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Paul Triller aus Berlin

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Summary

Gliomas are the most frequent brain tumors and are characterized by a poor prognosis despite extensive research in recent years. Microglia constitute the main population of immune cells in the central nervous system and ensure the compliance of homeostasis under physiologic conditions but they have been found to play a pivotal role in tumor progression. After tumors recruit microglia and peripheral macrophages to the tumor bulk, glioma cells induce a phenotype switch. These glioma-associated microglia/macrophages (GAMs) contribute up to 50 % to the tumor bulk. The polarization includes the upregulation of microglial matrix metalloproteases (MMPs) via TLR2 activation. MMP2, MMP9 and MMP14 amongst others catalyze the destruction of the extracellular matrix in the CNS, a process that favors glioma invasiveness.
3-D structural analysis of TLR2 revealed a loop within an intracellular adapter domain that is essential for consecutive signaling and can be blocked by o-vanillin due to its molecular shape.
We used o-vanillin to inhibit the glioma-induced TLR2 activation and reduce the overexpression of glioma promoting MMPs in microglia/macrophages. An o-vanillin treatment significantly mitigated glioma growth in murine brain slice cultures, and it reduced the expression of tumor-supporting genes in murine microglia and the MMP RNA levels of monocytes isolated from fresh human glioma samples.
Our findings suggest a specific inhibition of glioma essential TLR2 signaling by o-vanillin in glioma-associated microglia/macrophages that makes it a promising candidate for a complementary targeted glioma treatment.

Zusammenfassung

Gliome sind die häufigsten Tumore des zentralen Nervensystems und trotz intensiver Forschung bleibt die Prognose düster. Als Hauptgrund hierfür stellte sich das bereits früh extrem invasive Wachstum der Gliome heraus, zu dem Mikroglia maßgeblich beitragen. Mikroglia sind Zellen des angeborenen Immunsystems und wahren unter physiologischen Umständen als „Wächter des Gehirns" in diesem immunprivilegierten Raum den Status quo. Doch nach intensiver Rekrutierung durch Turmorzellen wird ein Phänotyp-Wechsel in den Immunzellen über eine Toll-like-Rezeptor 2 (TLR2) Aktivierung forciert, woraus unter anderem die vermehrte Sekretion von Matrix-Metallo-Proteasen (MMPs) resultiert. Diese Enzyme katalysieren den Abbau der extrazellulären Matrix um den Tumor und ermöglichen dessen Invasion ins umliegende Parenchym.
Durch Strukturanalysen von TLR2 wurde eine intrazelluläre Domäne identifiziert, deren Blockade durch das Molekül o-vanillin die konsekutive Signalvermittlung unterbindet.
Wir konnten zeigen, dass o-vanillin das Wachstum von Gliomen in Gehirnschnittkulturen, die gliom-induzierte Überexpression von MMP9 und MMP14 in murinen und humanen GAMs und die Proliferation von tumorassoziierten Mikroglia/Makrophagen signifikant reduziert.

Zusammenfassend halten wir o-vanillin aufgrund seiner molekularen Eigenschaften als selektiver Inhibitor eines wichtigen Rezeptors in der Gliom-Mikroglia/Makrophagen-Interaktion für eine vielversprechende potentielle Erweiterung bestehender Therapieregime in der Gliombehandlung.

Introduction

Definition

Microglia are the innate immune cells of the central nervous system [2] and account for 5% of all cells in the human brain [3, 4]. Their primary function as yolk sack derived monocytes in an immune privileged environment is the maintenance of a state of homeostasis by continuously screening the entire brain for emerging irregularities. In the case of stimuli-detection, microglia can launch a broad spectrum of immune responses ranging from inflammatory to immune modulatory, depending on the identified pathogen [5-7]. Recent studies demonstrate that microglial cells also contribute to more complex processes. They modulate neuroplasticity as well as the formation of neurons in the developing brain and are involved in the pathology of complicated and yet hardly understood neurodegenerative diseases such as Alzheimer and Schizophrenia [8-10].

History

Rudolf Virchow, in 1856 was the first person to mention glia cells as a cell population distinct from neurons. He referred to the ancient Greek term "γλια", which translates as "glue" or "plaster" [11]. At this point it was not clear whether glia cells represent an independent cell population or rather consist of a fibrillar matrix with embedded, independent, solitary nuclei. The dispute about the nature of glia continued into the early 20th century, when Pio del Río Hortega first distinguished between astrocytes, oligodendrocytes and microglia, introducing a concept of glial cells that remains valid until today.

As early as 1919, Hortega postulated pioneering axioms of microglial attributes that were way ahead of his time. He claimed that microglial cells enter the brain in early stages of development in an amoeboid shape originating from the mesoderm and invade the central nervous system using blood vessels and white matter tracks as guiding structures. Universally distributed throughout the whole brain, they change to a ramified morphology after arriving from the periphery. When in contact with pathogens, microglial cells transform into an amoeboid shape that resembles their morphology in early development and gain the abilities to migrate, proliferate and phagocyte [12, 13]. Valid until today, the only hypothesis that needed to be revised was the one about the origin of microglia. However, due to various apparent similarities between microglia and other macrophage populations, Hortega´s idea of a myeloid mesodermal origin was strengthened and accepted as a doctrine in microglia research during the following decades. Admittedly, this was a research area that was considered a subject of limited interest [14]. In the following 70 years the

importance of microglia slowly started to emerge and the discussion whether microglial cells are mesodermal macrophages that invade the brain during early development as Hortega suggested or instead are derived from circulating monocytes that infiltrate the brain as claimed by other scientists in the second half of the 20th century flared up again [15].

Origin of Microglia

Despite the pleasure that the scientific controversy about those two ideas of "*The Origin of Microglia*" provided, it was Alliot et al. in 1999 who discovered that already at embryonic day (ED) 8 highly proliferative microglia precursor cells populate the murine primordium of the brain and thereby set a suggestive scene for the dispute [16, 17]. These findings forced researchers to investigate microglia further in the context of embryonic hematopoiesis. The review by Orkin and Zon highlights that the activity of hematopoietic stem cells (HSC) as the source of all individual hematopoietic lineages including macrophages starts at ED 11 and takes place in the fetal liver, three days after microglia have already been verified in the rudiment brain [18]. In elegant fate mapping studies using chimeric animals, yolk sac progenitor cells could be determined as the primary source of microglia in the brain and subsequently, primitive myeloid monocytes were excluded as contributors to the population of central nervous immune cells under physiological conditions [5]. It took 100 years to revise Hortega' s postulate, but finally, the question about the origin of microglia could be answered. Prinz et al. illustrate the exact states of microglia establishment in the early stages of brain development [19].

Microglia Function

The understanding of microglial functions in the central nervous systems has deepened in recent years. While Hortega postulated that microglia, similar to tissue macrophages, are above all part of inflammatory immune responses in the case of pathogen appearance, today scientific consensus considers microglia to fulfill a role as guardians of brain homeostasis [13, 20]. Microglia continuously scan the whole brain with extremely motile and highly ramified processes without disturbing neuronal networks to ensure this state of homeostasis and distinguish between pathological and physiological conditions [21]. Microglia know what is going on in the brain, anywhere and at any time

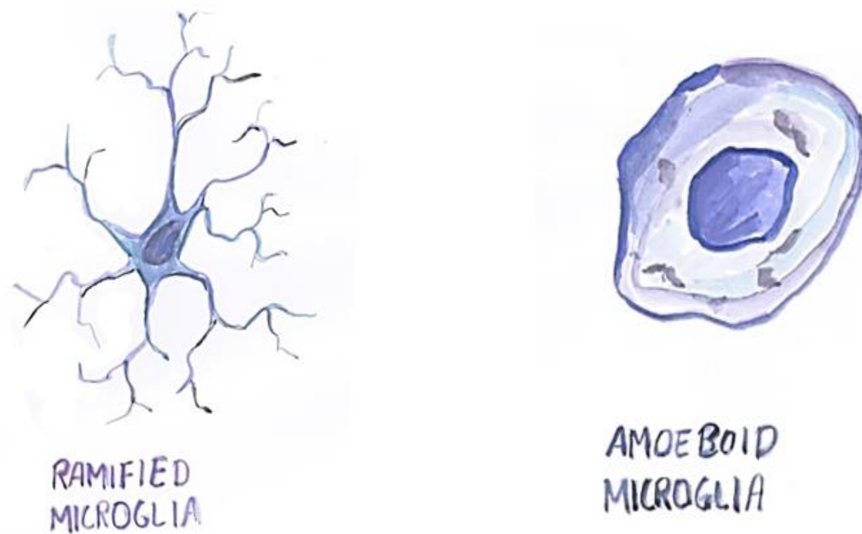


Figure 1. **Schematic depiction of a resting microglial cell A) and a microglial cell in an activated state B).** While resting, a microglia cell is histologically characterized by long, motile processes and small cell somata with dense nuclei A). The cells turn into an amoeboid shape after pathogen contact. This amoeboid shape features an increased ratio of nucleus to soma.

These observations make it necessary to revise the idea of ramified microglia as resting, inactive cells. Microglia move relentlessly and track everything that does not work as it is supposed to. Coverage of the whole brain is ensured by a ubiquitous distribution of microglial cells in a mostly non-overlapping fashion throughout every part of the encephalon. Each cell covers a volume of 50 000 μm^3 [22, 23]. If harmful stimuli are detected, the highly plastic ramified cells shift into an activated amoeboid state and migrate towards the origin of the disturbance. Equipped with a variety of receptors, microglia cover a broad spectrum of recognizable patterns that range from calcium currents to components of bacterial cell walls [19]. Microglia change their phenotype to adapt to specific irregularities, a process that is called polarization.

In the early years of intensified microglia research, it was hypothesized that there is a limited range of microglial activation status, above all the monophasic either-or concept of an M1 or M2 polarization [24]. In this dated concept, the M1 polarization state represents a tissue macrophage resembling a response to pathogens after infection or injury characterized by an increased expression of pro-inflammatory cytokines and phagocytic activity. The M2 polarization, in contrast, followed the initial destructive M1 response and was characterized by its positive and remodeling influence to reestablish neuronal function after cellular damage or disease [24].

But while the monophasic M1/M2 model of microglia activation is a suitable instrument to investigate microglia functions in a limited artificial in vitro environment, especially to quantify its immune response

capacities, the model reaches its limits as soon as processes become more complex within the development of neurologic or psychiatric diseases that manifest organically, e.g. stroke, gliomas or depression [24-29]. The augmentation of knowledge about pathogenesis and the mechanisms of these diseases demands a revision of microglia phenotyping.

Today we assume that microglial reactions to disturbances in the homeostasis of the brain and the concomitant phenotype changes are a result of the sum of processed stimuli. The presence or absence of specific pathogens defines the microglial polarization, creating an immense number of possible phenotypes characterized by an extreme functional variety adapted to particular situations and necessities [6, 30].

In the case of infection, microglia react by secretion of different cytokines: Interleukin-1 (IL-1), TNF-α, IL-6 and transforming growth factor-β1 (TGFβ1), chemokines (such as CXCL8, CCL2 and CCL5) and components of the complement system, that initiate and promote an inflammatory response while secreted enzymes like iNOS (nitric oxide synthetase) directly expose pathogens to cellular stress and induce apoptosis. Microglia cells also express adhesion molecules to recruit and accumulate other immune cells and upregulate the expression of receptors that increase their phagocytic activity [31].

In contrast to destructive responses after infections, microglia can also regulate damage and restructure tissue after harmful events. If they detect cell debris or myelin, e.g., after demyelination in acute multiple sclerosis lesions or apoptosis during inflammation, their reaction is tolerogenic phagocytosis and the downregulation of proinflammatory cytokines to prevent further brain damage [32, 33]. One recently discovered function of microglia in the developing brain is the support of neurogenesis and neuroprotection via interferon-γ (INF-γ) and IL-4, cytokines that are associated with the activity of modulating T helper cells [20]. They also influence synaptic pruning and the early vascularization of the brain, and they participate in the removal of the large numbers of apoptotic cells that accumulate in early stages of neurodevelopment [34].

Gliomas

Introduction

Glioblastomas (GBM) are poorly differentiated malignant glial tumors that remain incurable despite various attempts including surgery, irradiation and chemotherapy. They are characterized by a relatively high incidence, rapid progression and an aggressive infiltrative growth into surrounding healthy tissue [35, 36]. This aspect remains a significant problem in the therapy of GBMs, considering that patients´ prognosis still depends to a large extent on an optimal surgical resection. Moreover, even though the median survival can be prolonged from 3 months without therapy to more than 20 months, there has been no breakthrough in glioma therapy [37-40]. The WHO efforts to include molecular markers such as the MGMT methylation status or the IDH1 phenotype, both crucial prognostic factors, into the conventional histological

classification of gliomas in 2016 reflect the enormous gain of knowledge and simultaneously illustrate the absence of an appropriate curative therapeutic approach [41-43].

Origin of Gliomas

In a healthy brain, every cell has a particular function that is always accompanied by a high level of differentiation and inversely correlated with the cell's potential to proliferate. Neuronal stem cells (NSC) sit at the apex of the cellular hierarchy in proliferative niches and have the potential to generate all neuroepithelial lineages by developing into precursor cells that can differentiate into glial cells or neurons. The inverse correlation between proliferation and differentiation is partly decoupled in gliomas, which results in highly proliferative cells that resemble differentiated neuroepithelial cells. This explains one main characteristic of gliomas: their large variety in morphological appearance. High-grade gliomas can resemble astrocytes, oligodendrocytes and even hybrids of both cell populations [41, 44, 45].

Two main theories about the origin of gliomas coexist nowadays: the cell-of-origin and the cell-of-mutation theory. Both theories postulate that specific mutations in tumor suppressor genes and oncogenes are required to induce a malignant change by the upregulation of glioma promoting pathways. A prominent example is the RTK/RAS/PI-3-K pathway. Malfunctions can include alterations in growth receptors such as EGFR, ERB2, PDGFRA, the tumor suppressor genes NF1 and PTEN or dysfunction of regulatory processes concerning the cell cycle controlling genes p53 or RB1. Though both theories postulate the same genetic aberrations as requirements for the development of gliomas, mainly in the genes named above that control the cell cycle, the secretion of growth factors or suitable receptors that confine cell division, they differ about which cell is affected first [46-48].

The cell-of-origin theory is an appealing attempt that explains all GBM characteristics with a small population of malignant cancer stem cells (CSCs) descending from mutated neuronal stem cells (NSCs). These are capable of self-renewal, proliferation and differentiation into multiple neuroectodermal lineages, which, in the case of specific mutations, leads to malignancies [49].

The second hypothesis anticipates further differentiated cells, especially astrocytes and oligodendrocyte progenitor cells, to be the source of gliomas. It was published that specific mutations in cancer-driving genes could induce dedifferentiation and proliferation in these usually dormant cells [23, 50]. Nevertheless, completely differentiated cells such as astrocytes do not transform immediately even if the requirements are met. They instead acquire lineage-restricted abilities and turn malignant over time [51].

Lately, striking evidence supporting the cell-of-origin theory has emerged. Lee et al. identified astrocyte-like NSCs in the subventricular zone (SVZ) that carry glioma driving mutations and evolve clonally to high-grade malignancies after migration to distant brain regions [52].

Glioma Characteristics

The glioblastoma multiforme, as the name suggests, is a tumor characterized by its genetic and morphological heterogeneity. Though gliomas share some vital characteristics with other malignancies, including the sustainment of proliferative signaling, the evasion of immune system and growth suppressors, resistance to cell death, replicative immortality and the induction of angiogenesis, summarized as hallmarks of cancer [53, 54], we want to focus on a different quality: their aggressive invasiveness - a characteristic predetermined for interventional approaches.

In contrast to most malignancies, gliomas do not metastasize, via either intravascular or lymphatic routes. The tumors instead infiltrate the surrounding brain parenchyma around the tumor core through the extracellular space [55].

Two necessary prerequisites need to be met to accomplish directed movement in a dense matrix: first, the synchronous activity of adhesion of the leading cell edge and detachment of the trailing end and second, the remodeling of the extracellular matrix (ECM). The degradation of the ECM is performed by matrix metalloproteases (MMP), an enzyme class we will specify later on.

This particular migratory behavior results in early glioma satellites and renders a complete surgical resection impossible [36, 56].

