Tumor Microenvironment Remodeling by Intratumoral Oncolytic Vaccinia Virus Enhances the Efficacy of Immune-Checkpoint Blockade

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Abstract

Purpose: Cancer immunotherapy is a potent treatment modality, but its clinical benefit depends on the tumor’s immune profile. Here, we used mIX-594 (IX), a targeted and GM-CSF–armed oncolytic vaccinia virus, as a strategy to remodel the tumor microenvironment (TME) and subsequently increase sensitivity to αPD-1 and/or αCTLA-4 immunotherapy.

Experimental Design: The remodeling of the TME was determined using histologic, flow-cytometric, and NanoString immune profiling analyses. IX was intratumorally injected into implanted Renca kidney tumors or MMTV-PyMT transgenic mouse breast cancers with or without αPD-1 and/or αCTLA-4. Various combination regimens were used to evaluate immunotherapeutic anticancer responses.

Results: Intratumoral injection of IX remodeled the TME through dynamic changes in the immune system, as shown by increased tumor-infiltrating T cells and upregulation of immune-related gene signatures. This remodeling induced conversion of a noninflamed tumor into an inflamed tumor. IX virotherapy led to enhanced abscopal effects in distant tumors, with increased intratumoral infiltration of CD8+ T cells. A depletion study revealed that GM-CSF is an indispensable regulator of anticancer efficacy of IX. Dual-combination therapy with intratumoral IX and systemic αPD-1 or αCTLA-4 further enhanced the anticancer immune response, regardless of various treatment schedules. Of note, triple combination immunotherapy with IX, αPD-1, and αCTLA-4 elicited the most potent antitumor immunity and induced complete tumor regression and long-term overall survival.

Conclusions: Our results show that intratumoral IX treatment induces dramatic remodeling of the TME and more potently suppresses cancer progression with immune-checkpoint blockades by overcoming resistance to immunotherapy.

Introduction

Cancer immunotherapy with immune-checkpoint inhibitors (ICI) targeting PD-1 or CTLA-4 has demonstrated a potent and durable therapeutic efficacy and emerged as a new weapon in the war on cancer (1–6). However, the clinical efficacy of ICIs is confined to tumors with a T cell–inflamed tumor microenvironment (TME; refs. 7, 8). In poorly immunogenic tumors with few tumor-infiltrating lymphocytes (TILs), TME lacks the type I interferon signature and chemokines for T-cell recruitment (9, 10). Moreover, tumor vasculatures and stromal components may pose a barrier against intratumoral trafficking of T cells and their effector functions on tumor cells (11–13). Therefore, additional therapeutic interventions are required for these non–T cell–inflamed tumors to appropriately remodel the TME to render these tumors more sensitive to ICI treatments (8, 14).

Oncolytic viruses have been proposed as a novel class of antitumor therapy, and viruses with different backbones and transgenes are currently being evaluated in clinical trials (15–17). Although the success of oncolytic viruses was initially predicted during the past decade based on their faster replication and enhanced oncolytic capability, they are now beginning to be recognized as an immunotherapeutic because the strongest and most durable responses after oncolytic virotherapy are coupled with successful induction of antitumor immunity with increased tumor-specific effector and memory T cells (16, 18–21). Nonetheless, because the therapeutic efficacy of oncolytic viruses was greatly hindered by the immunosuppressive TME, releasing the brakes of the immune system is critical to maximize the immunotherapeutic efficacy of oncolytic viruses (22–25). Therefore, the combination of oncolytic viruses and ICIs is a rational and appealing strategy to overcome the poorly immunogenic and immunosuppressive TME.

IX-594 (pexastimogene devacirepvec, Pexa-vec) is an oncolytic vaccinia virus that is engineered to express an immune-activating transgene, GM-CSF, and that has the viral thymidine kinase gene disrupted (26, 27). IX-594 showed impressive anticancer activity with low toxicity in preclinical and clinical studies. It has become
**Translational Relevance**

Cancer immunotherapies, such as immune-checkpoint inhibitors (ICIs), have demonstrated potent therapeutic efficacy. However, many cancer patients have immunosuppressive tumors, leading to resistance against immunotherapy and consequently limited therapeutic response. JX-594 (Pexa-vec) is one of the most promising oncolytic virus platforms in clinical development as one of the few oncolytic viruses in phase III clinical trials. Here, we show that a murine version of JX-594 (JX) remodels the tumor microenvironment by facilitating the accumulation of T cells. As a result, poorly immunogenic tumors become sensitive to ICIs, augmenting the immunotherapeutic efficacy. Of note, the triple combination therapy of JX, oPD-1, and oCTLA-4 maximizes anticancer immunity and induces durable regression with improved overall survival. These findings demonstrate the potential of JX in combination with ICIs for improving antitumor immune responses.

one of the most feasible and promising oncolytic virus platforms in clinical development as one of the few oncolytic viruses in phase III clinical trials (27–30). In addition to its oncolytic and vascular disrupting activity, JX-594 is proposed to exert an *in situ* cancer vaccination effect because it can elicit the adaptive immune response against tumor antigens for selective tumor disruption and subsequent additional tumor antigen release (31, 32). Although JX-594 is now in a phase III randomized clinical trial (NCT02562755) in advanced hepatocellular carcinoma (33), few studies have characterized its immune modulatory functions in primary TME as well as distant lesions after JX-594 treatment (34). Moreover, the optimal combination of JX-594 with immunotherapeutics such as ICIs has not yet been pursued and verified.

