Aus dem Max Delbrück Centre for Molecular Medicine

DISSERTATION

The role of microglia in glioma expansion and progression

zur Erlangung des akademischen Grades
Doctor of Philosophy in Medical Neurosciences
(International Graduate Program Medical Neurosciences)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Katyayni Vinnakota

aus Hyderabad, Indien

Gutachter:

1. Prof. Dr. Helmut Kettenmann
2. Prof. Dr. Marco Prinz
3. Prof. Dr. Frank Kirchoff

Datum der Promotion: 01.02.2013

ACKNOWLEDGEMENTS

I sincerely thank Prof. Dr. Helmut Kettenmann for giving me the opportunity to work in his lab as a PhD student and for his mentorship that has helped me to develop intellectually. Heartfelt thanks to Dr. Susanne Wolf, Prof. Dr. Rainer Glass and Dr. Darko Markovic for their profound guidance and supervision during my entire study period. Many thanks to Birgit Jarchow who made every struggle in the lab smooth and easy. I sincerely thank my collaborators Prof. Uwe-Karsten Hanisch who introduced me to the fascinating world of TLR signaling and Dr. Seija Lehnardt for supporting me with her scholarly inputs and with TLR knock-out mice. Earnest gratitude to the skillful, hard-working and ever-friendly Irene Haupt for primary microglia cell preparations, Regina Piske and Nadine Scharek for help in cell/ molecular biology and immunohistochemistry experiments. I am grateful to the Deutscher Akademischer Austausch Dienst (DAAD, Bonn) for financial help and to the office of International PhD program of Medical Neurosciences, Charité, Berlin for all administrative assistance during my doctoral study period. Thank you very much Petya Georgieva and Julia Parnis, for being with me through my thick and thin. I also sincerely thank my past and current colleagues – Dr. Anika Langenfurth, Feng Hu, Minchi Ku, Pina Knauff, Frank Szulzewsky, Sridhar Reddy, Jitender Kumar, Kristin Stock, Adriana Rocha, Larisa Bulavina, Sabrina Lehmann, Karen Rosenberger, Tatyana Pivneva and everyone else who supported me in many ways. Meenakshi Sharma, Vignesh Sakthivelu, Anup Arumugham, Maliha Shah, Fraufi, Steven Sievers, Hannelore Drechsler, Birgit Cloos, Marietta Zille, Aditya Mungee, Raghu and Smita Bhushan, Britta Morich, Tanja Butzek, Manisha Juneja, Sudhir Tattikota– thank you all for being such great friends and for your timely help and endless caring ways at each step of my life in Berlin and in Germany.

I am deeply grateful for the love and support I received from my parents and sister. *MaNPa* and *Deepu*, this thesis is dedicated to you. "*Thanks*" is a small word for believing in me and for making me what I am today. And last but not the least, I owe a lot to Shyam, for being my friend, philosopher and guide and for his belief that I too can make it.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii
TABLE OF CONTENTS iv
LIST OF FIGURES ii
LIST OF TABLES iii
LIST OF ABBREVIATIONS v
1. INTRODUCTION 1
1.1. Cancer 1
1.1.1. *What is Cancer* 1
1.1.2. *The distinctive features of cancer* 2
1.1.3. *Sustenance of growth-promoting cues* 2
1.1.4. *Resistance to anti-proliferative and growth-inhibiting signals* 3
1.1.5. *Ability to escape cell death mechanisms* 3
1.1.6. *Infinite replicative ability* 4
1.1.7. *Activation of an angiogenic switch* 4
1.1.8. *Initiation of tumor invasion and metastasis* 5
1.2. Emerging hallmarks of cancer progression 5
1.3. The immune system of the brain 6
1.3.1. *Microglia cells - immune sensors of CNS* 8
1.3.2. *Microglia- role in physiology and pathology* 10
1.4. Brain tumors 12
1.5. Role of microglia in brain tumors 15
1.5.1. *Immune cell infiltration of intrinsic intracranial tumors* 15
1.5.2. *Microglia-glioma cross talk* 16
1.5.3. *Immune tolerance by gliomas* 16
1.6. Matrix metalloproteinases (MMPs) 18
1.6.1. *Role of MMPs in tumors* 21
1.7. Toll-like receptors – sentinels of immune system 22
1.7.1. *Toll-like receptor signaling* 23
1.7.2. *Mediators of Toll-like receptor signaling* 25
1.7.3. *Toll-like receptors in cancer* 28
1.7.4. *Toll-like receptors in neuroprotection* 28

1.8. Role of Minocycline in pathology ... 29

2. OBJECTIVES ... 31

2.1. Project 1 ... 31

2.2. Project 2 ... 32

3. MATERIAL & METHODS ... 33

3.1. Material ... 33

3.1.1. Reagents and Chemicals ... 33
3.1.2. Commercial Kits ... 34
3.1.3. Devices and Equipment ... 35
3.1.4. Plasticware and other tools ... 36
3.1.5. Primary Antibodies ... 36
3.1.6. Secondary Antibodies ... 37
3.1.7. Buffers for Immunohistochemistry ... 37
3.1.8. Buffers for Western blotting ... 38
3.1.9. Buffers for PCR ... 38
3.1.10. Media and Solutions for cell culture/ organotypic brain slices ... 38
3.1.11. Software ... 39

3.2. Methods ... 40

3.2.1. Animals and Anesthesia (Projects 1+2) ... 40
3.2.2. Cell Culture (Projects 1+2) ... 41
3.2.3. Generation of EGFP-GL261/mCHERRY-GL261 cells (Projects 1+2) ... 42
3.2.4. Preparation of glioma conditioned medium (Projects 1+2) ... 42
3.2.5. Genotyping of the TLR KO mice (Project 1) ... 43
3.2.6. TLR sub-type specific agonists (Project 1) ... 45
3.2.7. TLR2 specific agonists (Project 1) ... 46
3.2.8. Minocycline treatment (Project 2) ... 46
3.2.9. Quantification of gene expression changes (Projects 1+2) ... 47
3.2.10. Quantification of protein expression (Projects 1+2) ... 50
3.2.11. MT1-MMP Activity Assay (Project 2) ... 51
3.2.12. Determination of protein localization by IHC (Projects 1 & 2) ... 52
3.2.13. Organotypic Brain Slice Cultures to study tumor growth ex vivo *(Projects 1 & 2)* 53
3.2.14. In vivo *tumor studies (Projects 1 & 2)* ... 55
3.2.15. Analysis of tumor volume by Magnetic Resonance Imaging (Project 1) ... 56
3.2.16. Analysis of survival in vivo *(Project 1)* ... 57
3.2.17. Microscopy (Projects 1 & 2) ... 57

3.2.18. Analysis and Statistics (Projects 1 & 2) 58

4. RESULTS (PROJECT 1) 59

TLR signaling in microglia supports glioma growth and progression **59**

4.1. MT1-MMP expression in microglia is differentially regulated by TLR sub-type specific ligands 59

4.2. Toll-like receptor 2 is required for inducing MT1-MMP expression in glioma – associated microglia 61

4.3. Deletion of the Toll-like receptor 2 reduced glioma-induced microglial MT1-MMP expression in vitro 62

4.4. Glioma-induced MT1-MMP expression is not reduced in the MyD88-dependent TLR7 KO microglia 65

4.5. Reduction in MT1-MMP protein expression in TLR2 KO microglia stimulated with glioma conditioned medium 66

4.6. Toll-like receptor 2 interferes with glioma growth in an organotypic brain slice culture model 67

4.7. The MT1-MMP expression in glioma-associated microglia is reduced in vivo in TLR2 KO mice 68

4.8. Deletion of TLR2 led to reduction in glioma expansion 71

4.9. TLR2 KO mice implanted with glioma tumors had improved chances of survival 72

RESULTS (PROJECT 2) 73

Pharmacological intervention of the microglia-glioma interaction by Minocycline **73**

4.10. Minocycline blunts the pro-tumorigenic effect of microglial MT1-MMP expression in gliomas 73

4.11. Minocycline interferes with glioma growth ex vivo 77

4.12. Oral administration of Minocycline reduced glioma growth in vivo 78

4.13. MT1-MMP expression in glioma-associated microglia is reduced after Minocycline administration in vivo 80

5. DISCUSSION 82

PROJECT 1 ..... **83**

5.1. Microglial TLR signaling is required for glioma growth and progression 83

PROJECT 2 ..... **87**

5.2. Minocycline serves as a potential therapeutic candidate for glioma treatment 87

6. FUTURE OUTLOOK 89

7. SUMMARY 91

8. ZUSAMMENFASSUNG 93

9. REFERENCES 96

CURRICULUM VITAE 123

PUBLICATIONS 124

MEETINGS AND PRESENTATIONS 125

TALKS **125**

POSTERS **125**

ERKLÄRUNG 127

LIST OF FIGURES

Figure 1.1 Six fundamental traits of cancer cells 2

Figure 1.2 New features contributing to cancer development and progression 6

Figure 1.3 Myeloid cells in the brain perivascular region 7

Figure 1.4 Microglia cells- discovery and origin pseudopodic 9

Figure 1.5 Developmental stages of microglia

Figure 1.6 Distribution of All Primary Brain and CNS Gliomas by histology sub-types

Figure 1.7 Genetic pathways of gliomagenesis 14

Figure 1.8 Glioblastoma multiforme 15

Figure 1.9 Cascade of matrix metalloproteinase (MMP) activation at the cell surface 18

Figure 2 TLR mediated signaling 22

Figure 2.1 TLRs and their exogenous ligands 23

Figure 2.2 TLRs and their endogenous ligands 24

Figure 2.3 The chemical structure of minocycline and tetracycline 26

Figure 3. Microglia- glioma cross-talk 29

Figure 4.1 Differential gene expression changes in MT1-MMP in microglia upon stimulation with TLR ligands 59

Figure 4.2 Differential gene expression of MT1-MMP in microglia after stimulation with TLR2 ligands 60

LIST OF TABLES

Table 1. Biological fundtions of MMPs 18

Table 2. Classification of matrix metalloproteinases 20

Table 3. List of reagents and chemicals 34

Table 4. List of commercial kits 34

Table 5. List of devices and equipments 35

Table 6. List of plasticware and other tools 36

Table 7. List of Primary Antibodies used in IHC and WB 37

Table 8. List of Secondary Antibodies used in IHC and WB 37

Table 9. List of buffers for IHC 37

Table 10. List of buffers for WB 38

Table 11. List of buffers for PCR 38

Table 12. List of media & solutions used for cell culture/ OBSC 39

Table 13. List of software 39

Table 14. Genotyping PCR primers for TLRs 1, 2 & 6 43

Table 15. PCR reaction composition for genotyping TLR mice strains 44

Table 16. TLR sub-type specific agonists 45

Table 17. TLR2 specific agonists 46

Table 18. qRT-PCR composition for MT1-MMP and β-Actin 48

Table 19. qRT-PCR conditions for MT1-MMP and β-Actin 48

Table 20. Semi-quantitative PCR conditions for MT1-MMP and β-Actin 49

Table 21. qRT-PCR primer sequences of MT1-MMP and β-Actin 49

Table 22. Semi-quantitative RT-PCR primer sequences of MT1-MMP and β-Actin 50

LIST OF ABBREVIATIONS

Aqua distilled water (aq. H_2O)

Blood brain barrier (BBB)

Carbon dioxide (CO_2)

Central Nervous System (CNS)

Clodronate liposomes (CL)

Damage/Danger Associated Molecular Patterns (DAMPs)

Deoxyribonucleic acid (DNA)

Enhanced Green Fluorescent protein (EGFP)

Enzyme-linked immunosorbent assay (ELISA)

Extracellular matrix (ECM)

Fibroblast Growth factor (FGF)

Fluorescence activated cell sorting (FACS)

For example, exempla gratia (e.g.)

Glioma conditioned medium (GCM)

Horseradish peroxidase (HRP)

Hour (h)

Intra-peritoneal (i.p.)

Immunohistochemistry (IHC)

Knock-out (KO)

Lipopolysaccharide (LPS)

Major histocompatibility complex (MHC)

Matrix metalloproteinase (MMP)

Matrix metalloproteinase 2 (MMP2)

Matrix metalloproteinase 9 (MMP9)

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

Mitogen activated protein kinase (MAPK)

Nitric Oxide (NO)

Organotypic Brain Slice Cultures (OBSC)

Pattern Recognition Receptors (PRR)

Pathogen Associated Molecular Patterns (PAMP)

Polymerase Chain Reaction (PCR)

Phosphatase and Tensin homolog (PTEN)

Platelet-derived growth factor (PDGF)

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Retinoblastoma (RB)

Reverse transcriptase PCR (RT-PCR)

Room temperature (RT)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Thrombospondin-1 (TSP-1)

Tissue Inhibitor of metalloproteinase 2 (TIMP2)

Toll-like receptor (TLR)

Vascular Endothelial Growth factor (VEGF)

Western blotting (WB)

Wild-type (WT)

World Health Organization (WHO)

1. INTRODUCTION

1.1. Cancer

1.1.1. What is Cancer

Cancer includes a large group of diseases that can affect any part of the body and arise due to loss of growth control in normal tissue. As a result of accumulation of different genetic and epigenetic alterations/mutations during the process of cell cycle division, cells get transformed forming either a solid mass of proliferating cells (*benign tumor*) or may further spread via the blood and lymphatic systems to invade other organs by a process referred to as metastases (*metastatic tumor*). Unfortunately, the leading cause of death due to cancer around the world is metastatic dissemination of tumor cells. Today, cancer is a major disease across the globe. The World Health Organization (WHO) reported that in the year 2008 alone, cancer accounted for nearly 7.6 million deaths (around 13% of all deaths) world-wide, and predicts that the mortality rate would increase further beyond 11 million until the year 2030.

The affected genes are mainly of 2 types- the *oncogenes* (genes controlling cell division and growth) and the *tumor suppressor genes* (genes which inhibit cell division and survival). A cancer arises in several genes from a multistep process either via mutations and/or “Turn–on” signals in new oncogenes, or by atypical over-expression of normal oncogenes, or due to “Turn-off” signals such as less than- normal expression or disabling of tumor suppressor genes’ functions. The risk factors associated with cancer disease development and progression are manifold, ranging from environment, diet, chemicals, radiation, genetics, infections, hormones and others. Timely screening and proper diagnosis post-surgery, chemotherapy and/or radiation therapy can reduce mortality rates and prevent, if not completely cure the patient of cancer. However, further advances in basic and clinical research are warranted for developing alternate and effective therapeutic strategies that will complement the current treatment modalities to combat and treat cancer globally. Over decades of intense research, cancer biologists Douglas Hanahan and Robert Weinberg, have re-defined the six typical hallmarks of cancer that are incipient to neoplastic transformation of normal cells into a malignant phenotype (Hanahan and Weinberg 2000; Hanahan and

Weinberg 2011). They propose that tumors are not just a mass of proliferating cells of a single kind, but intricate ecosystems of multiple cell-types that are constantly interacting with one another in a heterotypic fashion to contribute to tumorigenesis.

1.1.2. The distinctive features of cancer

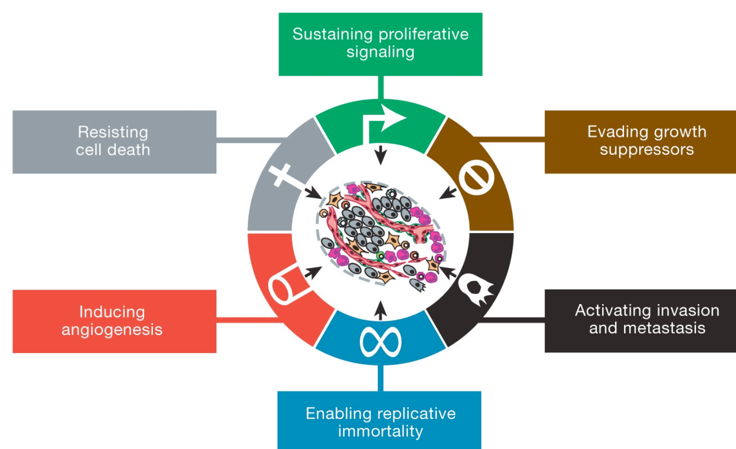


Figure 1. 1 Six fundamental traits of cancer cells. Taken from Hanahan and Weinberg 2000.

1.1.3. Sustenance of growth-promoting cues

By far the most important feature of cancer cells is their ability to maintain a persistent state of propagation and multiplication. While normal cells carefully orchestrate homeostasis of cell division through the influence of external or internal mitogenic cues, cancer cells circumvent this process by manipulating these very growth-promoting signals that would otherwise lead to the activation and propagation of intracellular mechanisms required for proper cell division, growth and progression. In turn, cancer cells either initiate a constitutive autocrine mechanism, wherein they release these growth-inducing cues themselves or alternately recruit non-transformed cells in their microenvironment to provide them

with the required growth factors for sustained proliferation and growth (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011).

1.1.4. Resistance to anti-proliferative and growth-inhibiting signals

Apart from balancing the positive effects of growth-enhancing signals on their proliferative state, cancer cells must also evade the negative effects of growth-suppressing cues, mediated mainly by tumor suppressor genes. The two canonical tumor suppressor genes or gatekeepers of cell-cycle progression, RB (Retinoblastoma) and TP53 proteins normally regulate cell division and proliferation or alternately induce cell senescence and apoptosis. Malfunctioning of these critical functions in either the RB-or TP53 pathways in cancer cells leads to constant cell proliferation and thereby tumor progression (Hanahan and Weinberg 2000; Weinberg 1995)

1.1.5. Ability to escape cell death mechanisms

A very important feature of many cancer cells is not only their ability to proliferate uncontrollably and resist the effects of anti-growth soluble factors, but also their capability to escape programmed cell death- inducing mechanisms like apoptosis. Cancer cells evade apoptosis through various molecular means, the most common being mutation or functional inactivation of the tumor suppressor gene p53 (Harris 1996a; Harris 1996b). A further support to the cancer cells in this context is received through over-activation of the signaling pathways AKT/PKB/mTOR, which transduce signals to escape death induced by apoptosis (Hanahan and Weinberg 2011) and confer survival benefits to many cancer cells, including gliomas (Evan and Littlewood 1998). Moreover, mutation and deletion of another tumor suppressor gene PTEN (Phosphatase and Tensin Homolog) which negatively regulates the AKT/PKB pathway, also leads to rapid cell proliferation and reduced or absent cell death in many cancers, including glioblastomas (Merlo and Bettler 2004).

1.1.6. Infinite replicative ability

Normal cells often go through a limited number of sequential steps of cell growth- and division cycles, due to the involvement of two critical barriers to cell proliferation- *Senescence* (irreversible entry into a non-proliferative but viable state) and *Crisis* (state of cell death). Evidence indicates that telomeres (multiple tandem hexanucleotide repeats) found on the ends of chromosomes play a vital role in regulating unlimited proliferation (Blasco 2005; Shay and Wright 2000). After each cell cycle, the telomeres shorten progressively, and in due course lose their ability to protect the chromosomal ends, thereby threatening cell viability and eventually inducing either senescence or cell death. A DNA polymerase enzyme called *Telomerase*, which adds telomere repeats to the telomeric ends of chromosomal DNA, has been found to be expressed at functionally high levels (~90%) in the vast majority of human cancers. Presence of high telomerase activity in cancer cells confers resistance to senescence and crisis/apoptosis and thereby promotes limitless proliferating abilities to the cancer cells (Bryan and Cech 1999; Hanahan and Weinberg 2011).

1.1.7. Activation of an angiogenic switch

Just like normal tissues that derive nourishment and oxygen from their environment and maintain proper physiology by getting rid of metabolic wastes, cancer cells are also sustained through the constitutively active process of *angiogenesis*, sprouting new vessels that incessantly feed expanding tumor masses (Hanahan and Folkman 1996). Angiogenesis is regulated by growth factors released by tumor cells that either induce (*Vascular Endothelial Growth Factor/VEGF*) or inhibit it (*Thrombospondin-1/TSP-1*). Other pro-angiogenic factors like Platelet-derived growth factor (*PDGF*) and Fibroblast growth factor (*FGF*) are also associated with aberrant tumor neo-vasculature which is often associated with capillary sprouting, excessive vessel branching, distorted and enlarged vessels, erratic blood flow, micro-hemorrhaging, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis (Baluk et al. 2005; Nagy et al. 2010).

1.1.8. Initiation of tumor invasion and metastasis

Cancer results from malignant transformation of normal cells that ultimately spread and disseminate as single foci to other organs in the body to form new tumors (Sporn 1996). This process of invasion and metastasis is the major cause of death among cancer patients and is facilitated either by the loss of cell-cell communication or by extracellular matrix digesting proteases (Hanahan and Weinberg 2000). One of the best examples of deregulated cell-cell communication is the loss of function of the cell adhesion molecule E-Cadherin in many cancers originating in epithelial cells. This molecule normally induces an anti-growth and anti-metastatic phenotype in coupled cells (Christofori and Semb 1999), but a mutational inactivation, transcriptional repression or proteolysis of its extracellular domain lead to its inefficacy in many cancers. Breakdown of the extracellular matrix is aided by activation of proteases like MMPs or suppression of protease inhibitors (TIMPs). Tumor cells manipulate stromal or inflammatory cells in their environment to secrete active proteases or the necessary intermediates for protease synthesis, which in turn assist in invasion and metastasis (Le et al. 2003; Markovic et al. 2005; Markovic et al. 2009; Werb 1997).

1.2. Emerging hallmarks of cancer progression

The importance of the tumor microenvironment in contributing to tumor proliferation, survival and spread apart from the role of above-mentioned transforming capabilities inherent to most cancer cells has been extensively highlighted recently (Hanahan and Weinberg 2011). Tumors are complex, heterogeneous systems comprising not only of tumor cells but also of different interacting partners like stromal, endothelial cells, fibroblasts and finally cells of the innate and adaptive immune system, all of which promote malignancy by diverse means (Bissell and Radisky 2001). Cancer cells can induce surrounding stromal cells to lose functional p53 and thereby get transformed into a malignant phenotype (Hill et al. 2005).

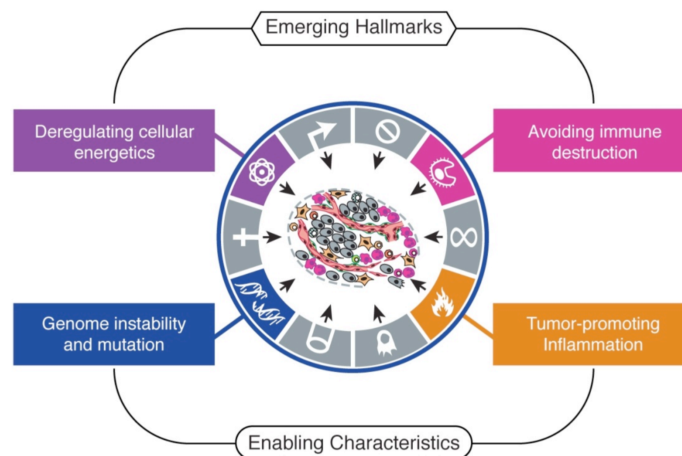


Figure 1. 2 New features contributing to cancer development and progression. Taken from Hanahan and Weinberg 2011.

Apart from cellular players that regulate tumorigenesis, compelling evidence suggests a role for over-active metabolic processes that chronically fuel cell growth and proliferation (Colotta et al. 2009; Luo et al. 2009; Negrini et al. 2010). The other potential characteristic is the ability of cancer cells to evade an attack and elimination by immune cells, a feature that emphasizes the divergent roles played by immune cells and inflammation not only to antagonize but also to support tumor growth and progression in most cancers, including tumors that arise in the central nervous system.

1.3. The immune system of the brain

Historically the brain and other organs of the CNS were thought to be physiologically isolated from the peripheral immune system. However, new studies indicate that despite its own anatomical and structural properties, the CNS is monitored continuously by modified immune surveillance mechanisms (Romo-Gonzalez et al. 2012). The concept of an "*immune-privileged*" CNS arose from early studies in tissue transplantation. Medawar observed that the immune response against heterologous skin tissue implanted into the brain parenchyma was rather weak. Medawar said *"it is concluded that skin homografts transplanted to the brain submit to but cannot elicit an immune state"* (Medawar 1948).

This rather impaired immune response was thought to be due to the presence of the blood-brain-barrier (BBB) which sequestered antigens and impaired T-cell migration across it, and thus prevented an efficacious immune response. Moreover, the lack of a proper immunity in the CNS was also attributed to a less effective lymphatic drainage from the CNS extracellular space into the peripheral lymphoid tissues (Cserr et al. 1992; Cserr and Knopf 1992; Knopf et al. 1995; Reese and Karnovsky 1967). The 'immune privilege' of the central nervous system (CNS) is indispensable for damage limitation during inflammation in a sensitive organ with poor regenerative capacity. It is a longstanding notion which, over time, has acquired several misconceptions and a lack of precision in its definition (Bechmann et al. 2007; Galea et al. 2007). Moreover, the limited expression of major histocompatibility complex (MHC) molecule class one (Risau and Wolburg 1990; Wekerle 2002) was also thought to contribute to a reduced immune response in the CNS. The cerebrovascular endothelia form tight junctions which helps the blood-brain-barrier to form a structural obstacle to the transfer of blood-borne leucocytes, antibodies, complement factors and select cytokines (Risau and Wolburg 1990). Hence, the intact BBB is a barricade to cells of the immune system, antibodies and other immunity-related molecules (Hickey 1991; Lassmann 1997).

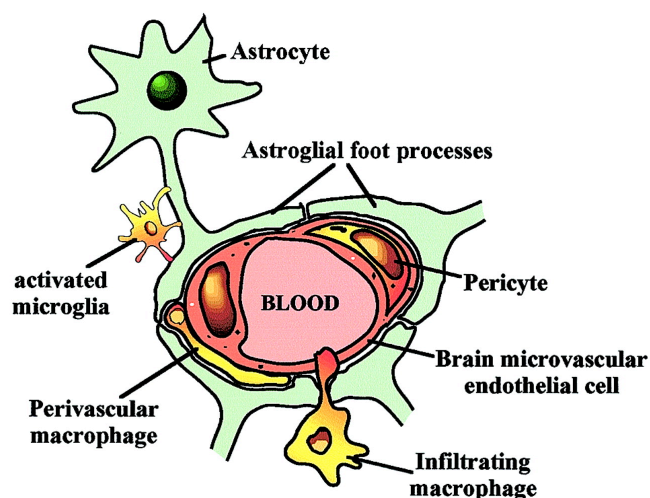


Figure 1. 3 Myeloid cells in the brain perivascular region. Simplified scheme taken from Guillemin and Brew 2004.

Of all the CNS cell sub-population, only the astrocytes and microglia could be induced to express MHC class II molecules *in vitro* (Lassmann et al. 1991). Under physiological conditions, only activated T-lymphocytes are capable of crossing the BBB to migrate into the brain (Dewey et al. 1999; Lassmann 1997). They depart the brain if they do not encounter antigen presentation (Fabry et al. 1994; Lassmann 1997). During acute or chronic brain damage or diseases, activated leukocytes migrate into the brain even when the BBB is intact (Brown 2001). The processes underlying these effects are still not clearly understood. Apart from blood derived activated leukocytes, the brain resident astrocytes, endothelial cells and microglia are also found at the site of brain injury.

However, only microglia cells are immunologically competent (Benveniste 1997; Gehrmann et al. 1995). Microglia cells not only share the surface molecules with peripheral macrophages but are also capable of antigen presentation, phagocytosis and secretion of cytokines, chemokines and cytotoxins (Farber and Kettenmann 2005; Gehrmann and Banati 1995; Kettenmann et al. 2011; Prinz et al. 1999). However, under pathological conditions, astrocytes are also capable of secreting cytokines and chemokines (Asensio and Campbell 1999; Maiese 2011; Monnet-Tschudi et al. 2011) and can thus also contribute to the immunological responses in the brain.

1.3.1. Microglia cells - immune sensors of CNS

Microglia cells are resident macrophages and immunomodulatory cells of the central nervous system (Kettenmann et al. 2011), associated with general brain physiology and pathology. Although the exact origin of microglia is still debatable, most researchers postulate that microglia cells are derived from the mesoderm (Ashwell 1990; Ashwell 1991; Boya et al. 1979; Kettenmann et al. 2011; Streit et al. 2005). Myeloid progenitor cells of microglia populate the CNS parenchyma early during embryonic development (Chan et al. 2007). The cells called fetal macrophages can be detected in the developing neuroectoderm at embryonic day 8 in rodents (Streit et al. 2005; Takahashi et al. 1989). Similarly, the origin of adult

microglial cells remains controversial. However, a study by Ginhoux et al (Ginhoux et al. 2010) showed that postnatal hematopoietic progenitors do not significantly contribute to microglia homeostasis in the adult brain. They conducted *in vivo* lineage tracing and established that adult microglia were derived from primitive myeloid progenitors that arose before embryonic day 8.

In 1932 Pio del-Rio Hortega discovered specific places in the embryonic brain where pial cells aggregated, which he called "*fountains of microglia*". Having introduced the concept of *microglia* through his seminal observations in a book chapter titled "Microglia" written for *Cytology and Cellular Pathology of the Nervous System*, Hortega postulated some of the earliest observations about the origin and functional characteristics of microglial cells (Hortega 1932).

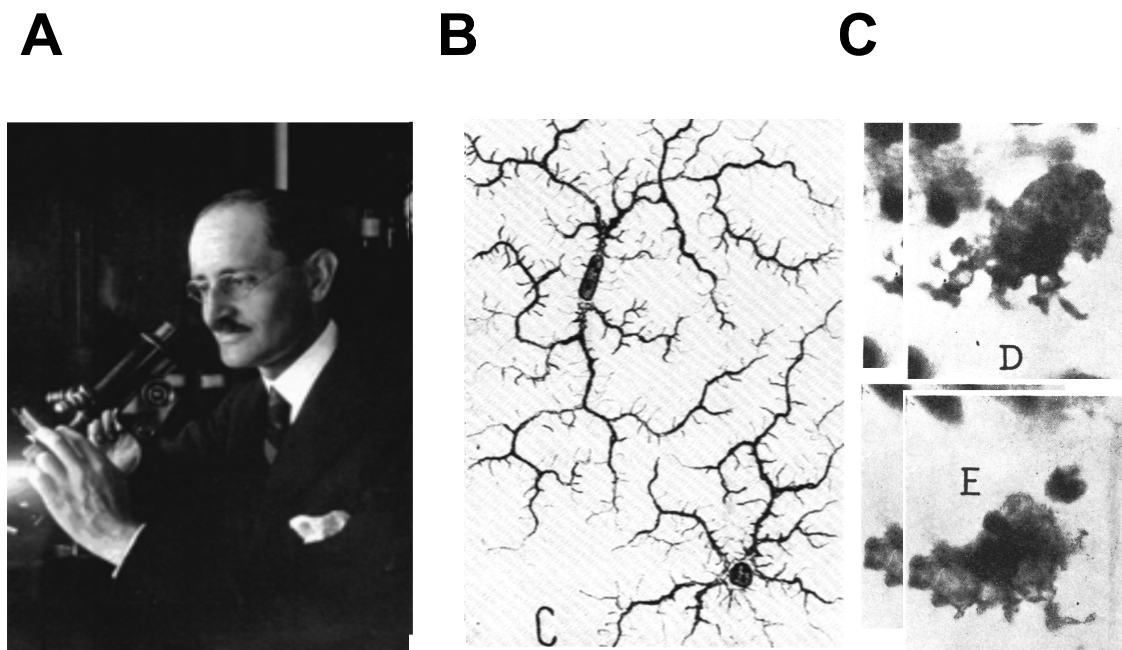


Figure 1. 4 Microglial cells- discovery and origin. (A) Pio del-Rio Hortega (1882-1945). (B) resting microglia drawn by Rio Hortega. (C) earliest photomicrographs of activated and pseudopodic microglia. Adapted from Hortega 1932.

