Aus dem Institut für Mikrobiologie und Tierseuchen

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Development and characterization of a new *in vitro* method to study the cellular immune response of microglia and astrocytes and the underlying crosstalk

Inaugural-Dissertation

zur Erlangung des Grades

eines Doctor of Philosophy (PhD) of Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von

Laura Zelenka

Mikrobiologin

aus Gütersloh

Berlin 2024

Journal-Nr.: 4431

Gedruckt mit Genehmigung

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Uwe Rösler
Erster Gutachter:	Univ.-Prof. Dr. Marcus Fulde
Zweite Gutachterin:	Prof. Dr. Seija Lehnardt
Dritte Gutachterin:	Univ.-Prof. Dr. Sonja Bröer

Deskriptoren (nach CAB-Thesaurus): neuroglia, microglia (MeSH), astrocytes (MeSH), cell culture, purity, viability, toll-like receptor 2, interleukin 10

Tag der Promotion: 10.01.2024

Table of contents

List of figures and tables

List of abbreviations

°C	Celsius
Acod1	aconitate decarboxylase 1
ACSA-2	astrocyte cell surface antigen-2
AD	Alzheimer‘s disease
ALDH1L1	aldehyde dehydrogenase 1 family, member L1
ALS	amyotrophic lateral sclerosis
AraC	cytosine arabinoside
ATP	adenosine triphosphate
ATP1B	sodium/potassium-transporting ATPase subunit beta-1
Aβ	amyloid-beta
BBB	blood-brain barrier
C1q	complement component 1, subcomponent q
C3	complement component 3
C3aR	complement component C3a receptor
Ca^{2+}	calcium ions
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD300d2	cd300 molecule like family member d2
CD300f	cd300 molecule like family member f
CNS	central nervous system
CSF-1R	colony-stimulating factor 1 receptor
CXCL	C-X-C motif ligand
DAMP	danger-associated molecular pattern
DCs	dendritic cells
DEG	differentially expressed gene
e.g.	exempli gratia
ECs	endothelial cells
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticula
EV	extracellular versicle

FACS	fluorescence-activated cell sorting
FESEM	field emission scanning electron microscopy
GFAP	glial fibrillary acidic protein
GO	gene ontology
h	hour
H_2O_2	hydrogen peroxide
HO-1	heme oxygenase-1
Iba1	ionized calcium-binding adapter molecule 1
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon-gamma inducible protein-10
Irg1	immunoresponsive gene 1
KO	knockout
L-AAA	L-α-aminoadipic acid
LCN2	lipocalin-2
LCN2R	lipocalin-2 receptor
LME	L-leucine methyl ester
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Ly6C	lymphocyte antigen complex 6C
Ly6G	lymphocyte antigen 6 complex locus G6D
M-CSF	macrophage colony-stimulating factor
M-CSFR	macrophage colony-stimulating factor receptor
MACS®	magnetic-activated cell sorting
MCP-1	monocyte chemoattractant protein-1
min	minutes
miRNA	microRNA
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

NO	nitric oxide
NOX2	NADPH oxidase 2
NVU	neurovascular unit
ORM2	orosomucoid-2
P	postnatal day
P2RY1	purinergic receptor P2Y1
PAI-1	plasminogen activator inhibitor type 1
PAMP	pathogen-associated molecular pattern
PD	Parkinson’s disease
PNS	peripheral nervous system
PRR	pattern recognition receptor
PTX3	pentraxin 3
Rab6a	ras-related protein Rab-6A
RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROS	reactive oxygen species
S100β	S100 calcium-binding protein beta
Sall1	spalt-like transcription factor 1
SN	supernatants
STAT	signal transducer and activator of transcription
TBI	traumatic brain injury
TGFβ	transforming growth factor-beta
TLR	Toll-like receptor
Tmem119	transmembrane protein 119
TNF	tumor necrosis factor
Tnfrsf9	tumor necrosis factor receptor superfamily member 9
TNT	tunneling nano tube
WT	wild-type
α-syn	alpha-synuclein

1 Introduction

"Hitherto, gentlemen, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system, it is extremely important to have a knowledge of that substance which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or lesser degree."

Quote from a lecture given by Rudolf Virchow (Charité Hospital, Berlin, 3rd April 1858).

1.1 Glia – the "glue" of the central nervous system (CNS)

These words by the German pathologist Rudolf Virchow during one of his medical lectures marked the birth of the concept "nerve connective tissue", which was named after the German word "*Nervenkitt*" or so-called "nerve glue". Today, this term is known as "neuroglia", "glial cells" or simply "glia". Over time, we learned that the cell type glia is more complex than initially described by Virchow in 1858. The discovered features and important functions exceed the capability of a "glue" holding the brain together. However, more than 150 years later, many questions remain unanswered despite pioneering neurobiologists having first asked them decades ago. In the 21th century, the function of the cell type glia and its role in the brain are at the forefront of research and are just beginning to be understood (Virchow 1858; Somjen 1988; Chvátal and Verkhratsky 2018; Verkhratsky et al. 2019).

Neuroglia lie between two broad classifications: glia of the central nervous system (CNS) and peripheral nervous system (PNS) (Jäkel and Dimou 2017; Butt and Verkhratsky 2018). As depicted in **Figure 1**, PNS glia can be further broken down into Schwann cells, satellite glial cells, enteric glia as well as olfactory ensheathing cells (Verkhratsky and Butt 2013; Butt and Verkhratsky 2018). The major cells populating the mammalian CNS are sub-classified into neurons and glia, which are highly heterogeneous coming from different origins and having diverse structures and functions (Verkhratsky et al. 2019; Tremblay 2020). CNS glia comprise the two subgroups microglia and macroglia. The latter includes astrocytes, oligodendrocytes as well as NG2 cells (also named as oligodendrocyte progenitor cells or polydendrocytes) (Reemst et al. 2016; Butt and Verkhratsky 2018; Greenhalgh et al. 2020). The main difference between microglia and macroglia is their origin: Unlike microglia, which derive from embryonic yolk sac progenitors entering the CNS during early embryogenesis, macroglia originate from a common neuroectodermal embryonic lineage (Curtis et al. 1988; Butt 1991; Ginhoux et al. 2010; Schulz et al. 2012; Thion and Garel 2017).

In the next chapters of this work, we will focus on the two main relevant glial cell types, namely *microglia* and *astrocytes*.

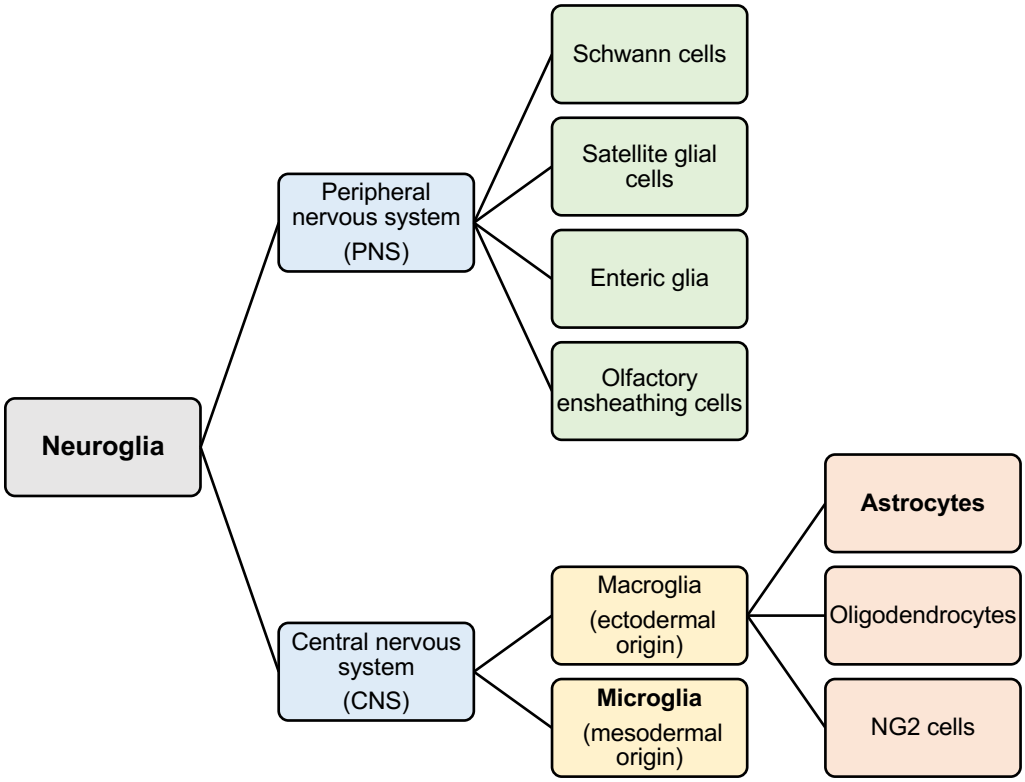


Figure 1: Classification of neuroglia. Illustration adapted from (Verkhratsky et al. 2019).

1.1.1 Microglia

Microglia are the tissue-resident macrophages of the CNS. They make up approximately 5-15% of all human brain cells, depending on the brain region and the quantification method (Aguzzi et al. 2013; Reemst et al. 2016; Li and Barres 2018) and are replenished via self-renewal (Ajami et al. 2007; Sheng et al. 2015). As the so-called “macrophages of the brain”, microglial cells are phagocytically active by constantly scanning their environment for any kind of brain insult with the help of their highly motile processes. If they sample homeostatic imbalance in the CNS, they quickly are activated and respond with morphological and functional transformation (Davalos et al. 2005; Nimmerjahn et al. 2005). Emerging evidence indicates that microglia activity is not only indispensable under pathological conditions: Recently, microglia have been recognized as highly dynamic cells that use their scavenging function to balance tissue homeostasis even under physiological conditions (Reemst et al. 2016; Kierdorf and Prinz 2017). Aside from their well-established immune functions in the CNS, microglia play a role in the maturation and shaping of neural circuits (Antony et al. 2011; Zhu et al. 2020), the modulation of neuronal plasticity by synaptic pruning and remodeling (Schafer et al. 2012; Kettenmann et al. 2013; Weinhard et al. 2018) as well as neurogenesis (Diaz-Aparicio et al. 2020; Pérez-Rodríguez et al. 2021). Furthermore, microglial cells are involved in supportive functions such as the regulation of myelination (Lampron et al. 2015; Hughes and Appel 2020). Beyond this, they are also part of the functional and structural unit called the

neurovascular unit (NVU) (**Figure 2**). The NVU is an ensemble of the microvasculature and a wide array of brain cells such as astrocytes and oligodendrocytes, neurons, microvessels like endothelial cells (ECs) from the blood-brain barrier (BBB) as well as extracellular components, to reciprocally orchestrate brain homeostasis (del Zoppo 2010; Muoio et al. 2014; Iadecola 2017; Liu et al. 2020; Schaeffer and Iadecola 2021). As an important partner of the NVU, microglia are responsible for the maintenance of vascular (Halder and Milner 2019; Dudiki et al. 2020) and BBB integrity (Haruwaka et al. 2019). The function of microglia and their interaction with astrocytes in the NVU has been recently reviewed by Liu and colleagues (Liu et al. 2020).

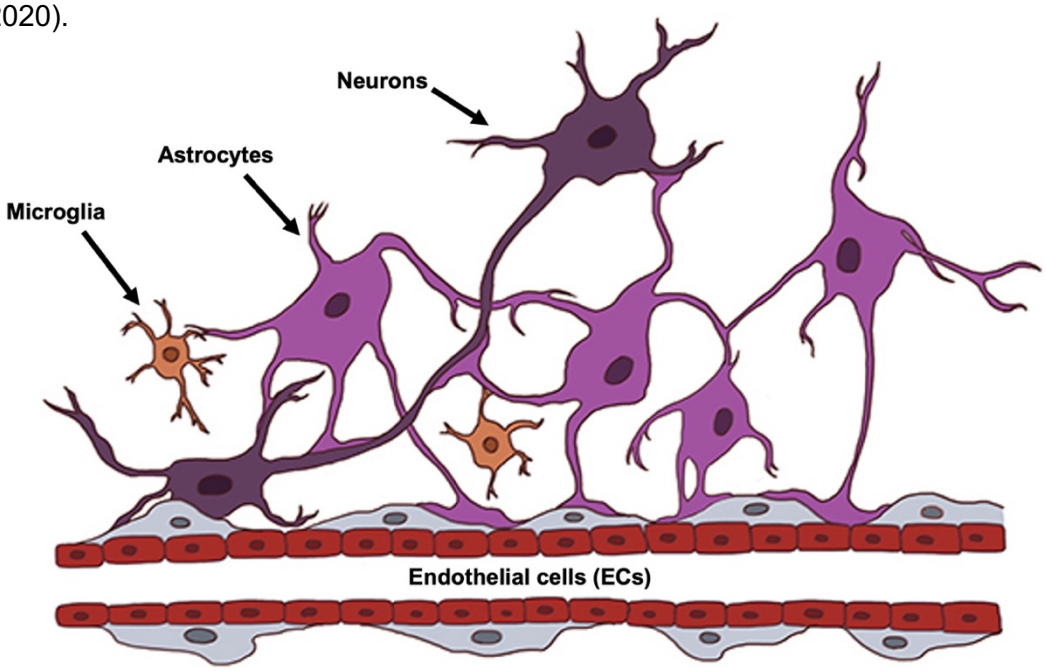


Figure 2: Association of microglia and astrocytes in the neurovascular unit (NVU). Illustration adapted from (Liu et al. 2020).

Depending on their developmental and functional state, microglia show a wide range of morphological phenotypes (Davis et al. 1994). At the beginning of the 20th century, the Spanish neuroscientist Pío del Río Hortega depicted that ramified microglia under balanced homeostatic brain conditions are considered as inactive “resting microglia”, which upon activation are transformed into amoeboid, phagocytic “active microglia” (Sierra et al. 2016). Recently, new evidence has suggested that “resting microglia” are also constantly highly active by sampling their microenvironment and interacting with other cortical elements (Nimmerjahn et al. 2005; Martinez and Gordon 2014; Ransohoff 2016; Stratoulias et al. 2019; Paolicelli et al. 2022). For the simplification of the rather complex and controversial microglia phenotype classification, the dichotomy of pro- (M1) and anti- (M2) inflammatory macrophages was assigned to microglia. For further in-depth elaboration on the diversity of microglial subtype classification, which is accompanied by semantic terminology issues, readers should refer to the latter cited reviews.

1.1.2 Astrocytes

The glial cell type astrocytes populates the entire CNS and outnumbers neurons by being abundantly expressed in the human brain (von Bartheld et al. 2016). They execute crucial functions by interacting with neural as well as non-neural brain cells. Traditionally, astrocytes were associated with passive cells giving neurons mechanic as well as metabolic and trophic support (Pellerin and Magistretti 1994; Gonzalez-Perez et al. 2015). However, like microglia, astrocytes possess immunocompetent functions within the CNS and are responsible for the proper development and function of the brain (Linnerbauer et al. 2020; Liu et al. 2020).

As already mentioned earlier, astrocytes are an important element of the NVU (**Figure 2**). They are involved in water and ion homeostasis (Walz 2000; Simard and Nedergaard 2004; Haj-Yasein et al. 2012), the regulation of blood flow (Howarth 2014; Takahashi 2022) as well as the maintenance of the BBB (Abbott et al. 2006; Iadecola and Nedergaard 2007). Beyond this, astrocytes release and take up neuro-transmitters (Volterra and Meldolesi 2005; Chiareli et al. 2021). In the late 1990s, Araque and colleagues coined the term “tripartite synapses”. This was the first concept proposing that astrocytes are active players in the CNS. They dynamically interact with the presynaptic and postsynaptic membrane to control neuronal activity and synaptic transmission by responding with feedback signals such as calcium ions (Ca^{2+}) (Araque et al. 1999). Recently, Semyanov and Verkhratsky introduced a new concept, called “the active milieu”, which addresses multiple theories of the interaction between neurons and astrocytes in the CNS. The active milieu is a morphofunctional concept, which unifies communication and interaction between neuronal, non-neuronal, vasculature, and extracellular space as well as extracellular matrix (Semyanov and Verkhratsky 2021). For a more detailed description of this concept and the astrocytes’ role in it, readers should refer to the review mentioned above.

Astrocytes are a very heterogenous glial cell type and can be further classified by their morphology, function, brain regions, physiological properties, developmental origins, and response to disease (Westergard and Rothstein 2020). Addressing all of them is far beyond the scope of this thesis, so readers are referred to the latter mentioned review on astrocyte heterogeneity. One simple and well-established classification is the subtype division into fibrous astrocytes and protoplasmic astrocytes. Fibrous astrocytes are found mainly in the white matter and have many long fiber-like processes. In contrast to this, protoplasmic astrocytes possess numerous bushy processes and are distributed throughout the gray matter (Andriezen 1893; Miller and Raff 1984). In pathological conditions, quiescent astrocytes respond with a process called “reactive astrogliosis”, through which astrocytes undergo morphological, molecular, and functional changes in response to environmental stimuli (Escartin et al. 2021). Traditionally, “reactive astrocytes” are considered as harmful by showing

hypertrophy and strongly upregulating glial fibrillary acidic protein (GFAP), the principal element of astrocytic intermediate filaments (Escartin et al. 2021; Kumar et al. 2021; Moulson et al. 2021). However, a recent published consensus statement by Escartin and colleagues emphasizes the non-existence of a prototypical reactive astrocyte. They disclaim fixed classifications of reactive astrocytes into binary “A1” and “A2” reactive phenotypes. They propose that reactive astrocytes can adopt numerous phenotypes even at the same time, ranging from losing homeostatic to gaining protective functions. The sum of these changes and the nature of the pathological situation determine if the outcome is beneficial or detrimental for the CNS (Escartin et al. 2021).

1.2 Neuroinflammation

The brain possesses different features to cope with CNS insult and thereby limit neuroinflammation. Yet, the CNS has been regarded for a long time as an immune-privileged organ due to its BBB (Galea et al. 2007; Matejuk et al. 2021), that serves as a highly regulated border between the blood and the brain (Galea 2021). Nowadays, emerging evidence implies that the CNS is actively interacting with the immune system for modulating the communication between the peripheral immune system and the CNS e.g. by the invasion of peripheral immune cells like T and B lymphocytes or the active recruitment of CNS-resident cells such as microglia and astrocytes (Carson et al. 2006). Upon neuroinflammation, microglia and astrocytes secrete a wide range of pro- and anti-inflammatory molecules reciprocally modulating the innate immune response of the brain (Jha et al. 2019; Yang et al. 2020).

1.2.1 Glial crosstalk

The intercellular communication between microglia and astrocytes, also termed as “crosstalk”, plays a crucial role in the dynamic process of neuroinflammation (Jha et al. 2019; Bernaus et al. 2020), and in the context of neurodegenerative disorders like Alzheimer’s disease (AD), Parkinson’s disease (PD) and others (Rostami et al. 2021; Mohamed et al. 2023; Wu and Eisel 2023; Zhang et al. 2023). However, the intimate relationship between these glial cell types is still at the forefront of glia research. The pathology of brain disorders and the glial cells incorporated within are just beginning to be understood (Bernaus et al. 2020; Sun et al. 2023).

The molecular conversation is enabled via the release of a wide range of different soluble mediators such as cytokines, chemokines, mitogenic factors, neurotransmitters and gliotransmitters, tissue damage molecules such as extracellular adenosine triphosphate (ATP) or growth factors, by which they determine each other’s fate (Jha et al. 2019; Matejuk et al. 2021). Microglia are usually the first cells to respond to pathological stimuli (Matsumoto et al. 1992; Kreutzberg 1995), mostly derived from either microbial constituents (pathogen-

associated molecular patterns, PAMPs) or non-microbial endogenous ligands (danger-associated molecular patterns, DAMPs) (Okun et al. 2009; Lehnardt 2010; Fiebich et al. 2018). In contrast to microglia providing the first line of defense (Kreutzberg 1995), astrocytes are usually delayed in responding, indicating that microglia play a critical role in the frame of reactive astrogliosis (Graeber and Kreutzberg 1988; McCann et al. 1996). To date, most of the evidence of an existing crosstalk between microglia and astrocytes has been obtained from *in vitro* studies.

A study by Chen and colleagues showed that lipopolysaccharide (LPS)-stimulated astrocyte cultures are only capable of releasing the pro-inflammatory factors tumor necrosis factor (TNF; also formerly referred to as TNF-α) and inducible nitric oxide synthase (iNOS) in the presence of microglia (0.5-1%), indicating an existing crosstalk between microglia and astrocytes (Chen et al. 2015). In accordance with this study, Izumi and others confirmed the crosstalk among microglia and astrocytes by showing that the release of reactive oxygen species (ROS) as well as nitric oxide (NO) upon LPS activation, disappeared in microglia-depleted astrocyte cultures (Iizumi et al. 2016). In addition, in the context of traumatic brain injury (TBI) in mice, pro-inflammatory cytokines like interleukin (IL)-1β, TNF-α, and IL-6 derived from microglia, promote a neuroprotective response of astrocytes *in vitro* and *in vivo* via the downregulation of the critical astrocyte purinergic receptor P2Y1 (*P2ry1*) (Shinozaki et al. 2017). Astrocyte reactivity can also be induced by microglial derived IL-1α, TNF, and complement component 1, subcomponent q (C1q), turning astrocytes into a harmful “A1” phenotype, which facilitates the death of neurons and oligodendrocytes after acute CNS injury (Liddelow et al. 2017). A study by Hou and co-authors identified microglial NADPH oxidase 2 (NOX2)-generated hydrogen peroxide (H_2O_2) as a critical component in the regulation of reactive astrogliosis in a signal transducer and activator of transcription (STAT)-1/3-dependent manner, using an experimental PD mouse model (Hou et al. 2017).

Apart from this, recent studies also demonstrate that astrocytes secrete signals that modulate the innate functions of microglia: The acute phase protein lipocalin-2 (LCN2) (Jha et al. 2015) is expressed in activated astrocytes using rodent ischemic stroke or vascular dementia models (Jin et al. 2014; Wang et al. 2015; Kim et al. 2017), while its receptor LCN2R is primarily expressed in neurons and microglia (Kim et al. 2017). In contrast to this, orosomucoid-2 (ORM2), an acute-phase protein belonging to the lipocalin subfamily immunocalin (Fournier et al. 2000; Logdberg and Wester 2000), is mainly secreted by astrocytes in the late phase of inflammatory conditions, acting as an anti-inflammatory mediator (Jo et al. 2017). Consequently, astrocytic ORM2 can downregulate microglial activation by blocking its C-C chemokine receptor (CCR)5, which is critical for C-X-C motif ligand (CXCL)-4-CCR5 interaction during brain inflammation (Jo et al. 2017). Furthermore, Norden et al. demonstrated

a dynamic cytokine-mediated interaction between microglia and astrocytes under inflammatory conditions: Upon LPS stimulation, microglia produce the anti-inflammatory cytokine IL-10. This promotes the downregulation of pro-inflammatory cytokines and the upregulation of transforming growth factor-beta (TGFβ) in astrocytes, which as a consequence diminish the activation of microglia (Norden et al. 2014). Beyond this, other mediators released by astrocytes modulating microglial activation are monocyte chemoattractant protein-1 (MCP-1)/CCL2 and interferon-gamma inducible protein-10 (IP-10)/CXCL10 in the context of the demyelinating disease multiple sclerosis (MS) (Tanuma et al. 2006). Studies by Jeon and colleagues showed that plasminogen activator inhibitor type 1 (PAI-1) and pentraxin 3 (PTX3) in activated glial cell cultures, most likely derived from astrocytes, selectively regulate microglial migration and phagocytosis (Jeon et al. 2010; Jeon et al. 2012). In an AD mouse model, astrocytes release complement component 3 (C3) upon amyloid-beta (Aβ) exposure, that interacts with microglial complement component C3a receptor (C3aR), which subsequently modulates microglial phagocytosis (Lian et al. 2016). A study by Seele and co-authors demonstrated in a bacterial meningitis infection model that primary microglia release significantly more NO in the presence of astrocytes, indicating a crosstalk between microglia and astrocytes (Seele et al. 2016).

Even though many *in vitro* glial isolation protocols have been established, emerging evidence is pointing to the issue that these protocols are not qualified to generate 100% pure microglia and astrocytes. In fact, the impact of lacking a suitable isolation protocol on entire *in vitro* experiments has been underestimated for a long time (Van Zeller et al. 2022). In particular, the activation of astrocytes can be mistakenly attributed to contaminating microglia, making it challenging to reliably investigate the crosstalk among microglia and astrocytes (Saura 2007; Losciuto et al. 2012). This issue and the available glial isolation methods, their benefits and drawbacks are discussed in detail in chapter *1.3 Methods for the isolation of primary microglia and astrocytes*.

1.2.2 Toll-like receptor 2 (TLR2)

As previously mentioned, microglia and astrocytes can respond to the pathological stimuli DAMPs and PAMPs, which get recognized by pattern recognition receptors (PRRs), eliciting either a pro- or anti-inflammatory response by the production of different cytokines and chemokines (Akira et al. 2006; Fiebich et al. 2018; Li et al. 2021a). Toll-like receptors (TLRs) represent a major group of PRRs, which are expressed by both microglia (Olson and Miller 2004) as well as astrocytes (Bowman et al. 2003), and play a major role in the context of glial crosstalk (Li et al. 2021a). Firstly, TLRs have been discovered in *Drosophila* (Lemaitre et al. 1996), and thus far there have been thirteen *Tlr* genes identified, of which ten are encoded in

humans (*TLRs* 1-10) and twelve in mice (*Tlrs* 1-9 and 11-13) (Fitzgerald and Kagan 2020). TLRs can be characterized depending on the region of cellular engagement with the respective PAMP ligands: TLRs 1, 2, 4, 5, 6, and 11 are located on cell surfaces. These mainly recognize proteins, lipids, and lipoproteins derived from microbial membrane components, whereas TLRs 3, 7, 8, and 9 are secreted in intracellular vesicles like endoplasmic reticula (ER), lysosomes or endolysosomes, sensing microbial nucleic acids (Kawai and Akira 2010).

One of the best characterized TLRs is TLR2, which is generally known as a sensor of bacterial lipoproteins (Akira and Takeda 2004). However, TLR2 can also interact with the widest array of components derived from bacteria (peptidoglycan and lipoteichoic acid (LTA)), mycoplasma (lipoarabinomannan), fungi (zymosan), and viruses (e.g. hemagglutinin protein from measles virus) (Kawai and Akira 2010; Takeuchi and Akira 2010). For the recognition and association of distinct agonists, TLR2 requires heterodimerization partners, namely TLR1 and 6 or the co-stimulatory molecules cluster of differentiation (CD)14 and CD36 (Oliveira-Nascimento et al. 2012). The heterodimer TLR2/1 recognizes triacylated lipoproteins or synthetic ligands such as Pam3CSK4, while the recognition of diacylated lipoproteins or synthetic ligands like Pam2CSK4 requires the heterodimerization of TLR2 with TLR6 (Ozinsky et al. 2000; Alexopoulou et al. 2002; Morr et al. 2002; Buwitt-Beckmann et al. 2006; Takeuchi and Akira 2010). On one hand, heterodimer formation allows recognition of a broader range of bacterial lipoproteins. On the other hand, rising evidence suggests that the induced immune response depends on the respective TLR2 heterodimer formation, which subsequently determines the outcome of infection and inflammation (DePaolo et al. 2008; Long et al. 2009; Rolf et al. 2015).

TLR2 belongs to the myeloid differentiation primary response 88 (MyD88)-dependent receptor family (Akira et al. 2006). This adapter protein is associated with various TLRs (Medzhitov et al. 1998), whose activation leads to the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transduction pathway (Sen and Baltimore 1986), resulting in the expression of innate immunity associated genes such as costimulatory molecules and inflammatory cytokines (Oliveira-Nascimento et al. 2012). Several studies demonstrated the critical role of TLR2 in the CNS immune response using mouse models of bacterial meningitis (Echchannaoui et al. 2002; Koedel et al. 2003; Hoffmann et al. 2007) or bacterial brain abscess (Kielian et al. 2005b; Stenzel et al. 2008). Of note, many *in vivo* studies (Edelson and Unanue 2002; Torres et al. 2004; Letiembre et al. 2005) as well as studies from our laboratory (unpublished data) have shown that TLR2-deficient neonatal mice which were infected with *Listeria monocytogenes* or other CNS pathogens, were characterized by an attenuated CNS immune response. This was accompanied by higher bacterial burden as well as lower survival rates, in comparison to wild-type (WT) neonates (our own unpublished data), further demonstrating a crucial role for TLR2 in the control of CNS infections in the neonatal host

(Echchannaoui et al. 2002; Du et al. 2011; Lalancette-Hébert et al. 2017). Moreover, TLR2 has been shown to play an age-related role in murine brains by impairing the production and signaling of TLR2-associated cytokines (Boehmer et al. 2005; Liang et al. 2009; Boyd et al. 2012; Shaw et al. 2013). In particular, the TLR2-dependent immune response by neonatal mice is primarily mediated by microglial cells under both physiological as well as pathological conditions (Lalancette-Hébert et al. 2017).

Since microglia serve as the first line of defense against invading pathogens (Kreutzberg 1995), they express most of the known *TLRs*, which has been well-studied in the context of neurodegenerative diseases (Guillot-Sestier and Town 2018). Human as well as murine microglia express *TLR2* constitutively (Bsibsi et al. 2002; Jack et al. 2005; Lehnardt et al. 2006), especially in the murine choroid plexus (Laflamme et al. 2001). Many studies describe the microglial response to different stimuli such as LPS (Kielian et al. 2005a), the main component Aβ involved in AD (Jana et al. 2008; Richard et al. 2008; Caldeira et al. 2017) or the ligand neuronal aggregated protein alpha-synuclein (α-syn) associated with PD (Beraud et al. 2011; Kim et al. 2013; Daniele et al. 2015) with an elevated TLR2 mRNA and protein expression. Furthermore, microglial *Tlr2* expression has been shown to play a critical role during stroke in a focal cerebral ischemia mouse model (Lehnardt et al. 2007). However, the regulation of TLR2 may depend on the microglial isolation method and the experimental conditions which might lead to the activation of microglia *in vitro* (Lehnardt 2010).

In contrast to microglia, *Tlr2* levels in unstimulated primary murine astrocytes are detectable but do not exceed background *Tlr2* levels (Bowman et al. 2003; Esen et al. 2004; Carpentier et al. 2005; Lehnardt et al. 2006; Holm et al. 2012). In one study, human astrocytes were shown to express *TLR2* (Bsibsi et al. 2002), however, the contrary was demonstrated in other studies (Farina et al. 2005; Jack et al. 2005). One of the reasons for these contradictory results could be the different experimental conditions such as the applied glial isolation protocol or the type and age of the selected species (Owens 2005; Kielian 2006). Recently, astrocytes received greater attention as contributor to the innate immune response in the context of TLR2-associated CNS pathologies such as PD (Lee et al. 2010; Kim et al. 2018), amyotrophic lateral sclerosis (ALS) (Faraco et al. 2007; Casula et al. 2011; Martinotti et al. 2015), TBI (Park et al. 2008; Burda et al. 2016) or bacterial meningitis (Phulwani et al. 2008; Zheng et al. 2011) by releasing a wide range of pro-inflammatory mediators including TNF as well as inducing an inflammatory gene expression profile (Li et al. 2021a). However, results from experiments using primary astrocyte culture should be taken with caution due to the lack of methods generating 100% pure astrocytes, as mentioned earlier (Saura 2007; Losciuto et al. 2012).

1.3 Methods for the isolation of primary microglia and astrocytes

1.3.1 “Indirect” glial isolation

To understand the function of the CNS under physiological as well as pathological conditions, a wide range of methods to isolate and purify primary microglia and astrocytes have been published. However, studies on the crosstalk between glial cells have been hampered by the need of highly viable and pure microglia and astrocytes. Small amounts of contaminating cells can already have an impact on the results of *in vitro* studies, which are critical for understanding the CNS and the incorporated cells within on a cell-specific level. Particularly, the generation of microglia-free astrocyte cultures has been a major challenge in the field of glial crosstalk research as residual microglia in astrocyte cultures sense and respond to the stimulus while proliferating (Saura 2007; Losciuto et al. 2012; Van Zeller et al. 2022). Therefore, the use of highly pure microglia and astrocytes coming from adequate isolation protocols allows the discrimination between immune signals and cytokines derived from the different glial cell types to understand the mechanisms behind the molecular crosstalk.

One of the most powerful and widely used tools is the isolation of microglia and astrocytes from cell cultures normally derived from rodent brain tissue (Guttenplan and Liddelow 2019). In 1972, Booher and Sensenbrenner were the pioneers who provided the basis for this “indirect approach”, which was a few years later modified by McCarthy and de Vellis. This basic protocol describes the isolation of astrocytes and oligodendrocytes from neonatal rodent tissue, namely the mixed cell culture, by seeding enzymatically digested and mechanically dissociated brain tissue into cell culture flasks (Booher and Sensenbrenner 1972; McCarthy and de Vellis 1980). Based on this, Giulian and Baker demonstrated that it is also possible to harvest microglia from the mixed glial culture (Giulian and Baker 1986). After plating the mixed glial culture, on the bottom of the flask a monolayer starts to form due to the tight adherence of the astrocytes, while microglia and oligodendrocytes stay in the medium or adhere more loosely on the astrocytes’ monolayer. After cultivation, cell cultures are shaken at least once to mechanically remove microglia and astrocytes. Finally, the remaining astrocytes are collected by enzymatic dissociation. The benefit of this indirect approach is that microglia can be harvested multiple times from the same mixed glial culture after a brief cultivation time (Guttenplan and Liddelow 2019; Aktories et al. 2022). However, one major shortcoming is that isolated microglia and astrocytes may contain contaminating CNS cells if no additional purification step is carried out, which is a problem, especially in astrocyte cultures (Saura 2007; Losciuto et al. 2012; Liddelow and Barres 2017; Van Zeller et al. 2022).

To tackle this problem, different pharmacological reagents have been developed to ablate unwanted cells from primary glial cultures (Jäkel and Dimou 2017; Ware et al. 2022).

Proliferating glial cells can be, for instance, depleted by the application of the well-established mitotic blocker cytosine arabinoside (AraC) which unspecifically affects all dividing cells (Kufe and Major 1982). Another way of microglial ablation is targeting colony-stimulating factor 1 receptor (CSF-1R), which is expressed by myeloid lineage cells including microglia and is involved in cellular proliferation and differentiation (Elmore et al. 2014; Van Zeller et al. 2022). Another approach is the addition of the lysosomotropic agent L-leucine methyl ester (LME), which causes lysosomal disruption and thereby cell apoptosis in microglia and macrophages (Thiele et al. 1983; Hamby et al. 2006). To date, the only method to ablate astrocytes is the toxin L-α-aminoadipic acid (L-AAA), a glutamate homologue that is known to specifically deplete astrocytes (Takada and Hattori 1986; Brown and Kretzschmar 1998; Nishimura et al. 2000; Jäkel and Dimou 2017). Whereas the impact of these wide array of pharmacological reagents has not yet been fully understood, still many of these ablation methods are accompanied by some major drawbacks. For example, studies demonstrated that relatively low concentrations of the antimitotic agent AraC can have severe effects on astrocytes such as cellular activation involving morphological changes and GFAP upregulation (Patel et al. 1988; Ahlemeyer et al. 2002; Ahlemeyer et al. 2003; Hui et al. 2016). Additionally, this antimitotic agent only targets proliferating microglia while non-proliferating microglial cells are excluded from ablation (Kumamaru et al. 2012). Moreover, studies have demonstrated that, under certain circumstances, LME may have toxic effects on astrocytes and affect the astrocytic adhesion and reactivity capacity (Guillemin et al. 1997; Hamby et al. 2006; Kumamaru et al. 2012).

1.3.2 “Direct” glial isolation

Microglia and astrocytes can also be directly isolated from whole brain tissue instead of cell cultures. The major disadvantage of these “direct” approaches is that microglia can only be harvested once (Aktories et al. 2022). One of these methods is called immunopanning, which was originally developed for rodents by Ben Barres in the 1980s. This concept involves petri dishes that are coated with specific antibodies targeting the cell of interest e.g. microglia or astrocytes (Barres et al. 1988). Firstly, an enzymatically dissociated, mixed glial cell suspension is prepared. Then, it is passed over a series of antibody-coated panning dishes to remove the unwanted cells. In a last step, the cells of interest are collected from the final dish (Barres et al. 1988; Foo et al. 2011; Barres 2014; Scholze et al. 2014; Bohlen et al. 2019). Lately, this method has been also adapted for the isolation of microglia and astrocytes from human brain tissue (Zhang et al. 2016; Nolle et al. 2021).

Another direct isolation method involves a density gradient centrifugation step, which was first described for the isolation of glial cells from mouse cerebellum by Campbell and co-authors in

1977 (Campbell et al. 1977). This centrifuge-based isolation method has been modified over the years, yet the basic principle remains the same: In the first step, brain tissue is mechanically and enzymatically dissociated, it is then layered on top of a pre-formed density gradient. Next, high-speed centrifugation is applied to separate the cells of interest from oligodendrocyte-derived myelin, blood cells as well as debris, which are enclosed in the interphase (Cardona et al. 2006; Zuiderwijk-Sick et al. 2007; Moussaud and Draheim 2010; Lee and Tansey 2013; Chew et al. 2014; Mizee et al. 2017). This method has been improved by combining it with immunomagnetic cell sorting, which is another direct isolation method, to enhance glial cell viability and purity (de Haas et al. 2007; Nikodemova and Watters 2012; Olah et al. 2012; Mizee et al. 2017; Grabert and McColl 2018; Stark et al. 2018; Pan and Wan 2020).

In 1990, Miltenyi and colleagues improved and patented magnetic-activated cell sorting (MACS®) (Miltenyi et al. 1990), which was originally adapted from Molday and MacKenzie (Molday and MacKenzie 1982), who first described cell separation by ferro-magnetic particles. The concept of immunomagnetic cell sorting has been adapted and improved by a lot of research groups, however, the basic steps stayed unchanged: First, a single-cell suspension derived from the brain region of interest is incubated with magnetic microbeads conjugated to antibodies against e.g., surface proteins of microglia or astrocytes, and then passed through a column surrounded by a strong magnetic field. The labeled cells of interest remain on the column while the non-targeted cells flow through. Subsequently, the retained cells, as the positive fraction, are removed from the column in a final elution step (Marek et al. 2008; Losciuto et al. 2012; Bedi et al. 2013; Harms and Tansey 2013; Holt and Olsen 2016; Batiuk et al. 2017; Bohlen et al. 2017; Sarkar et al. 2017; Guneykaya et al. 2018; He et al. 2018; Holt et al. 2019; Sugiyama et al. 2020; Schroeter et al. 2021). It is worth noting that the purification of microglia and astrocytes with magnetic microbeads also has its downsides. So far, protocols using magnetic microbeads have been primarily developed for neonatal animals (postnatal day (P)7) (Holt and Olsen 2016) and further require an antibody targeting extracellular protein (Schroeter et al. 2021).

Another approach to directly enrich microglia and astrocytes from brain tissue is purification with the help of fluorescence-activated cell sorting (FACS) using fluorescence-labeled antibodies, that separate cells depending on their size and molecular phenotype (Guez-Barber et al. 2012; Hickman et al. 2013; Bennett et al. 2016; Bohlen et al. 2019). Glial cell isolation via FACS has the benefits of being more suitable for multiple marker profiles as well as more precise for downstream applications such as RNA sequencing (RNA-seq) than using magnetic microbeads (Sutermaster and Darling 2019; Pan and Wan 2020).

A recently published study by Pan and Wan directly compared MACS- with FACS-isolated microglia and astrocytes from dissociated whole mouse brains which were prior treated with a Percoll density gradient centrifugation step (Pan and Wan 2020). They revealed that the viability of MACS-purified microglia and astrocytes was superior to the FACS-purified glial cells due to higher shear stress in the process of FACS purification since MACS is a gravity-driven process (Sutermaster and Darling 2019; Pan and Wan 2020). Moreover, MACS-purified microglia (>90%) and astrocytes (94.5%) also demonstrated superior purities than the FACS-purified glial cells (Pan and Wan 2020). Further benefits of using MACS instead of FACS to isolate glial cells are that it can be performed under sterile conditions in a cell culture hood and that it is less time-consuming. Moreover, MACS can be carried out in parallel only restricted by the number of available magnets achieving greater cell yields and faster recovery time. Despite its speed and efficiency, MACS does not require expensive machinery like a flow-activated sorter and no specialized training is needed, making it a more inexpensive and easier to use approach than FACS (Marek et al. 2008; Li et al. 2013; Holt and Olsen 2016; Bowles et al. 2019; Holt et al. 2019; Sutermaster and Darling 2019; Pan and Wan 2020; Schroeter et al. 2021; Buenaventura et al. 2022).

However, all these indirect and direct *in vitro* methods to isolate and purify primary microglia and astrocytes from rodent brains also have their limits. While most of these methods already achieve satisfying microglial purities of >99% (Timmerman et al. 2018), protocols for the isolation of astrocytes devoid of contaminating cells are lagging far behind (Van Zeller et al. 2022). With special regard to investigate the crosstalk among glial cells on a cell-specific level, there is an urgent need of a protocol generating ~100% viable and pure microglia and astrocytes.

1.4 Aims of the study

Although there is emerging evidence for the crucial role of microglia and astrocytes as major CNS players in development, homeostasis, and disease, knowledge of the mechanisms of the crosstalk between these two glial cell types is rather scant. Despite an array of developed *in vitro* methods to isolate primary microglia and astrocytes from brain tissue, these protocols do not meet the standards required to investigate the crosstalk on a cell-specific level. In particular, the development of isolation protocols generating nearly 100% astrocytes, has lagged far behind those of microglia making it challenging to assess the individual responsiveness of astrocytes.

The aim of this project was to develop a novel protocol to optimize the *in vitro* cultivation and purification of microglia and astrocytes derived from neonatal mice using MACS. In doing so, we aim to reduce animal experiments by harvesting microglia multiple times from the same mixed culture and subsequent modified MACS application of cultivated primary glial cell suspensions (**Publication I, Figure 3**). Finally, highly viable and pure glial cells generated with our novel method ultimately enable the investigation of the crosstalk between microglia and astrocytes on a cell-specific level by discriminating their immune signals with special regard to pro- and anti-inflammatory cytokines in the context of TLR2/1 activation (**Publication II**).

2 Publications

2.1 Publication I

Novel protocol for the isolation of highly purified neonatal murine microglia and astrocytes

Laura Zelenka (L.Z.), Dennis Pägelow (D.P.), Christina Krüger (C.K.), Jana Seele (J.S.), Friederike Ebner (F.E.), Sebastian Rausch (S.R.), Manfred Rohde (M.R.), Seija Lehnardt (S.L.), Kira van Vorst (K.v.V.), Marcus Fulde (M.F)

Journal of Neuroscience Methods. Volume 366, 15 January 2022, 109420

DOI: 10.1016/j.jneumeth.2021.109420

https://doi.org/10.1016/j.jneumeth.2021.109420

Authors' contributions:

L.Z., D.P., K.v.V. and M.F. planned, designed, conducted, and analyzed the glial cell experiments. L.Z., D.P., C.K., J.S. and S.L. established the isolation and purification protocol. M.R. conducted electron microscopic analysis. D.P., F.E. and S.R. analyzed and evaluated FACS data sets. S.L. provided technical support. D.P., K.v.V. and M.F. supervised the study and L.Z., K.v.V. and M.F. wrote the manuscript. All authors read and approved the final manuscript.

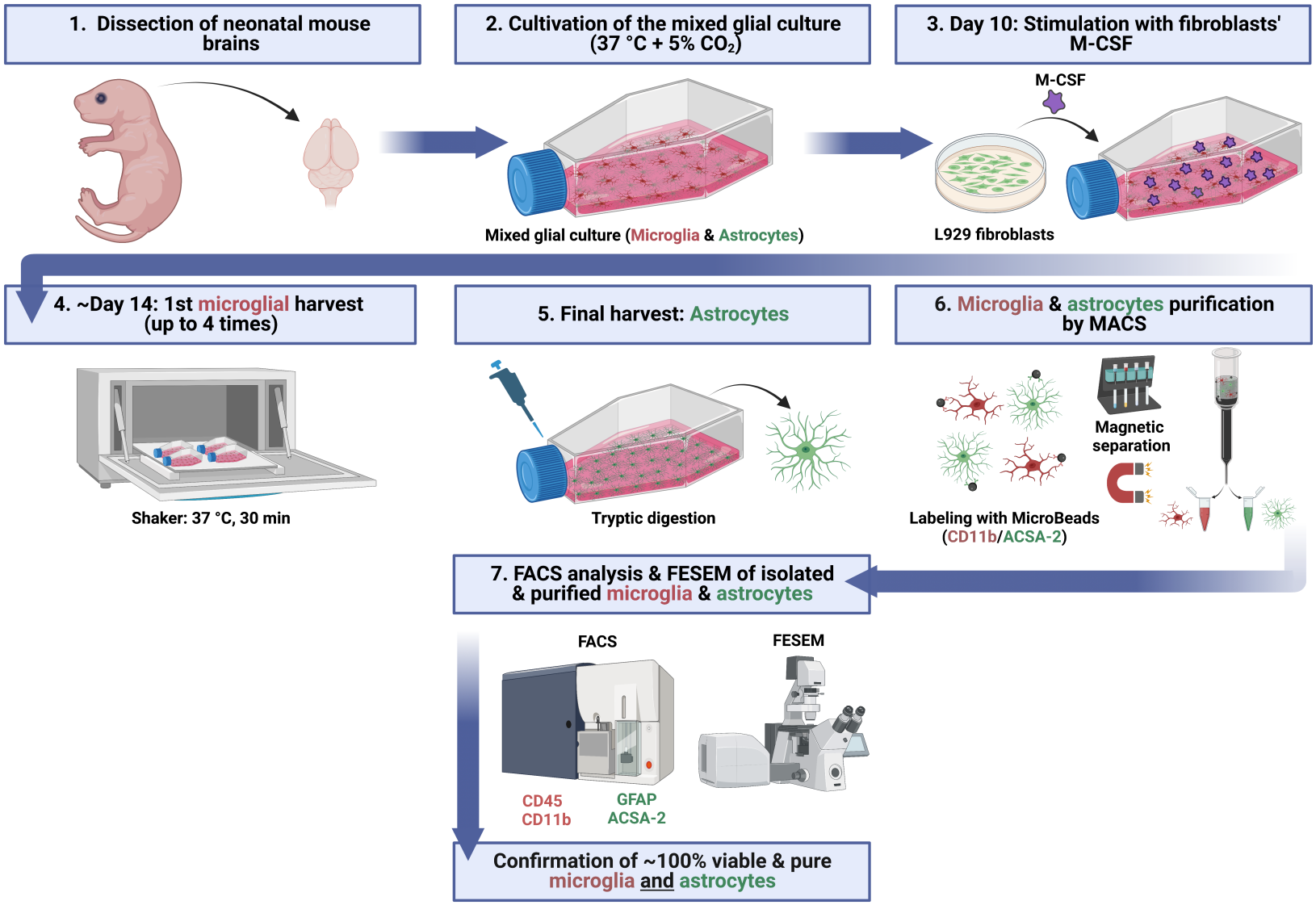


Figure 3: Graphical abstract of Publication I. Illustration created with BioRender.com.

Journal of Neuroscience Methods 366 (2022) 109420

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Novel protocol for the isolation of highly purified neonatal murine microglia and astrocytes

Laura Zelenka [a], Dennis Pägelow [a], Christina Krüger [b], Jana Seele [c], Friederike Ebner [d], Sebastian Rausch [d], Manfred Rohde [e], Seija Lehnardt [b,f], Kira van Vorst [a,1], Marcus Fulde [a,1,*]

[a] Institute of Microbiology and Epizootics, Centre of Infection Medicine, Freie Universität Berlin, Robert-von-Ostertag-Straße 7-13, 14163 Berlin, Germany
[b] Institute of Cell Biology and Neurobiology, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany
[c] University Medical Center Göttingen, Institute of Neuropathology, Göttingen, Germany
[d] Freie Universität Berlin, Institute of Immunology, Berlin, Germany
[e] Central Facility for Microscopy, Helmholtz Centre for Infection Research, Braunschweig, Germany
[f] Department of Neurology, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

ARTICLE INFO

Keywords:
Glial cells
Microglia
Astrocytes
Primary cell culture
Magnetic-activated cell sorting (MACS®)
Purity
Viability

ABSTRACT

Background: The crosstalk and reactivity of the cell type glia, especially microglia and astrocytes, have progressively gathered research attention in understanding proper brain function regulated by the innate immune response. Therefore, methods to isolate highly viable and pure glia for the analysis on a cell-specific level are indispensable.

New method: We modified previously established techniques: Animal numbers were reduced by multiple microglial harvests from the same mixed glial culture, thereby maximizing microglial yields following the principles of the 3Rs (replacement, reduction, and refinement). We optimized Magnetic-activated cell sorting (MACS®) of microglia and astrocytes by applying cultivated primary glial cell suspensions instead of directly sorting dissociated single cell suspension.

Results: We generated highly viable and pure microglia and astrocytes derived from a single mixed culture with a purity of ~99%, as confirmed by FACS analysis. Field emission scanning electron microscopy (FESEM) demonstrated integrity of the MACS-purified glial cells. Tumor necrosis factor (TNF) and Interleukin-10 (IL-10) ELISA confirmed pro- and anti-inflammatory responses to be functional in purified glia, but significantly weakened compared to non-purified cells, further highlighting the importance of cellular crosstalk for proper immune activation.

