Supporting Information

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SI Text

Cell Culture and Media. Medium-1 consisted of Dulbecco minimum essential medium with 4,500 mg/L glucose and L-glutamine supplemented with 10% heat-inactivated FCS (FBS), 0.2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Medium-2 consisted of Dulbecco minimum essential medium containing 25% heat-inactivated horse serum, 50 mM sodium bicarbonate, 2% glutamine, 25% HBSS (Hanks balanced salt solution, without $CaCl_2$ and $MgCl_2$), 1 μg/mL insulin, 2.46 mg/mL glucose, 0.8 μg/mL vitamin C, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5 mM Tris (Tris-aminomethane; all from Gibco).

Cell Culture Technique for Microglia. Microglial cultures were prepared from cerebral cortices of newborn C57/Bl6 mice (WT, transgenic, or knockouts) or from rat pups as described previously (1). Animals were decapitated, and skin and skull were removed simultaneously. Then, the brain was dissected from the brainstem and transferred to cold HBSS. The cerebellum and olfactory bulb were taken off. Then, the blood vessels and meninges were carefully removed from the hemispheres. The tissue was collected in a 15-mL flask and washed 3 times with HBSS. After the final wash, 200–300 μM trypsin/DNase and 200 μM DNase were applied and left at room temperature (RT) for 4 min. The reaction was blocked with 2 mL of medium-1. The tissue was mixed carefully 10 times with a plastic Pasteur pipette and then 5 times with a fire-polished glass Pasteur pipette. Medium was added to fill the 15-mL Falcon tube and centrifuged for 10 min at 100 *g* at 4 °C. The supernatant was discarded and the pellet was re-suspended with 2 mL medium and plated in T 75 culture flasks (2–3 mouse brain preparations per flask; TPP). Cultures were incubated at 37 °C, 5% CO_2, and the next day adherent cells were washed 5 times with PBS solution. After culturing for 1 week, 10 mL medium-1 and 5 mL of L929 (fibroblast cell line)-conditioned medium was applied for 2 d. The microglia are now seen as floating cells or as semi-adherent cells on top of an astrocytic monolayer. Microglia are harvested by shaking the culture flask at 37 °C, 15 *g*, for 30 min and centrifuging the medium at 100 *g* at 4 °C for 10 min. The cells were seeded in 6-well plates at a density 10^6 cells/well. Cultures usually contain >95% microglial cells, which can be stained with isolectin B4, a marker for microglia. For experiments cultures were used 1 to 5 d after plating.

Cell Culture Technique for Glioma Cell Lines. The GL261 cell line was obtained from American Type Culture Collection, the C6 cell line was purchased from National Cancer Institute, and U373 cells were from the European Collection of Cell Cultures. GL261, C6, and U373 glioma cells were grown in medium-1 in T 25 tissue culture flasks. The mouse GL261 cell line was selected for its isogenicity to the donor animals (C57/BL6 mice) of the brain slices; C6 cells are derived from rat and were used for experiments with rat microglia; U373 is a human astrocytoma line that was inoculated into immunodeficient (i.e., SCID) mice. The medium was changed every 2 d and cells were passaged when the cell density in the flask reached confluence. Cell cultures were maintained in the incubator at 37 °C in humidified and 5% CO_2 conditioned atmosphere.

GCM. GCM was then prepared from the 80% confluent GL261 cultures (or from 80% confluent C6 cells in the rat model) by cultivation in medium-1 overnight. Then the GCM was collected and sterile-filtered through 0.2 μm filter mesh and applied immediately on microglia cultures.

Glioma Transfection. The ORFs for EGFP were sub-cloned from pEGFP-N1 vector (Clontech) into the vector pMP71GPRE via the NotI and EcoRI restriction sites. Stable producer cell clones were generated by co-transfection of 293–10A1 packaging cells with either of the retroviral vector plasmids and the plasmid pWLneo (Stratagene). Retroviral titers were as follows: 5×10^6 to 1×10^7 cfu/mL. Transduced cells glioma cells were purified by FACS.

Microglia Transfection. Primary microglial cultures were transfected with jetPEI macrophage transfecting reagent according to manufacturers instructions (Polyplus). Briefly, microglia were plated in a 6-well plate at a density of 8×10^5 cells/well. On the second day, the mixture of 1.2 μg MT1-MMP luciferase and 0.3 μg Renilla luciferase plasmids for reporter assay experiments or 3 μg of MT1-MMP shRNA for MT1-MMP activity assay or gelatin zymography were incubated with 100 μL of NaCl (150 mM) for 10 min, and then the 100 μL of NaCl (150 mM) containing 3 μL of jetPEI reagent was added to plasmid mixture and let to incubate at RT for 30 min. The complexes of plasmid DNA and jetPEI were than added to microglia containing 2 mL culturing medium for 48 h.

Organotypic Brain Slice Model. The procedure to establish organotypic brain slice cultures was descibed previously (5). Brain tissue was derived from 16-d-old male C57BL/6 mice (animal breeding facility; Charles River). For the organotypic brain slice culture preparations, these mice were decapitated and the skin on the head was cut and removed. Then, the skull was opened and the brain was taken out of the brain cavity using a spatula and placed in ice-cold PBS solution. The cerebellum was cut off with a razor blade. The cerebrum was glued with cyanoacrylate glue onto a cutting block on its caudal side and supported from the back side by a 2% agar block. Coronal sections of 250 μm were then cut with a vibratome. The brain slices were collected with a glass pipette (with its wider opening) and transferred onto a 0.4-μm polycarbonate membrane in the upper chamber of a transwell tissue insert (Falcon cell culture inserts, 0.4 μm, 6-well format; BD Labware), which was inserted into a 6-well plate (BD Labware). Excess PBS was removed in a laminar flow bench. The brain slices were cultivated at the air-liquid interface in 1 mL of medium-1 per well. After overnight equilibration of the brain slices in medium-1, medium-1 was exchanged for medium-2.

Organotypic Brain Slice Model Liposomes. Clodronate-loaded liposomes were obtained from the Department of Molecular Cell Biology of the Free University of Amsterdam. For the preparation of clodronate liposomes, 86 mg of phosphatidylcholine (EPC; Lipoid) and 8 mg of cholesterol (Sigma) were combined with 10 mL of a clodronate solution (0.7 M; Roche Diagnostics) and gently sonicated. The resulting liposomes were then washed to eliminate free drug. For control experiments, PBS solution-filled liposomes were prepared under the same conditions. All liposomes were passed through a 12-μm filter immediately before use to eliminate large lipid aggregates.

