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Neoadjuvant use of oncolytic herpes virus G47Δ prevents local recurrence after insufficient resection in tongue cancer models

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A complete resection of tongue cancer is often difficult. We investigate the usefulness of administering G47Δ (teserpaturev), a triple-mutated oncolytic herpes simplex virus type 1, prior to resection. G47Δ exhibits good cytopathic effects and replication capabilities in all head and neck cancer cell lines tested. In an orthotopic SCCVII tongue cancer model of C3H/He mice, an intratumoral inoculation with G47Δ significantly prolongs the survival. Further, mice with orthotopic tongue cancer received neoadjuvant G47Δ (or mock) therapy with or without "hemilateral" resection, the maximum extent avoiding surgical deaths. Neoadjuvant G47Δ and resection led to 10/10 survival (120 days), whereas the survivals for G47Δ alone and resection alone were 6/10 and 5/10, respectively: all control animals died by day 11. Furthermore, 100% survival was achieved with neoadjuvant G47Δ therapy even when the resection area was narrowed to "partial," providing insufficient resection margins, whereas hemilateral resection alone caused death by local recurrence in half of the animals. G47Δ therapy caused increased number of tumor-infiltrating $CD8^+$ and $CD4^+$ cells, increased $F4/80^+$ cells within the residual tongues, and increased expression of immune-related genes in and around the tumor. These results imply that neoadjuvant use of G47Δ is useful for preventing local recurrence after tongue cancer surgery.

INTRODUCTION

Oral squamous cell carcinoma occurs most often in the lining of the tongue. Although surgical resection remains the first treatment choice, the time from diagnosis to surgery, often more than 1 month, allows for primary tumor growth and/or metastasis leading to disease advancement.[1] The surgery aims for complete excision of the tumor with no residual cancer cells, which is achieved by setting appropriate surgical margins[2]; however, this is often difficult because an extensive resection leads to impaired tongue functions and decreased quality of life (QOL). To prevent tumor progression before surgery, neoadjuvant therapy should be useful, but there is currently no effective neoadjuvant chemotherapy established for head and neck cancer.[3]

Oncolytic virus therapy has recently been recognized as a new therapeutic option for cancers. An oncolytic virus can selectively replicate in and kill cancer cells without harming the normal tissues.[4] Several oncolytic viruses have been shown to be effective against head and neck cancers in clinical and preclinical studies.[5–10] One of the more promising oncolytic viruses is G47Δ (teserpaturev), a triple-mutated, third-generation oncolytic herpes simplex virus type 1 (HSV-1), of which the safety and efficacy have been enhanced by deletion of the genes *γ34.5* and *α47* and inactivation of the gene *ICP6*.[11] In normal cells undergoing viral infection, double-stranded RNA-dependent protein kinase is activated early during the infection process, which inhibits viral protein translation primarily by phosphorylating eIF2α.[12] The *γ34.5* gene functions to block this host-cell-induced shutoff of protein synthesis,[13] which prevents the virus from replicating in normal cells. Because RNA-dependent protein kinase is universally reduced in tumor cells,[14] a virus deficient in the *γ34.5* gene retains its ability to replicate in tumor cells. The *ICP6* gene encodes the large subunit of ribonucleotide reductase, a key enzyme for viral DNA synthesis in non-dividing cells but not in dividing cells like tumor cells.[15] Deletion of the *α47* gene places the late *US11* gene under the control of the immediate-early *α47* promoter, which enhances the growth of $\gamma 34.5^-$ mutants in tumor cells by precluding the shutoff of protein synthesis.[11] Furthermore, because the *α47* gene functions to antagonize host cell transporters associated with antigen presentation,[16] deletion of the *α47* gene precludes the downregulation of major histocompatibility complex class I expression, which enhances stimulation of specific antitumor immune responses.[11]

Preclinical studies in a variety of cancers, including brain tumors, have shown that intratumoral administration of G47Δ induces robust antitumor activity via a direct cytopathic effect in the course of viral replication, accompanied by an induction of systemic antitumor

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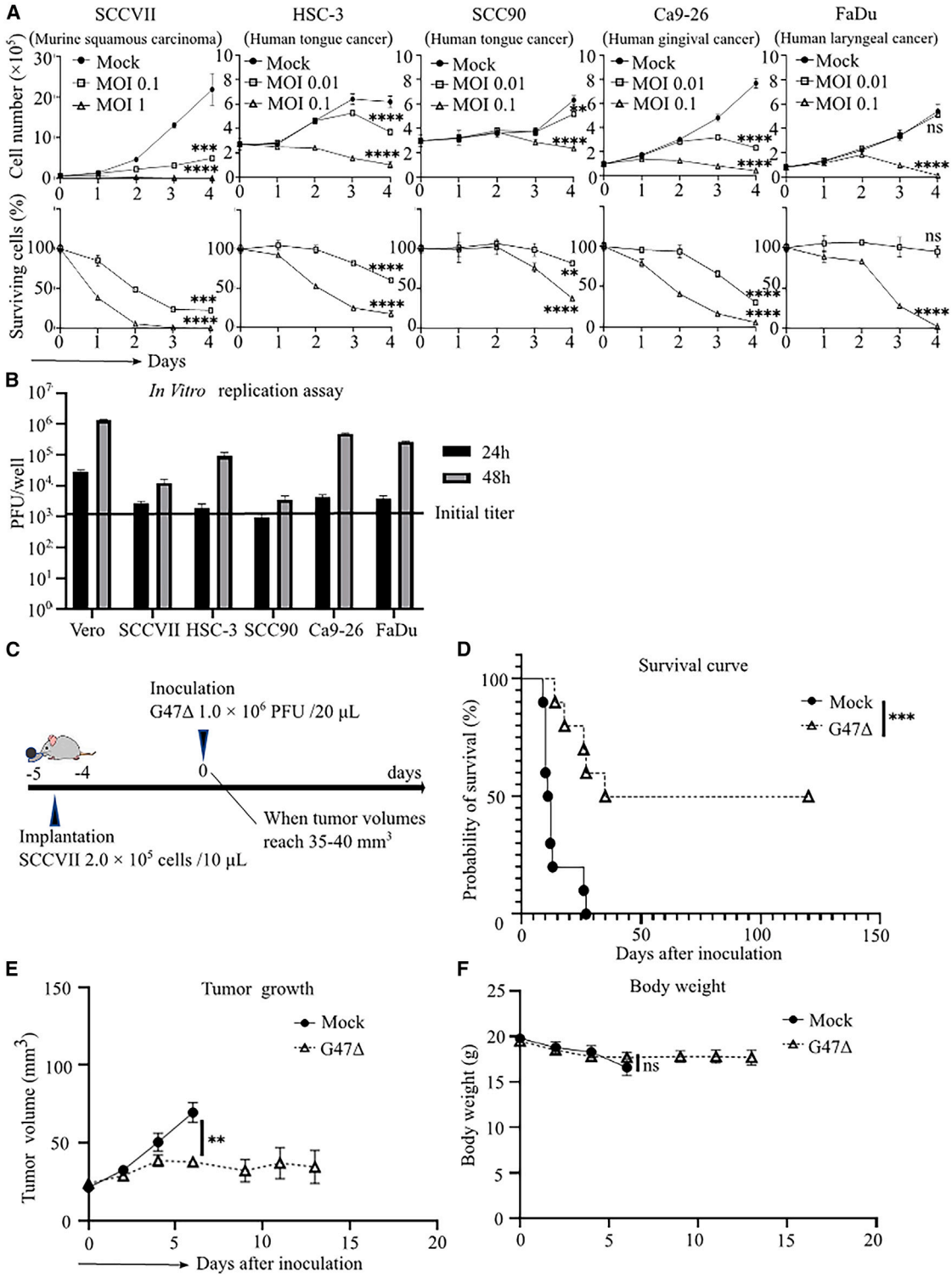

