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In vitro and *in vivo* Evidence on Intra-tumor Injection of Allogeneic Serum for Immunotherapy in a Mouse Model of Colon Cancer

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ABSTRACT

It is believed that preformed antibodies are responsible for blood transfusion reactions and transplant rejections. In order to remove a tumor, the tissue must be rejected. On the basis of transfusion reaction and transplantation immunology, we hypothesized that allogeneic serum can inhibit tumor growth when injected intra-tumor.

Initially, an in vitro cytotoxicity test was conducted using the C57BL/6 serum (intact or decomplemented) in combination with the BALB/c-originating CT26 cell line. The CT26 cell line was used to establish a mouse model of colon cancer. When the tumor was palpable, C57BL/6 serum was injected intra-tumor. In addition to tumor size, hypoxia, metastatic capacity, angiogenesis, and metabolic and inflammatory status, we evaluated matrix metalloproteinase-2 (MMP)-2 and 9, vascular endothelial growth factor (VEGF)-A, Cluster of Designation (CD) 31, CD38 and interleukin (IL)-10.

An in vitro experiment showed that heat-inactivated C57BL/6 serum had significantly lower cytotoxic effects on BALB/c-derived CT26 cells than intact C57BL/6 serum or BALB/c serum. In vivo experiments revealed that tumor size, HIF-1α, MMP-2, and MMP-9 levels were significantly lower in the experimental group than in the control group.. In contrast, to control animals, allogeneic serum treatment led to marked reductions in CD31, VEGF-1, CD38, and IL-10 levels.

A new approach to serum or plasma therapy and allogeneic vaccines for cancer is the intra-tumor injection of allogeneic serum. In light of the ease and availability of allogeneic immunotherapies, allogeneic serum and plasma therapy could potentially be used as an alternative monotherapy or in combination with other therapies.

Keywords: Allogeneic serum; Angiogenesis; Cluster of designation 38; Hypoxia-inducible factor 1; Alpha subunit; Interleukin-10; Matrix metalloproteinases

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INTRODUCTION

Recent advances in the last decade in the treatment of cancer using passive immunotherapy, surgery, radiotherapy, and chemotherapy have led to a promising

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outcome. However, cancer is considered an incurable disease. To devise new approaches, there has been a focus on affecting tumor metabolism and inducing inflammation to combat tumor hypoxia, angiogenesis, and metastasis.[1] Passive immunotherapy using antibodies relies on the administration of antibodies that can bind to their corresponding tumor antigens. Tumor-specific antibodies can induce the complement system activation, promote antibody-dependent cell-mediated cytotoxicity (ADCC), or block signaling pathways involved in growth leading to reduced proliferation or apoptosis.[2]

Rejection of organ transplantation can occur due to the binding of preformed antibodies to the corresponding antigen leading to activation of the complement system and cell lysis.[3,4] Preformed antibodies have been found against major histocompatibility complexes (MHC), minor histocompatibility complexes (mhc), and blood group antigens.[5-9] Such preformed antibodies and their corresponding antigens are an essential obstacle in transplantation.[3] Nevertheless, this is a downside of the clinical aspect of preformed antibodies. We hypothesized that such a natural phenomenon might also have an upside feature to be utilized for tumor treatment. There are reports indicating the expression of some blood group antigens, mhc , and even MHC in tumor cells.[10-16] It prompted us to benefit from such a natural process of inflammation induction by preformed antibodies at the tumor loci. On this basis, allogeneic serum from *C57*BL/6 donor mice was administered via the intra-tumor route to a BALB/c mouse model of colon cancer. To evaluate the hypothesis, the tumor size, hypoxia, angiogenesis, metastatic capacity, and metabolic and inflammatory status are represented by Hypoxia Inducible Factor-1α (HIF-1α), a cluster of designation 31(CD31) and vascular endothelial Growth Factor (VEGF)-A, Matrix metalloproteinase (MMP)-2 and 9, CD38, and interleukin 10 (IL-10), respectively, were evaluated. Tumor growth is accompanied by the development of hypoxic conditions, which help the acquisition of metastatic ability and favor the progression of dysfunctional vascularization.[17-24] In addition, hypoxia affects tumor metabolism, making an immunosuppressive microenvironment within tumor loci, promoting more tumor growth and invasion.[19,25-27]

