UNIVERSITÀ DEGLI STUDI DI MILANO
PhD in Pharmacological Biomolecular Sciences, Experimental and Clinical
Department of Pharmacological and Biomolecular Sciences
XXXIV Cycle

BIO/14

PhD thesis

MICROGLIA-TO-OLIGODENDROCYTE PRECURSOR CELLS COMMUNICATION AFTER STROKE: MOLECULAR MECHANISMS AND IMPLICATIONS FOR MYELIN REPAIR

Candidate: **Stefano RAFFAELE**
Student number: **R12196**

Tutor: **Prof. Marta FUMAGALLI**

Coordinator: **Prof. Giuseppe Danilo NORATA**

Academic year: 2020/2021

INDEX

PREFACE

The present thesis is submitted to the doctorate school in Pharmacological and Biomolecular Sciences, Experimental and Clinical, of the University of Milan, Milan, Italy.

The experimental work was carried out at the Department for Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy, in the period from October 1st, 2018, to September 30th, 2021, under the supervision of prof. Marta Fumagalli and prof. Maria Pia Abbracchio. Part of the experiments were performed during a secondment period at the Department of Neurobiology, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark, in the period from March 19th to August 31st, 2021, thanks to the kind collaboration of prof. Kate Lykke Lambertsen and prof. Roberta Brambilla.

The PhD scholarship was fully funded by the University of Milan. Experiments were supported by Fondazione Cariplo, Italy (grant number 2015-0910), Università degli Studi di Milano (Piano di Sostegno alla Ricerca, 2015-2017), and by the Italian Ministry of University and Research-MIUR project "Dipartimenti di Eccellenza 2018-2022" to Department of Pharmacological and Biomolecular Sciences. The secondment period was also supported by a European Molecular Biology Organization (EMBO) Scientific Exchange Grant (grant number 9043).

Part of the results of this thesis are included in an original paper published in the journal *Molecular Therapy* (doi: 10.1016/j.ymthe.2020.12.009). The remaining data are included in two original papers currently in preparation for submission.

The thesis consists of three main parts. The first part is an introduction to stroke and current knowledge in the field of microglia-oligodendrocyte interaction during post-stroke remyelination and neuroinflammation. The second part describes the aims of the thesis and the results obtained. The last part includes a discussion of the results in relation to the current literature in the research field, put in the perspective of how future research may take the findings even closer to the development of new stroke treatments.

.

.

ABSTRACT (ENGLISH)

Ischemic stroke is a neurological disorder representing a leading cause of death and permanent disability world-wide, for which effective regenerative treatments are still missing. Oligodendrocyte degeneration and consequent myelin disruption are considered major contributing factors to stroke-associated neurological deficits. Therefore, fostering myelin reconstruction through the generation of new myelinating oligodendrocytes has emerged as a promising therapeutic approach. Of note, the subpopulation of oligodendrocyte precursor cells (OPCs) expressing the P2Y-like receptor GPR17 has been shown to actively react to ischemic damage by increasing their proliferation and migration toward the lesion. However, their spontaneous differentiation capability remains very limited, likely due to non-permissive local inflammatory environment, which is mainly sustained by brain-resident microglia and blood-borne macrophages. Accumulating evidence has indeed shown that, immediately after stroke, microglial cells exert beneficial functions by promoting OPC recruitment toward the ischemic lesion and preserving myelin integrity, but these protective features are lost during lesion progression, contributing to remyelination failure. Thus, new strategies restoring microglia pro-remyelinating properties and unveiling the underlying mechanisms might provide important opportunities for both reducing myelin damage and promoting its regeneration by OPCs. On this basis, the general aim of the present thesis was to investigate the molecular mechanisms involved in the communication between microglia and OPCs after stroke, as well as the consequent implications for myelin repair.

Given that secretion of extracellular vesicles (EVs) represents a key mechanism through which microglia influence the functions of neighboring cells, the first part of this study was aimed at understanding how exogenous microglia-derived EVs orchestrate the response of recipient microglia and OPCs following ischemic stroke. GPR17-iCreER^{T2}:CAG-eGFP reporter mice were employed to trace the fate of GPR17-expressing OPCs, labeled by the green fluorescent protein (GFP), after permanent middle cerebral artery occlusion (pMCAo). Infusion of exogenous regenerative microglia-derived EVs in the ipsilateral corpus callosum of mice at day 14 post-ischemia, corresponding to the late disease stage, restored protective microglia/macrophages functions, limiting their "senescence", and enhanced the maturation of the pool of GFP^{+} OPCs accumulating at lesion borders, thus

resulting in ameliorated neurological functionality. In vitro experiments showed a direct beneficial effect of pro-regenerative microglial EVs on OPC maturation, which is partly mediated by EV-carried transmembrane TNF. Moreover, gene expression profiling of OPCs exposed to microglial EVs shedded light on the early molecular changes responsible for enhanced maturation. Specifically, results highlighted that the protective action exerted by microglial EVs on recipient cells involves reprogramming of OPC energy metabolism, suggesting that manipulation of specific metabolic pathways in OPCs may provide novel therapeutic opportunities to promote remyelination in brain disorders.

In the second part of this thesis, we evaluated the role of TNFR2, a transmembrane receptor previously implicated in regulating the pro-regenerative functions of microglia, in shaping microglial response after stroke, and its impact on remyelination and post-stroke recovery. To address this goal, conditional microglia-specific TNFR2 knockout mice and wild-type littermates have been subjected to pMCAo, and the effects of microglial TNFR2 ablation on stroke-induced motor disability, microglia phenotype, and OPC maturation have been assessed. Results unveiled signs of impaired motor performance in microglial TNFR2 knockout mice, accompanied by reduced expression of pro-regenerative microglial genes and by a significant decrease in OPC response to ischemic damage. These data suggest a role of TNFR2 signaling in driving the post-stroke microglia protective functions necessary to create the permissive local environment required for efficient remyelination.

Altogether, the results of this thesis provide entirely new molecular mechanisms underpinning the beneficial interaction between microglia and OPCs, paving the way for developing new therapeutic strategies to promote myelin repair and functional recovery in neurodegenerative diseases.

ABSTRACT (ITALIANO)

L'ischemia cerebrale è un disturbo neurologico di origine cardiovascolare che rappresenta una delle principali cause di morte e disabilità permanente nel mondo, per il quale mancano terapie rigenerative efficaci. La degenerazione degli oligodendrociti e il conseguente deterioramento della guaina mielinica sono considerati tra i principali fattori che contribuiscono ai deficit neurologici associati a tale patologia. Pertanto, promuovere la riparazione della guaina mielinica attraverso la generazione di nuovi oligodendrociti mielinizzanti potrebbe rappresentare un approccio terapeutico promettente. A tale riguardo, è stato dimostrato che la sottopopolazione di precursori oligodendrocitari (OPC) esprimenti il recettore P2Y-simile GPR17 reagisce attivamente al danno ischemico aumentando il proprio tasso di proliferazione e migrando verso il sito di lesione. Tuttavia, il differenziamento spontaneo di queste cellule risulta molto limitato, probabilmente a causa dell'ambiente infiammatorio locale non permissivo, sostenuto principalmente dalla microglia residente nel sistema nervoso centrale e dai macrofagi provenienti dalla circolazione periferica. Prove crescenti mostrano infatti come le cellule microgliali inizialmente svolgano funzioni benefiche, promuovendo il reclutamento degli OPC verso la lesione ischemica e preservando l'integrità della mielina. Tuttavia, questi tratti protettivi vengono persi durante la progressione della lesione ischemica, contribuendo al fallimento del processo di rimielinizzazione. Pertanto, nuove strategie in grado di ripristinare le funzioni pro-rimielinizzanti della microglia e di elucidare i meccanismi alla base di tali proprietà benefiche potrebbero garantire nuove opportunità per ridurre il danno alla mielina e promuoverne la rigenerazione da parte degli OPC. Su queste basi, l'obiettivo generale di questa tesi è stato quello di indagare i meccanismi molecolari coinvolti nella comunicazione tra microglia e OPC dopo ischemia cerebrale, analizzando le conseguenti implicazioni per la riparazione della mielina.

Dato che il rilascio di vescicole extracellulari (EV) rappresenta un importante meccanismo attraverso il quale la microglia influenza le funzioni delle cellule circostanti, la prima parte di questo studio ha avuto lo scopo di valutare gli effetti esercitati da EV microgliali esogene sulla risposta della microglia ricevente e degli OPC presenti ai margini della lesione ischemica. L'utilizzo di topi reporter GPR17-iCreER^{T2}:CAG-eGFP ha permesso di tracciare, attraverso l'espressione della proteina fluorescente verde (GFP), il destino degli OPC

esprimenti il recettore GPR17 dopo l'occlusione permanente dell'arteria cerebrale media (pMCAO). L'infusione intracerebrale di EV esogene prodotte da microglia con fenotipo pro-rigenerativo a partire dal giorno 14 post-ischemia ha ripristinato le funzioni protettive di microglia e macrofagi presenti ai margini della lesione ischemica, limitandone la "senescenza", e favorito il differenziamento del pool di OPC GFP^+ a cellule mielinizzanti, con conseguente miglioramento della funzionalità neurologica. Ulteriori esperimenti in vitro hanno evidenziato come le EV prodotte da cellule microgliali con fenotipo pro-rigenerativo siano in grado di esercitare un effetto benefico diretto sulla maturazione dell'OPC, il quale è risultato essere in parte mediato dal TNF trasportato sulla transmembrana delle EV. Inoltre, un'analisi trascrittomica ha permesso di fare luce sui primi cambiamenti molecolari indotti negli OPC dall'esposizione a EV microgliali e responsabili del loro effetto pro-differenziante. Nello specifico, i risultati ottenuti hanno evidenziato come l'azione protettiva esercitata dalle EV microgliali sulle cellule riceventi comporti la riprogrammazione del metabolismo energetico degli OPC, suggerendo che la manipolazione di specifiche vie metaboliche negli OPC potrebbe aprire nuove prospettive terapeutiche per promuovere la rimielinizzazione in condizioni neuropatologiche.

Nella seconda parte di questa tesi, è stato invece valutato il ruolo di TNFR2, un recettore transmembrana precedentemente implicato nella regolazione delle funzioni pro-rigenerative della microglia, nella regolazione della risposta microgliale dopo ischemia cerebrale, insieme al conseguente impatto sulla rimielinizzazione e sul recupero funzionale post-ischemico. Per raggiungere questo obiettivo, topi knockout microglia-specifici per TNFR2 e animali wild-type di controllo sono stati sottoposti a pMCAO con lo scopo di valutare gli effetti dell'ablazione di TNFR2 microgliale sulla disfunzione motoria post-ischemica, sul fenotipo della microglia e sulla maturazione dell'OPC. I risultati hanno rivelato segni di alterazione delle prestazioni motorie nei topi knockout per il TNFR2 microgliale, accompagnati da una ridotta espressione di geni associati a funzioni pro-rigenerative della microglia e da una significativa diminuzione della risposta degli OPC al danno ischemico. Questi dati suggeriscono un possibile ruolo di TNFR2 nel guidare le funzioni protettive della microglia dopo ischemia cerebrale, necessarie alla creazione di un ambiente locale permissivo che consenta un'efficiente rimielinizzazione.

Complessivamente, i risultati inclusi nella presente tesi forniscono nuovi meccanismi molecolari alla base dell'interazione benefica tra microglia e OPC, aprendo la strada allo sviluppo di nuove strategie terapeutiche in grado di promuovere la riparazione della mielina e il recupero funzionale in condizioni neurodegenerative.

1. INTRODUCTION

1.1 Ischemic stroke

1.1.1 The pathophysiology of ischemic stroke

Ischemic stroke, also referred to as cerebral ischemia, is a neurological disorder caused by the interruption of the blood supply to a given region of the brain, mainly due to occlusion of an afferent artery by an embolus or local thrombosis (Figure 1.1). It represents the second cause of death in Western countries, and is the leading reason for long-term disability, generating a consistent social and economic burden (Campbell et al., 2019; Virani et al., 2021).

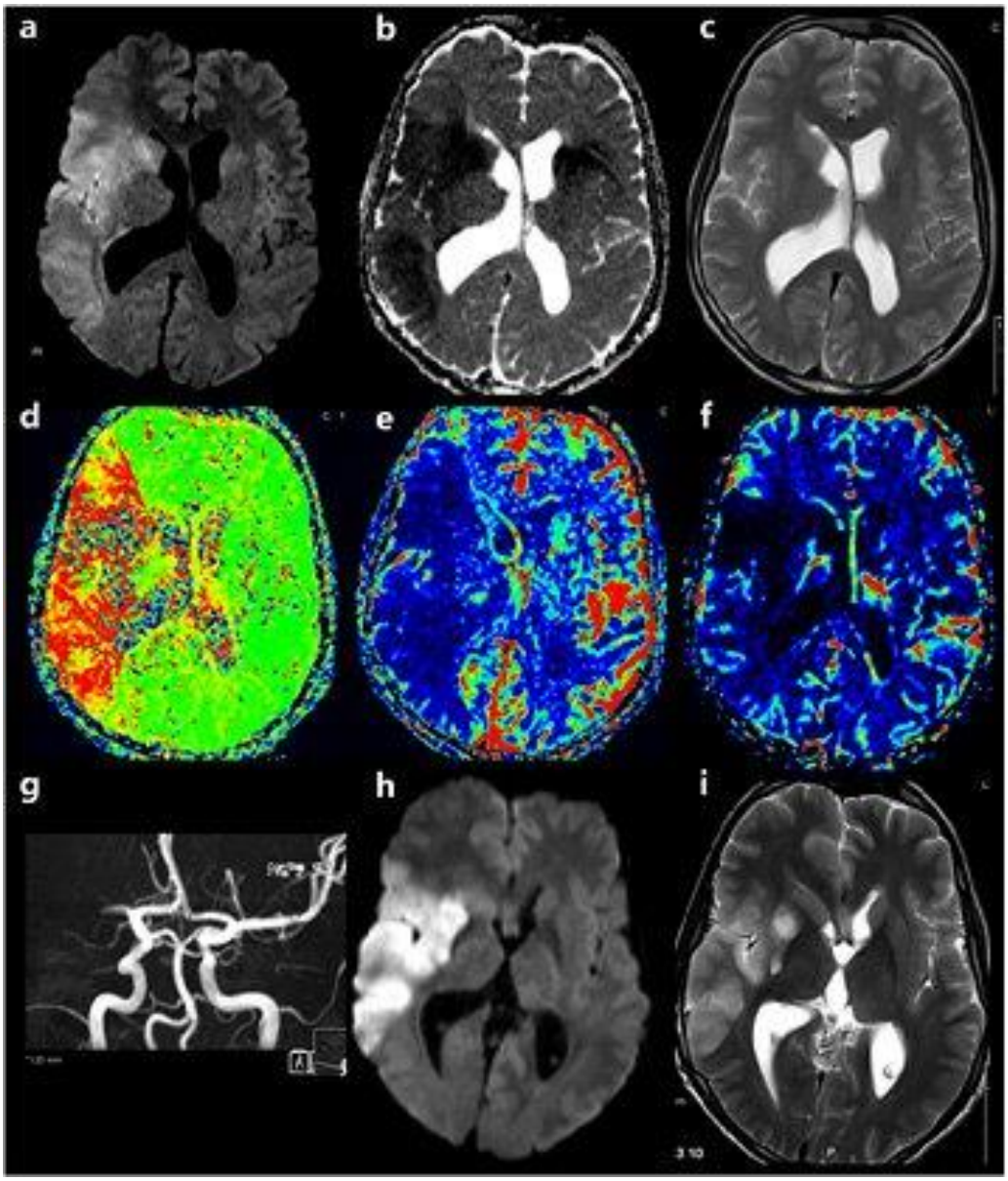

Figure 1.1 Magnetic resonance imaging (MRI) pictures showing a case of human ischemic stroke in the right brain hemisphere due to middle cerebral artery (MCA) occlusion*. (a-c) MRI images showing the area of acute ischemia as a direct bright signal and the restricted water diffusion as a low signal. (d-f) MRI-processed images showing*

the ischemic area characterized by low cerebral blood flow (red area surrounded by green normally perfused tissue), accompanied by diminished cerebral blood volume (seen as dark blue signal in the corresponding heat map). (g) Image obtained using the time-of-flight MR-Angiography technique, showing the occlusion of the MCA. (h-i) MRI images showing the ultimate extent of the brain infarction 24 hours after the acute event. Image adapted from (El-Koussy et al., 2014).

In the brain region affected by a stroke, the reduction of oxygen and nutrient supply causes a strong metabolic stress, progressively leading to necrosis of neurons and glial cells, including astrocytes and oligodendrocytes (OLs). This pathological process is generally known as ischemic damage (Barthels and Das, 2020). The extent and duration of the ischemic event determine the severity of the injury, which may involve not only the brain area where the primary damage occurs, but possibly also the surrounding tissue. In fact, if the vascular flow is not promptly restored, an increase in capillary permeability is observed with consequent formation of edema. The development of cerebral edema plays an important role in the evolution of ischemic damage, as the liquids leaking from the circulatory bed compress the brain tissue and capillaries, inducing a further reduction in local blood flow, with a worsening of the ischemic condition (Liebeskind et al., 2019).

The clinical picture following cerebral ischemia displays a high variability in relation to the cause, duration, location, and severity of the event; it is also necessary to evaluate the contribution of age, ethnicity, and any comorbidities (Hankey, 2017). An important factor to consider is the spontaneous reperfusion of the affected tissue, which can occur in the first hours after the ischemic injury and is documented in 17% of cases (Kassem-Moussa and Graffagnino, 2002). The extent of brain damage resulting from a focal ischemic event also depends on the presence of a collateral capillary circulation capable of ensuring a partial blood supply in the ischemic area (Ginsberg, 2018).

The symptoms caused by ischemic stroke are variable and characterized by the temporary or permanent loss of certain brain functions, depending on the extent and location of the injured area. The most common symptoms are insensitivity or unilateral paralysis, confusion, aphasia, and loss of muscular strength, balance, and coordination. In addition to acute neurological symptoms attributable to the affected brain area, consequences not directly related to the damaged region may also emerge, such as cognitive impairment and neuropsychiatric disorders (Makin et al., 2013; Robinson and Jorge, 2016).

The therapeutic options currently available for stroke treatment are limited to thrombolytic strategies, either surgical or pharmacological, aimed at restoring blood perfusion in the

injured brain area. These treatments, together with improved neurointensive care practices, display significant efficacy in reducing death rate in patients (Campbell et al., 2019). However, the temporal window in which they can be used is very narrow, being restricted to the first few hours after stroke occurrence. In addition, they are effective in limiting early disease progression, but have no impact on the repair of ischemic lesions, leaving a significant portion of patients with lifelong motor and cognitive disability. On this basis, the development of regenerative strategies able to restore proper brain functions, limiting stroke-induced functional deficits, is an urgent and still unmet medical need (Barthels and Das, 2020).

An important component of stroke pathogenesis is brain damage evolution, that can be explained by the concept of *penumbra*, which is the viable tissue around the ischemic core (Figure 1.2). A reduction in blood flow below 15-20% of the basal level indeed leads in a few minutes to the formation of an irreversibly damaged lesion *core*, characterized by neuronal depolarization and necrosis. On the contrary, the blood flow in the tissue surrounding the ischemic *core* is reduced to a lesser extent, compromising neuronal functions even if the structural integrity is maintained. If proper blood flow is not restored, this tissue is bound to become part of the necrotic area. Attention has been focused on this region, called *penumbra*, because its degeneration can potentially be prevented, until the functionality is fully recovered (Yang and Liu, 2021). In animal models of cerebral ischemia, the extension of the *penumbra* can be measured with various non-invasive techniques and is therefore known (it is about 80% of the infarcted area), while in humans no precise data are available (Yang and Liu, 2021). From a clinical point of view, this uncertainty represents an important limitation, since it is precisely towards this area, where the cells are still viable, that the neuroprotective pharmacological strategies are targeted. Without an appropriate and timely pharmacological treatment, the *penumbra* undergoes a progressive cell death, induced by the release of numerous mediators and transmitters triggering an inflammatory and cytotoxic process (Yang and Liu, 2021).

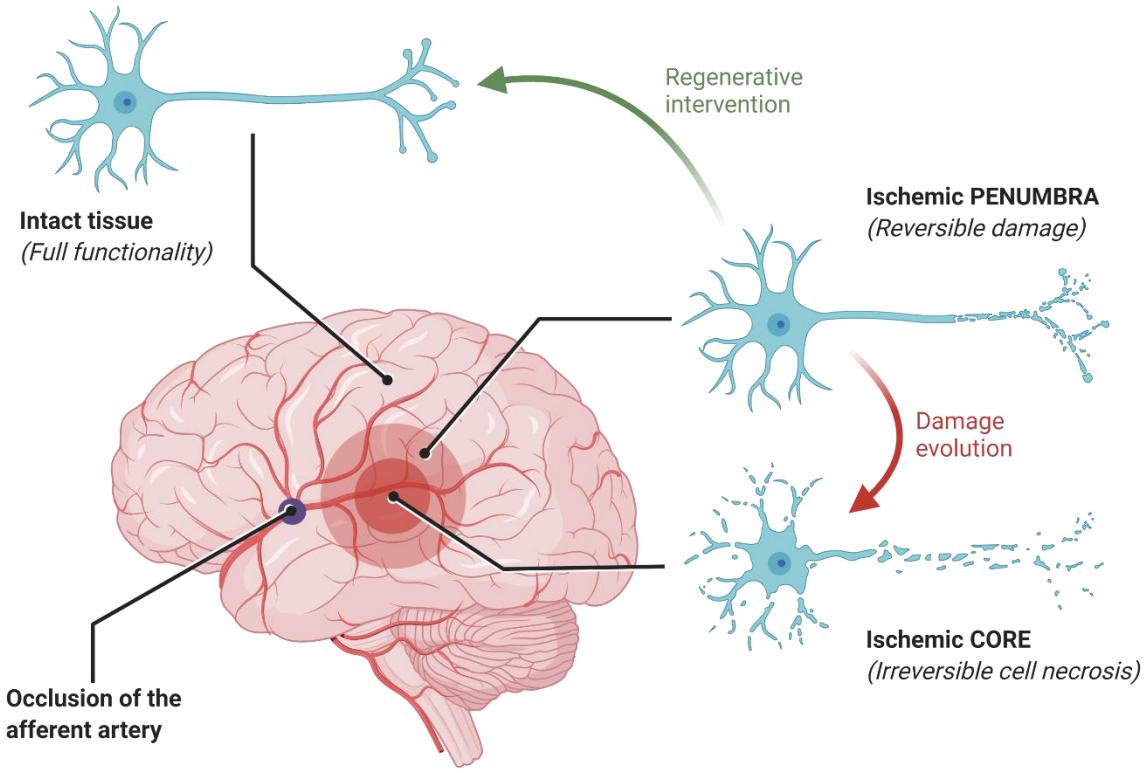

***Figure 1.2 The different stages of ischemic damage evolution**. Cerebral ischemic lesions are characterized by a core of irreversibly injured necrotic tissue, surrounded by a region called ischemic penumbra, in which cellular functionality is only partially compromised. The ischemic penumbra is committed to progressively become part of the ischemic core; however, timely therapeutic interventions could restore the full functionality of this area by promoting cell survival and tissue repair. Figure created with BioRender.com*

1.1.2 Mechanisms of stoke-induced cytotoxicity

The global energy demand of the brain is comparable to that of other metabolically active organs, such as the liver and the kidney. However, the central nervous system (CNS) is characterized by low abundance of energy reserves, such as glucose, glycogen, and ATP, and mostly relies on aerobic glycolytic metabolism, that is strictly dependent on oxygen and glucose supply from the bloodstream, to produce energetic substrates (Iadecola and Anrather, 2011).

During an ischemic event, the lack of glucose and oxygen due to reduced blood perfusion causes the blockade of aerobic metabolism, with consequent total and sudden depletion of ATP reserves. The immediate effect is the loss of functionality of the ATP-dependent membrane ion pumps, causing uncontrolled cell depolarization that initiates the necrotic process in the ischemic *core* (Iadecola and Anrather, 2011). This phenomenon, called anoxic depolarization, is followed by cellular edema, due to the osmotic water influx inside the cell, leading to cell lysis. Cellular depolarization caused by the increase in intracellular

cations (especially Na^+) is also transmitted to the neurons of the neighboring areas, triggering the massive release of excitatory neurotransmitters, such as glutamate and aspartate, from the synaptic terminals in toxic concentrations (Lai et al., 2014). Moreover, during the ischemic event, the activity of glutamate reuptake transporters expressed by glial cells is also impaired, further contributing to excitotoxicity (Lai et al., 2014). Ultimately, an accumulation of intracellular calcium cations (Ca^{2+}) occurs, due to voltage-gated channels (VOC) opened by membrane depolarization, glutamate-activated ionotropic receptors, and inositol triphosphate (IP3)-mediated release from intracellular deposits (Lai et al., 2014). The increased cytosolic levels of Ca^{2+} that occurs following ischemic injury trigger several processes involved in the so called 'secondary damage', including the activation of nucleases and proteases, and the production of reactive oxygen species (ROS) and inflammatory mediators (Mifsud et al., 2014).

1.1.3 Demyelination and neuroinflammation following ischemic injury

The ischemic pathology affects not only neurons, but also OLs, the glial cells responsible to form the myelin sheath in the CNS (paragraph 1.2). These cells have indeed some characteristics that make them even more vulnerable to hypoxia than other glial cells and neurons (Kuhn et al., 2019). Following cerebral ischemia, OLs are rapidly and severely damaged through many pathways such as the hyperactivation of glutamate and purinergic receptors, oxidative stress, and the impairment of mitochondrial function. These events lead to demyelination, namely the disruption of the myelin sheath, leaving axons denuded and prone to degeneration for the severe effects on their function, transport, structure, metabolism, and integrity. Therefore, clinically, demyelination strongly contributes to the functional deficits associated with stroke (Mifsud et al., 2014).

Recent studies also focused on the dualism of the inflammatory process which follows the ischemic event. Indeed, inflammation has been shown to contribute to the neurological outcome after stroke both negatively, sustaining a detrimental process which promotes ischemic secondary damage, and positively, limiting brain damage and supporting regenerative processes (Jayaraj et al., 2019; Lambertsen et al., 2019). Post-ischemic inflammation is initially triggered by the generation of ROS and other factors, hence called damage-associated molecular patterns (DAMPs), by necrotic cells (Anrather and Iadecola,

2016). Once released, these inflammation promoters lead to the activation of microglia, the brain-resident innate immune cells (paragraph 1.3) (Fumagalli et al., 2015b). Subsequently, microglia facilitate the recruitment of circulating immune cells within the lesion, by producing several chemokines, inducing chemotaxis of the circulating cells in the ischemic area, and pro-inflammatory cytokines which in turn lead to the induction of adhesion molecules in the cerebral blood vessels, mediating the adhesion of leukocytes to the vascular endothelium (Iadecola and Anrather, 2011). Following these early events, recruited immune cells release several cytotoxic agents, such as matrix metalloproteases (MMPs), nitric oxide (NO) and ROS. These molecules further contribute to damaging brain cells and cause the disruption of the extracellular matrix and the leakage of the blood brain barrier (BBB). Some of these responses can be harmful since they facilitate cell death, but others are beneficial for the ischemic area, as immune cells are also capable of secreting neurotrophic factors and removing necrotic debris, allowing the reorganization of the local environment for subsequent repair (Kawabori and Yenari, 2015).

Based on the results described in this paragraph, demyelination and neuroinflammation emerge as key mechanisms contributing to the progression of the ischemic brain damage and neurological disability. Therefore, promising therapeutic approaches should be aimed at promoting myelin regeneration and modulating the neuroinflammatory process. Novel strategies able to dampen the detrimental role of immune cells and to restore their beneficial functions, may support post-stroke tissue repair and functional recovery.

1.2 Oligodendrocyte lineage cells and post-stroke repair

1.2.1 The process of oligodendrogenesis

During an ischemic event, the lack of oxygen leads to OL death followed by demyelination (Jia et al., 2019). Since myelin, the lipid structure that enwraps axons, is essential for the conduction of the electrical impulse and for the trophic support of neurons, its loss contributes significantly to long-term sensory-motor and cognitive deficits (Shi et al., 2015). OL formation, termed oligodendrogenesis, is a late developmental process that occurs only after the generation of neurons and astrocytes (Waly et al., 2014). Within the developing forebrain, the entire population of OLs is generated by several waves of proliferation and migration of OL precursor cells (OPCs). Recent evidence indicated that OPCs, generated

during different waves, are capable of myelinating distinct regions of the brain, suggesting the existence of different functional subpopulations of progenitors, which can perform different functions (Tripathi et al., 2011). Indeed, although the myelin sheath is thought to consist of a homogeneous population of OLs (Kessaris et al., 2006), these cells were originally described as morphologically heterogeneous (Pérez-Cerdá et al., 2015). It is not yet clear whether OLs morphologically diversify during maturation, through interactions with the local environment, or whether intrinsic functional heterogeneity exists (Bechler et al., 2015; Tomassy and Fossati, 2014; Tripathi et al., 2011). To shed light on these questions, a recent single-cell RNA sequencing study analyzed the transcriptome of OLs isolated from 10 different regions of the antero-posterior and dorsal-ventral axis of the CNS of young and adult mice. This analysis led to the identification of 12 different subpopulations, distributed at different stages of maturation from OPCs to myelinating OLs (Marques et al., 2016). Interestingly, the initial differentiation of OLs appears to be homogeneous within the CNS, while increasing heterogeneity appears in the postnatal phases and in a region-specific manner (Marques et al., 2016). Although some OL subpopulations are widely present in all brain regions, others are restricted only to specific areas. Certain OL populations may represent transition states between different subsets or may have specific functions in youth and then disappear in adulthood (Marques et al., 2016). It therefore remains to be verified whether the different OL populations perform distinct functions within the brain (Foerster et al., 2019). In this respect, a recent work identified two functionally distinct populations of OPCs in the zebrafish spinal cord. One subset, retained near neuronal somas and dendrites, has been found to establish elaborate process networks and to exhibit remarkably high calcium activity, being apparently involved in the modulation of neuronal transmission. These cells rarely differentiate into mature OLs but retain the capacity to divide in an activity- and calcium-dependent manner, producing a second OPC subpopulation characterized by higher motility, capable of migrating toward axon-rich regions and differentiate into myelinating OLs (Marisca et al., 2020).

During development, OPCs are generated in confined regions, and, thanks to their high migratory capacity, they can populate the brain and spinal cord to generate mature OLs and to myelinate the entire CNS during the postnatal period. Furthermore, a small percentage (5-9%) of OPCs generated during development and distributed in all brain

regions are maintained in a slowly proliferating 'quiescent' stage up to adulthood (Dawson et al., 2003). After birth and in the adult brain, OPCs are also continuously produced in the subventricular zone (SVZ) from neural stem cells (NSCs) (Menn et al., 2006). Due to the combination of slow proliferation and new generation in the SVZ, OPC density in the adult brain is maintained relatively constant.

The maturation of OPCs into mature myelinating OLs involves a series of intermediate stages characterized by the expression of specific proteins and changes in cell morphology (Figure 1.3) (Elbaz and Popko, 2019; Emery, 2010).

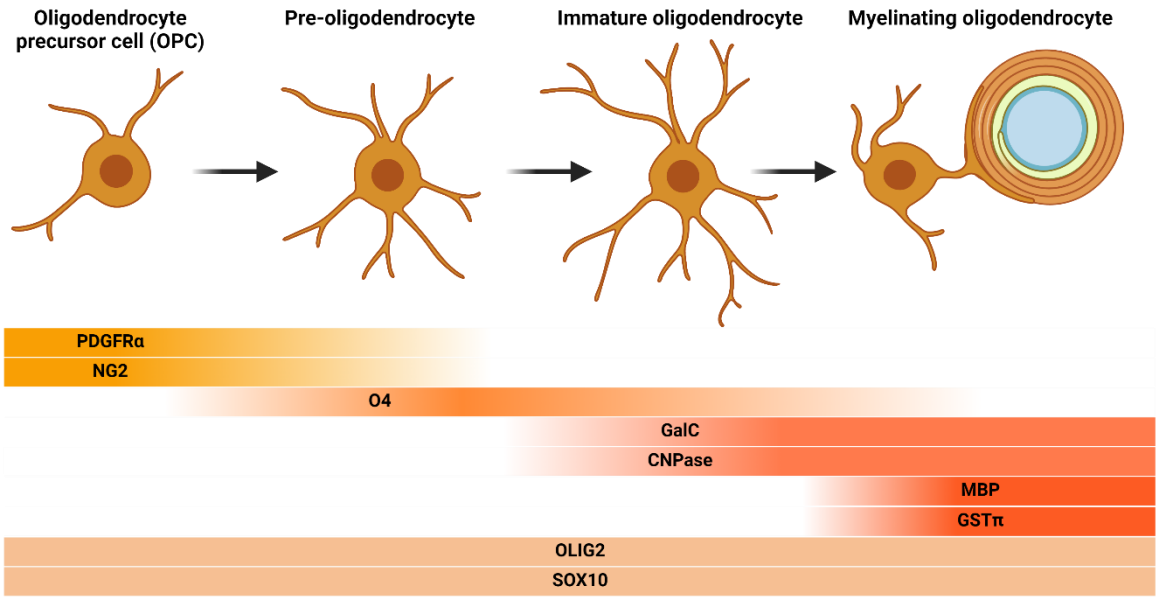

Figure 1.3 The multi-step program of oligodendrocyte differentiation. *Schematic illustration of the progressive morphological and molecular rearrangements that occur during the differentiation of oligodendrocyte precursor cells (OPCs) into myelinating oligodendrocytes (OLs). Specific markers of the different stages of OL differentiation are reported in the bottom part of the figure. Figure created with BioRender.com*

Initially, OPCs exhibit a bipolar morphology and are characterized by the expression of typical markers like the platelet-derived growth factor receptor alpha (PDGFRα), which is the most potent mitogen and survival factor for OPCs (Pringle et al., 1992). Other markers expressed in this stage are the NG2 proteoglycan, the DM-20 isoform of the proteolipid protein (PLP) and the gangliosides A2B5 and GD3 (Baumann and Pham-Dinh, 2001).

Subsequently, OPCs differentiate into pre-OLs, assuming a more branched morphology. At this stage of differentiation, the pre-OLs are characterized by the expression of the cell surface marker O4, loose the mitogenic response to PDGF and have less migratory capacity

(Sommer and Schachner, 1981). Then, the pre-OLs become immature OLs, post-mitotic cells with long ramified branches, that begin to express the galactocerebroside C (GalC) and the first myelin proteins, such as the cytoplasmic enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Scherer et al., 1994). Fully mature OLs are finally characterized by the production of myelin proteins, which include myelin basic protein (MBP), expressed on the cytoplasmic surface of the plasma membrane, the long transmembrane isoform of PLP, myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) (Barateiro and Fernandes, 2014). MBP and MAG appear first between postnatal day 5 and 7 in mouse CNS OLs, while MOG appears one or two days later (Kuhn et al., 2019). Widely used markers of mature OLs also include the cytosolic isoenzyme glutathione S-transferase pi (GSTπ) (Kuhn et al., 2019).

Other important markers are Olig1 and Olig2, genetically related but functionally different transcription factors present throughout the development and maturation process of OLs (Zhou and Anderson, 2002; Zhou et al., 2000). Olig2 is essential for the specification of neural precursors in OPCs, and its overexpression was demonstrated to increase OPC migration rate and differentiation capability, leading to precocious developmental myelination and enhanced remyelination of demyelinating lesions (Wegener et al., 2015). The role of Olig1, on the other hand, is less clear: it has a minor role in the differentiation of OPCs and has a greater impact on their terminal maturation in fully myelinating OLs; however, spinal cord OPCs are less dependent on this transcription factor than their brain counterparts (Dai et al., 2015; Xin et al., 2005). Other transcription factors involved in OL maturation are SOX10, essential for the differentiation of neural precursors toward the OL lineage during development (Claus Stolt et al., 2002; Pozniak et al., 2010; Stolt et al., 2004), and Nkx2.2, which promotes and regulates the timing of OL differentiation (Qi et al., 2001; Zhu et al., 2014).

It is important to mention that OL differentiation involves not only progressive transcriptomic and morphological changes, but also a profound bioenergetic rearrangement (Rosko et al., 2019). Indeed, OPCs were shown to mostly rely on mitochondrial oxidative phosphorylation to produce the high amount of ATP necessary to sustain their high proliferation rate, while, during differentiation, OLs shift into a primarily glycolytic metabolism, favoring the anabolic processes involved in the synthesis of

macromolecules. Accordingly, glycolysis has been associated with reduced production of ROS and increased synthesis of fatty acids and cholesterol required for myelin maintenance and turnover (Rao et al., 2017; Rosko et al., 2019). Even though mature OLs are characterized by enhanced anabolic requirements to support myelin synthesis and axonal ensheathment, they still retain the capacity of responding to situations of metabolic stress, like those consequents to cerebral ischemia, by temporarily retrieving an oxidative metabolism, sacrificing myelination to sustain their own survival through more efficient ATP production (Rao et al., 2017). On this basis, the manipulation of specific metabolic pathways might restore the proper myelinating capacity of OLs in disease conditions and increase the intrinsic predisposition of OPCs to differentiate and myelinate.

The maturation of OPCs into mature myelinating cells is an essential process, during development as well as in adults, both in physiological conditions and in pathological situations. In fact, white matter lesions are observed in a considerable variety of CNS pathologies such as demyelinating diseases like multiple sclerosis (MS), stroke, trauma to the brain or spinal cord, and neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Ogata, 2019). It is therefore important to identify new therapeutic strategies capable of stimulating remyelination by inducing an increase in the number of myelinating OLs and by allowing a remyelination supportive microenvironment (Franklin and Ffrench-Constant, 2017), (see also paragraph 1.2.3.).

1.2.2 Mechanisms of myelin formation and injury

The myelination process is an example of dynamic specialization of the plasmatic membrane, involving cell-cell and cell-extracellular matrix interactions. Myelin is indeed a biological membranous structure, rich in proteins and lipids, that enwraps neuronal axons allowing fast saltatory conduction of the action potential, mechanical protection of axonal integrity, and exchange of trophic substrates from OLs to neurons (Nave and Werner, 2014). The importance of proper myelin sheath formation is illustrated by the detrimental consequences of its absence or loss in pathological conditions such as cerebral ischemia or MS. In addition, increasing evidence associates myelination with neuronal plasticity and learning, suggesting that neuro-psychiatric disorders and age-dependent cognitive decline

are also related to the dysregulation of myelin homeostasis (McKenzie et al., 2014; Yeung et al., 2014).

