Intratumoral expression of IL-7 and IL-12 using an oncolytic virus increases systemic sensitivity to immune checkpoint blockade

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The immune status of the tumor microenvironment is a key indicator in determining the antitumor effectiveness of immunotherapies. Data support the role of activation and expansion of tumor-infiltrating lymphocytes (TILs) in increasing the benefit of immunotherapies in patients with solid tumors. We found that intratumoral injection of a tumor-selective oncolytic vaccinia virus encoding interleukin-7 (IL-7) and IL-12 into tumor-bearing immunocompetent mice activated the inflammatory immune status of previously poorly immunogenic tumors and resulted in complete tumor regression, even in distant tumor deposits. Mice achieving complete tumor regression resisted rechallenge with the same tumor cells, suggesting establishment of long-term tumor-specific immune memory. Combining this virotherapy with anti–programmed cell death-1 (PD-1) or anti–cytotoxic T lymphocyte antigen 4 (CTLA4) antibody further increased the antitumor activity as compared to virotherapy alone, in tumor models unresponsive to either of the checkpoint inhibitor monotherapies. These findings suggest that administration of an oncolytic vaccinia virus carrying genes encoding for IL-7 and IL-12 has antitumor activity in both directly injected and distant noninjected tumors through immune status changes rendering tumors sensitive to immune checkpoint blockade. The benefit of intratumoral IL-7 and IL-12 expression was also observed in humanized mice bearing human cancer cells. These data support further investigation in patients with non-inflamed solid tumors.

INTRODUCTION

Immunotherapies have provided remarkable clinical benefit to patients with different types of cancer. Systemic immune checkpoint blockade with anti–programmed cell death-1 (PD-1)/PD-L1 and anti–cytotoxic T lymphocyte antigen 4 (CTLA4) antibodies has demonstrated durable clinical responses and prolonged survival in patients with solid tumors (1–4). Combination therapy with anti–PD-1 antibody and anti–CTLA4 antibody in a metastatic melanoma clinical study showed a synergistic clinical response compared to the corresponding monotherapies, although the efficacy was still limited and toxicities related to systemic immune activation were of concern (5, 6). To optimize clinical response with minimum toxicity, further evaluation is needed using different immune checkpoint inhibitors, agonistic agents for stimulatory molecules, inflammatory cytokines, and their combinations (7, 8).

Local immunotherapies are a rational approach toward minimizing unexpected systemic toxicity. Among these, tumor-selective oncolytic viruses are promising candidates with potential for in situ vaccination against cancer (9, 10). Oncolytic viruses, which selectively replicate in the tumor microenvironment, are able to destroy cancer cells, exposing tumor antigens to antigen-presenting cells. Damage- or pathogen-associated molecular patterns are coincidentally released, which enhances antitumor immunity (10). It is worth noting that oncolytic viruses with tumor selectivity can be vectors for the delivery of immunomodulators into tumors for further modification of the tumor microenvironment. Treatment with tumor-selective oncolytic viruses carrying genes encoding inflammatory cytokines is expected to enhance antitumor immune responses in the tumor microenvironment, and a number of oncolytic viruses have been evaluated for several cancers (11). Talimogene laherparepvec (T-VEC), which was approved in the United States in 2015 as a first-in-class oncolytic agent for metastatic melanoma, is an engineered herpes simplex virus with insertion of genes encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) as an immune adjuvant (12–14). Pexastimogene devacirepvec (Pexa-Vec, JX-594), which also encodes GM-CSF (15, 16), has been evaluated in a phase 3 clinical trial in patients with advanced hepatocellular carcinoma. However, it is unclear whether further immune activation by transgenes results in better clinical outcomes because the enhanced inflammatory status potentially accelerates the exclusion of oncolytic viruses and decreases oncolytic activity and transgene expression (17–20).

Here, we propose intratumoral immune status changes by dual expression of interleukin-7 (IL-7) and IL-12 using an oncolytic vaccinia virus platform as both a monotherapeutic approach and a promising partner in combination with immune checkpoint blockade. IL-12 is a cytokine that activates both innate and adaptive immunity, partially due to interferon-γ (IFN-γ) secretion from natural killer (NK) cells, CD8+ T cells, and CD4+ T cells (21). Although in a past clinical study systemic administration of IL-12 caused severe adverse events, IL-12 remains an attractive candidate for cancer immunotherapy, and several clinical trials that include locally controlled IL-12 therapies are now underway (22–24). IL-7 is crucial for T cell homeostasis and, importantly, causes synergistic stimulation of T cells in vitro when combined with IL-12 (25, 26). In our evaluation, vaccinia virus encoding both IL-7 and IL-12 completely changed the tumor immune microenvironment by boosting the inflammatory immune status, showed beneficial systemic antitumor efficacy, and
markedly improved the sensitivity of solid tumors to systemic anti-PD-1 and anti-CTLA4. These findings provide strategies for overcoming tumors resistant to immunotherapies and provide a rationale for further evaluation in humans.

