Molecular Therapy
Oncology
Original Article

Oncolytic vaccinia virus harboring *CLEC2A* gene enhances viral replication and antitumor efficacy

Chunqing Gao,[1,2] Qi Ying,[1,2] Yufeng Qiu,[1] Ningbo Ren,[1] Kan Chen,[1] Yanrong Zhou,[1] Ting Ye,[1] and Gongchu Li[1]

[1]College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, China

In the field of innovative cancer treatment strategies, oncolytic vaccinia virus (VV)es have gained traction as promising vectors. In the current study, we inserted the human *C-type lectin domain family 2 member A* (*CLEC2A*) gene into VV, creating a replicating therapeutic, oncoVV-CLEC2A. The findings reveal that oncoVV-CLEC2A effectively suppresses colorectal proliferation of mouse xenografts and a range of human cancer cell lines by augmenting viral reproduction capabilities, including the lung cancer H460 cell line, colorectal cancer cell lines (HCT116 and SW620), and hepatocellular carcinoma HuH-7 cell line. Moreover, it is evident that oncoVV-CLEC2A can induce antitumor immunity by boosting cytokine production but not antivirus response, and enhancing calreticulin expression. Further investigation indicates that oncoVV-CLEC2A can enhance antitumor capabilities by activating natural killer cells to produce interferon-γ and induce M1-like macrophage polarization. These findings shed light on the antitumor mechanisms of oncoVV-CLEC2A, provide a theoretical basis for oncolytic therapies, and lay the groundwork for novel strategies for modifying VVs.

INTRODUCTION

Oncolytic virotherapy constitutes an innovative cancer therapy strategy that may provide potential clinical benefits for patients with advanced-stage cancer.[1,2] Currently, a variety of genetically engineered oncolytic viruses based on vaccinia virus (VV), human herpesvirus 1, and adenovirus are undergoing preclinical research or clinical trials, with VV emerging as a highly promising candidate for intravenous oncolytic virotherapy.[1–3] VV is a DNA virus possessing a genome of around 192 kb, allowing for extensive genetic modifications and the inclusion of transgenes of at least 25 kb in size.[4] Its relatively large size makes it easy to manipulate, and the viral particles preferentially deposit in tumors where new vascular permeability is increasing.[5] Additionally, VV has notable advantages, such as its safety record used as a live vaccine for smallpox, rapid replication in the cytoplasm, and ability to induce effective immune responses. Numerous recombinant VVs have been extensively studied in clinical experiments.[6] One example of an oncolytic VV is TG6002, which has undergone genetic modifications by removing the *thymidine kinase* (*TK*) gene and ribonucleotide reductase genes, and inserting the suicide gene *FCU1*.[7] TG6002 has already been administered to the first patient with colorectal cancer who had liver metastasis in a phase 1/2a clinical trial. Another VV, vvDD-CDSR, shows promising oncolytic activity in refractory and metastatic childhood solid tumors, with a phase 1 trial for colorectal cancer currently underway.[8] Furthermore, a phase 2 clinical trial of olvimulogene nanivacirepvec constructed with VV has demonstrated positive overall response rates, progression-free survival, and manageable safety profiles in platinum-resistant and -refractory ovarian cancer patients.[9] Collectively, these findings indicate that oncolytic VVs exhibit robust antitumor responses and present promising clinical outcomes.

To augment the antitumor efficacy of oncolytic virotherapy, researchers often make viruses harbor genes that promote viral replication or boost immune response. For instance, transgenes can be expressed in the oncolytic virus to stimulate antitumor immunity, including immune checkpoint inhibitors, tumor-associated antigens, cytokines and bispecific T cell engagers.[10] Previous studies have provided evidence that incorporating marine lectins into VV oncolytic can inhibit tumor growth by promoting viral replication, suggesting that lectins could be a potential gene pool for oncolytic virotherapy.[11,12] The human *C-type lectin domain family 2 member A* (CLEC2A) gene encodes keratinocyte-associated C-type lectin (KACL), which is known to act as a ligand for activating natural killer (NK) cell receptor NKp65.[13] NK cells fulfill essential functions in tumor surveillance and are regulated via a range of receptors.[14] NKp65 binding with KACL can stimulate the cytotoxicity of NK cells and the release of proinflammatory cytokines.[13] Gonçalves-Maia et al.[15] have shown that the absence of CLEC2A in fibroblasts of xeroderma pigmentosum and cancer-associated fibroblasts could potentially lead to immune dysfunction. Additionally, research has reported that overexpression of C-type lectin molecule NKG2D ligands on tumor cells can activate NK cells to eliminate malignant cells, resulting in

Received 17 January 2024; accepted 31 May 2024;
https://doi.org/10.1016/j.omton.2024.200823.

[2]These authors contributed equally

Correspondence: Ting Ye, College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, China.
E-mail: yeting@zstu.edu.cn
Correspondence: Gongchu Li, College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, China.
E-mail: lgc@zstu.edu.cn

Published by Elsevier Inc. on behalf of The American Society of Gene and Cell Therapy.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

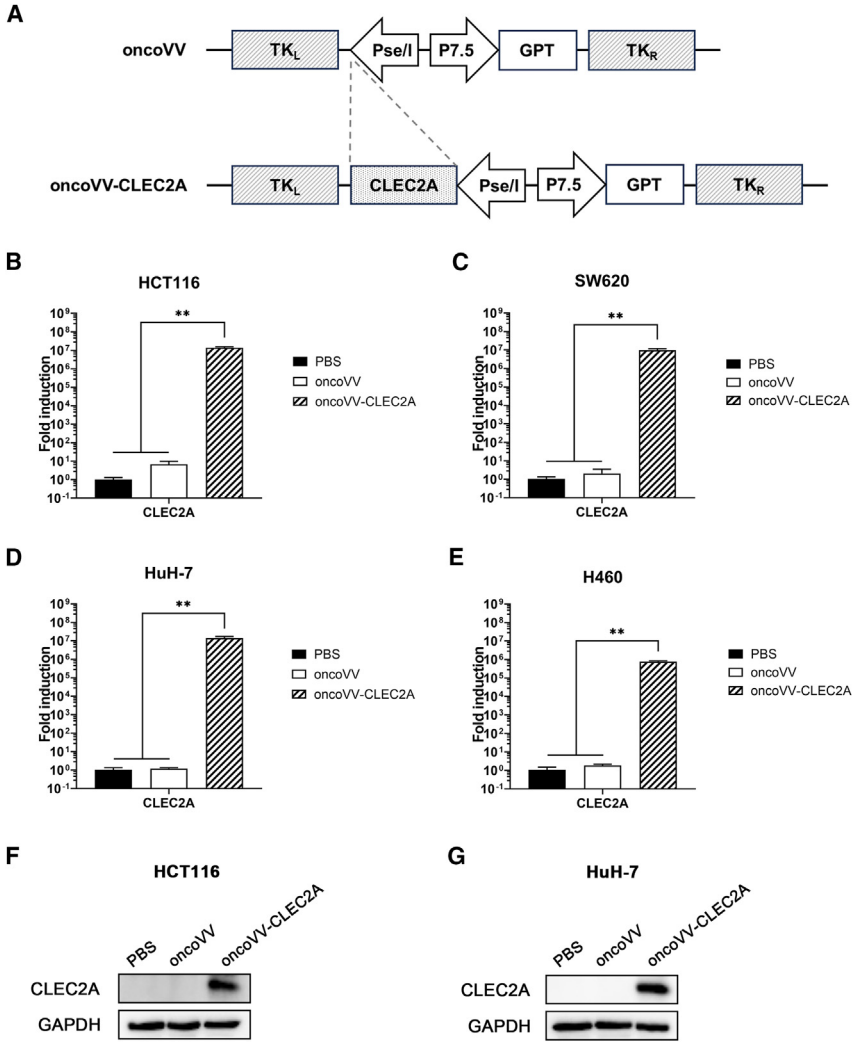


Figure 1. The high expression of CLEC2A in oncoVV-CLEC2A treatment

(A) Schematic of engineering the oncoVV-CLEC2A recombinant. (B–E) The expression of CLEC2A in HCT116 cells (B), SW620 cells (C), HuH-7 cells (D), and H460 cells (E) at 36 h after infection with oncoVV-CLEC2A or oncoVV. The mRNA level of CLEC2A was identified by qPCR assay. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$). (F and G) The expression levels of CLEC2A in HCT116 cells (F) and HuH-7 cells (G) after treatment with oncoVV-CLEC2A in cells. GAPDH was used as the internal control.

tumor growth retardation or rejection.[14] CLEC2A also coordinates with related molecules through T cell receptors to regulate and enhance the amplification of T cells.[16] However, there is currently no utilization of CLEC2A in oncolytic virotherapy.

