Macrophages Loaded *Newcastle Disease Virus* Enhance Anti-Tumor Efficacy of Oncolytic Newcastle Disease Virus Lasota Strain in Mouse Model of Cervical Cancer

Aezam Rasekhi Kazeruni[1], Nahid Babaei[1*], Hadi Esmaeili Gouvarchin Ghaleh[2], Abbas Doosti[3], Mahdieh Farzanehpour[2]

[1]Department of Molecular Cell Biology and Genetics, Bushehr Branch, Islamic Azad University, Bushehr, Iran.

[2]Applied Virology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical sciences, Tehran, Iran.

[3]Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Corresponding authors: Nahid Babaei (Nahid.babaei@iau.ac.ir)

Abstract

Today, cervical cancer (CC) is one of the most common cancers in women. Oncolytic viruses (OVs), especially those from non-human hosts, have anti-cancer properties but are rapidly eliminated by the immune system. This study aims to investigate the effect of macrophages (MQ) loaded with Newcastle disease virus (NDV) on their anti-tumor efficacy. In this study, TC1 cells were introduced into female C57BL/6 mice (6 to 8 weeks, n = 40) to induce tumor formation. After tumors developed, the mice were divided into four treatment groups. Three groups were treated with NDV, Doxo, and B.MQ-NDV, while the control group received PBS. After the last treatment, half of the mice were euthanized to evaluate the immune response, and the remaining half were observed until they naturally passed away. The findings revealed that mice treated with B.MQ-NDV had better survival rates and slower tumor growth compared to the control group.

B.MQ-NDV treatment increased the production of nitric oxide (NO) and lactate dehydrogenase (LDH), while elevating levels of cytokines IFN-γ, TNF-α, and IL-12, and decreasing IL-4 and TGF-β levels. Additionally, Bax and p53 expression increased in NDV, B.MQ.NDV, and Doxo groups. The findings suggest that the use of NDV loaded with MQs can improve the effectiveness of NDV in a mouse model of cervical cancer. This indicates that this strategy may provide an additional treatment option for cervical cancer.

Keywords: Cellular carriers, Cervical cancer, Macrophage, *Newcastle disease virus*, Oncolytic virus

1. Introduction

Cervical cancer (CC) is the fourth most common type of cancer worldwide and the fourth leading cause of cancer-related deaths among women. Over half of those diagnosed do not survive. The main cause is the Human Papillomavirus (HPV), transmitted through sexual contact, which can lead to cancer (1). CC can be treated through various methods, including cryotherapy, laser therapy, ring electrosurgery, antiviral drugs like interferons and podophyllin, immunomodulatory drugs, chemotherapy, and immunotherapy. However, due to the complex nature of the disease and its tendency to recur, a definitive treatment has not yet been found (2). Using products of the immune system to fight cancer and diseases has recently become a promising approach to cancer treatment due to its effectiveness in overcoming the immune evasion tactics used by cancer cells. One of the methods utilized in immunotherapy is oncolytic viruses (OVs). OVs specifically target and infect tumor cells, which helps suppress tumor growth while sparing normal cells. Additionally, OVs can stimulate the host's immune system, leading to an immune response that not only has antitumor effects but also helps limit the activity of the virus itself. Immunotherapy using OVs offers several significant advantages over conventional tumor immunotherapy, such as enhanced targeting and lethality, reduced side effects, and decreased drug resistance. The approach also allows for genetic modifications of the viruses to improve their effectiveness against tumors. This can involve altering the virus to specifically target certain tumors and incorporating genes that suppress tumor growth and induce inflammation (3). By enhancing the expression of antigens that the virus can bind to tumor cells, OVs can more effectively infect and eliminate these tumor cells. This process causes the virus to be released into nearby infected cells, increasing the overall destruction of tumor cells. Additionally, when OVs enter the tumor

environment, phagocytosis and antigen presentation by MQs can create a synergistic effect that further inhibits tumor growth. By adjusting the number of MQs, OVs can be encouraged to display anti-tumor activity. MQs can also eliminate OV particles through the secretion of interferon type I (IFN-I) and by utilizing phagocytosis. OVs boost the body's ability to combat tumors by activating the antiviral immune response. Upon entering the system, the immune system releases antiviral cytokines like IFNs, TNF-α, and IL-12, which collectively trigger an antiviral response (4). One notable OV is NDV, which possesses unique characteristics that enhance its efficacy in cancer treatment. A significant study conducted in 1992 at the University of Illinois, USA, demonstrated that NDV can selectively replicate within human cancer cells, leading to cancer cell death while sparing normal body cells. This selective targeting makes NDV a promising candidate for cancer therapy. The impact of the NDV-73T strain on various human cancer cells has been studied, revealing that this virus can effectively replicate in bone tumor cells, CC, bladder cancer, cerebral cortex cancer, and several other types of cancer. Importantly, oncolytic viruses like NDV do not harm normal cells, highlighting their potential for targeted cancer therapy (5). When it comes to treating systemic metastatic tumors, intravenous injection is a widely used method. However, this approach has its limitations, primarily due to various factors that hinder the injected OVs from reaching the tumor site. These factors include the formation of immune complexes and the presence of antibodies, which can obstruct the effectiveness of the treatment. One of the significant challenges in using naked viruses for cancer treatment is the host immune system, which can reduce the therapeutic effect. To overcome this issue, researchers have proposed using cellular carriers. Additionally, utilizing cells that naturally migrate within the tumor microenvironment can enhance treatment efficacy (6). To address this challenge, MQs can serve as carriers for OVs, facilitating their transport to tumor sites. Research has demonstrated that monocytes and macrophages present in peripheral blood can effectively transport live viral particles to tumors. This unique capability of MQs to transport viruses can be leveraged as a treatment approach for cancer therapy, potentially enhancing the delivery and efficacy of OVs therapies. This study seeks to investigate the therapeutic benefits of MQs loaded with NDV to enhance the anti-tumor efficacy of oncolytic NDV in a mouse model of CC.

2. Materials and Methods

2.1. Cell Lines and Mice

TC-1 cells were sourced from the Pasteur Institute of Iran in Tehran and were cultured in DMEM-F12 medium enriched with 10% fetal bovine serum (FBS) under optimal conditions of 37°C, 5% CO2, and humidity. Female C57BL/6 mice, aged between 6 to 8 weeks and weighing 20 to 25 grams, were also obtained from the same institute. The Newcastle disease virus, Lasota strain, was obtained with a specific title from the Virology Research Center of Baqiyatallah University of Medical Sciences. The experimental procedures received approval from the Animal Ethics Committee of Bushehr University, in accordance with the guidelines established by the Iranian Ministry of Health (IR.BPUMS.REC.1401.209).

2.2.Experimental Design

Prior to the subcutaneous injection of 1×10^5 live tumor cells into the mice, they were acclimatized for one week. After tumor establishment, the mice were randomly assigned to four groups, each containing ten mice. The first group acted as the control and received 100 µl of PBS. The second group was administered 10^8 PFU of NDV in 100 µl. The third group received 10^5 NDV-infected bone marrow macrophages (B. MQs-NDV) along with TC-1 tumor cell lysate, also delivered in 100 µl. The fourth group was treated with 10 mg/kg of Doxorubicin (Doxo) weekly. Tumor growth was closely monitored, and tumor volume was calculated using a specific formula (Height × Width × Length × 0.5236). One week after the last treatment, half of the mice were euthanized via intraperitoneal injection of a mixture of Ketamine and Xylazine for thorough assessment of their immune responses.

2.3.Preparation of B. MQs

To produce B. MQs, we followed the procedure described by Zhang et al. in their 2019 research. This involved harvesting bone marrow cells from the femur, filtering them through a cell strainer, and suspending them in a DMEM solution containing 10% fetal bovine serum (FBS) and 50 ng/ml macrophage colony-stimulating factor (M-CSF). The cells were then cultured for 7 days at 37 °C in a 5% CO2 environment, without any media changes. The identity of the cells was verified by measuring cytokine levels.

2.4.Investigating the cytotoxic effects of Lasota strain NDV on MQs

The cells were exposed to various multiplicities of infection (MOIs) of 1, 5, 10, 20, and 40 NDV for 1 hour in DMEM-F12. After incubation, the viral supernatant was removed and replaced with

DMEM culture medium containing 1% FBS. Following an additional 72 hours of incubation, 20 µl of MTT solution (5 mg/ml) was added to each well, and the cells were incubated at 37 °C for another 4 hours. Subsequently, the culture medium was discarded, and 150 µl of DMSO was added to dissolve the purple formazan crystals. The optical density of the samples was then measured at a wavelength of 550 nm.

2.5.Adjacent MQs with tumor cell lysates and NDV

The MQs produced from the previous step were treated with 100 µl of tumor cell lysate (20 µg/ml) and *Newcastle oncolytic virus* (MOI 5) for 72 hr. After the treatment, the MQs were trypsinized and counted for further use in subsequent experiments.

2.6.Preparation of splenocyte culture, investigation of proliferation index and measurement of cytokines in splenocyte culture supernatant

Seven days after the last injection, half of the mice were anesthetized, and splenocytes were harvested using sterile techniques and mixed with a medium containing 10% FBS. At this point, red blood cells were removed using RBC lysis buffer (ACK). The resulting cell suspension, containing 2×10^6 cells/ml, was combined with tumor antigen obtained through freezing and thawing (20 µg/ml) and placed in a 24-well plate. After 72 hours, the culture supernatants were collected and analyzed for the presence of IFN-γ, IL-4, IL-12, TNF-α, and TGF-β using the ELISA method, following the protocols provided by Zell Bio GmbH, Germany. This procedure was carried out to evaluate immune responses and cytokine production under controlled conditions.