Comparison with existing method(s): Unlike previous studies that either isolated a single type of glia or displayed a substantial proportion of contamination with other cell types, we achieved isolation of both microglia and astrocytes at an increased purity (99–100%).

Conclusions: We have created an optimized protocol for the efficient purification of both primary microglia and astrocytes. Our results clearly demonstrate the importance of purity in glial cell cultivation in order to examine immune responses, which particularly holds true for astrocytes. We propose the novel protocol as a tool to investigate the cell type-specific crosstalk between microglia and astrocytes in the frame of CNS diseases.

1. Introduction

Diseases of the central nervous system (CNS) and the role of the involved specific cell types have been at the forefront in neuroscience research (Holt et al., 2019). Glia represent more than half of the CNS cells and apart from neurons, these cells are the major players in the development as well as progression of CNS diseases (Greenhalgh et al., 2020). Microglia and astrocytes belong to the cell type glia, which is

* Corresponding author.
E-mail address: Marcus.Fulde@fu-berlin.de (M. Fulde).
[1] Kira van Vorst and Marcus Fulde should be considered joint senior author

https://doi.org/10.1016/j.jneumeth.2021.109420
Received 25 August 2021; Received in revised form 2 November 2021; Accepted 11 November 2021
Available online 20 November 2021

indispensable for the function of the developing and mature brain (Jha et al., 2019).

Microglia are the main immunocompetent cells and considered as the first line of defense in the brain. Their fast reaction to signals such as non-microbial endogenous ligands (danger-associated molecular patterns, DAMPs) or microbial constituents (pathogen-associated molecular patterns, PAMPs) promote a pro- or an anti-inflammatory response (Jafarzadeh et al., 2019; Xu et al., 2016). Beyond this, microglial cells also maintain tissue homeostasis depending on area-specific needs in collaboration with other CNS cells, e.g. astrocytes (Erny and Prinz, 2020; Masuda et al., 2020). Astrocytes are involved in multiple processes, i.e. providing structural support and nutrients for neuronal cells, as well as shaping neuronal activity (Li et al., 2021). They have barrier functions to restrict the spread of infections to the CNS parenchyma, as well as pro- and anti-inflammatory properties (Allen and Lyons, 2018; Westergard and Rothstein, 2020). Microglia and astrocytes are well-orchestrated cell populations, which are subjected to a tight mutual control of the function in the brain (Jha et al., 2019).

Recently, the crosstalk between microglia and astrocytes received greater attention due to emerging evidence of their fate-determining, reciprocal conversation in the context of brain development and neurodegenerative diseases (Matejuk and Ransohoff, 2020). Among others, Toll-like receptors (TLRs), a major group of pattern recognition receptors (PRRs) expressed on both microglia and astrocytes, recognize DAMPs and PAMPs. TLRs play a pivotal role in the glial crosstalk by eliciting and modulating the innate immune response (Fiebich et al., 2018; Frederiksen et al., 2019). During this bidirectional talk, microglia and astrocytes release a wide range of signal molecules such as adenosine triphosphate (ATP), growth factors, and cytokines, which determine the fate of the corresponding cell type (Jha et al., 2019; Matejuk and Ransohoff, 2020).

Pioneering work by Booher and Sensenbrenner in the early 1970s and the subsequent modification by McCarthy and de Vellis enabled for the isolation of rodent astrocytes and oligodendrocytes from enzymatically digested and mechanically dissociated neonatal brain tissues seeded into cell culture flasks (Booher and Sensenbrenner, 1972; McCarthy and de Vellis, 1980). Later, Giulian and Baker showed that microglia can also be harvested from mixed glial cultures (Giulian and Baker, 1986). By seeding mixed glial culture into a cell culture flask, astrocytes form a monolayer by adhering tightly to the bottom of the flask. Microglia remain in the medium or adhere loosely to the astrocytes' monolayer. Microglia and astrocytes can then be mechanically isolated by shaking. The remaining astrocytes can be dissociated by enzymatic digestion. An advantage of this "indirect" approach is that microglia can be harvested more than once from a mixed rodent glial culture (Guttenplan and Liddelow, 2019). However, the sole use of these protocols does not accomplish microglia-free astrocyte cultures (Saura, 2007).

Besides this indirect isolation method, there is also the approach of "direct" isolation of microglia and astrocytes from whole brain tissue with the major disadvantage that microglia cannot be harvested multiple times from one mixed glial cell suspension. One example is the application of a density gradient centrifugation step of mechanically and enzymatically dissociated brain tissue. For this, the prepared cell suspension is layered on a pre-formed density gradient. By high-speed centrifugation, cells of interest are separated from interphase layers containing unwanted myelin derived by oligodendrocytes, debris, or blood cells (Cardona et al., 2006; Chew et al., 2014; Mizee et al., 2017; Olah et al., 2012; Zuiderwijk-Sick et al., 2007). Another direct method is called immunopanning. The first step involves the preparation and dissection of a mixed glial cell suspension followed by an enzymatic dissociation step. After this, the mixed glial cell suspension is incubated on a series of antibody-coated dishes to deplete unwanted cells. In the final step, the purified cells of interest are removed from the dishes (Barres, 2014; Bohlen et al., 2019; Cahoy et al., 2008; Foo et al., 2011; Guttenplan and Liddelow, 2019; Scholze et al., 2014).

Glial cells can be purified by fluorescence-activated cell sorting (FACS) based on their size and molecular phenotype using fluorescence-labeled antibodies (Bennett et al., 2016; Bohlen et al., 2019; Guez-Barber et al., 2012). Another way to directly enrich glial cells is the separation by magnetic-activated cell sorting (MACS®) based on magnetic microbeads, which are, for instance, labeled with antibodies targeting microglia or astrocytes surface proteins (Bohlen et al., 2019; Feldmann et al., 2014; Guttenplan and Liddelow, 2019; Holt and Olsen, 2016; Holt et al., 2019; Mizee et al., 2017; Nikodemova and Watters, 2012).

Unfortunately, none of these methods yields a purity required to investigate cell type-specific effects, e.g. recognition of invading pathogens or contribution to immune cell responses in the brain. Even though some of the protocols achieve high microglial yields, none of them generated nearly 100% highly viable and pure astrocytes from the same glial culture. The generation of highly purified glial cultures is crucial to discriminate between immune signals and cytokines released from different cellular sources, and to unravel mechanisms in cellular crosstalk. Herein, we describe a novel method to optimize *in vitro* cultivation and purification of neonatal murine primary microglia and astrocytes. Unlike the "direct" protocols described above, we optimized MACS purification of microglia and astrocytes by applying cultivated primary glial cell suspensions instead of directly applying dissociated single cell suspensions. Microglial yields were increased by the stimulation with macrophage colony-stimulating factor (M-CSF). In addition to this, the number of animal experiments could be reduced, because microglia can be harvested and purified from one glial culture by MACS up to four times. With this optimized protocol, we generated microglia and astrocytes with a viability and purity of nearly 100%, as confirmed by FACS, which particularly holds true for astrocytes, where MACS purification increased cell purity from ~ 46% to up to 99%. In the next step, we stimulated non-purified and MACS-purified microglia and astrocytes with TLR agonists to determine their pro- and anti-inflammatory cytokine response. Non-purified microglia and astrocytes elicited higher IL-10 and TNF protein levels than the MACS-purified cells, which might be due to signal molecules released during the glial crosstalk.

In summary, our novel protocol accomplishes the isolation of microglia and astrocytes from a single mixed cell culture. In compliance with the rules of the 3Rs (replacement, reduction, and refinement), the method helps to reduce animal experiments. Cell cultures generated with this method are applicable for subsequent cell-specific analysis due to their high viability and purity. This might be an essential milestone, especially for the *in vitro* cultivation of microglia-free astrocytes. These viable and pure glial cells constitute the cornerstone for various downstream cell-specific applications and allow the precise investigation of the crosstalk between microglia and astrocytes, as well as the co-culturing of glia derived from different genetic backgrounds in future approaches.

2. Methods

2.1. Animals and ethical statement

Male and female 0 to 2-day-old wild-type (WT, C57BL/6 n) mice were used for isolation of primary microglia and astrocytes. Animals were bred in-house in individually ventilated cages (IVCs) under specific-pathogen-free (SPF) conditions at the Freie Universität Berlin. Water and food were provided *ad libidum*. All animals were maintained in compliance with the German animal protection law (TierSchG) and the local animal welfare committee. Organ harvesting was approved by the institutional review committee Landesamt für Gesundheit und Soziales Berlin, Germany (approval T0284/15).

2.2. Isolation and cultivation of primary microglia and astrocytes

Fig. 1 illustrates the workflow of the novel protocol. Albeit, the protocol has been proven to be very robust, immune cells of the CNS are fragile and have to be handled with care at every step. Common mistakes resulting in lower cell yields, decreased viability or improper purity and general advices on how to avoid them are depicted in the supplement (Table S1). As pictured in Fig. S6, we performed phase-contrast microscopy to visualize the different stages of the mixed glial culture using a ZEISS Axio Vert.A1 inverted microscope (Carl Zeiss AG, Germany) as well as Zeiss imaging software version 2.3 (Carl Zeiss AG, RRID: SCR_013672).

Whole brains from 0 to 2-day-old neonatal mice (P0–2) were isolated. After removing meninges and blood vessels gently, brain tissue was minced into approximately 1 mm^3 pieces and transferred into Leibovitz' L-15 Medium (Gibco, Germany) on ice. Brain tissues were washed twice with Leibovitz' L-15 Medium and treated with 3 ml trypsin (2.5%; Gibco, Germany,) for 25 min at 37 °C with 5% CO_2. Trypsin activity was stopped by the addition of 4 ml heat-inactivated fetal calf serum (FCS_{HI}) (Biochrom, Germany). Brains were further dissociated using a 5 ml-pipette. 100 µL DNase I (1 mg/ml) (Roche, Germany) was added, mixed gently followed by the incubation for 1 min at room temperature. The suspension was centrifuged (270 x *g*, 5 min, 4 °C), the supernatant removed, and the pellet resuspended in 2.5 ml Dulbecco's Modified Eagle Medium (DMEM; Gibco, Germany) supplemented with 10% FCS_{HI}, 1% L-Glutamine (200 mM; Gibco, Germany) and 0.5% Penicillin-Streptomycin (10,000 U/ml; Gibco, Germany), referred to as conditioned medium, per brain. The suspension was homogenized by repeatedly pipetting up and down with a 10- and 5 ml-pipette. Cell clumps were eliminated from the mixed glial culture by passing the suspension through a 70 µm-cell strainer (VWR, Germany), and 5 ml were seeded into a Poly-D-lysine (PDL) (Sigma-Aldrich, Germany)-pre-coated 75 cm^2-cell culture flask (Corning, USA) containing 10 ml of the conditioned DMEM and stored at 37 °C with 5% CO_2. On day 2, mixed cultures were washed twice with Phosphate Buffered Saline (PBS) (pH 7.5) (Gibco, Germany) to remove unattached cells and debris, and 15 ml of supplemented DMEM were added. One day later, medium was replaced by 15 ml fresh DMEM.

2.3. Preparation of L929 supernatants

Since it is know that supernatants derived from L929 fibroblast cultures contain M-CSF, we cultivated L929 cells in DMEM (Gibco, Germany) supplemented with 5% FCS_{HI} + 2 mM L-Glutamine in 175 cm^2-flasks (Thermo Fisher, Germany) at 37 °C with 5% CO^2 (Sawada et al., 1990). M-CSF promotes development and proliferation of microglia, which express the corresponding receptor M-CSFR (Kierdorf and Prinz, 2019). After 7 days, cells were grown to confluency, medium was removed and centrifuged twice (3230 x *g*, 5 min, 4 °C) to obtain fibroblast-free supernatants, which were stored at −20 °C for later applications.

2.4. Stimulation and harvest of microglia & astrocytes

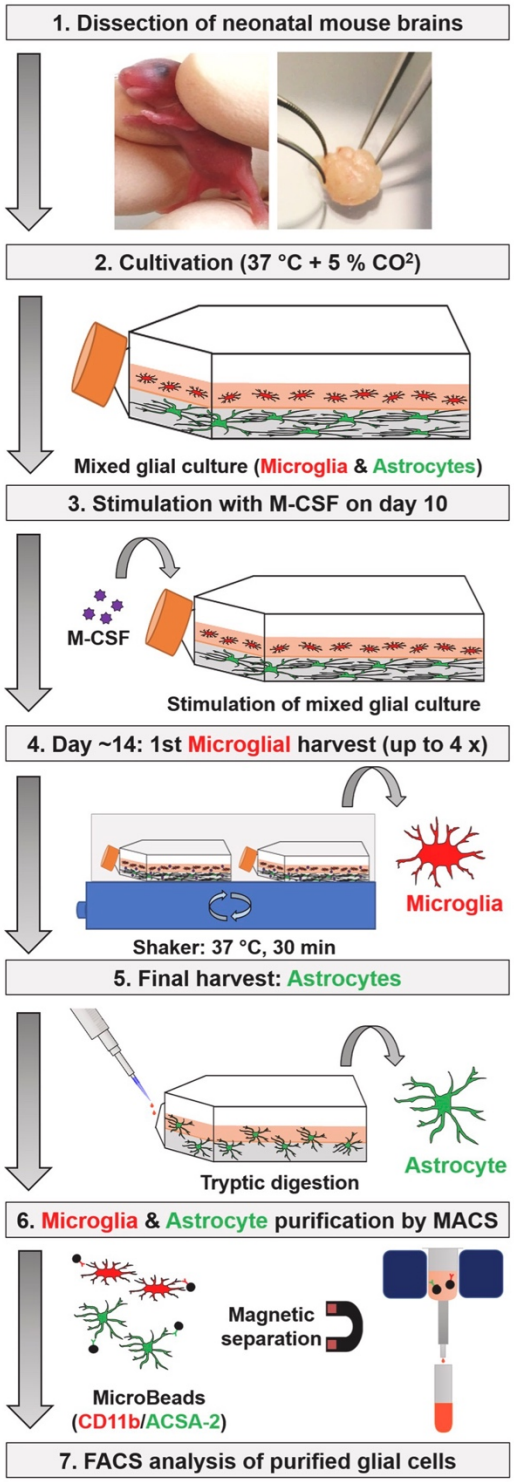
After 10 days of mixed glial cell cultivation, medium was removed and 10 ml fresh medium, as well as 5 ml of the fibroblast's supernatants, were added to stimulate the mixed glial culture with M-CSF (Fig. 1). After 4–5 days of stimulation, the supernatant comprising detached cells expected to be enriched in microglia was harvested for the first time. To that end, the culture flasks were kept at 37 °C, 5% CO_2 in a shaking incubator (Gesellschaft für Labortechnik, Germany), (150 rpm) for 30 min. The detached cells were harvested and cells remaining attached to the culture flask, received fresh conditioned medium supplemented with fibroblast supernatants as described above. The harvested mixed glial cell culture suspension was centrifuged (270 x *g*, 5 min, 4 °C). Supernatant was discarded and the pellet resuspended in 2 ml DMEM (Gibco, Germany), supplemented with FCS_{HI} and L-Glutamine per cell culture flask. Microglia harvest was performed up to 4 times in intervals of two and five days, respectively and finally the remaining attached cells, expected to be enriched in astrocytes were harvested. To this end, flasks were washed with PBS and treated with 4 ml 2.5% trypsin/0.5 mM EDTA (Carl Roth, Germany) for 10 min at 37 °C with 5% CO_2. Remaining cells were entirely removed from the bottom of the flasks with the help of a cell scraper (Corning, USA). Before centrifugation (270 x *g*, 5 min, 4 °C), 10 ml of the conditioned DMEM without Penicillin-Streptomycin were added to each flask, collected and passed through a 70 µm-cell strainer. The supernatant was discarded and the pellet resuspended in 2 ml DMEM without Penicillin-Streptomycin per cell culture flask for subsequent steps.

2.5. Purification by MACS

Microglia and astrocytes were purified using CD11b MicroBeads (Miltenyi Biotec, Germany) and the Anti-ACSA-2 MicroBead Kit (Miltenyi Biotec, Germany), respectively (Fig. 1). Magnetic labeling and magnetic separation were carried out following the manufacturer's protocol with some modifications: Before determining the cell number, microglia and astrocytes were passed through 40 µm- (VWR, Germany) and 70 µm-cell strainers, respectively. Glia were resuspended in MACS buffer containing PBS (Gibco, Germany) supplemented with 0.5% bovine serum albumin (BSA) (Carl Roth, Germany) and 10 mM (microglia) or 0.5 mM EDTA (astrocytes) (pH 7.5). Glial cells were magnetically labelled according to the manufacturer's protocols and loaded on Miltenyi Biotec's MS columns (Miltenyi Biotec, Germany), followed by separation using a MACS separator (Miltenyi Biotec, Germany). The MS columns were loaded with the maximum number of labeled microglia according to the manufacture's protocol and astrocytes were limited to a maximum number of 5×10^6 cells/column to prevent column clogging. Unlabeled cells flowed through the column and only labeled cells were retained. These were collected by flushing the columns with MACS buffer, after removing the columns from the magnetic separator. Finally, the cell numbers were determined by Trypan blue staining (0.4%) (Merck, Germany) using a Neubauer-improved counting chamber (Paul Marienfeld, Germany).

2.6. FACS analysis

FACS was performed based on non-purified as well as MACS-purified primary WT microglia and astrocytes (Fig. 1). Of each group, approximately 3×10^5 cells were resuspended in FACS buffer (PBS, 2% BSA, 2 mM EDTA, pH 8) containing anti-Fcγ receptor II/III (1:20) (Thermo Fisher Scientific Cat# 14–0161–82, RRID:AB_467133) and incubated with the respective antibodies for 10 min on ice. Cells were stained with anti-CD45-PacBlue (1:100) (BioLegend Cat# 103125, RRID: AB_493536), anti-CD11b-PE-Cy5 (1:100) (BioLegend Cat# 101210, RRID:AB_312793), anti-ACSA-2-PE (1:25) (Miltenyi Biotec Cat# 130–123–284, RRID:AB_2811488), anti-GFAP-AF647 (1:100) (BioLegend Cat# 837511, RRID:AB_2734610), and Fixable Viability Dye eFluor™ 780 (1:1000) (Invitrogen, Germany). Microglia were incubated for 10 min at 4 °C in the dark, washed with FACS buffer (300 x *g*, 5 min, 4 °C), and supernatants were removed. Subsequently, 0.5% paraformaldehyde (PFA) (Carl Roth, Germany) in PBS was added to the pellets. Astrocytes were incubated and washed followed by resuspension in 3% PFA/PBS for 10 min at 4 °C in the dark. Astrocytes were then washed (300 x *g*, 5 min, 4 °C) with eBioscience™ Permeabilization Buffer (Invitrogen, Germany) and intracellularly stained with anti-GFAP-AF647 (1:100) in eBioscience™ Permeabilization Buffer for 10 min at 4 °C in the dark. Astrocytes were washed again with permeabilization buffer (300 x *g*, 5 min, 4 °C) and resuspended in FACS buffer. Cells were acquired on a BD FacsCanto II Flow Cytometer (Becton Dickinson, USA) and analyzed with FlowJo v10.6.1 (FlowJo, RRID: SCR_008520). The general gating strategy is exemplary shown in Fig. S3.



(caption on next column)

Fig. 1. Workflow depicting the single steps of the novel protocol. **Step 1:** Brains of neonatal mice were dissected. **Step 2:** The mixed glial cells suspension was seeded into cell culture flasks. **Step 3:** After 10 days of cell cultivation (37 °C + 5% CO_2), mixed glial cells were stimulated by the addition of M-CSF derived from fibroblasts' supernatant. **Step 4:** 4–5 days after M-CSF stimulation, microglia were harvested for the first time by using a shaking incubator (37 °C, 30 min). **Step 5:** After 3–4 microglial harvests, astrocyte monolayer was detached from the cell culture flask by tryptic digestion. **Step 6:** Microglial and astrocyte cultures were purified by MACS using anti-CD11b and anti-ACSA-2 MicroBeads detecting microglia and astrocytes, respectively. **Step 7:** FACS analyzed the viability and purity of non-purified and MACS-purified microglia and astrocytes.

Viable single microglia and astrocyte populations were subsequently gated using the pan-myeloid marker CD11b, the common leukocyte marker CD45, as well as the astrocytes markers ACSA-2 and GFAP. The gating strategy for microglia and astrocytes is presented in Figs. S4–5.

2.7. Field emission scanning electron microscopy (FESEM)

Microglia and astrocytes were cultivated and purified as stated before. Next, 2×10^5 cells/ml were seeded into PDL (Sigma-Aldrich, Germany) pre-coated CellBIND® 24-well plates (Corning, USA) coverslips (Thermo Fisher, Germany). Microglia were incubated for 24 h to allow cell recovery, and astrocytes for 72 h to reach confluency (37 °C, 5% CO_2). After 24 h or 72 h of incubation, medium was replaced by fresh one and incubated for another 24 h at 37 °C with 5% CO_2. Subsequently, samples were fixed with 5% PFA and 2% glutaraldehyde in HEPES buffer and kept at 7 °C. Then, samples were washed with TE (TRIS-EDTA) buffer and dehydrated with a graded series of acetone (10, 30, 50, 70, 90%) on ice. Samples were allowed to reach room temperature and were further dehydrated twice with 100% acetone before performing a critical point drying (Leica, CPD300). Finally, samples were sputter-coated with gold/palladium (Bal-Tec SCD5009) before examination in a Zeiss field emission scanning electron microscope Merlin at an acceleration voltage of 5 kV applying the Everhart-Thornley SE-detector and Inlens SE-detector at a 25:75 ratio.

2.8. Stimulation with TLR ligands

Non-purified and MACS-purified primary microglia (harvests 1–3) and astrocytes derived from neonatal WT mice were resuspended in conditioned DMEM. Prior seeding, MACS-purified cells were washed by another centrifugation step (300 x *g*, 5 min (astrocytes) or 10 min (microglia), 4 °C) to remove the MACS buffer, and were resuspended in 1 ml conditioned DMEM without Penicillin-Streptomycin. Subsequently, cells were seeded (2×10^5 cells/ml) into 24-well plates. Prior stimulation, microglia were incubated for 24 h to allow cell recovery and astrocytes for 72 h to reach confluency (37 °C, 5% CO_2). Medium was aspirated, and cells were stimulated with fresh pre-warmed medium, containing either the TLR2 agonist Pam3CSK4 (100 ng/ml) (InvivoGen, USA) acting mainly through TLR2/1 heterodimeric receptors, or 1 μM of unmethylated cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODNs), a TLR9-specific agonist (CpG 1668 ODN, [5′-TsCsC sAsTsg sAsCsg sTsTsC sCsTsg sAsTsg sCsT-3′ 20 mer]; TIB MOLBIOL Syntheselabor GmbH, Germany), respectively. The ligand concentrations were adapted from previous work by Rosenberger and colleagues (Rosenberger et al., 2014). After removing the media from the untreated controls, fresh pre-warmed conditioned DMEM without Penicillin-Streptomycin was added to the wells. Microglia and astrocytes were stimulated with CpG ODN or Pam3CSK4, or were left untreated, for 24 h. Finally, supernatants were centrifuged (800 x *g*, 10 min, 4 °C) to remove contaminating cells and stored at − 80 °C until use.

2.9. Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-10 concentrations in cell culture supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA), using the ELISA MAX™ Deluxe Set Mouse IL-10 and TNF-α (BioLegend, USA) according to the manufacturer's manual. The expected minimum detectable concentration of TNF-α is 4 and for IL-10 less than 16 pg/ml according to the manufacture's protocol.

2.10. Statistical analysis

Statistics as indicated in the Figure legends were calculated using GraphPad Prism version 9.0.1 (GraphPad Prism, RRID:SCR_002798). The Mann-Whitney test was used for comparison of two groups. Differences were considered statistically significant when lower $p < 0.05$ ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****).

3. Results

3.1. Effect of M-CSF on glial cell yields

To investigate the effect of M-CSF, yields of microglia and astrocytes isolated from unstimulated glial cultures were compared to those from cultures treated with M-CSF, respectively (Fig. 2). The addition of M-CSF to the mixed glial cell culture increased microglial cell yields from an average of 1.3×10^7 to 3.2×10^7 cells/mouse in total (Fig. 2(a)). In particular, the second and third harvests were significantly enhanced from 2.3×10^4 to 2.2×10^5 cells/mouse (second) and 3.7×10^4 to 1.2×10^5 cells/mouse (third) in average, respectively (Fig. S1(a)). Stimulation of the mixed glial cultures with M-CSF did not have a significant effect ($p = 0.9637$) on the average astrocyte yield (3.4×10^7 cells/mouse), in comparison to astrocytes isolated from unstimulated mixed glial cultures (5.2×10^7 cells/mouse) (Fig. 2(b); S1(b)). Thus, the addition of M-CSF to the mixed glial culture acted as a "booster" on the yield of microglia, but not on the one of astrocytes.

3.2. Purification by MACS

Using a modified protocol from Miltenyi Biotec, glial cells were magnetically selected after every harvest. Total yields were compared for non-purified and MACS-purified microglia and astrocytes (Fig. 3). For microglia, total yields significantly decreased after MACS purification from an average of 3.2×10^7 to 4.6×10^6 cells/mouse (Fig. 3(a)). The highest average cell yield per mouse was accomplished for the second harvest of microglia (in average of 2.2×10^5 cells/mouse; Fig. S2 (a)). Purification by MACS MicroBeads had a significant effect on the second microglial harvest per mouse with a reduction from an average of 2.2×10^5 to 1.5×10^5 cells/mouse (Fig. S2(a)). Similar tendencies were shown for the fourth microglial harvest (not significant). MACS purification also had a significant impact on astrocyte yields (Fig. 3(b); S2(b)). In detail, total astrocyte yields dropped from 3.4×10^7 to 3.9×10^6 cells/mouse after MACS purification (Fig. 3(b)). Taken together, both total microglia and astrocyte yields were reduced by MACS purification.

3.3. Confirmation of viability and purity by FACS and FESEM

We assessed the viability and purity of purified microglia and astrocytes via FACS analysis. Figs. 4 and 5 exemplary show one representative FACS blot for non-purified as well as MACS-purified microglia (Fig. 4) and astrocytes (Fig. 5). Populations were identified as microglia, when they were positive for CD45, CD11b, or both markers (Table 1), and as astrocytes, when they expressed ACSA-2, GFAP, or both.

Evaluation of non-purified microglia (Fig. 4(a); Table 1) showed that MACS did not affect microglial cell viability. The mean value of live cells dropped negligibly from an average of 99.2% for non-purified microglia to 98.9% for MACS-purified microglia (Fig. 4(b); Table 1). Further analyses of the viable microglia revealed two main populations: An average of 74.7% of the cells were $CD45^-CD11b^+$, whereas 24.9% were $CD45^+CD11b^+$. Just a small population of an average of 0.3% were neither positive for CD45 nor CD11b as summarized in Table 1. This $CD45^-CD11b^-$ population was absent after MACS purification. Here, most of the microglial cells displayed a $CD45^-CD11b^+$ expression pattern (in average 95.9%), and a small fraction was positive for both CD45 and CD11b (in average 4.1%) (Table 1). About 100% of the cells were CD11b-positive and hence identified as (presumably) microglia. In summary, MACS did not affect microglial viability, but eliminated cells which neither expressed CD45 nor CD11b.

In contrast to this, astrocyte viability increased from an average of 97.8–99.0% after MACS purification (Fig. 5(a)-(b); Table 2). Of the non-purified astrocytes, more than half (in average 54.3%) of the cells expressed none of the astrocyte markers. An average of 28.5% of the cells expressed both markers, 12.2% were $ACSA\text{-}2^+GFAP^-$, and, on average, 5.0% solely expressed GFAP, as shown in Table 2. In contrast to this, an average of 75.6% of the purified astrocytes were $ACSA\text{-}2^+GFAP^+$, 22.7% $ACSA\text{-}2^+GFAP^-$, and 0.9% $ACSA\text{-}2^-GFAP^+$ (Fig. 5(b); Table 2). In comparison to the non-purified astrocytes (Fig. 5(a)), only a small population (in average 1.7%) of the purified astrocytes (Fig. 5(b)) were negative for both astrocyte markers ($ACSA\text{-}2^-GFAP^-$), indicating that an average of 99.2% of the cells were either positive for one or both astrocyte markers, respectively (Table 2). In the next step, the MACS-purified $ACSA\text{-}2^-GFAP^-$ population was sub-gated for expression of the pan-myeloid marker CD11b and the common leukocyte marker CD45 to unravel the proportion of residual microglia contamination in astrocyte cultures (Fig. 5(a)-(b); Table 3). This revealed that in total 88% of the $ACSA\text{-}2^-GFAP^-$ population of the non-purified astrocyte culture was either positive for CD11b or both CD45 and CD11b. After MACS purification, almost all of the $ACSA\text{-}2^-GFAP^-$ cells, accounting for 1.7% of the total cell population, were also negative for both microglia markers CD45 and CD11b (in average 98.4%; Table 3). Only a small fraction (in average 1.5%) was identified as $CD45^-CD11b^+$ cells (Table 3). Overall, MACS slightly improved astrocyte viability and led to a notably improved purity, whereas a remarkable high proportion of residual

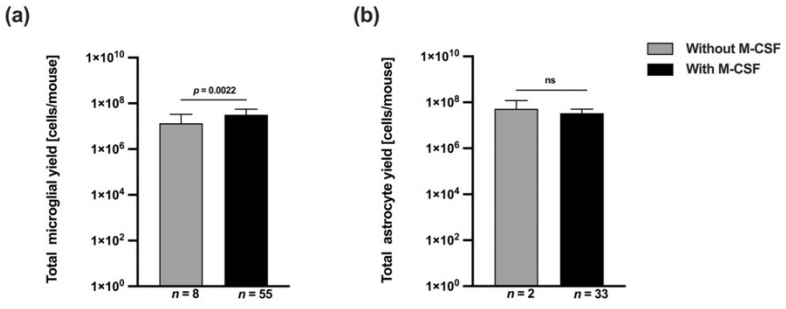


Fig. 2. Stimulation of a mixed murine primary glial culture with M-CSF promotes proliferation of microglia, but not astrocyte proliferation. Glial cells were isolated from 0 to 2-day-old neonatal mice (C57BL/6 n). Mixed primary glial cultures were either stimulated with M-CSF or were left untreated. Supernatants derived from the fibroblast cell line L-929, containing M-CSF, were added 10 days after brain tissue preparation, and after every harvest. Total cell yields per mouse were determined for microglia (a) and astrocytes (b). Results are expressed as mean + SD. Mann-Whitney test was applied to calculate statistical significances as indicated; a p-value > 0.05 is indicated as not significant (ns).

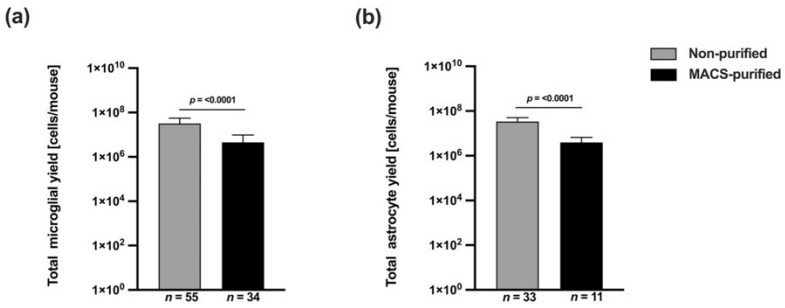


Fig. 3. Total cell yields of microglia and astrocytes were significantly reduced by MACS purification. Glial cells were isolated from 0 to 2-day-old neonatal mice (C57BL/6 n), and mixed primary glial cultures were stimulated with M-CSF derived from fibroblasts' supernatant, as described above. Purification was performed with MACS MicroBeads for microglia (anti-CD11b) and astrocytes (anti-ASCA-2). Total cell yields per mouse were determined for microglia (a) and astrocytes (b). Results are expressed as mean with + SD. Mann-Whitney test was applied to calculate statistical significances as indicated; a p-value > 0.05 is indicated as not significant (ns).

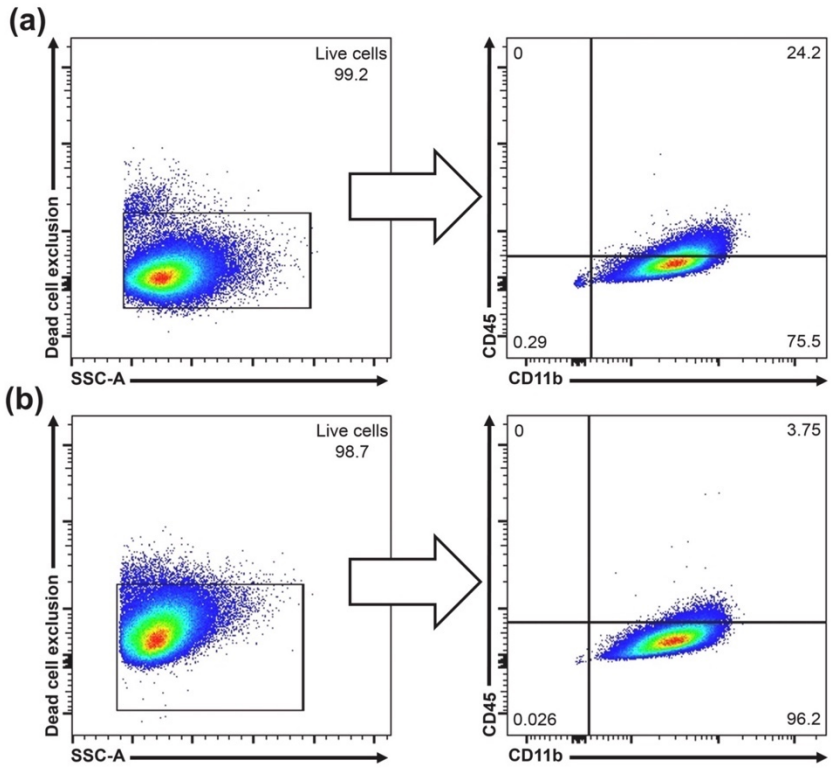


Fig. 4. Characterization of non-purified and MACS-purified microglia. Glial cells derived from 0 to 2-day-old mice (C57BL/6 n) were isolated. Non-purified cells (a) and MACS-purified cells (3×10^5) using MicroBeads (anti-CD11b, (b)) were analyzed by FACS. The single cells selected previously (see Fig. S3) were gated on pan-myeloid marker CD11b, as well as the common leukocyte marker CD45. Non-purified ($n = 6$) and MACS-purified ($n = 8$) microglia were isolated from 2 different litters.

microglia was examined without MACS purification prior to FACS analysis.

As summarized in Tables 1 and 2, the viability of both glial cell types was not decreased by MACS purification. This method guaranteed an average viability of approximately 98.9% for microglia and 99.0% for astrocytes. The purity of microglia was considerably high, irrespective of purification, yet MACS yielded slightly higher purity (100.0%) than non-purified microglial cultures (99.6%). In contrast to this, MACS dramatically improved the purity of astrocytes from 45.7% (without MACS) to 99.2% (with MACS).

In addition to this, FESEM confirmed the viability of the primary microglia and astrocytes generated with our novel method (Fig. 6): The microscopy images show that cell integrity of both microglia and astrocytes is still preserved after MACS purification. Both cell walls and processes were still intact and did not show any signs of perforation or disruption. Morphological differences between microglia derived from the first and second harvest were not observed.

3.4. Cytokine expression of non-purified and MACS-purified microglia and astrocytes upon TLR activation

To analyze the impact of our novel protocol on the cytokine expression of microglia and astrocytes, we measured the protein expression of a pro-inflammatory (TNF) and an anti-inflammatory (IL-10) cytokine by ELISA. To this end, non-purified, as well as MACS-purified microglia (Fig. 7 (a), (c)) and astrocytes (Fig. 7 (b), (d)) were stimulated either with the TLR2 ligand Pam3CSK4 or the TLR9 ligand CpG ODN for 24 h.

Cytokine concentrations are summarized in Tables 4 and 5 as means plus standard deviations (+ SD) of at least two representative ELISA analyses. Non-purified microglia and astrocytes produced significantly more TNF than their respective purified counterpart (Fig. 7(a)-(b);

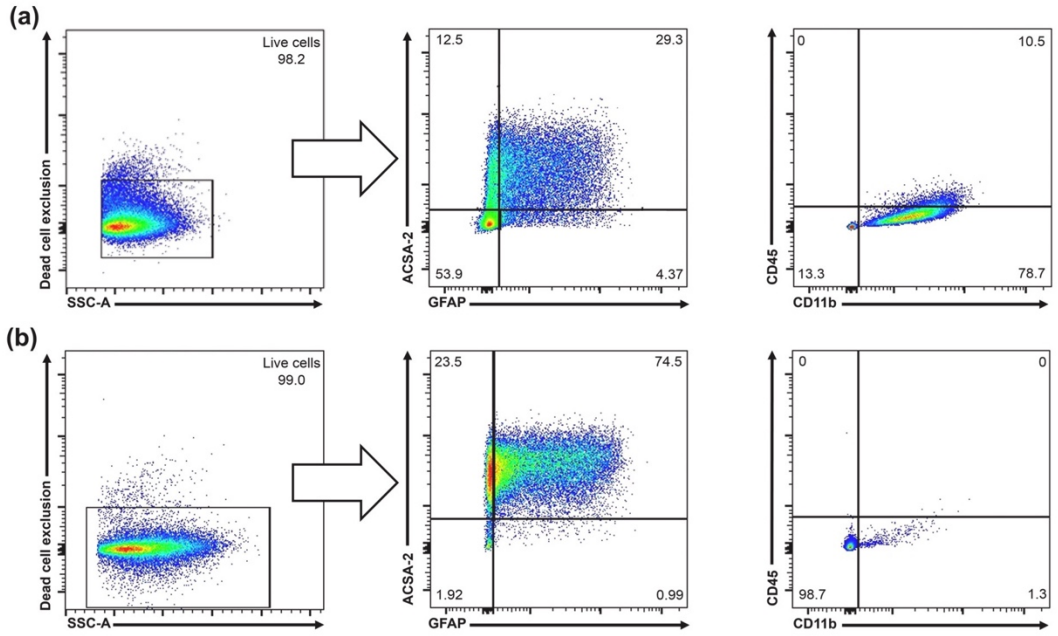


Fig. 5. Characterization of non-purified and MACS-purified astrocytes. The isolation of glial cells from 0 to 2-day-old neonatal mice (C57BL/6 n) was conducted as stated earlier. FACS of 3×10^5 non-purified (a) or MACS-purified (b) astrocytes using anti-ACSA-2 MicroBeads was performed. The single cells selected previously (Fig. S3) were gated on the astrocytes markers ACSA-2 and GFAP. The ACSA-2$^-$GFAP$^-$ population was additionally sub-gated on CD11b, as well as CD45. Non-purified ($n = 8$) and MACS-purified ($n = 8$) astrocytes derived from 2 different litters.

Table 1
Cellular subpopulations of non-purified and MACS-purified microglia. Glial cells were isolated from 0 to 2-day-old neonatal mice (C57BL/6 n), as described earlier. Microglia were MACS-purified using anti-CD11b MicroBeads detecting microglia, and analyzed via FACS with respect to the identification of cellular subpopulations according to their marker profile. Cells expressing CD45, CD11b, or both markers were considered as "microglia". Representation of 3 technical replicates as mean values in percentage [%] of 3 samples from one non-purified and one MACS-purified microglial culture, respectively.

Mean [%]	Live cells	Microglia				Others
		CD45$^+$CD11b$^-$	CD45$^+$CD11b$^+$	CD45$^-$CD11b$^+$	Total	CD45$^-$CD11b$^-$
Non-purified	99.2	0	24.9	74.7	**99.6**	0.3
MACS-purified	98.9	0	4.1	95.9	**100.0**	0

Table 2
Marker expression of non-purified and MACS-purified astrocytes. Glial cells were isolated from 0 to 2-day-old neonatal mice (C57BL/6 n). MACS purification was performed with MACS MicroBeads detecting astrocytes (anti-ASCA-2), applied for FACS studies, and analyzed as described above. Cells being positive for ACSA-2, GFAP, or both markers were considered as "astrocytes". Representation of 3 technical replicates as mean values in percentage [%] of 3 samples from one non-purified and one MACS-purified astrocyte culture, respectively.

Mean [%]	Live cells	Astrocytes				Others
		ACSA-2$^+$GFAP$^-$	ACSA-2$^+$GFAP$^+$	ACSA-2$^-$GFAP$^+$	Total	ACSA-2$^-$GFAP$^-$
Non-purified	97.8	12.2	28.5	5.0	**45.7**	54.3
MACS-purified	99.0	22.7	75.6	0.9	**99.2**	1.7

Table 3
Sub-gating of the ACSA-2$^-$GFAP$^-$ cell population. The remaining non-astrocyte population without (non-purified) and following (MACS-purified) anti-ACSA-2 MicroBead selection was sub-gated depending on CD45 and CD11b expression, considered as potentially contaminating microglia. CD45$^+$, CD11b$^+$, and cells positive for both markers were considered as potentially remaining microglia within the ACSA-2$^-$GFAP$^-$ population. Representation of 3 technical replicates as mean values in percentage [%] 3 samples from one non-purified and one MACS-purified astrocyte culture, respectively.

Mean [%]	ACSA-2$^-$GFAP$^-$				
	CD45$^+$CD11b$^-$	CD45$^+$CD11b$^+$	CD45$^-$CD11b$^+$	Total Microglia	CD45$^-$CD11b$^-$
Non-purified	0	10.2	77.8	**88.0**	12.1
MACS-purified	0	0	1.5	**1.5**	98.4

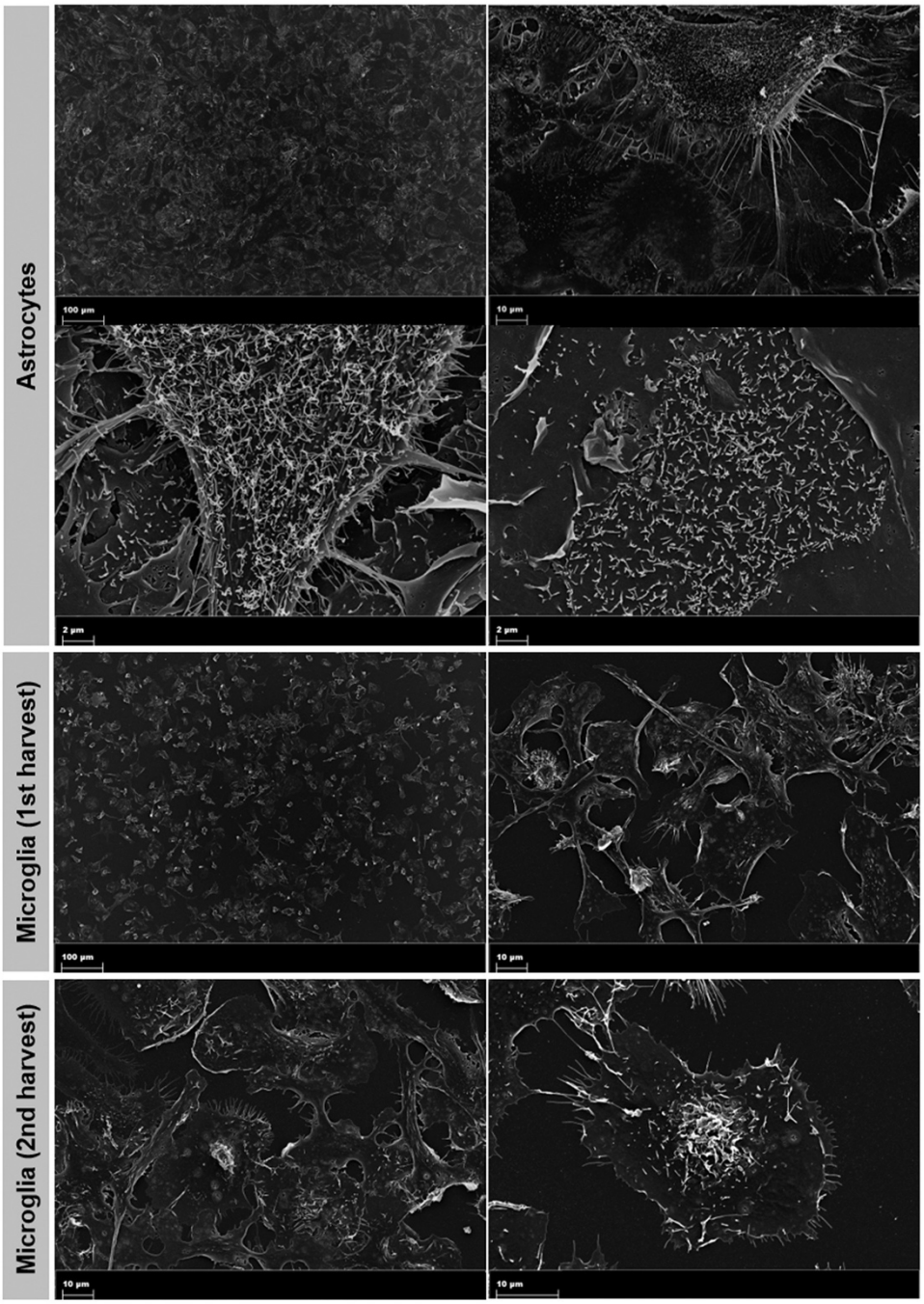


Fig. 6. Morphological integrity of microglia and astrocytes. Cells were generated with the novel glia isolation method, as assessed by FESEM. Microglia and astrocytes were isolated and MACS-purified as stated earlier, and seeded into 24-well plates (2×10^5/ml). Cells were fixed with paraformaldehyde and glutaraldehyde (see *Methods*). Images depict purified astrocytes, purified microglia derived from the first, and microglia from the second harvest.

Tables 4–5). Stimulation by Pam3CSK4 achieved TNF concentrations of up to 4709 (+ SD 250) pg/ml in non-purified microglia (Table 4). MACS significantly reduced the release of the pro-inflammatory cytokine to 1667 (+ SD 415) pg/ml. Stimulation with CpG ODN of non-purified microglia led to a TNF release of 2905 (+ SD 799) pg/ml on average, whereas purified microglia released 1193 pg/ml (+ SD 888) TNF. TLR2 stimulation triggered a significantly decreased TNF release from 361 (+ SD 145) pg/ml in non-purified to 26 (+ SD 13) pg/ml in MACS-purified

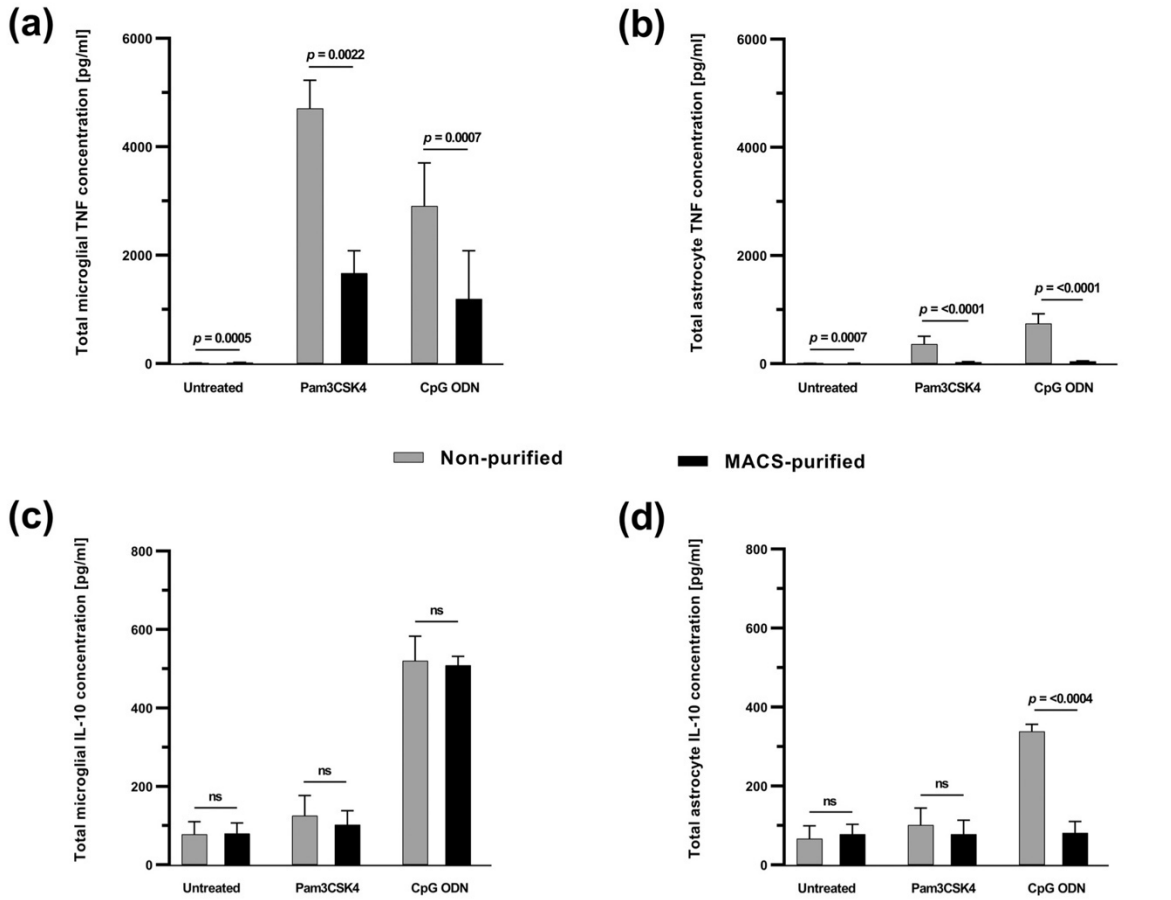


Fig. 7. Non-purified microglia and astrocytes expressed more TNF than the respective MACS-purified cell population. Isolated microglia and astrocytes derived from 0 to 2-day-old neonatal mice (C57BL/6 n) were either MACS-purified or non-purified, and seeded into 24-well plates (2×10^5/ml). Subsequently, cells were incubated with Pam3CSK4 (100 ng/ml), CpG ODN (1 μM), or were left untreated (control). Medium served as negative control (untreated). Supernatants were collected 24 h after stimulation and analyzed for TNF (a)-(b) and IL-10 (c)-(d) by sandwich ELISA. Results are expressed as mean + SD. Mann-Whitney test was applied of means of triplicates, derived from different harvests from biological duplicates, which were performed in at least 2 representative ELISA analyses. Statistical significances as indicated; *p*-values > 0.05 are indicated as not significant, ns. Microglia and astrocytes derived from 7 different litters in total, each consisting of 10–14 pups.

