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*J Immunol* 2008; 181:6720-6729; doi: 10.4049/jimmunol.181.10.6720

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TLR Ligands in the Local Treatment of Established Intracerebral Murine Gliomas

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Local TLR stimulation is an attractive approach to induce antitumor immunity. In this study, we compared various TLR ligands for their ability to affect murine GL261 cells in vitro and to eradicate established intracerebral murine gliomas in vivo. Our data show that GL261 cells express TLR2, TLR3, and TLR4 and respond to the corresponding TLR ligands with increasing MHC class I expression and inducing IL-6 secretion in vitro, while TLR5, TLR7, and TLR9 are essentially absent. Remarkably, CpG-oligonucleotides (CpG-ODN, TLR9) appeared to inhibit GL261 cell proliferation in a cell-type specific, but CpG-motif and TLR9-independent manner. A single intratumoral injection of CpG-ODN most effectively inhibited glioma growth in vivo and cured 80% of glioma-bearing C57BL/6 mice. Intratumoral injection of Pam3Cys-SK4 (TLR1/2) or R848 (TLR7) also produced a significant survival benefit, whereas poly(I:C) (TLR3) or purified LPS (TLR4) stimulation alone was not effective. Additional studies using TLR9+/+ wild-type and TLR9−/− knockout mice revealed that the efficacy of local CpG-ODN treatment in vivo required TLR9 expression on nontumor cells. Additional experiments demonstrated increased frequencies of tumor-infiltrating CD4+ and CD8+ effector T cells and a marked increase in the ratio of CD4+ effector T cells to CD4+FoxP3+ regulatory T cells upon CpG-ODN treatment. Surviving CpG-ODN treated mice were also protected from a subsequent tumor challenge without further addition of CpG-ODN. In summary, this study underlines the potency of local TLR treatment in antitumor therapy and demonstrates that local CpG-ODN treatment most effectively restores antitumor immunity in a therapeutic murine glioma model. The Journal of Immunology, 2008, 181: 6720–6729.

Malignant gliomas are highly aggressive brain tumors characterized by intense heterogeneity, high proliferative activity and local invasiveness. These tumors have developed multiple mechanisms to escape from immune surveillance (1, 2). We have recently demonstrated that the suppression of antitumor immune responses is strongly associated with the intratumoral accumulation of CD4+FoxP3+ regulatory T cells (Treg)3 (3). Several clinical phase I/II dendritic cell (DC) vaccination trials have been conducted with the objective to break immune tolerance against high grade gliomas (4–6). Although all studies led to the induction of antitumor CTLs and lymphocyte infiltration into tumors in situ, survival benefit remained low. Objective tumor regressions appeared to relate to the absence of a bulky tumor mass secreting TGF-β2 and, importantly, on the maturation status of DCs inside and around the tumor (7, 8). Therapeutic strategies combining abrogation of local immunosuppression with potent immune stimulation are therefore of particular interest in the search for efficient treatments of malignant gliomas.

The CNS is considered as a unique immunological site. The presence of the blood-brain barrier, graft acceptance, lack of conventional lymphatics, low T cell trafficking, and low but inducible MHC expression levels all point to low immune reactivity to prevent accidental inflammation within the CNS (9). However, in case of a CNS infection strong immune responses against the invading pathogens develop indicating that potent immune responses can occur.

The immune system is equipped with a panel of innate receptors that sense a broad spectrum of pathogens and alert cells to respond accordingly. The best characterized receptors are the TLRs that recognize a set of conserved molecular structures, so called pathogen-associated molecular patterns shared by large groups of microorganisms (10, 11). Currently, 13 TLR have been identified. TLR1–9 are expressed in both mice and humans, TLR10 is present in mice only as a degenerate pseudogene, and TLR11–13 are expressed in mice but lack human orthologs. TLR2 can associate with TLR1 and TLR6, and recognizes bacterial lipoproteins, peptidoglycan, and lipoteichoic acid. TLR3 recognizes viral double-stranded RNA and synthetic double-stranded RNAs, such as polyinosinic-polycytidylic acid (poly[I:C]). TLR4 binds LPS from Gram-negative bacteria and viral envelope proteins, while TLR5 recognizes flagellin. TLR7 and TLR8 recognize viral single stranded RNA and synthetic molecules like imidazoquinoline or derivatives. TLR9 recognizes unmethylated CpG motifs within bacterial and viral DNA. The specific ligand of TLR10 is currently unknown. The recently discovered TLR11 recognizes urapathogenic bacteria (12, 13).
TLRs are widely expressed by immune cells, but can also be found on nonimmune epithelial and endothelial cells, and tumor cells (14–16). Most cell-types show preferential expression of certain TLRs, either expressed in a constitutive or induced way. Phagocytic cells including APCs and monocytes/macrophages display the broadest spectrum of TLRs, but TLR expression is depending on the cellular subset and differentiation state (17, 18). TLR ligands provide danger signals for the innate as well as the adaptive arms of the immune system. TLR triggering on innate immune cells results in the induction of proinflammatory cytokines, phagocytosis, and subsequent innate effector mechanisms. APCs, such as DC, up-regulate costimulatory molecules, secrete immunomodulatory cytokines, such as IL-12, and increase Ag processing and presentation to B and T lymphocytes in response to TLR signals (19, 20). In addition, certain TLR ligands have been shown to directly potentiate the induction of an immune response by inhibiting the function of suppressor cells, like Tregs (21, 22). As such, the local administration of TLR ligands is of particular interest for immunotherapy aiming at the eradication of brain tumors.

