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KHYG-1 Cells With EGFRvIII-specific CAR Induced a Pseudoprogression-like Feature in Subcutaneous Tumours Derived from Glioblastoma-like Cells

TSUTOMU NAKAZAWA[1,2*], TOSHIHARU MURAKAMI[1*], ATSUSHI NATSUME[3], FUMIHIKO NISHIMURA[1], TAKAYUKI MORIMOTO[1], RYOSUKE MATSUDA[1], MITSUTOSHI NAKAMURA[1,4], SHUICHI YAMADA[1], ICHIRO NAKAGAWA[1], YOUNG-SOO PARK[1], YASUSHI MOTOYAMA[1], TAKAHIRO TSUJIMURA[4], TOSHIHIKO WAKABAYASHI[3] and HIROYUKI NAKASE[1]

[1]*Department of Neurosurgery, Nara Medical University, Kashihara, Japan;*
[2]*Grandsoul Research Institute for Immunology, Inc., Uda, Japan;*
[3]*Department of Neurosurgery, Nagoya University, Graduate School of Medicine, Nagoya, Japan;*
[4]*Clinic Grandsoul Nara, Uda, Japan*

Abstract. *Background/Aim: We previously established a novel type of epidermal growth factor receptor variant III (EGFRvIII)-specific chimeric antigen receptor (CAR)-expressing natural killer (NK) cell line, designated EvCAR-KHYG-1, which inhibited the growth of glioblastoma (GBM) cells in vitro via apoptosis. Materials and Methods: We investigated the cytokine-producing effect of EvCAR-KHYG-1 cells on GBM-like cell lines and their antitumour effect using in vivo xenograft assays. Results: EvCAR-KHYG-1 cells produced interleukin-2, interferon-γ, and tumour necrosis factor-α on EGFRvIII-expressing U87MG cells. In vivo xenograft assays showed that EvCAR-KHYG-1 cells did not reduce the volume of subcutaneous tumours derived from EGFRvIII-expressing U87MG cells but did reduce tumour cell occupancy. Conclusion: EvCAR-KHYG-1 cells led to expression of cellular immunity-related cytokines on EGFRvIII-expressing U87MG in vitro but did not inhibit tumour progression due to the induction of a pseudo progression-like pathological feature. Future studies investigating the effect of different conditions in vivo are required to study the inhibition of tumour progression in GBM.*

*These Authors contributed equally to this study.

Correspondence to: Tsutomu Nakazawa, Department of Neurosurgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara, Japan. Tel: +81 744298866, Fax: +81 744290818, e-mail: nakazawa@naramed-u.ac.jp

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Glioblastoma (GBM) is one of the most common and aggressive types of primary malignant central nervous system (CNS) tumours occurring in adults (1-3). Despite chemotherapy, radiation, with/without surgical resection, few patients survive for more than 5 years (1, 3). Thus, developing an effective therapy to treat GBM is warranted. Immunotherapy is a promising alternative to conventional treatments, with the possible long-term benefit of generating a sustained antitumour response and potentially targeting both localized and infiltrating tumour cells. Natural killer (NK) cells are potent effector cells in cell-based cancer immunotherapy. They respond rapidly to abnormal cells and represent a crucial effector cell population in adoptive immunotherapy (4). In addition, donor-derived NK cells such as NK-92 and KHYG-1 are being developed for clinical applications (5-8).

Chimeric antigen receptor (CAR) is an artificially modified fusion protein consisting of an extracellular antigen-recognition domain fused to an intracellular signalling domain (9, 10). CAR redirects the specificity and function of T-lymphocytes and other immune cells. The general premise for the use of CAR cells in cancer immunotherapy is the rapid generation of tumour-targeted T-cells, bypassing the barriers and incremental kinetics of active immunization (11, 12). CAR T-cells are effectively used to treat refractory chronic lymphocytic leukaemia and acute lymphoblastic leukaemia (ALL) (13, 14). Specifically, CD19-targeting CAR T-cells have been reported to result in complete response rates of 70-90% in ALL patients (15). However, CAR T-cells exhibited poor therapeutic efficacy against solid tumours (16-18). A clinical trial showed that epidermal growth factor receptor variant III (EGFRvIII)-targeted CAR T-cells infiltrated GBM but without objective

response. Furthermore, EGFRvIII expression vanished in GBM, helping GBM cells escape EvCAR T-cells (19). These findings indicate that strategies targeting a single antigen cannot inhibit the progression of GBM with heterogeneous expression of cancer-related antigens.

