Transcriptome-guided optimization of *in vitro* culture conditions to study microglia in health and disease

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Transcriptoom-geleide optimalisatie van *in vitro* kweekcondities om microglia in gezondheid en ziekte te bestuderen

(met een samenvatting in het Nederlands)

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Raissa Timmerman

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Promotor
Prof.dr. R. E. Bontrop

Copromotor
Dr. J.J. Bajramovic

Assessment Committee
Prof.dr. E.M. Hol
Prof.dr. H.E. de Vries
Prof.dr. D. Salvatori
Prof.dr. J.A.M. Langermans
Dr. I. Canals

"If the human brain were so simple that we could understand it,
we would be so simple that we couldn't."

Emerson M. Pugh

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Chapter 1

General introduction and thesis outline

Adapted from:

An overview of *in vitro* methods to study microglia

Raissa Timmerman[1], Saskia M. Burm[2] and Jeffrey J. Bajramovic[1]

[1]Alternatives Unit, Biomedical Primate Research Centre, Rijswijk, The Netherlands

[2]Genmab B.V., Utrecht, The Netherlands

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Neurodegenerative diseases are characterized by the progressive loss of neurons in the central nervous system (CNS). Ways to slow the progression of these diseases have focused chiefly on targeting neurons to prevent cell loss, but with limited success. This lack of progress has encouraged researchers to look instead at non-neuronal cells, including microglia, the immune cells of the CNS. These studies have amongst others discovered that microglia-specific pathways are affected in these diseases and that microglia activation is a hallmark of all neurodegenerative diseases[1,2]. Additionally, genome-wide association studies have identified multiple risk genes for neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), that are highly expressed by microglia[3,4]. Consequently, microglia are now positioned as key players in the pathogenesis of neurodegenerative diseases, which has resulted in the increased appreciation of microglia as druggable targets. Detailed knowledge of their cell biology is therefore of pivotal importance, and *in vitro* models provide an excellent means to obtain such knowledge. However, recent studies have demonstrated that the morphology and transcriptome (**Box 1**) of *in vitro* microglia only partially recapitulate that of *in vivo* microglia[5,6]. Yet, our knowledge of cues that shape the identity of *in vivo* microglia are limited and is mainly derived from rodent studies. In this thesis, a combination of *in silico* and *in vitro* approaches using primary rhesus macaque (*Macaca mulatta*) microglia is employed to i) optimize microglia *in vitro* culture conditions and to ii) identify microglia-based regulatory opportunities to modulate neuroinflammation.

Box 1. Transcriptomics

Transcriptomics is the study of all ribonucleic acid (RNA) molecules that are present in a sample at a given time. RNA is copied from deoxyribonucleic acid (DNA) segments, a process called transcription. Generally, only a part of the complete DNA code of a cell is transcribed. This process is regulated by epigenetic mechanisms and the interaction of transcription factors[7,8]. There are different types of RNA, and each type has different roles. For instance, messenger RNA (mRNA) is produced as an intermediary between DNA and proteins, while non-coding RNAs are involved in a variety of cellular functions[9-11]. RNA sequencing is a high-throughput method to map and quantify transcripts in a tissue or cell population[12]. This method has become an indispensable tool to discover novel genes, to profile cell-specific transcriptomes and to determine gene expression changes that are for instance associated with aging and disease.

Microglia

Origin

Microglia are the tissue-resident macrophages of the brain. They were first described by Pío del Río-Hortega early in the 20th century as non-neuronal elements that derive from oligodendroglia and astroglia[13]. Despite intensive research, the origin of microglia has long remained a controversial issue. Researchers described microglia as cells derived from mesodermal pial elements, from pericytes, and from neuroectodermal macroglia[14]. Whereas it was already proposed that microglia derive from yolk sac macrophages in 1999[15], conclusive evidence was only provided a decade later when it was shown that microglia originate from yolk sac primitive myeloid progenitor cells[16,17]. In contrast to most other macrophages, microglia maintenance relies on local self-renewal without replenishment from hematopoietic progenitors[18]. Thereby, they serve as their own progenitors and form a distinct population from circulating blood monocytes and hematopoietic macrophages.

Identity

The rapidly growing field of 'omics' technologies has resulted in an extensive interest in characterizing differences between microglia and other tissue-resident macrophages or CNS cells[6,19-24]. Expression data of freshly isolated microglia revealed a set of 'signature genes' that are uniquely expressed in microglia and that are not, or at much lower levels, expressed by other macrophages or CNS cell types. These genes include *GPR34, HEXB, OLFML3, P2RY12, P2RY13,* and *TMEM119*, both in rodents and humans[6,20,25]. It should be noted that these signature genes are highly expressed under homeostatic conditions, but that their expression is reduced during microglia activation[26], neurodegenerative disease[27], and also when microglia are brought in culture[5,28]. Engrafting of cultured microglia back into an intact CNS environment has been described to, at least partly, reverse the loss of signature genes expression[5]. This demonstrates that the CNS environment is important for microglia identity. However, as macrophages of different origin lack the expression of microglia signature genes when introduced to the CNS environment, the CNS environment is not solely responsible for microglia identity[29,30]. Taken together, this suggests that the identity of microglia is shaped by the unique combination of its origin and environment[6,31].

Microglia identity is established and maintained by factors expressed during different developmental stages (**Figure 1**). During early development, the transition of erythromyeloid progenitor-derived yolk sac macrophages to A1 myeloid progenitors ($CD45^{+}$, $CD117^{low}$ and $CX3CR1^{-}$) is regulated by PU.1 and RUNX1[16,32]. Subsequently, interferon regulatory factor (IRF) 8 and RUNX1 drive the maturation of A1 myeloid progenitors to A2 myeloid progenitors ($CD45^{+}$, $CD117^{-}$, $CX3CR1^{+}$ and $F4/80^{high}$)[32]. Around E9.5 (in mice), A2 myeloid progenitors migrate to the CNS, which is regulated by the interleukin (IL)-34-colony stimulating factor 1 receptor (CSF1R) pathway[33,34], and proliferate abundantly to achieve sufficient microglia numbers that are well distributed throughout the CNS. Environmental factors IL-34, macrophage colony-stimulating

factor (M-CSF) and transforming growth factor β (TGF-β) are essential for further microglia maturation[20,35-38]. In the postnatal CNS, crosstalk with other cells of the CNS subsequently shapes the unique identity of microglia and contributes to microglia homeostasis[31,39,40].

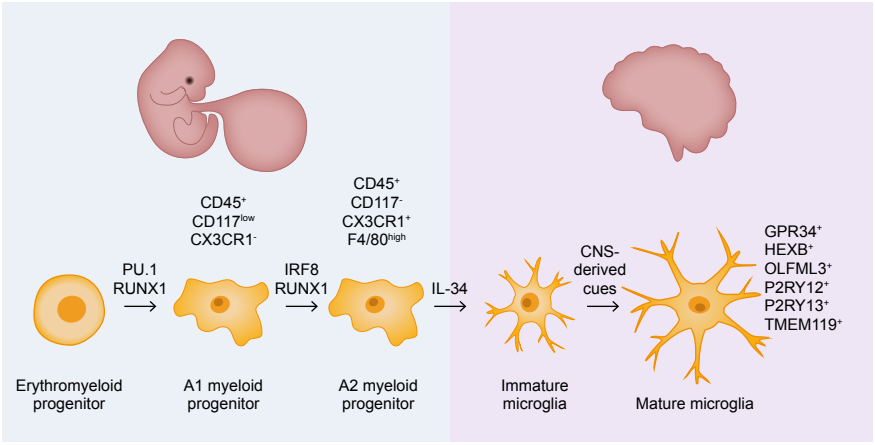


Figure 1. Microglia origin and development. Microglia derive from erythromyeloid progenitors, stem cells that are formed during primitive hematopoiesis. These cells start to upregulate CD45, which is regulated by PU.1 and RUNX1 (A1 myeloid progenitor). Later, IRF8 and RUNX1 expression differentiate these cells to A2 myeloid progenitors, which is characterized by the expression of myeloid cell markers CX3CR1 and F4/80. Migration of A2 myeloid progenitors to the central nervous system (CNS) is regulated by IL-34. Subsequently, CNS-derived cues are essential for further microglia maturation. Microglia reside in the brain throughout life and are thought to sustain the microglial population locally.

Function

Under homeostatic conditions, microglia continuously sample the CNS environment with their highly motile processes[41]. Microglia secrete neurotrophic and growth factors that regulate the proper development and homeostasis of oligodendrocytes, astrocytes and neuronal progenitors[42-47]. During CNS development and adult neurogenesis, microglia contribute to the clearance of superfluous neurons and control the size of the neuronal pool by engulfing neuronal progenitors[48-51]. Furthermore, microglia are involved in synapse modulation and synaptic pruning, which is required to establish efficient neuronal networks, both during development and in adulthood[52-56]. These actions should be tightly regulated as disruptions in microglia-mediated synaptic pruning are associated with neurodevelopmental and neuropsychiatric disorders, as autism and schizophrenia[57-59]. Finally, microglia are involved in inflammatory processes in the CNS.

Microglia and neuroinflammation

As the resident innate immune cells of the brain, microglia provide the first line of defense against invading pathogens, such as viruses, bacteria and prions[60,61]. Like other macrophages, microglia are phagocytic cells that can secrete a wide range of chemokines and pro- and anti-inflammatory cytokines[62].

Microglia activation

Initially, microglia were either categorized as resting (M0) under homeostatic conditions, classically activated (M1) under pro-inflammatory conditions or alternatively activated (M2) under anti-inflammatory conditions[63,64]. More recent studies have demonstrated that microglia can display a wide spectrum of phenotypes, both *in vitro* as well as *in vivo*[65]. The interplay between the local tissue environment, previous exposure to stress signals and the nature of the challenge all have impact on the microglia response[66]. The impact of 'nature and nurture' on microglia innate immune responses is discussed in **chapter 4**.

TLR signaling

Toll-like receptors (TLRs) are involved in the recognition of both pathogen-associated molecular patterns and damage/danger-associated molecular patterns. They form the most extensively studied family of pattern recognition receptors. For human and non-human primates (NHP), ten members of the TLR family (TLR1-10) have been described[67-69], of which the gene products of nine TLRs (TLR1-8 and TLR10) are observed in *ex vivo* microglia[70,71]. TLRs are expressed on the plasma membrane with the exception of TLR3, 7, 8 and 9 which are intracellularly expressed and localized to the endoplasmic reticulum, endosomes and lysosomes (**Figure 2**)[72]. Ligand binding to TLRs initiates an inflammatory immune response through the activation of transcription factors nuclear factor (NF)-κB, activator protein (AP)-1 and IRF3 causing synthesis of pro- and anti-inflammatory cytokines[73]. Inflammatory cytokines produced by microglia include IL-1α, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α and TGF-β. Multiple TLRs are constitutively expressed on microglia or can be induced during infections, CNS auto-inflammatory responses or neurodegenerative diseases[74-79] .

Purinergic signaling

Microglia are highly sensitive to subtle changes in the CNS environment. In response to pathological or damage-related events, cells in the CNS release nucleotides (e.g., ATP and UTP)[81,82]. These released molecules act as danger signals and can trigger signaling through two families of purinergic receptors, P1 and P2, that are expressed by many types of immune cells, including microglia. The family of P1 receptors includes four subtypes of G protein-coupled adenosine receptors, whereas the family of P2 receptors includes seven subtypes of ATP-gated ionotropic P2X receptors, and eight subtypes of G-protein-coupled metabotropic P2Y receptors. Purinergic receptor-

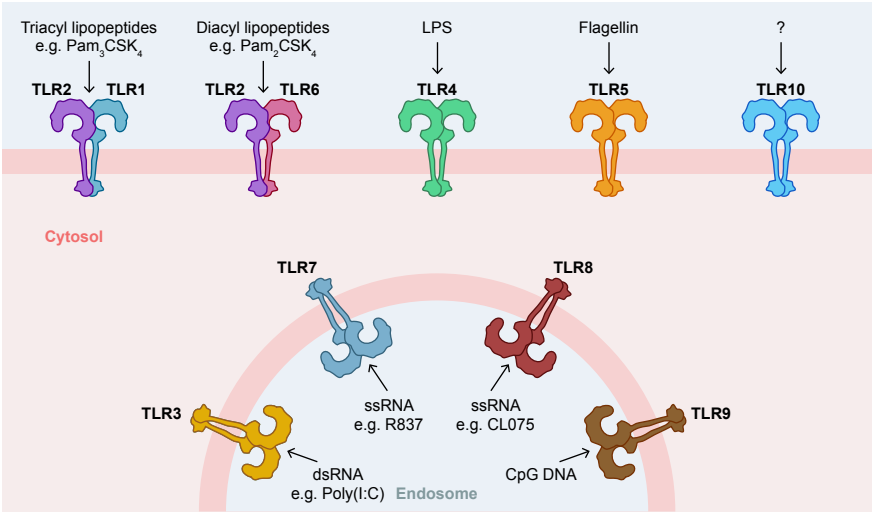


Figure 2. Cellular distribution of Toll-like receptors (TLRs) and their respective ligands. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface, while TLR3, 7, 8 and 9 are expressed intracellularly. Of note, ligands recognized by TLR10 are unknown[80].

mediated signaling can modulate migration, phagocytosis, apoptosis and the secretion of cytokines and chemokines by the expressing cell[83,84].

Interestingly, soluble inflammatory mediators, such as extracellular ATP and UTP, can influence inflammatory responses in microglia[85-87]. In **chapter 5**, we describe how signaling through the purinergic receptor P2RY6 modulates TLR-induced pro-inflammatory responses in microglia.

Microglia and neurodegenerative diseases

Neurodegeneration is defined as the progressive loss of functional neurons. This can be the selective loss of a particular neuronal subtype, such as occurs in diseases as PD and amyotrophic lateral sclerosis (ALS), or the widespread loss of many neuronal subtypes, such as occurs in AD and Huntington's disease (HD). Although all classified as neurodegenerative diseases, the underlying CNS pathologies are different. PD pathology is characterized by the formation of Lewy bodies in dopaminergic neurons consisting of fibrillar α-synuclein[88,89], whereas ALS is characterized by protein-rich cytoplasmic inclusions in motor neurons of the spinal cord[90,91]. AD pathology is characterized by the intracellular accumulation of hyperphosphorylated tau protein and neurofibrillary tangles and by the extracellular deposition of amyloid β (Aβ) in senile plaques[92,93]. HD pathology is characterized by neuronal intranuclear inclusions

consisting of mutant huntingtin protein[94]. Although the progress, etiology and symptoms of these diseases differ, neuroinflammation is a common hallmark of all of them. How neuroinflammation contributes to the progression of neurodegenerative diseases is still unclear as it can either be the cause or the consequence of neuronal cell death. It is, however, generally accepted that persistent inflammation of the CNS is detrimental to neurons. Intriguingly, some molecules that are associated with the pathology of neurodegenerative diseases, such as Aβ and α-synuclein, can induce or modulate inflammatory responses via receptors of the innate immune system thereby providing a molecular link between both processes[95-98]. Although microglia responses are thought to be primarily neuroprotective, they may also lead to tissue injury and neurodegeneration by the production of pro-inflammatory cytokines and reactive oxygen and nitrogen species[99-102]. There is a large body of evidence for a role of activated microglia in the pathogenesis of neurodegenerative disorders[103-105]. In the substantia nigra of PD patients, reactive microglia are found along with Lewy bodies[106] and large numbers of activated microglia can be observed in the CNS and spinal cords of human ALS patients as well as in ALS mouse models[107,108]. Microglia that surround plaques in AD change their morphology from ramified to amoeboid and stain positive for several activation markers, including CD68 and major histocompatibility complex II (MHCII)[109].

Microglia plasticity

Recently, the realization that microglia are not a uniform cell type has become a topic of interest to the scientific community. Research into microglia phenotypes as well as microglia heterogeneity across regions, age, sex, and disease has increased significantly since the emergence of (single cell) 'omics' methodologies.

Regional

Microglia are not uniformly distributed and exhibit morphological and transcriptomic differences across brain regions[110-114]. In humans, genes enriched in microglia from the gray matter are associated with cytokine-mediated signaling and microglia homeostasis, whereas the transcriptome of microglia from the white matter is more linked to chemotaxis, inflammatory responses and oligodendrocyte maintenance[115-118]. Genes enriched in microglia isolated from the subventricular zone are involved in biological processes related to hormonal signaling and interferon response[118]. Additionally, cerebellar microglia exhibit increased expression of several chemokines[119]. These region-specific microglia transcriptomes indicate that microglia may have region-specific functions. As we were aware of this phenomenon, all microglia isolations in this thesis were derived from a similar brain region, the frontal subcortical white matter.

Aging

Microglia in the aging CNS are characterized by changes in morphology, including hypertrophy of the soma and reduction of process length[120,121]. This phenotype is in line with the idea that microglia acquire a more activated state (also known as 'primed microglia') during aging. This is also characterized by an enriched expression of pro-inflammatory genes and a reduced expression of microglia signature genes[122,123]. In addition, genes of which the expression is enriched during aging were associated with phagocytosis and lipid homeostasis, whereas genes of which the expression was reduced during aging include actin cytoskeleton-associated genes, sensome cell surface receptors (receptors used by microglia to sense changes in their environment), and cell adhesion molecules (all in humans)[19,25,119,123-126]. The age-dependent microglia profile is linked to regulators TGF-β, RUNX1, IRF8 and PU.1[127,128], which are known for their role in establishing and maintaining microglia identity. Between mouse and human microglia, differences in the aging transcriptome have been reported, indicating that mouse and human microglia age differently[25,129]. It is important to take this into account when studying aging in mice or when data is used from mice aging studies. Species differences, differences in life span and in the history of inflammatory cues possibly all contribute to this lack of concordance.

Sex

Differences in morphological profiles of microglia have been reported between female and male mice. Microglia from male mice show a more globular morphology, which is associated with a pro-inflammatory phenotype[124,130-132]. In humans, sex-specific morphological and transcriptomic differences in microglia have been much less pronounced. Gene expression differences are mostly related to X- and Y-linked genes[6,118]. Sex-specific differences observed in cultured microglia derived from rhesus macaques will be briefly discussed in **chapter 2**.

Disease

Microglia in diseased brains exhibit a variety of morphologies, depending on (the stage of) the disease and the location of microglia[121,133-136]. Several studies have reported changes in the transcriptomes of microglia in neurodegenerative transgenic mouse models, including AD models, as compared to control mice. Depending on the study, these microglia were named either disease-associated microglia (DAM)[137], activated response microglia (ARM)[138] or microglia associated with neurodegenerative disease (MGnD)[139]. These cells are characterized by increased expression levels of genes related to immune responses, lipid metabolism, endocytosis, and phagocytic pathways. On the other hand, the expression levels of genes associated with microglia homeostasis were reduced. To simplify reading, we will further use the term DAM to define the neurodegenerative-associated microglia state, as many other groups have also chosen to do[27,123,140-144]. Whether the DAM transcriptome is associated with a neuroprotective or a neurotoxic phenotype is not well understood. An increasing number of studies are currently investigating the presence of DAM in humans.

Many studies report on the discrepancies in the DAM transcriptomic profiles of mice and men[145-149]. This underlines the challenges to extrapolate findings in rodent models to human neurodegenerative diseases[150,151]. Human stem cell-derived microglia, or primary microglia from humans or NHP provide researchers with viable alternatives to study the role of microglia in neurodegenerative diseases[152,153].

Non-human primate microglia

Because of their close resemblance to humans, NHP are used in biomedical research to study human biology and disease. Morphological and transcriptomic analysis of microglia across different animal species has demonstrated that microglia of NHP and humans are very much alike[70,154]. Importantly, the expression of AD and PD-associated genes in human microglia showed a high correlation with microglia derived from macaques. This correlation was much lower between human and rodent microglia[154]. In addition, studies in rhesus macaques indicated a similar microglia aging pattern as in humans[155]. Together, these studies demonstrate the potential of rhesus macaques as a model to study the role of microglia during health, aging and neurodegenerative diseases.

Microglia *in vitro* models

Over the years various microglia *in vitro* models have been developed, including microglia cell lines, stem cell-derived microglia cultures and primary dissociated cell cultures. Each of these models has specific advantages and limitations.

Microglia cell lines

Microglia cell lines are available from mouse, rat, macaque and human origin (**Table 1**). Most of these lines stem from primary microglia cultures derived from the brain or the spinal cord, which were immortalized by viral transduction with oncogenes (e.g., v-myc, v-raf, v-mil, SV40 T antigen). Non-transformed microglia cell lines that stem from primary microglia precursor cell cultures have been described as well. Advantages of cell lines include their ease of maintenance and their abundant availability due to their unrestricted proliferative capacity. However, a major disadvantage is their susceptibility to dedifferentiation. Furthermore, viral transformation or immortalization may alter the microglia phenotype. Indeed, recent studies have pointed out that microglia cell lines differ both genetically and functionally from primary microglia and *ex vivo* microglia[20,156-158]. In addition, microglia cell lines obtained from neonatal or embryonic CNS sources are unlikely to reflect the phenotype of adult or elderly microglia. Despite these limitations, microglia cell lines are suitable for e.g., biochemical and molecular approaches as well as for high-throughput screening assays which all require high cell numbers.

Table 1. Overview of available microglia cell lines of mouse, rat, rhesus macaque and human origin. In italics the CHME-5 cell line, of which the exact origin is currently uncertain.

Species	Cell line	Donor age	Brain area	Immortalization procedure	Citations till 2022	Reference
Mouse	BV2	Neonatal	Cerebral cortex	Transformed, *v-raf/v-myc* oncogene	>3700	159
	C8-B4	Neonatal	Cerebellum	Spontaneous	25	160
	EOC-2, EOC-13.31, EOC-20	Neonatal	Whole brain	Spontaneous, M-CSF-dependent clones	31	161
	IMG	Adult	Whole brain	Transformed, *v-raf/v-myc* oncogene	8	162
	MG5	Neonatal	Cerebral cortex	Transformed, microglia derived from p53-deficient mice	20	163
	MG6	Neonatal	Whole brain	Transformed, *c-myc* oncogene	27	164
	MG20	Neonatal	Whole brain	Transformed, *c-myc* oncogene	4	165
	Muμglia	Adult	Cortex	Transformed, SV40 large T antigen (and hTERT)	1	166
	N3, N9, N11, N13	Embryonic	Whole brain	Transformed, *v-myc* or *v-mil* oncogenes, clones	>400	167
	RA2	Neonatal	Whole brain	Non-enzymatic and non-virus transformed, GM-CSF-dependent	15	168
	SIM-A9	Neonatal	Cerebral cortex	Spontaneous	26	169
Rat	HAPI	Neonatal	Cerebral cortex	Spontaneous	64	170
	MLS-9	Neonatal	Neocortex	Spontaneous	17	171
Macaque	Mqμglia	Adult	Cerebral cortex	Transformed, SV40 large T antigen (and hTERT)	1	166
Human	*CHME-5*	*Embryonic*	*Spinal cord/ cortex*	*Transformed, transfection with SV40 large T antigen*	29	172
	HMO6	Embryonic	Telencephalon	Transformed, *v-myc* oncogene	17	173
	Huμglia	Adult	Cortex	Transformed, SV40 large T antigen (and hTERT)	1	166

Stem cell-derived microglia

Stem cell technology not only holds great promise for regenerative medicine, it can also provide scientists with an unlimited availability of cells for *in vitro* purposes. The two types of stem cells described in the context of microglia are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are derived from the inner cell mass of a blastocyst, whereas iPSCs can be generated from adult cells (e.g., fibroblasts) by reprogramming them via overexpression of just four transcription factors[174]. A major advantage of the iPSC approach is that it allows comparisons of iPSC-derived cells from healthy donors and patients with neurological disorders, also

while donors are still alive. In this way, the genetic background of these patients is recapitulated in their iPSC-derived neurons and glial cells.

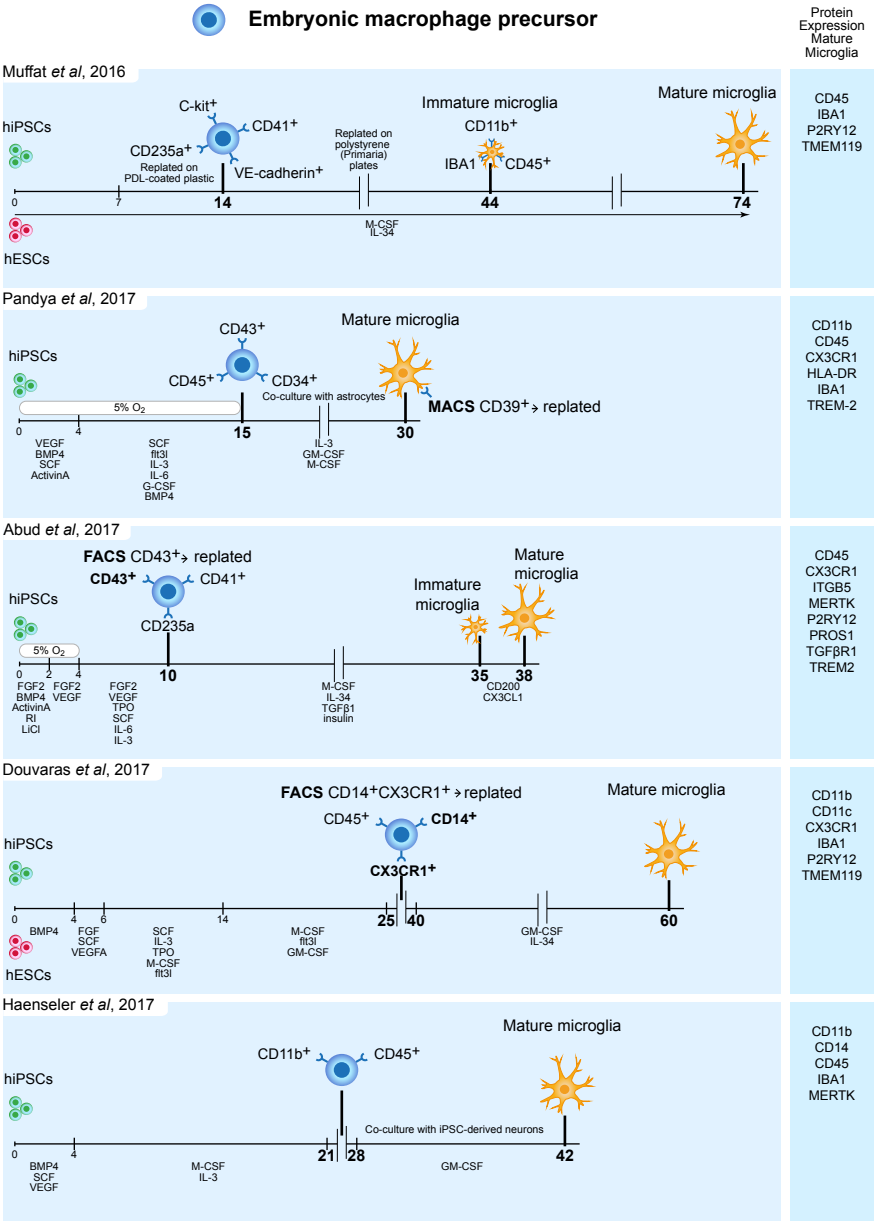
Many protocols have been established to differentiate ESCs and iPSCs to specific neuronal lineages, such as neurons, astrocytes and oligodendrocytes[175-177]. Microglia have, however, proven to be amongst the most difficult cells to differentiate from stem cells, partly because their exact origin remained obscure until 2010. The first microglia-related stem cell studies described methods to differentiate mouse ESCs to microglia by directing ESCs through neuronal differentiation pathways[178,179]. Since lineage tracing studies in mice revealed that microglia originate from primitive yolk sac macrophages[16], more recent protocols directly differentiate ESCs or iPSCs to embryonic macrophage precursors first, before skewing these toward a microglia phenotype by adding CNS-derived cues (**Figure 3**)[180-187]. In addition, a novel approach that rapidly converts human iPSCs into microglia by forced expression of transcription factors *SPI1* and *CEBPA* has recently been described[188].

As this is a new, rapidly emerging field, there is no consensus on methodology to generate iPSC or ESC-derived microglia yet. A variety of different culture media and culture conditions have been used, and comparative studies and harmonization are necessary to further validate the most reliable and reproducible approaches. Although stem cell technology can provide researchers with a readily available source of microglia, it should also be taken into account that these cells have never been exposed to the CNS microenvironment. In addition, most neurodegenerative disorders develop at adult or elderly age, and it is therefore important to recapitulate age-related characteristics in microglia when studying neurodegenerative disorders. However, reprogrammed iPSCs from adult donors have had their aging signature, such as telomere attrition and cellular senescence, reset[189]. Direct reprogramming of somatic cells to microglia might tackle this problem by avoiding passage through the stem cell phase, and it has been demonstrated that direct reprogramming retains aging-associated transcriptomic signatures[190,191].

Primary microglia

Methods to generate dissociated single cell cultures of primary microglia have been described for mice, rats, NHP and humans (**Table 2**). Most methods start with mechanical and enzymatic dissociation of the donor brain tissue followed by a density gradient centrifugation step to separate the myelin from the cells. Dependent on the density gradient used, this can either be sufficient to obtain microglia cultures with a purity of > 99% or it is followed by additional purification steps[192,193]. Other purification steps used to isolate microglia include labeling of cells with antibody-coated magnetic beads followed by magnetic-activated cell sorting (MACS)[28,194], labeling of cells with fluorescently labeled antibodies followed by fluorescence-activated cell sorting (FACS)[24,195] or specific shaking procedures[196].

Primary microglia from mice and rats are generally derived from brain tissue of neonatal animals[197], although more studies are now reporting the use of adult animals as well[5,20]. The advantage of using rodent primary microglia is that these animals form


Embryonic macrophage precursor
Protein Expression Mature Microglia
Muffat et al, 2016
hiPSCs
C-kit+
CD41+
CD235a+
Replated on PDL-coated plastic
VE-cadherin+
Replated on polystyrene (Primaria) plates
Immature microglia
CD11b+
IBA1
CD45+
Mature microglia
0
7
14
44
74
M-CSF
IL-34
hESCs
CD45
IBA1
P2RY12
TMEM119
Pandya et al, 2017
hiPSCs
CD43+
CD45+
CD34+
Co-culture with astrocytes
Mature microglia
MACS CD39+ replated
5% O2
0
4
15
30
VEGF
BMP4
SCF
ActivinA
SCF
flt3l
IL-3
IL-6
G-CSF
BMP4
IL-3
GM-CSF
M-CSF
CD11b
CD45
CX3CR1
HLA-DR
IBA1
TREM-2
Abud et al, 2017
FACS CD43+ replated
CD43+
CD41+
CD235a
hiPSCs
Immature microglia
Mature microglia
5% O2
0
2
4
10
35
38
FGF2
BMP4
ActivinA
RI
LiCl
FGF2
VEGF
FGF2
VEGF
TPO
SCF
IL-6
IL-3
M-CSF
IL-34
TGFβ1
insulin
CD200
CX3CL1
CD45
CX3CR1
ITGB5
MERTK
P2RY12
PROS1
TGFβR1
TREM2
Douvaras et al, 2017
FACS CD14+CX3CR1+ replated
CD45+
CD14+
CX3CR1+
hiPSCs
Mature microglia
0
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BMP4
FGF
SCF
VEGFA
SCF
IL-3
TPO
M-CSF
flt3l
M-CSF
flt3l
GM-CSF
GM-CSF
IL-34
hESCs
CD11b
CD11c
CX3CR1
IBA1
P2RY12
TMEM119
Haenseler et al, 2017
CD11b+
CD45+
hiPSCs
Mature microglia
Co-culture with iPSC-derived neurons
0
4
21
28
42
BMP4
SCF
VEGF
M-CSF
IL-3
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CD14
CD45
IBA1
MERTK

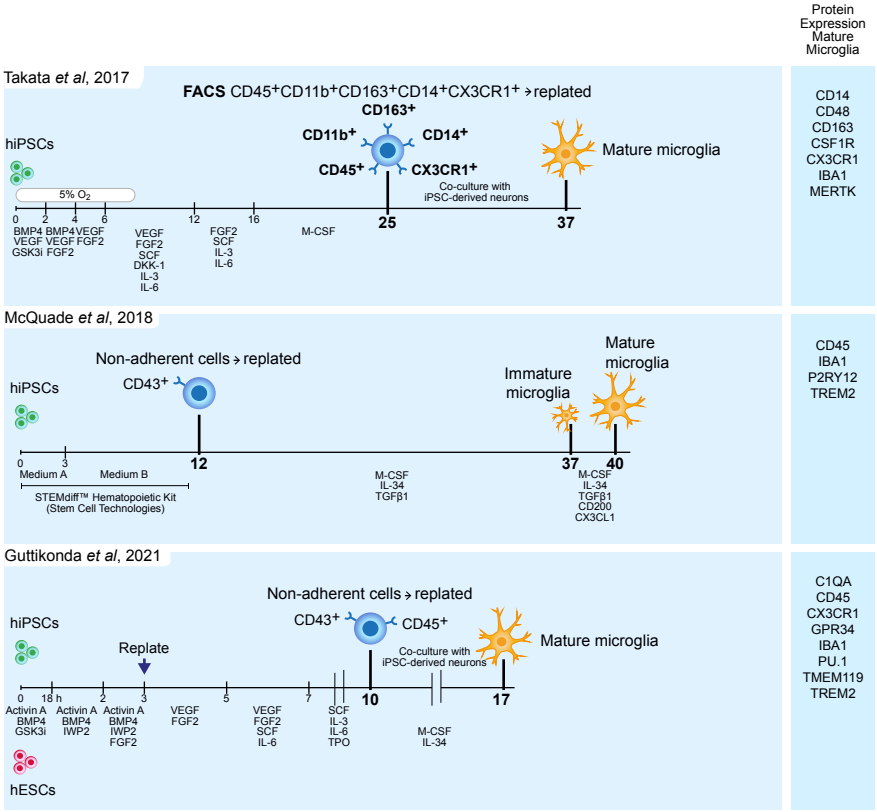

Figure 3. Overview of protocols to differentiate human iPSC and ESC to microglia-like cells. hiPSC and hESC are first differentiated to embryonic macrophage precursor cells, which is consistent with the *in vivo* developmental lineage of microglia. Subsequently, these precursor cells are differentiated into mature microglia-like cells. Timelines are indicated in days.

a genetic homogenous, specific pathogen free (SPF) population where *ante-mortem* conditions and *post-mortem* delay can be tightly controlled. The use of primary microglia derived from transgenic mice has been instrumental in delineating the role of specific genes in microglia activation. Limitations of rodent primary microglia include their evolutionary divergence from humans and lack of heterozygosity due to inbreeding and their aseptic housing conditions[150]. Such differences have been described to hamper translation of rodent (neuro)immunological studies to the clinic[76,150,198].

Dissociated cultures of human primary microglia can either be derived from fetal tissue that becomes available after abortion, surgical tissue from epilepsy patients that undergo neurosurgery, or from *post-mortem* brain tissue that becomes available from deceased human donors[157,199-201]. Microglia derived from patients who suffered from

Table 2. Comparison of human, non-human primate, and rodent primary microglia cell culture features.

	Human	Non-human primate	Rodent
Genetic distance to humans	None	Close evolutionary proximity to humans	Considerable evolutionary divergence from humans
Breeding	Outbred	Outbred	Inbred
Environment	Non-SPF	Non-SPF	SPF
***Ante-mortem* conditions**	Uncontrollable and often unknown	Controllable and well described	Controllable and well described
***Post-mortem* delay**	4-24 h at best	None	None
Donor age (most often)	Fetal or aged adults	Adult	Fetal/neonatal and adult
Donor characteristics (most often)	Neurological disease, shortage of non-diseased donors	Free of neurological disease	Free of neurological disease
Availability	Limited: brain banks	Limited: primate centers	Widely available
Microglia yields	0.1-0.5 $*10^6$ cells/gram wet brain tissue; 5-15 gram available[28]	0.3-0.6 $*10^6$ cells/gram wet brain tissue; > 35 gram available[193]	0.3-1$*10^6$ cells/brain; can be pooled from multiple brains of inbred animals[208]
Availability other tissues from the same donor	Limited	Good	Good

neurological disease may provide new insights into the role of microglia in the pathogenesis[103,202-204]. To enable research using human brain tissue, brain banks have been set up worldwide. Human primary microglia are derived from different individuals thus reflecting the genetic variability within a population, and translation of results is not hampered by the use of a genetically divergent species[150]. Limitations of human primary microglia include the limited availability of (healthy) human brain tissue, and the limited control over the *ante-mortem* conditions and *post-mortem* delay, which might affect the microglia phenotype. For example, CD11b expression, a marker for microglia activation, showed a significant positive correlation with *post-mortem* delay in gray matter microglia[28].

To bridge the gap between rodents and humans, primary microglia cultures derived from NHP may be considered. Protocols have been developed for the rhesus macaque[193]. To isolate such cells, the presence of a research center with NHP facilities is a requirement. Advantages on the other hand are that microglia are isolated from outbred individuals that are in close evolutionary proximity to humans[70] with much control over *ante-mortem* conditions and *post-mortem* delay. Comparison of primary microglia with primary bone marrow-derived macrophages from the same donors has been instrumental in uncovering microglia specific features of innate immune responses[205-207], demonstrating the utility of this methodology.

Motivated by the usefulness and multiple advantages of primary microglia from NHP in advancing our knowledge of microglia biology, studies throughout this thesis were performed using primary microglia from rhesus macaques. No animals were exclusively sacrificed for the aim of our studies, since tissue was used from animals

that were sacrificed for other purposes. Thereby, the application of our *in vitro* cultures contributes to the active program to replace, reduce and refine the use of experimental animals at the Biomedical Primate Research Centre.

Ex vivo microglia versus *in vitro* microglia

Efforts to study microglia biology *in vitro* have been hampered by the lack of microglia culture models that recapitulate the features of homeostatic *in vivo* microglia. When maintained in culture, microglia generally exhibit an amoeboid morphology, thereby resembling activated microglia[209]. Transcriptome analysis of *in vitro* microglia have brought to light that currently no microglia *in vitro* system fully matches the transcriptome of homeostatic *in vivo* microglia. When brought in culture, microglia upregulate genes associated with disease, inflammation and stress, whereas genes that are associated with homeostasis and maturation were reduced as compared to *ex vivo* microglia[5,6,20,28,210] (**Figure 4**). These observations are mirrored by changes in the epigenetic landscape of primary microglia[6]. Nevertheless, studies that determined to which degree microglia cell lines, stem cell-derived microglia and primary microglia express the microglia signature gene profile showed that the transcriptomes of primary microglia best resembled that of adult *ex vivo* microglia[20,211,212].

Like *ex vivo* microglia, primary microglia are characterized by a substantial transcriptomic heterogeneity amongst single cells. However, the distinct states differ from those observed *ex vivo*. *In vitro* microglia states were rather linked to either proliferative, interferon-related, LPS-related or neurodegenerative processes[210]. Together, these studies demonstrate the challenges associated with culturing microglia that resemble *in vivo* microglia. To gain a better insight into microglia cell biology in health and disease, optimization of *in vitro* microglia is instrumental.

Variables to be considered to optimize microglia *in vitro* culture conditions

Supplements and growth factors

For the culture of microglia, many different cell culture media combined with a diversity of growth factors have been tested to maintain or induce a more *in vivo*-like phenotype.

Traditionally, microglia cell lines and primary microglia are cultured in medium that contains fetal calf serum (FCS) to support proliferation[193,213,214]. Besides the fact that microglia in a healthy CNS are not exposed to serum –let alone FCS–, the use of serum in cell culture media comes with more disadvantages. Serum is a poorly defined cell culture component and batch-to-batch variability negatively contributes to reproducibility. Another important motivation to eliminate serum from cell culture protocols is that the acquisition of FCS is associated with animal suffering.

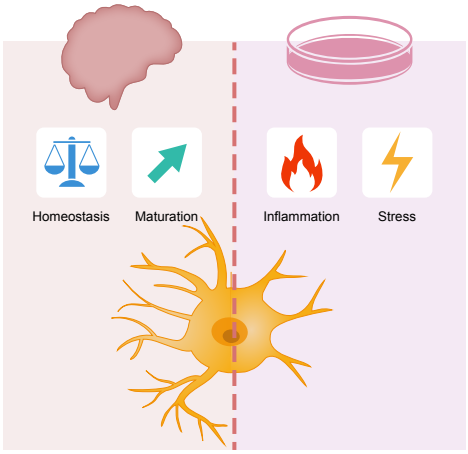

Figure 4. *Ex vivo* microglia versus *in vitro* microglia. *Ex vivo* microglia in the healthy central nervous system are characterized by a ramified morphology and a gene expression profile associated with microglia homeostasis and maturation (left). *In vitro* microglia exhibit a more amoeboid morphology and show increased expression of genes associated with inflammation and stress (right).

Serum elimination from microglia cell line cultures did not affect the morphology of microglia[215], which is probably the consequence of their sustained active and proliferative state. Stem cell-derived microglia protocols, apart from the differentiation protocol of Pandya and colleagues[180], use serum-free culture protocols and acquire a ramified morphology. This ramified morphology, which resembles the morphology of homeostatic microglia *in vivo,* was also observed when primary microglia from rat were cultured under serum-free conditions[5]. In **chapter 2**, we tested if this serum-free culture protocol can optimize *in vitro* primary microglia derived from rhesus macaques.

Studies using knock-out mice have demonstrated the importance of CSF1R signaling for microglia survival and proliferation[35], which has led to the inclusion of CSF-1 (M-CSF) in most microglia media. The discovery of IL-34 as a second brain-specific ligand for CSF1R, has inspired researchers to experiment with this factor as well[216]. In **chapter 2**, we examine whether the transcriptomes of primary microglia exposed to either growth factor are different. Since in rodents, the TGF-β pathway has been identified as important for microglia homeostasis, both *in vivo* and *in vitro*[5,20,217,218], we also investigate the effects of TGF-β exposure on primary rhesus macaque microglia.

Intercellular communication

In vivo, microglia are chronically exposed to many inhibitory signals from the CNS environment and, in the absence of strong activating signals, will remain in a homeostatic state. For example, neuronal cell surface proteins CD47, CD200, and CD22 interact with CD172, CD200 receptor and CD45 on microglia respectively, providing

inhibitory signals[76]. Loss or disruption of constitutive inhibitory signaling leads to a more activated microglia phenotype[217,219-221], which is characterized by the increased expression of activation markers, such as CD11b and CD45. Together these findings demonstrate that intercellular signals from the CNS microenvironment are required to maintain microglia specification. This has been supported by the notion that engraftment of *in vitro* microglia into a CNS parenchyma can partly reverse the loss of microglia signature gene expression[5,222].

Thus far, it has been demonstrated that microglia cell-cell contact with neurons and/or astrocytes are important for microglia homeostasis[39,40,223]. In **chapter 2**, we investigate the contribution of cell-cell contact with other glia cells, including oligodendrocytes and radial glia, on microglia homeostasis. In addition, in **chapter 3**, we examine the effects of exposure to conditioned medium that is derived from different cultured CNS cell types. Furthermore, we use a computational method to uncover CNS-derived cues that are important for microglia homeostasis, and test if exposure to these cues indeed improve primary microglia cultures.

Extracellular matrix

The extracellular space of the brain is composed of a 3D molecular network, called the extracellular matrix (ECM), that occupies ±20% of the adult brain volume[224,225]. The brain ECM consists largely of proteoglycans (including chondroitin sulphate proteoglycans and heparan sulfate proteoglycans), glycoproteins (including tenascins and laminins) and glycosaminoglycans (including the abundant hyaluronan)[225-227]. Additional ECM proteins found in the brain are collagen and fibronectin, which are expressed in relative low levels[224]. The brain ECM provides both biological and biophysical cues that are essential for many cellular processes, including migration, differentiation, proliferation and survival[228,229]. Studies have investigated the effect of ECM coatings, including laminin, on microglia morphology and function[5,230-232]. In **chapter 3**, we examined if laminin-coated substrates can optimize microglia *in vitro* cultures[233].

Thesis outline

Microglia *in vitro* models are instrumental to acquire fundamental biological knowledge of this cell type during health and disease. Primary microglia cultures, although laborious and relatively short-lived, have proven to be especially useful in this aspect. It however remains challenging to recapitulate the resting, homeostatic phenotype of *in vivo* microglia. In this thesis we aimed to i) optimize microglia *in vitro* culture conditions by uncovering and testing potential CNS-derived cues, and to ii) identify microglia-based regulatory opportunities to modulate neuroinflammation.

In **chapter 2**, we extensively characterize the morphology and transcriptomes of *ex vivo* and *in vitro* microglia that were cultured under different conditions. We propose a new, partly serum-free, microglia culture protocol yielding confluent cultures of

microglia with a ramified morphology. In **chapter 3**, we combine *in silico* with *in vitro* modeling to uncover (CNS-derived) cues that are important to induce or maintain the *ex vivo* microglia reference transcriptome.

As both ontogeny and CNS environment are important for microglia identity, we reviewed the impact of 'nature and nurture' on microglial innate immune responses in **chapter 4**. In this review we also address the current knowledge on the impact of aging, epigenetics and the CNS microenvironment. In **chapter 5**, we describe that microglia in particular are sensitive to P2RY6-mediated amplification of the production of TLR-induced pro-inflammatory cytokines as compared to bone marrow-derived macrophages from the same donors. These differences may be associated with tissue-specific adaptations and is a good example of the impact of 'nature and nurture' on innate immune responses.

Finally, **chapter 6** provides an overall discussion of our findings and future perspectives regarding the optimization and use of microglia *in vitro* models to study health and disease.

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Chapter 2

Transcriptome analysis reveals the contribution of oligodendrocyte and radial glia-derived cues for maintenance of microglia identity

Raissa Timmerman[1], Ella A. Zuiderwijk-Sick[1], Nynke Oosterhof[2,3], Anke E.J. 't Jong[1], Jennifer Veth[1], Saskia M. Burm[1], Tjakko J. van Ham[2] and Jeffrey J. Bajramovic[1]

[1] Alternatives Unit, Biomedical Primate Research Centre, Rijswijk, The Netherlands
[2] Department of Clinical Genetics, Erasmus MC, University Medical Center Rotterdam, The Netherlands
[3] European Research Institute for the Biology of Ageing, University Medical Center Groningen, The Netherlands

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Abstract

Microglia are increasingly being recognized as druggable targets in neurodegenerative disorders, and good *in vitro* models are crucial to address cell biological questions. Major challenges are to recapitulate the complex microglial morphology and their *in vivo* transcriptome. We have therefore exposed primary microglia from adult rhesus macaques to a variety of different culture conditions including exposure to soluble factors as M-CSF, IL-34 and TGF-β as well as serum replacement approaches, and compared their morphologies and transcriptomes to those of mature, homeostatic *in vivo* microglia.

This enabled us to develop a new, partially serum-free, monoculture protocol, that yields high numbers of ramified cells. We also demonstrate that exposure of adult microglia to M-CSF or IL-34 induces similar transcriptomes, and that exposure to TGF-β has much less pronounced effects than it does on rodent microglia. However, regardless of culture conditions, the transcriptomes of *in vitro* and *in vivo* microglia remained substantially different. Analysis of differentially expressed genes inspired us to perform 3D-spherical co-culture experiments of microglia with oligodendrocytes and radial glia. In such spheres, microglia signature genes were strongly induced, even in the absence of neurons and astrocytes. These data reveal a novel role for oligodendrocyte and radial glia-derived cues in the maintenance of microglial identity, providing new anchor points to study microglia in health and disease.

Introduction

Microglia, the resident tissue macrophages of the central nervous system (CNS), are key players during brain development, homeostasis and disease[1-4]. Activation or dysfunction of microglia is linked to classical neuroinflammatory diseases such as viral encephalitis and multiple sclerosis (MS), but also to neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis[5-10]. In addition, many of the genes recently identified as risk factors for the development of AD in genome-wide association studies, such as *APOE*, *TREM2* and *CD33*, are expressed by microglia[11-13]. Microglia therefore represent promising cellular targets for therapeutic intervention in neuroinflammatory and neurodegenerative diseases.

