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Immunotherapy targeting fibroblast activation protein inhibits tumor growth and increases survival in a murine colon cancer model

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Murine studies have shown that immunological targeting of fibroblast activation protein (FAP) can elicit protective immunity in the absence of significant pathology. Fibroblast activation protein is a product overexpressed by tumor-associated fibroblasts (TAF) and is the predominant component of the stoma in most types of cancer. Tumor-associated fibroblasts differ from normal adult tissue fibroblasts, and instead resemble transient fetal and wound healing-associated fibroblasts. Tumor-associated fibroblasts are critical regulators of tumorigenesis, but differ from tumor cells by being more genetically stable. Therefore, in comparison to tumor cells, TAF may represent more viable therapeutic targets for cancer immunotherapy. To specifically target TAF, we constructed a DNA vaccine directed against FAP. This vaccine significantly suppressed primary tumor and pulmonary metastases primarily through $CD8^+$ T-cell-mediated killing in tumor-bearing mice. Most importantly, tumor-bearing mice vaccinated against FAP exhibited a 1.5-fold increase in lifespan and no significant pathology. These results suggest that FAP, a product preferentially expressed by TAF, could function as an effective tumor rejection antigen. (*Cancer Sci* 2010; 101: 2325–2332)

The long-term benefits of immunotherapy in cancer patients are threatened by the escape of tumor cells from the immune system due to the inherent genetic instability of tumor cells.[1] For example, mutations in tumor-specific antigens and downregulation of MHC class I antigen can lead to escape from immune surveillance.[2–4] Additionally, defects in apoptotic signaling pathways or upregulation of apoptosis inhibitors can confer resistance to T-cell-mediated killing.[5] Therefore, to increase the efficacy of cancer immunotherapy, targeting of more genetically stable cells in the tumor stroma may prove beneficial.[5–7]

The crucial role of the tumor stroma in tumorigenesis and invasion is becoming more widely recognized.[8–10] Early studies revealed that stromal cells can stimulate the transformation of normal epithelial cells and can produce growth factors, cytokines and chemokines that induce the selection and expansion of neoplastic cells.[8,9] In support of this role, tumor cells inoculated in suspension are less tumorigenic than fragments of solid tumors containing the stroma.[11] Importantly, recent reports have suggested that modulation of tumor–stromal fibroblasts[12] or disturbance of the tumor–stromal network can lead to tumor rejection.[13,14] Tumor-associated macrophages and fibroblasts appear to contribute to the local immunosuppressive microenvironment.[15–18]

Tumor-associated fibroblasts (TAF) synthesize both collagen type I and fibroblast activation protein (FAP), a type II membrane-bound serine protease that exhibits dipeptidyl peptidase and collagenase activities implicated in extracellular matrix remodeling.[7,19,20] Some reports have indicated that overexpression of FAP leads to promotion of tumor growth.[21] Consistently, high levels of stromal FAP have been associated with aggressive disease progression and metastasis or recurrence in colon cancer,[22] and abrogation of FAP enzymatic activity can attenuate tumor growth.[23] The murine FAP gene is comprised of a 2285-bp sequence that encodes an 88 kDa protein that is 96% homologous to the human protein and exhibits a similar tissue distribution.[21,22,24–26] Fibroblast activation protein is expressed at high levels in reactive stromal fibroblasts in >90% of human epithelial carcinomas.[20] In contrast, FAP expression in non-cancerous tissues has only been observed in the developing fetus and in healing wounds.[20,27]

Together, these studies indicate that FAP is selectively expressed in TAF, where it is required for maintenance of the tumor microenvironment. Importantly, FAP has also been shown to stimulate FAP-specific cytotoxic T lymphocyte (CTL) responses from human peripheral blood mononuclear cells *in vitro*.[28] These observations suggest that FAP may represent a ''universal'' antigen that could be targeted in >90% of patients with epithelial carcinomas. We explored this hypothesis by demonstrating that an effective $CD8^+$ T-cell-mediated antitumor immune response could be induced by treatment with a DNA vaccine directed against FAP. Furthermore, vaccination against FAP significantly suppressed primary tumor formation and inhibited metastatic tumor growth in the CT26 mouse colon cancer model.

Materials and Methods

Animals and cell lines. Female BALB/c mice, 6–8 weeks in age, were purchased from the West China Experimental Animal Center (ChengDu, China). The CT26 colon carcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). CT26 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Vaccine preparation. A cDNA encoding murine FAP was purchased from Open Biosystems (Huntsville, AL, USA). Primers used to amplify the FAP fragment were as follows: 5′-CGGGATCCAAAAT GAAGACATGGCTGAAAACT-3′ and 5′-ACGCGTCGACTCAGTCTGATAAAGAAAA GCATTG-3′ (underlined sequences indicate introduced *Bam*HI and *Sal*I sites, respectively). Amplified products were subcloned into the PVITRO2-neo-mcs (pVector; InvivoGen, San Diego, CA, USA) to yield the pFAP vector, which was verified by sequencing (Invitrogen, Carlsbad, CA, USA). DNA for vaccinations and transfections was prepared using EndoFree Kits (Qiagen, Valencia, CA, USA).