Organotypic Brain Slice Model Glioma Cell Injection. T 25 culture flasks confluent with EGFP-expressing glioma cells were trypsinized and centrifuged, and the pellet was resuspended into

200 μL medium-1. The cell number was adjusted to $10^4/\mu L$. Five thousand GL261 glioma cells within a defined injection volume (0.5 μL) were inoculated into the slices by using a 1-μL syringe (Mikrolitерspritze 7001N; Hamilton) with a blunt tip mounted to a self-constructed micromanipulator. To ensure identical experimental conditions, the gliomas were always inoculated into the same anatomical area. Therefore, the tip of the syringe was always placed at the same defined region on the slice surface. The EGFP-expressing glioma cells were inoculated below the corpus callosum into the globus pallidus. Using the micromanipulator, an injection canal was formed, reaching 150 μm deep into the 250-μm-thick slice. Then, the needle was retracted by 50 μm, leaving an injection cavity of approximately 50 μm. The cell suspension was slowly injected over a period of 30 s and subsequently the syringe was slowly pulled out in 10-μm increments over a period of 60 s. Directly after injection, the glioma cells remained at the inoculation site, which could therefore mark the point of origin for all further movements of these cells. Careful control of the injection procedure ensured that no cells spilled onto the surface of the slices, which could then migrate over this surface rather than invade through the tissue.

Organotypic Brain Slice Model Immunofluorescence. The primary antibodies were applied at the indicated dilutions overnight in TBS++ buffer (4 °C). This was followed by 3 washing cycles in TBS+. Then, the appropriate fluorescent conjugated secondary antibody was applied (1:125 ratio) for 3 h at RT. Finally, the slides were then washed 3 times in TBS+ and mounted with mounting medium.

Organotypic Brain Slice Model Quantification of Glioma Cell Invasion. Fluorescence microscopy images obtained at ×10 optical magnification were stored as tagged image files (.tif) with a resolution of 72 pixels/inch. As one tumor was usually too large to be photographed with only one picture, series of micrographs were made to cover the complete tumor. With Photoshop software (Adobe Systems), the images were merged into one picture that represented the whole tumor. Finalized images were analyzed with Image Pro plus software (Media Cybernetics). This software allowed us, after calibration, to measure the distance of the single glioma cells from the injection site or the surface of the tumors. Then, the values relating to the area of the single tumors were collected and exported into Excel (Microsoft). We used at least 8 tumors from each experimental group from 3 independent experiments for statistical evaluation.

Organotypic Brain Slice Model Time-Lapse Imaging. Time-lapse recordings of glioma cell migration in the brain slice cultures were performed with a 2-photon laser scanning microscope (TILL-Photonics), controlled by FluoView software (Olympus). The light source was a Chameleon Ultra II tuneable Ti:Sapphire laser (Choherent), operating at 920 nm. Image stacks (15–25 optical sections spaced at 5 μm) were acquired every 10 min with a ×40 water-immersion objective (0.8 NA, LUMPlanFL/IR; Olympus) for 6–8 h.

Analysis of the cell migration was performed using custom-made software, written in MatLab (Mathworks), and consisting of the following steps: each image was filtered with non-linear band-pass filter, cell positions were identified in each stack by computing local maxima in the distance transform, and cell trajectories were reconstructed from these positions. Straight-line velocities were determined by dividing the straight-line distances, represented by the shortest line connecting the start and the end positions of the cell migration trajectory, by the time of recording.

Reporter Gene Assay. A full-length fragment (7.2 kbp) of the human MT1-MMP promoter region (2) was cloned in front of luciferase gene and the resulting construct was transiently transfected into microglia using jetPEI macrophage transfecting reagent (Polyplus). The activity of MT1-MMP-Luc was compared with mutated MT1-MMP-Luc promoter constructs in which the Sp-1 site had been altered by site directed mutagenesis (3). The Sp-1 binding consensus sequence GGGGCGGGGA was replaced by non-functional GGGCCATGGT. Transfection efficiency was determined by co-transfecting the promoter-luciferase construct with pRL-TK control plasmid containing the herpes thymidine kinase promoter linked to the Renilla luciferase gene (Promega). Increase in luciferase levels was compared with microglia cultures transfected with a promoter-less pGL3-Basic plasmid (Promega).

The Dual Luciferase assay (Promega) was used according to manufacturer instructions. Briefly, cells were seeded at 10^5 cells per well of a 6-well plate, incubated in jetPEI transfecting reagent for 48 h, stimulated with GCM for 6 h, then washed with PBS solution and agitated gently for at least 20 min with 500 μL passive lysis buffer. The cell lysate was cleared by centrifugation and 20 μL was added to 100 μL of luciferase assay reagent in a luminometer tube. The relative light units were measured immediately in a Berthold-2000 luminometer. The average of 10 relative luciferase units measurements taken at 2-s intervals was recorded.

Western Blotting.
Sample buffer was 1% SDS, 1% Tx-100 with complete proteinase inhibitor (Roche) in TBS (pH 7.4).

The 10X running buffer was 30 g Tris, 140 g glycine, and 10 g SDS added to 1 L H_2O.

Transfer buffer was 2.93 g glycine, 5.81 g Tris, and 0.375 g SDS added to 800 mL H_2O and 200 mL methanol.

Lower gel buffer was 72.7 g Tris and 1.6 g SDS added to 400 mL H_2O (pH 8.8).

Upper gel buffer was 18.2 g Tris and 1.2 g SDS added to 300 mL H_2O (pH 6.8).

Western Blot Sample Preparation. The cells were quickly washed 2 times with ice-cold PBS solution and then all PBS solution was carefully removed. Sample buffer was applied to the cells (10 $\mu L/cm^2$) and the cells were scraped with a rubber policeman. Cell lysates were collected in a 1.5-mL tube and incubated on ice for 15 min. Then, the samples were centrifuged at 25,000 *g* for 20 min. The supernatant was collected and the pellet discarded. The protein concentration was determined with the BCA protein assay kit (Pierce). In all samples, the protein concentration was equilibrated with sample buffer. Mercapto-ethanol (2%) and glycerol (20%) were added to the samples, and they were incubated at 95 °C for 15 min.

Western Blot SDS-PAGE. The 10% polyacrylamide gels were cast in glass plates. The gels were loaded with 10 μL molecular weight marker and 20 μL of each sample. The gel was then run for 10 min at 100 V, then for 70 min at 150 V.

Western Blotting: Semi-Dry Trans-Blotting. Following electrophoresis, the gels were equilibrated in transfer buffer for 10 min. Meanwhile, a PVDF membrane (Hybond-P; Amersham Biosciences) was activated by incubation in methanol for 5 min, and equilibrated in transfer buffer for 5 min. Simultaneously, blotting paper was incubated in transfer buffer for 5 min. Then, the moist blotting paper was placed on the lower electrode (i.e., anode), the PVDF membrane onto the blotting paper, and then the separating gel onto the membrane and covered with another moist blotting paper. This "sandwich" was covered with the upper electrode (i.e., cathode) and electro-blotted at 15 V for 60 min.