RESULTS

Increased TILs after oncolytic vaccinia virus administration

To characterize the effects of oncolytic vaccinia virus, we evaluated its use in three different immunocompetent mouse models. The oncolytic vaccinia virus we used here is a recombinant virus based on an attenuated vaccine strain, LC16mO, with functional deletion of vaccinia virus growth factor (VGF) and O1L for selective replication in cancer cells (27–30), and with further modification of B5R membrane protein for reduced antigenicity (31, 32); hereafter, it will be referred to as Cont-VV. Intratumoral administration of Cont-VV demonstrated antitumor activity in all three models tested, with tumor growth inhibitions of 92.9% for the B16-F10 melanoma model, 53.3% for the CT26.WT colon carcinoma model, and 53.3% for the LLC lung carcinoma model (Fig. 1A). There was no correlation between in vivo efficacy and in vitro cytotoxicity against these murine cell lines (fig. S1), suggesting that in mouse models the direct oncolytic activity is not the only contributor to the antitumor effect and that other mechanisms might be involved, such as immune responses in the tumor microenvironment. As we expected, intratumoral infiltration of CD8+ T cells, CD4+FoxP3− conventional T cells (T_{conv}), and NKT cells was increased after the administration of Cont-VV in all three models (Fig. 1, B to D). In addition, increases in the numbers of intratumoral CD4+FoxP3+ regulatory T cells (T_{regs}) were observed in all models, whereas the percentages of T_{regs} in CD4+ T cells were

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Fig. 1. Oncolytic vaccinia virus shows antitumor responses and increases in tumor-infiltrating lymphocytes. (A) Mice were subcutaneously inoculated with B16-F10, CT26.WT, or LLC cells. When tumors reached about 50 mm³, tumors were directly injected with PBS or Cont-VV (4 × 10⁷ pfu) every other day, for a total of three injections. Tumor growth in mice treated with PBS or Cont-VV is shown (n = 12 for B16-F10 and CT26.WT, n = 8 for LLC per group). *P < 0.05 and ***P < 0.001 by unpaired t test. (B to G) Eleven days after the last treatment, tumors were collected and analyzed by flow cytometry to calculate the percentages of TILs in tumor cells. (B to D) Intratumoral CD8+ T cells, T_{conv}, NKT cells, and NK cells in B16-F10 (B), CT26.WT (C), and LLC tumor models (D). (E to G) Intratumoral T_{regs} and the percentages of T_{regs} in CD4+ T cells in B16-F10 (E), CT26.WT (F), and LLC tumors (G). n = 11 to 12 for B16-F10 and CT26.WT and n = 8 for LLC. **P < 0.01 and ***P < 0.001 by Mann-Whitney U test. ns, not significant. Mean ± SEM is shown.
Immune status changes with improved efficacy by IL-7 and IL-12

Although Cont-VV demonstrated antitumor activity with intratumoral immune responses in immunocompetent mouse models, the tumor growth inhibition observed in CT26.WT and LLC models was partial. Because we hypothesized that further up-regulation of inflammatory status in the tumor microenvironment by exogenous inflammatory cytokines may improve the antitumor responses, we especially focused on the biological activity of IL-7 and IL-12 and examined the effects of their intratumoral expression, separately and together, using a vaccinia virus carrying human IL-7 (hIL-7-VV; human IL-7 is cross-reactive for mouse immune cells (33)) and a vaccinia virus carrying murine IL-12 (mIL-12-VV). In the LLC model, intratumoral administration of hIL-7-VV and mIL-12-VV in combination led to dose-dependent secretion of human IL-7 and murine IL-12 proteins in the injected tumors (fig. S2, A and B) with induction of increased intratumoral murine IFN-γ protein compared to Cont-VV (fig. S2C), indicating that intratumoral secretion of IL-7 and IL-12 promoted an inflammatory response in the tumor microenvironment. The combination of hIL-7-VV and mIL-12-VV elicited higher numbers of tumor-infiltrating CD8+ T cells, CD4+ T cells, NKT cells, and NK cells compared to either of the agents alone or to Cont-VV (Fig. 2A). This combination did not increase M1 macrophages or CD103+ dendritic cells (DCs) compared to either of the agents alone, whereas the combination of mIL-12-VV alone strongly up-regulated major histocompatibility complex (MHC) class II in M1 macrophages (Fig. 2, B and C). Four of seven mice treated with the combination achieved complete tumor regression (CR), whereas only one of seven mice achieved CR after treatment with mIL-12-VV alone (Fig. 2D). No mice achieved CR when treated with hIL-7-VV or Cont-VV alone. No weight loss was observed after treatment with the combination (fig. S3). Antitumor efficacy of this combination therapy correlated positively with dose (fig. S4). A combination of mIL-12-VV and mGM-CSF-VV, a vaccinia virus carrying a murine GM-CSF transgene, did not induce CR in the LLC model (fig. S5). A recombinant oncolytic vaccinia virus encoding both human IL-7 and murine IL-12 (hIL-7/mIL-12-VV) induced CR in the CT26.WT model (fig. S6) and B16-F10 model (Fig. 2E) and, moreover, suppressed tumor growth in the poorly immunogenic (34) TRAMP-C2 prostate adenocarcinoma model (Fig. 2F) and in large-sized (>160 mm3) CT26.WT tumors (Fig. 2G). Antitumor activity of hIL-7/mIL-12-VV against CT26.WT tumors was suppressed by depletion of CD8+ T cells, but not CD4+ T cells, indicating that CD8+ T cells drive treatment efficacy (Fig. 2H and fig. S7). Consistent with these results, hIL-7/mIL-12-VV increased intratumorally activated CD8+ T cells, characterized by high expression of granzyme B (Fig. 2I). To further examine the immune pathways up-regulated by hIL-7/mIL-12-VV, isolated RNA from treated tumors was analyzed using a NanoString PanCancer Immune Profiling panel. Treatment with hIL-7/mIL-12-VV up-regulated multiple immune-related pathways (Fig. 2J). Hierarchical clustering analysis indicated that up-regulation of the innate pathway and type I IFN pathway was due to the virus backbone, whereas genes related to T cell function and antigen processing were mainly up-regulated by IL-7 and IL-12 (fig. S8 and data files S1 and S2).

