The neurotrophic hepatocyte growth factor attenuates CD8+ cytotoxic T-lymphocyte activity

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Background: The prognosis of glioblastomas remains poor; therefore, effective therapeutic strategies need to be developed. An agonistic CD40 antibody was shown to activate anti-tumor effects and has been extensively targeted for immunotherapeutic purposes.

Methods: The expression of CD40 and CD40L mRNAs were examined in 86 cases of WHO grade IV glioblastomas and 36 cases of grade III gliomas and correlated with outcomes. CD40 signaling was employed to augment the efficacy of immunotherapy against gliomas. The efficacy of FGK45, an agonistic antibody for CD40, was examined by adding it to a tumor lysate-based subcutaneous vaccination against a GL261 glioma model and NSCL61 glioma-initiating cell-like cell tumor model.

Results: We demonstrated for the first time using quantitative PCR that grade III gliomas expressed higher levels of CD40 and CD40L than grade IV glioblastomas. The higher expression of CD40 and CD40L was associated with good prognoses in patients with glioblastomas. The addition of FGK45 to the subcutaneous vaccination significantly prolonged survival over that with the control vaccination in both tumor models. The efficacy of this treatment was limited to the NSCL61 model; therefore, we established combination immunotherapeutic strategies using FGK45 and OX86, an agonistic antibody for OX40. Combination immunotherapy significantly prolonged survival with synergistic effects. Apoptosis was increased and proliferation decreased in tumors treated with combination immunotherapy.

Conclusions: The high expression of CD40/CD40L can be used as a biomarker for better prognoses in patients with gliomas. Immunotherapy using FGK45 significantly prolonged survival and represents a potential therapeutic strategy for gliomas including glioma-initiating cells.
CD40/CD40L expression correlates with the survival of patients with glioblastomas and an augmentation in CD40 signaling enhances the efficacy of vaccinations and prolongs survival in intracranial glioma- and glioma-initiating cell-isografted tumor models

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Running Title
CD40 signaling in glioblastoma

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Abstract

Background: The prognosis of glioblastomas remains poor; therefore, effective therapeutic strategies need to be developed. An agonistic CD40 antibody was shown to activate anti-tumor effects and has been extensively targeted for immunotherapeutic purposes.

Methods: The expression of CD40 and CD40L mRNAs were examined in 86 cases of WHO grade IV glioblastomas and 36 cases of grade III gliomas and correlated with outcomes. CD40 signaling was employed to augment the efficacy of immunotherapy against gliomas. The efficacy of FGK45, an agonistic antibody for CD40, was examined by adding it to a tumor lysate-based subcutaneous vaccination against a GL261 glioma model and NSCL61 glioma-initiating cell-like cell tumor model.

Results: We demonstrated for the first time using quantitative PCR that grade III gliomas expressed higher levels of CD40 and CD40L than grade IV glioblastomas. The higher expression of CD40 and CD40L was associated with good prognoses in patients with glioblastomas. The addition of FGK45 to the subcutaneous vaccination significantly prolonged survival over that with the control vaccination in both tumor models. The efficacy of this treatment was limited to the NSCL61 model; therefore, we established combination immunotherapeutic strategies using FGK45 and OX86, an agonistic antibody for OX40. Combination immunotherapy significantly prolonged survival with synergistic effects. Apoptosis was increased and proliferation decreased in tumors treated with combination immunotherapy.

Conclusions: The high expression of CD40/CD40L can be used as a biomarker for better
prognoses in patients with gliomas. Immunotherapy using FGK45 significantly prolonged survival and represents a potential therapeutic strategy for gliomas including glioma-initiating cells.

**Keywords:** CD40, CD40L, prognosis, immunotherapy, glioma
**Introduction**

Glioblastoma, classified as grade IV glioma by the World Health Organization, are highly malignant primary brain tumors in adults. The current standard of care for patients with glioblastomas consists of maximal resection followed by fractionated radiation therapy and the administration of temozolomide.\(^1\) Despite recent extensive research and the current therapeutic interventions, the prognoses of patients with glioblastomas remain poor, with median survival of between 12 and 15 months.\(^1,2\) The resistance of malignant gliomas to various therapies has recently been attributed to cancer-initiating cells (CIC). CICs have the ability to perpetuate themselves through self-renewal and generate amplifying cancer cells.\(^3\) Thus, the development of CIC targeting therapy is critical to improve the outcomes of patients with malignant gliomas. Many novel strategies to improve treatments against glioblastomas are currently being investigated. Immunotherapy-based strategies are now expected to be effective approaches in the treatment of cancer.\(^4\)\(^-\)\(^7\)