In the present study, we engineered recombinant VV expressing *CLEC2A* gene (oncoVV-CLEC2A) and investigated the oncolytic efficiency of oncoVV-CLEC2A on non-small cell lung cancer cell line H460, colorectal cancer cell lines (HCT116 and SW620), and hepatocellular carcinoma cell line HuH-7, as well as the antitumor effects on a mouse xenograft model. Furthermore, we analyzed the underlying anti-tumor molecular mechanism of oncoVV-CLEC2A.

RESULTS

Harboring CLEC2A enhanced cell cytotoxicity of oncoVV

The oncoVV-CLEC2A was generated from the Western Reserve (WR) strain of VV, using the pCB plasmid as previously outlined.[17] Within this construct, human CLEC2A and GPT were integrated into the *TK* gene region under the control of the p7.5 promoter and the Pse/l promoter, respectively (Figure 1A). The accuracy of the insertions was confirmed through Sanger sequencing on the *TK* gene, which was amplified from the extracted genomic DNA. To verify the expression of CLEC2A in HCT116, SW620, HuH-7, and H460 cell lines, qPCR was conducted 36 h after oncoVV-CLEC2A infection. PBS and oncoVV served as controls. We observed that CLEC2A mRNA levels were overexpressed in the oncoVV-CLEC2A treatment compared with the PBS and oncoVV treatments (Figures 1B–1E). We further selected HCT116 and HuH-7 cells to assess CLEC2A protein expression using western blot analyses. Our observations unveiled that the oncoVV-CLEC2A notably escalated the levels of CLEC2A protein expression within the HCT116 and HuH-7 cells as compared with the controls (Figures 1F and 1G). Next, MTT assay was used to evaluate cell viability at 24, 48, and 72 h after infection with different multiplicities of infection (MOI) of oncoVV-CLEC2A. In the HCT116 cell lines, the cell viability of oncoVV (5 MOI) at 24 h, 48 h, and 72 h after infection decreased to 89.46%, 83.42%, and 87.80%, respectively, while the cell viability of oncoVV-CLEC2A (5 MOI) is 68.07%, 44.04%, and 10.27% correspondingly, indicating that oncoVV-CLEC2A (5 MOI) has good antitumor efficiency (Figure 2A). In SW620, HuH-7, and H460 cell lines, the oncoVV-CLEC2A group demonstrated a greater decrease in cell viability compared with the oncoVV group at the same dose over time (Figures 2B–2D). Furthermore, the cytotoxicity of oncoVV-CLEC2A on tumor cells with stem cell characteristics was assessed using tumor spheroids, and the spheroids of HCT116 cells were treated with oncoVV-CLEC2A (MOI of 5) for 5 days. It was observed that the oncoVV-CLEC2A group had smaller spheroids compared with the PBS group and oncoVV group (Figure 2E), indicating a robust antiproliferative effect of oncoVV-CLEC2A on tumor spheroids. In summary, these findings suggest that harboring CLEC2A enhances the cytotoxicity of oncolytic VV on tumor cells and tumor spheres.

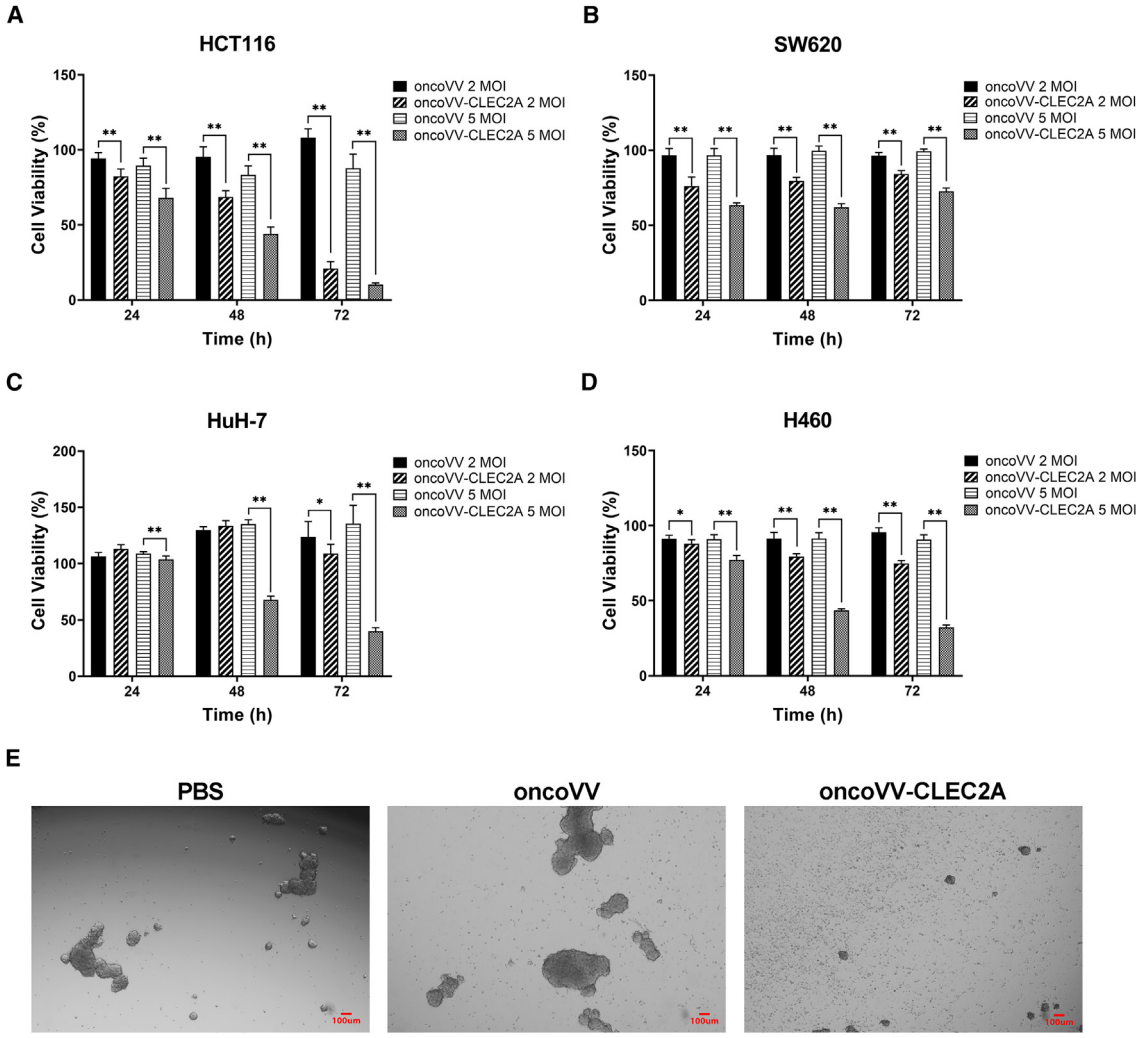


Figure 2. The cell cytotoxicity of oncoVV-CLEC2A on tumor cells and tumor spheroids

