PD-1 blockade augments CD8+ T cell dependent antitumor immunity triggered by Ad-SGE-REIC in *Egfr*-mutant lung cancer

Authors and affiliations:

[1]Takamasa Nakasuka MD, [2*]Kadoaki Ohashi, MD, PhD, [1]Kazuya Nishii, MD, [1]Atsuko Hirabae, MD, [1]Sachi Okawa, MD, [3]Nahoko Tomonobu, PhD, [1]Kenji Takada, MD, [1]Chihiro Ando, MD, [1]Hiromi Watanabe, MD, PhD, [2]Go Makimoto, MD, PhD, [1]Kiichiro Ninomiya, MD, PhD, [2]Masanori Fujii, MD, PhD, [4]Toshio Kubo, MD, PhD, [2]Eiki Ichihara, MD, PhD, [5]Katsuyuki Hotta, MD, PhD, MPH, [4]Masahiro Tabata, MD, PhD, [6]Hiromi Kumon, MD, PhD, [1]Yoshinobu Maeda, MD, PhD, and [2]Katsuyuki Kiura, MD, PhD.

1. Department of Hematology, Oncology and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
2. Department of Respiratory Medicine, Okayama University Hospital, Okayama, Japan
3. Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
4. Center for Clinical Oncology, Okayama University Hospital, Okayama, Japan
5. Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan
6. Innovation Center Okayama for Nanobio-targeted Therapy, Okayama University, Okayama, Japan.

Corresponding author: Kadoaki Ohashi

Okayama University Hospital, Department of Respiratory Medicine

2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8558, Japan

PHONE: +81-86-235-7227,

FAX: +81-86-232-8226,

E-mail: kohashi@cc.okayama-u.ac.jp

[1)]Abbreviations: Ad-REIC, genetically engineered adenovirus vector expressing the tumor suppressing gene REIC; Ad-CAG-LacZ, adenoviral vector carrying the LacZ gene with CAG; EGFR, epidermal growth factor receptor; FCM, flow cytometry; ICD, immunogenic cancer cell death; ICIs, immune checkpoint inhibitors; IHC, immunohistochemistry; NSCLCs, non-small cell lung cancers; PBS, phosphate buffered saline; PD-1, anti-programmed cell death-1; PD-L1, anti-PD-1 ligand 1; REIC, reduced expression in immortalized cells; TME, tumor microenvironment; TKIs, tyrosine kinase inhibitors.

Abstract

Objectives: No immunotherapeutic protocol has yet been established in never-smoking patients with lung cancer harboring driver oncogenic mutations, such as epidermal growth factor receptor *(EGFR)* mutations. The immunostimulatory effect of Ad-REIC, a genetically engineered adenovirus vector expressing a tumor suppressor gene, reduced expression in immortalized cells *(REIC)*, has been investigated in clinical trials for various solid tumors. However, the immunostimulatory effect of the Ad-REIC in *EGFR*-mutant lung cancer with a non-inflamed tumor microenvironment (TME) has not been explored.

Materials and methods: We used a syngeneic mouse model developed by transplanting *Egfr*-mutant lung cancer cells into single or double flanks of C57BL/6J mice. Ad-SGE-REIC, a 2nd-generation vector with an enhancer sequence, was injected only into the tumors from one flank, and its antitumor effects were assessed. Tumor-infiltrating cells were evaluated using immunohistochemistry or flow cytometry. The synergistic effects of Ad-SGE-REIC and PD-1 blockade were also examined.

Results: Injection of Ad-SGE-REIC into one side of the tumor induced not only a local antitumor effect but also a bystander abscopal effect in the non-injected tumor, located on the other flank. The number of PD-1^{+}CD8^{+} T cells increased in both injected and non-injected tumors. PD-1 blockade augmented the local and abscopal antitumor effects of Ad-SGE-REIC by increasing the number of CD8^{+} T cells in the TME of *Egfr*-mutant tumors. Depletion of CD8^{+} cells reverted the antitumor effect,

suggesting they contribute to antitumor immunity.

Conclusion: Ad-SGE-REIC induced systemic antitumor immunity by modifying the TME status from non-inflamed to inflamed, with infiltration of $CD8^+$ T cells. Additionally, in *Egfr*-mutant lung cancer, this effect was enhanced by PD-1 blockade. These findings pave the way to establish a novel combined immunotherapy strategy with Ad-SGE-REIC and anti-PD-1 antibody for lung cancer with a non-inflamed TME.

Keywords: *EGFR* mutation, non-small cell lung cancer, antitumor immunity, non-inflamed tumor, Ad-SGE-REIC, gene therapy, PD-1

1. Introduction

Immunotherapy with immune checkpoint inhibitors (ICIs), such as anti-programmed cell death-1 (PD-1) or anti-PD-1 ligand 1 (PD-L1), induces sustained cancer remission in a subset of non-small cell lung cancers (NSCLCs) [1]. The incidence of lung adenocarcinoma in patients who never smoke has been increasing in the past 20 years [2]. In these patients, the epidermal growth factor receptor (*EGFR*) is the most frequently mutated driver oncogene [3,4]; however, standard care with *EGFR*-tyrosine kinase inhibitors (TKIs) provides transient tumor remission for only 1–2 years in lung cancer harboring *EGFR* mutations [5–8]. Furthermore, clinical studies have revealed the poor effect of ICIs in lung cancer with *EGFR* mutations in contrast to that related to smoking [9]. Lung cancer with *EGFR* mutations is characterized by low immunogenicity or non-inflamed tumor microenvironment (TME), with only a small number of tumor-infiltrating $CD8^{+}$ T cells, and these characteristics might limit the effect of ICIs [10]. Oncogenic *EGFR* signaling contributes to the immunosuppressed TME status and its inhibition is crucial to induce antitumor immune responses [10,11]. However, clinical trials on lung cancers harboring *EGFR* mutations have not revealed promising results for the strategy based on the dual blockade of EGFR and PD-1/PD-L1 [12]. Therefore, an alternative approach is required to develop effective immunotherapies against *EGFR*-mutant lung cancers.

Currently, few gene therapies have been approved for use as anticancer drugs, including recombinant herpes virus modified to express *GM-CSF/CSF2*, talimogene laherparepvec for

malignant melanoma (in the United States and other countries), oncolytic herpes virus G47Δ for malignant glioma (in Japan), and recombinant adenovirus modified to express tumor suppressor gene p53 (Ad-p53) for head and neck cancer (in China) [13–15]. Pre-clinical studies revealed that ectopic induction of tumor suppressor genes not only exerted direct antitumor effects but also activated systemic antitumor immunity [16,17], suggesting that gene therapy may have the potential to transform non-inflamed TME into inflamed TME. Consequently, the combination of tumor-suppressing gene therapy and PD-1 blockade has been also explored in clinical trials for the treatment of solid tumors (NCT02842125 and NCT03544723). However, the efficacy of gene therapy in *EGFR*-mutant lung cancer with a noninflamed TME, has not been assessed.

