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Microglia/Macrophages Promote Glioma Progression

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Abstract

Gliomas are highly aggressive and accompanied by numerous microglia/macrophages (MG/MP) in and about the tumor. Little is known about what MG/MP do in this setting, or whether modulating MG/MP activation might affect glioma progression. Here, we used a glioma-microglia *in culture* system to establish the effects the tumor and microglia have on each other. We assessed glioma progression *in vivo* after MG/MP ablation or in the setting of exaggerated MG/MP activation. We show that glioma cells activate microglia but inhibit their phagocytic activities. Local ablation of MG/MP *in vivo* decreased tumor size and improved survival curves. Conversely, pharmacological activation of MG/MP increased glioma size through stimulating tumor proliferation and inhibiting apoptosis. In agreement with recent reports, expression of the chemokine CCL21 is enhanced after MG/MP activation and correlates with tumor growth. Taken together, our findings demonstrate that inhibition of MG/MP activation may constitute a new and effective contribution towards suppressing glioma proliferation.

Keywords

Introduction

Malignant gliomas are primary central nervous system (CNS) tumors arising from glial cells and are one of the deadliest cancers - median survival time is one year even with aggressive surgical resection combined with irradiation and chemotherapy. Although many therapeutic approaches have been explored, there has been no major improvement in survival over the last 30 years (DeAngelis, 2001; Legler et al., 1999).

Gliomas are infiltrated by MG/MP, and the extent of MG/MP infiltration correlates positively with malignancy (Morimura et al., 1990; Morris and Esiri, 1991; Roggendorf et al., 1996). Microglia are capable of antigen presentation to T cells patrolling the CNS (Kreutzberg, 1996). Upon injury, microglia undergo activation characterized by changes in morphology, gene expression, proliferation, phagocytic capacity, and migration towards the injury site (Kreutzberg, 1996; Streit et al., 1999).

The role of MG/MP in glioma progression remains controversial. Studies reported that the immune defensive functions of glioma-infiltrating MG/MP (GIMs) are compromised. Moreover, GIMs have been proposed to promote glioma growth by secreting growth factors,

immune-suppressive cytokines and angiogenic factors (Alterman and Stanley, 1994; Demuth et al., 2007; Galasso et al., 2000; Lafuente et al., 1999; Wagner et al., 1999; Wesolowska et al., 2008), thus stimulating interest in therapies that modulate MG/MP activity/function. However, such approaches yielded conflicting results: injection of CpG-containing oligonucleotides, which stimulate MG/MP, induced glioma apoptosis and prolonged survival times of tumor-bearing animals in one report, whereas the same approach caused increased animal tumor size in others (El Andaloussi et al., 2006; Ginzkey et al., 2009).

Here we investigate the consequences of interaction of MG/MP and glioma cells in culture using MG/MP activation and glioma cell proliferation as functional endpoints. We examine glioma progression in a mouse model using pharmacogenetics to locally ablate MG/MP, and a pharmacological approach to exaggerate MG/MP activation. We show that manipulation of MG/MP activation state appears to be a potentially promising novel interventional approach for gliomas.

Materials and methods

Cell Lines

GL261, a glioma cell line derived from C57BL/6 mice, and CRL-2541, a mouse astrocyte cell line, were obtained from ATCC. The cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum (FBS) and 1mM Na pyruvate (Fisher Mediatech). The cells were transfected with pEGFP-N1 using Lipofectamine (Invitrogen) and selected with 500μg/ml neomycin (Geneticin, Invitrogen) to generate the stable cell lines GL261-EGFP and CRL-2541-EGFP.

Animals

C57BL/6 mice (wild type, WT) were purchased from Jackson Laboratory. CD11b-HSVTK transgenic mice were described previously (Heppner et al., 2005). Female CD11b-HSVTK (+/−) mice were bred with male C57BL/6 mice and the offspring genotyped by PCR using primers 5′-GACTTCCGTGGCTTCTTGCTGC-3′ and 5′-GTGCTGGCATTACAGGCGTGAG-3′. All animal procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC). Mice were bred in-house under maximum isolation conditions on a 12:12 hour light: dark cycle with food *ad libitum*.

Primary microglia cultures

Mixed cortical cultures from newborn C57BL/6 mice (day 0–3) were made using standard protocols (Rogove and Tsirka, 1998). Ten days after plating, primary microglia were isolated by treatment with 15mM lidocaine (Sigma) and gentle rocking. Microglia were resuspended in an appropriate volume of DMEM with 1% FBS. After 24–48 hours of culture, microglia were used for experiments.

Microglia and glioma cell co-culture

GL261-EGFP cells were plated with rhodamine-labeled primary microglia. In brief, after isolation of primary microglia, the cells were resuspended at a density of 5×10^4 cells/ml in DMEM with 1% FBS and 20μg/ml mini-ruby (Invitrogen) (Ullrich et al., 2001). After 48 hours, the medium was removed and 2×10^4 GL261-EGFP cells seeded on top of the microglia in DMEM with 10% FBS and desired treatments, such as 150μg/ml tuftsin, MIF/TKP (Bachem), or 4μg/ml CCL21 neutralizing antibody (PeproTech). As controls, primary microglia were either switched to the same medium without the GL261-EGFP, or microglia were seeded with 2×10^4 CRL-2541-EGFP astrocytes. The microglia-glioma interactions

were followed by confocal imaging over 5 days. To evaluate the growth rate, cell numbers were counted by hemacytometer for 5 days. The experiments were repeated 3 times with duplicate samples per group.

Segregated microglia-glioma co-cultures were set as follows: 5×10^4 microglia were seeded on 0.4μm inserts (Millicell) in DMEM with 1% FBS. After 48 hours, the inserts were moved to 24-well plates containing 5,000 GL261 cells/well in DMEM with 10% FBS. 150μg/ml tuftsin or MIF/TKP was added to the medium above the inserts. Empty inserts with the same medium were used as control. GL261 proliferation was measured every other day. The experiments were repeated 3 times with duplicate samples per group.

Cell proliferation assay

The CCK-8 kit (Dojindo Molecular Technologies) was used for cell proliferation. In brief, equal numbers of GL261 cells were seeded in 96-well or 24-well plates (co-culture) with respective treatments (duplicate samples for each treatment). At each time point, the medium was changed to include a 1:10 dilution of CCK-8. After 2 hours at 37°C, absorbance was read at 450nm. Cell proliferation was plotted by normalizing the absorbance values at each time point over the ones at day 0.

