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Original Article

Activation of toll-like receptor 2 promotes invasion by upregulating MMPs in glioma stem cells

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Abstrac: Invasion is one of the deadly characteristics of malignant glioma with unknown underlying cellular and molecular mechanisms. In the present study, we investigated the role of toll-like receptor 2 (TLR2) in the invasiveness of malignant glioma. We enriched glioma stem cells (GSCs) from mouse GL261 cell line by means of tumor sphere formation, and found that GSCs expressed a significantly higher level of TLR2 than committed GL261 cells at the levels of mRNA and protein. Stimulation with Pam3CSK4, a ligand of TLR2, significantly increased the migration and invasion capability of GSCs. Knockdown of TLR2 attenuated the stimulating effect of Pam3CSK4 and the invasion capability of GSCs. An orthotopic allograft tumor model showed that TLR2-knockdown decreased the invasion capability of GSCs and prolonged survival span of tumor-bearing mice. The expressions of matrix metalloproteinases 2, 9 (MMP2 and MMP9) by GSCs were enhanced by treatment of Pam3CSK4 and decreased by TLR2 knockdown, implying that MMP2 and MMP9 were involved in TLR2-mediated invasion of GSCs. Our findings indicate that the activation of TLR2 up-regulates MMPs to promote invasion of GSCs, and suggest that TLR2 might be a potential therapeutic target for treatment of glioma patients.

Keywords: Glioma stem cells, invasion, matrix metalloproteinases, Pam3CSK4, toll-like receptor 2

Introduction

Glioma is the most common primary tumor of central nervous system. Malignant glioma always casts devastating effects on patients' life expectancy and quality although they only account for < 2% of all human cancer types [1]. Current therapies for glioma include maximal safe resection of the tumor mass followed by radio- and chemotherapies [2]. These treatments, however, hardly prevent the tumor from recurrences due to glioma high invasiveness and resistance to chemo- and/or radiotherapies, which results in the lower 5-year survival (< 3%) [3]. Therefore, the underlying cellular and molecular mechanisms need to be urgently elucidated.

During the recent years, cancer stem cell theory has been emerging, which hypothesizes that a small population of cells within tumors with stem cell properties may be responsible for tumorigenesis and give rise to a diversity of more differentiated cells and heterogeneous phenotypes [4]. Glioma appears to have a hierarchical cellular organization suggestive of a stem cell foundation. Up to now, glioma stem cells (GSCs) have been isolated and characterized by our group and others [5-8], which have been proposed to be responsible for highly invasive nature and chemo-/radiotherapy resistance of glioma [9]. Nevertheless, the molecular mechanisms remain unclear.

There is accumulating evidence that toll-like receptors (TLRs) are expressed not only by immune cells but also by human tumor cells. TLRs have been implicated in cancer cell proliferation, invasion, metastasis, and survival in a variety of tumors [10, 11]. For instance, TLR2 promotes invasion via activating NF-κB in MDA-MB-231 breast cancer cells. However, the role of TLR2 in controlling the invasion of glioma remains unknown.

In the present study, we demonstrated that TLR2 was highly expressed by GSCs derived

from murine GL261 glioma cell line and activation of TLR2 upregulated matrix metalloproteinases 2, 9 (MMP2 and MMP9) expression to promote tumor invasion, indicating that TLR2 signaling in GSCs is involved in the invasiveness of glioma.

Materials and methods

Glioma cell line and mice

Mouse GL261 glioma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, USA) supplemented with 10% fetal bovine serum (Gbico, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under atmosphere of 5% CO_2. For in vivo experiments, six- to eight-week-old female C57BL/6 mice were purchased from Animal Institute of Third Military Medical University. All animal experiments were approved by the Animal Experiments Committee of Third Military Medical University.

Enrichment of GSCs

GSCs were enriched by sphere formation method [5]. GL261 cells were seeded in 6-well plates at the density of 2000 cells/cm^2 in stem cell medium containing serum free DMEM/F12 medium (1:1, Gbico, USA), 20 ng/mL of epidermal growth factor (EGF, Sigma), 20 ng/mL of basic fibroblast growth factor (bFGF, Gbico), and B27 supplement (1 ×, Gbico). Cells were incubated at 37°C under atmosphere of 5% CO_2. Half of the medium was changed every three days. Tumor spheres containing 50~200 cells were isolated through cloning cylinders (Corning, USA). Each isolated tumor sphere was dissociated into single cell suspension and cultured in 6-well plates to generate secondary spheres. The morphology of the third passage of sphere colony was examined under a microscope and used for following experiments.

