Research Paper

Immunological Effects of Oncolytic Coxsackievirus A21 on the Mouse Model of Colorectal Cancer

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ABSTRACT

Introduction: Cancer is one of the most prevalent causes of death worldwide. In terms of global mortality, colorectal cancer (CRC) is the second most prevalent cancer form. Typically, the initial step in treating colon cancer is surgery to remove tumors. A referral for further treatments like chemotherapy and radiation therapy could also be made. However, because of medication resistance and a lack of focused treatments, it is constantly necessary to create new cancer therapy methods. This investigation examined the impact of the oncolytic coxsackievirus A21 (CVA21) on a mouse model of colorectal cancer.

Methods: Thirty BALB/c mice were divided into three equal groups randomly. 5×10^6 CT-26 cells, a colonic carcinoma cell line, were injected into the left flank of each animal to simulate colorectal cancer. Group A was treated with PBS once the palpable tumor was discovered (18 days later), group B was treated with the oncolytic CVA21 (10^6 TCID50/mL, twice at one-week intervals), and group C was treated with 5-fluorouracil (5-FU), 50 mg/kg, twice at one-week interval. The mice were euthanized ten days after the final injection, and the spleens were removed and examined under sterile circumstances to determine the lymphocyte proliferation index, LDH, NO, IL-4, IL-10, IFN-γ, and TGFβ production levels. A significant $P<0.05$ level was considered in all evaluations.

Results: The current study's findings showed that when compared to the control group, treatment with CVA21 increased NO (30.5±4.10 µM), LDH (58.18±4.61 %), and IFN-γ (44.82±3.72 pg/mL) levels and significantly decreased the secretion of IL-4 (30.07±3.34 pg/mL), IL-10 (62.11±5.69 pg/mL), and TGF-β (56.66±6.88 pg/mL).

Conclusion: The CVA21 treatment for colorectal cancer appears to have some potential benefits. In other words, the study's findings demonstrated that using oncolytic viruses also activates the innate immune system by raising levels of nitric oxide and the acquired immune system. The favorable benefits of the combination may also be attributable to immunological divergence in the current study from anti-inflammatory cytokines (such as IL-4, IL-10, and TGF-β) to pro-inflammatory cytokines, such as IFN-γ.

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1. Introduction

The second most prevalent cause of cancer fatalities is colorectal cancer (CRC), which accounts for around 10% of all cancers [1]. In 2020, 9.4% of cancer-related deaths were attributable to CRC. However, it is anticipated that by 2035, the incidence of CRC will double globally. Due to the enormous increase in the number of instances detected in the elderly population, developing nations are seeing the largest increases [2]. Numerous studies have revealed that factors like family history, chronic inflammation, and dietary and behavioral choices increase the risk of CRC. However, the most effective strategy to avoid CRC and reduce the fatalities linked to CRC in the general community is to screen those with average risk [3]. Surgery to eliminate the malignancy is typically the first step in treating colon cancer. There may also be a recommendation for further therapy like chemotherapy and radiation therapy. One of the main concerns of the global pharmaceutical community is the development of novel anticancer medications with high efficacy and low toxicity that selectively influence cells and are affordable [4]. The treatment of all forms of tumors is extremely difficult. Chemotherapy is now the most crucial cancer treatment, but it also has several negative side effects and can lead to a patient developing drug resistance. Certain natural substances have long been regarded as trustworthy and superior sources for creating anticancer medications. Natural substances can treat and prevent cancer and lessen the negative effects of radiation and chemotherapy due to being cost-effective [5]. One of the most cutting-edge methods for treating otherwise incurable cancers is oncolytic virotherapy. Despite recent positive discoveries, the small percentage of patients responding to treatment has shown the necessity to look for additional appropriate viruses [6]. The *Picornaviridae* family of viruses includes the non-enveloped coxsackievirus A21 (CVA21), which has an icosahedral shape and a single strand of positive sense RNA with a length of around 74 kb [7]. Along with several other Picornaviruses, CVA21 causes cell death by interrupting host cellular protein synthesis, preventing the transfer of cellular glycoproteins, inducing apoptosis, and proteolytic breaking down transcription factors [8]. Humans naturally contract CVA21 infections, which are typically asymptomatic and unrelated to serious illness. CVA21 is a unique contender with several excellent qualities [9]. The expression of certain viral receptors on the host cell's surface influences the targeted tissue tropism of most viruses, which are extremely selective [10]. The particular attachment of CVA21 and subsequent infection of the host cell are mediated through the cell surface receptors intercellular adhesion molecule-1 (ICAM-1) and/or decay-accelerating factor (DAF) [11]. If ICAM-1 is not also expressed on the cell surface, CVA21 cannot infect a cell even though it can bind to DAF expressed on the cellular membrane. As a result, under typical infection settings, ICAM-1 is regarded as a major factor responsible for CVA21 cell entry, unceasing, and reproduction [12]. Melanoma and many other diseased cells express ICAM-1 and DAF at comparatively high levels compared to most non-malignant cells, enabling selective CVA21 oncolysis. Numerous xenograft and syngeneic mouse tumor models demonstrate that CVA21 possesses broad anti-tumor action [13]. In addition to directly destroying tumor cells, the virus triggers a potent immune reaction against the tumor, greatly enhancing the effectiveness of the therapy. Depending on the viral strain, oncolytic CVA21's toxicity in healthy tissues varies [14]. This study aims to assess the impact of oncolytic coxsackievirus A21 on the mouse model of colorectal cancer due to the significance of utilizing innovative techniques in cancer treatment.