Western blotting of microglial markers

Primary microglia were seeded into 6-well plates at equal numbers in DMEM+1% FBS. After two days, the medium was replaced with DMEM+10% FBS or conditioned medium. Glioma-conditioned medium (GCM) was collected from an 80%-confluent GL261 plate and centrifuged at 10,000 rpm for 10 minutes to remove cell debris. Negative controls included GCM boiled for 10 minutes and astrocyte-conditioned medium (ACM) collected from a CRL-2541 plate. At different time points, cells were lysed and protein concentrations measured by the Bio-Rad Bradford detergent-compatible (DC) assay. 15% SDS-PAGE was used to separate protein samples (10μg), which were transferred to polyvinylidene difluoride (PVDF) membrane, probed with rabbit anti-mouse Iba1 antibody (1:1000, Wako) and mouse anti-mouse α-tubulin antibody (1:2000, Millipore) overnight at 4°C. Goat anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:5000. HRP activity was detected with LumiGLO Chemiluminescent Substrate System (KPL). Expression levels were quantified using SCION Image software, and Iba1 expression level normalized to the α-tubulin.

In vivo mouse glioma model and local drug delivery

12–16 week old male CD11b-HSVTK (+/−) mice weighing 25–30 grams were used. Wild type (WT) littermates [CD11b-HSVTK(−/−) mice] were used as controls. For the tuftsin group, 12–16 week old C57BL/6 mice were used. Mice were anesthetized with atropine (0.6mg/kg, i.p.) and 2.5% Avertin (0.02mg/g, i.p.), and then a midline incision made on the scalp. At stereotaxic coordinates of bregma, −1mm anteroposterior and +2mm mediolateral, a small burr hole was drilled on the skull. 3×10^4 GL261-EGFP cells were delivered in 1μl PBS at a depth of 3mm over 2minutes (modified from El Andaloussi et al., 2006).

Reagents were administered locally using mini-osmotic pumps (Alzet®, DURECT) containing different concentrations of ganciclovir (GCV) (Calbiochem), 250μg/ml tuftsin, or 250μg/ml MIF/TKP. Each pump was connected to plastic tubing and a 4mm guide cannula (PlasticsOne). The pumps were placed subcutaneously on the mouse back with the cannulas inserted to the injection site. The drugs were infused at 0.25μl/hr over 14 or 28 days. Pumps with normal saline inserted right after tumor cell injection were used as negative control. Pumps were inserted either immediately after (D0 pump), or five days after (D5 pump)

tumor injection. Animals accepting GCV pumps were administered 50μg/g GCV i.p. one day before tumor injection to ensure maximum ablation of MG/MP.

Tumor evaluation

Mice were deeply anesthetized with 2.5% Avertin and transcardially perfused with 50ml PBS followed by 50ml 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed in 4% PFA/PBS for 6 hours at 4°C, followed by 30% sucrose at 4°C until fully dehydrated. Brains were then frozen-embedded in optimal cutting temperature compound (Tissue-Tek) and cut using a Leica (Nussloch, Germany) cryostat. 10–15 series of 25μm coronal sections encompassing the entire tumor were generated for each animal. For general morphology, sections were stained with hematoxylin and eosin (H&E). GFP-fluorescent glioma cells were visualized using fluorescent microscopy (Nikon E600). To measure tumor size, randomly picked serial sections from each animal were used, and the GFP+ tumor area measured using NIS-Elements software. The tumor sizes were calculated as sum of (tumor area × section thickness) for each section with a tumor tissue. The sections were immunostained with Iba1 antibody or biotinylated tomato lectin (Sigma) to visualize MG/MP infiltration, Ki67 (Abcam) for proliferation, activated caspase-3 (Sigma) for apoptosis and CCL21 (PeproTech) to assess cytokine expression. For quantification, 3 randomly selected samples per group were used and at least 15 fields in different sections of the same sample captured by confocal microscopy.

Immunofluoresent staining

Primary microglia treated with GCM were fixed with 4% PFA/PBS for 15minutes at room temperature. Cells or brain sections were blocked in 5% goat serum (Sigma) in PBS-T for 1 hour at room temperature, and then incubated with Iba1 antibody (1:500), Ki67 (1:500), activated caspase-3 (1:500), CCL21 (1:500) and biotinylated tomato lectin (1:500) in PBS-T overnight at 4°C. The samples were incubated with corresponding secondary antibodies (Alexa Fluor-555 or 647, Invitrogen) for 1-hour at room temperature, and mounted with Fluoromount-G (Southern Biotech).

Statistical analysis

The statistical significance between two groups was determined by Student's t-test. For comparison of more than two groups, one-way ANOVA followed by a Bonferroni-Dunn test was used. The Kaplan-Meier survival curve was calculated with MedCalc software. Data were expressed as mean ± standard error of the mean (SEM). The statistical significance is either described in figure legends, or indicated as asterisks (*). *:P <0.05; **: P<0.01; ***:P< 0.001.

Results

Glioma cells produce soluble factors that activate microglia

We exposed primary microglia to glioma-conditioned medium (GCM) to determine whether soluble factors secreted by glioma cells alter microglial activation. Microglial activation can be assessed by morphology changes. Resting microglia appear rod-shaped in culture, and upon activation, they transform into an ameboid shape with pseudopodia (Siao and Tsirka, 2002). At time 0, resting microglia had elongated cell bodies and thin processes; no change in the morphology was obvious 24 h later in the same medium (microglia, DMEM+1% FBS) or in GL261 control medium (DMEM+10% FBS) (Fig. 1A, B). When exposed to GCM for 16–24 hours, however, the microglia retracted their processes and became ameboid in shape. Iba1 (ionized calcium binding adaptor molecule 1) is a MG/MP marker that is upregulated upon activation (Ito et al., 1998). Iba1 expression levels increased 3–4

fold with GCM treatment (Fig. 1B, C), concomitant with morphological changes. However, no increase in Iba1 was observed when the microglia switched from 1% to 10% FBS, or were cultured with boiled GCM or with astrocyte-conditioned medium (ACM), indicating that the secreted factor is glioma-specific and most likely protein in nature (Fig 1C).

Microglia in culture modestly affect glioma cell growth

Microglia play a major role in innate and adaptive immune responses in the CNS. As part of the innate immune response, they phagocytose pathogens and release proteases, cytokines and molecules that function to protect the host (Kreutzberg, 1996); this function may be compromised in a glioma-harboring environment (Hussain et al., 2006). To directly assess the effect of glioma cells on microglial function, we co-cultured rhodamine-labeled microglia and glioma GL261-EGFP cells. As control, the microglia were also plated alone or with the CRL-2541 astrocytes. In setting up this experiment, we first did a dose response by varying the microglial numbers. We used 4×10^3, 8×10^3, 2×10^4, 4×10^4, 8×10^4, 2×10^5, 4×10^5 cells and found that $4–8 \times 10^4$ was the optimal microglia concentration. If the microglia concentration was lower than 4×10^4 cells, they could not spread evenly in the well (they distributed along the margin of culture plates) and could not form sufficient contact with GL261-EGFP cells. If the concentration was higher than 8×10^4 cells, the density of microglia was too high and led to microglia forming a barrier against GL261-EGFP cell attachment. Then we kept the microglia concentration constant (5×10^4 cells) and varied the GL261-EGFP concentrations (5×10^3, 10^4, 2×10^4, 4×10^4, 8×10^4 cells). We found that 2×10^4 GL261-EGFP cells worked best in the co-culture system showing sufficient interactions with microglia and adequate growth in a well of a 12-well plate.

