Tumor Immunotherapy Targeting Fibroblast Activation Protein, a Product Expressed in Tumor-Associated Fibroblasts

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Abstract

Murine studies have shown that immunologic targeting of the tumor vasculature, a key element of the tumor stroma, can lead to protective immunity in the absence of significant pathology. In the current study, we expand the scope of stroma-targeted immunotherapy to antigens expressed in tumor-associated fibroblasts, the predominant component of the stroma in most types of cancer. Mice were immunized against fibroblast activation protein (FAP), a product up-regulated in tumor-associated fibroblasts, using dendritic cells transfected with FAP mRNA. Using melanoma, carcinoma, and lymphoma models, we show that tumor growth was inhibited in tumor-bearing mice vaccinated against FAP and that the magnitude of the antitumor response was comparable to that of vaccination against tumor cell–expressed antigens. Both s.c. implanted tumors and lung metastases were susceptible to anti-FAP immunotherapy. The antitumor response could be further enhanced by augmenting the translated FAP product into the class II presentation pathway, or by covaccination against FAP and a tumor cell–expressed antigen, tyrosinase-related protein 2. No morbidity or mortality was associated with anti-FAP vaccination except for a small delay in wound healing. The study suggests that FAP, a product which is preferentially expressed in tumor-associated fibroblasts, could function as a tumor rejection antigen in a broad range of cancers.

Introduction

The goal of active immunotherapy for the treatment of cancer is to stimulate immunity against tumor antigens that will lead to eradication of tumors in the absence of significant adverse effects to the patient. With the advent of increasingly effective antitumor vaccination strategies, the emergence of treatment-resistant tumor variants may become a major impediment in the development of effective and long-lasting therapies (1, 2).

One approach to reduce immune escape is to target the tumor stroma (3). Studies in murine models have shown that immunologic targeting of the tumor vasculature, a key element of the tumor stroma, can lead to protective immunity in the absence of significant pathology. Vaccination with fixed xenogeneic whole endothelial cells elicited protective immunity including regression of preexisting tumors, which correlated with a reduction in microvessel density within the tumors of the vaccinated animals (4). Vaccination against a specific endothelial product, vascular endothelial growth factor receptor 2 (VEGFR-2), encoded in a Salmonella vector led to reduced growth of established tumors in several tumor models, which was also associated with a slight delay in wound healing (5). Likewise, vaccination with dendritic cells loaded with VEGF-2 protein inhibited the development of pulmonary metastases in two tumor models but had a negative effect on the fertility of the immunized female mice (6). We have shown that vaccination of tumor-bearing mice with dendritic cells transfected with miRNA encoding several angiogenesis-associated products, including VEGFR-2, Tie-2, or VEGF, stimulated antitumor immunity in several tumor models and synergized with vaccination against “classic” tumor antigens (i.e., antigens expressed in the tumor cells themselves). Vaccination against VEGFR-2, but not against epidermal growth factor, was also associated with a transient inhibition of fertility (7).

The objective of the current study was to expand the scope of stroma-targeted immunotherapy to antigens expressed in tumor-associated fibroblasts. Fibroblasts are the predominant cellular component of the tumor stroma in most types of cancers (8–11). The tumor-associated fibroblasts, termed carcinoma-associated fibroblasts, reactive fibroblasts, or myofibroblasts, differ from normal adult tissue fibroblasts, resembling fibroblasts transiently present in the developing fetus and during wound healing. Like their wound or fetal counterparts, tumor-associated fibroblasts exhibit enhanced motility and proliferation and provide essential functions to promote the survival and growth of the tumor (8–11). Recent studies have also implicated fibroblasts in the early stages of carcinogenesis regulating the outgrowth of transformed cells from ostensibly normal tissues (12, 13).

Concurrent expression of β1-smooth muscle actin, vimentin, and fibroblast activation protein (FAP) are commonly used to distinguish tumor-associated fibroblasts from their normal counterparts (8–11). Expression profiling has revealed that over 170 genes are differentially expressed in the tumor-associated fibroblasts compared with normal skin– or liver-derived fibroblasts (14) and could therefore serve as antigens to stimulate antitumor immunity. Yet the fibroblast-expressed products are self-antigens and are likely to have triggered varying levels of tolerance which will inversely correlate with the frequency and avidity of cognate T cells available for activation by the vaccination protocol (15). To this end, we described an in vitro screening method to determine whether products which are overexpressed in tumor-associated fibroblasts are capable of stimulating CTL responses from human peripheral blood mononuclear cells. We have shown in the previous study that CTL responses could be consistently generated against FAP but not against two matrix metalloproteinases (MMP), MMP-9 and MMP-14 (16).

