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PROFILING OF MICROGLIA IN INFLAMMATION, AGING, AND THE TUMOR MICROENVIRONMENT

Lara Friess

Karolinska Institutet

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Profiling of microglia in inflammation, aging, and the tumor microenvironment
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Lara Friess

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Principal Supervisor:
Professor Bertrand Joseph
Karolinska Institutet
Institute of Environmental Medicine
Unit of Toxicology

Co-supervisor(s):
Professor Klas Blomgren
Karolinska Institutet
Department of Women's and Children's Health
Unit of Barnonkologi och Barnkirurgi

Opponent:
Professor Bart Eggen
University of Groningen
Department of Biomedical Sciences of Cells and Systems
Division of Molecular Neurobiology

Examination Board:
Associate Professor Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Sven Nelander
Uppsala Universitet
Department of Immunology, Genetics and Pathology
Division of Neuro-Oncology

Docent Magnus Olsson
Karolinska Institutet
Institute of Environmental Medicine

"*I knew exactly what to do. But in a much more real sense, I had no idea what to do*." –
Michael Scott

POPULAR SCIENCE SUMMARY OF THE THESIS

Our immune system is busy around-the-clock with keeping us safe from bacteria, viruses, and even internal threats such as tumor cells. In the central nervous system, which consists of the brain and the spinal cord, specialized immune cells called microglia take over the important part of being the body's guard. Microglia are already produced and in place during early development and have a vast variety of functions throughout life. This can range from maintenance, to dealing with a stroke, and even clearing out dead cells. Researchers found out decades ago that microglia can respond to different challenges by changing and adapting rapidly and with high plasticity, but to this day it is still not fully understood how these changes are controlled on a molecular level. We often call these changes the acquisition of an activated state.

Broadly speaking, there are two commonly researched activation states – pro- and anti-inflammatory activation. Pro-inflammatory activation often happens after events such as the invasion of pathogens. The microglia create an inflammatory microenvironment with the goal of combatting defective or damaging cells or components. On the other hand, anti-inflammatory microglia are, for example, beneficial for tissue repair after injury. In the aging brain, these functions can be significantly impaired or delayed, which in turn is thought to contribute to neurodegenerative diseases such as Alzheimer's or Parkinson's disease. We are therefore interested in finding mechanisms that control inflammatory microglia and in understanding their role in the aging brain.

Microglia also play an important role in brain tumors. Tumor cells attract microglia and reprogram them into a tumor-supportive phenotype. Instead of fighting off the malignant cells, microglia will help the tumor grow and invade more tissue. An additional part of this highly complex interplay are macrophages. These cells can be described as the counterpart of microglia outside of the central nervous system, they migrate into the brain and toward the tumor site, where their presence is associated with a worse clinical prognosis. We want to understand more about the interaction of these three cell types, what triggers the microglia or macrophages to become pro-tumoral and how to leverage this knowledge to find novel therapeutic options in the field of brain tumor research.

As you can now hopefully appreciate, microglia are involved in almost every aspect of brain health. It is therefore crucial to understand what drives microglia into their activation states, and what lessons we can learn to ultimately advance clinical research for the benefit of current, and future patients.

ABSTRACT

In this thesis I aim to present our ongoing efforts to elucidate activation states and how these are regulated in microglia cells. An extensive body of work is dedicated to understanding how these resident immune cells can acquire certain activation states, as their roles are crucial for a great range of functions in the central nervous system – from homeostasis to pathological occurrences. In summary, the work presented here underlines the importance of microglia cells in the context of inflammation, aging, and the tumor microenvironment.

In **project I** we identify a possible mechanism of regulation in microglia activation. Under normal circumstances, microglia are highly plastic and respond to a variety of stimuli in their microenvironment by drastically altering their transcriptome. As a result, cells acquire a pro-inflammatory phenotype after exposure to lipopolysaccharides. ATG7, the autophagy-related protein 7, is known to play a vital role in the formation of autophagosomes. Here, we show how the deficiency of *Atg7* significantly impacts the ability of microglia to respond to inflammatory stimuli on a transcriptional, as well as on a functional level. This effect was found to be attributed to reduced NF-κB-dependent signaling, which was demonstrated by impaired NF-κB nuclear translocation.

In **project II** we investigate microglia in the context of the aging brain. Aging is often accompanied by cellular senescence, which can be characterized by growth arrest, a senescence-associated altered secretion of cytokines, mitochondrial dysfunction, and apoptosis resistance. Cells with a senescent phenotype are often associated with areas of age-related pathology, and in aged microglia this can lead to, for example, accumulation of protein aggregates and altered response to neuroinflammatory stimuli. With long-term cultivation of microglia, we show that these cells do not enter age-associated proliferative senescence but present with a distinct transcriptomic state and altered response to inflammatory stimuli.

In **project III** we show that targeting the H3K27 histone methyltransferase, EZH2, in microglia can affect the invasion and cell death of pediatric glioma. Diffuse midline glioma H3K27M mutant, formerly known as diffuse intrinsic pontine glioma (DIPG), is associated with poor prognosis. This led to studies using EZH2 as a potential therapeutic target. Inhibition of EZH2 *in vivo* by other groups showed reduced tumor growth and extended survival, however *in vitro* studies carried out by us, and others, showed limited effects of EZH2 inhibitors on tumor growth and survival. Here, we present that the inhibition of EZH2 in microglia leads to a robust anti-tumoral microglial activation state reducing tumor cell migration and increasing microglia-mediated cell death and phagocytosis of tumor cells, highlighting the tumor microenvironment (TME) as a potential target for further research on glioma treatments.

In **project IV** we study the interplay of macrophages and microglia in the glioma microenvironment. Infiltrating and resident immune cells in the context of brain tumors are often grouped together as glioma-associated microglia/macrophages (GAMs). In this context, they are known to acquire phenotypes beneficial for tumor growth and progression, which makes them a particularly interesting subject researching treatment targets. Glioblastomas

carrying a mutation in an isocitrate dehydrogenase (IDH1) are known to have better clinical prognosis, and less infiltration of GAMs. Here, we show that the presence of macrophages impacts the tumor migration as well as the acquisition of an activated microglia phenotype.

LIST OF SCIENTIFIC PAPERS

I. **Lara Friess**, Mathilde Cheray, Lily Keane, Kathleen Grabert and Bertrand Joseph. (2021).

Atg7 deficiency in microglia drives an altered transcriptomic profile associated with an impaired neuroinflammatory response. *Mol Brain*, 14(1):87. doi: 10.1186/s13041-021-00794-7.

II. Martin Skandik, **Lara Friess**, Lily Keane, Kathleen Grabert, Mireia Cruz de Los Santos and Bertrand Joseph.

Aged microglia: development of immunosenescence without replicative senescence.
Manuscript.

III. Lily Keane, Mathilde Cheray, Dalel Saidi, Caoimhe Kirby, **Lara Friess**, Patricia Gonzalez-Rodriguez, Maren Elisabeth Gerdes, Kathleen Grabert, Barry W McColl and Bertrand Joseph. (2021).

Inhibition of microglial EZH2 leads to anti-tumoral effects in pediatric diffuse midline gliomas. *Neuro-Oncology Advances*, Volume 3, Issue 1, January-December 2021, vdab096, https://doi.org/10.1093/noajnl/vdab096

IV. **Lara Friess**, Lily Keane, Mercedes Posada-Pérez and Bertrand Joseph.

Understanding if the presence of peripheral macrophages in the context of glioblastoma influences the tumor-associated microglial phenotype.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATG	Autophagy-related protein/gene
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CAR T cell	Chimeric antigen receptor T cell
CCL2	C–C motif ligand
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CSFR1	Colony stimulating factor 1 receptor
CXCL12	C-X-C motif chemokine ligand 12
DAM	Disease-associated microglia
DAMP	Damage-associated molecular patterns
DIPG	Diffuse intrinsic pontine glioma
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EZH2	Enhancer of zeste homolog 2
GAM	Glioma-associated microglia and macrophages
GDNF	Glial cell–derived neurotrophic factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
GSEA	Gene set enrichment analysis
HDAC	Histone deacetylase
hMOF	Human ortholog of males absent on the first acetyltransferase

IBA1	Ionized calcium binding adaptor molecule 1
IDH	Isocitrate dehydrogenase
IFN	Interferon
IGF1	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
iNOS/NOS2	Inducible nitric oxide synthase 2
IRF	Interferon regulatory factor
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC3	Microtubule-associated protein 1A/1B-light chain 3
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSFR	Macrophage colony-stimulating factor receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NAM	Neurogenic-associated microglia
NF-κB	Nuclear factor κB
NO	Nitric oxide
NOX2	NADPH oxidase 2
NPC	Neuronal precursor cell
PCNA	Proliferating cell nuclear antigen
PE	Phosphatidylethanolamine
pHGG	Pediatric high-grade glioma
PRC2	Polycomb repressive complex 2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT qPCR	Real-time quantitative polymerase chain reaction
SAM	Synaptic region-associated microglia

scRNA-seq	Single cell RNA sequencing
SIRT1	Sirtuin 1 deacetylase
SVZ	Sub-ventricular zone
TAM	Tumor-associated microglia and macrophages
TGF	Transforming growth factor
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRRUST	Transcriptional regulatory relationships unraveled by sentence-based text mining
WHO	World health organization
YS	Yolk sac

1 INTRODUCTION

1.1 Physiology and function of microglia

1.1.1 Microglia origins

Microglia are the tissue-resident immune cells of the central nervous system (CNS). Pio Del Río-Hortega, a student of Santiago Ramón y Cajal, was the first to name this "third element" of the CNS microglia (Sierra et al., 2016). The term stems from the Greek *micro* (small) and *glia* (glue), as opposed to the other two elements being neuronal cells and neuroglia, or astrocytes. With the development of their histopathological staining techniques, they already hypothesized in the 1930's the microglia's phagocytotic abilities and their overall role in maintaining homeostasis (Rio-Hortega, 1939), laying an important foundation to today's research. Since then, these specialized macrophages have been the focus of immense research leading to thousands of published articles.

The ontogeny of microglia was long debated, with an initial hypothesis from Río-Hortega himself postulating a mesodermal origin (Sierra et al., 2016). More recent studies found that microglia in mice originate from distinct progenitor cells in the embryonic yolk sac (YS) (Ginhoux et al., 2010). Through fate mapping experiments, the authors show that the pool of microglia, once established, is not significantly affected by circulating monocytes and suggest an ontogenically well-defined population. These YS progenitors circulate in the embryo during a short developmental time window, and populate tissues to form the basis of the innate immune system (Stremmel et al., 2018). The YS hypothesis of the origin of microglia has also been shown in zebrafish (Herbomel et al., 2001) and quails (Cuadros et al., 1993), suggesting an evolutionarily conserved mechanism. Circulating monocytes and other bone marrow-derived cells, in either steady-state or even during inflammation, are additionally described as a less significant source of post-natal microglia, as reviewed in detail elsewhere (Ginhoux et al., 2013).

1.1.2 Homeostatic functions

The role of microglia in the developing, healthy brain has many facets, such as impacting the amount of neuronal precursor cells (NPC) via phagocytosis in the sub-ventricular zone (SVZ) (Cunningham et al., 2013). Their main contribution to CNS development is protecting and shaping the microenvironment. Other work has shown how endogenous microglia can regulate not only cortical precursor cells, but also astrocytes. A knockout mouse model using PU.1$^{-/-}$ animals, which have a lack of microglia, showed decreased proliferation of the precursor cells, as well as decreased astrogenesis (Antony et al., 2011). Microglia have also been found to play a significant role in the maturation of synapses, contributing to synaptic pruning by engulfment of synaptic material (Paolicelli et al., 2011). This process of maintaining certain synapses and pruning others is crucial for brain development. Different groups have hypothesized that synaptic engulfment is mediated by the complement pathway, which is classically involved in tagging dead cells or cellular debris for phagocytosis by microglia or macrophages. Steven *et*

al. showed that C1q- and C3b-tagged (activated form of complement 3) synapses are likely phagocytosed by microglia, which express the C3 receptor, and are therefore contributing to synaptic maintenance in the CNS (Stevens et al., 2007). An alternative explanation was recently published by Nguyen *et al.*, showing that synaptic plasticity can be promoted through remodeling of the extracellular matrix (ECM) (Nguyen et al., 2020). In the hippocampus, the brain region responsible for memory formation and consolidation of memory, neurons were shown to secrete interleukin-33 (IL-33). This IL-33-mediated neuron-microglia signaling was shown to impact the formation of new synapses as well as memory precision, measured by contextual fear discrimination. The same work further showed that the observed synaptic plasticity was likely due to microglia engulfing ECM components. Within the hippocampus, microglia have also been shown to be involved in the formation of learning-dependent synapses through brain-derived neurotrophic factor (BDNF) (Parkhurst et al., 2013), the differentiation of NPCs (Aarum et al., 2003), or the regulation of spatial memory and even altered social behaviors (Torres et al., 2016). Overall, these findings strongly suggest an impact of microglia on important memory functions in the healthy brain.

