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ORIGINAL ARTICLE

Anti-tumor effects of DNA vaccine targeting human fibroblast activation protein α by producing specific immune responses and altering tumor microenvironment in the 4T1 murine breast cancer model

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Abstract Fibroblast activation protein α (FAPα) is a tumor stromal antigen overexpressed by cancer-associated fibroblasts (CAFs). CAFs are genetically more stable compared with the tumor cells and immunosuppressive components of the tumor microenvironment, rendering them excellent targets for cancer immunotherapy. DNA vaccines are widely applied due to their safety. To specifically destroy CAFs, we constructed and examined the immunogenicity and anti-tumor immune mechanism of a DNA vaccine expressing human FAPα. This vaccine successfully reduced 4T1 tumor growth through producing FAPα-specific cytotoxic T lymphocyte responses which could kill CAFs, and the decrease in FAPα-expressing CAFs resulted in markedly attenuated expression of collagen I and other stromal factors that benefit the tumor progression. Based on these results, a DNA vaccine targeting human FAPα may be an attractive and effective cancer immunotherapy strategy.

Keywords FAPα · CAF · Immune suppression · DNA vaccine · Immunotherapy

Abbreviations
CAFs   Cancer-associated fibroblasts
ECM   Extracellular matrix
FAPα   Fibroblast activation protein α
FGF-2   Fibroblast growth factor-2
HGF   Hematopoietic growth factor
PDGF   Platelet-derived growth factor
SDF-1   Stromal cell-derived factor-1
VEGFα   Vascular endothelial growth factor alpha

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Introduction

At present, the incidence of malignant breast cancer ranks in first place among various tumor types and is the number two cause of death in females in the estimated new cases in developed countries, seriously affecting the lives and health of women [1]. Tumor vaccines, which are gaining increasing attention as a popular treatment approach for cancer [2], typically utilize tumor-associated antigens (TAAs) as targets for immunotherapy and induce specific immune responses to kill the tumor cells. At least two necessary conditions are required for a successful outcome, induction of high numbers of antigen-specific T cells and the entry of effector T cells into tumors by overcoming the effects of immunosuppressive cells and factors [3]. As simultaneously achieving these two goals will increase the probability of success of a cancer vaccine, finding a suitable and effective tumor antigen is highly important [4].

Fibroblast activation protein α (FAPα) is a type II membrane-bound serine protease with collagenase and dipeptidyl peptidase activities implicated in extracellular matrix remodeling [5, 6]. The human FAPα gene is comprised of a 2281-bp sequence encoding an 87-kDa protein that is 90 % homologous to the murine FAPα protein [7, 8]. In normal tissues, FAPα has also been found to be expressed in embryonic tissues and wounds [6, 9]. In tumor tissues, FAPα is overexpressed by cancer-associated fibroblasts (CAFs), which are the main components of the stroma in most types of cancer, and is not expressed on tumor cells [10, 11]. Overexpression of FAPα has been shown to

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promote tumor growth and tumor metastasis, whereas treatment with anti-FAPα antibodies or pulsed dendritic cells was found to inhibit tumor growth [12, 13]. Importantly, a vaccine based on FAPα has also been shown to stimulate FAPα-specific cytotoxic T lymphocyte (CTL) responses in mice [14] and human peripheral blood mononuclear cells in vitro [15].

In recent studies, CAFs were demonstrated to contribute to the local immunosuppressive microenvironment [16]. CAFs are different from normal fibroblast tissues and can produce growth factors and cytokines to change the extracellular matrix (ECM) to affect the biological behaviors of tumor cells [17, 18]. CAFs can secrete ECM proteins such as collagen I which surround the tumor cells and separate them away from T effectors and chemotherapy drugs. This role protects tumor cells from immune attack and promotes immunosuppression [19]. Moreover, compared with transformed tumor cells, CAFs are regarded as being more genetically homogeneous and thus have become an attractive target for cancer therapy. The long-term benefits of immunotherapy are threatened by the escape of tumor cells from the immune system due to their inherent genetic instability [20]. Therefore, FAPα-based vaccines can be used to elicit FAPα-specific CTLs to kill CAFs and disrupt the immunosuppressive components of the tumor microenvironment [19, 21–25]. Meanwhile, it may reduce the risk of immune escape, which is an advantage that TAAs do not possess.

Studies to date have indicated that FAPα is an appropriate antigen for vaccine design which satisfies the two above-mentioned requirements of induction of FAPα-specific CTL responses and elimination of the immunosuppressive environment [26]. In this study, we constructed and examined the immunogenicity of a DNA vaccine against human FAPα in healthy and 4T1 tumor-bearing mice. When given in both a prophylactic and a therapeutic setting, the DNA FAPα vaccine caused marked inhibition of 4T1 tumor growth, which was attributed to induction of FAPα-specific immune responses that ultimately killed FAP+ CAFs and altered the tumor immunosuppressive microenvironment.

Materials and methods

Construction, expression and preparation of plasmids

Human FAPα cDNA was amplified from total RNA of human colorectal cancer tissue by RT-PCR using the primer pair: 5′-GGAAGATCTGTCGACATGAAGACTTGGGT AAAAATCGTAT-3′ and 5′-CGGGGTACCGTCGACGG ATCCTTAGTCTGACAAAGAGAAACACTGC-3′. After DNA sequencing, the amplified fragments were cloned into

the *Bam*H I/*Bgl* II sites of the VR1012 (VR) vector which contains a CpG motif in front of the promoter, generating the plasmid CpVR-FAP. To increase the safety of the antigen, a point mutation was created in FAPα, S624A [27], which was verified by DNA sequencing. Expression of FAPα was confirmed by Western blot using a human FAPα antibody (cat no. NBP1-67557, NOVUS). The plasmid was amplified in *Escherichia coli* Top10 cells and extracted by using a Qiagen Maxi purification kit with the purity of 90 % for supercoiled DNA and 1 EU/mg of endotoxins.

Mice and murine 4T1 model

Female BALB/c mice (6–8 weeks) were purchased from Beijing Huafukang Biology Technology Co. Ltd. Mice were maintained in microisolator cages under pathogen-free conditions, and animal care conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council). Murine B16, CT26 and 4T1 cells were preserved by the National Engineering Laboratory for AIDS Vaccine, Jilin University. Mice were challenged subcutaneously with these three different types of tumor cells (1×10^6) for FAPα expression detection. BALB/c mice were challenged by subcutaneous injection into the right lower flank with 2×10^4, 5×10^4 or 1×10^5 4T1 cells and monitored for tumor development every 2 days. The width and length of tumors were measured using a caliper, and volumes of tumors were calculated using the formula: (length $\times$ width^2)/2(mm^3). Finally, we confirmed that all mice injected with at least 2×10^4 cells developed tumors successfully.

