# Oncolytic avian reovirus induces autophagy by triggering the TLR3-IRF3-NF-κB and IFN-γ-JAK-STAT1 signaling pathways

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**Abstract**

Oncolytic avian reovirus (ARV) has been identified as a virus capable of selectively infecting and inducing cell death in various cancer cell lines. This study investigates the role of ARV in activating innate immune responses in B16-F10 murine melanoma cells, focusing on the TLR3-IRF3-NF-κB and IFN-γ-JAK-STAT1 pathways. Our results demonstrate that the ARV σC protein interacts with TLR3 in the cytoplasm, leading to the upregulation of TLR3 and IFN-γ expression, as confirmed by quantitative real-time reverse transcription and polymerase chain reaction (qRT-PCR), ELISA, and Western blot assays. Suppression of TLR3,NF-κB, and IRF3 demonstrated the involvement of the TLR3-IRF3-NF-κB pathway in IFN-γ activation. Furthermore, depletion of STAT1 by shRNA and inhibition of JAK by inhibitors revealed that ARV triggers autophagy by the IFN-γ-JAK-STAT1 pathway. Immunofluorescence staining and LC3-mCherry transfection further confirmed ARV induces autophagy by triggering the TLR3-IRF3-NF-κB and IFN-γ-JAK-STAT1 signaling pathways. This study highlights the potential of ARV as a novel oncolytic virotherapy through immune pathway activation in cancer cells.

**Keywords** avian reovirus, Toll like receptor 3, autophagy, B16-F10 melanoma cells, IRF3, NF-κB, IFN-γ, STAT1

## 1.Introduction

Avian reovirus (ARV), a member of the Reoviridae family, is a segmented double-stranded RNA virus that primarily infects poultry, particularly chickens. ARV infection leads to various clinical manifestations, including viral arthritis, tenosynovitis, enteritis, and systemic symptoms, often resulting in lameness, anorexia, weight loss, and growth retardation (Franzo et al., 2024). These symptoms not only reduce production efficiency but also increase mortality rates and rearing costs, causing significant economic losses to the poultry industry. ARV exhibits a complex structural organization, with a genome comprising 10 segments of double-stranded RNA that encode multiple structural and non-structural proteins (Benavente and Martinez-Costas, 2007). The outer capsid proteins, including σC, σB, and λA, play crucial roles in viral attachment and host cell recognition. Among them, the σC protein functions as a spike protein that facilitates viral entry by binding to specific host cell receptors, thereby triggering host immune responses (Huang et al., 2023; Wu et al., 2024). This mechanism not only underscores ARV as a significant avian pathogen but also highlights its potential immunomodulatory functions.

In recent years, several studies have demonstrated the strong oncolytic activity of ARV, leading to its designation as an oncolytic ARV. The oncolytic properties of ARV have garnered significant interest due to its ability to selectively induce tumor cell lysis, apoptosis, and autophagy, making it a promising candidate for cancer virotherapy. ARV exploits the immune evasion characteristics of tumor cells, selectively infecting and destroying malignant cells while exerting minimal effects on normal cells. Previous studies from our laboratory have confirmed that ARV proteins p17 and σA co-repress

mTOR Complex 2 (mTORC2) and the CDK2/cyclin A2 complex, thereby inducing autophagy (Huang et al., 2017). Additionally, the p17 protein positively regulates p53 and activates PTEN, leading to the inhibition of the PI3K/Akt/mTORC1 pathway, further promoting autophagy to enhance viral replication (Huang et al., 2015). Furthermore, our previous research demonstrated that ARV σC protein can activate immune cells and the Toll-like receptor 3 (TLR3) pathway in gastric cancer cells, ultimately inducing apoptosis (Wu et al., 2024). ARV σA protein has also been found to upregulate glycolysis and glutaminolysis through the HIF-1α/c-Myc/GLUT1 pathway, facilitating viral replication in cancer cell lines (Hsu et al., 2023). Collectively, these findings highlight the potential of ARV as an oncolytic virus, underscoring its relevance as a novel approach for cancer treatment.

Although our laboratory has demonstrated that ARV induces apoptosis in cancer cells by activating immune cells and the TLR3 pathway in gastric cancer cells, previous studies have primarily focused on the immune cell-cancer cell interactions. However, the precise role of ARV in modulating TLR3 signaling at the single-cell level remains unclear. Therefore, this study aims to investigate whether ARV infection activates the TLR3-IRF3-NF-κB signaling pathway to induce IFN-γ expression, which subsequently triggers apoptosis or autophagy through the IFN-γ-JAK-STAT1 pathway.

