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Utilizing VEGF165b mutant as an effective immunization adjunct to augment antitumor immune response

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ABSTRACT

Compelling evidence has shown that blocking VEGF via monoclonal antibodies may be beneficial in that it not only inhibits tumor angiogenesis but also reduces immune suppression and promotes T cell infiltration into tumors. Herein, we determined whether our recently generated VEGF165b mutant could be used as a co-immunization adjunct to augment the peptide cancer-vaccine- induced immune response in a mouse model of breast cancer. When co-immunized mVEGF165b with the peptide-based cancer vaccine (MUC1, a T-cell epitope dominant peptide vaccine from Mucin1), the VEGF antibody titers increased approximately 600,000-fold in mice. Moreover, the anti-VEGF antibody also reduced the frequency of regulatory T cells (Tregs) in both preventive and therapeutic scenarios. Mechanistically, the decrease of the Tregs population was associated with a remarkably increased MUC-1-specific IFN-γ-producing $CD8^+$ T cells and anti-MUC1 humoral response. Finally, this combination co-immunization produced a superior antitumor response and significantly prolonged survival of tumor-bearing mice. In conclusion, our findings suggest that mVEGF165b may be an ideal immunization adjunct to enhance the immune efficacy of peptide-based tumor vaccines by overcoming immune tolerance.

1. Introduction

Cancer-associated antigens can be designed as tumor vaccines to generate durable immune responses by eliciting the patient's own immune system to treat or prevent cancer recurrence after surgery [1]. However, the antitumor effect induced by tumor vaccines is often hampered by tumor-induced immune tolerance and immune suppression [2]. Among the immune suppression factors produced by tumor and tumor-associated cells, VEGF (vascular endothelial growth factor, VEGF-A) is considered to play an essential role in tumor-induced immune suppression [3], although VEGF-A is commonly considered to be a pro-angiogenic factor. There is accumulated evidence showing that VEGF-A can inhibit dendritic cell maturation [4] and promote infiltration of immuno-suppressive cells, such as tumor-associated macrophages (TAM) and regulatory T cells [4,5], contributing to the overall immuno-suppressive tumor microenvironment. Additionally, VEGF-A has also been shown to suppress the migration, activation, and cytotoxicity of effector T cells [6]. Conversely, evidence from animal experiments and clinical studies have shown that blocking VEGF via monoclonal antibodies or small molecular inhibitors not only causes inhibition of tumor angiogenesis but also contributes to the breaking of immune tolerance by decreasing or inhibiting the functions of the Tregs and other immunosuppressive cells [7,8]. However, the small molecular inhibitors, such as sunitinib, acted as anti-angiogenic agents but showed some severe side effects [9], and Bevacizumab (a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF A) is very costly and needs to be repeatedly administrated. For this reason, using patient's own immune system to continuously produce the antibody targeting VEGF is considered as an extremely cost-effective and attractive regimen.

VEGF-A gene generates different VEGF-A isoforms by mRNA splicing, and all of them share the exons 1–5 [10,11], which means vaccination with any VEGF isoform protein could generate antibodies targeting all the VEGF-A isoforms. mVEGF165b is a VEGF165b mutant which recently generated in our lab through using pichia yeast expression system, in which the plasmin cleavage site is inactivated by changing VEGF165b Arg110 to Pro110. This alteration extended its half-life in rats to 10 times that of its native counterpart without any change in bioactivities [12]. Theoretically, the prolonged half-life mVEGF165b could allow it to interact with B cell receptors for a longer period and so produce more antibody than the native counterpart. In our preliminary

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experiments, the full-length mVEGF165b protein was able to produce higher titer antibody targeting the native VEGF proteins without any visible side effects.

Hence, we hypothesized that immunization with mVEGF165b in combination with a tumor-associated antigen could not only inhibit tumor angiogenesis by neutralizing circulating VEGF-A but also augmenting immune response induced by targeting the specific tumor-associated antigen by decreasing immune tolerance. Blocking VEGF and other associated antigens has shown promise for cancer treatment in animal experiments. Kaumaya et al. (2012) used the B conformational epitope from HER2 and VEGF as a vaccine to treat breast cancer, and it showed promising results [13]. Singh et al. (2013) also found that a combined blockade of HER2 and VEGF prepared by using VEGF-Trap binding to VEGF-A and the anti-HER2 antibody trastuzumab exerts greater growth inhibition of HER2-overexpressing gastric cancer xenografts than individual blockades [14]. However, the above-mentioned researchers mainly focused on the combined effect of the anti-angiogenic effect on tumor growth generated by the VEGF-A blockade and the anti-tumor response induced by the corresponding tumor-associated antigens and did not investigate the VEGF blockade effect on decreasing the immune suppression.

Among tumor vaccines, peptide-based cancer vaccine was chosen for assessment of the co-immunization effect because of its weaker immunogenicity [15]. Some peptide vaccines, especially the short peptide vaccines can cause immunological tolerance of the immunizing antigens, rather than immunity [16]. Here, we chose an extensively studied peptide from mucin-1 as the cancer-associated antigen. Mucin-1 is a single pass type I transmembrane glycoprotein that is physiologically expressed on various mammalian cells [17]. However, mucin-1 is overexpressed and aberrantly glycosylated in greater than 90% of human breast cancers and other cancers [18]. In addition, its under-glycosylation in cancer exposes a variable number of tandem repeat regions (VNTR) on the peptide backbone. In recent years, mucin-1 has been considered a good cancer-associated antigen for the development of cancer vaccines [19]. Furthermore, the immuno-dominant peptides from the VNTR region could be recognized by cytotoxic T lymphocytes (CTLs), which are capable of destroying tumor cells expressing the under-glycosylated form of MUC1 [20]. Several mucin-1-based peptide vaccines have been evaluated in various clinical trials [21,22]. An example is the liposome cancer vaccine termed L-BLP25, which contains a peptide sequence (STAPPAHGVTSAPDTRPAPGSTAPP, we named the peptide as MUC1) derived from the VNTR region of mucin-1 and has shown promising efficacy in experimental mouse and clinical trials [23]. Although the MUC1 peptide was designed to elicit a cell-based immune response [20], there are also reports that it could also stimulate a weak humoral response [24].

In the present study, we investigated whether immunization with mVEGF165b as an adjunct could augment the immune response for the peptide MUC1 through breakage of the immune tolerance in a murine prophylaxis and therapeutic breast cancer model.

2. Materials and methods

2.1. Animals and cell lines

Female BALB/c mice ($H2^d$), 6-to-8-week-old, body weight 20 g, were purchased from Charles River Laboratories (Beijing, China) and raised in our laboratory animal room under specific pathogen-free conditions. Animals were housed in a constant room temperature of 20–25 °C and constant humidity of 35–40% with a 12 h light-dark cycle throughout the experiment and given free access to food and water. All procedures in the animal experiments were carried out in accordance with the current regulations and standards of the Animal Ethics Study Committee of Xinxiang Medical University (Xinxiang, China).