2.7.Proliferation index (PI)

After counting the cells, 10^5 cells were added to each well of a 96-well plate in 100 µl and cultured for 72 hours in a CO2 incubator with 5% CO2 and 20 µg/ml of tumor cell lysis. For each sample, three replicates with and without antigen were included, and three empty wells served as negative controls. Following incubation, 20 µl of MTT solution (5 mg/ml) was added to each well and left for 4 hours. The viable cells reduced MTT, forming formazan crystals, which were then dissolved by adding 150 µl of DMSO. The color intensity was measured at a wavelength of 550 nm.

2.8.LDH measurement

Cellular toxicity was evaluated using the LDH kit from Takara and a colorimetric method. This assay leverages the stability of the LDH enzyme found in various cell types. After washing the effector cells (spleen cells) and target cells (TC1 cell line) with RPMI-1640 medium, they were co-cultured at a 50:1 ratio for 6 hours at 37 °C. The supernatants were then centrifuged and transferred to flat-bottom plates. To initiate the LDH detection process, 100 µl of the LDH reagent was added to each well and incubated for 30 minutes at 25 °C. The optical density was measured at a wavelength of 490 nm. The degree of cytotoxicity was calculated based on the results, providing insights into cell damage and treatment effectiveness.

Cytotoxicity (%) = (experimental release–spontaneous target release–spontaneous effector release) / (maximal target release–spontaneous target release) × 100%.

2.9.NO measurement

The production of NO by spleen cells was assessed by measuring NO levels in the culture supernatant using the Natrix™ kit. Cell-free supernatants were mixed with Griess reagent and incubated in the dark for 10 minutes. Absorbance was measured at a wavelength of 540 nm using an ELISA reader.

2.10. RNA extraction

To isolate RNA from tumor tissue, 1 g of the sample was collected and ground into a fine powder using liquid nitrogen. This powder was mixed with 1 ml of RNX-plus in a microtube to create a homogeneous solution. Following this, 250 µl of chloroform was added, and the mixture was shaken vigorously before centrifuging at 13,000 rpm for 20 minutes. The resulting clear phase was transferred to a new microtube, and an equal volume of isopropanol was added, followed by another centrifugation at the same speed. After discarding the supernatant, 700 µl of 70% ethanol was added, and the mixture was centrifuged again. The supernatant was removed, and the RNA pellet was allowed to air-dry for 5 minutes. Finally, 50 µl of DEPC-treated water was added, and the mixture was incubated at 55 °C for 10 minutes to dissolve the RNA. The quality and quantity of the extracted RNA were assessed using a Nanodrop spectrophotometer. To eliminate any potential contamination from genomic DNA, a DNase treatment was performed, enhancing the RNA's purity for subsequent analyses. This extraction method is crucial for obtaining high-quality RNA.

2.11.cDNA synthesis

cDNA synthesis was carried out using the AddScript cDNA synthesis kit from Adbio-Kore. In a 0.2 ml microtube, 1 µg of RNA was mixed with 1 µl of reverse transcriptase (RT), 10 µl of reaction buffer, 1 µl of oligodT primer, 1 µl of random hexamers, and 2 µl of dNTPs. The total volume was adjusted to 20 µl with DEPC-treated water. The reaction was incubated at 25 °C for 10 minutes, followed by 1 hour at 50 °C, and concluded with a 5-minute incubation at 85 °C to stop the reaction. The resulting cDNA was stored at -70 °C for later use. This synthesis process is essential for converting RNA into complementary DNA, facilitating subsequent applications like quantitative PCR and gene expression analysis. The careful choice of primers and precise incubation conditions ensure high efficiency and specificity in cDNA production, setting the stage for dependable molecular investigations.

2.12.Real-time PCR

Real-time PCR was conducted to evaluate the relative expression levels of the Bax, Bcl-2, p53, and Mmp2 genes. β-actin was utilized as a normalization control, and a no-template control (NTC) was included in all runs to eliminate any potential contamination. Each PCR experiment was performed in duplicate. In each microtube, a mixture was prepared containing 1 µl of cDNA, 1 µl of forward primers, 1 µl of reverse primers (as detailed in Table 1A and B), and 10 µl of 2X Prime Q-Master Mix with SYBR Green (Ampliqon-Denmark). After an initial heating step at 95 °C for 15 minutes, 40 cycles of three amplification phases were carried out.

Table 1A. Primer sequences, annealing temperatures, and PCR product size was showed in the (5′-3′) Direction.

Accession number	Genes		Primer sequences (5′-3′)	Length (bp)	Annealing temp (°C)	Product size (bp)
NM_017059.2	*Bax*	F	CAGCGGCAGTGATGGAC	17	57	109
		R	TCCTGGATGAAACCCTGTAG	20		

NM_016993.2	*Bcl-2*	F	GAGTACCTGAACCGGCATCT	20	58	102
		R	GAAATCAAACAGAGGTCGCA	20		

194

195 **Table 1B.** Primer sequences, annealing temperatures, and PCR product size was showed in the
196 (5′-3′) Direction.

Accession number	Genes		Primer sequences (5′-3′)	Length (bp)	Annealing temp (°C)	Product size (bp)
NM_001429996	*p53*	F	AGGGAGTGCAAAGAGAGCAC	20	59	137
		R	CCTCATTCAGCTCTCGGAAC	20		
NM_031144.3	*β-actin*	F	TAAGGCCAACCGTGAAAAGA	20	58	109
		R	GGTACGACCAGAGGCATACA	20		
NM_031054.2	*Mmp2*	F	AGAAGGCTGTGTTCTTCGCA	20	60	128
		R	AAAGGCAGCGTCTACTTGCT	21		

197

198 **2.13.Statistical analysis**

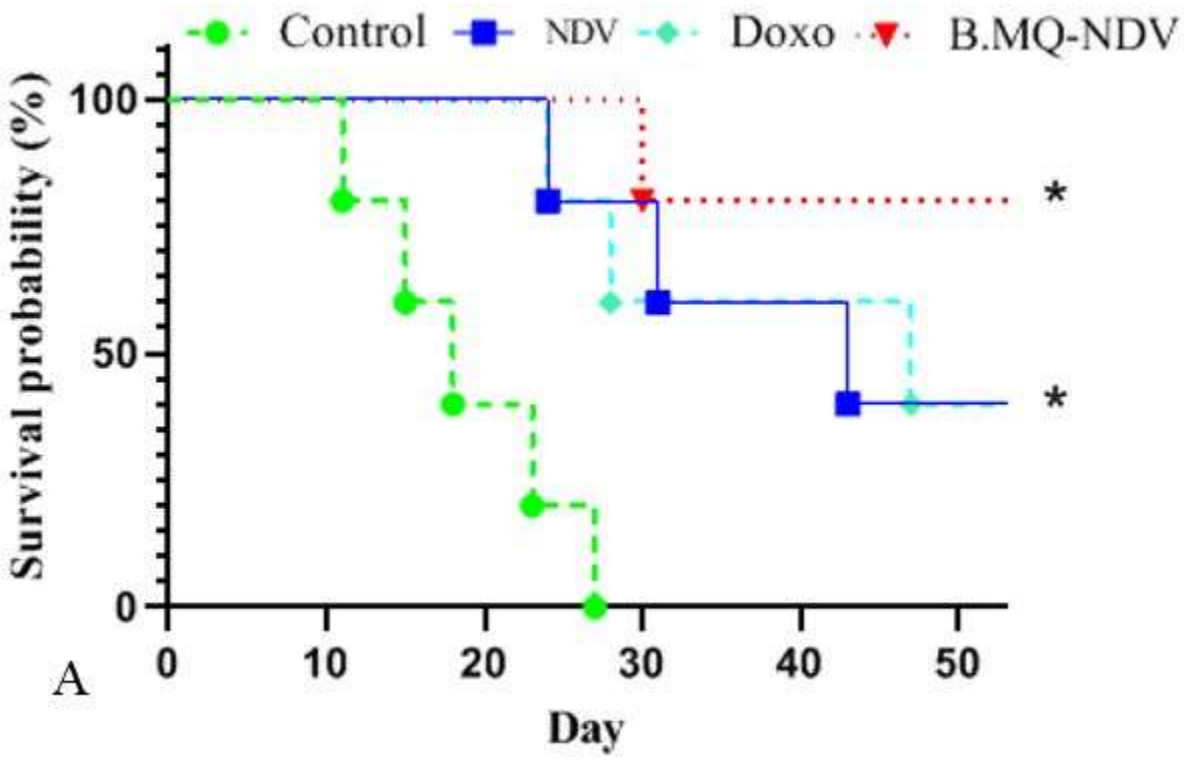
199 This research presented quantitative findings that included means and standard deviations,
200 utilizing ANOVA alongside LSD post-hoc tests for group comparisons. The statistical analysis
201 and table creation were conducted with SPSS version 19, whereas the figures were generated

using GraphPad version 8.2.1. To evaluate survival functions based on lifespan data, the Kaplan-Meier estimator was employed, with significance set at $P<0.05$. Furthermore, the analysis of relative gene expression was carried out using the Relative Expression Software Tool (REST) version 2.0.13.

3. Results

3.1. Survival probability and Tumor size

The results showed that mice treated with B. MQs-NDV had a higher survival rate than those in the control, NDV, and Doxo groups ($P<0.05$), with the B.MQ-NDV group exhibiting the highest survival rate. Additionally, mice receiving B. MQ-NDV experienced slower tumor growth compared to the other groups, and tumor size significantly decreased in all treatment groups compared to the control group ($P<0.05$). Both Doxo and B.MQ-NDV demonstrated similar efficacy in reducing tumor volume.