Glioma Classification

The urge to classify malignancies of the central nervous system dates back to 1926. Bailey et al. created a system to categorize gliomas on a histogenetic basis and even correlated the patients´ prognosis with tumor histology [57]. The classification was further extended by Nils Ringer, who introduced a grading system for astrocytoma. He referred to cellular anaplasias defined by histological criteria such as pleomorphism, cellularity and frequency of mitosis [58].

The modern glioma classification was introduced by the WHO in 1979 and revised four times, last in 2016. The last revision adds a spectrum of well-established molecular markers to the previous purely histologic classification [41].

A refinement of diagnostic criteria became mandatory due to two relevant limitations of the previous grading system. Firstly, because histological assessments are subjective processes, facing significant interobserver variabilities. Secondly, because it lacked precision in giving patients´ prognosis [59]. The lack of actual prognostic probability indicates that previous criteria were fragmentary. As a result, the WHO included genetic aberrations to improve prognostic accuracy and address an individualized therapy regime. Here we introduce three genetic parameters with a high impact on clinical practice.

Astrocytic/Oligodendroglial Tumors	Tumor Grade
Pilocytic Astrocytoma	I
Diffuse Astrocytoma *IDH-mutant/-wt; NOS*	II
Anaplastic Astrocytoma *IDH-mutant/-wt; NOS*	III
Oligodendroglioma *IDH-mutant and 1q/19p-codeleted; NOS*	II
Anaplastic Oligodendroglioma *IDH-mutant and 1q/19p-codeleted; NOS*	III
Glioblastoma *IDH-mutant/-wt; NOS*	IV
Oligoastrocytoma, NOS	II
Anaplastic Oligoastrocytoma, NOS	III

Table 1. **Exemplary overview of WHO 2016 Glioma classification**

Summary of the most prominent glioma entities sorted by their histological phenotype, molecular markers and tumor grade.

The MGMT Promoter Methylation

One ability of gliomas that complicates adjuvant chemotherapy is their partially strongly pronounced resistance to alkylating agents. The O^6-methylguanine-DNA methyltransferase (MGMT) gene encodes the MGMT DNA-repair enzyme that annuls the guanine alkylation by the alkylating chemotherapy temozolomide (TMZ), the first line chemotherapy treating gliomas, in a self-consuming process. The epigenetic silencing of the MGMT promoter region by methylation is a favorable prognostic marker in 40% of gliomas and is associated with tumor regression and prolonged survival [42, 60].

The IDH Mutation

The isocitrate dehydrogenase (IDH) catalyzes the conversion of isocitrate to α-ketoglutarate in almost all cells in aerobic organisms. This process generates chemical energy through oxidation of carbohydrates, fats and proteins within the citric acid cycle (CAC) [61, 62]. A mutation in the IDH gene is present in a high percentage of low-grade gliomas (LGG) and results in the accumulation of the oncometabolite 2-

hydroxyglutarate (2HG) and steady cellular dedifferentiation. The slow accumulation of glioma promoting metabolites excludes fast growing primary GBMs de facto. Hence, the glioma progression promoting IDH mutation is a positive prognostic marker, suggesting slow growth. Interestingly, the induction of this mutation in mature astrocytes does not result in a malignant transformation and cannot be considered a sufficient criterion of gliomagenesis [63, 64].

The 1p/19q Deletion

The co-deletion of the complete 1p and 19q chromosomal arms is the result of an unbalanced translocation and has recently been regarded as the hallmark of oligodendroglial tumors. The inclusion of the 1p/19q deletion criterium was one of the most prominent refinements in the revised 2016 WHO glioma classification and allows the clear differentiation of oligoastrocytomas in astrocytomas and oligodendrogliomas.

The co-deletion is considered a positive prognostic factor in oligodendrogliomas and indicates a beneficial response to chemotherapy [41, 65, 66].

Symptoms and Diagnosis

Most glioma symptoms result from an indirect compression of adjacent structures and an increased intracranial pressure. Hence, the central problem clinicians face in diagnosing gliomas is the late appearance of clinical symptoms and their vague character. The most common manifestation is a dull and constant tension such as headache in 56 % of patients, associated with nausea and vomiting [67]. Other symptoms are memory loss (36 %), language deficits (33 %), personality changes (23 %), motoric deficits (33 %) and seizure (32 %) next to B symptoms such as night sweat, fever of unknown origin and unintended weight loss as well as tiredness and fatigue [68-70]. All these symptoms are strict indications for neuroimaging. The imaging of choice is contrast-enhanced MRI. High-grade gliomas are usually hypointense on T1-weighted images, enhance contrast infusion and present an increased T2 and FLAIR signal intensity [71].

A tissue sample is required to verify the diagnosis of glioma. Stereotactic image-guided brain biopsies can be performed with MRI or PET assistance to obtain suitable samples but are associated with a procedure-related mortality of 1-2% [72].

Therapy

The alarmingly bad median prognosis for patients with a newly diagnosed GBM of several months without any treatment is an imperative to act. Today´s therapy regimen contains a multimodal approach consisting of surgical resection, radiation and chemotherapy [40].

Preoperative Imaging

Determining the exact localization and dimension of gliomas is crucial for complete resection. Considerable progress in the field of neuroimaging enables radiologists not only to locate the tumor bulk but also to visualize adjacent eloquent structures. This improvement facilitates a maximal resection with the preservation of essential neurological functions [73]. Additional navigated transcranial magnetic stimulation (nTMS) combined with diffusion tensor imaging (DTI), two approaches to depict motor fibers, help preoperatively to analyze the relation between tumors and cortical motor representation to minimize postoperative motor deficits [74].

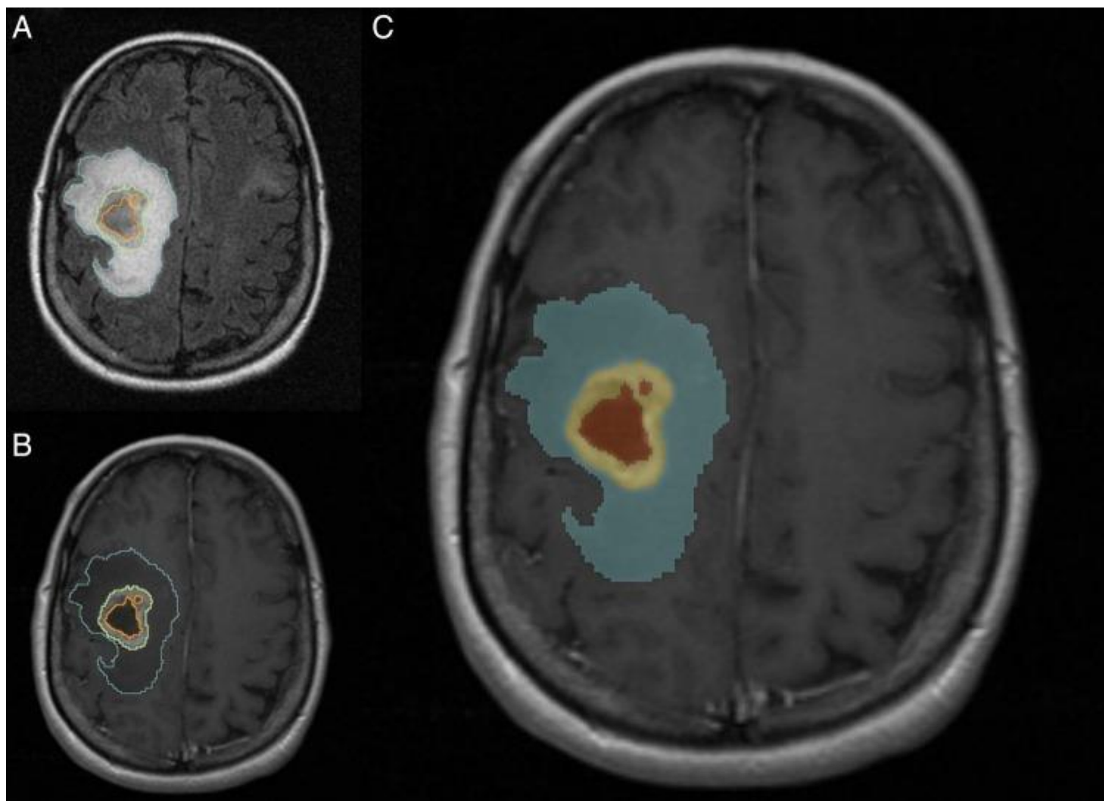


Figure 2. **Female patient with right frontoparietal GBM.**

In **A)** we see an axial FLAIR MR image with a hyperintense region indicating edema or glioma invasion. **B)** shows an axial postcontrast T1 weighted MR image that demonstrates the segmentation of viable tumor and necrosis. **C)** is an axial label map image. The blue area represents edema/tumor invasion, the yellow area the contrast enhanced tumor and the orange area the area of necrosis [1].

Surgical Resection

In 1884 Mr. Rickmann J. Godlee performed in London the first recorded complete resection of a primary brain tumor and laid the foundation for the rise of modern brain surgery. Despite several refinements and improvements, the procedure has remained the unquestioned baseline therapy for newly diagnosed gliomas until today [75]. It is common sense that resection is the most beneficial therapy.

It ensures a release of intracranial pressure, reduces mass effects, contributes to long-term disease control and eliminates tumor areas that are impenetrable by chemotherapy alone [38, 39, 76].
While the procedure itself was never controversial, opinions diverge on the extent of resection (EOR) required. Lacroix et al. published in 2001 that a resection of at least 98 % is crucial to improve patients´ survival and is most beneficial for the general postoperative performance. This dictum of neurosurgery has been questioned recently [77]. The idea of a maximal EOR was qualified and the resection outcome was linked instead to the location of the tumor and situational circumstances. The overall survival (OS) was averaged to 22,3 months for resections > 90 %, best possible care presumed. Moreover, even with an EOR of only 70-80 %, the OS was still 13,2 months [37]. Information about the median survival varies considerably and must be looked at critically in the context of each publication. To further improve the postoperative neurological performance of patients with tumors in eloquent areas, intraoperative language and neurologic examinations have made their way into clinical practice. These relatively safe techniques facilitates the resection in functional areas, reduces intraoperative brain damage and accelerates postoperative recovery [78, 79]. Though the advantages of a complete tumor resection are apparent, especially older patients with many comorbidities benefit from differentiated decision making. A conservative approach can be the preferable option assuming a high age, an acceptable neurological performance and potential perioperative complications [80].

Adjuvant radiation, chemotherapy and alternative approaches

The latest fundamental improvement in glioma therapy was introduced by Stupp et al. in 2005. They introduced the still valid main postoperative glioblastoma therapy regimen consisting of a fractionated focal radiation dose of 2 Gy given once per day between Monday and Friday over a period of 6 of weeks in combination with a concomitant and adjuvant temozolomide application. Temozolomide is an alkylating agent that predominantly targets guanine residues and consequently causes DNA damage and cell death [81]. It is applied at a dose of 75 mg/m^2 of body surface for the whole period of radiation therapy seven days per week. One month after the radiation therapy is completed, patients receive six cycles of adjuvant temozolomide according to the standard scheme with an increased dose of 150 mg/m^2. The addition of temozolomide to previous treatment regimens increased the median survival by 2,5 months in clinical trials [40, 82].
A more contemporary approach in the treatment of most malignancies is targeted therapies. This concept comprises sophisticated and less aggressive techniques using substances that interfere with tumor-specific molecular processes and reduce growth and invasion. Olson et al. give a factual overview of the present situation of targeted therapy in GBM treatment [83]. Unfortunately, no targeted attempt has yet achieved a clinical benefit compared to standard treatment.

The once most promising medication, Bevacizumab, a monoclonal humanized anti-vascular endothelial growth factor antibody, failed to improve patients´ overall survival. Other clinical trials that have investigated the effects of retinoids to regulate gliomas´ enzyme and growth factor synthesis, diazepam to modulate the mitochondrial metabolism and a variety of protein kinase C inhibitors have also failed to fulfill expectations. All these attempts illustrate the need for new substances in the field targeted therapies [83-85].

Co-medication

Most patients receive dexamethasone and anticonvulsants in addition to their radiation and chemotherapy to prevent postoperative complications such as edema or seizures. Though these substances actively interfere with patients´ immune responses, there is little to no evidence about the effects of those substances on glioma cells, the tumor micromilieu or interactions between co-medication and the actual anti-tumor-therapy. While the influence of anticonvulsants on the immune system has only been analyzed in general, it has been shown that even low doses of dexamethasone possibly antagonize tumor suppressing immune functions and significantly decrease the overall survival of patients suffering from gliomas [86, 87]. Further investigations on the interaction between tumor, antitumor therapy and concomitant medicaments are necessary.

Microglia Glioma Interaction

The importance of microglia/macrophages for the maintenance, promotion and invasion of gliomas has come to the fore in recent years. We want to introduce the underlying mechanisms and demonstrate how we can transfer this knowledge into therapeutic progress.

Microglia Recruitment

Every interaction starts with contact. Gliomas secrete a mixture of various factors to recruit microglia/macrophages at a yet unknown early point in glioma development. The directed migration is mediated by a complex mélange of β-chemokines, growth factors and other signaling molecules.

The group of chemokines β-chemokines, characterized by two adjacent cysteines and therefore also referred to as C-C-chemokines, is reported to induce migration in monocytes, microglia and NK cells. It was shown that gliomas secrete considerable amounts of those chemokines. One of the most prominent C-C-chemokines is the chemokine ligand 2 (CCL2), also known as monocyte attractant protein 1 (MCP1). While it has been debated if MCP1 or another representative of the C-C-chemokines, MCP-3 is the most potent chemoattractant, both are essential for microglia/macrophage recruitment by gliomas and strongly correlated with the grade of malignancy [88-93].

Another species of chemo-attractants is growth factors. The impact of vascular endothelial growth factor VEGF on microglia/macrophage recruitment has been discussed controversially. While Annovazzi et al. include VEGF into the spectrum of chemoattractants in their review, Turkowski et al. observed a somewhat opposite effect of glioma secreted VEGF on microglia/macrophage migration in experiments with VEGF overexpressing gliomas [94, 95].
So far only shown in vitro, hepatocyte growth factor and scatter factor also work as microglia attracting molecules as well as the glial cell-derived neurotrophic factor (GDNF) and colony stimulating factor (CSF), both released by glioma cells. The primary mechanisms of glioma recruitment have been compactly summarized by Kettenmann et al. [96].
These extensive efforts to recruit microglia result in a tumor bulk that consists of up to 50 % of microglia/macrophages [64]. Though microglia represent the predominant population of immune cells in the healthy brain, Chen et al. compared the contribution of microglia and peripheral monocytes to glioma-associated monocytes (GAM). Interestingly, only 15 % of GAMs displayed features of resident microglia, while 85 % of the analyzed cells were assigned to the population of peripheral macrophages. Further analyses revealed that peripheral macrophages locate in early stages of gliomagenesis in perivascular niches, while microglia settle preferentially in peritumoral regions [64].

Microglia Polarization

The term polarization describes a phenotype change in microglia. After recruitment, we would expect microglia/macrophages as innate immune cells to commence an immediate antitumor process. However, the opposite happens. Complex glioma signaling results in a unique change in the microglia/macrophage phenotype, characterized by the upregulation of a variety of tumor promoting genes. The RNA expression profiles of GAMs do not resemble any in vitro observed archaic microglia polarization scheme.
On the contrary, microglia/monocytes present a remarkable degree of plasticity in the creation of an individual tumor supportive micromilieu that promotes tumor growth, immune evasion, neovascularization and glioma invasiveness [96]. Though the exact mechanisms of microglia/macrophage reprogramming remain elusive, several prominent factors that contribute to the glioma supportive phenotype in GAMs have been identified. It was shown that the colony stimulating factors (CSF) 1 and 2 contribute to differentiation, accumulation and proliferation of GAMs. Another factor that remodels microglia/macrophages in a tumor favoring manner is TGF-β. TGF-β promotes an immunosuppressive phenotype in GAMs and protects tumors from immune responses by other cell populations [97].
Szulzewsky et al. analyzed glioma mediated gene expression clusters in human GAMs and highlighted the upregulation of genes that either control the “mitotic cell cycle” and “wound healing” or contribute to processes in the “extracellular matrix” in associated immune cells. All these processes are essential for the development of gliomas [98, 99].

Tumor supportive micromilieu

The portrayed changes create a niche that promotes glioma progression. This niche is populated by microglia and macrophages (GAMs), the cell population we are focusing on, but also by myeloid suppressor cells (MDSC), $CD4^+$ cells and regulatory T-cells, all highly characteristic of gliomas. All these cells as well as domestic glial cells interact with neoplastic cells and define an individual tumor milieu [97].
Especially GAMs play an essential role in this relationship and ensure comprehensive all-inclusive tumor support.