Figure 1. Oncolytic activities of G47Δ *in vitro* and *in vivo*

(A) *In vitro* cytotoxicity assay. Murine and human head and neck cancer cell lines were seeded in six-well plates at 2×10^5 cells/well except for SCCVII cells (2×10^4 cells/well). After incubation overnight, the cells were inoculated with G47Δ at various MOIs (0.01, 0.1, or 1). The number of surviving cells was counted daily and is expressed as the raw number (top) or as the percentage of the number of mock-infected control cells on each day (bottom). Data are presented as the mean of triplicates ± SD. (B) *In vitro* viral

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immune responses[17–21] A first-in-human clinical trial in patients with progressive glioblastoma (UMIN000002661) demonstrated the safety of G47Δ inoculated into the human brain.[22] A subsequent investigator-initiated phase II clinical trial in patients with residual or recurrent supratentorial glioblastoma (UMIN000015995) has shown a remarkable survival benefit and a good safety profile, which led to the approval of G47Δ as a new drug for malignant glioma in Japan.[23]

Antitumor immune responses induced by an oncolytic virus facilitate the elimination of remaining tumor cells even after the virus itself has been cleared,[6] which synergizes well with the goal of neoadjuvant therapy, because the therapeutic benefit of oncolytic virus therapy can persist following surgical removal of the treated tumor mass.[24] Previous studies have shown that neoadjuvant oncolytic virus therapy can induce antitumor immune responses that prevent tumor recurrence[25,26]; however, whether and how neoadjuvant oncolytic virus therapy suppresses residual tumor cells after insufficient tumor resection remain largely unknown.

Here, we investigate the efficacy of G47Δ used prior to resection of tongue tumors in immunocompetent mice and examine the immune responses in resected tumors as well as in the residual tongue. Neoadjuvant G47Δ therapy significantly improves the survival of animals with tongue cancer, even after insufficient tumor resection, not only via G47Δ's direct oncolytic activities but also due to an induction of antitumor immune responses that prevent local recurrences.

RESULTS

Oncolytic activities of G47Δ *in vitro*

To evaluate direct cytopathic effects and replication capabilities of G47Δ *in vitro*, G47Δ was inoculated into one murine squamous cell carcinoma cell line (SCCVII) and four human head and neck cancer cell lines (HSC-3, SCC90, Ca9-26, and FaDu). In all five cell lines, G47Δ at a higher multiplicity of infection (MOI) tested killed more than 70% of the cells within 4 days (Figure 1A). In addition, in all five cell lines, the yields of progeny virus increased at 48 h after 2×10^5 cells were infected at an MOI of 0.01 (Figure 1B), although the increase was minimal for SCC90, presumably due to the low rate of SCC90 cell proliferation until day 3 (Figure 1A). Viral replication assay was further performed for SCCVII, HSC-3, and SCC90 using a high MOI of 0.1, and, again, the yields of progeny virus increased at 48 h in all three cell lines (Figure S1).

Treatment of orthotopic tongue cancer with G47Δ alone in immunocompetent mice

The efficacy of G47Δ was evaluated for orthotopic tongue tumors generated in immunocompetent mice (Figure 1C). SCCVII cells (2×10^5 cells) were implanted in the tongue of syngeneic C3H/He mice, and when tumors reached a volume of 35–40 mm^3 (day 0), they were treated with a single intratumoral inoculation with G47Δ (1×10^6 plaque-forming units [pfu]) or mock (PBS). G47Δ treatment significantly prolonged the survival of tumor-bearing mice compared with control ($p = 0.0002$, log-rank test; Figure 1D) and also significantly suppressed the tumor growth compared with control ($p = 0.0061$, t test; Figure 1E). All mice in the control group died by day 27 due to tumor growth and dysphagia, whereas five (50%) of the G47Δ group survived the observation period (120 days) (n = 10 per group; Figure 1D). Tumors of mice that survived all regressed completely.

Planning of the extent of tongue resection for neoadjuvant G47Δ therapy

It has been reported that the extent of surgical resection is an important prognostic factor for tongue cancer patients.[2] In mice, however, extensive resection of the tongue often causes death due to post-surgical dysphagia. To generate a model that mimics the clinical setting, we first sought to identify the maximum extent of resection a mouse could withstand without the deed of resection itself affecting the well-being of the animal. C3H/He (5-week-old) mice were subjected to five types of resection currently used in clinical practice: partial, hemilateral, subtotal, near total, and total resection (n = 10 per group; Figure 2A).[27] All mice that received partial or hemilateral resection survived through the observation period (120 days). On the other hand, seven mice with subtotal resection, none with near-total resection, and none with total resection survived. In the latter two groups, all showed a rapid decrease in body weight and died by day 11 (Figures 2B and 2C). Hence, for the purpose of this study, we adopted hemilateral resection as the maximum resection model to be used in subsequent *in vivo* experiments.

We further used the model to assess the efficacy of neoadjuvant G47Δ therapy (Figure 2D). In mice with untreated tumors, the surgical margin is expected to be 0–0.5 mm. Because neoadjuvant G47Δ therapy should suppress the tumor growth, allowing a larger surgical margin, the margin is expected to be 1–1.5 mm for mice with G47Δ-treated tumors. To investigate the histopathological difference between these resection lines, we examined the respective resection margins in C3H/He mice bearing SCCVII tongue tumors (Figure 2E). A resection with a margin of 1–1.5 mm resulted in almost complete removal of microtumors in the remaining tongue. In contrast, residual microtumors were observed in the remaining tongue after resection with a margin of 0–0.5 mm, which should increase the potential for local recurrence.[28]

replication assay. Cells were seeded in six-well plates at 2×10^5 cells/well. Triplicate wells were infected with G47Δ at an MOI of 0.01. At 24 and 48 h after inoculation, the cells were collected and progeny virus titered by plaque-formation assay using Vero cells. Data are presented as the mean of triplicates ± SEM. A horizontal line indicates the initial number of virus added to each well (2×10^3 pfu/well). (C) Treatment of orthotopic tongue cancer with G47Δ alone in immunocompetent mice. C3H/He mice with established SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) when tumor volumes reached 35–40 mm^3 (day 0). (D) Efficacy of G47Δ assessed by survival curve. (E) Efficacy of G47Δ assessed by tumor growth. (F) Body weight of animals treated with mock or G47Δ. Data are presented as the mean ± SEM; n = 10 per group. Two-tailed Student's t test at day 4 (A) and at day 6 (E and F) and log-rank test (D). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.