## 2.Materials and methods

### *2.1. Cells and viruses*

The ARV vaccine strain S1133 was propagated in Vero cells. When cells reached 80-90% confluence, they were washed with PBS and inoculated with the virus in fresh

MEM containing FBS. Cells were incubated at 37°C with 5% $CO_2$, and cytopathic effects (CPE) were monitored. At ~50% CPE, the culture supernatant and cells were transferred to a new dish. When CPE reached 90%, cells were harvested and stored at -20°C.

African green monkey kidney epithelial cells (Vero) and murine melanoma cells (B16-F10) were co-cultured in modified Kaighn's F-12K medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 mM 4-(2-ethyl)-1-2-ethyl ester (HKES). Cells were maintained in a humidified incubator at 37°C with 5% $CO_2$.

*2.2. Reagents and antibodies*

The σC monoclonal antibodies were produced by our laboratory (Hsu et al., 2006). Anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) antibodies were purchased from Kirkegaard & Perry Laboratories (KPL, Washington, USA). The catalog numbers and dilution factor of the respective antibodies used in this study are shown in Table 1.

*2.3. shRNAs used in this study*

The pLKO-AS1-puro plasmid encoding shRNA comes from the National RNAi Core Facility of Academia Sinica, Taiwan. The shRNA sequence of this experiment is shown in Table 2. B16-F10 cells were transfected with their respective shRNAs for 6 hours, and then infected with ARV at MOI of 10 for 24 hours. Collect whole cell lysates for Western blot analysis.

*2.4. Quantitative real-time reverse transcript and polymerase chain reaction RT-PCR*

*analysis*

To investigate whether ARV infection affects the expression of TLRs, interferons, and cytokines in B16-F10 mouse melanoma cells, cells were infected with ARV at a multiplicity of infection (MOI) of 10. At 24 hours post-infection, cell lysates were collected. Total RNA was extracted from virus-infected or transfected cells using TRIzol reagent (Thermo Fisher Scientific, USA), following the manufacturer's protocol. qRT-PCR was performed using the iQ™ SYBR® Green Supermix kit (Bio-Rad, Hercules, CA, USA), as previously described (Chi et al., 2018; Hsu et al., 2023). The specific primer pairs used in this study are listed in Table 3.

*2.5. Transient transfection*

For transfection experiments, cells were seeded into 6-well plates. When the cells reached approximately 70% confluence, they were transfected with the TransIT-X2® transfection reagent (Mirus Bio, Madison, USA) according to the manufacturer's instructions. In this study, cancer cell lines were transfected with the respective shRNAs for 6 hours, followed by infection with ARV at a multiplicity of infection (MOI) of 10 for 24 hours. A scramble plasmid was used as a negative control. Cell viability was assessed following transfection with the respective genes or shRNAs. Transfection efficiency was evaluated using either Western blot or immunofluorescence staining, ensuring an efficiency of 80-90%.

*2.6. Plaque assay.*

Vero cells ($1\times10^5$/well) were seeded in a 24-well plate and incubated for 24 h. After washing with PBS, cells were infected with 500 μL of 10-fold serially diluted

140 virus and incubated at 37°C with 5% $CO_2$ for 24 h. The supernatant was removed, and
141 cells were stained with Trypan Blue (Merck, Burlingtong, USA). Plaques were
142 observed under a microscope to determine the viral titer. Viral particles were released
143 by freeze-thaw cycles, then centrifuged at 12,000 × g for 10 min to remove debris. The
144 supernatant was collected and stored at -80°C.
145
146 *2.7. Electrophoresis and Western blot assays*
147 Cells were seeded in a 6-well cell culture dish one day before infection with the
148 virus or transfection with the plasmid, as described above. The collected cells were
149 washed twice with 1× PBS and lysed using lysis buffer (Cell Signaling Technology,
150 Danvers, USA). The protein concentration in the cell lysates was determined using the
151 Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, USA), following the
152 manufacturer's protocol. Equal amounts of protein samples were mixed with 2.5×
153 Laemmli loading buffer and boiled in a water bath at 100°C for 10 minutes. The samples
154 were then subjected to electrophoresis on a 10% or 12% sodium dodecyl sulfate-
155 polyacrylamide gel (SDS-PAGE) and transferred onto a polyvinylidene difluoride
156 (PVDF) membrane (GE Healthcare Life Sciences, USA). Protein expression was
157 detected using the appropriate primary antibody followed by a horseradish peroxidase
158 (HRP)-conjugated secondary antibody. The membrane was incubated with an enhanced
159 chemiluminescence (ECL Plus) reagent (Amersham Biosciences, Waukesha, UK), and
160 the signals were visualized using X-ray film (Kodak, Rochester, USA). The intensity
161 of the target protein bands was quantified using ImageJ software.
162
163 *2.8. Immunofluorescence assay*