Microglia plated alone remained rod-like throughout the 5-day experiment (Fig. 2A). Microglia co-cultured with CRL-2541-EGFP cells exhibited modest signs of morphological activation and phagocytic activity throughout the 5-day period (Fig. 2B, arrows and insets where the red–microglia- channel has been removed to display the green (astrocytic) fluorescence within microglial cells, indicative of phagocytic events). In contrast, within one day of being co-plated with GL261 cells, the microglia exhibited activated morphology and their pseudopodia were in contact with the GL261 cells (Fig. 2C). At days 2 and 3, a few phagocytic microglia containing GFP fluorescence (arrows in figure 2C–D) could be observed. However, by day 4, phagocytosis of GL261 cells was observed only rarely.

We next examined the growth rate of GL261 and microglia in the co-culture system by counting the cell numbers of each population every 24-hours (Fig. 2E). Switching primary microglia into DMEM+10% FBS (the GL261 medium) did not stimulate proliferation, nor was there an effect of adding GL261 cells. In contrast, when GL261 cells were cultured together with microglia, the GL261 growth rate was modestly higher than that observed for GL261 cells cultured alone (15.3% more cells on day 5, slope of growth curve without microglia, 4.2; with microglia, 4.9). These results suggest that the innate phagocytic response of microglia in culture is inhibited by GL261 cells, while microglia promote glioma growth.

Establishment of an *in vivo* mouse glioma model with local MG/MPablation

To better understand the *in vivo* microglial response to a developing glioma, we employed a model of local microglial ablation. The CD11b-HSVTK transgenic mice express the herpes simplex thymidine kinase (HSVTK) protein in monocytic cells such as MG/MP (Heppner et al., 2005). When these animals are exposed to ganciclovir (GCV), the cells that express HSVTK are eliminated. Systemic administration (100μg/g GCV i.p. every 2days) resulted in death of all proliferating monocytic cells, which led to severe anemia and death around 10days after the GCV injection (Heppner et al., 2005). We modified this protocol by

performing local infusion of GCV (Mirrione et al., 2010) at the tumor injection site, which ablated MG/MP in and around the tumor.

To establish the optimal dose of GCV, we examined whether GCV affected GL261-EGFP growth in culture and *in vivo*. GL261-EGFP cells treated with 2.5μg/ml and 10μg/ml of GCV (every other day for 8days) exhibited no difference in proliferation in comparison to the vehicle-treated control group, whereas cell growth was significantly inhibited at 25μg/ml (data not shown). In the *in vivo* setting, concentrations up to 1.25mg/ml had no significant effect on tumor size at day 14 after tumor cell injection (Fig. 3A). Higher concentrations appeared to inhibit the rate of tumor growth, however (data not shown). These results suggested that sufficiently high concentrations of GCV affect GL261-EGFP growth, since all cells have intrinsic thymidine kinases; however the HSV thymidine kinases are ~1000-fold more sensitive to GCV. For subsequent *in vivo* experiments we thus used 1mg/ml GCV.

To investigate the role of MG/MP in the temporal progression of glioma, we treated CD11b-HSVTK mice with GCV: one group received GCV immediately after tumor injection (D0 GCV group), and the other was treated with GCV five days after tumor implantation (D5 GCV group) to mimic an early stage glioma. Control animals were either WT treated with saline or with GCV or CD11b-HSVTK mice treated with saline.

MG/MPablation inhibits glioma progression

Administration of clodronate for depletion of microglia was reported to attenuate glioma invasion in organotypic brain slices (Markovic et al., 2005), potentially by depriving the glioma cells of microglia-produced MT1-MMP (Markovic et al., 2009). We next performed a detailed evaluation of tumor growth in the presence and absence of microglia. Using the experimental and control groups described above, we found that the tumor size in control mice (WT receiving saline or GCV, or CD11b-HSVTK receiving saline) was similar at 14 days after GL261 injection, whereas the tumors were dramatically reduced in MG/MP-depleted mice (P<0.001; Figure 3B). At day 14 after tumor implantation, the average tumor sizes in the D0 GCV group and D5 GCV group of CD11b-HSVTK mice were respectively 87.5% and 76.3% less than that of CD11b-HSVTK mice receiving only saline (1.6mm^3 and 2.9mm^3 versus 12.38mm^3). Long-term effects of MG/MP depletion on glioma progression were also followed. Twenty days after tumor inoculation, WT mice were morbid with an average tumor size of 55.6mm^3 (Figure 3C), and exhibited extensive brain swelling, hemorrhage, and weight loss. In contrast, tumors in HSVTK mice treated with D0 GCV were very small (~0.15mm^3) 28 days after inoculation, and undetectable by day 42.

Survival curves demonstrated no difference between WT and CD11b-HSVTK mice treated with saline (median survival time 24 days and 22 days respectively, Fig. 3D). In contrast, all of the CD11b-HSVTK mice receiving GCV concomitant with tumor inoculation (HSVTK D0 GCV) survived. These results indicate that glioma growth is dependent on the presence of MG/MP in this model system.

Tumor morphology was evaluated using a combination of H&E-staining (Figure 3E), GFP-fluorescence to visualize tumor cells (Figures 3F–I), and anti-Iba1immunostaining to identify MG/MP (Figures 3H–I). Both saline- and GCV-treated WT mice as well as saline-treated CD11b-HSVTK (labeled as HSVTK) mice displayed intact glioma tissue with numerous MG/MP in and around the tumor (Figure 3H, I). GCV treatment of CD11b-HSVTK mice significantly decreased MG/MP, especially in the animals that received D0 pumps (although there was a significant decrease in those with the D5 pump as well). The decrease of MG/MP was accompanied by a reduction of tumor size as indicated by the limited amount of residual GFP signal. At late time points, day 20 and day 42, the tumor was almost undetectable.

The number of MG/MP, after an initial decrease at earlier time-points, increased later (day 28–42), and the microglial morphology indicated that MG/MP were activated, as they presumably were recruited to the injury site to clean up tissue debris (Figure 3H, I).

Exaggerated activation of MG/MPand glioma growth

Tuftsin (threonine-lysine-proline-arginine, TKPR), which is derived from the proteolytic degradation of immunoglobulin G (Nishioka et al., 1973), is a potent stimulator of MG/MP, enhancing the phagocytic activity, migration and antigen presentation of monocytic cells (Siemion and Kluczyk, 1999). Tuftsin has been widely used as an anti-tumor agent in animal cancer models (Banks et al., 1985; Noyes et al., 1981; Wleklik et al., 1986). GL261-EGFP cells cultured with tuftsin did not exhibit alterations in cell proliferation (data not shown). We thus examined whether tuftsin stimulation of microglia affected growth of GL261-EGFP cells, using a tissue-culture insert system to restrict the effects to soluble factors released by microglia. Microglia growing in inserts were placed in wells containing GL261-EGFP cells and control or tuftsin-supplemented medium, and the glioma cells counted every second day. When compared to GL261-EGFP alone, GL261-EGFP growth was modestly slower in the presence of microglia (Figure 4A); but after day 6 of co-culture, glioma proliferation was accelerated ($P<0.01$). Using a co-culture system with physical interaction between microglia and glioma, growth was increased at all timepoints (Figure 4B). Similarly incubation with tuftsin resulted in increased glioma cell numbers at the same rate as in the presence of microglia alone, therefore in this culture system tuftsin does not provide additional stimulation to microglial activation.

