Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia

Eric Tran,¹ Dhanalakshmi Chinnasamy,¹ Zhiya Yu,¹ Richard A. Morgan,¹ Chyi-Chia Richard Lee,² Nicholas P. Restifo,¹ and Steven A. Rosenberg¹

¹Surgery Branch and ²Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Fibroblast activation protein (FAP) is a candidate universal target antigen because it has been reported to be selectively expressed in nearly all solid tumors by a subset of immunosuppressive tumor stromal fibroblasts. We verified that 18/18 human tumors of various histologies contained pronounced stromal elements staining strongly for FAP, and hypothesized that targeting tumor stroma with FAP-reactive T cells would inhibit tumor growth in cancer-bearing hosts. T cells genetically engineered with FAP-reactive chimeric antigen receptors (CARs) specifically degranulated and produced effector cytokines upon stimulation with FAP or FAP-expressing cell lines. However, adoptive transfer of FAP-reactive T cells into mice bearing a variety of subcutaneous tumors mediated limited antitumor effects and induced significant cachexia and lethal bone toxicities in two mouse strains. We found that FAP was robustly expressed on PDGFR-α⁺, Sca-1⁺ multipotent bone marrow stromal cells (BMSCs) in mice, as well as on well-characterized, clinical-grade multipotent human BMSCs. Accordingly, both mouse and human multipotent BMSCs were recognized by FAP-reactive T cells. The lethal bone toxicity and cachexia observed after cell-based immunotherapy targeting FAP cautions against its use as a universal target. Moreover, the expression of FAP by multipotent BMSCs may point toward the cellular origins of tumor stromal fibroblasts.

However, FAP is also expressed in healing wounds and in fibrotic conditions such as fibrosis of the liver and lung, in Crohn’s disease, in arthritis, and on various sarcomas (Kelly et al., 2012). The seemingly limited normal tissue expression, and the fact that FAP expression is found in >90% of epithelial cancers (Garin-Chesa et al., 1990), makes FAP an attractive molecule for targeting tumor stromal fibroblasts.

Targeting FAP genetically, or with vaccines or pharmacological agents, has been shown to impair tumor progression in several preclinical cancer models (Lee et al., 2005; Loeffler et al., 2006; Ostermann et al., 2008; Liao et al., 2009; Santos et al., 2009; Kraman et al., 2010; Wen et al., 2010). Unfortunately, targeting FAP in human cancer patients with the monoclonal antibodies F19 and its humanized version Sibrotuzumab (Welt et al., 1994; Hofheinz et al., 2003;
Scott et al., 2003), or the FAP enzyme-inhibitor Talabostat (Narra et al., 2007; Eager et al., 2009a,b), has not demonstrated clinical efficacy. Despite this, favorable biodistribution of the FAP-specific antibodies has been reported, with selective uptake in sites of metastatic disease in patients (Welt et al., 1994; Scott et al., 2003). The general lack of clinical efficacy in these trials could be due to the possibility that binding to or inhibiting FAP activity alone is not sufficient to impact tumor stromal fibroblast function (Kelly et al., 2012).

Adoptive cell therapy (ACT) using ex vivo expanded tumor-infiltrating lymphocytes (TIL) or T cells genetically engineered with antitumor TCRs or chimeric antigen receptors (CARs) can cure some patients with metastatic cancers, demonstrating that T cells can be potent weapons against cancer (Rosenberg, 2012). CARs are typically composed of an extracellular antigen-recognition domain derived from a tumor-reactive monoclonal antibody (scFv) fused to intracellular T cell signaling domains, which, unlike conventional TCRs, allows T cells expressing CARs to directly recognize cell surface proteins and kill target cells in an MHC-independent fashion (Dotti et al., 2009; Sadelain et al., 2009). However, the decision of which antigen to target is a critical parameter of CAR design, as CAR-modified T cells can mediate significant “on-target, off-tumor” toxicities if the antigen being targeted is expressed on normal tissues (Dotti et al., 2009; Sadelain et al., 2009).