The murine breast cancer strain EMT-6 was purchased from the American Tissue Culture Collection (Manassas, VA, USA) and routinely maintained in RPMI1640 (Gibco, Bra, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sangon Biotech, Shanghai, China) at 37 °C with 5% CO_2 and 95% air humidified incubator. Mucin 1 cDNA (isoform 9, GenBank accession NM_001204285.1) was synthesized by Genewiz (Suzhou, China) and subcloned into pcDNA3.1 ^{plu}. EMT6 clones stably overexpressing the mucin 1 isoform 9 were transected with lipofectamine 3000 and screening with 600 μg/ml G418, we then used limited dilution to obtain the single clones. The level of relative expression of mucin1 of each screened single clone were quantified by semi-Q-PCR (Supplementary Fig S1) and fluorescence microscope (Supplementary Fig S2) (using the mucin1 antibody and according fluorescence labeled secondary antibody). The screened EMT6 clones expressing mucin1 (clone M5) were chosen as the experimental cell line and preserved in our laboratory, the d transfection and screening method were taken from a previous work [25].

The HUVECs and ECM medium were obtained from Sciencell (San Diego, CA, US), VEGF165b mutant protein were expressed via pichia yeast and purified in our Lab. VEGF165 protein was purchased from Sino Biological Inc (Beijing, China).

2.2. Immunization procedure

Thirty-five 6–8-week-old BAL B/c mice were divided randomly into five groups (PBS group (P), adjuvant group (A), mVEGF165b group (V), MUC1 peptide group (M) and mVEGF165b combination with MUC1 peptide group (V + M), n = 7 per group). The P and A groups were injected with PBS and adjuvant (complete Freund's adjuvant (product# F5881)) and incomplete Freund's adjuvant (product#F5506) were from SIGMA-ALDRICH (USA) alone, the V group received 50 μg mVEGF165b (expressed and purified by our laboratory, the dose of mVEGF165b as antigen was established in our preliminary experiment. we set a series of doses dose, specifically 15, 25, 50, 75 and 100 μg in our pre-experiment, the results also showed the anti-body targeting VEGF165 titer did not increase with the additional increase in the dose of mVEGF165b (50 μg), so 50 μg mVEGF165b was thought to be a suitable dose). The M group received 7.5 μg MUC1 peptide (synthesized by China Peptides (Shanghai, China), the amount of MUC 1 peptide based on the molar ratio VEGF165: MUC1 = 1:1), the V + M group received a mixture of 50 μg mVEGF165b and 7.5 μg MUC1. Each antigen was solubilized in 100 μl PBS and mixed with adjuvant to a final volume of 200 μl. The mix was injected subcutaneously in the lymph node area of the four armpits once a week for 4 consecutive weeks. Each injection site received 50 μl volume (first immunization with complete Freund's adjuvant (CFA), the remaining time using incomplete Freund's adjuvant (IFA)). Blood samples were obtained via fundus venous plexus.

Sera were collected weekly for immunoassay after the initial immunization for later analysis of antibodies and other assays.

2.3. Detection antibodies against VEGF165 and MUC1

An ELISA was carried out to detect the anti-VEGF165 and anti-MUC1 antibody titer in the immune sera, as described by Yankai et al. [26]. Briefly, 96-well flat-bottomed ELISA plates (Costar, USA) were coated with 100 μl/well VEGF165 proteins (10 μg/well) and 100 μl/well BSA-MUC1 (10 μg/well, the MUC1 peptide coupled with BSA), then incubated overnight at 4 °C, at the same time, the

wells coated with BSA served as negative controls. Plates were blocked with PBS containing 5% (w/v) nonfat milk powder (Sangon Biotech, Shanghai, China) and then incubated with 100 μl/well different dilution folds of sera collected from immunized animals in the different vaccinated groups and the controls. A secondary HRP-conjugated goat anti-mouse IgG (Solarbio Life Science, Beijing, China) was used for substrate reaction, Absorbance was measured at a wavelength of 450 nm. Each measurement was performed in duplicate.

Western blot analysis was performed to assess the anti-sera targeting VEGF165 in the corresponding groups according the conventional methods excepting that the loading buffer containing VEGF165 was added without reducing agents, while the loading buffer containing BSA-MUC1 contain reducing agents.

2.4. ADCC assay

HUVECs were seeded in a 96-well plate (1×10^4 cells/well) and cultured in M199 medium (100 μl/well) containing 5% FBS plus 50 ng VEGF165 (Sino Biological Inc, Beijing, China) overnight. Then, 1 μl, 5 μl, and 10 μl of the immune sera from the groups containing mVEGF165b and the PBS group were added, followed by incubation at 37 °C for 24 h and then 10 μl guinea pig serum was added and taken as the source of complement. At the end of incubation, 20 μl 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added. Cells were cultured for 24 h at 37 °C. Finally, the medium was discarded and 100 μl DMSO was added for cell lysis to measure absorbance at 570 nm. Each experiment was carried out in triplicate and the mean value was taken as the representative value for each experiment. The inhibition rate (IR) was calculated according to the following formula: IR (%) = [1 - (absorption value of experimental group / absorption value of control group)] × 100%. EMT-6 cells were used as the parallel control.

2.5. Preventive scenario

One week after the final immunization as described in immunization procedure, BALB/c mice were implanted subcutaneously with 5×10^5 EMT-6 cells overexpressing Mucin1 (isoform 9) on the right flank. When tumors were palpable, tumor volumes were measured with Vernier caliper every other day until death occurred. Tumor volumes were calculated using the following formula: tumor volume (cm^3) = length × $width^2$ × 0.52. The life span of the animals was also recorded.

2.6. Therapeutic scenario

BALB/c mice were implanted subcutaneously with 1×10^5 EMT-6 cells overexpressing MUC1 (isoform 9) on the right flank, and then divided randomly into five groups (n = 7 per group). Five days later, when the tumor volume was approximately 4–5 mm^3, the mice were immunized with the same dose and treatments as in the preventive procedure, three days later after every immunization, each mouse received 100 μl PD1-PDL1inhibitor 1 (concentration 500 μg/ml; i.p.) (MCE Cat. No. HY-19991), and the tumor size was measured by Vernier caliper every other day until death occurred. Apart from the PD1-PDL1 inhibitor, the immunization procedure was similar as in the preventive scenario. Tumor volumes were calculated using the following formula: tumor volume (cm^3) = length × $width^2$ × 0.52. The life spans were monitored.

2.7. Flow cytometry

Mice were immunized four times using the same doses and same way as in the preventive scenario, one week after the third and fourth immunization, the mice were sacrificed and the spleen cells were homogenized and suspended in RPMI1640 complete medium supplemented with 5% FBS.

One hundred microliter fresh blood per mouse in 1.5 ml anticoagulation tube was obtained from the tumor-bearing mice one week after the second and the third immunization in the therapeutic scenario and incubated with red blood lysis buffer at room temperature for 3 min (eBioscience 1 × lysis buffer), centrifuged at 4 °C (300*g* for 5 min), then the supernatant was discarded, sample was washed with 400 μl RPMI1640 medium containing 3% FBS, and centrifuged 300*g* for 5 min at 4 °C. The supernatant was discarded again; the freshly isolated white blood cells were washed with RPMI1640 and finally resuspended in 100 μl.