With the aim of reinforcing tumor-specific immunity, we focused our research on CD40, a co-stimulatory molecule. CD40 is a member of the tumor-necrosis factor (TNF) receptor (R) family, and is expressed on the surface of immune cells, including B cells, monocytes, macrophages, dendritic cells (DCs), as well as non-immune cells such as endothelial cells, epithelial cells, and also malignant tumors.\(^8\)\(^,\)\(^9\) The cognate ligand, CD40 ligand (CD40L), is expressed on activated T cells, platelets, and macrophages. Cross-linking of CD40 with CD40L has been shown to induce B cells to proliferate, differentiate, and form germinal centers.\(^8\) Interactions between CD40 and CD40L activate B cells and dendritic cells. The agonistic anti-CD40 antibody, FGK45 was previously reported to activate antigen-presenting cells (APC) and promote antitumor T cell responses.\(^10\) Thus, CD40L has been extensively targeted for immunotherapeutic purposes.
In the present study, we first examined the expression of CD40 and CD40L in glioma tissues, and correlated them to the outcomes of patients with glioblastomas. *In vivo* immunotherapy involving the addition of FGK45 to a tumor cell lysate-based subcutaneous vaccination against the glioblastoma cell line, GL261, or glioma-initiating cell-like cell line, NSCL61, an intracranial isografted tumor model, was conducted. In order to enhance anti-tumor efficacy and establish potent immunotherapy, even against glioma-initiating cells, combination therapies using FGK45, DCs, and an agonistic antibody for another co-stimulatory molecule, OX40, were examined using the NSCL61 mouse model.

**Materials and Methods**

*Patients, tumor specimens, and cell lines*

Patients with primary glioblastomas (n= 86) and patients with grade III gliomas (n= 36) underwent surgery at our institution. Their tissue specimens were stored at -80°C until use in experiments. Informed consent was obtained from all patients, and the Ethics Committee of Tohoku University Graduate School of Medicine approved the study. The mouse glioma cell line, GL261, kindly provided by Dr. Masaki Toda (Keio University, Tokyo, Japan), and mouse glioma-initiating cell-like cell line, NSCL61, kindly provided Dr. Toru Kondo (RIKEN, Kobe, Japan), were used for the mouse intracranial tumor models. FGK45 hybridoma cells were kindly provided by Dr. Antonius G. Rolink (Basel University, Basel, Switzerland). Cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Gibco, Grand Island, NY).

*RNA Isolation and Quantitative RT-PCR*
Total RNA was extracted from frozen human glioma primary specimens using the RNeasy Lipid Tissue Mini Kit (Qiagen Science, Germantown, MD) according to the manufacturer’s instructions. Reverse transcription was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Inc., Carlsbad, CA). cDNA was subjected to PCR amplification. The mRNA expression of CD40, CD40L, and the internal control, β-actin, was measured using probes purchased from the TaqMan Gene Expression Assays library (Applied Biosystems). The primers were as follows: CD40, Assay ID: Hs01002913_g1, CD40L, Assay ID: Hs 00163934_m1, and β-actin Control Reagents, Assay ID: Hs99999903_m1, respectively (Applied Biosystems). A mixture of 1 μl of cDNA, 10 μl of the TaqMan Fast Advanced Master Mix (Applied Biosystems), and each probe, was amplified by StepOnePlus Real-Time PCR Systems (Applied Biosystems) as described by the manufacturer’s instructions and quantitative analysis of mRNA was performed. Relative mRNA levels were calculated based on cycle threshold (Ct) values and were corrected for the expression of β-actin according to the following equation 13: \[2^{-\Delta Ct} \quad (\Delta Ct = Ct(\text{CD40, CD40L}) - Ct(\text{β-actin}))\].

**Immunohistochemistry**

Paraffin-embedded sections of human glioblastoma tissue were autoclaved with citrate buffer solution (pH 7.0) for 5 min at 120°C and used for CD40 and CD40L staining using an anti-CD40 rabbit polyclonal antibody (1: 25) (C-20: [sc]-975, Santa Cruz, California) and anti-CD154 (CD40L) rabbit polyclonal antibody (1: 25) (C-20: [sc]-978, Santa Cruz, California). Then, sections were incubated with rabbit polyclonal anti-rabbit antibody (1:200) (Vector laboratories Inc., CA, USA) for 30 min at room temperature. Sections were then
visualized with the Metal Enhanced 3, 3’-diaminobenzidine (DAB) Substrate kit (Thermo Fisher Scientific Inc., Rockford, Illinois) for 3 min.