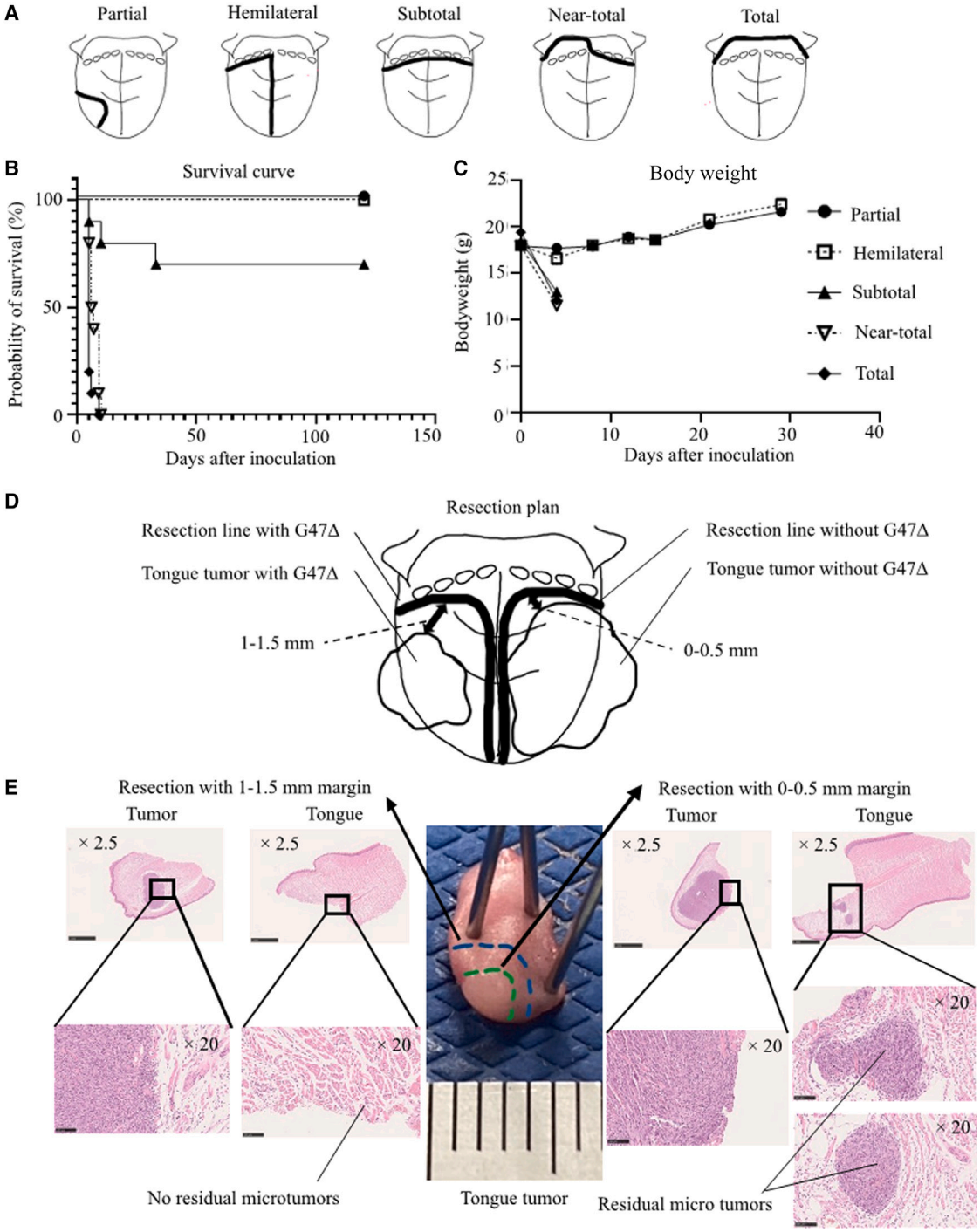
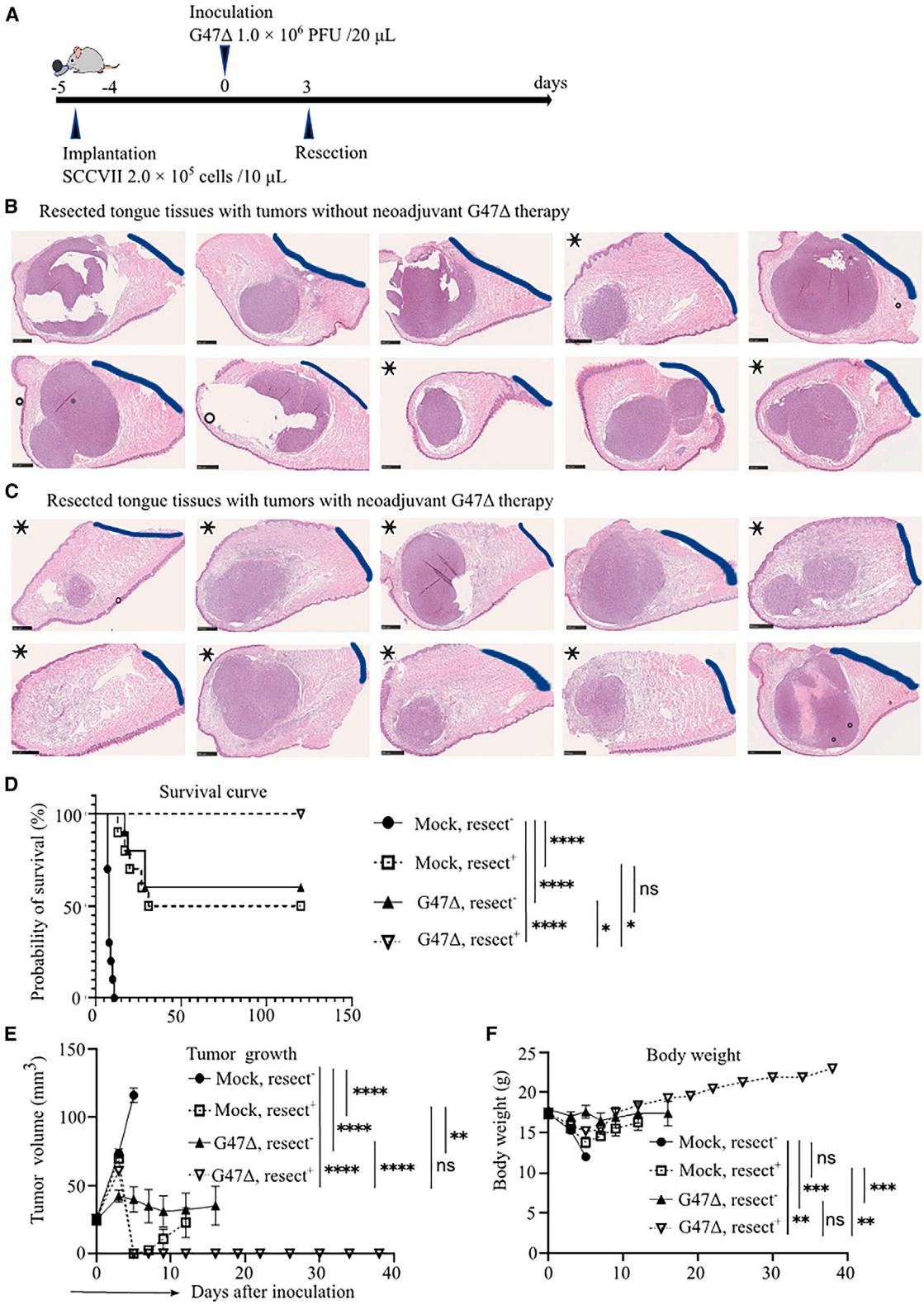


Figure 2. Planning of the extent of tongue resection for neoadjuvant G47Δ therapy

(A) Schematic drawings showing the areas of resection of the tongue according to the five resection types. Black lines indicate the resection lines. (B) Survival curve of C3H/He mice according to the resection type (n = 10 per group). (C) Body-weight changes in C3H/He mice according to the resection type (n = 10 per group). Results represent mean ± SEM. (D) Resection plans of tongue tumors for assessing the efficacy of neoadjuvant G47Δ therapy. (E) Histological evaluation of resected tumor and residual tongue based on the proposed resection margins. C3H/He mice with established SCCVII tongue tumors were subjected to resection and the resulting tissues were examined for residual microtumors. A resection with a margin of 1–1.5 mm results in almost complete removal of microtumors in the remaining tongue. In contrast, residual microtumors are observed in the remaining tongue after resection with a margin of 0–0.5 mm. Scale bars: ×2.5, 1 mm; ×20, 100 μm.



