Suppression of Antitumor Immunity by Stromal Cells Expressing Fibroblast Activation Protein –α

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fitness costs involve more rarely used codons, this is true for only a limited subset of mutations (Fig. 3E and table S4). Second, we could not find any overall correlation between relative codon usage and fitness effects, using either all *S. typhimurium* codons or only those used in ribosomal protein genes (Fig. 3E and table S4). This is also consistent with our previous experimental work in which substitution of the *S. typhimurium rpsT* and *rplA* genes with genes from other species with different codon usage caused very small fitness effects (*24*).

These small fitness costs suggest that the fitness constraints on the mRNA for the two ribosomal protein genes are highly conserved between related bacterial species and that this functional conservation is largely independent of codon usage. Selection coefficients determined from the competition experiments were plotted as a function of the absolute values of the predicted free energy change for the mRNA of the synonymous mutants. A weak but significant correlation [correlation coefficient (r) = 0.47, P = 0.0027, n = 38 synonymous mutants] was found, indicating a general connection between changed mRNA structure and fitness (Fig. 3F and table S5). However, no significant changes in mRNA levels could be detected by quantitative real-time fluorescence polymerase chain reaction for synonymous mutants with large fitness costs (SOM text). Studies of synonymous substitutions usually involve large changes in codon usage or particular examples of substitutions with large effects. Mutagenesis studies of single proteins rarely include the use of high-sensitivity assays of fitness and analysis of synonymous substitutions (SOM references).

Studies of fitness effects of defined base substitutions in viruses have focused on the DFE at the whole-genome level, whereas we studied two specific bacterial genes. However, the viruses examined are small and encode only 5 to 11 genes, meaning that there are many independently engineered mutations for each virus gene and that the DFE can also be studied at the level of the individual genes (*13*, *20*, *21*). Comparing the shape of the distributions obtained here with those from similar experiments in viruses reveals two differences that are valid both when the viral DFEs are analyzed at the level of the whole genome and of individual genes. First, for viruses, the most frequently found mutational type was lethal (up to 40%) (*13*, *20*, *21*), whereas most of the mutations examined here had only small effects on fitness (91% had s values between –0.003 and –0.03). Thus, the compact virus genomes appear to be highly constrained with regard to which sequence changes are acceptable for phage viability (*13*).

The second difference is the rarity of apparently neutral mutations found here as compared to the viruses examined (*13*, *20*, *21*). For the ribosomal protein genes, 6 of 126 mutants (4.8%) had $|s| < 0.003$, whereas for the viruses, 25% appeared neutral. One reason for this difference could be that the higher sensitivity of our fitness assays allows mutations with small fitness effects to be distinguished from neutral mutations and that a similar peak of weakly deleterious mutations might also exist in the viral systems. Thus, it is conceivable that the relatively high frequencies of apparently neutral mutations observed in certain experimental systems (*13*, *20*, *21*) are mainly a consequence of the limited sensitivity of the assays and that the proportion of deleterious mutations is very high even when synonymous substitutions are included.

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Supporting Online Material

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Suppression of Antitumor Immunity by Stromal Cells Expressing Fibroblast Activation Protein–α

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The stromal microenvironment of tumors, which is a mixture of hematopoietic and mesenchymal cells, suppresses immune control of tumor growth. A stromal cell type that was first identified in human cancers expresses fibroblast activation protein–α (FAP). We created a transgenic mouse in which FAP-expressing cells can be ablated. Depletion of FAP-expressing cells, which made up only 2% of all tumor cells in established Lewis lung carcinomas, caused rapid hypoxic necrosis of both cancer and stromal cells in immunogenic tumors by a process involving interferon-γ and tumor necrosis factor–α. Depleting FAP-expressing cells in a subcutaneous model of pancreatic ductal adenocarcinoma also permitted immunological control of growth. Therefore, FAP-expressing cells are a nonredundant, immune-suppressive component of the tumor microenvironment.

Almost 20 years ago, an important advance in tumor immunology was the discovery that a human melanoma may express an unmutated tumor-associated antigen that spontaneously elicits a $CD8^+$ T cell response (*1*). However, therapeutic vaccination with such antigens has only rarely been effective in controlling tumor growth. Some studies suggest that cancers induce systemic tolerance (*2*) or lose antigen expression as they progress (*3*, *4*), but these explanations cannot account for the findings that systemic immune responses occur in patients immunized with such antigens (*5*, *6*) and that these responses do not induce or maintain tumor regression, despite persistent expression of antigen and major histocompatibility complex (MHC) class I

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by tumors (*5*). These observations indicate that immune suppression within the tumor microenvironment may be a major determinant of the poor outcome of therapeutic vaccination.