Pioneering experiments from Carpentier et al. (23) have shown that direct injections of synthetic phosphorothioate-stabilized CpG-oligonucleotides (CpG-ODN) in neuroblastosomas induced complete tumor rejection in the majority of mice and triggered a long-term immunity. Further studies have confirmed the antitumor effects of CpG-ODN in different intracranial models of syngeneic glioma (24, 25). Moreover, a phase I trial utilizing convection-enhanced intratumoral delivery of CpG-28 has recently been completed in the setting of recurrent glioblastoma multiforme resulting in minor responses in two patients without displaying serious adverse events (26).

The current study was designed to evaluate various TLR ligands in their potency to effect murine GL261 glioma cells in vitro and to eradicate established intracerebral murine gliomas in vivo. In this study, we show that the reactivity of murine GL261 glioma cells to TLR ligands correlates with the expression of the corresponding TLR in vitro. We further observed that increasing CpG-ODN concentrations displayed a direct toxic effect on GL261 glioma cells which was cell-specific, independent of the CpG-motif, and TLR9 independent. In vivo, local application of CpG-ODN was most effective in the eradication of established intracerebral murine gliomas, which was predominately mediated through a TLR9-dependent immune activation leading to intratumoral accumulation of IFN-γ producing effector T cells (Teff) and long-term protective immunity. Thus, these findings support the application of local TLR ligand delivery as a strategy for breaking tumor-related immune suppression in glioma patients.

Materials and Methods

Mice and tumor cell lines

Eight- to ten-week-old female C57BL/6 mice were obtained from Charles River Laboratories. TLR9−/− mice were obtained from S. Akira (Department of Host Defense, Osaka University, Osaka, Japan). The wild-type littermates could be generated, and one was selected for the experiments based on its proliferative capacity, the potential to grow intracerebrally, and its ability to stimulate BZAT T cell hybridoma cells specific for the OVA257–264 CTL epitope LNFKELK in H-2Kb, which carry a β-galactosidase construct driven by NF-AT elements from the IL-2 promoter (27). Additional experiments revealed that GL261-OVA cells were also able to stimulate CD8+ transgenic OT-1 cells. Moreover, GL261 OVA cells could be lysed by OVA257–264 peptide-specific CTLs. The melanoma cell line B16-F10 was obtained from the American Type Culture Collection and cultured in complete medium. Cells were regularly monitored for mycoplasma contamination by PCR.

Brain tumor model

Before injection, GL261 glioma cells were harvested, washed twice in PBS, counted, and adjusted to 2 × 10^6 cells in 5 μl of PBS in a 26-gauge Hamilton syringe (rechallenge with 1 × 10^6 cells). Mice were anesthetized with isoflurane. After shaving the scalp and making an incision, a burr hole was made in the skull 2 mm lateral to the midline and 2 mm anterior to the bregma using a dental drill. Then, GL261 glioma cells were injected over 1 min at a depth of 2.5 mm below the dura matter into the right cerebral hemisphere. Animals were observed daily and sacrificed by cervical dislocation when characteristic symptoms such as hunched posture, reduced mobility, and significant weight loss (>20%) occurred. Animals without such symptoms were regarded as long-term survivors after 90 days.

Abs and flow cytometry

Directly labeled mAbs used for staining were anti-CD4 alloporphocyanin (RM4-4), CD8b alloporphocyanin (53–6.72), INF-γ-PE (25723.11), Vα2-PE (B20.1), and Vß5.1-PE (MRB94). For staining of MHC class I, a biotinylated anti-MHC class I Ab (28–8–6) was used followed by streptavidin-PE. All Abs and isotype controls were obtained from BD PharMingen. Rat anti-mouse FoxP3-biotin (clone FJK, staining according to instructions by the manufacturer) was obtained from eBioscience. Analysis of cell surface markers on lymphocytes was performed using a FACS Calibur (BD Bioscience) and CELLQuest software.