2. Methods

CT26 cell line

CT26.WT was provided by the Pasteur Institute, Iran (ATCC CRL-2638). The cells were grown in monolayers in DMEM (Merk-Germany) with 10% FBS (Sigma-Aldrich) and were maintained at 37°C in a humid environment with 5% CO_2.

Coxsackievirus A21

The Applied Virology Research Center of Baqiyatallah University of Medical Sciences provided the CVA21 (10^6 TCID50/mL). Briefly put, human lung fibroblast cells (MRC-5, CCL-171) were cultured and infected with a CVA21 virus stock at a different dilution after 24 hours, and the cell lysate was harvested about 3–4 days after infection at a cytopathic effect (CPE) of >90%. Then, TCID50/mL of virus was calculated using the Reed–Muench method.

Experimental design, mice and tumor induction

The Pasteur Institute provided female BALB/c mice that were 6-8 weeks old and weighed 25–30 g. The CT26 cell line was used to generate colon cancer in an animal model seven days following cell culture. Before receiving an injection of CT26 cells, these mice were kept in the animal's home for two weeks so they could

Table 1. The characteristics of the studied groups

Group	Abbreviation	Characteristics (in 100 µL of PBS)
Control	Control	PBS
Coxsackievirus A21	Cox	10^6 TCID50/mL, twice at one-week interval
5-fluorouracil	5-FU	50 mg/kg

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biologically adjust to their environment. Then, according to the guidelines for experimental animals, 5×10^6 CT26 cells were enumerated and subcutaneously injected into the left flank of mice in 100 µL of PBS. After the injection, the tumor cells showed up around 18 days later. The mice were then randomly divided into three equal groups (Table 1). When the palpable tumor was discovered (18 days later), group A received PBS; group B received the oncolytic CVA21 (10^6 TCID50/mL, twice at one-week intervals), and group C received 5-fluorouracil (5-FU), (50 mg/kg, twice at one-week intervals).

The proliferation level of splenocytes

To determine the level of splenocyte proliferation, the MTT test was performed. Mice spleen cells were isolated aseptically. Splenocytes were generated as single-cell suspensions in DMEM medium supplemented with 10% FBS, and red blood cells (RBCs) were eliminated using RBC lysis buffer. Next, 96-well plates with cell suspensions (10^5 cells/100 µL/well) were incubated while being activated by the freezing and thawing of tumor cell antigens (20 µg/mL). The cultures were stimulated with 20 µL of the MTT (Sigma-Aldrich) [15] solution (5 mg/mL) for four hours at 37°C. Using 20 µL of the MTT solution (5 mg/mL) for four hours at 37°C, the cultures were stimulated after 72 hours of incubation. To dissolve the formazan crystals, 100 µL of DMSO was added, after which the mixture was vigorously agitated. An ELISA reader was used to measure the optical density (OD) at 492 nm (Dynatech, Denkendorf, Germany). Evaluations were administered in three-set groups.