For the Tregs analysis, the freshly isolated spleen cells in the preventive setting and white blood cells in the therapeutic scenario were inoculated with surface marker (Anti-Mouse CD4 BV605 (Biolegend Cat. No. 100548 (San Diego, CA, US)), Anti-Mouse CD25 APC (eBioscience Cat. No. 17-0251-82 (eBioscience, Ltd., UK)), Anti-Mouse CD3 PerCP-eFluor® 710 (eBioscience Cat. No. 46-0032-82 (eBioscience, Ltd., UK)) 30 min at 37 °C, and then the cells were fixed and permeabilized using Fixation and Permeabilization Buffer Set (eBioscience Cat. No. 88-8824), then stained with Anti-Mouse/Rat Foxp3 PE (eBioscience Cat. No. 12-5773-82 (eBioscience, Ltd., UK)) according to the manufacturer's protocol, the fluorescence and the data were analyzed using FlowJo software (version 7.6).

For the CD8 IFN-γ secreting cells: the freshly isolated spleen cells from the preventative scenario were cultured in IMDM medium with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Next, cells were stimulated in the presence of Brefeldin A, MUC1 peptide, and mIL2 (Sino Biological Inc (Beijing, China)) 24 h, then stained with the surface marker Anti-Mouse CD8a PE-Cyanine7 (eBioscience Cat. No. 25-0081(eBioscience, Ltd., UK)), and fixed and permeabilized, finally stained with PE anti-mouse IFN-γ (eBioscience Cat. No.12-7311, (eBioscience, Ltd., UK). Fluorescence and the data were analyzed using FlowJo software (version 7.6).

2.8. CTLS killing assay

Splenocytes from different vaccinated groups after three-rounds of vaccination as in the preventive scenario were stimulated with MUC1 (1×10^{-3} μmol/ml) for 4 days in the presence of 20 U/ml of recombinant mIL-2 (Sino Biological Inc., Beijing, China) and used as effector cells. Mucin1-expressing EMT6 cells were used as target cells. Effector and target cells were mixed at various ratios (100:1, 50:1, 25:1, 1:1) in a final volume of 100 μl. After incubation for 4 h at 37 °C, 120 μl of the cultured media was collected to assess the amount of lactate dehydrogenase (LDH) using LDH Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China).

2.9. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC)

Three weeks after tumor cell inoculation, tumors in preventative procedures (n = 3 per group) were extracted and then fixed with 4% paraformaldehyde, dehydrated and paraffin-embedded, and then cut into 4-μm-thick sections. For immunohistochemistry, the sections were finally deparaffinized and rehydrated into PBS. Sections were stained with H&E (Zhongshan Biotech, Beijing, China) according to the manufacturer's instructions. H&E sections were examined by independent observers from Department of Pathophysiology of the Xinxiang Medical University (Xinxiang, China). For IHC staining, antigen retrieval was carried out with citrate buffer (pH 6.5) for 30 min at 92–98 °C. The sections were stained to identify CD8 T Cells with anti-$CD8^+$ antibodies

(bs-0648R, Bioss Antibodies, Beijing, China); followed by goat anti–rabbit IgG antibody. Antibody binding was visualized using DAB (3,3′-diaminobenzidine) (Zhongshan biotech, Beijing, China) and signal amplification was achieved via the avidin-biotin complex. Tissue was designated into three distinct compartments: benign glands, tumor centers, and tumor interfaces. Six randomly selected fields (original magnification 40×,) from each compartment were then captured from each slide with NIS-Elements software (Nikon). Representative mounted sections stained for $CD8^+$ T cells in the tumor center were counted randomly (n = 3 per group, 3 representative slides per tumor) for further analysis. Positive cells were counted in the tumor centers using Image Pro Plus 5.0 software, and cell counts per view were determined.

2.10. Influence of immunization with mVEGF165b plus peptide MUC1 combination on wound healing and fertility

Six female mice were vaccinated with mVEGF165b and MUC1 peptide combination as described above in the preventive scenario, with six other female mice left untreated and used as control. One week after the final vaccination, three mice from each group from the vaccinated and untreated groups were anesthetized and then sprayed with 75% ethanol to prevent infection, and about 0.5 cm^2 wounds were made on the hind limbs with eye scissors in a sterile environment. Wounds were wrapped with a sterile bandage, and days of wound healing were monitored daily.

The other three vaccinated female mice were mated with male mice and were checked for their farrowing rate.

2.11. Statistical analysis

The statistical analysis was carried out using the GraphPad software (version 6.0). Data were analyzed by one-way or two-way ANOVA with the same software and Bonferroni post-tests was used for all other comparisons. The results are expressed as mean ± SEM. *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. Immunization with mVEGF165b as adjunct generates a high-titer anti-VEGF antibody targeting native VEGF165

To determine the impact of immunization with mVEGF165b on anti-VEGF antibody, we used Western blot and ELISA to evaluate the immune sera from the corresponding immunization groups. ELISA results showed that one week after the third immunization the preventive scenario, the anti-VEGF level attained plateau (peak), approximately 600,000-fold (and almost same in the V and V + M group) (Fig. 1a). In the therapeutic scenario, the titer targeting VEGF165 achieved beyond 200,000-fold in mVEGF165b group and mVEGF165b + MUC1 group after one week after the fourth immunization (Fig. 1b).

Western blot results showed that the immune sera from the groups that contained mVEGF165b in the preventive scenario, even upon 1000-fold dilution, had a visible apparent band indicative of binding with native VEGF165 (Supplementary Fig. S3).