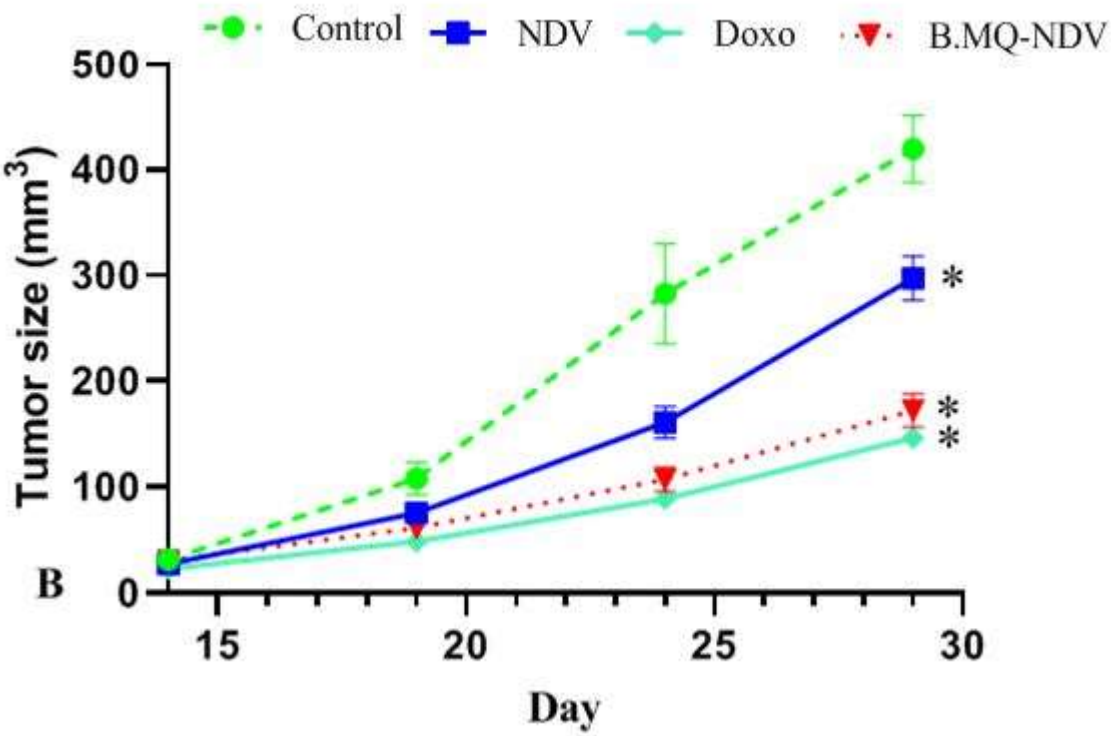


Figure 1. Effects of the oncolytic treatment on (A) survival probability and (B) tumor size in the CC mouse model (* indicates $P<0.05$).

3.2.Cytotoxic effects of NDV on B. MQs

As shown in **Figure 2**, NDV exhibited cytotoxic effects on cells at concentrations of 10 ($P<0.05$), 20 ($P<0.001$), and 40 ($P<0.001$) MOIs compared to the control group. In contrast, no cytotoxic effects were observed at concentrations of 1 and 5 MOIs ($P>0.05$) compared to the control group. Consequently, the concentration of 5 MOI was selected as a non-cytotoxic and effective dose for further evaluations. These findings highlight the dose-dependent nature of NDV's cytotoxicity, indicating that higher concentrations significantly impact cell viability, while lower concentrations remain safe for cellular assays. This selection of the 5 MOI concentration allows for continued investigation into the therapeutic potential of NDV without compromising cell health.

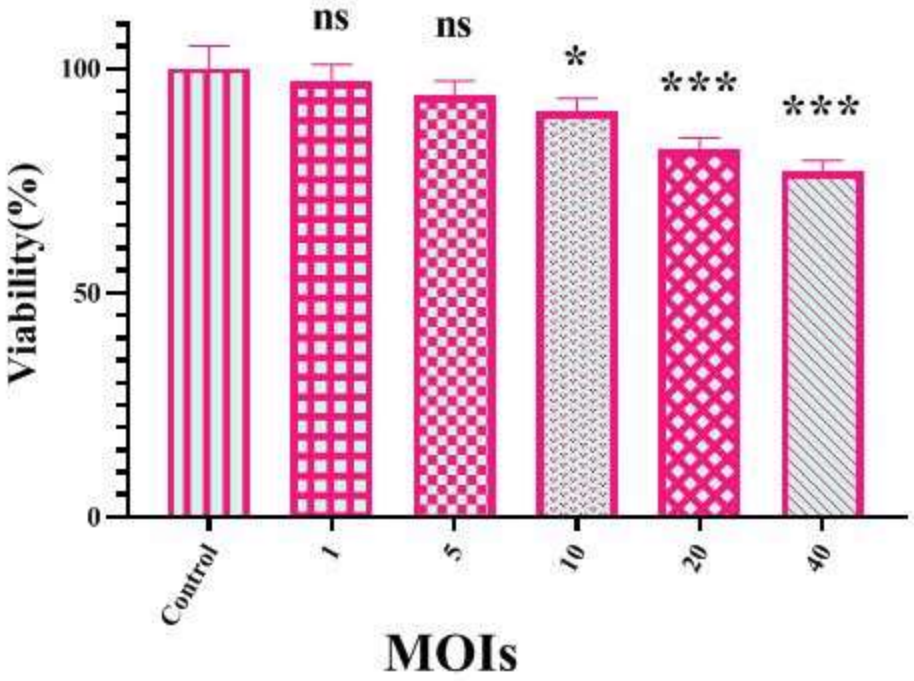
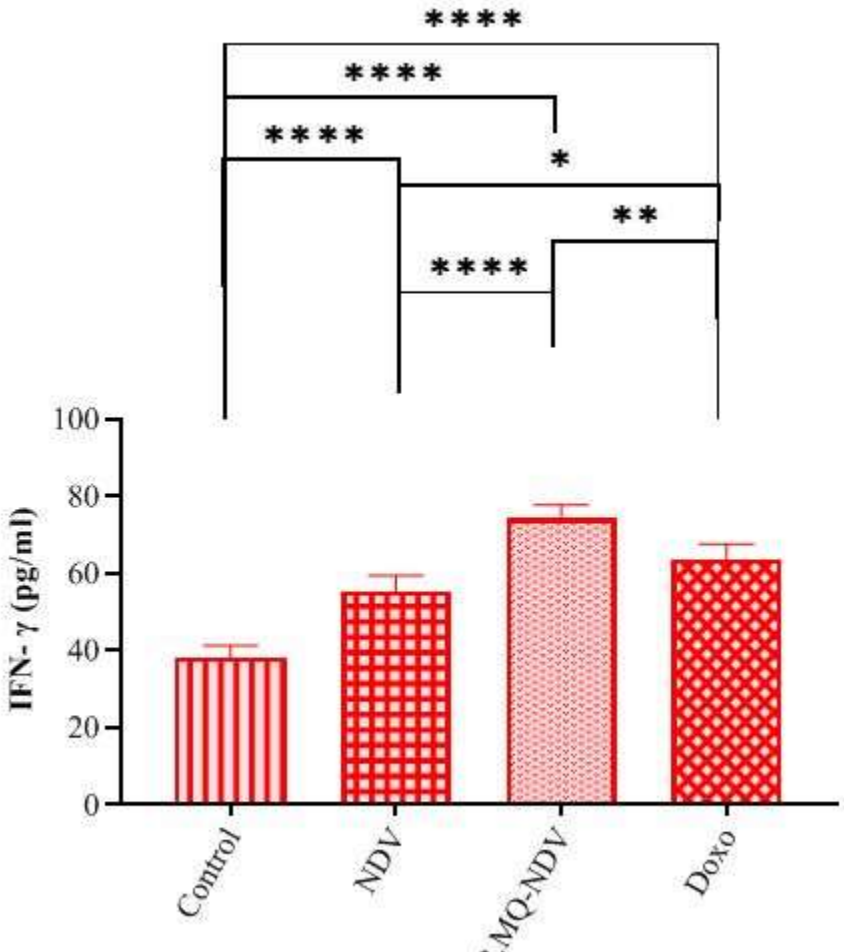