Quantitative RT-PCR

RT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems), and analyzed on the ABI Prism 7000 Sequence Detection System (SDS). Total RNA was isolated from tumor cells using TRIzol reagent (Invitrogen) and either treated with or without DNase I (Sigma-Aldrich). RNA content was photometrically determined and equal amounts of RNA were reverse transcribed into cDNA by AmpliTaq Gold DNA Polymerase according to the manufacturer’s instructions. For analysis of TLR expression, the minimum primer concentrations giving the lowest threshold cycle were determined for each primer pair. The absence of nonspecific amplification was confirmed by analyzing the PCR amplification products by agarose gel electrophoresis and the generation of a dissociation curve. The primers were synthesized by Sigma-Aldrich, including: GAPDH 5′-GTC GAG ATT GTT GCC ATC AAC G-3′ (sense), 5′-CAG TGG ATG CAG GGA TGA TGT TCT G-3′ (antisense); TLR2 5′-TCA GAC ACT GGG GGT GAT AAC ATC-3′ (sense), 5′-CGG ATC GAC TTT GCT CTT TGG G-3′ (antisense); TLR3 5′-GGG CTC GAA GAC AAC CT-3′ (sense), 5′-CCG AGA ACT TTA GAT G-3′ (antisense); TLR4 5′-GCC TCT CAG CAG ATT AAG CTC C-3′ (sense), 5′-AGA TCA ACC GAT CCG CAG G-3′ (antisense); TLR5 5′-TCA GAC GCC AGG ATA GCC TTT-3′ (sense), 5′-AAT GAT CCA GAT AGC ATA CTG G-3′ (antisense); TLR7 5′-TCT CAC CCT CTT CAT CAA GCA CA-3′ (sense), 5′-CCG CAC TAG AAC AGG TAC ACA-3′ (antisense); TLR9 5′-ACT CCG ACT TCC ACC TTA-3′ (sense), 5′-GGC TCA ATG GTC TGG G-3′ (antisense). The manufacturer’s RT-PCR protocol was used to determine relative quantities of TLR mRNA against GAPDH as an internal control after 40 PCR cycles. TLR mRNA detected after more than 35 cycles was regarded false positive (cut-off CT value).

ELISA

IL-6 and IL-12p70 ELISA were performed according to the manufacturer’s protocol (R&D Systems). The plates were read at 450 nm wavelength by the ELISA reader (Dynatech). The experiments were performed in triplicates.
TLR ligands

The following TLR ligands were used: PAM3Cys-SK4 (PAM) for TLR1/2 stimulation (EMC Microcollections), poly(I:C) for TLR3 stimulation (Amersham Biosciences), and R848 for TLR7 stimulation (PharmaTech). Escherichia coli derived LPS for TLR4 stimulation was obtained from Sigma-Aldrich and subsequently purified as described in Ref. 28. For TLR9 stimulation, various CpG-ODN were purchased commercially (Sigma-Aldrich). All CpG-ODN had a nucleoside-resistant phosphorothioate backbone and were confirmed to have undetectable endotoxin content before use. The CpG-ODN designated 1668 (5′-TCC ATG AGC TTC CTG ATG CT-3′), the CpG-ODN designated 1826 (5′-TCC ATG AGC TTC AGG ACC GGA GAT GAT-3′) and the CpG-ODN M352 (5′-TCC TCG AAC GGT CGA GAT CAT-3′) were used as immunostimulatory agents. CpG-ODN 1668, a non-CpG-containing ODN (5′-TCC ATG AGC TTC CTG ATG CT-3′) were used as controls for CpG-ODN1668.

Treatment of GL261-bearing mice

TLR ligands were delivered intratumorally on day 5 after tumor cell inoculation through the same burr hole which was used for the intracerebral implantation of GL261 glioma cells. TLR ligands were dissolved in PBS and 5 μl were injected locally. PBS alone was used as vehicle control. TLR ligand concentrations were selected on their in vitro potency to activate immune cells such as DC and previous in vivo experiments using TLR ligands locally to eradicate established murine melanomas or neuroblastomas (23, 29, 30). For PAM and LPS 5 μg/mouse, for R848 and CpG-ODN 20 μg/mouse, and for poly(I:C) 50 μg/mouse were used for the experiments.