Table 4
TNF and IL-10 protein expression of non-purified and MACS-purified microglia. Glial cells were isolated from 0 to 2-day-old neonatal mice (C57BL/6 n). Microglia were either MACS-purified by anti-CD11b MicroBeads or were left unpurified, and seeded into 24-well plates (2×10^5/ml). Cells were stimulated with Pam3CSK4 (100 ng/ml), CpG ODN (1 μM), or were left untreated (-). Supernatants were collected after 24 h and analyzed for TNF and IL-10 by sandwich ELISA. Cytokine concentrations are represented as means of triplicates + SD, derived from different harvests from biological duplicates, which were performed in at least two representative ELISA analyses.

Mean [%]	Non-purified microglia			MACS-purified microglia		
Stimulation	–	Pam3CSK4	CpG ODN	–	Pam3CSK4	CpG ODN
TNF [pg/ml]	9 (+ SD 6)	4709 (+ SD 520)	2905 (+ SD 799)	16 (+ SD 8)	1667 (+ SD 415)	1193 (+ SD 888)
IL-10 [pg/ml]	77 (+ SD 33)	126 (+ SD 51)	520 (+ SD 63)	80 (+ SD 27)	102 (+ SD 36)	509 (+ SD 23)

Table 5
TNF and IL-10 protein expression of non-purified and MACS-purified astrocytes. Glial cells were isolated from 0 to 2-day-old neonatal mice (C57BL/6 n). Astrocytes were either MACS-purified by anti-ACSA-2 MicroBeads or were left unpurified, and seeded into 24-well plates (2×10^5/ml). Cells were stimulated with Pam3CSK4 (100 ng/ml), CpG ODN (1 μM), or were left untreated (-). Supernatants were collected after 24 h and analyzed for TNF and IL-10 by sandwich ELISA. Cytokine concentrations are represented as means of triplicates + SD, derived from different harvests from biological duplicates, which were performed in at least two representative ELISA analyses.

Mean [%]	Non-purified astrocytes			MACS-purified astrocytes		
Stimulation	-	Pam3CSK4	CpG ODN	-	Pam3CSK4	CpG ODN
TNF [pg/ml]	7 (+ SD 2)	361 (+ SD 145)	741 (+ SD 180)	4 (+ SD 2)	26 (+ SD 13)	44 (+ SD 8)
IL-10 [pg/ml]	67 (+ SD 32)	101 (+ SD 43)	339 (+ SD 18)	78 (+ SD 25)	78 (+ SD 36)	81 (+ SD 30)

astrocytes. Likewise, TNF expression in astrocytes after purification by MACS upon TLR9 stimulation was affected, as non-purified astrocytes released 741 (+ SD 180) pg/ml, while MicroBead-purified cells released only 44 (+ SD 8) pg/ml (Fig. 7(b); Table 4). In general, TNF production was higher in microglia (Fig. 7(a); Table 3) compared to astrocyte cultures (Fig. 7(b); Table 4).

Stimulation with the TLR ligands revealed a different impact of MACS on IL-10 production in microglia and astrocytes (Fig. 7(c)-(d)). In detail, MACS purification of microglia did not significantly reduce their IL-10 release. Similar results were assessed for CpG ODN-stimulated microglia. Here, IL-10 concentration ranged from 520 (+ SD 63) pg/ml (non-purified) to 509 (+ SD 23) pg/ml in MACS-purified microglial cultures. In astrocytes, only CpG ODN-treated, non-purified cells produced notable amounts of IL-10 (339 (+ SD 18) pg/ml) compared to the unstimulated control, which was significantly reduced by MACS purification (81 (+ SD 30) pg/ml) (Fig. 7(d); Table 4).

Taken together, microglia and astrocytes generated with our novel method are applicable for TLR-activation experiments, releasing pro- as well as anti-inflammatory cytokines. The process of purification affected the resulting immune response mediated by microglia and astrocytes, as cytokine expression in non-purified microglia and astrocytes was elevated compared to the respective purified cells. In particular, MACS-purified astrocyte cultures showed a significantly higher cytokine release, which was no longer detectable after MACS purification. TNF-release upon TLR2 and TLR9 stimulation was higher in comparison to the IL-10 production by microglia and astrocytes.

4. Discussion

Over the last decades, various glial cell isolation techniques have been published to facilitate their characterization in brain function, CNS infections, and immune responses. Nevertheless, there has been a lack of *in vitro* methods achieving the isolation of highly viable and pure microglia and astrocytes to study their crosstalk on a cell-specific level. Hence, the first aim of this study was to establish a protocol filling this gap and optimizing future studies on glial cell interaction (Fig. 1). In a second step, we validated our new method and determined the response of purified glial cells to TLR stimulation to unravel the contribution of an individual glial cell type, i.e. microglia and astrocytes in our case, to pro- and anti-inflammatory cytokine responses.

As expected, stimulation with M-CSF had a positive effect on microglial proliferation and differentiation, and in turn on yields of harvested microglia (Elmore et al., 2014; Ginhoux et al., 2010; Hagemeyer et al., 2017; Zuiderwijk-Sick et al., 2007). When comparing our results to other studies, one needs to keep in mind, that the effectiveness of the glial isolation procedures depends on the brain regions used (Batiuk et al., 2020; Li et al., 2019; Savchenko et al., 1997). Isolation from whole brains, multiple harvests from the same mixed glial cell culture, and the addition of M-CSF were key factors combined in our optimized protocol to maximize yields of microglia. In turn, this procedure is beneficial in terms of the animal testing principles of the 3Rs (Kirk, 2018), as less mice have to be sacrificed.

In the next step, we established a modified MACS protocol to purify M-CSF-stimulated microglia and astrocytes after every harvest. Performing MACS purification with CD11b and ACSA-2 MicroBeads specific for CD11b expressed by microglia and ACSA-2 by astrocytes, we were able to enhance the purity of the cells. This was validated by FACS analysis, showing that CD11b-negative cells, still present in non-purified microglial cultures, were eliminated by MACS purification (Fig. 4; Table 1). The effect of MACS purification on astrocytes was even more striking (Fig. 3(b); S2(b)) as in purified cultures, the total amount of ACSA-2-negative cells was reduced from an average of 59.3% to only 2.6% (Table 2). It is possible that microglial cells located below or between the astrocyte layers were not entirely eliminated by shaking the flasks (Saura et al., 2003). Proliferation of microglia can be prevented by the addition of different reagents, such as the anti-mitotic agent cytosine arabinoside (Ara-C) (Holm et al., 2012). We did not use reagents that kill or inhibit glial cells, since Ara-C has been previously shown to have profound effects on the function and fate of astrocytes, even at low concentrations, which also involve morphological changes and an increased GFAP level due to cellular activation (Ahlemeyer et al., 2003; Patel et al., 1988).

FACS partly revealed residual contaminating cell populations before and after MACS and helped to demonstrate the efficiency of the novel method with respect to purity of the glial yields (Figs. 4–5). As summarized in Table 1, non-purified microglial populations, were already highly pure, even without selection through MicroBeads, and purity levels barely improved after MACS. Purification affected microglial viability only slightly (Table 1). This suggests that the developed method is already suitable for cell-specific applications. Other protocols, such as Georgieva and colleagues published, have the major advantage of isolating > 96% pure microglia from a mixed glial culture after only 4 days of incubation. Yet, we need to stress that protocols like this did not manage isolating microglia and astrocytes from the same mixed glial culture with comparable viability and purity (Georgieva et al., 2018). Furthermore, our results clearly indicate that even slightest contaminations with other cell types might skew the immune response of the glial cultures. Consequently, a higher purity is indispensable to study cell type-specific properties in future approaches. Since we radically modified the MACS MicroBeads manufacturer's standard protocol, we cannot directly compare our results with the existing literature. In comparison to our highly pure yields of microglia, microglial fractions that were conventionally isolated by following the original CD11b MACS Miltenyi Biotec protocol with minor modifications, exhibited final purities of only ~95–97% (Crain et al., 2009; Harms and Tansey, 2013). Most of the MACS-purified microglial cultures consisted of $CD45^{-}CD11b^{+}$ cells and a small fraction of $CD45^{+}CD11b^{+}$ cells. These two distinct cell populations differing in their CD45 expression level were also observed in other studies (Bedi et al., 2013; Carson et al., 1998; Hickman et al., 2013; Nikodemova and Watters, 2012; Rangaraju et al., 2018). Many myeloid cell types share overlapping surface marker profiles, making it challenging to distinguish them, especially to discriminate microglia from monocytes. Therefore, we chose to use the CD11b MicroBeads from Miltenyi Biotec, which were specifically designated for the positive isolation of microglia from murine brain tissue and not Miltenyi Biotec's CD11b MicroBeads selecting murine CD11b expressing monocytes/macrophages from cell suspensions of spleen, lymph node, peritoneal cavity, liver, muscle tissue, or blood. High CD45 expression is rather characteristic for blood monocytes than for microglia (Jeong et al., 2010). However, the CD45 expression of microglia depends on the pathological state, i.e. CNS injury and/or disease (Bennett et al., 2016; Bohlen et al., 2019). Upon infection or injury, monocytes and neutrophils expressing CD11b can enter the CNS via the blood-brain barrier (BBB), whereas under physiological conditions, only microglia express CD11b (Jeong et al., 2013). Microglia are able to switch from a pro-inflammatory to a neuroprotective phenotype (Pierre et al., 2017; Timmerman et al., 2018). In an activated state, microglia upregulate CD11b and CD45 expression (Schroeter et al., 2021; Timmerman et al., 2018). Thus, since we isolated glial cells from intact brains, we assume that the major population of $CD45^{-}CD11b^{+}$ cells might have been microglia, while the minor $CD45^{+}CD11b^{+}$ population were considered as highly activated microglia (Benakis et al., 2014; Rangaraju et al., 2018; Schroeter et al., 2021; Wlodarczyk et al., 2014). However, since we did not perfuse the mice, it is also possible that the $CD45^{+}CD11b^{+}$ population comprises monocytes and/or monocyte-derived macrophages derived from blood (Benakis et al., 2014; Yamasaki et al., 2014; Yang et al., 2014). However, Nikodemova and Watters obtained microglia from perfused mice using anti-CD11b MicroBeads and, in line with our results, determined two CD11b-positive populations in the healthy brain, differing in their CD45 expression level (Nikodemova and Watters, 2012). Since these two $CD11b^{+}$ populations differing in their CD45 expression state were observed in perfused mice as well, we assume that these cell populations might be microglia within two different activation states, rather than blood-derived monocytes. However, further markers, such as lymphocyte antigen complex 6 C (Ly6C), are needed to assign the $CD45^{+}CD11b^{+}$ population to distinct cell types and confirm their identity as highly activated microglia (Crotti and Ransohoff, 2016; Lewis

et al., 2014). Volden and co-authors isolated microglia from a single-cell suspension derived from murine adult hypothalamus and cerebellum using a papain-based enzymatic digestion kit and subsequently identified two subpopulations via FACS as well. One population was $CD45^{+}CD11b^{+}$, presumably representing perivascular microglia, whereas another population was $CD45^{-}CD11b^{+}$, suggested being parenchymal microglia. These findings indicate that microglia derived from different brain regions can differ in their CD45 expression. However, we did not distinguish between different brain regions, as we used the entire brain of neonatal mice. Volden et al. also showed that microglia were not significantly activated by the used magnetic-based enrichment method based on the detection of minimal *Iba1* expression, an established microglial activation marker (Volden et al., 2015). In addition, they examined expression of CD11c, which is upregulated in 'primed' microglia before they get fully activated (Remington et al., 2007; Sato-Hashimoto et al., 2019; Volden et al., 2015; Wlodarczyk et al., 2014). Yet, one has to keep in mind that microglia cultivated in vitro over several weeks are exposed to microenvironmental conditions, which are not present in the healthy CNS (e.g. medium supplemented with serum) (Timmerman et al., 2018). Consequently, microglia can lose their signature genes like *Tmem119* or *P2ry12,* and resemble microglia under pathological brain conditions lacking an intact microenvironment (Bohlen et al., 2019, 2017; Timmerman et al., 2018).

The improvements of our newly developed glia isolation method mainly concern astrocytes (Fig. 5; Table 2). While the viability of the astrocyte population was just slightly increased by MACS, its purity was tremendously heightened (Table 2). Previous studies on the isolation of astrocytes yielded notably lower viabilities and purities. For instance, Batiuk and colleagues revealed an astrocyte' viability of only 86% and a FACS purity of > 95% of MACS-purified astrocytes isolated with minor modifications to Miltenyi Biotec's recommended protocol (Batiuk et al., 2017). These lower percentages of viability and purity might be due to the application of the Miltenyi Biotec papain-based enzymatic protocol for the dissociation of the brain tissues. The authors suggested that ACSA-2 binds to sodium/potassium-transporting ATPase subunit beta-1 (ATP1B), a single-pass transmembrane protein, which is presumably papain-sensitive (Batiuk et al., 2017). This strongly supports the importance of the non-enzymatic papain-free approach of our established method.

We chose to identify astrocytes based on the astrocyte markers ACSA-2 and GFAP, which is a well-established marker for mature, as well as reactive astrocytes (Eng et al., 2000; Zarei-Kheirabadi et al., 2020). Before MACS purification, more than half of the cells were neither positive for ACSA-2 nor for GFAP (Fig. 5(a); Table 2). A range of contaminating cell types can be present before purification via anti-ACSA-2 MicroBeads, for instance oligodendrocytes and neurons, as well as their precursor cells, endothelial cells, or microglia (Batiuk et al., 2017). The other three populations of the non-purified cells expressing at least one of the astrocyte markers are presumably astrocytes, which altogether only account for 45.7% of the whole population (Table 2). Other studies also found that murine primary astrocytes do not necessarily express GFAP due to resting subpopulations (Chandrasekaran et al., 2016). Further sub-gating of the non-purified $ACSA\text{-}2^{-}GFAP^{-}$ population revealed that most of the cells were CD11b-, or CD45- and CD11b-positive (Fig. 5(a); Table 3). These two cell populations might be microglia differing in their activation state, or other blood-derived cells, as discussed previously (Benakis et al., 2014; Rangaraju et al., 2018; Wlodarczyk et al., 2014; Yamasaki et al., 2014; Yang et al., 2014). A small fraction of the sub-gated $ACSA\text{-}2^{-}GFAP^{-}$ cells did not express neither one of the two microglial markers (Table 3). These cells, potentially representing further cell populations such as astrocyte sub-types, cannot be identified with our applied FACS markers. (Feldmann et al., 2014).

Following MACS purification, the viability and purity of the astrocyte fraction was determined again via FACS (Fig. 5(b); Table 2). Here, the proportions of the cell populations shifted, and in total, an average of 99.2% of the cells were identified as presumably astrocytes (Table 2). The remaining small population of $ACSA\text{-}2^{-}$ $GFAP^{-}$ cells after purification might be due to the unspecific aggregation of astrocytes to non-labeled cells. Non-myelinating Schwann cells, the glial cells of the peripheral nervous system (PNS), also express GFAP (Chen et al., 2021; Triolo et al., 2006). However, only under pathological conditions associated with demyelination, such as multiple sclerosis, Schwann cells have been detected in the CNS (Chen et al., 2021; Franklin et al., 2017; Itoyama et al., 1983). Sub-gating of the small $ACSA\text{-}2^{-}GFAP^{-}$ population of MACS-purified astrocytes revealed that almost all of these cells were negative for both CD45 and CD11b and only a fraction of 1.5% was identified as $CD45^{-}CD11b^{+}$ cells, likely representing residual microglia (Table 3). Although we used two astrocyte markers, it is possible that ACSA-2 and GFAP do not cover all subpopulations of astrocytes, since they are considered a very heterogenetic cell type (Feldmann et al., 2014; Westergard and Rothstein, 2020; Yu et al., 2020). There are other astrocytes markers, including S100 beta or GLAST (EAAT-1), which may be useful for a more precise FACS analysis of different astrocyte populations, and should hence be considered in future approaches (Feldmann et al., 2014).

TLRs are main drivers of pathogen-associated and endogenous ligand recognition in the CNS. The distinct role of TLRs in microglia and astrocytes is still at the forefront of glia research. To date, 13 *Tlr* genes have been identified, although the last three TLR proteins are not expressed in humans (Kawai and Akira, 2010; Takeuchi and Akira, 2010). Murine and human microglia, as well as mouse astrocytes, constitutively express *Tlr1–9* (Fiebich et al., 2018; Lehnardt, 2010; Li et al., 2021). TNF, known as the major cytokine released by microglia upon TLR stimulation, is produced abundantly in the first 24 h after receptor stimulation (Ebert et al., 2005; Jha et al., 2019; Lehmann et al., 2012; Rosenberger et al., 2014). To exclude activation of glia upon MACS purification, we determined cytokine release of microglia and astrocytes with and without purification. Hence, we investigated the production of the pro-inflammatory cytokine TNF and the anti-inflammatory cytokine IL-10, released after stimulation with the TLR2 ligand Pam3CSK4 or the TLR9 agonist CpG ODN (Fig. 7; Tables 4–5). Interestingly, non-purified microglia released significantly more TNF than MACS-purified microglia (Fig. 7(a); Table 4). Two explanations might be taken into account for this observation. First, highly activated microglia with a $CD45^{+}CD11b^{+}$ expression profile, which were found in higher abundances in the non-purified culture than in the one after MACS purification, are the main source of TNF in primary microglial cultures, as suggested earlier (Brás et al., 2020). Second, release of TNF by microglia might be promoted by signaling molecules derived from astrocytes during their constant bidirectional crosstalk. The increased TNF release in non-purified astrocytes further points to the importance of crosstalk between microglia and astrocytes for the release of cytokines (Fig. 7(b); Table 5). It is also conceivable that microglia as the main contaminating cells in the non-purified astrocyte cultures, account for the increased cytokine release as previously discussed (Benakis et al., 2014; Yamasaki et al., 2014; Yang et al., 2014) (Fig. 5(b); Table 3). This further highlights the need for protocols that help to isolate highly pure astrocyte fractions, being free of contaminating cells such as microglia. On one hand, the bidirectional communication between microglia and astrocytes plays an important role for the development and function of the CNS, as well as brain hemostasis. On the other hand, miscommunication between these cells can lead to neuroinflammation (Bernaus et al., 2020; Jha et al., 2019; Liu et al., 2020; Matejuk and Ransohoff, 2020). There has been emerging evidence that contaminating microglia in primary astrocytes cultures are responsible for the observed major cytokine response of astrocytes (Crocker et al., 2008; Lange et al., 2012; Li et al., 2021; Saura, 2007; Sola et al., 2002). Chen et al. demonstrated that astrocytes alone fail to release sufficient TNF and nitric oxide (NO) upon lipopolysaccharide (LPS) stimulation, whereas in the presence of as low as 0.5–1% microglia, they succeed to produce adequate levels of these pro-inflammatory

factors (Chen et al., 2015). Izumi et al. stimulated pure primary murine astrocytes, as well as astrocyte cultures contaminated with microglia, with LPS and observed a significant increase in the cytokine response in the latter (Iizumi et al., 2016). Holm and colleagues observed that the response of astrocytes to endogenous ligands of TLR2, 3 and 4 depends on the presence of microglia. Thereby, LPS treatment led to the release of soluble mediators such as CC-chemokine ligand 2 (CCL2), which in turn activated or facilitated the astrocyte response (Holm et al., 2012). Shinozaki et al. observed that microglia-derived cytokines can determine the fate of the astrocyte phenotype. They showed *in vitro* and *in vivo* that microglia release a cytokine mixture (IL-6, IL-1β, TNF) in response to traumatic brain injury. In turn, these cytokines induced a neuroprotective phenotype and reactive astrogliosis by downregulating the $P2Y_1$ receptor in astrocytes (Shinozaki et al., 2017). Liddelow and colleagues also stated that LPS-activated microglia can induce a switch in astrocytes from the neurotrophic phenotype into a neurotoxic state by the secretion of IL-1α, TNF, and complement component 1, subcomponent q (C1q) *in vitro* (Liddelow et al., 2017).

Stimulation with the synthetic TLR2 ligand Pam3CSK4 did not evoke a high IL-10 response in neither microglia nor astrocytes. This indicates that IL-10 release by microglia and astrocytes is independent of TLR2 (Fig. 7(c)-(d)). For further confirmation, this experiment could be repeated with different TLR2 ligands, such as zymosan from yeast cell wall, or with bacterial cell wall components such as peptidoglycan or lipoteichoic acid (LTA), since TLR2 binds the widest range of PAMPs (Lehnardt, 2010). However, treatment with the TLR9 agonist CpG ODN significantly reduced the IL-10 release in non-purified astrocytes (Fig. 7 (d); Table 5), but not in microglia (Fig. 7(c); Table 4). It is possible that microglia do not depend on astrocytes for IL-10 expression upon TLR9 stimulation, whereas vice versa the presence of microglia is crucial for astrocytes to release IL-10 upon CpG ODN stimulation. It follows that contaminating microglia account for the significantly increased IL-10 release in the non-purified astrocyte culture (Fig. 7(d); 5(b); Table 3).

In summary, our newly developed protocol is suitable for culturing and isolating highly viable and pure primary murine neonatal microglia and astrocytes, which are qualified for various downstream cell-specific applications. This might be an essential milestone, especially with respect to the cultivation of primary microglia-free astrocytes. These viable and pure glial cell cultures now allow the precise investigation of the crosstalk between microglia and astrocytes, as well as the co-culturing of glia derived from mice with different genetic backgrounds. Furthermore, the novel method might also be of potential interest when studying CNS diseases affecting distinct brain regions to different extents, like various forms of epilepsy or Alzheimer's disease. In these animal disease models, glia from different brain compartments require separate isolation, most likely accompanied by increasing animal numbers to obtain sufficient glial cell yields.

CRediT authorship contribution statement

Laura Zelenka: Investigation, Methodology, Visualization, Writing – original draft. **Dennis Pägelow:** Investigation, Conceptualization, Methodology, Supervision, Funding acquisition. **Christina Krüger:** Methodology. **Jana Seele:** Methodology. **Friederike Ebner:** Formal analysis, Resources. **Sebastian Rausch:** Formal analysis, Resources. **Manfred Rohde:** Formal analysis, Visualization. **Seija Lehnardt:** Writing – review & editing, Project administration, **Kira van Vorst:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Marcus Fulde:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

M.F. and K.v.V. were supported by the German Federal Ministry of Education and Research (BMBF) within the consortium InfectControl 2020 (Project NeoBiom, grant ID 03ZZ0829C). M.F., K.v.V. and D.P. were supported by the national platform for zoonosis research (Project NAMPatIn, grant ID 01KI1906). M.F. received support by the Freie Universität Berlin within the Excellence Initiative of the German Research Foundation. We thank Ina Schleicher (Department of Medical Microbiology, Helmholtz Center for Infection Research, Braunschweig, Germany) for excellent technical support.

Author Contributions

L.Z., D.P., K.v.V. and M.F. planned, designed, conducted and analyzed the glial cell experiments. L.Z., D.P., C.K., J.S. and S.L. established the isolation and purification protocol. M.R. conducted electron microscopic analysis. D.P., F.E. and S.R. analyzed and evaluated FACS data sets. S.L. provided technical support. D.P., K.v.V. and M.F. supervised the study and L.Z., K.v.V. and M.F. wrote the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2021.109420.

References

Ahlemeyer, B., Kolker, S., Zhu, Y., Hoffmann, G.F., Krieglstein, J., 2003. Cytosine arabinofuranoside-induced activation of astrocytes increases the susceptibility of neurons to glutamate due to the release of soluble factors. Neurochem. Int. 42, 567–581. https://doi.org/10.1016/s0197-0186(02)00164-x.

Allen, N.J., Lyons, D.A., 2018. Glia as architects of central nervous system formation and function. Science 362, 181–185. https://doi.org/10.1126/science.aat0473.

Barres, B.A., 2014. Designing and troubleshooting immunopanning protocols for purifying neural cells. Cold Spring Harb. Protoc. 2014, 1342–1347. https://doi.org/10.1101/pdb.ip073999.

Batiuk, M.Y., de Vin, F., Duque, S.I., Li, C., Saito, T., Saido, T., Fiers, M., Belgard, T.G., Holt, M.G., 2017. An immunoaffinity-based method for isolating ultrapure adult astrocytes based on ATP1B2 targeting by the ACSA-2 antibody. J. Biol. Chem. 292, 8874–8891. https://doi.org/10.1074/jbc.M116.765313.

Batiuk, M.Y., Martirosyan, A., Wahis, J., de Vin, F., Marneffe, C., Kusserow, C., Koeppen, J., Viana, J.F., Oliveira, J.F., Voet, T., Ponting, C.P., Belgard, T.G., Holt, M. G., 2020. Identification of region-specific astrocyte subtypes at single cell resolution. Nat. Commun. 11 (1220), 8. https://doi.org/10.1038/s41467-019-14198-.

Bedi, S.S., Smith, P., Hetz, R.A., Xue, H., Cox, C.S., 2013. Immunomagnetic enrichment and flow cytometric characterization of mouse microglia. J. Neurosci. Methods 219, 176–182. https://doi.org/10.1016/j.jneumeth.2013.07.017.

Benakis, C., Garcia-Bonilla, L., Iadecola, C., Anrather, J., 2014. The role of microglia and myeloid immune cells in acute cerebral ischemia. Front. Cell. Neurosci. 8, 461. https://doi.org/10.3389/fncel.2014.00461.

Bennett, M.L., Bennett, F.C., Liddelow, S.A., Ajami, B., Zamanian, J.L., Fernhoff, N.B., Mulinyawe, S.B., Bohlen, C.J., Adil, A., Tucker, A., Weissman, I.L., Chang, E.F., Li, G., Grant, G.A., Hayden Gephart, M.G., Barres, B.A., 2016. New tools for studying microglia in the mouse and human CNS. Proc. Natl. Acad. Sci. U.S.A. 113, E1738–E1746. https://doi.org/10.1073/pnas.1525528113.

Bernaus, A., Blanco, S., Sevilla, A., 2020. Glia crosstalk in neuroinflammatory diseases. Front. Cell. Neurosci. 14, 209. https://doi.org/10.3389/fncel.2020.00209.

Bohlen, C.J., Bennett, F.C., Bennett, M.L., 2019. Isolation and culture of microglia. Curr. Protoc. Immunol. 125, e70 https://doi.org/10.1002/cpim.70.

Bohlen, C.J., Bennett, F.C., Tucker, A.F., Collins, H.Y., Mulinyawe, S.B., Barres, B.A., 2017. Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures. Neuron 94 (759–73), e8. https://doi.org/10.1016/j.neuron.2017.04.043.

Booher, J., Sensenbrenner, M., 1972. Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. Neurobiology 2, 97–105.

Brás, J.P., Bravo, J., Freitas, J., Barbosa, M.A., Santos, S.G., Summavielle, T., Almeida, M. I., 2020. TNF-alpha-induced microglia activation requires miR-342: impact on NF-kB signaling and neurotoxicity. Cell Death Dis. 11, 415. https://doi.org/10.1038/s41419-020-2626-6.

Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., Zamanian, J.L., Christopherson, K.S., Xing, Y., Lubischer, J.L., Krieg, P.A., Krupenko, S.A., Thompson, W.J., Barres, B.A., 2008. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new

resource for understanding brain development and function. J. Neurosci. 28, 264–278. https://doi.org/10.1523/JNEUROSCI.4178-07.2008.
Cardona, A.E., Huang, D., Sasse, M.E., Ransohoff, R.M., 2006. Isolation of murine microglial cells for RNA analysis or flow cytometry. Nat. Protoc. 1, 1947–1951. https://doi.org/10.1038/nprot.2006.327.
Carson, M.J., Reilly, C.R., Sutcliffe, J.G., Lo, D., 1998. Mature microglia resemble immature antigen-presenting cells. Glia 22, 72–85.
Chandrasekaran, A., Avci, H.X., Leist, M., Kobolak, J., Dinnyes, A., 2016. Astrocyte differentiation of human pluripotent stem cells: new tools for neurological disorder research. Front. Cell. Neurosci. 10, 215. https://doi.org/10.3389/fncel.2016.00215.
Chen, C.Z., Neumann, B., Forster, S., Franklin, R.J.M., 2021. Schwann cell remyelination of the central nervous system: why does it happen and what are the benefits? Open Biol. 11, 200352 https://doi.org/10.1098/rsob.200352.
Chen, S.H., Oyarzabal, E.A., Sung, Y.F., Chu, C.H., Wang, Q., Chen, S.L., Lu, R.B., Hong, J.S., 2015. Microglial regulation of immunological and neuroprotective functions of astroglia. Glia 63, 118–131. https://doi.org/10.1002/glia.22738.
Chew, L.J., DeBoy, C.A., Senatorov Jr., V.V., 2014. Finding degrees of separation: experimental approaches for astroglial and oligodendroglial cell isolation and genetic targeting. J. Neurosci. Methods 236, 125–147. https://doi.org/10.1016/j.jneumeth.2014.08.017.
Crain, J.M., Nikodemova, M., Watters, J.J., 2009. Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia. J. Neuroinflamm. 6, 24. https://doi.org/10.1186/1742-2094-6-24.
Crocker, S.J., Frausto, R.F., Whitton, J.L., Milner, R., 2008. A novel method to establish microglia-free astrocyte cultures: comparison of matrix metalloproteinase expression profiles in pure cultures of astrocytes and microglia. Glia 56, 1187–1198. https://doi.org/10.1002/glia.20689.
Crotti, A., Ransohoff, R.M., 2016. Microglial physiology and pathophysiology: insights from genome-wide transcriptional profiling. Immunity 44, 505–515. https://doi.org/10.1016/j.immuni.2016.02.013.
Ebert, S., Gerber, J., Bader, S., Muhlhauser, F., Brechtel, K., Mitchell, T.J., Nau, R., 2005. Dose-dependent activation of microglial cells by Toll-like receptor agonists alone and in combination. J. Neuroimmunol. 159, 87–96. https://doi.org/10.1016/j.jneuroim.2004.10.005.
Elmore, M.R., Najafi, A.R., Koike, M.A., Dagher, N.N., Spangenberg, E.E., Rice, R.A., Kitazawa, M., Matusow, B., Nguyen, H., West, B.L., Green, K.N., 2014. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. Neuron 82, 380–397. https://doi.org/10.1016/j.neuron.2014.02.040.
Eng, L.F., Ghirnikar, R.S., Lee, Y.L., 2000. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem. Res. 25, 1439–1451. https://doi.org/10.1023/a:1007677003387.
Erny, D., Prinz, M., 2020. How microbiota shape microglial phenotypes and epigenetics. Glia. https://doi.org/10.1002/glia.23822.
Feldmann, M., Pathipati, P., Sheldon, R., Jiang, X., Ferriero, D., 2014. Isolating astrocytes and neurons sequentially from postnatal murine brains with a magnetic cell separation technique. J. Biol. Methods 1, 11. https://doi.org/10.14440/jbm.2014.33.
Fiebich, B.L., Batista, C.R.A., Saliba, S.W., Yousif, N.M., de Oliveira, A.C.P., 2018. Role of microglia TLRs in neurodegeneration. Front. Cell. Neurosci. 12, 329. https://doi.org/10.3389/fncel.2018.00329.
Foo, L.C., Allen, N.J., Bushong, E.A., Ventura, P.B., Chung, W.S., Zhou, L., Cahoy, J.D., Daneman, R., Zong, H., Ellisman, M.H., Barres, B.A., 2011. Development of a method for the purification and culture of rodent astrocytes. Neuron 71, 799–811. https://doi.org/10.1016/j.neuron.2011.07.022.
Franklin, R.J.M., Ffrench-Constant, C., Regenerating, C.N.S., 2017. myelin - from mechanisms to experimental medicines. Nat. Rev. Neurosci. 18, 753–769. https://doi.org/10.1038/nrn.2017.136.
Frederiksen, H.R., Haukedal, H., Freude, K., 2019. Cell type specific expression of toll-like receptors in human brains and implications in Alzheimer's disease. Biomed. Res. Int. 2019, 7420189. https://doi.org/10.1155/2019/7420189.
Georgieva, M., Leeson-Payne, A., Dumitrascuta, M., Rajnicek, A., Malcangio, M., Huang, W., 2018. A refined rat primary neonatal microglial culture method that reduces time, cost and animal use. J. Neurosci. Methods 304, 92–102. https://doi.org/10.1016/j.jneumeth.2018.04.017.
Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R., Samokhvalov, I.M., Merad, M., 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330, 841–845. https://doi.org/10.1126/science.1194637.
Giulian, D., Baker, T.J., 1986. Characterization of ameboid microglia isolated from developing mammalian brain. J. Neurosci. 6, 2163–2178.
Greenhalgh, A.D., David, S., Bennett, F.C., 2020. Immune cell regulation of glia during CNS injury and disease. Nat. Rev. Neurosci. 21, 139–152. https://doi.org/10.1038/s41583-020-0263-9.
Guez-Barber, D., Fanous, S., Harvey, B.K., Zhang, Y., Lehrmann, E., Becker, K.G., Picciotto, M.R., Hope, B.T., 2012. FACS purification of immunolabeled cell types from adult rat brain. J. Neurosci. Methods 203, 10–18. https://doi.org/10.1016/j.jneumeth.2011.08.045.
Guttenplan, K.A., Liddelow, S.A., 2019. Astrocytes and microglia: models and tools. J. Exp. Med. 216, 71–83. https://doi.org/10.1084/jem.20180200.
Hagemeyer, N., Hanft, K.M., Akriditou, M.A., Unger, N., Park, E.S., Stanley, E.R., Staszewski, O., Dimou, L., Prinz, M., 2017. Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood. Acta Neuropathol. 134, 441–458. https://doi.org/10.1007/s00401-017-1747-1.
Harms, A.S., Tansey, M.G., 2013. Isolation of murine postnatal brain microglia for phenotypic characterization using magnetic cell separation technology. Methods Mol. Biol. 1041, 33–39. https://doi.org/10.1007/978-1-62703-520-0_5.
Hickman, S.E., Kingery, N.D., Ohsumi, T.K., Borowsky, M.L., Wang, L.C., Means, T.K., El, Khoury, J., 2013. The microglial sensome revealed by direct RNA sequencing. Nat. Neurosci. 16, 1896–1905. https://doi.org/10.1038/nn.3554.
Holm, T.H., Draeby, D., Owens, T., 2012. Microglia are required for astroglial Toll-like receptor 4 response and for optimal TLR2 and TLR3 response. Glia 60, 630–638. https://doi.org/10.1002/glia.22296.
Holt, L.M., Olsen, M.L., 2016. Novel applications of magnetic cell sorting to analyze cell-type specific gene and protein expression in the central nervous system. PLoS One 11, e0150290. https://doi.org/10.1371/journal.pone.0150290.
Holt, L.M., Stoyanof, S.T., Olsen, M.L., 2019. Magnetic cell sorting for in vivo and in vitro astrocyte, neuron, and microglia analysis. Curr. Protoc. Neurosci. 88, e71 https://doi.org/10.1002/cpns.71.
Iizumi, T., Takahashi, S., Mashima, K., Minami, K., Izawa, Y., Abe, T., Hishiki, T., Suematsu, M., Kajimura, M., Suzuki, N., 2016. A possible role of microglia-derived nitric oxide by lipopolysaccharide in activation of astroglial pentose-phosphate pathway via the Keap1/Nrf2 system. J. Neuroinflamm. 13, 99. https://doi.org/10.1186/s12974-016-0564-0.
Itoyama, Y., Webster, H.D., Richardson Jr., E.P., Trapp, B.D., 1983. Schwann cell remyelination of demyelinated axons in spinal cord multiple sclerosis lesions. Ann. Neurol. 14, 339–346. https://doi.org/10.1002/ana.410140313.
Jafarzadeh, A., Nemati, M., Khorramdelazad, H., Mirshafiey, A., 2019. The toll-like receptor 2 (TLR2)-related immunopathological responses in the multiple sclerosis and experimental autoimmune encephalomyelitis. Iran. J. Allergy Asthma Immunol. 18, 230–250. https://doi.org/10.18502/ijaai.v18i3.1117.
Jeong, H.K., Ji, K., Min, K., Joe, E.H., 2013. Brain inflammation and microglia: facts and misconceptions. Exp. Neurobiol. 22, 59–67. https://doi.org/10.5607/en.2013.22.2.59.
Jeong, H.K., Ji, K.M., Kim, B., Kim, J., Jou, I., Joe, E.H., 2010. Inflammatory responses are not sufficient to cause delayed neuronal death in ATP-induced acute brain injury. PLoS One 5, e13756. https://doi.org/10.1371/journal.pone.0013756.
Jha, M.K., Jo, M., Kim, J.H., Suk, K., 2019. Microglia-astrocyte crosstalk: an intimate molecular conversation. Neuroscientist 25, 227–240. https://doi.org/10.1177/1073858418783959.
Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat. Immunol. 11, 373–384. https://doi.org/10.1038/ni.1863.
Kierdorf, K., Prinz, M., 2019. Microglia: Same, but different. J. Exp. Med. 216, 2223–2225. https://doi.org/10.1084/jem.20191535.
Kirk, R.G.W., 2018. Recovering the principles of humane experimental technique: the 3Rs and the human essence of animal research. Sci. Technol. Hum. Values 43, 622–648. https://doi.org/10.1177/0162243917726579.
Lange, S.C., Bak, L.K., Waagepetersen, H.S., Schousboe, A., Norenberg, M.D., 2012. Primary cultures of astrocytes: their value in understanding astrocytes in health and disease. Neurochem. Res. 37, 2569–2588. https://doi.org/10.1007/s11064-012-0868-0.
Lehmann, S.M., Kruger, C., Park, B., Derkow, K., Rosenberger, K., Baumgart, J., Trimbuch, T., Eom, G., Hinz, M., Kaul, D., Habbel, P., Kalin, R., Franzoni, E., Rybak, A., Nguyen, D., Veh, R., Ninnemann, O., Peters, O., Nitsch, R., Heppner, F.L., Golenbock, D., Schott, E., Ploegh, H.L., Wulczyn, F.G., Lehnardt, S., 2012. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat. Neurosci. 15, 827–835. https://doi.org/10.1038/nn.3113.
Lehnardt, S., 2010. Innate immunity and neuroinflammation in the CNS: the role of microglia in toll-like receptor-mediated neuronal injury. Glia 58, 253–263. https://doi.org/10.1002/glia.20928.
Lewis, N.D., Hill, J.D., Juchem, K.W., Stefanopoulos, D.E., Modis, L.K., 2014. RNA sequencing of microglia and monocyte-derived macrophages from mice with experimental autoimmune encephalomyelitis illustrates a changing phenotype with disease course. J. Neuroimmunol. 277, 26–38. https://doi.org/10.1016/j.jneuroim.2014.09.014.
Li, L., Acioglu, C., Heary, R.F., Elkabes, S., 2021. Role of astroglial toll-like receptors (TLRs) in central nervous system infections, injury and neurodegenerative diseases. Brain Behav. Immun. 91, 740–755. https://doi.org/10.1016/j.bbi.2020.10.007.
Li, Q., Cheng, Z., Zhou, L., Darmanis, S., Neff, N.F., Okamoto, J., Gulati, G., Bennett, M. L., Sun, L.O., Clarke, L.E., Marschallinger, J., Yu, G., Quake, S.R., Wyss-Coray, T., Barres, B.A., 2019. Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell RNA sequencing, 207-23 e10 Neuron 101. https://doi.org/10.1016/j.neuron.2018.12.006.
Liddelow, S.A., Guttenplan, K.A., Clarke, L.E., Bennett, F.C., Bohlen, C.J., Schirmer, L., Bennett, M.L., Munch, A.E., Chung, W.S., Peterson, T.C., Wilton, D.K., Frouin, A., Napier, B.A., Panicker, N., Kumar, M., Buckwalter, M.S., Rowitch, D.H., Dawson, V. L., Dawson, T.M., Stevens, B., Barres, B.A., 2017. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541, 481–487. https://doi.org/10.1038/nature21029.
Liu, L.R., Liu, J.C., Bao, J.S., Bai, Q.Q., Wang, G.Q., 2020. Interaction of microglia and astrocytes in the neurovascular unit. Front. Immunol. 11, 1024. https://doi.org/10.3389/fimmu.2020.01024.
Masuda, T., Sankowski, R., Staszewski, O., Prinz, M., 2020. Microglia heterogeneity in the single-cell era. Cell Rep. 30, 1271–1281. https://doi.org/10.1016/j.celrep.2020.01.010.
Matejuk, A., Ransohoff, R.M., 2020. Crosstalk between astrocytes and microglia: an overview. Front. Immunol. 11, 1416. https://doi.org/10.3389/fimmu.2020.01416.

McCarthy, K.D., de Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Cell Biol. 85, 890–902. https://doi.org/10.1083/jcb.85.3.890.

Mizee, M.R., Miedema, S.S., van der Poel, M., Adelia, Schuurman, K.G., van Strien, M.E., Melief, J., Smolders, J., Hendrickx, D.A., Heutinck, K.M., Hamann, J., Huitinga, I., 2017. Isolation of primary microglia from the human post-mortem brain: effects of ante- and post-mortem variables. Acta Neuropathol. Commun. 5, 16. https://doi.org/10.1186/s40478-017-0418-8.

Nikodemova, M., Watters, J.J., 2012. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. J. Neuroinflamm. 9, 147. https://doi.org/10.1186/1742-2094-9-147.

Olah, M., Raj, D., Brouwer, N., De Haas, A.H., Eggen, B.J., Den Dunnen, W.F., Biber, K.P., Boddeke, H.W., 2012. An optimized protocol for the acute isolation of human microglia from autopsy brain samples. Glia 60, 96–111. https://doi.org/10.1002/glia.21251.

Patel, A.J., Hunt, A., Seaton, P., 1988. The mechanism of cytosine arabinoside toxicity on quiescent astrocytes in vitro appears to be analogous to in vivo brain injury. Brain Res. 450, 378–381. https://doi.org/10.1016/0006-8993(88)91579-x.

Pierre, W.C., Smith, P.L.P., Londono, I., Chemtob, S., Mallard, C., Lodygensky, G.A., 2017. Neonatal microglia: the cornerstone of brain fate. Brain Behav. Immun. 59, 333–345. https://doi.org/10.1016/j.bbi.2016.08.018.

Rangaraju, S., Raza, S.A., Li, N.X., Betarbet, R., Dammer, E.B., Duong, D., Lah, J.J., Seyfried, N.T., Levey, A.I., 2018. Differential phagocytic properties of CD45(low) microglia and CD45(high) brain mononuclear phagocytes-activation and age-related effects. Front. Immunol. 9, 405. https://doi.org/10.3389/fimmu.2018.00405.

Remington, L.T., Babcock, A.A., Zehntner, S.P., Owens, T., 2007. Microglial recruitment, activation, and proliferation in response to primary demyelination. Am. J. Pathol. 170, 1713–1724. https://doi.org/10.2353/ajpath.2007.060783.

Rosenberger, K., Derkow, K., Dembny, P., Kruger, C., Schott, E., Lehnardt, S., 2014. The impact of single and pairwise Toll-like receptor activation on neuroinflammation and neurodegeneration. J. Neuroinflamm. 11, 166. https://doi.org/10.1186/s12974-014-0166-7.

Sato-Hashimoto, M., Nozu, T., Toriba, R., Horikoshi, A., Akaike, M., Kawamoto, K., Hirose, A., Hayashi, Y., Nagai, H., Shimizu, W., Saiki, A., Ishikawa, T., Elhanbly, R., Kotani, T., Murata, Y., Saito, Y., Naruse, M., Shibasaki, K., Oldenborg, P.A., Jung, S., Matozaki, T., Fukazawa, Y., Ohnishi, H., 2019. Microglial SIRPalpha regulates the emergence of CD11c(+) microglia and demyelination damage in white matter. Elife 8. https://doi.org/10.7554/eLife.42025.

Saura, J., 2007. Microglial cells in astroglial cultures: a cautionary note. J. Neuroinflamm. 4, 26. https://doi.org/10.1186/1742-2094-4-26.

Saura, J., Tusell, J.M., Serratosa, J., 2003. High-yield isolation of murine microglia by mild trypsinization. Glia 44, 183–189. https://doi.org/10.1002/glia.10274.

Savchenko, V.L., Nikonenko, I.R., Skibo, G.G., McKanna, J.A., 1997. Distribution of microglia and astrocytes in different regions of the normal adult rat brain. Neurophysiology 29, 343–351. https://doi.org/10.1007/BF02463354.

Sawada, M., Suzumura, A., Yamamoto, H., Marunouchi, T., 1990. Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C. Brain Res. 509, 119–124.

Scholze, A.R., Foo, L.C., Mulinyawe, S., Barres, B.A., 2014. BMP signaling in astrocytes downregulates EGFR to modulate survival and maturation. PLoS One 9, e110668. https://doi.org/10.1371/journal.pone.0110668.

Schroeter, C.B.H., Bock, A.M., Vogelsang, S., Eichler, A., Albrecht, S., Meuth, P., Ruck, S. G., 2021. One Brain-All Cells: A Comprehensive Protocol to Isolate All Principal CNS-Resident Cell Types from Brain and Spinal Cord of Adult Healthy and EAE Mice. Cells 10, 10030651. https://doi.org/10.3390/cells10030651.

Shinozaki, Y., Shibata, K., Yoshida, K., Shigetomi, E., Gachet, C., Ikenaka, K., Tanaka, KF., Koizumi, S., 2017. Transformation of Astrocytes to a Neuroprotective Phenotype by Microglia via $P2Y_1$ Receptor Downregulation. Cell Rep. 19 (6), 1151–1164. https://doi.org/10.1016/j.celrep.2017.04.047, 28494865.

Sola, C., Casal, C., Tusell, J.M., Serratosa, J., 2002. Astrocytes enhance lipopolysaccharide-induced nitric oxide production by microglial cells. Eur. J. Neurosci. 16, 1275–1283. https://doi.org/10.1046/j.1460-9568.2002.02199.x.

Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805–820. https://doi.org/10.1016/j.cell.2010.01.022.

Timmerman, R., Burm, S.M., Bajramovic, J.J., 2018. An overview of in vitro methods to study microglia. Front. Cell. Neurosci. 12, 242. https://doi.org/10.3389/fncel.2018.00242.

Triolo, D., Dina, G., Lorenzetti, I., Malaguti, M., Morana, P., Del Carro, U., Comi, G., Messing, A., Quattrini, A., Previtali, S.C., 2006. Loss of glial fibrillary acidic protein (GFAP) impairs Schwann cell proliferation and delays nerve regeneration after damage. J. Cell Sci. 119, 3981–3993. https://doi.org/10.1242/jcs.03168.

Volden, T.A., Reyelts, C.D., Hoke, T.A., Arikkath, J., Bonasera, S.J., 2015. Validation of flow cytometry and magnetic bead-based methods to enrich cns single cell suspensions for quiescent microglia. J. Neuroimmune Pharm. 10, 655–665. https://doi.org/10.1007/s11481-015-9628-7.

Westergard, T., Rothstein, J.D., 2020. Astrocyte diversity: current insights and future directions. Neurochem. Res. 7. https://doi.org/10.1007/s11064-020-02959-.

Wlodarczyk, A., Lobner, M., Cedile, O., Owens, T., 2014. Comparison of microglia and infiltrating CD11c(+) cells as antigen presenting cells for T cell proliferation and cytokine response. J. Neuroinflamm. 11, 57. https://doi.org/10.1186/1742-2094-11-57.

Xu, L., He, D., Bai, Y., 2016. Microglia-mediated inflammation and neurodegenerative disease. Mol. Neurobiol. 53, 6709–6715. https://doi.org/10.1007/s12035-015-9593-4.

Yamasaki, R., Lu, H., Butovsky, O., Ohno, N., Rietsch, A.M., Cialic, R., Wu, P.M., Doykan, C.E., Lin, J., Cotleur, A.C., Kidd, G., Zorlu, M.M., Sun, N., Hu, W., Liu, L., Lee, J.C., Taylor, S.E., Uehlein, L., Dixon, D., Gu, J., Floruta, C.M., Zhu, M., Charo, I. F., Weiner, H.L., Ransohoff, R.M., 2014. Differential roles of microglia and monocytes in the inflamed central nervous system. J. Exp. Med. 211, 1533–1549. https://doi.org/10.1084/jem.20132477.