Epitope prediction and synthesis

For the human FAPα sequence, T cell epitopes were determined by prediction of binding to H-2Db MHC molecules using the IEDB Analysis Resource (http://tools.immuneepitope.org/main/tcell/) and by prediction of the optimal proteasomal cleavage sites using the PAProC algorithm (www.paproc.de) [28, 29]. The three highest scoring SYFPEITHI peptides, F105-113 (LSPDRQFVY), F275-283 (VGPQEVPVP) and F542-550 (GGPCSQSVR), were chosen and synthesized by Shanghai GL Peptide Ltd. at 95 % purity.

Quantitative real-time PCR (qRT-PCR)

The procedure and cycling conditions for qRT-PCR were previously published [30]. Primers for *β-actin* were 5′-CGTG GACATCCGCAAAGACC-3′ (forward) and 5′-GGACTCGT CATACTCCTGCTTGC-3′ (reverse). Primers for *FAPα* were 5′-CCCAGGAAGTGCCTGTTCCAGCA-3′ (forward) and 5′-GCTCCTGGGTCTTTGGACAATCCC-3′ (reverse). Gene

expression was calculated by the $\Delta\Delta Ct$ method. Other primers for genes encoding stromal cell-derived factor-1 (SDF-1), hematopoietic growth factor (HGF), interferon-γ (IFN-γ), granulocyte–macrophage CSF (GM-CSF), vascular endothelial growth factor alpha (VEGFα), fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor (PDGF) were used in the same protocol as cited above.

Immunization schedule

In immunogenicity assays, BALB/c mice (five per group) were immunized three times by intramuscular injection with 100 μg of plasmid into both legs every 2 weeks, and blood was collected 1 week after each immunization for a total of four times, including the time point before immunization. Negative control mice were given 100 μl phosphate-buffered saline (PBS). In a prophylactic setting, BALB/c mice (ten per group) were immunized three times with 100 μg of plasmid every 2 weeks. One week after the final immunization, the mice were challenged subcutaneously with 2×10^4 4T1 viable cells in the right lower flank and monitored daily for tumor development. Three or 4 weeks after challenge with tumor cells, all mice were killed to detect humoral and cellular immune responses. In a therapeutic setting, BALB/c mice (10 per group) were immunized three times with 100 μg of plasmid or 100 μg empty vector every week after challenge with 2×10^4 4T1 cells. All mice were killed on day 25.

Detection of humoral responses

The procedure for detecting humoral responses was performed as previously published [31]. ELISA plates were coated with FAPα protein at 0.1 μg/well. Sera diluted at 1:125 and a horseradish peroxidase (HRP)-conjugated secondary antibody diluted at 1:3000 (goat anti-mouse IgG, Jackson) were used.

ELISPOT assay

ELISPOT assays were performed as previously published [32]. Briefly, splenocytes (1×10^6) were stimulated with R10 medium [RPMI1640 with 10 % fetal bovine serum (FBS)] or an unrelated protein (survivin) or FAPα protein (99aa–499aa) at a concentration of 2 μg/ml for 24 h.

Cytokine detection by ELISA

Splenocytes (1×10^7) from vaccinated tumor-bearing mice or healthy mice were stimulated with the FAPα protein (99aa–499aa) at a concentration of 5 μg/ml for 5 days in a 96-well U plate, and then, the cell culture supernatants were collected to detect secretion of IL-2 by using the Mouse IL-2 ELISA MAX™ Standard Set (Biolegend). Culture medium was diluted 1:3 in PBS containing 1 % bovine serum albumin. All steps were conducted by following the manufacturer's recommended protocol.

In vitro cytotoxicity assay

In vitro cytotoxicity was determined as previously reported [33]. Target cells were incubated with FAPα peptides (LSPDRQFVY, 105-113; VGPQEVPVP, 275-283; GGPCSQSVR, 542-550) or unrelated peptides [hSurvivin (80–88), hSurvivin (5–13) and hSurvivin (53–61)] as non-specific controls.

CD8$^+$ detection

Tumors were minced and dissociated using Liberase (Roche) at 0.04 mg/mL for 1 h. After passing through a 0.45-mm nylon mesh, samples were immunostained with anti-mouse CD3 (FITC) (Biolegend) and anti-mouse CD8 (APC) (Biolegend) at 4 °C for 30 min. Flow cytometry analysis was performed on a BD Accuri™ C6 flow cytometer.

Mouse Ig isotyping by ELISA

A 96-well flat-bottom microplate was coated with 0.2 μg/well of full-length truncated FAPα (99aa–499aa) protein. The remaining steps of the procedure were performed according to a previously published method [30].

Immunohistochemistry

FAPα expression was assessed by immunohistochemistry in normal and cancerous 4T1 tissue from female BALB/c mice. Sections of 5 μM thickness were cut and stained with an anti-FAPα primary antibody (Fitzgerald) at a 1:100 dilution. A biotin–streptavidin–HRP detection kit (MaiXin Bio) was used to detect primary antibodies according to the manufacturer's recommended protocol. Anti-mouse collagen type 1 antibody (Millipore) was used (1:150) to detect the expression of collagen type 1 in the 4T1 tumor using the same protocol mentioned above. Hematoxylin and eosin (H&E) staining was conducted as described previously [33].