Quantitative RT-PCR

Total RNA was isolated by TRIzol™ reagent (TaKaRa, China). One μg RNA of each sample was used for reverse transcription by cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's protocol. Quantitative PCR to amplify target genes was performed using SYBR (Fermentas, Canada) according to the manufacturer's instruction. Primers used in this study were listed in Table S1.

Western blotting

Cells were washed with PBS and lysed with protein extraction reagent (Thermo, USA). The lysates were then centrifuged at 4°C, 15,000 × g for 15 min and supernatants were collected. Concentrations of proteins were measured using a bicinchoninic acid procedure. Equal amounts of proteins were separated by 10% SDS-PAGE, and transferred to PVDF membranes (Millipore, USA). PVDF membranes were blocked in 5% non-fat dry milk for two hours at room temperature and then incubated with primary antibodies (anti-TLR2 antibody and anti-β-actin antibody) (Abcam, USA) in blocking solution at 4°C for 16 hrs. After washing three times with PBST (containing 0.1% Tween-20) at 15-min intervals, PVDF membranes were probed with secondary antibodies in blocking solution for two hours at room temperature. Proteins were detected by ECL detection reagent (Thermo, USA). β-actin was used as loading control.

Knockdown of TLR2

TLR2 shRNA lentiviral particles were established by Shanghai SBO Medical Biotechnology Company (Shanghai, China). The transfection was carried out with TLR2-specific shRNA (TLR2-KD) or scrambled shRNA (Mock). Briefly, GL261 glioma cells (1.25×10^4 cells/well) were cultured in 24-well plates and infected with lentivirus of 3×10^6/well for stable expression. Then cells expressed GFP were selected by flow cytometry. The efficiency of TLR2 knockdown was determined by quantitative RT-PCR and western blotting.

Cell invasion assay

Inner bottoms of Transwell™ inserts (8.0 μm pore size) (Millipore, USA) were coated with a layer of Matrigel™ (BD Biosciences, USA). Cells suspended in serum-free DMEM medium containing 0.2% BSA were seeded in the compartments inside the inserts at the density of 50,000 cells each chamber, and the outer compartment of inserts were added with 500 μL DMEM containing 10% FBS. Cells were allowed to invade through the Matrigel™ for 24 hrs. Then, the cells in the upper chambers were removed using cotton swabs, and the cells remained on the outside surface of membranes were fixed with 4% paraformaldehyde, stained