Most of the available knowledge regarding the ultrastructure of the myelin sheath is based on electron microscopy studies. Myelin is a multilayer of uniformly thick membranes with a characteristic periodic structure of alternating electron-dense and light layers, respectively called major dense line and intraperiod line: the first is formed by the cytoplasmic surfaces of the OL processes, while the second is constituted by the superposition of the outer surfaces of OL membranes (Aggarwal et al., 2011; Salzer and Zalc, 2016). Compact myelin provides the high electrical resistance and low capacity that are essential for the propagation of the electric impulse. Furthermore, the myelin sheath is not continuous but, at regular intervals, displays interruptions rich in Na^+ channels, called nodes of Ranvier, in which the action potential is regenerated. The impulse conduction is therefore not continuous, but jumps from one node to the following, and is thus defined as saltatory conduction (Monje, 2018). Most OLs generate between 20 and 60 myelinating processes with internodal lengths of approximately 20-200 μm and up to 100 membrane turns (Chong et al., 2012).

Recent evidence indicates that the myelin sheath is more than an inert insulating membrane, being rich of cytoplasmic channels and membrane transporters involved in the transfer of short chain energy metabolites, such as pyruvate and lactate, from OLs to neurons. These substrates are then metabolized and contribute to the neuronal synthesis of ATP (Philips and Rothstein, 2017). It is therefore evident that, if myelin loses its normal structure, the lack of these energy substrates within the neurons could cause axonal degeneration (Simons and Nave, 2016).

The molecular composition of the myelin sheath is unique in its kind. Myelin is a poorly hydrated structure, which contains only 40% water compared to the 80% of the gray matter. It contains 70-80% lipids by dry weight and there is only a small group of proteins that reside within the compact structure of myelin, among which the most abundant are MBP and PLP (Simons and Nave, 2016). This imbalance towards lipids is necessary to perform the function of electrical insulator and to ensure the saltatory propagation of the nerve impulse.

Another important feature of myelin is its extraordinary stability, due to the lipid composition which includes high levels of long-chain saturated fatty acids, glycosphingolipids (about 20% of the total lipids) and cholesterol (about 40% of the total lipids). The Van der Waals dispersion forces, which are generated by the interaction between the methylene groups of these long and saturated hydrocarbon chains, constitute the main forces that allow the structural maintenance of myelin (Simons and Nave, 2016).

The process of myelination occurs in three consecutive phases: differentiation of OLs (process characterized by the formation of highly branched processes, described in paragraph 1.2.1), establishment and stabilization of OL-axon interactions with consequent formation of the primary winding, and finally formation of the mature and compact myelin sheath.

To ensure the correct formation of the myelin sheath, OLs undergo prominent morphological changes mediated by the cytoskeleton, which is composed of polarized polymers called microtubules (MT) and microfilaments (MF). The MFs are located immediately below the plasma membrane and mediate the initial phases of cellular process leakage, while the MTs confer greater mechanical stability to the process itself. Furthermore, both MTs and MFs are needed for the transport of cell organelles, vesicles, proteins, and mRNA granules. In addition to the cytoskeleton, an important modification is the formation of membrane microdomains called lipid rafts, which anchor the MTs to the growth zone, with the aim of mediating the transport of myelin proteins to the growing myelin sheet, when the contact between the prolonging process and the axon occurred (Fitzner et al., 2006; Schafer et al., 2004; Vinson et al., 2003). The last phase of the myelination process is the compaction of the myelin sheath, which involves the extrusion of the cytoplasm and the formation of both intracellular and extracellular interconnections by specialized proteins such as PLP (Bauer et al., 2009).

Post-mortem analyses carried out on brain tissue samples from stroke patients revealed that the disruption of white matter architecture is one of the main characteristic signs of cerebral ischemia (Marin and Carmichael, 2018). This is partly due to the high susceptibility of OLs to oxidative stress, excitotoxic damage and inflammatory cytokines that often accompany trauma, injury, or infection. The intrinsic functions of OLs contribute to their high sensitivity to damage conditions: these cells display the highest metabolic activity among

all brain cells, to produce and maintain the incredible volume of their membranes (up to 100 times the weight from the cell) which will concentrically envelop axons. Moreover, these cells are paradoxically poor in glutathione, which is one of the main antioxidant molecules. Therefore, OLs are predisposed to cell death under conditions of metabolic stress (Waly et al., 2014).

OLs are also particularly susceptible to excitotoxicity (Benarroch, 2009). Indeed, glutamate plays an important role in pathologies affecting the white matter, for example its concentration is increased in the plaques and white matter of MS patients (Srinivasan et al., 2005; Werner et al., 2001). In low energy conditions, like those of the ischemic area, the reversal of amino acid transporters (EAAT) can lead to the release of glutamate, contributing to glutamate-induced cytotoxicity and, ultimately, OL apoptosis (Benarroch, 2009).

This susceptibility of OLs explains, at least in part, their involvement in many CNS lesions and pathologies that are therefore characterized by demyelination. As previously mentioned in paragraph 1.1.2, cerebral ischemia causes cell death induced by hypoxia-related cascades. In accordance with the susceptibility mechanism of OLs, it is understandable that these cells are very vulnerable to ischemia. The swelling and vacuolation of the OLs appear within 3 hours after middle cerebral artery occlusion (MCAo), followed by process retraction and cell death within 24 hours (McIver et al., 2010; Pantoni, 2006).

1.2.3 Remyelination: the regeneration of myelin lesions

In the 1970s, numerous studies performed on autoptic samples from MS patients showed that demyelination can be followed by a spontaneous repair process of the myelin sheath called remyelination, namely the formation of new myelin sheath around demyelinated axons (Patrikios et al., 2006). Accordingly, *in vivo* studies performed using rodent models of cerebral ischemia have shown that, few days after stroke, the number of OLs in the areas surrounding the ischemic lesion is increased, suggesting that demyelinated axons could be remyelinated by immature cells, which respond to demyelination by differentiating into myelinating OLs (Mifsud et al., 2014).

The main steps of the remyelination process have been well defined (Figure 1.4). In response to damage, remyelination begins with the transition of OPCs in the vicinity of the lesion from a quiescent state to a regenerative phenotype (Moyon et al., 2015). This allows the progenitors to populate and expand within the injured area through a combination of proliferation and migration; finally, they undergo differentiation, a process that culminates in the formation of the new myelin sheath (Tripathi et al., 2010; Zawadzka et al., 2010).

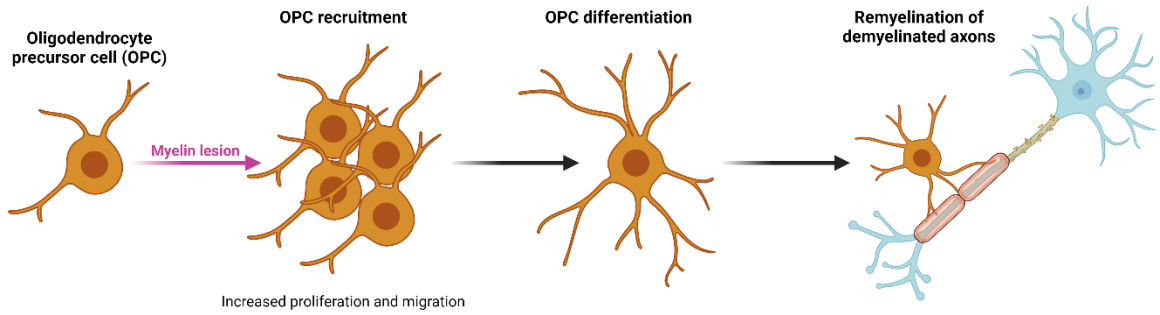

Figure 1.4 The phases of OPC response during remyelination. *Oligodendrocyte precursor cells (OPCs), that are present in a quiescent state within the adult brain parenchyma, respond to myelin injury by increasing their proliferation rate and migratory capacity to accumulate at the site of demyelination. Following recruitment, OPCs start to differentiate in the attempt to replace degenerating oligodendrocytes (OLs) with newly formed myelinating cells, and to reconstitute the functional myelin sheath around denuded axons. Figure created with BioRender.com*

This activation involves not only morphological changes (Levine and Reynolds, 1999; Reynolds et al., 2002), but also the upregulation of several genes, many of which are associated with the generation of OLs during development, such as those encoding the transcription factors Olig2, Nkx2.2, MYT1 and SOX2 (Franklin and Ffrench-Constant, 2008). In this context, it is possible that OPCs are activated by acute damage-induced modifications in microglia and astrocytes, two cell types that are very sensitive to changes in tissue homeostasis (Glezer et al., 2006; Rhodes et al., 2006), and not necessarily from primary demyelination (Nielsen et al., 2006). Microglia and astrocytes, activated by myelin and neuronal injury, are in fact the main source of factors that induce the rapid proliferative response of OPCs and migration toward the demyelinating lesion (Franklin and Ffrench-Constant, 2008). This response is modulated by the endogenous levels of the cell cycle regulatory protein p27Kip1 (Crockett et al., 2005) and is promoted by the growth factors PDGF and fibroblast growth factor (FGF) (Murtie et al., 2005), in addition to other factors associated with inflammatory lesions which have mitogenic effects on OPCs (Franklin and Ffrench-Constant, 2008).

During developmental myelination, there is a clear relationship between the thickness (and length) of the myelin sheath and the diameter of the axon. Instead, when remyelination occurs, the newly formed myelin is thinner and shorter than that generated during development (Franklin and Ffrench-Constant, 2017). Although remodeling of the new myelin internodes occurs, the original size is achieved only by fibers with a small diameter (Powers et al., 2013). On this basis, it is evident that remyelination has a neuroprotective role, as it limits the axonal degeneration that follows demyelination. However, it has been proven that the efficiency of this process progressively decreases with advancing age or in certain pathological conditions (Gruchot et al., 2019; Skaper, 2019). This decline in remyelinating abilities may be due to a defect in the recruitment of OPCs or in their differentiation capability. The second appears to be the more complex of the two processes, so it is the one that is most likely to be inefficient for the lack of pro-differentiating factors and/or presence of inhibiting cues (Waly et al., 2014). This suggests that not only OL-intrinsic regulators, but also the surrounding microenvironment plays a fundamental role in the success of remyelination. In this respect, growing evidence indicate that the concerted action of different cell types (i.e., astrocytes, microglia, peripheral immune cells, and pericytes) is critical for remyelination efficiency, due to the simultaneous modulation of several mechanisms, including the clearance of debris from the lesioned area, the remodeling of the extracellular matrix (ECM) and scar formation, the release of trophic factors, and the exchange of metabolic substrates (De La Fuente et al., 2017; Lloyd and Miron, 2019; Rawji et al., 2020a; Tanabe and Yamashita, 2020).

Promoting remyelination after trauma, cerebral ischemia or in demyelinating diseases, can be a promising therapeutic strategy to improve functional recovery (Plemel et al., 2014). In the context of a classical demyelinating condition such as MS, the main therapy currently employed in the early disease stages is the administration of immunosuppressants to counteract the inflammatory and immune-mediated components of this disease (Comi et al., 2017). However, in the progressive phase of MS, characterized by the constant increase in irreversible disability, this treatment is very far from being optimal (Rovaris et al., 2006). The failure observed in the use of immunosuppressants in patients with progressive MS suggests that, once the cascade of events leading to neuronal and axonal loss has been established, even effective management of inflammation does not protect against the

progression of the disease. Stimulation of undifferentiated OPCs, present in areas of chronic demyelination in MS patients, could instead lead to the repair of demyelinated lesions, restoring axonal protection (Fumagalli et al., 2016). Similarly, regarding cerebral ischemia, it is currently recognized that an ideal therapeutic intervention to decrease stroke-associated disability should include neuroprotective and neuroregenerative approaches fostering local spontaneous repair mechanisms (Fumagalli et al., 2016). Promoting endogenous myelin repair has therefore emerged as a new therapeutic approach for this pathology. Indeed, the discovery that parenchymal OPCs are recruited and proliferate in the ischemic penumbra suggests the possibility of repairing ischemic lesions by implementing endogenous spontaneous remyelination mediated by these cells (Zhang et al., 2013). This evidence indicates that the manipulation of OPCs could be a promising therapeutic strategy to enhance the endogenous mechanisms of remyelination and repair (Fumagalli et al., 2016).

1.2.4 The subpopulation of oligodendrocyte lineage cells expressing the GPR17 receptor

Remyelination represents an important process to block axonal degeneration and restore neuronal function. However, in pathological conditions such as cerebral ischemia and MS, this spontaneous reparative attempt often fails, leaving the axons devoid of myelin and making them vulnerable to possible damage, thus contributing to the progression of the disease (Franklin and Ffrench-Constant, 2008). In this respect, OPCs that persist in the adult brain and spinal cord are very important for therapeutic purposes. These cells, that in physiological conditions are normally quiescent, under specific circumstances can be stimulated to differentiate and generate newly formed mature myelinating OLs. These findings paved the way for innovative therapeutic opportunities, aimed at restoring axonal integrity by promoting efficient OL differentiation and myelination (Franklin and Ffrench-Constant, 2017; Fumagalli et al., 2016).

Recently, the balance between inhibitory and activating factors that regulate myelin formation, its plasticity, and the mutual interactions between myelinating glial cells and axons has been object of extensive studies, recently reviewed in (Saab and Nave, 2017). Among the variety of signals involved in oligodendrogenesis, ATP emerges as an important signaling molecule profoundly influencing both OPCs and mature OLs, and functional

purinergic receptors are found to be expressed on these cells. Of note, the expression of some of them appear to be restricted to specific stages of differentiation, suggesting that there may be a coordinated interplay between different receptors in regulating OPC maturation and myelination. The expression of several purinergic receptors was also found to be altered in demyelinating conditions, suggesting that their dysregulation may contribute to disease progression and that some of them may represent novel targets for remyelinating therapies (Fumagalli et al., 2016).

In this respect, the metabotropic Gi-coupled receptor GPR17 is of particular interest, since its role in regulating OL differentiation and response to CNS damage has been extensively investigated (Lecca et al., 2020). GPR17 has high structural similarity with his cognate P2Y receptors (Abbracchio et al., 2006) and was found to respond to purine nucleotides, like uridine diphosphate (UDP), UDP-glucose, and UDP-galactose, at concentrations in the micromolar range (Benned-Jensen and Rosenkilde, 2010; Ciana et al., 2006; Daniele et al., 2014; Lecca et al., 2008). GPR17 is also activated by cysteinyl-leukotrienes (CysLTs, such as LTC4, LTD4 and LTE4), inflammatory mediators that are not chemically or structurally related to nucleotides (Ciana et al., 2006). In addition, it has been reported that GPR17 can be activated by distress signals such as oxysterols, like other related receptors involved in inflammatory responses (Sensi et al., 2014).

After its initial characterization in recombinant systems, it has been shown that GPR17 is widely expressed in the CNS, particularly in OPCs (Boda et al., 2011; Chen et al., 2009; Fumagalli et al., 2011; Lecca et al., 2008), leading to an increasing number of studies aimed at understanding its possible role in CNS remyelination. *In vitro* studies carried out on purified rat postnatal OPC cultures showed that the expression of GPR17 is restricted to a specific time window of the OL differentiation process (Figure 1.5). Indeed, the receptor begins to be expressed in $NG2^+$ and $PDGFR\alpha^+$ bipolar early precursors, reaches a peak of expression in immature OLs co-expressing the marker O4, but is then progressively downregulated in mature OLs that begin to express myelin proteins such as MBP (Fumagalli et al., 2011). In particular, the downregulation of GPR17 in the terminal stages of differentiation represents a fundamental step to allow the achievement of the myelinating OL stage (Fumagalli et al., 2015a).

Based on this peculiar expression, GPR17 is now accepted as a new marker of the intermediate stage of OL differentiation (Crociara et al., 2013; Mitew et al., 2014; Nakatani et al., 2013). In agreement with these data, *in vivo* studies have shown that transgenic mice over-expressing GPR17 under the CNPase promoter, a marker of an advanced stage of OL maturation, display defective myelination, motor disabilities, tremors, and early death within the second week of life, probably due to the dysregulation of OL terminal maturation (Chen et al., 2009). Therefore, any spatial-temporal alteration of the normal expression kinetic of GPR17 seem to be able to completely impair the OL differentiation program. This hypothesis is in line with data showing that, in conditions in which the terminal maturation of OPCs is compromised, such as in demyelinating diseases, GPR17 expression is significantly upregulated (Bonfanti et al., 2020; Fumagalli et al., 2015a; Tyler et al., 2011).

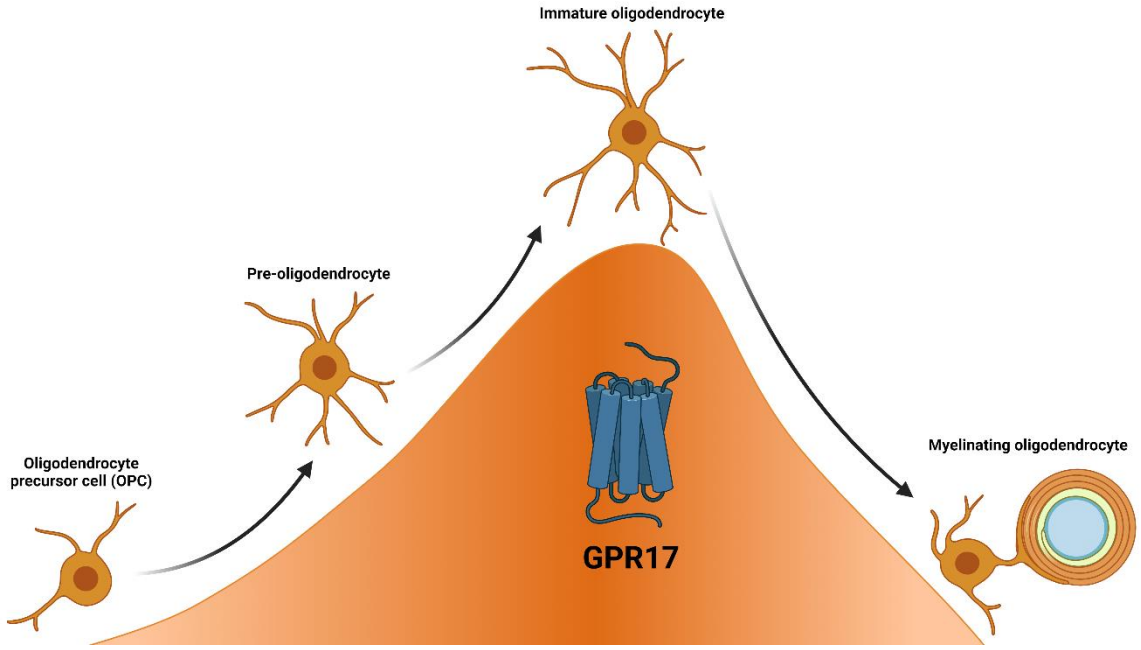

Figure 1.5 Kinetic of GPR17 expression during OPC differentiation. *Schematic illustration showing the transient GPR17 receptor expression during OPC differentiation. The peak of GPR17 expression corresponds to the immature oligodendrocyte stage, then GPR17 has to be downregulated to allow the complete terminal maturation into myelinating cells* (Fumagalli et al., 2011, 2015a). *Figure created with BioRender.com*

In addition to its role in regulating the OL differentiation, GPR17 has been also proposed to represent a sensor of damage in different pathological conditions (Lecca et al., 2008). At the basis of this hypothesis is the fact that large quantities of GPR17 endogenous ligands, namely nucleotides and CysLTs, are released from activated immune cells or escape from damaged or dead cells in response to CNS injury (Gelosa et al., 2017; Lecca and Ceruti,

2008). Therefore, both nucleotides and CysLTs are likely to activate GPR17 under these conditions.

Very early pathological GPR17 upregulation has been reported in degenerating OPCs inside demyelinating lesions, which has been linked to the activation of apoptotic processes within these cells (Ou et al., 2016). Accordingly, both genetic overexpression and pharmacological activation of GPR17 in primary OLs induced upregulation of pro-apoptotic genes and downregulation of pathways involved in cell survival (Ou et al., 2016), in line with previous findings showing a link between GPR17 expression and reduced OPC survival in response to pathologically increased extracellular ATP levels (Ceruti et al., 2011). Besides its contribution in regulating OL survival, GPR17 has been shown to play a pivotal role in tissue remodeling after chronic injury. Indeed, emergency molecules are released upon CNS injury and stimulate GPR17 expression to resume quiescent OPCs differentiation (Lecca et al., 2020). However, aberrant GPR17 upregulation has been reported in several experimental models of neurological disorders characterized by myelin disruption, such as brain ischemia (Ciana et al., 2006; Lecca et al., 2008), toxin-induced demyelination (Coppolino et al., 2018; Nyamoya et al., 2019), traumatic brain injury and Alzheimer's disease (Boda et al., 2011), the experimental autoimmune encephalomyelitis (EAE) model of MS (Chen et al., 2009; Coppolino et al., 2018), and the SOD1G93A murine model of ALS (Bonfanti et al., 2020). Notably, significant GPR17 upregulation has been also found in patients affected by MS (Angelini et al., 2021; Chen et al., 2009), traumatic brain injury (Franke et al., 2013), and congenital leukoencephalopathy (Satoh et al., 2017), thus confirming the results obtained in experimental models.

These data suggest that, when damage occurs, repair signaling molecules acting as emergency signals are released to stimulate GPR17 expression and initiate OPC differentiation and remyelination. However, if the production of these extracellular signals is prolonged over time due to severe or chronic injury, their stimulatory activity result in pathological GPR17 overexpression, which prevents its physiological downregulation required for OL maturation to myelinating phenotypes (Lecca et al., 2020). Accordingly, under all the neurodegenerative paradigms described above, GPR17-expressing OPCs do invariably show morphological features typical of immature phenotypes, suggesting that these cells are blocked at pre-myelinating stages (Fumagalli et al., 2015a). Consequently,

these cells are no longer able to remyelinate and are then committed to apoptosis (Fumagalli et al., 2017).

From these data, the GPR17 receptor assumes a pivotal importance in the remyelination process, triggering the initial reactivity of OPCs to damage. However, the prolonged upregulation of GPR17 which accompanies disease conditions represents an obstacle on the hard path toward remyelination. To identify new therapeutic strategies, it is therefore important to further study the pool of GPR17-expressing OPCs, to implement its remyelinating capabilities. To investigate the identity and fate of these cells in the postnatal and adult brain, a transgenic reporter mouse line has been generated, expressing the Cre-recombinase enzyme fused with the estrogen receptor (iCreER^{T2}) under the control of the GPR17 promoter (Viganò et al., 2016). This line was then crossed with the murine CAG-enhanced green fluorescent protein (eGFP) reporter line (Nakamura et al., 2006) to visualize, following the administration of tamoxifen, the cells expressing GPR17 at the very moment of induction thanks to the expression of GFP (Viganò et al., 2016). Experiments carried out on this transgenic line, named GPR17-iCreER^{T2}:CAG-eGFP, showed the lack of co-localization of GFP with the microglial marker Iba1 and with the astrocyte marker glial fibrillary acidic protein (GFAP), indicating selective GPR17 expression within cells of the OL lineage (Viganò et al., 2016). Moreover, the co-localization of GFP with the early OPC marker NG2 was also characterized, confirming that GPR17-expressing cells represent only a limited subset of all OPCs (Viganò et al., 2016). These results are in line with recent findings, showing that GPR17 expression appears to be restricted to a subpopulation defined as differentiation committed OPCs and newly formed OLs (Marques et al., 2016), and is peculiarly present in OPCs spatially located in proximity of neuronal axons and ready to myelinate (Marisca et al., 2020). Therefore, the pool of GPR17-expressing OPCs represents a particular subset among the OL lineage, which retains elevated differentiation and myelination capacity.

Further data obtained in the GPR17-iCreER^{T2}:CAG-eGFP reporter line indicated that, following ischemic damage, the density of OPCs double positive for NG2 and GFP in the ipsilateral hemisphere is significantly greater than in the intact contralateral one, thus confirming the role exerted by the GPR17 receptor in the activation of OPCs in response to damage (Viganò et al., 2016). Accordingly, a subsequent study revealed accumulation of

GPR17-expressing OPCs, labeled by GFP, in the regions surrounding the ischemic damage starting from 72 hours after MCAo, suggesting a regenerative role for these cells. In particular, the proliferation rate of GFP^+ cells was shown to reach its maximum during the first week after MCAo, indicating that this pool of OPCs is extremely responsive to damage (Bonfanti et al., 2017). In the same work, it was shown that cerebral ischemia sequentially induces morphological changes in GPR17-expressing OPCs, consistent with their migration towards the lesion site, followed by a rapid proliferative response of GFP^+ cells, a subsequent increase in the number of cells at intermediate stages of maturation, and finally the progression of some of these cells towards the myelinating phenotype (Bonfanti et al., 2017). Notably, a similar spatiotemporal gradient has also been described in post-mortem brain samples of patients who have suffered traumatic brain damage (Franke et al., 2013).
As previously mentioned, following the initial response of GPR17-expressing OPCs to CNS injury, the GPR17 receptor must be downregulated to allow complete maturation to myelinating OLs (Fumagalli et al., 2015a). However, the spontaneous differentiation capabilities of GFP^+ OPCs after cerebral ischemia, despite being present, appear to be very limited and insufficient to achieve complete remyelination (Bonfanti et al., 2017). In this respect, it has been recently showed that the maturation of GFP^+ OPCs could be dampened by the concomitant presence of excessive inflammation, as happens in the EAE model, while these cells managed to fully differentiate in the low inflammatory conditions that characterize cuprizone-induced demyelination model (Coppolino et al., 2018). The local inflammatory milieu, mainly regulated by microglia, may therefore assume a pivotal role in determining the final fate of GFP^+ OPCs recruited at the borders of the ischemic lesion.

1.3 The impact of microglia during post-stroke remyelination

1.3.1 Microglia homeostatic functions and response to ischemic injury

Microglia are the resident immune cells of the CNS, representing about 10-20% of all glial cells. Unlike all the other cells of the CNS, that are of ectodermal embryonic derivation, microglia derive from primitive mesodermal progenitors of the myeloid lineage and migrate in the developing CNS at an early stage of embryogenesis (Ginhoux et al., 2010; Li and Barres, 2018).

By colonizing the CNS early during development, microglia regulate the migration of neural precursors, the survival of neurons in the cortex, and the pruning of non-essential synapses after synaptic maturation is complete, therefore controlling the proper formation of the neuronal circuits required for cognition, learning, and memory (Aarum et al., 2003; Paolicelli et al., 2011; Schafer et al., 2012; Torres et al., 2016; Ueno et al., 2013). In addition, microglia were shown to orchestrate developmental oligodendrogenesis and myelination, by inducing OPC differentiation and axon ensheathing and by removing excessive and ectopic myelinated tracts, with a mechanism resembling synaptic pruning. These immune cells were also found to tightly regulate the number of quiescent OPCs in the adult white matter (Hagemeyer et al., 2017; Hughes and Appel, 2020).

As cells of the innate immune system, one of the major functions of microglia is to monitor the surrounding environment to check for signs of damage, such as DAMPs released by dead or stressed cells upon injury or infections. To this aim, under physiological conditions, microglia have a specific morphology characterized by a small cell body with very thin and mobile processes (Kettenmann et al., 2013). This state of restless movement allows them to continuously examine the surrounding extracellular space and to interact with neighboring cells and blood vessels. Interestingly, recent data indicate a central role of the purinergic receptor P2Y12 in the regulation of microglia process motility and surveillance, controlling their capacity to quickly respond to CNS insults (Bernier et al., 2019; Cserép et al., 2020).

After encountering damage signals in the CNS microenvironment, microglia undergo morphological transformations, characterized by cell body hypertrophy and retraction of cell processes, and rapidly respond through the induction of specific genes, necessary to initiate an inflammatory process with the aim of containing damage evolution and favoring repair (Prinz et al., 2019). Transformations of microglia morphology, phenotype and function have been observed in most neuropathological conditions, including neurodegenerative diseases, infections, ischemia, tumors, and trauma (Colonna and Butovsky, 2017; Nayak et al., 2014).

In the past, two opposite activation states of microglia have been hypothesized, a M1 pro-inflammatory and a M2 pro-regenerative phenotype. This dualistic classification has been questioned and recently replaced by a new proposal that includes numerous plastic and three-dimensional functional phenotypes (Amici et al., 2017; Ransohoff, 2016; Stratoulias et

al., 2019). Indeed, activation signals coming from the surrounding environment can drive microglia to acquire specific functional states to promote damage or facilitate repair (Fumagalli et al., 2018).

In the context of cerebral ischemia, microglia are promptly activated and can have both beneficial and harmful effects, depending on the specific stage of ischemic damage progression (Hu et al., 2012) (Figure 1.6).

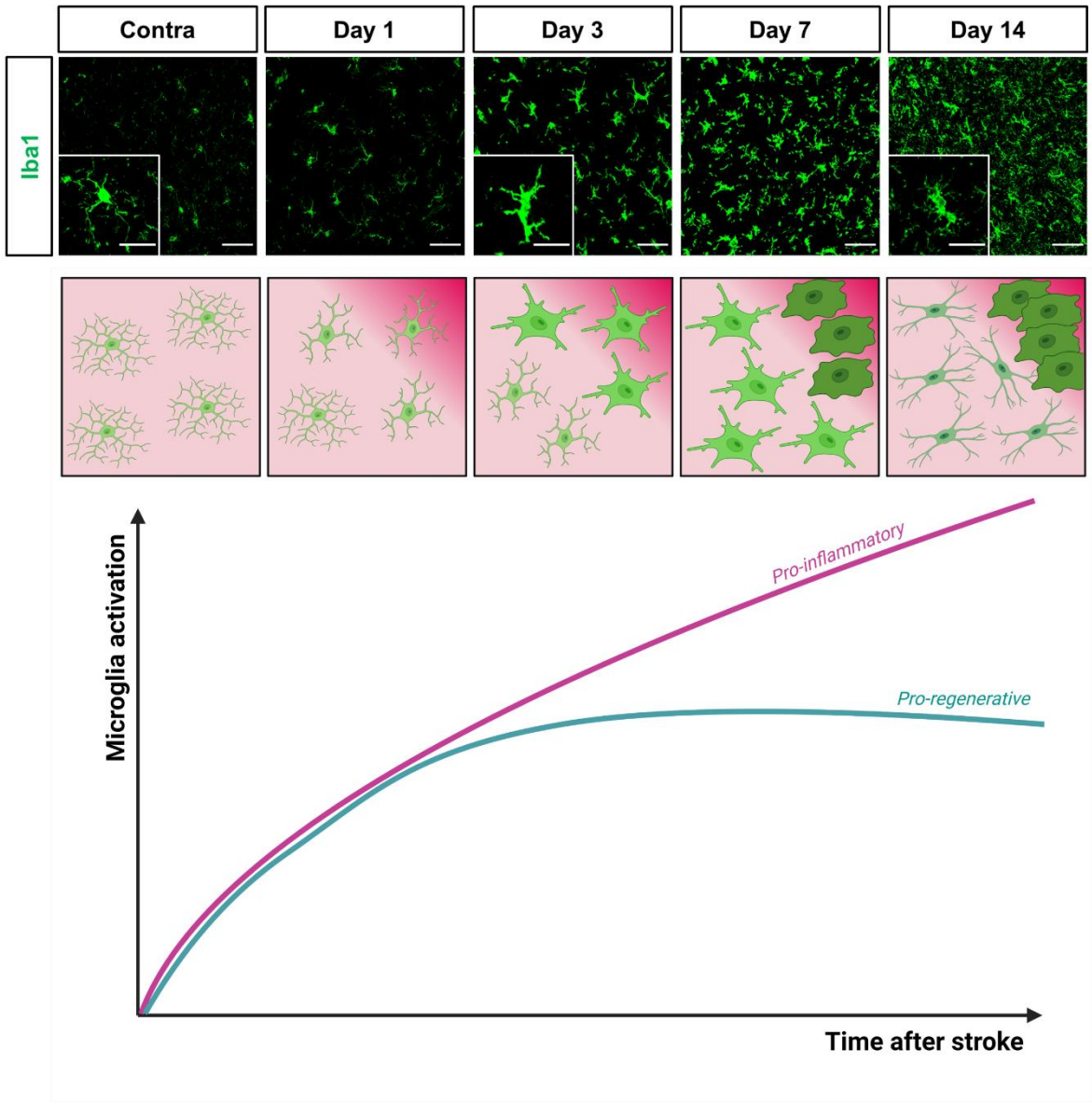

***Figure 1.6 Microglia activation over time after stroke**. In the intact brain, microglia display highly ramified morphology and fast process motility to surveil the surrounding tissue, as sentinels for danger signals. This allows microglial cells to rapidly respond to an ischemic event by undergoing profound morphological, molecular, and functional modifications. Two distinct temporal windows in microglial response following stroke have been defined. In the early phase (day 3 post-MCAo), microglial cells accumulating at the border of the ischemic lesion (decorated by Iba1 staining) appear ameboid and hypertrophic, express both pro-inflammatory and pro-regenerative markers, and have high phagocytic*

capacity. Conversely, at late stage after stroke (day 14 post-MCAo), dystrophic, senescent-like, pro-inflammatory microglia dominate the peri-infarct area. Figure adapted from (Raffaele et al., 2021)*, using BioRender.com*

In the MCAo model, it has been shown that microglia rapidly change their morphology and migrate to the lesion site within the first three days after stroke, with their number increasing up to 14 days post-MCAo (Raffaele et al., 2021). Once recruited at lesion boundaries, microglia increase the expression of pro-inflammatory genes, such as tumor necrosis factor (TNF), interleukin 1β (IL-1β), interferon γ (IFN -γ), IL-6, inducible nitric oxide synthase (iNOS), and proteolytic enzymes (MMP9, MMP3) (Yenari et al., 2010), but at the same time they also contribute to tissue repair and remodeling by removing debris and producing anti-inflammatory cytokines, pro-angiogenic factors, and growth factors, such as IL-10, transforming growth factor β (TGF-β), insulin-like growth factor (IGF1), and vascular endothelial growth factor (VEGF) (Ma et al., 2017; Ponomarev et al., 2013). This dualistic role of microglia appears to be associated with the post-stroke disease stage. In fact, at early stages after ischemic injury, microglia have been shown to exert protective functions, by containing detrimental astrocyte activation (Jin et al., 2017), limiting cytotoxic neutrophil infiltration within the lesion (Otxoa-de-Amezaga et al., 2019), and reducing excitotoxic injury to neurons (Szalay et al., 2016). On the contrary, at later time points, microglia acquire a detrimental pro-inflammatory phenotype hindering brain repair (Hu et al., 2012; Rajan et al., 2019). Accordingly, at later stages after ischemia, prolonged overstimulation by chronic inflammation was shown to induce microglia immunosenescence, similarly to that observed in chronic neurodegenerative diseases and aging (Raffaele et al., 2021; Rawji et al., 2016; Savage et al., 2019). Microglia immunosenescence has been associated with the acquisition of a dystrophic morphology, reduced process motility, and impaired capacity of exerting pro-regenerative and neuroprotective functions, including remyelination support (Rawji et al., 2016, 2020b; Savage et al., 2019). These data suggest that, to promote structural and functional recovery at late stages after stroke, therapeutic approaches should be aimed at selectively restoring microglial regenerative properties rather than simply suppressing their overall activation, which inevitably leads to the loss of their beneficial effects (Fumagalli et al., 2018).

1.3.2 The pro-remyelinating properties of microglia

Recent studies have shown that microglia can contribute to the remyelination process by creating an environment that supports the recruitment of OPCs and their subsequent differentiation into mature myelinating OLs. Indeed, microglial cells facilitate the OPC response by phagocytosing myelin debris, secreting regenerative factors, and modulating ECM composition (Lloyd and Miron, 2019) (Figure 1.7).

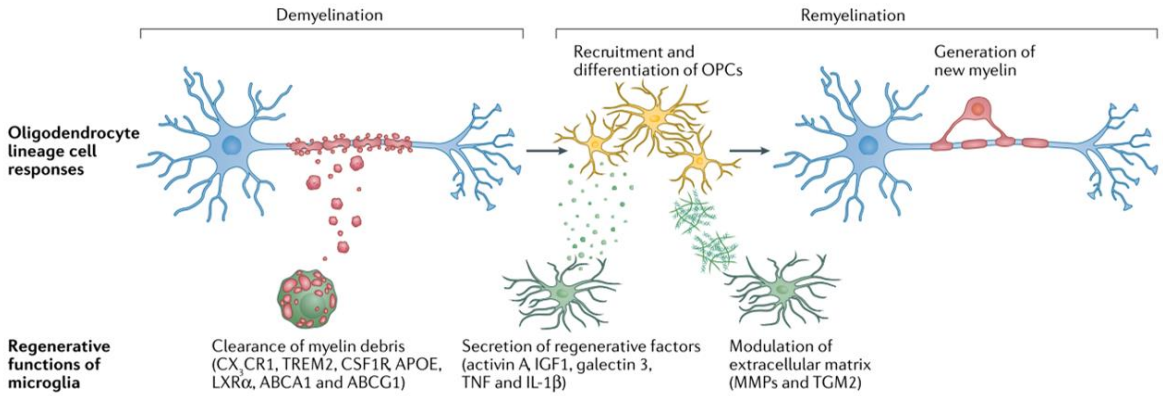

Figure 1.7 How microglia drive remyelination*. A supportive microglia functional phenotype is a pivotal determinant of the success or failure of remyelination. Indeed, microglia have a great impact on myelin regeneration by phagocytosing myelin and cell debris, modulating the composition of the extracellular matrix, and releasing soluble factors that promote the chemotaxis, survival, and maturation of OPCs. Hence, these protective functions of microglia contribute to promote a permissive local environment for proper recruitment of OPCs toward the lesion and their differentiation into myelinating cells. Adapted from* (Lloyd and Miron, 2019).