In the present study, we tested whether targeting tumor stromal fibroblasts using T cells genetically engineered with FAP-reactive CARs could inhibit tumor growth in various mouse tumor models. We found that adoptive transfer of T cells modified with highly reactive anti-FAP CARs had little impact on tumor progression in a variety of syngeneic mouse tumor implantation models, an observation that could be due to the relatively low stromal content in these tumors. Nonetheless, and more importantly, we found that high doses of FAP-reactive T cells induced severe cachexia and dose-limiting bone toxicity. This toxicity appeared to be the result of T cell targeting of FAP-expressing multipotent BM stromal cells (BMSCs). Thus, FAP is not only expressed by tumor stromal fibroblasts, but also by multipotent BMSCs. The expression of FAP on multipotent BMSCs not only cautions against strategies that can target and destroy FAP-expressing cells, but also may provide biological insight into the cellular origins of tumor stromal fibroblasts.

RESULTS AND DISCUSSION

Expression of FAP by human tumor stromal cells

FAP is reported to be highly expressed on tumor stromal fibroblasts from many different tumor types. Thus, we first assessed the expression of FAP in a variety of human tumors by immunohistochemistry (IHC) using the FAP-specific monoclonal antibody FAP5 (Ostermann et al., 2008). FAP staining was positive in all tumors tested (melanoma, n = 9; colorectal, n = 3; pancreatic, n = 3; and breast, n = 3), with the vast majority of samples showing strong FAP staining localized in the fibrotic stromal bands of the tumors (Fig. 1, A–D; and Table S1). In many tumors, thick, intensely FAP-positive stromal bands comprised a large component of the tumor. Thus, in accord with prior reports, FAP is strongly expressed in the reactive stroma of many solid cancers, which makes FAP a potentially attractive antigen for targeting the tumor stroma.

In vitro function of FAP-reactive CARs

To target tumor stromal fibroblasts with T cells, we genetically engineered T cells with CARs specific for FAP. For our preclinical studies, we generated two CAR constructs: (1) FAP5-CAR, which is comprised of the scFv from the FAP-specific antibody FAP5 (Ostermann et al., 2008) used in the aforementioned IHC studies, and is reactive to both mouse and human FAP, and (2) Sibro-CAR, which is comprised of the scFv from Sibrotuzumab, a FAP-reactive antibody that was previously used in clinical trials (Hofheinz et al., 2003; Scott et al., 2003) and is only reactive to human FAP. Both scFv fragments were genetically fused to the CD28, 4-1BB, and CD3ζ intracellular signaling domains (Fig. 1, E and F; and Tables S2 and S3), and cloned into the MSGV1 retroviral vector. Transduction efficiencies of FAP5-CAR in mouse T cells were between ~70 and 90% (Fig. 1 G), whereas Sibro–CAR-transduced human T cells demonstrated transduction efficiencies between ~45–65% (Fig. 1 H) as determined by flow cytometry. Both FAP5- and Sibro–CAR–transduced T cells specifically degranulated and produced cytokines when stimulated with plate-bound recombinant FAP or when co-cultured with HEK293 stably expressing FAP, but not when stimulated with irrelevant protein, or when co-cultured with the FAP-negative HEK293 cell line stably expressing eGFP (Fig. 1, I and J for mouse FAP5-CAR; Fig. 1, K and L for human Sibro-CAR). Thus, FAP-reactive CARs were generated and expressed in T cells, and they demonstrated specific in vitro activity.

Attempts to target tumor stromal cells

with FAP5-CAR–transduced T cells in vivo

We next tested whether targeting FAP with FAP5-CAR–transduced T cells could mediate antitumor effects in various mouse tumor models. We resorted to the B16 melanoma, MC38 colon cancer, MC17-51 fibrosarcoma, 4T1 breast cancer, CT26 colon cancer, and Renca kidney cancer cell lines, which all did not express FAP in vitro, with the exception of MC17-51, where a small subset of cells demonstrated very low levels of FAP expression (Fig. 2 A). However, when these tumor cells were subcutaneously implanted into syngeneic mice and allowed to grow to treatment size (~50 mm³), IHC staining demonstrated FAP positivity in all of these tumors, although the staining intensity was generally much weaker and more diffuse than in human tumors (Fig. 2, B–G, vs. Fig. 1, A–D). Moreover, these murine tumors lacked the thick stromal banding pattern seen in human tumors. The differences in FAP expression level and tumor stromal architecture between these murine tumors and human tumors likely reflects differences in the tumor initiation and development process, in combination with the intrinsic ability of each tumor–cell type to recruit and activate stromal elements. Nonetheless, although weaker, these in vivo murine tumor masses were
generally positive for FAP, and thus we evaluated whether targeting FAP with FAP5-CAR–transduced T cells could inhibit tumor growth in vivo.