Yang, J., Zhang, L., Yu, C., Yang, X.F., Wang, H., 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark. Res. 2, 1. https://doi.org/10.1186/2050-7771-2-1.

Yu, X., Nagai, J., Khakh, B.S., 2020. Improved tools to study astrocytes. Nat. Rev. Neurosci. 21, 121–138. https://doi.org/10.1038/s41583-020-0264-8.

Zarei-Kheirabadi, M., Mirsadeghi, S., Vaccaro, A.R., Rahimi-Movaghar, V., 2020. Kiani SJMBR Protoc. Purif. Cult. Astro.: Useful Not. only 2 days Post. but also adult Rat. brain 2020 2 doi: 10.1007/s11033-020-05272-.

Zuiderwijk-Sick, E.A., van der Putten, C., Bsibsi, M., Deuzing, I.P., de Boer, W., Persoon-Deen, C., Kondova, I., Boven, L.A., van Noort, J.M., Hart, B.A., Amor, S., Bajramovic, J.J., 2007. Differentiation of primary adult microglia alters their response to TLR8-mediated activation but not their capacity as APC. Glia 55, 1589–1600. https://doi.org/10.1002/glia.20572.

2.2 Publication II

Crosstalk of Highly Purified Microglia and Astrocytes in the Frame of Toll-like Receptor (TLR)2/1 Activation

Laura Zelenka (L.Z), Michael Jarek (M.J), Dennis Pägelow (D.P.), Robert Geffers (R.G.), Kira van Vorst (K.v.V), Marcus Fulde (M.F)

Neuroscience. Volume 526, 21 August 2023, Pages 256-266

DOI: 10.1016/j.neuroscience.2023.05.001

https://doi.org/10.1016/j.neuroscience.2023.05.001

Authors' contributions:

L.Z., D.P., K.v.V. and M.F. planned, designed, conducted and analyzed the glial cell experiments. M. J. and R.G. conducted and analyzed the transcriptome analysis L.Z., and M.F. evaluated the RNA sequencing data. M.F. supervised the study and L.Z., K.v.V. and M.F. wrote the manuscript. All authors read and approved the final manuscript.

NEUROSCIENCE
RESEARCH ARTICLE
L. Zelenka et al. / Neuroscience 526 (2023) 256–266

Crosstalk of Highly Purified Microglia and Astrocytes in the Frame of Toll-like Receptor (TLR)2/1 Activation

Laura Zelenka,[a] Michael Jarek,[b] Dennis Pägelow,[a] Robert Geffers,[b] Kira van Vorst[a] and Marcus Fulde[a]*

[a] *Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany*

[b] *Helmholtz Centre for Infection Research, Research Group Genome Analytics (GMAK), Braunschweig, Germany*

Abstract—The major immune cells of the central nervous systems (CNS) are microglia and astrocytes, subsets of the glial cell population. The crosstalk between glia via soluble signaling molecules plays an indispensable role for neuropathologies, brain development as well as homeostasis. However, the investigation of the microglia-astrocyte crosstalk has been hampered due to the lack of suitable glial isolation methods. In this study, we investigated for the first time the crosstalk between highly purified Toll-like receptor (TLR)2-knock out (TLR2-KO) and wild-type (WT) microglia and astrocytes. We examined the crosstalk of TLR2-KO microglia and astrocytes in the presence of WT supernatants of the respective other glial cell type. Interestingly, we observed a significant TNF release by TLR2-KO astrocytes, which were activated with Pam3CSK4-stimulated WT microglial supernatants, strongly indicating a crosstalk between microglia and astrocytes after TLR2/1 activation. Furthermore, transcriptome analysis using RNA-seq revealed a wide range of significant up- and down-regulated genes such as *Cd300*, *Tnfrsf9* or *Lcn2*, which might be involved in the molecular conversation between microglia and astrocytes. Finally, co-culturing microglia and astrocytes confirmed the prior results by demonstrating a significant TNF release by WT microglia co-cultured with TLR2-KO astrocytes. Our findings suggest a molecular TLR2/1-dependent conversation between highly pure activated microglia and astrocytes via signaling molecules. Furthermore, we demonstrate the first crosstalk experiments using ~100% pure microglia and astrocyte mono-/co-cultures derived from mice with different genotypes highlighting the urgent need of efficient glial isolation protocols, which particularly holds true for astrocytes.

Key words: crosstalk, microglia, astrocytes, toll-like receptor (TLR)2, tumor necrosis factor (TNF), interleukin-10 (IL-10).

INTRODUCTION

Microglia and astrocytes are the principal, non-neuronal cells of the central nervous system (CNS) innate immune response, and represent sub-populations of the glial cell type (Kwon and Koh, 2020). Microglia and astrocytes contribute to many essential functions in the CNS including homeostasis, the maintenance or the development and function of the brain (Greenhalgh et al., 2020). Whereas microglia, as the macrophages of the brain, are the first cells to respond to brain insult with phagocytic activity, astrocytes form a physical barrier to protect the cell parenchyma from inflammatory cells (Rodgers et al., 2020).

All cells of the CNS are equipped with Toll-like (TLR) receptors, which is a major family of pattern recognition receptors (PRRs), playing a critical role in the innate immune response of microglia and astrocytes (Fiebich et al., 2018; Rodgers et al., 2020; Li et al., 2021). The molecular conversation among murine microglia and astrocytes during health and disease is mainly orchestrated by the PRR *Tlr1-9* (Okun et al., 2009; Lehnardt, 2010; Fiebich et al., 2018). The engagement of TLRs is triggered by danger-associated molecular patterns (DAMPs), which are released during cell damage or by conserved pathogen-associated molecular patterns (PAMPs) derived from bacteria, fungi or viruses (Lehnardt, 2010; Fiebich et al., 2018). TLR stimulation leads to activation of downstream signaling pathways, and subsequently the production of cytokines is promoted, eliciting either a pro- or anti-inflammatory immune response (Bernaus et al., 2020).

Increasing evidence indicates a crosstalk between microglia and astrocytes via the release of a wide range of signaling molecules such as cytokines, chemokines, growth factors or adenosine triphosphate (ATP),

*Corresponding author. Address: Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Robert-von-Ostertag-Str. 7, 14163 Berlin, Germany.
E-mail address: Marcus.Fulde@fu-berlin.de (M. Fulde).

Abbreviations: CNS, central nervous system; DAMPs, danger-associated molecular patterns; IL-10, interleukin-10; IVCs, individually ventilated cages; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; SPF, specific-pathogen-free; TLR, Toll-like receptors; TLR2-KO, TLR2-knock out; TNF, tumor necrosis factor; WT, wild-type.

https://doi.org/10.1016/j.neuroscience.2023.05.001
0306-4522/

involved in reciprocal influences on the differentiation of the other cell type (Jha et al., 2019; Bernaus et al., 2020; Liu et al., 2020; Matejuk and Ransohoff 2020; Yang et al., 2020; Matejuk et al., 2021; Rostami et al., 2021). In particular, emerging studies strongly pointing to that TLRs as key sensors are playing a crucial role in the crosstalk among microglia and astrocytes as well as other cells such as neurons or CNS immune cells (Fiebich et al., 2018; Li et al., 2021). For example, Holm and colleagues showed that purified astrocyte cultures require the presence of microglial cells for being able to respond to TLR2, 3 and 4 ligation upon LPS treatment, probably induced by the release of microglial soluble factors such as the monocyte chemoattractant protein 1 (MCP1) (Holm et al., 2012).

Despite an array of developed *in vitro* methods to isolate primary microglia and astrocytes from brain tissue, these protocols do not meet the standards required to investigate the crosstalk on a cell-specific level. While most of these methods already achieve satisfying microglial purities of >99% (Timmerman et al., 2018), protocols for astrocytes are lagging far behind as even small amounts of contaminating cells can affect the outcome of *in vitro* studies (Saura 2007; Losciuto et al., 2012). Therefore, we recently developed a novel protocol generating ~100% microglia and astrocytes from a mixed murine glial cell culture using Miltenyi Biotec's anti-CD11b and anti-ACSA-2 MicroBeads to investigate crosstalk on a cell-specific level (Zelenka et al., 2022). In contrast to this, studies with minor modifications to Miltenyi Biotec's recommended protocol achieved notably lower microglial (~90–97%) (Crain et al., 2009; Kremer et al., 2010; Williamson et al., 2011; Harms and Tansey 2013; Cho et al., 2015; Jin et al., 2015; Bordt et al., 2020; Pan and Wan, 2020) and astrocyte (<97%) purities (Batiuk et al., 2017; Kantzer et al., 2017; Pan and Wan, 2020; Sugiyama et al., 2020), as judged by fluorescence activated cell sorting (FACS).

However, to best our knowledge there are no such studies investigating the crosstalk between primary murine microglia and astrocytes upon TLR2 activation using highly purified glial cells. Moreover, we are the first group analyzing the inflammatory protein response of 99–100% pure co-incubated microglia and astrocytes isolated from mice with different genetic backgrounds.

In the present study, we examined the crosstalk between microglia and astrocytes under inflammatory conditions using highly pure primary cells derived from wild-type (WT) and TLR2-knock out (TLR2-KO) mice. We demonstrate a crosstalk between microglia and astrocytes after TLR2/1 stimulation, determined by TNF and IL-10 release as well as a wide range of significantly up- and down-regulated genes. Co-culture of microglia and astrocytes from different genetic backgrounds (WT, TLR2-KO) confirmed the crosstalk, likely driven by microglial soluble signaling molecules released upon TLR2/1 activation.

EXPERIMENTAL PROCEDURES

Animals and ethical statement

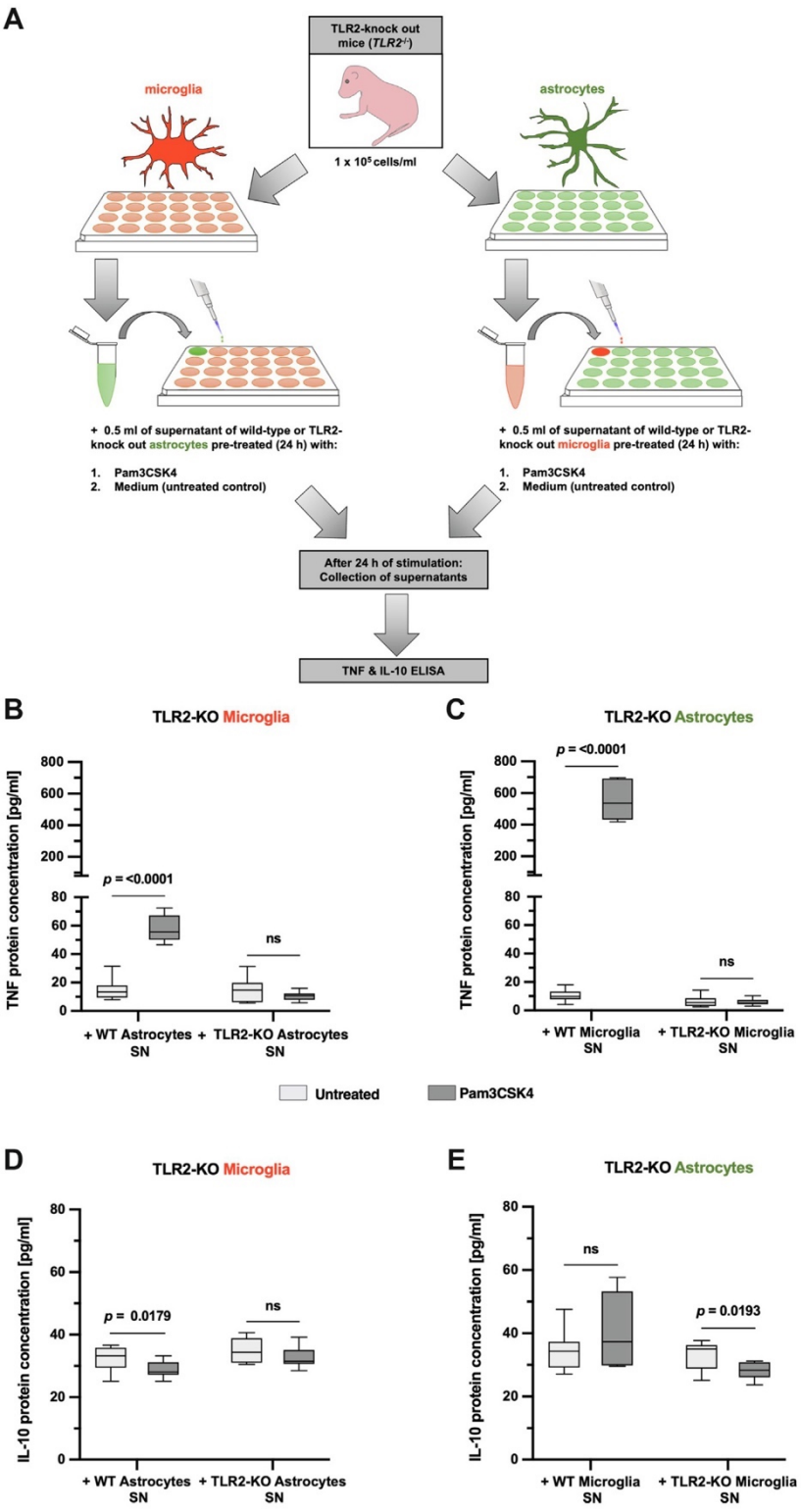
Male and female 0 to 2-day-old wild-type (WT, C57BL/6n) and TLR2-knock out (TLR2-KO, *Tlr2*$^{-/-}$, C57BL/6.129-Tlr2tm1Kir/J) mice were used for the isolation of primary microglia and astrocytes. Animals were bred in-house in individually ventilated cages (IVCs) under specific-pathogen-free (SPF) conditions at the Freie Universität Berlin. Water and food were provided *ad libidum*. All animals were maintained in compliance with the German animal protection law (TierSchG) and the local animal welfare committee. Organ harvesting was approved by the institutional review committee Landesamt für Gesundheit und Soziales Berlin, Germany (approval T0284/15).

Isolation and purification of primary microglia and astrocytes

Microglia and astrocytes were isolated from whole brains of 0 to 2-day-old mice and purified via magnetic-activated cell sorting (MACS®) using our previously described protocol (Zelenka et al., 2022), and seeded at either 1×10^5 or 2×10^5 cells/ml into 24-well plates (Corning, USA). Microglia were incubated for 24 h to allow cell recovery and astrocytes for 72 h to reach confluence (37 °C, 5% CO_2). Glia derived from our protocol showed viability and purity of 99–100%.

Activation of TLR2-KO microglia and astrocytes after TLR2/1 stimulation

As depicted in Fig. 1(**A**), TLR2-KO microglia and astrocytes (1×10^5 cells/ml) were pre-incubated with 0.5 ml supernatants (SN) derived from either Pam3CSK4 (100 ng/ml) stimulated or unstimulated (control) WT microglial and astrocyte monocultures, respectively for 24 h (37 °C, 5% CO_2). In the next step, TLR2-KO microglia and astrocytes (1×10^5 cells/ml) were stimulated with 1 ml SN derived from Pam3CSK4 stimulated or unstimulated (control) WT microglial and astrocytes monocultures, respectively, for 2 h (37 °C, 5 % CO_2), as shown in Fig. 2(**A**). Finally, the SN were removed and replaced with fresh, pre-warmed medium, and glial cells were incubated for another 24 h (37 °C, 5% CO_2). Pam3CSK4 is a synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial lipopeptides (Lombardi et al., 2008; Brandt et al., 2013). We used this ligand for the following experiments as it activates the TLR2/1 heterodimer leading to the induction of the transcription factor NF-κB, which is involved in inflammatory and immune responses (Aliprantis et al., 1999; Ozinsky et al., 2000). By treating TLR2-KO glial monocultures with Pam3CSK4-stimulated SN, we were aiming to avoid the direct activation of glial cells by the TLR2/1ligand Pam3CSK4.



Co-culture of microglia and astrocytes from different genetic backgrounds upon TLR2/1 stimulation

WT or TLR2-KO astrocytes (1×10^5 cells/ml) were grown to confluence (37 °C, 5 % CO_2). After 48 h, WT or TLR2-KO microglia (2×10^4 cells/ml) were added in a ratio of 1:5 (von Bartheld et al., 2016; Keller et al., 2018). 24 h later, cells were either stimulated with Pam3CSK4 (100 ng/ml) or fresh media (control) was added for an additional 24 h (37 °C, 5% CO_2) (Fig. 3(**A**)).

Enzyme-linked immunosorbent assay (ELISA)

At the indicated time points, SN were collected and centrifuged (800 × *g*, 10 min, 4 °C) to remove contaminating cells and stored at −80 °C until use. TNF and IL-10 concentrations in cell culture SN were determined by sandwich enzyme-linked immunosorbent assay (ELISA), using the ELISA MAX™ Deluxe Set Mouse IL-10 and TNF-α (BioLegend, USA) according to the manufacturer's instructions.

Fig. 1. Activation of TLR2-knock out (TLR2-KO) microglia and astrocytes with wild-type (WT) and TLR2-KO supernatants (SN). (A) Workflow showing the single steps of the experiment. Microglia **(B-D)** and astrocytes **(C-E)** were purified from TLR2-knock out (TLR2-KO, *Tlr2*$^{-/-}$, C57BL/6.129-Tlr2tm1Kir/J) 0 to 2-day-old neonatal mice and seeded into 24-well plates (1×10^5/ml). Subsequently, microglia and astrocytes were incubated with 0.5 ml supernatant (SN) derived from either Pam3CSK4 (100 ng/mL) stimulated or unstimulated (control) wild-type (WT, C57BL/6n) microglial and astrocyte monocultures (24 h), respectively for 24 h. Medium served as negative control (untreated). SN were collected after the indicated time points and analyzed for TNF and IL-10 by sandwich ELISA. Boxplots display the median as the middle line, the top and bottom of the box are the 25th and 75th percentiles (quartiles), and the ends of the whiskers are the 5th and 95th percentiles. Mann-Whitney test was applied of means of triplicates, derived from different harvests from biological duplicates, which were performed in at least 2 representative ELISA analyses. Statistical significances as indicated; *p*-values > 0.05 are indicated as not significant, ns.

RNA isolation

At the indicated time points, cells were detached from well plates by addition of 400 µl TRIzol™ Reagent (Thermo Fisher Scientific, USA) to the wells followed by vigorous pipetting, and the cell suspensions were collected and stored at −80 °C until use. Total RNA was isolated using the Direct-zol™ RNA Microprep Kit with the the Zymo-Spin IC Columns (both Zymo Research Europe GmbH, Germany) according to the manufacturer's protocol with minor modifications. All steps were centrifuged at 15 s (16,000 × *g*) except the second Direct-zol™ RNA PreWash step as well as the elution (30 s; 16,000 × *g*). Isolated RNA was stored at −80 °C until use.

Transcriptome analysis using RNA-seq

Quality and integrity of total RNA was controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Inc., USA). The RNA sequencing library was generated from 100 ng total RNA using NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina® (New England Biolabs, USA) according to manufacturer's protocols. The

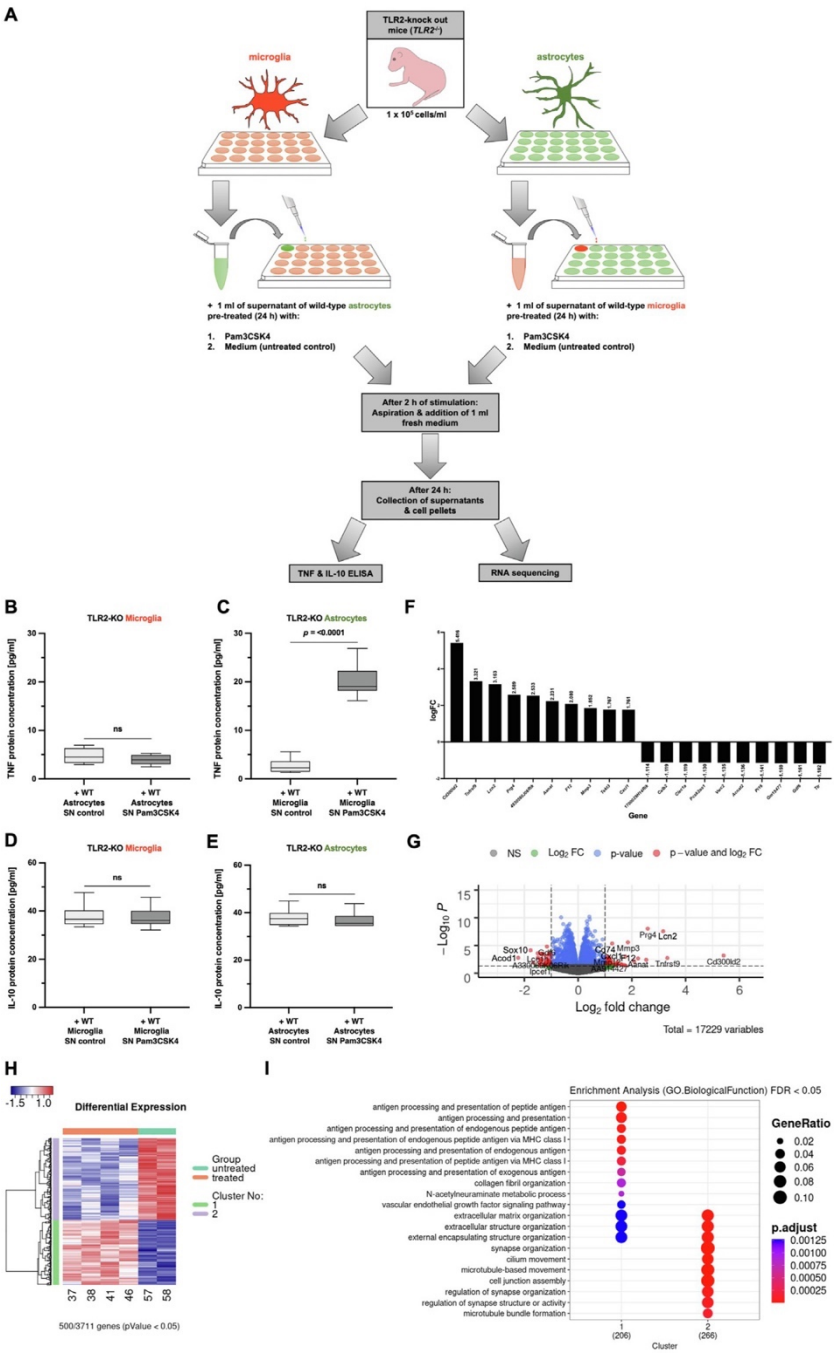
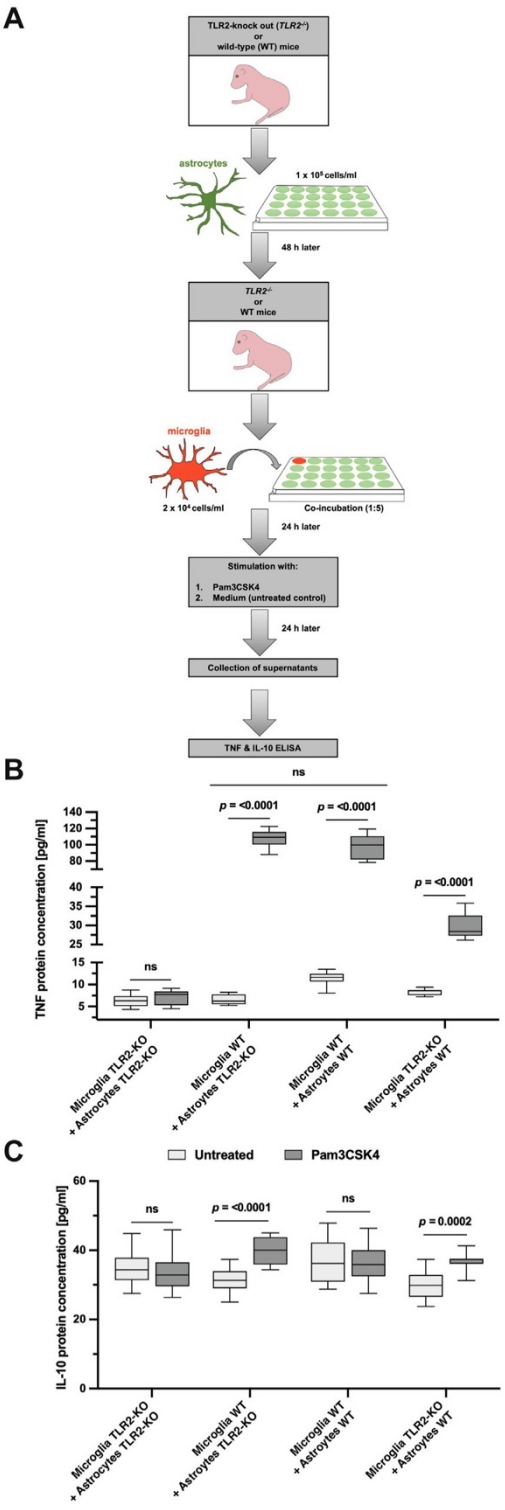


Fig. 2. Crosstalk between wild-type (WT) microglia and TLR2-knock out (TLR2-KO) astrocytes upon TLR2/1 activation. (A) Workflow showing the single steps of the experiment. Microglia **(B/D)** and astrocytes **(C/E)** were purified from TLR2-knock out (TLR2-KO, *Tlr2*$^{-/-}$, C57BL/6.129-Tlr2tm1Kir/J) 0 to 2-day-old neonatal mice and seeded into 24-well plates (1×10^5/ml). Subsequently, microglia and astrocytes were incubated with 1 ml supernatant (SN) derived from either Pam3CSK4 (100 ng/mL) stimulated or unstimulated (control) wild-type (WT, C57BL/6n) microglial and astrocyte monocultures (24 h), respectively for 2 h. Then, SN were taken off the wells, fresh pre-warmed medium was added and glial cells were incubated for another 24 h. Medium served as negative control (untreated). SN were collected after 24 h and analyzed for TNF and IL-10 by sandwich ELISA. Boxplots display the median as the middle line, the top and bottom of the box are the 25th and 75th percentiles (quartiles), and the ends of the whiskers are the 5th and 95th percentiles. Mann-Whitney test was applied of means of triplicates, derived from different harvests from biological duplicates, which were performed in at least two representative ELISA analyses. Statistical significances as indicated; *p*-values > 0.05 are indicated as not significant, ns. **(F)** RNA-seq revealed the top ten up- and downregulated genes of the 8,463 identified differentially expressed genes (DEGs) of TLR2-KO astrocytes stimulated with Pam3CSK4 activated WT microglial SN *versus* (vs). untreated WT microglial SN (control). **(G)** Volcano plot of the transcriptome analysis. **(H)**. Heat map of transcriptome analysis via RNA-seq shows 500 of the 3,711 identified DEGs revealing two distinct clusters between treated vs. untreated samples. Statistical significances as indicated; *p*-values <0.05. **(I)** Gene Ontology (GO) enrichment determining the biological functions of the two distinct clusters derived from the Heat map of transcriptome analysis (**H**); FDR < 0.05.

libraries were sequenced on Illumina NovaSeq (Illumina, Inc., USA) (100 cycles, paired end run) with an average of 5 × 10^7 reads per RNA sample. Each FASTQ file gets a quality report generated by FASTQC tool (Andrews, 2010). Before alignment to reference genome each sequence in the raw FASTQ files were trimmed on base call quality and sequencing adapter contamination using Trim Galore! wrapper tool (Krueger 2012). Reads shorter than 20 bp were removed from FASTQ file. Trimmed reads were aligned to the reference genome using open source short read aligner STAR (https://code.google.com/p/rna-star/) with default settings (Dobin et al., 2013). Feature counts were determined using R package "Rsubread". Only genes showing counts greater 5 at least two times across all samples were considered for further analysis (data cleansing) (Liao et al., 2014). Gene annotation was done by R package "BioMart" (Durinck et al., 2005). Before starting the statistical analysis steps, expression data was log2 transform and TMM normalized ("edgeR"). Differential gene expression was calculated by R package "edgeR" (Robinson et al., 2010). Functional analysis was performed by R package "clusterProfiler" (Yu et al., 2012).



Statistical analysis

Statistics as indicated in the Figure legends were calculated using GraphPad Prism version 9.5 (GraphPad Prism, USA). Statistical tests were performed depending on the normality distribution of the values using the D'Agostino & Pearson test ($p = < 0.05$). Where appropriate, the Mann-Whitney test was used for comparison of two groups. Differences were considered statistically significant when lower $p = < 0.05$ ($p = < 0.05$ *, $p = < 0.01$ **, $p = < 0.001$ ***, and $p = < 0.0001$ ****). Details on the statistical results are depicted in the supplementary material.

RESULTS

Activation of microglia and astrocyte monocultures with SN after TLR2/1 stimulation

First, we examined the crosstalk upon TLR2/1 activation between microglia and astrocytes by stimulating highly pure TLR2-KO microglia and astrocytes monocultures

Fig. 3. Co-cultures of highly purified microglia and astrocytes from different genetic backgrounds confirm crosstalk upon TLR2/1 activation. (A) Workflow showing the single steps of the experiment. Microglia and astrocytes were purified from wild-type (WT, C57BL/6n) and TLR2-knock out (TLR2-KO, *Tlr2*$^{-/-}$, C57BL/6.129-Tlr2tm1Kir/J) 0 to 2-day-old neonatal mice and co-cultured into 24-well plates (ratio 1:5). Subsequently, microglia and astrocytes were either stimulated with Pam3CSK4 (100 ng/ml) or left unstimulated (control) for 24 h. Medium served as negative control (untreated). Supernatants (SN) were collected after 24 h and analyzed for TNF **(B)** and IL-10 **(C)** by sandwich ELISA. Boxplots display the median as the middle line, the top and bottom of the box are the 25th and 75th percentiles (quartiles), and the ends of the whiskers are the 5th and 95th percentiles. Mann-Whitney test was applied of means of triplicates, derived from different harvests from biological duplicates, which were performed in at least 2 representative ELISA analyses. Statistical significances as indicated; *p*-values > 0.05 are indicated as not significant, ns.

with Pam3CSK4 treated or untreated WT supernatants (SN) of the respective other glial cells type (Fig. 1(**A**)). To confirm the TLR2-KO in microglia and astrocytes, we also treated these cells either with Pam3CSK4 stimulated SN or untreated SN derived from TLR2-KO microglia and astrocytes, respectively. The IL-10 and TNF protein concentrations and statistical results are depicted in **Table S1-S2**.

The addition of SN derived from Pam3CSK4 stimulated TLR2-KO glial cells did not have a significant effect on either TLR2-KO microglia or astrocytes in comparison to their untreated controls. An exception is seen in for IL-10 response by TLR2-KO astrocytes ($U^{n1=10,\ n2=12} = 25$, $p = 0.0193$), which was significantly reduced in comparison to the untreated control (Fig. 1(**E**)). In general, the anti-inflammatory IL-10 response (Fig. 1(**D/E**)) was less pronounced than the pro-inflammatory TNF release (Fig. 1(**B/C**)). TLR2-KO microglia and astrocytes which were stimulated with Pam3CSK4 WT SN showed a significantly increased TNF protein production in comparison to the controls (Fig. 1(**B/C**)).

In the next step, we wanted to rule out that secreted TNF or IL-10 protein concentrations in the SN were measured in the monocultures by adding the corresponding WT SN only for 2 h, replacing the SN-containing medium with fresh medium for another 24 h and determining the TNF and IL-10 protein release (**Table S3-4**) as depicted in Fig. 2(**A**). For IL-10 we found no significant effects by the addition of the SN, either for microglia (Fig. 2(**D**)) or for astrocytes (Fig. 2(**E**)) and did not observe the highly significant effect we found for TLR2-KO microglia with this experiment (Fig. 2(**B**)). Only TLR2-KO astrocytes showed a significantly increased TNF protein release by the addition of WT microglial SN upon Pam3CSK4 stimulation in comparison to their untreated control ($U^{n1=24,\ n2=19} = 0$, $p = <0.0001$) (Fig. 2(**C**)).

Subsequently, we further analyzed this sample (Fig. 2(**C**)) by performing transcriptome analysis using RNA-seq (Fig. 2(**F-I**)) to identify candidate mediators involved in the crosstalk. We identified 8,767 up- and 8,463 downregulated and differentially expressed genes (DEGs) in TLR2-KO astrocytes stimulated with Pam3CSK4 activated WT microglial SN (treated) in comparison to the control (TLR2-KO astrocytes stimulated with untreated WT microglial SN) (Fig. 2(**G**)). The top ten up- and down-regulated genes are depicted in Fig. 2(**F**): The highest significantly upregulated genes in comparison to the untreated control were *cd300 molecule like family member D2* (*Cd300d2*), *tumor necrosis factor receptor superfamily member 9* (*Tnfrsf9*) and *lipocalin2* (*Lcn2*). Heat map enrichment analysis revealed two distinct clusters among all genes distinguishing between TLR2-KO astrocytes, which were stimulated with activated microglial SN, and the untreated control (Fig. 2(**H**)). Moreover, these clusters were analyzed in more detail using Gene Ontology (GO) enrichment (Fig. 2(**I**)), showing cluster 1 to be mostly enriched for genes involved in inflammatory functions in contrast to cluster 2.

Co-culture of microglia and astrocytes from different genetic backgrounds upon TLR2/1 stimulation

In the last experiment, we investigated the TNF and IL-10 protein production of co-cultured microglia and astrocytes derived from neonatal mice coming from different genetic backgrounds, namely WT and TLR2-KO (Fig. 3(**A**)). Therefore, we co-cultured highly pure WT and TLR2-KO microglia and astrocytes (ratio 1:5), treated them with TLR2/1 ligand Pam3CSK4 and determined the TNF (Fig. 3(**B**)) and IL-10 (Fig. 3(**C**)) protein release (**Table S5-S6**). WT microglia co-cultured with TLR2-KO astrocytes and stimulated with Pam3CSK4 ($U^{n1=8,\ n2=10} = 0$, $p = <0.0001$) showed a similar TNF release as the treated co-culture of WT microglia and WT astrocytes ($U^{n1=11,\ n2=10} = 0$, $p = <0.0001$) (Fig. 3(**B**)). Both showed significant differences in comparison to their untreated control, but were not significantly different with respect to each other. Moreover, Pam3CSK4 activation of the co-culture consisting of TLR2-KO microglia and WT astrocytes had a highly significant effect ($U^{n1=14,\ n2=12} = 0$, $p = <0.0001$) on the TNF release in comparison to the control (Fig. 3(**B**)).

DISCUSSION

As the investigation of the glial crosstalk on a cell-specific level were hampered due to the lack of suitable methods, we have addressed this issue by determining the immune response of activated highly pure microglia and astrocytes generated with our recently published protocol (Zelenka et al., 2022). In particular, we examined the TNF and IL-10 protein release in response to the TLR2/1 ligand Pam3CSK4, delivering the first evidence of a TLR2/1-dependent crosstalk in highly pure primary microglial and astrocyte mono-/co-cultures derived from mice with different genotypes.

First, we investigated the glial crosstalk of WT and TLR2-KO microglia and astrocyte monocultures by adding Pam3CSK4 treated SN (WT or TLR2-KO) derived from the respective other glial cell type and determining their TNF and IL-10 protein production (Fig. 1(**A**)).

As expected, the immune response by TLR2-KO microglia and astrocyte monocultures were not significantly affected by the addition of SN derived from Pam3CSK4 stimulated TLR2-KO SN (Fig. 1(**B-D**)). However, one exception is the IL-10 response by TLR2-KO astrocyte monocultures, which was significantly reduced in comparison to the untreated control (Fig. 1(**E**)). Since Pam3CSK4 is a ligand for the heterodimer TLR2/TLR1, it is conceivable that – despite the knock-out of *Tlr2* – microglia secret soluble factors driven by TLR1 stimulation, which in turn lead to the downregulation of IL-10 in TLR2-KO astrocytes. However, further studies need to be conducted to confirm this theory, for example by repeating the experiments by using a different ligand, such as the TLR2/6 ligand Pam2CSK4 (Okun et al., 2009).

As expected, microglia as well as astrocytes depicted a weaker anti-inflammatory (Fig. 1(**D/E**)) than pro-inflammatory immune response (Fig. 1(**B/C**)), which we already observed in our previous studies (Zelenka et al., 2022). The TNF production by TLR2-KO microglia and astrocytes which were stimulated with Pam3CSK4 WT SN, was significantly increased in comparison to the controls (Fig. 1(**B/C**)). In studies by Chen *et al*., it was shown that contaminating microglia in stimulated astrocyte cultures are the major source of TNF protein release, leading to the proposal that astrocytes require the interaction with microglia for their TNF induction (Chen et al., 2015). Here, we demonstrate that the presence of microglia is not necessarily required, since astrocytes were activated by the addition of stimulated microglial SN (Fig. 1(**C**)). However, it is conceivable that the SN of the microglial cells, which were stimulated by Pam3CSK4, contained secreted signaling molecules which in turn activated TLR2-KO glial cells. As our prior experiments showed, astrocytes might rely on microglial signaling to properly respond to TLR stimulation, pointing to an existing crosstalk between microglia and astrocytes (Zelenka et al., 2022).

Next, we wanted to further pursue the question as to whether TLR2-KO astrocytes were activated by microglial soluble molecules to release cytokines alone, or if remaining microglial TNF (Fig. 2(**B**)) and IL-10 (Fig. 2(**C**)) proteins in the SN were responsible for these significant effects. Surprisingly, we did not observe the highly significant effect we found for TLR2-KO microglia with this experiment (Fig. 1(**B/2B**)). This supports our suggestion that secreted TNF in the SN derived from WT astrocytes was responsible for the significant TNF increase in TLR2-KO microglia (Fig. 2(**B**)). In contrast to this, TLR2-KO astrocytes were significantly activated by the addition of WT microglial SN upon Pam3CSK4 stimulation in comparison to their untreated control (Fig. 2(**C**)), confirming the presence of highly purified astrocytes, devoid of any microglial contamination, generated with our protocol (Zelenka et al., 2022). However, with this experiment we can not rule out that the media change after 2 h had any negative effects on the cells which might have caused the lack of microglial response (Fig. 2(**B**)). Nevertheless, microglia as the first line of defense (Kreutzberg, 1995; He et al., 2021) usually secret TNF – known as the first cytokine released by microglia upon TLR stimulation – abundantly in the first 24 h (Ebert et al., 2005; Lehmann et al., 2012; Rosenberger et al., 2014; Jha et al., 2019), indicating that the microglial response was hampered due to the lack of adequate and/or sufficient signaling mediators released by Pam3CSK4 activated WT astrocytes (Fig. 2(**B**)) since astrocytes showed a significant TNF response at 2 h of stimulation (Fig. 2(**C**)). This experiment shows that TLR2-KO astrocytes, even though they are not able to recognize TLR2, are activated by the addition of Pam3CSK4 stimulated microglial SN alone, consistent with the idea of an unidirectional crosstalk from microglia to astrocytes.

In an effort to identify additional candidate mediators involved in the crosstalk, we further analyzed these samples (Fig. 2(**C**)) by performing transcriptome analysis using RNA-seq (Fig. 2(**F-I**)). The highest significantly upregulated gene in comparison to the untreated control is *cd300 molecule like family member D2* (*Cd300d2*) (log2 = 5.416; FDR = 0.016) a member of the CD300 family of immunoreceptors in humans and mice, which forms extracellular homo- and hetero-signaling complexes in myeloid cells (Comas-Casellas et al., 2012; Peluffo et al., 2012; Borrego, 2013) (Fig. 2(**F**)). Whereas the other members are well characterized, e.g. CD300f is known to be an activating immune receptor, the function of *Cd300d2* is still largely unknown (Peluffo et al., 2012; Lima et al., 2017). A study by Comas-Casellas *et al.* proposes that human CD300d might be responsible for changing the activation state of myeloid cells due to its role in the regulation and/or formation of other CD300 molecules on the cell surface (Comas-Casellas et al., 2012). This suggestion correlates well with the highly increased *Cd300d2* expression by activated astrocytes upon microglial SN stimulation in our experiments. Studies by Lima and colleagues revealed that primary astrocytes derived from neonatal rats with a purity of 98% modulate the anti-inflammatory immune response in the CNS by expressing CD300f in a neuroprotective manner *in vitro* (Lima et al., 2017). Based on this, it might be conceivable that CD300d also has immunomodulatory functions including pro-inflammatory properties leading to the significant high TNF protein release in astrocytes, which were treated with stimulated microglial SN (Fig. 2(**C**)).

The second highest significantly upregulated gene in comparison to the untreated control, is *tumor necrosis factor receptor superfamily member 9* (*Tnfrsf9*) (log2 = 3.321; FDR = 0.030), also referred to as *Cd137* (Fig. 2(**F-H**)). This receptor has been found to be expressed by microglia, astrocytes, and neurons derived from primary human brain cells under normal conditions, whereas the expression of its ligand *CD137-L* is limited to astrocytes and neurons (Reali et al., 2003). Moreover, Reali and colleagues concluded that CD137 is responsible for the activation of microglia and the secretion of TNF-α as well as the inhibition of IL-10 release, based on observations Langstein *et al.* made in monocytes (Langstein et al., 1998; Langstein et al., 2000; Reali et al., 2003). Following the model of these research groups, it is conceivable that upon Pam3CSK4 stimulation, WT microglia become activated and release CD137 as well as TNF, whereas IL-10 release is inhibited. In turn, these signaling factors might stimulate TLR2-KO astrocytes to produce significantly more TNF than the control (Fig. 2(**C**)) and inhibit the release of IL-10 protein (Fig. 2(**E**)). We also confirmed the inhibited release of the anti-inflammatory cytokine IL-10 reported by Reali *et al*. and Langstein and co-authors in our experiment, since *Il-10* expression was downregulated in TLR2-KO astrocytes which had been stimulated with TLR2 activated microglial SN (Langstein et al., 2000; Reali et al. 2003) (Fig. 2(**F-I**)).

Another interesting finding is that *lipocalin2* (*Lcn2*) (log2 = 3.163; FDR = 0.000) was the third highest significantly upregulated gene compared to the control. The gene *Lcn2* encodes a member of the lipocalin

family involved in the transport by binding small hydrophobic molecules (Lee et al., 2009; Kim et al., 2011; Lee et al., 2011; Berard et al., 2012; Jin et al., 2014). It has been shown that the acute phase protein LCN2 (Liu and Nilsen-Hamilton, 1995) is expressed by microglia as well as astrocytes (Lee et al., 2011). Several studies describe the upregulation of *Lcn2* by astrocytes upon different inflammatory pathologies of the CNS such as ischemic stroke (Jin et al., 2014; Wang et al., 2015; Zhao et al., 2019). LCN2 is involved in the activation, the morphological change as well as the migration of astrocytes along with glial fibrillary acidic protein (GFAP) expression, particularly in the process of reactive astrocytosis, by the secretion of soluble mediators such as chemokine CXCL10 (Lee et al., 2009; Kim et al., 2011; Lee et al., 2011). Ranjbar Taklimie and co-authors examined the mRNA expression of different pro- and anti-inflammatory cytokines of hypoxic cultured astrocytes derived from cerebral cortex of WT and LCN2-deficient mice. They observed that the expression of the pro-inflammatory cytokine *Tnf* was upregulated with an enhanced *Lcn2* expression in WT astrocytes, whereas the anti-inflammatory cytokine *Il-10* was only significantly expressed in LCN2-deficient astrocytes (Ranjbar Taklimie et al., 2019). This is consistent with our observations, since the significant upregulation of *Lcn2* (Fig. 2(F)) coincided with the significant TNF (Fig. 2(**C**)) and the unchanged IL-10 (Fig. 2(**E**)) protein release in TLR2-KO astrocytes stimulated with activated microglial SN.

Based on the findings in the prior monoculture experiments (Fig. 1/Fig. 2), we investigated the TNF and IL-10 protein production of co-cultured microglia and astrocytes derived from neonatal mice coming from different genetic backgrounds (WT and TLR-KO) (Fig. 3 (**A**)). Interestingly, the co-culture of WT microglia and TLR2-KO astrocytes which was stimulated with Pam3CSK4 produced similar TNF protein levels as the treated co-culture of WT microglia and WT astrocytes (Fig. 3(**B**)). Particularly, both co-cultures were significantly different in comparison to their untreated control but did not show any significant differences with respect to each other (Fig. 3(**B**)). This effect in the co-culture of WT microglia and TLR2-KO astrocytes might be due to the the missing negative feedback by astrocytes which protect the brain from an exaggerated immune response. Activated astrocytes control the excessive pro-inflammatory microglial response by producing regulatory factors (Min et al., 2006; Norden et al., 2014).

The Pam3CSK4 activated co-culture containing TLR2-KO microglia and WT astrocytes also depicted a highly significant TNF increase in comparison to the control. However, the protein release was lower and therefore not comparable to that of co-cultured WT astrocytes and microglia. This attenuated TNF protein release by TLR2-KO microglia monocultures which were stimulated with astrocyte SN was already seen in Fig. 2 (**B**) and might be due to the fact that microglia-derived TNF acts as a stimulus in an autocrine/paracrine manner to augment TLR2/1 expression in microglia, similar to the effects described by Syed and colleagues in primary microglia upon bacterial challenge (Syed et al., 2007).

This phenomenon could also be observed for the IL-10 production from the co-culture of WT microglia and TLR2-KO astrocytes as well as TLR2-KO microglia co-cultivated with WT astrocytes (Fig. 3(**C**)). However, one needs to keep in mind that the prediction of the behavior on *in vitro* microglia has its limits as studies have demonstrated that within a few hours *in vitro* cultivated microglia significantly downregulate their signature genes like *Tmem119* and *P2ry12* leading to a reduced protein expression. In addition to this, they upregulate genes which are typical for *in vivo* microglia or associated with pathological conditions (Butovsky et al., 2014; Bohlen et al., 2017; Gosselin et al., 2017; Bohlen et al., 2019). In addition, this also applies for *in vitro* astrocytes cultured in serum-containing medium since in the healthy CNS astrocytes are shielded from the serum by the blood–brain barrier (BBB). In contrast to this, astrocytes cultured under serum-free conditions showed transcriptome profiles similar to *in vivo* astrocytes (Foo et al., 2011; Zhang et al., 2016; Holt et al., 2019). Moreover, in these co-culture experiments, soluble factors might not be the only main driver of the crosstalk. As microglia and astrocytes are in very close vicinity of each other, this could facilitate direct cell-to-cell contact e.g. via tunneling nano tubes (TNTs), which has recently been described in pathological microglia and astrocytes co-culture experiments by Rostami and co-authors (Rostami et al., 2021).

Our results show that although microglia or astrocytes are lacking TLR2, they can become activated through soluble mediators derived from the respective other WT glial cell type, indicating an existing crosstalk among microglia and astrocytes in the frame of TLR2/1 activation. However, to further confirm our theory and to test whether the crosstalk is unidirectional or not will require further study. For example, the addition of an IL-10 inhibitor to our WT/TLR2-KO co-culture systems would shed more light on our theory of an TLR2/1-dependent crosstalk between microglia and astrocytes.

Overall, these results confirm that microglia and astrocytes generated with our novel protocol (Zelenka et al., 2022) meet the standards of the required purity to study the crosstalk between microglia and astrocytes from different genetic backgrounds.

In summary, our data demonstrate that the TNF response of TLR2-KO astrocytes can be restored by the addition of Pam3CSK4 stimulated microglial WT SN, suggesting an existing crosstalk between microglia and astrocytes upon TLR2/1 activation most likely via a wide range of secreted molecules, as determined by transcriptome analysis. The co-culture studies of WT microglia and TLR2-KO astrocytes confirms our theory of a crosstalk, further highlighting the significance of our novel isolation protocol for co-culturing microglia and astrocytes coming from different genetic backgrounds, which should be considered in future glial co-culture experiments (Zelenka et al., 2022). Further studies need to be conducted to shed more light on the existence of a TLR2/1-dependent crosstalk between microglia and

astrocytes to gain a better understanding of their intimate molecular conversation.

AUTHOR CONTRIBUTIONS

L.Z., D.P., K.v.V. and M.F. planned, designed, conducted and analyzed the glial cell experiments. M. J. and R.G. conducted and analyzed the transcriptome analysis L.Z., and M.F. evaluated the RNA sequencing data. M.F. supervised the study and L.Z., K.v.V. and M.F. wrote the manuscript.

FUNDING

K.v.V., and M.F. were supported by the German Federal Ministry of Education and Research (BMBF) within the consortium InfectControl 2020 (Project NeoBiom, grant ID 03ZZ0829C). D.P., K.v.V. and M.F. were supported by the national platform for zoonosis research (Project NAMPatIn, grant ID 01KI1906). M.F. received support by the Freie Universität Berlin within the Excellence Initiative of the German Research Foundation.

ACKNOWLEDGEMENTS

We thank Prof. Simone Bergmann (Institute of Microbiology, Technische Universität Braunschweig, Braunschweig, Germany) and Dr. Karsten Tedin (Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany) for critically reading the manuscript. We acknowledge support by the Open Access Publication Initiative of Freie Universität Berlin.

REFERENCES

Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, et al. (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285:736–739. https://doi.org/10.1126/science.285.5428.736.

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data.