Immune Evasion

All cells that inhabit the direct tumor surroundings reshape their epigenetic profile according to tumor-secreted stimuli. GAMs provide a tumor-supportive immune environment after they have been polarized into a glioma-promoting state by the secretion of two popular immune regulatory messengers: IL-10 and TGF-β. These signaling molecules inhibit the activation and proliferation of naïve T-cells, which results in an insufficient cytotoxic T-cell response. Another mechanism that attenuates the effectiveness of killer T-cells is the recruitment of regulatory T-cells and dendritic cells.
TGF- β attenuates the proliferation of B-cells and modulates their antibody production. By reducing the activity of killer T-cells and B-cells, gliomas create a cellular and humoral shield against intrinsic anti-tumor actions [97, 100, 101].

Tumor Growth

GAMs do not only assist gliomas in evading the immune system; they also contribute to tumor growth.
Assessing the impact of single GAM released growth stimulus has proved to be a particular challenge. Multifunctional factors that also contribute to microglia polarization and recruitment, e.g. M-CSF, TGF-β as well as growth factors such as endothelial growth factor (EGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), secreted by both GAMS and glioma cells, induce proliferation in para- and autocrine feedback loops. The answer to the question of cause and consequence in these complex reciprocal interactions remains elusive [102].
The proof of concept was established by Markovic et al., who induced GL261 gliomas in murine brain slice cultures. The selective depletion of microglia via clodronate-filled liposomes resulted in significantly decreased tumor size in slice cultures devoid of microglia. Hence, we assume that GAMs actively contribute to glioma growth [103].

Vascularization

Unphysiological, excessive and partly microglia/macrophage-driven glioma growth without the installation of concomitant adequate vascular structures results in a lack of essential nutrients and oxygen. Therefore, gliomas, directly and indirectly, induce progressive vascularization to compensate for this deficiency. Due to fast and undirected angiogenesis, the formation of new tumor vessels is characterized by an unorganized architecture and the absence of pericyte covers.

Brandenburg et al. performed microglia depletion experiments to investigate the direct effects of GAMs on glioma vascularization. A significant reduction of vessel density, as well as tumor growth, was observed after depletion of microglia/macrophages. It was shown that glioma-associated microglia rather than macrophages settle in perivascular niches, in contrast to microglia in healthy tissue, and continuously express a pro-vascular genetic signature that consists of a panel of vascular development modulating molecules such as the well-known potential pro-angiogenic factors Ccl2, Cxl2 and VEGFa. The expression of these is stimulated by glioma-secreted hypoxia-induced transcription factor 1 α (HIF) and results in neovascularization and angiogenesis. In summary, all these findings indicate that adequate glioma neovascularization depends on the utilization of GAMs [104].

Tissue Invasion

GAMs facilitate glioma progression in various ways, as we outlined earlier. One process that is especially detrimental to patients´ overall survival is the extensive and early invasion of healthy tissue and the subsequent impracticability of complete surgical resection. A fundamental requirement for the infiltration of surrounding brain parenchyma via the extracellular space is the degradation of the extracellular matrix. The ECM is composed of different proteoglycans, collagens and other stabilizing elements characterized by a highly dynamic continuous degradation and reorganization. These intricate remodeling processes are balanced under physiological conditions and mediated by MMPs [105, 106]. We want to illustrate the underlying mechanisms and contextualize how gliomas exploit microglial MMPs in the following paragraph

Matrix Metallo Proteases (MMPs)

MMPs include a diverse multitude of proteases. One main function is the conversion of the ECM, for example, during tissue remodeling, regulatory processes in inflamed or diseased tissue and organ development [107]. Most MMPs are expressed as inactive precursors, so-called proMMPs, and need to be activated by proteolytic cleavage. The adequate and balanced function of MMPs in healthy tissue is defined by the enzymatic activation on one hand and the presence of inhibitors, mostly tissue inhibitors of matrix

metalloproteases (TIMPs) on the other hand. Under physiological conditions microglial cells constitute the main fraction of MMP expressing cells in the brain [108].

Hence, microglia hold an exclusive monopoly on the modulation of the central nervous ECM. Gliomas take advantage of this unique characteristic and utilize it to intensify the ECM degradation in a tumor invasion promoting manner in two ways [109].

They induce the expression of high levels of MMP2 and MMP9, both capable of degrading the ECM, and they promote glioma growth and invasion [103, 110].

We analyzed TCGA glioma data and were able to show that the expression of MMP2 in the tumor bulk is inversely correlated with the patients´ median survival (data not shown).

However, tumor-associated microglia/macrophages do not only provide the required proteases to enhance glioma invasion directly. They also express high levels of MMP14. MMP14 is a membrane-bound MMP that is absent in microglia/macrophages under physiological conditions but is massively upregulated after glioma contact. Microglial MMP14 proteolytically activates glioma secreted proMMP2, an inactive MMP2 precursor. The activation of MMP2 amplifies the degradation of ECM and is strongly correlated with patients´ median survival [103, 110].

Pattern Recognition Receptors

To illustrate how gliomas initiate all these tissue destructing processes, we have to introduce a receptor class that was an object of interest throughout the last century: toll-like receptors (TLR). The discovery of microbes and immune reactions of multicellular organisms suggested that immune cells were capable of pathogen recognition. However, it was firstly in 1995 that the toll gene and its association with antifungal and antibacterial effects were found, and this in Drosophila by Christiane Nüsslein, who screamed “toll”, the German word for “great”, when she discovered the gene. Further experiments uncovered a class of toll-like receptors in mammals that play a pivotal role in recognizing conserved structural motifs in pathogens, so-called pathogen-associated molecular patterns (PAMPs). The recognition of and reaction to PAMPs is a foundation pillar of the innate immune system [111, 112].

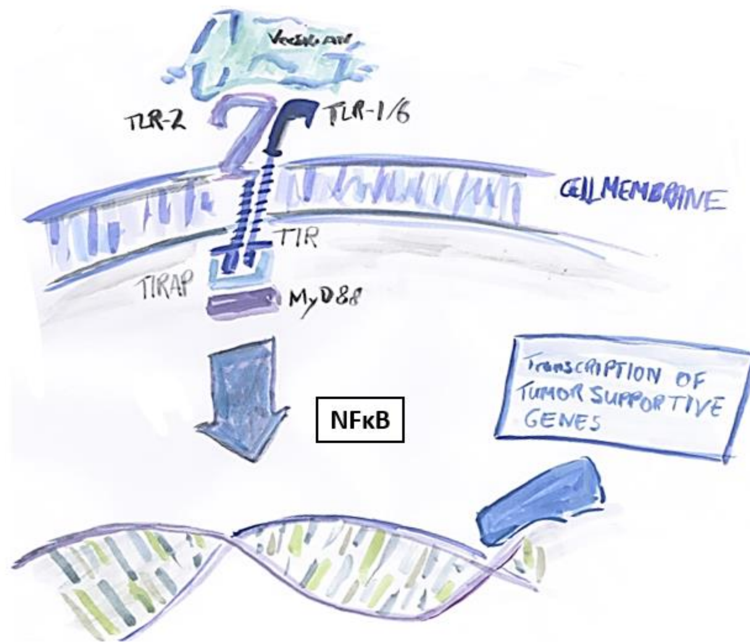


Figure 3. **Simplified depiction of TLR2 signaling.**

The binding of an agonist initiates the heterodimerization of TLR2 and TLR1 or TLR6 and results in the creation of an intracellular TIR docking domain that enables further MyD88 dependent signaling and results in the activation of NFκB. The overexpression of tumor supportive genes is a direct consequence.

One prominent representative of its class is toll-like receptor 2 (TLR2). TLR2 is a membrane-bound receptor that recognizes a broad spectrum of PAMPs, including lipoproteins and peptidoglycans. These molecules are embedded in cell walls of gram-positive and gram-negative bacteria but also constitute a small part of the ECM [106]. Depending on the detected lipopeptide, TLR2 and either TLR1 or TLR6 dimerize and activate the myeloid differentiation primary response gene dependent pathway. Both dimerizing toll-like receptors possess the Toll/IL1-receptor resistance (TIR) domain that enables a consecutive protein coupling. The dimerization of TIR domains creates a docking site for the adapter protein Myd88 and its associated kinase (IRAK). IRAK and the tumor necrosis factor receptor-associated factor (TRAF) create a binding site for the NFκB inducing kinase (NIK). NIK phosphorylates the IκB kinase (IKK), which results in the reduction of this NFκB degrading counterpart and an increase of activity of NFκB. Consecutively, the expression of a multitude of genes that regulate a variety of immune functions is upregulated. The importance of MyD88 binding and signaling for various processes in immune responses and tumorigenesis has been highlighted and summarized by several groups [113]. One group of proteins that are highly upregulated by the activation of the TLR2 pathway are microglial MMPs [96].

The depletion of the TLR2 adapter protein MyD88 in microglia results in a downregulation of MMP14. Markovic et al. were able to show that the reduction of MMP14 in GAMs by shRNA results in decreased

levels of active glioma derived MMP2. They also proved in MyD88 knock-out experiments that reduced TLR2 dependent MyD88 signaling leads to a significantly reduced MMP14 expression and tumor size in mice [114]. An indirect in vivo inhibition of TLR2 signaling in animals significantly increased the survival of mice suffering from gliomas. The animals were treated with minocycline, a broad spectrum tetracyclic antibiotics which generally blocks microglia activation and TLR2 related downstream signaling [115].
After TLR2 was identified as an essential mediator for the upregulation of MMP14 expression in GAMs, a TLR2 knockout in glioma-bearing mice was performed and led to significantly smaller tumors and reduced expression of MMP14 in GAMs. The TLR2 expression in human gliomas was shown to be inversely correlated with patients´ overall survival [116].
In summary, we know that gliomas can increase the MMP14 expression in GAMs by TLR2 signaling and thereby increase the expression of MMP14 and induce the activation of MMP2. However, how do gliomas facilitate these changes in the microglial transcriptome?

Versican

The simple answer to this question is that they do this by simulating a mismatch in the composition of the ECM that results in a non-inflammatory activation of macrophages/microglia and overexpression of MMPs. Gliomas secrete versican to initiate this process. Versican is a large chondroitin sulfate proteoglycan and a natural component of the neuronal ECM [117, 118]. It is overexpressed in tissues with a high turnover and acts as endogenous TLR2 agonist in monocytes. While vacant in the mature brain, glioma-derived versican and the following TLR2 activation lead to a glioma growth promoting degradation of the ECM by the overexpression of MMP14 in GAMs [96].
At this point, we want to highlight the importance of TLR2 signaling in microglia once again. The tumor increases the release of microglial MMPs by simulating an ostensible physiological degradation process [116, 119].
A 3-D analysis of the molecular structure of TLR2 identified an intracellular loop in the TIR motif that is essential for receptor dimerization and consecutive signaling. Hence, this highly conserved domain provides an opportunity to inhibit glioma promoting TLR2 signaling by inducing a simple blockade, and because of its specific shape, the small molecule o-vanillin exactly meets the requirements to do this [120].

Ortho Vanillin

O-vanillin is an isomer of vanillin, the main component of natural vanilla. It was already known and valued by the Aztecs more than 400 years ago for its flavor. Vanillin was first isolated by Ferdinand Tiemann in 1876 and became a popular food supplement in the following years [121].
Apart from its use in the food industry, vanillin was tested as an inhibitor of PIK3 and NFκB signaling in a variety of cancer entities and has been shown to have protective effects in Parkinson disease. It proved itself as a natural suppressor of tumor growth and metastasis in several different experiments [122-124].
While the mechanisms remained opaque, vanillin was tested and tolerated in animal experiments in daily doses up to 100 mg/kg and was used in in vitro experiments in concentrations ranging from 250 µM to 4 mM [120, 125, 126].
The LD50 was quoted as 1330 mg/kg per day in mice by Santa Cruz Biotechnology and is far from the doses used in previous experiments and needed for a sufficient treatment [127].
It has also been shown that vanillin is not only a suppressor of tumor growth but is also able to penetrate the blood-brain barrier (BBB). The BBB penetrability represents one major obstacle in the treatment of CNS diseases in general and is thought to be one main reason for the failure of several therapeutic attempts, for example, in the treatment of gliomas with antibodies [122, 128, 129].
We analyzed recent publications that used vanillin and noticed that most of them reported a potential TLR2 downstream target as affected by the treatment. The structural analysis of TLR2 described above offers an appealing explanation for the observed effects: vanillin and its isomer o-vanillin disable the toll-like receptor 2 dimerization due to its molecular structure by blocking an essential loop in the intracellular TIR motif. The blockade attenuates downstream signaling via the Myd88 or PIK3 pathway [115, 120, 130].
We want to adopt all these findings into the glioma context. We evaluate the penetration of the BBB and the inhibition of glioma promoting TLR2 activation in microglia/macrophages as highly promising for a GAM targeting attempt in glioma treatment.

Objectives

So far, we have introduced microglia cells as guardians of the central nervous system, the malignant characteristics of gliomas and the subsequent complications in today´s therapy. We have also summarized the recruitment of microglia to the glioma site, the polarization of GAMs into a tumor-supportive phenotype and the effects of polarization on the tumor´s ability to evade the immune system, grow and invade the surrounding tissue. One important driver of the glioma-induced degradation of the ECM, essential for invasive growth, is microglial MMPs. Next to MMP2 and MMP9 that proteolytically cleave extracellular glycans, MMP14 is highly upregulated. MMP14 activates tumor-secreted inactive precursor proMMP2 and is strongly correlated with the patients´ overall survival. The upregulation of microglial

MMP14 is induced by glioma-derived versican. Versican is an endogenous proteoglycan that acts as a TLR2 agonist. TLR2 is a pattern recognition receptor and highly expressed by cells of the innate immune system, e.g., microglia/macrophages, and can activate the MyD88 pathway. This pathway culminates in the upregulation of a broad variety of partially tumor-supporting immune modulatory factors and MMPs.

The analysis of the crystal structure of a cytoplasmatic domain of TLR2, TIR, identified a small pocket in the BB loop of this motif that is essential for the heterodimerization and binding of consecutive adapter proteins.

O-vanillin blocked the identified pocket due to its molecular shape and was able to attenuate the effects of TLR2 downstream signaling in preliminary experiments with murine macrophages.

We want to extend the therapeutic spectrum in glioma treatment by targeting microglia/macrophages as an important glioma supporting element and focus on their role as a promoter of glioma invasiveness: the TLR2 mediated upregulation of MMPs in GAMs. Recent studies indicate that o-vanillin is a suitable and well-tolerated candidate for an elegant inhibition of TLR2 signaling.

We want to evaluate the effects of o-vanillin on murine and human microglia in a glioma context: Can o-vanillin reduce glioma growth and be beneficial in complementing today´s glioma therapy?

