Aus dem Max Delbrück Centrum für Molekulare Medizin

DISSERTATION

Dissecting the role of Toll-like receptors in microglia-glioma crosstalk

zur Erlangung des akademischen Grades

Medical Doctor - Doctor of Philosophy (MD/PhD)

im Rahmen des

International Graduate Program Medical Neurosciences

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

Feng Hu/ 胡峰

aus Huanggang, China/中国 黄冈

Datum der Promotion: 05.12.2014

To my beloved family

献给我挚爱的家人

Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Aqua distilled water (aq. H_2O)

Blood brain barrier (BBB)

Brain Cancer-propagating Cells (BCPC)

Carbon dioxide (CO_2)

Central Nervous System (CNS)

Clodronate liposomes (CL)

Colony stimulating factor-1 (CSF-1)

Damage/Danger Associated Molecular Patterns (DAMPs)

Deoxyribonucleic acid (DNA)

Enhanced Green Fluorescent protein (EGFP)

Epidermal Growth Factor Receptor (EGFR)

Enzyme-linked immunosorbent assay (ELISA)

Experimental autoimmune encephalomyelitis (EAE)

Extracellular matrix (ECM)

Fibroblast Growth factor (FGF)

Fluorescence activated cell sorting (FACS)

For example, exempla gratia (e.g.)

Glial-derived neurotrophic factor (GDNF)

Glioma-associated Microglia/brain macrophages (GAMs)

Glioma conditioned medium (GCM)

Glycosaminoglycan (GAG)

Granulocyte/macrophage colony-stimulating factor (GM-CSF)

Hypoxia-inducible Factor (HIF)

Horseradish peroxidase (HRP)

Hour (h)

Intra-peritoneal (i.p.)

Immunohistochemistry (IHC)

Isocitrate Dehydrogenase (IDH)

Knock-out (KO)

Lipopolysaccharide (LPS)

Major histocompatibility complex (MHC)

Matrix metalloproteinase (MMP)

Membrane-type one-matrix metalloproteinase (MT1-MMP)

Mitogen activated protein kinase (MAPK)

Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-3 (MCP-3)

Nitric Oxide (NO)

Neurofibromatosis Type I (NF-1)

Organotypic Brain Slice Cultures (OBSC)

Pattern Recognition Receptors (PRR)

Pathogen Associated Molecular Patterns (PAMP)

Polymerase Chain Reaction (PCR)

Phosphatase and Tensin homolog (PTEN)

Phosphoinositol 3–Kinase (PI3K)

Platelet-derived growth factor (PDGF)

Platelet-derived growth factor receptor-A (PDGFRA)

Reverse transcriptase PCR (RT-PCR)

Room temperature (RT)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The Cancer Genome Atlas (TCGA)

Thrombospondin-1 (TSP-1)

Tissue Inhibitor of metalloproteinase 2 (TIMP2)

Toll-like receptor (TLR)

Tumor–Initiating Cells (TICs)

Vascular Endothelial Growth factor (VEGF)

Western blotting (WB)

Wild-type (WT)

World Health Organization (WHO)

1a. Abstract (English)

Background Malignant gliomas are the most frequent primary tumors of the brain with poor clinical prognosis. Infiltrating peripheral macrophages and resident microglia contribute significantly to the tumor mass. We have previously shown that microglia as the intrinsic immune competent brain cells, promote glioma expansion through the up-regulation of membrane-type 1 matrix metalloprotease (MT1-MMP) through microglial Toll-like receptor (TLR) and its adaptor protein molecule myeloid differentiation primary response gene 88 (MyD88). This effect is induced by the soluble factors released by glioma cells.

Methods Using *in vitro*, *ex vivo* and *in vivo* techniques I identified the important roles of microglial TLR2 and its endogenous ligand versican in microglia-glioma crosstalk.

Results The implantation of mouse GL261 glioma cells into TLR2 knock-out (KO) mice resulted in significantly reduced MT1-MMP expression and enhanced survival rates as compared to wild-type (WT) control mice. TLR2 is highly expressed in human gliomas and its expression inversely correlates with patient survival. I also found that the endogenous TLR2 ligand versican is released by glioma cells and triggers microglial/brain macrophage MT1-MMP expression. The splice variants V0/V1 of versican, but not V2, are highly expressed in mouse and human glioma tissue. Versican silenced gliomas induced less MT1-MMP expression in microglia both *in vitro* and *in vivo*. Implanting versican silenced GL261 cells into mouse brain resulted in smaller tumors and longer survival rates compare to controls. Using organotypic brain slices I found that the impact of versican signaling on glioma growth depended on the presence of microglia. Microglia but not astrocyte conditioned medium could induce glioma versican up-regulation. Additionally, an established TLR2 neutralizing antibody reduced glioma induced microglial MT1-MMP expression as well as glioma growth *ex vivo*. Moreover, I found that TLR2 expression is upreguated in glioma associated microglia, but not in astrocytes.

I also demonstrate that another important member of the MMPs family, maxtrix-metalloprotease 9 (MMP-9) is predominantly expressed by glioma associated microglia/macrophages in mouse and human glioma tissue but not by the glioma cells.

Supernatant from glioma cells induced the expression of MMP-9 and TLR2 in cultured microglial cells. I identified Toll-like receptor 2/6 as the signaling pathway for the glioma induced upregulation of microglial MMP-9. Both, the upregulation of MMP-9 and TLR2 were attenuated by the antibiotic minocycline *in vitro*.

Conclusion The results thus show that activation of TLR2 converts microglia into a glioma supportive phenotype, this signaling cascade might be a novel target for glioma therapies.

1b. Abstract (German)

Hintergrund Maligne Gliome sind die am häufigsten auftretenden primären Gehirntumore und mit einer schlechten klinischen Prognose assoziiert. Infiltrierende periphere Makrophagen und residente Mikroglia tragen signifikant zur Tumormasse bei. Wir haben in früheren Studien zeigen können, dass Mikroglia als inhärente immunkompetente Gehirnzellen die Ausbreitung von Gliomen unterstützen. Dies geschieht durch die verstärkte Expression der Membrane-type 1 Matrix Metalloprotease (MT1-MMP), welche durch den mikroglialen Toll-like Rezeptor (TLR) und dem Adapterprotein „Myeloid differentiation factor 88“ (MyD88) reguliert wird. Dieser Effekt wird von löslichen Faktoren induziert, die wiederrum von Gliomzellen abgegeben werden.

Methoden Mit Hilfe von *in vitro*, *ex vivo* und *in vivo* Techniken habe ich die wesentlichen Rolle von Mikroglia assoziierten TLR2 und ihrem endogenen Liganden Versican innerhalb der Mikroglia-Gliom-Interaktion identifiziert.

Ergebnisse Das Implantieren von murinen GL261-Gliomzellen in TLR2 „knockout“ (KO)-Mäuse hatte, im Vergleich zum Wildtyp (WT), eine signifikant verringerte MT1-MMP-Expression und eine erhöhte Überlebensrate zur Folge. TLR2 ist in humanen Gliomen stark exprimiert, wobei sich die Expressionsrate umgekehrt proportional zur Überlebenschance der Patienten verhält. Ich habe außerdem gezeigt, dass der endogene TLR2-Ligand Versican von Gliomzellen abgegeben wird und die MT1-MMP-Expression in Mikroglia/Gehirnmakrophagen einleitet. Die Spleißvarianten von Versican V0/V1, aber nicht V2, sind im hohen Maße in murinem und humanem Gliomgewebe exprimiert. In *in vitro* und *ex vivo* Experimenten führte die Abschaltung von Versican in Gliomen zu verringerter MT1-MMP-Expression der Mikroglia. Im Vergleich zu Kontrollen führte das Implantieren von für Versican stummgeschalteten GL261 Zellen zu kleineren Tumoren und verbesserten Überlebensraten. Mit Hilfe von organotypischen Gehirnschnitten konnten wir zeigen, dass der Einfluss von Versican auf das Gliomwachstum von Mikrogliapräsenz abhängig ist. Mikroglia-, jedoch nicht

Astrozyten-konditioniertes Medium, bedingt Gliom assoziierte Versican Hochregulierung. Zusätzlich verringert ein TLR2 neutralisierender Antikörper Gliom-induzierte mikrogliale MT1-MMP-Expression und Gliomwachstum *ex vivo*. Darüber hinaus konnten ich zeige, dass die TLR2-Expression in Gliom-assoziierten Mikroglia, jedoch nicht in Astrozyten erhöht ist.

Ich konnte außerdem demonstrieren, dass ein weiteres wichtiges Mitglied der MMP-Familie, die Matrix-Metalloprotease 9 (MMP-9), vorwiegend in murinen und humanen Gliom-assoziierten Mikroglia/Makrophagen exprimiert wird, allerdings nicht in Gliomzellen. Der Überstand von Gliomzellen induzierte die Expression von MMP-9 und TLR2 in kultivierten Mikrogliazellen. Der Toll-like Rezeptor 2/6 konnte von uns als Signalweg für die gliominduzierte Hochregulierung von mikroglialem MMP-9 identifiziert werden. Das Antibiotikum Minocyclin hat die Hochregulierung von MMP-9 und TLR2 *in vitro* verringert.

Fazit Meine Ergebnisse zeigen daher, dass die Aktivierung von TLR2 Mikroglia in den Gliom unterstützenden Phänotyp umwandelt. Diese Signalkaskade könnte ein neuer Ansatzpunkt für die Therapie von Gliomen darstellen.

2. Introduction

2.1 Gliomas

2.1.1 Epidemiologic and etiological features

Gliomas are the most common malignant brain tumors in the central nervous system, which account for around 70% of the newly diagnosed cases in adults (Wen and Kesari 2008). They comprise approximately 30% of all brain tumors, and approximately 60% of gliomas occur in the four lobes of the brain. Overall, brain tumors are relatively rare events, the average annual age-adjusted incidence rates of all malignant brain tumors ranged from 4.95 to 8.97 per 100,000, while non-malignant brain tumors ranged from 8.90 to 19.02 per 100,000, and males are more frequently affected with gliomas than females (Dolecek et al. 2012). Like most malignant cancers, the etiology of gliomas is still unclear, although it has been reported that professions, life styles and environmental carcinogens are associated with a high risk of gliomas, but the only unequivocal factor identified so far is therapeutic X-irradiation (Little et al. 1998; Ohgaki and Kleihues 2005).

WHO Grade	WHO designation	Histopathology
I	Pilocytic astrocytoma	Bipolar, "piloid" cells, Rosenthal fibers, eosinophilic granular bodies
II	Low grade astrocytoma	Neoplastic fibrillary, or gemistocystic astroyctes; nuclear atypia
III	Anaplastic astrocytoma	Neoplastic fibrillary, or gemistocystic astrocytes; nuclear atypia, mitotic activity
IV	Glioblastoma multifome	Cellular anaplasia, nuclear atypia, mitoses, vascular proliferation, necrosis

Table 1. Wortd Health Organization (WHO) grading system for astrocytoma

Modified from (Kleihues et al. 1995)

2.1.2 Histologic and molecular classifications

Classically gliomas are classified by their histological characteristics. Cytologic atypia, mitotic activity, high cellularity, vascular proliferation, and necrosis are the most significant indicators of anaplasia in gliomas. According to these features, gliomas were classified as 4 grades (Table 1.1, modified from Kleihues et al. 1993). The first well accepted grading scheme was edited and published by Kleihues in 1993 (Kleihues et al.

1993), and more genetic profiles were added by Kleihues and Cavenee in 2000 (Kleihues, Cavenee 2000). The latest version of the glioma grading system was established by 25 pathologists and geneticists in the German Cancer Research Center in Heidelberg in 2006, and a few new entities were defined and well described in this version (Louis et al. 2007).

Although we can distinguish gliomas from their morphologies within the same entity (e.g. GBM), patients may have a completely different prognosis after the same therapies. This indicates GBMs are genetically different although they share similar morphologies. Genetic analyses of gliomas, most of all GBM, have been carried out in the past decades. Major mutations such as epidermal growth factor receptor (EGFR) (Libermann et al. 1985) and, *TP53* tumor suppressor gene (van Meyel et al. 1994) were identified. However, the most recent and exciting achievement in the field was from The Cancer Genome Atlas (TCGA) (Verhaak et al. 2010), by analyzing a total of 202 primary, untreated human GBM specimens with cross-platform, multilevel (DNA, mRNA, microRNA) analyses, they uncovered novel genetic alterations and found GBM could be divided into 4 subgroups according to their genetic properties (Figure 1). The classical subtype of GBM was identified by frequent events such as Chromosome 7 amplification paired, chromosome 10 loss (93%) and focal losses on chromosome 9p21.3 (95%), which leads to the increase of *EGFR* expression and lack of *TP53* mutations. Classical GBM also demonstrates responsiveness to the classical radiation and chemotherapies. The second subtype was named “Mesenchymal”, which has frequent inactivation of the *NF* (37%), *TP53* (32%) and *PTEN* (23%) genes. Mesenchymal GBM shows responsiveness to aggressive radiotherapies and may benefit from Ras and PI3K, and angiogenesis inhibitor treatments. The third subtype “Proneural” has two major features: alterations of *PDGFRA* and point mutations in *IDH1.* In this group, patients are younger and survival time was slightly better than in the other three subtypes. The last group is “Neural” with a gene expression pattern that was most similar to those found in normal brain tissue. Though this subtype is less well defined, their expression signature is suggestive of cells with a differentiated phenotype. These findings lead us to a substantial understanding of genetics and ontogeny of GBM, and I believe new research

as well as therapies on GBM will benefit from this in the near future (Van Meir et al. 2010; Verhaak et al. 2010).

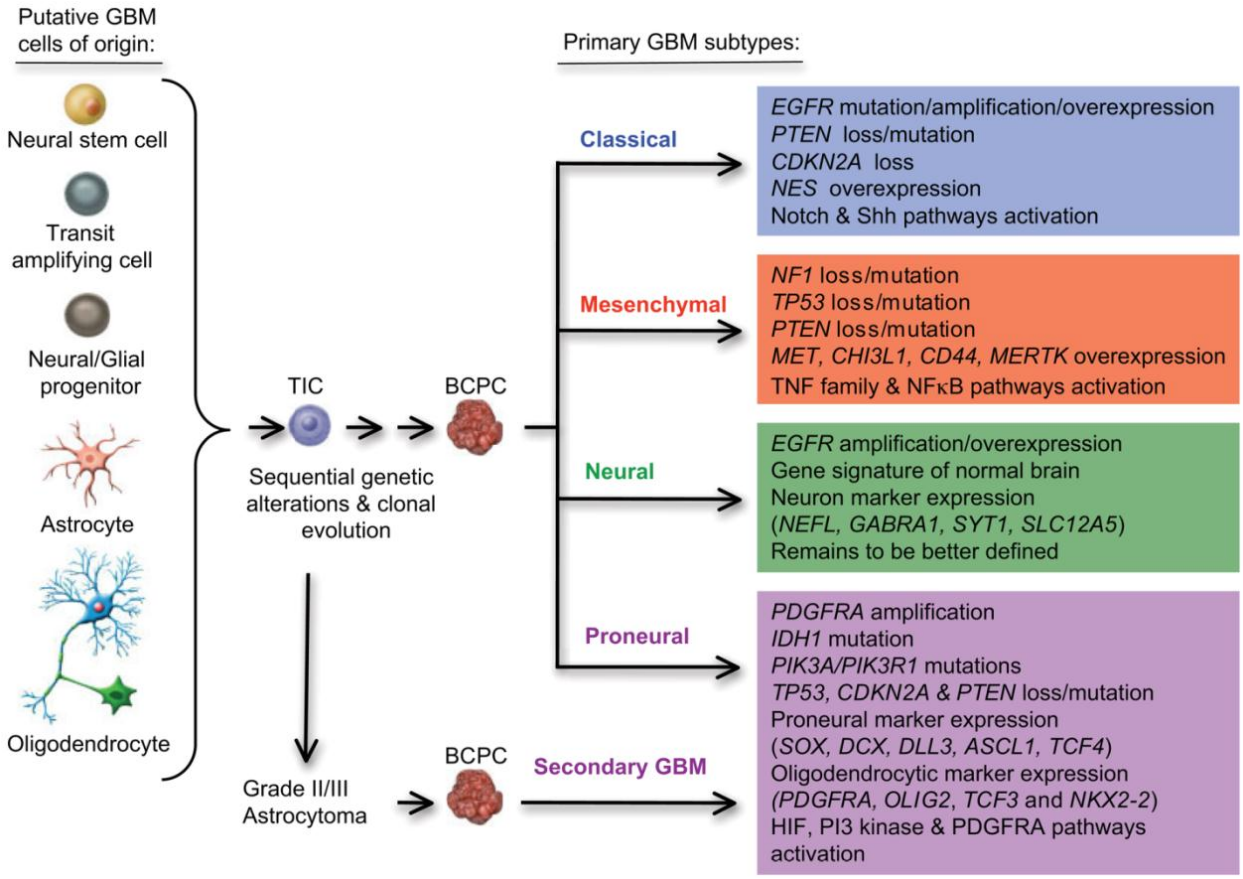

Figure 1. New molecular classifications and characteristics of glioblastoma.

Gliomas may derive from different cell types, these cells give rise to tumor–initiating cells (TICs), which can further become brain cancer-propagating cells (BCPC). BCPC are responsible for the formation of GBMs. According to the genetic analysis by TCGA, GBMs are divided into 4 subtypes Classical, Meshenchymal, Neural and Proneural with different genetic characters. GBM indicates glioblastoma multiforme; EGFR, epidermal growth factor receptor; PTEN, phosphatase and tensin homolog; TNF, tumor necrosis factor; PDGFRA, platelet-derived growth factor receptor–A; IDH, isocitrate dehydrogenase; PI3K, phosphoinositol 3–kinase; HIF, hypoxia-inducible factor. Adapted from (Van Meir et al. 2010)

2.1.3 Clinical presentation, diagnosis and treatments

Patients with gliomas may present with different symptoms such as headaches, seizures and, focal neurological deficits among others, which are mainly due to the mass effect, while a few of them were even discovered accidentally, for example by medical examinations after a car accident. Diagnoses are always made by magnetic resonance

imaging (MRI) or computed tomography (CT); functional MRI may help the surgeon to define the relationship between speech or motor area and tumor, so that it could aid in planning the surgery. The standard therapies for newly diagnosed malignant gliomas involve surgical resection, radiotherapy and chemotherapy. Temozolomide (TMZ), a methylating agent that was approved in 1999 by FDA for treatment resistant anaplastic astrocytoma, showed controversial therapeutic effect in treating malignant gliomas at the beginning (Batchelor 2000). Excitingly, Hegi *et al*. found out later that only patients suffering GBM containing a methylated *MGMT* gene promoter benefited from temozolomide while those who did not could not have such a benefit (Hegi et al. 2005). This key finding leads to the test of MGMT methylation in GBM samples as a routine for all newly diagnosed GBM patients. Other newly developed techniques like MRI-guided neuronavigation, intraoperative MRI, intraoperative mapping and fluorescence-guided surgery increased the extent of resection of lesions safely and thus may improve the quality of life or survival rate of GBM patients (Van Meir et al. 2010).

2.2 Microglia

2.2.1 Origin and properties of microglia

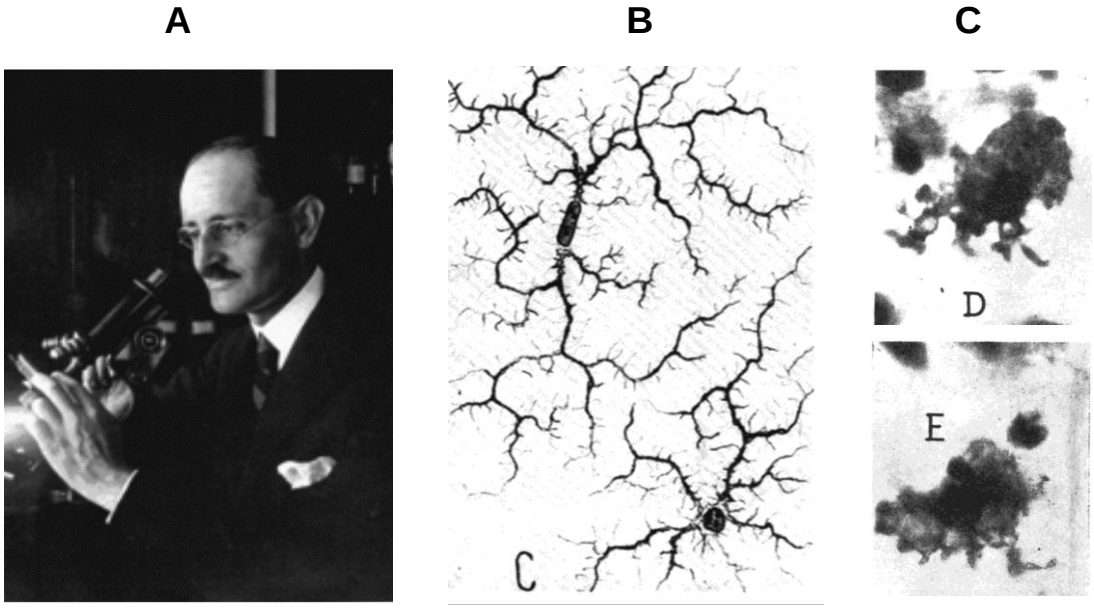

Figure 2. Discovery of microglial cells.
(A) Pio del-Rio Hortega (1882-1945). (B) Resting microglia drawn by Rio Hortega. (C) Earliest photomicrographs of activated and pseudopodic microglia. Adapted from (Del Rio-Hortega, 1932).

Microglial cells are resident immune cells in the CNS. There were first described by Pio del Rio-Hortega in a landmark publication *Cytology and Cellular Pathology of the Nervous System*, edited by Wilder Penfield in 1932 (Del Rio-Hortega, 1932) (Figure 2). The origin of microglia has been debated for a long time. We now know from recent studies that microglia arise from yolk sac macrophages that fill up the brain at a very early stage during development (Ginhoux et al. 2010; Kettenmann et al. 2011). These "invaded" microglial cells maintain themselves till adulthood via local proliferation during post-natal development as well as CNS inflammation (Ginhoux et al. 2013) (Figure 3). It is now well accepted that in the steady state of the brain, bone marrow derived monocytes do not enter the CNS, however, during pathological changes in the brain, such as neurodegenerative diseases or tumor growth, monocytes from bone marrow engraft into the CNS and contributed a proportion of mononuclear phagocytes together with resident microglial cells. But a few questions still remain unclear: Are they distinguishable by markers in rodents and human? Do these "microglia-like" cells have the same functions as resident microglia?

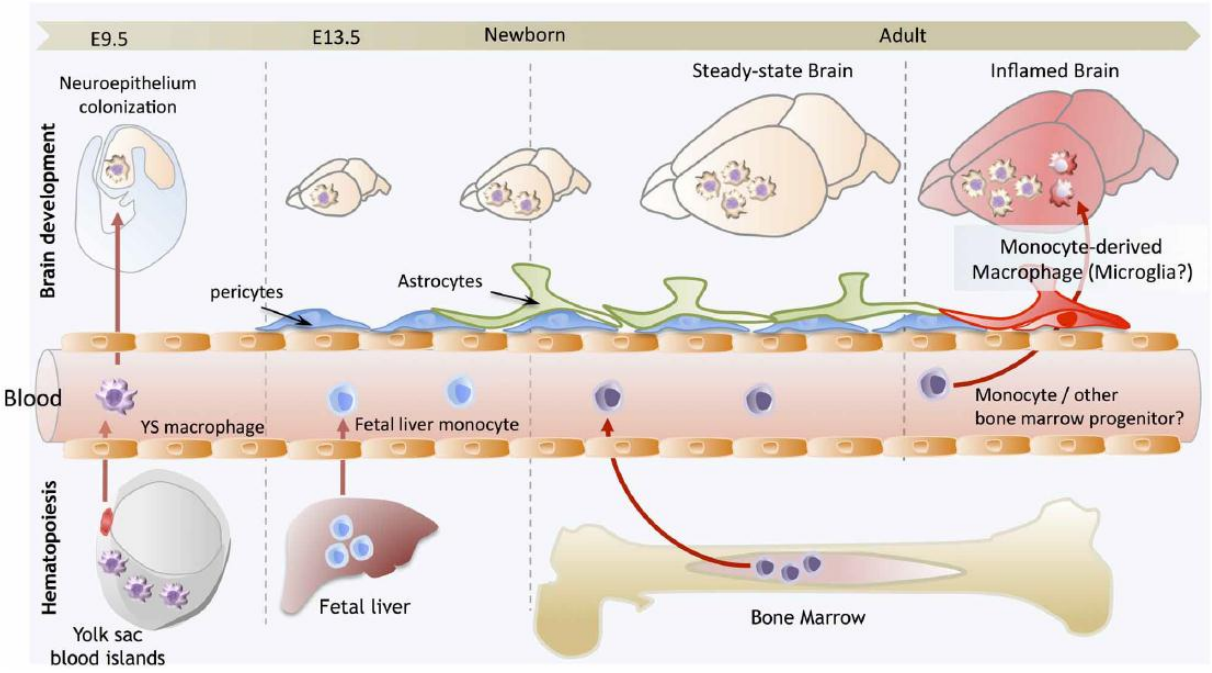

Figure 3. Microglia in brain development.
Primitive macrophages exit yolk sac and colonize neuroepithelium at E9.5 that give rise to microglia. Blood brain barrier (BBB) starts to form at E13.5, which isolate microglia from the periphery. Microglia keep expanding untill adulthood and under certain pathologies, the BBB is damaged so that monocytes from bone marrow could infiltrate into the brain and supplement the microglial population. Adapted from (Ginhoux et al. 2013)

As immune cells of the CNS, microglial cells not only respond to any kind of pathology by contributing to both innate and adaptive immune responses, they also play important roles in the maintenance of brain homeostasis. According to the morphology and functional state, microglia are divided into “resting microglia” with a ramified appearance and “activated microglia” with an ameboid shape. Numerous investigations have shown that activated microglia have various functions in different pathologies while during the physiological state, until recently, the roles of microglia in healthy brain were still overlooked. Actually these “resting” microglia are not quiescent, they are consistently and actively screening their microenvironment with their motile processes. Once they detect any (even very tiny, like a death of a neuron) damage, they will become or prepare to transform to an “activated” state and will also migrate rapidly to the area of the injury (Hanisch and Kettenmann 2007).

2.2.2 Glioma associated microglia/macrophages (GAMs)

Microglia, along with blood-borne macrophages, contribute as much as 30% to the total glioma mass and are positively associated with histopathological grade, malignancy and invasiveness of gliomas (Morimura et al. 1990; Roggendorf et al. 1996). Despite their cytotoxic and phagocytic function, glioma-associated microglia/brain macrophages (GAMs) promote rather than suppress glioma expansion (Markovic et al. 2005; Zhai et al. 2011). One important tumor-supportive mechanism is the up-regulation of MT1-MMP in GAMs by glioma-derived soluble factor(s) that activate the TLR adaptor molecule myeloid differentiation primary response gene (88) (MyD88) and p38 mitogen activated protein kinase (MAPK) in microglia. Blocking this pathway has therapeutic benefits in gliomas (Markovic et al. 2009; Markovic et al. 2011). Other mechanisms of GAMs assisted glioma invasion were also demonstrated by several research groups. Yeh et al. reported that microglia released IL-18 which could enhance C6 glioma invasion through NO/cGMP pathway (Yeh et al. 2012). A study by Wesolowska et al. showed that TGF-β1 which originates from microglia is crucial for the promotion of glioma invasion (Wesolowska et al. 2008). Another study by Jacobs showed gliomas induce microglial MMP-9 expression for tumor invasion, and this could be inhibited by treatment with propentofylline (Jacobs et al. 2012). More interestingly, microglia were also found to

interact with the minor population of gliomas, glioma stem-like cells. A study by Ye et al. demostrated microglial/macrophage derived TGF-β1 enhaces the invasiveness of CD133+ gliomas (glioma stem-like cells) by up regulation of MMP-9 in these cells (Ye et al. 2012). Taken together, most of the evidences supports GAMs as glioma promoting populations, and interfering with GAMs may have potential threrapeutic benefits for gliomas.

2.3 Toll-like receptors

2.3.1 Toll-like receptor signaling

TLRs belong to the superfamily of pattern recognition receptors (PRRs) that mediate responses in innate immune cells by recognizing invading pathogens and activating inflammatory pathways. However, when the *Toll* gene was first identified by Anderson et al. in 1985, this publication only demonstrated that it is important for the dorso-ventral patterning of the developing embryo of Drosophila (Anderson et al. 1985). Until 1989, when Charles Janeway predicted that PRRs may recognize microbial products and initiate immune response, TLRs have been increasingly investigated in the context of immunity (Janeway 1989). One of the milestone findings of TLRs in innate immunity was in 1996, when Bruno Lemaitre, a member from Jules Hoffman's lab, found activation of *Toll* could regulate Drosomycin, the antifungal peptide in *Drosophila* (Lemaitre et al. 1996). Later in 1998, Beutler and his associates discovered *lps* gene, which is responsible for the defective response of mouse strain CH3/HeJ to bacterial endotoxin, encoded a murine member of the TLR family. This provided the first evidence that TLRs recognize microbial products (LPS) such as PPRs (Poltorak et al. 1998). These two key findings by Hoffmann and Beutler led them to win the Nobel Prize for Physiology or Medicine in 2011.

