Oncolytic reovirus enhances the effect of CEA immunotherapy when combined with PD1-PDL1 inhibitor by reducing the immunosuppressive tumor microenvironment in a colorectal cancer model

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Research Article

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Abstract

Immunotherapy is a type of tumor treatment that increases anti-tumor immunity to inhibit tumor growth. By enhancing the immune response through the lysis of tumor cells with an oncolytic virus and inhibiting the immune system's inhibitory reactions, the effectiveness of immunotherapy can be improved. In this study on a mouse model of colorectal cancer, the efficacy of oncolytic reovirus in a combined treatment with an adenovector expressing carcinoembryonic antigen (CEA) and PD-1/PD-L1 inhibitor was evaluated. The tumorized mice with CEA-expressing CT26 cells were immunized with a constructed adenovector expressing CEA along with PD-1/PD-L1 inhibitor. Then three doses of reovirus were injected into the tumor. On day of 26th, all mice were sacrificed, and tumor size, histopathological findings, and immune response to tumor antigens were compared among treatment groups. The results showed that immunization with CEA, combined with treatment with reovirus and PD-1/PD-L1 inhibitor, resulted in the lowest tumor growth among the treated groups. Additionally, the combined treatment group exhibited the highest level of cytotoxic immunity. This treatment also led to a decrease in Foxp3 in the tumor microenvironment and TNF-α secretion compared to other groups. Furthermore, through the production of IFN-γ and increased cytotoxic effect, it was demonstrated that the cellular immune system works more efficiently. Histopathological evaluations revealed the lowest number of mitosis and the highest amount of tumor-infiltrating lymphocytes (TILs) in this group. In conclusion, although the combination of tumor vaccines with oncolytic viruses improves treatment efficacy, inhibiting the PD-1/PD-L1 interaction can further enhance immunovirotherapy efficacy by reducing immunosuppressive effects boosted by the virus activity, and stimulating the immune system. This approach, in combination with other treatment methods, shows promise in controlling tumor growth.

Background

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide with high morbidity and mortality (1). Despite improvements in CRC treatment, the prognosis of patients with advanced CRC remains poor and the development of more effective therapeutic strategies is needed for these patients. The utilization of cancer immunotherapy as a therapeutic vaccine has emerged with the potential to stimulate and activate immune response through the detection of Tumor-associated antigens (TAA) and Tumor-specific antigens (TSA) (2). Many cancer patients suffer from the absence of an immune response against tumors as a result of immunological tolerance toward TAAs (3). Indeed, TAAs are overexpressed in tumor cells but often fail to induce sufficient immune responses. Thus, one of the main purposes in the cancer vaccine fields is to enhance TAA-specific cellular immune responses by allowing the delivery of different TAAs and decreasing the inhibitory signals and immunosuppressive conditions of the tumor environment that can stimulate effective antitumor immunity.

One of the TAAs is the carcinoembryonic antigen (CEA) which is overexpressed in CRC (4). Different cancer vaccines including recombinant adenovirus expressing CEA vaccine were developed to overcome this immune tolerance (5, 6). The adenoviral platform was chosen since it has been used as a gene delivery vehicle and vaccine design due to its genetic stability, high expression of encoded genes,

induction of strong immunity as well as safety in people (7). It has been demonstrated that CEA molecules play functional roles in cell adhesion, cancer progression, inflammation, signaling, angiogenesis and metastasis. Therefore, this protein is considered an attractive therapeutic target for tumor immunotherapy and activation of tumor-specific cytotoxic T lymphocytes (CTLs) (8). Antitumor responses rely on immune cell infiltration, especially infiltration of T cells, capable of modifying the natural disease progression and increasing the efficacy of immunotherapy. Based on modern scientific understanding, tumor types can be categorized as either hot or cold based on the level of immune cell infiltration. Hot tumors have a higher expression of neoantigens on their cell surface, making them more likely to be recognized by the immune system and to provoke a strong antitumor immune response. Numerous tumor-infiltrating lymphocytes (TILs) are present in this type of tumor. In contrast, non-immunogenic cold tumors are characterized by poor T-cell infiltration, low expression of neoantigens, low major histocompatibility complex (MHC) class I expression and low programmed cell death ligand 1 (PD-L1) (9, 10). Thus, promoting the conversion of cold tumors to hot tumors through various strategies can help reduce resistance to immunotherapy and enhance T-cell infiltration into tumors.

Although immunotherapy has emerged as a powerful tumor therapy, CRC is one of the cancers that respond poorly to current immunotherapies. To improve the antitumor effect in patients with CRC, combinations of immunotherapy and other treatments such as radiotherapy, chemotherapy, and targeted therapy are currently being explored (11).

Combination therapy with other treatment modalities such as oncolytic viruses can also maximize the efficiency of tumor cell killing and induce an antitumor immune response (12, 13). Reovirus, which is one of the oncolytic viruses, has proved to be a candidate in cancer treatments (14). Recent molecular biology research has revealed that reovirus relies on an activated Ras signaling pathway to replicate in tumor cells, leading to cell death through various mechanisms including apoptosis, necrosis, and immune-mediated pathways. Mutations in the ras proto-oncogene have been found in about 50% of colorectal cancers. Therefore, reovirus may have potential broad applicability in the treatment of cancers. In addition to direct lysis of cancer cells, reovirus can also release tumor-associated antigens and trigger an immune response in the tumor microenvironment (TME), potentially enhancing immunotherapy. It facilitates the priming of CTLs by presenting TAAs to CD4 + and CD8 + T cells and enhances TILs (15).

Another way to stimulate specific T cells is using immune checkpoint inhibitors which are effective against a variety of tumors (16). Some tumor cells can activate immune checkpoint molecules excessively and by taking advantage of this phenomenon, they can escape surveillance and clearance of immune cells, thus, promoting tumor progression (17). Programmed cell death protein 1- programmed cell death ligand 1 (PD-1/PD-L1) inhibitors have been used as a groundbreaking strategy in the immunotherapy of colorectal cancer and it has demonstrated that if there are TILs in the TME, the use of immune checkpoint inhibitors is more effective in the treatment of cancers (18, 19).In this study, we hypothesized that priming anti-tumor immunity by CEA overexpression and boosting with reovirus-induced immunity against TAAs, along with potentiating cellular immunity through inhibition of PD-1/PD-

L1 interaction, could induce greater anti-tumor immune activity and cytokine secretion than each one alone in a mouse colorectal cancer model.