Materials and Methods

Materials

Chemicals

Object Description	Name	Company
Agar-Agar	Agar-Agar, Kobe I	Roth, Karlsruhe, Germany
Alamar Blue	alamarBlue Cell Viability Reagent	Invitrogen, Carlsbad, USA
Calcium chloride	calcium chloride dihydrate	Roth, Karlsruhe, Germany
CD11 b Microbeads	CD11b (Microglia) MicroBeads, mouse/human	Miltenyi Biotec, Bergisch Gladbach, Germany
DAPI	4´,6-Diamidino-2phenylindole dihydrochloride	Sigma Aldrich, St. Louis, USA
DMEM	Dulbecco´s Modified Eagle Medium	Life Technologies, Carlsbad, USA
DMSO	Dimethylsulphoxide Hybri Max	Sigma Aldrich, St. Louis, USA
EDTA	Ethylenediaminetetraacetic acid	Sigma Aldrich, St. Louis, USA
Ethanol	Ethanol, verg. 1% Petrolether	Berkel AHK, Ludwigshafen, Germany
FCS	Fetal Calf Serum	Life Technologies, Carlsbad, USA
Glucose	D-Glucose anhydrous	Roth, Karlsruhe, Germany
HBBS	Hank´s Balanced Salt Solution	Life Technologies, Carlsbad, USA
HBBS with	Hank´s Balanced Salt Solution with Calcium and Magnesium	Life Technologies, Carlsbad, USA
Isopropanol	Isopropanol 99,9%	Berkel AHK, Ludwigshafen, Germany
LPS	LPS from E. coli Serotype R515 Alexis	Enzo Life Sciences, Inc., Farmingdale, USA
Methanol	Methanol 99,8%	Th. Geyer, Renningen, Germany
mMCSF	Recombinant Murine M-CSF	PeproTech, Rocky Hill, USA

Mounting Medium	Aqua PolyMount	Polysciences, Inc., Warrington, USA
o-vanillin	ortho-vanillin	Sigma Aldrich, St. Louis, USA
Pam3CSK4	Pam3CSK4	InvivoGen, San Diego, USA
PBS	Dulbecco´s Phosphate Buffer Saline	Life Technologies, Carlsbad, USA
Percoll	Percoll	GE Healthcare, Little Chalfont, England
PFA	Paraformaldehyde	Sigma Aldrich, St. Louis, USA
Phalloidin	Phalloidin, Fluorescein Isothiocyanate Labeled	Sigma Aldrich, St. Louis, USA
PI	Propidium Iodide	Life Technologies, Carlsbad, USA
Potassium Chloride	Potassium chloride	Roth, Karlsruhe, Germany
PSG	PenStrepGlut	Life Technologies, Carlsbad, USA
RNase free water	Water	Sigma Aldrich, St. Louis, USA
Sodium Azide	Sodium Azide	Sigma Aldrich, St. Louis, USA
Sodium Chloride	Sodium Chloride	Roth, Karlsruhe, Germany
Sodium Di-Hydrogen Phosphate Monohydrate	Sodium Di-Hydrogen Phosphate Monohydrate	Roth, Karlsruhe, Germany
SYBR Green	SYBR Select Master Mix	Life Technologies, Carlsbad, USA
TRIS	Tris-hydrochloride	Roth, Karlsruhe, Germany
Trypan Blue	Trypan Blue Solution 0,4%	Sigma Aldrich, St. Louis, USA
Tween	Polyethylene glycol sorbitan monolaurate	Sigma Aldrich, St. Louis, USA

Buffers

ACK Lysis Buffer

Ingredients	Concentration, g/l
NH_4Cl	8,29
$KHCO_3$	1
Na_2-EDTA	37,2
pH	7,2 – 7,4

Myelin Gradient Buffer

Ingredients	Concentration, g/l
$NaH_2PO_4\ H_2O$	0,78
$Na_2HPO_4\ 2H_2O$	3,56
NaCl	8
KCl	0,4
Glucose	2

Percoll Solution

Ingredients	ml
Myelin Gradient Buffer	22,5
Percoll	6,6
1,5 M NaCl	0,72

Other Buffers

	MACS Buffer	FACS Buffer	Washing Buffer
Basis	PBS	PBS	PBS
% FCS	1	2	/
mM EDTA	2	1	/
% sodium azide	/	0,1	/
% Tween	/	/	0,05
pH	7,2-7,4	7,2-7,4	7,2-7,4

Media

Cell Culture

Murine Primary Culture Medium/Complete Growth Medium:

DMEM supplemented with 10 % FCS, 0,2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (PSG).

Human Primary Glioma Culture Medium:

Complete growth medium supplemented with 1 µg/ml EGF and 1 µg/ml FGF.

Organotypical Slice Culture

Working Medium:

Complete growth medium

Cultivation Medium:

DMEM supplemented with 25 % FCS, 25 % HBSS without Ca^{2+} and Mg^{2+}, 100 U/ml penicillin, 100 µg/ml streptomycin, 500 µM Tris, 0,8 mg/ml Vitamin C, 1 µg/ml Insulin, 2,4 mg/ml Glucose, 0,4 mM glutamine, 50 mM Na_2HCO_3

Conditioned Media

L929 Conditioned Medium:

Mouse L929 fibroblast cells were grown till confluency of 80 %. 30 ml of complete growth medium were added for 48 hours. Then the medium was collected, filtered and stored frozen in aliquots until needed.

Glioma Conditioned Medium, GCM:

Murine GL261 wild-type glioma cells were grown till 80 % confluent in a T75 culture flask. The cells were overlaid with 10 ml of complete growth medium for 18-20 hours. The next day the medium was collected and strained through a 0,4 µM filter, aliquoted and stored at -20°C until usage.

Primers

All primers were designed using the RefSeq sequences of the UCSC genome browser (http://genome.ucsc.edu/). To create primers, we used the Primer-Blast tool (http://www.ncbi.nlm.nih.gov/tools/primerblast/). We tried to minimalize the risk of amplification of unintended targets by testing the generated primers via BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To avoid undesirable secondary structures and Primer-Dimer, we used the IDT oligo analyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) [99].

Mouse Primers

Target	forward	reverse
beta-Actin	CGT GGG CCG CCC TAG GCA CCA	CTT AGG GTT CAG GGG GGC
iNOS	TCACGCTTGGGTCTTGTTCA	TGAAGAGAAACTTCCAGGGGC
MMP14	GTG CCC TAT GCC TAC ATC CG	CAG CCA CCA AGA AGA TGT CA
MMP9	CATTCGCGTGGATAAGGAGT	ACCTGGTTCACCTCATGGTC
TBP	AAGGGAGAATCATGGACCAG	CCGTAAGGCATCATTGGACT

Human Primers

Target	forward	reverse
GAPDH	GTC AGT GGT GGA CCT GAC CT	AGG GGA GAT TCA GTG TGG TG
MMP14	CGCTACGCCATCCAGGGTCTCAAA	CGGTCATCATCGGGCAGCACAAAA
MMP9	AAGGCGCAGATGGTGGAT	TCAACTCACTCCGGGAACTC
TLR2	TCTCCCATTTCCGTCTTTTT	GGTCTTGGTGTTCATTATCTTC

Kits

Name	Company
Brain Tumor Dissociation Kit	Miltenyi Biotec, Bergisch Gladbach, Germany
Cell Proliferation ELISA, BrdU (colorimetric)	Roche, Basel, Switzerland
PrimeScript RT reagent Kit (Perfect Real Time)	TAKARA BIO INC., Kyoto, Japan
Diff-Quik Staining Set	Medion Diagnostics International Inc., Miami, USA
Mouse IL-6 R alpha DuoSet ELISA	R&D Systems, Minneapolis, USA
ReliaPrep RNA Cell Miniprep System	Promega, Fitchburg, USA

Equipment and Devices

Object Description	Name	Company
0,2 µM Filter	MiniSart Syringe Filters	Sartorius, Göttingen, Germany
15ml Falcon Tube	15 mL Polypropylene Conical Tube	Falcon, New York, USA
50ml Falcon Tube	50 mL Polypropylene Conical Tube	Falcon, New York, USA
70 µM Strainer	Pre-Seperation Filters, 70 µm	Miltenyi Biotec, Bergisch Gladbach, Germany
Adhesive Film	MicroAmp Optical Adversive Film	applied biosystems, Foster City, USA
Bench-Top Centrifuge	Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Boyden Chamber	48-Well Micro Chemotxis Chamber	Neuro Probe Inc, Gaithersburg, USA
Centrifuge	Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Cover Slips	Deckgläser 24x50 mm	DIAGONAL GMBH & Co. KG, Münster, Germany
Confocal microscope	Zeiss LSM 710 confocal microscope	Zeiss, Oberkochen, Germany
Culture Inserts	0,4 µm Diameter Cell Culture Inserts, Millicell	Merck Millipore, Burlington, USA
Flow sorter	BD LSRFortessa Special Order Research Product	BD Biosciences, Franklin Lakes, USA
Fluorescent microscope	Zeiss AxioVert 135 microscope	Zeiss, Oberkochen, Germany
Gasket	Gasket for AP48 chamber	Neuro Probe Inc, Gaithersburg, USA
gentleMACS Dissociator	gentleMACS Octo Dissociator with heaters	Miltenyi Biotec, Bergisch Gladbach, Germany
Glue	UHU Sekundenkleber	UHU GmbH & Co. KG, Bühl/Baden, Germany
Hamilton Syringe	Microliter Syringe 7000 series	Hamilton, Reno, USA
Incubator	Forma Ster-Cult CO_2 Incubator	Thermo Fisher Scientific Inc., Waltham, USA

Membrane	8µm Polycarbonate Filters	Neuro Probe Inc, Gaithersburg, USA
NanoDrop	NanoDrop8000 Spectrophotometer	Thermo Fisher Scientific Inc., Waltham, USA
Neubauer Chamber	Neubauer improved	Marienfeld, Lauda-Königshofen, Germany
Object Slice	Menzel Gläser Super Frost	Thermo Fisher Scientific Inc., Waltham, USA
Plate reader	Infinite M200	Tecan, Männedorf, Switzerland
qPCR machine	7500 Fast Real-Time PCR System	Life Technologies, Carlsbad, USA
qPCR Plate	MicroAmp Fast Optical 96-Well Reaction Plate	Life Technologies, Carlsbad, USA
RT-PCR machine	T3000 Thermocycler	Biometra GmbH, Göttingen, Germany
Separation Columns	MACS Separation Columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Shaker	3D Rocking Platform STR9	Start Scientific, Austin, USA
Syringe	1ml Omnifix-F	Braun, Krondorf, Germany
Thermomixer	Thermomixer compact	Eppendorf, Hamburg, Germany
Vibratome	Microm HM 650 V	Thermo Fisher Scientific Inc., Waltham, USA
Vortexer	Vortex Genie 2	Life Technologies, Carlsbad, USA
Water Bath	Water Bath	GFL, Burgwedel, Germany

Software

Name	Company
EndNote X8	Clarivate Analytics, New York City, USA
Graphpad Prism 6	GraphPad Software, Inc., La Jolla, USA
Image J/FIJI	NIH, Maryland, USA
FlowJo	Treestar, Ashland, USA
Office 2016	Microsoft, Redmond, USA
Windows 10	Microsoft, Redmond, USA
ZEN Zeiss	Zeiss, Oberkochen, Germany

Methods

Animals

We cultivated primary microglial cell cultures and organotypic brain slice cultures (OBSC) from C57/Bl6 wild-type mice (Charles River Laboratories, Wilmington, MA, USA) for all in vitro experiments. The mice were bred and maintained in the animal housing facility of the Max Delbrück Center as per the rules of the local governmental institutions. The animals lived according to a 12/12 dark-light rhythm and had access to water and food ad libidum.

Microglia Cell Culture

We used complete growth medium for all in vitro cell culture experiments. All cells were incubated at 37°C, a CO_2 concentration of 5 % and a relative humidity of 80 % unless stated otherwise. One day old C57/Bl6 wild type mice were sacrificed and decapitated. The skull was fixed and cut from the foramen magnum to the nose. The crania were carefully taken off and the whole brain was transferred to cold HBSS. The cerebellum, the olfactory bulbs and the meninges were removed cautiously and the brains were collected in HBSS filled 15 ml Falcon tubes. The brains were washed with PBS. Trypsin/DNAse was added for enzymatic lysis for 2 minutes. The reaction was stopped with complete growth medium. The suspension was triturated with a glass pipette and filled up with complete growth medium. The cells were centrifuged at 800 rpm for 10 minutes at 4°C and resuspended in complete growth medium. The suspension was distributed to T75 flasks and stored in an incubator.

After two days, the flasks were washed 4 times with PBS and filled with fresh complete growth medium. On the 7th day, the cell layer had grown confluent and we changed the medium. L929 fibroblast conditioned medium was added. Two days later the microglial cells were harvested. The flasks were shaken for 30

minutes at 37°C and 100 rpm to shake off the cells. The suspended microglial cells were collected in a 15 ml Falcon tube and centrifuged at 800 rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in complete growth medium. Then the cells were counted in a Neubauer counting chamber according to the manufacturer´s instructions and plated as required for each experiment. We refilled the T75 flasks with complete growth medium and L929 fibroblast conditioned medium and shook off microglial cells two more times every two days.

Astrocyte Cell Culture

C57/Bl6 mice were processed as described above. After the third microglia shake off, we washed the confluently grown adherent astrocyte layer thoroughly with PBS. Trypsin/EDTA was used to detach the cells. We stopped the reaction by adding complete growth medium and centrifuged the detached cells at 500xg for 5 minutes at 4°C. Then the cells were counted in a Neubauer chamber according to the manufacturer´s instructions and plated as required for each experiment.

Cell Lines

Glioma Cells

The murine glioma cell line GL261 was obtained from the Charles River Laboratories, Wilmington, USA. The cells were grown in T75 flasks in complete growth medium.

Green fluorescent GL261 cells for OBSC experiments were generated according to the protocol of Vinnakota et al. [116]. The cells were cultured as described above. All GL261 cell lines were discarded after 15 passages.

Glioma Conditioned Medium (GCM)

GL261 cells were grown to a confluence of 70 % and incubated in complete growth medium for 24 hours. The GCM was collected, briefly centrifuged and filtered. Aliquots of pooled GCM were stored at -20°C for a maximum of 3 months.

Oligodendrocytes

The immortalized oligodendrocyte progenitor cell line OLN93 was created according to Jung et al. [131]. The cells were passaged in T75 flasks in complete growth medium and split when confluent.

Human Glioma Samples

All human glioma samples were kindly provided by the Department of Neurosurgery, University Hospital Schleswig-Holstein and the Department of Neurosurgery, HELIOS Hospital Berlin Buch. After the Department for Pathology confirmed the working hypothesis of malignant glioma, the cells were prepared. All patients were informed and gave their permission for scientific processing of the tissue samples.

In brief, all tissue samples were resected while patients were anesthetized. Samples for CD11 B isolation were immediately transferred to culture medium. The consecutive analysis of brain tissue was performed according to the Charité´s ethical committee guidelines (Charité, EA4/098/11).

Magnetic cell separation of human brain tumor tissue

Resected human tissue samples were stored on ice no longer than 24 hours and washed in cold PBS before processing. Before we isolated glioma associated brain microglia/macrophages, necrotic areas were removed and the samples were enzymatically dissociated using a neural tissue dissociation kit following the manufacturer´s instructions. After creating a single cell suspension, cells were centrifuged at 300xg for 8 minutes at 4°C and resuspended in 5 ml cold ACK for 15 minutes. 15 ml cold PBS were added to wash the redundant ACK out. The cells were centrifuged and the supernatant was discarded. Redundant myelin was removed using a Percoll buffer.

The cells were suspended in 25 ml of Percoll buffer and cold PBS was slowly pipetted onto the cells, creating two phases. By centrifugation at a 950xg for 25 minutes at 4°C without acceleration and break, the myelin was separated in a layer between the Percoll buffer and PBS and could be carefully aspirated. The remaining cell pellet was suspended in 90 µl MACS buffer containing 10 µl CD11 B magnetic microbeads and was incubated for 15 minutes at 4°C to label tumor associated monocytes.

The cells were washed with 3 ml MACS buffer and centrifuged as described above. The cell pellet was resuspended in 500 µl MACS buffer. A MS separator column was placed in a magnetic field (MACS separator) and washed three times with 500 µl MACS buffer before use. The cell suspension was applied to the washed column.

The magnetic cell separation distinguished a CD11 B negative (flow-through) and a CD11 B positive enriched cell population. Both fractions were collected and processed. The CD11 B negative cells were cultured as human glioma cell lines for future experiments; the CD11 B positive cells were plated and studied. The CD11 B positive cells were cultivated for a maximum of 12 hours. The cell viability was evaluated microscopically and by an alamarBlue viability assay performed according to the manufacturer´s instructions (results not shown).

Flow Cytometry

Murine microglia were plated at a density of 500 000 cells per well in 6-well plates. We treated the cells with increasing concentrations of o-vanillin (1-100 µM) for 24 hours. After that, the cells were washed, detached and dissolved in cold FACS buffer containing 2,5 µg/ml fluorescent labeled propidium iodide (PI). The cells were incubated for 15 minutes to accomplish sufficient staining.

The number of dead cells was determined as mean fluorescence intensity exceeding a predefined threshold oriented to healthy cultured murine microglia. The flow cytometry was performed using a BD FACS Aria and data were quantified and analyzed in FlowJo.

