Targeting Fibroblast Activation Protein in Tumor Stroma with Chimeric Antigen Receptor T Cells Can Inhibit Tumor Growth and Augment Host Immunity without Severe Toxicity

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Abstract
The majority of chimeric antigen receptor (CAR) T-cell research has focused on attacking cancer cells. Here, we show that targeting the tumor-promoting, nontransformed stromal cells using CAR T cells may offer several advantages. We developed a retroviral CAR construct specific for the mouse fibroblast activation protein (FAP), comprising a single-chain Fv FAP [monoclonal antibody (mAb) 73.3] with the CD8α hinge and transmembrane regions, and the human CD3ζ and 4-1BB activation domains. The transduced muFAP-CAR mouse T cells secreted IFN-γ and killed FAP-expressing 3T3 target cells specifically. Adoptively transferred 73.3-FAP-CAR mouse T cells selectively reduced FAP+ stromal cells and inhibited the growth of multiple types of subcutaneously transplanted tumors in wild-type, but not FAP-null immune-competent syngeneic mice. The antitumor effects could be augmented by multiple injections of the CAR T cells, by using CAR T cells with a deficiency in diacylglycerol kinase, or by combination with a vaccine. A major mechanism of action of the muFAP-CAR T cells was the augmentation of the endogenous CD8+ T-cell antitumor responses. Off-tumor toxicity in our models was minimal following muFAP-CAR T-cell therapy. In summary, inhibiting tumor growth by targeting tumor stroma with adoptively transferred CAR T cells directed to FAP can be safe and effective, suggesting that further clinical development of anti-human FAP-CAR is warranted. Cancer Immunol Res; 2(2); 154–66. ©2013 AACR.

Introduction
One approach to adoptive T-cell therapy has been to transfer patient-derived blood lymphocytes with chimeric antigen receptor (CAR) genes that combine the effector functions of T lymphocytes with high specificity of single-chain antibody fragments (scFv) that recognize predefined surface antigens in a non-MHC-restricted manner (1, 2). With recent modifications and improvements, CAR T-cell therapy is showing great promise in the clinic (3–5).

To minimize on-target/off-tumor toxicity, target antigens should be expressed at high levels on the surface of tumor components with minimal or no expression on “essential” normal tissues. To date, potential targets have largely been antigens on cancer cells, such as CD19, ErbB2, CEA, or GD2 (3–6). We postulate that noncancer cell components of the tumor may also be attractive targets for CAR-based immunotherapy. First, the genetic instability of tumor cells contributes to their immune escape, whereas stromal cells are more genetically stable. Second, the tumor stroma contributes to tumor growth and resistance to therapy by (i) forming a physical barrier to tumor-targeting agents; (ii) supporting tumor cell growth, invasion, and angiogenesis through the production of growth factors, chemokines, and matrix (7–11); and (iii) exerting an immunosuppressive influence by secreting factors that attract immunosuppressive cells, by producing factors that regulate T-cell functions and modulate the phenotype of myeloid cells (7, 12), and by expressing inhibitory surface molecules PD-L1 and PD-L2 (13). Finally, the mechanisms by which the cancer stroma supports tumorigenesis are shared among various stromal cell types, so that therapies targeting such mechanisms are likely to have therapeutic implications across a broad spectrum of cancers.

One attractive stromal cell target is the fibroblast activation protein (FAP), a transmembrane serine protease highly expressed in the cancer-associated stromal cells (CASC) of...
virtually all epithelial cancers (14–18), FAP is also expressed during embryonic development, in tissues of healing wounds, and in chronic inflammatory and fibrotic conditions such as liver cirrhosis and idiopathic pulmonary fibrosis (19–22). However, FAP has not been detected by immunohistochemistry in benign tumors nor in most normal quiescent adult stromal cells (23–25). FAP+ cells have been studied using Bac transgenic mice containing the FAP promoter driving the GFP or the human diphertheria toxin (DT) receptor to conditionally eliminate FAP+ cells (26, 27). Although the elimination of FAP+ cells caused the decreased growth of immunogenic OVA-expressing Lewis lung carcinoma tumors and augmented the efficacy of an OVA vaccine, the complete ablation of FAP+ cells was associated with anemia and cachexia, thought to result from the loss of FAP+ stromal cells in the bone marrow and muscle. Some FAP expression was also seen in the pancreas.

Therapeutically, FAP+ cells have been successfully targeted using vaccines and immunoconjugate therapy resulting in some tumor growth inhibition without obvious toxicity or effects on wound healing (28–30). However, given the increased efficacy of adoptive T-cell transfer, we and others have hypothesized that anti-FAP-CAR T cells may prove more effective. Since the submission of this manuscript, three contrasting articles have been published describing the use of anti-FAP-CAR T cells. Tran and colleagues, using anti-FAP-CAR mouse T cells [comprising the scFv from the FAP-5 monoclonal antibody (mAb; ref. 30) that targets human and mouse FAP] in combination with total body irradiation and interleukin-2 (IL-2) injections, showed limited antitumor efficacy that was associated with severe bone marrow toxicity and cachexia (31). In contrast, Kakarla and colleagues showed antitumor efficacy without toxicity using anti-FAP-CAR human T cells [comprising the scFv from the M036 mAb (32) that targets both human and mouse FAP] in an immunodeficient mouse model of human lung cancer (33). Similarly, Schuberth and colleagues showed a survival advantage in an intraperitoneal mesothelioma xenograft model after administering FAP-CAR human T cells [using a scFv from the human-specific F19 mAb (18)] together with FAP-expressing human mesothelioma cells (34). In this study, the CAR-mediated on-target/off-tumor toxicity could not be evaluated because the F19 anti-human FAP antibody does not cross-react with the mouse FAP.