To obtain detailed cellular biological knowledge of microglia, good *in vitro* models are instrumental. Different *in vitro* systems have been developed over time, ranging from cell lines to primary microglia to stem cell-derived microglia-like cells, all with their own characteristic features[14]. Primary microglia cultures, although laborious and relatively short-lived, have amongst others been used to provide insight into tissue-specific features of innate immune responses[15-17]. Recent studies have however demonstrated that there are important differences in the morphology and the transcriptome of primary *in vitro* microglia as compared to those of mature homeostatic *ex vivo* microglia, both in humans and in rodents[18-21]. The expression levels of a variety of gene products were found to differ and most importantly, expression of many of the microglia signature genes[13,18,20,22-26], such as *P2RY12, TMEM119* and *GPR34* was lost *in vitro*.

Multiple studies have been devoted to the optimization of primary microglia cultures. Gene knockout studies have demonstrated the importance of colony stimulating factor-1 (CSF-1) receptor-induced signaling for microglial proliferation and survival[27]. There are two reported ligands for the CSF-1 receptor, macrophage colony-stimulating factor (M-CSF) and interleukin (IL)-34, and at present it is not known whether primary microglia exposed to either growth factor develop differently. In addition, transcriptome comparisons of *ex vivo* and primary rodent microglia have pinpointed the importance of exposure to transforming growth factor beta (TGF-β) to induce the expression of the adult microglial gene expression profile[18], but its importance for primary microglia of primate origin remains to be established. Finally, it was recently reported that exposure of primary microglia to serum profoundly alters their gene expression profile, and a new serum-free culture medium has been developed[19]. Despite these advances, important challenges remain to culture microglia that resemble the complex ramified morphology of *in vivo* microglia and express the signature genes that determine microglial identity.

We here analyzed the cellular morphologies and the transcriptomes of primary microglia that were cultured under different conditions, and compared these to *ex vivo* microglia. To bridge the gap between rodents and humans, we isolated primary microglia from rhesus macaques, outbred animals that are evolutionary close to

humans[15,16,28,29]. We exposed adult rhesus microglia to M-CSF and IL-34, both in the presence and absence of TGF-β. We also explored different serum exposure and washout culture regimes, and finally, we have experimented with a co-culture system containing microglia, oligodendrocytes and radial glia. We report the development of a new, partially serum-free, monoculture protocol, that yields high numbers of ramified cells. In addition, our co-culture system revealed an unexpected role for oligodendrocyte and radial glia-derived cues in the maintenance of microglial identity.

Materials and methods

Animals

Brain tissue was obtained from adult rhesus macaques (*Macaca mulatta*) without neurological disease that became available from the outbred breeding colony. No animals were sacrificed for the exclusive purpose of microglia isolation. Better use of experimental animals contributes to the priority 3Rs program of the Biomedical Primate Research Centre. Individual identification data of the animals are listed in **Table 1.**

Primary cell isolation

Ex vivo microglia isolation was carried out as described previously[22]. Frontal subcortical white matter samples were collected in *ex vivo* microglia medium (EMM) comprised of HBSS (Gibco Life Technologies, Bleiswijk, The Netherlands) supplemented with 15 mM HEPES (Lonza, Cologne, Germany) and 0.6% (wt/vol) glucose (Sigma-Aldrich, Saint Louis, MO). Meninges and blood vessels were removed manually. The brain tissue was dissociated in a glass tissue homogenizer and filtered using a 300 µm sieve followed by a 106 µm sieve to obtain a single-cell suspension. Cells were pelleted by centrifugation at 220 *g* for 10 min at 4 °C. The pellet was resuspended in 22% (vol/vol) Percoll (Cytiva, Uppsala, Sweden), 37 mM NaCl and 75% (vol/vol) myelin gradient buffer (5.6 mM NaH_2PO_4, 20 mM Na_2HPO_4, 140 mM NaCl, 5.4 mM KCl, 11 mM glucose, pH 7.4). A layer of PBS (Gibco) was added on top, and this gradient was centrifuged at 950 *g* for 20 min at 4 °C (minimal acceleration, no brake). The myelin layer and the remaining supernatant were carefully removed and the pellet was resuspended in a solution of 60% Percoll, which was overlaid with 30% Percoll and layered with PBS, respectively, and centrifuged at 800 *g* for 25 min at 4 °C (minimal acceleration, no brake). The cell layer at the 60–30% Percoll interphase was collected with a Pasteur pipette, washed with EMM and centrifuged at 600 *g* for 10 min at 4 °C. The final pellet was resuspended in HBSS without phenol red (Gibco) supplemented with 15 mM HEPES, 0.6% glucose and 1 mM EDTA (Invitrogen; Life technologies). Fc receptors were blocked with human Fc receptor binding inhibitor (eBioscience, Thermo Fisher Scientific, Cat#14-9161-73, RRID:AB_468582, Waltham, MA) for 15 min on ice. For fluorescence-activated cell sorting, cells were incubated for 25 min on ice, in the dark, with anti-human CD11b-PE (1:25, Clone: ICRF44, BioLegend, Cat#301306,

Table 1. Individual identification data of rhesus macaques.

Donor nr.	Monkey ID nr.	Age (years)	Sex	Weight (kg)	Origin	Condition
1	R11065	5	Male	11,0	India	*In vitro*
2	R14033	2	Male	2,7	India	*In vitro*
3	R09080	7	Female	6,0	India	*In vitro*
4	R12102	4	Female	4,0	India	*In vitro*
5	R09105	7	Female	5,6	India	*Ex vivo*
6	R08072	8	Male	10,6	India	*Ex vivo*
7	R08094	8	Male	11,6	India	*Ex vivo*
8	R07015	9	Male	12,2	India	*Ex vivo*
9	R11110	7	Female	7,8	India	*In vitro*
10	R15031	3	Female	4,3	India	*In vitro*
11	R15028	3	Male	6,3	India	*In vitro*
12	R14052	4	Male	6,4	India	*In vitro*
13	R04030	15	Female	6,4	India	*In vitro*
14	R08124	11	Female	5,1	India	*In vitro*
15	R08130	11	Female	5,5	India	*In vitro*
16	R08007	12	Female	6,3	Mix	*In vitro*
17	R05080	14	Female	5	India	*In vitro*
18	R06005	13	Female	8,2	India	*In vitro*
19	R97062	22	Female	7,5	India	*In vitro*

RRID:AB_314158, Uithoorn, The Netherlands) and anti-rhesus CD45-FITC (1:25, Clone: MB4-6D6, Miltenyi Biotec, Cat# 130-091-898, RRID:AB_244324 Bergisch Gladbach, Germany). Subsequently, cells were washed with HBSS without phenol red and centrifuged at 300 *g* for 3 min at 4 °C. The cells were passed through a 35 µm nylon mesh, collected in round-bottom tubes (Corning Costar Europe, Badhoevedorp, the Netherlands) and sorted using a Beckman Coulter MoFloAstrio cell sorter. Cells were sorted based on $CD11b^{high}/CD45^{int}$ expression and negative staining for DAPI or the LIVE/DEAD Fixable Red Dead Cell stain (Thermo Fisher Scientific) and collected in RNAlater (Qiagen GmbH, Hilden, Germany). Sorted cells were centrifuged at 5000 *g* for 10 min and pellets were lysed in RLT-Plus buffer (Qiagen) for RNA extraction.

Microglia for primary cell cultures were isolated as described previously with a few modifications[15,29,30]. In short, frontal subcortical white matter samples were collected in primary microglia medium (PMM) comprised of 1:1 v/v DMEM (high glucose)/HAM

F10 Nutrient mixture (Gibco) supplemented with 10% v/v heat-inactivated FBS (TICO Europe, Amstelveen, The Netherlands), 2 mM glutamax, 50 units/mL penicillin and 50 µg/mL streptomycin (all from Gibco). Microglia isolations were initiated from cubes of ~4.5 g tissue that were depleted of meninges and blood vessels manually. Tissue was chopped into cubes of less than 2 mm^2 by using gentleMACS™ C tubes (Miltenyi Biotec) and incubated at 37 °C for 20 min in PBS containing 0.25% (w/v) trypsin (Gibco) and 1 mg/mL bovine pancreatic DNAse I (Sigma-Aldrich) and mixed every 5 min. The supernatant was discarded (no centrifugation), the pellet was washed in PMM and passed over a 100 µm nylon cell strainer (Falcon; Becton Dickinson Labware Europe) and centrifuged for 7 min at 524 *g*. The pellet was resuspended in 22% (vol/vol) Percoll, 37 mM NaCl and 75% (vol/vol) myelin gradient buffer (5.6 mM NaH_2PO_4, 20 mM Na_2HPO_4, 137 mM NaCl, 5.3 mM KCl, 11 mM glucose, 3 mM BSA Fraction V, pH 7.4). A layer of myelin gradient buffer was added on top, and this gradient was centrifuged at 1561 *g* for 30 min (minimal brake). The pellet was washed in PMM and centrifuged for 7 min at 524 *g*. Cells were plated at a density of 6.5 * 10^4 cells/cm^2 in tissue-culture treated well plates (Corning Costar Europe) in PMM. For sphere cultures, cells were plated at a density of 10.5 * 10^4 cells/cm^2 in ultra-low attachment plates (Corning Costar Europe) and placed on a Hi/Lo shaker (IBI Scientific, Dubuque, IA) at 15 rpm.

Cell culture

Microglia monoculture: After overnight incubation at 37 °C in a humidified atmosphere containing 5% CO_2, unattached cells and myelin debris were removed by washing with PBS and replaced by fresh standard medium (SM; **Table S1**) comprised of 1:1 v/v DMEM (high glucose)/HAM F10 Nutrient mixture supplemented with 10% v/v heat-inactivated FBS, 2 mM glutamax, 50 units/mL penicillin and 50 µg/mL streptomycin supplemented with 20 ng/mL (≥ 4 units/mL) M-CSF (PeproTech, London, UK) or 100 ng/mL IL-34 (Peprotech), with or without 12.5 ng/mL TGF-β1 (Miltenyi Biotec) or replaced by fresh serum-free microglial culture medium (SFM; **Table S1**) comprised of DMEM/F12 (Gibco) supplemented with 2 mM glutamax, 50 units/mL penicillin, 50 µg/mL streptomycin, 5 µg/mL N-acetyl-L-cysteine (Sigma-Aldrich), 5 µg/mL insulin (Sigma-Aldrich), 100 µg/mL apo-transferrin (Sigma-Aldrich), 100 ng/mL sodium selenite (Sigma-Aldrich), 20 ng/mL (≥ 4 units/ml) M-CSF, 12.5 ng/mL TGF-β1, 1.5 µg/mL ovine wool cholesterol (Avanti Polar Lipids, Alabaster, AL), 1 µg/mL heparan sulfate (Galen Laboratory Supplies, North Haven, CT), 0.1 µg/mL oleic acid (Cayman Chemical, Ann Arbor, MI), 1 ng/mL gondoic acid (Cayman Chemical).

SM culture: All cells were kept in culture for 8 days without passaging. Half of the medium was replaced by fresh SM containing new growth factors every 3 days.

SFM culture: All cells were kept in culture for 8 or 22 days without passaging. Half of the medium was replaced by fresh SFM medium containing new growth factors every 2–3 days.

SM culture followed by SFM culture: All cells were kept in culture for 8, 15 or 22 days without passaging. Cells were exposed to SM for the first 4 days. At day 4, cells were washed twice with PBS and replaced by SFM medium. From day 4, half of the medium was replaced by fresh SFM medium containing new growth factors every 2–3 days.

Microglia sphere culture: cells were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2, whereafter 20 ng/mL M-CSF was added. At day 4, spheres were carefully transferred to a new well containing SFM and were kept in culture for 11 additional days without passaging. Half of the culture medium was carefully replaced by fresh SFM containing new growth factors every 2-3 days.

RNA extraction and library synthesis

Total cellular RNA was isolated using the RNeasy minikit (Qiagen) according to manufacturer's protocol. For donor nr. 1-12 (**Table 1**) the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) was used to prepare and process the samples. Briefly, mRNA was isolated from total RNA using oligo(dT) magnetic beads. After fragmentation of the mRNA, cDNA synthesis was performed followed by ligation of sequencing adapters and PCR amplification. The quality and yield after sample preparation were measured with a fragment analyzer (Agilent Technologies, Amstelveen, The Netherlands). Clustering and sequencing using the Illumina NextSeq 500 was performed according to manufacturer's protocols.

For donor nr. 13-16 (**Table 1**) The NEBNext Low Input RNA Library Prep Kit for Illumina (New England Biolabs) was used to process the samples. The sample preparation was performed according to manufacturer's protocol. Briefly, cDNA was synthesized and amplified from poly A tailed mRNA followed by ligation with the sequencing adapters and PCR amplicification. The quality and yield after sample preparation was measured with the Fragment Analyzer (Agilent Technologies). Clustering and sequencing using the Illumina NovaSeq 6000 was performed according to manufacturer's protocols.

Prior to alignment, the reads were trimmed for adapter sequences using Trimmomatic v0.30. Presumed adapter sequences were removed from the read when the bases matched a sequence in the adapter sequence set (TruSeq adapters) with 2 or less mismatches and an alignment score of at least 12.

The *Macaca mulatta* genomic reference (Macaca_mulatta.Mmul_8.01.dna.toplevel.fa) was used for alignment of the reads for each sample. The reads were mapped to the reference sequence using a short read aligner based on Burrows-Wheeler Transform (Tophat v2.0.14) with default settings. SAMtools v1.3 package (http://htslib.org/, RRID:SCR_002105) was used to sort and index the BAM files. Based on the mapped locations in the alignment file the frequency of how often a read was mapped on a transcript was determined with HTSeq v0.6.1p1 (http://htseq.readthedocs.io/en/release_0.9.1/, RRID:SCR_005514). The counts were saved to count files, which served as input for downstream RNA sequencing analysis.

Bioinformatics

BiomaRt Bioconductor Package (https://bioconductor.org/packages/release/bioc/html/biomaRt.html, RRID:SCR_019214) was used to annotate the genes and to generate a gene symbol list[31,32]. The accession number for the gene level RNA-sequencing data from freshly isolated microglia and cultured primary microglia from rhesus macaques reported in this paper is GEO: GSE171476.

Data were inspected using principal component analysis (PCA) and heatmaps generated with heatmap.2 of Bioconductor package gplots. Differential gene expression analysis was performed with Bioconductor package EdgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html, RRID:SCR_012802)[33]. An overview of all performed differential gene expression analyses can be found in **Table S2**. Qiagen's Ingenuity Pathway Analysis (Qiagen, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis, RRID:SCR_008653) was used to perform pathway analysis. The Molecular Signatures Database (MsigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp, RRID:SCR_016863) was used to perform gene ontology analysis and canonical pathway analysis[34,35].

To infer the relative abundance of different CNS cell types in spheres, we used CIBERSORT (https://cibersort.stanford.edu/, RRID:SCR_016955)[36], a bioinformatic algorithm that allows calculation of cell types composition from gene expression profiles. For the signature gene expression file, expression levels of neurons, astrocytes, microglia and oligodendrocytes of GEO: GSE73721 was used[13]. For the expression of radial glia signature genes, the transcriptome dataset of Pollen and colleagues was used[37]. All CIBERSORT analyses had p-values of less than 0.05. The proportion of the CNS cell types in spheres are displayed in bar plots.

Immunofluorescence and morphological analysis of monocultured microglia

Cells grown on glass coverslips were fixed for 30 min at RT in 2% PFA in PBS (Affymetrix, Santa Clara, CA), rinsed with PBS and PBS+0.02% Tween20 (Sigma-Aldrich) respectively, and nonspecific binding was blocked by incubation for 30 min in PBS containing 2% normal donkey serum (Abcam, Cambridge, UK). Samples were incubated overnight at 4 °C with CX3CR1 antibody (1:400, Abcam, Cat#ab8021, RRID: AB_306203) in PBS containing 0.1% BSA (Sigma-Aldrich), rinsed with PBS+0.02% Tween20, and incubated for 1 h at RT with donkey anti rabbit-FITC (1:400, Jackson ImmunoResearch Laboratories, Weste Grove, PA, Cat#711-095-152, RRID:AB_2315776) in PBS containing 0.1% BSA. After extensive washes with PBS, coverslips were mounted using ProLong™ Diamond Antifade + DAPI (Thermo Fisher Scientific) and images were acquired using a Leica DMI6000 fluorescence microscope and LASX software.

Microglial complexity was analyzed using Sholl and fractal analysis. In brief, CX3CR1-positive cell branches were traced using the Simple Neurite Tracer plugin[38] in ImageJ (https://imagej.nih.gov/ij/, RRID:SCR_002285) and skeletonized. Of these skeletonized traces, branch intersections with concentric circles per 5 µm steps from the nucleus were counted using a Sholl analysis plugin[39]. Numbers of intersections were averaged per donor and plotted. Subsequently, the area under the curve (AUC) was extracted in

which higher AUC values reflect a higher complexity. For fractal analysis the FracLac plugin (https://imagej.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm) for ImageJ was used. Skeletonized images were converted to outlines and fractal dimensions (D_B) of each cell were analyzed as described earlier[40,41]. FracLac calculated the D_B using a box counting protocol, summarized in the reference guide provided for FracLac for ImageJ: https://imagej.nih.gov/ij/plugins/fraclac/FLHelp/BoxCounting.htm. In brief summary, a cell is more complex as D_B approaches 2.

Immunofluorescence of brain tissue and spheres

For formalin-fixed, paraffin-embedded brain tissue of adult rhesus macaques, 5 µm sections were collected on Superfrost Plus glass slides (VWR international, Leuven, Belgium), dried at 37 °C, deparaffinized in xylene and rehydrated through a graded series of ethanol concentrations. Endogenous peroxidase activity was quenched by incubating the slides for 20 minutes in 0.3% hydrogen peroxide, followed by rinsing in PBS and antigen retrieval by steaming in antigen retrieval buffer at pH 7.5 (IHC world, Woodstock, MD). Slides were cooled to room temperature and rinsed in PBS again. To block nonspecific binding, slides were incubated with 10% normal donkey serum (Abcam) in PBS for 20 min at RT.

For Tissue-Tek embedded spheres, 2% PFA-fixed 8 µm sections were collected on Superfrost Plus glass slides (VWR), fixed in acetone for 10 min at RT, and washed in PBS. Nonspecific binding was blocked by incubation with 2% normal donkey serum (Abcam) in PBS for 20 min at RT.

Brain tissue and sphere sections were incubated overnight at 4 °C with primary antibodies in PBS containing 0.1% BSA (Sigma-Aldrich). Primary antibodies used were anti-Tenascin C (1:50, R and D Systems, Minneapolis, MN, Cat#MAB2138, RRID: AB_2203818), anti-GFAP (1:50, Sigma-Aldrich, Cat#SAB5201104, RRID:AB_2827276), anti-IBA1 (1:50, Wako Pure Chemical Industries, Osaka, Japan, Cat#019-19741, RRID:AB_839504) and anti-MBP (1:100, Novus Biologicals, Centennial, CO, Cat#MAB4269, RRID:AB_10552058). Next, sections were rinsed with PBS and incubated with either Alexa-488 or-594-labeled secondary antibodies (1:400, Jackson ImmunoResearch Laboratories, Cat#712-546-153, RRID:AB_2340686; Cat#711-545-152, RRID:AB_2313584; Cat#715-585-150, RRID:AB_2340854) for 90 min at RT. After extensive washes with PBS, slides were mounted using Prolonged Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were acquired using a Leica DMI6000 fluorescence microscope and LASX software.

Statistics

GraphPad Prism 8.0 (http://www.graphpad.com/, RRID:SCR_002798) (GraphPad Software, San Diego, CA) was used for statistical analysis. Sholl analysis curves from which area under the curve data were deduced were created and analyzed using Graphpad Prism 8.0. Statistical details of experiments can be found in the figure legends.

Results

Exposure of primary primate microglia to IL-34 and TGF-β has only moderate effects on their signature gene expression profile

Recent findings have demonstrated that exposure of primary rodent microglia to IL-34 and TGF-β supports cell cultures that better resemble *in vivo* microglia than exposure to M-CSF only does, which is the standard protocol. To assess whether this also applies to primary microglia from adult outbred primates, we analyzed cell morphologies and profiled the transcriptomes of primary microglia from four adult rhesus macaques that were exposed for eight days to either M-CSF or IL-34, with or without TGF-β.

Microglial morphologies were highly similar for all different culture conditions (data not shown). Transcriptome analysis suggested that sex, and not culture condition, was the most important component to explain variance (15%) in the dataset (**Figure 1A**). Unsupervised hierarchical clustering using Spearman's correlation, further showed that samples from individual donors clustered together rather than samples from similar sex or similar culture conditions (**Figure 1B**). This demonstrates that the origin of the donor had more impact on the gene expression profiles of individual samples than the culture conditions did. Although variance is normal when working with outbred animals, we had anticipated that *in vitro* exposure for eight days to a similar culture regime would have had more profound skewing effects.

We next performed in-depth analyses of the effects of the different culture variables on microglial gene expression. When comparing M-CSF and IL-34-exposed microglia, not a single significant differentially expressed gene (DEG; FC ≥2, FDR <0.05) was found (**Table 2**). This implicates that M-CSF and IL-34 induce or sustain an almost identical gene expression profile in adult macaque primary microglia, which is consistent with data from adult human primary microglia[42]. When we focused on the effects of TGF-β, we found that exposure to TGF-β was the second important component to explain variance (13%) in our data set (**Figure 1A**). DEG analyses reveal that the expression levels of 297 genes differed significantly between M-CSF and M-CSF + TGF-β-exposed microglia, and that the expression levels of 168 genes differed significantly between IL-34 and IL-34 + TGF-β-exposed microglia (**Table 2**, **Figure S1** and **Table S2**). Since TGF-β has been described as pivotal for the expression of microglia signature genes in rodents[18,20], we analyzed the log-transformed RNA expression data of six described human and rodent microglia signature genes *GPR34, P2RY12, P2RY13, TMEM119, CX3CR1* and *OLFML3*. Surprisingly, only *CX3CR1* was significantly upregulated after TGF-β exposure, both in combination with M-CSF and IL-34

Table 2. Numbers of differentially expressed genes (DEG; FC ≥2, FDR <0.05) between primary microglia exposed for eight days to either M-CSF or IL-34, with or without TGF-β.

Comparisons	Total DEG
M-CSF vs IL-34	0
M-CSF vs M-CSF + TGF-β	297
IL-34 vs IL-34 + TGF-β	168
IL-34 + TGF-β vs M-CSF + TGF-β	0

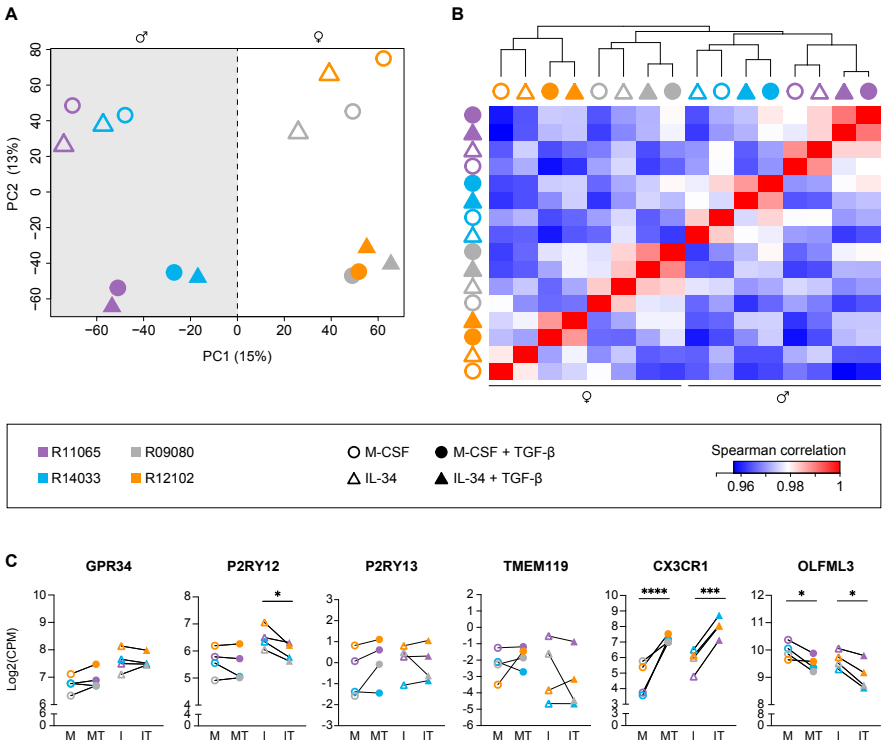


Figure 1. Effects of donor-donor variation and of M-CSF, IL-34, and TGF-β exposure on the transcriptomes of primary microglia. A) Principal component analysis of the transcriptomes of primary microglia from four adult donors cultured under four different conditions (M-CSF, M-CSF + TGF-β, IL-34 and IL-34 + TGF-β). The first principal component is responsible for 15% of the variance in the dataset, whereas the second principal component is responsible for 13% of the variance. The symbols in the gray area are derived from male donors, whereas the symbols in the white area are derived from female donors. **B)** Spearman's correlation heatmap of the transcriptome of cultured primary microglia. **C)** Log-transformed expression (CPM) values of microglial signature genes. M = M-CSF, MT = M-CSF + TGF-β, I = IL-34, IT = IL-34 + TGF-β. EdgeR false discovery rates (FDR) are used to display statistical differences. n=4 for each culture condition. * FDR <0.05, *** FDR <0.005, **** FDR <0.001.

(**Figure 1C**). *OLFML3*, a gene described to be positively regulated by TGF-β in mice[18,43], was even significantly downregulated after TGF-β exposure, and also *P2RY12* was downregulated when microglia were exposed to TGF-β in combination with IL-34. We used two pathway analysis applications (IPA and MsigDB) to verify that the TGF-β pathway had indeed been activated (**Table S3**).

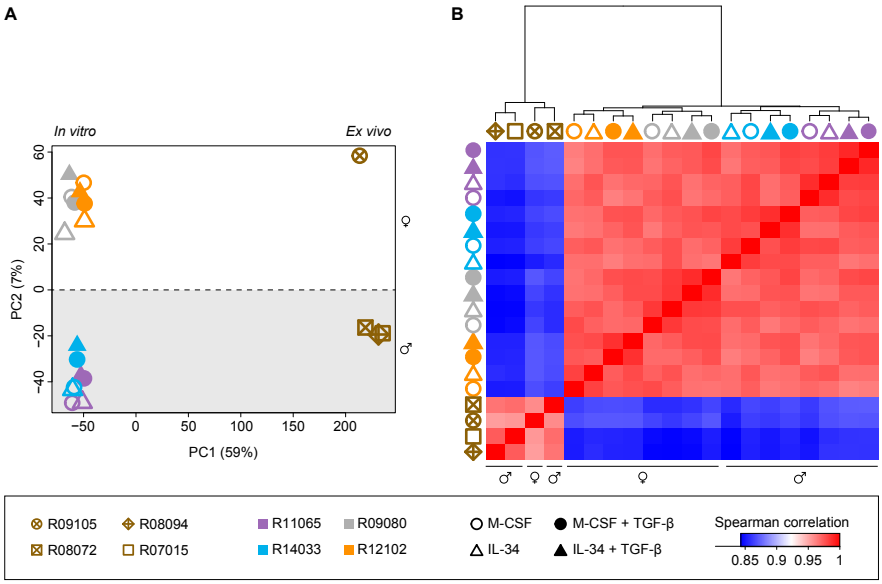
Regardless of culture conditions, *in vitro* microglia are characterized by a proliferative phenotype as compared to *in vivo* microglia

We next compared the transcriptomes of our *in vitro* samples with those of *ex vivo* microglia that were freshly isolated using FACS sorting. In line with published data, major differences in the gene expression profiles of *in vitro* and *ex vivo* microglia were observed, regardless of cell culture conditions (**Figure 2A** and **Table 3**). The most important component explaining 59% of the variance between the samples, was the *in vitro - ex vivo* parameter. The second important component explaining 7% of the variance, was as expected sex (**Figure 2A**). Hierarchical clustering of the *in vitro* and *ex vivo* samples demonstrates the profoundness of the *in vitro - ex vivo* difference (**Figure 2B**), whereas DEG analyses of *ex vivo* microglia and the different *in vitro* microglia cultures (**Figure S2** and **Table S2**) demonstrate that none of the culture regimes resulted in a transcriptome that better reflected the *ex vivo* transcriptome (**Table 3**), or resulted in a better mimic of their signature gene expression profile (**Figure S3**).

Table 3. Numbers of differentially expressed genes (DEG; FC ≥4, FDR <0.01) between different *in vitro* microglia cultures and *ex vivo* microglia.

Condition	# Genes up *in vitro*	# Genes down *in vitro*	Total DEG
M-CSF	1487	1771	3258
M-CSF + TGF-β	1410	1434	2844
IL-34	1499	1579	3077
IL-34 + TGF-β	1422	1685	3107

In order to gain more insight into the biological processes that were affected *in vitro*, we performed a gene set enrichment analysis for genes that were upregulated *in vitro*[34,35]. Genes upregulated *in vitro* were linked to gene ontology terms associated with the cell cycle, such as “mitotic cell cycle”, “cell cycle” and “cell cycle process”. In addition, evidence was found for biological processes associated with cell movement, adhesion and structure (**Figure 2C**). RNA expression data confirmed that six well-described genes associated with the cell cycle were indeed upregulated in *in vitro* microglia compared to *ex vivo* microglia (**Figure 2D**). These results are in line with recent data of primary adult rat microglia, that were reported to upregulate amongst others the cell cycle pathway when they were exposed to serum[19].



C

Gene Set Name	FDR q-value
Mitotic cell cycle	3.89E-59
Cell cycle	1.07E-57
Biological adhesion	3.37E-51
Cell cycle process	1.82E-48
Locomotion	5.11E-48
Extracellular structure organization	1.14E-47
Cell motility	6.20E-43
Cell division	2.31E-40
Regulation of cell population proliferation	1.29E-36
Cytoskeleton organization	7.39E-36

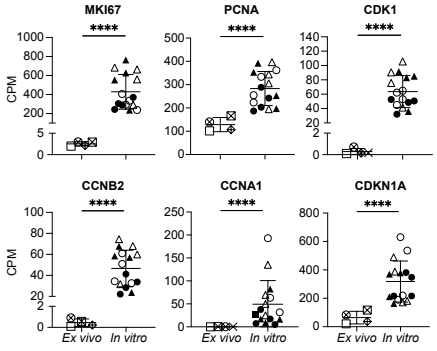
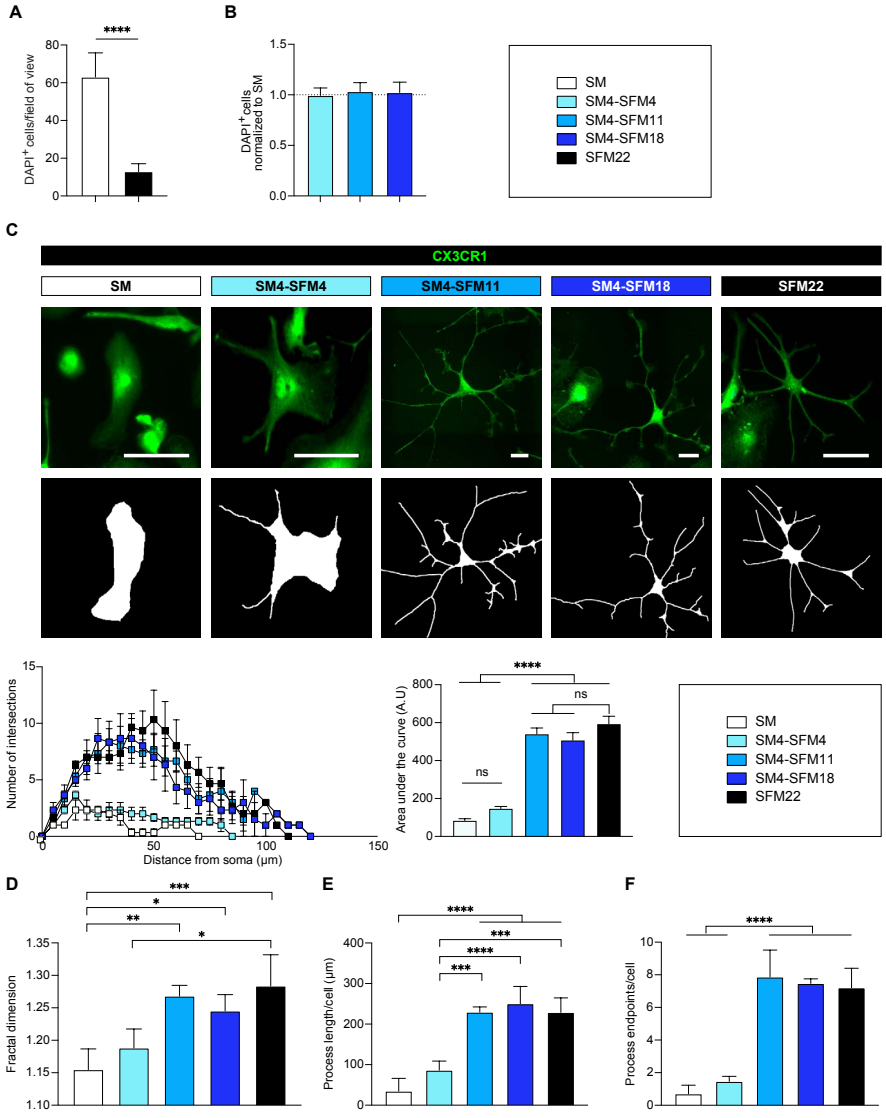


Figure 2. Transcriptome analysis of primary adult microglia and *ex vivo* microglia. A) Principal component analysis of the transcriptome of *ex vivo* and *in vitro* microglia cultured under four different culture conditions. *Ex vivo* and *in vitro* microglia were derived from different donors. The first principal component is responsible for 59% of the variance in the dataset, whereas the second principal component is responsible for 7% of the variance. The symbols in the gray area are derived from male donors, whereas the symbols in the white area are derived from female donors. **B)** Spearman's correlation heatmap of the transcriptomes of *in vitro* and *ex vivo* microglia. **C)** Gene ontology analysis of differentially expressed genes (DEG) upregulated in *in vitro* microglia as compared to *ex vivo* microglia. Biological processes associated with upregulated DEG were analyzed using the Molecular Signatures Database[34,35] FDR = false discovery rate. **D)** Expression values in counts per million (CPM) of well-known cell cycle genes of *in vitro* and *ex vivo* microglia. EdgeR false discovery rates (FDR) are used to display statistical differences. *In vitro* n=16, *ex vivo* n=4. Center lines indicate the mean, error bars represent SD, **** FDR <0.001.

Short-term serum exposure, followed by a serum-free washout period supports the outgrowth of high numbers of microglia with a complex, ramified morphology

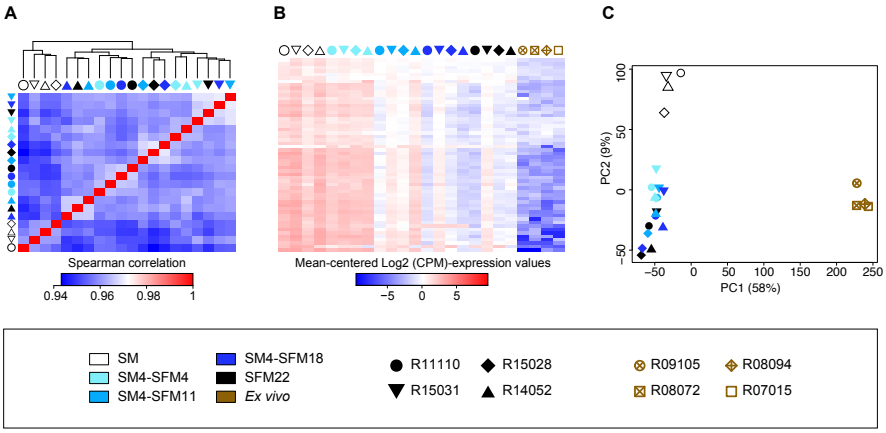
Bohlen and colleagues recently described a novel serum-free culture medium (SFM) containing M-CSF, TGF-β and cholesterol as the minimal supplements to allow survival of microglia in the absence of serum[19]. We tested this SFM for culture of primary adult rhesus macaque microglia. Of note, microglia cultured with SFM were still exposed to



serum during the isolation procedure and during the first 16 hours after plating of the cells to facilitate cell adhesion. Complete elimination of serum during isolation and plating did not yield viable microglia cultures. Despite the presence of TGF-β and cholesterol, total cell yields after 8 days of culture were around 4-fold lower than with our 10% fetal calf serum (FCS)-containing, standard microglia (SM) medium (**Figure 3A**). This decrease in cell number hampers downstream experimental analysis, and longer cell culture times did not improve cell yields (data not shown).

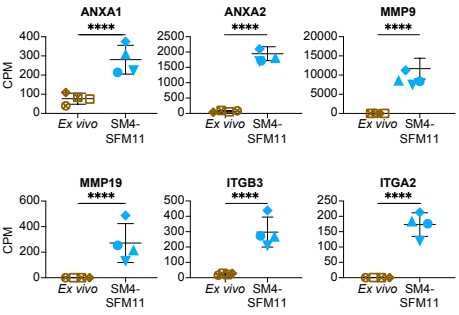
Consequently, we tested whether a short period of serum exposure, to facilitate initial survival and proliferation, followed by a period of serum-free washout could positively affect cell yields. We exposed microglia for 4 days to SM followed by exposure to SFM for 4, 11 or 18 days (**Figure S4**) and compared cell numbers to those of microglia exposed to SM for 8 days. We found that 4 days of serum exposure was sufficient to induce the outgrowth of cell numbers comparable to those obtained with SM, regardless of the duration of the serum-free washout period (**Figure 3B**). We also studied how different serum-free washout periods affected cellular morphology, using Sholl analysis. Serum-free washout periods of 11 and 18 days resulted in more complex cellular morphologies as compared to microglia cultured with SM and SM4-SFM4 (**Figure 3C**) or microglia cultured on SM for 22 days (data not shown). In addition, serum-free washout periods for 11 and 18 days supported outgrowth of microglia with a comparably complex morphology as microglia exposed to SFM for 22 days (SFM22). The increased morphological complexity of microglia exposed to serum-free washout periods of 11 and 18 days, as compared to microglia exposed to SM, was confirmed by fractal dimension analysis (**Figure 3D**) where a higher fractal dimension is associated with a more complex cellular morphology[41]. We also quantified the number of microglia process length and endpoints per cell. Serum-free washout periods of 11 and 18 days resulted in a higher total process length per cell (**Figure 3E**) and in higher numbers of

◀ **Figure 3. Effects of short-term serum exposure and serum-free washout regimes on microglia morphology.** **A)** Numbers of DAPI$^+$ primary microglia cultured in SM or SFM medium for 8 days. Cell counting was performed in 20 random fields of view from three donors for each condition. Statistical differences were examined by paired Student's t-test. Error bars represent SD, **** p <0.001. **B)** Numbers of DAPI$^+$ primary microglia after 4 days SM exposure followed by a serum-free washout of 4 (SM4-SFM4), 11 (SM4-SFM11) and 18 (SM4-SFM18) days. DAPI$^+$ cell numbers were normalized to DAPI$^+$ cell numbers of microglia cultured with serum for 8 days (SM). Dashed line represents the average DAPI$^+$ cells of SM cultured microglia. Statistical differences were examined by paired Student's t-test, n=5 per culture condition. Error bars represent SD. **C)** Representative pictures of microglia cultured under five different culture conditions (specified in **Figure S4**) and immunostained for the microglial marker CX3CR1. Scale bars are 50 µm. CX3CR1$^+$ cells were skeletonized and the number of intersections per 5 µm steps from the nucleus were analyzed using Sholl analysis. Number of intersections were analyzed for 10 random cells and averaged per donor and plotted. Error bars represent SD, n=3 for each condition. Sholl analysis-derived area under the curve was quantified (arbitrary units: A.U.) in which higher area under the curve (AUC) values reflect a higher morphological complexity. Statistical AUC differences were examined by one-way ANOVA with Tukey's multiple comparisons test. Error bars represent standard error of the mean. **** p <0.001. **D)** Fractal dimension, **E)** process length/cell and **F)** process endpoints/cell of microglia cultured under different conditions. Analyses were performed on 10 random cells for each condition and averaged per donor. Statistical differences were examined by one-way ANOVA with Tukey's multiple comparisons test. n=3, error bars represent SD, * p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.001.



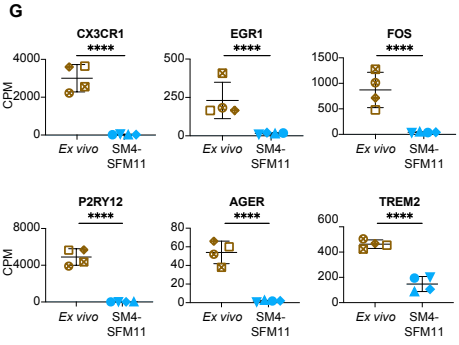
D

Gene Set Name	FDR q-value
Biological adhesion	7.6E-62
Locomotion	6.38E-58
Extracellular structure organization	1.05E-51
Cell motility	1.05E-51
Cellular component morphogenesis	5.61E-43
Cytoskeleton organization	5.36E-40
Anatomical structure formation involved in morphogenesis	7.59E-39
Regulation of cell population proliferation	3.69E-37
Neurogenesis	1.2E-35
Response to oxygen containing compound	1.42E-35



F

Gene Set Name	FDR q-value
Positive regulation of multicellular organismal process	1.05E-23
Cell cell signaling	1.38E-23
Regulation of cell differentiation	6.24E-22
Protein phosphorylation	1.22E-21
Locomotion	1.42E-19
Neurogenesis	2.36E-19
Positive regulation of signaling	3.52E-19
Inflammatory response	5.71E-19
Biological adhesion	5.71E-19
Regulation of immune system process	3.8E-18



process endpoints (**Figure 3F**) as compared to microglia exposed to SM conditions or to a serum-free washout period of 4 days. In fact, total process length per cell and the number of process endpoints were comparable to microglia that were exposed to SFM for 22 days.

◀ **Figure 4. Transcriptome analysis of serum-free cultured microglia and *ex vivo* microglia. A)** Spearman's correlation heatmap of the transcriptomes of SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22 cultured microglia. **B)** Heatmap of mean-centered Log2(CPM)-expression values of cell cycle genes of SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22 cultured microglia and *ex vivo* microglia. **C)** Principal component analysis of the transcriptomes of SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22 cultured microglia and *ex vivo* microglia. *Ex vivo* and *in vitro* microglia were derived from different donors. The first principal component explains 58% of the variance in the dataset, whereas the second principal component explains 9% of the variance. **D)** Biological processes associated with differentially expressed genes (DEG) upregulated in SM4-SFM11 microglia compared to *ex vivo* microglia. Processes were analyzed using the Molecular Signatures Database (MsigDB)[34,35]. FDR = false discovery rate. **E)** Expression values (CPM) of extracellular matrix-related genes (annexins, matrix metallopeptidases and integrins) of *ex vivo* and SM4-SFM11 microglia. EdgeR false discovery rates (FDR) are used to display statistical differences, n=4, center line indicates the mean, error bars represent SD, **** FDR <0.001. **F)** Biological processes associated with DEG downregulated in SM4-SFM11 microglia compared to *ex vivo* microglia. Processes were analyzed using the Molecular Signatures Database. FDR = false discovery rate. **G)** Expression values (CPM) of genes associated with microglia crosstalk of *ex vivo* and SM4-SFM11 microglia. EdgeR false discovery rates (FDR) are used to display statistical differences, n=4, center line indicates the mean, error bars represent SD, **** FDR <0.001.

Despite strongly reduced expression of cell cycling-associated gene products, the transcriptomes of serum-free microglia do not better mimic those of *ex vivo* microglia

Analysis of the transcriptomes by Spearman's correlation heatmap and DEG analyses demonstrated that the transcriptomes of SM4-SFM11 and SM4-SFM18 cultured microglia did not differ significantly from the transcriptomes of SFM22 microglia (**Figure 4A, Table 4** and **Figure S5**). Furthermore, the expression levels of cell cycle genes were similar to those of SFM22 microglia (**Figure 4B,** in high resolution **Figure S6**). In addition, the cell cycle gene expression profiles of SM4-SFM11 and SM4-SFM18 microglia better mimicked those of *ex vivo* microglia than those of SM or SM4-SFM4 microglia.

We next compared the whole transcriptomes of SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22 cultured microglia (**Figure S4**) to the reference transcriptomes of *ex vivo* microglia that we used previously. The most important component to explain variance (58%) in our dataset was the *ex vivo-in vitro* parameter, whereas exposure to serum was the second principal component explaining 9% of the variance (**Figure 4C**). Overall, the number of DEG (FC ≥4, FDR <0.01) between *ex vivo* samples and the different culture conditions was comparable (**Table 5**), regardless of whether cells had been exposed to serum or not. Heatmaps of the DEG between *ex vivo* microglia and the different culture conditions show that SM and SM4-SFM4 conditions cluster together, while SM4-SFM11, SM4-SFM18 and SFM22 rather cluster by donor (**Figure S7**). 80% of the DEG between the SM condition and *ex vivo* microglia overlapped with the DEG between the SFM4-SFM11 condition and *ex vivo* microglia (data not shown). The expression of microglia signatures genes was comparably different between all different culture conditions and *ex vivo* microglia (**Figure S8**). Although the use of SFM thus improved microglial cell morphology and the expression of cell cycling-associated gene products, their RNA expression profiles did not better mimic those of *ex vivo* microglia.

Table 4. Numbers of differentially expressed genes (DEG; FC ≥2, FDR <0.05) between the *in vitro* conditions SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22.

	SM	SM4-SFM4	SM4-SFM11	SM4-SFM18	SFM22
SM	-	1500	1715	2028	2334
SM4-SFM4	1500	-	354	903	871
SM4-SFM11	1715	354	-	3	26
SM4-SFM18	2028	903	3	-	0
SFM22	2334	871	26	0	-

Table 5. Numbers of differentially expressed genes (DEG; FC ≥4, FDR <0.01) between different *in vitro* microglia cultures and *ex vivo* microglia. See Figure S4 for an overview of the culture conditions.

Condition	# Genes up *in vitro*	# Genes down *in vitro*	Total DEG
SM	1589	1610	3199
SM4-SFM4	1710	1522	3232
SM4-SFM11	1619	1438	3057
SM4-SFM18	1601	1477	3078
SFM22	1721	1492	3213

A multicellular environment is important for maintenance of the *in vivo* microglia transcriptome

As the SM4-SFM11 protocol yielded high cell numbers with a ramified morphology and with a transcriptome indicative of reduced proliferative activity, we continued with this protocol as our reference. We performed new gene set enrichment analyses to gain further insights into the biological processes involved in the differences between *in vitro* and *ex vivo* microglia. We first analyzed the genes that were upregulated *in vitro* (**Figure 4D**) and observed that biological processes linked to cell movement, adhesion, morphology and structure organization were most affected. For example, annexins, integrins and matrix metalloproteinases (MMPs) related genes were all upregulated (**Figure 4E**). The upregulation of genes linked to these biological processes was not specific for microglia cultured under serum-free conditions, as it was also observed for microglia that had been exposed to serum (**Figure 2C**). Genes that were downregulated *in vitro* as compared to *ex vivo* were amongst others associated with the positive regulation of multicellular organismal processes, cell-cell signaling, regulation of cell differentiation and neurogenesis (**Figure 4F**). The lack of signals from other CNS cells might have contributed to this effect as microglial genes involved in crosstalk with other brain cells, such as *CX3CR1*[44], *EGR1*[45], *FOS*[46], *P2RY12*[47], *AGER*[48] and *TREM2*[49] are

prominently present on this list of downregulated genes *in vitro* (**Figure 4G**). These findings were again not related to exposure to serum, as genes associated with these biological processes were also downregulated in microglia that had been exposed to serum (**Table S4**).