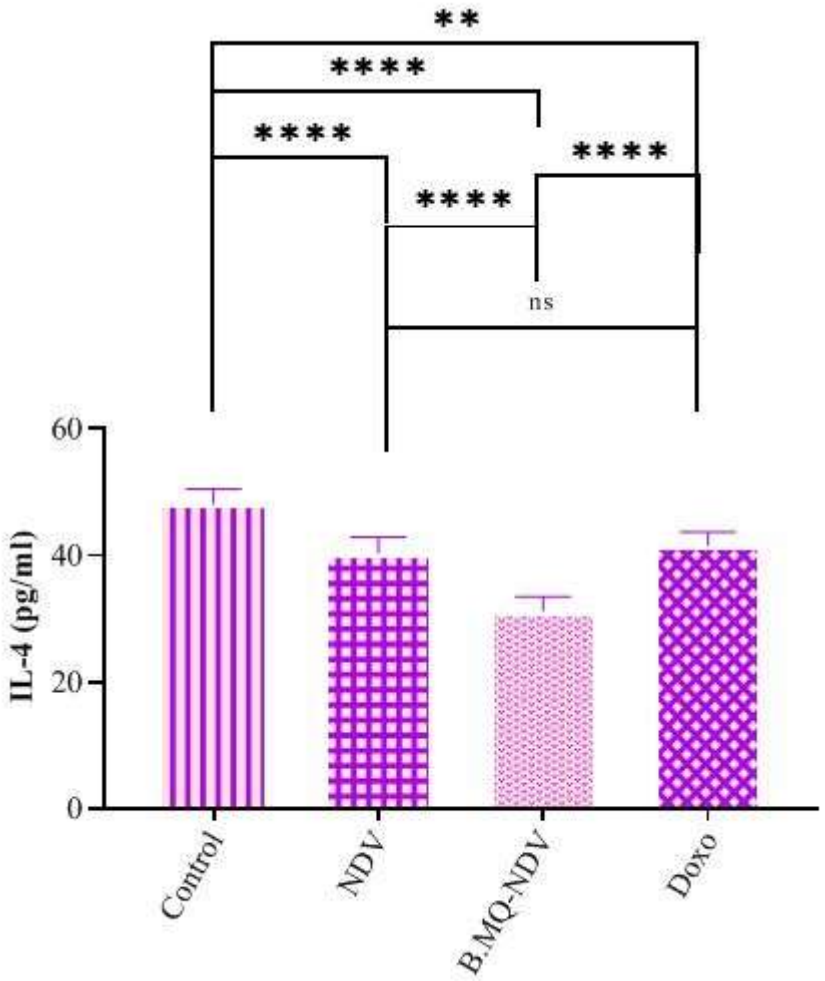
Figure 2. Effects of the NDV on B. MQs viability (* indicates *P*<0.05, ** indicates *P*<0.01, *** indicates *P*<0.001, **** indicates *P*< 0.0001, ns indicates non-significance).

3.3.Cytokine assay

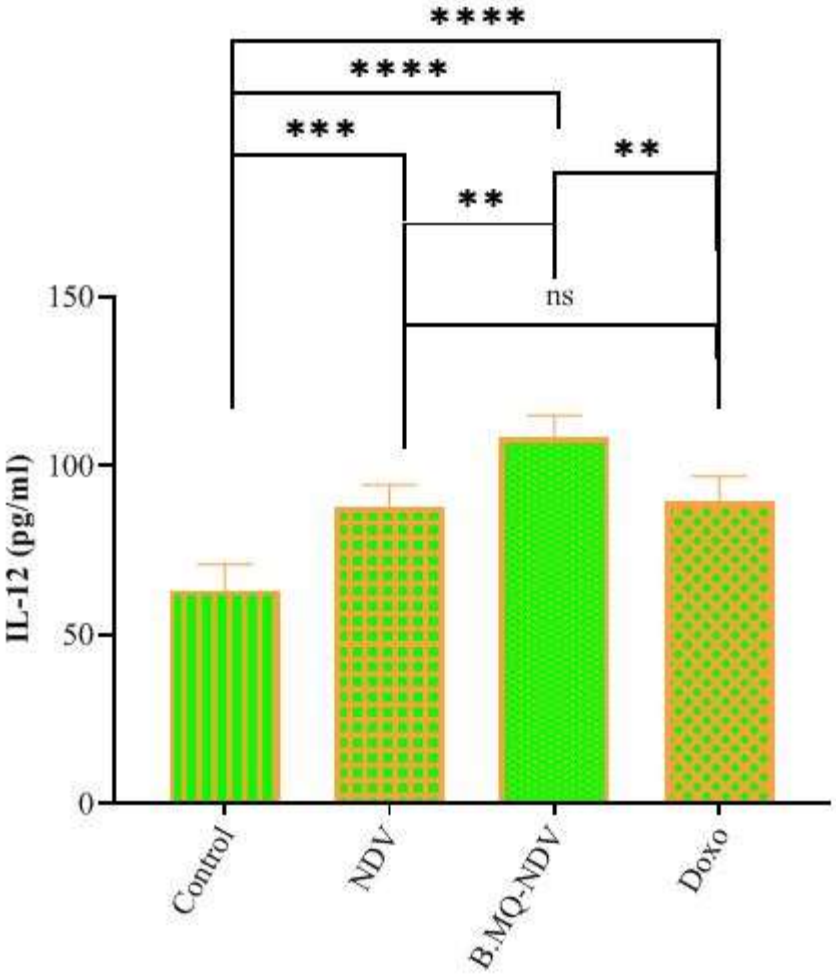
The cytokine assay results indicated that the levels of IL-12, IFN-γ, and TNF-α were significantly elevated in all treatment groups compared to the control group (P<0.05), with the B.MQ-NDV group showing the highest concentrations (Figure 3). Additionally, the treatment groups exhibited a significant reduction in IL-4 and TGF-β levels compared to the control group (P<0.05), with the B.MQ-NDV group having the lowest levels.



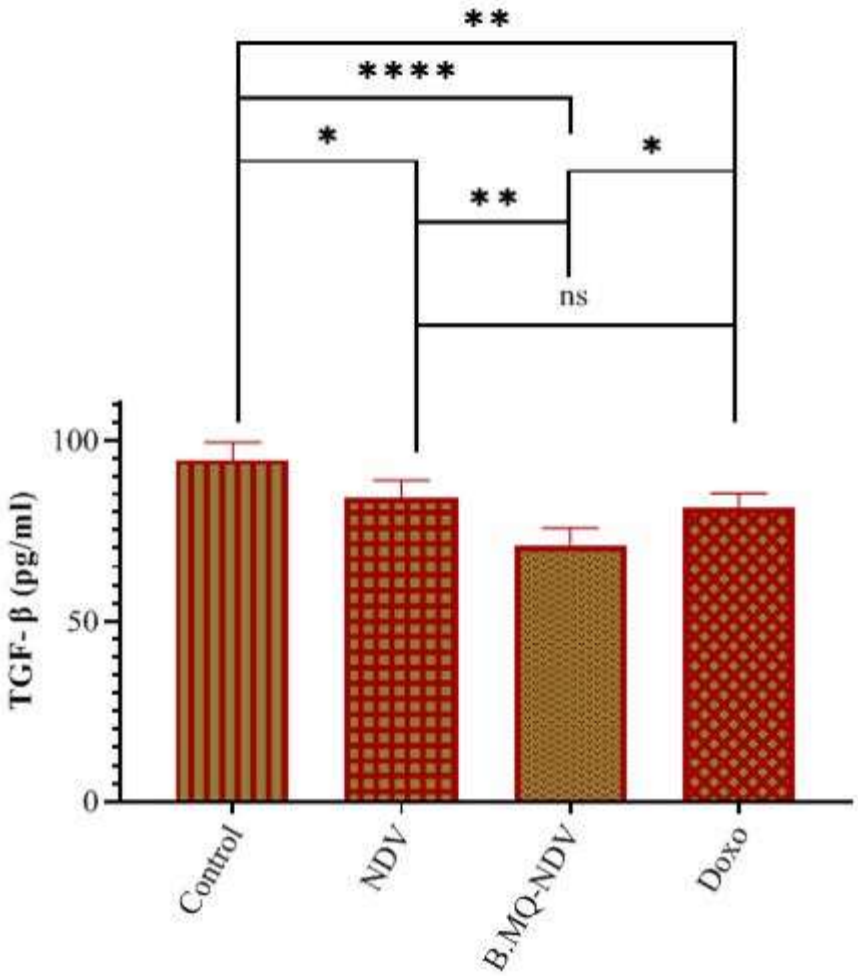
237



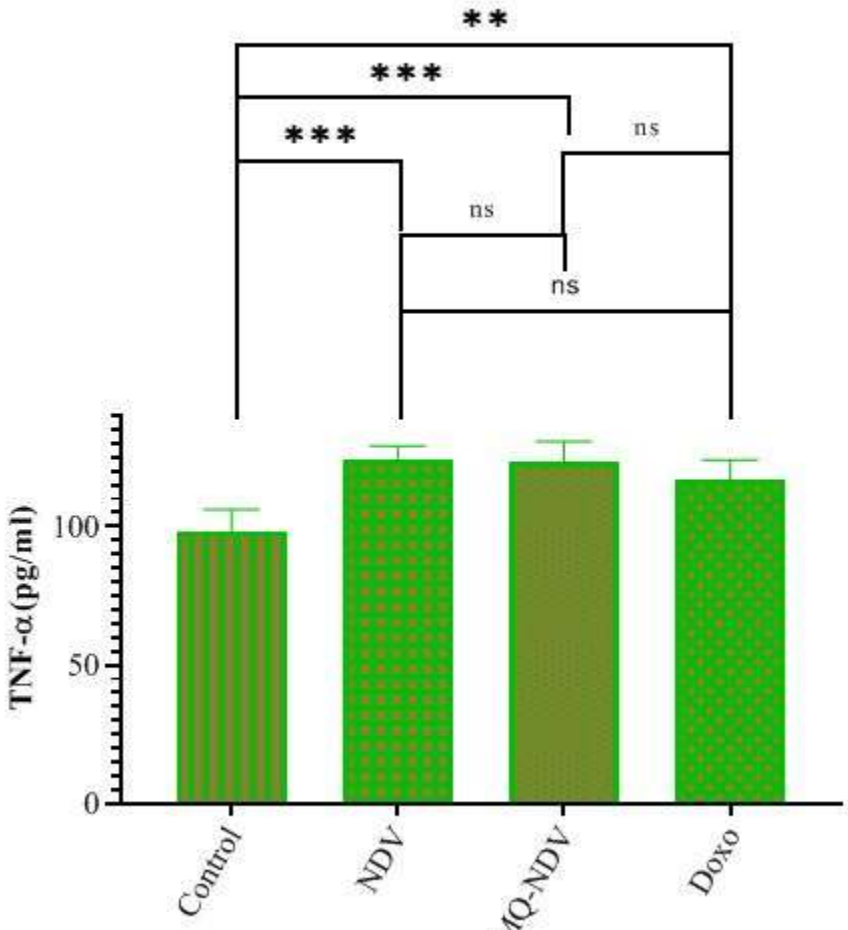
238



239



240



241

242 **Figure 3.** Effects of the oncolytic treatment on production of cytokines TGF-β, IL-4, IL-12, IFN-
243 γ, and TNF-α in the CC mouse model (* indicates $P<0.05$, ** indicates $P<0.01$, *** indicates
244 $P<0.001$, **** indicates $P< 0.0001$, ns indicates non-significance).