Although suppression may be mediated by cancer cells (*7*), "the paradoxical finding that antigenically foreign cell clones can develop into a tumor in an animal and are not automatically eliminated by the immune response" (*8*), as well as the occurrence of concomitant immunity (*9*), indicates that stromal cells have a major role in immune suppression. Of the two general types of nonvascular stromal cells, hematopoietic and mesenchymal, the former, which includes myeloid-derived suppressor cells (*10*, *11*), M2 macrophages (*12*), certain natural killer T cells (*13*), and $CD4^+$ $Foxp3^+$ regulatory T cells (*14*), has been considered more often in this context than mesenchymal cells, which have usually been studied as human carcinoma–associated fibroblasts in xenografted, immune-deficient mice (*15*). Nevertheless, a tumoral stromal cell of apparent mesenchymal origin—identifiable by its expression of the type II membrane dipeptidylpeptidase fibroblast activation protein–α (FAP) (*16*)—is associated with other biological processes in which immune suppression may occur, such as the gravid uterus and chronic, noninfected inflammatory lesions (*17*, *18*).

To assess the immune suppressive function of FAP^+ stromal cells in a tumoral microenvironment, we created two transgenic (Tg) mouse lines with bacterial artificial chromosomes (BACs) containing the murine *fap* gene modified by insertion of a cassette encoding either enhanced green fluorescent protein (EGFP) or the primate diphtheria toxin receptor (DTR) (fig. S1) (*19*). We subcutaneously injected EGFP Tg mice with cells of the LL2 Lewis lung carcinoma line, removed tumors after 16 days, and assessed frozen sections by confocal microscopy. In tumoral stromal cells, there was colocalization of EGFP and staining by an antibody against FAP (anti-FAP), demonstrating that the modified BACs contained the *fap* transcriptional elements necessary for appropriate expression of the inserted cassettes (Fig. 1A). Further characterization of $EGFP^+$ stromal cells by confocal microscopy showed that they are composed of both $CD45^+$ and $CD45^-$ cells, all staining with antibody to α-smooth muscle actin (α-SMA) and some with antibody to collagen type I (Col I) (Fig. 1A). Phenotyping FAP^+ stromal cells by flow cytometry revealed that the $CD45^-$ subset expressed CD34 and Sca-1 and that the $CD45^+$ subset expressed CD11b, MHC class II, and Sca-1, but not Gr-1 (fig. S2). Thus, the $CD45^-$ subset shares markers with the mesenchymal stem cells (MSCs) (*20*), as do human FAP^+ cells (*21*), and the $CD45^+$ subset resembles the $CD11b^+$/class II^+/Col I^+/α-SMA^+ fibrocyte (*22*). In LL2 tumors rendered immunogenic by expression of ovalbumin (LL2/OVA), $CD45^-$ and $CD45^+$ FAP^+ stromal cells each made up ~1% of all tumoral cells; the LL2/OVA cells and $CD45^+$/FAP^- cells represented 66 and 30% of the cells, respectively (Fig. 1B). We also found