Oligodendrocyte- and radial glia-derived cues induce the expression of microglia signature genes

Our initial cell suspension contains microglia as well as varying numbers of oligodendrocytes and CNS precursor cells, and we selectively favor the outgrowth of a >98% pure primary microglia population by washing away non-adherent cells at day 1[29]. We hypothesized that by plating our initial cell suspension in ultra-low attachment plates under continuous shaking, we might allow for survival and outgrowth of other cells. Indeed, this resulted in the formation of spheres (**Figure S9**). To determine their cellular composition, we isolated RNA and analyzed the expression levels of CNS cell type-specific genes. These were then compared to their expression levels in SM4-SFM11 microglia derived from the same four adult rhesus macaques. We observed that microglial genes such as *TYROBP, GPR84* and *PTPRC* were highly and comparably expressed in both conditions. On the other hand, oligodendrocytic, astrocytic and radial glial genes were expressed in spheres but virtually absent in monocultures of microglia (**Figure 5A**), demonstrating that sphere formation indeed allowed for the survival of multiple brain cell types. To estimate the abundance of CNS cell types in the spheres, we used CIBERSORT, a computational tool to quantify relative levels of distinct cell types within a complex gene expression admixture[36]. The RNA expression profiles of microglia, neurons, oligodendrocytes, astrocytes and radial glia were used to create a signature gene expression matrix[13,37]. Use of this matrix in a CIBERSORT analysis leads to the estimation that the spheres consist for ±50% of microglia, ±30% of oligodendrocytes and ±10% of radial glia (**Figure 5B**), and that astrocytes and neurons are not present. To validate the CIBERSORT analysis, we visualized the expression of IBA1 (as a marker for microglia), MBP (as a marker for oligodendrocytes) and GFAP (as a marker for radial glia) (**Figure 5C**). Stainings were validated in the rhesus macaque source tissue (**Figure S10A**). Quantification confirmed that with ±70% microglia were the most abundant cell type in spheres followed by approximately 30% oligodendrocytes, and ±10% radial glia (**Figure 5D**). As GFAP is expressed both by astrocytes and by radial glia, we also analyzed the expression of Tenascin C (TNC), which is more selectively expressed by radial glia and by astrocytes precursor cells[37,50]. Colocalization of GFAP and TNC in spheres (**Figure S10B**) are consistent with the idea that the GFAP-positive cells are radial glia.

Importantly, we observed that microglial signature genes *C1QC, CX3CR1, GPR34, HEXB, P2RY12, P2RY13* and *TREM2* were significantly upregulated in spheres as compared to microglia monocultures (**Figure 5E**). It is noteworthy that bulk RNA-sequencing was used, meaning that the microglia in the spheres are likely to express even higher levels of these microglial-specific genes. Intriguingly, the expression levels of microglial genes *P2RY12*, *P2RY13* and *CX3CR1* are thought to be regulated by

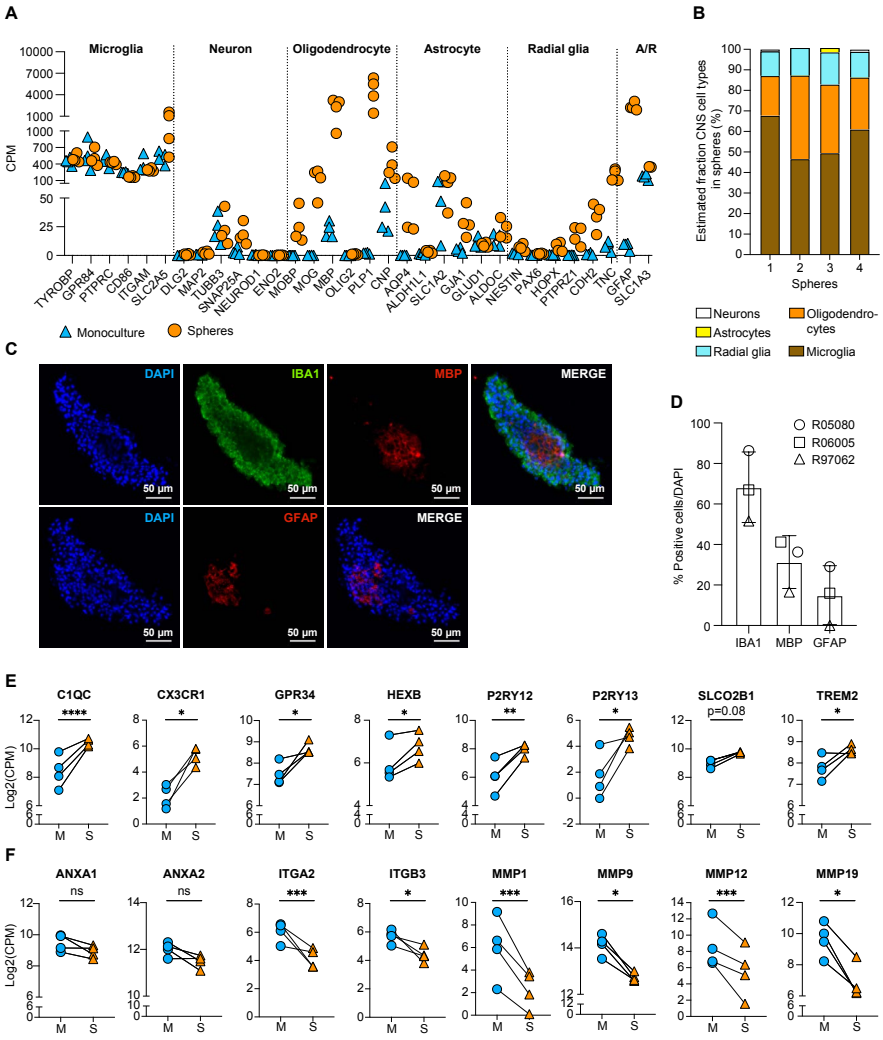


Figure 5. Gene and protein expression characterization of spheres. A) Expression values (CPM) of microglia, neuron, oligodendrocyte, astrocyte and radial glia genes of SM4-SFM11 monocultured microglia and spheres (n=4). A/R genes are genes both expressed by astrocytes and radial glia. **B)** CIBERSORT[36] quantification analysis of the estimated numbers of neurons, astrocytes, radial glia, oligodendrocytes and microglia in spheres, n=4. **C)** One representative example of a sphere (R06005) immunostained with anti-IBA1 (as a microglia marker), with anti-MBP (as an oligodendrocyte marker), and with anti-GFAP (as a radial glia marker). Cell nuclei (blue) were visualized using 4′,6-diamidino-2-phenylindole (DAPI). Scale bars represent 50 µm. **D)** Estimated percentages of the different cell types in spheres plotted in a graph. Each symbol represents a donor. n=3, error bars represent SD. **E)** Log-transformed expression (CPM) values of microglia signature genes of microglia monocultures and spheres. EdgeR false discovery rates (FDR) are used to display statistical differences, n=4, * FDR <0.05, ** FDR <0.01, **** FDR <0.001. M = Monoculture, S = Spheres. **F)** Log-transformed expression (CPM) values of extracellular matrix-related genes of microglia monocultures and spheres. EdgeR false discovery rates (FDR) are used to display statistical differences, n=4, * FDR <0.05, *** FDR <0.01. M = Monoculture, S = Spheres.

neuron-microglia crosstalk, whereas we have no indications that neurons were present in our spheres. In addition, we analyzed the expression of neuronal progenitor genes. Although we found a significant upregulation of *SOX2* and *NES* in spheres (**Figure S11**), these two genes are also expressed by radial glia. As other neuronal progenitor genes, including *PAX6, OCT4, DCX, ASCL1,* and *MSI1* were not significantly upregulated in spheres, we found no evidence for the presence of neuronal progenitors in our spheres. Finally, we observed that the expression levels of *ITGA2, ITGB3, MMP1, MMP9, MMP12* and *MMP19* were significantly downregulated in spheres as compared to microglia monocultures (**Figure 5F**).

The gene expression profile of microglia in spheres better resembles that of *ex vivo* microglia

To gain a broader insight in the gene expression profile of microglia in spheres, we performed further transcriptome analyses (**Figure 6A**). As a first approach, we analyzed the expression of the top 500 most abundantly expressed genes in *ex vivo* microglia (listed in **Table S5**) and compared their expression levels to those in monocultured microglia and to those in spheres (**Figure S12**). Interestingly, Spearman's correlation analysis of these 500 genes showed a higher correlation between spheres and *ex vivo* microglia than between monocultured microglia and *ex vivo* microglia (**Figure 6B** and **6C**). As a second approach, we analyzed the expression of the 3057 genes that were differentially expressed (DEG; FC≥4, FDR<0.01) between monocultured microglia (SM4-SFM11) and *ex vivo* microglia (**Table 5**). Again, Spearman's correlation analysis revealed an improvement of the expression of these 3057 genes in spheres for all four *in vitro* donors (**Figure 6D** and **6E**). DEG analysis showed that of these 3057 genes, 159 genes were no longer differentially expressed (FC≤4, FDR>0.01) between spheres and *ex vivo* microglia. Gene set enrichment analysis showed that the majority of biological processes associated with these 159 genes are linked to neuronal processes, such as neuron differentiation, neuron development and neurogenesis (**Table S6**). Together these data strongly suggest that microglia in spheres better mimic the gene expression profile of *ex vivo* microglia than monocultured microglia do.

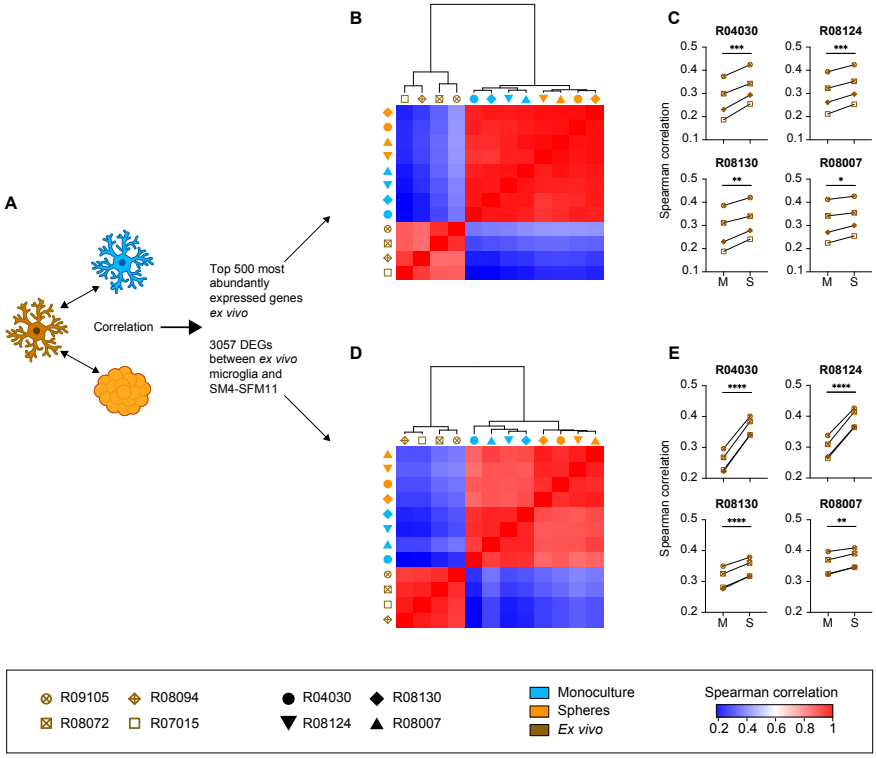


Figure 6. In-depth transcriptome analyses of microglia gene expression in spheres. A) Overview of the transcriptome analytical approaches to gain further insight in the gene expression profile of microglia in spheres. **B)** Spearman's correlation heatmap of the top 500 most abundantly expressed genes in *ex vivo* microglia. **C)** Spearman's correlation values of Figure 6B between *ex vivo* microglia and monocultured microglia, and *ex vivo* microglia and spheres. n=4, graph for each *in vitro* donor is displayed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. M = Monoculture, S = Spheres. **D)** Spearman's correlation heatmap of the 3057 genes that were differentially expressed between SM4-SFM11 microglia and *ex vivo* microglia. **E)** Spearman's correlation values of Figure 6D between *ex vivo* microglia and monocultured microglia, and *ex vivo* microglia and spheres. n=4, graph for each *in vitro* donor is displayed. ** $p < 0.01$, **** $p < 0.001$. M = Monoculture, S = Spheres.

Discussion

The increasing recognition of microglia as druggable cellular targets for a variety of neurodegenerative disorders, has spurred research into the determinants of microglial identity and into the development of *in vitro* methodology[18-20]. In this study, we have exposed primary microglia from adult rhesus macaques to a variety of different cell culture regimes to shed light on the relative contribution of different cell culture methods and conditions in shaping microglial identity, and to further cell culture innovations.

The importance of the CSF-1 receptor for microglial proliferation, survival and homeostasis has been firmly established by different studies[27,51-53]. The CSF-1 receptor has two reported ligands, M-CSF and IL-34, that lack similarity in terms of protein sequence and that are expressed in a largely non-overlapping manner in the brain[54-56]. The idea that unique requirements exist for either M-CSF or IL-34 in the development, colonization and homeostasis of microglia, stems from observations made in depletion studies in rodents[55,57,58] and zebrafish[59,60]. However, our results show that cell numbers and cellular morphologies (data not shown), as well as the transcriptomes, of primary microglia from adult primates exposed to either M-CSF or IL-34 were indistinguishable. These results are in line with those of a recent study in which the transcriptomes of primary microglia from adult humans exposed to either M-CSF or IL-34, were also reported to be almost identical[42]. Taken together, these studies suggest similar roles for M-CSF and IL-34 in postnatal microglia homeostasis. It remains to be established though whether exposure of microglia to M-CSF or IL-34 can have an impact on the polarization towards a pro- or anti-inflammatory phenotype[61], or on the susceptibility for infection with HIV[62], as has been described for human primary monocytes. If such effects were to be found in microglia as well, the heterogeneous expression of M-CSF and IL-34 in the brain[56-58,63] could lead to regional differences in microglia biology.

We also studied the effects of exposure to TGF-β, which is, both *in vitro* and *in vivo,* an important factor for microglia homeostasis and survival[18,19,64-66]. Whereas *in vitro* exposure of microglia to TGF-β induces the expression of a transcriptome that better resembles that of mouse *ex vivo* microglia and, in addition, the expression of microglia signature genes[18], these effects were not reproduced in our system. This was not attributable to a lack of engagement of TGF-β-induced signaling. Analysis of the expression of microglia signature genes demonstrates that only the expression of *CX3CR1* was upregulated upon exposure to TGF-β, confirming the positive regulation of CX3CR1 by TGF-β[67,68]. The expression levels of *GPR34, P2RY13* and *TMEM119* were unaffected, and expression levels of *P2RY12* and *OLFML3* were even downregulated after exposure to TGF-β. These data are in line with a study reporting on the modest effects of TGF-β exposure on primary cultured microglia from adolescent humans as compared to the effects on primary cultured microglia of 7-10 weeks old mice[20]. Similar to our results, exposure to TGF-β did not result in a better match to the *ex vivo* transcriptome of human microglia. These important differences might be attributable to species-specific effects of TGF-β, as is also supported by the reported differences in

TGF-β-mediated inhibition of IFN-γ-induced MHC class I expression in human and murine microglia[69]. It is at present unclear where these differences originate, as the TGF-β superfamily is well conserved between rodents and humans[70,71].

We further observed that sex as a variable better explained variance in gene expression profiles than different culture conditions did. This is relevant given the sex-specific differences in the incidence, prevalence and pathogenesis of neurological diseases such as AD and MS[72-77]. Interestingly, sex-specific features in microglial function in health and disease have been identified in rodents[78-81]. In line with earlier reported sex-specific DEGs in *ex vivo* human transcriptome data[20], analysis of the sex-specific DEGs in our *in vitro* transcriptome datasets shows that these were all localized to either the X- or the Y-chromosome (data not shown). In addition to sex, we observed considerable donor-donor variation in microglia transcriptomes. Although this is normal when working with material from an outbred population, and in line with reported variation in the transcriptomes of *ex vivo* human microglia[20,82-84], we had not expected to find such effects after prolonged *in vitro* culture periods under different regimes. This would suggest that donor-specific gene expression profiles in microglia remain relatively stable, which might hinder analyses of e.g. culture specific effects. It is at present not clear whether these sex and donor-specific differences can be related to differences in microglia biology or function.

Gene set enrichment analysis of our data uncovered that differences between the *in vitro* and *ex vivo* transcriptomes of microglia were for a considerable part attributable to gene transcripts associated with cell cycling, which could have been caused by *in vitro* exposure to serum. We therefore tested a recently described serum-free medium for rat microglia[19] on our primary rhesus macaque microglia in which it induced a complex, ramified, cell morphology accompanied by the reduced expression of genes associated with proliferation. As the lack of proliferation negatively impacted the number of cells available for further *in vitro* experiments, we optimized a cell culture regime that combines a short-term, 4 days serum exposure with a serum-free washout period of at least 11 days. Although microglia are not exposed to serum in the healthy CNS, our results demonstrate that microglial responses to serum exposure are relative short-lived and appear to be for most part reversible. A minimum serum-free washout period of 11 days is sufficient to yield microglia with a highly complex, ramified morphology and with reduced expression levels of cell cycle-associated genes. In spite of these advances, the transcriptomes of microglia subjected to this new *in vitro* protocol still differed significantly from those of *ex vivo* microglia. Gene ontology analyses of the DEGs suggested that further improvements were amongst others to be found in exposure to the CNS microenvironment, in line with other studies[19,85].

We therefore facilitated the outgrowth of other brain-derived cells by plating our initial cell suspension in ultra-low attachment plates, which resulted in the formation of spheres. In such spheres, neuronal gene products could not be detected, whereas microglia, oligodendrocyte, astrocyte and radial glia-specific gene products were easily detectable. Given the Percoll gradient-based isolation procedure we used, we think it is unlikely that astrocytes are present in our initial cell suspension. We favor the idea

that the detection of astrocyte-specific gene products as *AQP4, GJA1* and *GFAP* stems from neuronal or glial precursor cells, such as radial glia, which was also in line with the CIBERSORT analysis we performed and was further supported by immunofluorescence microscopical analysis.

In-depth transcriptome analyses demonstrate that the gene expression profile of microglia in spheres better resembles that of *ex vivo* microglia than those of monocultured microglia do. However, further studies, such as single cell RNA-sequencing, are required to confirm this. Most interestingly, the expression levels of many microglia signature genes were significantly enhanced in spheres, even of those genes thought to be regulated by neuron-microglia and astrocyte-microglia crosstalk. Whether this is the result of cell-cell contact, or of cell-derived soluble factors, warrants further investigation. At present, very little is known on the role of oligodendrocyte-derived and radial glia-derived soluble factors on microglia[86], and this co-culture system can help to disentangle the intercellular communication that establishes and maintains microglial identity. As it has already been convincingly demonstrated that neuron-microglia and astrocyte-microglia communication positively affect microglia signature gene expression levels[45,48,87,88], the addition of neurons and/or astrocytes to our spheres might even further increase the expression of microglial signature genes. Such a complex CNS culture model bears resemblance to the recently described cerebral organoids that were generated from induced pluripotent stem cells and that surprisingly also contained microglia[89].

Taken together, our results provide new biological insights in cues that are important for adult primate microglial identity and for the development and optimization of novel *in vitro* methodology.

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Supplemental information

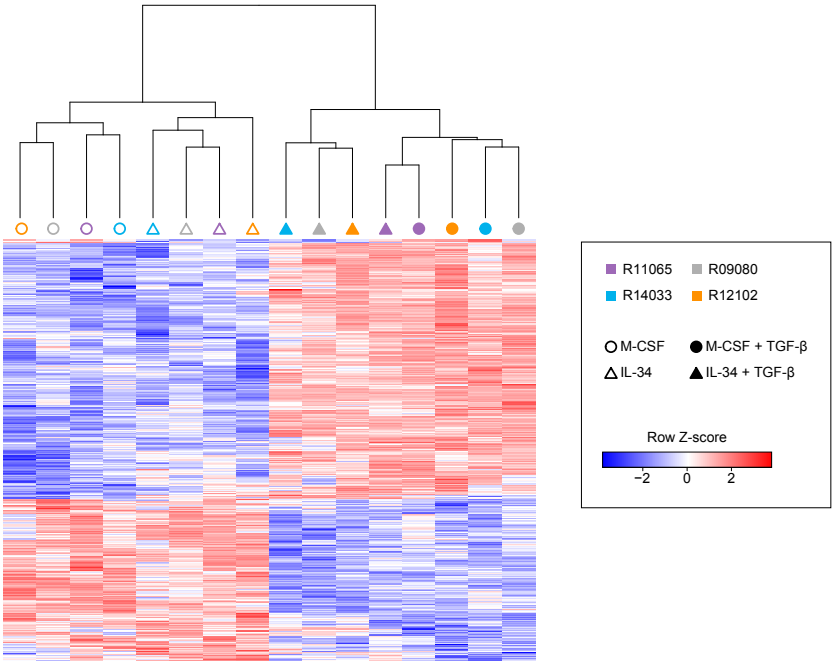


Figure S1. Heatmap of DEGs of microglia exposed to either M-CSF, M-CSF + TGF-β, IL-34 or IL-34 + TGF-β. DEGs (FC ≥2, FDR <0.05) between all culture condition were analyzed and z-scores from log-transformed CPM expression values of each gene were calculated and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different donors whereas the symbols represent the different culture conditions.

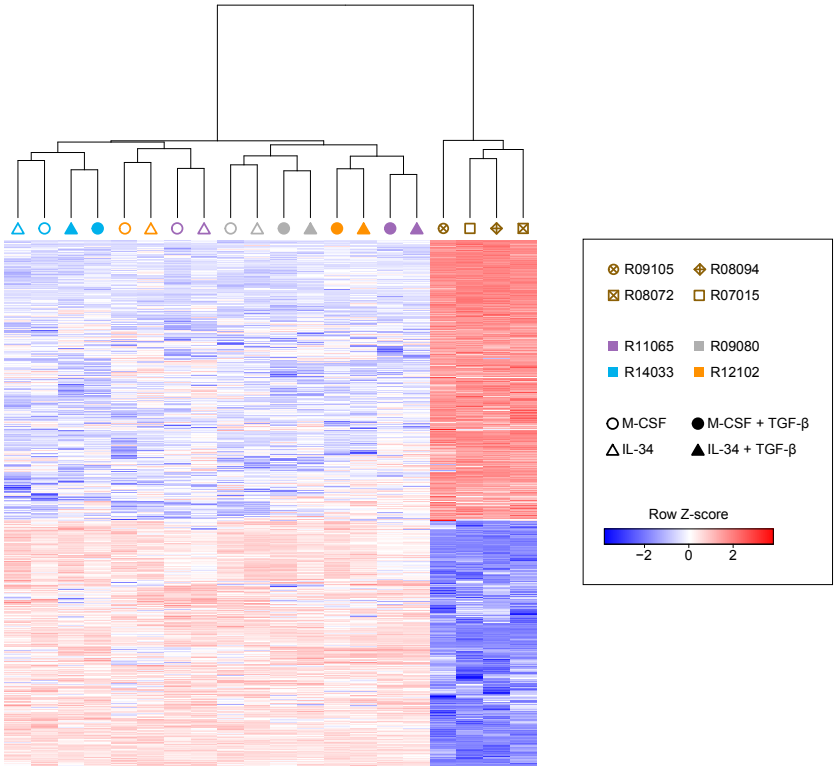


Figure S2. Heatmap of DEGs of *ex vivo* microglia and microglia exposed to either M-CSF, M-CSF + TGF-β, IL-34 or IL-34 + TGF-β. Differentially expressed genes (DEGs; FC ≥4, FDR <0.01) between *ex vivo* microglia and all different culture condition were analyzed and z-scores from log-transformed CPM expression values of each gene were calculated and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different *in vitro* donors whereas the symbols represent the different culture conditions or different *ex vivo* donors.

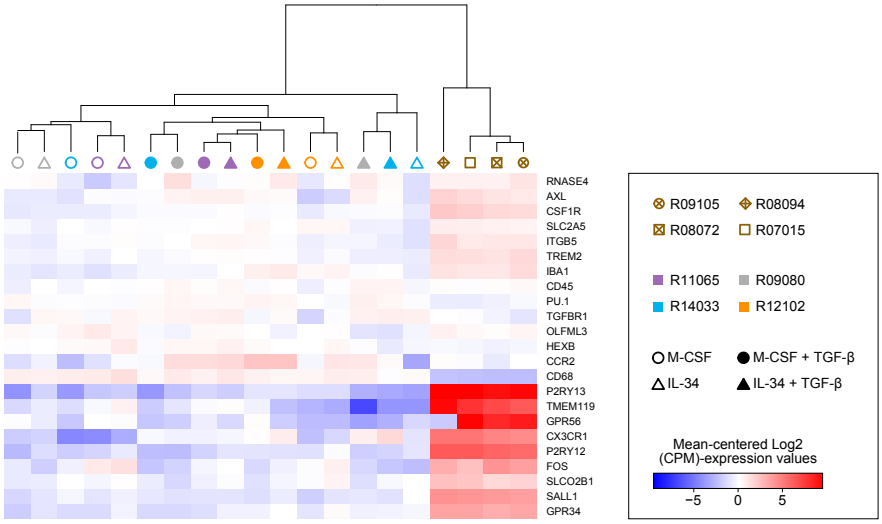


Figure S3. Expression of microglia signature genes. Heatmap of microglia signature gene expression of primary microglia cultured under 4 different conditions and *ex vivo* microglia. Log-transformed CPM values of each gene were mean-centered and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different *in vitro* donors whereas the symbols represent the different culture conditions or different *ex vivo* donors.

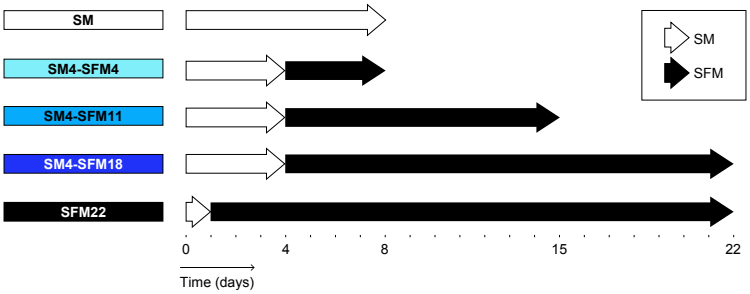


Figure S4. Schematic overview of primary microglia cultures exposed to SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22.

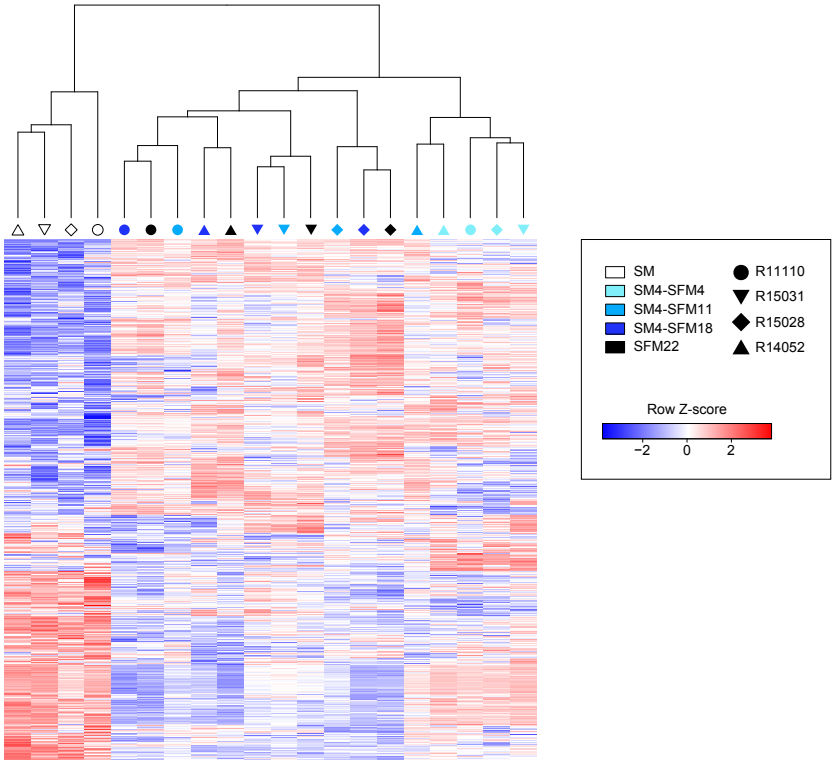


Figure S5. Heatmap of DEGs of microglia exposed to either SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 and SFM22. DEGs (FC ≥2, FDR <0.05) between all different culture condition were analyzed and z-scores from log-transformed CPM values of all genes were calculated and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different conditions, whereas the symbols represent the different donors.

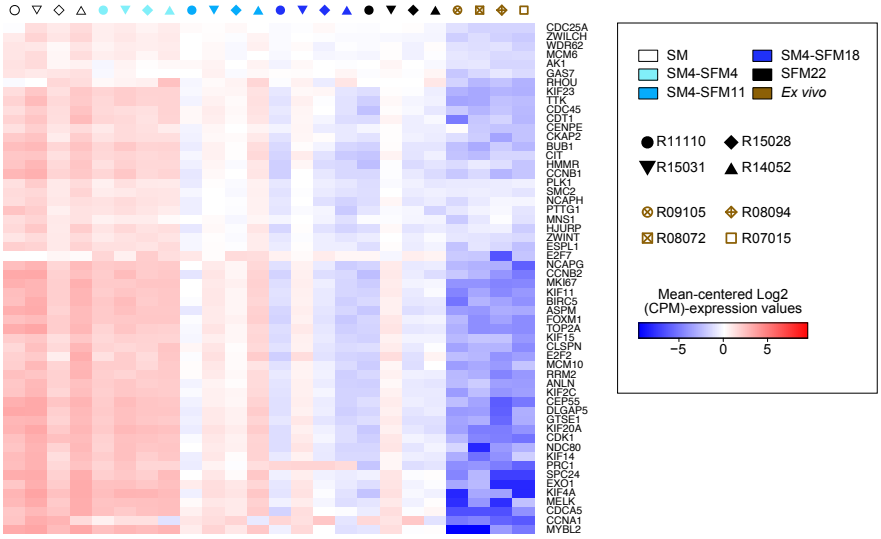


Figure S6. High resolution image of Figure 4B including the gene names for each row. Heatmap of mean-centered Log2(CPM)-expression values of cell cycle genes of microglia cultured under five different culture conditions and *ex vivo* microglia. Each row represents a gene and each column represents a condition. Colors represent the different conditions, whereas the symbols represent the different donors.

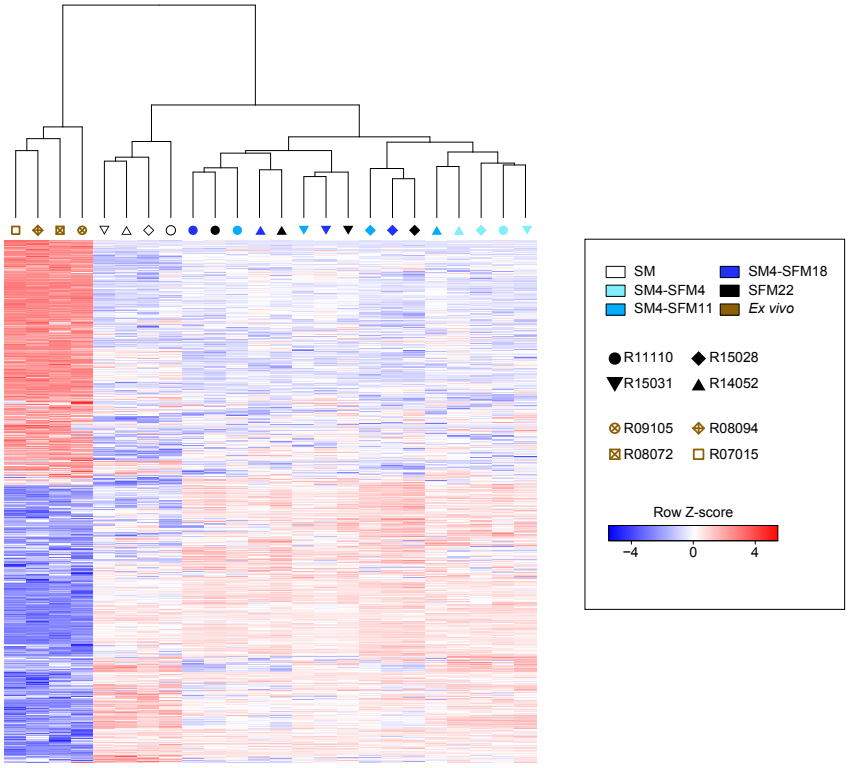


Figure S7. Heatmap of DEGs of *ex vivo* microglia and microglia exposed to either SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 or SFM22. DEGs (FC ≥4, FDR <0.01) between *ex vivo* microglia and all different culture condition were analyzed and z-scores from log-transformed CPM values of all genes were calculated and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different conditions whereas the symbols represent the different donors.

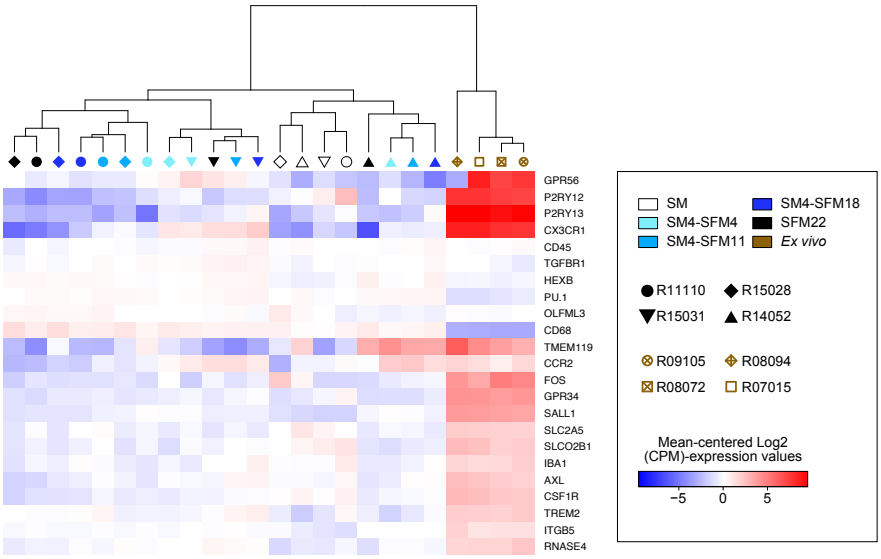


Figure S8. Effect of SFM culture on microglia signature genes. Heatmap of microglia signature gene expression of primary microglia exposed to SM, SM4-SFM4, SM4-SFM11, SM4-SFM18 or SFM22, and *ex vivo* microglia. Log-transformed CPM values of each gene were mean-centered and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different conditions whereas the symbols represent the different donors.

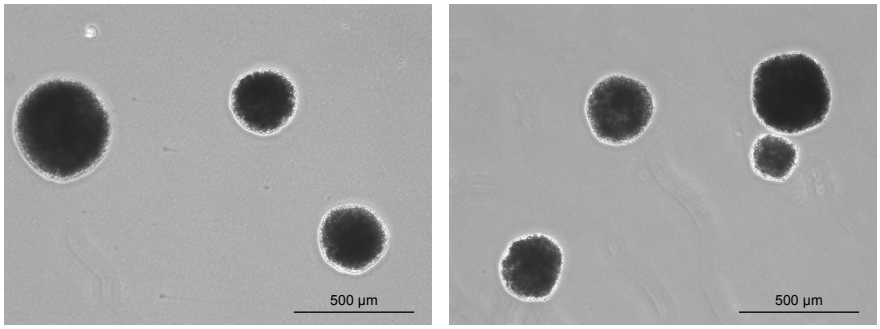


Figure S9. Brightfield pictures of spheres. After the isolation procedure, the cell suspension was plated in ultra-low attachment plates and cultured in SM4-SFM11 medium under continuous shaking which resulted in the formation of spheres. Pictures were taken at day 15. Scale bars = 500 µm.

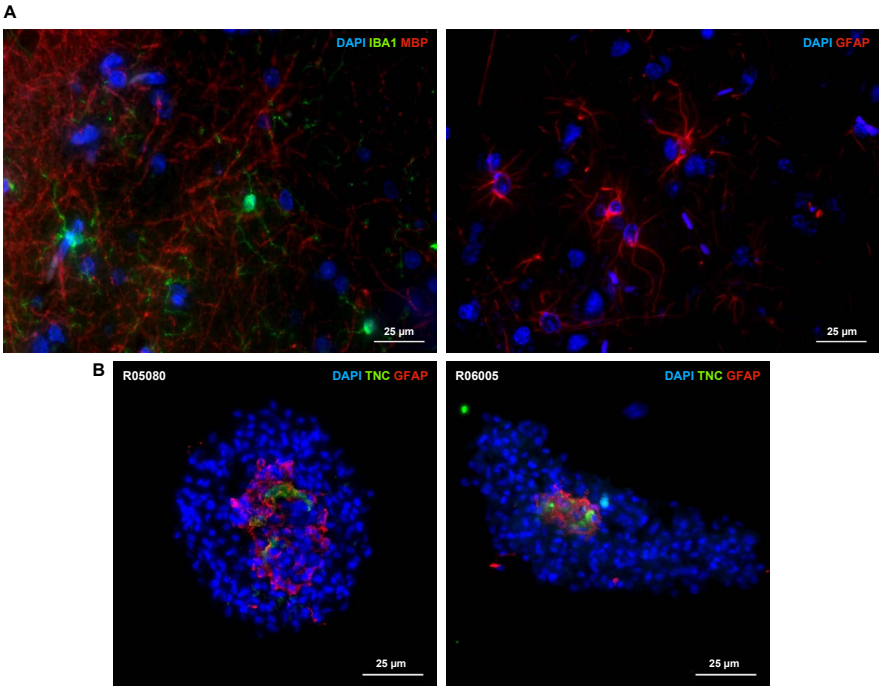


Figure S10. Immunofluorescence staining of rhesus macaque brain tissue and spheres. A) PFA fixed brain tissue of adult rhesus macaques were immunostained for microglia marker IBA1, oligodendrocyte marker MBP and radial glia marker GFAP. Cell nuclei (blue) were visualized using 4′,6-diamidino-2-phenylindole (DAPI). Scale bars = 25 µm. **B)** Expression of GFAP and TNC in spheres. Cell nuclei (blue) were visualized using DAPI. Scale bars = 25 µm.

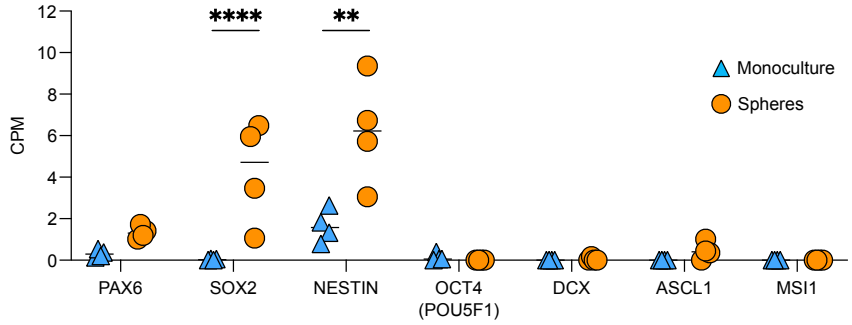


Figure S11. Neuronal progenitor gene expression in monocultured microglia and spheres. Expression values (CPM) of neuronal progenitor genes of SM4-SFM11 monoculture microglia and spheres. EdgeR false discovery rates (FDR) are used to display statistical differences. n=4 for each condition. ** FDR <0.01, **** FDR <0.001.

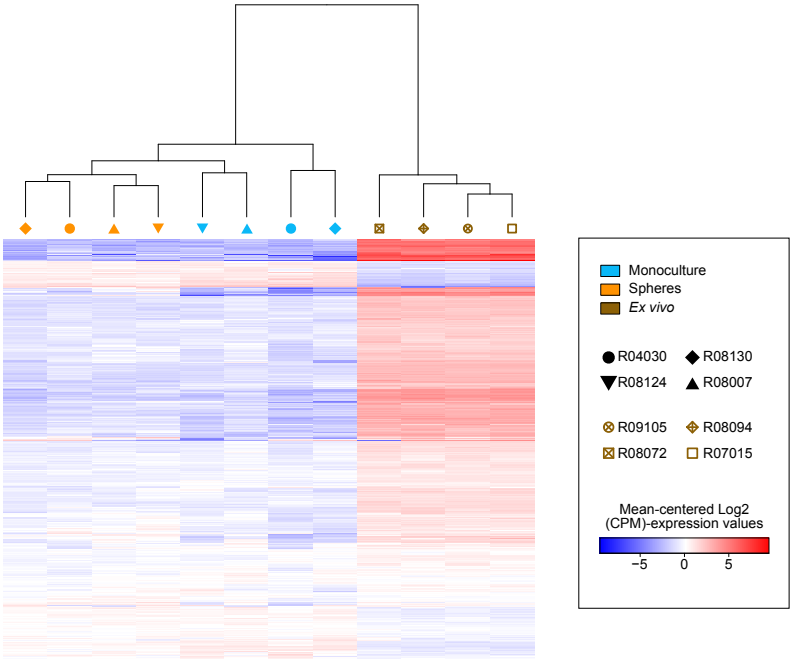


Figure S12. Heatmap of the gene expression of the top 500 most abundantly expressed *ex vivo* microglia genes in monocultured microglia, spheres and *ex vivo* microglia. Log-transformed CPM values of each gene were mean-centered centered and plotted in the heatmap. Each row represents a gene, and each column represents a condition. Colors represent the different condition whereas the symbols represent the different donors.

Table S1. Composition of cell culture media.

SM	Supplier	SFM	Supplier
50% DMEM (high glucose)	Gibco	DMEM/F12 (1:1)	Gibco
50% HAM F10 nutrient mixture	Gibco	2 mM glutamax	Gibco
10% FBS	TICO Europe	50 units/mL penicillin	Gibco
2 mM glutamax	Gibco	50 µg/mL streptomycin	Gibco
50 units/mL penicillin	Gibco	5 µg/mL N-acetyl cysteine	Sigma-Aldrich
50 µg/mL streptomycin	Gibco	100 ng/mL sodium selenite	Sigma-Aldrich
20 ng/mL M-CSF	PeproTech	5 µg/mL insulin	Sigma-Aldrich
		100 µg/mL apo-transferrin	Sigma-Aldrich
		20 ng/mL M-CSF	PeproTech
		12,5 ng/mL TGF-β	Miltenyi Biotec
		1,5 µg/mL ovine wool cholesterol	Avanti Polar Lipids
		1 µg/mL heparan sulfate	Galen Laboratory Supplies
		0,1 µg/mL oleic acid	Cayman Chemical
		0,001 µg/mL gondoic acid	Cayman Chemical

For **Table S2. Excel file with an overview of all performed differential gene expression analyses;**

see https://onlinelibrary.wiley.com/doi/10.1002/glia.24136
or scan the QR-code.

Table S3. Pathway analysis of differentially expressed genes between M-CSF vs. M-CSF + TGF-β exposed microglia. A) Top 5 canonical pathways that are associated with genes differentially expressed between M-CSF vs. M-CSF + TGF-β exposed microglia. The Molecular Signatures Database (MsigDB) was used for this analysis[34, 35]. FDR = False discovery rate. **B)** Top 5 upregulated canonical pathways that are associated with genes differentially expressed between M-CSF vs. M-CSF + TGF-β exposed microglia. Data were analyzed using IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

A

Top canonical pathways	**FDR q-value**
Naba Matrisome	2,26E-07
KEGG TGF-β signaling pathway	3,65E-07
PID BMP pathway	7,97E-06
Reactome signaling by interleukins	7,97E-06
Reactome signaling by TGF-β family members	1,48E-05

B

Top upregulated canonical pathways	**p-value**
TGF-β signaling	5,50E-08
PD-1, PD-L1 cancer immunotherapy pathway	1,48E-06
T Cell exhaustion signaling pathway	1,38E-05
Osteoarthritis pathway	6,80E-05
BMP signaling pathway	2,24E-04

Table S4. Gene set enrichment analysis shows the biological processes associated with genes downregulated (FC ≥4, FDR <0.01) in SM cultured microglia compared to *ex vivo* microglia. FDR = False discovery rate.

Gene Set Name	FDR q-value
Neurogenesis	3.06E-36
Neuron differentiation	2.10E-30
Protein phosphorylation	2.10E-30
Regulation of cell differentiation	4.30E-30
Cell-cell signaling	4.30E-30
Positive regulation of multicellular organismal process	3.27E-28
Regulation of transport	1.95E-27
Regulation of intracellular signal transduction	2.46E-26
Positive regulation of molecular function	1.41E-25
Positive regulation of signaling	2.34E-24

For **Table S5. Excel file with the names and the expression of the top 500 abundant expressed genes in *ex vivo* microglia. The expression in monocultured microglia, spheres and *ex vivo* microglia is shown;**

see https://onlinelibrary.wiley.com/doi/10.1002/glia.24136
or scan the QR-code.

Table S6. Biological processes associated with the 159 genes not differentially expressed (FC≤4, FDR >0.01) in spheres compared to *ex vivo* microglia, but that are differentially expressed (FC ≥4, FDR <0.01) between monocultured microglia and *ex vivo* microglia. FDR = False discovery rate.

Gene Set Name	FDR q-value
Neuron differentiation	8.58E-5
Neuron development	8.58E-5
Neurogenesis	8.58E-5
Cell part morphogenesis	3.34E-4
Positive regulation of developmental process	3.34E-4
Cell morphogenesis	3.92E-4
Synaptic signaling	3.92E-4
Presynaptic endocytosis	4.56E-4
Cellular component morphogenesis	6.58E-4
Synaptic vesicle recycling	9.92E-4

Chapter 3

In silico-in vitro modeling to uncover cues involved in establishing microglia identity: TGF-β3 and laminin can drive microglia signature gene expression

Raissa Timmerman[1], Ella A. Zuiderwijk-Sick[1], Wia Baron[2] and Jeffrey J. Bajramovic[1]

[1]Alternatives Unit, Biomedical Primate Research Centre, Rijswijk, The Netherlands
[2]Department of Biomedical Sciences of Cells & Systems,
Section Molecular Neurobiology, University of Groningen,
University Medical Center Groningen, Groningen, The Netherlands

Manuscript in preparation

Abstract

Microglia are the resident macrophages of the central nervous system (CNS) and play a key role in CNS development, homeostasis, and disease. Good *in vitro* models are indispensable to study their cellular biology, and although much progress has been made, *in vitro* cultures of primary microglia still only partially recapitulate the transcriptome of *ex vivo* microglia. In this study, we explored a combination of *in silico* and *in vitro* methodologies to gain insight into cues that are involved in the induction or maintenance of the *ex vivo* microglia reference transcriptome.

First, we used the *in silico* tool NicheNet, to investigate which CNS-derived cues could underlie the differences between the transcriptomes of *ex vivo* and *in vitro* microglia. Modeling on basis of genes that were found to be upregulated *in vitro,* predicted that HMGB2- and interleukin (IL)-1β-associated signaling pathways were driving their expression. A lack of specific inhibitors hampered *in vitro* verification of these results. Modeling on basis of the genes that were found to be downregulated *in vitro,* were hampered by low ligand-activity scores.

In a second approach, primary microglia were exposed to conditioned medium from different CNS cell types. Conditioned medium from spheres composed of microglia, oligodendrocytes, and radial glia, increased the mRNA expression levels of microglia signature gene *P2RY12.* NicheNet analyses of ligands expressed by oligodendrocytes and radial glia predicted TGF-β3 and LAMA2 as drivers of microglia signature gene expression, and *in vitro* exposure to TGF-β3 increased the mRNA expression levels of the microglia signature gene *TREM2*. Furthermore, microglia that were cultured on laminin-coated substrates were characterized by reduced mRNA expression levels of extracellular matrix-associated genes *MMP3* and *MMP7,* and by increased mRNA expression levels of the microglia signature genes *GPR34* and *P2RY13*. Together, our results suggest to explore inhibition of HMGB2- and IL-1β-associated pathways in *in vitro* microglia to reduce the expression of upregulated genes. In addition, exposure to TGF-β3 and cultivation on laminin-coated substrates are suggested to improve current microglia *in vitro* culture conditions.

