Intratumoral Delivery of a PD-1–Blocking scFv Encoded in Oncolytic HSV-1 Promotes Antitumor Immunity and Synergizes with TIGIT Blockade

Chaolong Lin, Wenfeng Ren, Yong Luo, Shaoqin Li, Yating Chang, Lu Li, Dan Xiong, Xiaoxuan Huang, Zilong Xu, Zeng Yu, Yingbin Wang, Jun Zhang, Chenghao Huang, and Ningshao Xia

ABSTRACT

Oncolytic virotherapy can lead to systemic antitumor immunity, but the therapeutic potential of oncolytic viruses in humans is limited due to their insufficient ability to overcome the immunosuppressive tumor microenvironment (TME). Here, we showed that locoregional oncolytic virotherapy upregulated the expression of PD-L1 in the TME, which was mediated by virus-induced type I and type II IFNs. To explore PD-1/PD-L1 signaling as a direct target in tumor tissue, we developed a novel immunotherapeutic herpes simplex virus (HSV), OVH-aMPD-1, that expressed a single-chain variable fragment (scFv) against PD-1 (aMPD-1 scFv). The virus was designed to locally deliver aMPD-1 scFv in the TME to achieve enhanced antitumor effects. This virus effectively modified the TME by releasing damage-associated molecular patterns, promoting antigen cross-presentation by dendritic cells, and enhancing the infiltration of activated T cells; these alterations resulted in antitumor T-cell activity that led to reduced tumor burdens in a liver cancer model. Compared with OVH, OVH-aMPD-1 promoted the infiltration of myeloid-derived suppressor cells (MDSC), resulting in significantly higher percentages of CD155+ granulocytic-MDSCs (G-MDSC) and monocytic-MDSCs (M-MDSC) in tumors. In combination with TIGIT blockade, this virus enhanced tumor-specific immune responses in mice with implanted subcutaneous tumors or invasive tumors. These findings highlighted that intratumoral immunomodulation with an OV expressing aMPD-1 scFv could be an effective stand-alone strategy to treat cancers or drive maximal efficacy of a combination therapy with other immune checkpoint inhibitors.

Introduction

Cancer immunotherapy has achieved great therapeutic success over the past several years. Only a subset of patients benefits from immunotherapeutic regimens (1). The tumor microenvironment (TME) in many tumor types that do not respond to immunotherapy lacks infiltration of tumor-specific immune cells, lacks neoantigen expression and costimulatory signaling, and exhibits coinhibitory signaling, which restricts the efficacy of cancer therapy (2). Reversing the immunosuppressive TME is the most important challenge in the development of immunotherapeutics (3). Oncolytic viruses (OV) can selectively replicate in tumor cells and provoke a virus-specific or tumor-specific inflammatory response in the TME (4). OV can elicit T-cell migration to tumor tissue and T-cell activation, ultimately mediating local and distant immunotherapeutic efficacy (5). Oncolytic virotherapy is promising therapeutic strategies for cancer, further preclinical studies are needed to maximize therapeutic efficacy (6). The antitumor efficacy of oncolytic virotherapy is significantly enhanced antitumor when combined with systemic immune checkpoint blockade, such as CTLA-4 and PD-1 blockade (7–10). However, this raises several concerns in terms of increased toxicities for patients and medical costs to healthcare systems (11, 12). To resolve these issues, investigators have designed various strategies to augment the antitumor immunity of oncolytic virotherapy, such as engineering OV expressing cytokines, costimulatory factors, and immunomodulatory agents (13–15).

In this study, we analyzed the TME alterations in response to intratumoral virotherapy to select a specific immune target to guide our design our multiplexed antitumor OV vector. We identified that the PD-1/PD-L1 pathway can be targeted to improve an oncolytic herpes simplex virus (OVH), thus constructed a recombinant OVH virus encoding a single-chain variable fragment (scFv) against PD-1 (aMPD-1 scFv), OVH-aMPD-1. We hypothesized that the intratumoral injection of OVH-aMPD-1 would induce potent oncolytic effects and revitalize intratumoral T cells, inducing antitumor activity. OVH-aMPD-1 increased the infiltration of CD155+ myeloid-derived suppressor cells (MDSC) within the TME. TIGIT blockade improved the antitumor efficacy of OVH-aMPD-1. In summary, we demonstrated that OVH-aMPD-1 exhibited robust antitumor activity activity and prolonged the survival of tumor-bearing mice in different multiple models. This strategy significantly augmented the efficacy of oncolytic virotherapy, providing evidence for the rational design of therapies employing this strategy for clinical investigation.

Materials and Methods

Mice

C57BL/6 mice and BALB/c nu/nu mice were purchased from the Shanghai Slack Laboratory Animal Co., Ltd., bred and housed under specific pathogen-free conditions in the Animal Facility of Xiamen University (Xiamen, China). The mice used in studies were 4–6 weeks old unless otherwise indicated. All animal protocols were approved by the Shanghai Slack Laboratory Animal Co., Ltd.
the Institutional Animal Care and Use Committee at Xiamen University for animal welfare (XMULAC20150016).

**Cells**

HEK293T, Hepa1-6, and U-2 OS cells were purchased from the ATCC (2015). MC38 cells were purchased from the China Infrastructure of Cell Line Resources (Beijing, China, 2018). 293T, U-2 OS, and Hepa1-6 cells were cultured in DMEM supplemented with 10% (v/v) FBS (Invitrogen). MC38 cells were cultured in 1640 medium supplemented with 10% (v/v) FBS. All cells were maintained at 37°C and 5% CO2. Hepa1-6 PD-L1− cells were generated by using the CRISPR/Cas9 method. In brief, PD-L1-targeting sgRNAs synthesized by Sangon (Shanghai, China; sgRNA1 5'-GTATGGCACAAAGTCAGA-3'; sgRNA2 5'-GCTTGGGTAGTGTTGATC-3'; and sgRNA3 5'-GGTCCAGCTCCGTTCTACA-3') were cloned into the lentivirus vector (v2 vector) (52961, Addgene). HEK293T cells were transfected with packaging plasmids (pSPAX2 (12260) and PMD2.G (12259, Addgene) and the lentiviral vector (v2 plasmid) by using Lipofectamine 2000 reagent (15668019, Invitrogen) according to the manufacturer's instructions. Virus-containing supernatants were harvested 48 hours posttransfection. Hepa1-6 cells were transduced with the virus supernatant for 48 hours. To obtain PD-L1− cells, these cells were seeded in 6-cm dishes at 105 cells/dish and infected with the indicated virus (0.1 PFU/cell) or mock infected. For each time point, the infected cells were harvested and thereafter subjected to virus titration.

**Cytotoxicity assay**

In total, 6 × 10⁵ cells were seeded in 6-cm dishes and infected with the indicated virus (1 PFU/cell) or mock infected. For each time point, cell viability was measured by detecting lactate dehydrogenase activity in the lysates using a Cytotoxicity Assay kit (G1780, Promega) according to the manufacturer's instructions.