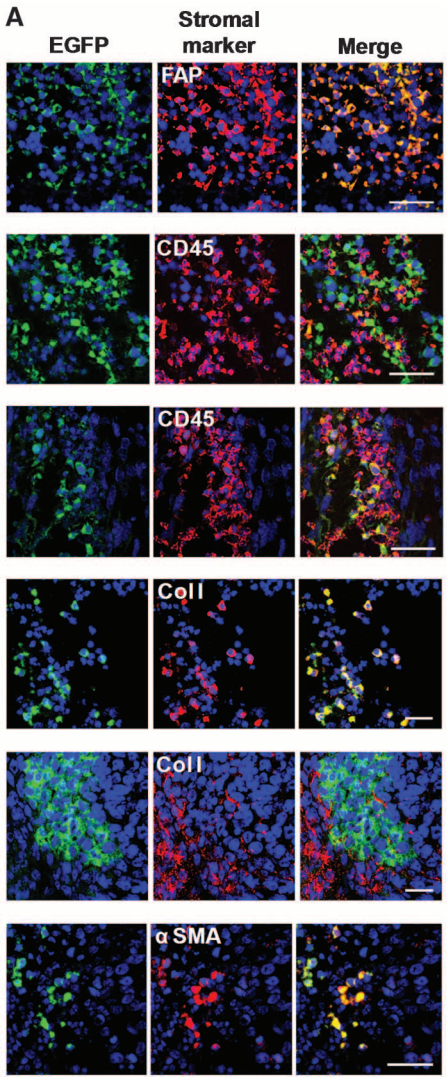

Fig. 1. Characterization of FAP^+ stromal cells. (**A**) Frozen sections of an established subcutaneous LL2 tumor taken from an EGFP Tg mouse were evaluated by confocal microscopy for native EGFP fluorescence and staining with antibodies specific for FAP, CD45, Col I, and α-SMA, respectively. Images are representative of multiple sections and tumors. Scale bars, 50 μm. (**B**) The cellular composition of enzyme-dispersed, rat $Thy1.1^+$ LL2/OVA tumors was assessed by flow cytometry. Closed circles represent individual tumors; horizontal lines denote means.

$CD45^+$ and $CD45^-$ FAP^+ cells in the bone marrow (fig. S3A), which is the origin of MSCs and fibrocytes. The functionality of the DTR Tg was shown by the decreased number of FAP^+ cells in both LL2/OVA tumors and bone marrow from DTR Tg mice after treatment with diphtheria toxin (DTX) (fig. S3). The loss of FAP^+ stromal cells did not abolish tumoral FAP expression because LL2 cells express FAP (fig. S2A), which is relevant because inhibition of FAP peptidase activity can impair nonimmunogenic tumor growth (*23*).

To determine if FAP^+ stromal cells contribute to the resistance of an immunogenic tumor to therapeutic vaccination, we first validated the efficacy of prophylactic vaccination. We immunized non-Tg mice with vaccinia virus–expressing OVA (VaxOVA) or influenza nucleoprotein (VaxNP) 2 weeks before subcutaneous injection of LL2/OVA cells. LL2/OVA outgrowth was delayed only in mice that had received VaxOVA, showing that prophylactic immunization is effective (Fig. 2A). Three groups of mice were assessed for the efficacy of therapeutic immunization on day 12 when tumors were palpable: non-Tg mice that did or did not receive VaxOVA and DTR Tg mice that received VaxOVA. DTX was given to all mice beginning on day 12. Therapeutic vaccination did not slow the growth of established LL2/OVA tumors unless it was combined with FAP^+ cell ablation, which fully suppressed tumor growth (Fig. 2B). Seven days after therapeutic vaccination, the frequencies of peripheral blood $CD8^+$ T cells that were H-2Kb/SIINFEKL(OVA)-specific in non-Tg mice and DTR Tg mice were nearly equivalent (1.4 ± 0.5% and 0.7 ± 0.3%, respectively, $P > 0.05$) (fig. S4, A and B), excluding an effect of FAP^+ cells on the priming of OVA-specific $CD8^+$ T cells.

FAP^+ cell ablation suppressed LL2/OVA growth by 48 hours (Fig. 2B), before a vaccine-induced immune response could have occurred, indicating that either the tumor itself induced an anti-OVA response (the effects of which were locally suppressed by FAP^+ cells) or the tumor-promoting effect of FAP^+ stromal cells did not have an immunological basis. To explore these possibilities, we examined the effect of FAP^+ cell ablation on the growth of established LL2/OVA tumors without therapeutic vaccination. The rate