Introduction

Tissue-resident macrophages (TRMs) are innate immune cells that play a role in tissue development, homeostasis, and damage responses through characteristic macrophage functions such as phagocytosis and inflammatory signaling[1]. In addition to these generic functions, TRM populations vary considerably between tissues in terms of gene expression profiles and fulfill specialized functions, which is partly a consequence of tissue- and niche-specific adaptations[2]. Microglia, the resident macrophages of the central nervous system (CNS), derive from a different progenitor than other TRMs[3]. In the CNS, microglia continuously receive signals from their microenvironment that contribute to homeostasis. For example, transforming growth factor beta (TGF-β) is constitutively expressed in the CNS and suppresses microglia activation both *in vitro* and *in vivo*[4-6]. In addition, neighboring neurons express CD47, CD200 and CX3CL1 that interact with CD172, CD200R and CX3CR1 on microglia respectively, providing inhibitory signals[7,8]. Loss of constitutive inhibitory signaling leads to a more activated microglia phenotype, characterized by an amoeboid morphology, increased expression of activation markers and loss of microglia signature genes (genes that are highly expressed by microglia and not, or at very low levels, expressed by other macrophages and other CNS cell types)[6,9-11]. This activated phenotype is also observed when primary microglia are isolated from the CNS environment and are brought in culture[11-13]. Transcriptome studies have demonstrated that the gene expression profile of *in vitro* microglia differs considerably from that of *ex vivo* microglia[4,12,13], and analysis of the differentially expressed genes (DEGs) suggests that this is attributable to the lack of CNS-specific cues. This suggestion is strengthened by the observation that loss of microglia signature markers can be partially reversed by engrafting of primary microglia back into a CNS environment or by culturing microglia together with other CNS cell types[11-13]. However, which CNS environmental cues contribute to the *in vivo* microglia gene expression profile that defines their identity is poorly understood.

In this study, we used NicheNet[14], an *in silico* (computational) method that predicts ligand-target links between interacting cells, to uncover (CNS-derived) ligands that drive the DEGs between *ex vivo* and *in vitro* microglia. If possible, we tested the effects of identified candidates and pathways *in vitro.* In a second approach, we exposed microglia to conditioned medium derived from different CNS cell types and analyzed the effects on microglia signature gene expression. Lastly, we exposed microglia to TGF-β3 and cultured microglia on laminin-coated substrates, and examined the effects on the expression of microglia signature genes.

Materials and methods

Animals

Brain tissue was obtained from adult rhesus macaques (*Macaca mulatta*) of either sex without neurological disease that became available from the outbred breeding colony or from other studies (all studies were ethically reviewed and approved by the Ministry of Agriculture, Nature and Food Quality of the Netherlands). No animals were sacrificed for the exclusive purpose of the initiation of microglia cell cultures. Better use of experimental animals contributes to the priority 3Rs program of the Biomedical Primate Research Centre. Individual identification data of the animals are listed in **Table 1.**

Table 1. Individual identification data of rhesus macaques.

Monkey ID nr.	Age (years)	Sex (F/M)	Weight (kg)	Origin
R01068	19	M	13	India
R01085	21	F	7	India
R04016	18	F	5	India
R04025	16	F	5	India
R06012	16	F	8	India
R06054	15	F	7	India
R08033	14	F	5	India
R09153	11	M	17	India
R12124	9	F	7	India
R13152	8	M	13	India
R13169	8	M	15	India
R14079	8	F	9	Mix
R14143	6	F	5	India
R15009	6	M	10	India
R15025	6	F	6	India
R17023	5	M	8	India
R17045	3	M	8	India
R18015	3	M	5	India

Reagents

5 μM inflachromene (ICM; Cayman Chemical, Ann Arbor, MI), 10 ng/mL human recombinant interleukin (IL)-1β (Invivogen, San Diego, CA), 250 ng/mL human IL-1 receptor antagonist (IL-1Ra; PeproTech, London, UK), 100 ng/mL lipopolysaccharide (LPS; Invivogen), 50 ng/mL human transforming growth factor (TGF)-β3 (Miltenyi Biotec, Bergisch Gladbach, Germany).

Microglia isolation and cell culture

Primary microglia were isolated and cultured as described previously[12]. In short, microglia isolations were initiated from cubes of ~4.5 g frontal subcortical white matter tissue that were depleted of meninges and blood vessels manually. Tissue was chopped into cubes of less than 2 mm^2 by using gentleMACS™ C tubes (Miltenyi Biotec) and incubated at 37 °C for 20 min in PBS containing 0.25% (w/v) trypsin (Gibco, Life Technologies, Bleiswijk, The Netherlands) and 1 mg/mL bovine pancreatic DNAse I (Sigma-Aldrich, Saint Louis, MO) and mixed every 5 min. The supernatant was discarded (no centrifugation), the pellet was washed and passed over a 100 µm nylon cell strainer (Falcon; Becton Dickinson Labware Europe, Vianen, The Netherlands) and centrifuged for 7 min at 524 *g*. The pellet was resuspended in 22% (vol/vol) Percoll (Cytiva, Uppsala, Sweden), 37 mM NaCl and 75% (vol/vol) myelin gradient buffer (5.6 mM NaH_2PO_4, 20 mM Na_2HPO_4, 137 mM NaCl, 5.3 mM KCl, 11 mM glucose, 3 mM BSA Fraction V, pH 7.4). A layer of myelin gradient buffer was added on top, and this gradient was centrifuged at 1561 *g* for 30 min (minimal brake). The pellet was washed and centrifuged for 7 min at 524 *g*. For laminin coating experiments, tissue culture-treated well plates (Corning Costar Europe, Badhoevedorp, The Netherlands) were coated with 10 µg/mL laminin-111 (Sigma-Aldrich) for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Plates were washed two times with PBS before plating the cells. Cells were plated at a density of 6.5 * 10^4 cells/cm^2 in serum-containing microglia medium (SM) comprised of 1:1 v/v DMEM (high glucose)/HAM F10 Nutrient mixture (Gibco) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; TICO Europe, Amstelveen, The Netherlands), 2 mM glutamax, 50 units/mL penicillin and 50 µg/mL streptomycin (all from Gibco). After overnight incubation at 37 °C in a humidified atmosphere containing 5% CO_2, unattached cells and debris were removed by washing with PBS twice and replaced by fresh SM medium supplemented with 20 ng/mL macrophage colony-stimulating factor (M-CSF; PeproTech). At day 4, cells were washed twice with PBS and replaced by serum-free microglia culture medium (SFM) comprised of DMEM/F12 supplemented with 2 mM glutamax, 50 units/mL penicillin, 50 µg/mL streptomycin (all from Gibco), 5 µg/mL N-acetyl cysteine, 5 µg/mL insulin, 100 µg/mL apo-transferrin, 100 ng/mL sodium selenite (all from Sigma-Aldrich), 20 ng/mL M-CSF, 12.5 ng/mL TGF-β1 (Miltenyi Biotec), 1.5 µg/mL ovine wool cholesterol (Avanti Polar Lipids, Alabaster, AL), 1 µg/mL heparan sulfate (Galen Laboratory Supplies, North Haven, CT), 0.1 µg/mL oleic acid and 1 ng/mL gondoic acid (both from Cayman Chemical). All cells were kept in culture for 15 days total without passaging. From day 4, half of the medium was replaced by fresh SFM medium containing new growth factors every 2-3 days.

Oligodendrocyte- and sphere-conditioned medium

Oligodendrocyte-conditioned medium (OCM) was collected from cultured primary rat oligodendrocyte precursor cells (OPC) or mature oligodendrocytes (mOLG) isolated from neonatal non-cortical areas[15,16]. Cells were cultured in defined SATO medium comprised of DMEM supplemented with 5 µg/mL bovine insulin, 50 µg/mL human

holo-transferrin, 100 µg/mL bovine serum albumin fraction V, 62 ng/mL progesterone, 16 µg/mL putrescine, 5 ng/mL sodium selenite, 400 ng/mL T3, 400 ng/mL T4 (all from Sigma-Aldrich), 4 mM L-glutamine, 100 units/mL penicillin and streptomycin (all from Gibco) and 27.5 µM 2-mercaptoethanol (Sigma-Aldrich). For OPC culture, 10 ng/mL platelet-derived growth factor-AA and 10 ng/mL fibroblast growth factor-2 (both from PeproTech) were added to SATO. For mOLG culture, cells were cultured for 2 days in SATO medium supplemented with PDGF-AA and FGF2, followed by differentiation upon growth factor withdrawal and culturing for 6 days in SATO supplemented with 0.5% FBS. For OCM experiments, OCM was mixed with fresh SFM medium at a volume ratio of 1:2 (OCM:SFM).

Sphere-conditioned medium (SCM) was collected from 3D-spherical co-cultures composed of microglia, oligodendrocytes, and radial glia[12]. For SCM experiments, SCM was mixed with fresh SFM medium at a volume ratio of 1:1. Microglia were exposed to conditioned medium from day 4 of cell culture.

NicheNet and Ingenuity Pathway Analysis (IPA)

The computational tool NicheNet uses gene expression data as input. It combines these with existing ligand-receptor, signaling and gene regulatory data sources, allowing for predictions on ligand-receptor interactions that drive gene expression changes in cells of interest[14]. We used NicheNet to identify ligands that regulate the expression of differentially expressed genes between *in vitro* and *ex vivo* microglia (**Figure 1**). NicheNet analyses were performed according to the code deposited in GitHub (https://github.com/saeyslab/nichenetr).

For this analysis, we selected ligands expressed on neurons, astrocytes, oligodendrocytes, and microglia, and also included an analysis with all ligands of the NicheNet database. For the expression of neuronal, astrocyte and oligodendrocyte ligands, the adult human transcriptome dataset (GSE73721) of Zhang and co-authors was used[17], where genes with an expression of ≥ 1.0 FPKM for astrocytes and oligodendrocytes, and ≥ 0.5 FPKM for neurons in at least one donor were selected. For the expression of microglial ligands and receptors, we used the in-house generated adult rhesus macaque microglia transcriptome dataset (GSE171476)[12], where genes with an expression of ≥ 5 CPM in all four *ex vivo* donors were selected. Next, we selected the target genes, which are the differentially expressed genes (FC ≥ 4; FDR < 0.01) between *ex vivo* and *in vitro* microglia.

For NicheNet analyses we studied the ligand-target interactions per CNS cell type separately and distinguished between target genes that were significantly upregulated or downregulated *in vitro*. First, we defined a set of potentially active ligands. This are ligands expressed by CNS cell types that can bind to a receptor that is expressed by microglia. These ligand-receptor links were gathered from NicheNet's ligand-receptor data sources. Second, a ligand-target activity analysis was performed to assess how well ligand-receptor induced activation can predict the expression of the microglial target genes. The 20 ligands with the highest Pearson correlation coefficients (measure used to define ligand-target activity) based on the presence of their target genes were

used for further NicheNet analyses. Lastly, ligand-target analyses of the top 20 ligands were performed and displayed in the ligand-target heatmap. In these heatmaps the regulatory potential scores for interaction between the top 20 ligands and their target genes are displayed. Of note, regulatory potential scores were set as 0 if the score was below a predefined threshold, which was here the 0.25 quantile of scores of interactions between the ligands and each of their respective top targets. Some regulatory potential scores were below the predefined threshold for some ligands and its targets. As a consequence, these ligands and targets were removed from the heatmaps. NicheNet does not process information on whether interactions are positively or negatively regulated. To accommodate for that information, we used IPA (Spring release March 2020; QIAGEN, https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) to reveal the signaling networks and signaling regulations of the ligand-target interactions.

RNA extraction and quantitative RT-PCR

Total cellular RNA was isolated using the RNeasy minikit (Qiagen) according to manufacturer's protocol. Subsequently, mRNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA synthesis kit according to the manufacturer's protocol (Fermentas; Thermo Fisher Scientific, Waltham, MA). RT-PCRs were performed on the CFX96™ Real-time PCR detection system (Bio-rad Laboratories, Hercules, CA) using primer (Invitrogen; Life technologies) and probe (human Exiqon probe library, Roche, Woerden, the Netherlands) combinations listed in **Table 2**, and iTaq Universal Probes Supermix (Biorad). Relative mRNA expression was standardized to housekeeping gene ACTB using the Pfafll method[18].

Statistics

GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. Statistical details of experiments can be found in the figure legends.

Results

***In silico* modeling of DEGs that were upregulated *in vitro* predicts that HMGB2- and IL-1β-associated signaling pathways are activated in *in vitro* primary microglia**

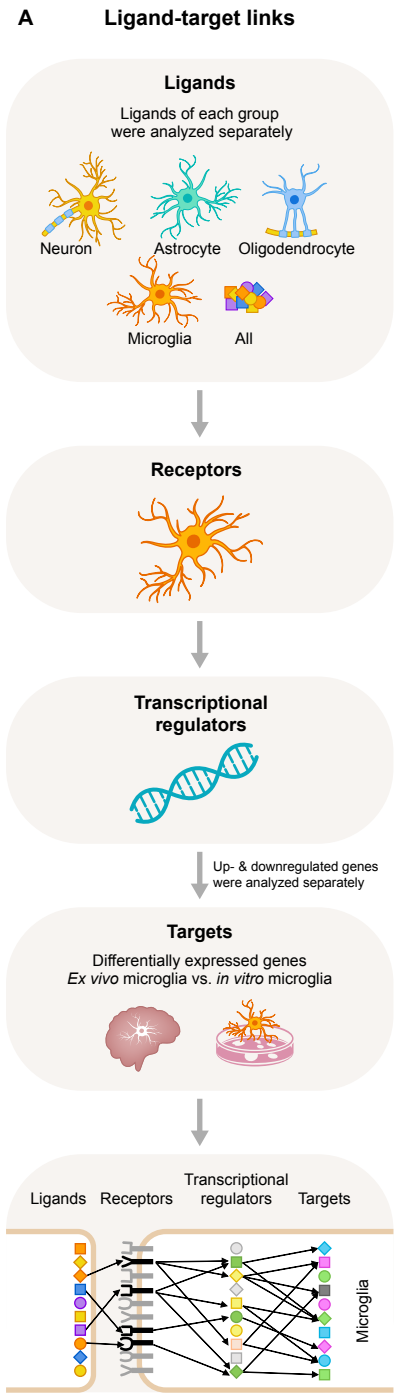
We used NicheNet[14], an *in silico* tool to study intercellular communication, to uncover CNS-derived cues that contribute to the gene expression profile that is characteristic for *in vivo* microglia. NicheNet uses gene expression data as input and combines these with existing ligand-receptor, signaling and gene regulatory data sources, allowing for predictions on ligand-receptor interactions that drive gene expression changes in cells of interest (**Figure 1**).

We started our analyses by filtering for i) ligands expressed by CNS cells (neurons, astrocytes, oligodendrocytes, and microglia), ii) receptors expressed by microglia and iii) target genes, which are the DEGs between *ex vivo* and *in vitro* microglia[12].

Table 2. Overview of primer/probe combinations used for RT-PCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Probe
ACTB	GCCCAGCACGATGAAGAT	CGCCGATCCACACAGAGTA	AGGAGGAG
CCL2	CAGCACTTCTGTGCCTGCT	GGGGCATTGATTGCATCT	GGCTGAAG
CCNF	GGGAAGATTCGAGTCCCCAC	GTGCAGCAGGGAGAGCTC	CAGAGGAA
CX3CR1	TGATTTTCCTGGAGACGCTTA	TCAGATCCCTCCTCATGTCA	TTCCCAGT
E2F7	GCTCGCCATGGTTCTTTCAA	AGTAGCCACCTGATCCTTGT	CCCAGCAG
FOS	GGGATGGCATCAAGGTACCC	CCCTTCTTCCCCTCCGAAAC	TCTGGAGC
GPR34	TGACGACAACTTCAGTCAGCA	GGTTGGTCGCTATGACTGGT	CTCCTCCC
IL6	ACAAAAGTCCTGATCCAGTTCC	GTCATGTCCTGCAGCCACT	CAGCAGGC
IL12p40	CCACATTCCTACTTCTCCCTGA	ACCGTGGCTGAGGTCTTGT	TCCAGGTC
KIF2C	GACACATACTATGGGCGGAGA	CCGGTAGCAGGGTTGATTCT	CTTCCTCC
LCN2	CCCAGGACTCCAGCTCAG	CATACCACTTCCCCTGGAAC	TCTGCTGC
MKI67	ACACTCCACCTGTCCTGAAGA	GTGCCTTCACTTCCACACTG	TGAGGCTGT
MMP3	CCTGACGTTGGTCACTTCAC	AATCTCGTGTATAATTCACAATCCTG	TTCCTGGC
MMP7	GCTCATGCCTTTGCACCT	GCGTTGCAGCATACAGGA	CTCCTCCA
MMP9	ACAAGCTCTACGGCTTCTGC	GAAGGTGAAGGGGAAAACG	CAGCTCCC
MMP14	CCAAGACCCTCCCGTTGT	GGCAGGTAGCCATACTGCTG	GGGAGCAG
P2RY12	TCCATTCAAAATTCTTAGTGATGC	CGGAGGTAACTTGACACACAAA	TTCCCAGT
P2RY13	ACTGAGTATCCTCCCAAAGGTG	CGGTCAAGAAAACCACTGTGT	CCCAGCCC
PHF19	TGGAAGGACATACAGCATGC	ACACTTCCCGCAGATGAGGA	CTTCCCCA
SHCBP1	GCAATTGAGCATGTCAGATTTTTC	CGAGGTTCAACACATCTGACA	CATCCTCC
TACC3	CCAGAAAGCCCTGAGACCA	TCCGCTGAGGCTGAATGCAG	GGAGCCAG
TNF-α	AAGCCTGTAGCCCATGTTGT	GCTGGTTATCTGTCAGCTCCA	CCAGGAGG
TREM2	CCGGCTGCTCATCTTACTCT	AGGACACCTGTAGGGACTGG	TCTGGAGC
TYMS	AAAACCAACCCTGACGACAGA	CACCACATAGAACTGGCAGAG	CTGCCTCC

We then performed NicheNet analyses for each CNS cell type separately, and for the up- and downregulated target genes separately. We also included an unbiased analysis where all ligands of the NicheNet database were used to include possible CNS intercellular signaling that has not been described yet or that are mediated by other CNS cells. A schematic overview of the NicheNet workflow is shown in **Figure 1A.** Next, we performed a ligand-target activity analysis to assess how well each ligand may regulate the expression of the target genes (**Figure 1B**). We selected the top 20 ligands with the highest Pearson correlation coefficients (PCC; measure of the ligand-target activity), and analyzed the regulatory potential scores for the top 20 ligands to interact with the target genes (**Figure 1C**). Of note, a predefined threshold was set for the regulatory potential scores. As a consequence, some ligands were removed from the analyses (see materials and methods). The remaining ligands and their PCC scores and regulatory potential scores with the up- or downregulated targets are displayed in **Figure S1**-**Figure S3**, respectively. We observed that the PCC scores of the ligands and the downregulated genes *in vitro* were very low (<0.05; **Figure S1**). This implies that



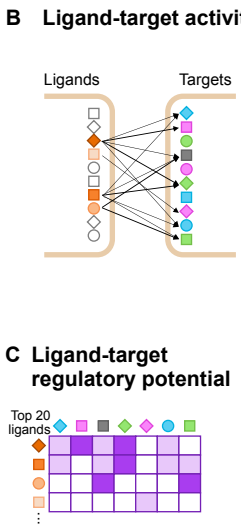


Figure 1. Schematic overview of the NicheNet workflow. A) We selected ligands expressed on neurons, astrocytes, oligodendrocytes, and microglia, and also included an analysis with all ligands of the NicheNet database. Next, we selected receptors expressed by microglia. We used NicheNet's ligand-receptor data sources to analyze ligand-receptor interactions. Of note, Ligands of each group were analyzed separately. Subsequently, information from NicheNet's signaling data sources were used to analyze which transcriptional regulators are activated by the ligand-receptor interactions. Lastly, NicheNet's gene regulatory data sources were used to further analyze which target genes (the differentially expressed genes between *ex vivo* and *in vitro* primary microglia from adult rhesus macaques) are regulated by the transcriptional regulators. Of note, upregulated and downregulated genes were analyzed separately. **B)** After the ligand-target links were determined we performed a ligand-target activity analysis to rank the ligands based on the presence of its target genes. The more target interactions, the higher the ranking of the ligand. **C)** The 20 ligands with the highest ligand-activity scores based on the presence of their target genes were displayed in the ligand-target heatmaps. In these heatmaps the regulatory potential scores for interaction between the top 20 ligands and target genes is displayed.

the ranking of the ligands would not be much better than random prediction[14]. For this reason, we decided not to continue with the analysis of the downregulated target genes and to only focus on the ligands that are predicted to drive the expression of the upregulated target genes, as the PCC scores of these ligands were much higher.

When we compared all ligand-target heatmaps of the upregulated *in vitro* target genes, we observed that some predicted ligands were present in multiple heatmaps. Furthermore, some target genes were predicted to be regulated by multiple upstream ligands (**Figure S2**). For this reason, we selected the 10 ligands from the ligand-target heatmaps that together regulate most target genes. Of note, as multiple ligands regulate the same target genes, we also took the regulatory scores into account. We named these selected ligands: ligands of interest (LOI; **Table S1**) and performed new NicheNet analyses with these ligands. Of note, for these analyses no predefined threshold was used as we were interested in all ligand-target interactions. The PCC scores of the LOI and the upregulated *in vitro* target genes are shown in **Figure 2A**.

High mobility group box 2 (HMGB2) was predicted as an upstream regulator for a specific selection of upregulated *in vitro* target genes (**Figure 2B**). The majority of the other LOI were predicted to regulate almost all remaining target genes in the heatmap. Transforming growth factor beta 1 (TGF-β1) showed high potential regulatory scores. However, this factor is already present in our culture medium. As the potential regulatory scores of IL-1β and its target genes were higher compared to the other ligands, we selected HMGB2 and IL-1β as potential ligands that regulate the expression of the upregulated genes *in vitro*. As NicheNet does not provide information on whether predicted ligands regulate their associated targets positively or negatively, we used QIAGEN Ingenuity Pathway Analysis (IPA) to examine this. IPA did not confirm all NicheNet's predicted ligand-target interactions, but did predict that inhibition of HMGB2- (**Figure S4**) and IL-1β-mediated signaling (**Figure S5**) would reduce the mRNA expression levels of most target genes.

mRNA expression levels of target genes are not affected by non-specific inhibition of HMGB- and IL-1R-mediated signaling

Next, we wanted to examine the effects of inhibiting HMGB2- and IL-1β-mediated signaling in cultured primary microglia. However, as far as we are aware of, specific blockers for HMGB2- and IL-1β-induced signaling have not been described yet. We therefore chose an approach with non-specific blockers.

To inhibit HMGB2-mediated signaling, we added inflachromene (ICM), a recently described inhibitor of both HMGB1 and HMGB2, to the microglia culture medium[19]. We confirmed the activity of ICM by demonstrating that ICM exposure reduced the mRNA expression levels of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines IL-6, IL-12p40 and TNF-α (**Figure S6A**), as reported by Lee and colleagues[19]. Subsequently, we analyzed the mRNA expression levels of *TACC3, CCNF, TYMS, E2F7, PHF19, MKI67, SHCBP1* and *KIF2C*, as selected target genes of which the expression was predicted to be regulated by HMGB2-mediated signaling (**Figure 2C**), and observed that exposure to ICM did not affect the mRNA expression levels of these genes.

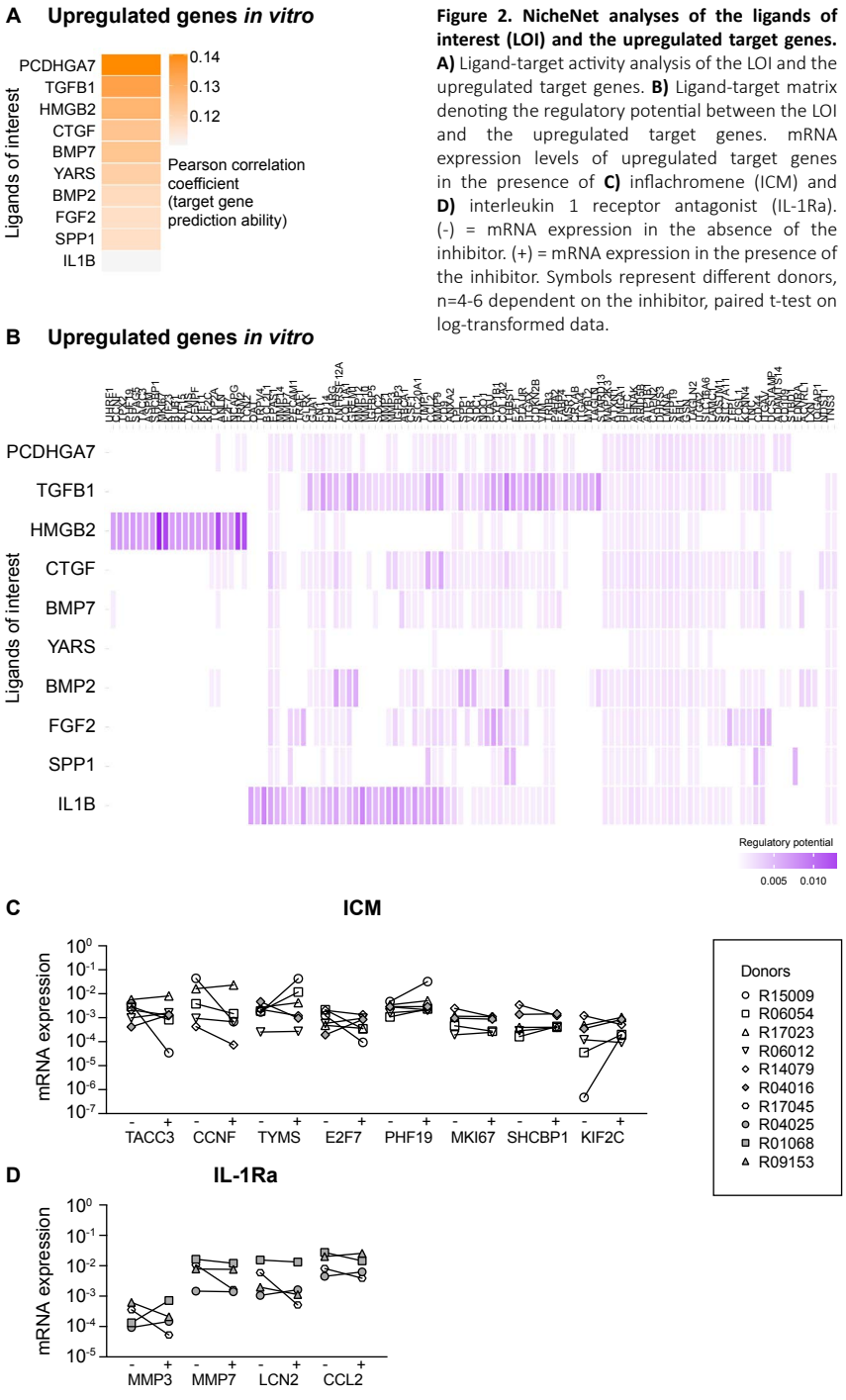


Figure 2. NicheNet analyses of the ligands of interest (LOI) and the upregulated target genes. **A)** Ligand-target activity analysis of the LOI and the upregulated target genes. **B)** Ligand-target matrix denoting the regulatory potential between the LOI and the upregulated target genes. mRNA expression levels of upregulated target genes in the presence of **C)** inflachromene (ICM) and **D)** interleukin 1 receptor antagonist (IL-1Ra). (-) = mRNA expression in the absence of the inhibitor. (+) = mRNA expression in the presence of the inhibitor. Symbols represent different donors, n=4-6 dependent on the inhibitor, paired t-test on log-transformed data.

To block IL-1β-mediated signaling, cultured primary microglia were exposed to IL-1 receptor antagonist (IL-1Ra). Importantly, IL-1Ra inhibits the activity of both IL-1α and IL-1β by competitively blocking their binding to type I and type II receptors[20]. The activity of IL-1Ra was confirmed by demonstrating that IL-1β-induced IL-6 mRNA expression levels were reduced in the presence of IL-1Ra (**Figure S6B**)[21]. We analyzed the mRNA expression levels of *MMP3, MMP7, LCN2* and *CCL2,* as selected target genes of which the expression was predicted to be regulated by IL-1β-mediated signaling. However, we did not observe a reduction in mRNA expression levels of these target genes in the presence of IL-1Ra (**Figure 2D**).

Together, these data demonstrate that non-specific inhibition of HMGB2- and IL-1β-mediated signaling does not reduce the mRNA expression levels of the analyzed target genes. Specific blockers of these pathways are needed to test the predictions from the NicheNet analyses.

Exposure to conditioned medium of mixed glia cell spheres increased the mRNA expression levels of P2RY12 in cultured primary microglia

In a second approach to gain insight into cues that could potentially optimize microglia *in vitro* culture conditions*,* we exposed microglia to conditioned medium derived from different CNS cell types. Earlier data from our group demonstrated that microglia cultured in spheres, together with oligodendrocytes and radial glia, are characterized by the increased expression of microglia signature genes[12]. To determine if these effects were attributable to factors secreted from cells in the spheres, we exposed primary cultured microglia to sphere-conditioned medium (SCM). We analyzed the mRNA expression levels of the microglia signature genes *CX3CR1, FOS, GPR34, P2RY12, P2RY13* and *TREM2*, as their expression was increased in spheres compared to monocultured microglia (**Figure S7**). Exposure to SCM indeed increased the mRNA expression levels of *P2RY12,* and showed a trend towards increased mRNA expression levels of *CX3CR1* (**Figure 3A**).

Since, after microglia, oligodendrocytes are the most abundant cell type in the spheres[12], we examined whether secreted factors by cultured oligodendrocytes could induce the expression of microglia signature genes. We therefore supplemented the microglia culture medium with conditioned medium derived from cultured primary rat oligodendrocyte precursor cells (OPC CM) or from cultured primary rat mature oligodendrocytes (mOLG CM) isolated from non-cortex brain tissue. We find that neither exposure to OPC CM (**Figure 3B**) nor to mOLG CM (**Figure 3C**) led to an increase of microglia signature gene expression levels. mOLG CM even significantly reduced the mRNA expression levels of *CX3CR1* and *FOS*.

Exposure to TGF-β3 increased the mRNA expression levels of microglia signature gene TREM2

In a further attempt to identify the ligands in SCM that were responsible for the induction of microglia signature genes, we performed a NicheNet analysis with expressed ligands in human oligodendrocytes[17] and human radial glia[22], and the six

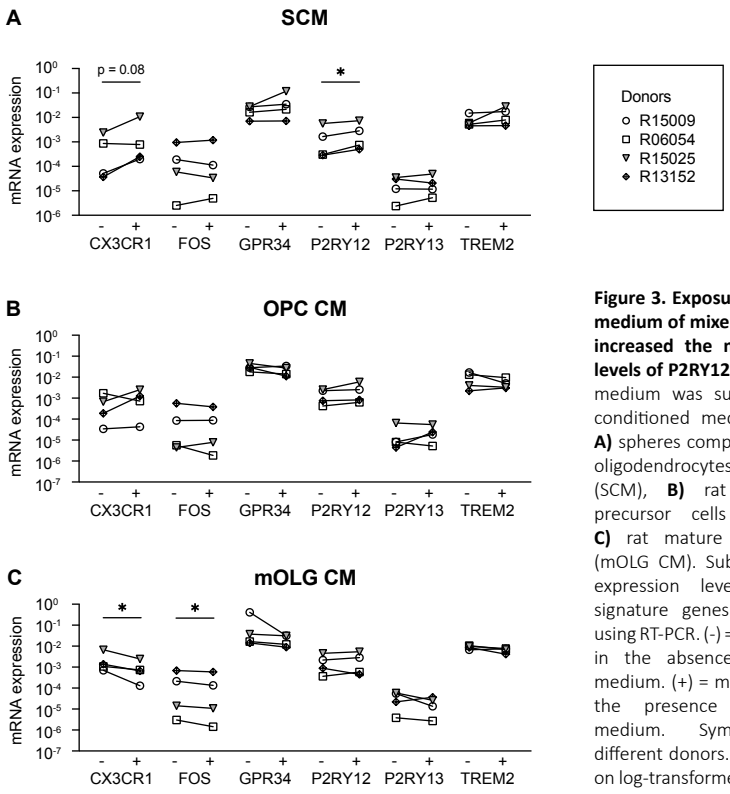


Figure 3. Exposure to conditioned medium of mixed glia cell spheres increased the mRNA expression levels of P2RY12. Microglia culture medium was supplemented with conditioned medium from either **A)** spheres composed of microglia, oligodendrocytes, and radial glia (SCM), **B)** rat oligodendrocyte precursor cells (OPC CM) or **C)** rat mature oligodendrocytes (mOLG CM). Subsequently, mRNA expression levels of microglia signature genes were measured using RT-PCR. (-) = mRNA expression in the absence of conditioned medium. (+) = mRNA expression in the presence of conditioned medium. Symbols represent different donors. n=4, paired t-test on log-transformed data, * $p < 0.05$.

microglia signature genes as target genes. Surprisingly, NicheNet did not predict that ligands expressed by oligodendrocytes or radial glia were drivers of *CX3CR1* or *P2RY12* expression levels (**Figure 4A** and **4B**). Nevertheless, multiple oligodendrocyte and radial glia ligands, including TGF-β3, were predicted to drive the expression of *FOS*. IPA analysis confirmed this and further predicted that TGF-β3 positively regulates the expression of *FOS* (**Figure S8**). TGF-β3 is an interesting ligand in this context, since the expression levels of TGF-β3 were significantly higher in the spheres as compared to monocultured microglia (**Figure 4C**)[12]. We hypothesized that TGF-β3 contributes to the increased expression of microglia signature genes in spheres. To test this, we supplemented the culture medium with TGF-β3. Of note, TGF-β1, another member of the TGF-β family, is already present in our standard culture medium. While it was predicted that TGF-β3 regulates *FOS*, we did not observe this. However, supplementation of the culture medium with TGF-β3 led to increased mRNA expression levels of *TREM2* and showed a trend towards increased expression of *GPR34* (**Figure 4D**).

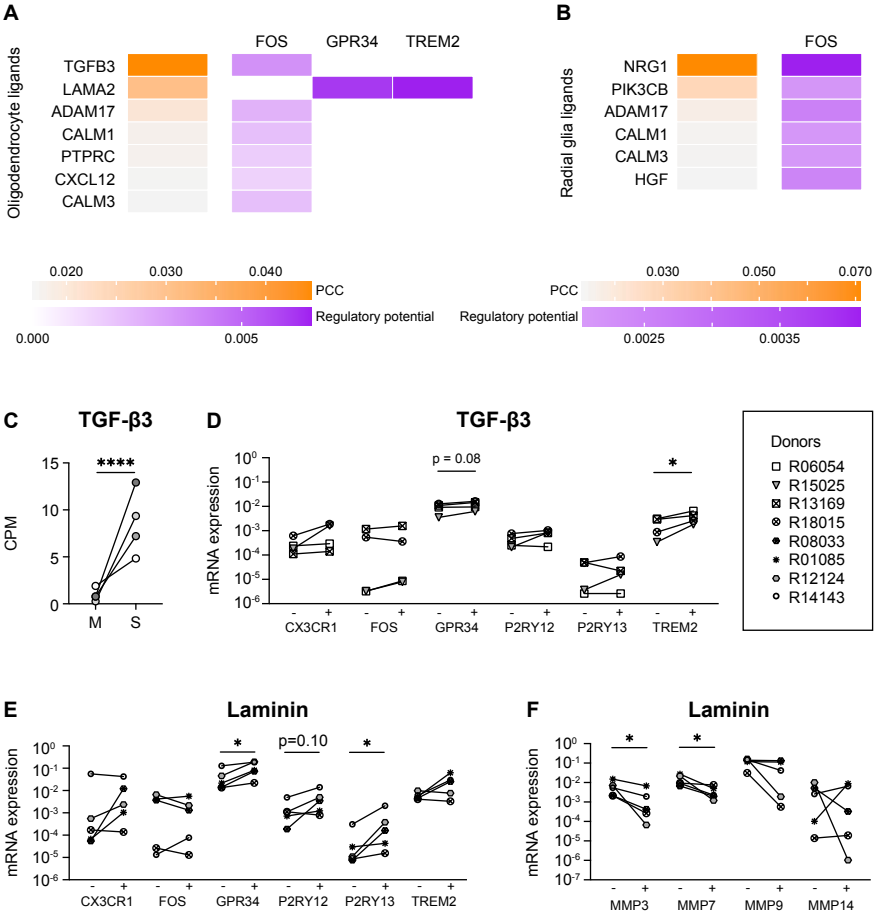


Figure 4. TGF-β3 and laminin exposure increased the mRNA expression levels of microglia signature genes. Ligand-activity and ligand-target matrix of **A)** oligodendrocyte ligands and **B)** radial glia ligands. PCC = Pearson correlation coefficient. **C)** Gene expression (CPM) of transforming growth factor beta 3 (TGF-β3) in monocultured microglia (M) and spheres (S), n=4, EdgeR false discovery rate (FDR) was used to display statistical differences, **** FDR < 0.001. **D)** mRNA expression levels of microglia signature genes of microglia cultured in the absence (-) or presence (+) of TGF-β3. Symbols represent different donors. n=4, paired t-test on log-transformed data, * $p < 0.05$. mRNA expression levels of **E)** microglia signature genes and **F)** matrix metalloproteinases of microglia cultured in the absence (-) or presence (+) of laminin. Symbols represent different donors, n=5, paired t-test on log-transformed data, * $p < 0.05$,

Laminin coating reduced the mRNA expression levels of matrix metalloproteinases and increased the mRNA expression levels of microglia signature genes

Interestingly, the non-soluble oligodendrocyte ligand LAMA2 was predicted by NicheNet to drive the expression of *GPR34* and *TREM2* (**Figure 4A**). The gene *LAMA2* encodes for a laminin subunit protein[23], which is part of the brain extracellular matrix (bECM)[24]. We therefore analyzed the expression levels of microglia signature genes of microglia cultured on laminin-coated substrates. As predicted, the mRNA expression levels of *GPR34* were indeed significantly increased, as were the mRNA expression levels of *P2RY13* (**Figure 4E**). In addition, we observed a trend towards increased expression of *P2RY12.*

Interestingly, the expression of ECM-associated genes is highly increased in *in vitro* microglia compared to *ex vivo* microglia (**Figure S9**)[12]. This is consistent with the reported increased expression of ECM-associated genes, including cathepsins and matrix metalloproteinases (MMPs), during microglia activation[25-27]. As MMPs were among the ECM-associated genes with the most robust increased expression in *in vitro* microglia, we analyzed the effect of laminin on the mRNA expression levels of MMP genes. Indeed, we found that the mRNA expression levels of *MMP3* as well as *MMP7* were reduced when microglia were cultured on laminin-coated substrates (**Figure 4F**).

Discussion

In this study, we combined *in silico-in vitro* modeling to uncover cues that drive the expression of DEGs between *ex vivo* and *in vitro* primary microglia, with a specific focus on microglia signature genes. Our aim was not only to gain insight into the determinants of microglial identity, but also to implement this knowledge to optimize microglia cell culture protocols.

Results from our first approach using the *in silico* tool NicheNet led us to select HMGB2 and IL-1β as upstream regulators of DEGs that were upregulated *in vitro*. IPA analysis predicted that blocking of HMGB2- and IL-1β-mediated signaling would reduce the expression of numerous upregulated *in vitro* target genes. HMGB2 and IL-1β are both associated with microglia pro-inflammatory processes[19,28-30] and blocking of these signaling pathways is in line with studies demonstrating that such pro-inflammatory gene products are upregulated *in vitro*[13,31]. In this study, inflachromene (ICM) and IL-1 receptor antagonist (IL-1Ra) were used to block HMGB2- and IL-1β-mediated signaling, respectively. It is important to note that ICM binds to both HMGB1 and HMGB2[19], and that IL-1Ra inhibits the activity of both IL-1α and IL-1β by competitively blocking their binding to type I and type II receptors[20]. Although the inhibitors are non-specific for our targets, they may have provided insight into the relative contribution of HMGB2- and IL-1β-mediated pathways in the regulation of the upregulated *in vitro* target genes. Regretfully, neither inhibition of HMGB- nor of IL-1R-

mediated signaling did reduce the mRNA expression levels of the predicted target genes. Whether this is attributable to the lack of specificity of the inhibitors used or to poor predictive power of NicheNet remains to be elucidated in future studies as our results are non-conclusive.

The NicheNet results for the DEGs that were downregulated *in vitro* were non-conclusive as well, due to low PCC scores of ligands and their target genes. This might in part be caused by the fact that NicheNet's ligand-receptor, signaling and gene regulatory information is gathered from data sources that include data from different species and cell types. Differences between primate microglia and rodent microglia have been described[32,33], as well as differences in biological mechanisms between microglia and other macrophages[34-36].

In a second approach to optimize microglia *in vitro* culture conditions, we experimented with exposure to conditioned medium derived from different CNS cell types. We had demonstrated in a previous study that microglia cultured in spheres, together with oligodendrocytes and radial glia, were characterized by the increased expression of microglia signature genes[12]. Exposure to conditioned medium of these spheres (SCM) increased the mRNA expression levels of microglia signature gene *P2RY12* and showed a trend towards increased expression of *CX3CR1*. Interestingly, the expression of *P2RY12* and *CX3CR1* is thought to be regulated by neuron-microglia and astrocyte-microglia crosstalk[8,11,37]. Our results suggest a novel role for oligodendrocyte and/or radial glia secreted factors in the regulation of these genes. Of note, the spheres (Ø ± 250 µm) were cultured in relatively high volumes (2.5 mL), which means that the secreted factors are considerably diluted. The effects of the secreted factors on microglia present in the spheres might therefore be much more robust as observed in the SCM experiments. Further research is needed to examine if exposure to concentrated SCM indeed generated more positive effects.

Recently, a role for oligodendrocyte precursor cells (OPCs) in maintaining the microglia homeostatic state has been described[38]. However, we observed that exposure to OPC- or mature oligodendrocyte-conditioned medium did not induce the mRNA expression levels of microglia signature genes. Of note, these oligodendrocytes were derived from rat, and it is not known if rat- and rhesus-derived oligodendrocytes secrete similar factors. In addition, the indirect effects that other cells in the spheres might have had on the oligodendrocyte secretome could not be tested either.

Although exposure to conditioned medium (CM) is a useful approach to analyze the effects of cell-secreted cues on microglia signature gene expression, CM is less suitable as a supplement for cell culture. The components in CM are mostly not defined and CM is notoriously associated with batch-to-batch variation, rendering standardization of culture conditions challenging. We performed new NicheNet analyses to gather more detailed information about ligands expressed by human oligodendrocytes and radial glia with the potential to regulate the expression of microglia signature genes. NicheNet predicted multiple oligodendrocyte and radial glia ligands as drivers of the microglia signature gene *FOS,* including TGF-β3, ADAM17, NRG1 and PIK3CB. Although the TGF-β signaling pathway has been reported earlier as an important driver of

microglia homeostasis and identity[4,6,9,11], TGF-β3 is a new and interesting candidate. Of note, TGF-β1 was already present in our standard culture medium. While addition of TGF-β3 did not induce the expression of *FOS,* it did increase the mRNA expression levels of *TREM2* and did show a trend towards increased expression of *GPR34*. There are three known isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) and they are all expressed in the human brain[39]. TGF-β1 and TGF-β2 share 71% protein sequence similarity[40], whereas TGF-β3 shares 80% of amino acid sequence with TGF-β1 and TGF-β2. Although all isoforms function through the same receptor signaling pathways[41,42], biological activity differences between TGF-β isoforms are described[43], and it is unclear if these differences affect microglia homeostasis. As far as we are aware of, microglia culture medium is supplemented with either isoform TGF-β1 or TGF-β2[4,11-13,44]. The effects of exposure to combinations of different TGF-β isoforms warrants further investigation. In addition, it should be tested whether exposure to higher concentrations of TGF-β1 could simulate the effects of exposure to TGF-β1 + TGF-β3.

NicheNet further predicted the oligodendrocyte ligand LAMA2 as a driver of *GRP34* and *TREM2* expression. As the gene *LAMA2* encodes a laminin subunit, we cultured microglia on laminin-coated substrates. Interestingly, primary microglia cultured on laminin-coated substrates were characterized by increased mRNA expression levels of microglia signature genes and by reduced mRNA expression levels of matrix metalloproteinases. In this study, laminin-111 (previous named laminin-1) with the chain composition α-1/β-1/γ-1 was used (for the most recent laminin nomenclature see[45]). In total, sixteen laminin isoforms have been described and some of them have specific function[46]. The effect of each laminin isoform to microglia homeostasis remains to be determined. Multiple integrins serve as receptors for laminin[47] but are also receptors for other extracellular matrix components, including fibronectin, vitronectin and collagen[48,49]. The role of other brain extracellular matrix components in the regulation of microglia signature genes warrants further study.

Taken together, our results suggest that HMGB2- or IL-1β-associated pathways are targets to specifically inhibit upregulated genes *in vitro* to better mimic *in vivo* microglia. In addition, exposure to TGF-β3 and cultivation on laminin-coated substrates are suggested as improvement over current culture practices. As the transcriptomes of primary *in vitro* microglia and stem cell-derived microglia are highly similar[50,51], our data might also be applied to optimize cell culture conditions of stem cell-derived microglia.