Similarly to CAR T-cells, CAR-NK cells were found to exhibit improved tumour-specific targeting and cytotoxicity against cancer cells in both *in vitro* and *in vivo* (20, 21). Importantly, NK cells have many advantages over T-cells in CAR-targeted immunotherapy (22, 23). NK cells recognize tumour cells that rely on a set of stimulatory and inhibitory receptors. Several receptors can sense whether a proximal cell expresses a ligand profile associated with oncogenic transformation. Cells expressing a tumour-associated profile trigger NK cell activation and targeted cell killing, suggesting that unlike T-cells, NK cells recognize multiple oncogenic antigens. Thus, CAR-expressing NK cells would be an attractive strategy for GBM with functional heterogeneity. We previously reported on the development of a third generation of CAR cells specific for EGFRvIII (24). Moreover, in our previous study, we generated the human NK cell line KHYG-1, which expressed EGFRvIII-specific CAR (EvCAR-KHYG-1) and evaluated the antitumour effects of this cell line on GBM cells *in vitro*. (25). This study aimed to investigate the cytokine production ability and *in vivo* antitumour effects of EvCAR-KHYG-1 cells in order to build upon the findings given in our previous report.

Materials and Methods

Cell lines. The human GBM-like cell line U87MG and human embryonic kidney-derived cell line HEK293T-cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). U87MG cells expressing EGFRvIII (Ev-U87MG) were provided by Atsushi Natsume of the Nagoya University School of Medicine, Japan. GBM cell lines were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; MP Biomedicals, Santa Ana, CA, USA). KHYG-1 cells were from the Japanese Collection of Research Bioresources Cell Bank (Suita, Osaka, Japan). The previously established EvCAR-expressing KHYG-1 cells (25) were maintained in RPMI-1640 (Thermo Fisher Scientific Inc.) with 150 IU/ml recombinant human interleukin-2 (Novartis Pharma AG, Basel, Switzerland) supplemented with 20% FBS. Each culture medium used was supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific Inc). The cell lines were cultured under standard culture conditions at 37°C in a humidified atmosphere containing 5% CO_2.

Cytokine detection. A Human Cytometric Bead Array Flex Set System (BD Biosciences, San Jose, CA, USA) was used to determine cytokine production by EvCAR-KHYG-1 cells. Briefly, the GBM cell line was co-cultured with the EvCAR-KHYG-1 cell line at ratios of 0:1, 1:0, 1:1, and 1:5 for 24 h, and the co-culture supernatant was obtained to measure the levels of interferon-γ (IFNγ), tumour necrosis factor-α (TNFα), IL2, IL4, IL6, and regulated on activation, normal T-cells expressed and secreted (RANTES). The experiment was performed three times. The assays were performed according to the manufacturer's instructions. Data were acquired on a BD FACSMelody flow cytometer (BD Biosciences).

In vivo xenograft assay. The *in vivo* xenograft assay was performed as described previously (26). Briefly, nonobese diabetes (NOD)/severe combined immunodeficiency (SCID)/γc^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Nara Medical University (approval number: 12137). A total of 10^6 Ev-U87MG cells were subcutaneously injected into the backs of 6-week-old female NOG mice. The mice were then randomly assigned to two subcutaneous (*s.c.*) injection groups (each n=4): Control (PBS) and EvCAR-KHYG-1 cells (107), respectively (Figure 1A). Tumour size was recorded weekly by external measurement of the diameter of *s.c.* tumour xenografts using a Vernier calliper. Tumour volumes were calculated weekly for 4 weeks, as previously reported (26), using the following equation: Tumour volume (mm^3)=π/6 × length × $(width)^2$.