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Preparation of cationic liposomes. Cationic liposomes were prepared as previously described by our laboratory.[29] Briefly, the cationic lipid DOTAP (*N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride; Avanti Polar Lipids, Alabaster, AL, USA) was mixed with neutral lipid cholesterol (Sigma; St Louis, MO, USA) at equimolar concentrations and dissolved in chloroform. The solution was rotated on a Buchi rotary evaporator at 30°C for 30 min. The resulting thin film was dried under vacuum for 15 min, followed by hydration in 5% dextrose in water to yield a final concentration of 7 mM DOTAP and 7 mM cholesterol, referred to as 7 mM DOTAP:chol. The hydrated lipid film was rotated in a water bath at 50°C for 45 min and then at 35°C for 10 min. The mixture was allowed to stand in a parafilm-covered flask at room temperature overnight, after which the mixture was sonicated at low frequency for 5 min at 50°C, transferred to a tube and heated for 10 min at 50°C. The mixture was then sequentially extruded through Millipore polycarbonate membranes (Billerica, MA, USA) of decreasing size using a syringe (five times through a 0.2 μM membrane and three times through a 0.1 μM membrane). Liposomes were stored under argon gas at 4°C.

Assays to evaluate *in vitro* expression and *in vivo* function of the recombinant plasmid. The PVITRO2-neo-mcs-FAP (pFAP) and PVITRO2-neo-mcs (pVector) constructs were transfected into CT26 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommended instructions. The FAP expression was analyzed in transfected CT26 cells by western blot analysis as previously described.[30–32] Blots with the anti-h-β-actin antibody (1:1000; Sigma) were used as loading controls.

Female BALB/c mice ($n = 5$ per group) were challenged with 1×10^5 untransfected, pVector-transfected or pFAP-transfected CT26 cells injected s.c. in the right flank. Tumor volumes were measured at 2 day intervals using calipers and were calculated according to the following formula:

$$\text{Tumor volume} = \text{width}^2 \times \text{length} \times 0.52.$$

Dipeptidyl peptidase (DPP) activity assay for recombinant murine FAP. The DPP activity assays were performed as described previously.[21,23] Briefly, an immunocapture assay was performed using Ala-Pro-7-amido-4-trifluoromethylcoumarin (Ala-Pro-AFC) as a substrate (Bachem, Torrance, CA, USA). Fluoronunc MaxiSorb 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 g/mL anti-FAP antibodies (Bender MedSystems, Vienna, Austria) and washed with phosphate-buffered saline containing 0.1% Tween-20 (PBST), followed by blocking in 5% bovine serum albumin for 1 h at room temperature. Total protein from pFAP-transfected, pVector-transfected and untransfected CT26 cells was extracted using detergent protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's recommended instructions. Protein concentrations were quantified by the bicinchoninic acid assay (Pierce Biotechnology). Approximately 1 mg of total protein extracts was added to each well and incubated for 1 h, followed by 10 washes with PBST. The DPP activity was assessed by cleavage of 0.25 mmol/L Ala-Pro-AFC for 1 h at room temperature. Release of the free AFC fluorescent substrate was detected using a cytofluor fluorimeter (Labsystems, Helsinki, Finland) with 396 nm excitation and 490 nm emission wavelengths.

Subcutaneous immunization and tumor cell challenge. For prophylactic experiments, female BALB/c mice ($n = 7$ per group) were immunized with 20 μg of DNA vaccine per injection, consisting of pFAP, pVector or normal saline (NS) encapsulated in cationic liposomes (plasmid:nanoliposome, 1:3). Immunization was performed by s.c. injection in both hind flanks six times at 1-week intervals. Seven days after the last immunization, the mice were challenged with 1×10^5 tumor cells s.c. in the right flank or i.v. in the tail vein. Tumor volume was measured as described above. Lungs were weighed, examined for metastases and scored by visual evaluation of the percentage of lung surface covered by fused metastases as follows: 0, 0%; 1, <20%; 2, 20–50%; 3, >50%.

***In vivo* adoptive transfer.** Spleens were collected on day 7 after the last vaccination. T lymphocytes were isolated from single-cell suspensions using Nylon Fiber Column T (L-Type, WAKO, Richmond, VA, USA).[31] Freshly isolated T lymphocytes (1×10^7) were injected into recipient BALB/c mice through the tail vein on the second day after mice were challenged with 1×10^5 tumor cells.

Assessment of $CD8^+$ T cell cytotoxicity. To explore whether FAP-specifc cytotoxicity was mediated by CTL, a 6 h ^{51}Cr release assay was performed as previously described.[30,31,33] Briefly, untransfected, pFAP-transfected and pVector-transfected CT26 cells were used as specific target cells. Splenocytes were isolated from immunized mice as described in a previous study.[31] Effector and target cells were seeded into a 96-well microtiter plate at the indicated effector/target ratios. Samples were then harvested and CTL activity was calculated via the following formula:

$$\%\ \text{cytotoxicity} = [(\text{Release}_{\text{experimental}} - \text{Release}_{\text{spontaneous}})/ (\text{Release}_{\text{max}} - \text{Release}_{\text{spontaneous}})] \times 100.$$

***In vivo* depletion of immune cell subsets.** Immune cell subsets were depleted as previously described.[31,33,34] Briefly, mice were injected i.p. with 500 μg anti-CD4 (cloneGK1.5, ATCC) or anti-CD8 (clone2.43, ATCC) rat monoclonal antibodies or anti-asialoGM1 polyclonal rabbit antibodies directed against natural killer cells (WAKO) 1 day prior to immunization, and then twice per week for 3 weeks. Mice were immunized with pFAP, pVector or NS twice at 1-week intervals, and four times at biweekly intervals. Seven days after the last immunization, the mice were challenged with 1×10^5 tumor cells injected s.c. in the right flank or i.v. in the tail vein.[35] Tumor volume was measured and lung metastases were assessed as described above.