Cells were cultured on 18 × 18 mm coverslips and fixed with 10% formaldehyde at room temperature for 30 minutes, followed by PBS washes. For immunostaining, cells were permeabilized with 0.01% Triton X-100 for 15 minutes, blocked with Block™ Blocking Buffer (Visual Protein, Taipei, Taiwan) for 90 minutes, and incubated overnight at 4°C with primary antibodies (1:250). The next day, cells were washed with 0.1% PBST, incubated overnight at 4°C with TRITC-conjugated secondary antibodies, and counterstained with 0.1 μg/mL DAPI. Coverslips were mounted with antifade medium, sealed with nail polish, and stored at 4°C. Fluorescence images were acquired using an inverted fluorescence microscope (ZEISS Axio Observer 3, Thornwood, USA) under light-protected conditions. Image processing was performed using Photoshop Mix.

*2.9. Proximity ligation assay (PLA)*

Cells were cultured on 18×18 mm coverslips, treated as required, and fixed with 10% formaldehyde at room temperature for 30 minutes, followed by PBS washes. Permeabilization was performed using 0.01% Triton X-100 for 15 minutes. After blocking with Block™ Blocking Buffer for 90 minutes at room temperature, cells were incubated overnight at 4°C with primary antibodies. The PLA assay was performed using the Duolink® PLA Multicolor Reagent Pack (Sigma-Aldrich, Louis, USA). After primary antibody removal, coverslips were washed with buffer A and incubated with diluted PLA probes at 37°C for 1 hour. Following two washes with buffer A, ligation was carried out using diluted ligase buffer for 30 minutes at 37°C. Cells were then washed and incubated with polymerase buffer at 37°C for 100 minutes to amplify the signal. After final washes with buffer B, cells were counterstained with 0.1 μg/mL

DAPI for 15 minutes, mounted with antifade medium, and sealed with nail polish. Fluorescence images were acquired using an inverted fluorescence microscope.

*2.10. Analysis of IFN-γ levels by ELISA*

The IFN-γ levels in ARV- infected cells were measured using the mouse IFN-γ uncoated ELISA Kit (Thermo Fisher Scientific, Vienna, Austria). ELISA plates were coated with 100 μL/well of capture antibody and incubated overnight at 4°C. After blocking with 1× ELISA/ELISPOT Diluent for 1 hour at room temperature, 100 μL/well of samples were added and incubated overnight at 4°C. The next day, wells were washed, then sequentially incubated with diluted detection antibody (1 hour), Streptavidin-HRP (30 minutes), and 1× TMB solution (15 minutes) at room temperature, with washes in between. The reaction was stopped, and absorbance at 405 nm was measured using a spectrophotometer.

*2.11. Statistical analysis.*

The Student's t-test was used to evaluate the statistical significance of all the data obtained in this study. The data is expressed as the average of three independent experiments. P values less than 0.05 are considered statistically significant.

## 3. Results

*3.1. Analysis of the mRNA levels of ARV-regulated TLR, interferons, and cytokine*

To investigate whether ARV infection regulates TLR signaling pathways in B16-F10 cells, we analyzed virus-associated TLRs (TLR3, TLR4, TLR7, TLR8, and TLR9)

as well as immune checkpoint-related proteins (DR4, DR5, and PD-L1) in these cells. B16-F10 cells were infected with ARV at a multiplicity of infection (MOI) of 10, and protein samples were collected at 24 and 48 hour post-infection. The mRNA expression levels of TLRs and immune checkpoint-related proteins were assessed by qRT-PCR. Results showed that TLR3 mRNA expression was significantly upregulated by 2- to 2.5-fold compared to the control group, confirming that ARV can regulate TLR3 expression in B16-F10 cells (Fig. 1A).

Previous studies in our laboratory demonstrated that ARV can induce Th1 cytokine responses in the AGS gastric cancer cell line (Wu et al., 2023; Wu et al., 2024). To verify whether ARV infection of B16-F10 cells induces Th1-related immune proteins, we analyzed the mRNA expression levels of Th1-related cytokines (IL-2, IL-5, IL-6, IL-7, IL-12, and IL-17) and interferons (IFN-α, IFN-β, and IFN-γ). B16-F10 cells were infected with ARV at an MOI of 10, and protein samples were collected at 24 and 48 hours post-infection. qRT-PCR results indicated a marked upregulation of IFN-γ mRNA expression (4- to 5-fold) and IL-12 mRNA expression (4-fold) compared to the control group, demonstrating that ARV regulates IFN-γ and IL-12 expression in B16-F10 cells (Fig. 1B).