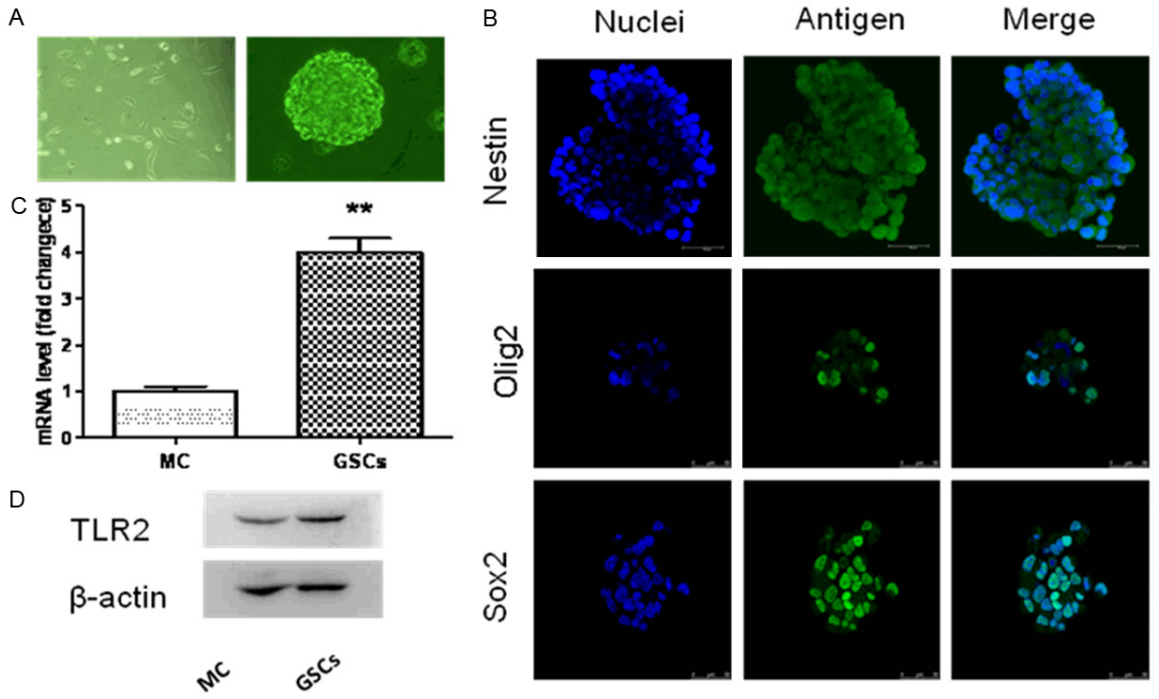

Figure 1. Sphere cells derived form GL261 cells expressed stemness related genes and highly expressed TLR2. A. Monolayer cells of GL261 (MC, left panel) and tight tumor spheres formed from GL261 under stem cell culture conditions (right panel). B. Tumor sphere cells expressed stemness related gene Nestin, Olig2 and Sox2 detected by immunofluorescence staining. C. Tumor sphere cells (GSCs) highly expressed TLR2 mRNA detected by quantitative RT-PCR. D. GSCs highly expressed TLR2 protein detected by western blotting. Data are presented as the mean ± SD. Student *t* test was performed to evaluate the difference. $^{**}p < 0.01$.

with crystal violet and counted under a microscope at 200 × magnification.

Immunofluorescence analysis

Cells were cultured on poly-L-lysine-coated glass cover slips overnight at 37°C, and then fixated with 4% paraformaldehyde for 10 min. After three washes with PBS, cells were permeabilized using 0.5% Triton X-100 in PBS solution for 15 min. After three washes with PBS, cells were blocked in 1% BSA for 30 min at room temperature, and then incubated with primary antibodies in 1% BSA solution for two hours at 37°C. The primary antibodies used were as follows: Sox2 (rabbit anti-human, 1:200, Novus, USA), nestin (mouse anti-human, 1:200, Millipore, USA), Olig2 (rabbit anti-human, 1:200, Millipore, USA). Then cells were washed with PBS and probed with fluorescence-conjugated secondary antibodies (goat anti-rabbit for Sox2 and Olig2, goat anti-mouse for nestin) for one hour at 37°C, and were counterstained with 5 μg/mL DAPI (Sigman, USA) for two minutes to identify nuclei. Slides were mounted with Fluoromount™ mounting medium, and then visualized and photographed under a laser confocal scanning microscope (SP-5, Leica, Germany).

Orthotopic allografts

Cells were collected, washed twice in PBS, counted, and adjusted to 4×10^4 cells in 10 μL of PBS in a 25-μL microliter syringe. Six-week-old C57BL/6 mice were anesthetized with 1% pentobarbital sodium (10 mL/kg). A hole was drilled in the skull 2 mm lateral to the midline and 2 mm anterior to the bregma using a syringe needle. Then, cells were injected orthotopically over three minute at a depth of 2.5 mm below the dura mater into the right cerebral hemisphere. The survival time was defined as the interval between the date of implantation and the date of death for any reason. Overall survival (OS) was obtained with complete observation until all mice of experimental groups were dead. Brains of dead mice were collected for further pathological examination. Mice in vehicle control group were sacrificed