Immunohistochemistry and cell staining. Fibroblast activation protein expression was assessed by immunohistochemistry in normal and cancerous colon tissue from female BALB/c mice. Sections of 5 μM were cut and stained with an anti-FAP primary antibody (Bender MedSystems) at a 1:800 dilution (v/v). A biotin-streptavidin-horseradish peroxidase detection system (BioGenex, San Ramon, CA, USA) was used to detect primary antibodies according to the manufacturer's recommended protocol. To assess levels of apoptosis in CT26 tumors, *in situ* terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling staining was performed on tumor sections according to the manufacturer's recommended instructions (Promega, Madison, WI, USA). For hematoxylin and eosin (HE) and Sirius Rose BB (Sigma) staining, paraffin-embedded tumor tissues were deparaffinized in xylene, followed by successive dehydration in 100%, 95%, 85% and 75% ethanol and rehydration in PBS (pH 7.5). HE staining was performed as described previously.[34] Sirius Rose BB staining was performed according to the manufacturer's protocol. To stain $CD8^+$ T cells, tumor sections were stained with Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 antibodies according to the manufacturer's recommended instructions (eBioScience, San Diego, CA, USA).

Statistical analysis. SPSS version 11.5 (Chicago, IL, USA) was used for statistical analyses. The Student's *t*-test and analysis of variance (ANOVA) were used to assess statistical

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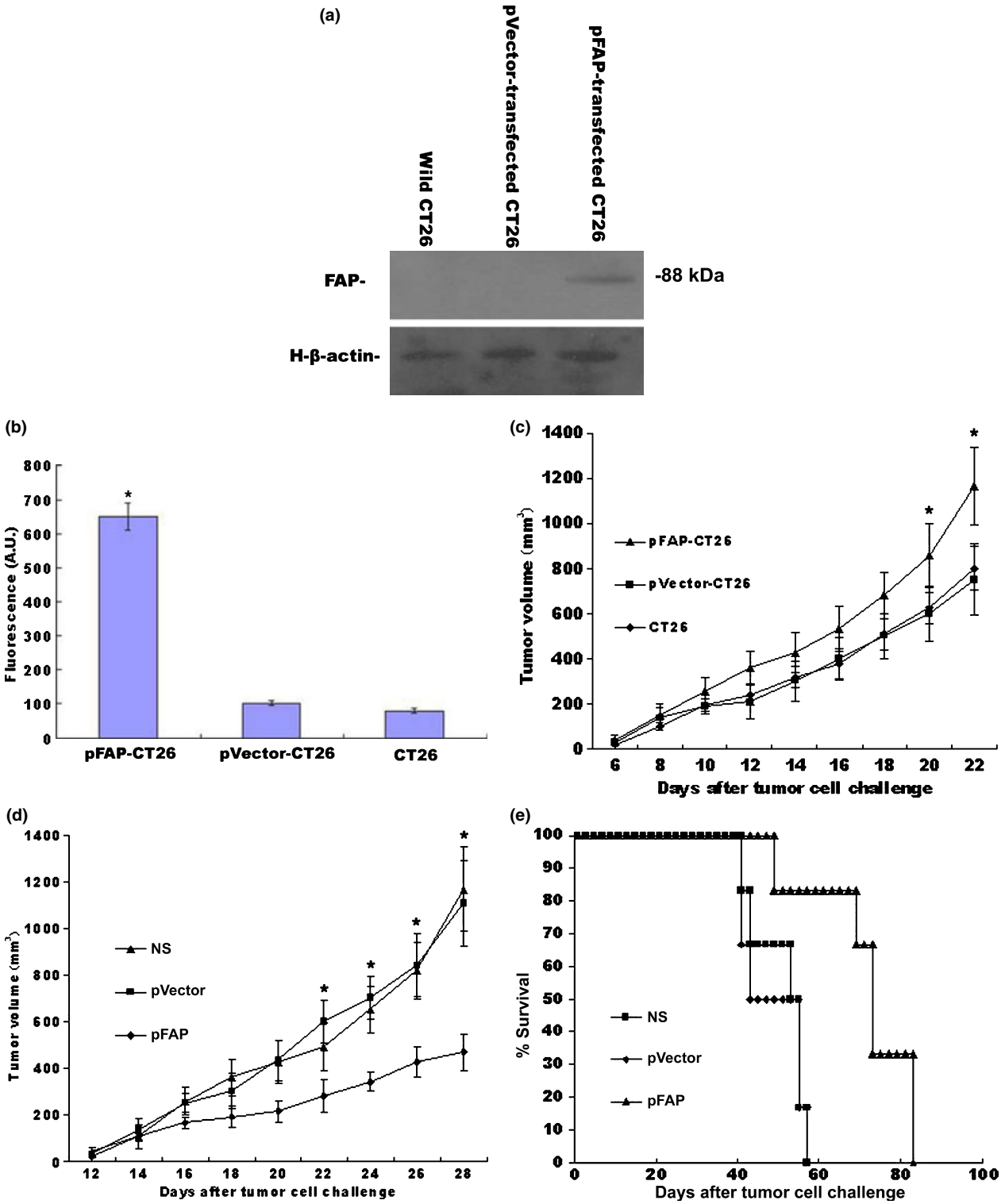
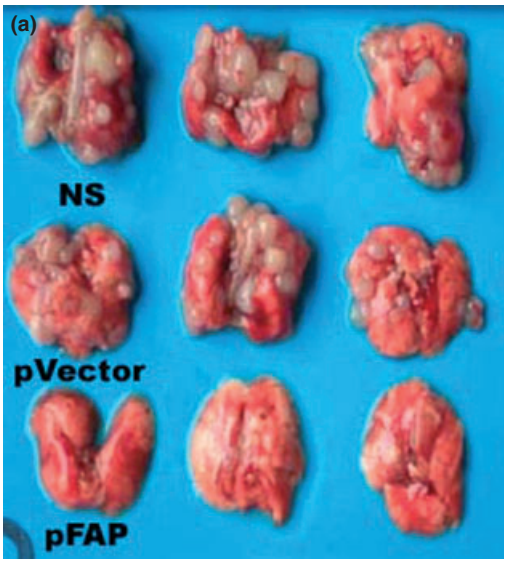
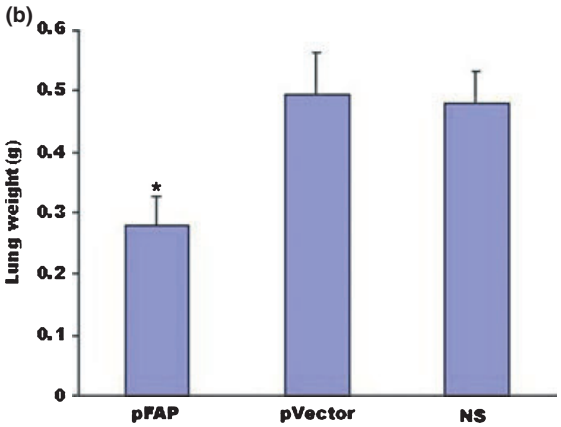


