Research Article

Cancer Immunology Research

STING Contributes to Antiglioma Immunity via Triggering Type I IFN Signals in the Tumor Microenvironment

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Abstract

Although type I IFNs play critical roles in antiviral and antitumor activity, it remains to be elucidated how type I IFNs are produced in sterile conditions of the tumor microenvironment and directly affect tumor-infiltrating immune cells. Mouse *de novo* gliomas show increased expression of type I IFN messages, and in mice, $CD11b^+$ brain-infiltrating leukocytes (BIL) are the main source of type I IFNs that are induced partially in a STING (stimulator of IFN genes)-dependent manner. Consequently, glioma-bearing $Sting^{Gt/Gt}$ mice showed shorter survival and lower expression levels of *Ifns* compared with wild-type mice. Furthermore, BILs of $Sting^{Gt/Gt}$ mice showed increased $CD11b^+$ $Gr\text{-}1^+$ immature myeloid suppressor and $CD25^+$ $Foxp3^+$ regulatory T cells (Treg) and decreased IFNγ-producing $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells that received direct type I IFN signals showed lesser degrees of regulatory activity and increased levels of antitumor activity, respectively. Finally, intratumoral administration of a STING agonist (cyclic diguanylate monophosphate; c-di-GMP) improved the survival of glioma-bearing mice associated with enhanced type I IFN signaling, *Cxcl10* and *Ccl5*, and T-cell migration into the brain. In combination with subcutaneous OVA peptide vaccination, c-di-GMP increased OVA-specific cytotoxicity of BILs and prolonged their survival. These data demonstrate significant contributions of STING to antitumor immunity via enhancement of type I IFN signaling in the tumor microenvironment and suggest a potential use of STING agonists for the development of effective immunotherapy, such as the combination with antigen-specific vaccinations. *Cancer Immunol Res; 2(12); 1199–208. ©2014 AACR.*

Introduction

Gliomas are the most common primary malignant brain tumors and carry a dismal prognosis despite current treatments, and new therapies are needed. Immunotherapies are promising in this regard. However, the successful development of immunotherapy for gliomas requires detailed understanding of factors critical for antiglioma immunity.

In addition to the ability of type I IFNs to interfere with viral infection, they also enhance antitumor host immunity. Indeed, loss of type I IFN signaling promotes tumorigenesis in a variety of tumor types, such as sarcomas (1), melanomas (2, 3), and gliomas as we have reported previously (4). Although a growing body of evidence suggests that endogenously produced type I IFNs participate in antitumor immune responses at the level of host hematopoietic cells (5, 6), the molecular mechanisms responsible for inducing the type I IFN in the sterile tumor microenvironment remain elusive. Furthermore, the impact of type I IFN on immune cell populations participating in the antitumor response *in vivo* needs to be elucidated. In this regard, $CD8\alpha^+$ dendritic cells (DC) have been shown to require type I IFNs for effective antitumor immunity (2, 3). Type I IFNs directly enhance *in vivo* clonal expansion of $CD4^+$ T cells following immunizations against lymphocytic choriomeningitis viruses (7), promote the survival of $CD8^+$ T cells, and stimulate the development of cytolytic functions, including the production of IFNγ (8). Although we have previously demonstrated a critical role of type I IFNs on maturation of glioma-infiltrating $CD11c^+$ DCs (4), it still remains to be elucidated how type I IFNs are induced in the glioma microenvironment and whether they directly affect T-cell functions.

Stimulator of IFN genes (STING) has recently been identified as one of the critical adaptors for cytosolic DNA sensing. It

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plays a critical role in host defense against viral and intracellular bacteria by regulating type I IFN signaling and innate immunity (9–12). STING is stimulated downstream of DNA sensors, such as helicase DDX41 (DExD/H-box helicases 41; ref. 13), and cyclic dinucleotides (CDN), such as c-di-GMP, c-di-AMP, cGMP-AMP (cGAMP), or 10-carboxymethyl-9-acridanone (CMA; refs. 14–18), thereby leading to production of type I IFNs. STING-deficient mice or cells show increased susceptibility to infection by several microbes and diminished levels of type I IFNs in response to several microbes and CDNs (19).

Considering that there are abundant dying tumor cells that release their genomic (g)DNA in the tumor microenvironment (20), we evaluated our hypothesis that STING-mediated DNA sensing is involved in type I IFN production in the glioma microenvironment, and stimulation of STING with its agonist enhances antiglioma immunity including T-cell responses.

Materials and Methods

Mice

Wild-type (WT) C57BL/6J (H-2K^b) and C57BL/6-background *Sting*$^{Gt/Gt}$ mice (C57BL/6J-*Tmem173*^{gt}/) were purchased from The Jackson Laboratory. B6.129(Cg)-Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J mice ("tdTomato" mice) were generated by breeding B6.Cg-Tg(Mx1-cre)1Cgn/J mice with B6.129(Cg)-Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J mice (21). All mice were maintained and handled in accordance with the Animal Facility at the University of Pittsburgh (Pittsburgh, PA) per an Institutional Animal Care and Use Committee–approved protocol.

Antibodies and the synthetic peptide

The following monoclonal antibodies (mAb) were obtained from BD Biosciences: anti-CD11c (HL3), anti-CD11b (M1/70), and anti-Gr-1 (RB6-8C5). The following mAbs were obtained from eBioscience: anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD3 (145-2C11), anti-CD19 (eBio1D3), anti-IFNγ (XMG1.2), anti-CD25 (7D4), and anti-FoxP3 (NRRF-30). The H-2K^b-binding OVA$_{257–264}$ (SIINFEKL) peptide was synthesized in the University of Pittsburgh Peptide Synthesis Facility. For Western blotting, an ISG54-specific polyclonal antibody (22) and actin-specific mAbs (Sigma-Aldrich) were used. For positive control, WT macrophages treated with 25 μg/mL of polyI:C (for 48 hours) were used.

De novo glioma induction

The procedure for intracerebroventricular DNA injection has been described previously (23). Briefly, the following DNA plasmids were mixed with *in vivo* compatible DNA transfection reagent, In vivo-JetPEI (Polyplus Transfection): pT2/C-Luc//PGK-SB100 (0.06 μg/mouse), Sleeping beauty (SB) transposon-flanked pT2/CAG-NRasV12 (0.12 μg/mouse), and pT2/shp53/mPDGF (0.12 μg/mouse), and injected into the right lateral ventricle of neonate. Intracranial injection of glioma cell lines has been described previously (24).