Here, we comprehensively dissected the dynamic remodeling of the TME with a mouse variant of JX-594 (mJX-594, WR. TK* mGm-CSF, hereafter referred to as JX) and investigated its immunotherapeutic potential to provide a rational combinatorial strategy with ICIs in poorly immunogenic tumor models.

**Materials and Methods**

**Mice and cell lines**

Male BALB/c mice between 6 and 8 weeks of age were purchased from Orient Bio Inc., and female MMTV-PyMT transgenic mice (FVB/N) were purchased from The Jackson Laboratory (#002374). Mice were housed in a specific pathogen-free animal facility at CHA University (Seongnam, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC, #170025) of CHA University and were carried out in accordance with the approved protocols. The Renca murine renal cancer cell line and the CT26 murine colon cancer cell line were obtained from the ATCC (#CRL-2947) and Korean Cell Line Bank (#KBC009). The human cancer cell lines HeLa S3 and U-2 OS were also originally obtained from ATCC (#CCL-2.2 and #HTB-96). These cells were maintained in RPMI-1640 medium or Dulbecco’s modified Eagle medium (DMEM), each supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and were incubated at 37°C, 5% CO₂ in an incubator. All cell lines were used within 10 passages and confirmed to be Mycoplasma free using the MycoAlert Mycoplasma Detection Kit.

**Generation and quantification of viruses**

mJX-594 (IX), provided by SillaJen, Inc., is a Western Reserve strain of vaccinia virus encoding murine GM-CSF in the vaccinia thymidine kinase gene locus under the control of the p7.5 promoter (35, 36). This virus was amplified in HeLa S3 cells prior to purification. In brief, HeLa S3 cells were infected and incubated with recombinant vaccinia virus for 3 days, collected by centrifugation, then homogenized and centrifuged once more. The virus-containing supernatant was layered onto a 36% sucrose cushion and centrifuged at 32,000 × g, and the purified viral pellet was resuspended in 1 mmol/L Tris, pH 7.0. To determine the viral titer, serially diluted virus in serum-free DMEM was applied onto a monolayer of U-2 OS cells for 2 hours, and then 1.5% carboxymethylcellulose in DMEM supplemented with 2% FBS was added.

After 72 hours, cells were stained with 0.1% crystal violet and plaques were counted.

**Tumor models and treatment regimens**

Tumors were implanted by subcutaneous injection of 2 × 10⁵ Renca cells into the right flank of wild-type BALB/c mice. When tumors reached >50 mm³, mice were treated with either PBS or 1 × 10⁶ plaque-forming units (pfu) of JX by intratumoral injection every 3 days. For the bilateral tumor model, 2 × 10⁵ Renca cells were implanted subcutaneously into the right flank, and 1 × 10⁵ Renca or CT26 cells were implanted subcutaneously into the left flank 4 days later. For the cell depletion study, antibodies against CD4 (200 μg, clone GK1.5; Bio X Cell), CD8 (200 μg, clone MP1-22E9; Bio X Cell), or GM-CSF (200 μg, clone MIP1-22E9; Bio X Cell) were intraperitoneally injected along with intratumoral JX treatment. For immune-checkpoint blockade, anti–PD-1 (10 mg/kg, clone 43B; Bio X Cell) and/or anti–CTLA-4 (4 mg/kg, clone 9D9; Bio X Cell) antibodies were injected intraperitoneally every 3 days depending on the dosing schedule. Tumors were measured every 2 or 3 days using a digital caliper, and tumor volumes were calculated using the modified ellipsoid formula \(V = \frac{1}{2} (length \times width^2)\). On day 50, the surviving mice with complete tumor regression were rechallenged with 2 × 10⁵ Renca or CT26 cells in the left flank and monitored for tumor growth and survival. Mice were euthanized when tumors reached 1.5 cm in diameter or when mice became moribund. Female MMTV-PyMT transgenic mice were purchased from The Jackson Laboratory. Starting at 9 weeks after birth, the volume of every palpable tumor node (≥20 mm³) was measured, and the total volume of all tumors combined was used to calculate the tumor burden per mouse. MMTV-PyMT mice were randomized according to their initial tumor burden, and were treated with 4 × 10⁶ pfu of JX with or without anti–PD-1 (10 mg/kg) or anti–CTLA-4 (4 mg/kg) antibodies at the indicated time points. In particular, JX was injected intratumorally (1 × 10⁷ pfu per tumor) in 4 randomly selected palpable tumors. After 4 weeks of treatment, mice were anesthetized, and tissues were harvested for further analyses. Analyses for MMTV-PyMT were performed as previously described (37, 38).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software) and PASW statistics 18 (SPSS). Values are represented as mean ± standard error of the mean unless otherwise indicated. Statistical differences between means...
were tested using unpaired Student t tests. Survival curves were generated using the Kaplan–Meier method, and statistical differences between curves were analyzed using the log-rank test. The level of statistical significance was set at $P < 0.05$.