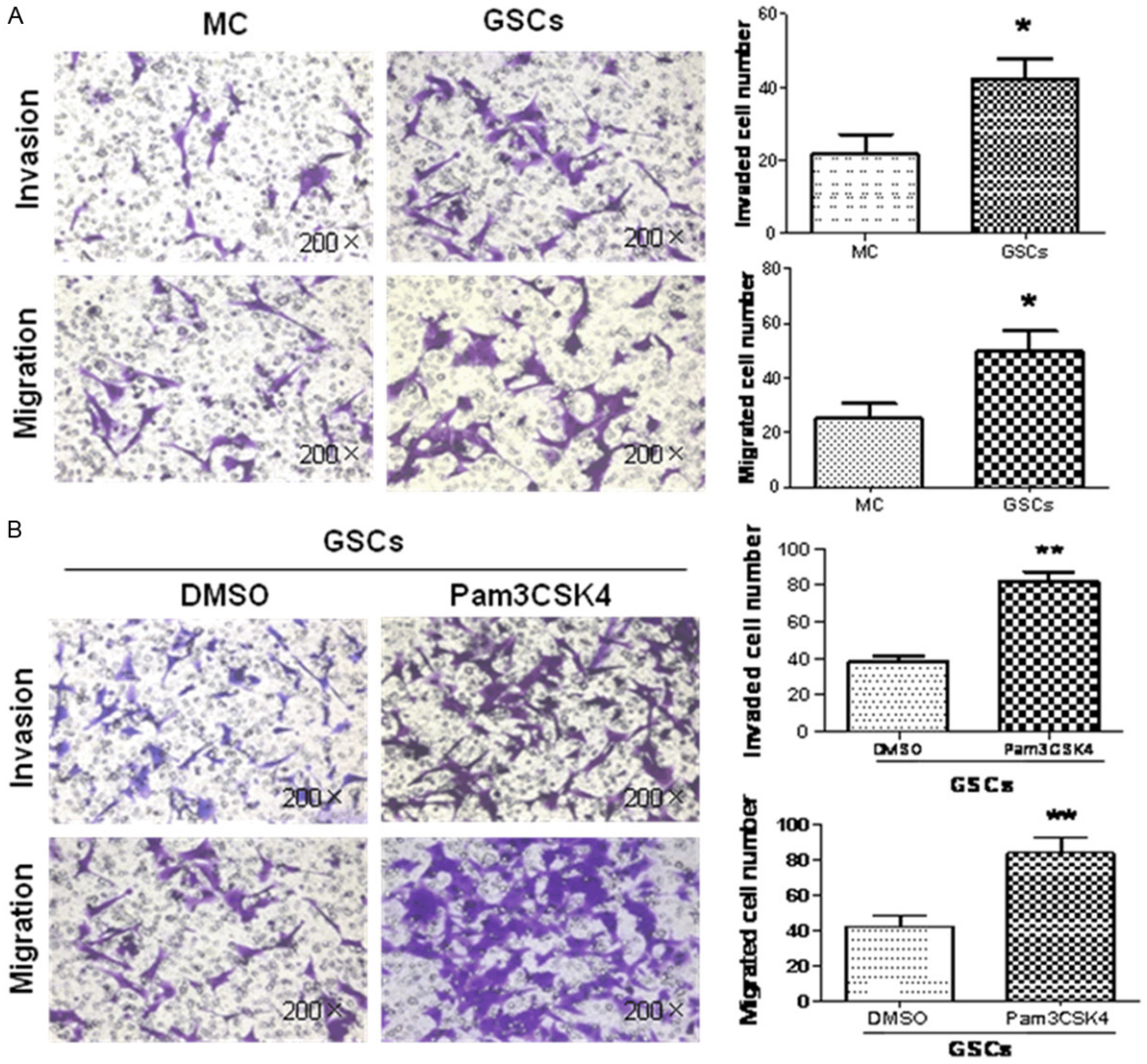

Figure 2. TLR2 agonist enhanced invasion and migration of GSCs. A. GSCs exhibited higher ability of invasion and migration than that of MC. B. Stimulation with TLR2 agonist Pam3CSK4 for 12 h significantly enhanced the invasion and migration of GSCs. Data are presented as the mean ± SD. Student *t* test was performed to evaluate the difference. *p < 0.05, **p < 0.01.

after 50 days post-implantation by cervical dislocation.

Statistical analysis

The unpaired Student's *t*-test or one-way ANOVA was performed for the unpaired two-group comparison and multiple comparisons, respectively. Kaplan-Meier curves and log-rank analysis were conducted for in vivo study. SPSS10.0 statistical software (SAS, Chicago, IL) was utilized for statistical analysis. All experiments were carried out independently at least three times on triplicate samples. Data were presented as the mean ± SD. $P < 0.05$ (*) and $P < 0.01$ (**) were considered statistically significant.

Results

GL261 tumor sphere cells possess stem cell properties

In order to investigate the role of TLR2 in the invasiveness of GSCs, we first enriched and characterized GSCs from GL261 cells by means of tumor sphere culture. After culture in stem cell medium at low density for 10 days, GL261 cells formed tight tumor spheres (**Figure 1A**).