To assess effects on glioma growth *in vivo*, tuftsin was delivered either at day 0 or 5 after tumor injection to the implantation site. Tumor size was analyzed 14 days after tumor inoculation. Tuftsin at day 0 (immediately after tumor implantation) significantly increased the tumor size (mean=$21.0mm^3$) compared to the average tumor size of the control group (mean=$11.7mm^3$) ($P<0.05$), while tuftsin infusion starting 5 days after tumor injection had no significant effect (Table 1, Fig. 5A). Immunofluorescence for Iba1 revealed that tuftsin activated MG/MP around the infusion site in the absence of glioma cell implantation (supplementary figure 1). MG/MP numbers and Iba1 intensity increased in and around the tumor by tuftsin administration immediately after tumor cell injection (Figure 5B, C). When the proliferation (Ki67) and apoptosis (activated caspase-3) in the saline and D0 tuftsin group were quantified, tuftsin infusion increased the proliferation in tumor tissue volume and cell numbers compared to the saline control group (Table 2), and decreased the apoptosis of both tumor cells and GIMs (Table 2).

MIF/TKP decreases the growth of glioma cells in the presence of microglia

Based on our results and previous findings (Ginzkey et al., 2009), the size of gliomas *in vivo* appears to moderately increase upon administration of stimulators of MG/MP. We used therefore an inhibitor of MG/MP, MIF/TKP, to evaluate whether it would reduce glioma growth. MIF/TKP (tuftsin fragment 1–3) is a tripeptide, that blocks the activity of monocytic cells *in vitro* and *in vivo* (Thanos et al., 1993; Rogove and Tsirka, 1998; Bhasin et al., 2007). The infusion of MIF/TKP to the site of excitotoxic injury inhibits microglia activation, and MIF/TKP treatment of primary microglia inhibits lipopolysaccharide (LPS)-mediated TNF-α release (Rogove and Tsirka, 1998). As shown above, an indirect (segregated) and direct (physical interaction) co-culture system was utilized to assess the effects of MIF/TKP on glioma growth. In the indirect system, in the presence of MIF/TKP and microglia the growth of GL261-EGFP cells slightly slowed down 6 days after co-culture compared to that of GL261-EGFP cells with microglia only ($P<0.05$) (Figure 4A). In the direct co-culture in the presence of microglia, MIF/TKP treatment had a very modest effect on GL261-EGFP growth 2 days after co-culture compared to that of GL261-EGFP cells with microglia only

(P<0.01) (Figure 4B). MIF/TKP-treated microglia resulted in lower total GL261-EGFP cell numbers than GL261 alone at day 4 (P<0.01). Since MIF/TKP had no effect on GL261-EGFP growth in the absence of microglia (not shown), the MIF/TKP-mediated inhibitory effect on glioma cell proliferation in microglial presence was probably due to attenuated microglial activation. These results were then explored *in vivo*: delivery of MIF/TKP 5 days after tumor injection inhibited tumor growth (Figure 5A) and MG/MP infiltration. The quantification of Ki67 (cell proliferation) and activated caspase-3 (cell death) in saline control mice and D5 MIF/TKP-treated mice revealed that MIF/TKP treatment inhibited glioma cell proliferation, but had little effect on glioma cell apoptosis (Figures 5B, C). However MIF/TKP induced apoptosis of GIMs, which may contribute to the lower level of MG/MP infiltration (Table 2).

Tuftsin and MIF/TKP treatment had different effects on survival time of tumor-bearing mice

Since D0 tuftsin and D5 MIF/TKP treatment can affect glioma progression, we examined the survival time of glioma-bearing mice with D0 tuftsin and D5 MIF/TKP pumps, which continuously delivered the drugs for 28 days. The Kaplan-Meier survival curve (Figure 6) revealed that the D0 tuftsin pump group survived significantly shorter time than the control group animals, while the D5 MIF/TKP group survived significantly longer (P<0.001). These results suggest that exaggerated MG/MP activation had a positive effect on glioma progression, while the inhibition of MG/MP was beneficial to the survival of mice with gliomas.

Tuftsin and MIF/TKP treatment had different effects on CCL21 expression in glioma tissue

The chemokine CCL21 is expressed by many cancers, including breast cancer and melanoma, which was recently shown to function as an immune suppressive chemokine, facilitating the immune escape of tumors (Shields et al., 2007; 2010). Here we examined whether the tuftsin or MIF/TKP treatment could modulate CCL21 expression in glioma tissue. The immunofluorescent staining of CCL21 revealed that primarily stromal and some glioma cells expressed CCL21 (supplementary figure 2), while the adjacent normal brain tissue didn't show any expression (Figure 7A). The D0 tuftsin group had more CCL21^{+} cells in glioma tissue compared to saline control group. In contrast, the D5 MIF/TKP group revealed a significant decrease in the number of CCL21^{+} cells (Figures 7A, B).

To further confirm the immunosuppressive role of CCL21 in glioma progression, we performed a microglia-glioma co-culture experiment in the presence of 4μg/ml CCL21 neutralizing antibody (CCL21 ab) and counted the GL261-EGFP cell numbers over 5 days (Figure 7C). When GL261-EGFP cells were cultured alone, the inhibition of CCL21 had no effect on GL261-EGFP growth. However, the presence of CCL21 ab in microglia-glioma co-culture abolished the glioma-promoting effect of microglia, indicating that CCL21 is a key regulator of the interactions between microglia and glioma cells.

Taken together, these results suggest that modulating microglial activation, potentially using MIF/TKP, may be beneficial for the treatment of glioma by attenuating the immune microenvironment surrounding the gliomas.

Discussion

The components of tumor mass are tumor and stromal cells, which interact with each other and ultimately affect tumor growth. Among the stromal cells, macrophages have been shown to have dual roles, which are both tumor-rejecting and tumor-promoting (Allavena et al., 2008; Solinas et al., 2009). Macrophages secrete anti-tumor cytokines and interact with T cells to destroy tumor cells (Galani et al., 2009). On the other hand they can be recruited

by tumor-released chemokines and switch to tumor-supporting behavior in the tumor microenvironment (Demuth and Berens, 2004; Pollard, 2004).