Given the recent data that FAP-expressing cells may play an important role in regulating antitumor immunity (35), we developed a system to study advanced-generation FAP-CAR T-cell therapy in wild-type (WT) mice with fully intact immune systems. Using a different anti-FAP scFv (mAb 73.3) linked to both human and murine cytoplasmic domains, we generated muFAP-CAR mouse T cells that elicited antitumor efficacy mediated by the activation of endogenous immune responses in multiple tumor models. The anti-FAP-CAR T cells demonstrated enhanced antitumor response when combined with a tumor vaccine. Our FAP-CAR constructs induced minimal toxicity with no anemia or weight loss, suggesting that further clinical development of anti-human FAP-CAR T cells may be feasible.

Materials and Methods

Cell lines

Mouse AE17.ova mesothelioma cells (expressing chicken ovalbumin) were provided by Delia Nelson (University of Western Australia, Crawley, WA, Australia; ref. 36). TC1 lung cancer cells were derived from mouse lung epithelial cells immortalized with human papillomavirus (HPV) HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (37). The mouse LKR cell line was derived from an explant of a pulmonary tumor from an activated K-rasG12D–mutant mouse generated in the laboratory of Tyler Jacks at MIT (38). Mouse 4T1 mammary carcinoma cells, CT26 colon cancer cells, and 3T3Balb/C cells were purchased from the American Type Culture Collection. Mouse FAP-expressing 3T3BALB/C (3T3.FAP) cells were created by lentiviral transduction of the FAP-3T3 parental line with murine FAP (data not shown). All cell lines were used for Mycoplasma: except for the protein expression of the transgenes, no additional authentication was performed. See Supplementary Methods for more details.

Antibodies

Specific antibodies used are listed in the Supplementary Methods.

Generation of 73.3 hybridoma

FAP-null mice (39) were immunized and twice boosted with FAP-expressing 3T3 cells intraperitoneally. Three days after the final boost, splenocytes were harvested and fused to myeloma cells. Hybridoma supernatants were screened for mAbs that reacted specifically with 3T3.FAP cells and activated primary WT fibroblasts, but not the parental 3T3 cells or the activated primary fibroblasts from FAP-null mice. mAb 73.3 with this reactivity profile was purified and characterized by ELISA and by immunoblotting as specific for mouse FAP based on the reactivity with the purified recombinant mouse FAP extracellular domain (rFAP-ECD), its reactivity with a single protein with an apparent molecular weight of approximately 95 kDa in the protein extracts of FAP+ but not FAP− cells and tissues, and lack of reactivity with cells expressing human FAP [human FAP transduced 3T3, human foreskin fibroblasts, or primary human tumor-associated stromal cell (data not shown)]. Biacore studies (see Supplementary Methods) showed that the affinity of mAb 73.3 for purified rFAP-ECD protein was less than 1 nmol/L and estimated to be between 0.1 and 0.3 nmol/L (data not shown).

Generation of anti-muFAP-CAR constructs

Total RNA from 73.3 hybridoma cells was isolated and reverse transcribed into cDNA. Variable heavy (VH) and light (VL) chains of the 73.3 immunoglobulin were PCR-amplified and sequenced (Fig. 1A and B). The VH and VL sequences were fused with a CAR construct that is being evaluated in clinical trials [CD8z hinge, CD8z transmembrane domain, and two human intracellular signaling domains (ICD) derived from 4-1BB and CD3ε; ref. 40]. This CAR was then inserted into an internal ribosome entry site (IRES)-containing retroviral
MigR1 vector (Fig. 1C) that also expresses GFP for tracking purposes (41). A fully mouse construct of FAP-CAR and FAP-CAR-m28z was also created by coupling the same 73.3 scFv with the murine CD3ζ chain and the murine CD28 ICD. This construct was inserted into another retroviral vector MSGV (Fig. 1D; ref. 42). Infective particles were generated from the supernatants of 293T cells transfected with the retroviral plasmid and helper plasmids using Lipofectamine 2000 (Invitrogen), as described previously (42, 43). Two control FAP-CAR constructs, one with human CD3ζ and the other with mouse CD3ζ ICDs, were also generated to evaluate the function of the costimulatory domains (see Supplementary Methods).

Isolation, transduction, and expansion of primary mouse T lymphocytes

Primary murine splenic T cells were isolated and transduced as previously described (43). See Supplementary Methods.

