An engineered oncolytic virus expressing PD-L1 inhibitors activates tumor neoantigen-specific T cell responses

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Oncolytic viruses offer an in situ vaccination approach to activate tumor-specific T cell responses. However, the upregulation of PD-L1 expression on tumor cells and immune cells leads to tumor resistance to oncolytic immunotherapy. In this study, we generate an engineered oncolytic virus that coexpresses a PD-L1 inhibitor and GM-CSF. We find that the oncolytic virus is able to secrete the PD-L1 inhibitor that systemically binds and inhibits PD-L1 on tumor cells and immune cells. Importantly, the intratumoral injection with the oncolytic virus overcomes PD-L1-mediated immunosuppression during both the priming and effector phases, provokes systemic T cell responses against dominant and subdominant neoantigen epitopes derived from mutations, and leads to an effective rejection of both virus-injected and distant tumors. In summary, this engineered oncolytic virus is able to activate tumor neoantigen-specific T cell responses, providing a potent, individual tumor-specific oncolytic immunotherapy for cancer patients, especially those resistant to PD-1/PD-L1 blockade therapy.
**Cancer** is a genetic disease, with the growth of tumor cells initiated by mutations that activate oncogenic drivers and disable tumor suppressors. Recent studies have demonstrated that tumor neoantigens can be derived de novo from the expression of genetic mutations and presented in major histocompatibility complexes (MHC) on tumor cells, and endogenous T cell responses against neoantigens can be naturally activated in cancer patients. However, only a small number of nonsynonymous mutations expressed in tumors can be adequately presented as neoantigens for which the T cell response can be mounted.

Compounding this problem of inefficient neoantigen presentation is the immunosuppressive tumor microenvironment that inhibits antitumor T cell responses by immune checkpoint molecules, such as PD-1/PD-L1. Immune checkpoint blockade effectively augments endogenous T cell responses against tumor neoantigens and led to the enduring responses in patients with advanced malignancies, including complete responses in various types of cancer, such as melanoma, metastatic lung, kidney, and bladder carcinoma. Responses to PD-1 inhibition are highly correlated with the presence of CD8+ T cells at the invasive margin and within the tumor lesions, which define the so-called inflamed “hot” tumors. However, the majority of cancer patients are resistant to PD-1/PD-L1 blockade. One reason for treatment failures is attributed to the so-called “cold” tumors, which might have low mutational burden and neoantigen load, poor MHC presentation, and poor capacity to attract T cell infiltration.

Increasing the response rates to PD-1 blockade therapy remains an important challenge, given that the majority of tumors fail to spontaneously provoke T cell responses against tumor mutant neoantigens and are resistant to PD-1 blockade. Recently, intensive efforts have been devoted to activating neoantigen-specific T cell responses. Neoantigen-specific T cells can be activated by comprehensive sequencing and the identification of individual mutations, the computational prediction of neoantigen epitopes, and vaccination with neoantigen epitopes for each patient. In contrast to this cumbersome strategy, oncolytic viruses possess the potential to offer a simpler in situ vaccination approach to activate T cell responses by locoregional immune activation, immunogenic oncolytic tumor cell death, mutant neoantigen release and presentation, and alteration of the immunosuppressive tumor microenvironment.

**Results**

**Generation and characterization of an armed oncolytic VV coexpressing a PD-L1 inhibitor and GM-CSF (VV-iPD-L1/GM).** We generated an engineered oncolytic VV coexpressing a murine soluble PD-1 extracellular domain fused with IgG1 Fc as a PD-L1 inhibitor (i.e., iPDL1) and murine GM-CSF (VV-iPDL1/GM), in the backbone of a tumor-selective double-deleted oncolytic VV, in which thymidine kinase (TK) and vaccinia virus growth factor viral genes had been deleted. A recombinant oncolytic VV-GM expressing murine GM-CSF and a recombinant oncolytic VV-RFP expressing the marker RFP were also generated and produced. High levels of both GM-CSF and iPDL1 (soluble PD-1-IgG Fc) proteins in a dimer were produced and efficiently released from VV-iPDL1/GM-infected tumor cells in vitro and in vivo, as detected by western blot and enzyme-linked immunosorbent assay (ELISA; Fig. 1b–d, Supplementary Fig. 1). Importantly, high levels of iPDL1 were detected in the sera of VV-iPDL1/GM-treated tumor-bearing mice for a long period of time (over 15 days) after intratumor injection (Fig. 1d).