Tumor suppressor genes with reduced expression in immortalized cells (*REIC*) are generally downregulated in a variety of cancers, including *EGFR*-mutant lung cancer [18,19]. Previous preclinical studies revealed that in prostate cancer, malignant mesothelioma and lung squamous cell carcinoma cells, ectopic expression of *REIC* leads to endoplasmic reticulum (ER) stress-induced apoptosis and immunogenic cancer cell death (ICD), inducing a systemic antitumor immune response *in vivo* [19–23]. Ad-REIC is a 1st-generation vector which consists of a gene-engineered replication-deficient adenovirus expressing the *REIC* gene. An early phase clinical study assessing its anticancer efficacy in patients with advanced prostate cancer showed its potential to activate systemic antitumor immunity [24]. Subsequently, a 2nd-generation vector with enhancer sequence Ad-super-gene-

expression (SGE)-REIC was created; its levels of expression of *REIC* gene were substantially higher than those of the 1st-generation Ad-REIC [25]. The safety and antitumor effects of Ad-SGE-REIC alone have been evaluated in patients with liver cancer or malignant glioma [26,27], and the combination therapy of Ad-SGE-REIC and anti-PD-1 antibody, nivolumab, has been investigated in patients with malignant mesothelioma (NCT04013334). Previous pre-clinical studies confirmed the direct antitumor effect of Ad-SGE-REIC in *EGFR*-mutant lung cancer cells [19,28]; however, its immunostimulatory effect has not yet been investigated *in vivo*.

Currently, few *in vivo* preclinical models can be used to assess antitumor immunity in lung cancer harboring *EGFR* mutations. Thus, we previously developed a genetically engineered mouse (GEM) that spontaneously develops lung adenocarcinoma owing to *EGFR* mutations in type II pneumocytes [29]. We also developed a syngeneic mouse model by the subcutaneous transplantation of lung tumor cells from GEM into wild-type C57BL/6 mice [30]. Consequently, using the syngeneic *Egfr*-mutant lung cancer mouse model, we sought to investigate the immunostimulatory effect of Ad-SGE-REIC and the effect of the combination of Ad-SGE-REIC and anti-PD-1 antibody.

2. Materials and methods

2.1 Reagents and antibodies

For the *in vivo* experiments, anti-PD-1 antibody (clone 29F.1A12) was purchased from BioXcell

(Lebanon, NH, USA). Anti-CD8α (53-6.7) and rat IgG2a (RTK2758) antibodies were purchased from BioLegend (San Diego, CA, USA). The primary antibody for Western blotting against dkk3 (EPR15611) was purchased from Abcam (Cambridge, UK). GAPDH (14C10) and caspase-3 (#9662) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit IgG horseradish peroxidase-linked whole antibody (donkey; NA934) was purchased from GE Healthcare Biosciences (Piscataway, NJ, USA). The primary antibodies for immunohistochemistry (IHC) against CD8α (EPR21769) were purchased from Abcam. CD11c (D1V9Y), PD-L1 (D5V3B), PD-L2 (D6L5A), and Granzyme B (E5V2L) were purchased from Cell Signaling Technologies. EnVision+ System-labeled polymer-horseradish peroxidase anti-rabbit antibody (DakoCytomation, Glostrup, Denmark) was used as secondary antibody. Flow cytometry (FCM) antibodies against CD8α (53-6.7), CD4 (GK1.5), CD25 (PC61.5), CD3 (17A2), Foxp3 (FJK-16s), and PD-1 (RMP1-30) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Zombie NIR Fixable Viability Kit (#423105) and rat IgG2b (RTK4530) were purchased from BioLegend.

2.2 Adenovirus vectors

For *in vivo* experiments, we used Ad-SGE-REIC, which is an adenoviral vector expressing the tumor suppressor, with *reduced expression in immortalized cells (REIC)/Dickkopf-3 (Dkk-3)*, based on the CMV promoter-driven SGE system [25]. Ad-CAG-LacZ, an adenoviral vector carrying the LacZ gene with CAG (cytomegalovirus immediate early enhancer/beta-actin promoter), was used as the control

vector. Ad-SGE-REIC and Ad-CAG-LacZ were provided by Momotaro-Gene Inc. (Okayama, Japan) under material transfer agreement.

2.3 Syngeneic *Egfr*-mutant lung cancer mouse models

Female C57BL/6J mice aged 6–8 weeks were purchased from Jackson Laboratory Japan, Inc. (formerly Charles River Laboratories Japan, Inc.) (Yokohama, Japan). All mice were provided with sterilized food and water and were housed in a barrier facility maintained at an air-conditioned temperature of 22 ± 2 °C with constant humidity under a 12/12-h light/dark cycle. We previously generated GEM expressing the *delE748-A752* mutant version of mouse *EGFR* driven by the SP-C promoter, which is equivalent to the *delE746-A750* mutation found in lung cancer patients [29]. We also developed a syngeneic mouse model by subcutaneously transplanting lung tumor cells from GEM into wild-type C57BL/6 mice [30]. Subcutaneous *Egfr*-mutant lung cancer tumors were passaged using C57BL/6J mice. The model was created as described previously [11,30]. *Egfr*-mutant lung cancer cells were subcutaneously transplanted into the left flank of C57BL/6J mice. Mice with tumors were randomly assigned to each group when the average volume reached approximately 50-100 mm^3. The mice were treated with phosphate-buffered saline (PBS) (100 μL, injected intratumorally [i.t.]), Ad-SGE-REIC (1.0×10^9 inclusion forming units [ifu], i.t.), Ad-CAG-LacZ (1.0×10^9 ifu, i.t.), the combination of PBS and anti-PD-1 antibody (200 μg/body, injected intraperitoneally [i.p.]), or the combination of Ad-SGE-REIC and anti-PD-1 antibody. *Egfr*-mutant lung cancer cells were

transplanted subcutaneously into the contralateral right flank of mice after the initiation of treatment. For the depletion of $CD8^+$ cells, either anti-mouse CD8α antibody (250 μg/body, i.p.) or IgG2a isotype control (250 μg/body, i.p.) was administered. Tumor volume ($width^2 \times length/2$) was determined periodically. The experimental protocol was approved by the Animal Care and Use Committee of the Okayama University, Okayama, Japan (OKU-2021073). All animal experiments complied with the provisions of ARRIVE guidelines and were carried out in accordance with Guideline for Proper Conduct of Animal Experiments.

2.4 Western blotting analysis

Mouse tumors and lung tissues were collected and immediately frozen in liquid nitrogen. The frozen tissue was pulverized using a cryo-press and incubated for 30 min in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerol phosphate, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate-containing protease-inhibitor tablets (Roche Applied Sciences, Mannheim, Germany)]. The suspension was centrifuged at 4 °C and 15,000 rpm for 30 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto membranes, which were then incubated with the indicated primary and secondary antibodies. Chemiluminescence was detected using the Prime western blotting Detection Reagents (Cytiva, Marlborough, MA, USA). Bands were detected using Amersham ImageQuant 800 (Cytiva).

2.5 Immunohistochemistry analysis (IHC)

IHC was conducted as previously described [11]. The sections were incubated overnight at 4° C with an antibody, as described above, followed by incubation with a secondary antibody for 20 min. Finally, the sections were counterstained with hematoxylin. The specimens were photographed using a BZ-8100 microscope (KEYENCE, Osaka, Japan). The number of cells and percentage of positive areas were measured using the ImageJ software (version 1.53).

2.6 Flow cytometry (FCM) analysis

The tumor tissues were collected from the mice and dissociated into single-cell suspensions using a Tumor Dissociation Kit mouse (catalog no. 130-096-730, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Red blood cells were removed using the red blood cell lysis solution. All cells, including tumor-infiltrating lymphocytes (TILs) and tumor cells, were stained with the indicated fluorescence-labelled antibodies, and subjected to FCM analysis. Cells were washed and incubated with monoclonal antibodies for 30 min at 4 °C in 2 mM EDTA and PBS containing 0.5% (w/v) bovine serum albumin (FACS staining buffer). The monoclonal antibodies described above were used for cell surface marker and intracellular Foxp3 staining. Cells were processed for intracellular staining using the Foxp3 staining buffer set (Thermo Fisher Scientific). The Zombie NIR Fixable Viability Kit was used to assess the live or dead status. The samples were analyzed using a MACS Quant flow cytometer (Miltenyi Biotec) and the data were analyzed using

FlowJo software (version 10.8.1, TreeStar, Ashland, OR, USA).