Effects on noninjected distant tumors

Next, to examine whether localized activation of immune responses by hIL-7/mIL-12-VV affects systemic disease, we conducted a bilateral CT26.WT model in which mice had subcutaneous CT26.WT tumors inoculated into both flanks. After the injection of hIL-7/mIL-12-VV into the tumors in the right flank, increases in local tumor infiltration with CD8+ T cells, tumor antigen–specific T cells (gp70-tetramer+CD8+ T cells), Tconv, NKT cells, and NK cells were observed in the injected right tumors, compared to Cont-VV. Moreover, a higher number of Treg cells were also observed, whereas the ratios of Treg/CD4+ T cells were identical to mice injected with Cont-VV (Fig. 3A). Monocytic and granulocytic myeloid-derived suppressor cells (MDSCs) were also increased in the injected tumors by hIL-7/mIL-12-VV (fig. S9A). Identical changes in immune status were observed in the noninjected contralateral tumors, characterized by infiltration of the same immune cells as those seen in the injected right-sided tumors (Fig. 3B and fig. S9B). Injected right and noninjected left tumors both showed higher concentrations of murine IFN-γ protein after treatment with hIL-7/mIL-12-VV compared to Cont-VV (Fig. 3C), and up-regulation of PD-L1 was also observed (Fig. 3D). All tumors that had been directly injected with hIL-7/mIL-12-VV disappeared, and the contralateral tumors in three of six mice also completely regressed (Fig. 3E), supporting the potential efficacy of this viral therapy against distant metastatic disease. Unlike the injected tumors, viral genome DNA was not detected in the contralateral tumors on the day after injection (Fig. 3F), suggesting that viral infection in this model is limited to the injected tumors and that the antitumor response in the contralateral tumors is not directly due to the systemically transferred hIL-7/mIL-12-VV but to immune responses caused by tumor-infiltrating lymphocytes (TILs). The abscopal antitumor effect of hIL-7/mIL-12-VV indicates strong antigen presentation to distant lymphoid organs. As we expected, up-regulation of MHC class II in M1 macrophages in the distant tumors and spleen was observed, as well as activation of CD11b+ DCs in the spleen (Fig. 3, G and H). Abscopal antitumor effects of hIL-7/mIL-12-VV were also observed in a bilateral LLC model (Fig. 3I; 95.0 and 43.1% of tumor growth inhibition in the injected and contralateral tumors, respectively), and as in the bilateral CT26.WT model, immune status changes were observed in both tumors after unilateral treatment with hIL-7/mIL-12-VV (fig. S10, A and B). In the bilateral LLC model, unlike CT26.WT, viral genome DNA was detected in contralateral tumors the day after the administration of 2 × 10^7 plaque-forming units (pfu) of hIL-7/mIL-12-VV (fig. S10C). The finding that the number of viral copies in the contralateral tumors was less than or equal to the amount in the tumors injected with 2 × 10^5 pfu of hIL-7/mIL-12-VV (fig. S10D), which is insufficient for antitumor efficacy (fig. S10E), also suggests that the transferred virus in the contralateral tumors is not the sole factor responsible for the abscopal effects in the LLC model.