The first step for efficient remyelination is the clearance of myelin debris from the demyelinated area, setting the basis for subsequent OPC recruitment and differentiation (Lampron et al., 2015). This is a complex process, involving the internalization of myelin debris within the cell, their degradation into lysosomes, and the final recycling of myelin breakdown products, including cholesterol (Bosch-Queralt et al., 2021). To fulfill this function, following demyelination, microglia express genes related to phagocytosis and lysosomal pathways, like the scavenger receptor CD68, and gain the ability of internalizing myelin debris. This capacity was shown to decrease with age, and this decrease is associated with reduced remyelination efficiency (Cantuti-Castelvetri et al., 2018; Safaiyan et al., 2016). Recent studies identified several microglial receptors regulating myelin clearance capacity, including triggering receptor expressed on myeloid cells 2 (TREM2). In TREM2 knockout mice, myelin degradation after demyelination was found to be impaired, leading to accumulation of myelin debris. This in turn was associated with decreased OPC

recruitment, prolonged demyelination, and axonal degeneration (Cantoni et al., 2015; Poliani et al., 2015). Of note, not only general knockout, but also TREM2 haploinsufficiency was accompanied by defective myelin debris clearance, and TREM2 stimulation using an agonistic antibody rescued such defect, leading to improved OL differentiation and remyelination (Cignarella et al., 2020). After internalization, myelin debris must be degraded and recycled outside of the cell, to avoid saturation of the lysosomal compartment and subsequent inability of microglia to support remyelination due to cholesterol overload (Bosch-Queralt et al., 2021; Cantuti-Castelvetri et al., 2018). Therefore, genes involved in cholesterol metabolism and recycling, like the cholesterol carrier ApoE, the receptor LXRα, and the efflux transporters Abca1 and Abcg1, assume a great importance for the clearance capacity of microglia around demyelinated lesions, and may represent attractive therapeutic targets (Bosch-Queralt et al., 2021).

Inflammation-induced release of soluble factors by microglia is another important component of remyelination, as indicated by several studies showing beneficial roles of microglia-secreted factors in this context, reviewed in (Miron, 2017). In this respect, one of the most studied is IGF-1. Interestingly, expression and release of IGF-1 were found to be restricted to a particular subset of microglia, characterized by the expression of the integrin CD11c, representing a consistent fraction of all microglial cells populating white matter regions during developmental myelination and in response to myelin injury (Wlodarczyk et al., 2017). Of note, the myelination-supporting properties of $CD11c^+$ microglia subset are lost after IGF-1 ablation, suggesting a pivotal role of this factor in microglia-OL interaction (Wlodarczyk et al., 2017). Another important factor involved in microglia-dependent remyelination is TNF, a pleiotropic cytokine existing both in a soluble form (solTNF), mainly driving detrimental processes by activating the receptor TNFR1, and in a trans-membrane form (tmTNF), promoting trophic and regenerative responses by interacting with the receptor TNFR2 (Probert, 2015). A recent paper showed that TNF released by microglial cells is required for the generation of new myelinating cells within demyelinated areas, suggesting direct effects of this cytokine on OPCs (Cunha et al., 2020). This is in line with previous findings showing that TNF finely regulates OL functions. While solTNF has been associated with increased OL necroptosis (Ofengeim et al., 2015), tmTNF/TNFR2 axis was shown to sustain OL maturation and remyelination (Arnett et al., 2001; Madsen et al.,

2016a). Microglia-dependent pro-myelinating effects have been also attributed to the expression of galectin-3 (Gal-3), a member of the family of b-galactoside-binding lectins (Thomas and Pasquini, 2018). Following myelin injury, expression of Gal-3 by microglia was shown to favor a pro-regenerative microglial phenotype, fostering myelin debris phagocytosis through TREM2 activity (Hoyos et al., 2014). Accordingly, genetic ablation of Gal-3 compromised myelin integrity and neurological functionality (Hoyos et al., 2014; Pasquini et al., 2011). Moreover, Gal-3 can be cleaved and released by microglia to directly interact with recruited OPCs in transition to the immature OL stage, enhancing actin assembly which is required for increased branching (Pasquini et al., 2011; Thomas and Pasquini, 2018).

Finally, also ECM molecules deposited into demyelinated lesions represent inhibitory cues hindering OPC recruitment and differentiation (Marangon et al., 2020). Thus, another way by which microglia facilitate OPC-dependent remyelination is through ECM remodeling (Lloyd and Miron, 2019). For instance, microglia-derived matrix metalloproteinases (MMPs) were found to degrade fibronectin and chondroitin sulfate proteoglycans (CSPGs), which are known to inhibit OPC recruitment and differentiation (Pu et al., 2018; Wang et al., 2018). Another mechanism of microglia-mediated ECM modification is through secretion of transglutaminase-2, which crosslinks laminin to regulate OPC proliferation and differentiation (Giera et al., 2018).

Furthermore, for efficient myelin reconstitution, microglia need to acquire a peculiar functional state, allowing them to accomplish their regenerative duties. In experimental models of focal demyelination, the onset of OL differentiation and remyelination has been found to be associated with a particular pro-regenerative phenotype of microglia, characterized by the expression of markers like arginase 1 (Arg1), IGF1, and CD206. This transition in microglial activation represents a rate-limiting step in remyelination, as the absence of CD206-expressing microglia, due to selective depletion in young mice or reduced formation occurring in old mice, impairs myelin regeneration (Miron et al., 2013). Accordingly, our recent data show that, at early stages after ischemic stroke, microglia acquire a beneficial phenotype characterized by the simultaneous expression of the inflammatory marker CD16/32 and of the regenerative marker YM1, promoting the recruitment of GPR17-expressing OPCs toward the ischemic lesion and preserving myelin

integrity. However, these protective features are lost during disease progression and replaced by a pro-inflammatory senescent-like state that is no longer able to sustain GPR17-expressing OPC remyelinating attempts (Raffaele et al., 2021). On this basis, identifying factors supporting this 'pro-remyelinating' phenotype of microglia might help to contrast myelin disruption and to promote its regeneration by OPCs.

In this respect, several mechanisms have been associated with the regenerative activation and functions of microglia (Fumagalli et al., 2018). The ionotropic purinergic receptor P2X4, whose expression is increased in microglia during the remission phase of EAE, was shown to suppress microglial pro-inflammatory activation while increasing the levels of CD206 (Zabala et al., 2018). Pharmacological inhibition of P2X4 receptor was shown to impair the clearance capacity of microglia and to worsen clinical outcome, whereas its potentiation significantly induced the regenerative activation of microglia and improved motor function and remyelination (Zabala et al., 2018). In addition, TNF signaling has been demonstrated to play a pivotal role in regulating microglial functions after injury (Raffaele et al., 2020). Relevant for our topic, microglial TNFR2 activation by tmTNF was shown to regulate the expression and release of several neuroprotective factors (Veroni et al., 2010). Accordingly, microglia-specific genetic ablation of TNFR2 exacerbated the pro-inflammatory activation of these cells, compromising their pro-regenerative functions, including tissue surveillance and phagocytosis (Gao et al., 2017). Consequently, knockout of microglial TNFR2 resulted in earlier disease onset and increased demyelination in EAE (Gao et al., 2017). Hence, these results suggest that tmTNF/TNFR2 signaling may be required for the pro-resolving properties of microglia, with possible important implications also in a stroke-related context. Indeed, conditional genetic ablation of solTNF with preservation of tmTNF resulted in reduced infarct volume and functional disability after MCAo (Madsen et al., 2016b). However, the contribution of microglial TNFR2 in myelin protection and repair after stroke has never been evaluated.

In general, the contribution of microglia to remyelination is to provide a favorable environment for myelin regeneration (Lloyd and Miron, 2019). However, despite the support of microglia, there are numerous pathological conditions characterized by remyelination failure, caused by impaired activation, recruitment, and differentiation of OPCs. Factors that are intrinsic to the OL lineage, such as epigenetic changes, and extrinsic

cues, such as an unfavorable environment and alterations in microglial responses are critical for successful OPC maturation (Franklin and Ffrench-Constant, 2017; Stangel et al., 2017). To design effective therapies supporting myelin repair, besides OL-targeted approaches, it is therefore necessary to understand the mechanisms underlying the pro-remyelinating properties of microglia and their communication with remyelinating OPCs.

1.3.3 The role of extracellular vesicles in the crosstalk between microglia and oligodendrocytes

As reported above, activated microglia can exert harmful effects on myelin-producing cells, by releasing pro-inflammatory mediators that can damage glia and nearby neurons. Despite this, it also plays a crucial role in damage repair and remyelination (Peferoen et al., 2014). Previous studies have shown that, by removing or blocking the action of microglia in different experimental models of CNS degeneration, the myelin repair process is impaired, suggesting the importance of the interaction between microglia and OLs during remyelination (Li et al., 2005; Miron et al., 2013; Raffaele et al., 2021; Tanaka et al., 2013). This also happens during aging, when impaired myelin turnover has been correlated with a reduction in the recruitment of microglia, in their activation towards a pro-regenerative phenotype, and their phagocytic potential (Rawji et al., 2016). In disease conditions, OLs can also directly participate in modulating microglial response, by releasing factors which stimulate the capacity of microglia to clear myelin debris and assume a pro-remyelinating phenotype (Boccazzi et al., 2021; Kirby and Castelo-Branco, 2021). Given the importance of the communication between microglia and OLs, new therapeutic strategies are focusing on the modulation of this interaction to regulate neuroinflammation and promote repair (Peferoen et al., 2014).

One of the means by which microglia communicate with other cells and exert their beneficial or harmful effects is the release of extracellular vesicles (EVs) (Paolicelli et al., 2019; Prada et al., 2013; Turola et al., 2012).

EVs are nanoparticles formed by a double layer of phospholipids resembling the cellular membrane, which can signal to adjacent cells or travel very long distances and deliver complex messages to distant cells (Van Niel et al., 2018). The cargo of EVs include both lipophilic components present in the membrane fraction, including lipids and

transmembrane proteins, and hydrophilic molecules retained in the cytosolic compartment, such as soluble proteins and nucleic acids, which are protected from enzymatic degradation by the vesicular membrane (Van Niel et al., 2018). Another important advantage of EVs-mediated signaling is the capacity to create a biologically active concentration of signaling molecules near target cells, which instead would not induce any effect if diluted in the extracellular environment (Fitzgerald et al., 2018).

EVs can be classified, depending on the size and intracellular origin, in apoptotic bodies, microvesicles and exosomes (Van Niel et al., 2018). The former usually have dimensions ranging from 500 to 2000 nm and are released during the cell death process. Microvesicles (MVs) or ectosomes (100-1000 nm) instead originate from the plasma membrane and are then released into the extracellular environment upon cell activation through a process called shedding (Turola et al., 2012). Their release occurs in response to specific stimuli, such as the change in ATP levels. In cells expressing the purinergic P2X7 receptor, such as microglia, it has been demonstrated that an increase in the extracellular concentration of ATP induces activation of the P2X7 receptor, leading to massive release of MVs from the plasma membrane (Bianco et al., 2005; Cocucci and Meldolesi, 2015). MVs contain distinct regions of the cell membrane of origin which include receptors, proteins, and genomic material. One of the most abundant markers on the surface of MVs is phosphatidylserine, a phospholipid capable of interacting and binding specific receptors present on the surface of target cells (Frey and Gaipl, 2011). As for the exosomes, they are the smallest vesicles (50-200 nm) and are generated by the trafficking of multivesicular bodies (MVBs) from the cytosol to the plasma membrane (Basso and Bonetto, 2016; Colombo et al., 2014). Despite their different sizes and origins, MVs and exosomes share similar release and fusion mechanisms with recipient cells (Cocucci and Meldolesi, 2015). The general interaction of EVs with target cells demonstrates that they act as platforms for signal transduction, inducing stimulation or inhibition of their targets. Once in contact with the target cell, exosomes and ectosomes roll onto the cell surface. Immediately afterwards, the vesicles stop, most likely due to binding with proteins and cell surface receptors, and transfer material inside the cells. In many cases, EVs release their contents into the cytoplasm of the target cell by fusing directly with the plasma membrane, with a mechanism involving the binding of specific surface proteins with the respective receptor. However, the uptake of

EVs can also occur through a type of endocytosis that does not involve membrane fusion (Cocucci and Meldolesi, 2015). Exosomes and small ectosomes follow a clathrin-dependent process, while larger ectosomes employ other internalization processes, such as macropinocytosis and phagocytosis (Tian et al., 2014). Upon uptake into the endosome, EVs finally fuse with the organelle membrane and discharge their contents into the cytoplasm (Bissig and Gruenberg, 2014). Alternatively, the vesicles can be retained within the endosomal lumen or merge with the lysosomes and be completely digested (Cocucci and Meldolesi, 2015). The interaction is neither random nor non-specific, as the vesicles recognize the target cells (Lösche et al., 2004) through the activation of specific membrane receptors (Bianco et al., 2005; Gasser and Schifferli, 2004) or through the transfer of membrane receptors (Mack et al., 2000).

The content of the microglial EVs was shown to reflect the activation state of the donor cell (Garzetti et al., 2014; Grimaldi et al., 2019; Prada et al., 2018). Their production increases significantly during brain inflammation, reflecting the extent of microglial activation (Lombardi et al., 2019). Following interaction with recipient cells, microglial vesicles activate contact-mediated signaling pathways (Antonucci et al., 2012; Gabrielli et al., 2015) and/or carry genetic information (Drago et al., 2017; Prada et al., 2018; Verderio et al., 2012), thus profoundly influencing the molecular configuration and function of target cells. MVs and exosomes released from glial cells are known to contain pro-inflammatory cytokines IL-1β and TNF (Bianco et al., 2005; Raffaele et al., 2020), angiogenic factors FGF-2 and VEGF with respective mRNAs (Proia et al., 2008), and the matrix metalloproteases MMP-2 and MMP-9 (Sbai et al., 2010).

In recent years, microglial EVs have been identified as mediators of inflammation and neurodegeneration, playing a pivotal role in spreading pathological misfolded protein aggregates (Crotti et al., 2019; Delpech et al., 2019; Joshi et al., 2014; Pérez et al., 2019) and in triggering harmful responses in recipient neurons and glial cells (Lombardi et al., 2019; Prada et al., 2018). On the other hand, they have been also described as vehicles of pro-regenerative molecules, promoting remyelination and brain repair (Casella et al., 2018; Delpech et al., 2019; Li et al., 2021; Lombardi et al., 2019). Recently, microglia-derived EVs have been shown to play a role in directing OLs towards maturation (Lombardi et al., 2019). EVs generated by microglia polarized towards a pro-regenerative phenotype (IL-4 EVs)

have been found to foster remyelination when injected into focal demyelinating lesions in vivo, and to directly enhance the proliferation, migration, differentiation, and myelination capacities of primary cultured OLs in vitro (Lombardi et al., 2019).

Microglial EVs were also found to be able to modulate the activation state of the microglia residing in the damaged tissue, resuming their protective functions (Casella et al., 2018; Grimaldi et al., 2019). Indeed, it has been shown that microglial cells are able to capture and internalize EVs, suggesting the possibility that the different microglial phenotypes can influence each other through EV release (Van den Broek et al., 2020; Casella et al., 2018; Lecuyer et al., 2021).

Based on these results, microglia-derived EVs emerge as potential regenerative tools to enhance CNS repair in pathological conditions, such as cerebral ischemia, characterized by demyelination and excessive neuroinflammation. Furthermore, the identification of the molecular components responsible for the beneficial effects produced by microglial EVs could reveal new pharmacological targets to be exploited for remyelinating therapies. However, the role of microglial EVs in the regulation of reparative processes following the ischemic event, in which the pool of GPR17-expressing OPCs is primarily involved (Bonfanti et al., 2017), has never been investigated.

2. AIMS

Ischemic stroke is a neurological disorder representing a leading cause of death and permanent disability world-wide, for which effective regenerative treatments are still missing (Barthels and Das, 2020). OL degeneration and consequent myelin disruption are considered major contributing factors to stroke-associated neurological deficits. Therefore, fostering myelin repair by promoting OPC maturation has emerged as a promising therapeutic approach (Fumagalli et al., 2016). In particular, the subpopulation of OPCs expressing the P2Y-like receptor GPR17, which is still present in the adult brain, has been shown to specifically represent a reserve pool maintained for repair purposes (Lecca et al., 2020). Previous data demonstrated that the GPR17-expressing cells actively react to ischemia and accumulate at injury borders by increasing their proliferation and migratory ability, but their spontaneous maturation appears very limited, likely due to the non-permissive local inflammatory environment (Bonfanti et al., 2017). A pivotal role in regulating remyelination is played by microglia, the brain-resident macrophages. Early after stroke, microglial cells exert beneficial functions, promoting OPC recruitment toward the ischemic lesion and preserving myelin integrity. However, the protective features of microglia are lost during disease progression, contributing to remyelination failure (Raffaele et al., 2021). Of note, activated microglia can affect neighboring cells by releasing EVs, small membranous particles able to transfer specific proteins, lipids, and microRNAs between cells (Budnik et al., 2016). EVs derived from pro-regenerative microglia have been recently shown to exert protective effects on endogenous immune cells and differentiating OPCs surrounding myelin lesions (Casella et al., 2018; Lombardi et al., 2019), emerging as promising tools to promote brain repair. However, the impact of microglial EVs in the context of brain ischemia, as well as the molecular mechanisms responsible for their beneficial effects on OL maturation, remain to be elucidated.

On this basis, the specific aims of this thesis were:

1. to study the effects mediated by microglial EVs on endogenous microglia activation, GPR17-expressing OPC differentiation, and functional recovery of ischemic mice, by infusing microglial-derived EVs in the ipsilateral corpus callosum of GPR17-iCreER^{T2}:CAG-eGFP mice at late stage after MCAo.

2. to investigate the molecular mechanisms underlying EV-mediated beneficial effects on OPCs, by performing immunocytochemical and transcriptomic analysis in primary OPC cultured exposed to microglial EVs during differentiation.

In addition, since the inflammatory microenvironment has been shown to play a pivotal role in determining the efficiency of remyelination after stroke (Raffaele et al., 2021), unveiling the mechanisms driving the pro-remyelinating properties of microglia might provide important opportunities for both reducing myelin damage and promoting its regeneration by OPCs. In this respect, the activation of microglial TNFR2, the main receptor for tmTNF, has been previously implicated in regulating the pro-regenerative functions of microglia (Gao et al., 2017). Nevertheless, the contribution of microglial TNFR2 in myelin protection and repair after stroke has never been evaluated.
Therefore, the aim of the last part of my PhD work was:

3. to evaluate the role of TNFR2 in shaping microglial response after stroke, as well as the consequent impact on remyelination and post-stroke recovery, by using microglia-specific TNFR2 conditional knockout mice subjected to MCAo.

3. RESULTS

3.1 Infusion of microglia-derived EVs modifies the phenotype of microglia at the border of the ischemic lesion

To evaluate the impact of microglial EVs on post-stroke repair and functional recovery, primary microglia were stimulated in vitro toward a pro-inflammatory (i-MG) or pro-regenerative phenotype (IL-4 MG) for 48 hours, and the expression of specific markers for these two activation states has been confirmed by qPCR (Figure 3.1A-C). Then, i-MG and IL-4 MG have been exposed to ATP for 30' to maximize EV production (Lombardi et al., 2019), and microglial EVs have been collected from the supernatant by differential centrifugation. Notably, no differences were found in terms of size distribution and quantity between EVs produced by i-MG (i-EVs) and IL-4 MG (IL-4 EVs), as analyzed by tunable resistive pulse sensing (TRPS, Figure 3.1D, E).

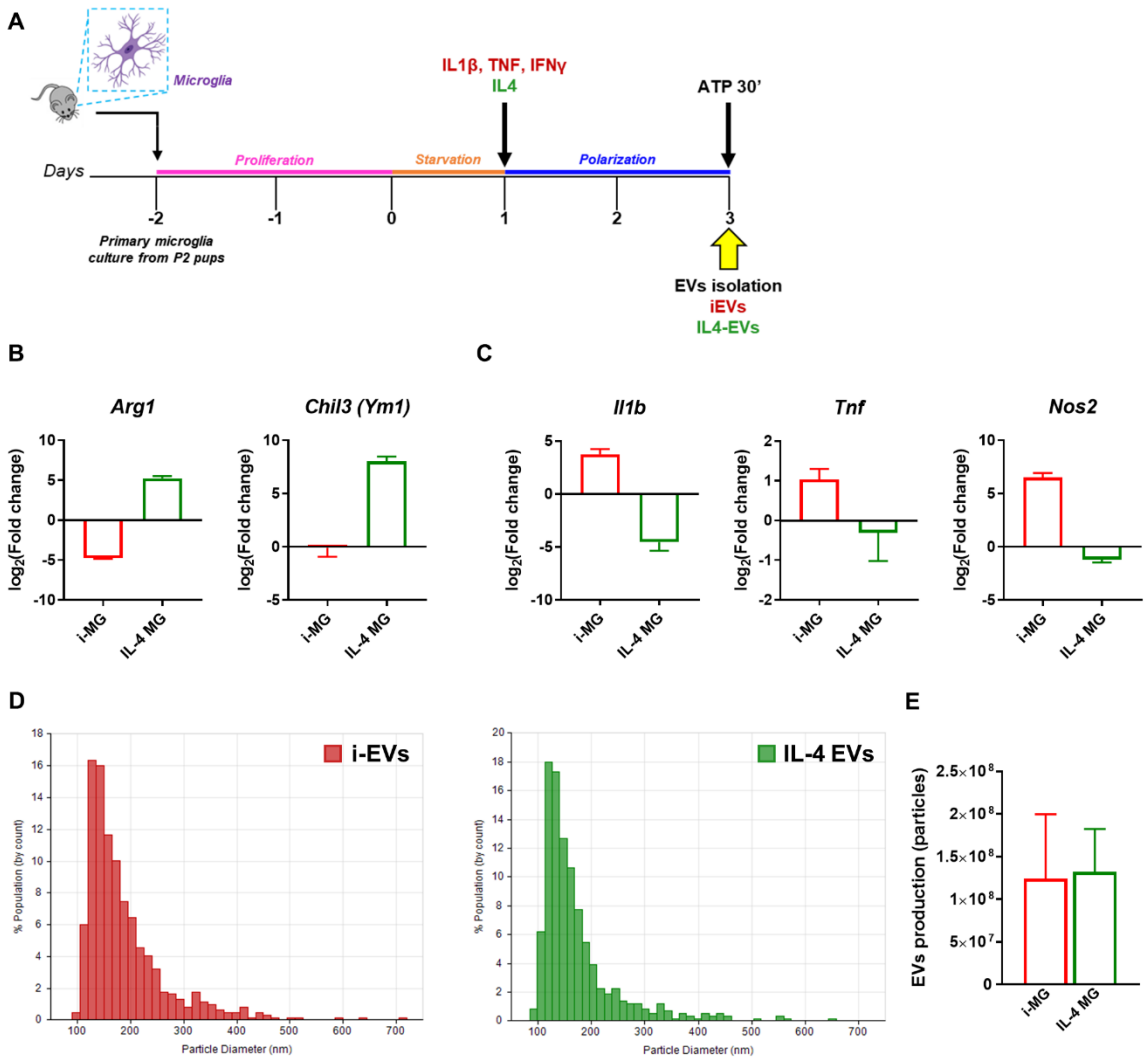

Figure 3.1 Characterization of polarization and EV release of murine microglia exposed to pro-inflammatory (i-MG) or pro-regenerative (IL-4 MG) stimuli. (A) Schematic representation of the experimental protocol utilized for

the preparation and analysis of microglia-derived EVs. (B) Gene expression of pro-regenerative markers in primary microglia exposed to pro-inflammatory (i-MG) or pro-regenerative (IL-4 MG) stimuli with respect to non-stimulated cells (NS-MG) set to 0. Data are shown as mean ± SE. (C) Gene expression of pro-inflammatory markers in primary microglia exposed to pro-inflammatory (i-MG) or pro-regenerative (IL-4 MG) stimuli with respect to non-stimulated cells (NS-MG) set to 0. Data are shown as mean ± SE. (D) Size distribution graphs relative to EVs released by i-MG (i-EVs) and IL-4 MG (IL-4 EVs) upon ATP simulation. EV size was measured by Tunable Resistive Pulse Sensing (TRPS) technique. (E) Quantification of EVs produced by i-MG (i-EVs) and IL-4 MG (IL-4 EVs) upon ATP simulation. Data are expressed as mean ± SE

After isolation, approximately $2x10^8$ i-EVs or IL-4 EVs, produced by $1.5x10^6$ microglia, or vehicle alone were chronically infused for 1 week into the ipsilateral corpus callosum of GPR17-iCreER^{T2}:CAG-eGFP reporter mice through osmotic minipumps, starting from day 14 post-MCAo.(Figure 3.2A).

Immunohistochemistry (IHC) analysis was performed at day 28 post-MCAo to evaluate the impact of the infusion of microglial EVs on endogenous Iba1$^+$ cells surrounding the ischemic lesion. Of note, although Iba1 labels both activated microglia and infiltrating macrophages, the two cell populations can be distinguished based on their morphology and spatial distribution. Indeed, peripheral macrophages display a clear rounded morphology and accumulate within the lesion core, while microglial cells acquire a more ramified morphology and represent the prevalent cell type in the peri-infarct region analyzed in the present thesis (Fumagalli et al., 2015b).

Results show no variations in global Iba1$^+$ microglial cell density after infusion of either IL-4 EVs, i-EVs or vehicle alone (Figure 3.1B-D). Furthermore, to get more insights on the molecular signature of microglia, double staining with Iba1 and specific microglial phenotypic markers has been performed. To this goal, the co-localization of Iba1 with the Fc gamma receptors CD16/32 or the chitinase 3-like 3 Ym1, decorating pro-inflammatory and pro-regenerative cells respectively (Perego et al., 2016; Villa et al., 2018), has been quantified. Notably, infusion of i-EVs resulted in a slight but significant increase in the density of pro-inflammatory microglial cells co-expressing Iba1 and CD16/32 as compared to vehicle administration (Figure 3.2B, D), whereas IL-4 EVs administration led to a significantly higher density of Iba1$^+$&Ym1$^+$ pro-regenerative microglia compared to i-EVs and vehicle treatment (Figure 3.2C, D).

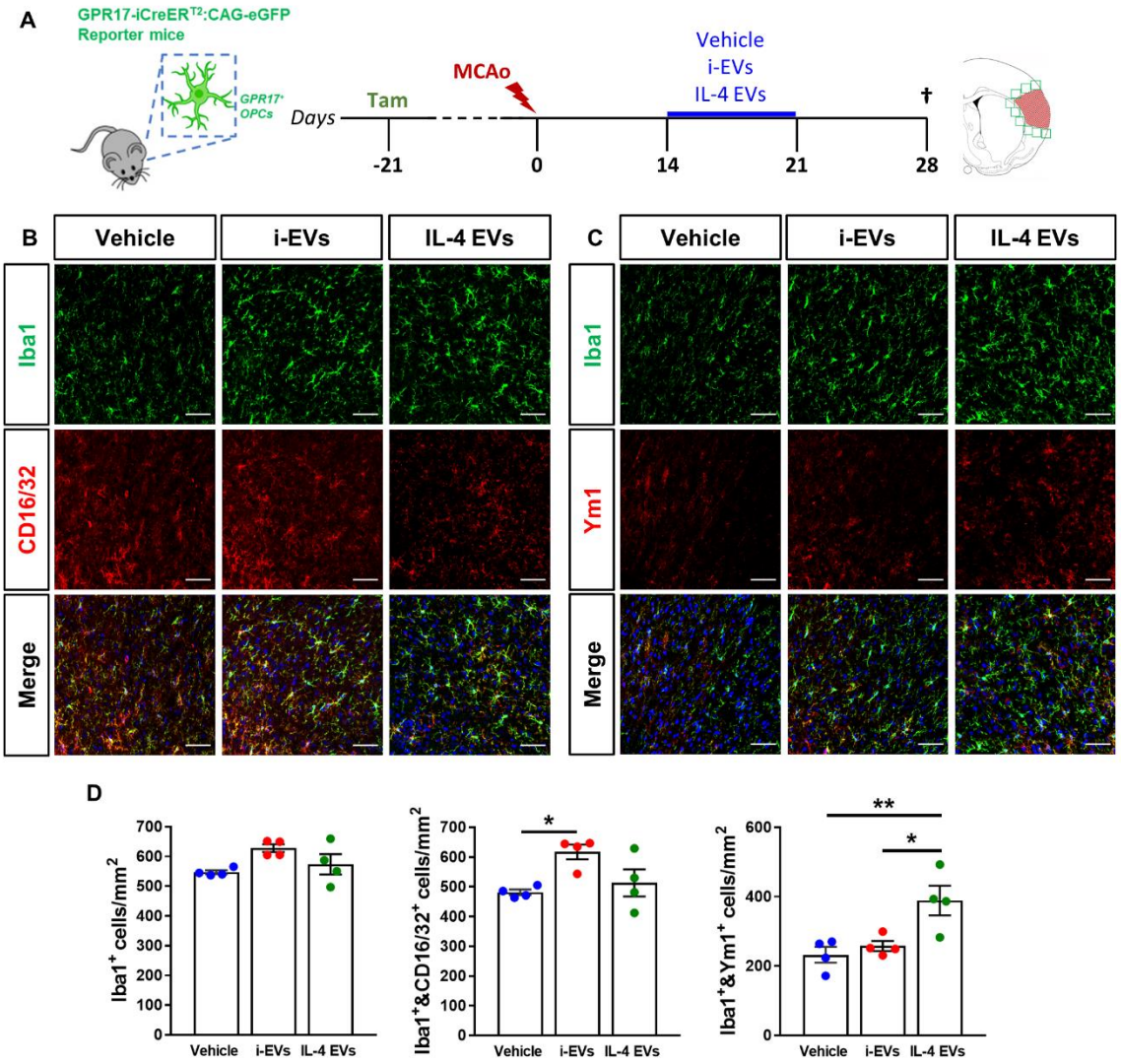

Figure 3.2 Infusion of pro-regenerative microglia-derived EVs at late stages after ischemia promotes a beneficial polarization of endogenous Iba1$^+$ cells. *(A) Schematic representation of the experimental protocol exploited for the infusion of microglia-derived EVs after MCAo. (B) Representative images of cells stained for Iba1 and CD16/32 at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. (C) Representative images of cells stained for Iba1 and Ym1 at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. (D) Quantification of the density of total Iba1$^+$, Iba1$^+$&CD16/32$^+$ and Iba1$^+$&Ym1$^+$ cells at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (n=4). Data are expressed as mean ± SE. * p<0.05, ** p<0.01; One-way ANOVA followed by Tukey's post-hoc analysis.*

Recent data suggest that not only the expression of specific markers, but also changes in morphological features help to characterize different microglia functional states (Heindl et al., 2018). On this basis, a detailed morphological analysis of Iba1⁺ cells has been implemented by means of the semi-automated program 3DMorph (York et al., 2018), revealing prominent modifications of Iba1⁺ cell branching induced by microglial EVs when compared to vehicle-treated animals (Figure 3.3A, B). An increase in the number of branchpoints and ramification index, indicative of a recovery from a dystrophic shape to a functional one (Savage et al., 2019), was observed after treatment with both i-EVs or IL-4 EVs (Figure 3.3C). In addition, exposure to IL-4 EVs only was able to induce a marked increase of Iba1⁺ cell volume and surveilled cell territory compared to vehicle and i-EVs (Figure 3.3C), suggesting a switch of microglial cells towards pro-resolving functions. Globally, these data demonstrate that infusion of IL-4 EVs had a significant impact on activated microglia at ischemic lesion boundaries, favoring their pro-regenerative activation.

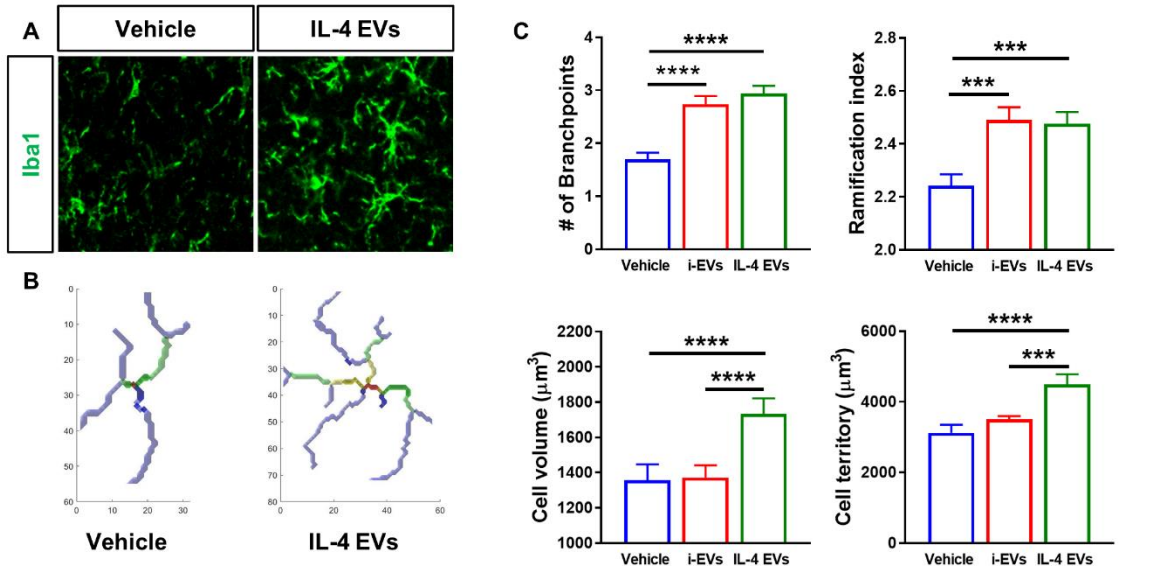

Figure 3.3 Administration of IL-4 EVs induces morphological changes of microglia surrounding the ischemic lesion. *(A) Representative images of cells stained for Iba1 at the boundary of ischemic lesion (0-500 µm) at day 28 post-MCAo, following infusion of IL-4 EVs or vehicle, displaying different cell morphology. (B) Representative 3D skeleton images showing major branching of Iba1⁺ cells after infusion of IL-4 EVs or vehicle. Colors indicate order of connectivity (red = primary, yellow = secondary, green = tertiary, and blue = connected to endpoint). (C) Quantification of Iba1⁺ microglia number of branchpoints, ramification index, cell volume, and cell territory, at the boundary of ischemic lesion (0-500 µm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (130-150 cells from 3-4 animals/experimental condition have been analyzed). Data are expressed as mean ± SE. *** p<0.001; **** p<0.0001; Kruskal-Wallis test followed by Dunn's post-hoc analysis.*

3.2 Infusion of pro-regenerative microglia-derived EVs promotes GPR17-expressing OPC maturation after cerebral ischemia

Besides modulating the functions of endogenous microglia, recent evidence indicates that microglia-derived EVs regulate remyelination also by directly influencing OPCs surrounding myelin lesions (Lombardi et al., 2019). In this respect, the subpopulation of OPCs expressing the GPR17 receptor has been shown to retain an elevated regenerative potential among the OL lineage (see paragraph 1.2.4), which could be specifically enhanced by the exposure to microglial EVs.

On this basis, the impact of the infusion of microglial EVs on the differentiation of GPR17-expressing OPCs in the peri-infarct area, labelled by GFP expression in GPR17-iCreER^{T2}:CAG-eGFP reporter mice utilized in this study, has been analyzed. At day 28 post-MCAo, the infusion of IL-4 EVs resulted in significantly increased density of GFP$^+$ cells at lesion borders as compared to littermates receiving either i-EVs or vehicle alone (Figure 3.4A, B). Moreover, the expression of differentiation stage-specific OL markers in GFP$^+$ cells has been quantified. The fraction of GFP$^+$ OPCs co-expressing the early differentiation marker NG2 did not differ among all the experimental conditions (Figure 3.4C, D). Conversely, the infusion of IL-4 EVs was able to significantly increase the percentages of GFP$^+$ cells co-expressing GPR17 (indicative of a preOL stage) or the mature myelinating OL marker GSTπ compared to vehicle or i-EVs treated groups (Figure 3.4E-H). Finally, administration of IL-4 EVs was found to result in a significant increase of the area covered by FluoroMyelin stain, labeling intact myelin structures, in the ipsilateral corpus callosum of ischemic mice (Figure 3.4I, J), suggesting that efficient myelin repair occurred.

Globally, these results suggest that the infusion of microglial IL-4 EVs at late stages after MCAo was able to promote the response to ischemic damage of the pool of OPCs expressing GPR17 and to foster their terminal maturation, contributing to enhanced remyelination.