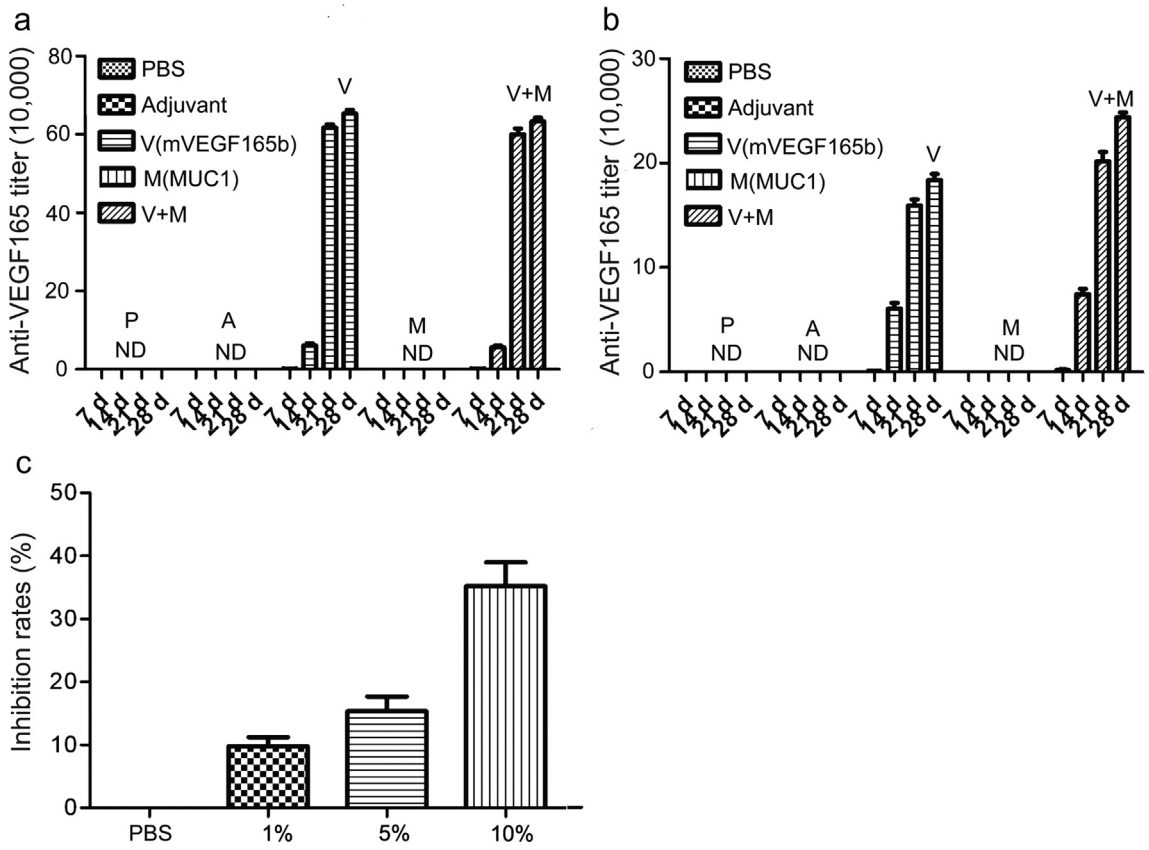

Fig. 1. Determination and function analysis of anti-sera elicited by mVEGF165b. (a) IgG antibody titer against human VEGF165 in mice immunized with mutant VEGF165b by ELISA the preventive scenario. ND; not detected. (b) IgG antibody titer against human VEGF165 in mice immunized with mutant VEGF165b by ELISA the therapeutic scenario. Each column represents the average titer calculated from duplicate samples of individual mice (n = 3) and the values represent mean ± SEM. ND; not detected. (c) In vitro antisera-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) against HUVECs proliferation. PBS group; 1% immune sera; 5% immune sera; 10% immune sera.

3.2. Immune sera from groups containing mVEGF165b inhibit VEGF165-mediated HUVEC proliferation

To evaluate the inhibitory effect of sera from immunized mice with mVEGF165b groups on the proliferation of HUVECs *in vitro*, cultured HUVECs were incubated with 1%, 5%, and 10% immunized serum from the mice immunized with mVEGF165b and mVEGF165b + MUC1 groups. MTT results showed that anti-sera at 1%, 5% and 10% could inhibit the VEGF165-mediated HUVEC proliferation (Fig. 1c). Taking the inhibition rate from PBS group as 0%, the rates of inhibition were 9.8% (1% anti-sera), 15.3% (5% anti-sera) and 35.2% (10% anti-sera), respectively. However, the anti-VEGF sera also showed there was no significant inhibition against the proliferation of EMT6 expressing-Mucin 1 compared to sera from the PBS and the adjuvant group, although we found the proliferation of EMT6 cells could be induced by VEGF autocrine pathway [25].

3.3. Immunization with mVEGF165b decreases the frequency of $CD3^+CD4^+CD25^+Foxp3$ Tregs

To determine whether the higher titer anti-sera targeting VEGF165 elicited by mVEGF165b could decrease Treg frequency, flow cytometry was used to monitor the number of Tregs in the spleens among the vaccinated and control group in the preventative scenario. As shown in Fig. 2a and 2b, compared with the vaccinated groups without mVEGF165b (PBS, Adjuvant and MUC1 groups), mice immunized with the mVEGF165b alone or combined with MUC1 showed that the splenic Tregs frequencies decreased significantly after the third immunization in the preventative scenario. Additionally, Tregs frequencies in the blood in the therapeutic scenario significantly reduced after the second immunization (Fig. 2c and d).

3.4. Co-immunization mVEGF165b as an adjunct augment the immune response elicited by MUC1

In order to evaluate whether the immunization with mVEGF165b could enhance the immune response induced by MUC1, mice were treated as described in the Methods section and flow cytometry was used to monitor IFN-γ secreting $CD8^+$ T cell frequencies of the splenocytes in the vaccinated groups' mice. Results showed that only mice in the group vaccinated with mVEGF165b + MUC1 could induce a significantly higher IFN-γ-secreting $CD8^+$ T cell frequencies compared with the other groups ($P < 0.05$) (Fig. 3a and b). Additionally, anti-MUC1 sera titers

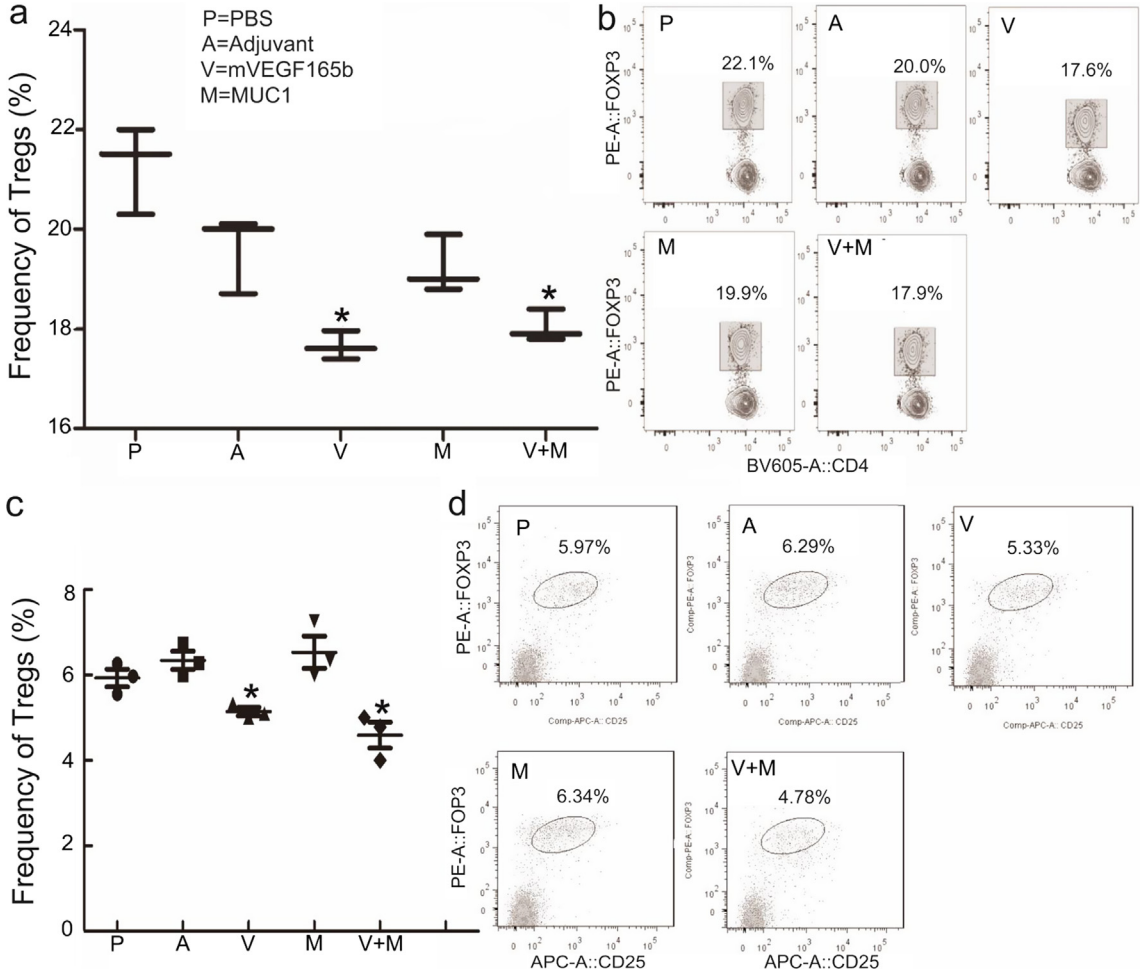

Fig. 2. Frequency of Tregs in splenocytes and blood from experimental groups in preventative and therapeutic scenarios determined by flow cytometry. Mice (n = 3/group) were treated as described in Methods. Splenocytes and white blood cells were harvested from the euthanized mice and incubated with anti-CD4-BV605, anti-CD25-APC and anti-Foxp3-PE for Tregs ($CD4^+CD25^+FoxP3^+$) separately according to the protocol (eBioscience Cat. No. 12-5773-82). Representative Treg cells in $CD4^+$ splenocytes (a) and blood white cells (c) (%) in the indicated groups are shown, representative dot plots of three individual experiments in each group are shown (b and d). The values are presented as mean ± SEM. *P* was determined by independent *t* test when compared to the mice immunized with PBS. Asterisks (*) indicate statistical significance, where $^*P < 0.05$.