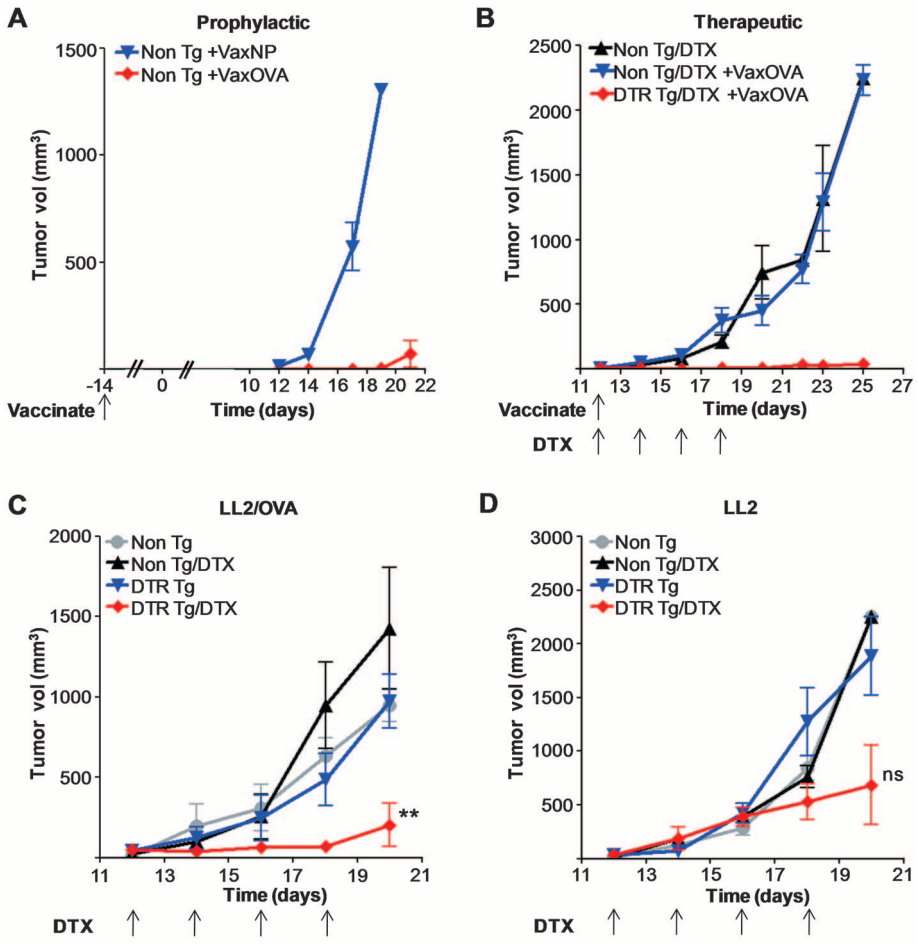

Fig. 2. Combining ablation of FAP$^+$ stromal cells with a therapeutic vaccine controls tumor growth. (**A**) Mice were prophylactically vaccinated with VaxNP or VaxOVA 14 days before subcutaneous injection of LL2/OVA tumors, and tumor sizes were measured thereafter. (**B**) Non-Tg and DTR Tg mice were injected with LL2/OVA cells. Twelve days later, when tumors were palpable, all mice began alternate-day treatment with DTX, and the indicated groups were therapeutically vaccinated with VaxOVA. (**C**) Non-Tg and DTR Tg mice were injected with LL2/OVA cells; 12 days later, the indicated groups began alternate-day DTX treatment. (**D**) Same as in (C), except that mice were injected with LL2 cells. Tumor sizes are presented as mean ± SEM (error bars). The curves describing tumor growth were compared for differences using the "compareGrowthCurves" permutation test [$^{**}P < 0.01$; not significant (ns), $P > 0.05$; representative of two replicate experiments; cohorts contained four or more mice].

of expansion of LL2/OVA tumors in DTR Tg mice was significantly suppressed by DTX treatment, as compared with that of the control groups of non-Tg mice with or without DTX and DTR Tg mice without DTX (Fig. 2C). Growth arrest was apparent by 48 hours after DTX. At day 20, 0.4 ± 0.2% and 0.2 ± 0.2% ($P > 0.05$) of splenic CD8$^+$ T cells were H-2Kb/SIINFEKL(OVA)-specific in the DTX-treated non-Tg mice and DTR Tg mice, respectively (fig. S4C), indicating that the LL2/OVA tumors had induced an immune response, as has been reported by Nelson *et al*. (*24*). The same analysis of the growth curves of nonimmunogenic LL2 tumors in non-Tg mice and DTR Tg mice with or without DTX did not reveal significant differences (Fig. 2D); however, diminished LL2 growth in DTR Tg mice given DTX did seem to eventually occur after 6 to 8 days of treatment. Therefore, the loss of FAP$^+$ stromal cells causes immediate growth arrest of a tumor that has induced an immune response, but not of a nonimmunogenic tumor. Although there may be a nonimmunological function for the FAP$^+$ cell, we elected to focus on its immune-suppressive activity because of a potential relation to the poor efficacy of tumor vaccines.