To this end, we adoptively transferred FAP5-CAR–transduced or untransduced T cells into lymphodepleted mice bearing established subcutaneous tumors, followed by 6 doses of IL-2. As seen in Fig. 2 H, the antitumor effect of FAP5-CAR–transduced T cells on C57BL/6 mice bearing B16 melanomas was weak. Similar results were seen in C57BL/6 mice bearing MC38 colon cancers (Fig. 2 I), and no antitumor effects were seen in mice bearing MC17-51 fibrosarcomas (Fig. 2 J). Adoptive transfer of FAP5-CAR–transduced T cells also mediated no significant antitumor effects in BALB/c mice bearing 4T1 breast or CT26 colon tumors, and a modest antitumor effect against Renca kidney tumors (Fig. 2, K–M). Thus, in a variety of established, subcutaneous murine tumors, targeting FAP with FAP5-CAR–transduced T cells generally mediated weak antitumor effects at best, although it should be noted that these tumor masses had sparse stroma, and demonstrated only weak to moderate FAP expression.

Transfer of FAP5-CAR–transduced T cells is associated with BM toxicity and cachexia

In addition to the limited antitumor activity of FAP5-CAR–transduced T cells seen in some in vivo tumor models, we also found that infusion of FAP5-CAR–transduced T cells (1–2 × 10^7) led to morbidity or mortality in the majority of these mice (unpublished data). To better understand this toxicity, non–tumor-bearing wild-type C57BL/6 mice were treated with 2 × 10^7 FAP5-CAR–transduced or untransduced T cells. 7 d later, these mice underwent a comprehensive necropsy by a veterinary pathologist blinded to the treatment conditions. Severe BM hypocellularity and necrosis was seen in mice treated with FAP5-CAR–transduced T cells; this was not seen in mice treated with untransduced T cells (Fig. 3, A and B).

Corroborating these pathological observations, the total number of live BM and osteogenic (OS) cells isolated from the femurs and tibias of mice treated with 2 × 10^7 FAP5-CAR–transduced or untransduced T cells, generated and used to transduce mouse and human T cells, respectively, and flow cytometry was used to assess transduction efficiency at day 2 after transduction for FAP5-CAR (G) and day 8–10 after transduction for Sibro-CAR (H). Solid line is isotype control and filled histogram is FAP5 or SibroZuzumab stained. Day 5-stimulated untransduced (UnTd) and FAP5-CAR–transduced (Td) mouse T cells were assessed for reactivity against plate-bound BSA, α-CD3 mAb, and recombinant human FAP (r-huFAP), and against HEK293 cell lines expressing or not expressing FAP. After an overnight stimulation, supernatants were assessed for IFN-γ with an IFN-γ ELISA (I), and cells were further assessed for cell surface CD107a expression, and production of IFN-γ and TNF by ICS (J). For ICS, cells are gated on FAP5-CAR Td cells. Day ~14-stimulated UnTd or Sibro-CAR Td human T cells were assessed for in vitro reactivity as described for mouse. IFN-γ ELISA (K), and ICS results gated on Sibro-CAR Td T cells (L) are shown. Mean ± SD. All results are representative of at least three independent experiments.

Figure 1.  IHC staining for FAP in various human tumors, and design and in vitro activity of FAP-reactive CARs. Representative IHC staining for FAP in human melanoma (A), colorectal (B), pancreatic (C), and breast (D) adenocarcinomas. Isotype stains were negative (not depicted). Bars: 400 µm (A); 200 µm (B–D). Schematic of the FAP-reactive CAR constructs FAP5-CAR (E) and Sibro-CAR (F). LS, GM-CSFR leader sequence; V_h and V_l, variable heavy and light chains; L, 218 linker; CD8, transmembrane domain; CD28, 4–1BB, and CD3–ζ, intracellular signaling domains; m, murine; h, human. Both constructs were cloned into the MSGV1 retroviral vector. Retrovirus containing FAP5-CAR or Sibro-CAR constructs were...
mice treated with $2 \times 10^7$ untransduced T cells (Fig. 3, C and D). In addition to the bone toxicity, mice treated with $2 \times 10^7$ FAP5-CAR T cells appeared cachectic and lost $\sim 20\%$ of their body weight by 7 d after treatment (Fig. 3 E).