Two-photon excitation microscopy

The procedure has been described previously (24).

In vivo bioluminescent intensity measurement

The procedure has been described previously (24). Luciferin was obtained from Caliper Life Sciences.

Tumor cell culture

The GL261 mouse glioma cell line was kindly provided by Dr. Robert Prins (University of California Los Angeles, Los Angeles, CA). The GL261-luc cell line was generated by transfection of GL261 cells (24) with a plasmid vector pcDNA3.1 encoding *luciferase* cDNA, followed by selection with G418 (Sigma), limiting dilution and selection of a clone based on the highest luciferase expression level using luminometer in the presence of luciferin in culture. Survival of syngeneic mice bearing GL261-luc cells was confirmed to be comparable with survival of those bearing parental GL261 cells (not shown). The Quad-GL261 cell line, kindly provided by Dr. John R. Ohlfest (University of Minnesota, Minneapolis, MN), expresses OVA$_{257–264}$, OVA$_{323–339}$, human gp100$_{25–33}$, and mouse I-Eα$_{52–68}$ (25). Stable expression of transgenes was maintained by G418 in the culture, and monitored every 3 months by evaluating their susceptibility against antigen-specific cytotoxic T lymphocytes, such as Pmel-1 cells, which were derived from B6.Cg-*Thy1a*/Cy Tg(TcraRcrb)8Rest/J mice (The Jackson Laboratory). The RMA-S mouse thymoma cell line was kindly provided by Dr. Walter J. Storkus (University of Pittsburgh). All cell lines were tested to be *Mycoplasma* free. No other authentication assay was performed.

Quantitative real-time PCR

Primers and probes for the following genes were obtained from Applied Biosystems: *Ifna6* (Mm01703458_s1), *Ifnb1* (Mm00439552_s1), *Foxp3* (Mm00475162_m1), *Tgfb1* (Mm01178820_m1), *Tbx21* (Mm00450960_m1), *Ifng* (Mm01168134_m1), *Ccl5* (Mm01302427_m1), *Cxcl10* (Mm00445235_m1), and *Gapdh* (Mm99999915_g1). In some experiments, following primers were used: mouse pan *Ifna* forward: CCTGAGAAGAGAAGAACACAGCC, reverse: GGCTCTCCAGACTTTCTGCTCTG; mouse pan *Ifnb* forward: CCGAGCA GAGATCTTCAGGAA; reverse: CCTGCAACCACCACTCATTCT; mouse *Gapdh* forward: TCACCACCATGGAGAAGGC, reverse: GCTAAGCAGTTGGTGGTGCA. *Gapdh* was used as an internal control. Relative expression levels compared with control samples were calculated in each experiment using the $\Delta\Delta C_t$ method.

Stimulation of CD11b$^+$ cells with gDNA *in vitro*

gDNA was isolated from GL261 and NIH 3T3 cell lines using the Wizard Genomic DNA Purification Kit from Promega. The final gDNA suspension was made in TE buffer (10 mmol/L Tris–Cl and 1 mmol/L EDTA). Aliquots of CD11b$^+$ bone marrow–derived macrophage cells (5×10^5 cells/mL) from WT or *Sting*$^{Gt/Gt}$ mice were stimulated with gDNA (1 or 5 μg/mL). At 48 hours, cells were harvested and total RNA was extracted. Quantitative real-time PCR (qRT-PCR) analyses were performed with SsoFast EvaGreen Supermix (Bio-Rad), and data were analyzed with CFX manager 2.0 software from Bio-Rad.

BIL isolation and flow cytometry

These procedures have been described previously (24).

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Suppression assay

GFP-positive or GFP-negative $CD4^+$ T cells derived from draining lymph nodes of glioma-bearing tdTomato mice were sorted by MoFlo AstrosTM (Beckman Coulter). $CD8^+$ T cells were isolated from non–glioma-bearing WT mice and labeled with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) for 10 minutes in the incubator. After washing with medium, $CD8^+$ T cells were cocultured with GFP-negative or GFP-positive $CD4^+$ T cells in the presence of Dynabeads (Gibco by Life Technologies). After 60-hour incubation, samples were evaluated by BD Accuri C6.

Cytotoxicity assay

OVA-specific cytotoxicity of brain-infiltrating leukocytes (BIL) was measured by 4-hour ^{51}Cr-release assay as described previously (4, 24). Briefly, freshly isolated BILs were incubated with ^{51}Cr-labeled GL261 cells loaded with or without $OVA_{257–264}$ peptide (10 μg/mL) for 4 hours. For reverse antibody-dependent cell-mediated cytotoxicity (ADCC) of lymphocytes, GFP-positive or GFP-negative $CD8^+$ T cells derived from draining lymph nodes of glioma-bearing mice were sorted by MoFlo Astrios (Beckman Coulter), then incubated with ^{51}Cr-labeled Fc receptor–positive RMA-S cells pretreated with or without anti-CD3 mAb (10 μg/mL, 145-2C11; BD PharMingen) for 4 hours. The percentage of cytotoxicity was calculated as described previously (26).

Treatment with c-di-GMP and vaccination with the OVA peptide in glioma-bearing mice

C-di-GMP (InvivoGen) was dissolved in physiologic water per the manufacturer's instruction. Mice bearing gliomas received intracranial injections of either c-di-GMP (4 μg/2 μL/dose) or mock treatment with solvent alone. Some mice received subcutaneous vaccinations with $OVA_{257–264}$ peptide (100 μg/dose) emulsified in incomplete Freund Adjuvant (Difco Laboratories) on the same day as the c-di-GMP treatment.

Statistical analyses

The statistical significance of differences between two groups was determined by the Student *t* test; one-way ANOVA with the Holm *post hoc* test was conducted for multiple group comparisons. The log-rank test was used to determine statistically significant differences in survival curves among groups. All mouse data were analyzed by R Environment version 2.10.1.

Results

Induction of type I IFN messages in mouse gliomas

We first evaluated type I IFN mRNA levels in the mouse glioma microenvironment by qRT-PCR. Murine brain hemispheres bearing *de novo* glioma expressed significantly higher levels of *Ifna6*, *Ifna8*, and *Ifnb1* compared with non–tumor-bearing contralateral hemispheres (Fig. 1).