Increased sensitivity to anti–PD-1 and anti-CTLA4 antibody treatment

We hypothesized that altered immune status in the hIL-7/mIL-12-VV–injected and contralateral tumors, characterized by increases in TILs and up-regulation of PD-L1 expression by cancer cells, would sensitize tumors to immune checkpoint blockade. In the bilateral CT26.WT model, anti–PD-1 or anti-CTLA4 antibody was administered with or without previous hIL-7/mIL-12-VV treatment into tumors on one side (Fig. 4A). Monotherapies with anti–PD-1 antibody did not change in the B16-F10 model (Fig. 1E) and decreased in the CT26.WT and LLC models (Fig. 1, F and G). NK cells were increased in the B16-F10 and CT26.WT tumors (Fig. 1, B and C).
or anti-CTLA4 antibody showed no or weak efficacy and induced no CR in tumors on either side in mice not pretreated with hIL-7/mIL-12-VV (Fig. 4, B and C). In contrast, hIL-7/mIL-12-VV alone, the combination of hIL-7/mIL-12-VV with anti–PD-1 antibody, or the combination of hIL-7/mIL-12-VV with anti-CTLA4 antibody all induced CR in almost all mice in the virus-injected tumors (Fig. 4B). In the contralateral tumors, the combination of hIL-7/mIL-12-VV with anti–PD-1 antibody or anti-CTLA4 antibody induced a much higher rate of CR than viral monotherapy (Fig. 4C). Administration of hIL-7/mIL-12-VV before immune checkpoint blockade increased CD8+ T cells, Tconv, and Tregs with reduction of the ratios of Treg/CD4+ T cells in both injected and distant tumors (Fig. 4, D and E). This result indicates that local injection of hIL-7/mIL-12-VV has the potential to sensitize tumors resistant to immune checkpoint inhibitors at both virus-injected and distant sites. No weight loss was observed during the experiments (fig. S11).
Fig. 3. Intratumoral administration of hIL-7/mIL-12-VV changes immune status of noninjected distant tumors and inhibits tumor growth. (A and B) Mice were subcutaneously inoculated with CT26.WT cells in both flanks. After establishment of tumors, PBS, 2 × 10^7 pfu of Cont-VV, or 2 × 10^7 pfu of hIL-7/mIL-12-VV were injected into the tumors in the right flanks (injected tumors) every other day, for a total of three times. Twelve days after the last treatment, TILs in injected tumors (A) and distant tumors (B) were analyzed by flow cytometry (n = 9 to 12). (C) Concentration of intratumoral murine IFN-γ (mIFN-γ) the next day after the treatment (n = 6). (D) Murine PD-L1 expression on CT26.WT cells 7 days after the treatment (n = 10). *P < 0.05, **P < 0.01, and ***P < 0.001 by Mann-Whitney U test. (E) Growth of injected tumors and distant tumors in bilateral CT26.WT model (n = 6). ***P < 0.001 versus Cont-VV by unpaired t test. (F) Viral DNA in tumors measured by qPCR the next day after the treatment (n = 5 to 6). ND, not detected. (G and H) Expression of MHC class II in antigen-presenting cells in distant CT26.WT tumors (G) and spleens (H) was analyzed (n = 7 to 8). **P < 0.01 and ***P < 0.001 by Mann-Whitney U test. (I) Growth of injected tumors and distant tumors in the LLC model (n = 12). ***P < 0.001 versus Cont-VV by unpaired t test. Mean ± SEM is shown.
Fig. 4. Intratumoral administration of hIL-7/mIL-12-VV sensitizes tumors to anti–PD-1 and anti-CTLA4 antibodies. (A) Treatment scheme. CT26.WT cells were subcutaneously implanted into both flanks on the same day 7 days before treatments. Tumors on the right flank were treated with vehicle solution or $2 \times 10^7$ pfu of hIL-7/mIL-12-VV, for a total of three times (on days 1, 3, and 6). From the day of the last injection of hIL-7/mIL-12-VV, 100 $\mu$g of anti–PD-1 antibody or 200 $\mu$g of anti-CTLA4 antibody was intraperitoneally administered twice a week during the experiment. (B and C) Tumor growth of individual injected tumors (B) and distant tumors (C) is shown with ratios of mice that achieved CR ($n = 10$ per group). (D and E) In a separate study, 7 days after the last viral treatment, infiltrating CD8$^+$ T cells, Tconv, and Treg in injected tumors (D) and distant tumors (E) were analyzed by flow cytometry ($n = 6$ to 10). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Mann-Whitney $U$ test. Mean ± SEM is shown.
Establishment of long-term antitumor memory
We evaluated whether the intratumoral administration of hIL-7/mIL-12-VV contributes to the establishment of antitumor memory. Splenocytes from mice in which hIL-7/mIL-12-VV had caused CR of CT26 WT tumors were collected, and the immune response against CT26 WT cells was analyzed. These splenocytes stimulated with CT26 WT cells secreted a higher concentration of IFN-γ than did those stimulated with 4T1 breast cancer cells or Renca renal carcinoma cells, whereas splenocytes of treatment-naïve mice showed no detectable response to any stimulation (Fig. 5A), indicating that the intratumoral treatment with hIL-7/mIL-12-VV generated tumor-specific memory in the splenocytes. To examine the long-term persistence of antitumor memory, mice cured of CT26 WT tumors by hIL-7/mIL-12-VV were rechallenged with the same tumors 3 months after the last treatment. Tumors in the cured mice were completely rejected within 4 weeks, whereas all age-matched tumor-naïve control mice developed tumors (Fig. 5B). This protective effect against tumor rechallenge was also observed in the LLC model (fig. S12A). Mice rejecting an LLC tumor rechallenge showed higher percentages of CD44+CD62L+ central memory T cells in their spleens (fig. S12, B and C). It was also observed that mice previously cured of subcutaneous CT26 WT tumors by hIL-7/mIL-12-VV rejected intracranially inoculated CT26 WT cells (Fig. 5C), suggesting the establishment of long-term antitumor memory.