Mice bearing B16, MC38, MC17-51, 4T1, CT26, and Renca tumors treated with $5 \times 10^6$ or $10^7$ FAP5-CAR–transduced T cells also lost similar amounts of weight, but the majority of those treated with $5 \times 10^6$ FAP5-CAR–transduced T cells did not succumb to treatment–related toxicities (unpublished data). Nonirradiated, lymphoreplete mice receiving high doses of FAP5-CAR–transduced T cells also suffered from similar bone toxicities and cachexia (Fig. 3, C, D, and F).

These toxicities were not limited to non–tumor-bearing mice and mice bearing tumors with low FAP expression, as the adoptive transfer of FAP5-CAR–transduced T cells into Rag1-deficient mice bearing a stroma-rich, intensely FAP-positive

Figure 2. FAP expression in mouse tumors, and in vivo activity of FAP5-CAR–transduced T cells against various murine tumors. In vitro cultured B16, MC38, MC17-51, 4T1, CT26, and Renca murine tumors were assessed for FAP expression by flow cytometry with the FAP-specific antibody FAP5 (A). Solid line is isotype control and filled histogram is FAP5 stained. Results are representative of at least two independent experiments. Established (~11–16 d) subcutaneously implanted B16 (B), MC38 (C), MC17-51 (D), 4T1 (E), CT26 (F), and Renca (G) tumors were harvested from mice (irradiated before harvest) and assessed for FAP expression by IHC using biotinylated–FAP5 antibody. Bars, 400 µm. Representative of at least two independent experiments. C57BL/6 mice bearing established B16 (H), MC38 (I), MC17-51 (J) tumors, and BALB/c mice bearing established 4T1 (K), CT26 (L), or Renca (M) tumors were left untreated (No Tx) or treated with $10^7$ UnTd or $10^7$ FAP5-CAR Td T cells, and the perpendicular diameters of the tumors were measured over time. Mean ± SEM. Results are representative of at least two independent experiments for H–J and one experiment for K–M with initially five mice per group. *, $P < 0.05$ by Wilcoxon rank-sum test for UnTd versus FAP5-CAR Td T cells.
pancreatic cancer xenograft (Fig. 3 G) also led to cachexia and significant morbidity (Fig. 3 H and not depicted). Thus, FAP-reactive T cells can induce major toxicities in both the absence and presence of a highly FAP-positive tumor stroma.

### Mouse and human multipotent BMSCs express FAP

The observed bone toxicity suggested that the FAP5-CAR–transduced T cells were targeting some aspect of bone. Given that most, if not all, cells of hematopoietic origin do not express FAP (Bae et al., 2008), it was unlikely that direct targeting of hematopoietic cells by FAP5-CAR–transduced T cells was responsible for the bone toxicity. Thus we tested whether FAP5-CAR–transduced T cells were targeting other non-hematopoietic compartments of bone. To that end, we first assessed the expression of FAP on freshly isolated BM and OS cells from the tibias and femurs of mice. As expected, BM and OS cells of hematopoietic or erythroid origin (Lin+ cells: CD45+/TER119+) were largely FAP− (Fig. 4 A and B). However, in contrast, OS cells of nonhematopoietic origin (Lin− cells: CD45−/TER119−) were markedly enriched in FAP+ cells (Fig. 4 B). Given that multipotent, mesenchymal BMSCs are known to be enriched in Lin− OS cells, we stained OS cells with antibodies specific for PDGFR-α and Sca-1, which are markers ascribed to murine multipotent BMSCs (Morikawa et al., 2009), and found that cells that co-expressed these markers were uniformly positive for FAP (Fig. 4 B). Cells expressing PDGFR-α alone were also uniformly FAP+, whereas the Sca-1 single-positive, and Sca-1/PDGFR-α double-negative populations displayed more heterogeneous expression of FAP (Fig. 4 B). Thus, freshly isolated OS cells of nonhematopoietic origin, including multipotent BMSCs, express FAP and therefore may potentially be targeted by FAP5-CAR–transduced T cells.