Western Blotting: Immunoblotting. The membranes were first blocked with Western blot blocking buffer [TBS Tween-20 (TBST): 100 mM Tris, 150 mM NaCl, pH 7.4, 0.05% Tween-20; blocking buffer, 5% fat-free milk powder in TBS Tween-20] for 30 min. Then, the primary antibody was applied in blocking buffer overnight at 4 °C on the shaker. After that, the membrane was washed 3 times in TBST and incubated with HRP-conjugated secondary antibody for 1 h at RT. After 3 washes for 20 min in TBST, the ECL reagent (Hyperfilm ECL; Amersham Biosciences) was applied for 5 min. The membrane was dried from excess ECL reagent and placed on the transparent foil. The membranes were placed in a film cassette and exposed to ECL films (in the dark room) for various time periods (e.g., 15 s, 30 s, 1 min, 5 min), and the films were developed in the film developing machine.

MT1-MMP Activity Assay. This ELISA-based assay measures amounts of active MT1-MMP (i.e., matrix metalloproteinase-14; Biotrak activity assay system; GE Healthcare). It was performed according to manufacturer instructions in a 96-well plate with the anti-MT1-MMP antibody immobilized onto microplate. The assay uses the pro-form of a detection enzyme (i.e., pro-urokinase) that can be activated only by captured active MT1-MMP, into an active detection enzyme, through a single proteolytic event. The natural activation sequence in the pro-detection enzyme has been replaced using protein engineering, with an artificial sequence recognized only by active MT1-MMP. The activated detection enzyme can then be measured by using a specific chromogenic peptide substrate (S-2444 peptide). Standards and samples are incubated in the plate, and any MT1-MMP present is bound to the immobilized antibody. Active MT1-MMP is detected through activation of the modified pro-detection enzyme (pro-urokinase) and the subsequent cleavage of its chromogenic peptide substrate. The resultant color is read at 405 nm in a microplate reader (Perkin Wallac).

Gelatin Zymography. Sample buffer was 2.5 mL 0.5 M Tris-HCl (pH 6.8), 2.0 mL glycerol, 4.0 mL 10% SDS, 100 μL 10% Tx-100, 100 μL and 1% bromphenol blue, with H_2O added to 10 mL.

5X running buffer was 125 mM Tris HCl (pH 8.3), 1.23 M glycine, 0.5% SDS for 1 L: 15.1 g Tris, 94 g glycine, and 5 g SDS.

Developing buffer was 50 mM Tris HCl (pH 7.6), 10 mM $CaCl_2\times$ $2H_2O$, 50 mM NaCl, 0.05% Brij 35 for 1 L: 6.06 g Tris, 1.47g $CaCl_2$, 2.92 g NaCl, 0.5g Brij 35 (pH 7.6),

0.5M Tris (pH 6.8) for 1 L: 60.55 g Tris in 800 mL H_2O with pH adjusted to 6.8 with HCl, then adding H_2O to 1 L.

1.5 M Tris (pH 8.8) for 1 L: 181.65 g Tris in 800 mL H_2O, with pH adjusted to 8.8 with HCl, then adding H_2O to 1 L.

Gelatin stock solution was made by vortexing 20 mg gelatin with 1 mL H_2O in a 2-mL Eppendorf tube, and shortly boiling in a microwave.

Washing buffer was 2.5% Tx-100 in H_2O, with dilution of 25 mL of Tx-100 into 975 mL H_2O.

Coomasie staining solution was made by diluting 0.5 g of Coomassie blue stain in 40% methanol, 10% acetic acid solution.

Destaining solution was 40% methanol and 10% acetic acid solution.

Activity of gelatinases (MMP-2 and MMP-9) was analyzed with the gelatin zymography procedure (4). Briefly, conditioned media from microglia cultures were mixed with sample buffer, and the samples were loaded on 7.5% SDS/PAGE containing 1% gelatin. After electrophoresis (4 °C, 90 V), the gel was washed 2 times for 15 min in 2.5% Tx-100 washing solution, and incubated overnight in developing buffer. Next, the gel was stained for 30 min in 0.5% Coomassie blue solution and then destained in 40% methanol and 10% acetic acid. The gel was wrapped between 2 foils of cellophane and dried in the gel dryer for 1 h. Enzymatic activity resulted in gelatin degradation, which was directly visible as clear bands on the dark blue background. The surface of these clear bands directly correlated to the amount of the active gelatinase. The different gelatinase types were identified by their corresponding molecular weights.

RT-PCR. RNA was isolated with an RNeasy-kit (Qiagen) and all oligonucleotides were from MWG-Biotech. First-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) by using 1 μg RNA and oligo-dT primer. PCR was performed with a High Fidelity kit (Invitrogen).

Primers were as follows: MT1-MMP forward, 5′-GGATACCCAATGCCCATTGGCCA-3′; reverse, 5′-CCATTGGGCATCCAGAAGAGA-3′; and for actin, forward, 5′-CCCTGAAGTACCCCATTGAA-3′; reverse, 5′GTGGACAGTGAGGCCAAGAT'3′.

The thermal cycling conditions were as follows: 95 °C for 2 min, 95 °C for 45 sec, followed by 25 cycles of 45 sec at 95 °C for denaturation and 2 min at 72 °C for annealing and 10 min at 72 °C for extension.

Quantitative Real-Time PCR. Quantitative real-time PCR was carried out using SYBR Green chemistry and the ABI Prism 7500 sequence detection system (Applied Biosystems). Total RNAs isolated from 4 independent experiments were used for these analyses. cDNAs were synthesized by extension of oligo$(dT)_{15}$ primers with 200 U of M-MLV reverse transcriptase in a mixture containing 1 μg of total RNA in 20 μL. An aliquot of each RT reaction (cDNA equivalent to 50 ng RNA) was amplified in duplicate in 20 μL reaction volume containing 1× SYBR Green PCR master mix (Applied Biosystems) and 0.9 μM of each primer. The following primers were used:

rat 18S sense, 5′-GTAACCCGTTGAACCCCATT-3′;
antisense, 5′-CCATCCAATCGGTAGTAGCG-3′;
rat MT1-MMP sense, 5′-TGTCCCAGATAAGCCCAGAA-3′;
antisense, 5′-TATTCCTCACCCGCCAGAAC-3′.

The thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. The specificity of the PCR was confirmed by a single peak in a dissociation curve. To correct for sample-to-sample variation, an endogenous control (18S rRNA) was amplified with the target and served as an internal reference to normalize the data. The threshold cycle (Ct) was determined after automatic setting the threshold in the linear amplification phase of the PCR and averaged for each sample assayed in duplicates. Fold change was calculated as $2^{-\Delta\Delta Ct}$.