Antigen- or antibody-coated beads

rFAP-ECD (see Supplementary Methods), bovine serum albumin (BSA; Fisher Scientific), or anti-CD3ε/anti-CD28 antibodies (eBioscience) were chemically cross-linked to tosylactivated 4.5 μm Dynabeads (Invitrogen; #140-13) as per the manufacturers’ instructions.

Immunoblotting

FAP-CAR–transduced T cells were incubated either with BSA or FAP-ECD–coated beads (at 2:1 bead to T-cell ratio), or with anti-CD3ε antibody for 10 minutes. Lysates were then prepared and immunoblotted for phosphorylated extracellular signal–regulated kinase (ERK), phosphorylated AKT, phosphorylated inhibitor of 1kB kinase (IKK)-α/β, or β-actin.

Cytotoxicity and IFN-γ ELISA

Parental 3T3 and 3T3.FAP cells were transduced with luciferase as described previously (44). T cells and target 3T3 cells were cocultured at the indicated ratios, in triplicate, in 96-well round-bottomed plates. After 18 hours, the culture supernatants were collected for IFN-γ analysis using an ELISA (mouse IFN-γ; BD OpEIA). Cytotoxicity of transduced T cells was determined by detecting the remaining luciferase activity from the cell lysate using a previously described assay (43).

CAR T-cell transfer into mice bearing established tumors

Mice were injected subcutaneously with 2 × 10^6 AE17.ova (C57BL/6 mice), 1 × 10^6 TC1 (C57BL/6 mice), 2 × 10^6 LKR (C57BL/6 crossed with 129 pF/j), 0.5 × 10^6 4T1 (BALB/c mice), or 1 × 10^6 CT26 (BALB/c mice) tumor cells into the dorsal–lateral flank. Mice bearing established tumors (100–150 mm³) were randomly assigned to receive either FAP-CAR T cells or MigR1-transduced T cells or remained untreated (minimum, 5 mice per group, each experiment repeated at least once). A total of 1 × 10^7 T cells was administered through the tail vein. Body weight and tumor size were measured by electronic scales and calipers, respectively. At the end of the experiment, tumors and spleens were harvested for flow-cytometric analyses.

Flow-cytometric analyses

Tumors were harvested 3 and 8 days after adoptive transfer of FAP-CAR T cells to analyze intratumoral cells by flow cytometry as described previously (42). Cell acquisition was performed on LSR-II using FACSDiva software (BD Bioscience). Data were analyzed using FlowJo (TreeStar).

Statistical analyses

For flank-tumor studies comparing two groups, the Student t test was used. For comparisons of more than two groups, we
used one-way ANOVA with appropriate post hoc testing. Differences were considered significant when $P$ value was less than 0.05. Data are presented as mean ± SEM.

**Results**

**In vitro evaluation of mouse FAP-CAR T cells**

From our primary retroviral CAR construct (containing the scFv from anti-murine FAP antibody 73.3 coupled to the human CD3ζ and 4-1BB cytoplasmic domains that we have used previously in murine models; ref. 42) and a control virus expressing only GFP (Fig. 1) were used to transduce activated mouse T cells resulting in greater than 60% of the T cells expressing GFP (MigR1) or GFP plus FAP-CAR (Fig. 2A).

To verify functionality, mouse T cells expressing FAP-CAR were stimulated for 18 hours with beads coated with either BSA (negative control), or recombinant FAP protein, or anti-CD3/anti-CD28 antibodies (positive control). The FAP-coated beads activated FAP-CAR T cells, as shown by increased CD69 expression above that of the negative control (Fig. 2B).

To further evaluate intracellular signaling, lysates from bead-stimulated T cells were electrophoresed and immunoblotted. In comparison with BSA-coated beads, FAP-coated beads induced the phosphorylation of AKT, ERK, and IKK-β (Fig. 2C).

To assess effector functions, transduced mouse T cells were cocultured with 3T3 fibroblasts (which do not express FAP) or with 3T3 fibroblasts transduced to express mouse FAP (3T3.FAP; data not shown). After 18 hours, T cells expressing the FAP-CAR construct (but not the control GFP construct) effectively killed 3T3.FAP fibroblasts (Fig. 2D) and secreted IFN-γ (Fig. 2E) in a dose-dependent manner, but had no effect on parental 3T3 cells.

**Injection of mouse FAP-CAR T cells reduces tumor growth in a FAP-specific fashion**

We next explored the capability of FAP-CAR mouse T cells to inhibit tumor growth using three different tumor lines that do not express FAP: AE17.ova mesothelioma cells, TC1, and LKR lung cancer cells. Cells were injected into the flanks of syngeneic mice and allowed to form established tumors. The tumors had an easily detectable number of FAP-expressing cells with the majority of the FAP+ cells being CD45−/CD90+ stromal cells (~3% of total tumor cells), and only a small minority being CD45+ hematopoietic cells (~0.2% of total tumor cells; Table 1 and Supplementary Fig. S1).