FAP is a type II membrane-bound serine protease exhibiting dipetidyl peptidase and collagenase activities (17, 18). FAP expression is highly restricted to tumor-associated fibroblasts...
Materials and Methods

Mice
Five- to six-week-old C57BL/6 mice (H-2b) and BALB/c (H-2d) were obtained from Charles River Laboratories (Wilmington, MA). In conducting the research described in this article, the investigators adhered to the Guide for the Care and Use of Laboratory Animals as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National for the Care and Use of Laboratory Animals as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke vivarium are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Murine Tumor Cell Lines and Fibroblast Cultures
The F10.9 clone of the B16 melanoma (26) and 4T1 breast carcinoma (27) cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL; all from Invitrogen, Carlsbad, CA). The EL4 thymoma cell line (28) was cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. Fibroblasts from day 13.5 C57BL/6 embryos were obtained from Dr. Rachel Rempel (Duke University, Durham, NC). EL4 tumor-associated fibroblasts were obtained from s.c. implanted EL4 tumor by collagenase treatment following separation by meshing through a nylon filter and plastic adherence. Fibroblasts were cultured in DMEM supplemented with 20% heat-inactivated FBS, penicillin, and streptomycin and passed every 3 to 5 days. After five passages, over 90% of cells were positive for vimentin and β-smooth muscle actin as measured by immunostaining (data not shown).

Generation of murine bone marrow–derived dendritic cells.
Murine bone marrow–derived dendritic cells were generated from bone marrow progenitors as previously described (7). Briefly, bone marrow progenitors isolated from tibias and femurs of C57BL/6 or BALB/c mice were harvested followed by treatment with ammonium chloride Tris buffer for 3 minutes at 37°C to deplete the RBC. Progenitors (1 × 10^7) were cultured in RPMI 1640 with 5% heat-inactivated FBS, penicillin, streptomycin, sodium pyruvate (1 mmol/L), MEM nonessential amino acids (1 mmol/L), HEPES (10 mmol/L), γ-glutamine (2 mmol/L), MEM amino acids (1 mmol/L), and β-mercaptoethanol (5 × 10^-2 mmol/L; all from Invitrogen), as well as granulocyte macrophage colony-stimulating factor (GM-CSF; 15 ng/mL) and interleukin 4 (IL-4; 10 ng/mL; both from Peprotech, Rocky Hill, NJ), at 37°C in a humidified atmosphere with 5% CO2. On day 3, nonadherent cells were removed and adherent cells replenished with the fresh complete medium including GM-CSF and IL-4. On day 6, nonadherent cells were harvested and used as immature dendritic cells.

Isolation of Total RNAs from Tumor Cell Lines and Tumors
RNA was isolated from in vitro cultured tumor cell lines and s.c. implanted tumors using a RNeasy Mini kit (Qiagen, Valencia, CA) following the instructions of the manufacturer and frozen at -20°C before use.

Murine Fibroblast Activation Protein Reverse Transcription-PCR
Reverse transcription was done with SuperScript III reverse transcriptase (Invitrogen) following the instructions of the manufacturer. The PCR reaction volume was 30 μL, containing 0.2 μmol/L deoxyxynucleotide triphosphate mixture, 1.5 mmol/L MgCl2, 2.5 units of Platinum Tag DNA polymerase (Invitrogen), 1.5 μL reverse transcription reaction mixture as templates, and 1 μmol/L each of various primers: murine FAP forward primer 5’-TGGGTGTCCAGTGAAACGATGTAG-3’ and reverse primer 5’-TGTATTTCTTGGTCTGTGTCGGCC-3’, murine tyrosinase-related protein 2 (TP2-2) forward primer 5’-GGATGACCTGAGAATGGCTGCCC-3’ and reverse primer 5’-GCTTGATACCAGGTACC-3’, and reverse primer 5’-CAAACATGATCAGTCGATCTTT-3’ (Invitrogen). The GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT) was used with an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds, 55.3°C for 30 seconds, and 72°C for 1 minute, and a final elongation step at 72°C for 7 minutes. PCR products were separated on 1% agarose gels containing ethidium bromide.