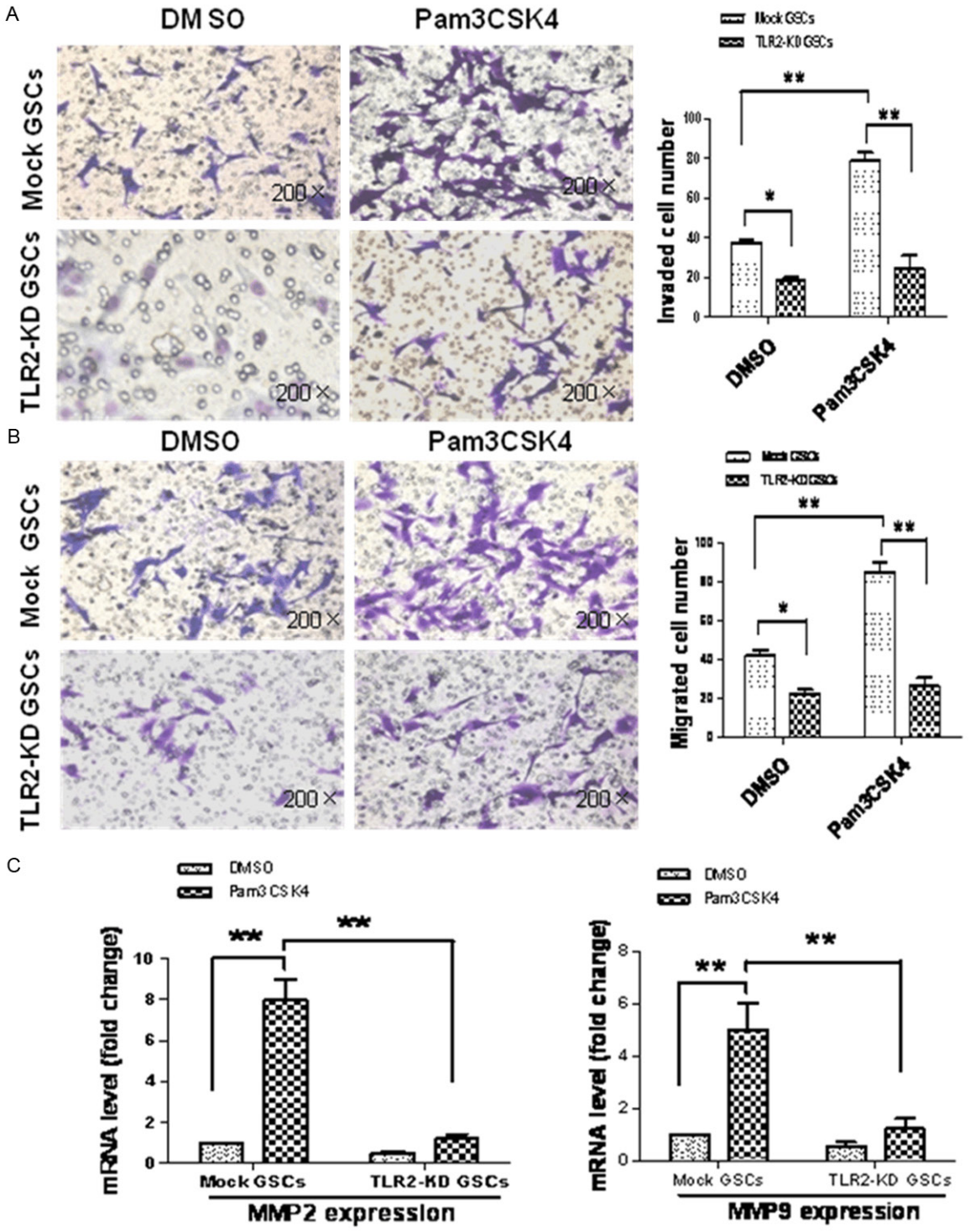

Figure 3. Knockdown of TLR2 expression inhibited the invasion and migration and decreased expression of MMP2 and MMP9 in GSCs. A. Invasive abilities of Mock GSCs and TLR2-KD GSCs were compared. The invasive cell number of GSCs was decreased by TLR2 knockdown at presence of Pam3CSK4 stimulation or not. B. Migration abilities of Mock GSCs and TLR2-KD GSCs with or without Pam3CSK4 stimulation were compared. C. The expression of MMP2 and MMP9 responded to TLR signaling. TLR2 knockdown in GSCs attenuated the expression of MMP2 and MMP9. Data are presented as the mean ± SD. Student *t* test was performed to evaluate the difference. *$p < 0.05$, **$p < 0.01$.