When tumors reached approximately 100 to 150 mm3 (7–14 days after tumor cell inoculation), 10^7 T cells were injected intravenously and the tumors were measured with
calipers. FAP-CAR T cells, but not MigR1 T cells, significantly (P < 0.05) reduced the growth of TC1 tumors (Fig. 3A), LKR tumors (Fig. 3B), and AE17.ova tumors (Fig. 3C) by 35% to 50%.

To confirm specificity, we inoculated AE17.ova cells into FAP-null C57BL/6 mice and treated the tumors as described above. In contrast with the effect on AE17.ova tumors in WT C57BL/6 mice (Fig. 3C), FAP-CAR T cells had no effect on the growth of AE17.ova tumors in FAP-null mice (Fig. 3D).

Given the differences between our efficacy data and those of Tran and colleagues’ data (31), we also treated two of the same tumor lines, CT26 and 4T1, they reported. In contrast to their findings, our FAP-CAR construct induced a

Table 1. Depletion of FAP$^+$ cells in flank tumors after FAP-CAR treatment

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<tr>
<th></th>
<th>Untreated</th>
<th>FAP-CAR</th>
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<tr>
<td>AE17.ova</td>
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<td>CD45-CD90$^+$</td>
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<td>1.15</td>
<td>0.001$^a$</td>
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<td>CD45$^+$</td>
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<td>0.08</td>
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<tr>
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<td>0.03$^a$</td>
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<tr>
<td>CD45-CD90$^+$</td>
<td>1.72</td>
<td>0.60</td>
<td>0.04$^a$</td>
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<td>CD45$^+$</td>
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<td>0.04</td>
<td>0.066</td>
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<tr>
<td>Average tumor volume, mm$^3$</td>
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<td>140</td>
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<th></th>
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<tr>
<td>CD45-CD90$^+$</td>
<td>2.68</td>
<td>1.84</td>
<td>0.009$^a$</td>
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<td>CD45$^+$</td>
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<td>0.15</td>
<td>0.035$^a$</td>
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<tr>
<td>Average tumor volume, mm$^3$</td>
<td>410</td>
<td>260</td>
<td>0.02$^a$</td>
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NOTE: The values above indicate the averages of percentage FAP$^+$ cells per total tumor population. Tumors were harvested 7–9 days after T-cell infusion, and there were 5 mice in each group. Student paired t test was performed to evaluate FAP$^+$ cell depletion, as well as change in tumor volume, after adoptive T-cell therapy.

$^a$Statistical significance between untreated and FAP-CAR–treated samples; P < 0.05.

Figure 3. Antitumor activities of FAP-CAR T cells in mice bearing flank tumors. Syngeneic mice bearing (A) TC1, (B) LKR, (C) AE17.ova, (E) CT26, and (F) 4T1 tumors were injected intravenously with 10 million FAP-CAR or MigR1 T cells when the tumors reached approximately 100 to 150 mm$^3$. Tumor measurements followed. D, to test the target specificity of FAP-CAR T cells, AE17.ova tumor cells were also injected into FAP-null C57BL/6 mice. FAP-CAR T cells were given 7 days later. $^*$, Statistical significance between untreated, MigR1–, and FAP-CAR–treated samples; P < 0.05.
significant reduction in tumor size (Fig. 3E and F), although the changes were smaller than those seen in Fig. 3A–D.

**Effect of the injection of mouse FAP-CAR T cells on FAP**

To evaluate the effect of the T cells on the FAP**+** stromal cells, we harvested tumors 7 to 9 days after T-cell infusion and analyzed the dissociated cells by flow cytometry. As shown in Table 1, at this time point, the FAP**+/CD45**−/CD90**+** and FAP**+/CD45**− populations were decreased by about 50% in comparison with those in the untreated group, whereas the amount of FAP**+** cells remaining in the MigR1 group was similar to that in the untreated controls (data not shown).

We characterized this depletion in more detail using the AE17.ova model and evaluated the FAP**+** cells 3 days after T-cell transfer. At this earlier time point, we saw larger decreases in FAP**+/CD90**+** stromal cells (a reduction of 82%) and FAP**+/CD45**− leukocytes (a reduction of 56%; Supplementary Fig. S2A). Moreover, in control animals, we could identify both low- and high-FAP—expressing cells in both the CD45**+** and CD45**− populations (Supplementary Fig. S1). When we gated on these specific populations, we noted that the FAP-CAR T cells selectively depleted the FAP-high—expressing cells, with little effect on FAP-low—expressing cells (Supplementary Fig. S2B and S2C).

**Kinetics of FAP-CAR T-cell persistence**

We assessed the number of intratumoral FAP-CAR T cells in the AE17.ova model at 3, 7, and 10 days after adoptive transfer and found that the number peaked at day 3 after injection and diminished at the 7 and 10 day time points by approximately 65% (Supplementary Fig. S3A).

To determine whether this rapid loss of T cells was a consequence of abnormal function of the human 4-1BB and CD90**+** populations were decreased by about 50% in comparison with those in the untreated group, whereas the amount of FAP**+** cells remaining in the MigR1 group was similar to that in the untreated controls (data not shown).