MATERIALS AND METHODS

Animals

Female 8-10 week old BALB/c and C57BL/6 mice (Royan Institute, Karaj, Iran) were used to induce mice tumor model of colon cancer and prepare allogeneic serum from *C57*BL/6 mice donors, respectively. The mice were given free access to food and water, kept in standard conditions, and housed for one week before the experiments started. All experiments were approved by the Student Research Committee of Alborz University of Sciences, under reference number IR.ABZUMS.REC.1399.266, and performed according to the Animal Care and Use Protocol of Alborz University of Medical Sciences**.**

Cell Line and Preparation of the Mouse Colon Cancer Model

The CT26 mouse colon cancer cell line was purchased from Pasteur Institute, Cell Bank of Iran (NCBI, Tehran, Iran). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine (Thermo Fisher Scientific), and 100 IU/mL streptomycin and 100 IU/mL penicillin at 37°C in 5% CO2 atmosphere.

To do in vitro experiment and evaluate the cytotoxic effects of allogeneic serum on the CT26 tumor cell line, a reaction between BALB/c-originating CT26 cell line and C57BL/6 serum was set up on a plate and incubated at 37°C for 30 min. A similar reaction was set with heat-inactivated C57BL/6 serum (56°C:30 min). As a control, a BALB/c-originating CT26 cell line treated with intact or heat-inactivated serum from BALB/c mice was also established.

To establish the BALB/c mouse tumor model, a 0.10 ml suspension containing 7×10^5 CT26 cells was injected subcutaneously into the dorsal flank regions.[28] After the tumor mass was palpable, cages were coded, and animals were divided randomly into two groups (n=5), including test and control groups treated with allogeneic serum from C57BL/6 mice donor or phosphate buffer saline (PBS), respectively. Tumor size was measured every other day for all mice using a caliper. Growth curves were prepared based on tumor volume calculated based on the following formula:

$$Tumor\ Volume=(length\times width^2)/2$$

Tissue Preparation for Immunohistochemical Staining

After completion of the treatment program (Figure 1), the mice were anesthetized using a mixture of ketamine and xylazine, and the tumors were removed. The dissected tumors were fixed in 10% neutral buffered formalin. This was followed by paraffin embedding, and 5 µm-thick sections were prepared on a rotary microtome (Leica, Germany). The sections were placed on polylysine-coated slides and underwent immunohistochemical staining.[29] The tissue sections were blocked with 0.3% Triton X-100 and 10% goat serum in PBS (pH 7.3) for 30 min. Primary antibodies, including anti-cluster of differentiation 31 (CD31), anti-CD38, and anti-interleukin-10 (IL-10) (Biorbyt, Cambridge, UK), all of which originated from rabbits, were then added. The slides were incubated overnight at room temperature. After washing with 0.01M phosphate-buffered saline (PBS), the tissue sections were incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Biorbyt, Cambridge, UK) diluted in 0.01-M PBS as the secondary antibody for 2 h at room temperature. After rinsing with 0.01 M PBS, the sections were stuck to glass slides and observed using a fluorescence microscope. 4'-6-diamidino-2-phenylindole (DAPI) was used for nuclei staining in each section. Quantification and analysis of the immunohistochemically stained tissue sections were performed after taking digitized images using a Zeiss Axioplan 2 fluorescence microscope. ImageJ software (version: 1.52 h) was used to analyze the digitized images by an observer blinded to the origin of the sample.

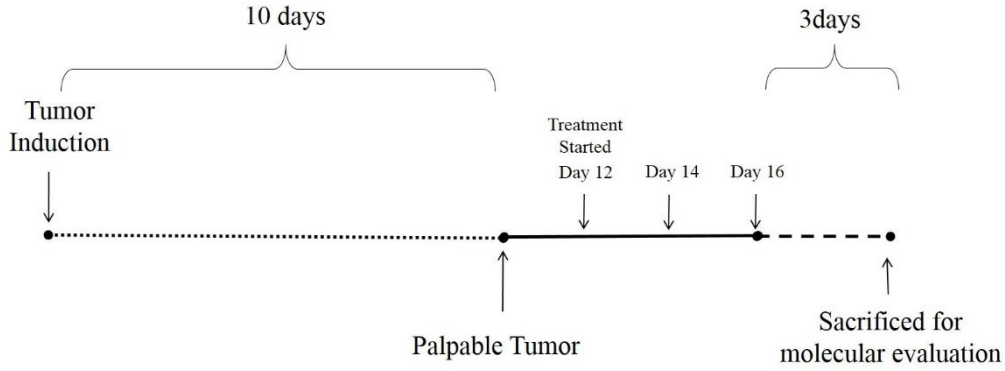

Figure 1. Protocol of intra-tumor injection of allogeneic serum. Ten days after tumor implantation using the CT26 cell line in BALB/c mice, the animals were treated with allogeneic serum prepared from C57BL/6 mice. Treatment with allogeneic serum was performed via the intra-tumor route every other day in the tumor center. Three days after the last treatment, the animals were euthanized and molecular evaluations were performed.