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Efficacy of tumor resection with neoadjuvant G47Δ therapy in orthotopic tongue cancer model

The efficacy of tumor resection with neoadjuvant G47Δ therapy was evaluated according to the above resection plan (Figure 2D) using the orthotopic SCCVII tongue cancer model. Tumors received intratumoral inoculations with G47Δ (1×10^6 pfu) or mock (PBS) on day 0 and were resected on day 3 (Figure 3A). As planned, the hemilateral type was used for all resections. We defined resection margins that secured a distance over 1 mm from the resection line and the tumor boundary as "sufficient margins." Those with sufficient margins are indicated by asterisks in Figures 3B and 3C. The resection provided sufficient margins, where all microtumors were considered included, in 8 of 10 G47Δ-treated mice. In contrast, the hemilateral resection led to sufficient margins in only 3 of 10 mock-treated controls. Thus, one of the advantages of neoadjuvant G47Δ therapy shown was to allow sufficient resection margins, presumably reducing the number of microtumors left in the residual tongue.

In this model, the hemilateral resection alone resulted in 5 of 10 mice surviving120 days, whereas G47Δ therapy alone resulted in 6 of 10 mice surviving. However, when G47Δ therapy was combined with resection, all 10 mice survived, showing that neoadjuvant G47Δ therapy prior to resection is significantly more efficacious than either therapy alone ($p = 0.011$ vs. resection alone and $p = 0.029$ vs. G47Δ therapy alone, log-rank test; Figure 3D). All mock-treated mice without resection died by day 11 as a result of rapid tumor growth accompanied by body-weight loss (Figures 3D, 3E, and 3F). The necropsy of those that received resection only and died (5/10) revealed that four of the five died of local recurrence, and one died of regional metastases.

Efficacy of neoadjuvant G47Δ therapy prior to resections with insufficient margins

When the hemilateral type was applied to all resections regardless of neoadjuvant G47Δ therapy, those that received G47Δ tended to have larger resection margin areas, because G47Δ caused tumor growth suppression. To eliminate this margin factor, using the same orthotopic SCCVII tongue cancer model, we adjusted the resection plan so that an insufficient resection margin of 0–0.5 mm was applied to all resections, i.e., smaller areas of the tongue were resected for tumors receiving neoadjuvant G47Δ than for those not receiving G47Δ (Figures 4A, 4B, and 4C). All 10 mice of the neoadjuvant G47Δ group survived for 120 days, whereas only 5 of 10 mice of the resection-only group survived, despite the areas of tongue resection being smaller for the former group than for the latter group ($p = 0.034$, log-rank test; Figure 4D). Five of 10 mice of the resection-only group died of local recurrence. All control mice without treatment died by day 13. The trends for tumor growth and body weight were similar to those observed in the previous experiment (Figures 4E and 4F). The results imply that neoadjuvant G47Δ therapy works to prevent local recurrence even in cases with insufficient resection margins.

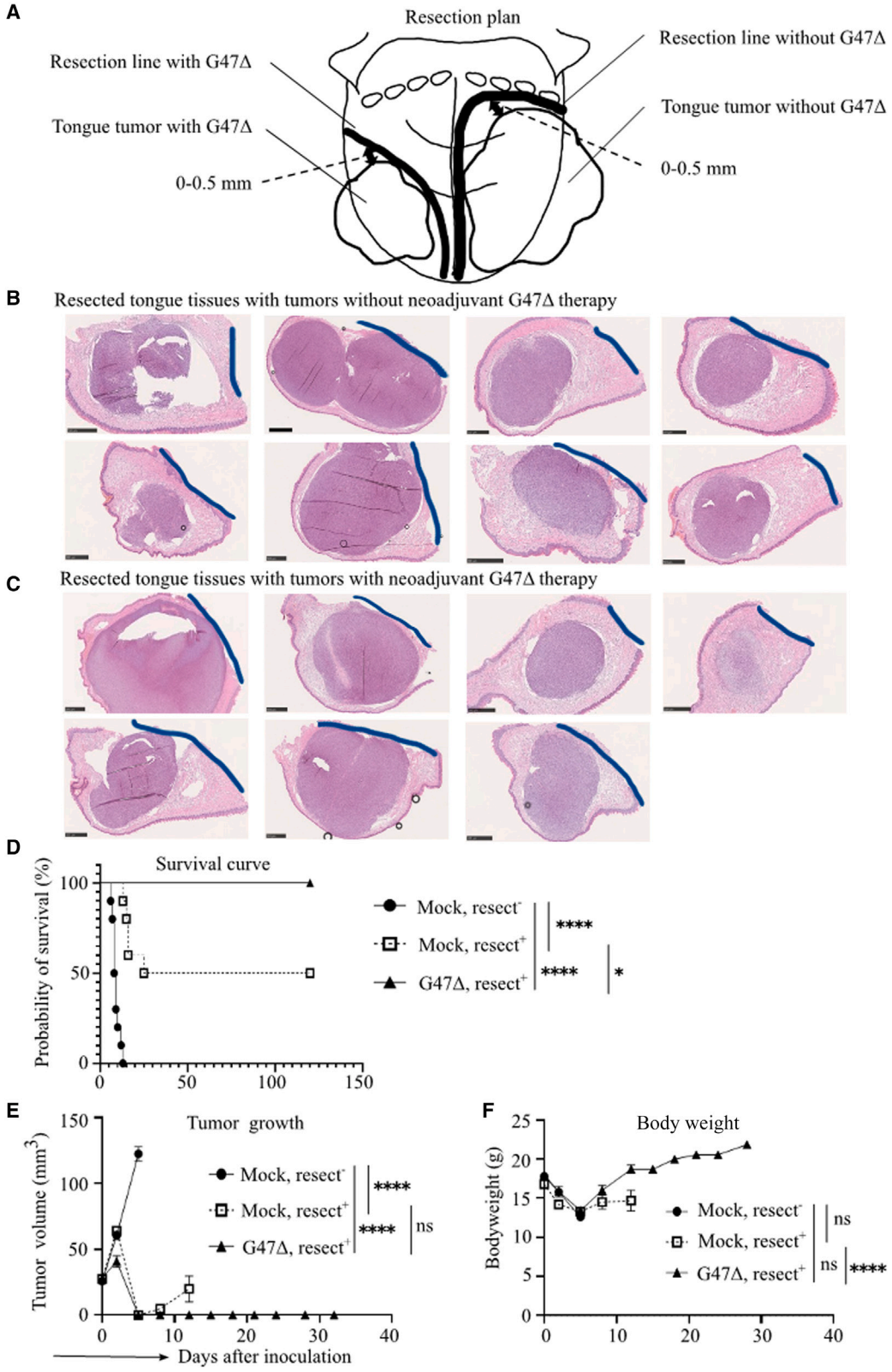
Intratumoral administration of G47Δ stimulates the host immune response

To investigate the immune induction by G47Δ, we used flow cytometry to examine the immune cell infiltration into orthotopic SCCVII tongue tumors in C3H/He mice. Tumors were inoculated intratumorally with G47Δ (1×10^6 pfu) or mock (PBS) on day 0 and collected on day 7 (Figure 5A). Responders were defined as mice with tumors that were reduced in size on day 7 compared with day 0, and non-responders were defined as mice with tumors that did not change or increased in size. Of 15 G47Δ-treated animals, 7 were responders and 8 non-responders. Responders and non-responders (n = 4 for each group randomly selected) both showed significantly more tumor-infiltrating $CD8^+CD4^-$ T cells compared with mock-treated mice (n = 4; Figures 5B and S2). Activated $CD8^+$ T cells that express immune-checkpoint proteins, such as PD-1 and TIM-3, are related to immune tolerance. Tumor-infiltrating $CD8^+PD\text{-}1^+$ and $CD8^+TIM\text{-}3^+$ cell populations were comparable among the three groups. The median expression rate of CD39 in tumor cells, an index of exhausted T cells, tended to be low in G47Δ responders, although there were no statistical differences among the three groups (Figure 5C).