Batiuk MY, de Vin F, Duque SI, Li C, Saito T, Saido T, Fiers M, Belgard TG, et al. (2017) An immunoaffinity-based method for isolating ultrapure adult astrocytes based on ATP1B2 targeting by the ACSA-2 antibody. J Biol Chem 292:8874–8891. https://doi.org/10.1074/jbc.M116.765313.

Berard JL, Zarruk JG, Arbour N, Prat A, Yong VW, Jacques FH, Akira S, David S (2012) Lipocalin 2 is a novel immune mediator of experimental autoimmune encephalomyelitis pathogenesis and is modulated in multiple sclerosis. Glia 60:1145–1159. https://doi.org/10.1002/glia.22342.

Bernaus A, Blanco S, Sevilla A (2020) Glia Crosstalk in Neuroinflammatory Diseases. Front Cell Neurosci 14:209. https://doi.org/10.3389/fncel.2020.00209.

Bohlen CJ, Bennett FC, Bennett ML (2019) Isolation and Culture of Microglia. Curr Protoc Immunol 125:e70.

Bohlen CJ, Bennett FC, Tucker AF, Collins HY, Mulinyawe SB, Barres BA (2017) Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures. Neuron 94(759–773):e758.

Bordt EA, Block CL, Petrozziello T, Sadri-Vakili G, Smith CJ, Edlow AG, Bilbo SD (2020) Isolation of Microglia from Mouse or Human Tissue. STAR Protoc 1. https://doi.org/10.1016/j.xpro.2020.100035.

Borrego F (2013) The CD300 molecules: an emerging family of regulators of the immune system. Blood 121:1951–1960. https://doi.org/10.1182/blood-2012-09-435057.

Brandt KJ, Fickentscher C, Kruithof EK, de Moerloose P (2013) TLR2 ligands induce NF-kappaB activation from endosomal compartments of human monocytes. PLoS One 8:e80743.

Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, Koeglsperger T, Dake B, et al. (2014) Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. Nat Neurosci 17:131–143. https://doi.org/10.1038/nn.3599.

Chen SH, Oyarzabal EA, Sung YF, Chu CH, Wang Q, Chen SL, Lu RB, Hong JS (2015) Microglial regulation of immunological and neuroprotective functions of astroglia. Glia 63:118–131. https://doi.org/10.1002/glia.22738.

Cho SH, Chen JA, Sayed F, Ward ME, Gao F, Nguyen TA, Krabbe G, Sohn PD, et al. (2015) SIRT1 deficiency in microglia contributes to cognitive decline in aging and neurodegeneration via epigenetic regulation of IL-1beta. J Neurosci 35:807–818. https://doi.org/10.1523/JNEUROSCI.2939-14.2015.

Comas-Casellas E, Martinez-Barriocanal A, Miro F, Ejarque-Ortiz A, Schwartz Jr S, Martin M, Sayos J (2012) Cloning and characterization of CD300d, a novel member of the human CD300 family of immune receptors. J Biol Chem 287:9682–9693. https://doi.org/10.1074/jbc.M111.279224.

Crain JM, Nikodemova M, Watters JJ (2009) Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia. J Neuroinflammation 6:24. https://doi.org/10.1186/1742-2094-6-24.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, et al. (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21. https://doi.org/10.1093/bioinformatics/bts635.

Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, Huber W (2005) BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics 21:3439–3440. https://doi.org/10.1093/bioinformatics/bti525.

Ebert S, Gerber J, Bader S, Muhlhauser F, Brechtel K, Mitchell TJ, Nau R (2005) Dose-dependent activation of microglial cells by Toll-like receptor agonists alone and in combination. J Neuroimmunol 159:87–96. https://doi.org/10.1016/j.jneuroim.2004.10.005.

Fiebich BL, Batista CRA, Saliba SW, Yousif NM, de Oliveira ACP (2018) Role of Microglia TLRs in Neurodegeneration. Front Cell Neurosci 12:329. https://doi.org/10.3389/fncel.2018.00329.

Foo LC, Allen NJ, Bushong EA, Ventura PB, Chung WS, Zhou L, Cahoy JD, Daneman R, et al. (2011) Development of a method for the purification and culture of rodent astrocytes. Neuron 71:799–811. https://doi.org/10.1016/j.neuron.2011.07.022.

Gosselin D, Skola D, Coufal NG, Holtman IR, Schlachetzki JCM, Sajti E, Jaeger BN, O'Connor C, et al. (2017) An environment-dependent transcriptional network specifies human microglia identity. Science 356. https://doi.org/10.1126/science.aal3222.

Greenhalgh AD, David S, Bennett FC (2020) Immune cell regulation of glia during CNS injury and disease. Nat Rev Neurosci 21:139–152. https://doi.org/10.1038/s41583-020-0263-9.

Harms AS, Tansey MG (2013) Isolation of murine postnatal brain microglia for phenotypic characterization using magnetic cell separation technology. Methods Mol Biol 1041:33–39. https://doi.org/10.1007/978-1-62703-520-0_5.

He Y, Taylor N, Yao X, Bhattacharya A (2021) Mouse primary microglia respond differently to LPS and poly(I:C) *in vitro*. Sci Rep 11:10447. https://doi.org/10.1038/s41598-021-89777-1.

Holm TH, Draeby D, Owens T (2012) Microglia are required for astroglial Toll-like receptor 4 response and for optimal TLR2 and TLR3 response. Glia 60:630–638. https://doi.org/10.1002/glia.22296.

Holt LM, Stoyanof ST, Olsen ML (2019) Magnetic Cell Sorting for *In Vivo* and *In Vitro* Astrocyte, Neuron, and Microglia Analysis. Curr Protoc Neurosci 88:e71.

Jha MK, Jo M, Kim JH, Suk K (2019) Microglia-Astrocyte Crosstalk: An Intimate Molecular Conversation. Neuroscientist 25:227–240. https://doi.org/10.1177/1073858418783959.

Jin LW, Horiuchi M, Wulff H, Liu XB, Cortopassi GA, Erickson JD, Maezawa I (2015) Dysregulation of glutamine transporter SNAT1 in Rett syndrome microglia: a mechanism for mitochondrial dysfunction and neurotoxicity. J Neurosci 35:2516–2529. https://doi.org/10.1523/JNEUROSCI.2778-14.2015.

Jin M, Kim JH, Jang E, Lee YM, Soo Han H, Woo DK, Park DH, Kook H, et al. (2014) Lipocalin-2 deficiency attenuates neuroinflammation and brain injury after transient middle cerebral artery occlusion in mice. J Cereb Blood Flow Metab 34:1306–1314. https://doi.org/10.1038/jcbfm.2014.83.

Kantzer CG, Boutin C, Herzig ID, Wittwer C, Reiss S, Tiveron MC, Drewes J, Rockel TD, et al. (2017) Anti-ACSA-2 defines a novel monoclonal antibody for prospective isolation of living neonatal and adult astrocytes. Glia 65:990–1004. https://doi.org/10.1002/glia.23140.

Keller D, Ero C, Markram H (2018) Cell Densities in the Mouse Brain: A Systematic Review. Front Neuroanat 12:83. https://doi.org/10.3389/fnana.2018.00083.

Kim H, Lee S, Park HC, Lee WH, Lee MS, Suk K (2011) Modulation of glial and neuronal migration by lipocalin-2 in zebrafish. Immune Netw 11:342–347. https://doi.org/10.4110/in.2011.11.6.342.

Kremer M, Henn A, Kolb C, Basler M, Moebius J, Guillaume B, Leist M, Van den Eynde BJ, et al. (2010) Reduced immunoproteasome formation and accumulation of immunoproteasomal precursors in the brains of lymphocytic choriomeningitis virus-infected mice. J Immunol 185:5549–5560. https://doi.org/10.4049/jimmunol.1001517.

Kreutzberg GW (1995) Microglia, the first line of defence in brain pathologies. Arzneimittelforschung 45:357–360.

Krueger F (2012) Trim Galore!: A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files, with some extra functionality for MspI-digested RRBS-type (Reduced Representation Bisufite-Seq) libraries.

Kwon HS, Koh SH (2020) Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. Transl Neurodegener 9:42. https://doi.org/10.1186/s40035-020-00221-2.

Langstein J, Becke FM, Sollner L, Krause G, Brockhoff G, Kreutz M, Andreesen R, Schwarz H (2000) Comparative analysis of CD137 and LPS effects on monocyte activation, survival, and proliferation. Biochem Biophys Res Commun 273:117–122. https://doi.org/10.1006/bbrc.2000.2889.

Langstein J, Michel J, Fritsche J, Kreutz M, Andreesen R, Schwarz H (1998) CD137 (ILA/4-1BB), a member of the TNF receptor family, induces monocyte activation via bidirectional signaling. J Immunol 160:2488–2494.

Lee S, Kim JH, Kim JH, Seo JW, Han HS, Lee WH, Mori K, Nakao K, et al. (2011) Lipocalin-2 Is a chemokine inducer in the central nervous system: role of chemokine ligand 10 (CXCL10) in lipocalin-2-induced cell migration. J Biol Chem 286:43855–43870. https://doi.org/10.1074/jbc.M111.299248.

Lee S, Park JY, Lee WH, Kim H, Park HC, Mori K, Suk K (2009) Lipocalin-2 is an autocrine mediator of reactive astrocytosis. J Neurosci 29:234–249. https://doi.org/10.1523/JNEUROSCI.5273-08.2009.

Lehmann SM, Kruger C, Park B, Derkow K, Rosenberger K, Baumgart J, Trimbuch T, Eom G, et al. (2012) An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci 15:827–835. https://doi.org/10.1038/nn.3113.

Lehnardt S (2010) Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58:253–263. https://doi.org/10.1002/glia.20928.

Li L, Acioglu C, Heary RF, Elkabes S (2021) Role of astroglial toll-like receptors (TLRs) in central nervous system infections, injury and neurodegenerative diseases. Brain Behav Immun 91:740–755. https://doi.org/10.1016/j.bbi.2020.10.007.

Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30:923–930. https://doi.org/10.1093/bioinformatics/btt656.

Lima TZ, Sardinha LR, Sayos J, Mello LE, Peluffo H (2017) Astrocytic Expression of the Immunoreceptor CD300f Protects Hippocampal Neurons from Amyloid-beta Oligomer Toxicity *In Vitro*. Curr Alzheimer Res 14:778–783. https://doi.org/10.2174/1567205014666170202121709.

Liu LR, Liu JC, Bao JS, Bai QQ, Wang GQ (2020) Interaction of Microglia and Astrocytes in the Neurovascular Unit. Front Immunol 11:1024. https://doi.org/10.3389/fimmu.2020.01024.

Liu Q, Nilsen-Hamilton M (1995) Identification of a new acute phase protein. J Biol Chem 270:22565–22570. https://doi.org/10.1074/jbc.270.38.22565.

Lombardi V, Van Overtvelt L, Horiot S, Moussu H, Chabre H, Louise A, Balazuc AM, Mascarell L, et al. (2008) Toll-like receptor 2 agonist Pam3CSK4 enhances the induction of antigen-specific tolerance via the sublingual route. Clin Exp Allergy 38:1819–1829. https://doi.org/10.1111/j.1365-2222.2008.03056.x.

Losciuto S, Dorban G, Gabel S, Gustin A, Hoenen C, Grandbarbe L, Heuschling P, Heurtaux T (2012) An efficient method to limit microglia-dependent effects in astroglial cultures. J Neurosci Methods 207:59–71. https://doi.org/10.1016/j.jneumeth.2012.03.010.

Matejuk A, Ransohoff RM (2020) Crosstalk between astrocytes and microglia: an overview. Front Immunol 11:1416. https://doi.org/10.3389/fimmu.2020.01416.

Matejuk A, Vandenbark AA, Offner H (2021) Cross-talk of the CNS with immune cells and functions in health and disease. Front Neurol 12. https://doi.org/10.3389/fneur.2021.672455 672455.

Min KJ, Yang MS, Kim SU, Jou I, Joe EH (2006) Astrocytes induce hemeoxygenase-1 expression in microglia: a feasible mechanism for preventing excessive brain inflammation. J Neurosci 26:1880–1887. https://doi.org/10.1523/JNEUROSCI.3696-05.2006.

Norden DM, Fenn AM, Dugan A, Godbout JP (2014) TGFbeta produced by IL-10 redirected astrocytes attenuates microglial activation. Glia 62:881–895. https://doi.org/10.1002/glia.22647.

Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV (2009) Toll-like receptors in neurodegeneration. Brain Res Rev 59:278–292. https://doi.org/10.1016/j.brainresrev.2008.09.001.

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 97:13766–13771. https://doi.org/10.1073/pnas.250476497.

Pan J, Wan J (2020) Methodological comparison of FACS and MACS isolation of enriched microglia and astrocytes from mouse brain. J Immunol Methods 486. https://doi.org/10.1016/j.jim.2020.112834 112834.

Peluffo H, Ali-Ruiz D, Ejarque-Ortiz A, Heras-Alvarez V, Comas-Casellas E, Martinez-Barriocanal A, Kamaid A, Alvarez-Errico D, et al. (2012) Overexpression of the immunoreceptor CD300f has a neuroprotective role in a model of acute brain injury. Brain Pathol 22:318–328. https://doi.org/10.1111/j.1750-3639.2011.00537.x.

Ranjbar Taklimie F, Gasterich N, Scheld M, Weiskirchen R, Beyer C, Clarner T, Zendedel A (2019) Hypoxia Induces Astrocyte-Derived Lipocalin-2 in Ischemic Stroke. Int J Mol Sci 20. https://doi.org/10.3390/ijms20061271.

Reali C, Curto M, Sogos V, Scintu F, Pauly S, Schwarz H, Gremo F (2003) Expression of CD137 and its ligand in human neurons, astrocytes, and microglia: modulation by FGF-2. J Neurosci Res 74:67–73. https://doi.org/10.1002/jnr.10727.

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. https://doi.org/10.1093/bioinformatics/btp616.

Rodgers KR, Lin Y, Langan TJ, Iwakura Y, Chou RC (2020) Innate Immune Functions of Astrocytes are Dependent Upon Tumor

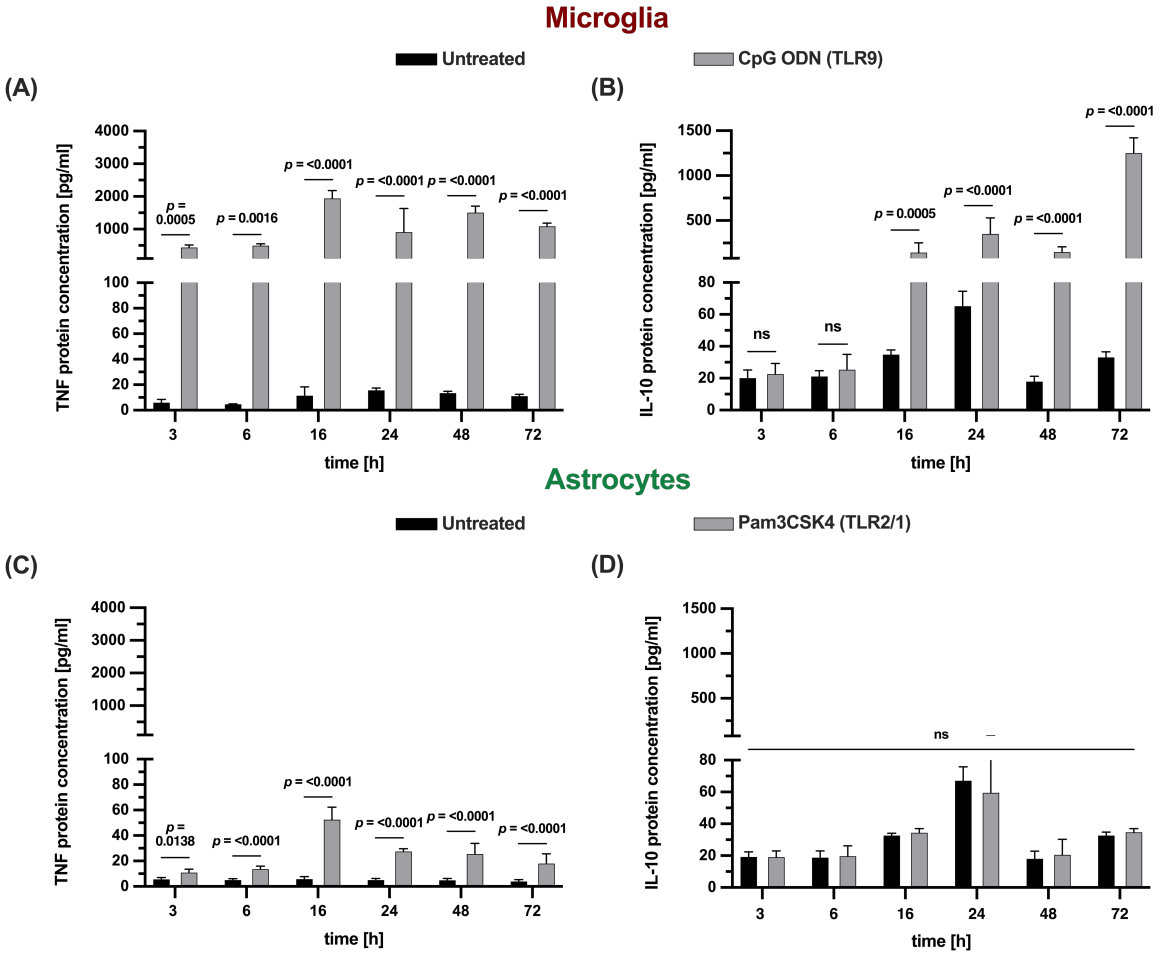
Necrosis Factor-Alpha. Sci Rep 10:7047. https://doi.org/10.1038/s41598-020-63766-2.
Rosenberger K, Derkow K, Dembny P, Kruger C, Schott E, Lehnardt S (2014) The impact of single and pairwise Toll-like receptor activation on neuroinflammation and neurodegeneration. J Neuroinflammation 11:166. https://doi.org/10.1186/s12974-014-0166-7.
Rostami J, Mothes T, Kolahdouzan M, Eriksson O, Moslem M, Bergstrom J, Ingelsson M, O'Callaghan P, et al. (2021) Crosstalk between astrocytes and microglia results in increased degradation of alpha-synuclein and amyloid-beta aggregates. J Neuroinflammation 18:124. https://doi.org/10.1186/s12974-021-02158-3.
Saura J (2007) Microglial cells in astroglial cultures: a cautionary note. J Neuroinflammation 4:26. https://doi.org/10.1186/1742-2094-4-26.
Sugiyama M, Banno R, Yaginuma H, Taki K, Mizoguchi A, Tsunekawa T, Onoue T, Takagi H, et al. (2020) Hypothalamic glial cells isolated by MACS reveal that microglia and astrocytes induce hypothalamic inflammation via different processes under high-fat diet conditions. Neurochem Int 136. https://doi.org/10.1016/j.neuint.2020.104733 104733.
Syed MM, Phulwani NK, Kielian T (2007) Tumor necrosis factor-alpha (TNF-alpha) regulates Toll-like receptor 2 (TLR2) expression in microglia. J Neurochem 103:1461–1471. https://doi.org/10.1111/j.1471-4159.2007.04838.x.
Timmerman R, Burm SM, Bajramovic JJ (2018) An overview of *in vitro* methods to study microglia. Front Cell Neurosci 12:242. https://doi.org/10.3389/fncel.2018.00242.
von Bartheld CS, Bahney J, Herculano-Houzel S (2016) The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. J Comp Neurol 524:3865–3895. https://doi.org/10.1002/cne.24040.
Wang G, Weng YC, Han X, Whaley JD, McCrae KR, Chou WH (2015) Lipocalin-2 released in response to cerebral ischaemia mediates reperfusion injury in mice. J Cell Mol Med 19:1637–1645. https://doi.org/10.1111/jcmm.12538.
Williamson LL, Sholar PW, Mistry RS, Smith SH, Bilbo SD (2011) Microglia and memory: modulation by early-life infection. J Neurosci 31:15511–15521. https://doi.org/10.1523/JNEUROSCI.3688-11.2011.
Yang L, Zhou Y, Jia H, Qi Y, Tu S, Shao A (2020) Affective immunology: the crosstalk between microglia and astrocytes plays key role? Front Immunol 11:1818. https://doi.org/10.3389/fimmu.2020.01818.
Yu G, Wang LG, Han Y, He QY (2012) clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16:284–287. https://doi.org/10.1089/omi.2011.0118.
Zelenka L, Pägelow D, Krüger C, Seele J, Ebner F, Rausch S, Rohde M, Lehnardt S, et al. (2022) Novel protocol for the isolation of highly purified neonatal murine microglia and astrocytes. J Neurosci Methods 366. https://doi.org/10.1016/j.jneumeth.2021.109420 109420.
Zhang Y, Sloan SA, Clarke LE, Caneda C, Plaza CA, Blumenthal PD, Vogel H, Steinberg GK, et al. (2016) Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. Neuron 89:37–53. https://doi.org/10.1016/j.neuron.2015.11.013.
Zhao N, Xu X, Jiang Y, Gao J, Wang F, Xu X, Wen Z, Xie Y, et al. (2019) Lipocalin-2 may produce damaging effect after cerebral ischemia by inducing astrocytes classical activation. J Neuroinflammation 16:168. https://doi.org/10.1186/s12974-019-1556-7.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2023.05.001.

(Received 21 July 2022, Accepted 2 May 2023)
(Available online 28 June 2023)

2.3 Unpublished data

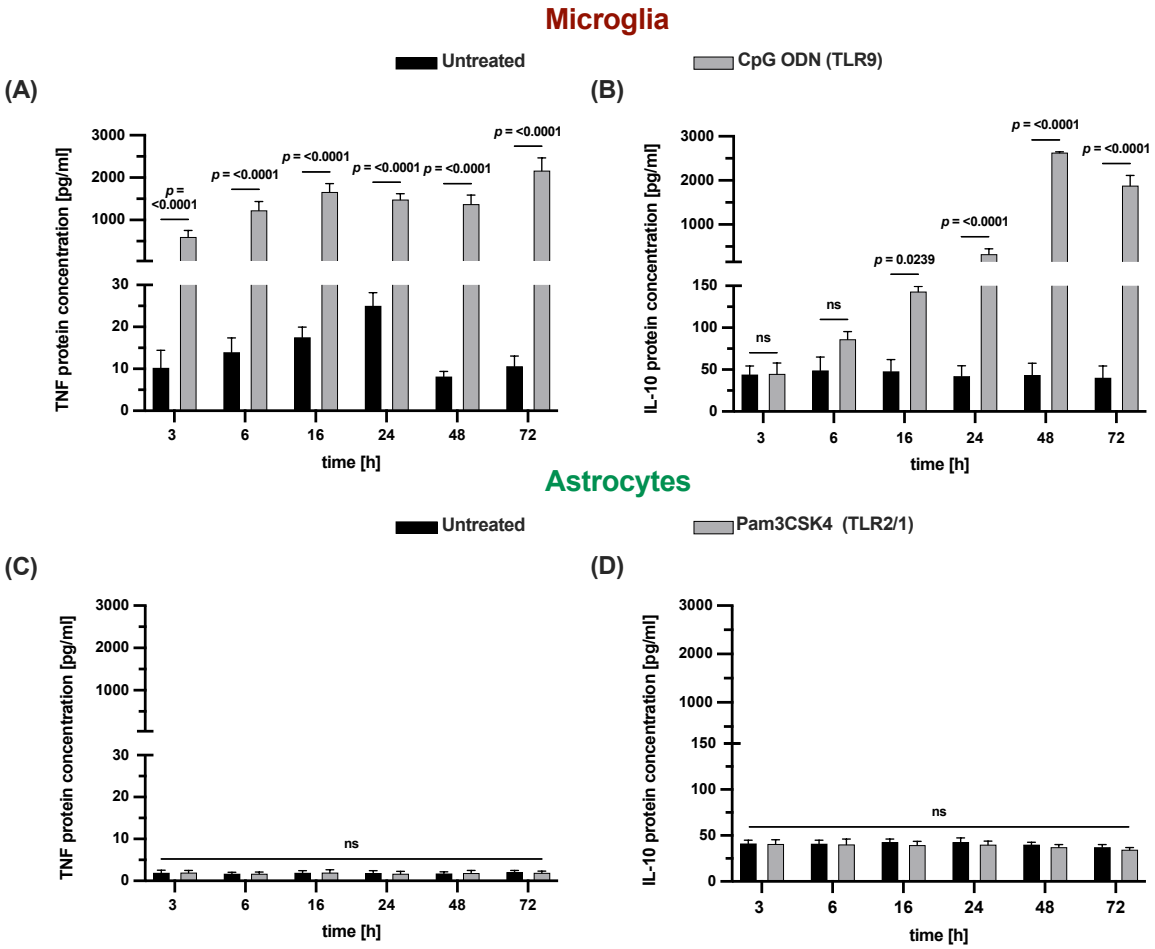


Unpublished data figure 1: Kinetics of wild-type (WT) microglia and astrocytes upon TLR stimulation.

Isolated microglia and astrocytes derived from WT (wild-type, C57BL/6n) 0 to 2-day-old neonatal mice were seeded into 24-well plates (2 x 10^5/ml). Subsequently, microglia were incubated with CpG ODN (1 µM) **(A/B)** and astrocytes with Pam3CSK4 (100 ng/ml) **(C/D)**, or were left untreated (control) for 3, 6, 16, 24, 48, and 72 h. Medium served as negative control (untreated). Supernatants (SN) were collected after the indicated time points and analyzed for TNF **(A/C)** and IL-10 **(B/D)** amounts by sandwich ELISA. Results are expressed as mean + SD. An ANOVA was performed followed by Bonferroni's *post hoc* test by means of triplicates, derived from different harvests from biological duplicates, which were performed in at least 2 representative ELISA analyses. Statistical significances as indicated; *p* values >0.05 are indicated as not significant, ns. F values for each panel are as followed **(A)** Time: (5, 122) = 26.16 (*p* = <0.0001); treatment: F (1, 122) = 512.8 (*p* = <0.0001); time x treatment: F (5, 122) = 25.74 (*p* = <0.0001). **(B)** Time: F (5, 113) = 183.5 (*p* = <0.0001); treatment: F (1, 113) = 574.1 (*p* = <0.0001); time x treatment: F (5, 113) = 173.6 (*p* = <0.0001). **(C)** Time: F (5, 87) = 73.34 (*p* = <0.0001); treatment: F (1, 21) = 343.2 (*p* = <0.0001); time x treatment: F (5, 87) = 68.79 (*p* = <0.0001). **(D)** Time: F (5, 97) = 128.7 (*p* = <0.0001); treatment: F (1, 22) = 0.04295 (ns); time x treatment: F (5, 97) = 1.819 (ns).

Unpublished data table 1: TNF and IL-10 protein release of wild-type (WT) microglia and astrocytes upon CpG ODN (microglia) and Pam3CSK4 (astrocytes) activation, respectively.
Microglia and astrocytes were isolated from 0 to 2-day-old wild-type (WT, C57BL/6n) neonatal mice and seeded into 24-well plates (2 x 10^5/ml). Microglia were stimulated with CpG ODN (1 µM), astrocytes with Pam3CSK4 (100 ng/ml), or were left untreated (-). Supernatants were collected after 3, 6, 16, 24, 48, and 72 h and analyzed for TNF and IL-10 amounts by sandwich ELISA. Cytokine concentrations are represented as means of triplicates + SD, derived from different harvests from biological duplicates, which were performed in at least two representative ELISA analyses.

WT												
Time point [h]	3		6		16		24		48		72	
Microglia												
Treatment	-	CpG ODN	-	CpG ODN	-	CpG ODN	-	CpG ODN	-	CpG ODN	-	CpG ODN
TNF [pg/ml]	6 (+ SD 3)	441 (+ SD 74)	5 (+ SD 0)	497 (+ SD 57)	11 (+ SD 7)	1939 (+ SD 241)	16 (+ SD 2)	911 (+ SD 723)	13 (+ SD 1)	1508 (+ SD 197)	11 (+ SD 1)	1087 (+ SD 98)
IL-10 [pg/ml]	20 (+ SD 1)	23 (+ SD 7)	21 (+ SD 4)	25 (+ SD 10)	35 (+ SD 3)	143 (+ SD 108)	65 (+ SD 9)	350 (+ SD 180)	18 (+ SD 3)	149 (+ SD 60)	33 (+ SD 3)	1251 (+ SD 170)
Astrocytes												
Treatment	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4
TNF [pg/ml]	6 (+ SD 2)	11 (+ SD 3)	5 (+ SD 1)	14 (+ SD 2)	6 (+ SD 2)	53 (+ SD 10)	5 (+ SD 1)	27 (+ SD 2)	5 (+ SD 2)	26 (+ SD 8)	4 (+ SD 1)	18 (+ SD 8)
IL-10 [pg/ml]	19 (+ SD 3)	19 (+ SD 4)	19 (+ SD 4)	20 (+ SD 7)	33 (+ SD 1)	34 (+ SD 3)	67 (+ SD 9)	56 (+ SD 6)	18 (+ SD 5)	21 (+ SD 10)	33 (+ SD 2)	35 (+ SD 2)



Unpublished data figure 2: Kinetics of TLR2-knockout (TLR2-KO) microglia and astrocytes upon TLR stimulation.

Isolated microglia and astrocytes derived from TLR2-knockout (TLR2-KO, *Tlr2*$^{-/-}$, C57BL/6.129-Tlr2tm1Kir/J) 0 to 2-day-old neonatal mice were seeded into 24-well plates (2 x 10^5/ml). Subsequently, microglia were incubated with CpG ODN (1 µM) **(A/B**) and astrocytes with Pam3CSK4 (100 ng/ml) **(C/D)**, or were left untreated (control) for 3, 6, 16, 24, 48, and 72 h. Medium served as negative control (untreated). Supernatants (SN) were collected after the indicated time points and analyzed for TNF **(A/C)** and IL-10 **(B/D)** amounts by sandwich ELISA. Results are expressed as mean + SD. An ANOVA was performed followed by Bonferroni's *post hoc* test of means of triplicates, derived from different harvests from biological duplicates, which were performed in at least 2 representative ELISA analyses. Statistical significances as indicated; *p* values >0.05 are indicated as not significant, ns. F values for each panel are as followed **(A)** Time: (5, 75) = 79.69 (*p* = <0.001); treatment: F (1, 22) = 1544 (*p* = <0.001); time x treatment: F (5, 75) = 79.03 (*p* = <0.001). **(B)** Time: F (5, 60) = 1159 (*p* = <0.001); treatment: F (1, 12) = 3671 (*p* = <0.001); time x treatment: F (5, 25) = 1201 (*p* = <0.001). **(C)** Time: F (5, 89) = 1741 (ns); treatment: F (1, 22) = 0.001162 (ns); time x treatment: F (5, 89) = 0.4874 (ns). **(D)** Time: F (5, 93) = 7669 (*p* = <0.001); treatment: F (1, 22) = 4273 (ns); time x treatment: F (5, 93) = 0.6684 (ns).

Unpublished data table 2: TNF and IL-10 protein release of TLR2-knockout (TLR2-KO) microglia and astrocytes upon CpG ODN (microglia) and Pam3CSK4 (astrocytes) activation.

Microglia and astrocytes were isolated from 0 to 2-day-old TLR2-knockout (TLR2-KO, *Tlr2*$^{-/-}$, C57BL/6.129-Tlr2tm1Kir/J) neonatal mice, and seeded into 24-well plates (2 x 10^5/ml). Microglia were stimulated with CpG ODN (1 µM), astrocytes with Pam3CSK4 (100 ng/ml), or were left untreated (-). Supernatants were collected after 3, 6, 16, 24, 48, and 72 h and analyzed for TNF and IL-10 amounts by sandwich ELISA. Cytokine concentrations are represented as means of triplicates + SD, derived from different harvests from biological duplicates, which were performed in at least two representative ELISA analyses.

TLR2-KO												
Time point [h]	3		6		16		24		48		72	
Microglia												
Treatment	-	CpG ODN	-	CpG ODN	-	CpG ODN	-	CpG ODN	-	CpG ODN	-	CpG ODN
TNF [pg/ml]	10 (+ SD 4)	595 (+ SD 156)	14 (+ SD 3)	1227 (+ SD 207)	17 (+ SD 2)	1657 (+ SD 200)	25 (+ SD 3)	1481 (+ SD 138)	8 (+ SD 1)	1373 (+ SD 214)	11 (+ SD 2)	2165 (+ SD 300)
IL-10 [pg/ml]	44 (+ SD 10)	45 (+ SD 13)	48 (+ SD 16)	86 (+ SD 9)	48 (+ SD 14)	143 (+ SD 6)	42 (+ SD 12)	323 (+ SD 128)	43 (+ SD 14)	2627 (+ SD 22)	40 (+ SD 14)	1880 (+ SD 234)
Astrocytes												
Treatment	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-	Pam3CSK4	-
TNF [pg/ml]	2 (+ SD 1)	2 (+ SD 1)	2 (+ SD 0)	2 (+ SD 0)	2 (+ SD 1)	2 (+ SD 1)	2 (+ SD 1)	2 (+ SD 1)	2 (+ SD 0)	2 (+ SD 1)	2 (+ SD 0)	2 (+ SD 0)
IL-10 [pg/ml]	41 (+ SD 4)	41 (+ SD 5)	41 (+ SD 4)	40 (+ SD 6)	43 (+ SD 3)	39 (+ SD 4)	43 (+ SD 5)	40 (+ SD 4)	40 (+ SD 3)	37 (+ SD 3)	37 (+ SD 3)	34 (+ SD 2)

3 Discussion

3.1 Development of the novel glial isolation protocol

In the first publication of this thesis, we improved existing glial *in vitro* protocols to isolate primary microglia and astrocytes from neonatal mouse brains. Subsequently, these isolated glial cells were directly purified using a modified MACS protocol (**Publication I, Figure 3**).

For the cultivation of primary microglia and astrocytes we optimized a direct isolation approach based on the previously mentioned mixed glial cultivation protocols (Booher and Sensenbrenner 1972; McCarthy and de Vellis 1980; Giulian and Baker 1986). To enhance glial cell yields, we stimulated mixed glial culture with macrophage colony-stimulating factor (M-CSF) derived from L929 fibroblast cultures, based on earlier findings (Boltz-Nitulescu et al. 1987; Ladner et al. 1988; Sawada et al. 1990).

M-CSF binds to the tyrosine kinase receptor (M-CSFR), which is expressed by a range of mononuclear phagocytes including microglia (Akiyama et al. 1994; Du Yan et al. 1997). This activation results in proliferation, differentiation, and survival of these cells (Wang et al. 2012; Elmore et al. 2014; Kierdorf and Prinz 2019; Priller and Prinz 2019). Consequently, mice lacking M-CSFR or the inhibition of M-CSFR in mice leads to a lack of microglia in the entire brain during all developmental stages (Zuiderwijk-Sick et al. 2007; Ginhoux et al. 2010; Elmore et al. 2014; Hagemeyer et al. 2017). To understand the effect of M-CSF on glial yields, we compared the microglial and astrocyte yields before and after every harvest with and without M-CSF (**Publication I**). As expected, we saw a positive effect of M-CSF on the microglial yields due to enhanced microglial proliferation and differentiation rates in line with the published data (Ginhoux et al. 2010; Elmore et al. 2014).

Nevertheless, the addition of M-CSF to mixed glial cultures can also influence microglial morphology (Sawada et al. 1990; Suzumura et al. 1990; Suzumura et al. 1991; Liu et al. 1994; Fujita et al. 1996) and activation (Imai and Kohsaka 2002; Smith et al. 2013). Future experiments could be conducted to shed more light on the impact of M-CSF on the morphology and the activation status of isolated microglial cells. For instance, immunofluorescence could be used to assess the morphology and phagocytosis assays to analyze the activation status (Hu et al. 2022), since activated microglia display enhanced phagocytic activity (Stence et al. 2001). However, Hu et al. recently demonstrated that the stimulation of primary mixed glial cultures derived from neonatal rats with fibroblast-conditioned medium (25%) containing growth factor including M-CSF, significantly improved the viability and purity of microglial cultures. Consequently, the total incubation time in comparison to unstimulated cultures was

reduced (Hu et al. 2022), which further confirms our theory of M-CSF acting as a “booster” of microglial yields (**Publication I**). In addition to this, microglia from stimulated mixed glial cultures displayed no major differences regarding phagocytic function or baseline phenotype markers and remained responsive to different stimuli in comparison to microglia isolated from unstimulated mixed glial cultures (Hu et al. 2022). More importantly, with special regard to our microglial activation experiments (**Publication I and II**), Hu and co-authors also demonstrated that M-CSF did not have an impact on microglial receptor gene expression, including *Tlr2* (Hu et al. 2022). The differences between the individual M-CSF stimulated microglial yields (**Publication I**) could be explained by the fact that the harvests differed in their age, which resulted in the shortening of the telomeres: Studies have shown that in contrast to *in vitro* astrocyte cultures, microglial cultures are subject to replicative senescence upon mitogen stimulation which is characterized by telomeres shortening (Flanary and Streit 2004; Streit 2006).

The addition of M-CSF had a negative effect on astrocytes, as yields were lower here in comparison to the unstimulated astrocyte cultures. To the best of our knowledge, the influence of M-CSF on *in vitro* culturing of astrocytes has not been described yet. However, in contrast to microglia, astrocytes do not express M-CSFR on their surface (Sawada et al. 1990). Therefore, it is conceivable that astrocytes must compete for nutrition and space with the growing number of microglia as astrocytes do not survive more than a few days in nutrient-poor cultures (Hao et al. 1991). To avoid this effect, it would be feasible to stimulate only some of the cell culture flasks with M-CSF to maintain a maximum of astrocyte yields in the unstimulated flasks. However, due to the significantly enhanced microglial yields, the addition of M-CSF was one important key stone of our aim reducing animal experiments in compliance with the animal testing principles of the 3Rs, namely replacement, reduction, and refinement (Kirk 2018).

One important limitation of *in vitro* glial isolation protocols which are based on the traditional “indirect” mixed glial cultivation approach (McCarthy and de Vellis 1980; Giulian and Baker 1986) is that glial cells are cultivated for weeks under microenvironmental conditions which do not reflect the ones in the healthy CNS. This especially holds true for media supplemented with serum since under normal healthy brain conditions microglia and astrocytes are shielded from serum by the BBB (Timmerman et al. 2018; Guttenplan and Liddelow 2019). In comparison to the manufacturer’s standard MACS purification protocol, which works without the cultivation of mixed glial cultures and applies whole brains instead, this is a drawback of our presented novel protocol (**Publication I**). Moreover, it has been shown that the transcriptome profiles, as well as the morphology, of *in vitro* astrocytes cultivated in serum-free medium were more similar to *in vivo* astrocytes, than to astrocytes cultured in the presence of

serum (Cahoy et al. 2008; Doyle et al. 2008; Foo et al. 2011; Zhang et al. 2016; Holt et al. 2019; Prah et al. 2019). This also applies for microglia as studies have demonstrated that within a few hours *in vitro* microglia cultivated in serum-exposed medium significantly downregulated their signature genes like transmembrane protein 119 (*Tmem119*) and *P2ry12* leading to reduced protein expression. In addition to this, they upregulated genes which are only typical for *in vivo* microglia in pathological conditions and are associated with enhanced phagocytic activity (Butovsky et al. 2014; Bohlen et al. 2017; Gosselin et al. 2017; Bohlen et al. 2019). Thus, it should be considered that the cultivation of primary microglia and astrocytes under certain conditions, such as the usage of serum-conditioned medium, limits the ability to predict the behavior of *in vitro* microglia and astrocytes. Despite the aforementioned shortcomings of serum-exposed microglial cultures, murine microglia cultivated in serum-free systems still lose their signature genes such as *Tmem119* and spalt-like transcription factor 1 (*Sall1*), which has been also observed in microglia isolated from human brain tissue (Bohlen et al. 2017; Gosselin et al. 2017). Moreover, although “direct” isolation methods like immunopanning or immunomagnetic cell sorting from whole brain tissue are serum-free approaches, they have the big drawback of generating lower glial yields due to lower cell proliferation rates and thereby are more cost-intensive than the *in vitro* glial cell culturing protocol suggested here (**Publication I**) (Bohlen et al. 2017; Bohlen et al. 2019; Guttenplan and Liddelow 2019). Of note, higher glial cell yields due to supplemented serum has the crucial benefit of sacrificing less mice, which is in compliance with the principles of the 3R’s (Kirk 2018), as discussed earlier. We decided to isolate microglia and astrocytes from whole brain tissue as we wanted to maximize glial yields (**Publication I**). For other experimental set-ups such as studying brain diseases affecting distinct brain regions like AD, higher animal numbers might be required to generate sufficient glial cell numbers from the individual brain regions.

One important challenge when using *in vitro* protocols, based on the findings by McCarthy and de Vellis and Giulian and Baker (McCarthy and de Vellis 1980; Giulian and Baker 1986), is the contamination of astrocyte cultures with microglial cells. Apart from amoeboid microglia on top of the astrocyte monolayer, which can be removed by shaking, there are also ramified microglial cells which might be shielded under the astrocytic monolayer (Saura 2007; Crocker et al. 2008; Holm et al. 2012). As mentioned earlier, an approach to target these proliferating cells would be the addition of mitotic blockers like AraC when astrocytes become confluent around 10-14 days of cultivation (Holm et al. 2012; Lange et al. 2012). However, due to the previously mentioned profound negative effects on glial cell cultures, such as the activation of astrocytes, we did not add any chemical reagents to our mixed glial cultures. Instead, as an important element of our new approach, we purified cultivated microglia and astrocytes by using a modified MACS protocol with the help of anti-CD11b (microglia) and anti-ACSA-2

(ACSA-2: astrocyte cell surface antigen-2) (astrocytes) MicroBeads. As expected, the additional selection step reduced total microglial and astrocyte yields in comparison to the respective non-purified cultures. The impact of MACS on microglial and astrocyte viability and purity, was subsequently validated by FACS after every harvest (**Publication I**).

One major challenge distinguishing between the different CNS cells is the lack of suitable pan-markers for microglia and astrocytes (Jurga et al. 2020; Jurga et al. 2021). Therefore, to identify microglial cell populations we used two FACS markers. First, we used common leukocyte antigen CD45, a marker for nucleated hematopoietic cells (Thomas 1989; Trowbridge and Thomas 1994) as it was conventionally shown that microglia express low CD45 levels under physiological brain conditions (Sedgwick et al. 1991; Ford et al. 1995; Campanella et al. 2002). Secondly, the pan-myeloid marker CD11b was used, which is an activation marker for microglia in the intact brain (Chakrabarty et al. 2010; Jeong et al. 2013). Cell populations that were positive for CD45, CD11b or both markers were identified as microglia. FACS revealed that microglial viability and purity were nearly 100% high before performing MACS purification indicating that our novel protocol is already highly efficient for the isolation of viable and pure microglial cultures even without the additional purification step. In contrast to this, studies using the conventional anti-CD11b MACS Miltenyi Biotec protocol with minor modifications achieved microglial purity of only ~90-97%, as judged by FACS (Crain et al. 2009; Kremer et al. 2010; Williamson et al. 2011; Harms and Tansey 2013; Cho et al. 2015; Jin et al. 2015; Bordt et al. 2020; Pan and Wan 2020).

We observed two distinct $CD11b^+$ subpopulations differing in their CD45 expression before and after MACS purification of the microglial cultures. As expected, most of the cells were characterized as $CD45^-CD11b^+$ cells and were thus presumably resident microglia and not infiltrating hematogenous myeloid cells (Sedgwick et al. 1991; Ford et al. 1995; Becher and Antel 1996). Interestingly, the second smaller fraction consisted of $CD45^+CD11b^+$ cells. However, even though this subpopulation decreased with MACS purification, it was still present after this step. There are multiple possible explanations for the occurrence of this small $CD45^+CD11b^+$ cell subpopulation, which has been observed previously (Nikodemova and Watters 2012; Bedi et al. 2013; Wlodarczyk et al. 2014; Volden et al. 2015; Rangaraju et al. 2018; Honarpisheh et al. 2020; Schroeter et al. 2021): First of all, it has been shown that if microglia are activated, for example upon brain insult, they change their phenotype along with an enhanced activation marker expression, such as CD45 and CD11b (Timmerman et al. 2018; Schroeter et al. 2021). This was also confirmed by observations from Rangaraju et al. since they identified two distinct $CD11b^+$ populations differing in their CD45 expression in murine brains showing that the gene expression of the small $CD45^+CD11b^+$ population resembled the ones from activated microglia (Rangaraju et al. 2018). Yet, we cannot rule out

whether microglial cell activation occurred during the enzymatic and mechanical dissociation procedures of our protocol. However, Volden et al. showed that MACS-purified microglial cells were not activated by magnetic selection (Volden et al. 2015). As they could only detect minimal expression of the microglial activation marker ionized calcium-binding adapter molecule 1 (*Iba-1*), they concluded that MACS-purified microglia represent most likely a quiescent, resting phenotype (Volden et al. 2015). Moreover, this observation was confirmed by He and colleagues who showed that MACS using anti-CD11b MicroBeads was a suitable approach to keep the microglial transcriptome in a relatively quiescent state (He et al. 2018). In addition, a recent published study by Ocañas et al. confirmed that the isolation of microglia by neither MACS nor by FACS induced an *ex vivo* activational phenotype (Ocañas et al. 2022). Furthermore, as an alternative to the supplementation with transcription/translation inhibitors, they recommend to prevent *ex vivo* activation by performing the non-enzymatic steps at low temperatures, which is in accordance with our isolation protocol (**Publication I**) (Ocañas et al. 2022).

In line with our results, Volden et al. also identified two $CD11b^+$ cell subpopulations differing in their CD45 expression after enriching microglia from murine adult hypothalamus and cerebellum via anti-CD11b MicroBeads (Volden et al. 2015). In contrast to our assumption, they suggest that the $CD45^+CD11b^+$ population consists of perivascular microglia whereas the $CD45^-CD11b^+$ population might be parenchymal microglia. Nevertheless, since we did not discriminate between different brain regions, we cannot confirm these observations by Volden and co-authors. As we isolated microglia from intact mouse brains, the $CD45^+CD11b^+$ cell fraction might also be other myeloid cells such as infiltrating monocytes or monocyte-derived macrophages (Sedgwick et al. 1991; Ford et al. 1995; Campanella et al. 2002; Jeong et al. 2010; Martin et al. 2017) due to the high CD11b and CD45 expression, as we did not perfuse the mice. However, studies by Nikodemova and Watters showed the presence of two distinct subpopulations comprising of $CD45^+$ and $CD45^-$ cells even though microglia have been isolated and MACS-purified with anti-CD11b MicroBeads from perfused mice (Nikodemova and Watters 2012). Since these observations are in line with our results, it is more likely that the small fraction of $CD45^+CD11b^+$ cells were rather highly activated microglia, as discussed earlier, and not CNS-infiltrating myeloid cells. Moreover, Volden et al. observed the expression of *Cd11c* in MACS-purified microglial cultures (Volden et al. 2015) which is a marker for dendritic cells (DCs) as well as for pre-activated, so-called “primed”, microglia (Holtman et al. 2015; Benmamar-Badel et al. 2020). Their results are in accordance with a study by Wlodarczyk et al. showing an increased CD45 expression of a $CD11c^+$ microglial subpopulation during inflammation. Yet, this subpopulation was still discriminable from highly CD45 expressing blood-infiltrating cells (Wlodarczyk et al. 2014).

In conclusion, FACS revealed two distinct $CD11b^+$ subpopulations, differing in their CD45 expression which most likely represent microglia in different activation states (**Publication I**). These studies highlight the limitations of using CD45 as a FACS expression marker to sort microglia and discriminate them from peripherally derived myeloid populations in experimental mouse models. Besides these well-established markers, lately there have been also promising new microglia-specific markers developed such as Tmem119 (Bennett et al. 2016; Jurga et al. 2020). Yet, a recently published study by Honarpisheh et al. demonstrated in several *in vivo* mouse models that a subpopulation of activated adult rodent microglia can upregulate CD45 and downregulate microglial markers Tmem119 as well as P2RY12 after they were sorted by FACS (Honarpisheh et al. 2020). Therefore, to confirm our assumption of the $CD45^+CD11b^+$ cell fraction being highly activated microglia, it would be necessary to utilize additional markers. For instance, lymphocyte antigen complex 6C (Ly6C), lymphocyte antigen 6 complex locus G6D (Ly6G) (Getts et al. 2008; Saederup et al. 2010; Benakis et al. 2014; Ritzel et al. 2015; Haage et al. 2019; Schroeter et al. 2021) or the blood-derived leukocyte marker CCR2 (Mizutani et al. 2012; Wlodarczyk et al. 2017; Shen et al. 2022) could be used to more precisely discriminate between microglia and CNS-infiltrating myeloid cells in future studies. Another possibility would be to use CD11c as an additional marker to investigate whether the $CD45^+CD11b^+$ subpopulation consisted of “primed” microglia, as discussed previously (Wlodarczyk et al. 2014; Volden et al. 2015; Shen et al. 2022).