TLRs are type I trans-membrane proteins, characterized by an extracellular domain of leucine-rich repeats and an intracellular Toll/interleukin-1 receptor domain. A total of 13 mammalian TLR orthologs (11 in humans and 13 in mice) have been described so far

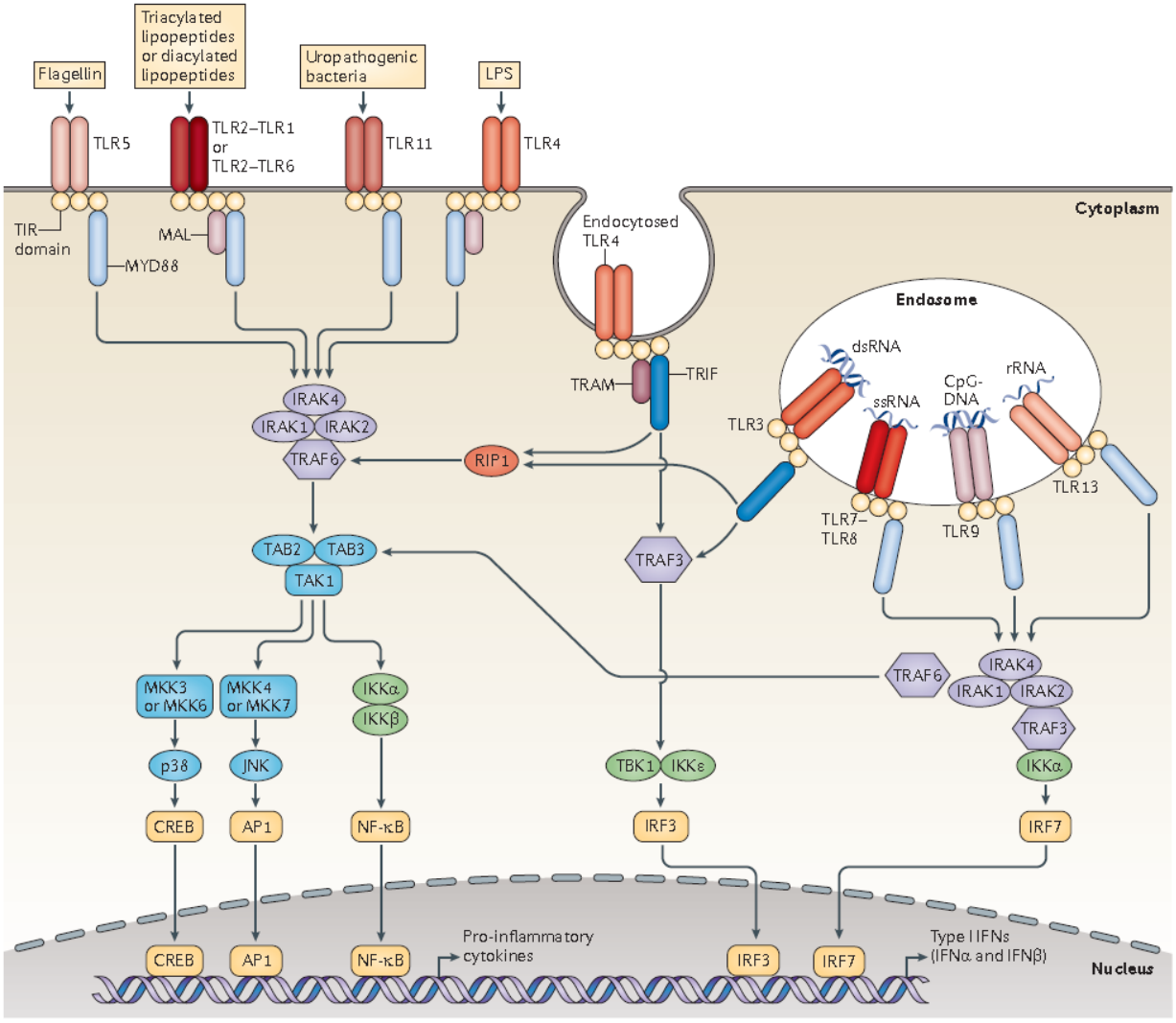

Figure 4. Toll like receptor signaling pathway.
TLRs locate both on plasma membrane and intracellularly. TLR signaling is initiated by binding of ligands, following this, adaptor molecules such as MyD88, MYD88-adaptor-like protein (MAL), or TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) were recruited and downstream signaling was activated. Two main transcription factors that are activated downstream of TLR signaling are nuclear factor-κB (NF-κB) and the interferon-regulatory factors (IRFs). These activations lead to the pro-inflammatory cytokines release. Adapted from (O'Neill et al. 2013)

(Takeda et al. 2003). TLRs 1, 2, 4, 5 and 6 localize to the plasma membrane while TLRs 3, 7, 8 and 9 are found in the intracellular compartments of endosomes and lysosomes. TLRs form dimers to initiate their signaling, either by forming homodimers with themselves or heterodimers with other TLR subtypes (Farhat et al. 2008). While most of the TLRs function as homodimers, TLR2 can heterodimerize with either TLR1 or TLR6.

TLRs may also need co-receptors for full ligand sensitivity, such as in the case of TLR4 binding of LPS, which requires MD-2 (Shimazu et al. 1999) and CD14 (Regen et al. 2011). After the binding of different ligands, TLRs form dimers and recruit adaptor proteins such as myeloid differentiation factor 88 (MyD88). While most of the TLRs are activated in a MyD88 dependent way, TLR3 transduces its signals mainly through the MyD88-independent pathway and TLR4 could have both possibilities. Activation of TLRs may trigger multiple cellular phenomena like nuclear factor-κB (NF-κB), MAPKs and interferon regulatory factors (IRFs). These activations may induce pro-inflammatory cytokine release (Figure 4).

2.3.2 Exogenous and endogenous Toll-like receptors ligands

TLRs are first identified by specifically detecting components from evading pathogens, which are referred to as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov 2002), the exogenous mediators of TLRs include lipoproteins, lipids, proteins and nucleic acids from all kinds of microbes like bacteria, viruses and fungi. One of the classic examples is lipopolysaccharide (LPS), derived from outer membrane of gram-negative bacteria, which can activate TLR4 on macrophages or microglia and massively induce pro-inflammatory cytokines release. Other best characterized microbial ligands of different subtypes of TLRs are as follows: bacterial lipoproteins and lipotechoic acid stimulate TLR1, TLR2 and TLR6; double-stranded RNA stimulate TLR3; RNA bacterial flagellin stimulate TLR5; single-stranded RNA stimulate TLR7 and TLR8; unmethylated CpG motifs present in DANN stimulate TLR9 (Kawai and Akira 2010) (Figure 4).

Besides infection, sterile inflammation may also generate immune responses. Thus PAMPs may not cover all the mediators that trigger the pattern recognition receptors of the innate immune system, in particular, Toll-like receptors. Damage-associated molecular patterns (DAMPs) were then proposed to describe all kinds of molecules signaling the threat of either infection or injury to the organism, which includes PAMPs (Non-self) and endogenous mediators derived upon tissue injury or tumor growth (Self) (Figure 5). For example, during the injury of the CNS, Heat shoct protein 60 (HSP60) is

released by the dying CNS cells which activate microglia through a MyD88- and TLR4-dependent pathway (Lehnardt et al. 2008). High mobility group box 1 (HMGB1) released by necrotic tumors was reported to serve as a signal of TLR2 on dendritic cells after immune therapies (Curtin et al. 2009). Interestingly, let-7, a miRNA up-regulated in Alzheimer's patients, triggers TLR7 on neurons for neurodegeneration (Lehmann et al. 2012). However, the crystal structures of TLR-endogenous ligand complexes have not been deeply investigated so far (Piccinini and Midwood 2010).

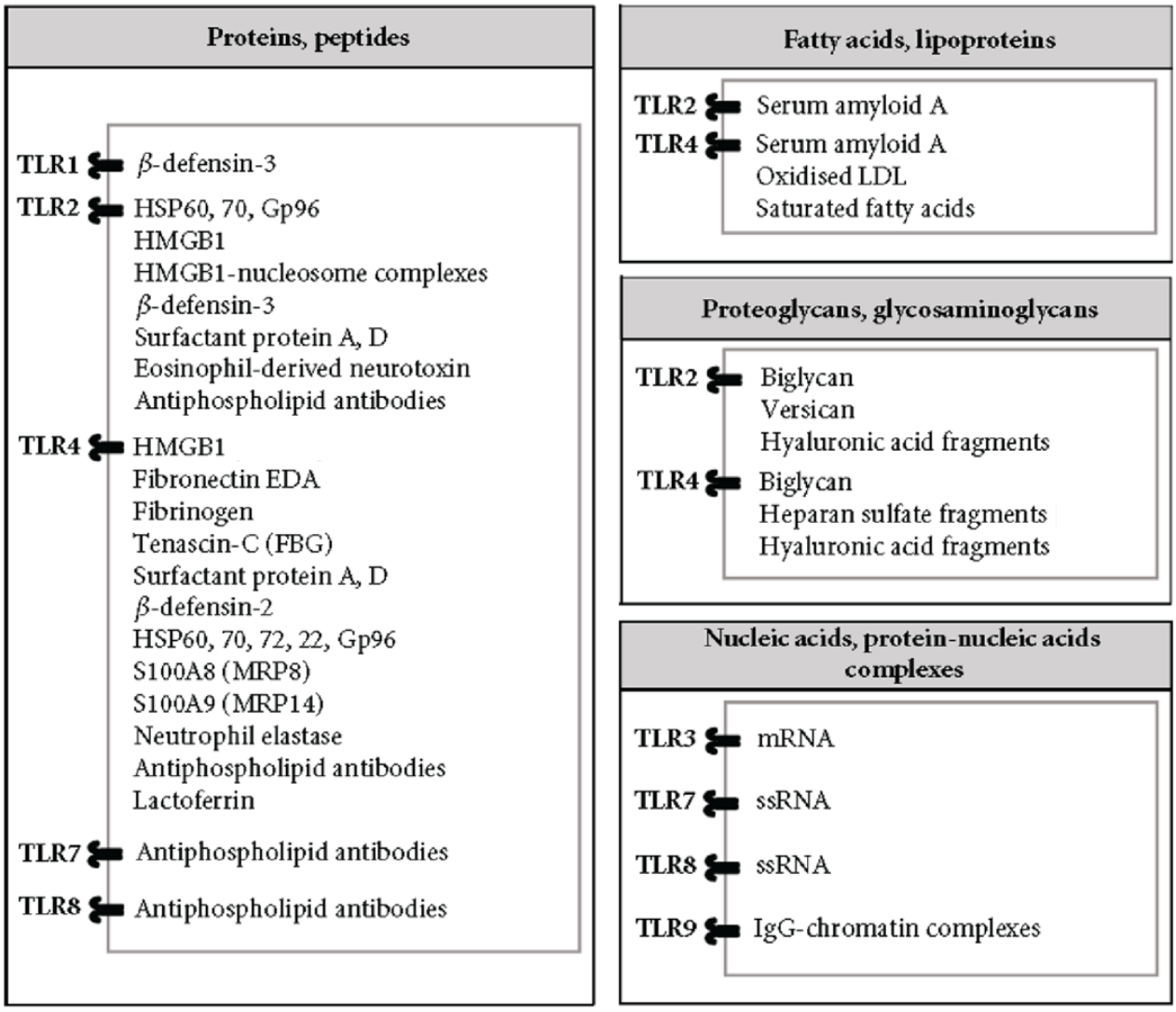

Figure 5. Endogenous ligands of TLRs.
TLRs are activated by damage-associated molecular patterns (DAMPs). Known endogenous TLR mediators are listed based on their biochemical nature. Adapted from (Piccinini and Midwood 2010)

2.3.3 The structure and role of Versican

Versican, also known as CSPG2, is a member of the large chondroitin sulfate proteoglycans that belong to extracellular matrix components. Due to RNA splicing, there are at least 4 isoforms identified so far, V0, V1, V2, V3, respectively (Naso et al. 1994). Sequence analyses revealed different isoforms contains different glycosaminoglycan (GAG) domains. V0, the largest isoform contains two alternatively spliced GAG attachment domains designated as GAG-α and GAG-β, whereas V1 only contains GAG-β and V2 comprises GAG-α only. V3 consists only of the globular domains. Versican is able to regulate cell proliferation, migration, adhesion through direct or indirect interaction with cells and molecules (Figure 6).

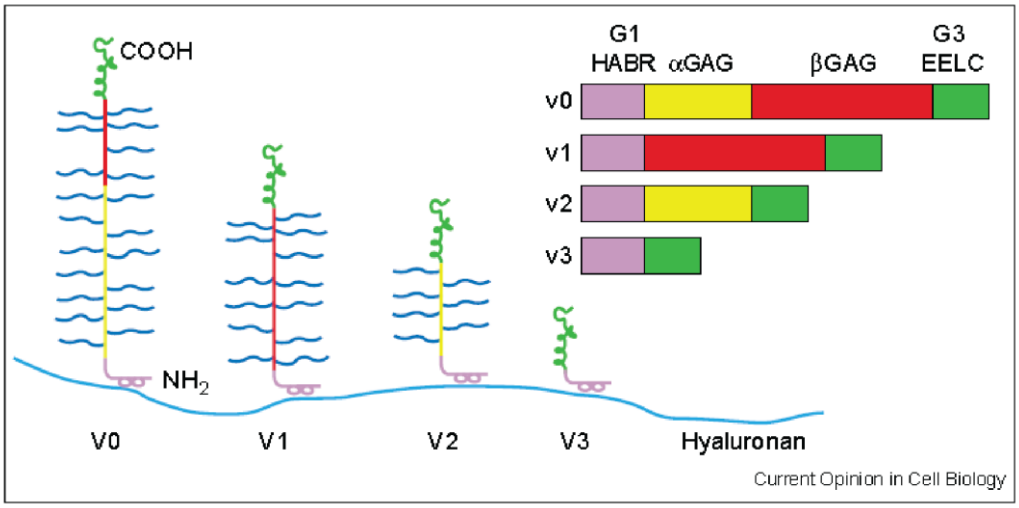

Figure 6. Structures of versican isoforms.
All isoforms interact with hyaluronan and thus are capable of forming different sized versican–hyaluronan aggregates, which in turn determines, in part, tissue volume. Different colors denote specific domains in the gene and in the protein product. Purple = hyaluronan binding region (HABR); yellow = the α GAG exon and protein product; red = β GAG exon and the protein product; green = two epidermal growth factor repeats (EE), a lectin bindingdomain (L) and a complement regulatory region. The glycosaminoglycan chains are shown in blue. Adapted from (Wight 2002)

Versican is highly expressed in tissue compartments undergoing active cell proliferation and migration, such as smooth muscle tissues and cartilage. V2 is the abundant isoform which accumulates at the nodes of Ranvier in the adult central nervous system and it is expressed mainly by oligodentrocyte lineage cells after injury (Asher et al. 2002; Schmalfeldt et al. 1998). Versican isoforms showed distinct functions in the brain. V1

induces neuronal differentiation and promotes neurite outgrowth while V2 is a potent inhibitor of axonal growth (Schmalfeldt et al. 2000; Wu et al. 2004). It has been also found that versican expression is elevated in several cancers including brain, lung, breast and ovarian cancers (Kim et al. 2009; Paulus et al. 1996; Ricciardelli et al. 2002). Interestingly, besides its role in cell proliferation, migration and adhesion, versican V1 was also reported as an endogenous ligand of TLR2 on macrophages. Cancer cells released versican V1 to activate macrophage TLR2 to induce cytokines release for tumor invasion and metastasis (Kim et al. 2009; Li et al. 2013).

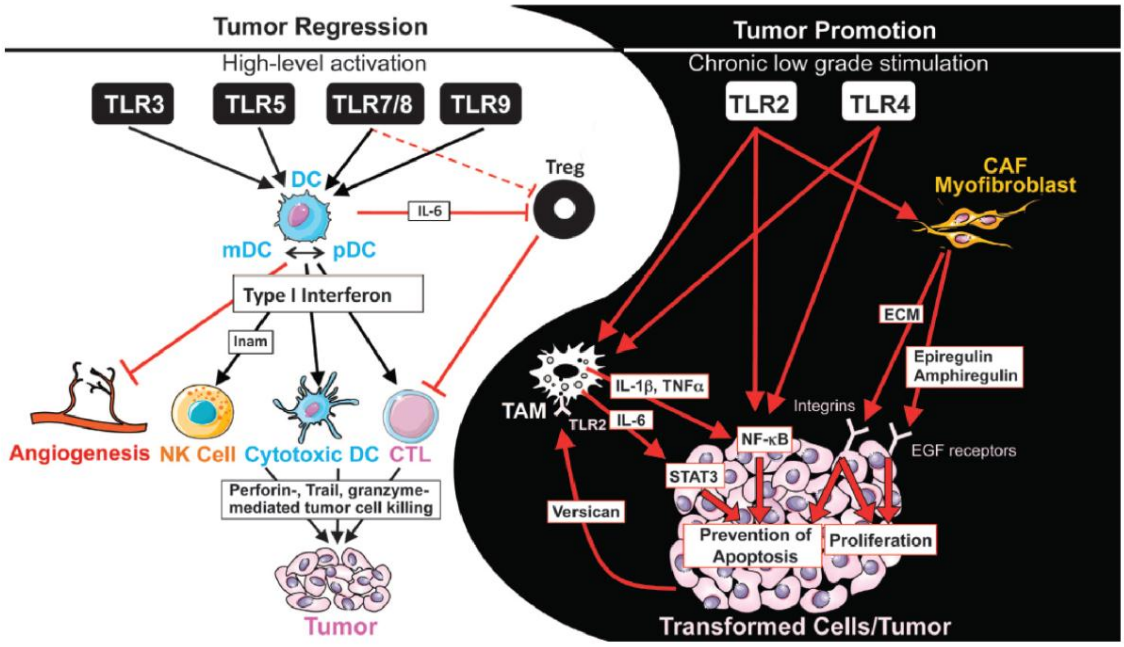

Figure 7. Yin and Yang of TLRs in cancer.
High level activation of TLRs may achieve antitumor effects by converting immune tolerance into antitumor immunity. Chronic low grade stimulation of TLRs by endogenous ligands may lead to tumor-promoting inflammation and inhibition of tumor apoptosis. Adapted from (Pradere et al. 2013)

2.3.4 Toll-like receptors in gliomas

As TLR could serve as neurotoxic as well as neuroprotective mediators in the CNS (Hanisch et al. 2008), TLRs in gliomas could also be a double-edged sword. Activation of TLRs in tumors may have diverse effects on tumor survival, as shown in Figure 7,

TLR mediated tumor regression involves high level activation, while upon chronic low grade stimulations by endogenous ligands released from cancers, TLR activation may induce tumor-promoting inflammation and prevention of tumor apoptosis (Pradere et al. 2013). TLR expression in gliomas have not been systematically investigated, a study from El Andaloussi et al. showed a murine glioma cell line GL261 expresses TLR1 to TLR9 except TLR6 and 2 human glioma cells U87 and U251 express TLR1, TLR2, TLR4, TLR5, TLR7, TLR8 and TLR9 at the mRNA level by RT-PCR (El Andaloussi et al. 2006). Meng et al. showed TLR9 expression was detected at variable levels in 37 primary human GBM samples by qPCR while in 3 human glioma cell lines, TLR9 was relative low (Meng et al. 2008). TLR9 is the most well studied subtype of all TLRs in gliomas, it has been demonstrated that TLR9 expression in glioma tissues is correlated with malignancy of glioma by tissue microarray, TLR9 expression also correlates to glioma progression and the prognosis of GBM patients (Wang et al. 2010). Additionally, by intratumoral injection of various TLR agonists to mice bearing gliomas, only the TLR9 ligand, CpG-oligonucleotides was most effective at inhibiting glioma growth *in vivo,* and this effect was dependent on the TLR9 expression on nontumor cells (Grauer et al. 2008).

2.4 Matrix metalloproteinases (MMPs)

2.4.1 The structure of MMPs

The MMPs comprise a family of zinc-dependent endopeptidases participating in ECM-degradation. So far at least 23 human MMPs have been identified, MMPs were previously grouped into collagenases, gelatinases, stromelysins and matrilysins on the basis of their specificity for ECM components, since the list of MMPs substrates is growing, MMPs are now classified according to their structures: 5 secreted and 3 membrane-type MMPs (Kessenbrock et al. 2010) (Figure 8). MMPs are synthesized in an inactive form (Pro-MMP), and their expression and activities could be regulated by cytokines, hormones, as well as other MMPs and MMP inhibitors. A classic example of MMP activation is MMP2, it needs both MT1-MMP and Tissue inhibitor of

metalloproteinase (TIMP2) to form a trimolecular complex to form a mature MMP2 (Figure 9).

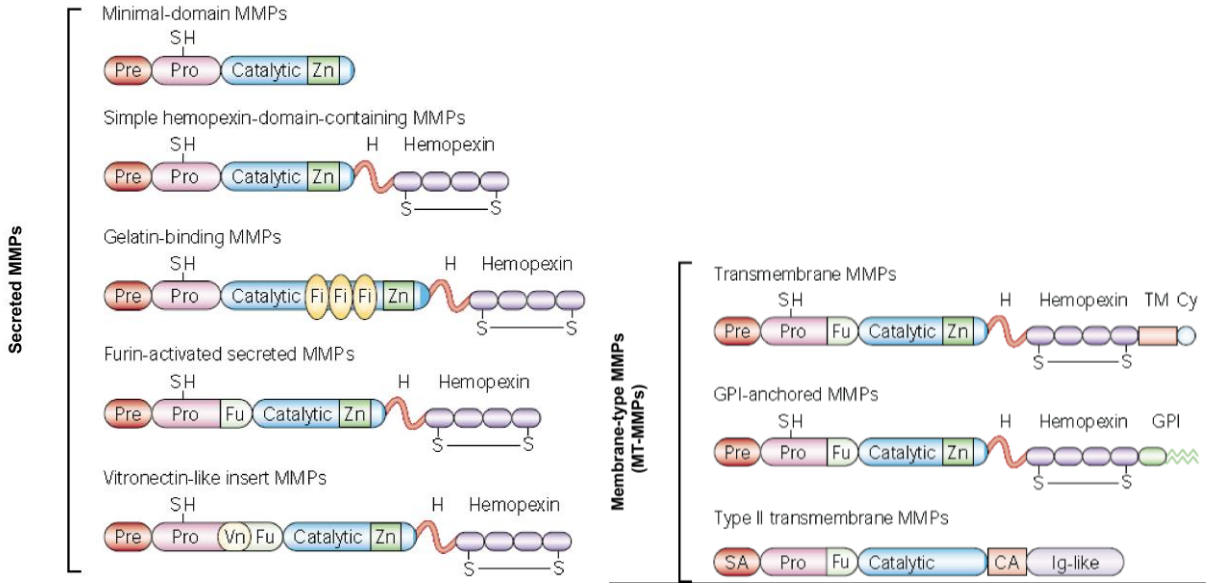

Figure 8. MMP composition and classification.
MMPs comprised of different subdomains, all of them have a similar domain in common which contains three regions: an amino-terminal signal sequence (Pre) to be cleaved by the signal peptidase during entry into the endoplasmic reticulum, a pro-domain (Pro) containing a thiol-group (-SH) and a furin cleavage site, and the catalytic domain with a zinc-binding site (Zn^{2+}). MMPs can be principally divided into secreted (MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -19, -20, -21, -22, -27, -28) and membrane-anchored proteinases (MMP-14, -15, -16, -17, -23, -24, -25). Adapted and modified from (Sternlicht and Werb 2001)

2.4.2 The role of MMPs in physiology and cancer

Physiologically, MMPs play crucial roles in tissue remodeling and organ development (Page-McCaw et al. 2007). They are excreted by a number of cell types including macrophages, microglia, fibroblasts, osteoblasts, endothelial cells and, neutrophils. Expression and activity of MMPs are up-regulated in almost every solid cancer. Basic and clinical data supported the fact that MMPs are a positive factor in cancer progression and metastasis. Malignant gliomas are extremely invasive tumors in the CNS, and MMPs are maybe responsible for this highly invasive behavior. It has been shown that MMP-2, MMP-9 and MT1-MMP are the most predominant MMPs expressed in malignant gliomas (Forsyth et al. 1999), their active form can degrade ECM components for tumor invasion. Besides activation of MMP-2, MT1-MMP also involves in

tumor angiogenesis, it can degrade the fibrin matrix that surrounds newly formed vessels around tumor tissue so that endothelial cells invade further into the tumor core (Hiraoka et al. 1998), MT1-MMP and MMP9 deficient mice showed impaired angiogenesis during development which also indicates their important roles in vascular formation (Egeblad and Werb 2002). MMP-9 levels were also significantly correlated with the histological grade of malignancy in gliomas (Rao et al. 1993). Recent evidence indicated that MMP-9 has a distinct role in tumor angiogenesis mainly in regulating the bioactivity of vascular endothelial growth factor (VEGF), the most promising factor in interfering with tumor angiogenesis and thus a new therapeutic target (Du et al. 2008; Lee et al. 2005).

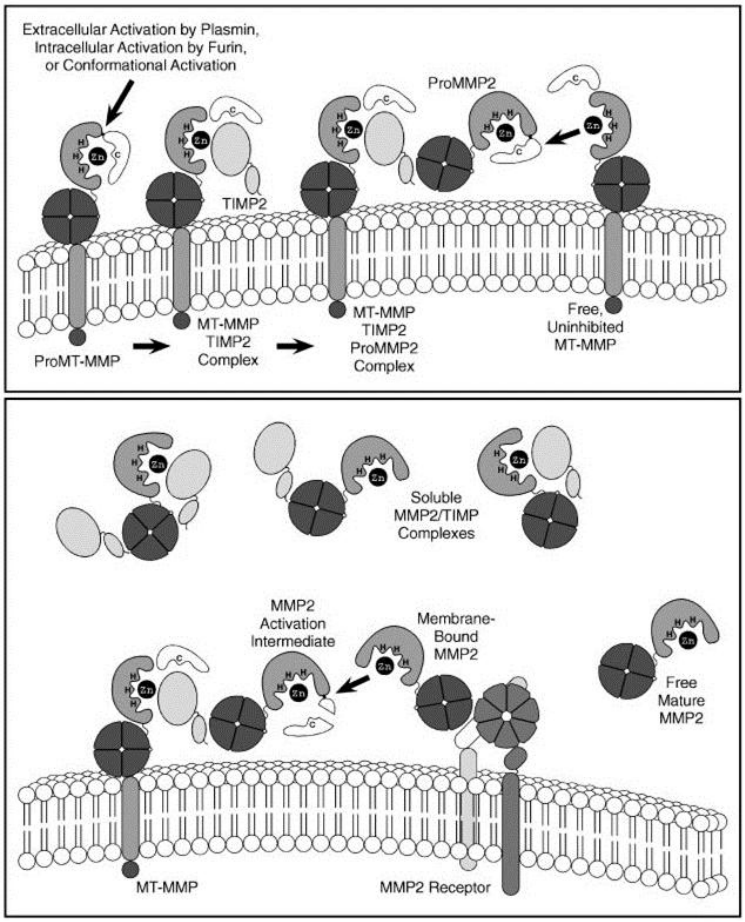

Figure 9. Activation cascade of MMP-2.
After the activation of MT-MMP, it is then inhibited by TIMP2 and the hemopexin domain of ProMMP2 to the C-terminal of TIMP2 form a trimolecular complex. The remaining portion of the propeptide is removed by a separate MMP2 molecule at the cell surface to yield fully active mature MMP2. Adapted and modified from (Sternlicht and Werb 2001)

2.4.3 Link between TLRs and MMPs

The signaling pathways that lead to the expression of MMPs are still not fully understood, it has been shown that mitogen-activated protein kinases (MAPK) and Nuclear factor-kB (NFkB) transcription factors may be involved in MMP expression in some cell types (Markovic et al. 2009; Nakanishi and Toi 2005). Inhibition of NFkB reduces MMP-1, MMP-3 and MMP-9 production by vascular smooth muscle cells (Bond et al. 2001). TLRs are downstream receptors of MAPK and NFkB signaling pathways, it was reported that MMP-9 expression is related to TLR signaling, e.g. in influenza infection (Bradley et al. 2012), we have also shown that glioma released soluble factors exploit microglia to induce MT1-MMP for tumor invasion through TLR2-p38 MAPK signaling pathways (Markovic et al. 2009). Inhibition of this pathway reduced glioma associated microglial MT1-MMP expression and may have potential therapeutic benefits in glioma treatment (Markovic et al. 2011).

2.5 Minocycline

Minocycline is a tetracycline based antibiotic and an FDA approved drug to treat chronic inflammatory conditions such as rosacea, skin and respiratory tract infections (Yong et al. 2004). It is a small, highly lipophilic molecule (495kDa), readily absorbed by the gut after oral intake and is capable of crossing the intact blood-brain barrier. Apart from bactericidal effects, it has been shown that minocycline demonstrated neurorestorative as well as neuroprotective properties in various models of neurodegenerative diseases. In a double-blind, randomized clinical study, minocycline treatment showed improvement in negative symptoms and executive functioning of early-phase schizophrenia patients (Levkovitz et al. 2010). Because of its anti-inflammatory properties, minocycline can alleviate the severity of symptoms of intra-cerebral ischemia and hemorrhage (Hess and Fagan 2010; Rosenberg et al. 2007), spinal-cord injury, Parkinson's disease and Huntington's disease (Yong et al. 2004). We and others demonstrated that minocycline could also inhibited glioma growth and invasion alone or together with other therapies in different animal models (Liu et al. 2013; Liu et al. 2011; Markovic et al. 2011).

2.6 Aim of the dissertation

Gliomas are primary malignant brain tumors with poor prognosis. The mechanisms of glioma proliferation, invasion and angiogenesis have been deeply investigated. However, crosstalk among gliomas and other brain resident cells (glial cells) are not well understood. Microglial cells are brain immune cells, which are screening the brain environment in healthy conditions. During tumor growth, microglia are activated and accumulate around and within the tumor. They are actively communicating with tumor cells. Such interactions may lead to the proliferation, neo-vascularization, invasion and progression of gliomas. Our previous work showed that gliomas released soluble factor(s) that induce an induction of MT1-MMP expression in glioma associated microlgia via TLR2-P38 MAPK signaling pathways for glioma expansion (Markovic et al. 2009; Vinnakota et al. 2013).

The aim of this study is to investigate the roles of microglial TLRs in glioma progression.

Specific questions are:

1. How are TLRs expressed and regulated in microglia and gliomas *in vitro* and *in vivo*?
2. Does the expression of TLRs in gliomas correlate with the malignance of gliomas and survival rates of patients?
3. Do deficiencies in TLRs affect glioma growth *in vivo*?
4. What kinds of soluble factors are released by gliomas (GL261 murine model)
5. Among these factors, what control microglial MT1-MMP expression?
6. How about MMP-9 in GAMs? Is it expressed and up regulated by GAMs?
7. If gliomas could also induce microglial MMP-9 expression, what is the mechanism behind it?
8. Does a TLR2 neutralizing antibody inhibit microglial MT1-MMP expression? Could it be used as a potential drug for treating mouse gliomas?