Next, using the same orthotopic tongue cancer model, we performed immunohistochemical examination of the residual tongue extracted on day 15 after inoculating the tumors with G47Δ (1×10^6 pfu) or mock on day 0 and resecting the tumor on day 3 according to the insufficient-margin plan (Figure 5D). The residual tongues were immunostained for CD4, CD8, F4/80, and FoxP3, and quantitative evaluations were conducted separately for the residual tumor areas and the areas surrounding the residual tumors (Figures 5E and S3). Significantly higher numbers of infiltrating $CD4^+$ and $CD8^+$ cells were observed in the residual tumor areas of G47Δ-treated animals compared with mock-treated ones. In the surrounding areas, a significantly higher number of F4/80 cells (mature macrophages) were observed in G47Δ-treated animals compared with mock-treated ones. The numbers of $FoxP3^+$ cells (regulatory T cells) within the

Figure 3. Efficacy of tumor resection with neoadjuvant G47Δ therapy

(A) C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0 when tumor volumes reached 35–40 mm^3, and the tongues harboring tumors were partially resected on day 3 by hemilateral resection. (B and C) Representative histopathology of resected tongue tissues with tumors. (B) Resected tongue tissues with tumors without neoadjuvant G47Δ therapy. (C) Resected tongue tissues with tumors with neoadjuvant G47Δ therapy. Representative histopathology from each mouse is presented. Asterisks indicate tumor resections with sufficient margins defined as >1 mm between the resection line and the tumor boundary. Hematoxylin and eosin staining. Blue lines depict actual resection lines. Scale bars: 500 μm. (D) Efficacy of tumor resection with neoadjuvant G47Δ therapy assessed by survival curve (n = 10 per group). (E) Efficacy of tumor resection with neoadjuvant G47Δ therapy assessed by tumor growth (n = 10 per group). Results are the mean ± SEM. (F) Body-weight changes in mice with or without tumor resection and with or without neoadjuvant G47Δ therapy (n = 10 per group). Results are the mean ± SEM. Log-rank test (D) and two-way ANOVA followed by Tukey's multiple comparison test (E and F). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.



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tumor areas and the surrounding areas were scarce in both G47Δ- and mock-treated mice.

Gene expression is significantly changed in responders to G47Δ

To elucidate the mechanism of immune cell infiltration into tumors, we investigated gene expression in the tumor microenvironment by RNA-sequencing (RNA-seq) analysis. C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0. Tumors and adjacent tongue tissues were collected on day 5 and subjected to RNA-seq analysis (Figure 6A). In this experiment, responders were defined as mice with tumors that were reduced in size on day 5 compared with day 0 and non-responders as those that did not change or increased in size. Of 20 mice, 11 were categorized as responders and 9 as non-responders. Gene expression was significantly altered in responders compared with that in mock and non-responders (n = 5 for each group randomly selected). The principal-component analysis (PCA) showed that inoculation with G47Δ into the healthy tongue did not alter the gene expression profile compared with the normal tongue tissue (Figure 6B). Three of five responders (R3, R4, and R5) were grouped in a gene expression profile different from that of mock and non-responders. We normalized relative log gene expression based on lead count value using the DESeq2 software and extracted differentially expressed genes (DEGs). The expression levels of immune-related genes, including *Trav12-3*, *Ctsg*, *Gm4841*, and *CxCl9*, were increased in responders compared with mock-treated mice, whereas *Trav 12-3* alone showed a remarkably higher expression level in responders compared with non-responders (Figure 6C). Ingenuity Pathway Analysis showed several gene groups, associated with proliferation of immune cells, immune response, antiviral response, activation of natural killer cells, functions of helper lymphocytes, and pathogen-induced cytokine signaling, top-ranked in a comparison of responders with mock (Figure S4). In contrast, STAT3 signaling was the only pathway that was top-ranked in a comparison of responders with non-responders. Similar to the PCA results, the *k*-means clustering heatmap revealed different genetic compositions in three responders compared with mock and non-responders (Figure 6D). Close observation of tumor volume changes revealed that the three responders were those that showed temporal increases on day 3 before shrinkage on day 5 (Figure S5). The gene groups (clusters 2 and 3) that were expressed especially highly in responders included not only immune-related genes such as *Stk39*, *Icam1*, *Cebpb*, *Stat3*, *Tnfrsf21*, *Zfp335*, *Crkl*, *Trim56*, *Ticam1*, and *E1f4*, but also genes related to viral infection, such as *Trim11*, *Pcbp1*, and *Hyal2* (Table S2). Gene ontology analysis of responders vs. mock uncovered several significantly enriched terms, including cellular responses to interferon-β and interferon-γ, positive regulation of natural killer cell-mediated cytotoxicity, immune response, and adaptive immune response (Figure 6E). None were significantly enriched in the analysis of responders vs. non-responders.

DISCUSSION

The current principal treatment for tongue cancer is surgical resection. Although neoadjuvant therapy has been suggested, several induction chemotherapy protocols have shown no efficacy over surgery alone.[29–31] In contrast, more extensive resections and longer operation time may be required for patients that receive neoadjuvant chemotherapy.[32] In addition, neoadjuvant therapies may interfere with later chemotherapeutic agents.[33] In recent years, there have been several clinical trials of neoadjuvant immunotherapy using immune-checkpoint inhibitors in patients with head and neck cancer,[34] including those that report survival benefits.[35,36] However, accompanying issues, such as immune-mediated toxicity, hyperprogression, and post-operative wound healing, remain,[37,38] which prevent it from becoming the standard of care.[38]

Oncolytic virus therapy is an attractive candidate for neoadjuvant therapy, because of its function as an efficient *in situ* cancer vaccination that provides long-term therapeutic benefits.[39] Oncolytic viruses are capable of destroying cancer cells not only by their direct cytopathic effects but also by activating the host antitumor immune responses.[40–42] A third-generation oncolytic HSV-1, G47Δ, is specifically designed to exhibit enhanced stimulation of specific antitumor immunity[22,23] and has in fact shown long-term efficacy via elicitation of antitumor immune responses in glioblastoma patients.[22,23] Several preclinical studies have shown that neoadjuvant use of oncolytic virus therapy can improve therapeutic outcomes.[25,43]

Using immunocompetent C3H/He mice harboring orthotopic SCCVII tongue tumors, we demonstrate that neoadjuvant therapy with intratumoral inoculations of G47Δ followed by hemilateral resections of the tongue leads to prolongation of survival. The significance of this model is that it mimics the clinical setting, where most patients receive resection of a portion of the tongue together with the tumor. Generation of tongue cancer treatment models in mice has been difficult, because resection of the tongue often causes post-operative dysphagia and death. In this study, we found that implanting the tumor in the lateral apex of the tongue and restricting the extent of tongue resection to "hemilateral" can keep the mice alive, reproducing the treatment situation in clinics. The main reason for the

Figure 4. Efficacy of neoadjuvant G47Δ therapy prior to resections with insufficient margins

(A) Modified resection plan of tongue tumors. Tumors treated with G47Δ were resected with purposely insufficient margins of 0–0.5 mm. (B and C) Representative histopathology of resected tongue tissues with tumors. (B) Resected tongue tissues with tumors without neoadjuvant G47Δ therapy. (C) Resected tongue tissues with tumors with neoadjuvant G47Δ therapy. Hematoxylin and eosin staining. Blue lines depict actual resection lines. Scale bars: 500 μm. (D) Survival curve. C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0 when tumor volumes reached 35–40 mm^3, and the tongues harboring tumors were partially resected according to the modified resection plans on day 3 (n = 10 per group). (E) Efficacy of neoadjuvant G47Δ therapy assessed by tumor growth (n = 10 per group). Results are the mean ± SEM. (F) Body-weight changes in mice with or without neoadjuvant G47Δ therapy (n = 10 per group). Results are the mean ± SEM. Log-rank test (D) and two-way ANOVA followed by Tukey's multiple comparison test (E and F). *$p < 0.05$; ****$p < 0.0001$; ns, not significant.