**Results**

**JX converts immunosuppressive noninflamed tumors into inflamed tumors**

To determine the immunomodulatory potential of the oncolytic virus JX, we extensively examined temporal changes in TME after single intratumoral injections of JX into the Renca tumors, which are resistant to ICIs (39). The tumoral level of JX was already high at day 1, peaking at day 3, but was barely detectable at day 7 after the injection (Fig. 1A and B). Conversely, tumor vessel density was markedly reduced between days 1 and 3 but was recovered at day 7 and thereafter after the injection (Fig. 1A and B; Supplementary Fig. S1B and S1C), indicating that JX is a potent but transient tumor vessel disruptor. Of note, the population of CD8$^+$ cytotoxic T cells within the tumor, which comprise the most critical aspect of anticancer immunity, began to increase strikingly at day 5, peaking at day 7, and remaining at a high density at 2 weeks after injection (Fig. 1A and B), demonstrating distinct and long-lasting conversion of the noninflamed tumor into a T cell–inflamed tumor by JX. By comparison, CD11c$^+$ dendritic cells (DC) transiently emerged at day 3 and then decreased in tumors. However, DCs accumulated in draining lymph nodes from day 5 where they interacted with CD8$^+$ T cells (Fig. 1A; Supplementary Fig. S1D). In addition, the level of PD-L1 was low at day 0 and upregulated after JX treatment (Fig. 1A and B). Intriguingly, the PD-L1 upregulation followed just after a massive influx of CD8$^+$ TILs (Fig. 1C), indicating activation of the PD-1/PD-L1 axis in an attempt to negatively regulate T cell–mediated immunity. Most PD-L1$^+$ cells were cytokeratin$^+$ tumor cells, and some were CD11b$^+$ myeloid but were not T cells (Fig. 1D). Thus, JX is not only a transient tumor vessel disruptor but also a potent and durable anticancer immunity enhancer.

To elucidate the cancer immune pathways modulated by JX, we further analyzed changes in expression of 750 immune-related genes in the Renca tumor following JX monotherapy, using a PanCancer Immune Profiling panel. Of note, expression levels of the genes (~100 genes) related to immune modulation, including activation of type I IFN signaling, DC maturation, and T-cell activation, were significantly different between control- and JX-treated tumors (Fig. 1E and F). In particular, the genes related to inhibitory immune checkpoints (Pd-1, Pd-1L, Cila-4, and Lag-3) and agonistic immune checkpoints (Icos, Gitr, and Cd27), Th1 and Th2 responses, and M1 macrophage polarization (Nos2 and Cdb6) were upregulated in JX-treated tumors compared with control-treated tumors (Fig. 1G). These results indicate that JX elicits long-term immune activation through dynamic changes in the TME to remodel noninflamed tumors into T cell–inflamed tumors that can respond to ICIs.

**JX augments intratumoral infiltration of CD8$^+$ T cells and induces myeloid cell repolarization**

JX-induced delay of tumor growth was dose-dependent (Supplementary Fig. S2A). In parallel, JX-induced increases in CD8$^+$ T-cell infiltration in both peritumoral and intratumoral regions were also dose-dependent (Supplementary Fig. S2B). Indeed, flow-cytometric subset analysis of the lymphoid cell compartment revealed that the JX-induced increase in absolute numbers of intratumoral CD8$^+$ and CD4$^+$ T cells was dose-dependent (Supplementary Fig. S2C and S2D). Although the number of CD4$^+$Foxp3$^+$CD25$^+$ regulatory T cells increased following the triple administration of JX (Supplementary Fig. S2E), the ratio of CD8$^+$ T cells to regulatory T cells was 5.3-fold higher compared with that of control treatment (Supplementary Fig. S2E), implicating an overall increase in T-cell effector function in the TME by JX treatment. Additionally, the expression of ICOS and granzyme B (GzB), which are costimulatory and T-cell activation markers, was increased in CD8$^+$ T cells following JX treatment (Supplementary Fig. S2F). To confirm the presence of tumor-specific T cells that were recruited into the tumor after JX treatment, IFN-$\gamma$ ELISPOT assays were performed with isolated TILs and splenocytes. The result was a marked increase in IFN-$\gamma$-secreting T cells against Renca tumor cells within tumors and spleens of JX-treated mice compared with control mice. This finding indicated the presence of tumor-specific CD8$^+$ T cells in tumors as well as in the lymphoid organ (Supplementary Fig. S2G). Further subset analysis of the myeloid compartment revealed no significant change in CD11b$^+$Gr1$^+$ myeloid cell fraction in tumors treated with JX (Supplementary Fig. S2H). However, the CD11b$^+$Ly6G$^+$Ly6C$^+$ monocytic myeloid cell fraction was increased, whereas the CD11b$^+$Ly6G$^+$Ly6C$^+$ granulocytic myeloid cell fraction was reduced, indicating polarization of myeloid cells following JX treatment (Supplementary Fig. S2I). These findings demonstrate that repeated JX administration enhances anticancer immunity, leading to increased infiltration of activated T cells and repolarization of myeloid cells.