245 **3.4.Proliferation index of splenocytes**

246 After measuring the cellular proliferation index in the spleen, the results showed that the lowest
247 level of proliferation was observed in the control and Doxo groups. All treatment groups exhibited
248 an increase in proliferation levels, particularly in the B.MQ-NDV and NDV groups, which was
249 statistically significant (P<0.0001). The average proliferation index of splenocytes was highest in
250 the B.MQ-NDV group and lowest in the control and Doxo groups. A statistically significant
251 difference was observed between the NDV and Doxo groups (P<0.001) (Figure 4).

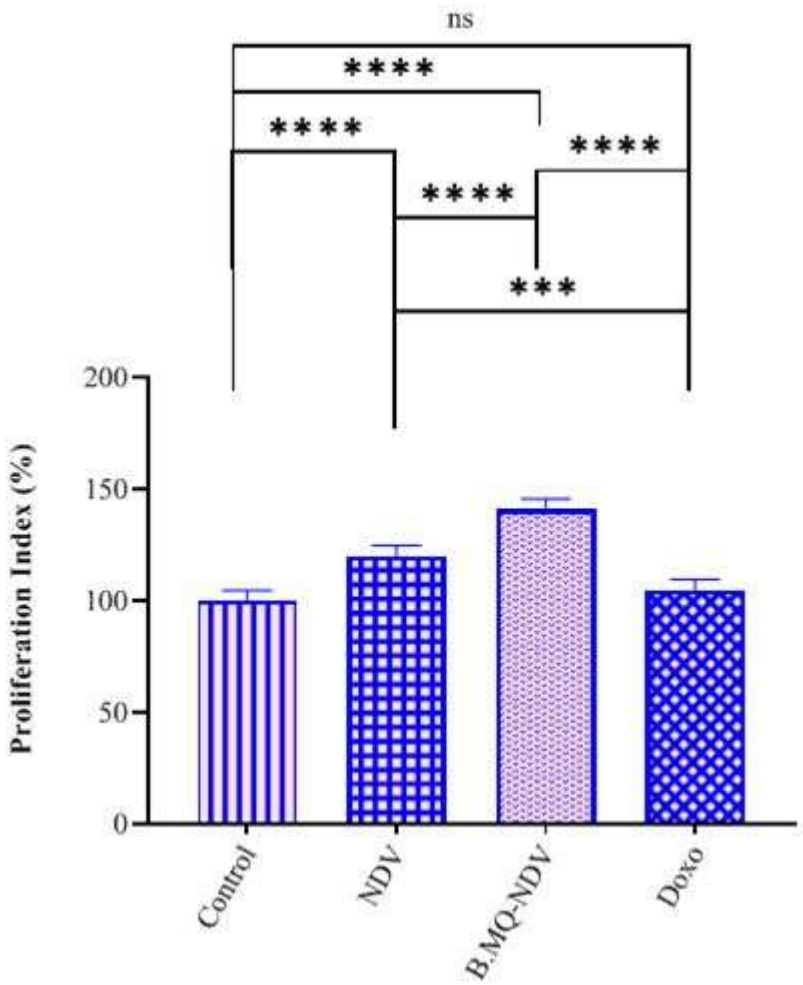


Figure 4. Effects of the oncolytic treatment on proliferation index of splenocytes in the CC mouse model (* indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, **** indicates $P<0.0001$, ns indicates non-significance).

3.5.LDH assay

All treatment groups showed a significant increase in LDH production compared to the control group ($P<0.05$), with the B.MQ-NDV group exhibiting the highest levels. Additionally, there was a statistically significant difference in LDH production between the B.MQ-NDV group and the NDV and Doxo groups (**Figure 5**). These findings indicate that B.MQ-NDV not only enhances LDH production, which is often associated with cell damage and cytotoxicity, but also suggests a stronger effect compared to the other treatment groups.

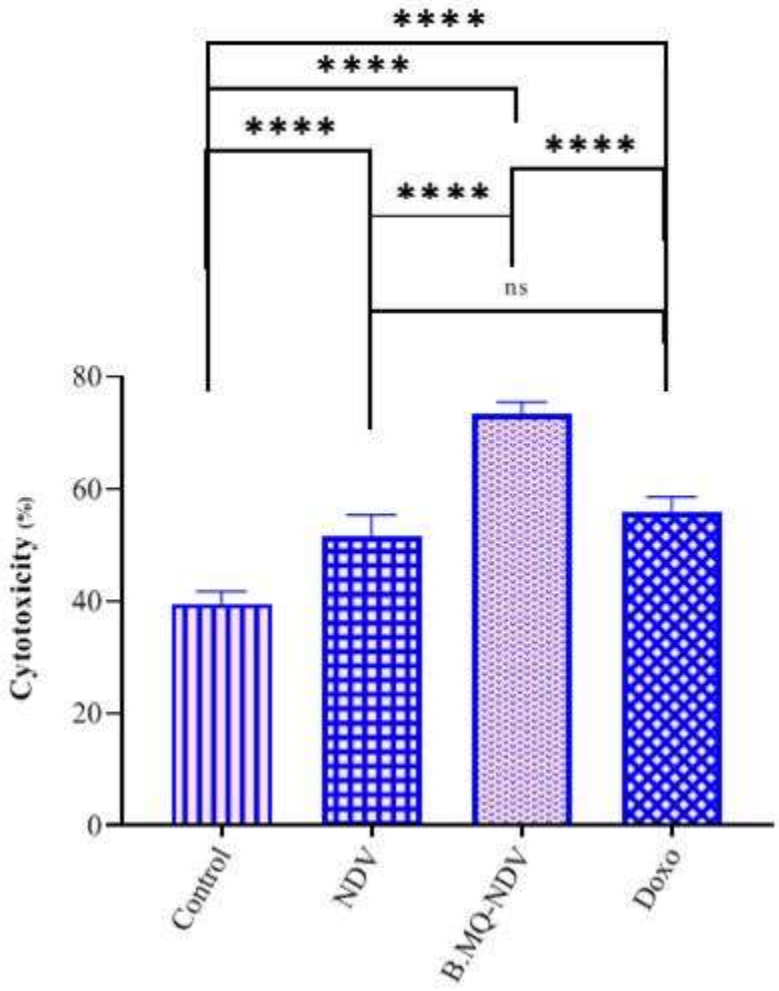


Figure 5. Effects of the oncolytic treatment on LDH release in the CC mouse model (* indicates *P*<0.05, ** indicates *P*<0.01, *** indicates *P*<0.001, **** indicates *P*< 0.0001, ns indicates non-significance).

3.6.NO assay

The results of this study indicated that all treatment groups exhibited a significant increase in NO production compared to the control group (P<0.05). The B.MQ-NDV group recorded the highest NO levels, showing a significant difference from both the NDV group (P<0.0001) and the Doxo group (P<0.05). Additionally, a significant difference was observed between the NDV group and the control group (P<0.001) (Figure 6).