Evaluation of wound healing and side effects

The procedure evaluating wound healing was previously published [34]. Livers and kidneys from vaccinated mice were stained with H&E for assessment of toxicity.
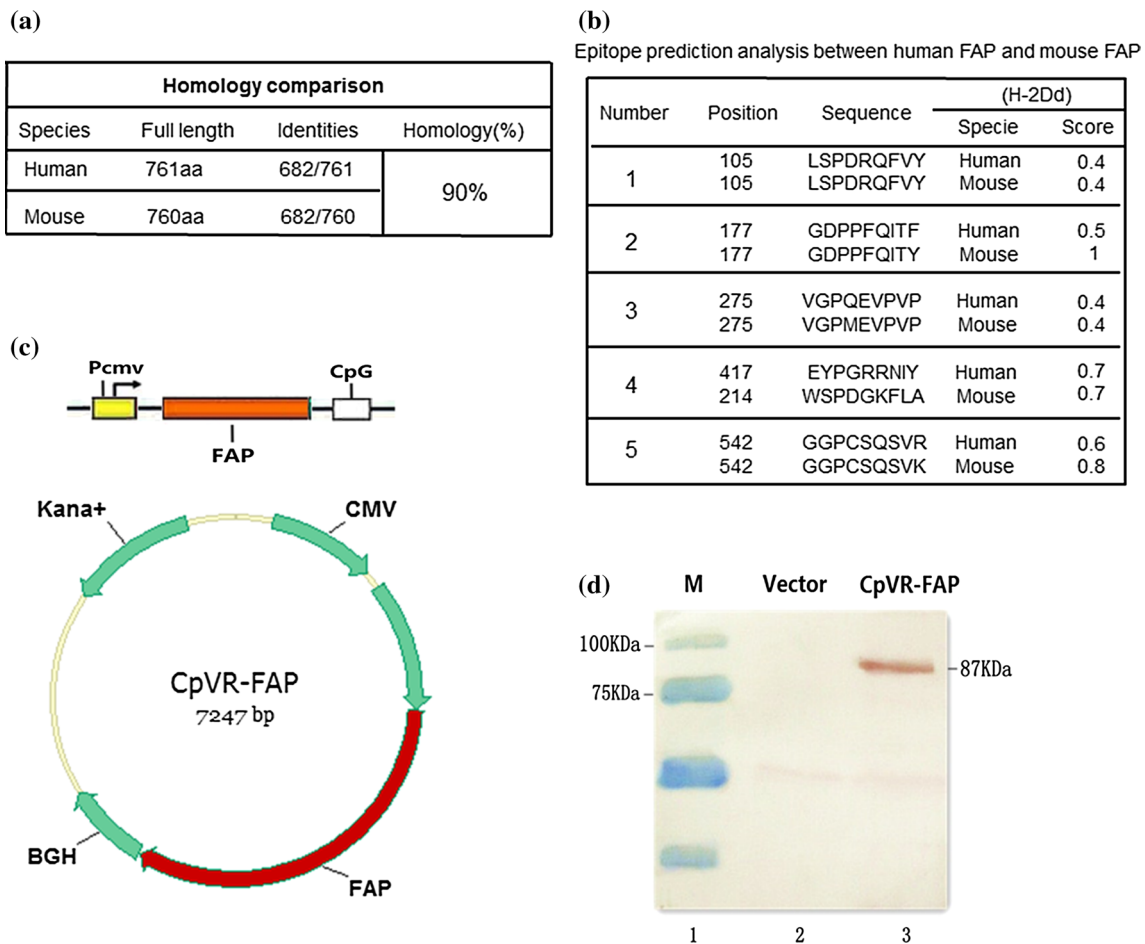
(a)

Homology comparison			
Species	Full length	Identities	Homology(%)
Human	761aa	682/761	90%
Mouse	760aa	682/760	

(b)

Epitope prediction analysis between human FAP and mouse FAP

Number	Position	Sequence	(H-2Dd)	
			Specie	Score
1	105	LSPDRQFVY	Human	0.4
	105	LSPDRQFVY	Mouse	0.4
2	177	GDPPFQITF	Human	0.5
	177	GDPPFQITY	Mouse	1
3	275	VGPQEVPVP	Human	0.4
	275	VGPMEVPVP	Mouse	0.4
4	417	EYPGRRNIY	Human	0.7
	214	WSPDGKFLA	Mouse	0.7
5	542	GGPCSQSVR	Human	0.6
	542	GGPCSQSVK	Mouse	0.8

(c)

(d)

Fig. 1 Homology analysis and comparison of predicted mouse and human FAPα epitopes, and characterization of human FAPα construct. **a** Homology between mouse FAPα and human FAPα. **b** Highest scoring peptides predicted by using the IEDB analysis resource (http://tools.immuneepitope.org/main/tcell/). **c** Schematic representation of eukaryotic expression vector with full-length human FAPα (S624A), verified by nucleotide sequencing. **d** Protein expression was verified by Western blotting after transient transfection of 293T cells

Statistical analysis

All in vivo and in vitro experiments were performed at least three times. Data were analyzed using one-way ANOVA. Differences between the groups were assessed for statistical significance using the unpaired t test. All statistical analyses were performed with GraphPad Prism software.
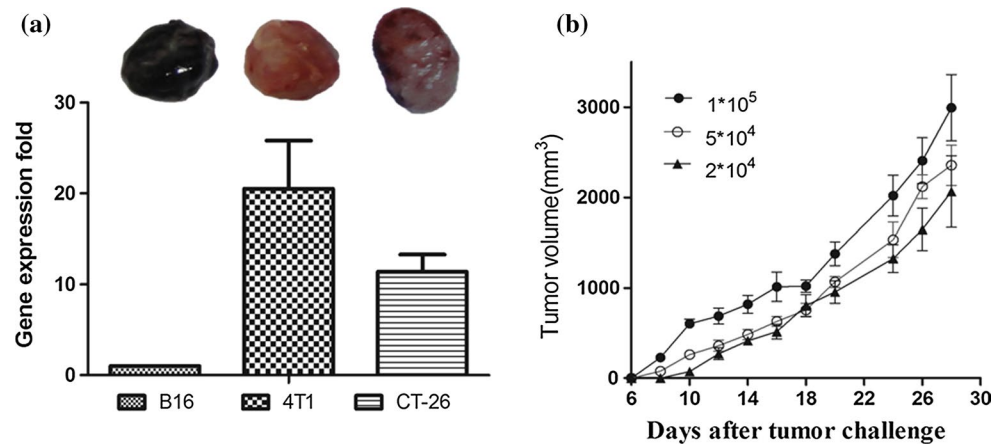
Results

Homology analysis between human FAPα and mouse FAPα, prediction of epitope peptides and construction and expression of plasmid CpVR-FAP

Amino acid sequences of human FAPα and mouse FAPα were used as queries in BLAST searches. The results showed that the murine FAPα gene encodes a protein which is 90 % homologous to the human protein (Fig. 1a). Among the five highest scoring predicted peptides from mouse and human FAPα, the best scoring peptide was the same between the species, and three of the remaining four predicted peptides were similar to only one amino acid difference (Fig. 1b). As these results suggested that human FAPα peptides can be used to stimulate human-specific CTL which can kill mouse FAP[+] CAFs, human FAPα was used as an antigen target to treat murine carcinoma. To avoid the acceleration of tumor growth, a point mutation S624A was made in FAPα to eliminate its enzymatic function [27]. After constructing the eukaryotic expression vector CpVR-FAP as described in "Materials and methods" section (Fig. 1c), the correct expression of the 87-kDa FAPα protein was verified by Western blotting of cell lysates from transiently transfected 293T cells which do not express FAPα (Fig. 1d).