Type I IFNs directly signal in T cells in mice that are developing glioma

To determine the effects of type I IFN expression in the glioma microenvironment, we used a novel reporter mouse model, in which type I IFN signaling induces the *Mx1* (IFN-induced GTP-binding protein) promoter-driven Cre recombinase, which turns the expression of *loxp*-flanked *tdTomato* off, and turns *GFP* expression on, thereby enabling us to monitor the induction of IFN signaling in the glioma microenvironment. Under two-photon microscopy, glioma tissues showed higher levels of GFP signals compared with the normal (non–glioma-bearing) brain (Fig. 2A), further substantiating IFN induction in the glioma microenvironment. Using flow cytometry, we evaluated the percentage of GFP^+ cells, in which IFN signaling has turned GFP signal on. In each of $CD11b^+$ $Gr\text{-}1^+$, $CD11b^+$ $CD11c^+$, $CD19^+$, and $CD3^+$ BIL subpopulations, glioma-bearing brains revealed a higher percentage of GFP^+ cells compared with the spleen or inguinal lymph nodes (iLN; Fig. 2B and C). GFP^+ RFP^+ double-positive cells are thought to be the ones that have been exposed to IFN but still retain residual RFP. Because non–glioma-bearing brains do not contain sufficient numbers of BILs, we were unable to evaluate them. Nonetheless, the spleen and iLNs derived from non–glioma-bearing mice showed percentages of GFP^+ cells that were similar to those derived from glioma-bearing mice (data not shown), suggesting that type I IFNs produced locally in the glioma tissue transmit their signals in BILs, but do not have significant impacts on cells in the spleen or iLN.

Type I IFNs directly affect T-cell functions in mice that are developing glioma

We and others have previously demonstrated a critical role of type I IFN pathway in the function of tumor-infiltrating

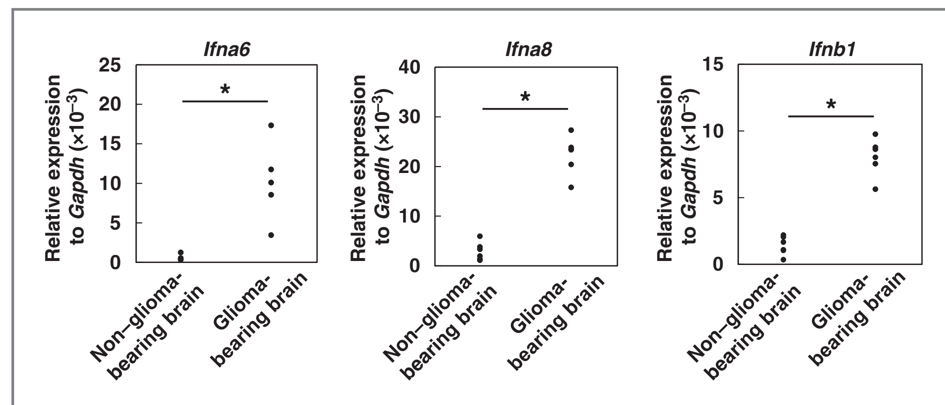

Figure 1. Type I IFNs are detected in glioma tissues of mice. Mice bearing *SB*-induced glioma in the right hemisphere were sacrificed between days 40 and 50 (with tumors of similar size at ~1 × 10^7 luciferase units). Total RNA was extracted from the left (non–glioma-bearing) and right (glioma-bearing) hemispheres of each brain separately (5 mice) and evaluated for expression of *Ifna6* (left), *Ifna8* (middle), and *Ifnb1* (right) by qRT-PCR. Each experiment was performed at least twice. *, $P < 0.05$, based on the Student *t* test.

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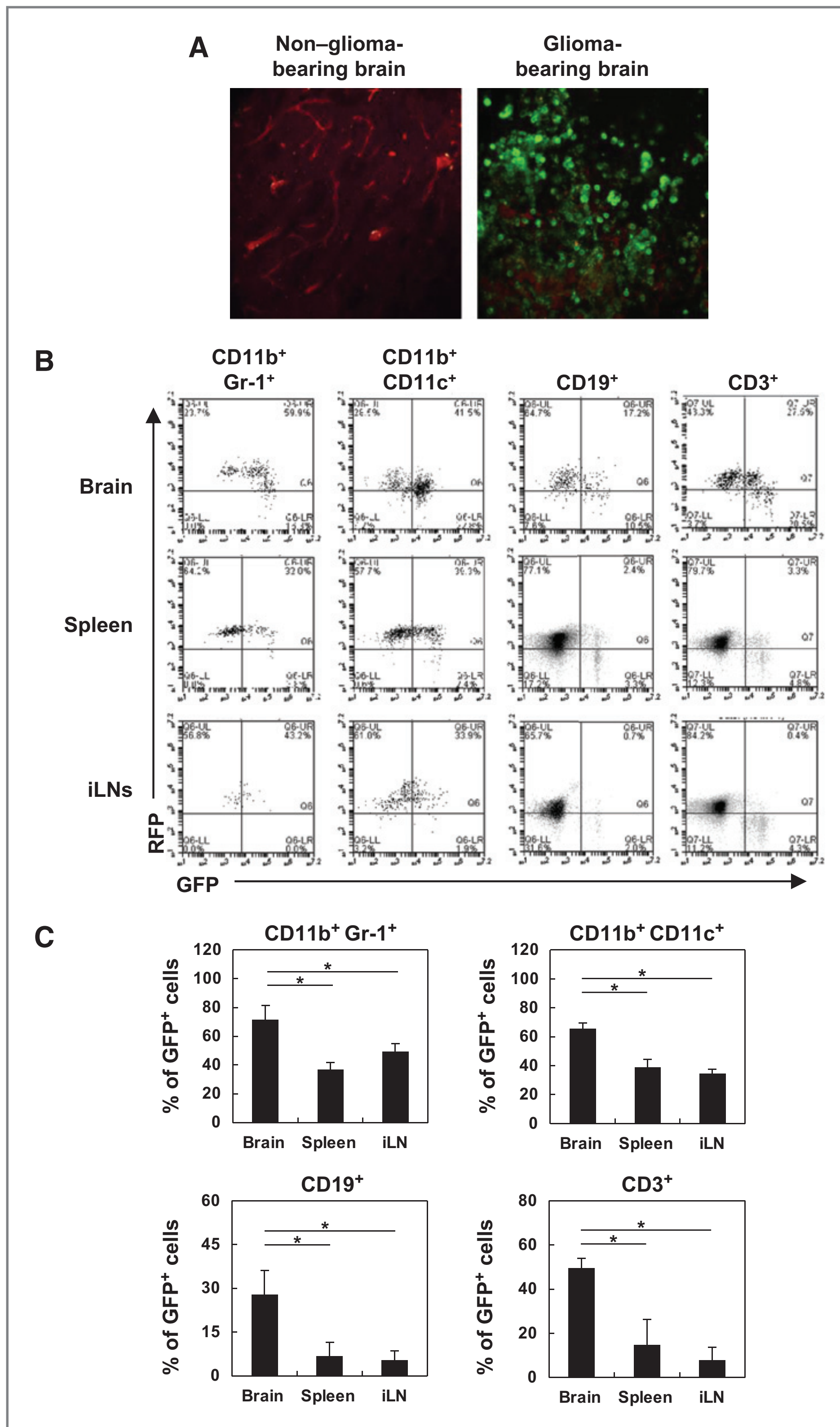