Histochemical analysis. Tumour-bearing mice were sacrificed at 28 days after *s.c.* injection of Ev-U87MG. The *s.c.* tumours were fixed with 10% neutral-buffered formalin and embedded in paraffin. Then, 5-μm-thick sections were placed on glass slides and stained with haematoxylin and eosin (HE). Photographs were taken using an IX83 microscope (Olympus, Osaka, Japan) at 40×, 100×, and 200× magnification with a DP80 digital camera (Olympus, Osaka, Japan). Data were acquired by CellSens Dimensions imaging software (Olympus, Osaka, Japan). Tumour cell occupancy, *i.e.* the percentage of area occupied by tumour cells in subcutaneous tumour tissue, was calculated using CellSens imaging software (Dimension package) according to the manufacturer's instructions.

Statistical methods. Values are shown as the means±standard deviation (SD). Statistical significance of differences was determined using a *t*-test or one-way analysis of variance followed by Tukey's test and accepted at $p<0.05$.

Results

EvCAR-KHYG-1 cells produce cytokines upon recognition of target cells. We determined the cytokine levels produced by KHYG-1 and EvCAR-KHYG-1 (effector) cells upon exposure to U87MG and Ev-U87MG (target) cells. IL2 was weakly (with range of 6-18 pg/ml and 5-7 pg/ml, respectively) detected in the supernatants of Ev-U87MG cells co-cultured with EvCAR-KHYG-1 cells (at 1:1 and 1:5). The IFNγ levels in the supernatants from EvU87MG co-cultured with EvCAR-KHYG-1 cells were in the range of 11-42 pg/ml and 456-781 pg/ml, respectively, using the same target:effector ratios. The TNFα level in the supernatants from cultures of Ev-U87MG and EvCAR-KHYG-1 cells were in the range of 0-7 pg/ml and 98-234 pg/ml, respectively. IL4 was not detected in the supernatant.

Table I. *Cytokine production by epidermal growth factor receptor variant III (Ev)-specific chimeric antigen receptor (CAR)-expressing natural killer cell line (EvCAR-KHYG-1) cultured with Ev-expressing U87MG cells. Glioblastoma-like cell lines (U87MG and Ev-U87MG) were cultured alone and with natural killer cell lines (KHYG-1 and EvCAR-KHYG-1) at the indicated ratios for 24 hours. The concentrations of interferon-γ (IFNγ), tumour necrosis factor α (TNFα), interleukin-2 (IL2), IL4, IL6, and regulated on activation, normal T-cells expressed and secreted (RANTES) were then determined in the supernatant. Values are shown as the means±SD (n=3). Statistical significance of differences was determined using one-way analysis of variance, followed by Tukey's test.*

		Concentration					
	Culture ratio	IFNγ (pg/ml)	TNFα (pg/ml)	IL2 (pg/ml)	IL4 (pg/ml)	IL6 (ng/ml)	RANTES (ng/ml)
LOQ	-	1.8	0.7	11.2	1.4	1.6×10^{-3}	2×10^{-6}
U87MG	-	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.1	0.0±0.0
KHYG-1	-	0.1±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.5±0.6[a]
U87MG:KHYG-1	1:1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.4±0.1	2.3±0.2[a]
	1:5	0.6±0.5	0.0±0.0	0.0±0.0	0.0±0.0	2.9±0.2[b]	3.9±0.4[abc]
EvCAR-KHYG-1	-	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.9±0.1
U87MG:EvCAR-KHYG-1	1:1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.9±0.1	1.3±0.3[a]
	1:5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.4±0.1	3.2±0.6[abc]
Ev-U87MG	-	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.7±0.3	0.0±0.0
Ev-U87MG:KHYG-1	1:1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.2±0.3	1.7±0.5[a]
	1:5	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	3.0±0.4[b]	3.8±0.4[abc]
Ev-U87MG:EvCAR-KHYG-1	1:1	21.8±14.6	3.1±2.9	10.2±5.4[ab]	0.0±0.0	9.2±2.0[ab]	1.9±0.3[a]
	1:5	611.1±133.2[abc]	153.4±58.1[abc]	3.8±2.9	0.0±0.0	11.3±1.4[ab]	4.4±0.3[abc]

Significantly different at $p<0.05$ from: [a]Target alone, [b]effector alone, and [c]1:1 target:effector ratio.