In the immunohistochemical study of CD4 and CD8, mice were euthanized and their brains were harvested and frozen in Optimal Cutting Temperature (OCT) compound (Funakoshi Inc., Tokyo, Japan). Frozen brain sections (7 μm) were cut and fixed in 10% formalin for immunohistochemistry. After blocking by normal goat serum (Nichirei Biosciences Inc., Tokyo, Japan), sections were incubated with either an anti-CD4 (1:500) or anti-CD8a (1:200) antibody (eBioscience, San Diego, CA) for 2 h at 4°C. After washing, sections were incubated with anti-rat IgG Alexa 546 (1:200) for 30 min at room temperature.

VECTASHIELD Mounting Medium with 4’, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., CA) was used for nuclear staining.

For Ki-67 staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, brains harvested from mice were processed to paraffin-embedded sections at a thickness of 4 μm. Slices for Ki-67 staining were autoclaved with histofine antigen activating solution (pH 9.0) (Nichirei Biosciences Inc., Tokyo, Japan). Then slices were incubated with an SP6 rabbit anti-mouse Ki-67 antibody (1: 200) (Nichirei Biosciences Inc., Tokyo, Japan) for 24h at 4°C. The endogenous peroxidase was blocked using methanol with 1% hydrogen peroxide water. Sections were then visualized with simple stain solution for a mouse tissue (Nichirei Biosciences Inc., Tokyo, Japan). The total cell number, number of Ki-67-positive cells, and average ratio of Ki-67-positive cells were calculated. To detect apoptotic cells, sections were stained with the TUNEL method using an In situ Cell Death Detection Kit (Roche, Penzberg, Germany) in accordance with the manufacturer’s instructions. As a negative control, the terminal transferase was omitted from staining.

Mouse strains
Six- to eight-week-old C57BL/6 female mice and seven- to eight-week-old BALB/c male nude mice were purchased from SLC Japan, Inc. (Hamamatsu, Shizuoka, Japan). The protocols used in the animal studies were approved by the Institute for Animal Experimentation of Tohoku University Graduate School of Medicine.

Brain Tumor Models

GL261 and NSCL61 cell lines were used as a mouse intracranial tumor model. After anesthesia using ketamine and xylazine, mice were placed in a stereotactic apparatus (Narishige Inc., Tokyo, Japan). A burr hole was drilled in the skull; 0.5 mm forward and 2.5 mm lateral from the bregma, 3.5 mm ventral from the dura. A total of $2 \times 10^6$ GL261 cells or $1 \times 10^4$ NSCL61 cells in 2 μl PBS was then injected into the right striatum using a 2-μl Hamilton syringe (Hamilton Company, Nevada) with a 26-gauge needle.

Dendritic cell culture

C57BL/6 mouse bone marrow-derived dendritic cells (DCs) were harvested and cultured. The cells in both the femur and tibia were harvested and cultured in media supplemented with 10 ng/ml purified recombinant mouse granulocyte-macrophage colony-stimulating factor (Miltenyi Biotec Inc., CA). After 4 days of cultivation, half of the medium was changed and cytokines were again added. After 7 days of cultivation, cells were harvested and CD11c-positive cells were selected using a magnet bead-conjugated anti-CD11c antibody (Miltenyi Biotec Inc., CA) and MACS cell isolation system (Miltenyi Biotec Inc., CA).
These cells were then cultured with the tumor lysates of $2 \times 10^6$ GL261 and $1 \times 10^4$ NSCL61 for 3 h and again collected for use in the vaccination therapy.

**CD40 agonistic antibody, FGK45 refining**

FGK45 was refined using FGK45 hybridoma cells from seven- to eight-week-old BALB/c male nude mice. Pristane 0.5ml was administrated to BALB/c male nude mice twice and $1 \times 10^6$ hybridoma cells were implanted into the intraperitoneal. One- to two week later, the ascites was obtained for FGK refining. This sample was eluted in the HiTrap Protein A HP Columns (GE Healthcare, Uppsala, Sweden) by Elusion buffer. Finally, the concentration of FGK45 was calculated by Nano Drop 2000 (Thermo Fisher Scientific Inc., Waltham, MA USA) and FGK45 was stored at -80°C until use in experiments.