Figure 2. Type I IFN signaling in the tumor microenvironment. tdTomato mice bearing *SB*-induced glioma were sacrificed between days 40 and 50. A, brain sections were evaluated by two-photon microscopy for GFP$^+$ and RFP$^+$ cells. Original magnification, ×60. B, BILs, splenocytes, and iLN cells were evaluated for the percentages of GFP$^+$ cells. Representative flow histograms are shown. C, percentages of GFP$^+$ cells in the glioma-bearing brain, spleen, and iLNs (3 mice/group). *, $P < 0.05$, based on the Student *t* test.

CD11c$^+$ DCs as antigen-presenting cells (2–4). However, whether local production of type I IFNs directly affects the T cells in the glioma-bearing mice remains to be elucidated. The tdTomato mouse model allowed us to address this question *in vivo* during glioma progression. We sorted CD4$^+$ and CD8$^+$ T-cell populations from draining lymph nodes based on

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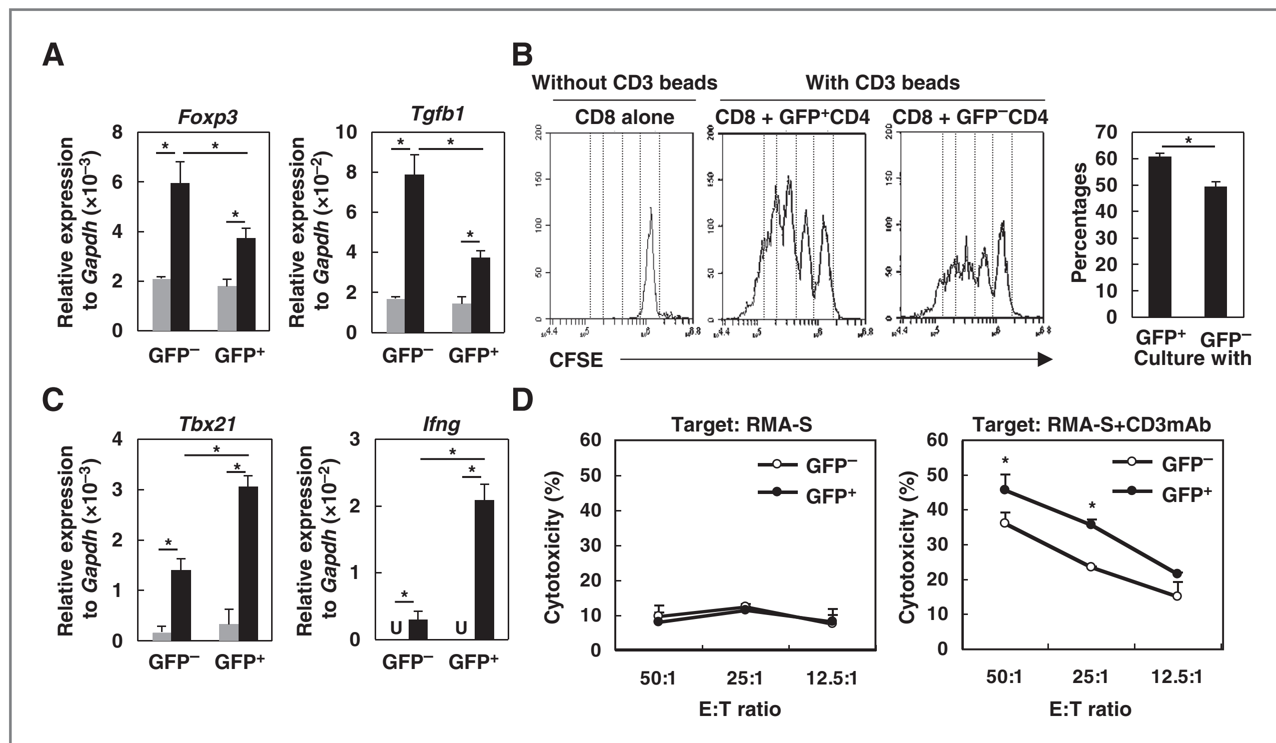

Figure 3. Type I IFNs directly affect T-cell functions in glioma-developing mice. A, CD4$^+$ cells from draining lymph nodes derived from glioma-developing tdTomato mice were sorted into GFP$^-$ or GFP$^+$ cells and incubated with (black bars) or without (gray bars) anti-CD3mAb. After 4 hours, total RNA was extracted for evaluation of *Foxp3* and *Tgfb1* mRNA levels by qRT-PCR. B, CFSE-labeled WT CD8$^+$ T cells were cocultured with GFP$^-$ or GFP$^+$ CD4$^+$ T cells in the presence of CD3 beads. After 60 hours, division of CFSE-labeled CD8$^+$ T cells gated by reactivity to PE-Cy7–condjugated anti-CD8mAb was evaluated by CFSE intensity. As a negative control, CFSE-labeled WT CD8$^+$ T cells were cultured without any stimulation (left). Histograms are representative of two independent experiments. The bar graph shows the percentage of CD8$^+$ cells that divided at least twice in each of two stimulation conditions ($N = 4$/group; *, $P < 0.05$). C, GFP$^-$ or GFP$^+$ CD8$^+$ T cells were incubated with (black bar) or without (gray bar) anti-CD3mAb. After 4 hours, total RNA was extracted for evaluation of *Tbx21* and *Ifng* mRNA expression levels by qRT-PCR (U: undetected). D, cytotoxic activity of GFP$^-$ and GFP$^+$ CD8$^+$ T cells was evaluated by ^{51}Cr-release assay. RMA-S cells untreated (left) or pretreated (right) with anti-CD3mAb (10 µg/mL) were used as target cells. Each experiment was performed at least twice. *, $P < 0.05$, compared at the same E:T ratio.

their GFP expression. CD4$^+$ T cells that received the type I IFN signal (GFP$^+$ cells) expressed significantly lower levels of *Foxp3* and *Tgfb1* compared with CD4$^+$ T cells that did not receive the type I IFN signal (GFP$^-$ cells; Fig. 3A), suggesting that the GFP$^-$ population contains more regulatory T cells (Treg). Indeed, GFP$^-$ cells inhibited CD8$^+$ T-cell proliferation more profoundly than GFP$^+$ cells in the coculture assay (Fig. 3B). Among the CD8$^+$ T cells, GFP$^+$ cells expressed significantly higher levels of *Tbx21* and *Ifnγ* (Fig. 3C), suggesting that the type I IFN signal skews CD8$^+$ cells toward type I effector cells. Accordingly, GFP$^+$ CD8$^+$ cells showed higher cytotoxic activity than GFP$^-$ cells (Fig. 3D). Taken together, these results indicate that type I IFN signaling directly enhances antitumor activity of T cells in glioma-bearing mice.