**Western blot analysis**

Cell lysates were prepared in RIPA lysis buffer containing a protease inhibitor cocktail (04693132001, Roche), and the protein content of the generated cell lysates was determined using the BCA Protein Assay (752806, BioLegend) for 24 hours and stained with an anti-PD-L1 antibody (124319, BioLegend), and the PD-L1−negative cells were sorted into single-cell clones. Knockout clones were confirmed by flow cytometry analysis for PD-L1. Hepa1-6-mRuby3 and MC38-mRuby3 cells were generated by transduction with a lentiviral vector (17477, Addgene) encoding mRuby3 or OVA. Positive clones were selected in medium containing 1 μg/mL puromycin (ant-pr-5, InvivoGen), and fluorescent protein expression was confirmed by flow cytometry analysis. Cell lines were not authenticated in the past year and cultured for fewer than 8 passages in indicated medium. All cell lines were routinely tested using a Mycoplasma contamination detection kit (rep-p1, InvivoGen).

**Viruses and virus generation**

OVH was constructed on the backbone of KOS, in which both copies of the ICP34.5 and ICP0 coding sequences were replaced by the eGFP gene and the ICP27 promoter was replaced with a core hTERT promoter previously constructed in our laboratory. The gene encoding aMPD-1 scFv consisted of a secretion signal sequence (SP), variable light chain (VL), 3 × G8S, variable heavy chain (VK), and His tag (His), which were sequentially spliced from the CDNAs sequence of a rat anti-mouse PD-1 antibody (clone 32D6; ref. 16) and assembled into pcDNA3.1 (Invitrogen) under the control of the human cytomegalovirus promoter, named pcDNA3.1-aMPD-1 scFv (Supplementary Fig. S1A and S1B). OVH-aMPD-1 was constructed on the backbone of OVH, in which both copies of the eGFP coding sequences were replaced by the gene encoding aMPD-1 scFv. OVH-aMPD-1-Luc was constructed on the backbone of OVH-aMPD-1, in which the gene coding luciferase was inserted into the genome between the UL37 and UL38 regions (17). The generation of recombinant virus was performed using a cell-based recombinant method as described previously (17).

**Virus titration and replication assay**

The titers of amplified viruses were determined on U-2 OS monolayers using a classical plaque assay as described previously (18). Viral titers (PFU/mL) were calculated using the following formula: titer = plaque numbers × dilution fold × 2. For a virus replication assay, cells were seeded in 6-cm dishes at 10⁵ cells/dish and infected with the indicated virus (0.1 PFU/cell) or mock infected. For each time point, the infected cells were harvested and thereafter subjected to virus titration.

**Potentiation of TIGIT Blockade by OV Expressing a PD-1 scFv**

For the production of the aMPD-1 scFv recombinant protein, the expression plasmid pcDNA3.1-aMPD-1 scFv was transfected into HEK293T cells using PEI transfection reagents in Expi293 Expression Medium (A1435101, Invitrogen). The medium was harvested 5 days after transfection, filtered through a 70-μm nylon filter, concentrated using ammonium sulfate precipitation, and stored at 4°C. aMPD-1 scFv was purified by using Ni-NTA chromatography (17–5318-00, GE Healthcare), Primary antibodies were used for probing: gD (21719, Santa Cruz Biotechnology), ICP0 (56985, Santa Cruz Biotechnology), GFP (32146, Abcam), β-actin (47778, Santa Cruz Biotechnology), cleaved PARP (9541S, Cell Signaling Technology), cleaved caspase-3 (9664T, Cell Signaling Technology), as well as polyclonal anti-ICP34.5 antibodies.

**In vitro infection experiments**

Cells were cultured in 6-well dishes at 6 × 10⁵ cells/well and infected with OVH at the indicated MOIs. The infected cells were collected for PD-L1 surface labeling at 36 hours postinfection and analyzed by flow cytometry analysis. The supernatants of the infected and noninfected cells were centrifuged for 5 minutes at 3,000 rpm to remove cellular cell debris. The supernatants from the infected cells were UV inactivated with a UV Stratalinker 2400 instrument (Stratagene, 360 mJ/cm²) for 5 minutes, which is sufficient to completely eliminate live virus (Supplementary Fig. S2). For supernatant transfer experiments, the inactivated supernatant was diluted 1:2 in fresh complete medium containing 10% FBS and added to fresh cells in 6-well plates. The infected cells were collected for PD-L1 surface labeling at 24 hours postinfection and analyzed by flow cytometry. For cytokine treatment, cells were treated with 2,000 U/mL mouse IFNα (12100, R&D SciBiotech) and stored at −20°C. aMPD-1 scFv proteins were subjected to SDS-PAGE. The proteins separated on the gel were visualized by silver staining (24600, Pierce) according to the manufacturer’s instructions.
ELISA analysis

The protein expression of aMPD-1 scFv was determined by an indirect chemiluminescence immunoassay (CEIA). Briefly, 96-well plates were coated with 100 ng/well mPD-1-Fc protein (1021-PD, Sino Biological), and nonspecific binding was blocked with PBS containing 20% CBS. Purified aMPD-1 scFv protein, supernatants from OVH-aMPD-1–infected cells, or samples from OVH-aMPD-1–treated mice were added to the wells for a 1-hour incubation, followed by washing and reaction with an anti-His-HRP antibody (HRP-66005, ProteinTech). After the addition of 100 μL luminol substrates (Wantai BioPharm) for 5 minutes, the plates were measured with a chemiluminescence reader (ORION II, Berthod). For detection of aMPD-1 scFv in tumors, tumors were weighed and homogenized in 2 mL of sterile PBS in gentleMACS M tubes (130-096-335, Miltenyi Biotec) using a gentleMACS dissociator and with the running program Protein_01 (Miltenyi Biotec). The homogenates were centrifuged for 5 minutes at 12,000 rpm and the supernatants were assessed by the above method. Empty medium served as standards. The quantity of each experimental sample was determined using a standard curve.