During late embryogenesis and peri-natal period, blood monocytes colonize the brain from the pia mater, evenly distribute throughout the brain and mature into microglia (Ling and Wong 1993). In peri-natal stages they exhibit an amoeboid

morphology with little cell processes indicating a rather active state phenotype with high proliferation rates (Farber and Kettenmann 2005). These cells are often referred to as microglial progenitors (Streit et al. 2005). During the peri-natal period microglia have one important role in CNS maturation, which is to regulate the specific neuronal fractions by differential induction of apoptosis (Marin-Teva et al. 2004) and by phagocytosing the apoptotic neurons (Ling and Wong 1993; Moore and Thanos 1996). Further, during brain development these amoeboid microglia differentiate into ramified microglia with a small cell body and a large number of fine and long membrane protrusions (Streit et al. 2005; Streit and Kincaid-Colton 1995)

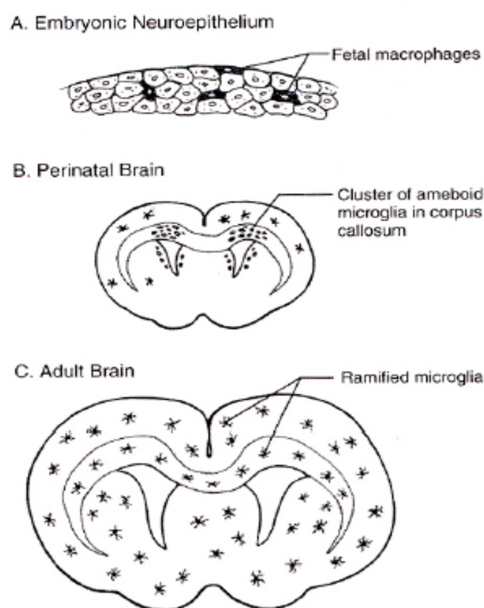


Figure 1. 5 Developmental stages of microglia. (A) Fetal macrophages are found in the neuroectoderm of 8 day old embryonic brains. (B) Groups of amoeboid microglia are found in the perinatal brain. (C) Ramified microglia reside in the adult brain. Adapted from Neuroglia, 2nd ed, Oxford University Press 2005.

1.3.2. Microglia- role in physiology and pathology

1.3.2.1. Resting or ramified microglia

Microglial cells (along with astrocytes) are distributed in large non-overlapping regions of the brain and spinal cord and constitute upto 20% of all glial cell population in the adult brain (Altman 1994; Nimmerjahn et al. 2005; Perry 1994).

Microglial cells were earlier presumed to be in the "resting" state in adult brain. Microglia in the "resting state" are characterized by a ramified morphology and by the expression of certain cell surface antigens, like complement receptor C3b (Wu et al. 1994). The "resting state" earlier suggested that microglial cells were non-active. However, this stage does not reflect the original microglial cell profileration (Raivich 2005). Lawson et al demonstrated that resting microglia proliferate at a low rate even in normal, non- pathologically altered brain (Lawson et al. 1992). These ramified or "resting microglia" constantly scan their environment by means of their motile processes. Moreover, the recent two-photon motion-picture study by Nimmerjahn (Nimmerjahn et al. 2005) demonstrated an active role of these microglial cells in the mouse brain, a state in which they continuously and dynamically examine and interact with their microenvironment. Under physiological conditions, microglia cells move their ramified processes very diligently without moving their cell body. The Nimmerjahn studies suggest that microglia survey or scan every area of the brain by moving their processes within short time periods. In the pathological state, microglial cells migrate within minutes to the site of injury, travelling more than 100 μm distances. At the site of the injury, microglial cells rapidly shrink their fine processes and enlarge their cell bodies, a morphological change characteristic of activated microglial cells. When there is brain damage or injury, microglial cells assume the "amoeboid" phenotype and get activated in a multistage pattern.

1.3.2.2. Activated or amoeboid microglia

"*Activated microglia*" is a term used to describe the change in the physiological properties of microglial cells in response to different pathological conditions in the brain such as viral or bacterial infections, CNS injuries or other diseases (Town et al. 2005). Activation of microglial cells is followed by morphological and functional transformations (Gehrmann and Banati 1995; Streit et al. 1988). The ramifications get shortened, their number reduced and the cell body gets enlarged, a state termed as *amoeboid microglia* (Kreutzberg 1996). Microglia activation increases gradually and can be further sub-characterized by increased cell motility,

proliferation, phagocytosis (Gehrmann et al. 1995; Hurley et al. 1999; Kreutzberg 1996; Streit et al. 2005; Streit et al. 1988; Town et al. 2005) and also by changed electrophysiological properties like established inward and outward rectifying potassium currents (Farber and Kettenmann 2005) and increased basal Ca^{2+} levels (Hoffmann et al. 2003). Activated microglia can release a diverse set of cytotoxic substances like reactive oxygen radicals, nitric oxide (NO) and non-specific proteases which are important for the activation of cytokines by cytokine shedding (Banati et al. 1993; Kreutzberg 1996; Town et al. 2005; Zielasek et al. 1996). Primary microglial cells in culture are neither "*resting*" nor "*activated*". They show a rather amoeboid morphology with little ramified processes and possess inward rectifying potassium currents, whereas microglia in acute brain slices are characterized by a ramified morphology and the absence of potassium currents (Farber and Kettenmann 2005). Interestingly, when microglial cells are cultured with astrocyte conditioned medium they regain their ramified morphology (Eder et al. 1998). This emphasizes their responsiveness to environmental cues and points out that microglia research must be multi-sided and as close to *in vivo* setting as possible to avoid artifacts of experimental approaches (Kettenmann et al. 2011; Nimmerjahn et al. 2005) .

1.4. Brain tumors

Gliomas are the most abundant primary tumors of the central nervous system. They appear with an incidence of 5 in 100,000 (Friese et al. 2004) and constitute 30-40% of all brain tumors, thus representing the largest group of brain neoplasms in adults. Classically, gliomas were thought to arise as a result of neoplastic transformation of glial cell sub-types, namely the astrocytes (Astrocytoma), oligodendrocytes (Oligodendroglioma), ependymal cells (Ependymoma).

Gliomas were classified in 1993, 2000 and 2007 by the World Health Organization (WHO) and listed in the "WHO Blue Books" into mainly four grades based on the presence of certain histo-pathological, clinical and genetic characteristics which include nuclear atypia, high mitotic activity, necrosis and neo-vascularization

(Kleihues et al. 1993; Kleihues et al. 2002; Kleihues et al. 1995; Louis et al. 2007). WHO Grade I are low grade, pediatrical tumors; WHO Grade II are low malignant oligodendrogliomas, diffuse-astrocytomas and ependymomas; WHO Grade III are anaplastic-astrocytomas, oligodendrogliomas and ependymomas; WHO Grade IV are highly aggressive glioblastomas (common in adults) and malignant gliomas-like pineoblastomas and medulloblastomas (common in children).

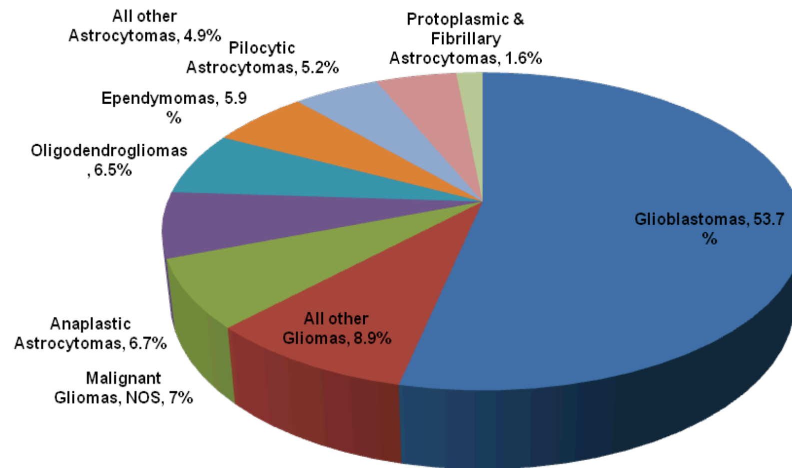


Figure 1. 6 Distribution of All Primary Brain and CNS Gliomas by histology sub-types. Modified from CBTRUS Statistical Report: NPCR and SEER Data from 2004-2008.

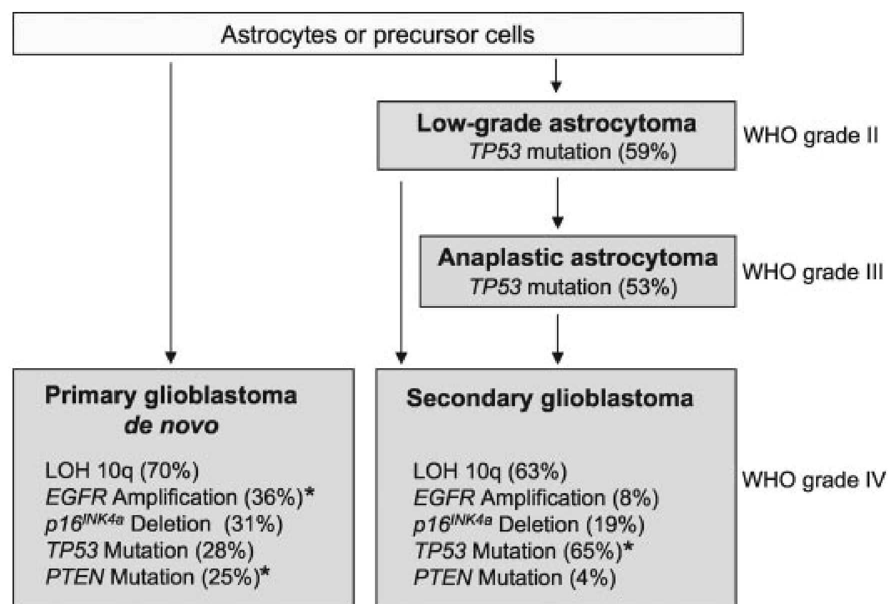


Figure 1. 7 Genetic pathways of gliomagenesis. Accumulation of different gene transformations (mutations and amplification of tumor suppressor genes and oncogenes) in astrocytes or neural precursor cells leads to the development of progressive astrocytomas (seconday GBMs) or *de novo* malignant astrocytomas (primary GBMs). Adapted from Ohgaki et al. 2004.

In adults, two thirds of primary brain tumors arise from structures above the tentorium (supratentorial), whereas in children, two thirds of brain tumors arise from structures below the tentorium (infratentorial). The most common type of gliomas are astrocytomas, which range from low grade, relatively indolent juvenile pilocytic astrocytomas to the highly invasive and fatal glioblastoma multiformae. Generally, the diffusely infiltrating astrocytomas of the cerebral hemispheres tend to progress towards a more malignant phenotype. It is still unknown whether gliomas originate from immature astrocytes, mature astrocytes, or neuroectodermal stem cells located in the adult brain. Nevertheless, the pathological diagnosis relies on comparison of the gliomas with non-neoplastic mature glial cells (Weller 2003).

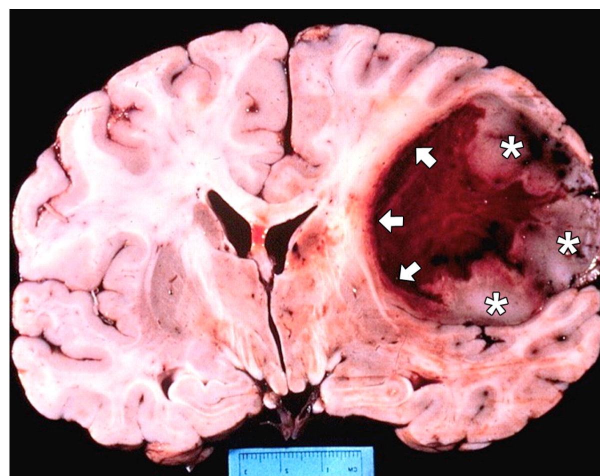


Figure 1. 8 Glioblastoma multiforme. Photograph of a coronal section shows the outer cortical region of the tumor with the more typical, thick irregular rim (*) and shaggy inner margin and the relatively smooth, thin, deep inner margin (arrows). Within the neoplasm is a region of hemorrhagic necrosis. Scale is in centimeters. Taken from Smirniotopoulos et al 2007.

1.5. Role of microglia in brain tumors

1.5.1. Immune cell infiltration of intrinsic intracranial tumors

Immune cells, with the exception of microglia, do not infiltrate gliomas in large numbers. Strik et al reported in a study of 67 intracranial neoplasms from which 18 were glioblastoma multiformae (GBM) that 2% of the cells are tumor associated leukocytes, labeled for leukocyte marker LCA (Strik et al. 2004), whereas 15% are microglia (labeled for CD68). They describe the morphology of these tumor-associated microglia as mostly amoeboid. To characterize CNS microglia Sedgwick et al. used flow cytometry by simultaneous CD11b/c and CD45 labeling (Sedgwick et al. 1991). Microglia cells differ from macrophages in their low content of CD45. Therefore, microglia cells are identified as $CD45^{low}$ $CD11b/c^{high}$ cells and macrophages as $CD45^{high}$ $CD11b/c^{low}$. Using this method Badie et al. showed that microglia were detected in high number in the brain tissue surrounding the tumors (Badie and Schartner 2001; Badie and Schartner 2000) in contrast to macrophages which were only found inside the tumors. Along that line, Roggendorf et al reported in a neuropathological study that a vast number of amoeboid microglia are located in the peripheral tumor areas, where the tumor shows diffuse infiltration into surrounding brain tissue (Roggendorf et al. 1996). The exact resource of microglia in brain tumors remains unclear. A variety of growth factors and chemokines can be released directly from the tumor cells or as a result of local tissue injury due to tumor growth and metastases (Badie and Schartner 2001; Badie et al. 2001). This can result in the recruitment of microglia and macrophages from two main sources: resident brain microglia or perivascular macrophages that can become activated and migrate toward brain tumors (Watters et al. 2005). Furthermore, CNS parenchyma can be populated by trafficking hematopoetic cells such as monocytes that can assume typical microglial cell morphology upon entry into the CNS (Flugel et al. 2001). Regardless of their origin, these microglia and macrophages can continuously infiltrate brain tumors and influence tumor growth (Gabrusiewicz et al. 2011; Villeneuve et al. 2005; Zhai et al. 2011).

1.5.2. Microglia-glioma cross talk

A number of studies have demonstrated the intense communication between glioma cells and microglia. Microglia are attracted by several glioma secreting chemoattractants: Monocyte chemoattractant protein-1 [MCP-1; (Prat et al. 2000)], acts on microglia receptor CCR2 (Galasso et al. 2000), colony stimulating factor-1 (Papavasiliou et al. 1997), granulocyte colony-stimulating factor [G-CSF; (Stan et al. 1994)] and hepatocyte growth factor/scatter factor (HGF/SF) acts on the HGF/SF receptor c–Met (Koochekpour et al. 1997). Further interaction of microglia and gliomas is complex and intensely researched. It is presently debated whether the presence of microglia in and around tumors is an attempt by the immune response to combat the tumor, or whether microglia are recruited by tumors to promote their growth and proliferation. On one hand, microglia may act against the tumor by releasing anti-tumorigenic cytokines like TNFα, or behave as antigen presenting cells (APC) by expressing MHC II and B7.1 and B7.2 molecules. However, there is little evidence that the release of cytotoxic cytokines is the main action of tumor infiltrating microglia. Furthermore, microglial cells are weak antigen presenting cells in gliomas due to the abundance of IL-10 in the tumor (Stan et al. 1994; Wagner et al. 1999). On the other hand, it is possible that microglia promote glioma proliferation and invasion via pro tumor secreted cytokines.

1.5.3. Immune tolerance by gliomas

Patients with malignant gliomas show decreased cellular immunity (as assessed by delayed-type cutaneous reactions) and a reduced number of circulating T cells due to the selective depletion of T helper cells (Brooks et al. 1972; Brooks et al. 1977; Mahaley et al. 1977). A characteristic feature of glioblastomas is that they do not metastasize systemically (Schweitzer et al. 2001; Stark et al. 2005). Secondary extraneural metastases from the lung or breast have however been reported, showing tumor growth under limited immune-surveillance. After withdrawal of immunosuppressive drugs extraneural glioma cells were eradicated

in several cases, suggesting an immunological control of glioma cells outside the CNS, while the CNS milieu supports tumor growth and inhibits immune responses (Schweitzer et al. 2001). Hao et al quantitatively analyzed the expression of 53 cytokines and cytokine receptors in human gliomas and glioma cell lines (Hao et al. 2002). The results of this quantitative study indicate that the strongly immunosuppressive cytokine response greatly predominates in both human solid tumors and in glioma cell lines. For example, the cytokines interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin-M (OSM), TGF ß and their respective receptors were strongly expressed in nearly all glioblastomas and cell lines tested, whereas pro-inflammatory cytokines interferon γ (IFNγ), tumor necrosis factor α (TNFα), IL-2 and IL-12 family members and their receptors were virtually absent in both tumors and cell lines (Hao et al. 2002). Others have observed similar expression patterns of immunosuppressive cytokines in gliomas. All IL-6 type cytokines (IL-1, IL-11, CNTF, CT-1, LIF, and OSM) have been detected at the protein and mRNA levels in glioma cell lines (Goswami et al. 1998; Halfter et al. 1998a; Halfter et al. 1998b; Hao et al. 2002; Murphy et al. 1995) as have been all members of the TGF ß cytokine family (Constam et al. 1992; Hao et al. 2002; Olofsson et al. 1992). TGF ß for example inhibits the proliferation of microglia and the production of cytokines *in vitro* (Suzumura et al. 1993), whereas the cytokines IL-6 and IL-10 have been postulated to promote glioma cell proliferation (Huettner et al. 1997); (Goswami et al. 1998). The immunosuppressive cytokine IL-10 not only promotes glioma cell proliferation, but also enhances their ability to migrate *in vitro* (Huettner et al. 1997), further supporting the importance of microglia–glioma cross talk. Immunosuppressive effects on leukocyte apoptosis have also been observed. Badie and colleagues demonstrated previously that leukocyte infiltration into subcutaneously (SC)-propagated GL261 mouse gliomas was much greater than leukocyte infiltration into intracranially (IC)-propagated tumors, again suggesting that the brain microenvironment plays an important immunosuppressive role (Badie et al. 2001). Furthermore, they postulate that the threefold increased expression of FasL by monocytes in IC gliomas compared to SC gliomas is pro-apoptotic for circulating, glioma infiltrating leukocytes.

1.6. Matrix metalloproteinases (MMPs)

Components of extracellular matrix (ECM) are required during the process of tissue morphogenesis and development and contribute to the normal homeostasis and turn-over of tissues regulated by proteolytic enzymes. Matrix metalloproteases, collectively called matrixins, are calcium-dependent zinc-ion containing endopeptidases participating in the ECM-degradation (Nagase and Woessner 1999; Sternlicht and Werb 2001). MMP activities are physiologically regulated by hormones, cytokines and growth factors at the level of transcription, activation of the inactive zymogen forms, interaction with specific ECM components, and inhibition by endogenous inhibitors. Endogenous MMP inhibitors (MMPIs) and tissue inhibitors of MMPs (TIMPs) control the activation of these enzymes strictly. MMPs are released by a variety of connective tissues and pro-inflammatory cells including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes (Verma and Hansch 2007). MMPs also regulate cell survival, angiogenesis, inflammation and various mitogenic signaling pathways (Baay et al. 2011; Chakraborti et al. 2003).

Physiological processes	Pathological processes	
Angiogenesis	Arthritis	Osteoarthritis (OA)
Apoptosis	Alzheimer’s disease	Periodontal disease
Blastocyst implantation	Atherosclerosis	Rheumatoid arthritis
Bone remodeling	Breakdown of blood–brain barrier	Skin ulceration
Cervical dilation	Cancer	Sorby’s fundus disease
Embryonic development	Cardiovascular disease	Vascular disease
Endometrial cycling	Central nervous system disorders	Multiple sclerosis
Hair follicle cycling	Corneal ulceration	Nephritis
Immune response	Emphysema	Neurological disease
Inflammation	Fibrotic lung disease	
Nerve growth	Gastric ulcer	
Organ morphogenesis	Guillian-Barre disease	
Ovulation	Liver cirrhosis	
Postpartum uterine involution	Liver fibrosis	
Wound healing	Metastasis	

Table 1. Biological fundtions of MMPs. Modified from Webster and Crowe 2006.

Presently, there are at least 26 known human MMPs. They have been classified based on the type of substrate they degrade (collagenases, gelatinases, stromelysins and matrilysins), their structure and on their trans-membrane localizations.

Nr.	MMP Nr.	Class	Enzyme
1	MMP-1	**Collagenases**	Collagenase-1
2	MMP-8		Neutrophil collagenase
3	MMP-13		Collagenase-3
4	MMP-18	**Gelatinases**	Collagenase-4
5	MMP-2		Gelatinase-A
6	MMP-9		Gelatinases-B
7	MMP-3	**Stromelysins**	Stromelysin-1
8	MMP-10		Stromelysin-2
9	MMP-11		Stromelysin-3
10	MMP-27		Homology to stromelysin-2 (51.6%)
11	MMP-7	**Matrilysins**	Matrilysin (PUMP)
12	MMP-26		Matrilysin-2
13	MMP-14	**MT-MMP**	MT1-MMP
14	MMP-15		MT2-MMP
15	MMP-16		MT3-MMP
16	MMP-17		MT4-MMP
17	MMP-24		MT5-MMP
18	MMP-25		MT6-MMP
19	MMP-12	**Other enzymes**	Macrophage metalloelastase
20	MMP-19		RASI 1
21	MMP-20		Enamelysin
22	MMP-21		MMP identified on chromosome 1
23	MMP-22		MMP identified on chromosome 1

24	MMP-23		From human ovary cDNA
25	MMP-28		Epilysin
26	MMP-29		Unnamed

Table 2. Classification of matrix metalloproteinases. Modified from Verma and Hansch 2007.

Some MMP family members are covalently linked to the cell membrane-membrane type MMPs (MT-MMP) and are specialized for localized and controlled proteolysis of other MMPs (Badie and Schartner 2001). Most of the MMPs are secreted into the extracellular space as inactive zymogens (pro MMPs). Their activation requires proteolytic removal of the pro-peptide region where a zinc atom present in the catalytic domain is bound to a cysteine residue. Activating factors disrupt the cysteine–zinc interaction (`cysteine switch') and thus expose the catalytic site; the result is a partially active intermediate form that can cleave the pro-peptide region by autocatalysis and render the enzyme fully active. An important physiological activator of pro-MMPs is plasmin, a serine proteinase that is generated from plasminogen by the action of tissue- or urokinase-plasminogen activator (uPA). Activation of the MT-MMPs also requires removal of a pro-peptide, but this is catalyzed by a serine proteinase, furin. Activation pathways can co-operate, leading to the activation of additional downstream MMPs such as MMP-9. Once secreted and activated, they are inhibited by a family of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs; (Woessner 1991); (Greene et al. 1996). The balance between the levels of activated MMP and free inhibitors thus determines the overall MMP activity and function (Mohanam et al. 1995). As a result of their potent proteolytic activity, abnormal MMP function leads to pathological conditions and diseases such as arthritis, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers, and fibrosis (Hotary et al. 2003; Yong et al. 1998). MMP dysregulation is also associated with neurodegenerative and neuroinflammatory diseases such as stroke, alzheimer's disease, multiple sclerosis, brain injury/trauma, amyotrophic lateral sclerosis to name a few (Yong et al. 1998).

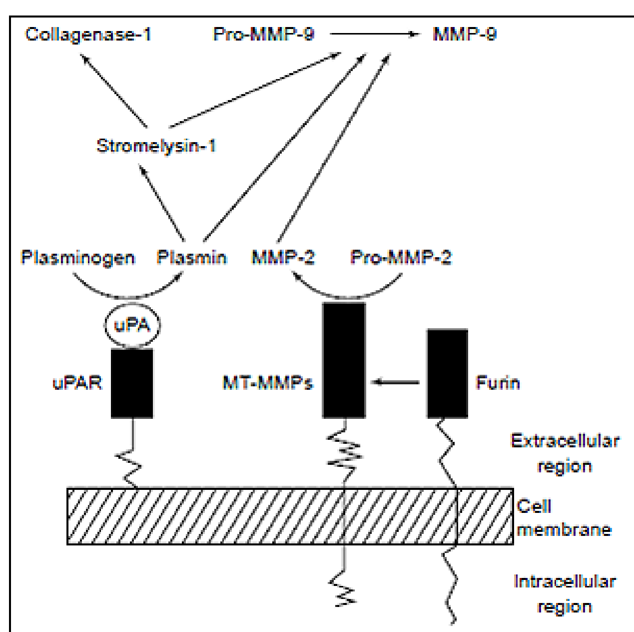


Figure 1. 9 Cascade of matrix metalloproteinase (MMP) activation at the cell surface.

The co-ordinate activation of several MMPs is initiated by the formation of plasmin. Plasmin is produced from plasminogen by the action of urokinase plasminogen activator (uPA) that is anchored by its receptor, uPAR. Plasmin can activate MMP-9 and stromelysin-1, and the latter can in turn activate other MMPs, including MMP-9 and collagenase-1, thus amplifying and broadening the activation cascade. MMP-2 is activated by membrane-type MMPs (MT-MMPs) that are activated by furin proteinases. Taken from Yong et al. 1998.

1.6.1. Role of MMPs in tumors

The classical area of MMP research is tumor invasion and metastasis of systemic cancers. Invasion requires at least two changes in cell behavior: first, the affinity of cells, either for each other or for the ECM, must decrease in order to allow the release of cells from the primary tumor; second, the surrounding ECM must be remodeled by the local production of MMPs to allow cellular migration. Various MMPs are elevated in many types of human cancers including breast, colon, prostate, bladder, ovarian and brain neoplasms (Langers et al. 2012; Liu et al. 2012; Mentlein et al. 2012; Shen et al. 2012; Zheng et al. 2012), and several synthetic inhibitors of MMPs have been shown to reduce tumor invasion and metastasis in animal models. Furthermore, the over-expression of MMPs (including MT-MMPs) in cells increases their metastatic activity, whereas the over-expression of several TIMPs reduces their invasiveness. Surprisingly, the

increased MMP expression in most tumors is derived from host stromal cells rather than the tumor cells themselves, highlighting the importance of tumor–stromal interactions. Local dissemination of tumors is a major cause of high morbidity and mortality in malignant brain tumor patients; MMPs are responsible for this highly invasive behavior of gliomas. A strong correlation can be found between the invasiveness of glioma cells *in vitro* or *in vivo*, and their production of MMP-2, MMP-9 or MT1-MMP (Friedberg et al. 1998; Levicar et al. 2003; Markovic et al. 2005; Markovic et al. 2009; Raithatha et al. 2000).

It has been shown that the expression of MMP-2 by glioma cells correlates with their invasive rate and, in a few patients, accounts for metastasis outside the CNS (Lee and Tsygankov 2010). In contrast to MMPs, TIMP-1 and -2 concentrations can be low in malignant gliomas (Mohanam et al. 1995), suggesting that decreased inhibition of MMP could contribute to their dysregulation in these cells. Whether or not all four forms of TIMPs are altered in gliomas remains to be determined. Furthermore, the potential effects of MMPs (or TIMPs) on glioma proliferation could also be important. Finally, excessive MMP activity might also increase the angiogenic capacity of these highly vascularized tumors. The expression of several MMPs is upregulated in malignant gliomas, and this could contribute to the robust invasive phenotype of glioma cells. Inhibitors of MMPs might be efficient in patients with malignant gliomas, and are currently being tested in several clinical trials (Fu et al. 2011; Nuti et al. 2011). Whether these will be effective alone or in combination with cytotoxic drugs or radiotherapy remains to be determined.

1.7. Toll-like receptors – sentinels of immune system

Immune responses in vertebrates can be classified into two broad groups- the innate immune responses and the adaptive immune responses. The evolutionarily conserved innate immune system controls the early onset of infection. Its main functions include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signaling cascades, and apoptosis (Medzhitov 2001). The innate immune system uses non-clonal sets of

recognition molecules, called pattern recognition receptors (PRRs). Pattern recognition receptors bind conserved molecular structures found in large groups of pathogens, termed pathogen-associated molecular patterns or PAMPs (Medzhitov and Janeway 1997). There are various groups of pattern recognition receptors, which can be secreted, expressed on the cell surface, or reside in intracellular compartments (Medzhitov 2001). The Toll-like receptors (TLRs) are one of the most important pattern recognition receptor families and play a major role in immune defense mechanisms.

The discovery of Toll-like receptors (TLRs) nearly a decade ago laid the foundation to our understanding of how the innate immune system functions, more specifically, of how the body's immune cells could recognize a huge repertoire of evolutionarily conserved "*non-self*" or exogenous (ie. microbial) as well as "*self*" or endogenous (ie. host-derived) molecules, thereby providing the first line of defense not only against invading microorganisms but also in response to tissue trauma and insult, ultimately leading to the development of antigen-specific adaptive immunity. Targeting the TLRs could offer potential new therapeutic strategies to treat a number of acute and chronic inflammatory conditions, ranging from different autoimmune and allergic diseases, ischemia, trauma, neurodegenerative diseases and cancer.

1.7.1. Toll-like receptor signaling

Toll was first identified as a receptor required for the dorso-ventral patterning of the developing embryo of Drosophila (Anderson et al. 1985). Later in 1996, it was discovered that Drosophila *Toll* also conferred anti-fungal properties in the adult fly (Lemaitre et al. 1996), thus contributing to the immune responses of Drosophila. Studies by Gay and Keith showed that the intracellular domain of Drosophila *Toll* had a striking similarity to the intracellular domain of the mammalian interleukin-1 (IL-1) receptor (Gay and Keith 1991). Different human homologues of Drosophila *Toll* were identified and shown to induce activation of NF-κB upon over-expression, revealing that TLRs and IL-1 receptors shared common signal transduction cascades (Medzhitov et al. 1997;

Rock et al. 1998). It was only in 1998, that Poltorak et al. (Poltorak et al. 1998) discovered that the *lps* gene in the lipopolysaccharide (LPS)-nonresponsive mouse strain CH3/HeJ encoded a murine member of the TLR family, providing the first clue of a functional pattern recognition receptor role in mammalian TLRs. This led to the identification of Toll-like receptors (TLRs) in mammals, with TLR4 being the first mammalian homolog of Drosophila *Toll.*

Till date, 11 Toll-like receptors have been identified in humans and 13 in mice (Leulier and Lemaitre 2008; Okun et al. 2009). TLRs are expressed in innate immune cells (macrophages, dendritic cells, NK cells), in adaptive immune cells (B and T lymphocytes) and in non-immune cells (epithelial cells, endothelial cells, fibroblasts) (Delneste et al. 2007). TLRs are type I integral membrane receptors with an extracellular trans-membrane and an intracellular domain. They are either localized to the cell surface (TLRs 1, 2, 4 and 6) or expressed in intracellular compartments such as endosomes or lysosomes (TLRs 3, 7, 8 and 9) (Heil et al. 2003). The cytoplasmic portion of the TLRs shows high similarity to that of the Interleukin-1 receptor family and is also known as the Toll/IL-1 receptor (TIR) domain. However, the extracellular portion of both these receptors is different- while IL-1 receptor is characterized by an Ig-like domain, the TLRs have leucine-rich repeats in their extracellular domain. Leucine-rich repeats are found in both cytoplasmic and transmembrane proteins and are involved in ligand recognition and signal transduction (Kobe and Deisenhofer 1995).

Microbial recognition of TLRs aids their dimerization, mainly formation of homodimers, with the exception of TLR2, which forms a heterodimer either with TLR1 or TLR6 or TLR10 (Akashi-Takamura and Miyake 2008). Dimerization of TLRs triggers the activation of downstream signaling pathways from the conserved cytoplasmic TIR domain-containing adaptor, myeloid differentiation factor 88 (MyD88) which is required for the induction of inflammatory cytokines such as TNF-α, IL-6 and IL-12 by all TLRs, except TLR3 (Akira 2006). Most of the TLRs signal in a MyD88-dependent manner with the exception of TLRs 3 (Alvarez 2005; Okun et al. 2009) and sometimes TLR4 (Jiang et al. 2005; Kenny and O'Neill 2008; Yamamoto et al. 2003).