CD11b$^+$ cells express higher levels of *type I Ifn* than CD11c$^+$ cells in a STING-dependent manner

Next, we focused on identifying the specific cells that are primarily responsible for producing IFN in glioma as a means to define the signaling mechanism of IFN induction in the "sterile" tumor microenvironment. As it was previously reported that CD11b$^+$ and CD11c$^+$ cells are responsible for IFN production (4), we isolated CD11b$^+$ and CD11c$^+$ cells from BILs derived from *SB* glioma-bearing mice. As shown in Fig. 4A, CD11b$^+$ cells showed higher levels of *Ifna6* and *Ifnb1* expression than CD11c$^+$ cells by qRT-PCR.

We next focused on the stimulus and the signaling pathway responsible for the observed *Ifn* induction. We excluded RNA sensors from our evaluation because, based on the literature (27), we thought it unlikely that high levels of immunostimulatory RNA, which can stimulate IFN production, would be induced in the glioma microenvironment. Although other receptors, such as high-mobility group protein B1 (28) and inflammasomes (29), have been implicated in antitumor immunity, these receptors do not directly cause strong IFN induction. We therefore hypothesized that gDNA derived from dying or dead cells can induce type I IFNs through STING-mediated signaling in glioma-infiltrating macrophages because both human and mouse glioma tissues contain necrotic areas that are heavily infiltrated by macrophages (23, 30). Indeed, we found upregulation of *Sting* and *Ifi16*, which is involved in DNA virus sensing (Supplementary Fig. S1; ref. 31). Another DNA sensor, *Aim2*, which is responsible for inflammasome activation in response to DNA (32), was not upregulated. We first tested this hypothesis *in vitro* by stimulating

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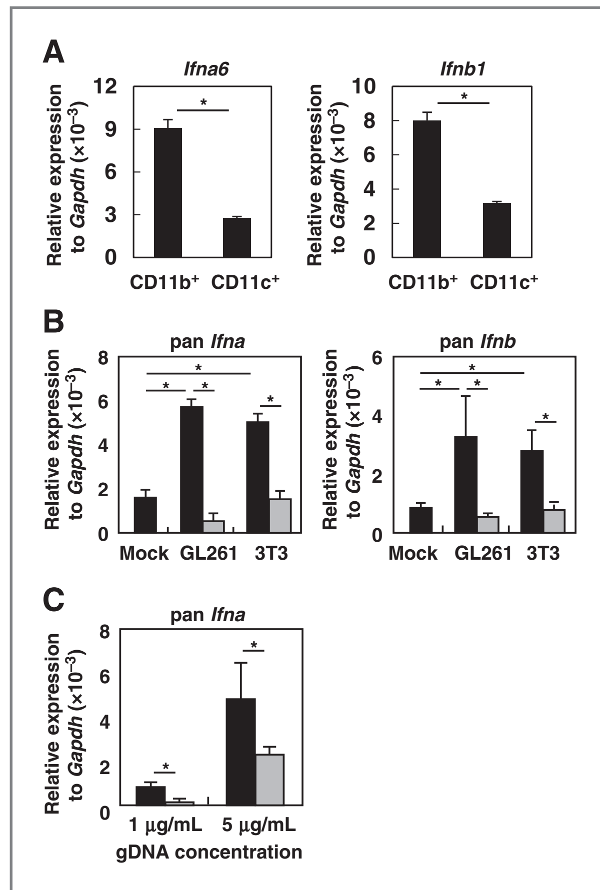

Figure 4. CD11b$^+$ cells produce type I IFNs in response to genomic DNA in a partially STING-dependent manner. A, total RNA was extracted from magnetic bead-enriched CD11b$^+$ and CD11c$^+$ BIL populations (purity was >85% and >80%, respectively) and evaluated for *Ifna6* and *Ifnb1* by qRT-PCR. B, bone marrow–derived macrophages from WT mice were stimulated with gDNA derived from GL261 or NIH3T3 cell lines, or solvent only as mock treatment with (gray bars) or without (black bars) pretreatment with DNase. After 48 hours, total RNA was extracted and evaluated for pan *Ifna* and pan *Ifnb* by qRT-PCR. C, BMDM from WT (black bars) or *Sting*$^{Gt/Gt}$ (gray bars) mice were stimulated with GL261-derived gDNA (1 or 5 µg/mL). After 48 hours, total RNA was extracted and evaluated for pan *Ifna* by qRT-PCR. Each experiment was performed at least twice. *, $P < 0.05$, based on the Student *t* test.

CD11b$^+$ macrophages with gDNA derived from either GL261 glioma or NIH3T3 cells *in vitro*. We detected enhanced expression of pan *Ifna* and pan *Ifnb* at similar levels, which was abrogated by DNases (Fig. 4B). The induction of pan *Ifna* was partially abrogated in STING-deficient cells (Fig. 4C). These data indicate cell-derived gDNA, from either nonmalignant or malignant cells, induces type I IFNs at least partially in a STING-dependent manner, and led us to further investigate the role of STING in antiglioma immunity.