Glioma cells release factors such as colony stimulating factor-1, granulocyte-macrophage stimulating factor, monocyte chemoattractant protein-1, and hepatocyte growth factor/scatter factor, which recruit and promote the growth of tumor-infiltrating MG/MP (Alterman and Stanley, 1994; Badie et al., 1999; Kielian et al., 2002; Leung et al., 1997; Nitta et al., 1992). Moreover, tumor-infiltrating MG/MP are commonly activated and proliferate in gliomas (Klein and Roggendorf, 2001). In our study, we show that glioma conditioned medium, but not astrocyte-derived medium, activated microglia, as previously reported (Klein and Roggendorf, 2001). In the GL261 mouse glioma model, as in human glioma specimens, activated MG/MP infiltrated within and around the tumor, and the density of MG/MP in the tumor area was higher than the MG/MP density that surrounded relatively normal brain tissue.

MG/MP are crucial to CNS innate and adaptive immune responses. Patients with gliomas often have deficiency in immunologic responses against the tumor (Morford et al., 1997). Although up to one third of all cells in glioma specimens were MG/MP, T cells were rarely seen in gliomas (Morimura et al., 1990; Roggendorf et al., 1996). Independent studies showed that the microglial antigen-presenting function was compromised in gliomas, since the major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules exhibited decreased expression levels (Badie et al., 2002; Flugel et al., 1999). The studies on GIM innate immune responses demonstrated that GIMs expressed Toll-like receptors (TLR) and mediated phagocytosis, but lacked IL-1β and TNF-α production, which are important cytokines for tumor rejection (Frei et al., 1994; Hussain et al., 2006). Moreover in a CD11b-HSVTK glioma model, Markovic et al. (2009) reported that activation of MMPs in glioma is promoted via activation of TLRs and MT1-MMP in microglia. Our data shed light on the MG/MP innate immune response against gliomas. When microglia encountered glioma cells in culture, they first became activated, but at later timepoints phagocytosis was rarely observed and the growth of glioma cells was unaffected by microglia, suggesting the presence of immunosuppressive environment within gliomas. The components contributing to this suppression could be anti-inflammatory cytokines released by glioma cells, such as interleukin (IL)-6, IL-11, leukemia inhibitory factor, oncostatin-M, and TGF-β, which can inhibit cytotoxic T-cell activation and MG/MP activation (Constam et al., 1992; Goswami et al., 1998; Halfter et al., 1998; Hao et al., 2002; Murphy et al., 1995).

On the other hand, glioma infiltrating microglia/macrophages (GIMs) release many factors, such as cytokines, growth factors and proteases, which directly or indirectly influence tumor progression (Graeber et al., 2002). It has been shown that GL261 cell migration occurred earlier and faster in the presence of primary microglia or microglia-conditioned medium, which indicated that microglia-released soluble factors can promote glioma migration (Bettinger et al., 2002). GIMs are the major source of IL-10, an immunosuppressive cytokine that promotes glioma proliferation and migration (Huettner et al., 1997; Wagner et al., 1999). Moreover, the glioma- and GIM-derived TGF-β has multiple effects on glioma progression: it can inhibit microglia proliferation and release of cytokines *in vitro* (Suzumura et al., 1993), can also induce metalloproteinase (MMPs) expression and promote the invasion of glioma (Markovic et al., 2009; Wesolowska et al., 2008; Wick et al., 2001). Both GIMs and glioma cells secrete vascular endothelial growth factor (Lafuente et al., 1999; Tsai et al., 1995) that mediates angiogenesis. These findings suggested that gliomas recruit MG/MP, inhibit the immune response and induce the MG/MP production of tumor survival factors, which in turn facilitates glioma growth and malignancy.

Our experiments further supported this hypothesis and provide new evidence of the tumor-promoting function of GIMs. *In vivo*, we demonstrated that GIM depletion led to significant decrease in glioma size and prolonged survival of tumor-bearing mice. The results presented here agree with recent data (Markovic et al., 2009), but provide more detailed characterization of the model. Since GIMs are a significant source of tumor survival factors, GIM loss may result in decrease of immunosuppressive cytokines, growth factors and proteases, which are essential for glioma growth.

It was shown that macrophage depletion in CD11b-TK^{mt-30} mice via systemic GCV injection resulted in glioma growth increase (Galarneau et al., 2007). These results agree with the early-time points of our data, but the final outcome is different. The difference may lie in the experimental system (administration route of GCV, reconstitution with bone-marrow derived wild-type cells).

The recent treatments for glioma are–among others - manipulations of the activity of tumor-associated macrophages, including inhibition of their recruitment to and survival in tumor tissue and restoration of their anti-tumor immunity (Allavena et al., 2005; Sessa et al., 2005; Wu et al., 2009). However, the development of therapeutic anti-glioma options by modulating the activity of GIM has not been thoroughly investigated. Glioma cells used in animal studies are of different immunogenicity and thus probably a reason for controversial results in the literature. For example, the injection of CpG ODN to GL261 mouse glioma cells resulted in contradictory data in a 9L rat glioma model (El Andaloussi et al., 2006; Ginzkey et al., 2009), which suggests that MG/MP stimulators may have different effects on MG/MP activities depending on the tumor microenvironment. Our results suggest that tuftsin will not be an effective treatment option for glioma patients. Although tuftsin has been widely used in anticancer studies (Banks et al., 1985; Noyes et al., 1981; Wleklik et al., 1986), its infusion into the glioma model presented here exaggerated tumor growth and shortened the survival time of tumor-bearing mice. Tuftsin activates monocytes and macrophages leading to IL-1, TNF-α and nitric oxide release (Cillari et al., 1994; Robey et al., 1987; Wleklik et al., 1987), which possibly contributed to the initial slowing down of glioma growth. However, due to the immunosuppressive environment within gliomas, the activation effects of tuftsin on GIMs may switch them towards a tumor-promoting phenotype at later stages. Similar results were obtained in a mouse model of multiple sclerosis, where tuftsin infusion altered the host immune response towards favoring the expression of immunosuppressive Th2 genes (Bhasin et al., 2007).

In contrast, the presence of MIF/TKP (tuftsin fragment 1–3) and microglia slowed down glioma cell growth. Delivery of MIF/TKP to the tumor inhibited tumor proliferation and induced GIM apoptosis, in agreement with the current trend of tumor therapy (Allavena et al., 2005; Meng et al., 2010; Miselis et al., 2008). Similarly, it was shown that MIF/TKP delivery to the injury site after intracerebral hemorrhage (ICH) inhibited MG/MP infiltration and activation, and afforded neuroprotection (Wang and Tsirka, 2005).