3. Materials and Methods

3.1 Materials

3.1.1 Devices and Equipment

Product	Company
Balances BL610	Sartorius (Göttingen, Germany)
Centrifuge 5403, 5417R, 5810R	Eppendorf (Hamburg, Germany)
Confocal microscope TSC SPE	Leica Microsystems (Wetzlar, Germany)
Counting Chamber Neubauer	LaborOptic (Bad Homburg, Germany)
Cryostat CM 3050S	Leica Microsystems (Wetzlar, Germany)
Flow Cytometry LSR Fortessa 5Laser	(BDBiosciences,Erembodegem, Belgium)
Gel documentation G-Box	Syngene (Cambridge, United Kingdom)
Gel electrophoresis device	Biometra (Göttingen, Germany)
Cell culture incubater Heracell	Heraeus Instruments (Hanau, Germany)
Microliter syringe 7001N	Hamilton (Bonaduz, Switzerland)
Microplate reader Infinite M200	Tecan (Männedorf, Switzerland)
Microtome AM2000R	Leica Microsystems (Wetzlar, Germany)
Perfusion system WAS02	DITEL (Prague, Czech Republic)
pH meter CG840	Schrott (Mainz)
Pipette boy Accu-jet	Brand (Wertheim, Germany)
SDS-PAGE Protean II electrophoresis unit	Bio-Rad Laboratories (Munich, Germany)
Spectrophotometer Nanodrop ND-1000	Thermo Scientific (Schwerte, Germany)
Stereotactic head holder	David Kopf Instruments (Tujunga, USA)
Sterile hood	Heraeus Instruments (Hanau, Germany)
Thermocycler T3000 (RT-PCR)	Biometra, (Göttingen, Germany)
Thermocycler FAST 7500 Real-Time PCR System (qPCR)	Applied Biotsystems (Foster City, USA)
Weighing scales	Sartorius (Göttingen, Germany)
Vibratome VT1000S	Leica Microsystems (Wetzlar, Germany)
Vortex	Janke & Kunkel, Germany
Water bath 1008	GFL (Brugwedel, Germany)

Table 2. Devices and Equipments

3.1.2 Reagents and Chemicals

Reagents/Chemicals	Company
Acetic Acid	Carl Roth (Karlsruhe, Germany)
Acrylamide/bis-Acrylamide 30%	Sigma-Aldrich (Munich, Germany)
Agarose	Peglab (Erlangen, Germany)
Ammonium persulphate (APS)	Merck (Darmstadt, Germany)
Aqua Poly/mount	Poly sciences Europe (Eppelheim,Germany)
Beta-Mercaptoethanol	Sigma-Aldrich (Munich, Germany)
Brilliant Blue R®	Sigma-Aldrich (Munich, Germany)
Bovine serum albumin (BSA)	Carl Roth (Karlsruhe, Germany)
Clodronate-liposomes©	Clodronate Liposomes Foundation (CLF) (Amsterdam, The Netherlands)
cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets	Roche Diagnostics (Mannheim, Germany)
dNTP (deoxyribonycleoside triphospahtes)	Invitrogen (Karlsruhe, Germany)
Donkey serum	Sigma-Aldrich (Munich, Germany)
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen (Darmstadt, Germany)
ECL Western Blotting Substrate	Thermo Fisher Scientific (Rockford, USA)
Enhanced Green Fluorescent Protein (EGFP)	Clontech, Heidelberg, Germany
Ethylene glycol	Carl Roth (Karlsruhe, Germany)
Ethidium Bromide (EtBr)	Carl Roth (Karlsruhe, Germany)
Ethanol	Carl Roth (Karlsruhe, Germany)
FastStart SYBR green master	Roche Diagnostics (Mannheim, Germany)
Fetal calf serum (FCS)	Invitrogen (Darmstadt, Germany)
Gel blotting paper	Carl Roth (Karlsruhe, Germany)
Gelatin from porcine skin, type A	Sigma-Aldrich (Munich, Germany)
Glycine	Merck (Darmstadt, Germany)
Glucose	Carl Roth (Karlsruhe, Germany)
Glycerol	Sigma-Aldrich (Munich, Germany)
Hank's balanced salt solution (HBSS)	Invitrogen (Darmstadt, Germany)
Hybond-P PVDF membrane	GE Healthcare (Munich, Germany)
Isopropanol	Carl Roth (Karlsruhe, Germany)
L-glutamine	Biochrom AG (Berlin, Germany)
Lipofectamine 2000 transfection reagent	Invitrogen (California, USA)
Lipopolysaccharide (LPS)	Alexis Biochemicals (Lausen, Switzerland)

Magnesium Chloride (MgCl2)	Invitrogen (Darmstadt, Germany)
Minocycline hydrochloride	Sigma-Aldrich (Munich, Germany)
Methanol	Carl Roth (Karlsruhe, Germany)
Nacoren (Pentobarbital)	Merial (Hallbergmoos, Germany)
Oligo(dT)12-18 primers	Invitrogen (Karlsruhe, Germany)
Pam3Csk4	Cayla- InvivoGen (Toulouse, France)
PageRuler™ Prestained Protein ladder	Fermentas (Germany)
Paraformaldehyde (PFA)	Merck (Darmstadt, Germany)
PCR High Fidelity Supermix®	Invitrogen (Karlsruhe, Germany)
Penicillin/Streptomycin	Biochrom AG (Berlin, Germany)
Phosphate Buffered Saline (DPBS)	I Invitrogen (Darmstadt, Germany)
Restore™ Plus Western blot stripping buffer	Pierce (Thermo Fischer Scientific), Bonn, Germany
RIPA buffer	Sigma-Aldrich (Munich, Germany)
Scrambled non-targeted siRNA	Dharmacon (Chicago, USA)
siRNA versican (on target plus SMARTpool)	Dharmacon (Chicago, USA)
siRNA GAPDH (SMARTpool)	Dharmacon (Chicago, USA)
Sodium chloride (NaCl)	Carl Roth (Karlsruhe, Germany)
Sodium dodecyl Sulphate (SDS)	Sigma-Aldrich (Munich, Germany)
Sucrose	Merck (Darmstadt, Germany)
Superscript II reverse transcriptase	Invitrogen (Karlsruhe, Germany)
SuperSignal® west	Pierce (Thermo Fischer Scientific) (Bonn, Germany)
Sutures	Johnson&Johnson (Langhorne, USA)
TEMED (N, N, N', N' Tetramethyl-Ethylene Diamine)	Amresco (USA)
Tris base	Carl Roth (Karlsruhe, Germany)
Triton X-100	Merck (Darmstadt, Germany)
Trypsin/EDTA	Biochrom AG (Berlin, Germany)
Tween-20	Merck (Darmstadt, Germany)

Table 3. Reagents and Chemicals

3.1.3 Commercial Kits

Kits	Company
BCA Protein assay	Thermo Fisher Scientific (Rockford, USA)
InviTrap® Spin Universal RNA mini kit	Invitek (Stratec) (Berlin, Germany)
Mouse Total MMP-9 Quantikine ELISA Kit	R&D Systems (Abingdon, United Kingdom)
RNease mini RNA isolation kit	Qiagen (Hilden, Germany)

Fast Western Blot Kit, SuperSignal West Pico	Thermo Fisher Scientific (Rockford, USA)
SuperScript® III cDNA synthesis kit	Invitrogen (Darmstadt, Germany)
pSUPER puro RNAi system	Oligoengine, Seattle, USA

Table 4. Commercial Kits

3.1.4 Plastic ware and other tools

Product name	Company
96-well skirted PCR plates	VWR International (Darmstadt, Germany)
96-, 24-, 12- and 6-well cell culture plates	BD Biosciences (Heidelberg, Germany)
BD Falcon™ Cell culture inserts for 6-well plates (0.4 µm pores)	Becton Dickinson (Heidelberg,Germany)
Cuvettes for measuring protein	Eppendorf (Hamburg, Germany)
Double-edged razor blades	Thermo Fischer Scientific (Walldorf, Germany)
Falcon™ tubes (15 ml, 50 ml)	Becton Dickinson (Heidelberg, Germany)
Menzel glass cover slips (24x40, 24x50, 24x60 mm)	Thermo Fischer Scientific, Walldorf, Germany
Menzel SuperFrost plus microscopic slides	Thermo Fischer Scientific, Walldorf, Germany
MicroAmp™ optical adhesive films	Applied Biosystems (Foster City, USA)
Minisart filter units (0.2, 0.45 µm)	Sartorius Stedim Biotech (Göttingen, Germany)
Parafilm	Pechiney Plastic Packaging (Chicago, USA)
Saran wrap (transparent foil)	Dow Chemical (USA)
Secure-Seal™ spacers for cell culture	Invitrogen, USA
Stericup® and Steritop® vacuum filtration and storage units	Millipore-Merck (Darmstadt, Germany)
Tissue culture dishes (60 mm, 30 mm)	BD Biosciences (Heidelberg,Germany)
Tissue culture flasks (25 cm^2, 75 cm^2)	BD Biosciences (Heidelberg,Germany)

Table 5. Plastic ware and other tools

3.1.5 Primary antibodies

3.1.5.1 Primary antibodies for Western Blot (WB) and Immunohistochemistry (IHC*)*

Epitope	origin	Dilution	Provider
Mouse versican V0/V1	Rabbit	1:1000 (WB) 1:250 (IHC)	Prof. Dieter R. Zimmermann (University of Zurich, Switzerland)
Mouse versican V0/V2	Rabbit	1:1000 (WB) 1:250 (IHC)	
Human versican V0/V1	Rabbit	1:1000 (WB) 1:250 (IHC)	
Human versican V0/V2	Rabbit	1:1000 (WB) 1:250 (IHC)	
MMP-9 (ab38898)	Rabbit	1:1000 (WB) 1:200 (IHC)	Abcam (Cambridge, UK)
MMP-14 (ab51074)	Rabbit	1:1000 (WB) 1:200 (IHC)	Abcam (Cambridge, UK)
Iba1 (ab5076)	Goat	1:500 (IHC)	Abcam (Cambridge, UK)
Iba1	Rabbit	1:250 (IHC)	Wako Pure Chemicals (Japan)
β-Actin (HRP conjugated)		1:25,000 (WB)	Sigma-Aldrich (Munich, Germany)

Table 6. Primary antibodies for WB and IHC

3.1.5.2 Primary Antibodies for flow cytometry

Product name	Dilution	Provider
Anti-mouse GLAST APC	1:11	Miltenyi Biotec (Bergisch Gladbach, Germany)
Anti-mouse TLR6 PE	1:60	R&D Systems (Abingdon, United Kingdom)
Anti-mouse CD14 PE	1:100	Biolegend (San Diego, USA)
Anti-mouse TLR7 PE	1:200	
Anti-mouse CD11b PE-Cy7	1:400	eBioscience (San Diego, USA)
Anti-human CD11b FITC	1:200	
Anti-mouse CD45 eFlour®450	1:200	
Anti-mouse Ly-6G FITC	1:600	
Anti-mouse Ly-6C PreCP-Cy5.5	1:200	
Anti-mouse TLR1 PE	1:200	
Anti-mouse TLR2 PE	1:200	
Anti-mouse TLR4 PE	1:200	
Anti-mouse TLR9 PE	1:200	

Table 7. Primary antibodies for flow cytometry

3.1.5.3 Isotype controls for flow cytometry antibodies

Product name	Dilution	Provider
Rat IgG2a K Isotype Control PE (TLR1,TLR6,CD14)	As primary antibody	eBioscience (San Diego, USA)
Rat IgG2b K Isotype Control PE (TLR2)		
Mouse IgG1 K Isotype Control PE (TLR4)		
Rat IgG2a K Isotype Control FITC (TLR9)		

Table 8. Isotype controls for flow cytometry antibodies

3.1.6 Secoundary antibodies for WB and IHC

Product name	Dilution	Provider
DyLight-488 conjugated donkey anti-rabbit IgG	1:200	Jackson Immunoresearch Laboratories, USA
Cy3 conjugated donkey anti-rabbit IgG	1:200	Dianova (Hamburg, Germnay)
DyLight-488 conjugated donkey anti-goat IgG	1:200	Dianova (Hamburg, Germnay)
Rhodamine Red conjugated donkey anti-goat IgG	1:200	Dianova (Hamburg, Germnay)
Cy 5-conjugated Streptavidin	1:200	Dianova (Hamburg, Germnay)
DAPI	1:200	Sigma-Aldrich (Munich, Germany)
Biotin SP conjugated donkey anti-rabbit IgG	1:200	Dianova (Hamburg, Germnay)
HRP conjugated anti-mouse IgG	1:2000	Cell signaling (Danvers, USA)
HRP conjugated anti-Rabitt IgG	1:2000	Cell signaling (Danvers, USA)

Table 9. Secondary antibodies for WB and IHC

3.1.7 Buffers

3.1.7.1 Buffers for PCR

Buffer name	Composition
Gel loading buffer	60 mM Tris-HCl pH 7.5, 30 mM sodium acetate, 12 mM EDTA, 60% (w/v) glycerin, 0.12% (w/v) Xylene cyanol blue
50X Tris-Acetate-EDTA (TAE)	242 g Tris-base, 57.1 ml acetic acid, 0. 5 M EDTA, 1 L distilled water, pH 8 For use 50X TAE was diluted in H_2O 1:50

Table 10. Buffer for PCR

3.1.7.2 Buffers for Immunohistochemistry

Buffer name	Composition
Blocking buffer (TBS+)	0.1%Trition X-100, 5% donkey serum in TBS
Paraformaldehyde (PFA) (For perfusion and fixation)	4 g PFA in 1 L aqua distilled water, heated for total dissolution
Sodium Citrate Buffer (pH 6) (For antigen retrieval)	2.94 g tri-sodium citrate dihydrate in1 L aqua distilled water
1X Phosphate Buffered Saline (PBS)	8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4. in 1 L aqua distilled water, pH 7.4
1X Tris Buffered Saline (TBS)	1.21 g Tris, 8.76 g NaCl, dissolve in 1 L aqua distilled water, pH 7.4
Cryoprotection buffer (CPS)	25% glycerol, 25% ethyleaglycol in 0.05M phosphate buffer
30% Sucrose	Sucrose (300g/L) in 0.05M phosphate buffer

Table 11. Buffers for IHC

3.1.7.3 Buffers for Gelatin Zymography

Buffer name	Composition
Gel washing Buffer	25ml Triton X-100 in 975 ml aqua distilled water
10x Gel development Buffer	500 mM Tris-HCl pH 7.8, 2M NaCl, 50 mM $CaCl_2$, 0.2% v/v Brij 35
Coomassie blue staining solution	0.125g Coomassie brilliant blue R-250, 1 ml acetic acid, 45ml ethanol in 54 ml aqua distilled water
Gel destaining solution	125ml ethanol, 50 ml acetic acid in 325 ml aqua distilled water

Table 12. Buffers for Gelatin Zymography

3.1.7.4 Buffers for Western Blot

Buffer name	Composition
Sample Lysis Buffer	10 ml RIPA, 1 tablet cOmplete proteinase inhibitor
5X Laemmli Buffer	1 ml glycerol, 1 g SDS, 6.25 ml Tris HCL 0.5 M pH 6.8, 2.5 ml ß- Mercaptoethanol, 1 ml Bromophenol blue 0.5%
10% Ammonium Persulphate (APS)	100 mg APS in 1 ml aqua distilled water
10% Sodium dodecylsulphate (SDS)	1 g SDS in 10 ml aqua distilled water
1.5 M Tris-Cl resolving gel buffer (4X)	36.3 g Tris, 150 ml aqua distilled water, adjust pH, to 8.8 with I N HCl,

	make up volume to 200 ml
0.5 M Tris-Cl stacking gel buffer (4X)	3 g Tris, 40 ml aqua distilled water, adjust pH to 6.8 with 1 N HCl, make up volume to 50 ml
5X Electrophoresis Buffer	15.1 g Tris base, 72 g glycine, 5 g SDS, 1 L aqua distilled water
1X Wet transfer buffer	6.06 g Tris base, 28.08 g glycine, 400 ml Methanol, make upto 2 L with aqua distilled water
Wash buffer (TBST)	0.1% Tween 20, 1 L TBS
Blocking buffer	5% Bovine serum albumin (BSA) in wash buffer

Table 13. Buffers for WB

3.1.8 SDS-PAGE

3.1.8.1 SDS-PAGE for Western blot

Separating gel (10%)		Stacking gels (5%)	
components	10(ml)l total	components	4(ml) total
ddH2O	4.0	ddH2O	2.2
30% acrylamide	3.3	30% acrylamide	0.67
1.5 M Tris	2.5	0.5 M Tris	1.0
10% SDS	0.1	10% SDS	0.04
10% APS	0.1	10% APS	0.04
TEMED	0.004	TEMED	0.004

Table 14. SDS-PAGE for WB and IHC

3.1.8.2 SDS-PAGE for Gelatin Zymography

Separating gel (8%)		Stacking gels (5%)	
components	10(ml)l total	components	4(ml) total
ddH2O	3.26	ddH2O	2.2
30% acrylamide	2.7	30% acrylamide	0.6
1.5 M Tris	2.5	0.5 M Tris	1.0
10% SDS	0.4	10% SDS	0.04
10% APS	0.2	10% APS	0.04
TEMED	0.004	TEMED	0.004
1% Gelatin	1.5		

Table 15. SDS-PAGE for Gelatin Zymography

3.1.9 Media and Solutions for cell culture/ organotypic brain slices

Fetal calf serum (FCS) was always heat-inactivated in the water bath for 30 min at 60°C before further use.

Name	Composition
Trypsin/DNase	10 mg Trypsin, 0.5 mg DNase / ml PBS
Complete growth medium	10% FCS, 100 U/ml penicillin, 100 µg/ ml streptomycin, 0.2 mM L-glutamine in DMEM
Glioma conditioned medium (GCM) Microglial conditioned medium (MCM) Astrocyte conditioned medium (ACM)	Mouse GL261 cells at 80% confluence were overlaid with complete growth medium for 18-20 h.The GCM was harvested the next day, briefly centrifuged and filtered, aliquoted and stored frozen until usage
L929 conditioned medium	Mouse L929 fibroblast cells at 80% confluence were overlaid with 30 ml complete growth medium. After 2 days conditioned medium was collected, filtered and stored frozen until usage
Medium-1 (for OBSC)	complete growth medium
Medium-2 (for OBSC)	25% heat inactivated FCS, 50 mM sodium bicarbonate, 2% L-glutamine, 255 HBSS, 1µg/ml insulin, 2.46 mg/ml glucose, 0.8 µg/ml Vitamin C, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mM Tris in DMEM

Table 16. Media for cell culture and OBS

3.1.10 Recombinant proteins and neutralizing antibody

Recombinant MMP9	R&D Systems (Abingdon, United Kingdom)
Recombinant HMGB-1	HMGBiotech （Milan, Italy）
Recombinant versican V1	From Dr. Carmela Ricciardelli
Anti-Human/Mouse CD282 (TLR2) Functional Grade Purified	eBioscience (San Diego, USA)
Mouse IgG1 isotype control for anti-TLR2	eBioscience (San Diego, USA)

3.1.11 Software

Software	company
Adobe Illustrator CS6	Adobe systems (Munich, Germany)
Adobe Photoshop CS6	Adobe systems (Munich, Germany)
Image J	NIH (Bethesda, USA)
Leica LAS AF	Leica (Wetzlar, Germany)
Microsoft Office 2010	Microsoft (Berlin, Germany)
Microsoft Windows 7 professional	Microsoft (Berlin, Germany)
SPSS 11.5	SPSS Inc., IBM, Chicago, USA
Stereo Investigator® system	MicroBrightField (Magdeburg, Germany)
Syngene G-Box gel documentation	system Imgen Technologies (Virginia, USA)
Thomson Reuters EndNote X6 ®	Thomson Reuters (Carlsbad, USA)

Table 17. Software

3.2 Methods

3.2.1 Animals and Anesthesia

All *in vitro*, *ex vivo* and *in vivo* experiments were carried out using C57Bl/6 WT mice (Charles River Laboratories, Sulzfeld, Germany) and TLRs 1, 2, 6, 7 and 9 KO mice on a C57Bl/6 back ground. The TLR KO mice were generated by Dr. Shizuo Akira and colleagues from the Osaka University, Japan and obtained from Oriental BioServices Inc., Japan(Hemmi et al. 2002; Hemmi et al. 2000; Takeuchi et al. 1999; Takeuchi et al. 2001; Takeuchi et al. 2002). The mice were bred and maintained in the animal house facilities of the Max Delbrueck Center and Charité University hospital (Berlin, Germany) as per rules of the local governmental institutions (TVV G0343_10). The mice were housed with a 12 h/12 h light-dark cycle and received food and water ad libitum. For all in vivo tumor inoculations, mice were anesthetized by intra-peritoneal (i.p.) injections of Ketamine and Rompun. For perfusions followed by immunohistochemistry, tumor-bearing mice were anesthetized by i.p. injections of Narcoten.

3.2.2 Cell culture

3.2.2.1 Mouse cell culture

Murine GL261 glioma cells (National Cancer Institute, USA) were grown in DMEM with 10% FCS, 200 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Germany).

Microglial cells were prepared from neonatal WT and TLRs 1, 2, 6, 7 and 9 KO mice according to previously established protocols (Markovic et al. 2005; Prinz and Hanisch 1999). Briefly, the brains of new born C57BL/6 mice (P0-P3) were removed and placed in HBSS. Brain meninges, vessels and cerebellum were carefully removed. After 3 times washing with HBSS, the cortical tissues were incubated with the Trypsine/DNase for 2 min. The reaction was stopped by adding complete culture medium. Finally, the cell mixture was, dissociated with a fire-polished pipette and washed twice. Mixed glial cells were then cultured for 9 to 12 days in complete culture medium in 75 cm^2 flasks until cells were confluent. The cultures were washed carefully every 3 days to remove dead cell debris by several replacements of the medium with PBS and strong shaking. After

establishment of the astrocytic monolayer, inspected by morphology under the microscope, the medium was changed to complete DMEM containing 30% L929 conditioned medium and incubated for 3 days. Microglia cells were then separated from the underlying astrocytic cell layer by gentle shaking of the flask for one hour at 37 °C in a shaker-incubator at a speed of 100 rpm. The cells were then seeded in 6-well plates at a density of $1x10^6$ cells/ well. Cultures usually contained more than 95 % microglia cells.

3.2.2.2 Human cell culture

The human glioblastoma cells were derived from human tumor resections which were obtained from glioblastoma patients without any prior clinical history, according to governmental and internal (Charité) rules and regulations (Charité, EA4/098/11).U373 cell line and primary GBM cells were cultured in RPMI 1640 with 10% FCS and antibiotics. GBM 1-4 were then established as primary cultures to exclude non-tumor cells.

All cells mentioned above were maintained in a 37°C incubator with a 5% CO_2 humidified atmosphere.

3.2.3 Generation of EGFP-GL261 and mCherry-GL261 cells

GL261 glioma cells were transfected with the pEGFP-N1 vector for the stable expression of enhanced green fluorescent protein (EGFP; Clonetech, Germany) using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer´s instructions. The pEGFP-N1 vector contains the human cytomegalovirus promoter, which drives high level expression of the EGFP in transfected cells. Using the Genetecin- G418 selection method (600 µg/ml; Gibco, USA) stably transfected clones of GL261 cells were established. Viable cells with bright fluorescence were selected by FACS analysis. These cells were used for glioma inoculation in organotypic brain slices. On the day of injection 90%- 95% of cells were fluorescent.

3.2.4 Versican knockdown with siRNA and shRNA approach

For transient silencing versican expression, GL261 cells were transfected with siRNA versican (on target plus SMARTpool, Dharmacon) and control scrambled non-targeted siRNA with Dharmafect4 (Dharmacon) according the manufacturer’s instructions. After

24 hrs of transfection, cells were seeded into 6-well plate again with same numbers for generating conditioned medium. siGAPDH (control SMARTpool, Dharmacon) was used as a positive control and transfection efficiency was tested by real-time PCR and western blot.

For stable versican knockdown, shRNA was generated with pSUPER.retro system (Oligoengine, seattle) according to the instructions. shRNA sequences are listed below, and empty vector was used as a negative control. Retroviral supernatant to transduce versican genes to GL261 or EGFP GL261 cells were generated by transfections of Phoenix cells as described (Schmitt et al. 2000). Infected cells were selected for 3 days with 2 µg/ml puromycin.

A 5'-GATCCCC**ACTGCGGAGCACACGTAAATA**TTCAAGAGATA**TATTTACGTGTGCTCCGCAGT**TTTTTA-3'
B 5'-GATCCCC**GGTGGCCCAGAACGGAAATAT**TTCAAGAGATA**ATATTTCCGTTCTGGGCCACC**TTTTTA-3'
(Bold sequences are sense and antisense of target gene)

After selection of puromycin, cell proliferation of versican knocked down GL261 and control cells were analyzed by cell counting or AlamarBlue assay according the manufacturer's instructions.

3.2.5 Microglia/macrophage isolation from mice and human glioma patients

Glioma associated microglia/macrophages (GAMs) were acutely isolated from naïve and tumor-bearing WT and TLR2 KO mice 2 weeks after tumor inoculation for RNA isolation using magnetic beads as per the manufacturer's instructions (Miltenyi Biotec, Germany). Briefly, after anesthetization and decapitation of the mice, brains were removed, weighed and enzymatically digested into a single cell suspension using Neural Tissue Dissociation Kit (Miltenyi Biotec, Germany). The tissue was further dissociated and debris was removed by applying a 40 µm cell strainer (Miltenyi Biotec, Germany). After removal of myelin with anti-myelin beads, cell suspensions were incubated with CD11b microbeads in MACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) for 10 minutes. The cells were then loaded onto a MACS column (Miltenyi Biotec, Germany),

after washing the column with MACS buffer. The CD11b-positive cells were eluted from the column. Then a fraction of the isolated cells were stained with CD11b antibody for FACS analysis to verify cell purity. The pure populations of CD11b-positive cells were then used for investigating gene expression by real-time PCR. GAMs isolation from human materials has similar procedure according to the manufacturer's instructions.

3.2.6 Molecular biology

3.2.6.1 Total RNA isolation and cDNA synthesis

Total RNA was isolated by using an InviTrap® Spin Universal RNA Mini Kit according to the manufacturer's instructions. The concentration of isolated RNA was determined photometrically and 250ng was used for cDNA synthesis using SuperSript II reverse transcriptase kit.

3.2.6.2 Semiquantitative PCR

Semiquantitative PCR was carried out using a TaKaRa Tag PCR system in a thermocycler. H_2O was used as a negative control and β-actin was used as internal control.

Component	Volume (µl)
TaKaRa Tag™	0.2
10xPCR Buffer	2.5
dNTP Mixture	0.5
Primer (forward, 10µM)	0.5
Primer (reverse, 10µM)	0.5
Sterillized distilled water	17.3
TOTAL	23

Table 18. RT-PCR reaction form

50ng of cDNA (2µl) was added to the PCR master mixture

PCR program was used as follows:

Lid Temperature 99°C		
1	95°C for 2 min	
2	95°C for 45 sec	**40 cycles**
3	X °C for 45 sec	
4	72°C for 1 min	
5	72°C for 5 min	
6	4°C for ∞	

Table 19. RT-PCR program

After PCR reactions, products were loaded with loading buffer and electrophoretically separated on an agarose gel (2% agarose in TAE buffer, 10μl ethidiumbrominde per 100ml buffer) at 90 V for about 45min. The gel was then exposed to UV light (254 nm) in a gel documentation system to visualize DNA bands.

RT-PCR Primer sequences are listed below:

Gene product	Primer sequences	Annealing Tempreture
Human *β-actin*	5'-AGGCACCAGGGCGTGAT-3' 5'-GCCCACATAGGAATCCTTCTGAC-3'	58°C
Human *versican V0*	5'-TCAACATCTCATGTTCCTCCC-3' 5'-TTCTTCACTGTG GGTATAGGTCTA-3'	57-60°C
Human *versican V1*	5'-GGCTTTGACCAGTGCGATTAC-3' 5'-TTCTTCACTGTGGGTATAGGTCTA-3'	57-60°C
Human *versican V2*	5'-TCAACATCTCATGTTCCTCCC-3' 5'-CCAGCCATAGTCACATGTCTC-3'	57-60°C
Human *versican V3*	5'-GGCTTTGACCAGTGCGATTAC-3' 5'-CCAGCCATAGTCACATGTCTC-3'	57-60°C

Table 20. RT-PCR primers

3.2.6.3 Quantitative PCR

Quantitative real-time PCR was carried out in at least duplicates by using SYBR PCR mix in a FAST 7500 real-time PCR system. H_2O was used as negative control and β -actin was used as reference.

Component	Volume (µl)
SYBR PCR mix (2x)	10
Primer (forward, 10µM)	1
Primer (reverse, 10µM)	1
Sterillized distilled water	6
TOTAL	18

Table 21. Real-time PCR reaction form

20-50ng cDNA (2µl) was added to the PCR master mixture

PCR program was used as follows:

Lid Temperature 99°C		
1	95°C for 2 min	
2	95°C for 45 sec	**40 cycles**
3	X °C for 60 sec	
4	72°C for 1 min	
5	72°C for 5 min	
6	4°C for ∞	

Table 22. Real-time PCR program

Real-time PCR Primer sequences are listed below:

Gene product	Primer sequences	Annealing Temperature
Mouse *β-actin*	5'-CCCTGAAGTACCCCATTGAA-3' 5'-GTGGACAGTGAGGCCAAGAT-3'	60°C
Mouse *MT1-MMP*	5'-GTGCCCTATGCCTACATCCG-3' 5'-CAGCCACCAAGAAGATGTCA-3'	60°C
Mouse *MMP-9*	5'-CATTCGCGTGGATAAGGAGT-3' 5'-ACCTGGTTCACCTCATGGTC-3'	60°C
Mouse all *versican*	5'-CCACCTCACAAGCATCCTTTCT-3' 5'-TGAGGCCGATCCACTGGT -3'	58-60°C
Mouse *versican V0*	5'-TGAGGTCAGAGAAAACAAGACA-3' 5'-CTGCAAGGTTCCTCTTCTTTAGATTC-3'	58-60°C
Mouse *versican V1*	5'-CAGATTTGATGCCTACTGCTTTAAAC-3' 5'-CTGCAAGGTTCCTCTTCTTTAGATTC-3'	58-60°C
Mouse *versican V2*	5'-TGAGGTCAGAGAAAACAAGACA-3' 5'-GATAACAGGTGCCTCCGTTGA-3'	58-60°C
Mouse *versican V3*	5'-CAGATTTGATGCCTACTGCTTTAAAC-3' 5'-GATAACAGGTGCCTCCGTTGA-3'	58-60°C
Human *MMP-9*	5'-AAGGCGCAGATGGTGGAT-3' 5'-TCAACTCACTCCGGGAACTC-3'	58-60°C
Human *GAPDH*	5'-ACAGTCAGCCGCATCTTCTT-3' 5'-ACGACCAAATCCGTTGACTC-3'	60°C

Table 23. Real-time PCR primers

3.2.7 Protein detection assays

3.2.7.1 Gelatin zymography

Gelatin zymography has been used to detect the inactive and active forms of gelatinases (MMP-2 and MMP-9) from tissues and cells based on the molecular weight. Briefly, conditioned medium from cell culture was collected and mixed with loading buffer, the samples are then loaded on to an 8% SDS-PAGE gel containing 1% gelatin. 25 ug Recombinant MMP-9 was used as a positive control. After electrophoresis (4°C, 100V), the gel was carefully removed and washed 3 times with 50ml gel washing buffer. The gel

was incubated with development buffer in a container at 37°C for overnight. The gel was stained with Coomassie blue solution for 1h at room temperature, with constant agitation on a rotary shaker. The gel was then destained with destaining solution for about 30min till the bands were seen on the gel. After the appearance of seen clear bands, the gel was dried and pictures were taken for analysis.

3.2.7.2 Western Blot

Total cellular lysates from cultured cells were obtained by RIPA buffer containing EDTA-free protease inhibitor cocktail at 4 °C. Cellular debris was removed by centrifugation, and protein quantification was performed using the bicinchoninic acid (BCA) assay. Proteins were mixed with Laemmli Buffer containing β- mercaptoethanol, after boiling for 5 min at 95 °C, samples were cooled and loaded on 10% SDS-PAGE gels, and immunoblotting was performed using the following primary antibodies, Anti-rabbit HRP-conjugated secondary antibody (Cell signalling) was used and bands were visualized by using enhanced chemiluminescence (Amersham Biosciences).

3.2.7.3 Enzyme-linked immunosorbent assay (ELISA)

Supernatant collected from mouse primary cultured microglia (from WT, TLRs 1, 2, 4, 6, 7 and 9 KO mice) determined for total MMP-9 by ELISA kits according to the manufacturer's protocols (R&D systems). The colometric reaction was read on a Tecan Infinite F-500 photometer. Results were presented as picograms of MMP-9 per $1x10^6$ cells.