Astrocytes were identified using ACSA-2 as well as the most popular astrocytic marker GFAP. The latter is specific to reactive astrocytes as well as mature astrocytes since GFAP levels increase progressively throughout adult lifespan (Eng et al. 2000; Preston et al. 2019; Jurga et al. 2021) (**Publication I**). In comparison to the microglial cultures, the effect of MACS purification was even more striking on astrocyte culture purity. Whereas astrocyte viability was only slightly improved to 99% by the additional selection step, purity increased from only 45.7% on average to nearly 100% (**Publication I**). In contrast to our results, studies using anti-ACSA-2 MicroBeads with minor modifications to Miltenyi Biotec’s recommended protocol achieved notably lower viabilities (85-88%) and purities (<97%) of astrocytes, as judged by FACS (Batiuk et al. 2017; Kantzer et al. 2017; Pan and Wan 2020; Sugiyama et al. 2020). These lower values might be due to the additional papain-based enzymatic digestion step of whole brain tissue recommended by the manufacturer. Batiuk and colleagues suggested that the so far unknown target of ACSA-2 might be sodium/potassium-transporting ATPase subunit beta-1 (ATP1B), a single-pass transmembrane protein whose epitope is supposedly sensitive to papain (Batiuk et al. 2017). It is worth noting that our approach does not depend on this enzymatic dissociation step since we performed MACS purification of cultivated primary mixed glial cell suspensions instead of whole brain tissue, as recommend by Miltenyi Biotec. This in turn, might be another

benefit of our novel non-enzymatic protocol. In addition, by using field emission scanning electron microscopy (FESEM), we were subsequently able to provide evidence that the processes of MACS-purified astrocytes were retained and the cell integrity of their complex morphology preserved comparable to the one of the non-purified astrocytes, in accordance with the aforementioned studies by Holt and co-authors (Holt and Olsen 2016; Holt et al. 2019) (**Publication I**).

Before the selection of the astrocyte cultures via anti-ACSA-2 MicroBeads, we observed three sub-populations, positive for ACSA-2, GFAP or both which made up 45.7% in total. More than half of the cells (54.3%) did not express any of the two astrocyte markers, suggesting that they were contaminating cells of different cell types such as neurons and oligodendrocytes, as well as their precursor cells, microglia or ECs (Batiuk et al. 2017). Interestingly, further sub-gating of the ACSA-2$^-$GFAP$^-$ population of the non-purified astrocyte culture identified mostly CD11b$^+$, or CD45- and CD11b-positive cells. Due to their expression profile, these contaminating cells could be microglia in different activation states (CD45$^{-/+}$CD11b$^+$) or other CNS-resident cells derived from blood as discussed earlier for the microglial cultures (Sedgwick et al. 1991; Ford et al. 1995; Campanella et al. 2002; Jeong et al. 2010; Nikodemova and Watters 2012; Bedi et al. 2013; Benakis et al. 2014; Wlodarczyk et al. 2014; Rangaraju et al. 2018; Honarpisheh et al. 2020; Schroeter et al. 2021). The contaminating microglia might have been ramified microglial cells trapped under the astrocyte monolayer, which were shielded from the mechanical shaking forces (Saura 2007; Crocker et al. 2008; Holm et al. 2012).This further highlights the need of additional purification steps of cultivated astrocyte cultures. Sub-gating of the ACSA-2$^-$GFAP$^-$ population revealed a small CD45$^-$CD11b$^-$ sub-population which we could not identify with the applied FACS markers. Since the glial cell type astrocytes is a very heterogenous cell type these cells might be other astrocyte subtypes which need to be characterized by using additional FACS markers (Batiuk et al. 2020; Sofroniew 2020; Westergard and Rothstein 2020). Apart from GFAP, which mainly targets white matter astrocytes, the most common astrocytic markers are cytosolic S100 calcium-binding protein beta (S100β), which is mostly expressed by grey matter astrocytes, and aldehyde dehydrogenase 1 family member L1 (ALDH1L1), a new marker for astrocytes and astrocytic precursors (Cahoy et al. 2008; Yu et al. 2020; Jurga et al. 2021).

After MACS purification, >99% of the population were identified as presumably astrocytes, while only a small cell population of ACSA-2$^-$GFAP$^-$ cells remained unknown by the application of our astrocyte FACS markers (**Publication I**). It is conceivable that this population might be non-labeled cells caused by unspecific aggregation of astrocytes. Another possibility would be that this small fraction of cells are non-myelinating Schwann cells which are known to express GFAP (Triolo et al. 2006). Yet, Schwann cells have been only observed in the CNS in

association with demyelination diseases such as MS, neuromyelitis optica or spinal cord injury (Itoyama et al. 1983; Franklin and Ffrench-Constant 2017; Chen et al. 2021). Further characterization of this small ACSA-2$^-$GFAP$^-$ MACS-purified sub-population identified 1.5% of these cells as CD45$^-$CD11b$^+$, suggesting minimal contamination by presumably microglia. In sum, these results highlight once more the need for additional FACS markers in order to more precisely identify all astrocyte subtypes, as well as their contaminating cells, since pan-markers are still scant. Thus, developing new potential markers for cell type identification is at the forefront of glial science. One promising candidate might be for example the novel pan-specific astrocyte marker ras-related protein Rab-6A (Rab6a) (Melzer et al. 2021).

As with microglia, we cannot rule out whether astrocyte activation occurred during the enzymatic and mechanical dissociation procedures of our novel protocol. However, as previously mentioned, studies by Holt and colleagues showed that the complex cell integrity of astrocytes was preserved after MACS purification (Holt and Olsen 2016; Holt et al. 2019). Due to this as well as our own FESEM results (**Publication I**), we conclude that our protocol did not have an impact on the activation status of either astrocytes or microglia, as discussed earlier (Volden et al. 2015; He et al. 2018; Ocañas et al. 2022). In future experiments, the activation status of glial cells should be determined, for instance by comparing the transcriptomic profile of non-purified and MACS-purified microglia and astrocytes, respectively.

In conclusion, FACS analysis of the microglial and astrocyte cultures generated with our novel protocol demonstrated that the selection via MACS MicroBeads is an indispensable step for achieving glial viability and purity of nearly 100%. All things considered, it seems reasonable to assume that our novel protocol is an efficient and gentle approach for the isolation, purification, and cultivation of highly viable and pure microglia and astrocytes from neonatal mice. Pursuing this further, in the next step, we assessed whether the MACS-purified glial cells were functional and qualified for cell-specific downstream applications such as investigating the crosstalk between microglia and astrocytes by analyzing their immune responses.

3.2 Validation of the novel protocol

In the next step, in order to validate cell functionality and to exclude activation by MACS purification, we measured the pro- (TNF) and anti- (IL-10) inflammatory cytokine response of microglia and astrocytes before and after MACS purification upon TLR stimulation (**Publication I**).

Interestingly, non-purified microglia exhibited a significantly higher TNF protein release than MACS-purified microglia after TLR2 and 9 activation. One explanation might be that the CD45$^+$CD11b$^+$ (24.9%) subpopulation we identified in the non-purified microglial cultures (**Publication I**), which presumably consisted of highly activated microglia as discussed earlier,

accounted for the significant TNF protein release. Secondly, it is conceivable that the remaining $CD45^{-}CD11b^{-}$ (0.3%) subpopulation in the non-purified microglial culture composed of astrocytes which enabled a bidirectional crosstalk between microglia and astrocytes via signaling molecules (**Publication I**).

Like the microglial cultures, non-purified astrocyte cultures displayed significantly increased TNF protein levels compared to the MACS-purified astrocytes. Sub-gating of the non-purified astrocyte cultures revealed that most of the remaining $ACSA\text{-}2^{-}GFAP^{-}$ cells (54.3%) were microglia. Thus, we can conclude that contaminating microglia in these cultures were most likely responsible for the significantly enhanced TNF protein release in comparison to MACS-purified astrocytes (**Publication I**). It is conceivable that the presence of these contaminating microglia promoted a cycle of overactivation by activating astrocytes, which resembled a chronic inflammatory state and was further accompanied by an enhanced TNF protein release (Saijo et al. 2009; Vallejo et al. 2010; Liu et al. 2011; Losciuto et al. 2012). Overall, this highly significantly increased TNF protein release upon TLR stimulation by non-purified microglia and astrocytes monocultures in contrast to the MACS-purified cultures reinforces our hypothesis of an existing microglia-astrocyte crosstalk. As already mentioned in chapter *1.2.1 Glial crosstalk*, there are emerging studies providing evidence of an existing crosstalk between microglia and astrocytes (Chen et al. 2015; Iizumi et al. 2016; Liddelow et al. 2017; Shinozaki et al. 2017). The observed effect of contaminating glial cells, and especially microglia in alleged "pure" astrocyte cultures, emphasizes once again the need for protocols for the isolation of highly pure microglia and astrocytes, which has been underestimated for too long. Due to the emerging awareness of this issue, there has been rising evidence of the presence of contaminating microglial cells in astrocyte cultures and their significant impact on the outcome of the cytokine release. Consequently, there has been a growing demand for stating the viability and purity of the cells incorporating within the glial experiment (Sola et al. 2002; Saura 2007; Crocker et al. 2008; Lange et al. 2012; Losciuto et al. 2012).

In contrast to this, we could not confirm this highly significant TNF increase in the non-purified cultures in comparison to the MACS-purified cultures for the anti-inflammatory response. More precisely, the stimulation with the TLR9 ligand CpG ODN evoked a significant reduction of IL-10 only in non-purified astrocytes in comparison to its control, which we could not detect in the microglial cultures (**Publication I**). An explanation might be that upon CpG ODN stimulation, microglia are able to release IL-10 independently of astrocytes, while the presence of microglia is indispensable for the IL-10 protein production in astrocyte cultures. Thus, remaining contaminating microglial cells might be responsible for the highly significant IL-10 increase in our non-purified astrocyte cultures. These observations further point to the existence of a molecular conversation between microglia and astrocytes.

Stimulation with Pam3CSK4 did not induce a significantly elevated IL-10 protein response, neither in microglia nor astrocytes in comparison to their controls. Therefore, it might be conceivable that these glial cells release IL-10 independent from TLR2. This assumption is also in line with our own kinetic experiments using wild-type (WT) as well as TLR2-knockout (TLR2-KO) astrocyte monocultures: Pam3CSK4-activated WT astrocyte monocultures did not show significantly elevated IL-10 protein levels in comparison to their untreated controls at any of the investigated time points (3, 6, 16, 24, 48, 72 h) (**Unpublished data figure 1 and table 1**). In addition, we could also confirm this observation for TLR2-KO astrocytes (**Unpublished data figure 2 and table 2**). Interestingly, it has been demonstrated that isolated microglia and astrocytes express *Il-10* in a delayed manner and release detectible protein concentrations at 24 h in response to different TLR ligands or pathogens (Burmeister and Marriott 2018). In contrast to this, we could not detect any significant IL-10 protein levels in astrocytes in comparison to their controls even at 48 and 72 h (**Unpublished data figure 1 and table 1**). Nevertheless, as we investigated the TLR2 response using only the synthetic ligand Pam3CSK4 which is only one of many, our assumption needs to be confirmed using for example other TLR2 ligands such as zymosan, peptidoglycan or LTA (Kawai and Akira 2010). Moreover, the IL-10 response by TLR2/1-activated microglia needs to be further elucidated at time points later than 24 h and by using a wider array of TLR2 ligands in future experiments to confirm our theory of a TLR2-independent IL-10 release in glial monocultures (**Publication I**).

In conclusion, as validated above, primary microglia and astrocytes generated with our novel protocol are highly viable and pure. Therefore, they are suitable for downstream applications on a cell-specific level such as the co-culture of glial cells coming from different genetic backgrounds. The stimulation of these cells with different TLR ligands demonstrated the potential need for efficient glial isolation protocols, with special regard to microglia-free astrocyte cultures. These experiments provided preliminary evidence for the existence of a crosstalk among microglia and astrocytes as demonstrated for the TNF protein release (**Publication I**). To further confirm our theory, we subsequently activated highly pure and viable microglia and astrocytes generated with our protocol in different mono- as well as co-culture experiments (**Publication II**).

3.3 Application of the novel protocol to investigate the microglia-astrocyte crosstalk

3.3.1 TLR2/1 activation of highly pure glial monocultures

In the first step of **Publication II**, we investigated the pro- (TNF) and the anti- (IL-10) inflammatory immune response upon TLR2/1 stimulation of WT as well as TLR2-KO microglial and astrocyte monocultures, which were generated with our novel protocol, as described earlier (**Publication I**).

For this, we stimulated highly pure TLR2-KO microglia and astrocytes monocultures with Pam3CSK4-treated or untreated WT or TLR2-KO supernatants (SN) of the respective other glial cell type. Expectedly, the addition of Pam3CSK4-activated TLR2-KO glial SN to TLR2-KO microglial and astrocyte monocultures did not induce a significant cytokine response in comparison to their controls. As an exception, TLR2-KO astrocytes, which were stimulated with SN derived from Pam3CSK4-activated TLR2-KO microglia, depicted a significantly lower IL-10 protein response in comparison to the untreated control (**Publication II**). Zheng and co-authors also observed that the release of pro-inflammatory mediators by primary murine TLR2-KO astrocytes following bacterial challenge was not completely dampened (Zheng et al. 2011) which was possibly due to TLR2 heterodimer formation with TLR1 and/or 6 (Ozinsky et al. 2000; Kirschning and Schumann 2002). Therefore, it is conceivable that Pam3CSK4 as a ligand for the heterodimer TLR2/1, was able to induce the release of inflammatory signaling molecules in TLR2-deficient microglia through TLR1 engagement, which in turn downregulated the IL-10 protein release in TLR2-KO astrocytes. However, these assumptions need to be reinforced in future experiments, for instance by repeating the experiments with the synthetic TLR2/6 ligand Pam2CSK4 (Okun et al. 2009) as we only used the TLR2/1 agonist Pam3CSK4. Furthermore, for this reason, we cannot compare these results one-to-one to our prior observed *in vivo* data from our lab (unpublished data) using *Tlr2*$^{-/-}$ mice as this KO does not allow for TLR2/1 or TLR2/6 signaling. Hence, as already mentioned, the outcome of the induced immune response depends on the type of TLR2 heterodimer formation (DePaolo et al. 2008; Long et al. 2009; Rolf et al. 2015).

Consistent with our prior TLR activation experiments (**Publication I**), the pro-inflammatory response was more pronounced than the anti-inflammatory response in microglial as well as astrocyte cultures (**Publication II**). This could be explained with our kinetic experiments as discussed earlier (**Unpublished data figure 1 and table 1)**: As pro-inflammatory TNF cytokines were released abundantly in the first 24 h including a peak at 16 h, subsequently the secretion of the anti-inflammatory cytokine IL-10 was induced in a time-dependent manner which has been shown earlier (Burmeister and Marriott 2018). Furthermore, by stimulating TLR2-KO microglial and astrocyte monocultures with TLR2/1-activated WT SN, we observed that the TNF protein release was highly upregulated in comparison to their untreated controls (**Publication II**). There are two possible explanations for this effect: Firstly, TLR2-KO microglia and astrocytes can be activated by the addition of SN derived from the respective other WT glial cell type, which further supports our theory of the existence of an intimate glial crosstalk. According to this, it is likely that the Pam3CSK4-activated SN derived from WT glial cells contained signaling molecules which in turn induced a pro-inflammatory immune response in the TLR2-KO glial monocultures. Secondly, there is the possibility that secreted TNF proteins

in the SN derived from the WT glial cells accounted for this elevated pro-inflammatory response. This assumption is reinforced by our earlier findings (**Publication I**) as well as by studies by Chen and co-authors since microglia, as the main contaminating cell type in stimulated astrocyte cultures, were found to be primarily responsible for the TNF-α protein release (Chen et al. 2015).

With the next experiment, we were aiming to rule out the possibility of remaining cytokines in the SN being the main source of the significantly increased TNF protein release in the TLR2-KO glial cultures. For this, TLR2-KO glial monocultures were stimulated with WT SN for 2 h and the SN-containing medium was exchanged with fresh medium for another 24 h (**Publication II**). Here, we could not detect the highly significantly enhanced TNF protein release in TLR2-KO microglia anymore, which were stimulated with Pam3CSK4 WT astrocyte SN for 24 h. Yet, we cannot exclude that the lack of microglial response was induced by the media change after 2 h. However, TNF is known to be one of the first cytokines abundantly released in the first 24 h by microglia (Ebert et al. 2005), which are the first line of defense (Kreutzberg 1995; He et al. 2021). Another possibility would be that microglia are highly dependent on TLR2-signaling, as we could not detect TNF release in TLR2-depleted microglia, even after stimulation with WT astrocyte SN (**Publication II**).

Interestingly, in TLR2-KO astrocyte cultures we still observed the highly significant TNF protein rise by the addition of Pam3CSK4-activated microglial WT SN in comparison to the untreated control, in accordance with the results from the latter activation experiment (**Publication II**). Consequently, in comparison to activated TLR2-KO astrocytes, which released significantly more TNF than their control, the pro-inflammatory response by TLR2-KO microglia may be impaired by inadequate and/or insufficient mediator secretion by Pam3CSK4-activated WT astrocytes. Given these observations, we can conclude that the remaining TNF in the astrocyte SN most likely induced the highly significant TNF protein increase in TLR2-KO microglia in comparison to the control (**Publication II**). As previously mentioned, astrocytes have been shown to be involved in TLR2-associated neuropathologies (Li et al. 2021a): A study by Zheng and co-authors, using a meningitis model, showed that the *Tlr2* mRNA expression was upregulated in primary activated murine WT astrocytes following bacterial challenge. Furthermore, a wide range of pro-inflammatory mediators including TNF-α were released, while in TLR2-deficient astrocytes the TNF protein release was significantly impaired (Zheng et al. 2011). In contrast to these results, we demonstrated that even though astrocytes are lacking TLR2 they were able to significantly release TNF solely by stimulation with Pam3CSK4-activated WT microglial SN. Thereby, confirming our theory of an existing crosstalk among microglia and astrocytes (**Publication II**).

In sum, these experiments deliver the first evidence of a unidirectional crosstalk from microglia to astrocytes, however, this assumption needs to be investigated in future glial crosstalk experiments. These may include the application of different ELISA assays to investigate other potential cytokines being involved in the microglia-astrocyte crosstalk such as IL-1β or IL-6 (Matejuk and Ransohoff 2020) or the application of different TLR2 ligands, as discussed earlier (Okun et al. 2009). Moreover, with this experiment, we did not observe any significant effects on the IL-10 protein release by TLR2-KO glial monocultures in comparison to their controls (**Publication II**). The IL-10 response rather resembled the one from our previous experiment where non-purified and MACS-purified microglia and astrocytes were directly stimulated with the TLR2/1 ligand Pam3CSK4 (**Publication I**).

Next, in order to identify the secreted signaling molecules which enabled the significant TNF protein release in TLR2-KO astrocyte monocultures from the latter experiment, we performed transcriptome analysis using RNA-seq. We identified in TLR2-KO astrocytes stimulated with Pam3CSK4-activated WT microglial SN (treated) 8,767 up- and 8,463 downregulated differentially expressed genes (DEGs), in contrast to the control (TLR2-KO astrocytes stimulated with untreated WT microglial SN). Using heat map enrichment analysis, we further identified two distinct clusters among all genes between the treated sample and the untreated control. Interestingly, unlike cluster 2, cluster 1 was mostly enriched for genes which compromise inflammatory functions, as characterized by Gene Ontology (GO) enrichment (**Publication II**).

Among these identified genes, cd300 molecule like family member D2 (*Cd300d2)* was the most significantly upregulated gene compared to the untreated control. *Cd300d2* belongs to the CD300 human and murine family of immunoreceptors forming extracellular homo- and hetero-signaling complexes in myeloid cells (Comas-Casellas et al. 2012; Peluffo et al. 2012; Borrego 2013). However, its function remains unknown while other genes of the family are known to be activating immune receptors such as CD300f (Peluffo et al. 2012; Lima et al. 2017). Comas-Casellas and colleagues suggested that human CD300d might be associated with the regulation and the trafficking of other CD300 complexes on the cell surface (Comas-Casellas et al. 2012). Studies by Lima and co-authors demonstrated that *in vitro* cultivated rat astrocytes express *hCd300f* (the human isoform of CD300f) against Aβ toxicity in a neuroprotective manner (Lima et al. 2017). According to this, it is conceivable that the significantly upregulated *Cd300d2* gene expression, in comparison to the untreated control, was involved in the significant TNF protein release we could detect for TLR2-KO astrocytes that were stimulated with WT microglial SN in comparison to their controls (**Publication II**).

The second most significantly upregulated gene in comparison to the untreated control was tumor necrosis factor receptor superfamily member 9 (*Tnfrsf9*), which is also called *Cd137*. Reali et al. demonstrated that astrocytes and neurons derived from human brain tissue both express *CD137* as well as its ligand (*CD137-L*), while microglia only express *Cd137* mRNA (Reali et al. 2003). Additionally, based on observations in primary monocytes by Langstein et al. (Langstein et al. 1998), it was hypothesized that CD137 is associated with the activation of microglial cells as it was shown to enhance the induction of pro-inflammatory cytokines including TNF-α while the secretion of IL-10 was inhibited (Langstein et al. 2000; Reali et al. 2003). Therefore, it is conceivable that WT microglia became activated upon Pam3CSK4 stimulation and induced a pro-inflammatory response by secreting TNF and CD137, while the anti-inflammatory IL-10 release was inhibited, as shown in our first TLR experiments (**Publication I**). Subsequently, the addition of these activated microglial WT SN to the TLR2-KO astrocyte monocultures induced a significantly enhanced TNF protein release in comparison to the control, while the IL-10 release was hampered as shown in our glial monoculture activation experiments (**Publication II**). Moreover, our RNA-seq data are in line with these observations (Langstein et al. 2000; Reali et al. 2003), as the *Il-10* expression was significantly downregulated in TLR2-KO astrocytes which had been stimulated with TLR2/1-activated microglial SN in comparison to the control (**Publication II**).

The third most significantly upregulated gene compared to the control was lipocalin2 (*Lcn2*) which belongs to the lipocalin family and is responsible for the transport of small hydrophobic molecules (Lee et al. 2009; Kim et al. 2011; Lee et al. 2011; Berard et al. 2012; Jin et al. 2014). The upregulation of *Lcn2* by astrocytes has been shown to be associated with a wide array of CNS diseases including ischemic stroke (Jin et al. 2014; Wang et al. 2015; Kim et al. 2017; Zhao et al. 2019). As discussed previously, the release of the acute phase protein LCN2 (Liu and Nilsen-Hamilton 1995) is associated with the crosstalk among microglia and astrocytes since it is expressed in activated astrocytes (Lee et al. 2011), while its receptor LCN2R is expressed in neurons and microglia (Kim et al. 2017; Jha et al. 2019). A study by Ranjbar Taklimie and co-authors showed in WT hypoxic astrocyte cultures that *Tnf* mRNA was significantly upregulated coinciding with the induction of *Lcn2*. In contrast to this, LCN2-deficient astrocytes depicted a significantly enhanced *Il-10* expression (Ranjbar Taklimie et al. 2019). This is in line with our observation that TLR2-KO astrocytes which have been stimulated with activated microglial SN significantly upregulated *Lcn2* accompanied by a significant TNF protein increase and steady IL-10 protein release in comparison to their controls (**Publication II**).

Interestingly, among all expressed *Tlrs,* the second highest was *Tlr9*, indicating that TLR9 can be independently induced of TLR2 (**Publication II**). This result is in line with the significantly

increased TNF protein response after every time point by TLR2-KO microglia which were stimulated with the TLR9 ligand CpG ODN in comparison to their controls (**Unpublished data figure 2 and table 2**). Kinetic studies by Rosenberger and co-authors also demonstrated a time-dependent TNF protein release in purified microglial monocultures in response to sole as well as pairwise Pam3CSK4 and CpG ODN stimulation, which was just slightly enhanced by pairwise activation of TLR2 and 9 (Rosenberger et al. 2014).

The most significantly downregulated gene in comparison to the untreated control was aconitate decarboxylase 1 (*Acod1*), also known as immunoresponsive gene 1 (*Irg1*). Its expression is induced in immune cells like macrophages, monocytes or microglia during infection and inflammation (Lee et al. 1994; Michelucci et al. 2013; Wu et al. 2020). A recent study by Kuo et al. showed in an ischaemic stroke mouse model, that microglia were the main cell type in the CNS highly inducing *Irg1.* Consequently, this led to an increased expression of protective heme oxygenase-1 (*Ho-1*) restraining ischaemic brain injury (Kuo et al. 2021). However, in contrast to microglia, *Irg1* was found to be barely detectable *in vitro* in stimulated murine primary astrocytes (Li et al. 2018), which is in line with our results as *Irg1* was the most significantly downregulated gene of all sequenced genes in comparison to the control.

3.3.2 TLR2/1 activation of glial co-cultures

Subsequent to our glial monoculture experiments, we additionally wanted to pursue the question of whether glial crosstalk also occurs in TLR2/1-activated microglial and astrocyte co-cultures (**Publication II**). Therefore, we co-cultured Pam3CSK4-stimulated microglia and astrocytes coming from mice with different backgrounds generated with our novel protocol (**Publication I**).

Significantly, the TNF protein release of the Pam3CSK4-treated WT microglia and TLR2-KO astrocyte co-cultures was comparable to the pro-inflammatory response from the co-cultures consisting of WT microglia and astrocytes (not significant). These similar TNF protein levels in both co-cultures might have been caused by the noticeable protein release derived from activated WT microglia which in turn superimposed the astrocytic TNF release. However, further studies are urgently needed in order to clarify their individual contribution and signaling molecules to the immune response in the CNS. For instance, other anti-inflammatory cytokines as mediators apart from IL-10, such as TGF-β, IL-4Rα or fractalkine receptor CX3CR1 (Norden et al. 2014), may have affected the TNF protein release. In addition to this, TNF protein levels were significantly increased in comparison to their untreated controls in both co-cultures, which was especially remarkable for the co-culture of WT microglia and TLR2-KO astrocytes. It might be conceivable that the microglial pro-inflammatory immune response was lacking soluble

feedback factors by astrocytes which normally prevent an exaggerated immune response (Min et al. 2006; Norden et al. 2014).

The Pam3CSK4 activation of TLR2-KO microglia co-cultured with WT astrocytes significantly enhanced the TNF protein release in comparison to the untreated control. However, this pro-inflammatory response was approximately two thirds lower than in the latter mentioned co-cultures and therefore not comparable to them. We already observed this attenuated TNF protein release by the TLR2-KO microglia and WT astrocyte co-cultures in the prior monoculture experiments for microglial TLR2-KO which were stimulated with Pam3CSK4-activated WT astrocyte SN (*3.3.1 TLR2/1 activation of highly pure glial monocultures*) (**Publication II**). Nevertheless, even though the TNF secretion in WT astrocytes is generally weaker than in WT microglia upon activation, as discussed earlier (**Publication I and II**), the addition of activated microglial SN was sufficient to induce a significantly higher TNF protein response in Pam3CSK4-activated astrocytes in comparison to the control. This, once again, highlights the importance of the glial interplay and cellular crosstalk since one glial cell type can activate the respective other one which results in the induction of an inflammatory response. Furthermore, this observation might be also explained by a study by Syed and co-authors which revealed that microglial derived TNF-α acts in an autocrine/paracrine manner as a potent stimulus upon bacterial stimulation to augment microglial *Tlr2* expression (Syed et al. 2007). According to this, it might be conceivable that the hampered TNF release by TLR2-KO microglia was due to their TLR2-deficiency. The anti-inflammatory response resembled the pro-inflammatory cytokine response as the WT microglia/TLR2-KO astrocytes, as well as the TLR2-KO microglia/WT astrocytes co-cultures, showed a significantly upregulated IL-10 protein release upon Pam3CSK4 stimulation in comparison to their untreated controls (**Publication II**).

Murine glial cell density values depend on many factors such as the brain region of interest, age, gender, mouse strain as well as the applied counting methods. We decided to co-culture microglia and astrocytes in a ratio of 1:5, which reflects the cell density of the cortex, one of the best studied brain regions, in accordance with the latest published review (Keller et al. 2018) (**Publication II**). Our co-culture model is also suitable to study the role of the microglia-astrocyte crosstalk in the context of different CNS diseases, such as AD or PD (Rostami et al. 2021; Wu and Eisel 2023; Zhang et al. 2023). These neurodegenerative diseases affect brain regions which are characterized by distinct glial subpopulations (Matejuk and Ransohoff 2020). Of note, modifications of our protocol such as the usage of mouse strains or ratios others than ours require careful validation and might be accompanied with an enhanced animal number in order to generate sufficient glial cell numbers, as already mentioned earlier (**Publication I**).

Nevertheless, we cannot fully clarify from these experiments whether this crosstalk is of a uni- or bi-directional nature. Moreover, we cannot exclude key drivers for the crosstalk other than signaling molecules secreted by the glial cells, since co-culturing of microglia and astrocytes also enables constant cell-to-cell interaction. For instance, due to their close proximity, tunneling nano tubes (TNTs) or other membrane structures might be also key driver of the microglia-astrocyte crosstalk (Rostami et al. 2021). Another form of intracellular communication is extracellular versicles (EVs) e.g., exosomes, macrovesicles, or apoptotic bodies, which are derived from different cellular compartments (Paschon et al. 2016). EVs deliver specific cargos including proteins, genetic material such as mRNAs, or microRNAs (miRNAs) thereby modulating the gene expression of the recipient cells (Gupta and Pulliam 2014; Budnik et al. 2016; Li et al. 2021b). Both microglia and astrocytes secret EVs in the extracellular CNS environment under physiological conditions and increasingly upon glial cell activation in response to different stimuli (Guo et al. 2021; Zhao et al. 2021; Chen et al. 2022; Szpakowski et al. 2023). For instance, it has been shown that exosomes derived from LPS-stimulated microglia induced a dose-dependent activation of astrocytes by upregulating the expression of pro-inflammatory markers including *Il1β*, *Il6* or *iNOS* (Verderio et al. 2012). Moreover, miRNAs derived from exosomes can modulate the innate immunity by specifically acting on TLR signaling pathways (Paschon et al. 2016; Lehnardt et al. 2019; Guo et al. 2020). Nevertheless, whether EVs like exosomes were key drivers of the microglia-astrocyte crosstalk by acting on TLR2/1 signaling needs to be elucidated in future experiments. For instance, microscopy imaging could be used to stain exosomal membrane proteins and lipids like endosome-specific tetraspanins or proteins which are involved in membrane fusion and transport e.g. annexins (Gupta and Pulliam 2014; Chuo et al. 2018).

Taken together, the results of our co-culture experiments provide substantial evidence that both microglia and astrocytes can be activated solely by signaling molecules derived from the other Pam3CSK4-activated WT glial cell type, even though they are lacking TLR2. These findings further confirm our theory of an existing crosstalk between microglia and astrocytes in the frame of TLR2/1. Above all, our results confirm that primary microglia and astrocytes generated with our novel protocol are suitable to investigate the glial crosstalk on a cell-specific level (**Publication I and II**).

3.4 Concluding remarks and outlook

While there is emerging evidence for the crosstalk between microglia and astrocytes and its role in the emergence and progression of a wide range of neuropathologies (Matejuk et al. 2021), the development of suitable methods to isolate primary microglia and astrocytes from brain tissue has lagged far behind. To date, the developed isolation protocols do not meet

the required standards to investigate the crosstalk among microglia and astrocytes on a cell-specific level. In particular, the isolation of primary microglia-free astrocyte cultures has been a major challenge in glial research as even minor amounts of contaminating microglial cells can have major effects on entire *in vitro* experiments (Saura 2007; Losciuto et al. 2012; Van Zeller et al. 2022). Due to this, the understanding of glial crosstalk and the cells involved has been hindered by the need for suitable isolation methods.

This thesis suggests a novel protocol for the optimized *in vitro* cultivation of mixed glial cultures from neonatal brain tissue and the subsequent isolation of primary microglia and astrocytes. We were able to reduce animal experiments by harvesting microglia from the same culture, which is a major advantage of our proposed protocol (**Figure 3**). As confirmed by FACS, cells generated with this novel method are applicable for following cell-specific analysis due to their high viability and purity. By applying these cells in different monoculture experiments, we produced evidence for the molecular conversation between microglia and astrocytes coming from different genetic backgrounds in response to TLR2/1. Finally, we confirmed these results in co-cultured microglia und astrocytes, thereby further providing evidence of an existing crosstalk among these two glial cell types.

Consequently, we have shown our novel protocol to be a highly useful, quick and easy tool which will facilitate the analysis of specific cell-type involvement in the frame of CNS diseases. With this new efficient tool, the possibilities to study microglia and astrocytes in different experimental settings are extended as we have shown in our mono- and co-culture experiments. In future experiments, investigating brain diseases affecting distinct brain regions might require additional animal numbers to generate sufficient glial cells from the individual brain regions. Another interesting approach would be to investigate the pathogenesis of AD and PD and their underlying microglia-astrocyte crosstalk on a cell-specific level. For instance, the protein aggregate transfer of Aβ and α-syn between these two glial cell types could be analyzed in order to identify new therapeutic targets. It seems obvious that, apart from microglia and astrocytes, other CNS cells such as neurons or oligodendrocytes are also playing a critical role in the function of the brain as well as in the development of CNS diseases. Certainly, it would be conceivable to add highly pure primary neurons to our *in vitro* system. This triple co-culture model would potentially allow to study the crosstalk among neurons and glial cells and their cellular contribution to neuroinflammatory processes by closely mimicking the *in vivo* CNS microenvironment. Nevertheless, our new approach represents one important milestone at the forefront of glial research to overcome the lack of suitable methods to properly comprehend the microglia-astrocyte crosstalk and the mechanisms behind it in future approaches.

4 Zusammenfassung

Entwicklung und Charakterisierung einer neuen *in vitro*-Methode zur Untersuchung der zellulären Immunantwort von Mikroglia und Astrozyten sowie den zugrunde liegenden „crosstalk“

Erkrankungen des zentralen Nervensystems (ZNS) stellen eine globale Gesundheitsbedrohung für Mensch und Tier dar, mit hohen Mortalitätsraten und häufigem Auftreten von Rekonvaleszenzen, aber auch gelegentlichen Langzeitfolgen. Somit steht die Rolle der verschiedenen ZNS-Zelltypen im Vordergrund, um die Entwicklung, Funktion und Erkrankungen des Gehirns zu verstehen. Der ZNS-Zelltyp Glia umfasst Mikroglia und Astrozyten, die beide entscheidende Akteure im Gehirn sind und an der Abwehr von Pathogenen, der Aktivierung des angeborenen Immunsystems sowie der Signalübertragung beteiligt. Ihr enger „*crosstalk*“ hält das empfindliche Gleichgewicht zwischen Homöostase und der schnellen Detektion von eindringenden Pathogenen aufrecht und hat zunehmend die Aufmerksamkeit der Forschung erlangt. Folglich ist die Verbesserung von Methoden zur Isolierung, Kultivierung und Aufreinigung unterschiedlicher ZNS-relevanter Zelltypen mit hoher Vitalität und Reinheit unerlässlich, um die individuellen Beiträge von Zellpopulationen zur einwandfreien Funktion des Gehirns zu untersuchen.

Das Ziel dieser Studie war die Entwicklung eines *in vitro*-Protokolls für die Isolierung, Kultivierung und Aufreinigung hochvitaler und reiner Mikroglia und Astrozyten aus neonatalen Mäusen sowie die anschließende Untersuchung ihrer zelltypspezifischen Effekte und ihres Beitrages zur Immunantwort des ZNS sowie des zellulären *crosstalk*.

Im ersten Schritt wurden bestehende Techniken zur *in vitro*-Isolierung und Kultivierung von Mikroglia und Astrozyten aus ganzen neonatalen Mäusegehirnen optimiert, um ein Protokoll zur Isolierung von Gliazellen zu entwickeln. Die Ausbeute an Mikrogliazellen wurde maximiert, indem mit dem Makrophagen-Kolonie-stimulierenden Faktor (M-CSF) stimuliert sowie mehrere Mikroglia-Ernten aus derselben gemischten Glia-Kultur gewonnen wurden. Anschließend wurde ein Reinigungsprotokoll für *magnetic-activated cell sorting* (MACS®) verbessert, indem kultivierte primäre Glia-Suspensionen anstelle von direkt-sortierten dissoziierten Einzelzell-Suspensionen verwendet wurden. Dadurch konnten Mikroglia und Astrozyten erfolgreich isoliert, kultiviert und zu 99-100% aufgereinigt werden, was durch *fluorescence-activated cell sorting* (FACS) bestätigt werden konnte. Darüber hinaus waren die mit unserem neuen Protokoll generierten Gliazellen hoch lebensfähig (~100%) und wiesen eine intakte Morphologie auf, was durch Rasterelektronenmikroskopie gezeigt wurde. Anschließend wurden nicht aufgereinigte und MACS-aufgereinigte Mikroglia- und Astrozyten-Monokulturen mit verschiedenen TLR-Liganden stimuliert. Ihre pro- und anti-inflammatorische

Reaktion wurde durch *enzyme-linked immunosorbent assays* (ELISA) für Tumor-Nekrose-Faktor (TNF) und Interleukin-10 (IL-10) bestimmt. Tatsächlich zeigten diese Experimente eine signifikant abgeschwächte Proteinantwort in den MACS-aufgereinigten Mikroglia- und Astrozyten-Monokulturen im Vergleich zu den nicht aufgereinigten Glia-Monokulturen und lieferten damit den ersten Hinweis auf einen Mikroglia-Astrozyten *crosstalk*.

Im nächsten Schritt wurde unsere Hypothese eines vorhandenen *crosstalk* bekräftigt, indem TLR2-Knockout (TLR2-KO) Mikroglia- und Astrozyten-Monokulturen mit Wildtyp (WT)-Überständen (ÜS) aus den jeweils anderen Glia-Zelltypen stimuliert wurden. Hierbei wurde eine signifikante Ausschüttung von TNF-Proteinen in TLR2-KO Astrozyten-Monokulturen beobachtet, die mit Pam3CSK4-aktivierten ÜS aus WT Mikroglia stimuliert wurden, im Vergleich zur unbehandelten Kontrolle. Interessanterweise deckte eine Transkriptom-Analyse mittels *RNA sequencing* (RNA-seq) eine Vielzahl signifikant hoch- und herunterregulierter Gene im Vergleich zu unbehandelten Kontrollen auf, die potenzielle Mediatoren des engen molekularen *crosstalk* sein könnten. Schließlich wurden Co-Kultur-Experimente mit Mikroglia und Astrozyten aus verschiedenen genetischen Hintergründen durchgeführt. Interessanterweise wurde eine signifikante Freisetzung von TNF-Proteinen in Pam3CSK4-aktivierten Co-Kulturen aus WT Mikroglia und TLR2-KO Astrozyten, im Vergleich zur unbehandelten Kontrolle, beobachtet, was die Ergebnisse der vorherigen Monokultur-Experimente bestätigte.

In dieser Arbeit beschreiben wir ein neues Protokoll zur Optimierung der *in vitro*-Isolierung, Kultivierung und Reinigung von neonatalen murinen primären Mikroglia und Astrozyten. Mit unserer vorgeschlagenen Methode haben wir die Mikroglia-Ausbeute maximiert, mit dem großen Vorteil, so wenig Mäuse wie möglich gemäß den Prinzipien der 3R (Ersatz, Reduktion und Verfeinerung) zu nutzen. Durch die Verwendung von ~100% reinen Glia-Mono-/Co-Kulturen aus Mäusen mit unterschiedlichen Genotypen konnten wir den Mikroglia-Astrozyten *crosstalk* auf zellulärer Ebene analysieren. Im Gegensatz zu früheren Protokollen haben wir gezeigt, dass die Immunantwort während des *crosstalk* einem TLR2/1-abhängigen Mechanismus unterliegt. Folglich postulieren wir unser neues Protokoll als eine geeignete und effiziente Methode, um zelltypspezifische Effekte zu untersuchen, die zur Immunmodulation sowie zum zellulären *crosstalk* in zukünftigen Ansätzen beitragen. Hierdurch haben wir die Möglichkeiten zur Untersuchung von Gliazellen in verschiedenen experimentellen Fragestellungen zur Hirnfunktion sowie zu ZNS-Infektionen erweitert.

5 Summary

Development and characterization of a new *in vitro* method to study the cellular immune response of microglia and astrocytes and the underlying crosstalk

Diseases of the central nervous system (CNS) are a global health threat to both humans and animals with high mortality rates and frequent occurrence of convalescence, occasionally also including long-term sequela. Thus, the role of the different CNS cell types involved is at the forefront of comprehending the development, function, and diseases of the brain. The CNS cell type glia includes microglia and astrocytes, both are crucial players in the brain and are involved in pathogen defense, innate immune activation, and signaling. Their intimate crosstalk maintains the delicate balance between homeostasis and rapid detection of invading pathogens and has progressively gathered research attention. Therefore, the improvement of methods for the isolation, cultivation, and purification of distinct CNS-derived cell types at a high viability and purity is indispensable in order to study the individual contributions of cellular populations to the proper function of the brain.

The objective of this study was to develop an *in vitro* protocol for the isolation, cultivation, and purification of highly viable and pure microglia and astrocytes from neonatal mice and subsequently investigate their cell type-specific effects as well as their contribution to the CNS immune response and cellular crosstalk.

In the first step, a glial isolation protocol was developed by optimizing pre-existing techniques for the *in vitro* isolation and cultivation of microglia and astrocytes from whole neonatal murine brains. Microglial cell yields were maximized by stimulating with macrophage colony-stimulating factor (M-CSF) and applying multiple microglial harvests derived from the same mixed glial culture. Then, a purification protocol for magnetic-activated cell sorting (MACS®) was improved by using cultivated primary glial cell suspensions instead of directly sorting dissociated single cell suspensions. Thereby, microglia and astrocytes were successfully isolated, cultivated, and MACS-purified at a purity of 99-100%, as confirmed by fluorescence-activated cell sorting (FACS) analysis. Furthermore, glial cells generated with our novel protocol are highly viable (~100%) and show a preserved integrity, as demonstrated by field emission scanning electron microscopy (FESEM). Subsequently, non-purified and MACS-purified microglial and astrocyte monocultures were stimulated with different TLR ligands. Their pro- and anti-inflammatory response was determined by using Tumor necrosis factor (TNF) and Interleukin-10 (IL-10) enzyme-linked immunosorbent assays (ELISA). In fact, these experiments demonstrated a significantly weakened protein response in MACS-purified microglia and astrocytes in contrast to the non-purified glial monocultures, providing the first hint of a microglia-astrocyte crosstalk.

In the next step, our hypothesis of an existing crosstalk was reinforced by stimulating TLR2-knockout (TLR2-KO) microglial and astrocyte monocultures with wild-type (WT) supernatants (SN) derived from the respective other glial cell type. Here, a significant TNF protein release by TLR2-KO astrocyte monocultures was observed, which were activated with microglial WT SN in response to Pam3CSK4, in comparison to the untreated control. Interestingly, transcriptome analysis using RNA sequencing (RNA-seq) unraveled a wide array of significantly up- and down-regulated genes in comparison to their untreated controls, which may be candidate mediators of the intimate molecular glial conversation. Finally, co-culture experiments with microglia and astrocytes coming from different genetic backgrounds were performed. Interestingly, a significant TNF protein release by Pam3CSK4-activated WT microglia co-cultured with TLR2-KO astrocytes was detected in comparison to the untreated control, confirming the results from the prior monoculture experiments.

In this thesis, we describe a novel protocol to optimize *in vitro* isolation, cultivation, and purification of neonatal murine primary microglia and astrocytes. With our proposed method, we maximized microglial yields with the big advantage of sacrificing as few mice as possible following the principles of the 3Rs (replacement, reduction, and refinement). By using ~100% pure glial mono-/co-cultures derived from mice with different genotypes, we were able to analyze the microglia-astrocyte crosstalk on a cell-specific level. Moreover, unlike previous protocols, we showed that the immune response during the crosstalk underlies a TLR2/1-dependent mechanism. Consequently, we postulate our novel protocol to be a suitable and efficient method to investigate cell type-specific effects which contributes to immune modulation as well as cellular crosstalk in future approaches. Thereby, we further extended the possibilities to study glial cells in different experimental issues on brain function as well as CNS infections.

6 References

Abbott, N. J., L. Ronnback and E. Hansson (2006):

Astrocyte-endothelial interactions at the blood-brain barrier.

Nat Rev Neurosci 7: 41-53. DOI: 10.1038/nrn1824.

Aguzzi, A., B. A. Barres and M. L. Bennett (2013):

Microglia: scapegoat, saboteur, or something else?

Science 339: 156-161. DOI: 10.1126/science.1227901.

Ahlemeyer, B., S. Kolker, Y. Zhu, G. F. Hoffmann and J. Krieglstein (2002):

Increase in glutamate-induced neurotoxicity by activated astrocytes involves stimulation of protein kinase C.

J Neurochem 82: 504-515. DOI: 10.1046/j.1471-4159.2002.00994.x.

Ahlemeyer, B., S. Kolker, Y. Zhu, G. F. Hoffmann and J. Krieglstein (2003):

Cytosine arabinofuranoside-induced activation of astrocytes increases the susceptibility of neurons to glutamate due to the release of soluble factors.

Neurochem Int 42: 567-581. DOI: 10.1016/s0197-0186(02)00164-x.

Ajami, B., J. L. Bennett, C. Krieger, W. Tetzlaff and F. M. Rossi (2007):

Local self-renewal can sustain CNS microglia maintenance and function throughout adult life.

Nat Neurosci 10: 1538-1543. DOI: 10.1038/nn2014.

Akira, S. and K. Takeda (2004):

Toll-like receptor signalling.

Nat Rev Immunol 4: 499-511. DOI: 10.1038/nri1391.

Akira, S., S. Uematsu and O. Takeuchi (2006):

Pathogen recognition and innate immunity.

Cell 124: 783-801. DOI: 10.1016/j.cell.2006.02.015.

Akiyama, H., T. Nishimura, H. Kondo, K. Ikeda, Y. Hayashi and P. L. McGeer (1994):

Expression of the receptor for macrophage colony stimulating factor by brain microglia and its upregulation in brains of patients with Alzheimer's disease and amyotrophic lateral sclerosis.

Brain Res 639: 171-174. DOI: 10.1016/0006-8993(94)91779-5.

Aktories, P., P. Petry and K. Kierdorf (2022):

Microglia in a Dish-Which Techniques Are on the Menu for Functional Studies?

Front Cell Neurosci 16: 908315. DOI: 10.3389/fncel.2022.908315.

Alexopoulou, L., V. Thomas, M. Schnare, Y. Lobet, J. Anguita, R. T. Schoen, R. Medzhitov, E. Fikrig and R. A. Flavell (2002):

Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice.

Nat Med 8: 878-884. DOI: 10.1038/nm732.

Andriezen, W. L. (1893):

The Neuroglia Elements in the Human Brain.

Br Med J 2: 227-230. DOI: 10.1136/bmj.2.1700.227.

Antony, J. M., A. Paquin, S. L. Nutt, D. R. Kaplan and F. D. Miller (2011):

Endogenous microglia regulate development of embryonic cortical precursor cells.

J Neurosci Res 89: 286-298. DOI: 10.1002/jnr.22533.

Araque, A., V. Parpura, R. P. Sanzgiri and P. G. Haydon (1999):

Tripartite synapses: glia, the unacknowledged partner.

Trends Neurosci 22: 208-215. DOI: 10.1016/s0166-2236(98)01349-6.

Barres, B. A., B. E. Silverstein, D. P. Corey and L. L. Chun (1988):

Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning.

Neuron 1: 791-803. DOI: 10.1016/0896-6273(88)90127-4.

Barres, B. A. (2014):

Designing and troubleshooting immunopanning protocols for purifying neural cells.

Cold Spring Harb Protoc 2014: 1342-1347. DOI: 10.1101/pdb.ip073999.

Batiuk, M. Y., F. de Vin, S. I. Duque, C. Li, T. Saito, T. Saido, M. Fiers, T. G. Belgard and M. G. Holt (2017):

An immunoaffinity-based method for isolating ultrapure adult astrocytes based on ATP1B2 targeting by the ACSA-2 antibody.

J Biol Chem 292: 8874-8891. DOI: 10.1074/jbc.M116.765313.

Batiuk, M. Y., A. Martirosyan, J. Wahis, F. de Vin, C. Marneffe, C. Kusserow, J. Koeppen, J. F. Viana, J. F. Oliveira, T. Voet, C. P. Ponting, T. G. Belgard and M. G. Holt (2020):

Identification of region-specific astrocyte subtypes at single cell resolution.

Nat Commun 11: 1220. DOI: 10.1038/s41467-019-14198-8.

Becher, B. and J. P. Antel (1996):

Comparison of phenotypic and functional properties of immediately *ex vivo* and cultured human adult microglia.

Glia 18: 1-10. DOI: 10.1002/(SICI)1098-1136(199609)18:1<1::AID-GLIA1>3.0.CO;2-6.

Bedi, S. S., P. Smith, R. A. Hetz, H. Xue and C. S. Cox (2013):

Immunomagnetic enrichment and flow cytometric characterization of mouse microglia.

J Neurosci Methods 219: 176-182. DOI: 10.1016/j.jneumeth.2013.07.017.

Benakis, C., L. Garcia-Bonilla, C. Iadecola and J. Anrather (2014):

The role of microglia and myeloid immune cells in acute cerebral ischemia.

Front Cell Neurosci 8: 461. DOI: 10.3389/fncel.2014.00461.

Benmamar-Badel, A., T. Owens and A. Wlodarczyk (2020):

Protective Microglial Subset in Development, Aging, and Disease: Lessons From Transcriptomic Studies.

Front Immunol 11: 430. DOI: 10.3389/fimmu.2020.00430.