**Intratumoral injection of JX leads to systemic and cancer-specific immune responses**

To determine whether local injection of JX could induce a systemic immune response for regulating noninjected distant tumors, we administered JX into the right-side tumor after implantation of Renca tumor cells into both side flanks. This treatment suppressed the growth of both right and left (opposite, not injected side) Renca tumors (Fig. 2A). In line with tumor growth inhibition on both sides, infiltrations of CD8$^+$ T cells at intratumoral regions were increased by 7.9- and 5.5-fold in both right and left Renca tumors (Fig. 2B), suggesting that local JX virotherapy can activate systemic anticancer immunity.

Next, to exclude the possibility of direct viral spread to the distant tumors through systemic circulation after the local virotherapy, we examined the presence of JX in the left, noninjected Renca tumors and found no immune-detective JX in the left tumors (Supplementary Fig. S3). This finding indicated that the anticancer activity of JX in distant tumors was systemically immune-mediated and not a result of systemic viral spread.

To evaluate whether the observed systemic immune response was tumor-specific, we performed a similar experiment using mice implanted with Renca tumors on the right flank and CT26 tumors on the left flank. Intratumoral treatment of the right, Renca tumor with JX markedly decreased the growth of the injected tumor, whereas the growth of the left, untreated CT26 tumor was unaffected (Fig. 2C). Moreover, the number of CD8$^+$ T cells was not changed in CT26 tumors but was highly increased in Renca tumors (Fig. 2D), indicating that JX virotherapy induces a...
Figure 1.

JX converts immunosuppressive noninflamed tumors into inflamed tumors. Renca tumors were implanted subcutaneously (s.c.) into BALB/c mice and treated with a single intratumoral injection of $1 \times 10^7$ plaque-forming units (pfu) of mJX-594 (JX) when tumors reached $>50 \text{mm}^3$. A, Representative images of Renca tumors treated with JX. Tumor sections were stained for JX, CD31, CD8, CD11c, and PD-L1. B, Quantifications of the JX⁺ area, CD31⁺ blood vessels, CD8⁺ cytotoxic T cells, CD11c⁺ dendritic cells, and PD-L1⁺ cells. *, $P<0.05$ versus day 0. C, Temporal changes in JX, CD8, and PD-L1 in the TME after JX treatment. D, Images showing upregulated PD-L1 expression (red) in various cell types (green) within the TME after JX treatment. Note that the expression of PD-L1 was observed mainly in Pan-CK⁺ tumor cells (arrowheads), and some CD11b⁺ myeloid cells (arrow) also occasionally expressed PD-L1, whereas CD3⁺ T cells did not. E, NanoString immune-related gene-expression heat map. Red and green colors represent upregulated and downregulated genes, respectively. F, Volcano plot showing changes in immune-related gene expression in JX-treated tumors. Red line, $P<0.05$. G, Comparisons of gene expressions related to inhibitory immune checkpoints (IC), agonistic ICs, Th1 response, Th2 response, TME, and myeloid cell. Pooled data from two experiments with 5 animals per group. Values are mean ± SEM. *, $P<0.05$ versus control. Two-tailed Student t-test was used. Scale bars, 50 µm.
Chon et al.