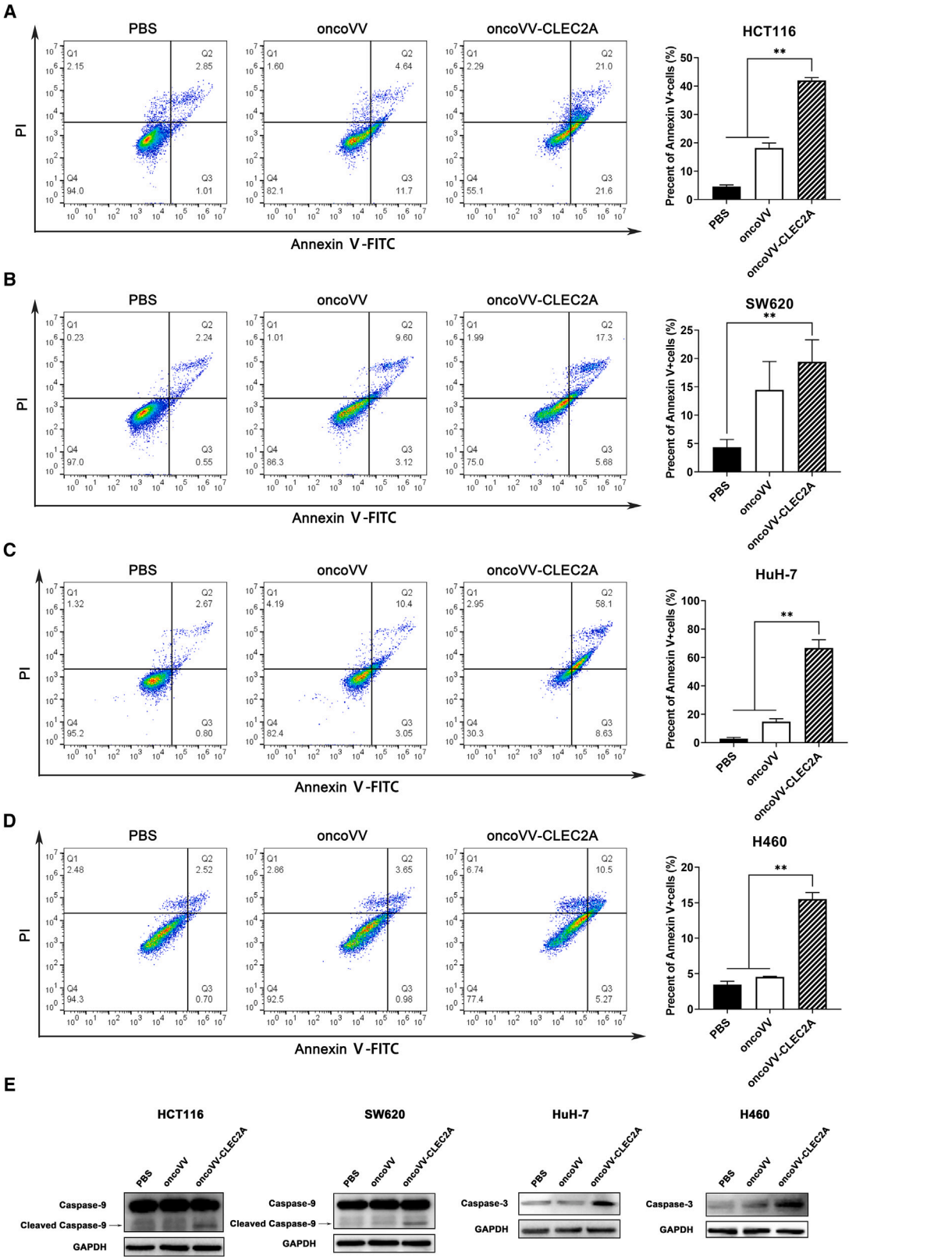
(A–D) Cell viability in HCT116 (A), SW620 (B), HuH-7 (C), and H460 (D) cell lines. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$). (E) Morphology of the HCT116 tumor spheroids observed by inverted microscope at day 5 after infection with 5 MOI of oncoVV or oncoVV-CLEC2A.

OncoVV-CLEC2A enhanced the cytotoxicity by inducing apoptosis

To explore the underlying mechanism of oncoVV-CLEC2A on antitumor effects, flow cytometry and western blot analyses were employed to examine apoptotic processes. The results demonstrated a remarkable increase in the percentage of apoptotic cells in the oncoVV-CLEC2A group in comparison to the control in HCT116, SW620, HuH-7 and H460 cell lines (Figures 3A–3D). Caspases are essential proteins involved in the execution of apoptosis. They are divided into two subclasses: the caspase-9 subclass (caspase-9, -10, -8, and -2) as initiators and the caspase-3 subclass (caspase-3, -6, and -7) as executors. We examined the expression of caspases and found that the oncoVV-CLEC2A group upregulated cleaved caspase-9 in both HCT116 cells and SW620 cells compared with the PBS group and oncoVV group (Figure 3E). In addition, caspase-3 was upregulated after treatment with oncoVV-CLEC2A in HuH-7 cells and H460 cells. These data suggest that oncoVV harboring CLEC2A enhances the cytotoxicity of oncolytic VV by inducing apoptosis.

OncoVV-CLEC2A prompted viral replication

Viral replication is the key to oncolytic therapy. In both HCT116 cells and HuH-7 cells, the viral yields in the oncoVV-CLEC2A treatment were noticeably higher compared with those in the oncoVV infection treatment at 12 h, 24 h, and 36 h after virus infection (Figures 4A and 4B). Furthermore, we observed an upregulation of A27L, a viral membrane protein,[18] in the oncoVV-CLEC2A treatment compared with the oncoVV and PBS groups (Figures 4C and 4D). These data unveil that oncoVV-CLEC2A strengthens the viral



(legend on next page)

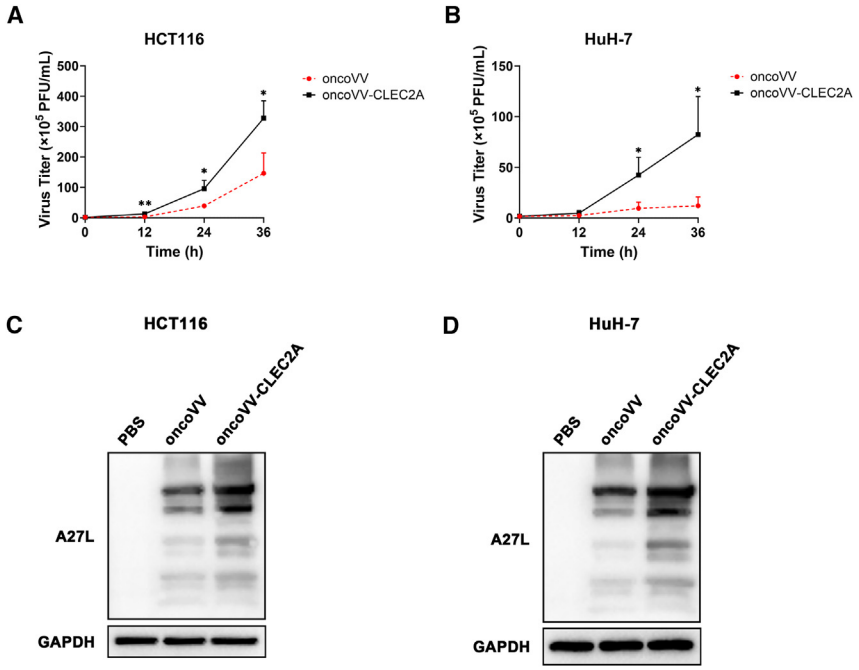


Figure 4. The promotion of oncoVV-CLEC2A in viral yields

(A and B) The replicative capacity of oncoVV and oncoVV-CLEC2A was assessed using $TCID_{50}$ assay in HCT116 cells (A) and HuH-7 cells (B). Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$). (C and D) The levels of A27L in HCT116 (C) and HuH-7 (D) cells at 36 h after infection with oncoVV or oncoVV-CLEC2A were determined using Western blot. GAPDH was used as the internal control.

replication capacity, with higher concentrations of A27L resulting in greater viral yields.

OncoVV-CLEC2A promoted the cytokine production of tumor cells

Interferons (IFNs) are glycoproteins known for their remarkable abilities in combating viral infections and restraining the proliferation of tumor cells.[19] Inflammatory factors, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α, also have pivotal roles in cancer therapy.[20] To demonstrate the cytokine production triggered by oncoVV-CLEC2A, the mRNA expression levels of type I IFNs (IFN-α/-β), IL-6 and TNF-α were quantified using qPCR in HCT116 and HuH-7 cell lines at 36 h after infection. As shown in Figure 5, oncoVV-CLEC2A significantly promoted the transcription of type I IFNs, TNF-α, and IL-6 compared with the PBS group and oncoVV group in HCT116 and HuH-7 cells.

In HCT116 cells, the expression of IFN-α was upregulated by more than 7-fold, IFN-β and TNF-α were increased by more than 9-fold, and IL-6 showed a greater than 13-fold increase. In HuH-7 cells, the levels of IFN-α, IFN-β, and TNF-α were upregulated more than 6-fold, and IL-6 increased more than 8-fold. These observations illustrate that oncoVV-CLEC2A efficiently promotes cytokine production in both HCT116 and HuH-7 cells. IRF3/7 and AP-1 translocate into nucleus and stimulate the transcription of cytokines.[21] To explore whether the transcriptional activities of AP-1, IFN-stimulated responsive element (ISRE) and nuclear factor κB (NF-κB) are activated by oncoVV-CLEC2A, the dual-luciferase reporter assay was performed at 36 h after infection in HCT116 and HuH-7 cells. Compared with the PBS group and oncoVV group, oncoVV-CLEC2A infection significantly enhanced the transcriptional activity of AP-1 in both cell lines, while there was no significant difference in the transcriptional activity of NF-κB between oncoVV-CLEC2A group and the other groups (Figures 6A–6C). AP-1 is primarily composed of c-Fos and c-Jun.[22] The expression of phospho-c-Jun and phospho-c-Fos were upregulated in oncoVV-CLEC2A treatment compared with the oncoVV and PBS group in both HuH-7 cells and HCT116 cells (Figure 6D). Taken together, these results reveal that oncoVV-CLEC2A infection stimulates the transcriptional activity of AP-1 by enhancing the expression and phosphorylation of c-Fos and c-Jun, thereby triggering inflammatory responses.