IL6 was abundantly present in supernatant from U87MG cells co-cultured with KHYG-1 cells (at 1:5, range=2,668-3,182 pg/ml), EvU87MG with KHYG-1 cells (at 1:5, range=1,350-1,547 pg/ml), and Ev-U87MG with EvCAR-KHYG-1 cells (at 1:5, 9,452-12,816 pg/ml). In addition, it was detected in the supernatant from U87MG cells when cultured alone (522-759 pg/ml and 1,378-2,419 pg/ml). RANTES was detected in the supernatants from culture of effector cells alone (562-3,326 pg/ml). RANTES was also detected in the supernatants of cells with an target:effector ratio of 1:1 relative to effector-only conditions, and the supernatants of cells with an target:effector ratio of 1:5 showed an approximately 1.5 to 5-fold increase in RANTES levels compared to effector-only conditions. RANTES was barely detected in the supernatants from target cells when cultured alone.

In vivo xenograft assay. We then studied the antitumour effects of EvCAR-KHYG-1 on Ev-U87MG in NOG mice *in vivo*. NOG mice were subcutaneously inoculated with 10^6 Ev-U87MG cells and then topically injected three times (days 7, 10 and 13) with PBS (control) and EvCAR-KHYG-1 (10^7). The tumour size was recorded weekly (Figure 1A). The tumour volumes (means±SD) of mice that had received PBS, and EvCAR-KHYG-1 were 6,407±3,497 mm^3 and 7,733±2,565 mm^3, respectively, at 4 weeks post-treatment. EvCAR-KHYG-1 treatment showed no significant effect on the volume of Ev-U87MG-derived subcutaneous tumours (Figure 1B). The results of the histochemical analysis showed that tumours from both groups exhibited a pseudo-palisading pattern of tumour cells surrounding necrosis, which is a human GBM-like histological feature (Figure 1C). In terms of tumour cell occupancy, the EvCAR-KHYG-1-treated group exhibited a significantly higher mean occupancy of 46.6±9.1% compared with 23.2±4.4% for the PBS-treated group.

Discussion

We previously reported that our established EvCAR-KHYG-1 cell line elicited an antitumour effect on GBM *in vitro via* apoptosis in an EvCAR- and EGFRvIII-dependent manner. EvCAR enhanced the inhibition of Ev-U87MG cell growth by KHYG-1 cells (25). As an extension of our previous study, the present study investigated the cytokine-producing ability and *in vivo* antitumour effect of EvCAR-KHYG-1 cells on GBM cells.

The results of the cytokine detection experiment revealed that IL2, IFNγ, TNFα, and IL6 were produced when Ev-U87MG cells were exposed to EvCAR-KHYG-1 cells. IL4 was not detected under any conditions. IL2 and IFNγ production indicated that EvCAR-KHYG-1 cells are characterized by cellular immunity-inducing cells, which can lead the attack against intracellular pathogens, such as viruses, and fight cancer cells *in vivo* (27, 28). Thus, not only may EvCAR-KHYG-1 cells elicit direct cytotoxicity on GBM cells,