The reactivity of aMPD-1 scFv against PD-1 protein of human origin or mouse origin (Sino Biological) was determined by CEIA as described previously (16). To compare the blocking activity of the commercial anti-PD-1 antibodies (clone RMP1-14 and J43, BioXcell), 32D6 antibodies and aMPD-1 scFv, a blocking CEIA was set up. Binding was blocked with PBS containing 20% CBS. The reactivity of aMPD-1 scFv against PD-1 protein of human origin or mouse origin (Sino Biological) was determined by CEIA as described previously (16). To compare the blocking activity of the commercial anti-PD-1 antibodies (clone RMP1-14 and J43, BioXcell), 32D6 antibodies and aMPD-1 scFv, a blocking CEIA was set up. Binding was blocked with PBS containing 20% CBS. The reactivity of aMPD-1 scFv against PD-1 protein of human origin or mouse origin (Sino Biological) was determined by CEIA as described previously (16). To compare the blocking activity of the commercial anti-PD-1 antibodies (clone RMP1-14 and J43, BioXcell), 32D6 antibodies and aMPD-1 scFv, a blocking CEIA was set up. Binding was blocked with PBS containing 20% CBS. The reactivity of aMPD-1 scFv against PD-1 protein of human origin or mouse origin (Sino Biological) was determined by CEIA as described previously (16). To compare the blocking activity of the commercial anti-PD-1 antibodies (clone RMP1-14 and J43, BioXcell), 32D6 antibodies and aMPD-1 scFv, a blocking CEIA was set up. Binding was blocked with PBS containing 20% CBS.

Assays for detecting immunogenic cell death determinants

MC38 and Hepa1-6 cells were infected with OVH or OVH-aMPD-1 at an MOI of 1 PFU/cell. After 48 hours of infection, the cells were collected and stained with an Alexa Fluor 647–conjugated anti-calreticulin antibody (bs-5913B-A647, Bioss) and subjected to flow cytometry to analyze calreticulin-positive cells. Briefly, a monolayer cell was washed with PBS after trypan digestion, and single-cell suspensions were washed twice with PBS, followed by centrifugation at 1,000 rpm for 5 minutes. Cells were then stained with antibodies for 1 hour in Brilliant Stain Buffer (563794, BD Biosciences) on ice in the dark. Following this incubation period, stained cells were washed with PBS and then centrifuged at 1,000 rpm for 5 minutes. The supernatant was then aspirated and the resulting cells were resuspended in Brilliant Stain Buffer and samples were run on a BD LSFRFortessa X-20 according to manufacturer’s recommendations, and data were analyzed by FlowJo 10. The ATP level in the supernatant was measured by the Enhanced ATP Assay Kit (S0027, Beyotime), and the HMGB1 level in the supernatant was measured by an HMGB1 ELISA kit (STS0101, TECAN) according to the manufacturer’s instructions.

Dendritic cell purification and phagocytosis assays

To generate bone marrow–derived dendritic cells (BMDC), single-cell suspensions of bone marrow from wild-type C57BL/6 mice were obtained according to standard protocols with minor modifications (19). Briefly, head extractions of C57BL/6 mice were collected, soft tissues removed, and bones rinsed in 70% ethanol. After cutting the ends of femurs and tibias, bone marrow was flushed out with RPMI1640 medium and collected. Red cells were lysed with RBC lysis buffer (420301, BioLegend). The plates were assayed using the above method. The isolated DCs were cultured in RPMI1640 medium supplemented with 10% FBS, 50 ng/mL GM-CSF (576304, BioLegend), and 25 ng/mL IL4 (204-IL, R&D Systems) for 7 days. CD11c+ dendritic cells (DC) were purified using a Dynabeads Mouse DC Enrichment kit (11429D, Thermo Fisher Scientific) according to the manufacturer’s instructions. The isolated DCs were cultured overnight with recombinant GM-CSF and Hepa1-6-mRuby3 or MC38-mRuby3 cells pretreated with vehicle or virus for 24 hours. The efficiency of DC phagocytosis is expressed as the percentage of CD11c+ mRuby3+ cells among all CD11c+ CD45+ cells, which were counted by flow cytometry analysis.

Tumor-infiltrating lymphocyte isolation and flow cytometry analysis

Tumor analysis was performed as described previously (20). For isolation and analysis of tumor-infiltrating lymphocytes (TIL), mice were sacrificed, and tumors were harvested for analysis 7 days after two doses of the indicated treatment were given. The tumors were removed using forceps and surgical scissors and weighed. Tumors were minced with scissors and incubated with 1 mg/mL Collagenase D (11284932001, Sigma) and 0.5 mg/mL DNase I (11088866001, Roche) and 100 μg/mL DNase I (11284932001, Sigma) in RPMI1640 medium supplemented with 2% FBS for 1.5 hours with continuous agitation. The digestion mixture was homogenized by repeated pipetting and filtered through a 70-μm nylon filter. The single-cell suspensions were washed twice with a Brilliant Stain Buffer (563794, BD Biosciences) and stained with the Zombie Aqua Fixable Viability Kit (423102, BioLegend) to eliminate dead cells according to the manufacturer’s instructions. After washing twice, the cells were stained with the corresponding antibodies, incubated for 30 minutes at 4°C, and then subjected to flow cytometry analysis with a BD LSFRFortessa X-20. Data were analyzed by FlowJo 10. The antibodies used for flow cytometry are listed below: anti-CD45.2 APC/Cy7 (clone 104, 109824), anti-CD3 ε Brilliant Violet 421 (clone 145-2C11, 100341), anti-CD8α
Potential of TIGIT Blockade by OV Expressing a PD-1 scFv

Subcutaneous xenograft model
An inoculum of $5 \times 10^5$ Hepa-1-6 cells in 100 μL of sterile PBS was injected subcutaneously (i.s.c.) into the flank of 5-week-old female BALB/c nu/nu mice. After 10 days, the Hepa-1-6 tumors reached an average size of approximately 100 mm$^3$. The mice were randomized into treatment groups immediately prior to treatment. Virus in 50 μL of sterile PBS was administered via intratumoral injection every 3 days for three doses in total. Tumor growth was monitored every 3 days by measurement with a caliper (06-664-16, Thermo Fisher Scientific). Twenty-one days after the last treatment, the mice were measured a final time. Tumor volume was calculated according to the formula: (length × width$^2$)/2.

Syngeneic murine cancer model
For the establishment of subcutaneous Hepa-1-6 tumors, an inoculum of $5 \times 10^5$ murine Hepa-1-6 cells or Hepa-1-6-OVA cells in 100 μL of sterile PBS was injected subcutaneously into each flank of 6-week-old female C57BL/6 mice. The mice were randomized into treatment groups on day 7 or 10 following tumor inoculation, immediately before treatment. Virus in 50 μL of sterile PBS was administered via intratumoral injection every 3 days for three doses in total. Tumor growth was monitored every 3 days as described above. The overall survival of the mice was monitored over a 90-day period. The tumor-free incidence is presented as the percentage of tumor-free mice among the total treated mice.

For a mixed competition assay, equal numbers of EGFP- and mRuby3-positive cells were mixed together, and $5 \times 10^5$ cells in 100 μL of sterile PBS were injected subcutaneously into the flank of 6-week-old female C57BL/6 mice. After 10 days, when the tumors reached an average size of approximately 100 mm$^3$, OVH was administered via intratumoral injection at a dose of $1 \times 10^7$ PFU and in 50 μL of sterile PBS. Seven days after virus injection, the percentage of CD45$^+$ fluorescent cells in the tumors was calculated by flow cytometry analysis.