Materials and Methods

Construction and preparation of Ad ΔE1/E3 CEA

Human CEACAM5/CEA/CD66e Gene ORF cDNA clone expression plasmid, C-GFP Spark tag (Cat Number: HG11077-ACG) was purchased from Sino Biological (Beijing, China). Adenovirus vector expressing CEA was constructed as described previously (20). Briefly, The CEA gene (2838bp) was inserted into the transfer plasmid. pAdenoVator ΔE1/E3 (AdenoVator system, Qbiogene) is a deficient E1 and E3 deleted human adenovirus type 5 backbone vector whose functions can be complemented in the Human Embryonic Kidney 293A (293A) cells. pAdenoVator is co-transformed with the transfer plasmid expressing CEA into E.coli strain BJ5183 by electroporation method.

To produce the recombinant adenovirus, the pAdenovector construct was cleaved with Pac I to linearize and expose its ITR (Inverted Terminal Repeats). Then, the linear plasmid was transfected using polyethylenimine (PEI) reagent into 293A cells. After 7 days when full CPE was achieved, the cells were collected and lysed by three freeze/thaw cycles at -80°C/37°C. The supernatant was transduced into the 293A cells in 25cm2 flask which was followed by incubation at 37°C for 72 h. The cells were analyzed for GFP expression using a fluorescence microscope. Furthermore, to confirm CEA protein production, the supernatant of the infected cells was measured for CEA by the electrochemiluminescence immunoassay (CLIA) (Elecsys CEA, Roche). Finally, the produced recombinant adenoviruses were further purified using gradient ultracentrifugation, tittered and kept in -70°C until further use.

Oncolytic reovirus propagation

L929 cells (mouse fibroblast cell line) were cultured in DMEM media which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% streptomycin (100 µg/ml)/penicillin (100 U/ml) and incubated at 37°C in a humidified atmosphere consisting of 5% CO2. Reovirus type 3 Dearing strain (T3D), (kindly provided by Dr soleimanjahi, Tarbiat Modares University, Tehran, Iran) was propagated in L929 cells and the virus titration was determined by tissue culture infectious dose 50 (TCID50) assay. For this purpose, a serial dilution of the reovirus was prepared and transduced to L929 cells cultured in the 96-well plate. After 48 h, the wells were checked for cytopathic effect (CPE), and virus titer was calculated with the Reed-Muench method.

Production of the stable CT26 cell line expressing CEA

Lenti-X 293T cells for lentivirus production and the mouse CRC cell line, CT26, were obtained from the Pasteur Institute (Tehran, Iran). As previously described, the CEA gene was cloned into the mammalian expression plasmid pCDH and according to the protocol, the recombinant lentivirus was produced. To generate the CT26 cells stably express CEA, the lentivirus was transduced to CT26 cell lines. The stable expression of CEA was confirmed in different passage numbers of the cells. The ability of cells to

establish tumors in mice was confirmed with GPF imaging of tumorized mice as described previously (21).

Tumor model establishment

For tumor treatment studies, male BALB/C mice, 6–8 weeks old and weighing 18-22g were purchased from the Institute of Royan Laboratory Animal Sciences (Tehran, Iran) and handled according to the guidelines and the ethical protocols of Tarbiat Modares University animal care Committee with code number of IR.TMU.REC.1396.726. Briefly, three mice were injected with 3×10^6 CT26 expressing CEA cells subcutaneously in the right flank. Once the tumor grew to approximately 600 mm^3, the mice were sacrificed and the tumor was removed under the sterile conditions and gently cut into approximately 1mm^3 sized fragments for use in implantation. About 40 mice were anesthetized with intraperitoneal injection of 20µl of a mixture of ketamine 10% (Bremer pharma GMBH, Germany) and xylene 20mg/ml (Bio beta, Czeh rep). After confirming the authenticity of the tumor cells in the tumor center sections through GFP expression and histopathology, the skin on the right flank of each mouse was incised with sterile scissors and the fragments were implanted. Six days after tumor implantation, when the tumors were palpable, the tumor-bearing mice were randomly divided into seven groups (n = 5 per group) according to Table 1.

Table 1
Grouping of mice and treatment administered.

Groups	Treatments
A	Adenovirus expressing CEA (Ad + CEA)
B	Adenovirus expressing CEA/Reovirus (Ad + CEA/Reo)
C	Adenovirus expressing CEA/Reovirus/PD-1/PD-l1 inhibitor (Ad + CEA/Reo/Adj)
D	Adenovirus empty (Ad control)
E	Adenovirus empty/ Reovirus (Ad control/ Reo)
F	Reovirus (Reo)
G	phosphate-buffered saline (PBS)

Mice Treatments

In groups A, B, and C, the mice received subcutaneous injections of adenovirus expressing CEA (Ad + CEA), and group D was treated with empty adenovirus as control at 1×107 TCID50 in 100µL PBS with two times per week intervals. To evaluate whether reovirus could inhibit tumor growth, Tumor-bearing mice received an intratumoral injection of reovirus at 1×107 TCID50 three times at 3-day intervals. To determine the additive effect of PD-1/PD-L1 inhibitor (Solarbio, China), a checkpoint inhibitor anticancer peptide that blocks the activity of PD-1 and PD-L1 immune checkpoint proteins present on the surface of cells, 50µg/dose of inhibitor was intratumoral injected into the mice according to the Table 2. In addition,

100μL of PBS was administered directly into the tumor-bearing mice in group G on days 10, 13, and 16 as blank control. Tumor growth was assessed by measuring two dimensions using digital calipers every 3 days and calculated as follows: Volume (mm^3 = (l x w^2) / 2 where the short dimension is “w”. The mice were sacrificed 10 days after the last injection (day 26). Spleens and tumors were removed under sterile conditions.

Table 2
Mice inoculation and experimental design.