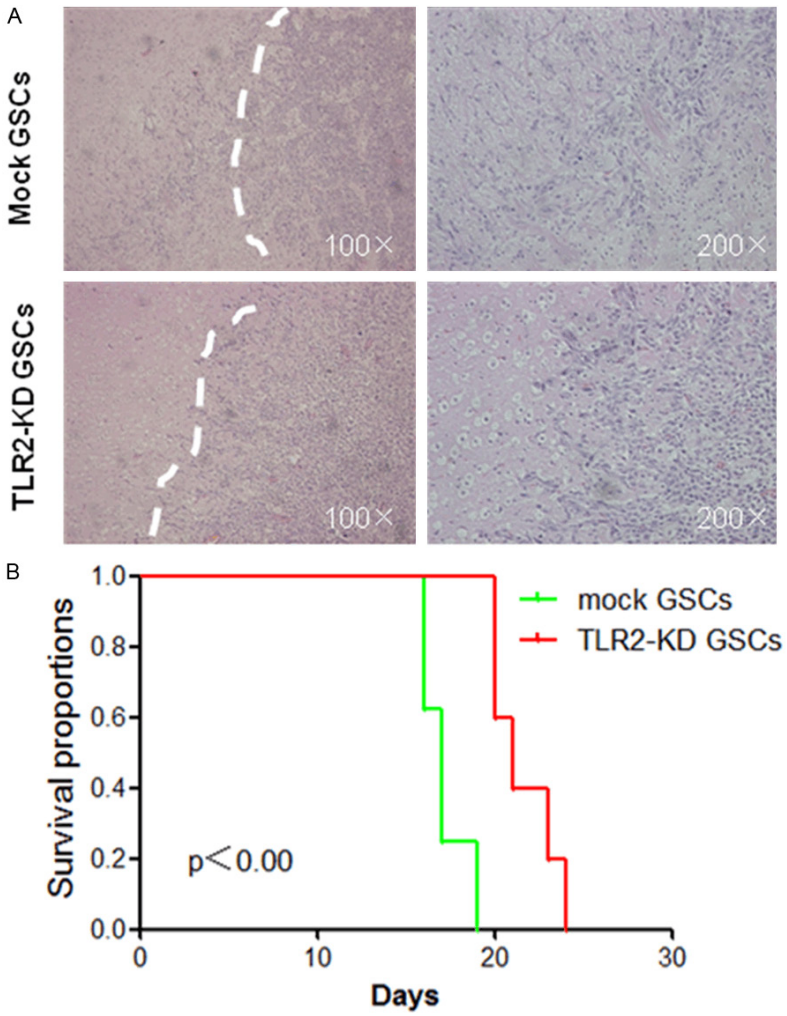

Figure 4. Knockdown of TLR2 attenuated the invasion of allograft tumors derived from GSCs and prolonged the survival time of tumor-bearing mice. A. Survival curves of tumor-bearing mice in orthotopic allograft models of GSCs showed that TLR2-KD group exhibited longer survival span than that of Mock group ($p < 0.01$, $N = 6$). B. H&E staining showed that the invasion of allograft tumors derived from TLR2-KD GSCs was weaker than that of Mock GSCs.

The tumor spheres could continuously generate new spheres by the passage up to fifth generations at least, and increasing number of tumor spheres were formed in new generations of culture. Since the expression of progenitor/stem cell genes is the important trait of GSCs, we further examined the expressions of nestin, Olig2 and Sox2 in GL261 tumor spheres. Among these genes, nestin is relatively specific marker of brain CSCs [12], whereas Olig2 and Sox2 are not only widely accepted as CSCs markers of epithelial malignancies, but also considered to be essential for glioblastoma propagation [13]. As shown in **Figure 1B**, tumor spheres were positively stained for nestin, Olig2 and Sox2. These data indicate that tumor sphere cells derived from GL261 possess the properties of GSCs.

TLR2 is preferentially expressed by GSCs

Quantitative RT-PCR analysis was performed to systematically analyze the expression of all TLRs in GL261 glioma cells, and revealed that TLR2, TLR3, TLR9 and TLR12 were relatively high expressed (data not shown). Among those expressed TLRs, TLR2 was higher than others. We further compared the expression of TLR2 between GSCs and GL261 monolayer cells (MC), and found that TLR2 expression was significantly higher in GSCs than MC at the levels of both mRNA (**Figure 1C**) and protein (**Figure 1D**).

Activation of TLR2 by its ligand enhances migration and invasion of GSCs

Since invasion is an important biological feature of GSCs, we reasonably hypothesized that GL261-derived GSCs might have more invasive and migrating capabilities. Matrigel™ invasion assay showed that GSCs exhibited significantly enhanced invasive ability (**Figure 2A**, upper panel) and increased migrating capability (**Figure 2A**, lower panel) as compared to MC.

It has been previously reported that expression of TLR2 in breast cancer cell lines positively correlated with invasive capability [10]. We presumed that TLR2 might also be a potential invasive factor in glioma cells, especially in GSCs. Pam3CSK4 (Invivogen, CA), a specific ligand of TLR2, was used to examine whether invasion of GSCs is responsive to TLR2 signaling. After

stimulation with Pam3CSK4 at 1 μg/mL for 48 hrs, the number of invaded cells was increased for at least two folds ($P < 0.01$, **Figure 2B**, upper panel). Similar results were obtained from migration experiments of GSCs ($P < 0.01$, **Figure 2B**, lower panel). These results imply that the invasive and migration features of GSCs are by some means dependent on TLR2 activation.

