From the Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

# STUDIES ON MICROGLIA IN TUMOR BIOLOGY AND NEUROBIOLOGY

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*Cover*: microglia (white), glioma cells (yellow), nuclei (blue) in mouse GL261 tumor model
Artwork by Mattias Karlén based on a confocal image taken by Xianli Shen


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# Studies on Microglia in Tumor Biology and Neurobiology

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**Friday June 16th, 2017 at 09:00**

By


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To my parents

君子学以聚之，问以辨之，宽以居之，仁以行之。

——《周易·乾卦·文言》

# ABSTRACT

Microglia are innate immune cells that reside in the central nervous system (CNS). Their activities are critical for ensuring the correct microenvironment for brain development and for maintaining homeostasis in the brain after birth. However, during the lifetime, the neuronal and the glial cells in the brain suffer from multiple challenges that require microglia to react and to execute different functions. Under diverse pathological conditions, polarized microglia have detrimental effects by either promoting neuronal death in neurodegenerative diseases, or shaping the microenvironment to enhance brain tumor growth and invasiveness. Although microglia contribute to the maintenance of brain homeostasis and the pathogenesis of brain tumors, the molecular mechanisms behind their polarization towards selective phenotypes remains elusive.

In the first study, we describe a novel molecular mechanism employed by glioma cells to polarize microglia towards a tumor-supportive phenotype. We demonstrate that decreased basal caspase-3 activity in microglia is a necessary condition for their polarization into a tumor-supportive phenotype. We reveal that this process relies on the inhibition of microglial thioredoxin-2 denitrosylation activity, which in turn leads to increased S-nitrosylation of caspase-3. Furthermore, we demonstrate that microglial thioredoxin-2 becomes inactive due to nitric oxide (NO) originating from the glioma nitric oxide synthase-2 (NOS2) activity. Using a syngeneic glioma tumor model in immunocompetent mice, and through different strategies including the generation of a $Casp3^{flox/flox}Cx3cr1^{CreERT2}$ mouse model, we validated that interfering with this glioma-microglia signaling pathway impacted on the recruitment of microglia towards the tumor and also the tumor growth.

Our current findings, together with previous report from our lab, uncover a central role for distinct caspase-3-dependent signaling pathways in the regulation of different microglia phenotypes. Previously it was established that the sequential activation of caspase-8 and caspase-3/7 is of importance for the pro-inflammatory polarization of microglia. In the present study, we demonstrate a role for thioredoxin-2 mediated repression of caspase-3 in promoting a tumor-supportive phenotype in microglia. Mechanisms promoting a tumor-supportive phenotype in immune cells are of extraordinary importance, given the strong correlation between this phenotype and tumor malignancy. Our research work suggests that caspase-3 may function as a rheostat which modulates microglial polarization states in response to various stimuli. More specifically, we show that highly elevated activity of caspase-3 causes cell death, while moderate induced activity and reduced basal activity of caspase-3 regulates the pro-inflammatory and the tumor-supportive microglial polarization states, respectively.

Brain injury is commonly followed by neuroinflammation, and microglia are critical cellular elements of the brain mediating this process. In the second study, neural stem cells (NSCs) were exposed to conditioned medium originating from non-stimulated microglia, or stimulated microglia exhibiting pro- or anti-inflammatory phenotype. We found that NSCs grown in conditioned medium collected from anti-inflammatory microglia had better survival, enhanced migration and lower astrocytic differentiation compared to NSCs kept in conditioned medium deriving from pro-inflammatory microglia. This study demonstrates that pro- and anti-inflammatory microglia regulate NSCs functions differentially, and they induce chemokine CCL2 expression in differentiated NSCs.

# LIST OF SCIENTIFIC PAPERS

I. **Glioma-induced inhibition of caspase-3 in microglia promotes a tumor-supportive phenotype.**
**Shen X***, Burguillos MA*, Osman AM, Frijhoff J, Carrillo-Jiménez A, Kanatani S, Augsten M, Saidi D, Rodhe J, Kavanagh E, Rongvaux A, Rraklli V, Nyman U, Holmberg J, Östman A, Flavell RA, Barragan A, Venero JL, Blomgren K, Joseph B.
*Nature Immunology*. 2016, **17**(11):1282-1290.

II. **The secretome of microglia regulate neural stem cell function.**
Osman AM, Rodhe J*, **Shen X***, Dominguez CA, Joseph B, Blomgren K.
*Submitted*

*The authors contributed equally to this work.

# RELATED PUBLICATION, NOT INCLUDED IN THE THESIS

**Guilt by association, caspase-3 regulates microglia polarization.**
**Shen X**, Burguillos MA, Joseph B.
*Cell Cycle*. 2017, **16**(4):306-307.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 2-HG | 2-hydroxyglutarate |
| α-KG | α-ketoglutarate |
| AGM | Aorta-gonad-mesonephros |
| APCs | Antigen presenting cells |
| BBB | Blood-brain barrier |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| cGMP | Cyclic guanosine monophosphate |
| cIAPs | Cellular inhibitor of apoptosis proteins |
| CNS | Central nervous system |
| CSF-1 | Colony-stimulating factor-1 |
| CSF-1R | Colony-stimulating factor-1 receptor |
| DCs | Dendritic cells |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| GBM | Glioblastoma multiforme |
| GEM | Genetically engineered mouse |
| GFAP | Glial fibrillary acidic protein |
| GSC | Glioma stem cell |
| GSH | Glutathione |
| GSNOR | S-nitrosoglutathione reductase |
| HSCs | Hematopoietic stem cells |
| IDH | Isocitrate dehydrogenase |
| IFN-γ | Interferon-γ |
| IGF-1 | Insulin growth factor-1 |
| IKK | IκB kinase |
| IL | Interleukin |

| | |
|---|---|
| IRF-8 | Interferon regulatory factor-8 |
| LOH | Loss of heterozygosity |
| LPS | Lipopolysaccharide |
| MDSCs | Myeloid-derived suppressor cells |
| MEFs | Mouse embryonic fibroblasts |
| MMP | Matrix metalloprotease |
| NGF | Nerve growth factor |
| NK | Natural killer |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NSCs | Neural stem cells |
| PDGFR | Platelet-derived growth factor receptor |
| PI3K | Phosphoinositide 3-kinase |
| PKC-δ | Protein kinase C-δ |
| RNR | Ribonucleotide reductase |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |
| SGZ | Subgranular zone |
| SVZ | Subventricular zone |
| TAMs | Tumor-associated macrophages |
| TGF-β | Transforming growth factor-β |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor-α |
| Trx | Thioredoxin |
| TrxR | Thioredoxin reductase |
| Txnip | Thioredoxin-interacting protein |
| YS | Yolk sac |

# 1 INTRODUCTION

## 1.1 MICROGLIA

Microglia are innate immune cells, which reside in the central nervous system (CNS). They are distributed throughout the brain parenchyma and they constitute about 5-10% of the entire cell population in the adult brain (Turrin and Rivest, 2006). The microglial population serves as the key regulator of immune response in the CNS. Historically, microglia were discovered and characterized in the early 20$^{th}$ century (Del Río Hortega, 1919). However, the functional profiles of microglia have remained veiled for a long time since their discovery. The vast majority of microglial research has been emerging only since two decades ago and the research field is approaching a dynamic stage nowadays.

Microglia reside in close proximity to other types of cells in the brain. Of note, they directly interact with neuronal cells and other glial cells during development of the healthy brain (Frost and Schafer, 2016; Nayak *et al.*, 2014). After birth, microglial cells contribute to the homeostasis in the mature brain. Their functions are partially understood in physiological conditions, while their central roles in the development of pathological events are well demonstrated in brain disorders including ischemic brain injury, neurodegenerative diseases and brain tumors (Perry *et al.*, 2010; Watters *et al.*, 2005; Weinstein *et al.*, 2010). In response to various microenvironmental cues, microglia can promote neuronal cell death or tumor cell growth during disease progression, but they can also be beneficial to the brain parenchyma by defending invading pathogens and by facilitating tissue recovery process following brain damages. Thus, microglia has been an ideal target for therapeutic interventions in brain diseases.

### 1.1.1 THE ORIGIN AND HOMEOSTASIS OF MICROGLIA

The origin of microglia is different from other brain cell types. Therefore, they are considered as unique and specialized tissue macrophages. Recent RNA-sequencing transcriptome studies identified the molecular signatures of unstimulated microglia, which distinctly separate them from other CNS cells, from peripheral myeloid cells, as well as from microglia deriving from mouse model with neurodegenerative disease (Chiu *et al.*, 2013; Gautier *et al.*, 2012; Zhang *et al.*, 2014). Nevertheless, microglia share certain characteristics with other myeloid cell types, for instance surface markers and phagocytic activity.

However, the precise origin, cell lineage and mechanisms underlying homeostasis of microglia has been under debate for a long time (Ling and Wong, 1993). Alliot and colleagues proposed that microglia progenitors derive from the yolk sac (YS) and they appear in the murine brain rudiment since early embryonic stage. Furthermore, they observed that the number of microglia increased within the first two postnatal weeks (Alliot *et al.*, 1999; Alliot *et al.*, 1991). Recent fate mapping analysis performed on mice conclusively confirmed that the primitive YS macrophages contribute predominately to the resident microglia population (Ginhoux *et al.*, 2010). Currently it is accepted that the production of

hematopoietic stem cells (HSCs) initially takes place in murine embryonic aorta-gonad-mesonephros (AGM) region, event that occurs shortly after generation of primitive macrophages in YS. Subsequently, the HSCs will give rise to all the myeloid cells and lymphoid cells later during development. (Cumano and Godin, 2007). Before the establishment of blood-brain barrier (BBB), the YS-primitive macrophages colonize the embryonic neuroepithelium where they generate microglia. Thereafter, embryonic microglia invade the CNS and colonize all the regions in there.

Notably, microglia have a long lifespan and they renew themselves by local proliferation in the CNS since late embryonic stage of development (Ajami *et al.*, 2007; Lawson *et al.*, 1992). Nevertheless, under certain pathological conditions, bone marrow-derived cell populations can penetrate the BBB and undergo differentiation to macrophage/microglia (Malm *et al.*, 2005; Simard *et al.*, 2006). In particular, when the BBB is injured the peripheral monocytes can migrate to the diseased brain, in reaction to inflammations (Ajami *et al.*, 2011). Several molecular factors regulating the homeostasis of microglia have been identified. The absence of colony-stimulating factor-1 (CSF-1) or of the corresponding receptor CSF-1R and interleukin-34 (IL-34) results in a substantial reduction of microglia numbers in the mouse brain (Ginhoux *et al.*, 2010; Wang *et al.*, 2012; Wegiel *et al.*, 1998). In addition, one recent study revealed that the steady number of microglia is maintained by balanced microglial proliferation and cell death in the adult brain during the lifetime, and CSF-1R is necessary for controlling this process in microglial homeostasis (Askew *et al.*, 2017). Moreover, the PU.1 transcription factor and its interacting partner interferon regulatory factor-8 (IRF-8) are important for microglial development. Deficiency of PU.1 or IRF-8 leads to decrease of microglial density in the mouse brain (Kierdorf *et al.*, 2013).