During normal development, fractalkine signaling and the release of trophic factors by microglia, such as insulin-like growth factor 1 (IGF1), are involved in supporting neuronal survival (Ueno et al., 2013). During early postnatal development, microglial CD11b, as well as the DAP12/TREM2 pathway, are required for neuronal homeostasis (Wakselman et al., 2008). CD11b, together with CD18, forms the complement receptor 3 that is involved in phagocytosis through the complement signaling cascade. Microglia also contribute to normal neuronal apoptosis through the production of reactive oxygen species (ROS). ROS in high concentrations can become cytotoxic by oxidizing cellular components and molecules and its associated cell damage is also reported to be involved in aging and neurodegenerative diseases. Apart from this detrimental effect, ROS has also been shown to be involved in neuronal and synaptic plasticity, connectivity, or other processes in neuronal development (Oswald et al., 2018). In an evolutionary conserved mechanism, microglia have also been shown to initiate apoptosis and finally engulf Purkinje cells, likely as part of developmentally programmed cell death (Marín-Teva et al., 2004). Lastly, the removal of cellular debris after apoptosis is part of the homeostatic functions of microglia. This phagocytic approach, which does not induce an inflammatory reaction, is mediated via a complex interplay of so called “find me”, “eat me” or “don’t eat me” signals and through receptors such as CX_3CR1, triggering receptor expressed on myeloid cells 2 (TREM2) or SIGLEC, respectively (Butler et al., 2021). Clearing the CNS of debris, dying or dead cells is an important homeostatic function of these resident phagocytotic cells.

1.1.3 Common microglia markers

Markers that were found in early research to characterize microglia under steady-state conditions were, among others, CD45, CD11b, MHC class I and class II. Other general cell markers are further cluster of differentiation receptors such as CD68, CD80 or colony stimulating factor 1 receptor (CSFR1), cell surface glycoprotein F4/80, fractalkine receptor

CX_3CR1, as well as ionized calcium binding adaptor molecule 1 (IBA1) (Jurga et al., 2020). Fractalkine/CX_3CR1 signaling in particular is important during developmental time windows of synaptic pruning, and CX_3CR1-deficient mice present with impaired neuronal circuit integrity, regulation of neuronal precursor cells and neurogenesis (Kierdorf & Prinz, 2013). Many of the above-mentioned markers however are not specific to microglia or can be upregulated in microglia upon activation. This can present a challenge when trying to differentiate microglia from, for example, infiltrating macrophages. To discriminate between the different cell types, it can be useful to consider the amount of a certain marker protein expressed. For example, CD45 is also present on other monocytes and expressed at a higher level compared to microglia under steady-state conditions, although it should be noted the expression of this marker can also increases with microglia activation (Butovsky & Weiner, 2018; Masliah et al., 1991). TMEM119, a transmembrane protein of currently unknown function, and P2RY12, a purinoceptor, are commonly described as unique markers for microglia cells (Bennett et al., 2016; Butovsky et al., 2014), see also **figure 1**. However, more recent work has challenged this view, suggesting that the activation of microglia alters and downregulates the expression of homeostatic microglia signature genes (DePaula-Silva et al., 2019). Comparisons to other tissue-resident macrophages found that genes encoding for proteins such as SIGLEC-H, TREM2, and transcription factors like jun-B, jun-D or SALL1 maintain microglia identity in the microenvironment of the CNS (Buttgereit et al., 2016, p. 1; the Immunological Genome Consortium et al., 2012).

1.2 Microglia activation

1.2.1 Inflammatory activation states

Such a variety of functions in a highly complex and plastic environment as the developing or adult brain presupposes that microglia are capable of responding to a long list of challenges including injury, inflammation, or tumors. In traumatic CNS injury, microglia rapidly respond and migrate towards the lesion site, where they have been reported to contribute to secondary injury events by exacerbating an inflammatory reaction, recruiting more immune cells to the injury site and releasing pro-inflammatory cytokines (Dusart & Schwab, 1994). Long-term microglia activation due to traumatic injury can lead to chronic effects and can even be seen in humans, posing a potential risk for post-traumatic neurodegeneration (Gentleman et al., 2004). Using $Cx_3cr1^{GFP/+}$ mice with green fluorescent protein (GFP)-expressing microglia, a fast chemotactic response towards a two-photon laser ablation showcased the plasticity of their processes reacting to an injury site (Davalos et al., 2005). The same work furthermore showed that individual cells could even react towards multiple laser ablation sites nearby, and that their response is mediated through extracellular adenosine triphosphate (ATP). Other publications support these findings by demonstrating how purinergic receptor P2RY12, which are key players in ATP-mediated signaling, are crucial for directional process extension as well as migration (Haynes et al., 2006), and how microglia processes are triggered by ATP-mediated neuron-microglia communication (Dissing-Olesen et al., 2014). This remarkable plasticity and the ability to undergo structural changes based on their environment or challenge is one of the cells' hallmarks of how a fast and targeted immune response can react to CNS injuries. Even in a resting state, microglia have been shown to be highly motile and undergo morphological changes, dynamically interacting with other cell types and surveilling the microenvironment through direct contact and their surface receptors (Nimmerjahn, 2005).

Opposed to the non-inflammatory pathway clearing apoptotic cells, microglia utilize a second kind of phagocytosis to remove pathogens via toll-like receptors (TLR). Exposure to microorganisms, but also cytokines in a microenvironment of neuronal damage, induce a pro-inflammatory response characterized by the upregulation of many pro-inflammatory mediators such as nitric oxide synthase 2 (NOS2), tumor necrosis factor alpha (TNF) or interleukins IL-6 and IL-1β. In cell culture experiments, this is often demonstrated using lipopolysaccharide (LPS), a compound derived from the surface membrane of Gram-negative bacteria. A physiologically slightly more relevant approach using mediators such as TNFα or interferon gamma (IFNγ), and therefore mimicking a more sterile form of inflammation, was found to elicit a similar, yet less elevated inflammatory response in microglia (Lively & Schlichter, 2018). This suggests that, as with all *in vitro* set ups, results should be interpreted with caution regarding context and relevancy. Pro-inflammatory microglia can also produce superoxides through NADPH oxidases (NOX). NOX2, the catalytic unit, will transfer electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to oxygen, reducing it to superoxide (Lambeth, 2004). Microglia can furthermore produce nitric oxide (NO) through the inducible nitric oxide synthase (iNOS) after pro-inflammatory stimulation with LPS or cytokines, which

under healthy conditions is not expressed in microglia (Béchade et al., 2014, p. 2). Pioneering experiments showed early on how this mechanism can lead to neurotoxicity, and while NO can act as a signaling molecule, it is most often correlated with its anti-proliferative and pro-apoptotic effect during the immune response (Boje & Arora, 1992; Guix et al., 2005).

Apart from such pro-inflammatory functions, microglia can also act as neuro-protective in several experimental conditions. In ischemic stroke studies, microglia appear to be protective against induced neuronal damage (Lalancette-Hebert et al., 2007). Further investigations found that said neuroprotective functions are likely due to microglia engulfing invading neutrophils, as well as releasing the cytokine TNFα (Lambertsen et al., 2009; Neumann et al., 2008). In an experimental set up of spinal cord injury in rats, transplanted microglia also showed a beneficial effect of the growth of neuronal processes. This beneficial influence is likely due to the production and release of cytokines and growth factors, such as transforming growth factor beta (TGF-β), which is also found in the lesioned spinal cord (Rabchevsky & Streit, 1997). The production of TGF-β by microglia is thought to be crucial for an anti-inflammatory response and inducing a deletion of its receptor gene, *Tgfbr2*, was shown to significantly impair microglia homeostasis and increase the expression of multiple activation-associated markers (Zöller et al., 2018).

1.2.2 Aging and neurodegeneration

Microglia in the aging brain present with cytoplasmic abnormalities, such as de-ramification and fragmentation, that are thought to be a sign of a senescent phenotype and are coined dystrophic microglia (Streit et al., 2004). These cells seem to be activated, since expression of MHC II increases with age, as shown in early studies on non-human primates (Sheffield & Berman, 1998). Aged microglia have a slower response rate towards injury, reduced phagocytosis, as well as an altered production of inflammatory cytokines (Damani et al., 2011; Koellhoffer et al., 2017). Models of accelerated aging propose that aged microglia differ by having an increased expression of inflammatory genes as well as an increased activation in response to LPS, which is described as microglia priming as coined by Holtman et al (Holtman et al., 2015; Spittau, 2017). The overall shift towards a more pro-inflammatory environment in the aging brain leads to priming, where response to stimuli or stress are exaggerated and prolonged, ultimately leading to increased release of inflammatory mediators and longer recovery periods (Sparkman & Johnson, 2008).

Microglia in the adult brain are not just involved in the process of aging *per se* but play vital roles in multiple neurodegenerative disorders. Phagocytosis of neurotoxic protein aggregates, myelin, neurons, or other cell debris is an important function of microglia. The consensus of multiple animal disease models and aging experiments is that the phagocytotic capabilities of microglia, similar to macrophages, decreases with age (Gabandé-Rodríguez et al., 2020). In a meta-analysis, Holtman and colleagues described a distinct microglia phenotype hypersensitive to pro-inflammatory stimuli, representing primed microglia (Holtman et al., 2015). They specifically found a downregulation of common microglia markers such as TMEM119, P2RY12 or CX_3CR1 in aged microglia, as well as enrichment of genes involved in the

phagocytic and antigen-presenting signaling pathways, and genes of Alzheimer's Disease (AD) signaling. As aging is also the most important risk factor for neurodegenerative disorders such as AD, understanding the processes that microglia undergo in the aging brain may hold the key to developing novel therapeutic approaches for many age-related diseases (Bullain & Corrada, 2013).

AD is a common neurodegenerative disorder with an estimated prevalence of 6-8% in the population of 85 years and older (Mayeux & Stern, 2012). It is characterized by accumulation of amyloid-β peptides as well as hyper-phosphorylated tau forming intraneuronal fibril tangles (Selkoe, 2001). AD susceptibility genes are often known microglia markers such as TREM2 (Jonsson et al., 2013) or PU.1 (Rustenhoven et al., 2018), and are associated with the phagocytic clearing pathway (the Alzheimer's Disease Neuroimaging Initiative et al., 2011). In support of these findings, experiments have shown how early recruitment of microglia is initially neuroprotective, but can become pro-inflammatory and neurotoxic, at least in some instances, as pathological burden increases and clearance is impaired (Hickman et al., 2008; Krabbe et al., 2013). Especially the AD risk factor CD33 is implicated in the phagocytosis of amyloid-β by microglia, suggesting a significant involvement of these cells in the pathology of AD likely due to impaired phagocytic functions (Griciuc et al., 2013). However, an *in vivo* AD model with ablation of microglia showed no significant difference in either formation, or maintenance of amyloid-β pathology, raising further questions regarding their exact involvement in disease progression (Grathwohl et al., 2009). Synaptic loss is a hallmark of neurodegenerative disorders, even before the onset of pathological plaque formations. Gene expression analysis found elevated levels of complement 3 (C3) in aged retinal microglia, and it is known that C3-dependent signaling is involved in the phagocytosis and synapse loss in AD (Stephan et al., 2012).

Neuroinflammation has been identified as a disease risk factor, or risk-escalating factor for AD, yet anti-inflammatory drugs fail to halt disease progression. Epidemiological studies have shown a potential effect of non-steroidal anti-inflammatory drugs on the onset of AD, however, the discrepancy to non-successful clinical trials may be due to the longer time periods of administration necessary to elicit a response (Rivers-Auty et al., 2020). Microglia-mediated inflammation is typically initiated via damage-associated molecular patterns (DAMP), like aggregated or misfolded proteins. This can lead to chronic neuroinflammation, where aggregate clearance is impaired and neurotoxic factors are produced and released by microglia (Block & Hong, 2007). This chronic activation is subject to clinical research, for example targeting microglia via nanoparticles in Alzheimer's or Parkinson's Disease, with one nanoparticle study currently being in a phase I clinical trial for AD (H. Liu et al., 2020; N. Zhao et al., 2020). Disease-associated microglia (DAMs), a transcriptionally distinct microglial activation state in neurodegeneration, specifically AD, as described by Keren-Shaul and colleagues, show elevated expression levels for AD risk factor genes such as *Apoe* and *Trem2* (Keren-Shaul et al., 2017). The latter is further orchestrating the transition of microglia in the aging brain towards the DAM phenotype. As shown by Kraseman and colleagues, this in turn is modulated by apolipoprotein E (APOE), describing the TREM2-APOE pathway as a regulator of the

DAM transcriptome and a potential target to rescue the homeostatic microglia phenotype in an AD mouse model (Krasemann et al., 2017). Microglia have also been shown to be implicated in amyloid-β as well as tau pathologies. The activation of NLRP3 via amyloid-β leads to a pro-inflammatory and neurotoxic activation of microglia (Halle et al., 2008). This has also been shown to impact the pathogenesis of AD *in vivo*, where NLRP3 deficiency led to decreased deposition and overall levels of amyloid-β, and an increased microglia phagocytosis of amyloid-β in an APP mouse model of AD (Heneka et al., 2013). The activation of the NLRP3 inflammasome in microglia by tau could also be shown in a mouse model for fronto-temporal dementia overexpressing human tau, suggesting a similar mechanism as seen in the AD model (Ising et al., 2019). Microglia furthermore seem to be involved in the seeding, and therefore propagation, of amyloid-β pathology in AD models (Venegas et al., 2017). Even though the past years have brought tremendous advances for the knowledge on microglia in aging and neurodegeneration, there is still the need to find suitable therapeutic options for not just disease onset, but also progression of AD and other disorders. Additionally, the limited lifespan of mice provides a challenge when researching aging or age-related diseases. Therefore, exploring novel models of microglia in aging could help understand mechanisms underlying not only healthy aging, but also the development of neurodegenerative diseases.