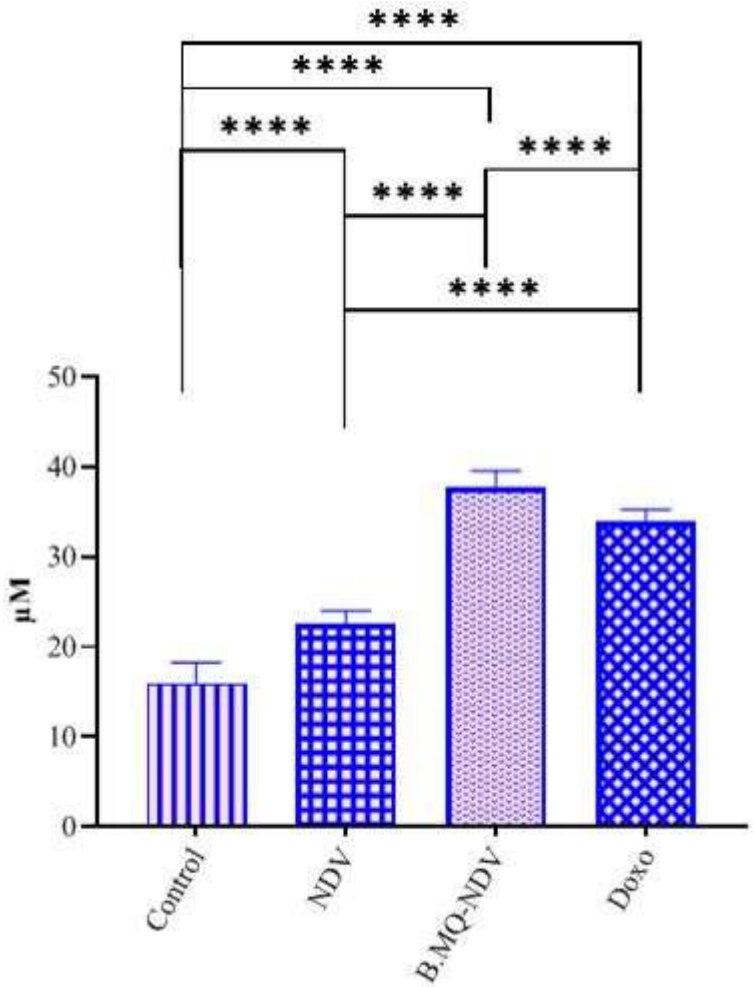
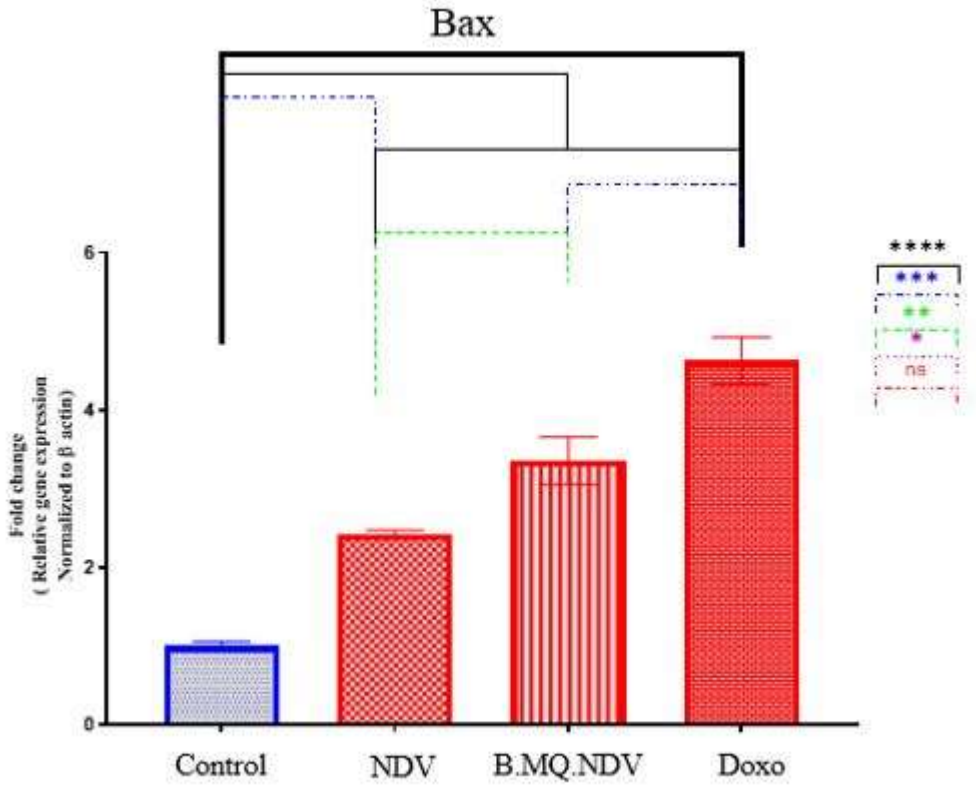


Figure 6. Effects of the oncolytic treatment on NO production in the CC mouse model (* indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, **** indicates $P< 0.0001$, ns indicates non-significance).

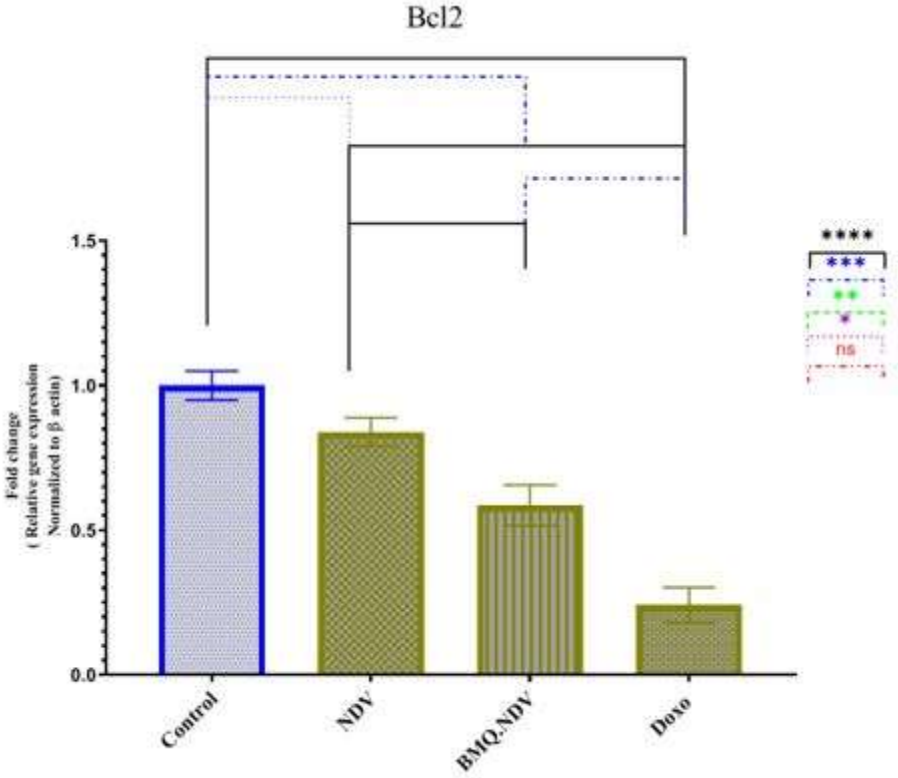
3.7.Real-time PCR

The expression of Bax and p53 significantly increased in the NDV, B.MQ.NDV, and Doxo groups compared to the control group. Bcl2 significantly decreased in the NDV, B.MQ.NDV, and Doxo groups. Additionally, the expression of Mmp2 significantly decreased in the NDV, B.MQ.NDV, and Doxo groups compared to the control group. Details of the changes in the expression of the genes Bax, Bcl2, p53, and Mmp2 across the different groups are illustrated in **Figure 7.** These results suggest that the treatments may induce apoptosis through the upregulation of pro-apoptotic

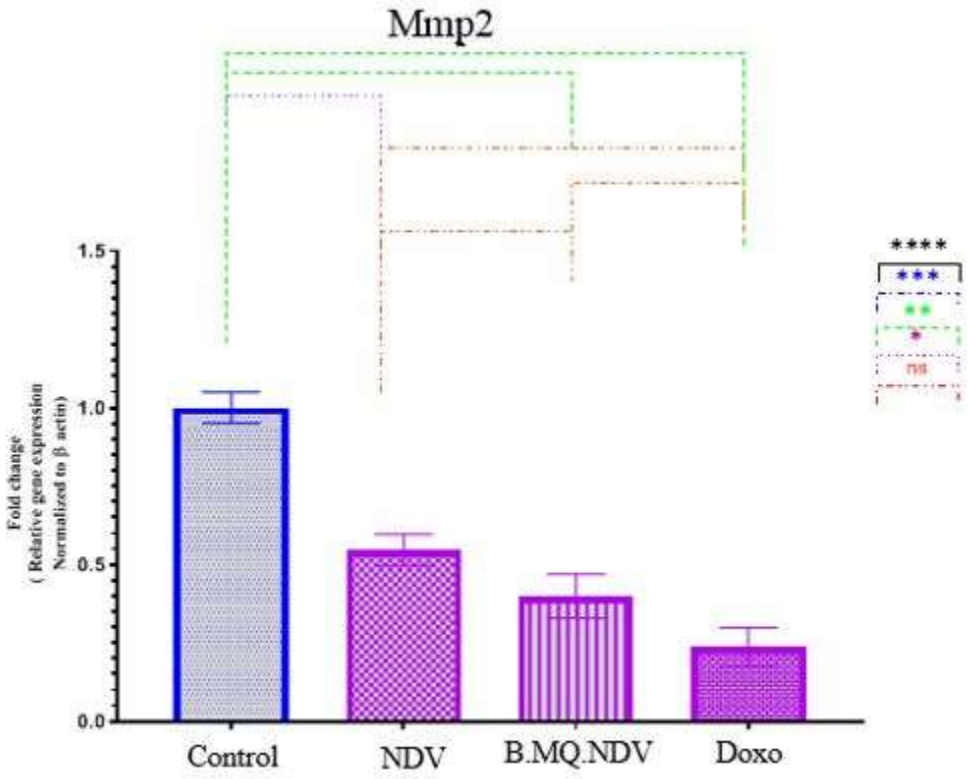
287 factors like Bax and p53, while downregulating anti-apoptotic factors like Bcl2, which could be
288 crucial for enhancing the therapeutic effects against tumors (**Figure 7**).