Fig. 1. Effects of fibroblast activation protein (FAP) expression and vaccination on tumor growth and lifespan of tumor-bearing mice. (a) Western blot analysis of FAP expression and (b) dipeptidyl peptidase (DPP) activity in untransfected, PVITRO2-neo-mcs (pVector)-transfected and PVITRO2-neo-mcs-FAP (pFAP)-transfected CT26 cells. pFAP-transfected CT26 cells exhibited significantly increased FAP expression and DPP activity compared with controls (*$P < 0.05$). (c) BALB/c mice ($n = 5$) were challenged by s.c. injection with 1×10^5 untransfected, pVector-transfected or pFAP-transfected CT26 cells. Mice challenged with pFAP-transfected CT26 cells (pFAP-CT26 mice) exhibited a significant increase in tumor volume in comparison with control groups (*$P < 0.05$) (c). (d–e) Seven days after the last of six vaccinations administered at 1-week intervals, BALB/c mice ($n = 7$ per group) were challenged with a lethal dose of 1×10^5 CT26 cells delivered by s.c. injection. (d) A significant decrease in tumor volume (*$P < 0.05$) was present in pFAP-immunized mice compared with control mice immunized with pVector or normal saline (NS). (e) A significant increase in survival occurred in pFAP-immunized mice in comparison with control mice ($P < 0.05$, log-rank test). Experiment was performed in duplicate for each group. Error bars indicate mean ± SD.





(c)

	Metastasis scores
NS	3,3,3,3,2,2
pVector	3,3,3,2,2,2
pFAP	2,2,1,1,1,1

Fig. 2. Inhibition of lung metastasis in mice immunized with fibroblast activation protein (FAP). BALB/c mice were immunized with PVITRO2-neo-mcs-FAP (pFAP), PVITRO2-neo-mcs (pVector) or normal saline (NS) six times at 1-week intervals, injected i.v. with 1×10^5 CT26 cells and killed on day 23 after injection. (a) Representative images of mouse lungs. (b) Average lung weights. Lungs from pFAP-immunized mice were significantly smaller than those of the control groups (*$P < 0.05$). Error bars indicate mean ± SD. (c) Percentage of lung surface covered by fused metastases.

significance. Survival curves were compared using the log-rank test. P-values <0.05 were deemed statistically significant.

Results

Recombinant pFAP expression plasmid drives expression of active pFAP *in vitro* and increases tumorigenicity of CT26 cells *in vivo*. We initially assessed expression of FAP in CT26 cells transfected with the recombinant pFAP expression vector. Western blot analysis revealed that a band corresponding to the 88 kDa murine FAP protein was present in pFAP-transfected CT26 cells (Fig. 1a), but not in untransfected cells or cells transfected with the empty pVector. Furthermore, exogenously expressed FAP was active because significantly higher levels of DPP activity were present in pFAP transfected cells compared with controls ($P < 0.05$, Fig. 1b).

To assess the tumorigenicity of pFAP-transfected CT26 cells, female BALB/c mice ($n = 5$) were challenged with untransfected, pVector-transfected or pFAP-transfected CT26 cells. Importantly, injection with pFAP-transfected CT26 cells resulted in a larger average tumor volume than injection with pVector-transfected CT26 cells or negative control cells ($P < 0.05$, Fig. 1c), indicating that expression of FAP increases the tumorigenicity of CT26 cells *in vivo*.

Tumor growth is inhibited and survival is increased in CT26 tumor-bearing mice immunized against FAP. Seven days after the last of six immunizations administered at 1-week intervals, BALB/c mice ($n = 7$ per group) were challenged with CT26 colon carcinoma cells. Subcutaneous administration of the DNA vaccine encoding murine FAP suppressed growth of primary tumors induced by CT26 cells ($P < 0.05$, Fig. 1d). In contrast, no significant antitumor immunity was detected in mice immunized with pVector or NS. Significantly increased survival was also observed in pFAP-immunized mice compared with the control groups ($P < 0.05$, Fig. 1e). Therefore, these data suggest that the FAP-based DNA vaccine can elicit a potent protective antitumor immune response, consistent with a previous study.[7]

Vaccination against FAP reduces growth of established metastases in a prophylactic setting. To assess whether vaccination with the recombinant FAP plasmid inhibits or abrogates growth of metastases, we used the CT26 lung metastasis model. Seven days after the last of six immunizations, we challenged different groups of BALB/c mice ($n = 6$ per group) i.v. with CT26 cells. On day 23, the lungs were weighed, examined for metastases and scored by visual evaluation to assess the percentage of lung surface covered by fused metastases. A significant increase in lung weight was observed in the control groups, consistent with the presence of extensive metastases and severe signs of tumor cachexia. Mice in the pFAP-vaccinated group showed a significant decrease in the number of metastases on the lung surface, as indicated by metastasis scoring and lung weight ($P < 0.05$, Fig. 2).