Generation of Fibroblast Activation Protein and Fibroblast Activation Protein/Lympho-associated Membrane Protein mRNA
Cloning of pSP73-Sph/FAP/A64.
Forward primer 5’-TATATCTGCAGC-CACCATGGAAGATGGCTGAAAATCTGTCG-3’ and reverse primer 5’-TATAGTGCAGCTGCTGATAAAGAAGCCCCG-3’ were used to amplify the entire coding region of murine FAP from a cDNA library generated from a s.c. grown B16/F10.9 tumor. The FAP cDNA was cloned into the pLon-Pal II sites of the transcription vector pSP73-Sph/A64 (Promega, Madison, WI).

Cloning of pSP73-Sph-gp96SS/FAP/LAMP/A64.
The gp96 signal sequence was amplified from the plasmid pGEM4Z/HTERT/LAMP/A64 (29) with the primers 5’-ATATAAGCCTGCCATAGGACGGCCTGGGT-3’ and 5’-GATAATAGCCCCAGGTCACATACTTATTCGACG-3’. The lyosomal targeting signal sequence of lysosome-associated membrane protein-1 (LAMP-1) was amplified from the same plasmid using the primers 5’-CTATATGCCCTGTTGGCGCTATCCCGATGTTGGG-3’ and 5’-AATAAGAATTCTCATGAGCTCGTGTCGG-3’. The FAP coding sequence not including signal sequences was amplified from the plasmid pSP73-Sph/FAP/A64 using the primers 5’-TATATGCCCATTTGGGGCCCTTAAACACCTGAAAGAAAACAC-3’ and 5’-TATATAGCACAAGGGCCTGTGCTGTAAGAAAAGCATTG-3’. The resulting PCR products were digested with SfiI and ligated using T4 DNA ligase. The product was digested with HindIII and EcoRI and cloned into the HindIII and EcoRI sites of pSP73-Sph/A64/Not (11).

In vitro Transcription and Translation of mRNA
Plasmids were linearized with SpeI and in vitro transcribed using the mMESSAGE mMACHINE T7 RNA transcription kit (Ambion, Austin, TX). The in vitro transcribed RNA was isolated using RNeasy kit (Qiagen) after digestion of the DNA templates with DNase I. The in vitro transcribed RNAs and total RNAs from tumor cell lines were amplified into immature dendritic cells as previously described with minor modifications (7). Briefly, 5 × 10^5 immature dendritic cells suspended in 200 μL Opti-MEM (Invitrogen) were mixed with 15 μg in vitro transcribed RNAs or 25 μg total RNAs in 2-mm cuvettes and were electrophorated at 300 V for 500 μs using an Electro Square Porator ECM 830 (BTX, San Diego, CA). Transfected cells were incubated in GM-CSF– and IL-4-containing medium for 24 hours. Lipopolysaccharide (100 ng/mL; Sigma-Aldrich, St. Louis, MO) was added for 4 hours to induce dendritic cell maturation.

Tumor Immunotherapy
B16/F10.9 melanoma (4 × 10^5) or EL4 thymoma (2 × 10^5) cells were implanted s.c. into the right flank of a C57BL/6 mouse and 3 × 10^5 4T1 cells were implanted s.c. into the right flank of a BALB/c mouse. Three and 10 days after tumor implantation, the mice were immunized i.p. with mRNA-transfected 4 × 10^5 mature dendritic cells in 200 μL PBS. Mice received two immunizations, 1 week apart. Tumor growth was evaluated every other day after the second immunization by measuring tumor diameter using a...
caliper. Tumor volume was defined as (smallest diameter)$^2 \times$ (longest diameter). Mice were scored as tumor positive when tumors were palpable and longest diameter reached 5 mm. Mice were sacrificed when tumor diameter reached 2 cm.