3.2.7.4 Immunohistochemistry of brain sections (free floating sections)

The PFA perfused and cryoprotected brains were rapidly frozen by dry ice and mounted onto a microtome. 40µm sections were prepared and collected into a CPS filled 24-well plate. Sections were stored in -20°C. Brain sections were washed three times in TBS-0.05% Tween 20(TBST), pH 7.4 followed by antigen retrieval using Sodium Citrate buffer, pH 6 at 80°C for 20 min in a water bath. The sections were washed again with TBST and incubated in blocking buffer (5% donkey serum in TBST) for 1h for permeabilization. The sections were then incubated o/n at 4°C with primary antibody. After 3 times washing with TBST, the sections were further incubated with a fluorophore-

labeled antibody or a Biotin-SP-conjugated secondary and a fluorophore-streptavidin labeled tertiary antibody at room temperature. After the final wash, the slices were mounted in Aqua-Poly/Mount mounting medium onto microscope slides and stored at 4°C until used for microscopic analysis.

3.2.8 Organotypic Brain Slice cultures (OBS)

3.2.8.1 Preparation of organotypic brain slices

Organotypic brain slices were derived from 16-day-old mice as previous described (Markovic, 2005). Mice were decapitated, brains removed within 2-3 minutes and placed in ice-cold PBS under sterile conditions. The forebrain was dissected from the brainstem and glued with cyanoacrylate glue onto a magnetic block and cut in the coronal plane into 250 µm sections with a vibratome. Brain slices were transferred with a glass pipette into cell culture inserts of 0.4 µm pore that were fitted into wells of a 6-well plate. Thereafter, 1 ml of culture medium containing was added into each well and the brain slices were incubated in the inserts at an air-medium interface. After overnight equilibration, the culture medium was exchanged for cultivation medium.

3.2.8.2 Depletion of microglia in organotypic brain slices

Selective depletion of microglia in OBS was achieved following a previously established protocol (Markovic, 2005) by adding liposome-encapsulated clodronate diluted with culture medium (1:10) to the slices. The slices were left with clodronate for 24 h which led to a 90% ablation of microglia. After 24 h, the culture medium containing clodronate was replaced by fresh cultivation medium and slices were left undisturbed for 72 h.

3.2.8.3 Glioma implantation into organotypic brain slices

5000 EGFP- GL261 glioma cells in a volume of 0.1 µl were inoculated into brain slices using a 1 µl syringe mounted on a micromanipulator. This device allowed placement of the tip of the syringe consistently at the same defined region on the slice surface. An injection canal was formed that reached 150 µm deep into the 250 µm thick slice. The needle was then retracted by 50 µm, leaving a cavity of approximately 50 µm in length. The cell suspension was injected slowly into this canal in the slices. To ensure identical

experimental conditions, glioma cells were always inoculated into the same area of the slices, into the globus pallidus in the cortex. Directly after glioma injection, tumor cells remained at the inoculation site. Careful control of the injection procedure ensured that no cells spilled onto the surface of the slices, which could migrate over the surface rather than invade through the tissue.

3.2.9 *In vivo* studies

3.2.9.1 *In vivo* glioma implantation

Mice were anesthetized by intraperitoneal injections with a 0.1% xylazyne (Rompun) and 1.5% ketamine hydrochloride (Ketanest) mixture in 0.9% NaCl. 8ul of mixture was injected per g of mouse body weight. The eyes of the mice were carefully covered with glycerin fat to avoid cornea drying. Mice were then immobilized and mounted onto a stereotactic head holder in the flat-skull position. After skin incision 1 mm anterior and 1.5 mm lateral to the bregma, the skull was carefully drilled with a 20G needle tip. A 1µl syringe with a blunt tip was inserted to a depth of 4 mm and retracted to a depth of 3 mm from the dural surface into the right caudate putamen. Over 2 minutes, 1 µl ($2x10^4$ cells/µl) of glioma cell suspension was slowly injected into the brain. The needle was then carefully retracted from the injection canal and the skin was sutured with a surgical sewing cone. After surgery the mice were kept warm until awake and their post-operative condition was monitored daily.

3.2.9.2 Unbiased stereology for tumor volume estimation

Tumor size quantification was determined in at least 6 mice per group. Mice were anesthetized with Narcoten 14 days after tumor inoculation, brains were perfused and fixed, and resulting brain slices were subsequently used to analyze tumor expansion in vivo. The tumor volume in Hematoxylin & Eosin (H&E) stained brain slices of was quantified according to the Cavalieri principle by determining tumor area in every 12th 40 µm thick brain slice and then multiplying this area by the factor 12 x 40µm.

3.2.9.3 Survival studies

All the mice were used in the long term in vivo studies were intracranially implanted with glioma cells. Briefly, 2x 10^4 cells/µl of glioma cell suspension was slowly injected into the brain as stated previously. Analysis of cumulative mean survival time by Kaplan-Meier plots in tumor-implanted mice was based on the end-point event (when mice showed clinical symptoms).

3.2.10 Statistical analysis

All data represent the average of at least 3 independent experiments. Error bars represent standard error of the mean. Data sets were analyzed statistically by SPSS11.5 software and tested for normality by Shapiro-Wilks test. For non-parametric analysis, the Mann-Whitney-U or paired t test was used. Parametric testing was done with Student-t test. Comparisons between multiple groups were done using one-way ANOVA with Scheffé post-hoc test. Statistical significance was determined at p values < 0.05 (*) and <0.01 (**) while n.s. implied a non-significant p value.

4. Results

4.1 The expression and regulation of TLRs in gliomas

4.1.1 TLR2-deficient tumor bearing mice show reduced microglial MT1-MMP expression and enhanced survival rate

We have shown previously that deletion of *TLR2* gene locus resulted in smaller tumor volume (Vinnakota et al. 2013). I then determined MT1-MMP expression *ex vivo* in microglia/brain macrophages freshly isolated from naïve and glioma-bearing WT and TLR2 KO mice by real-time PCR (Fig. 10A, n=3). MT1-MMP expression in GAMs from WT mice was increased 4.08-fold (±0.204; p=0.002), relative to naïve microglia from WT mice. In GAMs derived from TLR2 KO mice, MT1-MMP expression was 2.19-fold (±0.26; p=0.02) up-regulated, relative to naïve microglia from TLR2 KO mice. Overall, MT1-MMP expression in GAMs from TLR2 KO mice was significantly lower (p=0.045) than that in GAMs from WT mice.

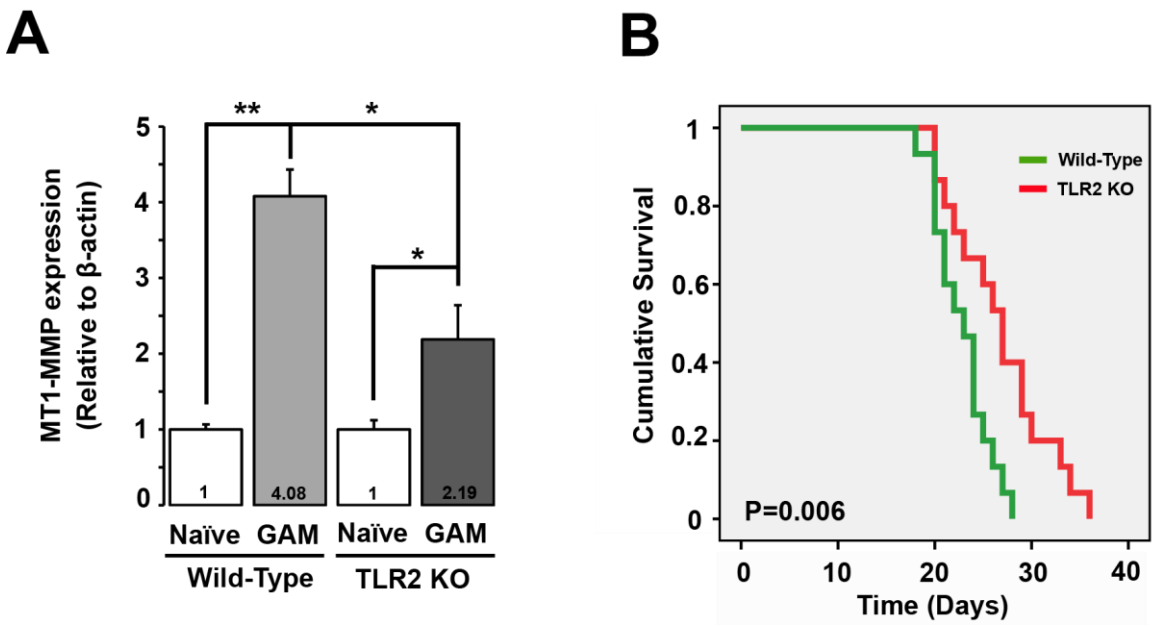

Figure 10. Absence of TLR2 results in decrease in MT1-MMP expression and increased survival.
(A) WT and TLR2 KO mice were intracerebrally implanted with glioma tumors, and glioma associated microglia/macrophages were isolated 14 days after glioma cell injection. Naïve tumor-free brain was used as a control. Data was collected from 3 different mice in each group. Differences in MT1-MMP expression were analyzed by $2^{(-\Delta\Delta CT)}$ method. MT1-MMP expression in WT GAMs is 4.08-fold up-regulated (±0.204; p= 0.002), compared to WT naïve microglia, while in TLR2 KO GAMs, MT1-MMP expression was 2.19-fold up-regulated (±0.26; p=0.02). MT1-MMP expression in TLR2 KO GAMs is significantly decreased compared to WT GAMs (p=0.045). **(B)** The Kaplan-Meier curve represents the cumulative survival of WT and TLR2 KO mice after glioma cell injection. The p value is 0.006.

To investigate the survival rates of glioma-bearing WT and TLR2 KO mice, I inoculated GL261 glioma cells into WT and TLR2 KO mice (n=15 in each group) and observed the survival time of mice bearing glioma. As seen in Fig. 10B, deletion of the *TLR2* gene locus led to significantly increased survival rate (p=0.006) in TLR2 KO mice as compared to WT mice suggesting that TLR2 expression relates to poor prognosis.

4.1.2 TLR1, TLR4 and TLR6 deficiency does not interfere with tumor growth *in vivo*

TLR2 signaling can be mediated by heterodimerization with TLR1 and TLR6. To determine the contribution of TLR1 and TLR6 in tumor growth *in vivo*, I implanted tumor cells in TLR1 KO (n=5), TLR6 (n=6) KO mice and gender, age matched WT mice to observe the tumor volume. As seen in Fig. 11A, there was no significant difference between KO and WT groups (p>0.05 in both group), suggesting that single deficiency of either TLR1 or TLR6 does not interfere with tumor growth *in vivo*. Since TLR4 is also an important subtype of TLRs in several diseases, I also compared the tumor size of TLR4KO mice (n=7) bearing glioma and WT mice, there was also no significant difference (p>0.05), indicating TLR4 is not involved in glioma growth *in vivo* (Fig. 11B).

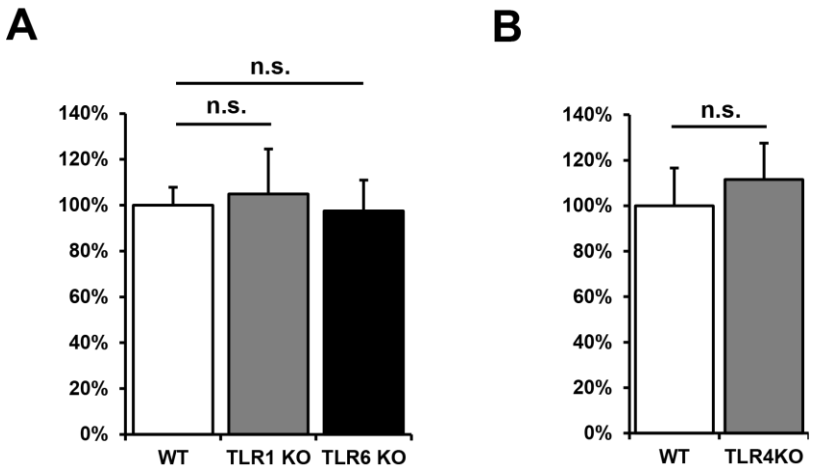

Figure 11. Absence of TLR1, 4 or 6 does not interfere with glioma growth *in vivo*.
GL261 cells were intra-cerebrally implanted into WT and TLR1, 6 (A, B) or 4 KO (C) mice. After 2 weeks of tumor growth, tumor size was evaluated based on unbiased stereology.

4.1.3 TLR7 depletion does not interfere with glioma growth *ex vivo*

It has been showed by us that TLR2 deletion and microglia ablation interfere with glioma growth *ex vivo* (Vinnakota et al. 2013). To test if any other TLR(s) apart from TLR2 was involved in glioma growth *ex vivo*, I prepared organotypic brain slices from WT and TLR7 KO mice (p16). In both types of mice, tumors were significant smaller when microglial cells were depleted (Fig. 12A, B, $p<0.001$ in WT and $p<0.01$ in TLR7KO), but there was no difference in the tumor size between WT and TLR7 KO groups ($p=0.45$), indicated that TLR7 was unnecessary for glioma growth.

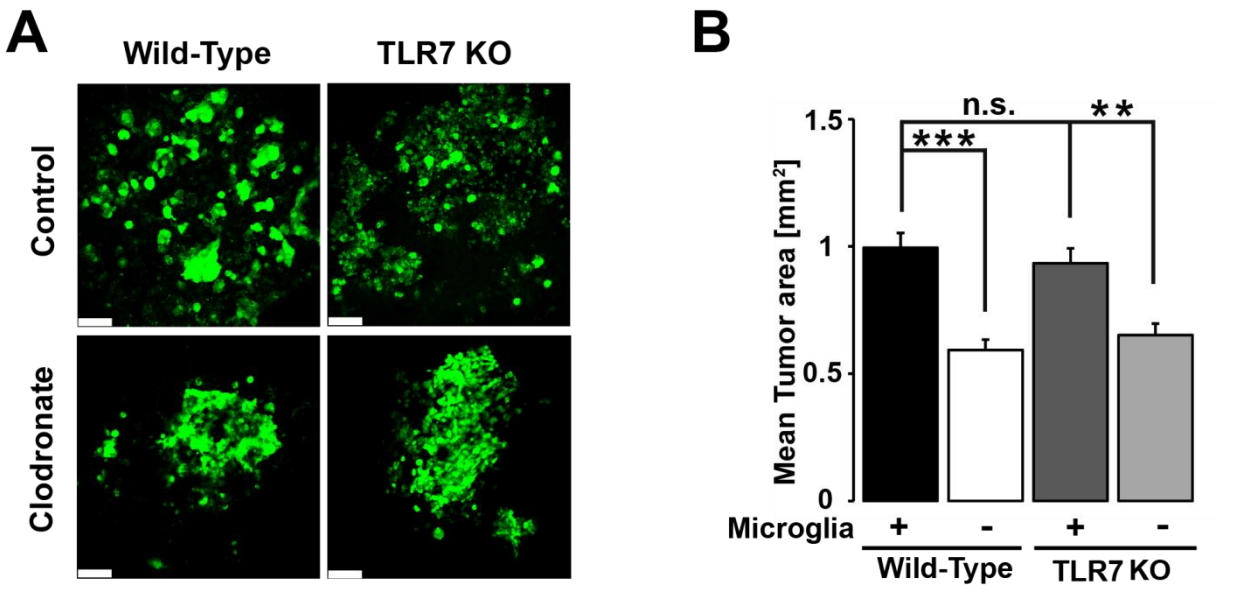

Figure 12. TLR7 does not interfere with glioma growth *ex vivo*.
Brain slices from 16-day-old WT and TLR7 KO mice were implanted with 5000 EGFP-GL261 glioma cells and the area occupied by the glioma cells was measured after 5 days (A) The fluorescence micrograph of EGFP-labeled glioma cells is shown for microglia-containing (+) and clodronate-treated microglia-depleted (-) brain slices from WT (left) and TLR7 KO mice (right). Scale bar is 10μM. (B) Tumor area was quantified in microglia-containing (+) and microglia-depleted (-) slices from WT and TLR7 KO mice.

4.1.4 TLR2 is highly expressed in human gliomas and its expression inversely correlates with patient survival

After showing some evidences of TLRs in the mouse glioma model, I used the REMBRANDT database to analyze whether high TLR2 expression correlates with clinical data from human glioma patients. Indeed, TLR2 is highly expressed in all gliomas (n=454) as compared to 28 cases of non-tumor tissue (Fig. 13A). Comparing the probability of survival in all glioma cases, patients with up-regulated TLR2 revealed a

lower survival rate (Fig. 13B). This may indicate that patients with high TLR2 expression usually suffer from a higher-grade glioma and that patients with lower TLR2 expression have a lower-grade glioma. Moreover, even survival rates within a homogenous group of tumors (i.e. astrocytomas) correlated with the expression of TLR2. Patients with high TLR2 expression levels presented with reduced survival as compared to patients having lower TLR2 expression (Fig. 13C).

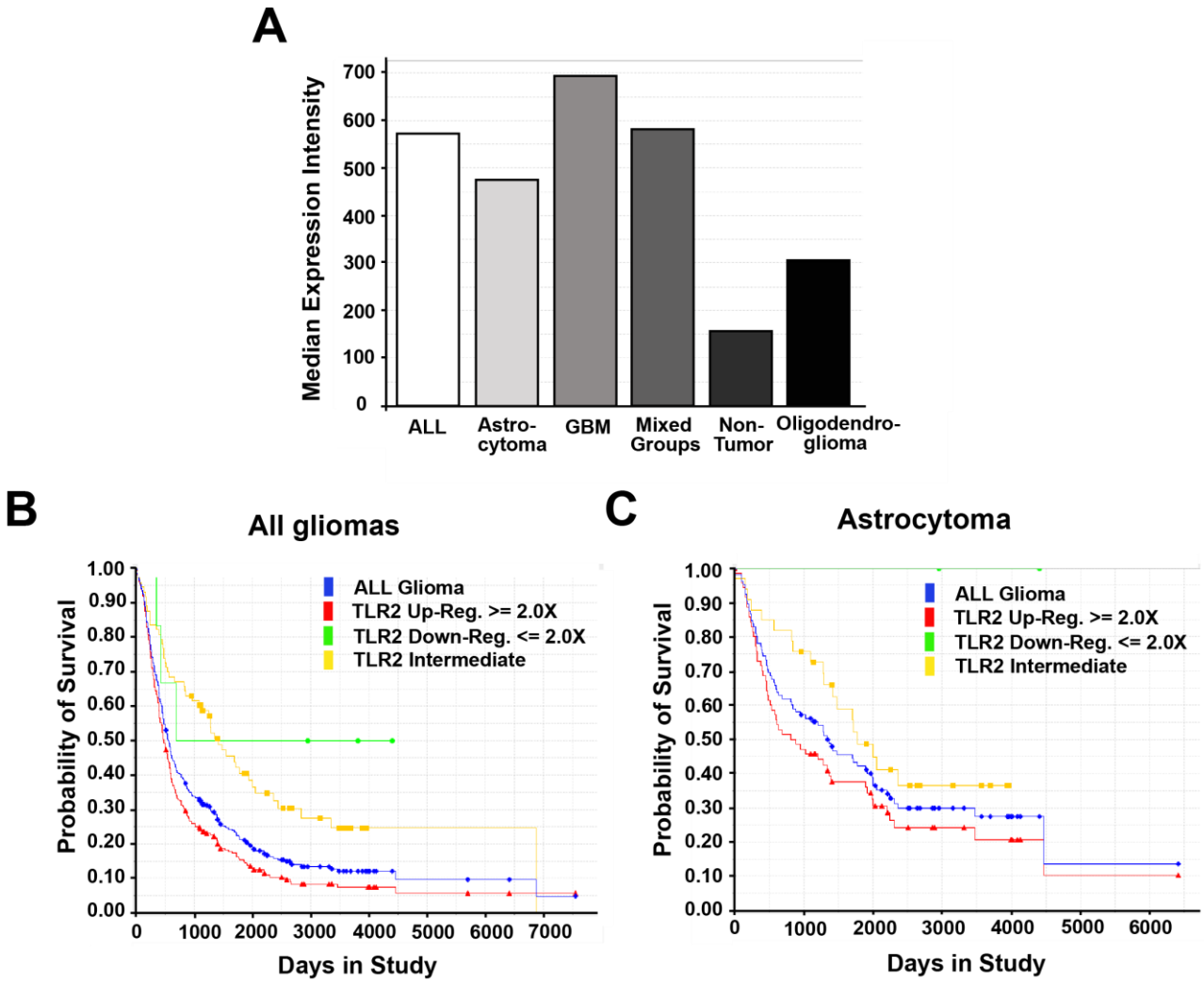

Figure 13. TLR2 is highly expressed in human gliomas and its expression is inversely correlated to patient survival.
(A) Clinical data from the Rembrandt database showed that TLR2 is highly expressed in all human glioma cases compared to non-tumor tissue samples. (B) TLR2 expression in human gliomas inversely correlates with patient survival. Log-rank p-values of each group are: Up-regulated versus Intermediate (9.41E-7); Up-regulated versus Down-regulated (0.035); Up-regulated versus all other samples (1.834E-7). (C) TLR2 expression in human asctrocytomas also inversely correlates with patient survival. Log-rank p values of each group are: Up-regulated versus Intermediate group (0.046); Up-regulated versus all other samples (0.019).

4.1.5 TLR2 but not TLR1 and TLR6 expression is up-regulated by GCM stimulation *in vitro*

It is clear that TLR2 is an important mediator in gliomagenesis. I then addressed the question of whether microglial TLR2 expression is affected by GCM. Primary cultured microglia were incubated with GCM for 24 hours, and microglial TLR2 expression levels were analyzed by flow cytometry. GCM stimulated microglia had higher levels of TLR2 as compared to the unstimulated controls (Fig.14A). I quantified the data by analyzing the mean fluorescence intensity (normalized to isotype); microglial TLR2 expression is significantly (1.58±0.13 folder, $p<0.05$) up-regulated after GCM treatment (n=6). Since TLR1 and TLR6 are heterodimers of TLR2, I also determined TLR1 and TLR6 expression under GCM stimulation. However, I could not see any significant difference between control and GCM stimulated microglia (Fig. 14B, C).

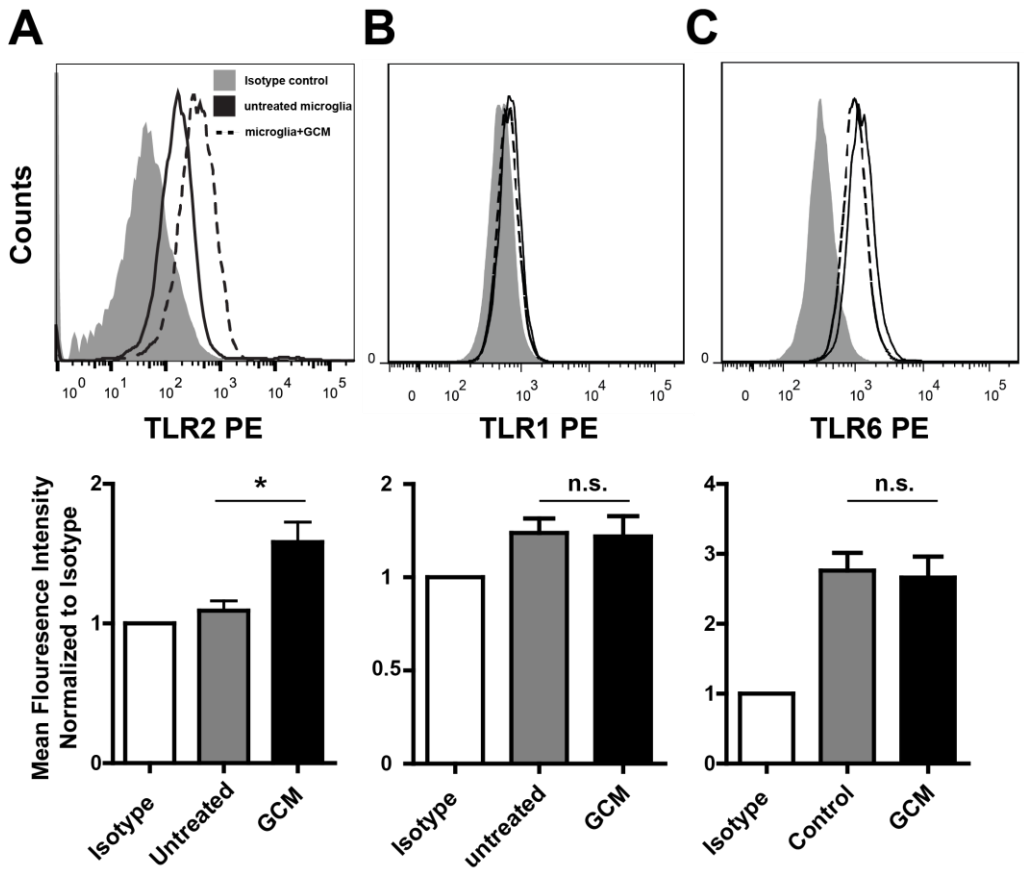

Figure 14. Microglial TLR2 is up-regulated by GCM stimulation.
(A) TLR2 expression of microglia upon GCM stimulation for 24 h was analyzed by flow cytometry. Representative histogram of flow cytometry was shown (upper panel), mean fluorescence intensity (normalized to isotype control) was used to quantify the expression of

TLR2 (lower panel). Representative histograms of TLR1 (B) and TLR6 (C) expression on microglia after GCM stimulation. Data was quantified by relative mean fluorescence intensity. Bars represent the mean±s.e.m. from at least 3 independent experiments.

Fig. 4.1.6, Fig. 4.1.7 and Fig. 4.1.8 are a collaboration with medical student Omar Dildar a Dzaye.

4.1.6 TLR expression on GL261 cells

Expression of TLRs in glioma cells is not well documented. Only TLR9 was confirmed to be expressed by GL261 cells (Grauer et al. 2008). To explore the TLR expression in different cell types in glioma microenvironment, I first analyzed TLR expression in our glioma model GL261 cell line. As seen in Fig. 15 only TLR6 and CD14 are expressed, while TLR1, TLR2, TLR4 are not expressed by GL261 cells, indicating tumor cells may not the predominant cell type expressing TLR2 in glioma tissue.

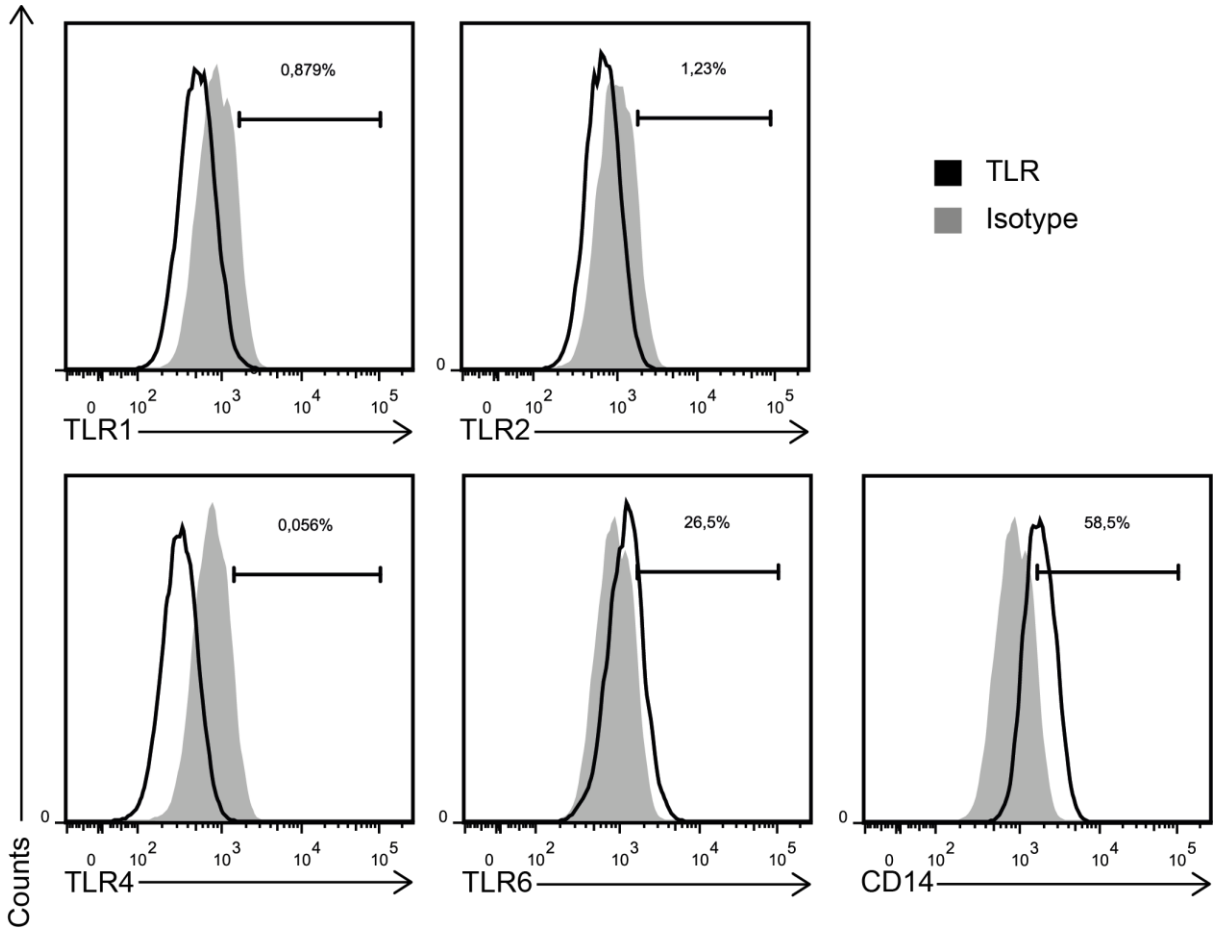

Figure 15. TLRs expression on GL261 cell line.