Lactate dehydrogenase assay

LDH Cytotoxicity Detection Kit (Abcam-UK) was used to investigate the cytotoxic activity. In this test, cytotoxicity is determined using the practical, rapid colorimetric method of measuring the activity of LDH generated by injured cells. Most cells contain the cytoplasmic enzyme LDH, which is constant. The target cells were the CT-26 cell type, whereas the effector cells were splenocytes. After being cleaned in the test medium of DMEM with 1% FBS, the effector and target cells were co-cultured on 96-well round-bottomed plates for six hours at 37°C at a ratio of 50 effector cells to one target cell. The supernatants were then transferred from the centrifuged plates to 96-well fat-bottomed plates. The LDH detection mixture was then poured into each well and allowed to stand at room temperature for 30 minutes before being placed in the refrigerator. An ELISA analyzer (Dynatech, Denkendorf, Germany) was used to measure the OD at 492 nm.

Measurement of NO in splenocytes

Using the Griess reagent, the nitrite content of the splenocyte culture supernatants was determined to gauge the capability for NO production. After the splenocytes had been cultivated, 50 µL of the cell-free supernatants were removed, and they were combined with 50 µL of Griess reagent, which contains 0.1% sulfanilamide, 0.3% phosphoric acid, and 0.1% N-(1-naphthyl) ethylenediamine. The final combination was given for ten minutes at a room temperature and with no light. After incubation, the absorbance at 492 nm was gauged using an ELISA reader (Dynatech, Denkendorf, Germany).

Cytokine assay

Mice were euthanized a week after the last agent therapy to assess the cytokines that splenocytes generated. Splenocytes were removed from the animals in an aseptic setting to make single-cell suspensions of the splenocytes in DMEM media with 10% FBS. After that, RBCs were eliminated using ACK (ammonium-chloride-potassium) lysing buffer. Then, cell suspensions (2×10^6 cells/mL) were used to pre-treat 24-well plates before injecting tumor antigens. 20 µL of freezing and thawing of the tumor cells yielded these antigens [15]. It has been established that tumor antigen has been created. It took 72 hours to collect the growing supernatants. The manufacturer's instructions for the ELISA kit (Abcam-UK) were followed to quantify IFN-γ, IL-4, IL-10, and TGF-β.

Statistical analysis

The quantitative variables' Means±SD indicators were measured in the current study. An LSD post hoc test and analysis of variance were employed to compare the groups. GraphPad Prism software, version 8 was used to plot the graphs, and SPSS software, version 24 was used for statistical analysis. A student's t-test was used to examine the differences. P<0.05 was statistically significant.

3. Results

Splenocyte cell proliferation (MTT assay)

Inflammatory cells of the innate immune system's arm created nitric oxide. Compared to the control group, the findings show that coxsackievirus A21 (P<0.05) and 5-FU (P<0.001) treatment factors enhance nitric oxide production. Nitric oxide generation was also considerably greater (P<0.01) in the 5-FU treatment group compared to the coxsackievirus A21 treatment group (Figure 1).

Nitric oxide production rate

Inflammatory cells of the innate immune system's arm created nitric oxide. The findings showed that, compared to the control group, both coxsackievirus A21 (P<0.05) and 5-FU (P<0.001) treatment factors enhance nitric oxide production. Nitric oxide generation was also considerably greater (P<0.01) in the 5-FU treatment group compared to the coxsackievirus A21 treatment group (Figure 2).

Lactate dehydrogenase production rate

A biomarker for damaged cell membranes is lactate dehydrogenase. According to the findings of our investigation, compared to the control group, the levels of LDH release were highest in the splenocytes treated with 5-FU (P<0.001) and lowest in those treated with coxsackievirus A21 (P<0.05). The 5-FU and coxsackievirus A21 groups both showed a significant rise (P<0.01) in the infection rate (Figure 3).

Splenocytes supernatant cytokines

The expression level of CD markers or the degree of cytokine production can be assessed to determine the direction of immune system responses and the lymphocyte population specific to tumors. We looked at IFN-γ as a representation of Th1 cells, IL-4 as a representative of Th2 lymphocytes, TGF-β as a representative of Treg lymphocytes, and IL-10 as a representative of Treg and Th2 lymphocytes in this work. Figure 4 demonstrates that coxsackievirus A21 (P<0.05) and 5-FU (P<0.001) significantly boosted IFN-γ levels and decreased IL-4, TGF-β, and IL-10 levels when compared to the control group.