1.2.3 Transcriptomics of activation states

In recent years, it has been shown that microglia exhibit huge spatial and temporal heterogeneity, and more and more subsets of microglia throughout developmental phases are identified based on, *e.g.*, their transcriptome (Li et al., 2019; Masuda et al., 2020). This vast heterogeneity of microglia is reflected in the number of activation states and even subtypes that have been identified to date. A subtype, as opposed to an activation state, describes an intrinsic microglia population that is found alongside canonical microglia in the CNS, without the impact of environmental factors, such as external inflammatory stimuli. These subtypes include morphologically specialized “satellite” microglia interacting with the axon initial segment to support homeostasis in the healthy brain, KSPG-microglia which appear to be insult-related, or CD11c-microglia promoting neurogenesis and myelination (Baalman et al., 2015; Bertolotto et al., 1993; Wlodarczyk et al., 2017).

Some activation patterns may indeed reflect certain subtypes; however, this remains to be proven. One aspect of said heterogeneity is the difference in features based on the CNS region the microglia are found in. Some examples, as reviewed extensively, have different morphologies, densities, and expression of markers such as CX_3CR1, TREM2 or immune-related genes (Y.-L. Tan et al., 2020). It is mostly unclear yet if and how these regional differences can impact the functional signatures of microglia. One study approached this question with a genome-wide analysis of microglia from distinct regions, as well as comparing young versus aged animals. The extracted cells showed regionally heterogeneous transcriptomes, suggesting different immune-vigilant states depending on the distinct brain regions (Grabert et al., 2016).

More recent advances in bulk and single cell RNA sequencing (scRNA-seq) methods have made their contributions to the field by elucidating the transcriptomic profiles of activated or disease-associated phenotypes. In the human brain, scRNA-seq identified region- and age-dependent microglia clusters, demonstrating high heterogeneity of transcriptome signatures (Sankowski et al., 2019). Throughout development, the highest heterogeneity as analyzed in mice seems to occur during early development, and even certain microglial marker genes such as *P2ry12*, *Tmem119* or *Cx$_3$cr1* show altered expression levels during developmental stages (Hammond et al., 2019). The same study also identified microglia clusters with upregulated inflammatory response genes in the aged mouse brain, which may contribute to the onset or progression of age-related disorders (Hammond et al., 2019).

In aged human microglia, RNA-seq analysis resulted in a distinct transcriptomic profile including susceptibility genes associated with AD (Olah et al., 2018). The same study furthermore showed that the transcriptome of aging microglia was associated with a gain of function for genes in the amyloid fiber formation pathway, suggesting that certain, but not all neurodegenerative disorders might be mediated through aged microglia. Interestingly, microglia that were *Cx$_3$cr1*-deficient, which is usually a highly expressed microglia marker, presented with a transcriptome and morphology similar to that of aged microglia, suggesting that the receptor protein is involved in regulating microglia aging (Gyoneva et al., 2019). A comparison between aged human and aged mouse microglia found little overlap of their transcriptomes, indicating a difference in how these cells age between species even though the core signature at steady-state was found to be similar (Galatro et al., 2017; Gosselin et al., 2017). Through scRNA-seq, Keren-Shaul and colleagues found that DAMs are enriched for genes that are known risk factors for AD or are involved in phagocytic pathways, and that their activation is orchestrated by TREM2 (Keren-Shaul et al., 2017). On the other hand, studies performed on human post-mortem tissue failed to identify subsets with DAM signature genes in AD brain samples (Alsema et al., 2020). More recently, studies in zebrafish identified two additional microglia subpopulations, synaptic region-associated microglia (SAM) and neurogenic-associated microglia (NAM) (Silva et al., 2021). These activation states show transcriptionally distinct profiles regarding the brain region, as well as their function, with SAMs locating predominantly in the synapse-rich hindbrain where they engulf synapses as a form of pruning, and NAMs presenting in the optic tectum, a region of significant neurogenesis.

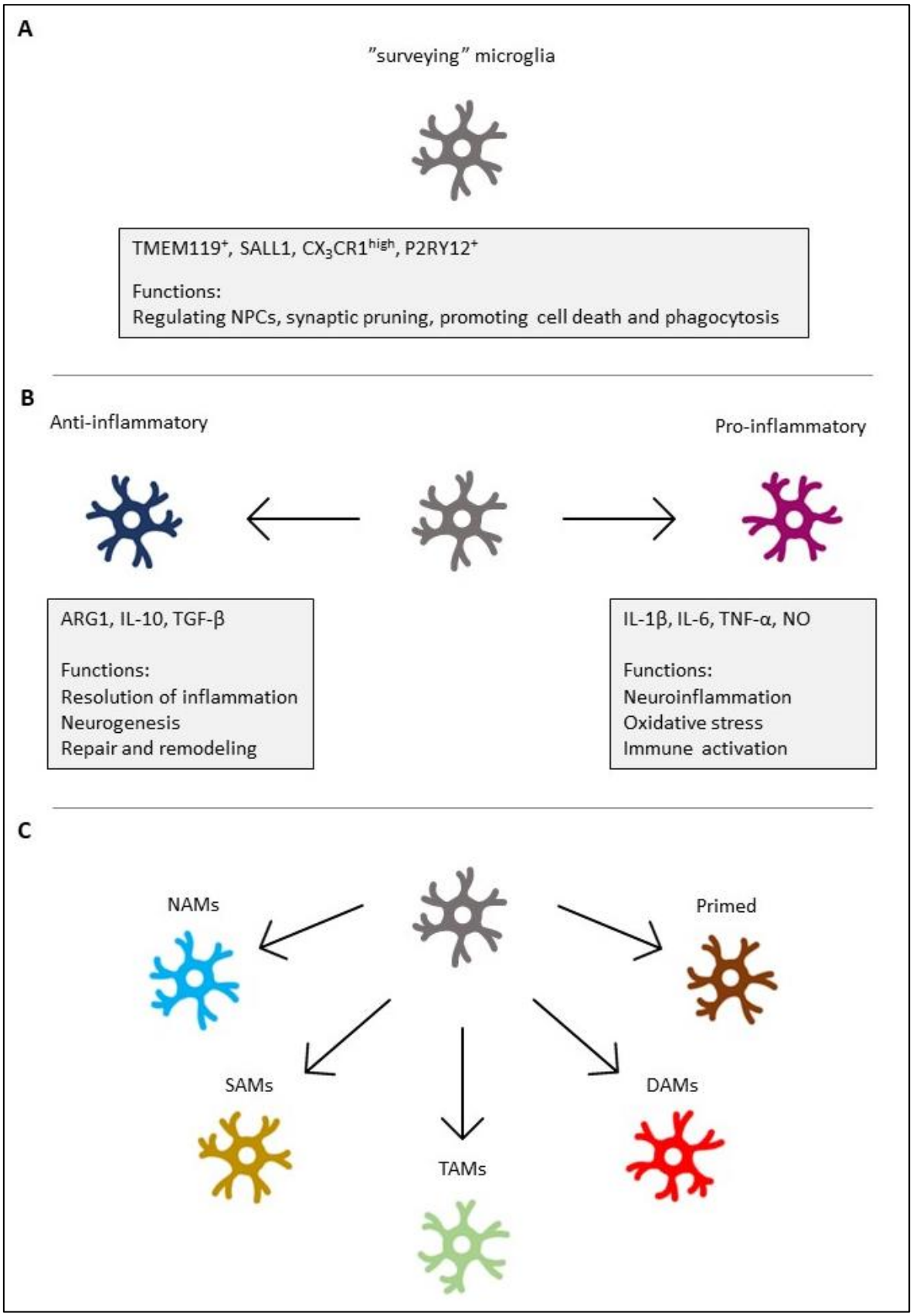

Figure 1: *Microglia can acquire different phenotypes depending on their environment or activating stimulus.* ***(A)*** *Surveying microglia fulfill roles throughout development as well as in the healthy adult brain, ranging from regulating neuronal precursor cells to pruning synapses, promoting beneficial cell death, or phagocytosing debris in the CNS. General microglia*

markers include TMEM119- and P2RY12-positivity, expression of transcription factor SALL1 or a high expression of CX_3CR1 (Bennett et al., 2016; Buttgereit et al., 2016; Jurga et al., 2020). ***(B)*** *In a highly simplified model, microglia are often classified as classically or alternatively activated, speaking of pro- or anti-inflammatory phenotypes. Anti-inflammatory phenotypes will show upregulated markers such as ARG1, IL-10 or TGF-β and are involved in tasks like repair after traumatic injury and neurogenesis (Cherry et al., 2014). Pro-inflammatory phenotypes on the other hand are involved in stress- and inflammatory-mediated reactions and produce cytokines IL-1β, IL-6 and TNF-α, as well as nitric oxide (J. A. Smith et al., 2012).* ***(C)*** *Depending on physical microenvironment, age, or disease occurrence, microglia will acquire distinct morphological and transcriptomic signatures. Some examples shown here include neurogenic associated microglia (NAM), synaptic-region associated microglia (SAM), tumor-associated microglia (TAM), disease-associated microglia (DAM), or microglia primed through the normal aging process (Hammond et al., 2019; Keren-Shaul et al., 2017; Sa et al., 2020; Silva et al., 2021).*

As presented in these large number of studies, sequencing techniques can be used to identify manifold microglia states or possible microglia subtypes based on their transcriptomic profiles extending far beyond the traditionally labeled pro- or anti-inflammatory states (**Figure 1**). However, a caveat of most current advances using single cell sequencing is that the actual functions of these identified subsets of microglia still need to be elucidated to draw more relevant conclusions. Methodologically, there is also variation in the analysis of sequencing data such as different available clustering methods, introducing challenges when comparing data. Additional discussion points are the comparison between human and mouse microglia, as well as *in vivo* versus *in vitro* transcriptomes. In a study by Gosselin and colleagues, a well-conserved transcriptomic signature was found between human and mouse cells, although certain genes involved in the development of brain structure or the complement pathway were distinctly different between the species (Gosselin et al., 2017). Changes were also observed *in vitro* compared to *ex vivo*, especially in genes associated with an acute inflammatory response, likely due to the loss of signals from the brain environment. It is now abundantly clear that microglia are highly plastic cells with the ability to respond to a great deal of stimuli with a wide range of adaptable cellular functions. This remarkable ability for a single cell type begs the question of how these phenotypes can be regulated and how they can be differentiated, however, caveats in possible differences due to methods used, species analyzed, or the cells' microenvironment are all factors to take into consideration.

1.3 Microglia regulation

1.3.1 Transcription factors associated with microglia homeostasis or activation

Microglia and their activation states can be regulated by a number of pathways and transcription factors, depending on the activating stimulus. Analysis of the binding sites and regulatory elements of genes of interest expressed in activated microglia states has given insight into how these phenotypes may be regulated. Nuclear factor κB (NF-κB) signaling is of particular importance to the regulation of immune cell responses and the initiation of inflammation (T. Liu et al., 2017). It represents a family of transcription factors including p65 (or RelA), p50 (NF-κB1), p52 (NF-κB2), c-Rel and RelB (Verma et al., 1995). Upon activation and dimerization in homo- or heterodimers, the subunits translocate into the nucleus where they bind DNA at recognized κB binding sites. As reviewed comprehensively elsewhere, the lists of inducers for NF-κB are extensive and include exposure to viral or bacterial infection, oxidative stress, environmental stress, or different receptor ligands. Similarly, there are hundreds of target genes, often such that are required for immune response and cell survival (Pahl, 1999). In macrophages, NF-κB signaling is known to be crucial for the polarization of these cells mostly towards a pro-inflammatory phenotype. Through receptors such as TLR4, which recognizes LPS as a ligand, and the sequential activation of kinases downstream of TLR4, NF-κB induces genes necessary for the inflammatory response (T. Liu et al., 2017). The pathway is furthermore activated by the presence of α-synuclein or amyloid-β, where it promotes a pro-inflammatory cascade contributing to the pathogenic involvement of microglia in neurodegenerative disorders (Chen et al., 2005, p. 1; Hoenen et al., 2016). LPS-induced signaling mediated through TLR4 can also act via mitogen-activated protein kinase (MAPK) pathways, leading to the activation of the transcription factor family of activator protein 1 (AP-1) (W. Liu et al., 2009). In macrophages and dendritic mouse cells, pro-inflammatory expression of cytokines such as IL-23 have been shown to involve NF-κB as well as AP-1, and the interaction of these transcription factors in the regulation of pro-inflammatory responses has also been shown in microglia (Jeong et al., 2020; W. Liu et al., 2009).