2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, CA, USA). Two-sided Student's t-test was used for comparison of the means of data between two groups, and one-way ANOVA with post-hoc Tukey's test was used for comparisons among multiple independent groups, unless otherwise specified. Statistical significance was set at P levels < 0.05.

3. Results

3.1 Ad-SGE-REIC exerted local antitumor effects in *Egfr*-mutant lung cancer

First, we examined the expression of the REIC protein in the syngeneic *Egfr*-mutant lung cancer mouse model. Consistent with the findings in clinical studies on lung cancer tissues [19], the expression of the REIC protein was substantially lower in the *Egfr*-mutant lung cancer subcutaneous tumors than in lung tissue from C57BL/6J mice (Suppl. Fig. S1A). Intra-tumoral injection of Ad-SGE-REIC induced a higher expression of the REIC protein than PBS or Ad-CAG-LacZ (Suppl. Fig. S1B). Next, we assessed the local antitumor effect of Ad-SGE-REIC *in vivo*. *Egfr*-mutant lung cancer cells were transplanted subcutaneously into the left flank of C57BL/6J mice; PBS or Ad-SGE-RIEC was injected into the tumors, and tumor growth was subsequently observed (Fig. 1A). The treatment with Ad-SGE-REIC significantly suppressed the tumor growth compared with PBS or Ad-CAG-LacZ (Fig.

1B and Suppl. Fig. S1C-D).

Second, the impact of Ad-SGE-REIC on the TME was assessed using IHC or FCM analysis. $CD8^+$ cells, $CD11c^+$ cells broadly expressed in murine dendritic cells [31], or granzyme B as a marker of T cell activation were evaluated by IHC analysis, revealing that the number of $CD8^+$ cells, or the positive area of CD11c or granzyme B, were significantly increased in tumors treated with Ad-SGE-REIC compared with those treated with PBS (Fig. 1C-D and Suppl. Fig. S2A-B). FCM analysis showed that the number of $CD8^+$ T cells or $PD\text{-}1^+CD8^+$ T cells and the ratio of $PD\text{-}1^+CD8^+$ T cells/$PD\text{-}1^+$Tregs was significantly increased in the tumors treated with Ad-SGE-REIC compared with those treated with PBS (Fig. 1E, Suppl. Fig. S2C), suggesting clonal expansion of tumor reactive $CD8^+$ T cells [32,33]. IHC analysis showed that PD-L1 expression was not increased in tumor or stromal cells of tumors treated with Ad-SGE-REIC compared with those treated with PBS. In contrast, PD-L2 expression was increased in the TME of tumors treated with Ad-SGE-REIC compared to those treated with PBS (Fig. 1C-D). IHC staining of serial sections indicated that in Ad-SGE-REIC-treated tumors, the locations of $PD\text{-}L2^+$ and $CD11c^+$ cells were almost identical; in contrast, the location of $PD\text{-}L2^+$ cells was not close to that of the tumor or $CD8^+$ cells (Suppl. Fig. S3A). Considering the TME altering induced by Ad-SGE-REIC and the previous reports showing that Ad-SGE-REIC induced immune activation following apoptosis in non-*Egfr*-mutant cancer models [19,23,28], we evaluated the expression of cleaved caspase-3, a marker of apoptosis. Consistent with previous studies, the apoptosis marker was

increased in the tumors treated with Ad-SGE-REIC, compared with those treated with PBS (Suppl. Fig. S3B). Taken together, these results suggest that in the *Egfr*-mutant lung cancer model, the apoptosis and the infiltration of TME with PD-1^{+}CD8^{+} T cells and PD-L2^{+}CD11c^{+} dendritic cell-like cells might be involved in the local antitumor effect of Ad-SGE-REIC.

3.2 Local injection of Ad-SGE-REIC induced abscopal antitumor effects in *Egfr*-mutant lung cancer

Next, we investigated whether local injection of Ad-SGE-REIC in the tumor could induce an abscopal effect in *Egfr*-mutant lung cancer. The *Egfr*-mutant lung cancer cells were transplanted subcutaneously into the contralateral right flank of mice (defined as 2nd tumor: non-injected tumor) after initiation of Ad-SGE-REIC treatment of the tumor in the left flank of the mice (defined as 1st tumor: injected tumor). PBS or Ad-SGE-RIEC was injected into the 1st tumor, and growth of the 2nd tumor was observed (Fig. 2A). The growth of 2nd tumor was significantly lower in mice bearing the Ad-SGE-REIC-injected 1st tumor than in those bearing the PBS- or Ad-CAG-LacZ-injected 1st tumor (Fig. 2B and Suppl. Fig. 4A-B). Next, 2nd tumors were collected to assess the TME without direct injection of reagents. FCM analysis revealed that the number of CD8^{+} T cells or PD-1^{+}CD8^{+} T cells, and the ratio of PD-1^{+}CD8^{+} T cells/PD-1^{+}Tregs was significantly higher in the 2nd tumor of mice bearing the Ad-SGE-REIC-injected 1st tumor than in those bearing the PBS-injected 1st tumor (Fig. 2C). IHC analysis also showed that the number of CD8^{+} or CD11c^{+} cells was significantly higher in the 2nd tumor of mice

bearing the Ad-SGE-REIC-injected 1st tumor than in those bearing PBS-injected 1st tumor (Fig. 2D-E). Additionally, PD-L2 but not PD-L1 expression, was increased and the locations of PD-L2$^+$ and CD11c$^+$ cells were almost identical in the 2nd tumor of mice bearing Ad-SGE-REIC-injected 1st tumor compared with those in the 2nd tumor of control groups (Fig. 2D-E). Thus, similar findings were identified in both 1st and 2nd tumors, even the former received direct treatment while the latter did not. Consequently, in the *Egfr*-mutant lung cancer mouse model, local injection of Ad-SGE-REIC induced the infiltration of CD8$^+$ cells and exerted an antitumor effect in distant tumors.

3.3 PD-1 blockade augments CD8$^+$ T cell-dependent responses triggered by Ad-SGE-REIC in *Egfr*-mutant lung cancer

Given that the presence of PD-1$^+$CD8$^+$ T cells or PD-1$^+$CD8$^+$ T cells/PD-1$^+$Tregs ratio predicts the beneficial effect of ICIs in patients with NSCLC [33,34], we investigated the effect of PD-1 blockade in *Egfr*-mutant lung cancer tumors treated with Ad-SGE-REIC. Anti-PD-1 antibody was administered intraperitoneally to mice bearing *Egfr*-mutant lung cancer tumors in which PBS or Ad-SGE-RIEC were injected, and tumor growth was observed (Fig. 3A). Consistent with a previous report [11], anti-PD-1 antibody with/without intra-tumoral injection of PBS showed no antitumor effect in *Egfr*-mutant lung cancer tumors. (Fig. 3B and Suppl. Fig. S5A-B). In contrast, the combination of Ad-SGE-REIC and anti-PD-1 antibody induced a significant local antitumor effect compared to monotherapy with PBS, Ad-SGE-REIC, or the combination of PBS and anti-PD-1 antibody (Fig. 3B). To assess the TME

status, tumors were collected from mice treated with PBS +/- anti-PD-1 antibody or Ad-SGE-REIC+/- anti-PD-1 antibody. The number of $CD8^+$ cells was significantly increased in tumors treated with a combination of Ad-SGE-REIC and anti-PD-1 antibody compared with those treated with PBS injection +/- anti-PD-1 antibody or Ad-SGE-REIC injection alone (Fig. 3C-D). FCM analysis also showed that the number of $CD8^+$ T cells increased significantly in tumors treated with the combination of Ad-SGE-REIC and anti-PD-1 antibody compared to those treated with PBS injection or Ad-SGE-REIC injection alone (Fig. 3E). To investigate the antitumor effect of $CD8^+$ cells in the syngeneic mouse model, we assessed the impact of $CD8^+$ cell depletion in tumors treated with a combination of Ad-SGE-REIC and anti-PD-1 antibody. As expected, the administration of the anti-CD8 antibody diminished the tumor inhibitory effect in mice treated with the combination of Ad-SGE-REIC and anti-PD-1 antibody compared with the isotype control (Fig. 3F). Consequently, these results indicate that the addition of anti-PD-1 antibody to Ad-SGE-REIC augmented a local antitumor effect in *Egfr*-mutant lung cancer by enhancing $CD8^+$ cell-related immunity.