*3.2. Western blot assays of TLR3, NF-κB, and IRF3 induced by avian reovirus*

To confirm whether ARV infection increases the expression level of TLR3 in B16-F10 cancer cells, the expression of levels TLR3, NF-κB, and IRF3 were analyzed. B16-F10 cells were infected with ARV at an MOI of 10, and protein samples were collected at 24 and 48 hours post-infection. Western blot analysis revealed that TLR3 and IRF3 protein levels increased by 2- to 3-folds and 2- to 10-folds, respectively, compared to

the control group (Fig. 2A, B).

To further examine whether NF-κB is activated in ARV-infected cells, cytoplasmic and nuclear proteins were fractionated after ARV infection (24 hours, MOI 10), followed by Western blot analysis. The nuclear translocation of NF-κB subunits p65 and p50 was observed, with an 8-fold increase compared to the control group (Fig. 2C, D).

*3.3. Analysis of autophagy and apoptosis induced by ARV*

Previous studies have shown that viral infection of cancer cells often triggers both autophagy and apoptosis (Wu et al., 2023). To investigate whether ARV infection affects autophagy or apoptosis in B16-F10 cells, we analyzed autophagy-related protein LC3 and apoptosis-related protein caspase 3. Western blot analysis revealed that LC3-II protein levels increased 2-fold at 24 hours post-infection but showed no significant changes at 48 hours. In contrast, the levels of cleaved-caspase 3 were elevated by 5- to 8-fold at both 24 and 48 hours compared to the control group (Fig. 2E, F).

*3.4. Interaction between ARV σC protein and cytoplasmic TLR3*

Previous studies indicated that TLR3 is primarily located on the surface of endosomes but may also be expressed on the cell membrane (Mielcarska et al., 2020). Our team has demonstrated that the ARV σC protein interacts with TLR3 on the surface of $CD8^+$ TIL cells (Wu et al., 2024). To determine whether TLR3 is expressed on the plasma membrane of B16-F10 cells, both cytoplasmic and membrane proteins were fractionated 24 hour post infection with an MOI of 10 and analyzed by Western blot. Results showed that TLR3 was expressed in the cytoplasm but not on the plasma

membrane of B16-F10 cells (Figure 3A).

To confirm the interaction between ARV σC and cytoplasmic TLR3, proximity ligation assay was performed. A strong red fluorescent signal was observed in the infected group compared to the control group, indicating an interaction between ARV σC and cytoplasmic TLR3 (Fig. 3B).

*3.5. Regulation of TLR3 pathway and induction of autophagy or apoptosis by ARV*

TLR3 activation is known to induce downstream nuclear translocation of NF-κB, IRF3, and AP-1, leading to the production of interferons, cytokines, and immune-related proteins (Kawasaki and Kawai, 2014). To examine whether ARV infection activates the TLR3-NF-κB-IRF3 pathway, B16-F10 cells were treated with the TLR3 inhibitor CU-CPT 4a (27 μM) before infection. Western blot analysis revealed that inhibition of TLR3 significantly reduced the levels of IRF3, LC3-II, and cleaved-caspase 3 by 2.5-, 3.5-, and 3-fold, respectively, compared to ARV-infected cells without inhibitor treatment (Fig. 4A, B).

Furthermore, nuclear translocation of NF-κB subunits p50 and p65 was reduced by 2.5- and 2-fold, respectively, in the TLR3-inhibited group, confirming that TLR3 is required for effective NF-κB activation during ARV infection (Fig. 4C, D).

*3.6. TLR3-IRF3-NF-κB pathway and IFN-γ production*

To investigate the role of the TLR3-IRF3-NF-κB pathway in IFN-γ production, B16-F10 cells were treated with the TLR3 inhibitor, NF-κB inhibitor (BAY 11-7082), or IRF3-shRNA, followed by ARV infection with an MOI of 10. ELISA results showed a significant 2- to 3-fold reduction in IFN-γ levels in all three inhibitor- or shRNA-

treated groups compared to the ARV-only group, demonstrating the critical role of the TLR3-IRF3-NF-κB pathway in IFN-γ production (Fig. 5).

