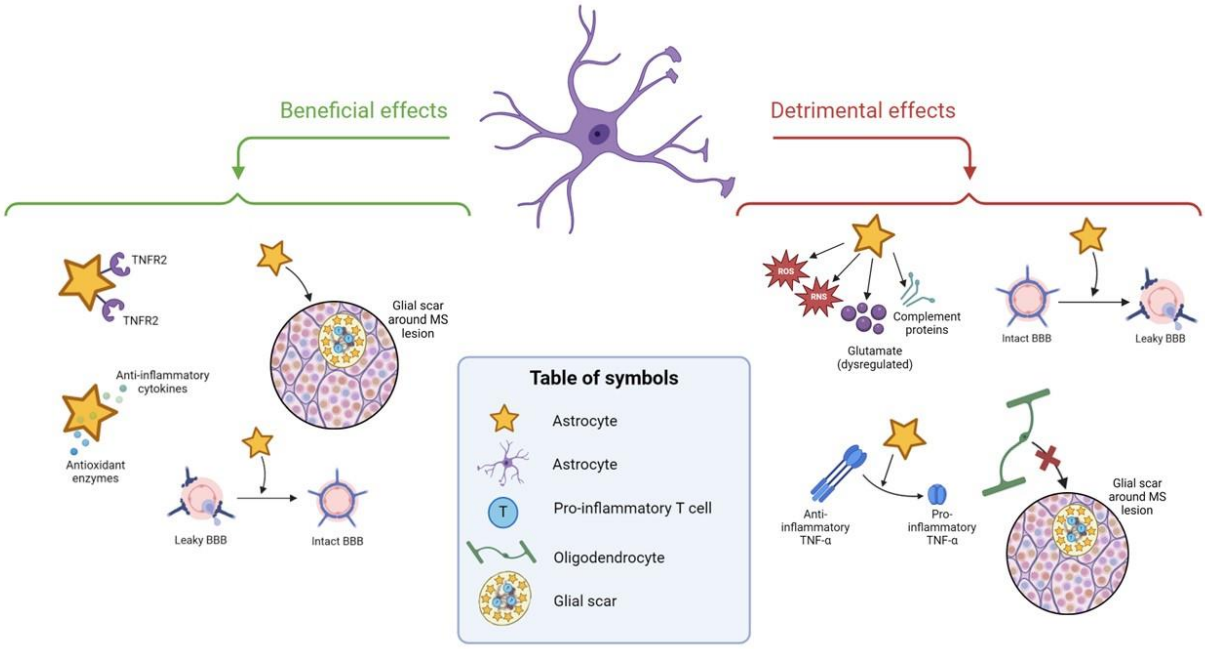

Figure 3. Overview of the roles of astrocytes in MS. On the left-hand side, the beneficial effects of astrocytes in MS are shown. These include the release of antioxidant enzymes and anti-inflammatory cytokines, as well as colocalized TNFR2 and GFAP expression and astrocytes possibly mediating the restoration of the BBB and the formation of a glial scar. On the right-hand side, detrimental effects of astrocytes in MS are shown. These include the release of reactive species and other harmful compounds, as well as preventing access of oligodendrocytes into the demyelinated area by forming a glial scar and astrocytes mediating pro-inflammatory effects such as the cleavage of membrane-bound TNF-α into soluble TNF-α and creating a leaky BBB. In the table of symbols, an overview of the different symbols used is shown. Figure created by using BioRender.com.

8 INTERACTIONS OF GLIAL CELLS IN MS

As will be discussed in this final chapter, interactions between glial cells can shape the disease outcome of MS. Oligodendrocytes, the cells responsible for myelination of axons in the CNS, and remyelination of demyelinated axons in MS also interact with microglia and astrocytes in multiple ways (Peferoen et al., 2014). In the next sections, interactions among microglia, oligodendrocytes and astrocytes will be discussed. An overview of these interactions is shown in figure 4.

8.1 CROSS-TALK BETWEEN MICROGLIA AND OLIGODENDROCYTES

Oligodendrocytes are the cells responsible for myelination in the CNS. This chapter will focus on interactions between microglia and oligodendrocytes due to the function of oligodendrocytes both in the healthy CNS and in MS. When demyelination occurs, oligodendrocytes get activated to remyelinate the unmyelinated axons. To do so, myelin debris must first be cleaned up. Oligodendrocytes benefit from microglial support in multiple ways. However, microglia can also severely damage them (Peferoen et al., 2014). This chapter will focus on the interactions between these two cell types.