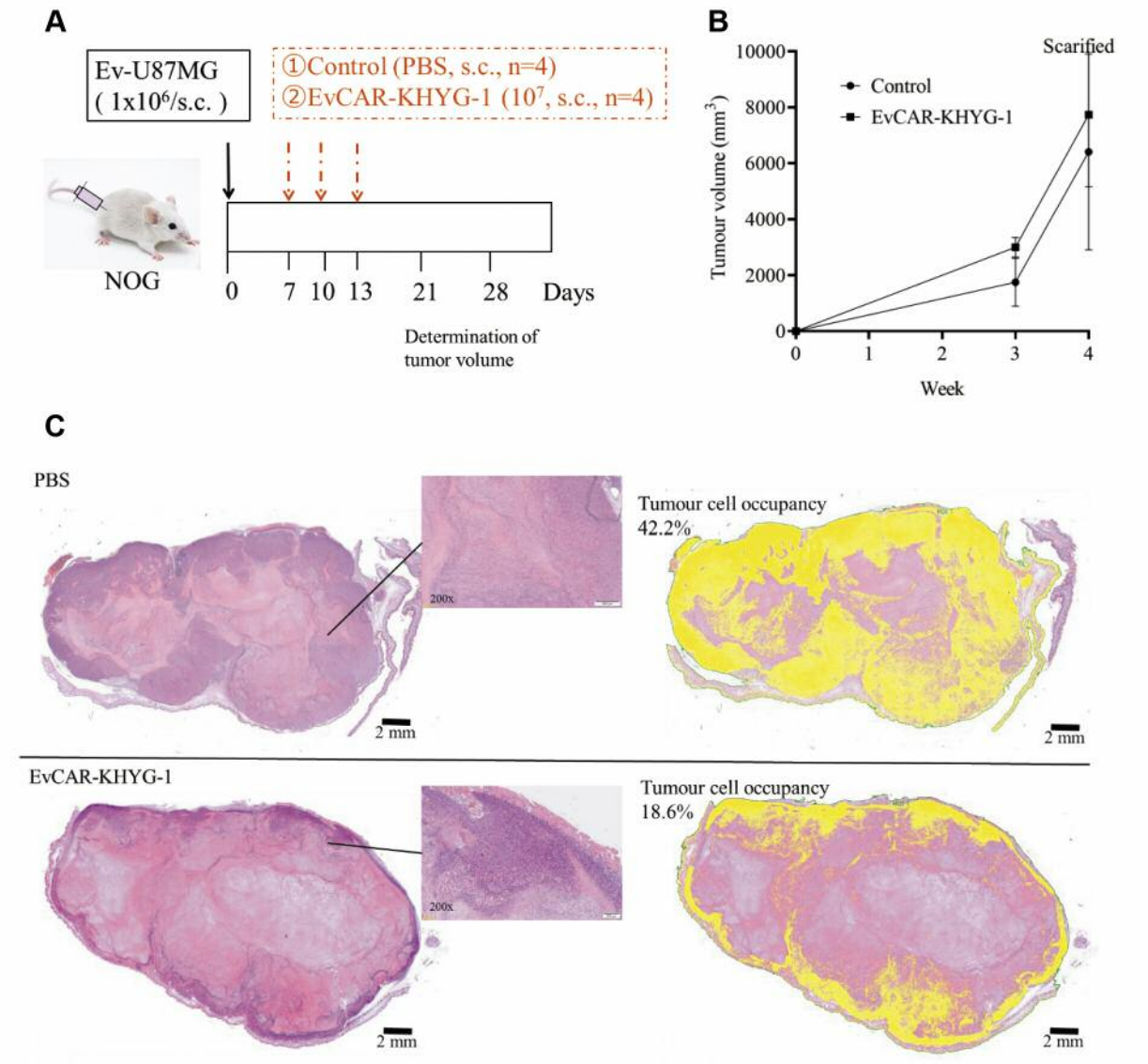

Figure 1. *In vivo antitumor effect of epidermal growth factor receptor variant III (Ev)-specific chimeric antigen receptor (CAR)-expressing natural killer cell line (EvCAR-KHYG-1) cells against subcutaneous tumour xenograft derived from epidermal growth factor receptor variant III-expressing U87MG (Ev-U87MG) cells. A: Schematic representation of the experimental setting for the control (group 1) and treated (group 2) animals. PBS: Phosphate-buffered saline. B: Volume (mean±SD) of Ev-U87MG cell-derived subcutaneous tumour in control and EvCAR-KHYG-1 cell-treated groups (n=4). C: Representative haematoxylin and eosin staining of Ev-U87MG cell-derived subcutaneous tumour (left) and tumour cell occupancy (right) in control and EvCAR-KHYG-1-treated mice. Tumour cells are shown as yellow regions in the images.*

they may also indirectly exert a tumour-regression effect *via* cellular immunity in humans *in vivo*. Moreover, EvCAR-KHYG-1 cells release TNFα. A previous study showed that the INFγ and TNFα produced by NK cells were functionally linked to and induce target cell cytolysis through up-regulation of intercellular adhesion molecule-1 (29). Together, the data support the inhibition of GBM growth *in vivo*. RANTES was increased in all conditions when the target:effector ratio was 1:5, implying that it was spontaneously released by KHYG-1 and EvCAR-KHYG-1 cells and not in response to U87MG-expressing ligands, including EGFRvIII. RANTES accumulates in T-cells and regulates inflammation in several diseases, playing an active role in recruiting a variety of leukocytes into inflammatory sites, such as T-cells, macrophages, eosinophils and basophils. In collaboration with certain cytokines released by T-cells, such as IL2 and IFNγ, RANTES also induces the activation and proliferation of particular NK cells to generate chemokine-activated killer cells