*3.7. Analysis IFN-γ-JAK-STAT1 pathway in induction of autophagy*

IFN-γ is a critical immunomodulatory and antiviral protein that activates the JAK-STAT1 pathway upon binding to its receptor, IFNGR, leading to downstream regulation of autophagy and apoptosis-related genes (Ivashkiv, 2018; Li et al., 2012). To investigate whether the IFN-γ-JAK-STAT1 pathway mediates autophagy, B16-F10 cells were treated with the STAT1 shRNA or the JAK1 inhibitor (upadacitinib) prior to ARV infection. Western blot analysis revealed that inhibition of STAT1 or JAK1 significantly reduced LC3-II expression by 2-fold, indicating the importance of the IFN-γ-JAK-STAT1 pathway in autophagy (Fig. 6). To further confirm these findings, mCherry-LC3-transfected B16-F10 cells were observed under fluorescence microscopy. Cells infected with ARV showed distinct LC3 puncta, while cells treated with STAT1 shRNA or JAK1 inhibitor lacked LC3 puncta, consistent with Western blot results (Fig. 7). In conclusion, ARV σC protein interacts with cytoplasmic TLR3 in B16-F10 cells, activating the TLR3-IRF3-NF-κB pathway to induce IFN-γ production. IFN-γ subsequently triggers autophagy through the IFN-γ-JAK-STAT1 pathway (Fig. 8).

## Discussion

Cancer remains a major global health challenge, accounting for nearly 10 million deaths annually, making it the second leading cause of death worldwide after cardiovascular diseases, according to the World Health Organization (Sung et al., 2021).

306 The incidence of cancer continues to rise globally, driven by aging populations and the
307 widespread adoption of unhealthy lifestyles. This is particularly concerning in low- and
308 middle-income countries, where inadequate healthcare infrastructure and insufficient
309 cancer screening mechanisms exacerbate the burden. Characterized by uncontrolled
310 cellular proliferation, invasion of adjacent tissues, and metastasis to distant sites, cancer
311 is notoriously difficult to manage clinically. Current therapeutic modalities, including
312 chemotherapy and radiotherapy, exhibit limited efficacy in advanced cancer stages and
313 are often accompanied by significant side effects. Consequently, the development of
314 more effective and specific therapeutic strategies has become a central focus of cancer
315 research. Innovative approaches such as immunotherapy, gene therapy, and oncolytic
316 virotherapy have shown promising progress in recent years, but their clinical
317 application remains challenging, underscoring the urgent need for further research to
318 enhance safety and specificity (Fares et al., 2019).

319 ARV, a non-enveloped double-stranded RNA virus of the family Reoviridae,
320 primarily infects poultry, causing conditions such as viral arthritis, enteritis, and
321 tenosynovitis. However, recent studies have highlighted ARV's potential to selectively
322 target and destroy tumor cells, making it a candidate for oncolytic virotherapy (Kozak
323 et al., 2017; Chiu et al., 2018; Cai et al., 2019; Robles-Planells et al., 2020; Hsu et al.,
324 2023; Hsu et al., 2024; Wu et al., 2024). ARV's antitumor mechanisms involve its
325 ability to selectively infect tumor cells, inducing apoptosis and autophagy without
326 affecting normal cells. The σC protein of ARV has been identified as a key determinant
327 of tumor cell selectivity. Previous studies in our laboratory demonstrated that ARV,
328 through its σC protein, activates immune cells and triggers apoptosis in gastric cancer
329 cells by interacting with membrane-localized TLR3 (Wu et al., 2024). In this study, we

further confirmed that the σC protein binds to cytoplasmic TLR3 in B16-F10 murine melanoma cells, inducing both apoptosis and autophagy. While our findings align with previous studies, TLR3 localization differs across cell types: it is primarily membrane-associated in immune and gastric cancer cells but localized intracellularly in B16-F10 cells. This discrepancy may arise from cell-specific functional demands, microenvironmental differences, or variations in double-stranded RNA (dsRNA) recognition mechanisms.

TLR3, a key component of the innate immune system and a member of the pattern recognition receptor (PRR) family, is predominantly expressed in immune, neuronal, and epithelial cells. It recognizes viral dsRNA and initiates immune responses (Durinova et al., 2023). Within the tumor microenvironment, TLR3 exhibits a dual role: it can inhibit tumor progression by inducing immune responses and promoting tumor cell apoptosis, but in certain contexts, it may facilitate tumor growth and metastasis (Zheng et al., 2021). In its tumor-suppressive role, TLR3 activates the TRIF signaling pathway, which subsequently stimulates downstream molecules such as TRAF3, TBK1, IRF3, and NF-κB. This cascade ultimately leads to the production of interferons and pro-inflammatory cytokines (Yang and Shu, 2020). Our experiments demonstrated that ARV infection activates TLR3 in B16-F10 cells, leading to the phosphorylation and nuclear translocation of NF-κB and IRF3, which regulate the production of IFN-γ. This finding is consistent with previous studies indicating that these factors not only exhibit antiviral properties but also enhance the immunogenicity of cancer cells, promoting apoptosis and autophagy. Moreover, TLR3-induced IFN-γ and other cytokines recruit natural killer (NK) cells and cytotoxic T lymphocytes, further inhibiting tumor growth. These results highlight the multifaceted role of TLR3 activation in enhancing antitumor

354 immune responses within the tumor microenvironment.