Another transcription factor of interest is PU.1, which is found in functional macrophages and is constitutively expressed in quiescent and activated rodent (Walton et al., 2000), as well as human microglia (A. M. Smith et al., 2013). PU.1 has been shown to be important in microglia activation regulating gene expression. Specifically, PU.1 expression seems to spike after hypoxic-ischemic insults, suggesting its involvement in immune activation (Walton et al., 2000). It is also known that PU.1 is involved in the regulation of microglial genes associated with AD (Rustenhoven et al., 2018). With PU.1 being a genetic risk for AD itself, interesting research has emerged pinpointing how PU.1 is not only regulating pro-inflammatory gene expression, but is also crucial for the cells' phagocytic ability (Pimenova et al., 2021). PU.1 has been found to be regulated via a positive feedback loop with the transcription factor interferon regulatory factor 8 (IRF8) (N. Zhou et al., 2019). The latter in turn is known to regulate homeostatic microglia signatures such as CD11b or CX_3CR1, as well as regulating

microglial motility-related genes or the activation of microglia specifically in the context of injury-induced neurodegeneration (Masuda et al., 2012, 2014; Minten et al., 2012).

1.3.2 Epigenetic regulation

Lastly, epigenetic mechanisms can impact microglia plasticity and their functions in homeostasis or even CNS disorders (Cheray & Joseph, 2018). Epigenetics refers to changes altering the physical structure of DNA, and subsequently impacting gene expression by increasing or decreasing DNA accessibility. This includes histone modifications, DNA methylation and even non-coding RNA. Such epigenetic changes can depend on a number of factors ranging from age, to exposure to toxins or infection, and even prenatal exposure to nutritional starvation (Chandran et al., 2015; Heijmans et al., 2008; Heyn et al., 2012; McCartney et al., 2018).

Histones, around which DNA is tightly coiled to form packaging units known as nucleosomes, are subject to post-translational modifications – acetylation, methylation, or phosphorylation of the histone tails are the most common and can result in direct structural changes or impact the DNA binding of other proteins (Bannister & Kouzarides, 2011). Histone acetylation is mediated by two types of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). In microglia, a switch towards the glioma-induced pro-tumoral phenotype was correlated to increased H4K16 acetylation mediated by the HDAC sirtuin 1 (SIRT1) and the HAT hMOF, resulting in increased expression of genes associated with a tumor-supporting microglial phenotype (Saidi et al., 2018). The epigenetic regulation of lineage-determining transcription factors such as PU.1 or SALL1 can therefore determine the DNA accessibility and expression of microglia signature genes. Increased acetylation of histone H4 at the PU.1 promotor region, for example, leads to decreased PU.1 expression, mediated via the inhibition of HDACs (Laribee & Klemsz, 2005). The pharmacological inhibition or genetic targeting of HDACs has also been tested after spinal cord injury and in LPS-activated mouse microglia, leading to a decreased inflammatory response (Alireza Abdanipour et al., 1996; Kannan et al., 2013).

Histone methylation of H3K27 as well as of H3K4 have been shown to be in positive correlation with microglia activation. The inhibition of the enhancer of zeste homolog 2 (EZH2) histone-methyltransferase within the polycomb repressive complex 2 (PRC2) modulates the transcription factors IRF8 and STAT1, which are implicated in the expression of inflammatory-related genes (Arifuzzaman et al., 2017). H3K27me3, and PRC2, were also associated with the repression of genes related to clearance activity in mice, impacting neuronal morphology and function (Ayata et al., 2018). Furthermore, H3K4 methylation was associated with DAM-specific microglial genes marking promotor and enhancer regions (Keren-Shaul et al., 2017). In an effort to elucidate how amyloid-β accumulation may impact microglia transcriptomic changes via epigenetics, Wendeln and colleagues found increased levels of H3K4me1 in mouse microglia after pro-inflammatory stimulation with LPS, as well as in APP23 mice, a model for AD (Wendeln et al., 2018). This particularly affected genes involved in phagocytic functions, but also the transcription factor HIF-1α which influences cellular

metabolism, mitochondrial respiration and response to hypoxic conditions as can be observed in the AD brain (Ashok et al., 2017).

1.3.3 Autophagy in microglia

Autophagy, or components of the autophagic pathway, are also gaining interest as potential regulators of microglia and overall processes in the CNS such as aging, neurodegenerative diseases or epilepsy models (Alam et al., 2021; Plaza-Zabala et al., 2017; Su et al., 2016). Derived from the Greek words for ”self” (*auto*) and ”eating” (*phag-*), autophagy is primarily the selective bulk degradation of proteins and organelles to maintain cellular homeostasis and can be induced by a multitude of stimuli, such as amino acid- and nitrogen-starvation, ER-stress, hypoxia, as well as other stress-related pathways (Ecker et al., 2010; Mazure & Pouysségur, 2010; Onodera & Ohsumi, 2005; Rashid et al., 2015). In general, one can differentiate between three main types of autophagy. During micro-autophagy, cytosolic components are, usually non-selectively, internalized directly into lysosomes. (Parzych & Klionsky, 2014). Chaperone-mediated autophagy uses the recognition of specific amino acid motif sequences of substrate proteins. The substrate is bound and translocated by so-called chaperone proteins to a lysosome for degradation, hence the name of the process (Cuervo & Wong, 2014). Macro-autophagy on the other hand utilizes double-membrane vesicles, formed *de novo* around components to be degraded, followed by fusion with enzyme-carrying lysosomes. There is furthermore a selective, organelle-specific type of macro-autophagy which aims to remove damaged components of the cells, such as mitophagy for degrading mitochondria (Swerdlow & Wilkins, 2020). In the following text, the term autophagy will however simply refer to the process of macro-autophagy.

The process of autophagy is mediated by a number of autophagy-related genes and proteins and is characterized as sequential formation of double-membrane vesicles engulfing cargo to be degraded. In an initial step of induction and nucleation, the phagophore expands and forms the autophagosome by closing into a vesicle. In mammals, this step is mainly controlled by the interaction of ATG4 and MAP1LC3 (microtubule associated protein 1 light-chain 3, hereafter referred to as LC3) (Xie et al., 2008, p. 8; Y. Zhou et al., 2021). LC3 is cleaved and sequentially conjugated to phosphatidylethanolamine (PE), which creates the membrane-bound LC3-II. This step is coordinated through the activation of, sequentially, ATG7, ATG3, and the ATG12/ATG5/ATG16L1-complex, acting as E1-like, E2-like and E3-like enzymes, respectively (Y.-K. Lee & Lee, 2016). ATG7, similar to ATG10, also plays the role of an activating E1-like enzyme in the conjugation of ATG12 with ATG5, before they then associate to ATG16L1 to form this complex (Walczak & Martens, 2013). These key aspects make ATG7 an interesting target when studying autophagy. After the autophagosome fuses with a lysosome carrying enzymes such as hydrolases, the vesicle becomes an autolysosome. Following the enzymatic degradation of the vesicle cargo into molecules like amino acids, autophagy-related proteins such as ATG22 are involved in the release of the molecules back into the cytosol (Yang et al., 2006). The steps of autophagy as described above, and the roles of ATG7 specifically, are summarized in **Figure 2**.

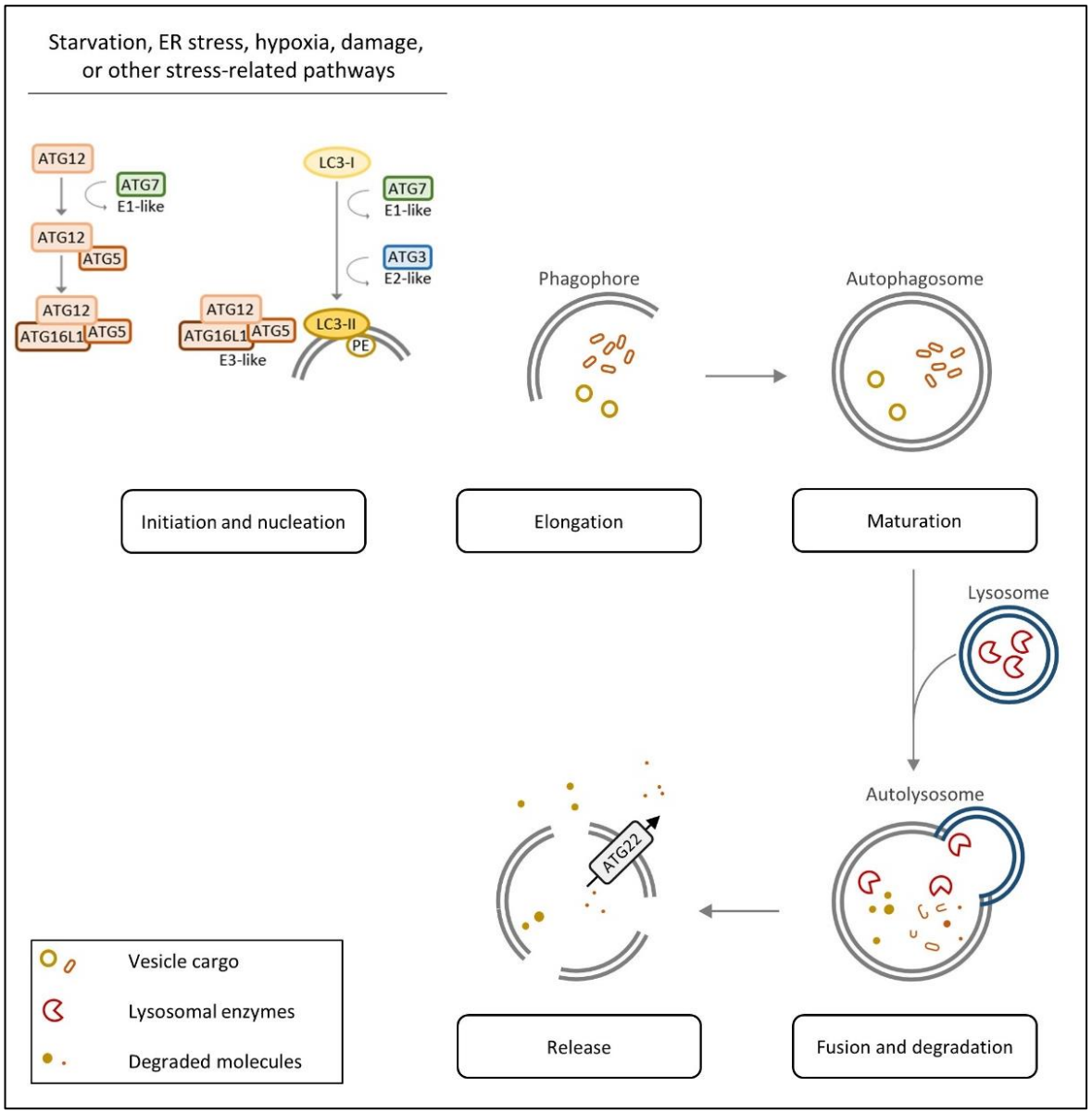

Figure 2: *Simplified schematic of the autophagy process. Different stimuli, such as nutrient starvation or other stressors, initiate autophagy. During the first step, the double-lipid membrane starts to form to incapsulate the cargo. The elongation of the phagophore is finalized in the autophagosome, which then fuses with enzyme-carrying lysosomes. The then formed autolysosome degrades its cargo into molecules and releases these to be used in anabolic synthesis of, e.g., proteins or cell components. ATG7 plays an important role during autophagy in mainly two functions. First, it acts as an E1-like enzyme in the conjugation process of ATG12 and ATG5, which then form a complex together with ATG16L1. ATG7 furthermore activates and facilitates the transfer of LC3 to ATG3. The E3-like enzyme complex of ATG12/ATG5/ATG16L1 then localizes to the expanding membrane of the phagophore to facilitate the conjugation of LC3 with PE.*

Autophagy-related proteins can exhibit roles outside of their function in the autophagy process. Multiple autophagy-related proteins, especially the ones involved in LC3 conjugation, are

implicated in the release of viral particles via exocytosis, as well as in inflammation-associated endocytosis (Münz, 2021). In murine macrophages, ATG16L1 was linked to the control of the endotoxin-associated inflammatory immune response by regulating the inflammasome activation (Saitoh et al., 2008). An additional implication in the immune response is that ATG5 regulated MyD88-dependent signaling, which is an adaptor protein for TLRs and therefore crucial for the signaling cascades following TLR stimulation (Inomata et al., 2013). ATG7 as well as the complex ATG12/ATG5/ATG16L1 are required for the IFNγ-mediated antiviral host defense in mouse macrophages, where the activation of IFNγ was dependent on the complex without the induction of autophagy itself (Hwang et al., 2012). ATG7-deficiency is also linked to an accumulation of p62, and the interplay of p62 and NF-κB forms an intrinsic feedback loop regulating inflammatory responses (Zhong et al., 2016). Specifically in microglia, ATG7-deficiency has been shown to disrupt the clearance of tissue debris or amyloid-β fibrils in mouse disease models such as for multiple sclerosis or AD, exacerbating neuroinflammation (Berglund et al., 2020; Cho et al., 2014). ATG7 has also been implicated in transcriptional regulation, which makes it an interesting target to research regarding the activation of certain cell types, such as microglia. One example in nutrient-deprived mouse fibroblasts showed that ATG7 could bind the transcription factor p53 and therefore regulate the transcription of genes implicated in cell cycle arrest (I. H. Lee et al., 2012). Conversely, the study found induced expression of pro-apoptotic genes mediated by p53 in *Atg7*-deficient cells. ATG7 was also found to bind to the transcription factor FOXO1, which is implicated in induction of autophagy itself and autophagic cell death (Y. Zhao, Yang, et al., 2010).