iPDL1 protein purified from the supernatants of VV-iPDL1/GM-infected tumor cells was able to bind to PD-L1+ tumor cells, but not to PD-L1-knocked down tumor cells in vitro (Fig. 1e). In addition, it was shown that MAGEA3-IgG Fc fusion proteins failed to bind to PD-L1+ tumor cells, further ruling out the non-specific binding of IgG Fc domains to tumor cells. iPDL1 had a comparable IC50 value with the commercial anti-PD-L1 antibody in blocking PD-1/PD-L1 interaction, as manifested by a competitive ELISA assay (Fig. 1f). It was found that iPDL1, but not IgG1 Fc, efficiently mediated antibody-dependent cell-mediated cytotoxicity (ADCC) against IFNα-treated, PD-L1-expressing tumor cells (Fig. 1g). Supernatants derived from VV-GM- or VV-iPDL1/GM-infected MC38 tumor cells also had GM-CSF functionality in driving bone marrow (BM)-derived monocytes to differentiate into CD11c+ DCs (Fig. 1h). Moreover, the insertion of iPDL1 gene into the oncolytic VV did not interfere with the infection and replication of VV-iPDL1/GM in vitro and in vivo (Fig. 1i, j, Supplementary Fig. 2). Taken together, these data demonstrate that the armed oncolytic virus VV-iPDL1/GM can infect tumor cells to produce and secrete high levels of functional iPDL1 and GM-CSF proteins.
of PD-L1 expression in the tumors injected with VV-iPD1L/GM, compared to VV-RFP, suggesting that the secreted iPD1L bound to PD-L1 (Fig. 2c). Indeed, the binding of secreted iPD1L (PD-1-IgG Fc) to PD-L1 on VV-iPD1L/GM-treated CD45− non-leukocyte cells was detected (Fig. 2c). Importantly, iPD1L (PD-1-IgG Fc) secreted from treated primary tumors also bound to PD-L1 on CD45− cells in untreated, distant tumors (Fig. 2d). These data indicate that the secreted iPD1L binds to PD-L1 on CD45− tumor and stromal cells in VV-iPD1L/GM-treated primary and untreated, distant tumors in autocrine and paracrine manners.

We further examined whether the iPD1L secreted from VV-iPD1L/GM-treated tumors was able to bind PD-L1 on immune cells in vivo. The upregulation of PD-L1 expression on CD45− hematopoietic cell infiltrates, including DCs, MDCSs, and T cells, was observed in both VV-RFP-treated and untreated tumors, compared to PD-L1 expression in PBS-treated tumors (Fig. 2e, f). Lower levels of PD-L1 expression on CD45− hematopoietic cell infiltrates in VV-iPD1L/GM-injected tumors and distant tumors were detected compared to the PD-L1 expression in VV-RFP or VV-GM-injected tumors and distant tumors (Fig. 2e, f). Figure 2g shows the binding of secreted iPD1L to PD-L1 on immune cells from VV-iPD1L/GM-treated and untreated distant tumors. Furthermore, we investigated the infection and secretion of iPD1L after intratumor injections of VV-iPD1L/GM in tumor-bearing mice (Supplementary Fig. 3). Supplementary Figs. 4–6 show the efficient infection (RFP+) of tumor cells (CD45− CD31− Ter119−) by VV-iPD1L/GM in vivo. Supplementary Figs. 7 and 8 show the

![Image](https://doi.org/10.1038/s41467-020-15229-5)
secretion of the iPDL1 dimer from the isolated tumor cells after intratumoral injections with the binding activity to PD-L1 on tumors. We evaluated the antitumor activity of VV-iPDL1/GM rechallenged homologous B16-F10 tumors, compared to VV-GM and VV-RFP. Furthermore, intratumoral injections with VV-iPDL1/GM were more potent in inhibiting the growth of rechallenged homologous Py230 tumors compared to VV-GM and VV-RFP. In vivo CD8 T cell depletion significantly abolished the systemic antitumor activity in VV-iPDL1/GM-treated tumor-bearing mice (Fig. 4i).

Enhanced antitumor activities against primary and distant tumors. We evaluated the antitumor activity of VV-iPDL1/GM using a luciferase+ B16-F10 melanoma syngeneic transplant mouse model, which was weakly immunogenic. Tumor-bearing mice received intratumoral injections of various VVs or PBS as described in Methods section. Although intratumoral injections with VV-RFP or VV-GM drastically inhibited tumor growth, both bioluminescence monitoring (Fig. 3a, b) and caliper measurement (Fig. 3c) showed that VV-iPDL1/GM was more potent in inhibiting B16-F10 tumor growth. Intratumoral injections of the recombinant VVs also drastically inhibited the growth of Py230 breast cancer and MC38 colon adenocarcinoma (Fig. 3d, e).

We then tested if an intratumoral injection with VV-iPDL1/GM is able to provoke a systemic antitumor response. Groups of C57BL/6 mice bearing B16-F10 tumors were treated with various VVs, and then inoculated with luciferase+ B16-F10 tumors on the contralateral flank. Bioluminescence imaging (Fig. 4a, b), caliper measurement (Fig. 4c), and survival curve (Fig. 4d) showed that VV-iPDL1/GM was more potent in inhibiting the growth of rechallenged homologous B16-F10 tumors, compared to VV-GM and VV-RFP. Furthermore, intratumoral injections with VV-iPDL1/GM were also more potent in inhibiting the growth of rechallenged homologous Py230 tumors and MC38 tumors, compared to the intratumoral injections with VV-GM or VV-RFP (Fig. 4e–h). In vivo CD8 T cell depletion significantly abolished the systemic antitumor activity in VV-iPDL1/GM-treated tumor-bearing mice (Fig. 4i).
We further tested if an intratumoral injection with VV-iPDL1/GM is able to provoke a systemic antitumor response against established tumor growth. B16-F10 melanoma cells were implanted to the left and right flanks of C57B/6 mice. When tumor volumes reached ~100 mm$^3$ (counted as day 0), the mice were intratumorally injected with 50 μL of VV-RFP, VV-GM, or VV-iPDL1/GM (5 × 10$^7$ pfu per tumor) or PBS at days 0, 3, and 7. Bioluminescence monitoring a, b and caliper measurement c of B16-F10-Luc cells were performed on the indicated days. Data are presented as the means ± SD (n = 5 mice). Significant differences are indicated as *P < 0.05 determined by two-tailed Student’s t-test. d, e Py230 d or MC38 e tumor volume was monitored by caliper measurement using the same treatment schedule as in a-c. Data are presented as the means ± SD (n = 5 mice).