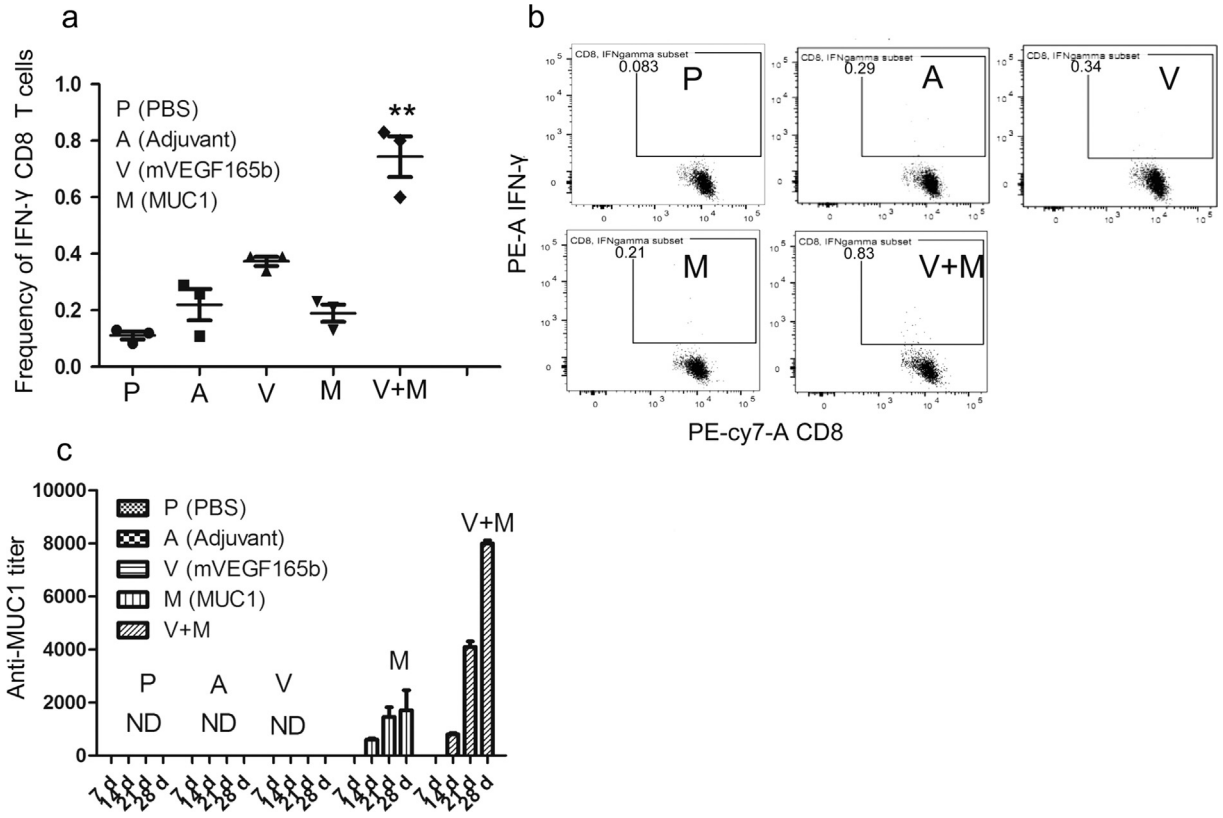

Fig. 3. Frequency of IFN-γ-secreting CD8$^+$ in splenocytes from experimental groups in preventative procedure determined by flow cytometry. Mice (n = 3/group) were treated as described the Methods. Splenocytes were harvested from the euthanized mice on day 21 and stimulated with the presence of Brefeldin A, MUC1 peptide, and mIL 2 24 h, then stained with the surface marker anti-Mouse CD8a PE-Cyanine7, and fixed and permeabilized, finally stained with PE anti-mouse IFN-γ. Representative dot plots of three individual experiments in each group were shown (a). IFN-γ-secreting CD8 in CD8+ splenocytes (%) in the indicated groups are shown (b). The values are presented as mean ± SEM. P was determined by independent *t* test when compared to the mice immunized with PBS group. (c) IgG antibody titer against BSA-MUC1 in mice immunized with mutant VEGF165b + MUC. Each point represents the average titer calculated from duplicate samples of individual mice (n = 3) and the values represent mean ± SEM.ND; not detected. Asterisks (*) indicate statistical significance, where $^*P < 0.05$, $^{**}P < 0.01$.

detected in the V + M group much were higher than the MUC1 group in the preventive scenario. That also show co-immunization with mVEGF165b also could also enhance the humoral response elicited by MUC (Fig. 3c). The ELISA results showed the titer to MUC1 was approximately 8000-fold in V + M group and while 2000 in the M group after the fourth immunization in the preventive scenario (Fig. 3c).

To verify the cellular response induced by co-immunized VEGF165 with MUC1, the direct cytolytic activity of splenocytes of mice from different groups was analyzed 1 week after the third immunization using LDH release assay. The splenocytes from mice immunized with mVEGF165 + MUC1 group demonstrated a significantly higher level of cytotoxic activity against EMT6 cells stably expressing Mucin1 than those from the mice in other groups ($P < 0.05$) (Fig. 4).

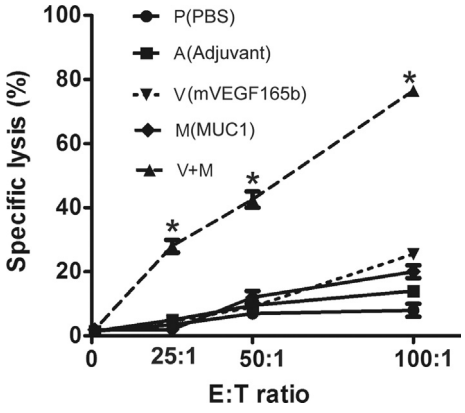

Fig. 4. . CTL activity against mucin1-expressing EMT6 cells. Mice were vaccinated three times as described in preventive scenario, then spleen cells from each vaccinated group were used as effector cells. Mucin1-expressing EMT6 cells were used as target cells. In vitro cytotoxicity was assessed with LDH release method (Asterisks (*) indicate statistical significance, where $^*P < 0.05$.). One representative experiment of two is shown.