**In vivo Depletion of CD4+ and CD8+ T Cells**

In vivo depletion of CD4+ and/or CD8+ T cells was done as previously described with minor modifications (30). Briefly, antimouse CD4 antibody (ATCC hybridoma GK1.5) or antimouse CD8 antibody (ATCC hybridoma 53.6.72) was administered i.p. at a dose of 150 and 200 μg/mouse, respectively, on days $-2, 0,$ and $+2$ of tumor implantation and dendritic cell immunization, resulting in over 95% depletion of CD4+ or CD8+ T cells. Mouse immunoglobulin G (200 μg/mouse) was used as control in mice not receiving the depleting antibodies.

**Wound Healing Study**

Wound healing experiments were done as previously described with minor modifications (31). Briefly, mice were immunized twice, 1 week apart, with mRNA-transfected dendritic cells as described above. Eight days after the last immunization, mice were anesthetized and 6-mm biopsy punch (Fray, Buffalo, NY) was applied to generate two full-thickness superficial wounds by folding the back skin and punching through the skin. Wounds were measured daily in two dimensions with a caliper to calculate wound area.

**Statistical Analysis**

The difference of tumor volume and wound healing among experimental groups was compared using the two-tailed Student’s $t$ test. Significance of tumor-free survival was determined by the log-rank test. A probability of $<0.05\ (P < 0.05)$ was used for statistical significance.

**Results**

**Expression of fibroblast activation protein in tumor-associated fibroblasts.** FAP was shown to be expressed in tumor-associated fibroblasts but not in tumor cells (17, 23–25). To confirm that FAP exhibits a similar expression pattern in the experimental systems used in this study, we measured FAP expression in tumor cell lines grown in vitro and in stroma-containing tumors isolated from mice. Three tumor models were used in this study: the B16/F10.9 melanoma (26), the EL4 thymoma (28), and the 4T1 breast carcinoma (27). RNA was isolated from either in vitro grown tumor cells or from s.c. implanted tumors and FAP expression was determined by semiquantitative reverse transcription-PCR (RT-PCR; murine FAP antibodies are not available for measuring protein expression). RNA from embryonic fibroblasts was used as a positive control (17, 23) and RNA from L929 fibroblasts was used as a negative control for FAP expression (24).

As shown in Fig. 1, FAP expression was readily detected in embryonic fibroblasts but not in L929 fibroblasts. FAP expression was not detected in either B16/F10.9 or EL4 tumor cells grown in vitro. A trace amount of FAP expression was, however, seen in the in vitro grown 4T1 tumor cells. By contrast, FAP expression was readily detected when RNA was isolated from in vivo grown B16/F10.9, EL4, and 4T1 tumors, most likely due to the presence of tumor-associated fibroblasts. As expected, TRP-2 transcripts were detected in B16/F10.9 tumor–derived RNA. TRP-2, an endogenous tumor antigen corresponding to a nonself-mutated melanocytic product, was expressed in the B16/F10.9 tumor cells (32). The experiment shown in Fig. 1 is therefore consistent with previous observations showing that FAP is expressed in the tumor stroma but not in tumor cells.

**Inhibition of tumor growth in B16/F10.9 melanoma tumor–bearing mice immunized against fibroblast activation protein.** We used stringent therapeutic tumor models to determine whether vaccination against FAP engenders protective tumor immunity. In the experiments shown in Fig. 2, C57BL/6 mice were implanted s.c. with B16/F10.9 melanoma, a low MHC class I–expressing, highly metastatic, and poorly immunogenic tumor of C57BL/6 (H-2b) origin (26). Three days later, mice were immunized twice, 1 week apart, with dendritic cells transfected with mRNA encoding actin, TRP-2, or FAP. Actin served as a negative control and TRP-2 served as a positive control for a conventional tumor cell–expressed antigen (32). Tumor progression was determined by measuring time to tumor appearance. As shown in Fig. 2, tumor growth was significantly retarded in mice vaccinated against FAP ($P < 0.0001$). Interestingly, vaccination against the stromal antigen FAP was comparably effective to vaccination against the tumor cell–expressed antigen TRP-2. This was similar to a previous observation in which vaccination of B16 tumor–bearing mice against VEGFR-2, a stromal product expressed in the tumor vasculature, was not less effective than vaccination against TRP-2 (7). Vaccination against TRP-2 or FAP led to retardation of tumor growth but no long-term regressions were seen. One reason for this seemingly modest antitumor effect is the stringency of the therapeutic model used in this experiment; the B16/F10.9 clone is a low MHC class I–expressing, highly aggressive, and fast-growing tumor and mice received only two vaccinations targeting a single antigen. Another reason could be that the cytoplasmic expression of the mRNA-translated FAP antigen is deficient in stimulating an effective CD4+ T-cell response, which could also play an important role in engendering protective immunity (33, 34). Wu et al. have shown that appending the lysosomal targeting signal from LAMP-1 to the carboxyl end of the endogenously expressed antigen can enhance class II presentation and induction of CD4+ T-cell responses (35,36).