3.4 PD-1 blockade augments the abscopal effect triggered by Ad-SGE-REIC in *Egfr*-mutant lung cancer

Lastly, using the syngeneic mouse model, we investigated whether PD-1 blockade had an inhibitory effect on the growth of 2nd tumor in mice bearing Ad-SGE-REIC-injected 1st tumor. PBS or Ad-SGE-RIEC were injected into the 1st tumor, and an anti-PD-1 antibody was administered intraperitoneally

to C57BL/6J mice (Fig. 4A). In mice bearing the PBS-injected 1st tumor, administration of the anti-PD-1 antibody did not show a superior inhibitory effect on the growth of the 2nd tumor when compared with the lack of its administration; however, in mice bearing the Ad-SGE-REIC-injected 1st tumor, the anti-PD1 antibody significantly suppressed the growth of the 2nd tumor compared with the lack of its administration (Fig. 4B). No body weight loss was observed in mice treated with combination therapy (Suppl. Fig. S6). IHC analysis revealed that in the 2nd tumor collected from the mice bearing the Ad-SGE-REIC-injected 1st tumor, the number of CD8^{+} cells significantly increased after treatment with anti-PD-1 antibody compared with the lack of its administration (Fig. 4C-D). To determine whether CD8^{+} cells contributed to the abscopal effect against the 2nd tumor, we examined the impact of CD8^{+} cell depletion on the growth of the 2nd tumor in mice bearing Ad-SGE-REIC-injected 1st tumor treated with anti-PD-1 antibody. As expected, the administration of the anti-CD8 antibody diminished the antitumor effect against the 2nd tumor in these mice compared with the isotype control (Fig. 4E). Consequently, these results indicate that in *Egfr*-mutant lung cancer model, PD-1 blockade augmented the abscopal effect induced by Ad-SGE-REIC through enhancement of CD8^{+} cell-related antitumor immunity.

4. Discussion

No immunotherapeutic protocol has been established yet for the treatment of lung cancer harboring

EGFR mutations, which is the subset of lung adenocarcinoma with the highest prevalence in patients that never smoked. To the best of our knowledge, this is the first report to demonstrate that gene therapy activates systemic antitumor immunity in *Egfr*-mutant lung cancer *in vivo*. Additionally, we found that PD-1 blockade augmented not only local tumor inhibition, but also the abscopal effect induced by Ad-SGE-REIC in non-inflamed tumors.

The cancer-immunity cycle is a fundamental process in activating cancer immunity [35]. Considering the characteristics of *EGFR*-mutant lung cancer - low tumor mutation burden and non-inflamed TME, the step of "recognition of cancer cells by T cells" might not be satisfactory in the patient with this subset of lung cancer. Consistently with this hypothesis, a previous study revealed that resident memory $CD8^+$ T-like cells were significantly lower in *EGFR*- mutant human lung cancer than in *EGFR* wild-type lung cancer [36]. Therefore, gene therapy that induces ICD and prompts immune cells to recognize cancer antigens may be a reasonable strategy for this subset of lung cancer. A clinical study showed that Ad-REIC induced long-term sustained systemic antitumor effects for over 2 years in patients with chemotherapy-refractory, metastatic castration-resistant prostate cancer [24]. Moreover, a multiple S-cationized antigen bead array assay confirmed that serum antibodies against cancer testis antigens, such as MAGE2, were increased following treatment with Ad-REIC [37], suggesting that gene therapy with Ad-REIC could prompt the cancer-immunity cycle in clinical practice as well as in preclinical settings. In addition, Ad-SGE-REIC showed the potential inducer of ICD in several types

of cancer models, including lung squamous cancer or malignant mesothelioma *in vivo* [19,23]. In the present study, the number of PD-1^{+}CD8^{+} T cells was increased in tumors treated with Ad-SGE-REIC; thus, in *Egfr*-mutant lung cancer, gene therapy modified the TME status from non-inflamed to inflamed. High expression of PD-1 on CD8^{+} T cells indicates the clonal expansion of tumor-reactive CD8^{+} T cells [32,33] and strongly predicts a survival benefit in patients with NSCLC treated with PD-1 inhibitors [33,34]. Consistently, addition of anti-PD-1 antibody to Ad-SGE-REIC gene therapy augmented antitumor immunity, and the depletion of CD8^{+} cells reversed the antitumor effect in our mouse model, suggesting that PD-1^{+}CD8^{+} T cells contributed to the antitumor effect and were not in a terminally differentiated exhausted state [38]. Taken together, our study suggests that Ad-SGE-REIC gene therapy could promote the "cancer-immunity cycle" in *Egfr*-mutant lung cancer tumors with non-inflamed TME.

Our study had several limitations. First, we did not verify the antitumor effect of Ad-SGE-REIC in other types of *EGFR*-mutant cancers, although the type of mutations or co-occurring mutations, such as *p53*, could affect the efficiency of immunotherapy. Second, we assessed only a subset of immune cells, CD8^{+} T cells but did not comprehensively evaluate the role of other immune cells or stromal cells in the TME, although Ad-SGE-REIC could affect the complex interactions of the immune network. In addition, we did not completely characterize the phenotype of these cells in the TME. Single-cell analysis may provide further details or insight into the complex interactions of immune

cells, leading to CD8$^+$ T-cell activation, and clarify the characteristics of immune cells (e.g. exhausted state of CD8$^+$ T cells or phenotype of CD11c$^+$ cells) in the TME. Furthermore, the local and abscopal effect induced by the combination of Ad-SGE-REIC and anti-PD-1 antibody did not result in radical cure, suggesting that more potent combinations of drugs or treatment schedules may exist. However, our lung cancer model reproduced the clinical features of *EGFR*-mutant lung cancer compared to existing models (e.g., minimum PD-L1 expression, poor effect of ICI monotherapy, and development of acquired resistance to EGFR-TKI owing to *Egfr* mutations similar to human lung cancers [11,30]); thus, this study has a potential for clinical applicability in patients with *EGFR* mutant lung adenocarcinoma.