Groups	Days post-transplantation				
	0	7	10	13	16
A	CEA Adenoviral vector	CEA Adenoviral vector			
B	CEA Adenoviral vector	CEA Adenoviral vector	Reovirus	Reovirus	Reovirus
C	CEA Adenoviral vector PD1-PDL1 inhibitor	CEA Adenoviral vector PD1-PDL1 inhibitor	Reovirus	Reovirus PD1-PDL1 inhibitor	Reovirus
D	Adenovirus control	Adenovirus control			
E	Adenovirus control	Adenovirus control	Reovirus	Reovirus	Reovirus
F			Reovirus	Reovirus	Reovirus
G	PBS	PBS	PBS	PBS	PBS

T cell cytotoxicity assay

To determine the T lymphocyte cell cytotoxicity of the spleen tissue, it was assessed by measuring Lactate dehydrogenase enzyme release from the damaged cells into the culture medium. The assay was performed using the LDH- CYTOX TM Assay Kit (BioLegend, USA) following the manufacturer’s instructions. Briefly, 2×104 CT26 cells expressing CEA as target cells were cultured in RPMI medium with 10% FBS in 96 cell culture plates and incubated for 24 hours at 37°C. For each well, 2×105 isolated lymphocytes from spleens as effector cells (effector/target ratio: 10/1) were co-cultured with CT26 cells for 6 h at 37°C. The absorbance was measured at reference wavelengths of 450 and 630 nm. It should be noted that all samples (5 mice in each group) were performed in triplicates.

Histopathological examination

To evaluate tumor histopathological changes such as; the rate of tumor infiltrating lymphocytes (TILs), mitotic count (per 10 high power field), and nuclear pleomorphism (Score 1–3), tumors were fixated in 10% formaldehyde, routinely embedded in paraffin wax, and sectioned into 4-μm thick. The slides were deparaffinized and stained with hematoxylin and eosin (H&E) using a standard method (22).

Immunohistochemistry

To determine the expression of CD8 and Foxp3 in tumor tissue, immunohistochemistry (IHC) was performed. Following deparaffinization of sections in ethanol and xylene, to retrieve antigens, the slides were incubated in citrate acid buffer (pH = 6) for 10 min at 95˚c and to block endogenous peroxidase activity were incubated in 10% hydrogen peroxide for 10 min. Then, the slides were stained with Foxp3+ (Mouse monoclonal antibody sc-166212, Santa Cruz Biotechnology, USA) and CD8+ (Mouse monoclonal antibody SP16, MAD-000318QD, Master diagnostic, Spain) primary antibody for 50 min and after washing, incubated with secondary antibody (HRP polymer, master diagnostic) for 40 min. The sections were detected with the Master Plus Detection System kit (HRP, DAB included; ref. MAD-000237QK) and the stained slides were visualized under a Zeiss fluorescence microscope (Germany). The number of lymphocytes and tumor cells reactivated with the antibodies was measured per 10HPF.

Cytokine assay

The secretory IFN-γ and TNF-α of the spleen lymphocytes were quantitatively analyzed according to manufacturer instructions ELISA kits (Mouse Tumor Necrosis Factor-alpha, Karmania, IRAN, Cat No: KPG-HTNF and Mouse IFN gamma ELISA Ready-SET-Go, eBioscience-USA, Cat No: 88–731). For this purpose, a suspension of spleen cells from each mouse was prepared and to remove the red blood cells, the lysis buffer was added. After centrifugation at 2500rpm for 5 min, the supernatant was collected, and performed cell count. The number of 4×10^6 cells was seeded in the 24-well plate in 1 mL of RPMI 1640 medium, supplemented with 10% FBS. To boost response, CT26 cells lysate (10μg/mL) was added to the wells and incubated for 72 hours at 37°C. After incubation, the supernatants were collected and the cytokines were measured by ELISA method.

Statistical analysis

Data were presented as the mean ± standard deviation (SD) and statistically were assessed by one-way ANOVA and analyzed using GraphPad Prism (version 8). A p-value less than 0.05 was considered statistically significant.

Results

Evaluation the treatments effects in inhibiting tumor growth in a colorectal cancer mouse model

Six days post-transplantation, when the tumor became palpable, the mice were treated with different agents and tumor growth was evaluated on days 12, 15, 18, 21, and 26 (Fig. 1). The obtained data were analyzed using ANOVA statistical test. In comparison with the groups treated with PBS and Ad control, the tumor volume of the Ad + CEA/ Reo/Adj treated group was restricted ($p < 0.03$). The group that received PBS showed a significantly enhanced tumor volume compared to the other groups.

Figure 1. The mice transplantation and tumor volume. After the mice were anesthetized by intraperitoneal injection ketamine/xylene, a small fragment (~ 1mm^3) of the tumor was transplanted into the right flank of animals subcutaneously. The tumor volume of mice monitored for 26 days (a). Mean ± SD values of tumor volume variation during treatment in different groups. Mean tumor volumes in the mice group treated with Ad + CEA/ Reo/ Adj (401.6 mm^3) and also the group treated with Adeno + CEA/ Reo (459.3mm^3) was smaller compared to the others bur significantly these two groups had difference with groups PBS, Adeno control and Adeno control/Reo (P: <0.05). The highest tumor volume was observed in the PBS group (1528.5 mm^3) and after that in the Adeno control group (1025.3 mm^3) in 26th day (b).

Cytotoxic effects of tumor specific CD8 + T cells

To determine functional cytotoxic T-cell responses in mouse models, we performed the LDH cytotoxicity release assay (Fig. 2). The cytotoxicity analysis revealed that the treated mice with Ad + CEA/Reo/Adj express higher lytic activity against tumor cells (61.8%) as compared to other groups. Overall, the result demonstrated that Ad + CEA/Reo/Adj (P: 0.006) and Ad + CEA/Reo (P: 0.01) groups could increase the higher cytolytic immune responses against tumor cells compared to the PBS group. No significant cytotoxicity differences were observed when the mice received Ad control, reovirus or PBS.

Figure 2. Induction of CTL-mediated tumor specific cytotoxicity following the various treatments. The results showed that the lysis rate of target cells in treated groups with Ad control/Reo (50.9%) (P: 0.029), Adeno + CEA (51.7%) (P: 0.014), Adeno + CEA/Reo (58.7%) (P: 0.01), Adeno + CEA/Reo/Adj (61.8%) (P: 0.006) groups had a significant difference in cytotoxicity when compared to the PBS (33.6%) control group. Also, the percentage of cytotoxicity in treated groups with Adeno + CEA/Reo/Adj was higher than the Reo (34.5%) group (P: 0.046). There was no significant difference in lysis rate among other groups.