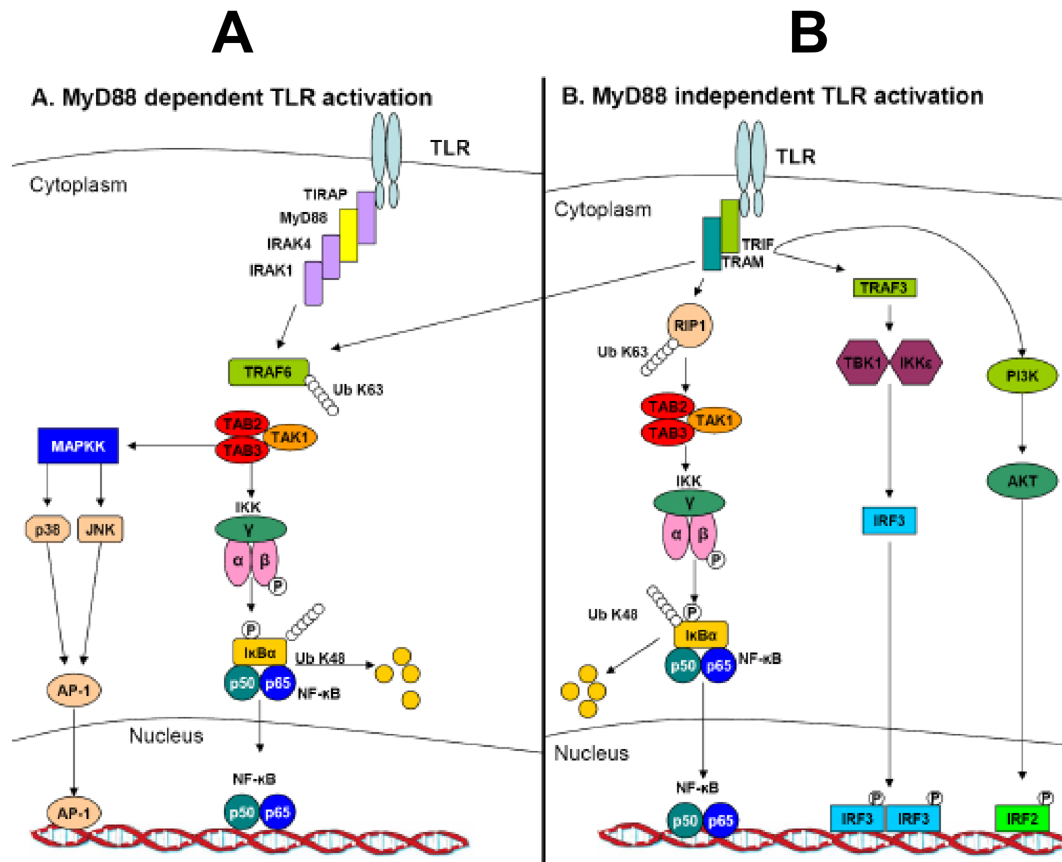


Figure 2 TLR mediated signaling.

(A) Myd88 mediated pathway is shared by all TLRs except TLR3 and TLR4. MyD88 recruits TRAF6 and members of the IRAK family. TRAF6, along with Uev1A and Ubc13 activates the TAK1 complex by a K63 linked ubiquitination. The TAK1 complex then activates the IKK complex that consists of IKKα, IKKβ and IKKγ, that further catalyzes IκB proteins phosphorylation. This in turn facilitates IκB proteins degradation by a proteasome-dependent manner, which allows NFκB translocation to the nucleus. TAK1 also activates the MAPK pathway which culminates in AP-1 activation. The combination of AP-1 and NFκB controls inflammatory responses mediated by inflammatory cytokines. (B) MyD88 independent starts when TRIF associates with TRAF3 which binds to TBK1 and IKKε. This binding culminates in IRF3 phosphorylation that facilitates IRF3 dimerization and translocation into the nucleus and transcription regulation. TRIF can also interact with TRAF6 which along with RIP1 mediates NFκB activation. Adapted from Okun et al. 2009.

1.7.2. Mediators of Toll-like receptor signaling

The exact nature and type of ligand that recognizes different TLRs is determined by using TLR-deficient cells derived from a TLR KO mouse or a natural mutant and/or cells transfected with TLR cDNAs (Takeda and Akira 2003). Anti-TLR antibodies can also be used if the TLR is present on the cell surface, although the inhibition maybe partial. The sub-cellular localization of different TLRS is very important for effective recognition of different ligands. Receptors such as TLR 1, 2,

4 and 6 are located on the cell surface and are hence capable of recognizing and interacting with their cognate ligands at the cell surface. On the other hand, TLRs 3, 7, 8 and 9 interact with their putative ligands in endosomal or lysosomal compartments inside the cells (Kaisho and Akira 2003; Latz et al. 2004; Matsumoto et al. 2003). Each TLR recognizes a specific PAMP in order to initiate and trigger innate immune responses through induction of gene expression of important pro-inflammatory cytokines. Based on the type of PAMPs they recognize, TLRs are divided into 3 groups (Akira 2006; O'Neill 2006). The first group comprises of TLR2 (as a heterodimer with TLR1 or TLR6) and TLR4 (as homodimer) recognizing lipid-based ligands or lipoteichoic acid (TLR2; (Takeuchi et al. 1999; Takeuchi et al. 2002)) and LPS (TLR4; (Poltorak et al. 1998)) respectively. The second group consists of TLRs 3, 7, 8 and 9 which recognize bacterial and viral nucleic acids such as dsRNA (TLR3;(Alexopoulou et al. 2001)), ssRNA (TLR7 and 8; (Lund et al. 2004) ; (Heil et al. 2004) and non-methylated CpG DNA (TLR9; (Hemmi et al. 2000). The third group comprising of TLRs 5 and 11 recognize microbial proteins such as Flagellin (TLR5; (Hayashi et al. 2001) and Profilin (murine TLR11; (Yarovinsky et al. 2005)). No ligand has been identified for TLR10 as yet.

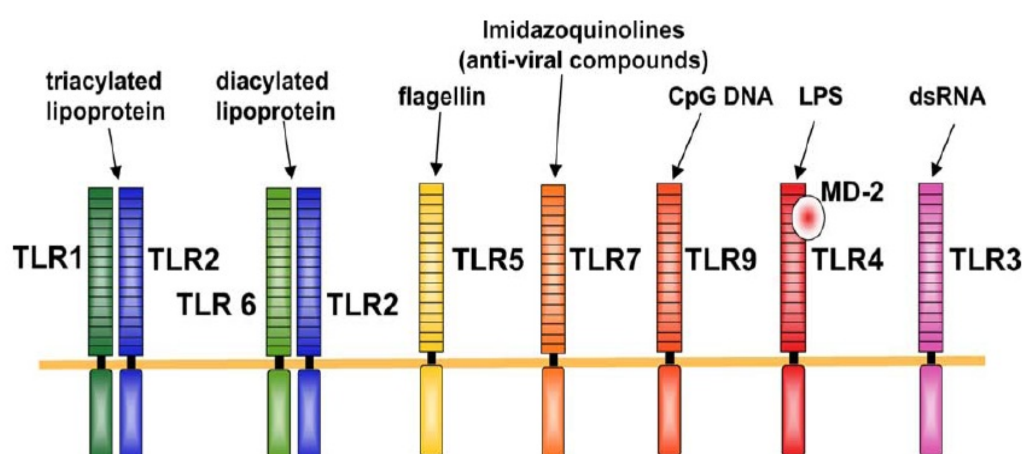


Figure 2. 1 TLRs and their exogenous ligands.

TLR1–TLR7 and TLR9 have been characterized to recognize microbial components. TLR2 is essential for the recognition of microbial lipopeptides. TLR1 and TLR6 associate with TLR2, and discriminate subtle differences between triacyl- and diacyl lipopeptides, respectively.TLR4 recognizes LPS. TLR9 is the CpG DNA receptor, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 is a receptor for flagellin. Taken from Akira 2006.

This grouping is rather over-simplified because many TLRs are promiscuous, in particular the TLRs 2 and 4, which recognize a wide repertoire of various microbial and mammalian proteins and also several endogenous molecules (Tsan and Gao 2004; Tsan and Gao 2007). This “promiscuity” of the TLRs could be explained according the danger theory (Matzinger 2002a; Matzinger 2002b) which proposes that the immune system not only recognizes external and internal danger signals but also non-self degradation products of endogenous macromolecules released as a result of tissue injury, trauma, necrosis, infection, and/or tissue remodeling (Johnson et al. 2003), hence the name Damage or Danger-associated molecular patterns (*DAMPs*).

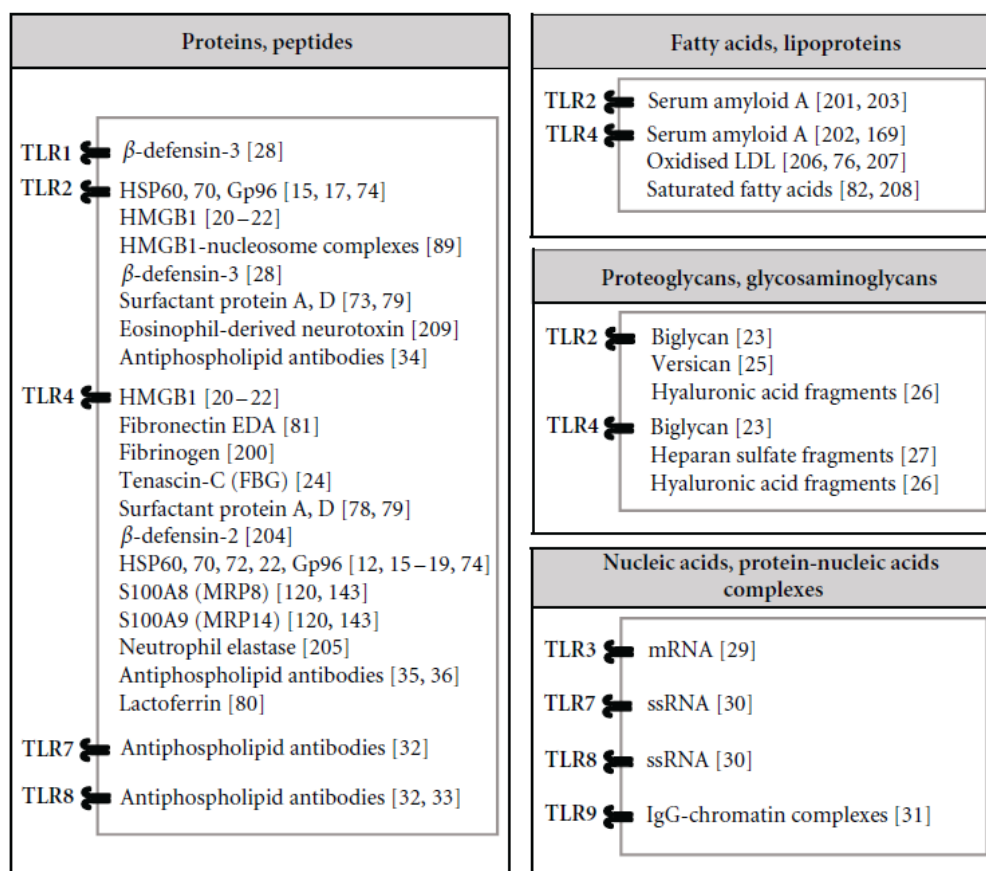


Figure 2. 2 TLRs and their endogenous ligands.

TLRs are activated by damage-associated molecular patterns (DAMPs) including intracellular molecules released in the extracellular milieu by activated or necrotic cells and extracellular matrix molecules either upregulated upon injury or degraded following tissue damage. Known endogenous TLR activators are listed based on their biochemical nature.Taken from Piccinini and Midwood 2010.

1.7.3. Toll-like receptors in cancer

Increasing evidence indicates that engagement of TLRs can promote cancer cell survival and proliferation, induce tumor immune evasion, and enhance tumor metastasis and chemoresistance (Yu et al. 2011). The various endogenous molecules or DAMPs released from damaged/necrotic tissues are capable of activating TLRs and thus the endogenous ligand-mediated TLR signaling is implicated in tumor development and progression and thereby affects the therapeutic outcome of tumors. Future studies and immune-based therapies could thus be developed to address the pathological effect exerted by different TLRs in the etiology of cancers such as esophageal squamous cell carcinoma (Sheyhidin et al. 2011), malignant melanoma (Gast et al. 2011), cervical cancer (DeCarlo et al. 2011), intestinal epithelial cancer (Marques and Boneca 2011), gliomas to name a few (El Andaloussi et al. 2006; Haghparast et al. 2011; Hussain et al. 2006).

1.7.4. Toll-like receptors in neuroprotection

The discovery of mammalian TLRs has greatly altered our understanding of how the innate immune system recognizes and responds to diverse microbial pathogens. The expression of TLRs in CNS was mainly studied in astrocytes and microglial cells (Bsibsi et al. 2002; Okun et al. 2009; Olson and Miller 2004). However, new evidence indicates that these receptors may also play an important role in neuronal homeostasis (Okun et al. 2011; Rolls et al. 2007). TLR signaling also mediates host defense responses during several CNS infections such as meningitis (Hoffmann et al. 2007; Lehnardt et al. 2006), cerebral malaria (Coban et al. 2007; Mishra et al. 2009) The expression of TLRs in the CNS is variable and can be modulated by multiple factors, including pro-inflammatory molecules, which in turn increase the expression of TLRs in CNS cells and contribute to diseases progression of several brain inflammatory pathologies like cerebral ischemia/stroke, trauma and hemorrhage, pathogen infection and autoimmune

diseases (Kong and Le 2011). Not only is their expression elevated in inflammatory diseases, but TLRs are also involved in several aspects of neurodegenerative disease development and progression (Arroyo et al. 2011). Owing to their presence and immune-regulatory role within the brain, TLRs represent an attractive therapeutic target for numerous CNS disorders and infectious diseases (Hanke and Kielian 2011). Hence in order to develop potential therapeutic strategies to target TLRs, a thorough understanding of the signals governing specific CNS disorders would have to be achieved so as to develop customized therapies for various diseases involving the TLRs.

1.8. Role of Minocycline in pathology

Minocycline is a broad spectrum semi-synthetic second generation derivative of the antibiotic tetracycline, with bacteriostatic functions and has been approved for over 30 years by the FDA to treat chronic inflammatory conditions such as acne, rosacea, skin and respiratory tract infections (Seukeran et al. 1997; Yong et al. 2004). It is a small, highly lipophilic molecule (495kDa), readily absorbed from the gut after oral intake and capable of crossing the intact blood-brain barrier (Seukeran et al. 1997).

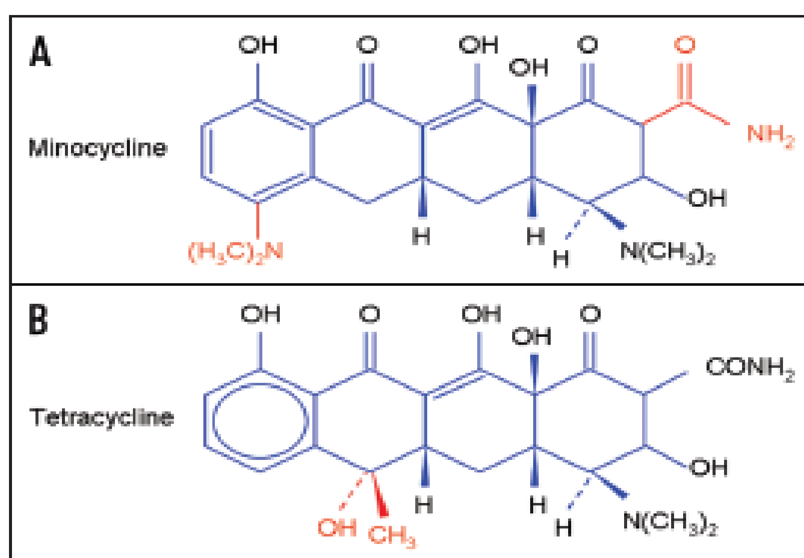


Figure 2. 3 Chemical structures of minocycline and tetracycline.

Apart from its bactericidal effects, the role of Minocycline in other pathologies, especially those involving neuroinflammation and neurodegeneration has proved its efficacy as a neuroprotective agent. The ability of minocycline to alleviate disease symptoms of various neurological disorders in animals is being increasingly recognized. Minocycline has been demonstrated to block p38 MAP kinase pathway in microglia cells and to thereby counteract microglia cell activation into a pro-inflammatory phenotype (Suk 2004).

A large number of studies have shown that Minocycline can alleviate the severity of symptoms of various neurological disorders ranging from depression (Dean et al. 2012; Pae et al. 2008), schizophrenia (Levkovitz et al. 2007; Miyaoka 2008; Miyaoka et al. 2012), neuroinflammatory conditions such as intra-cerebral ischemia and hemorrhage (Hess and Fagan 2010; Rosenberg et al. 2007), neurodegenerative disorders like stroke, multiple sclerosis, spinal-cord injury, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Yong et al. 2004) and also in brain tumors like gliomas (Daginakatte and Gutmann 2007; Frazier et al. 2003; Markovic et al. 2011; Weingart et al. 1995). Although the exact molecular mechanisms of the neuroprotective role of Minocycline are still unclear, new studies are required to unravel its mode of action However, Minocycline certainly is a potential therapeutic drug for treating CNS disorders.

2. OBJECTIVES

Gliomas are a group of extremely heterogeneous tumors mainly due to the presence of different non-transformed parenchymal cell types, often intermingled with tumor cells. This sub-population of cells comprising of neural precursor/stem cells, endothelial cells, stromal cells, tumor-associated macrophages and microglia play a pivotal role in controlling the course of glioma progression and development. Such interactions often contribute to neo-vascularization, invasion, survival, proliferation and progression of gliomas. From previous studies (Markovic et al. 2005; Markovic et al. 2009), it was shown that soluble factor(s) released by gliomas induce an increase in expression and activity of MT1-MMP in microglia closely associated with the invasive edge of gliomas. This up-regulation takes place via the activation of p38 MAPK pathway and the signal is transduced via the Toll-like receptor (TLR) pathway, since deletion of the crucial adaptor molecule of TLRs, MyD88, not only led to a reduction in MT1-MMP expression and activity but also caused attenuation of tumor growth and invasion *in vivo*.

2.1. Project 1

Project 1 was designed to investigate the role of MyD88-dependent TLR signaling pathways in glioma-infiltrating microglial cells. The specific aims of the project were thus to:

1. Examine if Toll-like receptors (TLRs) regulate glioma-microglia interaction, with special emphasis on identifying specific TLR sub-type(s) in glioma associated microglial cells;

2. Investigate the effect on MT1-MMP gene expression of different TLR sub-type specific agonists in primary microglial cells *in vitro*;

3. Identify possible TLR(s) involved in upregulating MT1-MMP expression in glioma-associated microglia *in vitro*;

4. Examine how the identified TLR(s) might interfere with tumor expansion and MT1-MMP expression using *in vivo* and *ex vivo* experimental mouse models

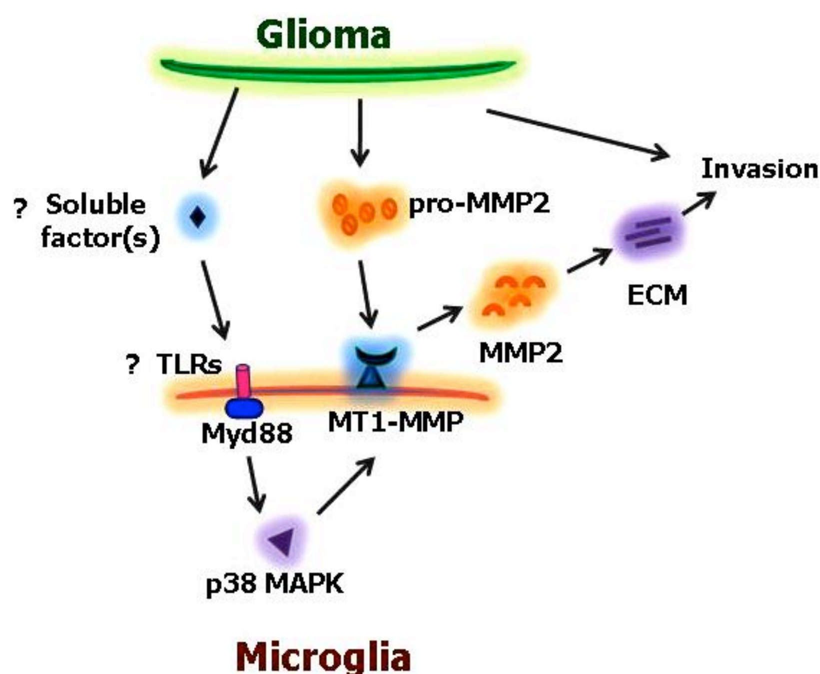


Figure 3. Microglia- glioma cross-talk. Modified from Markovic et al. 2005, 2009. .

2.2. Project 2

Project 2 was designed to explore if tumor promoting effects of microglia on glioma expansion and invasion could be pharmacologically intervened. A commonly used antibiotic Minocycline was used to address this question. Minocycline is a known blocker of p38 MAPK phosphorylation and microglial cell activation. The specific goals of this project were to:

1. Analyze effect of Minocycline on glioma- associated microglia *in vitro*;

2. Examine if Minocycline could reduce glioma-induced MT1-MMP expression in microglial cells and if it could also interfere with tumor expansion under *ex vivo* and *in vivo* conditions.

3. MATERIAL & METHODS

3.1. Material

3.1.1. Reagents and Chemicals

Reagents/Chemicals	Company
Acetic Acid	Carl Roth GmbH, Karlsruhe, Germany
Acrylamide/bis-Acrylamide 30%	Sigma-Aldrich, Munich, Germany
Agar agar	Carl Roth GmbH, Karlsruhe, Germany
Ammonium persulphate (APS)	Merck, Darmstadt, Germany
Aqua Polymount	Polysciences Europe GmbH, Eppelheim, Germany
ßeta-Mercaptoethanol	Sigma-Aldrich, Munich, Germany
Brij 35®	Sigma-Aldrich, Munich, Germany
Brilliant Blue R®	Sigma-Aldrich, Munich, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH, Karlsruhe, Germany
Clodronate-liposomes©	Clodronate Liposomes Foundation (CLF), Amsterdam, The Netherlands
Cyanoacrylate glue	UHU GmbH, Bühl, Germany
cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets	Roche Diagnostics, Mannheim, Germany
DAB (3, 3' DiaMinocyclinebenzidine Tetrahydrochloride) tablets	Sigma-Aldrich, Munich, Germany
dNTP (deoxyribonycleoside triphospahtes)	Invitrogen, Karlsruhe, Germany
Donkey serum	Sigma-Aldrich, Munich, Germany
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, Darmstadt, Germany
Enhanced Green Fluorescent Protein (EGFP)	Clontech, Heidelberg, Germany
Ethylene glycol	Carl Roth GmbH, Karlsruhe, Germany
Ethidium Bromide (EtBr)	Carl Roth GmbH, Karlsruhe, Germany
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
FastStart SYBR green master	Roche Diagnostics, Mannheim, Germany
Fetal calf serum (FCS)	Invitrogen, Darmstadt, Germany
Flagellin	Cayla- InvivoGen, Toulouse, France
Gel blotting paper	Carl Roth GmbH, Karlsruhe, Germany
Gelatin from porcine skin, type A	Sigma-Aldrich, Munich, Germany
Geneticin G418	GIBCO®, Maryland, Germany
Glycine	Merck, Darmstadt, Germany
Glucose	Carl Roth GmbH, Karlsruhe, Germany
Glycerol	Sigma-Aldrich, Munich, Germany
Hank's balanced salt solution (HBSS)	Invitrogen, Darmstadt, Germany
Hybond-P PVDF membrane	GE Healthcare Europe GmbH, Munich, Germany
Isopropanol	Carl Roth GmbH, 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
MALP2	Cayla- InvivoGen, Toulouse, France
Minocycline hydrochloride	Sigma-Aldrich, Munich, Germany
Methanol	Carl Roth GmbH, Karlsruhe, Germany
Oligo(dT)12-18 primers	Invitrogen, Karlsruhe, Germany
Pam3Csk4	Cayla- InvivoGen, Toulouse, France
PageRuler™ Prestained Protein ladder	Fermentas GmbH, Germany
Paraformaldehyde (PFA)	Merck, Darmstadt, Germany
PCR High Fidelity Supermix®	Invitrogen, Karlsruhe, Germany
Penicillin/Streptomycin	Biochrom AG, Berlin, Germany
Phosphate Buffered Saline (DPBS)	Invitrogen, Darmstadt,Germany
Poly (I:C)	Cayla- InvivoGen, Toulouse, France
Poly (U)	Cayla- InvivoGen, Toulouse, France
Restore™ Plus Western blot stripping buffer	Pierce (Thermo Fischer Scientific), Bonn, Germany
Roti® -load DNA-small (6X gel loading buffer with Xylene Cyanol)	Carl Roth GmbH, Karlsruhe, Germany
Roti® pUC19 marker ready to use	Carl Roth GmbH, Karlsruhe, Germany
SeaKem® LE Agarose	Lonza, Cologne, Germany
Sodium chloride (NaCl)	Carl Roth GmbH, 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
TEMED (N, N, N', N' Tetramethyl-Ethylene Diamine)	Amresco, USA
Tris base	Carl Roth GmbH, Karlsruhe, Germany
Triton X-100	Merck, Darmstadt, Germany
Trypsin/EDTA	Biochrom AG, Berlin, Germany
Tween-20	Merck, Darmstadt, Germany

Table 3. List of reagents and chemicals

3.1.2. Commercial Kits

Kits	Company
BCA Protein assay kit	Pierce (Thermo Fischer Scientific), Bonn, Germany
InviTrap® Spin Universal RNA mini kit	Invitek (Stratec), Berlin, Germany
Mouse TLR2 agonist kit	Cayla-InvivoGen, Toulouse, France
MMP-14 Biotrak activity assay kit	GE Healthcare Europe GmbH, Munich, Germany
peqLAB DirectLyse tail kit (Genotyping)	peqLAB, Erlangen,Germany

Table 4. List of commercial kits

3.1.3. Devices and Equipment

Device	Company
CCD Camera	Proscan, Germany
Centrifuges 5403, 5417R, 5810R	Eppendorf, Hamburg, Germany
Clean bench	Biowizard, USA
Neubauer cell counting chambe	LaborOptik, Germany
Axioplan Flourescence microscope	Carl Zeiss, Jena, Germany
BioPhotometer plus	Eppendorf, Hamburg, Germany
Thermomixer	Eppendorf, Hamburg, Germany
TCS SPE confocal microscope	Leica, Wetzlar, Germany
Microplate reader infinite M200	Tecan, Männedorf, Switzerland
Model 1025 Monitoring & Gating system	SA Instruments Inc., Stony Brook, NY, USA
Axiovert microscope 25	Carl Zeiss, Jena, Germany
Realplex2 Mastercycler	Eppendorf, Hamburg, Germany
Stuart see-saw rocker SSM4	Bibby Scientific Ltd, Staffordshire, UK
small animal MRI system (9.4 T)	Biospec 94/20, Bruker Biospin, Ettlingen, Germany
Thermocycler T3000	Biometra, Göttingen, Germany
G-box gel documentation system	Syngene, UK
SDS-PAGE Protean II electrophoresis unit	Bio-Rad Laboratories GmbH, Munich, Germany
Gradient cycler	Eppendorf, Hamburg, Germany
Incubator cell-culture	Labotect, Germany
Powerpac 300 electrophoresis power supply	Bio-Rad Laboratories GmbH, Munich, Germany
Weighing scales	Sartorius AG, Göttingen, Germany
Stereotactic head holder	David Kopf Instruments, Tujunga, USA
Mini Trans-Blot electrophoretic transfer cell unit	Bio-Rad Laboratories GmbH, Munich, Germany
Vortex	Janke & Kunkel, Germany
Water bath	GFL, Germany
NanoDrop 1000	PeqLab Biotechnologie GmbH, Erlangen, Germany
pH 211R microprocessor pH meter	Hanna Instruments®, Kehl am Rhein, Germany
Perkin Wallac microplate Reader	Perkin-Elmer/Wallac, Freiburg, Germany

Table 5. List of devices and equipments

3.1.4. Plasticware and other tools

Equipments	Company
96-well skirted PCR plates	VWR International, Darmstadt, Germany
96-, 24-, 12- and 6-well cell culture plates	BD Biosciences, Heidelberg, Germany
Cuvettes for measuring protein	Eppendorf, Hamburg, Germany
Double-edged razor blades	Thermo Fischer Scientific, Walldorf, Germany
Single-edged razor blades	Apollo Herkenrath, Solingen, Germany
Surgical sewing cone	Johnson & Johnson, New Jersey, USA
BD Falcon™ Cell culture inserts for 6-well plates (0.4 µm pores)	Becton Dickinson GmbH, Heidelberg, Germany
Falcon™ tubes (15 ml, 50 ml)	Becton Dickinson GmbH, Heidelberg, Germany
1 µl syringes 7101KH	Th. Geyer, Renningen, Germany
Minisart filter units (0.2, 0.45 µm)	Sartorius Stedim Biotech GmbH, Göttingen, Germany
Stericup® and Steritop® vacuum filtration and storage units	Millipore-Merck, Darmstadt, Germany
MicroAmp™ optical adhesive films (for PCRs)	Applied Biosystems, Foster City, USA
Secure-Seal™ spacers for cell culture	Invitrogen, USA
Menzel SuperFrost plus microscopic slides	Thermo Fischer Scientific, Walldorf, Germany
Menzel glass cover slips (24x40, 24x50, 24x60 mm)	Thermo Fischer Scientific, Walldorf, Germany
Parafilm	Pechiney Plastic Packaging, Chicago, USA
Saran wrap (transparent foil)	Dow Chemical Co., USA
Tissue culture dishes (60 mm, 30 mm)	BD Biosciences, Heidelberg,Germany
Tissue culture flasks (25 cm2, 75 cm2	BD Biosciences, Heidelberg,Germany

Table 6. List of plasticware and other tools

3.1.5. Primary Antibodies

Antigen	Host	Dilution	Company
anti-MMP14 (clone id: EP1264Y)	Rabbit	1:100 (IHC); 1:1000 (WB)	Epitomics, California,USA
anti-Iba1	Goat	1:500 (IHC)	Abcam, Germany
anti-β-Actin-peroxidase		1:25,000 (WB)	Sigma-Aldrich, Germany
anti-MMP14	Mouse	1:100 (IHC)	Calbiochem, Darmstadt, Germany

anti-Iba1	Rabbit	1:250(IHC)	Wako Pure Chemicals, Japan

Table 7. List of Primary Antibodies used in IHC and WB

3.1.6. Secondary Antibodies

Antigen	Host	Conjugate	Dilution	Company
Goat IgG	Donkey	Rhodamine Red	1:200 (IHC)	Jackson Immunoresearch Laboratories, USA
Goat IgG	Donkey	DyLight-488©	1:200 (IHC)	Jackson Immunoresearch Laboratories, USA
Rabbit-IgG	Donkey	Biotin-SP	1:200 (IHC)	Jackson Immunoresearch Laboratories, USA
Mouse-IgG	Donkey	Biotin-SP	1:125 (IHC)	Jackson Immunoresearch Laboratories, USA
Rabbit-IgG	Donkey	Rhodamine Red	1:200 (IHC)	Jackson Immunoresearch Laboratories, USA
Rabbit IgG		HRP (WB)	1:2000 (WB)	Cell Signaling, USA
Substrate	**Conjugate**		**Dilution**	**Company**
Streptavidin	Peroxidase		1:1000	Jackson Immunoresearch Laboratories, USA
Streptavidin	Cy5		1:200 (IHC)	Jackson Immunoresearch Laboratories, USA
DAPI			1:200 (IHC)	Sigma-Aldrich, Germany

Table 8. List of Secondary Antibodies used in IHC and WB

3.1.7. Buffers for Immunohistochemistry

Name	Composition
0.9% Saline	0.9 g NaCl in 1 L aqua distilled water
4% 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 Na_2HPO_4, 0.24 g KH_2PO_4. 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
PBS-Tween 20 (PBST)	1 L PBS, 0.0025% Tween 20 (Wash buffer)
TBS-Tween 20 (TBST)	1 L TBS, 0.0025% Tween 20 (Wash buffer)
Blocking buffer	3% Donkey serum, PBST/TBST

Table 9. List of buffers for IHC

3.1.8. Buffers for Western blotting

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	0.1% Tween 20, 1 L PBS/ TBS
Blocking buffer	5% Bovine serum albumin (BSA) in wash buffer

Table 10. List of buffers for WB

3.1.9. Buffers for PCR

Name	Composition
Roti®-6X gel loading dye	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

Table 11. List of buffers for PCR

3.1.10. 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)	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 12. List of media & solutions used for cell culture/ OBSC

3.1.11. Software

Software	Company
Mastercycler ep realplex	Eppendorf, Hamburg, Germany
Image J Macbiophotonics	http://www.macbiophotonics.ca/index.htm
Stereo Investigator® system	MicroBrightField Europe, Magdeburg, Germany
Leica LAS AF	Leica, Wetzlar, Germany
SPSS 11.5	SPSS Inc., IBM, Chicago, USA
Thomson Reuters EndNote ®	Thomson Reuters, Carlsbad, USA
Adobe Photoshop CS	Adobe Systems Inc., San Jose, USA
Microsoft Office 2007®	Microsoft, Redmond, USA
mipav Image analyzer	http://mipav.cit.nih.gov

Table 13. List of software

3.2. Methods

The Methods chapter in the thesis describes the details of both the projects either separately or combined wherever appropriate and as indicated.