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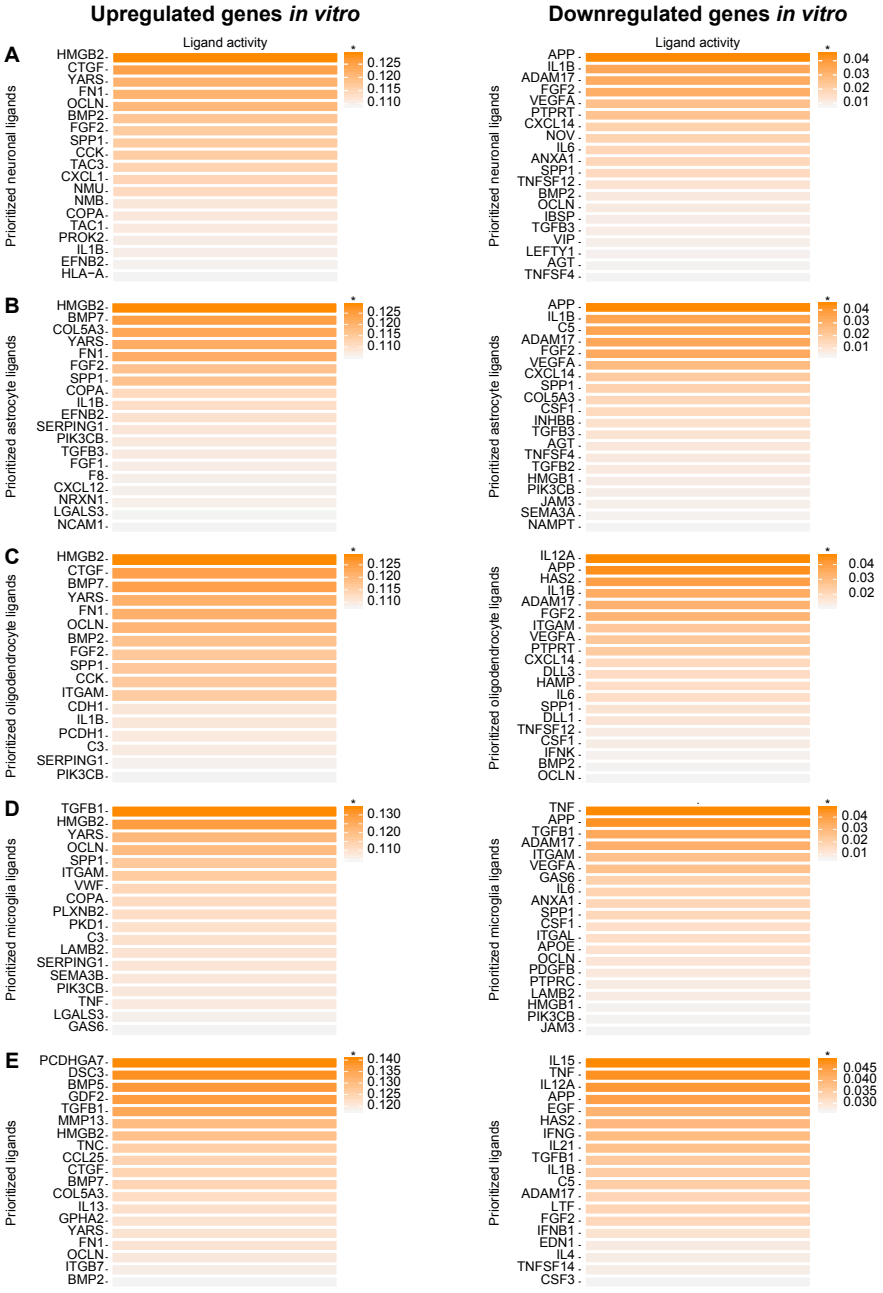
Figure S1. NicheNet upstream ligand-target activity analysis for differentially expressed genes between *ex vivo* and *in vitro* microglia. Top potential upstream ligands expressed by either **A)** neurons, **B)** astrocytes, **C)** oligodendrocytes or **D)** microglia based on their potential to regulate significantly (FC ≥ 4; FDR < 0.01) upregulated (left) or downregulated (right) genes between *ex vivo* and *in vitro* primary microglia. **E)** We also included an unbiased approach where all ligands of the database were used. As the ligand-target activity ranking metric, the Pearson correlation coefficient is used. This metric indicates the ability of each ligand to predict the expression of the differentially expressed genes, and better predictive ligands are thus ranked higher. ▶

Supplemental information



*Pearson correlation coefficient (target gene prediction ability)

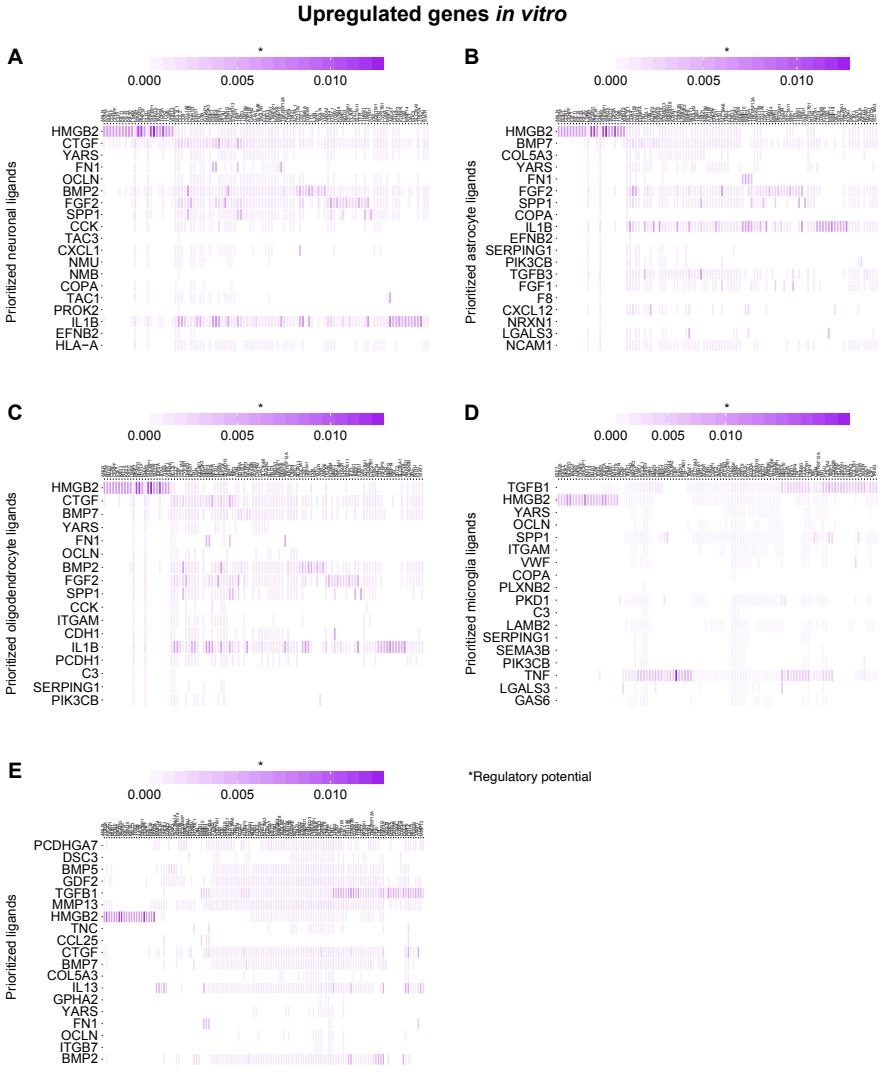


Figure S2. Ligand-target network matrix of the top-ranked ligands that potentially regulate genes upregulated in *in vitro* microglia compared to *ex vivo* microglia. NicheNet's ligand-target matrix denoting the regulatory potential between ligands expressed by **A)** neurons, **B)** astrocytes, **C)** oligodendrocytes or **D)** microglia, and significantly upregulated *in vitro* microglia target genes. **E)** We also included an unbiased analysis where all ligands of the NicheNet database were used.

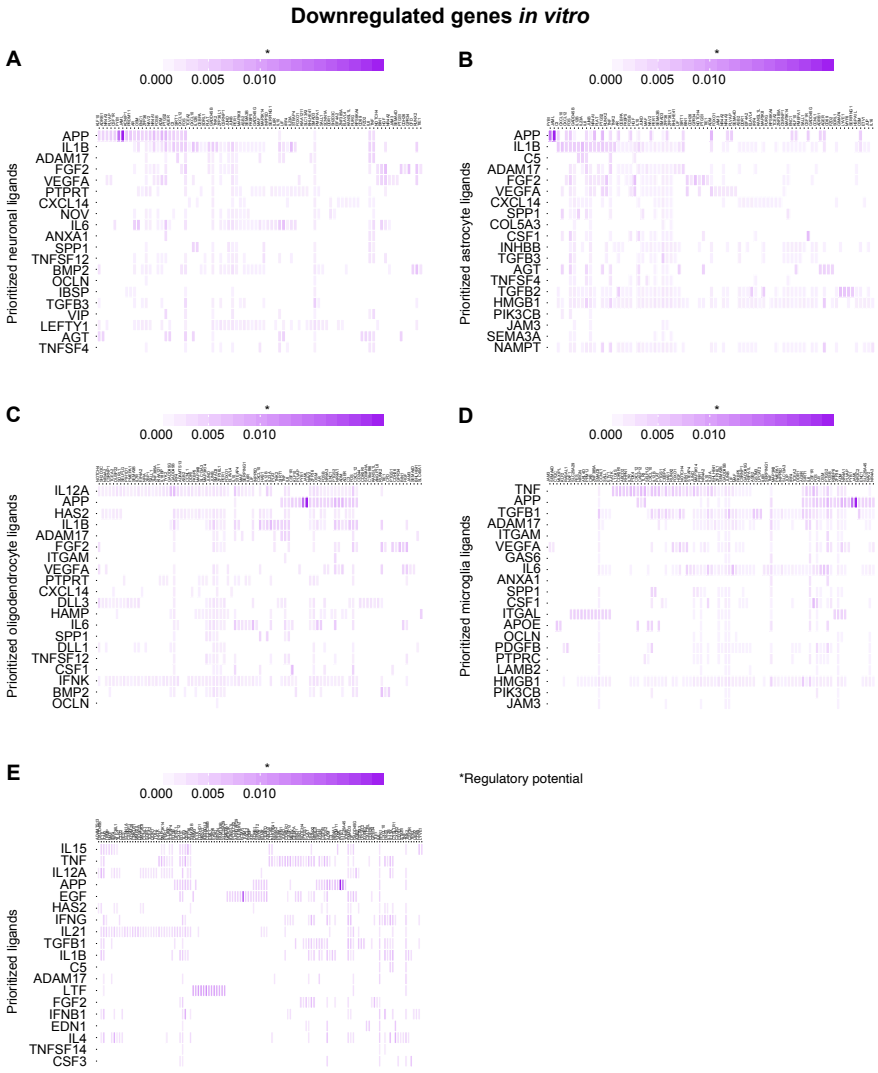
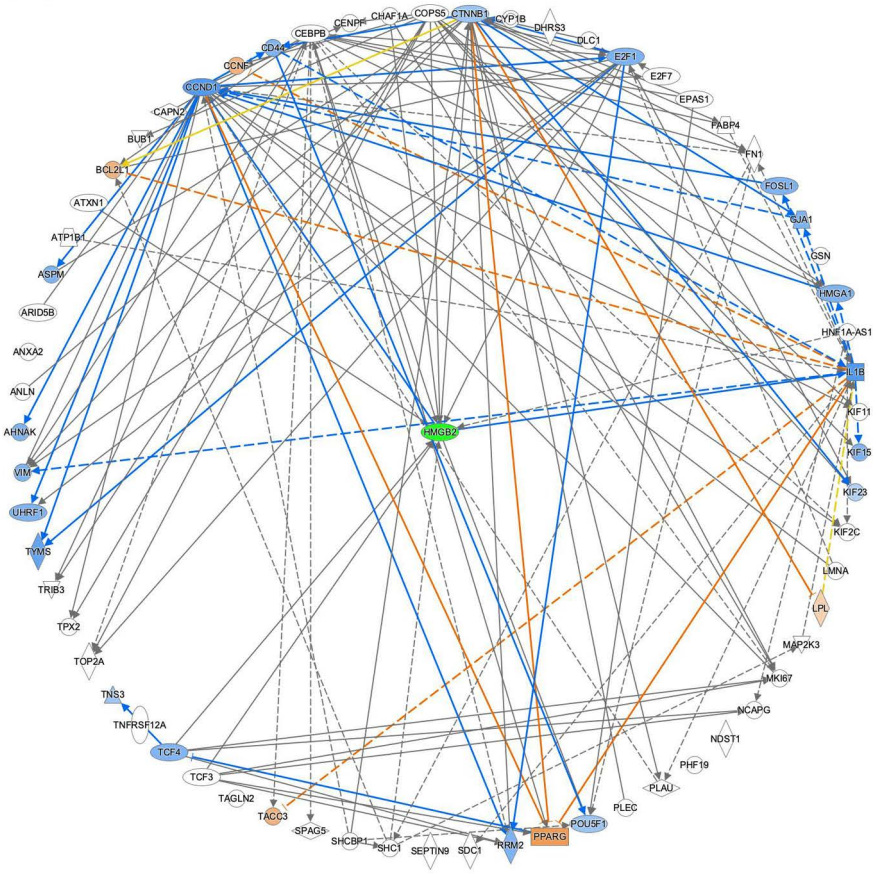


Figure S3. Ligand-target network matrix of the top-ranked ligands that potentially regulate genes downregulated in *in vitro* microglia compared to *ex vivo* microglia. NicheNet's ligand-target matrix denoting the regulatory potential between ligands expressed by **A)** neurons, **B)** astrocytes, **C)** oligodendrocytes or **D)** microglia, and significantly downregulated *in vitro* microglia target genes. **E)** We also included an unbiased analysis where all ligands of the NicheNet database were used.



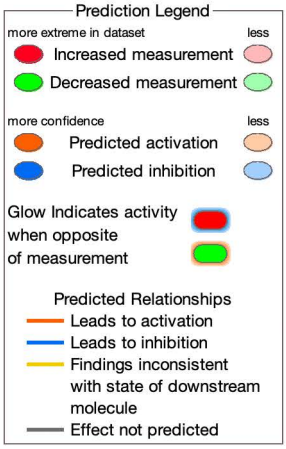


Figure S4. Predicted ingenuity pathways analysis (IPA) interactions of HMGB2 and its target genes.

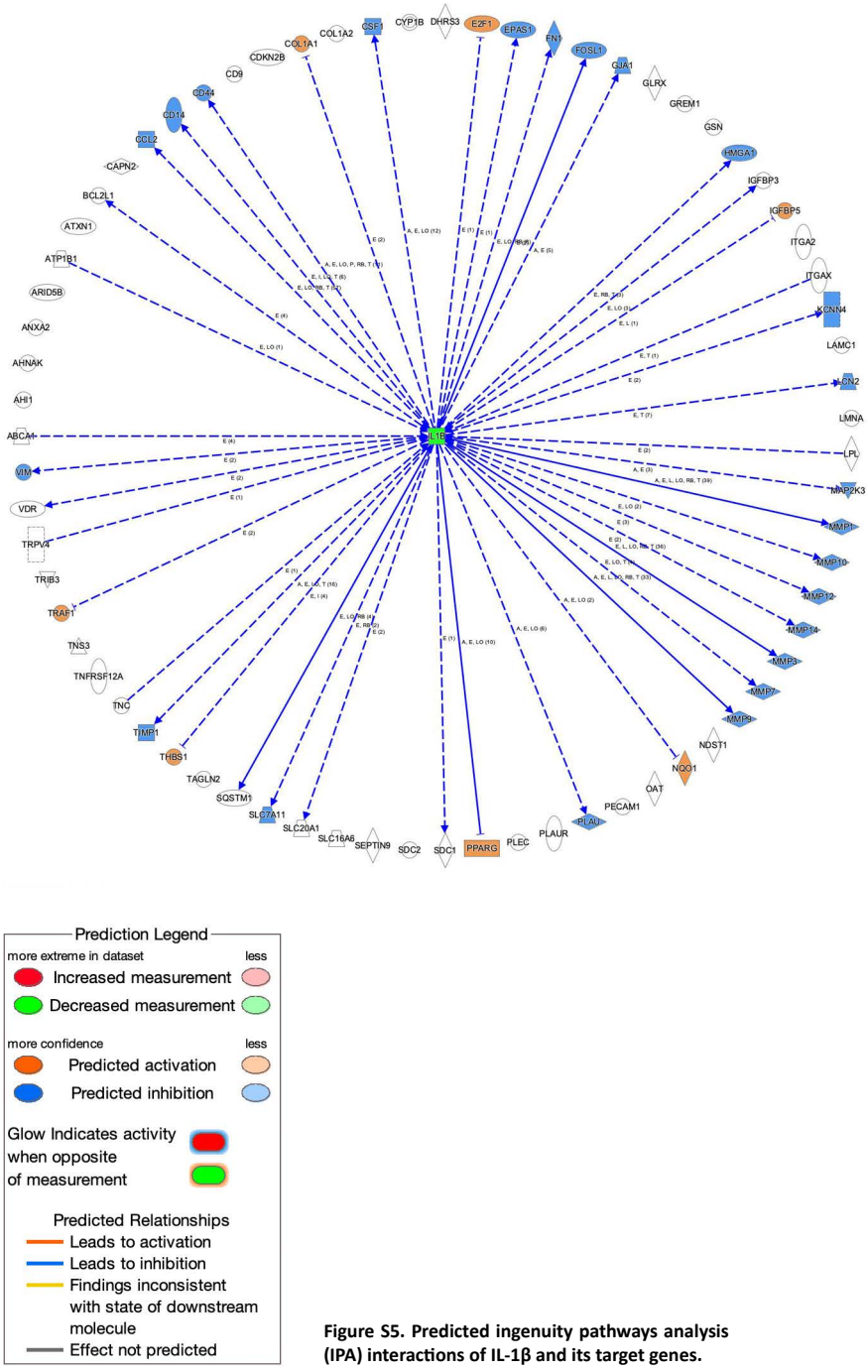


Figure S5. Predicted ingenuity pathways analysis (IPA) interactions of IL-1β and its target genes.

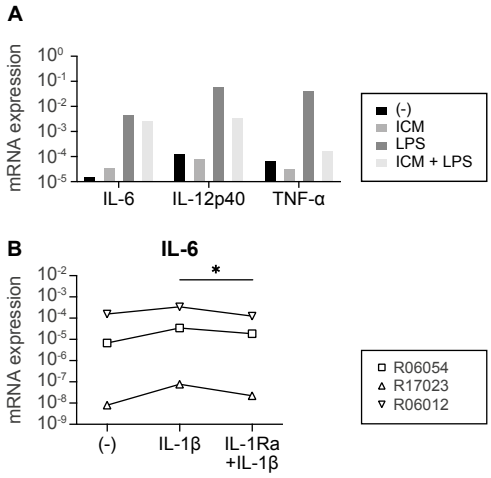


Figure S6. Activity of ICM and IL-1Ra in *in vitro* primary microglia. A) *In vitro* primary microglia were cultured in the absence or presence of 5 µM inflachromene (ICM), and at day 14 of culture exposed for 16 h to 100 ng/mL lipopolysaccharide (LPS). mRNA expression levels of IL-6, IL-12p40 and TNF-α were analyzed. n=1. **B)** *In vitro* primary microglia were cultured in the absence or presence of 250 ng/mL IL-1 receptor antagonist (IL-1Ra) and at day 14 of culture exposed for 16 h to 10 ng/mL interleukin (IL)-1β. mRNA expression levels of IL-6 were analyzed. Each symbol represents a donor, paired t-test on log-transformed data, n=3, * < p 0.05. Relative gene expression was standardized to housekeeping gene ACTB.

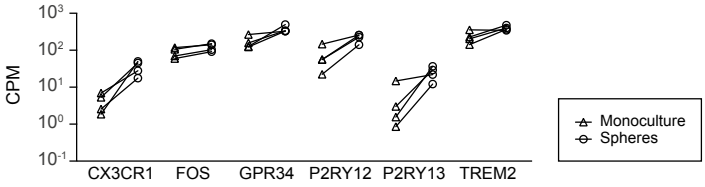


Figure S7. Gene expression values in counts per million (CPM) of microglia signature genes in monocultured microglia and in spheres.

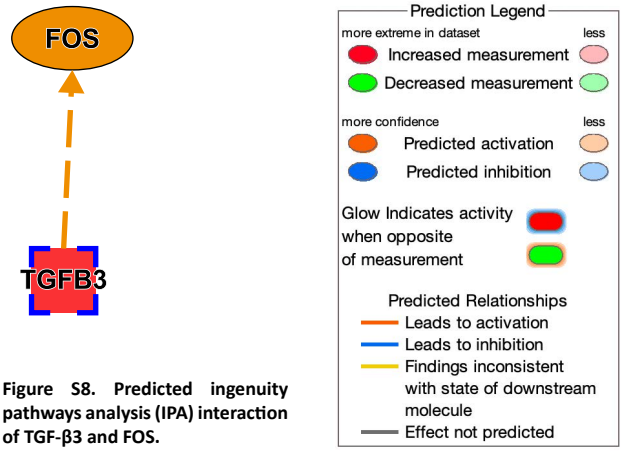


Figure S8. Predicted ingenuity pathways analysis (IPA) interaction of TGF-β3 and FOS.

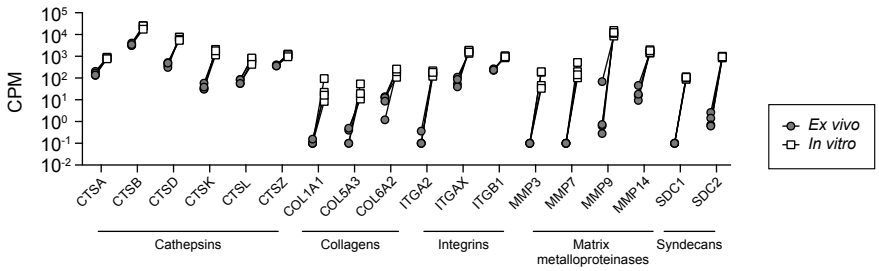


Figure S9. Gene expression values in counts per million (CPM) of extracellular matrix-associated genes in *ex vivo* microglia and *in vitro* microglia.

Table S1. Ligands of interest.

Ligands of interest
BMP2
BMP7
CTGF
FGF2
HMGB2
IL1B
PCDHGA7
SPP1
TGFB1
YARS

Chapter 4

Tissue-specific features of microglial innate immune responses

Raissa Timmerman[1], Saskia M. Burm[2] and Jeffrey J. Bajramovic[1]

[1] Alternatives Unit, Biomedical Primate Research Centre, Rijswijk, The Netherlands

[2] Genmab B.V., Utrecht, The Netherlands

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Abstract

As tissue-resident macrophages of the brain, microglia are increasingly considered as cellular targets for therapeutical intervention. Innate immune responses in particular have been implicated in central nervous system (CNS) infections, neuro-oncology, neuroinflammatory and neurodegenerative diseases. We here review the impact of 'nature and nurture' on microglial innate immune responses and summarize documented tissue-specific adaptations. Overall, such adaptations are associated with regulatory processes rather than with overt differences in the expressed repertoire of activating receptors of different tissue-resident macrophages. Microglial responses are characterized by slower kinetics, by a more persistent nature and by a differential usage of downstream enzymes and accessory receptors. We further consider factors like aging, previous exposure to inflammatory stimuli, and differences in the microenvironment that can modulate innate immune responses. The long-life span of microglia in the metabolically active CNS renders them susceptible to the phenomenon of 'inflammaging', and major challenges lie in the unravelling of the factors that underlie age-related alterations in microglial behavior.

Introduction

Tissue-resident macrophages (TRM) form a heterogeneous population of immune cells that are present in most vertebrate tissues where they fulfill functions of immunological, regulatory, trophic and regenerative nature[1-5]. TRM can derive from the bone marrow, mesenchymal progenitor cells, the fetal liver or the yolk sac (**Table 1**)[6-10], and these differences in origin might affect their functionality. In addition, functional differences between TRM might also reflect tissue-specific adaptations[11].

Microglia are the resident macrophages of the central nervous system (CNS) and play important roles in brain development, homeostasis and pathology[12-15]. They secrete neurotrophic factors, stimulate proliferation of other brain cells, contribute to axon growth, vascularization and myelination[16], and initiate synapse elimination or synaptic pruning which is pivotal to establish efficient neuronal networks[17,18]. Importantly, microglia are immunocompetent cells that are equipped with a variety of activating receptors. Like for other TRM, microglial identity is shaped by a combination of 'nature and nurture'. In this review we will focus on tissue-specific features of microglial innate immune responses.

Table 1. Overview TRM

Anatomical niche	Macrophage name	Origin	References
Adipose tissue	Adipose tissue macrophages	YS, BM	19,20
Bone	Osteoclasts	YS, BM	21-23
Bone marrow	Bone marrow macrophages	YS, FL	8,24
Central nervous system	Microglia	YS	7,25
Granulomas	Epithelioid cells	BM	26
Gut	Intestinal macrophages	YS, FL, BM	9,27,28
Heart	Cardiac macrophages	YS, FL, BM	29
Kidney	Kidney macrophages	YS, FL, BM	25,27,30,31
Liver	Kupffer cells	YS, FL, BM	25,32
Lungs	Alveolar macrophages	YS, FL	10,27,33
Lymph nodes	Subcapsular sinus macrophages	YS, BM	34,35
Pancreas	Pancreatic islet macrophage	YS, FL, BM	25,36
Peritoneal cavity	Peritoneal macrophages	FL, BM	8,9,27
Placenta	Hofbauer cells	YS, BM, MPC	37,38
Skin	Langerhans cells	YS, FL	39
Spleen	Red pulp macrophages	YS, FL	25,27

BM, bone marrow; FL, fetal liver; MPC, mesenchymal progenitor cell; YS, yolk sac.

Microglia origin and environment

Microglia were first described by Pío del Río-Hortega as non-neuronal elements distinct from astroglia and oligodendroglia[40]. Despite intensive research, the origin of microglia has long remained a controversial issue[41]. Over the last decade, fate mapping experiments have demonstrated that microglia arise independently of monocyte input, originating prenatally from yolk sac progenitors[7,25]. Throughout life, microglia are maintained by local self-renewal without replenishment from hematopoietic progenitors[42]. Thereby, they form a distinct population from circulating blood monocytes and hematopoietic macrophages.

The importance of the local environment in shaping the function and phenotype of TRM has received much attention over the past few years[43-50]. High-throughput technologies for studying the transcriptome, epigenome and proteome have been used to characterize various populations of TRM, including microglia, Kupffer cells (from the liver), alveolar, splenic, intestinal and peritoneal macrophages[43,44,51]. These studies show that TRM acquire features that are tailored for assisting tissue homeostasis. Local homeostasis can vary considerably, because tissues differ in metabolic and mechanical activities, environmental exposure to nutrients or microbiota, and the existence of physical barriers[45]. Microglia are chronically exposed to the highly metabolic active CNS environment, which is secluded from peripheral blood by the blood-brain barrier (BBB). They are subject to many inhibitory signals from the CNS environment and, in the absence of strong activating signals, will remain in a homeostatic state. For example, transforming growth factor-β (TGF-β) is constitutively expressed in the CNS and inhibits microglia activation[51-53]. In addition, neuronal cell surface proteins CD47, CD200, and CD22 interact with CD172, CD200 receptor and CD45 on microglia respectively, providing inhibitory signals[54]. Loss or disruption of constitutive inhibitory signaling leads to a more activated microglia phenotype[52,55-57], characterized by an increased expression of activation markers, such as CD45 and CD11b.

The differentiation and tissue-specific activation of macrophages requires precise regulation of gene expression, a process governed by the use of specific transcription factors and epigenetic mechanisms such as DNA methylation, histone modification and chromatin structure[44]. Multiple studies show that changes in the identity and global expression profiles of TRM are dependent on tissue-specific transcription factors[44,48,58,59]. For example, microglia are characterized by the expression of TGF-β regulated transcription factors *Sall1* and *Mef2c*[44,51,60,61]. Accordingly, microglia have a specific epigenetic profile that differs from other TRM and monocytes and that corresponds to their gene expression profile[43,44,62]. TRM that share a similar environment, e.g. macrophages from small and large intestines or different types of peritoneal macrophages, are highly similar in their gene expression and epigenetic landscape[43,44]. Despite their differences in environment, microglia cluster together with other TRM rather than with monocytes and dendritic cells (DCs) in both gene expression and epigenetic studies[44,51,63]. This CNS-resident microglial phenotype can however not be

fully adopted by cells from a different origin. Adult monocyte-derived macrophages that are engrafted in the brain only partially adapt to the transcriptional and chromatin features of microglia and respond differentially to inflammatory stimuli[64-66]. In conclusion, microglial identity is shaped by both nature and nurture.

The microglial innate immune receptor repertoire

As the TRM of the brain, microglia protect the CNS against invading pathogens and injury[67-70]. Microglia express various pattern recognition receptors (PRRs) that recognize pathogen- or danger-associated molecular patterns (PAMPs or DAMPs respectively). Toll-like receptors (TLR) are the most extensively studied PRR in microglia. Ligand binding to TLRs triggers the activation of transcription factors NF-κB, AP1 and IRF3 via multiple intracellular signaling cascades. This subsequently leads to the production of pro-inflammatory mediators and to the upregulation of molecules that are implicated in activation of the adaptive immune system[71-74]. Multiple TLRs are constitutively expressed on microglia or can be induced during viral and bacterial infections, CNS auto-inflammatory responses or neurodegenerative diseases[54].

Microglia also express NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) [54,75-78]. NLRs and RLRs can directly induce the transcription of a range of antiviral or pro-inflammatory cytokines[79-81]. In addition, NLRs can also form large intracellular multiprotein complexes called inflammasomes[82] which are key components of the innate immune system. They link perturbances in cellular homeostasis, sensed by NLRs, to caspase-dependent processing and secretion of pro-inflammatory cytokines such as interleukin (IL)-1β[83]. The final processing step can be triggered by exposure to DAMPs such as high concentrations of extracellular ATP, that can be detected by the P2X7 receptor.

Furthermore microglia express multiple receptors that facilitate phagocytosis of pathogens and cellular debris such as Fc receptors, complement receptors and triggering receptor expressed on myeloid cells 2 (TREM2)[84].

Microglia activation

Classically, activation of microglia has been associated with retraction of their processes, leading to an amoeboid-like morphology[84,85]. Recent studies have however shown that microglia can display a spectrum of morphologies after activation, ranging from hyper-ramified to amoeboid shapes[86-88]. A direct correlation between microglia morphology and activation state is lacking, complicating research aimed at uncovering the activating stimuli.

Activated microglia have initially been categorized similar to other macrophages into M1 and M2 phenotypes. Exposure to interferon (IFN)-γ and lipopolysaccharide (LPS) *in vitro* induces a pro-inflammatory M1 phenotype[89,90] which has long been

associated with neuroinflammation. *In vitro* exposure of microglia to anti-inflammatory cytokines such as IL-4, can induce an alternative, M2 phenotype[89,90]. However, other studies that demonstrate that microglia and other macrophages display a wide range of intermediate phenotypes, both *in vitro* and *in vivo*[88,91-95], have casted doubt on the usefulness of dichotomous characterization methods of microglia activation. Instead, a spectrum of activation states may be observed along with a variety of responses with which microglia can contribute to CNS pathologies.

CNS infections

Infection with neurotrophic viruses can lead to a diversity of pathologies with asymptomatic to lethal outcomes[96]. The pathogenesis is dependent on the viral species as well as on the speed and degree of the inflammatory response that leads to encephalitis. Acutely upon viral infection, microglia produce and secrete a range of chemokines and pro-inflammatory cytokines, such as IL-6, IL-12, tumor necrosis factor (TNF)-α, IFN-β and-γ[67,97]. The IFNs comprise a family of cytokines that play a key role in defense against viral infections in particular[98]. Binding of viral-specific structures, including single-stranded RNA and double-stranded RNA, to PRRs results in induction of IFNs by microglia. IFN-mediated signaling is critical in controlling viral infections by blocking viral processes such as viral entry, translation and efflux[97]. In addition, microglia enhance the expression of antigen presenting proteins[72,99,100]. Compared to other antigen presenting cells (APC), such as DCs and B cells, microglia do not express major histocompatibility complex II (MHCII) constitutively, but only after activation[101,102].

Bacterial infections of the brain can also lead to a variety of CNS pathologies. For example, infection with *Streptococcus pneumoniae* is most commonly associated with meningitis, whereas *Staphylococcus aureus* causes brain abscesses instead. During bacterial infections, microglia are capable of detecting multiple bacterial components[67] of which LPS is the most widely studied. LPS is the primary component of the outer membrane of gram-negative bacteria and initiates TLR4-mediated microglial secretion of IL-6, IL-12, TNF-α, chemokines and prostaglandins *in vitro* as well as *in vivo*[103,104]. Interestingly, prolonged exposure to LPS leads to a different inflammatory response than short-term LPS exposure does[105], and previous bacterial infections could alter microglial responsiveness to subsequent ones, a phenomenon described as 'trained immunity'[106].

Gliomas

Gliomas comprise one of the most aggressive tumor forms in humans. They can develop a complex tumor microenvironment, of which microglia form a significant part, that influences tumor proliferation, invasion and metastasis. Approximately 30% of gliomas are composed of microglia and other glioma infiltrating macrophages, together called 'GAMs'[107-109]. Gliomas release factors, such as monocyte chemoattractant protein (MCP)-1, macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) that recruit GAMs to the tumor[110-112].

GAMs play an important role during glioma pathogenesis by affecting the capacity to activate anti-tumor effector T cells[113] and by promoting the degradation of the extracellular matrix (ECM) via secretion of a.o. several matrix metalloproteinases, pentraxin 3 and versican[114-116]. Moreover, GAMs promote angiogenesis and glioma proliferation through secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-6, epidermal growth factor (EGF) and IL-10[109,117,118]. GAMs are therefore often characterized as pro-tumorigenic [119].

Neuroinflammation

Demyelinated plaques in the white and gray matter of the CNS, also known as lesions, are the hallmark of multiple sclerosis (MS) pathology[120]. Both microglia and peripheral macrophages contribute to the pathogenesis of MS by phagocytosis of myelin debris[121,122]. In early active lesions myelin phagocytosing microglia are predominant, whereas in more mature lesions the majority of myelin phagocytosing macrophages derive from the periphery[123,124], which is in line with the progressive recruitment of peripheral macrophages during the pathogenesis of MS[92,123]. A number of studies have reported that microglia and macrophages differ in their capacity to phagocytose myelin[125-128]. It is noteworthy that activation of macrophages has been demonstrated to impact their phagocytic ability, as classically activated macrophages appear less capable of myelin phagocytosis than alternatively activated macrophages[125]. *In vitro,* microglia are characterized by a better ability for myelin debris uptake than other macrophages, but the underlying mechanism is unclear yet[125,126,129,130].

Although the exact cause of MS remains elusive, there is direct and indirect evidence for the contribution of innate immune responses to MS pathogenesis. Activated microglia are observed in all types of MS lesions[74,131] and the expression levels of TLR2, 3 and 4 are upregulated on those microglia[74,132-134]. In addition, they produce a wide range of pro-inflammatory cytokines, including IL-23 and TNF, and chemokines including MCP-1, MIP-1α and MIP-1β[135-138]. It is unclear yet whether activation is caused by an activating signal or rather by the loss of inhibitory signals. The expression levels of inhibitory signals as CD200 and CD47 are reduced in MS lesions[139,140], and microglia of MS patients are characterized by the expression of low levels of CD200R[141]. The clinical relevance of these inhibitory pathways is underlined by studies in rodent experimental autoimmune encephalomyelitis (EAE), the animal model for MS, that demonstrate that blocking of CD200R, or CD200 deficiency, enhances inflammation and results in increased EAE diseases scores[55,142].

There is also evidence for inflammasome involvement in MS pathogenesis as IL-1β levels are elevated in the cerebrospinal fluid (CSF) and peripheral blood of MS patients and correlate with disease susceptibility, severity and progression[143-146]. In rodent EAE, the expression levels of inflammasome components NALP1, NLPR3, ASC and caspase 1 were increased in the spinal cord[147,148]. In addition, in rhesus macaque EAE, IL-1β was prominently expressed in the CNS and its expression was mainly localized to perivascular lesions[149]. A further role for the inflammasome in MS pathogenesis is indirectly

supported by the delayed onset of EAE and the less severe clinical symptoms in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ or *caspase 1*$^{-/-}$ transgenic mice[147,150,151]. However, while there is consensus on the abundant expression of IL-1β in the brain during EAE, reports on IL-1β expression in MS lesions[152-154] are by no means unequivocal[122,155] and warrant further investigation.

Neurodegeneration

There is a growing body of evidence for the involvement of microglial innate immune responses in neurodegeneration[156,157]. We will focus here on the two most prevalent neurodegenerative diseases, Alzheimer's disease (AD) and Parkinson's disease (PD). Microglial activation has been detected in close proximity to amyloid β (Aβ) aggregates in AD patients[158] and in the substantia nigra of PD patients[159-161]. In addition, TLR expression levels are upregulated in the CNS during AD and PD[74,132-134] and inflammasome activation in microglia has been correlated to AD and PD[162-164].

It has been hypothesized that an imbalance in the production and clearance of Aβ in AD might lead to neuroinflammation-induced neurodegeneration[165,166]. Microglia-mediated phagocytosis of Aβ, cellular debris and apoptotic neurons is mainly accomplished by cell surface receptors, such as TM2D3[167] and TREM2[168], and uptake of Aβ by microglia has been demonstrated in multiple *in vitro* and *in vivo* models[169-173]. Recent genome-wide association studies (GWAS) and transcriptome analyses have uncovered that many of the genes correlated to AD risk encode for proteins that are expressed by microglia[174,175]. One of the best known examples is the *TREM2* gene[176], of which the variant R47H is a risk factor for the development of late-onset AD[177,178]. Mutations in *TREM2,* as well as loss-of-function of TREM2, impair microglial phagocytosis of Aβ and apoptotic neurons. The strongest genetic risk factor for AD is the apolipoprotein *E (APOE)* ε4 allele[179,180]. APOE is involved in the metabolism of lipids, such as cholesterol. The APOE4 isoform affects many microglial processes and is associated with increased activation, impaired metabolic activity, reduced capacity to phagocytose Aβ and deficient clearance of debris[181-185]. Other microglial genes that are identified as risk factors for the development of AD are *ACA7*, *PICALM*, *CD33*, *TM2D3* and *TLR4*[186-190].

As already mentioned, multiple studies have correlated inflammasome activation to AD. In addition to the local production of IL-1β near Aβ aggregates, increased levels of IL-1β are detected in CSF and serum of AD patients[163,191-193]. *In vitro* data demonstrate that phagocytosis of fibrillar Aβ by microglia and macrophages activates the NLRP3 inflammasome and leads to caspase 1 activation and IL-1β secretion[194-196]. In line with these results, expression of IL-1β and increased levels of the inflammasome components NLRC4, ASC and active caspase 1 are observed in brain tissue of AD patients[163]. In APP/PS1 mice, an animal model for AD, *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ animals are protected from memory deficits and are characterized by less neurobehavioral disturbances. This is associated with reduced caspase 1 and IL-1β activation and with enhanced Aβ clearance in the brain[163]. In Tau22 mice, an animal model for frontotemporal dementia (FTD), *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ mice are characterized by reduced

levels of tau pathology and by improved spatial memory functions. In addition, injection of Aβ-containing brain homogenate in the hippocampus of Tau22 mice induced tau pathology, but not when *Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$ Tau22 mice were used[162]. These observations are all in line with an important role for microglia and NLRP3 inflammasome activation in AD pathogenesis. IL-1β-induced signaling can further enhance Aβ production and tau phosphorylation in neurons[197-199], which may lead to a self-sustaining mechanism of inflammasome-mediated activation in AD pathology.

During PD pathogenesis, microglia have been demonstrated to clear dopaminergic cell debris and to engulf α-synuclein, which is among others regulated by TLR4[84,200-202]. Exposure to α-synuclein can directly contribute to neurodegeneration by affecting microglial phagocytic functions[203,204]. In addition, apoptotic neurons and protein aggregates can trigger TLR2, TLR4 and TLR6-mediated inflammation[205]. Studies have demonstrated that *in vitro* exposure of microglia to α-synuclein induces the secretion of IL-1, IL-6 and TNF-α in a dose-dependent manner[206-208], like Aβ does. IL-1β is thought to contribute to dopaminergic neuronal cell death, linking inflammasome activation to PD pathogenesis[209-211]. Aggregated α-synuclein induces TLR2-mediated synthesis of pro-IL-1β and activates the NLRP3 inflammasome leading to caspase 1-dependent IL-1β secretion in *in vitro* cultures of monocytes and macrophages[212,213]. In line with these results, expression of IL-1β is increased in brain tissue of PD patients[211,214]. In MPTP-treated mice, an animal model for PD, NLRP3 inflammasome activation and elevated IL-1β levels have also been described[215]. Interestingly, α-synuclein, tau and Aβ aggregates can all be spread from cell to cell, spreading associated pathology throughout the brain, which is a hallmark of neurodegenerative diseases[216-220] and it has recently been shown that inflammasome activation is involved in the seeding and spreading of Aβ in AD patients[221].

Transcriptomic studies of sorted, purified microglia in AD animal models have identified a novel microglia phenotype, now known as disease-associated microglia (DAM)[222]. Such DAMs are also observed in disease models of amyotrophic lateral sclerosis (ALS)[223], tauopathies[224,225] and MS[226]. The DAM profile is enriched for the expression of genes associated with immune responses, lipid metabolism, phagocytosis and lysosomal processes[224,227,228], while genes associated with homeostasis are downregulated[222,224]. It however remains to be demonstrated how well these data apply to microglia in human neurodegenerative diseases. A recent paper profiled the gene expression levels of human AD-associated microglia and demonstrated that it differed significantly from the DAM profile[229]. This clearly indicates that more studies are needed to shed further light on the specific mechanisms of microglia activation during AD pathogenesis.

Tissue-specific features of microglial innate immune responses

In order to study tissue-specific features of innate immune responses, comparative research using microglia and other populations of macrophages is instrumental. One of the first detailed studies of the microglial receptor repertoire has revealed a variety of transcripts that are highly enriched in microglia as compared to other macrophages[230]. These genes include purinergic receptors (*P2rx7*, *P2ry12*, *P2ry13* and *P2ry6),* chemokine receptors (*Ccr5* and *Cx3cr1*) and phagocytosis receptors (*Trem2, Fcgr1* and *Fcgr3)*. By contrast, the expression levels of *P2rx4*, *Ccr1*, *Cxcr7*, *Ifitm2*, *Ifitm3*, *Ifitm6* and *Tlr8* were significantly higher in macrophages. Although differences in expression levels of innate immune receptors like TLRs have been described between TRM[51,230,231] (see **Figure 1** for a modified heatmap of mouse TRM receptor repertoires based on data from Lavin et al., 2014), the main differences appear to be found in genes that are involved in the regulation of innate immune responses. This is in accordance with the idea that TRM need to fine-tune their innate immune responses in a tissue-specific manner. For microglia this is also reflected by distinct expression profiles of unique silencing modules, such as molecules from the IL-10 and the CD200 axis[44,51,230]. These may critically define the sensitivity and activation threshold of distinct populations of TRM. As stated earlier, microglia receive many inhibitory signals by exposure to TGF-β and via cell-cell interactions with neurons, e.g. CD200R-CD200 interactions[51,54,232], of which the loss can lead to a more activated microglia phenotype[51,55]. Single nucleotide polymorphisms in the *TGFB1* and *TGFB2* gene are associated with AD and PD respectively[233-235], and in the human brain TGF-β2 is mainly expressed by microglia[176]. In culture, exposure of rodent microglia to TGF-β induces the uptake of Aβ[236,237], suggesting a beneficial role for TGF-β in AD. Reduced or deficient TGF-β signaling in mice results in degeneration of the nigrostriatal system and in loss of dopaminergic neurons respectively[238,239], and postnatal silencing of TGF-β signaling results in microglia activation and priming[52]. However, TGF-β signaling is involved in both survival as well as in apoptotic processes and modulatory effects may strongly depend on the timing of the intervention[240-242]. Similar to MS, loss of inhibitory signaling might also contribute to AD and PD pathogenesis. In AD patients, CD200 and CD200R mRNA expression is decreased in the hippocampus and the inferior temporal gyrus[243], indicating that CD200-CD200R signaling may be affected during AD. In a rat model for PD, blocking of CD200R resulted in an increased loss of dopaminergic neurons, in more severe PD-like movement dysfunction and in a significant greater number of activated microglia[244].

Studies from our lab have uncovered some functional differences between inflammasome responses of microglia and those of bone marrow- and blood-derived macrophages (**Figure 2**). We compared the expression profiles of NLRs, adaptor proteins and caspases of microglia and hematopoietic macrophages, and characterized inflammasome activation and regulation in detail[75]. Whereas primary microglia responded to the same innate stimuli as hematopoietic macrophages do, their

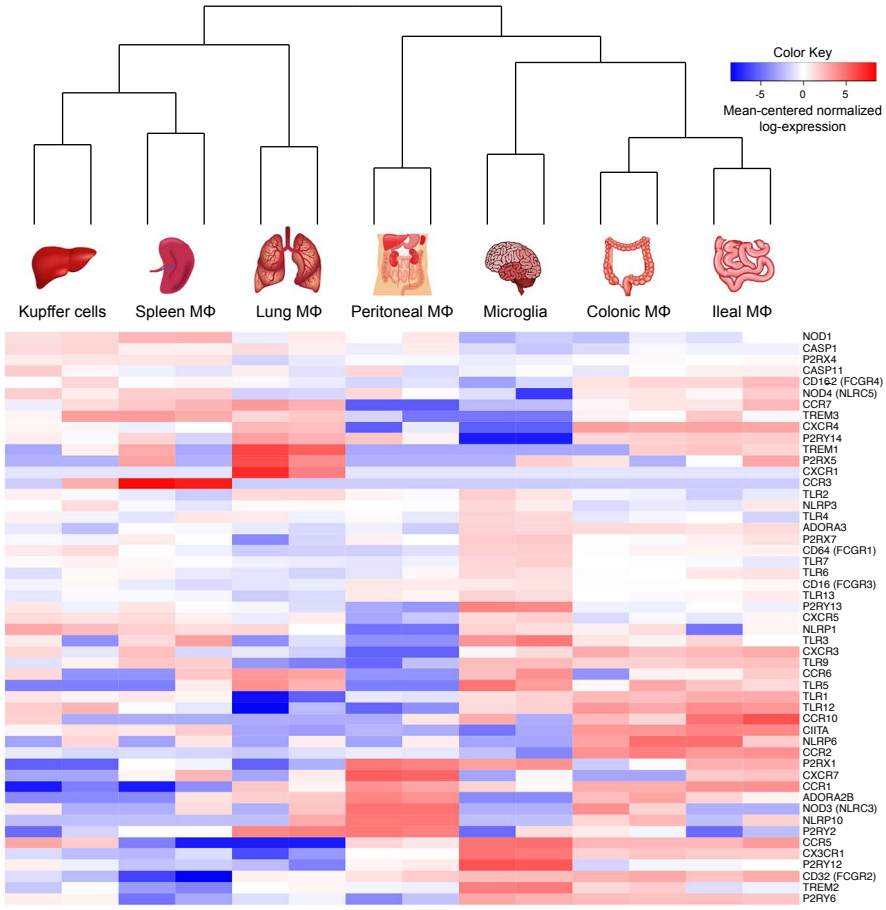

Figure 1. Heatmap of mean-centered, normalized log-expression values of the innate immune receptor repertoire and related genes (adenosine receptors, chemokine receptors, inflammatory caspases, NLRs, phagocytic receptors, purinergic receptors and TLRs) of different populations of TRM as derived from mice (n=2). A normalized RNAseq dataset (GSE63340) of was used[44]. Only genes with an average expression level of ≥ 10 in at least one of the macrophage populations were included for further analysis.

responses were characterized by slower kinetics and by a more persistent nature. The latter was attributable to a lack of negative regulation on pro-IL-1β expression. More importantly, while caspase 1, 4 and 5 were found to be pivotal for inflammasome-mediated IL-1β secretion of hematopoietic macrophages, microglial secretion of IL-1β was only partially dependent on these inflammatory caspases. This suggests that an additional mechanism is involved in the processing of IL-1β in microglia specifically. In another comparative study we demonstrated that microglia respond differently to exposure to ATP than hematopoietic macrophages do[245]. Triggering of ATP-induced

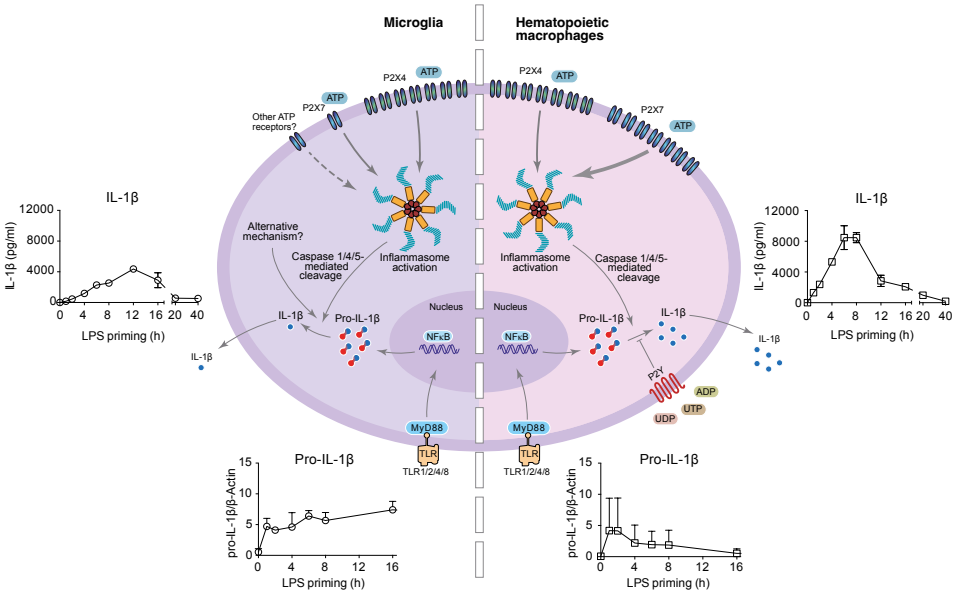

Figure 2. Overview of differences in inflammasome-mediated activation of microglia and hematopoietic macrophages. TLR-mediated signaling strongly induces pro-IL-1β mRNA expression in both microglia and hematopoietic macrophages. However, the kinetics of this response differ between cell types. Whereas pro-IL-1β expression is negatively regulated in hematopoietic macrophages already after 2 h of exposure to LPS, microglia are characterized by persistent expression of pro-IL-1β transcripts and by delayed negative regulation. In both microglia and hematopoietic macrophages, inflammasome activation by e.g. silica, monosodium urate crystals or ATP, leads to activation of caspase 1, 4 and 5, which subsequently induces cleavage of pro-IL-1β to bioactive and secreted IL-1β. Again, the kinetics of this response differ between cell types. In comparison to hematopoietic macrophages, microglial IL-1β secretion is slower and has a more chronic character. Furthermore, while caspase 1, 4, and 5 are pivotal for inflammasome-induced IL-1β secretion by hematopoietic macrophages, microglial secretion of IL-1β is only partially dependent on these inflammatory caspases. This suggests that an additional mechanism is involved in the processing of IL-1β in microglia specifically. Alternative mechanisms for IL-1β processing and secretion include inflammasome-independent mechanisms, such as matrix metalloproteinases, cathepsins and serine proteases and warrant further investigation. Finally, one of our key findings is that ATP-induced IL-1β secretion in microglia is restricted when compared to hematopoietic macrophages. We hypothesize that this may be due to significant lower expression levels of P2X7 on microglia. When P2X7 expression levels are too low, ATP-induced P2X7-mediated signaling might be insufficient to trigger IL-1β secretion and the contribution of other receptors may become more important. Whereas in hematopoietic macrophages ATP-induced IL-1β secretion appeared to be fully dependent on P2X7-induced signaling, microglial ATP-induced IL-1β secretion was the result of combined activation of the P2X4, P2X7 and possibly other – yet unknown – ATP receptors.