In Vivo Inoculation of Glioma Cells into Mouse Brain

Anesthesia. Mice were anesthetized with i.p. injections of 2% xylazine (Rompun; Bayer) and 10% ketamine hydrochloride (WDT) mixture in 0.9% NaCl. Ten microliters of the anesthetic mixture was injected per 1 g of mouse body weight. The eyes of the mice were carefully covered with glycerin fat to avoid cornea drying.

Glioma Inoculation into the Mouse Brain. Anesthetized mice were immobilized and mounted onto a stereotactic head holder (Kopf Instruments) in the flat-skull position. The skin of the skull was dissected with a scalpel blade and the skull surface was disinfected with 10% KI (potassium iodide) solution. One millimeter anterior and 1.5 mm lateral to the bregma, the skull was carefully drilled with a 20-gauge needle tip. Then a 1-μL syringe with a blunt tip was inserted to a depth of 4 mm and retracted to a depth of 3 mm from the dural surface. During 2 min, 1 μL (2×10^4 cells/μL) of glioma cell suspension was slowly injected. The needle was then slowly taken out from the injection canal and the

skin was sutured with a surgical sewing cone (Johnson & Johnson).

Paraformaldehyde Fixation. The mice were euthanized by 10% ketamine i.p. injection and perfused with an intra-cardiac injection of 0.9% NaCl solution. Then, the NaCl perfusate was replaced by freshly prepared 4% paraformaldehyde (Merck) and each mouse was perfused with 30 mL of 4% paraformaldehyde. The brain was then carefully removed and post-fixed for an overnight period in 4% paraformaldehyde. Finally, the brains were cryopreserved in 30% sucrose solution (48 h incubation minimum).

Immunohistochemistry of Brain Sections (Floating Sections)

Immunolabeling reagents were as follows.

TBS+ was 100 mM Tris, 150 mM NaCl (pH 7.4), 0.1% Tx100; and

TBS++ was 100 mM Tris, 150 mM NaCl (pH 7.4), 0.1% Tx-100, pH 7.4, and 3% donkey serum.

The paraformaldehyde-perfused cryoprotected brains were rapidly frozen in dry ice and mounted onto a sliding microtome (SM 2000R; Leica). Sections 40 μm thick were collected into CPC cryoprotecting solution (25% glycerol and 25% ethylene glycol in 0.05 M phosphate buffer). Before immunolabeling, the sections were first washed 3 times in TBS+ and subsequently incubated in TBS++ (i.e., blocking buffer) for 1 h. Then, the sections were incubated 48–72 h at 4 °C with primary antibodies. Sections were washed 3 times in TBS+ and incubated overnight with fluorescently conjugated secondary antibodies. They were then washed 3 times in TBS and finally mounted onto microscope glass slides, covered with coverslips, and stored at 4 °C until they were used for microscopic analysis.

Immunolabeling of Tissue Arrays. Brain glioma tissue arrays (GL801–5-BX; US Biomax) containing 47 cores (5 μm core thickness each), with matched or unmatched adjacent normal tissue for immunohistochemistry. The primary antibodies were anti-MT1-MMP (mouse monoclonal, 1:2,000 dilution) and anti-Iba1 (rabbit polyclonal, 1:500 dilution).

The tissue array was baked at 60 °C for 2 h to remove the extra-thin layer of paraffin per manufacturer recommendations. This was followed by de-paraffinization of the array (i.e., mouse paraffin sections) with xylol and rehydration with graded series of 100%, 90%, and 70% alcohol and finally with water.

For MT1-MMP, antigen retrieval was carried out by heat treatment of the deparaffinized tissue array/mouse paraffin sections in a water bath at 92 °C for 30 min in Tris-EDTA (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0). This was followed by cooling and washing the slides in running tap water and subsequent washes with TBS+ 0.025% Triton X-100 (pH 7.4) buffer. The arrays/sections were further treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidases followed by washes with TBS buffer. The TBS+ buffer was used to block background staining for 1 h. The sections/array were incubated with the primary antibodies overnight at 4 °C in a humid chamber.

The next day, incubation was performed with anti-rabbit Rhodamine Red (1:125) for Iba1 and further amplification of the MT1-MMP signal using the Alexa fluor-488 tyramide signal amplification kit from Invitrogen/Molecular Probes per manufacturer instructions. Finally, the sections/array were stained with Hoechst stain for nuclear staining, mounted, and analyzed by fluorescence microscopy.

Confocal Microscopy. All confocal microscopy was performed using a spectral confocal microscope (TCS SP2; Leica). Appropriate gain and black level settings were determined on control tissues stained with secondary antibodies alone. Overview images were processed with Photoshop (version CS; Adobe Systems) and co-localization images with Volocity software (version 2.6.1; Improvision).

Fluorescence Microscopy. Immunohisto-cytochemical preparations were also visualized with a fluorescence microscope. Fluorescence microscopy was further used to evaluate transfection rates, which were measured by a corresponding EGFP expression in the glioma cultures. Live cultures were analyzed for fluorescence using an inverted fluorescence microscope (Axiovert 100; Zeiss).

Statistical Analysis. Statistical analyses were performed using SPSS software. Statistical significance was determined at the $P < 0.05$ level. The results are expressed as mean ± SEM. Comparisons among the groups were performed with the paired-samples t test and the Wilcoxon signed-rank test.