Enhanced tumor infiltration and activation of immune cells. We analyzed the tumor infiltration of immune cells after intratumoral injections of VV-iPDL1/GM. Groups of MC38 tumor-bearing mice were treated with various VVs via intratumoral injections. One group of MC38-bearing mice was i.p. injected with anti-PD-L1 Ab (clone 10F.9G2) for comparison. VV-intratumoral injections significantly enhanced the tumor infiltration of CD45$^+$ hematopoietic cells, especially the injections of VV expressing GM-CSF (Fig. 5a). VV-GM injection enhanced composition of MDSC-containing cells (CD11b$^+$Gr-1$^+$, 46%) in the CD11b$^+$ population. In contrast, VV-iPDL1/GM injection greatly reduced MDSCs to 23% of the CD11b$^+$ population (Fig. 5a), which was consistent with the reduced absolute MDSC numbers of VV-iPDL1/GM-treated or distant tumors (Fig. 5b, c), suggesting the ability of VV-iPDL1/GM to block the PD-1/PD-L1 interaction and decrease tumor-associated immune suppressive cells. Moreover, VV-iPDL1/GM significantly enhanced dendritic cell (DC; CD11c$^+$) content in the infiltrates compared with control VV-RFP (Fig. 5b, c).

We subsequently analyzed infiltrating lymphocytes in VV-treated tumors. Consistent with the published studies, VV injections enhanced the overall lymphocyte infiltration into tumor tissues (Fig. 5a). However, the double-armed VV-iPDL1/GM enhanced the percentages of CD8$^+$ T cells, and CD4$^+$ T cells, and PD-1$^+$CD8$^+$ T cells in the CD45$^+$ infiltrates more significantly in comparison to control VV or single-armed VV-GM (Fig. 5a, Supplementary Fig. 11). The injection of VV-GM alone did not significantly affect Treg cells (CD4$^+$FoxP3$^+$) in tumor infiltrates, but the injection of VV-iPDL1/GM reduced Treg cells to a level lower than that in PBS-treated tumors.
resulting in a robustly enhanced CD8$^+$ T cells/Treg ratio (Fig. 5b). We further analyzed infiltrating lymphocytes in distant, untreated tumors. Figure 5c also shows that the intratumoral injection with VV-iPDL1/GM enhanced the tumor infiltration and activation of lymphocytes and other immune cells in distant, untreated tumors. Moreover, tumor-infiltrating CD8$^+$ effector T cells were more efficiently activated by VV-iPDL1/GM injections, as manifested by an enhanced expression of IFN-γ, TNF-α, and CD107a in response to the stimulation with tumor lysate-pulsed DCs (Fig. 5d, e). Altogether, these findings demonstrate that the double-armed VV-iPDL1/GM has the ability to alter the tumor microenvironment by enriching the tumor infiltration of immune cells, reducing immune suppressive cells in the tumors, and activating tumor-infiltrating effector T cells.

**Enhanced T cell responses against dominant and subdominant neoantigen epitopes.** We tested whether an intratumoral injection of VV-iPDL1/GM is able to generate neoantigen-specific T cell responses. Recently, Yadav et al. identified MHC-I-restricted
neopeptides in MC38 tumor cells using whole-exome and transcriptome sequencing analysis combined with mass spectrometry. MC38 tumor-bearing mice were intratumorally treated with various VVs. Ten days after the last viral injection, splenocytes were harvested and analyzed for the neopeptides-specific immune responses. Eleven mutant neoantigen epitopes were synthesized and used for this study (Supplementary Table 1). Neopeptides 1–6 were detected on the cell surface by the membrane protein purification and mass spectrometry method, while neopeptides 7–11 were not detected on the cell surface, probably due to the sensitivity of the detection method, or poor peptide processing and presentation. After intratumoral injections with VVs, the tumor-bearing mice exhibited an enhanced proliferation and cytokine (IFN-γ) secretion of splenic T cells compared with that in PBS-treated mice in response to stimulation with the 11 neopeptides mixture. However, the most potent splenic T cell responses against the neopeptides mixture were detected in the VV-iPDL1/GM-treated mice (Fig. 6a). Importantly, systematical (i.p.) administration of anti-PD-L1 antibody (200 μg) did not significantly induce neopeptide-specific T cell responses in the tumor-bearing mice. These data indicate the superior potency of VV-iPDL1/GM to activate neoantigen-specific T cell responses.