3.5. The intratumor tissue from the mVEGF165b + MUC1 combination shows greater CD8$^+$ T cell infiltration

Considering that inhibition of VEGF could modulate immune cell infiltration [27], we next investigated whether the anti-VEGF sera elicited by mVEGF165b could promote the T cell infiltration into tumors. Since we found that immunization with mVEGF165b + MUC1 combination could yield a greater percentage antigen-specific IFN-γ-secreting CD8$^+$ cells in the spleen, we explored whether there existed a corresponding higher CD8$^+$ distribution in the tumors. We found that there was no difference on the CD8$^+$ distribution between benign glands and tumor interfaces among each vaccinated group; however, there was a significantly greater number of CD8$^+$ in the tumor centers of mVEGF165b + MUC1 group compared with A, P and M groups ($P < 0.01$), V groups ($P < 0.05$) (Fig. 5). Additionally, the tumor from the mVEGF165b group also showed a significantly greater T cell infiltration compared with A, P and M groups ($P < 0.05$).

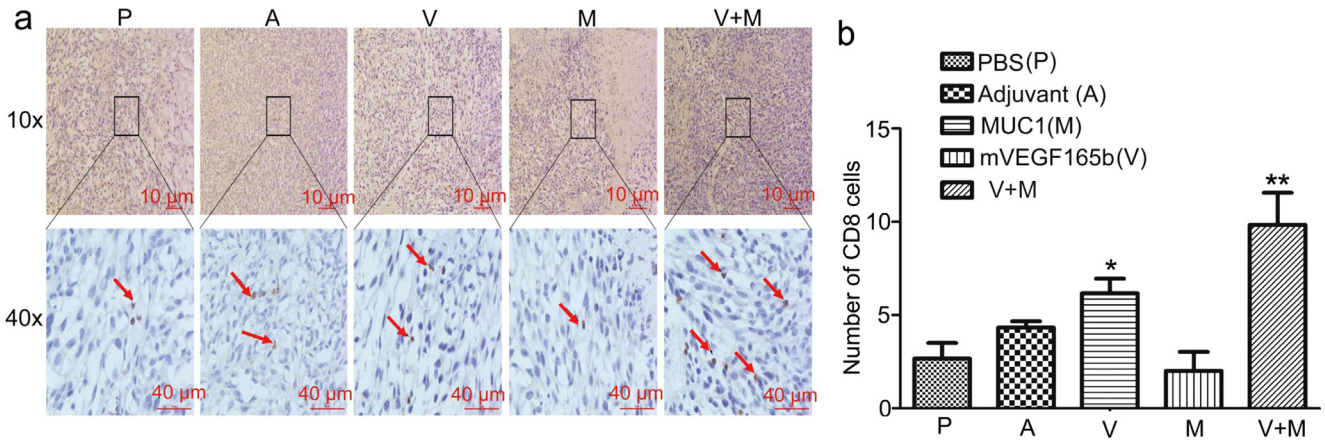

Fig. 5. The CD8 T cell infiltration into tumor centers. CD8 T cell staining of murine breast cancers overexpressing MUC1, showing infiltration of CD8 -lymphocyte clusters infiltrating in the center of breast cancers of various experimental groups. The arrow denotes the $CD8^+$-positive cells, only cells along or within tumor centers were counted. Asterisks (*) indicate statistical significance, where $^*P < 0.05$, $^{**}P < 0.01$. Scale bars under 100-fold magnification are 10 μm, Scale bars under 400-fold magnification are 40 μm.

3.6. mVEGF165b + MUC1 combination immunization induces a stronger anti-tumor effect in both the preventative and therapeutic scenarios

Given that MUC1 immunization has been used to treat Mucin1-overexpressing tumors in animal experiments and clinical trials to treat Mucin1-overexpressing tumors, we evaluated whether co-immunization with mVEGF165b could induce a robust anti-tumor response in both the preventative and therapeutic scenarios. As depicted in Fig. 6a and 6b, MUC1 vaccination alone was associated with no apparent anti-tumor activity, while immunization with mVEGF165b + MUC1 combination showed a stronger inhibitory effect in both scenarios compared with the control groups and those immunized with mVEGF165b or MUC1 alone and also prolonged the life-span of the tumor–bearing mice (for the therapeutic scenario, an extra PD1-PDL1 inhibitor injected 3 days after each immunization) (Fig. 6c and 6d). We also found that immunization with mVEGF165b + MUC1 could significantly reduce the tumor area in a mouse breast lung metastasis model under preventive scenario compared with other groups ($P < 0.05$) (Supplementary Fig S5).

3.7. Using mVEGF165b as immunization adjunct does not affect wound healing and fertility in mice

Considering that the angiogenesis plays a vital role in the process wound healing and embryonic implantation, we evaluated the impact of immunization with mVEGF165b on the process of wound healing and embryonic implantation. Compared with the control group, we found no obvious changes on the wound healing timespan and farrowing rate between the vaccinated groups containing mVEGF165b and the control group. Furthermore, there were also no obvious changes on the fur, body weight, and appetite in the two groups. Results from H&E sections from the main organs including heart, lung, stomach and liver also showed no pathomorphological changes or differences between the two groups. However, the volumes of the spleens from the mVEGF165-vaccinated groups were significantly larger than in those lacking mVEGF165b (Supplementary Fig. S4).

4. Discussion

The efficacy of cancer vaccines can be hampered by tumor immune suppression. VEGF has been shown to exhibit immunosuppressive properties in addition to its pro-angiogenic activities [4,6,8,28]. In this way, neutralizing circulating VEGF or inhibiting the VEGF-VEGFR signal axis have been demonstrated to be beneficial in counteracting tumor-induced immune suppression and inhibiting tumor angiogenesis [7]. Terme et al. (2013) and WADA et al (2009) found that using bevacizumab, a monoclonal antibody targeting VEGF-A specifically, could inhibit tumor-induced regulatory T cell proliferation [29,7]. There is growing amount of evidence that reducing Tregs ratio and attenuating their suppressive activity can promote the antitumor immune response [30,31]. In the present study, Herein, mVEGF165b was used as an adjunct to yield a high-titer anti-VEGF antibody, the latter lower immune tolerance by decreasing Tregs frequencies and augment the frequencies of antigen-specific IFN-ɤ secreting T cells and humoral immune response elicited by peptide tumor vaccine MUC1. Additionally, the antibody targeting VEGF generated by mVEGF165b also promoted the infiltration of T cells. All in all, using mVEGF165b as an adjunct improve the anti-tumor immune response.

Admittedly, the percentage of Treg cells is fairly constant in naïve mice, and only after the third immunization in the preventative scenario did the frequency of Tregs decrease. Nevertheless, in the therapeutic scenario, after the second immunization, the frequency of Treg cells of the tumor-bearing mice decreased. This is consistent with a previous report that showed the percentage of Tregs tended to decrease in the tumor-bearing mice [7]. It may explained that VEGFR-1 or -2 is only expressed in a small proportion of Tregs in healthy mice, while their expression was elevated in tumor-bearing mice [7].

VEGF has also been shown to be a main molecule in the immuno-suppressive tumor microenvironment by inhibiting dendritic cell maturation [4], immunosuppressive accumulation of tumor-associated macrophages (TAM) and Tregs [32,22]. Additionally, VEGF-A has also been shown to suppress the migration and activation of CTLs [6]. In theory, VEGF antibody elicited by mutant VEGF165b should have an inhibitory effect on the above-mentioned immuno-suppressive cells in tumors not just on Tregs; However, additional studies are needed to address this hypothesis.