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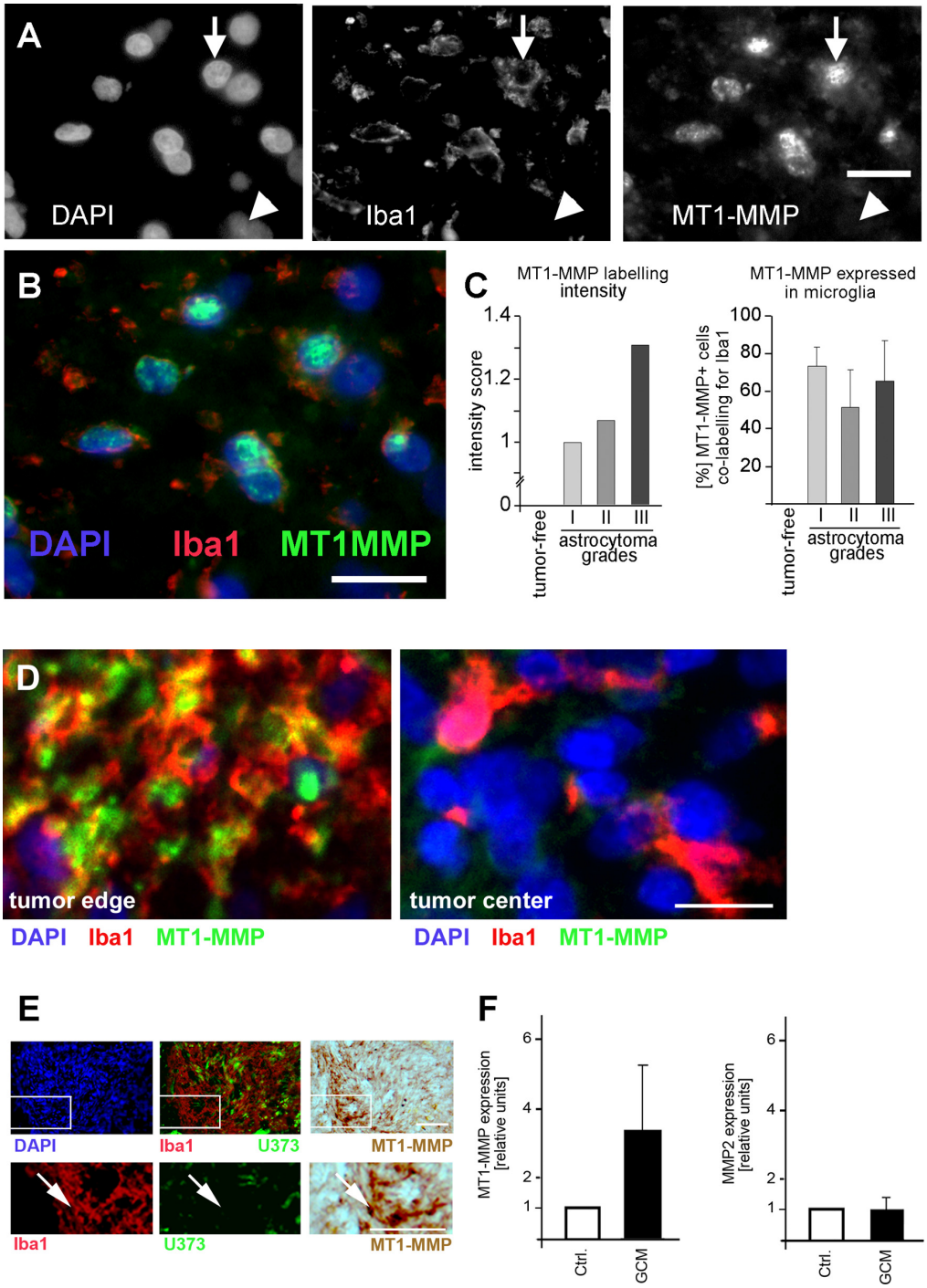


Fig. S1. Glioma induce MT1-MMP expression in human and rat microglia. A human tissue array containing 47 different biopsy specimens (37 from astrocytomas of various grades and 10 tumor-free specimens; see Table S1) was immuno-labeled for MT1-MMP and Iba1. A representative example is highlighted; note that Iba1-labeled cells abundantly express MT1-MMP(arrow), whereas Iba1-negative cells are void of MT1-MMP labeling (*A* and *B*). (*C*) Immuno-labeling intensity for MT1-MMP was scored in all samples and scores are averaged for astrocytomas of various grades (0, no staining; 1, moderate labeling; 2, intense labeling). The fraction of microglial cells among all MT1-MMP-expressing cells was quantified and is indicated for tumors of various grades. (*D*) Human glioma biopsy specimens from the tumor edge or tumor center were immunostained for Iba1 and MT1-MMP and counterstained with DAPI; note that material from the tumor edge labels much more intensely for MT1-MMP than tissue from the glioma core. (*E*) EGFP-expressing U373 cells (a human cell line) were intracerebrally inoculated into immunocompromised mice (nude mice, which have a T cell defect and hence no adaptive immune response). Fourteen days after the operation, we immunostained the brains for MT1-MMP and Iba1 and applied nuclear counterstaining (DAPI). The boxed area (*Upper*) was magnified (*Lower*) and indicates that intratumoral areas overexpressing MT1-MMP abundantly contain microglia, but not tumor cells (arrow). (*F*) Rat microglial cultures were stimulated with GCM (from rat glioma; C6 cells) or left untreated (*Ctrl*) and the mRNA expression of MT1-MMP and MMP-2 was subsequently quantified by real-time PCR ($n = 4$ independent cultures, $P < 0.05$ by one-way ANOVA). (Scale bars: 10 μm in *A* and *B*, 100 μm in *D*.)

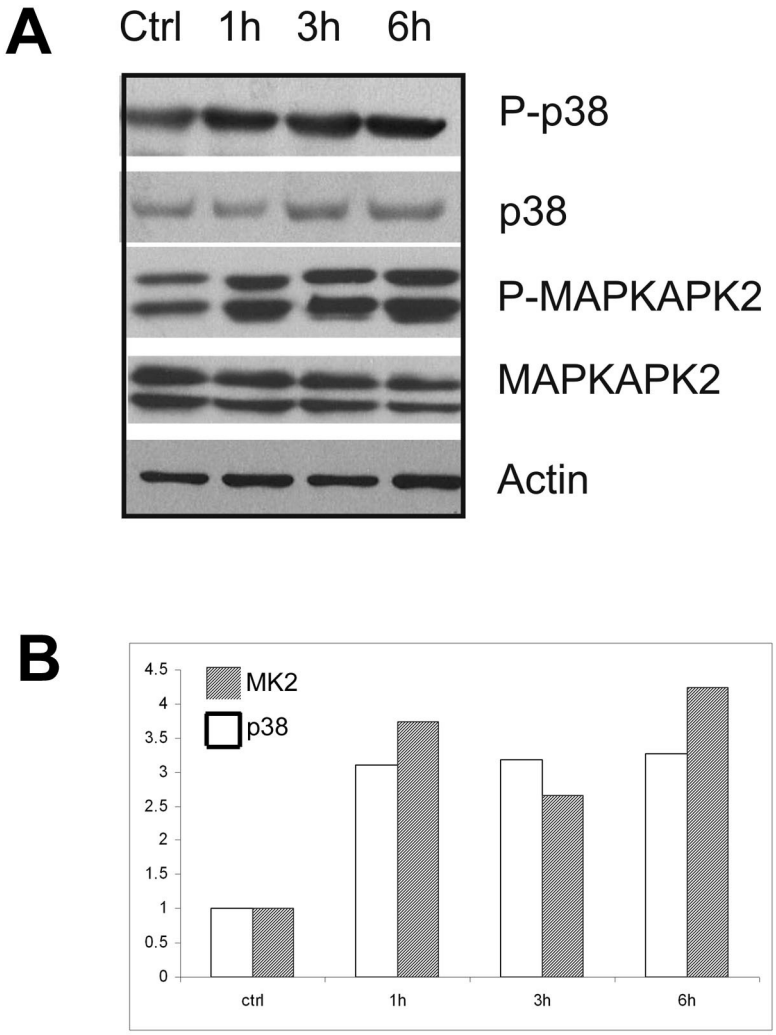


Fig. S2. Glioma cell-conditioned medium induces p38 MAPK signaling in microglia. (*A*) Activation of the p38 MAPK pathway was observed in primary microglia treated for a maximum of 6 hours with GCM. The cell lysates were analyzed for activated (i.e., phosphorylated) p38 MAPK (*p*-p38) and total p38 MAPK (p38). The membranes were then re-probed with anti-phosho-MAPKAPK-2 (*p*-MK-2) and total MK-2 antibodies, showing the activation of p38 MAPK downstream kinase (MK-2; actin serves as loading control). (*B*) The ratio of activated p38-MAPK to total p38-MAPK and of activated MK-2 to total MK-2 was determined by densitometry.