For tumor rechallenge experiments, naïve C57BL/6 mice and Hepa-1-6 tumor-free C57BL/6 mice treated by virotherapy that survived for 90 days were subcutaneously rechallenged with $5 \times 10^5$ Hepa-1-6 cancer cells in the different sites as the primary tumors. The incidence of secondary challenge rejection is presented as the percentage of tumor-free mice among the total rechallenged mice.

For comparing the therapeutic efficacy of OVH plus anti-PD-1 blockers with OVH-aMPD-1 therapy, virus ($1 \times 10^7$ PFU) and 10 μg of aMPD-1 scFv in 50 μL of sterile PBS was intratumorally injected at day 10 and every 3 days thereafter until three doses were administered.

For combinatorial therapy, virus ($1 \times 10^7$ PFU) was intratumorally injected in a volume of 50 μL, and 200 μg of anti-Tigit antibody (clone 1G9, BioXcell) or rat IgG isotype control antibodies in 100 μL of sterile PBS were intraperitoneally (i.p.) injected at day 14 and every 3 days thereafter until three doses were administered.

For the establishment of intraperitoneal MC38 tumors, an inoculum of $1 \times 10^5$ murine MC38 cells in 200 μL of sterile PBS was injected intraperitoneally into 6-week-old female C57BL/6 mice. The mice were randomized into treatment groups on day 7 following tumor inoculation, immediately before treatment. Virus ($1 \times 10^7$ PFU) in 100 μL of sterile PBS and anti-Tigit antibodies (200 μg) in 100 μL of sterile PBS were administered via intraperitoneal injection every 3 days for three doses in total. The overall survival of the mice was monitored over a 100-day period.
Depletions
Depletion of immune cells was performed with corresponding depleting rat mAbs against different immune markers. When the tumors reached 130 mm³, all depleting antibodies (anti-CD86 (clone 2.43, BioXcell), anti-CD4 (clone GK1.5, BioXcell) and rat IgG isotype control antibodies) were intraperitoneally administered beginning 2 days before initiation of therapy, at a dose of 400 μg per antibody every 2 days in 100 μL of sterile PBS for four dosages and thereafter every 5 days until the end of the experiment. Virus (5 × 10⁶ PFU) in 50 μL of sterile PBS was administered via intratumoral injection into the right flank tumors every 3 days for three doses in total. The tumor size was monitored for 24 days as described above.

Bioluminescence imaging
Mice that received OVH-aMPD-1 therapy were imaged every day until day 6. Mice were injected retro-orbitally with 50 μL of 40 μg/mL luciferin (E1605, Promega) and imaged immediately using the IVIS Imaging System (Caliper Life Sciences). Cells infected with OVH-aMPD-1 at different MOIs were incubated with 1 μg/mL luciferin in PBS and imaged immediately using the IVIS Imaging System (Caliper Life Sciences).

IHC
Tumors were collected at sacrifice and kept in 10% buffered formalin. The fixed tissues were histologically analyzed by IHC staining of indicated markers. Anti-CD86t (98941) and anti-Ki67 (12202) were obtained from Cell Signaling Technology. Anti-CD155 (LS-B10536) was obtained from LSbio. IHC staining was performed using an Ultravision SP kit (KIT-9720, Maxim) and a DAB detection kit (DAB-0031, Maxim) according to the manufacturer’s instructions. Images were taken with a research-level upright microscope (BX51, Olympus) and data were analyzed by cellSens Standard Ver.1.4 software.

Statistical analysis
Statistical significance was calculated using Student t test or repeated-measure ANOVA, as indicated in the figure legends. Data for survival were analyzed by the log-rank (Mantel–Cox) test. For all statistical analyses, differences were considered significant when the P value was less than or equal to 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant). Statistical analyses were performed using GraphPad Prism 7. The numbers of animals included in the study are discussed in each figure.

Results
OVH upregulated PD-L1 expression in the TME
To characterize the immunomodulatory effect of intratumoral OVH therapy, we used a Hepa-1-6 liver cancer model and analyzed the TILs within the tumor tissue after treatment (Fig. 1A). Analysis of virus-injected tumors revealed an increased inflammatory response in the tumors, showing increased infiltration of CD45⁺ leukocytes and CD3⁺ lymphocytes (Supplementary Fig. S3A–S3C). Notably, there were substantial increases in the absolute numbers of CD4⁺ and CD8⁺ T cells (Fig. 1B). CD45⁺ tumor cells, MDSCs, and DCs isolated from the injected and distant tumors had increased expression of PD-L1 (Fig. 1C and D); tumor-associated macrophages (TAM) isolated from the injected tumors, but not those from distant tumors, also has increased PD-L1 expression (Supplementary Fig. S4A–S4C). Given the existing upregulation of PD-L1 expression with OVH treatment, we chose to explore whether PD-L1 on CD45⁺ tumor cells could directly attenuate the increased immune response induced by OVH, resulting in the resumption of rapid tumor cell growth. We used a mixed competition assay to test this idea by injecting C57BL/6 mice with mixtures of equivalent numbers of Hepa-1-6 wild-type cells that stably expressed mRuby3 and Hepa-1-6 PD-L1 knockout (KO) cells that stably expressed EGFP. At 7 days post OVH injection, we measured the cellular composition of the tumors by assessing the fluorescence of the different markers ex vivo (Fig. 1E; Supplementary Fig. S5A and S5B). We hypothesized that if PD-L1 was critical for the direct suppression of the CD8⁺ T-cell cytotoxicity mediated by OVH, then the Hepa-1-6 PD-L1 KO cells would be selectively depleted. Indeed, the PD-L1 KO cells were selectively reduced in tumors (Fig. 1F). These findings provided rationale for targeting the PD-1/PD-L1 axis directly in tumors, which was further supported by studies demonstrating that PD-L1 blockade can potentiate the efficacy of oncolytic virotherapy (7, 8).