**Vaccination therapy**

Heavily irradiated tumor cells were used as tumor lysates. Irradiation of 5,000 rad was administered for $2 \times 10^5$ GL261 cells and 7,000 rad for $1 \times 10^4$ NSCL61 cells. In the standard vaccination, mice received a subcutaneous injection of tumor lysates in combination with 250 μg of an IgG control antibody. To observe the additive effects of triggering OX40 or CD40, 250 μg OX86, an anti-OX40 agonistic antibody, or 100 μg FGK45, an anti-CD40 agonistic antibody, was injected instead of the IgG antibody. Vaccinations were administrated twice at a 5-day interval. To assess the tumor therapy used, each tumor model; GL261 and NSCL61; was treated with tumor lysates in combination with FGK45, DCs, OX86, or the IgG control antibody. Mice were monitored daily for survival and general health.
ELISA

Lymphocytes were harvested from the spleens of mice vaccinated with FGK45, OX86, DCs, and IgG. Red blood cells were lysed with ammonium-chloride-potassium lysing buffer (Gibco, Grand Island, NY). CD4 T cells were selected using Mouse CD4 MicroBeads (Miltenyi Biotec Inc., CA) and the MACS cell isolation system (Miltenyi Biotec Inc., CA). A total of $1 \times 10^5$ CD4 T cells were cultured for 24 h, and the supernatants were used for ELISA to detect mouse interferon (IFN)-γ (BD OptEIA; BD Biosciences, Franklin Lakes, NJ).

Microscopy and image capture

Regarding optical and fluorescence microscopy, sections were imaged with a BZ9000 microscope (Keyence, Tokyo, Japan). Images were captured and archived using appropriate software (Keyence, Tokyo, Japan).

Statistical analyses

In the rodent study, data were collected from either three independent experiments or 10 mice. Significance was determined using the Mann-Whitney $U$ test for comparison between two groups. Comparison between more than three groups was determined using One-way ANOVA test. The log-rank test was used for the analysis of Kaplan-Meier survival curves. All statistical analyses were performed with GraphPad Prism 5.0.3. software (GraphPad Software, San Diego, CA). All statistical studies were two-sided, and $P$-values $< .05$ were considered significant.
Results

Expression of CD40 and CD40L in glioma tissues and its association with outcomes of gliomas

The frozen tissue samples of 122 patients with WHO Grade III (n=36) or IV (n=86) gliomas were collected retrospectively. The expression levels of human CD40 and CD40L mRNA were investigated by quantitative PCR. The expression of CD40 and CD40L by Grade III gliomas was significantly higher than that by grade IV glioblastomas (Fig. 1A and C). The expression of the CD40 and CD40L proteins was confirmed with immunohistochemical staining in high mRNA-expressing tissues (Fig. 1B and D). We subsequently evaluated the relationship between the mRNA expression levels of CD40 and CD40L and progression-free survival (PFS) and overall survival (OS). We subdivided patients with glioblastomas into a high CD40 (CD40L) expression group and low CD40 (CD40L) expression group. A CD40 value higher than 0.01 (relative mRNA level) was defined as high expression and lower as low expression. Similarly, a CD40L value higher than 0.001 (relative mRNA level) was defined as high expression and lower as low expression. The higher expression of CD40 and CD40L correlated with prolonged PFS (Fig. 2A and C) and OS (Fig. 2B and D). These results suggested that the high expression of CD40 and CD40L could be used as a prognostic factor of glioblastomas.

Effects of CD40 signaling on the GL261 glioma cell line

Since the higher expression of CD40 and CD40L correlated with prolonged OS and PFS, we
hypothesized that the stimulation of CD40 signaling could induce stronger anti-tumor immunity against gliomas. To investigate the effects of CD40 signaling on the survival of an intracranial mouse glioma model, an intracranial glioma model was established using the GL261 glioma cell line. Mice were randomly divided into three groups. One of these three groups was observed as non-treated group. For the other two groups, starting from five days after the inoculation of tumor cells, IgG, a control, or FGK45, a CD40 agonistic antibody, were administrated subcutaneously with irradiated GL261 tumor lysates as vaccines twice at a 5-day interval (Fig. 3A). Mice vaccinated with IgG and tumor lysates survived longer than the control non-treated group ($P = .0162$, Fig. 3B). However, its efficacy was only modest. Mice vaccinated with FGK45 and tumor lysates survived significantly longer than mice vaccinated with IgG and tumor lysate ($P < .0001$, Fig. 3B). This result suggested that the administration of tumor lysates and FGK45 induced significant anti-tumor effects.