Previously discussed studies mentioned that microglia are a key player in the clearing up of myelin debris. Additionally, microglia can secrete neurotrophic factors, such as IGF-1, which supports OPC maturation and myelination (Lloyd & Miron, 2019; Peferoen et al., 2014). Moreover, microglia can secrete factors that degrade molecules inhibiting OPC-differentiation and proliferation (Lloyd & Miron, 2019).

However, microglia can also negatively contribute to oligodendrocyte function. Oligodendrocytes respond to ROS, RNS and pro-inflammatory cytokines secreted by microglia/macrophages. These secreted factors can result in oligodendrocytes performing hypomyelination, a low-quality form of remyelination (Peferoen et al., 2014). Additionally, ROS and RNS can directly kill oligodendrocytes and damage myelin sheaths (Smith et al., 1999). As mentioned earlier, microglia can produce TNF-α, and astrocytes upregulate an enzyme which cleaves membrane-bound TNF-α into soluble TNF-α, the form more associated with pro-inflammatory TNFR1 binding (Brambilla, 2019; Klinkert et al., 1997). It is thought that activation of TNFR1 in oligodendrocytes contributes to apoptosis of oligodendrocytes (Peferoen et al., 2014). Furthermore, Selmaj & Raine (1988) showed that TNF-α can damage not only oligodendrocytes but myelin as well *in vitro*, possibly exacerbating MS.

As discussed in previous chapters, NO is a RNS that can induce damage in other cells, such as neurons, astrocytes and oligodendrocytes (Dong & Yong, 2022; Fischer et al., 2012; Haider et al., 2011; Hickman et al., 2018; Ohl et al., 2015). Microglia can excrete NO while being resistant to its toxicity (Mitrovic et al., 1994). Mitrovic et al. (1994) mention that NO can inhibit mitochondrial enzyme function, forcing cells to switch to glycolysis. They hypothesized that oligodendrocytes might suffer more from NO toxicity compared to astrocytes due to astrocytes having a greater ability of switching the metabolic profile, as one of their functions in the CNS is to provide glucose to neurons. Indeed, Almeida et al. (2001) found that astrocytes switch to glycolysis in the presence of NO, but neurons do not. In addition, Lan et al. (2018) confirm that NO in oligodendrocytes harms mitochondria, resulting in impaired metabolism of oligodendrocytes. Furthermore, Lan et al. (2018) describe how oligodendrocytes transfer lactate to neurons for axonal support. This could possibly mean that oligodendrocytes cannot generate enough ATP to sustain themselves when its

mitochondria are impaired. Altogether, NO produced in MS by microglia severely impacts oligodendrocytes and their ability to supply axons with lactate.

8.2 CROSS-TALK BETWEEN ASTROCYTES AND OLIGODENDROCYTES

Similar to microglia, astrocytes can interact with oligodendrocytes in multiple ways. Although these interactions have not been as extensively studied as microglia-oligodendrocyte interactions, astrocytic support can both support and destroy oligodendrocytes. This chapter will focus on the crosstalk between astrocytes and oligodendrocytes.

Molina-Gonzalez & Miron (2019) showed that increased GFAP expression reduced demyelination and neurodegeneration in cuprizone mice. Similarly, the authors showed that depletion of GFAP-producing reactive astrocytes inhibited remyelination. This could indicate that GFAP-producing reactive astrocytes provide beneficial factors to oligodendrocytes in cuprizone mice (Molina-Gonzalez & Miron., 2019). Indeed, Barnett & Linington (2013) describe the evidence of astrocytes being a source of differentiation factors for OPCs. In addition, Colón Ortiz & Eroglu (2024) recently showed that astrocytes provide OPCs with essential factors. Without astrocytes, OPCs cannot differentiate and mature into oligodendrocytes. In MS, astrocytes can be impaired, resulting in a deficiency in the release of these essential factors for OPCs (Colón Ortiz & Eroglu, 2024). This can delay or inhibit remyelination of axons in MS. In addition to playing a pivotal role in differentiation and maturation of OPCs, astrocytes also provide oligodendrocytes with lipids such as cholesterol, which are essential for the formation of myelin sheaths. In EAE mice, cholesterol synthesis decreased in astrocytes in certain regions of the CNS currently undergoing demyelination (Colón Ortiz & Eroglu, 2024).