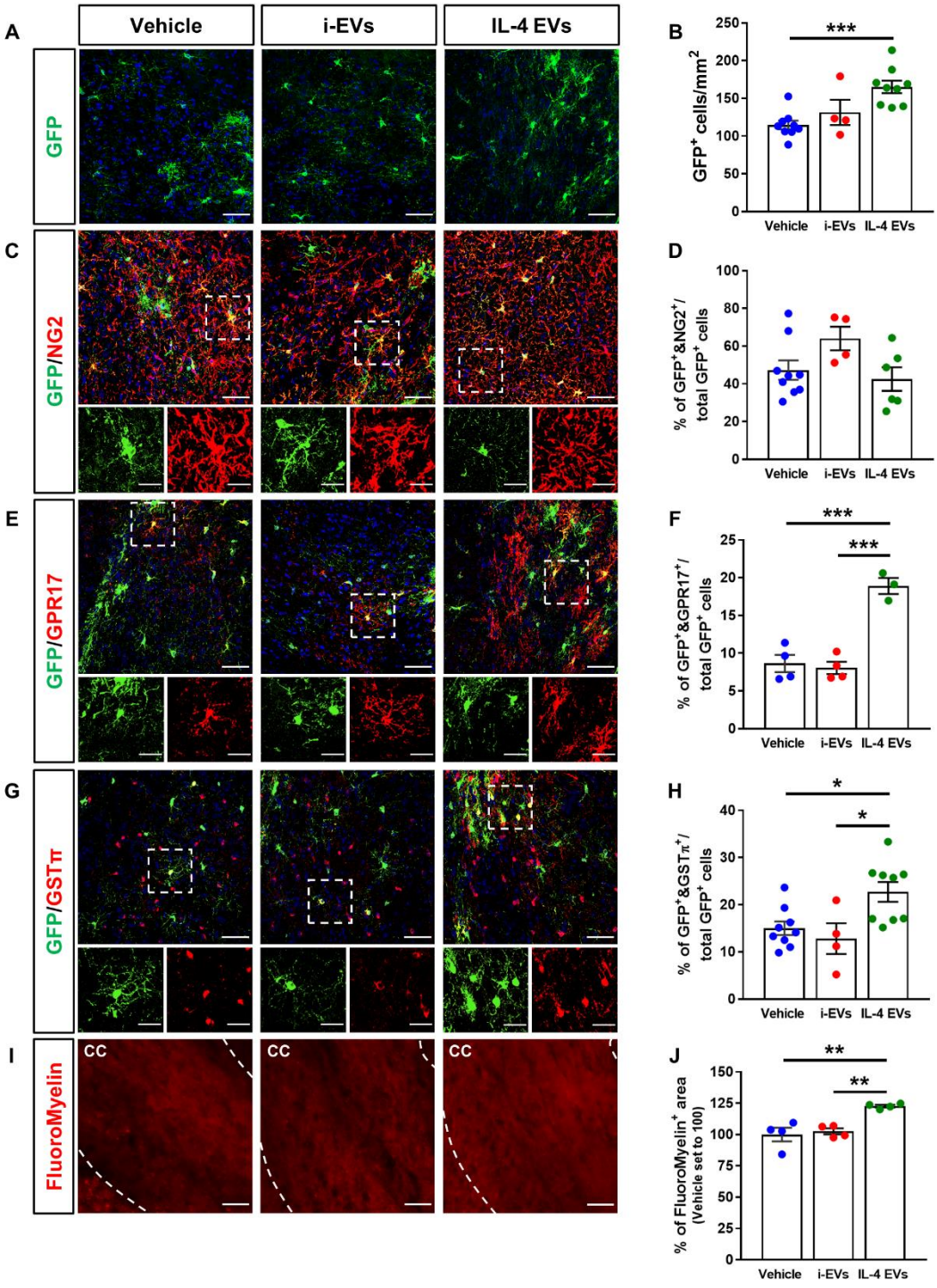

Figure 3.4 Infusion of pro-regenerative microglia-derived EVs at late stages after ischemia enhances GPR17-expressing OPC differentiation. *(A) Representative images of GFP⁺ OPCs at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. (B) Quantification of the density of GFP⁺ OPCs at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (n=4-9). Data are expressed as mean ± SE. *** p<0.0001; One-way ANOVA followed by Tukey's post-hoc analysis. (C) Representative images of cells stained for GFP and NG2 at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. Magnifications show cells co-expressing GFP and NG2. Scale bar: 25 μm. (D) Quantification of the percentage of GFP⁺ OPCs co-expressing NG2 at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (n=4-9). Data are expressed as mean ± SE. (E) Representative images of cells stained for GFP and GPR17 at the boundary of*

*ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. Magnifications show cells co-expressing GFP and GPR17. Scale bar: 25 μm. (F) Quantification of the percentage of GFP⁺ OPCs co-expressing GPR17 at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (n=3-4). Data are expressed as mean ± SE. *** p<0.0001; One-way ANOVA followed by Tukey's post-hoc analysis. (G) Representative images of cells stained for GFP and GSTπ at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. Magnifications show cells co-expressing GFP and GSTπ. Scale bar: 25 μm. (H) Quantification of the percentage of GFP⁺ OPCs co-expressing GSTπ at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (n=4-9). Data are expressed as mean ± SE. * p<0.05; One-way ANOVA followed by Tukey's post-hoc analysis. (I) Representative images of myelin visualized using FluoroMyelin Red stain in the corpus callosum (CC, delimited by white dashed lines) at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle. Scale bar: 50 μm. (J) Quantification of the percentage of FluoroMyelin⁺ area in the corpus callosum at the boundary of ischemic lesion (0-500 μm) at day 28 post-MCAo, following infusion of i-EVs, IL-4 EVs or vehicle (n=4). Data are expressed as mean ± SE and normalized to vehicle set to 100. ** p<0.01; One-way ANOVA followed by Tukey's post-hoc analysis.*

3.3 Infusion of pro-regenerative microglia-derived EVs fosters functional recovery of ischemic mice

The final goal of pro-regenerative therapeutic interventions in patients affected by cerebral ischemia is to limit the long-lasting persistence of cognitive and motor disability (Stinear, 2010, 2017). However, a proper evaluation of long-term functional deficits in ischemic mice is very challenging, due to fast spontaneous recovery occurring within the first week after stroke, as well as to compensatory mechanisms involving the intact contralateral hemisphere (Balkaya et al., 2018). While most of the common behavioral tests are therefore not adequate at late time points after cerebral ischemia, ischemic mice have been reported to manifest a persisting turning preference toward the ipsilateral side, with a consequent reduction in the percentage of contralateral turns, which could be utilized as a behavioral readout of post-stroke disability (Balkaya et al., 2018).

On this basis, the impact of IL-4 EVs on the turning preference of ischemic mice has been analyzed by performing the Y-maze test (Saadoun et al., 2010) at day 14 post-MCAo, before minipump implantation, and at day 28 post-MCAo, after IL-4 EVs or vehicle infusion (Figure 3.5A, B). At day 14 post-MCAo, ischemic animals showed a significant reduction in the percentage of contralateral turns as compared to sham-operated controls, while no significant differences in locomotor activity and spontaneous alternation were detected (Figure 3.5C-E). Interestingly, the turning preference was maintained in vehicle-treated animals at day 28 post-MCAo, while the infusion of IL-4 EVs significantly restored mice turning behavior at the level of sham-operated littermates (Figure 3.5F). Of note, no

abnormalities in locomotor activity and spontaneous alternation among the three experimental groups were found also at this time point (Figure 3.5G, H).

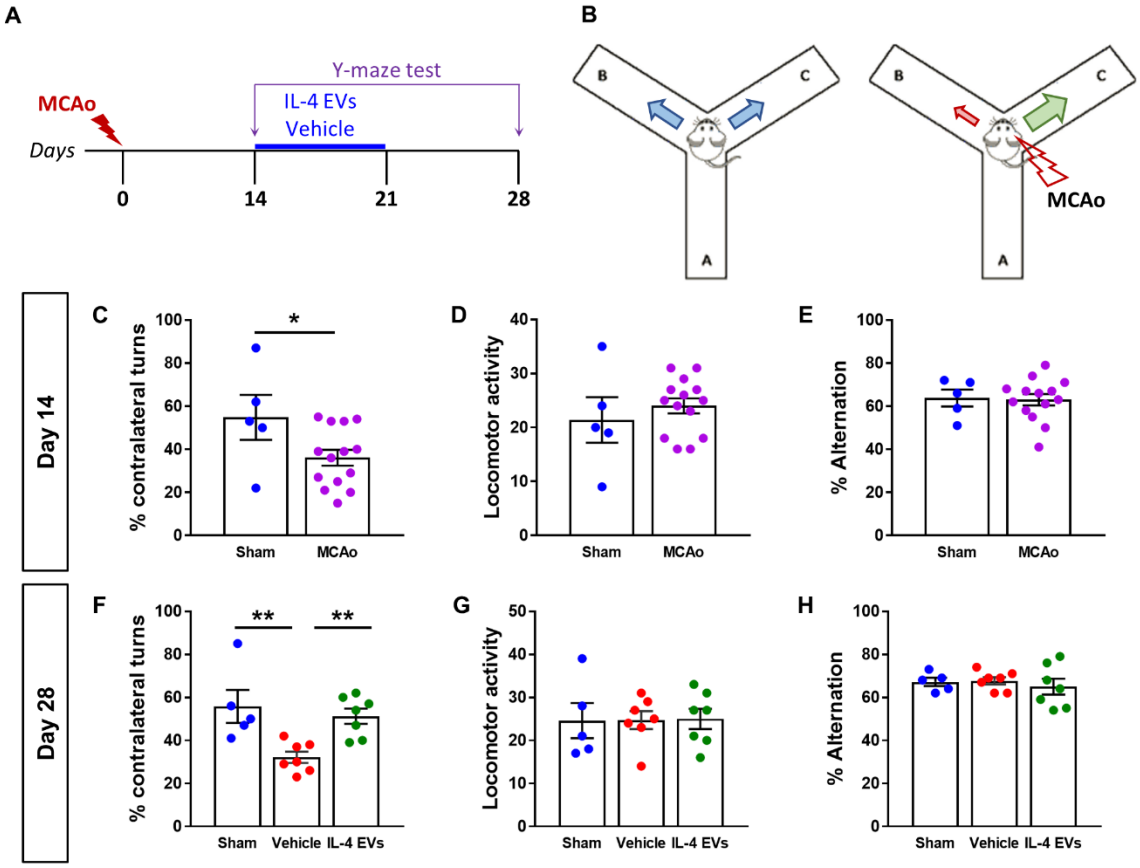

Figure 3.5 Infusion of pro-regenerative microglia-derived EVs at late stages after ischemia promotes functional recovery of ischemic mice. *(A) Schematic representation of the experimental protocol exploited for behavioral analysis before and after the infusion of microglia-derived EVs after MCAo. (B) Schematic representation of the Y-maze test to evaluate the turning preference of ischemic mice. (C) Quantification of the percentage of contralateral turns made in the Y-maze test at day 14 post-MCAo by MCAo mice (n=14) and sham-operated controls (n=5). Data are expressed as mean ± SE. * p<0.05; Student's t-test. (D) Quantification of the locomotor activity of MCAo mice (n=14) and sham-operated controls (n=5) during the Y-maze test at day 14 post-MCAo. Data are expressed as mean ± SE. (E) Quantification of the percentage of spontaneous alternations made in the Y-maze test at day 14 post-MCAo by MCAo mice (n=14) and sham-operated controls (n=5). Data are expressed as mean ± SE. (F) Quantification of the percentage of contralateral turns made in the Y-maze test at day 28 post-MCAo by sham-operated controls (n=5) and MCAo mice after infusion of IL-4 EVs (n=7) or vehicle (n=7). Data are expressed as mean ± SE. ** p<0.01; One-way ANOVA followed by Tukey's post-hoc analysis. (G) Quantification of the locomotor activity of sham-operated controls (n=5) and MCAo mice after infusion of IL-4 EVs (n=7) or vehicle (n=7) during the Y-maze test at day 28 post-MCAo. Data are expressed as mean ± SE. (H) Quantification of the percentage of spontaneous alternations made in the Y-maze test at day 28 post-MCAo by sham-operated controls (n=5) and MCAo mice after infusion of IL-4 EVs (n=7) or vehicle (n=7). Data are expressed as mean ± SE.*

In parallel, the impact of microglial EVs on neuronal integrity in the peri-infarct region, whose damage might be involved in turning behavior, has been evaluated by immunofluorescence for the neuronal marker NeuN and hematoxylin/eosin (HE) staining. Notably, at day 28 post-MCAo, the infusion of IL-4 EVs resulted in a significant reduction

of the percentage of ischemia-induced neuronal tissue loss as compared to vehicle-treated mice (Figure 3.6A-D), possibly contributing to the functional amelioration observed.

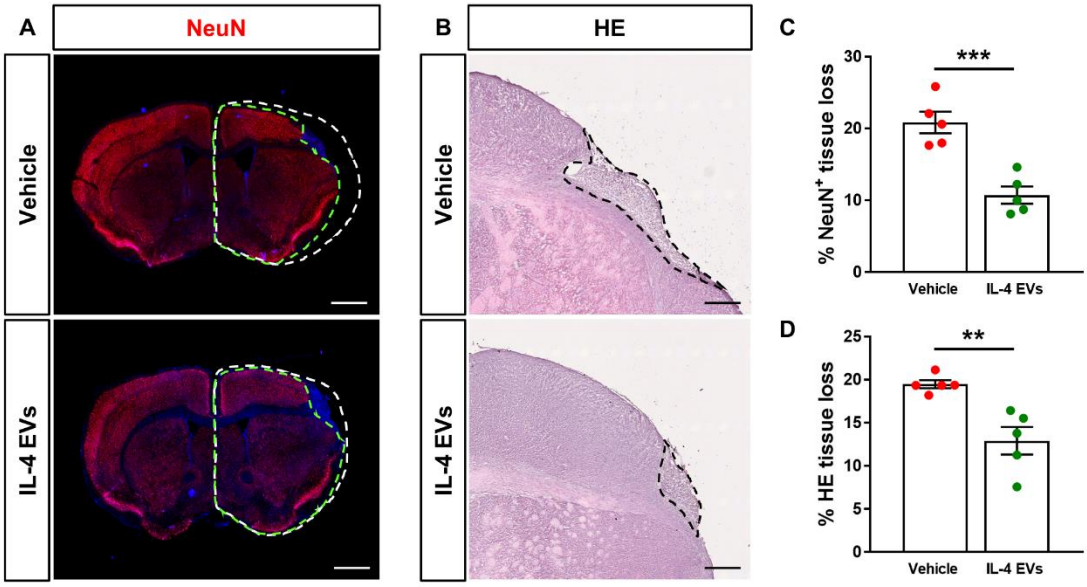

*Figure 3.6 IL-4 EVs administration limits ischemia-induced loss of neuronal tissue. (A) Representative images of NeuN⁺ tissue at day 28 post-MCAo, following infusion of IL-4 EVs or vehicle. Green dashed lines delineate the area of NeuN staining in the ipsilateral hemisphere. White dashed lines correspond to the projection of NeuN⁺ area in the intact contralateral hemisphere. Scale bar: 1 mm. (B) Representative images of the ischemic region in hematoxylin-eosin (HE) stained sections at day 28 post-MCAo, following infusion of IL-4 EVs or vehicle. Black dashed lines delineate the ischemic core. Scale bar: 400 µm. (C) Quantification of the percentage of NeuN⁺ tissue loss at day 28 post-MCAo after infusion of IL-4 EVs or vehicle (n=5). Data are expressed as mean ± SE. *** p<0.001; Student's t-test. (D) Quantification of the percentage of HE-labeled tissue loss at day 28 post-MCAo after infusion of IL-4 EVs or vehicle (n=5). Data are expressed as mean ± SE. ** p<0.01; Student's t-test.*

3.4 Microglia-derived EVs exert direct beneficial effects on GPR17-expressing OPC maturation

To explore the direct effects of IL-4 EVs on the pool of GFP⁺ cells, OPCs were isolated from GPR17-iCreER^{T2}:CAG-eGFP mice and cultured in presence of either IL-4 EVs or medium alone (CTRL) for 72 hours in differentiating conditions (Figure 3.7A). Notably, after *in vitro* exposure to 4-hydroxytamoxifen (OH-TAM), no significant differences were detected in the percentage of recombinant GFP⁺ cells between CTRL and IL-4 EVs-treated cultures (Figure 3.7B, C).

Immunofluorescence analysis showed a significant increase in the percentage of mature MBP⁺ cells in cultures exposed to IL-4 EVs compared to CTRL (Figure 3.7C, D), demonstrating a direct pro-differentiating effect of IL-4 EVs. Moreover, the fraction of GFP⁺

cells co-expressing MBP was found to be significantly higher in cultures exposed to IL-4 EVs with respect to CTRL, while no significant changes were observed in the GFP negative pool of progenitors (Figure 3.7E). These data suggest that the subpopulation of GFP⁺ OPCs, namely the GPR17-expressing pool of cells, is more prone to differentiate and more responsive to the pro-differentiating action of microglial EVs. Accordingly, after exposure to either CTRL or IL-4 EVs, the percentages of MBP⁺ cells were found to be significantly higher in the GFP⁺ OPC subset as compared to the GFP negative one (Figure 3.7E).

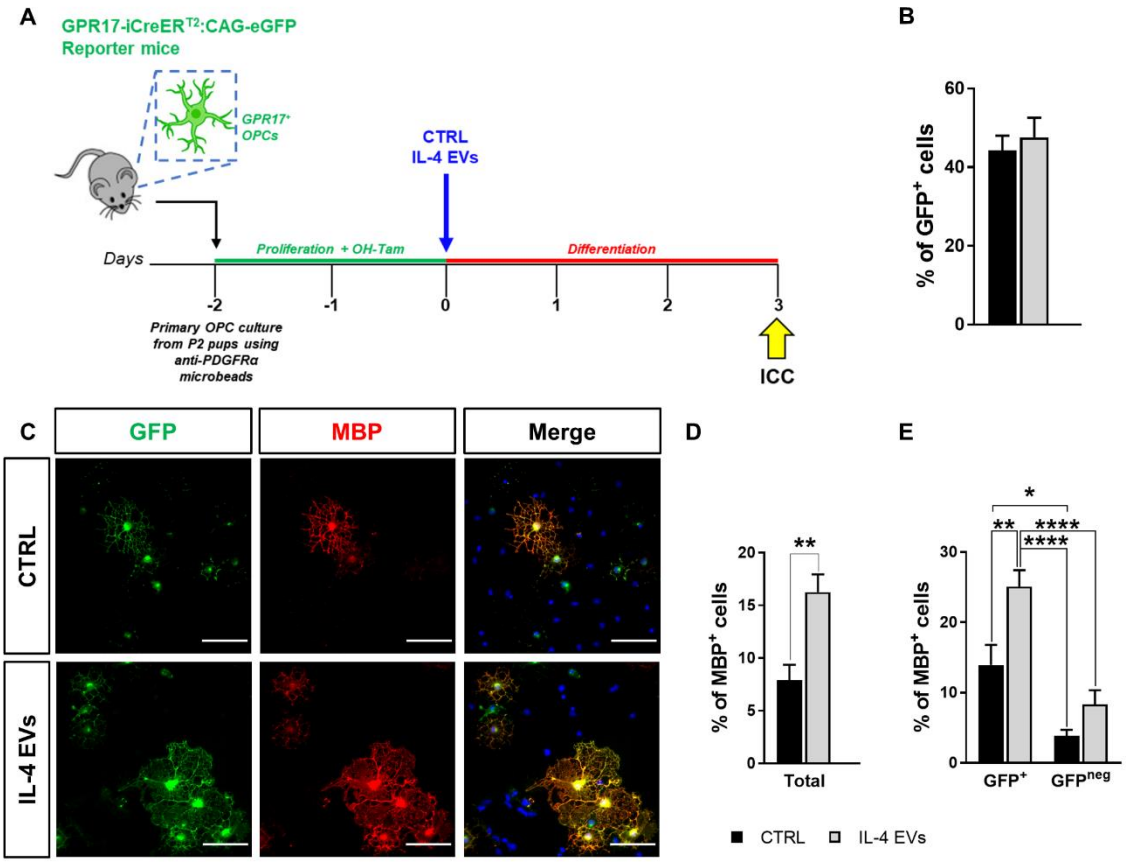

Figure 3.7 Direct effects of microglial IL-4 EVs on GFP+ OPC maturation in vitro. *(A) Schematic representation of the experimental protocol utilized to evaluate the effect of microglial EVs on primary cultured OPCs in vitro. (B) Quantification of the percentage of GFP⁺ cells in primary OPC cultures from GPR17-iCreER^{T2}:CAG-eGFP mice exposed to IL-4 EVs or CTRL. Data are expressed as mean ± SE (n = 9 coverslips from 3 independent experiments). (C) Representative images showing cells expressing GFP and MBP in primary OPC cultures from GPR17-iCreER^{T2}:CAG-eGFP mice exposed to IL-4 EVs or medium alone (CTRL). Scale bars, 50 μm. (D) Quantification of the total percentage of MBP⁺ cells in primary OPC cultures from GPR17-iCreER^{T2}:CAG-eGFP mice exposed to IL-4 EVs or CTRL. Data are expressed as mean ± SE (n = 9 coverslips from 3 independent experiments). ** p<0.01; Student's t test. (E) Quantification of the percentage of GFP⁺ and GFPneg cells co-expressing MBP in primary OPC cultures from GPR17-iCreER^{T2}:CAG-eGFP mice exposed to IL-4 EVs or CTRL. Data are expressed as mean ± SE (n = 9 coverslips from 3 independent experiments). * p<0.05, ** p<0.01, **** p<0.0001; One-way ANOVA followed by Tukey's post hoc analysis.*

3.5 Transmembrane tumor necrosis factor (tmTNF) is involved in the beneficial effects exerted by microglial EVs on OPC maturation

Recent evidence indicates that TNF is increased in EVs derived from activated microglia and monocyte-derived macrophages (Raffaele et al., 2020). EVs released by these myeloid cells are indeed particularly enriched in tmTNF, which has been associated with increased OL differentiation and remyelination both *in vitro* and *in vivo* by engaging the TNFR2 receptor (Desu et al., 2021; Madsen et al., 2016a). It is therefore reasonable to postulate that tmTNF may play a role in the pro-differentiating effects of IL-4 EVs.

To verify this hypothesis, primary OPCs were exposed to IL-4 EVs in the presence of the selective solTNF inhibitor XPro™1595 (XPro) or the non-selective solTNF and tmTNF blocker etanercept (ETN). Interestingly, results from these experiments showed that, while specific inhibition of solTNF by XPro did not affect the increase in the percentage of MBP^+ cells induced by IL-4 EVs, simultaneous blockade of tmTNF by ETN neutralized the pro-differentiating action of IL-4 EVs (Figure 3.8). These data provide concrete clues of a possible involvement of tmTNF/TNFR2 axis in the mechanism underlying the beneficial effect of microglial EVs on OPC maturation.

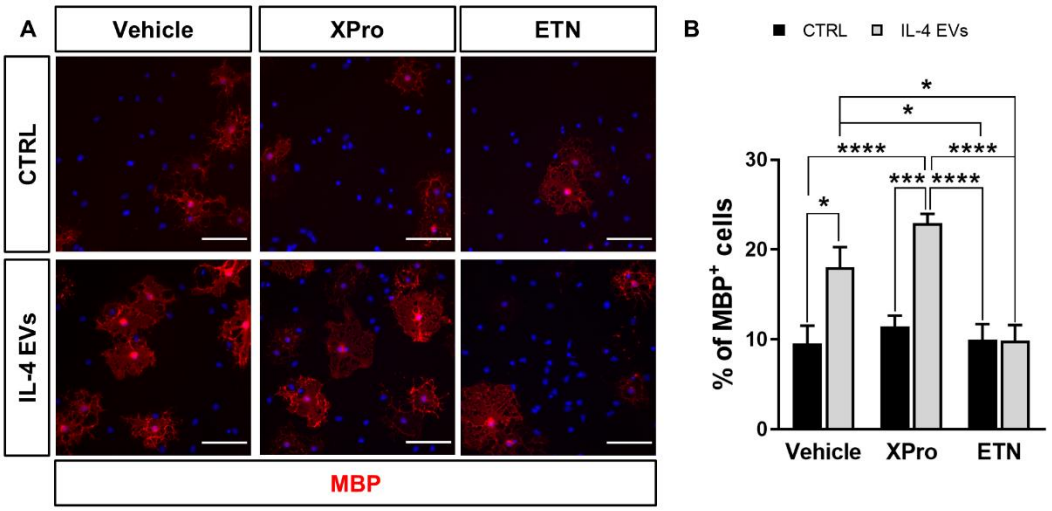

Figure 3.8 Involvement of tmTNF in the pro-differentiating properties of IL-4 EVs. *(A) Representative images showing MBP^+ cells in primary OPC cultures exposed to IL-4 EVs or CTRL in the presence or absence of the selective soluble TNF (solTNF) inhibitor XPro1595 (XPro) and of the nonselective TNF inhibitor etanercept (ETN). Scale bars, 50 μm. (B) Quantification of the percentage of MBP^+ cells in primary OPC cultures exposed to IL-4 EVs or CTRL in the presence or absence of XPro or ETN. Data are expressed as mean ± SE (n = 9 coverslips from 3 independent experiments). * p<0.05, *** p<0.001, **** p<0.0001; One-way ANOVA followed by Tukey's post hoc analysis.*

3.6 Microglial EVs enhance OPC maturation by transcriptionally regulating cellular energy metabolism and stress response

To detect the early molecular changes induced by microglial EVs which may be responsible for enhanced OPC maturation, a transcriptomic profiling of primary OPCs exposed to microglial IL-4 EVs, i-EVs or vehicle (CTRL) for 24 hours in differentiating conditions has been performed (Figure 3.9A).

Results revealed prominent transcriptional changes induced by IL-4 EVs compared to CTRL, while i-EVs were less potent (1143 DEGs IL-4 EVs vs CTRL, 103 DEGs i-EVs vs CTRL; Figure 3.9B). Interestingly, among the genes significantly modulated by IL-4 EVs, several were found to be involved in cellular energy and lipid metabolism, including among the others *Fasn* encoding for the enzyme fatty acid synthase, *Lrp1* and *Lrp6* encoding for low-density lipoprotein receptors, *Sdhc* and *Sdhd* encoding for subunits of the succinate dehydrogenase (also known as complex II of the mitochondrial electron transport chain), and *Ldhb* encoding for lactate dehydrogenase (Figure 3.9C). Moreover, several genes encoding for pro-apoptotic proteins (i.e., Caspase 3 and SIVA1 Apoptosis Inducing Factor) and factors implicated in the response to oxidative stress (i.e., peroxiredoxins 3 and 4, glutathione S-transferase, carbonyl reductase 1) were found to be significantly downregulated after exposure to IL-4 EVs (Figure 3.9D). Of particular interest, the expression of *Chd7*, encoding for a helicase known to control OPC differentiation through chromatin opening and transcriptional activation of key regulators like *Sox10* and *Gpr17* (Marie et al., 2018), was significantly increased in cells after exposure to IL-4 EVs (Figure 3.9E), suggesting that also epigenetic mechanisms might partially underpin the differentiation effects induced by microglial EVs.

To identify the biological processes affected by exposure to IL-4 EVs, functional enrichment analysis has been performed using the Ingenuity Pathway Analysis (IPA) software. Results unveiled several biological processes significantly modulated by IL-4 EVs, which were found to be mostly related to mitochondrial ATP synthesis, histone deacetylation, DNA repair machinery, and protein synthesis (Figure 3.9F). Taken together, these data suggest that microglial EVs exert their pro-differentiating effect on target OPCs by improving the metabolic state of recipient cells, placing them in the proper bioenergetic conditions for differentiation (Rosko et al., 2019).

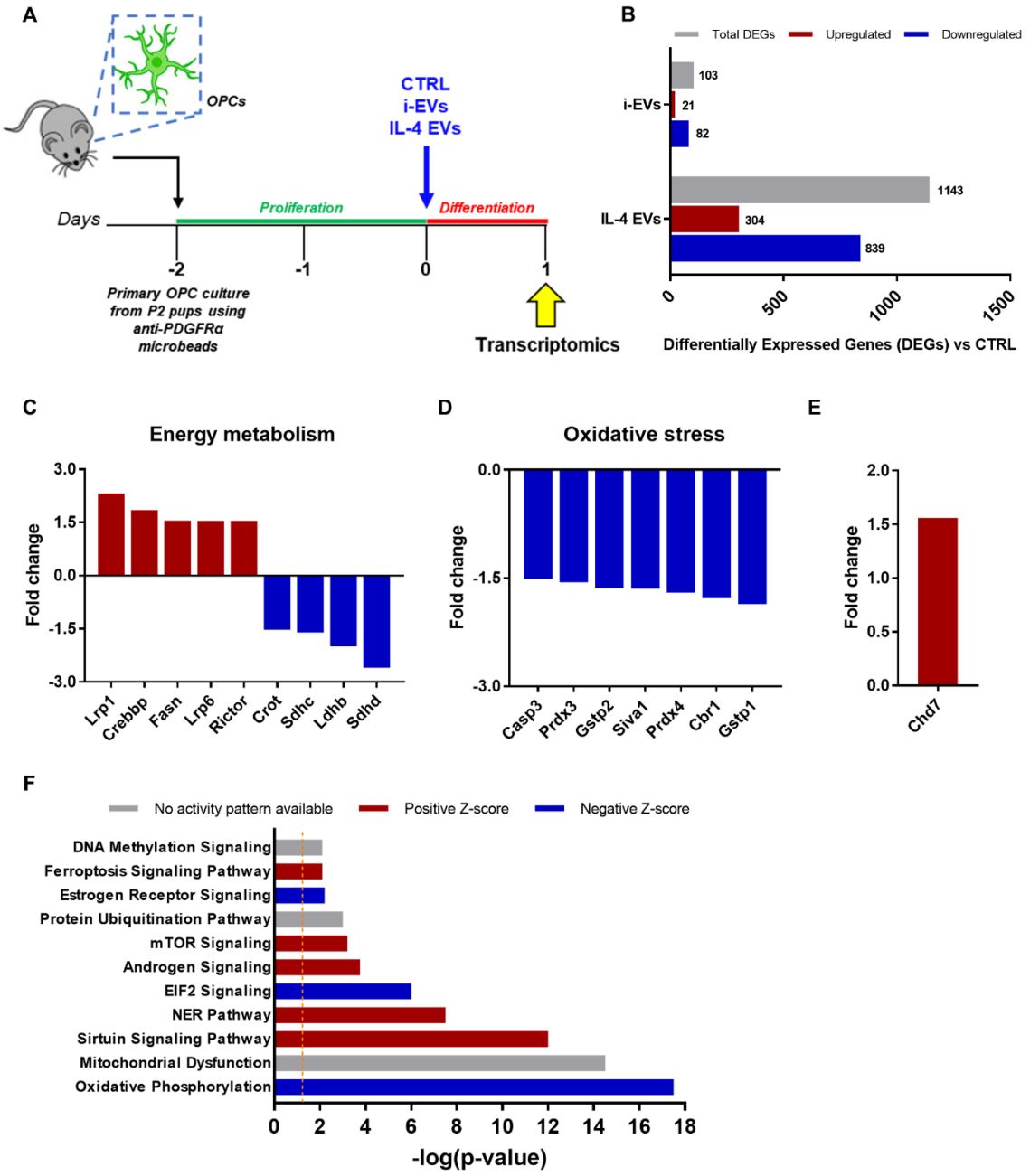

Figure 3.9 Early transcriptional changes underpinning enhanced OPC maturation after exposure to IL-4 EVs. *(A) Schematic representation of the experimental protocol utilized to evaluate the transcriptomic profile of primary cultured OPCs exposed to IL-4 EVs, i-EVs or CTRL in vitro. (B) Histogram showing the number of total differentially expressed genes (DEGs, in gray), upregulated DEGs (in red), and downregulated DEGs (in blue), in primary OPCs exposed to i-EVs or IL-4 EVs as compared to CTRL (n=3). The analysis was carried out on 65.956 mouse genes. Criteria for defining DEGs included fold change>1.5 and FDR-corrected p-value<0.05. (C) Histogram showing the average fold change of specific DEGs involved in cellular energy metabolism in primary OPCs exposed to IL-4 EVs compared to CTRL. (D) Histogram showing the average fold change of specific DEGs involved in oxidative stress response in primary OPCs exposed to IL-4 EVs compared to CTRL. (E) Histogram showing the average fold change of Chd7 gene expression in primary OPCs exposed to IL-4 EVs compared to CTRL. (F) Graph showing the biological processes significantly modulated in primary OPCs exposed to IL-4 EVs compared to CTRL. Positive Z-score (in red) indicates that the pathway is globally activated, negative Z-score (in blue) indicates that the pathway is globally inhibited.*

By exploiting the Upstream Regulator tool of IPA, it was also possible to unravel the cascade of upstream transcriptional regulators potentially responsible for the transcriptional changes observed (Table 3.1). Notably, many of them have been previously implicated in cellular energy metabolism (RICTOR, CPT1B, CLPP, STK11), potentially representing novel therapeutic targets to enhance OPC differentiating capacity.

Table 3.1 Predicted upstream regulators involved in the transcriptional effects of microglial IL-4 EVs

Upstream Regulator	Molecule Type	Activation z-score
RICTOR	other	6,325
CPT1B	enzyme	3,790
KDM5A	transcription regulator	3,742
NR4A1	ligand-dependent nuclear receptor	2,887
CLPP	peptidase	2,646
Tcf7	transcription regulator	2,000
MYCL	transcription regulator	-2,178
NFE2L1	transcription regulator	-2,236
MLXIPL	transcription regulator	-2,331
MIR17HG	other	-2,433
PKD1	ion channel	-2,433
FOXO1	transcription regulator	-2,828
HBA1/HBA2	transporter	-3,162
Hbb-b1	transporter	-3,162
NFE2L2	transcription regulator	-3,233
RB1	transcription regulator	-3,742
MYC	transcription regulator	-3,742
STK11	kinase	-4,642

3.7 TNFR2 regulates protective microglia activation at early stages after stroke

The identification of the mechanisms regulating the pro-remyelinating properties of microglia *in vivo* may help to create a permissive niche, facilitating OPC recruitment and differentiation for efficient remyelination. In this respect, TNFR2, the main receptor for tmTNF, was shown to control microglial pro-regenerative functions, including the release of neuroprotective factors, tissue surveillance, and phagocytosis (Gao et al., 2017). However, the contribution of microglial TNFR2 in myelin protection and repair after stroke has never been investigated.

To fill this gap, conditional knockout CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice, namely transgenic animals in which microglia-selective TNFR2 ablation can be achieved by exploiting the different origins and turnover rates of microglia versus other CX3CR1-expressing peripheral myeloid cells (Gao et al., 2017), have been utilized. To evaluate if TNFR2 deficiency affects microglia activation after stroke, female microglial TNFR2 knockout mice and control littermates have been subjected to MCAo and sacrificed at day 1 and 5 post-stroke for molecular analyses (Figure 3.10A).

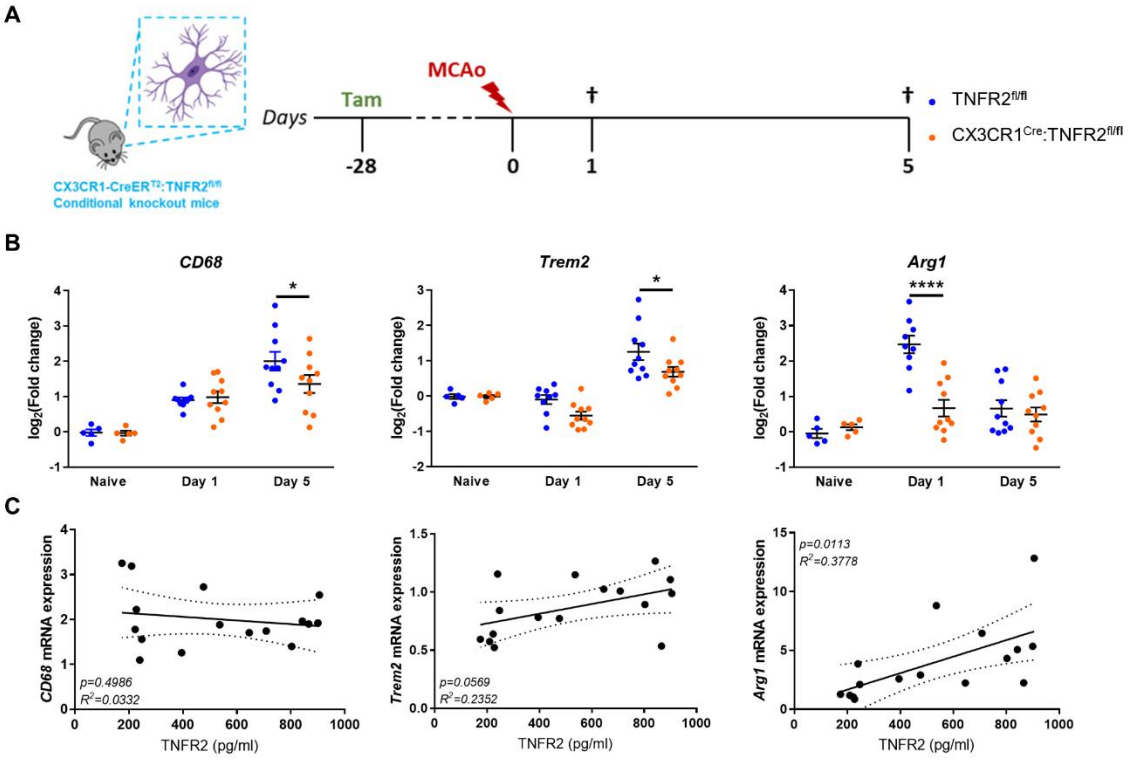

Figure 3.10 Microglial TNFR2 ablation decreases the expression of pro-regenerative microglial genes at early stages after stroke. *(A) Schematic representation of the experimental protocol exploited to study the role of microglial TNFR2 after MCAo. (B) Gene expression of markers involved in microglia phagocytosis (CD68 and Trem2) and pro-*

*regenerative activation (Arg1) in bulk brain lysates from CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and wildtype littermates (TNFR2$^{fl/fl}$) in naïve conditions (n=5) and at day 1 and 5 after MCAo (n=10). Data are shown as mean ± SE and normalized to naïve TNFR2$^{fl/fl}$ mice set to 0. *p<0.05, **** p<0.0001; Two-way ANOVA followed by Tukey's post hoc analysis. (C) Scatterplot representation of the linear correlation between the protein levels of TNFR2 (x axis) and CD68, Trem2, or Arg1 mRNA expression (y axis) in bulk brain lysates from CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and wildtype littermates (TNFR2$^{fl/fl}$) at day 1 after MCAo. For correlation analysis, two-tailed Pearson test was used.*

The expression levels of genes involved in microglia phagocytosis (*CD68* and *Trem2*) and regenerative polarization (*Arg1*) in bulk brain tissue have been quantified by qPCR, revealing a significant reduction in TNFR2-deficient mice compared to control littermates (Figure 3.10B) and a linear correlation between the expression of these genes and TNFR2 protein levels (Figure 3.10C). These data suggest that the pro-regenerative functions of microglia observed at early stages after MCAo, necessary to initiate OPC recruitment and remyelination (Raffaele et al., 2021), are impaired in the absence of microglial TNFR2.

To further characterize the effects of TNFR2 ablation on the functional phenotype of microglia at the borders of the ischemic lesion, an immunofluorescence analysis was carried out in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and control littermates at day 5 post-MCAo (Figure 3.11A, B). A morphological analysis revealed a significant reduction of the cell size and surveilled area of Iba1^{+} microglial cells (Figure 3.11C, D), suggesting reduced activation of microglia in TNFR2 knockout mice compared to controls. Accordingly, double staining for Iba1 and the scavenger receptor CD68 or the regenerative marker Gal-3, both required for microglial beneficial functions after stroke (see paragraph 1.3.2), showed a significant reduction of the area fraction decorated by these two markers and of their co-localization with Iba1 at the border of the ischemic lesion (Figure 3.11E, F).