289


Bcl2
Fold change
(Relative gene expression
Normalized to β actin)
1.5
1.0
0.5
0.0
Control
NDV
BMQ.NDV
Doxo
****
***
**
*
ns

290


Mmp2
Fold change
(Relative gene expression
Normalized to β actin)
1.5
1.0
0.5
0.0
Control
NDV
B.MQ.NDV
Doxo
****
***
**
*
ns

291

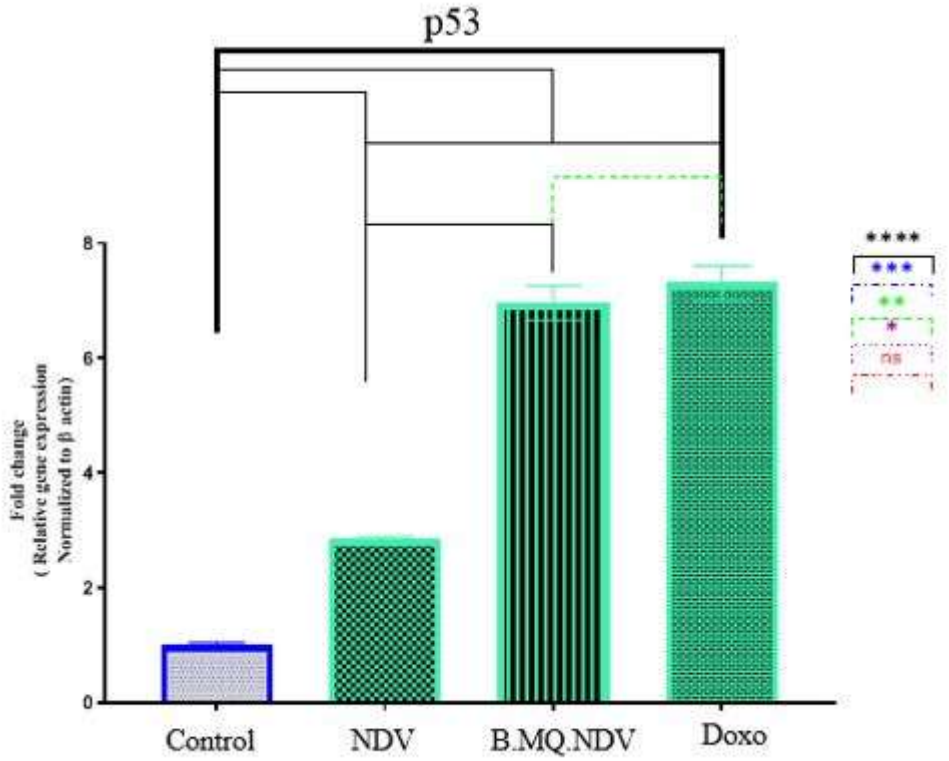


Figure 7. Effects of the oncolytic treatment on expression of selected genes in the CC mouse model (* indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, **** indicates $P<0.0001$, ns indicates non-significance).

4. Discussion

The aim of this study was to investigate the therapeutic effects of MQs carrying NDV in the experimental treatment of CC. This research focuses on evaluating how these treatments can influence cancer progression and potentially improve therapeutic outcomes in CC models. By examining various biological markers and pathways, the study seeks to provide insights into the efficacy of MQs with NDV in combating this type of cancer. The findings indicated that mice treated with MQs carrying the virus exhibited better survival rates and slower tumor growth compared to the control group of tumor-bearing mice.

Kwan et al., (2022) showed that HSV1716 OVs can be transported inside the cells of MQs as a carrier and MQs-HSV1716 led to tumor shrinkage, increased animal survival, and slowed the growth of the tumor in murine mammary cancers. Additionally, the amount of IL-12 production increased from 48% to 74% after treatment with MQs-HSV1716 (7). Burke et al., 2020 revealed

that NDV can infect MQs, which can act as vectors to mediate the infection of tumor cells. A potential mechanism suggests that MEDI5395 is delivered intravenously to tumor lesions. Circulating monocytes infected with MEDI5395 may contribute to this effect when they exit the bloodstream and differentiate into MQs and dendritic cells (DC) in the tumor microenvironment (TME). MEDI5395 enhances immune cell activation and pro-inflammatory responses. OVs infect cells through various mechanisms. The *measles virus* uses CD46 on the cell as a receptor for entry, while *herpes simplex* utilizes receptors such as NECTIN for cell entry. Both the *vaccinia* virus and NDV lack specific binding receptors and primarily enter host cells through the process of endocytosis, utilizing internalization mechanisms (8). NDV infection promotes the release of cytokines and chemokines, as well as the activation and recruitment of immune cells, particularly enhancing the cytotoxicity of T cells. The encapsulation of the NDV virus using thiolated chitosan nanoparticles enhances the immune protection of the virus by concealing it and improving its bioavailability in CC (9). Cytokines are proteins that play a crucial role in maintaining the balance of the immune system. They act as signaling molecules that facilitate communication between immune cells, helping to regulate immune responses, inflammation, and the overall homeostasis of the immune system. By coordinating the activities of various immune cells, cytokines. They can either attach to cell membranes or be released into the bloodstream, facilitating communication between cells. Both innate and adaptive immune cells produce cytokines in response to cancer cells and pathogens. Each cytokine has a distinct effect on the immune system, influenced by factors such as its concentration, the expression patterns of its receptors, and the interaction of various immune response pathways. Cytokines can inhibit tumor growth directly through anti-proliferative or pro-apoptotic actions, or indirectly by activating immune cells to target tumor cells. For instance, interferons are chemical signals produced in response to pathological threats that help normal cells become resistant to similar infections. They also regulate angiogenesis and possess immunomodulatory properties, making them a promising treatment option for cancer (10). IL-4 is a versatile cytokine that significantly influences the immune response by regulating various processes such as lymphocyte development, differentiation, and survival. It can also support the growth and longevity of different types of cancer cells. High levels of IL-4 have been detected in several human cancers, including ovarian, lung, breast, pancreatic, colon, and bladder cancers. On the other hand, IL-10 is crucial for controlling inflammation and maintaining cellular homeostasis. It acts by inhibiting the release

of several cytokines, including IL-1, IL-18, IL-12, IL-6, and TNF-α (11). Cytokines are essential proteins that play a key role in maintaining the balance of the immune system. They act as signaling molecules, facilitating communication between immune cells and coordinating immune responses and inflammation. Cytokines are vital for the body's defense against infections and diseases, as well as for facilitating the healing process. An imbalance in cytokines can lead to immune disorders. Among cytokines, IL-12 is recognized as a potent pro-inflammatory cytokine that has garnered attention in cancer immunotherapy. IL-12 enhances the activity of T cells and NK cells, stimulating the production of IFN-γ, which boosts the immune response against tumors. Therefore, IL-12 is viewed as a promising candidate for combination therapies in cancer treatment. IFN-γ is a crucial element in triggering cellular immunity, playing a key role in activating the immune response against tumors. Its properties, which include being cytostatic, pro-apoptotic, and anti-proliferative, position it as a strong candidate for use in adjuvant immunotherapy for various cancers. IFN-γ plays an important role in inhibiting the formation of new blood vessels in tumors, thereby slowing down tumor growth. By preventing the development of blood vessels that supply nutrients and oxygen to the tumor, IFN-γ helps reduce tumor size. Additionally, this protein enhances the activity of M1 inflammatory macrophages, which play a role in the immune response against cancer cells. These macrophages not only help eliminate tumor cells but also stimulate the immune system further by releasing cytokines (12). TGF-β, produced by tumor cells, is associated with tumor development and the immune response in cancer. This protein regulates the SMAD signaling pathway and influences metastasis. Additionally, by generating regulatory T cells, it suppresses anti-tumor immunity (13). Hammad et al., (2020) neural stem cells (NSC) were used to deliver the oncolytic virus CF33 (*orthopoxvirus*) to ovarian cancer cells. The results showed that infection increased in the tumor tissue of mice treated with NSC.CF33 compared to those treated with CF33 alone. These findings indicate that the NSC cell line effectively enhances the distribution of the oncolytic virus CF33 to the tumor site. This increased distribution may be due to the unique characteristics of NSC cells, which allow them to access tumor tissue more effectively. As a result, this leads to a decrease in the viability of cancer cells, as the CF33 virus directly attacks and eliminates tumor cells (14). Jafari et al., (2017), reported that tumor-bearing mice that received a combination of treatments showed a higher survival rate and slower tumor growth compared to control mice. The combination of vaccinations in this study had a significant impact on the production of NO and

the cytotoxicity of natural killer (NK) cells. The increase in NO production indicates a higher activity of the immune system in combating tumors. Measurement of LDH activity in splenocyte cultures showed that this therapeutic combination not only improved the performance of NK cells but also led to increased levels of IFN-γ. IFN-γ is a key cytokine in regulating the immune response and can enhance the activity of immune cells. At the same time, this combined immunotherapy reduced the levels of IL-4 and TGF-β, both of which can contribute to weakening the immune response and tumor progression (15). Tavakoli et al., (2021) revealed that the pairing of propranolol with a heated extract of 4T1 showed synergistic effects, leading to better survival rates and slower tumor progression than other treatment options. This combination therapy not only significantly increased the production of IFN-γ, NO, and LDH in the splenocyte cultures of tumor-bearing mice but also demonstrated a positive impact on enhancing the immune response. On the other hand, the reduction in IL-10 and TGF-β levels in splenocytes indicates the ability of this therapy to decrease immunosuppressive factors (16). JalaliKondori et al., (2022) demonstrated that the combined application of copper nanoparticles, the NDV, radiotherapy, and hyperthermia leads to decreased survival, increased apoptosis, and elevated LDH levels in colorectal cancer cells (17). In present study, MQs carrying the virus significantly enhanced the secretion of pro-inflammatory cytokines like IFN-γ, TNF-α, and IL-12, promoting immune activation. Simultaneously, they reduced IL-4 and TGF-β levels, shifting the immune response away from immunosuppression, thereby creating a more effective environment for targeting tumor cells.