In the therapeutic scenario, we used the mVEGF165b combined with MUC1, resulting in significant retardation of the tumor growth compared with the PBS, MUC1, and adjuvant groups. However, this mVEGF165b and MUC1 combination did not significantly augment mice survival rates. Meanwhile extra i.p. administration of PD1-PDL1 inhibitors 3 days after each vaccination significantly increased the survival rates of the mVEGF165b group and mVEGF165b + MUC1 group. We deduced that without PD1-PDL1

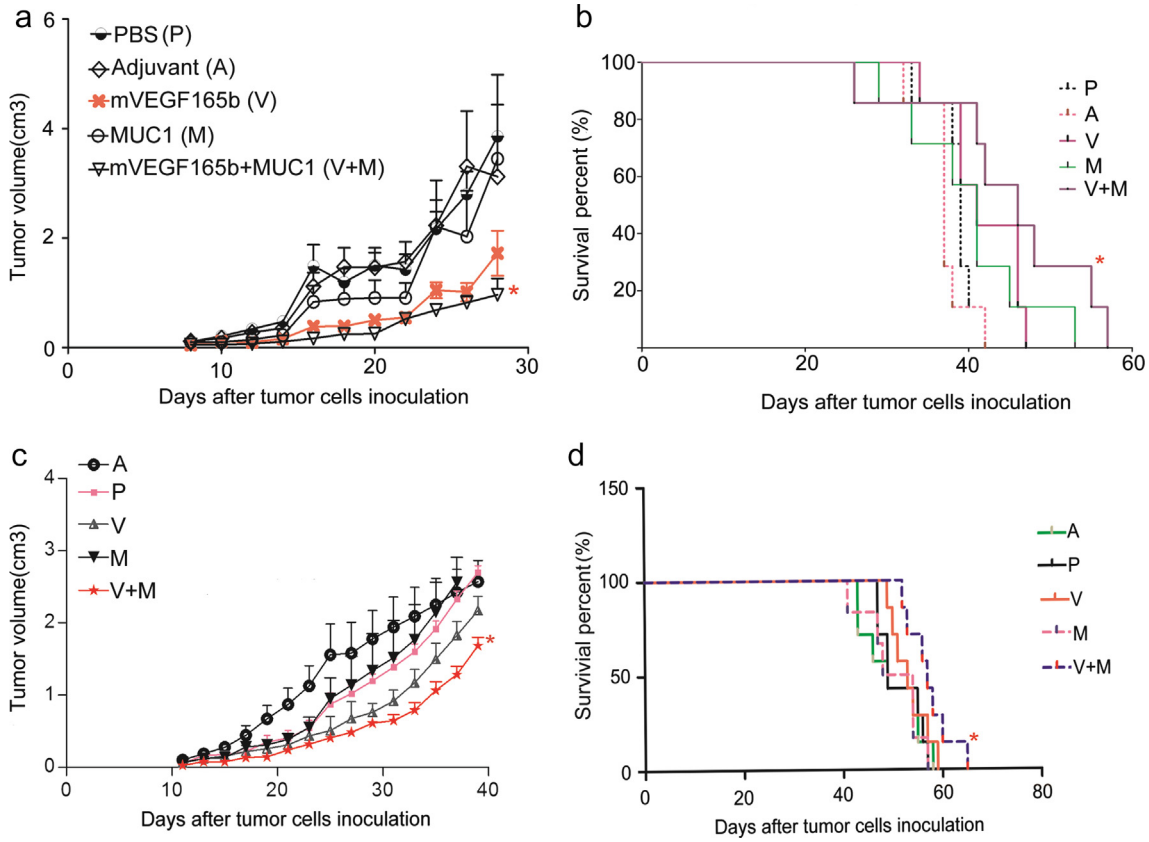

Fig. 6. Administration of the mVEGF165b and MUC1 significantly slow down tumor growth and prolong the survival rates in both the preventative and therapeutic scenarios. (a) Tumor mean volume kinetics + standard deviation of the mean (+SEM) in the preventive scenario (n = 7). (b) Survival rates various groups in the preventive scenario. (c) Tumor mean volume kinetics + standard deviation of the mean (+SEM) (n = 7) in the therapeutic scenario. (d) Survival rates various groups in the therapeutic scenario. Asterisks (*) indicate statistical significance, where $^{*}P < 0.05$.

inhibitors, the CTLs elicited by vaccine could be exhausted or become anergic by the tumor immuno-suppressive microenvironment, whereas PD1-PDL1 signaling inhibition prolonged the bioactivity of cytotoxic T cells. Duraiswamy *et al.* (2014) demonstrated that dual blockade of PD1 and CTLA-4 combined with a tumor vaccine could effectively restore the T cell rejection function in tumors [33]. Further, Moynihan *et al.* (2016) combined IL-2 and anti-PD1 and a powerful T cell vaccine in an optimized order and eradicated large, established tumors in mice [34]. These results indicate that blockage of PD1 function after tumor vaccine immunization can achieve a significant therapeutic benefit. Therefore, successful immune therapy not only requires the induction of an effective T cell response but also a response that can maintain the activity of T cells in the tumor deposits. Slingluff (2011) also suggested that a peptide vaccine combined with other immunologically active agents to block immunoregulatory mechanisms might be an essential prerequisite for successful immune therapy [35].

Unexpectedly, the vaccination with the combination of mVEGF165b and MUC1 did not attain optimal anti-tumor efficacy because our main aim was to explore whether mVEGF165b could be used an adjunct to decrease the immuno-suppressive status with a peptide vaccine. We only optimized the amount of mVEGF165b but not the amount of MUC1 in the combination. In addition, in theory, immunization with any VEGF-A-related isoforms or mutants should achieve similar effects to those of mVEGF165b because all VEGF-A isoforms possess exons 1–5; thus, their differences lie in the presence or absence of exons 6–8 [36].

In conclusion, our study offers direct evidence that co-immunized mVEGF165b with a peptide-based vaccine could augment the MUC1 induced T cell response and humoral immune response through decrease of the immuno-tolerance and enhancement of the infiltration of $CD8^{+}$ cells into the tumor center. The other advantage of using mVEGF165b as an adjunct to generate anti-VEGF antibody to decrease Tregs percentages could be in its prevention of the occurrence of autoimmune-mediated side effects often associated with total Treg depletion by other small molecule inhibitors. In the future, we consider that mVEGF165b or other VEGF A isoforms could be used as an ideal adjunct with other types of tumor vaccines due to its dual anti-tumor efficacy. In all, our study provides a potentially promising combinatorial regimen for cancer immunotherapy.

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Author contribution statement

Zhang Huiyong and Zhu Wuling conceived and designed research. Zhang Huiyong, Jia Enchao, Xia Wenjiao, Lu Chengui and Xu Zhenping conducted experiments and analyzed the data. Zhang Huiyong wrote the MS with the help of Zhu Wuling. All authors read and approved the manuscript.

Conflict of interest

All the authors declare that they have no conflicts of interest.

Ethical approval

All animal experiment procedures in this study were approved by the current regulations and standards of the Animal Ethics Study Committee of Xinxiang Medical University.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.055.