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**Role of the acquired immune system in the efficacy of FAP-CAR T cells**

To evaluate the role of the acquired immune system in the FAP-CAR T cell—mediated antitumor response, we injected AE17.ova tumor cells into the flanks of either WT C57BL/6 or immunodeficient NSG mice, followed by one injection of 10**7** FAP-CAR T cells. AE17.ova tumors grew more rapidly in NSG than in WT mice (Fig. 5A vs. B), reflecting the endogenous antitumor activity in WT mice that was lost in the NSG mice. In contrast to their efficacy in WT mice (Fig. 5A), the mouse FAP-CAR T cells had no antitumor effects on AE17.ova tumors in the immunodeficient NSG mice (Fig. 5B). This loss in activity was not due to the loss of FAP expression in the NSG tumor microenvironment, as we confirmed that AE17.ova tumors had a similar level of FAP expression in the NSG mice as in the immune-competent C57BL/6 mice (data not shown).

**Approaches to enhance FAP-CAR T-cell therapy**

Because our mouse CAR T cells persisted for only a short period of time in vivo, we hypothesized that giving a second infusion of FAP-CAR T cells might enhance therapeutic efficacy. AE17.ova tumor cells were injected into the flanks of C57BL/6 mice. When the tumors reached approximately 100 mm**3**, a first dose of FAP-CAR T cells was given intravenously. One week later, we randomly divided the FAP-CAR–treated animals into two groups, one treated with an additional dose of control MigR1 T cells (Single dose; Fig. 4A) and one treated with a second dose of FAP-CAR T cells (Double dose; Fig. 4A). At 2 weeks, tumors in mice given two doses of FAP-CAR T cells were significantly smaller (P < 0.05) than those in mice given only one dose of FAP-CAR T cells.

Another, not mutually exclusive, explanation for the lack of tumor eradication might be the suboptimal CAR signaling in mouse T cells and/or functional suppression in the tumor microenvironment. As we recently reported that mesothelin—targeted CAR mouse T cells deficient in the inhibitory enzyme diacylglycerol kinase-ζ (DGKζ) had enhanced effector functions in vitro and in vivo and increased persistence (43), we compared the efficacy of comparably transduced FAP-CAR splenic T cells isolated from WT C57BL/6 versus DGKζ-null mice. DGKζ-deficient FAP-CAR T cells were more efficient in lysing 3T3.FAP cells (Supplementary Fig. S4A) and in secreting IFN-γ (Supplementary Fig. S4B) with retention of specificity in vitro. The DGKζ-deficient FAP-CAR T cells were also more effective (P < 0.05 on day 11) after being injected into AE17.ova-bearing mice (Fig. 4B). The increased efficacy was associated with greater persistence of the DGKζ-knockout compared with WT FAP-CAR T cells (GFP**+** cells; Supplementary Fig. S4C). Thus, the enhanced antitumor efficacy was likely due to both increased T-cell activity and to increased persistence.
Figure 4. Enhanced therapeutic responses of FAP-CAR T cells. Mice with AE17.ova flank tumors were injected intravenously with FAP-CAR T cells when tumors were approximately 100 mm³. A, the overall efficacy of FAP-CAR T cells was enhanced when a second dose of FAP-CAR T cells was given a week later. The gray arrow indicates the injection time of the second dose of FAP-CAR T cells. B, efficacy could also be enhanced after injection of FAP-CAR T cells lacking the negative intracellular regulator DGKζ. #, Statistical significance between untreated and FAP-CAR-treated samples; P < 0.05. * Statistical significance between single dose FAP-CAR-treated group versus double dose group or DGKζ knockout (KO) FAP-CAR-treated group. C, FAP-CAR T cells enhance efficacy of cancer vaccine. TC1 tumor cells were inoculated into the right flanks of C57BL/6 mice. When tumors reached 200 mm³, one dose of Ad.E7 (10⁹ pfu) was given to the mice contralaterally to their flank tumors (black arrow). FAP-CAR T cells (10 million cells) were given 4 days later (gray arrow). Tumor measurements followed. The values are expressed as the mean ± SEM (n = 5). * Significant difference between untreated and the combo groups (P < 0.05).

To determine the mechanisms of this immune response, we repeated this experiment in AE17.ova tumor-bearing mice and analyzed the endogenous (non-GFP-expressing) T cells 3 and 8 days after T-cell injection. Consistent with the previous findings by Kraman and colleagues (26), who used a genetic approach to ablate FAP⁺ cells, the number of intratumoral T cells was similar between all three groups 3 days after adoptive transfer (Fig. 5C and E). Tumors from the FAP-CAR-treated mice had a significant (P = 0.015) increase in the numbers of E7-specific T cells (Fig. 5D) or ova-specific T cells within the TC-1 and AE17.ova tumors, respectively (Fig. 5F).