In conclusion, in *Egfr*-mutant lung cancer mouse model, Ad-SGE-REIC modified the TME status from non-inflamed to inflamed, with infiltration of CD8$^+$ T cells, and induced direct and indirect antitumor effects. PD-1 blockade augmented CD8$^+$ T cell-dependent antitumor immunity triggered by Ad-SGE-REIC; conversely, monotherapy with anti-PD-1 antibody had little effect on *Egfr*-mutant tumor. These findings pave the way to establish a novel immunotherapy strategy utilizing the combination of gene therapy and ICIs for the treatment of *EGFR*-mutant lung cancer with non-inflamed TME. Although the administration of Ad-SGE-REIC into the lung is highly challenging, clinical trials for other solid cancers have shown the feasibility of administering the Ad-REIC formula into tumors using ultrasound or CT guidance [26,39] (NCT04013334). We plan to conduct a clinical

trial assessing the effect of the combination of Ad-SGE-REIC and anti-PD-1 antibody, in *EGFR*-mutant lung cancer.

Acknowledgements

We are grateful to Ms. Hiromi Nakashima, Ms. Kyoko Maeda from Department of Hematology, Oncology and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. We also thank our laboratory colleagues for the useful discussions.

Funding

This work was supported by a specific grant from JSPS Grant-in-Aid for Scientific Research [Scientific Research (C): KAKEN 19K08625 to K.O. and K.K.], JSPS Grants-in-Aid for Scientific Research [Scientific Research (B): KAKEN 19H03667 to K.K. and K.O.] and JSPS Grants-in-Aid for Scientific Research [Scientific Research (B): KAKEN 22H03078 to K.O. and K.K.]. This work was also supported by Ryobi Teien Memory Foundation (K.O.), Okayama Medical Foundation (K.O.), Kobayashi Foundation for Cancer Research (K.O.), Takeda Science Foundation (K.O.), Project Mirai Cancer Research Grants (K.O.), Sanyo Broadcasting Foundation (K.O.), and Momotaro-Gene Inc (K.K).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

T. Nakasuka: Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. K. Ohashi: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, visualization, methodology, writing–original draft, project administration, writing–review and editing. K. Nishii: Investigation, writing–review and editing. A. Hirabae: Investigation, writing–review and editing. S. Okawa: Investigation, writing–review and editing. N. Tomonobu: Validation, methodology, writing–review and editing. K. Takada: Resources, validation, writing–review and editing. C. Ando: Resources, writing–review and editing. H. Watanabe: Resources, writing–review and editing. G. Makimoto: Resources, writing–review and editing. K. Ninomiya: Resources, writing–review and editing. M. Fujii: Resources, writing–review and editing. T. Kubo: Resources, writing–review and editing. E. Ichihara: Resources, writing–review and editing. K. Hotta: Resources, writing–review and editing. M. Tabata: Resources, writing–review and editing. H. Kumon: Conceptualization, resources, supervision, writing–review and editing. Y. Maeda: Resources, supervision, project administration, writing–review and editing. K. Kiura: Conceptualization, Resources, supervision,

funding acquisition, project administration, writing–review and editing.

References

[1] M. Reck, J. Remon, M.D. Hellmann, First-Line Immunotherapy for Non–Small-Cell Lung Cancer, Journal of Clinical Oncology. (2022). https://doi.org/10.1200/JCO.21.01497.

[2] L. Pelosof, C. Ahn, A. Gao, L. Horn, A. Madrigales, J. Cox, D. McGavic, J.D. Minna, A.F. Gazdar, J. Schiller, Proportion of Never-Smoker Non–Small Cell Lung Cancer Patients at Three Diverse Institutions, JNCI J Natl Cancer Inst. 109 (2017) djw295. https://doi.org/10.1093/jnci/djw295.

[3] M. Saito, K. Shiraishi, H. Kunitoh, S. Takenoshita, J. Yokota, T. Kohno, Gene aberrations for precision medicine against lung adenocarcinoma, Cancer Sci. 107 (2016) 713–720. https://doi.org/10.1111/cas.12941.

[4] K. Nishii, K. Ohashi, T. Tamura, K. Ninomiya, T. Matsubara, S. Senoo, H. Kano, H. Watanabe, N. Oda, G. Makimoto, H. Higo, Y. Kato, T. Ninomiya, T. Kubo, H. Yamamoto, S. Tomida, K. Hotta, M. Tabata, S. Toyooka, Y. Maeda, K. Kiura, Detection of epidermal growth factor receptor mutations in exhaled breath condensate using droplet digital polymerase chain reaction, Oncol Lett. 20 (2020) 393. https://doi.org/10.3892/ol.2020.12256.

[5] K. Ohashi, Y.E. Maruvka, F. Michor, W. Pao, Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease, J Clin Oncol. 31 (2013) 1070–1080. https://doi.org/10.1200/JCO.2012.43.3912.

[6] K. Ninomiya, K. Ohashi, G. Makimoto, S. Tomida, H. Higo, H. Kayatani, T. Ninomiya, T. Kubo, E. Ichihara, K. Hotta, M. Tabata, Y. Maeda, K. Kiura, MET or NRAS amplification is an acquired resistance mechanism to the third-generation EGFR inhibitor naquotinib, Sci Rep. 8 (2018) 1955. https://doi.org/10.1038/s41598-018-20326-z.

[7] K. Ohashi, K. Ninomiya, H. Yoshioka, A. Bessho, T. Shibayama, K. Aoe, N. Ishikawa, T. Kozuki, H. Kawai, S. Kuyama, S. Miyoshi, K. Fujitaka, H. Obata, Y. Tsubata, Y. Awaya, M. Inoue, K. Inoue, N. Horita, H. Yanai, K. Hotta, K. Kiura, Impact of HER2 expression on EGFR-TKI treatment outcomes in lung tumors harboring EGFR mutations: A HER2-CS study subset analysis, Lung Cancer. 150 (2020) 83–89. https://doi.org/10.1016/j.lungcan.2020.09.024.

[8] H. Kayatani, K. Ohashi, K. Ninomiya, G. Makimoto, K. Nishii, H. Higo, H. Watanabe, H. Kano, Y. Kato, T. Ninomiya, T. Kubo, K. Rai, E. Ichihara, K. Hotta, M. Tabata, Y. Maeda, K. Kiura, Beneficial effect of erlotinib and trastuzumab emtansine combination in lung tumors harboring EGFR mutations, Biochem Biophys Res Commun. 532 (2020) 341–346. https://doi.org/10.1016/j.bbrc.2020.07.055.

[9] C.K. Lee, J. Man, S. Lord, W. Cooper, M. Links, V. Gebski, R.S. Herbst, R.J. Gralla, T. Mok, J.C.-H. Yang, Clinical and Molecular Characteristics Associated With Survival Among Patients Treated With Checkpoint Inhibitors for Advanced Non–Small Cell Lung Carcinoma: A

Systematic Review and Meta-analysis, JAMA Oncology. 4 (2018) 210–216. https://doi.org/10.1001/jamaoncol.2017.4427.

[10] E. Sugiyama, Y. Togashi, Y. Takeuchi, S. Shinya, Y. Tada, K. Kataoka, K. Tane, E. Sato, G. Ishii, K. Goto, Y. Shintani, M. Okumura, M. Tsuboi, H. Nishikawa, Blockade of EGFR improves responsiveness to PD-1 blockade in EGFR-mutated non-small cell lung cancer, Sci Immunol. 5 (2020) eaav3937. https://doi.org/10.1126/sciimmunol.aav3937.

[11] K. Nishii, K. Ohashi, S. Tomida, T. Nakasuka, A. Hirabae, S. Okawa, J. Nishimura, H. Higo, H. Watanabe, H. Kano, C. Ando, G. Makimoto, K. Ninomiya, Y. Kato, T. Kubo, E. Ichihara, K. Hotta, M. Tabata, S. Toyooka, H. Udono, Y. Maeda, K. Kiura, CD8+ T-cell responses are boosted by dual PD-1/VEGFR2 blockade after EGFR inhibition in Egfr-mutant lung cancer, Cancer Immunol Res. (2022) cir.21.0751. https://doi.org/10.1158/2326-6066.CIR-21-0751.