In summary, the activation of microglia phenotypes is regulated by a vast number of factors, ranging from signature transcription factors to epigenetic modulations, and potentially even proteins such as ATG7, which are previously thought to govern completely different functions.

1.4 High-grade glioma

Malignancies of the brain are amongst the most severe and difficult to treat diseases occurring in adults as well as in children. In a statistical report from the Central Brain Tumor Registry of the United States (CBTRUS) it was shown that primary brain and CNS tumors are the most common cancer type in children under 15 years of age, and the third most common cancer type in adults under 40 years (Ostrom et al., 2019). The same report stated that, of the malignant CNS tumors, gliomas make up over 80% of this category and glioblastomas are the most common subtype of malignant CNS tumor.

Historically, CNS tumors were classified on the consensus of histopathological opinions. In order to implement internationally accepted standards of diagnosis, the international agency of research on cancer (IARC) and its group for the classification of tumors at the world health organization (WHO) publishes a series of guides based on pathological features and associated genetic alterations. These WHO Classification of Tumors are currently in their 5th edition for most tumor groups. The classification for CNS tumors is currently available as a revised 4th edition of the 2016 report, however, a summary of major changes in the 5th edition is also available as a separate publication (Louis et al., 2016, 2021). A summary of selected tumor types discussed in this thesis, namely with key diagnostic genes being *IDH1*, *IDH2*, or alterations in histone modification on H3, is shown in **table 1**. Astrocytomas (*IDH*-mutant) do now include glioblastoma *IDH*-mutant tumors and range in their WHO grading from grade 2 to 4. While grade 1 and grade 2 are characterized as low-grade with slow growth and limited infiltration, grade 3 is classified as malignant and infiltrative, sometimes anaplastic, and grade 4 tumors are described as having aggressive and infiltrative growth, with often rapid recurrence and being prone to necrosis (Louis et al., 2007). Specific pediatric brain stem tumors, previously termed diffuse intrinsic pontine gliomas (DIPG), are now included in the group of diffuse midline gliomas. They are furthermore specified as H3K27-altered, as mutations affecting the histone modification status are recognized as classification criteria for these tumors. Furthermore, H3-wildtype and *IDH*-wildtype diffuse pediatric-type high-grade gliomas are now newly recognized as their own classification.

WHO tumor classification			Former nomenclature	WHO grade
Gliomas, glioneuronal tumors, and neuronal tumors	Adult-type diffuse gliomas	Astrocytoma, *IDH*-mutant	Glioblastoma, *IDH* mutant	2, 3, 4
		Glioblastoma, *IDH*-wildtype		4
	Pediatric-type diffuse high-grade gliomas	Diffuse midline glioma, H3 K27-altered	Diffuse intrinsic pontine glioma or Diffuse midline glioma, H3 K27 mutant	4
		Diffuse pediatric-type high-grade glioma, H3-wildtype, and *IDH*-wildtype		4

Table 1: *Selected tumor classifications relevant to this thesis, and their current nomenclature based on a summary of the 2021 WHO Classification of Tumors of the Central Nervous System (Louis et al., 2021).*

1.4.1 Pediatric glioma

In pediatric diffuse high-grade gliomas, variations in the genes encoding for histone H3 are of importance to classify and stage the tumor types. One common mutation resulting in the substitution of lysine to methionine (H3K27M) has been shown to be an important prognostic marker for worse survival compared to wildtype tumors (Khuong-Quang et al., 2012). The mutation leads to a global reduction of the methylated histone mark, H3K27me3, and alters gene expression profiles in combination with DNA hypomethylation (Bender et al., 2013). The same study, as well as others, additionally found that certain gene loci also show increased local methylation such as on the loci for cyclin dependent kinase inhibitor 2A (CDKN2A), leading to a complex altered program of transcriptional activation and repression at different loci, which may ultimately drive tumorigenesis (Chan et al., 2013). Part of this interplay in H3K27 mutant tumors is the increased recruitment of the PRC2 to H3K27me3 and enzymatic inhibition of its catalytic subunit, and therefore repressing the function of PRC2. Expression of

EZH2, the histone-methyltransferase of PRC2, is increased in H3K27 mutant gliomas, and was therefore considered a promising target for this highly aggressive pediatric cancer. Studies have tested small-molecule inhibitors for EZH2 with contradicting results *in vivo* and *in vitro* (Mohammad et al., 2017; Wiese et al., 2016). This suggests that targeting the TME and associated cell types may play an important role in tumor progression and the search for viable targets in clinical research.

1.4.2 IDH-mutant glioma

IDH1 and 2, or isocitrate dehydrogenase type 1 and 2, are single-gene enzymes and located in the cytoplasm and mitochondria, respectively. They have similar functions, providing a cell with NADPH catalyzed from the co factor $NADP^+$. The reduced form, NADPH, is a crucial electron donor in numerous anabolic reactions and implicated in the genesis and progression of different cancer types, whereas it can likely impact metabolism and oxidative stress (Ju et al., 2020). Mutations in *IDH2* are much less frequently found in gliomas and seem to be mutually exclusive with mutations in *IDH1* (Cohen et al., 2013). A commonly found missense mutation affecting amino acid residue 132 (R132H) on IDH1, replacing the arginine residue with histidine, has been shown to lead to neo-enzymatic activities of mutant IDH1. Dramatically elevated levels of 2-hydroxyglutarate (2HG) in glioma as a result of this gain-of-function mutation have been linked to epigenetic changes. 2HG can act as an inhibitor for histone and DNA demethylases, and therefore cause an increase in their methylation levels. This in turn is hypothesized to inhibit the binding of certain transcription factors, leading to altered gene expression and potentially cancer progression (Raineri & Mellor, 2018). It is therefore somewhat surprising that the mutation status of IDH1 in grade 2 to 4 gliomas is an important, independent and favorable prognostic biomarker (Sanson et al., 2009). In lower grade gliomas, a significant correlation between *IDH1*-mutation and hypermethylation of the promotor for the O^6-methylguanine-DNA methyltransferase (MGMT*)* has been found, leading to favorable overall survival due to higher sensitivity to certain therapeutic agents (Pandith et al., 2021). This suggests a more extensive and complicated epigenetic landscape in these tumors that needs to be further explored.

1.4.3 Microglia and macrophages in the TME

A different angle of research studies indicates that immune cells in the TME play a role in oncogenesis, progression, diagnosis, and treatment. For example, in IDH-wildtype glioblastoma, which present with a less favorable disease progression, significantly more glioblastoma-associated microglia and macrophages are found compared to the mutant tumor (Poon et al., 2019). Tumor-initiating cells can recruit microglia to the tumor site, where microglia in turn will upregulate the release of IL-6 benefiting the tumor growth by supporting glioma stem cells (a Dzaye et al., 2016). Recruitment of microglia, but also infiltrating macrophages, is mediated by the release of chemo-attractants such as C–C motif ligand 2 (CCL2), C-X-C motif chemokine 12 (CXCL12), glial cell–derived neurotrophic factor (GDNF), or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hambardzumyan et al., 2016). Once recruited to the tumor site, tumor-associated (TAM) or more specifically

glioma-associated microglia and macrophages (GAM) have been reported to promote glioma growth and invasion by releasing growth factors. Glioma invasion and migration are aided by intricate signaling initiated by the secretion of microglial TGF-β, which involves a cascade of matrix metalloproteinases to modulate the extracellular matrix, and can be enhanced further by microglia-activating factors such as GM-CSF (Bettinger et al., 2002; Wick et al., 2001). TGF-β, which is produced by glioma cells as well as TAMs, can furthermore inhibit T lymphocyte-mediated tumor cytotoxicity by suppressing gene expression and ultimately hindering tumor clearance (Thomas & Massagué, 2005). Hypoxia further increases the recruitment and polarization of macrophages towards glioma, and immunosuppressive hypoxic effects such as upregulation of TAM-expressed macrophage colony-stimulating factor receptor (M-CSFR) or glioma-expressed TGF-β are regulated by transcription factors STAT3 and HIF (Guo et al., 2016; Wei et al., 2011). In a preclinical study, STAT3 inhibition showed limited activity which was partly due to molecular heterogeneity of glioblastoma, and additional challenges such as low penetration rates of the blood-brain barrier (M. S. Y. Tan et al., 2019). The above-described events can be summarized as escaping the immune surveillance and creating an immune-suppressive TME. Clinical trials for immune therapies such as checkpoint inhibitors show promising results in other cancers, but have unfortunately not yet been successful in glioblastoma trials (Reardon et al., 2020). Other therapeutic approaches such as utilizing chimeric antigen receptor (CAR) T cell therapies in pediatric high-grade glioma (pHGG) also remain to show efficiency, and limitations include the immune-suppressive TAM microenvironment as well as penetration rates and persistence of the CAR T cells (Haydar et al., 2021).

Immune cells in the glioma microenvironment have been targeted by several studies aiming to find successful treatment approaches, with most approaches following strategies to inhibit the recruitment of TAMs, depleting TAMs or reprogramming TAMs (Zhang et al., 2020). For example, inhibition of CXCR4, the receptor for CXCL12, led to decreased immune cell infiltration post-radiation in mice, impacting vasculogenesis of the tumor (Kioi et al., 2010). A different approach aims to target the immune evasion of glioma cells via the upregulation of the anti-phagocytic cell surface protein CD47. In the TME, CD47 is recognized by the receptor signal regulatory protein SIRPα expressed on microglia and macrophages, leading to a “don’t eat me” signaling cascade. In xenograft models of pediatric brain tumors, such as pediatric glioblastoma, the use of an anti-CD47 antibody disrupted anti-phagocytic signaling in macrophages, and CD47 blockade in glioblastoma models showed enhanced tumor phagocytosis by microglia (Gholamin et al., 2017; Hutter et al., 2019). Inhibiting CSF-1R seems to show beneficial effects on glioma progression and proliferation by modulating and targeting the immuno-suppressive activation of the TAMs, strengthening the hypotheses of the TME playing a crucial role in finding novel treatment strategies (Yan et al., 2017). CSF-1R inhibition in preclinical glioblastoma mouse studies resulted in increased tumor sensitivity to irradiation and depletion of $CD11b^+$ cells (Stafford et al., 2016). However, other studies found that there is a potential to acquire long-term resistance as tumors recur eventually, stressing the

need for further investing contributing pathways and potential combination treatments to combat resistances (Quail et al., 2016).

A challenge presented to researchers is how to properly distinguish tumor-associated microglia from infiltrating macrophages, especially considering that their transcriptomic signatures will change once recruited to the tumor site (Klemm et al., 2020). Apart from full sets of genetic signature genes differentially expressed in microglia versus macrophages, reports suggest CD49d as a distinguishing marker in human and mouse samples that is stable at both steady state and during activation, see also **figure 3** (Bowman et al., 2016; Haage et al., 2019). TMEM119 and P2RY12 were also found to be robust markers to distinguish microglia from macrophages, whereas other markers were upregulated in tumor-associated microglia and not just found in macrophages (Woolf et al., 2021).