Given the effects of FAP⁺ tumor cell depletion on antitumor immunity, we hypothesized that combining a tumor vaccine with FAP-CAR T-cell administration might enhance antitumor efficacy compared with either approach alone. HPV-E7-expressing TC1 tumor cells were injected subcutaneously into C57BL/6 mice; when the tumors reached approximately 200 mm³, saline or one subcutaneous dose of a vaccine consisting of 10⁵ plaque-forming units (pfu) of an adenovirus-expressing HPV-E7 (Ad.E7; black arrow) was administered to boost the endogenous T-cell response against E7-expressing cells. Four days after injection with saline or the Ad.E7 vaccine, FAP-CAR T cells (10⁷ cells; gray arrow) were given intravenously. Both the Ad.E7 cancer vaccine and the FAP-CAR T cells alone had only modest effects on these large
established tumors (Fig. 4C). However, the combination regimen induced tumor regressions and suppressed tumor growth for up to 2 weeks before further tumor progression.

Toxicity

Because FAP is an endogenous protein and toxicity (especially weight loss and anemia) was recently reported after depletion of FAP⁺ cells either by genetic ablation (26, 27) or FAP-CAR-T-cell administration (31), we assessed off-tumor/on-target adverse effects after administration of our FAP-CAR T cells. In contrast with these reports, we observed no clinical toxicity or anemia in any of the FAP-CAR T-cell studies described above. The body weight of tumor-bearing mice remained constant or increased throughout each experiment (Supplementary Fig. S5).

To further evaluate toxicity, necropsies were performed and visceral organs (heart, lungs, pancreas, liver, spleen, kidneys, skeletal muscle, and bone marrow) were harvested, sectioned, stained, and analyzed in a blinded fashion 8 days after T-cell injection in mice treated with one dose of WT FAP-CAR T cells and 8 days after a second dose of WT-FAP-CAR T cells from the mice from the experiment shown in Fig. 4A. When compared with control tumor-bearing mice, no abnormalities were observed in the mice given WT FAP-CAR T cells. Specifically, this included a lack of bone marrow hypoplasia (Supplementary Fig. S6A–S6C) as reported by Tran and colleagues (31), or any change in skeletal muscle (data not shown).

We also preformed necropsies on mice 8 days after injection of the hyperactive DGKα-deficient FAP-CAR T cells from the experiment depicted in Fig. 4B. No abnormalities were noted, except in the pancreatic sections that showed some mild focal perivascular and peri-islet lymphocytic infiltration (Supplementary Fig. S6F). These changes were not seen in mice injected with WT FAP-CAR T cells (Supplementary Fig. S6E).

Discussion

In this study, we investigated the antitumor efficacy and safety of CAR-transduced T cells targeted to cells expressing...
FAP, which is highly upregulated in the tumor stroma. Given that CASCs may have a major immune-modulating effect on both innate and acquired immunity (13, 26), we used fully immune-competent mice, without bone marrow ablation, so that we could evaluate the role of the acquired immune system in FAP-CAR T-cell-mediated antitumor response.

We demonstrate in multiple mouse models with established mesothelioma and lung cancer that our mouse FAP-CAR T cells exhibited antigen-specific cytotoxicity against FAP\textsuperscript{+} stromal cells and markedly reduced the rare subset of FAP\textsuperscript{+}/CD45\textsuperscript{+}/F4/80\textsuperscript{+} myeloid cells and the more prevalent FAP\textsuperscript{+}/CD90\textsuperscript{+} stromal cells (Table 1). A single treatment with the FAP-CAR T cells resulted in approximately 80% depletion of the FAP\textsuperscript{hi} stromal cells 3 days after treatment of FAP-CAR T cells (Supplementary Fig. S2A), leaving white blood cells and FAP\textsuperscript{lo} cells relatively unaffected (Supplementary Fig. S2B and S2C). The depletion of FAP\textsuperscript{+} cells was associated with a significant inhibition (35%-50%) of tumor growth compared with untreated and vector control–transduced CAR T-cell (MigR1)-treated tumors (Fig. 3A-C, E, and F). Importantly, the antitumor activity of the FAP-CAR T cells was lost in the FAP-null mice (Fig. 3D) indicating that the antitumor activity is dependent on FAP expression on host-derived cells.

The antitumor efficacy of the FAP-CAR T cells was also lost in immunodeficient mice (Fig. 5B), indicating the importance of the acquired immune system in these tumor models. To delineate this effect, we evaluated the endogenous T cells within the tumors at 3 and 8 days after CAR T-cell infusion. At the earlier time point, we did not see an increase in T-cell infiltration or CD8 T-cell activation. However, there was an increase in the number of TNF-\(\alpha\)-producing CD4\textsuperscript{+} T cells (Fig. 6B). Similarly, Kraman and colleagues showed that there was no difference in the numbers of CD4\textsuperscript{+} or CD8\textsuperscript{+} cells in the tumors 48 hours after genetically ablating FAP\textsuperscript{+} cells with DT (26), but they did note an increase in the levels of TNF-\(\alpha\) mRNA. In contrast, at the later time point, we observed an increase in total CD8\textsuperscript{+} T cells as well as antigen-specific CD8\textsuperscript{+} T cells in both the AE17.ova and E7-positive TC1 tumors. More IFN-\(\gamma\)–producing CD8\textsuperscript{+} T cells, as well as CD69\textsuperscript{+} T cells, were also found at this time point (Fig. 6C and E). We postulate that the FAP-CAR T cells enter the tumors, deplete the FAP\textsuperscript{+} T cells, and by an as yet unknown mechanism activate the endogenous CD4\textsuperscript{+} T cells to produce TNF-\(\alpha\). The high levels of TNF-\(\alpha\) may induce tumor cell apoptosis and a temporary tumor vasculature shut down that may limit early infiltration by endogenous T cells. It seems that by 8 days after FAP-CAR T-cell treatment, activated CD8\textsuperscript{+} cells enter the tumor and function to further limit tumor growth. It should be noted that the tumors in our mouse models are relatively immunogenic with few fibroblasts, and therefore the contribution of the immune-mediated mechanisms may be more prominent than the contribution of the nonimmune-mediated mechanisms (i.e., alterations in matrix and/or angiogenesis)
in nonimmunogenic and fibroblast-rich tumors. Preliminary studies using more desmoplastic, nonimmunogenic mouse tumor models and human xenografts support this idea.