Microscopic examination

To determine the anti-tumor activity of the treatments in vivo, the tumor sections were stained with hematoxylin and eosin (H&E). Histopathological, and morphological analyses were evaluated using pleomorphism and mitosis scoring in the studied samples. Nuclear pleomorphism is one of the earliest hallmarks of cancer progression and a feature characteristic of malignant neoplasms and dysplasia (23). In this study, cancer control groups (Ad control, PBS) had prominent nuclear pleomorphism and frequent mitosis (> 3/10 HPF) with score 3. In other groups with score 2 a lower pleomorphism was found. (Fig. 3a).

Furthermore, mitotic activity is a widely used criterion in cancer evaluation and is used to predict the disease (24). There was a significant difference between the PBS control group and other groups. The lowest mitotic count was observed in treated mice with Ad + CEA/Reo/Adj. (Fig. 3b)

The number of TILs was also examined in the pathology slides of all tumors. As shown in Fig. 3c There was a significant increase in the mean (SD) of TILs in Ad + CEA/Reo/Adj and Ad + CEA/Reo groups

compared to others and the lowest TILs count was observed in PBS treated mice and after that in Ad control group.

Figure 3. Evaluation of tumor tissue pathology by hematoxylin and eosin staining. After removing the tumor tissue of each mice group and preparing the slides by H&E staining, the slides were examined for mitotic count, pleomorphism and the amount of tumor TILs under a microscope (magnification, ×40) in 10 high field of view (10HPF). The yellow arrows in each image indicate cell dividing cells which are reported as mitotic count, the green arrows indicate TILs, and the black arrows indicate nuclear pleomorphism and the data were then compared statistically by mean + SD (a). The graph shows the analysis of mitotic counts and a significant difference was observed when comparing groups with each other (b). The graph shows the analysis of number of TILs (c). The significant results are represented as follows: *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$

In addition, the mean number of two markers CD8 and Foxp3 in tumor tissue of each mouse group were investigated by immunohistochemistry technique. We obtained statistically ambiguous results when each factor was analyzed separately. However, when the ratio of CD8 to Foxp3 was measured, it was observed that combination therapy of recombinant Adenovirus with Reovirus and PD-1/PD-L1 inhibitor had the highest ratio of CD8/Foxp3 in tumor tissue compared to other mice groups (Fig. 4) which shows the decrease of inhibitory conditions in tumor microenvironment.

Figure 4. Statistical analysis of CD8/Foxp3 ratio. Immune system stimulation in tumor tissue was evaluated through measuring the ratio of CD8 to Foxp3 markers on T cells. Immunostaining of $CD8^+$ (right image) and $Foxp3^+$ (left image) immune cells. In each image the positive lymphocytes have been marked with a black arrow (a). The graph shows the analysis of the obtained data from the ratio of the two markers (CD8/Foxp3) in the mice groups with different treatments (b). The results showed that the Adeno + CEA/Reo/Ad group had a significant difference with Adeno control (P: 0.026), Adeno control/Reo (P: 0.025), and PBS (P: 0.024) groups. Although Adeno + CEA had the highest ratio of $CD8^+$ to Foxp3 after Adeno + CEA/Reo/Ad group, there was no significant difference between this group and the other groups.

The effects of treatments on IFN-γ and TNF-α production in mice

To measure the amount of cytokines secreted from spleen cells in each mice group, the spleen cells were exposed to CT26 cells lysate. The supernatant was collected after 72 hours and IFN-γ and TNF-α cytokines concentration were measured using ELISA kit. From this analysis, the group that received Ad + CEA/Reo/Adj, and after that, the Ad + CEA/Reo group produced the lowest level of TNF-α while, the highest level of this cytokine was observed in the PBS group. There was no statistically significant difference between mice injected with Reovirus, Reovirus along with adenovirus control, adenovirus control, and adenovirus expressing CEA.

Analysis of data from IFN-γ cytokine assay showed that mice injected with Ad + CEA/Reo/Adj and after that Ad + CEA/Reo group produced the highest and PBS group produced the lowest level of this cytokine. As shown in the diagram, although there was a significant difference in level of IFN-γ in all groups compared to mice injected with PBS, but in the treatment of the mouse group with a combination of adenovirus expressing the CEA gene and Reovirus, a much higher difference was observed. In addition, the injection of PD-1/PD-L1 inhibitor also confirmed its effective role in stimulating further production of this cytokine (Fig. 5).

Figure 5. Evaluation of cytokine levels. Diagram shows TNF-α concentration in studied mice and a significant difference was observed when comparing the level of TNF between the group which received Ad + CEA/Reo/Adj and Ad + CEA/Reo with other groups ($P < 0.001$) in these two mice groups the level of TNF-α was lower than others. Also, There was a significant difference between PBS and other groups (P: <0.001) which the mice injected with PBS had a higher amount of TNF-α cytokine than other mice (a). Diagram shows IFN-γ concentration in each mouse group with different treatments. The results showed a significant difference between Ad control and Reo groups (P: 0.001), Ad control/Reo and Reo groups (P: 0.02), Ad + CEA and Reo (P: 0.018) and other groups (P:<0.001). There was no significant difference between Ad control and Ad + CEA groups (b).

Discussion

In this study, the efficiency of oncolytic reovirus, and PD-1/PD-L1 inhibitor in combination with adenovector vaccine expressing CEA, was evaluated in induction of anti-tumor response in a colorectal tumor of mice model.

Numerous studies have explored strategies to enhance the response of colorectal cancer to immunotherapy, improve T cell priming, and facilitate their homing to the tumor bed for more effective therapeutic protocols. Immune tolerance to TAAs represents a significant barrier to antitumor responses. To address this challenge, researchers have investigated the use of tumor antigen-expressing recombinant virus vaccines in human clinical trials. A previous study involving metastatic colorectal cancer demonstrated that the CEA ETBX-011 (Ad5-CEA) vaccine exhibited antitumor activity and showed potential evidence of a survival benefit (25). Other research has indicated that immunization with CEA peptides can trigger an immune response to eliminate cancer cells, potentially improving survival, breaking immune tolerance to CEA, and inducing CEA-specific T cell responses in transgenic mice and colon cancer patients (26, 27). However, immunologic tolerance has also been observed following the subcutaneous injection of CEA-expressing cell lines into mice. Subsequent investigations revealed that despite the presence of anti-CEA antibodies, the tumors were still able to grow (28, 29).