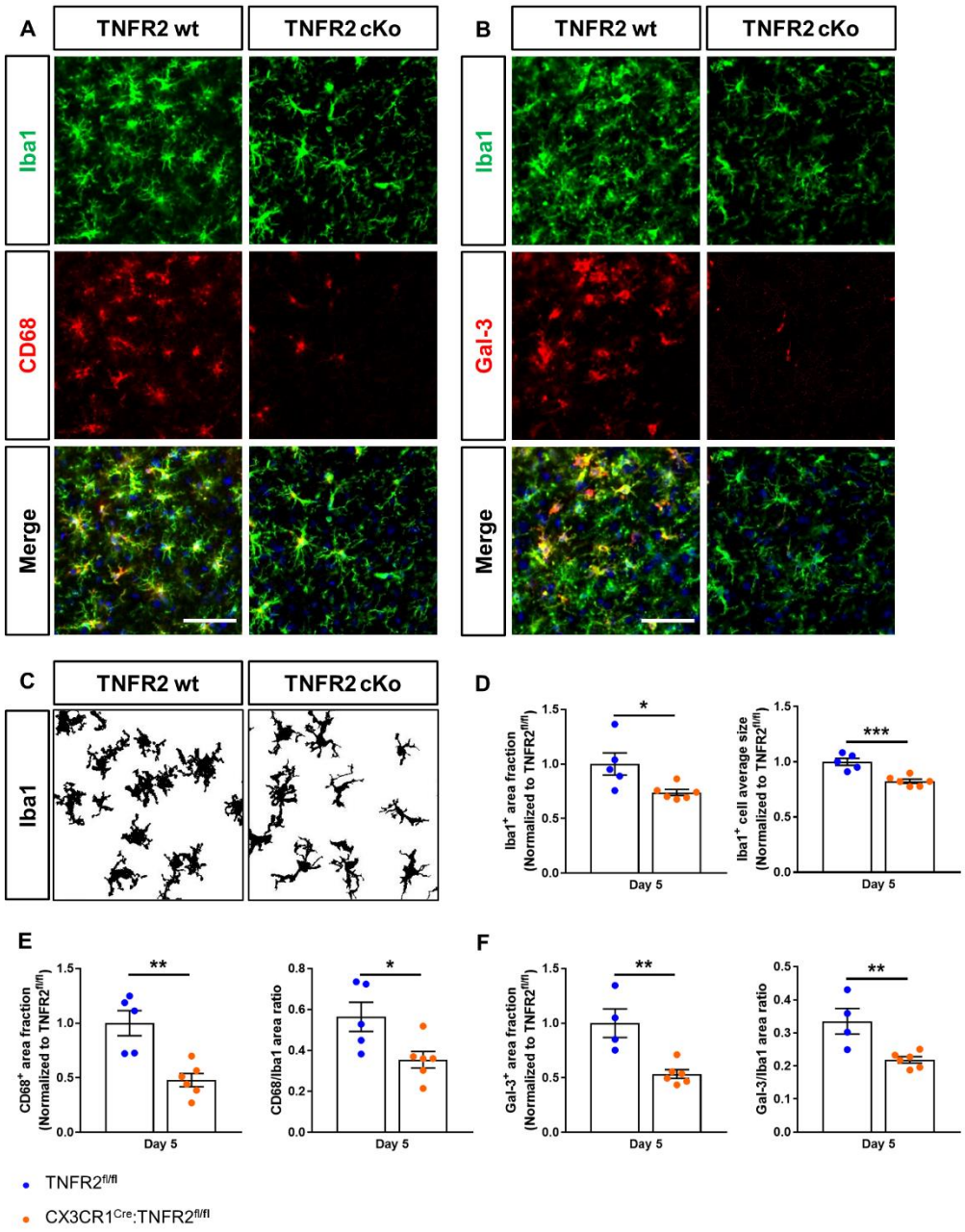

Figure 3.11 TNFR2 ablation affects microglial morphology and functional phenotype at the border of the ischemic lesion. *(A) Representative images of cells stained for Iba1 and CD68 at the boundary of ischemic lesion (0-500 µm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ (TNFR2 cKo) mice and TNFR2$^{fl/fl}$ wildtype littermates (TNFR2 wt) at day 5 post-MCAo. Scale bar: 50 µm. (B) Representative images of cells stained for Iba1 and galectin-3 (Gal-3) at the boundary of ischemic lesion (0-500 µm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ (TNFR2 cKo) mice and TNFR2$^{fl/fl}$ wildtype littermates (TNFR2 wt) at day 5 post-MCAo. Scale bar: 50 µm. (C) Representative images of the binary mask of Iba1 staining at the boundary of ischemic lesion (0-500 µm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ (TNFR2 cKo) mice and TNFR2$^{fl/fl}$ wildtype littermates (TNFR2 wt) at day 5 post-MCAo, utilized to quantify microglia morphology by particle analysis. (D) Quantification of Iba1^{+} area fraction and average cell size at the boundary of ischemic lesion (0-500 µm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and TNFR2$^{fl/fl}$ wildtype littermates at day 5 post-MCAo (n=5-6). Data are expressed as mean ± SE and normalized to TNFR2$^{fl/fl}$ set to 1. * p<0.05, *** p<0.001; Student's t-test. (E) Quantification of CD68^{+} area fraction and CD68/Iba1 area ratio at the boundary of ischemic lesion (0-500 µm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and TNFR2$^{fl/fl}$ wildtype littermates at day 5 post-MCAo (n=5-6). Data are expressed as mean ± SE. * p<0.05, ** p<0.01; Student's t-test. (F) Quantification of Gal-3^{+} area fraction and Gal-3/Iba1 area ratio at the boundary of ischemic lesion (0-500 µm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and TNFR2$^{fl/fl}$ wildtype littermates at day 5 post-MCAo (n=4-6). Data are expressed as mean ± SE. ** p<0.01; Student's t-test.*

Finally, a pilot flow cytometry experiment has been performed to quantify the total number of microglia and infiltrating immune cells in the ipsilateral cortex of CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and control littermates at day 5 post-MCAo. Preliminary data suggest that microglial TNFR2 ablation resulted in increased infiltration of lymphocytes and monocytes from the blood circulation, which are believed to exert detrimental effects on ischemic damage evolution (Iadecola and Anrather, 2011), and in a reduction of the global number of microglial cells as compared to wildtype mice (Figure 3.12A, B). Of note, the number of CD11c^{+} microglia, a subpopulation of microglial cells specifically associated with developmental myelination and strongly activated in severe demyelinating conditions (Wlodarczyk et al., 2015, 2017), was instead found to be increased in microglial TNFR2-ablated mice with respect to controls (Figure 3.12B).

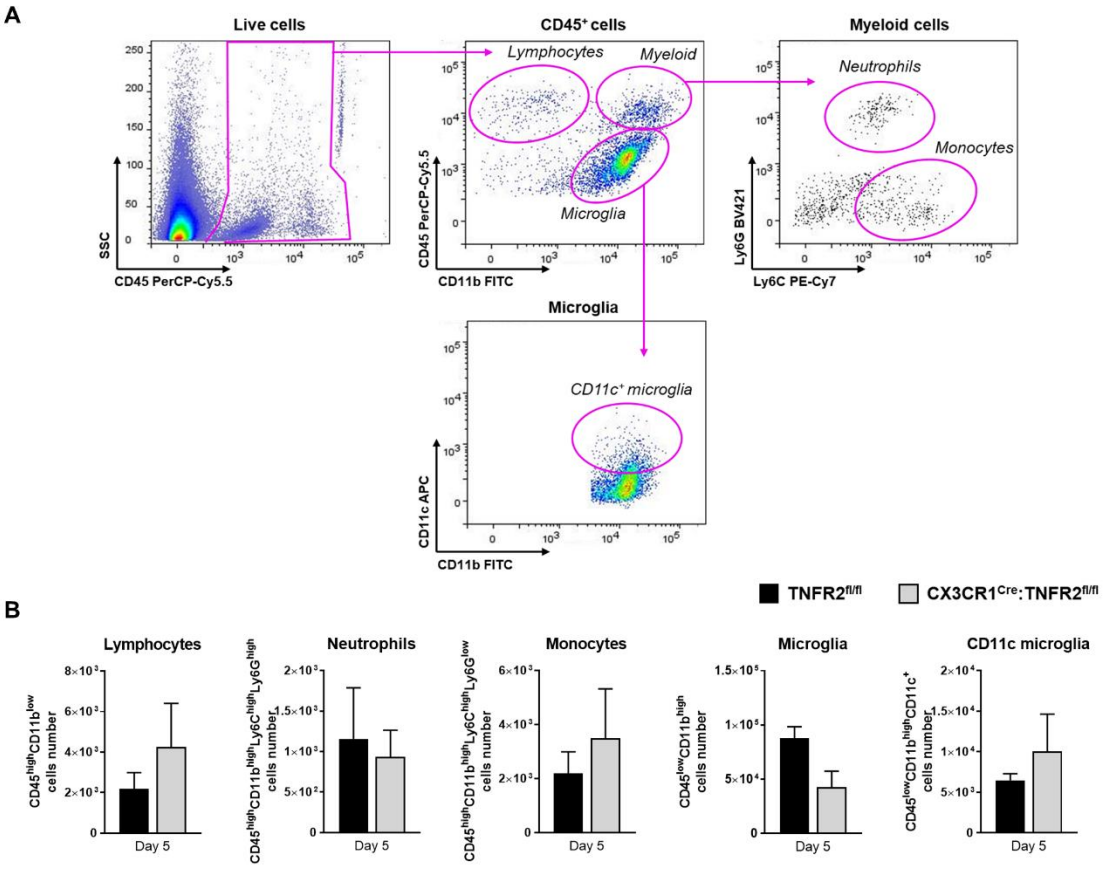

Figure 3.12 Effects of microglial TNFR2 ablation on immune cell infiltration and microglia numbers at early stages after stroke. *(A) Gating strategy used in the flow cytometry experiment for CD45^{+} cells, CD45^{high}CD11b^{low} lymphocytes, CD45^{high}CD11b^{high} myeloid cells, CD45^{high}CD11b^{high}Ly6C^{high}Ly6G^{high} neutrophils, CD45^{high}CD11b^{high}Ly6C^{high}Ly6G^{low} monocytes, CD45^{low}CD11b^{high} microglia, and CD45^{low}CD11b^{high}CD11c^{+} microglia. (B) Quantification of the number of CD45^{high}CD11b^{low} lymphocytes, CD45^{high}CD11b^{high}Ly6C^{high}Ly6G^{high} neutrophils, CD45^{high}CD11b^{high}Ly6C^{high}Ly6G^{low} monocytes, CD45^{low}CD11b^{high} microglia, and CD45^{low}CD11b^{high}CD11c^{+} microglia in the ipsilateral cortex of CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and wildtype littermates (TNFR2$^{fl/fl}$) at day 5 after MCAo (n=3).*

3.8 Microglial TNFR2 ablation negatively impacts OPC remyelinating attempts and myelin integrity

To evaluate the effects of microglial TNFR2 ablation on OPC response after stroke, the expression levels of *Ng2* and *Bcas1*, markers of OPCs and remyelinating OLs respectively, have been measured in bulk brain lysates from microglial TNFR2 knockout mice and wildtype littermates. Results showed that *Ng2* and *Bcas1* expression were significantly lower in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ at day 1 post-MCAo (Figure 3.13A). However, no significant correlation was found between *Ng2* and *Bcas1* mRNA expression and TNFR2 protein levels (Figure 3.13B).

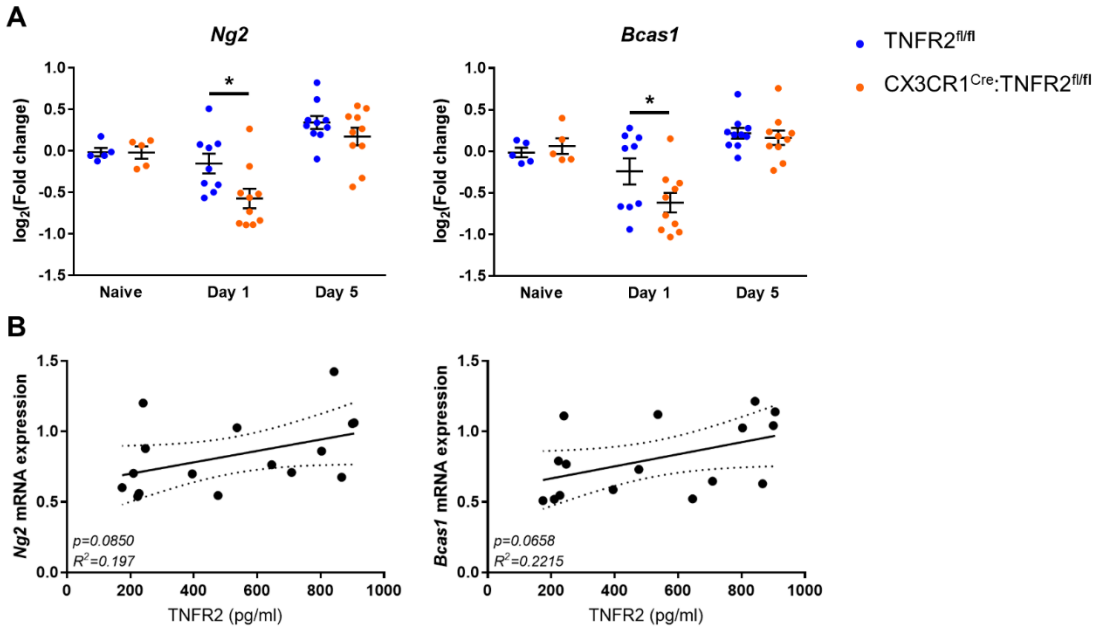

Figure 3.13 Expression of genes related to remyelinating OPCs is reduced in microglial TNFR2-ablated mice at day 1 post-MCAo. *(A) Gene expression of the OPC marker Ng2 and the remyelinating OL marker Bcas1 in bulk brain lysates from CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and wildtype littermates (TNFR2$^{fl/fl}$) in naïve conditions (n=5) and at day 1 and 5 after MCAo (n=10). Data are shown as mean ± SE and normalized to naïve TNFR2$^{fl/fl}$ mice set to 0. *p<0.05; Two-way ANOVA followed by Tukey's post hoc analysis. (B) Scatterplot representation of the linear correlation between the protein levels of TNFR2 (x axis) and Ng2 or Bcas1 mRNA expression (y axis) in bulk brain lysates from CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and wildtype littermates (TNFR2$^{fl/fl}$) at day 1 after MCAo. For correlation analysis, two-tailed Pearson test was used.*

Next, a novel protocol has been set up and utilized to analyze OL differentiation after stroke by flow cytometry (Figure 3.14A). Results of this pilot experiment suggest a tendency to reduction in the number of OPCs and immature OLs in the ipsilateral cortex of CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice compared to TNFR2$^{fl/fl}$ littermates at day 5 post-MCAo (Figure 3.14B). Hence, gene expression and flow cytometry data indicate that OPC reactivity to ischemic damage might be reduced in the absence of microglial TNFR2.

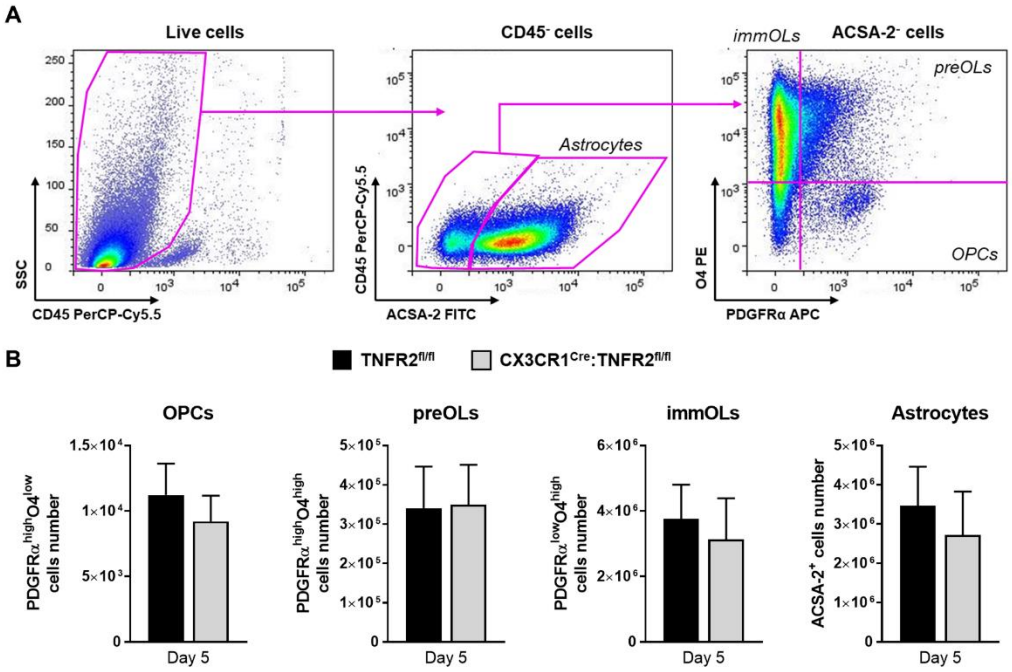

Figure 3.14 Ablation of TNFR2 in microglia results in reduced numbers of OPCs and immature oligodendrocytes (immOLs) in the ipsilateral cortex. *(A) Gating strategy used in the flow cytometry experiment for CD45⁻ cells, ACSA-2⁺ astrocytes, PDGFRα^{high}O4^{low} OPCs, PDGFRα^{high}O4^{high} pre-oligodendrocytes (preOLs), and PDGFRα^{low}O4^{high} immature oligodendrocytes (immOLs). (B) Quantification of the number of PDGFRα^{high}O4^{low} OPCs, PDGFRα^{high}O4^{high} preOLs, PDGFRα^{low}O4^{high} immOLs, and ACSA-2⁺ astrocytes in the ipsilateral cortex of CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and wildtype littermates (TNFR2$^{fl/fl}$) at day 5 after MCAo (n=3).*

Then, to evaluate if microglial TNFR2 ablation affected myelin integrity at the borders of the ischemic lesion, an immunofluorescence analysis for the myelin marker MBP has been performed (Figure 3.15). Notably, a significant reduction of the area covered by MBP⁺ myelinated fibers and of the integrated density of MBP staining was found in the peri-lesion cortex of TNFR2-knockout mice at day 5 post-stroke as compared to controls (Figure 3.15B).

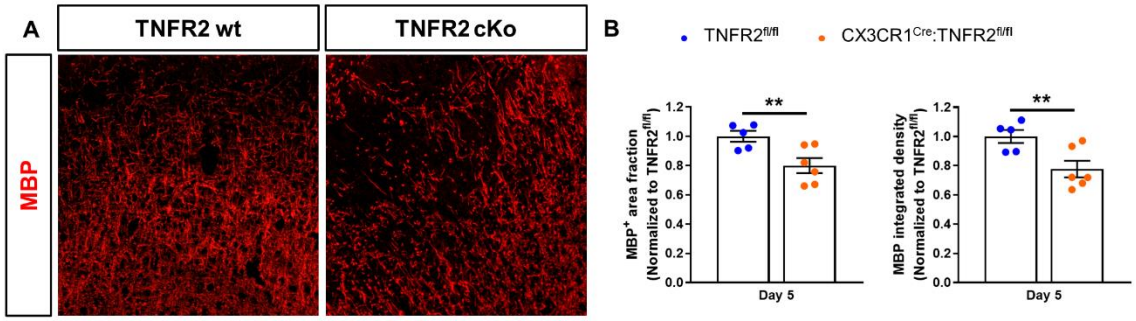

Figure 3.15 Peri-infarct myelin integrity is reduced in microglial TNFR2 knockout mice. *(A) Representative images of MBP⁺ myelinated fibers at the boundary of ischemic lesion (0-500 μm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ (TNFR2 cKo) mice and TNFR2$^{fl/fl}$ wildtype littermates (TNFR2 wt) at day 5 post-MCAo. (B) Quantification of MBP⁺ area fraction and integrated density at the boundary of ischemic lesion (0-500 μm) in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and TNFR2$^{fl/fl}$ wildtype littermates at day 5 post-MCAo (n=5-6). Data are expressed as mean ± SE and normalized to TNFR2$^{fl/fl}$ set to 1. ** p<0.01; Student's t-test.*

Finally, the effects of microglial TNFR2 knockout on mice motor functionality have been assessed at baseline, day 3, and day 5 post-MCAo using the rotarod test. Interestingly, results show signs of impaired performance in microglial TNFR2-ablated mice compared to controls (Figure 3.16), which might be, at least partially, related to the myelination defects observed.

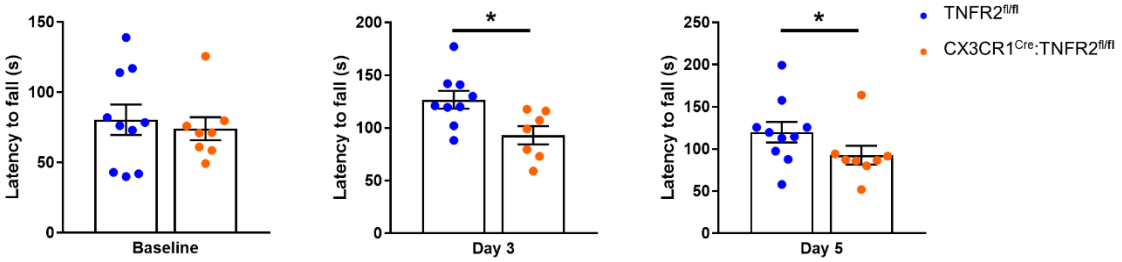

*Figure 3.16 Microglial TNFR2 ablated mice display worse motor performance. Quantification of the latency to fall from the rotarod in CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice and TNFR2$^{fl/fl}$ wildtype littermates at baseline, day 3, and day 5 post-MCAo (n=8-10). Data are expressed as mean ± SE. * p<0.05; Student's t-test.*

Taken together, these results support a role of TNFR2 in promoting a myelin-protective activation of microglia during the early phase after stroke. Accordingly, ablation of microglial TNFR2 compromises OPC remyelinating attempts and myelin integrity, contributing to increased behavioral deficits.

4. DISCUSSION

Thanks to recent advances in emergency care, the survival rate of ischemic stroke patients is significantly increasing (Campbell et al., 2019). However, due to the lack of effective therapies able to reduce brain damage and stimulate tissue repair, these patients suffer of long-lasting neurological disabilities (Shi et al., 2015). Recognizing the mechanisms underlying progressive damage after ischemic stroke is therefore necessary to identify new therapies improving long-term neurological functions (Barthels and Das, 2020). Besides degeneration of neuronal cells within the gray matter, recent studies highlighted the equal importance of axonal demyelination and white matter disruption in the functional deficits caused by ischemic injury (Matute et al., 2013). On this basis, promoting remyelination is currently recognized as a promising therapeutic strategy to improve post-stroke sensory-motor capacities (Fumagalli et al., 2016; Plemel et al., 2014). In this respect, the subpopulation of OPCs expressing the P2Y-like receptor GPR17 represents an attractive target for remyelinating therapies, given their elevated regenerative potential and capacity to react to ischemic injury (Bonfanti et al., 2017; Lecca et al., 2020). Nevertheless, the spontaneous differentiation capabilities of GPR17-expressing OPCs after stroke appear to be limited (Bonfanti et al., 2017), suggesting that the inflammatory lesion environment, mainly regulated by microglia, might be a key determinant of remyelination efficiency (Lloyd and Miron, 2019). Accordingly, our recent data show that microglia initially acquire a pro-regenerative phenotype to promote GPR17-expressing OPC recruitment to the ischemic lesion and to preserve myelin integrity (Raffaele et al., 2021). However, the protective functions of microglia are lost at later stages after stroke, leading to remyelination failure due to impaired differentiation of GPR17-expressing cells (Raffaele et al., 2021). Therapeutic interventions capable, at the same time, of directly promoting OPC maturation and of sustaining a pro-regenerative local microenvironment may therefore represent the best option to achieve efficient remyelination and functional recovery.

4.1 The therapeutic potential of microglial EVs for stroke treatment

The release of EVs, microscopic cellular structures containing multiple molecular signals, by microglia recently attracted the attention of researchers in the field. Besides their role as crucial mediators of intercellular communication (Budnik et al., 2016), microglial EVs emerged as biomarkers of disease progression and patient's response to pharmacological

treatment, due to the possibility to acquire information on damage evolution by detecting and quantifying them in biological fluids (Verderio et al., 2012). Notably, the molecular composition of microglial EVs strictly reflects the activation state of donor cells, thus conveying signals able to activate protective mechanisms or to sustain detrimental processes (Garzetti et al., 2014; Nigro et al., 2016; Prada et al., 2018). In the present thesis, attention has been focused on the effects of EVs released by pro-regenerative activated microglia (IL-4 EVs), based on previous studies showing their ability to significantly resume protective functions in recipient microglia at the site of CNS injury (Casella et al., 2018; Grimaldi et al., 2019), and to directly promote OPC differentiation and myelination both *in vitro* and following lysolecithin-induced demyelination *in vivo* (Lombardi et al., 2019). The results collected for this thesis demonstrated that the infusion of IL-4 EVs in the ipsilateral corpus callosum of ischemic mice starting from day 14 post-MCAo, corresponding to the late stage after stroke, provided multifaceted benefits by acting on both microglia and GPR17-expressing OPCs, labeled by GFP expression in this study, at the borders of the ischemic lesion, ultimately leading to improved functional recovery of ischemic mice.

In detail, IL-4 EVs were found to promote a shift of recipient pro-inflammatory and dystrophic microglial cells at the boundary of the ischemic lesion toward a pro-regenerative phenotype, as highlighted by the increased expression of the protective marker Ym1 and by morphological rearrangements, indicative of a recovery from a senescent-like phenotype to a functional one (Savage et al., 2019), contributing to create a permissive environment for remyelination. Conversely, i-EVs further increased the pro-inflammatory activation of recipient microglia, suggesting that the immunomodulatory role of microglial EVs is strictly dependent on the phenotype of donor cells. This is in line with previous studies demonstrating a role of microglial EVs in spreading inflammatory signals within the injured CNS, amplifying detrimental responses originated at the site of lesion (Verderio et al., 2012). Since excessive neuroinflammation and aberrant microglia activation represent common pathogenetic mechanisms to different neurological disorders, the identification of the molecular components responsible for the beneficial effects of IL-4 EVs on microglial phenotype, which goes beyond the aims of the present thesis, might provide novel candidate targets to sustain the regenerative functions of microglia during CNS repair.

Of note, IL-4 EVs were also able to increase the number of GFP^+ OPCs at lesion boundaries and to enhance their maturation, as indicated by the higher percentage of cells co-expressing GFP and GPR17, herein used as a marker for the intermediate stage of OL differentiation, or the mature OL marker GSTπ. These results are also supported by *in vitro* data, obtained using primary cultures of OPCs isolated from GPR17-iCreER^{T2}:CAG-eGFP mice, demonstrating that microglial IL-4 EVs have a direct effect on GFP^+ OPCs, promoting their differentiation into MBP-expressing cells. Accordingly, infusion of IL-4 EVs significantly increased myelin integrity in the ipsilateral corpus callosum as showed by FluoroMyelin staining, suggesting that more efficient remyelination occurred. On the other end, the infusion of i-EVs did not induce significant changes in the number of GFP^+ OPCs recruited at lesion borders nor in their degree of maturation. In this respect, previous studies demonstrated the capacity of i-EVs to cause a clear impairment of OL maturation following lysolecithin-induced focal demyelination *in vivo*, which was attributed to their negative impact on astrocyte harmful phenotype (Lombardi et al., 2019). Notably, astroglial functions are known to depend on the nature of the damaging stimulus (Hasel et al., 2021; Liddelow et al., 2017). While, in LPS-induced neuroinflammation, astrocytes were found to assume a detrimental phenotype, they were shown to maintain a beneficial and protective function following experimental stroke (Zamanian et al., 2012). This could explain the absence of harmful effects of i-EVs observed in our experiments.

To understand whether the combined effect of the infusion of IL-4 EVs on OPCs and microglia translates into effective functional recovery, ischemic mice were subjected to a behavioral test. Individuals affected by an ischemic event display impaired motor function and spatial cognition, resulting in a difficulty to explore the space contralateral to the lesion and in a reduced perception of the stimuli coming from contralateral body districts, a condition known by the medical term of *neglect* (Vallar and Calzolari, 2018). Frequently, such deficit manifests itself in the inability to direct attention and to move toward the contralateral side, with a consequent preference to turn toward the ipsilateral one (Ringman et al., 2004). To evaluate this functional aspect of the ischemic pathology in the MCAo model, the Y-maze test has been used, an apparatus generally utilized to study spontaneous alternation and turning preference in rodents (Saadoun et al., 2010). Results showed that, following MCAo, ischemic mice display a greater tendency to turn to the side commanded

by the undamaged cerebral hemisphere, while sham-operated mice have no significant preference. Notably, following intracerebral infusion of IL-4 EVs, ischemic mice no longer showed any turning preference and become comparable to sham-operated controls, while vehicle-treated littermates displayed a worsening of behavioral symptoms. Hence, these data indicate that the infusion of IL-4 EVs, by restoring microglia pro-resolving features and enhancing remyelination, was able to promote the long-term recovery of stroke-induced disability.

An increasing body of evidence indicate that EVs represent ideal candidates as biomarkers, drug carriers and mediators for a variety of therapies in oncology, immunotherapy, and regenerative medicine (Lener et al., 2015; Yáñez-Mó et al., 2015). The ability to transport multiple functional molecules in different body districts, through biological fluids, without showing immunogenicity, makes EVs a good alternative to liposomes or artificial nanoparticles, paving the way for new therapeutic strategies for different applications (Casella et al., 2018; Wiklander et al., 2019). For instance, multidisciplinary studies are focusing on the construction of nanosystems for future joint applications of diagnosis and therapy (Fais et al., 2016). This approach has great potential in the field of personalized medicine, as it allows for the detection and monitoring of a disease in individual patients, as well as the targeted delivery of drugs at the site of the disease (Fais et al., 2016). In the specific case of cerebral ischemia, the analysis of EVs in liquid biopsies might represent a potent tool for diagnosis and for the prediction and monitoring of post-stroke recovery (Gualerzi et al., 2021). Furthermore, several EV-based therapies obtained from different cellular sources proved effective in promoting lesion repair e functional recovery in rodent stroke models (Gualerzi et al., 2021; Tian et al., 2018; Yang et al., 2017, 2020; Zhang et al., 2019), and the use of EVs derived from mesenchymal stem cells (MSC) is currently under evaluation in clinical trials on stroke patients (Bang and Kim, 2019; Otero-Ortega et al., 2019).

Due to their ability to positively influence several different targets, also microglial EVs could represent a useful therapeutic strategy to enhance post-stroke recovery, especially in combination with standard pharmacological therapy, such as thrombolysis with tissue plasminogen activator, and with motor neurorehabilitation (Gualerzi et al., 2021). However, different administration routes must be validated for microglial EV delivery,

since intracerebral infusion is not applicable in patients due to high invasiveness. In this respect, intravenous infusion is currently employed for EV administration, due to the high capacity of EVs to cross the blood brain barrier, but still holds limitations because the therapeutic efficacy depends on EV biodistribution (Wiklander et al., 2019). Moreover, intranasal delivery of EVs is gaining increasing attention, due to the possibility to directly reach the CNS in a non-invasive way by diffusion through the nasal mucosae (Losurdo et al., 2020; Zhuang et al., 2011).

Another limitation of our study is that the isolation of EVs from microglia may prove impractical or non-reproducible in the context of large-scale therapy. Future research should therefore be directed toward the identification of the bioactive molecular components of microglial EVs, to design engineered EVs for the targeted delivery of pro-regenerative molecules. Different procedures could be used for the preparation of these therapeutic tools, including cellular engineering techniques, such as gene manipulation, which could allow to change the surface protein expression and the content of EVs derived from standardized human cell lines (Mentkowski et al., 2018). A further possibility involves the creation of synthetic analogues that mimic the characteristics of endogenous EVs, allowing a precise selection of the vesicular content and a scalable and well-characterized drug delivery system (Mentkowski et al., 2018).

4.2 Microglial EVs enhance OL maturation by reprogramming cellular energy metabolism in recipient OPCs

Identifying the molecular mechanisms underpinning the beneficial effects of microglial EVs on OPC maturation might pave the way for the development of innovative remyelinating therapies. In this study, attention has been initially focused on TNF, since recent studies demonstrate that the protein levels of this cytokine are significantly increased within microglial EVs (Yang et al., 2018), indicating its possible role in the interaction between microglia and surrounding cells. Moreover, it has been shown that damage signals, such as ATP, redirect the intracellular trafficking of TNF, inhibiting the release of solTNF in favor of the accumulation of tmTNF on EV surface (Soni et al., 2019). Once released, tmTNF can interact with its main receptor TNFR2 on OPCs, fostering their differentiation into myelinating cells (Madsen et al., 2016a). On this basis, we evaluated whether tmTNF could

be partly responsible for the effects induced by IL-4 EVs on OPCs by using the non-selective TNF inhibitor etanercept and the selective solTNF blocker XPro1595. Results showed that the pro-differentiating effect of IL-4 EV on OPCs is maintained in the presence of XPro1595, while it was abolished by concomitant administration of etanercept, confirming that the tmTNF/TNFR2 axis plays, at least in part, a role in mediating the pro-regenerative effect of microglial EVs. Selective TNFR2 agonists are currently under development for the treatment of many inflammatory and autoimmune diseases (Atretkhany et al., 2020; Fischer et al., 2019, 2020; Torrey et al., 2020), and may therefore hold great promise also as remyelinating drugs. However, given the complexity of the cellular processes regulated by TNF and its receptors (Atretkhany et al., 2020; Probert, 2015; Raffaele et al., 2020), the selectivity of these molecules for the desired TNF receptor subtype, target cell population, and body district should be carefully addressed to avoid undesired side effects.

An alternative approach to unravel the biological processes responsible for the pro-differentiating effects of microglial EVs on OPCs has been to focus on the early transcriptional changes induced by EVs on recipient OPCs. In this respect, exposure to IL-4 EVs was found to induce significant transcriptional modifications in primary cultured OPCs, while only few genes were modulated by i-EVs, highlighting once again the importance of the molecular signature of donor cells for the final effect of microglial EVs.

Among the genes significantly modulated by IL-4 EVs, several are involved in the regulation of cellular energy metabolism. Genes involved in fatty acid and cholesterol homeostasis, like *Lrp1* and *Fasn*, were found to be significantly upregulated, in line with previous studies indicating the importance of lipid metabolism for proper OL maturation and myelin generation (Dimas et al., 2019; Lin et al., 2017). Several genes involved in Krebs cycle and mitochondrial respiration (*Sdhc* and *Sdhd*), and β-oxidation (*Crot*) were instead downregulated, suggesting that metabolic rearrangements are taking place in differentiating OPCs after exposure to IL-4 EVs. Accordingly, functional enrichment analysis highlighted oxidative phosphorylation and mitochondrial dysfunction among the top biological processes significantly modulated by IL-4 EVs. It is worth noting that the main functions of OLs, namely myelination and trophic support to neurons, have been shown to strongly depend on cellular energy metabolism. Indeed, during development, OPCs mainly rely on oxidative phosphorylation to produce the ATP necessary to sustain

their proliferation, while, during differentiation, OLs shift into a primarily glycolytic metabolism, reducing the production of reactive oxygen species associated with mitochondrial respiration and enhancing their anabolic synthesis of myelin components (Beyer et al., 2018; Meyer and Rinholm, 2021; Rosko et al., 2019). This is particularly relevant in the case of demyelinating conditions, like cerebral ischemia, that are characterized by metabolic dysfunction and elevated oxidative stress (Marangon et al., 2020; Rosko et al., 2019). The importance of targeting OL metabolic dysfunction to achieve remyelination is further corroborated by recent evidence showing that the efficacy of several established remyelinating molecules depends on off-target metabolic effects (Hubler et al., 2018; Rankin et al., 2019). It is therefore plausible that IL-4 EVs increase OPC maturation by speeding up the conversion from oxidative metabolism to a glycolytic one, putting cells in the proper bioenergetic state for differentiation. Further experiments evaluating the metabolomic profile and enzymatic activity of OPCs exposed to IL-4 EVs will help to verify this hypothesis

Noteworthy, sirtuin signaling pathway appeared among the top biological processes significantly activated by IL-4 EVs. Sirtuins are a family of NAD^+-dependent deacetylases, which can sense changes in cell metabolic state and consequently regulate cellular functions such as gene expression, metabolism, telomere activity, cell cycle, differentiation, apoptosis, proliferation, DNA repair, senescence, and oxidative stress response (O'Callaghan and Vassilopoulos, 2017). The role of sirtuins in OL lineage cells is still unclear. While Sirt2 has been demonstrated to control OL differentiation by promoting both arborization and downstream expression of myelin-specific genes (Ji et al., 2011; Thangaraj et al., 2017), Sirt1 has been proven to regulate OPC proliferation, although with apparently contrasting effects (Jablonska et al., 2016; Rafalski et al., 2013). Given their ability to interact with different protein families in several cell compartments, sirtuins might represent the ideal effector molecules to translate the metabolic changes induced by IL-4 EVs into epigenetic and morphological modifications necessary for OL differentiation. On the other end, sirtuins are also known as master regulators of cell metabolism (Houtkooper et al., 2012), and it is therefore possible that they contribute to establishing a positive feedback loop, propagating external inputs into cell-wide metabolic rearrangements that push OLs toward maturation.

Among the predicted upstream regulators which might be responsible for the transcriptional changes induced by IL-4 EVs, the highest activation z-score was predicted for Rictor, an essential component of the mTORC2 complex whose overexpression was found to improve lesion regeneration in a rodent model of spinal cord injury (Chen et al., 2020), while its silencing in OLs significantly delayed myelination (Grier et al., 2017). The lowest z-score was instead reported for serine/threonine kinase 11 (STK11), also known as liver kinase B1 (LKB1), a master kinase involved in regulating mitochondrial metabolism in immune and tumoral cells via AMPK signaling (Kottakis et al., 2016; Lizcano et al., 2004). The role of STK11 in OLs has never been investigated so far but based on literature data it could be postulated that it can work in concert with Rictor to modulate OL metabolism during differentiation. Interestingly, predicted upstream regulators also include carnitine palmitoyl transferase 1 (CPT1), the rate-limiting enzyme in the fatty acid β-oxidation process (Schlaepfer and Joshi, 2020), pointing once again at lipid metabolism as a central pathway regulating OL differentiation. Further studies are required to validate the role of the predicted upstream regulators during OL differentiation, which might potentially unveil novel putative targets for remyelinating therapies.

4.3 TNFR2 regulates the pro-remyelinating functions of microglia after stroke

As discussed above, OL-targeted remyelinating approaches might prove ineffective if a permissive local environment, allowing efficient OPC recruitment and maturation, is not concomitantly provided. Is therefore essential to uncover the mechanisms driving the pro-remyelinating functions of microglia, to design therapies able to support myelin regeneration with a favorable immune cell phenotype (Fumagalli et al., 2018). Here, for the first time, we investigated the contribution of microglial TNFR2 in myelin protection and repair after stroke.