Bennett, M. L., F. C. Bennett, S. A. Liddelow, B. Ajami, J. L. Zamanian, N. B. Fernhoff, S. B. Mulinyawe, C. J. Bohlen, A. Adil, A. Tucker, I. L. Weissman, E. F. Chang, G. Li, G. A. Grant, M. G. Hayden Gephart and B. A. Barres (2016):

New tools for studying microglia in the mouse and human CNS.

Proc Natl Acad Sci U S A 113: E1738-1746. DOI: 10.1073/pnas.1525528113.

Berard, J. L., J. G. Zarruk, N. Arbour, A. Prat, V. W. Yong, F. H. Jacques, S. Akira and S. David (2012):

Lipocalin 2 is a novel immune mediator of experimental autoimmune encephalomyelitis pathogenesis and is modulated in multiple sclerosis.

Glia 60: 1145-1159. DOI: 10.1002/glia.22342.

Beraud, D., M. Twomey, B. Bloom, A. Mittereder, V. Ton, K. Neitzke, S. Chasovskikh, T. R. Mhyre and K. A. Maguire-Zeiss (2011):

α-Synuclein Alters Toll-Like Receptor Expression.

Front Neurosci 5: 80. DOI: 10.3389/fnins.2011.00080.

Bernaus, A., S. Blanco and A. Sevilla (2020):

Glia Crosstalk in Neuroinflammatory Diseases.

Front Cell Neurosci 14: 209. DOI: 10.3389/fncel.2020.00209.

Boehmer, E. D., M. J. Meehan, B. T. Cutro and E. J. Kovacs (2005):

Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway.

Mech Ageing Dev 126: 1305-1313. DOI: 10.1016/j.mad.2005.07.009.

Bohlen, C. J., F. C. Bennett, A. F. Tucker, H. Y. Collins, S. B. Mulinyawe and B. A. Barres (2017):

Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures.

Neuron 94: 759-773 e758. DOI: 10.1016/j.neuron.2017.04.043.

Bohlen, C. J., F. C. Bennett and M. L. Bennett (2019):

Isolation and Culture of Microglia.

Curr Protoc Immunol 125: e70. DOI: 10.1002/cpim.70.

Boltz-Nitulescu, G., C. Wiltschke, C. Holzinger, A. Fellinger, O. Scheiner, A. Gessl and O. Forster (1987):

Differentiation of rat bone marrow cells into macrophages under the influence of mouse L929 cell supernatant.

J Leukoc Biol 41: 83-91. DOI: 10.1002/jlb.41.1.83.

Booher, J. and M. Sensenbrenner (1972):

Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures.

Neurobiology 2: 97-105.

Bordt, E. A., C. L. Block, T. Petrozziello, G. Sadri-Vakili, C. J. Smith, A. G. Edlow and S. D. Bilbo (2020):

Isolation of Microglia from Mouse or Human Tissue.

STAR Protoc 1. DOI: 10.1016/j.xpro.2020.100035.

Borrego, F. (2013):

The CD300 molecules: an emerging family of regulators of the immune system.

Blood 121: 1951-1960. DOI: 10.1182/blood-2012-09-435057.

Bowles, K. R., J. Tcw, L. Qian, B. M. Jadow and A. M. Goate (2019):

Reduced variability of neural progenitor cells and improved purity of neuronal cultures using magnetic activated cell sorting.

PLoS One 14: e0213374. DOI: 10.1371/journal.pone.0213374.

Bowman, C. C., A. Rasley, S. L. Tranguch and I. Marriott (2003):

Cultured astrocytes express toll-like receptors for bacterial products.

Glia 43: 281-291. DOI: 10.1002/glia.10256.

Boyd, A. R., P. Shivshankar, S. Jiang, M. T. Berton and C. J. Orihuela (2012):

Age-related defects in TLR2 signaling diminish the cytokine response by alveolar macrophages during murine pneumococcal pneumonia.

Exp Gerontol 47: 507-518. DOI: 10.1016/j.exger.2012.04.004.

Brown, D. R. and H. A. Kretzschmar (1998):

The glio-toxic mechanism of α-aminoadipic acid on cultured astrocytes.

J Neurocytol 27: 109-118. DOI: 10.1023/a:1006947322342.

Bsibsi, M., R. Ravid, D. Gveric and J. M. van Noort (2002):

Broad expression of Toll-like receptors in the human central nervous system.

J Neuropathol Exp Neurol 61: 1013-1021. DOI: 10.1093/jnen/61.11.1013.

Budnik, V., C. Ruiz-Canada and F. Wendler (2016):

Extracellular vesicles round off communication in the nervous system.

Nat Rev Neurosci 17: 160-172. DOI: 10.1038/nrn.2015.29.

Buenaventura, R. G., A. C. Harvey, M. P. Burns and B. S. Main (2022):

Sequential Isolation of Microglia and Astrocytes from Young and Aged Adult Mouse Brains for Downstream Transcriptomic Analysis.

Methods Protoc 5. DOI: 10.3390/mps5050077.

Burda, J. E., A. M. Bernstein and M. V. Sofroniew (2016):

Astrocyte roles in traumatic brain injury.

Exp Neurol 275 Pt 3: 305-315. DOI: 10.1016/j.expneurol.2015.03.020.

Burmeister, A. R. and I. Marriott (2018):

The Interleukin-10 Family of Cytokines and Their Role in the CNS.

Front Cell Neurosci 12: 458. DOI: 10.3389/fncel.2018.00458.

Butovsky, O., M. P. Jedrychowski, C. S. Moore, R. Cialic, A. J. Lanser, G. Gabriely, T. Koeglsperger, B. Dake, P. M. Wu, C. E. Doykan, Z. Fanek, L. Liu, Z. Chen, J. D. Rothstein, R. M. Ransohoff, S. P. Gygi, J. P. Antel and H. L. Weiner (2014):

Identification of a unique TGF-β-dependent molecular and functional signature in microglia.

Nat Neurosci 17: 131-143. DOI: 10.1038/nn.3599.

Butt, A. and A. Verkhratsky (2018):

Neuroglia: Realising their true potential.

Brain Neurosci Adv 2: 2398212818817495. DOI: 10.1177/2398212818817495.

Butt, A. M. (1991):

Macroglial cell types, lineage, and morphology in the CNS.

Ann N Y Acad Sci 633: 90-95. DOI: 10.1111/j.1749-6632.1991.tb15598.x.

Buwitt-Beckmann, U., H. Heine, K. H. Wiesmuller, G. Jung, R. Brock, S. Akira and A. J. Ulmer (2006):

TLR1- and TLR6-independent recognition of bacterial lipopeptides.

J Biol Chem 281: 9049-9057. DOI: 10.1074/jbc.M512525200.

Cahoy, J. D., B. Emery, A. Kaushal, L. C. Foo, J. L. Zamanian, K. S. Christopherson, Y. Xing, J. L. Lubischer, P. A. Krieg, S. A. Krupenko, W. J. Thompson and B. A. Barres (2008):

A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function.

J Neurosci 28: 264-278. DOI: 10.1523/JNEUROSCI.4178-07.2008.

Caldeira, C., C. Cunha, A. R. Vaz, A. S. Falcao, A. Barateiro, E. Seixas, A. Fernandes and D. Brites (2017):

Key Aging-Associated Alterations in Primary Microglia Response to Beta-Amyloid Stimulation.

Front Aging Neurosci 9: 277. DOI: 10.3389/fnagi.2017.00277.

Campanella, M., C. Sciorati, G. Tarozzo and M. Beltramo (2002):

Flow cytometric analysis of inflammatory cells in ischemic rat brain.

Stroke 33: 586-592. DOI: 10.1161/hs0202.103399.

Campbell, G. L., M. Schanchner and S. O. Sharrow (1977):

Isolation of glial cell-enriched and -depleted populations from mouse cerebellum by density gradient centrifugation and electronic cell sorting.

Brain Res 127: 69-86. DOI: 10.1016/0006-8993(77)90380-8.

Cardona, A. E., D. Huang, M. E. Sasse and R. M. Ransohoff (2006):

Isolation of murine microglial cells for RNA analysis or flow cytometry.

Nat Protoc 1: 1947-1951. DOI: 10.1038/nprot.2006.327.

Carpentier, P. A., W. S. Begolka, J. K. Olson, A. Elhofy, W. J. Karpus and S. D. Miller (2005):

Differential activation of astrocytes by innate and adaptive immune stimuli.

Glia 49: 360-374. DOI: 10.1002/glia.20117.

Carson, M. J., J. M. Doose, B. Melchior, C. D. Schmid and C. C. Ploix (2006):

CNS immune privilege: hiding in plain sight.

Immunol Rev 213: 48-65. DOI: 10.1111/j.1600-065X.2006.00441.x.

Casula, M., A. M. Iyer, W. G. Spliet, J. J. Anink, K. Steentjes, M. Sta, D. Troost and E. Aronica (2011):

Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue.

Neuroscience 179: 233-243. DOI: 10.1016/j.neuroscience.2011.02.001.

Chakrabarty, P., K. Jansen-West, A. Beccard, C. Ceballos-Diaz, Y. Levites, C. Verbeeck, A. C. Zubair, D. Dickson, T. E. Golde and P. Das (2010):

Massive gliosis induced by interleukin-6 suppresses Aβ deposition *in vivo*: evidence against inflammation as a driving force for amyloid deposition.

FASEB J 24: 548-559. DOI: 10.1096/fj.09-141754.

Chen, C. Z., B. Neumann, S. Forster and R. J. M. Franklin (2021):

Schwann cell remyelination of the central nervous system: why does it happen and what are the benefits?

Open Biol 11: 200352. DOI: 10.1098/rsob.200352.

Chen, S. H., E. A. Oyarzabal, Y. F. Sung, C. H. Chu, Q. Wang, S. L. Chen, R. B. Lu and J. S. Hong (2015):

Microglial regulation of immunological and neuroprotective functions of astroglia.

Glia 63: 118-131. DOI: 10.1002/glia.22738.

Chen, Y., J. Zhu, J. Ji, Z. Liu and G. Ren (2022):

The role of microglial exosomes in brain injury.

Frontiers in Cellular Neuroscience 16. DOI: 10.3389/fncel.2022.1003809.

Chew, L. J., C. A. DeBoy and V. V. Senatorov, Jr. (2014):

Finding degrees of separation: experimental approaches for astroglial and oligodendroglial cell isolation and genetic targeting.

J Neurosci Methods 236: 125-147. DOI: 10.1016/j.jneumeth.2014.08.017.

Chiareli, R. A., G. A. Carvalho, B. L. Marques, L. S. Mota, O. C. Oliveira-Lima, R. M. Gomes, A. Birbrair, R. S. Gomez, F. Simao, F. Klempin, M. Leist and M. C. X. Pinto (2021):

The Role of Astrocytes in the Neurorepair Process.

Front Cell Dev Biol 9: 665795. DOI: 10.3389/fcell.2021.665795.

Cho, S. H., J. A. Chen, F. Sayed, M. E. Ward, F. Gao, T. A. Nguyen, G. Krabbe, P. D. Sohn, I. Lo, S. Minami, N. Devidze, Y. Zhou, G. Coppola and L. Gan (2015):

SIRT1 deficiency in microglia contributes to cognitive decline in aging and neurodegeneration via epigenetic regulation of IL-1β.

J Neurosci 35: 807-818. DOI: 10.1523/JNEUROSCI.2939-14.2015.

Chuo, S. T., J. C. Chien and C. P. Lai (2018):

Imaging extracellular vesicles: current and emerging methods.

J Biomed Sci 25: 91. DOI: 10.1186/s12929-018-0494-5.

Chvátal, A. and A. Verkhratsky (2018):

An Early History of Neuroglial Research: Personalities.

Neuroglia 1: 245-257. DOI: https://doi.org/10.3390/neuroglia1010016.

Comas-Casellas, E., A. Martinez-Barriocanal, F. Miro, A. Ejarque-Ortiz, S. Schwartz, Jr., M. Martin and J. Sayos (2012):

Cloning and Characterization of CD300d, a Novel Member of

the Human CD300 Family of Immune Receptors.

J Biol Chem 287: 9682-9693. DOI: 10.1074/jbc.M111.279224.

Crain, J. M., M. Nikodemova and J. J. Watters (2009):

Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia.

J Neuroinflammation 6: 24. DOI: 10.1186/1742-2094-6-24.

Crocker, S. J., R. F. Frausto, J. L. Whitton and R. Milner (2008):

A novel method to establish microglia-free astrocyte cultures: comparison of matrix metalloproteinase expression profiles in pure cultures of astrocytes and microglia.

Glia 56: 1187-1198. DOI: 10.1002/glia.20689.

Curtis, R., J. Cohen, J. Fok-Seang, M. R. Hanley, N. A. Gregson, R. Reynolds and G. P. Wilkin (1988):

Development of macroglial cells in rat cerebellum. I. Use of antibodies to follow early *in vivo* development and migration of oligodendrocytes.

J Neurocytol 17: 43-54. DOI: 10.1007/BF01735376.

Daniele, S. G., D. Beraud, C. Davenport, K. Cheng, H. Yin and K. A. Maguire-Zeiss (2015):

Activation of MyD88-dependent TLR1/2 signaling by misfolded α-synuclein, a protein linked to neurodegenerative disorders.

Sci Signal 8: ra45. DOI: 10.1126/scisignal.2005965.

Davalos, D., J. Grutzendler, G. Yang, J. V. Kim, Y. Zuo, S. Jung, D. R. Littman, M. L. Dustin and W. B. Gan (2005):

ATP mediates rapid microglial response to local brain injury *in vivo*.

Nat Neurosci 8: 752-758. DOI: 10.1038/nn1472.

Davis, E. J., T. D. Foster and W. E. Thomas (1994):

Cellular forms and functions of brain microglia.

Brain Res Bull 34: 73-78. DOI: 10.1016/0361-9230(94)90189-9.

de Haas, A. H., H. W. Boddeke, N. Brouwer and K. Biber (2007):

Optimized isolation enables *ex vivo* analysis of microglia from various central nervous system regions.

Glia 55: 1374-1384. DOI: 10.1002/glia.20554.

del Zoppo, G. J. (2010):

The neurovascular unit in the setting of stroke.

J Intern Med 267: 156-171. DOI: 10.1111/j.1365-2796.2009.02199.x.

DePaolo, R. W., F. Tang, I. Kim, M. Han, N. Levin, N. Ciletti, A. Lin, D. Anderson, O. Schneewind and B. Jabri (2008):

Toll-Like Receptor 6 Drives Differentiation of Tolerogenic Dendritic Cells and Contributes to LcrV-Mediated Plague Pathogenesis.

Cell Host & Microbe 4: 350-361. DOI: https://doi.org/10.1016/j.chom.2008.09.004.

Diaz-Aparicio, I., I. Paris, V. Sierra-Torre, A. Plaza-Zabala, N. Rodriguez-Iglesias, M. Marquez-Ropero, S. Beccari, P. Huguet, O. Abiega, E. Alberdi, C. Matute, I. Bernales, A. Schulz, L. Otrokocsi, B. Sperlagh, K. E. Happonen, G. Lemke, M. Maletic-Savatic, J. Valero and A. Sierra (2020):

Microglia Actively Remodel Adult Hippocampal Neurogenesis through the Phagocytosis Secretome.

J Neurosci 40: 1453-1482. DOI: 10.1523/JNEUROSCI.0993-19.2019.

Doyle, J. P., J. D. Dougherty, M. Heiman, E. F. Schmidt, T. R. Stevens, G. Ma, S. Bupp, P. Shrestha, R. D. Shah, M. L. Doughty, S. Gong, P. Greengard and N. Heintz (2008):

Application of a translational profiling approach for the comparative analysis of CNS cell types.

Cell 135: 749-762. DOI: 10.1016/j.cell.2008.10.029.

Du, X., B. Fleiss, H. Li, B. D'Angelo, Y. Sun, C. Zhu, H. Hagberg, O. Levy, C. Mallard and X. Wang (2011):

Systemic stimulation of TLR2 impairs neonatal mouse brain development.

PLoS One 6: e19583. DOI: 10.1371/journal.pone.0019583.

Du Yan, S., H. Zhu, J. Fu, S. F. Yan, A. Roher, W. W. Tourtellotte, T. Rajavashisth, X. Chen, G. C. Godman, D. Stern and A. M. Schmidt (1997):

Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease.

Proc Natl Acad Sci U S A 94: 5296-5301. DOI: 10.1073/pnas.94.10.5296.

Dudiki, T., J. Meller, G. Mahajan, H. Liu, I. Zhevlakova, S. Stefl, C. Witherow, E. Podrez, C. R. Kothapalli and T. V. Byzova (2020):

Microglia control vascular architecture via a TGFbeta1 dependent paracrine mechanism linked to tissue mechanics.

Nat Commun 11: 986. DOI: 10.1038/s41467-020-14787-y.

Ebert, S., J. Gerber, S. Bader, F. Muhlhauser, K. Brechtel, T. J. Mitchell and R. Nau (2005):

Dose-dependent activation of microglial cells by Toll-like receptor agonists alone and in combination.

J Neuroimmunol 159: 87-96. DOI: 10.1016/j.jneuroim.2004.10.005.

Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli and R. Landmann (2002):

Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation.

J Infect Dis 186: 798-806. DOI: 10.1086/342845.

Edelson, B. T. and E. R. Unanue (2002):

MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity.

J Immunol 169: 3869-3875. DOI: 10.4049/jimmunol.169.7.3869.

Elmore, M. R., A. R. Najafi, M. A. Koike, N. N. Dagher, E. E. Spangenberg, R. A. Rice, M. Kitazawa, B. Matusow, H. Nguyen, B. L. West and K. N. Green (2014):

Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain.

Neuron 82: 380-397. DOI: 10.1016/j.neuron.2014.02.040.

Eng, L. F., R. S. Ghirnikar and Y. L. Lee (2000):

Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000).

Neurochem Res 25: 1439-1451. DOI: 10.1023/a:1007677003387.

Escartin, C., E. Galea, A. Lakatos, J. P. O'Callaghan, G. C. Petzold, A. Serrano-Pozo, C. Steinhauser, A. Volterra, G. Carmignoto, A. Agarwal, N. J. Allen, A. Araque, L. Barbeito, A. Barzilai, D. E. Bergles, G. Bonvento, A. M. Butt, W. T. Chen, M. Cohen-Salmon, C. Cunningham, B. Deneen, B. De Strooper, B. Diaz-Castro, C. Farina, M. Freeman, V. Gallo, J. E. Goldman, S. A. Goldman, M. Gotz, A. Gutierrez, P. G. Haydon, D. H. Heiland, E. M. Hol, M. G. Holt, M. Iino, K. V. Kastanenka, H. Kettenmann, B. S. Khakh, S. Koizumi, C. J. Lee, S. A. Liddelow, B. A. MacVicar, P. Magistretti, A. Messing, A. Mishra, A. V. Molofsky, K. K. Murai, C. M. Norris, S. Okada, S. H. R. Oliet, J. F. Oliveira, A. Panatier, V. Parpura, M. Pekna, M. Pekny, L. Pellerin, G. Perea, B. G. Perez-Nievas, F. W. Pfrieger, K. E. Poskanzer, F. J. Quintana, R. M. Ransohoff, M. Riquelme-Perez, S. Robel, C. R. Rose, J. D. Rothstein, N. Rouach, D. H. Rowitch, A. Semyanov, S. Sirko, H. Sontheimer, R. A. Swanson, J. Vitorica, I. B. Wanner, L. B. Wood, J. Wu, B. Zheng, E. R. Zimmer, R. Zorec, M. V. Sofroniew and A. Verkhratsky (2021):

Reactive astrocyte nomenclature, definitions, and future directions.

Nat Neurosci 24: 312-325. DOI: 10.1038/s41593-020-00783-4.

Esen, N., F. Y. Tanga, J. A. DeLeo and T. Kielian (2004):

Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the Gram-positive bacterium *Staphylococcus aureus*.

J Neurochem 88: 746-758. DOI: 10.1046/j.1471-4159.2003.02202.x.

Faraco, G., S. Fossati, M. E. Bianchi, M. Patrone, M. Pedrazzi, B. Sparatore, F. Moroni and A. Chiarugi (2007):

High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration *in vitro* and *in vivo*.

J Neurochem 103: 590-603. DOI: 10.1111/j.1471-4159.2007.04788.x.

Farina, C., M. Krumbholz, T. Giese, G. Hartmann, F. Aloisi and E. Meinl (2005):

Preferential expression and function of Toll-like receptor 3 in human astrocytes.

J Neuroimmunol 159: 12-19. DOI: 10.1016/j.jneuroim.2004.09.009.

Fiebich, B. L., C. R. A. Batista, S. W. Saliba, N. M. Yousif and A. C. P. de Oliveira (2018):

Role of Microglia TLRs in Neurodegeneration.

Front Cell Neurosci 12: 329. DOI: 10.3389/fncel.2018.00329.

Fitzgerald, K. A. and J. C. Kagan (2020):

Toll-like Receptors and the Control of Immunity.

Cell 180: 1044-1066. DOI: 10.1016/j.cell.2020.02.041.

Flanary, B. E. and W. J. Streit (2004):

Progressive telomere shortening occurs in cultured rat microglia, but not astrocytes.

Glia 45: 75-88. DOI: 10.1002/glia.10301.

Foo, L. C., N. J. Allen, E. A. Bushong, P. B. Ventura, W. S. Chung, L. Zhou, J. D. Cahoy, R. Daneman, H. Zong, M. H. Ellisman and B. A. Barres (2011):

Development of a method for the purification and culture of rodent astrocytes.

Neuron 71: 799-811. DOI: 10.1016/j.neuron.2011.07.022.

Ford, A. L., A. L. Goodsall, W. F. Hickey and J. D. Sedgwick (1995):

Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct *ex vivo* antigen presentation to myelin basic protein-reactive $CD4^+$ T cells compared.

J Immunol 154: 4309-4321.

Fournier, T., N. N. Medjoubi and D. Porquet (2000):

Alpha-1-acid glycoprotein.

Biochim Biophys Acta 1482: 157-171. DOI: 10.1016/s0167-4838(00)00153-9.

Franklin, R. J. M. and C. Ffrench-Constant (2017):

Regenerating CNS myelin - from mechanisms to experimental medicines.

Nature Reviews Neuroscience 18: 753-769. DOI: 10.1038/nrn.2017.136.

Fujita, H., J. Tanaka, K. Toku, N. Tateishi, Y. Suzuki, S. Matsuda, M. Sakanaka and N. Maeda (1996):

Effects of GM-CSF and ordinary supplements on the ramification of microglia in culture: a morphometrical study.

Glia 18: 269-281. DOI: 10.1002/(sici)1098-1136(199612)18:4<269::aid-glia2>3.0.co;2-t.

Galea, I., I. Bechmann and V. H. Perry (2007):

What is immune privilege (not)?

Trends in Immunology 28: 12-18. DOI: https://doi.org/10.1016/j.it.2006.11.004.

Galea, I. (2021):

The blood-brain barrier in systemic infection and inflammation.

Cell Mol Immunol 18: 2489-2501. DOI: 10.1038/s41423-021-00757-x.

Getts, D. R., R. L. Terry, M. T. Getts, M. Muller, S. Rana, B. Shrestha, J. Radford, N. Van Rooijen, I. L. Campbell and N. J. King (2008):

Ly6c$^+$ “inflammatory monocytes” are microglial precursors recruited in a pathogenic manner in West Nile virus encephalitis.

J Exp Med 205: 2319-2337. DOI: 10.1084/jem.20080421.

Ginhoux, F., M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov and M. Merad (2010):

Fate mapping analysis reveals that adult microglia derive from primitive macrophages.

Science 330: 841-845. DOI: 10.1126/science.1194637.

Giulian, D. and T. J. Baker (1986):

Characterization of ameboid microglia isolated from developing mammalian brain.

J Neurosci 6: 2163-2178.

Gonzalez-Perez, O., V. Lopez-Virgen and A. Quiñones-Hinojosa (2015):

Astrocytes: Everything but the glue.

Neuroimmunol Neuroinflamm 2: 115-117. DOI: 10.4103/2347-8659.153979.

Gosselin, D., D. Skola, N. G. Coufal, I. R. Holtman, J. C. M. Schlachetzki, E. Sajti, B. N. Jaeger, C. O'Connor, C. Fitzpatrick, M. P. Pasillas, M. Pena, A. Adair, D. D. Gonda, M. L. Levy, R. M. Ransohoff, F. H. Gage and C. K. Glass (2017):

An environment-dependent transcriptional network specifies human microglia identity.

Science 356. DOI: 10.1126/science.aal3222.

Grabert, K. and B. W. McColl (2018):

Isolation and Phenotyping of Adult Mouse Microglial Cells.

Methods Mol Biol 1784: 77-86. DOI: 10.1007/978-1-4939-7837-3_7.

Graeber, M. B. and G. W. Kreutzberg (1988):

Delayed astrocyte reaction following facial nerve axotomy.

J Neurocytol 17: 209-220. DOI: 10.1007/BF01674208.

Greenhalgh, A. D., S. David and F. C. Bennett (2020):

Immune cell regulation of glia during CNS injury and disease.

Nat Rev Neurosci 21: 139-152. DOI: 10.1038/s41583-020-0263-9.

Guez-Barber, D., S. Fanous, B. K. Harvey, Y. Zhang, E. Lehrmann, K. G. Becker, M. R. Picciotto and B. T. Hope (2012):

FACS purification of immunolabeled cell types from adult rat brain.

J Neurosci Methods 203: 10-18. DOI: 10.1016/j.jneumeth.2011.08.045.

Guillemin, G., F. D. Boussin, J. Croitoru, M. Franck-Duchenne, R. Le Grand, F. Lazarini and D. Dormont (1997):

Obtention and characterization of primary astrocyte and microglial cultures from adult monkey brains.

J Neurosci Res 49: 576-591. DOI: 10.1002/(SICI)1097-4547(19970901)49:5<576::AID-JNR8>3.0.CO;2-8.

Guillot-Sestier, M. V. and T. Town (2018):

Let's make microglia great again in neurodegenerative disorders.

J Neural Transm (Vienna) 125: 751-770. DOI: 10.1007/s00702-017-1792-x.

Guneykaya, D., A. Ivanov, D. P. Hernandez, V. Haage, B. Wojtas, N. Meyer, M. Maricos, P. Jordan, A. Buonfiglioli, B. Gielniewski, N. Ochocka, C. Comert, C. Friedrich, L. S. Artiles, B. Kaminska, P. Mertins, D. Beule, H. Kettenmann and S. A. Wolf (2018):

Transcriptional and Translational Differences of Microglia from Male and Female Brains.

Cell Rep 24: 2773-2783 e2776. DOI: 10.1016/j.celrep.2018.08.001.

Guo, H. Y., A. C. Cheng, M. S. Wang, Z. Q. Yin and R. Y. Jia (2020):

Exosomes: Potential Therapies for Disease via Regulating TLRs.

Mediators Inflamm 2020: 2319616. DOI: 10.1155/2020/2319616.

Guo, M., Y. Hao, Y. Feng, H. Li, Y. Mao, Q. Dong and M. Cui (2021):

Microglial Exosomes in Neurodegenerative Disease.

Front Mol Neurosci 14: 630808. DOI: 10.3389/fnmol.2021.630808.

Gupta, A. and L. Pulliam (2014):

Exosomes as mediators of neuroinflammation.

J Neuroinflammation 11: 68. DOI: 10.1186/1742-2094-11-68.

Guttenplan, K. A. and S. A. Liddelow (2019):

Astrocytes and microglia: Models and tools.

J Exp Med 216: 71-83. DOI: 10.1084/jem.20180200.

Haage, V., M. Semtner, R. O. Vidal, D. P. Hernandez, W. W. Pong, Z. Chen, D. Hambardzumyan, V. Magrini, A. Ly, J. Walker, E. Mardis, P. Mertins, S. Sauer, H. Kettenmann and D. H. Gutmann (2019):

Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma.

Acta Neuropathol Commun 7: 20. DOI: 10.1186/s40478-019-0665-y.

Hagemeyer, N., K. M. Hanft, M. A. Akriditou, N. Unger, E. S. Park, E. R. Stanley, O. Staszewski, L. Dimou and M. Prinz (2017):

Microglia contribute to normal myelinogenesis and to oligodendrocyte progenitor maintenance during adulthood.

Acta Neuropathol 134: 441-458. DOI: 10.1007/s00401-017-1747-1.

Haj-Yasein, N. N., V. Jensen, I. Ostby, S. W. Omholt, J. Voipio, K. Kaila, O. P. Ottersen, O. Hvalby and E. A. Nagelhus (2012):

Aquaporin-4 regulates extracellular space volume dynamics during high-frequency synaptic stimulation: a gene deletion study in mouse hippocampus.

Glia 60: 867-874. DOI: 10.1002/glia.22319.

Halder, S. K. and R. Milner (2019):

A critical role for microglia in maintaining vascular integrity in the hypoxic spinal cord.

Proc Natl Acad Sci U S A 116: 26029-26037. DOI: 10.1073/pnas.1912178116.

Hamby, M. E., T. F. Uliasz, S. J. Hewett and J. A. Hewett (2006):

Characterization of an improved procedure for the removal of microglia from confluent monolayers of primary astrocytes.

J Neurosci Methods 150: 128-137. DOI: 10.1016/j.jneumeth.2005.06.016.

Hao, C., A. Richardson and S. Fedoroff (1991):

Macrophage-like cells originate from neuroepithelium in culture: characterization and properties of the macrophage-like cells.

Int J Dev Neurosci 9: 1-14. DOI: 10.1016/0736-5748(91)90067-v.

Harms, A. S. and M. G. Tansey (2013):

Isolation of murine postnatal brain microglia for phenotypic characterization using magnetic cell separation technology.

Methods Mol Biol 1041: 33-39. DOI: 10.1007/978-1-62703-520-0_5.

Haruwaka, K., A. Ikegami, Y. Tachibana, N. Ohno, H. Konishi, A. Hashimoto, M. Matsumoto, D. Kato, R. Ono, H. Kiyama, A. J. Moorhouse, J. Nabekura and H. Wake (2019):

Dual microglia effects on blood brain barrier permeability induced by systemic inflammation.

Nat Commun 10: 5816. DOI: 10.1038/s41467-019-13812-z.

He, Y., X. Yao, N. Taylor, Y. Bai, T. Lovenberg and A. Bhattacharya (2018):

RNA sequencing analysis reveals quiescent microglia isolation methods from postnatal mouse brains and limitations of BV2 cells.

J Neuroinflammation 15: 153. DOI: 10.1186/s12974-018-1195-4.

He, Y., N. Taylor, X. Yao and A. Bhattacharya (2021):

Mouse primary microglia respond differently to LPS and poly(I:C) *in vitro*.

Sci Rep 11: 10447. DOI: 10.1038/s41598-021-89777-1.

Hickman, S. E., N. D. Kingery, T. K. Ohsumi, M. L. Borowsky, L. C. Wang, T. K. Means and J. El Khoury (2013):

The microglial sensome revealed by direct RNA sequencing.

Nat Neurosci 16: 1896-1905. DOI: 10.1038/nn.3554.

Hoffmann, O., J. S. Braun, D. Becker, A. Halle, D. Freyer, E. Dagand, S. Lehnardt and J. R. Weber (2007):

TLR2 mediates neuroinflammation and neuronal damage.

J Immunol 178: 6476-6481. DOI: 10.4049/jimmunol.178.10.6476.

Holm, T. H., D. Draeby and T. Owens (2012):

Microglia are required for astroglial Toll-like receptor 4 response and for optimal TLR2 and TLR3 response.

Glia 60: 630-638. DOI: 10.1002/glia.22296.

Holt, L. M. and M. L. Olsen (2016):

Novel Applications of Magnetic Cell Sorting to Analyze Cell-Type Specific Gene and Protein Expression in the Central Nervous System.

PLoS One 11: e0150290. DOI: 10.1371/journal.pone.0150290.

Holt, L. M., S. T. Stoyanof and M. L. Olsen (2019):

Magnetic Cell Sorting for *In Vivo* and *In Vitro* Astrocyte, Neuron, and Microglia Analysis.

Curr Protoc Neurosci 88: e71. DOI: 10.1002/cpns.71.

Holtman, I. R., D. D. Raj, J. A. Miller, W. Schaafsma, Z. Yin, N. Brouwer, P. D. Wes, T. Moller, M. Orre, W. Kamphuis, E. M. Hol, E. W. Boddeke and B. J. Eggen (2015):

Induction of a common microglia gene expression signature by aging and neurodegenerative conditions: a co-expression meta-analysis.

Acta Neuropathol Commun 3: 31. DOI: 10.1186/s40478-015-0203-5.

Honarpisheh, P., J. Lee, A. Banerjee, M. P. Blasco-Conesa, P. Honarpisheh, J. d'Aigle, A. A. Mamun, R. M. Ritzel, A. Chauhan, B. P. Ganesh and L. D. McCullough (2020):

Potential caveats of putative microglia-specific markers for assessment of age-related cerebrovascular neuroinflammation.

J Neuroinflammation 17: 366. DOI: 10.1186/s12974-020-02019-5.

Hou, L., X. Zhou, C. Zhang, K. Wang, X. Liu, Y. Che, F. Sun, H. Li, Q. Wang, D. Zhang and J. S. Hong (2017):

NADPH oxidase-derived H_2O_2 mediates the regulatory effects of microglia on astrogliosis in experimental models of Parkinson's disease.

Redox Biol 12: 162-170. DOI: 10.1016/j.redox.2017.02.016.

Howarth, C. (2014):

The contribution of astrocytes to the regulation of cerebral blood flow.

Front Neurosci 8: 103. DOI: 10.3389/fnins.2014.00103.

Hu, J., P. Wang, Z. Wang, Y. Xu, W. Peng, X. Chen, Y. Fang, L. Zhu, D. Wang, X. Wang, L. Lin and L. Ruan (2022):

Fibroblast-Conditioned Media Enhance the Yield of Microglia Isolated from Mixed Glial Cultures.

Cell Mol Neurobiol. DOI: 10.1007/s10571-022-01193-9.

Hughes, A. N. and B. Appel (2020):

Microglia phagocytose myelin sheaths to modify developmental myelination.

Nat Neurosci 23: 1055-1066. DOI: 10.1038/s41593-020-0654-2.

Hui, C. W., Y. Zhang and K. Herrup (2016):

Non-Neuronal Cells Are Required to Mediate the Effects of Neuroinflammation: Results from a Neuron-Enriched Culture System.

PLoS One 11: e0147134. DOI: 10.1371/journal.pone.0147134.

Iadecola, C. and M. Nedergaard (2007):

Glial regulation of the cerebral microvasculature.

Nat Neurosci 10: 1369-1376. DOI: 10.1038/nn2003.

Iadecola, C. (2017):

The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease.

Neuron 96: 17-42. DOI: 10.1016/j.neuron.2017.07.030.

Iizumi, T., S. Takahashi, K. Mashima, K. Minami, Y. Izawa, T. Abe, T. Hishiki, M. Suematsu, M. Kajimura and N. Suzuki (2016):

A possible role of microglia-derived nitric oxide by lipopolysaccharide in activation of astroglial pentose-phosphate pathway via the Keap1/Nrf2 system.

J Neuroinflammation 13: 99. DOI: 10.1186/s12974-016-0564-0.

Imai, Y. and S. Kohsaka (2002):

Intracellular signaling in M-CSF-induced microglia activation: role of Iba1.

Glia 40: 164-174. DOI: 10.1002/glia.10149.

Itoyama, Y., H. D. Webster, E. P. Richardson, Jr. and B. D. Trapp (1983):

Schwann cell remyelination of demyelinated axons in spinal cord multiple sclerosis lesions.

Ann Neurol 14: 339-346. DOI: 10.1002/ana.410140313.

Jack, C. S., N. Arbour, J. Manusow, V. Montgrain, M. Blain, E. McCrea, A. Shapiro and J. P. Antel (2005):

TLR signaling tailors innate immune responses in human microglia and astrocytes.

J Immunol 175: 4320-4330. DOI: 10.4049/jimmunol.175.7.4320.

Jäkel, S. and L. Dimou (2017):

Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation.

Front Cell Neurosci 11: 24. DOI: 10.3389/fncel.2017.00024.

Jana, M., C. A. Palencia and K. Pahan (2008):

Fibrillar Amyloid-β Peptides Activate Microglia via TLR2: Implications for Alzheimer's Disease.

J Immunol 181: 7254-7262. DOI: 10.4049/jimmunol.181.10.7254.

Jeon, H., S. Lee, W. H. Lee and K. Suk (2010):

Analysis of glial secretome: the long pentraxin PTX3 modulates phagocytic activity of microglia.

J Neuroimmunol 229: 63-72. DOI: 10.1016/j.jneuroim.2010.07.001.

Jeon, H., J. H. Kim, J. H. Kim, W. H. Lee, M. S. Lee and K. Suk (2012):

Plasminogen activator inhibitor type 1 regulates microglial motility and phagocytic activity.

J Neuroinflammation 9: 149. DOI: 10.1186/1742-2094-9-149.

Jeong, H. K., K. M. Ji, B. Kim, J. Kim, I. Jou and E. H. Joe (2010):

Inflammatory responses are not sufficient to cause delayed neuronal death in ATP-induced acute brain injury.

PLoS One 5: e13756. DOI: 10.1371/journal.pone.0013756.

Jeong, H. K., K. Ji, K. Min and E. H. Joe (2013):

Brain inflammation and microglia: facts and misconceptions.

Exp Neurobiol 22: 59-67. DOI: 10.5607/en.2013.22.2.59.

Jha, M. K., S. Lee, D. H. Park, H. Kook, K. G. Park, I. K. Lee and K. Suk (2015):

Diverse functional roles of lipocalin-2 in the central nervous system.

Neurosci Biobehav Rev 49: 135-156. DOI: 10.1016/j.neubiorev.2014.12.006.

Jha, M. K., M. Jo, J. H. Kim and K. Suk (2019):

Microglia-Astrocyte Crosstalk: An Intimate Molecular Conversation.

Neuroscientist 25: 227-240. DOI: 10.1177/1073858418783959.

Jin, L. W., M. Horiuchi, H. Wulff, X. B. Liu, G. A. Cortopassi, J. D. Erickson and I. Maezawa (2015):

Dysregulation of glutamine transporter SNAT1 in Rett syndrome microglia: a mechanism for mitochondrial dysfunction and neurotoxicity.

J Neurosci 35: 2516-2529. DOI: 10.1523/JNEUROSCI.2778-14.2015.

Jin, M., J. H. Kim, E. Jang, Y. M. Lee, H. Soo Han, D. K. Woo, D. H. Park, H. Kook and K. Suk (2014):

Lipocalin-2 deficiency attenuates neuroinflammation and brain injury after transient middle cerebral artery occlusion in mice.

J Cereb Blood Flow Metab 34: 1306-1314. DOI: 10.1038/jcbfm.2014.83.

Jo, M., J. H. Kim, G. J. Song, M. Seo, E. M. Hwang and K. Suk (2017):

Astrocytic Orosomucoid-2 Modulates Microglial Activation and Neuroinflammation.

J Neurosci 37: 2878-2894. DOI: 10.1523/JNEUROSCI.2534-16.2017.

Jurga, A. M., M. Paleczna and K. Z. Kuter (2020):

Overview of General and Discriminating Markers of Differential Microglia Phenotypes.

Front Cell Neurosci 14: 198. DOI: 10.3389/fncel.2020.00198.

Jurga, A. M., M. Paleczna, J. Kadluczka and K. Z. Kuter (2021):

Beyond the GFAP-Astrocyte Protein Markers in the Brain.

Biomolecules 11. DOI: 10.3390/biom11091361.

Kantzer, C. G., C. Boutin, I. D. Herzig, C. Wittwer, S. Reiss, M. C. Tiveron, J. Drewes, T. D. Rockel, S. Ohlig, J. Ninkovic, H. Cremer, S. Pennartz, M. Jungblut and A. Bosio (2017):

Anti-ACSA-2 defines a novel monoclonal antibody for prospective isolation of living neonatal and adult astrocytes.

Glia 65: 990-1004. DOI: 10.1002/glia.23140.

Kawai, T. and S. Akira (2010):

The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.

Nat Immunol 11: 373-384. DOI: 10.1038/ni.1863.

Keller, D., C. Ero and H. Markram (2018):

Cell Densities in the Mouse Brain: A Systematic Review.

Front Neuroanat 12: 83. DOI: 10.3389/fnana.2018.00083.

Kettenmann, H., F. Kirchhoff and A. Verkhratsky (2013):

Microglia: new roles for the synaptic stripper.

Neuron 77: 10-18. DOI: 10.1016/j.neuron.2012.12.023.

Kielian, T., N. Esen and E. D. Bearden (2005a):

Toll-like receptor 2 (TLR2) is pivotal for recognition of *S. aureus* peptidoglycan but not intact bacteria by microglia.

Glia 49: 567-576. DOI: 10.1002/glia.20144.

Kielian, T., A. Haney, P. M. Mayes, S. Garg and N. Esen (2005b):

Toll-like receptor 2 modulates the proinflammatory milieu in *Staphylococcus aureus*-induced brain abscess.

Infect Immun 73: 7428-7435. DOI: 10.1128/IAI.73.11.7428-7435.2005.

Kielian, T. (2006):

Toll-like receptors in central nervous system glial inflammation and homeostasis.

J Neurosci Res 83: 711-730. DOI: 10.1002/jnr.20767.

Kierdorf, K. and M. Prinz (2017):

Microglia in steady state.

J Clin Invest 127: 3201-3209. DOI: 10.1172/JCI90602.

Kierdorf, K. and M. Prinz (2019):

Microglia: Same same, but different.

J Exp Med 216: 2223-2225. DOI: 10.1084/jem.20191535.

Kim, C., D. H. Ho, J. E. Suk, S. You, S. Michael, J. Kang, S. Joong Lee, E. Masliah, D. Hwang, H. J. Lee and S. J. Lee (2013):

Neuron-released oligomeric α-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia.

Nat Commun 4: 1562. DOI: 10.1038/ncomms2534.

Kim, C., B. Spencer, E. Rockenstein, H. Yamakado, M. Mante, A. Adame, J. A. Fields, D. Masliah, M. Iba, H. J. Lee, R. A. Rissman, S. J. Lee and E. Masliah (2018):

Immunotherapy targeting toll-like receptor 2 alleviates neurodegeneration in models of synucleinopathy by modulating α-synuclein transmission and neuroinflammation.

Mol Neurodegener 13: 43. DOI: 10.1186/s13024-018-0276-2.

Kim, H., S. Lee, H. C. Park, W. H. Lee, M. S. Lee and K. Suk (2011):

Modulation of glial and neuronal migration by lipocalin-2 in zebrafish.

Immune Netw 11: 342-347. DOI: 10.4110/in.2011.11.6.342.

Kim, J. H., P. W. Ko, H. W. Lee, J. Y. Jeong, M. G. Lee, J. H. Kim, W. H. Lee, R. Yu, W. J. Oh and K. Suk (2017):

Astrocyte-derived lipocalin-2 mediates hippocampal damage and cognitive deficits in experimental models of vascular dementia.

Glia 65: 1471-1490. DOI: 10.1002/glia.23174.

Kirk, R. G. W. (2018):

Recovering The Principles of Humane Experimental Technique: The 3Rs and the Human Essence of Animal Research.

Sci Technol Human Values 43: 622-648. DOI: 10.1177/0162243917726579.

Kirschning, C. J. and R. R. Schumann (2002):

TLR2: cellular sensor for microbial and endogenous molecular patterns.

Curr Top Microbiol Immunol 270: 121-144. DOI: 10.1007/978-3-642-59430-4_8.

Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H. W. Pfister and C. J. Kirschning (2003):

Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis.

J Immunol 170: 438-444. DOI: 10.4049/jimmunol.170.1.438.

Kremer, M., A. Henn, C. Kolb, M. Basler, J. Moebius, B. Guillaume, M. Leist, B. J. Van den Eynde and M. Groettrup (2010):

Reduced immunoproteasome formation and accumulation of immunoproteasomal precursors in the brains of lymphocytic choriomeningitis virus-infected mice.

J Immunol 185: 5549-5560. DOI: 10.4049/jimmunol.1001517.

Kreutzberg, G. W. (1995):

Microglia, the first line of defence in brain pathologies.

Arzneimittelforschung 45: 357-360.

Kufe, D. W. and P. P. Major (1982):

Studies on the mechanism of action of cytosine arabinoside.

Med Pediatr Oncol 10 Suppl 1: 49-67. DOI: 10.1002/mpo.2950100708.

Kumamaru, H., H. Saiwai, K. Kobayakawa, K. Kubota, N. van Rooijen, K. Inoue, Y. Iwamoto and S. Okada (2012):

Liposomal clodronate selectively eliminates microglia from primary astrocyte cultures.

J Neuroinflammation 9: 116. DOI: 10.1186/1742-2094-9-116.

Kumar, A., I. C. Fontana and A. Nordberg (2021):

Reactive astrogliosis: A friend or foe in the pathogenesis of Alzheimer's disease.

J Neurochem. DOI: 10.1111/jnc.15565.

Kuo, P. C., W. T. Weng, B. A. Scofield, D. Furnas, H. C. Paraiso, I. C. Yu and J. H. Yen (2021):

Immunoresponsive gene 1 modulates the severity of brain injury in cerebral ischaemia.

Brain Commun 3: fcab187. DOI: 10.1093/braincomms/fcab187.

Ladner, M. B., G. A. Martin, J. A. Noble, V. P. Wittman, M. K. Warren, M. McGrogan and E. R. Stanley (1988):

cDNA cloning and expression of murine macrophage colony-stimulating factor from L929 cells.

Proc Natl Acad Sci U S A 85: 6706-6710. DOI: 10.1073/pnas.85.18.6706.

Laflamme, N., G. Soucy and S. Rivest (2001):

Circulating cell wall components derived from gram-negative, not gram-positive, bacteria cause a profound induction of the gene-encoding Toll-like receptor 2 in the CNS.

J Neurochem 79: 648-657. DOI: 10.1046/j.1471-4159.2001.00603.x.

Lalancette-Hébert, M., J. Faustino, S. S. Thammisetty, S. Chip, Z. S. Vexler and J. Kriz (2017):

Live imaging of the innate immune response in neonates reveals differential TLR2 dependent activation patterns in sterile inflammation and infection.

Brain Behav Immun 65: 312-327. DOI: 10.1016/j.bbi.2017.05.020.

Lampron, A., A. Larochelle, N. Laflamme, P. Prefontaine, M. M. Plante, M. G. Sanchez, V. W. Yong, P. K. Stys, M. E. Tremblay and S. Rivest (2015):

Inefficient clearance of myelin debris by microglia impairs remyelinating processes.

J Exp Med 212: 481-495. DOI: 10.1084/jem.20141656.

Lange, S. C., L. K. Bak, H. S. Waagepetersen, A. Schousboe and M. D. Norenberg (2012):

Primary cultures of astrocytes: their value in understanding astrocytes in health and disease.

Neurochem Res 37: 2569-2588. DOI: 10.1007/s11064-012-0868-0.

Langstein, J., J. Michel, J. Fritsche, M. Kreutz, R. Andreesen and H. Schwarz (1998):

CD137 (ILA/4-1BB), a member of the TNF receptor family, induces monocyte activation via bidirectional signaling.

J Immunol 160: 2488-2494.

Langstein, J., F. M. Becke, L. Sollner, G. Krause, G. Brockhoff, M. Kreutz, R. Andreesen and H. Schwarz (2000):

Comparative analysis of CD137 and LPS effects on monocyte activation, survival, and proliferation.

Biochem Biophys Res Commun 273: 117-122. DOI: 10.1006/bbrc.2000.2889.

Lee, C. G., J. Demarquoy, M. J. Jackson and W. E. O'Brien (1994):

Molecular cloning and characterization of a murine LPS-inducible cDNA.

J Immunol 152: 5758-5767. DOI: Not available.

Lee, H. J., J. E. Suk, C. Patrick, E. J. Bae, J. H. Cho, S. Rho, D. Hwang, E. Masliah and S. J. Lee (2010):

Direct Transfer of α-Synuclein from Neuron to Astroglia Causes Inflammatory Responses in Synucleinopathies.

J Biol Chem 285: 9262-9272. DOI: 10.1074/jbc.M109.081125.

Lee, J. K. and M. G. Tansey (2013):

Microglia isolation from adult mouse brain.

Methods Mol Biol 1041: 17-23. DOI: 10.1007/978-1-62703-520-0_3.

Lee, S., J. Y. Park, W. H. Lee, H. Kim, H. C. Park, K. Mori and K. Suk (2009):

Lipocalin-2 is an autocrine mediator of reactive astrocytosis.

J Neurosci 29: 234-249. DOI: 10.1523/JNEUROSCI.5273-08.2009.

Lee, S., J. H. Kim, J. H. Kim, J. W. Seo, H. S. Han, W. H. Lee, K. Mori, K. Nakao, J. Barasch and K. Suk (2011):

Lipocalin-2 Is a chemokine inducer in the central nervous system: role of chemokine ligand 10 (CXCL10) in lipocalin-2-induced cell migration.

J Biol Chem 286: 43855-43870. DOI: 10.1074/jbc.M111.299248.

Lehnardt, S., P. Henneke, E. Lien, D. L. Kasper, J. J. Volpe, I. Bechmann, R. Nitsch, J. R. Weber, D. T. Golenbock and T. Vartanian (2006):

A mechanism for neurodegeneration induced by group B streptococci through activation of the TLR2/MyD88 pathway in microglia.

J Immunol 177: 583-592. DOI: 10.4049/jimmunol.177.1.583.