We have previously shown that LAMP-1-modified carboxyembryonic antigen or telomerase reverse transcriptase (TERT) mRNA–transfected human dendritic cells stimulated enhanced CD4+ T-cell responses in vitro without compromising the generation of CD8+ CTL responses (37,38). We therefore tested whether a lysosomal targeting signal–modified FAP (FAP/LAMP) antigen will be more effective in stimulating antitumor immunity. As shown in Fig. 2, vaccination of the B16/F10.9 tumor–bearing mice with FAP/LAMP was significantly more effective than vaccination with unmodified FAP antigen in retarding tumor progression ($P = 0.0354$) and also resulted in the long-term regression of tumors in a fraction of the vaccinated animals.

We have previously shown that covaccination against a tumor cell–expressed antigen, TRP-2, and a stromal antigen, VEGFR-2,
was synergistic compared with vaccination against either antigen alone (7). We therefore tested whether the concept of concurrent vaccination against tumor cell–expressed and stromal antigens also applies to vaccination against FAP. In the experiment shown in Fig. 2B, tumor growth was again delayed in the B16/F10.9 tumor–bearing mice vaccinated against either TRP-2 or FAP/LAMP and vaccination against FAP/LAMP was more effective than vaccination against TRP-2. However, in this experiment, none of the FAP/LAMP–vaccinated mice remained tumor-free as seen in Fig. 2A.

Importantly, when mice were covaccinated against both TRP-2 and FAP/LAMP, tumor growth was significantly delayed compared with mice vaccinated against TRP-2 alone (P = 0.0345) or FAP/LAMP (P = 0.0489) alone. This observation confirms and extends our previous findings that concomitant vaccination against tumor cell–expressed and stromal antigens is synergistic, enhancing the therapeutic outcome of the vaccination protocol.

To determine whether metastatic growth is also susceptible to FAP immunotherapy, FAP-immunized mice were challenged with B16/F10.9 melanoma cells i.v. and the appearance of lung metastasis was monitored. As shown in Fig. 3A, lung metastasis was significantly inhibited in mice immunized against the FAP/LAMP antigen (P = 0.0038), which was comparable or superior to that of vaccination against the tumor cell–expressed TRP-2 antigen. Figure 3B shows that reduced metastasis also correlated with a significant extension of survival of the FAP/LAMP– and TRP-2–vaccinated groups (both P < 0.0001).

Role of CD4+ and CD8+ T cells in fibroblast activation protein– and fibroblast activation protein/lysosome-associated membrane protein–mediated tumor immunity. To determine whether CD4+ T cells contributed to enhanced protective
immunity in mice vaccinated with the FAP/LAMP antigen as shown in Fig. 2, FAP- or FAP/LAMP–vaccinated mice were depleted of either CD4+ or CD8+ T-cell subsets using antibodies. As shown in Fig. 4, inhibition of tumor growth in mice vaccinated against FAP was mediated by CD8+ T cells because depletion of CD8-expressing cells significantly enhanced tumor growth (P = 0.015). However, tumor growth was not significantly affected by depletion of CD4-expressing cells (P = 0.12), suggesting that the contribution of CD4+ T cells to protective immunity was small at best. In contrast, when mice were vaccinated with FAP/LAMP, depletion of either CD4-expressing (P = 0.029) or CD8-expressing (P = 0.003) cells significantly enhanced tumor growth. In addition, depletion of CD4+ T cells in FAP/LAMP–vaccinated mice had a more pronounced effect on tumor growth compared with FAP-vaccinated mice (P = 0.003). Thus, mice vaccinated with the FAP/LAMP antigen stimulated a CD4+ T-cell response that contributed to the protective antitumor response. This observation is consistent with previous studies showing that appending lysosomal targeting signal sequences to cytoplasmic antigens targets the antigens into the class II presentation pathway to generate CD4+ T-cell responses that augment protective antitumor immunity (35).