**Efficacy of stimulating CD40 signaling in tumor immunotherapy**

To investigate the mechanism underlying the anti-tumor effects of the FGK45-mediated vaccination, brain sections from mice 4 days after the treatment, i.e. second vaccination, were stained immunohistochemically with an anti-CD4, anti-CD8 antibody (Fig. 3C, D, and E). The infiltration of CD4 and CD8 T cells in the FGK45-vaccinated group was significantly stronger than that in the IgG-treated group. Furthermore, the size of germinal centers in the spleen was larger in the FGK45-vaccinated group than in the IgG-treated group (Fig. 3F). This result suggested that the strong immune stimulatory effects of FGK45 induced the formation of large germinal centers in the spleen. We also demonstrated using ELISA that the FGK45 vaccination induced significantly larger amounts of IFN-$\gamma$ proteins than the IgG vaccination (Fig. 3G).
Combination immunotherapy in Cancer-Initiating Cell (CIC) model mice

The same treatment was tested in the NSCL61 tumor model. Starting from five days after the inoculation of tumor cells, IgG, a control, or FGK45, a CD40 agonistic antibody was administrated with irradiated NSCL61 tumor lysates subcutaneously as vaccines twice at a 5-day interval, similar to the study using GL261 (Fig. 3A). FGK45-vaccinated mice survived significantly longer than IgG-treated mice (data not shown); however, its efficacy was not as prominent as that observed in the GL261 model. Therefore, a combination treatment with dendritic cell vaccination therapy was performed. An intracranial glioma model was established using the NSCL61 cell line. Mice were randomly divided into following four groups. With the same method as Fig 3A, group 1 received vaccination of IgG and tumor lysate as subcutaneous vaccines; group 2 received FGK45 and tumor lysate as subcutaneous vaccines; group 3 received DCs, IgG, and tumor lysates as subcutaneous vaccines; group 4 received DCs, FGK45, and tumor lysates as subcutaneous vaccines. The survival of intracranial model mice was longer in the group 2, 3, and 4, \(P < .0001, = .0001, \text{and} < .0001\) for each group versus group 1 in the log-rank test, Fig. 4A). However, the prolongation of survival in the combination group, i.e. group 4, was not significantly different from that in the FGK45 group, i.e. group 2 \(P < .083, \text{Fig.} 4A\). The production of IFN-\(\gamma\) from CD4 lymphocytes harvested from the spleens of treated mice was synergistically induced in the combination group (Fig. 4B). Subsequently, the combination of FGK45 and OX86, an agonistic antibody for another costimulatory molecule OX40, was tested. Intracranial isografted tumor model mice were randomly divided into 4 groups. With the same method as Fig 3A, group I received vaccination of IgG and tumor lysate as subcutaneous vaccines; group II received FGK45 and tumor lysate as subcutaneous vaccines; group III received
OX86, and tumor lysates as subcutaneous vaccines; group IV received FGK45, OX86, and tumor lysates as subcutaneous vaccines. Survival was prolonged in both the group II and group III (P = .0001 and .0002 for each group versus group I in the log-rank test, Fig. 4C). Furthermore, combination therapy, group IV, significantly prolonged survival than group II (P = .0382) and than group III (P = .0313, Fig. 4C). The synergistic induction of IFN-γ from CD4 lymphocytes harvested from the spleens of treated mice was also observed (Fig. 4D).

*Immunotherapy downregulated proliferation and promoted apoptosis of tumor cells*

Proliferation and cell death were evaluated in mouse brains harvested 4 days after the second vaccination. The proliferation of tumor cells was evaluated by Ki-67 staining (Fig. 5A). The percentage of Ki-67-positive cells in the FGK45 group, FGK45 and DCs group, and FGK45 and OX86 group was significantly lower than that in the control group (*P < .05, **P < .01, ***P < .0001, Fig. 5C). This result suggested that CD40 signaling significantly inhibited the proliferation of tumors in the NSCL61 model. To investigate the mechanism underlying the anti-tumor effects of the FGK45-mediated vaccination, brain sections from mice after the treatment were stained using the TUNEL method. The total cell number, number of TUNEL-positive cells, and average ratio of TUNEL-positive cells were calculated. The FGK45 vaccination induced markedly larger numbers of apoptotic cells than the IgG or OX86 vaccinations (Fig. 5B). A significant difference was observed in the percentage of TUNEL-positive NSCL61 between these groups. The percentage of TUNEL-positive cells was significantly higher in the FGK45 group, FGK45 and DCs group, and FGK45 and OX86 group than in the control group (*P < .05, **P < .01, ***P < .0001, Fig. 5D). This result suggested that immunotherapy augmented by stimulation of CD40 signaling significantly induced apoptosis in the NSCL61 model.
DISCUSSION