Expression of TLRs and CD14 on GL261 cells were analysed by flow cytometry**.**

4.1.7 Identification of different cell populations in the GL261 glioma model by flow cytometry

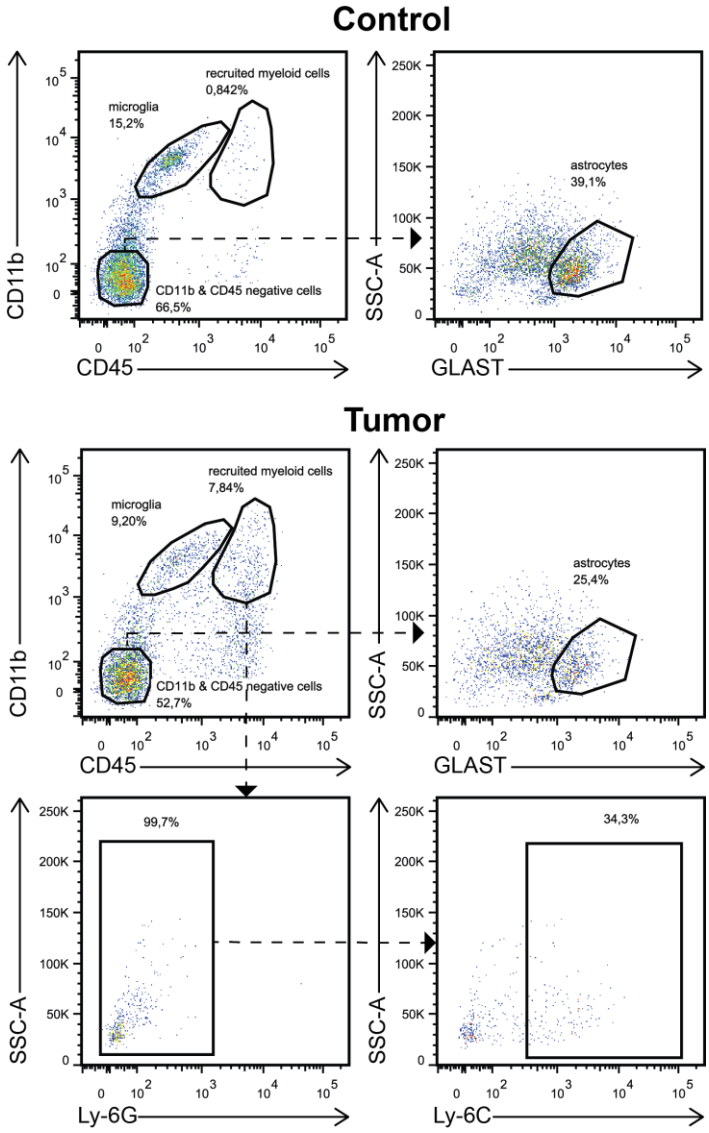

Figure 16. Gating strategies of flow cytometry

Control and tumor tissue was dissociated and analysed by flow cytometry. Cell differentiation was based on the cell markers CD45, CD11b, GLAST, Ly6C and Ly6G. Microglia: $CD11b^{+}CD45^{low}$; infiltrative macrophage: $CD11b^{+}CD45^{high}Ly\text{-}6G^{-}$ $Ly\text{-}6C^{+}$; astrocyte: $CD11b^{-}$ $CD45^{-}GLAST^{+}$

It has been shown that CD45 could be used as a marker to distinguish microglia from macrophages by flow cytometry (Gabrusiewicz et al. 2011). Control tissue and tumor tissue were dissociated and flow cytometry was performed. As shown in Fig. 16, microglia, macrophages and astrocytes could be identified by surface markers. Thus

receptors expression on different cell populations could be further analyzed by flow cytometry.

4.1.8 TLR2 is up-regulated in glioma associated microglia but not astrocyte *ex vivo*.

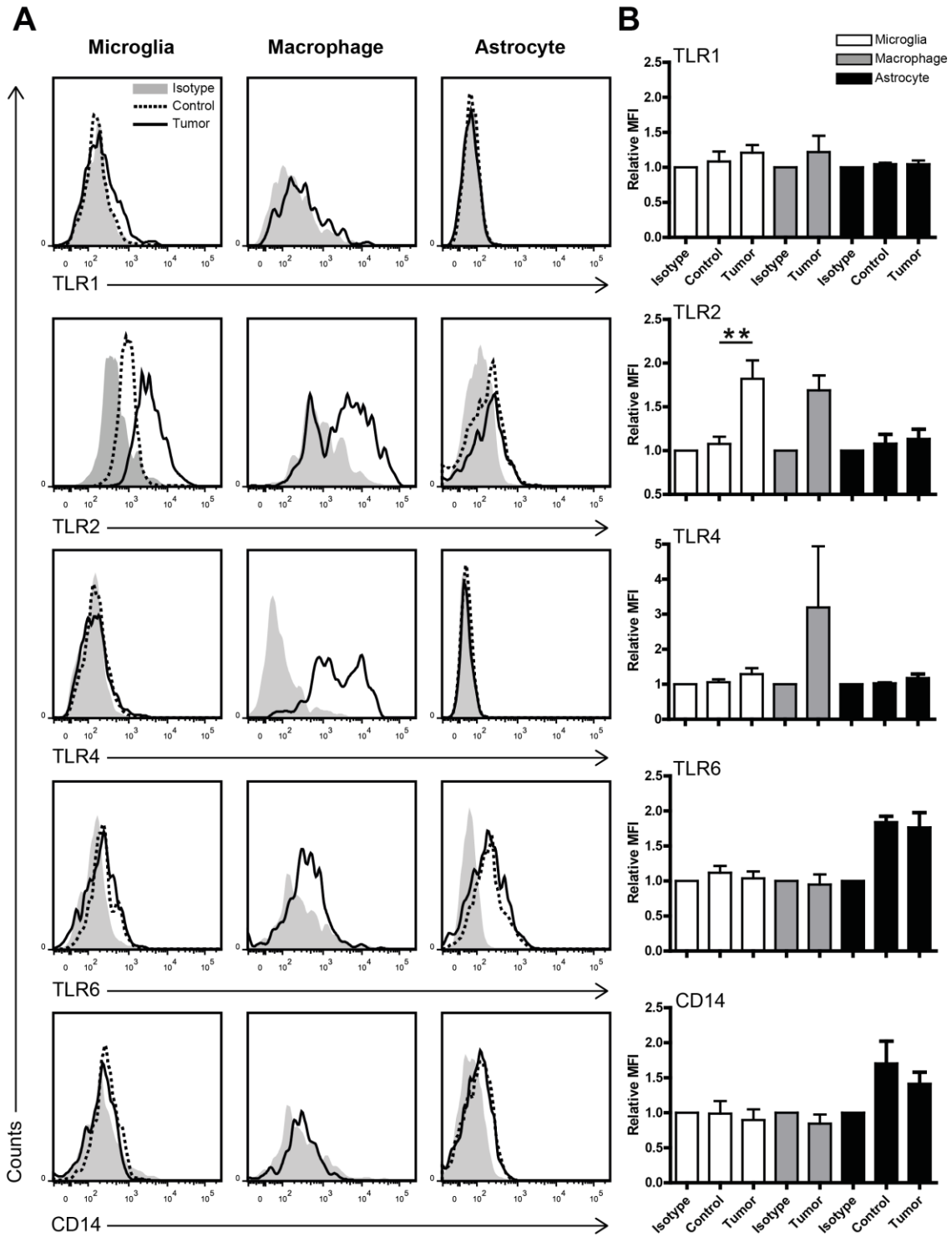

Figure 17. TLRs expression in GAMs and glioma associated astrocytes

Naïve mouse brain or tumor tissue was dissociated immediately after resection. Erythrocytes were lysed by adding ammonium chloride solution and myelin was removed by Percoll gradient centrifugation, cell pellets were further analyzed by flow cytometry. Representative histograms of TLR1, TLR2, TLR4, TLR6 and CD14 expression on microglia, macrophages and astrocytes in both control and glioma bearing mice (left panel, $n \geqslant 3$). Median Fluorescence Intensity (MFI) of control and tumor was normalized to isotype and quantified by Flowjo software on the right panel.

We have shown in the previous results that microglial TLRs are regulated under the influnce of gliomas *in vitro*. To investigate how TLRs are expressed and regulated in differnet cell types of gliomas, we used flow cytometry to detect TLR expression on different cell populations in the GL261 glioma model. I implanted GL261 cells into the mouse brain, after 2 weeks of tumor growth, tumor and control brain tissue were dissociated and stained with different surface markers and TLR antibodies. As shown in Fig. 17, in the naïve mciroglia, none of the TLRs were significantly expressed compared to the isotype control. However, in the tumor tissue, only TLR2 was significantly up-regulated compared to the naïve controls (data was quantified by MFI, $p<0.05$). Since TLR2 forms heterodimers with either TLR1 or TLR6, we observed a slight but not significant induction in TLR1 but not TLR6. For infiltrative macrophages, due to the difficulties of finding a proper control, I could only demonstrate here that TLR2 and TLR4 are expressed in glioma associated macrophages. Additionally, TLR2, TLR6 and CD14 were seen to be expressed on naïve astrocyte, however, I did not observe any significant difference of TLRs expression between naïve astrocyte and tumor associated astrocyte. Taken together, TLR2 is the only subtype of TLRs up regulated in GAMs but not glioma associated astrocyte.

4.2 Identify soluble factor(s) released from glioma regulate microglial MT1-MMP expression

4.2.1 Mass spectrometry of conditioned medium from GL261 cells

To screen the soluble factors released by GL261cells, serum-free conditioned medium of GL261 cells was generated and sent for mass spectrometry analysis. A few reported endogenous ligands of TLRs were identified in the conditioned medium of GL261 as listed in Table.24.

Protein name	Targeted TLRs
Heat shock protein 60, 70, 90 (HSP)	TLR2, TLR4
High mobility group box 1 (HMGB1)	TLR2, TLR4
Versican (CSPG2)	TLR2
Fibronectin	TLR4

Table 24. Reported endogenous ligands of TLRs in GL261 conditioned medium by mass spectrometry

4.2.2 HMGB1 is expressed in microglia, astrocytes and GL261 cells and it does not induce microglial MT1-MMP expression

HMGB1 has been identified as an endogenous ligand of both TLR2 and TLR4 (Yu et al. 2006), to verify its expression in GL261 cells, I performed the western blot as shown in Fig. 18A that GL261 expresses HMGB1. However, I also found microglia and astrocytes express the protein. To further investigate its role in regulating microglial MT1-MMP, recombinant HMGB1 was applied to microglia, after 6h incubation, MT1-MMP was analyzed by real-time PCR. It showed that 200nM and 400nM HMGB1 could not induce microglial MT1-MMP expression *in vitro*, a positive control Heat killed *Listeria monocytogenes* (HKLM) as a ligand for TLR2 induced significant up-regulation of MT1-MMP. These data indicated HMGB1 is not exclusively expressed by GL261 cells, and it could not induce microglial MT1-MMP induction.

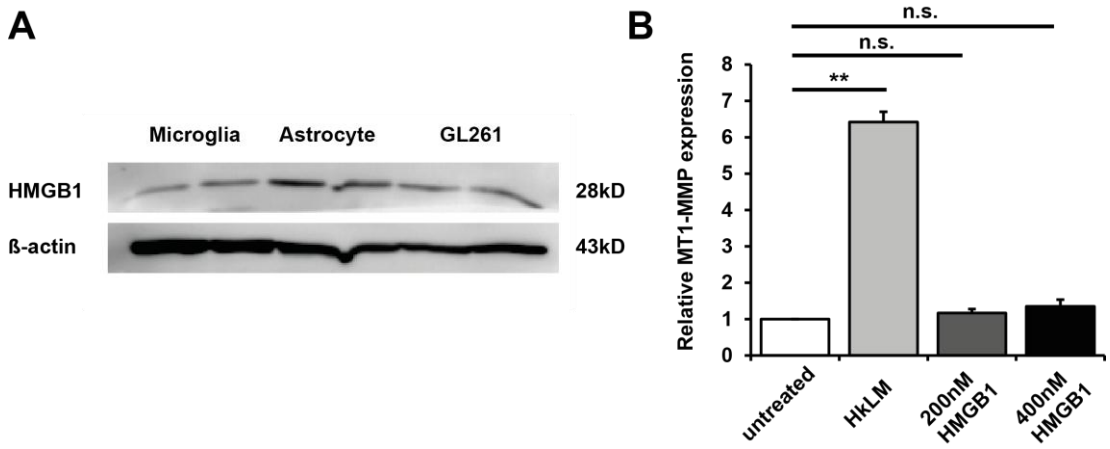

Figure 18. HMGB1 expression in microglia, astrocytes and GL261 cells, it could not induce microglial MT1-MMP induction.

(A)Expression of HMGB1 in microglia, astrocytes and GL261 cells by western blot. (B) Microglia was stimulated with 200nM and 400nM HMGB1, MT1-MMP expression was analyzed by real-time PCR. HKLM was used as a positive control.

4.2.3 Versican V0/V1 is highly expressed by gliomas but not by microglia

To confirm the data from mass spectrometry, expression of versican isoforms were analyzed in microglia, whole adult brain lysate and GL261 cells at the mRNA level by real-time PCR. Gliomas expressed predominately V0, V1 and V3 but not V2, while microglial cells did not express any versican isoforms (Fig.19A). Western blot and immunohistochemistry also showed Gl261 cells only expressed V0/V1 but not V2 (Oligodendrocyte as a positive control for V2) at the protein level (Fig. 19B, C). To further verify the expression of versican *in situ*, mouse glioma tissue and human GBM tissue were stained with V0/V1 antibody, I could observed a colocalization of mCherry glioma cells (red) and versican V0/V1 (blue) in a mouse glioma tissue (Fig. 19D). In a human GBM tissue, versican V0/V1 expression (red) was also found in non-Iba1 positive cells, and its expression was even found with close contact to microglia/macrophages (Fig. 19E, left insert). Additionally, a human glioma cell line U373 and 4 primary human GBM samples were also analyzed by RT-PCR, versican isoforms were expressed by all these cells (Fig.19E).

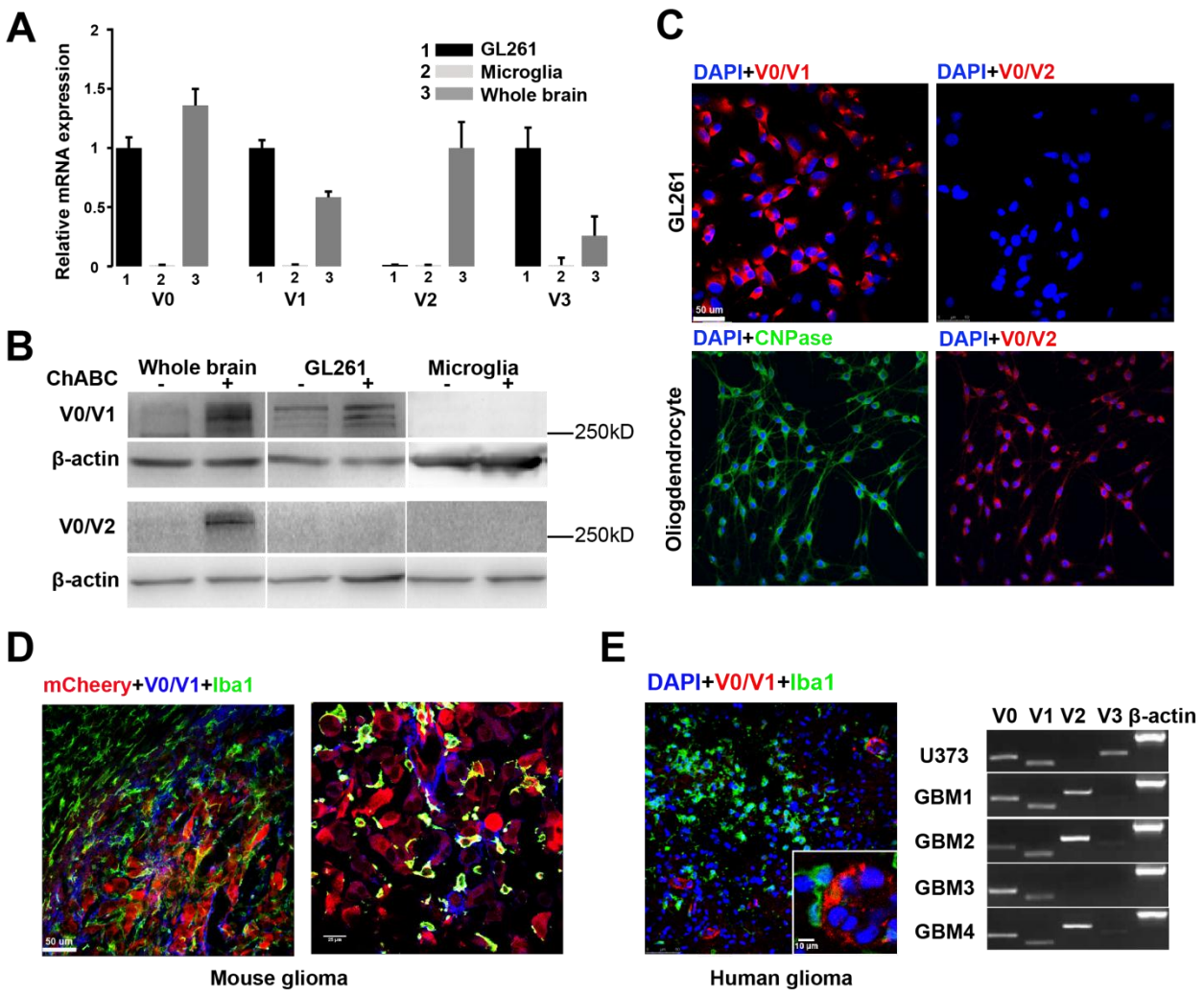

Figure 19. Versican isoform expression in gliomas.
(A) Expression of V0, V1, V2, and V3 in GL261 glioma cells by real-time PCR. (B) Western blot using antibodies against the versican isoforms V0/V1 (top) and against V0/V2 (bottom) shows the expression of V0/V1 in glioma cells. (C) Immunohistochemistry shows the expression of V0/V1 but not V0/V2 in mouse glioma, oligodentrocyte cell line was used as positive control of V0/V2. (E) Expression of the versican isoforms in human glioma tissues by immunohistochemistry (left) and RT PCR with a human glioma cell line (U373) and cells cultured from human glioma samples (GBM1-4).

4.2.4 Versican silenced gliomas induced less MT1-MMP expression in microglia *in vitro*

To investigate the role of versican on microglial MT1-MMP regulation, versican expression in GL261 cells was knocked down by siRNA (Fig.20A), and glioma conditioned medium were collected from both no-target siRNA transfected GL261 and versican siRNA transfected GL261. Primary cultured microglia were then stimulated by conditioned medium from both for 3h and 6h, microglial MT1-MMP expression was analyzed by real-time PCR. After 6h stimulation, microglial MT1-MMP expression was significantly increased in the no-target siRNA group (GCM) while it decreased in the versican siRNA group (TCM), indicating versican is an important factor released by gliomas in microglial MT1-MMP regulation (Fig. 20B).

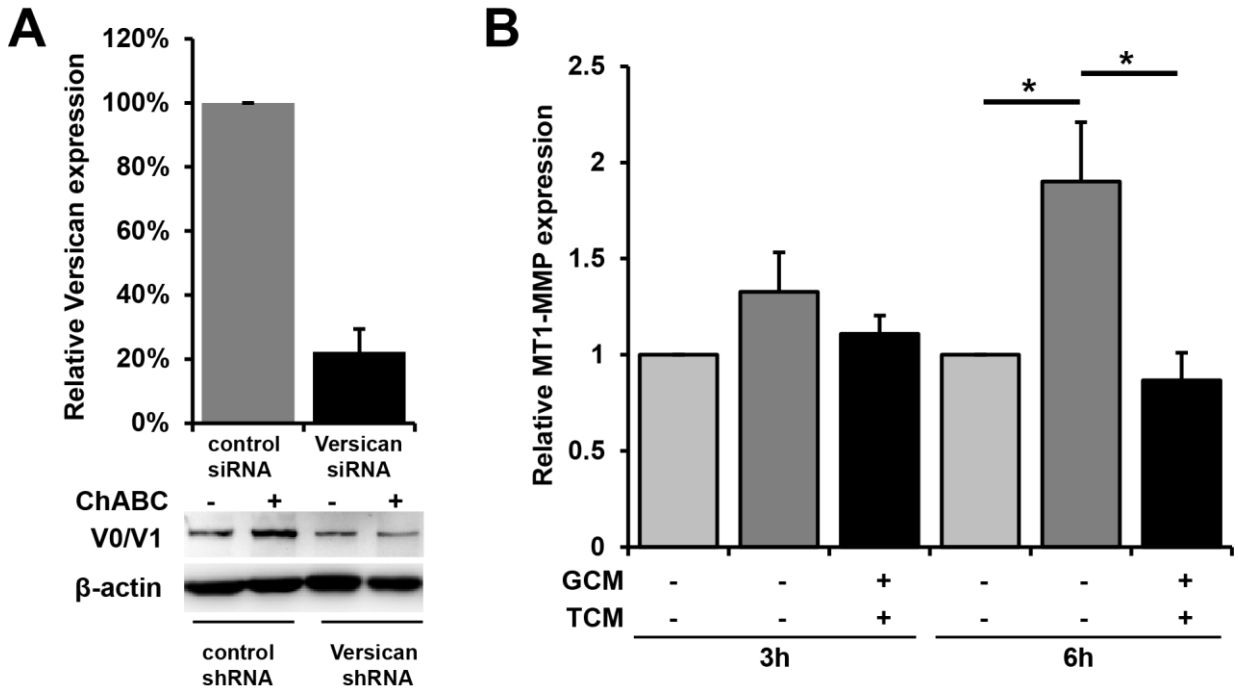

Figure 20. Silencing of versican in gliomas induce less microglial MT1-MMP *in vitro*.
(A) Down-regulation of versican expression by the siRNA and shRNA approach on mRNA (upper panel) and protein level (lower panel). (B) Primary microglia were stimulated with conditioned medium from siRNA-Versican (TCM) and non-target transfected GL261 cells (GCM) and microglial MT1-MMP expression was analyzed by real-time PCR (n=5).

4.2.5 Versican silenced gliomas induced less MT1-MMP expression in microglia *in vivo*

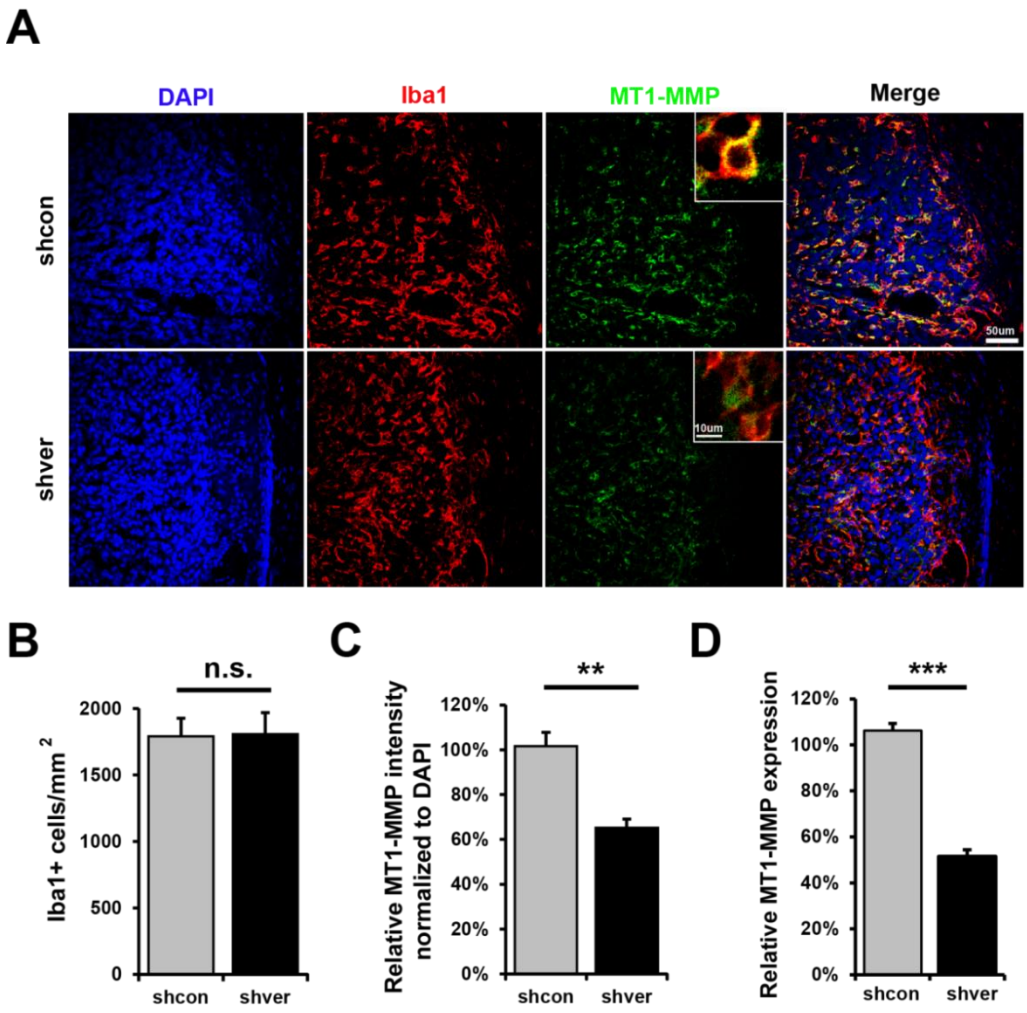

Figure 21. Silencing of versican in gliomas induce less microglial MT1-MMP *in vivo*.
(A) Versican knocked-down (shver) and control (shcon) GL261 were implanted into WT mice, Iba 1 positive cells and MT1-MMP expression were quantified (B, C) by Image J (n=6 in each group).(D)GAMs were isolated from shcon and shver GL261 injected mice, and MT1-MMP was analyzed by real-time PCR.

To further verify this effect *in situ*, I generated control and versican knocked down GL261 cells with control shRNA (shcon) or versican shRNA (shver). These two cell lines were implanted into mice brain (n=7 in each group), after 2 weeks tumor growth, mice were sacrificed and brain tissue were analyzed by immunohistochemistry for Iba-1 and MT1-MMP expression. GAMs were identified by immunolabelling with microglia/macrophage-specific antibody Iba1 (Fig. 21A). MT1-MMP fluorescence intensity was analyzed by Image J by being normalized to the intensity of DAPI, I

observed a significant decrease in MT1-MMP immunoreactivity in GAM in the shver group compared to the shcon group (Fig.21C). Iba 1 positive cells density was also analyzed and I did not observe any difference between two groups, indicating versican is not the factor in recruiting microglial/macrophages to the tumor (Fig.21B). To further verify the role of versican in regulating MT-1MMP expression *in vivo*, I implanted shcon and shver GL261 cells into the WT mice brain, after 2 weeks of tumor growth, I freshly isolated GAMs by MACS (CD11b+), total RNA was collected and qPCR of MT1-MMP was performed (Fig. 21D). MT1-MMP expression in shver injected tumor was 51% less compared to the shcon injected tumor (±3%, $p<0.001$), indicating tumor derived versican indeed regulates microglial MT1-MMP level *in situ*.

4.2.6 Recombinant Versican induces microglial MT1-MMP expression through TLR2

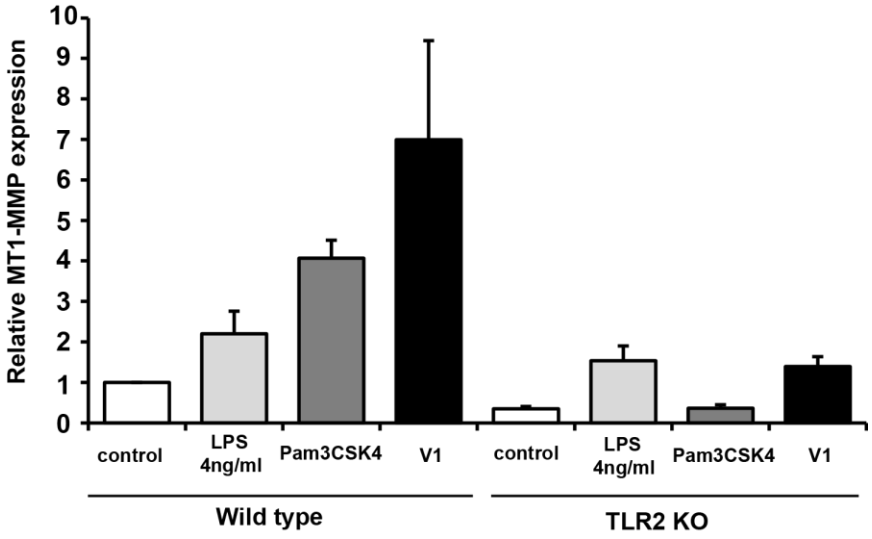

Figure 22. Recombinant V1 up regulates microglial MT1-MMP via TLR2

Primary cultured microglia from WT and TLR2 KO animals were stimulated with recombinant versican V1, LPS and Pam3CSK4 for 6h. Microglial MT1-MMP expression was analyzed by real-time PCR. (n=2).

To verify versican V1 triggers microglial TLR2 for MT1-MMP induction, recombinant versican V1 was applied to primary microglia from both WT and TLR2 KO animals.

Since purified V1 was contaminated with endotoxin (2-4ng/ml) (Ween et al. 2011), LPS was used as a control while Pam3CSK was used as a positive control. As shown in Fig. 22, although LPS also induced microglial MT1-MMP induction, V1 could induce higher level of MT1-MMP. And this effect was abolished in TLR2 KO microglia, indicating Versican V1 is the ligand of TLR2. However, due to lack of TLR2 KO microglia, more experiments are needed for statistical analysis.

4.2.7 Knockdown of versican slightly decreases proliferation of glioma cells

It has been shown that versican also plays a role in proliferation of different cell types including gliomas. To investigate the role versican in glioma proliferation, I seeded the same number of shcon GL261 and shver GL261, after different time points, cell proliferation rates were analyzed by the counting of cell numbers and alamar blue assay. As seen Fig. 22A, after 3 days of growth, shver GL261 cells proliferate significantly less compared to shcon GL261 cells ($p<0.05$). This result was confirmed by cell counting, after 4 days growth, shcon GL261 got significant larger numbers compared to the shver GL261. These results indicated versican also regulates cell proliferation.

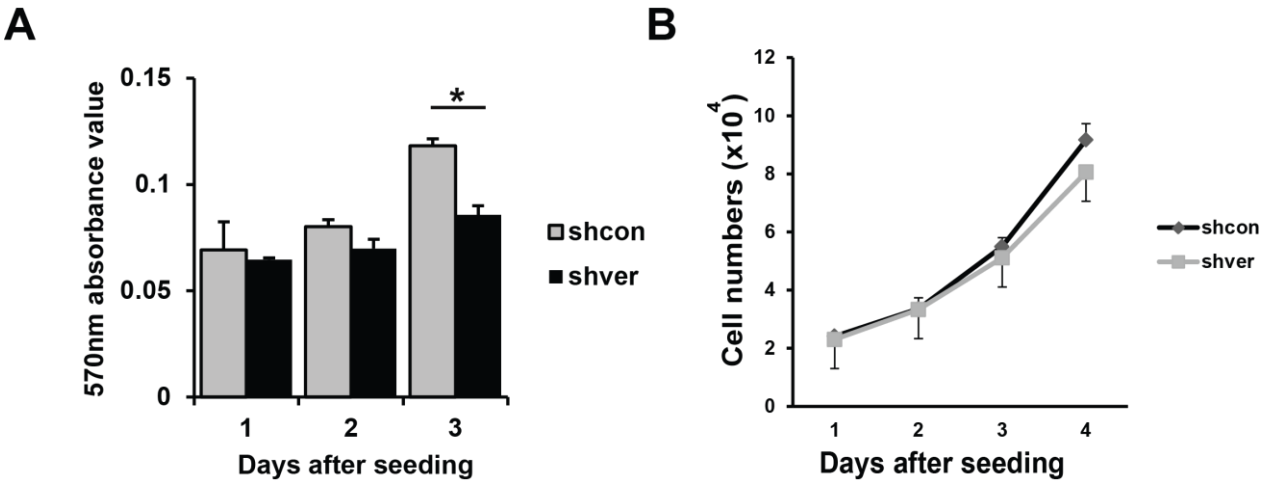

Figure 22. Silencing of versican decrease proliferation of GL261 cells *in vitro*.
(A) Cell proliferation rate in shcon and shver GL261 cells was determined by amalar blue assay. (B) $2X10^4$ shcon and shver GL261 cells were seeded, cell numbers were counted after 1, 2, 3 and 4 days.