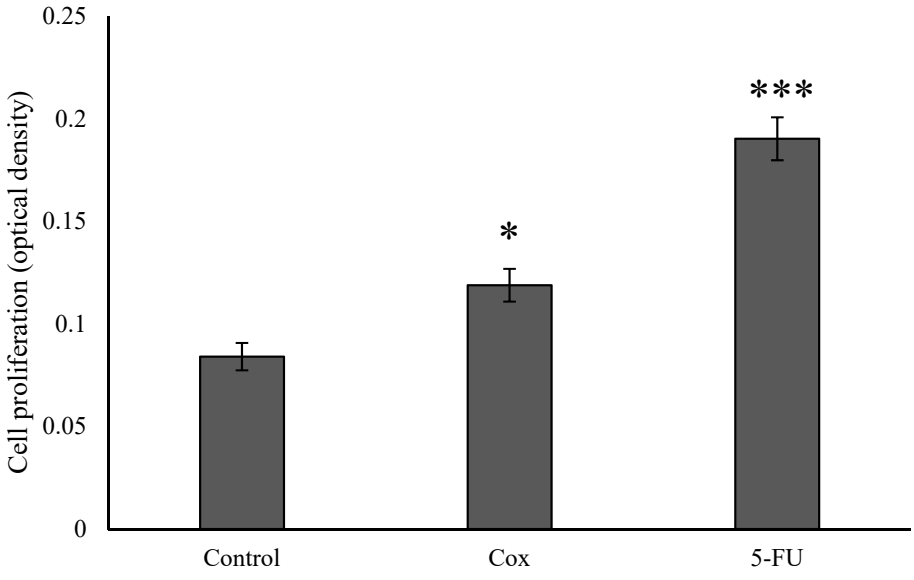


Figure 1. Effects of coxsackievirus A21 treatment on the proliferation level of splenocytes (OD)
*P<0.05, **P<0.01, ***P<0.001.

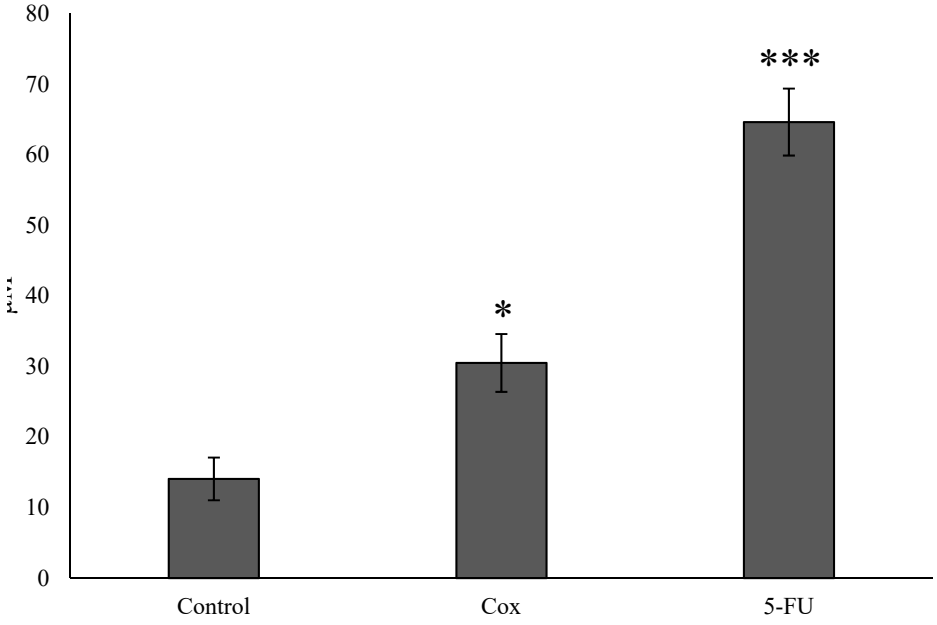


Figure 2. Effects of coxsackievirus A21 treatment on the NO production (µM)
*P<0.05, **P<0.01, ***P<0.001.

4. Discussion

The second leading cause of cancer-related death is colon (colorectal) cancer, which ranks third globally in terms of cancer incidence. Colorectal cancer's specific etiology is unknown, although studies have revealed that certain risk factors raise a person's likelihood of developing the disease [16]. The proper use of medications in cancer treatment is crucial for this reason. Given that several genetic alterations are required to produce a malignant version of a cell [17]. Chemotherapy is one of the most often used cancer therapies. Chemotherapy