RNA Extraction and Real-time PCR

Total RNA was extracted from frozen tumors using TRIzol™ Reagent (Invitrogen) according to the standard protocol and a previous report.[30] Briefly, TRIzol™ Reagent and chloroform were added to the tissue, vortexed to homogenize, and centrifuged for 15 min at 12000 RPM at 4°C. The supernatant was decanted, isopropanol was added, incubated for 10 min, and centrifuged for 10 min at 12000 RPM at 4°C. The supernatant was discarded, and the pellet was resuspended in 70% ethanol, vortexed briefly, and centrifuged for 5 min at 7500 RPM at 4°C. The supernatant was removed, and the pellet was air-dried and resuspended in RNase-free water. NanoDrop 2000c (Eppendorf, Germany) was used to determine the quality and quantity of RNA concentrations. mRNA expression for hypoxanthine phosphoribosyl transferase (HPRT), HIF-1α, VEGF-A, MMP-2, and 9 were determined using an ABI Step One Plus (Applied Biosystems, Sequences Detection Systems, Foster City, CA) thermocycler and SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Paisley, United Kingdom) according to the manufacturer's instructions. Each reaction contained a 10 µL master mix, 1 µL (100 nM) primers for HPRT, HIF-1α, VEGF-A, MMP-2 and 9, and 1 µL (200 ng) template cDNA synthesized with cDNA kits (Parstous, Tehran, Iran) and 8 µL diethylpyrocarbonate (DEPC) water. The sequences for primers are presented in Table 1 supplementary material. The primers' efficiency,

specificity, and fidelity of real-time PCR and melting curve analysis were determined as before.[30] Thermocycler conditions included an initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 56-63°C for 30 sec (the annealing temperature of each primer), and 72°C for 30 sec. The HPRT gene was chosen as the internal control against which the mRNA expression of the target gene was normalized.[31,32] The resultant gene expression level was presented as $2^{-\Delta\Delta Ct}$, in which ΔCt was the difference between Ct values of the target gene and reference gene.[30]

Statistical Analysis

Statistical operations were performed using GraphPad Prism software (GraphPad Software, San Diego, CA) to analyze the data using the t-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc procedure to compare two or multiple groups, respectively. The tumor size results were analyzed using two-way ANOVA and the Bonferroni post hoc procedure. Differences were considered statistically significant when the *p* value was less than 0.05.

RESULTS

Complement-mediated Tumor Cell Lysis

Initially, an in vitro experiment was performed to evaluate the cytotoxic effects of allogeneic serum on the CT26 tumor cell line. To do so, a reaction between BALB/c-originating CT26 cell line with C57BL/6 serum or heat-inactivated C57BL/6 serum was set. As a control, the CT26 cell line originating from BALB/c was also considered with intact serum or heat-inactivated serum from BALB/c mice. The percentage of cell viability was determined using trypan blue staining. Results demonstrated that, unlike the intact allogeneic serum, treatment of the CT26 tumor cell line in heat-inactivated form did not exert significant cytotoxic effects on the cells ($p<0.001$, Figure 2).