355 Autophagy, an intracellular degradation and recycling mechanism, plays a critical
356 role in maintaining cellular energy homeostasis and survival. It involves the formation
357 of autophagosomes that encapsulate damaged organelles, misfolded proteins, or other
358 intracellular debris, which are subsequently degraded in lysosomes. In cancer therapy,
359 autophagy is often leveraged to drive cancer cells toward death rather than survival.
360 Conversely, apoptosis, a tightly regulated cell death mechanism, is crucial for
361 maintaining organismal stability and health. Apoptosis is orchestrated through two
362 primary pathways: the intrinsic pathway, often triggered by mitochondrial dysfunction
363 under conditions of oxidative stress or genetic damage, and the extrinsic pathway,
364 initiated by the activation of death receptors such as Fas and TNF receptors. These
365 pathways converge on the activation of caspase cascades, ultimately leading to
366 programmed cell death (Taylor et al., 2008). Notably, autophagy and apoptosis exhibit
367 complex crosstalk, with autophagy potentially delaying apoptosis during early stress
368 responses by clearing damaged cellular components. However, prolonged stress may
369 lead to a switch from autophagy to apoptosis, mediated by interactions between Bcl-2
370 family proteins and autophagy regulators like Beclin-1. In our study, ARV infection of
371 B16-F10 cells induced early-stage autophagy, as evidenced by increased LC3-II
372 expression, followed by late-stage apoptosis characterized by elevated caspase-3 levels.
373 These observations are consistent with previous findings that ARV infection induces
374 early- to mid-stage autophagy and late-stage apoptosis in Vero and DF1 cells (Lin et al.,
375 2015).

376 IFN-γ, a critical cytokine secreted by activated T cells and NK cells, plays a central
377 role in host defense against viral infections, bacteria, and tumors. It mediates its effects

primarily through the JAK-STAT1 signaling pathway. While IFN-γ's role in tumor immunomodulation and its underlying mechanisms are well-documented, its autocrine effects within tumor cells remain underexplored. Studies have shown that some cancer cells autonomously secrete IFN-γ, independent of immune cells, with varying biological consequences. For example, in hepatocellular carcinoma, autocrine IFN-γ activates the JAK-STAT pathway, upregulating autophagy-related genes such as Beclin-1 and LC3 (Li et al., 2012). Similarly, in pancreatic cancer cells, autocrine IFN-γ induces apoptosis through the STAT1 pathway by upregulating caspase-3 and Bax (Detjen et al., 2001). In colorectal cancer, autocrine IFN-γ has been shown to induce both autophagy and apoptosis, with autophagy initially depleting cancer cell resources, followed by apoptosis, enhancing tumor suppression (Wang et al., 2018). These studies underscore the dual role of autocrine IFN-γ in regulating tumor cell fate. In our research, we demonstrated that ARV-induced IFN-γ enhances autophagy-related protein LC3-II expression in B16-F10 cells through the JAK-STAT1 pathway, although apoptosis was not directly mediated by IFN-γ.

This study revealed that ARV effectively activates cytoplasmic TLR3 in B16-F10 murine melanoma cells via its σC protein, leading to the activation of the TLR3-IRF3-NF-κB signaling axis and the subsequent induction of autophagy through IFN-γ-JAK-STAT1 signaling (Fig. 8). These findings highlight ARV's potential as an oncolytic virus and provide mechanistic insights into its ability to induce tumor cell autophagy. While TLR3 activation was explored in this study, apoptosis induction appeared to be mediated through alternative signaling pathways independent of TLR3 and IFN-γ. Further research is warranted to elucidate these pathways, including the potential involvement of proteins such as p53, FasL, and Bcl-2 family members. Additionally,

evaluating ARV's efficacy in other cancer cell types will help determine its broader applicability and therapeutic potential. To optimize ARV as an oncolytic virus, future studies should focus on enhancing its selectivity and safety through genetic engineering, such as targeting tumor-specific markers. Overall, this study lays the groundwork for developing ARV as a novel therapeutic approach for challenging cancer types, emphasizing its role in augmenting endogenous antitumor immunity.


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Zheng, X., Li, S., Yang, H., 2021. Roles of toll-like receptor 3 in human tumors. Front Immunol 12, 667454.