**Discussion**

In the current study, we have shown that FAP, a product expressed in tumor-associated fibroblasts but not in tumor cells, can function as a tumor rejection antigen (i.e., stimulating immunity against FAP engenders protective immunity in the absence of significant pathology). Previous studies have shown the feasibility of antistromal immunotherapy by targeting products expressed in the tumor vasculature, primarily VEGFR-2 (5–7). In this study, we extended this concept to products expressed in a second key component of the tumor stroma, fibroblasts.

It is well established that beyond a minimal size, tumor progression is critically dependent on a stroma consisting of vascular cells, inflammatory cells, fibroblasts, and other cell types (8, 29, 39). Because stromal cells, unlike tumor cells, are diploid, genetically stable, and exhibit limited proliferative capacity, stimulating immune responses against products expressed in the stromal cells could substantially reduce the incidence of immune evasion (3). In addition, because expression of many stromal products is ubiquitous, they could be targeted in most, if not all, cancer patients. The recognition that the tumor stroma plays a key role in tumor progression has led to the development of small molecules or monoclonal antibodies designed to inhibit essential stromal functions (40, 41). Despite earlier predictions (42), emergence of treatment-resistant stromal cells was seen, most
likely due to overlap or redundancy in the targeted functions (43). It is tempting to speculate that because immune recognition is solely dependent on the expression of the target antigen and will not be affected by the expression of other products of redundant function, the emergence of treatment-resistant stromal cells by active immunotherapy will be further reduced compared with the current approaches.

A main criterion in choosing candidate self-antigens for cancer immunotherapy, whether expressed in the tumor cells or the tumor stroma, is their differential expression in the tumor versus normal tissue. Murine studies are then used to provide proof-of-concept that vaccination against such antigen can stimulate antitumor activity in the absence of significant autoimmunity. Differential expression of self-antigens in the tumor milieu is, however, not the sole determinant of their suitability as tumor rejection antigens. Self-antigens are normal nonmutated gene products which are likely to have triggered various degrees of tolerance, manifested by reduced numbers and avidity of cognate T cells that can be activated to stimulate effective immunity (15). Because the pool of differentially expressed products is often large, we suggested that screening for products which triggered less tolerance, by measuring the ability to stimulate CTL responses in vitro from the peripheral blood mononuclear cells of cancer patients, should precede animal studies assessing their ability to engender protective immunity and autoimmune pathology in murine tumor models (16). For example, expression profiling has revealed that >170 products are overexpressed in tumor-associated versus normal fibroblasts (14). Of three products, we found that FAP, but not MMP-9 or MMP-14, was able to consistently stimulate CTL responses from peripheral blood mononuclear cells (16), suggesting that FAP has triggered less tolerance in humans than did MMP-9 or MMP-14. We therefore

Figure 6. Effect of anti-FAP immunotherapy on wound healing. C57BL/6 mice (n = 5 in each group) were immunized twice, 1 week apart, with actin, FAP, or FAP/LAMP mRNA–transfected dendritic cells or mock immunized with PBS. Eight days after the last immunization, mice were anesthetized and 6-mm biopsy punch was applied to generate two full-thickness superficial wounds by folding the back skin and punching through the skin (30). *, P < 0.05; †, P < 0.02. Experiments were done twice.

Figure 5. Inhibition of 4T1 breast carcinoma and EL4 thymoma growth in mice immunized with FAP/LAMP mRNA–transfected dendritic cells. A, BALB/c mice (n = 10 in each group) were implanted s.c. with 3 × 10^3 4T1 tumor cells and, 3 days later, immunized twice, 1 week apart, with dendritic cells transfected with actin or FAP/LAMP mRNA or with RNA isolated from in vitro grown 4T1 tumor cells as described in Materials and Methods. Left, time to tumor appearance; right, tumor volume at day 24. P < 0.0001, FAP/LAMP– versus actin-vaccinated groups; P = 0.1, FAP/LAMP versus 4T1 vaccinated groups. Experiments were done twice. B, same as (A) except that C57BL/6 mice (n = 10 in each group) were implanted s.c. with 2 × 10^4 EL4 thymoma cells. P = 0.007, FAP/LAMP– versus actin-vaccinated groups; P > 0.1, FAP/LAMP– versus EL4-vaccinated groups. Experiments were done thrice.
chose to evaluate FAP as a candidate antigen to stimulate protective immunity in the murine tumor models.