T cell proliferation and in vivo T cell proliferation

Spleen and inguinal lymph nodes from OT-I and OT-II transgenic mice were isolated and mashed into single cell suspensions. Spleens were mashed, filtered, and T cells were purified using anti-mouse-CD4 or anti-mouse CD8 Microbeads (MACS, Miltenyi Biotec) resulting in a 95% pure CD4+ or CD8+ T cell population as measured by flow cytometry. Subsequently, freshly sorted OT-I transgenic CD8+ T cells and OT-II transgenic CD4+ T cells were CFSE labeled (5 μM) and were injected into the right retro-orbital venous plexus (2×10^6 each per mouse). After 2 days, mice were challenged i.c. with 1×10^7 GL261 glioma cells or 1×10^5 GL261-OVA cells in the presence or absence of CpG1668 (20 μg/mouse). Four days later, cervical lymph nodes, mesenterial lymph nodes, and spleens were isolated and flow cytometry was done on the transgenic T cell population (using anti-CD4 allophycocyanin, anti-V85.1-PE mAbs, and propidium iodide to identify transgenic OT-I cells) to assess T cell proliferation, reflected by halving of CFSE fluorescence intensity in daughter cells produced with each round of proliferation.

Analysis of tumor-infiltrating lymphocytes (TIL)

After tumor challenge, mice were sacrificed at indicated time points, and TIL were isolated as previously described (3). TILs were stained for flow cytometry with various anti-CD4 allophycocyanin or anti-CD8 allophycocyanin, rat anti-mouse Foxp3-biotin/Streptavidin-Percp (BD Pharmingen), and anti-IFN-γ-PE. For INF-γ production, TILs were replated in a 96-well round-bottom plate and incubated for 16–18 h with 50 ng/ml PMA and ionomycin (500 ng/ml) in the presence of 5 μg/ml Brefeldin A (Sigma-Aldrich). Intracellular INF-γ was stained after fixation and permeabilization using the intracellular staining kit from eBioscience. TILs were analyzed by flow cytometry for the expression of CD3, CD4, Foxp3+, and IFN-γ. The ratio of IFN-γ-producing CD4+ or CD8+ Teffs to CD4+ Foxp3+ Tregs within the TIL population of a tumor-bearing mouse was calculated as: % of INF-γ+ CD4+ or CD8+ T cells/TIL divided by % of CD4+ Foxp3+ Treg/TIL.

Generation of bone-marrow derived dendritic cells (BMDC)

Murine bone marrow cells were prepared and cultured in six-well plates at 0.5–1×10^6 cell/well in complete medium supplemented with 20 ng/ml recombinant mouse GM-CSF plus 20 ng/ml IL-4 (provided by G. Zarawska, DNAX, Palo Alto, CA). Fresh cytokines were given on days 3 and 6. Nonadherent and loosely adherent clusters of proliferating DCs were harvested on day 8, resuspended in fresh complete medium and stimulated with various TLR ligands for 24 h. For each TLR ligand, the optimal concentration was used to stimulate BMDC as tested in preceding titration experiments (Pam3Cys-SK4 1 μg/ml, poly(I:C) 20 μg/ml, LPS 0.5 μg/ml, R848 2 μg/ml, CpG1668 1 μg/ml).

Statistical analysis

A two-tailed Student t test was used to analyze for significant differences between two treatment groups. One-way ANOVA was used to analyze differences between three and more groups. A post test was performed with p values <0.05. Differences in survival between two treatment groups were analyzed for significance using the log-rank test. Data were analyzed by GraphPad Software 4.0.

Results

GL261 glioma cells respond differently to TLR ligands in vitro

Recent studies have indicated that not only immune cells, but also tumor cells, can express TLRs (16). To assess the importance of TLR expression by murine GL261 glioma cells in our setting, we determined the expression of TLRs in G261 glioma cells and in murine BMDC that are known to express high levels of TLRs. As many TLR genes consist of a single exon, RNA samples were either used as such or treated with DNase to eliminate contaminating genomic DNA. As shown in Fig. 1A, GL261 glioma cells express high amounts of TLR3 and significant amounts of TLR2 and TLR4, but little or no TLR5, TLR7, or TLR9. Interestingly, the level of TLR3 expression in GL261 glioma cells was 5-fold higher than in BMDC. All other TLRs are expressed at much lower levels in GL261 as compared with BMDC. As DNase treatment increased the CT-value for TLR5, TLR7, and TLR9 above 35, we regard their expression as non-functional in GL261 glioma cells.