One possible mechanism contributing to the above results could be the change of CCL21 expression level in glioma tissue. A recent report (Shields et al., 2010) showed that melanoma cells could shift their microenvironment to an immune tolerant one by expressing CCL21. This led to the formation of lymphoid-like stromal structures, increased the levels of TGF-β1 and the number of myeloid-derived suppressor cells. Melanoma cells upregulated CCL21 in stromal cells, and this induced expression was a major contributor to an immunotolerant microenvironment, which resulted in promotion of melanoma progression. Consistently, we found that addition of CCL21 neutralizing antibody to microglia-glioma co-culture inhibited the glioma-promoting effects of microglia, suggesting that CCL21 is an important mediator of glioma growth. Previously it has been shown that CCL21 is up-

regulated in neurons after injury, which activates microglia through the chemokine receptor CXCR3 and induces microglia migration (Biber et al., 2001; de Jong et al., 2005; Rappert et al., 2002). We found here that glioma and surrounding stromal cells expressed CCL21 two weeks after glioma inoculation, which suggested that the glioma microenvironment is immune suppressive. Moreover, tuftsin treatment increased CCL21 levels in the glioma tissue, whereas MIF/TKP treatment had the opposite effect. In GCV-treated CD11b-HSVTK mice, the expression of CCL21 was not detectable (data not shown), indicating that the modulation of GIM activities can possibly change the tumor microenvironment and modulate tumor progression.

Taken together, our data demonstrate that gliomas activate microglia and suppress immune responses (phagocytosis) by microglia, even when microglia are activated. On the other hand we find that microglia only modestly stimulate glioma growth in culture, even when activated (or inhibited); nonetheless, glioma growth *in vivo* is profoundly affected by microglia activation. We think that this effect is due to the environment, as CCL21 is upregulated to immunoprotect tumors, while in absence of microglia (or microglial activation), no immune protection is afforded to the tumor. GIMs promote glioma growth and invasion, and, GIMs depletion or pharmacological inhibition is in the longer-term beneficial to glioma-bearing animals. Thus, inhibiting GIM activity by local delivery of factors such as MIF/TKP may be a potential novel interventional, adjuvant regimen to tackle gliomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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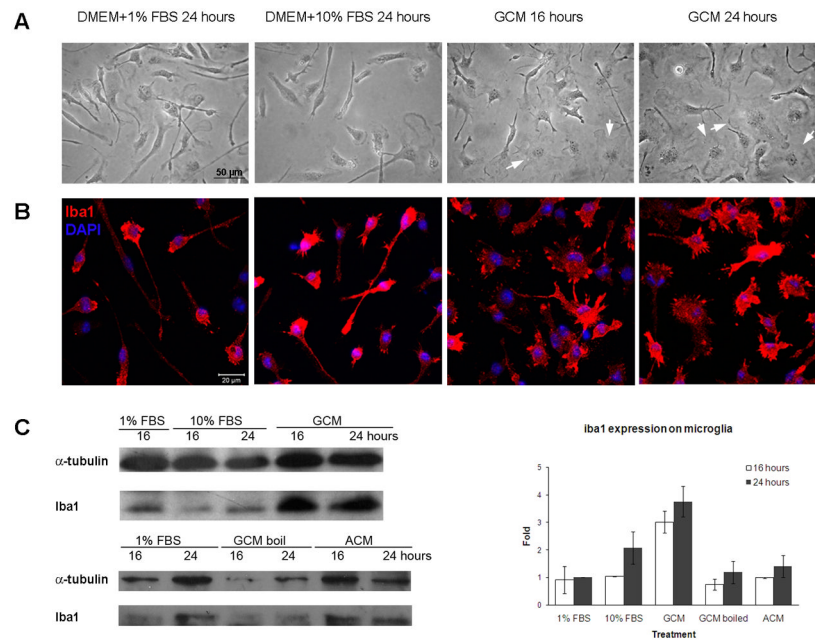
Figure 1. GCM activated primary microglia
Microglia were treated with GCM or DMEM+1% FBS (microglial medium) or 10% FBS (glioma medium) as controls. Cells were observed under bright field (A), or immunofluorescently stained with Iba1 antibody and labeled with DAPI (B), or lysed to obtain protein extracts for western blotting (C). Iba1 expression was normalized to α-tubulin. The experiment was repeated 3 times. Arrows: amoeboid microglia.
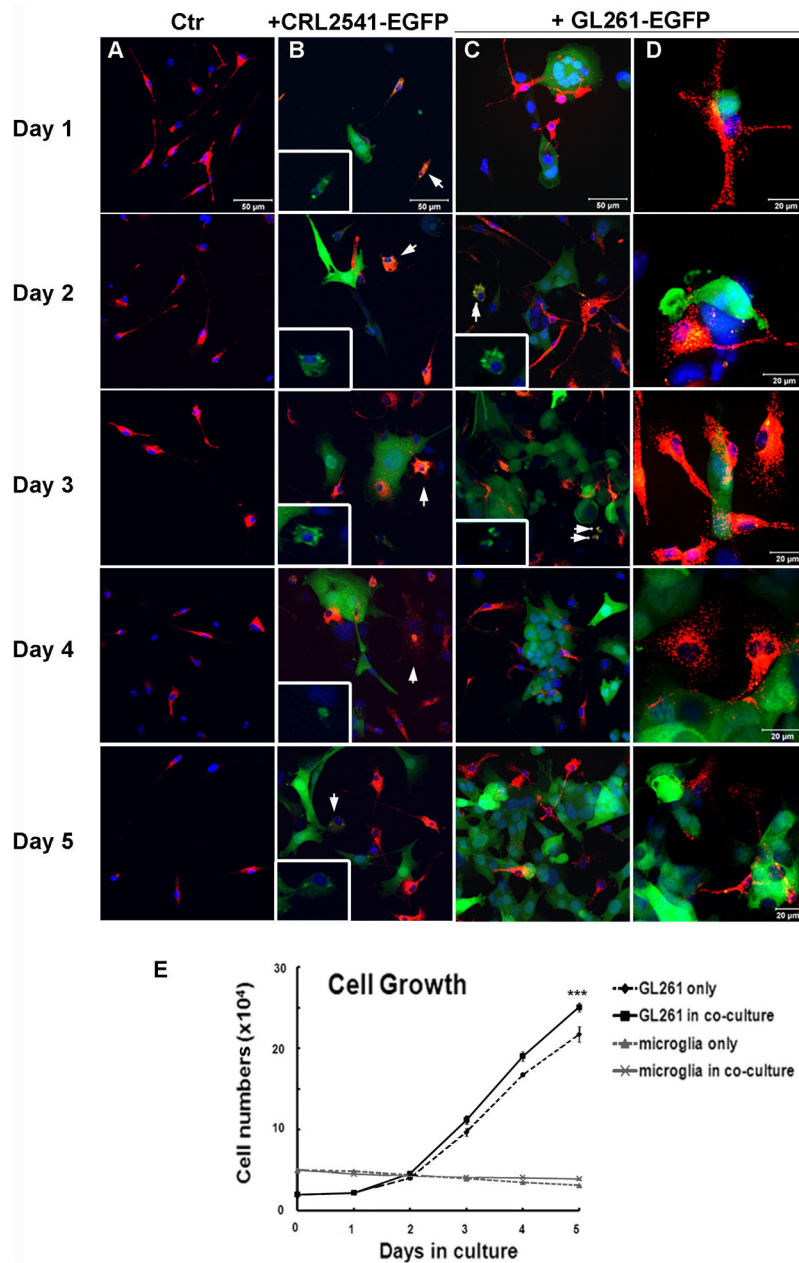
Figure 2. Microglia-glioma co-culture