4.2.8 Silencing of Versican by shRNA in GL261 reduced the tumor size and prolonged survival time of mice bearing glioma

To investigate if ablation of versican in gliomas interfered with tumor expansion *in vivo*, shcon GL261 cells and shver GL261 cells were inoculated into the mouse brain, and after two weeks, tumor volumes were quantified by unbiased stereological estimation (Cavalieri method). Tumor volumes were significantly reduced in versican shRNA GL261 implanted mice compared to control mice (Fig. 23A). Furthermore, I also investigated survival rates of mice bearing control shRNA glioma and versican shRNA glioma. As seen in Fig. 23B, deletion of the versican in GL261 cells led to a significantly increased survival rate (p=0.002).

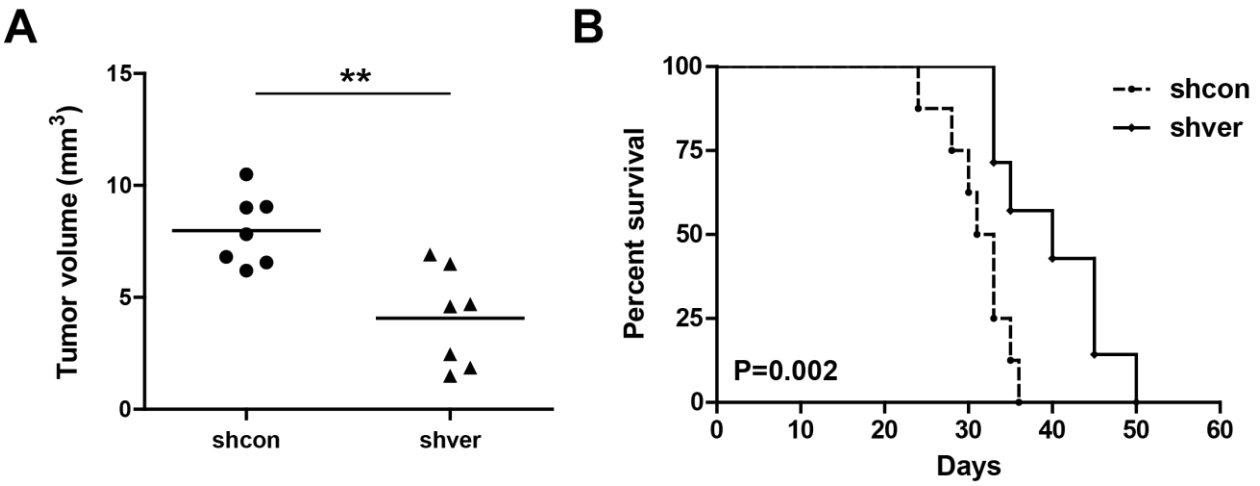

Figure 23. Versican regulates tumor growth and survival rate of tumor-bearing mice *in vivo*

(A) Versican knocked-down and control GL261 were inoculated into WT mice, after 2 weeks of tumor growth, tumor volume was evaluated based on unbiased stereology (n=7). (B) The Kaplan-Meier curves represents the cumulative survival of mice after versican knocked-down (shver) or control (shcon) GL261 cell injection (n=8, p=0.002).

4.2.9 Versican silenced GL261 resulting in a smaller tumor is dependent on the presence of microglia

Since versican is involved in cell proliferation, next I wanted to know whether versican silenced GL261 cells resulting in smaller tumors are dependent on tumor proliferation or its interaction with microglia. I prepared organotypic brain slices, and microglia in the slices were depleted using liposome-encapsulated clodronate followed by inoculation of

either control shRNA EGFP-GL261 glioma cells or versican shRNA EGFP-GL261 cells into the slices. I analyzed the area occupied by glioma cells 5 days later, versican knocked down glioma resulted in significant smaller tumor (shcon pbs: 1.1±0.36mm^2; shver pbs: 0.79±0.25mm^2; p<0.0001) in the group with the presence of microglia (PBS treatment), while a slight but not significant reduction of tumor was observed in the microglia depleted group (shcon cl: 0.65±0.26mm^2; shver cl: 0.59±0.2mm^2; p=1.0), indicating the role of versican in tumor growth is mainly dependent on the presence of microglia. Also as I found before, depletion of microglia in both groups led to a significantly smaller tumor.

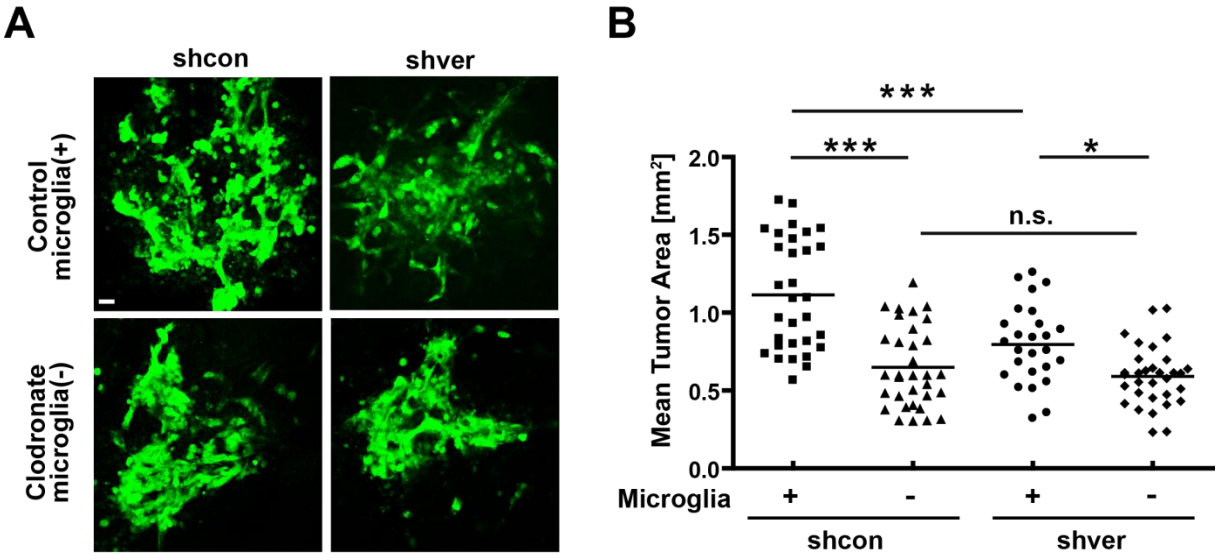

Figure 24. Versican regulation of tumor growth is dependent on the present of microglia. Brain slices from 16 day old WT mice were implanted with 5000 shcon or shver EGFP-GL261 cells. Additionally those cells were also implanted in slices which had been depleted of microglia by treatment with clodronate filled liposomes. (A) The representative fluorescence micrograph of EGFP-labeled glioma cells in both microglia-containing and -depleted brain slices injected with shcon (left) and shver (right) cells. Scale bar is 10 μM. (B) The area occupied by glioma cells was quantified after 5 days in both microglia-containing (+) and -depleted (-) slices injected with shcon and shver glioma cells.

4.2.10 Conditioned medium from microglia but not astrocytes induces glioma versican expression *in vitro*.

It has been shown that macrophages could induce lung cancer versican expression in a co-culture system (Said et al. 2012). To investigate whether microglia may also influence glioma versican expression, Gl261 cells were stimulated with the conditioned medium

from microglia and astrocytes. Interestingly, only microglia could induce the expression of all versican isoforms except V2 in glioma cells, since it was previously shown V2 is not expressed by gliomas at all. As seen in Fig. 25A, microglial conditioned medium (MCM) induced GL261 cells almost 2 times higher in all versican (AV) (1.94±0.1, $p<0.001$), 1.5 times higher in V0 (1.44±0.07, $p<0.01$), 1.6 times higher in V1 (1.58±0.09, $p<0.01$) and 1.5 times higher but not significant in V3 (1.49±0.2, $p>0.05$). To test if this effect is specifically from microglia, I also simulated GL261 with astrocyte conditioned medium (ACM), versican isoform expression was analyzed by real-time PCR, interestingly, none of the versican isoforms were significantly changed after 24 hours of treatment (Fig. 25B). These data suggested factors released from microglia but not astrocytes could induce glioma versican expression, these data also indicated that microglia not only receive signals from gliomas but also give feedback to gliomas for active interaction.

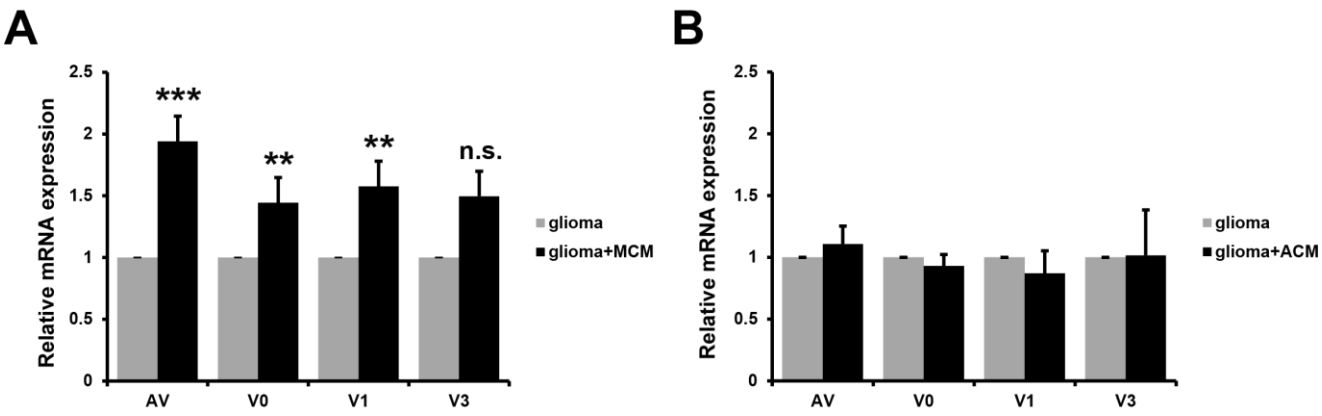

Figure 25. Microglia but not astrocytes regulate glioma versican expression.
GL261 cells were stimulated with either conditioned medium from (A) microglia (MCM) or from (B) astrocyte (ACM) for 24h, versican isoforms expression were analyzed by real-time PCR.

4.3 Inhibition of microglial MT1-MMP expression and tumor growth by a TLR2 neutralizing antibody

All the previous data indicated that TLR2 is a vital receptor for glioma-microglia crosstalk. TLR2 monoclonal antibody T2.5 has been showed the capacity to treat gastric cancer by blocking TLR2 (Tye et al. 2012). The next part of the result will demonstrate

whether this antibody could also interfere with microglial MT1-MMP expression and glioma growth, and the work was collaboration with master's student Alexander Hahn.

4.3.1 TLR2 neutralizing antibody blocked microglial TLR2 functionally *in vitro*

To investigate if the monoclonal antibody T2.5 (mAb T2.5) could functionally block TLR2 and determine the optimum concentration of the antibody, we first tested whether it could impair TLR2 ligand induced microglial IL-6 and MT1-MMP expression. Microglial cells were stimulated for 6 h with the TLR2 ligand Pam_3Csk_4 alone, together with the mAb T2.5 or with isotype control. Changes in IL-6 and MT1-MMP expression levels were determined by Real-time PCR. As shown in Fig. 26A Pam_3Csk_4 could induce dramatic expression of IL-6 (157±54.6 fold, $p<0.01$), when microglia were treated by Pam_3Csk_4 together with mAbT2.5, IL-6 induction was significantly reduced in both 5 µg/ml (18.48±8, $p<0.05$) and 10 µg/ml (18.18±11.77) concentrations compared to the same concentration of isotype control (141±48 in 5 µg/ml and 135±29.3 in 10 µg/ml).

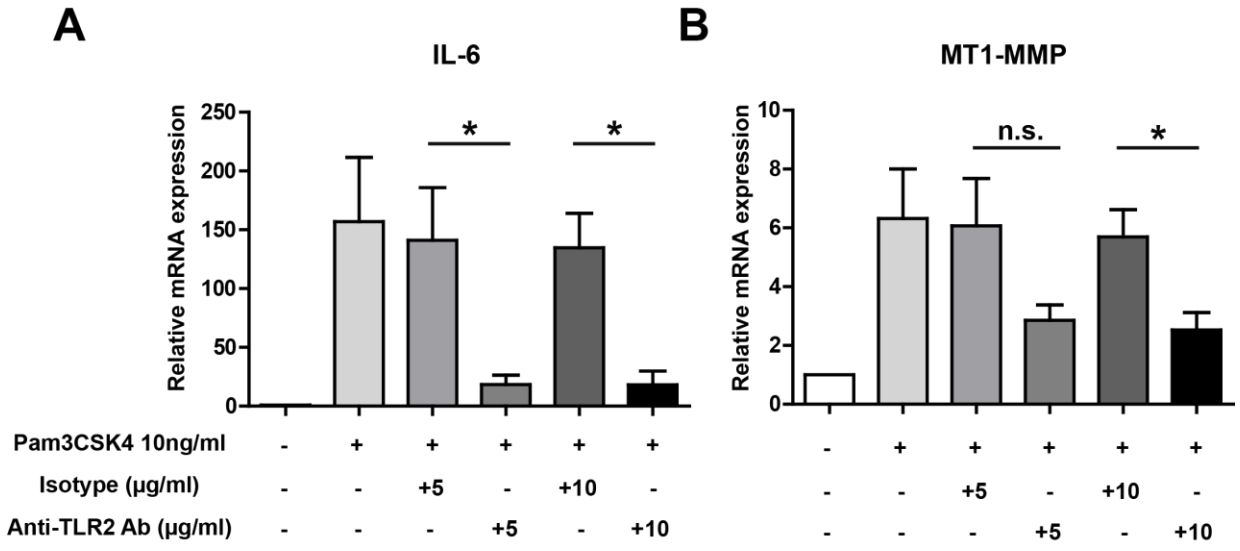

Figure 26.TLR2 monoclonal antibody T2.5 downregulates Pam3Csk4 induced MT1-MMP and IL6 expression.
Primary mouse microglial cells were stimulated for 6 h either with the TLR1/2 ligand Pam3Csk4 alone, Pam3Csk4 together with the isotype or Pam3Csk4 with mAb T2.5. MT1-MMP/IL6 expression was analyzed by real-time PCR. (A) IL6 expression under mAb T2.5 treatment was significantly lower with both concentrations (5 and 10 µg/ml) compared to the isotype treatment. (B) No significant reduction of MT1-MMP expression was shown between isotype and mAb T2.5 treatment with a concentration of 5 µg/ml. However, a concentration of 10 µg/ml of mAb T2.5 resulted in significantly lower expression of MT1-MMP. Bars represent mean ±SEM from 4 independent experiments.

Similar results were seen in MT1-MMP, upon the stimulation of Pam_3Csk_4, although with a concentration of 5 µg/ml mAb T2.5 treatment did not lead to a significant decrease of MT1-MMP (2.85±0.53, p=0.06), 10 µg/ml mAb T2.5 treatment significantly reduced microglial MT1-MMP expression (2.53±0.6, p=0.028) compared to the isotype control (6.1±1.6 in 5 µg/ml and 5.7±0.9 in 10 µg/ml). These results showed that mAb T2.5 could block microglial TLR2 efficiently *in vitro* and 10µg/ml of mAb T2.5 will be used for the following experiments.

4.3.2 TLR2 neutralizing antibody inhibits glioma induced microglial MT1-MMP expression and reduces tumor growth *ex vivo*

To investigate if blocking microglial TLR2 with mAb T2.5 could impair glioma induced microglial MT1-MMP and interfere with tumor growth. Microglial were stimulated either with GCM in combination with mAb T2.5, or GCM with isotype for 6h, MT1-MMP was analysed by real-time PCR. As seen if Fig. 27A, microglial MT1-MMP was significantly upregulated upon GCM treatment with isotype compared to the untreated control (3.8±0.7, p=0.03). However, when microglial TLR2 was blocked by mAb T2.5, this induction was abolished from 3.8 to 1.7 folders (±0.3, p=0.03), suggesting mAb T2.5 could block microglial TLR2 in the context of glioma *in vitro*. To further test if mAb T2.5 could have clinical relevance in the treatment of glioma, an organotypic slice model was used. Brain slices were prepared and EGFP GL261 cells were injected as previous described. During the tumor growth, mAb T2.5 or isotype was applied into the cultured medium, and tumor size was quantified after 5 days of tumor injection. As shown in Fig. 27B, after mAb T2.5 treatment, tumor volume was dramatically reduced to 0.28 (±0.02, p<0.001) mm^2 compared to isotype control (0.65±0.04 mm^2). These results indicated mAb T2.5 could inhibit glioma growth *ex vivo*, and it may provide an alternative for glioma therapies.

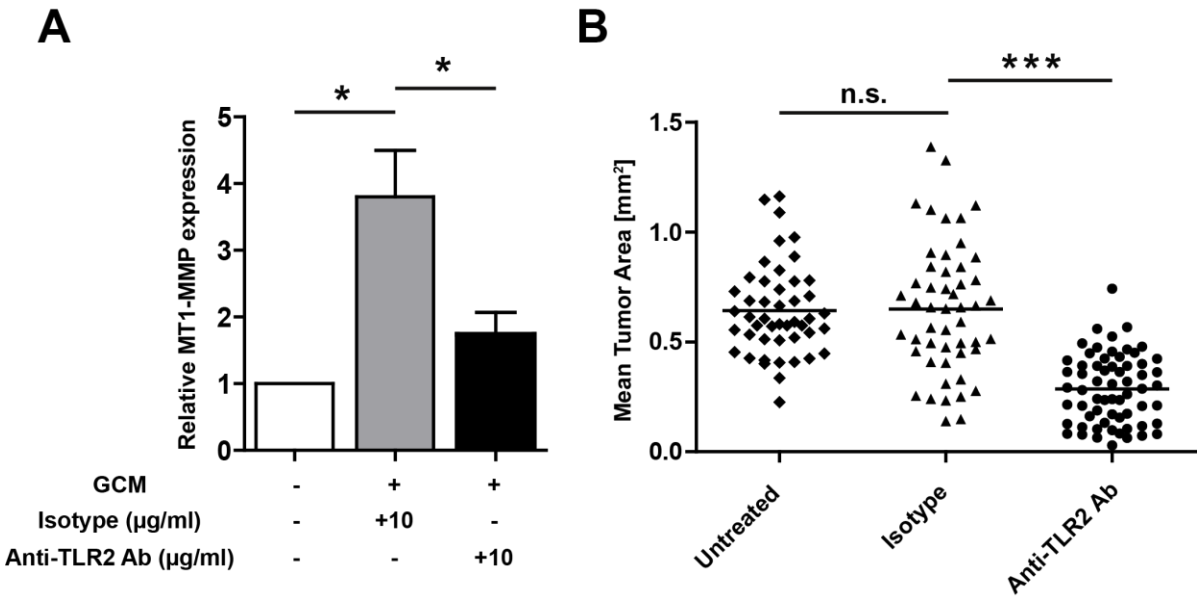

Figure 27. TLR2 monoclonal antibody T2.5 inhibited GCM driven microglial MT1-MMP expression as well as glioma growth *ex vivo*.
(A) Primary microglial cells were stimulated with GCM and isotype or GCM together with mAb T2.5 for 6h, MT1-MMP was analyzed by real-time PCR. (B) Organotypic brain slices of 16 day old C57BL/6 mice were inoculated with 5000 EGFP-GL261 cells and treated with isotype or mAb T2.5. The tumor area was determined 5 days after injection of glioma cells.

4.4 Glioma induce microglial MMP-9 expression through TLR2/6 signaling

4.4.1 Microglial MMP-9 is up-regulated by glioma supernatant and in the glioma environment

To test whether glioma cells also trigger MMP-9 up-regulation in microglia, mouse primary microglial cell cultures were treated with normal medium (as controls) or glioma conditioned medium (GCM). After 3, 6, or 24 h, cells were lysed and MMP-9 RT-PCR was performed. Compared to control medium, microglial MMP-9 mRNA was up-regulated upon GCM treatment already after 3 h and was further increased after 6 and 24 h (Fig. 28A). In GL261 cells, MMP-9 expression could not be detected. We further performed real-time PCR and found that after 24 h of GCM treatment, microglial MMP-9 mRNA level was increased 17 times fold (±6, p=0.02) when compared to non-treated controls (Fig. 28A). This effect was further verified by western blot and gelatin zymography where we observed a significant increase in MMP-9 production and secretion, respectively, upon GCM treatment (Fig.28B). To examine the MMP-9

expression in an experimental glioma model, Gl261 cells were inoculated into mouse brains. Mice were sacrificed after two weeks of glioma growth, and brain sections were stained with Iba-1 and MMP-9 antibodies. As shown in Fig.28C, we detected strong immunolabelling for MMP-9 in the glioma tissue, but not in the tumor free area. The glioma associated Iba-1-positive microglia/brain macrophages were the dominant cell population expressing MMP-9.

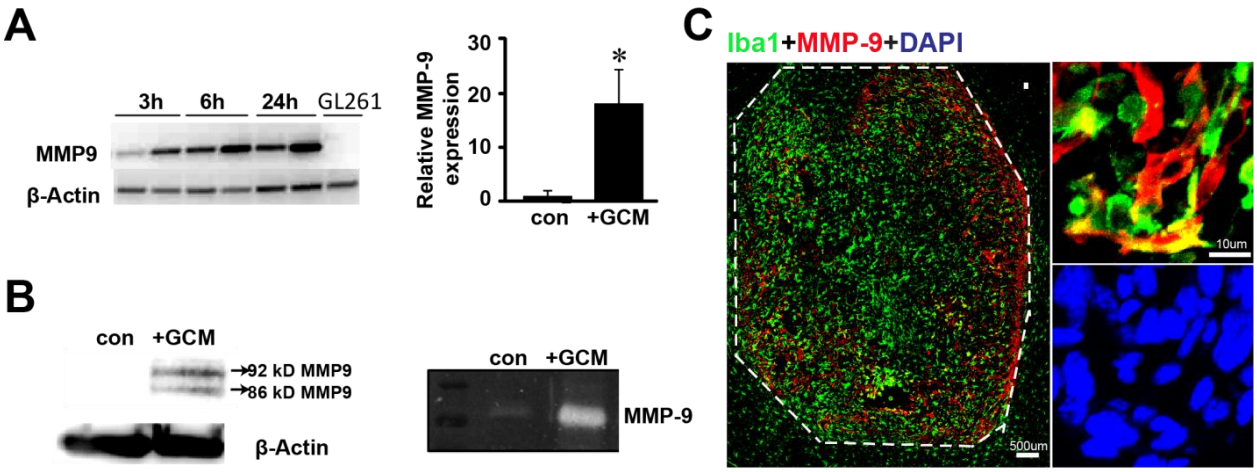

Figure 28. Microglial cells are up regulating MMP9 when associated with gliomas
(A) MMP-9 gene expression in microglia stimulated with GCM for 3h, 6h and 24h was analyzed by RT-PCR (left). GL261 cells were also analyzed for MMP-9 expression. β-actin serves as a loading control. Microglial MMP9 expression was quantified with qRT-PCR after 24h stimulation with GCM (right). Bars represent the mean±s.e.m. from 3 independent experiments. (B) Western blot from both cell lysate (left panel) and gelatin zymography from supernatant (right panel) showed MMP9 induction in microglia upon GCM stimulation for 24h. (C) Mouse brains injected with GL261 glioma cells were stained for microglial marker Iba-1(green) and for MMP-9 (red). In the tumor free area the level of MMP-9 was low (left, out of the dash line) while within the tumor MMP9 is expressed mainly in Iba-1 positive cells (right).

4.4.2 Microglia are the main source of MMP-9 in mouse and human glioma tissue

It has been reported that glioma cell lines as well as primary GBM tissue expressed MMP-9 (Lakka et al. 2004; Song et al. 2009), while for a mouse glioma model the main source of MMP-9 were glioma infiltrating CD45+ cells, which was essential and sufficient to initiate angiogenesis by increasing VEGF activity (Du et al. 2008). We analyzed the MMP-9 expression in human GBM patient tissue by immunohistochemistry. As seen in Fig. 29B, MMP-9 was predominantly expressed by Iba1+ cells, indicating glioma associated microglia/brain macrophages are the predominant cells expressing MMP-9.

We also isolated these cells by MACS directly after tumor resection and analyzed MMP9 gene expression in CD11b+ cells (i.e. glioma associated microglia/macrophage) and flow-through cells (i.e. mainly glioma cells). The MMP-9 expression was normalized to the level of the expression in CD11b+ cells, and the level of MMP-9 expression in flow through cells was compared to that. In the human glioma samples MMP-9 expression was predominately from GAMs as determined by real-time PCR from MACS isolated cells and flow through cells (Fig. 29C). When we compared MMP-9 expression by RT-PCR in 8 different established primary human glioma cell lines, we found MMP-9 expression in only one (Fig. 29A). Moreover, the Gl261 mouse glioma cell line also did not express MMP-9 (Fig. 28A).

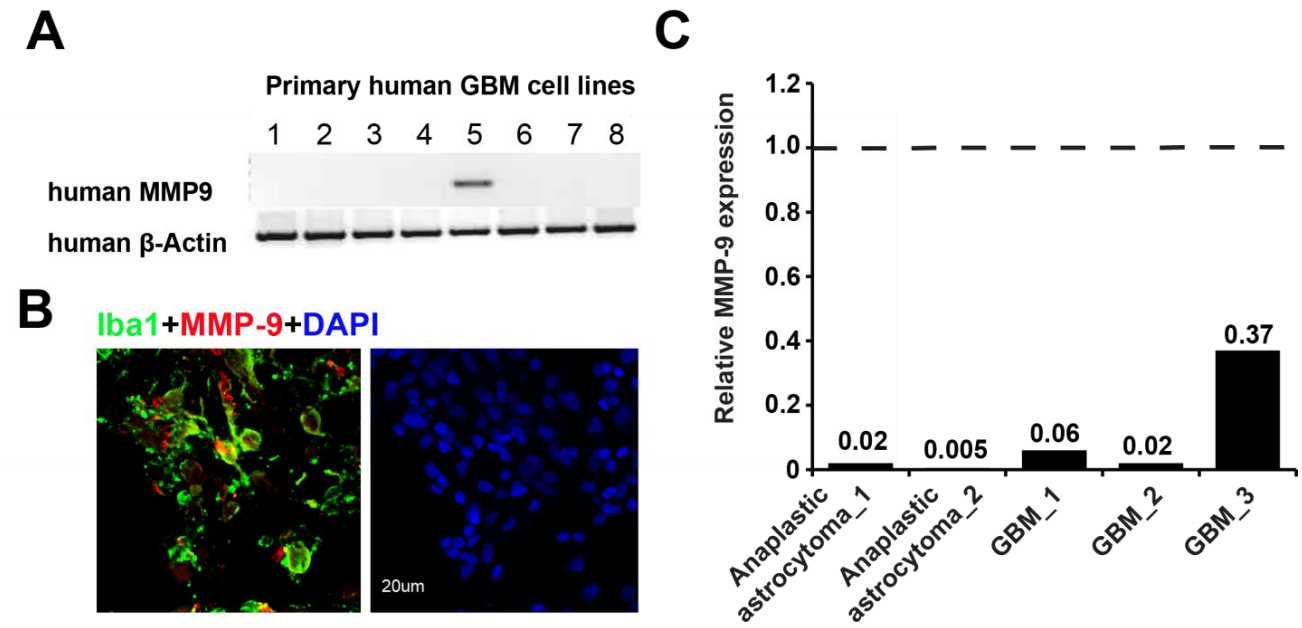

Figure 29. Glioma associated microglia/macrophages but not gliomas are the main MMP-9 producing cells
(A) 8 primary cultured human GBM cell lines were analyzed for MMP-9 expression in mRNA level by RT-PCR (β-Actin serves as a loading control). In only one line MMP-9 was detected. (B) Human GBM tissues were analyzed by immunohistochemistry showing that Iba1 positive cells (green) express MMP-9 (red). (C) MACS freshly isolated CD11b+ cells and flow-through (i.e. mainly glioma cells) from 3 GBM and 2 anaplastic astrocytomas were analyzed for MMP-9 expression by qRT-PCR (Dash line represents MMP-9 expression in CD11b+ cells in each sample, solid bars represent folder changes of MMP-9 expression in flow through cells compared to CD11b+cells).

4.4.3 Glioma released factors induced microglial MMP-9 expression through Myd88-TLR2 signaling.

We have previously shown that glioma-released factor(s) induced microglial MT1-MMP production mediated through the TLR2 signaling pathway. To check if microglial MMP-9 induction is also regulated by TLRs, we cultured microglial cells from WT mice and from mice deficient for TLR1, TLR2, TLR4, TLR6, TLR7, TLR9 and the intracellular adaptor molecule Myd88. The microglial cells were stimulated for 24 h with GCM and incubated for another 24 hours with serum free cultivation medium. MMP-9 expression in the cell supernatant was analyzed by gelatin zymography, Elisa and Western blot. As seen in Fig. 30A, GCM induced MMP-9 functional expression as revealed by zymograpy in WT and TLR1KO, TLR4 KO, TLR7 KO, TLR9 KO, but not in the Myd88, TLR2 and TLR6 deficient microglia. These effects were further verified by Elisa (Fig. 30B); GCM stimulated microglia released significant higher amount of MMP-9 compared to the untreated control (Control: 0.33±0.17ng/ml, GCM: 3.95±0.46ng/ml, $p=0.002$), and this expression level was not altered when TLR1 (control: 0.05±0.003ng/ml, GCM: 3.68±1.06ng/ml, $p=0.026$), TLR4 (control: 0.17±0.08ng/ml, GCM: 4.16±0.68ng/ml, $p=0.004$), TLR7 (control: 0.35±0.15ng/ml, GCM: 4.89±0.06ng/ml, $p<0.001$) or TLR9 (control: 0.55±0.24ng/ml, GCM: 4.65±0.41ng/ml ,$p=0.001$) was absent. In contrast, microglial MMP-9 induction by GCM was impaired from mice deficient for Myd88 (control: 0.08±0.001ng/ml, GCM: 0.08±0.006ng/ml, $p=0.378$), TLR2 (control: 0.14±0.023ng/ml, GCM: 0.3±0.17ng/ml, $p=0.409$) or TLR6 (control: 0.12±0.07ng/ml, GCM: 0.238±0.08ng/ml, $p=0.107$). Cell lysate analyzed by Western blot confirmed that the GCM induced microglial MMP-9 up-regulation depends on TLR2 signaling since increased protein level was observed in WT microglia, but not in TLR2 KO microglia (Fig. 30C). To confirm that microglial MMP-9 secretion can be induced by TLR2 activation, primary microglia from WT mice were stimulated for 24 h with the TLR2 agonists Pam3CSK4, PG-LPS from P. gingivalis and HKLM (heat-killed Listeria monocytogenes). Subsequently cell supernatant was collected and gelatin zymography was performed. As shown in Fig. 31A, all three agonists induced the expression of MMP-9.