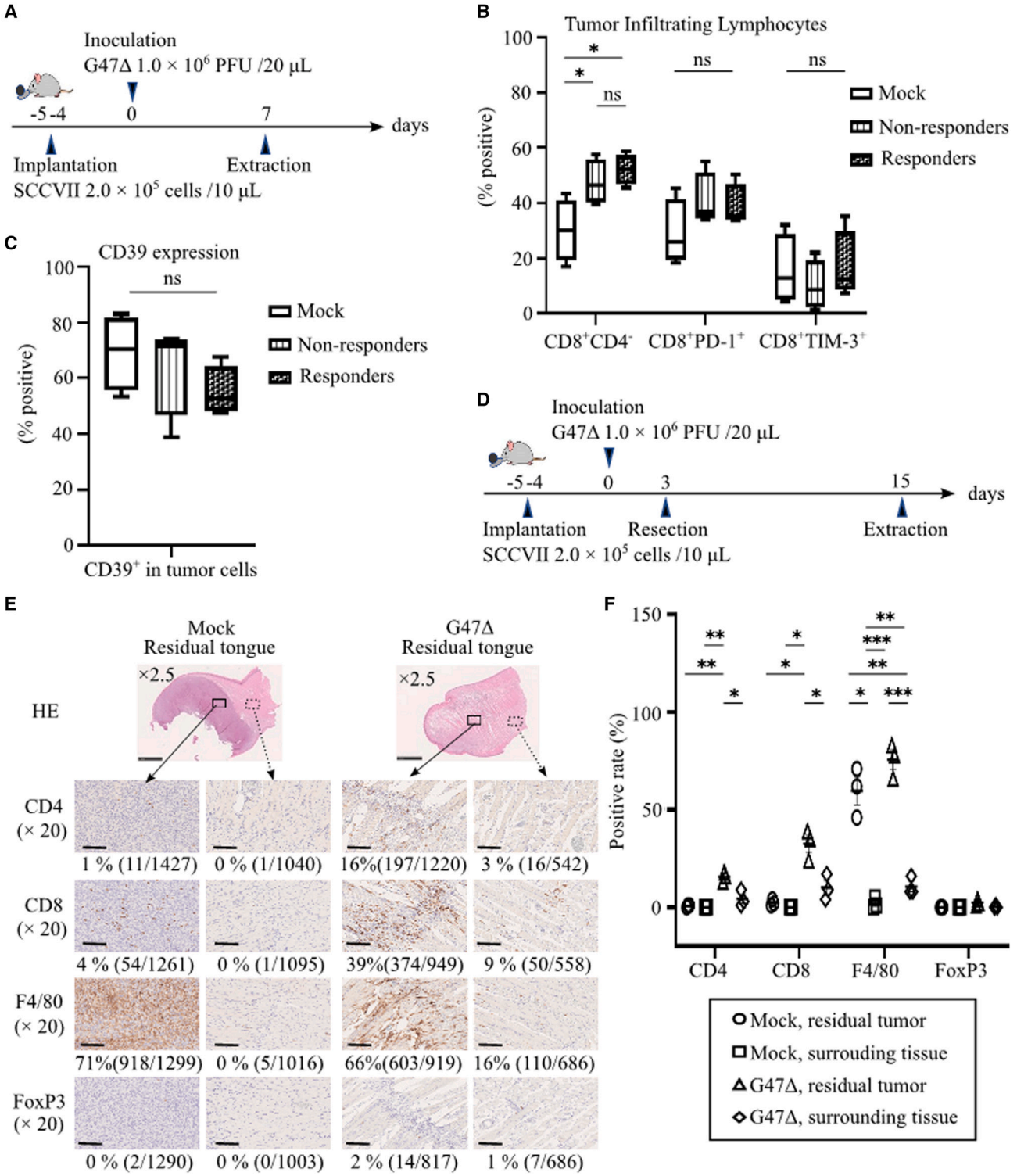

Figure 5. Intratumoral administration of G47Δ stimulates the host immune response

(A) C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated intratumorally with G47Δ (1×10^6 pfu) or mock (PBS) on day 0, and tumors were collected on day 7. Immune cells infiltrating the tumors were evaluated by flow cytometry. Responders were defined as mice with tumors that were reduced in size on day 7 compared with day 0, and non-responders were defined as mice with tumors that did not change or increased in size. (B) Tumor-infiltrating lymphocyte populations.

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survival prolongation was assumed to be that neoadjuvant G47Δ therapy suppressed the tumor growth and allowed wider and sufficient resection margins than no-neoadjuvant controls. Naturally, the wider the resection margin, the lower the chance of leaving microtumors behind. Surprisingly, however, the survival prolongation effect of neoadjuvant G47Δ persisted even when resection areas were intentionally narrowed to give insufficient margins. Neoadjuvant G47Δ therapy therefore not only suppresses tumor growth prior to surgery, but also works to eliminate microtumors that are left in the residual tongue. We found that intratumoral G47Δ inoculations cause significantly more tumor-infiltrating $CD8^+CD4^-$ T cells compared with mock treatment by flow cytometry. We further found that G47Δ causes significantly higher numbers of infiltrating $CD4^+$ and $CD8^+$ cells in the residual tumors as well as significantly higher numbers of $F4/80^+$ cells in the tongue tissues surrounding the residual tumors by quantitative analyses of immunohistochemistry. We previously showed that G47Δ inoculated into tongue tumors swiftly traffics to the draining lymph nodes.[44] These observations imply that immune responses induced by neoadjuvant G47Δ therapy are likely involved in suppressing the local recurrence or regional metastases in the case of insufficient resection margins. The clinical implication of the current findings is quite significant: with neoadjuvant G47Δ therapy, the resection area of the tongue tumor can be kept to a minimum, not requiring, e.g., subtotal resection with free flap reconstruction, thereby preserving patients' speech and swallowing functions and QOL.[45,46] Furthermore, neoadjuvant G47Δ therapy confers on tongue cancer patients the possibility of a long-term survival regardless of the extent of resection margins.

We and others[47] used RNA-seq analysis accompanied by gene ontology annotation to analyze transcriptome data for tongue cancer models. RNA-seq analysis revealed a notable change in gene expression profile in the tumor microenvironment after intratumoral inoculation with G47Δ, and immune-related gene ontology terms were enriched in responders compared with the mock group. Interestingly, STAT3 signaling, the pathway that was top-ranked in the comparison of responders with non-responders by Ingenuity Pathway Analysis, has been reported to enhance oncolytic HSV-1 replication.[48.] It is noteworthy that healthy tongues inoculated with G47Δ and the normal tongue tissues showed very similar gene expression profiles, which likely reflects the high safety features of G47Δ. Whether the change in gene expression profile after intratumoral inoculation with G47Δ is related to the infiltration of immune cells is yet to be elucidated.