While overexpression of CEA at the tumor site could potentially overcome immune tolerance, the study results indicate that administering recombinant adenovirus expressing CEA alone in mice as a monotherapy is not effective in significantly breaking immune tolerance to CEA. This lack of effectiveness may be attributed to the tumor's immunosuppressive TME. A promising class of

anticancer treatments is oncolytic reovirus which helps to change immunologically cold tumors to hot tumors and causes the proliferation of immune cells in the tumor environment (30). Reovirus as an antitumor agent has been evaluated in many clinical trials (31). This virus preferentially replicates in patients possessing KRAS mutated cells induces apoptotic responses and downregulates angiogenesis-related genes, therefore, it can be an effective CRC treatment (32, 33). In addition to its oncolytic activity, it has been shown that reovirus can induce the release of antigens such as TAA from infected tumor cells into TME, which is important for inducing potent antitumor immunity (34).

Reovirus has the potential to enhance apoptotic signaling and make tumor cells more sensitive to chemotherapeutic agents. Several phase I and II clinical trials are currently investigating the effectiveness of the T3D strain reovirus in combination with chemotherapeutic agents or radiotherapy against various cancers. We hypothesized that priming the immune system with an adenovirus encoding CEA and then boosting it with reovirus could provoke a robust immune response against CEA.

When tumor cells are infected, they release viral progeny, cytokines, and TAA, which enhance adaptive anti-tumor immunity. On the other hand, the induction of anti-tumor immune responses by the virus leads to the activation of regulatory immune mechanisms, including the upregulation of immune checkpoints and increase the level of regulatory T cells which boosts the immune-suppressive nature of the tumor microenvironment. To further stimulate the immune system, a PD-1/PD-L1 inhibitor was administered to reduce the levels of regulatory T cells and immune checkpoints.

The experiments showed that injecting reovirus into the vaccinated mice decreased the ratio of CD8 to Foxp3. However, adding a PDL1/PDL1 inhibitor significantly increased this ratio. This indicates that a reducing agent for the tumor suppressive environment is necessary for the greater efficiency of combined immunovirotherapy treatment due to the fact that reovirus can upregulate immune checkpoint molecules and also promote the accumulation of Tregs which could impede CTL responses. It is well known that Treg cells express FOXP3 and suppress antitumor immune responses and in patients with CRC, the number of Treg cells is increased in the TME (35). The literature showed that a high CD8/Foxp3 ratio in the intra-tumoral is associated with improved survival and has a positive effect on prognosis in several tumors, including colorectal cancer and breast cancer (36, 37). A meta-analysis of TIL phenotyping indicated that individual lymphocytic subtypes could not predict survival and have less prognostic potential than CD8/Foxp3 (38, 39).

Like the previous data published on the effect of Foxp3 + cells in CRC, our study also confirmed that the evaluation of the presence of Foxp3 + cells alone in tumors had no prognostic value it is also important to note.

Decreases in the number of Tregs may allow the differentiation of other CD4 + T cells into effector T cells and increase the activity of tumor antigen-associated T cells. Therefore, we expected spleen cytolytic T cell function would enhance as the T cells foxp3 + decreased and the TILs increased. For this purpose, we performed the LDH cytotoxicity release assay and the results demonstrated that a stronger cytolytic immune response against tumor cells occurred in Ad + CEA/reovirus/PD1-PDL1 inhibitor and Ad +

CEA/reovirus groups than in other groups. In addition, IFN-γ interacts with T-cells to stimulate their differentiation toward the Th1 subset and stimulates its production in Th1 cells and since Tregs are inhibited by IFN-γ, these data are consistent with our result. So, it could be confirmed that cytolytic T-cell function was enhanced in combination therapy.

We found that administration of combined Ad + CEA/Reovirus/PD-1/PD-L1 inhibitor did result in reduced tumor size compared to Ad + CEA/Reovirus or Ad + CEA and reovirus as monotherapy. Although there was a statistically significant difference in gross tumor volume between Ad + CEA/Reovirus/PD-1/PD-L1 inhibitor combination and PBS or Ad + control, this difference was not significant with other groups which may be due to the infiltrating the immune cells and more cavity formation in test groups.

TNF-induced inflammation seems to play a key role in tumor-genesis of CRC and promoting oncogene expression levels. It was found that in patients with CRC, TNF-α mRNA overexpression and higher TNF-α serum levels have been associated with tumor progression and reduced patient survival (40–42). Conversely, high amounts of IFN-γ are required for an efficient antitumor response. The result of our experiments demonstrated a significant decrease in the TNF-α amount and an increase in the IFN-γ secretion after the combination therapy. It is noteworthy that the injection of PD-1/PD-L1 inhibitor also confirmed its effective role in stimulating further secretion of IFN-γ and reduction of TNF-α and providing these conditions in the TME may be more effective for the activity of Ad + CEA vaccine. By comparing the amount of these cytokines in the two groups of monotherapy (Ad + CEA and reovirus) there was no difference in the level of TNF-α, but the amount of IFN-γ was higher in the reovirus group.

TILs play a crucial role in interpreting tumor genesis and predicting a clinical biologic outcome. Herein, the highest increase in TILs among the groups was observed in the treatment group with Ad + CEA/Reovirus/PD-1/PD-L1 inhibitor which corresponds with other studies of adenovector encoding CEA and anti-PD-1 antibody in the treatment of colorectal cancer (43, 44).

In Conclusion, the antitumor effect of immunotherapy and virotherapy was investigated separately and in combination in colorectal cancer in the Balb/C mouse model. The results demonstrated that the administration of immunotherapy or virotherapy separately are not effective in stimulation of antitumor immune response and their combination is needed for the optimization of treatment. Regarding the induction of more inhibitory factors in the tumor microenvironment by the use of oncolytic reovirus, the addition of an agent for reducing the suppressive effect of TME improves the immunoviroterapy effects.