Fig. 3. Antitumor effects of the fibroblast activation protein (FAP) vaccine are mediated by $CD8^+$ T cells. (a–b) T cells (1×10^7) obtained from BALB/c mice immunized with PVITRO2-neo-mcs-FAP (pFAP), PVITRO2-neo-mcs (pVector) or normal saline (NS) were injected into syngeneic mice ($n = 6$) 1 day after inoculation with 1×10^5 CT26 cells. Suppression of tumor growth (*$P < 0.05$) (a) and a significant increase in survival ($P < 0.05$, log-rank test) (b) were observed in mice injected with T cells from pFAP-immunized mice compared with controls. (c–e) T lymphocytes from mice immunized with pFAP, pVector or NS were incubated with pFAP-transfected (c), pVector-transfected (d) or untransfected CT26 (e) cells at the indicated effector/target ratios, and *in vitro* cytotoxicity was measured as ^{51}Cr release. T cells isolated from mice immunized with pFAP exhibited increased cytotoxicity against FAP-positive target cells ($P < 0.05$), but not against FAP-negative target cells. A representative graph of triplicate samples from the experiment is shown. (f) $CD4^+$ or $CD8^+$ T lymphocytes or natural killer (NK) cells were depleted using corresponding antibodies in immunized tumor-bearing BALB/c mice ($n = 6$ per group), and tumor volume was assessed. Significant suppression of tumor growth was observed in pFAP, pFAP/anti-$CD4^+$ and pFAP/anti-NK mice, but not pVector, NS or pFAP/anti-$CD8^+$ mice (*$P < 0.05$). No significant difference was observed between pFAP and pFAP/anti-$CD4^+$ or pFAP/anti-NK mice. (g–i) Depletion of $CD8^+$ T lymphocytes inhibited the antitumor activity of the pFAP vaccine in the CT26 lung metastasis model ($n = 5$ per group). (g) Representative images of mouse lungs. (h) Average lung weights. The lungs from pFAP, pFAP/anti-$CD4^+$ and pFAP/anti-NK mice were significantly smaller than that of pFAP/anti-$CD8^+$ mice and the control groups (*$P < 0.05$). No significant difference in lung weight was observed between pFAP, pFAP/anti-$CD4^+$ or pFAP/anti-NK mice. Error bars indicate mean ± SD. (i) Percentage of lung surface covered by fused metastases.

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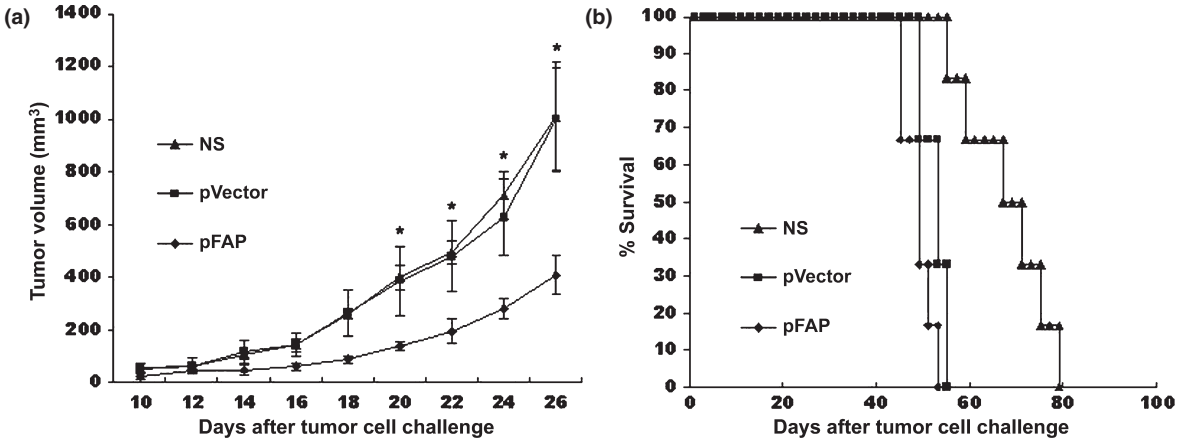