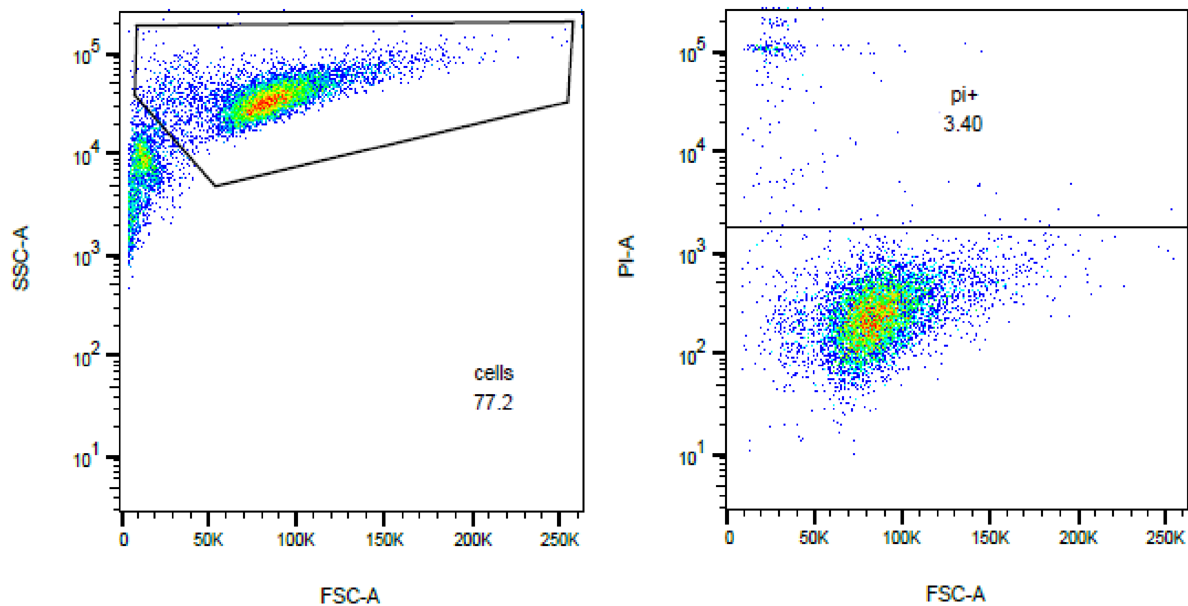


Figure 4. **FACS settings for PI assessment of o-vanillin treated microglia.**

On the left we define a cell population we consider as healthy microglia due to size distribution and density using the forward (FSC-A) and sideward scatter (SSC-A). On the right we define a threshold for a propidium iodide (PI-A) binding. Cells with a fluorescence intensity exceeding the predefined threshold were considered as PI positive and dead.

Plate reader

PI Analysis

20 000 murine microglia cells, 10 000 murine astrocytes, 5 000 GL261 and 5 000 OLN93 oligodendrocyte precursor cells were separately plated and processed the following day. All cells were treated with 100 µM o-vanillin for 24 or 48 hours, washed and stained with complete growth medium containing 2,5 µg/ml PI for 15 minutes at room temperature. The PI fluorescence intensity was determined at an excitation wavelength of 530 nm and a frequency of 25 flashes per sample. The emission was analyzed at 645 nm. We defined the fluorescence intensity of untreated cells of each population as 100 % viable cells. After analyzing the PI incorporation, we treated every condition with 10 % DMSO for 15 minutes to verify an increase of PI fluorescence in the case of dead cells present.

BrdU ELISA Proliferation Assay

We determined the number of proliferating cells by analyzing the incorporation of fluorescent labeled BrdU into the DNA in 24 hours. 20 000 C57/Bl6 microglial cells were plated in a 96 well plate and treated with 100 µM o-vanillin overnight. The old medium was discarded and new complete growth medium containing fluorescent labeled BrdU and 100 µM o-vanillin was added for another 24h.

We used cells that were treated with 10 % DMSO for 24 hours as a negative control. Murine macrophage colony-stimulating factor (mMCSF), a well-known inductor of microglia proliferation, stimulated cells were used as a positive control [132]. The concentration of BrdU was chosen according to the manufacturer´s instructions. Fixation and ELISA analysis of incorporated BrdU were performed following the manufacturer´s instructions. The absorption was analyzed at an excitation of 370 nm and the emission at 492 nm in a plate reader.

Boyden Chamber Migration Assay

The migration tests were performed in a 48-well Boyden Chamber. The upper and lower chambers were sealed with a silicone gasket and separated by a semi-permeable membrane.
1 000 000 freshly isolated microglial cells were suspended in 1 ml of complete growth medium and treated with increasing concentrations of o-vanillin (1-100 µM). 1 µg/ml ATP was used as a positive migration stimulus and plain complete growth medium was defined as physiological standard motility and used as a reference value [133]. We added different migration stimuli to the lower chambers and 50 000 o-vanillin treated microglia cells to the upper chamber. To imitate a tumor-induced migration stimulus, we investigated the migration toward glioma conditioned medium in the lower chamber. After 6 hours at 37°C, 5 % CO_2 and 80 % humidity, the membrane was removed from the chamber and fixed in the Diff-Quick fixation solution. After fixing, the membranes were stained with HE according to manufacturer´s instructions and washed in double distilled water. The membranes were dried overnight and mounted on object slides. After that, the redundant cells attached to the membrane surface were carefully scratched off with cotton sticks. Images were taken with a fluorescent microscope using the 10x magnification. The number of migrated cells was analyzed via the totalized surface area of stained cells in ImageJ FIJI.

Cytokine Release/IL6 ELISA

We assessed how the cytokine release was affected by an o-vanillin treatment with an IL6 ELISA. 20 000 microglial cells were plated in 96 well plates, treated with 100 µM o-vanillin and stimulated with Pam3CSK4 and GCM for 6 hours. LPS was used as a positive control. After stimulation, the medium was discarded and fresh complete growth medium was added for 12 hours and then stored at -20°C.
For further processing, the medium was diluted 1:10 in an ELISA buffer.

Before the quantification of IL6, the plates were washed three times, blocked for 1 hour and washed again. 100 µl of the diluted sample solution and standard solution were added to the prepared plates and incubated for 2 hours at room temperature. Afterwards, the sample solutions were aspirated and the plates were rewashed. The detection antibody was added for 2 hours at room temperature with the subsequent aspiration of the antibody and washing. After incubating the plates with 100 µl of the provided Streptavidin-HRP for 20 minutes, the substrate solution was added for 20 minutes. To stop the reaction, we mixed 50 µl of Stop Solution into each well. The optical density of each well was analyzed using a plate reader at 450 nm.

RNA Analysis

RNA Isolation

Samples used for total RNA extraction contained a minimum of 300 000 cells plated in 12-well plates. The primary murine microglia cells were treated with 100 µM o-vanillin for 6 hours. Human isolated CD11 B positive glioma-associated monocytes/macrophages were treated for 12 hours.

Total RNA was isolated using the Promega RNA Isolation Kit according to the manufacturer´s instructions. The isolated RNA was dissolved in 15 µl RNAse free water and stored at -20 °C. The RNA yield was quantified and assessed using a NanoDrop.

cDNA Synthesis

200 ng of already isolated RNA was transcribed into cDNA using the TaKaRa cDNA Synthesis Kit. 0,5 µl of random oligomers, 0,5 µl nucleotides, 0,5 µl of active enzyme and 2 µl of first strand buffer per sample were added to the RNA in a 1,5 ml Eppendorf tube. The mix was filled up to 10 µl with RNase free water and incubated for 15 minutes at 37°C. The enzymes were inactivated by heating them to 85°C for 10 seconds. The samples were stored for further use afterwards at -20°C afterward.

qPCR

We performed qPCR in 96 well real-time pPCR fast plates. A master mix containing 10 µl SYBR Green, 8 µl RNAse free water and 1 ng of cDNA was added to each well. The forward and reverse primer were added in a concentration of 10 pg/ml. The plate was covered with an adhesive film and centrifuged at 1000xg for 1 minute at 4°C. TATA-binding protein (TBP) was used as a housekeeping gene.

Organotypical Brain Slices

Preparation of Brain Slices

All practical steps were carried out after all the used material was disinfected with 70% EtOH for at least 30 minutes. The brain slices were obtained from 13 - to 16-day-old C57/Bl6 wild type mice. The mice were sacrificed by cervical dislocation and decapitated. The brains were carefully removed and immediately transferred to cold PBS. The cerebellum and olfactory bulbs were cut off. The brain was glued in an upward position with its caudal end to the cutting table of the vibratome and its ventral end to an already fixed agar block. The cutting table was fixed in a cooled cutting chamber and covered with cold PBS.

A vibratome was used to cut the fixed brains in 250 µm thin slices. The slices were directly transferred to cold HBSS. Up to 3 brain slices were plated on one semi-permeable membrane insert in a 6-well plate and covered with 1ml complete growth medium.

Tumor Injection

Green fluorescent GL261 glioma cells were grown confluent and harvested. We drew 100 000 cells up into a Hamilton 1 µl precision syringe and attached it to a customized micromanipulator.

24 hours after preparing the murine brain slices, the culture inserts were transferred to a working 6 well plate placed in the micromanipulator. All work steps were performed in 37°C warm complete growth medium. The precision syringe was adjusted 250 µm above the culture insert membrane and the brain slices were positioned. 10 000 GL261 cells were injected into the region of basal ganglia by slowly pushing the plunger. During injection, the syringe was slowly moved upwards. The GL261 cells were injected over a vertical range of 50 µm.

After injections of green fluorescent GL261, the culture inserts were transferred to a new plate filled with culture medium containing different concentrations of o-vanillin. The brain slices were incubated for 5 days. The medium was renewed every two days. On the 5th day, the brain slices were fixed in 4% PFA and carefully cut out of the culture inserts. After that, the brain slices were transferred to microscopy-slides, mounted with Aqua-Poly/Mount and covered with coverslips.

Confocal Microscopy

All micrographs were taken using a Zeiss LSM 710 confocal microscope with a 40 X oil objective.

Injected GL261*egfp* gliomas were visualized at an excitation wavelength of 488nm and analyzed at the depth of maximal surface area.

Microglial cells were treated with 100 µM o-vanillin for 24 hours to analyze the morphology of o-vanillin treated microglia. Afterwards, they were fixed with 4 % paraformaldehyde (PFA) and stained with 1 µg/ml phalloidin-FITC for 20 minutes and washed three times with PBS.

Statistical Analysis

All analyses were performed using Prism Graphpad 6 and Microsoft Excel 2016. Statistically significant differences were determined with the Student´s *t*-test for parametric testing. One-way ANOVA was used to compare multiple groups with Bonferroni posthoc test. Significance was defined at p values < 0.05 (*), <0.01 (**) and <0.001 (***).

Results

O-vanillin inhibits TLR2 mediated signaling in microglia

We stimulated primary cultured microglial cells with the TLR2 agonist Pam3CSK4 (1 µg/ml) to induce a TLR2 specific activation and verify that o-vanillin works as a selective inhibitor of TLR2 signaling [120]. We evaluated the response on a translational and functional level.

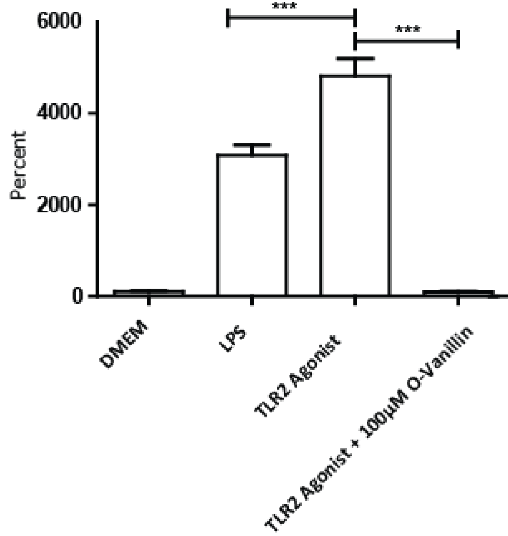
First, we analyzed the RNA expression of the already established TLR2 activation markers MMP14, MMP9 and iNOS [110, 116, 134]. We applied Pam3CSK4 for 6 hours and induced a significant increase in MMP9, MMP14 and iNOS on an RNA level.

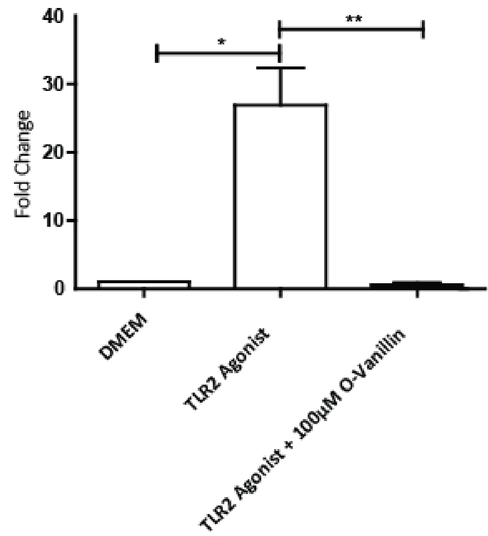
While the application of Pam3CSK4 increased the RNA expression of MMP14 by 2,4 times, we observed a much more pronounced effect in MMP9, which was increased by 26 times; the expression of iNOS increased 36-fold.

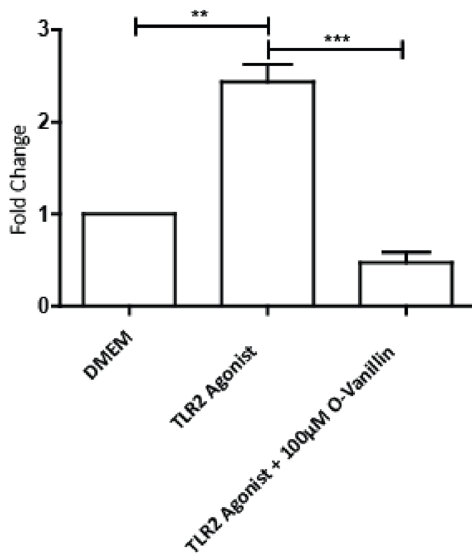
The effects on a functional level were similar. We observed a 48-fold increased IL-6 release.

The co-application of 100 µM o-vanillin prevented all stimulatory effects and even reduced the expression of TLR2 downstream targets compared to untreated controls.

Hence, the treatment with 100 µM o-vanillin inhibits the TLR2 dependent increase in predefined activation markers and results in regular expression levels. We were able to completely and significantly antagonize a specific TLR2 stimulation in microglia using o-vanillin.







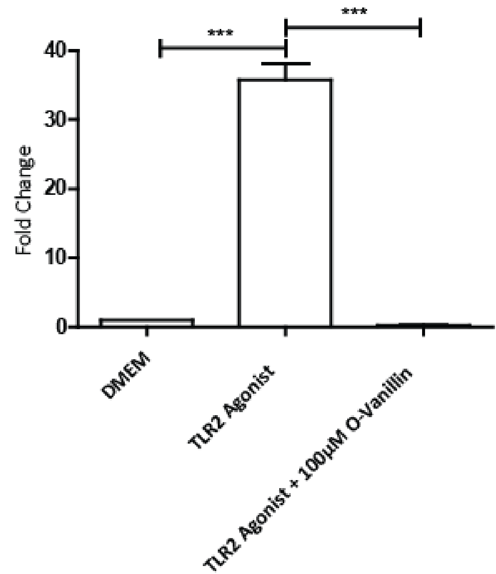


Figure 5. O-vanillin inhibits the agonistic effects of TLR2 stimulation by TLR2 agonist Pam3CSK4 on established TLR2 downstream targets in primary cultured microglia.

We stimulated murine microglial cells with a TLR2 agonist for 6 hours and studied the effects of a treatment with 100 µM o-vanillin on a functional level on **A)** IL6 and on the RNA expression level of **B)** MMP14 and **C)** MMP9 and **D)** iNOS. O-vanillin significantly reduces all downstream TLR2 effects.

O-vanillin is not cytotoxic to glial cells

Prior to the possible use of o-vanillin in a treatment process, we evaluated its cytotoxicity. So far, it has been reported that moderate doses of o-vanillin are not toxic to mice in general [125].

We want to broaden our knowledge by focusing on its effects in the CNS because the tolerability by native cells of the central nervous system is the basis of its suitability as possible glioma therapy.

We exposed primary cultured murine microglia and astrocytes, an immortalized oligodendrocyte precursor cell line (OLN93) and GL261 glioma cells to increasing doses of o-vanillin (1-100 µM) for 24 hours and analyzed the propidium iodide (PI) incorporation as a marker for permeable membranes in dead cells in a plate reader at 530/620 nm.

As can be seen in Figure 6., there are no differences in the PI fluorescence intensity between o-vanillin treated cells and those of an untreated control group. The application of o-vanillin in concentrations up to 100 µM was well tolerated by all the exposed cell types. The number of PI-positive cells was constant. The observed fluctuations never exceeded 5 % and were neither dose dependent nor significant. After the measurement, we incubated all cell samples with 10 % DMSO for 15 minutes to induce cell death. The increase in PI fluorescence intensity was essential and significant. The rate of PI-positive cells at least doubled, independently of cell type and o-vanillin concentration after DMSO exposure.