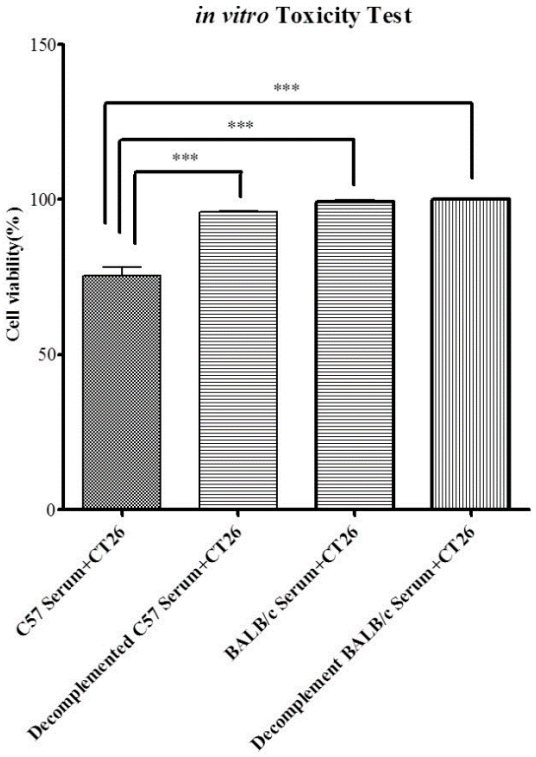

Figure 2. ***In vitro*** **cytotoxicity assay of allogeneic serum prepared from C57BL/6 mice against BALB/C mice-originating CT26 cell line. Reactions were set in triplicate, including the C57BL/6 mice serum+CT26 cell line, heat-inactivated C57BL/6 mice serum+CT26 cell line, BALB/c mice serum+CT26 cell line, and heat-inactivated BALB/c mice serum+CT26 cell line. The last two reactions were used as control. In each case, a serum pool was prepared from 3 mice. The percentage of cell viability was calculated as [number of viable cells/(number of dead cells + viable cells)] × 100. *p value<0.05; data are expressed as the means±SEM.**

Tumor Size, Hypoxia, and Metastatic Potential

Measurement of tumor size is one of the methods available to assess the efficacy of immunotherapy.[17,18] Evaluation of tumor size (Figure 3a) during the course of the study revealed that intra-tumor administration of allogeneic serum could potently slow down tumor growth compared with the control group on day 7 (p<0.01) and 10 (p<0.0001) after treatment started.

Measurement of tumor size alone is insufficient to assess the effects of treatment on cancers.[18] It is also important to evaluate tumor hypoxia and its metastatic ability. In parallel with tumor size, hypoxia, represented by HIF-1α, was markedly decreased after treatment with allogeneic serum (p<0.0001; Figure 3b). Hypoxia helps develop the epithelial-mesenchymal transition process, resulting in cell mobility and metastasis.[19] MMP-2 and MMP-9 were also evaluated as indicators of metastatic potential.[20] The levels of MMP-2 and MMP-9 mRNA were markedly decreased in animals treated with an intra-tumor injection of allogeneic serum compared to control animals (p<0.001; Figures 3c and d).