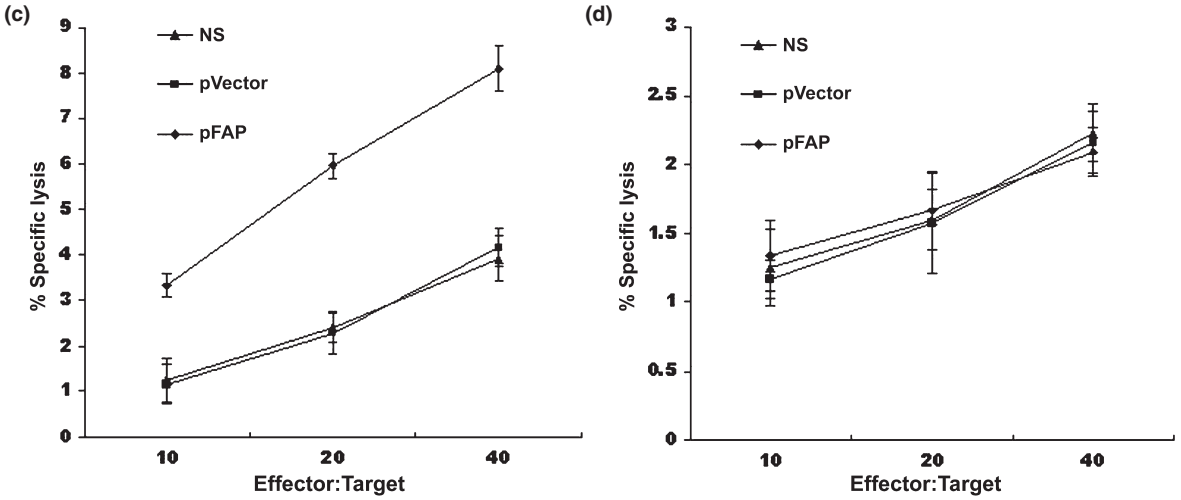

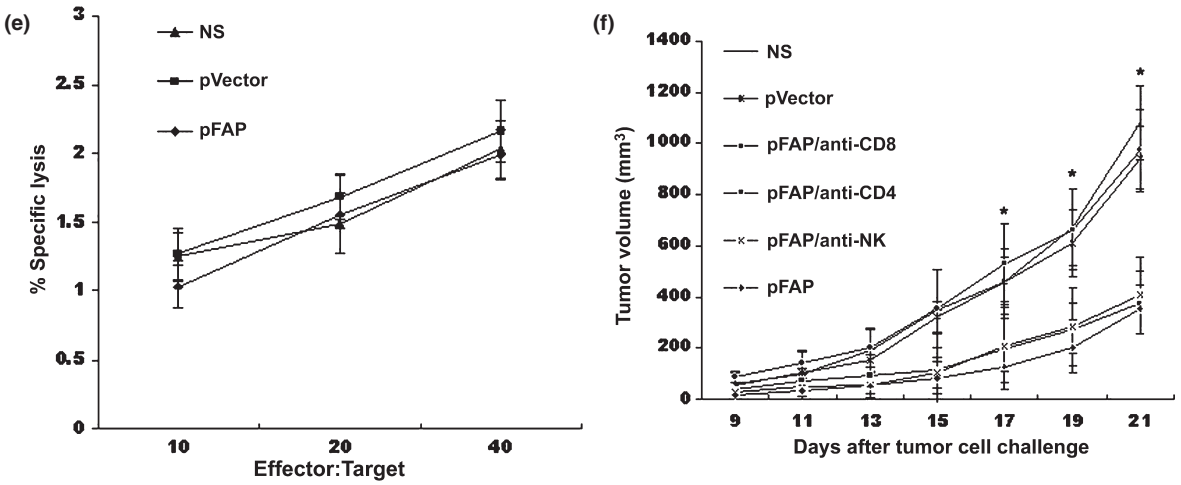

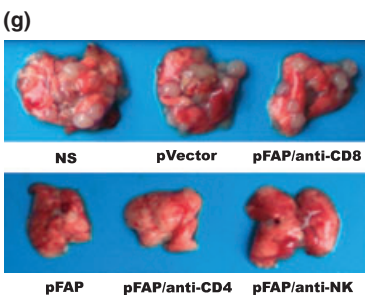

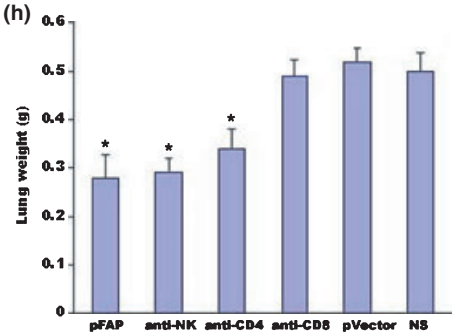

(i)

	Metastasis scores
NS	3,3,3,2,2
pVector	3,3,3,2,2
pFAP	2,2,1,1,1
pFAP/anti-CD4	2,2,1,1,1
pFAP/anti-CD8	3,3,3,3,1
pFAP/anti-NK	2,2,2,1,1

Antitumor immune response is mediated by $CD8^+$ T cells in FAP-immunized mice. To explore the possible mechanism underlying the antitumor activity of pFAP, T cells were isolated from immunized mice and transferred i.v. into BALB/c mice that had been inoculated with CT26 cells on the previous day. Tumor growth was significantly reduced ($P < 0.05$, Fig. 3a) and survival was increased in mice that received T cells from pFAP-immunized mice, as opposed to pVector- or NS-immunized mice ($P < 0.05$, Fig. 3b). These results suggest that antitumor immunity is due to the action of T cells in immunized mice.

To further characterize the requirement for CTL in antitumor immunity, splenocytes from immunized mice were used in a conventional ^{51}Cr-release assay. Splenocytes from mice immunized with pFAP, pVector or NS were incubated for 6 h with pFAP-transfected, pVector-transfected or untransfected CT26 cells as specific targets, and the percentage of cell lysis was calculated. Incubation with splenocytes from pFAP-vaccinated mice led to substantially increased lysis of FAP-positive target cells in comparison with splenocytes from pVector- and NS-immunized mice at all target-to-effector ratios (Fig. 3c). The specificity of FAP targeting was indicated by the results showing that splenocytes from pFAP-vaccinated mice did not selectively lyse FAP-negative target cells (Fig. 3d,e).