3.2.1. Animals and Anesthesia (Projects 1+2)

Control C57Bl/6 wild-type (WT) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All *in vivo*, *in vitro* and *ex vivo* experiments were done using specific TLR knock-out (KO) mice. Dr. Seija Lehnardt from Charité, Berlin provided the TLR 2, 7 and 9 KO mice (Takeuchi, O et al 1997), while TLR 1 (Takeuchi, O et al 2002) and 6 (Takeuchi, O et al 2001) KO mice were purchased from Oriental BioServices Inc., Japan. All the TLR KO mice were generated by Dr. Shizuo Akira and colleagues from Osaka University, Japan. The TLR KO mice are characterized by deletion of the respective gene loci.

All animals were bred and maintained in the respective institutional animal facilities of the Max Delbrück Centre (Berlin, Germany) and the Charité-Universitätsmedizin (Berlin, Germany). The animal experiments were approved by the Office for Health, Protection and Technical Safety of the regional government of Berlin (LaGeSo) and followed the guidelines of the European Community Council Directive. The Tierversuchsverordnung or TVV nummer (animal experiment regulation number) for *in vivo* tumor implantation studies was G0268/10; for *in vitro* and *ex vivo* experiments was T0014_08; for genotyping of tail cuts was O 0416-9 and for animal perfusion and further analysis of the brain was O 0360. The mice were housed with a 12 h/ 12 h light-dark cycle and received food and water *ad libitum*. For all *in vivo* experiments, mice were anesthetized by intra-peritoneal (i.p.) injections of Ketamine (80 µg/kg) and Rompun (12 µg/kg) mix. 150 µl- 200 µl of the anesthetic drug mixture was injected into each mouse. The eyes of the mice were carefully covered with glycerin fat to avoid corneal drying. Due to restrictions in breeding I used age and sex-matched male and female mice for all my experiments in the study.

3.2.2. Cell Culture (Projects 1+2)

Murine GL261 glioma cells were purchased from National Cancer Institute, NCI-Frederick (MD, USA). The cells were grown in Dulbecco's Modified Eagle Medium with 10% heat inactivated fetal calf serum, 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Germany) in 75 cm^2 tissue culture flasks (Falcon model 3023, Becton Dickinson, Lincoln Park, NJ, USA). The GL261 cells were chosen for all *in vivo* and *ex vivo* studies as they were homologous to the donor C57Bl/6 animals. The medium of the cells was changed every 2 days and cells were passaged when the cell density in the flask reached a confluence level of 80%. Cell cultures were maintained at 37°C in a humidified 5% CO_2 / 95% air incubator (Heraeus, Hanau, Germany).

Primary microglial cells were prepared from cerebral cortices of p0-p2 neonatal C57Bl/6 and TLRs 1, 2, 6, 7 and 9 KO mice as per the protocol of Dr. Marco Prinz (Prinz et al. 1999). The whole isolation process was carried out on ice. Brains of newborn mice were collected in Hank's Balanced Salt Solution (HBSS). Forebrains were carefully freed of blood vessels and meninges. After three washes with HBSS, the cortical tissue was incubated with a trypsin/DNase mix for 2 min. The reaction was stopped by the addition of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Finally, the cell mixture was incubated with DNase, dissociated with a fire-polished pipette and washed twice. Mixed glial cells were then cultured for 9 to 12 days in complete DMEM 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. Microglial 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 > 95 % microglial cells; the purity was assessed by

immunostaining the cell cultures with tomato lectin, a marker for microglia. Cultures were used for experiments 1 to 2 days after plating.

3.2.3. Generation of EGFP-GL261/mCHERRY-GL261 cells (Projects 1+2)

GL261 glioma cells were transfected with the pEGFP-N1 vector for stable expression of enhanced green fluorescent protein (EGFP) using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. The pEGFP-N1 vector contains the human cytomegalovirus promoter, which drives high level expression of the enhanced green fluorescent protein in transfected cells. An amount of 600 µg/ml Genetecin- G418 (Gibco®, Maryland, USA) selection method yielded stably transfected clones of GL261 cells. Viable cells with bright fluorescence were selected by fluorescence activated cell sorting (FACS). 90% to 95% of cells were stable for fluorescence labeling on the day of ex *vivo* and *in vivo* injections.

The mCherry-GL261 cell line was generated by transfecting OmicsLink™ non targeted shRNA tagged with mCherry (GeneCopoeia, Maryland, USA) according to the manufacturer's instructions. Transfected GL261 cells were then selected by treating with 5 µg/ml Puromycin and used for further *in vivo* tumor implantation experiments.

3.2.4. Preparation of glioma conditioned medium (Projects 1+2)

Conditioned medium from GL261 glioma cells (GCM) was prepared as described. Cells were seeded at a density of $1x10^6$ cells in 75 cm^2 tissue culture flasks and grown to 80% confluence. They were then replenished with fresh complete growth medium which was left on the cells for 16-18 h before being collected. The GCM was then harvested, briefly centrifuged at 4°C, 1000 rpm for 8 min to remove cell debris and filtered using a 0.2 µm filter unit (Sartorius Stedim Biotech GmbH, Germany). Aliquots of the conditioned medium were stored at -20°C or used immediately for further experiments.

3.2.5. Genotyping of the TLR KO mice (Project 1)

All TLR KO mice were genotyped according to protocols established in the lab of Dr. Shizuo Akira from the Osaka University, Japan. DNA for genotyping was isolated from tail cuts of the respective TLR KO mice by using the peqLABDirectLyse tail kit according to the manufacturer's instructions. Briefly, 100 µl DirectLyse together with 10 µl Proteinase K was added to the tail tip and incubated for 3 h at 55°C followed by incubation for 45 min at 85°C to inactivate the enzymes.

The following primers were used for genotyping:

TLR1 wild-type (WT)	
Name	**Sequence**
TLR1a	5'-GAT GGT GAC AGT CAG CAG AAC AGT ATC-3'
TLR1b	5'-AAG GTG ATC TTG TGC CAC CCA ACA GTC-3'
TLR1 knock-out (KO)	
Name	**Sequence**
TLR1b	5'-AAG GTG ATC TTG TGC CAC CCA ACA GTC-3'
TLR1c	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'

TLR2	
Name	**Sequence**
TLR2 KO a	5'-GTT TAG TGC CTG TAT CCA GTC AGT GCG-3'
TLR2 KO b	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'
TLR2 KO c	5'-TTG GAT AAG TCT GAT AGC CTT GCC TCC-3'

TLR6 wild-type (WT)	
Name	**Sequence**
TLR6 wild	5'-GAA ATG TAA ATG AGC TTG GGG ATG GCG-3'
TLR6 extra	5'-TTA TCA GAA CTC ACC AGA GGT CAA ACC-3'
TLR6 knock-out (KO)	
Name	**Sequence**
TLR6 extra	5'-TTA TCA GAA CTC ACC AGA GGT CAA ACC-3'
TLR6 neo	5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3'

Table 14. Genotyping PCR primers for TLRs 1, 2 & 6

The composition of the genotyping PCR reaction for TLRs 1, 2 and 6 are as follows:

For TLRs 1 and 6:

Knock-out		**Wild-type**	
H_2O	17.75 µl	H_2O	17.75 µl
10 X PCR buffer	2.5 µl	10 X PCR buffer	2.5 µl
$MgCl_2$	1.5 µl	$MgCl_2$	1.5 µl
dNTPs	0.5 µl	dNTPs	0.5 µl
Fwd primer (10 pmol/µl)	0.5 µl	Fwd primer (10 pmol/µl)	0.5 µl
Rev primer (10 pmol/µl)	0.5 µl	Rev primer (10 pmol/µl)	0.5 µl
Taq DNA Polymerase	0.25 µl	Taq DNA Polymerase	0.25 µl
Sub-total	**23.5 µl**	**Sub-total**	**23.5 µl**
DNA	1.5 µl	DNA	1.5 µl
PCR product size			
TLR1	1300 bp		1300 bp
TLR6	300 bp		300 bp

For TLR2:

Knock-out		**Wild-type**	
H_2O	19.85 µl	H_2O	17.75 µl
10 X PCR buffer	2.5 µl	10 X PCR buffer	2.5 µl
dNTPs	0.5 µl	dNTPs	0.5 µl
primer b	0.5 µl	primer b	0.5 µl
primer c	0.5 µl	primer a	0.5 µl
Taq DNA Polymerase	0.15 µl	Taq DNA Polymerase	0.15 µl
Sub-total	**25 µl**	**Sub-total**	**25 µl**
DNA	2µl	DNA	2 µl
PCR product size			
TLR2	1000 bp		1000 bp

Table 15. PCR reaction composition for genotyping TLR mice strains

The PCR amplification reactions were set up as follows:

For TLRs 1 & 6

Lid Temp 99°C

1. 94°C 3min
2. 94°C 30sec ⎫
3. 67°C 30sec ⎬ X 30 cycles
4. 72°C 1min ⎭
5. 72°C 10min
6. 4°C ∞

For TLR2

Lid Temp 99°C

1. 94°C 3min
2. 94°C 30sec ⎫
3. 67°C 30sec ⎬ X 34 cycles
4. 74°C 1min ⎭
5. 74°C 10min
6. 4°C ∞

3.2.6. TLR sub-type specific agonists (Project 1)

To analyze the effect of different Toll-like receptor agonists on MT1-MMP gene expression, primary microglia from C57Bl/6 WT mice were stimulated with receptor-specific ligands obtained from Prof. Dr. Uwe Karsten Hanisch from the University of Göttingen, Germany. Microglial cells were stimulated with specific ligands for 6 h followed by analysis of MT1-MMP gene expression changes by qRT-PCR. Following final concentrations of the sub-type specific agonists were used:

Agonist	TLR sub-type	Stock concentration	Final working concentration
Pam_3CSK_4	TLR1/2	0.5 mg/ml	10 ng/ml
Poly(I:C)	TLR3	1 mg/ml	10 µg/ml
LPS (*E. coli* R515)	TLR4	1 mg/ml	100 ng/ml
Flagellin	TLR5	50 µg/ml	500 ng/ml
MALP-2	TLR6/2	0.1 mg/ml	10 ng/ml
Poly(U)	TLR7/TLR8	1 mg/ml	5 µg/ml

Table 16. TLR sub-type specific agonists

3.2.7. TLR2 specific agonists (Project 1)

The effect of different TLR2 specific agonists on the expression of MT1-MMP was analyzed using a Mouse TLR2 Agonist kit (Cayla-InvivoGen, Toulouse, France). Primary microglial cells from C57Bl/6 WT mice were stimulated with 2 of these ligands for 6 h, total RNA was isolated, reverse-transcribed into cDNA and analyzed for changes in MT1-MMP gene expression by quantitative real-time SYBR green PCR. Following final concentrations of the TLR2- specific agonists were used:

Agonist	TLR	Stock concentration	Final working concentration
Pam3CSK4	TLR1/2	100 µg/ml	10 ng/ml
LPS P. gingivalis (PG-LPS)	TLR2	1 mg/ml	1 µg/ml
HKLM (Heat Killed Lysteria Monocytogenes)	TLR2	10^7 cells/ml	10^5 cells/ml

Table 17. TLR2 specific agonists

3.2.8. Minocycline treatment (Project 2)

To study if glioma-induced microglial MT1-MMP over-expression and activity could be pharmacologically abrogated, Minocycline, a broad-spectrum antibiotic and inhibitor of microglial cell activation was used. The drug, Minocycline Hydrochloride (#M9511, Sigma-Aldrich, Germany) was always freshly prepared in sterile water and used at a final concentration of 200 nM *in vitro* and 10 ng/ml *in vivo*. For all *in vitro* experiments, microglial cells were pre-treated with 200 nM Minocycline for 30 min at 37°C in the cell culture incubator, followed by co-incubation with GCM or only complete growth medium for 3 and 6 h. For *in vivo* studies, a 10 ng/ml working solution of Minocycline was freshly prepared in distilled water every day and administered through drinking water to tumor implanted mice for the entire 2 week period of tumor growth. There were 2 groups of mice that received Minocycline through drinking water- the first group received Minocycline <u>immediately</u> after tumor implantation, while the second group received

Minocycline 1 week after tumor implantation. A change in glioma expansion upon oral administration of Minocycline in these 2 groups of mice was estimated 2 weeks after tumor implantation according to the Cavalieri's principle.

3.2.9. Quantification of gene expression changes (Projects 1+2)

For Project 1, total RNA was isolated from ligand and GCM treated microglia from control and treated microglia derived from C57BL/6 WT and TLRs 1, 2, 6, 7 and 9 KO mice. To analyze MT1-MMP gene expression changes in primary microglia from C57Bl/6 WT treated with TLR sub-type specific agonists and TLR2 specific agonists, microglia were seeded into 6-well plates at a density of $1x10^6$ cells/ well and allowed to adhere for 24 h before the actual experiment was performed.

Microglia were stimulated the next day with only complete growth medium (DMEM control), GCM or appropriate working dilutions of TLR sub-type specific agonists or TLR2 specific agonists. Similarly, to investigate the effect of GCM on MT1-MMP gene expression in microglia derived from WT and TLR1, 2, 6, 7 and 9 KO mice, microglia were stimulated with either complete growth medium (DMEM) or GCM. All stimulations were carried out for 3 and 6 h. At the end point of the experiments, total RNA was isolated using the Invitrap Spin Universal RNA mini kit (Invitek GmbH, Berlin, Germany) as per the manufacturer's instructions. The quality and yield were determined using the NanoDrop 1000 spectrophotometer (PeqLab Biotechnologie GmbH, Germany). cDNA was synthesized using 250 ng total RNA which was incubated with 0.5 µg/µl Oligo(dT)12-18 primers and 0.5 mM dNTPs in a total volume of 12 µl for 5 min at 65°C. After adding First-Strand buffer, 10 mM dithiothreitol (DTT), 100 U/µl RNase inhibitor and 200 U/µl SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) to a final reaction volume of 20 µl, the reaction was incubated further at 42°C for 50 min followed by an inactivation at 70°C for 15 min.

In Project 2, to study the effect of Minocycline on MT1-MMP expression in GCM-treated primary microglia from Bl/6 WT mice, microglia cells were pre-treated with Minocycline for 30 min, followed by co-incubation with 200 nM Minocycline-

containing GCM or with just complete growth medium for 3 and 6 h. At the end point of treatment, microglia cells were processed for total RNA as stated earlier.

The qRT-PCR reaction for MT1-MMP was prepared to a total volume of 20 µl as follows-

Components	Reaction Volume (µl)
SYBR Green PCR mix	10
Fwd primer (10 pmol/ml)	1
Rev primer (10 pmol/ml)	1
DEPC treated Rnase free H_2O	6
cDNA	2
Sub-total	**20**

Table 18. qRT-PCR composition for MT1-MMP and β-Actin

Gene amplification was done in triplicates using SYBR Green PCR mix (Roche Diagnostics GmbH, Mannheim, Germany). The qRT-PCR and semi-quantitative RT-PCR amplification reactions for MT1-MMP and ß-Actin were done using the Realplex Mastercycler (Eppendorf, Hamburg, Germany) and the Thermocycler T3000 (Eppendorf, Hamburg, Germany) respectively under the following conditions-

Quantitative RT-PCR of MT1-MMP and β-Actin:

Lid Temperature 99°C		
1	95°C for 2 min	
2	95°C for 15 sec	X 35 cycles
3	68°C for 20 sec	X 35 cycles
4	72°C for 30 sec	X 35 cycles
5	72°C for 3 min	
6	4°C for ∞	

Table 19. qRT-PCR conditions for MT1-MMP and β-Actin

Relative quantification of gene expression was determined using the comparative $2^{(-\Delta\Delta CT)}$ method. Expression levels were related to the house-keeping gene β-Actin. Total RNA from 4 independent biological experiments were used for determining significant changes in MT1-MMP gene expression.

Semi-quantitative RT-PCR of MT1-MMP and β-Actin

MT1-MMP		β-Actin	
Lid Temperature 99°C		**Lid Temperature 99°C**	
1 95°C for 2 min		1 94°C for 3 min	
2 95°C for 45 sec	**X 28 cycles** (steps 2–4)	2 94°C for 30 sec	**X 26 cycles** (steps 2–4)
3 55°C for 45 sec		3 55°C for 30 sec	
4 72°C for 2 min		4 72°C for 1 min	
5 72°C for 10 min		5 72°C for 10 min	
6 4°C for ∞		6 4°C for ∞	
MT1-MMP : 432 bp		**β-Actin** : 238 bp	

Table 20. Semi-quantitative PCR conditions for MT1-MMP and β-Actin

The sequences of the mouse primers used for qRT-PCR reaction are

MT1-MMP	sense	5’-GTGCCCTATGCCTACATCCG-3’
MT1-MMP	anti-sense	5’-CAGCCACCAAGAAGATGTCA-3’
β-Actin	sense	5'-CCCTGAAGTACCCCATTGAA-3'
β-Actin	anti-sense	5'-GTGGACAGTGAGGCCAAGAT'-3’

Table 21. qRT-PCR primer sequences of MT1-MMP and β-Actin

The sequences of the mouse primers used in semi-quantitative RT-PCR reaction are

MT1-MMP	sense	5´-GGATACCCAATGCCCATTGGCCA-3´
MT1-MMP	anti-sense	5´- CCATTGGGCATCCAGAAGAGA-3´
β-Actin	sense	5'-CCCTGAAGTACCCCATTGAA-3'
β-Actin	anti-sense	5'-GTGGACAGTGAGGCCAAGAT'-3'

Table 22. Semi-quantitative RT-PCR primer sequences of MT1-MMP and β-Actin

3.2.10. Quantification of protein expression (Projects 1+2)

Project 1: whole-cell protein extracts were prepared from microglia obtained from C57Bl/6 WT and TLR 2 KO mice after stimulation with GCM for 6 h.

Project 2: protein extracts for analyzing effect of Minocycline on GCM-induced MT1-MMP expression in microglia were prepared from C57Bl/6 WT microglial cells which were stimulated with only GCM or GCM in combination with 200 nM Minocycline for 3 and 6 h, following a pre-incubation only with Minocycline for 30 min. Briefly, microglial cells were washed thrice with ice-cold PBS, and lysed using ice-cold RIPA lysis buffer (Sigma-Aldrich, Steinheim, Germany) containing EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). The lysates were cleared using a 1 µl syringe, fitted with a 21” gauge needle to obtain a homogenous solution, incubated for 15 min at 4°C, and finally centrifuged at 4°C, 14,0000 rpm for 20 min. Supernatants were collected and protein concentration in each sample was determined by the BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany). Following protein concentration estimation, the samples were mixed with 5X Laemmli Buffer containing β-mercaptoethanol (Sigma-Aldrich, Germany), boiled for 5 min at 95°C, cooled and loaded on the gel. 20 µg of each protein sample was resolved on a 10% denaturing SDS-PAGE gel system from BioRad, Germany. Along with the unknown proteins, a standard pre-stained protein ladder (Fermentas, Germany)

was also loaded on the gels. The proteins were transferred after complete separation onto a PVDF membrane (Amersham GE, Germany) by wet-transfer method at 4°C, 325 mA for 65 min. Post-transfer, the membranes were checked for efficient protein transfer by staining the PVDF membranes with the reversible dye Ponceau-S red (Sigma-Aldrich, Germany). The membranes were washed with PBS-0.1% Tween 20, pH 7.4 (PBST) while blocking was done using 5% Bovine Serum Albumin (BSA; Carl-Roth GmbH, Germany) prepared in PBST. Primary antibodies were diluted in PBST-1% BSA and the membranes were incubated overnight at 4°C with gentle agitation. The antibody recognizing Rabbit anti-MT1-MMP monoclonal antibody was purchased from Epitomics, California, USA and anti-β-Actin−Peroxidase conjugated antibody (A3854) was purchased from Sigma-Aldrich, Schnelldorf, Germany. Concentration of primary antibodies used was as follows: MT1-MMP 1:1000; β-Actin−Peroxidase 1:25,000. The secondary antibody anti-rabbit HRP conjugated IgG (Cell Signaling Technology, USA) was diluted in PBST and used at a concentration of 1:2000. The membranes were incubated with secondary antibody at room temperature for 1 h and subsequently developed with a chemiluminiscent substrate SuperSignal West Pico Chemiluminiscence substrate kit (Thermo Fisher Scientific, Bonn, Germany) and visualized by the Molecular Imager Gel Doc XR system using the QuantityOne™ software (Bio-Rad Laboratories GmbH, Munich, Germany).

3.2.11. MT1-MMP Activity Assay (Project 2)

To study the effect of Minocycline on MT1-MMP activity in microglia treated with GCM, microglia from Bl/6 WT mice were pre-treated with Minocycline for 30 min, followed by co-incubation with 200 nM Minocycline-containing GCM or with just complete growth medium for 3 and 6 h. The cells were then washed with ice-cold PBS followed by lysis with a Lysis buffer provided in the assay kit. An ELISA based activity assay (Matrix Metalloproteinase-14 Biotrak Activity Assay System, GE Healthcare, Germany) in a 96 well plate format was used to determine the amount of active MT1-MMP released by GCM-treated primary microglia cells upon treatment with Minocycline. The assay is based on a detection enzyme (pro-urokinase) which is activated only by active MT1-MMP through a single proteolytic

event. The activated urokinase was detected by a specific chromogenic peptide substrate (S-2444™ peptide) and the color reaction was quantified at 405 nm in a microplate reader (Perkin Wallac, Freiburg, Germany).

3.2.12. Determination of protein localization by IHC (Projects 1 & 2)

In Project 1 to visualize MT1-MMP protein expression *in vivo*, immunohistochemical labeling was performed on 40 µm thick free-floating brain sections obtained from C57Bl/6 WT and TLR 2 KO mice. Similarly in Project 2, brain sections from tumor-implanted WT mice that were orally administered with Minocycline through drinking water were also immunohistochemically labeled for MT1-MMP and microglia/macrophage marker Iba1.

Project 1: Briefly, the brain sections were washed thrice in PBS- 0.025% Tween 20 (PBST), pH 7.4 followed by antigen retrieval using Sodium Citrate buffer, pH 6 at 80°C for 20 min in a water bath. Non-specific staining in sections was controlled by blocking with 3% donkey serum prepared in PBST for 1 h at room temperature followed by overnight incubation at 4°C with primary antibodies for goat anti-Iba1 (1:500; Abcam, Cambridge, UK) and rabbit anti-MT1-MMP (1:200; Epitomics, California, USA). The secondary antibodies used were Donkey anti-rabbit Biotin-SP conjugated IgG (1:200; Jackson Immunoresearch/Dianova, Hamburg, Germany), Streptavidin conjugated Cy5 (1:200; Jackson Immunoresearch/Dianova, Hamburg, Germany) and Donkey anti-goat Rhodamine Red (1:200; Jackson Immunoresearch/Dianova, Hamburg, Germany). Nuclear staining was visualized by DAPI (1:200; Sigma-Aldrich, Hamburg, Germany). A Hematoxylin-Eosin staining was done to delineate the tumor morphology. The brain sections from Bl/6 WT and TLR2 KO mice were mounted onto microscopic slides and stained with Meyer's Hematoxylin for 2 min first and finally counterstained with alcoholic eosin. GL261 glioma cells were identified by stable fluorescence expression of mCherry (red).

Since Project 2 was done at a different time, brain sections from the Minocycline group were immunolabelled by a different protocol. Brain sections were washed thrice in PBS- 0.025% Tween 20 (PBST), pH 7.4 followed by antigen retrieval

using Sodium Citrate buffer, pH 6 at 80°C for 30 min in a water bath. Endogenous peroxidases were quenched by incubating the brain sections in 3% Hydrogen Peroxide in PBS for 30 min at room temperature. Following washes in PBST, non-specific staining in the sections was blocked using 3 % donkey serum prepared in PBST for 1 h at room temperature. This was followed by overnight incubation at 4°C with primary antibodies to detect Rabbit anti-Iba1 (1:250; Wako Pure Chemicals, Japan) and Mouse anti-MT1-MMP (1:100; Calbiochem, Darmstadt, Germany). The secondary antibodies used for detecting Iba1 and MT1-MMP were Donkey anti-Rabbit Rhodamine Red (1:200; Jackson Immunoresearch/Dianova, Hamburg, Germany) and Donkey anti-Mouse Biotin-SP conjugated IgG (1:125; Jackson Immunoresearch/Dianova, Hamburg, Germany) for 2 h at room temperature followed by incubation with Strepatividin-conjugated Peroxidase (1:200 Jackson Immunoresearch/Dianova, Hamburg, Germany) for 2 h at room temperature. A DAB (3, 3'-DiaMinocyclinebenzidine) staining was done to visualize localization of MT1-MMP in the brain sections. Nuclear staining was visualized by DAPI (1:200; Sigma-Aldrich, Hamburg, Germany). GL261 glioma cells were identified by stable fluorescence expression of EGFP (green).

3.2.13. Organotypic Brain Slice Cultures to study tumor growth **ex vivo** *(Projects 1 & 2)*

The procedure to establish organotypic brain slice cultures (OBSC) is a modification of previously published protocols (Markovic et al. 2005; Markovic et al. 2009). For both projects, brain tissue was derived from 16 day old mice (Project 1- C57BL/6 WT and TLR2 KO mice; Project 2- C57BL/6 WT mice).

Mice were decapitated and brains were removed within 2 to 3 min and placed in ice-cold phosphate-buffered saline (PBS) under sterile conditions. The forebrain was removed and the hindbrain was glued onto a magnetic platform using cyanoacrylate glue (Uhu, Germany) and cut in the coronal plane into 250 µm sections with a vibratome (Leica VT1000S; Leica Co., Heidelberg, Germany). The brain slices were transferred into 0.4 µm polycarbonate membrane of a transwell tissue insert (Falcon model 3090; Becton Dickinson), which was fitted into a 6-well

plate (Falcon model 3502; Becton Dickinson). Thereafter, the brain slices were incubated at an air-medium interface in 1 mL of complete growth medium containing DMEM supplemented with 10% heat inactivated fetal calf serum (FCS), 0.2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Medium-1). After overnight equilibration in medium-1, the brain slices were exchanged for cultivation medium (Medium- 2), added directly into the wells and not into the inserts. Medium-2 contained 25% heat-inactivated FCS, 50 mM sodium bicarbonate, 2% L-glutamine, 25% Hank's balanced salt solution (HBSS), 1 mg/mL insulin (all from Invitrogen, Germany), 2.46 mg/mL glucose (Braun Melsungen, Germany), 0.8 mg/mL Vitamin C (Sigma-Aldrich, Germany), 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma- Aldrich, Germany), and 5 mM Tris in DMEM (Invitrogen, Germany).

To assess the pro-tumorigenic effect of microglia in OBSCs, the brain slices in both projects were selectively depleted of microglia by the application of liposome-encapsulated clodronate (CL; purchased from Nico van Rooijen, Amsterdam, The Netherlands). Briefly, CL was diluted in Medium-1 at a concentration of 1:10 and 1 ml of the working solution was directly added into the wells of the 6-well plate which were fitted with OBSC-containing tissue inserts and left in the wells for 24-29 h. Liposome-encapsulated PBS (PL) was used as a control at the same dilution (1:10) in Medium-1. The microglia sub-population was ablated when CL was taken up from the lower surface of the OBS through capillary action. After 24 h, the medium containing CL or PL was removed and replaced with 1 ml of Medium-2 and the OBSCs were left undisturbed for 72 h in the incubator at 37°C. After 3 days, slices were injected with EGFP-GL261 cells to assess the effect of microglia on tumor expansion in the presence (PL control) and absence (CL) of microglia in brain slices either from Bl/6 WT and TLR2 KO mice (Project 1) or in brain slices derived from Bl/6 WT mice treated with 200 nM Minocycline (Project 2). To study the effect of Minocycline on tumor growth in organotypic brain slices, Minocycline was always prepared freshly in medium-2 to a final concentration of 200 nM and added directly into each well of the 6-well plate and was changed every alternate day during the 5 days after tumor inoculation into the slices.

Approximately 5000 EGFP-transfected GL261 glioma cells in a volume of 0.1 µL were inoculated into the globus pallidus in the cortex of the brain slices using a Hamilton syringe (Mikroliterspritze 7001N, Hamilton, Switzerland) mounted to 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 an injection cavity of approximately 50 µm. The cell suspension was injected slowly into this cavity. To ensure identical experimental conditions, glioma cells were always inoculated into the same area on the slice. Tumor cells remained at the inoculation site directly after glioma injection, which therefore marked the point of origin for all further movements and growth of the tumor cells. Careful control of the injection procedure ensured that no cells spilled onto the surface of the slices, which could otherwise migrate over the surface rather than invade through the tissue. The tumors were allowed to grow for 5 days, after which the slices were fixed with 4 % PFA, mounted and the area occupied by the tumors in microglia-containing and microglia depleted slices was evaluated using the Stereo Investigator™ software (MicroBrightField Europe, Magdeburg, Germany).

3.2.14. In vivo *tumor studies (Projects 1 & 2)*

Mice were handled as described earlier in the Methods section. C57BL/6 WT and TLR2, 7 and 9 KO mice were used in Project 1 while C57Bl/6 WT mice were used for Project 2 for all *in vivo* immunohistochemical and tumor volume measurements.

Briefly, mice were anesthetized, immobilized and mounted onto a stereotactic head holder (David Kopf Instruments, USA) 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. Then a 1 µl syringe with a blunt end (Mikroliterspritze 7001N, Hamilton, Switzerland) 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 min, 1 µl containing $2x10^4$ cells/µl of EGFP-GL261 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 (Johnson & Johnson International, USA). After surgery the mice were kept warm until awake and their post-operative condition was monitored daily. To analyze changes in MT1-MMP expression 14 days after surgery, the C57Bl/6 WT and TLR2, 7 and 9 KO (Project 1) as well as C57Bl/6 mice from the Minocycline group (Project 2) were euthanized with Ketamine and Rompun mix, brains perfused and fixed (4% paraformaldehyde), cryopreserved in 30% sucrose and immunohistochemically labeled for MT1-MMP and Iba1 antibodies as stated previously. Further-more, DAPI nuclear staining was done to demarcate the tumor for further analysis of tumor volume in both the projects.

3.2.15. Analysis of tumor volume by Magnetic Resonance Imaging (Project 1)

To investigate changes in tumor expansion in TLR2 KO mice, mice from C57Bl/6 WT and TLR2 KO strains were implanted with tumors and changes in tumor expansion were monitored by MR imaging. Anesthesia to the mice was induced with 2.8% isoflurane in an oxygen/air mixture (2:1) with a flow rate of 750 ml/min and maintained at 1.5% to 2% for the rest of the experiments. Respiration rate and body temperature were continuously monitored (Model 1025, SA Instruments Inc., USA). Using a heated circulating water system the body temperature was maintained at 37°C throughout the experiment.

Two groups of mice (n=11/ group of TLR2 KO & C57Bl/6 WT; 22 mice in total) were imaged 21 days after intra-cerebral inoculation of GL261 glioma cells. All MR imaging was performed on a horizontal bore 9.4 T small animal MRI system (Biospec 94/20, Ettlingen, Germany). T2-weighted images [RARE, effective echo time (TE) = 60 ms; repetition time (TR) = 3268 ms; RARE factor = 12] were acquired with the same slice geometry [field of view (FOV) = 18 mm, matrix size = 350 x 350, slice thickness = 270 µm] in-plane spatial resolution = 51 µm, 21 coronal slices covering a brain region of 5.67 mm starting at the frontal end of the cerebral cortex approximately 3.56 to -2.11 mm from the bregma (Paxinos G et al 2001). T2-contrast images were optimized beforehand in pilot experiments to

achieve good tumor delineation. The total experimental time, including animal preparation, was approximately 70 min per animal. The tumor volumes were calculated by manual segmentation using the software mipav (http://mipav.cit.nih.gov). A region of interest (ROI) following the tumor borders was drawn on the T2-weighted images along with the exclusion of the needle path (stemming from the tumor cell injection). The T2-weighted images offer superior contrast (tumor is hyper-intense while the needle path is black). The whole tumor volume was calculated in *mipav* (http://mipav.cit.nih.gov) by adding up the voxel volumes within the ROIs of all image slices.

3.2.16. *Analysis of survival* **in vivo** *(Project 1)*

In order to investigate if deletion of the TLR2 gene locus could offer any survival benefits to mice intra-cerebrally implanted with glioma tumor, adult female C57Bl/6 WT (n=10) and TLR2 KO (n=10) mice were implanted with gliomas in a procedure as stated above. Briefly, 1x 10^4 cells/µl of GL261 glioma cell suspension was slowly injected into the brain in a manner as explained earlier. The tumor-bearing TLR2 KO and WT mice were monitored daily until the end-point of the study (survival). The cumulative survival time of WT and TLR2 KO mice was determined by a log-rank Kaplan Meier test based on this end point.