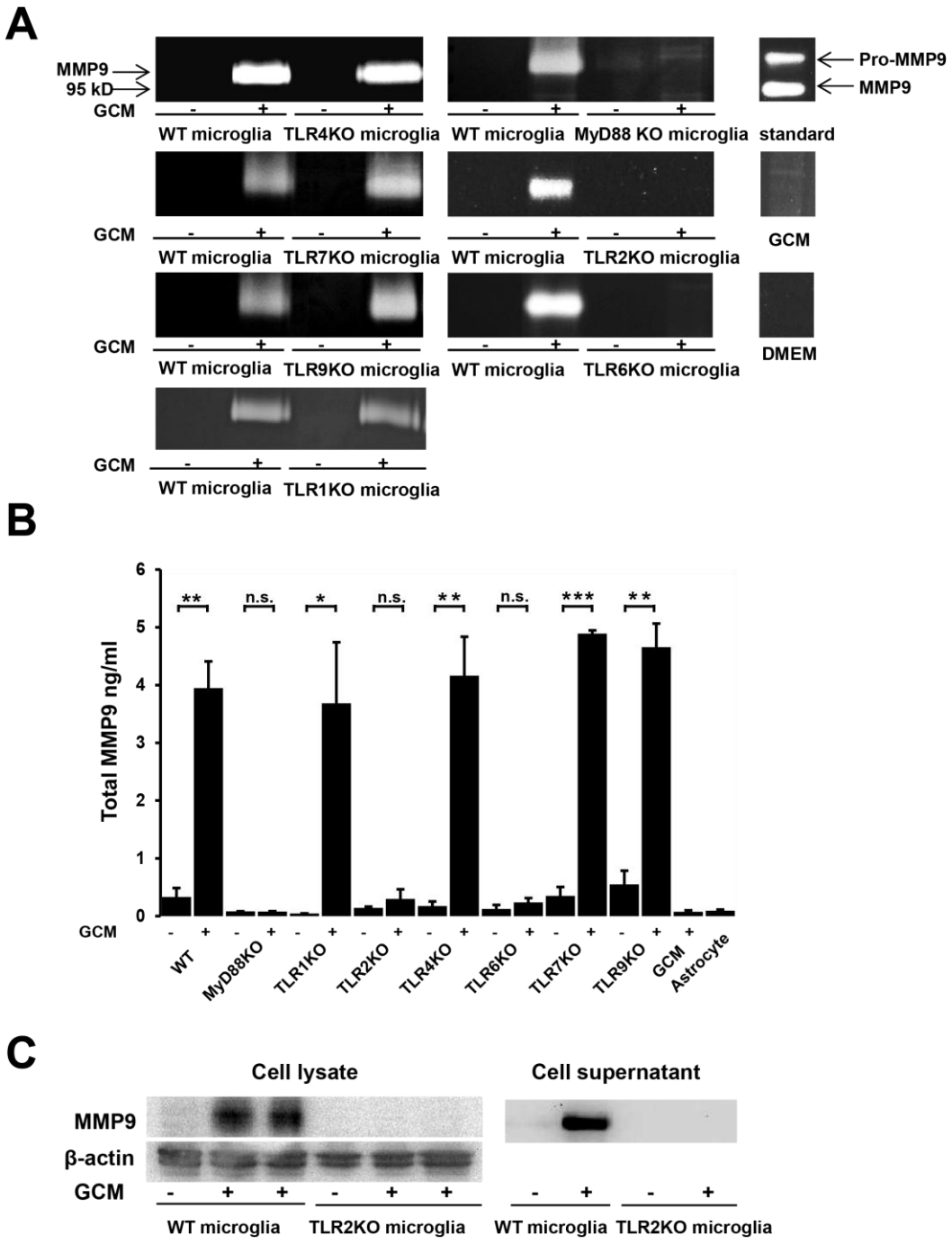

Figure 30. TLR2 signaling triggers glioma associated microglial MMP-9 up-regulation.
(A) Microglia from WT and MyD88 or TLR 1, 2, 4, 6, 7 and 9 deficient mice were stimulated with GCM for 24h, cell supernatant was collected for gelatin zymography. Recombinant MMP-9 was used as a standard, GCM and DMEM were loaded as controls. Images were representative from 3 independent experiments. (B) Elisa was performed on cell supernatant for quantification of total MMP9 release. GCM and conditioned medium from primary cultured astrocytes were used as controls. Bars represent the mean±s.e.m. from 3 independent experiments. (C) Cell lysate and supernatant from WT and TLR2 KO microglia treated with GCM for 24h were further analyzed by western blot and compared to an untreated control (β-actin served as a loading control).

4.4.4 TLR2-deficient mice show reduced MMP-9 *in vivo*

We used an experimental mouse glioma model to study MMP-9 expression in GAMs in vivo. Cells of the mouse glioma cell line GL261 were inoculated into WT and TLR2 KO mice. After 2 weeks of glioma growth, brain tissue was analyzed by immunohistochemistry for MMP-9 expression. MMP-9 fluorescence intensity was quantified and normalized to DAPI with Imag J software. As shown in Fig. 4B, we observed an increase in MMP-9 immunoreactivity within the tumor in WT mice, whereas in TLR2 KO mice, the immunoreactivity of MMP-9 was significantly lower compared to the WT controls (WT 121%±10%, TLR2KO 76%±8%, p=0.008).

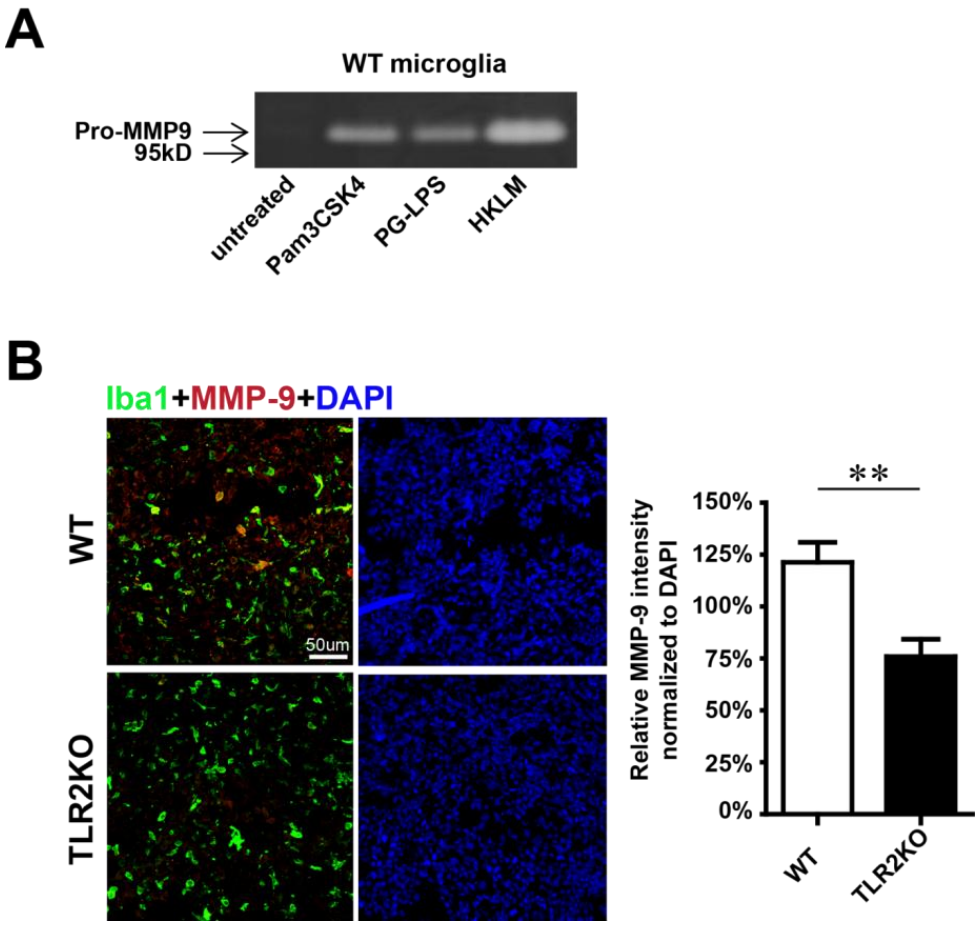

Figure 31. Microglial MMP-9 as well as TLR2 is regulated by GCM stimulation.
(A) The influence of TLR2 ligands on MMP-9 expression was determined in primary microglia from WT mice. Microglial cells were stimulated with 3 different TLR2 agonists for 24h and levels of MMP-9 in the supernatant were analyzed by gelatin zymography. (B) Slices from glioma inoculated WT and TLR2 KO mice were immunohistologically labeled. Microglia/brain macrophages were identified by the expression of Iba1 (green) and MMP-9 by immunolabelling in red (left), fluorescence intensity was quantified by ImagJ and normalized to DAPI (right).

4.4.5 Minocycline attenuated glioma-induced MMP-9 and TLR2 expression *in vitro*

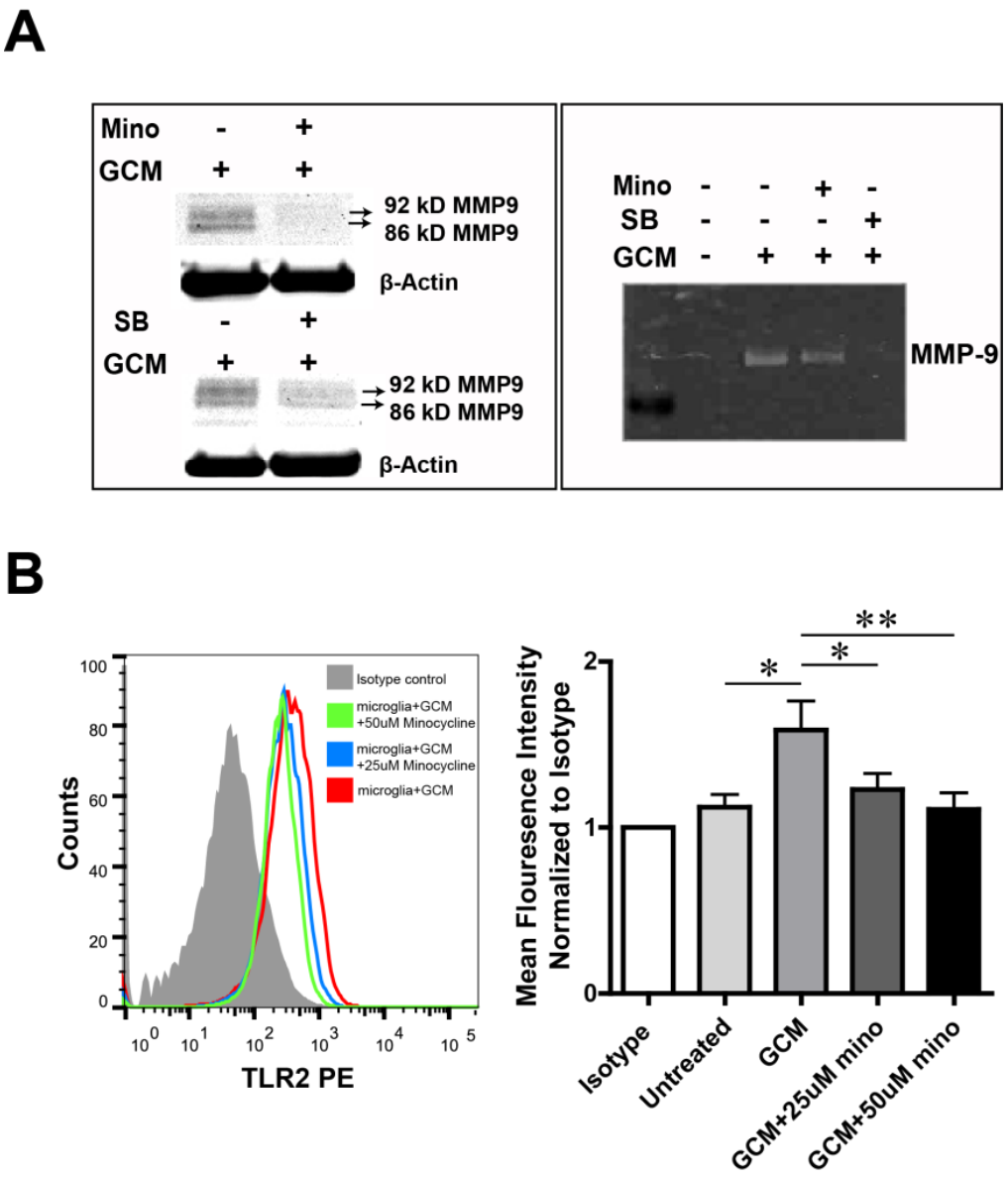

Figure 32. Minocycline interfers with glioma associated microglial MMP9 and TLR2 induction

(A) MMP-9 expression is shown by Western blot in the lysate (left) and Gelatin zymography in the supernatant (right) obtained from primary cultured microglia. On the left, cells were stimulated with GCM alone or with GCM combined with either 50 uM minocycline (Mino, upper panel) or 10uM MAPK inhibitor SB202190 (SB, lower panel) for 24h. Similarly on the right panel MMP-9 expression is shown for the supernatant obtained from cells stimulated with GBM alone or combined with minocycline and SB. (B) Microglia were either treated with GCM or GCM together with 25uM or 50uM minocycline for 24 h and TLR2 in microglia was analyzed by flow cytometry (left panel: representative histogram). On the right panel, mean fluorescence intensity was quantified. Bars represent the mean±s.e.m. from 5 independent experiments.

Our previous data have shown that minocycline and the p38 MAPK inhibitor SB202190 inhibit MT1-MMP expression in glioma associated microglia (Markovic et al. 2009; Markovic et al. 2011). We therefore tested whether minocycline and the p38 MAPK inhibitor SB202190 can also interfere with the induction of MMP-9 expression. Mouse microglial cells were first pretreated with either minocycline or SB202190 for 3h, following 24 hours treatment either with GCM alone or GCM together with SB202190 or minocycline. Cells were further incubated for another 24 hours with serum free medium. Cell lysate was then collected to perform Western blot and supernatant to perform gelatin zymography. 50 μM minocycline and 10 μM p38 MAPK inhibitor (SB202190) reduced MMP-9 production from GCM treated microglia as analyzed in Western blot and gelatin zymography (Fig. 32A). We also tested whether minocycline would affect the up regulation of TLR2 by GCM. As shown in Fig. 32B, we detected significantly lower levels of microglial TLR2 expression when GCM-stimulated cells were treated with minocycline (Mean fluorescence intensity was normalized to isotype, 25uM: 1.23±0.1 $p=0.02$; 50uM: 1.11±0.1 $p<0.001$) compared to the cells treated with GCM only (GCM: 1.59±0.18).

5. Discussion

5.1 The role of microglia in gliomagenesis

Microglia, as the dominating immune cells in the CNS, were initially regarded as brain defenders to fight against glioma when they accumulated around and within the tumor. However, untill now direct evidence for the cytotoxic effect of microglia on glioma is limited. Including the studies from our group, emerging results indicated that tumor associated microglia/macrophage show a tumor-supportive phenotype (da Fonseca and Badie 2013; Li and Graeber 2012; Markovic et al. 2009; Zhai et al. 2011).

As in some other neurodegenerative diseases, during glioma growth, abundant macrophages infiltrate the CNS from the bone marrow. Since microglia share a lot of similarities with macrophages including most of the cell surface markers, a more general term “microglia/brain macrophages” has been used instead of microglia alone. Although a few new markers were defined in the past years, CD11b, CD68, F4/80, and the lectin binding protein Iba-1 are used as general markers of microglia/macrophages. However, by using a chimera rat model, Sedgwick et al. identified for the first time that CD11b/CD45 could be used as a marker to distinguish resident microglia ($CD11b^{+}/Cd45^{low}$) from infiltrating macrophages ($CD11b^{+}/Cd45^{high}$) (Sedgwick et al. 1991). More researchers started to use this method to differentiate microglia from macrophages. However, a few weaknesses of this method are raised: 1. This discrimination is only based on the FACS analysis and sorting, it is hard to quantify low/high expression of CD45 with a immunohistochemical assay like staining. 2. A lot of tissue (which means a lot of animals) is needed for FACS sorting to get enough cell numbers for the RNA or protein extraction. 3. This is not the case with human material. Just recently, Mizutani et al. found out that CX3CR1, a fratalkine receptor was exclusively expressed by adult microglia but not inflamed monocytes, thus by using a CX3CR1 GFP-CCR2 RFP mouse model, resident microglia could be distinguished from infiltrated macrophages in an EAE model *in situ* (Mizutani et al. 2012). It will be interesting if this model could be also used in glioma studies to identify the roles of different myeloid cells in the glioma microenvironment.

According to the classification of macrophages, microglia can also be defined as an M1 (classic activation) and M2 (alternative activation) phenotype (Colton 2009). In the presence of interferon-gama (IFN-γ), lipopolysaccharide (LPS) and other microbial products, microglia are converted to an M1 state by up-regulating the expression of MHC II so that microglial antigen presenting ability to T cells is enhanced. They could also secrete a wide range of pro-inflammatory cytokines, like TNF-alpha, IL-6 and, IL-1beta. On the other hand, M2 microglia are characterized by up-regulating arginase 1 and immunosuppressive cytokines IL-10, TGF-beta, down-regulating pro-inflammatory cytokine expression and phagocytic activity. The phenotype of GAMs has been well reviewed by Li et al, they summarized and concluded that polarization of GAMs is toward an M2 state (Li and Graeber 2012). Supporting evidence include: 1 gliomas release several immunosupressive cytokines such as IL4, IL10 and, TGF-beta which are well known M2 promoting cytokines (Stein et al. 1992). 2. A few M2 microglial markers such as Arginase 1, cd163 and cd204 are up regulated in GAMs (Gabrusiewicz et al. 2011; Komohara et al. 2008). 3. GAMs shows down regulation of pro-inflammatory cytokines. However, the M1/M2 definition is mainly based on *in vitro* studies of macrophages, while GAMs represent immune cells in a specialized pathological situation. The role and phenotype of GAMs may change during different stages of tumor growth or in different subtypes of gliomas. More studies of GAMs are urgently needed for a more accurate definition of GAM phenotypes.

In the current study, I reported that glioma derived versican activates microglial TLR2, which recruits its adaptor protein MyD88, activates P38 MAPK and results in the up-regulation of MT1-MMP. This membrane bound MMP further cleaves glioma released Pro-MMP-2 to active MMP-2 for ECM degradation (Fig. 33). Microglia could also in turn induce glioma versican expression, indicating glioma-microglia interaction is bidirectional. Overall, the current study confirmed again that GAMs show a glioma-supportive phenotype through TLR2.

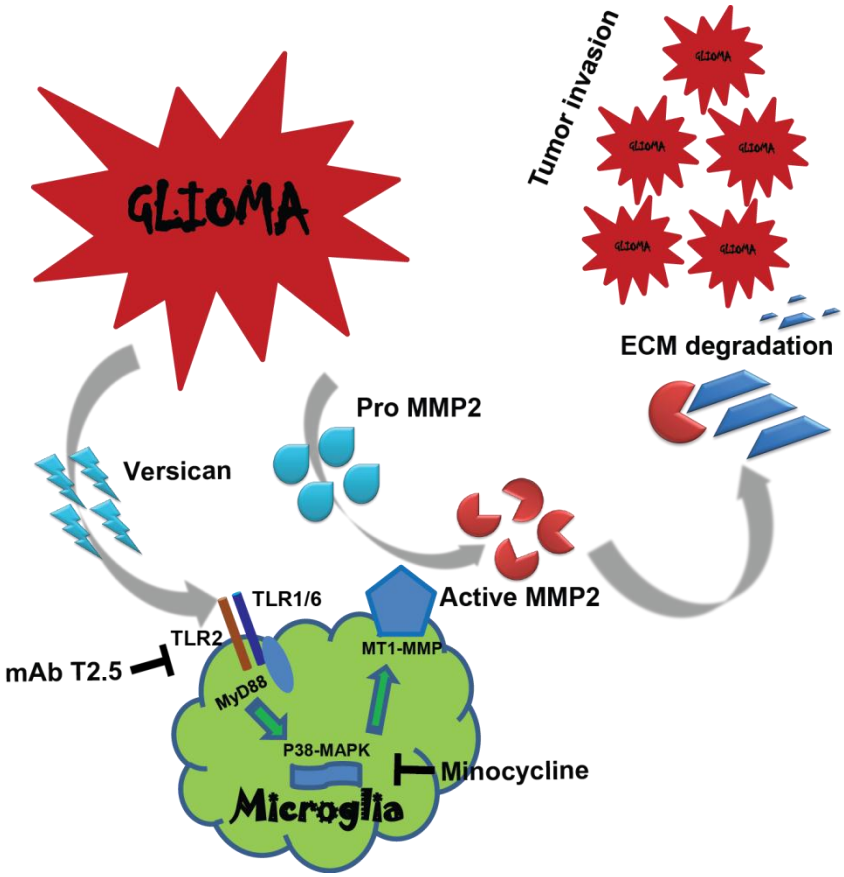

Figure 33. Microglia-glioma crosstalk

5.2 The expression and function of Toll-like receptors in gliomas

By recognizing DAMPs, TLRs regulate a wide range of biological events including immune responses in carcinogenesis. TLRs have been shown to be a tumor promoting factor as well as an anti-tumor regulator. As Pradere et al. summarized in a recent review, most of the anti-tumor effects of TLRs are based on exogenous stimuli like TLR agonists treatment. This may lead microglia to a high level activation state with strong immune responses that shape an anti-tumor environment. On the other hand, during tumor growth or therapies, different endogenous TLR ligands could be released by dying cancer cells or necrotic tissue to activate TLRs, this moderate or chronic activation may induce further cytokine or chemokine release for tumor invasion or angiogenesis (Pradere et al. 2013). In gliomas, a few studies have shown that activation of TLR9 by CpG may reduce tumor growth *in vivo* and also prolonged the survival of glioma bearing

mice (El Andaloussi et al. 2006; Grauer et al. 2008). This therapeutic effect depends on the expression of TLR9 on tumor cells as well as in host cells.

The expression and regulation of TLRs in gliomas are poorly understood. As introduced at the beginning of the thesis, Andaloussi et al. showed some evidences that TLRs are expressed in gliomas. However, all the data are from RT-PCR which is based on the mRNA level. Among so many subtypes of TLRs, only TLR9 expression in gliomas was confirmed by flow cytometry and RT-PCR, and TLR9 expression correlated to glioma malignancy as well as prognosis of glioma patients (Meng et al. 2008; Wang et al. 2010). Since microglial cells are immune cells in the brain, one would speculate how TLRs are expressed on these cells. Hussain et al. examined TLR expression on human GAMs by flow cytometry and found out TLR2, 3, 4 are expressed by human GAMs (Hussain et al. 2006). However, due to the limited sample numbers the expression analysis of TLRs in GAMs was not systematic.

We have shown previously that microglial TLR2 is a crucial factor in regulating MT1-MMP expression to activate ProMMP-2 for glioma expansion. Thus how TLR2 and other TLRs are regulated in gliomas will be a very interesting question. By digging out the Rembrandt database, I found TLR2 is highly expressed in gliomas compared to non-tumor tissue, and it also inversely correlated to patient survival. However, the expression of TLRs in this database was from the whole tumor tissue, this could not indicate which specific cell type are TLR expressing cells. By using different cell markers, I analyzed different TLR expression in GL261 cells, GAMs as well as glioma associated astrocytes. By using cell culture and *in vivo* glioma models, I found TLR2 was the only up-regulated subtype of TLRs in tumor associated microglial cells while astrocytes and GL261 cells do not highly express TLR2. These data confirmed that TLR2 is the unique subtype of TLRs in both murine and human gliomas.

5.3 Versican, the endogenous ligand of TLR2

The endogenous agonists of TLRs can be proteins, fatty acids, proteoglycans or nucleic acids (Piccinini and Midwood 2010). These intrinsic ligands derived mainly from damage tissues, e.g. trauma or neoplasm formation. Versican is a component of extracellular

matrix and is found in a variety of tumor tissues including gliomas. Just recently it was found by Kim et al. that versican also behaves as a ligand of macrophage TLR2 in lung cancer metastasis. By using mass spectrometry, versican was identified to be released by lung cancers that induced macrophage TNF-alpha and IL-6 induction for tumor progression. When recombinant veriscan V1 was applied to wild type macrophage, a robust induction of IL-6 and TNF-alpha was observed while this effect was totally abolished when the same recombinant versican was applied to TLR2 KO macrophages, indicating that versican activates macrophage through TLR2 (Kim et al. 2009). Another study by Li et al. also reported ovarian cancer derived versican V1 could activate macrophage to express hCAP18 as a TLR2 ligand by using a TLR2 neutralizing antibody (Li et al. 2013).

In the present study, I first screened the soluble factors released by GL261 cells by mass spectrometry. A few reported TLR ligands were identified, then I confirmed that the versican variant V0/V1 but not V0/V2 is expressed on both murine and human gliomas at the mRNA and protein level. When versican expression was knocked down in GL261 cells, it induced less microglial MT1-MMP expression *in vitro* and *in vivo* compared to the control vector transfected cells. Implanting versican silenced GL261 cells into mouse brain resulted in smaller tumors and longer survival rates. Since it is known that versican also plays role in cell proliferation and migration, studies on glioma injected organotypic brain slices confirmed that the impact of versican signaling on glioma growth depended on the presence of microglia. Finally, by applying recombinant versican, I could show that versican is the ligand of TLR2 that could induce microglial MT1-MMP expression (more experiments are needed for statistical analysis). Taken together, versican is a glioma derived endogenous TLR2 mediator which regulates microglial MT1-MMP expression for tumor expansion.

5.4 GAMs are main source of MMP-9 in gliomas and it is mediated by TLR2 signaling

There are several studies which highlight the importance of MMP-9 for glioma growth and invasiveness (Hagemann et al. 2012). It has been reported that silencing of MMP-9 either by shRNA or antisense RNA approach in the human glioma cell lines diminishes

its proliferation, tumor growth and neovascularization both *in vitro* and *in vivo* (Sun et al. ; Zhao et al.). There is also evidence that glioma associated microglia or infiltrating myeloid cells are major MMP-9 producers (Du et al. 2008; Jacobs et al. 2012). Treatment with a microglia inhibitor (propentophyline) down-regulated microglial MMP-9 expression and decreased glioma growth and invasiveness in a rat glioma model (Jacobs et al. 2012). Hagemann et al. showed that human GBM samples expressed high level of MMP-9 in passage 1, while the expression successively decreased and almost disappeared at passage 10 (Hagemann et al. 2010). This could indicate that primary samples contain MMP-9 expressing microglia, which are diluted with each passage until they disappear at late passages. My data support the statement that MMP-9 is predominantly expressed in glioma-associated microglia/macrophages and considerably less in glioma cells. This expression pattern was similar in mouse and human glioma tissue from which I was able to isolate and separate the two cell populations, microglia and glioma cells.

We have recently demonstrated that TLR2 is an important signaling pathway for inducing MT1-MMP in GAMs. Here I demonstrate that TLR2 is equally important for the induction of MMP-9 in these cells thus sharing a similar pathway. While for the induction of MT1-MMP both heterodimers TLR1/TLR2 and TLR2/TLR6 play a role, I show in the present study that the induction of MMP-9 predominantly requires the presence of TLR2/TLR6 only. TLR1, 4, 7 and 9 did not play a significant role as in cells deficient in these TLRs as the induction of MMP-9 by GCM was not different compared to control cells. I could confirm the importance of TLR2 since I could mimic the effect of GCM on the induction of MMP-9 by specific TLR2 agonists. Moreover, these results were also supported by *in vivo* data, since I observed lower levels of MMP-9 in TLR2KO animals injected with glioma cells compared to wild type animals. I show that glioma cells increase the expression of TLR2 on GAMS and utilize this signaling pathway to upregulate MMP-9 and thus provide the substrate of extracellular matrix degradation to promote glioma cell invasion.

5.5 The molecular mechanism of Minocycline in treating murine glioma

Minocycline is a tetracycline based antibiotic and a FDA approved drug. It has been demonstrated that minocycline treatment showed therapeutic benefits in different neurological diseases like EAE (Popovic et al. 2002), Parkinson's disease (Yang et al. 2003), ischemia (Yrjanheikki et al. 1998), gliomas (Liu et al. 2013; Markovic et al. 2011) and so on. The mechanisms behind this effect are still not completely understood. However, in glioma studies, anti-tumor effects by minocycline are mainly based on two aspects: glioma itself and microglia. Liu et al. have shown recently that minocycline could serve as a promising drug for glioma treatment by inducing glioma cell death. The authors found that minocycline induced glioma cell death by autophagy but not apoptosis through reducing activation of AKT/mTOR/p70s6K pathway and activating ERK1/2 pathway (Liu et al. 2011). In the latest publication by the same group, they further identified minocycline induced glioma autophagy by inducing ER stress (Liu et al. 2013). On the other hand, studies showed that minocycline could inhibit microglial activation through p38 MAPK in cultured neurons exposed to glutamate as well as in a model of Parkinson's disease (Tikka et al. 2001; Tikka and Koistinaho 2001). Interestingly, minocycline was also found to decrease MMPs expression during intracerebral hemorrhage and in an EAE model (Brundula et al. 2002; Power et al. 2003). Since our group demonstrated glioma exploits microglia to express MT1-MMP through TLR-P38 MAPK signaling pathway (Markovic et al. 2009), it will be interesting to investigate whether minocycline affects glioma induced microglia MT1-MMP expression and thereby influence glioma expansion. Indeed, a study from our group found that minocycline could reduce microglial MT1-MMP expression and in turn decrease tumor volume *ex vivo* and *in vivo* (Markovic et al. 2011).

As described previously, MMP-9 is another important member of MMPs family which is up-regulated on GAMs through TLR2/6 signaling. Minocycline has been shown not to only down-regulate MMP expression but also may impede microglial TLR2 expression during infections (Henry et al. 2008; Kielian et al. 2007). To investigate whether minocycline could have the same effect on GAMs, I treated GAMs with minocycline and analyzed MMP-9 and TLR2 expression by different methods. I found that minocycline attenuated the deleterious up-regulation of TLR2 and subsequently MMP-9 in GAMs. This finding revealed another mechanism that minocycline could inhibit microglia

assisted glioma expansion. Finally, the University of Utah has started a clinical study using repeated radiation, minocycline and VEGF monoclonal blocking-antibody Bevacizumab (Avastin®) in treating recurrent glioblastomas (http://clinicaltrials.gov/ct2/show/NCT01580969?term=minocycline+glioma&rank=1). Thus minocycline has the potential to become a standard element of glioma therapies.

5.6 TLR2 neutralizing antibody, another "bevacizumab"?

Among all subtypes of TLRs, TLR2 seems to be the most crucial receptor in glioma-microglia crosstalk. Inhibition of this pathway may have potential benefit for glioma treatment. A humanized anti-TLR2 antibody (OPN-301 and OPN-305) has been shown to inhibit TLR2 mediated inflammation in a murine model of renal transplantation (Farrar et al. 2012). Since inflammation has been regarded as one of most significant features of cancer (Hanahan and Weinberg 2011), inhibition of inflammation via TLR2 may give rise to cancer therapies. Tye et al. have recently used this neutralizing antibody to block TLR2 in a gastric cancer model and found out therapeutic targeting of TLR2 inhibited gastric tumorigenesis (Tye et al. 2012). More importantly, this antibody has already been tested in a phase I clinical trial showing its safety and tolerability on healthy subjects, and a phase II study in renal transplantation is ongoing (Reilly et al. 2013).

In my present study, I first showed a TLR2 antibody could impede TLR2 ligand induced microglial MT1-MMP and IL-6 induction indicating it could functionally block TLR2. Then I verified that GCM induced microglial MT1-MMP up-regulation could also be abolished by the antibody treatment, which showed a similar result as the previous study that GCM induced microglial MT1-MMP via TLR2 signaling (Vinnakota et al. 2013). Finally, by using an organotypic brain slice model, I could show that the TLR2 antibody has potential therapeutic benefits in glioma therapies.