Efficacy of intratumoral immune responses in humanized mice
Studies in immunocompetent mouse models demonstrated the marked effectiveness of hIL-7/mIL-12-VV against mouse tumors as described above in nonhumanized mice. To assess the effect of oncolytic activity and immune activation potential against human tumors, we engineered a vaccinia virus carrying human IL-7 and human IL-12 genes (hIL-7/hIL-12-VV) because murine IL-12 is not cross-reactive with human immune cells. Human cancer cell lines of various organs including skin, brain, pharynx, liver, esophagus, colon, gastric, bladder, kidney, ovary, cervix, breast, prostate, lung, and pancreas were completely killed by hIL-7/hIL-12-VV in vitro, with varying degrees of sensitivity (Fig. 6A). Intratumoral administration of hIL-7/hIL-12-VV in immunocompromised mice bearing human cancer cell lines resulted in regression of tumors of Detroit 562 head and neck cancer, U-87 MG glioblastoma, and HCT 116 colon cancer (57, 20, and 5% regression, respectively, 23 days after viral treatment) (Fig. 6B). Culture supernatant of A549 cells infected with hIL-7/hIL-12-VV showed the ability to induce proliferation of human peripheral blood mononuclear cells (PBMCs) and to induce IFN-γ secretion, whereas culture supernatant of A549 cells infected with Cont-VV showed no influence on PBMCs (Fig. 6C), indicating that the stimulatory effects on PBMCs are due to human IL-7 and IL-12.

Last, to examine the ability of hIL-7/hIL-12-VV to change the tumor immune microenvironment and boost antitumor efficacy, we evaluated hIL-7/hIL-12-VV in tumor-bearing humanized mice into which human CD34+ hematopoietic stem cells had been previously grafted to establish a human immune system. Injection of hIL-7/hIL-12-VV into subcutaneously inoculated NCI-H1353 human tumors caused secretion of human IL-7 and human IL-12 proteins in the injected tumors as expected, whereas Cont-VV induced no detectable production of these cytokines (fig. S13). Increased numbers of tumor-infiltrating CD8+ T cells, CD4+ T cells, NKT cells, and NK cells were observed in mice treated with hIL-7/hIL-12-VV compared to mice treated with Cont-VV (Fig. 7A). Cont-VV showed antitumor activity in this model, but hIL-7/hIL-12-VV demonstrated even higher efficacy and induced tumor regression (Fig. 7B). No weight loss was observed in mice treated with hIL-7/hIL-12-VV (Fig. 7C). The activity of hIL-7/hIL-12-VV in the humanized mouse model is consistent with that of hIL-7/mIL-12-VV observed in immunocompetent mouse models, suggesting an important role for intratumoral expression of IL-7 and IL-12.

**DISCUSSION**
Various immunotherapeutic approaches have been attempted in patients to treat solid tumors. Anti–PD-1/PD-L1, anti-CTLA4, and their combination are becoming standards of care for some cancers. Efficacy is limited to a subset of patients likely due to the multiple immunologic pathways involved in the tumor microenvironment and the toxicities related to unwanted systemic immune activation (4, 35). Therefore, identification of an
appropriate therapeutic approach to further increase the clinical response rate with a good safety profile is needed. Here, we demonstrated that intratumoral dual expression of IL-7 and IL-12 by an oncolytic vaccinia virus changed not only the immune status in the tumor microenvironment but also the systemic immune status in LLC and CT26.WT syngeneic mouse models refractory to treatment with anti–PD-1 and anti-CTLA4 antibodies. Further, our data demonstrated sensitization of these tumors to immune checkpoint blockade without toxicities.

Recently, the abilities of IL-7 and IL-12 to treat cancers through immune activation in humans have been separately evaluated. Subcutaneous administration of recombinant human IL-7 or its glyco-sylated form, CYT107, was reported to increase infiltration of CD8+ cytotoxic T cells and tumoral expression of IL-12 in patients with metastatic melanoma (37, 38). Intra-tumoral expression of IL-12 in patients with metastatic melanoma was reported to increase infiltration of CD8+ cytotoxic T cells and CD45RO+ memory T cells into tumors (38). Our nonclinical data showed that intratumoral secretion of IL-7 and IL-12 induced by hIL-7/mIL-12-VV synergistically activated and expanded T cells, NK cells, and NKT cells in directly treated tumors in a poorly immunogenic LLC model that was fully resistant to anti–PD-1 antibody (39). It is plausible that the increase of TILs further enhanced secretion of inflammatory cytokines and chemokines, which further recruited immune cells into tumors, resulting in an anti-tumor efficacy superior to monotherapy with hIL-7-VV or mIL-12-VV. This mechanism of action is assumed to contribute to the tumor regression observed in the humanized mouse model treated with hIL-7/mIL-12-VV. Greater inflammation might result in more rapid exclusion of virus from the body and consequently limit the oncolytic and immunotherapeutic activity of virotherapy (18, 20).

However, the short replication cycle of vaccinia virus (20, 40, 41) likely allows for sufficient secretion of inflammatory cytokines for tumor growth inhibition before the virus’ elimination from the injected tumor while minimally exposing the host immune system to viral proteins.