Anticancer immunity plays a critical role in the overall therapeutic efficacy of JX

To determine which components of the immune system are responsible for the therapeutic efficacy of JX, we examined its effect on tumors in mice treated with neutralizing antibodies against CD8, CD4, or GM-CSF (Supplementary Fig. S4A). Of special note, depletion of either CD8\(^+\) or CD4\(^+\) T cells abrogated the effective tumor growth inhibition by JX (Supplementary Fig. S4B and S4C), emphasizing the importance of an immune-mediated mechanism rather than direct oncolysis in JX-induced tumor growth inhibition. Intriguingly, depletion of CD4\(^+\) T cells at the time of JX injection reduced intratumoral infiltration of CD8\(^+\) T cell (Supplementary Fig. S4D), indicating that CD4\(^+\) T cells are involved in the activation and recruitment of CD8\(^+\) T cells in the TME. However, depletion of CD8\(^+\) T cells did not significantly alter infiltration of CD4\(^+\) T cells (Supplementary Fig. S4D), indicating that CD8\(^+\) T cells did not affect CD4\(^+\) T cells in the TME. These findings indicate that intratumoral JX treatment induces priming of CD8\(^+\) and CD4\(^+\) T cells, which may interact with each other to mediate anticancer immunity.

Previous virotherapy based on herpes and vaccinia viruses used GM-CSF as an immune-activating transgene, which recruits and activates antigen-presenting cells that subsequently trigger T-cell response (40). However, the use of GM-CSF is still controversial because of its potential immunosuppressive roles in tumor progression, such as inducing proliferation of myeloid-derived suppressor cells (18). Therefore, we explored whether GM-CSF is required for the therapeutic effect of JX. Interestingly, depletion of GM-CSF negated the antitumor effect of JX and reduced both CD8\(^+\) and CD4\(^+\) T cell levels, suggesting that GM-CSF is critical for the immunotherapeutic efficacy of JX (Supplementary Fig. S4C and S4D). Thus, both CD8\(^+\) and CD4\(^+\) T cells are indispensable mediators of the anticancer effect of JX, and GM-CSF is an essential regulator of T-cell activation for the JX treatment.

Combination of JX with immune-checkpoint blockade elicits an enhanced anticancer effect with augmented infiltration of T lymphocytes into the tumor

As shown earlier, whereas JX inflames the TME by enhancing the recruitment of CD8\(^+\) T cells, it concomitantly increases the expression of PD-L1, which hinders the anticancer effects of cytotoxic T cells. On the other hand, ICI monotherapy is ineffective in noninflamed, T cell–insufficient tumors (8). Therefore, we sought to combine the two modalities to compensate for their respective weaknesses.

The combination of anti–PD-1 antibody (aPD-1) and JX reduced tumor growth by 70%, whereas aPD-1 and JX mono-therapy delayed tumor growth by 23% and 44%, respectively (Fig. 3A). In support of these findings, CD8\(^+\) T cells were more highly infiltrated in both peritumoral (2.5-fold) and intratumoral (2.4-fold) regions of the tumors treated with combination therapy than those treated with JX (Fig. 3B and C). Furthermore, CD31\(^+\) tumor blood vessels were decreased by combination therapy compared with control (peritumoral and intratumoral regions, 1.8-fold and 2.6-fold, respectively; Fig. 3B and C; Supplementary Fig. S5A), and tumor apoptosis was most severely induced in tumors treated with combination therapy compared with all other groups (Fig. 3B and C; Supplementary Fig. S5B). Similarly to our initial findings (Fig. 1A–C), although the PD-1-L1 expression was minimal in control tumors, it was upregulated by 2.1- to 3.7-fold in both peritumoral and intratumoral regions of JX-treated
Combination of JX with αPD-1 elicits an enhanced anticancer effect with augmented infiltration of T lymphocytes into the tumor. Renca tumor-bearing mice were treated with or without JX and αPD-1 on the indicated days (arrows). A, Comparisons of tumor growth. Mean and individual tumor growth curves over time. B and C, Representative images (B) and comparisons (C) of CD8⁺ T cells, CD31⁺ blood vessels, activated caspase-3 (casp-3)⁺ apoptotic cells, and PD-L1⁺ cells in the peritumoral and intratumoral regions. D, Diagram depicting the mechanism by which the immunosuppressive TME is overcome by a combination therapy of JX and ICI. Pooled data from two experiments with 7 animals per group. Values are mean ± SEM. *P < 0.05 versus control; #, P < 0.05 versus JX; $, P < 0.05 versus αPD-1; ns, not significant. Two-tailed Student t test was used. Scale bars, 100 μm.
tumors (Fig. 3B and C; Supplementary Fig. S5B), implying that PD-L1 involves an adaptive negative feedback mechanism that dampens anticancer immunity after oncolytic virotherapy.

Next, to determine whether combination therapy is effective against distant untreated tumors as well as injected tumors, we treated mice carrying bilateral Renca tumors with JX and/or αPD-1 (Supplementary Fig. S5C). The combination therapy more potently suppressed the growth of distant untreated tumors compared with JX or αPD-1 monotherapy.

Therefore, our findings indicate that combining JX and ICIs not only potentiates the systemic immunotherapeutic effect of JX virotherapy but also overcomes resistance against ICIs monotherapy through enhanced anticancer immunity by increasing CD8⁺ T-cell infiltration (Fig. 3D).