Increasing the treatment time up to 48h did not result in a further increase in PI fluorescence in o-vanillin incubated microglial cells (results not shown).

We additionally analyzed the PI incorporation in microglial cells after 24 hours of o-vanillin treatment using fluorescence-activated cell sorting (FACS) to validate these results. We exposed the cells to increasing concentrations from 1 µM up to 100 µM o-vanillin. Congruent with the PI fluorescence measured in the plate reader (Fig. 6), there were no significant changes in the PI incorporation sorting of the treated microglia samples via FACS (results not shown).

The rate of PI-positive cells that we considered as dead was constant and independent of the o-vanillin concentration, the exposition time and the method. O-vanillin has no cytotoxic effects on murine microglia, astrocytes, oligodendrocytes and GL261.

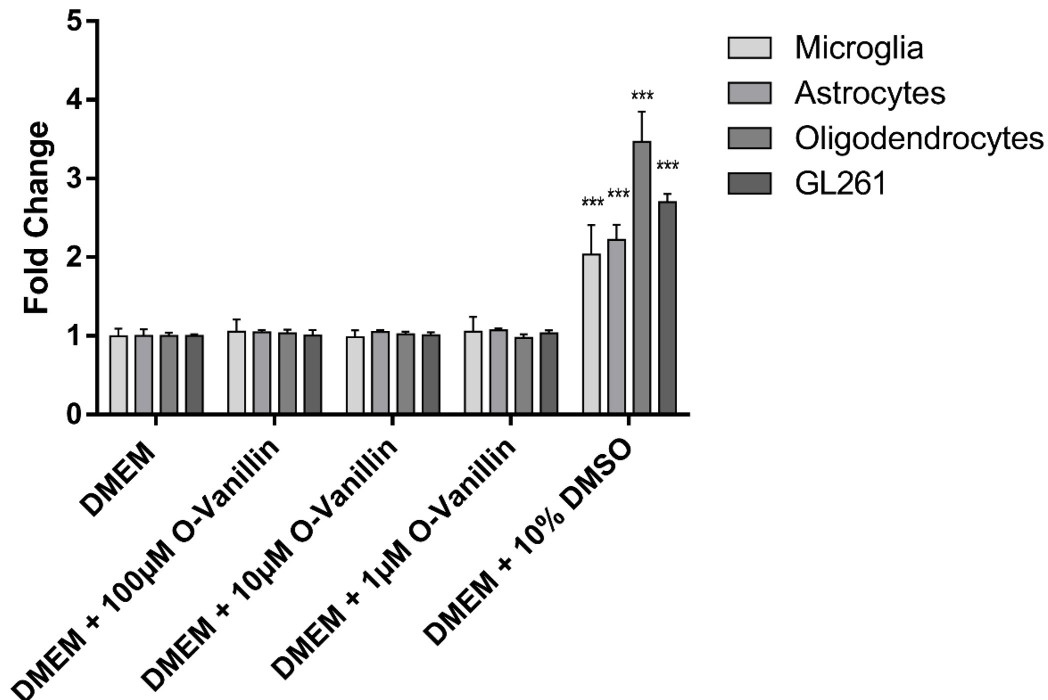


Figure 6. **Treatment with o-vanillin has no significant effects on the PI incorporation in murine microglial cells, astrocytes, oligodendrocytes and GL261 cells.**

Microglia, astrocytes, oligodendrocytes and GL261 glioma cells were treated with o-vanillin in increasing concentrations (1 µM-100 µM) for 24 hours. PI was added subsequently. The incorporation of PI was not changed by an o-vanillin treatment. After measurement, cells were incubated for 15 minutes in 10% DMSO as a positive control. The DMSO incubation led to a significant increase in PI fluorescence in every cell type and concentration. There was no evidence for cytotoxic effects of o-vanillin.

O-vanillin reduces glioma growth in organotypical brain slices

The recruitment and utilization of microglia/macrophages by gliomas is a crucial driver of tumor progression [103]. One mechanism already identified in the complex interaction is the upregulation of microglial MMPs due to TLR2 stimulation by glioma-derived versican [119].

After we demonstrated that o-vanillin is a potent inhibitor of glioma driving TLR2 signaling (Fig. 5), we then wanted to investigate how this inhibition affects tumor growth.

We applied o-vanillin to test whether tumor growth can be restricted by blocking TLR2 signaling in glioma-associated microglia/macrophages.

We injected green fluorescent GL261 cells into 250 µm thick murine organotypical brain slices to imitate the development of gliomas ex vivo. The tumor bearing brain slices were treated with different concentrations of o-vanillin (1 µM, 10 µM, 100 µM) for 5 days. The slices were fixed after treatment. We used a confocal microscope to determine the tumor surface in the microscopical sectional planes with the maximal glioma surface.

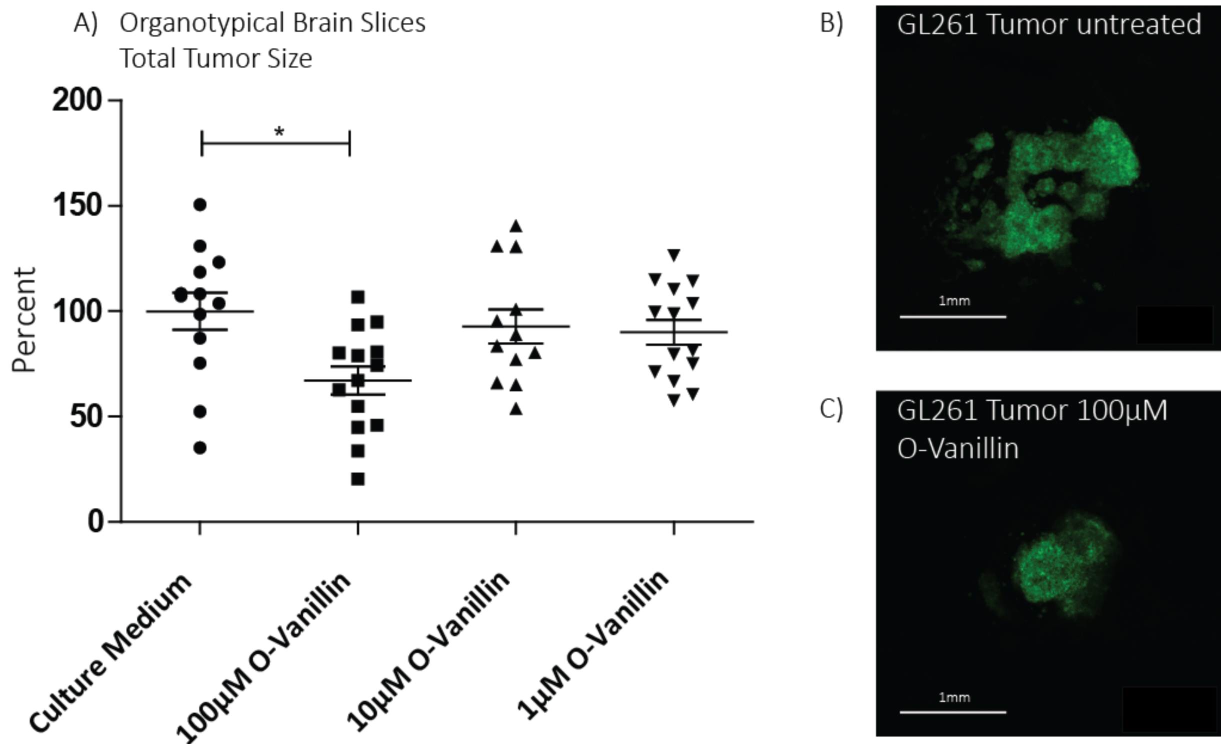


Figure 7. **Reduction of total tumor growth in organotypical brain slices after o-vanillin treatment.**

In **A)** the exemplary tumor surface in murine brain slice cultures was analyzed after injection of fluorescent labeled GL261 cells with or without o-vanillin treatment. Tumor cells were injected and slices were treated for 5 days with increasing concentrations (1 µM-100 µM) of o-vanillin. We defined the average tumor size in the untreated control group as 100% and compared it with the o-vanillin treated groups. The average tumor size in the group treated with 100 µM o-vanillin was significantly reduced by 31 %. The reduction in tumor size in brain slices treated with 1 µM or 10 µM o-vanillin was not significant.
B) is a fluorescent micrograph of Gl261 *egfp* cells after 5 days in a p14 C57/Bl6 brain slice culture without any treatment in comparison to **C)**, a GL261 *egfp* tumor treated with 100 µM o-vanillin.
There is a distinct tendency in the untreated tumors toward a faster and more heterogenous growth.

The treatment with 100 µM o-vanillin significantly reduced the tumor surface by 31 % compared to an untreated control. In slices treated with 1 µM and 10 µM o-vanillin, there was also a tendency toward

smaller tumors, but the fluorescent glioma surface was not altered significantly compared to that of the untreated control (Fig. 7 A).
Additionally, the tumors differed not only in size but also in shape. It became apparent that tumors in slices incubated with 100 µM o-vanillin appeared with fewer processes and a more homogenous distribution compared with untreated tumors, which showed an irregular undirected growth and an incoherent tumor mass (Fig. 7 B, C).
In summary, we can state that the treatment with 100 µM o-vanillin significantly reduces the total tumor size and modulates tumor morphology in a way that is most likely favorable.

Microglia migration is not affected by o-vanillin

The interaction between gliomas and microglia/macrophages always starts with the recruitment of the innate immune cells that contribute to up to 50 % of the glioma bulk [29, 64].
In Fig. 7 A we demonstrated that a treatment with o-vanillin significantly decreases the size of induced gliomas in slice cultures. We used a Boyden Chamber to quantify tumor-directed migration and evaluate whether previous findings can be attributed to a reduction of migrated microglia/macrophage.
We used glioma conditioned medium as a tumor imitating migratory stimulus. The distance between the upper and lower chambers functioned as a measure for the migratory affinity of microglia towards gliomas. The undirected migration in an untreated control without migration stimulus was defined as a reference value and the number of migrated cells determined after 6 hours was defined as 100 % physiologic migratory activity. After 6 hours in the Boyden Chamber, cells were fixed and HE stained. The overall surface was determined in the object field (20x magnification) of maximal cell count in a fluorescent microscope and quantified.
GCM was compared with ATP as a known positive migration stimulus [133]. We treated microglia with increasing concentrations of o-vanillin (1 µM, 10 µM, 100 µM) to investigate the effects of TLR-2 inhibition on migration.
The treatment with o-vanillin did not decrease the migration of microglia toward the glioma conditioned medium. There was rather a tendency toward the opposite effect (Fig. 8 A).
Unfortunately, neither ATP nor GCM had the statistical power to induce a significant migration as a positive control.

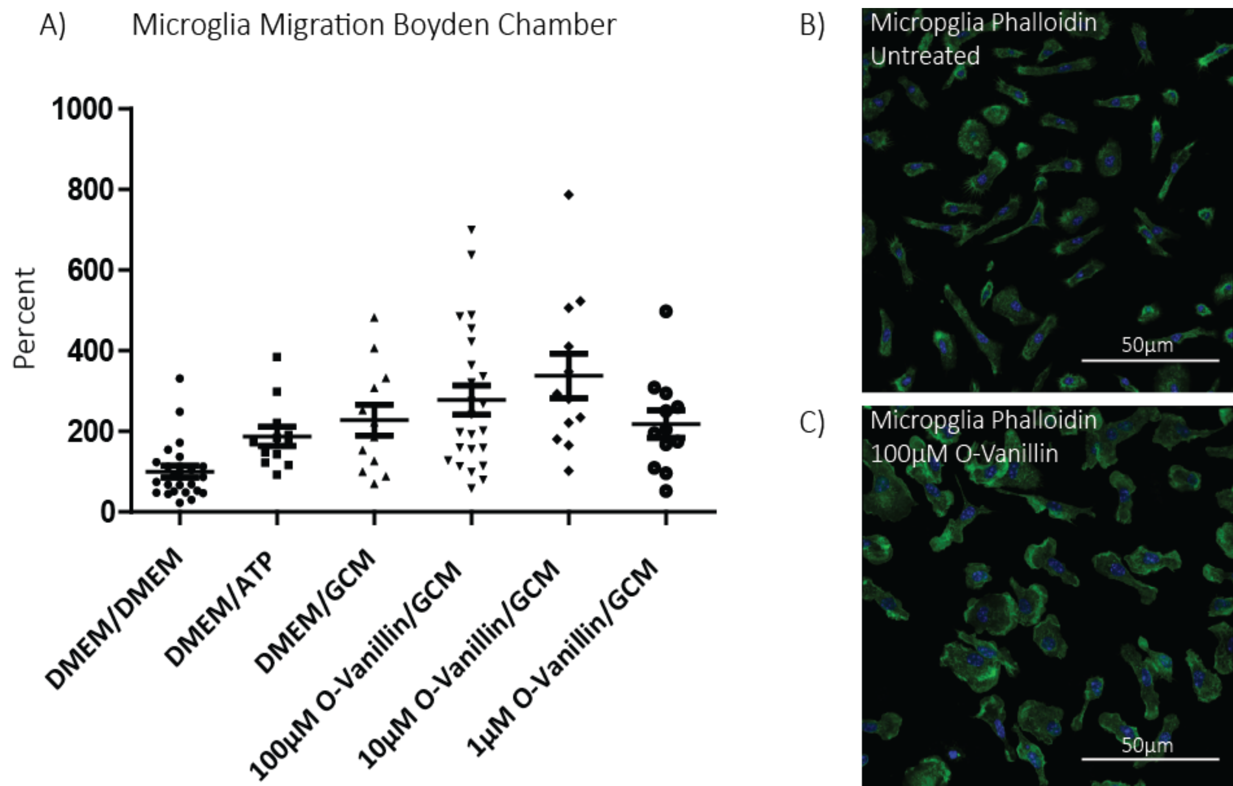


Figure 8. **The treatment with o-vanillin does not affect the microglia migration toward glioma conditioned medium in a Boyden Chamber.**

In **A)** the relative amount of migrating microglial cells was analyzed in a Boyden chamber. There were no significant dose dependent effects of o-vanillin (1 µM-100 µM) on the migration toward GCM in a 6 hour assay.
Neither ATP nor GCM induced a significant microglia migration.
B) is a micrograph of Phalloidin/DAPI costained untreated murine primary cultured microglia. **C)** is a micrograph of Phalloidin/DAPI stained primary cultured microglia treated with 100 µM o-vanillin with dominant membrane associated actin clusters hinting toward a migratory activity.

We also stained actin filaments of primary cultured microglial cells with fluorescent tagged Phalloidin to assess the migratory behavior of microglia on a histological level. We compared the morphology of an untreated control group with microglia incubated with 100 µM o-vanillin for 24 hours.
The o-vanillin treatment resulted in an accumulation of membrane-associated actin agglomerates (membrane ruffling) as well as in more pronounced lamellipodia. These observations can be interpreted as histological manifestations of increased migratory activity and could substantiate the tendencies from the Boyden chamber experiments (Fig.8 A)[133].
Due to high variances and hard to objectify histologic criteria, we are not able to make a clear statement about the effects of o-vanillin on the migration of microglia in a glioma context.

Glioma-induced microglia proliferation is reduced by o-vanillin

Emphasizing once more the importance of GAMs for glioma growth [96], we want to understand how a microglia/macrophages targeting o-vanillin treatment affects tumor size (Fig. 7 A).

In "Microglia migration is not affected by o-vanillin" we excluded a mitigated recruitment as a cause for the smaller tumors.

Therefore, we want to evaluate an insufficiently studied aspect of glioma-microglia interaction: proliferation. Do gliomas induce proliferation in microglia/macrophages and thereby amplify their own growth and is the TLR2 pathway involved and can its involvement be inhibited?