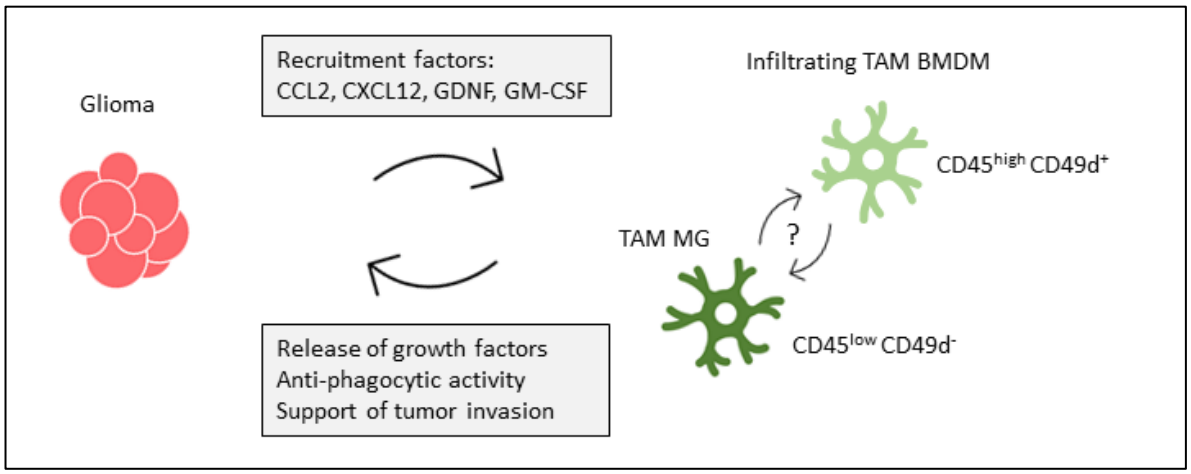

Figure 3: *Interaction between glioma cells, resident- and infiltrating immune cells in the TME. To recruit microglia or infiltrating macrophages, glioma cells will secrete factors such as CCL2, CXCL12, GDNF or GM-CSF. TAMs in turn release cytokines and growth factors supporting the tumor growth and invasion and decrease their phagocytic activity. The interplay of tumor-associated microglia with macrophages is yet to be fully understood. Markers to distinguish the two cell types to investigate their distinct functions in models include CD45 and CD49d.*

Relatively little is known about the underlying differences of microglia versus infiltrating macrophages within the TME, or the transcriptomic differences of TAMs between different types of gliomas. In an effort to compare TAMs between DIPG and adult glioblastoma samples, RNA-seq on human samples revealed that DIPG-associated TAMs show a significantly less inflammatory phenotype, emphasizing the need for targeted strategies depending on tumor and TME heterogeneity (Lin et al., 2018). In glioma mouse models, TAM microglia showed distinct transcriptomic activation compared to TAM macrophages (Bowman et al., 2016). There, Bowman et al. found enrichment of immune effector genes as well as some genes associated with immune suppression in TAM macrophages, while TAM microglia showed enrichment for chemokines involved in the pro-inflammatory response. TAM signatures of

microglia as well as infiltrating macrophages could even be differentiated between *IDH* wildtype and *IDH* mutant glioblastoma, and signature gene expression profiles influenced by the underlying type of tumor could even be used as a predictive marker for disease outcome (Klemm et al., 2020). In a recent study, a higher ratio of microglia in TAMs in glioblastoma was found to be beneficial for survival, affirming the need to analyze these cell populations individually (Woolf et al., 2021).

Targeting TAMs in the context of glioma presents to be promising for therapeutic interventions, however, the distinct roles of microglia and recruited macrophages need to be better understood in order to achieve higher target specificity and a more personalized approach based on the underlying tumor type and composition of TME. Since macrophages acquire a distinct transcriptomics profile compared to microglia in the context of glioma, and the number of infiltrating macrophages versus resident microglia can be associated with disease progression, it is furthermore of interest to elucidate the possible interactions between these immune cells in the TME.

To briefly summarize: understanding the transcriptomic signatures and associated functional activation states or subtypes of microglia could help answer a wide range of research questions. Insights into how these specialized immune cells adapt throughout development and aging, how they become dysfunctional in neurodegeneration and how they impact the composition and cell communication within the TME could enable us to harvest that potential by modulating or reprogramming them. We should furthermore aim to understand the regulation and pathways involved explaining differences depending on the spatial-, time-, stimuli- or disease-dependent context to ultimately provide a solid basis for clinical research leading to novel treatment strategies.

2 RESEARCH AIMS

Overall, this thesis aims to increase knowledge and support the gain of insights into microglial activation and regulation. The projects presented as published papers or manuscripts are focused on individual aspects of microglia phenotypes in the context of inflammation, the TME including tumor-associated microglia and macrophages, and the aging brain. The research aims are specified as follows:

Paper I – *Atg7* deficiency in microglia drives an altered transcriptomic profile associated with an impaired neuroinflammatory response

Distinct profiles of gene expression have been reported in microglia activated towards certain phenotypes. Using stimuli such as LPS, we provoked a pro-inflammatory response and characterized their transcriptome and altered functions. Remarkably, *Atg7*-deficient microglia showed diminished activation on a transcriptional, as well as on a functional level. To elucidate the mechanisms behind such altered transcriptomic signatures, we aim to identify a relevant modulator of microglial activation selectively towards an inflammatory phenotype.

Paper II/*Manuscript* – Aged microglia: development of immunosenescence without replicative senescence

Microglia present altered transcriptomic profiles and functional phenotypes in the healthy aging brain as well as in the context of neurodegenerative disorders. Cellular senescence is characterized by mitochondrial dysfunction, senescence-associated secretion of pro-inflammatory cytokines or metabolic imbalances leading to protein aggregates, which likely contributes to the overall process of the aging brain. In this study, we aim to model cellular aging with a long-term cultivation of microglia and perform functional cell assays, as well as transcriptomic analyses.

Paper III – Inhibition of microglial EZH2 leads to anti-tumoral effects in pediatric diffuse midline gliomas

Pediatric diffuse midline gliomas are known to be aggressive tumors of the brain stem with a significant lack of treatment options. In this study, we aim to understand the effect of EZH2 inhibition on the tumor-associated microglia, leading to an anti-tumoral phenotype. We furthermore wanted to characterize an epigenetic mechanism behind the tumor-induced activation of microglia, elucidating their impact on the TME and their potential use as future therapeutic targets in glioma research.

Paper IV/*Manuscript* – Understanding if the presence of peripheral macrophages in the context of glioblastoma influences the tumor-associated microglial phenotype

Tumor-associated microglia and macrophages are often described and researched as a group of cells with significant impact on the TME. In this project however, we aim to specifically elucidate the individual contributions of microglia and infiltrating macrophages in the glioblastoma context, hypothesizing that an initial anti-tumoral microglia phenotype can be reverted by the presence of recruited macrophages.

3 MATERIALS AND METHODS

Cell Lines: The studies presented in this thesis make use of a variety of established cell lines from mouse, rat, or human origin. *In vitro* approaches provide a good basis for investigating molecular mechanisms in an initial and simplified form, without the need of laboratory animals. However, cell culture also poses challenges such as lack of other cell types usually present in the microenvironment, or potential differences in gene expression between primary cells and established cell lines. With awareness of the *in vitro* downsides, we have opted to use functional assays, reproducing findings in multiple cell lines, using primary patient-derived cell lines, or utilizing human tissues through external collaborations to validate our findings. The following cell models were used:

BV2 microglia: they are of a murine, C57BL/6J background and immortalized using a -raf/v-myc oncogene carrying retrovirus (Blasi et al., 1990). These cells have been found to be a suitable model compared to primary cells and show similar, even though less pronounced, reaction to inflammatory stimuli (Henn, 2009).
To study the impact of *Atg7*, we received shControl and sh*Atg7* BV2 as a generous gift from Dr. Seong-Woon Yu, Daegu Gyeongbuk Institute of Science and Technology, Republic of Korea. The cells were generated using lentiviruses (J.-W. Lee et al., 2019).

HMC3 microglia: HMC3 are human fetal brain-derived primary microglia cells, immortalized using simian virus 40 (Janabi et al., 1995). These cells were obtained from ATCC (CRL-3304) and used to validate findings from rodent cell lines in human-derived cells.

RAW 264.7 macrophages: this murine macrophage cell line was generated using Abelson murine leukemia virus-transformed cells from a BALB/c mouse strain (Raschke et al., 1978). The cells were obtained from ATCC (TIB-71).

MN9D neurons: cells were a kind gift from Dr. Alfred Heller, University of Chicago, USA. These cells represent dopaminergic neurons and were generated by somatic cell fusion using a 14-day-old embryonic C57BL/6J mouse (Choi et al., 1991). Neuronal cells were used for a functional assay investigating induced neurotoxicity.

C6 glioma: Cells were obtained from ATCC (CCL-107) and are a rat glioma cell line that was first induced using N-nitrosomethylurea (Benda et al., 1968). This cell line was chosen for functional assays as it can overall simulate glioblastoma multiforme as a good model (Giakoumettis et al., 2018).

SF188 glioblastoma: This human pHGG cell line was used as a control line to SF8628. The line is primary patient-derived and was a kind gift from Stefan M. Pfister, German Cancer Research Center.

SF8628 glioma: This DIPG cell line carries a substitution mutation on histone H3.3 (lysine 27 to methionine, K27M). It is a primary patient-derived line and was obtained from Merck (Cat. #SCC127)

U87 glioblastoma: Human cell line of male origin, likely derived from malignant glioblastoma patient tissues (Pontén & Macintyre, 2009). Allen et al. showed a different DNA profile of currently distributed cells from ATCC compared to the original cells, likely to be glioblastoma of unknown origin (Allen et al., 2016). Our cells were obtained from a stock from B. Westermark originator's laboratory, Uppsala University, however, this information demands caution when comparing literature using this cell line. Apart from this wildtype U87MG cell line, we also studied the effect of the commonly reported IDH1 mutation. Therefore, we used a human glioma cell line with knock-in IDH1 mutation (IDH1R132H), generated using CRISPR/Cas9 gene editing technology (ATCC, HTB-14IG).

U251 glioblastoma: This cell line was originally derived from a male patient with malignant astrocytoma, presented in the same study as the original U87MG cells (Pontén & Macintyre, 2009). The cells used in this thesis were obtained from the same laboratory (B. Westermark) as the U87 cell line.

U1242 glioblastoma: This cell line originated from a malignant human glioma of (Lubitz et al., 1980) and was chosen for the analysis of a highly aggressive and invasive tumor compared to U87MG, as shown by xenograft invasion experiments (Y. Zhao, Xiao, et al., 2010). We obtained our cells from the same laboratory (B. Westermark) as the U87 and U251 cell lines.

Immunoblotting: Protein analysis using the Western blot method allowed to study the presence, molecular size, and quantity of specific proteins present in the cell samples. In brief, cell lysates were collected, sonicated, and boiled, before separated by gel electrophoresis in SDS–polyacrylamide gels with appropriate percentages. Subcellular fractionation, instead of whole cell lysates, was used for some experiments to show the distribution of proteins in the cytoplasm compared to the nucleus. After blocking and incubating with the indicated antibodies, membranes were imaged using the LI-COR Biosciences Odyssey CLx infrared imaging system and images were quantified using ImageJ software.

Immunohistochemistry: Immunohistochemistry (IHC) was performed to visualize the location and amount of specifically labeled proteins in cells, using confocal microscopy. Cells seeded on glass coverslips were fixed with paraformaldehyde solution, incubated with blocking solution and afterwards with the indicated antibodies and DNA stain. Imaging was performed with the Zeiss LSM800 confocal laser scanning microscope, and analysis using the ZEN software.

Electron microscopy: Cells were fixed in 2.5 % glutaraldehyde in phosphate buffer, rinsed, and post-fixed in phosphate buffered 2% osmium tetroxide. The cells were then stepwise dehydrated in ethanol followed by acetone and embedded in LX-112. Ultrathin sections were cut and contrasted with uranyl acetate followed by lead citrate. The sections were examined in a Hitachi HT7700 transmission electron microscope.

Real-time quantitative Polymerase Chain reaction (RT qPCR): RT qPCR allows for the detection and relative quantification of gene transcripts. RNA was isolated from cell lysates

using the Qiagen RNeasy Plus kit, converted to cDNA and amplified with specific primers and the SYBR green method. Applied Biosystems' StepOne plus instrument was used to analyze the samples, and the ΔΔCT method used to examine relative changes in gene expression.

RNA sequencing and bioinformatic analysis: RNA sequencing was performed by the core facility Bioinformatics and Expression Analysis (BEA) at Karolinska Institutet. Details can be found in the materials and methods section of the published paper I (Friess et al., 2021). In brief, Illumina HiSeq 2000 sequencing system was used, followed by differential gene expression analysis with R/Bioconductor. For further analysis and visualization, tools such as Gene Ontology (GO), Metascape, Gene Set Enrichment Analysis (GSEA), Transcriptional regulatory relationships unraveled by sentence-based text mining (TRRUST) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used.

Chromatin Immunoprecipitation (ChIP): ChIP was performed to analyze the interaction between specific pulled down proteins with DNA sequences of interest. Using the Diagenode iDeal ChIP-qPCR kit, chromatin immunoprecipitation was done after cells were cross-linked and fixed, incubating the samples with the respective antibody of interest and finally purifying DNA to perform RT qPCR.

Lysosomal assays: Senescence-associated β-Galactosidase assay using a detection kit (Abcam) was performed according to the manufacturer's instructions. Acridine Orange at a final concentration 10 µg/ml was incubated for 20 minutes at 37° C. Fluorescent signal was detected by inverted fluorescent microscope Axio Observer 3 (Zeiss) using Zen blue software.