Malignant gliomas are the most common type of primary brain tumor and still have a poor prognosis. In the present study, we demonstrated for the first time that the expression of CD40 and CD40L was associated with the outcomes of gliomas. Grade III gliomas expressed higher levels of CD40 and CD40L than the grade IV gliomas, GBM (Fig. 1A and C). The high expression levels of CD40 and CD40L were also associated with better PFS and OS of GBM patients who underwent gross total removal of the tumor (Fig. 2A, B, C, and D). This result suggested that CD40 and CD40L could be useful biomarkers for gliomas.

As the higher expression of CD40 and CD40L in GBM was associated with improved survival, we hypothesized that triggering CD40/CD40L induced both immune stimulatory and anti-tumor effects. In order to develop effective treatments based on these results, FGK45, an agonistic antibody for CD40, was added to a tumor cell lysate vaccination against a rodent glioma model. Immunotherapy using FGK45 significantly induced a larger amount of IFN-γ (Fig. 3G) from CD4 T cells than that using IgG and prolonged survival (Fig. 3B) of the GL261 model as well as the NSCL61 glioma-initiating cell-like cell model (Fig. 4A, B, C, and D).

Although the vaccination using FGK45 markedly prolonged the survival of the GL261 glioma model, its efficacy against NSCL61 was not as strong. Therefore, we attempted to augment its efficacy by combining it with another immunostimulant. Antigen-presenting cells, dendritic cells, and another costimulation molecule, OX40, were selected as candidates. We initially used a FGK45 and DC-based vaccination. Combination immunotherapy induced large amounts of IFN-γ from CD4 T cells (Fig. 4B). However, the synergistic effects of the combination immunotherapy were significantly different from those in the FGK45-treated
group. We then used a combination of FGK45 and OX86, an agonistic antibody against OX40. The combination immunotherapy induced a large amount of IFN-γ from CD4 T cells (Fig. 4D) and prolonged survival with significant synergistic effects (Fig. 4C). Ki-67 staining revealed that immunotherapy inhibited the proliferation of tumor cells, especially when CD40 was stimulated (Fig. 5A and C). TUNEL staining showed that immunotherapy induced apoptosis against tumor cells (Fig. 5B and D). Furthermore, the stimulation of CD40 signaling significantly induced apoptosis against tumor cells (Fig. 5B and D). CD40 signaling has been shown to stimulate B cells, monocytes, macrophages, and DCs, while OX40 signaling stimulates CD4 T cells, CD8 T cells, and natural killer T cells. Therefore, it is likely that combination immunotherapy using FGK45 and OX86 stimulated a broad range of molecules that mediate anti-tumor effects and resulted in enhanced efficacy. In addition, IFN-γ was previously shown to enhance the expression of CD40 in glioma cells. Since the significant induction of IFN-γ was observed in CD4 lymphocytes following the combined treatment with FGK45 and OX86, combination immunotherapy appeared to potently induce synergistic effects.

Kosaka A, et al. recently reported the efficacy of a CD40 agonist against glioma intracranial models. They administrated FGK45 i.p. and demonstrated synergistic effects with a Cox-2 inhibitor. To the best of our knowledge, no other study has demonstrated the efficacy of the CD40 stimulation against gliomas. We here showed the efficacy of the addition of a CD40 agonistic antibody to a vaccine against glioma models. In a previous Phase 1 trial, a cytokine storm was unexpectedly induced by the i.v. systemic administration of an anti-CD28 monoclonal antibody. Since CD28 is a co-stimulatory molecule for T-cell activation, similar to CD40, a similar cytokine reaction may limit the dose of the CD40 agonistic antibody when delivered systemically. From this point of view, subcutaneous administration as an additive to a vaccine may provide a safer strategy when considering
clinical development.