### 1.1.2 THE ROLE OF MICROGLIA IN BRAIN PHYSIOLOGY

Our understanding of microglial functions are primarily based on their roles in pathological conditions. Their physiological functions have been partly characterized by developmental biologists, although their functions in adult brain remain elusive. Microglia maintain homeostasis in the brain under physiological conditions, whereas they conduct phagocytosis and mediate neuroinflammation, tissue repair and immune responses in reaction to brain injury (Mallat *et al.*, 2005; Wynn *et al.*, 2013). Microglial cells are never resting, but constantly moving their ramified processes to survey and scan their surroundings (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). In the healthy brain, microglial cells are able to rapidly detect any disturbance in the brain parenchyma. Moreover, microglia are well known for their capacity of engulfing apoptotic cells in the developing brain (Ferrer *et al.*, 1990). But microglial functions go beyond of their phagocytic capacity as the cleaners of the brain. Interestingly, microglia can induce apoptosis on the target cells which they will engulf later on. It has been shown during embryonic development that microglia can release nerve growth factor (NGF) or reactive oxygen species (ROS) to induce death of neurons (Frade and Barde, 1998; Wakselman *et al.*, 2008). Furthermore, microglia phagocytose non-apoptotic neural

precursor cells in order to regulate their population size in the developing brain, but microglial ablation or inhibition abrogates this regulation (Cunningham *et al.*, 2013).

Microglia are not only capable of regulating neuronal apoptosis during development but also supporting cell survival and proliferation. Several studies on microglial conditioned medium have supported that microglia release soluble trophic factors to increase neuronal proliferation and promote neuronal differentiation *in vitro* (Jonakait *et al.*, 1996; Morgan *et al.*, 2004; Nagata *et al.*, 1993). In addition, one recent *in vivo* investigation demonstrated that microglia-derived insulin growth factor-1 (IGF-1) is necessary for cortical neurons survival during early postnatal development (Ueno *et al.*, 2013).

Another study reported the enhancement of both neurogenesis and oligodendrogenesis requires activated microglia-released pro-inflammatory cytokines (Shigemoto-Mogami *et al.*, 2014). Growing evidences extend our understanding of microglia's functions as they can also promote astrogliogenesis from neural stem cells (NSCs) and that effect is impaired in the mice lacking resident microglia (Antony *et al.*, 2011; Nakanishi *et al.*, 2007; Zhu *et al.*, 2008). Hence, microglia control the integrity and activity of the developing brain by regulating CNS cell death, survival, proliferation and differentiation. Furthermore, microglia regulate the construction of synaptic network in prenatal stage, and they refine the network in an activity dependent fashion to eliminate overproduced or incorrect synaptic contacts and to support the formation of necessary new ones in postnatal stage (Parkhurst *et al.*, 2013; Schafer *et al.*, 2012; Stevens *et al.*, 2007; Wake *et al.*, 2009).

### 1.1.3 THE ROLE OF MICROGLIA IN TUMOR BIOLOGY

As the CNS resident immune cells, microglia essentially protect the brain from damages. Once exposed to pathogens, microglia polarize to a pro-inflammatory phenotype and produce a wide variety of inflammatory factors to defend against the infections, followed by elimination of pathogens and debris (Mariani and Kielian, 2009). The pro-inflammatory microglia possess potent anti-tumor properties, but they might become immunosuppressive cells in the course of glioma progression (Wei *et al.*, 2013). An early study on human glioblastoma specimens reported infiltrating microglia/macrophages account for a significant population in tumor mass ranging from 8% to 78% (Morantz *et al.*, 1979). Likewise, $CD68^+$ microglia/macrophages are found in the majority of low grade astrocytomas (Yang *et al.*, 2011). The microglia/macrophages infiltration in glioma correlates with malignancy grade and high vascularity (Nishie *et al.*, 1999). In addition, it has been shown that the percentage of microglia/macrophages displaying anti-inflammatory properties correlates with the grade of human glioma (Komohara *et al.*, 2008). Hence, accumulation of microglia/macrophages in gliomas indicates that they do not only skip tumor surveillance, but they also play a role in promoting tumor progression.

Since microglia and macrophages share many surface markers, it is difficult to differentiate them through conventional approaches. Therefore, it is not clear which cell type plays the major role over the other one in glioma biology. A recent study on chimeric mice

demonstrated that the monocytes-derived macrophages start to infiltrate the brain only in the late stage of glioma progression, representing 25% of all glioma-infiltrating myeloid cells in mice (Muller *et al.*, 2015). In this study, mice were subjected to total body irradiation or head-protected irradiation, followed by transplantation of $GFP^+$ bone marrow cells. Thereby, the naïve monocytes were damaged and replaced by their $GFP^+$ counterparts. At the same time, the brain intrinsic microglia and the blood-brain barrier (BBB) were preserved in the mice with head-protected irradiation, preventing non-physiological infiltration of peripheral monocytes in healthy brain. Subsequently, the GL261 glioma cells were intracranial inoculated in chimeric mice. It was shown the $GFP^+$ monocytes/macrophages did not infiltrate tumor mass until 21 days post tumor engraftment. Moreover, this study revealed that glioma-associated microglia are capable to increase the expression of CD45, comprising a portion of $CD45^{high}$ cells which are commonly identified as macrophages (Muller *et al.*, 2015). High grade gliomas are characterized by abnormal vascularity. The selective depletion of microglia results in 50% reduction of blood vessels density in experimental GL261 glioma, which is similar to the consequence from ablation of all $CD11b^+$ myeloid cells, implying microglia play a predominant role in promoting glioma angiogenesis (Brandenburg *et al.*, 2016).

Different experimental approaches have been utilized to explore the role of microglia in glioma biology. One *in vitro* study reported that microglial cells and microglia-conditioned medium significantly support and promote GL261 glioma cells migration, whereas oligodendroglia and endothelial cells show only weak promoting effect on motility of glioma cells (Bettinger *et al.*, 2002). Similarly, it has been shown that murine tumor infiltrating microglia/macrophages can promote $CD133^+$ glioma stem cells invasiveness through a transforming growth factor-β (TGF-β) signaling axis (Ye *et al.*, 2012). In organotypic brain slice cultures, the invasiveness of glioma cells are substantially reduced upon selective depletion of endogenous microglia by treating the slices with liposomes harboring clodronate (Markovic *et al.*, 2005). Also the eradication of intrinsic microglia by ganciclovir treatment led to a reduction in tumor volume in a glioma mouse model (Brandenburg *et al.*, 2016). Taken together, these data demonstrate that microglia are central in glioma progression, particularly in promoting glioma growth and invasion.

Microglia promote glioma invasion through degradation of the extracellular matrix (ECM) by activating matrix metalloprotease (MMP) in a cooperative manner. Glioma cells produce inactive pro-MMP2 which needs further cleavage to become active. Once in contact with glioma cells, microglia enhance MMP14 (MT1-MMP) expression. Subsequently, microglial MMP14 cleaves glioma pro-MMP2 enabling it become into active MMP2. The stepwise activation of MMPs is necessary for glioma cells, since overexpression of MMP14 in glioma cells *per se* induce cell death (Markovic *et al.*, 2009).

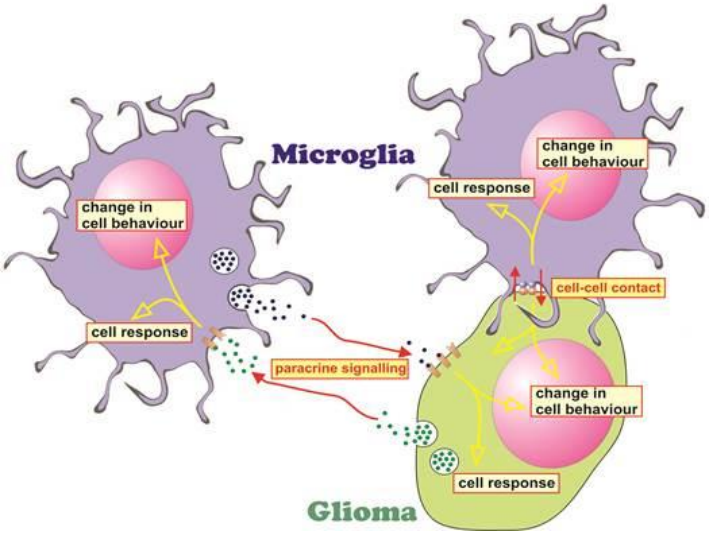


Figure 1. Cross-talk between microglia and glioma cells.

Glioma cells recruit and induce microglia polarization towards a tumor-supportive phenotype through cell-cell communications (**Figure 1**). Several paracrine loops have been identified underlying these processes. For instance, human glioma cells secrete chemokine (C-C motif) ligand 2 (CCL2) that attract microglia towards the tumor. At the same time, they also stimulate the CCL2 receptor (CCR2) on microglia which in turn produce IL-6 to promote glioma invasion (Zhang *et al.*, 2012). Another report demonstrated that glioma cells constitutively produce CSF-1 which is a known chemoattractant factor. Glioma CSF-1 activates microglial CSF-1 receptor (CSF-1R) signaling, which triggers the release of epidermal growth factor (EGF). Next, microglial EGF acts on glioma EGF receptor (EGFR) leading to increase of glioma proliferation and invasiveness (Coniglio *et al.*, 2012).

In contrast, one recent study showed that microglia still possess the anti-tumor ability when in contact with glioma. In a coculture setting, the proliferation of glioma stem cells from glioblastoma patient was mitigated by naïve microglia from non-glioma epilepsy brain, but not curbed by the glioma-associated microglia. Furthermore, the cytokine profiling revealed pro-inflammatory factors (e.g. CCL2, IL-8) were released by naïve microglia but not by glioma-associated microglia (Sarkar *et al.*, 2014). However, the timing at which microglia are compromised and switched to the tumor-supportive phenotype during glioma progression remains unclear. Although no conclusive findings are available, most likely microglial cells skew their functions towards tumor-supporting in the early period upon exposure to gliomas.

### 1.1.4 MICROGLIA POLARIZATION

In physiological conditions, microglia are surveying cells that inspect the brain parenchyma through moving their numerous long ramified processes (Nimmerjahn *et al.*, 2005). But under pathological conditions, microglia undergo morphological changes: they display fewer and shorter ramified process, and eventually adopt an amoeboid phenotype. Along with the morphological changes, microglia alter their gene expression profiles and motility, leading to changes of their activities and functions according to distinct conditions (Hanisch, 2002;

Henry *et al.*, 2009; Rawji *et al.*, 2016). Microglia possess high sensitivity and great plasticity, and differentially react to the microenvironmental cues accompanied by polarization to diverse states (Hanisch and Kettenmann, 2007). Microglia polarization is a progressive and dynamic process which is dependent on stimuli under different conditions.