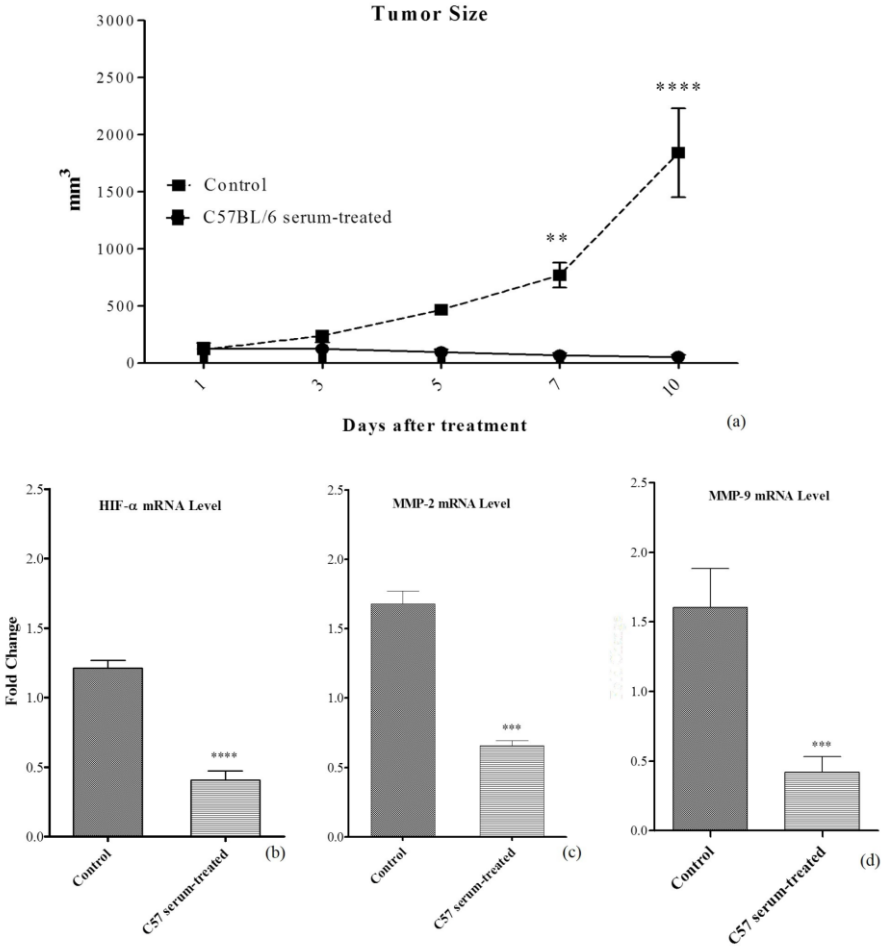

Figure 3. Effect of intra-tumor administration of allogeneic serum on tumor size (a), hypoxia (b), and metastatic potential (c). Injection of allogeneic serum via the intra-tumor route was performed in three doses every other day. Tumor dimensions were measured using a caliper (vernier) every other day, and tumor size was calculated as described in the Materials and Methods section. Three days after the last injection, the animals were sacrificed, tumors were isolated, RNA was extracted, and cDNA was synthesized. Real-time PCR using cyber green was performed, and the quantification of each gene was normalized against HPRT as the reference gene. Hypoxia and metastatic capacity were evaluated based on mRNA expression of Hypoxia Inducible Factor (HIF)-1α or Matrix Metalloproteinase (MMP)-2 and 9, respectively, in tumor loci from the control and treatment groups. *p value<0.05; data are expressed as the means±SEM.

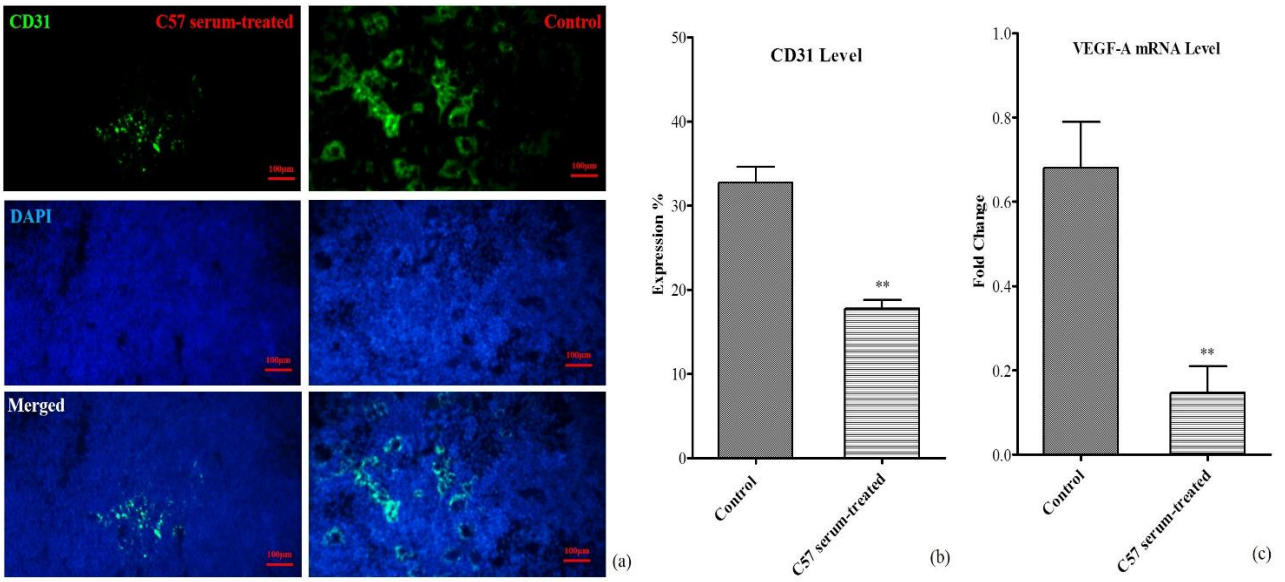

Figure 4. Effect of intra-tumor injection of allogeneic serum on angiogenesis at tumor sites. Cluster of Designation (CD)31 was detected by immunohistochemical staining of serial tumor sections using a corresponding antibody (a). Expression levels of CD31 were evaluated in tumor tissue sections (b). To quantify Vascular Endothelial Growth Factor (VEGF)-A mRNA level, hypoxanthine phosphoribosyl transferase (HPRT)was used as the reference gene (c). *p value<0.05; data are expressed as the means±SEM.