Fig. 2 Selection and establishment of a tumor model. **a** Relative *FAPα* mRNA expression in B16, 4T1 and CT-26 tumors. *β-actin* was used as an endogenous control. **b** Female BALB/c mice ($n = 5$) were challenged with 2×10^4, 5×10^4 or 1×10^5 4T1 cells, and tumor volume was measured for 28 days after tumor challenge as described in "Materials and methods" section

Establishment of tumor model

The expression levels of FAPα in several types of rectal cancer were tested (Fig. 2a), and the results indicated that FAPα was expressed most highly in 4T1 tumor tissue among the three types of tumors (approximately twofold higher expression compared with the CT26 tumor and 20-fold higher expression compared with the B16 tumor). Here, we only detected the expression of FAPα in tumor tissues since it is only found on CAFs and not on tumor cells. Moreover, the 4T1 tumor model was chosen for subsequent animal experiments as it is enriched for CTL target antigens. To ensure a tumor formation rate and size that would be suitable for animal experiments, mice were injected subcutaneously into the right lower flank with three different numbers of 4T1 cells (2×10^4, 5×10^4 and 1×10^5). Tumor growth was monitored for 28 days after inoculation, and tumor size was measured as described in "Materials and methods" section (Fig. 2b). A 100 % tumor formation rate was observed with all three cell doses, and the tumor growth rate was positively correlated with the number of 4T1 tumor cells. For tumor challenge in the subsequent prophylactic setting, the tumor cell dose of 2×10^4 was chosen as it was the lowest number of cells tested which would not significantly impair the health and immune system of the animals.

## Analysis of immunogenicity of CpVR-FAP DNA vaccine

To determine the immunogenicity of the CpVR-FAP DNA vaccine, female BALB/c mice ($n = 5$) were immunized with CpVR-FAP or PBS control three times every 2 weeks. Two weeks after the final immunization, all mice were killed (Fig. 3a), and the blood and spleens were collected for testing humoral and cellular immune responses. The isolated splenocytes were assessed by using a cytotoxicity assay and ELISPOT assay. The results showed that the

CpVR-FAP group induced higher specific CTL responses in mice than the PBS group at three different target-to-effector ratios (100:1, 50:1, 25:1). Splenocytes from mice vaccinated with CpVR-FAP were able to lyse significantly more targets cells (P815) than those from PBS controls at the target-to-effector ratio of 1:100 (Fig. 3b, $P < 0.05$). More than threefold higher frequencies of antigen-specific IFN-γ-secreting T cells, as detected in an ELISPOT assay, were induced by the CpVR-FAP group than the PBS group (Fig. 3c, $P < 0.05$). Cytokine IL-2 expression in the culture medium secreted by splenocytes was also detected by ELISA. The results showed an approximately sixfold higher expression of IL-2 in the CpVR-FAP group than the PBS group with FAP protein stimulation (Fig. 3d, $P < 0.001$). By ELISA, the FAP group showed threefold higher antibody titers than the PBS group after the second and third immunizations (Fig. 3e, $P < 0.05$). Therefore, the CpVR-FAP DNA vaccine successfully induced strong immune effects in healthy mice.

CpVR-FAP DNA vaccine exerts anti-4T1 effects by FAP-specific immune responses in mice in a prophylactic setting

According to the immunization schedule shown in Fig. 4a, female BALB/c mice ($n = 10$) were immunized with CpVR-FAP or PBS as a control three times every 2 weeks. At 1 week after the final immunization, all mice were inoculated subcutaneously with 2×10^4 4T1 cells into the right lower flank. Tumor growth was monitored for 24 days after inoculation. As shown in Fig. 4b, the tumor growth rate was inhibited significantly from 16 days onward after tumor challenge. When the mice were killed, the tumors were removed, stripped and weighed. Based on tumor weights (Fig. 4c), the CpVR-FAP strategy showed a tumor inhibition rate of 22 % relative to the PBS group. To investigate the anti-tumor mechanism of the vaccine, CTL activities of splenocytes from immunized BALB/c mice (Fig. 4d) were