Over time, one useful concept facilitating our understanding of microglia polarization has been the M1/M2 model. In this model, microglia adopt a pro-inflammatory phenotype, so-called M1, upon stimulation of lipopolysaccharides (LPS) or interferon-γ (IFN-γ) *in vitro*, resulting in production of inflammatory factors including IL-1β, tumor necrosis factor-α (TNF-α) and nitric oxide (NO). On the other hand, microglia polarize to an anti-inflammatory phenotype, referred to as M2, when stimulated by IL-4 or IL-13, leading to production of factors like IL-10, arginases-1 that promote tissues remodeling. It is worthy to notice that this is a simplified dichotomy model reflecting *in vitro* experimental settings, and also microglia are assumed to expose to limited number of stimuli. However, recent investigations indicate that this model fails to describe the actual diverse polarization states *in vivo* (Martinez and Gordon, 2014; Ransohoff, 2016). For instance, the proposed M2 phenotype and its subtypes (M2a, M2b and M2c) cannot define exclusively the tumor-associated microglia (Szulzewsky *et al.*, 2015).

The notion of microglia M1/M2 classification was inspired by macrophage polarization paradigm (Mills *et al.*, 2000). However, the M1/M2 terms do not faithful reflect macrophage activation and polarization neither (Ginhoux *et al.*, 2016). One recent comprehensive study demonstrated the complex nature of macrophage activation states. In this study, human macrophage were exposed to 29 *in vitro* conditions, and subsequent transcriptome profiling of those macrophage revealed a multidimensional model rather than a linear spectrum model underlying macrophage polarization (Xue *et al.*, 2014). Moreover, since macrophage activation is stimulus-dependent, it has been suggested to describe macrophage activations with stimulation scenarios (Murray *et al.*, 2014). Microglia/macrophages tailor their polarization to a large extent in different context. Hence, it is of importance to understand the functions regardless of the nomenclature.

## 1.2 GLIOMAS

Occurrences of uncontrolled abnormal cell growth in the brain inevitably initiate the formation of brain tumors. Primary brain tumors derive from the transformed cellular elements in the brain parenchyma and meninges; whilst metastatic brain tumors are the cancer cells spread from other parts of the body, mostly lung and breast. The latter occurs more frequently than the primary types (Vecht, 1998). Among all malignant primary brain tumors, gliomas represent the most common form and constitute 70% in adults (Ricard *et al.*, 2012). The most common symptoms presented by glioma patients include headache, nausea, seizures, focal neurological deficits and raised intracranial pressure (Behin *et al.*, 2003). The conventional treatment protocol for high-grade gliomas is surgery typically followed by fractionated radiation therapy and temozolomide chemotherapy aiming to remove the tumors as much as possible. Despite the standard treatment, the median survival of patients diagnosed with high-grade gliomas is less than 15 months (Stupp *et al.*, 2005).

### 1.2.1 CLASSIFICATION OF GLIOMAS

Gliomas consist of astrocytomas, oligodendrogliomas and ependymomas which are named based on the type of glial cell the tumor originates from. Gliomas are graded as following: Grade I (pilocytic astrocytoma), Grade II (astrocytoma, oligodendroglioma and mixed oligoastrocytoma), Grade III (anaplastic astrocytoma, anaplastic oligodendroglioma and anaplastic oligoastrocytomas) and Grade IV (glioblastoma multiforme), based on the World Health Organization (WHO) classification (Louis *et al.*, 2007). High-grade gliomas (Grade III and IV) are defined as malignant types which grow rapidly. The WHO classification is based on histopathological features. Grade III gliomas are featured by the presence of mitotic activity and nuclear atypia, and Grade IV gliomas by necrosis or microvascular proliferation.

### 1.2.2 GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM) is known as the most malignant and frequently occurring glioma. Glioblastomas arise *de novo* as primary tumors or from lower grade gliomas as secondary tumors. Primary GBMs are detected in broad brain regions and commonly in patients over 50 years-old, which accounts for the majority (95%) of all cases. Secondary GBMs comprise the remainder (5%) of the cases and they occur in the frontal lobe and typically in younger patients preceded from lower grade gliomas over years (Ohgaki and Kleihues, 2005).

Like other types of cancers, malignant transformation and following glioma progression result from the accumulation of genetic alterations, chromosome instability and unregulated growth factor signaling pathways (Ohgaki and Kleihues, 2007). Primary GBMs are characterized by loss of heterozygosity (LOH) at chromosome 10q, deletion of *CDKN2* family, mutations of tumor suppressor genes *PTEN*, *NF1*, *Rb* and *p53*, as well as dysregulation of signaling pathways involving phosphoinositide 3-kinase (PI3K)/Akt and receptor tyrosine kinase (RTK)/growth factor (Dunn *et al.*, 2012). Cellular growth factor receptors like EGFR, MET receptor and platelet-derived growth factor receptor (PDGFR) are

commonly mutated in glioblastomas, leading to acceleration of tumor cell proliferation (Furnari *et al.*, 2007). Notably, amplification of EGFR and its variant EGFRvIII are observed in approximately 50% of primary GBMs, but seldom in secondary GBMs or in normal tissue (Schlegel *et al.*, 1994). EGFRvIII, the most commonly mutated variant of EGFR, is deficient for the ligand-binding domain but is constitutively activated, and has been suggested as a prognostic factor and therapeutic target for inhibitors and antibody treatment (Sathornsumetee *et al.*, 2007; Shinojima *et al.*, 2003).

On the other hand, younger patients with lower-grade gliomas often develop *IDH1* mutations in early stage of tumorigenesis. Subsequently, they acquire multiple other gene mutations like *p53* and *Rb*, and also overexpress PDGFR, and they progress to higher-grade gliomas or directly to secondary GBMs over a period of years. The mutant isocitrate dehydrogenase 1 (IDH1) is associated with an improved prognosis for glioma patients (Dimitrov *et al.*, 2015). In human cells, there are three IDH isoforms including cytosolic IDH1 and mitochondrial IDH2 and IDH3. IDH1 and IDH2 normally convert isocitrate to α-ketoglutarate (α-KG), coupled with reduction of $NADP^+$. However, mutant IDH1/IDH2 catalyzes the reduction of α-KG to R-2-hydroxyglutarate (2-HG) in a NADPH dependent manner. Interestingly, 2-HG has been identified as an onco-metabolite, and its excess accumulation in human leads to the malignant transformation and progression of gliomas (Dang *et al.*, 2009).

Malignant gliomas display inter-tumor heterogeneity among the group of same WHO grade gliomas. Glioblastomas are well known for the high invasive capability and heterogeneous nature. Utilizing transcriptome profiling, two landmark studies have fundamentally modified our view of molecular classification of glioblastomas (Phillips *et al.*, 2006; Verhaak *et al.*, 2010). In total, four GBM molecular subtypes including proneural, neural, classical and mesenchymal were identified through comprehensive analysing of the gene expression patterns. The gene expression profile of GBM neural subtype were found however similar to that of the normal brain tissues. Despite the neural subtype, the proneural, classical and mesenchymal subtypes have unique genetic aberrations characterized by abnormal expression of genes *PDGFRA/IDH1*, *EGFR* and *NF1*, respectively. Interestingly, the majority of younger patients are classified as the proneural subtype in which mutations of *IDH1* was almost exclusively identified. Moreover, the patients manifesting *IDH1* mutations do not harbor *PDGFRA* alterations and *vice versa* (Verhaak *et al.*, 2010).

Furthermore, one recent study using a more comprehensive approach identified six distinct methylation groups of diffuse gliomas which are segregated into two macro-groups according to *IDH* status. More specifically, this study showed that *IDH1/IDH2* mutation and *IDH* wildtype were enriched for lower grade gliomas (Grades II and III) and GBM, respectively. In addition, the *IDH* mutant group displayed genome-wide higher methylation level than the *IDH* wildtype group (Ceccarelli *et al.*, 2016).

### 1.2.3 GLIOBLASTOMA MODELS

Numerous approaches have been employed for developing GBM animal models. These models mainly include chemically induced syngeneic models, genetically engineered models and human cell based xenograft models. From historical perspective, the first attempts at generating these models can be traced back to several decades ago (Brinster *et al.*, 1984; Greene and Arnold, 1945; Seligman *et al.*, 1939). During the past years, a vast number of new models have been established utilizing the same strategies. These models essentially provided us with important insights in glioma biology and to some extent yielded information on therapeutic principles. The models have been used to identify the genetic alterations contributing to glioma initiation and progression, and to validate molecular mechanisms underlying the glioma proliferation and invasion, as well as to evaluate therapeutic targets in preclinical investigations.

However, each model has its own limitations of recapitulating the disease. GBMs are thought to start from abnormal genetic events followed by dysregulation of signaling pathways in a small cell population, which contribute to glioma initiation and malignant transformation. During the tumor progression, glioma can possess histological inter- and intra-tumor heterogeneity. Simultaneously, the gliomas undergo interactions with the surrounding stromal cells, immune cells in particular, and modulations of the microenvironment, leading to gliomas expand and infiltrate into the brain parenchyma. At present there is no model that fully recapitulates the human GBM characteristics. Nevertheless, good utilization of models allows studying glioma biology in appropriate settings.

*Chemically induced syngeneic models*

Spontaneous gliomas are seldom reported in rodents. In early studies, the glioma models were induced in animals by treating them with DNA alkylating agents such as N-nitrosomethylurea (Schmidek *et al.*, 1971). The alkylation of DNA bases results in base mispairing, generation of a damaged DNA template and further gene point mutations. These models have been primarily generated in rats including 9L, C6 and CNS-1, also some in mice like GL261 (Stylli *et al.*, 2015). However, chemically induced gliomagenesis may vary to a large degree in the occurrence, incidence and malignancies.

The rodent glioma cell lines derived from these models have been widely used for *in vitro* studies and for constructing syngeneic models with high reproducibility. The cell lines normally are transplanted to a syngeneic immunocompetent host by an orthotopic approach. It is therefore a great advantage of these models that allows the developing tumor to interact with the intact immune system. These models are particularly valuable for the experimental studies on the mechanisms behind immune responses during glioma progression and invasion. For instance, one study using GL261 model reported the inhibition of transforming growth factor-β (TGF-β) reverses glioma induced immunosuppression (Ueda *et al.*, 2009). Also the GL261 model has been widely adopted for testing of experimental immunotherapy in preclinical studies (Maes and Van Gool, 2011). It is worth noting that the vigorous induction of systematic immune reaction can lead to tumor rejections in the model. In one

study, all the rats survived after receiving C6 glioma cells both in the flank and brain, in contrast to 11% survival rate in those only implanting C6 cells in the brain (Parsa *et al.*, 2000).

*Human cell based xenograft models*

The xenograft models are the most common models used for preclinical studies on glioma growth and progression. The establishment of such models involves the intracranial transplantation of human glioma cell lines or patient biopsy spheroid into the immunodeficient mice. Biopsy samples from GBM patients are subjected to tissue culture in medium containing serum for several passages to establish human monolayer cell lines. The vast quantities of glioma cells yielded by culture of human cell lines are sufficient for experimental use, especially for studies involving a large cohort of mice. Moreover, the human cell lines are genetically more relevant to human gliomas than the chemically induced cells. Yet, human cell lines have been adapted to the *in vitro* culture selection and the cell population became more homogeneous in culture. Besides, studies reported the genomic landscapes of glioma cell lines differ from that of original GBMs (Allen *et al.*, 2016; Li *et al.*, 2008).