3.2.17. *Microscopy (Projects 1 & 2)*

Immunohistochemical preparations from both the projects were visualized and analyzed with a fluorescence microscope (Axioplan, Zeiss, Germany) and a Confocal laser scanning microscope (TCS SPE, Leica, Germany). Images were visualized and acquired using 20X and 40X oil objectives. For Project 1, images were taken from at least 3 random fields of interest (microglia in close association to tumor periphery) from WT and TLR2, 7 and 9 KO mice. Positively labeled Iba-1 cells were counted in both WT and TLR2 KO mice using the ImageJ software (NIH, USA). The labeling intensity of MT1-MMP immunostaining was also measured by ImageJ keeping the digital gain constant over all the sections.

Fluorescent images were processed using the Leica LAS AF software and Adobe Photoshop ©.

3.2.18. *Analysis and Statistics (Projects 1 & 2)*

An unbiased stereological method using the Stereo Investigator software for estimating the tumor volumes *in vivo* was done using the Cavalieri principle, by determining the tumor area in every 12th 40µm thick brain slice and then multiplying this area by the factor 12 x 40µm (Markovic et al. 2005) to calculate the tumor volume in the TLR2 KO and C57Bl/6 WT mice (Project 1) and in both the groups of Minocycline administered C57BL/6 WT mice with tumor implantations (Project 2).

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, Illinois, USA) software and Microsoft® Excel 2007. Changes in MT1-MMP gene expression were calculated according to the $2^{(-\Delta\Delta Ct)}$ method and expressed as means ± standard error of mean (SEM) while statistical significance was determined by the non-parametric Mann Whitney U test. Differences between groups in *in vivo* experiments (tumor volumes) were estimated using unpaired Wilcoxon sum-rank test. Survival analysis was done using the Kaplan-Meier method and significant differences were compared by a log-rank test. Statistical significance was determined at p values <0.05 (*) and < 0.01 (**).

4. RESULTS (PROJECT 1)

TLR signaling in microglia supports glioma growth and progression

Growing evidence from several studies suggests a correlation between Toll-like receptor signaling, inflammation, cancer development and progression (Creagh and O'Neill 2006; Huang et al. 2007; Ilvesaro et al. 2007; Yu et al. 2011) . Previous studies from our lab (Markovic, D et al 2005, 2009) have shown that microglia are required for promoting invasiveness in gliomas. Glioma cells release various types of growth factors and chemo-attracting cytokines like MCP-1, HGF/SF, MIP-1 etc. which recruit microglia in and around the tumor vicinity (Locasale et al. 2012; Magge et al. 2009; Xie et al. 2011). Further-more, soluble factor(s) released by glioma cells induce over-expression of MT1-MMP in microglia cells via the recruitment of the Toll-like receptor signaling adaptor molecule MyD88 and activation of p38 MAPK pathway. The upregulated MT1-MMP then activates the pro-form of MMP2 released by glioma cells and thereby facilitates the breakdown of the extracellular matrix components and the escape and invasion of glioma cells to other regions of the brain. Since TLR signaling in tumor-associated macrophages promotes metastasis in different types of cancers, I wanted to investigate if Toll-like receptor signaling is involved in the tumor-promoting effect exerted by microglia on gliomas.

4.1. MT1-MMP expression in microglia is differentially regulated by TLR sub-type specific ligands

The enhanced expression of MT1-MMP in glioma-associated microglial cells is mediated by MyD88 and the activation of p38 MAPK (Markovic et al. 2009). Based on this, I wanted to first identify if at all the Toll-like receptors were involved and which TLR sub-type (s) might trigger MT1-MMP gene expression in glioma-associated microglial cells.

To address this question, I stimulated primary microglia from C57Bl/6 WT mice with ligands specific for TLR sub-types and compared the MT1-MMP expression in ligand- versus GCM -stimulated microglia after 6 h. Microglial cells were incubated with agonists or GCM for 6 h, and changes in gene expression of MT1-MMP were assessed by semi-quantitative and qRT-PCR. MT1-MMP gene expression changes were normalized to the expression of β-Actin. Representative images from 4 independent biological experiments are shown in figure 4.1 A and B.

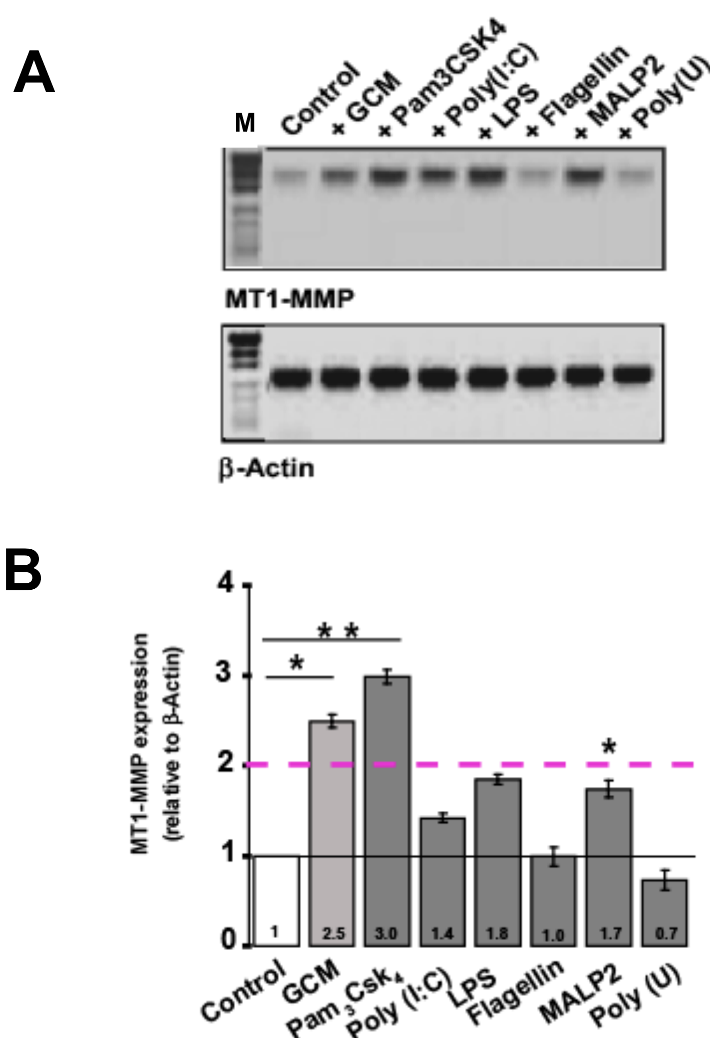


Figure 4. 1 Differential gene expression of MT1-MMP in microglia upon stimulation with TLR ligands *in vitro*.

Toll-like receptor subtype specific ligands and GCM trigger and enhance MT1-MMP gene expression in microglia from Bl/6 WT mice. Microglia were stimulated with different concentrations of ligands for 6 h and the fold changes in MT1-MMP expression were analyzed by semi-quantitative (A) and quantitative RT-PCR (B) by the $2^{(-\Delta\Delta Ct)}$ method relative to the house-keeping gene β-Actin. In (A) amplicon size of MT1-MMP is 432 bp and β -Actin is 238 bp. In (B) values of MT1-MMP fold changes are listed in the figure. Data are represented as mean ±SEM, $p<0.05$(*) and $p<0.01$(**). GCM (glioma conditioned medium), LPS (Lipopolysachcharide), M (Molecular weight marker).

The tri-acylated polypeptide ligand Pam3Csk4 that recognizes the TLR1/2 heterodimer induced a 3-fold (±0.075 SEM) increase in MT1-MMP expression at 6 h relative to the non-stimulated control while in the GCM-stimulated microglia there was a 2.5-fold (±0.07 SEM) increase in MT1-MMP expression. MT1-MMP expression in microglia cells was not significantly altered by TLR3 specific agonist Poly (I:C), and resulted in a 1.4-fold (±0.051 SEM) increase in microglia treated with TLR3 specific agonist Poly (I:C) after 6 h while with the TLR4 specific ligand lipopolysaccharide (LPS) there was an insignificant but moderate upregulation in MT1-MMP expression by 1.8-fold (±0.055 SEM) compared to the non-stimulated control. Incubation with TLR 2/6 di-acylated polypeptide agonist MALP2 induced a significant increase by 1.7 fold (±0.096 SEM) in MT1-MMP expression. Treatment with TLR5-specific agonist Flagellin and TLR 7/8-specific PolyU resulted in no change or caused down-regulation of MT1-MMP expression (not significant) respectively. These results indicate a potential link between MT1-MMP expression and toll-like receptor signaling and specify the role of one or more toll-like receptor(s) in the microglia-glioma cross talk, particularly the TLR2 in association with TLR 1 and/or 6.

4.2. Toll-like receptor 2 is required for inducing MT1-MMP expression in glioma –associated microglia

Based on results using different receptor sub-type specific agonists on microglial MT1-MMP expression, I wanted to further examine if Toll-like receptor 2-indeed specifically mediated glioma-induced microglial MT1-MMP over-expression. To address this question, I used two known TLR2 agonists apart from Pam3Csk4 to stimulate primary microglia. The microglial cells were treated with the specific agonists LPS from P. gingivalis (1 µg/ml) and HKLM (10^5 µg/ml) for 6 h and subsequently analyzed for changes in expression levels of MT1-MMP by semi-quantitative and qRT-PCR.

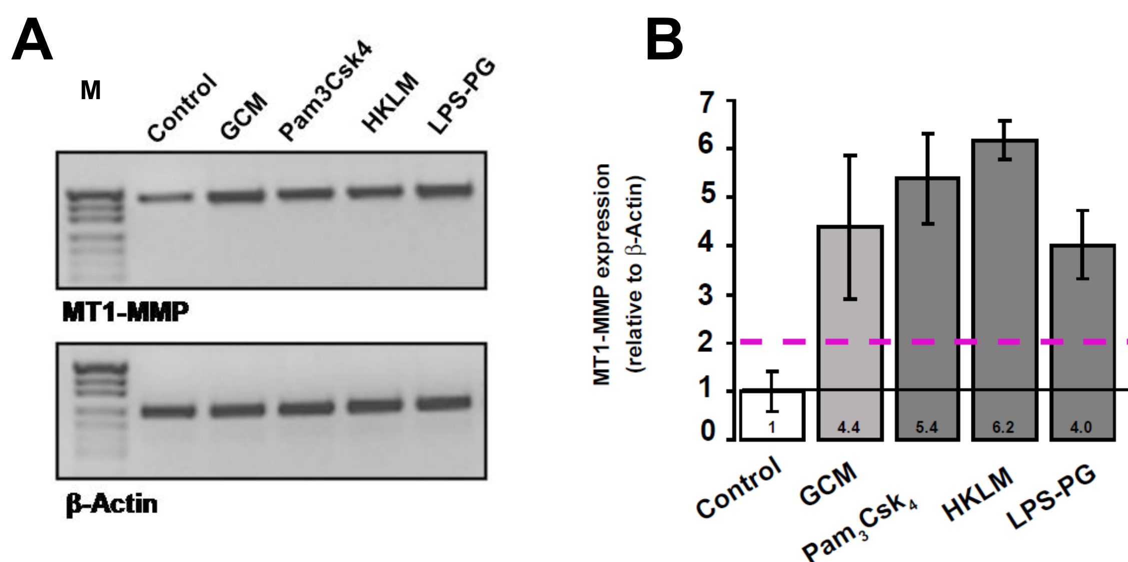


Figure 4. 2 Upregulation in MT1-MMP gene expression in microglia after stimulation with TLR2 ligands *in vitro*.

Microglia stimulated with TLR2 agonists LPS-PG and HKLM for 6 h showed a robust induction of MT1-MMP gene expression as seen by semi-quantitative (A) and quantitative RT-PCR (B) by the $2^{(-\Delta\Delta Ct)}$ method relative to the house-keeping gene β-Actin. In (A) amplicon size of MT1-MMP is 432 bp and β -Actin is 238 bp. In (B) values of MT1-MMP fold changes are listed in the figure. Data are represented as mean ±SEM.GCM (glioma conditioned medium), LPS-PG (Lipopolysachcharide from P.gingivalis), HKLM (Heat Killed Lysteria Monocytogenes), M (Molecular weight marker).

Based on these findings, I found that different TLR2 agonists induced varied levels of up-regulation in MT1-MMP gene expression levels in microglia, thereby indicating a link between TLR2 signaling and the activation of MT1-MMP gene expression. These results indicated that MT1-MMP expression in microglia is mediated via MyD88-dependent TLR2 signaling.

4.3. Deletion of the Toll-like receptor 2 reduced glioma-induced microglial MT1-MMP expression in vitro

Since TLR2 ligands induced robust induction of MT1-MMP gene expression, I wanted to examine the effect of silencing of TLR2 gene locus on glioma-induced MT1-MMP over-expression in microglia. To address this question, I stimulated primary microglia derived from C57Bl/6 WT control and TLR2 KO mice with GCM for 6 h and analyzed changes in MT1-MMP transcript levels by semi-quantitative Figure 4.3 (A) and quantitative RT-PCRs (B). Indeed, knocking-out the TLR2 gene

caused attenuation in MT1-MMP mRNA levels after stimulation with GCM as observed through semi-quantitative (A) and quantitative RT-PCR analyses (B). While in WT control microglia, there was a 2.5-fold amplification in MT1-MMP expression after 6 h of stimulation with GCM, there was no observable enhancement in MT1-MMP gene expression in TLR2 KO microglia stimulated with GCM at either after 6 h.

Since TLR2 is known to dimerize with either TLR1 or TLR6 to mediate it's signaling via the MyD88 adaptor molecule, primary microglial cells from TLR1 and 6 KO mice were stimulated with GCM for 6 h to investigate any putative alteration in MT1-MMP gene expression by quantitative RT-PCR (Figure 4.3 C and D).

MT1-MMP expression was reduced in both TLR1 and 6 KO microglia stimulated with GCM compared to WT microglia stimulated with GCM for 6 h (Figure 4 C and D). While MT1-MMP expression was reduced both at 6 h in the GCM stimulated TLR6 KO microglia compared to the non-stimulated TLR6 KO, there was a modest but insignificant decrease in MT1-MMP expression at 6 h. This indicates a possible role played by either TLR 1 or 6 or both along with TLR2 in mediating MT1-MMP expression in glioma-associated microglia.

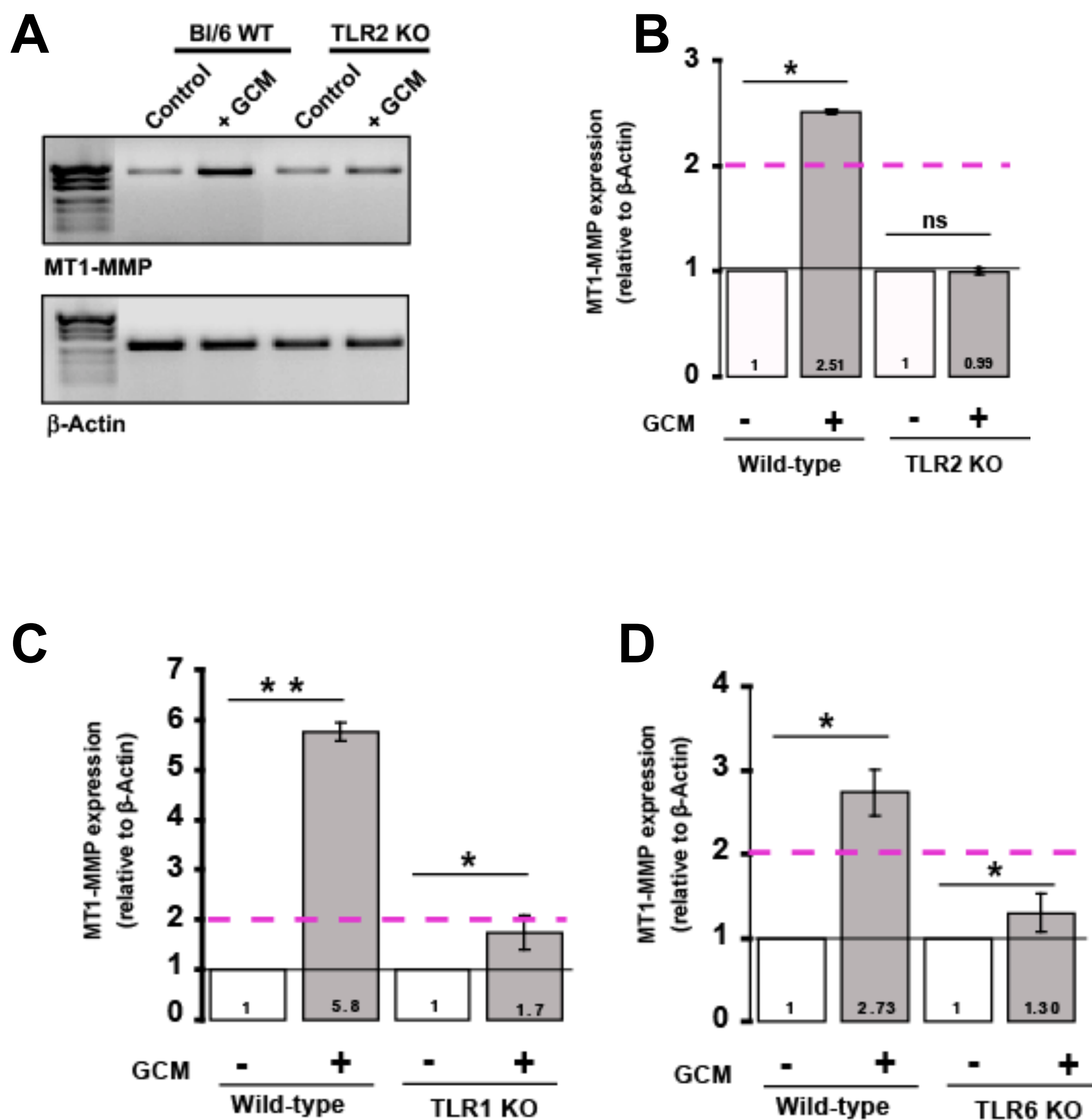


Figure 4. 3 MT1-MMP expression is reduced in glioma-associated microglia cells from TLR2, 1 and 6 KO mice *in vitro*.

TLR2 is essential for inducing glioma-associated microglial MT1-MMP expression. Microglia from Bl/6 WT and TLR2 KO mice were stimulated with GCM for 6 h and MT1-MMP expression was analyzed by semi-quantitative (A) and qRT-PCR (B) by the $2^{(-\Delta\Delta Ct)}$ method relative to the house-keeping gene β-Actin. MT1-MMP gene expression was also significantly reduced in GCM-stimulated microglia from TLRs 1 and 6 KO mice as seen in (C) and (D). In (A) amplicon size of MT1-MMP is 432 bp and β -Actin is 238 bp. In (B) the values of MT1-MMP fold changes are listed in the figures. Data are represented as mean ±SEM, p<0.05(*) and p<0.01(**).GCM (glioma conditioned medium), M (Molecular weight marker).

4.4. Glioma-induced MT1-MMP expression is not reduced in the MyD88-dependent TLR7 KO microglia

In order to address if any other MyD88-dependent TLR is involved in up-regulating MT1-MMP expression I also analyzed MT1-MMP expression in glioma-associated microglia from TLR7 KO mice which were stimulated with GCM for 6 h and changes in transcript levels of MT1-MMP were analyzed by qRT-PCR (A & B). Interestingly, stimulation of TLR7 KO microglia with GCM did not cause any decrease but rather a strong enhancement in MT1-MMP expression as compared to the control non-stimulated microglia.

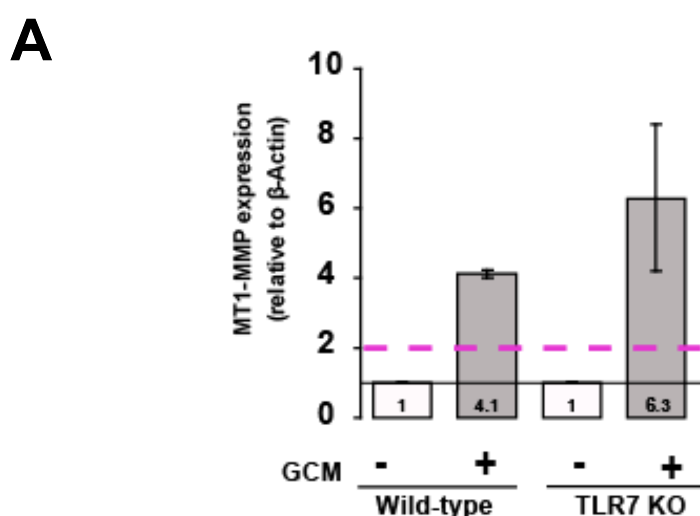


Figure 4. 4 MT1-MMP expression is not affected in GCM-stimulated microglia from TLR7 KO mice.

In microglia derived from TLR7 KO mice stimulation with GCM induced a robust MT1-MMP expression compared to WT microglia as seen in (A). MT1-MMP expression was analyzed by qRT-PCR by the $2^{(-\Delta\Delta Ct)}$ method relative to the house-keeping gene β-Actin. The values of MT1-MMP fold changes are listed in the figures. Data are represented as mean ±SD, GCM (glioma conditioned medium), SD (Standard Deviation).

When primary microglial cells from TLR7 KO mice were stimulated with GCM, there was an enhanced increase in MT1-MMP gene expression, indicating that despite MyD88 being a common adaptor molecule that transduces signals via different TLRs, the specific effect exerted by factor(s) released by glioma cells on MT1-MMP expression in glioma-associated microglial cells is mediated by TLR2 and one or more of its heterodimeric partners.

4.5. Reduction in MT1-MMP protein expression in TLR2 KO microglia stimulated with glioma conditioned medium

To verify changes in MT1-MMP expression in TLR2 KO microglia stimulated with GCM, I analyzed protein levels of MT1-MMP in TLR2 KO microglia after stimulation with GCM for 6 h. Whole cell extracts were prepared and resolved by western blotting and probed for detecting MT1-MMP protein expression.

As observed in Figure 4.5 (A) and quantified in (B), while GCM induced an up regulation in MT1-MMP expression in C57BL/6 WT microglia, on the contrary it led to a decrease in MT1-MMP in TLR2 KO microglia after 6 h.

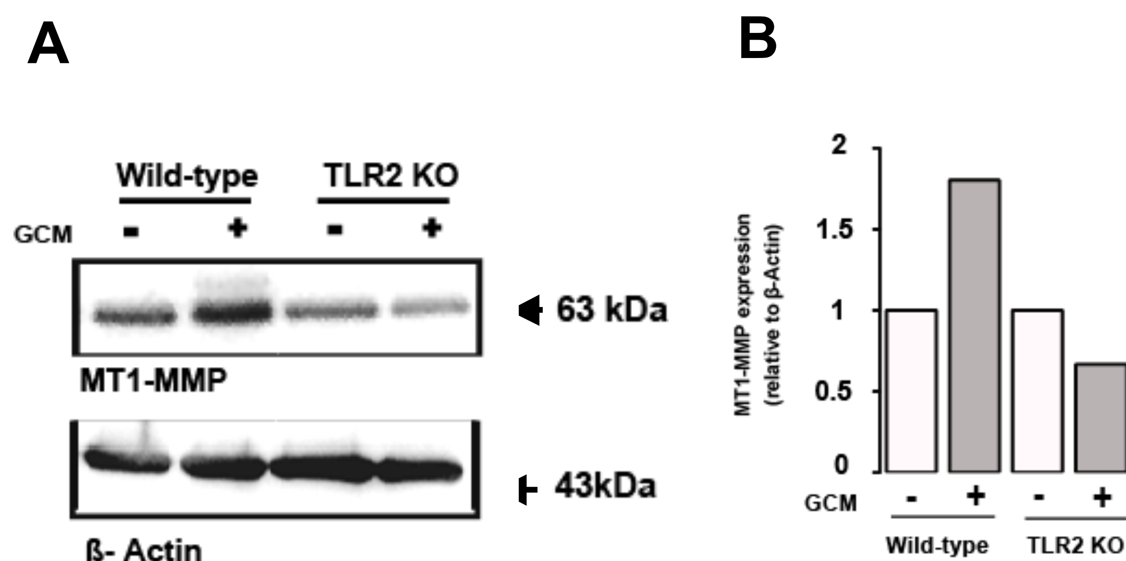


Figure 4. 5 MT1-MMP protein expression is also reduced in TLR2 microglia stimulated with GCM.

MT1-MMP protein expression was also reduced in TLR2 KO microglia treated with GCM as compared to WT controls on the western blot (A and B), indicating that TLR2 is required to mediate glioma-induced MT1-MMP expression in microglia. GCM (glioma conditioned medium), KO (Knock-out), kDa (Kilo Dalton).

4.6. Toll-like receptor 2 interferes with glioma growth in an organotypic brain slice culture model

In order to analyze if deletion of the TLR2 gene locus could have any significant effect on glioma expansion in an *ex vivo* model of organotypic brain slice culture, I injected EGFP labeled GL261 cells into the slices and analyzed the area occupied by the glioma cells 5 days after injection. Moreover, in order to investigate the knock-out effect of TLR2 in the brain sub-population of microglial cells on glioma growth, I depleted microglial cells in the organotypic slices with liposomes-encapsulated clodronate.

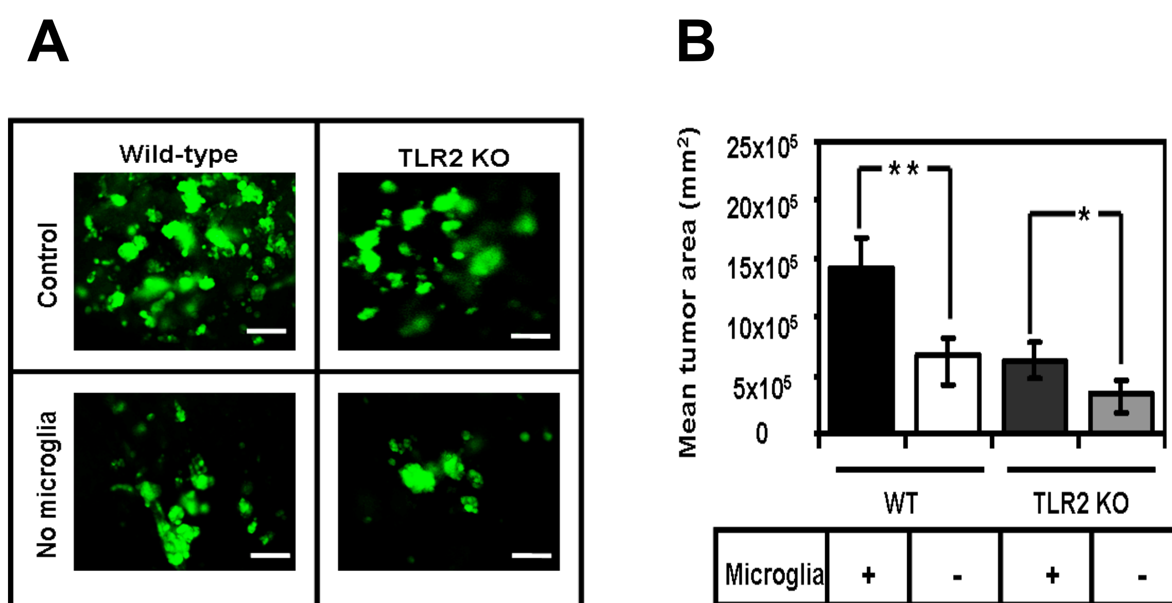


Figure 4. 6 TLR2 interferes with glioma growth in an organotypic brain slice culture model.

TLR2 expressed by microglia contributes to tumor expansion in an *ex vivo* model of microglia-glioma interaction. Organotypic brain slices from 16-day-old WT and TLR2 KO mice were implanted with 5000 EGFP-GL261 glioma cells and the area occupied by the tumor cells was measured stereologically after 5 days in microglia containing and depleted. Data are represented as mean ±SEM, p<0.05(*) and p<0.01(**). Scale bar is 10µM.

As expected, the area occupied by the glioma cells after 5 days post inoculation into the organotypic brain slices was reduced in the TLR2 KO slices compared to the slices from the wild-type mice as seen in (A). Further-more, ablation of

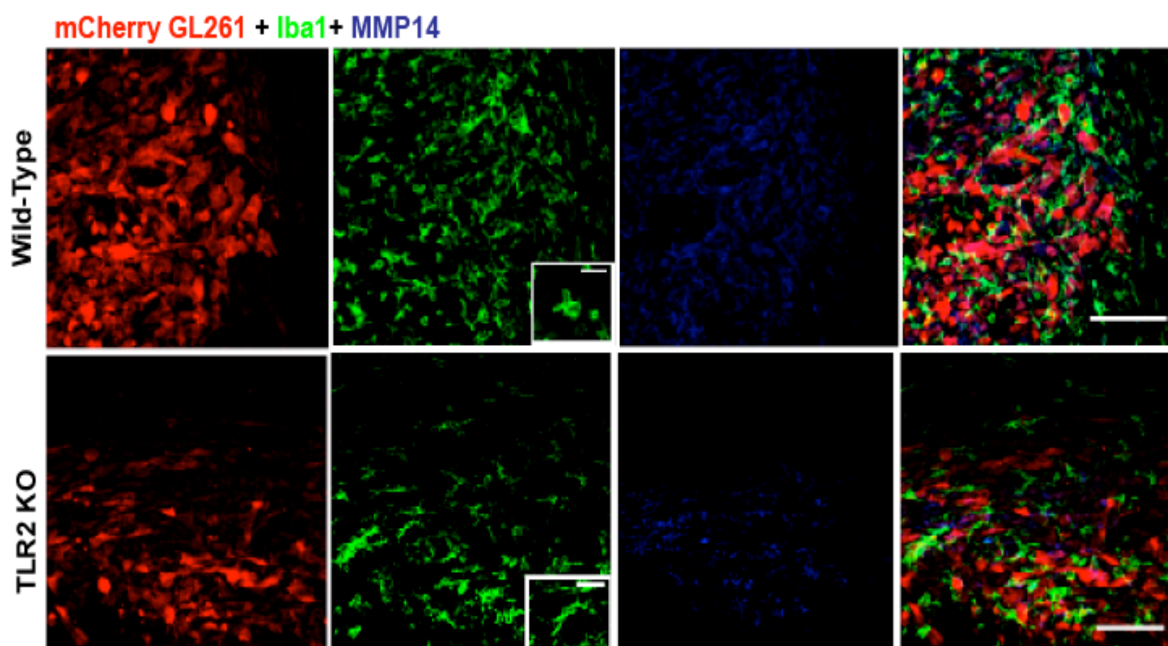
microglia from the organotypic brain slices caused a significant reduction in the tumor area occupied by glioma cells not just in the WT controls but also in the slices from the TLR2 KO mice. Compared to the control organotypic brain slices, microglia depleted slices from wild-type mice had a nearly 30% reduction in tumor size ($p=0.0031$). Indeed, the knock-out of the TLR2 gene reduced the average size occupied by glioma cells by nearly 33% ($p=0.026$) relative to tumor size in control slices as seen in (B). This suggests the presence of additional TLR2-independent cellular mechanisms sustaining glioma growth *ex vivo*. Overall, the data indicate that TLR2 is an important mediator of tumor-promoting activity of microglia. Statistical significance was analyzed by Wilcoxon sum-rank test and significance was taken at $p<0.05$(*) and $p<0.01$(**).

4.7. The MT1-MMP expression in glioma-associated microglia is reduced in vivo in TLR2 KO mice

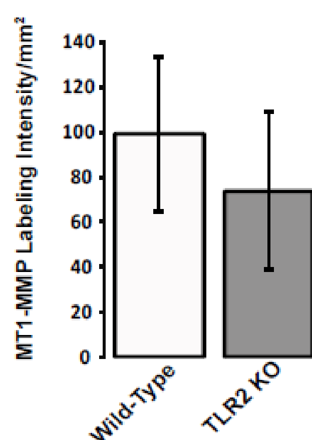
In order to evaluate changes in MT1-MMP expression *in vivo*, adult C57Bl/6 WT and TLR2 KO mice were intra-cranially implanted with GL261 glioma cells expressing the red fluorophore mCherry. The tumors were allowed to grow for two weeks and subsequently mice were sacrificed and the brain tissue was processed for immunohistochemical analysis of MT1-MMP expression. The microglial cells were identified by labeling with the microglia/macrophage- specific antibody against Iba1.