5×10^4 rhodamine-labeled microglia (M) were plated in DMEM+1% FBS (microglial medium) for 48 hours before 2×10^4 CRL-2541-EGFP (B) or GL261-EGFP (C, D) were plated in the same well. DMEM+10% FBS was used as negative control. Confocal images (A–D) were taken and cell numbers of each population (E) were counted over 5 days. (A–D) Red cells: primary microglia; green cells: CRL-2541-EGFP or GL261-EGFP as indicated. Arrows: phagocytic microglia with GFP protein in cell body. (E) *** indicates the statistical analysis of GL261 alone and GL261 cell numbers in co-culture. The experiment was repeated 3 times. The scale bar in panels A–C is 50μm, in D 20μm.
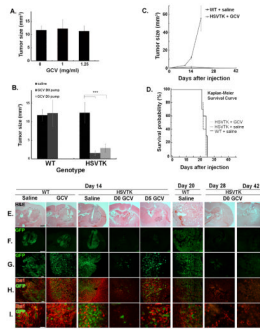
Figure 3. MG/MP ablation decreased glioma size and slowed down glioma progression
(A) WT mice were treated with GCV 14 days after GL261 injection (n= 5). (B) Tumor sizes in WT and HSVTK mice were measured 14 days after GL261-EGFP injection and infusion of 1mg/ml GCV or saline (WT saline and GCV group: n=5; HSVTK saline and D0 GCV group: n=6; HSVTK D5 GCV group: n=4). (C) Tumor sizes were measured at different time points after GL261-EGFP injection and infusion of GCV or saline (WT saline group: day10 n=6, day14 n=5, day20 n=8; HSVTK D0 GCV group: day14 n=6, day28 n=3, day42 n=2). (D) Kaplan-Meier survival curves of tumor-bearing mice. WT saline group: n=10, HSVTK saline and D0 GCV groups: n=5. Saline group of WT and HSVTK mice: no significant difference; HSVTK GCV group vs. saline groups: P<0.01. (E–I) Tumor morphology and MG/MP infiltration was evaluated at each time point. Representative images of (E) H&E staining (scale bar=1000μm; the tumor is demarcated by the dashed line); (F–G) GFP fluorescence (F: scale bar=1000μm; G: scale bar=100μm); (H–I) Iba1 immunofluorescence (H: scale bar=100μm; I: scale bar=50μm).
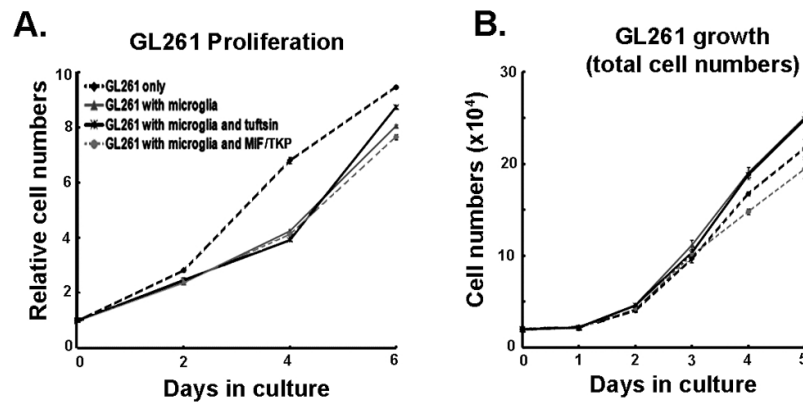
Figure 4. Different effects of tuftsin and MIF/TKP on GL261 growth in the presence of microglia
(A) Primary microglia on inserts were treated with 150μg/ml tuftsin or MIF/TKP. The inserts were placed in wells containing GL261 cells. GL261 proliferation was measured every 2 days. (B) GL261-EGFP cells were cultured with primary microglia in the presence of 150μg/ml tuftsin or MIF/TKP, and GL261-EGFP cell number was counted every day for 5 days. Experiments were repeated 3 times.
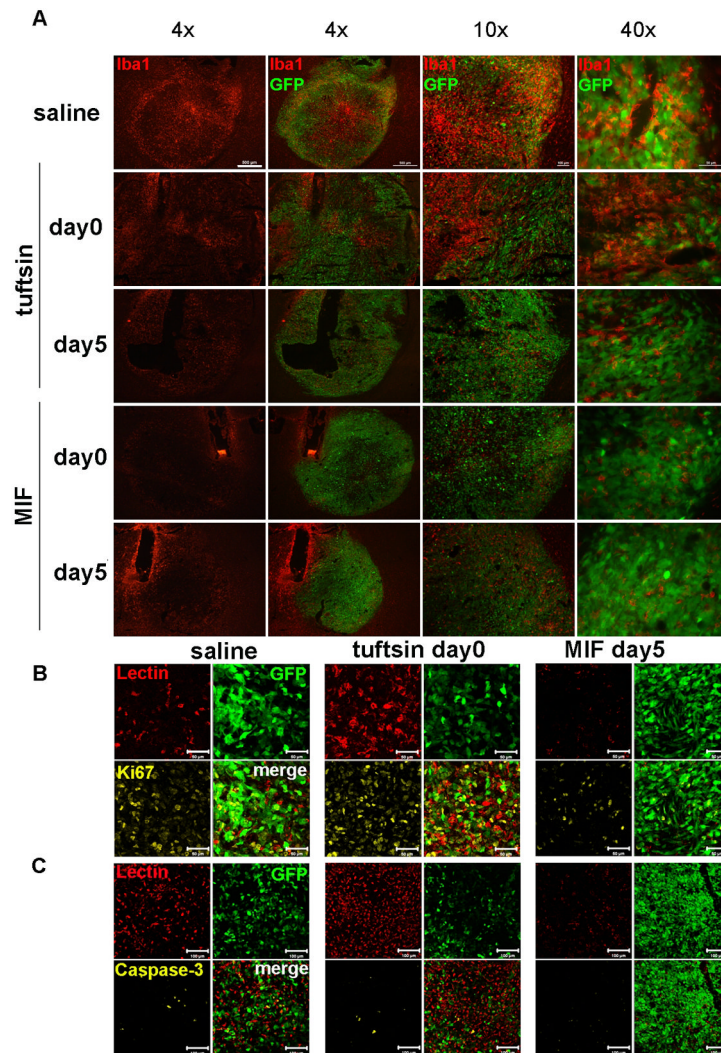
Figure 5. Tuftsin and MIF/TKP treatment affect glioma progression *in vivo*
(A) Tumor sizes and MG/MP infiltration (Iba1^{+} cells) were evaluated 14 days after GL261-EGFP injection and infusion of 250μg/ml tuftsin (TF), MIF/TKP or saline (scale bar 500μm). The proliferation and apoptosis of tumor cells and GIM in saline group, D0 tuftsin and D5 MIF/TKP groups were quantified with (B) Ki67 (scale bar 50μm) and (C) activated caspase-3 (scale bar 100μm) immunofluorescent staining (n = 3).