In order to determine the precise effector cell population that accounts for the observed antitumor immune response, mice were immunized with pFAP, pVector or NS twice at 1-week intervals, followed by four times at biweekly intervals. During the effector phase, the mice were depleted of specific effector cells using antibodies directed against $CD4^+$ or $CD8^+$ T cells or natural killer (NK) cells. Seven days after the last immunization, the mice were challenged with 1×10^5 tumor cells injected s.c. in the right flank or i.v. into the tail vein. Importantly, antibody-mediated depletion of $CD8^+$ T cells, but not $CD4^+$ or NK cells, resulted in loss of the antitumor immune response, evident as increases in tumor volume, lung weight and metastasis scoring, similar to that of controls (Fig. 3f–i). This requirement for $CD8^+$ T cells has previously been observed in a similar study using a different DNA-based vaccine.[6,7]

Fibroblast activation protein immunization is associated with increases in the numbers of $CD8^+$ T cells and apoptotic cells and decreases in FAP and collagen expression in tumors. Based on data suggesting a requirement for $CD8^+$ T cells in antitumor immunity, we next evaluated whether increased lymphocyte infiltration was present in tumors in immunized mice. HE staining was performed on tumor sections from mice vaccinated with pFAP, pVector or NS. A $35.4\% \pm 3.04\%$ increase in lymphocyte infiltration was observed in mice vaccinated with pFAP compared with control groups (Fig. 4a–c). To determine whether infiltrating lymphocytes included $CD8^+$ T cells, tumor sections from vaccinated mice were stained with FITC-anti-mouse CD8 antibodies. Compared with controls (Fig. 4d,e), a $42.4\% \pm 2.54\%$ increase in $CD8^+$ T cell infiltration was observed in mice vaccinated with pFAP (Fig. 4f). In order to determine whether the increase in $CD8^+$ T cells is associated with apoptosis of tumor cells, we performed TUNEL staining on tumor sections from vaccinated mice. These results revealed that the number of apoptotic tumor cells was increased in mice vaccinated with pFAP compared with controls (Fig. 4g–i). These results suggest that $CD8^+$ T cells play a key role in induction of apoptosis as part of the antitumor response in FAP-vaccinated mice.

To evaluate whether the antitumor immune response leads to downregulation of FAP and collagen in the tumor stroma, tumor sections from vaccinated mice were stained with anti-FAP antibodies and Sirius Rose BB. Compared with mice vaccinated with pVector or NS, significantly decreased expression of both FAP (Fig. 4j–l) and collagen (Fig. 4m–o) were observed in mice vaccinated with pFAP. Similarly, normal female BALB/c colon tissues also exhibited significantly decreased expression of FAP (Fig. 4p). These results are consistent with previous studies suggesting that expression of FAP and collagen in the tumor stroma are important for tumor growth.[7,21]

Discussion

We have demonstrated that subcutaneous injection of a DNA-based vaccine targeting the tumor stromal antigen FAP can elicit T-cell-mediated anti-cancer immune responses. In comparison with therapies directed against tumor cell-specific antigens, this approach has several well-documented advantages. First, stromal fibroblasts are more genetically stable than tumor cells,[2–4] and may represent more reliable targets for immunotherapy. Second, antigen presentation by stromal fibroblasts to the T cell receptor complex is not impaired by downregulation of MHC class I antigen expression.[7] Third, tumor cells often become increasingly resistant to T-cell-mediated killing due to defects in apoptotic signaling pathways that can include upregulation of anti-apoptotic proteins or immunosuppressive effects on CTL.[1,5] Finally, initial results from clinical trials targeting FAP enzymatic activity[36,37] and results demonstrating specific overexpression of FAP in more than 90% of colon, breast and lung carcinomas[26] suggest that targeting of FAP could be an effective therapeutic approach for a broad array of patients with different malignancies.

Colorectal cancer is the third most common cancer with regard to incidence and lethality. Clinical data have shown that stromal FAP expression is inversely correlated with the clinical stage in human colon cancer and that increased intensity of FAP staining is associated with decreased survival for advanced disease.[22] Additionally, small phase I and II trials using monoclonal antibodies directed against FAP have had promising results,[36–38] suggesting that FAP may represent a viable therapeutic target for treatment of metastatic disease.

Our experimental data demonstrate that immune tolerance against the FAP self-antigen can be inhibited through delivery of FAP cDNA as a subcutaneous DNA vaccine. In prophylactic experiments, the $CD8^+$ T-cell-mediated antitumor immune response induced by pFAP vaccination inhibited tumor growth (Fig. 1d), significantly suppressed growth of pulmonary metastases (Fig. 2) and prolonged the lifespans of vaccinated mice (Fig. 1e), consistent with a previous study.[7] Similar results were also observed for adaptive immunity induced by adoptive transfer of T cells from pFAP-immunized mice (Fig. 3a,b). Non-specific immune responses were unlikely, because the cytotoxic effects mediated by $CD8^+$ T cells *in vitro* were restricted to target cells overexpressing the FAP antigen (Fig. 3c–e), consistent with other studies.[6,7] Selective *in vivo* depletion of effector cells indicated that $CD8^+$ T cells, but not $CD4^+$ T cells or NK cells, were required for the antitumor response (Fig. 3f–i). The specificity of the response was also supported by the observation that decreased FAP (Fig. 4l) and collagen (Fig. 4o) expression were observed in the tumor stroma in pFAP-vaccinated mice, similar to normal mouse colon tissues. Based on these data, we hypothesize that decreases in tumor growth in FAP-immunized mice are related to decreased numbers of TAF that synthesize FAP and collagen type I[7,19,20] and act as key regulators of tumorigenesis.[7]