To assess the impact of FAP5-CAR–transduced T cells on FAP+ OS cells in vivo, we adoptively transferred $2 \times 10^7$ FAP5-CAR–transduced or untransduced T cells into mice. 7 d later, we assessed FAP expression on various populations of freshly isolated OS cells. As shown in Fig. 4 C and D, there was a marked decrease in FAP expression in the OS cell populations isolated from mice treated with FAP5-CAR–transduced T cells compared with mice treated with untransduced T cells. This was associated with a concomitant decrease in the mean fluorescence intensity (MFI) of FAP in these populations, with the greatest fold decrease consistently seen in the PDGFR-α single-positive, and multipotent Sca-1/PDGFR-α double-negative populations displayed more heterogeneous expression of FAP (Fig. 4 B). Thus, freshly isolated OS cells of nonhematopoietic origin, including multipotent BMSCs, express FAP and therefore may potentially be targeted by FAP5-CAR–transduced T cells. This was associated with a concomitant decrease in the mean fluorescence intensity (MFI) of FAP in these populations, with the greatest fold decrease consistently seen in the PDGFR-α single-positive, and multipotent Sca-1/PDGFR-α double-positive cell populations (Fig. 4 E). Thus, the adoptive transfer of FAP5-CAR–transduced T cells in mice is associated with a loss of OS cells expressing high levels of FAP.
Since multipotent BMSCs provide essential environmental support for hematopoiesis by secreting factors that promote the maintenance of hematopoietic stem cells (Méndez-Ferrer et al., 2010; Ding et al., 2012), and by differentiating into key niche elements such as osteoblasts, adipocytes, chondrocytes, and CXCL12-abundant reticular cells (Nombela-Arrieta et al., 2011), their destruction may contribute to the observed bone toxicity. Therefore, we next tested whether FAP5-CAR–transduced T cells could directly recognize BMSCs in vitro. Cultured murine BMSCs (passage 5) were first assessed for FAP expression by flow cytometry. As seen in Fig. 5 A (left), some of these murine BMSCs expressed FAP and, consistent with the aforementioned results, the Sca-1/PDGFR-α double-positive population displayed uniform expression of FAP (Fig. 5 A, middle and right). Importantly, co-culture assays revealed that these murine BMSCs were specifically recognized by FAP5-CAR–transduced T cells (Fig. 5, B and C). We also assessed FAP expression on highly pure, well-characterized, clinical-grade multipotent human BMSCs from three different donors (Fig. 5 D). Multipotent BMSCs from all three donors were uniformly and strongly FAP-positive as determined by flow cytometry with the two FAP-specific antibodies Sibrotuzumab and FAP5 (Fig. 5, E and F). Similar to mouse, human T cells transduced with the human FAP–reactive Sibro-CAR...
Given that FAP is expressed on multipotent BMSCs, which are essential for hematopoiesis, it is intriguing that no significant toxicities were reported in previous preclinical studies targeting FAP (Lee et al., 2005; Loeffler et al., 2006; degranulated and produced effector cytokines upon co-culture with human BMSCs (Fig. 5, G and H). Thus, both murine and human multipotent BMSCs express FAP and can be targeted by FAP-CAR–transduced T cells.

Figure 5. Murine and human multipotent BMSCs express FAP and are recognized by T cells expressing FAP-reactive CARs. Passage-5 in vitro–expanded murine BMSCs were stained with antibodies specific for Sca-1, PDGFR-α, and FAP, and assessed by flow cytometry (A). "Q" represents quadrant. Solid lines are isotype controls and filled histograms are FAP stained. UnTd or FAP5-CAR Td T cells were cultured overnight with murine BMSCs and supernatants were assessed for IFN-γ by ELISA (B), and cells were further analyzed for expression of CD107a and production of IFN-γ and TNF by ICS (C). Mean ± SD. Data are representative of two independent experiments. Flow cytometric phenotype of in vitro–expanded human BMSCs derived from three different donors (D). BMSCs from D were stained with the FAP-specific monoclonal antibodies Sibrotuzumab (E) and FAP5 (F) and assessed by flow cytometry. Solid lines are isotype or secondary antibody controls and filled histograms are FAP or Sibrotuzumab stained. UnTd or Sibro–CAR Td T cells were cultured overnight with BMSCs and the supernatants assessed for IFN-γ by ELISA (G), and cells were further analyzed for CD107a expression and IFN-γ and TNF production by ICS (H). Mean ± SD. Similar results were seen with two additional T cell donors.
cellular origins of tumor stromal fibroblasts are incompletely elucidated and our finding that FAP is expressed by both tumor stromal fibroblasts and multipotent BMSCs is consistent with the idea that BMSCs may be a source of tumor stromal cells (Mishra et al., 2008). It seems plausible that some tumor stromal cells may simply be multipotent MSCs recruited into the tumor microenvironment, and thus the promising strategy of targeting these normal tumor components must be approached with great care to avoid potentially life threatening collateral damage to these essential regenerative cells.