IL-1β secretion in microglia was limited when compared to hematopoietic macrophages, and differences were found in the regulation of inflammasome activation by purinergic receptors. Whereas in hematopoietic macrophages ATP-induced IL-1β secretion was fully dependent on P2X7-induced signaling, microglial ATP-induced IL-1β secretion was the result of the combined activation of the P2X4, P2X7 and possibly other – yet unknown – ATP receptors. As microglia are regularly exposed to extracellular ATP, this

might reflect a CNS-specific adaptation to avoid excessive inflammasome activation in this vulnerable and vital organ.

Overall, these adaptations probably contribute to tissue homeostasis as they increase the activation threshold for microglia, rendering initiation of inflammatory processes less likely. However, if microglia do become activated they appear to be relatively refractory to negative regulation. Low level and persistent activation may be the result. Such an inflammatory phenotype is consistent with the chronic nature of neurodegenerative diseases.

Additional considerations

The CNS is not a static, homogeneous environment and factors like aging, previous exposure to inflammatory stimuli, and differences in the microenvironment have all been reported to modulate innate immune responses. The process of 'inflammaging' is a well-known chronic low-grade inflammation that develops with advanced age, and is believed to accelerate the process of biological aging and to worsen many age-related diseases[246,247]. Aged microglia have indeed been described to differ substantially from their younger counterparts[248-253], and age-associated microglial effects are reversible by replacement of aged microglia with younger ones[254,255]. Aged or senescent microglia are characterized by a dystrophic morphology, by reduced process motility, and by reduced migration and phagocytosis capacities[256-260]. This is in line with the observed downregulation of genes that are involved in the sensing of apoptotic neurons, such as *Trem2* and *Dap12*[230]. In addition, aged microglia are characterized by an increase of basal production levels of pro-inflammatory cytokines, such as IL-6 and IL-1β[261-263], and by a decline in the production levels of anti-inflammatory cytokines, such as IL-10 and TGF-β[264,265]. Also expression levels of MHCII and TLRs are increased[266,267]. Upon inflammatory challenge, the responses of aged microglia are exaggerated and prolonged when compared to that of adult microglia[268-271]. Importantly, aged microglia show impairments in returning to a homeostatic state, which might contribute to the chronic inflammation which is a hallmark of neurodegenerative diseases. The high mobility group box 1 (HMGB1) protein has been shown to play a role in determining the phenotype of aged microglia as blocking of HMGB1 reversed the phenotype and could prevent exaggerated immune responses[272]. Interestingly, a recent publication has shown a similar potential for reversibility of the aged phenotype by targeting CD22, a negative regulator of phagocytosis of which the expression levels are enhanced on aged microglia. Blocking of CD22 promoted the clearance of myelin debris, Aβ oligomers and α-synuclein fibrils *in vivo*[273].

During aging, there is a decline in total sialic acid content and sialoglycoproteins in the brain[274]. Moreover, glycoprotein patterns in the brain of rats are markedly altered by aging[275]. Interestingly, key proteins involved in AD, such as TREM2, APP, BACE1 and PrP^c are sialoglycoproteins[276], and changes in the sialylation of the pathogenic form of the prion protein resulted in an increase of inflammatory responses of microglia[277].

In addition, glycomics studies have revealed a change in glycans of several glycoproteins in AD patients[278,279] that might well affect microglial innate immune responses.

Also, significant epigenetic changes at all levels of chromatin and DNA organization have been described in aged microglia[280]. The expression of the deacetylase sirtuin 1 (SIRT1), that plays a key role in both senescence and inflammation[281], is reduced in aged microglia which was associated with memory deficits that were mediated by upregulation of IL-1β[282]. Epigenetics also underlie the earlier mentioned phenomenon of 'trained immunity', often referred to as 'priming'. Originally described for monocytes[283], it has now been convincingly demonstrated that also microglia respond differently to innate immune stimuli based on previous exposure to similar stimuli. Priming of microglia with the TLR4 agonist LPS leads to a different, suppressed response to a second exposure[284] and this suppressive effect can be long-lasting[285]. These acquired alterations in innate immune responses have also been observed *in vivo*[286-290], and add another layer of complexity.

Furthermore, there are indications that even the local microenvironment in the CNS can have an impact on innate immune responses[291-293]. Microglia that were isolated from different regions of the CNS were characterized by differences in immunoregulatory protein expression levels[294,295], and when gene expression profiles of microglia that reside in the myelinated white matter of the CNS were compared to those of microglia that reside in the gray matter, higher expression levels of NF-κB pathway genes and lower expression of type I IFN genes were found in white matter microglia[296-298]. The presence or absence of contact with immune modulatory moieties in myelin or neurons in the direct vicinity might underlie such differences. Finally, recent single-cell RNA sequencing studies have revealed considerable heterogeneity within microglia populations[94,252,293,299-302]. It is at present unclear whether different clusters of microglia are indeed relatable to different functions, but it is clear that the viewpoint of microglia as a relatively homogenous population of cells across the entire CNS is too simplistic.

Concluding remarks

Over the last decade we have made substantial progress in unraveling the factors that determine microglial identity. Pinpointing the origin of microglia has clarified the 'nature' of this specific type of TRM. The 'nurture' of microglia is a booming, rapidly expanding research field in which much remains to be discovered. The application of state-of-the-art technology is yielding many surprising results, of which the interpretation is still ongoing. Research into the role of microglial innate immune responses during neurodegenerative diseases should ideally take into account that 'nurture' affects microglia as well as their direct surroundings in many different ways. Mimicking such effects *in vitro* presents us with major challenges, especially if attempted via stem cell approaches. Whether some of the challenges can be circumvented by using primary microglia from adult source material remains an outstanding question.

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Chapter 5

P2Y6 receptor-mediated signaling amplifies TLR-induced pro-inflammatory responses in microglia

Raissa Timmerman[1], Ella A. Zuiderwijk-Sick[1] and Jeffrey J. Bajramovic[1]

[1]Alternatives Unit, Biomedical Primate Research Centre, Rijswijk, The Netherlands

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Abstract

TLR-induced signaling initiates inflammatory responses in cells of the innate immune system. These responses are amongst others characterized by the secretion of high levels of pro-inflammatory cytokines, which are tightly regulated and adapted to the microenvironment. Purinergic receptors are powerful modulators of TLR-induced responses, and we here characterized the effects of P2Y6 receptor (P2RY6)-mediated signaling on TLR responses of rhesus macaque primary bone marrow-derived macrophages (BMDM) and microglia, using the selective P2RY6 antagonist MRS2578. We demonstrate that P2RY6-mediated signaling enhances the levels of TLR-induced pro-inflammatory cytokines in microglia in particular. TLR1, 2, 4, 5 and 8-induced responses were all enhanced in microglia, whereas such effects were much less pronounced in BMDM from the same donors. Transcriptome analysis revealed that the overall contribution of P2RY6-mediated signaling to TLR-induced responses in microglia leads to an amplification of pro-inflammatory responses. Detailed target gene analysis predicts that P2RY6-mediated signaling regulates the expression of these genes via modulation of the activity of transcription factors NFAT, IRF and NF-κB. Interestingly, we found that the expression levels of heat shock proteins were strongly induced by inhibition of P2RY6-mediated signaling, both under homeostatic conditions as well as after TLR engagement. Together, our results shed new lights on the specific pro-inflammatory contribution of P2RY6-mediated signaling in neuroinflammation, which might open novel avenues to control brain inflammatory responses.

Introduction

Toll-like receptors (TLR) comprise a family of pattern recognition receptors that are involved in pathogen recognition by innate immune cells in particular[1]. For human and non-human primates, ten members of the TLR family have been described[2,3]. TLR activation initiates a cascade of intracellular signaling events that culminate amongst others in the activation of transcription factors nuclear factor (NF)-κB, activator protein (AP)-1 and interferon regulatory factors (IRFs), which in turn induce the expression of inflammatory soluble mediators such as the cytokines interleukin (IL)-1α, IL-6, IL-8, IL-12 and tumor necrosis factor (TNF)-α[4,5]. TLR-induced responses must be strictly regulated since uncontrolled activation can amongst others lead to chronic inflammation[6].

During inflammatory conditions, including TLR activation, extracellular levels of adenosine and other nucleotides, such as uridine diphosphate (UDP), rapidly rise[7,8]. These molecules can trigger autocrine or paracrine signaling through two families of purinergic receptors, P1 and P2 respectively, that are expressed by many types of immune cells including macrophages. The family of P1 receptors includes four subtypes of G protein-coupled adenosine receptors, whereas the family of P2 receptors includes seven subtypes of ATP-selective ligand-gated ion-conducting P2X receptors, and eight subtypes of G-protein-coupled P2Y receptors. Signaling by purinergic receptors can modulate the secretion of cytokines, migration, phagocytosis and apoptosis by the expressing cell[9,10]. It is therefore not surprising that purinergic signaling is involved in the pathophysiology of multiple disorders, such as the neurological diseases Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis[11-13]. In this context, many studies have reported on the potential of purinergic receptor-mediated signaling to modulate TLR-induced responses in microglia, the resident macrophages of the brain[14-19]. For example, in rodents, inhibition of P2Y6 receptor (P2RY6)-mediated signaling by deletion or antagonist approaches reduced lipopolysaccharide (LPS)-induced neuronal loss in the substantia nigra and the production of pro-inflammatory cytokines, respectively[16,19].

The contribution of P2RY6-mediated signaling to the release of pro-inflammatory cytokines in microglia has been attributed to ERK1/2, calcium/NFAT, MAP kinases and NF-κB activation[16,20,21], but the exact mechanisms by which P2RY6-mediated signaling contributes to TLR-induced neuroinflammatory processes remain largely unknown. As studies emphasize the importance of P2RY6-mediated signaling in neuro-inflammation, we choose to directly compare the effects of P2RY6-mediated signaling on TLR-induced immune responses in microglia to those in bone marrow-derived macrophages (BMDM). We isolated primary cells from rhesus macaques, outbred animals that are evolutionary close to humans[22,23], and used the selective P2RY6 antagonist MRS2578 to demonstrate that P2RY6-mediated signaling broadly amplifies the production of TLR-induced pro-inflammatory cytokines in microglia, while such effects were much less pronounced in BMDM. Transcriptome analysis reveals the breadth of the pro-inflammatory contribution of P2RY6-mediated signaling to TLR-

induced responses in microglia, and predicts that enhanced activation of the transcription factors NFAT, IRFs and NF-κB is a likely explanation for this. Interestingly, we also observed that P2RY6-mediated signaling strongly inhibits the mRNA expression levels of heat shock proteins (HSP), both under homeostatic conditions as well as after TLR engagement. As this phenomenon was observed both in BMDM as well as in microglia, it is probably not directly related to the P2RY6-mediated amplification of pro-inflammatory responses but may well be relevant when considering the therapeutical use of P2RY6 inhibitors.

Materials and methods

Reagents

P2RY6 antagonist *N*,*N*''-1,4-Butanediyl*bis*[*N*'-(3-isothiocyanatophenyl)thiourea (MRS2578) (Tocris Bioscience, Bristol, UK) was reconstituted in DMSO at a concentration of 50 mM. MRS2578 is a selective – yet not entirely specific – antagonist of P2Y6 nucleotide receptors with an IC_{50} value of 37 nM at human P2RY6. It displays insignificant activity at P2Y1, P2Y2, P2Y4 and P2Y11 receptors (IC_{50} > 10 μM). DMSO controls were included in all assays where MRS2578 was used. TLR agonists used were Pam_3CSK_4 (TLR1/2), LPS (TLR2/4), ultrapure LPS (TLR4), Flagellin (TLR5), and CL075 (TLR8; all Invivogen, San Diego, CA). Used concentrations of the TLR agonists can be found in the figure legends.

Animals

Brain tissue and bone marrow were obtained from adult rhesus macaques (*Macaca mulatta*) of either sex without neurological disease that became available from the outbred breeding colony or from other studies (all studies were ethically reviewed and approved by the Ministry of Agriculture, Nature and Food Quality of the Netherlands). No animals were sacrificed for the exclusive purpose of the initiation of primary cell cultures. Better use of experimental animals contributes to the priority 3Rs program of the Biomedical Primate Research Centre. Individual identification data of the animals are listed in **Table 1**.

Primary cells isolation and cell culture

Rhesus macaque primary microglia were isolated as described previously[22,23]. In short, Frontal subcortical white matter samples were collected in primary microglia medium (PMM) comprised of 1:1 v/v DMEM (high glucose)/HAM F10 Nutrient mixture (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% v/v heat inactivated FBS (TICO Europe, Amstelveen, The Netherlands), 2 mM glutamax, 50 units/mL penicillin and 50 μg/mL streptomycin (all from Gibco). Microglia isolations were initiated from cubes of ~4.5 g tissue that were depleted of meninges and blood vessels manually. Tissue was chopped into cubes of less than 2 mm^2 using gentleMACS™ C tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated at 37 °C for

Table 1. Individual identification data of rhesus macaques

Monkey ID nr.	Age (years)	Sex	Weight (kg)	Origin
R00043	21	F	11,6	India
R01085	21	F	6,7	India
R02008	19	M	14,2	India
R02046	19	F	11,2	India
R02060	18	F	8,2	India
R03098	17	F	7,3	India
R06050	15	F	10,9	India
R06054	15	F	7,0	India
R07110	13	M	10,7	India
R12016	9	M	8,1	India
R13169	8	M	15,0	India
R14143	6	F	4,8	India
R15009	6	M	10,0	India
R15143	6	M	6,6	India
R15150	6	M	9,2	India
R17023	5	M	8,0	India
R18015	3	M	4,9	India

20 min in PBS containing 0.25% (w/v) trypsin (Gibco) and 1 mg/mL bovine pancreatic DNase I (Sigma-Aldrich, Saint Louis, MO) and mixed every 5 min. The supernatant was discarded (no centrifugation), the pellet was washed in PMM and passed over a 100 µm nylon cell strainer (Falcon; Becton Dickinson Labware Europe) and centrifuged for 7 min at 524 *g.* The pellet was resuspended in 22% (vol/vol) Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 37 mM NaCl and 75% (vol/vol) myelin gradient buffer (5.6 mM NaH_2PO_4, 20 mM Na_2HPO_4, 137 mM NaCl, 5.3 mM KCl, 11 mM glucose, 3 mM BSA Fraction V (Sigma-Aldrich), pH 7.4). A layer of 100% myelin gradient buffer was added on top and centrifuged at 1561 *g* for 30 min (minimal brake). The pellet was washed in PMM and centrifuged for 7 min at 524 *g.* Cells were plated at a density of 6.5 * 10^4 cells/cm^2 in tissue-culture treated well plates (Corning Costar Europe, Badhoevedorp, the Netherlands) in PMM. After overnight incubation at 37 °C in a humidified atmosphere containing 5% CO_2, unattached cells and myelin debris were removed by washing with PBS twice and attached cells were cultured in fresh PMM supplemented with 20 ng/mL macrophage colony-stimulating factor (M-CSF; PeproTech, London, UK). Cells were kept in culture for 8 days without passaging. Half of the medium was replaced by fresh medium containing M-CSF every 3-4 days.

Rhesus macaque primary bone marrow-derived macrophages were isolated by flushing the bone marrow from the femur (~4 cm) with PBS, followed by passing the suspension over a 100 µm nylon cell strainer (Falcon) and density gradient centrifugation using Lymphoprep (Axis Shield PoC AS, Oslo, Norway) according to manufacturer's protocol. Cells were plated at a density of 1.3 * 10^5 cells/cm^2 in tissue-culture treated well plates (Corning Costar Europe) in RPMI 1640 (Gibco) supplemented with 10% v/v heat inactivated FBS (Tico), 2 mM glutamax, 50 units/ml penicillin, 50 *µg*/ml streptomycin (all Gibco) and 20 ng/ml M-CSF (Peprotech). Half of the medium was replaced by fresh medium containing M-CSF at day 4. Cells were kept in culture for 8 days without passaging.

Knockdown of P2RY6 in primary microglia

Transfection of siRNAs in adult rhesus macaque primary microglia was performed using the Glial-Mag kit (OZ Biosciences, San Diego, CA) as described by Carrillo-Jimenez and colleagues[24], with a few modifications. The described method here refers to a 24-well plate format. For one well, 72 nM siRNA (Horizon Discovery, Waterbeach, UK) was added to a microcentrifuge tube (for siP2RY6, 18 nM of each siRNA was used). 100 µl DMEM (Gibco) without supplements was added to the tube with siRNA and mixed by vortexing. The contents of this tube were added to a new microcentrifuge tube containing 0.6 µl Glial-Mag (OZ Biosciences) and mixed gently by pipetting up and down five times. The mixture was incubated for 20 min at room temperature. 100 µl culture medium was removed from the well to assure a final volume of 400 µl. 100 µl siRNA + Glial-Mag mixture was added drop by drop to the well. Subsequently, 5 µl Glial-Boost (100x) (OZ Biosciences) was added to the well. To ensure even distribution, the culture plate was moved back and forward a few times. The culture plate was placed on top of the magnetic plate (provided by the Glial-Mag kit) inside the cell incubator for 30 min. The magnetic plate was removed, and the culture plate was placed in the incubator for 3 more hours at 37 °C. After 3 h incubation, culture medium was replaced for fresh PMM medium containing 20 ng/mL M-CSF. Stimulation experiments with 10 µg/mL uLPS were performed 24 h after the medium change. The different siRNAs used for this study were siGLO Green Transfection Indicator (#D-001630-01-05), ON-TARGETplus Non-targeting Pool (D#001810-10-05) and 4x Custom ON-TARGETplus P2RY6 siRNA specifically designed for rhesus macaques (all from Horizon Discovery). All siRNAs were reconstituted in 1x siRNA Buffer (Horizon Discovery). The sequences of the different siRNAs are provided in **Table 2**.

Table 2. Sequences of the different small interfering RNAs (siRNAs)

siRNA	Sequence
siRNA non-targeting (1)	UGGUUUACAUGUCGACUAA
siRNA non-targeting (2)	UGGUUUACAUGUUGUGUGA
siRNA non-targeting (3)	UGGUUUACAUGUUUUCUGA
siRNA non-targeting (4)	UGGUUUACAUGUUUUCCUA
siP2RY6 (1)	UUACGCUGGAUGCCUGUGGUU
siP2RY6 (2)	UUUGGCUGUGAGUUUCUGUUU
siP2RY6 (3)	AGUCGCUUGAAGUUCUCGCUU
siP2RY6 (4)	UAGCGCUGGAAGCUGAUGCUU

RNA isolation and quantitative RT-PCR

Total cellular RNA was isolated using the RNeasy minikit (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol. Subsequently, mRNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA synthesis kit according to the manufacturer's protocol (Fermentas; Thermo Fisher Scientific). RT-PCRs were performed on the CFX96™ Real-time PCR detection system (Bio-rad Laboratories, Hercules, CA) using primer (Invitrogen; Thermo Fisher Scientific) and probe (human Exiqon probe library, Roche, Woerden, The Netherlands) combinations listed in **Table 3**, and iTaq Universal Probes Supermix (Bio-rad Laboratories). Relative gene expression was standardized to ACTB using the Pfafll method[25].

Table 3. Overview of primer/probe combinations used for RT-PCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Probe
ACTB	GCCCAGCACGATGAAGAT	CGCCGATCCACACAGAGTA	AGGAGGAG
CRYAB	ATGGCAAACATGAAGAGCGC	GTAATGGTGAGAGGGTCCACA	TCTCCAGG
DNAJA4	TTTCAGATCCAAAGAAAAGGGATATTT	ATGTCCATGGGTGAAGAGAAGCTG	GCTGCCTG
DNAJB1	CATCGAAGTGAAGAAGGGGTG	AACAAAGACGATATCAGCTGGAA	GGAAGGAG
HSP90AA1	CATGAAGACTCACAAAATCGGAAG	TTTCCTTCATTCTGGTGCAGT	CTGCCTCT
HSPA5	TCCGGTCTACTATGAAGCCC	AATTCGAGTCGAGCCACCAA	CTTCCAGC
HSPB1	CGGACGAGCTAACGGTTAAG	GTGAAGCACCGGGAGATGTAG	CTCCACCA
HSPD1	GTTGGTCTTCAGGTTGTGGC	CTCCAAACACTGCACCACCA	CCTGGAGC
HSPH1	CTCACAGTCTCCCCCTTCAC	GGCAGCTCGACATTCACCA	GGAGGCTG
IL-1α	AATAACCTGGAGGCCATCG	GCTAAAAGGTGCCGACCTG	CAGAGGAA
IL-6	ACAAAAGTCCTGATCCAGTTCC	GTCATGTCCTGCAGCCACT	CAGCAGGC
IL-8	TCTGTGTAAACATGACTTCCAAGC	CACTCCTTGGCAAAACTGC	CAGAGGAA
IL-12p40	CCACATTCCTACTTCTCCCTGA	ACCGTGGCTGAGGTCTTGT	TCCAGGTC
P2RY6	AGCTGTCTTTGCTGCCACA	GGGCTGAGGTCATAGCAAAC	GCTGGATG
TNF-α	AAGCCTGTAGCCCATGTTGT	GCTGGTTATCTGTCAGCTCCA	CCAGGAGG

Cytokine analysis

Old world monkey sandwich ELISA kits for IL-6, IL-8, IL-12p40 and TNF-α, (U-CyTech, Utrecht, The Netherlands) were used for quantification of the cytokines in cell culture supernatants according to manufacturer's instructions. Cytokine levels were analyzed using the ELx800™ Absorbance Microplate Reader (Biotek, Winooski, VT).

Next generation RNA sequencing

The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) was used to prepare and process the total RNA samples. Briefly, mRNA was isolated from total RNA using oligo(dT) magnetic beads. After fragmentation of the mRNA, cDNA synthesis was performed followed by ligation of sequencing adapters and PCR amplification. The quality and yield after sample preparation were measured with a fragment analyzer (Agilent Technologies, Amstelveen, The Netherlands). Clustering and sequencing using the Illumina NovaSeq 6000 was performed according to manufacturer's protocols. Prior to alignment, the reads were trimmed for adapter sequences using fastp v0.20[26], using default parameters. The *Macaca mulatta* genomic reference (Mmul_10) was used for alignment of the reads for each sample. The reads were mapped to the reference sequence using a short-read aligner based on Burrows-Wheeler Transform (STAR2 v2.5.4) with default settings. SAMtools v1.10 package (http://htslib.org/, RRID:SCR_002105) was used to sort and index the BAM files. Based on the mapped locations in the alignment file the frequency of how often a read was mapped on a transcript was determined with HTSeq v0.11.0 (https://htseq.readthedocs.io/en/release_0.11.1/, RRID:SCR_005514). Only unique reads that fall within exon regions were counted. The counts were saved to count files, which were served as input for downstream RNA sequencing analysis.

Cell viability assay

To assess the cytotoxicity of MRS2578 on primary bone marrow-derived macrophages and microglia, cell viability was investigated using the live/dead viability/cytotoxicity kit (Thermo Fisher Scientific). In short, cells were rinsed twice with PBS. Subsequently, 2 µM of calcein AM and 4 µM of ethidium homodimer-1 (both part of the live/dead assay kit) in PBS were added to each well and incubated for 45 min at room temperature protected from light. Samples were rinsed with PBS and the nuclei were stained by incubation with 1 µM Hoechst 33342 (Thermo Fisher Scientific) in PBS for 10 min at room temperature. Samples were rinsed with PBS and fixed with 2% paraformaldehyde in PBS (Affymetrix, Santa Clara, CA) for 30 min at room temperature. Samples were rinsed twice with PBS and live and dead cells were visualized using a Leica DMI6000 fluorescence microscope and LASX software.

TUNEL assay

To label fragmented DNA of apoptotic cells, the DeadEnd Fluorometric TUNEL System kit (Promega, Madison, WI) was used according to the manufacturer's protocol. Briefly, cells grown on coverslips were fixed in 2% paraformaldehyde solution in PBS for 30 min at room temperature, rinsed twice with PBS and then treated with 0.2% Triton X-100 in PBS for 5 min at room temperature. To generate positive controls, the samples were incubated in DNase I buffer (40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$) for 5 min and treated with DNase I (± 6 units/mL; Qiagen GmbH) in DNase I buffer for 10 min. After two washes with PBS, samples were incubated in equilibration buffer (part of the TUNEL assay kit) for 10 min at room temperature. The reaction mix was prepared according to manufacturer's protocol. Staining was carried out for 1 h at 37 °C in a humidified chamber protected from light. The reaction was arrested by triple incubation in 2x SSC (part of the TUNEL assay kit) for 5 min. Samples were rinsed twice with PBS and mounted using ProLong™ Diamond Antifade + DAPI (Thermo Fisher Scientific). Images were acquired using a Leica DMI6000 fluorescence microscope and LASX software.

Bioinformatics

BiomaRt Bioconductor Package (https://bioconductor.org/packages/release/bioc/html/biomaRt.html, RRID:SCR_019214) was used to annotate the genes and to generate a gene symbol list[27,28]. The accession number for the RNA-sequencing data from cultured primary microglia from rhesus macaques exposed to 10 µg/mL uLPS in the presence or absence of 5 µM P2RY6 antagonist MRS2578 reported in this paper is GSE195866. Data were inspected using principal component analysis and heatmaps generated with heatmap.2 of Bioconductor package gplots. Differential gene expression analysis was performed with Bioconductor package EdgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html, RRID:SCR_012802)[29]. The Molecular Signatures Database (MsigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp, RRID:SCR_016863) was used to perform gene ontology analysis, canonical pathway analysis and transcription factor target analysis[30,31].

Statistics

GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. Statistical details of experiments can be found in the figure legends.

Results

Microglia are particularly sensitive to P2RY6-mediated amplification of the production of TLR-induced pro-inflammatory cytokines

It has been reported that P2RY6-mediated signaling modulates LPS (TLR2/4)-induced cytokine responses in resident macrophages[16,32], but studies that compare modulation of such responses in different subpopulations of macrophages are lacking. We therefore analyzed the involvement of P2RY6-mediated signaling on pro-inflammatory cytokine production as induced by a broad range of TLR agonists in primary BMDM and microglia from rhesus macaques. Engagement of TLR1/2 (by Pam_3CSK_4), TLR2/4 (by LPS), TLR4 (by ultrapure (u)LPS), TLR5 (by Flagellin), and TLR8 (by CL075) potently induced IL-6, IL-8 and IL-12p40 production in both cell types (**Figure 1A** and **1B**), while exposure to TLR1/2 agonist induced the production of TNF-α in microglia only. Exposure of BMDM to the selective P2RY6 antagonist MRS2578 inhibited the production of TLR8-induced IL-6 and TLR5-induced IL-12p40, whereas TLR2/4-induced IL-8 was enhanced (**Figure 1A**). By comparison, exposure of microglia to MRS2578 had much more potent effects and reduced the TLR1/2-, TLR4- and TLR8-induced production of IL-6, the TLR1/2-, TLR2/4-, TLR5- and TLR8-induced production of IL-8, the TLR1/2-, TLR2/4-, TLR4-, TLR5- and TLR8-induced production of IL-12p40, and the TLR5- and TLR8-induced production of TNF-α (**Figure 1B**). Mean log fold changes of **Figure 1A** and **Figure 1B** are presented in **Table S1**. Of note, we intended to analyze IL-1α production as well, but this was not possible since commercial ELISA reagents are not available for non-human primates. Inhibition of TLR-induced cytokine responses could not be attributed to MRS2578-associated cytotoxicity. Exposure to up to 5 μM MRS2578 did not affect the numbers of viable cells, neither for BMDM nor for microglia (**Figure S1**). Only when cells were exposed to concentrations as high as 25 μM MRS2578, decreases in cell viability were observed. We next questioned whether the differential sensitivity to P2RY6-mediated signaling between BMDM and microglia could be attributed to different P2RY6 mRNA expression levels, but these were comparable between BMDM and microglia (**Figure S2**).

To investigate whether the reduced production levels of TLR-induced pro-inflammatory cytokines in microglia in the presence of P2RY6 antagonist were correlated to reduced mRNA expression levels we used a real-time PCR approach. Engagement of different TLRs potently induced the mRNA expression levels of IL-6, IL-8, IL-12p40, TNF-α and IL-1α (**Table S2**), and exposure to P2RY6 antagonist significantly reduced the mRNA expression levels of TLR2/4- and TLR4-induced IL-6 (**Figure 1C**). In addition, TLR2/4-, TLR4-, TLR5- and TLR8-induced IL-12p40 and TLR1/2-, TLR2/4-, TLR4-, TLR5- and TLR8-induced IL-1α mRNA expression levels were also significantly reduced in the presence of P2RY6 antagonist. Although we observed some minor discrepancies between production (ELISA) and mRNA expression (RT-PCR) levels, the overall data clearly demonstrate that P2RY6-mediated signaling broadly affects TLR-induced pro-inflammatory cytokine production and mRNA expression levels, in microglia in particular.

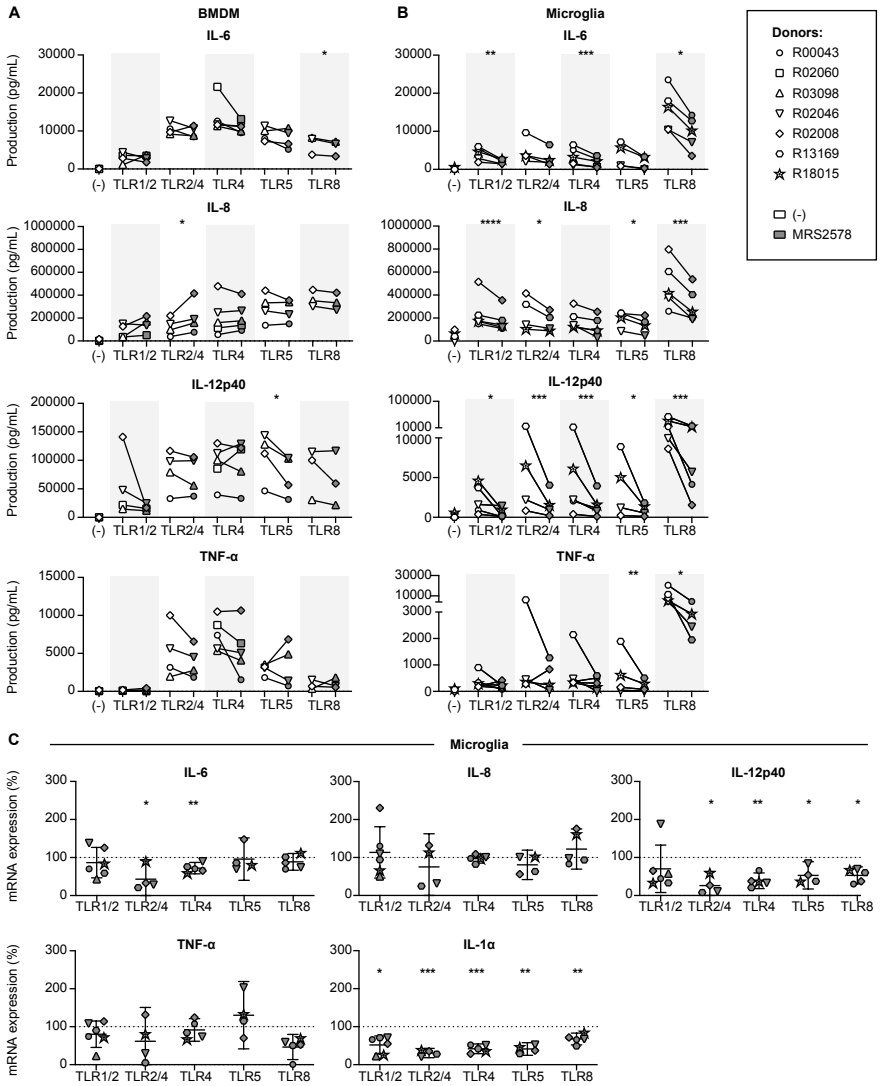


Figure 1. P2RY6-mediated signaling is broadly involved in microglia TLR-induced pro-inflammatory cytokine production and mRNA expression. Primary bone marrow-derived macrophages (BMDMs) and microglia from rhesus macaques were exposed for 16 h to different TLR ligands in the absence (white symbols) or presence (gray symbols) of 1 h pre-incubation of 5 μM P2RY6 antagonist MRS2578. TLR ligands used were 1 μg/mL PAM_3CSK_4 (TLR1/2), 10 ng/mL LPS (TLR 2/4), 10 ng/mL ultrapure LPS (TLR4), 100 ng/mL Flagellin (TLR5) or 1 μg/mL CL075 (TLR8). IL-6, IL-8, IL-12p40 and TNF-*α* production levels of **A)** BMDM and **B)** microglia are shown in pg/mL. Symbols represent different donors. n=3-5, paired t-test on log-transformed data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. **C)** Microglia mRNA expression levels of pro-inflammatory cytokines in the presence of 5 μM MRS2578 are expressed relative to mRNA expression after exposure to each TLR ligand alone (dotted line = 100%). Symbols represent different donors. Horizontal lines indicate mean values with 95% confidence intervals. n=4-6, paired t-test on log-transformed data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

To further characterize the effects of P2RY6-mediated signaling on TLR-induced responses, we continued with the robust and specific TLR4 agonist uLPS. Microglia were stimulated with uLPS in the presence of escalating concentrations of MRS2578. Exposure to uLPS strongly induced the production of IL-6, IL-8, IL-12p40 and TNF-α (**Table S3**), whereas exposure to MRS2578 inhibited the uLPS-induced production of IL-6 and IL-12p40 in a dose-dependent manner (**Figure 2A**). This was confirmed at the transcription level. IL-6, IL-8, IL-12p40, TNF-α and IL-1α mRNA expression levels were strongly induced after uLPS exposure (**Figure S3**), and in the presence of MRS2578, uLPS-induced mRNA gene expression levels of IL-6, IL-12p40 and IL-1α were dose-dependently inhibited (**Figure 2B**).

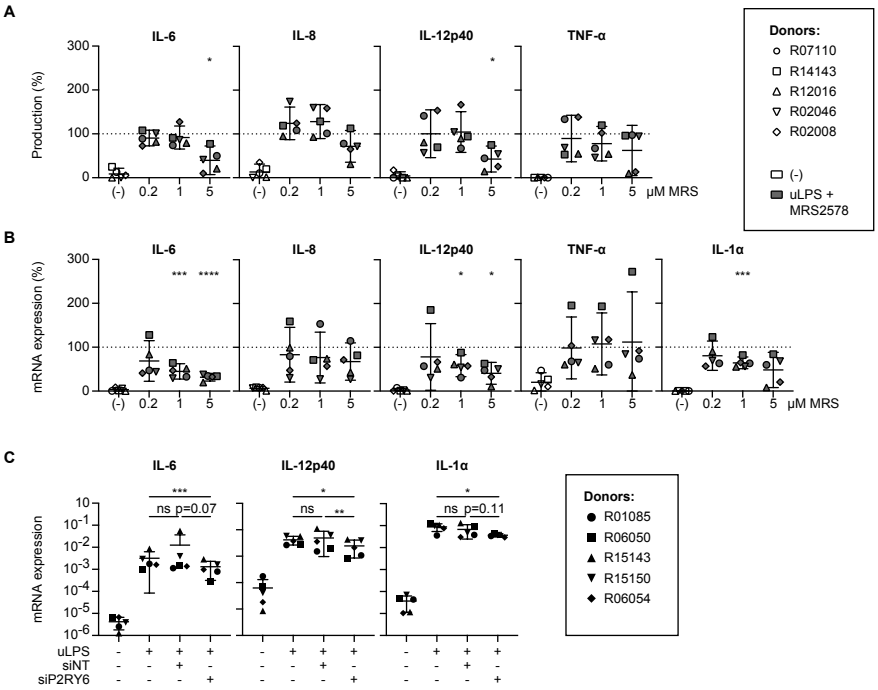


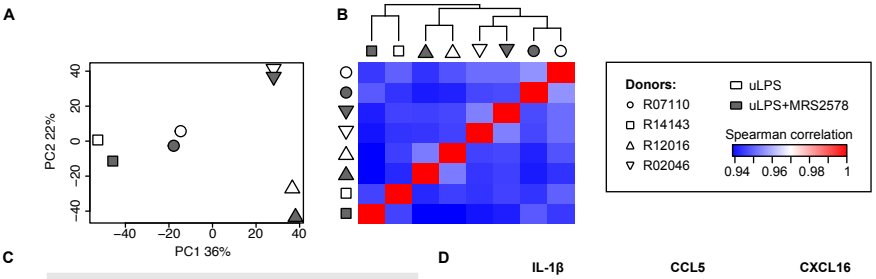
Figure 2. uLPS-induced IL-6, IL-12p40 and IL-1α expression levels are dependent on P2RY6-mediated signaling. Primary microglia from rhesus macaques were exposed for 16 h to 10 μg/mL uLPS (TLR4) with or without 1 h pre-incubation of 0.2, 1 or 5 μM P2RY6 antagonist MRS2578. The effects of P2RY6 antagonist on uLPS-induced IL-6, IL-8, IL-12p40, TNF-α and IL-1α were analyzed. Graphs show the effects of P2RY6-mediated signaling on TLR4-induced **A)** cytokine production and **B)** mRNA expression levels. Levels of all graphs are expressed relative to levels after exposure to uLPS alone (dotted line =100%). Symbols represent different donors. Horizontal lines indicate mean values with 95% confidence intervals. n=5, paired t-test on log-transformed data, * $p < 0.05$, *** $p < 0.005$, **** $p < 0.001$. **C)** Effects of P2RY6 knockdown (siP2RY6) on uLPS-induced IL-6, IL-12p40 and IL-1α mRNA expression levels. siNT = transfection with non-targeting control siRNA. Symbols represent different donors. n=5, one-way ANOVA on log-transformed data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

To confirm that our results were attributable to the selective inhibition of P2RY6-mediated signaling, we silenced the expression of P2RY6 in microglia with P2RY6-targeting siRNAs and analyzed IL-6, IL-12p40 and IL-1α mRNA expression levels after exposure to uLPS. P2RY6 knockdown decreased P2RY6 mRNA expression by ± 15-fold as confirmed by RT-PCR (**Figure S4** and **Table S4**). In comparison to untransfected microglia or to microglia that were transfected with non-targeting control siRNAs, uLPS-induced IL-12p40 mRNA levels in microglia transfected with P2RY6-targeting siRNAs were significantly inhibited (**Figure 2C** and **Table S4**). In addition, TLR4-induced IL-1α and IL-6 mRNA levels were also reduced, although not significantly. Mean log fold change mRNA expression values of **Figure 2C** are presented in **Table S5**.

RNA transcriptome analysis reveals that P2RY6-mediated signaling broadly amplifies uLPS-induced pro-inflammatory responses

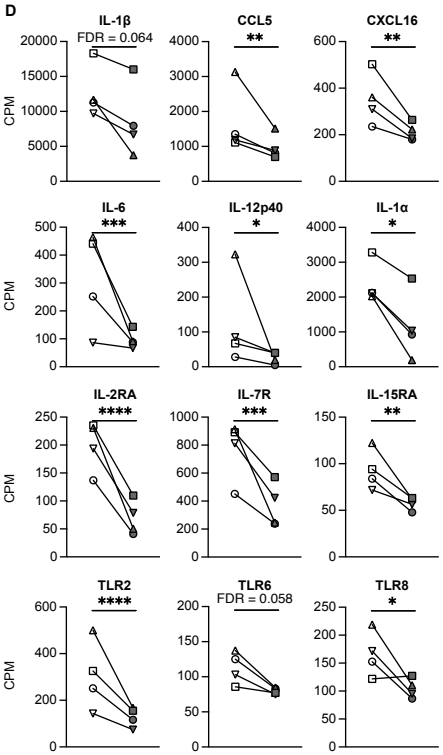
To get a broader overview of the P2RY6-mediated effects, we compared the RNA transcriptomes of microglia exposed to the TLR4 agonist uLPS in the presence of MRS2578 to those exposed to uLPS only. Of note, for this analysis we selected four donors that were also used in the previous section (**Figure 2**). Principal component analysis and heatmap analysis show that the transcriptomes from individual donors cluster together, rather than samples that were exposed to similar stimulation conditions (**Figure 3A** and **3B**). Such large donor-donor variability is not uncommon when working with samples derived from outbred individuals[22]. Nevertheless, differential gene expression analysis demonstrates that 302 gene products were expressed at significantly different levels when cells were exposed to P2RY6 antagonist (FDR < 0.05; **Table S6**). Gene ontology analysis showed that the differentially expressed genes (DEG) are associated with biological processes such as immune response (93 genes), defense response (76 genes) and response to cytokine (55 genes; **Figure 3C**). It should be noted that only the top 10 biological processes are presented and that genes can be associated with multiple biological processes. The DEG associated with each biological process can be found in **Table S7**. Indeed, transcripts encoding for cytokine IL-1β (not significant) and the chemokines CCL5 and CXCL16 (both significant) were decreased when P2RY6-mediated signaling was inhibited (**Figure 3D**). Importantly, transcriptome analysis confirmed the downregulation of IL-6, IL-12p40 and IL-1α mRNA expression levels in the presence of P2RY6 antagonist. In addition to cytokine and chemokine transcripts, we observed that IL-2, IL-7 and IL-15 receptor transcripts were also decreased in the presence of P2RY6 antagonist. Furthermore, TLR6 (not significant), TLR2 and TLR8 (both significant) expression levels were also markedly reduced. These data demonstrate the broad pro-inflammatory contribution of P2RY6-mediated signaling to uLPS-induced responses.

In order to gain insight into the molecular underpinnings of the P2RY6-mediated effects on uLPS-induced responses, we analyzed which transcription factor targets were shared between the downregulated genes. This analysis identifies NFAT, IRFs and NF-κB, as potential modulated targets by P2RY6-mediated signaling (**Figure 3E**). Of note, only the top 10 transcription factor targets are presented. The downregulated



C

Gene Set Name	FDR q-value
Immune response	1.19E-49
Regulation of immune system process	8.19E-46
Biological process involved in interspecies interaction between organisms	1.45E-37
Cell activation	4.85E-37
Defense response	3.40E-36
Regulation of immune response	6.50E-35
Regulation of cell activation	2.16E-34
Programmed cell death	6.61E-34
Response to cytokine	2.96E-32
Positive regulation of multicellular organismal process	7.64E-31



E

Gene Set Name	FDR q-value
TGGAAA NFAT Q4 01	2.20E-10
ISRE 01	2.20E-10
STTTCRNTTT IRF Q6	6.53E-10
ICSBP Q6	1.74E-7
IRF1 01	1.74E-7
NFKAPPAB 01	1.74E-7
IRF7 01	1.74E-7
AACTTT unknown	1.98E-7
WGTTNNNNNAAA unknown	7.16E-7
CTTTGA LEF1 Q2	9.24E-7

F

Gene Set Name	FDR q-value
Cytokine signaling in immune system	3.63E-23
Innate immune system	6.61E-18
Cellular responses to stimuli	8.94E-15
Signaling by interleukins	1.24E-14
HSF1-dependent transactivation	2.55E-11
Attenuation of the heat shock transcriptional response	4.94E-11
Cellular response to heat stress	6.25E-11
Regulation of HSF1-mediated heat shock response	2.41E-9
HSF1 activation	5.51E-9
Adaptive immune system	2.47E-8

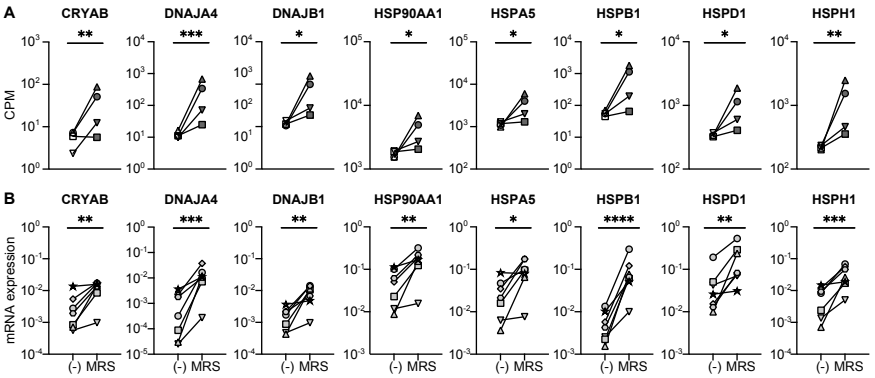
Figure 3. Effects of P2RY6-mediated signaling on the transcriptomes of uLPS-exposed primary microglia. **A)** Principal component analysis and **B)** Spearman correlation heatmap of the transcriptomes of primary microglia from four adult rhesus macaques exposed to 10 µg/mL uLPS (TLR4), with (gray symbols) or without (white symbols) 1 h pre-incubation with 5 µM P2RY6 antagonist MRS2578. n=4, symbols represent different donors. **C)** Biological processes associated with the 302 differentially expressed genes were analyzed using the molecular signatures database (MSigDB). FDR = false discovery rate. **D**) Gene expression levels (CPM) of several genes associated with immune responses. Microglia were exposed to uLPS, with (gray symbols) or without (white symbols) 1 h pre-incubation with MRS2578. n=4, EdgeR false discovery rates (FDR) are used to display statistical differences, * FDR < 0.05 ** FDR < 0.01, *** FDR < 0.005, **** FDR < 0.001. **E)** Transcription factor target analysis of the downregulated genes in the presence of P2RY6 antagonist analyzed using MSigDB. FDR = false discovery rate. **F)** Canonical pathways associated with the 302 differentially expressed genes were analyzed using MSigDB. FDR = false discovery rate.

genes associated with the transcription factor targets listed in **Figure 3E** can be found in **Table S8**. The possible involvement of NF-κB is in line with the notion that the promoter regions of IL-1α, IL-6 and IL-12p40 all share binding sites for NF-κB[33-35]. Finally, we performed a canonical pathway analysis of the 302 DEG using the Reactome Pathway Database. Besides cytokine and interleukin signaling pathways (42 genes), we unexpectedly observed that multiple heat shock transcription factor 1 (HSF1)-mediated pathways (12 genes) were present in this list (**Figure 3F**). Again, only the top 10 pathways are presented. The DEG associated with the top 10 pathways are displayed in **Table S9**.