We then analyzed T cell responses in VV-treated mice against individual neopeptides. VV-RFP enhanced the proliferation and cytokine production of splenic T cells of treated mice in response to neopeptides 2, 4, and 5, compared to only neopeptide 2 or 4 in the PBS or anti-PD-L1 antibody-treated mice (Fig. 6b, Supplementary Fig. 12). VV-GM significantly enhanced the T cell responses to neopeptides 2, 4, and 5, and also additionally triggered T cell responding to neopeptide 9 and slightly to neopeptide 11. Compared with VV-GM, VV-iPDL1/GM further strengthened T cell responses against neopeptides 2, 4, and 5, as well as the subdominant neopeptides 9 and 11 (Fig. 6b, Supplementary Fig. 12). Furthermore, splenic T cells from the VV-iPDL1/GM-treated mice showed responses against dominant neopeptide 2, 4, or 5 even at a very low peptide concentration (0.1 μg/mL or 1 μg/mL), and also showed responses against subdominant neopeptide 9 or 11 at a low concentration (10 μg/mL), in which splenic T cells from other VV-treated mice didn’t show detectable responses (Fig. 6c, Supplementary Fig. 12). Given the prominent neopeptide 4-specific T cell response detected in various VV-treated mice, neopeptide 4 peptide-MHC H-2I^b^ labeled pentamers were synthesized and used to analyze tumor-infiltrating neoantigen-specific T cells. Among the groups, the VV-iPDL1/GM-treated mice had maximal CD45^+^CD8^+^pentamer^+^ T cells in tumor infiltrates (Fig. 6d, e), indicative of VV-iPDL1/GM injections being most efficacious in activating neopeptide 4-specific T cells in the tumor-bearing mice.

Even 40 days after the last VV injection when all tumors were gone, splenocytes of the VV-iPDL1/GM-treated mice showed the strongest response to neopeptide 4-loaded DC restimulation (Fig. 6f). These results demonstrate the ability of VV-iPDL1/GM to activate T cell responses against dominant and subdominant neoantigen epitopes.

Enhanced tumor-infiltrating DC maturation and neoantigen presentation. We further explored the mechanisms of the double-armed VV-iPDL1/GM to activate neoantigen-specific T cell responses. DCs are professional antigen-presenting cells with the ability to prime antigen-specific T cell responses. Thus, we compared the immunostimulatory potency of tumor-infiltrating DCs from various VV-treated mice. Tumor-infiltrating CD11c^+^ DCs isolated from VV-treated MC38 tumors were pulsed with neopeptides 4 (dominant), 9, and 11 (subdominant), and then cocultured with neoantigens-primed T cells isolated from mice immunized with the 11 neopeptide peptides mixture formulated with adjuvants. Tumor-infiltrating DCs from VV-iPDL1/GM-treated MC38 tumors had the enhanced potency to stimulate neoantigens-primed T cells (Fig. 7a). In comparison, tumor-infiltrating DCs from MC38 tumor-bearing mice receiving anti-PD-L1 antibody (i.v.) alone only had a much weaker stimulatory potency. We also observed that VV-iPDL1/GM significantly promoted tumor-infiltrating DC maturation, as evidenced by an increased expression of MHCII, CD80, CD86, and CD40 (Fig. 7b). A recent study revealed that CD103^+^ DCs are the main intratumoral myeloid cell population that transports antigens to the tumor-draining lymph nodes for activating T cells. The analysis of surface markers on the DC population showed that VV-iPDL1/GM injection significantly increased tumor-infiltrating CD103^+^ DCs, compared to VV-GM or VV-RFP (Fig. 7c, Supplementary Fig. 13). IL-12 is known to be an important cytokine in cross talk between DCs and T cells. Chemokines CXCL9 and CXCL10 direct effector T cell trafficking and tumor infiltration. The expression of IL-12, CXCL9, and CXCL10 in CD103^+^ DCs from VV-iPDL1/GM-treated tumors was elevated (Fig. 7d, e). These data demonstrate that VV-iPDL1/GM injections likely enhanced tumor-infiltrating DC maturation and neoantigen presentation.