Fig. 3 Immunogenicity of CpVR-FAP DNA vaccine in non-tumor-bearing mice. **a** BALB/c mice ($n = 5$) were injected with PBS or CpVR-FAP intramuscularly at 2-week intervals and then killed according to the schedule. **b** Cytolytic activity of splenocytes from immunized mice was determined by measuring specific killing of target cells (CFSE high) pulsed with mixtures of predicted peptides or unrelated peptides (survivin, not shown) relative to control non-pulsed target cells (CFSE low). E/T ratio = ratio of effector cells to target cells. **c** Splenocytes from vaccinated mice were stimulated with FAPα protein, and the frequencies of IFN-γ-producing T cells were measured by ELISPOT (*$P < 0.05$; **$P < 0.01$). *R10* indicates RPMI1640 with 10 % FBS, and *Pro* indicates FAPα protein. **d** Splenocytes from vaccinated mice were stimulated with FAPα protein for 5 days, and then, the culture medium was collected for testing IL-2 secretion by using an ELISA MAX™ Set (***$P < 0.001$). **e** Serum was collected every week after each immunization for four times in total, including the first time before immunization. Specific antibodies against FAPα were detected by ELISA
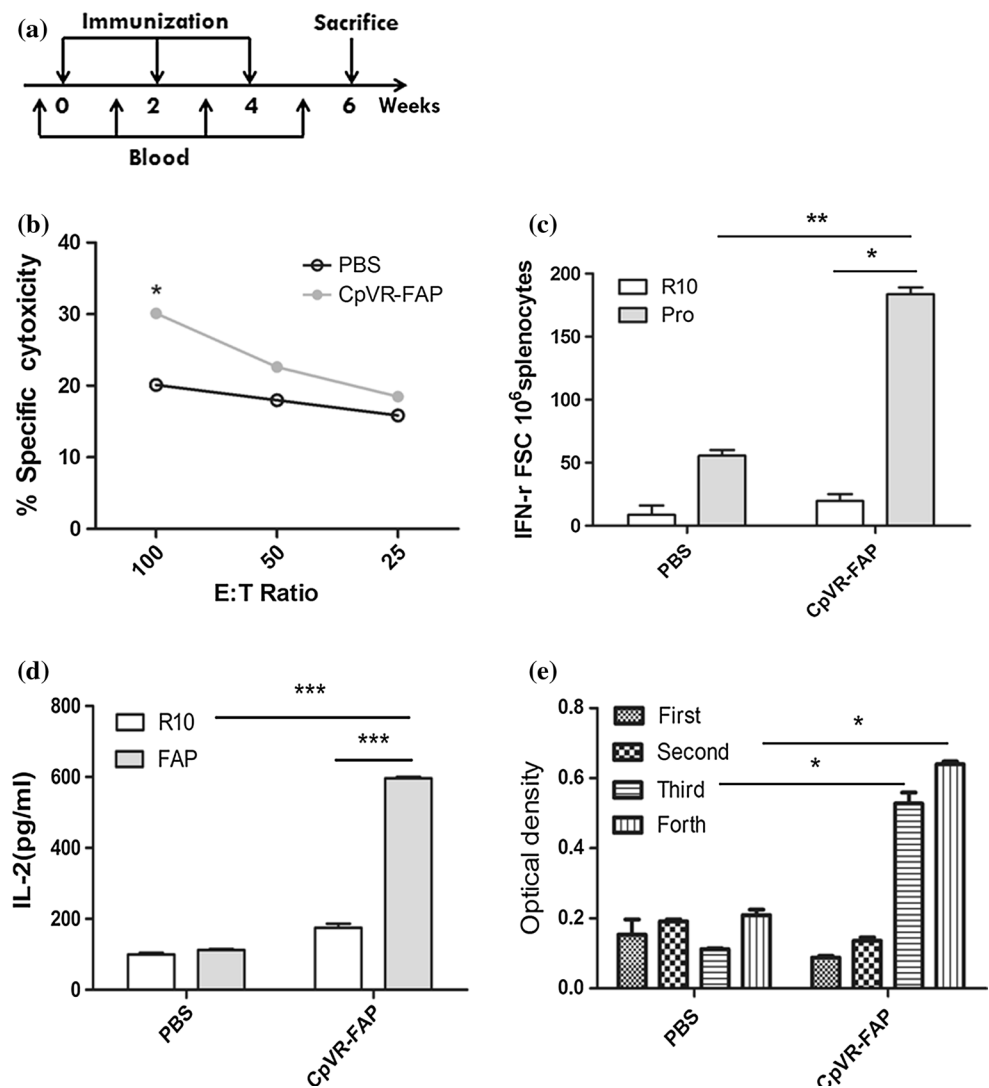
assessed against MHC-matched tumor cells (P815 cells) labeled with H-2 Db-restricted FAPα peptides. The results showed that a stronger specific CTL response was induced by the CpVR-FAP DNA vaccine than PBS ($P < 0.05$). The ELISPOT results (Fig. 4e) indicated that FAPα-specific CD8^{+} T cells releasing IFN-γ were well induced in mice immunized with CpVR-FAP ($P < 0.05$). By ELISA, the amount of IL-2 cytokine secreted by splenocytes into culture medium was found to be nearly threefold higher in the CpVR-FAP group than the PBS group (Fig. 4f, $P < 0.005$). More infiltrated CD8^{+} lymphocytes were detected by flow cytometric analysis in tumors of the CpVR-FAP group (Fig. 4g, $P < 0.05$). Meanwhile, qRT-PCR analysis showed that the expression of *IFN-γ* mRNA in the tumor was enhanced by the CpVR-FAP vaccine (Fig. 4h, $P < 0.01$). We further investigated the in vivo effects of the CpVR-FAP vaccine on the modulation of antigen-specific antibody responses by favoring the development of Th1 versus Th2 cells. As shown in Fig. 4i, mice vaccinated with

CpVR-FAP presented a higher IgG2a/IgG1 ratio than that of the PBS group ($P < 0.05$). These results suggest that the CpVR-FAP DNA vaccine generated specific anti-tumor immunity effects targeting FAPα which could inhibit the tumor growth.