Type I IFNs are known to activate ISRE and drive the transcription of hundreds of IFN-stimulated genes (ISGs), which are essential in virus defense and the antiviral response.[23] In HCT116 cells, there was no statistical difference in ISRE transcriptional activity between the oncoVV-CLEC2A group and the oncoVV control. A similar result was seen in HuH-7 cells, suggesting that oncoVV-CLEC2A stimulates IFN production, but does not induce an antiviral response.

OncoVV-CLEC2A suppressed tumor growth in mice

The mouse model of subcutaneous tumorigenesis using HCT116 cells was established to assess the effectiveness of oncoVV-CLEC2A in inhibiting tumor growth *in vivo*. The results, as shown in Figure 7A, demonstrated that oncoVV-CLEC2A effectively repressed tumor growth compared with the control treatment that was injected with

Figure 3. OncoVV-CLEC2A promoted apoptosis

(A–D) The proportion of apoptotic cells was determined using flow cytometry in HCT116 (A), SW620 (B), HuH-7 (C), and H460 (D) cell lines at 36 h after infection with oncoVV-CLEC2A or oncoVV. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$). (E) The expression levels of cleaved caspase-9, caspase-9, and caspase-3 after treatment with oncoVV-CLEC2A in cells. GAPDH was used as the internal control.

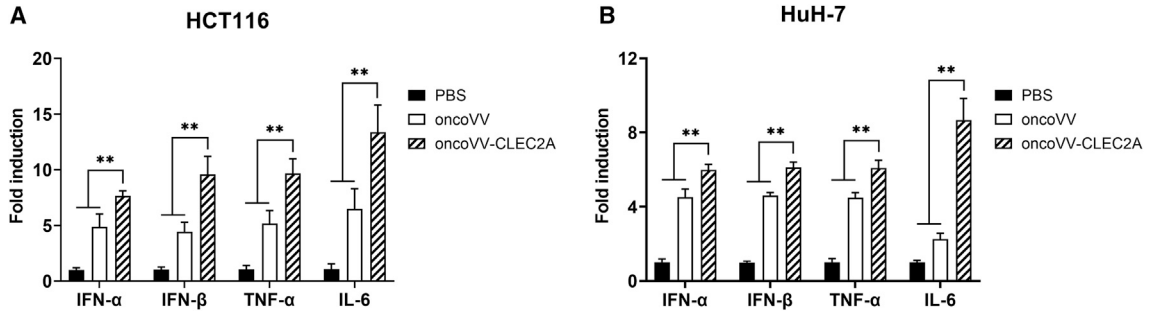


Figure 5. Inflammatory response mechanism induced by oncoVV-CLEC2A

(A and B) The transcription of cytokines in HCT116 cells (A) and HuH-7 cells (B) at 36 h after infection with oncoVV or oncoVV-CLEC2A. The mRNA levels of IFN-α, IL-6 IFN-β and TNF-α were measured using qPCR. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$).

0.9% NaCl or oncoVV alone. The findings revealed that the tumor size of the oncoVV-CLEC2A group was considerably reduced in comparison to the 0.9% NaCl group or the oncoVV group (Figure 7C). Additionally, the proportion of tumor weight to body weight in the oncoVV-CLEC2A group exhibited a significant decrease compared with that in the 0.9% NaCl group or the oncoVV group (Figure 7B). To further explore the effects of oncoVV-CLEC2A on tumor growth, we conducted hematoxylin and eosin (HE) staining. The results revealed that, compared with the control groups, the majority of tumor cells in the oncoVV-CLEC2A group exhibited a loose arrangement, changes in morphology, and intracellular nuclei shrinkage and karyorrhexis, indicating that oncoVV-CLEC2A effectively inhibited tumor growth *in vivo* (Figure 7D). Immunohistochemistry (IHC) using a primary antibody specific for VV (A27L) was conducted to detect the distribution and replication extent of the virus in tumor tissue. The IHC assay revealed a higher expression of A27L, which resulted in a dark brown cytoplasm, indicating a substantial virus proliferation in the oncoVV-CLEC2A treatment (Figure 7E).

Calreticulin (CALR) is a major determinant of cellular adjuvanticity. Surface-exposed CALR, rather than endoplasmic reticulum-CALR, delivers a major "eat me" signal to immune cells.[24] The translocation of CALR on the outer surface of the plasma membrane can be induced by oncolytic viruses.[25] Accumulating evidence suggests that the expression level of CALR is down-regulated in colon neoplasms, and higher CALR levels correlate with improved patient overall survival.[25,26] To detect the change of CALR protein expression, we used an IHC analysis. As evidenced in Figure 7F, the oncoVV-CLEC2A group demonstrated enhanced CALR levels in contrast to the PBS and oncoVV groups, indicating that oncoVV-CLEC2A has a therapeutic effect on mice with colon-transplanted tumors. In summary, this study revealed that the antitumor effectiveness of oncoVV-CLEC2A was greater compared with oncoVV in mouse models with subcutaneous xenografts of HCT116 cells.

OncoVV-CLEC2A enhanced the cytotoxicity of NK cell

NK cells are a principal generator of IFN-γ, influencing the T helper 1 immune pathway, which is crucial for regulating the immune system and promoting effective antitumor reactions by enhancing macrophage activity and increasing the levels of antigen presentation-associated proteins.[27] To explore whether VV-CLEC2A can activate NK cells, we administered female BALB/c mice with 0.9% NaCl, oncoVV, or oncoVV-CLEC2A, and examined the levels of IFN-γ and TNF-α in the serum using ELISA. The data disclosed that, in comparison with the oncoVV group, the levels of IFN-γ in the oncoVV-CLEC2A treatment tremendously increased by approximately 76-fold (Figure 8A), while the levels of TNF-α slightly improved (Figure 8B). The proportion of activated NK cells (marked as IFN-γ^+ CD49b$^+$) in myeloid cells of the oncoVV-CLEC2A group increased by approximately 130% compared with the control group, suggesting that the oncoVV-CLEC2A group indeed activates the NK cells (Figure 8B). Furthermore, because IFN-γ could induce the polarization of macrophages (M) to M1 type, we determined the macrophage types. As shown in Figure 8C, the results showed that the proportion of M1 macrophages (marked as IFN-γ^+ F4/80$^+$) in the myeloid cells approximately increased by two times in the oncoVV-CLEC2A group compared with the oncoVV group, while the percentage of M2 macrophages (marked as IL-4$^+$ F4/80$^+$) were slightly altered in the oncoVV-CLEC2A (Figure 8D). Taken together, these data suggest that oncoVV-CLEC2A can enhance anti-tumor capabilities by activating NK cells to produce IFN-γ and altering macrophages to an M1 type.

DISCUSSION

Based on the 2020 Global Cancer Statistics Report issued by the International Agency for Research on Cancer, cancer has become the dominant global cause of death in recent years. As per the report, an estimated 19.3 million new cancer cases were diagnosed worldwide, leading to approximately 10 million cancer-related deaths. Notably, lung cancer, liver cancer, and colorectal cancer exhibit significantly higher mortality rates compared with other types of cancer.[28] Traditional therapeutic approaches for cancer treatment have limitations, including inadequate effectiveness, lack of specificity, and severe side effects. Oncolytic virotherapy that selectively targets and eliminates cancer cells while minimizing harm to healthy cells offers a promising and innovative method for treating cancer. The antitumor effect mediated by oncolytic virus mainly relies on two actions,