Accumulating evidence, both preclinical and clinical, suggests that G47Δ therapy causes immunologically cold tumors to turn hot,[19–23] so the efficacy of neoadjuvant G47Δ therapy is potentially enhanced when combined with immune-checkpoint inhibitors. Neoadjuvant use of nivolumab or nivolumab plus ipilimumab in untreated oral cavity squamous cell carcinoma patients was reportedly feasible with modest efficacy.[49] G47Δ armed with murine interleukin-12 (IL-12) in combination with an anti-CTLA-4 antibody or an anti-PD-1 antibody has shown improvement of survival in a mouse glioma model.[50] The phase II part of a clinical trial in upfront patients with malignant melanoma using G47Δ-based oncolytic HSV-1 armed with human IL-12 (T-hIL12) in combination with nivolumab is ongoing, at the time of this writing, in Japan (jRCT2033190086). The pivotal study of G47Δ in glioblastoma patients (UMIN000015995) that led to drug approval in Japan showed the high safety profile of G47Δ when injected repeatedly six times into brain tumors at 1×10^9 pfu/dose.[23] Therefore, neoadjuvant therapy using G47Δ with or without immune-checkpoint inhibitors in patients with tongue cancer may be not only reasonable but promising.

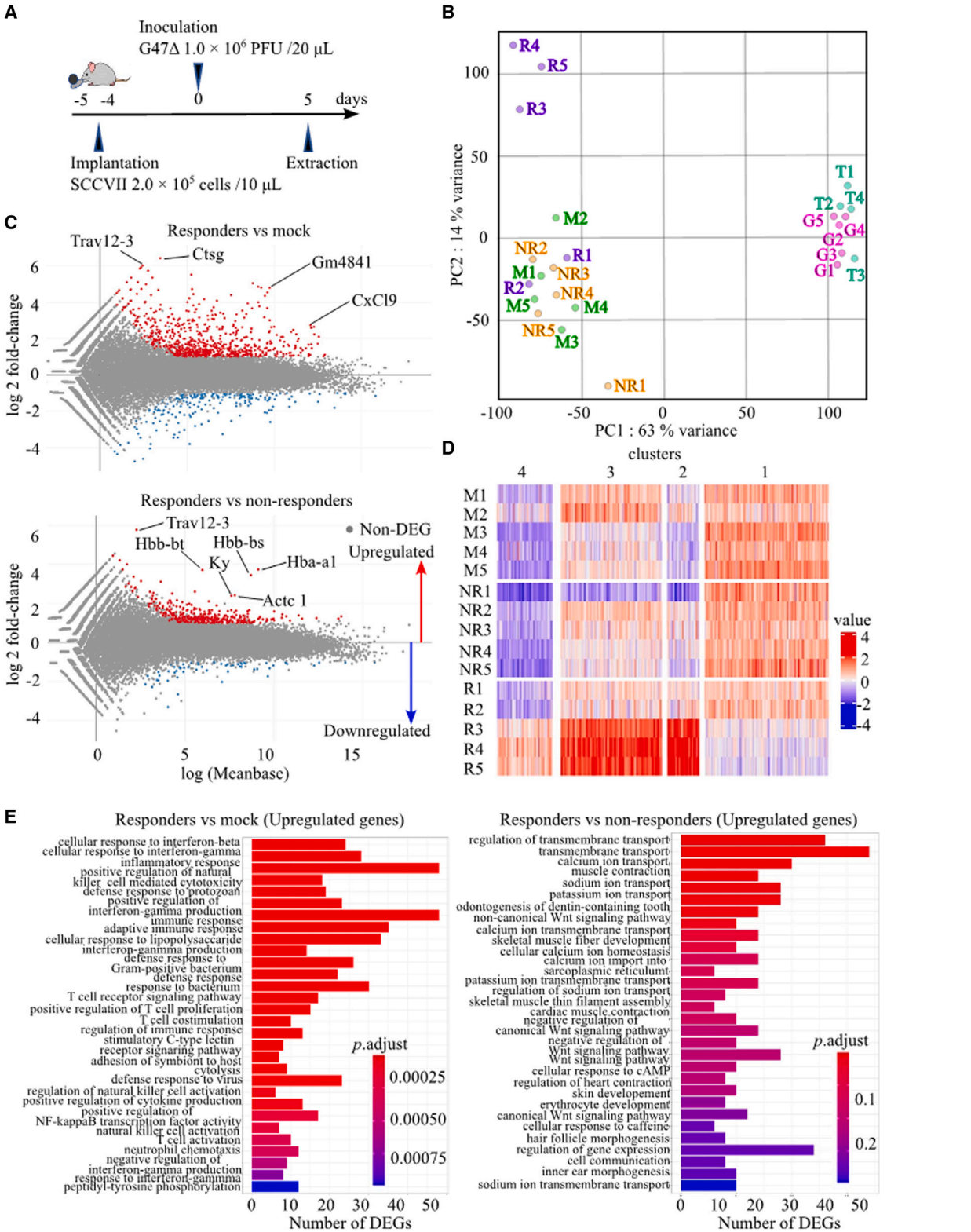
In conclusion, neoadjuvant G47Δ therapy in patients with tongue cancer not only suppresses tumor growth prior to surgery but also works to eliminate microtumors that are left in the residual tongue. Neoadjuvant G47Δ therapy may therefore be a useful strategy for the treatment of tongue cancer, in which an effective suppression of both local recurrence and regional metastases can be expected after surgery regardless of the extent of resection margins.

MATERIALS AND METHODS

Cell lines and virus

Vero cells (African green monkey kidney), two human oral squamous cell carcinoma cell lines (HSC-3 and SCC90), and one human head and neck cancer cell line (FaDu) were purchased from the American Type Culture Collection (Rockville, MD, USA). A human oral squamous cell carcinoma (SCC) cell line, Ca9-26, was purchased from Japanese Cell Resource Bioresources (Osaka, Japan). A mouse SCC cell line, SCCVII, derived from the C3H/He mouse strain was provided by Professor Yoshiaki Yura (Second Department of Oral and Maxillofacial Surgery, Osaka University, Osaka, Japan). All cell lines were cultured according to the directions provided by the suppliers. G47Δ is an oncolytic HSV-1 that was grown, purified, and titered by plaque assay using Vero cells as described previously.[11]

Responders and non-responders both showed significantly greater numbers of tumor-infiltrating $CD8^+CD4^-$ T cells compared with mock-treated mice (n = 4 per group). Tumor-infiltrating $CD8^+PD\text{-}1^+$ and $CD8^+TIM\text{-}3^+$ cell populations were comparable among the three groups. (C) CD39 expression in tumor cells. The median expression rate of CD39 in tumor cells tended to be low in G47Δ responders, although there were no statistical differences among three groups. (D) C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated intratumorally with G47Δ (1×10^6 pfu) or mock (PBS) on day 0, and tumors were resected on day 3 and residual tongues extracted on day 15. Immune cells in the residual tongues were evaluated by immunohistochemistry. (E) Sections of residual tongue were immunostained with indicated antibodies. Immunohistochemistry (IHC)-positive rates (the number of IHC-positive cells/total cells) for each immune cell type inside the residual tumor area (solid line) or outside of the area (dashed line) in mock- or G47Δ-treated mice were calculated automatically using PatholoCount (version 1.0; Mitani, Tokyo, Japan). Results of representative areas are shown. HE, hematoxylin and eosin. Scale bars: ×2.5, 1 mm; ×20, 100 μm. (F) Immunopositive immune cell counts inside or outside of the residual tumor area in mock- or G47Δ-treated mice. Results are mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test (B and C) and two-tailed Student's t test (F). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant.