Silencing TLR2 reverses the invasion of GSCs stimulated by TLR2 ligand

As mentioned above, TLR2 might be a potential invasive and migration factor in GSCs. Thus, inhibition of TLR2 signaling should be able to reverse invasion and migration capabilities of GSCs. To reveal whether TLR2 is critical for GSCs invasion, we performed a TLR2 knockdown (KD) experiment with a lentivirus-based approach in GL261 cells. After infection of GL261 cells with lentivirus of TLR2 shRNA (TLR2-KD), reduction of TLR2 expression was observed in TLR2-KD cells as compared with Mock or wild-type cells (Figure S1). No significant difference was observed in the tumor sphere formation among TLR2-KD, Mock or wild-type cells (date not shown), but the invasion (**Figure 3A**) and migration (**Figure 3B**) capability of TLR2-KD GSCs was impaired with or without Pam3CSK4 stimulation. These results confirm that TLR2 is involved in the invasion of GSCs.

Expressions of both MMP2 and MMP9 are regulated by TLR2 in GSCs

MMPs such as MMP-2 and MMP-9 are known to be associated with glioma invasion [4, 9, 14]. We further explored whether MMPs were involved in TLR2-mediated invasion process of GSCs. By Pam3CSK4 stimulation, the mRNA levels of both MMP-2 and MMP-9 in Mock GSCs were dramatically increased (**Figure 3C**), whereas the expressions of both MMP-2 and MMP-9 in TLR2-KD GSCs were less responsive to Pam3CSK4 stimulation (**Figure 3C**). These results indicate that both MMP-2 and MMP-9 are involved in TLR2-mediated invasion of GSCs.

Silencing TLR2 expression of GSCs decreases the invasion of allograft tumors and prolongs survival span of tumor-bearing mice

To further elucidate the role of TLR2 in glioma invasion, a GSCs-derived orthotopic allograft tumor model was established in C57BL/6 mice [15]. The cells dissociated from TLR2-KD and Mock spheres were orthotopically injected into C57BL/6 mouse cerebral hemisphere respectively. At day 14 after implantation, severe weight loss, trembling behavior and hunched posture were observed in mice implanted with Mock sphere cells (Mock GSCs), but not in the animals implanted with TLR2-KD sphere cells (TLR2-KD GSCs). The behaviors of animals in vehicle control (PBS) group were normal up to 50 days after transplantation. Survival curves estimated by Kaplan–Meier algorithm were shown in **Figure 4A**. Compared with the mice implanted with GSCs, the mice implanted with TLR2-KD GSCs exhibited significantly prolonged survival time by four to five days (median survival = 21.6 days *vs* 17.125 days, $p < 0.00$). The invasion of allograft tumors into surrounding normal brain tissue was examined with H&E staining. As shown in **Figure 4B**, the invasion of allograft tumors derived from TLR2-KD GSCs, which exhibited an irregular edge of infiltration, was significantly decreased as compared to that of Mock GSCs-derived tumors. Thus, our data demonstrate that the interruption of TLR2 signaling pathway leads to the decreased invasive capability of GSCs cells in vivo.

Discussion

The ability of cancer cells extensively invading to normal brain tissue, which prevents surgical treatment, contributes to the deadly characters of malignant glioma [16]. Therefore, a fundamental challenge presented in glioma therapy is the insidious invasiveness of the glioma cells [17]. Thus the factors involved in the glioma invasion need to be further explored and exploited as novel anti-glioma targets.

Recent studies have shown that functional TLRs are expressed not only by immune cells, but also by cancer cells. Many efforts have been undertaken to elucidate the role of TLRs in tumor biology. Accumulating evidence suggests that TLRs function as double-edged swords, with both pro- and anti-tumor consequences [18]. The function of TLR2 is cell type-dependent. TLR2 involved in innate immunity orchestrates an adaptive anti-brain tumor immune response. TLR2-stimulation activates traffics of dendritic cells therefore prolongs survival in cancer patients who relapse under chemotherapy [18]. On the other hand, TLR2 promotes gastric carcinoma metastasis [19] and human colorectal carcinoma cell invasion [20], implying a malignant property of TLR2.

In this study, we analyzed the expression of all TLRs in GL261 mouse glioma cells. TLR2 was not only highly expressed by GL261 cells as compared to other TLRs, but also more highly expressed by GL261 stem cells. The invasion and migration capabilities of GSCs were enhanced by stimulating with TLR2 ligand, but impaired by interrupting TLR2 expression in vitro. Silencing TLR2 decreased invasive capability of allograft tumors in a mouse model. Our data strongly suggest a role of TLR2 in invasion of glioma.