Local injection of hIL-7/mIL-12-VV increased infiltration of immune cells, enhanced MHC class II expression on antigen-presenting cells, up-regulated multiple immune-related pathways, and induced regression of tumors in noninjected distant tumors in which the viral genome was not detected by quantitative polymerase chain reaction (qPCR). This supports the hypothesis that activated and expanded immune cells in the treated tumors migrate to distant tumors via chemokines derived from distant cancer cells and immune cell surface receptors. The contribution of the immune response in distant tumors is quite powerful considering the low numbers of infiltrating immune cells. It is also plausible that the tumor-specific lymphocytes migrate to the secondary lymphoid organs such as the spleen and lymph nodes and are maintained as memory T cells. This then would contribute to the rejection of cutaneously or intracranially introduced tumor rechallenges. However, we were not able to confirm the infiltration of tumor-specific memory T cells into rechallenged CT26.WT tumors, which were completely rejected during our experiments. It has been reported that in clinical studies of immunotherapy, the extent of inflammation in the tumor microenvironment represented by the presence of infiltrating lymphocytes and the establishment of antitumor memory correlates with prognosis and affects the overall survival and progression-free survival (42–44). This, together with our findings, suggests that further investigation in clinical studies is warranted.
It is of note that hIL-7/mIL-12-VV up-regulated PD-L1 expression on cancer cells in both directly treated and distant tumors, and it was likely induced by intratumoral IFN-γ (45, 46). Although PD-L1 generally down-regulates T cell activity (47, 48), many reports have shown that patients with high baseline expression of immune-related genes, including PD-1/PD-L1 or preexisting intratumoral CD8+ T cells, appear to respond preferentially to anti–PD-1/PD-L1 and anti-CTLA4 antibodies (49–53). We demonstrated that hIL-7/mIL-12-VV administered before anti–PD-1 or anti-CTLA4 antibody sensitized CT26.WT tumor to these antibodies, suggesting that hIL-7/mIL-12-VV turned tumors “hot,” that is, sensitive to immune checkpoint blockade. Moreover, our results support the concept that both the activation of immune responses and the prevention of immunosuppressive responses are essential for therapeutic benefit (4). It should be noted that the combination of hIL-7/mIL-12-VV and immune checkpoint blockade was well tolerated without any symptoms of a cytokine storm, despite the magnitude of antitumor effect, which is likely due to the tumor selectivity of the viral replication related to the functional deletion of VGF and O1L and the local viral delivery by intratumoral administration.

We acknowledge that there are several limitations to this study that prevent the full profiling of this virotherapy. First, although we demonstrated immune status changes in tumor microenvironments triggered by IL-7 and IL-12, focusing on the characterization of the effector cell subsets, it would be important to further investigate the behavior and roles of various intratumoral cells, including Treg, antigen-presenting cells, and stromal cells (54), considering the anticipated broad applicability of IL-7 and IL-12 in this virotherapy (26, 55). The contribution of these transgenes to adaptive immunity needs to be deeply examined through detailed gene expression analysis and T cell receptor repertoire analysis. Further, it is necessary to elucidate what types of tumors are expected to be more sensitive to this virotherapy by developing panels of biomarkers. Third, whereas we started anti–PD-1 and anti-CTLA4 antibody administration on the last day of hIL-7/mIL-12-VV treatment based on the previous reports that the checkpoint inhibitors can hinder the replication of vaccinia virus (56), further optimization of the dosing schedule may be important for improved clinical efficacy. Ongoing clinical studies for some oncolytic viruses in combination with immune checkpoint inhibitors, such as T-VEC followed by pembrolizumab, should provide valuable data for our combinational virotherapy (57).

Together, our data demonstrate that intratumoral expression of IL-7 and IL-12 by an oncolytic vaccinia virus enhances immune responses in injected and in noninjected distant tumors and sensitizes poorly immunogenic tumors to immune checkpoint inhibition. This IL-7 and IL-12 combination virotherapy may provide a clinical benefit as monotherapy and has potential to effectively combine with immunotherapies for various types of solid tumors.

**MATERIALS AND METHODS**

**Study design**

The overall objective of this study was to characterize the therapeutic potential of intratumoral expression of IL-7 and IL-12 induced by an oncolytic vaccinia virus and to evaluate the benefit of this virotherapy before immune checkpoint inhibition. Antitumor activity and immune responses were evaluated in several mouse models, including a humanized mouse model. Animals were randomly allocated to experimental groups such that mean tumor volumes and mean body weights were similar in each group, but experimenters were not blinded. The number of animals per group in each experiment was determined to ensure statistical power. Six to 12 animals per group were used for antitumor efficacy studies. More than five animals per group were typically used for flow cytometry analyses. The number of animals per group and statistical tests used for each experiment are described in the figure legends.

**Viruses**

Recombinant vaccinia viruses used in this study were based on the LC16mO strain, which was described previously (58–60). Cont-VV was constructed using a plasmid to express Discosoma sp. red fluorescent protein (DsRed) from the p7.5 promoter and a plasmid to express luciferase from a synthetic vaccinia virus promoter (SP)
supplemented with 10% heat-inactivated fetal bovine serum and essential medium [American Type Culture Collection (ATCC)] maintained in RPMI 1640 medium, DMEM, or Eagle’s minimum 

When signs of deterioration or acute weight loss were observed, or when the total tumor volume reached 3000 mm³, animals were euthanized. When tumor was not detected by palpation, mice were defined as having achieved CR.