**MATERIALS AND METHODS**

**Ethics statement.** All patient-derived material used in this study was approved by the Institutional Review Board of the National Cancer Institute (NCI). All animal studies were performed in accordance with the Animal Care and Use Committee guidelines of the National Institutes of Health and were conducted under protocols approved by the Animal Care and Use Committee of the NCI.

**IHC.** Human melanoma and mouse tumors were embedded in OCT compound (Tissue-Tek) and frozen at −80°C before sectioning onto positively charged slides. Frozen human breast, colorectal, and pancreatic tumor slides were provided by the Cooperative Human Tissue Network, which is funded by the NCI. Tissue sections were stained using standard IHC procedures. In brief, slides were air dried for 20 min, and then fixed with cold acetone for 10 min at 4°C. Sections were then air dried (20 min), washed with PBS (three times), and incubated with 0.3% hydrogen peroxide for 10 min to block endogenous peroxidase. After PBS wash (two times), sections were blocked with protein block solution (Dako), washed with PBS (two times), and then further blocked with Biotin/Avadin block reagent (Invitrogen). After PBS wash (three times), sections were incubated with primary antibody or isotype control antibody for 45 min at room temperature in a humidified chamber. For human tissues, the FAP-specific antibody FAP5 (Ostermann et al., 2008) or mouse IgG2a isotype control antibody (BioLegend) were used at 0.625 µg/ml, and developed with the mouse Dako EnVision+ System-HRP (DAB) kit as recommended. For mouse tissues, FAP5 or mouse IgG2a isotype control antibodies were biotinylated (EZ-Link NHS PEG-biotin; Thermo Fisher Scientific) and used at ~2 µg/ml. Sections were developed with ABC reagent (Vector Laboratories) and DAB substrate (Dako). All slides were counterstained with hematoxylin, washed, dehydrated through graded alcohol and xylen, and then mounted. Stained tissue sections were evaluated by a pathologist for: % stroma (of tumor section), % stromal cells positive for FAP (either >50% or <50%), and FAP staining intensity (0 = no staining, 1+ = strong staining).

**Cell lines and media.** The tumor cell lines B16-F10 (mouse melanoma), MC17-51 (mouse fibrosarcoma), 4T1 (mouse mammary carcinoma), CT26 (mouse colon adenocarcinoma), Renca (mouse renal adenocarcinoma), and HPAC (human pancreatic adenocarcinoma) were maintained in RPMI supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 25 mM Hepes buffer (all from Invitrogen). The mouse colon adenocarcinoma MC38 and the retrovirus packaging line 293GP were maintained in DMEM (Quality Biologies Inc.) with the same supplements as described for RPMI.

To generate FAP-expressing cell lines, FAP-negative HEK293 cells were transduced with retrovirus encoding mouse or human FAP cDNA (Open Biosystems), or eGFP as control. All cloning was done using the Fast Cloning Pack and FastDigest restriction enzymes (both from Fermentas). The DNA sequence of mouse and human FAP was verified by standard Sanger sequencing (Macrogen). Retroviral supernatants were made and used to transduce HEK293 cell (described in following section). Transduced HEK293 cells were selected with 1 mg/ml G418 (CellGro). HEK293 cells stably expressing eGFP, mouse FAP, or human FAP are designated as HEK-eGFP, HEK-mFAP, and HEK-hFAP, respectively.
Mouse T cell media composed of RPMI containing 10% heat-inactivated FBS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 2 mM l-glutamine (all from Invitrogen), and 60 IU/ml of rlhl-2 (Chiron). Human T cell media was comprised of RPMI supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products), 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 2 mM l-glutamine. Human clinical-grade BMSCs were generated by the National Institutes of Health Department of Transfusion Medicine, as previously described (Sabatino et al., 2012), and cultured in α-MEM (Lonza) supplemented with 20% FBS (Sigma-Aldrich) and 10 µg/ml gentamicin (Lonza).