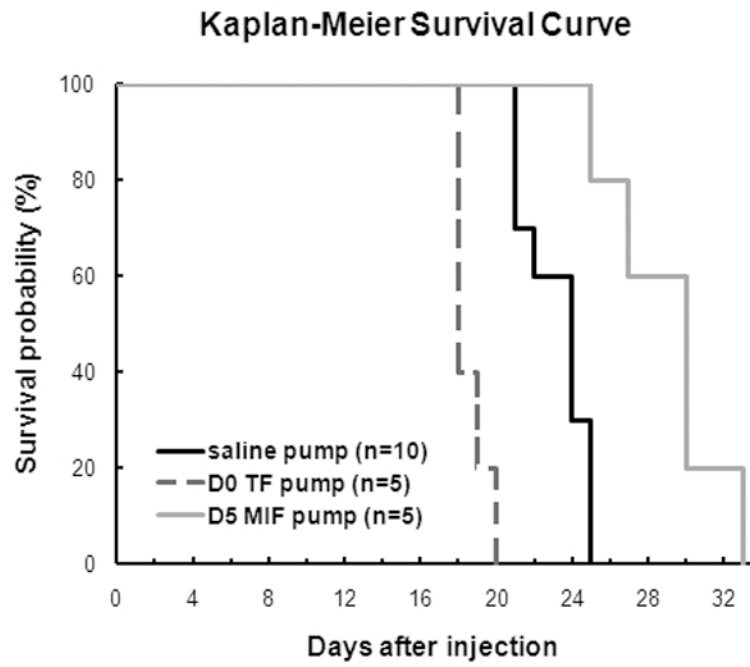## Kaplan-Meier Survival Curve

Figure 6. Kaplan-Meier survival curves of tumor-bearing mice with tuftsin or MIF/TKP (tuftsin fragment 1–3) treatment
The survival time of tumor-bearing mice infused with D0 tuftsin pumps (n=5) and D5 MIF/TKP pumps (n=5) was compared to the D0 saline pumps (n=10). (P < 0.001)
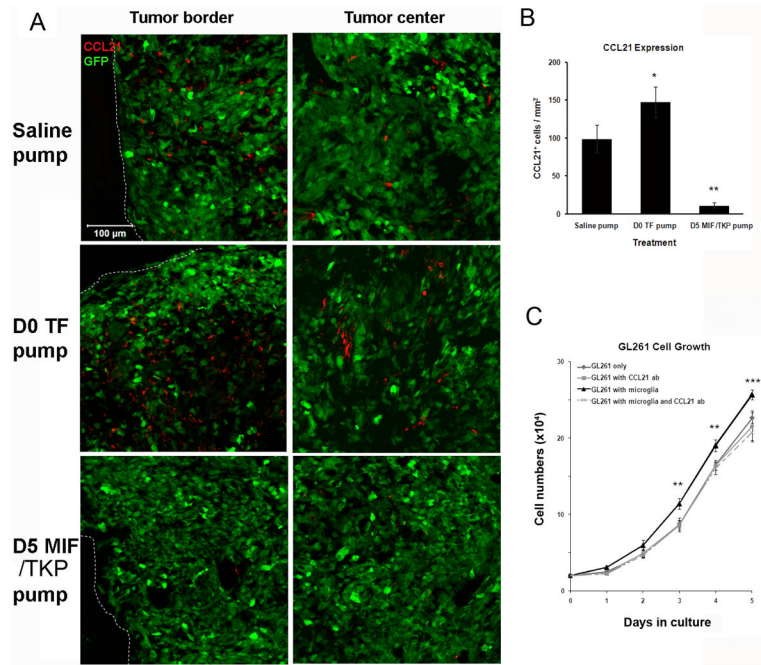
Figure 7. Tuftsin and MIF/TKP treatment had different effects on CCL21 expression in glioma tissue

14 days after GL261-EGFP injection and infusion of 250μg/ml tuftsin (TF), MIF/TKP or saline, CCL21 expression was quantified by counting CCL21^{+} cells within the tumor tissue (B) (n=3). Representative images from tumor border and center shown in (A). Dashed lines indicate the tumor borders. (C) The tumor-promoting function of CCL21 was confirmed in microglia-glioma co-culture. (n = 4) ** and *** indicates the statistical significance between the cell numbers of GL261 in co-culture with and without 4μg/ml CCL21 antibody (ab). There is no significant difference among the cell numbers of GL261 alone, or cultured with 4μg/ml CCL21 antibody, or in co-culture with microglia and CCL21 antibody.

Table 1

Tumor size after tuftsin and MIF/TKP treatment

Treatment	Tumor size at Day 14 (mm^3)
Saline	11.16±2.01
Tuftsin Day0 pump	20.99±3.91 [***]
Tuftsin Day5 pump	9.86±2.57
MIF/TKP Day0 pump	17.97±17.06
MIF/TKP Day5 pump	5.46±0.69 [**]

Tumor sizes were measured 14 days after GL261-EGFP injection and infusion of 250μg/ml tuftsin, MIF/TKP or saline.

[**] Data are presented as mean±SEM. and

[***] indicate the statistical analysis between the experimental group and saline control group.

Table 2

Quantification of Glioma Infiltrating Microglia/Macrophages (GIMs) density, proliferation and apoptosis

Tumor treatment	Saline	tuftsin	MIF/TKP
Measurement			
GIM density (lectin$^+$ cells/mm^2)	687±23.9	1014±176.8[*]	495±51.7[**]
Total proliferation (Ki67$^+$ cells/mm^2)	2354.7±225.0	2839.5±164.0[*]	1386.0±219.7[**]
Tumor cell proliferation (Ki67$^+$GFP$^+$ cells/mm^2)	1848.1±130.3	2207.6±78.5[**]	1322.7±267.9[*]
Total apoptosis (activated caspase 3$^+$/mm^2)	48.6±4.6	28.0±0.9[**]	60.4±5.2[*]
Tumor cell apoptosis (activated caspase 3$^+$GFP$^+$ clls/mm^2)	20.9±3.8	13.4±0.8[*]	15.2±2.1
GIM apoptosis (activated caspase 3$^+$lectin$^+$ cells/mm^2)	4.2±0.5	2.8±0.5[*]	17.3±4.1[**]

GIM density was measured by counting MG/MP number per field inside tumor area. The proliferation and apoptosis of tumor tissue, tumor cells and GIM in saline group, D0 tuftsin group and D5 MIF/TKP group were quantified by Ki67 and activated caspase-3 immunofluorescent staining (n = 3)

[*] Data are presented as mean±SEM. and

[**] indicate the statistical analysis between the experimental group and saline control group.