Genetic ablation of TNFR2 from microglia resulted in a significant reduction of the capacity of these cells to react to the ischemic damage, as suggested by the reduced expression of pro-regenerative and phagocytic markers at both gene and protein level and by the aberrant morphological features unveiled by particle analysis. These data are in line with previous findings showing that microglial TNFR2-ablation exacerbates the pro-inflammatory activation of these cells and impairs their capacity of sensing the environment and of

clearing debris, contributing to worse disease outcome in the EAE model of MS (Gao et al., 2017). Moreover, conditional genetic ablation of solTNF with preservation of tmTNF, the main ligand for TNFR2, resulted in reduced infarct volume and functional disability after experimental stroke in mice (Madsen et al., 2016b). On this basis, it can be proposed that TNFR2 plays a pivotal role in shaping microglial functions in response to external inflammatory stimuli, with the aim of preventing excessive inflammation and promoting repair mechanisms. Accordingly, our data demonstrated that the capacity of microglia to orchestrate the inflammatory response after stroke is loss upon TNFR2 ablation, as suggested by the increased infiltration of cytotoxic peripheral immune cells and by the reduced number of microglial cells detected by flow cytometry analysis.

It is worth mentioning that the subpopulation of CD11c-expressing microglial cells was instead found to be increased in microglial TNFR2 knockout mice compared to wildtype littermates. This data appears controversial, given the established role of $CD11c^+$ microglia in supporting myelin generation and repair during development and demyelinating conditions (Wlodarczyk et al., 2017, 2019). However, since the appearance of $CD11c^+$ microglia in the adult brain has been clearly associated with myelin damage and neuroinflammation (Wlodarczyk et al., 2015, 2019, 2021), it can be suggested that increased cell number somehow reflects an attempt to cope with a larger white matter lesion. Furthermore, the protective functions of $CD11c^+$ microglia have been shown to heavily rely on TREM2 activity (Kamphuis et al., 2016), which was found to be downregulated upon TNFR2 ablation. It therefore remains to be clarified if $CD11c^+$ microglia accumulating in the ipsilateral cortex of TNFR2 knockout mice are still fully functional, or if the elevated number observed represent a pathological mechanism to compensate for the reduced cell regenerative potential.

The full activation of microglia has been demonstrated to be essential for myelin clearance and oligodendrogenesis in the initial phase of remyelination (Cunha et al., 2020), and our previous data uncovered their beneficial role for the recruitment of GPR17-expressing OPCs and myelin protection at early stages after stroke (Raffaele et al., 2021). Interestingly, microglial TNFR2 ablation resulted in reduced mRNA expression of the OPC marker *Ng2* and of *Bcas1*, a gene specifically labelling a subpopulation of newly-formed actively remyelinating OLs (Fard et al., 2017), at day 1 post-MCAo. These early changes were also

reflected by a lower number of OPCs and immOLs in the ipsilateral cortex at day 5 post-MCAo and, most importantly, by a significant reduction of MBP^+ myelinated fibers in the peri-lesion area, likely contributing to the worsening of motor performance observed. Hence, these data suggest that OPC-mediated remyelinating attempts are significantly impaired in the absence of microglial TNFR2, supporting the involvement of this receptor in regulating the pro-remyelinating phenotype of microglia.

Upregulation of TNFR2 has been recently described in the peri-lesion area of human stroke patients (Clausen et al., 2020), indicating that TNFR2-targeted therapeutic approaches for the regeneration of ischemic lesions might retain high translational potential. In this respect, TNFR2 has been not only implicated in regulating microglial functions but was also found to be involved in addressing OPCs toward differentiation (Madsen et al., 2016a) and in regulating the immunomodulatory functions of OL lineage cells (Desu et al., 2021; Madsen et al., 2020). TNFR2-based treatments therefore represent ideal remyelinating strategies simultaneously acting on multiple OL-intrinsic and immune-related mechanisms. Moreover, EV-based tools may be perfect carriers for the targeted delivery of these compounds to the desired cells in the CNS, due to their established role in the release of TNF signaling molecules and their well characterized capacity to interact with both microglia and OPCs at sites of myelin injury, reducing the risk of off-target effects.

Taken together, the results described in the present thesis provide entirely new molecular mechanisms underpinning the beneficial interaction between microglia and OPCs, which could be useful for developing innovative therapeutic strategies to promote myelin repair and functional recovery after ischemic stroke, as well as in other neurodegenerative diseases characterized by demyelination and detrimental neuroinflammation.

5. MATERIALS AND METHODS

5.1 Animals and experimental procedures

The experimental procedures described in this thesis, concerning animal care, surgery, and sacrifice, have been carried out in line with national (D.L. n.26, 2014) and international (EU Directive 2010/63/EU) legislations, under formal approval of the local ethical committees (Italian National Ministry of Health-University of Milan Committee, approval number 868/2016-PR; The Danish Animal Inspectorate under the Ministry of Food and Agriculture, approval number 2019-15-0201-01620), and in the respect of ARRIVE guidelines.

GPR17-iCreER^{T2}:CAG-eGFP conditional reporter mice, allowing to follow the fate of GPR17-expressing OPCs by GFP labeling (Viganò et al., 2016), have been bred, housed, and utilized at the animal facility of the Department of Pharmacological and Biomolecular Sciences, University of Milan, Italy. Genotyping has been performed on DNA extracted from tail biopsies (Viganò et al., 2016), and only double transgenic GPR17-iCreER^{T2}:CAG-eGFP mice of eleven weeks of age were treated with 10 mg tamoxifen (Sigma-Aldrich, Taufkirchen, Germany), dissolved in 10% ethanol and 90% corn oil, three times by oral gavage once every second day to induce recombination. After 21 days of wash-out, mice were anesthetized and underwent permanent MCAo as previously described (Bonfanti et al., 2017; Lambertsen et al., 2005; Storini et al., 2006). Briefly, the skin between the orbit and the ear was vertically incised with a scalpel, the temporal muscle was dissected, and a 1 mm hole was created in the skull using a drill to expose the MCA. The right MCA was then permanently occluded by means of microbipolar coagulation just proximal to the origin of the olfactory branch. The same surgical procedures with exception of artery occlusion were performed on sham-operated mice. In all the experiments involving GPR17-iCreER^{T2}:CAG-eGFP mice, male and female animals have been equally utilized.

CX3CR1^{Cre}:TNFR2$^{fl/fl}$ conditional knockout mice, in which consistent TNFR2 ablation is achieved specifically in microglia (Gao et al., 2017), and control TNFR2$^{fl/fl}$ littermates with normal TNFR2 expression, have been bred, housed, and utilized at the Biomedical Laboratory of the University of Southern Denmark, Odense, Denmark. After genotyping performed as described (Gao et al., 2017), Cre recombinase was induced by five daily intraperitoneal tamoxifen injections (2 mg/mouse/day) followed by a 28-day waiting period to allow drug wash-out and full replenishment of TNFR2-expressing monocyte-derived macrophages. Control littermates received the same treatment. Mice were then subjected to

permanent left MCAo as detailed above. In all the experiments involving CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice, eleven weeks old female animals have been utilized.

5.2 Primary microglia cultures

Primary microglia were prepared starting from mixed glial cell cultures, obtained from dissected brain tissue of C57/Bl6 wild-type pups at postnatal day (P) 2 (Charles River, Lecco, Italy) and maintained for 10 days in culture medium containing South American fetal bovine serum (Life Technologies, Monza, Italy) that optimizes microglia expansion. Then, microglia were harvested by orbital shaking for 40 min at 1300 rpm and re-plated on poly-l-ornithine-coated tissue culture dishes (50 μg/ml, Sigma-Aldrich, Taufkirchen, Germany). Pure microglia cultures (> 98%) were obtained, expanded for two days, and kept in low-serum (1%) medium for 24 hours to minimize spontaneous activation. Microglia polarization was then induced by stimulating cells for 48 hours with a cocktail of pro-inflammatory cytokines, including 20 ng/ml IL-1β (Peprotech, Milan, Italy), 20 ng/ml TNF (Peprotech, Milan, Italy), and 25 ng/ml IFN-ɣ (Sigma-Aldrich, Taufkirchen, Germany), or with 20 ng/ml IL-4 (R&D, Milan, Italy) to respectively induce pro-inflammatory (i-MG) or pro-regenerative (IL-4 MG) activation (Prada et al., 2018). Non-stimulated microglia, exposed to regular medium without cytokines, have been used as control. Finally, cells have been washed and stimulated with ATP to increase EV production (see paragraph 5.4). Cell supernatant was collected for EV isolation, while real-time qPCR has been performed on donor microglia, as described below, to check the expression of pro-inflammatory and pro-regenerative markers.

5.3 RNA isolation and gene expression analysis in primary microglia cultures

Donor microglia were lysed using TRIZOL® reagent (Life Technologies, Monza, Italy) and total RNA was extracted by Direct-zol™ RNA Micro-Prep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. RNA was then subjected to RQ1 DNase (Promega, Milan, Italy) treatment to avoid contaminations with genomic DNA. For the retro-transcription of 400 ng RNA/sample, the SensiFAST™ cDNA synthesis kit (Bioline, London, UK) has been utilized. Then, several reaction mixes were prepared according to the number of interested genes, each including Master Mix 2x (Life Techonologies, Monza,

Italy), 250 nM probe (*Arg1* Mm00475988_m1; *Chil3 (Ym1)* Mm00657889_mH; *Il1b* Mm00434228_m1; *Tnf* Mm00443258_m1; *Nos2* Mm00440502_m1; *Rpl13a* Mm05910660_g1; Life Technologies, Monza, Italy), and 20 ng of cDNA. TaqMan® Gene Expression Assay was employed to quantify gene expression using a CFX96 real-time PCR system (BioRad Laboratories, Segrate, Italy) following the manufacturer's protocol. *Rpl13a* has been used as housekeeping gene for internal normalization. Data were analyzed using the $\Delta\Delta C_t$ method and are presented as mean of $\log_2$(fold change) ± SE.

5.4 Isolation and quantification of microglia-derived EVs

Polarized microglia were stimulated with 1 mM ATP (Sigma-Aldrich, Taufkirchen, Germany) for 30 min in KRH (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH_2PO, 2 mM $CaCl_2$, 6 mM D-glucose, and 25 mM HEPES/NaOH, pH 7.4) to maximize the production and release of EVs (Bianco et al., 2005). The culture supernatant was then collected, centrifugated at 300 g for 10 min to eliminate detached cells and debris, and total EVs containing both microvesicles and exosomes were pelleted at 100.000 g for 1 hour as previously described (Gabrielli et al., 2015). EV pellets were resuspended and used immediately after isolation.

EV concentration and size distribution were measured by Tunable-Resistive Pulse Sensing (TRPS), using an Izon qNano instrument, equipped with a NP300 nanopore (Izon, Christchurch, New Zealand). Izon EV reagent kit (Izon, Christchurch, New Zealand) was used for both pre-treating the pore and resuspending EVs to prevent EV binding to the pore or spontaneous EV aggregation. For quantification, EVs produced by 1×10^6 microglia in 1 hour were re-suspended in a volume of 100 µl, and CPC200 carboxylated polystyrene particles (Izon, Christchurch, New Zealand), diluted following manufacturer's protocol, were used as standards. During data recording and analysis, performed using Izon Control Suite software (version V3.3), constant parameters (applied voltage, pressure, and pore stretch values) were set for all samples and calibration particles.

5.5 Intracerebral infusion of microglial EVs

Approximately 2×10^8 EVs, produced by 1.5×10^6 microglia, were resuspended in 150 µl of sterile saline and infused in the ipsilateral corpus callosum of GPR17-iCreER^{T2}:CAG-eGFP

mice at day 14 post-MCAo (coordinates from bregma: 1.0 mm rostral, 1.0 mm lateral, 2 mm deep) using osmotic mini-pumps (1007D equipped with Brain Infusion kit 3, Alzet, Cupertino, CA, USA) over one week at 0.5 μl/h delivery rate. Mice were divided in three experimental groups, receiving IL-4 EVs, i-EVs or saline solution alone (vehicle). Published data already demonstrated that microglial EVs loaded into the minipumps are stable over time. Moreover, GFP-labelled EVs administrated with this method have been previously detected within the tissue and were shown to interact with both microglia and OPCs at the site of myelin lesions (Lombardi et al., 2019). Mice were euthanized at day 28 post-MCAo, 2 weeks after minipump implantation, for immunofluorescence analysis.

5.6 Behavioral evaluation of mice turning preference

The turning preference of ischemic mice has been evaluated before and after infusion of IL-4 EVs or vehicle using the Y-maze test (Saadoun et al., 2010). Briefly, mice have been allowed to freely explore a Y-shaped maze, consisting of three equal plastic arms at 120° angle, for 8 minutes, during which a blinded observer recorded the number and sequence of movements in each arm. One entry was considered as valid only when made with all four limbs, and one spontaneous alternation was counted when the animal entered all three different arms consecutively. After each trial, locomotor activity (total number of arm entries), % alternation (number of spontaneous alternations/number of total triads*100), and % of contralateral turns (number of turns toward the contralateral side when entering a new arm/number of total turns*100) have been calculated.

First, stroke-induced alterations in turning behavior have been quantified on ischemic mice (MCAo; n=14) at day 14 post-MCAo, before EV administration, and compared to sham-operated mice (n=5). After the first behavioral analysis, MCAo animals have been randomized in two experimental groups, receiving infusion of IL-4 EVs (n=7) or vehicle (n=7) as described above. At day 28 post-MCAo, the Y-maze test has been repeated to evaluate the impact of IL-4 EVs infusion on mice turning preference with respect to both vehicle-treated animals and sham-operated controls.

5.7 Assessment of motor performance by rotarod test

Stroke-dependent effects on motor coordination, endurance, and balance of $CX3CR1^{Cre}$:$TNFR2^{fl/fl}$ mice (n=8) and $TNFR2^{fl/fl}$ littermates (n=10) have been quantified using the rotarod apparatus (LE8200 system, Panlab Harvard Apparatus, Barcelona, Spain) as previously described (Lambertsen et al., 2012). First, naive mice were pre-trained to stay and walk on the rod for 30 s at a constant speed of 4 rpm. Then, data recording has been performed before stroke induction (baseline) and at day 3 and 5 post-MCAo, when mice were placed on the rotarod, and the speed of the rotor was accelerated from 4 to 40 rpm over 5 min. The latency to fall (s) from the rod was recorded automatically during the trial. Four trials/mouse/time point have been performed, with 10 min resting time between each trial.

5.8 RNA isolation and gene expression analysis in bulk brain tissue

The analysis of relative gene expression *in vivo* has been performed on bulk brain tissue obtained from $CX3CR1^{Cre}$:$TNFR2^{fl/fl}$ mice and $TNFR2^{fl/fl}$ littermates prior to ischemia induction (naïve, n=5) and at day 1 (n=10) and day 5 (n=10) post-MCAo. Mice were deeply anaesthetized with pentobarbital (200 mg/ml) containing lidocaine (20 mg/ml), transcardially perfused with 20 ml of ice-cold 0.01 M phosphate buffered saline (PBS), and the brain has been dissected and snap frozen using carbon dioxide snow. Then, 30 μm-thick coronal sections have been cut using a microtome in six parallel series collected in tubes for further processing (1/6 of the brain in each tube).

To isolate total RNA from bulk brain tissue, 1 ml of TRIZOL® reagent was added to the samples and the tissue was homogenized. The samples were then allowed to reach room temperature (RT) to ensure complete dissociation of nucleoprotein complexes, and phase separation was performed with chloroform (Sigma-Aldrich, Taufkirchen, Germany). The upper aqueous phase containing RNA was then transferred to a new tube and the RNA was precipitated by adding isopropanol (Sigma-Aldrich, Taufkirchen, Germany). The RNA pellet was washed with 75% ethanol twice, air dried, and dissolved in nuclease free water. Synthesis of cDNA was performed using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instruction.

RT-qPCR was performed using the following primers (TAG, Copenhagen, Denmark): *Arg1* (Fw: ATGAAGAGCTGGCTGGTGTG; Rv: CCAACTGCCAGACTGTGGTC), *CD68* (Fw: GGTGGAAGAAAGGCTTGGGG; Rv: GAGACAGGTGGGGATGGGTA), *Trem2* (Fw: TGCTGGAGATCTCTGGGTCC; Rv: AGGTCTCTTGATTCCTGGAGGT), *Ng2* (Fw: TGTTCTCACACAGAGGAGCC; Rv: GGCCACCACTCGGAAGAAAT), *Bcas1* (Fw: GCTTCTTCAAGACACTGGTTTCAC; Rv: TGTCTGCCTTGGTTGCCTTC), *Hprt1* (Fw: TCCTCAGACCGCTTTTTGCC; Rv: TCATCATCGCTAATCACGACGC). Primers were designed to target exon-exon junctions whenever possible. Analysis was performed in a total volume of 12,5 µl containing 1x Maxima SYBR Green, 50 ng of template cDNA, and 600nM forward and reverse primers. SYBR Green Gene Expression Assay was utilized to quantify gene expression using a CFX96 real-time PCR system. All samples were analyzed in triplicates and the standard curve was prepared from a mixture of all tested samples with a 4-fold serial dilution. The standard curve samples were run in each assay together with the experiment samples, calibrator (a mixture of all tested samples in 6 wells) and no template control. Furthermore, the experimental samples were randomly distributed over assays and the relative transcript levels were calculated using the Pfaffl method (Pfaffl, 2001) and normalized to the housekeeping gene *Hprt1*. Data are presented as mean of $\log_2$(fold change) ± SE.

5.9 Immunohistochemistry (IHC) and cell counting

IHC analysis have been performed on GPR17-iCreER^{T2}:CAG-eGFP mice treated with IL-4 EVs (n=9), i-EVs (n=4) or vehicle (n=9) and sacrificed at day 28 post-MCAo, and on CX3CR1^{Cre}:TNFR2$^{fl/fl}$ mice (n=6) and TNFR2$^{fl/fl}$ littermates (n=5) euthanized at day 5 post-MCAo. Mice were deeply anaesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg), or with pentobarbital (200 mg/ml) containing lidocaine (20 mg/ml), and transcardially perfused with 0.01 M PBS followed by 4% paraformaldehyde (PFA) in 0.01 M PBS. Brains were dissected, post-fixed for 1 hour in 4% PFA and cryoprotected in a solution containing 30% sucrose in 0.01 M PBS at 4 °C until precipitation. Coronal sections of 20 µm thickness have been collected using a microtome and incubated with the following primary antibodies: chicken anti-GFP (1:1400; Aves Labs, Inc., Tigard, OR, USA); rat anti-CD68 (1:400; BioRad Laboratories, Segrate, Italy); rat anti-CD16/32 (1:200; BD Biosciences, Milan,

Italy); rabbit anti-GSTπ (1:500; MBL, Woburn, MA, USA), rabbit anti-Iba1 (1:400 for regular staining, 1:30000 for amplification kit; Wako, Japan); rabbit anti-NeuN (1:100; Cell Signaling Technologies, Danvers, MA, USA); rabbit anti-NG2 (1:100 for regular staining, 1:2000 for amplification kit; Millipore, Milan, Italy), rabbit anti-GPR17 (1:2500; custom antibody produced by Primm, Milan, Italy) (Bonfanti et al., 2020), rabbit anti-Ym1 (1:100; Stemcell Technologies, Cambridge, UK), rat anti-Gal-3 (1:300; clone M38, kind gift of prof. Tomas Deierborg) (Boza-Serrano et al., 2019), rat anti-MBP (1:200; Millipore, Milan, Italy). Incubation with primary antibodies was made overnight at 4 °C in 0.01 M PBS with 1% normal goat serum (Dako, Glostrup, Denmark) and 0.3% Triton-X 100. The sections were then exposed for 2 hours at room temperature to the corresponding secondary antibodies (all goat) conjugated with Alexa Fluor 488, 555, 594 or 633 (1:600; Life Technologies, Monza, Italy). For the experiments performed on GPR17-iCreER^{T2}:CAG-eGFP, the High Sensitivity Tyramide Signal Amplification kit (Perkin-Elmer, Monza, Italy) was used to enhance the signal intensity of rabbit anti-NG2, GPR17 and Iba1. Nuclei were labeled with Hoechst 33258 (0.06 μg/ml; Life Technologies, Monza, Italy). FluoroMyelin™ Red stain (1:300; Life Technologies, Monza, Italy) has been used following manufacturer's instructions to visualize myelinated structures. For the quantitative analysis, the peri-infarct region (0-500 μm from the boundary of the ischemic lesion) has been considered. Images were acquired using a confocal microscope (merge of 6-μm z-stack at 2-μm intervals; A1R, Nikon, Tokyo, Japan) and a fluorescence microscope (BX53 fluorescence microscope fitted with DP73 camera, Olympus, Tokyo, Japan). For the experiments performed on GPR17-iCreER^{T2}:CAG-eGFP mice, Iba1 staining was performed using 633 fluorophore, and the false color green has been applied in representative images to better display co-localization with other markers in red. To visualize brain damage, hematoxylin-eosin (HE; Sigma-Aldrich, Taufkirchen, Germany) staining has been performed and images acquired by means of a slide scanner (NanoZoomer S60, Hamamatsu Photonics, Hamamatsu City, Japan). Image analysis has been performed by a blinded investigator, using the software Fiji/ImageJ, on three slices per mouse taken from −1.00 to 0.00 mm from bregma. For analysis of tissue loss, the area of NeuN or HE staining has been manually measured for each slice both in the ipsilateral hemisphere and in the intact contralateral one and the percentage of tissue loss has been calculated as % tissue loss = (area contra – area ipsi)/area contra * 100.

5.10 Analysis of microglia morphology

The morphological analysis of microglia described in figure 3.3 has been performed on confocal micrographs, acquired from 20 μm-thick brain sections stained for Iba1, using the semi-automatic Matlab-based tool 3DMorph (York et al., 2018). 130-150 cells from 3-4 animals for each experimental condition have been analyzed. Morphological parameters considered were number of branchpoints and ramification index, calculated as cell surface area/(4π × ((3 × cell volume)/(4π))2/3 and gaining score 1 for round-shaped cells and higher values as cell ramification increases, both reflecting the complexity of cell branching. Moreover, cell volume, which gives indications about the intensity of cell activation, and cell territory, which is proportional to cell volume and to the number and the length of cell processes and is indicative of the brain area surveilled by each individual cell, have been quantified.

The quantification of microglia area fraction and average cell size described in figure 3.11 has been performed on fluorescent microscopy images, taken from 20 μm-thick brain sections stained for Iba1 and CD68 or Iba1 and Gal-3, using the particle analysis tool of the Fiji-ImageJ software as previously described (Magni et al., 2020). The area fraction covered by Iba1, CD68, and Gal-3 staining, and the average size of $Iba1^{+}$ cells, were automatically determined and are expressed as fold over values obtained in control mice set to 100.

5.11 Flow cytometry

Flow cytometry analysis have been performed on the ipsilateral cerebral cortex of $CX3CR1^{Cre}$:$TNFR2^{fl/fl}$ mice (n=3) and $TNFR2^{fl/fl}$ littermates (n=3) at day 5 post-MCAo. Mice were deeply anaesthetized with pentobarbital (200 mg/ml) containing lidocaine (20 mg/ml), transcardially perfused with 20 ml of ice-cold 0.01 M PBS, and the ipsilateral cortex was dissected and placed in cold RPMI-1640 (Gibco, Roskilde, Denmark). Samples were homogenized through a 70 μm cell strainer (AH Diagnostics, Aarhus, Denmark) and myelin and cell debris were removed using the Debris Removal Solution (Miltenyi Biotec, Bologna, Italy) according to manufacturer's protocol. Then, each sample was split in two equal parts and further processed to analyze immune or glial cells.

Immune cell analysis was carried out using the following panel: PerCP-Cy™5.5 Rat Anti-Mouse CD45 (clone 30-F11, BD Biosciences, Eysins, Switzerland), BB515 Rat Anti CD11b

(clone M1/70, BD Biosciences, Eysins, Switzerland), PE-Cy™7 Rat Anti-Mouse Ly-6C (clone AL-21, BD Biosciences, Eysins, Switzerland), BV421 Rat Anti-Mouse LY-6G (clone 1A8, BD Biosciences, Eysins, Switzerland), Biotinylated Hamster Anti-Mouse CD11c (clone HL3, BD Bioscience, Eysins, Switzerland), and their corresponding isotype controls. CD11c labeling was then completed using APC Streptavidin (BioLegend, San Diego, CA, USA).

Glial cell analysis was performed using the following panel: PerCP-Cy™5.5 Rat Anti-Mouse CD45, FITC REAfinity Anti-Mouse ACSA-2 (Miltenyi Biotec, Bologna, Italy), PE REAfinity Anti-Mouse O4 (Miltenyi Biotec, Bologna, Italy), APC REAfinity Anti-Mouse CD140a (PDGFRα; Miltenyi Biotec, Bologna, Italy), and their corresponding isotype controls.

Briefly, after myelin removal, the cell suspensions were stained for live/dead cells using Fixable Viability Dye eFlouro 506 (eBioscience, San Diego, CA, USA), washed, blocked to prevent non-specific staining using Purified Rat anti-mouse CD16/CD32 (Mouse BD Fc block; BD Biosciences, Eysins, Switzerland) and Syrian hamster gamma globulin (Jackson Immuno Research Europe Ltd, Cambridge, UK) for 30 min at 4°C, and stained for CD11c (immune panel) or ACSA-2/O4/PDGFRα (glia panel) for 20 min at 4°C. Cells where then washed and fixed with 2% PFA for 15 min. After fixation, the samples were blocked and stained for CD45/CD11b/Ly6C/Ly6G (immune panel) or CD45 (glia panel). Trucount™ beads (BD Biosciences, Eysins, Switzerland) were added to each sample for the quantitative determination of absolute cell numbers. Stained cells were run on a FACSAria™ III flow cytometer (BD Biosciences, Eysins, Switzerland), and approximately 50000-100000 events were acquired per sample using forward scatter (FSC) and side scatter (SSC). The analysis was performed using the Flowlogic software, determining positive staining based on the respective isotype and fluorescent minus one (FMO) control.

5.12 Primary OPC cultures

Primary OPC cultures have been prepared from total brain tissue of P2 GPR17-iCreER^{T2}:CAG-eGFP mice. Briefly, brains were dissected and preserved in Tissue Storage Solution® (Miltenyi Biotec, Bologna, Italy) at 4°C for 3 hours during genotyping (performed on tail biopsies as described above). Brains deriving from double transgenic mice were pulled together and dissociated into single cell suspensions using Papain-based Neural

Tissue Dissociation Kit (Miltenyi Biotec, Bologna, Italy). Then, PDGFRα^+ OPCs were isolated by magnetic activated cell sorting (MACS) using anti-CD140a microbeads (Miltenyi Biotec, Bologna, Italy) following the manufacturer's instructions. PDGFRα^+ cells have been plated on poly-DL-ornithine coated plates and cultured in OPC proliferation medium containing Neurobasal (Life Technologies, Monza, Italy), 2% B27 (Life Technologies, Monza, Italy), 1% L-glutamine (Euroclone, Pero, Italy), 1% penicillin/streptomycin (Euroclone, Pero, Italy), 10 ng/ml PDGF-AA (Sigma-Aldrich, Taufkirchen, Germany), 10 ng/ml FGF2 (Space Import Export, Milan, Italy), and 1μM 4-Hydroxitamoxifen (to induce GFP expression; Sigma-Aldrich, Taufkirchen, Germany). After two days of proliferation, cells were switched to differentiation medium containing DMEM (Euroclone, Pero, Italy), 1% N-2 supplement (Life Technologies, Monza, Italy), 2% B27, 0.01% BSA (Sigma-Aldrich, Taufkirchen, Germany), 1% L-glutamine, 1% penicillin/streptomycin and 10 ng/ml T3 (Sigma-Aldrich, Taufkirchen, Germany). After approximately 4 hours, medium alone (CTRL), i-EVs or IL-4 EVs resuspended in 50 μl of medium were added to the OPC culture (1:2 OPC-microglia ratio).

For immunofluorescence analysis, cells have been plated in 24-well plates at a density of 30.000 cells/well, differentiated for three days, and then fixed at room temperature with 4% PFA (Sigma-Aldrich, Taufkirchen, Germany) in 0.01 M PBS containing 0.12 M sucrose (Sigma-Aldrich, Taufkirchen, Germany). To evaluate the role of EV-carried TNF on OPC differentiation, the non-selective TNF inhibitor Etanercept (ETN) (200 ng/ml; Wyeth, Madison, NJ, USA) or XPro™1595 (200 ng/ml; Xencor, Monrovia, CA, USA), a selective solTNF inhibitor, were added to the differentiation medium the same day of EV exposure. For transcriptomic analysis, cells were plated in 6-well plates at a density of 300.000 cells/well, cultured as described above and collected after one day of differentiation. Under these culture conditions, contaminating astrocytes and microglia were routinely less than 1% each.

5.13 Immunocytochemistry

After fixation, cells were incubated overnight at 4°C with the following primary antibodies diluted in Goat Serum Dilution Buffer [GSDB: 450 mM NaCl (Sigma-Aldrich, Taufkirchen, Germany), 20 mM sodium phosphate buffer pH 7.4, 15% goat serum (Life Technologies, Monza, Italy), 0.3% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany)]: rat anti-MBP (1:200; Millipore, Milan, Italy), chicken anti-GFP (1:1400; Aves Labs, Inc., Tigard, OR, USA). The following day, cells were washed and incubated for 1 hour at room temperature with the following secondary antibodies: goat anti-rat conjugated to Alexa Fluor 555, goat anti-chicken conjugated to Alexa Fluor 488 (1:600 in GSDB; Life Technologies, Monza, Italy). Nuclei were labelled with Hoechst33258 (0.3 μg/ml; Life Technologies, Monza, Italy). After labeling, coverslips were mounted with a fluorescent mounting medium (Dako, Glostrup, Denmark) and stained cells were imaged using an inverted fluorescence microscope (200M; Zeiss, Jena, Germany) connected to a PC equipped with the Axiovision software (Zeiss, Jena, Germany). Cell counts have been performed using the Fiji/ImageJ software on 30 fields acquired at x20 magnification (0.07 mm^2/field; three coverslips for each experimental condition deriving from three independent experiments).

5.14 Transcriptomic profiling of primary OPCs

Total RNA was extracted from three independent samples per experimental condition by means of RNeasy Micro kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA quality control, processing and microarray hybridization were performed at the School of Medicine and Surgery, University of Milano-Bicocca, Italy. RNA concentration and purity was assessed by Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), total RNA integrity was assessed by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the RNA Integrity Number (RIN) was calculated. The quality of each sample was assured by a RIN ≥ 7 and visual confirmation of clear, distinct 28S and 18S rRNA peaks.

100 ng of RNA were used for the preparation of targets for Clariom™ D mouse arrays (Thermo Fisher Scientific, Waltham, MA, USA) according to the GeneChip™ WT Plus Reagent Kit manual. The Clariom™ D mouse arrays contain >66100 genes, >214900 transcripts, >498500 exons, >282500 exon-exon splice junctions, with >4895600 total probes.

The staining, washing, and scanning of the arrays were conducted using a Fluidics 450 station, Command Console Software and GeneChip® Scanner 3000 7G (Thermo Fisher Scientific, Waltham, MA, USA), generating .CEL files for each array. The images were scanned by ThermoFisher GeneChip Command Console (AGCC, Thermo Fisher Scientific, Waltham, MA, USA) and analyzed with the ThermoFisher GeneChip Expression Console (Thermo Fisher Scientific, Waltham, MA, USA). The quality control of the scanned data was first estimated by confirming the order of the signal intensities of the Poly-A and Hybridization controls using Expression Console Software (Thermo Fisher Scientific, Waltham, MA, USA). Raw expression values were imported as .CEL files into TAC 4.0 software (Thermo Fisher Scientific, Waltham, MA, USA). Raw expression values from the Clariom™ D mouse arrays were analyzed and normalized using the TAC 4.0 software, which includes the Preprocessing, Differentially Expressed Genes (DEGs) Finding, and Clustering modules.

For this experiment, a total of 9 .CEL files (3 CTRL, 3 i-EVs, 3 IL-4 EVs) were uploaded and normalized in perfect match (PM)-only conditions as a PM intensity adjustment. A Robust Multichip Analysis (RMA) quantification method (Irizarry et al., 2003) was used as a probe set summarization algorithm for log transformation with base 2 (log2) and the Quantile normalization method was chosen to evaluate the preliminary data quality in the Preprocessing module, which functions as a data quality control through the ThermoFisher Expression Console Software. The mean signal intensities of all genes were obtained using 3 chips from each group. After normalization, the differentially expressed genes (DEGs) satisfying the conditions of the fold change cutoff 1,5 and a FDR corrected p-value < 0,05 from all the genes probed in the array, were selected as DEGs.

5.15 Bioinformatic analysis

Ingenuity® Pathway Analysis (IPA®; Qiagen, Hilden, Germany; www.qiagen.com/ingenuity) software was used to perform functional enrichment of canonical pathways and upstream regulator analysis on DEGs identified in IL-4 EVs vs CTRL. For canonical pathways, positive Z-score indicates activation, whereas negative Z-score indicates inhibition of the biological process. For upstream regulator analysis, Z-score>2 indicates that the signaling guided by a transcriptional regulator is likely activated,

whereas Z-score<2 indicates that it is likely inhibited in the experimental condition considered.

5.16 Statistical analysis

For each experimental procedure, the proper sample size has been calculated by mean of the G*Power 3.1 software, estimating effect size and standard deviation based on previously published data and fixing the alpha value (type 1 error) at the level of 5% (p=0.05) and the power at 80%.

Data are expressed as mean ± standard error (SE). Statistical analysis was performed using the Prism 7 software (GraphPad, San Diego, CA, USA). Gaussian distribution of the values in each experimental group has been assessed using Shapiro-Wilk (for n<8) or D'Agostino-Pearson (for n>8) normality tests. For all comparisons between two groups with a normal distribution, unpaired Student's t-test was performed, while for groups without normal distribution nonparametric Mann-Whitney test was used. For multiple comparison testing, depending on the experimental design, one-way or two-way analysis of variance (ANOVA) accompanied by Tukey's post-hoc test was used for groups with normal distribution, whereas nonparametric Kruskal-Wallis test followed by Dunn's post-hoc analysis was performed when normal distribution of the values could not be assumed. For correlation analysis, two-tailed Pearson test was used. Differences were considered significant for p-value <0.05.

6. REFERENCES

Aarum, J., Sandberg, K., Haeberlein, S.L.B., and Persson, M.A.A. (2003). Migration and differentiation of neural precursor cells can be directed by microglia. Proc. Natl. Acad. Sci. U. S. A. *100*, 15983–15988.

Abbracchio, M.P., Burnstock, G., Boeynaems, J.M., Barnard, E.A., Boyer, J.L., Kennedy, C., Knight, G.E., Fumagalli, M., Gachet, C., Jacobson, K.A., et al. (2006). International Union of Pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: From molecular mechanisms and pathophysiology to therapy. Pharmacol. Rev. *58*, 281–341.

Aggarwal, S., Yurlova, L., and Simons, M. (2011). Central nervous system myelin: Structure, synthesis and assembly. Trends Cell Biol. *21*, 585–593.

Amici, S.A., Dong, J., and Guerau-de-Arellano, M. (2017). Molecular mechanisms modulating the phenotype of macrophages and microglia. Front. Immunol. *8*, 1520.

Angelini, J., Marangon, D., Raffaele, S., Lecca, D., and Abbracchio, M.P. (2021). The distribution of gpr17-expressing cells correlates with white matter inflammation status in brain tissues of multiple sclerosis patients. Int. J. Mol. Sci. *22*, 4574.

Anrather, J., and Iadecola, C. (2016). Inflammation and Stroke: An Overview. Neurotherapeutics *13*, 661–670.

Antonucci, F., Turola, E., Riganti, L., Caleo, M., Gabrielli, M., Perrotta, C., Novellino, L., Clementi, E., Giussani, P., Viani, P., et al. (2012). Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism. EMBO J. *31*, 1231–1240.

Arnett, H.A., Mason, J., Marino, M., Suzuki, K., Matsushima, G.K., and Ting, J.P.Y. (2001). TNFα promotes proliferation of oligodendrocyte progenitors and remyelination. Nat. Neurosci. *4*, 1116–1122.

Atretkhany, K.S.N., Gogoleva, V.S., Drutskaya, M.S., and Nedospasov, S.A. (2020). Distinct modes of TNF signaling through its two receptors in health and disease. J. Leukoc. Biol. *107*, 893–905.

Balkaya, M.G., Trueman, R.C., Boltze, J., Corbett, D., and Jolkkonen, J. (2018). Behavioral outcome measures to improve experimental stroke research. Behav. Brain Res. *352*, 161–171.

Bang, O.Y., and Kim, E.H. (2019). Mesenchymal Stem Cell-Derived Extracellular Vesicle Therapy for Stroke: Challenges and Progress. Front. Neurol. *10*, 211.

Barateiro, A., and Fernandes, A. (2014). Temporal oligodendrocyte lineage progression: In vitro models of proliferation, differentiation and myelination. Biochim. Biophys. Acta - Mol. Cell Res. *1843*, 1917–1929.

Barthels, D., and Das, H. (2020). Current advances in ischemic stroke research and therapies. Biochim. Biophys. Acta - Mol. Basis Dis. *1866*, 165260.

Basso, M., and Bonetto, V. (2016). Extracellular vesicles and a novel form of communication in the brain. Front. Neurosci. *10*, 127.

Bauer, N.G., Richter-Landsberg, C., and Ffrench-Constant, C. (2009). Role of the oligodendroglial cytoskeleton in differentiation and myelination. Glia *57*, 1691–1705.

Baumann, N., and Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol. Rev. *81*, 871–927.

Bechler, M.E., Byrne, L., and Ffrench-Constant, C. (2015). CNS Myelin Sheath Lengths Are an Intrinsic Property of Oligodendrocytes. Curr. Biol. *25*, 2411–2416.

Benarroch, E.E. (2009). Oligodendrocytes: Susceptibility to injury and involvement in neurologic disease. Neurology *72*, 1779–1785.

Benned-Jensen, T., and Rosenkilde, M.M. (2010). Distinct expression and ligand-binding profiles of two constitutively active GPR17 splice variants. Br. J. Pharmacol. *159*, 1092–1105.

Bernier, L.P., Bohlen, C.J., York, E.M., Choi, H.B., Kamyabi, A., Dissing-Olesen, L., Hefendehl, J.K., Collins, H.Y.,

Stevens, B., Barres, B.A., et al. (2019). Nanoscale Surveillance of the Brain by Microglia via cAMP-Regulated Filopodia. Cell Rep. *27*, 2895-2908.e4.

Beyer, B.A., Fang, M., Sadrian, B., Montenegro-Burke, J.R., Plaisted, W.C., Kok, B.P.C., Saez, E., Kondo, T., Siuzdak, G., and Lairson, L.L. (2018). Metabolomics-based discovery of a metabolite that enhances oligodendrocyte maturation. Nat. Chem. Biol. *14*, 22–28.