Decrease of Angiogenesis by Intra-tumor Injection of Allogeneic Serum

Hypoxia also helps the progression of dysfunctional vascularization.[19] VEGF-A and CD31 were evaluated as well-defined markers of angiogenesis. CD31 is established for the monitoring of vessel density in tumors, as it is highly expressed on the surface of endothelial cells and involved in angiogenesis. On this basis, CD31 has even been used as a prognostic marker.[21,22] VEGF-A is an important growth factor and signaling molecule involved in vasculogenesis and angiogenesis. The expression of VEGF-A has also been suggested to have prognostic significance.[23,24] Intra-tumor injection of allogeneic serum led to decreased CD31 expression compared to the control (p<0.01; Figures 4a and b). Consistently, the VEGF-A mRNA level was also decreased in the group treated with allogeneic serum via the intra-tumor route compared to the control group (p<0.01; Figure 4c).

CD38 and IL-10 Levels Following Intra-tumor Injection of Allogeneic Serum

Hypoxic conditions within tumor loci affect tumor cell metabolism.[19] CD38 is an ectoenzyme that participates in making an immunosuppressive microenvironment within tumors by maintaining the adenosine pathway.[25,26] Injection of allogeneic serum at the tumor center led to a significant decrease in CD38 level (p<0.001; Figures 5a and b). Consistently, IL-10 expression, as an anti-inflammatory cytokine promoting tumor growth and invasion,[27] showed significant downregulation in the animals treated with allogeneic serum compared to the control group (p<0.001; Figures 5c and d).