Therapeutic effects of FAP-based vaccine on 4T1 tumor

Female BALB/c mice ($n = 10$) were inoculated subcutaneously with 2×10^4 4T1 cells into the right lower flank and then treated with CpVR-FAP or CpVR (empty vector) on days 1, 8 and 15 (Fig. 5a). Tumor weights (Fig. 5b, $P < 0.05$) were measured after killing, and tumor volumes (Fig. 5c, $P < 0.05$) were recorded every 2 days until day 25. CTL (Fig. 5d, $P < 0.05$) and ELISPOT (Fig. 5e, $P < 0.05$) assays were then performed as described in "Materials and methods" section. The results demonstrated that FAP DNA vaccine could induce anti-4T1 immune responses which inhibited 4T1 growth in a therapeutic setting.
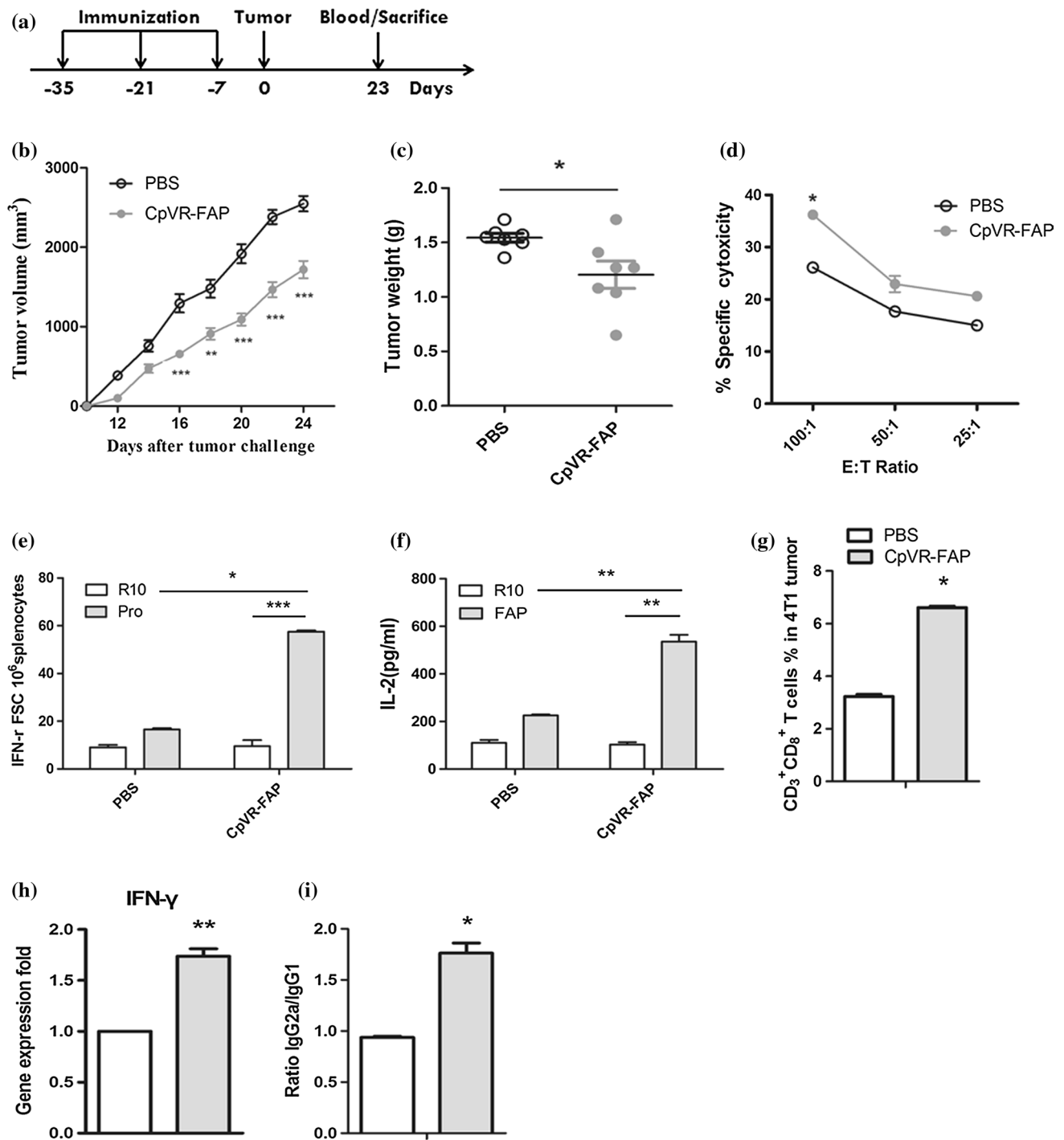
Fig. 4 Induction of prophylactic anti-4T1 immunity. **a** Prophylactic setting. 7 days after the last of three vaccinations at 2-week intervals, BALB/c mice ($n = 10$) were challenged with 2×10^4 4T1 cells. **b** Tumor volumes were measured for 24 days after tumor challenge (PBS group $= 2549 \pm 273.1$ mm^3, CpVR-FAP group $= 1719 \pm 308.6$ mm^3). **c** Tumor weights were measured on day 25 after tumor challenge (PBS group $= 1.544 \pm 0.1052$ g, CpVR-FAP group $= 1.204 \pm 0.3307$ g. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *ns* not significant). **d** In vitro CTL assays using P815 target cells pulsed with FAPα peptide mixtures or unrelated peptides (survivin, not shown) in different E/T ratios. **e** Splenocytes from vaccinated mice were stimulated with FAPα protein, and the frequencies of IFN-γ-producing T cells were measured by ELISPOT. **f** IL-2 secretion in culture medium was measured by ELISA. The mRNA expression of IFN-γ was confirmed by qRT-PCR. **g** Percentages of infiltrated CD8$^+$ T lymphocytes in CD3$^+$ cells of tumors were verified by flow cytometry. **h** The mRNA expression of IFN-γ was confirmed by qRT-PCR. **i** Ratios of IgG2a/IgG1 were detected by ELISA in serum collected upon killing of mice

Fig. 5 Induction of thera-
peutic anti-4T1 immunity. **a**
Therapeutic setting. BALB/c
mice ($n = 10$) were chal-
lenged with 2×10^4 4T1 cells
on day 0. Empty vector was
used as a control. **b** Tumor
weight was measured after
killing (*$P < 0.05$). **c** Tumor
volumes were measured for
25 days after tumor challenge
(***$P < 0.001$). **d** In vitro CTL
assays using P815 target cells
pulsed with FAPα peptide
mixtures or unrelated peptides
(survivin, not shown) in dif-
ferent E/T ratios (*$P < 0.05$).
e Splenocytes from vaccinated
mice were stimulated with
FAPα protein or unrelated pro-
tein (survivin), and the frequen-
cies of IFN-γ-producing T cells
were measured by ELISPOT
(*$P < 0.05$)
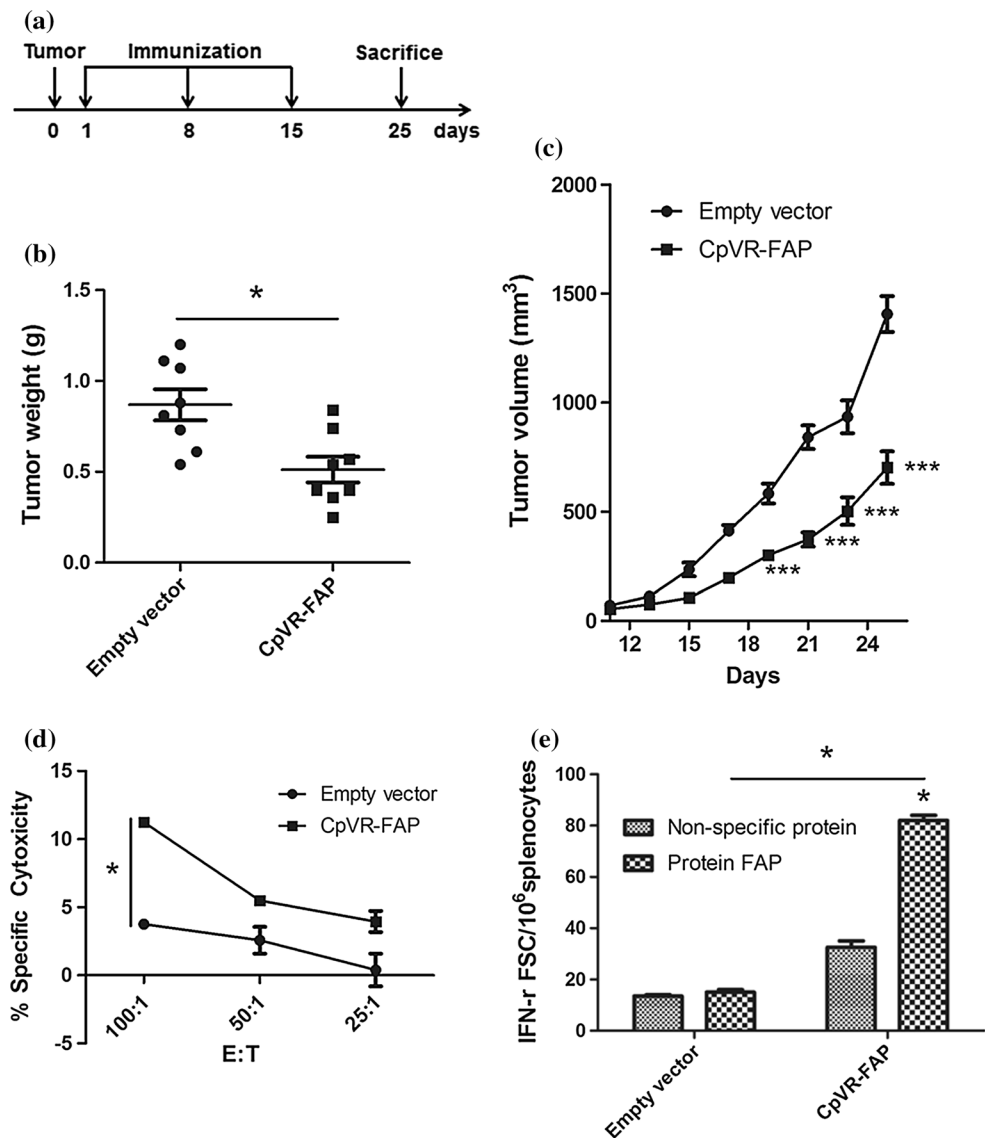
FAPα immunization is accompanied by decreases in FAPα, collagen type I expression and production of stromal cytokines but does not impair wound healing or safety