In the present study, we demonstrated for the first time that the expression of CD40/CD40L could be used as a prognostic factor for gliomas and their higher expression correlated with better survival. We then showed that CD40-based immunotherapy was effective against rodent glioma tumor models and a glioma-initiating cell-like cell tumor model. Combination immunotherapy using FGK45 and OX86 further prolonged survival with synergistic effects. Therefore, this combination immunotherapy can be used as an effective treatment strategy, even against glioma-initiating cells. We only vaccinated animals twice at a 5-day interval; therefore, modifying the administration schedule and the amount of antibodies may further induce stronger anti-tumor immunity. Clinical development is warranted.

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**Figure Legends**

Fig. 1. CD40/CD40L gene expression and immunohistochemistry in glioma tissues. (A) CD40 gene expression analyzed by quantitative PCR in glioma tissues was significantly higher in 36 cases of grade III gliomas than in 86 cases of grade IV glioblastomas (*P = .0334). (B) Representative immunohistochemical image of CD40 in glioblastoma tissues, which revealed the high expression of CD40 by quantitative PCR. CD40-positive glioblastoma cells were detected. Scale bar, 100 μm. (C) CD40L gene expression analyzed by quantitative PCR was significantly higher in grade III gliomas than in grade IV glioblastomas (*P < .0001). (D) Representative immunohistochemical image of CD40L in glioblastoma tissues, which revealed the high expression of CD40L by quantitative PCR. CD40L-positive glioblastoma cells were detected. Scale bar, 100 μm.

Fig. 2. Kaplan-Meier survival curves of patients with glioblastomas. (A) and (B). High expression levels of CD40 in patients with glioblastomas who underwent gross total resection of the tumor (2^ΔCt > .01; n=46) were associated with longer progression-free survival (PFS) and overall survival (OS) than those with low expression levels (2^ΔCt < .01; n=40). (C) and
(D), High expression levels of CD40L in patients with glioblastomas who underwent gross total resection of the tumor ($2^{-\Delta C_t} > .001; n=39$) were also associated with longer PFS and OS than those with low expression levels ($2^{-\Delta C_t} < .001; n=47$).

Fig. 3. Effects of triggering CD40 by FGK45 in the GL261 tumor model. (A) Schedule of vaccination therapy. (B) Intracranial glioma model established using the GL261 glioma cell line were randomly divided into three groups. One of these three groups was observed as a non-treated group. For the other two groups, IgG, a control, or FGK45, a CD40 agonistic antibody, were administrated subcutaneously with irradiated GL261 tumor lysates as vaccines. Mice vaccinated with IgG and tumor lysates (n=10) survived longer than the control non-treated group (n=10) ($P = .0162$, Fig. 3B). However, its efficacy was only modest. Mice vaccinated with FGK45 and tumor lysates (n=10) survived significantly longer than mice vaccinated with IgG and tumor lysate ($P<.0001$). (C) Mouse CD4 and CD8 positive T cells (red) in GL261 tumor models were counterstained with 4',6-diamino-2-phenylindole (DAPI, blue). Scale bars, 100 μm. Immunohistochemistry showed the stronger infiltration of CD4- and CD8-positive T cells in GL261 tumor models 4 days after the second vaccination with FGK45. (D) and (E), The number of cells that were positively stained for CD4 or CD8 were counted under ×400 magnification, Bars indicate the mean ± SD (*$P < .05$). (F) Hematoxylin and eosin (H&E) stain of mouse spleens from the FGK45 vaccination group and IgG vaccination group. The size of germinal centers (black triangles) in the FGK45 vaccination group was larger than that in the IgG vaccination group. Scale bars, 100 μm. (G) Production of the interferon (IFN)-γ protein by mouse CD4-positive T cells after the second subcutaneous vaccination with FGK45 and irradiated GL261 cells or IgG and irradiated GL261 cells, Bars indicate the mean ± SD. TLs, tumor lysates.
Fig. 4. Effects of the combined immunotherapy of FGK45, dendritic cells, and OX86 in the cancer-initiating model mouse. (A) Survival analysis of NSCL61 model mice treated with a subcutaneous injection of irradiated NSCL61 cells (1×10^4) as tumor lysates and IgG (group 1), tumor lysates and FGK45 (100 μg) (group 2), tumor lysates, dendritic cells (DCs) (1×10^6), and IgG (group 3), and tumor lysates, DCs (1×10^6), and FGK45 (100 μg) (group 4). FGK45 therapy (group 2), DCs therapy (group 3), and combination therapy (group 4) significantly prolonged survival compared to group 1 (P = < .0001, .0001, < .0001, log-rank test). The synergistic effects of the combination therapy (group 4) were detected compared to DCs therapy alone (group 3) (P=.0083, log-rank test), while not compared to FGK45 therapy alone (group 2). (B) Production of the IFN-γ protein by mouse CD4-positive T cells after the second subcutaneous vaccination as determined by ELISA. Comparisons between groups 1, 2, 3, and 4. Bars indicate the mean ± SD. (C) Survival analysis of NSCL61 model mice treated with a subcutaneous injection of irradiated NSCL61 cells (1×10^4) as tumor lysates and IgG (group I), tumor lysates and FGK45 (100 μg) (group II), tumor lysates and OX86 (250 μg) (group III), and tumor lysates, OX86 (250 μg), and FGK45 (100 μg) (group IV). FGK45 therapy (group II), OX86 therapy (group III), and combination therapy (group IV) significantly prolonged survival compared to group 1 (P = .0013, P < .0001, P <.0001, log-rank test). Synergistic effects of the combination therapy (group IV) were detected compared to FGK45 therapy alone (group II) (P=.0382, log-rank test), and OX86 therapy alone (group III) (P=.0313, log-rank test). (D) Production of the IFN-γ protein by mouse CD4-positive T cells after the second subcutaneous vaccination as determined by ELISA. Comparisons between groups I, II, III, and IV. Bars indicate the mean ± SD.