The level of MT1-MMP labeling was increased in microglia in close proximity to the tumor border and MT1-MMP immunoreactivity was particularly pronounced when the microglia were in close contact with glioma cells in the control WT mice. In contrast, the expression of MT1-MMP was significantly reduced in microglia in the proximity to the tumor in TLR2 KO mice, at two weeks after tumor inoculation. Furthermore, the density of microglia in the tumor boundary was decreased in the TLR2 KO mice as compared to the WT mice.

A



B



C

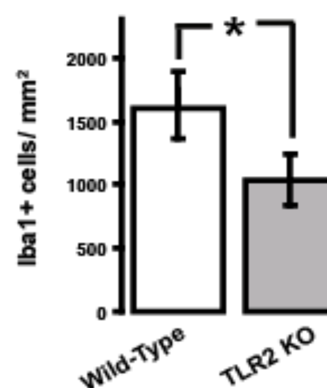


Figure 4. 7 Myd88-dependent TLR2 signaling mediates glioma-induced MT1-MMP expression *in vivo*.

C57Bl/6 WT and TLR2 KO mice were intra-cerebrally implanted with Gl261 cells and brain tissues were histochemically processed after 2 weeks of tumor growth. Glioma cells in the WT and TLR2 KO mice were identified by stable expression of mCherry (red), microglia/brain macrophages by the expression of Iba1 (green) and MT1-MMP labeling was in blue (A, upper and lower panels). MT1-MMP labeling intensity was quantified in WT and TLR2 KO mice around the tumor edge in both WT and TLR2 KO mice from 3 random fields of interest in the tumor (B). Further-more microglia density at the tumor-edge in WT and TLR2 KO mice was calculated as shown in (C). Phenotype of glioma-associated microglia in WT mice was amoeboid while in TLR2 KO mice was more ramified (A; insets). Data are represented as mean ±SEM, $p<0.05$(*) and $p<0.01$(**). Scale bar is 50μM.

To analyze the specificity of TLR2 signaling and MT1-MMP expression, I also analyzed if any other MyD88 dependent TLR might also be involved. Adult C57Bl/6 and TLR 7 and 9 KO mice were intra-cerebrally implanted with GL261 glioma tumors transfected for stable expression of EGFP. Tumors were allowed to grow for two weeks, after which the mice were sacrificed and immunohistochemical analysis to investigate MT1-MMP expression and tumor expansion were done on the brain tissue samples. Microglia cells were detected by the Iba1 antibody (in red) while MT1-MMP labeling was in blue (D & E).

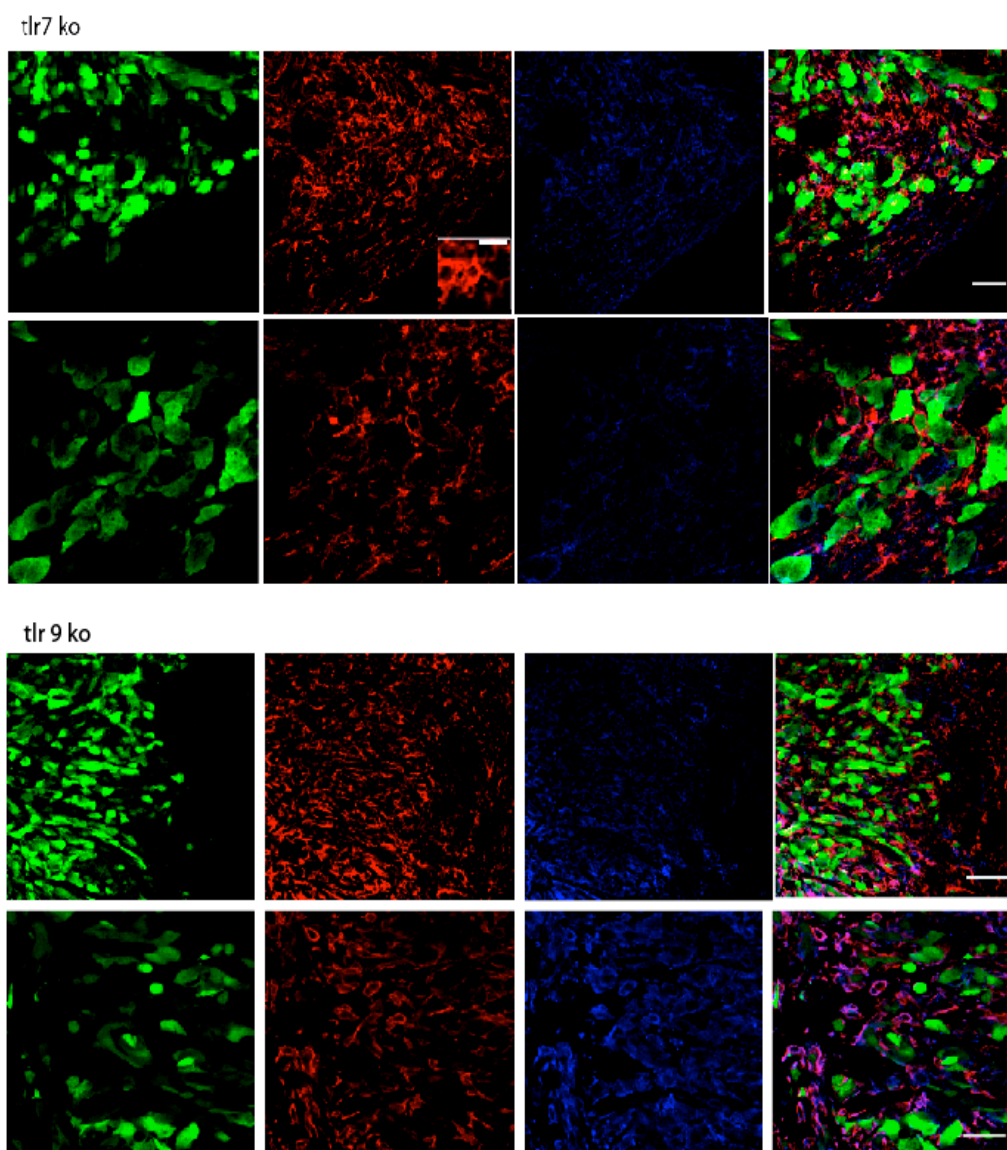


Figure 4. 7 Myd88-dependent TLR7 an 9 signaling mediates glioma-induced increase in MT1-MMP expression *in vivo*. Scale bar is 50µM.

4.8. Deletion of TLR2 led to reduction in glioma expansion

In order to investigate how deletion of the TLR2 gene locus might affect the tumor expansion in vivo, 2 groups of adult C57Bl/6 WT and TLR2 KO mice were implanted with tumors and analyzed after 3 weeks of tumor growth.

The first group of mice was analyzed for changes in tumor sizes by the Cavalieri principle while the second group of mice was analyzed by MR Imaging. Representative pictures used for both the methods are shown in (A & B).

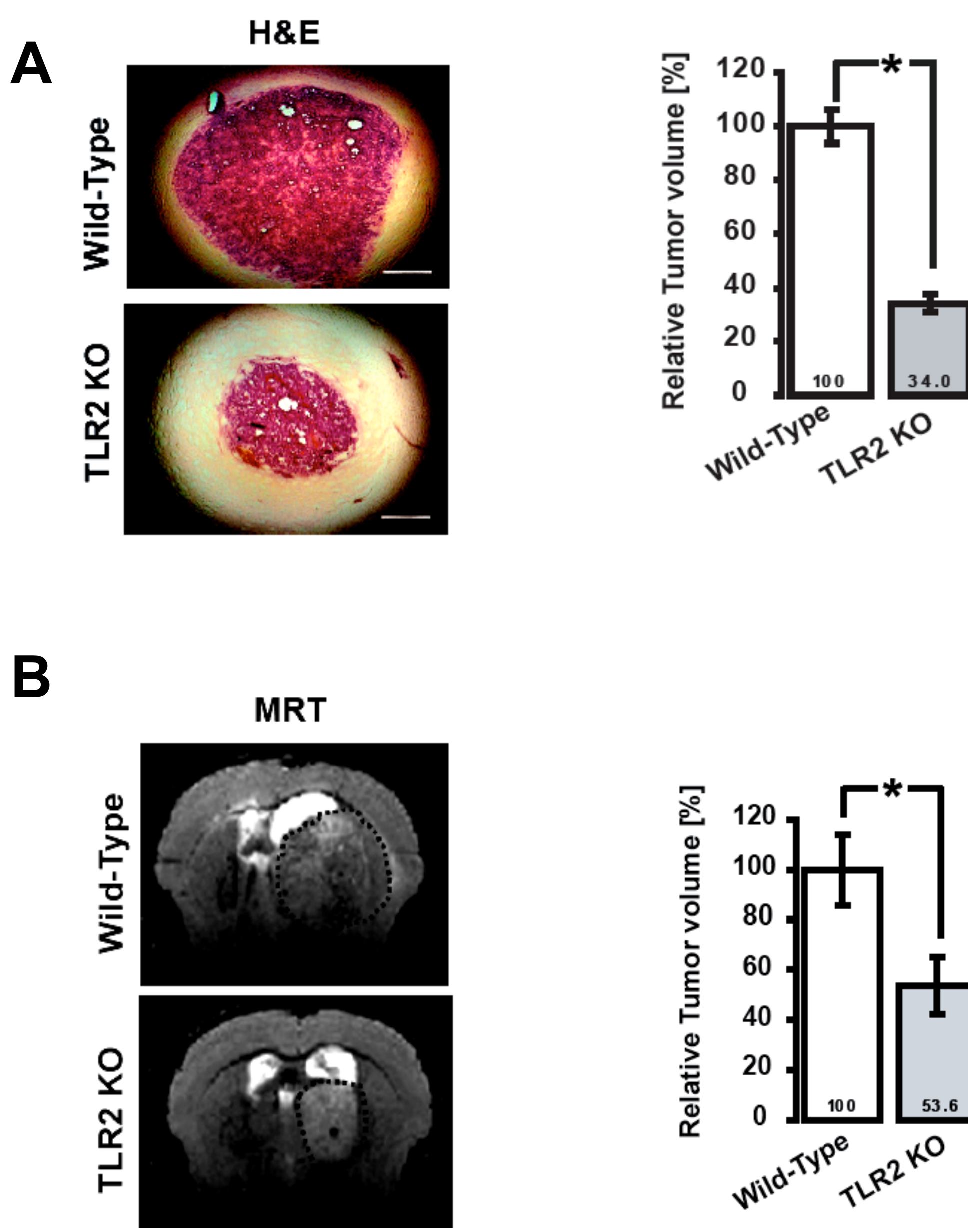


Figure 4. 8 Deletion of TLR2 gene locus results in reduced tumor expansion in glioma implanted mice.

The knock-out of TLR2 gene locus impacts tumor expansion *in vivo*. Mouse GL261 cells were intra-cerebrally implanted into two groups of TLR2 KO and Bl/6 WT mice. The changes in tumor volumes were analyzed in these groups by two different approaches- 1) by unbiased stereolgy and 2) by MRI scanning. For the stereological method, the WT and TLR2 KO mice (n=8/group, total 16) were implanted with GL261 tumors and histochemically processed for measuring tumor volumes after 14 days of tumor implantation. The brains sections were stained for Heamtoxylin & Eosin and tumor areas were measured according to the Cavalieri's principle as shown in (A). Scale bar is 500µM. For the second approach (B), Gl261 cells were implanted into WT and TLR2 KO mice (n=11/group, total 22) and tumor volumes were measured after 21 days by MRI. A representative MRI T_2-weighted image of a hyper-intense tumor (delineated by dotted black circle) from WT and TLR2 KO mice is shown in (B). Data are represented as mean ±SEM, $p<0.05$(*) and $p<0.01$(**).

4.9. TLR2 KO mice implanted with glioma tumors had improved chances of survival

Since MT1-MMP expression was reduced in TLR2 KO mice compared to C57Bl/6 WT mice with a concomitant reduction in the tumor volume, I investigated if deletion of the TLR2 gene locus could enhance survival in mice implanted with glioma tumors.

To address this aspect, ten adult C57Bl/6 WT and ten TLR 2 KO mice were inoculated with GL261 glioma tumors and the mice were monitored until the end point of the experiment, ie. time at which one or more mice survived. Based on the results obtained, the cumulative survival period for the Bl/6 WT and TLR2 KO mice was calculated at 59 days. As expected, TLR2 KO mice intra-cranially inoculated with gliomas showed a marginal but significant survival rate (p=0.039) compared to the Bl/6 WT counterparts

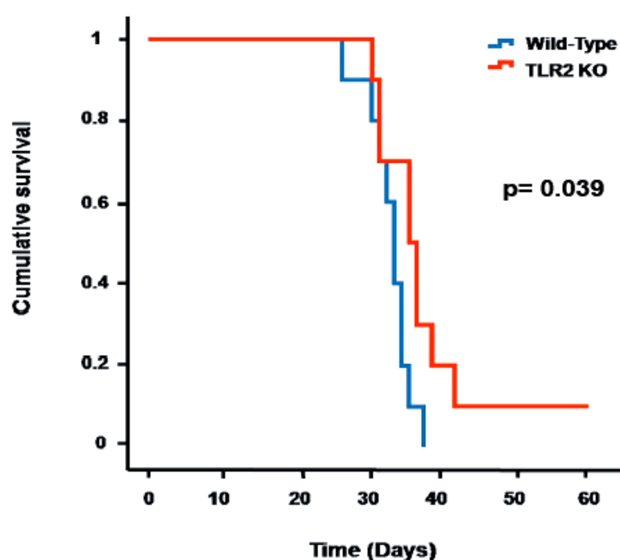


Figure 4. 9 Deletion of the TLR2 locus improved survival in mice implanted with glioma tumors.

Silencing of the TLR2 gene locus resulted in a marginal but significant survival benefit in glioma-implanted TLR2 KO mice compared to the WT controls.

RESULTS (PROJECT 2)

The project to investigate the role of Minocycline on glioma-microglia interaction was done by Darko Markovic and me and the results are published in a short communication with equal shared first authorship.

Pharmacological intervention of the microglia-glioma interaction by Minocycline

Microglia play a crucial role in supporting the growth and invasion of glioma tumors by various molecular and cellular mechanisms as has been reported by several investigators (Badie et al. 1999)The infiltrative nature of gliomas is a major concern and one way of overcoming it is to intervene with the microglia-glioma interaction by different genetic and pharmacologic approaches.

Minocycline, a second generation tetracycline derivative with a broad-spectrum antimicrobial activity, is a good drug candidate to interfere with the microglia-glioma communication. Minocycline can effectively cross the blood brain barrier and inactivate all forms of microglial cell activation and function. Moreover, Minocyclinecycline has also been reported to abrogate activity of function of several MMPs, including MMP2 and MMP9 and can also prevent activation of the MAPKs. Thus, Minocycline has the ability to confer neuroprotection under normal and pathological conditions.

4.10. Minocycline blunts the pro-tumorigenic effect of microglial MT1-MMP expression in gliomas

From previous studies (Markovic et al 2009), it has been shown that soluble factor(s) produced by glioma cells attract and recruit microglia in and around the tumors to promote invasion, proliferation and growth. Hence, to test if Minocycline would interfere *in vitro* with the increase in MT1-MMP expression and activity in microglia after treatment with glioma conditioned medium (GCM), primary microglia derived from C57Bl/6 WT mice were stimulated for 3 and 6 h with GCM and/or only complete growth medium containing 200 nM Minocycline.

From pilot *in vitro* experiments on primary microglia, the optimal working concentration of Minocycline was determined to be 200 nM (IC or Inhibitory Concentration of Minocycline at which it did not cause any adverse effects on the cells *in vitro but* induced reduction in release of pro-inflammatory cytokines).

Microglial cells were first pre-incubated for 30 min with 200 nM Minocycline and then co-incubated with Minocycline and GCM together. Total RNA was isolated from Minocycline treated microglia and reverse transcribed into cDNA. Any changes in MT1-MMP expression and activity due to Minocycline treatment were analyzed through semi-quantitative RT-PCR, qRT-PCR, western blotting and a MT1-MMP activity assay.

Drug treatment with Minocycline indeed led to alterations in GCM-induced microgial MT1-MMP gene expression at 3 and 6 h as observed by semi-quantitative (Figure 4-2-1 A) and quantitative RT-PCRs (Figure 4-2-1 B). Stimulation of microglia with GCM lead to an increase in MT1-MMP expression at 3 and 6 h compared to the control condition as seen in (A). However co-treatment of microglia with GCM and Minocycline lead to a complete abrogation of MT1-MMP gene expression. Moreover, a similar effect was observed by quantitative RT-PCR (B). An average 2-fold up-regulation in MT1-MMP gene expression was detected at 6 h after GCM stimulation which was further reduced to near baseline expression upon treatment with Minocycline (B).

Further-more, I wanted to investigate if Minocycline induced similar effects on MT1-MMP expression as seen at the mRNA level by comparing changes at protein levels. In order to address this, I stimulated microglia from C57Bl/6 WT mice with GCM in the presence and absence of 200 nM Minocycline for 3 and 6 h. Whole cell protein extracts from microglia stimulated with GCM alone or GCM and Minocycline were analyzed by western blotting for MT1-MMP protein expression.

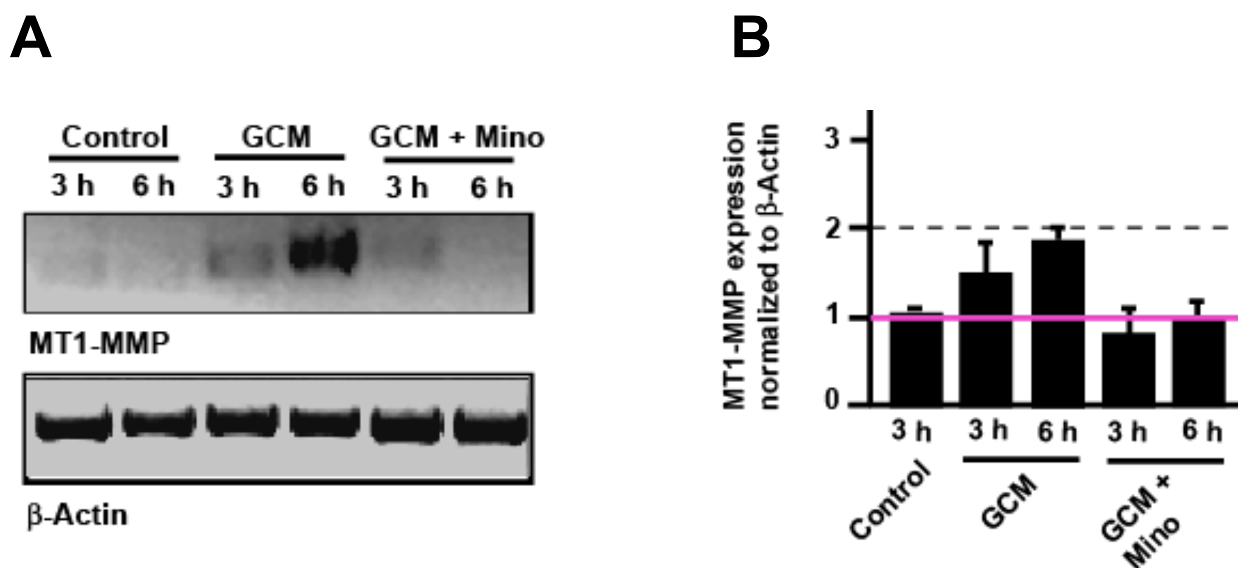
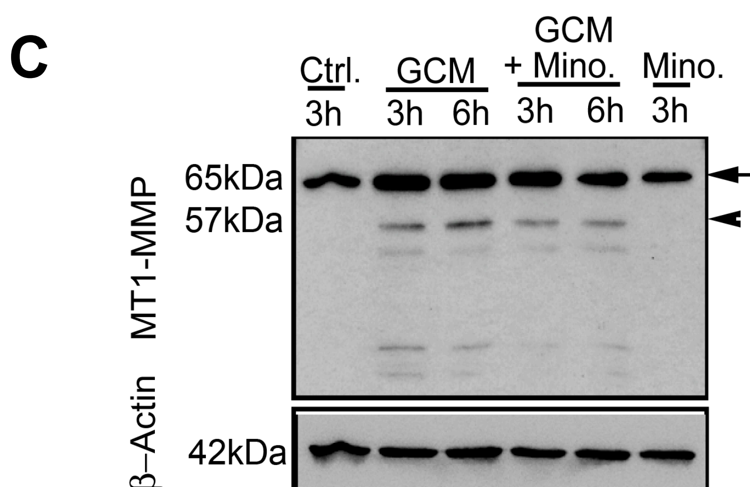


Figure 4. 10 Minocycline abrogates GCM induced-MT1-MMP gene expression in microglia.

(A) Semi-quantitative PCR analysis of MT1-MMP expression revealed changes in primary microglia stimulated with GCM alone or GCM and Minocycline after 3 and 6 h. MT1-MMP gene expression was reduced when microglia were stimulated with GCM containing 200 nM Minocycline. This was also corroborated through qRT-PCR analysis as seen in (B) where GCM treatment for 6 h alone lead up to a 2-fold increase in microglial MT1-MMP expression. After GCM stimulation together with Minocycline, the over-expression of MT1-MMP was abrogated and reduced to near basal levels.



GCM-induced increase in MT1-MMP expression in microglia is blunted by Minocycline.

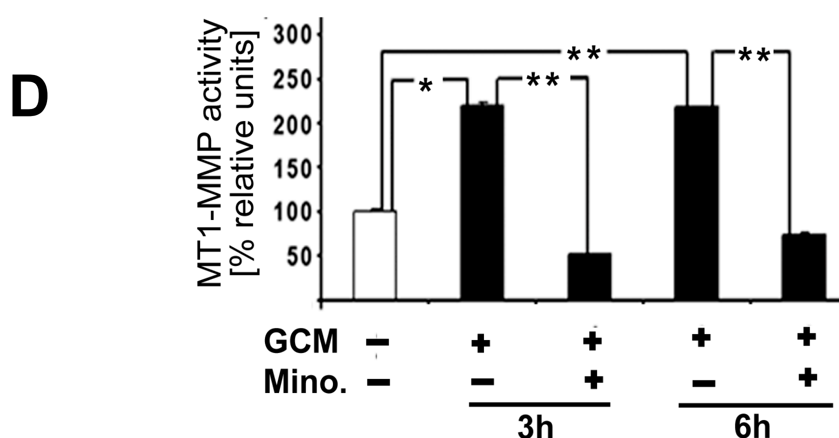
(C) Immunoblot analysis of MT1-MMP expression revealed differences in protein expression in primary microglia stimulated with GCM alone or GCM and 200 nM Minocycline after 3 and 6 h. While GCM induced an increase in expression of the active form of MT1-MMP after 3 and 6 h as compared to control microglia, Minocycline the formation of the active MT1-MMP after 3 and 6 h

(57 kDa band, arrowhead). Thus, Minocycline effectively interfered with GCM- stimulated microglial MT1-MMP expression.

As observed with the RT-PCR analysis, I found a similar enhanced MT1-MMP expression after 3 and 6 h of treatment with GCM. However, western blot analysis of the whole cell protein extracts revealed a decrease in MT1-MMP expression after they were stimulated with GCM and Minocycline together. GCM induced an increase in MT1-MMP expression after 6 h whereas co-treatment with 200 nM Minocycline prevented an increase in MT1-MMP expression as demonstrated by a decreased amount of active MT1-MMP product as seen in figure (C). This indicated a decreased turnover of MT1-MMP upon Minocycline treatment in microglia exposed to GCM.

Since Minocycline hindered the increase in MT1-MMP gene and protein expressions in primary microglia stimulated with GCM, I wanted to investigate if Minocycline could influence the activity of GCM-induced MT1-MMP in microglia.

Primary microglia from Bl/6WT mice were treated in a similar manner as explained earlier for 3 and 6 h. After the end-point of the experiment, the microglia cells were lysed and supernatants collected and analyzed by an ELISA-based MT1-MMP activity which measured the release of active MT1-MMP from biological samples in a 96-well plate format. Active MT1-MMP was normalized to controls (i.e., the baseline activity was measured in Minocycline non-treated control microglia). A * significance was assessed at $p < 0.05$ and ** significance at $p < 0.01$.



Minocycline reduces GCM-induced increase in MT1-MMP activity in microglia.

The MT1-MMP activity in microglia was significantly elevated after incubation with GCM (D).The enzymatic activity increased by 220% and 217% after 3 and 6 h respectively. The difference in

MT1-MMP activity between 3 and 6 h stimulation with GCM was however not significant and could be attributed to a saturation level reached towards the end of the assay. In the presence of Minocycline however, GCM no longer stimulated MT1-MMP activity in microglia and caused a significant reduction in MT1-MMP activity to 51% and 73% after 3 and 6 h respectively as compared to untreated control microglia. Image courtesy Darko Markovic.

Microglia stimulated with GCM for 3 and 6 h released significant amounts of active MT1-MMP. But co-treatment of the microglia with GCM and 200 nM Minocycline led to a major reduction in the release of active MT1-MMP from microglia cells as seen in (D). The MT1-MMP activity assay hence confirmed the results from the mRNA and protein expression experiments, namely an increase in microglial MT1-MMP activity after GCM treatment is inhibited by treatment with Minocycline

4.11. Minocycline interferes with glioma growth ex vivo

Based on the results from *in vitro* experiments using Minocycline, the next question to investigate was if Minocycline interfered with glioma expansion.

To address this question, organotypic brain slices (OBS) from 16 day old male Bl/6 WT mice were injected with EGFP labeled GL261 cells and subsequently analyzed for the area occupied by glioma cells after 5 days of inoculation. Cell-specific effect of Minocycline, mainly effect on microglia cells, was examined in two groups of OBS- microglia containing OBS and microglia-depleted OBS. Microglia were depleted by application of liposome encapsulated clodronate to organotypic brain slices. GL261 cells were inoculated after complete ablation of microglia in these organotypic brain slices according a previous protocol (Markovic, D 2005, 2009).

The results revealed that Minocycline treatment reduced the average tumor size occupied by glioma cells to 57% as compared to non-treated control slices. Compared to control slices, microglia depletion resulted in a reduction of tumor size to 58%. However, Minocycline alone did not drastically affect tumor size in microglia depleted slices and the effect it exerted was similar to the one seen in microglia-depleted slices without Minocycline treatment, namely a reduction of 56% in tumor size. These results indicated that Minocycline acts via microglia

closely associated with glioma tumors and thereby leads to a reduction in the tumor-promoting activity of microglia cells.

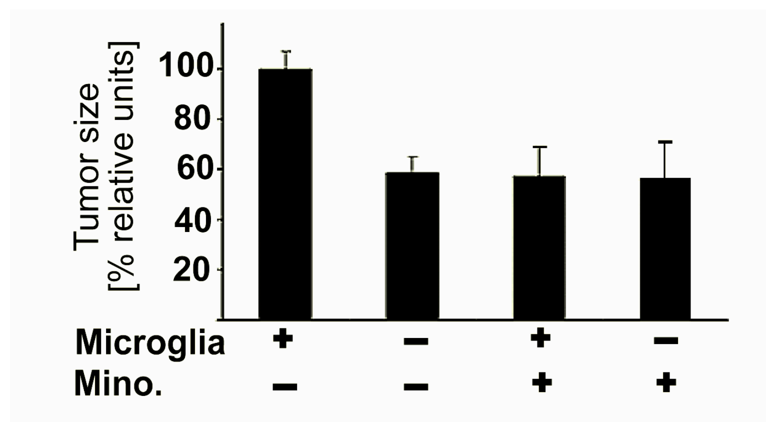


Figure 4. 11 Minocycline interferes with tumor growth and expansion *ex vivo*.

The effect of Minocycline on the growth of glioma tumor was assessed in an organotypic brain slice model. The brain slices were inoculated with GL261 glioma cells and the relative tumor sizes were measured in control and microglia depleted brain slices, with and without Minocycline treatment. By keeping tumor sizes in control slices to 100%, it was observed that Minocycline treatment caused a significant reduction in tumor size to nearly 60% relative to control. Image courtesy Darko Markovic.

4.12. Oral administration of Minocycline reduced glioma growth in vivo

Since Minocycline treatment in *ex vivo* organotypic brain slice cultures lead to a reduction in microglia-associated glioma growth, it was imperative to investigate if the same was true in the *in vivo* situation.

To approach this question, experimental gliomas were intra-cerebrally implanted into brains of 30-day old C57Bl/6 WT mice by stereotactic injection of EGFP labeled GL261 cells. The tumors were allowed to grow for two weeks after which the mice were sacrificed to analyze changes in tumor volumes associated after treatment with Minocycline. Oral administration of Minocycline through drinking water was preferred over daily intra-peritonial injections of Minocycline as the former approach minimized the extreme stress inflicted on the tumor-bearing mice which is unavoidable in the latter route of drug delivery.

Further-more, two different experimental designs were used to orally administer Minocycline to the mice. In the first type (group 1), C57Bl/6 WT mice were provided Minocycline immediately on the same day after glioma tumor implantation for the rest of the two week period. In the second approach (group 2), the implanted glioma tumors in Bl/6 WT mice were allowed to grow for one week after which the mice were orally administered Minocycline for the entire two weeks of the experiment. In both the approaches the drug was always prepared freshly in drinking water at a dosage of 10 ng/ml and administered to the mice while the control group received only pure drinking water. Mice were sacrificed after two weeks of Minocycline administration and the tumor volumes were estimated by unbiased Cavalieri method using the Stereo Investigator ® software.

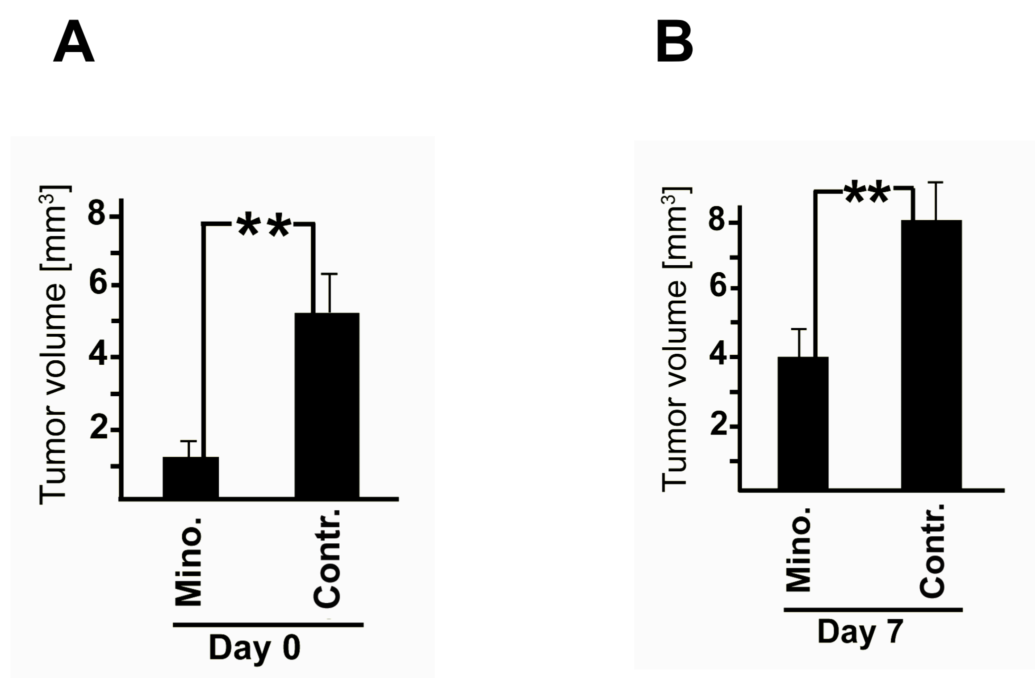


Figure 4. 12 Minocycline interferes with tumor expansion in a mouse model of experimental glioma *in vivo*.

The *in vivo* administration of Minocycline resulted in a decrease of tumor volume relative to the control group. Two groups of mice (control and experimental) were implanted with EGFP-GL261 glioma cells. While one group was administered Minocycline through drinking water from day 0 post tumor-implantation, the other group was administered Minocycline 1 week after tumor injection. In both groups, the relative volume of the tumor was significantly reduced upon Minocycline treatment as compared to the controls. Data are represented as mean tumor volumes, significance was calculated at $p < 0.01$. Image courtesy Darko Markovic.

In mice that received Minocycline during the entire period of tumor growth (group 1), the difference in average tumor volume was strongly reduced to 1.05 mm^3 as compared to 4.71 mm^3 in the control group (A). Also in the group 2 mice, Minocycline significantly reduced glioma growth to 4.07 mm^3 as compared to control mice where the average tumor volume was 7.52 mm^3 (B). Thus, based on the results from this study, Minocycline treatment resulted in decreased glioma growth in an experimental glioma mouse model, even in a situation when Minocycline was applied at a later time point during tumor growth, thus mimicking a clinical state where a patient already presents a tumor before receiving any form of treatment.