Inhibition of P2RY6-mediated signaling induces the expression of heat shock protein genes

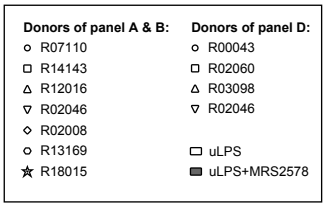
To our surprise, transcriptome analysis revealed that inhibition of P2RY6-mediated signaling in uLPS-exposed microglia led to a strong induction of the expression levels of multiple heat shock protein (HSP) genes, including CRYAB, DNAJA4, DNAJB1, HSP90AA1, HSPA5, HSPB1, HSPD1 and HSPH1 (**Figure 4A**). Interestingly, RT-PCR analysis showed that these genes were also upregulated in microglia exposed to P2RY6 antagonist alone (**Figure 4B**), suggesting a role for homeostatic P2RY6-mediated signaling in microglia. We performed a gene set enrichment analysis of the upregulated genes in uLPS-exposed microglia in the presence of P2RY6 antagonist and found that these genes were associated with biological processes such as protein folding, response to topologically incorrect protein and chaperone mediated protein folding (**Figure 4C**), which are all well described biological processes linked to HSP functions. The upregulation of HSP might indicate that the absence of P2RY6-mediated signaling results in cellular stress, as is also suggested by the DEG associated with programmed cell death in the presence of P2RY6 antagonist (**Figure 3C**). To analyze this in more detail, we performed TUNEL assays and observed that exposure to MRS2578 did not induce apoptosis in microglia (**Figure S5**). Even in combination with exposure to uLPS, the percentage of apoptotic microglia remained very low (<0.4%).

Finally, we questioned whether the amplification of pro-inflammatory responses and the inhibition of HSP expression levels by P2RY6-mediated signaling were related. If this were the case, one might expect that exposure of BMDM to P2RY6 antagonist might induce HSP expression levels to a lesser extent, as the pro-inflammatory effects of P2RY6-mediated signaling were much less pronounced in this cell type. We however observed that inhibition of P2RY6-mediated signaling in BMDM induced the expression levels of HSP genes to a similar extent as in microglia, both under homeostatic conditions as well as after TLR4 engagement (**Figure 4D**).



C

Gene Set Name	FDR q-value
Protein folding	1.35E-16
Response to topologically incorrect protein	1.44E-11
Response to abiotic stimulus	1.03E-8
Chaperone mediated protein folding	8.52E-8
Regulation of protein kinase activity	1.21E-7
Cellular response to stress	2.94E-7
Protein refolding	3.71E-7
Response to heat	7.67E-7
Response to temperature stimulus	7.67E-7
Regulation of cell death	1.45E-6



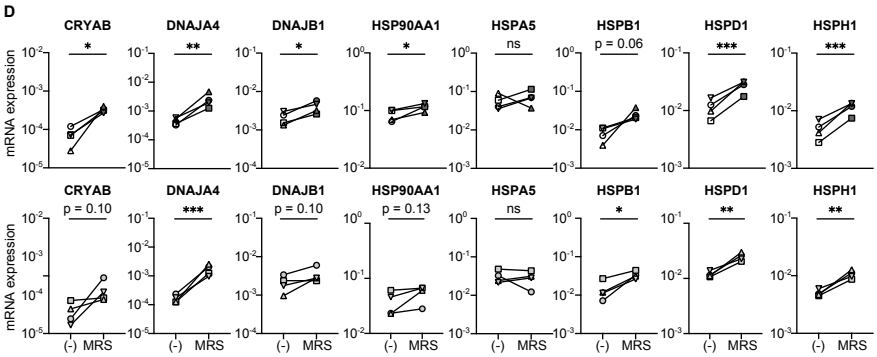


Figure 4. Exposure to P2RY6 antagonist MRS2578 induces the expression of heat shock protein genes. **A)** Gene expression levels (CPM) of heat shock protein genes in microglia exposed to uLPS (TLR4), with (gray symbols) or without (white symbols) 1 h pre-incubation with MRS2578. n=4, EdgeR false discovery rates (FDR) are used to display statistical differences, * FDR < 0.05 ** FDR < 0.01 *** FDR < 0.005. **B)** mRNA expression levels measured by RT-PCR of heat shock protein genes in homeostatic microglia and microglia exposed to 5 µM MRS2578. Symbols represent different donors. n=7, paired t-test on log-transformed data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. **C)** Biological processes associated with upregulated differentially expressed genes in uLPS exposed microglia in the presence of P2RY6 antagonist. FDR = false discovery rate. **D)** mRNA expression levels measured by RT-PCR of heat shock protein genes in homeostatic BMDM and BMDM exposed to 5 µM MRS2578, either in the absence or presence of 10 ng/mL uLPS. Symbols represent different donors. n=4, paired t-test on log-transformed data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Discussion

There is ample evidence for the involvement of P2RY6-mediated signaling in a broad range of central nervous system (CNS) disorders (reviewed in[36]). While the role of microglial P2RY6 as an essential regulator of phagocytosis in the brain has been widely acknowledged[37,38], P2RY6-mediated signaling in microglia can also affect cytokine and chemokine production[16,20]. In the present study we demonstrate that P2RY6-mediated signaling broadly amplifies TLR-induced pro-inflammatory signaling in microglia in particular. Modeling predicts that the transcription factors NFAT, IRFs and NF-κB are involved. Furthermore, we describe for the first time a prominent role for P2RY6-mediated signaling in the regulation of HSP gene expression levels.

Engagement of TLRs induced the mRNA expression levels and protein production of multiple pro-inflammatory cytokines and chemokines in both BMDM and microglia. When directly compared to BMDM, we find that in microglia P2RY6-mediated signaling is much more broadly involved in TLR-induced pro-inflammatory cytokine production. These cell type-specific differences are not attributable to differences in P2RY6 mRNA expression levels between BMDM and microglia, as these were comparable. Possibly, differences in P2RY6-mediated responses between different macrophage populations might rather be associated with tissue-specific adaptations of regulatory circuits. Alternatively, microglia might release higher levels of UDP upon TLR exposure when compared to BMDM, resulting in enhanced P2RY6-mediated signaling. Differences in innate immune responses between macrophage populations, including BMDM and microglia has been reported earlier[14,39] and the impact of 'nature and nurture' on BMDM and microglia innate immune responses has been the subject of many studies[40,41]. Our data show that, particularly in the CNS, P2RY6-mediated signaling in microglia most likely amplifies pro-inflammatory responses during inflammation.

The prominent contribution of P2RY6 to pro-inflammatory signaling was further characterized in microglia that were exposed to the TLR4 agonist uLPS and to escalating concentrations of P2RY6 antagonist MRS2578. MRS2578 is a selective – yet not entirely specific – antagonist of P2RY6, but the concentrations used in this study were well below reported IC_{50} values for other P2Y receptors. Whereas TLR4-induced IL-6, IL-12p40 and IL-1α responses were inhibited in the presence of P2RY6 antagonist, IL-8 and TNF-α responses were unaffected. This contrasts with data from a recent study. When mice microglia were exposed to a TLR2/4 agonist in the presence of P2RY6 antagonist, MIP-2 (the murine equivalent of human IL-8) and TNF-α were inhibited. Differences in species, P2RY6 protein-coding sequences (88% overlap between rhesus macaque and mouse), TLR agonists used or differences in sampling timepoints might have been responsible for these discrepant findings. We confirmed the involvement of P2RY6 in TLR4-induced IL-12p40 responses by silencing of P2RY6 gene expression with P2RY6-targeting siRNAs. Although this also affected TLR4-induced IL-1α and IL-6 responses, this was not significant. Transcriptome analysis revealed that the overall contribution of P2RY6-mediated signaling to TLR4-induced responses was of pro-inflammatory nature, as multiple pro-inflammatory cytokines, chemokines and

receptors were downregulated in the presence of P2RY6 antagonist. Whether P2RY6-mediated signaling also affected baseline levels of these target genes could not be concluded, as data from unexposed microglia is lacking. Transcription factor target analysis predicted the implication of NF-κB as a target of P2RY6-mediated signaling, which is supported by the notion that the promoter regions of IL-1α, IL-6 and IL-12p40 all share binding sites for NF-κB. Furthermore, NFAT and IRFs were also predicted to be affected by P2RY6-mediated signaling, warranting further detailed biochemical investigations.

Striking was the strong induction of HSP genes in the presence of P2RY6 antagonist, both in uLPS-exposed and in homeostatic microglia. HSPs are a large family of molecular chaperones that are induced by various stressors to provide protection against cellular damage by aiding the folding and assembly of proteins and to prevent protein aggregation[42,43]. The upregulation of multiple HSPs suggests that ablation of P2RY6-mediated signaling induces a stress response in microglia. Since exposure to DMSO (as a solvent control) did not induce the expression of HSP (data not shown), the effect is directly attributable to exposure to the P2RY6 antagonist MRS2578. Noteworthy is that we could not find evidence for MRS2578-induced apoptosis. Although MRS2578 is a potent inhibitor of P2RY6-mediated signaling, it should be tested whether MRS2578 has any off-target effects on microglia to further confirm and elucidate our findings.

HSPs have both pro- and anti-inflammatory effects depending on the cellular location of HSPs and the activation state of the cell (reviewed in[43,44]). One could speculate that P2RY6-mediated signaling modulates pro-inflammatory cytokine responses via the regulation of HSPs. However, as HSPs were also upregulated in BMDM in the presence of P2RY6 antagonist – and P2RY6-mediated signaling had little effects on TLR-induced pro-inflammatory cytokine responses in this cell type – we have no data to support this hypothesis.

HSPs, including HSP90AA1, HSPA8 and DNAJB1 (all upregulated in the presence of P2RY6 antagonist) are key players in chaperone-mediated autophagy (CMA), a form of autophagy that is impaired in aging and neurodegenerative diseases[45]. This suggests that P2RY6-mediated signaling is involved in CMA activity. Functional assays to measure CMA activity are needed to confirm this, but technical limitations associated with the use of primary cells render such assays very challenging[46].

Taken together, our data suggest that during homeostasis, tonic P2RY6-mediated signaling is a requirement for healthy microglia. Although the protocol and culture medium we used in this study have been characterized extensively to yield microglia that we would characterize as 'neutral', it should be taken into account that *in vitro* models for microglia always affect their phenotype and modeling homeostatic microglia remains a major challenge. Our data further suggests that under inflammatory conditions, P2RY6-mediated signaling amplifies TLR-induced pro-inflammatory responses supporting the idea that upregulation of P2RY6, as amongst others seen in neurodegenerative diseases, including AD and PD[16,47], might be partly responsible for excessive neuroinflammatory responses. In accordance with that idea, previous studies have shown that blocking of P2RY6 is beneficial in models of AD, PD and ischemic

stroke[16,18,19,38]. Based on our data and published literature, we propose that blocking of P2RY6-mediated signaling can indeed reduce neuroinflammation and might induce HSP expression and CMA activity (**Figure 5**), which are all considered advantageous during neurodegenerative diseases[48-51]. As P2RY6 is involved in multiple physiological and pathological cell functions, it should be noted that blocking of P2RY6-mediated signaling may induce both protective and harmful responses depending on the timing, dose and cellular target. Therefore, future research is pivotal to gain further insight in the diverse roles of P2RY6 during neuroinflammatory and neurodegenerative diseases and to determine how and in which context this pathway can be targeted.

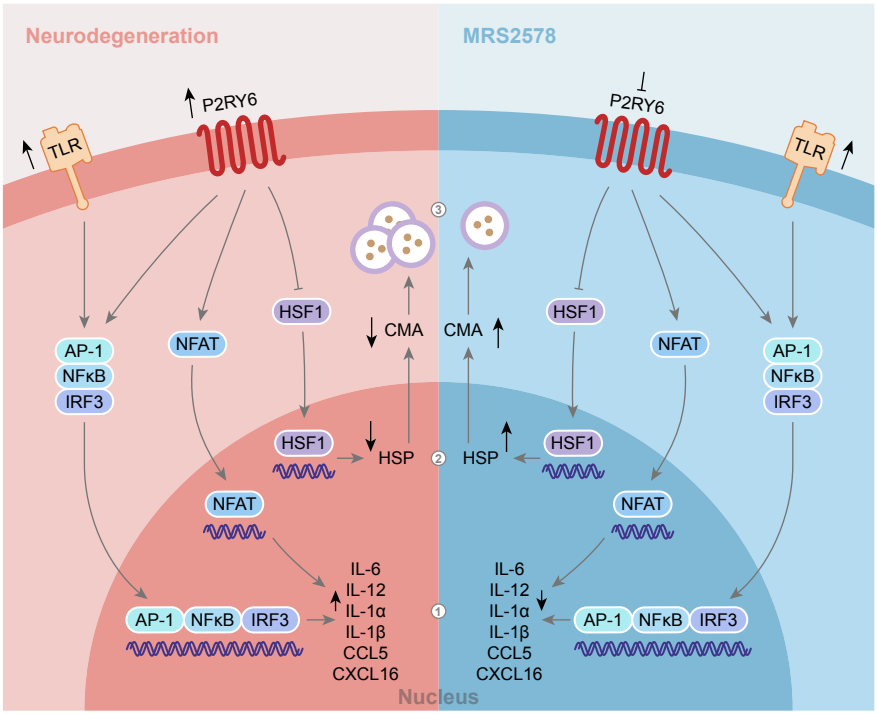


Figure 5. Schematic model of the contribution of P2RY6-mediated signaling during neuroinflammation. During neurodegeneration, TLRs and P2RY6 are described to be upregulated[16,47,52]. The upregulation of both TLRs and P2RY6 may contribute to 1) chronic neuroinflammation, 2) downregulation of HSP, and 3) autophagosome accumulation due to impaired chaperone-mediated autophagy (CMA) processes, which are all hallmarks of neurodegenerative diseases. Our data show that blocking of P2RY6 with P2RY6 antagonist MRS2578 can 1) reduce the expression of pro-inflammatory cytokines and chemokines and 2) induce the expression of HSPs, which 3) may lead to increased CMA activity.

Acknowledgments

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Supplemental information

Supplemental information

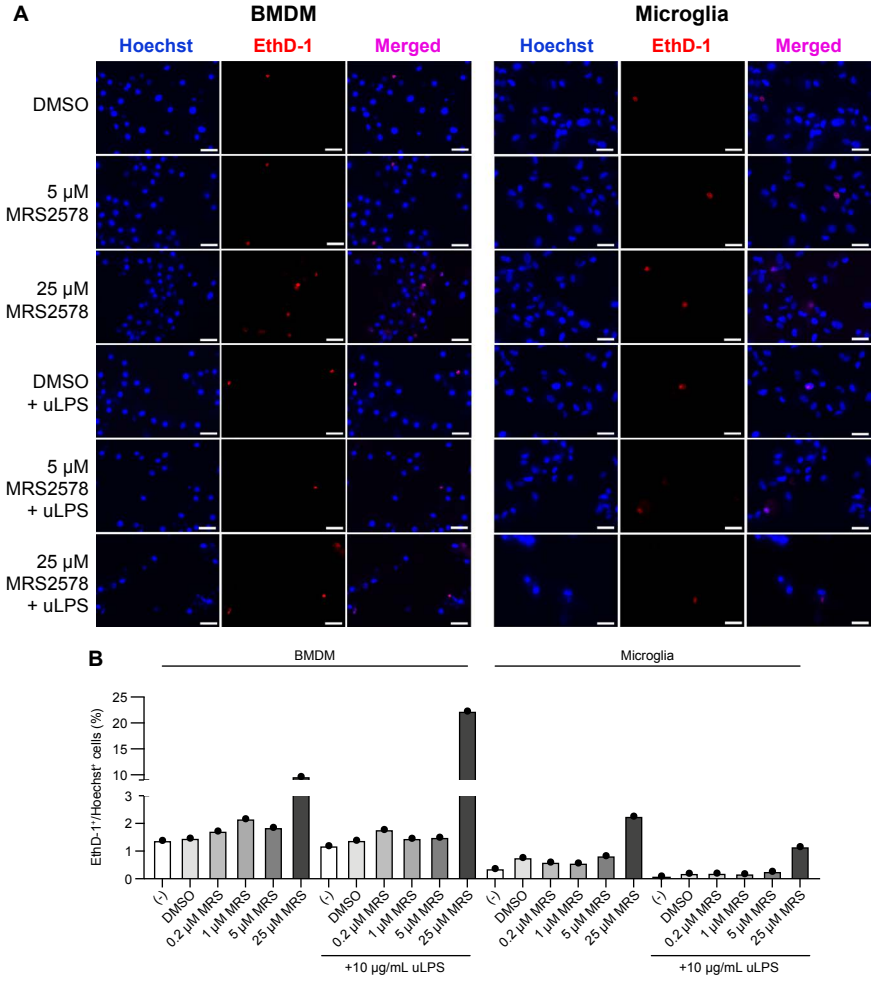


Figure S1. Effects of MRS2578 on the viability of bone marrow-derived macrophages and microglia. Bone marrow-derived macrophages (BMDM) and microglia were pre-incubated for 1 h with 0.2, 1, 5 or 25 µM MRS2578. Subsequently cells were stimulated with 10 µg/mL TLR4 agonist uLPS for 16 h. DMSO and unstimulated conditions were also included as a control. **A)** Images showing Hoechst+ and ethidium homodimer-1 (EthD-1)+ BMDM and microglia exposed to DMSO, 5 µM MRS2578 or 25 µM MRS2578 in the absence or presence of 16 h exposure to 10 µg/mL uLPS. Scale bars are 25 µm **B)** Cell viability was analyzed in 25 random fields of view. EthD-1+ nuclei were normalized to the number of Hoechst+ cells (n=1).

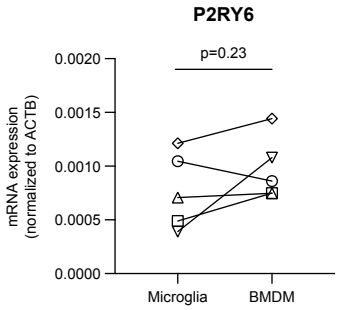


Figure S2. P2RY6 mRNA expression levels in microglia and BMDM. P2RY6 mRNA expression levels in primary microglia and bone marrow-derived macrophages were normalized to housekeeping gene ACTB. Symbols represent different donors, n=5, paired t-test on log-transformed data.

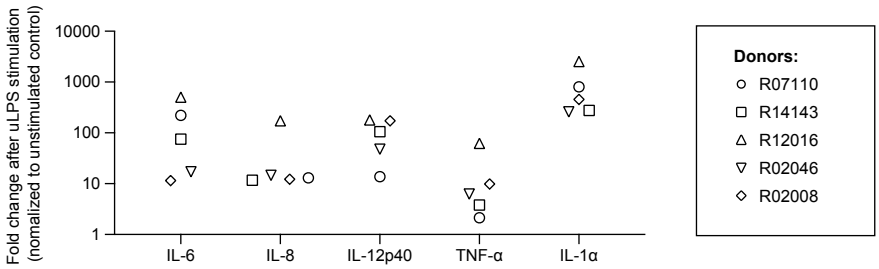


Figure S3. Fold change cytokine gene expression after uLPS stimulation. Microglia were stimulated with 10 µg/mL uLPS. After 16 h incubation the gene expression of IL-6, IL-8, IL-12p40, TNF-α and IL-1α was analyzed. The graph shows the fold change gene expression after uLPS stimulation normalized to its unstimulated control. Gene expression was normalized to ACTB. n=5, each symbol represents a donor.

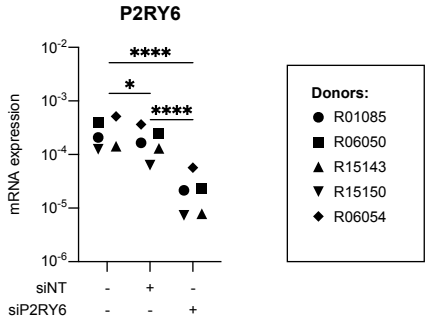


Figure S4. P2RY6 silencing in primary microglia. Primary microglia were transfected with either 72 nM non-targeting control siRNA (siNT) or 72 nM P2RY6-targeting siRNAs (siP2RY6). 40 h after transfection, P2RY6 mRNA levels were analyzed. P2RY6 expression levels were normalized to ACTB. Each symbol represents a donor. n=5, paired t-test on log-transformed data, * $p < 0.05$, **** $p < 0.001$.

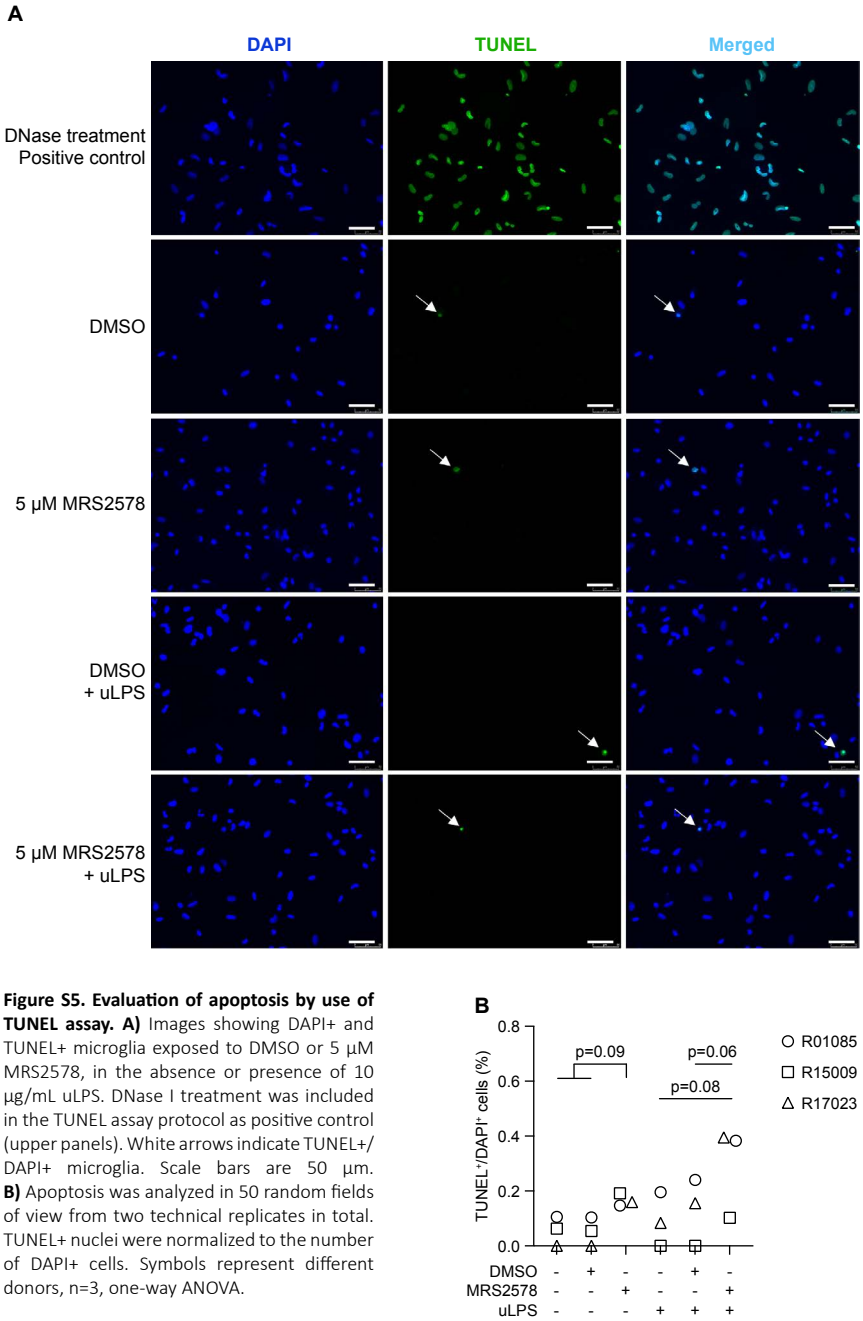


Figure S5. Evaluation of apoptosis by use of TUNEL assay. A) Images showing DAPI+ and TUNEL+ microglia exposed to DMSO or 5 µM MRS2578, in the absence or presence of 10 µg/mL uLPS. DNase I treatment was included in the TUNEL assay protocol as positive control (upper panels). White arrows indicate TUNEL+/DAPI+ microglia. Scale bars are 50 µm. **B)** Apoptosis was analyzed in 50 random fields of view from two technical replicates in total. TUNEL+ nuclei were normalized to the number of DAPI+ cells. Symbols represent different donors, n=3, one-way ANOVA.

Table S1. Mean log2 fold change (LogFC) cytokine production values of bone marrow-derived macrophages (BMDM) and microglia exposed several TLR agonists, in the presence versus absence of P2RY6 antagonist MRS2578 (raw data are presented in Figure 1A and Figure 1B)

IL-6	**BMDM**		**Microglia**	
	LogFC	**SD**	**LogFC**	**SD**
TLR1/2	0,09791423	1,14735583	*-0,9316018*	*0,3978378*
TLR2/4	-0,1022535	0,24505571	-0,6856904	0,49332544
TLR4	-0,2839329	0,28119094	*-0,9622986*	*0,353432*
TLR5	-0,2367002	0,3085705	-1,9566037	1,58541367
TLR8	*-0,2053414*	*0,0451123*	*-0,8028271*	*0,437632*

IL-8	**BMDM**		**Microglia**	
	LogFC	**SD**	**LogFC**	**SD**
TLR1/2	0,88063173	1,03529491	*-0,4312042*	*0,1057365*
TLR2/4	*0,74541259*	*0,29120097*	*-0,4653554*	*0,2019728*
TLR4	0,1979982	0,3466912	-0,717875	0,76932858
TLR5	-0,0834408	0,19636463	*-0,5240429*	*0,3065974*
TLR8	-0,1039932	0,0475027	*-0,6457286*	*0,2228286*

IL-12p40	**BMDM**		**Microglia**	
	LogFC	**SD**	**LogFC**	**SD**
TLR1/2	-1,2139681	1,26949067	*-2,5474273*	*1,6742343*
TLR2/4	-0,1139073	0,29014978	*-1,7964967*	*0,3912375*
TLR4	0,00396496	0,33236835	*-1,5806403*	*0,4409508*
TLR5	*-0,5793699*	*0,2852369*	*-1,5779896*	*0,6216272*
TLR8	-0,4155901	0,39706591	*-1,5807464*	*0,6228426*

TNF-α	**BMDM**		**Microglia**	
	LogFC	**SD**	**LogFC**	**SD**
TLR1/2	-0,297644	1,32440258	-0,7946214	1,35620328
TLR2/4	-0,2985793	0,57218531	-1,0833412	2,08604117
TLR4	-0,652677	0,92778218	-1,698678	2,51624274
TLR5	-0,2289576	1,19329679	*-1,3441206*	*0,3835932*
TLR8	0,35582761	1,85515577	*-1,5533014*	*0,7557958*

Positive values refer to an upregulation of the cytokine production in combination with P2RY6 antagonist, whereas negative values refer to a downregulation of the cytokine production in combination with P2RY6 antagonist. Values in italic represent values that are statistically significant in the presence of P2RY6 antagonist.

Table S2. Cytokine mRNA expression levels normalized to ACTB

IL-6/ACTB	R00043	R03098	R02046	R02008	R13169	R18015
(-)	0,00003546	0,00809091	0,000119649	0,000259471	0,000025070	0,000064453
PAM3CSK4	0,00505291	0,09402169	0,001432174	0,010555881	0,015537682	0,017049217
MRS2578 + PAM3CSK4	0,00354260	0,03986960	0,001986299	0,013266050	0,009149290	0,014251765
LPS	nd	nd	0,002010874	0,007635992	0,039023494	0,006479842
MRS2578 + LPS	nd	nd	0,000586456	0,002503999	0,008138432	0,006214852
uLPS	0,00215710	nd	0,000387123	0,002385828	0,005918083	0,007044204
MRS2578 + uLPS	0,00151713	nd	0,000349639	0,001561034	0,004486201	0,004105388
Flagellin	nd	nd	0,000336297	0,001106794	0,004612060	0,005982744
MRS2578 + Flagellin	nd	nd	0,000236460	0,001632871	0,003964269	0,004774585
CL075	0,01313472	nd	0,005542869	0,013215434	0,059942860	0,041287295
MRS2578 + CL075	0,00910964	nd	0,004138057	0,011380382	0,060751683	0,046098542
IL-8/ACTB	**R00043**	**R03098**	**R02046**	**R02008**	**R13169**	**R18015**
(-)	0,00676343	0,46428291	0,042480264	0,026033796	0,006932328	0,017944201
PAM3CSK4	0,14406385	3,07991283	0,320423221	0,098081411	0,258565436	0,215436542
MRS2578 + PAM3CSK4	0,18724558	1,52682675	0,354203953	0,355547152	0,243773833	0,141261101
LPS	nd	nd	0,425017396	0,304688363	4,039544043	0,024601169
MRS2578 + LPS	nd	nd	0,136703308	0,401130869	0,995533895	0,027736159
uLPS	0,11494742	nd	0,119011188	0,238626146	0,216448907	0,095866927
MRS2578 + uLPS	0,11206407	nd	0,120308380	0,260026965	0,177714434	0,092739914
Flagellin	nd	nd	0,147698854	0,235887710	0,546807580	0,036537710
MRS2578 + Flagellin	nd	nd	0,149964051	0,132458361	0,345863353	0,037237714
CL075	0,32821085	nd	0,382848136	0,374025668	3,569052413	0,156891751
MRS2578 + CL075	0,27242166	nd	0,376768576	0,657559471	3,339620871	0,252047561
IL-12p40/ACTB	**R00043**	**R03098**	**R02046**	**R02008**	**R13169**	**R18015**
(-)	0,00057756	0,00017687	0,000749509	0,000061276	0,000086144	0,000322478
PAM3CSK4	0,01002188	0,00268937	0,021635986	0,005355699	0,014873942	0,051242818
MRS2578 + PAM3CSK4	0,00446097	0,00153295	0,040808361	0,003473477	0,004924395	0,016804548
LPS	nd	nd	0,057416466	0,026742094	0,305821295	0,048845037
MRS2578 + LPS	nd	nd	0,006497211	0,007109192	0,023801902	0,028772254
uLPS	0,01257565	nd	0,017749187	0,008199787	0,054242274	0,050325566
MRS2578 + uLPS	0,00829204	nd	0,005951416	0,003096993	0,011150957	0,018128453
Flagellin	nd	nd	0,010431962	0,004940737	0,019247820	0,038043230
MRS2578 + Flagellin	nd	nd	0,008821423	0,002661590	0,007176620	0,013740936
CL075	0,21011839	nd	0,108311782	0,077890833	0,224764040	0,350902075
MRS2578 + CL075	0,06308783	nd	0,075680581	0,029184517	0,135129357	0,233385127
TNFA/ACTB	**R00043**	**R03098**	**R02046**	**R02008**	**R13169**	**R18015**
(-)	0,00001392	0,00083630	0,000037959	0,000010851	0,000063357	0,000053520
PAM3CSK4	0,00051133	0,00276131	0,000187339	0,000320112	0,000561454	0,000323389
MRS2578 + PAM3CSK4	0,00037943	0,00061963	0,000203916	0,000364483	0,000506409	0,000231879
LPS	nd	nd	0,000318122	0,000106244	0,015634313	0,000300679
MRS2578 + LPS	nd	nd	0,000096600	0,000139676	0,000749734	0,000240245
uLPS	0,00020675	nd	0,000169584	0,000074032	0,000293562	0,000306923
MRS2578 + uLPS	0,00022255	nd	0,000125898	0,000091913	0,000248107	0,000204246
Flagellin	nd	nd	0,000073184	0,000058024	0,000238400	0,000168100
MRS2578 + Flagellin	nd	nd	0,000149208	0,000040544	0,000273863	0,000223089
CL075	0,08710478	nd	0,000573749	0,000480253	0,002418964	0,001254224
MRS2578 + CL075	0,00047618	nd	0,000343933	0,000251236	0,001218810	0,000866307

IL-1α/ACTB	R00043	R03098	R02046	R02008	R13169	R18015
(-)	0,00009837	0,04198078	0,000196243	0,000062831	0,000020978	0,000055438
PAM3CSK4	0,02569045	0,325775226	0,046508566	0,024183643	0,016242005	0,032641548
MRS2578 + PAM3CSK4	0,01698758	0,071541166	0,033286588	0,013321629	0,011561838	0,008389118
LPS	nd	nd	0,064572037	0,037018368	0,090078563	0,017819370
MRS2578 + LPS	nd	nd	0,013610010	0,013124692	0,025125642	0,006772212
uLPS	0,02553467	nd	0,021496998	0,017350903	0,014089178	0,012322661
MRS2578 + uLPS	0,01332532	nd	0,011156551	0,004987339	0,005885766	0,004338258
Flagellin	nd	nd	0,022376019	0,011896503	0,017255870	0,035371350
MRS2578 + Flagellin	nd	nd	0,011882614	0,004418004	0,004991544	0,016246457
CL075	0,04094127	nd	0,050298625	0,043691685	0,113355118	0,077666500
MRS2578 + CL075	0,02697493	nd	0,034084199	0,031311564	0,055822039	0,065235912

Microglia were exposed to different TLR ligands for 16 h, with or without 1 h pre-incubation of the P2RY6 antagonist MRS2578 (5 µM). TLR ligands used are 1 µg/mL Pam_3CSK_4 (TLR1/2), 10 ng/mL LPS (TLR2/4), 10 ng/mL uLPS (TLR4), 100 ng/mL Flagellin (TLR5) and 1 µg/mL CL075 (TLR8).

Table S3. IL-6, IL-8, IL-12p40 and TNF-α levels in picograms per milliliter per donor

	R07110		R14143		R12016		R02046		R02008	
IL-6	**pg/mL**	**SD**	**pg/mL**	**SD**	**pg/mL**	**SD**	**pg/mL**	**SD**	**pg/mL**	**SD**
(-)	452	-	513	11	bd	bd	bd	bd	84	118
5 µM MRS2578	489	109	157	-	bd	bd	bd	bd	bd	bd
10 ug/mL uLPS	3520	523	2055	541	10596	869	2216	587	1481	787
5 µM MRS2578 + uLPS	1756	387	1587	541	2185	663	910	168	144	78
1 µM MRS2578 + uLPS	2598	142	1868	251	8376	147	1914	712	1888	338
0.2 µM MRS2578 + uLPS	3127	63	2219	90	8642	213	2249	1217	1075	622
IL-8										
(-)	19461	-	50277	18472	3151	602	bd	bd	98747	28861
5 µM MRS2578	19760	27945	114005	52059	14790	2208	bd	bd	121840	28101
10 ug/mL uLPS	186077	45736	256898	96282	237774	12445	143980	8400	283492	107849
5 µM MRS2578 + uLPS	144730	10420	289158	108317	73410	29306	102730	49935	184675	30380
1 µM MRS2578 + uLPS	188124	2894	330719	57937	220600	12645	230110	63003	449440	6835
0.2 µM MRS2578 + uLPS	201428	3763	305387	51779	225851	21678	250240	18667	352771	111647
IL-12p40										
(-)	bd	bd	bd	bd	bd	bd	86	31	52	7
5 µM MRS2578	bd	bd	bd	bd	bd	bd	74	3	bd	bd
10 ug/mL uLPS	680	83	2396	584	10153	997	2471	224	304	35
5 µM MRS2578 + uLPS	302	261	1796	1165	1453	44	1337	332	78	14
1 µM MRS2578 + uLPS	456	7	2254	350	9125	1244	2578	267	507	95
0.2 µM MRS2578 + uLPS	961	64	1670	960	8194	970	1407	17	467	117
TNF-α										
(-)	bd	bd	bd	bd	113	160	bd	bd	bd	bd
5 µM MRS2578	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd
10 ug/mL uLPS	419	136	1313	416	7928	1406	165	161	494	418
5 µM MRS2578 + uLPS	408	193	1262	927	791	76	440	57	65	0
1 µM MRS2578 + uLPS	281	33	1573	189	4292	1207	214	294	504	106
0.2 µM MRS2578 + uLPS	560	59	693	508	4326	95	318	36	684	159

The detection limits for IL-6, IL-12p40 and TNF-α were 8 pg/mL, the detection limit for IL-8 was 31 pg/mL. bd = below detection, nd = no data,- = no SD due to 1 measurement.

Table S4. P2RY6, IL-6, IL-12p40 and IL-1α mRNA expression levels normalized to ACTB

P2RY6/ACTB	**R01085**	**R06050**	**R14143**	**R15150**	**R06054**
(-)	0,002074117	0,003942705	0,001432201	0,001249353	0,005158663
72 nM siNT	0,001648237	0,002442381	0,001300708	0,000634292	0,003631725
72 nM siP2RY6	0,000214067	0,000228962	0,000078663	0,000073152	0,000570325
IL-6/ACTB	**R01085**	**R06050**	**R14143**	**R15150**	**R06054**
(-)	0,000025281	0,000065677	0,000012724	0,000039156	0,000143823
(-) + uLPS	0,017661622	0,009952204	0,085580582	0,029290366	0,016208134
72 nM siNT + uLPS	0,011331460	0,014389294	0,550316899	0,039067413	0,013647038
72 nM siP2RY6 + uLPS	0,007886920	0,002817470	0,028615537	0,016457138	0,009726473
IL-12p40/ACTB	**R01085**	**R06050**	**R14143**	**R15150**	**R06054**
(-)	0,001737157	0,000728342	0,000085712	0,000408871	0,000181550
(-) + uLPS	0,026604241	0,028845215	0,057045250	0,058016014	0,037350047
72 nM siNT + uLPS	0,015142563	0,019957635	0,110589963	0,063076055	0,035672200
72 nM siP2RY6 + uLPS	0,010763012	0,008455282	0,043292286	0,039226616	0,021020553
IL-1α/ACTB	**R01085**	**R06050**	**R14143**	**R15150**	**R06054**
(-)	0,000043445	0,000049981	0,000011743	0,000070075	0,000010360
(-) + uLPS	0,035587229	0,121438298	0,107656972	0,096557961	0,075797481
72 nM siNT + uLPS	0,037694923	0,089814780	0,131282181	0,046911438	0,029931014
72 nM siP2RY6 + uLPS	0,033539940	0,045640298	0,038851538	0,036757301	0,028843083

Microglia were untransfected (-), transfected with 72 nM non-targeting control siRNA (siNT) or transfected with 72 nM P2RY6-targeting siRNAs (siP2RY6). 24 h after transfection, microglia were stimulated with 10 µg/mL uLPS and mRNA expression levels were analyzed after 16 h.

Table S5. Mean log2 fold change (LogFC) cytokine mRNA expression values of microglia transfected with P2RY6-targeting siRNAs (siP2RY6) versus untransfected microglia, and versus microglia transfected with non-targeting control siRNAs (siNT) (raw data are presented in Figure 2C)

	siP2RY6 transfected microglia vs. Untransfected microglia		**siP2RY6 transfected microglia vs. siNT transfected microglia**	
	LogFC	**SD**	**LogFC**	**SD**
IL-6	*-1,226525167*	*0,468516752*	-1,775313365	1,584055946
IL-12p40	*-0,973581375*	*0,562253274*	*-0,906568382*	*0,371105356*
IL-1α	*-1,151001989*	*0,596477388*	-0,661417347	0,708394675

The negative values refer to a downregulation of the cytokine mRNA expression levels in microglia transfected with P2RY6-targeting siRNAs. Values in italic represent values that are statistically significant.

see https://www.frontiersin.org/articles/10.3389/fimmu.2022.967951/full#supplementary-material or scan the QR-code.

Table S6. Excel file with the Ensemble IDs, log fold changes (LogFC), false discovery rates (FDR), gene names, counts per million (CPMs) values and log CPM values of the 302 differentially expressed genes (FDR < 0.05) between 10 µg/mL uLPS and 10 µg/mL uLPS + 5 µM P2RY6 antagonist MRS2578 stimulated microglia.

Table S7. Excel file with the differentially expressed genes (DEG) associated with the biological processes displayed in Figure 3C.

Table S8. Excel file with the differentially expressed genes (DEG) associated with the transcription factor targets displayed in Figure 3E.

Table S9. Excel file with the differentially expressed genes (DEG) associated with the pathways displayed in Figure 3F.

Chapter 6

General discussion and future directions

Parts of this discussion will be integrated in a perspective review on *In vitro* microglia models: the era of engineered cell microenvironments

Neuroinflammation is a hallmark of all neurodegenerative diseases[1,2]. For that reason, modulation of neuroinflammation is increasingly being considered as an attractive strategy to beneficially affect the course of these diseases. As microglia are key players in neuroinflammatory responses, this has led to an increase in research in this particular cell type. Thorough cell biological knowledge of microglia is becoming of pivotal importance, and *in vitro* microglia models provide excellent tools to research and obtain such knowledge. However, challenges still exist in recapitulating the features of *in vivo* microglia, which hamper the *in vitro-in vivo* translation. For example, microglia in a healthy central nervous system (CNS) exhibit a ramified morphology. But when microglia are taken out of this microenvironment and are brought in culture, their morphology is characterized by a more amoeboid phenotype, which is associated with microglia activation[3,4]. In addition, the transcriptome of *in vitro* microglia differs considerably from that of *ex vivo* microglia[5-8]. Together, this provides a strong impetus to uncover and understand cues that determine the specific characteristics of *in vivo* microglia.

Over the course of this thesis, we have compared morphological features and transcriptomes of primary *ex vivo* microglia with those of primary *in vitro* microglia that were cultured under different conditions as a strategy to uncover such cues. These cues can be used for further optimization of microglia *in vitro* culture conditions, and might also lead to a better understanding of microglial identity.

Microglia-specific responses: what are the implications?

The unique combination of the origin and environment of microglia shapes microglia identity (**Box 1**). As a consequence, microglia have a gene expression profile that differs from other tissue-resident macrophages, monocytes and CNS cells[6,9,10]. This unique identity is amongst others reflected by specific features of microglia innate immune responses (**Chapter 4**). For example, microglia and bone marrow-derived macrophages (BMDM) show cell type-specific differences pertaining to i) inflammasome-mediated responses, ii) their dependence on inflammatory caspases and iii) purinergic receptor-induced interleukin (IL)-1β secretion[11,12]. In **chapter 5**, we further demonstrate that P2Y purinoreceptor 6 (P2RY6)-mediated signaling increased the expression levels of Toll-like receptor (TLR)-induced pro-inflammatory cytokines in microglia, whereas such effects were much less pronounced in BMDM. Adjusting the expression of P2RY6 may be a possible strategy to modulate excessive pro-inflammatory responses is microglia that may damage the CNS, while leaving pro-inflammatory responses in other tissue-resident macrophages intact. It is generally thought that microglia-specific responses represent adaptations to the vulnerable CNS environment. These cell type-specific responses underline that cell signaling pathways or regulatory circuits are complex and cannot simply be extrapolated from one cell type to another. This should for example be taken into account when computational methods are used to modulate cell signaling, as these often use data sources containing data from different cell types (**Chapter 3**).

Box 1. Microglia identity, plasticity, phenotypes, and heterogeneity: are the definitions as plastic as microglia themselves?

The identity of microglia is shaped by a complex interplay of developmental, origin-dependent transcriptional networks and CNS-derived environmental factors[6,13]. Features such as morphology (ramified), location (CNS), origin (yolk sac-derived) and the expression of microglia signature genes (e.g., *CX3CR1, P2RY12* and *TMEM119*) define *microglia identity*. These features are characteristics of microglia in homeostatic conditions. However, microglia are highly *plastic cells* and can – quickly – adapt to changes in their microenvironment, for example when microglia are brought in culture or when tissue homeostasis is perturbed, e.g., during injury or inflammation. During these conditions, microglia can lose some of the features that are associated with microglia identity. Nevertheless, these cells are still microglia, but exhibit a different *microglia phenotype* (state). To add another layer of complexity, single cell RNA sequencing (scRNAseq) studies have reported considerable *microglia heterogeneity* on the transcriptomic level during development, homeostasis and perturbations, but also across different CNS regions[14-17].

Whereas microglia identity can be seen as an intrinsic property (although it is reprogrammable under defined conditions[18,19]), microglia can (temporarily) exist in a range of different phenotypes, depending on the stimulus, and can transform from one phenotype into another (**Figure 1**).

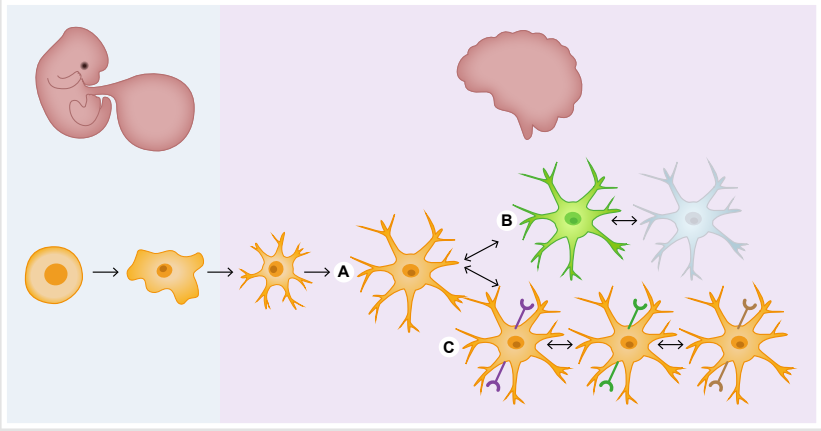


Figure 1. Microglia identity, plasticity, phenotypes, and heterogeneity. A) Microglia identity is shaped by the unique combination of its ontogeny (yolk sac-derived) and the CNS environment, and refers to microglia in homeostatic conditions. **B)** The highly plastic microglia can respond to changes in their environment and may therefore exhibit different phenotypes. Some of these phenotypes are characterized by the loss of features associated with microglia identity. However, when homeostasis is established again, microglia can regain the characteristics that are associated with microglia identity. **C)** On a single cell transcriptomic level, microglia are considerably heterogeneous during development, homeostasis, perturbations, but also across different CNS regions.

Which strategy to choose to optimize microglia culture conditions?

Since our final aim is to culture microglia that resemble *in vivo* microglia as close as possible, we have chosen to use *ex vivo* microglia as reference material (**Box 2**). We and many others have used bulk RNAseq data to compare the transcriptomes of *ex vivo* and *in vitro* microglia[5-8,20] (**Chapter 2**). scRNAseq approaches to compare *ex vivo* and *in vitro* microglia have been used as well, mainly to investigate the transcriptomes of *in vitro* microglia in co-culture systems[21,22]. Other platforms to compare *ex vivo* and *in vitro* microglia are epigenomic[6] and proteomic profiling[23]. In general, all 'omics' studies report on a loss of microglia homeostasis and on increased microglia activation in *in vitro* microglia. Although this consensus demonstrates that transcriptomics, epigenomics as well as proteomics are useful methods to characterize *ex vivo* and *in vitro* microglia for the optimization of culture conditions, the obtained data does not provide information about how the functional characteristics of *in vitro* microglia relate to those of *in vivo* microglia. The translatability and applicability of *in vitro* functional data will therefore initially have to be validated by *in vivo* studies. Even so, these data have intrinsic value to gain insights into microglial mechanistic responses and on how to connect 'omics' readouts to microglia function.

Box 2. The effect of isolation procedures on the microglia transcriptome

Ex vivo microglia isolation procedures may well affect the microglia transcriptome, leading to the possibility that reference *ex vivo* microglia expression data may not completely reflects the *in vivo* microglia transcriptome. For example, it has been reported that enzymatic dissociation of brain tissue at 37 °C caused an increase in the expression of inflammatory genes when compared to mechanical dissociation performed at low temperature[24-29]. Keeping the cells at low temperatures better preserves their transcriptional state due to cellular metabolic inactivation. However, enzymes that are used for enzymatic dissociation, including trypsin and collagenase, are not active at low temperatures. Ways to minimize artificial microglia activation during the enzymatic dissociation is the inclusion of transcriptional and translational inhibitors[24,27,30] or the use of proteases that are active at low temperatures[31].