STING contributes to antiglioma immunity through production of type I IFN in the glioma microenvironment

To determine the *in vivo* role of STING in glioma development, we induced *de novo SB* gliomas in WT or *Sting*$^{Gt/Gt}$ mice. Total RNA extracted from glioma-bearing brains of *Sting*$^{Gt/Gt}$ mice showed significantly lower levels of *Ifna6* and *Ifnb1* compared with total RNA derived from WT mice (Fig. 5A). Also, ISG54 protein, which is induced by type I IFNs (22), was detected at a lower amount in the right (i.e., glioma-bearing) hemisphere of *Sting*$^{Gt/Gt}$ mice than in the counterpart in WT mice (Fig. 5B), indicating partial loss of IFN signaling. SB glioma-bearing *Sting*$^{Gt/Gt}$ mice exhibited significantly shorter survival compared with WT mice (Fig. 5C). In BILs, *Sting*$^{Gt/Gt}$

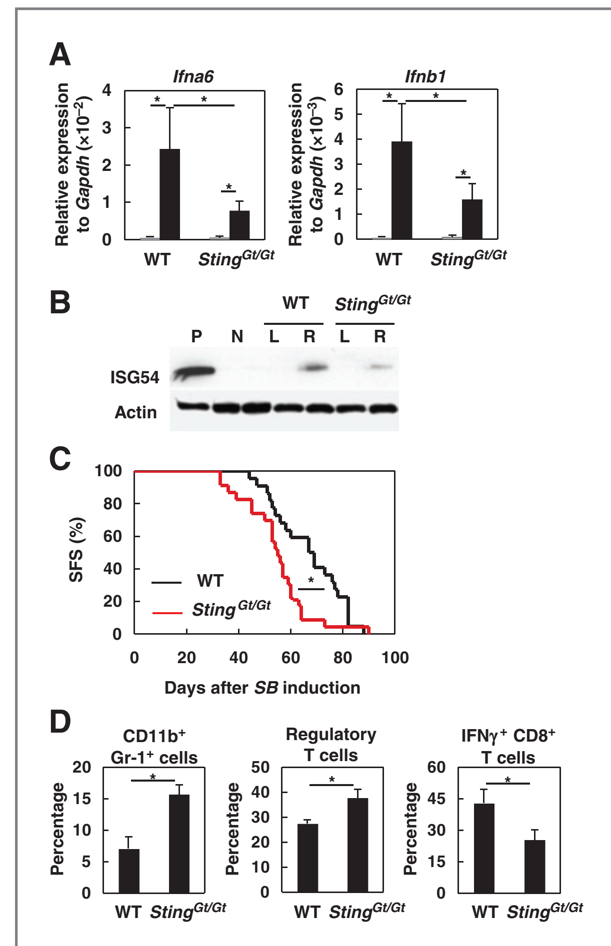

Figure 5. STING contributes to IFN production and antiglioma immunity. A, WT or *Sting*$^{Gt/Gt}$ mice bearing *SB*-induced gliomas were sacrificed between days 40 and 50 (with tumors of similar size at $\sim 1 \times 10^7$ luciferase units). Total RNA was extracted from the left (non–tumor-bearing; gray bars) and right (tumor-bearing; black bars) hemispheres of each brain separately (5–6 mice/group), and evaluated for expression of *Ifna6* (left) and *Ifnb1* (right) by qRT-PCR. *, $P < 0.05$, based on the Student *t* test. B, 50-µg protein extracts were analyzed by SDS-PAGE and Western blotting for detection of ISG54. "L" and "R" indicate samples from left and right hemispheres, respectively, of WT or *Sting*$^{Gt/Gt}$ mice bearing *SB*-induced glioma. WT-derived macrophage sample treated with p(I):p(C) was used as positive control (P). Glioma-free WT mouse brain was used as negative control (N). Actin was used as an internal control. C, gliomas were induced in WT (22 mice; black line) or *Sting*$^{Gt/Gt}$ mice (23 mice; red line) neonatal mice by the *SB* transposon system. Survival was monitored. *, $P < 0.05$, based on the log-rank test. D, percentages of CD11b$^+$ Gr-1$^+$, CD25$^+$ Foxp3$^+$ CD4$^+$ (Treg), and IFNγ-producing CD8$^+$ T BILs were compared between the two groups by flow cytometry. Each experiment except for C was performed at least twice. *, $P < 0.05$, based on the Student *t* test.

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mice exhibited more CD11b^{+} Gr-1^{+} immature myeloid cells, which are likely myeloid-derived suppressor cells (MDSC; ref. 33), and CD25^{+} Foxp3^{+} CD4^{+} Tregs than WT mice. Furthermore, *Sting*$^{Gt/Gt}$ mice had less IFNγ-producing CD8^{+} T cells compared with WT mice (Fig. 5D). These results suggest that STING is at least partially responsible for spontaneous type I IFN production, and affects the phenotype of a variety of BIL populations, including T cells, in the glioma microenvironment. These data also led us to evaluate whether augmentation of the STING-mediated signal via administration of a STING agonist would enhance the antiglioma immunity.

STING agonist enhances type I IFN signaling and antiglioma immunity

Among various ligands that have been reported to activate STING, structure–function studies have indicated that the CDNs have been the most authentic and robust activators of STING (34). When we administered c-di-GMP intratumorally in tdTomato mice bearing gliomas, BILs from c-di-GMP–treated mice showed increased numbers of GFP^{+}CD8^{+}, GFP^{+} CD4^{+}, and GFP^{+}CD11c^{+} cells compared with control mice treated with solvent alone (Supplementary Fig. S2). Treatment of glioma-bearing WT mice with c-di-GMP significantly prolonged survival (Fig. 6A), and upregulated *Ccl5* and *Cxcl10* levels compared with control treatment (Fig. 6B) in a STING-dependent manner. In BILs, c-di-GMP treatment also enhanced tumor-homing of CD4^{+} and CD8^{+} T cells as well as IFNγ-producing CD8^{+} T cells in a STING-dependent manner (Fig. 6C and D). In the *de novo* glioma model, administration of c-di-GMP also significantly inhibited glioma growth (Fig. 6E). These data indicate that direct intratumoral administration of c-di-GMP enhances antiglioma