Monoclonal antibodies (mAbs) have been used with increasing success against many tumors. Bevacizumab, also a humanized monoclonal antibody to vascular endothelial growth Factor (VEGF), was approved in 2009 by FDA in the United States for the treatment of recurrent glioblastoma. Thus a mAb targeting TLR2 may also be used as an adjuvant therapy in the treatment of glioma. However, one has to keep in mind that although in high grade gliomas the blood-brain barrier (BBB) is somehow ruptured so

that intravenously delivered mAb could flow into the brain, the BBB is still able to restrict the quantity of mAb that reaches the tumor.

5.7 Prospective of glioma research: Do not overlook microglia

Like other solid cancers, gliomas are heterogeneous and keep interacting with other “healthy” resident cells in the brain. Gliomas may not only derive from the healthy cells but after the initiation they start to influence these cells and convert them to a tumor supporting phenotype. Among different types of tumor associated parenchymal cell populations in the brain, glioma associated microglia/macrophages would be the most interesting subtype. Firstly, they shared a large proportion of gliomas with a contribution of up to 30% of tumor mass. Secondly, as the guardians in the brain, microglial cells constantly screen brain tissue using their motile processes, once an insult is found, they become activated and move to the lesions very rapidly. However, this property raises a few interesting questions: How do microglial cells behave when brain resident cells just start to transform to tumor cells? Do they sense these events? Do they accumulate around it? Do they phagocytose these transformed cells? These questions are still obscure. Last but not least, microglial cells are the immune cells of the brain, they express a wide range of receptors like neurotransmitter receptors, pattern-recognition receptors and cytokine and chemokine receptors (Kettenmann et al. 2011), by which they may easily get activated by tumor released factors or physical contact. And they could also release different types of cytokines and chemokines, which may shape the characteristics of gliomas. Glioma research has been extensively developed in the past decades; however, the majority mainly focus on the biology of the tumor itself without much concern about tumor microenvironment. These tumor-associated resident brain cells also contribute immensely to gliomagenesis, and more importantly, these cells may also play part in drug resistance in conventional chemotherapy.

How could glioma therapy become more advanced from the point of view of glioma associated microglia/macrophages? Herein I could propose three potential aspects.

1. Inhibition of microglial/macrophage chemoattraction

Researches have shown so far that GAMs aid glioma progression rather than inhibit it. Preventing microglial/macrophage accumulation around the tumor mass will be a possible approach to stop this assistance. Candidate chemoattractants of GAMs include monocyte chemoattractant protein-3 (MCP-3) (Okada et al. 2009), granulocyte/macrophage colony-stimulating factor (GM-CSF) (Sielska et al. 2013), Monocyte chemoattractant protein-1 (MCP-1) (Platten et al. 2003) and Glial-derived neurotrophic factor (GDNF) (Ku et al. 2013). Targeting these molecules by using antagonists may have potential therapeutic benefits in gliomas (Gong et al. 1997; van Nieuwenhuijze et al. 2013).

2. Reeducating GAMs

A few mechanisms demonstrating how GAMs promote glioma growth have been identified. Interfering with these pathways may lead to a turnover of tumor-promoting GAMs. A recent study by Sarka et al. reported that microglia from non-glioma subjects reduce glioma initiating cell formation, but GAMs failed to do so. Surprisingly, amphotericin B (AmpB), an antifungal drug could rectify this failure (Sarkar et al. 2014). Additionally, AmpB administration could reduce brain tumor initiating cells tumorigenicity thus reduce tumor growth *in vivo*. Another study by Pyonteck et al. showed inhibition of colony stimulating factor-1 (CSF-1) by an inhibitor could impair tumor supporting functions of GAMs, treatment with a CSF-1 inhibitor BLZ945 significantly increased survival and regressed established tumors in a proneural glioma model (Pyonteck et al. 2013). In the present study, I also found that minocycline as well as a TLR2 neutralizing antibody could influence GAM properties for tumor regression. Thus, reprogramming GAM by different approaches may correct the phenotype of GAM so that it may inhibit tumorigenicity or at least reduce or stop its assistance in promoting tumor growth.

3. Bone marrow derived macrophages: Drug delivery vehicle

Now it is clear that numerous bone marrow derived monocyte/macrophages infiltrate into the CNS during different brain pathologies including gliomas. These infiltrated cells could then be potentially used as delivery vehicles to carry drugs or gene therapy products to cure gliomas. Nanozyme, a colloidal nanoparticle with a diameter of 40-100nm could be rapidly taken up by bone marrow derived monocyte in 40-60 min. RNA interfering

products carried by nanozyme could be released later when they become activated, and this approach has been used successfully in treating Parkinson's disease and HCV in animal models (Batrakova et al. 2007; Wang et al. 2012). Hopefully, this novel technique may also shed a new light on glioma therapies.

6. Bibliography

Anderson KV, Jurgens G, Nusslein-Volhard C. 1985. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell 42:779-89.

Asher RA, Morgenstern DA, Shearer MC, Adcock KH, Pesheva P, Fawcett JW. 2002. Versican is upregulated in CNS injury and is a product of oligodendrocyte lineage cells. J Neurosci 22:2225-36.

Batchelor T. 2000. Temozolomide for malignant brain tumours. Lancet 355:1115-6.

Batrakova EV, Li S, Reynolds AD, Mosley RL, Bronich TK, Kabanov AV, Gendelman HE. 2007. A macrophage-nanozyme delivery system for Parkinson's disease. Bioconjug Chem 18:1498-506.

Bond M, Chase AJ, Baker AH, Newby AC. 2001. Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovasc Res 50:556-65.

Bradley LM, Douglass MF, Chatterjee D, Akira S, Baaten BJ. 2012. Matrix metalloprotease 9 mediates neutrophil migration into the airways in response to influenza virus-induced toll-like receptor signaling. PLoS Pathog 8:e1002641.

Brundula V, Rewcastle NB, Metz LM, Bernard CC, Yong VW. 2002. Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. Brain 125:1297-308.

Colton CA. 2009. Heterogeneity of microglial activation in the innate immune response in the brain. J Neuroimmune Pharmacol 4:399-418.

Curtin JF, Liu N, Candolfi M, Xiong W, Assi H, Yagiz K, Edwards MR, Michelsen KS, Kroeger KM, Liu C and others. 2009. HMGB1 mediates endogenous TLR2 activation and brain tumor regression. PLoS Med 6:e10.

da Fonseca AC, Badie B. 2013. Microglia and macrophages in malignant gliomas: recent discoveries and implications for promising therapies. Clin Dev Immunol 2013:264124.

Del Rio-Hortega P. Microglia. In: Cytology and Cellular Pathology of the Nervous System, edited by Penfield W. New York: Hoeber, 1932, p. 482–1924–534

Dolecek TA, Propp JM, Stroup NE, Kruchko C. 2012. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009. Neuro Oncol 14 Suppl 5:v1-49.

Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, Song H, Vandenberg S, Johnson RS, Werb Z and others. 2008. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 13:206-20.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161-74.

El Andaloussi A, Sonabend AM, Han Y, Lesniak MS. 2006. Stimulation of TLR9 with CpG ODN enhances apoptosis of glioma and prolongs the survival of mice with experimental brain tumors. Glia 54:526-35.

Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, Buwitt-Beckmann U, Roschmann K, Jung G, Wiesmuller KH and others. 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. J Leukoc Biol 83:692-701.

Farrar CA, Keogh B, McCormack W, O'Shaughnessy A, Parker A, Reilly M, Sacks SH. 2012. Inhibition of TLR2 promotes graft function in a murine model of renal transplant ischemia-reperfusion injury. FASEB J 26:799-807.

Forsyth PA, Wong H, Laing TD, Rewcastle NB, Morris DG, Muzik H, Leco KJ, Johnston RN, Brasher PM, Sutherland G and others. 1999. Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. Br J Cancer 79:1828-35.

Gabrusiewicz K, Ellert-Miklaszewska A, Lipko M, Sielska M, Frankowska M, Kaminska B. 2011. Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas. PLoS One 6:e23902.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER and others. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330:841-5.

Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. 2013. Origin and differentiation of microglia. Front Cell Neurosci 7:45.

Gong JH, Ratkay LG, Waterfield JD, Clark-Lewis I. 1997. An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. J Exp Med 186:131-7.

Grauer OM, Molling JW, Bennink E, Toonen LW, Sutmuller RP, Nierkens S, Adema GJ. 2008. TLR ligands in the local treatment of established intracerebral murine gliomas. J Immunol 181:6720-9.

Hagemann C, Anacker J, Ernestus RI, Vince GH. 2012. A complete compilation of matrix metalloproteinase expression in human malignant gliomas. World J Clin Oncol 3:67-79.

Hagemann C, Anacker J, Haas S, Riesner D, Schomig B, Ernestus RI, Vince GH. 2010. Comparative expression pattern of Matrix-Metalloproteinases in human glioblastoma cell-lines and primary cultures. BMC Res Notes 3:293.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144:646-74.

Hanisch UK, Johnson TV, Kipnis J. 2008. Toll-like receptors: roles in neuroprotection? Trends Neurosci 31:176-82.

Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10:1387-94.

Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L and others. 2005. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352:997-1003.

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 3:196-200.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K and others. 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408:740-5.

Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, Bailey MT, Sheridan JF, Godbout JP. 2008. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. J Neuroinflammation 5:15.

Hess DC, Fagan SC. 2010. Repurposing an old drug to improve the use and safety of tissue plasminogen activator for acute ischemic stroke: minocycline. Pharmacotherapy 30:55S-61S.

Hiraoka N, Allen E, Apel IJ, Gyetko MR, Weiss SJ. 1998. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell 95:365-77.

Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. 2006. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. Neuro Oncol 8:261-79.

Jacobs VL, Landry RP, Liu Y, Romero-Sandoval EA, De Leo JA. 2012. Propentofylline decreases tumor growth in a rodent model of glioblastoma multiforme by a direct mechanism on microglia. Neuro Oncol 14:119-31.

Janeway CA, Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 54 Pt 1:1-13.

Janeway CA, Jr., Medzhitov R. 2002. Innate immune recognition. Annu Rev Immunol 20:197-216.

Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11:373-84.

Kessenbrock K, Plaks V, Werb Z. 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52-67.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. 2011. Physiology of microglia. Physiol Rev 91:461-553.

Kielian T, Esen N, Liu S, Phulwani NK, Syed MM, Phillips N, Nishina K, Cheung AL, Schwartzman JD, Ruhe JJ. 2007. Minocycline modulates neuroinflammation independently of its antimicrobial activity in staphylococcus aureus-induced brain abscess. Am J Pathol 171:1199-214.

Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, Luo JL, Karin M. 2009. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature 457:102-6.

Kleihues P, Burger PC, Scheithauer BW. 1993. The new WHO classification of brain tumours. Brain Pathol 3:255-68.

Kleihues P, Cavenee WK (eds) (2000) World Health Organization ClassiWcation of Tumours. Pathology and genetics of tumours of the nervous system. IARC Press, Lyon

Kleihues P, Soylemezoglu F, Schauble B, Scheithauer BW, Burger PC. 1995. Histopathology, classification, and grading of gliomas. Glia 15:211-21.

Komohara Y, Ohnishi K, Kuratsu J, Takeya M. 2008. Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. J Pathol 216:15-24.

Ku MC, Wolf SA, Respondek D, Matyash V, Pohlmann A, Waiczies S, Waiczies H, Niendorf T, Synowitz M, Glass R and others. 2013. GDNF mediates glioblastoma-induced microglia attraction but not astrogliosis. Acta Neuropathol 125:609-20.

Lakka SS, Gondi CS, Yanamandra N, Olivero WC, Dinh DH, Gujrati M, Rao JS. 2004. Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. Oncogene 23:4681-9.

Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. 2005. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol 169:681-91.

Lehmann SM, Kruger C, Park B, Derkow K, Rosenberger K, Baumgart J, Trimbuch T, Eom G, Hinz M, Kaul D and others. 2012. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat Neurosci 15:827-35.

Lehnardt S, Schott E, Trimbuch T, Laubisch D, Krueger C, Wulczyn G, Nitsch R, Weber JR. 2008. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. J Neurosci 28:2320-31.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86:973-83.

Levkovitz Y, Mendlovich S, Riwkes S, Braw Y, Levkovitch-Verbin H, Gal G, Fennig S, Treves I, Kron S. 2010. A double-blind, randomized study of minocycline for the treatment of negative and cognitive symptoms in early-phase schizophrenia. J Clin Psychiatry 71:138-49.

Li D, Wang X, Wu JL, Quan WQ, Ma L, Yang F, Wu KY, Wan HY. 2013. Tumor-produced versican V1 enhances hCAP18/LL-37 expression in macrophages through activation of TLR2 and vitamin D3 signaling to promote ovarian cancer progression in vitro. PLoS One 8:e56616.

Li W, Graeber MB. 2012. The molecular profile of microglia under the influence of glioma. Neuro Oncol 14:958-78.

Libermann TA, Nusbaum HR, Razon N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature 313:144-7.

Little MP, de Vathaire F, Shamsaldin A, Oberlin O, Campbell S, Grimaud E, Chavaudra J, Haylock RG, Muirhead CR. 1998. Risks of brain tumour following treatment for cancer in childhood: modification by genetic factors, radiotherapy and chemotherapy. Int J Cancer 78:269-75.

Liu WT, Huang CY, Lu IC, Gean PW. 2013. Inhibition of glioma growth by minocycline is mediated through endoplasmic reticulum stress-induced apoptosis and autophagic cell death. Neuro Oncol 15:1127-41.

Liu WT, Lin CH, Hsiao M, Gean PW. 2011. Minocycline inhibits the growth of glioma by inducing autophagy. Autophagy 7:166-75.

Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P. 2007. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 114:97-109.

Markovic DS, Glass R, Synowitz M, Rooijen N, Kettenmann H. 2005. Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. J Neuropathol Exp Neurol 64:754-62.

Markovic DS, Vinnakota K, Chirasani S, Synowitz M, Raguet H, Stock K, Sliwa M, Lehmann S, Kalin R, van Rooijen N and others. 2009. Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion. Proc Natl Acad Sci U S A 106:12530-5.

Markovic DS, Vinnakota K, van Rooijen N, Kiwit J, Synowitz M, Glass R, Kettenmann H. 2011. Minocycline reduces glioma expansion and invasion by attenuating microglial MT1-MMP expression. Brain Behav Immun 25:624-8.

Meng Y, Kujas M, Marie Y, Paris S, Thillet J, Delattre JY, Carpentier AF. 2008. Expression of TLR9 within human glioblastoma. J Neurooncol 88:19-25.

Mizutani M, Pino PA, Saederup N, Charo IF, Ransohoff RM, Cardona AE. 2012. The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. J Immunol 188:29-36.

Morimura T, Neuchrist C, Kitz K, Budka H, Scheiner O, Kraft D, Lassmann H. 1990. Monocyte subpopulations in human gliomas: expression of Fc and complement receptors and correlation with tumor proliferation. Acta Neuropathol 80:287-94.

Nakanishi C, Toi M. 2005. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 5:297-309.

Naso MF, Zimmermann DR, Iozzo RV. 1994. Characterization of the complete genomic structure of the human versican gene and functional analysis of its promoter. J Biol Chem 269:32999-3008.

O'Neill LA, Golenbock D, Bowie AG. 2013. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol 13:453-60.

Ohgaki H, Kleihues P. 2005. Epidemiology and etiology of gliomas. Acta Neuropathol 109:93-108.

Okada M, Saio M, Kito Y, Ohe N, Yano H, Yoshimura S, Iwama T, Takami T. 2009. Tumor-associated macrophage/microglia infiltration in human gliomas is correlated with MCP-3, but not MCP-1. Int J Oncol 34:1621-7.

Page-McCaw A, Ewald AJ, Werb Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 8:221-33.

Paulus W, Baur I, Dours-Zimmermann MT, Zimmermann DR. 1996. Differential expression of versican isoforms in brain tumors. J Neuropathol Exp Neurol 55:528-33.

Piccinini AM, Midwood KS. 2010. DAMPening inflammation by modulating TLR signalling. Mediators Inflamm 2010.

Platten M, Kretz A, Naumann U, Aulwurm S, Egashira K, Isenmann S, Weller M. 2003. Monocyte chemoattractant protein-1 increases microglial infiltration and aggressiveness of gliomas. Ann Neurol 54:388-92.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C and others. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085-8.

Popovic N, Schubart A, Goetz BD, Zhang SC, Linington C, Duncan ID. 2002. Inhibition of autoimmune encephalomyelitis by a tetracycline. Ann Neurol 51:215-23.

Power C, Henry S, Del Bigio MR, Larsen PH, Corbett D, Imai Y, Yong VW, Peeling J. 2003. Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. Ann Neurol 53:731-42.

Pradere JP, Dapito DH, Schwabe RF. 2013. The Yin and Yang of Toll-like receptors in cancer. Oncogene.

Prinz M, Hanisch UK. 1999. Murine microglial cells produce and respond to interleukin-18. J Neurochem 72:2215-8.

Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, Olson OC, Quick ML, Huse JT, Teijeiro V and others. 2013. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. Nat Med 19:1264-72.

Rao JS, Steck PA, Mohanam S, Stetler-Stevenson WG, Liotta LA, Sawaya R. 1993. Elevated levels of M(r) 92,000 type IV collagenase in human brain tumors. Cancer Res 53:2208-11.

Regen T, van Rossum D, Scheffel J, Kastriti ME, Revelo NH, Prinz M, Bruck W, Hanisch UK. 2011. CD14 and TRIF govern distinct responsiveness and responses in mouse microglial TLR4 challenges by structural variants of LPS. Brain Behav Immun 25:957-70.

Reilly M, Miller RM, Thomson MH, Patris V, Ryle P, McLoughlin L, Mutch P, Gilboy P, Miller C, Broekema M and others. 2013. Randomized, double-blind, placebo-controlled, dose-escalating phase I, healthy subjects study of intravenous OPN-305, a humanized anti-TLR2 antibody. Clin Pharmacol Ther 94:593-600.

Ricciardelli C, Brooks JH, Suwiwat S, Sakko AJ, Mayne K, Raymond WA, Seshadri R, LeBaron RG, Horsfall DJ. 2002. Regulation of stromal versican expression by breast cancer cells and importance to relapse-free survival in patients with node-negative primary breast cancer. Clin Cancer Res 8:1054-60.

Roggendorf W, Strupp S, Paulus W. 1996. Distribution and characterization of microglia/macrophages in human brain tumors. Acta Neuropathol 92:288-93.

Rosenberg GA, Estrada EY, Mobashery S. 2007. Effect of synthetic matrix metalloproteinase inhibitors on lipopolysaccharide-induced blood-brain barrier opening in rodents: Differences in response based on strains and solvents. Brain Res 1133:186-92.

Said N, Sanchez-Carbayo M, Smith SC, Theodorescu D. 2012. RhoGDI2 suppresses lung metastasis in mice by reducing tumor versican expression and macrophage infiltration. J Clin Invest 122:1503-18.

Sarkar S, Doring A, Zemp FJ, Silva C, Lun X, Wang X, Kelly J, Hader W, Hamilton M, Mercier P and others. 2014. Therapeutic activation of macrophages and microglia to suppress brain tumor-initiating cells. Nat Neurosci 17:46-55.

Schmalfeldt M, Bandtlow CE, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR. 2000. Brain derived versican V2 is a potent inhibitor of axonal growth. J Cell Sci 113 (Pt 5):807-16.

Schmalfeldt M, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR. 1998. Versican V2 is a major extracellular matrix component of the mature bovine brain. J Biol Chem 273:15758-64.

Schmitt CA, Rosenthal CT, Lowe SW. 2000. Genetic analysis of chemoresistance in primary murine lymphomas. Nat Med 6:1029-35.

Sedgwick JD, Schwender S, Imrich H, Dorries R, Butcher GW, ter Meulen V. 1991. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc Natl Acad Sci U S A 88:7438-42.

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 189:1777-82.

Sielska M, Przanowski P, Wylot B, Gabrusiewicz K, Maleszewska M, Kijewska M, Zawadzka M, Kucharska J, Vinnakota K, Kettenmann H and others. 2013. Distinct roles of CSF family cytokines in macrophage infiltration and activation in glioma progression and injury response. J Pathol 230:310-21.

Song H, Li Y, Lee J, Schwartz AL, Bu G. 2009. Low-density lipoprotein receptor-related protein 1 promotes cancer cell migration and invasion by inducing the expression of matrix metalloproteinases 2 and 9. Cancer Res 69:879-86.

Stein M, Keshav S, Harris N, Gordon S. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 176:287-92.

Sternlicht MD, Werb Z. 2001. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463-516.

Sun C, Wang Q, Zhou H, Yu S, Simard AR, Kang C, Li Y, Kong Y, An T, Wen Y and others. Antisense MMP-9 RNA inhibits malignant glioma cell growth in vitro and in vivo. Neurosci Bull 29:83-93.

Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. Annu Rev Immunol 21:335-76.

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443-51.

Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S. 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 13:933-40.

Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 169:10-4.

Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. 2001. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 21:2580-8.

Tikka TM, Koistinaho JE. 2001. Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia. J Immunol 166:7527-33.

Tye H, Kennedy CL, Najdovska M, McLeod L, McCormack W, Hughes N, Dev A, Sievert W, Ooi CH, Ishikawa TO and others. 2012. STAT3-driven upregulation of TLR2 promotes gastric tumorigenesis independent of tumor inflammation. Cancer Cell 22:466-78.

Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. 2010. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J Clin 60:166-93.

van Meyel DJ, Ramsay DA, Casson AG, Keeney M, Chambers AF, Cairncross JG. 1994. p53 mutation, expression, and DNA ploidy in evolving gliomas: evidence for two pathways of progression. J Natl Cancer Inst 86:1011-7.

van Nieuwenhuijze A, Koenders M, Roeleveld D, Sleeman MA, van den Berg W, Wicks IP. 2013. GM-CSF as a therapeutic target in inflammatory diseases. Mol Immunol 56:675-82.

Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP and others. 2010. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17:98-110.

Vinnakota K, Hu F, Ku MC, Georgieva PB, Szulzewsky F, Pohlmann A, Waiczies S, Waiczies H, Niendorf T, Lehnardt S and others. 2013. Toll-like receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion. Neuro Oncol 15:1457-68.

Wang C, Cao S, Yan Y, Ying Q, Jiang T, Xu K, Wu A. 2010. TLR9 expression in glioma tissues correlated to glioma progression and the prognosis of GBM patients. BMC Cancer 10:415.

Wang Z, Liu H, Yang SH, Wang T, Liu C, Cao YC. 2012. Nanoparticle-based artificial RNA silencing machinery for antiviral therapy. Proc Natl Acad Sci U S A 109:12387-92.

Ween MP, Hummitzsch K, Rodgers RJ, Oehler MK, Ricciardelli C. 2011. Versican induces a pro-metastatic ovarian cancer cell behavior which can be inhibited by small hyaluronan oligosaccharides. Clin Exp Metastasis 28:113-25.

Wen PY, Kesari S. 2008. Malignant gliomas in adults. N Engl J Med 359:492-507.

Wesolowska A, Kwiatkowska A, Slomnicki L, Dembinski M, Master A, Sliwa M, Franciszkiewicz K, Chouaib S, Kaminska B. 2008. Microglia-derived TGF-beta as an important regulator of glioblastoma invasion--an inhibition of TGF-beta-dependent effects by shRNA against human TGF-beta type II receptor. Oncogene 27:918-30.

Wight TN. 2002. Versican: a versatile extracellular matrix proteoglycan in cell biology. Curr Opin Cell Biol 14:617-23.

Wu Y, Sheng W, Chen L, Dong H, Lee V, Lu F, Wong CS, Lu WY, Yang BB. 2004. Versican V1 isoform induces neuronal differentiation and promotes neurite outgrowth. Mol Biol Cell 15:2093-104.

Yang L, Sugama S, Chirichigno JW, Gregorio J, Lorenzl S, Shin DH, Browne SE, Shimizu Y, Joh TH, Beal MF and others. 2003. Minocycline enhances MPTP toxicity to dopaminergic neurons. J Neurosci Res 74:278-85.

Ye XZ, Xu SL, Xin YH, Yu SC, Ping YF, Chen L, Xiao HL, Wang B, Yi L, Wang QL and others. 2012. Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-beta1 signaling pathway. J Immunol 189:444-53.

Yeh WL, Lu DY, Liou HC, Fu WM. 2012. A forward loop between glioma and microglia: glioma-derived extracellular matrix-activated microglia secrete IL-18 to enhance the migration of glioma cells. J Cell Physiol 227:558-68.

Yong VW, Wells J, Giuliani F, Casha S, Power C, Metz LM. 2004. The promise of minocycline in neurology. Lancet Neurol 3:744-51.

Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. 1998. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc Natl Acad Sci U S A 95:15769-74.

Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, Tracey KJ, Yang H. 2006. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock 26:174-9.

Zhai H, Heppner FL, Tsirka SE. 2011. Microglia/macrophages promote glioma progression. Glia 59:472-85.

Zhao Y, Xiao A, diPierro CG, Carpenter JE, Abdel-Fattah R, Redpath GT, Lopes MB, Hussaini IM. An extensive invasive intracranial human glioblastoma xenograft model: role of high level matrix metalloproteinase 9. Am J Pathol 176:3032-49.

Affidavit

"I, Feng Hu, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Dissecting the role of Toll-like receptors in microglia-glioma crosstalk". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of any eventual publications

Feng Hu had the following share in the following publications:

1. Vinnakota, Katyayni[#], Feng Hu[#], Min-Chi Ku, Petya B. Georgieva, Frank Szulzewsky, Andreas Pohlmann, Sonia Waiczies et al. "Toll-like receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion." Neuro-oncology 15, no. 11 (2013): 1457-1468.
   [#]Co-first author.
   Contribution: Designed and performed the experiments

2. Przanowski, Piotr, Michal Dabrowski, Aleksandra Ellert-Miklaszewska, Michal Kloss, Jakub Mieczkowski, Beata Kaza, Anna Ronowicz, Feng Hu et al. "The signal transducers Stat1 and Stat3 and their novel target Jmjd3 drive the expression of inflammatory genes in microglia." Journal of Molecular Medicine (2013): 1-16.
   Co-author
   Contribution: Organized TLRKO animals and performed experiments.

Signature, date and stamp of the supervising University teacher

Signature of the doctoral candidate

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of publications

1. **Hu, Feng**[#], Min-Chi Ku[#], Darko Markovic[#], Omar Dildar a Dzaye, Seija Lehnardt, Michael Synowitz, Susanne A. Wolf and Helmut Kettenmann. Glioma associated microglial MMP9 expression is up regulated by TLR2 receptor activation and sensitive to minocycline.
   [#] **Co-first author accepted by International Journal of Cancer**

2. Vinnakota, Katyayni[#], **Feng Hu**[#], Min-Chi Ku, Petya B. Georgieva, Frank Szulzewsky, Andreas Pohlmann, Sonia Waiczies et al. "Toll-like receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion." Neuro-oncology 15, no. 11 (2013): 1457-1468.
   [#]**Co-first author**

3. Przanowski, Piotr, Michal Dabrowski, Aleksandra Ellert-Miklaszewska, Michal Kloss, Jakub Mieczkowski, Beata Kaza, Anna Ronowicz, **Feng Hu** et al. "The signal transducers Stat1 and Stat3 and their novel target Jmjd3 drive the expression of inflammatory genes in microglia." Journal of Molecular Medicine (2013): 1-16.
   Co-author

4. Han, Qingdong, Shengwen Liu, Zhengwei Li, **Feng Hu**, Qiang Zhang, Min Zhou, Jingcao Chen, Ting Lei, and Huaqiu Zhang. "DCPIB, a potent volume-regulated anion channel antagonist, attenuates microglia-mediated inflammatory response and neuronal injury following focal cerebral ischemia." Brain research1542 (2014): 176-185.
   Co-author

5. Zhang, S. J., F. Wan, **F. Hu**, R. F. Xie, Y. Wang, B. F. Wang, F. Ye, D. S. Guo, and T. Lei. "[Differentiation treatment by all-trans retinoic acid reduces stemness of glioma stem cells]." Zhonghua yi xue za zhi 93, no. 1 (2013): 19-22.
   Co-author

6. Zhang, S. J., F. Ye, R. F. Xie, **F. Hu**, B. F. Wang, F. Wan, D. S. Guo, and T. Lei. "Comparative study on the stem cell phenotypes of C6 cells under different culture conditions." Chinese medical journal 124, no. 19 (2011): 3118-3126.
   Co-author

7. **Hu, Feng**, Omar Dildar a Dzaye, Yong Yu, Michael Synowitz, Susanne A. Wolf and Helmut Kettenmann. Glioma derived versican promotes tumor growth via glioma associated microglial/macrophage Toll-like receptor 2 signaling.
   First author manuscript in preparation

Acknowledgements

Foremost I would like to epxress my sincere gratitude to my supervisor Prof. Dr. Helmut Kettenmann for giving me the opportunity to work in his fantastic lab, leading me to the miraculous glial research and continuous support to my projects. I also would like to thank Dr. Susanne A. Wolf for the co-supervison and support during my study.

Special thanks to Dr. Katyayni Vinnakota and Birgit Jarchow who helped me most when I started my research work in the lab. Many thanks to my collabrators: Prof. Dr. Seija Lehnardt for providing TLR KO animals, FACS antibodies as well as fruitful discussions and suggestions; PD. Dr. Michael Synowitz for providing human glioma materials; Prof. Dr. Dieter Zimmermann for sharing versican antibodies and plasmids; Dr. Carmela Ricciardelli for providing recombinant versican and Dr. Yong Yu for the help of shRNA experiment. I also appreciated the excellent technical support from the technicians: Irene Haupt for primary microglia cell preparations; Regina Piske, Hanna Schmidt for organization of materials, animals and molecular biology experiments; Nadine Scharek for help in immunohistochemistry experiments. I am grateful to the China scholarship council (CSC) for financial support and to the office of International PhD program of Medical Neurosciences(Charité), the PhD office in MDC for all administrative assistance during my doctoral study period.

I also sincerely thank my friend and colleague Omar Dzaye for the contributions in my projects (TLR regulation) and wonderful time we spent together in the lab, my master student Alexander Hahn for the help in TLR2 antibody project, as well as my past and current colleagues –Dr. Maria Pannell, Dr. Min-chi Ku, Dr. Darko Macrovic, Songbo Guo, Le Dong, Dr. Julia Parnis, Nadine Richter, Petya Georgieva, Frank Szulzewsky, Dr. Kristin Stock, Dr. Adriana Rocha, Dr. Larisa Bulavina, Dr. Christiane Nolte, Dr. Vitali Matyash, Dr. Marina Matyash**,** Dr. Anaïs Djodari-Irani, Philipp Jordan, Daniele Mattei, Felipe Sassi, Laura Korvers, Karen Rosenberger, and everyone else who supported me in all possible ways. And all the friends from chinese community in MDC and Berlin– thank you all for the memorable time we spent together.

Last but not least, I thank my wife Yumin, for being with me, supporting me and believing in me. I also thank my parents for raising me up, for their endless love. Without my family, I could not finish my degree.