OVH-induced type I IFN led to upregulation of PD-L1 in tumor cells
We used GFP-expressing OVH as a tool to investigate the PD-L1 expression of virus-infected and noninfected cancer cells (Fig. 2A). We found that the infection of mouse tumor cell lines with OVH induced marked PD-L1 upregulation on the surface of both virus-infected and noninfected cells. The intensity of PD-L1 staining was higher in the fraction of virus-infected cells than in that of noninfected cells, suggesting that PD-L1 upregulation was mediated by two mechanisms: direct infection by the virus and an unknown indirect mechanism (Fig. 2B and C). We hypothesized that the upregulation of PD-L1 in noninfected cells after OVH infection was probably mediated by in situ secreted immune factors. Therefore, we collected UV-inactivated cell culture supernatants from the infected cells, transferred it to noninfected cells, and detected PD-L1 expression. We found that conditioned medium generated from different cancer cell lines by exposure to virus induced PD-L1 upregulation on the cell surface irrespective of the cancer cell type (Fig. 2D; Supplementary Fig. S6). These findings suggested that secreted immune factors may have promoted the upregulation of PD-L1 expression on other cells in a paracrine fashion. We hypothesized that cytokines may play a major role in PD-L1 upregulation after exposure to OVH, thus treated tumor cells with different cytokines. Treatment of cancer cells with IFNs resulted in efficient PD-L1 upregulation, with IFNγ inducing the most robust PD-L1 upregulation (Fig. 2E and F). To confirm that type I IFN was the regulator of the PD-L1 increase, we treated cells with UV-inactivated cell culture supernatants from infected cells in the presence of an antibody blocking IFNAR or a control antibody, IFNα but not IFNγ, was detected in the transferred supernatants from OVH-infected Hepa-1-6 cells (Supplementary Fig. S7A and S7B). IFNAR antibody blockade resulted in complete abrogation of PD-L1 upregulation by OVH treatment (Fig. 2G), confirming that type I IFN was responsible for the OVH-mediated PD-L1 upregulation observed in vitro. Furthermore, we used tumor cells and a T-cell coculture assay to confirm that type II IFN was the regulator of the PD-L1 increase. We cocultured cancer cells with inactivated T cells or activated T cells in the presence of an antibody blocking IFNγ or a control antibody (Supplementary Fig. S7C). IFNγ antibody blockade resulted in significantly reduced PD-L1 expression (Fig. 2H). We next sought to determine whether IFNγ could be induced by OVH in vivo (Fig. 2I). Intratumoral treatment of tumors with OVH resulted in the induction of IFNγ expression in the treated tumors (Fig. 2I). These findings highlighted the idea...
that OVH-induced IFN responses can drive PD-L1 expression and possibly elicit adaptive immune resistance.

**Generation of a recombinant OVH expressing an scFv against murine PD-1**

To target PD-1 directly within tumors, we engineered a recombinant OVH encoding an aMPD-1 scFv (OVH-aMPD-1; Fig. 3A). The blocking ability of aMPD-1 scFv in inhibiting the interaction between PD-1 and PD-L1 was similar to the other well-known commercial antibodies (RMP1-14 and J43), and aMPD-1 scFv specifically recognized mouse PD-1 (Supplementary Fig. S8A and S8B). The recombinant viruses were verified by examining viral genes and exogenous gene expression (Fig. 3B). Further *in vitro* characterization of OVH-aMPD-1 revealed that the virus was equivalent to the parental OVH strain in regards to its replicative capacity (Fig. 3C) and cell killing ability (Fig. 3D) in U-2 OS cells. OVH-aMPD-1 possessed significant cell-killing activity against Hepa-1-6 cells and MC38 cells (Supplementary Fig. S9). Infection of U-2 OS cells with OVH-aMPD-1 resulted in an over 1,000-fold increase in aMPD-1 scFv in the supernatant of the infected cells in a time-dependent fashion (Fig. 3E). The expression of aMPD-1 scFv was relatively higher in human cancer cells than in mouse cancer cells possibly due to the relatively low permissivity of mouse cancer cells to HSV-1 (Fig. 3F). As expression was most efficient in Hepa-1-6 cells, this cell line was selected as the primary model for *in vivo* studies. Intratumoral administration of OVH-aMPD-1 into Hepa-1-6 tumors resulted in significant aMPD-1 scFv expression in treated tumors in a time-dependent fashion (Fig. 3G). Finally, we confirmed the T-cell–stimulating property of recombinant...
aMPD-1 scFv purified from a virus-infected cell culture (Fig. 3H; Supplementary Fig. S10).

**OVH-aMPD-1 improved DC cell presentation and revived T lymphocytes.**

Studies indicate that PD-1 expression by TAMs significantly inhibits phagocytosis and antitumor immunity (21), suggesting that PD-1 blockade may restore phagocytosis. We wondered whether aMPD-1 scFv derived from OVH-aMPD-1 infected culture could promote phagocytosis, and thus we used an *in vitro* phagocytosis assay to test this idea using DCs. The results revealed that the phagocytosis of cancer cells by DCs was significantly increased when cancer cells were preinfected with virus, and OVH-aMPD-1 induced much stronger phagocytosis than OVH (Fig. 3I and J; Supplementary Fig. S11A). To determine whether aMPD-1 scFv secreted from OVH-aMPD-1-infected tumor cells could prevent CD3^+^ T-cell exhaustion by reversing PD-1-mediated immune inhibition, we used Hepa1-6 cells and a T-cell coculture assay. Compared with the OVH-treated supernatants, the OVH-aMPD-1–treated supernatants significantly increased the percentages of CD4^+^ and CD8^+^ T cells with upregulation of ICOS and CD69 expression (Fig. 3K and L). Compared with the OVH-treated supernatants, the OVH-aMPD-1–treated supernatants also significantly increased the IFNγ secretion of CD8^+^ T cells (Fig. 3M). These *in vitro* results indicated that aMPD-1 scFv expression from OVH-aMPD-1 led to an enhanced cytotoxic killing ability in T cells.

Next, we asked whether OVH-aMPD-1 enhanced phagocytosis could improve presentation by DCs. To analyze cross-presentation by APCs induced by OVH-aMPD-1 *in vivo*, tumors were harvested as described in Fig. 3N. Our results showed that OVH-aMPD-1 and OVH significantly increased the ability of APCs to present the MHCI–restricted OVA-derived SIINFEKL peptides (Fig. 3O; Supplementary Fig. S11B), which may be conducive to the activation of tumor-specific CD8^+^ T cells. OVH-aMPD-1 exhibited relatively excellent DC cell presentation *in vivo*. These results indicated that OVH-aMPD-1 treatment led to improved antigen presentation by DCs.

**OVH-aMPD-1 induced immunogenic cell death in murine cancer cells.**

OVH-aMPD-1–infected tumor cells promoted phagocytosis, thus improving antigen presentation by DCs. To understand why we first dissected the cell death pattern induced by OVs, we tested the activity of the apoptotic executioner caspase-3 and apoptotic marker PARP. The cleaved forms of both markers were increased after either OVH infection or OVH-aMPD-1 infection (Supplementary Fig. S12A), indicating that OV-induced oncoslysis could lead to apoptotic cell death. To determine the immunogenicity of OV-treated cancer cell lines, and the infected cells and supernatants were harvested and analyzed for expression of immunogenic cell death (ICD)–associated damage-associated molecular patterns (DAMP). The levels of secreted ATP, surface expressed calreticulin, and secreted HMGB1 were upregulated in the OVH-infected cancer cells and OVH-aMPD-1–infected cancer cells (Supplementary Fig. S12B–S12D). Our study revealed that OVH and OVH-aMPD-1 both induced ICD in murine cancer cells, thus leading to DC stimulation. We observed OVH-aMPD-1 induced stronger antigen presentation by DCs than OVH, which may be associated with the expression of aMPD-1 scFv.