Astrocytes and oligodendrocytes can also have physical contact with each other via gap junctions, allowing for direct communication between the two cells. Gap junctions consist of connexins, which are transmembrane proteins that assemble into gap junctions. It has been found that expression of connexins is altered in MS, possibly altering the cell-cell communication of astrocytes and oligodendrocytes (Markoullis et al., 2014; Colón Ortiz & Eroglu, 2024). Markoullis et al. (2014) hypothesized that it is likely that this altered cell-cell communication in MS is rather detrimental than beneficial, potentially contributing to MS progression.

8.3 CROSS-TALK BETWEEN ASTROCYTES AND MICROGLIA

Astrocytes and microglia can also interact with each other in several ways. In this section, cell-cell interactions of astrocytes and microglia will be discussed in terms of cytokine-receptor interactions, but also recruitment and phenotypic changes.

First, the phenotypic change will be discussed. Mainly A1 phenotypic astrocytes have been reported in MS lesions, which suggests that astrocytes promote neurotoxicity in MS lesions. In an experiment performed by Liddelow et al. (2017), mice lacking microglia failed to generate A1 astrocytes, in contrast to the control mice. Follow-up experiments showed that the presence of the cytokines IL-1α, TNF-α and complement protein C1q was sufficient to induce a phenotype in astrocytes almost identical to A1 astrocytes generated in the previous experiment (Liddelow et al., 2017). As mentioned earlier, TNF-α is released by microglia (Klinkert et al., 1997). Sanmarco et al. (2021b) found that TNF-α derived from microglia promotes glutamate release in astrocytes, which is neurotoxic. Indeed, Bezzi et al. (2001) discovered that this release is driven by CXCL12/SDF-1, a

chemokine abundantly present in astrocytes. This chemokine binds to CXCR4 receptors, which are present on astrocytes and microglia. CXCL12/SDF-1 binding to CXCR4 promotes glutamate release in astrocytes, but only in the presence of TNF-α (Bezzi et al., 2001). Moreover, Bezzi et al. (2001) found that TNF-α further exacerbates this pathway. The relevance of this signaling cascade lies in the fact that TNF-α is released by microglia in MS (Klinkert et al., 1997), as well as that CXCL12/SDF-1 levels in astrocytes are elevated in MS lesions (Calderon et al., 2006). These studies further highlight the importance of studying these cytokine-chemokine-receptor interactions in MS.

As discussed in previous chapters, the release of RNS by microglia impacts astrocytes. The release of NO by microglia can be toxic to astrocytes by decreasing the function of mitochondrial enzymes (Mitrovic et al., 1994). However, Mitrovic et al. (1994) show that unlike oligodendrocytes, astrocytic DNA is less prone to double stranded DNA breaks induced by NO, and therefore is more likely to survive NO-mediated oxidative stress.

However, cell-cell interactions in astrocytes and microglia can also exert anti-inflammatory effects. IL-10, an anti-inflammatory cytokine, is released by microglia. Astrocytes can respond to this cytokine in an anti-inflammatory way by inhibiting pro-inflammatory responses in microglia (Norden et al., 2014; Sanmarco et al., 2021b). Astrocytes can influence microglial behavior as well. For instance, astrocytes can secrete IL-33, a cytokine which is elevated in serum levels of MS patients. Although the role of IL-33 in MS is still unclear, it is evident that astrocyte-derived IL-33 promotes phagocytosis of synapses by microglia (Sanmarco et al., 2021b; Vainchtein et al., 2018).

Finally, the recruitment of microglia by astrocytes has been shown to contribute to disease progression as well as disease amelioration in MS animal models. In cuprizone mice, the interference of microglial recruitment to the site of demyelination by astrocytes delays phagocytosis of myelin debris, OPC proliferation and maturation and remyelination. This illustrates the importance of microglial recruitment by astrocytes, which is possibly facilitated by cell-cell crosstalk (Sen et al., 2022; Skripuletz et al., 2013). However, it has been found that in EAE mice, astrocytes produce increased levels of lactosyl ceramide. This glycosphingolipid can upregulate the production of a cytokine that recruits microglia/macrophages. It is hypothesized that this could contribute to MS disease progression (Kim et al., 2014; Mayo et al., 2014). Although these opposing effects could be attributed to the fact that different animal models were used, one could also argue that microglial recruitment both carries positive and negative effects in MS. The latter seems to be more likely, as the interactions of microglia in MS are complex, as has been highlighted in the first chapter.