We used stringent tumor models to show that vaccination against FAP, a product expressed preferentially in tumor-associated fibroblasts, exhibits a significant antitumor effect which has a magnitude comparable to that of vaccination against tumor cell–expressed antigens, such as TRP-2 expressed in B16/F10.9 melanoma or total tumor cell–derived antigenic mixtures from EL4 or 4T1 tumor cells (Figs. 2 and 5). Consistent with the broad distribution of FAP-expressing fibroblasts in tumors, anti-FAP immunotherapy was effective against three independently derived tumors of distinct origin, B16/F10.9 melanoma, 4T1 breast carcinoma, and EL4 thymoma (Figs. 2 and 5). Both s.c. implanted primary tumors and metastatic tumor were susceptible to anti-FAP vaccination (Figs. 2 and 3). Thus, FAP, as previously shown for another stromal product, VEGFR-2 (5–7), could serve as a "universal" antigen that can be targeted in many, if not all, cancer patients.

Notwithstanding the stringency of the tumor models used in this study, anti-FAP vaccination was only partially effective; tumor growth was significantly retarded but complete tumor regressions were not common. Whereas extending the immunization protocol beyond the two vaccinations used in these experiments would conceivably increase the "cure" rate of the treated animals, the purpose and main value of such murine tumor models is the comparative testing of strategies that would augment the therapeutic effect of an otherwise promising approach; in this instance, vaccinating against FAP. We illustrate this point with two examples: enhancing the CD4+ T-cell arm of the anti-FAP immune response and concomitant vaccination against tumor cell–expressed antigens. As shown in Fig. 2A, redirecting the mRNA-translated FAP product into the class II presentation pathway potentiated the vaccination-induced antitumor response. The antibody depletion experiment shown in Fig. 4 provides evidence that CD4+ T cells induced by the FAP/LAMP antigen were responsible for the enhanced antitumor response. In the second example, Fig. 2B shows that covaccination against FAP and the tumor cell–expressed TRP-2 leads to an enhanced antitumor effect. A similar enhancing effect was seen when mice were covaccinated against the endothelial VEGFR-2 product and TRP-2 (7). It is tempting to speculate that this additive effect is a result of simultaneous targeting and destruction of the tumor and its stroma.

The antigenic targets in the tumor stroma are normal non-mutated gene products (self-antigens) that are preferentially, not necessarily exclusively, expressed in the stromal cells. For example, FAP is up-regulated in fibroblasts of wound healing tissue (23) and bears 48% homology to dipeptidyl peptidase IV, which is widely expressed in normal tissues (44). This raises the concern that vaccination against a stromal product such as FAP will induce autoimmune pathology. New insights on how the immune system discriminates between self and nonself supported by animal studies suggest that vaccination against such normal products is capable of stimulating protective antitumor immunity in the absence of significant autoimmune (see discussions in refs. 3, 45–48). In this study, the FAP-vaccinated mice remained viable and appeared healthy. Nevertheless, consistent with FAP expression in wounds, induction of a FAP-specific immune response was associated with a small delay in wound healing (Fig. 6). Adverse effects of limited severity and transient in nature were also seen when mice were vaccinated against the endothelial product VEGFR-2 (5–7). Overall, these observations suggest that vaccination against stromal products such as FAP can elicit protective antitumor immunity in the absence of unacceptable levels of autoimmune pathology. However, additional studies employing increasing potent vaccination protocols are needed to address this important issue.

Successful application of FAP-targeted vaccination in cancer patients will require the development of increasingly potent vaccination protocols and testing in tumor models of increasing stringency and relevance. Future studies need to address the mechanism how a FAP-directed immune response affects tumor growth, whether by directly affecting the tumor-associated fibroblast or via collateral damage inducing a local inflammatory response. The demonstration that FAP, a stromal product, can stimulate protective antitumor immunity is a step toward the identification of tumor rejection antigens which (a) in combination will stimulate potent immunity, (b) can be targeted in most, if not all, cancer patients, and (c) can counter the propensity of tumors to escape immune elimination.

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