(30). IL6 was remarkably increased when EvCAR-KHYG-1 cells were co-cultured with EvU87MG cells. In the cytokine assay with EvCAR-KHYG-1 and Ev-U87MG cells, it was not possible to determine which cells produced IL6. Goswami *et al*. reported that U87MG cells expressed IL6 and IL6 receptors, while U87MG cells have an autocrine growth loop (31). Previously, IL6/signal transducer and activator of transcription 3 (STAT3) signalling was reported to support GBM cell growth and migration (32). An IL6/STAT3/hypoxia-inducible factor 1 subunit alpha autocrine loop has been observed in GBM. GBM cancer stem cells respond to perturbations by hypoxia, inhibition of STAT3 phosphorylation, and IL6 stimulation (33). Thus, IL6 is strongly involved in the formation and progression of GBM. Based on our findings, we considered that Ev-U87MG cells may have released IL6, although flow cytometry-based analysis of cytokine-producing cells is warranted to verify this. On the other hand, we observed that EvCAR-KHYG-1 elicits direct cytotoxicity and releases cellular immunity-related cytokines, which is contrary to the role of IL6. Therefore, we aimed to verify the antitumour effect of EvCAR-KHYG-1 *in vivo*.

The *in vivo* xenograft experiments showed that EvCAR-KYHG-1 cells did not elicit any distinct reduction in the volume of GBM cell-derived *s.c*. tumour. We considered that *in vivo* experiments carried out under a range of conditions are required to determine the conditions under which GBM tumour progression can be inhibited. Histochemical analysis revealed that the EvCAR-KHYG-1 injection increase the necrotic area of GBM tissue compared to the control. We consider this finding to be a progression-like pathological phenotype. This 'pseudoprogression' can occur in up to 20% of patients who receive chemoradiotherapy with temozolomide, which is an alkylating agent for GBM, and it can explain about half of all cases with increasing size of lesions after the end of the treatment in patients with GBM. The mechanisms behind these events have not yet been fully elucidated but it is likely that chemoradiotherapy causes a higher degree of (desired) tumour-cell and endothelial-cell killing. This increased cell killing can lead to secondary reactions such as oedema and abnormal vessel permeability in the tumoural area, mimicking tumour progression (34). We consider EvCAR-KHYG-1 cells to be able to induce strong tumour-cell killing similarly to the effect of temozolomide in GBM tumours. We also suspect that IL6 might be involved in this progression-like pathological phenotype. However, this experimental setting was in a subcutaneous model not an orthotopic one. Further orthotopic transplant experiments are required to obtain more accurate information on the antitumour effect and this pseudoprogression-like feature in GBM.

In summary, we demonstrated that EvCAR-KHYG-1 cells produce cellular immunity-related cytokines and TNFα on exposure to Ev-U87MG cells. However, EvCAR-KHYG-1 cells did not inhibit tumour progression *in vivo* in our study setting. Further studies are necessary to explore the conditions that maximize the antitumour effect of EvCAR-KHYG-1 without pseudoprogression in orthotopic brain tumours.

Conflicts of Interest

The Grandsoul Research Institute for Immunology and Clinic Grandsoul Nara provided the research location and equipment. The Grandsoul Research Institute for Immunology also provided monetary compensation in the form of salaries to Tsutomu Nakazawa.

Authors' Contributions

Tsutomu Nakazawa and Toshiharu Murakami conceptualized, designed, provided overall guidance for the experiments, performed and analyzed the experiments, as well as wrote, reviewed, and edited the article. Atsushi Natsume, Fumihiko Nishimura, and Takahiro Tsujimura conceptualized, reviewed, and edited the article. Takayuki Morimoto, Ryosuke Matsuda, Mitsutoshi Nakamura, Shuichi Yamada, Ichiro Nakagawa, Young-Soo Park, Yasushi Motoyama, Toshihiko Wakabayashi and Hiroyuki Nakase reviewed and edited the article. All Authors made important contributions to the experiments.

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