Enhanced neoantigen presentation on tumor cells, and CTL effector function. During the effector phase of the antitumor response, activated T cells need to recognize neoantigen-presented tumor cells for their effector function. A poor neoantigen presentation and the expression of PD-L1 can render tumor cells resistant to CTL-mediated cytolysis. We performed an
in vivo T cell proliferation assay, in which neoepitopes-primed T cells were adoptively transferred into the various VV-treated MC38-bearing mice. A higher efficiency in neoepitopes-primed T cell proliferation in vivo was observed in VV-iPDL1/GM-treated MC38-bearing mice (Fig. 7f). We tested the ability of neoantigen-specific T cells to recognize MC38 tumor cells infected with various VVs in vitro. MC38 tumor cells were infected with VV-iPDL1/GM or control VVs, and after washing, then cocultured with neoantigens-primed T cells isolated from mice immunized with the 11 neoepitope peptides mixture formulated with adjuvants. VV-iPDL1/GM-infected MC38 tumor cells were more potent in stimulating the proliferation and cytokine production of the neoepitopes-primed T cells (Fig. 7g), suggesting that the neoepitopes-primed T cells more efficiently recognize and interact with the neoepitopes-presented, VV-iPDL1/GM-infected tumor cells. We further tested the role of the secreted iPDL1 in enhancing tumor cell immunogenicity. MC38 tumor cells without VV infection were cocultured with the neoepitopes-primed T cells isolated from mice immunized with the 11 neoepitope peptides mixture in the presence of sera from tumor-bearing mice treated with various VVs. Figure 7h shows that only sera from VV-iPDL1/GM-treated mice were able to enhance the cytolytic
activity of neoantigens-primed T cells against various VV-infected MC38 tumor cells. Moreover, it was observed that higher IFN-γ+ frequency of PD-L1+ CD8+ T cells isolated from VV-treated MC38 tumor cell suspensions in the in vitro coculture with MC38 tumor cells in the presence of purified iPDL1 in comparison to the presence of control IgG (Fig. 7i), suggesting the role of secreted iPDL1 in overcoming the immunosuppression of PD-L1+ tumor cells. In addition, our preliminary data showed the upregulation of the expression of TNF signaling genes and protein processing genes in VV-iPDL1/GM-infected tumor cells by RNA-Seq and qRT-PCR (Supplementary Fig. 14, Supplementary Table 2). A possible role of neoantigen presentation...
Fig. 7 Enhanced neoantigen presentation and cytolytic activity of neoantigen-specific CTLs. a Enhanced stimulatory potency of tumor-infiltrating DCs. Tumor-infiltrating DCs from VV-treated mice were loaded with neopeptide 4, 9, or 11, and cocultured with the neoantigens-primed T cells from mice immunized with the 11 neopeptide mixture to assess IFN-γ production; n = 3 mice. Data presented as the means ± SD. *P < 0.05, **P < 0.001 by two-tailed Student’s t-test. b Enhanced maturation of tumor-infiltrating DCs. Using a similar treatment schedule as described in Fig. 5a, cell suspensions prepared from VV-treated tumors were analyzed by flow cytometry. c Enhanced tumor infiltration of CD103+ DCs. Using the same treatment schedule as in Fig. 5a, tumor cell suspensions from VV-treated mice were analyzed by FACS; n = 5 mice. Data presented as the means ± SD. **P < 0.01 by two-tailed Student’s t-test. d Intracellular staining of IL-12 and CXCL9 of CD103+ DCs from VV-treated tumors. e qRT-PCR analysis of CXCL10 mRNA levels in CD103+ DCs isolated from VV-treated tumors; n = 5 mice. Data presented as the means ± SD. **P < 0.01 by two-tailed Student’s t-test. f Neoantigens-primed T cells proliferated more efficiently in VV-iPDL1/GM-treated mice. The neoantigens-primed T cells were labeled with 5 μM CFSE and i.v. injected into VV-treated mice. Three days later, T cell proliferation was assessed by FACS. g Enhanced stimulatory effect of VV-iPDL1/GM-infected tumor cells. MC38 tumor cells infected with VVs at MOI = 1 were cocultured with the neoantigens-primed T cells. IFN-γ production (left) and T cell proliferation (right) were measured. Data presented as the means ± SD. *P < 0.05 by two-tailed Student’s t-test. h Serum of VV-iPDL1/GM-treated mice enhanced the cytolytic activity of neoantigens-primed T cells. MC38-Luc cells were cocultured with the neoantigen-specific T cells in the presence of the sera from treated MC38-bearing mice. Cytolytic activity was calculated using luciferase emission value. Data are presented as means ± SD. **P < 0.01 by two-tailed Student’s t-test. i PD-1+ CD8+ T cells isolated from VV-treated MC38 tumors were cocultured with MC38 cells in the presence of purified iPDL1 or IgG. IFN-γ frequencies of PD-1+ T cells were shown from one of two independent experiments.

enhanced by VV-iPDL1/GM infection still cannot be ruled out. These data demonstrate the enhanced neoantigen presentation on tumor cells by VV-iPDL1/GM infection for enabling neoantigen-specific CTL effector functions.

Discussion
T cells against mutant neoantigens that are individually tumor specific play a critical role in driving antitumor immunity. Each tumor harbors a unique repertoire of mutated neoantigenic peptides that are immunogenic and can potentially induce tumor-specific immune responses. T cells can be activated against shared, nonmutated tumor-associated self-antigens. NK cells and NKT cells also have antitumor activities. Thus far, the majority of cancer patients still fail to spontaneously activate neoantigen-specific T cells and are resistant to immune checkpoint blockade therapy, likely due to the poor presentation of tumor neoantigens and the immunosuppressive tumor microenvironment. Thus, the activation of tumor neoantigen-specific T cells is critical to enhancing the efficacy of tumor immunotherapy.
The results of this study demonstrate that the armed oncolytic virus coexpressing a PD-L1 inhibitor and GM-CSF (VV-iPDL1/GM) was able to produce the PD-L1 inhibitor and systematically bind to PD-L1+ tumor cells and immune cells. The intratumoral injections with VV-iPDL1/GM produced iPDL1, enhanced neoantigen presentation, and activated systemic T cell responses against dominant, as well as subdominant neoantigens, resulting in the effective rejection of both virus-injected and distant tumors. Thus, this double-armed oncolytic virus is capable of activating neoantigen-specific T cell responses by the likely synergistic action of PD-L1 inhibition on tumor cells and immune cells, viral replication, and GM-CSF stimulation.