Generation of mouse and human FAP-CARs, retrovirus production, and T cell transduction. The CAR constructs used in this study are illustrated in Fig. 1 (E and F). The mouse and human FAP-specific scFv used for generating the FAPS-CAR, was derived from the high-affinity mouse antibody FAP5 (Ostermann et al., 2008), which has a Kd of 0.6 and 5 nM for mouse and human FAP, respectively. The high-affinity (6 nM) human FAP-specific scFv used for generating Sibro-CAR was derived from the monoclonal antibody Sibrotuzumab (BIBH1; Scott et al., 2003). The scFv sequences of FAP5 and Sibrotuzumab were obtained from US Patent Application Publications US 2009/0304718 A1 and US 2003/0103968 A1, respectively. After codon optimization and synthesis (Blue Heron Technology), the scFv constructs were cloned in-frame into the MSGV1 retroviral vector containing the appropriate envelope encoding plasmid (ECO for mouse and RD114 for human; P. Garin-Chesa, Transfusion Medicine, as previously described (Sabatino et al., 2012), and cultured in α-MEM (Lonza) supplemented with 20% FBS (Sigma-Aldrich) and 10 µg/ml gentamicin (Lonza).

P. Garin-Chesa (Boehringer Ingelheim, Vienna, Austria). The secondary detection antibody was PE-conjugated AffiniPure F(ab’)2 fragment donkey anti–mouse IgG, and PE-conjugated AffiniPure F(ab’)2 fragment donkey anti–human IgG (both from Jackson ImmunoResearch Laboratories) used at 1:100 dilution to detect the FAPS and Sibrotuzumab antibodies, respectively. All FAP staining experiments contained a negative control (HEK-eGFP) and positive control (HEK-mFAP or HEK-hFAP) cell line. Mouse IgG2a isotype antibody was used as a control for FAP5 antibody in indicated experiments. To detect FAPS-CAR expression on mouse T cells, T cells were stained with the biotin-SP-conjugated AffiniPure goat anti–mouse IgG F(ab’)2 fragment specific antibody (Jackson ImmunoResearch Laboratories) at 1:20 dilution, followed by 2.5 µg/ml streptavidin-PE (BD). To detect Sibro-CAR expression on human T cells, T cells were stained with 2 µg/ml C-terminal His-tagged recombinant human FAP (GenWay Biotech Inc.) followed by 1:10 dilution of PE-conjugated anti-His antibody (Miltenyi Biotec). The following antibodies were used for staining of BM and OS cells: 0.15 µg/ml CD45-PE, 1.25 µg/ml TER119-PE, 5 µg/ml PDGF-R-α-APC, and 1 µg/ml Sca1-PE-Cy7 (all from eBioscience, except Sca1-PE-Cy7 [BD]), and biotinylated FAP5 antibody (5 µg/ml, described above) with the secondary detection reagent Alexa Fluor 488-conjugated streptavidin (2 µg/ml, Invitrogen).

The following isotype controls were used at the same concentrations as the corresponding test antibodies: PE-rat IgG2b, APC-rat IgG2a, and PE-Cy7 rat IgG2a (all from eBioscience), and biotinylated mouse IgG2a (control for biotinylated FAP5). For all flow cytometry experiments, cells were stained for 20–30 min at 4°C in the dark and washed two times with flow cytometry buffer (1X PBS + 1% FBS). For BM and OS cells, cells were additionally blocked with mouse Fc block (10–20 µg/ml BD) before antibody staining. Cells were stained with propidium iodide (PI) to demarcate live/dead cells before acquisition on either a FACSCalibur or a FACSComp II (BD). Data were analyzed with FlowJo software (Tree Star).