To link the TLR expression data of GL261 glioma cells to their ability to respond to TLR stimuli, cells were exposed to various TLR ligands for 48 h and examined by flow cytometry for the cell surface expression of MHC class I molecules. Addition of INF-γ to GL261 glioma cells was used as positive control. Supernatants were collected and analyzed for the presence of IL-6 by ELISA. Fig. 1B demonstrates that GL261 glioma cells express significant levels of MHC class I molecules, which can be further up-regulated by INF-γ stimulation. As depicted in Fig. 1, C and D, Pam3Cys-SK4 (TLR1/2 ligand), poly(I:C) (TLR3 ligand), and purified LPS (TLR4-ligand) both induced an up-regulation of MHC class I molecules and the secretion of IL-6, whereas R848 (TLR7 ligand) and CpG-ODN (Cpg1668, TLR9 ligand) failed to stimulate GL261 glioma cells in vitro. As additional control for TLR stimulation, murine BMDC were incubated with various TLR ligands for 24 h and analyzed for CD86 expression and IL-12/IFN-γ production by flow cytometry and ELISA, respectively. As expected, all TLR ligands, except poly(I:C), were able to activate murine BMDC demonstrating the high stimulatory potency of the TLR ligands used in our studies (Fig. 1, E and F).

Cpg-ODN inhibit GL261 glioma cell proliferation in a cell-type specific, but CpG-motif and TLR9-independent manner

In the process of studying the effects of the TLR ligands on GL261 glioma cells, we observed that GL261 glioma cells lost their ability to adhere to plastic and started to clump together after incubation with CpG-ODN (Cpg1668) (Fig. 2A). Further analysis showed that CpG-ODN significantly reduced proliferation of GL261 glioma cells in a dose-dependent manner (Fig. 2B). Proliferation of GL261 glioma cells was almost completely blocked at a CpG-ODN concentration of 5 μg/ml. FACs analysis revealed that GL261 glioma cells become necrotic with increasing CpG-ODN concentrations (Fig. 2C). We note that the other TLR ligands did not significantly affect the proliferation of GL261 glioma cells in vitro (data not shown).
B16-F10 melanoma cells (data not shown). Fig. 2D shows that all CpG-ODN as well as the control GpG-ODN markedly diminished the proliferation of GL261 glioma cells, whereas B16F10 melanoma cells were much less sensitive to the ODN tested. In addition to B16F10 melanoma cells, several other human and rat glioma cell lines were studied, but tumor cell proliferation was not affected by CpG-ODN (data not shown). These data demonstrate that the toxic effect of phosphorothioate-backbone ODNs are specific for GL261 glioma cells and are independent of a CpG-motif and significant TLR9 levels.

Intratumoral CpG-ODN treatment is superior to other TLR ligands in eradicating established intracerebral murine gliomas

Most murine glioma models primarily assessed the ability of immunotherapy to prevent the outgrowth of tumor in a prophylactic setting in which tumor cells are injected after treatment. So far, attempts to treat tumors in the therapeutic setting were of limited success. To study the effect of various immune potentiating TLR ligands in a therapeutic setting in vivo, mice were inoculated intracerebrally with \( 2 \times 10^4 \) GL261 glioma cells and then treated locally 5 days later with...
different TLR ligands or vehicle alone as a control. Mice were observed for the development of clinical symptoms. As shown in Fig. 3, intratumoral CpG-ODN treatment was superior in the eradication of established murine gliomas when compared with other TLR ligands. Over 80% of the CpG1668 treated animals were able to reject the tumor (CpG-ODN vs control: \( p < 0.0001 \), median survival 90 days vs 25 days). Interestingly, R848 (\( p < 0.0001 \), median survival = 36.5 days) also significantly prolonged the survival of glioma-bearing mice, but were less effective than CpG-ODN (CpG vs R848, \( p = 0.0126 \)). Pam3Cys-SK4 administration was almost as beneficial as R848 for the treatment of glioma-bearing mice (\( p < 0.0001 \), median survival = 34.5 days), whereas LPS and poly(I:C) did not show a significant effect on tumor outgrowth and survival (LPS vs control: \( p = 0.0649 \), median survival = 27 days, poly(I:C) vs control: \( p = 0.24 \), median survival = 27 days). Immediate adverse events were not observed after injection of the various TLR ligands at the concentrations used. None of the surviving animals exhibited neurological disabilities. Tumor growth was found to be the cause of death for all of the deceased animals.