Abbreviations

Ad: adenovirus

ANOVA: analysis of variance

CEA: carcinoembryonic antigen

ELISA: enzyme-linked immunosorbent assay

IFNg: interferon gamma

MHC: major histocompatibility complex

PBS: phosphate buffered saline

PD1: programmed death 1

PD-L1: programmed death ligand 1

TAA: tumor-associated antigen

TIL: tumor-infiltrating lymphocyte

TNFa: tumor necrosis factor alpha

Treg: regulatory T-cell

Declarations

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Ethics approval and consent to participate

All animal experiments were performed according to the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and were approved by Tarbiat modares University animal care Committee with approval code number of IR.TMU.REC.1396.726.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

There are no competing interests.

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Authors' contributions

Study concept and design: T. B. Acquisition of data: A. Y., S.A., N. H. Analysis and interpretation of data: A. Y., M.F., and T. B. prepared the manuscript: A. Y. Critical revision of the manuscript for important intellectual content: T. B. Technical and material support: T. B. and S. Y. H. Study supervision: T. B. and S. Y. H. All authors approved the final manuscript.

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Figures

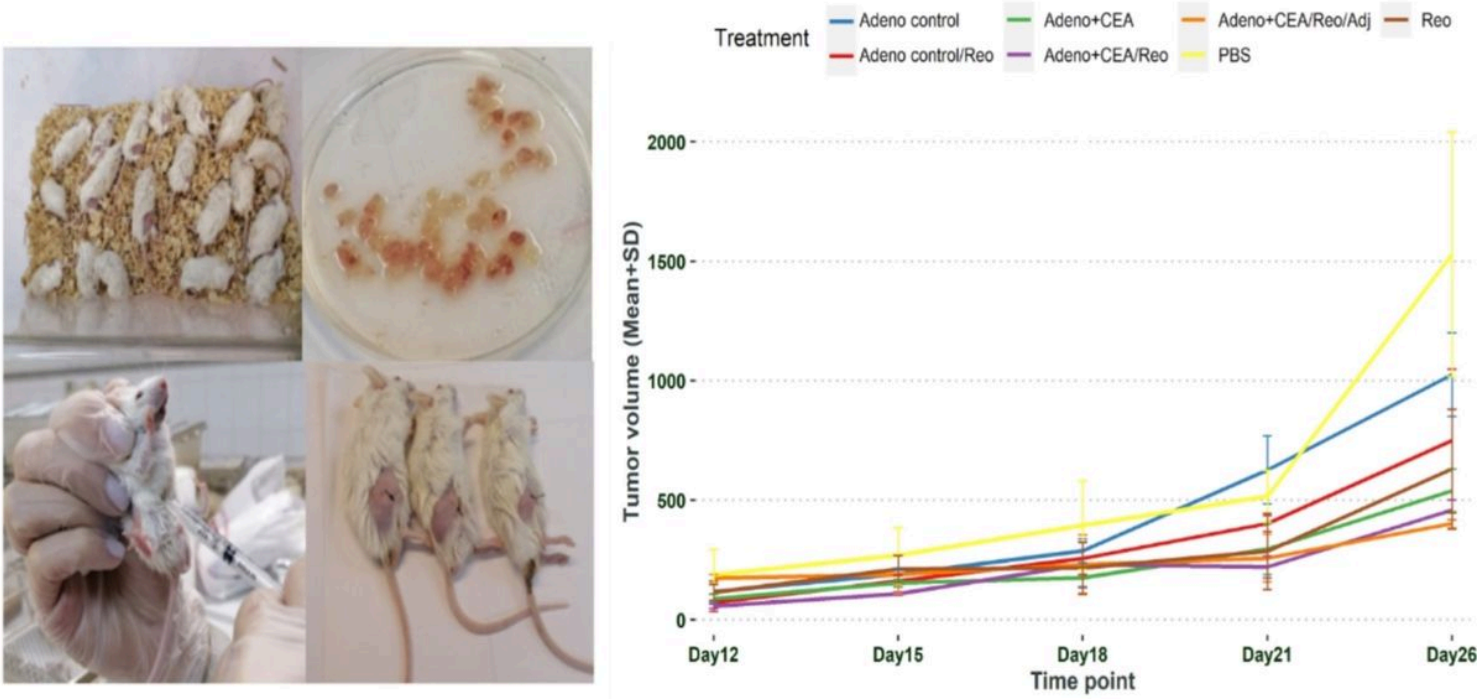

Figure 1

The mice transplantation and tumor volume. After the mice were anesthetized by intraperitoneal injection ketamine/xylene, a small fragment (~1mm^3) of the tumor was transplanted into the right flank of animals subcutaneously. The tumor volume of mice monitored for 26 days (a). Mean ± SD values of tumor volume variation during treatment in different groups. Mean tumor volumes in the mice group treated with Ad+CEA/ Reo/ Adj (401.6 mm^3) and also the group treated with Adeno+CEA/ Reo (459.3mm^3) was smaller compared to the others bur significantly these two groups had difference with groups PBS, Adeno control and Adeno control/Reo (P: <0.05). The highest tumor volume was observed in the PBS group (1528.5 mm^3) and after that in the Adeno control group (1025.3 mm^3) in 26th day (b).

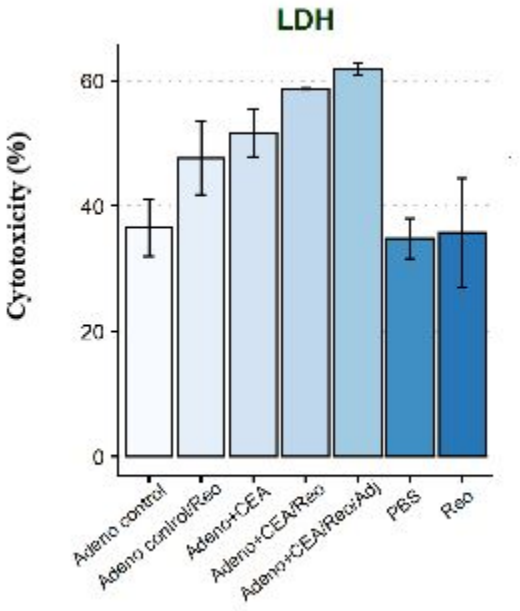

Figure 2

Induction of CTL-mediated tumor specific cytotoxicity following the various treatments. The results showed that the lysis rate of target cells in treated groups with Ad control/Reo (50.9%) (P: 0.029), Adeno+CEA (51.7%) (P: 0.014), Adeno+CEA/Reo (58.7%) (P: 0.01), Adeno+CEA/Reo/Adj (61.8%) (P: 0.006) groups had a significant difference in cytotoxicity when compared to the PBS (33.6%)control group. Also, the percentage of cytotoxicity in treated groups with Adeno+CEA/Reo/Adj was higher than the Reo (34.5%) group (P: 0.046). There was no significant difference in lysis rate among other groups.