Lehnardt, S., S. Lehmann, D. Kaul, K. Tschimmel, O. Hoffmann, S. Cho, C. Krueger, R. Nitsch, A. Meisel and J. R. Weber (2007):

Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia.

J Neuroimmunol 190: 28-33. DOI: 10.1016/j.jneuroim.2007.07.023.

Lehnardt, S. (2010):

Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury.

Glia 58: 253-263. DOI: 10.1002/glia.20928.

Lehnardt, S., T. Wallach, V. Gres and P. Henneke (2019):

Guardians of neuroimmunity – Toll-like receptors and their RNA ligands.

Neuroforum 25: 185-193. DOI: doi:10.1515/nf-2018-0032.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart and J. A. Hoffmann (1996):

The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in Drosophila adults.

Cell 86: 973-983. DOI: 10.1016/s0092-8674(00)80172-5.

Letiembre, M., H. Echchannaoui, F. Ferracin, S. Rivest and R. Landmann (2005):

Toll-like receptor-2 deficiency is associated with enhanced brain TNF gene expression during pneumococcal meningitis.

J Neuroimmunol 168: 21-33. DOI: 10.1016/j.jneuroim.2005.06.016.

Li, L., C. Acioglu, R. F. Heary and S. Elkabes (2021a):

Role of astroglial toll-like receptors (TLRs) in central nervous system infections, injury and neurodegenerative diseases.

Brain Behav Immun 91: 740-755. DOI: 10.1016/j.bbi.2020.10.007.

Li, Q., X. Zhang, Y. Peng, H. Chai, Y. Xu, J. Wei, X. Ren, X. Wang, W. Liu, M. Chen and D. Huang (2013):

Comparison of the sorting efficiency and influence on cell function between the sterile flow cytometry and immunomagnetic bead purification methods.

Prep Biochem Biotechnol 43: 197-206. DOI: 10.1080/10826068.2012.719846.

Li, Q. and B. A. Barres (2018):

Microglia and macrophages in brain homeostasis and disease.

Nat Rev Immunol 18: 225-242. DOI: 10.1038/nri.2017.125.

Li, T., X. Tan, S. Li, M. Al-Nusaif and W. Le (2021b):

Role of Glia-Derived Extracellular Vesicles in Neurodegenerative Diseases.

Front Aging Neurosci 13: 765395. DOI: 10.3389/fnagi.2021.765395.

Li, W., B. Viengkhou, G. Denyer, P. K. West, I. L. Campbell and M. J. Hofer (2018):

Microglia have a more extensive and divergent response to interferon-α compared with astrocytes.

Glia 66: 2058-2078. DOI: https://doi.org/10.1002/glia.23460.

Lian, H., A. Litvinchuk, A. C. Chiang, N. Aithmitti, J. L. Jankowsky and H. Zheng (2016):

Astrocyte-Microglia Cross Talk through Complement Activation Modulates Amyloid Pathology in Mouse Models of Alzheimer's Disease.

J Neurosci 36: 577-589. DOI: 10.1523/JNEUROSCI.2117-15.2016.

Liang, S., H. Domon, K. B. Hosur, M. Wang and G. Hajishengallis (2009):

Age-related alterations in innate immune receptor expression and ability of macrophages to respond to pathogen challenge *in vitro*.

Mech Ageing Dev 130: 538-546. DOI: 10.1016/j.mad.2009.06.006.

Liddelow, S. A. and B. A. Barres (2017):

Reactive Astrocytes: Production, Function, and Therapeutic Potential.

Immunity 46: 957-967. DOI: 10.1016/j.immuni.2017.06.006.

Liddelow, S. A., K. A. Guttenplan, L. E. Clarke, F. C. Bennett, C. J. Bohlen, L. Schirmer, M. L. Bennett, A. E. Munch, W. S. Chung, T. C. Peterson, D. K. Wilton, A. Frouin, B. A. Napier, N. Panicker, M. Kumar, M. S. Buckwalter, D. H. Rowitch, V. L. Dawson, T. M. Dawson, B. Stevens and B. A. Barres (2017):

Neurotoxic reactive astrocytes are induced by activated microglia.

Nature 541: 481-487. DOI: 10.1038/nature21029.

Lima, T. Z., L. R. Sardinha, J. Sayos, L. E. Mello and H. Peluffo (2017):

Astrocytic Expression of the Immunoreceptor CD300f Protects Hippocampal Neurons from Amyloid-β Oligomer Toxicity *In Vitro*.

Curr Alzheimer Res 14: 778-783. DOI: 10.2174/1567205014666170202121709.

Linnerbauer, M., M. A. Wheeler and F. J. Quintana (2020):

Astrocyte Crosstalk in CNS Inflammation.

Neuron 108: 608-622. DOI: 10.1016/j.neuron.2020.08.012.

Liu, L. R., J. C. Liu, J. S. Bao, Q. Q. Bai and G. Q. Wang (2020):

Interaction of Microglia and Astrocytes in the Neurovascular Unit.

Front Immunol 11: 1024. DOI: 10.3389/fimmu.2020.01024.

Liu, Q. and M. Nilsen-Hamilton (1995):

Identification of a new acute phase protein.

J Biol Chem 270: 22565-22570. DOI: 10.1074/jbc.270.38.22565.

Liu, W., C. F. Brosnan, D. W. Dickson and S. C. Lee (1994):

Macrophage colony-stimulating factor mediates astrocyte-induced microglial ramification in human fetal central nervous system culture.

Am J Pathol 145: 48-53.

Liu, W., Y. Tang and J. Feng (2011):

Cross talk between activation of microglia and astrocytes in pathological conditions in the central nervous system.

Life Sciences 89: 141-146. DOI: https://doi.org/10.1016/j.lfs.2011.05.011.

Logdberg, L. and L. Wester (2000):

Immunocalins: a lipocalin subfamily that modulates immune and inflammatory responses.

Biochim Biophys Acta 1482: 284-297. DOI: 10.1016/s0167-4838(00)00164-3.

Long, E. M., B. Millen, P. Kubes and S. M. Robbins (2009):

Lipoteichoic Acid Induces Unique Inflammatory Responses when Compared to Other Toll-Like Receptor 2 Ligands.

PLOS ONE 4: e5601. DOI: 10.1371/journal.pone.0005601.

Losciuto, S., G. Dorban, S. Gabel, A. Gustin, C. Hoenen, L. Grandbarbe, P. Heuschling and T. Heurtaux (2012):

An efficient method to limit microglia-dependent effects in astroglial cultures.

J Neurosci Methods 207: 59-71. DOI: 10.1016/j.jneumeth.2012.03.010.

Marek, R., M. Caruso, A. Rostami, J. B. Grinspan and J. Das Sarma (2008):

Magnetic cell sorting: a fast and effective method of concurrent isolation of high purity viable astrocytes and microglia from neonatal mouse brain tissue.

J Neurosci Methods 175: 108-118. DOI: 10.1016/j.jneumeth.2008.08.016.

Martin, E., M. El-Behi, B. Fontaine and C. Delarasse (2017):

Analysis of Microglia and Monocyte-derived Macrophages from the Central Nervous System by Flow Cytometry.

J Vis Exp. DOI: 10.3791/55781.

Martinez, F. O. and S. Gordon (2014):

The M1 and M2 paradigm of macrophage activation: time for reassessment.

F1000Prime Rep 6: 13. DOI: 10.12703/P6-13.

Martinotti, S., M. Patrone and E. Ranzato (2015):

Emerging roles for HMGB1 protein in immunity, inflammation, and cancer.

Immunotargets Ther 4: 101-109. DOI: 10.2147/ITT.S58064.

Matejuk, A. and R. M. Ransohoff (2020):

Crosstalk Between Astrocytes and Microglia: An Overview.

Front Immunol 11: 1416. DOI: 10.3389/fimmu.2020.01416.

Matejuk, A., A. A. Vandenbark and H. Offner (2021):

Cross-Talk of the CNS With Immune Cells and Functions in Health and Disease.

Front Neurol 12: 672455. DOI: 10.3389/fneur.2021.672455.

Matsumoto, Y., K. Ohmori and M. Fujiwara (1992):

Microglial and astroglial reactions to inflammatory lesions of experimental autoimmune encephalomyelitis in the rat central nervous system.

J Neuroimmunol 37: 23-33. DOI: 10.1016/0165-5728(92)90152-b.

McCann, M. J., J. P. O'Callaghan, P. M. Martin, T. Bertram and W. J. Streit (1996):

Differential activation of microglia and astrocytes following trimethyl tin-induced neurodegeneration.

Neuroscience 72: 273-281. DOI: 10.1016/0306-4522(95)00526-9.

McCarthy, K. D. and J. de Vellis (1980):

Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue.

J Cell Biol 85: 890-902. DOI: 10.1083/jcb.85.3.890.

Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh and C. A. Janeway, Jr. (1998):

MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways.

Mol Cell 2: 253-258. DOI: 10.1016/s1097-2765(00)80136-7.

Melzer, L., T. M. Freiman and A. Derouiche (2021):

Rab6A as a Pan-Astrocytic Marker in Mouse and Human Brain, and Comparison with Other Glial Markers (GFAP, GS, Aldh1L1, SOX9).

Cells 10. DOI: 10.3390/cells10010072.

Michelucci, A., T. Cordes, J. Ghelfi, A. Pailot, N. Reiling, O. Goldmann, T. Binz, A. Wegner, A. Tallam, A. Rausell, M. Buttini, C. L. Linster, E. Medina, R. Balling and K. Hiller (2013):

Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production.

Proc Natl Acad Sci U S A 110: 7820-7825. DOI: 10.1073/pnas.1218599110.

Miller, R. H. and M. C. Raff (1984):

Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct.

J Neurosci 4: 585-592.

Miltenyi, S., W. Muller, W. Weichel and A. Radbruch (1990):

High gradient magnetic cell separation with MACS.

Cytometry 11: 231-238. DOI: 10.1002/cyto.990110203.

Min, K. J., M. S. Yang, S. U. Kim, I. Jou and E. H. Joe (2006):

Astrocytes induce hemeoxygenase-1 expression in microglia: a feasible mechanism for preventing excessive brain inflammation.

J Neurosci 26: 1880-1887. DOI: 10.1523/JNEUROSCI.3696-05.2006.

Mizee, M. R., S. S. Miedema, M. van der Poel, Adelia, K. G. Schuurman, M. E. van Strien, J. Melief, J. Smolders, D. A. Hendrickx, K. M. Heutinck, J. Hamann and I. Huitinga (2017):

Isolation of primary microglia from the human post-mortem brain: effects of ante- and post-mortem variables.

Acta Neuropathol Commun 5: 16. DOI: 10.1186/s40478-017-0418-8.

Mizutani, M., P. A. Pino, N. Saederup, I. F. Charo, R. M. Ransohoff and A. E. Cardona (2012):

The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood.

J Immunol 188: 29-36. DOI: 10.4049/jimmunol.1100421.

Mohamed, W., J. Kumar, B. S. Alghamdi, A.-H. Soliman and Y. Toshihide (2023):

Neurodegeneration and inflammation crosstalk: Therapeutic targets and perspectives.

IBRO Neuroscience Reports 14: 95-110. DOI: https://doi.org/10.1016/j.ibneur.2022.12.003.

Molday, R. S. and D. MacKenzie (1982):

Immunospecific ferromagnetic iron-dextran reagents for the labeling and magnetic separation of cells.

J Immunol Methods 52: 353-367. DOI: 10.1016/0022-1759(82)90007-2.

Morr, M., O. Takeuchi, S. Akira, M. M. Simon and P. F. Muhlradt (2002):

Differential recognition of structural details of bacterial lipopeptides by toll-like receptors.

Eur J Immunol 32: 3337-3347. DOI: 10.1002/1521-4141(200212)32:12<3337::AID-IMMU3337>3.0.CO;2-#.

Moulson, A. J., J. W. Squair, R. J. M. Franklin, W. Tetzlaff and P. Assinck (2021):

Diversity of Reactive Astrogliosis in CNS Pathology: Heterogeneity or Plasticity?

Front Cell Neurosci 15: 703810. DOI: 10.3389/fncel.2021.703810.

Moussaud, S. and H. J. Draheim (2010):

A new method to isolate microglia from adult mice and culture them for an extended period of time.

J Neurosci Methods 187: 243-253. DOI: 10.1016/j.jneumeth.2010.01.017.

Muoio, V., P. B. Persson and M. M. Sendeski (2014):

The neurovascular unit - concept review.

Acta Physiol (Oxf) 210: 790-798. DOI: 10.1111/apha.12250.

Nikodemova, M. and J. J. Watters (2012):

Efficient isolation of live microglia with preserved phenotypes from adult mouse brain.

J Neuroinflammation 9: 147. DOI: 10.1186/1742-2094-9-147.

Nimmerjahn, A., F. Kirchhoff and F. Helmchen (2005):

Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*.

Science 308: 1314-1318. DOI: 10.1126/science.1110647.

Nishimura, R. N., D. Santos, S. T. Fu and B. E. Dwyer (2000):

Induction of cell death by L-alpha-aminoadipic acid exposure in cultured rat astrocytes: relationship to protein synthesis.

Neurotoxicology 21: 313-320.

Nolle, A., I. van Dijken, C. M. Waelti, D. Calini, J. Bryois, E. Lezan, S. Golling, A. Augustin, L. Foo and J. J. M. Hoozemans (2021):

Enrichment of Glial Cells From Human Post-mortem Tissue for Transcriptome and Proteome Analysis Using Immunopanning.

Front Cell Neurosci 15: 772011. DOI: 10.3389/fncel.2021.772011.

Norden, D. M., A. M. Fenn, A. Dugan and J. P. Godbout (2014):

TGFβ produced by IL-10 redirected astrocytes attenuates microglial activation.

Glia 62: 881-895. DOI: 10.1002/glia.22647.

Ocañas, S. R., K. D. Pham, H. E. Blankenship, A. H. Machalinski, A. J. Chucair-Elliott and W. M. Freeman (2022):

Minimizing the *Ex Vivo* Confounds of Cell-Isolation Techniques on Transcriptomic and Translatomic Profiles of Purified Microglia.

eNeuro 9. DOI: 10.1523/ENEURO.0348-21.2022.

Okun, E., K. J. Griffioen, J. D. Lathia, S. C. Tang, M. P. Mattson and T. V. Arumugam (2009):

Toll-like receptors in neurodegeneration.

Brain Res Rev 59: 278-292. DOI: 10.1016/j.brainresrev.2008.09.001.

Olah, M., D. Raj, N. Brouwer, A. H. De Haas, B. J. Eggen, W. F. Den Dunnen, K. P. Biber and H. W. Boddeke (2012):

An optimized protocol for the acute isolation of human microglia from autopsy brain samples.

Glia 60: 96-111. DOI: 10.1002/glia.21251.

Oliveira-Nascimento, L., P. Massari and L. M. Wetzler (2012):

The Role of TLR2 in Infection and Immunity.

Front Immunol 3: 79. DOI: 10.3389/fimmu.2012.00079.

Olson, J. K. and S. D. Miller (2004):

Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs.

J Immunol 173: 3916-3924. DOI: 10.4049/jimmunol.173.6.3916.

Owens, T. (2005):

Toll-like receptors on astrocytes: patterning for immunity.

J Neuroimmunol 159: 1-2. DOI: 10.1016/j.jneuroim.2004.10.015.

Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder and A. Aderem (2000):

The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.

Proc Natl Acad Sci U S A 97: 13766-13771. DOI: 10.1073/pnas.250476497.

Pan, J. and J. Wan (2020):

Methodological comparison of FACS and MACS isolation of enriched microglia and astrocytes from mouse brain.

J Immunol Methods 486: 112834. DOI: 10.1016/j.jim.2020.112834.

Paolicelli, R. C., A. Sierra, B. Stevens, M. E. Tremblay, A. Aguzzi, B. Ajami, I. Amit, E. Audinat, I. Bechmann, M. Bennett, F. Bennett, A. Bessis, K. Biber, S. Bilbo, M. Blurton-Jones, E. Boddeke, D. Brites, B. Brône, G. C. Brown, O. Butovsky, M. J. Carson, B. Castellano, M. Colonna, S. A. Cowley, C. Cunningham, D. Davalos, P. L. De Jager, B. de Strooper, A. Denes, B. J. L. Eggen, U. Eyo, E. Galea, S. Garel, F. Ginhoux, C. K. Glass, O. Gokce, D. Gomez-Nicola, B. González, S. Gordon, M. B. Graeber, A. D. Greenhalgh, P. Gressens, M. Greter, D. H. Gutmann, C. Haass, M. T. Heneka, F. L. Heppner, S. Hong, D. A. Hume, S. Jung, H. Kettenmann, J. Kipnis, R. Koyama, G. Lemke, M. Lynch, A. Majewska, M. Malcangio, T. Malm, R. Mancuso, T. Masuda, M. Matteoli, B. W. McColl, V. E. Miron, A. V. Molofsky, M. Monje, E. Mracsko, A. Nadjar, J. J. Neher, U. Neniskyte, H. Neumann, M. Noda, B. Peng, F. Peri, V. H. Perry, P. G. Popovich, C. Pridans, J. Priller, M. Prinz, D. Ragozzino, R. M. Ransohoff, M. W. Salter, A. Schaefer, D. P. Schafer, M. Schwartz, M. Simons, C. J. Smith, W. J. Streit, T. L. Tay, L. H. Tsai, A. Verkhratsky, R. von Bernhardi, H. Wake, V. Wittamer, S. A. Wolf, L. J. Wu and T. Wyss-Coray (2022):

Microglia states and nomenclature: A field at its crossroads.

Neuron 110: 3458-3483. DOI: 10.1016/j.neuron.2022.10.020.

Park, C., I. H. Cho, D. Kim, E. K. Jo, S. Y. Choi, S. B. Oh, K. Park, J. S. Kim and S. J. Lee (2008):

Toll-like receptor 2 contributes to glial cell activation and heme oxygenase-1 expression in traumatic brain injury.

Neurosci Lett 431: 123-128. DOI: 10.1016/j.neulet.2007.11.057.

Paschon, V., S. H. Takada, J. M. Ikebara, E. Sousa, R. Raeisossadati, H. Ulrich and A. H. Kihara (2016):

Interplay Between Exosomes, microRNAs and Toll-Like Receptors in Brain Disorders.

Mol Neurobiol 53: 2016-2028. DOI: 10.1007/s12035-015-9142-1.

Patel, A. J., A. Hunt and P. Seaton (1988):

The mechanism of cytosine arabinoside toxicity on quiescent astrocytes *in vitro* appears to be analogous to *in vivo* brain injury.

Brain Res 450: 378-381. DOI: 10.1016/0006-8993(88)91579-x.

Pellerin, L. and P. J. Magistretti (1994):

Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization.

Proc Natl Acad Sci U S A 91: 10625-10629. DOI: 10.1073/pnas.91.22.10625.

Peluffo, H., D. Ali-Ruiz, A. Ejarque-Ortiz, V. Heras-Alvarez, E. Comas-Casellas, A. Martinez-Barriocanal, A. Kamaid, D. Alvarez-Errico, M. L. Negro, N. Lago, S. Schwartz, Jr., A. Villaverde and J. Sayos (2012):

Overexpression of the Immunoreceptor CD300f Has a Neuroprotective Role in a Model of Acute Brain Injury.

Brain Pathol 22: 318-328. DOI: 10.1111/j.1750-3639.2011.00537.x.

Pérez-Rodríguez, D. R., I. Blanco-Luquin and M. Mendioroz (2021):

The Participation of Microglia in Neurogenesis: A Review.

Brain Sci 11. DOI: 10.3390/brainsci11050658.

Phulwani, N. K., N. Esen, M. M. Syed and T. Kielian (2008):

Toll-like receptor 2 (TLR2) expression in astrocytes is induced by TNF-α- and NF-κB-dependent pathways.

J Immunol 181: 3841-3849. DOI: 10.4049/jimmunol.181.6.3841.

Prah, J., A. Winters, K. Chaudhari, J. Hersh, R. Liu and S. H. Yang (2019):

A novel serum free primary astrocyte culture method that mimic quiescent astrocyte phenotype.

J Neurosci Methods 320: 50-63. DOI: 10.1016/j.jneumeth.2019.03.013.

Preston, A. N., D. A. Cervasio and S. T. Laughlin (2019):

Visualizing the brain's astrocytes.

Methods Enzymol 622: 129-151. DOI: 10.1016/bs.mie.2019.02.006.

Priller, J. and M. Prinz (2019):

Targeting microglia in brain disorders.

Science 365: 32-33. DOI: 10.1126/science.aau9100.

Rangaraju, S., S. A. Raza, N. X. Li, R. Betarbet, E. B. Dammer, D. Duong, J. J. Lah, N. T. Seyfried and A. I. Levey (2018):

Differential Phagocytic Properties of $CD45^{low}$ Microglia and $CD45^{high}$ Brain Mononuclear Phagocytes-Activation and Age-Related Effects.

Front Immunol 9: 405. DOI: 10.3389/fimmu.2018.00405.

Ranjbar Taklimie, F., N. Gasterich, M. Scheld, R. Weiskirchen, C. Beyer, T. Clarner and A. Zendedel (2019):

Hypoxia Induces Astrocyte-Derived Lipocalin-2 in Ischemic Stroke.

Int J Mol Sci 20. DOI: 10.3390/ijms20061271.

Ransohoff, R. M. (2016):

A polarizing question: do M1 and M2 microglia exist?

Nat Neurosci 19: 987-991. DOI: 10.1038/nn.4338.

Reali, C., M. Curto, V. Sogos, F. Scintu, S. Pauly, H. Schwarz and F. Gremo (2003):

Expression of CD137 and its ligand in human neurons, astrocytes, and microglia: Modulation by FGF-2.

J Neurosci Res 74: 67-73. DOI: 10.1002/jnr.10727.

Reemst, K., S. C. Noctor, P. J. Lucassen and E. M. Hol (2016):

The Indispensable Roles of Microglia and Astrocytes during Brain Development.

Front Hum Neurosci 10: 566. DOI: 10.3389/fnhum.2016.00566.

Richard, K. L., M. Filali, P. Prefontaine and S. Rivest (2008):

Toll-Like Receptor 2 Acts as a Natural Innate Immune Receptor to Clear Amyloid β_{1-42} and Delay the Cognitive Decline in a Mouse Model of Alzheimer's Disease.

J Neurosci 28: 5784-5793. DOI: 10.1523/JNEUROSCI.1146-08.2008.

Ritzel, R. M., A. R. Patel, J. M. Grenier, J. Crapser, R. Verma, E. R. Jellison and L. D. McCullough (2015):

Functional differences between microglia and monocytes after ischemic stroke.

J Neuroinflammation 12: 106. DOI: 10.1186/s12974-015-0329-1.

Rolf, N., A. Kariminia, S. Ivison, G. S. Reid and K. R. Schultz (2015):

Heterodimer-specific TLR2 stimulation results in divergent functional outcomes in B-cell precursor acute lymphoblastic leukemia.

European Journal of Immunology 45: 1980-1990. DOI: https://doi.org/10.1002/eji.201444874.

Rosenberger, K., K. Derkow, P. Dembny, C. Kruger, E. Schott and S. Lehnardt (2014):

The impact of single and pairwise Toll-like receptor activation on neuroinflammation and neurodegeneration.

J Neuroinflammation 11: 166. DOI: 10.1186/s12974-014-0166-7.

Rostami, J., T. Mothes, M. Kolahdouzan, O. Eriksson, M. Moslem, J. Bergstrom, M. Ingelsson, P. O'Callaghan, L. M. Healy, A. Falk and A. Erlandsson (2021):

Crosstalk between astrocytes and microglia results in increased degradation of α-synuclein and amyloid-β aggregates.

J Neuroinflammation 18: 124. DOI: 10.1186/s12974-021-02158-3.

Saederup, N., A. E. Cardona, K. Croft, M. Mizutani, A. C. Cotleur, C. L. Tsou, R. M. Ransohoff and I. F. Charo (2010):

Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice.

PLoS One 5: e13693. DOI: 10.1371/journal.pone.0013693.

Saijo, K., B. Winner, C. T. Carson, J. G. Collier, L. Boyer, M. G. Rosenfeld, F. H. Gage and C. K. Glass (2009):

A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death.

Cell 137: 47-59. DOI: 10.1016/j.cell.2009.01.038.

Sarkar, S., E. Malovic, B. Plante, G. Zenitsky, H. Jin, V. Anantharam, A. Kanthasamy and A. G. Kanthasamy (2017):

Rapid and Refined CD11b Magnetic Isolation of Primary Microglia with Enhanced Purity and Versatility.

J Vis Exp. DOI: 10.3791/55364.

Saura, J. (2007):

Microglial cells in astroglial cultures: a cautionary note.

J Neuroinflammation 4: 26. DOI: 10.1186/1742-2094-4-26.

Sawada, M., A. Suzumura, H. Yamamoto and T. Marunouchi (1990):

Activation and proliferation of the isolated microglia by colony stimulating factor-1 and possible involvement of protein kinase C.

Brain Res 509: 119-124. DOI: 10.1016/0006-8993(90)90317-5.

Schaeffer, S. and C. Iadecola (2021):

Revisiting the neurovascular unit.

Nat Neurosci 24: 1198-1209. DOI: 10.1038/s41593-021-00904-7.

Schafer, D. P., E. K. Lehrman, A. G. Kautzman, R. Koyama, A. R. Mardinly, R. Yamasaki, R. M. Ransohoff, M. E. Greenberg, B. A. Barres and B. Stevens (2012):

Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner.

Neuron 74: 691-705. DOI: 10.1016/j.neuron.2012.03.026.

Scholze, A. R., L. C. Foo, S. Mulinyawe and B. A. Barres (2014):

BMP signaling in astrocytes downregulates EGFR to modulate survival and maturation.

PLoS One 9: e110668. DOI: 10.1371/journal.pone.0110668.

Schroeter, C. B., A. M. Herrmann, S. Bock, A. Vogelsang, S. Eichler, P. Albrecht, S. G. Meuth and T. Ruck (2021):

One Brain-All Cells: A Comprehensive Protocol to Isolate All Principal CNS-Resident Cell Types from Brain and Spinal Cord of Adult Healthy and EAE Mice.

Cells 10. DOI: 10.3390/cells10030651.

Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S. E. Jacobsen, J. W. Pollard, J. Frampton, K. J. Liu and F. Geissmann (2012):

A lineage of myeloid cells independent of Myb and hematopoietic stem cells.

Science 336: 86-90. DOI: 10.1126/science.1219179.

Sedgwick, J. D., S. Schwender, H. Imrich, R. Dorries, G. W. Butcher and V. ter Meulen (1991):

Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system.

Proc Natl Acad Sci U S A 88: 7438-7442. DOI: 10.1073/pnas.88.16.7438.

Seele, J., R. Nau, C. K. Prajeeth, M. Stangel, P. Valentin-Weigand and M. Seitz (2016):

Astrocytes Enhance *Streptococcus suis*-Glial Cell Interaction in Primary Astrocyte-Microglial Cell Co-Cultures.

Pathogens 5. DOI: 10.3390/pathogens5020043.

Semyanov, A. and A. Verkhratsky (2021):

Astrocytic processes: from tripartite synapses to the active milieu.

Trends Neurosci 44: 781-792. DOI: 10.1016/j.tins.2021.07.006.

Sen, R. and D. Baltimore (1986):

Multiple nuclear factors interact with the immunoglobulin enhancer sequences.

Cell 46: 705-716. DOI: 10.1016/0092-8674(86)90346-6.

Shaw, A. C., D. R. Goldstein and R. R. Montgomery (2013):

Age-dependent dysregulation of innate immunity.

Nat Rev Immunol 13: 875-887. DOI: 10.1038/nri3547.

Shen, X., Y. Qiu, A. E. Wight, H. J. Kim and H. Cantor (2022):

Definition of a mouse microglial subset that regulates neuronal development and proinflammatory responses in the brain.

Proc Natl Acad Sci U S A 119. DOI: 10.1073/pnas.2116241119.

Sheng, J., C. Ruedl and K. Karjalainen (2015):

Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells.

Immunity 43: 382-393. DOI: 10.1016/j.immuni.2015.07.016.

Shinozaki, Y., K. Shibata, K. Yoshida, E. Shigetomi, C. Gachet, K. Ikenaka, K. F. Tanaka and S. Koizumi (2017):

Transformation of Astrocytes to a Neuroprotective Phenotype by Microglia via P2Y1 Receptor Downregulation.

Cell Rep 19: 1151-1164. DOI: 10.1016/j.celrep.2017.04.047.

Sierra, A., F. de Castro, J. Del Rio-Hortega, J. Rafael Iglesias-Rozas, M. Garrosa and H. Kettenmann (2016):

The "Big-Bang" for modern glial biology: Translation and comments on Pío del Río-Hortega 1919 series of papers on microglia.

Glia 64: 1801-1840. DOI: 10.1002/glia.23046.

Simard, M. and M. Nedergaard (2004):

The neurobiology of glia in the context of water and ion homeostasis.

Neuroscience 129: 877-896. DOI: 10.1016/j.neuroscience.2004.09.053.

Smith, A. M., H. M. Gibbons, R. L. Oldfield, P. M. Bergin, E. W. Mee, M. A. Curtis, R. L. Faull and M. Dragunow (2013):

M-CSF increases proliferation and phagocytosis while modulating receptor and transcription factor expression in adult human microglia.

J Neuroinflammation 10: 85. DOI: 10.1186/1742-2094-10-85.

Sofroniew, M. V. (2020):

Astrocyte Reactivity: Subtypes, States, and Functions in CNS Innate Immunity.

Trends Immunol 41: 758-770. DOI: 10.1016/j.it.2020.07.004.

Sola, C., C. Casal, J. M. Tusell and J. Serratosa (2002):

Astrocytes enhance lipopolysaccharide-induced nitric oxide production by microglial cells.

Eur J Neurosci 16: 1275-1283. DOI: 10.1046/j.1460-9568.2002.02199.x.

Somjen, G. G. (1988):

Nervenkitt: notes on the history of the concept of neuroglia.

Glia 1: 2-9. DOI: 10.1002/glia.440010103.

Stark, J. C., E. Wallace, R. Lim and B. Leaw (2018):

Characterization and Isolation of Mouse Primary Microglia by Density Gradient Centrifugation.

J Vis Exp. DOI: 10.3791/57065.

Stence, N., M. Waite and M. E. Dailey (2001):

Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices.

Glia 33: 256-266.

Stenzel, W., S. Soltek, M. Sanchez-Ruiz, S. Akira, H. Miletic, D. Schluter and M. Deckert (2008):

Both TLR2 and TLR4 are required for the effective immune response in *Staphylococcus aureus*-induced experimental murine brain abscess.

Am J Pathol 172: 132-145. DOI: 10.2353/ajpath.2008.070567.

Stratoulias, V., J. L. Venero, M. E. Tremblay and B. Joseph (2019):

Microglial subtypes: diversity within the microglial community.

EMBO J 38: e101997. DOI: 10.15252/embj.2019101997.

Streit, W. J. (2006):

Microglial senescence: does the brain's immune system have an expiration date?

Trends Neurosci 29: 506-510. DOI: 10.1016/j.tins.2006.07.001.

Sugiyama, M., R. Banno, H. Yaginuma, K. Taki, A. Mizoguchi, T. Tsunekawa, T. Onoue, H. Takagi, Y. Ito, S. Iwama, M. Goto, H. Suga, O. Komine, K. Yamanaka and H. Arima (2020):

Hypothalamic glial cells isolated by MACS reveal that microglia and astrocytes induce hypothalamic inflammation via different processes under high-fat diet conditions.

Neurochem Int 136: 104733. DOI: 10.1016/j.neuint.2020.104733.

Sun, M., H. You, X. Hu, Y. Luo, Z. Zhang, Y. Song, J. An and H. Lu (2023):

Microglia-Astrocyte Interaction in Neural Development and Neural Pathogenesis.

Cells 12. DOI: 10.3390/cells12151942.

Sutermaster, B. A. and E. M. Darling (2019):

Considerations for high-yield, high-throughput cell enrichment: fluorescence versus magnetic sorting.

Sci Rep 9: 227. DOI: 10.1038/s41598-018-36698-1.

Suzumura, A., M. Sawada, H. Yamamoto and T. Marunouchi (1990):

Effects of colony stimulating factors on isolated microglia *in vitro*.

J Neuroimmunol 30: 111-120. DOI: 10.1016/0165-5728(90)90094-4.

Suzumura, A., T. Marunouchi and H. Yamamoto (1991):

Morphological transformation of microglia *in vitro*.

Brain Res 545: 301-306. DOI: 10.1016/0006-8993(91)91302-h.

Syed, M. M., N. K. Phulwani and T. Kielian (2007):

Tumor necrosis factor-alpha (TNF-alpha) regulates Toll-like receptor 2 (TLR2) expression in microglia.

J Neurochem 103: 1461-1471. DOI: 10.1111/j.1471-4159.2007.04838.x.

Szpakowski, P., D. Ksiazek-Winiarek, J. Czpakowska, M. Kaluza, M. Milewska-Jedrzejczak and A. Glabinski (2023):

Astrocyte-Derived Exosomes Differentially Shape T Cells' Immune Response in MS Patients.

Int J Mol Sci 24. DOI: 10.3390/ijms24087470.

Takada, M. and T. Hattori (1986):

Fine structural changes in the rat brain after local injections of gliotoxin, alpha-aminoadipic acid.

Histol Histopathol 1: 271-275.

Takahashi, S. (2022):

Metabolic Contribution and Cerebral Blood Flow Regulation by Astrocytes in the Neurovascular Unit.

Cells 11: 813. DOI: https://doi.org/10.3390/cells11050813.

Takeuchi, O. and S. Akira (2010):

Pattern recognition receptors and inflammation.

Cell 140: 805-820. DOI: 10.1016/j.cell.2010.01.022.

Tanuma, N., H. Sakuma, A. Sasaki and Y. Matsumoto (2006):

Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis.

Acta Neuropathol 112: 195-204. DOI: 10.1007/s00401-006-0083-7.

Thiele, D. L., M. Kurosaka and P. E. Lipsky (1983):

Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester.

J Immunol 131: 2282-2290.

Thion, M. S. and S. Garel (2017):

On place and time: microglia in embryonic and perinatal brain development.

Curr Opin Neurobiol 47: 121-130. DOI: 10.1016/j.conb.2017.10.004.

Thomas, M. L. (1989):

The leukocyte common antigen family.

Annu Rev Immunol 7: 339-369. DOI: 10.1146/annurev.iy.07.040189.002011.

Timmerman, R., S. M. Burm and J. J. Bajramovic (2018):

An Overview of *in vitro* Methods to Study Microglia.

Front Cell Neurosci 12: 242. DOI: 10.3389/fncel.2018.00242.

Torres, D., M. Barrier, F. Bihl, V. J. Quesniaux, I. Maillet, S. Akira, B. Ryffel and F. Erard (2004):

Toll-like receptor 2 is required for optimal control of *Listeria monocytogenes* infection.

Infect Immun 72: 2131-2139. DOI: 10.1128/IAI.72.4.2131-2139.2004.

Tremblay, M. E. (2020):

A Diversity of Cell Types, Subtypes and Phenotypes in the Central Nervous System: The Importance of Studying Their Complex Relationships.

Front Cell Neurosci 14: 628347. DOI: 10.3389/fncel.2020.628347.

Triolo, D., G. Dina, I. Lorenzetti, M. Malaguti, P. Morana, U. Del Carro, G. Comi, A. Messing, A. Quattrini and S. C. Previtali (2006):

Loss of glial fibrillary acidic protein (GFAP) impairs Schwann cell proliferation and delays nerve regeneration after damage.

J Cell Sci 119: 3981-3993. DOI: 10.1242/jcs.03168.

Trowbridge, I. S. and M. L. Thomas (1994):

CD45: An Emerging Role as a Protein Tyrosine Phosphatase Required for Lymphocyte Activation and Development.

Annual Review of Immunology 12: 85-116. DOI: 10.1146/annurev.iy.12.040194.000505.

Vallejo, R., D. M. Tilley, L. Vogel and R. Benyamin (2010):

The role of glia and the immune system in the development and maintenance of neuropathic pain.

Pain Pract 10: 167-184. DOI: 10.1111/j.1533-2500.2010.00367.x.

Van Zeller, M., A. M. Sebastião and C. A. Valente (2022):

Microglia Depletion from Primary Glial Cultures Enables to Accurately Address the Immune Response of Astrocytes.

Biomolecules 12. DOI: 10.3390/biom12050666.

Verderio, C., L. Muzio, E. Turola, A. Bergami, L. Novellino, F. Ruffini, L. Riganti, I. Corradini, M. Francolini, L. Garzetti, C. Maiorino, F. Servida, A. Vercelli, M. Rocca, D. Dalla Libera, V. Martinelli, G. Comi, G. Martino, M. Matteoli and R. Furlan (2012):

Myeloid microvesicles are a marker and therapeutic target for neuroinflammation.

Ann Neurol 72: 610-624. DOI: 10.1002/ana.23627.

Verkhratsky, A. and A. M. Butt (2013):

Peripheral Glial Cells

In: Glial Physiology and Pathophysiology/A. V. a. A. Butt, 1st edition, 381-430

Hoboken: John Wiley & Sons, Ltd – 9781118402061.

Verkhratsky, A., M. S. Ho, R. Zorec and V. Parpura (2019):

The Concept of Neuroglia

In: Neuroglia in Neurodegenerative Diseases/A. Verkhratsky, M. S. Ho, R. Zorec and V. Parpura, p. 1-13

Singapore: Springer Singapore – ISBN 978-981-13-9913-8.

Virchow, R. (1858):

Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre: Zwanzig Vorlesungen, gehalten während der Monate Februar, März und April 1858 im pathologischen Institute zu Berlin.

1st edition, Berlin: August Hirschwald – ISBN not available.

Volden, T. A., C. D. Reyelts, T. A. Hoke, J. Arikkath and S. J. Bonasera (2015):

Validation of Flow Cytometry and Magnetic Bead-Based Methods to Enrich CNS Single Cell Suspensions for Quiescent Microglia.

J Neuroimmune Pharmacol 10: 655-665. DOI: 10.1007/s11481-015-9628-7.

Volterra, A. and J. Meldolesi (2005):

Astrocytes, from brain glue to communication elements: the revolution continues.

Nat Rev Neurosci 6: 626-640. DOI: 10.1038/nrn1722.

von Bartheld, C. S., J. Bahney and S. Herculano-Houzel (2016):

The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting.

J Comp Neurol 524: 3865-3895. DOI: 10.1002/cne.24040.

Walz, W. (2000):

Role of astrocytes in the clearance of excess extracellular potassium.

Neurochem Int 36: 291-300. DOI: 10.1016/s0197-0186(99)00137-0.

Wang, G., Y. C. Weng, X. Han, J. D. Whaley, K. R. McCrae and W. H. Chou (2015):

Lipocalin-2 released in response to cerebral ischaemia mediates reperfusion injury in mice.

J Cell Mol Med 19: 1637-1645. DOI: 10.1111/jcmm.12538.

Wang, Y., K. J. Szretter, W. Vermi, S. Gilfillan, C. Rossini, M. Cella, A. D. Barrow, M. S. Diamond and M. Colonna (2012):

IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia.

Nat Immunol 13: 753-760. DOI: 10.1038/ni.2360.

Ware, K., E. Wegman, A. Bedolla and Y. Luo (2022):

Major Caveats Discovered in the Genetic Cell Ablation Model Used to Study Microglia Functions.

Transl Stroke Res. DOI: 10.1007/s12975-022-01029-0.

Weinhard, L., G. di Bartolomei, G. Bolasco, P. Machado, N. L. Schieber, U. Neniskyte, M. Exiga, A. Vadisiute, A. Raggioli, A. Schertel, Y. Schwab and C. T. Gross (2018):

Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction.

Nat Commun 9: 1228. DOI: 10.1038/s41467-018-03566-5.

Westergard, T. and J. D. Rothstein (2020):

Astrocyte Diversity: Current Insights and Future Directions.

Neurochem Res 45: 1298-1305. DOI: 10.1007/s11064-020-02959-7.

Williamson, L. L., P. W. Sholar, R. S. Mistry, S. H. Smith and S. D. Bilbo (2011):

Microglia and memory: modulation by early-life infection.

J Neurosci 31: 15511-15521. DOI: 10.1523/JNEUROSCI.3688-11.2011.

Wlodarczyk, A., M. Lobner, O. Cedile and T. Owens (2014):

Comparison of microglia and infiltrating CD11c$^+$ cells as antigen presenting cells for T cell proliferation and cytokine response.

J Neuroinflammation 11: 57. DOI: 10.1186/1742-2094-11-57.

Wlodarczyk, A., I. R. Holtman, M. Krueger, N. Yogev, J. Bruttger, R. Khorooshi, A. Benmamar-Badel, J. J. de Boer-Bergsma, N. A. Martin, K. Karram, I. Kramer, E. W. Boddeke, A. Waisman, B. J. Eggen and T. Owens (2017):

A novel microglial subset plays a key role in myelinogenesis in developing brain.

EMBO J 36: 3292-3308. DOI: 10.15252/embj.201696056.

Wu, R., F. Chen, N. Wang, D. Tang and R. Kang (2020):

ACOD1 in immunometabolism and disease.

Cell Mol Immunol 17: 822-833. DOI: 10.1038/s41423-020-0489-5.

Wu, Y. and U. L. M. Eisel (2023):

Microglia-Astrocyte Communication in Alzheimer's Disease.

Journal of Alzheimer's Disease Preprint: 1-19. DOI: 10.3233/JAD-230199.

Yang, L., Y. Zhou, H. Jia, Y. Qi, S. Tu and A. Shao (2020):

Affective Immunology: The Crosstalk Between Microglia and Astrocytes Plays Key Role?

Front Immunol 11: 1818. DOI: 10.3389/fimmu.2020.01818.

Yu, X., J. Nagai and B. S. Khakh (2020):

Improved tools to study astrocytes.

Nat Rev Neurosci 21: 121-138. DOI: 10.1038/s41583-020-0264-8.

Zhang, W., L. Ding, H. Chen, M. Zhang, R. Ma, S. Zheng, J. Gong, Z. Zhang, H. Xu, P. Xu and Y. Zhang (2023):

Cntnap4 partial deficiency exacerbates α-synuclein pathology through astrocyte–microglia C3-C3aR pathway.

Cell Death & Disease 14: 285. DOI: 10.1038/s41419-023-05807-y.

Zhang, Y., S. A. Sloan, L. E. Clarke, C. Caneda, C. A. Plaza, P. D. Blumenthal, H. Vogel, G. K. Steinberg, M. S. Edwards, G. Li, J. A. Duncan, 3rd, S. H. Cheshier, L. M. Shuer, E. F. Chang, G. A. Grant, M. G. Gephart and B. A. Barres (2016):

Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse.

Neuron 89: 37-53. DOI: 10.1016/j.neuron.2015.11.013.

Zhao, N., X. Xu, Y. Jiang, J. Gao, F. Wang, X. Xu, Z. Wen, Y. Xie, J. Li, R. Li, Q. Lv, Q. Liu, Q. Dai, X. Liu and G. Xu (2019):

Lipocalin-2 may produce damaging effect after cerebral ischemia by inducing astrocytes classical activation.

J Neuroinflammation 16: 168. DOI: 10.1186/s12974-019-1556-7.

Zhao, S., S. Sheng, Y. Wang, L. Ding, X. Xu, X. Xia and J. C. Zheng (2021):

Astrocyte-derived extracellular vesicles: A double-edged sword in central nervous system disorders.

Neurosci Biobehav Rev 125: 148-159. DOI: 10.1016/j.neubiorev.2021.02.027.

Zheng, H., M. C. Punaro, M. Segura, C. Lachance, S. Rivest, J. Xu, M. Houde and M. Gottschalk (2011):

Toll-like receptor 2 is partially involved in the activation of murine astrocytes by *Streptococcus suis*, an important zoonotic agent of meningitis.

J Neuroimmunol 234: 71-83. DOI: 10.1016/j.jneuroim.2011.02.005.

Zhu, H., X. Qiao, W. Liu, C. Wang and Y. Zhao (2020):

Microglia Play an Essential Role in Synapse Development and Neuron Maturation in Tissue-Engineered Neural Tissues.

Front Neurosci 14: 586452. DOI: 10.3389/fnins.2020.586452.

Zuiderwijk-Sick, E. A., C. van der Putten, M. Bsibsi, I. P. Deuzing, W. de Boer, C. Persoon-Deen, I. Kondova, L. A. Boven, J. M. van Noort, B. A. t Hart, S. Amor and J. J. Bajramovic (2007):

Differentiation of primary adult microglia alters their response to TLR8-mediated activation but not their capacity as APC.

Glia 55: 1589-1600. DOI: 10.1002/glia.20572.

List of publications

Publication I:

Zelenka, L., Pägelow, D., Krüger, C., Seele, J., Ebner, F., Rausch, S., Rohde, M., Lehnardt, S., van Vorst, K. and Fulde, M. (2022):

Novel protocol for the isolation of highly purified neonatal murine microglia and astrocytes.

J Neurosci Methods 366: 109420. DOI: 10.1016/j.jneumeth.2021.109420.

Publication II:

Zelenka, L., Jarek, M., Pägelow, D., Greffers, R., van Vorst, K. and Fulde, M. (2023):

Crosstalk of Highly Purified Microglia and Astrocytes in the Frame of Toll-like Receptor (TLR)2/1 Activation

Neuroscience 526: 256-266. DOI: 10.1016/j.neuroscience.2023.05.001.

Further aspects of this thesis have been presented at national conferences as posters or oral presentations:

Zelenka, L., Pägelow, D., Krüger, C., Seele, J., Lehnardt, S. and Fulde, M. (2019):

In vitro Cultivation and Magnetic Cell Sorting of highly purified neonatal murine microglia and astrocytes.

Proceedings of the 12th DRS Doctoral Symposium, Berlin, Germany (2019)

Zelenka, L., Pägelow, D., Krüger, C., Seele, J., Lehnardt, S. and Fulde, M. (2019):

In vitro Cultivation and Magnetic Cell Sorting of highly purified neonatal murine microglia and astrocytes.

Proceedings of the DipDok-Meeting at the Robert Koch Institute, Berlin, Germany (2019)

Zelenka, L., Pägelow, D., Krüger, C., Seele, J., van Vorst, K., Ebner, F., Rausch, Hartmann, S., Lehnardt, S. and Fulde M. (2020):

Novel protocol for the *in vitro* cultivation and Magnetic-activated cell sorting (MACS) of highly purified neonatal murine microglia and astrocytes.

Proceedings of the International Symposium on Zoonoses Research, Berlin, Germany (2020)

Acknowledgement

First and foremost, I would like to thank my supervisor and mentor Prof. Dr. Marcus Fulde. Thank you for guiding me through this PhD, for the fruitful discussions and for having constant faith in my scientific capabilities – especially when I did not. I am really grateful for your support and encouraging words as well as for teaching me calmness throughout stressful situations.

Secondly, I would like to express my gratitude to my second mentor Prof. Dr. Seija Lehnardt, for providing the framework for my PhD by giving me opportunity to learn the glial isolation techniques in her institute, for her constructive criticism and successful collaborations. I would also like to thank Prof. Dr. Susanne Hartmann who volunteered as my third mentor, for her interest in my project.

Next, I would like to express my gratitude to the entire team of the Institute of Microbiology and Epizootics at the Veterinary faculty of the Freie Universität Berlin. A special thanks to Dr. Dennis Pägelow, for his supervision at the start of my PhD journey, for introducing me to the – at that time for me – completely unknown field of glial biology, for teaching me the necessary techniques essential for my PhD thesis, and his invaluable scientific advice. Furthermore, I would like to extend my thanks to Dr. Kira van Vorst, for being helpful, especially when it came to organizing the breed for the mice experiments. A special thanks also goes to all the other members of the “AG Fulde” and especially to all former and present PhD students that I met and spent time together with during my PhD, for the many laughs, your support, and comforting talks and relaxing breaks together (Antje Lapschies, Julian Brombach and Mareike Saathoff: I already miss our lunch breaks together!). Moreover, I would like to thank the lab technicians Peter Schwerk and Petra Krienke, whom I could always approach when I was facing any kinds of technical issues in the lab. Furthermore, I am very grateful to all people involved at various stages of the proofreading process, especially Dr. Anna-Lena Sander and Saoirse Walsh.

Next, I would like to thank all my collaborators whose work appears here in this thesis: Thank you Christina Krüger for patiently teaching me the glial isolation techniques. In addition, thanks to Dr. Jana Seele, for her scientific support in developing the glial isolation protocol. Moreover, I would like to thank Dr. Sebastian Rausch as well as Prof. Dr. Friederike Ebner for their immense help in analyzing the FACS data.

Finally, I owe my deepest gratitude to my friends and family: Thank you for supporting and encouraging me, distracting me when needed or just simply for enriching my life. Most importantly, I am really grateful for the unconditional support of my whole family, which not only morally but also financially backed me up and only made my studies, be it in Germany or abroad, possible in the first place. Thank you!

Finanzierungsquellen – Funding Sources

This work was in part financed by the Federal Ministry of Education and Research (BMBF) and the Federal Ministry of Food and Agriculture (BMEL) and carried out under the umbrella of the National Research Platform for Zoonoses grant #01KI1906 (NAMPatIn).

Interessenskonflikte – Conflict of Interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

Selbstständigkeitserklärung – Declaration of Independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated.

This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

Berlin, 10.01.2024 Laura Zelenka