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Cytotoxicity assay

In vitro cytotoxicity assays were performed as described previously.[42] Cells were seeded in six-well plates at optimal density (2×10^5 cells/well), except for SCCVII cells seeded at 2×10^4 cells/well to avoid early confluence. After incubation overnight, the cells were treated with mock (PBS) or G47Δ at various MOIs and grown in 1% inactivated fetal calf serum. Because human cells are generally more susceptible to HSV-1 than murine cells, MOIs of 0.01 and 0.1 were used for human cell lines and MOIs of 0.1 and 1 were used for SCCVII cells. The number of surviving cells was counted daily using a Coulter counter (Beckman Coulter, Fullerton, CA, USA) and expressed as raw number or as the percentage of the number of mock-infected control cells on each day.

In vitro virus replication assay

Cells were seeded in six-well plates at 2×10^5 or 1×10^5 cells/well. Triplicate wells were treated with G47Δ at an MOI of 0.01 or 0.1. At 24 or 48 h after inoculation, the cells were collected, and progeny virus was titered by plaque assay using Vero cells.

Mice

Female C3H/He mice (4–6 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). All animal studies were approved by the Ethics Committee for Animal Experimentation of the University of Tokyo.

Efficacy assessment of neoadjuvant G47Δ therapy in an orthotopic tongue cancer model

Orthotopic implantation of SCCVII cells (2×10^5 cells) in 10 μL of serum-free medium was performed into the right apex of the tongue of C3H/He mice under general anesthesia (intraperitoneal administration of ketamine and xylazine). When tongue tumor volumes reached 35–40 mm^3 (day 0), the tumors were inoculated with mock (PBS) or G47Δ (1×10^6 pfu) in 20 μL of PBS containing 10% glycerol. Survival was assessed every day; tumor volume (length × width × height) and body weight were assessed every other day. The survival was observed for 120 days.

Efficacy assessment of neoadjuvant G47Δ therapy with insufficient resection margins

C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0, followed by resection on day 3. All mice in the mock-treated group were given hemilateral resection with insufficient margins. Survival, tumor volume, and body weight were evaluated as described above.

Flow cytometric analysis of tumor-infiltrating cells

C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0, and tumors were collected on day 7. Suspensions of immune cells infiltrating the tumors were prepared with a Tumor Dissociation Kit (Miltenyi Biotec, Auburn, CA, USA). Red blood cells were removed with RBC lysis buffer (eBioscience, Santa Clara, CA, USA). The immune cells were stained with several antibodies (Table S1) and analyzed following a gating strategy (Figure S1). Flow cytometric analysis was performed on the CytoFLEX flow cytometer (Beckman Coulter, Pasadena, CA, USA).

Immunohistochemistry

C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0. Tumors were resected on day 3, and residual tongues were collected on day 15. Extracted tissues were fixed with formalin and embedded in paraffin. Sections were rehydrated through an alcohol gradient and cut to a thickness of 4 μm by using a microtome. Immunohistochemistry was performed using Bond-Max (Leica, Nussloch, Germany) according to the Bond Polymer Refine IHC protocols. Images were acquired with a NanoZoomer S60 digital slide scanner (C13210-01; Hamamatsu, Shizuoka, Japan). The NDP.View2 software was used for image acquisition (U12388-01; Hamamatsu). The following antibodies purchased from Cell Signaling Technology were used for immunohistochemistry: CD4 (1:50, 25229S), CD8 (1:200, 98941S), FoxP3 (1:800, 12653S), and F4/80 (1:200, 70076S). Antigen retrieval was performed by microwave heat-induced epitope retrieval with Bond Epitope Retrieval Solution 2 (AR9640; Vision BioSystems, Newcastle upon Tyne, UK). Quantitative analyses were performed by using PatholoCount (version 1.0; Mitani, Tokyo, Japan).

Extraction of RNA and RNA-seq analysis

C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0. Tumors and adjacent tongue tissues were collected on day 5 and snap-frozen in liquid nitrogen. The collected samples were homogenized, and RNA was extracted by using an RNeasy Mini Kit (Qiagen) and QIAcube. PCA was conducted on all of the detected genes by statistical software. Relative log expression was normalized based on lead

Figure 6. Inoculation with G47Δ significantly altered gene expression in the tumor microenvironment

(A) C3H/He mice with established orthotopic SCCVII tongue tumors were inoculated with G47Δ (1×10^6 pfu) or mock (PBS) on day 0, and tumors and adjacent tongue tissues were collected on day 5. Gene expression in the collected tissues was determined by RNA-seq. (B) Principal-component analysis. M1–M5, samples from mock-treated mice; R1–R5, samples from G47Δ responder mice; NR1–NR5, samples from G47Δ non-responder mice; G1–G5, samples from healthy tongue inoculated with G47Δ; T1–T4, samples from healthy tongue without G47Δ inoculation. (C) Microarray (MA) plots of differentially expressed genes (DEGs). Relative log expression was normalized based on lead count value using the DESeq2 software, and DEGs were extracted. One-way ANOVA was used to filter variable genes and the Wald test was used to extract DEGs. Genes with |log2 fold change| > 1 and $p < 0.05$ were selected. (D) Heatmap of DEGs in mock-treated mice (M1–M5), non-responders (NR1–NR5), and responders (R1–R5). Genes are categorized into four clusters. The gene labels of each cluster are described in Table S2. (E) Top 20 gene ontology enrichment terms of DEGs. Bar length indicates the number of DEGs in that gene class, and the color reflects the p value of the enriched term. The p value is higher from red to blue.

count value using DESeq2 software, and DEGs were extracted and then visualized by microarray (MA) plot. One-way ANOVA was used to filter variable genes, and the Wald test was used to extract DEGs. Genes with |log2 fold change| > 1 and $p < 0.05$ were selected. Data were analyzed using the Ingenuity Pathway Analysis software (version 01-20-04; Qiagen, Venlo, the Netherlands).

Statistical analysis

A two-tailed Student's test (for comparison of two groups) or one-way or two-way ANOVA followed by Tukey's multiple comparisons test was used to determine statistical significance. Survival curves were analyzed by the Kaplan-Meier method, and significance was evaluated by the log-rank test. In all cases, $p < 0.05$ was considered to indicate significance. All statistical analyses ware performed using GraphPad Prism8 (GraphPad Software, San Diego, CA, USA).

Data and code availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The RNA-seq analysis data are deposited in the Gene Expression Omnibus database under accession no. GSE227813 (GEO Accession viewer [nih.gov]).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.07.002.

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AUTHOR CONTRIBUTIONS

K.I. and H.I. were involved in the conception, design, and conduct of the experiments; statistical analyses; interpretation of results; and writing of the manuscript. M.I. assisted in designing and performing the experiments. M.T. and Y.M. were involved in the conception and design of the experiments. T.T. was involved in the conception, design, and conduct of the experiments; statistical analyses; interpretation of results; writing and editing of the manuscript; and funding acquisition.

DECLARATION OF INTERESTS

T.T. owns the patent right for G47Δ in Japan. The Project Division of Oncolytic Virus Development is endowed by Denka Company Ltd. (Tokyo, Japan) to T.T. and The Institute of Medical Science, The University of Tokyo. Denka Company is currently the commercial manufacturer of Delytact (G47Δ). M.T. belongs to the project division endowed by Denka Company.

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