We further validated our hypothesis by testing the efficacy of combination treatment with anti–CTLA-4 antibody (αCTLA-4) and JX. Although tumor growth was modestly inhibited by either JX (42.0%) or αCTLA-4 (20.0%) monotherapy, combination therapy displayed the most potent inhibitory effect (57.6%; Supplementary Fig. S6A). In addition, CD8⁺ T cells were more highly accumulated in both peritumoral (1.9-fold increase) and intratumoral (1.9-fold increase) regions of tumors treated with combination therapy compared with JX (Supplementary Fig. S6B and S6C). CD31⁺ tumor blood vessels were also disrupted in both peritumoral and intratumoral regions of combination therapy–treated tumors compared with control (2.1-fold and 3.8-fold reductions, respectively; Supplementary Fig. S6B and S6C). Furthermore, flow cytometry revealed that intratumoral infiltration of CD8⁺ and CD4⁺ T cells was also increased by JX and αCTLA-4 combination therapy (Supplementary Fig. S6D). Taken together, these results indicate that combination therapy using JX and ICIs can overcome the resistance against immunotherapy in immunosuppressive TMEs, resulting in enhanced anticancer effects.

The efficacy of combination immunotherapy with intratumoral JX and ICIs is not largely affected by treatment schedule

Because ICIs can negatively affect viral replication and lead to premature clearance of the oncolytic virus, previous studies explored the optimal schedules of treatment using combinations of systemic oncolytic virotherapy and ICIs and reported that some combination schedules could antagonize the therapeutic efficacy (22, 41). However, the dependency of local oncolytic virotherapy on the treatment schedule of ICIs has not been reported. To establish the optimal combination schedule for intratumoral JX and ICIs, we compared the following: (i) simultaneous administration of JX and ICI (schedule I); (ii) initiation of ICI 3 days after administration of JX (schedule II); and (iii) administration of JX 3 days after initiation of ICI (schedule III; Fig. 4A). All combination schedules delayed tumor growth by ~40% (Fig. 4B). Likewise, levels of tumor-infiltrating CD8⁺ and CD4⁺ T cells were increased by >8.0-fold and >4.0-fold, respectively, and the expression of ICOS and GzB in CD8⁺ T cells was remarkably increased compared with control regardless of the treatment schedule (Fig. 4C and D).

Similar to combination therapy with JX and αPD-1, the combination of JX and αCTLA-4 inhibited tumor growth by ~40% regardless of the treatment schedule (Supplementary Fig. S7A). Furthermore, intratumoral infiltration of CD8⁺ and CD4⁺ T lymphocytes (>7-fold and >7-fold increases, respectively) and GzB and ICOS expression in CD8⁺ T cells were greater regardless of the treatment schedule (Supplementary Fig. S7B and S7C).

The therapeutic efficacy of concurrent combination therapy with ICIs and oncolytic viruses varies depending on the virus administration route because viral clearance by adaptive immunity may differ when the oncolytic virus is injected either systemically (intravenous) or locally (intratumoral; refs. 22, 41). Therefore, we hypothesized that the administration route could affect the efficacy of concurrent combination therapy. To test this hypothesis, we compared intravenous versus intratumoral injection of JX concurrently with αPD-1 (Supplementary Fig. S8A and S8B). Intriguingly, in tumors treated with intravenous JX, JX tumoral levels were remarkably reduced with concurrent αPD-1 treatment. In contrast, in tumors treated with intratumoral JX, concurrent αPD-1 treatment had almost no effect on tumoral levels of JX. Therefore, concurrent αPD-1 treatment seems less likely to affect JX if JX is administered via intratumoral injection.

Collectively, combination therapy with intratumoral JX injection and systemic ICI led to an effective anticancer immunity regardless of treatment schedule, suggesting that intratumoral administration of JX could minimize the potential antagonism with systemic ICI treatment.

Triple combination of JX, αPD-1, and αCTLA-4 induces profound tumor regression and provides a long-term survival benefit in implanted kidney cancer

As dual combination of JX and ICIs did not induce complete tumor regression, we explored the effect of triple combination therapy using JX, αPD-1, and αCTLA-4. Although the dual combination of αPD-1 and αCTLA-4 delayed tumor growth by 14.5% and JX monotherapy inhibited tumor growth by 36.9%, the triple combination inhibited tumor growth by 76.5% (Fig. 5A). Of note, a few mice (~40%) of this triple combination group exhibited complete tumor regression, which was not observed in any other groups (Fig. 5B). Furthermore, mice with complete tumor regression were tumor-free for more than 14 weeks after treatment cessation. They were also fully protected against rechallenge with Renca tumor cells but were not immune to CT26 tumor cells, suggesting the establishment of an effective, long-term, and tumor-specific immune memory (Fig. 5C).