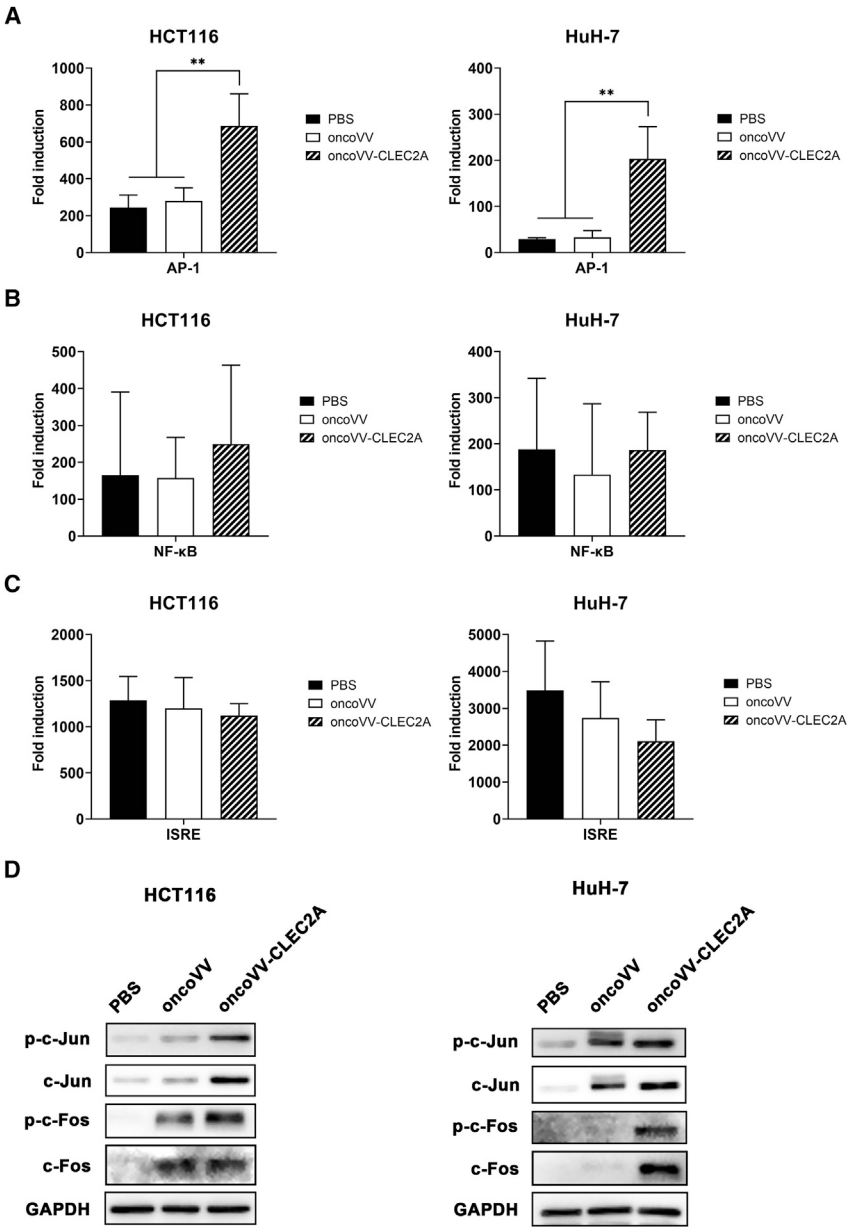


Figure 6. Inflammatory response mechanism induced by oncoVV-CLEC2A

(A–C) Transcriptional activity of AP-1 (A), NF-κB (B), and ISRE (C) were investigated by dual luciferase reporter gene assay in HCT116 and HuH-7 cells. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$). (D) The protein expression levels of c-Fos, p-c-Fos, c-Jun, and p-c -Jun in HCT116 and HuH-7 cells were quantified using western blot analysis at the 36-h time point after infection with oncoVV-CLEC2A or oncoVV. GAPDH was used as the internal control for loading normalization.

one is direct lytic function based on viral infection and replication; the second is immune-mediated tumor eradication through modulating the tumor microenvironment. In the current study, we found that arming VV with the human *CLEC2A* gene promoted viral replication, improved antitumor immune response, and enhanced the antitumor efficacy both *in vitro* and in tumor-bearing nude mice.

Several cytokines or co-stimulatory molecules have been inserted to oncolytic virus to enhance the immunotherapeutic effects in both laboratory and clinical research.[29] However, this strategy often faces challenges; a strong immune reaction may hinder the initial tumor invasion or eliminate the virus too rapidly. Finding a balance between induction of antitumor immunity and immune-mediated viral clearance is crucial for maximizing the effectiveness of cancer viral therapy. IFNs play a crucial role in the immune defense against both tumor development and viral infections, serving as the frontline of protection. IFN initiates the activation of the intracellular JAK/STAT pathway and produces a complex called ISG factor 3 (ISGF3). Subsequently, ISGF3 translocates to the nucleus and interact with the IFN-stimulated response element (ISRE) promoter, resulting in the transcription of ISGs that establish an antiviral state.[23,30] In our study, we found that oncoVV-CLEC2A treatment elicited an increase in intracellular IFN expression, but no stimulation of ISRE expression, indicating that oncolytic VV harboring *CLEC2A* gene suppressed the antiviral response of tumor cells. Moreover, we investigated that oncoVV-CLEC2A also had a stronger replication ability compared with oncoVV, indicate harboring *CLEC2A* gene can significantly promote viral replication, the potential mechanism requires further research. Although a number of cytokines, such as TNF, IL-2, and IFN, have been used in pre-clinical oncolytic VV therapy, they have not been effectively applied in a clinical setting, because they may induce direct antiviral effects.[29,31] Our study provides new opportunities to improve therapeutic effects of oncolytic VVs through expressing human CLEC2A.

Oncolytic viral therapy applies to strengthen the anti-tumor immune response primarily focused on attenuating pathogenicity and enhancing immune response. Oncolytic viruses modulate the tumor microenvironment and turn "cold" tumors to "hot" tumors through the production of cytokines, induction of immunogenic cell death, and infiltration of immune cells.[32] NK cells assume a pivotal part in scrutinizing tumors, shaping the immune reaction by interacting with dendritic cells, macrophages, and T cells.[33] Activation of NK cell-surface receptors has the potential to trigger NK cell cytotoxicity

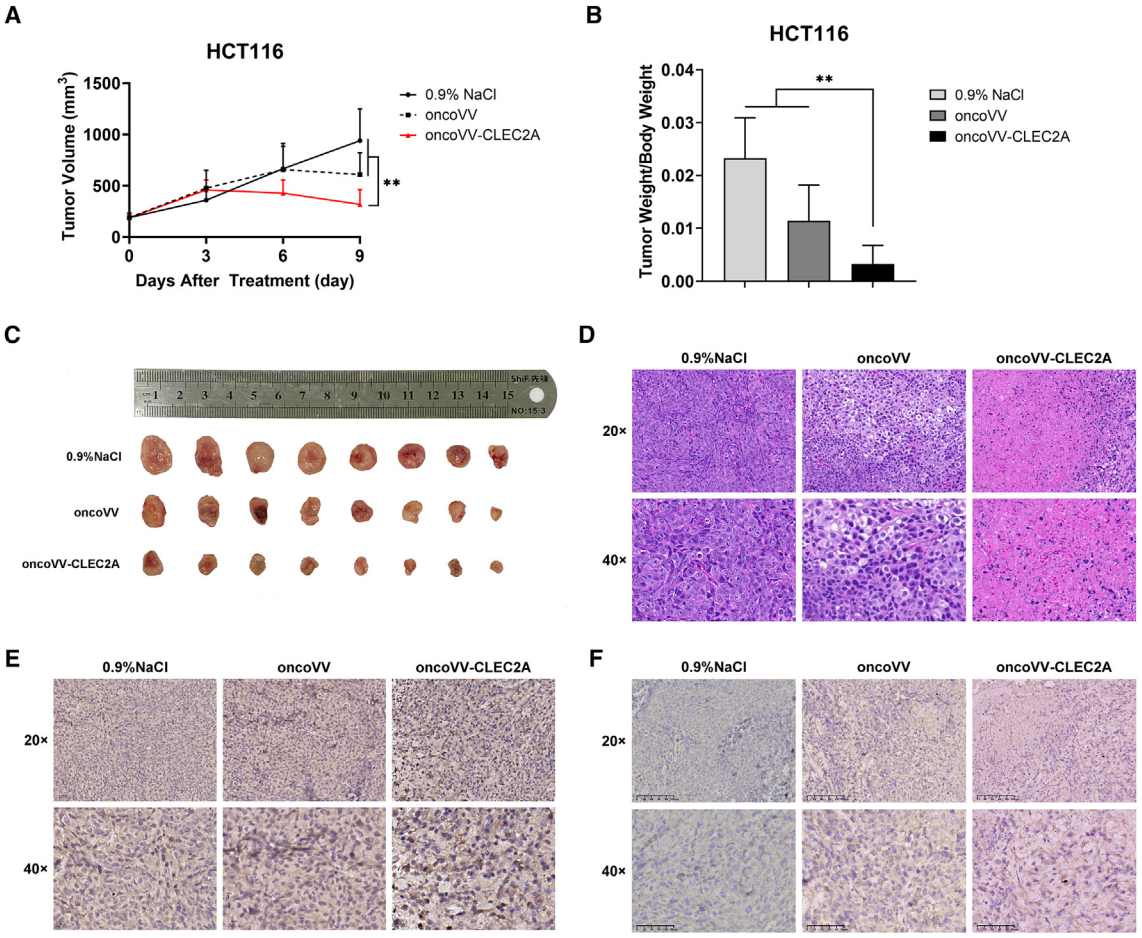


Figure 7. The antitumor efficacy of oncoVV-CLEC2A on HCT116 tumors

(A) The volume of the tumor was evaluated every 3 days after treatment. (B) Proportion of tumor weight in the body weight of mice. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$). (C) Photo of tumors taken from sacrificed mice. (D) HE staining of tumor tissue obtained from the mice treated with 0.9% NaCl, oncoVV or oncoVV-CLEC2A. (E and F) IHC detected A27L (E) and CALR (F) expression in the tumor tissue of mice treated with 0.9% NaCl, oncoVV or oncoVV-CLEC2A.