525 Table 1. The catalog numbers and dilution factor of the respective antibodies used in

526 this study

| Antibodies | Catalog numbers | Clone name | Dilution factor | Manufacture |
|---|---|---|---|---|
| Mouse anti-σC* | - | - | 4000 | Our laboratory |
| Rabbit anti-TLR3* | SBP3643 | - | 2000 | Sigma-aldrich |
| Rabbit anti-IRF3 | 4302 | D83B9 | 2000 | Cell Signaling |
| Rabbit anti-p-IRF3 (S379) | 79945 | E6F7Q | 2000 | Cell Signaling |
| Rabbit anti-p65 | 4764 | C22B4 | 2000 | Cell Signaling |
| Rabbit anti-p50 | 13586 | D4P4D | 2000 | Cell Signaling |
| Rabbit anti-Histone H2A* | 2578 | - | 2000 | Cell Signaling |
| Mouse anti-GAPDH | 8245 | | 10000 | Abcam Co |
| Rabbit anti-Cleaved caspase 3 | 9664 | 5A1E | 2000 | Cell Signaling |
| Rabbit anti-STAT1* | 9172 | - | 2000 | Cell Signaling |
| Rabbit anti-p-STAT1 (Tyr701) | 9167 | 58D6 | 2000 | Cell Signaling |
| Rabbit anti-Na, K ATPase* | 3010 | - | 2000 | Cell Signaling |
| Mouse anti-LC3-II | 83506 | E5Q2K | 2000 | Cell Signaling |
| Mouse anti-β-actin | MAB1501 | C4 | 10000 | Millipore |
| Goat anti-mouse IgG (H+L) HRP | 5220-0341 | - | 5000 | SeraCare |
| Goat anti-rabbit IgG (H+L) HRP | 5220-0336 | - | 5000 | SeraCare |

527 *Polyclonal antibodies

528

529

530 Table 2. shRNAs used in this study

| | Cat. No. | Sequence (5′-3′) |
|---|---|---|
| STAT1 | TRCN0000054926 | GCTGTTACTTTCCCAGATATT |
| IRF3 | TRCN0000085241 | CGAAGTTATTTGATGGCCTGA |

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535 Table 3. Primers used in this study for amplification of the respective targeted genes

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| DR4 | TGGCACACAGCAATGGGAACATAG | GAAACACACCCTGTCCATGCACTT |
| DR5 | CAGGTGTCAACATGTTGTCC | ATCGAAGCACTGTCTCAGAG |
| PD-L1 | GCTGTTGAAGGACCAGCTCT | TGGAGGATGTGCCAGAGGTA |
| TLR3 | GCATTTGTTTTCTCACTCTTT | TTAGCCACTGAAAAGAAAAAT |
| TLR4 | CGAGGAAGAGAAGACACCAGT | CATCATCCTCACTGCTTCTGT |
| TLR7 | AAACTCCTTGGGGCTAGATG | AGGGTGAGGTTCGTGGTGTT |
| TLR8 | CTGTGAGTTATGCGCCGAAGA | TGGTGCTGTACATTGGGGTTG |
| TLR9 | CGCCCTGCACCCGCTGTCTCT | CGGGGTGCTGCCATGGAGAAG |
| IL-2 | TGGAGCATCGCTATCACCAG | TTGCTGACTGCACTCCTTGA |
| IL-4 | TTCCTGTGGCAAGATGAACG | CTGCAGGTTCTTGTGGCAGT |
| IL-5 | TGACGAAAGCTGCATCAAAA | CTCTTGCCAGGTTTGCTGTG |
| IL-6 | GCTTCGACGAGGAGAAATGC | AGCACAGCGATTCGACATTC |
| IL-7 | CCTTGTTCTGTTGCCAGTAGC | CCAATTTCTTTCATGCTGTCC |
| IL-12 | TCCAAAGACTGGGCCAAAAG | CTCCAGCAGCAGAAGGCTCT |
| IFN-α | GCCTCCTCAACCAGATCCAG | TGATGGTGAGGTGAGGGTTG |
| IFN-β | CCGTTCTGGAAAGCAAGGAC | GTGTGCGTGGTCAATCCAGT |
| IFN-γ | ACCTGGCCAAGCTTCAGATG | TGGCTCCTTTTCCTTTTGGA |

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539 **Figure legend**

Fig 1. ARV infection upregulates TLRs, interferons, and cytokines in B16-F10 cells. (A–B) Cells were infected with ARV (MOI 10), and lysates were collected at 24 and 48 hpi for qRT-PCR analysis of TLRs, interferons, and cytokines mRNA levels. Data are presented as mean ± SD from three independent experiments. *P<0.05 **p<0.01

Fig 2. Western blot analysis of TLR3, IRF3, apoptotic, autophagic, and NF-κB signaling pathways following infection. (A) Western blot analysis of TLR3 and phosphorylated IRF3 at 24 and 48 hpi. (C) Western blot analysis of cleaved caspase-3, LC3-II, and phosphorylated STAT1 at 24 and 48 hpi. (E) Subcellular fractionation and Western blot analysis of NF-κB p65 and p50 at 24 hpi. Protein quantification was performed using ImageJ, normalized to β-actin or GAPDH. (B, D, F) Signals in all Western blots were quantified using Image J software and normalized to mock-treated β-actin. Values for mock-treated cells were considered 1-fold. Each value represents mean± SE from three independent experiments, determined by Duncan's Multiple Range Test. Similar alphabets (a, b, c) denote no significance at p<0.05. In this work, all original blots and images are shown in Fig. S1-S2.