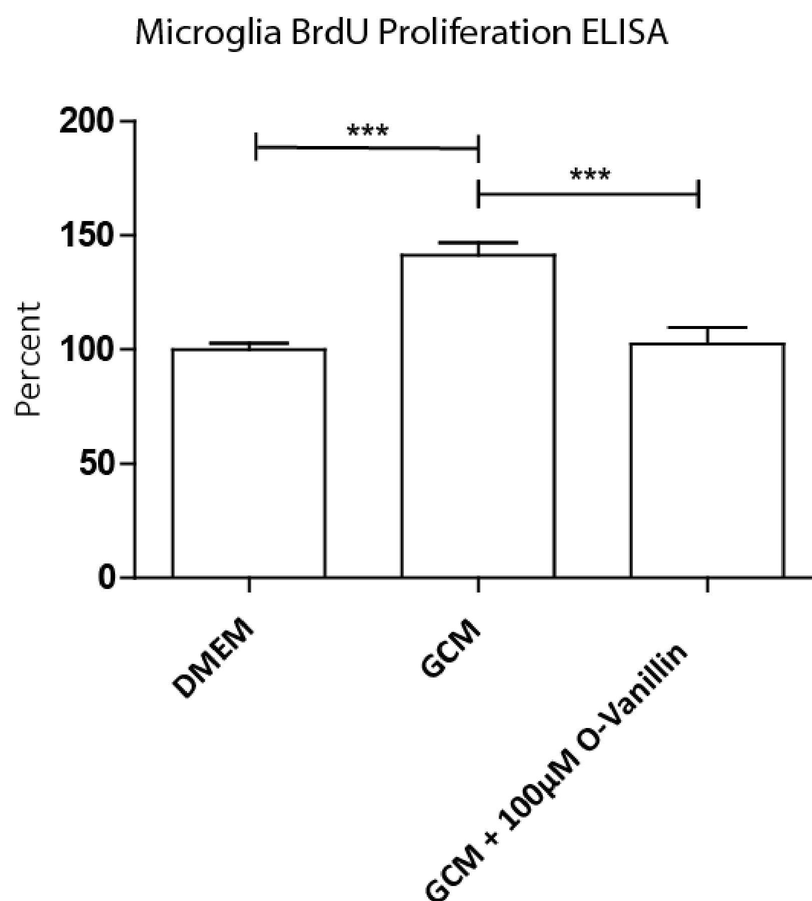


Figure 9. O-vanillin significantly reduces glioma induced microglia proliferation.

Murine primary microglia were stimulated with GCM and treated with 100 µM o-vanillin for 24 hours. The cells were simultaneously incubated with BrdU.

Stimulation with GCM significantly increased the proliferation by 41 %. The GCM induced increase of proliferation was significantly reduced back to normal levels by a treatment with o-vanillin.

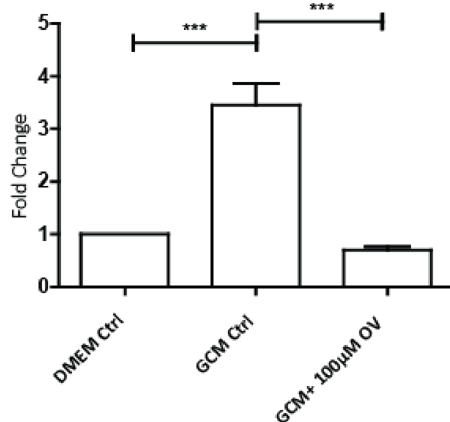
It is well known that microglia increase the proliferation rate of glioma cells, but reciprocal processes have only been evaluated to a limited extent [96].
To analyze the proliferation of microglia, we quantified the amount of fluorescent-labeled BrdU that was incorporated in 24 hours after previous overnight stimulation with GCM and treatment with o-vanillin. 1 µg/ml mMCS was used as a positive proliferation stimulus (results not shown) [132].
The stimulation of murine primary cultured microglia with glioma conditioned medium overnight induced a significant increase in the proliferation of microglial cells by 41 % (Fig.9) in 24 consecutive hours.
Hence, we were able to show that GCM significantly induces a notable proliferation in cultured microglia and that this effect can be inhibited by an o-vanillin treatment.

O-vanillin reduces the expression of microglial MMPs

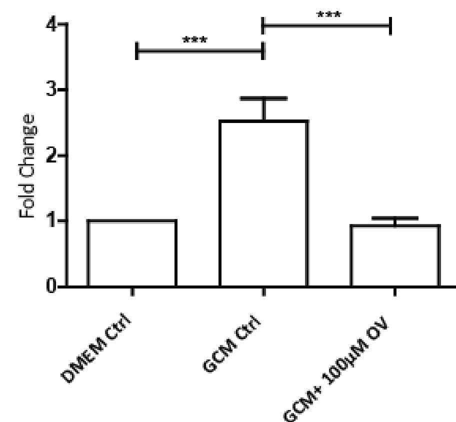
The importance of microglial MMPs for glioma invasion has been highlighted in various publications [96, 110, 114]. We investigated the effects of an o-vanillin treatment on the expression of the crucial MMPs 9 and -14. For this, we stimulated murine primary cultured microglia with GCM for 6 hours. The stimulation resulted in a significant increase by 3,5 times in MMP14 and by 2,5 times in MMP9 (Fig. 10 A, B). The co-application of 100 µM o-vanillin nullified these effects.
To transfer these findings into a more clinically orientated context, we isolated CD11 B positive monocytes (microglia and peripheral macrophages) from fresh human perioperative glioblastoma samples, cultivated and treated them.
We compared the expression of MMP9 and MMP14 after an overnight treatment with 100 µM o-vanillin with that of an untreated control. An untreated control was used as reference value. The RNA expression of MMP14 was significantly reduced by 24 % (Fig. 10 C), while the results in MMP9 (Fig. 10 B) showed only a strong tendency toward a reduction of MMP9 RNA expression in human CD11 B positive cells.
We were able to induce an increase in glioma-essential MMPs using GCM in murine microglia and significantly reduced the expression of glioma-induced MMP14 and MMP9 by an o-vanillin treatment.
Though partly limited by small numbers, we were able to adapt these results into human gliomas and reduced the expression of MMP14 in human GAMs using 100 µM o-vanillin.

Murine Primary Neonatal Cell Culture

A) Microglia MMP14 qPCR

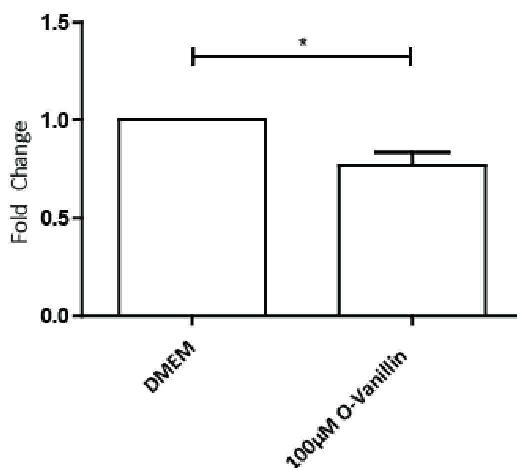


B) Microglia MMP9 qPCR



CD11b + Human Glioma Associated Monocytes

C) Monocytes MMP14 Expression



D) Monocytes MMP9 Expression

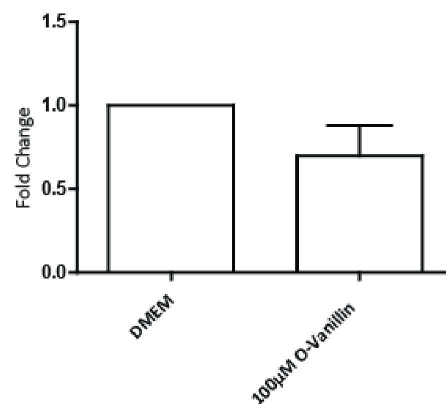


Figure 10. **O-vanillin significantly reduces the glioma induced increase of the TLR2 downstream targets MMP9 and MMP14 on RNA level in murine primary microglial culture and CD11b + human glioma associated monocytes.**

In A) and B) we induced a significant increase in the RNA expression levels of MMP14 (A) and MMP9 (B) in murine primary microglia cells by incubating the cells with GCM for 6 hours. The co-application of 100 µM o-vanillin led to a significant inhibition of this effect.
In C) and D) we treated CD11b + monocytes isolated from fresh human glioma samples overnight with o-vanillin and compared the expression with that of untreated controls. The expression of MMP14 (C) was significantly reduced by 24 %. In D) there is a strong tendency toward a reduction of MMP9 RNA levels after a o-vanillin treatment.

Discussion

O-vanillin specifically inhibits TLR2 signaling

Before we could start to evaluate a selective inhibition of TLR2 signaling in microglia/macrophages, we had to define this activation in a glioma centered context. We predefined the upregulation of TLR2 specific genes and cytokines as the condition for successful activation. The receptor stimulation with the TLR2 agonist Pam3CSK4 led to a significant increase in the TLR2 activation defining RNA expression panel in murine microglia. This panel includes MMP9 and MMP14 as indicators of a tumor invasion promoting ECM remodeling as well as iNOS and IL6 as characteristics of general inflammation in microglia/macrophages. We chose our panel to cover all aspects of TLR2 activation that we consider essential for malignant processes [114, 134-136].

We were able to nullify all the effects that resulted from the TLR2 stimulation in microglia by an o-vanillin treatment. The expression of TLR2 independent genes remained steady.

We used this experiment as proof of concept to adapt previous findings by Mistry et al., who were able to prevent a TLR2 facilitated increase of IL8 in peripheral macrophages using o-vanillin, into the context of central nervous monocytes and pathologies [120].

The TLR2 signaling pathway and the subsequent partly NFκB mediated reaction in TLR2 expressing cells, above all cells of the innate immune system, gains importance through the omnipresence of microglia/macrophages in cerebral inflammatory as well as the contribution of monocytes to malignant processes. While we already illustrated the impact of microglial MMPs for the invasiveness of gliomas, the TLR2 pathway has recently been found to have a tumor promoting effect in various malignancies and it is associated with bad prognoses in devastating tumors like ovarian carcinomas and melanomas [116, 137-139]. Because the evidence for TLR2 involvement in numerous different malignant processes has multiplied in recent years, it would be possible to apply the specific inhibition of TLR2 signaling not only in GAMs and a glioma context but in most tumors that either express TLR2 or interact with innate immune cells. It appears to be an attractive alternative to expensive anti-TLR2 antibodies in several set-ups, mainly due to its easy handling and its ability to penetrate the BBB [122].

O-vanillin is suitable to treat glial cell

After we had shown that o-vanillin works as a selective inhibitor of TLR2 signaling, we had to prove its tolerability before we could consider it as a potential glioma therapy in humans. Therefore, we tested the effect of o-vanillin on the viability of cells of the central nervous system. Previous experiments demonstrated a tolerance of even high concentrations of up to 2 mM vanillin on a cellular level and 100 mg/kg over one month in rodents without evidence for macroscopic anomalies or cell death [123, 126]. However, so far there has been no data about the effects of o-vanillin on cell populations of the central

nervous system. We chose a partly representative, orientating cross-section of affected cells in the CNS and evaluated the effects on different glial cells, namely microglia, oligodendrocytes and astrocytes to answer the simple question if o-vanillin is cytotoxic.
We chose the incorporation of PI as an established readout to differentiate between viable and dead cells and did not observe any harmful effects of an o-vanillin treatment in concentrations ranging from 1 µM to 100 µM in an incubation period of maximal 48 hours (data not shown).
Our findings slightly contradict the results of Martin et al., who reported reduced viability by 10 % after treating A375 melanoma cells for 48 hours with 250 µM [125]. Martin et al. treated the cells with higher concentrations and used an indirect XTT assay that is based on reductive processes to assess o-vanillin´s cytotoxicity [140]. We repeated our experiments using a similar, more comparable method to validate our results. We used alamarBlue, an assay that also visualizes a metabolic turnover and resembles Martin´s assay. The results were identical with our previous findings using PI as marker for cell viability, independent of the readout method.
In summary, we did not observe any toxic effects of o-vanillin in moderate concentrations up to 100 µM on glial cells and classify it with reservations, considering all available information, as harmless to cells of the CNS. Further experiments are required to substantiate our results.

Effects on tumor growth: o-vanillin mitigates glioma progression

One of the most important factors for patients´ overall performance and survival is, despite the grade of malignancy, the tumor size. It is not without reason radical resection remains the unquestioned therapy of choice [141].
To simulate a glioma developing situation *ex vivo* and test if the inhibition of TLR2 has a tumor suppressive effect, we injected GL261 cells in murine organotypical brain slice cultures and treated the slices with 100 µM o-vanillin over 5 days. The treatment led to a significant reduction in tumor size by 31 %. Based on these observations, we conclude that the inhibition of TLR2 signaling mitigates glioma growth. Under the premise that GAMs express high levels of TLR2, are vulnerable to TLR2 inhibition and massively contribute to the tumor bulk, we assign the observed reduced tumor size to a TLR2 inhibition in GAMs. Nevertheless, we need to consolidate our knowledge about the TLR2 expression in glioma cells and, even though a subject of controversial discussion, in astrocytes before we can clearly assign the observed effects to a TLR2 inhibition in GAMs [142, 143].
In addition to the question if glioma cells or other glial cell populations are affected by the inhibition of TLR2, previous research suggests an important role of microglial TLR2 signaling in glioma progression. We refer to Markovic et al., who already demonstrated that the inhibition of the TLR2 depending MyD88 pathway in microglia reduces the MMP14 expression and thereby glioma growth. The depletion of Myd88, TLR2 and microglia, in general, was shown to reduce the tumor size significantly [114].

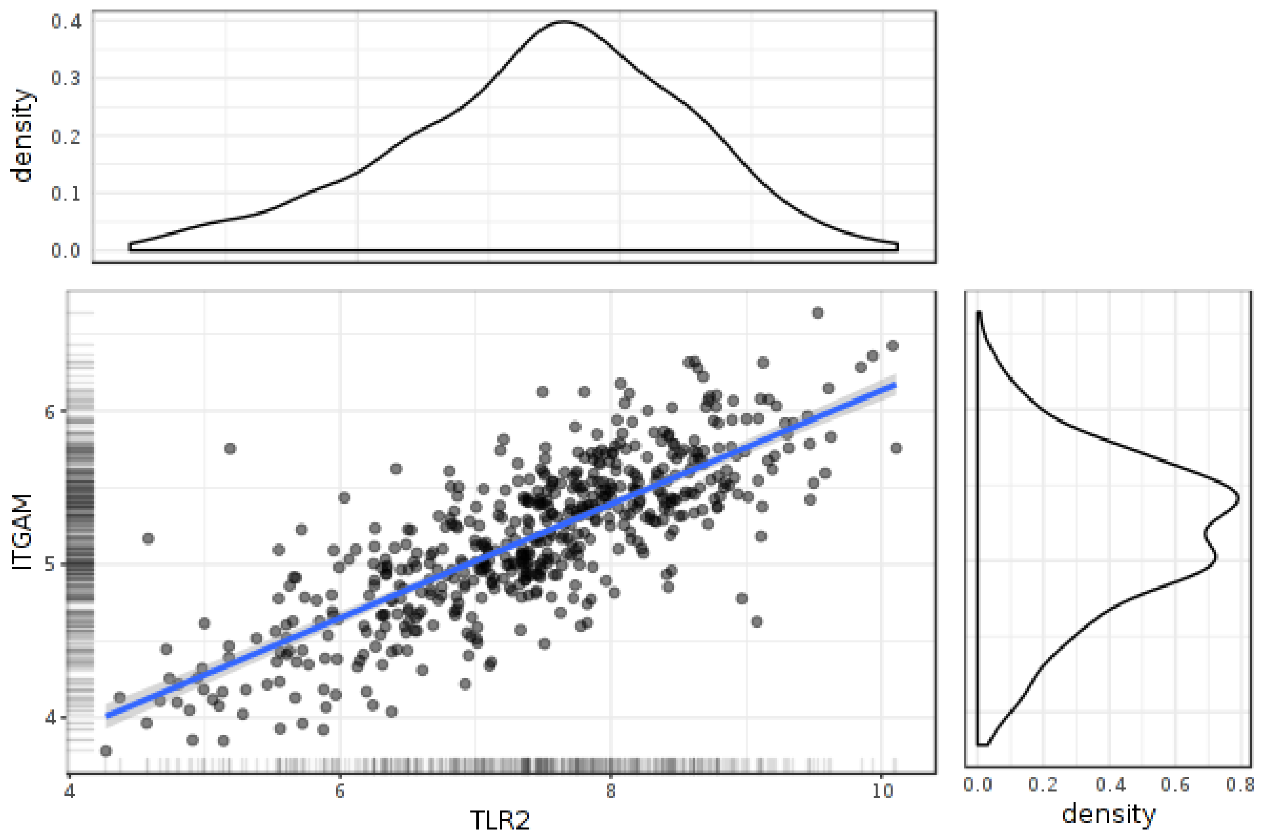


Figure 11. **Correlation between the RNA expression of CD11 B (ITGAM) and TLR2 in non-sorted human glioblastomas.**

The linear distribution of the macrophage/microglia specific marker CD11 B/ITGAM and TLR2 in human gliomas indicates that GAMs are one major contributor of TLR2 RNA.