[12] M. Qiao, T. Jiang, X. Liu, S. Mao, F. Zhou, X. Li, C. Zhao, X. Chen, C. Su, S. Ren, C. Zhou, Immune Checkpoint Inhibitors in EGFR-Mutated NSCLC: Dusk or Dawn?, Journal of Thoracic Oncology. 16 (2021) 1267–1288. https://doi.org/10.1016/j.jtho.2021.04.003.

[13] R.H.I. Andtbacka, H.L. Kaufman, F. Collichio, T. Amatruda, N. Senzer, J. Chesney, K.A. Delman, L.E. Spitler, I. Puzanov, S.S. Agarwala, M. Milhem, L. Cranmer, B. Curti, K. Lewis, M. Ross, T. Guthrie, G.P. Linette, G.A. Daniels, K. Harrington, M.R. Middleton, W.H. Miller, J.S. Zager, Y. Ye, B. Yao, A. Li, S. Doleman, A. VanderWalde, J. Gansert, R.S. Coffin, Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma, J Clin Oncol. 33 (2015) 2780–2788. https://doi.org/10.1200/JCO.2014.58.3377.

[14] T. Todo, H. Ito, Y. Ino, H. Ohtsu, Y. Ota, J. Shibahara, M. Tanaka, Intratumoral oncolytic herpes virus G47Δ for residual or recurrent glioblastoma: a phase 2 trial, Nat Med. 28 (2022) 1630–1639. https://doi.org/10.1038/s41591-022-01897-x.

[15] W.-W. Zhang, L. Li, D. Li, J. Liu, X. Li, W. Li, X. Xu, M.J. Zhang, L.A. Chandler, H. Lin, A. Hu, W. Xu, D.M.-K. Lam, The First Approved Gene Therapy Product for Cancer Ad-p53 (Gendicine): 12 Years in the Clinic, Hum Gene Ther. 29 (2018) 160–179. https://doi.org/10.1089/hum.2017.218.

[16] S. Chada, D. Wiederhold, K.B. Menander, B. Sellman, M. Talbott, J.J. Nemunaitis, H.M. Ahn, B.-K. Jung, C.-O. Yun, R.E. Sobol, Tumor suppressor immune gene therapy to reverse immunotherapy resistance, Cancer Gene Ther. 29 (2022) 825–834. https://doi.org/10.1038/s41417-021-00369-7.

[17] Y. Xiao, J. Chen, H. Zhou, X. Zeng, Z. Ruan, Z. Pu, X. Jiang, A. Matsui, L. Zhu, Z. Amoozgar, D.S. Chen, X. Han, D.G. Duda, J. Shi, Combining p53 mRNA nanotherapy with immune checkpoint blockade reprograms the immune microenvironment for effective cancer therapy, Nat Commun. 13 (2022) 758. https://doi.org/10.1038/s41467-022-28279-8.

[18] T. Tsuji, I. Nozaki, M. Miyazaki, M. Sakaguchi, H. Pu, Y. Hamazaki, O. Iijima, M. Namba,

Antiproliferative Activity of REIC/Dkk-3 and Its Significant Down-Regulation in Non-Small-Cell Lung Carcinomas, Biochemical and Biophysical Research Communications. 289 (2001) 257–263. https://doi.org/10.1006/bbrc.2001.5972.

[19] K. Suzawa, K. Shien, H. Peng, M. Sakaguchi, M. Watanabe, S. Hashida, Y. Maki, H. Yamamoto, S. Tomida, J. Soh, H. Asano, K. Tsukuda, Y. Nasu, H. Kumon, S. Miyoshi, S. Toyooka, Distant Bystander Effect of REIC/DKK3 Gene Therapy Through Immune System Stimulation in Thoracic Malignancies, Anticancer Res. 37 (2017) 301–307. https://doi.org/10.21873/anticanres.11321.

[20] F. Abarzua, Y. Kashiwakura, M. Takaoka, M. Watanabe, K. Ochiai, M. Sakaguchi, T. Iwawaki, R. Tanimoto, Y. Nasu, N. Huh, H. Kumon, An N-terminal 78 amino acid truncation of REIC/Dkk-3 effectively induces apoptosis, Biochemical and Biophysical Research Communications. 375 (2008) 614–618. https://doi.org/10.1016/j.bbrc.2008.08.079.

[21] F. Abarzua, M. Sakaguchi, M. Takaishi, Y. Nasu, K. Kurose, S. Ebara, M. Miyazaki, M. Namba, H. Kumon, N. Huh, Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase, Cancer Res. 65 (2005) 9617–9622. https://doi.org/10.1158/0008-5472.CAN-05-0829.

[22] Y. Kashiwakura, K. Ochiai, M. Watanabe, F. Abarzua, M. Sakaguchi, M. Takaoka, R. Tanimoto, Y. Nasu, N.-H. Huh, H. Kumon, Down-regulation of inhibition of differentiation-1 via activation of activating transcription factor 3 and Smad regulates REIC/Dickkopf-3-induced apoptosis, Cancer Res. 68 (2008) 8333–8341. https://doi.org/10.1158/0008-5472.CAN-08-0080.

[23] K. Araki, N. Yamamuro, N. Tomonobu, H. Kumon, REIC/Dkk-3 Gene Therapy Induces Immunogenic Cell Death in a Mouse Model of Malignant Mesothelioma, Anticancer Res. 41 (2021) 4837–4855. https://doi.org/10.21873/anticanres.15298.

[24] H. Kumon, K. Sasaki, Y. Ariyoshi, T. Sadahira, S. Ebara, T. Hiraki, S. Kanazawa, H. Yanai, M. Watanabe, Y. Nasu, Ad-REIC Gene Therapy: Promising Results in a Patient with Metastatic CRPC Following Chemotherapy, Clin Med Insights Oncol. 9 (2015) 31–38. https://doi.org/10.4137/CMO.S23252.

[25] M. Watanabe, M. Sakaguchi, R. Kinoshita, H. Kaku, Y. Ariyoshi, H. Ueki, R. Tanimoto, S. Ebara, K. Ochiai, J. Futami, S.-A. Li, P. Huang, Y. Nasu, N. Huh, H. Kumon, A novel gene expression system strongly enhances the anticancer effects of a REIC/Dkk-3-encoding adenoviral vector, Oncol Rep. 31 (2014) 1089–1095. https://doi.org/10.3892/or.2013.2958.

[26] A. Oyama, H. Shiraha, D. Uchida, M. Iwamuro, H. Kato, A. Takaki, F. Ikeda, H. Onishi, T. Yasunaka, Y. Takeuchi, N. Wada, Y. Iwasaki, M. Sakata, H. Okada, H. Kumon, A Phase I/Ib trial of Ad-REIC in liver cancer: study protocol, Future Oncol. 15 (2019) 3547–3554. https://doi.org/10.2217/fon-2019-0115.

[27] K. Kurozumi, K. Fujii, Y. Shimazu, Y. Tomita, T. Sasaki, T. Yasuhara, T. Hishikawa, M. Kameda,

H. Kumon, I. Date, Study protocol of a Phase I/IIa clinical trial of Ad-SGE-REIC for treatment of recurrent malignant glioma, Future Oncol. 16 (2020) 151–159. https://doi.org/10.2217/fon-2019-0743.