Relative decreases in mRNA expression of FAPα and col-
lagen type I by CpVR-FAP vaccination were observed in
tumor tissues (Fig. 6a, $P < 0.01$, $P < 0.05$). Meanwhile,
expression levels of FAPα and collagen were examined
by immunohistochemistry in tumor biopsies of the two
groups with the corresponding antibody (Fig. 6b). Com-
pared with mice vaccinated with PBS, the expression
of FAPα as well as collagen type I decreased in those
vaccinated with CpVR-FAP. CAFs and tumor-associ-
ated macrophages (TAMs) are prominent cells in the
tumor stroma, producing growth factors and cytokines
that support tumor growth and facilitate metastasis by

promoting angiogenesis. Therefore, we next explored
the effects of immune modulation by the CpVR-FAP
DNA vaccine on the expression of stromal factors in
the tumor microenvironment. The qRT-PCR analysis of
mRNA from tumors of vaccinated mice showed that the
relative expression levels of SDF-1, HGF, VEGFα, GM-
CSF, PDGF and FGF-2 were significantly reduced in the
CpVR-FAP group (Fig. 6c, $P < 0.01$, $P < 0.05$). Based
on these results, we hypothesize that the decrease in
tumor growth in FAPα-immunized mice was associated
with the decrease in number of CAFs that could synthe-
size FAPα collagen type I and decreased factors acting
as key regulators of tumorigenesis. The fact that FAPα
is overexpressed during wound healing led us to evalu-
ate whether a decrease in the number of cells expressing
FAPα could also lead to a prolongation of wound healing.
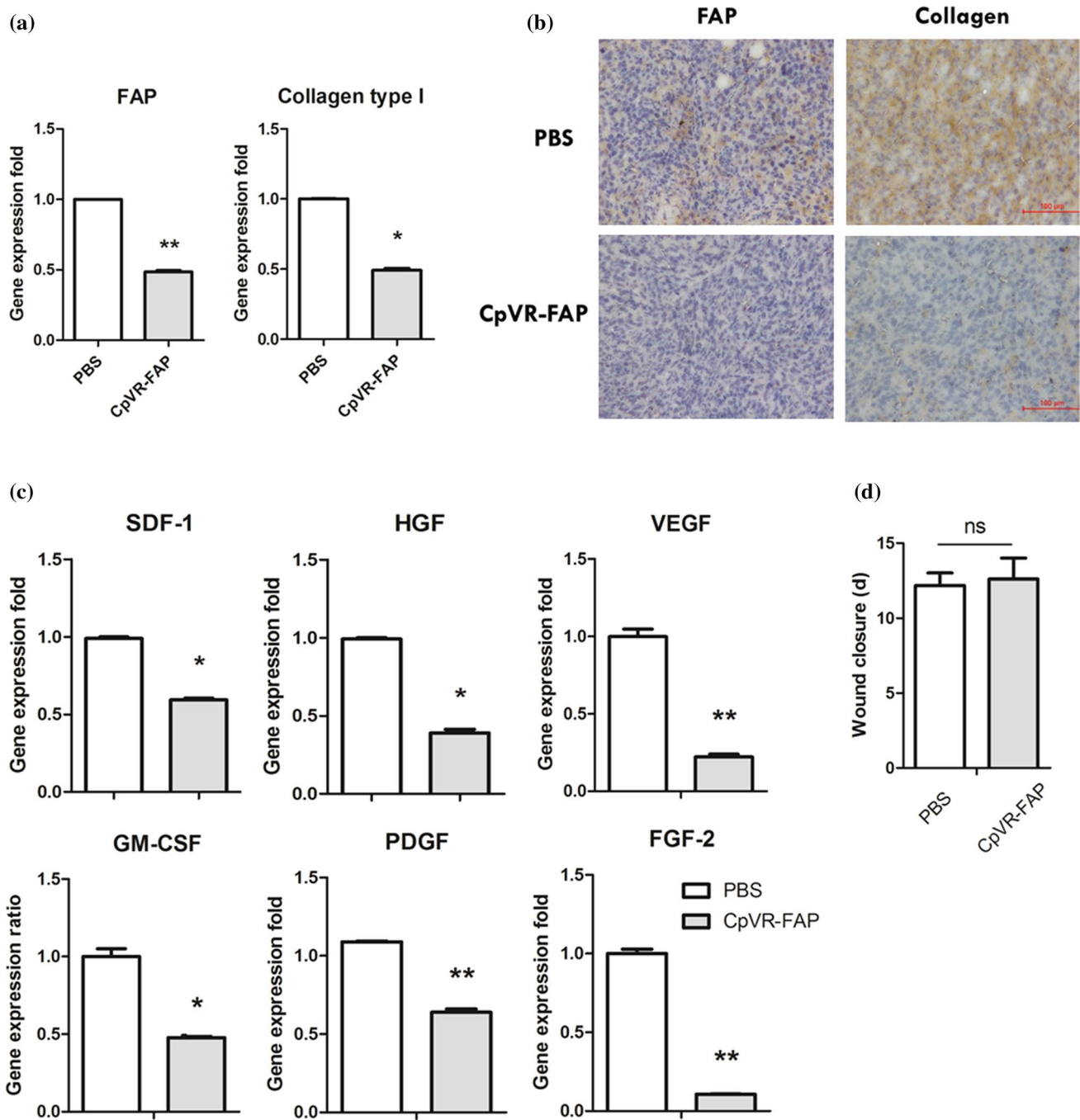After inflicting a 3-mm-diameter wound on the back skin