Modification has been made to culture the biopsy material as neurospheres in neurobasal medium with no serum. The cells isolated from the sphere culture displayed cancer stem cell characteristics (Galli *et al.*, 2004). The xenograft model inoculated with caner stem cells highly recapitulated the profiles of primary GBM (Lee *et al.*, 2006). However, it is reported the success rate of performing neurosphere assay on primary gliomas varies to a large extent (Wan *et al.*, 2010). Instead of a long term culture, the biopsy material from GBM patients can be minced with blade and then subjected to tissue culture in a short time allowing for the formation of spheroids. In addition, tumors that are difficult to grow *in vitro* can be passaged in nude mice. Such biopsy spheroids preserved many original tumor features as well as the genomic signatures of human GBM (De Witt Hamer *et al.*, 2008). The biopsy spheroid xenograft model is clinically relevant since it well resembles the original tumor. At the same time, these models are variable due to the intra- and inter-tumor heterogeneity nature of GBMs.

*Genetically engineered mouse models*

The genetically engineered mouse (GEM) is a valuable tool to study genetic alterations that contribute to glioma initiation and progression. In this model, the entire immune system is maintained, allowing it to faithfully reflect tumor immunology and microenvironment. The GEM model bears the tumor formed *in situ* which retains the etiological and pathological events as well as the histological traits of gliomas. GEM models are produced by genomic manipulations in a transgenic or gene knock-out manner to study gene functions. Several GEM models have been created on the thought of modulating the central signaling pathways perturbed in human gliomas. These include pathways such as PDGF, EGFR, TP53, Rb, PTEN, Ras and Akt (Holland *et al.*, 2000; Huszthy *et al.*, 2012; Uhrbom *et al.*, 1998). However, GEMs possess the altered genes of interest in all the cell types, which may cause

embryonic lethality in some models (Schmid *et al.*, 2012). The glial fibrillary acidic protein (GFAP) is almost exclusively expressed in astrocytes. Therefore, *GFAP* is widely used as the ideal promoter in transgenic mice to restrict genes modifications in astrocytes (Danks *et al.*, 1995). Similarly, the *S100b* promoter has been used to generate the mice expressing *v-erbB* which is a homologous oncogene to *EGFR*. The overexpression of *S100b-v-erbB* initiated low grade glioma in mice followed by its transition to high grade mediated by mutations in p53 pathway (Weiss *et al.*, 2003).

Gene manipulations can be performed not only on germline, but also on somatic cells. The somatic gene transfer enables the gene alterations occurring in a small number of cells, which mimic the natural process of tumor initiation. The frequently used methodology is to deliver selected oncogenes to the target cells by RCAS/tv-a system (Dai *et al.*, 2001; Holland *et al.*, 1998; Uhrbom *et al.*, 2002). The general idea is that the avian retrovirus RCAS can be constructed to encode exogenous oncogenes, while its receptor tv-a can be engineered into transgenic mice under any cell specific promoter (e.g. *GFAP* in Gtv-a mice). Subsequently, upon injection of the transfected cells producing RCAS viral particles, the tumor is initiated but restricted by cell type and location. However, it should be taken into account the GEM models may have long tumor-free latency periods and low penetrance rates.

### 1.2.4 THE IMMUNE MICROENVIRONMENT OF GLIOMA

The heterogeneity of glioma is not only attributed to the transformed malignant cells *per se* but also to various stromal cells. The glioma stroma includes cellular components such as numerous immune cells, astrocytes, neurons, endothelial cells and fibroblasts. In addition, there are non-cellular elements surrounding the glioma including cytokines, chemokines and brain extracellular matrix (ECM). Moreover, during glioma progression, the normal brain vasculatures are disrupted as evidenced by breakdown of the BBB and induction of tumor angiogenesis (Davies, 2002; Jain *et al.*, 2007). Massive traffic of a range of immune cells takes place from the periphery to the brain once the BBB is compromised in diseases (Weiss *et al.*, 2009). All these factors together create a unique microenvironment of glioma, which plays vital roles in glioma growth, invasion and resistances to treatments. Nevertheless, the glioma microenvironment is subjected to dynamic changes during glioma progression. In the initial and early stage of glioma, the brain vasculature remains intact and thus there is no recruitment of peripheral immune cells.

Numerous immune cells are present in glioma which primarily consist of microglia, myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), tumor-associated macrophages (TAMs) and lymphoid cells (**Figure 2**). The tumor-infiltrating cells of myeloid linage, microglia, TAMs and MDSCs, are thought to be converted to a tumor-supportive phenotype shaping the tumor immunosuppressive niche (Gabrilovich *et al.*, 2012; Wu *et al.*, 2010). Among all the infiltrating cells, microglia and TAMs together have been identified as the predominant population in brain tumors (Morantz *et al.*, 1979). A recent study demonstrated

that microglia ($CD11b^{+}CD45^{low}CD33^{-}$), MDSCs ($CD11b^{+}CD45^{med}CD33^{+}$) and TAMs ($CD11b^{+}CD45^{high}CD33^{-}$) represent approximately 40%, 40% and 20% of the myeloid population in human GBM tumor mass, respectively (Gabrusiewicz *et al.*, 2016).

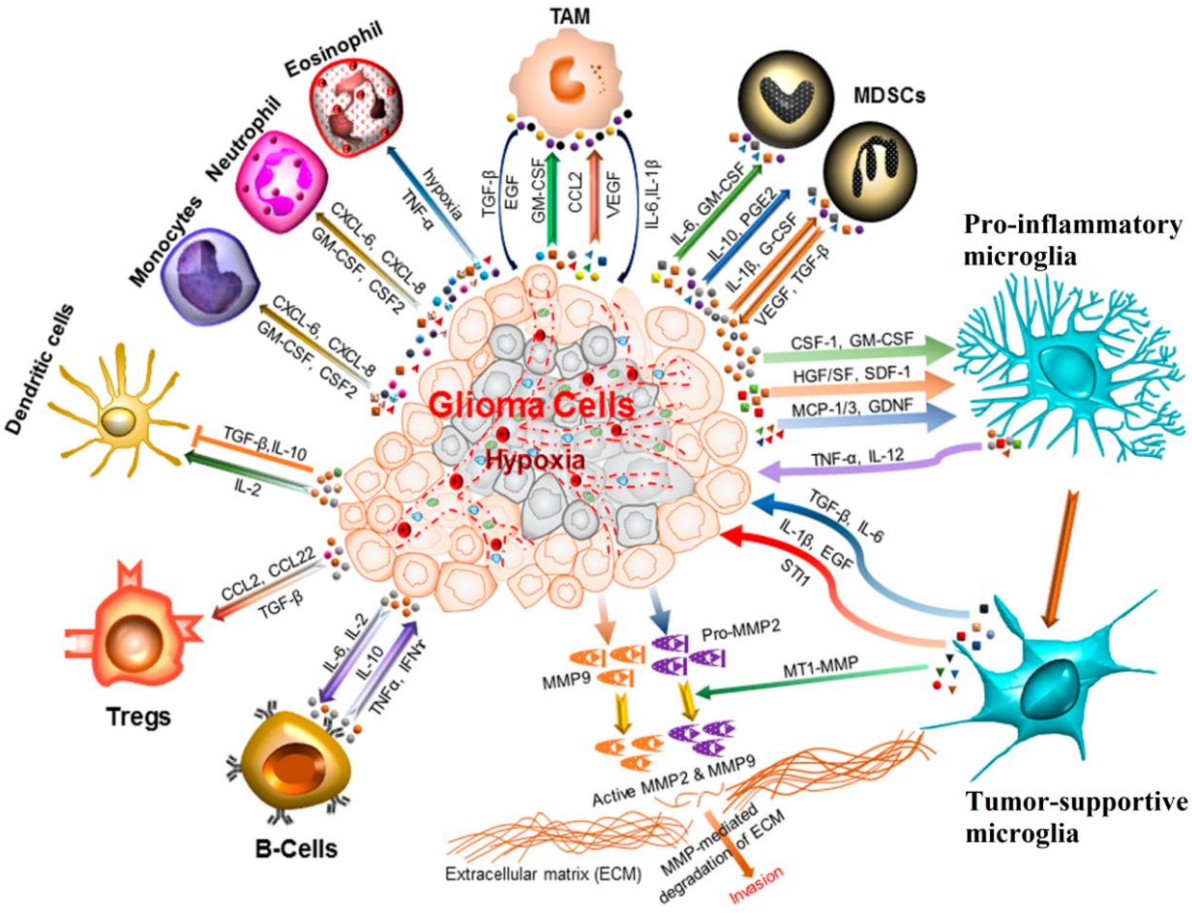


Figure 2. Illustration of the immune microenvironment of glioma.

*Adapted from a figure from an open access article (Mostofa et al., 2017).*

Under pathological conditions, bone marrow-derived monocytes traffic to the blood circulation and further migrate to the diseased tissues, giving rise to macrophages or dendritic cells (Shi and Pamer, 2011). As consequence of the disruption of the BBB, the circulating monocytes are recruited towards the brain and further differentiate into peripheral macrophages. In respect to gliomas, TAMs act in a similar way as microglia in promoting tumor progression. Yet, the functions of TAMs that distinguish them from microglia in tumor biology remains to be uncover. It has been difficult to segregate TAMs from microglia under pathological conditions due to the absence of highly specific markers. The expression level of CD45 is commonly used in flow cytometry to separate TAMs ($CD45^{high}$) and microglia ($CD45^{low}$) from mice model. A few promising markers distinguishing microglia from TAMs have been identified in recent years, which includes Tmem119, Siglec-H, Olfml3 and Cx3cr1 (Bennett *et al.*, 2016; Chiu *et al.*, 2013; Gautier *et al.*, 2012).

MDSCs are a heterogeneous myeloid progenitor and immature myeloid cell population deriving from bone marrow. Under physiological conditions, immature myeloid cells

differentiate into neutrophils, macrophages and DCs. But in pathological conditions like cancer, the regulated myeloipoiesis is perturbed, leading to the expansion of MDSCs which later migrate to the tumor site and promote tumor growth (Marvel and Gabrilovich, 2015). Indeed, MDSC accumulation was found in peripheral blood in GBM patients (Raychaudhuri *et al.*, 2011). Moreover, MDSCs were not detected in normal brain parenchyma but in resected human GBM tissue (Gabrusiewicz *et al.*, 2016). In mice models, MDSCs ($Gr\text{-}1^+$) population increase significantly 21 days post transplantation of GL261 cells in the brain, as accounting for about 3% brain cells and 20% blood cells (Alizadeh *et al.*, 2010). MDSCs have immunosuppressive functions in tumor microenvironment, which is to inhibit natural killer (NK) cells activities and to suppress the responses of helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells (Marvel and Gabrilovich, 2015). The T cell responses are dampened through a mechanism dependent on nitric oxide (NO) released from MDSCs (Raber *et al.*, 2014).

In the immune system, DCs function as the professional antigen presenting cells (APCs). DCs derive from bone marrow and have crucial functions in linking innate and adaptive immunity. They have the capacity to capture antigens, process them into peptides, present peptides on its cell surface to T cells, and eventually stimulate the adaptive immune response. The studies on DCs in gliomas have been mainly focused on DC based vaccines. In one early clinical trial, DCs were cocultured with autologous glioma lysate overnight, and then they were verified and injected into patients followed by administration of Toll-like receptor (TLR) agonists. The median overall survival of GBM patients receiving the DC vaccine was prolonged compared to historical controls, particularly of those bearing GBM mesenchymal subtype (Prins *et al.*, 2011).