**TLR9 expression on nontumor cells is required for the therapeutic efficacy of CpG-ODN treatment and the induction of long-term immunity**

To assess whether a direct toxic effect of CpG-ODN on GL261 glioma cells or the activation of the immune system via TLR9 is...
Local CpG-ODN treatment markedly increases the percentage of tumor-infiltrating INF-γ-producing CD4+ and CD8+ Teff

Recently, we provided direct evidence that CD4+ FoxP3+ Tregs infiltrate and accumulate in gliomas and suppress antiglioma immune responses in vivo. Treatment with anti-CD25 mAbs significantly reduced the number of these highly suppressive CD4+ FoxP3+ Treg within the growing tumor and provoked a CD4 and CD8 T cell dependent destruction of the glioma cells (3). To study the impact of local CpG-ODN treatment on Teffs and Tregs in gliomas, GL261 glioma cells were intracerebrally co-injected with CpG-ODN. TILs were isolated at indicated time points for flow cytometric analysis. The percentages of IFN-γ-producing CD4+ and CD8+ Teffs as well as FoxP3 expressing CD4+ Tregs were determined. As shown in Fig. 5A, local CpG-ODN, but not PBS treatment resulted in an increased recruitment of IFN-γ-producing CD4+ and CD8+ Teffs to the tumor side (CD4: CpG day 7 vs CpG day 12, *** p < 0.0001; PBS day 12 vs CpG day 12,

\[ \text{day 7} = \text{day 12} < 0.0001 \]

of the glioma-bearing TLR9−/− mice. As shown in Fig. 4A, glioma-bearing TLR9−/− mice were not protected against glioma growth after local CpG-ODN treatment, whereas 75% (3/4) of the glioma-bearing TLR9−/− wild-type mice survived. These results demonstrate that TLR9 engagement on immune cells is a prerequisite for survival after local CpG-ODN treatment. Although we cannot exclude that a direct toxic effect of phosphorothioate-stabilized CpG-ODN on glioma cells contributes to the efficacy of local CpG-ODN treatment in TLR9−/− mice in vivo, it is apparently not sufficient, as TLR9−/− do not survive and tumor growth is not delayed. Moreover, mice that had rejected their tumor after local CpG treatment were protected against a rechallenge with a 5-fold higher dose of GL261 glioma cells (1 × 10^5) at the left cerebral hemisphere (Fig. 4B). The finding that all mice survived the rechallenge without any further treatment demonstrates the induction of protective immunity after initial local CpG-ODN treatment of established intracerebral murine gliomas.

**FIGURE 5.** Effect of local CpG-ODN treatment on influx of CD4+ and CD8+ Teff and Treg after local CpG-ODN treatment. GL261 glioma cells were co-injected with CpG1668 (20 μg/mouse) or PBS into the right cerebral hemisphere. Mice were sacrificed 7 and 12 days after tumor challenge, and TIL were isolated as previously described (3). TILs were analyzed by flow cytometry for the expression of CD4, CD8, Foxp3+, and IFN-γ. For IFN-γ production, TIL were replated in a 96-well round-bottom plate and were stimulated directly ex vivo for 16–18 h with 50 ng/ml PMA and ionomycin (500 ng/ml) in the presence of brefeldin A (5 μg/ml). Intracellular IFN-γ was stained after fixation and permeabilization of the cells with the kit from eBioscience. A, The percentage of IFN-γ producing CD4+ and CD8+ T cells at days 7 and 12 after tumor challenge and CpG-ODN or PBS treatment are indicated (CD4: CpG day 7 vs CpG day 12, *** p < 0.0001; PBS day 12 vs CpG day 12, * p = 0.0284; CD8: CpG day 7 vs CpG day 12, ** p = 0.0067). B, The ratio of IFN-γ producing CD4+ Teffs to CD4+ FoxP3+ Tregs and IFN-γ producing CD8+ Teffs to CD8+ FoxP3+ Tregs within the TIL population was calculated on the basis of flow cytometric analyses and is shown (CD4: CpG day 7 vs CpG day 12, *** p = 0.0007). Data represent the mean value ± SEM of data pooled from two independent experiments (day 7: PBS n = 4/group, CpG n = 7/group; day 12: PBS n = 7/group, CpG n = 10/group).
FIGURE 6. Activation of Ag-specific T cells in the cervical lymph nodes after local CpG-ODN treatment. CD8-MACS sorted OT-I cells and CD4-MACS sorted OT-II cells were CFSE-labeled and i.v. injected into the retro-orbital venous plexus of recipient mice (2 × 10⁶ each). Two days later GL261 glioma cells or GL261-OVA cells (1 × 10⁵) were implanted i.c. into the right cerebral hemisphere with or without CpG1668 (20 μg/mouse). After 4 days, mice were sacrificed and cLN, mLN, and spleens were collected. Cell suspensions were made and stained with anti-CD4 allophycocyanin, anti-CD8 allophycocyanin, anti-Vα2-PE, and anti-Vβ5.1-PE mAbs. CD8⁺/Vα2⁺ cells and CD4⁺/Vβ5.1⁺ cells were gated and analyzed for CFSE dilution. The numbers in the left corner represent the percentage of proliferating OT-I (A) or OT-II cells (B).
Ag-specific activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the cervical lymph nodes after local CpG-ODN treatment