Fig 3. Interaction between ARV σC and cytoplasmic TLR3 in B16-F10 cells. (A) Western blot analysis of TLR3 in cytoplasmic and membrane fractions at 24 hpi, normalized to Na/K ATPase or GAPDH. (B) Proximity ligation assay (PLA) detecting σC-TLR3 interaction. Red fluorescence indicates interaction; blue represents DAPI nuclear staining (scale bar, 20 μm). Data are presented as mean ± SD from three independent experiments.

Fig 4. ARV regulates autophagy, apoptosis, IRF3 phosphorylation, and NF-κB via

TLR3. (A, C) Cells were pretreated with TLR3 inhibitor (CU-CPT 4a, 27 μM) for 6 h, then infected with ARV (MOI 10). Western blot analysis of cleaved caspase-3, LC3-II, phosphorylated IRF3, and NF-κB at 24 hpi. Data were quantified using ImageJ, normalized to β-actin, GAPDH, or Histone H2A. Mean ± SD from three independent experiments. (B, D) Signals in all Western blots in A-B were quantified using Image J software and normalized to mock-treated β-actin. Values for mock-treated cells were considered 1-fold. Each value represents mean± SE from three independent experiments, determined by Duncan's Multiple Range Test. Similar alphabets (a, b, c) denote no significance at $p<0.05$.

Fig 5. ARV regulates IFN-γ via the TLR3-IRF3-NF-κB pathway. (A) IFN-γ levels in culture supernatants at 12, 24, and 48 hpi, measured using an ELISA kit. (B) Cells were pretreated with TLR3 inhibitor (27 μM), NF-κB inhibitor (10 μM), or transfected with IRF3 shRNA, followed by ARV infection (MOI 10). IFN-γ levels in culture supernatants at 24 hpi were quantified by ELISA. Statistical significance: ***$p < 0.001$; ****$p < 0.0001$ (Student's t-test). Mean ± SD from three independent experiments.

Fig 6. ARV regulates autophagy via the IFN-γ-STAT1-JAK pathway. (A) Cells were transfected with scramble or STAT1-shRNA plasmids, then infected with ARV (MOI 10) for 24 h. Western blot analysis of cleaved caspase-3, LC3-II, and phosphorylated STAT1. (C) Cells were treated with JAK1 inhibitor (Upadacitinib, 50 nM) for 6 h, then infected with ARV (MOI 10) for 24 h. Western blot analysis of cleaved caspase-3, LC3-II, and phosphorylated STAT1. Protein quantification was performed using ImageJ, normalized to β-actin. Mean ± SD from three independent experiments. (B, D) Signals

588 in all Western blots were quantified using Image J software and normalized to mock-
589 treated β-actin. Values for mock-treated cells were considered 1-fold.
590
591 Fig 7. Fluorescence microscopy analysis of LC3-mCherry puncta and nuclear staining
592 in JAK1-inhibited and STAT1-knockdown B16-F10 cells following ARV infection (A)
593 B16-F10 cells were seeded in 6-well plates and treated with 50 nM JAK1 inhibitor
594 (Upadacitinib) or transfected with pLKO.-puro, scramble, pLKO.-puro-STAT1-shRNA,
595 and LC3-mCherry plasmids. After 6 hours, cells were infected with ARV (MOI 10) and
596 incubated for 24 hours. Fluorescence signals were observed using an inverted
597 fluorescence microscope (scale bar, 10 μm). Red fluorescence indicates mCherry
598 puncta, and blue fluorescence represents DAPI nuclear staining. (B) Signals in all
599 image in A were quantified using Image J software. Each value represents mean± SE
600 from three independent experiments, determined by Duncan's Multiple Range Test.
601 Similar alphabets (a, b, c) denote no significance at $p<0.05$.
602
603 Fig 8. Schematic model of ARV-induced autophagy via the TLR3-IRF3-NF-κB -IFN-γ
604 pathway. The ARV σC protein interacts with cytoplasmic TLR3 in B16-F10 cells,
605 activating the TLR3-IRF3-NF-κB pathway and inducing IFN-γ production. IFN-γ
606 subsequently triggers autophagy through the IFN-γ-JAK-STAT1 pathway.