Despite the predominant infiltration of myeloid-derived cells, lymphoid cells also infiltrate gliomas. NK cells are innate cytotoxic lymphocytes capable of eliminating microbe-infected cells and tumor cells (Vivier *et al.*, 2008). NK cells are not involved in low grade gliomas, but they represent a minor population (approximately 2%) of all infiltrating immune cells in GBM biopsies (Kmiecik *et al.*, 2013). One *ex vivo* study demonstrated activated NK cells can kill GBM stem-like cells that are isolated from patients (Castriconi *et al.*, 2009). However, NK cells activities are impaired in gliomas likely due to the suppression mediated by MDSCs and T regulatory cells (Ogbomo *et al.*, 2011). The number of both tumor infiltrating $CD4^+$ helper and $CD8^+$ cytotoxic T cells increase according to the grade of gliomas, while the $FoxP3^+$ T regulatory cells are present commonly in GBM specimens but rarely in low grade gliomas (Heimberger *et al.*, 2008). Both of the helper and cytotoxic T cells from GBM biopsies were manifested with a suppressed phenotype, while the $CD8^+CD28^-Foxp3^+$ T regulatory cells are present in GBM biopsies but absent in the control counterparts (Kmiecik *et al.*, 2013). However, the $CD4^+$ and $CD8^+$ T cells are not properly activated due to suppression by cytokines, such as TGF-β and IL-10, secreted in the niche and inhibition by other cells. In fact, malignant gliomas induce microglia and TAMs to produce cytokines as well as to recruit MDSCs and T regulatory cells to the tumor microenvironment (Perng and Lim, 2015).

## 1.3 CASPASES

Caspases (cysteine dependent aspartate directed proteases) are a group of proteases highly conserved among organisms. Caspases act as critical regulators of programmed cell death and inflammation. The catalytic active sites in caspases are cysteine residues which cleave the substrates by cutting peptide bonds specifically at certain aspartic acid residues. Caspases are recognized for their essential role in triggering cell apoptosis, resulting in DNA fragmentation, cell shrinkage and plasma membrane blebbing (Elmore, 2007). In recent years, several non-apoptotic roles of caspases have emerged with regard to regulate cellular functions.

### 1.3.1 CLASSIFICATION OF CASPASES

Based on function, human caspases can be divided into apoptotic and inflammatory caspases. Caspase-2, -3, -6, -7, -8, -9 and -10 are well known for their apoptosis related functions, while caspase-1, -4, -5 and -12 have inflammatory roles. According to the precise role in apoptosis, the apoptotic caspases can be divided into two subgroups: initiator caspases including caspase-2, -8, -9 and -10; effector/executioner caspases including caspase-3, -6 and -7. The upstream initiator caspases recognize and hydrolyze a few protein substrates including inactive effector caspases, whereas the downstream effector caspases cleave a much broader range of substrates.

### 1.3.2 ACTIVATION OF CASPASE-3 AND THE REGULATION

Caspases are produced in cells as inactive zymogens (monomer pro-caspases) that need to undergo dimerization and cleavage to become active. The activation of effector caspases depend on cleavage by initiator caspases. Initiator caspases are activated when dimerization occurs followed by stabilization through autocatalytic cleavage, which are dependent on the initiation by an intrinsic or extrinsic signaling pathway in the context of cell death (Boatright and Salvesen, 2003; Chang *et al.*, 2003). The intrinsic pathway is activated when either severe cell injury (e.g. DNA damage, viral infection) or absence of trophic factor occurs, in which the mitochondrial membrane barrier is disrupted, followed by cytochrome c releasing into cytosol. Afterwards, pro-caspase-9, cytochrome c and apoptotic protease-activating factor-1 (Apaf-1) are recruited to build the multi-protein apoptosome complex (Shiozaki *et al.*, 2002). The activation of caspase-9 in apoptosome leads to subsequent caspase-3 activation which triggers apoptosis. Alternatively, the extrinsic pathway is activated upon stimulation of death receptors like TNF and Fas, which results first in caspase-8 activation and latter caspase-3 activation.

Activation of caspase-3 has been linked with apoptosis for long time. However, one novel study altered our understanding of roles of caspase-3 plays in cell biology (Burguillos *et al.*, 2011). In this study, it was shown that stimulation of Toll-like receptor 4 (TLR-4) by lipopolysaccharide (LPS) leads to sequential activation of caspase-8 and caspase-3 but with absence of cell death. Caspase-3 cleaves protein kinase C-δ (PKC-δ) enabling it to become active, which in turn activates the IκB kinase (IKK) complex. Thereafter IKK phosphorylates

IκB protein, the inhibitor of transcription factor NF-κB, leading to its degradation. Once released from the inhibitor, NF-κB undergoes translocation into the nucleus which promote the expression of a range of pro-inflammatory factors such as NOS2, TNF-α and IL-1β. Thus, moderate induction of caspase-3 activity regulates microglia activation towards a pro-inflammatory phenotype and associated neurotoxicity (**Figure 3**) (Venero *et al.*, 2011).

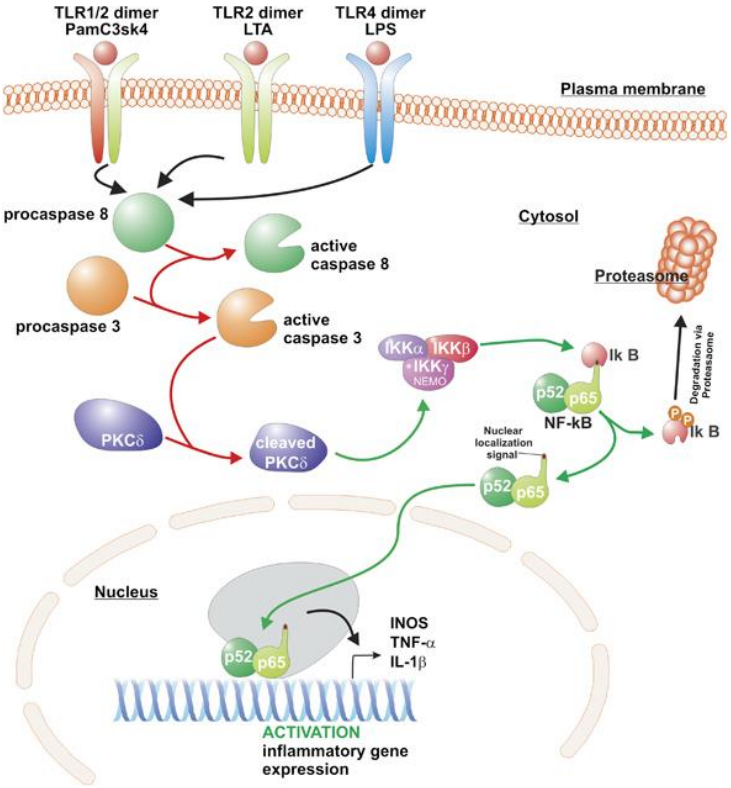


Figure 3. Caspases controls microglial pro-inflammatory polarization.

*Adapted from (Venero et al., 2011). Reprinted with permission from Nature Publishing Group.*

Moreover, it have been uncovered the mechanisms that restrict caspase-3 from committing cell death during microglia activation. As described above, pro-caspase-3 requires the cleavage by initiator caspases, caspase-8 in this context, for activation. However, this processing conducted by caspase-8 only generates active caspase-3 in the intermediate form p19/p12 complex which stays in the cytoplasm. Upon occurrence of further autocatalytic cleavage, caspase-3 (p19/p12) becomes fully active in the form p17/p12 leading to its relocation to nucleus to perfom its apoptotic function. The cellular inhibitor of apoptosis proteins (cIAPs) are a family of natural inhibitors for caspases that play a major role in regulating caspases activation (LeBlanc, 2003). During the pro-inflammatory microglia activation, the expression cIAP2 is upregulated which prevent the processing of caspase-3 subunit p19 to p17, thereby controling caspase-3 activity and translocation (Kavanagh *et al.*, 2014).

### 1.3.3 NON-APOPTOTIC ROLE OF CASPASES

Caspases have multiple physiological functions besides executing cell death. These include regulatory functions in immunity, cell proliferation, differentiation and migration. Caspase-1 is a cysteine protease recognized for its role in converting pro-IL-1β to IL-1β (Thornberry *et al.*, 1992). In response to infectious events, caspase-1 activation occurs through the inflammasome in macrophage that induce production of inflammatory factors IL-1β, IL-18 and IL-33 (Arend *et al.*, 2008; Nadiri *et al.*, 2006). Patients inherited homozygous caspase-8 deficiency manifest defects in activation of all types of lymphocytes resulting in immunodeficiency (Chun *et al.*, 2002). Knockout of caspase-8 causes embryonic death in mice, implying it is vital during embryonic development (Varfolomeev *et al.*, 1998). Mice with caspase-8 knockout specifically in T lymphocyte display defective T cell proliferation and impaired response to stimulation (Salmena *et al.*, 2003). In contrast, mice with homozygous caspase-3 knockout have enhanced proliferation of B cells (Woo *et al.*, 2003).

It has been shown that caspases facilitate cell terminal differentiation by removing nucleus in keratinocytes, lens cell, megakaryocytes and erythroid cells (Lamkanfi *et al.*, 2007). Activations of caspases are required for human monocytes to differentiate into macrophage upon stimulation by M-CSF (Sordet *et al.*, 2002). Moreover, caspase-3 deficient mice were found displaying defects in differentiation of skeletal muscle and osteoblasts (Fernando *et al.*, 2002; Miura *et al.*, 2004). It has been reported that mouse embryonic fibroblasts (MEFs) lacking caspase-8 exhibit deficiency in calpain proteolytic pathways which are responsible for cell motility (Helfer *et al.*, 2006). Caspase-8 expression level is frequently upregulated in tumor cells. One explanation is that caspase-8 can promote calpain activation independent of its catalytic activity, which in turn enhances tumor cell migration (Barbero *et al.*, 2009).

## 1.4 NITRIC OXIDE

Nitric oxide (NO) is a colorless gas with short half-life, and it is free radical harboring one unpaired electron (Hakim *et al.*, 1996). NO is soluble both in water and in organic solvents which allow it to pass easily between cells. NO is a potent molecule reacting rapidly with other components. It severs as an signaling molecule which play a physiological role of importance particularly in nervous system (neurotransmitter), cardiovascular system (control of blood pressure) and immune system (killing of microbes) (Hirst and Robson, 2011). However, excessive NO has detrimental effects on cells that can cause cell damage and apoptosis. Moreover, the controversial functions of NO during the course of tumor progression have been emerging in recent years (Choudhari *et al.*, 2013). The mechanism of intracellular NO signaling is mediated by cyclic guanosine monophosphate (cGMP) or based on redox modifications of cysteines (S-nitrosylation and denitrosylation) (Murad, 1994; Stamler *et al.*, 2001).