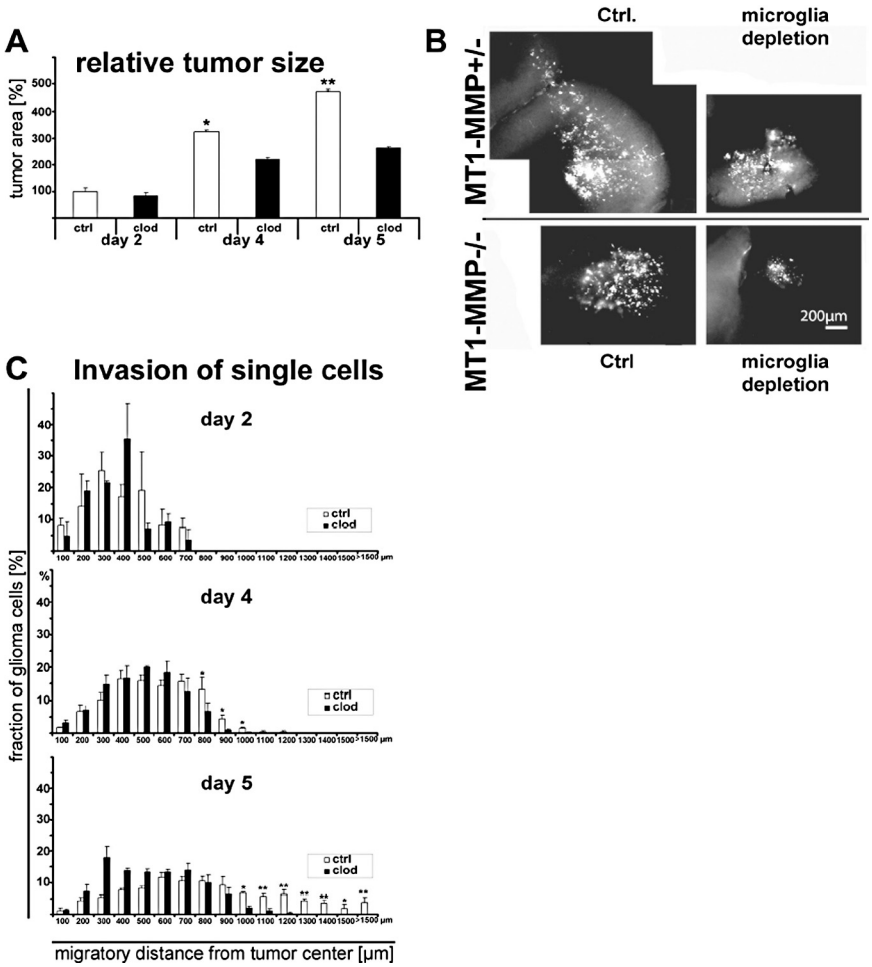


Fig. S3. Microglial MT1-MMP mediates increased tumor size. Invasion of glioma cells within organotypic brain slice cultures (over time courses of 2, 4, and 5 d after glioma inoculation) was measured by 2 different methods. Brain slices from MT1-MMP WT (MT1-MMP$^{+/+}$) were maintained under control conditions (*Ctrl*; containing microglia) or were microglia-depleted (by clodronate treatment; *clod*) before tumor injection. The total area covered by the tumor (*A*) or the number of cells migrating a certain distance away from the injection site (*B*) were determined from identical specimen. The distance measurements show that glioma cells in microglia-depleted slices and in control slices form an initial tumor mass with a maximum radius of 700 μm (at d 2). At d 5, the glioma cells in microglia-depleted slices still gather mainly within the confines of the initially established tumor, whereas the distribution of glioma cells in microglia-containing slices looks very different: here, on d 5 the majority of glioma cells are beyond the size of the initially established tumor (700 μm on d 2) and many glioma cells have even migrated more than 1.5 mm into the parenchyma (*C*). Recording the migratory distances of individual cells (as described earlier) has previously been established as a method to determine the invasive behavior of gliomas (5). Measuring the total tumor area (as in *A*) is a much simpler method to determine glioma expansion; however, for the present glioma model, this method delivered results that correlated well with the data obtained by recording individual migratory distances (as in *C*). Hence, for the present experimental paradigm, tumor surface measurements give a good correlation for glioma invasion. This view is supported by our in vivo data: reducing microglia number in glioma, as well as MT1-MMP expression and glioma size, by interfering with TLR signaling (i.e., MyD88 knockout) reduced glioma expansion as measured by total tumor size (see Fig. 5 in main text) and as measured by recording the invasion of individual glioma cells (see Fig. S5). Importantly, MyD88-knockout had no effect on glioma cell proliferation (see Fig. S5), which could mediate tumor expansion alternatively/additionally to glioma cell invasion. (*B*) Representative images for organotypic brain slices from MT1-MMP heterozygous (MT1-MMP$^{+/-}$) and knockout (MT1-MMP$^{-/-}$) mice 5 d after tumor injection are presented correspond to the samples quantified in Fig. 4*B*.