Individual tumors with numerous genetic mutations can contain high numbers of potentially immunogenic neoantigens. Despite the presence of immunogenic neoantigen epitopes in each tumor, spontaneous priming and activation of neoantigen-specific T cells are inefficient in the majority of cancer patients. Immunosuppressive tumor microenvironments, due to the lack of the “danger signals” of pathogen-associated molecular pattern (PAMP) molecules, and the expression of immune checkpoints, such as PD-L1, on tumor cells, T cells, and DCs, inhibit the priming or activation of T cell responses against tumor neoantigens. The engineered oncolytic virus generated in this study is able to produce the PD-L1 inhibitor, and bind to PD-L1+ tumor cells and immune cells. It is tempting to postulate that the secretory iPDL1 in combination with the viral oncolysis-mediated, immunogenic cell death and the release of viral PAMP molecules from infected cells may lead to the enhanced DC maturation and neoantigen presentation in the tumor microenvironment, and the systemic activation of tumor neoantigen-specific T cell responses. Thus, this study demonstrates that secretory PD-L1 inhibitors, GM-CSF, and viral oncolysis work synergistically to promote neoantigen presentation and activate tumor neoantigen-specific T cell responses, representing a potent, individual tumor-specific oncolytic immunotherapy.

An interesting finding of this study is the ability of the armed oncolytic virus to activate T cell responses against subdominant neoantigen epitopes. T cell responses are primed or activated by DCs, which present a repertoire of MHC-associated peptides. The tumor neoantigen repertoire derived from mutated gene products are presented to T cells after DCs capture and process antigens, derive neoantigen repertoire derived from mutated gene products DCs, which present a repertoire of MHC-associated peptides. The neoantigen epitopes. T cell responses are primed or activated by oncolytic virus to activate T cell responses against subdominant neoantigenic epitopes. The engineered oncolytic virus generated in this study should over- come this problem. Moreover, this oncolytic virus, which activates the neoantigen-specific T cell response by the synergistic action of PD-L1 inhibition, GM-CSF, and viral oncolysis in the tumor microenvironment may be advantageous to the therapies with PD-L1/PD-1 antibodies.

In summary, this engineered armed oncolytic virus with the ability to activate neoantigen-specific T cell responses by the synergistic action of viral immunogenic oncolysis, GM-CSF function, and PD-L1 inhibition on tumor cells and immune cells provides a potent, individual tumor-specific oncolytic immunotherapy, which could be therapeutically used alone or in combination with immune checkpoint inhibitors, targeted therapy, and chemotherapy for cancer patients, especially those resistant to PD-1/PD-L1 blockade therapy.

**Methods**

**Cell lines.** Human embryonic kidney cell line 293T, osteosarcoma HUTK-143B, monkey kidney fibroblasts CV1, murine adenocarcinoma Py230, murine melanoma B16-F10, and murine lymphoma EL4 were purchased from the American Type Culture Collection (ATCC). Murine colon adenocarcinoma cells MC38 was purchased from Kerafast. All the adherent cells were cultured in complete Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin–glutamine (100×) (Thermo, cat. no.: 10378016). T cells and splenocytes were grown in RPMI with 10% of heat-inactivated FBS, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 1% penicillin–streptomycin–glutamine, and 1× minimal essential medium nonessential amino acids. Cells were maintained in an incubator at 37 °C and 5% CO₂.

**Antibodies.** The antibodies used in the study included: anti-CD16/32 (clone: 93, Biologend, 1:100), anti-PD-L1 (APC or PE-cy7, clone: 10F.9G2, Biologend; clone: MH5.12, ebioscience, 1:100), anti-IGGa2a-Fc (Polyclonal, Thermo, 1:500), anti-CD45 (BV421 or PE, clone: 30-F11, Biologend, 1:500), anti-CD11c (APC or PE, clone: H13, BD Biosciences, 1:500), anti-CD11b (VF4 or PE-cy5, clone: M1/70, BD Biosciences, 1:100), anti-CD103 (FITC, clone: 2E7, Biologend, 1:100), viability dye (BV510 or UV-650, Tonbo Biosciences, 1:1000), anti-CD3 (FITC or Pacific Blue, clone: 17A2, Biologend, 1:1000), anti-CD4 (PE or PE-cy5, clone: RM-4.5, BD Biosciences, 1:500), anti-CD8 (FITC, APC, or APC-cy7, clone: 53-6.7, Biologend, 1:1000), anti-Gr-1 (PE or PE-cy7, clone: RB6-8C5, Biologend, 1:100), MHCI (FACSCalibur, BD, 1:1000), anti-CD11b (APC or PE, clone: 12–25C1, BD, 1:500), anti-FoxP3 (APC or PE, clone: FJK-16B, Biologend, 1:100), anti-IFN-γ (APC, clone: XMGL12.2, Biologend, 1:100), anti-CD4 (FITC, clone: 144B, Biologend, 1:1000), anti-IFN-γ (APC, clone: 12–25C1, BD, 1:500), anti-TNFα (PE, clone: MP-6XT2, BD, Biologend, 1:100), anti-IL-2 (PerCP-cy5.5, clone: JES6-5H4, Biologend, 1:100), anti-IL-12 (PE-cy7, clone: C12-6, Biologend, 1:100), anti-IL-10 (APC, clone: M1-70, BD Biosciences, 1:100), anti-CD80 (PE-cy5, clone: 16-10A1, Biologend, 1:100), anti-CD86 (PE-cy7, clone: GL-1, Biologend, 1:100), and anti-CD40 (PE, clone: 3/23, Biologend, 1:100), anti-CD31 (FITC, clone: 390, NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-15229-5 | www.nature.com/naturecommunications
Recombinant VV generation and purification. VV shuttle vector pSel-DsRed2N1 (refs. 25,26) was used to construct the recombinant shuttle vectors for coexpressing Recombinant VV generation and purification. BioXcell) Pentamer H-2Db-ASMTNMELM-PE was provided by ProImmune Inc. 800CW Goat anti-Mouse IgG Secondary Antibody (Polyclonal, Li-cor), anti-CD40 (cat. no. BP0016-2, Lot: 671717N1, BioXcell), and anti-PD-L1 (clone: 10F9.22, BioXcell) Pentamer H-2Db-ASMTNMELM-PE was provided by ProImmune Inc.