To establish that the potent anticancer effects induced by triple combination therapy could translate into a long-term survival benefit, we performed survival analyses of tumor-bearing mice (Fig. 5D). Mice treated with triple combination immunotherapy showed a remarkably better overall survival compared with results with monotherapy or dual-combination therapy. Intriguingly, the difference between dual and triple combination therapy was not remarkable early in the treatment period, but it increased over time and was maintained for a long time. Therefore, triple combination therapy is needed to induce durable immunotherapeutic effects and longer survival. In conclusion, these findings demonstrate that triple combination immunotherapy has the potential to induce complete tumor regression and long-term survival.

The triple combination therapy enhances anticancer immune responses in a spontaneous breast cancer model

To validate the long-term immunotherapeutic efficacy of the triple combination therapy in immune-resistant tumors, we used the MMTV-PyMT transgenic mouse model, which is a spontaneous breast cancer model with intrinsic resistance to immunotherapy (42). After 4 weeks of treatment, mice treated with the triple combination of JX, αPD-1, and αCTLA-4...
exhibited a significant reduction in overall tumor burden by 48.1% and a delay in the development of palpable tumor nodules compared with control mice (Fig. 6A–D). Furthermore, triple combination therapy led to a 48.1% reduction in average tumor nodule size and better overall survival compared with other treatments (Fig. 6E and F). Histologic analyses revealed less-invasive carcinoma with well-preserved tumor margins in the triple combination group, indicating that triple combination effectively delays tumor progression and invasion (Fig. 6G). Moreover, intratumoral recruitment of CD8+ T cells was further increased by 2.0-fold in tumors treated with triple combination therapy compared with those treated with JX monotherapy (Fig. 6H). However, tumor vascular density was similar among the treatment groups (Fig. 6H), indicating that the vascular disrupting effect is not long-lasting after repeated JX injections. Finally, the number of hematogenous lung metastases was significantly reduced in the triple combination group (Fig. 6I), indicating an effective antitumor action by the triple combination therapy. Taken together, these results demonstrated that triple combination immunotherapy with JX and ICIs can elicit a robust anticancer immune response even in a poorly immunogenic spontaneous breast cancer model.

Discussion

Here, we demonstrate that combination therapy with JX and ICIs is an effective therapeutic strategy for immune-resistant tumors. The combination therapy leads to an immunologic "boiling point" in which a cold, noninflamed tumor is sufficiently inflamed to enable the host immune system to eradicate tumor cells. The most profound effect was observed with triple immunotherapy with JX, αPD-1, and αCTLA4, which induced complete regression in ~40% of Renca tumors, one of the most resistant syngeneic tumors to immunotherapy. This strong efficacy can be explained by the mutually complementary cooperation of oncolytic virus and ICIs.

JX-594 is an oncolytic virus in the most advanced stage of clinical trials and acts through various mechanisms (27, 32, 33).
Although it can rapidly induce direct oncolysis and vascular disruption in tumors, these effects are transient and mostly diminish within 1 week of injection. Thereafter, CD8+ T cells extensively infiltrate the tumor to initiate anticancer immune responses. However, at the same time, tumors begin to evolve to avoid immune-mediated elimination by upregulating immune inhibitory checkpoint molecules such as PD-1, PD-L1, or CTLA-4 in the TME. Because the most potent and durable anticancer effects of an oncolytic virus are achieved when it is coupled with successful induction and maintenance of antitumor immunity, it is reasonable to combine ICIs with oncolytic virus to prevent early shutdown of oncolytic virotherapy–induced anticancer immunity (18).