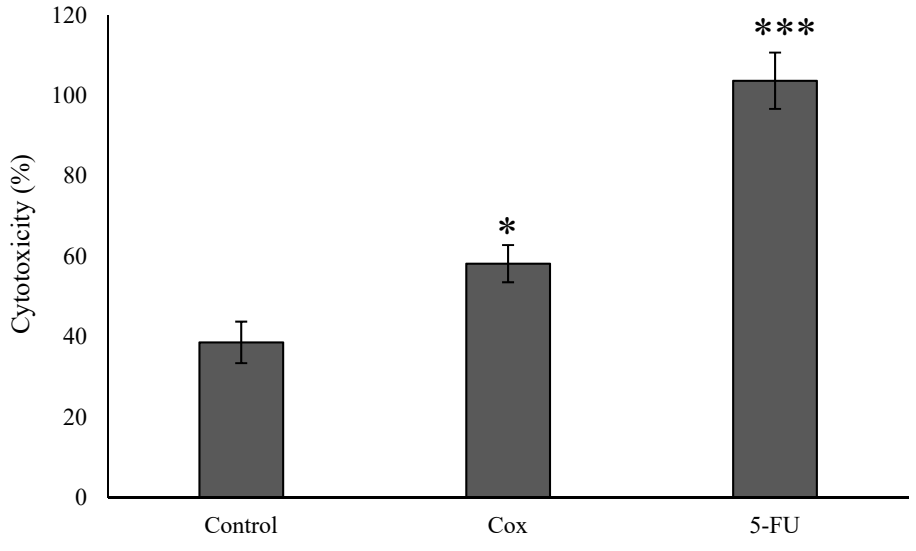
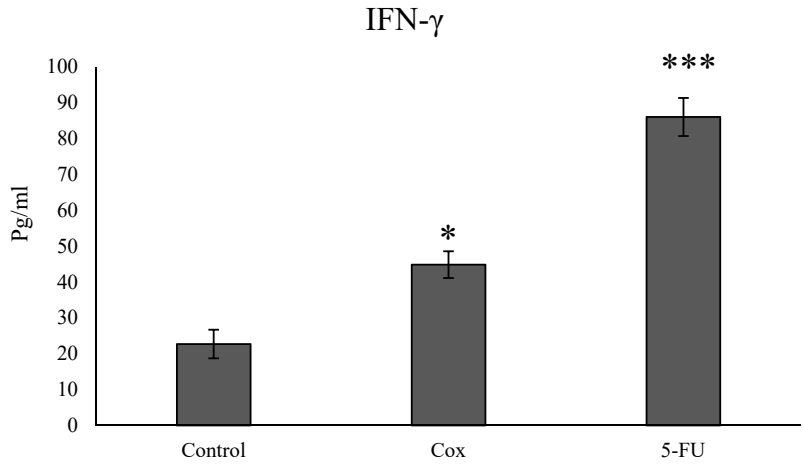


Figure 3. Effects of coxsackievirus A21 treatment on the LDH production (%)
*P<0.05, **P<0.01, ***P<0.001.