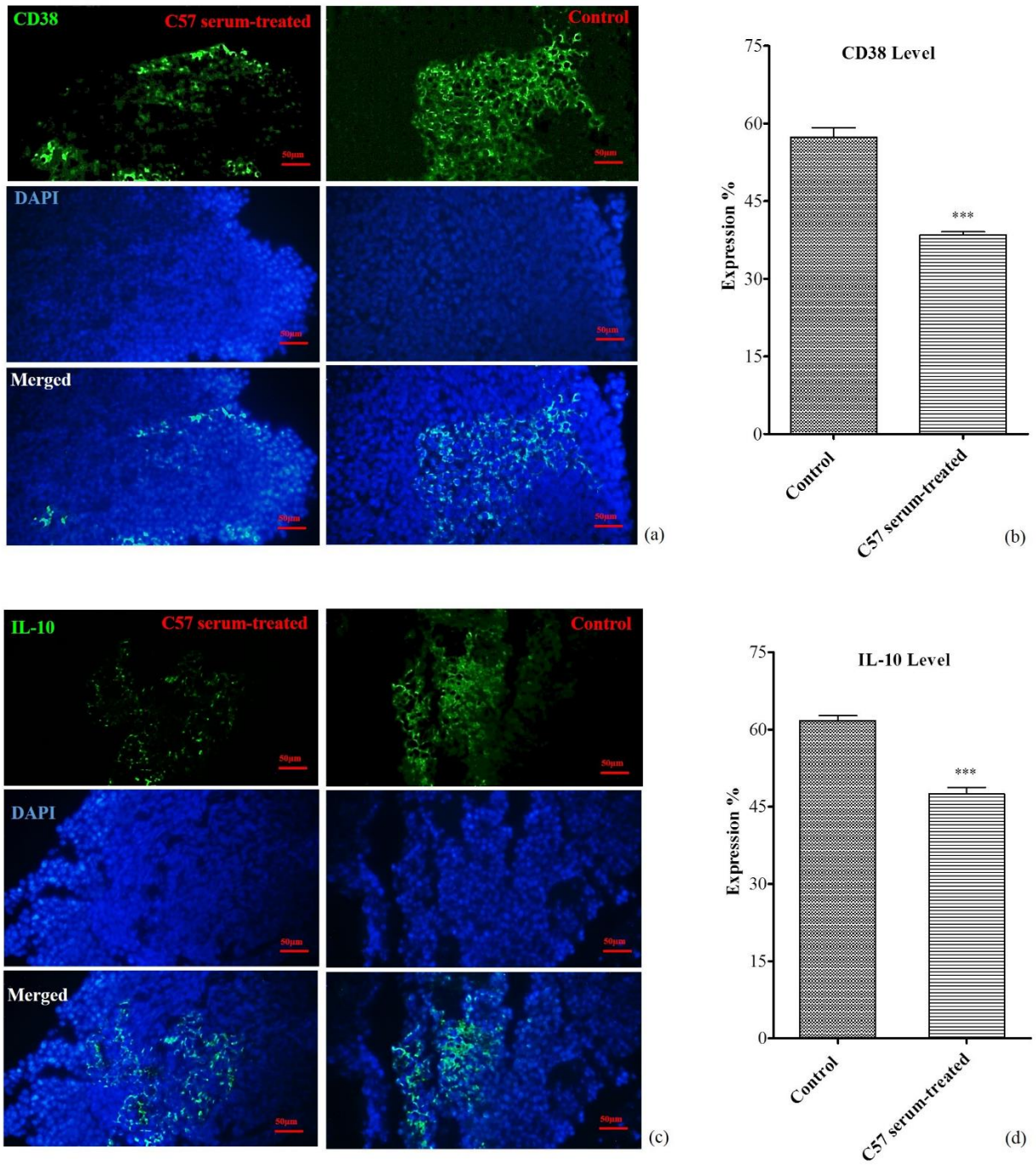

Figure 5. Impact of intra-tumor injection of allogeneic serum on tumor metabolic and anti-inflammatory status. A microscopic view of immunohistochemical staining of serial sections in tumor tissue using anti-CD38 or Interleukin (IL)-10 antibody is shown (a, c). The percentage of each molecule in the tumor was analyzed using ImageJ software (b, d). *p value<0.05; data are expressed as the means±SEM.

DISCUSSION

Passive immunotherapy using monoclonal antibodies has been applied against tumor antigens to promote inflammation based on their function through directly inducing programmed cell death upon binding to tumor targets and by antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP). Alternatively, antibodies specific to immune checkpoint inhibitors have also been applied to promote an inflammatory response. These approaches have achieved partial success in some cases, yet other issues remain.[33,34]

Organ transplantation between BALB/c and C57BL/6 mice has led to organ rejection.[35-43] Considering the tumor as a tissue we need to reject, we utilized allogeneic serum to induce inflammation at the tumor loci. By using this approach, we simulated a reverse transplantation rejection phenomenon in the tumor foci to make the host immune system reject the tumor.

In vitro evaluations demonstrated that the complement system plays an important role in CT26 tumor cell lysis. Although we do not have enough information on the mice blood group and mice are not regarded as a suitable model for ABO modeling,[44] there are preformed antibodies in mice,[45] as shown by in vitro evaluations performed in this study.

Intra-tumor injection of C57BL/6 serum to the tumor center in BALB/c mice led to a significant decrease in tumor growth rate. Allogeneic serum was injected into the tumor center because hypoxia is more intense in the tumor center with more cancer stem cells.[46] Injection into the tumor mass center prevented damage to the normal surrounding tissues. Consistent with tumor size, there was a significant decrease in hypoxia, represented by HIF-1α. Along with decreased hypoxia, a metabolic change was also observed, represented by a significant decrease in CD38 level. CD38 functions as an ectoenzyme.[25] It decreases extracellular nicotinamide adenine dinucleotide (NAD), alters calcium signaling pathways, and produces immunosuppressive adenosine. The upregulation of CD38 expression and its enzymatic activation within the tumor microenvironment leads to an increased adenosine level and the subsequent repression of the cytotoxic T cell response. Increased CD38 expression has been proposed to be associated with downregulation of p53 signaling and perhaps recruitment and survival of tumor-promoting macrophages favoring tumor growth. Based on data that largely suggest an immunosuppressive role for CD38 in solid tumors,[26,47,48] therapeutic approaches utilizing CD38 inhibitors have also been proposed. Pharmacological targeting via adenosine receptor inhibition to inhibit the adenosine pathway has been reported to reverse the immunosuppressive action mediated by CD38 upregulation.[26] However, CD38 is also expressed in multiple immune populations apart from enzymatic activity. Nevertheless, research on immune cells suggests that enzymatic and receptor functions of CD38 are independent of each other.[25] Connecting these two points, regulatory CD4 T cells accumulated in the tumor loci are more sensitive to NAD accumulation than antitumor T cells,[49] and blocking CD38 in a mouse model of lung cancer led to a significant reduction of regulatory CD4+ T cells within the tumor microenvironment.[26] Nevertheless, the issue of the role of CD38 in immune cells remains controversial as there are reports demonstrating the pro-inflammatory role of CD38 with regard to immune cells and its contribution to compromising antitumor response.[50] On this basis, in CD38-targeted immunotherapy of solid tumors, it is required to consider that CD38 is a highly complex molecule capable of numerous functions, and its inhibition would likely have unexpected effects. Combinational therapy involving CD38 has been discussed in different reports.[26,34] Apart from immune cells, endothelial cells express CD38 ligand–that is, CD31– which decreased in the animals treated with allogeneic serum. Accordingly, VEGF-A along with CD31 was also significantly decreased. This is consistent with a significant decrease in hypoxia, represented by HIF-1α, shown in this study and others.[51] In addition to angiogenesis, MMP-2, and MMP-9 were also significantly decreased. MMP-2 and MMP-9 are mainly secreted by tumor cells and stromal cells and play key roles in degrading extracellular matrices and promoting tumor metastasis and invasion.[52-54] These two molecules, as well as IL-10 and VEGF-A, are also highly produced by macrophages with M2 polarization found in the tumor. Interactions between macrophages and cancer cells make a significant contribution to the immunosuppressive condition established in the tumor foci.[55-58] Macrophages differentiate into populations with distinct inflammatory profiles depending on the tumor microenvironment. M2 macrophages significantly contribute to immunosuppression by