Bianco, F., Pravettoni, E., Colombo, A., Schenk, U., Möller, T., Matteoli, M., and Verderio, C. (2005). Astrocyte-Derived ATP Induces Vesicle Shedding and IL-1β Release from Microglia. J. Immunol. *174*, 7268–7277.

Bissig, C., and Gruenberg, J. (2014). ALIX and the multivesicular endosome: ALIX in Wonderland. Trends Cell Biol. *24*, 19–25.

Boccazzi, M., Van Steenwinckel, J., Schang, A.L., Faivre, V., Le Charpentier, T., Bokobza, C., Csaba, Z., Verderio, C., Fumagalli, M., Mani, S., et al. (2021). The immune-inflammatory response of oligodendrocytes in a murine model of preterm white matter injury: the role of TLR3 activation. Cell Death Dis. *12*, 1–12.

Boda, E., Viganò, F., Rosa, P., Fumagalli, M., Labat-Gest, V., Tempia, F., Abbracchio, M.P., Dimou, L., and Buffo, A. (2011). The GPR17 receptor in NG2 expressing cells: Focus on in vivocell maturation and participation in acute trauma and chronic damage. Glia *59*, 1958–1973.

Bonfanti, E., Gelosa, P., Fumagalli, M., Dimou, L., Viganò, F., Tremoli, E., Cimino, M., Sironi, L., and Abbracchio, M.P. (2017). The role of oligodendrocyte precursor cells expressing the GPR17 receptor in brain remodeling after stroke. Cell Death Dis. *8*, e2871.

Bonfanti, E., Bonifacino, T., Raffaele, S., Milanese, M., Morgante, E., Bonanno, G., Abbracchio, M.P., and Fumagalli, M. (2020). Abnormal Upregulation of GPR17 Receptor Contributes to Oligodendrocyte Dysfunction in SOD1 G93A Mice. Int. J. Mol. Sci. *21*, 2395.

Bosch-Queralt, M., Cantuti-Castelvetri, L., Damkou, A., Schifferer, M., Schlepckow, K., Alexopoulos, I., Lütjohann, D., Klose, C., Vaculčiaková, L., Masuda, T., et al. (2021). Diet-dependent regulation of TGFβ impairs reparative innate immune responses after demyelination. Nat. Metab. *3*, 211–227.

Boza-Serrano, A., Ruiz, R., Sanchez-Varo, R., García-Revilla, J., Yang, Y., Jimenez-Ferrer, I., Paulus, A., Wennström, M., Vilalta, A., Allendorf, D., et al. (2019). Galectin-3, a novel endogenous TREM2 ligand, detrimentally regulates inflammatory response in Alzheimer's disease. Acta Neuropathol. *138*, 251–273.

Van den Broek, B., Pintelon, I., Hamad, I., Kessels, S., Haidar, M., Hellings, N., Hendriks, J.J.A., Kleinewietfeld, M., Brône, B., Timmerman, V., et al. (2020). Microglial derived extracellular vesicles activate autophagy and mediate multi-target signaling to maintain cellular homeostasis. J. Extracell. Vesicles *10*, e12022.

Budnik, V., Ruiz-Cañada, C., and Wendler, F. (2016). Extracellular vesicles round off communication in the nervous system. Nat. Rev. Neurosci. *17*, 160–172.

Campbell, B.C.V., De Silva, D.A., Macleod, M.R., Coutts, S.B., Schwamm, L.H., Davis, S.M., and Donnan, G.A. (2019). Ischaemic stroke. Nat. Rev. Dis. Prim. *5*, 1–22.

Cantoni, C., Bollman, B., Licastro, D., Xie, M., Mikesell, R., Schmidt, R., Yuede, C.M., Galimberti, D., Olivecrona, G., Klein, R.S., et al. (2015). TREM2 regulates microglial cell activation in response to demyelination in vivo. Acta Neuropathol. *129*, 429–447.

Cantuti-Castelvetri, L., Fitzner, D., Bosch-Queralt, M., Weil, M.T., Su, M., Sen, P., Ruhwedel, T., Mitkovski, M., Trendelenburg, G., Lütjohann, D., et al. (2018). Defective cholesterol clearance limits remyelination in the aged central nervous system. Science (80-.). *359*, 684–688.

Casella, G., Colombo, F., Finardi, A., Descamps, H., Ill-Raga, G., Spinelli, A., Podini, P., Bastoni, M., Martino, G., Muzio, L., et al. (2018). Extracellular Vesicles Containing IL-4 Modulate Neuroinflammation in a Mouse Model of Multiple Sclerosis. Mol. Ther. *26*, 2107–2118.

Ceruti, S., Viganò, F., Boda, E., Ferrario, S., Magni, G., Boccazzi, M., Rosa, P., Buffo, A., and Abbracchio, M.P.

(2011). Expression of the new P2Y-like receptor GPR17 during oligodendrocyte precursor cell maturation regulates sensitivity to ATP-induced death. Glia *59*, 363–378.

Chen, N., Zhou, P., Liu, X., Li, J., Wan, Y., Liu, S., and Wei, F. (2020). Overexpression of Rictor in the injured spinal cord promotes functional recovery in a rat model of spinal cord injury. FASEB J. *34*, 6984–6998.

Chen, Y., Wu, H., Wang, S., Koito, H., Li, J., Ye, F., Hoang, J., Escobar, S.S., Gow, A., Arnett, H.A., et al. (2009). The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. Nat. Neurosci. *12*, 1398–1406.

Chong, S.Y.C., Rosenberg, S.S., Fancy, S.P.J., Zhao, C., Shen, Y.A.A., Hahn, A.T., McGee, A.W., Xu, X., Zheng, B., Zhang, L.I., et al. (2012). Neurite outgrowth inhibitor Nogo-A establishes spatial segregation and extent of oligodendrocyte myelination. Proc. Natl. Acad. Sci. U. S. A. *109*, 1299–1304.

Ciana, P., Fumagalli, M., Trincavelli, M.L., Verderio, C., Rosa, P., Lecca, D., Ferrario, S., Parravicini, C., Capra, V., Gelosa, P., et al. (2006). The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. EMBO J. *25*, 4615–4627.

Cignarella, F., Filipello, F., Bollman, B., Cantoni, C., Locca, A., Mikesell, R., Manis, M., Ibrahim, A., Deng, L., Benitez, B.A., et al. (2020). TREM2 activation on microglia promotes myelin debris clearance and remyelination in a model of multiple sclerosis. Acta Neuropathol. *140*, 513–534.

Claus Stolt, C., Rehberg, S., Ader, M., Lommes, P., Riethmacher, D., Schachner, M., Bartsch, U., and Wegner, M. (2002). Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. Genes Dev. *16*, 165–170.

Clausen, B.H., Clausen, B.H., Wirenfeldt, M., Wirenfeldt, M., Høgedal, S.S., Frich, L.H., Frich, L.H., Nielsen, H.H., Nielsen, H.H., Nielsen, H.H., et al. (2020). Characterization of the TNF and IL-1 systems in human brain and blood after ischemic stroke. Acta Neuropathol. Commun. *8*, 1–17.

Cocucci, E., and Meldolesi, J. (2015). Ectosomes and exosomes: Shedding the confusion between extracellular vesicles. Trends Cell Biol. *25*, 364–372.

Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. *30*, 255–289.

Colonna, M., and Butovsky, O. (2017). Microglia function in the central nervous system during health and neurodegeneration. Annu. Rev. Immunol. *35*, 441–468.

Comi, G., Radaelli, M., and Soelberg Sørensen, P. (2017). Evolving concepts in the treatment of relapsing multiple sclerosis. Lancet *389*, 1347–1356.

Coppolino, G.T., Marangon, D., Negri, C., Menichetti, G., Fumagalli, M., Gelosa, P., Dimou, L., Furlan, R., Lecca, D., and Abbracchio, M.P. (2018). Differential local tissue permissiveness influences the final fate of GPR17-expressing oligodendrocyte precursors in two distinct models of demyelination. Glia *66*, 1118–1130.

Crociara, P., Parolisi, R., Conte, D., Fumagalli, M., and Bonfanti, L. (2013). Cellular and Molecular Characterization of Multipolar Map5-Expressing Cells: A Subset of Newly Generated, Stage-Specific Parenchymal Cells in the Mammalian Central Nervous System. PLoS One *8*, e63258.

Crockett, D.P., Burshteyn, M., Garcia, C., Muggironi, M., and Casaccia-Bonnefil, P. (2005). Number of oligodendrocyte progenitors recruited to the lesioned spinal cord is modulated by the levels of the cell cycle regulatory protein p27Kip-1. Glia *49*, 301–308.

Crotti, A., Sait, H.R., McAvoy, K.M., Estrada, K., Ergun, A., Szak, S., Marsh, G., Jandreski, L., Peterson, M., Reynolds, T.L., et al. (2019). BIN1 favors the spreading of Tau via extracellular vesicles. Sci. Rep. *9*, 1–20.

Cserép, C., Pósfai, B., Lénárt, N., Fekete, R., László, Z.I., Lele, Z., Orsolits, B., Molnár, G., Heindl, S., Schwarcz, A.D., et al. (2020). Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. Science (80-.). *367*, 528–537.

Cunha, M.I., Su, M., Cantuti-Castelvetri, L., Müller, S.A., Schifferer, M., Djannatian, M., Alexopoulos, I., van der Meer, F., Winkler, A., van Ham, T.J., et al. (2020). Pro-inflammatory activation following demyelination is required for myelin clearance and oligodendrogenesis. J. Exp. Med. *217*.

Dai, J., Bercury, K.K., Ahrendsen, J.T., and Macklin, W.B. (2015). Olig1 function is required for oligodendrocyte differentiation in the mouse brain. J. Neurosci. *35*, 4386–4402.

Daniele, S., Trincavelli, M.L., Fumagalli, M., Zappelli, E., Lecca, D., Bonfanti, E., Campiglia, P., Abbracchio, M.P., and Martini, C. (2014). Does GRK-β arrestin machinery work as a "switch on" for GPR17-mediated activation of intracellular signaling pathways? Cell. Signal. *26*, 1310–1325.

Dawson, M.R.L., Polito, A., Levine, J.M., and Reynolds, R. (2003). NG2-expressing glial progenitor cells: An abundant and widespread population of cycling cells in the adult rat CNS. Mol. Cell. Neurosci. *24*, 476–488.

Delpech, J.C., Herron, S., Botros, M.B., and Ikezu, T. (2019). Neuroimmune Crosstalk through Extracellular Vesicles in Health and Disease. Trends Neurosci. *42*, 361–372.

Desu, H.L., Illiano, P., Choi, J.S., Ascona, M.C., Gao, H., Lee, J.K., and Brambilla, R. (2021). TNFR2 Signaling Regulates the Immunomodulatory Function of Oligodendrocyte Precursor Cells. Cells *10*, 1785.

Dimas, P., Montani, L., Pereira, J.A., Moreno, D., Trötzmüller, M., Gerber, J., Semenkovich, C.F., Köfeler, H.C., and Suter, U. (2019). Cns myelination and remyelination depend on fatty acid synthesis by oligodendrocytes. Elife *8*.

Drago, F., Lombardi, M., Prada, I., Gabrielli, M., Joshi, P., Cojoc, D., Franck, J., Fournier, I., Vizioli, J., and Verderio, C. (2017). ATP modifies the proteome of extracellular vesicles released by microglia and influences their action on astrocytes. Front. Pharmacol. *8*, 910.

El-Koussy, M., Schroth, G., Brekenfeld, C., and Arnold, M. (2014). Imaging of acute ischemic stroke. Eur. Neurol. *72*, 309–316.

Elbaz, B., and Popko, B. (2019). Molecular Control of Oligodendrocyte Development. Trends Neurosci. *42*, 263–277.

Emery, B. (2010). Regulation of oligodendrocyte differentiation and myelination. Science (80-.). *330*, 779–782.

Fais, S., O'Driscoll, L., Borras, F.E., Buzas, E., Camussi, G., Cappello, F., Carvalho, J., Cordeiro Da Silva, A., Del Portillo, H., El Andaloussi, S., et al. (2016). Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. ACS Nano *10*, 3886–3899.

Fard, M.K., Van der Meer, F., Sánchez, P., Cantuti-Castelvetri, L., Mandad, S., Jäkel, S., Fornasiero, E.F., Schmitt, S., Ehrlich, M., Starost, L., et al. (2017). BCAS1 expression defines a population of early myelinating oligodendrocytes in multiple sclerosis lesions. Sci. Transl. Med. *9*.

Fischer, R., Sendetski, M., del Rivero, T., Martinez, G.F., Bracchi-Ricard, V., Swanson, K.A., Pruzinsky, E.K., Delguercio, N., Rosalino, M.J., Padutsch, T., et al. (2019). TNFR2 promotes Treg-mediated recovery from neuropathic pain across sexes. Proc. Natl. Acad. Sci. U. S. A. *116*, 17045–17050.

Fischer, R., Kontermann, R.E., and Pfizenmaier, K. (2020). Selective Targeting of TNF Receptors as a Novel Therapeutic Approach. Front. Cell Dev. Biol. *8*, 401.

Fitzgerald, W., Freeman, M.L., Lederman, M.M., Vasilieva, E., Romero, R., and Margolis, L. (2018). A System of Cytokines Encapsulated in ExtraCellular Vesicles. Sci. Rep. *8*, 1–11.

Fitzner, D., Schneider, A., Kippert, A., Möbius, W., Willig, K.I., Hell, S.W., Bunt, G., Gaus, K., and Simons, M. (2006). Myelin basic protein-dependent plasma membrane reorganization in the formation of myelin. EMBO J. *25*, 5037–5048.

Foerster, S., Hill, M.F.E., and Franklin, R.J.M. (2019). Diversity in the oligodendrocyte lineage: Plasticity or heterogeneity? Glia *67*, 1797–1805.

Franke, H., Parravicini, C., Lecca, D., Zanier, E.R., Heine, C., Bremicker, K., Fumagalli, M., Rosa, P., Longhi, L., Stocchetti, N., et al. (2013). Changes of the GPR17 receptor, a new target for neurorepair, in neurons and glial cells in patients with traumatic brain injury. Purinergic Signal. *9*, 451–462.

Franklin, R.J.M., and Ffrench-Constant, C. (2008). Remyelination in the CNS: From biology to therapy. Nat. Rev. Neurosci. *9*, 839–855.

Franklin, R.J.M., and Ffrench-Constant, C. (2017). Regenerating CNS myelin - From mechanisms to experimental medicines. Nat. Rev. Neurosci. *18*, 753–769.

Frey, B., and Gaipl, U.S. (2011). The immune functions of phosphatidylserine in membranes of dying cells and microvesicles. Semin. Immunopathol. *33*, 497–516.

Fumagalli, M., Daniele, S., Lecca, D., Lee, P.R., Parravicini, C., Douglas Fields, R., Rosa, P., Antonucci, F., Verderio, C., Letizia Trincavelli, M., et al. (2011). Phenotypic changes, signaling pathway, and functional correlates of GPR17-expressing neural precursor cells during oligodendrocyte differentiation. J. Biol. Chem. *286*, 10593–10604.

Fumagalli, M., Bonfanti, E., Daniele, S., Zappelli, E., Lecca, D., Martini, C., Trincavelli, M.L., and Abbracchio, M.P. (2015a). The ubiquitin ligase Mdm2 controls oligodendrocyte maturation by intertwining mTOR with G protein-coupled receptor kinase 2 in the regulation of GPR17 receptor desensitization. Glia *63*, 2327–2339.

Fumagalli, M., Lecca, D., and Abbracchio, M.P. (2016). CNS remyelination as a novel reparative approach to neurodegenerative diseases: The roles of purinergic signaling and the P2Y-like receptor GPR17. Neuropharmacology *104*, 82–93.

Fumagalli, M., Lecca, D., Coppolino, G.T., Parravicini, C., and Abbracchio, M.P. (2017). Pharmacological properties and biological functions of the GPR17 receptor, a potential target for neuro-regenerative medicine. In Advances in Experimental Medicine and Biology, (Springer New York LLC), pp. 169–192.

Fumagalli, M., Lombardi, M., Gressens, P., and Verderio, C. (2018). How to reprogram microglia toward beneficial functions. Glia *66*, 2531–2549.

Fumagalli, S., Perego, C., Pischiutta, F., Zanier, E.R., and De Simoni, M.G. (2015b). The ischemic environment drive s microglia and macrophage function. Front. Neurol. *6*, 1–19.

Gabrielli, M., Battista, N., Riganti, L., Prada, I., Antonucci, F., Cantone, L., Matteoli, M., Maccarrone, M., and Verderio, C. (2015). Active endocannabinoids are secreted on extracellular membrane vesicles. EMBO Rep. *16*, 213–220.

Gao, H., Danzi, M.C., Choi, C.S., Taherian, M., Dalby-Hansen, C., Ellman, D.G., Madsen, P.M., Bixby, J.L., Lemmon, V.P., Lambertsen, K.L., et al. (2017). Opposing Functions of Microglial and Macrophagic TNFR2 in the Pathogenesis of Experimental Autoimmune Encephalomyelitis. Cell Rep. *18*, 198–212.

Garzetti, L., Menon, R., Finardi, A., Bergami, A., Sica, A., Martino, G., Comi, G., Verderio, C., Farina, C., and Furlan, R. (2014). Activated macrophages release microvesicles containing polarized M1 or M2 mRNAs. J. Leukoc. Biol. *95*, 817–825.

Gasser, O., and Schifferli, J.A. (2004). Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. Blood *104*, 2543–2548.

Gelosa, P., Colazzo, F., Tremoli, E., Sironi, L., and Castiglioni, L. (2017). Cysteinyl Leukotrienes as Potential Pharmacological Targets for Cerebral Diseases. Mediators Inflamm. *2017*.

Giera, S., Luo, R., Ying, Y., Ackerman, S.D., Jeong, S.J., Stoveken, H.M., Folts, C.J., Welsh, C.A., Tall, G.G., Stevens, B., et al. (2018). Microglial transglutaminase-2 drives myelination and myelin repair via GPR56/ADGRG1 in oligodendrocyte precursor cells. Elife *7*.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley,

E.R., et al. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science (80-.). *330*, 841–845.

Ginsberg, M.D. (2018). The cerebral collateral circulation: Relevance to pathophysiology and treatment of stroke. Neuropharmacology *134*, 280–292.

Glezer, I., Lapointe, A., and Rivest, S. (2006). Innate immunity triggers oligodendrocyte progenitor reactivity and confines damages to brain injuries. FASEB J. *20*, 750–752.

Grier, M.D., West, K.L., Kelm, N.D., Fu, C., Does, M.D., Parker, B., McBrier, E., Lagrange, A.H., Ess, K.C., and Carson, R.P. (2017). Loss of mTORC2 signaling in oligodendrocyte precursor cells delays myelination. PLoS One *12*, e0188417.

Grimaldi, A., Serpe, C., Chece, G., Nigro, V., Sarra, A., Ruzicka, B., Relucenti, M., Familiari, G., Ruocco, G., Pascucci, G.R., et al. (2019). Microglia-derived microvesicles affect microglia phenotype in glioma. Front. Cell. Neurosci. *13*, 41.

Gruchot, J., Weyers, V., Göttle, P., Förster, M., Hartung, H.P., Küry, P., and Kremer, D. (2019). The Molecular Basis for Remyelination Failure in Multiple Sclerosis. Cells *8*.

Gualerzi, A., Picciolini, S., Rodà, F., and Bedoni, M. (2021). Extracellular Vesicles in Regeneration and Rehabilitation Recovery after Stroke. Biology (Basel). *10*, 843.

Hagemeyer, N., Hanft, K.M., Akriditou, M.A., Unger, N., Park, E.S., Stanley, E.R., Staszewski, O., Dimou, L., and Prinz, M. (2017). Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. Acta Neuropathol. *134*, 441–458.

Hankey, G.J. (2017). Stroke. Lancet *389*, 641–654.

Hasel, P., Rose, I.V.L., Sadick, J.S., Kim, R.D., and Liddelow, S.A. (2021). Neuroinflammatory astrocyte subtypes in the mouse brain. Nat. Neurosci. 1–13.

Heindl, S., Gesierich, B., Benakis, C., Llovera, G., Duering, M., and Liesz, A. (2018). Automated morphological analysis of microglia after stroke. Front. Cell. Neurosci. *12*, 106.

Houtkooper, R.H., Pirinen, E., and Auwerx, J. (2012). Sirtuins as regulators of metabolism and healthspan. Nat. Rev. Mol. Cell Biol. *13*, 225–238.

Hoyos, H.C., Rinaldi, M., Mendez-Huergo, S.P., Marder, M., Rabinovich, G.A., Pasquini, J.M., and Pasquini, L.A. (2014). Galectin-3 controls the response of microglial cells to limit cuprizone-induced demyelination. Neurobiol. Dis. *62*, 441–455.

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R.K., Chen, S., Gao, Y., and Chen, J. (2012). Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke *43*, 3063–3070.

Hubler, Z., Allimuthu, D., Bederman, I., Elitt, M.S., Madhavan, M., Allan, K.C., Shick, H.E., Garrison, E., T. Karl, M., Factor, D.C., et al. (2018). Accumulation of 8,9-unsaturated sterols drives oligodendrocyte formation and remyelination. Nature *560*, 372–376.

Hughes, A.N., and Appel, B. (2020). Microglia phagocytose myelin sheaths to modify developmental myelination. Nat. Neurosci. *23*, 1055–1066.

Iadecola, C., and Anrather, J. (2011). The immunology of stroke: From mechanisms to translation. Nat. Med. *17*, 796–808.

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., and Speed, T.P. (2003). Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. *31*, e15.

Jablonska, B., Gierdalski, M., Chew, L.J., Hawley, T., Catron, M., Lichauco, A., Cabrera-Luque, J., Yuen, T., Rowitch, D., and Gallo, V. (2016). Sirt1 regulates glial progenitor proliferation and regeneration in white

matter after neonatal brain injury. Nat. Commun. *7*, 1–16.

Jayaraj, R.L., Azimullah, S., Beiram, R., Jalal, F.Y., and Rosenberg, G.A. (2019). Neuroinflammation: Friend and foe for ischemic stroke. J. Neuroinflammation *16*, 1–24.

Ji, S., Doucette, J.R., and Nazarali, A.J. (2011). Sirt2 is a novel in vivo downstream target of Nkx2.2 and enhances oligodendroglial cell differentiation. J. Mol. Cell Biol. *3*, 351–359.

Jia, W., Kamen, Y., Pivonkova, H., and Káradóttir, R.T. (2019). Neuronal activity-dependent myelin repair after stroke. Neurosci. Lett. *703*, 139–144.

Jin, W.N., Shi, S.X.Y., Li, Z., Li, M., Wood, K., Gonzales, R.J., and Liu, Q. (2017). Depletion of microglia exacerbates postischemic inflammation and brain injury. J. Cereb. Blood Flow Metab. *37*, 2224–2236.

Joshi, P., Turola, E., Ruiz, A., Bergami, A., Libera, D.D., Benussi, L., Giussani, P., Magnani, G., Comi, G., Legname, G., et al. (2014). Microglia convert aggregated amyloid-β into neurotoxic forms through the shedding of microvesicles. Cell Death Differ. *21*, 582–593.

Kamphuis, W., Kooijman, L., Schetters, S., Orre, M., and Hol, E.M. (2016). Transcriptional profiling of CD11c-positive microglia accumulating around amyloid plaques in a mouse model for Alzheimer's disease. Biochim. Biophys. Acta - Mol. Basis Dis. *1862*, 1847–1860.

Kassem-Moussa, H., and Graffagnino, C. (2002). Nonocclusion and spontaneous recanalization rates in acute ischemic stroke: A review of cerebral angiography studies. Arch. Neurol. *59*, 1870–1873.

Kawabori, M., and Yenari, M. (2015). Inflammatory Responses in Brain Ischemia. Curr. Med. Chem. *22*, 1258–1277.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., and Richardson, W.D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nat. Neurosci. *9*, 173–179.

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: New Roles for the Synaptic Stripper. Neuron *77*, 10–18.

Kirby, L., and Castelo-Branco, G. (2021). Crossing boundaries: Interplay between the immune system and oligodendrocyte lineage cells. Semin. Cell Dev. Biol. *116*, 45–52.

Kottakis, F., Nicolay, B.N., Roumane, A., Karnik, R., Gu, H., Nagle, J.M., Boukhali, M., Hayward, M.C., Li, Y.Y., Chen, T., et al. (2016). LKB1 loss links serine metabolism to DNA methylation and tumorigenesis. Nature *539*, 390–395.

Kuhn, S., Gritti, L., Crooks, D., and Dombrowski, Y. (2019). Oligodendrocytes in Development, Myelin Generation and Beyond. Cells *8*, 1424.

De La Fuente, A.G., Lange, S., Silva, M.E., Gonzalez, G.A., Tempfer, H., van Wijngaarden, P., Zhao, C., Di Canio, L., Trost, A., Bieler, L., et al. (2017). Pericytes Stimulate Oligodendrocyte Progenitor Cell Differentiation during CNS Remyelination. Cell Rep. *20*, 1755–1764.

Lai, T.W., Zhang, S., and Wang, Y.T. (2014). Excitotoxicity and stroke: Identifying novel targets for neuroprotection. Prog. Neurobiol. *115*, 157–188.

Lambertsen, K.L., Meldgaard, M., Ladeby, R., and Finsen, B. (2005). A quantitative study of microglial-macrophage synthesis of tumor necrosis factor during acute and late focal cerebral ischemia in mice. J. Cereb. Blood Flow Metab. *25*, 119–135.

Lambertsen, K.L., Gramsbergen, J.B., Sivasaravanaparan, M., Ditzel, N., Sevelsted-Møller, L.M., Oliván-Viguera, A., Rabjerg, M., Wulff, H., and Köhler, R. (2012). Genetic KCa3.1-Deficiency Produces Locomotor Hyperactivity and Alterations in Cerebral Monoamine Levels. PLoS One *7*, e47744.

Lambertsen, K.L., Finsen, B., and Clausen, B.H. (2019). Post-stroke inflammation—target or tool for therapy?

Acta Neuropathol. *137*, 693–714.

Lampron, A., Larochelle, A., Laflamme, N., Préfontaine, P., Plante, M.M., Sánchez, M.G., Wee Yong, V., Stys, P.K., Tremblay, M.È., and Rivest, S. (2015). Inefficient clearance of myelin debris by microglia impairs remyelinating processes. J. Exp. Med. *212*, 481–495.

Lecca, D., and Ceruti, S. (2008). Uracil nucleotides: From metabolic intermediates to neuroprotection and neuroinflammation. Biochem. Pharmacol. *75*, 1869–1881.

Lecca, D., Trincavelli, M.L., Gelosa, P., Sironi, L., Ciana, P., Fumagalli, M., Villa, G., Verderio, C., Grumelli, C., Guerrini, U., et al. (2008). The recently identified P2Y-like receptor GPR17 is a sensor of brain damage and a new target for brain repair. PLoS One *3*.

Lecca, D., Raffaele, S., Abbracchio, M.P., and Fumagalli, M. (2020). Regulation and signaling of the GPR17 receptor in oligodendroglial cells. Glia *2*, 1–11.

Lecuyer, M., Pathipati, P., Faustino, J., and Vexler, Z.S. (2021). Neonatal stroke enhances interaction of microglia-derived extracellular vesicles with microglial cells. Neurobiol. Dis. *157*, 105431.

Lener, T., Gimona, M., Aigner, L., Börger, V., Buzas, E., Camussi, G., Chaput, N., Chatterjee, D., Court, F.A., del Portillo, H.A., et al. (2015). Applying extracellular vesicles based therapeutics in clinical trials - An ISEV position paper. J. Extracell. Vesicles *4*, 30087.

Levine, J.M., and Reynolds, R. (1999). Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. Exp. Neurol. *160*, 333–347.

Li, Q., and Barres, B.A. (2018). Microglia and macrophages in brain homeostasis and disease. Nat. Rev. Immunol. *18*, 225–242.

Li, W.W., Setzu, A., Zhao, C., and Franklin, R.J.M. (2005). Minocycline-mediated inhibition of microglia activation impairs oligodendrocyte progenitor cell responses and remyelination in a non-immune model of demyelination. J. Neuroimmunol. *158*, 58–66.

Li, Z., Song, Y., He, T., Wen, R., Li, Y., Chen, T., Huang, S., Wang, Y., Tang, Y., Shen, F.X., et al. (2021). M2 microglial small extracellular vesicles reduce glial scar formation via the miR-124/STAT3 pathway after ischemic stroke in mice. Theranostics *11*, 1232–1248.

Liddelow, S.A., Guttenplan, K.A., Clarke, L.E., Bennett, F.C., Bohlen, C.J., Schirmer, L., Bennett, M.L., Münch, A.E., Chung, W.S., Peterson, T.C., et al. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. Nature *541*, 481–487.

Liebeskind, D.S., Jüttler, E., Shapovalov, Y., Yegin, A., Landen, J., and Jauch, E.C. (2019). Cerebral Edema Associated with Large Hemispheric Infarction: Implications for Diagnosis and Treatment. Stroke *50*, 2619–2625.

Lin, J.P., Mironova, Y.A., Shrager, P., and Giger, R.J. (2017). LRP1 regulates peroxisome biogenesis and cholesterol homeostasis in oligodendrocytes and is required for proper CNS myelin development and repair. Elife *6*.

Lizcano, J.M., Göransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., Hawley, S.A., Udd, L., Mäkelä, T.P., Hardie, D.G., et al. (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J. *23*, 833–843.

Lloyd, A.F., and Miron, V.E. (2019). The pro-remyelination properties of microglia in the central nervous system. Nat. Rev. Neurol. 29–34.

Lombardi, M., Parolisi, R., Scaroni, F., Bonfanti, E., Gualerzi, A., Gabrielli, M., Kerlero de Rosbo, N., Uccelli, A., Giussani, P., Viani, P., et al. (2019). Detrimental and protective action of microglial extracellular vesicles on myelin lesions: astrocyte involvement in remyelination failure. Acta Neuropathol. *138*, 987–1012.

Lösche, W., Scholz, T., Temmler, U., Oberle, V., and Claus, R.A. (2004). Platelet-derived microvesicles transfer

tissue factor to monocytes but not to neutrophils. Platelets *15*, 109–115.

Losurdo, M., Pedrazzoli, M., D'Agostino, C., Elia, C.A., Massenzio, F., Lonati, E., Mauri, M., Rizzi, L., Molteni, L., Bresciani, E., et al. (2020). Intranasal delivery of mesenchymal stem cell-derived extracellular vesicles exerts immunomodulatory and neuroprotective effects in a 3xTg model of Alzheimer's disease. Stem Cells Transl. Med. *9*, 1068–1084.

Ma, Y., Wang, J., Wang, Y., and Yang, G.Y. (2017). The biphasic function of microglia in ischemic stroke. Prog. Neurobiol. *157*, 247–272.

Mack, M., Kleinschmidt, A., Brühl, H., Klier, C., Nelson, P.J., Cihak, J., Plachý, J., Stangassinger, M., Erfle, V., and Schlöndorff, D. (2000). Transfer of the chemokine receptor CCR5 between cells by membrane- derived microparticles: A mechanism for cellular human immunodeficiency virus 1 infection. Nat. Med. *6*, 769–775.

Madsen, P.M., Motti, D., Karmally, S., Szymkowski, D.E., Lambertsen, K.L., Bethea, J.R., and Brambilla, R. (2016a). Oligodendroglial TNFR2 mediates membrane TNF-dependent repair in experimental autoimmune encephalomyelitis by promoting oligodendrocyte differentiation and remyelination. J. Neurosci. *36*, 5128–5143.

Madsen, P.M., Clausen, B.H., Degn, M., Thyssen, S., Kristensen, L.K., Svensson, M., Ditzel, N., Finsen, B., Deierborg, T., Brambilla, R., et al. (2016b). Genetic ablation of soluble tumor necrosis factor with preservation of membrane tumor necrosis factor is associated with neuroprotection after focal cerebral ischemia. J. Cereb. Blood Flow Metab. *36*, 1553–1569.

Madsen, P.M., Desu, H.L., Vaccari, J.P. de R., Florimon, Y., Ellman, D.G., Keane, R.W., Clausen, B.H., Lambertsen, K.L., and Brambilla, R. (2020). Oligodendrocytes modulate the immune-inflammatory response in EAE via TNFR2 signaling. Brain. Behav. Immun. *84*, 132–146.

Magni, G., Pedretti, S., Audano, M., Caruso, D., Mitro, N., and Ceruti, S. (2020). Glial cell activation and altered metabolic profile in the spinal-trigeminal axis in a rat model of multiple sclerosis associated with the development of trigeminal sensitization. Brain. Behav. Immun. *89*, 268–280.

Makin, S.D.J., Turpin, S., Dennis, M.S., and Wardlaw, J.M. (2013). Cognitive impairment after lacunar stroke: Systematic review and meta-analysis of incidence, prevalence and comparison with other stroke subtypes. J. Neurol. Neurosurg. Psychiatry *84*, 893–900.

Marangon, D., Boccazzi, M., Lecca, D., and Fumagalli, M. (2020). Regulation of Oligodendrocyte Functions: Targeting Lipid Metabolism and Extracellular Matrix for Myelin Repair. J. Clin. Med. *9*, 470.

Marie, C., Clavairoly, A., Frah, M., Hmidan, H., Yan, J., Zhao, C., Van Steenwinckel, J., Daveau, R., Zalc, B., Hassan, B., et al. (2018). Oligodendrocyte precursor survival and differentiation requires chromatin remodeling by Chd7 and Chd8. Proc. Natl. Acad. Sci. U. S. A. *115*, E8246–E8255.

Marin, M.A., and Carmichael, S.T. (2018). Stroke in CNS white matter: Models and mechanisms. Neurosci. Lett. *684*, 193–199.

Marisca, R., Hoche, T., Agirre, E., Hoodless, L.J., Barkey, W., Auer, F., Castelo-Branco, G., and Czopka, T. (2020). Functionally distinct subgroups of oligodendrocyte precursor cells integrate neural activity and execute myelin formation. Nat. Neurosci. *23*, 363–374.

Marques, S., Zeisel, A., Codeluppi, S., Van Bruggen, D., Falcão, A.M., Xiao, L., Li, H., Häring, M., Hochgerner, H., Romanov, R.A., et al. (2016). Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. Science (80-.). *352*, 1326–1329.

Matute, C., Domercq, M., Pérez-Samartín, A., and Ransom, B.R. (2013). Protecting white matter from stroke injury. Stroke *44*, 1204–1211.

McIver, S.R., Muccigrosso, M., Gonzales, E.R., Lee, J.M., Roberts, M.S., Sands, M.S., and Goldberg, M.P. (2010). Oligodendrocyte degeneration and recovery after focal cerebral ischemia. Neuroscience *169*, 1364–1375.

McKenzie, I.A., Ohayon, D., Li, H., De Faria, J.P., Emery, B., Tohyama, K., and Richardson, W.D. (2014). Motor skill learning requires active central myelination. Science (80-.). *346*, 318–322.

Menn, B., Garcia-Verdugo, J.M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D., and Alvarez-Buylla, A. (2006). Origin of oligodendrocytes in the subventricular zone of the adult brain. J. Neurosci. *26*, 7907–7918.

Mentkowski, K.I., Snitzer, J.D., Rusnak, S., and Lang, J.K. (2018). Therapeutic Potential of Engineered Extracellular Vesicles. AAPS J. *20*, 1–17.

Meyer, N., and Rinholm, J.E. (2021). Mitochondria in myelinating oligodendrocytes: Slow and out of breath? Metabolites *11*.

Mifsud, G., Zammit, C., Muscat, R., Di Giovanni, G., and Valentino, M. (2014). Oligodendrocyte Pathophysiology and Treatment Strategies in Cerebral Ischemia. CNS Neurosci. Ther. *20*, 603–612.

Miron, V.E. (2017). Microglia-driven regulation of oligodendrocyte lineage cells, myelination, and remyelination. J. Leukoc. Biol. *101*, 1103–1108.

Miron, V.E., Boyd, A., Zhao, J.W., Yuen, T.J., Ruckh, J.M., Shadrach, J.L., Van Wijngaarden, P., Wagers, A.J., Williams, A., Franklin, R.J.M., et al. (2013). M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat. Neurosci. *16*, 1211–1218.

Mitew, S., Hay, C.M., Peckham, H., Xiao, J., Koenning, M., and Emery, B. (2014). Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. Neuroscience *276*, 29–47.

Monje, M. (2018). Myelin plasticity and nervous system function. Annu. Rev. Neurosci. *41*, 61–76.

Moyon, S., Dubessy, A.L., Aigrot, M.S., Trotter, M., Huang, J.K., Dauphinot, L., Potier, M.C., Kerninon, C., Parsadaniantz, S.M., Franklin, R.J.M., et al. (2015). Demyelination causes adult CNS progenitors to revert to an immature state and express immune cues that support their migration. J. Neurosci. *35*, 4–20.

Murtie, J.C., Zhou, Y.X., Le, T.Q., Vana, A.C., and Armstrong, R.C. (2005). PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination. Neurobiol. Dis. *19*, 171–182.

Nakamura, T., Colbert, M.C., and Robbins, J. (2006). Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system. Circ. Res. *98*, 1547–1554.

Nakatani, H., Martin, E., Hassani, H., Clavairoly, A., Maire, C.L., Viadieu, A., Kerninon, C., Delmasure, A., Frah, M., Weber, M., et al. (2013). Ascl1/Mash1 promotes brain oligodendrogenesis during myelination and remyelination. J. Neurosci. *33*, 9752–9768.

Nave, K.A., and Werner, H.B. (2014). Myelination of the nervous system: Mechanisms and functions. In Annual Review of Cell and Developmental Biology, (Annual Reviews), pp. 503–533.

Nayak, D., Roth, T.L., and McGavern, D.B. (2014). Microglia development and function. Annu. Rev. Immunol. *32*, 367–402.

Van Niel, G., D'Angelo, G., and Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. *19*, 213–228.

Nielsen, H.H., Ladeby, R., Drøjdahl, N., Peterson, A.C., and Finsen, B. (2006). Axonal degeneration stimulates the formation of NG2+ cells and oligodendrocytes in the mouse. Glia *54*, 105–115.

Nigro, A., Colombo, F., Casella, G., Finardi, A., Verderio, C., and Furlan, R. (2016). Myeloid extracellular vesicles: Messengers from the Demented Brain. Front. Immunol. *7*, 1.