IFN-γ
Pg/ml
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90
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70
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50
40
30
20
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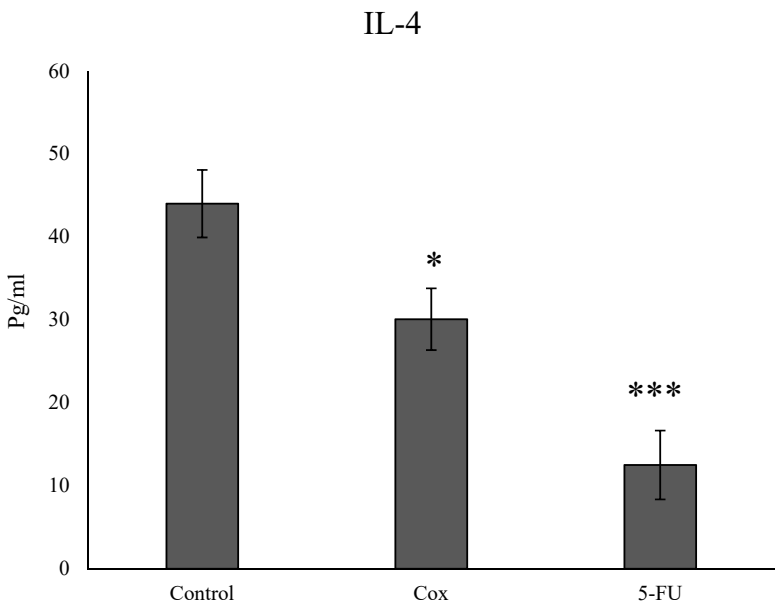
Control
Cox
5-FU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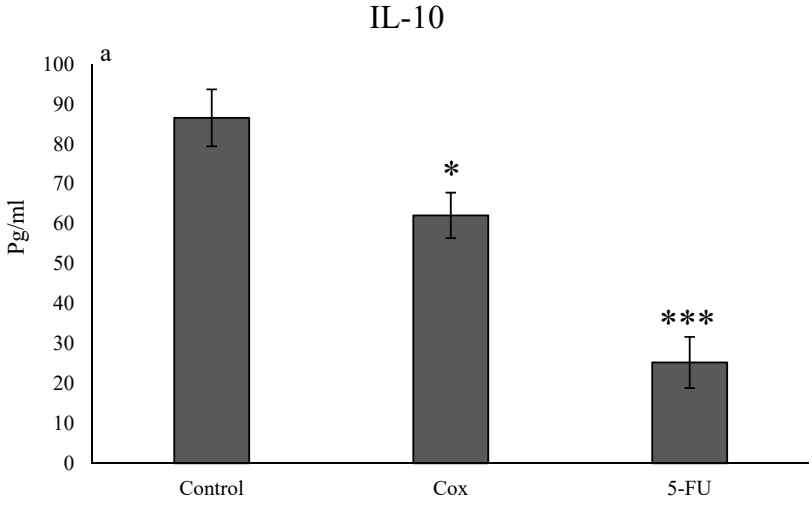
IL-10
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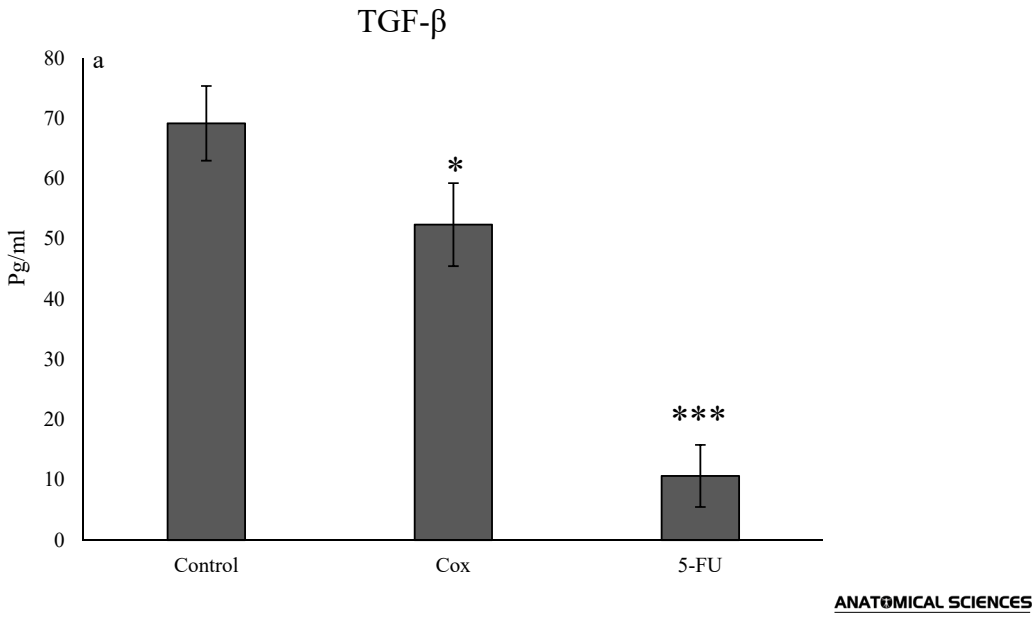

Figure 4. Effects of coxsackievirus A21 treatment on the cytokine production (pg/mL)
*P<0.05, **P<0.01, ***P<0.001.

can have a negative effect on the patient's quality of life and has various side effects, including tiredness, anemia, alopecia, nausea, and vomiting. This condition is treated with various chemotherapy regimens, all of which have 5-fluorouracil (5-FU) as their primary component. For more than 40 years, the first-line therapy for colorectal cancer metastases has been injection 5-FU [18]. The initial step in treating colon cancer is frequently surgery to remove tumors. Additional treatments, including chemotherapy and radiation therapy, could also be suggested. However, because of medication resistance and a lack of focused treatments, there is always a need to create innovative cancer therapy options [19].