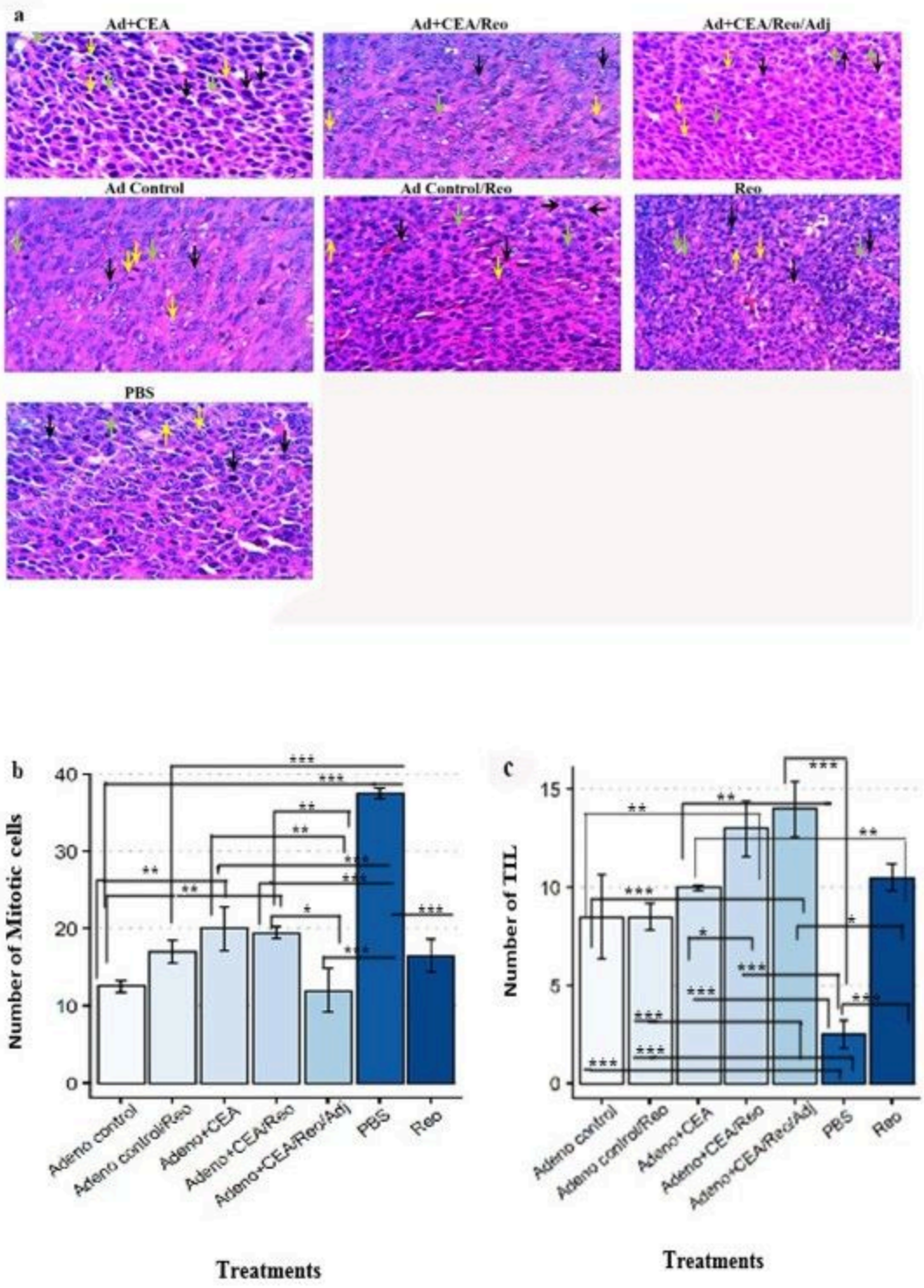

Figure 3

Evaluation of tumor tissue pathology by hematoxylin and eosin staining. After removing the tumor tissue of each mice group and preparing the slides by H&E staining, the slides were examined for mitotic count, pleomorphism and the amount of tumor TILs under a microscope (magnification, ×40) in 10 high field of view (10HPF). The yellow arrows in each image indicate cell dividing cells which are reported as mitotic count, the green arrows indicate TILs, and the black arrows indicate nuclear pleomorphism and the data

were then compared statistically by mean+SD (a). The graph shows the analysis of mitotic counts and a significant difference was observed when comparing groups with each other (b). The graph shows the analysis of number of TILs (c). The significant results are represented as follows: *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$