iPDL1 binding assay by flow cytometry. Tumor cells were infected with PBS, VV-Rfp, and VV-iPDL1/GM at MOI = 0.5. 24 h later, all cells were collected, and stained with anti-IgG Fc. For some experiments, tumor cells were first cultured overnight in the presence of IFN-γ (20 μg/mL) to enhance PD-L1 expression and then infected with PBS, VV-RFP, and VV-iPDL1/GM at MOI = 0.5. Forty hours later, all the cells were collected, and stained with anti-PD-L1 or anti-IgG Fc. PD-L1 RFP+ (virus-infected) cells and PD-L1+ RFP− (uninfected) cells were analyzed by flow cytometry. For detecting purified iPDL1 binding, wild-type MC38 cells and MC38 cells transduced with the recombinant lentiviral vector PD-L1shRNA/GFP were incubated with 50 μg/mL IgG or purified iPDL1 for 30 min on ice. Cells were then stained with anti-PD-L1 or anti-IgG Fc.

Inhibition of PD-1/PD-L1 interaction. Ninety-six-well ELISA plates were coated with 1 μg/well PD-L1 protein (Abcam, ab30039). A total of 50 μL mixture of 20 ng mouse PD-1–biotin (Sano Biological, 50124-M80H-B) and purified iPDL1, IgG control (Sigma, E5841), or anti-PD-L1 antibody control (Biolegend, 124301) at indicated concentration, or 50 μL assay buffer (blank) was added into wells, and incubated at RT for 2 h. Diluted streptavidin–HRP was added to each well after wash and incubated at RT for 1 h with slow shaking. After three times of wash, TMB substrate was added until blue color is developed in the positive control well. OD value at 450 nm UV was measured after 100 μL 2N sulfuric acid was added to stop reaction. OD of unknown (OD of positive control − OD of blank) represents the percent of inhibition activity.

ADCC assay. A total of 1 × 106 well target cells MC38 or IFN-stimulated MC38 cells were seeded in a 96-well plate 1 day before the experiment. On the day of experiment, different amounts of PBS, IgG Fc (Thermo, cat. no.: 31205) or purified iPDL1 were added into wells containing target MC38 cells followed by the addition of 6 × 104 ADCC bioassay effector cells per well that were provided in the ADCC Reporter Bioassays kit (Promega, Madison, WI). After incubation for 6 h at 37 °C, the plates were kept on the bench for 15 min. Then each well was added with 75 μL of Bio-Glo Luciferase reagent and kept at RT for 10 min. Luminescence values were measured using a plate reader with glow-type luminescence read capabilities.

Mouse experiments. All the animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of USC, and were bred and maintained in our institute-specific pathogen-free facilities. B16-F10, B16-F10-Luc, Py230, or MC38 tumors were established by subcutaneously injecting 5 × 105 of corresponding tumor cells into the left flank of C57BL/6 mice (N = 5 or 10 per group, the Jackson Laboratory). For the established tumor model, 1 × 105 B16-F10 cells were infected to the right flank simultaneously. When left flank tumor sizes reached ~100 mm3 or indicated sizes, tumors were intratumorally injected with 50 μL of the indicated VVs three times on days 0, 3, and 7 (5 × 105 pfu/tumor), or PBS with or without i.v. injections of 50 μL (200 μg/mL) of anti-PD-L1 antibody. Tumor sizes of treated primary tumors and untreated tumor on the contralateral side (distant tumor) were measured by caliper or monitored by bioluminescence imaging for B16-F10-Luc tumors. Tumor volumes were calculated according to the formula: width × length × 0.5. For tumor rechallenge assay, the treated mice were subcutaneously injected with 2.5 × 105 B16-F10 cells, 5 × 105 Py230, or 5 × 105 MC38 onto the right flank of each mouse at indicated days after the virus treatment. A group of naive mice were injected with tumor cells for control. The rechallenged tumors were monitored as above described. For CD8 T cell depletion experiment, anti-CD8 antibodies (clone: 2.43, Bio X cell, cat. no.: BP0906) were injected i.v. twice weekly starting one day prior to viral injection.

Ne ontogenetic-specific T cell response assays. Splenocytes were isolated from various VV-treated tumor-bearing C57BL/6 mice and cultured in a 96 round bottom well plate (1 × 106 cells/well) in the presence of single neopeptide or a mixture of neopeptides of MC38 at the indicated concentrations at 37 °C in 5% CO2. After 8 h incubation, 200 μL supernatants were collected from each well to evaluate IFN-γ via ELISA. [3H]Thymidine (1 μCi per well) was added and cultured for an additional 6 h. [3H]Thymidine incorporation was measured in TopCount Scintillation and Luminescence Counter. For flow cytometric analysis, splenocytes from the various VV-treated groups were cocultured with syngeneic monocye-degenerated DRGs (10:1) that were pulsed with neopeptides for 12 h in the presence of Golgi-plug65. Cells were stained with anti-CD8, anti-107a, anti-IFN-γ, anti-H2L, and anti-TNF-α, and analyzed by flow cytometry.
virus. A total of 500 μL homogenate were incubated on 143B TK cells and titers were determined24,25. Viral titers were standardized to tissue weight.