To evaluate Cont-VV, hIL-7/mIL-12-VV, hIL-7-VV, mIL-12-VV, mGM-CSF-VV, and their combinations in immunocompetent mice, unilateral B16-F10, CT26.WT, LLC, and TRAMP-C2 models were used. In these models, 2 × 10^5 to 7 × 10^5 cells of B16-F10, CT26.WT, and LLC or 3 × 10^6 cells of TRAMP-C2 were subcutaneously inoculated into the right flank. Unless otherwise noted, when tumors reached about 50 mm³, about 30 μl of phosphate-buffered saline (PBS) or virus suspension was intratumorally injected every other day for a total of three doses. For depletion of immune cell populations, 200 μg of anti-CD8 antibody per mouse (clone 53-6.72, ATCC) or 200 μg of anti-CD4 antibody per mouse (clone GK1.5, Bio X Cell) was used. To examine the abscopal effects of hIL-7/mIL-12-VV, bilateral CT26.WT and LLC models were used. CT26.WT cells (2 × 10^5 to 7 × 10^5 cells per injection site) were subcutaneously inoculated into the right and left flanks of mice at the same time. LLC cells were similarly inoculated into the flanks of C57BL/6 mice, but inoculation into the left flank was 2 days later than the inoculation into the right flank. When tumors reached about 50 mm³, unilateral intratumoral treatment commenced as above. To examine the effects of combinational therapy with hIL-7/mIL-12-VV and checkpoint inhibitors, a bilateral CT26.WT model was used, in which intraperitoneal administration of 100 μg of anti–PD-1 antibody per mouse (clone RMP1-14, Bio X Cell) or 200 μg of anti–CTLA4 antibody per mouse (clone 9D9, Bio X Cell) was initiated from the end of viral treatment and continued twice a week until the end of the experiment. In rechallenge studies, BALB/c mice, which had been previously cured of subcutaneous CT26.WT by hIL-7/mIL-12-VV, or age-matched treatment-naïve mice were inoculated with 5 × 10^6 cells of CT26.WT subcutaneously or 5 × 10^5 cells of luciferase-expressing CT26.WT intracranially. For the continuous in vivo monitoring of luciferase-expressing CT26.WT tumors in mouse brain, an IVIS Imaging System (PerkinElmer) was used. Just before in vivo imaging, mice bearing intracranial CT26.WT tumors received 200 μl of VivoGlo luciferin (15 mg/ml) (Promega). Bioluminescent signals and grayscale images of individual mice were fused using Living Image software (PerkinElmer). In the other rechallenge study, C57BL/6 mice previously cured of LLC by hIL-7/mIL-12-VV were subcutaneously inoculated with 5 × 10^5 cells of LLC.

For in vivo assessment of hIL-7/hIL-12-VV, immunocompromised mice bearing the human cancer cell lines Detroit 562, U-87 MG, or HCT 116 were used. Cells (3 × 10^6 per mouse) were subcutaneously inoculated into the right flank of nude mice. When tumors reached about 50 to 100 mm³, intratumoral treatment was initiated and repeated every other day for a total of three doses. To evaluate immune responses elicited by hIL-7/hIL-12-VV, humanized mice were developed by intravenously transplanting human cord blood CD34+ hematopoietic stem cells (Lonza) into NOD-Scid IL2Rγ−/− mice. Thirteen weeks later, the humanized mice were subcutaneously inoculated with 3 × 10^6 NCI-H1337 cells per mouse. When tumors reached about 50 to 100 mm³, a single intratumoral injection of PBS, Cont-VV, or hIL-7/hIL-12-VV was administered.

**Ex vivo analysis of immune responses**

Spleens were collected from treatment-naïve mice or mice that had previously been cured of CT26.WT tumors by hIL-7/mIL-12-VV. Spleenocytes were cultured with irradiated (100 Gy) CT26.WT, 4T1,
or Renca cells at a 50:1 ratio in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 55 μM 2-mercaptoethanol, and penicillin-streptomycin according to the manufacturer’s protocol. Three days later, the concentration of murine IFN-γ in the supernatants was measured using the Quantikine ELISA Mouse IFN-γ Immunoassay (R&D Systems) using an EnSpire multimode plate reader (PerkinElmer).

Flow cytometry of TILs and splenocytes

Tumor tissues were suspended with the Tumor Dissociation Kit, Mouse (Miltenyi Biotec). Intratumoral cells or splenocytes of mice were stained with the following antibodies: mouse CD3 V450, mouse CD8a V500, mouse CD11b allopoxycyanin (APC)–Cy7, mouse CD25 phycoerythrin (PE)–Cy7, mouse CD80 PE, mouse I-A/I-E peridinin chlorophyll protein (PerCP)–Cy5.5, and mouse Ly-6G fluorescein isothiocyanate (FITC) (BD Biosciences); mouse B220 FITC, mouse CD3 APC-Cy7, mouse CD4 FITC, mouse CD11c PE, mouse CD44 PE-Cy7, mouse CD45 Alexa Fluor 647, mouse CD45 APC, mouse CD45 Pacific Blue, mouse CD49b PE, mouse CD103 PE-Cy7, mouse CD206 APC, mouse F4/80 PE-Cy7, mouse Ly-6C PerCP-Cy5.5, mouse granzyme B Alexa Fluor 647, and mouse PD-L1 BV421 (BioLegend); mouse CD62L eFluor 450 and mouse FoxP3 PerCP-Cy5.5 (Thermo Fisher Scientific), and PE-labeled gp70 tetramer (Medical & Biological Laboratories). Cells obtained from the humanized mice were stained with the following antibodies: human CD45 PE-Cy7, human CD3 PerCP-Cy5.5, human CD4 APC-Cy7, human CD8 Alexa Fluor 488, and human CD56 BV421 (BD Biosciences). Zombie Aqua (BioLegend) or Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific) was used to distinguish dead cells. Cells were analyzed by flow cytometry using a MACSQuant Analyzer 10 (Miltenyi Biotec). Acquired flow cytometry data were analyzed using FlowJo version 10 (BD Biosciences).