Mitochondrial assays: Total mitochondrial mass was evaluated using 250 nM MitoTracker™ (Invitrogen) fluorescent probe in DMEM for 30 min at 37° C. Signal was detected by inverted fluorescent microscope and fluorescent mean and area were analyzed. Production of mitochondrial superoxide radical was detected using MitoSox™ (Invitrogen) fluorescent probe. 5 µM dye was prepared in HBSS and cells were cultivated for 10 minutes at 37° C. Fluorescent signal was detected at λex 535 nm/λem 595 nm by Spark reader (TECAN). Hydrogen peroxide at 100 µM for 10 min was used as positive control. TMRE (Invitrogen) fluorescent probe at 100 nm concentration in DMEM for 30 min was used to detect changes in mitochondrial membrane potential. Fluorescent signal was detected by Spark reader (TECAN) at λex 535 nm/λem 595 nm.

Cell death and Neurotoxicity assay: MN9D dopaminergic neuronal cells were stained with CellTracker Green CMFDA (Invitrogen, C7025) and washed, before microglia were plated as a direct co-culture. The cells were incubated with LPS for 24h, followed by the DNA stain Höchst and then counted regarding nuclear condensation and fragmented nuclei for quantification of cell death. Additionally, intracellular ROS production in microglia within this direct co-culture set up was analyzed using a general oxidative stress indicator kit. Samples were run with the BD LSR2 Cell analyzer and data analyzed using FlowJo (BD Biosciences, v10) from BD Biosciences.

Migration assay: Macrophages or microglia – naïve or pre-conditioned with other cell types – were seeded in medium with 10% serum. Using inserts with 8 µm pores, glioma cell lines in medium with 5% serum were placed above the macrophages or microglia, respectively. After the indicated time points, cells on the inside of the inserts were removed with a cotton swab. Remaining cells were fixed and stained using DAPI, the insert membranes imaged under the microscope and counted for absolute number of migrated cells.

Phagocytosis assays: Uptake of tumor cells, labelled with 1 µM of carboxyfluorescein succinimidyl (CFSE), by microglia was measured after 4h of co-culture in a seeding ration of 1:2. Cells were then removed, stained with CD11b-APC, and analyzed with the BD LSR2 cell analyzer and later with FlowJo. Uptake of bacteria was tested using microglia infected with S. pneumoniae at a multiplicity of infection (MOI) 10. After co-incubation, 200 µg/ml of gentamicin was added to kill extracellular bacteria. Subsequently, cells were lysed with 1% filtered saponin after indicated time points. Lysates were diluted 1:10 and 100 µl were plated overnight (37° C, 5% CO2). Colony forming units were then counted. Uptake of amyloid-β was tested using the Beta-Amyloid (1-42) Aggregation Kit (rPeptide) according to the manufacturer's instructions. Fluorescent signal was detected using Spark reader at λex 430 nm/λem 485 nm and immunoblot analysis confirmed aggregated protein products.

Cell cycle analysis: Fixed cells were stained in PBS containing 0.1% (v/v) TritonX-100, 10 ug/ml of propium iodide and 100 ug/ml of RNase for 15 minutes at 37◦C. Samples were then analyzed with the BD LSR2 cell analyzer and data evaluated using FlowJo (BD Biosciences, v10).

Cell proliferation assay: To measure the proliferation of tumor cells, we used the Click-IT EDU Alexa fluor 488 flow cytometry kit (Thermo Fisher, C10420) according to manufacturer's guidelines. Briefly, cells were stained with 10 µM of EdU diluted in culture media for 2 hours. Cells were washed, fixed in Click-iT fixative for 15 minutes, and then incubated with Click-iT reaction cocktail for 30 minutes. Samples were immediately analyzed with the BD LSR2 cell analyzer and later with FlowJo.

Statistical analysis: Statistical analysis was performed using the two tailed unpaired student's t-test when comparing two separate cell types at a given time point or condition or using two-way ANOVA when comparing multiple time points or conditions. All values are a mean of, as indicated, at least 3 independent experiments ± SEM. Results are considered significant for *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, or n.s., not significant, for the indicated comparison.

Ethical considerations: These *in vitro* set ups provide comparatively straightforward ways to investigate molecular mechanisms without the need of sacrificing laboratory animals following ethical principles of 3R. Future validation of key findings however might call for the involvement of animal studies. Human tissue samples from the UK Brain Bank were obtained after thorough review and approval of all ethical licenses.

4 RESULTS AND DISCUSSION

4.1 Paper I – *Atg7* deficiency in microglia drives an altered transcriptomic profile associated with an impaired neuroinflammatory response

It is well established that microglia can display a wide variety of activation states upon reaction to different stimuli. Microglia-dependent inflammation-mediated neurotoxicity plays a role in neurodegenerative disorders and it is important to identify modulators of such activation states to better understand their underlying mechanisms (Sarlus & Heneka, 2017). ATG7, as well as other components of the autophagy machinery, has multiple autophagy-independent roles (Galluzzi & Green, 2019), some of which include direct or indirect impact on transcription in different cell types.

Reduced *Atg7* levels do not prevent autophagy induction in microglia

To investigate how *Atg7* may impact the phenotypic activation of microglia, we used BV2 cells with shRNA targeting *Atg7*. Firstly, we show that this knockdown is stable and shows a reduction of approximately 80% in mRNA as well as protein levels, compared to the control cell line. This is furthermore confirmed by confocal immunofluorescence imaging. This knockdown did not *per se* incapacitate the cells to undergo autophagy. A reduced, but not completely abolished induction was shown by immunoblotting of LC3I versus LC3II. As reported for macrophages, *Atg7* is not essential for autophagy (Vujić et al., 2021).

***Atg7*-deficient microglia show an impaired response to LPS, but not to IL-4**

To test the acquisition of different phenotypes with a knockdown of *Atg7*, microglia cells were treated with either the bacteria-derived endotoxin LPS, or interleukin 4 (IL-4). LPS is a commonly used stimulus to induce a pro-inflammatory activation state, while IL-4 is considered a non-inflammatory stimulus. LPS-induced activation could be measured by a time-dependent upregulation of *Nos2*. However, this induction was significantly less pronounced in *Atg7*-knockdown microglia, suggesting an impaired activation. Interestingly, IL-4 stimulation led to an equal upregulation of *Arg1* between the two cell lines. *Atg7* might therefore be a selective modulator of some, but not other microglia activation states.

***Atg7* deficiency drives an altered transcriptome with a reduced immune response**

Based on the results described above, we decided to investigate the transcriptome of cell stimulated with LPS. In untreated cells, we found over a thousand differentially expressed genes (DEG) between BV2 sh*Atg7* and control cells. By investigating the GO term annotations, we could visualize that a great deal of genes downregulated in *Atg7*-deficient microglia are related to terms such as immune response, cytokine production or antigen processing. *Atg7*-deficient cells, that had been stimulated with LPS, showed very similar downregulated DEGs compared to stimulated control cells, suggesting that the lack of *Atg7* impairs the cells' immune

response. These results are in line with other publications showing the impact of *Atg7* on transcriptional regulation and pro-inflammatory cytokines (Wang et al., 2018).

Reduced NF-κB-dependent signaling and nuclear translocation in *Atg7*-deficient cells

NF-κB signaling is known to contribute to the regulation of both innate and adaptive immune responses, and its deregulation is associated with the development and progression of inflammatory diseases (T. Liu et al., 2017). The pathway consists of multiple transcription factor subunits, and we found that *Nfkb1* was a significant hit in the TRRUST analysis from the RNA-seq results. We further showed that the mRNA levels of several other subunits are similar in sh*Atg7* and control cells, however, we found that there is less nuclear translocation of p65 in knockdown cells. This result is supported by other reports showing inhibition of LPS-induced inflammation via blocking of NF-κB signaling (Hou et al., 2019; Subedi et al., 2019). Through ChIP analysis we also showed less enrichment of NF-κB to the *Nos2* promotor in LPS-treated sh*Atg7* microglia, providing a possible mechanism explaining our observed differences in the pro-inflammatory response.

***Atg7*-deficient microglia show reduced neurotoxicity after treatment with a pro-inflammatory stimulus**

In a functional assay cultured with dopaminergic neurons and stimulated with LPS, microglia show a certain degree of neurotoxicity characterized by the production of reactive oxygen species (ROS). Here, *Atg7*-deficient cells showed no significant neurotoxicity towards the cultured neurons. Flow cytometry analysis of intracellular ROS showed a robust drop in control cells after LPS stimulation, consistent with the cells releasing ROS and therefore contributing to oxidative stress in the microenvironment. This drop in intracellular ROS was not observed in sh*Atg7* cells. Taken together, the results presented in this paper show that a reduction in *Atg7* gene expression leads to an altered transcriptome and dampened pro-inflammatory response, likely mediated through the NF-κB signaling pathway. This suggests that *Atg7* is a selective modulator of the pro-, but not of the anti-inflammatory response of microglia.

4.2 Paper II/Manuscript – Aged microglia: development of immunosenescence without replicative senescence

Microglia acquire a distinct phenotype and transcriptome signature during aging and neurodegeneration, that can at times be associated with cellular senescence (C. E. Cho et al., 2019; Olah et al., 2018). With conflicting results on microglia aging *in vitro* versus *in vivo*, such as differences in telomerase activity, we identified a need for a model of long-term cultivated microglia cells to investigate transcriptomic changes and possible cellular senescence to gain a better understanding into physiological aging in microglia.

Long-term cultivated microglia are not associated with age-related cellular senescence

Cellular senescence can present as many factors, such as cell cycle perturbations or abnormal organelle morphology. In our model of culturing microglia for at least 100 days, we observed no difference in cell cycle phase distributions as compared to early passages, further referred to as young cells, and genes encoding for cell cycle repressors such as *Cdkn2a* and *Cdkn1a* were even downregulated in aged cells. Likewise, we found no observable difference in organelle morphologies in electron microscopy pictures, mitochondrial membrane potential, or staining for β galactosidase, a biomarker used for cellular senescence. We therefore conclude that these *in vitro* aged microglia cells do not enter a senescent state.

Long-term cultivation of microglia induces a distinct transcriptomic profile associated with reduced expression of immune-related genes

High-throughput bulk RNA-seq revealed a distinct transcriptomic signature in aged versus young cells, including over 1000 downregulated and over 600 upregulated genes in aged cells. GO term analysis of genes downregulated in aged microglia cells presented clusters associated with response to external stimuli, cytokine production, mediated immunity, and signaling pathways such as NF-κB. GO terms clustering upregulated genes on the other hand included telomere maintenance, which were unexpected regarding an aged phenotype. We could indeed confirm an increased length of telomeres in the aged cells. TRRUST analysis on the subset of downregulated genes in aged cells resulted in hits on transcription factors SPI1/PU.1, playing a crucial role in the development and homeostasis of microglia, as well as RELA/p65 and NFKB1, implicated in the regulation of immune response genes (Holtman et al., 2017).

Long-term cultivated microglia share transcriptomic similarities with primary microglia derived from aged mice

To evaluate the relevance of this proposed model, transcriptomic signatures were compared with studies on primary cells extracted from aging mice. We found an overlap of 140 genes, of which several were associated with a database of murine age-related genes, the AgingAtlas (Aging Atlas Consortium et al., 2021). We suggest that primary cells derived from aged animals are likely not completely unchallenged or primed in life, and therefore also compared the datasets to aged, LPS-challenged microglia to find an overlap of over 200 genes.

Long-term cultivated microglia show reduced neurotoxicity, but efficient uptake of amyloid-β

To investigate potential functional disturbances correlating with the significant downregulation of immune-related genes in the aged microglia, we carried out functional assays on neurotoxicity and the uptake of amyloid-β. The disrupted clearance of amyloid-β plaques and its associated neurotoxicity in the aging brain are hallmarks of neurodegeneration, specifically AD. In our model after co-culture with dopaminergic neurons, aged microglia stimulated with LPS resulted in decreased levels of neurotoxicity as compared to young LPS-treated microglia. We could not observe a difference in the uptake of amyloid-β, suggesting there may be cues from the microenvironment of the aging brain missing to induce such dysfunction or that the toxic accumulation of protein aggregated precedes an immunosenescent or primed-like microglia phenotype *in vivo*.

In summary, long-term cultivated microglia may present a novel model of aging cells that do not enter a senescent state yet show similarities to primary cells from aged animals. Our data indicate that such aged microglia have a reduced inflammatory response to stimuli, but the correlations between aging, pathological changes in neurodegeneration and acquisition of distinct microglia activation states remains to be fully elucidated.