4.13. MT1-MMP expression in glioma-associated microglia is reduced after Minocycline administration in vivo

The final goal of the project was to examine if the *in vitro* effect of Minocycline on MT1-MMP expression in glioma-associated microglia was reproducible. For this purpose, six-week old adult Bl/6 WT mice were intra-cranially injected with EGFP-GL261 cells; tumors were allowed to g row for two weeks and were orally administered with Minocycline in parallel.

The mice received 10 ng/ml Minocycline dissolved in drinking water daily for the entire two week period of tumor growth. After two weeks, the mice were sacrificed and the brain tissues were processed for immunohistochemical analysis of MT1-MMP expression. Microglia cells were identified by the expression of the microglia/macrophage-specific Iba1 antibody. MT1-MMP labeling was increased greatly in microglia cells which were in close proximity to the tumor border in the control WT mice. However in contrast, the expression of MT1-MMP in microglia closely associated with the invasive edge of the tumor was significantly reduced in mice which had received Minocycline through drinking water compared to control animals which received plain drinking water (Figure 4.13).

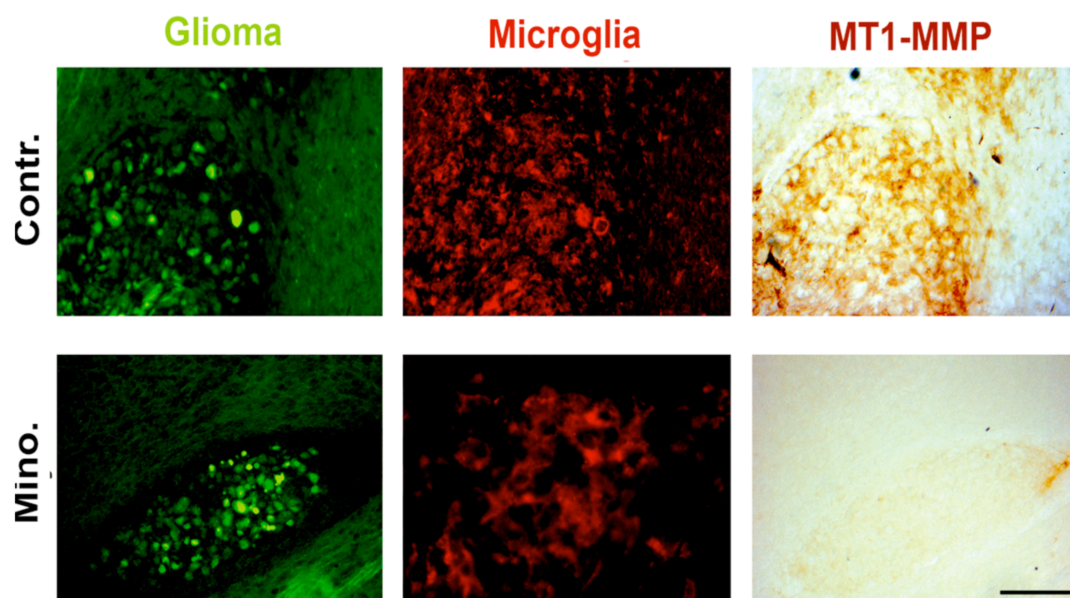


Figure 4. 13 Minocycline abrogates MT1-MMP expression in glioma-associated microglia *in vivo*.

Microglia cells labeled for Iba1 (in red) aggregated less at the tumor boundary after Minocycline treatment. MT1-MMP labeling shown in brown is less intense in the microglia cells associated with gliomas in Minocycline administered mice. A representative picture of the immunolabeling from the experimental and control mice is shown in this figure. Scale bar is 500 µm. Image courtesy Darko Markovic.

Most importantly, the *in vivo* administration of Minocycline not only reduced MT1-MMP expression in mice compared to the control mice but also lead to the simultaneous decrease in tumor size. Thus, the demonstrated downregulation of MT1-MMP *in vivo* furthermore indicated the strong therapeutical effect of continuous Minocycline treatment. Hence, in the glioma bearing mice treated with Minocycline, the MT1-MMP expression in microglia was lower as compared to the enhanced expression observed in control mice

5. DISCUSSION

Malignant astrocytic tumors (Glioblastoma multiformae; high grade or WHO grade IV gliomas) are the deadliest and most aggressive cancers arising in the CNS. All forms of current therapies have had limited success due to the complexity and heterogeneity of these tumors. Tumor progression to the malignant phenotype is greatly dependent on the permissive action of the microenvironment consisting of immune and non-immune cells like macrophages, dendritic cells, stromal, epithelial cells etc. The dynamic interaction between tumors and these cell types renders a huge support to several cellular features associated with tumor migration, survival, proliferation, angiogenesis, inflammation, metastasis, evasion of immune responses, resistance to apoptosis and invasion (Yu et al 2007; Hanahan and Weinberg 2011). One critical factor regulated by the tumor microenvironment, specifically by the brain macrophages or microglia, is the production of various pericellular milieu altering proteases like MMPs and their inhibitors (TIMPs). Previous studies have shown the association of such MMPs like MMP2 and MT1-MMP with enhanced glioma tumorigenicity (Markovic D et al 2005, 2009).

Gliomas are infiltrated with large number of microglia cells that constitute a major cell population of tumor microenvironment. Malignant gliomas are paradigmatic tumors because of their “immune privilege”. They have devised multiple mechanisms to escape immune attack by secretion of several cytokines such as transforming growth factor-ß (TGF-ß) and vascular endothelial growth factor (VEGF), which impair both antigen presenting capacity of immune cells and also tumor specific T-cell immunity (Vicari et al 2002). The balance between immunosuppressive and immunostimulatory signals in aggressive gliomas must be altered in order to facilitate tumor cell recognition and an effective anti-tumor response by microglia.

Toll-like receptors are a class of pattern recognition receptors best known for their role in host defense responses against microbial components. Emerging evidence also ascribes a role for the TLRs in maintaining tissue homeostasis by regulating inflammatory and tissue repair responses to injury or trauma in the host (Nahoum

and Medzhitov 2009; Yu Li et al 2011). Recent studies also focus on the role of the TLR signaling in contributing to neoplastic advancement and progression (Huang B et al 2008; Matijevic and Pavelic 2010). Moreover, pharmacological intervention using drugs such as Minocycline, to inhibit microglia-glioma interaction could offer new benefits to glioma patients in terms of better prognosis and survival.

PROJECT 1

5.1. Microglial TLR signaling is required for glioma growth and progression

Mammalian TLRs are sentinels of the immune system and were first identified in 1997 based on their homology with Drosophila *Toll*, and recognize diverse microbial pathogens to induce innate immune responses (Hanke and Kielian 2011). They are evolutionarily conserved type I integral membrane glycoproteins expressed in both immune and non-immune cells(Akira 2006). Recognition of conserved molecular patterns by TLRs (pathogen-associated molecular patterns or PAMPS), which are shared by large groups of microorganisms, induces a signaling response that leads to the production of several cytokines and inflammatory mediators that impact tissue homeostasis and pathology (Akira 2006). Moreover, TLRs also play a crucial role in non-infectious diseases/injuries and may generally serve to monitor the “well-being” in tissues (Johnson et al. 2003). Microglia are the predominant TLR expressing cells in the CNS (Olson and Miller 2004), and are constantly scanning the brain for pathological insults and damage (Nimmerjahn et al. 2005). Furthermore, our present study shows that microglia cells receive inputs from glioma cells via TLRs expressed on their membranes.

Gliomas can constitutively release heat-shock proteins, high mobility group box 1 protein and hyluronan (Guzhova et al. 2001; Tsan and Gao 2004) and these factor(s) have been shown to stimulate TLRs (Aravalli et al. 2008). In fact, there is a high probability of one or more of these soluble factor(s) to be found in our GCM, which was used to stimulate microglial MT1-MMP expression *in vitro*. Moreover, *in vivo* TLRs can also be activated by factors liberated due to sterile inflammation,

hypoxia or necrosis, which are frequent events associated with malignant and invasive gliomas. Additionally, TLR-signaling can be activated through degradation of the extracellular matrix (Johnson et al. 2003). In gliomas, this may lead to the generation of a vicious cycle, in which TLRs induce and enhance metalloproteinase activity that in turn leads to a positive regulation of the TLR signaling, bolstering glioma pathology.

Our previous study has shown that GCM upregulated microglial MT1-MMP expression and activity via the TLR-adaptor MyD88 and subsequent activation of the p38 mitogen activated protein kinase (MAPK) pathway, which is in agreement with studies showing that p38 MAPK activation is induced downstream of TLR and MyD88-signalling (Watters et al. 2007) and that MT1-MMP expression is regulated through p38 MAPK (Boyd et al. 2005; Munshi et al. 2004). Moreover, in our present study we found that stimulation of microglia with different agonists for specific TLR sub-types like 1/2, 3, 4, 5, 6/2 and 7/8 induced a differential response of MT1-MMP gene expression after 6 h of treatment. While TLR1/2 agonist Pam_3Csk_4 induced a 3.5 fold upregulation in MT1-MMP expression after 6 h compared to the un-stimulated control and GCM stimulated control, the other agonists of TLRs like LPS (TLR4) and MALP2 (TLR6/2) induced a modest increase of around 2 fold after 6 h stimulation or a slight decrease (Flagellin for TLR5 and Poly (U) for TLRs7/8) in MT1-MMP gene expression.

I then asked if TLR2 alone or in combination with other accessory TLR molecules could induce MT1-MMP expression in glioma-associated microglia cells and contribute to the disease progression and invasion. Moreover, lung carcinoma produced soluble factors activate cells of myeloid origin via TLR2 to contribute to metastasis according to a study by Kim et al (Kim et al. 2009).

When the gene for TLR2 locus was knocked out, microglia showed a decrease in MT1-MMP gene expression after stimulation with GCM compared to non-stimulated WT control microglia, indicating a possible role of TLR2 upstream of MyD88-induced activation of MT1-MMP in glioma-associated microglia cells. Similarly, microglia derived from TLRs 1 and 6 KO mice when stimulated with GCM showed a reduction in MT1-MMP expression after 6 h as compared to the WT un-stimulated and GCM –treated counterpart microglia. Since TLR2 mediates

its intracellular signaling in a MyD88-dependent manner by heterodimerizing either with TLRs 1 or 6 to broaden its ligand spectrum (Farhat et al. 2008), based on my qRT-PCR results, I hypothesized that TLRs 1 and 6 along with TLR2 may play differential roles, depending on specific and perhaps common stimuli derived from glioma cells, to enhance MT1-MMP expression in glioma-associated microglia. When Bl/6 WT and TLR2 KO microglia stimulated with GCM for 6 h were analyzed by immunoblotting for MT1-MMP protein expression, there was a marked reduction in the formation of the activated 57kDa form of the pro-form of MT1-MMP, at 6 h in the TLR2 KO microglia compared to the control and GCM-stimulated WT microglia.

The depletion of microglia via clodronate treatment of organotypic brain slices derived from Bl/6 WT and TLR2 KO mice highlighted the importance of microglia in mediating glioma tumor expansion in vivo. Interestingly, while deletion of the TLR2 gene locus alone led to a significant reduction in tumor growth ex vivo, the ablation of microglia in these slices also modestly complemented the TLR2 gene locus knockout in terms of the reduction in tumor expansion in these organotypic brain slices. In the in vivo situation, the TLR2 KO mice formed smaller tumors compared to their WT littermate controls 2 weeks post tumor implantation. Furthermore there was a marked reduction in MT1-MMP expression in the tumors formed in the TLR2 KO mice as compared to the WT mice, indicating perhaps a reduction in invasive potential of these tumors in the TLR2 KO mice compared to the WT mice

The data from this study highlight the observations that gliomas control tumor associated microglial cells via TLR signalling to increase MT1-MMP expression mediated effects and thereby support glioma expansion. Our results suggest that the glioma supportive role of microglia cells is extensive and emphasizes their role as an important target of novel brain tumor therapies. Therapeutic TLR blockade, which may be achieved with TLR subtype specific antagonists and/or blocking antibodies and/or drugs like Minocycline (Markovic et al. 2011) or Propentofylline (Jacobs et al.) could serve as future tools to attenuate microglia-assisted glioma progression.

Tumor growth and progression has long been associated with inflammation. Often increased macrophage density in solid tumors has been linked to poor prognosis.

Cancer is a disease linked to the inherent loss of normal tissue homeostasis and perpetual tissue stress and damage (Chang 2004; Dvorak 1986). Among the signals that perpetuate tissue damage are endogenously released TLR ligands. Several scientific studies point out to the classical protective functions of the Toll-like Receptor signaling against the devastating effects of various pathogens and damage-associated molecular patterns. However, recent research studies in different murine models of carcinoma revealed that the TLR signaling can also lead to tumor progression and metastasis (Harmey et al. 2002; Luo et al. 2004), and that the signaling was dependent on tumor-supportive cell types, namely the tumor-associated macrophages or TAMs. Although the exact role of these tumor-associated macrophages is yet to be clearly characterized, their presence within the tumor is linked to a worse clinical prognosis in a majority of tumor types (REF). Unfortunately, very few tumor-derived TLR ligands that activate TAMS to promote tumor progression and metastasis have been identified and characterized till date. Since macrophages are normally linked to the restoration of tissue integrity and homeostasis after tissue damage, many tumors exploit them for the soluble factors that promote tumor growth, angiogenesis and metastasis.

Initial studies that investigated the tumor-promoting role of TLR signaling used exogenous and synthetic TLR ligands. However, recent studies focus more on the role of microbial infections on tumorigenesis. A classic example is the effect of chronic Helicobacter pylori infection on the development of gastric cancer. Another study by Huang et al. in 2007 (Huang et al. 2007) found that the Gram-positive bacterium Listeria monocytogenes when injected into ectopically implanted H22 hepatocellular carcinoma cells lead to the growth and formation of large tumors, an effect that could be abrogated by silencing TLR2 but not TLR4 expression. Moreover, a study by Rakoff-Nahoum and Medzhitov in 2007 (Rakoff-Nahoum and Medzhitov 2007) showed that the crucial MyD88 signaling is important for tumor progression but not initiation in a chemical carcinogenesis model of interstitial tumorigenesis. A study by Kim et al. in 2009 (Kim et al. 2009) showed that the extracellular matrix protein versican produced by Lewis lung carcinoma cells induced the secretion of IL-6 and TNFα by macrophages through the TLR2/TLR6 signaling pathway and that abrogation of the TLR2/TLR6- MyD88

pathway enhanced survival and reduced metastases in a murine model of lung cancer.

PROJECT 2

5.2. Minocycline serves as a potential therapeutic candidate for glioma treatment

Previous studies have shown that microglia can promote tumor growth and invasion through various molecular and cellular mechanisms, one of which includes MT1-MMP expression in glioma-associated microglia cells (Markovic et al. 2009). In the present study, it was observed that Minocycline, a medically approved antibiotic, blunts the pro-tumorigenic effect of glioma associated microglia. Minocycline has been reported to block p38 MAP kinase activation in microglia (Nikodemova et al. 2006). From a previous report by Markovic, D. et al in 2009, it was shown that the upregulation of MT1-MMP is dependent on the p38 MAP kinase activation.

In an animal model of multiple sclerosis, Minocycline inhibited MMP activity, reduced production of MMP-9 and decreased the transmigration of T lymphocytes (Brundula et al. 2002). In vivo studies on intra-cerebral hemorrhage in rodents demonstrated that MMP-2 and MMP-9 (Machado et al. 2006) and MMP-12 (Power et al. 2003) are downregulated and inhibited in their activity and expression after treatment with Minocycline. Moreover, Minocycline can also affect other microglia cell functions since it was previously reported that it is also capable of suppressing chemokine secretion (Kremlev et al. 2004). The microglial MT1-MMP over-expression may not only promote glioma invasion, but may also contribute to revascularization of the tumor (Belien et al. 1999).

Recent findings suggest that Minocycline can be neuroprotective and anti-inflammatory in various neurodegenerative disorders (Yong et al. 2004). The neuroprotective action of Minocycline may include its inhibitory effect on 5-lipoxygenase, an inflammatory enzyme associated with brain aging, and is being studied for its therapeutic use in Alzheimer disease patients (Seabrook et al. 2006). Minocycline also confers neuroprotection in mouse models of amyotrophic

lateral sclerosis (Kriz et al. 2002) and Huntington's disease (Chen et al. 2002b; Wang et al. 2003) and has recently been shown to stabilize the course of Huntington's disease in humans over a 2-year period (Chen et al. 2002a). Based on results from the present study implies, Minocycline can target the pro-tumorigenic effect of glioma associated microglia and may thus serve as a potential candidate to concomitant adjuvant therapies along with current treatment modalities in glioma patients.

6. FUTURE OUTLOOK

Results from the current experimental glioma mouse model have highlighted two aspects of microglia- glioma cross talk. One part demonstrated the importance of TLR signaling in glioma-associated microglia cells, in particular TLR2 along with TLR1 and/or TLR6 in contributing to glioma invasion and expansion. Deletion of the gene loci of these receptors led to a simultaneous reduction in microglial MT1-MMP expression and also a decrease in tumor expansion, pointing out to their contribution to glioma growth and invasion. In the second part, the broad-spectrum antibiotic Minocycline could effectively reduce an increase in MT1-MMP expression and activity in glioma-associated microglia and also resulted in the formation of smaller tumors, thus underscoring its importance as a potential drug to be used in combination with alternate strategies to treat gliomas. Although the exact molecular mechanism of Minocycline action remains unclear, it is tempting to speculate a link between TLR signaling and Minocycline in inhibiting glioma-associated microglial MT1-MMP expression and glioma expansion based on recent studies by (Ref).

Future studies that would explore various aspects of TLR signaling in the context of tumor-promoting capabilities could hold a huge promise for clinical studies in human glioma patients. A better understanding of the role of microglial TLRs in glioma growth and invasion could be done by:

1. Isolating tumor-associated microglia/macrophages from TLR1, 2, 6, 7 and 9 KO mice implanted with GL261 tumors by Magnetic Associated Cell Sorting (MACS) and profiling them for MT1-MMP expression (qRT-PCRs, Western blots, activity assays) in comparison to microglia isolated from tumors implanted into wild-type mice.

2. To generate TLR1/2 and TLR2/6 double-knock out mice and investigate the effect of gliomas on MT1-MMP expression in microglia derived from these mice *in vitro* (qRT-PCRs, Western blots, activity assays).

3. To investigate if the heterodimerization of TLR1/2 and/ or TLR2/6 in these double knock-out mice could impact MT1-MMP expression in tumor-associated microglia and overall tumor expansion *in vivo.*

4. To identify putative TLR2 ligand(s) produced by glioma cells that induce MT1-MMP over-expression in microglia using different biochemical and mass spectrometry approaches.

5. To investigate the role of antagonists, small molecule inhibitors, blocking antibodies *in vivo* on the TLR2 signaling pathway as an alternative to immune-based strategies for abrogating tumor-supportive role of microglia.

A major challenge for the future would be to dissect the outcome of TLR stimulation of tumor initiation and progression. But targeting the TLRs is not so straight-forward since activation of TLR signaling may aid tumor development and progression. But harnessing the benefits of TLRs and Minocycline as effective anti-cancer adjuvants could open new avenues in the field of glioma immunotherapy and design of tumor vaccines.

7. SUMMARY

Malignant gliomas are aggressive tumors of the CNS and offer a poor prognosis with a median survival period of 12 months after diagnosis. Gliomas are highly resistant to apoptosis, escape immune surveillance, invade and expand in healthy brain parenchyma. Glioma infiltration is mainly facilitated by brain immune cells or microglia. Soluble factor(s) released by glioma cells attract microglia cells in and around the tumor vicinity to aid glioma diffusion. Infiltration of gliomas into parenchyma is further-more mediated by the action of extracellular matrix degrading enzymes like matrix metalloproteases (MMPs). Metalloproteases are secreted into the extracellular space as inactive pro-forms that are activated by membrane anchored metalloproteases or by autocatalysis. Glioma cells produce pro-MMP2 which is catalytically processed and activated by a membrane anchored MMP called MT1-MMP that is produced by microglia cells. Thus, glioma-associated microglia cells play a pro-neoplastic role by regulating the activity and expression of MT1-MMP via recruitment of Toll-like-receptor adaptor molecule MyD88 and stimulation of p38 MAPK pathway.

<u>Project 1:</u> The exact molecular mechanism by which gliomas induce MyD88 dependent-MT1-MMP over-expression in microglia via TLRs was unknown so far. Here for the first time, it is reported that glioma-released factor(s) induce MT1-MMP over-expression in microglia via the Toll-like receptor 2 (TLR2), possibly in association with its heterodimeric partners TLR1 and/or TLR6. Receptor sub-type specific ligands of TLRs induced variable changes in MT1-MMP gene expression, with TLR1/2 specific ligand Pam3Csk4 inducing the highest upregulation as compared to other agonists. Deletion of TLR2 gene caused an inhibition in MT1-MMP gene over-expression in microglia exposed to glioma conditioned medium (GCM) *in vitro*. Microglia derived from TLR1 and 6 knock-out mice also responded with a reduced MT1-MMP expression after stimulation with GCM. Knock-out of TLR7 and 9 gene loci did not decrease the GCM-induced MT1-MMP expression in other MyD88-dependent TLRs. Most importantly, a significant abrogation in MT1-MMP expression and reduction in tumor expansion were also observed in TLR2 knock-out mice intra-cerebrally implanted with tumors. Further-more, ablation of the TLR2 gene locus also led to a marginal but significant improvement of survival

in glioma-implanted TLR2 KO mice as compared to wild-type mice. These results highlight for the first time the role of TLR2, in association with either or both TLRs 1 and 6, in glioma-induced MT1-MMP over-expression in microglia cells. Targeting the TLR2 pathway could thus offer new insights into the molecular basis of microglia-promoted glioma development and evolution.

Project 2: Pharmacological intervention of microglia-glioma cross-talk using the broad spectrum antibiotic and anti-inflammatory drug Minocycline yielded encouraging results to support the development of anti-tumorigenic strategies to limit glioma invasion and growth. Minocycline, a second-generation derivative of the antibiotic tetracycline, and a blocker of microglia activation and p38 MAPK signaling could effectively abrogate glioma-induced MT1-MMP over-expression and activity in microglia at the mRNA and protein expression levels. When Minocycline was administered to *ex vivo* organotypic brain slice cultures inoculated with glioma cells, a significant decrease in tumor expansion was observed. An effect of Minocycline was not observed in microglia-depleted slices, emphasizing its specific effect on microglia cell sub-population. The *in vivo* oral administration of Minocycline not only led to a reduction in tumor expansion but also caused a decrease in MT1-MMP expression, suggesting that it could serve as a potential drug to target glioma-microglia interaction.

In summary, this study has highlighted the molecular mechanism of glioma-induced MT1-MMP over-expression in microglia via the MyD88-dependent TLR2 signaling pathway and also the promising use of Minocycline as adjuvant therapy to existing treatment modalities of malignant gliomas.

8. ZUSAMMENFASSUNG

Bösartige Gliome sind aggressive Tumore des zentralen Nervensystems (ZNS) und Patienten haben trotz bestmöglicher Behandlung nur eine durchschnittliche Überlebenszeit von 12 Monaten nach der Diagnose. Gliome sind sehr resistent gegen programmierten Zelltod, entziehen sich der Immunabwehr und dringen ins gesunde Hirnparenchym ein, was eine chirurgische Entfernung erschwert. Die Ausbreitung der Gliomzellen wird begünstigt durch die Immunzellen des ZNS – die Mikroglia. Von den Gliomzellen abgesonderte, lösliche Moleküle rekrutieren Mikroglia innerhalb oder in der Nähe des Tumors, welche ihrerseits die Invasion der Gliomzellen in das umliegende Parenchym unterstützen. Die Invasion der Gliomzellen in das Hirnparenchym wird durch die Produktion von Enzymen, welche die extrazelluläre Matrix degradieren, z.B. die Matrix-Metalloproteasen (MMPs), ermöglicht. Sie werden als inaktive Form sezerniert und anschließend aktiviert, entweder durch autokatalytische Spaltung oder via membranverankerte Metalloproteasen. Die von den Gliomzellen abgesonderten pro-MMP2-Moleküle werden durch die Mikroglia-produzierte, membrangebundene MT1-MMP katalytisch prozessiert und aktiviert. Folglich spielen gliom-assoziierte Mikroglia durch die Regulation der Aktivität von MMP2, vermittelt durch die Produktion von MT1-MMP - welche durch die Rekrutierung des Toll-like-Rezeptor Adaptormoleküls MyD88 und die Stimulation des p38 Signalwegs erfolgt, eine tumorfördernde Rolle.

Projekt 1: Der genaue molekulare Mechanismus, durch welchen Gliome die MyD88-abhängige MT1-MMP-Überexpression in Mikroglia durch die TLRs hervorrufen, war bis jetzt nicht bekannt. In dieser Arbeit wird erstmalig gezeigt, dass ein (oder mehrere) von Gliomen produzierter löslicher Faktor die Überexpression von MT1-MMP in Mikroglia über den Toll-like-Rezeptor 2 (TLR2) auslöst, möglicherweise durch die Heterodimerisierung mit TLR1 und/oder TLR6. Liganden, die spezifisch für die Toll-like-Rezeptor-Untertypen sind, induzierten Änderungen der Genexpression von MT1-MMP, wobei der TLR1/2 spezifische Agonist Pam3Csk4 die größte Überexpression bewirkte. Die Deletion des TLR2 Gens verhinderte die MT1-MMP Überexpression in Mikroglia, welche *in vitro* mit gliomkonditioniertem Medium (GKM) behandelt wurden. Mikroglia, welche aus

TLR1 und -6-Knockout-Mäusen isoliert wurden, zeigten ebenfalls eine verringerte Expression von MT1-MMP nach Behandlung mit GKM. Dieser Effekt wurde bei Zellen mit einer Deletion von TLR7 und TLR9 nicht beobachtet.
Ausserdem wurde eine signifikante Reduktion der MT1-MMP Expression und dem Tumorwachstum in TLR2-Knockout-Mäusen festgestellt, die intrazerebral mit Tumoren implantiert wurden. Hinzu kommt, dass die Deletion des TLR2-Gens zu einer geringfügigen, aber signifikanten Verlängerung des Überlebens von tumorimplantierten Knockout-Mäusen im Vergleich zu den Wildtyp-Mäusen führte. Diese Ergebnisse demonstrieren zum ersten Mal die Rolle von TLR2, in Verbindung mit TLR1 und TLR6, in der gliom-induzierten MT1-MMP Überexpression in Mikroglia. Der Fokus auf den TLR2-Signalweg könnte also neue Einblicke in die molekularen Grundlagen der mikroglia-unterstützten Gliomentwicklung und –evolution liefern.

Projekt 2: Pharmakologische Eingriffe in das Zusammenspiel von Mikroglia und Gliome durch den Einsatz von Minocyclin (Breitbandantibiotikum mit antiinflammatorischer Wirkung) lieferten ermutigende Ergebnisse, die für eine Entwicklung von therapeutischen Strategien gegen Gliominvasion und -wachstum anregend sein könnten. Minocyclin, ein Derivat des Antibiotikums Tetracyclin, das die Aktivierung von Mikroglia und spezifisch vom p38 MAPK Signalweg inhibiert, verhinderte die gliominduzierte Überexpression von MT1-MMP in Mikroglia, sowohl auf der mRNA- als auch auf der Proteinebene. Minocyclinbehandlung von *ex vivo* organotypischen Hirnschnitten, in die Gliomzellen injiziert wurden, führte zu einer signifikanten Reduktion der Tumorausbreitung. Dieser Effekt konnte jedoch nicht in Schnitten, die frei von Mikroglia waren, beobachtet werden, was auf einen mikrogliaspezifischen Effekt schließen lässt. Die Minocyclinbehandlung in einem experimentellen Gliommausmodells führte zu einer verringerten Tumorgröße, begleitet durch eine reduzierte MT1-MMP-Expression. Daraus lässt sich schließen, dass Minocyclin als potentielles Medikament eingreifend in das Zusammenspiel von Mikroglia und Gliome fungieren könnte.

Zusammenfassend hebt diese Studie den molekularen Mechanismus der gliominduzierten MT1-MMP Überexpression in Mikroglia mittels den MyD88-abhängigen TLR2 Signalweg hervor und wirft neues Licht auf den möglichen

Einsatz von Minocyclin als Begleitung der existierenden Therapienasätzen von bösartigen Gliomen.

9. REFERENCES

Akashi-Takamura S, Miyake K. 2008. TLR accessory molecules. Curr Opin Immunol 20(4):420-5.

Akira S. 2006. TLR signaling. Curr Top Microbiol Immunol 311:1-16.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413(6857):732-8.

Altman J. 1994. Microglia emerge from the fog. Trends Neurosci 17(2):47-9.

Alvarez JI. 2005. Inhibition of Toll Like Receptor immune responses by microbial pathogens. Front Biosci 10:582-7.

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(3):779-89.

Aravalli RN, Hu S, Lokensgard JR. 2008. Inhibition of toll-like receptor signaling in primary murine microglia. J Neuroimmune Pharmacol 3(1):5-11.

Arroyo DS, Soria JA, Gaviglio EA, Rodriguez-Galan MC, Iribarren P. 2011. Toll-like receptors are key players in neurodegeneration. Int Immunopharmacol 11(10):1415-21.

Asensio VC, Campbell IL. 1999. Chemokines in the CNS: plurifunctional mediators in diverse states. Trends Neurosci 22(11):504-12.

Ashwell K. 1990. Microglia and cell death in the developing mouse cerebellum. Brain Res Dev Brain Res 55(2):219-30.

Ashwell K. 1991. The distribution of microglia and cell death in the fetal rat forebrain. Brain Res Dev Brain Res 58(1):1-12.

Baay M, Brouwer A, Pauwels P, Peeters M, Lardon F. 2011. Tumor cells and tumor-associated macrophages: secreted proteins as potential targets for therapy. Clin Dev Immunol 2011:565187.

Badie B, Schartner J. 2001. Role of microglia in glioma biology. Microsc Res Tech 54(2):106-13.

Badie B, Schartner J, Klaver J, Vorpahl J. 1999. In vitro modulation of microglia motility by glioma cells is mediated by hepatocyte growth factor/scatter factor. Neurosurgery 44(5):1077-82; discussion 1082-3.

Badie B, Schartner J, Prabakaran S, Paul J, Vorpahl J. 2001. Expression of Fas ligand by microglia: possible role in glioma immune evasion. J Neuroimmunol 120(1-2):19-24.

Badie B, Schartner JM. 2000. Flow cytometric characterization of tumor-associated macrophages in experimental gliomas. Neurosurgery 46(4):957-61; discussion 961-2.

Baluk P, Hashizume H, McDonald DM. 2005. Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15(1):102-11.

Banati RB, Gehrmann J, Schubert P, Kreutzberg GW. 1993. Cytotoxicity of microglia. Glia 7(1):111-8.

Bechmann I, Galea I, Perry VH. 2007. What is the blood-brain barrier (not)? Trends Immunol 28(1):5-11.

Belien AT, Paganetti PA, Schwab ME. 1999. Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. J Cell Biol 144(2):373-84.

Benveniste EN. 1997. Cytokines: influence on glial cell gene expression and function. Chem Immunol 69:31-75.

Bissell MJ, Radisky D. 2001. Putting tumours in context. Nat Rev Cancer 1(1):46-54.

Blasco MA. 2005. Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet 6(8):611-22.

Boya J, Calvo J, Prado A. 1979. The origin of microglial cells. J Anat 129(Pt 1):177-86.

Boyd PJ, Doyle J, Gee E, Pallan S, Haas TL. 2005. MAPK signaling regulates endothelial cell assembly into networks and expression of MT1-MMP and MMP-2. Am J Physiol Cell Physiol 288(3):C659-68.

Brooks WH, Horwitz DA, Netsky MG. 1972. Evidence for tumor-specific immune response in patients with primary brain tumors. Surg Forum 23(0):430-2.

Brooks WH, Roszman TL, Mahaley MS, Woosley RE. 1977. Immunobiology of primary intracranial tumours. II. Analysis of lymphocyte subpopulations in patients with primary brain tumours. Clin Exp Immunol 29(1):61-6.

Brown KA. 2001. Factors modifying the migration of lymphocytes across the blood-brain barrier. Int Immunopharmacol 1(12):2043-62.