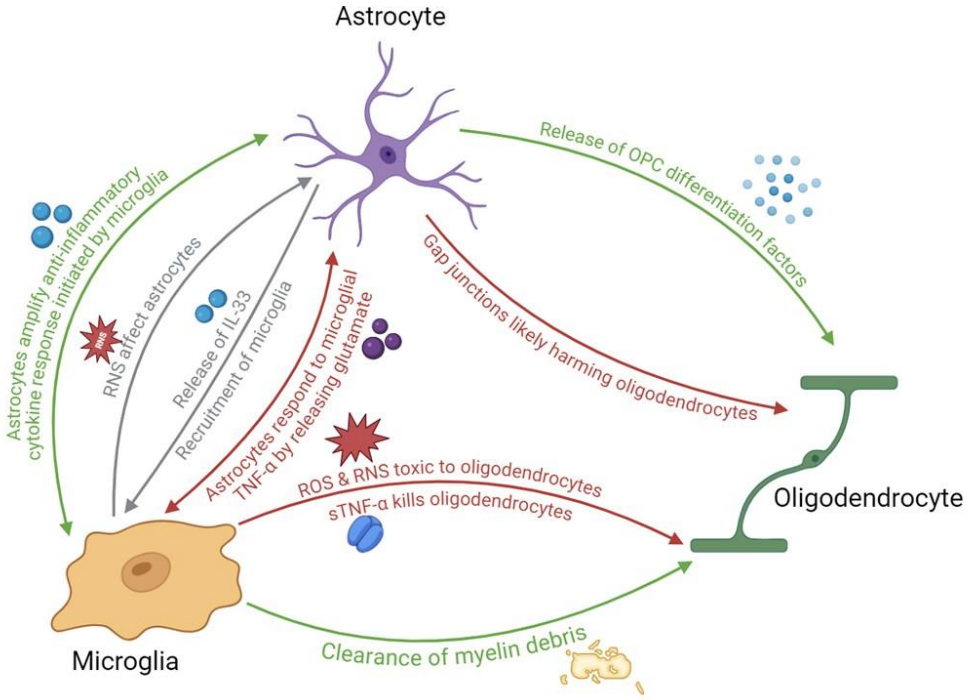

Figure 4. Overview of the interactions between astrocytes, oligodendrocytes or OPCs and microglia. In red, harmful interactions are shown. In green, advantageous interactions are shown. In gray, interactions of which the effects are either neutral, unknown or both beneficial and detrimental are shown. The direction of the arrow highlights which cell is benefited or harmed. Figure created by using BioRender.com.

9 DISCUSSION

The aim of this study was to investigate the behavior of microglia and astrocytes in MS lesions and their interactions with other glial cells, especially in the context of how this might affect MS disease progression. Recommendations for future research as well as potential therapeutics will be given.

In the first chapter, two of the most widely used MS models, EAE and cuprizone have been covered. Both models are deemed to be good models for MS, albeit with each model having its own limitations. This highlights the importance of carefully choosing which MS model will be chosen in an experiment. The increased levels of microglia and astrocytes in the models make both EAE and cuprizone accurate models to use for studying the roles of microglia and astrocytes in MS.

Microglia are involved in pro-inflammatory as well as anti-inflammatory pathways. Because of how microglia can undergo apoptosis when exposed to large amounts of pro-inflammatory cytokines, and because of the ability of microglia to phagocytose many different harmful molecules, the hypothesis presented in this thesis is that in general, microglia are more beneficial in MS than they cause harm. However, this might be lesion-dependent as more active lesions show more pro-inflammatory cytokine-secreting microglia and vice versa for inactive lesions (Guerrero & Sicotte, 2020). Additionally, microglia may be more harmful in certain stages of MS, and more beneficial in other stages (Hickman et al., 2018). Furthermore, different microglial phenotypes may also affect MS lesions in different ways. Finally, Heppner et al. (2005) showed that microglial paralysis inhibited the development of EAE, which indicates that microglia are responsible for the onset of the disease. However, in later stages of EAE, microglia exert anti-inflammatory effects. These studies highlight the importance of thoroughly investigating in which environments microglia express harmful phenotypes in MS to develop specific strategies and/or therapeutics that specifically inhibit these phenotypes or environments without wiping out the beneficial effects of microglia in MS. In addition to studying the specific phenotypes of microglia, this study highlights the importance of unassociating pro-inflammatory with harmful, as phagocytosis of myelin debris, a pro-inflammatory function of microglia, is essential for remyelination.