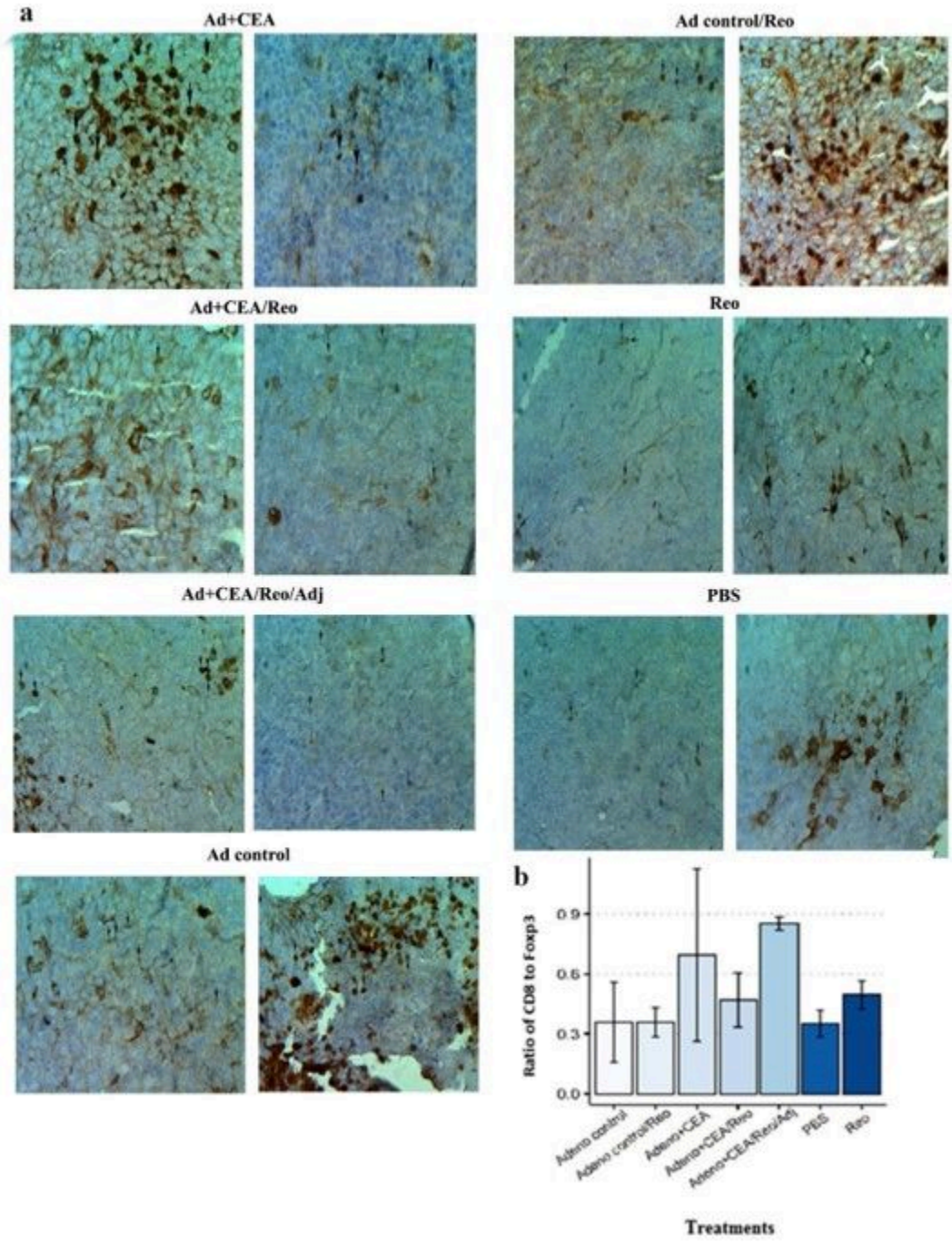

Figure 4

Statistical analysis of CD8/Foxp3 ratio. Immune system stimulation in tumor tissue was evaluated through measuring the ratio of CD8 to Foxp3 markers on T cells. Immunostaining of $CD8^+$ (right image) and $Foxp3^+$ (left image) immune cells. In each image the positive lymphocytes have been marked with a black arrow (a). The graph shows the analysis of the obtained data from the ratio of the two markers

(CD8/Foxp3) in the mice groups with different treatments (b). The results showed that the Adeno+CEA/Reo/Ad group had a significant difference with Adeno control (P: 0.026), Adeno control/Reo (P: 0.025), and PBS (P: 0.024) groups. Although Adeno+CEA had the highest ratio of $CD8^+$ to Foxp3 after Adeno+CEA/Reo/Ad group, there was no significant difference between this group and the other groups.

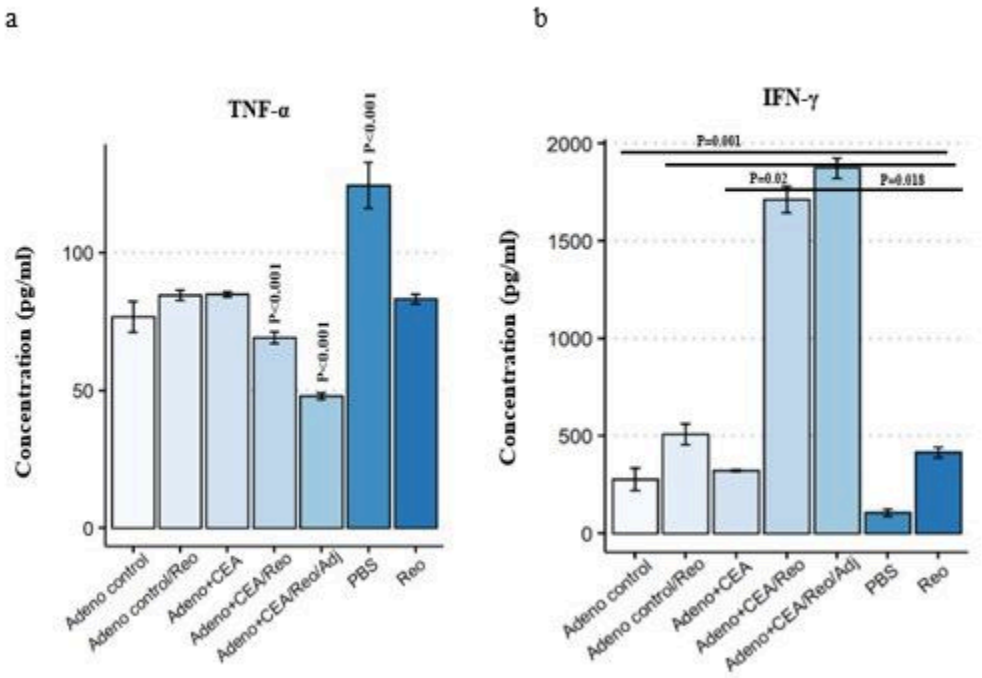

Figure 5

Evaluation of cytokine levels. Diagram shows TNF-α concentration in studied mice and a significant difference was observed when comparing the level of TNF between the group which received Ad+CEA/Reo/Adj and Ad+CEA/Reo with other groups (*P*<0.001) in these two mice groups the level of TNF-α was lower than others . Also, There was a significant difference between PBS and other groups (*P*: <0.001) which the mice injected with PBS had a higher amount of TNF-α cytokine than other mice (a). Diagram shows IFN-γ concentration in each mouse group with different treatments. The results showed a significant difference between Ad control and Reo groups (*P*: 0.001), Ad control/Reo and Reo groups (*P*: 0.02) , Ad+CEA and Reo (*P*: 0.018) and other groups (*P*:<0.001). There was no significant difference between Ad control and Ad+CEA groups (b).