T cell assays. FAP-CAR–transduced T cells were assessed for reactivity against the target antigen FAP by assessing culture supernatants for IFN-γ using IFN-γ ELISA, and by analyzing the cells using a flow cytometry-based degranulation/intracellular cytokine staining (ICS) assay. For plate-bound stimulations, 96-well flat bottom plates were coated overnight at 4°C with 1 µg/ml BSA, 1 µg/ml recombinant human FAP (GenWay Biotech or R&D Systems), 1 µg/ml anti–mouse CD3-ε antibody (clone 145-2C11; BioLegend), or 1 µg/ml OKT3 (Orthoclone), all diluted in PBS. For plate-bound antigen stimulation assays, cells were plated at 10^6 cells per well in 200 µl of media. For most co-culture experiments, 10^6 T cells were cultured with 10^5 target cells in 200 µl of media. For human co-culture experiments involving BMSCs, 3 × 10^5 T cells were cultured with 10^6 BMSCs in a 48-well plate (in 500 µl total volume). All T cell assays were conducted using media without IL-2. In select experiments, fluorochrome-conjugated anti-CD107a antibody was added to the wells at the beginning of the stimulation; for mouse, anti-CD107a-Alexa Fluor 647 (BioLegend) was used at 1.25 µg/ml and for human, anti-CD107a-FITC (BD) used at 20 µl antibody per ml of culture. At 20–22 h after stimulation, both Golgistop and GolgiPlug (BD) were added to cultures (each used at 1/2 the recommended dilution). After 4–6 h, supernatants were harvested and used in IFN-γ ELISAs (eBioscience), and cells were further processed for ICS. In brief, cells were harvested, stained for FAP-CAR (described above) and then fixed, permeabilized, and stained using the BD Cytofix/Cytoperm kit as directed. Mouse cells were stained with 5 µg/ml anti-mouse IFN-γ-FITC (BioLegend) and 4 µg/ml anti–mouse TNF-PerCP/Cy5.5 (BD). Human cells were stained with 1.25 µg/ml anti–human IFN-γ-Alexa Fluor 647 (BioLegend) and 2.5 µl/100 µl anti–human TNF-PerCP/Cy5.5 (BD).

In vivo experiments. Inbred female C57BL/6 mice were purchased from the National Cancer Institute–Frederick Cancer Research and Development Center (Frederick, MD). Female and male BALB/c, and female Rag1-deficient mice were purchased from The Jackson Laboratory. In adoptive cell transfer (ACT) models with syngeneic tumors, 8–12-wk-old mice were injected subcutaneously with 5 × 10^5 tumor cells and ~11 d later, when
tumors were typically 30–60 mm², mice were lymphodepleted with 5 Gy total body irradiation, and then treated with T cells via tail vein injection. For human pancreatic cancer xenograft experiments, Rag1-deficient mice were injected subcutaneously with 1.5–2 × 10⁶ HPAC cells and then ~21 d later, when tumors were typically 50–70 mm², were treated with T cells via tail vein injection. All mice receiving T cells were also given intraperitoneal injections of rhIL-2 b.i.d. (2 × 10⁵ IU/dose) for 6 doses total. Unless otherwise indicated, at least two independent experiments were performed with similar results. As a quality control, T cells used in every ACT experiment were assessed for FAP-CAR expression and specific in vitro activity (see T cell assays). In all ACT experiments, the adoptively transferred T cells demonstrated >70% transduction efficiency of the FAP5-CAR and specifically recognized plate-bound FAP and FAP-expressing cells. All experiments were performed in a blinded, randomized fashion, with each treatment group including a minimum of 5 mice. Blinded tumor measurements are the products of perpendicular diameters and are plotted with ± SEM. Tumor growth statistics were calculated using the Wilcoxon rank-sum test, based on linear slopes of the tumor growth curves at each data point. P-values of 0.05 or lower were considered significant.

Isolation of BM and OS Cells. The protocol used was derived from StemCell Technologies website. In brief, mice were sacrificed and femurs and tibias were harvested and cleaned of muscle. Epiphyses were removed with a scalpel and bones were further scraped clean using the scalpel. Cleaned bones were placed in a mortar containing cold buffer (PBS + 2% FBS + 1 mM EDTA). Bones were then very gently crushed with a pestle to release the BM, which was collected and filtered through a 70-µM strainer. To isolate OS cells, the bone fragments were washed multiple times with buffer and gentle agitation (approximately three to six times, or until the buffer is no longer red/pink), and then minced with a scalpel into 1–2-mm pieces in the presence of 0.25% collagenase I (Invitrogen) solution (in PBS + 2% FBS). Bones were digested in the 0.25% collagenase solution (5 ml per mouse) for 45 min in a 37°C shaking incubator, and then filtered through a 70-µM strainer and washed with cold buffer. Red blood cells in BM and OS were lysed with ACK buffer and washed. Cells were then counted using a hemocytometer with Trypan blue exclusion, and then stained for flow cytometry analysis as described in the Cell Surface Flow Cytometry section above.

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