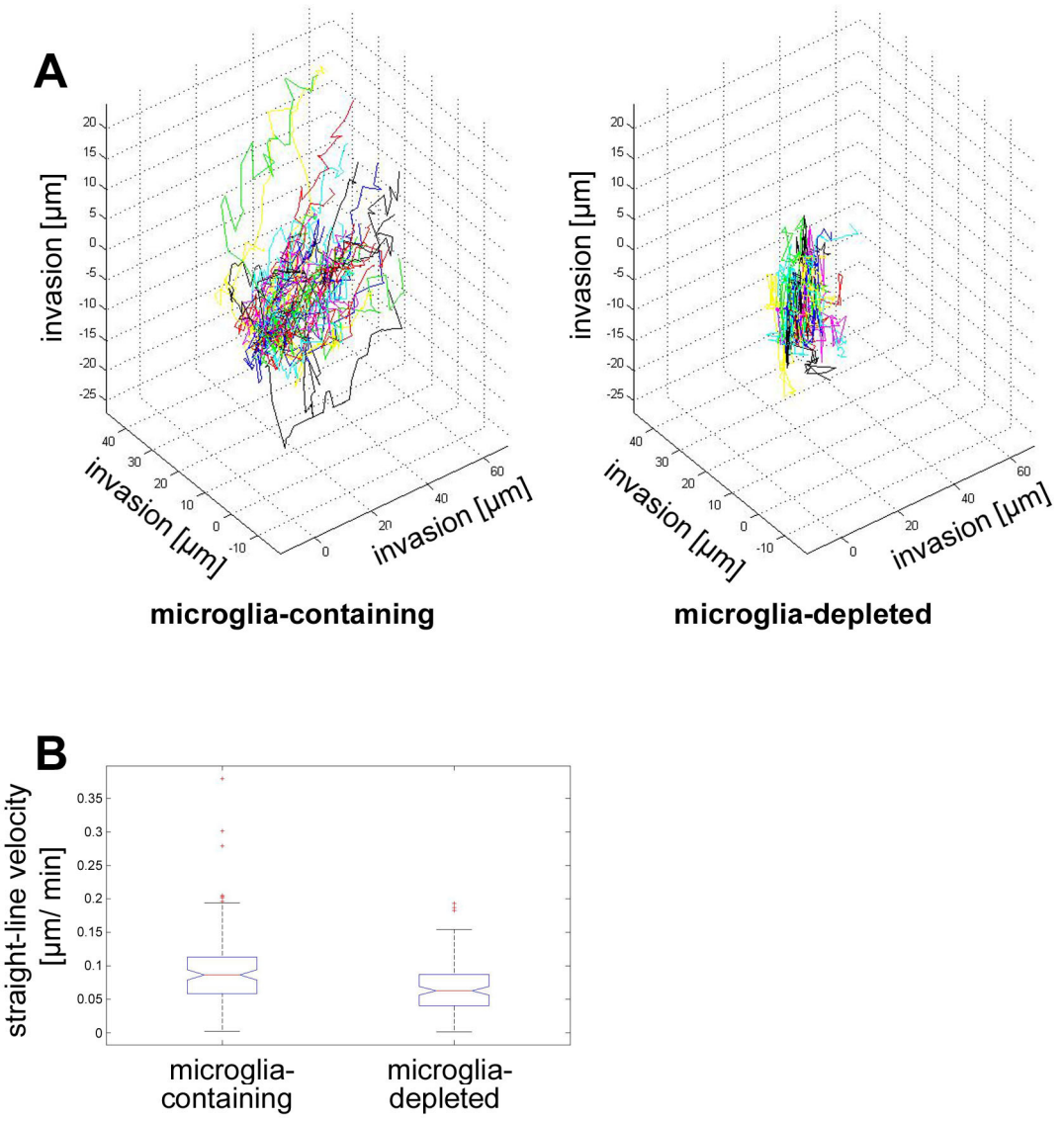


Fig. S4. Time-lapse microscopy of glioma-inoculated brain slices. Invasion of single glioma cells was recorded by 2-photon microscopy in microglia-containing and microglia-depleted brains slices over a time course; images were taken every 10 min. (*A*) A representative experiment showing the trajectories (of 3D movement) for single glioma cells, which were recorded (over a time course of 400 min) in cultivated brain slices containing microglia (50 glioma cells) and without microglia (34 glioma cells); note that gliomas cells invade much deeper into the parenchyma when microglia are present. (*B*) Box and whisker plot of the straight-line velocity of all recorded glioma cells (123 cells in microglia-containing slices in 5 independent experiments; 139 cells in microglia-depleted slices in 5 independent experiments; statistical difference is highly significant according to Mann-Whitney *U* test); note that, on average, glioma cells invade much faster in the presence of microglia; importantly, fast-invading cells ($>$0.2 μm/min) are seen only in microglia-containing slices.

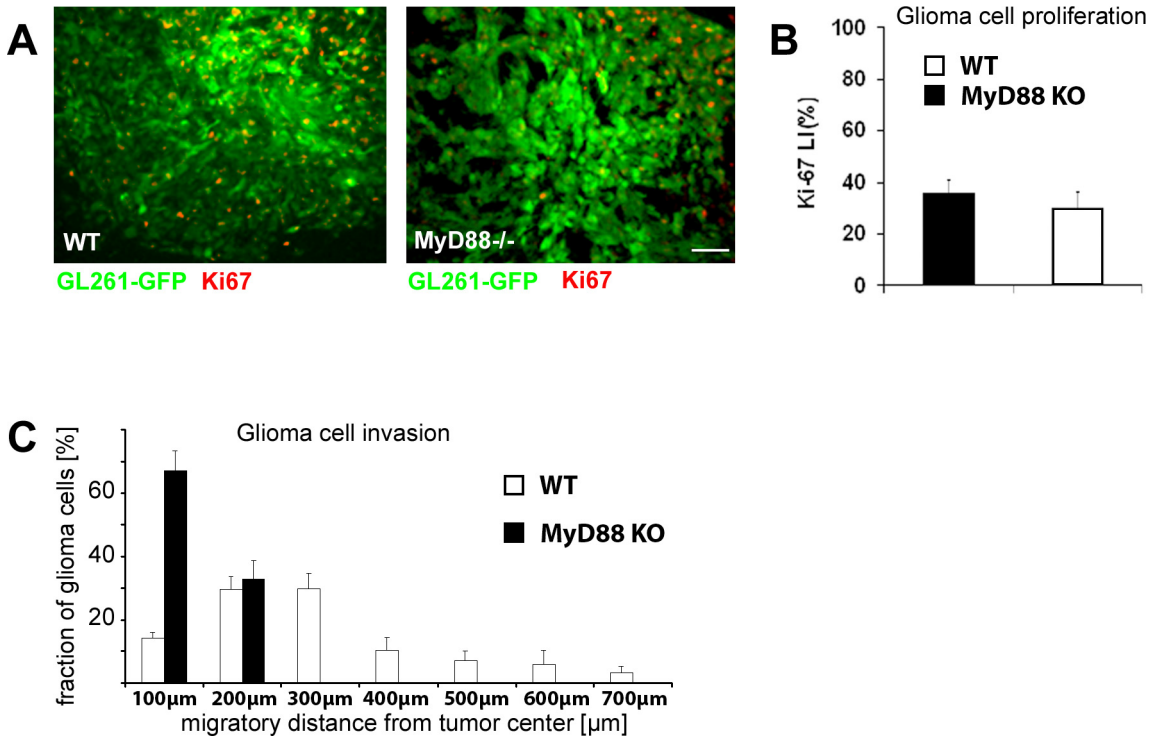
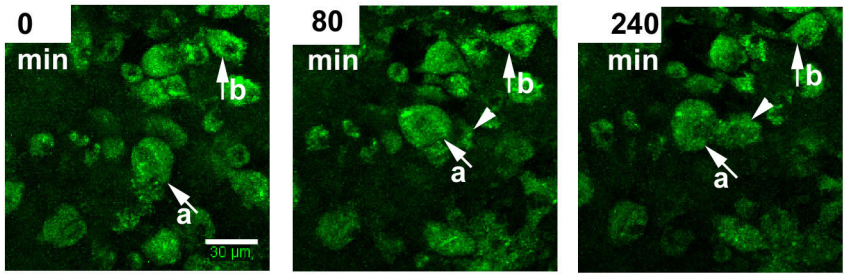
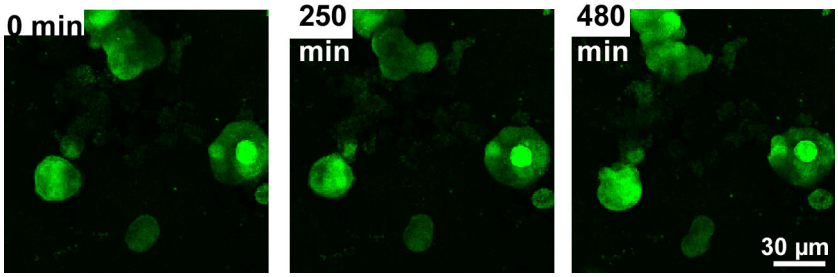