[28] K. Shien, N. Tanaka, M. Watanabe, J. Soh, M. Sakaguchi, K. Matsuo, H. Yamamoto, M. Furukawa, H. Asano, K. Tsukuda, Y. Nasu, N.-H. Huh, S. Miyoshi, H. Kumon, S. Toyooka, Anti-Cancer Effects of REIC/Dkk-3-encoding Adenoviral Vector for the Treatment of Non-small Cell Lung Cancer, PLOS ONE. 9 (2014) e87900. https://doi.org/10.1371/journal.pone.0087900.

[29] K. Ohashi, K. Rai, Y. Fujiwara, M. Osawa, S. Hirano, K. Takata, E. Kondo, T. Yoshino, M. Takata, M. Tanimoto, K. Kiura, Induction of lung adenocarcinoma in transgenic mice expressing activated EGFR driven by the SP-C promoter, Cancer Sci. 99 (2008) 1747–1753. https://doi.org/10.1111/j.1349-7006.2008.00875.x.

[30] H. Higo, K. Ohashi, G. Makimoto, K. Nishii, K. Kudo, H. Kayatani, H. Watanabe, H. Kano, K. Ninomiya, K. Hotta, Y. Maeda, K. Kiura, EGFR-TKI acquired resistance in lung cancers harboring EGFR mutations in immunocompetent C57BL/6J mice, Lung Cancer. 136 (2019) 86–93. https://doi.org/10.1016/j.lungcan.2019.08.019.

[31] M. Guilliams, F. Ginhoux, C. Jakubzick, S.H. Naik, N. Onai, B.U. Schraml, E. Segura, R. Tussiwand, S. Yona, Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny, Nat Rev Immunol. 14 (2014) 571–578. https://doi.org/10.1038/nri3712.

[32] A. Gros, P.F. Robbins, X. Yao, Y.F. Li, S. Turcotte, E. Tran, J.R. Wunderlich, A. Mixon, S. Farid, M.E. Dudley, K. Hanada, J.R. Almeida, S. Darko, D.C. Douek, J.C. Yang, S.A. Rosenberg, PD-1 identifies the patient-specific $CD8^+$ tumor-reactive repertoire infiltrating human tumors, J Clin Invest. 124 (2014) 2246–2259. https://doi.org/10.1172/JCI73639.

[33] S. Kumagai, Y. Togashi, T. Kamada, E. Sugiyama, H. Nishinakamura, Y. Takeuchi, K. Vitaly, K. Itahashi, Y. Maeda, S. Matsui, T. Shibahara, Y. Yamashita, T. Irie, A. Tsuge, S. Fukuoka, A. Kawazoe, H. Udagawa, K. Kirita, K. Aokage, G. Ishii, T. Kuwata, K. Nakama, M. Kawazu, T. Ueno, N. Yamazaki, K. Goto, M. Tsuboi, H. Mano, T. Doi, K. Shitara, H. Nishikawa, The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies, Nat Immunol. 21 (2020) 1346–1358. https://doi.org/10.1038/s41590-020-0769-3.

[34] D.S. Thommen, V.H. Koelzer, P. Herzig, A. Roller, M. Trefny, S. Dimeloe, A. Kiialainen, J. Hanhart, C. Schill, C. Hess, S. Savic Prince, M. Wiese, D. Lardinois, P.-C. Ho, C. Klein, V. Karanikas, K.D. Mertz, T.N. Schumacher, A. Zippelius, A transcriptionally and functionally distinct PD-1+ CD8+ T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade, Nat Med. 24 (2018) 994–1004. https://doi.org/10.1038/s41591-018-0057-z.

[35] D.S. Chen, I. Mellman, Oncology Meets Immunology: The Cancer-Immunity Cycle, Immunity. 39 (2013) 1–10. https://doi.org/10.1016/j.immuni.2013.07.012.

[36] J.-W. Cho, S. Park, G. Kim, H. Han, H.S. Shim, S. Shin, Y.-S. Bae, S.Y. Park, S.-J. Ha, I. Lee, H.R. Kim, Dysregulation of TFH-B-TRM lymphocyte cooperation is associated with unfavorable anti-PD-1 responses in EGFR-mutant lung cancer, Nat Commun. 12 (2021) 6068. https://doi.org/10.1038/s41467-021-26362-0.

[37] A. Miyamoto, T. Honjo, M. Masui, R. Kinoshita, H. Kumon, K. Kakimi, J. Futami, Engineering Cancer/Testis Antigens With Reversible S-Cationization to Evaluate Antigen Spreading, Front Oncol. 12 (2022) 869393. https://doi.org/10.3389/fonc.2022.869393.

[38] C.U. Blank, W.N. Haining, W. Held, P.G. Hogan, A. Kallies, E. Lugli, R.C. Lynn, M. Philip, A. Rao, N.P. Restifo, A. Schietinger, T.N. Schumacher, P.L. Schwartzberg, A.H. Sharpe, D.E. Speiser, E.J. Wherry, B.A. Youngblood, D. Zehn, Defining "T cell exhaustion," Nat Rev Immunol. 19 (2019) 665–674. https://doi.org/10.1038/s41577-019-0221-9.

[39] H. Kumon, K. Sasaki, Y. Ariyoshi, T. Sadahira, M. Araki, S. Ebara, H. Yanai, M. Watanabe, Y. Nasu, Feasibility of Neoadjuvant Ad-REIC Gene Therapy in Patients with High-Risk Localized Prostate Cancer Undergoing Radical Prostatectomy, Clin Transl Sci. 8 (2015) 837–840. https://doi.org/10.1111/cts.12362.

Figure legends

Fig. 1: Local antitumor effect of Ad-SGE-REIC in *Egfr*-mutant lung cancer

A. Schematic image of the experiment schedule. *Egfr*-mutant lung cancer cells were transplanted subcutaneously to the left flank of C57BL/6J mice, and PBS (100μL) or Ad-SGE-RIEC (1.0×10^9 ifu/100 μL) was injected intratumorally on day 1 (when tumors reached around 50-100 mm^3) and days 4 and 7, and subsequently the tumor growth was observed. i.t., intratumoral injection; FCM, flow cytometry; IHC, immunohistochemistry.

B. The growth curve of tumors treated with PBS (n=4) or Ad-SGE-REIC (n=5). Data are shown as the

mean ± standard error of the mean (SEM). ***p<0.001, Student's t-test.

C. Representative images of CD8, CD11c, PD-L2 and PD-L1 immunohistochemistry staining in the tumors treated with PBS or Ad-SGE-REIC. Tumors were collected at day 11. Magnification, x100. Scale bar, 100 μm.

D. The number of CD8 positive cells and the area percentage of CD11c, PD-L2, or PD-L1 positive cells in the field of view were quantified using ImageJ software. Data are shown as the mean ± SEM (n=6/group). ns, not significant. *p<0.05, **p<0.01, Student's t-test.

E. Flow cytometry analysis of $CD8^{+}$T cells ($CD3^{+}CD4^{-}CD8^{+}$), $PD\text{-}1^{+}CD8^{+}$Tcells ($CD3^{+}$ $CD4^{-}CD8^{+}$ $PD\text{-}1^{+}$) and the ratio of $PD\text{-}1^{+}CD8^{+}$T cells / $PD\text{-}1^{+}$Tregs ($CD3^{+}CD4^{+}CD8^{-}CD25^{+}Foxp3^{+}PD\text{-}1^{+}$) within the dissociated tumors treated with PBS (n=4) or Ad-SGE-REIC (n=5). Tumors were collected at day 11. Data are shown as the mean ± SEM. **p<0.01, ***p<0.001, Student's t-test.