OVs are a category of non-pathogenic DNA or RNA viruses that can infect and destroy cancer cells without reproducing in healthy cells or causing harm to them. This selective proliferation occurs because healthy cells activate antiviral pathways, leading to the secretion of IFN-I, which prevents the virus from surviving and proliferating. In contrast, cancer cells experience disruptions in these pathways, allowing the virus to replicate and ultimately lyse the cancer cells. One significant limitation of OVs is their rapid clearance by immune system responses. Thus, employing cell carriers for OVs delivery appears advantageous in cancer treatment (18, 19). Cancer therapy strategies utilizing OVs involve genetically engineered or naturally occurring strains that provoke a robust adaptive anti-tumor immune response by selectively infecting cancer cells while sparing normal tissue cells (20). Seyed-Khorrami et al., (2021) revealed that treatment with MSCs carrying reovirus effectively delivered the virus to the tumor site and showed a

significant reduction in tumor growth compared to treatment with reovirus alone (21). Keshavarz et al. (2020) discovered that the *NDV Lasota strain* decreases survival rates and inhibits the growth of the TC1 cell line (CC cells) by inducing apoptosis. This induction of apoptosis in the TC1 cell line occurs through an increase in the production of reactive oxygen species (ROS). The increase in ROS can lead to damage to the cell membrane and DNA, ultimately resulting in programmed cell death (apoptosis) and helping to reduce tumor growth (22). In a follow-up study in 2020, Keshavarz et al. (2020) utilized MSCs as carriers and transmitters of NDV to improve its efficacy and prevent its clearance by immune system responses. In these investigations, it was observed that MSCs carrying NDV not only help increase lifespan but also significantly reduce tumor volumes. These positive effects are attributed to the increased production of important cytokines such as IFN-γ, and IL-12, which play a vital role in regulating immune responses and combating tumors (23).

BCL2, MMP2, BAX, and P53 play critical roles in cancer. BCL2 acts as an anti-apoptotic protein, preventing cell death and supporting the survival of cancer cells (24). MMP2 facilitates invasion and metastasis by degrading the extracellular matrix (25). In contrast, BAX is a pro-apoptotic protein that activates apoptotic pathways, leading to cell death. P53, known as a tumor suppressor, responds to DNA damage and can trigger apoptosis. Together, these four proteins significantly influence the regulation of cell growth and death in cancer, highlighting their importance in cancer biology and potential therapeutic targets (24). In our study, the expression of Bax and p53 increased in the NDV, B.MQ.NDV, and Doxo groups, while Bcl2 decreased in the NDV, B.MQ.NDV, and Doxo groups. The expression of Mmp2 also decreased, indicating that the treatments may enhance apoptosis and therapeutic effects. NDV has the capability to target cancer cells through both direct and indirect mechanisms. However, a significant challenge in utilizing these viruses for cancer treatment is the host's immune system. Antibody-dependent neutralization by complement can diminish the treatment's effectiveness. To address this issue, scientists propose the use of cellular carriers, which can shield OVs from immune-mediated clearance or neutralization. Additionally, employing cells that can naturally migrate within the tumor microenvironment to deliver anticancer agents can enhance treatment outcomes. This strategy helps the virus evade detection by the immune system. Our research indicated that B. MQs could serve as a valuable tool for delivering NDV, as they induce specific antitumor responses and boost the production of TH1 cytokines, thereby amplifying the antitumor effect of

NDV with fewer side effects compared to chemotherapy. This approach may be particularly effective in treating CC. However, clinical studies are essential to validate these findings.

Acknowledgements

The current study is part of the Ph.D. thesis of Mrs. Aezam Rasekhi Kazeruni (project number:162673030). The article's authors express gratitude to those who helped us in this research.

Author contributions

Study concept and design: A.R.K, N.B, H.E.G.G, A.D, M.F

Acquisition of data: A.R.K, H.E.G.G

Analysis and interpretation of data: A.R.K, H.E.G.G, M.F

Drafting of the manuscript: A.R.K, N.B, H.E.G.G, A.D, M.F

Critical revision of the manuscript for important intellectual content: A.R.K, N.B, H.E.G.G, A.D, M.F

Statistical analysis: H.E.G.G, M.F

Administrative, technical, and material support: A.R.K, N.B, H.E.G.G, A.D, M.F

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Our university's ethical committee confirmed this study's protocols (Ethics: IR.BPUMS.REC. 1401 .209).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

References

1. Okunade KS. Human papillomavirus and cervical cancer. Journal of Obstetrics and Gynaecology. 2020 Jul; 40(5):602-608. doi: 10.1080/01443615.2019.1634030.
2. Burmeister CA, Khan SF, Schäfer G, Mbatani N, Adams T, Moodley J, et al. Cervical cancer therapies: Current challenges and future perspectives. Tumour Virus Res. 2022 Jun; 13:200238. doi: 10.1016/j.tvr.2022.200238.
3. Muthukutty P, Yoo SY. Oncolytic Virus Engineering and Utilizations: Cancer Immunotherapy Perspective. Viruses. 2023 Jul 28; 15(8):1645.
4. Müller E, Speth M, Christopoulos PF, Lunde A, Avdagic A, Øynebråten I, et al. Both Type I and Type II Interferons Can Activate Antitumor M1 Macrophages When Combined with TLR Stimulation. Front Immunol. 2018 Nov 2; 9:2520.
5. Lam HY, Yeap SK, Rasoli M, omar AR, Yussof K, Suraini AA, et al. Safety and clinical usage of Newcastle disease virus in cancer therapy. J Biomed Biotechnol. 2011:2011: 718710.
6. Ghasemi Darestani N, Gilmanova AI, Al-Gazally ME, Zekiy AO, Ansari MJ, Zabibah RS, et al. Mesenchymal stem cell-released oncolytic virus: an innovative strategy for cancer treatment. Cell Commun Signal. 2023 Feb 24;21(1):43.
7. Kwan A, Howard F, Winder N, Atkinson E, Jailani A, Patel PB, et al. Macrophage delivered HSV1716 is active against triple negative breast cancer. Future Pharmacol. 2022, 22, 444-459.
8. Yan Z, Zhang Z, Chen Y, Xu J, Wang J, Wang Z. Enhancing cancer therapy: the integration of oncolytic virus therapy with diverse treatments. Cancer Cell Int. 2024, 11; 24(1):242.
9. Kousar K, Naseer F, Abduh MS, Anjum S, Ahmad T. CD44 targeted delivery of oncolytic Newcastle disease virus encapsulated in thiolated chitosan for sustained release in cervical cancer: a targeted immunotherapy approach. Front immunol. 2023 May 22:14; 1175535.
10. Ghosh G, Parida P. Interferon therapy in lung cancer: current perspectives. Curr Cancer Ther Rev. 2017, 12, 237-245.
11. Nguyen KG, Vrabel MR, Mantooth SM, Hopkins JJ, Wagner ES, Gabaldon TA, et al. Localized interleukin-12 for cancer immunotherapy. Front immunol. 2020, 11, 575597.
12. Jorgovanovic D, Song M, Wang L, Zhang Y. Roles of IFN-γ in tumor progression and regression: A review. Biomark Res. 2020 Sep 29:8:49.
13. Maruyama T, Chen W, Shibata H. TGF-β and cancer Immunotherapy. Biol Pharm Bull. 2022; 45(2):155-161.

14. Hammad M, Cornejo YR, Covello JB, Majid AA, Burke C, Liu Z, et al. Neural stem cells improve the delivery of oncolytic chimeric orthopoxovirus in a metastatic ovarian cancer model. Mol Ther Oncolytics. 2020, 25; 18: 326-334.
15. Jafari S, Abtahi Froushani SM, Tokmachi A. Combined extract of heated 4T1 and a heat-killed preparation of Lactobacillus Casei in a mouse model of breast cancer. Iran J Med Sci. 2017 Sep; 42(5):457-464.
16. Tavakoli P, Abtahi Froushani SM, Aliyari A. Combination of propranolol and heated 4T1 cells elicits beneficial response against mouse model of breast cancer. Zahedan J Res Med Sc. 2021 Feb; 23(1):e94570.
17. JalaliKondori B, Hemadi SMH, Esmaeili Gouvarchinghaleh H, MilaniFard AM, Dorostkar R. Pharmacological study of the antitumor effect of Newcastle oncolytic virus in combination with copper nanoparticles, hyperthermia and radiation on malignant colorectal cancer cell line. J Med Chem Sci. 2022 5:457-467.
18. Lin Y, Wang W, Wang Y. Oncolytic activity of a coxsackievirus B3 strain in human endometrical cancer cell lines. Virol J. 2018 Apr 10; 15 (1):65.
19. Lemos de Matos A, Franco LS, McFadden G. Oncolytic Viruses and the Immune System: The Dynamic Duo. Mol ther Methods clin dev. 2020 Jan 15; 17:349-358. Doi: 10.1016/j.omtm.2020.01.001
20. Tian Y, Xie D, Yang L. Engineering strategies to enhance oncolytic viruses in cancer immunotherapy. Signal Transduct Target Ther. 2022 Apr 6; 7(1):117.
21. Seyed-Khorrami SM, Soleimanjahi H, Soudi S, Habibian A. MSCs loaded with oncolytic reovirus: migration and in vivo virus delivery potential for evaluating anti–cancer effect in tumor–bearing C57BL/6 mice. Cancer Cell Int. 2021, 1; 21(1):244.
22. Keshavarz M, Nejad ASM, Esghaei M, Bokharaei-Salim F, Dianat-Moghadam H, Keyvani H, et al. Oncolytic Newcastle disease virus reduces growth of cervical cancer cell by inducing apoptosis. Saudi J Biol Sci. 2020 Jan;27(1):47-52
23. Keshavarz M, Ebrahimzadeh MS, Miri SM, Dianat Moghaddam H, Ghorbanhosseini SS, Mohebbi SR, et al. Oncolytic Newcastle disease virus delivered by Mesenchymal stem cells-engineered system enhances the therapeutic effects altering tumor microenvironment. Virol J. 2020 Dec; 17(1):1-3.

24. Qian S, Wei Z, Yang W, Huang J, Yang Y, Wang J. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. Front Oncol. 2022 Oct 12;12:985363.
25. Wolosowicz M, Prokopiuk S, Kaminski TW. The Complex Role of Matrix Metalloproteinase-2 (MMP-2) in Health and Disease. Int J Mol Sci. 2024 Dec 21;25(24):13691.