and cytokine generation. Some NK receptors, represented by Ly49s, have the capability to recognize major histocompatibility complex (MHC) or MHC-like molecules, while others (NKp65, Nkrp1, and NKp80) attach to C-type lectin-like receptors.[34] The NK gene complex (NKC) CLEC2A-NKp65 is a part of the latter category, their interaction lead to the activation of NK cells. In this study, oncoVV-CLEC2A invigorates NK cell function, stimulating IFN-γ secretion in mice, supporting the idea that the NKC-encoded receptor-ligand partnership Nkrp1f-Clrg has been detected, as well as the evidence that mouse Nkrp1f and humanNKp65 share conserved residues that interact with human CLEC2A.[35] IFN-γ is predominantly secreted by activated lymphocytes such as T cells, NK T cells, and NK cells.[27] Due to nude mice lacking T cells, we infer that heightened IFN-γ levels in oncoVV-CLEC2A are primarily instigated by activated NK cells. Moreover, IFN-γ plays a pivotal role in adjusting the anti-tumor immune response, known for its ability to induce M1-like macrophage polarization.[36] Apparently consistent with the above, oncoVV-CLEC2A activated NK to induce IFN-γ production, leading to M1 polarization. Despite this antitumor model used in immunodeficient mice, the impact is conspicuous owing to NK cell activity and tumor suppression; hence, we intend to use humanized peripheral blood mononuclear cell mice for future investigations.

To summarize, the results presented herein offer a strategy for oncolytic virotherapy using VV harboring CLEC2A. In this series of studies, we have shown that oncoVV-CLEC2A could enhance the anti-tumor effects by promoting viral replication to directly oncolysis, activating NK cells to release IFN-γ, and inducing M1-like macrophage polarization to shape the tumor microenvironment.

MATERIALS AND METHODS

Cell culture

Five cell lines were acquired from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), including human

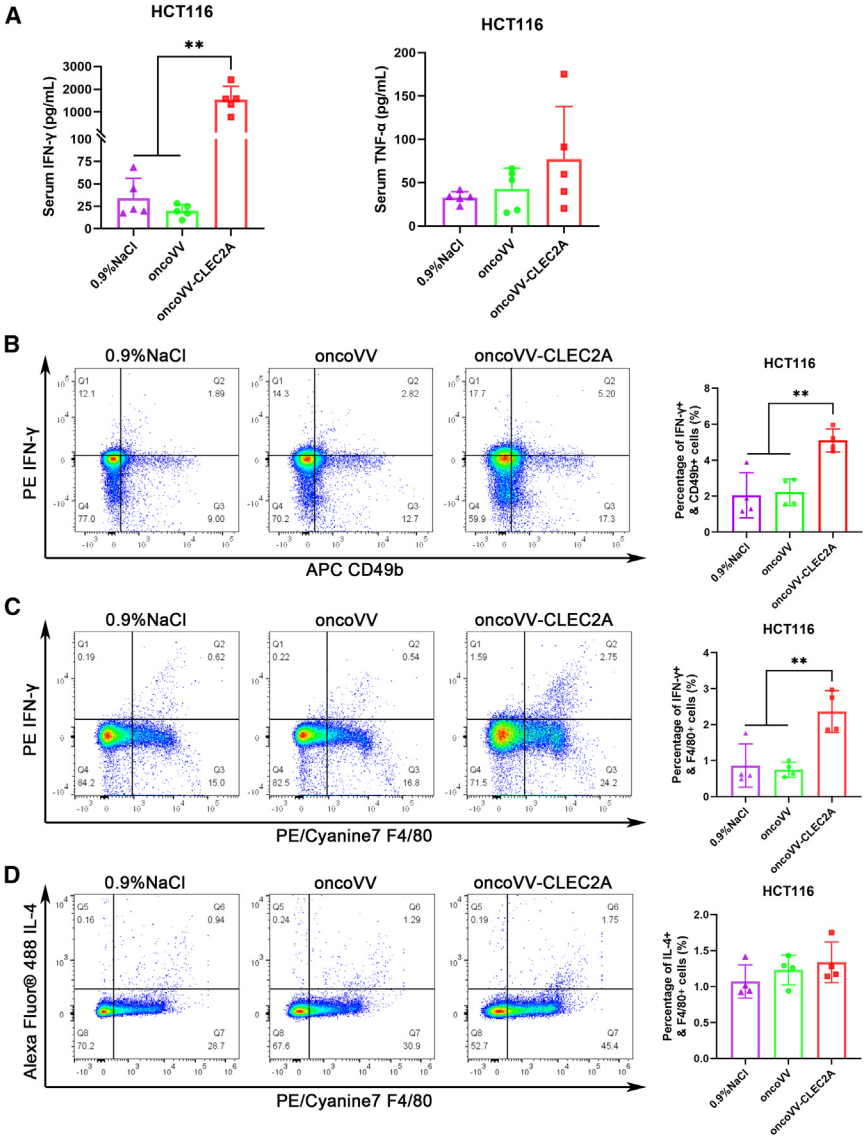


Figure 8. OncoVV-CLEC2A enhanced the cytotoxicity of NK cell

(A) The serum levels of IFN-γ and TNF-α in the mice. (B–D) FCM analysis and quantitative analysis of percentage of NK cells ($CD49b^+$ IFN-γ^+) (B), M1 macrophagocytes ($F4/80^+$ IFN-γ^+) (C) and M2 macrophagocytes ($F4/80^+$ IL-4^+) (D) of three groups. Data are shown as the mean ± SD (*$p < 0.05$, **$p < 0.01$).

embryonic kidney cell line HEK-293, the colorectal cancer cell lines HCT116 and SW620, hepatocellular carcinoma cell line HuH-7, and non-small cell lung cancer cell lines H460. HCT116 cells were incubated in 5A medium (Cienry, Zhejiang, China), while other cells were cultured in DMEM (high glucose) medium (Bio-channel, Nanjing, China), all these mediums were supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China) and 1% penicillin-streptomycin (Bio-channel). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

For cell spheroidization, HCT116 cells were cultured in serum-free Advanced DMEM/F12 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20 ng/mL epidermal growth factor (Thermo Fisher Scientific), 40 ng/mL basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), 2% B-27 (Thermo Fisher Scientific), and 1% *N*-2 (Thermo Fisher Scientific). Cells were seeded into 24-well ultra-low attachment plates with three replicates and 1×10^2 cells per well in 0.5 mL sphere media. After inoculation for 7 days, oncoVV-CLEC2A or oncoVV dilution was added to 24-well plates, and PBS was used as a negative control. Spheres were observed and photographed using microscope (Olympus, Tokyo, Japan, CKX53).

Generation of oncoVV-CLEC2A

The wild-type VV WR strain, purchased from the American Type Culture Collection (Manassas, VA, USA), serves as the backbone for our research. The shuttle vector pCB, which harbors the xanthine-guanine phosphoribosyl transferase gene controlled by the p7.5 promoter, facilitates the homologous recombination to delete vaccinia *TK* gene. Cloning *CLEC2A* (GenBank: NM_001130711.2) gene into pCB under the Pse/l promoter yielded pCB-CLEC2A plasmid. Subsequently, the recombinant pCB-CLEC2A plasmids were transfected into Vero cells previously infected with WR at a MOI of 0.1 using HighGene Transfection Reagent (Signagen Laboratories, Frederick, MD, catalog no. RMO9014). The virus was screened in the mixture of xanthine, hypoxanthine, and mycophenolic acid and then it was sequenced. The strain with TK deletion alone serves as the control.