### 1.4.1 BIOLOGICAL SYNTHESIS OF NITRIC OXIDE

In mammalian cells, nitric oxide synthase (NOS) produce NO by converting L-arginine to L-citrulline, which is dependent on NADPH and oxygen. Mammalian cells harbor genes encoding three isoforms of NOS: NOS1/nNOS, NOS2/iNOS and NOS3/eNOS. NOS1 and NOS3 are constitutively expressed in neurons and endothelial cells, respectively. NOS1 and NOS3 activities are dependent on $Ca^{2+}$ and regulated by posttranslational phosphorylation (Lee *et al.*, 2005). On the other hand, cellular NOS2 expression is induced by a wide spectrum of inflammatory stimuli such as LPS, TNF-α and IL-1β, whereas NOS2 induction is inhibited by factors like TGF-β, IL-4 and IL-10. The NOS2 activity is $Ca^{2+}$-independent but is subjected to regulation at the transcriptional level. Therefore, the induction of NOS2 usually leads to production of NO in large quantities over an extended period of time until the NOS2 expression is under control (Kleinert *et al.*, 2003). NOS2 is vital in mediating immune responses against infectious microbes and cancer, but it also has pathological effects when massive production of NO occurs.

### 1.4.2 NITRIC OXIDE IN TUMOR BIOLOGY

Elevated activity of NOS and increased level of NO have been detected in various types of cancer (Choudhari *et al.*, 2013). For instance, one early study demonstrated high levels of NOS expression and NADPH staining in astrocytic gliomas, along with the observation that the highest NOS activity is found in higher grade gliomas (Cobbs *et al.*, 1995). Likewise, glioma patients manifest enhanced expression of NOS1 in tumor cells and increased NOS1 according to the grade of tumor, also raised NOS3 expression in vascular endothelial cells (Broholm *et al.*, 2003). These findings indicate NO production is involved in tumor biology and shape of tumor microenvironment. Furthermore, it has been proposed NO can modulate several critical events in tumor progression such as angiogenesis, invasion and metastasis (Ying and Hofseth, 2007). However, the precise effect of NO in cancer has been under debate

with current notion that NO plays dual roles in tumor biology. It seems NO can both promote and prevent tumor cell growth and proliferation.

Special attention should be given to NOS2, since its expression is the most variable among all NOS isoforms. Upon stimulation, macrophage NOS2 are capable of killing tumor cells by producing large amount of NO (60μM) (Xu *et al.*, 1998). Mice lacking NOS2 developed substantially more intestinal adenomas than their control counterparts, indicating NOS2 may have an anti-tumorigenesis function (Scott *et al.*, 2001). In contrast, NOS2 has been revealed as a mediator of angiogenesis as NOS2 expression is positively associated with angiogenic activity in various types of tumors (Fukumura *et al.*, 2006). The possible mechanisms underlying NO anti- and support-tumor roles require further investigations. NO effect is largely dependent on concentrations as high dose of NO causes apoptosis but lower concentration (nanomolar) of it promotes tumor cell proliferation (Pervin *et al.*, 2007). There are several other variable factors involved in NO functions in tumor including the timing, duration and location of exposure, stage of the developing tumor as well as the overall redox state of tumor cells.

### 1.4.3 PROTEIN S-NITROSYLATION AND DENITROSYLATION

NO signal transduction regulates cellular functions partly by conducting posttranslational modifications on proteins. The covalent addition of NO to cysteine thiol (Cys-SH) is termed protein S-nitrosylation. The resultant protein harboring a cysteine nitrosothiol (Cys-SNO) is named S-nitrosylated protein (SNO-protein). Protein denitrosylation refers to the reverse process of S-nitrosylation, which is the conversion of Cys-SNO to Cys-SH. The reactions rates of nitrosylation and denitrosylation determine the level of S-nitrosylation occurs on any given protein (**Figure 4**).

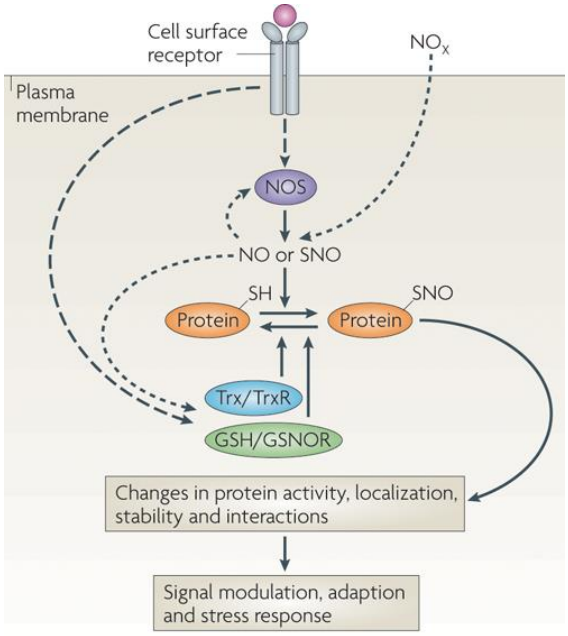


Figure 4. Mechanisms of protein S-nitrosylation and denitrosylation.

*Adapted from (Benhar et al., 2009). Reprinted with permission from Nature Publishing Group.*

Like phosphorylation and ubiquitylation, S-nitrosylation is a common modification that occurs on numerous proteins. A wide range of SNO-proteins are constitutively expressed at a low level in intact cells such as pro-caspase-3, GTPases and the ryanodine receptor (Hess *et al.*, 2005). S-nitrosylation essentially modifies protein activity, stability, interactions and its subcellular localization (Benhar *et al.*, 2009). The NO group attached to protein in S-nitrosylation is produced by endogenous NOS or from exogenous higher nitrogen oxides (e.g. $NO_2^-$ $NO_3^-$). It has been shown that endogenous protein nitrosylation is dependent on direct binding of NOS to proteins or subcellular localization of NOS (Iwakiri *et al.*, 2006; Kim *et al.*, 2005). Moreover, protein S-nitrosylation can occur in a manner of transnitrosylation between proteins. One transnitrosylation reaction involves a nitroso ($NO^+$) transfer between an SNO and an acceptor thiol.

On the other hand, protein denitrosylation is primarily mediated by S-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) systems. Glutathione (GSH) can denitrosylate SNO-protein by converting SNO to a reduced thiol, coupling with the formation of GSNO which are subsequently metabolized by GSNO in a NADH dependent fashion. However, GSNOR only denitrosylate GSNO but no other SNO-proteins, thus it acts indirectly in protein denitrosylation (Liu *et al.*, 2001). In contrast, the Trx system has been identified as the direct regulator on protein denitrosylation (Benhar *et al.*, 2008).

### 1.4.4 THE THIOREDOXIN SYSTEM

The Trx system comprise of Trx, Trx reductase (TrxR) and NADPH. Trx is a 12 kD redox protein and is present in all organisms conserved from bacteria to human (Lillig and Holmgren, 2007). Mammalian species possess two universally expressed Trxs, cytosolic Trx1 and mitochondrial Trx2, and the testis specific Trx3 (Jimenez *et al.*, 2004). Trx proteins have an active site Cys-Gly-Pro-Cys (CGPC) that is responsible for transferring electrons from NADPH to protein substrates, with involvement of TrxR. Thereby, Trx systems function as disulfide reductases for several cellular proteins (Lillig and Holmgren, 2007). For instance, Trx was originally characterized by its capacity of providing electron to ribonucleotide reductase (RNR) which is an enzyme central in synthesizing DNA (Holmgren, 1985).

Several studies have found that the Trx system also plays a major role in protein enzymatic denitrosylation (Benhar *et al.*, 2008; Sengupta *et al.*, 2007; Stoyanovsky *et al.*, 2005). It has been reported that the Trx system can restore NO-induced reduction of cellular protein activity (Kahlos *et al.*, 2003; Zhang *et al.*, 1998). One landmark study illustrated that Trx systems are capable of regulating both the constitutive basal and the stimulus-induced denitrosylation of caspase-3 (Benhar *et al.*, 2008). Trx can mediate protein denitrosylation through two likely biochemical mechanisms which generate intermediate product Protein-S-S-Trx or Trx-SNO.

However, some other studies proposed the concept that human Trx1 can promote protein nitrosylation through the non-active site $Cys^{69}$ or $Cys^{73}$ (Haendeler *et al.*, 2002; Mitchell *et al.*, 2007). This idea indicates Trx1 may be involved in protein transnitrosylation, but not Trx2 since it lacks these Cys residues. Similar to protein nitrosylation, denitrosylation can also occur in cellular compartments. For example, in lymphocytes most SNO-caspases are localized in mitochondria. Upon induction of apoptosis, SNO-caspase-3 undergoes denitrosylation by the upregulated Trx2 in mitochondria, therefore facilitating subsequent caspase-3 activation (Benhar *et al.*, 2008; Mannick *et al.*, 2001).

It has been raised the notion that activity of Trx systems are subjected to regulatory mechanisms according to cellular nitrosative stress such as the level of NO and SNO-proteins (Benhar *et al.*, 2009). Moreover, it was revealed Trx mediated denitrosylation is regulated by its endogenous inhibitor thioredoxin-interacting protein (Txnip), and Txnip itself is repressed by NO that allowing Trx system cope with nitrosative stress (Forrester *et al.*, 2009). Txnip is localized in the cytosol and in the nucleus where Txnip binds reduced Trx (Trx-$(SH)_2$) and oxidize it, while Txnip does not interact with oxidized Trx (Trx-$(S)_2$) (Patwari *et al.*, 2006). Interestingly, in response to $H_2O_2$ treatment or Txnip overexpression, Txnip can translocate from the nucleus to mitochondria and convert Trx2-$(SH)_2$ to Trx2-$(S)_2$ (Saxena *et al.*, 2010).

## 1.5 NEURAL STEM CELLS

Neural stem cells (NSCs) are multipotent cells capable of self-renewing and generating neurons, astrocytes and oligodendrocytes throughout life. In the postnatal brain, NSCs reside in two discrete neurogenic niches, the subventricular zone (SVZ) and the subgranular zone (SGZ), where they function as a source of neurons and glia cells in adult mammalian brain (Lois and Alvarez-Buylla, 1994; Luskin, 1993). Adult NSCs remain in quiescent state and they transit to active state upon stimulation by extracellular signals such as endothelial cell-derived growth factor (Sun *et al.*, 2010). Once activated, NSCs undergo either asymmetric division generating a progenitor and a NSC, or symmetric division giving rise to two progenitors (non-self-renewal). Progenitors subsequently differentiate into neuronal or glial cell types (Bond *et al.*, 2015). Adult radial glia-like NSCs in SVZ (type B cells) divide and yield transient amplifying progenitors (type C cells) followed by production of neuroblasts (type A cells) after a few divisions (Doetsch *et al.*, 1999). The neuroblasts migrate to the olfactory bulb in the form of chains and differentiate into mature interneurons. In the SGZ niche, NSCs (type 1 cells) generate intermediate progenitor cells which amplify themselves and give rise to neuroblasts, eventually maturing into dentate granule cells in hippocampus (Berg *et al.*, 2015; Seri *et al.*, 2001). Since the concept of adult neurogenesis was established, NSCs have been considered as an endogenous tool to replace damaged neurons and repair the diseased brain (Bellenchi *et al.*, 2013).