In our tumor models, the FAP-CAR T cells elicited significant but temporary inhibition of tumor growth. One explanation for the transient effect is the short persistence of the murine CAR T cells; the number of intratumoral murine FAP-CAR T cells rapidly decreased with time (Supplementary Fig. S3A). This is likely due to a number of well-known intrinsic differences between mouse and human T cells. After expanding human CAR T cells, the lymphocytes are “rested down” before injection and are less sensitive to immediate activation-induced cell death (AICD). In the mouse system, the injected cells are highly activated (as required for retroviral transduction). Although the human T cells persist and proliferate for weeks in tumors in immunodeficient mice (3, 44), the transduced mouse T cells have short lifespan and undergo AICD (Supplementary Fig. S7). There is relatively little information about the persistence of mouse CAR T cells, but data from this study and our previous work with mesothelin-CAR mouse T cells (43) are consistent with the results reported by Peng and colleagues (45) using in vitro expanded pmel-1 T cells that showed similar short persistence of mouse T cells in mice. In their study, pmel-1 T cells were undetectable in the peripheral blood by day 9 after adoptive transfer and by day 13 at the tumor site. To ensure that the short lifespan was not caused by the human CD3ζ and human 4-1BB activation domains, we constructed a fully mouse FAP-CAR comprised the 73.3 scFv coupled with the mouse CD3ζ chain and mouse CD28 domain (Fig. 1D). We found virtually equal efficacy in killing, cytokine production, persistence, and antitumor activity between the T cells expressing both constructs (Supplementary Fig. S3B–S3E). We also found that both CARs were equally susceptible to AICD (Supplementary Fig. S7B). We determined whether our FAP-CAR mouse T cells might be enhanced if we preconditioned the host by inducing lymphodepletion (46, 47), but no increase in efficacy was observed in mice irradiated before injection of our FAP-CAR T cells (data not shown). We have not yet tested whether the administration of IL-2 might enhance their efficacy.

Given the short persistence of our FAP-CAR T cells (Supplementary Fig. S3A), a second dose of T cells was administered 1 week after the first injection and showed added efficacy (Fig. 4A), which clearly indicated that enhanced persistence would augment efficacy. Our recent observation (43) indicates that blocking a T cell-intrinsic negative regulatory mechanism (upregulation of the enzyme DGK) augments the killing ability and persistence of murine CAR T cells. Similar to our observations with mouse CAR-T cells targeted to mesothelin (42), FAP-CAR T cells deficient in DGKζ had enhanced ability to kill FAP-expressing cells in vitro and were clearly more efficacious in vivo (Fig. 4B). Our data suggest that it will be advantageous to optimize both the persistence and potency of the T cells.

As in any cancer therapy where the target is not completely tumor specific, potential toxicity is a major concern. In previous studies using a vaccine against FAP and with immunoconjugate therapy (28–30), murine tumor growth was inhibited without obvious toxicity. Some of these studies also performed analyses and showed that there was no significant inhibition of wound healing (28, 29). In a phase I human trial, trace levels of 131I-labeled humanized anti-FAP antibody (sibrotuzumab) showed excellent tumor targeting with no detectable uptake in normal organs and no major toxicity at any dose level (48). However, as described above, the authors of two recent articles reported that ablation of FAP+ cells (using a genetic approach in ref. 27 or using FAP-CAR T cells in ref. 31) led to significant cachexia (weight loss) and anemia. We used flow cytometry to assess the percentage of FAP+ cells in the pancreas, lungs, tumors, and bone marrow collected from mice bearing different flank tumors. Despite different mouse strains used in each mouse tumor model, we found less than 0.1% of FAP+ stromal cells in the lungs and bone marrow (data not shown). In contrast and consistent with results reported by Roberts and colleagues (27), 3% to 5% of the dissociated cells from the pancreas expressed FAP (data not shown). However, when we compared FAP expression on those pancreatic stromal cells with that on the tumor-associated stromal cells isolated from the same hosts, we found that the CASCs expressed higher levels of FAP (Supplementary Fig. S8).