Fig. 5. Ki-67 and TUNEL staining in NSCL61 model mice treated with either IgG (control), FGK45, dendritic cells (DCs), OX86, DCs combined with FGK45, or OX86 combined with
FGK45. (A) Ki-67 staining. Scale bars, 100 μm. (B) TUNEL staining. Scale bars, 100 μm.

(C) The number of cells that positively stained for Ki-67 was counted under ×400 magnification. Bars indicate the mean ± SD.

(D) The number of cells that positively stained with TUNEL staining were counted under ×400 magnification. Bars indicate the mean ± SD.
Figure 1

A

CD40 mRNA expression

Grade III  |  Grade IV

n = 36  |  n = 86

P = 0.0334

B

Human CD40 expression

C

CD40L mRNA expression

Grade III  |  Grade IV

n = 36  |  n = 86

P < 0.0001

D

Human CD40L expression
Figure 2

A

Progression-free survival (%) vs. Months

CD40 high: n = 46
CD40 low: n = 40

P = 0.0085

B

Overall survival (%) vs. Months

CD40 high: n = 46
CD40 low: n = 40

P = 0.0027

C

Progression-free survival (%) vs. Months

CD40L high: n = 39
CD40L low: n = 47

P = 0.0001

D

Overall survival (%) vs. Months

CD40L high: n = 47
CD40L low: n = 39

P = 0.0006
Figure 3 FG

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Figure 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Ki-67</th>
<th>TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Group 1)</td>
<td><img src="image1.png" alt="Ki-67 Image" /></td>
<td><img src="image2.png" alt="TUNEL Image" /></td>
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<tr>
<td>FGK45 (Group 2)</td>
<td><img src="image3.png" alt="Ki-67 Image" /></td>
<td><img src="image4.png" alt="TUNEL Image" /></td>
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<tr>
<td>DCs (Group 3)</td>
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<td><img src="image6.png" alt="TUNEL Image" /></td>
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<tr>
<td>FGK+DCs (Group 4)</td>
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<td><img src="image8.png" alt="TUNEL Image" /></td>
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<tr>
<td>OX86 (Group III)</td>
<td><img src="image9.png" alt="Ki-67 Image" /></td>
<td><img src="image10.png" alt="TUNEL Image" /></td>
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<tr>
<td>FGK45+OX86 (Group IV)</td>
<td><img src="image11.png" alt="Ki-67 Image" /></td>
<td><img src="image12.png" alt="TUNEL Image" /></td>
</tr>
</tbody>
</table>

**A** Ki-67

**B** TUNEL

**C**

- KI-67 index
- Group 1: 70 ± 5
- Group 2: 60 ± 4
- Group 3: 50 ± 3
- Group 4: 40 ± 2
- Group III: 30 ± 1
- Group IV: 20 ± 0.5

**D**

- TUNEL positive cells
- Group 1: 80 ± 5%
- Group 2: 70 ± 4%
- Group 3: 60 ± 3%
- Group 4: 50 ± 2%
- Group III: 40 ± 1%
- Group IV: 30 ± 0.5%