Although ICI monotherapy revolutionized the treatment landscape of cancer, its dramatic therapeutic response is confined to a subset of patients (1, 43). This outcome gave rise to the concept of immunologically "hot" or "cold" tumors: hot tumors respond well to ICIs because they are immunologically inflamed with TILs and show high expression of PD-L1, whereas cold tumors are refractory to ICIs because of the paucity of CD8+ TILs and immunosuppressive TME (9, 24). Therefore, current efforts are focused on overcoming resistance to ICIs by converting...
Figure 6.
The triple combination therapy delays tumor growth and metastasis in a spontaneous breast cancer model. Growth of spontaneous mammary tumors of MMTV-PyMT mice was analyzed starting from 9 weeks after birth. Samples were harvested 13 weeks after birth. **A,** Diagram depicting the treatment schedule. Arrows indicate treatment with or without intratumoral delivery of JX and systemic delivery of αPD-1 (P) and αCTLA-4 (C). **B,** Representative image showing gross appearance of tumors. Dotted-line circles demarcate palpable mammary tumor nodules. **C,** Comparison of total tumor burden. Tumor burden was calculated by summing the volume of every tumor nodule per mouse. **D,** Comparison of volume of each tumor nodule. Each tumor nodule in MMTV-PyMT mice is plotted as an individual dot. **E,** Kaplan-Meier curves for overall survival. Log-rank test was used. **F,** H&E-stained tumor sections showing intratumoral regions. Acinar structures of JX and JX + P + C groups are early, less-invasive lesions (Ea) showing the distinct boundary with the surrounding mammary adipose tissue (Adi). Invasive ductal carcinoma regions (Ca) of Cont and P + C have massively invaded into the surrounding tissue and formed solid sheets of tumor cells with no remaining acinar structure. **H,** Representative images and comparisons of CD8⁰ T cells and CD3¹ tumor blood vessels in tumor. **I,** Representative lung sections stained with H&E and comparison of the number of metastatic colonies per lung section. Arrows indicated metastatic foci. Unless otherwise denoted, n = 8–9 for each group. Values are mean ± SD. * P < 0.05 versus control, † P < 0.05 versus JX, ‡ P < 0.05 versus αPD-1, § P < 0.05 versus αCTLA-4. ns, not significant. Two-tailed Student t test was used in C–E, H, and I. Scale bars, 200 μm.
immunologically cold tumor to hot tumors. In this respect, our result identifies JX as an ideal combination partner for ICIs. It can selectively replicate in tumor cells, destroy them, and release tumor antigens to stimulate the host immune system. Moreover, our study shows that JX can dramatically convert the TME from a cold to hot state by inducing intratumoral inflammatory responses: induction of Th1 responses along with activation and recruitment of T cells, upregulation of PD-L1, and polarization of myeloid cells toward M1. Intriguingly, the replication and spread of oncolytic viruses are more active in cold tumors where there are few immune cells to eliminate the virus, whereas hot tumors with ample resident TILs can induce premature clearance of virus and attenuate its therapeutic effects (24). Therefore, together with the results of this study, JX emerges as an optimal combination partner for ICIs, especially for noninflamed cold tumors with intrinsic resistance to immunotherapy.

GM-CSF is the most commonly used therapeutic genetic payload of oncolytic viruses (18, 44, 45). Two oncolytic viruses in the most advanced phases of clinical trials, T-Vec and Pexa-Vec (JX-594), are both armed with GM-CSF (46). Although GM-CSF is generally known to induce proliferation of various immune cells such as DCs, there is a concern regarding unwanted proliferation of immunosuppressive cells such as myeloid-derived suppressor cells (23, 47). In the present study, we revealed that JX did not significantly alter the fraction of intratumoral CD11b⁺Gr1⁺ cells. In addition, neutralization of GM-CSF ablated the therapeutic efficacy of JX, which was partly because of the reduction in CD8⁺ and CD4⁺ TILs, indicating that GM-CSF has an indispensable role in antitumor immunity elicited by JX.

Previous studies have reported that although the combination of an oncolytic virus and ICIs elicits an impressive immune response, the therapeutic efficacy can be affected by administration route and treatment schedule (22, 41, 48). In particular, when both the oncolytic virus and ICIs are systemically administered simultaneously, the combination could be antagonistic because of the ICI-induced antiviral immunity that can facilitate premature viral clearance, indicating the importance of an adequate time gap between treatments for the oncolytic virus to induce a successful antitumor immunity (41, 49). In the present study, local injection of JX consistently induced antitumor immunity without being significantly affected by administration sequences. We presume that this result is attributable to the intratumoral injection having provided the oncolytic virus a sufficient time lag to inflame the TME before being detected and eliminated by systemic antiviral immunity. Indeed, in tumors treated with intratumoral JX, concurrent αPD-1 treatment had almost no effect on the tumoral level of JX, in contrast to the markedly decreased level of JX in tumors treated with intravenous JX. Therefore, intratumoral virotherapy may be more suitable for designing clinical trials with the ICI and oncolytic virus combination compared with systemic virotherapy in terms of administration schedule.

In this study, we were not able to exclude the possibility that the immunogenicity of mouse model was affected by a tumor implantation–induced inflammatory reaction (50). Although we performed every treatment 10 or 12 days after tumor implantation to minimize inflammatory reaction, the level of the response to treatment that we observed in this study may not fully reflect the immune reaction in human cancer. Therefore, the findings of this preclinical study should be confirmed in clinical trials.

Several clinical trials are ongoing to investigate the efficacy of JX-594 in combination with αPD-1, αCTLA-4, or αPD-L1 to target various solid cancers, including liver, kidney, and colon cancers (ClinicalTrials.gov: NCT03071094, NCT02977156, NCT03294083, and NCT03206073). Thus, we will be able to verify the findings of this study in a clinical setting in the near future.

In conclusion, these results indicate that intratumoral injection of JX induces a profound remodeling of the TME from cold to hot state and elicits robust antitumor immunity in combination with ICIs, overcoming immunotherapy resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Potential of Immunotherapy by Oncolytic Virus