Cell viability assay

Cells were seeded into 96-well plates with six replicates and a cell density of 8×10^3 cells per well. After inoculation for 12 h, oncoVV-CLEC2A or oncoVV dilution (at MOI of 1, 2, 5, and 10) was added to the plates, and PBS of equal volume was used as a control, then the plates were incubated at 37°C and 5% CO_2 for a serious time. After infection finished, a 20 μL concentration of 5 mg/mL MTT solution (Beyotime Biotechnology, Shanghai, China) was added and incubated at 37°C for 4 h. The supernatant was then replaced with 150 μL DMSO to dissolve formazan completely. Finally, the absorbance of 570 nm was investigated by a microplate reader (Multiskan, Thermo Fisher Scientific).

Quantitative PCR assay

After a 12-h cell incubation, oncoVV-CLEC2A or oncoVV was added to the respective wells (MOI of 2 for HCT116 and MOI of 5 for SW620, HuH-7, and H460), while PBS was used as the negative control group. After infection for 36 h, the cells were collected, and total RNA was extracted with the RNA-Quick Purification Kit (YiShan Biotech, Shanghai, China). The extracted RNA was then reverse transcribed into cDNA using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The cDNA was amplified using the SYBR Green Real-time PCR Master Mix (TOYOBO). The expression levels of each gene were normalized by GAPDH and calculated by $2^{\wedge -\Delta\Delta CT}$. The primers used were as follows: CLEC2A-F, 5′-GGACTGGCTTG GAGTGAGAGAT-3′ and CLEC2A-R 5′-GTGCATATCAGTTCC TGCGTACC-3′; IFN-α-F, 5′-GTGAGGAAATACTTCCAAAGAAT CAC-3′and IFN-α-R 5′-TCTCATGATTTCTGCTCTGACAA-3′; IFN-β-F, 5′-CAGCAATTTTCAGTGTCAGAAGC-3′ and IFN-β-R, 5′-TCATCCTGTCCTTGAGGCAGT-3′. Other primers used were as previously described.[37]

Western blot analysis

The cells were collected and lysed using an ultrasonic cell disrupter system from (Scientz, Ningbo, China). The proteins extracted from the lysate were subjected to electrophoresis on SDS-PAGE gel and transferred onto a PVDF membrane. Primary antibodies (dilution, 1:1,000) were as follows: caspase-3 (Cat. NO. 9662), c-Jun (Cat. NO. 9165), p-c-Jun (Cat. NO.9261), GAPDH (Cat. NO. 2118), c-Fos (Cat. NO. 2250), and p-c-Fos (Cat. NO. 5348) were purchased from Cell Signaling Technology, caspase-9 (Cat. NO. ab202068) and A27L (Cat. NO. ab35219) were obtained from Abcam Inc. (Cambridge, UK), and CLEC2A was purchased from Affinity Biosciences (Jiangsu, China). As for secondary antibodies (1:4,000), goat anti-rabbit antibodies (Cat. NO. AS014) were obtained from ABclonal Technology (Wuhan, China).

Virus replication capacity assay

Cells were seeded in 24-well plates at a density of cells/well (HCT116) or cells/well (HuH-7) 12 h before oncoVV-CLEC2A or oncoVV infection. Samples were collected at different time points and stored at −80°C. Viral yields were tested using a $TCID_{50}$ assay in HEK293A cells after repeated freezing and thawing for three times.

Dual luciferase reporter gene assay

Cells were seeded in white 96-well plates and co-transfected with Ranilla luciferase reporter vector pRL-TK, Luc/Rluc recombinant plasmids containing target genes (AP-1, ISRE). After co-transfection for 24 h, oncoVV-CLEC2A or oncoVV was added to infect for 36 h, to measure luciferase activity, a chemiluminescence apparatus was used along with the Dual Luciferase Reporter Gene Assay Kit (E2920, Promega, Madison, WI, USA).

Animal experiments

Six-week-old female BALB/c nude mice used in this study were obtained from Shanghai SLAC Laboratory Animal Company Limited (Shanghai, China). HCT116 cells were injected subcutaneously into the mice on the back at 8×10^6 cells/mouse with Matrigel (Corning Life Sciences, Kennebunkport, ME, USA) of equal volume. Once the tumor volume approached approximately 200 mm^3, the mice were randomly divided into three groups. Each group was then injected with 1×10^7 plaque-forming units of oncoVV-CLEC2A, oncoVV alone, or an equal volume of saline. Tumor volumes were monitored every 3 days after virus injection and calculated in accordance with the following formula: V (mm^3) = $^1/_2$ × length (mm) × width (mm)2. After a total of 9 days, the mice were euthanized, and the tumors were collected and fixed with 4% paraformaldehyde for further analysis. For ELISA, 2 weeks after virus injection, blood samples were collected. We performed ELISA following the instructions, using the mouse IFN-γ ELISA Kit (EK280/3-48) and the mouse TNF-α ELISA Kit (EK282/4-48), purchased from Lianchuan Biotechnology Company Limited (Hangzhou, China).

HE staining and IHC

HE staining and IHC were conducted by RuChuang Biotechnology Company Limited (Shanghai, China). Mouse tumor was gathered and placed in neutral buffered 10% formalin, embedded in paraffin and cut into sections with a thickness of 5 μm. For HE staining, samples were stained with HE. For IHC assay, antibody against VV (Abcam, 1:100) and CALR (Abways, Shanghai, China, 1:100) were used. Stained slides were mounted and observed using microscope (Olympus, CKX53).

Flow cytometry determination

In the assessment of apoptosis, cells were cultured for 12 h before introducing oncoVV-CLEC2A or oncoVV (with an MOI of 2) into the designated wells. Meanwhile, the negative control group received an equivalent volume of PBS. After a 36-h infection period, the cells underwent collection and staining employing the Annexin V FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA).

To assess NK cells and macrophages, after a 2-week treatment, myeloid cells were extracted from the femurs and tibiae of donor mice, then underwent filtration through a 300-mesh nylon filter and were washed twice with PBS (1% fetal bovine serum). Removal of erythrocytes was accomplished through incubation with RBC lysis buffer (BioLegend, San Diego, CA, USA, 420302). The single-cell suspension was then subjected to staining using flow cytometry antibodies from BioLegend, which included APC anti-mouse CD49b antibody (103515), PE/cyanine7 anti-mouse F4/80 antibody (123113), PE anti-mouse IFN-γ antibody (163503), and Alexa Fluor 488 anti-mouse IL-4 antibody (504111). Stained cells were analyzed using a BD Fortessa flow cytometer (C6, BD Biosciences) along with FlowJo10 software.

Data analysis

The Student's t test was used for the data analysis, and statistical significance was determined at a threshold of a p value of <0.05 or p <0.01. Results were expressed as mean ± SD.

DATA AND CODE AVAILABILITY

All raw and analyzed data supporting the findings of this study are available upon request.

ACKNOWLEDGMENTS

This research was funded by Natural Science Foundation of Zhejiang Province, grant numbers LTGY23D060001 and LQ24D060001; National Natural Science Foundation of China, grant number 42376105; Zhejiang Provincial Department of Education, grant number Y202354004; and the State Key Laboratory of Microbial Technology Open Projects Fund (Project NO. M2023-04).