This study aimed to evaluate the effects of oncolytic CAV21 on the colorectal cancer mouse model. The results showed that CAV21 and 5-FU treatment groups caused significant increases in splenocyte proliferation, LDH and NO production, IFN-γ cytokine levels, and reduced IL-4, TGF-β, and IL-10 compared to the control group. Oncolytic viruses are appealing biological agents for the treatment of human cancer. Virotherapy is expected to be most successful in slow-growing tumors, as quickly developing tumors may avoid viral oncolysis if offspring virus distribution is inefficient [20]. CAV21, a naturally occurring human enterovirus, has been proven by researchers to be an efficient oncolytic agent against human melanoma cells in vitro and in vivo in numerous immune-deficient xenograft mice models [21]. CAV21 has previously been delivered to end-stage melanoma patients with no side effects, and additional human studies to assess safety are presently underway [22]. Oncolytic viruses have a direct lethal impact, but it is now well documented that the anticancer benefits of oncolytic viruses also result from the activation of innate and adaptive tumor-specific immunity and the immunogenicity of dying or dead cancer cells [23]. Kingston et al. (2022) discovered that CAV21, in addition to lowering tumor growth and enhancing survival, enhances cellular immunity and the number of NK cells in a colorectal cancer animal model [24]. CVA21 induced immunogenic apoptosis in bladder cancer cell lines, as evidenced by expression of the ICD determinant calreticulin, as well as HMGB-1 release and the ability to reject MB49 tumors in syngeneic mice after vaccination with MB49 cells undergoing CVA21-induced ICD [25]. According to Zhang et al., CAB3 exhibits oncolytic efficacy against colon cancer via gasdermin-e mediated pyroptosis, aided by reactive oxygen species (ROS) [26]. Tumors can avoid immune responses by secreting mediators such as IL-4, TGF-β, and IL-10. These cytokines have the ability to reduce key components of anti-tumor immunity, such as inflammatory macrophages and Th1 responses. In human breast cancer, IL-4 can directly increase tumor cell proliferation [27]. TGF-β and IL-10 tend to reduce lymphocyte and macrophage proliferation and activation and thus suppress cell-mediated immunity, which is required to limit tumor progression. Both cytokines have the ability to drive the production of regulatory T cells, which have been detected in a variety of malignancies,

as well as dampen T-cell responses to tumors. Surprisingly, TGF-β and IL-10 are also produced by regulatory T cells [28]. The current study findings showed that CAV21 strongly suppressed TGF-β, IL-10, and IL-4 levels compared to the control group. IFN-γ levels are directly associated with anti-tumor responses. Natural killer cells and macrophages are two essential innate immune effector cells in cancer defense. Natural killer cells have been shown in vitro and in vivo to be capable of eliminating tumor cells [29]. Natural killer cells can establish an immunological response against tumor cells by secreting cytokines like IFN-γ and directly inducing apoptosis in tumor cells [30]. The current study showed that CAV21 considerably enhanced the amount of IFN-γ compared to control mice. M1 macrophages have several anti-tumor actions, including the generation of nitric oxide, a lethal factor for malignancies. Unfortunately, malignant tumors impart a local state for tumor growth by promoting macrophages toward the M2 anti-inflammatory phenotype [31, 32]. Coxsackievirus promoted bone marrow production of inflammatory macrophages (M1) [33]. The current study found that nitric oxide generation rose much more in tumor-bearing mice treated with CAV21 than in control animals. Furthermore, the present study found that CAV21 cannot compete with 5-FU chemotherapy, and that the anti-cancer effects of 5-FU chemotherapy are about double those of CAV21. One of the study's limitations is the lack of investigation into the synergistic effects of CAV21 and 5-FU, so it is suggested that researchers investigate the synergistic effects of these two therapeutic agents to reduce drug dosage if they strengthen each other's effects.

5. Conclusion

According to the findings of this investigation, CVA21 treatment for colorectal cancer appears to be beneficial. In other words, the study's findings revealed that, in addition to boosting the acquired immune system, oncolytic viruses activate the innate immune system by raising the quantity of nitric oxide produced. Furthermore, in the current study, immunological divergence from anti-inflammatory cytokines (such as IL-4, IL-10, and TGF-β) to pro-inflammatory cytokine IFN-γ may contribute to the combination's favorable effects.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee of the Baqiyatallah University of Medical Sciences reviewed and approved the study protocol (Code: IR.BMSU.REC.1400.001).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflicts of interest.

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