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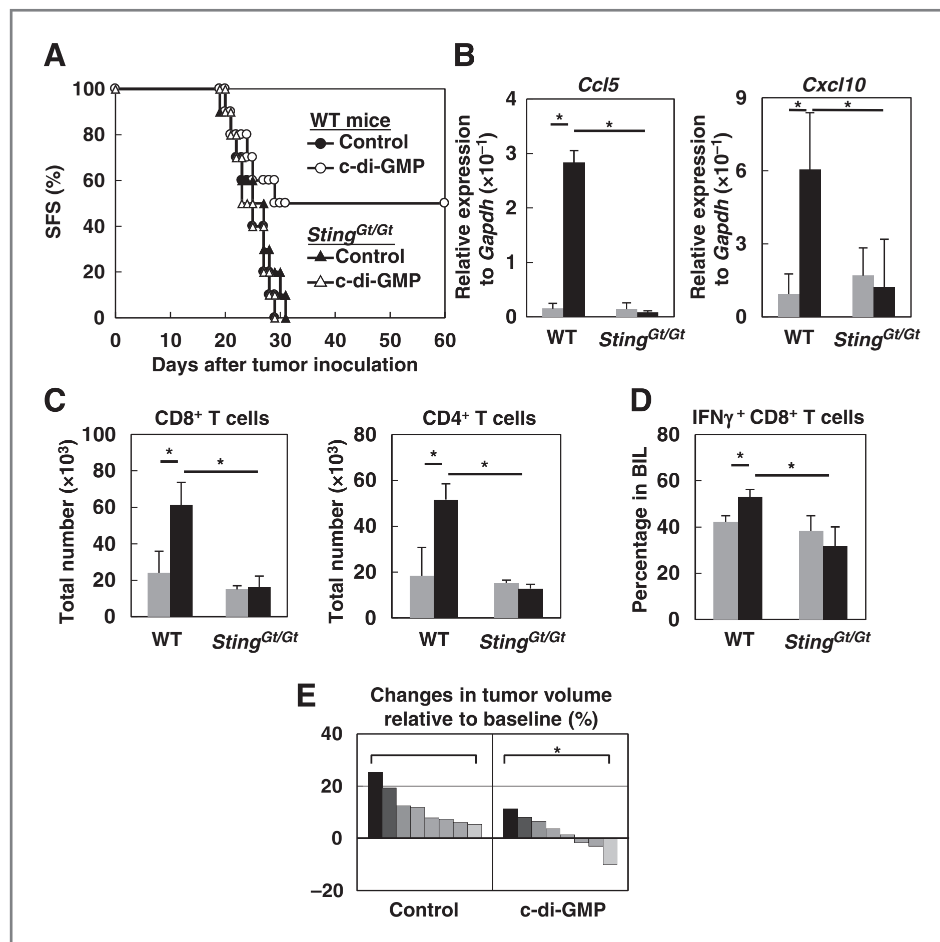

Figure 6. STING agonist enhances antiglioma immunity. A, WT and *STING*$^{Gt/Gt}$ mice bearing GL261-luc gliomas in the brain received intratumoral injection of control solvent (closed circle or closed triangle, respectively) or c-di-GMP (open circle or open triangle, respectively) on day 10 following tumor inoculation ($n = 10$/group). Survival was monitored. *, $P < 0.05$ based on the log-rank test. B, on day 5 following the control (gray bars) or c-di-GMP (black bars) treatment, WT and *Sting*$^{Gt/Gt}$ mice were sacrificed and their brains were harvested for qRT-PCR evaluation of *Ccl5* and *Cxcl10* expression levels. *, $P < 0.05$, based on the Student *t* test. C, at the same time as experiments in B, total numbers of CD8^{+} (left) and CD4^{+} (right) T cells in BILs of WT and *Sting*$^{Gt/Gt}$ mice were enumerated by flow cytometry. *, $P < 0.05$, based on the Student *t* test. D, IFNγ-producing CD8^{+} BILs were enumerated by flow cytometry. Each experiment was performed at least twice. *, $P < 0.05$, based on the Student *t* test. E, mice bearing *SB*-induced *de novo* gliomas received intracranial injection of c-di-GMP or control solvent ($N = 8$/group). The percentage of changes in tumor volume from the day before treatment (baseline) to day +7 is shown for each mouse as a waterfall plot. Tumor volume was evaluated by bioluminescent imaging. *, $P < 0.05$, based on the Fisher exact test.

immunity by enhancing the recruitment of T cells into the brain tumor site.

STING agonist enhances antitumor effects of peripheral vaccine

Finally, to investigate whether c-di-GMP treatment would enhance the efficacy of vaccinations targeting a tumor-specific antigen, using the mouse Quad-GL261 glioma cell line expressing $OVA_{257-264}$ (25), we evaluated a combination of c-di-GMP and the $OVA_{257-264}$ peptide vaccine. Although monotherapy with c-di-GMP alone significantly prolonged the survival of mice compared with vaccine alone or negative control with mock treatment ($P < 0.01$), the combination treatment further enhanced the survival benefit, with 7 of 10 mice surviving longer than 70 days ($P < 0.05$ compared with c-di-GMP alone; Fig. 7A). All 10 mice treated with $OVA_{257-264}$ peptide vaccine alone died by day 47. In BIL analyses (Fig. 7B), while c-di-GMP monotherapy significantly enhanced the tumor-homing of $CD8^+$ T cells compared with mice receiving mock treatment or vaccine alone, the combination regimen further enhanced the percentage of $CD8^+$ cells compared with c-di-GMP alone. BILs obtained from mice receiving c-di-GMP monotherapy showed a modest but significant cytotoxic activity against both $OVA_{257-264}$ peptide–pulsed and nonpulsed GL261 cells when compared with the control treatment, suggesting that c-di-GMP therapy induces cytotoxic responses against endogenous antigens in GL261 cells (Fig. 7C). Furthermore, BILs obtained from mice receiving the combination therapy demonstrated a significantly higher $OVA_{257-264}$ peptide–specific cytotoxic activity compared with ones from mice receiving c-di-GMP alone, vaccine alone, or mock treatment (Fig. 7C). These data strongly support the development of a combination strategy with vaccine and a STING agonist.

Discussion

This is, to our knowledge, the first study to describe the induction and roles of type I IFNs in the glioma microenvironment. These mechanistic evaluations also led us to demonstrate the efficacy of c-di-GMP as an adjuvant in glioma immunotherapy.

Mouse glioma tissues spontaneously expressed type I IFN mRNAs. Fuertes and colleagues (3) have demonstrated that tumor-resident $CD11c^+$ $CD8\alpha^+$ DCs are the source of type I IFNs and these DCs are critical for induction of tumor-reactive T-cell responses. In human melanoma, Wenzel and colleagues (35) have demonstrated a presence of strong type I IFN signals in regressive melanocytic skin lesions. Our studies using tdTomato mice indicate that the type I IFN signal in the glioma microenvironment indeed promotes type I T-cell responses while inhibiting Tregs. On the other hand, the development of gliomas in the brain did not affect immune cells in the spleen and iLNs, suggesting that spontaneous immune response in the glioma site does not induce systemic immune responses at least through the type I IFN signals.