The double faces of TLR2 in cancer progress disclose the intricacy of TLR2 signaling. Our data suggest that the neoplastic process may sabotage TLR signaling pathways to favor glioma progression. Page *et al* found that TLR2 regulated MMP-9 release from neutrophils [21]. Using mice deficient for different TLRs, Hu *et al* identified that TLR2 signaling pathway upregulated expression of MMP9 in microglia [22]. The implantation of GL261 glioma cells into TLR2 knockout mice resulted in reduced MT1-MMP expression [11]. These reports imply that MMPs might be regulated by TLR2 in glioma. Our previous report indicated the role of MM9 in glioma invasion [9]. We also revealed that MMP2 is associated with the metastasis capability of gastric cancer stem cells [23] and glioblastoma [24]. We therefore hypothesized that MMP2 and MMP9 might be downstream factors involving in the invasion of glioma stem cells. As expected, we demonstrated that both MMP2 and MMP9 responded to TLR2 signaling.

In summary, we illustrated a valuable insight into the mechanism of glioma invasion from the concept of CSCs. As an important factor of glioma invasion, TLR2 may be a potential target for glioma therapy.

Acknowledgements

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Disclosure of conflict of interest

No potential conflicts of interest were disclosed.

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Table S1. The sequences of primers for qPCR

GAPDH	sense	5'-GTG GAG ATT GTT GCC ATC AAC G-3'
	antisense	5'-CAG TGG ATG CAG GGA TGA TGT TCT G-3'
TLR1	sense	5'-CTC TGG TGA GCC TTA CCT TGA-3'
	antisense	5'-GCT ATG ATG CAA TCA GGG ATG G-3'
TLR2	sense	5'-AGT TTT CAC CAC TGC CCG TA-3'
	antisense	5'-CCC AGC TCG CTC ACT ACG TC-3'
TLR3	sense	5'-TGC CAA ATA CTC CCT TTG TTG AA -3'
	antisense	5'-CCC GTT CCC AAC TTT GTA GAT G-3'
TLR4	sense	5'-CTC TGG CAT CAT CTT CAT TG-3'
	antisense	5'-CCG TTT CTT GTT CTT CCT CT-3'
TLR5	sense	5'-GCC CCG TGT TGG TAA TAT CTC-3'
	antisense	5'-GCT ATC CTG CCG TCT GAA GA-3'
TLR6	sense	5'-AGC CAA GAC AGA AAA CCC ATC -3'
	antisense	5'-CCA GGG CGC AAA CAA AGT G-3'
TLR7	sense	5'-ATG TGG ACA CGG AAG AGA CAA-3'
	antisense	5'-ACC ATC GAA ACC CAA AGA CTC-3'
TLR8	sense	5'-CTA TCC TTG TGA CGA GAT AAG GC-3'
	antisense	5'-AGT TGA CGA TGG TTG CAT TCT G-3'
TLR9	sense	5'-ATG GTT CTC CGT CGA AGG ACT-3'
	antisense	5'-CAG GTG GTG GAT ACG GTT GG-3'
TLR11	sense	5'-CTC TGG TGA GCC TTA CCT TGA-3'
	antisense	5'-GCT ATG ATG CAA TCA GGG ATG G-3'
TLR12	sense	5'-CCT GGT CTC CCG CTA TTT CAC-3'
	antisense	5'-CCG AGG TAC AAC TTC CAA GGT -3'
TLR13	sense	5'-GTT GTA ACC TGG ATG CCT AAG AC-3'
	antisense	5'-GGC CTC TGT CAA GTT GGT GA-3'
MMP2	sense	5'-GGG TGG AAG GCT CGT CAA TG-3'
	antisense	5'-TGG GTT TTT AGG AGA CAC CAA TG-3'
MMP9	sense	5'-GCA GAG GCA TAC TTG TAC CG-3'
	antisense	5'-TGA TGT TAT GAT GGT CCC ACT TG-3'

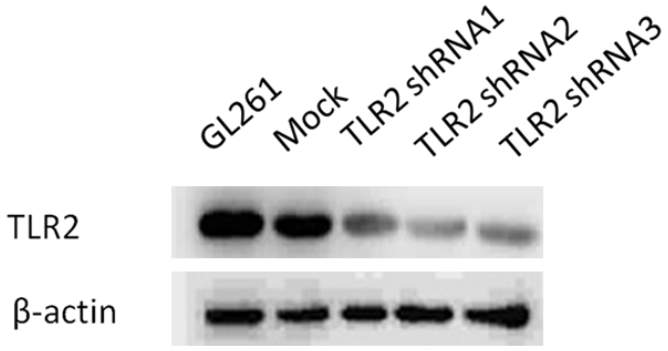

Figure S1. The efficacy of TLR2 knockdown by shRNA in GL261 cells. GL261 cells were transfected with three different TLR2 shRNAs or scrambled shRNA. After stably transfected, the protein levels of TLR2 expression were determined by western blotting.