Measurement of intratumoral cytokines

Tumor samples were collected and immediately frozen in liquid nitrogen. Then, samples were suspended in T-PER Buffer (Thermo Fisher Scientific) using the gentleMACS Dissociator (Miltenyi Biotec) and centrifuged at 10,000g for 10 min at room temperature. Intratumoral human IL-7 and murine IL-12 in immunocompetent mouse models were quantified using the RayBio Human IL-7 ELISA Kit (Ray Biotech) and Mouse IL-12 p70 Quantikine ELISA Kit (R&D Systems), respectively. Intratumoral murine IFN-γ was quantified using the Mouse IFN-γ Quantikine ELISA Kit or Cytometric Bead Array (CBA) Mouse IFN-γ Flex Set (BD Biosciences). Intratumoral human IL-7 and human IL-12 in the humanized mouse model were quantified using CBA Human IL-7 Flex Set and CBA Human IL-12p70 Flex Set (BD Biosciences), respectively. Concentrations of cytokines in tumor lysates were normalized to total protein extracted from each sample, which was assayed using Pierce BCA Protein Assay Reagent or Pierce 660-nm Protein Assay Reagent (Thermo Fisher Scientific). All procedures were performed according to the manufacturers’ directions. Values obtained by enzyme-linked immunoabsorbent assay (ELISA) kits and protein assays were measured on an EnSpire multimode plate reader or a SpectraMax plate reader (Molecular Devices). Values obtained by CBA kits were measured on a MACSQuant analyzer (Miltenyi Biotec).

qPCR of viral genome

Tumors were disaggregated using the gentleMACS Dissociator (Miltenyi Biotec), and DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen). DNA quantity was measured using NanoDrop (Thermo Fisher Scientific). DNA was subjected to standard qPCR using TaqMan Gene Expression Master Mix, the TaqMan MGB probe (5′-AGTGCCTGGTATAAAGGAG-3′) (Thermo Fisher Scientific), and primers specific to the vaccinia virus hemagglutinin J7R gene (forward, 5′-GATGATGCAACTCTATCATGTA-3′; reverse, 5′-GTATAATTACAAATACAAGACGTC-3′).

In vitro infection experiments

Cells (1 × 10^6) of human cancer lines were plated in 96-well plates and infected with hIL-7/hIL-12-VV at various MOIs. Six days after the infection, cell viabilities were assessed using CellTiter-Glo Luminescence Cell Viability Assay (Promega) according to the manufacturer’s recommended protocols. Similarly, 3 × 10^5 cells of murine cancer cell lines (B16-F10, CT26.WT, and LLC) were plated in 96-well plates and infected with Cont-VV, and 4 days after the infection, cell viabilities were assessed. To evaluate the influences of transgene derived from hIL-7/hIL-12-VV on human immune cells, we first prepared a supernatant from A549 cells, which had been infected with hIL-7/hIL-12-VV at MOI 1.0 and cultured for 2 days. Then, the supernatant was filtered through 0.1-μm polyvinylidene difluoride membrane (Millipore) and added to 1 × 10^5 human PBMCs (AllCells) in a 96-well plate. Seven days later, secretion of human IFN-γ from PBMCs was assessed using the Human IFN-γ Quantikine ELISA Kit (R&D Systems), and at the same time, the proliferation of PBMCs was assessed using CellTiter-Glo (Promega). Values were measured on an EnSpire multimode plate reader (PerkinElmer).

NanoString gene expression profiles

LLC tumor-bearing mice were treated with intratumoral injections of PBS, Cont-VV, or hIL-7/mIL-12-VV at a dose of 2 × 10^7 pfu. Two days after the treatment, the tumors were dissected and then RNA was extracted. Isolated RNA was hybridized with the PanCancer Mouse Immune Profiling panel (NanoString Technologies) and quantified using the Digital Analyzer (nCounter). Data were processed and normalized using nSolver Analysis Software and the Advanced Analysis module (NanoString).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7 and 8 (GraphPad Software). Procedures of comparison and the numbers of animals in the experiment are described in each figure. P values <0.05 were considered to be significant. Original data are in data file S4.


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Intratumoral expression of IL-7 and IL-12 using an oncolytic virus increases systemic sensitivity to immune checkpoint blockade

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A virus with benefits
Oncolytic viruses, which kill cancer cells, have received increasing attention in recent years as the field has advanced toward biological and immune-based approaches to fighting cancer. To help further these viruses’ cancer-fighting potential, Nakao et al. engineered an oncolytic vaccinia virus to deliver two cytokines that help stimulate antitumor immune responses. The authors successfully tested this approach in multiple immunocompetent mouse models, showing promising results in directly treated tumors and in distant tumors of the same type, as well as protection from rechallenge with tumor cells. The treatment was particularly effective in combination with immune checkpoint inhibitors, even in tumors unresponsive to checkpoint inhibitors alone.