Generation of neoepitopes–primed T cells. C57BL/6 mice (6–8 weeks) were injected intraperitoneally with a mixture of 11 peptides (10 μg each) formulated with the adjuvant system consisting of 100 μg anti-CD40 (Abclone PK45) and 100 μg poly (I:C; InovioGen) two times on days 0 and 14. On day 21, the splenocytes were harvested and in vitro stimulated with irradiated autologous naïve splenocytes preplus with the peptide mixture for two rounds. Expanded splenic cells were harvested for further experiments36,37.

Isolation of tumor-infiltrating immune cells. C57BL/6 mice were subcutaneously inoculated with MC38 cells (1 × 10^6) on one side flank. When the tumor sizes reached ~100 mm^3 or indicated sizes (counted as day 0), mice were intratumorally injected with 50 μL of PBS, VV-RFP, VV-GM, or VV-iPDL1/GM (5 × 10^5 pfu/tumor) on days 0 and 3. One group of mice were intraperitoneally injected with 200 μg of anti-PD-L1 antibody (clone 10F.9G2). At indicated days post viral treatment, tumors were collected, weighed, and digested with collagenase type I and DNase for 30 min at 37 °C. The tumor tissues were homogenized and then filtered through a 70-μm nylon strainer33. Single-cell suspensions were analyzed by FACS or used for other assays81.

In vitro and in vivo assays of neoepitopes–specific T cell responses. Tumor-infiltrating DCs from various VV-treated, tumor-bearing mice were isolated using CD11c MicroBeads UltraPure (Miltenyi Biotec, 130-108-338). The DCs were pulsed with indicated neoepitopes, and then cocultured with the neoantigens–primed T cells to assess cytokine production and T cell proliferation19.

To assess the immunogenicity of the VV-treated tumor cells, MC38 cells seeded in 96-well round bottom plates (5 × 10^4 per well) were infected with PBS, VV-RFP, VV-GM, or VV-iPDL1/GM at MOI = 1 for 2 h. Infected MC38 cells were extensively washed and then cocultured with 2 × 10^4 the neoantigens–primed T cells for 48 h. One of mock-infected MC38/CTL cocultures was added with 1 μg/mL anti-PD-L1 antibody, multiple observations at each level (for tumor volumes). Production via ELISA. Cells were harvested, immune stained with anti-CD3. T cell numbers were counted by adding precision counting beads (Biolegend, 424902).

To assess in vivo proliferation of the neoantigens–primed T cells in various VV-treated, tumor-bearing mice, MC38 tumor-bearing mice were treated with the indicated viruses (5 × 10^5 pfu), followed by adoptive transfer of CFSE-labeled 2 × 10^6 the neoantigens–primed T cells. Three days later, TDLNs were harvested and their proliferation was assessed based on CFSE dilution via flow cytometry34. Data shown are a representative histogram of two independent experiments.

CTL assay. Firefly Luciferase stably expressing cells were cocultured with effector T cells at the indicated ratios. Forty eight hours later, all the cells were spun down and resuspended in 100 μL media supplemented with 100 μg/mL Beetle Luciferin Potassium Salt and incubated at RT for 5 min. Cells were transferred to 96-well white opaque plate. Luciferase emission was measured on a TopCount Scintillation and Luminescence Counter. Killing lysis % = 1 – (blank – sample/blank) × 100%.

RNA sequencing. MC38 cells were infected with VV-iPDL1/GM. Cells were harvested at various times. Cellular RNAs were extracted from cell lysates using RNeasy Plus Mini Kit (Qiagen). Total RNA is enriched by oligo (dT) magnetic beads (rRNA removed). RNA-seq library preparation using KAPA Stranded RNA-Seq Library Prep Kit (Illumina). The libraries were sequenced on a HiSeq 4000 instrument using 2 × 150 bp pair-end sequencing (Arraystar Inc., Rockville, MD).

Software. Odyssey v3.0, MicroWin2000, Living Image v4.4, FACS DIVA 6.1.2, Illustrator CS6, Bowyer 4.0A, Graphpad prism 4.0, Microsoft excel 2011 for mac, Living Image v4.3,1, RNA-seq analysis was performed with the following software HTSeq v0.9.3, Solexa pipeline v1.8, FastQC software 0.11.7, Hisat2 software, StringTie 1.3.3, R 3.4.1, and Python 2.7.

Statistics. Statistical analysis was performed using GraphPad Prism 6. When passing the normality test, two-tailed Student’s t-test was used to compare the two groups. Otherwise, a Mann–Whitney U test was used. Repeated-measures two-way ANOVA with Bonferroni’s correction was used to compare the effect of multiple levels of factors within each (for multiple tumors with each level for tumor volumes). Animal survival is presented using Kaplan–Meier survival curves and was statistically analyzed using log rank test. The data presented in the figures are mean ± SD. P values < 0.05 were considered to statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The RNA-seq data have been deposited in the NCBI GEO database under the accession code GSE145823. The source data underlying Supplementary Fig. 14b are provided as a Source Data file. All the other data supporting the findings of this study are available within the article and its Supplementary Information files, and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file.

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