Importantly, the pFAP vaccine appears to avoid immune tolerance against the FAP self-antigen, similar to previous studies,[6,7,28] as pFAP-vaccinated mice exhibited increased tumor lymphocyte infiltration (Fig. 4c), $CD8^+$ T cell infiltration (Fig. 4f) and apoptosis of tumor cells (Fig. 4i) compared with controls. Vaccination against FAP did not cause any discernible autoimmune reactions or significant delays in wound healing,

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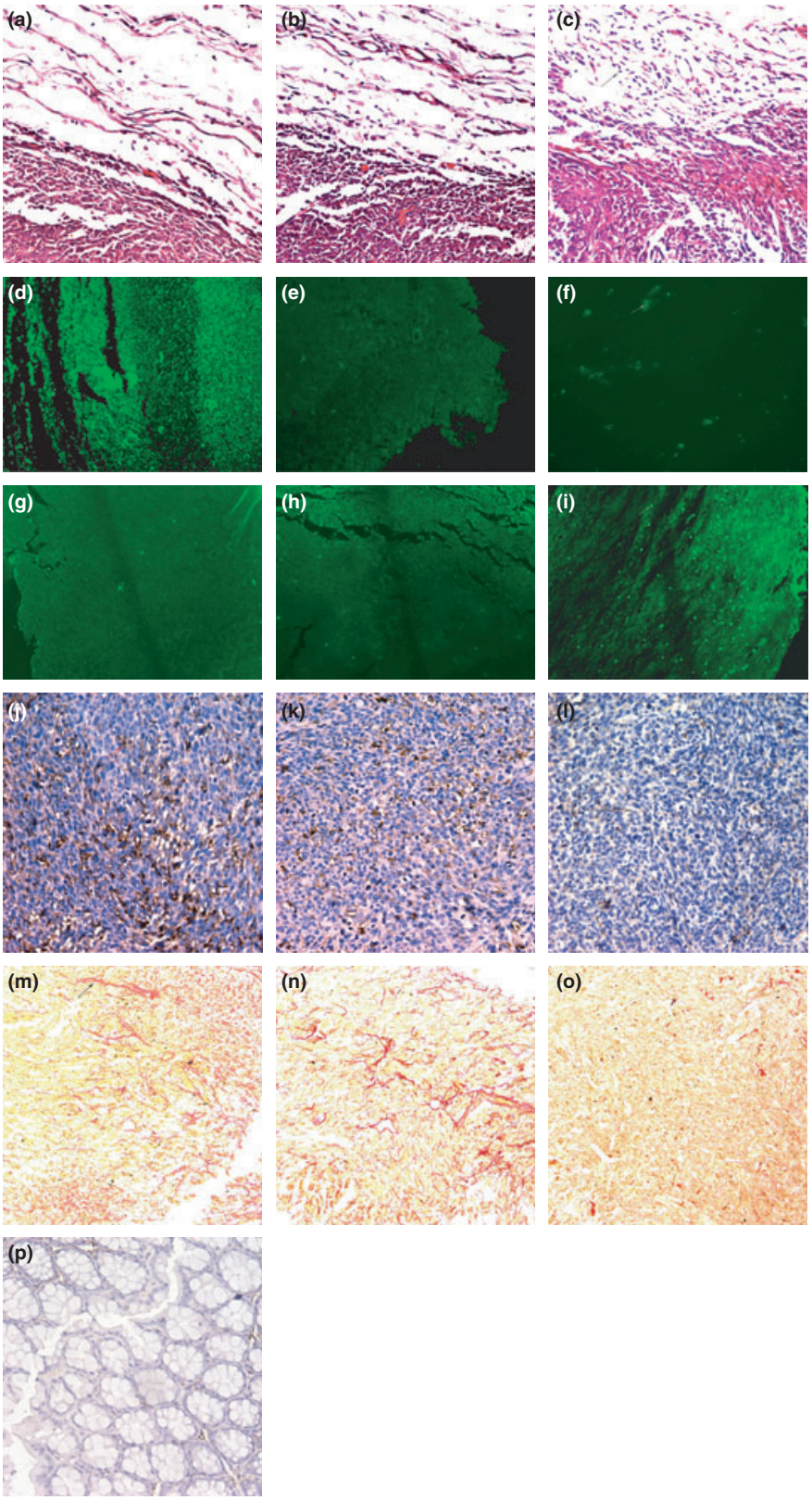

Fig. 4. Immunohistochemical characterization of tumor tissues in immunized mice. Tumor sections from mice immunized with normal saline (NS) (a,d,g,j,m), PVITRO2-neo-mcs (pVector) (b,e,h,k,n) or PVITRO2-neo-mcs-FAP (pFAP) (c,f,i,l,o), or colon tissue sections from control mice (p) were stained with HE (a–c), anti-CD8 antibodies (d–f), with the TUNEL assay (g–i), with antibodies directed against fibroblast activation protein (FAP) (j–k,p), and with Sirius Rose BB to stain for stromal collagen (m–o). In comparison with mice immunized with NS or pVector, increased lymphocyte and $CD8^+$ T cell infiltration (black and white arrows, respectively), increased numbers of apoptotic cells (white arrows), and decreased FAP and collagen expression (red and black arrows, respectively) were observed in tumors from pFAP-immunized mice. (a–c,j–o,p) ×40 magnification; (d–i) ×20 magnification.

consistent with previous studies.[6,7,23] Redundancy of proteins with overlapping functions may account for these observations.[39]

Together, our findings demonstrate the efficacy of a subcutaneous DNA vaccine in eliciting a $CD8^+$ T-cell-mediated immune response against the tumor-stromal antigen FAP in the absence of obvious pathology. We anticipate that this study and other related immunotherapy-based studies might lead to novel approaches and improvements in the clinical treatment of cancer.

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Disclosure Statement

The authors have no conflict of interest.

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