**OVH-aMPD-1 improved tumor control and enhanced effector T-cell function.**

To further evaluate the antitumor potential of OVH-aMPD-1 *in vivo*, we used a preclinical murine tumor model bearing bilateral Hepa-1-6 tumors (Fig. 4A), which were treated with three consecutive intratumoral OV injections. We first excluded the possibility of direct virus infection of untreated tumor on the distant flank (Supplementary Fig. S13A–S13G). It was observed that bilateral tumor growth was significantly inhibited both in the OVH-treated group and OVH-aMPD-1–treated group (Fig. 4B and C), and there was no significant difference in tumor size reduction between these two groups. However, more long-term tumor regression was observed in the OVH-aMPD-1–treated group than in the OVH-treated group (Fig. 4D–J). Long-term survivors that rejected primary tumors also rejected a second challenge with a larger amount of Hepa-1-6 tumor cells (Fig. 4K). These results showed that OVH-aMPD-1 exhibited relatively excellent therapeutic efficacy *in vivo*, which led to complete tumor rejection. However, both OVH-aMPD-1 and OVH were comparable in their ability to cause tumor regression in immunodeficient nude mice (Fig. 4L), suggesting that intratumoral aMPD-1 scFv–expressing OVH-aMPD-1 led to enhanced therapeutic effects only in immunocompetent tumor models. In addition, we compared the therapeutic efficacy of OVH plus anti-PD-1 blockers with OVH-aMPD-1 monotherapy. There was no significant difference in therapeutic efficacy between these two groups (Fig. 4M–O), further supporting the benefits of OVH-aMPD-1 as a single agent to treat tumor.

To investigate the molecular mechanisms underlying OVH-aMPD-1–mediated tumor control, we analyzed the inflammatory response within tumors (Fig. 5A). Increased infiltration of adaptive cells, including CD4^+^ and CD8^+^ lymphocytes, was observed in tumors (Fig. 5B and C), suggesting that abundant immune infiltrates were recruited into the injected tumors and distant tumors treated with either OVH or OVH-aMPD-1. When compared with those isolated from OVH-infected and distal tumors, the CD4^+^ and CD8^+^ T cells isolated from the OVH-aMPD-1–injected and distal tumors expressed significantly more of the activation markers ICOS and CD69 (Fig. 5D and E), suggesting that OVH-aMPD-1 enhanced effector T-cell function. It seemed that more pronounced ICOS^+^ CD8^+^ T cells were infiltrated in the distant tumors compared with the OVH-infected tumors (Fig. 5D and E). These results indicated that OVH-aMPD-1...
virotherapy could remodel the TME and lead to the activation of intratumoral T cells.

Despite the significant T-cell activation observed after intratumoral administration of OVH-aMPD-1, the magnitude of the effect was dependent on tumor size (Supplementary Fig. S1A and S1B), suggesting that additional inhibitory mechanisms within the TME prevent complete tumor rejection in large advanced tumors. We carried out IHC staining to analyze the histologic changes within the tumor tissue before and after treatment (Fig. 5F). OVH and OVH-aMPD-1 treatment induced CD8+ T cells infiltration into the virus-infected tumors and distant tumors (Fig. 5F). Significant upregulation of CD155 and significant reduction in proliferation signals (Ki67) were observed in the virus-infected tumors and distant tumors (Supplementary Fig. S15A and S15B), which suggested immune-mediated tumor inhibition

**OVH-aMPD-1 potentiated the efficacy of TIGIT blockade**

Analysis of TILs from virus-infected and distant tumors revealed that the immune infiltrates were characterized by an increase in CD11b+ Gr-1+ MDSCs (Fig. 6A; Supplementary Fig. S16A). Significantly higher amounts of MDSCs were observed in both the OVH-aMPD-1–injected and distant tumors compared with the OVH–injected and distal tumors. The percentage of intratumorally CD115+ granulocytic-MDSCs (G-MDSC) and CD115+ monocytic-MDSCs (M-MDSC) were significantly higher in both the OVH-aMPD-1–injected and distal tumors compared in the OVH–injected and distal tumors (Fig. 6B and C; Supplementary Fig. S17A and S17B). The upregulation of CD155 expression on tumor cells was not observed in either the OVH-treated group or the OVH-aMPD-1–treated group (Supplementary Fig. S17C).

We thus evaluated the efficacy of combination therapy using TIGIT blockade and virotherapy. Combination therapy with OVH-aMPD-1 and an anti-TIGIT antibody led to regression of the virus-infected tumors and distant tumors, which was superior to the combination of OVH and TIGIT blockade (Fig. 6D–F). To validate these findings in other tumor models, we used the peritoneal MC38 colon carcinoma model (Fig. 6G). MC38 cell line exhibited relatively lower sensitivity to OVH infection, resulting in lower expression of a MPD-1 scFv (Fig. 3F, Supplementary Fig. S18). In the MC38 model, the combination of OVH-aMPD-1 and TIGIT blockade was superior to both monotherapies (OVH or TIGIT blockade) and combination therapy (OVH with TIGIT blockade; Fig. 6H). OVH-aMPD-1 synergized with TIGIT blockade, leading to long-term management of the invasive tumors.

We carried out deletion experiments to analyze whether CD4+ or CD8+ T cells were critical for mediating antitumor effects in non–tumor-injected tumors (Fig. 6I; Supplementary Fig. S19). Deletion of CD8+ T cells or CD4+ T cells in vivo impaired the therapeutic efficacy of combination therapy with OVH-aMPD-1 and an anti-TIGIT antibody (Fig. 6J and K). When CD8+ T cells were depleted in the mice treated with the OVH, tumors progressed more rapidly when compared to mice with depletion of CD4+ T cells (Fig. 6L and M). These results demonstrated that CD4+ or CD8+ T cells were critical for mediating tumor regression of both injected tumors and distant tumors.