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(Pt 6):1297-308.

Bryan TM, Cech TR. 1999. Telomerase and the maintenance of chromosome ends. Curr Opin Cell Biol 11(3):318-24.

Bsibsi M, Ravid R, Gveric D, van Noort JM. 2002. Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol 61(11):1013-21.

Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. 2003. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253(1-2):269-85.

Chan WY, Kohsaka S, Rezaie P. 2007. The origin and cell lineage of microglia: new concepts. Brain Res Rev 53(2):344-54.

Chang DK. 2004. [Cancer epigenetics]. Korean J Gastroenterol 44(1):1-12.

Chen GJ, Jeng CH, Lin SZ, Tsai SH, Wang Y, Chiang YH. 2002a. Fetal striatal transplants restore electrophysiological sensitivity to dopamine in the lesioned striatum of rats with experimental Huntington's disease. J Biomed Sci 9(4):303-10.

Chen S, Ferrone FA, Wetzel R. 2002b. Huntington's disease age-of-onset linked to polyglutamine aggregation nucleation. Proc Natl Acad Sci U S A 99(18):11884-9.

Christofori G, Semb H. 1999. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci 24(2):73-6.

Coban C, Ishii KJ, Uematsu S, Arisue N, Sato S, Yamamoto M, Kawai T, Takeuchi O, Hisaeda H, Horii T and others. 2007. Pathological role of Toll-like receptor signaling in cerebral malaria. Int Immunol 19(1):67-79.

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. 2009. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30(7):1073-81.

Constam DB, Philipp J, Malipiero UV, ten Dijke P, Schachner M, Fontana A. 1992. Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. J Immunol 148(5):1404-10.

Creagh EM, O'Neill LA. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol 27(8):352-7.

Cserr HF, DePasquale M, Harling-Berg CJ, Park JT, Knopf PM. 1992. Afferent and efferent arms of the humoral immune response to CSF-administered albumins in a rat model with normal blood-brain barrier permeability. J Neuroimmunol 41(2):195-202.

Cserr HF, Knopf PM. 1992. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. Immunol Today 13(12):507-12.

Daginakatte GC, Gutmann DH. 2007. Neurofibromatosis-1 (Nf1) heterozygous brain microglia elaborate paracrine factors that promote Nf1-deficient astrocyte and glioma growth. Hum Mol Genet 16(9):1098-112.

Dean OM, Data-Franco J, Giorlando F, Berk M. 2012. Minocycline: therapeutic potential in psychiatry. CNS Drugs 26(5):391-401.

DeCarlo CA, Rosa B, Jackson R, Niccoli S, Escott NG, Zehbe I. 2011. Toll-like receptor transcriptome in the HPV-positive cervical cancer microenvironment. Clin Dev Immunol 2012:785825.

Delneste Y, Beauvillain C, Jeannin P. 2007. [Innate immunity: structure and function of TLRs]. Med Sci (Paris) 23(1):67-73.

Dewey RA, Morrissey G, Cowsill CM, Stone D, Bolognani F, Dodd NJ, Southgate TD, Klatzmann D, Lassmann H, Castro MG and others. 1999. Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in

survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. Nat Med 5(11):1256-63.

Dvorak E. 1986. Experimental design for vaccine preparations against human malignant tumors. Med Hypotheses 20(4):429-52.

Eder C, Klee R, Heinemann U. 1998. Involvement of stretch-activated Cl- channels in ramification of murine microglia. J Neurosci 18(18):7127-37.

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(6):526-35.

Evan G, Littlewood T. 1998. A matter of life and cell death. Science 281(5381):1317-22.

Fabry Z, Raine CS, Hart MN. 1994. Nervous tissue as an immune compartment: the dialect of the immune response in the CNS. Immunol Today 15(5):218-24.

Farber K, Kettenmann H. 2005. Physiology of microglial cells. Brain Res Brain Res Rev 48(2):133-43.

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(3):692-701.

Flugel A, Bradl M, Kreutzberg GW, Graeber MB. 2001. Transformation of donor-derived bone marrow precursors into host microglia during autoimmune CNS inflammation and during the retrograde response to axotomy. J Neurosci Res 66(1):74-82.

Frazier JL, Wang PP, Case D, Tyler BM, Pradilla G, Weingart JD, Brem H. 2003. Local delivery of minocycline and systemic BCNU have synergistic activity in the treatment of intracranial glioma. J Neurooncol 64(3):203-9.

Friedberg MH, Glantz MJ, Klempner MS, Cole BF, Perides G. 1998. Specific matrix metalloproteinase profiles in the cerebrospinal fluid correlated with the presence of malignant astrocytomas, brain metastases, and carcinomatous meningitis. Cancer 82(5):923-30.

Friese MA, Steinle A, Weller M. 2004. The innate immune response in the central nervous system and its role in glioma immune surveillance. Onkologie 27(5):487-91.

Fu Y, Zheng S, Huang R, An N, Zheng Y, Zhang Z, Liang A. 2011. A potential strategy for high-grade gliomas: combination treatment with lithium chloride and BmK CT. Biotechnol Lett 34(1):9-17.

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(8):e23902.

Galasso JM, Stegman LD, Blaivas M, Harrison JK, Ross BD, Silverstein FS. 2000. Experimental gliosarcoma induces chemokine receptor expression in rat brain. Exp Neurol 161(1):85-95.

Galea I, Bechmann I, Perry VH. 2007. What is immune privilege (not)? Trends Immunol 28(1):12-8.

Gast A, Bermejo JL, Claus R, Brandt A, Weires M, Weber A, Plass C, Sucker A, Hemminki K, Schadendorf D and others. 2011. Association of inherited variation in

Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS One 6(9):e24370.

Gay NJ, Keith FJ. 1991. Drosophila Toll and IL-1 receptor. Nature 351(6325):355-6.

Gehrmann J, Banati RB. 1995. Microglial turnover in the injured CNS: activated microglia undergo delayed DNA fragmentation following peripheral nerve injury. J Neuropathol Exp Neurol 54(5):680-8.

Gehrmann J, Matsumoto Y, Kreutzberg GW. 1995. Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev 20(3):269-87.

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(6005):841-5.

Goswami S, Gupta A, Sharma SK. 1998. Interleukin-6-mediated autocrine growth promotion in human glioblastoma multiforme cell line U87MG. J Neurochem 71(5):1837-45.

Greene J, Wang M, Liu YE, Raymond LA, Rosen C, Shi YE. 1996. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J Biol Chem 271(48):30375-80.

Guzhova I, Kislyakova K, Moskaliova O, Fridlanskaya I, Tytell M, Cheetham M, Margulis B. 2001. In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Res 914(1-2):66-73.

Haghparast A, Heidari Kharaji M, Malvandi AM. 2011. Down-regulation of CD14 transcripts in human glioblastoma cell line U87 MG. Iran J Immunol 8(2):111-9.

Halfter H, Kremerskothen J, Weber J, Hacker-Klom U, Barnekow A, Ringelstein EB, Stogbauer F. 1998a. Growth inhibition of newly established human glioma cell lines by leukemia inhibitory factor. J Neurooncol 39(1):1-18.

Halfter H, Lotfi R, Westermann R, Young P, Ringelstein EB, Stogbauer FT. 1998b. Inhibition of growth and induction of differentiation of glioma cell lines by oncostatin M (OSM). Growth Factors 15(2):135-47.

Hanahan D, Folkman J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86(3):353-64.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100(1):57-70.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144(5):646-74.

Hanke ML, Kielian T. 2011. Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. Clin Sci (Lond) 121(9):367-87.

Hao C, Parney IF, Roa WH, Turner J, Petruk KC, Ramsay DA. 2002. Cytokine and cytokine receptor mRNA expression in human glioblastomas: evidence of Th1, Th2 and Th3 cytokine dysregulation. Acta Neuropathol 103(2):171-8.

Harmey JH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C, Bouchier-Hayes D, Dong Z. 2002. Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. Int J Cancer 101(5):415-22.

Harris CC. 1996a. p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology, and cancer risk assessment. Environ Health Perspect 104 Suppl 3:435-9.

Harris CC. 1996b. Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. J Natl Cancer Inst 88(20):1442-55.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410(6832):1099-103.

Heil F, Ahmad-Nejad P, Hemmi H, Hochrein H, Ampenberger F, Gellert T, Dietrich H, Lipford G, Takeda K, Akira S and others. 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol 33(11):2987-97.

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303(5663):1526-9.

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(6813):740-5.

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(7 Pt 2):55S-61S.

Hickey JV. 1991. Cerebral circulation demystified. AACN Clin Issues Crit Care Nurs 2(4):657-64.

Hill R, Song Y, Cardiff RD, Van Dyke T. 2005. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell 123(6):1001-11.

Hoffmann A, Kann O, Ohlemeyer C, Hanisch UK, Kettenmann H. 2003. Elevation of basal intracellular calcium as a central element in the activation of brain macrophages (microglia): suppression of receptor-evoked calcium signaling and control of release function. J Neurosci 23(11):4410-9.

Hoffmann O, Braun JS, Becker D, Halle A, Freyer D, Dagand E, Lehnardt S, Weber JR. 2007. TLR2 mediates neuroinflammation and neuronal damage. J Immunol 178(10):6476-81.

Hortega d-R. 1932. Microglia. . Cytology and Cellular Pathology of the Nervous System.

Hotary KB, Allen ED, Brooks PC, Datta NS, Long MW, Weiss SJ. 2003. Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. Cell 114(1):33-45.

Huang B, Zhao J, Shen S, Li H, He KL, Shen GX, Mayer L, Unkeless J, Li D, Yuan Y and others. 2007. Listeria monocytogenes promotes tumor growth via tumor cell toll-like receptor 2 signaling. Cancer Res 67(9):4346-52.

Huettner C, Czub S, Kerkau S, Roggendorf W, Tonn JC. 1997. Interleukin 10 is expressed in human gliomas in vivo and increases glioma cell proliferation and motility in vitro. Anticancer Res 17(5A):3217-24.

Hurley SD, Walter SA, Semple-Rowland SL, Streit WJ. 1999. Cytokine transcripts expressed by microglia in vitro are not expressed by ameboid microglia of the developing rat central nervous system. Glia 25(3):304-9.

Hussain SF, Yang D, Suki D, Grimm E, Heimberger AB. 2006. Innate immune functions of microglia isolated from human glioma patients. J Transl Med 4:15.

Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, Selander KS. 2007. Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro. Prostate 67(7):774-81.

Jacobs VL, Landry RP, Liu Y, Romero-Sandoval EA, De Leo JA. Propentofylline decreases tumor growth in a rodent model of glioblastoma multiforme by a direct mechanism on microglia. Neuro Oncol 14(2):119-31.

Jiang Z, Georgel P, Du X, Shamel L, Sovath S, Mudd S, Huber M, Kalis C, Keck S, Galanos C and others. 2005. CD14 is required for MyD88-independent LPS signaling. Nat Immunol 6(6):565-70.

Johnson GB, Brunn GJ, Tang AH, Platt JL. 2003. Evolutionary clues to the functions of the Toll-like family as surveillance receptors. Trends Immunol 24(1):19-24.

Kaisho T, Akira S. 2003. Regulation of dendritic cell function through toll-like receptors. Curr Mol Med 3(8):759-71.

Kenny EF, O'Neill LA. 2008. Signalling adaptors used by Toll-like receptors: an update. Cytokine 43(3):342-9.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. 2011. Physiology of microglia. Physiol Rev 91(2):461-553.

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(7225):102-6.

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

Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK. 2002. The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 61(3):215-25; discussion 226-9.

Kleihues P, Soylemezoglu F, Schauble B, Scheithauer BW, Burger PC. 1995. Histopathology, classification, and grading of gliomas. Glia 15(3):211-21.

Knopf PM, Cserr HF, Nolan SC, Wu TY, Harling-Berg CJ. 1995. Physiology and immunology of lymphatic drainage of interstitial and cerebrospinal fluid from the brain. Neuropathol Appl Neurobiol 21(3):175-80.

Kobe B, Deisenhofer J. 1995. Proteins with leucine-rich repeats. Curr Opin Struct Biol 5(3):409-16.

Kong Y, Le Y. 2011. Toll-like receptors in inflammation of the central nervous system. Int Immunopharmacol 11(10):1407-14.

Koochekpour S, Jeffers M, Rulong S, Taylor G, Klineberg E, Hudson EA, Resau JH, Vande Woude GF. 1997. Met and hepatocyte growth factor/scatter factor expression in human gliomas. Cancer Res 57(23):5391-8.

Kremlev SG, Roberts RL, Palmer C. 2004. Differential expression of chemokines and chemokine receptors during microglial activation and inhibition. J Neuroimmunol 149(1-2):1-9.

Kreutzberg GW. 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19(8):312-8.

Kriz J, Nguyen MD, Julien JP. 2002. Minocycline slows disease progression in a mouse model of amyotrophic lateral sclerosis. Neurobiol Dis 10(3):268-78.

Langers AM, Verspaget HW, Hawinkels LJ, Kubben FJ, van Duijn W, van der Reijden JJ, Hardwick JC, Hommes DW, Sier CF. 2012. MMP-2 and MMP-9 in normal mucosa are independently associated with outcome of colorectal cancer patients. Br J Cancer.

Lassmann H. 1997. Basic mechanisms of brain inflammation. J Neural Transm Suppl 50:183-90.

Lassmann H, Zimprich F, Vass K, Hickey WF. 1991. Microglial cells are a component of the perivascular glia limitans. J Neurosci Res 28(2):236-43.

Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol 5(2):190-8.

Lawson LJ, Perry VH, Gordon S. 1992. Turnover of resident microglia in the normal adult mouse brain. Neuroscience 48(2):405-15.

Le DM, Besson A, Fogg DK, Choi KS, Waisman DM, Goodyer CG, Rewcastle B, Yong VW. 2003. Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. J Neurosci 23(10):4034-43.

Lee H, Tsygankov AY. 2010. c-Cbl regulates glioma invasion through matrix metalloproteinase 2. J Cell Biochem 111(5):1169-78.

Lehnardt S, Henneke P, Lien E, Kasper DL, Volpe JJ, Bechmann I, Nitsch R, Weber JR, Golenbock DT, Vartanian T. 2006. A mechanism for neurodegeneration induced by group B streptococci through activation of the TLR2/MyD88 pathway in microglia. J Immunol 177(1):583-92.

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(6):973-83.

Leulier F, Lemaitre B. 2008. Toll-like receptors--taking an evolutionary approach. Nat Rev Genet 9(3):165-78.

Levicar N, Nuttall RK, Lah TT. 2003. Proteases in brain tumour progression. Acta Neurochir (Wien) 145(9):825-38.

Levkovitz Y, Levi U, Braw Y, Cohen H. 2007. Minocycline, a second-generation tetracycline, as a neuroprotective agent in an animal model of schizophrenia. Brain Res 1154:154-62.

Ling EA, Wong WC. 1993. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia 7(1):9-18.

Liu A, Sha L, Shen Y, Huang L, Tang X, Lin S. 2012. [Experimental study on anti-metastasis effect of emodin on human pancreatic cancer]. Zhongguo Zhong Yao Za Zhi 36(22):3167-71.

Locasale JW, Melman T, Song S, Yang X, Swanson KD, Cantley LC, Wong ET, Asara JM. 2012. Metabolomics of Human Cerebrospinal Fluid Identifies Signatures of Malignant Glioma. Mol Cell Proteomics.

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(2):97-109.

Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 101(15):5598-603.

Luo J, Solimini NL, Elledge SJ. 2009. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 136(5):823-37.

Luo JL, Maeda S, Hsu LC, Yagita H, Karin M. 2004. Inhibition of NF-kappaB in cancer cells converts inflammation- induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. Cancer Cell 6(3):297-305.

Machado LS, Kozak A, Ergul A, Hess DC, Borlongan CV, Fagan SC. 2006. Delayed minocycline inhibits ischemia-activated matrix metalloproteinases 2 and 9 after experimental stroke. BMC Neurosci 7:56.

Magge SN, Malik SZ, Royo NC, Chen HI, Yu L, Snyder EY, O'Rourke DM, Watson DJ. 2009. Role of monocyte chemoattractant protein-1 (MCP-1/CCL2) in migration of neural progenitor cells toward glial tumors. J Neurosci Res 87(7):1547-55.

Mahaley MS, Jr., Gentry RE, Bigner DD. 1977. Immunobiology of primary intracranial tumors. J Neurosurg 47(1):35-43.

Maiese K. 2011. The Many Facets of Cell Injury: Angiogenesis to Autophagy. Curr Neurovasc Res.

Marin-Teva JL, Dusart I, Colin C, Gervais A, van Rooijen N, Mallat M. 2004. Microglia promote the death of developing Purkinje cells. Neuron 41(4):535-47.

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(9):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(30):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(4):624-8.

Marques R, Boneca IG. 2011. Expression and functional importance of innate immune receptors by intestinal epithelial cells. Cell Mol Life Sci 68(22):3661-73.

Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 171(6):3154-62.

Matzinger P. 2002a. The danger model: a renewed sense of self. Science 296(5566):301-5.

Matzinger P. 2002b. An innate sense of danger. Ann N Y Acad Sci 961:341-2.

Medawar PB. 1948. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 29(1):58-69.

Medzhitov R. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol 1(2):135-45.

Medzhitov R, Janeway CA, Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91(3):295-8.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388(6640):394-7.

Mentlein R, Hattermann K, Held-Feindt J. 2012. Lost in disruption: Role of proteases in glioma invasion and progression. Biochim Biophys Acta 1825(2):178-85.

Merlo A, Bettler B. 2004. Glioblastomas on the move. Sci STKE 2004(229):pe18.

Mishra BB, Gundra UM, Teale JM. 2009. Toll-like receptors in CNS parasitic infections. Curr Top Microbiol Immunol 336:83-104.

Miyaoka T. 2008. Clinical potential of minocycline for schizophrenia. CNS Neurol Disord Drug Targets 7(4):376-81.

Miyaoka T, Wake R, Furuya M, Liaury K, Ieda M, Kawakami K, Tsuchie K, Taki M, Ishihara K, Araki T and others. 2012. Minocycline as adjunctive therapy for patients with unipolar psychotic depression: An open-label study. Prog Neuropsychopharmacol Biol Psychiatry 37(2):222-6.

Mohanam S, Wang SW, Rayford A, Yamamoto M, Sawaya R, Nakajima M, Liotta LA, Nicolson GL, Stetler-Stevenson WG, Rao JS. 1995. Expression of tissue inhibitors of metalloproteinases: negative regulators of human glioblastoma invasion in vivo. Clin Exp Metastasis 13(1):57-62.

Monnet-Tschudi F, Defaux A, Braissant O, Cagnon L, Zurich MG. 2011. Methods to assess neuroinflammation. Curr Protoc Toxicol Chapter 12:Unit12 19.

Moore S, Thanos S. 1996. The concept of microglia in relation to central nervous system disease and regeneration. Prog Neurobiol 48(4-5):441-60.

Munshi HG, Wu YI, Mukhopadhyay S, Ottaviano AJ, Sassano A, Koblinski JE, Platanias LC, Stack MS. 2004. Differential regulation of membrane type 1-matrix metalloproteinase activity by ERK 1/2- and p38 MAPK-modulated tissue inhibitor of metalloproteinases 2 expression controls transforming growth factor-beta1-induced pericellular collagenolysis. J Biol Chem 279(37):39042-50.

Murphy GM, Jr., Bitting L, Majewska A, Schmidt K, Song Y, Wood CR. 1995. Expression of interleukin-11 and its encoding mRNA by glioblastoma cells. Neurosci Lett 196(3):153-6.

Nagase H, Woessner JF, Jr. 1999. Matrix metalloproteinases. J Biol Chem 274(31):21491-4.

Nagy JA, Chang SH, Shih SC, Dvorak AM, Dvorak HF. 2010. Heterogeneity of the tumor vasculature. Semin Thromb Hemost 36(3):321-31.

Negrini S, Gorgoulis VG, Halazonetis TD. 2010. Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol 11(3):220-8.

Nikodemova M, Duncan ID, Watters JJ. 2006. Minocycline exerts inhibitory effects on multiple mitogen-activated protein kinases and IkappaBalpha degradation in a stimulus-specific manner in microglia. J Neurochem 96(2):314-23.

Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308(5726):1314-8.

Nuti E, Casalini F, Santamaria S, Gabelloni P, Bendinelli S, Da Pozzo E, Costa B, Marinelli L, La Pietra V, Novellino E and others. 2011. Synthesis and biological evaluation in U87MG glioma cells of (ethynylthiophene)sulfonamido-based hydroxamates as matrix metalloproteinase inhibitors. Eur J Med Chem 46(7):2617-29.

O'Neill LA. 2006. How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol 18(1):3-9.

Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV. 2009. Toll-like receptors in neurodegeneration. Brain Res Rev 59(2):278-92.

Okun E, Griffioen KJ, Mattson MP. 2011. Toll-like receptor signaling in neural plasticity and disease. Trends Neurosci 34(5):269-81.

Olofsson A, Miyazono K, Kanzaki T, Colosetti P, Engstrom U, Heldin CH. 1992. Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. J Biol Chem 267(27):19482-8.

Olson JK, Miller SD. 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 173(6):3916-24.

Pae CU, Marks DM, Han C, Patkar AA. 2008. Does minocycline have antidepressant effect? Biomed Pharmacother 62(5):308-11.

Papavasiliou AK, Mehler MF, Mabie PC, Marmur R, Qingbin S, Keating RF, Kessler JA. 1997. Paracrine regulation of colony-stimulating factor-1 in medulloblastoma: implications for pathogenesis and therapeutic interventions. Neurosurgery 41(4):916-23.

Perry VH. 1994. Modulation of microglia phenotype. Neuropathol Appl Neurobiol 20(2):177.

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

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(5396):2085-8.

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(6):731-42.

Prat E, Baron P, Meda L, Scarpini E, Galimberti D, Ardolino G, Catania A, Scarlato G. 2000. The human astrocytoma cell line U373MG produces monocyte chemotactic protein (MCP)-1 upon stimulation with beta-amyloid protein. Neurosci Lett 283(3):177-80.

Prinz M, Kann O, Draheim HJ, Schumann RR, Kettenmann H, Weber JR, Hanisch UK. 1999. Microglial activation by components of gram-positive and -negative bacteria: distinct and common routes to the induction of ion channels and cytokines. J Neuropathol Exp Neurol 58(10):1078-89.

Raithatha SA, Muzik H, Rewcastle NB, Johnston RN, Edwards DR, Forsyth PA. 2000. Localization of gelatinase-A and gelatinase-B mRNA and protein in human gliomas. Neuro Oncol 2(3):145-50.

Raivich G. 2005. Like cops on the beat: the active role of resting microglia. Trends Neurosci 28(11):571-3.

Rakoff-Nahoum S, Medzhitov R. 2007. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science 317(5834):124-7.

Reese TS, Karnovsky MJ. 1967. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol 34(1):207-17.

Risau W, Wolburg H. 1990. Development of the blood-brain barrier. Trends Neurosci 13(5):174-8.

Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. 1998. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 95(2):588-93.

Roggendorf W, Strupp S, Paulus W. 1996. Distribution and characterization of microglia/macrophages in human brain tumors. Acta Neuropathol 92(3):288-93.

Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M. 2007. Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol 9(9):1081-8.

Romo-Gonzalez T, Chavarria A, Perez HJ. 2012. Central nervous system: A modified immune surveillance circuit? Brain Behav Immun.

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(1):186-92.

Schweitzer T, Vince GH, Herbold C, Roosen K, Tonn JC. 2001. Extraneural metastases of primary brain tumors. J Neurooncol 53(2):107-14.

Seabrook TJ, Jiang L, Maier M, Lemere CA. 2006. Minocycline affects microglia activation, Abeta deposition, and behavior in APP-tg mice. Glia 53(7):776-82.

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(16):7438-42.

Seukeran DC, Eady EA, Cunliffe WJ. 1997. Benefit-risk assessment of acne therapies. Lancet 349(9060):1251-2.

Shay JW, Wright WE. 2000. Implications of mapping the human telomerase gene (hTERT) as the most distal gene on chromosome 5p. Neoplasia 2(3):195-6.

Shen B, Zheng MQ, Xu XY, Mo FG, Zhang T, Feng JF. 2012. [Expression of MT1-MMP and RECK protein in human gastric carcinoma]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 25(5):364-7.

Sheyhidin I, Nabi G, Hasim A, Zhang RP, Ainiwaer J, Ma H, Wang H. 2011. Overexpression of TLR3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma. World J Gastroenterol 17(32):3745-51.

Sporn MB. 1996. The war on cancer. Lancet 347(9012):1377-81.

Stan AC, Walter GF, Welte K, Pietsch T. 1994. Immunolocalization of granulocyte-colony-stimulating factor in human glial and primitive neuroectodermal tumors. Int J Cancer 57(3):306-12.

Stark AM, Nabavi A, Mehdorn HM, Blomer U. 2005. Glioblastoma multiforme-report of 267 cases treated at a single institution. Surg Neurol 63(2):162-9; discussion 169.

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

Streit WJ, Conde JR, Fendrick SE, Flanary BE, Mariani CL. 2005. Role of microglia in the central nervous system's immune response. Neurol Res 27(7):685-91.

Streit WJ, Graeber MB, Kreutzberg GW. 1988. Functional plasticity of microglia: a review. Glia 1(5):301-7.

Streit WJ, Kincaid-Colton CA. 1995. The brain's immune system. Sci Am 273(5):54-5, 58-61.

Strik HM, Stoll M, Meyermann R. 2004. Immune cell infiltration of intrinsic and metastatic intracranial tumours. Anticancer Res 24(1):37-42.

Suk K. 2004. Minocycline suppresses hypoxic activation of rodent microglia in culture. Neurosci Lett 366(2):167-71.

Suzumura A, Sawada M, Yamamoto H, Marunouchi T. 1993. Transforming growth factor-beta suppresses activation and proliferation of microglia in vitro. J Immunol 151(4):2150-8.

Takahashi K, Yamamura F, Naito M. 1989. Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. J Leukoc Biol 45(2):87-96.

Takeda K, Akira S. 2003. Toll receptors and pathogen resistance. Cell Microbiol 5(3):143-53.

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(4):443-51.

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(1):10-4.

Town T, Nikolic V, Tan J. 2005. The microglial "activation" continuum: from innate to adaptive responses. J Neuroinflammation 2:24.

Tsan MF, Gao B. 2004. Endogenous ligands of Toll-like receptors. J Leukoc Biol 76(3):514-9.

Tsan MF, Gao B. 2007. Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors. J Endotoxin Res 13(1):6-14.

Verma RP, Hansch C. 2007. Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. Bioorg Med Chem 15(6):2223-68.

Villeneuve J, Tremblay P, Vallieres L. 2005. Tumor necrosis factor reduces brain tumor growth by enhancing macrophage recruitment and microcyst formation. Cancer Res 65(9):3928-36.

Wagner S, Czub S, Greif M, Vince GH, Suss N, Kerkau S, Rieckmann P, Roggendorf W, Roosen K, Tonn JC. 1999. Microglial/macrophage expression of interleukin 10 in human glioblastomas. Int J Cancer 82(1):12-6.

Wang X, Zhu S, Drozda M, Zhang W, Stavrovskaya IG, Cattaneo E, Ferrante RJ, Kristal BS, Friedlander RM. 2003. Minocycline inhibits caspase-independent and -dependent mitochondrial cell death pathways in models of Huntington's disease. Proc Natl Acad Sci U S A 100(18):10483-7.

Watters JJ, Schartner JM, Badie B. 2005. Microglia function in brain tumors. J Neurosci Res 81(3):447-55.

Watters TM, Kenny EF, O'Neill LA. 2007. Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. Immunol Cell Biol 85(6):411-9.

Weinberg RA. 1995. Prospects for cancer genetics. Cancer Surv 25:3-12.

Weingart JD, Sipos EP, Brem H. 1995. The role of minocycline in the treatment of intracranial 9L glioma. J Neurosurg 82(4):635-40.

Wekerle H. 2002. Immune protection of the brain--efficient and delicate. J Infect Dis 186 Suppl 2:S140-4.

Werb Z. 1997. ECM and cell surface proteolysis: regulating cellular ecology. Cell 91(4):439-42.

Woessner JF, Jr. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5(8):2145-54.

Wu CH, Wen CY, Shieh JY, Ling EA. 1994. Down-regulation of membrane glycoprotein in amoeboid microglia transforming into ramified microglia in postnatal rat brain. J Neurocytol 23(4):258-69.

Xie Q, Bradley R, Kang L, Koeman J, Ascierto ML, Worschech A, De Giorgi V, Wang E, Kefene L, Su Y and others. 2011. Hepatocyte growth factor (HGF) autocrine activation predicts sensitivity to MET inhibition in glioblastoma. Proc Natl Acad Sci U S A 109(2):570-5.

Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol 4(11):1144-50.

Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, Hieny S, Sutterwala FS, Flavell RA, Ghosh S and others. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science 308(5728):1626-9.

Yong VW, Krekoski CA, Forsyth PA, Bell R, Edwards DR. 1998. Matrix metalloproteinases and diseases of the CNS. Trends Neurosci 21(2):75-80.

Yong VW, Wells J, Giuliani F, Casha S, Power C, Metz LM. 2004. The promise of minocycline in neurology. Lancet Neurol 3(12):744-51.

Yu L, Wang L, Chen S. 2011. Exogenous or endogenous Toll-like receptor ligands: which is the MVP in tumorigenesis? Cell Mol Life Sci 69(6):935-49.

Zhai H, Heppner FL, Tsirka SE. 2011. Microglia/macrophages promote glioma progression. Glia 59(3):472-85.

Zheng LD, Yang CL, Qi T, Qi M, Tong L, Tong QS. 2012. Effects of resistin-like molecule beta over-expression on gastric cancer cells in vitro. World J Gastroenterol 18(8):754-66.

Zielasek J, Muller B, Hartung HP. 1996. Inhibition of cytokine-inducible nitric oxide synthase in rat microglia and murine macrophages by methyl-2,5-dihydroxycinnamate. Neurochem Int 29(1):83-7.

CURRICULUM VITAE

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

PUBLICATIONS

Markovic DS, Vinnakota K*, et al.

Gliomas induce and exploit microglial MT1-MMP expression for tumor expansion.

(* second author)

(Accepted, Proceedings of National Academy of Sciences, 2009)

Markovic DS*, **Vinnakota K***, et al.

Minocycline reduces glioma expansion and invasion by attenuating microglial MT1-MMP expression. (* equal contribution)

(Accepted, Brain, Behavior and Immunity, 2011)

Sielska M, Vinnakota K*, et al.

Tumor-derived granulocyte-macrophage colony stimulating factor drives microglia/macrophage infiltration and glioma progression. (* co-author on the paper)

(Manuscript under revision, Journal of Pathology)

Vinnakota K*, Hu F, Ku MC et al.

Toll-like-receptor 2 mediates microglia/brain macrophage MT1-MMP expression and glioma expansion. (* first author)

(Manuscript submitted)

MEETINGS AND PRESENTATIONS

TALKS

Vinnakota K*, Markovic DS, et al.

MT1-MMP expression in microglia facilitates glioma expansion and invasion.

MDC-FMP PhD retreat, September 2009, Berlin, Germany.

Vinnakota K*, Hu F, et al.

TLR signaling mediates glioma-induced MT1-MMP expression in microglia.

Berlin Brain Days, December 2011, Berlin, Germany.

POSTERS

Markovic DS, **Vinnakota K**, et al.

MT1-MMP expression in microglia facilitates glioma expansion and invasion.

Brain Tumor Meet, June 2008, Berlin, Germany.

Vinnakota K, Markovic DS, Glass R and Kettenmann H.

Minocycline attenuates the microglia-assisted glioma expansion and invasion.

Berlin Brain Days, Nov 1-3 2010, Berlin, Germany.

Vinnakota K, Markovic DS, Glass R and Kettenmann H.

Microglia-assisted glioma invasion and expansion is attenuated by Minocycline.

40th Neuroscience Congress (SfN), Nov 11-18 2010, USA.

Vinnakota K, Markovic DS, Glass R and Kettenmann H.

Minocycline attenuates the microglia-assisted glioma expansion and invasion.

Ninth Gottingen Meeting of German Neuroscience Society, Mar 2011, Göttingen, Germany.

Vinnakota K, Markovic DS, Glass R and Kettenmann H.

TLR signaling induces glioma-associated MT1-MMP expression in microglia.

Brain Tumor Meet, June 2011, Berlin, Germany.

ERKLÄRUNG

„Ich, Katyayni Vinnakota, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „*The role of microglia in glioma expansion and progression*“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, 16.05.2012 Katyayni Vinnakota