GBM contains a glioma stem cell (GSC) population that share several traits with stem cells and manifest capacity of self-renewing, maintaining proliferation and differentiating to multiple cell lineages (Galli *et al.*, 2004; Singh *et al.*, 2003; Stiles and Rowitch, 2008). These GSCs are thought to confer resistance to radiotherapy and chemotherapy (Bao *et al.*, 2006; Eramo *et al.*, 2006). The origin of GSCs remains elusive as they are proposed to be transformed from differentiated astrocytes, neural progenitor cells, or directly NSCs (Mangum and Nakano, 2012). In particular, SVZ NSCs has been thought of as the source that may give rise to some human gliomas (Lim *et al.*, 2007; Sanai *et al.*, 2005). One study utilizing transcriptome analysis revealed that human biopsy-derived GSCs and adult NSCs share common markers related to stem cells and lineages, but GSCs display a more heterogeneous gene expression profile than adult NSCs. Moreover, GSCs represent a spectrum of GBM molecular subtypes, while adult NSCs homogeneously correlate with the neural subtype (Sandberg *et al.*, 2013).

# 2 AIMS OF THE THESIS

Microglia are innate immune cells that reside in the CNS throughout life. They interact with other cellular elements in both healthy and diseased brain. The general aim of my studies was to investigate the functional roles of microglial cells in glioma biology and neural stem cell biology, as well as to study the molecular mechanisms behind the communications between microglia and glioma cells.

The specific aims of the present studies were:

**Paper I** To investigate caspase signaling pathways in glioma associated-microglia activation and to decipher the mechanisms underlying the polarization of microglia towards tumor-supportive phenotype controlled by glioma cells

**Paper II** To explore microglia that exhibit pro-inflammatory or anti-inflammatory phenotype impacts on neural stem cell function

# 3 RESULTS AND DISCUSSION

**Paper I Glioma-induced inhibition of caspase-3 in microglia promotes a tumor-supportive phenotype**

In this study, we firstly measured and quantified caspases activity in both *in vitro* and *in vivo* models. For the *in vitro* approach, we established a segregated cell coculture setting that was used for most of the coculture experiments throughout the study (Supplementary Fig. 1a). In the upper compartment we seeded microglia cells, while on the bottom of the dish we cultured a monolayer of glioma cell lines of various origins. Thereby, the microglia and glioma cells communicate with each other through soluble factors in the medium.

We detected a significant reduction in BV2 microglial DEVDase activity, which reflects caspase-3 like activity, upon segregated coculture with different glioblastoma cell lines for 6h and 24h (Fig. 1a). At the same time BV2 microglial LETDase activity, which accounts for caspase-8 activity, was marginally changed upon coculture with glioma cells for 6h and 24h (Supplementary Fig. 1b). Next, we found a similar decrease of caspase-3 like activity in microglia that were subjected to the joined coculture setting in which glioma cells physically contact with microglia (Fig. 1b). Furthermore, we confirmed the effect in human microglia and primary microglia segregated cocultured with glioma cells of distinct origins, as well as in BV2 microglia and primary murine microglia cultured with glioma-conditioned medium (Fig. 1c-e).

These observations indicate that glioma cells decrease caspase-3 like activity in microglia independent of microglial caspase-8, which is carried out effectively through glioma-derived soluble factors. It is worth to note that the pro-inflammatory polarization of microglia depends on the orderly activation of caspase-8 and caspase-3 upon stimulation of TLR-4 (Burguillos *et al.*, 2011). This implies that the glioma-associated microglial activation involves a caspase-3 signaling pathway which differs from that in pro-inflammatory microglia activation.

The reduction in microglial basal caspase-3 like activity correlates with a decrease of the cleaved form of caspase-3 (p19) and an increase of the procaspase-3 upon coculture for 6h (Fig. 1f-g). Currently it is known procaspase-3 is processed to p19 subunit, which is not fully active yet and requires to be further cleaved to become p17 to conduct apoptotic functions (Han *et al.*, 1997; Kavanagh *et al.*, 2014). Collectively, these data indicate that the cleavage of basal procaspase-3 and the corresponding caspase-3 activity were inhibited in microglia by glioma cells.

To validate the *in vitro* results, we established an *in vivo* mouse GL261 glioma model (Supplementary Fig. 1c). The GL261 glioma model is widely used for glioma experimental investigations, in particular adopted for studying immune cells functions in glioma biology since the model recapitulate the immune microenvironment. In addition, this mice model has been shown to exhibit limited infiltration by peripheral monocytes/macrophages, at the time points we used in our study (Muller *et al.*, 2015). We quantified the levels of microglial

cleaved caspase-3 in the regions inside the tumor and at the border of tumor, at different time points post tumor transplantation. We found that the florescence intensity of microglial cleaved caspase-3 within the tumor is much lower as compared to the microglia present in the border at one and two weeks after tumor transplantation (Fig. 1h-j).

To investigate the functional outcome of the decrease in basal caspase-3 like activity in microglia, gene expression profiling and functional assays were carried out in microglia subjected to caspase-3 knockdown. BV2 cells were transfected with siRNA against caspase-3 or scrambled control (Fig. 2a), and caspase-3 like activity indeed decreased in microglia transfected with siRNA caspase-3 (Fig. 2b), which enabled us to mimic the glioma effect on microglia caspases. Transfected microglia cells were included in the coculture experiments and later they were checked for the expression of genes that are central to wound-healing process. These gene products are involved in cell proliferation, tissue remodeling and immunosuppression, thus possessing pro-tumorigenic properties (Dvorak, 1986). The profiling analysis revealed that several genes were upregulated in microglial treatment groups compared with their control counterparts, which indicates both knockdown of caspase-3 in microglia and glioma effects on microglia can induce a microglial tumor-supporting phenotype. In addition, the silencing of microglial caspase-3 worked in synergy with glioma effects on microglia (Fig. 2c).

Of note, the most upregulated gene product was IL-6 in microglia. It has been showed that IL-6 functions in promoting glioma stem cell growth, maintaining microglial pro-tumoral functions, and correlated with poor survival of glioma patients (Wang *et al.*, 2009; Zhang *et al.*, 2012). Furthermore, we utilized quantitative PCR (qPCR) to validate mRNA expression levels of IL-6 along with assessment of CCL-22, Ym1, MMP14 and NOS2. We found all the tumor-supportive markers (IL-6, CCL-22, Ym1 and MMP14) were upregulated, whilst pro-inflammatory marker (NOS2) was downregulated, in microglia subjected to caspase-3 silencing or coculture with glioma cells or both conditions (Fig. 2d).

Finally, we observed that glioma cells migrate and invade further in the presence of siRNA caspase-3 transfected BV2 microglia as compared to siRNA control transfected ones (Fig 2.e), which strengthens the hypothesis that decrease in basal caspase-3 like activity is involved in the induction of microglial tumor-supportive phenotype.

The investigations until this step were based on microglia in surveying state. However, this scenario is rather marginal since during the formation of the GBM there is a massive recruitment of activated microglia/macrophages towards the tumor which subsequently polarized into a tumor-supportive phenotype (Voisin *et al.*, 2010; Zhai *et al.*, 2011). Thus, the next step was to study the strength of glioma cells-mediated inhibition of caspase-3 in activated microglia and the importance of that in inducing microglia tumor-supportive phenotype.

Previously our lab reported that LPS induces microglial caspase-3 like activity and that increase of the activity promotes microglial inflammatory response (Burguillos *et al.*, 2011).

We treated BV2 cells with LPS for 24h to let them fully polarized as pro-inflammatory phenotype, followed by a 6h coculture with glioma cells. We found that after 6h coculture, glioma cells effectively repressed LPS-induced microglial caspase-3 like activity and the expression of caspase-3 p19 subunit, as well as the inflammatory response induced by LPS (Fig. 3a, c, d). But LPS-induced caspase-8 activity in microglia was not affected by glioma cells (Fig. 3b).

Our next step was to decipher the mechanism that glioma cells employ to induce the inhibition of caspase-3 like activity in microglia. We found that this inhibitory effect on caspase-3 was independent of the initiator caspase-8 activity (Supplementary Fig. 1b), and not attributed by potential alterations of mRNA expression levels (Supplementary Fig. 4a, b). Thus, caspase-3 activity in microglia is likely inhibited through certain modifications at posttranslational level. In recent years, the role of nitric oxide (NO) has been demonstrated to be critical for the etiology of GBM (Badn and Siesjo, 2010; Eyler *et al.*, 2011). NO plays several roles in cell biology, one of which is to regulate caspases activity through S-nitrosylation (Li *et al.*, 1997; Melino *et al.*, 1997). In fact, procaspase-3 is regulated through S-nitrosylation at its catalytic site $Cys^{163}$ in a reversible manner (Benhar *et al.*, 2008; Mitchell and Marletta, 2005).

We initially examined the likelihood of NO involvement in the glioma cell-microglia communication leading to the decrease of basal caspase-3 like activity in microglia. We found that both L-NAME, a pan-inhibitor of NOS, and CPTIO, a NO scavenger, abrogated effectively the decrease of caspase-3 like activity in BV2 cells and primary microglia coculture with C6 glioma cells (Fig. 4a). Furthermore, we utilized two different techniques including biotin-switch method and in situ proximity ligation assay, and found that the level of SNO-procaspase-3 was increased in microglia cells after exposure to glioma cells in coculture (Fig. 4b, c). Finally we sought to find the origin of NO that causes S-nitrosylation of caspase-3. NOS2-specific inhibitor (1400W) but not pan-inhibitor of NOS (L-NAME) totally prevented the glioma-induced reduction of microglial caspase-3 activity (Fig. 4a). This data suggests NOS2-derived NO essentially act as a mediator in glioma cell-microglia communication.

However, it is unclear which cell produced NO as both of microglia and glioma cells could be the potential source. Our previous data showed almost complete inhibition of mRNA expression of microglial NOS2 upon coculture with C6 glioma cells (Fig. 2d). In contrast, the NOS2 mRNA level was increased significantly in glioma cells under coculture condition (Fig. 4d). Moreover, NOS2 siRNA transfected C6 glioma cells but not BV2 cells abrogated partially the glioma-induced repression of microglial caspase-3 like activity (Fig. 4e, f). Thus, we found that it is the NOS2 activity in glioma cells that triggers the inhibition of caspase-3 like activity in microglia.

The process of protein nitrosylation in cells is negatively regulated by a family of small proteins named thioredoxins (Trxs) including Trx1 and Trx2 (Benhar *et al.*, 2008). To identify the precise roles of each Trx in microglia, we selectively knocked down Trx1 or Trx2

(Supplementary Fig. 5a). BV2 cells transfected with siRNA Trx1 but not Trx2 manifested higher caspase-3 like activity than the control counterpart (Fig. 5a). In addition, glioma induced caspase-3 repression in BV2 cells transfected with siTrx2 were not as strong as in those with siTrx1 (Fig. 5a). Furthermore, the poor contribution of Trx1 to glioma induced effect on microglial caspase-3 was confirmed with using Trx1 specific inhibitor, PX-12, under coculture condition (Supplementary Fig. 5b).