**Combination therapy increased tumor-specific CD8+ T-cell responses**

To investigate the immune mechanisms underlying the antitumor efficacy of the combination therapy of TIGIT blockade and OVH-aMPD-1 virotherapy, we used a Hepa1-6-OVA cancer model and analyzed the infiltration of CD8+ T lymphocytes and tumor-specific CD8+ T lymphocytes in tumors and the spleen (Fig. 7A). Increased infiltration of CD8+ T lymphocytes was observed in the tumors treated with either OVH, TIGIT blockade, or combination therapy (Fig. 7B and C). TIGIT blockade could facilitate CD8+ lymphocyte infiltration regardless of whether OVs were administered, and the percentage of intratumorally infiltrated CD8+ T lymphocytes was significantly higher in the mice receiving combination therapy with TIGIT blockade and OVH-aMPD-1 virotherapy than those receiving combination therapy with TIGIT blockade and OVH virotherapy. In addition, compared with either monotherapy or combination therapy with TIGIT blockade and OVH virotherapy, combination therapy with TIGIT blockade and OVH-aMPD-1 virotherapy significantly increased the accumulation of tumor-specific CD8+ T lymphocytes (Fig. 7D; Supplementary Fig. S20). Increased accumulation of tumor-specific CD8+ T lymphocytes was observed in the tumors and spleens isolated from the mice treated with either OVH-aMPD-1 virotherapy or combination therapy with TIGIT blockade and OVH-aMPD-1 virotherapy (Fig. 7E and F). TIGIT blockade could facilitate CD8+ T lymphocyte infiltration into the spleens only when OVH-aMPD-1 was administered, and the percentage of splenic tumor-specific CD8+ T lymphocytes was slightly higher in the mice receiving combination therapy with TIGIT blockade and OVH-aMPD-1 virotherapy than in those receiving combination therapy with TIGIT blockade and OVH virotherapy. These *in vivo* results indicated that aMPD-1 scFv expression from OVH-aMPD-1, together with TIGIT blockade, led to significantly increased numbers of tumor-specific CD8+ lymphocytes, correlating to rejection of established tumors.

**Discussion**

Treatment options and their outcomes in several tumor indications, such as melanoma and small-cell lung cancer, have changed significantly. Immunotherapy has become the first-line therapy for several tumors and other cancer types. The current study provides unique insights into the antitumor efficacy of OVH-aMPD-1 virotherapy and the potential benefits of combining TIGIT blockade and a PD-1–specific scFv. We hypothesized that combining an antitumor scFv with TIGIT blockade would potentiate antitumor effects through increased T-cell infiltration and activation.

The results of this study indicate that OVH-aMPD-1 virotherapy, combined with TIGIT blockade, has the potential to remodel the TME and activate T lymphocytes. The combination therapy led to a significant increase in the infiltration of CD8+ T lymphocytes into tumors and distant tumors compared with monotherapy or combination therapy with TIGIT blockade and OVH virotherapy. This finding suggests that the combination therapy could enhance the antitumor efficacy of TIGIT blockade and virotherapy by inducing a greater immune response.
Figure 4. OVH-aMPD-1 improved both local and systemic tumor control. A, Treatment scheme. B and C, Growth of vehicle (PBS)-, OVH-, and OVH-aMPD-1–treated syngeneic Hepa1-6 tumors in immunocompetent C57BL/6 mice (n = 9). Tumor growth of injected (right flank) Hepa1-6 tumors (B) and distant (left flank) Hepa1-6 tumors (C). D–F, Individual tumor growth curves of vehicle (PBS)-, OVH- and OVH-aMPD-1–injected Hepa1-6 tumors. G–I, Individual tumor growth curves of distant Hepa1-6 tumors. J, Percentage of tumor-free mice in the Hepa1-6 liver cancer model. K, Survival of cured Hepa1-6 model survivors rechallenged with 5 × 10⁵ Hepa1-6 cells. L, Growth of vehicle (PBS)-, OVH-, or OVH-aMPD-1–treated Hepa1-6 xenografts in immunodeficient nude mice (n = 6). Data for survival were analyzed by the log–rank (Mantel-Cox) test (K). M, Treatment scheme for comparing the therapeutic efficacy of OVH plus anti-PD-1 blockers with OVH-aMPD-1 monotherapy. N and O, Tumor growth of injected and distant Hepa1-6 tumors. Data represent results from either one of three (B–K) or one of two (L, N, and O) independent experiments with n = 6 to n = 10 per group. All values are presented as the mean ± SEM. Statistical analysis was performed using repeated-measure ANOVA (B, C, L, N, and O); **, P < 0.01; and ***, P < 0.001; ns, not significant.
Figure 5.
OVH-aMPD-1 enhanced effector T-cell function. A, Treatment scheme. Mice bearing Hepa1-6 tumors were intratumorally injected with vehicle (PBS), OVH, or OVH-aMPD-1, and tumors were collected on day 10 following virus injection and analyzed by flow cytometry. The percentages of tumor-infiltrating CD4⁺ and CD8⁺ T cells isolated from the injected (B) and distant tumors (C), gated on the total CD45⁺ cell population. Expression of CD69 and ICOS on the surface of tumor-infiltrating CD4⁺ and CD8⁺ T cells in the vehicle-injected or virus-injected tumors (D) and distant tumors (E). F, IHC analysis of CD8⁺ T cells marker (CD8α) and CD155 in virus-injected tumor and distant tumor at 7 days after receiving intratumoral injection of two doses of OVH or OVH-aMPD-1 (1 × 10⁷ PFU per dose) or vehicle. Data represent results from either one of three (B–E) or one of two (F) independent experiments with n = 5 to n = 6 per group. All values are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA (B–E); *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. MFI, mean fluorescence intensity.
Figure 6.

TIGIT blockade potentiated the efficacy of OVH-aMPD-1 virotherapy. A, Increase in the proportions of MDSCs in the lymphocyte populations isolated from injected and distant tumors. MDSCs, CD11b+ Gr-1+ cells. Increase in the proportions of CD155+ G-MDSCs and M-MDSCs in injected tumors (B) and distant tumors (C). G-MDSC, granulocytic-MDSCs (CD11b+ Ly6C/Ly6G+); M-MDSC, monocytic-MDSCs (CD11b+ Ly6C/Ly6G+). D, Treatment scheme. E and F, Mice bearing Hepa-1-6 tumors received monotherapy or combination therapy. Growth of injected tumors (E) and distant tumors (F). G, Treatment scheme for establishment of intraperitoneal MC38 tumors. H, Overall survival was monitored over a 100-day period. I, Treatment scheme for depletion experiments. J and K, Mice bearing Hepa-1-6 tumors received combination therapy and indicated depletion antibodies or isotype antibodies. Growth of injected tumors (J) and distant tumors (K). Data represent results from either one of three (A-H) or one of two (J and K) independent experiments with n = 6 to n = 10 per group. Data for survival were analyzed by the log-rank (Mantel–Cox) test (H). All values are presented as the mean ± SEM. Statistical analysis was performed using repeated-measure ANOVA (E, F, J, and K) or one-way ANOVA (A–H); *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001; ns, not significant.
Combination therapy with TIGIT blockade and OVH-aMPD-1 virotherapy increased tumor antigen–specific CD8+ T-cell responses. A, Treatment scheme. Tumors isolated from mice receiving various treatments were dissociated and analyzed by flow cytometry. B, Percentages of CD8+ T cells in the live CD45+ cell population. C, Representative flow cytometry plots of CD45+CD8+ cells gated on the total live CD45+ cell population. D, Representative flow cytometry plots of OVA–specific (H-2 Kb/SIINFEKL tetramer+) CD8+ cells gated on the CD8+ cell population in the tumor. E, Percentages of H-2 Kb/SIINFEKL tetramer+ CD8+ T cells in the tumor CD8+ T-cell population. F, Percentage of H-2 Kb/SIINFEKL tetramer+ CD8+ T cells in the splenic CD8+ T-cell population. Data represent results from either one of three (B and C) or one of two (D–F) independent experiments with n = 6 per group. All values are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA (B–D); *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001.
subset of patients with a high tumor mutational burden, microsatellite instability, or PD-L1 expression, including melanoma, non–small-cell lung cancer (NSCLC), colorectal cancer, and urothelial cancer (24). However, the clinical efficacy of immunotherapy is still limited, and a large proportion of patients with advanced cancer do not benefit from current immunotherapeutic strategies (25). Combination therapy with anti-CTLA-4 and anti-PD-1 inhibitors has been suggested as a potential efficacious treatment option for advanced melanoma; however, the potential toxicities of this form of combinatorial immunotherapy is still the largest concern for its clinical application (26, 27). There is still an urgent need for improved agents for long-term tumor control. Therefore, the aim of this study was to use an engineered multiplexed OV to improve the potential of oncolytic virotherapy as a stand-alone therapeutic approach.