Fig. 2: Abscopal antitumor immunity induced by Ad-SGE-REIC in *Egfr*-mutant lung cancer

A. Schematic image of the experiment schedule. *Egfr*-mutant lung cancer cells were transplanted subcutaneously to the left flank of C57BL/6J mice, and PBS (100 μL) or Ad-SGE-RIEC (1.0×10^{9} ifu/100 μL) was injected in the left-side tumor (defined as 1st tumor) on day 1 (when the tumors reached around 50-100 mm^3) and days 4 and 7. Then, *Egfr*-mutant lung cancer cells were transplanted subcutaneously to the contralateral right flank of the mice on day 4 which is 3 days after the initiation of PBS or Ad-SGE-REIC treatment, and subsequently the growth of the right-side tumor (defined as

2nd tumor) was observed. Neither PBS nor Ad-SGE-REIC were injected into the 2nd tumor. i.t., intratumoral injection; FCM, flow cytometry; IHC, immunohistochemistry.

B. The growth curve of 2nd tumor in the mice bearing 1st tumor treated with PBS (n=7) or Ad-SGE-REIC (n=7). Data are shown as the mean ± SEM. **p*<0.05, Student's t-test.

C. Flow cytometry analysis of $CD8^{+}$T cells, $PD\text{-}1^{+}CD8^{+}$Tcells and the ratio of $PD\text{-}1^{+}CD8^{+}$T cells/$PD\text{-}1^{+}$Tregs using 2nd tumor from the mice bearing 1st tumor treated with PBS (n=5) or Ad-SGE-REIC (n=6). 2nd tumors were collected at day 11. Data are shown as the mean ± SEM. ***p<0.001, ****p<0.0001, Student's t-test.

D. Representative images of CD8, CD11c, PD-L2 and PD-L1 immunohistochemistry staining in the 2nd tumor in the mice bearing 1st tumor treated with PBS or Ad-SGE-REIC. Second tumors were collected at day 11. Magnification, x100. Scale bar, 100 μm.

E. The number of CD8 positive cells and the area percentage of CD11c, PD-L2, or PD-L1 positive cells in the field of view were quantified using ImageJ software. Data are shown as the mean ± SEM (n=6 / group). ns, not significant. ****p<0.0001, Student's t-test.

Fig. 3: Anti-PD-1 antibody boosted the local antitumor immunity induced by Ad-SGE-REIC in *Egfr*-mutant lung cancer

A. Schematic image of the experiment schedule. *Egfr*-mutant lung cancer cells were transplanted subcutaneously to the left flank of C57BL/6J mice, and PBS (100 μL) or Ad-SGE-RIEC (1.0×10^{9}

ifu/100 μL) was injected intratumorally on day 1 (when tumors reached around 50-100 mm^3) and days 4 and 7. Additionally, anti-PD-1 antibody (200 μg/body) was injected intraperitoneally on day 1, and subsequently, the tumor growth was observed. i.t., intratumoral injection; i.p., intraperitoneal injection; FCM, flow cytometry; IHC, immunohistochemistry.

B. The growth curve of tumors treated with PBS (n=22), combination of PBS and anti-PD-1 antibody (n=21), Ad-SGE-REIC (n=23) or combination of Ad-SGE-REIC and anti-PD-1 antibody (n=24). The results of three independent identical experiments were pooled. Data are shown as the mean ± SEM. ** $p<0.01$, one-way ANOVA with post-hoc Tukey's test.

C. Representative images of CD8 immunohistochemistry staining in the tumors treated with PBS, combination of PBS and anti-PD-1 antibody, Ad-SGE-REIC or combination of Ad-SGE-REIC and anti-PD-1 antibody. Tumors were collected at day 11. Magnification, x100. Scale bar, 100μm.

D. The number of CD8 positive cells in the field of view is quantified using ImageJ software. Data are shown as the mean ± SEM (n=6 / group). ns, not significant. * $p<0.05$, **** $p<0.0001$, one-way ANOVA with post-hoc Tukey's test.

E. Flow cytometry analysis of $CD8^{+}$T cells within the dissociated tumors treated with PBS (n=5), Ad-SGE-REIC (n=5) or combination of Ad-SGE-REIC and anti-PD-1 antibody (n=5). Tumors were collected at day 11. Data are shown as the mean ± SEM. * $p<0.05$, **** $p<0.0001$, one-way ANOVA with post-hoc Tukey's test.

F. The growth curve of tumors in mice treated with combination of Ad-SGE-REIC and anti-PD-1 antibody. The experiment schedule for the transplantation of cancer cells and treatments with Ad-SGE-REIC and anti-PD-1 antibody are the same as in Figure 3A. Anti-CD8 antibody (250 µg/body, n=8) or isotype control (250 µg/body, n=8) was intraperitoneally injected on days -3,1,4,7 and 10. In day -3, cancer cells were subcutaneously transplanted into the left flank. Data are shown as the mean ± SEM. *$p<0.05$, Student's t-test.

Fig.4: Anti-PD-1 antibody augmented the abscopal antitumor immunity induced by Ad-SGE-REIC in *Egfr*-mutant lung cancer

A. Schematic image of the experiment schedule. *Egfr*-mutant lung cancer cells were transplanted subcutaneously to the left flank of C57BL/6J mice, and PBS (100 µL) or Ad-SGE-RIEC (1.0×10^9 ifu/100 µL) was injected in the left-side tumor (defined as 1st tumor) on day 1 (when 1st tumor reached around 50-100 mm^3) and days 4 and 7. Additionally, anti-PD-1 antibody (200 µg/body) was injected intraperitoneally on day 1. Then, *Egfr*-mutant lung cancer cells were transplanted subcutaneously to the contralateral right flank of the mice on day 2 (1 day after the initiation of PBS or Ad-SGE-REIC treatment), and subsequently the growth of the right-side tumor (defined as 2nd tumor) was observed. Neither PBS nor Ad-SGE-REIC were injected into the 2nd tumor. i.t., intratumoral injection; i.p., intraperitoneal injection; IHC, immunohistochemistry.

B. The growth curve of 2nd tumor in the mice bearing 1st tumor treated with PBS (n=22), combination of PBS and anti-PD-1 antibody (n=21), Ad-SGE-REIC (n=23) or combination of Ad-SGE-REIC and anti-PD-1 antibody (n=24). PBS or Ad-SGE-REIC was injected to 1st tumor. The results of three independent identical experiments were pooled. Data are shown as the mean ± SEM. ***p<0.001, ****p<0.0001, one-way ANOVA with post-hoc Tukey's test.

C. Representative images of CD8 immunohistochemistry staining in the 2nd tumor in the mice bearing 1st tumor treated with PBS, combination of PBS and anti-PD-1 antibody, Ad-SGE-REIC or combination of Ad-SGE-REIC and anti-PD-1 antibody. The 2nd tumors were collected at day 11. Magnification, x100. Scale bar, 100 μm.

D. The number of CD8 positive cells in the field of view is quantified using ImageJ software. Data are shown as the mean ± SEM (n=6/group). ns, not significant. **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with post-hoc Tukey's test.

E. The growth curve of 2nd tumor in mice bearing 1st tumor treated with combination of Ad-SGE-REIC and anti-PD-1 antibody. The experiment schedule for transplantations of cancer cells and treatments with Ad-SGE-REIC and anti-PD-1 are the same as in Figure 4A. Ad-SGE-REIC was injected to 1st tumor. Anti-CD8 antibody (250 μg/body, n=8) or isotype control (250 μg/body, n=8) was intraperitoneally injected on days -3,1,4,7 and 10. In day -3, cancer cells were subcutaneously transplanted into the left flank. Data are shown as the mean ± SEM. **p<0.01, Student's t-test.