As major rejection Ags are not known in the GL261 glioma model, we used OVA as a model Ag to further analyze the mechanisms of the anti-glioma immune response after local CpG-ODN treatment. Purified naïve CD8<sup>+</sup> OT-I cells and CD4<sup>+</sup> OT-II cells from OVA-TCR transgenic mice were CFSE-labeled and i.v. injected into the retroorbital venous plexus of recipient mice (2 × 10<sup>6</sup> each). Two days later, GL261 glioma cells or GL261-OVA cells (1 × 10<sup>6</sup>) were implanted i.c. into the right cerebral hemisphere with or without CpG1668 (20 μg). After 4 days, mice were sacrificed and OVA-specific T cell priming was determined by assessing the proliferation of CFSE-labeled OT-I and OT-II cells in cervical lymph nodes (cLN), mesenterial lymph nodes (mLN) and spleen by flow cytometry. We note that similar to nontransfected GL261 glioma cells, GL261-OVA cells were also tested to be TLR9 negative (data not shown). Fig. 6 depicts that, in the absence of CpG-ODN treatment, already some proliferation of CD8<sup>+</sup> OT-I cells occurred in the cLN. However, local CpG-ODN treatment significantly enhanced the proliferation of CD8<sup>+</sup> OT-I cells and also induced CD4<sup>+</sup> OT-II cell proliferation in the cLN. In contrast, in the mLN and spleen, only a limited number of CD8<sup>+</sup> OT-I cells proliferated, whereas no significant proliferation of CD4<sup>+</sup> OT-II cells was observed when mice were treated with CpG-ODN. Inoculation of GL261 glioma cells with or without CpG-ODN addition was not sufficient to stimulate CD8<sup>+</sup> OT-I or CD4<sup>+</sup> OT-II cells. This experiment demonstrates that local CpG-ODN treatment of an intracerebral glioma specifically enhances the activation of both CD4<sup>+</sup> and CD8<sup>+</sup> Ag-specific T cells present in the cLN.

Discussion

A major issue in immunotherapy of cancer is to develop effective treatment protocols for established tumors that induce strong immune responses and counteract local immune-suppressive mechanisms. Malignant gliomas have developed multiple mechanisms to evade from an immune attack, among those the intratumoral accumulation of highly suppressive Tregs (3, 31). Therefore, a key issue to successfully treat gliomas is to attenuate Treg-mediated immune suppression and, concomitantly, to stimulate tumor-reactive immune-effector cells. As activation of the immune system by local TLR-stimulation has been shown to reverse the immunosuppressive tumor environment in several cancer models (23, 29, 32), this study was designed to explore the potency of various TLR ligands to induce immunity against established intracerebral murine GL261 gliomas in vivo and to determine their direct impact on these tumor cells in vitro.

Aside from immune cells, tumor cells have been reported to express TLRs themselves (16). Expression profiling of RNA samples of the GL261 glioma cells used in this study showed that they express high levels of TLR3, significant levels of TLR2 and TLR4 mRNA, and low-to-undetectable levels of TLR5, TLR7, and TLR9 mRNA. The virtual absence of TLR9 mRNA is surprising, as other data have suggested the presence of TLR9 GL261 glioma cells (33). Although it is difficult to exclude differences in the GL261 glioma cells, we would like to emphasize that our RNA samples were treated with DNase I to avoid accidental amplification of contaminating genomic DNA. In line with our expression data, TLR2-, TLR3-, and TLR4-ligation on GL261 glioma cells in vitro up-regulated MHC class I molecules and increased IL-6 secretion, whereas TLR7- and TLR9-ligation had no effect. These findings also match previous studies showing that murine and human astrocytes express TLR2, TLR3, and TLR4 and respond to bacterial and viral infections in the CNS by the production of proinflammatory cytokines and chemokines (34–40). Remarkably, while CpG-ODN did not increase MHC expression or IL-6 secretion, increasing CpG-ODN concentrations appeared toxic for GL261 glioma cells in vitro. This direct toxic effect on GL261 glioma cells could not be observed with other TLR ligands tested. The toxicity of CpG-ODN was GL261 glioma cell specific, as B16F10 melanoma cells were hardly affected by the CpG-ODN concentrations used. Moreover, toxicity was independent of the CpG-motif, because the control ODN containing a nonstimulatory GpG-motif was also effective. These results imply that the phosphorothioate backbone used for stabilization of the ODN is responsible for a TLR9-independent toxic effect on GL261 glioma cells and are in line with recent data showing that phosphorothioate containing ODN, but not those containing a phosphodiester backbone can induce cell death in different types of cancer cells (41, 42).