On the basis of our data with STING-deficient mice and cells, we surmised that STING is at least partially responsible for the production of type I IFNs responding to its ligands in the glioma microenvironment. On the basis of our data showing that both glioma (i.e., GL261)- and nontransformed

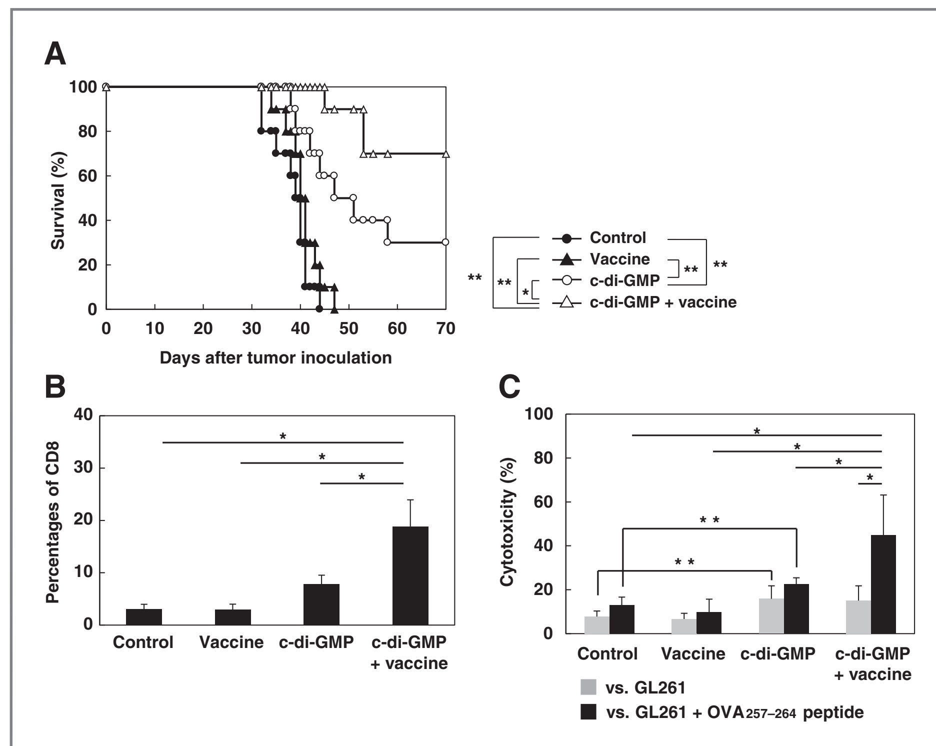

Figure 7. STING agonist enhances the efficacy of an OVA-targeted peripheral vaccine. A, mice bearing Quad-GL261 glioma in the brain received with control solvent ($N = 10$, closed circle), OVA peptide vaccination alone ($N = 10$, closed triangle), c-di-GMP alone ($N = 10$, open circle), or c-di-GMP with OVA peptide vaccination ($N = 10$, open triangle) on day 14 following tumor inoculation. Survival was monitored. *, $P < 0.05$; **, $P < 0.01$, based on the log-rank test. B, $CD8^+$ BILs were enumerated for each group by flow cytometry (5 mice/group). *, $P < 0.05$, based on ANOVA test. C, cytotoxic activity of freshly isolated BILs against the vaccine-targeted OVA epitope was evaluated by ^{51}Cr-release assay using control GL261 (gray bars) or $OVA_{257-264}$ peptide–pulsed GL261 cells (black bars) as target cells at E:T ratio 10:1 (5 mice/group). Each experiment was performed at least twice. *, $P < 0.05$, based on ANOVA test; **, $P < 0.05$, based on the Student t test.

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fibroblast (i.e., NIH3T3)-derived gDNA equally induced type I IFN mRNAs in myeloid cells in a STING-dependent manner, we postulate that gDNA released from either glioma or nontumor stroma cells could be ligands for STING signaling. Indeed, necrosis is often observed in mouse *de novo* as well as human glioma lesions (23, 30). It is likely that STING is activated downstream of specific DNA sensors that are activated by gDNA, such as cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS; ref. 36), *Ifi16*, and *DDX41* (13, 15–18, 37), in the glioma tissue. cGAS may play a major role as cGAS produces cGAMP, which binds to and activates the adaptor protein STING, thereby inducing type I IFNs and other cytokines (36, 38). Further investigations are warranted to gain better understanding of STING activation in gliomas.

Cyclic-di-GMP had been demonstrated to be an effective vaccine adjuvant (39, 40) before it was found to be a ligand for STING in 2011 (16). Before our data in the current report, other groups have also demonstrated the role of c-di-GMP as an effective adjuvant. Ebensen and colleagues (41) have demonstrated that intranasal administrations of c-di-GMP in combination with vaccines induce significantly stronger humoral and cellular immune responses than the administration of the antigen alone. Moreover, Hu and colleagues (42) have demonstrated that subcutaneous administrations of c-di-GMP plus *Staphylococcus aureus*–associated antigens induce enhanced humoral immune responses in mice, leading to prolonged survival after a challenge with cognate bacteria. These studies administered c-di-GMP three times to observe protective effects in their disease settings. On the other hand, in our glioma model, one c-di-GMP injection was sufficient to induce significant antitumor effects. This may be because our treatment was directed to the tumor site as local therapy, whereas systemic protection against infections requires systemic enhancement of the immune system. Interestingly, during the preparation of this article, Miyabe and colleagues (43) reported that subcutaneous administration of c-di-GMP delivered in liposome, but not c-di-GMP alone, can induce high-level IFNβ and antitumor immunity. It has also been demonstrated that intravenous administrations of c-di-GMP suppress vaccine-induced responses (44). Following the submission of our original article, Chandra and colleagues (45) have reported efficacy of intraperitoneally administered c-di-GMP in a metastatic 4T1 mammary adenocarcinoma model. As strategies to induce type I IFNs, we (46, 47) and others (48–50) have conducted cancer immunotherapy clinical trials using Toll-like receptor ligands. On the basis of our data in this study, early-phase clinical studies are warranted to evaluate the safety and efficacy of intratumoral administration of a STING agonist in patients with glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Ohkuri, A. Ghosh, A. Kosaka, M. David, S.N. Sarkar, H. Okada
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Ohkuri, A. Ghosh, A. Kosaka, S.N. Sarkar, H. Okada
Writing, review, and/or revision of the manuscript: T. Ohkuri, A. Kosaka, H. Okada
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.N. Sarkar, H. Okada
Study supervision: S.N. Sarkar, H. Okada

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