producing more MMP-2 and MMP-9, causing cancer progression.[55-60] In line with MMP-2 and MMP-9, IL-10 was also significantly decreased at tumor foci after intra-tumor injection of allogeneic serum. This is consistent with the critical role of IL-10 in tumor growth.[27] The influence of IL-10 on macrophage polarization and its association with promoting gastric and colorectal cancer cell invasion, motility, migration, angiogenesis, and proteolysis due to enhanced MMP-2 and MMP-9 activities has been previously reported.[61,62] These reports are consistent with the findings of this study, demonstrating a simultaneous decrease in MMP-2, MMP-9, IL-10, VEGF-A, and hypoxia after intra-tumor treatment with allogeneic serum. Notably, the significant decrease in IL-10 level as an M2 macrophage marker followed by injection of allogeneic serum into the tumor center may also be due to modulation of macrophage tumor cell crosstalk ,[63] leading to the control of tumor growth and progression.

So far, allogeneic vaccines for cancer have been devised solely based on tumor cells. These approaches are desirable because of their ease of production and accessibility.[64,65] This study provides initial evidence for allogeneic serum or plasma therapy as a new aspect of the allogeneic vaccine for cancer. As an advantage, allogeneic serum or plasma therapy requires a lower labor-intensive task to prepare than allogeneic tumor cell-based vaccine, though with a different origin. In addition, this approach is less invasive than approaches such as surgery and presumably has lower side effects than chemotherapy and radiotherapy.

Passive immunotherapy of a mouse model of colon cancer was performed using preformed antibodies in allogeneic serum. This was based on the natural phenomenon of transplant rejection. Treatment of the colon cancer cell line with allogeneic serum demonstrated the involvement of the complement system in the cytotoxic effects of allogeneic serum. This was followed by an intra-tumor injection of allogeneic serum. Allogeneic serum was injected into the tumor center to prevent damage to normal surrounding tissues. Passive immunotherapy using allogeneic serum led to a significant decrease in tumor size along with tumor hypoxia, metastatic capability, angiogenesis, metabolic status, and anti-inflammatory milieu, represented by HIF-1α, MMP-2, 9, CD31/VEGF-A, CD38, and IL-10, respectively. This study suggests intra-tumor application of preformed antibodies, such as blood group alloantibodies, as a candidate for passive immunotherapy. This study, for the first time, suggests allogenic serum or plasma therapy as the new aspect of allogeneic tumor vaccines, which have been solely devised based on tumor cells. This approach requires a lower labor-intensive task to prepare than allogeneic tumor cell vaccines, is less invasive than other approaches such as surgery, and presumably has lower side effects than chemotherapies and radiation therapy. It is worth to mention that our lack of knowledge on mice blood group system at the present was a limitation of this study. More research is required to shed light on more aspects of this therapeutic approach.

STATEMENT OF ETHICS

Research was approved by the Student Research Committee of the Alborz University of Sciences, and the foundation received was under reference number IR.ABZUMS.REC.1399.266.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors to declare.

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