Based on the evidence found in the articles, astrocytes seem to be able to react to MS lesions in both beneficial and detrimental ways. However, several of the attempts of astrocytes to repair the injured CNS seem to harm the damaged tissue even more. For instance, while the glial scar is supposed to limit the spread of pro-inflammatory lymphocytes, it essentially also prevents oligodendrocytes from remyelinating the damaged axons, which could result in neurodegeneration and eventually lead to more inflammation. In addition, given the fact that astrocytes regulate the permeability of the BBB (Qian et al., 2023), and given that the permeability of the BBB in MS is leaky and disrupted (Archie et al., 2021), it seems that astrocytes, even if they pose a repair mechanism to injuries in the CNS, harm the BBB more than they can repair it in MS. Therefore, it can be concluded that in general, astrocytes might contribute more to disease progression than disease termination.

A hypothesis explaining the detrimental role of astrocytes in MS is that the astrocytic response, which evolved in healthy humans into the pro-inflammatory state, may become maladaptive in the context of MS. This could explain why astrocytes do not pose a threat in healthy individuals, while astrocytes have been linked to exacerbation of the disease in MS patients and MS models. Further research into the exact mechanisms of astrocytes in MS could highlight where astrocytes prove to be beneficial, and where these cells must be put to a halt with the help of therapeutics to prevent exacerbation of the disease. Healy et al. (2022) reported that dimethyl

fumarate can increase expression of anti-inflammatory antioxidants in astrocytes as well as upregulate a gene which is associated with reducing BBB leakage. More research is needed to extensively study the effects of dimethyl fumarate to investigate its potential as a therapeutic in MS.

Although microglia can help oligodendrocytes with the remyelinating of axons (Lloyd & Miron, 2019; Mitrovic et al., 1994; Peferoen et al., 2014), they can also severely interfere with this process. The exact reaction from microglia is probably affected by environmental factors and microglial phenotype. Similarly, astrocytes can affect oligodendrocytes in both remyelination-stimulating and remyelination-inhibiting manners (Barnett & Linington, 2013; Colón Ortiz & Eroglu, 2024 Markoullis et al., 2014; Molina-Gonzalez & Miron., 2019). Microglia-astrocyte crosstalk has been investigated more thoroughly in previous studies (Bezzi et al., 2001; Liddelow et al., 2017; Mitrovic et al., 1994; Norden et al., 2014; Sanmarco et al., 2021b; Sen et al., 2022). Altogether, these studies show that microglia and astrocytes can exert pro-inflammatory as well as anti-inflammatory effects on each other. The exact response elicited by the receiving cell likely depends on factors such as MS lesion type, phenotype of the messenger cell and phenotype of the receiver. The complex interactions of glial cells highlight the importance of further research in this area in the context of MS. This study did not account for different glial phenotypes, which might have resulted in an oversimplification of interactions. Nevertheless, this study provides a base for future research to dive into the exact mechanisms and interactions of specific glial phenotypes.

10 CONCLUSION

This study highlights more beneficial roles of microglia and more harmful roles of astrocytes in MS; however, this might be dependent on its phenotype and its environment. In addition, both microglia and astrocytes have also been associated with harmful responses to oligodendrocytes. These responses largely differ per glial phenotype, which should heavily be considered. Furthermore, microglia can induce harmful phenotypes in astrocytes, whereas astrocytes can amplify anti-inflammatory responses induced by microglia. Given this, it seems as if microglia have control over the response of astrocytes, but not vice versa. Therefore, microglia pose a therapeutic challenge to stimulate anti-inflammatory effects while not compromising on their beneficial pro-inflammatory properties such as phagocytosis of myelin debris. However, the roles of microglia and astrocytes and their interactions in MS remain complex, therefore a general statement whether these cells are beneficial or detrimental in MS cannot be made as the answer will depend on environmental factors and the phenotype of the glial cell. Further research into these specific environmental factors could expose the triggers for specific phenotypes in MS. Moreover, future research that focuses on methods to transform harmful microglial phenotypes into protective microglial phenotypes could provide alleviation of symptoms in MS lesions without sacrificing protective microglial effects.

The absence of phenotypic differentiation of microglia and astrocytes can be deemed a limitation of this literature study. Moreover, the absence of classifications of MS lesions might have been a limiting factor as well. Furthermore, the limitations of animal models for MS need to be considered, as the literature studied in this literature review heavily relies on these models. Future research should focus on filling the gaps of this literature review to gain a fuller understanding of the roles of microglia and astrocytes in MS.

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