Fig. S5. Parenchymal TLR signaling mediates increased glioma invasion but has no effect on glioma proliferation. (*A*) Brains from WT mice and animals with defective TLR signaling (MyD88-/-) were harvested 14 d after intracerebral inoculation of syngenic gliomas (GL261 cells expressing GFP; GL261-GFP) and immunostained for the proliferation marker Ki67. (*B*) Glioma cell proliferation in both experimental groups was quantified; note that there is no evident change in tumor cell proliferation. (*C*) Invasion distances of individual cells were measured and the GL261 cell numbers per invasion distance were plotted; note that the glioma cell population in Myd88-/- remains within the area the glioma cells had initially been injected into, whereas in WT mice the majority of glioma cells distributes beyond the injection area and many cells deeply invaded into the parenchyma. (Scale bar: 25 μm.)



Movie S1. Typical motility of GFP-expressing glioma cells ina cultivated brain slice. Each frame is a maximum-intensity projection from stacks of fluorescence images, recorded every 10 min..

Movie S1 (AVI)



Movie S2. Typical mortility of GFP-expressing glioma cells in a cultured brain slice, injected with glioma cells and treated with clodronate to deplete microglial cells..

Movie S2 (AVI)

Table S1. In human glioma biopsies, MT1-MMP is up-regulated and predominantly expressed by microglia

Patient No.	Sex	Age (y)	Organ	Diagnosis	Grade	MMP14 expression [a]	MMP14+ co-labeling for Iba1 (%)
1	F	68	Brain	Tumor-free cerebrum	–	–	–
2	F	44	Brain	Tumor-free cerebrum	–	–	–
3	F	36	Brain	Tumor-free cerebrum	–	–	–
4	M	41	Brain	Tumor-free cerebrum	–	–	–
5	F	33	Brain	Normal cerebrum	–	–	–
6	F	37	Brain	Normal cerebrum	–	–	–
7	M	39	Brain	Normal cerebrum	–	–	–
8	M	52	Brain	Normal cerebrum	–	–	–
9	F	47	Brain	Normal cerebrum	–	–	–
10	M	72	Brain	Normal cerebrum	–	–	–
11	M	41	Brain	Astrocytoma (right frontal lobe)	I	1	76
12	M	39	Brain	Astrocytoma	I	–	–
13	M	36	Brain	Astrocytoma (frontal lobe)	I	1	62
14	F	30	Brain	Astrocytoma	I	1	82
15	M	52	Brain	Astrocytoma	I	–	–
16	F	68	Brain	Astrocytoma (right temporal lobe)	II	–	–
17	F	36	Brain	Astrocytoma (left temporal lobe)	II	–	–
18	IF	37	Brain	Astrocytoma (right temporal lobe)	II	1	71
19	F	42	Brain	Astrocytoma (left occipital parietal lobe	II	1	28
20	M	16	Brain	Astrocytoma (right occipital lobe)	II	1	84
21	M	17	Brain	Astrocytoma	II	1	57
22	M	31	Brain	Astrocytoma (left temporal lobe)	II	2	41
23	F	34	Brain	Astrocytoma	II	1	37
24	M	58	Brain	Astrocytoma (left temporal lobe)	II	–	–
25	M	33	Brain	Astrocytoma	II	1	56
26	F	48	Brain	Astrocytoma (right temporal lobe)	II	1	44
27	IF	30	Brain	Astrocytoma (fourth ventricle)	II	1	82
28	M	53	Brain	Astrocytoma (right temporal lobe)	II	1	26
29	IF	33	Brain	Astrocytoma (left tempo-parietal lobe)	II	1	76
30	F	36	Brain	Astrocytoma	II	1	48
31	M	53	Brain	Astrocytoma (left temporal lobe)	II	1	41
32	F	47	Brain	Astrocytoma	II	1	32
33	M	35	Brain	Astrocytoma (left temporal lobe)	II	–	–
34	IF	40	Brain	Astrocytoma (right tempo-parietal lobe)	III	–	–
35	F	40	Brain	Astrocytoma (frontal lobe)	III	1	88
36	M	65	Brain	Astrocytoma	III	1	32
37	M	32	Brain	Astrocytoma	III	1	31
38	IF	36	Brain	Astrocytoma	III	1	81
39	F	16	Brain	Astrocytoma (cerebellum)	III	2	89
40	M	39	Brain	Astrocytoma (left temporal lobe)	III	1	60
41	M	35	Brain	Astrocytoma	III	1	63
42	M	52	Brain	Astrocytoma (left occipital lobe)	III	1	42
43	F	47	Brain	Astrocytoma (left temporal lobe)	III	2	91
44	F	48	Brain	Astrocytoma (left temporal lobe)	III	1	83
45	M	68	Brain	Astrocytoma (right temporal lobe)	III	2	75
46	M	9	Brain	Astrocytoma (right temporal lobe)	IV	2	52
47	M	5	Brain	Astrocytoma	IV	1	64

A human tissue array containing 47 different biopsy specimens (37 from astrocytomas of various grades and 10 tumor-free specimens; from patients between 5 and 72 years of age and of both sexes) was immuno-labeled for MT1-MMP and Iba1 (see Fig. S1 *A* and *B*). Immuno-labeling intensity for MT1-MMP (i.e., MMP14 expression) was scored in all samples and scores are averaged for astrocytomas of various grades. The fraction of microglial cells among all MT1-MMP expressing cells (i.e., MMP14+ co-labeling for Iba1) was quantified and is indicated (as percentage values) for tumors of various grades.

[a], –, no staining; 1, moderate labeling; 2, intense labeling.