AUTHOR CONTRIBUTIONS

G.L. and T.Y. conceived and designed the experiment; C.G., Q.Y., Y.Q., N.R., and Y.Z. performed the experiments; C.G., T.Y., K.C., and G.L. analyzed the data; C.G. and T.Y. wrote the manuscript; G.L. supervised the research and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

1. Fukuhara, H., Ino, Y., and Todo, T. (2016). Oncolytic virus therapy: A new era of cancer treatment at dawn. Cancer Sci. *107*, 1373–1379.
2. Mondal, M., Guo, J., He, P., and Zhou, D. (2020). Recent advances of oncolytic virus in cancer therapy. Hum. Vaccines Immunother. *16*, 2389–2402.
3. Jefferson, A., Cadet, V.E., and Hielscher, A. (2015). The mechanisms of genetically modified vaccinia viruses for the treatment of cancer. Crit. Rev. Oncol. Hematol. *95*, 407–416.
4. Smith, G.L., and Moss, B. (1983). Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. Gene *25*, 21–28.
5. Breitbach, C.J., Burke, J., Jonker, D., Stephenson, J., Haas, A.R., Chow, L.Q.M., Nieva, J., Hwang, T.H., Moon, A., Patt, R., et al. (2011). Intravenous delivery of a multimechanistic cancer-targeted oncolytic poxvirus in humans. Nature *477*, 99–102.
6. Guo, Z.S., Lu, B., Guo, Z., Giehl, E., Feist, M., Dai, E., Liu, W., Storkus, W.J., He, Y., Liu, Z., and Bartlett, D.L. (2019). Vaccinia virus-mediated cancer immunotherapy: cancer vaccines and oncolytics. J. Immunother. Cancer *7*, 6.
7. Foloppe, J., Kempf, J., Futin, N., Kintz, J., Cordier, P., Pichon, C., Findeli, A., Vorburger, F., Quemeneur, E., and Erbs, P. (2019). The enhanced tumor specificity of TG6002, an armed oncolytic vaccinia virus deleted in two genes involved in nucleotide metabolism. Mol. Ther. Oncolytics *14*, 1–14.
8. Downs-Canner, S., Guo, Z.S., Ravindranathan, R., Breitbach, C.J., O'Malley, M.E., Jones, H.L., Moon, A., McCart, J.A., Shuai, Y., Zeh, H.J., and Bartlett, D.L. (2016). Phase 1 study of intravenous oncolytic poxvirus (vvDD) in patients with advanced solid cancers. Mol. Ther. *24*, 1492–1501.
9. Holloway, R.W., Mendivil, A.A., Kendrick, J.E., Abaid, L.N., Brown, J.V., LeBlanc, J., McKenzie, N.D., Mori, K.M., and Ahmad, S. (2023). Clinical activity of Olvimulogene Nanivacirepvec-primed immunochemotherapy in heavily pretreated patients with platinum-resistant or platinum-refractory ovarian cancer: the nonrandomized phase 2 VIRO-15 clinical trial. JAMA Oncol. *9*, 903–908.
10. Melcher, A., Harrington, K., and Vile, R. (2021). Oncolytic virotherapy as immunotherapy. Science *374*, 1325–1326.
11. Jiang, R., Qiu, Y., Zhang, X., Zhou, N., Jia, X., Chen, K., Zhou, Y., Ye, T., and Li, G. (2022). Oncolytic vaccinia virus harboring *Aphrocallistes vastus* lectin inhibits the growth of hepatocellular carcinoma cells. Mar. Drugs *20*, 378.
12. Zhou, Y., Wang, Q., Ying, Q., Zhang, X., Chen, K., Ye, T., and Li, G. (2023). Effects of oncolytic vaccinia viruses harboring different marine lectins on hepatocellular carcinoma cells. Int. J. Mol. Sci. *24*, 3823.
13. Spreu, J., Kuttruff, S., Stejfova, V., Dennehy, K.M., Schittek, B., and Steinle, A. (2010). Interaction of C-type lectin-like receptors NKp65 and KACL facilitates dedicated immune recognition of human keratinocytes. Proc. Natl. Acad. Sci. USA *107*, 5100–5105.
14. Lysenko, O., Schulte, D., Mittelbronn, M., and Steinle, A. (2013). BACL is a novel brain-associated, non-NKC-encoded mammalian C-type lectin-like receptor of the CLEC2 family. PLoS One *8*, e65345.
15. Gonçalves-Maia, M., Gache, Y., Basante, M., Cosson, E., Salavagione, E., Muller, M., Bernerd, F., Avril, M.F., Schaub, S., Sarasin, A., et al. (2020). NK cell and fibroblast-mediated regulation of skin squamous cell carcinoma invasion by CLEC2A is compromised in xeroderma pigmentosum. J. Invest. Dermatol. *140*, 1723–1732.
16. Huarte, E., Cubillos-Ruiz, J.R., Nesbeth, Y.C., Scarlett, U.K., Martinez, D.G., Engle, X.A., Rigby, W.F., Pioli, P.A., Guyre, P.M., and Conejo-Garcia, J.R. (2008). PILAR is a novel modulator of human T-cell expansion. Blood *112*, 1259–1268.
17. Li, G., Cheng, J., Mei, S., Wu, T., and Ye, T. (2018). *Tachypleus tridentatus* lectin enhances oncolytic vaccinia virus replication to suppress in vivo hepatocellular carcinoma growth. Mar. Drugs *16*, 200.
18. Lorenzo, M.M., Sánchez-Puig, J.M., and Blasco, R. (2019). Genes *A27L* and *F13L* as genetic markers for the isolation of recombinant vaccinia virus. Sci. Rep. *9*, 15684.
19. Lazear, H.M., Schoggins, J.W., and Diamond, M.S. (2019). Shared and distinct functions of type I and type III interferons. Immunity *50*, 907–923.
20. Grivennikov, S.I., and Karin, M. (2011). Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage. Ann. Rheum. Dis. *70*, i104–i108.
21. Hehlgans, T., and Pfeffer, K. (2005). The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology *115*, 1–20.
22. Wan, P., Zhang, S., Ruan, Z., Liu, X., Yang, G., Jia, Y., Li, Y., Pan, P., Wang, W., Li, G., et al. (2022). AP-1 signaling pathway promotes pro-IL-1β transcription to facilitate NLRP3 inflammasome activation upon influenza A virus infection. Virulence *13*, 502–513.
23. Smith, G.L., Talbot-Cooper, C., and Lu, Y. (2018). How does vaccinia virus interfere with interferon. Adv. Virus Res. *100*, 355–378.
24. Fridman, W.H., Zitvogel, L., Sautès-Fridman, C., and Kroemer, G. (2017). The immune contexture in cancer prognosis and treatment. Nat. Rev. Clin. Oncol. *14*, 717–734.
25. Fucikova, J., Spisek, R., Kroemer, G., and Galluzzi, L. (2021). Calreticulin and cancer. Cell Res. *31*, 5–16.
26. Peng, R.Q., Chen, Y.B., Ding, Y., Zhang, R., Zhang, X., Yu, X.J., Zhou, Z.W., Zeng, Y.X., and Zhang, X.S. (2010). Expression of calreticulin is associated with infiltration of T-cells in stage IIIB colon cancer. World J. Gastroenterol. *16*, 2428–2434.
27. Burke, J.D., and Young, H.A. (2019). IFN-γ: A cytokine at the right time, is in the right place. Semin. Immunol. *43*, 101280.
28. Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. *71*, 209–249.
29. Thorne, S.H. (2014). Immunotherapeutic potential of oncolytic vaccinia virus. Front. Oncol. *4*, 155.
30. Aaronson, D.S., and Horvath, C.M. (2002). A road map for those who don't know JAK-STAT. Science *296*, 1653–1655.
31. Wang, L.C.S., Lynn, R.C., Cheng, G., Alexander, E., Kapoor, V., Moon, E.K., Sun, J., Fridlender, Z.G., Isaacs, S.N., Thorne, S.H., and Albelda, S.M. (2012). Treating tumors with a vaccinia virus expressing IFNβ illustrates the complex relationships between oncolytic ability and immunogenicity. Mol. Ther. *20*, 736–748.
32. Zhu, Z., McGray, A.J.R., Jiang, W., Lu, B., Kalinski, P., and Guo, Z.S. (2022). Improving cancer immunotherapy by rationally combining oncolytic virus with modulators targeting key signaling pathways. Mol. Cancer *21*, 196.

33. Crinier, A., Narni-Mancinelli, E., Ugolini, S., and Vivier, E. (2020). Snapshot: natural killer cells. Cell *180*, 1280–1280.e1.

34. Yokoyama, W.M., and Plougastel, B.F.M. (2003). Immune functions encoded by the natural killer gene complex. Nat. Rev. Immunol. *3*, 304–316.

35. Li, Y., Wang, Q., Chen, S., Brown, P.H., and Mariuzza, R.A. (2013). Structure of NKp65 bound to its keratinocyte ligand reveals basis for genetically linked recognition in natural killer gene complex. Proc. Natl. Acad. Sci. USA *110*, 11505–11510.

36. Mily, A., Kalsum, S., Loreti, M.G., Rekha, R.S., Muvva, J.R., Lourda, M., and Brighenti, S. (2020). Polarization of M1 and M2 human monocyte-derived cells and analysis with flow cytometry upon mycobacterium tuberculosis infection. J. Vis. Exp. *163*, e61807.

37. Zheng, X., Xu, W., Ying, Q., Ni, J., Jia, X., Zhou, Y., Ye, T., Li, G., and Chen, K. (2022). Oncolytic vaccinia virus carrying Aphrocallistes vastus lectin (oncoVV-AVL) enhances inflammatory response in hepatocellular carcinoma cells. Mar. Drugs *20*, 667.