References

[1] Mannan S. Cancer vaccine clinical trials. Immunotherapy 2016;8(11):1263–4. https://doi.org/10.2217/imt-2016-0126.

[2] van der Sjoerd H, Burg RA, Ossendorp Ferry, van Hall Thorbald, Melief Cornelis JM. Vaccines for established cancer: overcoming the challenges posed by immune evasion. Nat Rev Cancer 2016;16(4):219–33. https://doi.org/10.1038/nrc.2016.16.

[3] Lapeyre-Prost A et al. Immunomodulatory activity of VEGF in cancer. Int Rev Cell Mol Biol 2017;330:295–342. https://doi.org/10.1016/bs.ircmb.2016.09.007.

[4] Gabrilovich DI et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med 1996;2(10):1096–103. https://doi.org/10.1016/bs.ircmb.2016.09.007.

[5] Gavalas NG et al. VEGF directly suppresses activation of T cells from ascites secondary to ovarian cancer via VEGF receptor type 2. Br J Cancer 2012;107(11):1869–75. https://doi.org/10.1038/bjc.2012.468.

[6] Ziogas AC et al. VEGF directly suppresses activation of T cells from ovarian cancer patients and healthy individuals via VEGF receptor Type 2. Int J Cancer 2012;130(4):857–64. https://doi.org/10.1002/ijc.26094.

[7] Terme M et al. VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. Cancer Res 2013;73(2):539–49. https://doi.org/10.1158/0008-5472.can-12-2325.

[8] Voron T et al. Control of the immune response by pro-angiogenic factors. Front Oncol 2014;4:70. https://doi.org/10.3389/fonc.2014.00070.

[9] Aparicio-Gallego G et al. New insights into molecular mechanisms of sunitinib-associated side effects. Mol Cancer Ther 2011;10(12):2215–23. https://doi.org/10.3389/fonc.2014.00070.

[10] Neufeld G et al. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13(1):9–22. , http://www.ncbi.nlm.nih.gov/pubmed/9872925.

[11] Woolard J et al. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. Cancer Res 2004;64(21):7822–35. https://doi.org/10.1158/0008-5472.can-04-0934.

[12] Zhang H et al. VEGF165b mutant with a prolonged half-life and enhanced anti-tumor potency in a mouse model. J Biotechnol 2018;284:84–90. https://doi.org/10.1016/j.jbiotec.2018.08.002.

[13] Kaumaya PT, Foy KC. Peptide vaccines and targeting HER and VEGF proteins may offer a potentially new paradigm in cancer immunotherapy. Future Oncol 2012;8(8):961–87. https://doi.org/10.2217/fon.12.95.

[14] Singh R et al. Combined blockade of HER2 and VEGF exerts greater growth inhibition of HER2-overexpressing gastric cancer xenografts than individual blockade. Exp Mol Med 2013;45:. https://doi.org/10.1038/emm.2013.111e52.

[15] Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 2008;8(5):351–60. https://doi.org/10.1038/nrc2373.

[16] Toes RE et al. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. Proc Natl Acad Sci USA 1996;93(15):7855–60. https://doi.org/10.1073/pnas.93.15.7855.

[17] Gendler SJ. MUC1, the renaissance molecule. J Mammary Gland Biol Neoplasia 2001;6(3):339–53. , http://www.ncbi.nlm.nih.gov/pubmed/11547902.

[18] Lau SK, Weiss LM, Chu PG. Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. Am J Clin Pathol 2004;122(1):61–9. https://doi.org/10.1309/9r6673qec06d86y4.

[19] Beatson RE, Taylor-Papadimitriou J, Burchell JM. MUC1 immunotherapy. Immunotherapy 2010;2(3):305–27. https://doi.org/10.2217/imt.10.17.

[20] Jerome KR et al. Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Cancer Res 1991;51(11):2908–16. https://doi.org/10.1093/intimm/9.5.791.

[21] Carmon L et al. Phase I/II study exploring ImMucin, a pan-major histocompatibility complex, anti-MUC1 signal peptide vaccine, in multiple myeloma patients. Br J Haematol 2015;169(1):44–56. https://doi.org/10.1111/bjh.13245.

[22] Butts C et al. Updated survival analysis in patients with stage IIIB or IV non-small-cell lung cancer receiving BLP25 liposome vaccine (L-BLP25): phase IIB randomized, multicenter, open-label trial. J Cancer Res Clin Oncol 2011;137(9):1337–42. https://doi.org/10.1007/s00432-011-1003-3.

[23] Sangha R, Butts C. L-BLP25: a peptide vaccine strategy in non small cell lung cancer. Clin Cancer Res 2007;13(15 Pt 2):s4652–4. https://doi.org/10.2165/00128413-200414320-00026.

[24] Jerome KR, Domenech N, Finn OJ. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. J Immunol 1993;151(3):1654–62. , http://www.ncbi.nlm.nih.gov/pubmed/8393050.

[25] Zhang H et al. Overexpression of VEGF183 promotes murine breast cancer cell proliferation in vitro and induces dilated intratumoral microvessels. Tumour Biol 2015;36(5):3871–80. https://doi.org/10.1007/s13277-014-3029-z.

[26] Yankai Z et al. Ten tandem repeats of beta-hCG 109–118 enhance immunogenicity and anti-tumor effects of beta-hCG C-terminal peptide carried by mycobacterial heat-shock protein HSP65. Biochem Biophys Res Commun 2006;345(4):1365–71. https://doi.org/10.1016/j.bbrc.2006.05.022.

[27] Roland CL et al. Inhibition of vascular endothelial growth factor reduces angiogenesis and modulates immune cell infiltration of orthotopic breast cancer xenografts. Mol Cancer Ther 2009;8(7):1761–71. https://doi.org/10.1158/1535-7163.mct-09-0280.

[28] Ohm JE et al. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. Blood 2003;101(12):4878–86. https://doi.org/10.1182/blood-2002-07-1956.

[29] Wada J et al. The contribution of vascular endothelial growth factor to the induction of regulatory T-cells in malignant effusions. Anticancer Res 2009;29(3):881–8. , http://www.ncbi.nlm.nih.gov/pubmed/19414323.

[30] Oh DS et al. Intratumoral depletion of regulatory T cells using CD25-targeted photodynamic therapy in a mouse melanoma model induces antitumoral immune responses. Oncotarget 2017;8(29):47440–53. , https://doi.org/10.18632/oncotarget.17663.

[31] Kobayashi M et al. Changes in peripheral blood immune cells: their prognostic significance in metastatic renal cell carcinoma patients treated with molecular targeted therapy. Med Oncol 2013;30(2):556. https://doi.org/10.1007/s12032-013-0556-1.

[32] Gabrilovich D et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. Blood 1998;92(11):4150–66. , https://www.ncbi.nlm.nih.gov/pubmed/9834220.

[33] Duraiswamy J, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors–response. Cancer Res 2014;74(2). https://doi.org/10.1158/0008-5472.can-13-2752. pp. 633–4; discussion 635.

[34] Moynihan KD et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. Nat Med 2016;22(12):1402–10. https://doi.org/10.1038/nm.4200.

[35] Slingluff Jr CL. The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? Cancer J 2011;17(5):343–50. https://doi.org/10.1097/ppo.0b013e318233e5b2.

[36] Robinson CJ, Stringer SE. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 2001;114(Pt 5):853–65. , https://www.ncbi.nlm.nih.gov/pubmed/11181169.