To prevent artifactual gene expression changes during tissue dissociation, we used a mechanical dissociation protocol at 4 °C to isolate microglia for the generation of *ex vivo* transcriptome data. Importantly, data of mechanical dissociation methods have in common that they result in considerably lower overall yields of viable cells compared to enzymatic dissociation methods[27,29]. Although such lower cell yields are not problematic for studies that aim to profile microglia directly *ex vivo*, for the *in vitro* microglia experiments substantially more microglia are – in general – required. Therefore, *in vitro* microglia studies still rely on enzymatic dissociation methods. However, a study demonstrated that, already

after 24 h in culture, primary microglia recovered from the initial stress that was associated with the isolation procedure[7]. Moreover, primary microglia that were cultured for 7 days demonstrated similar expression levels of cell surface markers that are indicative of activation as *ex vivo* microglia[32]. In line with this, we reported that serum-induced effects were for most part reversible in primary cultured microglia (**Chapter 2**). This is in line with the reported plasticity of microglia in adapting to changes in their microenvironment.

Which factors to consider when optimizing primary microglia culture conditions?

Biological cues

CSF1R signaling – does it matter to use M-CSF or IL-34?

Colony stimulating factor-1 receptor (CSF1R) signaling is involved in microglia survival, proliferation and homeostasis[7,33-35]. The two reported ligands for CSF1R are macrophage colony-stimulating factor (M-CSF)[36] and IL-34[37]. Unique contributions for either M-CSF or IL-34 in the development and colonization of microglia have been documented in depletion studies in rodents and zebrafish[38-42]. In **chapter 2**, we uncovered that adult primary rhesus macaque microglia exposed to either M-CSF or IL-34 for 7 days, are characterized by virtually identical transcriptomes. These data are in line with a recent study performed in adult primary human microglia, that did not detect any differential patterns of gene expression upon M-CSF or IL-34 exposure[43]. It is important to note that M-CSF interacts exclusively with CSF1R, while IL-34 is reported to signal through two additional receptors: receptor protein tyrosine phosphatase-ζ (PTPRZ1)[44] and CD138 (syndecan-1)[45,46]. Transcriptome analysis shows that *ex vivo* adult primate microglia do not express *PTPRZ1* or *CD138*. However, *in vitro* adult primate microglia do express *CD138*, regardless of the culture condition (data not shown). Although CD138-mediated signaling has been implicated in multiple cellular processes, including wound healing, cell adhesion, cell proliferation and apoptosis[47], our data do not indicate an effect of IL-34-CD138 signaling. Taken together, these data suggest similar roles for M-CSF and IL-34 in adult primary primate microglia cultures.

TGF-β signaling – do all TGFs signal equal?

In rodents, transforming growth factor beta 1 (TGF-β1) signaling is, both *in vitro* and *in vivo*, an important factor for microglia homeostasis and survival[5,7,48]. For instance, *in vitro* exposure of mouse microglia to TGF-β1 induced the expression of microglia signature genes and better recapitulated the transcriptomes of freshly isolated murine microglia[5,6]. However, we could not reproduce these effects in primary rhesus macaque microglia (**Chapter 2**). Our data are in line with studies reporting on the modest effects

of TGF-β1 exposure on primary human microglia[6,20]. This observation may reflect an important difference between the rodent and primate lineage[49]. However, as the TGF-β superfamily is highly conserved between rodents and humans, it is at present unclear where these differences originate[50,51]. It could be that the presence of other TGF-β isoforms, for instance induced by splicing, is necessary to induce microglia homeostasis in primary primate microglia. In **chapter 3**, we highlighted the increased expression of the microglia signature gene *TREM2* by microglia that were exposed to TGF-β1 and TGF-β3. Although all TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) function through the same receptor signaling pathways[52,53], differences in biological activity between TGF-β isoforms have been reported[54]. It is known that some researchers supplement microglia culture medium with either TGF-β1 or TGF-β2[5-7,20,21], and our results would lead to the suggestion to experiment with combinations of isoforms as well. The effects of TGF-β3 or combinations of TGF-β isoforms on the primary microglia transcriptome are currently not understood at all and represent an interesting and relevant research topic.

Inhibition of cell surface receptor-induced activation pathways – too complex to handle?

Primary cultured microglia exhibit an activated phenotype, which is amongst others characterized by the increased expression of genes indicative of activation, such as *CD14, CD68* and *CCL2*[7,55]. High mobility group box 2 (HMGB2) and IL-1β were, amongst others, identified as possible drivers of the upregulated gene expression profile in *in vitro* microglia (**Chapter 3**). Possible ways to optimize microglia *in vitro* culture conditions may therefore lie in the blocking of such activation pathways[55]. To block HMGB2- and IL-1β-mediated signaling, we exposed primary *in vitro* microglia to inflachromene (ICM) or IL-1 receptor antagonist (IL-1Ra), respectively. It is important to note that ICM binds to both HMGB1 and HMGB2[56], and that IL-1Ra inhibits the activity of both IL-1α and IL-1β by competitively blocking their binding to type I and type II receptors[57]. We observed no reduced expression of the examined upregulated *in vitro* microglia genes after exposure to ICM and IL-1Ra. We attributed this, for the time being, to the non-specific blocking effects of the factors. Future studies are needed to investigate if specific blocking of HMGB2- and IL-1β-mediated signaling can reduce the mRNA expression levels of the examined genes. A different approach to reduce microglia activation *in vitro* could also be found in the addition of factors that induce microglia homeostasis, also known as neuro-immune-regulators (NIREGs), such as CD200, CD47 and CX3CL1[58,59]. It may be that a combination of exposure to i) microglia activation pathways inhibitors and ii) NIREGs is beneficial in the optimization of microglia *in vitro* culture conditions.

Serum – pros and cons: a compromise for now

Gene set enrichment analysis of the upregulated genes in *in vitro* microglia demonstrated a robust association with proliferative cell processes. Fetal calf serum (FCS), a routinely used cell culture medium supplement, is a well described driver of cell proliferation *in vitro*[60-62]. We tested whether primary microglia exposed to a

recently described serum-free medium[7] better resembled *ex vivo* microglia. In line with other recently published studies[63,64], serum-free medium induced a complex, ramified cell morphology, which resembles the morphology of microglia in the healthy CNS[65,66]. In addition, serum-free medium reduced the expression of genes associated with proliferation (**Chapter 2**). Nevertheless, the lack of proliferative microglia in culture negatively impacted the number of cells available for further *in vitro* experiments. We therefore developed a cell culture regime that combines a 4-day serum exposure with a serum-free washout period of 11 days. This protocol was sufficient to yield high numbers of ramified microglia with reduced expression levels of genes driving proliferation. Additionally, the transcriptomes of microglia exposed to this protocol were similar to those of microglia that were cultured in serum-free medium only. Thus, microglia responses to serum exposure are relative short-lived and appear to be for most part reversible. This is supported by the notion that microglia are plastic cells that can quickly adapt to changes in their environment[55,67,68]. Besides the fact that microglia in a healthy CNS are not exposed to serum – let alone FCS –, the use of serum in cell culture media comes with more disadvantages. Serum is a poorly defined cell culture component and batch-to-batch variability negatively contributes to reproducibility. Another important motivation to eliminate serum from cell culture protocols is that the acquisition of FCS is associated with animal suffering. Therefore, serum-free media contribute to the replacement, reduction and refinement of animal experiments (3Rs)[69]. To completely replace the use of FCS in the microglia isolation and culture protocol, human platelet lysates (hPLs) may be considered as a 4-day supplement instead of FCS. hPLs are produced from expired donated human blood and demonstrated similar capacity in promoting adhesion, survival and proliferation as FCS did, at least to mesenchymal stromal cells[70-72].

It is known that different microglia phenotypes can respond differently to the same stimulus[32,73]. Along this line, culture media formulation, including serum-containing versus serum-free media, affected *in vitro* microglia innate immune responses (**Box 3**). Whether the immune functions of microglia exposed to serum-free medium better resemble the immune functions of microglia *in vivo* has yet to be determined, but this is likely since microglia are not exposed to serum in the healthy CNS.

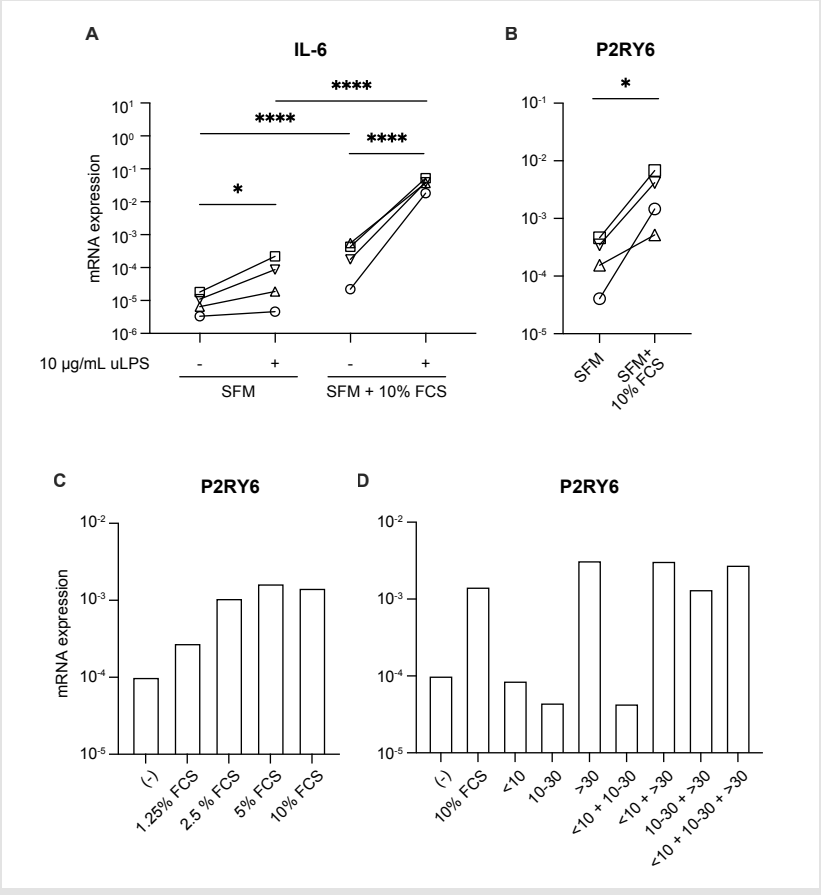
Box 3. Serum exposure affects P2RY6 mRNA expression levels and P2RY6-associated functions in primary microglia

Microglia cultured in serum-free medium show decreased phagocytic capacity, reduced TLR4-induced IL-6 responses (**Figure 2A**) and suppressed microglia activation as compared to microglia cultured in serum-containing medium[7,20,64]. Since the P2Y6 receptor is involved in all these processes[74-76], we hypothesized that serum exposure affects the expression of P2RY6.

Indeed, P2RY6 mRNA expression levels of microglia cultured in serum-free medium were significantly reduced when compared to those of microglia cultured in the same medium supplemented with 10% FCS (**Figure 2B**). In addition,

escalating concentrations of FCS induced the mRNA expression levels of P2RY6 in a dose-dependent manner (**Figure 2C**). To gain more insight into serum components responsible for this effect, we fractionated FCS through decreasing molecular weight cut off (MWCO) filters and collected <10 kDa proteins, 10-30 kDa proteins and >30 kDa proteins, and exposed microglia to these different serum protein fractions, both separately and in combination. We observed that the fraction containing >30 kDa proteins was responsible for the upregulation of P2RY6 mRNA expression levels, whereas exposure to <10 kDa proteins and 10-30 kDa proteins had no effect (**Figure 2D**). Bovine serum albumin (± 66 kDa), a major component of FCS, can activate microglia[77,78] and may (indirectly) increase the mRNA expression levels of P2RY6.

Taken together, these data demonstrate that serum exposure induces the expression levels of P2RY6 encoding mRNA, thereby rendering microglia more sensitive to P2RY6-mediated effects, such as the induction of TLR4-induced IL-6.



Co-culture + conditioned medium: standardization is key

The CNS environment plays an important role in the establishment and maintenance of adult microglia identity[13]. In this context, multiple studies that focused on the optimization of microglia *in vitro* models have experimented with co-culture systems of microglia, neurons and/or astrocytes, and conditioned medium from astrocytes[7,21,79-81]. These studies have demonstrated the importance of neuron- and astrocyte-derived cues for microglia identity. We pioneered with a 3D-spherical co-culture system of microglia with oligodendrocytes and radial glia, all derived from the same donor (**Chapter 2**). The gene expression profile of microglia, and the expression of microglia signature genes, in these spheres better resembled that of *ex vivo* microglia than those of monocultured microglia did. These data revealed a novel role for oligodendrocyte and radial glia-derived cues in the maintenance of microglia identity. In **chapter 3**, we followed up on this observation and exposed *in vitro* microglia to conditioned medium from the spheres. This showed increased expression of some microglia signature genes, but to a much lesser extent than microglia cultured in spheres. This suggests that both cell-cell contact, as well as soluble factors derived from spheres were – partly – responsible for the increased expression of microglia signature genes. We also tested if oligodendrocyte-conditioned medium could increase the expression of microglia signature genes, but without effect. Of note, these oligodendrocytes were derived from rat possibly introducing species-specific effects. In addition, the indirect effects that other cells in the spheres might have on the oligodendrocyte secretome could not be tested.

Interestingly, we observed high expression levels of 'myelin genes' such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein (PLP1) in spheres. This suggests that oligodendrocytes in the spheres produce myelin. In the healthy CNS, microglia are in contact with myelin sheaths[82], and it may be that myelin supports microglia homeostasis.

In general, very little is known about the role of oligodendrocytes, radial glia and myelin on microglia identity. Co-culture systems of oligodendrocytes, radial glia or myelinated neurons and microglia, and further oligodendrocyte- or radial glia-conditioned medium studies are instrumental to deepen our understanding on this topic.

◀ **Figure 2. Serum exposure affects the mRNA expression levels of IL-6 and P2RY6 in microglia. A)** IL-6 mRNA expression levels of microglia cultured in serum-free medium (SFM) or SFM + 10% fetal calf serum (FSC), in the absence or presence of 16 h exposure to 10 µg/mL ultrapure lipopolysaccharide (uLPS). Symbols represent different donors, n=4, paired t-test on log-transformed data, * $p < 0.05$, **** $p < 0.001$. **B)** P2RY6 mRNA expression levels of primary microglia cultured in SFM or SFM + 10% FCS medium. Symbols represent different donors, n=4, pared t-test on log-transformed data, * $p < 0.05$. **C)** P2RY6 mRNA expression levels of microglia exposed to escalating concentrations FCS, n=1. **D)** P2RY6 mRNA expression levels of microglia exposed to serum fractions with different molecular weight cut offs (<10 kDa, 10-30 kDa, > 30 kDa), n=1. For all graphs, microglia were cultured for 7 days total. Both IL-6 and P2RY6 mRNA expression levels were normalized to housekeeping gene ACTB.

Although co-culture and conditioned medium systems provide a more physiologically relevant environment, there are some important limitations that should be taken into account. For example, as each cell type requires a specific medium composition, choosing the right culture medium for co-culture systems is challenging[83]. In addition, cell observations and measurements, such as gene expression levels or immune responses, are typically easier to perform in monoculture systems than in co-culture systems, although scRNAseq approaches are a – costly – alternative to address this type of questions. Regarding studies with conditioned medium, the undefined composition of conditioned medium can lead to inconsistent outcomes in experiments due to batch-to-batch differences in their production. This makes it challenging to standardize culture conditions.

Thus, co-culture and conditioned medium systems are powerful tools to study cell-cell interactions or secretory factors that might be important for cell culture optimization. However, for standardization purposes it is thereafter recommended to add such factors as supplement to culture medium rather than to rely on conditioned medium.

Impact of the extracellular matrix: most certainly but difficult to mimic

The extracellular matrix (ECM) provides essential biological cues that are required for tissue morphogenesis, differentiation, and homeostasis[84,85]. In **chapter 2**, we observed that genes linked to biological processes associated with the ECM were strongly upregulated in *in vitro* microglia as compared to *ex vivo* microglia. This suggests a role for the ECM in microglia homeostasis. This idea was further supported by the prediction that LAMA2 (a laminin-associated protein) drives the expression of microglia signature genes *GPR34* and *TREM2* (**Chapter 3**). Primary microglia cultured on laminin-coated substrates were characterized by reduced mRNA expression levels of matrix metalloproteinases (MMPs; ECM-associated proteins) and increased mRNA expression levels of microglia signature genes. This suggests that laminin can also contribute to microglia homeostasis *in vitro*. The laminin family contains sixteen isoforms[86], and some of them have specific functions[87]. It would be interesting to determine the effects of different laminin isoforms, either alone or in combination, on microglia. In addition, integrins are receptors for laminin[88], and it would be worthwhile to gain more insight into integrin-mediated signaling for the optimization of microglia *in vitro* culture conditions. As integrins are also receptors for other extracellular matrix components, including fibronectin, vitronectin and collagen[89,90], it is likely that other (brain) ECM components can also be used to optimize microglia *in vitro* culture conditions.

Another holistic approach to expose microglia to brain ECM components is to decellularize whole brain tissue. This method removes all cells from the brain tissue while preserving brain ECM components[91,92]. Although certainly most promising, it should again be noted that batch-to-batch variation in the production of decellularized brain ECM may also negatively contribute to reproducibility.

Biophysical cues

Currently, the majority of microglia studies are carried out in 2D plastic culture ware systems. However, microglia experience a complex, 3D, and extremely soft environment *in vivo*. Changes in mechanical properties or patterning of the microglia culture environment affect microglia morphology, gene expression and function[93-99]. More biophysically accurate culture methods for microglia could therefore be of importance to improve microglia *in vitro* conditions.

To provide a more *in vivo*-like environment for microglia, we experimented – as mentioned earlier – with a 3D-spherical microglia co-culture system (**Chapter 2**). Although sphere cultures are more physiologically relevant than 2D cultures, it also comes with some challenges[100]. Sphere cultures are more difficult to initiate and the reproducibility of sphere cultures is more challenging as compared to 2D cell cultures (personal experiences). In addition, microscopic analysis and some types of measurements, such as immunostainings, can be difficult in spheres. Also, the access of oxygen, nutrients and metabolites to cells in the middle of the spheres can become limited and can lead to a necrotic core inside the sphere. In addition, uniform exposure to a molecule is challenging in spheres, which can hamper drug development studies. To bridge the gap between 2D cultures and sphere cultures, cells can also be cultured on 2.5D micro- or nanotopographies as well as on 3D biomimetic scaffolds[101,102]. Interestingly, adult primary rhesus macaque microglia cultured on 2.5D nanopillars (diameter = 0.2 µm, height = 2.5 µm, inter-pillar spacing = 1 µm) fostered a ramified morphology when compared to microglia cultured on flat substrates[102]. Importantly, the nanopillars did not only affect the topography and geometry of the substrate, but it also decreased the stiffness of the substrate as compared to the flat substrate. Stiffness of a material is measured by Young's modulus of elasticity, and is expressed in pascals (Pa). The stiffness of plastic cell culture ware (polystyrene) is around 10^6 kPa, which contrasts with the soft tissue of the brain, that is around 1 kPa[103,104]. Surprisingly, we and others find that microglia become less complex when cultured on softer substrates (**Figure 3**)[93,94], which is associated with a less homeostatic microglia phenotype.

Together, these data suggest that the topography and geometry of the nanopillars are responsible for the ramified morphology of microglia, rather than the reduced stiffness of the nanopillars. Furthermore, it is unclear if microglia cultured on these nanopillars reflect a more homeostatic gene expression profile. The broad effects of substrate topography, geometry and stiffness on microglia homeostasis remain open questions and warrant further investigation.

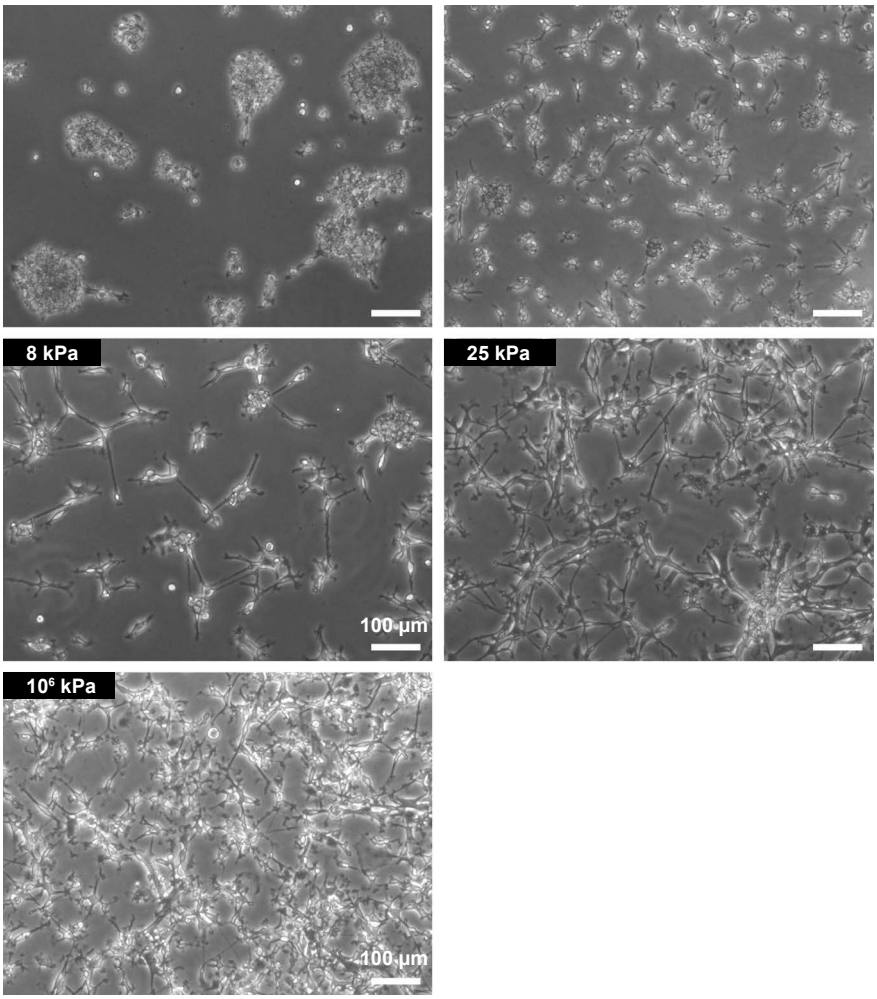


Figure 3. Substrate stiffness affects microglia morphology. Brightfield photos of microglia that were cultured on 0.5 kPa, 2 kPa, 8 kPa and 25 kPa hydrogel substrates and on plastic culture ware (± 10^6 kPa). All microglia were cultured for 4 days in serum-containing medium and subsequently cultured in serum-free medium for 11 days.

Future directions

The main objective of this thesis was to find cues that are important for microglia identity in order to optimize primary microglia *in vitro* conditions. To achieve this, we chose a transcriptomic approach where we compared the transcriptomes of primary *ex vivo* microglia with the transcriptomes of primary *in vitro* microglia.

Analyses of the differentially expressed genes between *ex vivo* and *in vitro* microglia primarily point to the lack of the CNS environment *in vitro*. This is in line with studies that describe the importance of the CNS environment for microglia identity and homeostasis[6,13,105]. Further optimization of microglia *in vitro* culture conditions may therefore be found in:

i. The addition of intercellular microglia signaling cues. It is still not clear which CNS cell-derived cues play a role in primate microglia identity and homeostasis. Our data hint to a combination of TGF-β isoforms, oligodendrocyte- and radial glia-derived factors, microglia activation pathway inhibitors and NIREGs.
ii. The exposure to ECM components. Our data suggest a role for laminin as a cue to optimize microglia culture conditions. Other (brain) ECM components may also be pivotal to optimize microglia culture conditions. A possible way to expose microglia to brain ECM components is the use of decellularized brain tissue.
iii. The generation of more biophysically accurate culture methods for microglia. 2.5D micro- or nanotopographies provide a more physiologically relevant microenvironment for microglia compared to flat substrates. Furthermore, the presence of micro- or nanopillars decrease the stiffness of the substrate. In addition, compared to 3D models, such as spheres, 2.5D systems have the advantage to facilitate experimental measurements.

The improvement of microglia *in vitro* models is pivotal for better drug development studies, a better understanding of microglia biology and for the reduction of animal studies. To date, *in vitro* models are not able to fully replace the need of laboratory animals, as only laboratory animals can demonstrate the broad effects of a disease, injury, treatment, or preventive measure on a complex organism. Nevertheless, *in vitro* models are highly useful to give answers to questions that are part of the big picture. Thus, throughout the development of knowledge, this approach eventually can lead to a reduction in animal experiments.

It is important to note that there is a large overlap of the differentially expressed genes between *ex vivo* and *in vitro* microglia from rhesus macaques and humans (data not shown). This suggests that the reported results are also applicable to primary microglia derived from humans. Related, as the differentially expressed genes between *ex vivo* and primary *in vitro* microglia, and *ex vivo* and human stem cell-derived microglia overlap[106,107], our results could possibly also be applicable to study human stem cell-derived microglia. It should however be noted that primary *in vitro* microglia are deprived of CNS-derived cues that are responsible to retain their identity, whereas

stem cell-derived microglia have never been exposed to the CNS environment at all. Whether this impacts the optimization of both *in vitro* models warrants further investigation.

It is important for researchers to carefully evaluate which *in vitro* microglia model is the most suitable to answer their scientific questions. For example, studies of age-associated diseases, including neurodegenerative diseases, are preferably performed using primary microglia from adult donors rather than microglia cell lines or stem cell-derived microglia. Furthermore, the trade-off between the physiological relevance of an *in vitro* microglia model and experimental control over conditions must also be assessed based on the scientific question.

The microglia studies in this thesis have contributed to the optimization of *in vitro* culture conditions to study microglia in health and disease. Using transcriptomic analyses, we have developed a partly serum-free culture protocol that yields high numbers of ramified microglia and a transcriptome profile similar to microglia that were cultured in serum-free medium only. The in-depth transcriptome analyses throughout this thesis provide powerful leads to further improve microglia *in vitro* conditions, thereby furthering our efforts to understand the biology of microglia and to reduce the need of animal experiments.

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Appendices

Summary

Neuroinflammation is a characteristic of almost all neurological diseases. Since microglia, the resident macrophages of the central nervous system (CNS), are key players in neuroinflammatory processes there is an increasing interest in this cell type as a therapeutical target to suppress neuroinflammation. Thorough biological knowledge of microglia is therefore of pivotal importance, and microglia *in vitro* models (in a culture dish) are excellent means to obtain such knowledge. However, there is currently no *in vitro* microglia model that recapitulates all the characteristic features of *in vivo* microglia (in a living organism), which hampers the *in vitro-in vivo* translation. For instance, microglia in the healthy CNS are characterized by a ramified morphology, whereas *in vitro* microglia are characterized by a more amoeboid (rounder) morphology. In addition, the gene expression profile of *in vitro* microglia differs considerably from the gene expression profile of *ex vivo* microglia (freshly isolated microglia). Importantly, the expression of microglia signature genes (genes that are highly expressed by microglia and not, or at very low levels, by other macrophages and cells in the CNS), is lost in *in vitro* microglia.

Several studies have reported that cues from the CNS environment are important for microglia to establish or maintain their identity. For example, loss of expression of microglia signature genes can be partially reversed by engrafting primary microglia (cultured microglia that were freshly isolated from the brain) back into an intact CNS environment or by culturing microglia together with neurons and/or astrocytes. However, which CNS environmental cues contribute to the *in vivo* microglia gene expression profile that defines their identity is poorly understood.

In this thesis, we used a transcriptomic-guided approach to uncover cues that shape microglia identity and investigated if they could be used to optimize *in vitro* culture conditions to study microglia in health and disease. Furthermore, we investigated opportunities to modulate microglia-induced neuroinflammation.

In **chapter 2**, we examined the effects of different *in vitro* culture conditions on the microglia transcriptome. First, we reported similar roles for macrophage colony-stimulating factor (M-CSF) and interleukin (IL)-34, the two ligands of the colony stimulating factor-1 receptor (CSF1R), in adult primary primate microglia cultures. Additionally, we analyzed the effects of transforming growth factor beta 1 (TGF-β1) exposure on the microglia *in vitro* transcriptome, as TGF-β signaling has been reported as an important factor for rodent microglia homeostasis and survival, both *in vitro* and *in vivo*. However, we could not reproduce these effects in primary rhesus macaque microglia.

We also compared the transcriptomes of primary *in vitro* microglia that were cultured under different conditions to those of primary *ex vivo* microglia. We observed major differences in the gene expression profiles of *in vitro* and *ex vivo* microglia and found that upregulated genes in *in vitro* microglia were associated with proliferative

cell processes. We hypothesized that this was due to the presence of fetal calf serum in the culture medium. Microglia cultured in serum-free medium indeed exhibited a more ramified morphology and were characterized by a reduced expression of genes that induce proliferation. However, the lack of proliferative microglia in culture negatively impacted the number of cells available for further *in vitro* experiments. We therefore developed a cell culture regime that combined a period of 4-days of serum exposure with a period of serum-free washout of 11 days. This protocol supported the outgrowth of high numbers of ramified microglia that were characterized by low expression levels of genes that are driving proliferation. Additionally, the transcriptomes of microglia exposed to this protocol were virtually identical to those of microglia that were cultured in serum-free medium only.

Although this was a significant improvement of cell culture conditions, differential gene expression analysis of the transcriptomes of *ex vivo* microglia and microglia cultivated with the partly serum-free protocol further underlined the lack of a CNS environment *in vitro*. We therefore pioneered with a 3D-spherical co-culture system of microglia with oligodendrocytes and radial glia, all derived from the same donor. The gene expression profile of microglia, and the expression of microglia signature genes, in these spheres better resembled that of *ex vivo* microglia than those of monocultured microglia did. Together, these data reveal a contribution of oligodendrocyte- and radial glia-derived cues for maintenance of microglia identity.

In **chapter 3**, we followed up on the findings described in chapter 2 and aimed to uncover CNS-derived cues that drive the expression levels of differentially expressed genes (DEGs) between *ex vivo* and *in vitro* microglia. NicheNet, an *in silico* (computational) analysis tool, identified amongst others high mobility group box 2 (HMGB2)- and IL-1β-mediated signaling as possible drivers of the upregulated gene expression profile in *in vitro* microglia. Due to the lack of specific inhibitors of HMGB2 and IL-1β, we were unable to confirm these results.

In a second approach to gain insight into cues that could potentially optimize microglia *in vitro* culture conditions, we exposed microglia to conditioned medium derived from different CNS cell types. We observed that conditioned medium from spheres composed of microglia, oligodendrocytes and radial glia could induce the mRNA expression levels of the microglia signature gene *P2RY12*. Next, we used NicheNet to find ligands expressed by oligodendrocytes and radial glia that might drive the expression of microglia signature genes. This analysis predicted amongst others TGFβ-3 and LAMA2 as drivers of the microglia signature gene expression. Exposure to TGFβ-3 induced the mRNA expression levels of the microglia signature gene *TREM2* in *in vitro* microglia. LAMA2 encodes a subunit of the laminin family, which is part of the extracellular matrix (ECM), and microglia that were cultured on laminin-coated substrates were characterized by reduced expression levels of matrix metalloproteinase genes (genes that are highly upregulated *in vitro*) and by increased expression levels of microglia signature genes. Together, our results suggest to investigate inhibition of

HMGB2- and IL-1β- associated pathways in *in vitro* microglia to reduce the expression of upregulated genes. In addition, exposure to TGF-β3 and cultivation on laminin-coated substrates are suggested to improve current microglia *in vitro* culture conditions.

In **chapter 4**, we described the impact of 'nature and nurture' on microglia innate immune responses and we summarized reported tissue-specific adaptation. For instance, microglial responses are characterized by slow kinetics and by a persistent nature compared to other macrophages. It is generally thought that microglia-specific responses represent adaptations to the vulnerable CNS environment. We also reviewed factors like aging, previous exposure to inflammatory stimuli, and differences in the microenvironment that can modulate microglia innate immune responses. In this chapter, we also pointed out that 'nurture' plays an important role in shaping microglia innate responses, which should be taken into account when studying microglia. Mimicking of 'nurture-induced' effects *in vitro* presents a major challenge, especially if attempted via stem cell approaches, since stem cell-derived microglia have never been exposed to a CNS environment.

In **chapter 5**, we characterized the role of purinergic receptor P2RY6-mediated signaling in Toll-like receptor (TLR)-induced pro-inflammatory responses in bone marrow-derived macrophages (BMDMs) and microglia. First, we demonstrated that P2RY6-mediated signaling enhanced the levels of TLR-induced pro-inflammatory cytokines in primary microglia, whereas such effects were much less pronounced in BMDMs from the same donors. These differences in P2RY6-mediated responses between BMDMs and microglia might be associated with tissue-specific adaptations of regulatory circuits. Transcriptome analysis demonstrated that the overall effects of P2RY6 on TLR4-induced responses in microglia were of pro-inflammatory nature. We further observed that blocking of P2RY6 in BMDMs and microglia induced the expression levels of multiple heat shock proteins, both in the absence and presence of inflammatory stimuli. Together, these results suggest that blocking of P2RY6 may be a promising strategy to suppress microglia-induced excessive neuroinflammatory responses, as seen in neurodegenerative diseases like Alzheimer's disease and Parkinson's disease.

Together, the results of this thesis contribute to the optimization of microglia *in vitro* culture conditions to study microglia in health and disease. Transcriptome analyses of *ex vivo* and *in vitro* microglia cultures have led to the development of a partly serum-free culture protocol that yields high numbers of ramified microglia with a transcriptome profile similar to that of microglia that were cultured in serum-free medium only. Additional transcriptome and *in silico* analyses throughout this thesis have provided powerful leads to further improve microglia *in vitro* culture conditions, for instance through i) the addition of intercellular microglia signaling cues, ii) the exposure to ECM components and iii) the generation of more biophysically accurate culture methods for

microglia. Importantly, there is a large overlap of the DEGs between *ex vivo* and *in vitro* microglia from rhesus macaques and humans. This suggests that the reported results are also applicable to primary microglia derived from humans. Related, as the DEGs between *ex vivo* and primary *in vitro* microglia, and *ex vivo* and stem cell-derived microglia overlap, our results could also be applicable to stem cell-derived microglia. Better microglia *in vitro* models are pivotal to i) further understand the biology of microglia, ii) to reduce the number of animal experiments and to iii) study opportunities to modulate microglia-induced neuroinflammation.

Nederlandse samenvatting

Ontstekingsprocessen in de hersenen zijn een kenmerk van bijna alle hersenaandoeningen. Microglia, ook wel bekend als de immuuncellen van de hersenen, spelen een belangrijke rol in het aan- en uitzetten van deze ontstekingsprocessen. Hierdoor zijn microglia een interessant celtype waarlangs ontstekingsprocessen in de hersenen onderdrukt zouden kunnen worden. Om dit te kunnen onderzoeken is goede fundamentele biologische kennis van microglia van cruciaal belang, en microglia kweeksystemen zijn uitstekende modellen om die kennis te verkrijgen.

Microglia in kweeksystemen verliezen echter belangrijke eigenschappen en kenmerken ten opzichte van microglia die in de hersenen voorkomen. In gezonde hersenen zijn microglia aanwezig in een vertakte staat, terwijl microglia in kweeksystemen een rondere vorm vertonen. Ook verschilt het zogenaamde 'transcriptoom' van microglia in een kweeksysteem aanzienlijk ten opzichte van de transcriptomen van microglia die aanwezig zijn in de hersenen. De term 'transcriptoom' verwijst naar de totaliteit aan eiwit-coderende moleculen in een cel op een bepaald moment. Ontwikkelingsstadia en veranderingen in de omgeving van een cel kunnen invloed hebben op de expressie van deze moleculen. Pas de laatste jaren hebben vooruitgang in biotechnologische technieken het mogelijk gemaakt om transcriptomen, die vele miljoenen moleculen groot zijn, met behulp van informatietechnologie te analyseren.

Verschillen tussen microglia in kweeksystemen en microglia in de hersenen kunnen de vertaalslag van kennis die is opgedaan in een kweeksysteem naar de situatie in een levend organisme belemmeren. Verschillende studies hebben aangetoond dat factoren in de hersenen belangrijk zijn voor het ontstaan en het behouden van de eigenschappen en kenmerken van microglia. Welke factoren in de hersenen dat precies zijn, is echter nog niet goed bekend.

In dit proefschrift hebben we onderzocht hoe we microglia kweeksystemen kunnen verbeteren. Dit deden we onder andere door de transcriptomen van microglia in de hersenen te vergelijken met die van microglia in kweeksystemen. Ook onderzochten we manieren om microglia-geïnduceerde ontstekingsprocessen te onderdrukken.

In **hoofdstuk 2** stelden we microglia in kweeksystemen bloot aan verschillende factoren die in de hersenen voorkomen. Vervolgens analyseerden we de transcriptomen van deze microglia. We analyseerden bijvoorbeeld de effecten van blootstelling aan transformerende groeifactor bèta 1 (TGF-β1), aangezien het is beschreven dat deze factor een belangrijke rol speelt bij het ontstaan en het behouden van de eigenschappen en kenmerken van microglia in knaagdieren. Wij konden deze effecten echter niet reproduceren in microglia van resusapen. Mogelijk spelen in primaten andere factoren een rol in het ontstaan en behouden van microglia eigenschappen.

In dit hoofdstuk vergeleken we ook de transcriptomen van microglia in de kweeksystemen met de transcriptomen van microglia in de hersenen. We brachten de verschillen in transcriptomen in kaart en vonden dat biologische processen die

geassocieerd zijn met celgroei en celdeling een veel belangrijkere rol speelden in microglia in kweeksystemen. Ook zagen we dat microglia in kweeksystemen veel meer geprikkeld waren dan microglia in de hersenen. Wij veronderstelden dat dit effect werd veroorzaakt door de aanwezigheid van serum (foetaal kalfsserum) in het kweekmedium. Wanneer we serum weghaalden uit het kweekmedium werden de microglia inderdaad veel minder geprikkeld en groeiden ze ook veel minder snel. Ook de vorm van de microglia veranderde van een ronde vorm naar een meer vertakte vorm, wat een kenmerk is voor gezonde, niet-geprikkelde microglia. Echter, doordat de microglia door het weghalen van serum minder snel groeiden, had dit een negatief effect op het aantal cellen dat beschikbaar was voor verdere experimenten. We ontwikkelden daarom een microglia kweekprotocol dat een 4-daagse blootstelling aan serum (om de groei van microglia te stimuleren) combineerde met een serumvrije uitwasperiode van 11 dagen (om prikkeling van microglia en biologische processen als celgroei en celdeling te remmen). Dit protocol zorgde voor hoge aantallen vertakte microglia die veel minder geprikkeld waren dan microglia die blootgesteld waren aan serum. Bovendien waren de transcriptomen van microglia blootgesteld aan dit protocol vergelijkbaar met die van microglia die alleen aan serumvrij medium waren blootgesteld.

Hoewel dit een aanzienlijke verbetering was van de microglia kweekcondities, bleken er nog flinke verschillen te zijn in de transcriptomen van microglia gekweekt met het gedeeltelijke serumvrije protocol en microglia in de hersenen. Nieuwe analyses wezen naar het gebrek van de hersenomgeving in het kweeksysteem. We besloten daarom om andere celtypes uit de hersenen, de zogenaamde oligodendrocyten en radiale glia cellen, toe te voegen aan het microglia kweeksysteem. We vonden dat microglia die gekweekt werden met de andere hersenceltypes beter de transcriptomen nabootsten van microglia in de hersenen. Samen tonen deze resultaten aan dat factoren afkomstig van oligodendrocyten en radiale glia cellen bijdragen aan het behouden van de celspecifieke eigenschappen en kenmerken van microglia.

In **hoofdstuk 3** zijn we verdergegaan met de bevindingen in hoofdstuk 2 en hebben we geprobeerd te ontdekken welke factoren in de hersenen de transcriptomen van microglia in een kweeksysteem kunnen verbeteren. Om deze factoren te ontdekken gebruikten we een geavanceerd computermodel, genaamd NicheNet. NicheNet identificeerde onder andere dat de factoren 'hoge mobiliteit groep box 2' (HMGB2) en interleukine (IL)-1β een rol spelen in biologische processen die te maken hebben met de overprikkelde kenmerken van microglia in de kweeksystemen. Door het gebrek aan specifieke remmers van HMGB2 en IL-1β konden wij deze resultaten echter niet bevestigen.

In een tweede benadering om inzicht te krijgen welke factoren microglia kweeksystemen kunnen verbeteren, stelden wij microglia bloot aan geconditioneerd medium afkomstig van verschillende gekweekte hersenceltypes. Geconditioneerd medium is kweekmedium waar cellen een bepaalde tijd aan zijn blootgesteld en die factoren bevat die door deze cellen zijn uitgescheiden. We vonden dat geconditioneerd

medium van een kweeksysteem bestaande uit microglia, oligodendrocyten en radiale glia cellen een positieve invloed had op de transcriptomen van microglia. Vervolgens gebruikten we NicheNet om te onderzoeken welke factoren, die door oligodendrocyten en radiale glia cellen worden gemaakt, dit effect kunnen veroorzaken. NicheNet voorspelde onder andere dat de factoren TGFβ-3 en LAMA2 mogelijk de transcriptomen van microglia in een kweeksysteem kunnen verbeteren. We stelden microglia in een kweeksysteem bloot aan deze factoren en vonden inderdaad een verbetering van de transcriptomen, die meer overeenkwamen met de transcriptomen van microglia in de hersenen. Samen suggereren de resultaten in dit hoofdstuk om te onderzoeken of remming van HMGB2 en IL-1β de overprikkeling van microglia in kweeksystemen kan remmen. Bovendien zou blootstelling aan TGF-β3 en LAMA2 de huidige microglia kweeksystemen kunnen verbeteren.

In bijna alle organen van het lichaam bevinden zich cellen die macrofagen genoemd worden. Macrofagen zijn erg goed in het herkennen van infecties en stress en in het opnemen van bacteriën, afval en dode cellen. Macrofagen ontstaan al vroeg in de embryonale fase en migreren dan naar organen waar ze vervolgens blijven. Microglia zijn de macrofagen van het brein en verschillen van macrofagen in andere organen. In **hoofdstuk 4** hebben we beschreven welke rol de herkomst en de omgeving (hersenen) spelen bij het ontstaan van de specifieke immuunfuncties van microglia. Microglia zijn beter in het opruimen van breinstofjes dan andere macrofagen en hebben bepaalde specifieke moleculen op hun celoppervlak om signalen in het brein te kunnen ontvangen. We beschrijven dat de meeste verschillen tussen macrofagen te vinden zijn in de regulering van hun immuunresponsen. In dit hoofdstuk werd ook ingegaan op welke rol veroudering speelt bij microglia immuunfuncties en beschreven we een aantal specifieke aanpassingen van microglia aan het brein. Deze aanpassingen lijken vooral als resultaat te hebben dat microglia niet zo snel en niet zo heftig reageren als macrofagen in andere weefsels. Gezien het belang van de hersenen is het te begrijpen dat we zuinig op ze zijn, en dat immuunreacties alleen op gang komen wanneer het echt niet anders kan. Ook wijzen we erop dat de omgeving (de hersenen) een belangrijke rol speelt bij de vorming van microglia immuunfuncties, waarmee rekening moet worden gehouden bij het bestuderen van microglia. Het nabootsen van ‘omgeving-geïnduceerde immuunfuncties’ in een kweeksysteem is een grote uitdaging, vooral als dit wordt geprobeerd via stamcelbenaderingen, aangezien microglia die gedifferentieerd zijn vanuit stamcellen nooit zijn blootgesteld aan de hersenomgeving.

Microglia houden de hersenen continu in de gaten en komen in actie wanneer er iets fout dreigt te gaan. Zodra microglia iets ongewensts tegenkomen, slaan ze alarm door verschillende signalen uit te zenden. Dit doen ze door het uitscheiden van boodschappermoleculen, zogenaamde cytokines. Dit alarmsysteem moet tot in de puntjes kloppen, want een teveel of tekort aan alarm kan schadelijk zijn. Het is daarom belangrijk om de werking van het alarmsysteem van microglia zo goed mogelijk in kaart te brengen. Een wirwar van eiwitten en factoren spelen een belangrijke rol in de

werking van het alarmsysteem. Een eiwitfamilie die afwijkende elementen en ziekteverwekkers kan herkennen is de familie van zogenaamde Toll-like receptoren (TLR). Naast microglia beschikken ook andere immuuncellen in het menselijk lichaam over deze eiwitten. In **hoofdstuk 5** onderzochten we of we de werking van TLR-eiwitten specifiek in microglia kunnen moduleren. Voor dit onderzoek gebruikten we microglia kweeksystemen en kweeksystemen met macrofagen uit het beenmerg. We vonden dat alarmeringen die via de TLR-eiwitten lopen geremd kunnen worden door de activiteit van een ander eiwit, namelijk P2RY6, te remmen. Dit effect vonden we echter alleen in microglia en niet in macrofagen uit het beenmerg. Het blijkt dus dat P2RY6 specifiek in microglia de alarmsignalen die uitgezonden worden door TLR-eiwitten reguleert. De resultaten in dit hoofdstuk suggereren dat remming van P2RY6 mogelijk een manier kan zijn om ontstekingsprocessen in de hersenen, zoals wordt gezien in Alzheimer en Parkinson, te onderdrukken.

De resultaten van dit proefschrift dragen samen aan tot het verbeteren van microglia kweeksystemen. Zo hebben we een nieuw, gedeeltelijk serumvrij, kweekprotocol voor microglia ontwikkeld dat resulteert in hoge aantallen vertakte microglia met transcriptomen die sterk lijken op die van microglia die alleen in serumvrij medium werden gekweekt. Verder hebben de resultaten in dit poefschrift aanknopingspunten opgeleverd om microglia kweeksystemen verder te verbeteren, bijvoorbeeld door remming van HMGB2 en IL-1β, of door blootstelling aan TGF-β3 en LAMA2.

Het is belangrijk om te benoemen dat de transcriptomen van resusaap microglia sterk overeenkomen met de transcriptomen van humane microglia. Dit suggereert sterk dat de resultaten beschreven in dit proefschrift ook van toepassing zijn op microglia van mensen. Daarnaast overlappen de verschillen in transcriptomen van resusaap microglia in kweeksystemen en resusaap microglia in de hersenen met de verschillen in transcriptomen van microglia die gedifferentieerd zijn vanuit humane stamcellen en humane microglia in de hersenen. Hierdoor kunnen onze resultaten mogelijk ook stamcel microglia kweeksystemen verbeteren. Betere microglia kweeksystemen zijn van cruciaal belang om i) de biologie van microglia beter te begrijpen, ii) het aantal dierproeven te verminderen, en iii) mogelijkheden te bestuderen om microglia-geïnduceerde ontstekingsprocessen te kunnen onderdrukken.

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Curriculum vitae

Raissa Timmerman was born on the 5th of June 1991 in Ommen, the Netherlands. In 2008 she obtained her HAVO diploma at Vechtdal College, Hardenberg. After, she started the bachelor Biology and Medical Laboratory Research at the Hanze University of Applied Sciences in Groningen. As part of her studies, she performed an internship in the research group of Dr. Ruud Toonen at the Center for Neurogenomics and Cognitive Research (CNCR), where she generated and validated new fluorescent tools to study synaptic transmission and the release of neuromodulators. Following her Bachelor's degree, she started with the research master Biomedical Sciences: Neurobiology (track Molecular Neurosciences) at the University of Amsterdam. During this master, she performed an internship at the Swammerdam Institute for Life Sciences in the group of Dr. Marco Hoekman, where she studied the link between circadian rhythms and stem cell maintenance in the adult hippocampus. For her second internship she moved to Lund (Sweden) to join the research group of Dr. Henrik Ahlenius. The project of this internship was focused on transcription factor programming of pluripotent stem cells to astrocytes for modeling of leukodystrophies. After her graduation, she started her PhD research under supervision of Dr. Jeffrey Bajramovic at the Alternatives Unit at the Biomedical Primate Research Centre in Rijswijk, the Netherlands. This research was focused on the optimization of microglia *in vitro* culture conditions, which is amongst others important for reducing animal experiments. The results of her PhD research are described in this thesis, which will be defended at Utrecht University with Prof. dr. Ronald Bontrop as promotor.

List of publications

Sharaf, A., **Timmerman, R.**, Bajramovic, J. J., & Accardo, A. (2023). In vitro microglia models: the era of engineered cell microenvironments. *Accepted for publication in Neural regeneration research*

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