Our analysis of TCGA data solidifies these findings. We were also able to show that the expression of TLR2 was significantly higher in gliomas that also expressed high levels of the GAM characterizing surface antigen CD11 B (ITGAM) (Fig. 11). This indicates that microglia/macrophages contribute heavily to overall TLR2 RNA levels of glioma and strengthens the assumption that o-vanillin inhibits especially microglia/macrophage TLR2 signaling.

It should also be noted that we analyzed not only the glioma surface but also the growth patterns in murine brain slice cultures. In this process, we discovered another aspect of the treatment: o-vanillin changes the macroscopic glioma morphology. An already elucidated challenge in glioma treatment is the early spreading of tumor satellites in the surrounding tissue. The histologic analogies to this problem are heterogenous polymorphous gliomas, morphologically characterized by processes and far spread glioma satellites [55]. The treatment with o-vanillin induced a change in this unfavorable growth profile. We observed smaller tumors with fewer processes and a more homogenous appearance. We interpret the attenuated invasive growth behavior as a result of reduced MMP expression due to the inhibitory effects

of an o-vanillin treatment. Based on these findings we consider o-vanillin as a neoadjuvant therapy that could improve the growth profile of gliomas and facilitate a better resection.

Microglia migration is not affected by o-vanillin

We already attributed the reduced glioma size at least partially to the effects of an o-vanillin mediated TLR2 inhibition in GAMs in "*Effects on tumor growth"*. We want to complement this result by answering a subsequently arising question: Are these effects caused only by a changed TLR2 signaling profile on a molecular level that was shown to be essential for glioma progression? Alternatively, does o-vanillin also influence the presence and accumulation of microglia/macrophages - keeping in mind that GAMs contribute to up to 50 % of the tumor mass [64]?

We injected *egfp* GL261 glioma cells into murine brain slices to evaluate the behavior of microglia in the presence of emerging gliomas. The use of microglia that express green fluorescent Iba1 allowed the visualization of microglial proliferation and migration. Unfortunately, it turned out that an exact quantification was rendered impossible due to a progressive maceration of microglia cells in cultured brain slices over days.

Hence, we approached this obstacle indirectly and evaluated glioma adjacent microglia/macrophages, focusing on the two events that we consider essential for microglia/macrophage accumulation: first, the migration toward the tumor and second, the proliferation of already present tumor-associated microglia/macrophages.

We used glioma conditioned medium and ATP as positive migratory stimuli to investigate the effects of TLR2 inhibition on migration and evaluated the effects of a TLR2 inhibition by o-vanillin.

The impact of TLR2 inhibition on directed migration turned out to be negligible. We instead found a tendency toward an increased migratory activity after TLR2 inhibition but with the addition that these results have to be assessed with reservations due to their extreme variability. However, our findings match previous studies by Ifuku et al., who were able to demonstrate a distinct role of TLR7 in directed migration, while TLR2 is responsible for undirected motility [144]. We hypothesize that the reduction of undirected motility results in an increased directed migration. Our histological findings support the theory of increased directed migration. Microglial cells exhibit characteristics of increased migratory activity following TLR2 inhibition, for example, membrane-associated actin agglomerates and pronounced lamellipodia [133]. To determine the influence of TLR2 inhibition on migration, we need to increase the number of experiments to gain statistical power and establish reliable histomorphological criteria to ensure an objective comparison.

At this point, we conclude that the inhibition of TLR2 does not affect microglia accumulation by a reduction of migratory processes toward the tumor. O-vanillin does not decrease the tumor size by the reduction of recruited microglia. This assumption correlates with the conclusions of Badie et al., who stated that the

effects of even massive chemoattraction, though very important for the gathering of GAMs, does not cover the presence of microglia/macrophages around gliomas to a full extent and can therefore hardly account for the observed effects of o-vanillin [145].

TLR2 inhibition reduces glioma-induced proliferation in microglia

Because we found no explanation for a reduced tumor size by a downregulated migration toward gliomas, we focus on the second approach to assess the microglial contribution to glioma progression: the proliferation of cells already recruited. Does TLR2 inhibition reduce the proliferation of glioma associated microglia/macrophages and is this effect responsible for a reduced tumor size as observed in brain slice culture experiments?

We already highlighted the processes that result in an increased glioma cell proliferation promoted by microglia derived growth stimuli. To improve our understanding of the complex microglia-glioma-interactions, it is essential to add that growth enhancement is bilateral. Not only microglia but also glioma cells provide growth factors that operate in para- and autocrine fashion. Though the vice-versa processes have not been extensively investigated, there is disagreement about the extent of glioma-induced microglia proliferation. While Badie et al. found increased Ki67 levels in GAMs that are equivalent to our results in cultured murine microglia, Ellert-Miklaszewska et al. did not find a significant increase in the BrdU incorporation in GCM stimulated microglia [145, 146]. Further experiments are required to clearly assign the effects of glioma signaling on microglia/macrophage growth.

However, while some factors, e.g., GM-CSF have been the subject of intensive research, a broad variety of proliferation-promoting factors in the mélange of glioma growth stimulating signaling remains unknown [28].

One of them is TLR2. The TLR2 pathway has hardly been investigated in terms of proliferation but a TLR2 induced proliferation has been verified in many different physiological contexts, for example, in intestinal epithelial cells, glomeruli cells and T-cell proliferation as well as in different pathologies [147, 148]. It turns out that the TLR2 dependent NFκB upregulation represents a primary trigger for the proliferation of malignancies of the lymphatic system [149].

We induced proliferation in murine cultured microglia by glioma conditioned medium that contains the TLR2 agonist versican [119].

The specific inhibition of TLR2 using 100 µM o-vanillin suppressed the proliferation inducing effect. We assume that a glioma-induced TLR2 mediated NFκB activation in microglia results in an increased glioma promoting proliferation of GAMs. This effect can be antagonized with o-vanillin as a specific TLR2 inhibitor. Though the exact mechanism remains a subject of speculation, the impact of o-vanillin as an inhibitor of glioma-induced microglia/macrophage proliferation adds a still insufficiently studied aspect to its suitability as glioma treatment.

We need more data for a comprehensive evaluation of the effects of direct TLR2 stimulation on proliferation using different TLR1/2 and TLR2/6 agonists to implement an understanding of this aspect of TLR2 signaling.

O-vanillin disturbs the microglia-glioma interaction

After we proved that the inhibition of TLR2 reduces the proliferation of glioma associated microglia and subsequently the glioma size, we wanted to turn our attention to another more particular mechanism: the TLR2 mediated upregulation of microglial MMPs.

The expression of TLR2 in glioma samples is inversely correlated with patients´ overall survival [116]. We complemented existing TCGA analyses by including prominent MMPs into the survival analysis and found a correlation not only between TLR2 but also MMP14 and MMP9 and overall survival as well as a correlation between TLR2 expression level and malignancy grade (data not shown). These findings support the idea of expanding an already established glioma therapy regimen by the inclusion of tumor-supporting cells as targets. However, so far, practical approaches are in their infancy. A clinical trial with Bevacizumab, a humanized monoclonal antibody that targets VEGFa, was not beneficial at all [84]. Right now, the University of Utah is recruiting patients to investigate the effects of minocycline on recurrent gliomas. Minocycline inhibits amongst others an MyD88 depended pathway in microglia and represents a similar approach as the o-vanillin treatment by aiming at GAMs instead of glioma cells, but the exact mechanism of action remains elusive in minocycline [150]. We managed to inhibit a glioma-induced increase in MMP9 and MMP14 by an o-vanillin treatment in order to reduce the glioma promoting effects of both MMPs.

The understanding of versican-TLR2 signaling as an essential pathway in microglia/macrophages-glioma interaction that results in a tumor-promoting increase of MMPs is the foundation of our new therapeutic attempt. The TCGA data supports our approach to alleviate glioma promoting effects of GAMs and suggests that patients suffering from gliomas with a high TLR2 expression could highly benefit from an o-vanillin treatment.

Adaptation into a human context

O-vanillin turned out to be a sufficient inhibitor of MMP overexpression in murine GAMs. It attenuated TLR2 signaling in murine microglia and significantly reduced the expression of tumor-promoting genes *in vitro* as well as tumor growth *ex vivo*. The next step was to repeat these effects in a more clinical environment and adapt them into a human glioma setting. For this, we isolated human glioma-associated microglia/macrophages using magnetic-activated cell sorting from fresh glioma samples and treated the isolated CD11 B positive glioma-associated monocytes.

We compared untreated controls with cells treated with 100 µM of o-vanillin and observed a significant decrease of MMP14 expression. We also performed alamarBlue cell viability assays on samples that we included in our data to exclude unviable cells.
Considering the small numbers of gliomas and the partly desolate condition of the cells after isolation, accompanied with a general decrease in gene expression after storage for several hours on ice, we are sure to reach a level of significant reduction for MMP9 by increasing the numbers.
Although not significant for all the tested MMPs, the collected data is highly suggestive of being adaptable from a murine to a human context.
The reduction of negative effects of microglial MMPs in patients suffering from gliomas using o-vanillin as a specific TLR2 inhibitor bears the potential to attenuate the invasive growth behavior and thereby improve the patients´ overall survival. Despite that, o-vanillin as a small lipophilic molecule could be a promising candidate facing an omnipresent problem of targeted therapy: the delivery of medication. The blood-brain-barrier shields the central nervous system efficiently from extrinsic factors and thereby aggravates the accumulation of drug doses needed for successful therapy.
While we have already observed promising results in glioma treatment in vitro using TLR2 specific antibodies, previous clinical trials faced recurring difficulties when it came establishing sufficient therapeutic concentrations in the brain. We hope to circumvent the obstacle of the blood-brain barrier with o-vanillin, which was shown to penetrate the barrier [122].
In summary, we were able to inhibit all glioma promoting TLR2 dependent activity in GAMs with an o-vanillin treatment and assess o-vanillin as a suitable molecule to complement existing glioma therapy because of its high tolerability, its specific inhibitory abilities and its reported penetration of the blood brain.

Outlook

So far, we were able to show that o-vanillin is a specific inhibitor of TLR2 signaling in microglia and that it reduces the tumor-induced increase in glioma promoting MMPs. We further showed that a treatment with o-vanillin reduces the growth of gliomas in murine slice cultures and that it reduces the glioma-induced increase in microglia proliferation. However, although these results suggest o-vanillin´s high potential as an additional therapy option in the treatment of gliomas, several questions remain unanswered.
To complement the research necessary to determine o-vanillin´s suitability as a glioma treatment, we would like to investigate further in three different experimental settings.
The first and in our opinion most important aspect is to evaluate in an animal experiment the biological availability including a dose-response curve of o-vanillin. We want to test the penetration of the blood-brain-barrier to estimate if a reasonable dose results in a sufficient concentration in the central nervous

system as described by Dhanalakshmi et al. [122]. In this trial, we also want to rule out significant adverse effects.

One important aspect we neglected in our research was the influence of o-vanillin on two other cell populations in the glioma bearing brain: neurons and the glioma cells.

We need to evaluate the effects of an o-vanillin treatment on those cells especially because TLR2 is overexpressed in gliomas and the expression correlates with patients´ overall survival [116]. Though we have continued our research on the effects of TLR2 inhibition in glioma cells, it is still unknown how neuronal networks are affected by o-vanillin.

The last missing aspect is an analysis of interferences. If we plan to use o-vanillin in addition to standard care, we need to find out how it interacts with current therapy regimens. Especially temozolomide and dexamethasone, part of the established glioma medication, are drugs with significant effects on gene expression, cell viability and metabolism. Dexamethasone in particular was reported to interfere with NFκB and little is known to what extent this interaction affects or is affected by the inhibition of TLR2 signaling [81, 87].

Therefore, we would like to deepen our knowledge of the interference between the essential glioma treatment and a concomitant TLR2 inhibition as the foundation of a responsible glioma therapy concept.

Limitations

The first and probably most important limitation is the use of murine cells, especially in cell culture. While there are many similarities between the genesis and characteristics of gliomas in mice and humans, we must not forget that there are vast differences in genotype profiles between an artificially generated murine glioma cell line such as GL261 and individual human malignancies. Szulzewsky et al. all provide a good overview of similarities and differences in essential genetic aberrations between human malignant gliomas and experimental murine gliomas [99]. This obstacle can be avoided by using microglia-like iPS cells and cultivated human gliomas - pilot experiments were performed in spring 2019 [151, 152].

We have already found evidence for the tolerability of o-vanillin in concentrations of up to 100 µM for up to 48 hours (data not shown). Now we need to increase both the concentration and the incubation time to create a dose- and time dependent tolerability curve, not only for the glial cells already investigated but also for all missing populations, especially neurons and inter-neuronal networks as well as for glioma cells that were reported to express TLR2 [119].

We also want to complete our results by adding in vivo data to analyze the biological availability. We observed a reduction in tumor size in treated brain slices when we evaluated the effects of o-vanillin on tumor growth, but we were unable to picture the microglia distribution around gliomas due to technical difficulties and could not determine the contribution of glioma-associated microglia to the glioma bulk. This

lack of information makes it more difficult to determine if the reduced tumor size is due to TLR2 inhibitory effects on microglia, glioma cells or a different cell type. We want to inject *cherry red* fluorescence labeled tumors into the brains of Iba1 *egfp* mice and analyze the distribution of microglia without previous cultivation to prevent these difficulties. In this setup, we would be able quantify the proportion of microglia and glioma cells and interrelate the proportions of a treated and an untreated group.

Hypothesizing the reduced tumor growth can at least be partially attributed to an attenuated proliferation of glioma-associated microglia/macrophages after TLR2 inhibition as indicated above, we need to investigate on the effects of TLR2 stimulation and inhibition on cell proliferation in vitro. Do TLR2 agonisms contribute amongst other already known factors in the tumor-secreted cocktail to increased proliferation in microglia? Moreover, if so, to what extent? Another approach to increase the reliability of our results is to include different markers and methods to quantify proliferation, for example, Ki67 on an RNA level or a FACS analysis of DAPI stained nuclei to determine the cell cycle phase of single cells.

The next experiment that needs to be regarded critically is our migration study. While analyzing the total surface areas of exemplary fields after migration of microglia, we observed a high variance. Additionally, we could analyze single round particles instead of a surface to improve the liability of our experiment.

Without statistical significance, it is impossible to interpret the migration tendency of microglia after TLR2 inhibition by o-vanillin. We need to increase the number of experiments in order to provide a clear statement and determine whether pursuing this question is worth both the time and effort.

A different aspect of this experiment that needs to be interpreted with caution is the assumptions regarding histologic cytoskeletal features. Based on the characteristics of migratory activity by Honda et al., these interpretations remain subjective to a certain degree and complicated to quantify [133].

The last aspect that we must treat with caution is the limited amount of literature about o-vanillin. Most experiments were performed with vanillin and it is hard to estimate the effects of slight molecular differences.

Additionally, the interindividual variance and differences in the quality and nature of human glioma samples complicated the analysis. First, we had to evaluate the quality of our tumor samples. Before we could process the glioma samples, the tissue was exposed to high extrinsic stress during surgery and transport for up to 24 hours. These stressors, especially the transport on ice, reduce the cell viability and thereby the general gene expression [153]. The size of the glioma samples was in many cases not sufficient to isolate an adequate number of CD11 B positive cells. To achieve a reliable RNA quality, we required at least 500 000 CD11 B positive cells per condition, which equals a tumor weight of approximately 500 mg and does not include the cells needed for obligate viability controls. Though we faced many difficulties, the results on an RNA level appear to be transferable and suggest that the beneficial effects of an o-vanillin treatment we observed in mice would be similar in humans.

Despite these limitations, we were able to answer our initial question: Can o-vanillin inhibit the upregulation of glioma promoting microglial MMPS by attenuating TLR2 signaling adequately in primary murine cultured cells and reduce glioma size? The answer is yes, at least in the context of murine microglia cultures and the limited human GAMs we had access to.

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Publications

Int. J. Mol. Sci. 2020

Paul Triller, Julia Bachorz, Michael Synowitz, Helmut Kettenmann and Darko Markovic

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