In fact, we found that increased S-nitrosylation occurred on Trx2 itself and that associated decreased Trx2 activity in microglia after coculture (Supplementary Fig. 5c, d). Moreover, both the Trxs and TrxRs activity in microglia were found reduced upon coculture with glioma cells (Supplementary Fig. 5d, e), indicating the glioma effects on microglial caspase-3 is coupled with the repression of Trx systems in microglia. In particular, knockdown of Trx2 in microglia mirrored the glioma effects on microglia with regard to the level of SNO-procaspase-3 (Fig. 5b), which underscores that the deregulation of microglial Trx2 is necessary for glioma cells to achieve the effects.

It has been reported protein nitrosylation can occur in cytosol and cellular compartments (Iwakiri *et al.*, 2006; Kim *et al.*, 2005). Next, we aimed to determine the subcellular location in microglia that glioma-induced SNO-procaspase-3 occurs. We found procaspase-3 was located in both cytosol and mitochondria, while cleaved caspase-3 was present only in cytosol which represented for the majority of cellular caspase-3 activity (Fig. 5c, d). In addition, upon coculture with glioma cells, caspase-3 like activity and the expression level of caspase-3 p19 subunit were decreased in microglial cytosol (Fig. 5c, d), whereas SNO-procaspase-3 was accumulated mainly in microglial mitochondria (Fig. 5e). Taken together, these data suggest glioma induce microglia pro-tumoral activation partly through repression of Trx2-regulated denitrosylation of SNO-procaspase-3 in microglia mitochondria.

Collectively, the data demonstrate that decrease of microglial caspase-3 like activity is required for its polarization into a tumor-supportive phenotype after exposure to glioma cells, and that this process depends on impaired Trx2-mediated denitrosylation of caspase-3 in microglia which is likely due to nitrosative stress origin from glioma cells.

Next, we sought to examine the proposed signaling pathway *in vivo*. We started with inhibition of glioma NOS2 *in vivo* which is the initial component in the upstream of the pathway. We utilized NOS2 shRNA to stably knockdown NOS2 in GL261 glioma cells (Fig. 6a). Targeting NOS2 in GL261 indeed abrogated the glioma effects on microglial caspase-3 like activity (Fig. 6b). We transplanted the GFP-GL261 cells expressing shRNA-control or shRNA-NOS2 into C57/BL6/J mice brain. Using confocal microscopy, we found the mice receiving shRNA-NOS2 glioma cells displayed a substantially reduced volume of tumor and much less recruitment of microglia cells compared to the control counterparts at time of 1-week and 2-week after tumor transplantation (Fig. 6c-f). Thus, it was validated *in vivo* the glioma-derived NOS2 is essential to recruit microglial cells towards tumor mass.

Ultimately, we attempted to validate the downstream of the signaling pathway involved in glioma cell-microglia crosstalk. The chemokine receptor CX3CR1 is a highly specific marker for microglia, allowing us to generate the conditional mice model bearing gene alterations restricted in microglia (Chiu *et al.*, 2013; Gautier *et al.*, 2012). We generated $Casp3^{flox/flox}Cx3cr1^{CreERT2}$ mice as the microglial caspase-3 knockout model upon tamoxifen treatment, and the $Casp3^{flox/flox}$ mice treated as control (Fig. 7a). Following the previous procedure, we implanted GFP-GL261 glioma cells in $Casp3^{flox/flox}$ mice and $Casp3^{flox/flox}Cx3cr1^{CreERT2}$ mice. The analysis performed on the brain tissues revealed the mice, subjected to deletion of microglial caspase-3, had developed tumors with greater volume than the control counterparts at 1-week and 2-week after tumor transplantation (Fig. 7b-e). Thus, it was confirmed that *in vivo* depletion of microglial caspase-3 induced a microglial tumor-supportive phenotype promoting glioma growth and invasion.

In summary, we identified glioma NOS2-produced NO leads to inhibition of Trx2 function in denitrosylating SNO-procaspase-3 in microglial mitochondria, further causes a decrease in microglial basal caspase-3 activity, ultimately results in the activation of microglia tumor-supportive phenotype (Supplementary Fig. 7a). This study shed light on the role that caspase-3 plays in the process of microglia polarization in the context of tumor biology. Deciphering the mechanism that regulates microglia pro-tumoral activation can provide us with suitable therapeutic options to prevent GBM expansion.

Together with previous finding in the lab (Burguillos *et al.*, 2011), we found that caspase-3 might work as a modulator that controls microglial activation states in response to various stimuli (Supplementary Fig. 7b). The surveying microglia exhibit basal caspase-3 like activity, while the dying microglia display significant elevated caspase-3 like activity. Moreover, moderate increased caspase-3 like activity and reduced caspase-3 like activity below basal level promotes microglia polarization towards pro-inflammatory and tumor-supporting phenotypes, respectively (**Figure 5**).

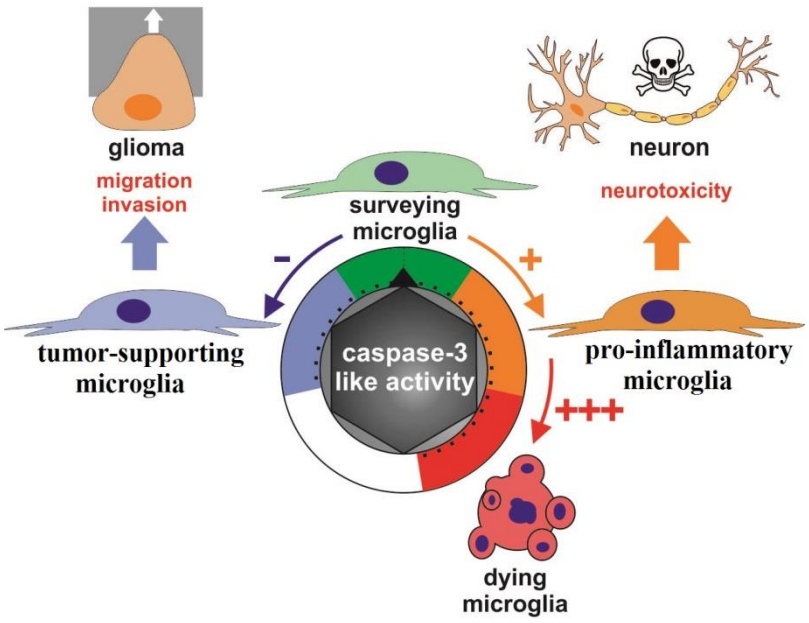


Figure 5. Caspase-3 like activity regulates microglia polarization. *From (Shen et al., 2017).*

**Paper II The secretome of microglia regulate neural stem cell function**

Microglia are essential cellular elements that reside in the neurogenic niches, they regulate adult neurogenesis, and they communicate with neurons (Gemma and Bachstetter, 2013). In response to brain injury, microglia undergo polarization and recruitment towards the affected area where they mediate tissue repair (Mallat *et al.*, 2005; Wynn *et al.*, 2013). In this study, we investigated the differential influences of microglia that displays distinct properties on neural stem cells (NSCs) functions.

We firstly stimulated BV2 microglial cells with LPS or IL-4 in order to induce microglial pro-inflammatory or anti-inflammatory phenotypes, respectively (Fig. 1c, d). Next, the validated multipotent NSCs (Fig. 1a, b) were exposed to conditioned medium collected from stimulated microglia, and subsequently NSCs were examined for gene expression profile and cellular functions in the aspect of survival, proliferation, migration, and differentiation.

We found that NSCs cultured in pro-inflammatory microglia conditioned medium showed worse survival (Fig. 2a, b), decreased migration (Fig. 3a-c) and higher astrocytic differentiation (Fig. 4c) compared to those grown in conditioned medium collected from anti-inflammatory microglia. The conditioned medium derived from different microglia phenotypes had similar influence on NSC proliferation (Fig. 2c) and neuronal or oligodendrocytic differentiation (Fig. 4a, b, d).

Furthermore, utilizing qPCR we demonstrated NSCs differentiated in microglial conditioned media expressing increased level of the pro-inflammatory chemokine CCL2 compared to the control counterpart (Fig. 5a), and that was more notable when NSCs were grown in the pro-inflammatory microglia conditioned medium. In summary, we found differentially polarized microglia impacts on NSCs functions distinctly.

# 4 CONCLUSIONS AND FUTURE PERSPECTIVES

The major findings of the present studies are listed as following.

- Glioma cells inhibit microglial caspase-3 for their expansion and invasion
- Glioma NOS2 activity induces S-nitrosylation of caspase-3 in microglia
- S-nitrosylated caspase-3 accumulates in microglial mitochondria due to glioma- induced inhibition of thioredoxin-2 activity
- Repression of glioma NOS2 reduces microglia recruitment and tumor expansion
- Depletion of microglial *Casp3* gene promotes glioma tumor growth
- Microglial regulations on neural stem cells are dependent on their polarization states

We uncovered a novel microglia-glioma cell-cell communication signaling pathway, wherein NO produced by NOS2 in glioma cells leads to an S-nitrosylation-dependent inhibition of Trx2 activity in microglial cells, which in turn results in increased S-nitrosylation and inhibition of caspase-3, an event which promotes the tumor-supportive polarization of microglia. Furthermore, appropriate mice models were used to validate glioma NOS2 and microglial caspase-3 as the upstream and downstream molecular components respectively of this signaling pathway. However, the signaling events in the downstream of caspase-3 remain unclear. It is worthy to investigate how microglial tumor-supportive phenotype is mechanistically induced when its caspase-3 activity is repressed below the basal level.

Caspase-3 is constitutively expressed at a low level which has several non-apoptotic roles. The inhibition of caspase-3 likely has negative regulations on microglial pro-inflammatory functions. On the other hand, the accumulation of SNO-procaspase-3 might have positive regulations on microglial pro-tumoral phenotype independent of caspase catalytic activity. Then, the majority of procaspase-3 and SNO-procaspase-3 were found present in cytosolic fraction. Under the experimental condition, the level of mitochondrial SNO-procaspase-3 was increased, but the origin of that is elusive. The increased SNO-procaspase-3 is likely to be of mitochondria origin, although it could also be transported from the cytosol. Further studies are required to explore potential dynamics of cellular SNO-procaspase-3. Finally, Txnip is the endogenous inhibitor for Trx, and it is able to translocate to cellular compartments. To examine the level of Txnip can provide insight of additional inhibitory mechanisms on Trx2. It is assumed the level of Txnip decreases in cytosol but increases in mitochondria in microglia exposed to glioma cells.

With respect to *in vivo* study, we generated a microglial caspase-3 knockout model which provided direct evidence for our hypothesis. The investigations can be extended as to determine the precise phenotype of glioma-associated microglia in both knockout mice and control group. This work can be conducted by utilizing conventional immunofluorescence

staining or by transcriptome profiling and analysis. Overall, these future studies could advance our understanding of mechanisms behind microglia-glioma cell communication system. On the other hand, within the niche microglia also cross-talk with NSCs and their influences on NSCs functions are dependent on polarization states. It is of interest to identify microglia-derived soluble factors that mediate the regulations on NSCs in future study.

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