Our rationale for constructing an armed OV that can mediate immune checkpoint blockade was that OVH induced strong upregulation of PD-L1 expression in the TME. A similar strategy has been successful in human patients with melanoma, where talmogene laherparepvec (T-VEC, armed with GM-CSF) improves antitumor efficacy mediated with GM-CSF-enhancing immune response (28). GM-CSF is an immune stimulator that promotes the differentiation of progenitor cells into DCs and shows a certain degree of antitumor efficacy in clinical trials. The combination of GM-CSF with oncolytic therapy may provide an in situ antitumor vaccine by enhancing tumor antigen presentation. Furthermore, intratumoral T-VEC therapy in combination with systemic anti-PD-1 therapy significantly increases overall response rate (62%) in patients with metastatic melanoma (10). However, in a randomized open-label phase III trial, 26.4% of the patients in the T-VEC alone arm had an objective response (29). Other studies also suggest that oncolytic virotherapy may improve the efficacy of immune checkpoint blockade by changing the TME (30). These findings highlight that combinatorial regimens may achieve efficacy superior to that of monotherapy. We thus hypothesized that constructing an OV, expressing a PD-1-blocking scFv, would provide combinatorial immunotherapy and localized delivery of aMPD-1 scFv in the TME.

It is intriguing that this antitumor activity was dependent on the expression of aMPD-1 scFv within the TME. The limits of this study were the inherent poor replication of OVH-aMPD-1 in murine cancer cells and thus the low production of aMPD-1 scFv in mouse tumors. A major reason for the short-term life cycle of OVH-aMPD-1 in treated mice, which was closely related to the in vivo dynamic kinetics of aMPD-1 scFv expression, is likely to be the relatively low permissiveness of immunocompetent mice, especially C57BL/6 mice, to HSV-1 infection (31, 32). This observation suggests that adequate replication of OVH-aMPD-1 reduces the expression of aMPD-1 scFv and thus restricts combinatorial antitumor effects. Despite this possible replicative defect, compared with its parental virus, OVH-aMPD-1 significantly reduced tumor sizes and extended survival.

Another possible concern was that aMPD-1 scFv reacted only with PD-1 of mouse origin due to the homology disparity between mouse and human PD-1 sequences, which share approximately 61.1% amino acid identity in the extracellular domains (33). To our knowledge, it is difficult to obtain an antibody that recognizes both human and mouse PD-1 with high affinity and blocking activity, and the activity of an antibody may determine its antitumor activity. Although, aMPD-1 scFv that can bind to PD-1 of both mouse and human origin exists, but whether this aMPD-1 scFv can execute immunomodulatory functions has not been fully addressed (34). For further clinical investigation, we should construct an OV arming with a humanized antibody that recognizes human PD-1.

In addition to inducing ICD, OVVs can induce the release of tumor antigens, which facilitates the initiation of a tumor-antigen–specific response within a tumor (35). Our study revealed that OVH-aMPD-1 could not only release immunogenic DAMPs but also significantly promote antigen cross-presentation by DCs. The importance of presentation efficiency by DCs in initiating a durable T-cell response has previously been demonstrated (36, 37), and in situ aMPD-1 scFv expression may enhance tumor-antigen–specific T-cell responses by promoting efficient presentation of antigens to T cells.

The highly immunosuppressive TME may require a more complex immunotherapeutic strategy (38). Although HSV-1 vectors are emerging as an effective therapeutic approach for cancer, it is ultimately cleared by the host immune system before complete tumor clearance (31). Thus, it is vital to develop rational combinatorial strategies to overcome the highly immunosuppressive TME (39, 40). Our rationale for designing combinatorial strategies using OVH-aMPD-1 armed for immune checkpoint blockade was that OVH-aMPD-1 induced significantly higher percentages of CD155+ G-MDSCs and M-MDSCs in tumors. Given that CD155-TIGIT signaling exerts potent inhibitory action in different subsets of immune cells (41, 42), this study points toward a promising therapeutic strategy to combine OVH-aMPD-1 with TIGIT-blocking agents. Our study revealed that the combination of a virus expressing aMPD-1 scFv with TIGIT blockade significantly improved therapeutic efficacy; however, TIGIT blockade did not improve the antitumor effect of OVH virotherapy. Therefore, in situ aMPD-1 scFv expression together with TIGIT blockade further enhanced the locoregional and systemic tumor-antigen–specific T-cell response. These findings suggest that the magnitude and efficacy of TME remodeling and T-cell activation induced by a multifaceted oncolytic vector may potentiate the efficacy of immune checkpoint blockade.

In summary, our data demonstrated that OVH-aMPD-1 virotherapy was an effective strategy for aMPD-1 scFv delivery and treatment, TME remodeling, improving antigen cross-presentation in DCs and inducing antitumor T-cell immunity. To further overcome the highly immunosuppressive TME, OVH-aMPD-1 synergized with TIGIT blockade, which lead to the long-term control of invasive tumors. The findings from this study provide a rationale for the combination of a novel OV armed with immunotherapeutics with immune checkpoint blockade for the treatment of advanced cancer.

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

Authors’ Contributions
Conception and design: C. Lin, W. Ren, C. Huang, N. Xia
Development of methodology: W. Ren, Y. Luo, S. Li, Y. Chang, L. Li, D. Xiong, X. Huang, Z. Xu, Z. Yu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Lin, S. Li, Y. Wang, J. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Huang, N. Xia
Writing, review, and/or revision of the manuscript: C. Lin, C. Huang, N. Xia
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Xia
Study supervision: C. Huang

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Chaolong Lin, Wenfeng Ren, Yong Luo, et al.


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