(a)

(b)

(c)

(d)

Fig. 6 CpVR-FAP vaccine reduces protein expression of FAPα and collagen type I and mRNA levels of tumor-associated factors but does not impair wound healing. **a** Tumors were isolated after killing, and total RNA was extracted to generate cDNA for qRT-PCR analysis. Gene expression was normalized to *β-actin*. Data for the CpVR-FAP vaccine group were calculated relative to the PBS group control for FAPα and collagen type I. **b** Immunohistochemical detection of FAPα and collagen type I expression in 4T1 tumors of vaccinated mice. **c** Relative gene expression levels for other tumor stromal factors (SDF-1, HGF, VEGFα, PDGF, FGF and GM-CSF) were also detected by qRT-PCR ($*P < 0.05$; $**P < 0.01$). **d** Circular wounds 3 mm in diameter were inflicted on the upper backs of vaccinated mice ($n = 5$), and the average time until complete wound closure was measured (*ns* not significant)

of vaccinated BALB/c mice ($n = 5$), no significant differences in wound healing times were observed between FAPα-vaccinated and PBS-vaccinated mice (Fig. 6d). Meanwhile, no adverse events or significant changes in

weight were seen throughout the experiment. Moreover, no differences were observed in the hepatic sinusoid, lobular architecture of livers and in the morphologic structure of kidneys (data not shown).

Discussion

Several studies have reported that targeting FAPα and CAFs which express FAPα inhibits tumor growth, indicating that it can become a reliable target for T cell-mediated cancer immunotherapy [12, 24, 34, 35]. DNA vaccination targeting tumor stromal antigen FAPα has been shown to elicit anti-tumor immune responses mainly mediated via CD8$^+$ T cells [14]. Indeed, targeting FAPα has several advantages over therapies directed against TAAs. FAPα-directed immunotherapy can weaken the microenvironment and immunosuppression which impair the ability of T effectors to enter into tumors and replicate. With the decrease in CAFs, the barrier for T effectors in approaching tumor cells is reduced, which helps to enhance the immune response and improve the efficacy of cancer vaccination [16]. In addition, more than 90 % of malignant epithelia such as colon, breast and lung carcinomas express FAPα [4, 6], suggesting that FAPα-targeting immunotherapy can have wide-ranging effects. These advantages support that targeting FAPα may be a potentially more effective therapeutic approach for patients with different malignancies than other ways of targeting TAAs.

Breast cancer is the most common cancer in women in the USA, being first with regard to incidence and second to lethality [1]. Clinical data have associated increased intensity of FAPα staining with decreased survival in advanced disease [36]. Additionally, using monoclonal antibodies directed against FAPα has yielded promising results in phase I and II trials [37–39], indicating that FAPα may be developed as a viable therapeutic target for treating metastatic disease. In our experiments, human FAPα presented as an effective target for 4T1 tumor immunotherapy due to its high homology, offering support and prospects for the development and application of this protein in future clinical cancer immunotherapies.

The DNA vaccine targeting human FAPα constructed in this study successfully stimulated CTLs and inhibited the rate of tumor growth and therefore has great potential for further development. Although human FAPα epitopes for stimulating immune responses still differ partially from mouse FAPα epitopes and may influence the anti-tumor immune response in mouse experiments, our findings support the feasibility of targeting human FAPα for clinical application. Moreover, other vaccine carriers such as adenoviruses are worth exploring in prime-boost strategies to enhance the relatively limited immunogenicity of the DNA vaccine. Interestingly, other studies have reported good results when combining a DNA vaccine with chemotherapies [34]. Therefore, synergistic effects are reasonably expected by coordinating a FAPα-targeted vaccine with other cancer treatment approaches such as immunotherapies or chemotherapies. Targeting CAFs and the tumor microenvironment may relieve the immunosuppression and ECM barrier [19], thereby exposing tumor cells to the direct immune attack stimulated by other TAA vaccines and chemotherapeutics.

Based on our data, we concluded that a DNA vaccine targeting human FAPα could elicit specific anti-tumor immune responses which reduced tumor growth in both a prophylactic setting and a therapeutic setting. The DNA vaccine targeting FAPα could stimulate an increase in the number of FAPα-specific CD8$^+$ T cells and improve the expression of IFN-γ in the spleen. Furthermore, the vaccine overcame the immunosuppressive environment and facilitated the increase in CD8$^+$ T cell numbers and higher levels of IFN-γ and IL-2 infiltrating into the tumor. The anti-tumor effects were also supported by decreased expression of FAPα, collagen and other stromal factors [6, 34, 40] in the tumor stroma in FAPα-vaccinated mice. Meanwhile, the destruction of the tumor CAFs and microvessels lifted the protective immunosuppression and nutrition provided to the tumor cells, thereby exposing them to the body's immune attacks. Even though FAPα can be found transiently overexpressed during wound healing, targeting this protein by vaccination did not impair wound healing, nor did it cause any discernible harmful reactions consistent with previous studies [12, 14, 34]. This study provides support for the potential clinical treatment of breast cancer using a DNA vaccine targeting human FAPα that can not only induce a high number of antigen-specific T cells against the 4T1 tumors but also break the barrier of the immunosuppressive microenvironment to allow CD8$^+$ T cells to more easily infiltrate tumors, demonstrating a great advantage for FAPα as a target over other TAAs.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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