4.3 Paper III – Inhibition of microglial EZH2 leads to anti-tumoral effects in pediatric diffuse midline gliomas

DIPG are highly aggressive pediatric brain malignancies, characterized by H3K27M mutational status which leads to a global loss of H3K27me3 expression (Khuong-Quang et al., 2012). In preclinical models, DIPG can be targeted by inhibiting the histone methyltransferase EZH2, even though *in vitro* experiments on DIPG cells yielded little to no effect on these cells (Wiese et al., 2016). The TME can be highly variable between different types of tumors and contribute to disease onset as well as progression and targeting tumor-associated microglia could provide further insight into the mechanisms behind the use of EZH2 inhibitors.

Pro-tumoral microglia activation is linked to a short-term decrease in H3K27me3

First, we examined the activation of microglia as mediated by the different glioma cell lines used in this study. DIPG and pHGG cell lines with and without the H3K27M mutation, respectively, could both induce a pro-tumoral microglia phenotype as seen by increased tumor migration after co-culture with BV2 microglia as well as a decrease of *Nos2* gene expression in the microglia cells. Additionally, we found a transient downregulation of H3K27me3 expression in microglia cells after 4h of co-culture with both cell lines. The expression levels returned to baseline expression after 24h. Such mechanisms of epigenetic control mediated by the tumor could be required to reprogram microglia towards a pro-tumoral activation state (Cheray & Joseph, 2018).

Repression of *EZH2* in pHGG has no impact on proliferation or cell death

Next, we tried to understand if EZH2 inhibitors were mediating their reported *in vivo* effects through targeting the tumor cell or the TME. To achieve that, we first targeted the *EZH2* gene expression by siRNA specifically in the tumor cells. We found that neither cell proliferation nor cell death, measured by proliferating cell nuclear antigen (PCNA) and markers of apoptotic cell death, were affected in DIPG or pHGG cells when *EZH2* was repressed. This suggests that genetic inhibition of *EZH2* in glioma cells has little to no effect on their basic cellular functions.

Repression of *Ezh2* in BV2 microglia leads to an anti-tumoral activation state

We then repressed *Ezh2* specifically in BV2 microglia cells. Knockdown of *Ezh2* in microglia led to a significant decrease of global H3K27me3 as expected. Gene expression of *Nos2* was increased by more than 3-fold, suggesting a reprogramming of microglia to a more anti-tumoral state when *Ezh2* was repressed in these cells. ChIP analysis showed a decrease of H3K27me3, and an increase of H3K27ac on the *Nos2* promotor, with both modifications being associated with a higher transcriptional activity. Investigation of the LPS-induced anti-tumoral cytokine *Il1b* showed increased gene expression after *Ezh2* knockdown in microglia, further supporting that these cells acquire a distinct phenotype by the repression of *Ezh2*. To test this hypothesis in a functional manner, we conducted invasion assays, measured phagocytotic activity and glioma cell death. We found that in both cell lines, as well as in primary patient-derived DIPG cells, glioma migration is significantly reduced after co-culture with si*Ezh2* microglia.

Phagocytosis, as well as glioma cell death, were on the other hand significantly increased after the genetic knockdown, further supporting that microglia become anti-tumoral when *Ezh2* is repressed. Taken together, our results support the hypothesis that EZH2 inhibition *in vivo* leads to reduction in tumor growth not by acting upon the glioma cells *per se*, but on the epigenetic landscape of microglia in the TME. These epigenetic changes result in a pro-tumoral reprogramming of microglia independent of the H3K27M status of the glioma cells. Overall, this argues for the role of microglia, and potentially other cell types within the TME, to have significant impact on tumor initiation and progression.

4.4 Paper IV/Manuscript – Understanding if the presence of peripheral macrophages in the context of glioblastoma influences the tumor-associated microglial phenotype

Glioblastoma carrying an IDH1 mutation are associated with less infiltration and a generally better prognosis compared to IDH1 wildtype tumors (Price et al., 2017). While microglia in the TME have been shown to be rather pro-inflammatory, infiltrating macrophages show a more upregulated anti-inflammatory transcriptome, which could explain the correlation between tumor-associated and macrophage amount with overall survival independently of IDH-status (Poon et al., 2019). A higher ratio of microglia was furthermore found to be beneficial for survival in glioblastoma, suggesting distinct roles of microglia and macrophages in the TME (Woolf et al., 2021). We hypothesized that the infiltration of macrophages impacts microglia, which initially exert pro-inflammatory or anti-tumoral functions, to create a tumor-supportive TME.

The presence of TAM macrophages as a negative prognostic marker in glioblastoma

The presence of an IDH1 mutation is associated with a better overall survival and carrying this mutation can be a prognostic marker for glioblastoma. Furthermore, patient samples carrying the IDH1 mutation showed a low gene expression of the macrophage markers *ITGA4* and *CD163*, while having a higher gene expression for microglia marker *P2RY12*. Overall survival in glioblastoma was positively correlated with the expression of *P2RY12*, while expression of *CD163* and *ITGA4* on the other hand is in negative correlation with overall survival.

Glioblastoma carrying IDH1 mutation induces an anti-tumoral microglial activation state

We identified the IDH1 mutational status of the cell lines used by protein analysis via Western blotting. U87 MG IDH1R132H, as characterized, carried the IDH1 mutation, while U87 MG, U251 and U1242 did not show any sign of the mutation. The latter present glioblastoma cells with higher rates of aggression and invasion. We utilized glioma cell migration as a functional assay to test the result of a potential activation of microglia. In IDH1 mutant glioblastoma cells, a significant reduction of migration as compared to wildtype glioblastoma cells was observed. U1242 cells showed slightly less migration after co-culture with microglia, however, this effect was not as strong as seen in IDH1 mutant cells.

The presence of microglia decreases proliferation rate of glioblastoma cells

We then analyzed proliferation rates of the glioblastoma cell lines after co-culture with microglia cells and found a decrease for both wildtype and IDH1 mutant cells. Preliminary data of one replicate suggests a similar trend for U251 and U1242 cell lines. We preliminarily conclude that microglia exert an anti-tumoral phenotype towards all glioblastoma cell lines, which seems to be more prominent in glioblastoma with IDH1 mutational status.

Microglia and macrophages increase anti-tumoral gene expression with glioblastoma cells

Microglia show an increased gene expression of *Tnf* and *Nos2* after co-culture with some, but not all glioblastoma cell lines. These results represent at the time three independent replicates and do not show statistical significance. However, macrophages present upregulated gene expression in a similar manner and co-culture with the highly aggressive cell line U1242 leads to significant increase of *Tnf* and *Nos2*. Further characterization of the anti-tumoral activation of both microglia and macrophages in the presence of glioblastoma will be performed.

Presence of macrophages induces a pro-tumoral microglial activation state in co-culture with glioblastoma cells

In an effort to model the presence of both, microglia and macrophages, in the TME of glioblastoma, we performed a triple co-culture exposing microglia to both macrophages and glioblastoma. We then transferred these primes microglia to perform a sequential migration assay with glioblastoma cells to analyze the impact of both macrophages and glioblastoma present on microglia-mediated tumor cell migration. Interestingly, we found that in the described set up, microglia seemed to activate towards a more pro-tumoral phenotype, represented by increased tumor cell migration and upregulation of *Tnf* and *Mmp14*. These results represent only one replicate at this point and are currently being repeated.

Taken together, preliminary results of our currently ongoing research work suggest that microglia present an initial anti-tumoral phenotype when cultured with only glioblastoma cells. In the additional presence of macrophages however, microglia seemingly transition towards a pro-tumoral phenotype, increasing tumor migration and upregulating a pro-tumoral gene expression profile.

5 CONCLUSIONS AND POINTS OF PERSPECTIVE

The here presented studies and manuscripts provide novel insights into microglial activation and function within different contexts.

- Knocking down *Atg7*, otherwise implicated in the process of autophagy, highly affects the transcriptomic signature of microglia and shifts them towards a reduced immune response profile. This regulation is likely mediated via the NF-κB signaling pathway.

- *Atg7*-deficient microglia cells are impaired when responding to a challenge with a pro-inflammatory stimulus and show a distinct, decreased function in assays assessing neurotoxicity. This diminished response was not observed after an anti-inflammatory stimulus, suggesting a selected modulation.

- In the context of aging, we aim to provide a novel model of long-term cultivated microglia that show transcriptomic similarities with primary microglia extracted from aging mice. We show that these *in vitro* aged cells have a distinct transcriptomic signature, including downregulation of immune-related genes and pathways, but do not enter cellular senescence.

- Inhibition of the histone methyltransferase EZH2 in the DIPG TME does not affect the tumor cells or their ability to recruit TAMs *per se*, but rather leads to an anti-tumoral activation state of the microglia. This highlights the importance of microglia, and the heterogeneity of the TME, as a potential therapeutic target in pHGG.

- Preliminary experiments show that microglia exert anti-tumoral activation towards glioblastoma as represented by reduced tumor cell migration. This is potentially switched towards a pro-tumoral activation when microglia are exposed to not just glioblastoma cells, but also macrophages, suggesting that infiltrating immune cells impact the TME and potentially reprogram microglia. However, more work remains to be done with this project.

In summary, we addressed research questions regarding the inflammatory response of microglia, a potential new model for cellular immunosenescence and the role of microglia in the TME of glioblastoma. Such examples and follow-up studies will be beneficial in elucidating the role of microglia, and macrophages, and how they adapt their functions depending on stimuli from their microenvironment by modulating the underlying transcriptomics signatures. To really bring these findings into the clinic however, a lot of work remains to be done.

Regarding future perspectives, one subject of research to come will surely be to understand the unique features and functions of microglia in their distinct activation types. Especially advances in fields such as single cell RNA sequencing hold a great deal of information describing new transcriptomically distinct population of microglia. However, more effort should be invested into elucidating how these manifold subsets, or even subtypes, functionally differ from each other to draw useful conclusions. Many studies are conducted on microglia in the context of different diseases or disorders, yet with such plastic cells it remains often difficult to investigate the exact time points or circumstances that are crucial for microglia activation states. Within the TME especially, it remains unclear how the different cell types involved really communicate with each other, including the mechanisms behind microglia and macrophage reprogramming.

Secondly, we need to gain further understanding of how microglia activation states are regulated. Knowledge of how to potentially manipulate a microglia cell *in vivo* into, for example, a more phagocytic active cell taking up and clearing higher loads of accumulated neurotoxic protein aggregates, could hold enormous potential for clinical advances. Signals that drive cellular change are often well established. However, signaling cascades with their complex interconnections between different pathways and interactions between regulatory transcription factors are often only described on a high level with the need for further clarifying research. Other regulatory components such as epigenetic changes or post-translational modifications, impacting how microglia cells function and communicate with other cell types, could also serve as promising new targets for therapeutics. The potential impact of external challenges on microglia activation states, such as how stress or infections throughout the span of a life could prime or affect microglia towards certain functions in old age or during neurodegeneration, is an additionally interesting aspect of future research. Another example is addressed by newly emerging research on the interaction of the gut microbiome with the CNS, contributing findings on how the gut-brain-axis can be linked to microglia development and function and providing exciting new hypotheses and insights into alternative modulators of microglia activation states (Abdel-Haq et al., 2019).

A third perspective addresses potential technical drawbacks and challenges that are present in the field of microglia research. As with any experiments, especially in the neurosciences, it is important to be aware of the limitations of cell culture lacking the structural and chemical environment of other cell types involved. With microglia in particular, we know that they exhibit different transcriptomic signatures between *ex vivo* and *in vitro*, as well as differences depending on the brain region, independently of other stimuli (Gosselin et al., 2017; Grabert et al., 2016). However, basic findings can still be established in simple set ups and, if applicable, validated in other models. With advances in scRNA-seq and similar methods, more and more clusters and transcriptionally distinct cell groups are analyzed, yet their implications are mostly unclear. Methodological hurdles present furthermore in standardizing sample preparation and extraction, sequencing protocols, and particularly the bioinformatics pipelines and analysis. And lastly, even though key proteins in research findings might be evolutionarily conserved, comparisons between species such as mice and humans are challenging. Regarding, as one

example, research in microglia aging, one needs to keep the vastly different life expectancies or natural processes of aging in mind. Finding novel models and characterizing them extensively to evaluate their use if therefore crucial to confidentially translate *in vitro* into *in vivo* findings.

Finally, points of perspectives include the chances and challenges of therapeutic advances in the field of microglia research. Understanding the underlying mechanisms of how microglia activation states are induced and regulated could hold the key in finding novel treatment approaches to ultimately bring basic research findings into the clinics for the patients' benefit. Many approaches to, for example, deplete microglia are either not selective enough, result in other deficiencies or lack efficiency and CSF1R inhibitors seem to propose a somewhat promising route to study microglia (Green et al., 2020). It will be helpful to stop addressing microglia as a single entity and consider their vast heterogeneity to be able to target specific populations when needed. The reprogramming of such cells will likely be more promising and successful than simply depleting them, however, this requires in-depth knowledge about regional, spatial, and functional heterogeneity of microglia across the healthy lifespan, as well as in the context of diseases.

The current microglia research field is as active as the cells themselves, and I am excited to see what groundbreaking findings are just waiting to be uncovered.

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