Comparison of the various TLR ligands for their therapeutic efficacy in the local treatment of established intracerebral murine gliomas revealed that a single CpG-ODN injection was far superior over the other TLR ligands and cured 80% of the mice. Survival benefit was also observed for the TLR ligands R848 (TLR7 ligand) and Pam3Cys-SK4 (TLR1/2 ligand), while poly(I:C) (TLR3 ligand) and LPS (TLR4 ligand) were not effective in this therapeutic glioma model.

It has been demonstrated that the initiation of immune responses in the brain is promoted by local DC (43). R848 is known to be a potent immune response modifier that induces the maturation of DC and the secretion of proinflammatory Th1 cytokines (44). Our data show that R848 can induce DC maturation and the secretion of IL-12 at similar levels as CpG-ODN in vitro, but has no effect on glioma cells in vitro. Pam3Cys-SK4 was almost as effective as R848 in the local glioma treatment. Pam3Cys-SK4 both modulated glioma cell function and activated DC to secrete proinflammatory cytokines. IL-6, which is secreted by glioma cells and DC after Pam3Cys-SK4 stimulation, was previously shown to inhibit the expansion and inhibit the suppressor effect of Treg cells by rescuing conventional T cells from Treg-mediated suppression (45). Moreover, we recently demonstrated that Pam3Cys-SK4 is also able to directly manipulate Treg function (21).

The reasons for the absence of therapeutic efficacy of poly(I:C) and LPS is not clear, but might be related to the specific environment in the brain. Poly(I:C) was able to activate glioma cells, but failed to induce DC maturation and cytokine production in vitro. These results are in accordance with recent findings indicating that TLR3 is predominantly expressed on the surface of astrocytes, whereas TLR3 in APC is expressed intracellularly (46, 47). LPS was both a potent stimulator of glioma cells and DC in vitro. However, other studies showed that LPS-treated mice lose their APC function in the CNS in vivo and that glioma-infiltrating APC are refractory to LPS-induced production of proinflammatory cytokines (48). In addition, LPS-induced TLR4 signaling in tumor cells...
can result in the resistance of tumor cells to CTL attack (16), and can induce Ag-specific, IL-10 producing regulatory Tr1 cells (49).

We indicate that higher amounts of LPS (> 5 μg/mouse) were not used in this study to minimize the potential risk of acute neurotoxicity (50, 51). Moreover, as previously demonstrated in a different murine glioma model, even higher amounts of LPS (100 μg/mouse) only showed modest antitumoral effects after local administration and failed to induce long-term survivors (52). Finally, it should be noted that CpG-ODN has been optimized to improve its in vivo stability, while poly(I:C) and R848 are not and therefore may have unfavourable pharmacokinetics in vivo. Therefore, it will be interesting to compare stabilized forms of poly(I:C) (e.g., enclosed in cationic liposomes or poly-lysine stabilized) and R848 or otherTLR7-ligands (e.g., phosphorothioate-stabilized immunostimulatory RNA) in this setting (53–56).

In general, TLR responsiveness of GL261 glioma cells in vitro did not correlate with the therapeutic efficacy of the various TLR ligands in the local treatment of intracerebral murine gliomas. As CpG-ODN did act on the tumor cells and on immune cells, we determined the importance of both in the efficacy of local CpG-ODN treatment. Comparing CpG-ODN in TLR9 \(^{+/+}\) wild-type and TLR9 \(^{-/-}\) knockout mice showed that its therapeutic efficacy was TLR9 dependent. TLR9 \(^{-/-}\) knockout mice succumbed to glioma growth, indicating that a direct toxic effects on GL261 glioma cells is essential for this response. A single intratumoral injection of CpG-ODN was sufficient to induce Teff associated glioma eradication and to induce protective antitumor immunity. The potency of local TLR treatment of gliomas is further underlined by our observation that the activation of the immune system using this approach is far more effective in a therapeutic setting than repeated peripheral vaccinations with tumor lysate-loaded DC (62). Therefore, it will be interesting to explore the local delivery of TLR ligands as an immunotherapeutic strategy to break local immune suppression in glioma patients.

**Disclosures**

The authors have no financial conflict of interest.

**References**


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