Nyamoya, S., Leopold, P., Becker, B., Beyer, C., Hustadt, F., Schmitz, C., Michel, A., and Kipp, M. (2019). G-Protein-Coupled Receptor Gpr17 Expression in Two Multiple Sclerosis Remyelination Models. Mol. Neurobiol. *56*, 1109–1123.

O'Callaghan, C., and Vassilopoulos, A. (2017). Sirtuins at the crossroads of stemness, aging, and cancer. Aging Cell *16*, 1208–1218.

Ofengeim, D., Ito, Y., Najafov, A., Zhang, Y., Shan, B., DeWitt, J.P., Ye, J., Zhang, X., Chang, A., Vakifahmetoglu-Norberg, H., et al. (2015). Activation of necroptosis in multiple sclerosis. Cell Rep. *10*, 1836–1849.

Ogata, T. (2019). Therapeutic Strategies for Oligodendrocyte-Mediated Remyelination. In Advances in Experimental Medicine and Biology, (Springer, Singapore), pp. 265–279.

Otero-Ortega, L., Laso-García, F., Gómez-de Frutos, M.C., Fuentes, B., Diekhorst, L., Díez-Tejedor, E., and Gutiérrez-Fernández, M. (2019). Role of Exosomes as a Treatment and Potential Biomarker for Stroke. Transl. Stroke Res. *10*, 241–249.

Otxoa-de-Amezaga, A., Miró-Mur, F., Pedragosa, J., Gallizioli, M., Justicia, C., Gaja-Capdevila, N., Ruíz-Jaen, F., Salas-Perdomo, A., Bosch, A., Calvo, M., et al. (2019). Microglial cell loss after ischemic stroke favors brain neutrophil accumulation. Acta Neuropathol. *137*, 321–341.

Ou, Z., Sun, Y., Lin, L., You, N., Liu, X., Li, H., Ma, Y., Cao, L., Han, Y., Liu, M., et al. (2016). Olig2-targeted G-protein-coupled receptor Gpr17 regulates oligodendrocyte survival in response to lysolecithin-induced demyelination. J. Neurosci. *36*, 10560–10573.

Pantoni, L. (2006). White matter ischemia: Time to begin integrating experimental and clinical data. Eur. Neurol. *56*, 71–73.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. Science (80-.). *333*, 1456–1458.

Paolicelli, R.C., Bergamini, G., and Rajendran, L. (2019). Cell-to-cell Communication by Extracellular Vesicles: Focus on Microglia. Neuroscience *405*, 148–157.

Pasquini, L.A., Millet, V., Hoyos, H.C., Giannoni, J.P., Croci, D.O., Marder, M., Liu, F.T., Rabinovich, G.A., and Pasquini, J.M. (2011). Galectin-3 drives oligodendrocyte differentiation to control myelin integrity and function. Cell Death Differ. *18*, 1746–1756.

Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., Sorensen, P.S., Brück, W., Lucchinetti, C., and Lassmann, H. (2006). Remyelination is extensive in a subset of multiple sclerosis patients. Brain *129*, 3165–3172.

Peferoen, L., Kipp, M., van der Valk, P., van Noort, J.M., and Amor, S. (2014). Oligodendrocyte-microglia cross-talk in the central nervous system. Immunology *141*, 302–313.

Perego, C., Fumagalli, S., Zanier, E.R., Carlino, E., Panini, N., Erba, E., and De Simoni, M.G. (2016). Macrophages are essential for maintaining a M2 protective response early after ischemic brain injury. Neurobiol. Dis. *96*, 284–293.

Pérez-Cerdá, F., Sánchez-Gómez, M.V., and Matute, C. (2015). Pío del Río hortega and the discovery of the oligodendrocytes. Front. Neuroanat. *9*, 92.

Pérez, M., Avila, J., and Hernández, F. (2019). Propagation of tau via extracellular vesicles. Front. Neurosci. *13*.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. *29*, E45.

Philips, T., and Rothstein, J.D. (2017). Oligodendroglia: Metabolic supporters of neurons. J. Clin. Invest. *127*, 3271–3280.

Plemel, J.R., Keough, M.B., Duncan, G.J., Sparling, J.S., Yong, V.W., Stys, P.K., and Tetzlaff, W. (2014). Remyelination after spinal cord injury: Is it a target for repair? Prog. Neurobiol. *117*, 54–72.

Poliani, P.L., Wang, Y., Fontana, E., Robinette, M.L., Yamanishi, Y., Gilfillan, S., and Colonna, M. (2015). TREM2

sustains microglial expansion during aging and response to demyelination. J. Clin. Invest. *125*, 2161–2170.

Ponomarev, E.D., Veremeyko, T., and Weiner, H.L. (2013). MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. Glia *61*, 91–103.

Powers, B.E., Sellers, D.L., Lovelett, E.A., Cheung, W., Aalami, S.P., Zapertov, N., Maris, D.O., and Horner, P.J. (2013). Remyelination reporter reveals prolonged refinement of spontaneously regenerated myelin. Proc. Natl. Acad. Sci. U. S. A. *110*, 4075–4080.

Pozniak, C.D., Langseth, A.J., Dijkgraaf, G.J.P., Choe, Y., Werb, Z., and Pleasure, S.J. (2010). Sox10 directs neural stem cells toward the oligodendrocyte lineage by decreasing Suppressor of Fused expression. Proc. Natl. Acad. Sci. U. S. A. *107*, 21795–21800.

Prada, I., Furlan, R., Matteoli, M., and Verderio, C. (2013). Classical and unconventional pathways of vesicular release in microglia. Glia *61*, 1003–1017.

Prada, I., Gabrielli, M., Turola, E., Iorio, A., D'Arrigo, G., Parolisi, R., De Luca, M., Pacifici, M., Bastoni, M., Lombardi, M., et al. (2018). Glia-to-neuron transfer of miRNAs via extracellular vesicles: a new mechanism underlying inflammation-induced synaptic alterations. Acta Neuropathol. *135*, 529–550.

Pringle, N.P., Mudhar, H.S., Collarini, E.J., and Richardson, W.D. (1992). PDGF receptors in the rat CNS: During late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. Development *115*, 535–551.

Prinz, M., Jung, S., and Priller, J. (2019). Microglia Biology: One Century of Evolving Concepts. Cell *179*, 292–311.

Probert, L. (2015). TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. Neuroscience *302*, 2–22.

Proia, P., Schiera, G., Mineo, M., Ingrassia, A.M.R., Santoro, G., Savettieri, G., and Di Liegro, I. (2008). Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor. Int. J. Mol. Med. *21*, 63–67.

Pu, A., Stephenson, E.L., and Yong, V.W. (2018). The extracellular matrix: Focus on oligodendrocyte biology and targeting CSPGs for remyelination therapies. Glia *66*, 1809–1825.

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J., and Qiu, M. (2001). Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. Development *128*, 2723–2733.

Rafalski, V.A., Ho, P.P., Brett, J.O., Ucar, D., Dugas, J.C., Pollina, E.A., Chow, L.M.L., Ibrahim, A., Baker, S.J., Barres, B.A., et al. (2013). Expansion of oligodendrocyte progenitor cells following SIRT1 inactivation in the adult brain. Nat. Cell Biol. *15*, 614–624.

Raffaele, S., Lombardi, M., Verderio, C., and Fumagalli, M. (2020). TNF Production and Release from Microglia via Extracellular Vesicles: Impact on Brain Functions. Cells *9*, 2145.

Raffaele, S., Gelosa, P., Bonfanti, E., Lombardi, M., Castiglioni, L., Cimino, M., Sironi, L., Abbracchio, M.P., Verderio, C., and Fumagalli, M. (2021). Microglial vesicles improve post-stroke recovery by preventing immune cell senescence and favoring oligodendrogenesis. Mol. Ther. *29*, 1439–1458.

Rajan, W.D., Wojtas, B., Gielniewski, B., Gieryng, A., Zawadzka, M., and Kaminska, B. (2019). Dissecting functional phenotypes of microglia and macrophages in the rat brain after transient cerebral ischemia. Glia *67*, 232–245.

Rankin, K.A., Mei, F., Kim, K., Shen, Y.A.A., Mayoral, S.R., Desponts, C., Lorrain, D.S., Green, A.J., Baranzini, S.E., Chan, J.R., et al. (2019). Selective estrogen receptor modulators enhance CNS remyelination independent of estrogen receptors. J. Neurosci. *39*, 2184–2194.

Ransohoff, R.M. (2016). A polarizing question: Do M1 and M2 microglia exist. Nat. Neurosci. *19*, 987–991.

Rao, V.T.S., Khan, D., Cui, Q.L., Fuh, S.C., Hossain, S., Almazan, G., Multhaup, G., Healy, L.M., Kennedy, T.E., and Antel, J.P. (2017). Distinct age and differentiation-state dependent metabolic profiles of oligodendrocytes under optimal and stress conditions. PLoS One *12*, e0182372.

Rawji, K.S., Mishra, M.K., Michaels, N.J., Rivest, S., Stys, P.K., and Yong, V.W. (2016). Immunosenescence of microglia and macrophages: Impact on the ageing central nervous system. Brain *139*, 653–661.

Rawji, K.S., Gonzalez Martinez, G.A., Sharma, A., and Franklin, R.J.M. (2020a). The Role of Astrocytes in Remyelination. Trends Neurosci. *43*, 596–607.

Rawji, K.S., Young, A.M.H., Ghosh, T., Michaels, N.J., Mirzaei, R., Kappen, J., Kolehmainen, K.L., Alaeiilkhchi, N., Lozinski, B., Mishra, M.K., et al. (2020b). Niacin-mediated rejuvenation of macrophage/microglia enhances remyelination of the aging central nervous system. Acta Neuropathol. *139*, 893–909.

Reynolds, R., Dawson, M., Papadopoulos, D., Polito, A., Di Bello, I.C., Pham-Dinh, D., and Levine, J. (2002). The response of NG2-expressing oligodendrocyte progenitors to demyelination in MOG-EAE and MS. J. Neurocytol. *31*, 523–536.

Rhodes, K.E., Raivich, G., and Fawcett, J.W. (2006). The injury response of oligodendrocyte precursor cells is induced by platelets, macrophages and inflammation-associated cytokines. Neuroscience *140*, 87–100.

Ringman, J.M., Saver, J.L., Woolson, R.F., Clarke, W.R., and Adams, H.P. (2004). Frequency risk factors, anatomy, and course of unilateral neglect in an acute stroke cohort. Neurology *63*, 468–474.

Robinson, R.G., and Jorge, R.E. (2016). Post-stroke depression: A review. Am. J. Psychiatry *173*, 221–231.

Rosko, L., Smith, V.N., Yamazaki, R., and Huang, J.K. (2019). Oligodendrocyte Bioenergetics in Health and Disease. Neuroscientist *25*, 334–343.

Rovaris, M., Confavreux, C., Furlan, R., Kappos, L., Comi, G., and Filippi, M. (2006). Secondary progressive multiple sclerosis: Current knowledge and future challenges. Lancet Neurol. *5*, 343–354.

Saab, A.S., and Nave, K.A. (2017). Myelin dynamics: protecting and shaping neuronal functions. Curr. Opin. Neurobiol. *47*, 104–112.

Saadoun, S., Waters, P., Bell, B.A., Vincent, A., Verkman, A.S., and Papadopoulos, M.C. (2010). Intra-cerebral injection of neuromyelitis optica immunoglobulin G and human complement produces neuromyelitis optica lesions in mice. Brain *133*, 349–361.

Safaiyan, S., Kannaiyan, N., Snaidero, N., Brioschi, S., Biber, K., Yona, S., Edinger, A.L., Jung, S., Rossner, M.J., and Simons, M. (2016). Age-related myelin degradation burdens the clearance function of microglia during aging. Nat. Neurosci. *19*, 995–998.

Salzer, J.L., and Zalc, B. (2016). Myelination. Curr. Biol. *26*, R971–R975.

Satoh, J.I., Kino, Y., Yanaizu, M., Tosaki, Y., Sakai, K., Ishida, T., and Saito, Y. (2017). Expression of GPR17, a regulator of oligodendrocyte differentiation and maturation, in Nasu-Hakola disease brains. Intractable Rare Dis. Res. *6*, 50–54.

Savage, J.C., Carrier, M., and Tremblay, M.È. (2019). Morphology of Microglia Across Contexts of Health and Disease. In Methods in Molecular Biology, (Humana Press Inc.), pp. 13–26.

Sbai, O., Ould-Yahoui, A., Ferhat, L., Gueye, Y., Bernard, A., Charrat, E., Mehanna, A., Risso, J.J., Chauvin, J.P., Fenouillet, E., et al. (2010). Differential vesicular distribution and trafficking of MMP-2, MMP-9, and their inhibitors in astrocytes. Glia *58*, 344–366.

Schafer, D.P., Bansal, R., Hedstrom, K.L., Pfeiffer, S.E., and Rasband, M.N. (2004). Does Paranode Formation and Maintenance Require Partitioning of Neurofascin 155 into Lipid Rafts? J. Neurosci. *24*, 3176–3185.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., and Stevens, B. (2012). Microglia Sculpt Postnatal Neural Circuits in an

Activity and Complement-Dependent Manner. Neuron *74*, 691–705.

Scherer, S.S., Braun, P.E., Grinspan, J., Collarini, E., Wang, D. y., and Kamholz, J. (1994). Differential regulation of the 2′,3′-cyclic nucleotide 3′-phosphodiesterase gene during oligodendrocyte development. Neuron *12*, 1363–1375.

Schlaepfer, I.R., and Joshi, M. (2020). CPT1A-mediated Fat Oxidation, Mechanisms, and Therapeutic Potential. Endocrinol. (United States) *161*.

Sensi, C., Daniele, S., Parravicini, C., Zappelli, E., Russo, V., Trincavelli, M.L., Martini, C., Abbracchio, M.P., and Eberini, I. (2014). Oxysterols act as promiscuous ligands of class-A GPCRs: In silico molecular modeling and in vitro validation. Cell. Signal. *26*, 2614–2620.

Shi, H., Hu, X., Leak, R.K., Shi, Y., An, C., Suenaga, J., Chen, J., and Gao, Y. (2015). Demyelination as a rational therapeutic target for ischemic or traumatic brain injury. Exp. Neurol. *272*, 17–25.

Simons, M., and Nave, K.A. (2016). Oligodendrocytes: Myelination and axonal support. Cold Spring Harb. Perspect. Biol. *8*, a020479.

Skaper, S.D. (2019). Oligodendrocyte precursor cells as a therapeutic target for demyelinating diseases. In Progress in Brain Research, (Elsevier), pp. 119–144.

Sommer, I., and Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: An immunocytological study in the central nervous system. Dev. Biol. *83*, 311–327.

Soni, S., O'Dea, K.P., Tan, Y.Y., Cho, K., Abe, E., Romano, R., Cui, J., Ma, D., Sarathchandra, P., Wilson, M.R., et al. (2019). ATP redirects cytokine trafficking and promotes novel membrane TNF signaling via microvesicles. FASEB J. *33*, 6442–6455.

Srinivasan, R., Sailasuta, N., Hurd, R., Nelson, S., and Pelletier, D. (2005). Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T. Brain *128*, 1016–1025.

Stangel, M., Kuhlmann, T., Matthews, P.M., and Kilpatrick, T.J. (2017). Achievements and obstacles of remyelinating therapies in multiple sclerosis. Nat. Rev. Neurol. *13*, 742–754.

Stinear, C. (2010). Prediction of recovery of motor function after stroke. Lancet Neurol. *9*, 1228–1232.

Stinear, C.M. (2017). Prediction of motor recovery after stroke: advances in biomarkers. Lancet Neurol. *16*, 826–836.

Stolt, C.C., Lommes, P., Friedrich, R.P., and Wegner, M. (2004). Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. Development *131*, 2349–2358.

Storini, C., Bergamaschini, L., Gesuete, R., Rossi, E., Maiocchi, D., and De Simoni, M.G. (2006). Selective inhibition of plasma kallikrein protects brain from reperfusion injury. J. Pharmacol. Exp. Ther. *318*, 849–854.

Stratoulias, V., Venero, J.L., Tremblay, M., and Joseph, B. (2019). Microglial subtypes: diversity within the microglial community. EMBO J. *38*.

Szalay, G., Martinecz, B., Lénárt, N., Környei, Z., Orsolits, B., Judák, L., Császár, E., Fekete, R., West, B.L., Katona, G., et al. (2016). Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. Nat. Commun. *7*.

Tanabe, S., and Yamashita, T. (2020). Function of Lymphocytes in Oligodendrocyte Development. Neuroscientist *26*, 74–86.

Tanaka, T., Murakami, K., Bando, Y., and Yoshida, S. (2013). Minocycline reduces remyelination by suppressing ciliary neurotrophic factor expression after cuprizone-induced demyelination. J. Neurochem. *127*, 259–270.

Thangaraj, M.P., Furber, K.L., Gan, J.K., Ji, S., Sobchishin, L., Doucette, J.R., and Nazarali, A.J. (2017). RNA-binding protein quaking stabilizes Sirt2 mRNA during oligodendroglial differentiation. J. Biol. Chem. *292*, 5166–5182.

Thomas, L., and Pasquini, L.A. (2018). Galectin-3-mediated glial crosstalk drives oligodendrocyte differentiation and (Re)myelination. Front. Cell. Neurosci. *12*, 1–16.

Tian, T., Zhu, Y.L., Zhou, Y.Y., Liang, G.F., Wang, Y.Y., Hu, F.H., and Xiao, Z.D. (2014). Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. J. Biol. Chem. *289*, 22258–22267.

Tian, T., Zhang, H.X., He, C.P., Fan, S., Zhu, Y.L., Qi, C., Huang, N.P., Xiao, Z.D., Lu, Z.H., Tannous, B.A., et al. (2018). Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. Biomaterials *150*, 137–149.

Tomassy, G.S., and Fossati, V. (2014). How big is the myelinating orchestra? Cellular diversity within the oligodendrocyte lineage: Facts and hypotheses. Front. Cell. Neurosci. *8*, 201.

Torres, L., Danver, J., Ji, K., Miyauchi, J.T., Chen, D., Anderson, M.E., West, B.L., Robinson, J.K., and Tsirka, S.E. (2016). Dynamic microglial modulation of spatial learning and social behavior. Brain. Behav. Immun. *55*, 6–16.

Torrey, H., Kühtreiber, W.M., Okubo, Y., Tran, L., Case, K., Zheng, H., Vanamee, E., and Faustman, D.L. (2020). A novel TNFR2 agonist antibody expands highly potent regulatory T cells. Sci. Signal. *13*.

Tripathi, R.B., Rivers, L.E., Young, K.M., Jamen, F., and Richardson, W.D. (2010). NG2 glia generate new oligodendrocytes but few astrocytes in a murine experimental autoimmune encephalomyelitis model of demyelinating disease. J. Neurosci. *30*, 16383–16390.

Tripathi, R.B., Clarke, L.E., Burzomato, V., Kessaris, N., Anderson, P.N., Attwell, D., and Richardson, W.D. (2011). Dorsally and ventrally derived oligodendrocytes have similar electrical properties but myelinate preferred tracts. J. Neurosci. *31*, 6809–6819.

Turola, E., Furlan, R., Bianco, F., Matteoli, M., and Verderio, C. (2012). Microglial microvesicle secretion and intercellular signaling. Front. Physiol. *3 MAY*, 149.

Tyler, W.A., Jain, M.R., Cifelli, S.E., Li, Q., Ku, L., Feng, Y., Li, H., and Wood, T.L. (2011). Proteomic identification of novel targets regulated by the mammalian target of rapamycin pathway during oligodendrocyte differentiation. Glia *59*, 1754–1769.

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., and Yamashita, T. (2013). Layer v cortical neurons require microglial support for survival during postnatal development. Nat. Neurosci. *16*, 543–551.

Vallar, G., and Calzolari, E. (2018). Unilateral spatial neglect after posterior parietal damage. In Handbook of Clinical Neurology, (Elsevier B.V.), pp. 287–312.

Verderio, C., Muzio, L., Turola, E., Bergami, A., Novellino, L., Ruffini, F., Riganti, L., Corradini, I., Francolini, M., Garzetti, L., et al. (2012). Myeloid microvesicles are a marker and therapeutic target for neuroinflammation. Ann. Neurol. *72*, 610–624.

Veroni, C., Gabriele, L., Canini, I., Castiello, L., Coccia, E., Remoli, M.E., Columba-Cabezas, S., Aricò, E., Aloisi, F., and Agresti, C. (2010). Activation of TNF receptor 2 in microglia promotes induction of anti-inflammatory pathways. Mol. Cell. Neurosci. *45*, 234–244.

Viganò, F., Schneider, S., Cimino, M., Bonfanti, E., Gelosa, P., Sironi, L., Abbracchio, M.P., and Dimou, L. (2016). GPR17 expressing NG2-Glia: Oligodendrocyte progenitors serving as a reserve pool after injury. Glia *64*, 287–299.

Villa, A., Gelosa, P., Castiglioni, L., Cimino, M., Rizzi, N., Pepe, G., Lolli, F., Marcello, E., Sironi, L., Vegeto, E., et

al. (2018). Sex-Specific Features of Microglia from Adult Mice. Cell Rep. *23*, 3501–3511.

Vinson, M., Rausch, O., Maycox, P.R., Prinjha, R.K., Chapman, D., Morrow, R., Harper, A.J., Dingwall, C., Walsh, F.S., Burbidge, S.A., et al. (2003). Lipid rafts mediate the interaction between myelin-associated glycoprotein (MAG) on myelin and MAG-receptors on neurons. Mol. Cell. Neurosci. *22*, 344–352.

Virani, S.S., Alonso, A., Aparicio, H.J., Benjamin, E.J., Bittencourt, M.S., Callaway, C.W., Carson, A.P., Chamberlain, A.M., Cheng, S., Delling, F.N., et al. (2021). Heart Disease and Stroke Statistics-2021 Update A Report from the American Heart Association. Circulation *143*, E254–E743.

Waly, B. El, Macchi, M., Cayre, M., and Durbec, P. (2014). Oligodendrogenesis in the normal and pathological central nervous system. Front. Neurosci. *8*, 1–22.

Wang, P., Gorter, R.P., de Jonge, J.C., Nazmuddin, M., Zhao, C., Amor, S., Hoekstra, D., and Baron, W. (2018). MMP7 cleaves remyelination-impairing fibronectin aggregates and its expression is reduced in chronic multiple sclerosis lesions. Glia *66*, 1625–1643.

Wegener, A., Deboux, C., Bachelin, C., Frah, M., Kerninon, C., Seilhean, D., Weider, M., Wegner, M., and Nait-Oumesmar, B. (2015). Gain of Olig2 function in oligodendrocyte progenitors promotes remyelination. Brain *138*, 120–135.

Werner, P., Pitt, D., and Raine, C.S. (2001). Multiple sclerosis: Altered glutamate homeostasis in lesions correlates with oligodendrocyre and axonal damage. Ann. Neurol. *50*, 169–180.

Wiklander, O.P.B., Brennan, M., Lötvall, J., Breakefield, X.O., and Andaloussi, S.E.L. (2019). Advances in therapeutic applications of extracellular vesicles. Sci. Transl. Med. *11*, 1–16.

Wlodarczyk, A., Cédile, O., Jensen, K.N., Jasson, A., Mony, J.T., Khorooshi, R., and Owens, T. (2015). Pathologic and protective roles for microglial subsets and bone marrow- and blood-derived myeloid cells in central nervous system inflammation. Front. Immunol. *6*, 463.

Wlodarczyk, A., Holtman, I.R., Krueger, M., Yogev, N., Bruttger, J., Khorooshi, R., Benmamar-Badel, A., Boer-Bergsma, J.J., Martin, N.A., Karram, K., et al. (2017). A novel microglial subset plays a key role in myelinogenesis in developing brain. EMBO J. *36*, 3292–3308.

Wlodarczyk, A., Benmamar-Badel, A., Cédile, O., Jensen, K.N., Kramer, I., Elsborg, N.B., and Owens, T. (2019). CSF1R stimulation promotes increased neuroprotection by CD11c+ microglia in EAE. Front. Cell. Neurosci. *12*, 523.

Wlodarczyk, A., Khorooshi, R., Marczynska, J., Holtman, I.R., Burton, M., Jensen, K.N., Blaabjerg, M., Meyer, M., Thomassen, M., Eggen, B.J.L., et al. (2021). Type I interferon-activated microglia are critical for neuromyelitis optica pathology. Glia *69*, 943–953.

Xin, M., Yue, T., Ma, Z., Wu, F.F., Gow, A., and Lu, Q.R. (2005). Myelinogenesis and axonal recognition by oligodendrocytes in brain are uncoupled in Olig1-null mice. J. Neurosci. *25*, 1354–1365.

Yáñez-Mó, M., Siljander, P.R.-M., Andreu, Z., Zavec, A.B., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. J. Extracell. Vesicles *4*, 1–60.

Yang, S.H., and Liu, R. (2021). Four Decades of Ischemic Penumbra and Its Implication for Ischemic Stroke. Transl. Stroke Res. *1*, 1–9.

Yang, J., Zhang, X., Chen, X., Wang, L., and Yang, G. (2017). Exosome Mediated Delivery of miR-124 Promotes Neurogenesis after Ischemia. Mol. Ther. - Nucleic Acids *7*, 278–287.

Yang, J., Wu, S., Hou, L., Zhu, D., Yin, S., Yang, G., and Wang, Y. (2020). Therapeutic Effects of Simultaneous Delivery of Nerve Growth Factor mRNA and Protein via Exosomes on Cerebral Ischemia. Mol. Ther. - Nucleic Acids *21*, 512–522.

Yang, Y., Boza-Serrano, A., Dunning, C.J.R., Clausen, B.H., Lambertsen, K.L., and Deierborg, T. (2018).

Inflammation leads to distinct populations of extracellular vesicles from microglia. J. Neuroinflammation *15*, 1–19.

Yenari, M.A., Kauppinen, T.M., and Swanson, R.A. (2010). Microglial Activation in Stroke: Therapeutic Targets. Neurotherapeutics *7*, 378–391.

Yeung, M.S.Y., Zdunek, S., Bergmann, O., Bernard, S., Salehpour, M., Alkass, K., Perl, S., Tisdale, J., Possnert, G., Brundin, L., et al. (2014). Dynamics of oligodendrocyte generation and myelination in the human brain. Cell *159*, 766–774.

York, E.M., Ledue, J.M., Bernier, L.P., and Macvicar, B.A. (2018). 3dmorph automatic analysis of microglial morphology in three dimensions from ex vivo and in vivo imaging. ENeuro *5*.

Zabala, A., Vazquez-Villoldo, N., Rissiek, B., Gejo, J., Martin, A., Palomino, A., Perez-Samartín, A., Pulagam, K.R., Lukowiak, M., Capetillo-Zarate, E., et al. (2018). P2X4 receptor controls microglia activation and favors remyelination in autoimmune encephalitis. EMBO Mol. Med. *10*, 1–20.

Zamanian, J.L., Xu, L., Foo, L.C., Nouri, N., Zhou, L., Giffard, R.G., and Barres, B.A. (2012). Genomic analysis of reactive astrogliosis. J. Neurosci. *32*, 6391–6410.

Zawadzka, M., Rivers, L.E., Fancy, S.P.J., Zhao, C., Tripathi, R., Jamen, F., Young, K., Goncharevich, A., Pohl, H., Rizzi, M., et al. (2010). CNS-Resident Glial Progenitor/Stem Cells Produce Schwann Cells as well as Oligodendrocytes during Repair of CNS Demyelination. Cell Stem Cell *6*, 578–590.

Zhang, H., Wu, J., Wu, J., Fan, Q., Zhou, J., Wu, J., Liu, S., Zang, J., Ye, J., Xiao, M., et al. (2019). Exosome-mediated targeted delivery of miR-210 for angiogenic therapy after cerebral ischemia in mice. J. Nanobiotechnology *17*, 1–13.

Zhang, R., Chopp, M., and Zhang, Z.G. (2013). Oligodendrogenesis after cerebral ischemia. Front. Cell. Neurosci. *7*, 201.

Zhou, Q., and Anderson, D.J. (2002). The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell *109*, 61–73.

Zhou, Q., Wang, S., and Anderson, D.J. (2000). Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron *25*, 331–343.

Zhu, Q., Zhao, X., Zheng, K., Li, H., Huang, H., Zhang, Z., Mastracci, T., Wegner, M., Chen, Y., Sussel, L., et al. (2014). Genetic evidence that Nkx2.2 and Pdgfra are major determinants of the timing of oligodendrocyte differentiation in the developing CNS. Dev. *141*, 548–555.

Zhuang, X., Xiang, X., Grizzle, W., Sun, D., Zhang, S., Axtell, R.C., Ju, S., Mu, J., Zhang, L., Steinman, L., et al. (2011). Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Mol. Ther. *19*, 1769–1779.

SUMMARY OF PhD ACTIVITIES

Publications

Boccazzi M, **Raffaele S**, Fumagalli M. Not only myelination: the immune-inflammatory functions of oligodendrocytes. Neural Regeneration Research. Manuscript under revision. IF: 5.135.
Angelini J, Marangon D, **Raffaele S**, Lecca D, Abbracchio MP. The Distribution of GPR17-Expressing Cells Correlates with White Matter Inflammation Status in Brain Tissues of Multiple Sclerosis Patients. International Journal of Molecular Sciences. 2021 Apr 27;22(9):4574. doi: 10.3390/ijms22094574. IF: 5.923
Raffaele S, Boccazzi M, Fumagalli M. Oligodendrocyte Dysfunction in Amyotrophic Lateral Sclerosis: Mechanisms and Therapeutic Perspectives. Cells. 2021 Mar 5;10(3):565. doi: 10.3390/cells10030565. IF: 6.600
Raffaele S, Gelosa P, Bonfanti E, Lombardi M, Castiglioni L, Cimino M, Sironi L, Abbracchio MP, Verderio C, Fumagalli M. Microglial vesicles improve post-stroke recovery by preventing immune cell senescence and favoring oligodendrogenesis. Molecular Therapy. 2021 Apr 7;29(4):1439-1458. doi: 10.1016/j.ymthe.2020.12.009. IF: 11.454
Raffaele S, Lombardi M, Verderio C, Fumagalli M. TNF Production and Release from Microglia via Extracellular Vesicles: Impact on Brain Functions. Cells. 2020 Sep 23;9(10):2145. doi: 10.3390/cells9102145. IF: 6.600
Bonfanti E, Bonifacino T, **Raffaele S**, Milanese M, Morgante E, Bonanno G, Abbracchio MP, Fumagalli M. Abnormal Upregulation of GPR17 Receptor Contributes to Oligodendrocyte Dysfunction in SOD1 G93A Mice. International Journal of Molecular Sciences. 2020 Mar 31;21(7):2395. doi: 10.3390/ijms21072395. IF: 5.923
Lecca D, **Raffaele S**, Abbracchio MP, Fumagalli M. Regulation and signaling of the GPR17 receptor in oligodendroglial cells. Glia. 2020 Oct;68(10):1957-1967. doi: 10.1002/glia.23807. IF: 7.452
Marangon D, **Raffaele S**, Fumagalli M, Lecca D. MicroRNAs change the games in central nervous system pharmacology. Biochemical Pharmacology. 2019 Oct;168:162-172. doi: 10.1016/j.bcp.2019.06.019. IF: 4.960

Manuscripts in preparation
Raffaele S, Lombardi M, Verderio C, Fumagalli M. Gene expression profiling unveils metabolic reprogramming of differentiating oligodendrocytes induced by microglia-derived vesicles. Biomedicines. Manuscript in preparation.
Lund MC, Ellman DG, Nielsen PV, **Raffaele S**, Fumagalli M, Guzman R, Degn M, Brambilla R, Meyer M, Clausen BH, Lambertsen KL. Selective inhibition of soluble tumor necrosis factor alters the inflammatory response after moderate spinal cord injury in mice. Manuscript in preparation.

Congresses And Seminars

Date	Title	Place
10-14/10/2021 (Accepted)	Oral presentation entitled "Unraveling the contribution of TNFR2 in the pro-remyelinating properties of microglia after stroke".	18th TNF Superfamily Meeting, Les Diablerets, Switzerland.

05–09/07/2021	Poster presentation entitled "Investigating the transcriptional changes underpinning enhanced oligodendrocyte maturation after exposure to microglia-derived vesicles".	XV European Meeting on Glial Cells in Health and Disease (GLIA 2021), online congress.
09-13/03/2021	Invited oral presentation entitled "Abnormal up-regulation of P2Y-like receptor GPR17 contributes to oligodendrocyte dysfunction in a murine model of ALS".	40th National meeting of the Italian Society of Pharmacology (SIF), online congress.
04-05/02/2021	Oral presentation entitled "Microglial vesicles improve post-stroke recovery by preventing immune cell senescence and favoring oligodendrogenesis".	Annual meeting of the Italian Purine Club, online congress.
25-26/11/2020	Video-poster presentation entitled "Microglia-derived extracellular vesicles promote brain repair and functional recovery after stroke".	3rd BRAYN - Brainstorming Research Assembly for Young Neuroscientists, online congress.
25-28/06/2020	Oral presentation entitled "Microglia-to-oligodendrocyte precursor cells communication via extracellular vesicles: molecular mechanisms involved and implications for myelin repair".	PhD Spring School, 4th edition, Chiesa in Valmalenco (SO), Italy.
27/01/2020	Oral presentation entitled "The GPR17 receptor as a new potential pharmacological target to restore oligodendroglial dysfunction in amyotrophic lateral sclerosis".	Annual meeting of the Italian Purine Club, Ferrara, Italy.
20-23/11/2019	Oral presentation entitled "The GPR17 receptor as a new potential pharmacological target to restore oligodendroglial dysfunction in amyotrophic lateral sclerosis".	39th National meeting of the Italian Society of Pharmacology (SIF), Florence, Italy.
14-16/11/2019	Poster presentation entitled "Effects of activated microglia-derived extracellular vesicles on GPR17-expressing oligodendrocyte precursor cells and post-stroke recovery" and co-chairman selected among the abstracts submitted for the session "Neuroinflammation".	2nd BRAYN - Brainstorming Research Assembly for Young Neuroscientists, Milan, Italy.
18/09/2019	Oral presentation entitled "Effects of activated microglia-derived extracellular vesicles on GPR17-expressing oligodendrocyte precursors cells and post-stroke recovery".	Next Step 10: la giovane ricerca avanza, Milan, Italy.
04-06/09/2019	Invited oral presentation entitled "Activated microglia regulate the response of oligodendrocyte progenitors expressing the P2Y-like receptor GPR17 following cerebral ischemia".	First European Purine Meeting, Santiago de Compostela, Spain.

10-13/07/2019	Poster presentation entitled "Time-dependent effects of microglia/macrophages partial depletion on oligodendrocyte precursors in brain ischemia".	XIV European Meeting on Glial Cells in Health and Disease (GLIA 2019), Porto, Portugal.
11-14/04/2019	Oral presentation entitled "Microglia-to-oligodendrocyte precursor cells communication via extracellular vesicles: role of TNFα signalling".	PhD Spring School, 3rd edition, Chiesa in Valmalenco (SO), Italy.
18/01/2019	Oral presentation entitled "Activated microglia regulate the response of oligodendrocyte precursors expressing the P2Y-like receptor GPR17 following stroke".	Annual meeting of the Italian Purine Club, Florence, Italy.

Awards

Year	Description of award
2021	TRAVEL GRANT to attend the 18th TNF Superfamily Meeting, 10-14/10/2021, Les Diablerets, Switzerland.
2021	SCIENTIFIC EXCHANGE GRANT from European Molecular Biology Organization (EMBO) – to work for 6 months in the lab headed by prof. Kate Lykke Lambertsen at the Institute of Neurobiology Research, University of Southern Denmark, Odense, DK, on the project entitled "Unraveling the contribution of TNFR2 signaling in the pro-remyelinating properties of microglia after stroke".
2019	TRAVEL GRANT from Italian Purine Club – to attend the First European Purine Meeting, 4-6/09/2019, Santiago de Compostela, Spain

Secondment

03-08/2021	GUEST PhD STUDENT at the Institute of Neurobiology Research, University of Southern Denmark, Odense, DK – experimental activities related to the project entitled "Unraveling the contribution of TNFR2 signaling in the pro-remyelinating properties of microglia after stroke", under the supervision of prof. Kate Lykke Lambertsen.

Outreach Activity

18/03/21: Author of the post entitled "È possibile riparare lesioni ischemiche cerebrali favorendo l'interazione tra microglia e oligodendrociti?", published on RicercaMix, the dissemination blog of the Department of Pharmacological and Biomolecular Sciences of the University of Milan. Link: http://www.ricercamix.org/2021/03/18/e-possibile-riparare-lesioni-ischemiche-cerebrali-favorendo-linterazione-tra-microglia-e-oligodendrociti/
25/05/2020: Author of the post entitled "Nuovi orizzonti per il trattamento della SLA: gli oligodendrociti e il recettore GPR17", published on RicercaMix, the dissemination blog of the Department of Pharmacological and Biomolecular Sciences of the University of Milan. Link: http://www.ricercamix.org/2020/05/25/nuovi-orizzonti-per-il-trattamento-della-sla-gli-

oligodendrociti-e-il-recettore-gpr17/
From 2019: Author of "Literature highlights" for PuriNews, the quarterly newsletter of the Italian Purine Club. Link: http://italianpurineclub.altervista.org/purinews.html

Editorial Activity

From 2021: Reviewer for the journal *Scientific Reports*, Nature Publishing Group. IF: 4.379
From 2021: Reviewer for the journal *Biomedicines*, MDPI. IF: 6.081
From 2021: Member of the Reviewer Board for the journal *International Journal of Molecular Sciences*, MDPI. IF: 5.923

Tutoring

CO-TUTOR of the following candidates during the preparation of the graduation thesis
Camilla Stucchi, Master's degree in Pharmacy, 18/12/2020, theoretical dissertation entitled: La disfunzione degli oligodendrociti nella sclerosi laterale amiotrofica: meccanismi patogenetici e potenziali approcci terapeutici.
Benedetta Mercuri, Bachelor's degree in Biotechnology, 17/12/2020, experimental thesis entitled: Allestimento e caratterizzazione di colture primarie di oligodendrociti da midollo spinale di topi SOD1G93A, un modello sperimentale di sclerosi laterale amiotrofica.
Giulia Margiotta, Master's degree in Pharmacy, 19/03/2020, experimental thesis entitled: Effetti di microvescicole microgliali sui precursori oligodendrocitari GPR17-positivi e sul recupero funzionale in un modello murino di ischemia cerebrale.
Caterina Sperandio, Master's degree in Pharmacy, 19/03/2020, theoretical dissertation entitled: Il controllo della neuroinfiammazione come potenziale strategia terapeutica per il trattamento della sclerosi laterale amiotrofica.