In contrast with these reports (27, 31) and consistent with the findings of Kakarla and colleagues (33) and Schuberth and colleagues (34), administration of one dose or even two doses of our FAP-CAR T cells did not cause any weight loss (Supplementary Fig. S5), nor did they cause any decrease in hematocrit or increase in serum amylase (data not shown). Furthermore, detailed histologic analyses of necropsy samples showed no microscopic abnormalities, and specifically no damage to the muscle, the pancreas (Supplementary Fig. S6D and S6E), or the bone marrow (Supplementary Fig. S6A–S6C).

The reasons for these differences in toxicity are not completely understood. It is possible that the total body irradiation before adoptive T-cell transfer used by Tran and colleagues (31) somehow contributed to the side effects. Although our main construct had the 4-1BB costimulatory molecule rather than CD28, in vitro studies showed no differences in their killing ability or IFN production (Supplementary Fig. S3B and S3C) or in vivo activity (Supplementary Fig. S3E). A likely explanation relates to the different properties of the scFv antibodies used. The affinities of the scFv antibodies for mouse FAP are similar. The affinity of the FAP-5 antibody for mouse FAP was reported to be 0.6 nmol/L (31). Our biacore measurement showed that the affinity of the 73.3 mAb for mouse FAP protein was less than 1 nmol/L (0.1–0.3 nmol/L). However, we found that FAP-5 and 73.3 reacted with distinct epitopes on FAP in antibody competition experiments (Supplementary Fig. S9), consistent with the specificity of the 73.3 mAb for murine FAP compared with that of FAP-5 for an epitope shared by murine and human FAP. The distinct epitope specificity seems to allow the FAP-CAR T cells expressing the 73.3 scFv to efficiently eliminate the FAP expressing cells, while sparing cells expressing lower levels of FAP (Supplementary Fig. S2), such as cells in the pancreas, and presumably cells in the bone marrow and muscle. The position of the binding epitope of a scFv in a CAR T cell has previously been shown to determine the efficacy of activation (49). Data from the studies...
conducted by Roberts and colleagues and Tran and colleagues suggest that complete ablation of FAP+ cells may cause such side effects as fatigue and anemia (27, 31). However, our data show that it is possible to partially deplete tumor-associated FAP+ cells while retaining antitumor efficacy, but without eliciting severe side effects. The only instance in which we observed any histologic abnormalities was in the pancreas (Supplementary Fig. S6F) but only when we used the hyperactive DGKζ-deleted cells. Data from Kakarla and colleagues support this idea (33).

Further studies will be needed to determine why we and two other groups (33, 34) induced significant antitumor efficacy, whereas Tran and colleagues (31) did not. Although the constructs are all slightly different, we showed antitumor activity with CARs containing either human or mouse cytoplasmic domains (Supplementary Fig. S3E). Our data suggest that endogenous immune responses may be important for the activity of our CARs. Because Tran and colleagues (31) used total body irradiation, they may have lost some of that effect. Alternatively, irradiation might have impacted the tumor stroma, thus limiting the effect or antistromal therapy. Furthermore, the amount of stroma was very low in the small mouse tumors studied by Tran and colleagues (ref. 31; data not shown). In any case, we were able to show clear antitumor efficacy albeit to a lesser extent in two of the tumor cell lines used by Tran and colleagues (ref. 31; Fig. 3A–3C, E, and F).

The optimal utility of FAP-CAR T cells may be in combination with other therapies, including immuno- and chemotherapy, in which stromal disruption could enhance drug delivery. Kakarla and colleagues (33) augmented efficacy by combining FAP-CAR T cells with CAR T cells targeting EphA2 on tumor cells. In the current study, we combined FAP-CAR T cells with an Ad.E7 cancer vaccine that elicits E7-specific adaptive immune response against TC1 cells. As expected, neither the cancer vaccine nor the FAP-CAR T cells alone worked well on large, established tumors (Fig. 4C); however, in combination, there was an additive or synergistic effect against TC1 tumors.

In summary, we showed that FAP-CAR T cells reduce tumor growth in vivo in an antigen-specific manner. Targeting tumor stromal cells with CAR T cells augmented antitumor immunity. Although we did not eradicate tumors completely in our animal models, we believe that the efficacy of FAP-CAR T cells could be enhanced by improving the persistence of T cells, generating more highly active T cells, administering multiple doses of FAP-CAR-T cells, and by combining with other types of immuno- or chemotherapy. We did not observe toxicity under the dosing conditions tested and in otherwise healthy tumor-bearing animals.

We believe that data from Karkala and colleagues (33), Schuberth and colleagues (34), and in this report support the development of anti-human FAP-CAR for potential translation to the clinic. Potential toxicities, such as anemia and/or cachexia, should they occur, are tolerable and similar to those seen in most conventional chemotherapy treatments. Importantly, our current clinical approach using biodegradable mRNA-transduced CAR T cells (50) will allow us to determine whether these toxicities may only be temporary. It may well be that the FAP-elimination approach will best be used in combination with other therapies and may only need to be given for short periods of time.

Disclosure of Potential Conflicts of Interest

C.H. June has received commercial research support from Novartis. No potential conflicts of interest were disclosed by the other authors.

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