We analyzed LL2/OVA tumors taken from non-Tg and DTR Tg mice 48 hours after initiating DTX to characterize the changes that were

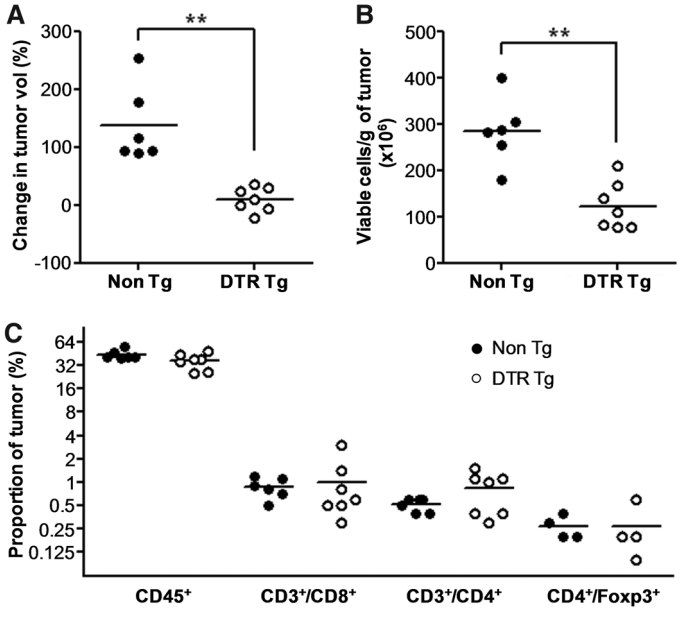

Fig. 3. The acute effects of ablating FAP$^+$ stromal cells on LL2/OVA tumors. Non-Tg and DTR Tg mice bearing established LL2/OVA tumors were given DTX; 48 hours later, tumors were assessed for (**A**) growth by comparison to tumor size before DTX, (**B**) number of viable cells, and (**C**) immune cellular composition [$^{**}P < 0.01$; representative of replicate experiments; closed and open circles represent individual tumors; horizontal lines denote means].

specific to the immunogenic tumor. LL2/OVA tumor size in non-Tg mice doubled during this period, whereas growth in the DTR Tg mice ceased (Fig. 3A). Growth arrest was associated with a 60% decrease in the number of viable cells per gram of tumor (Fig. 3B). Loss of viability must have occurred among both LL2/OVA cancer cells and CD45$^+$ stromal cells, because their relative proportions did not change (Fig. 3C). FAP$^+$ cell ablation did not alter the proportions of CD4$^+$ or CD8$^+$ T cells or of CD4$^+$ Foxp3$^+$ regulatory T cells, suggesting that tumor cell death did not involve a rapid increase of effector T cells or decrease of suppressive T cells. There was also

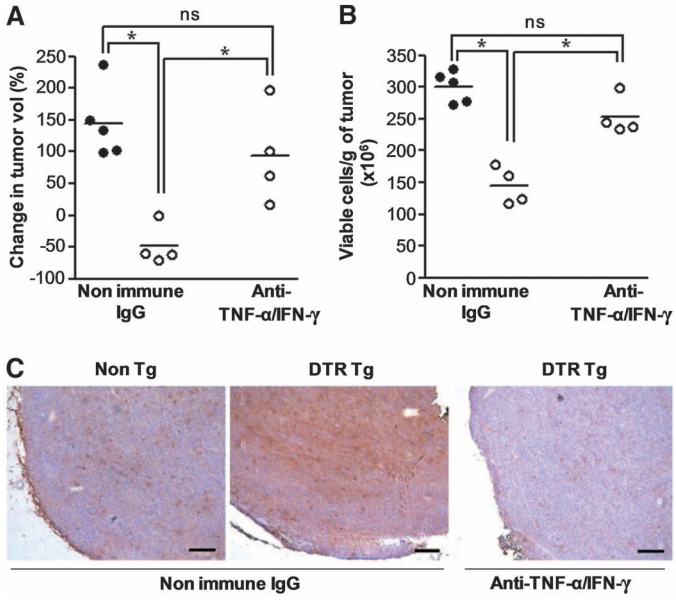

Fig. 4. Protection of LL2/OVA tumors from the effects of ablating FAP$^+$ stromal cells by neutralizing antibodies to TNF-α and IFN-γ. Non-Tg and DTR Tg mice bearing established LL2/OVA tumors were given control immunoglobulin G (IgG) or neutralizing antibodies to TNF-α and IFN-γ 24 hours before treatment and at 0 hours, the time of the first day of DTX treatment. Forty-eight hours later, tumors were assessed for (**A**) growth by comparison to size before DTX and (**B**) number of viable cells (representative of replicate experiments; closed and open circles represent individual tumors from non-Tg and DTR Tg mice, respectively; horizontal lines denote means). (**C**) Tumors were assessed for the occurrence of hypoxia, as detected in frozen sections by immunoperoxidase staining of stable protein adducts formed with reductively activated pimonidazole (*$P < 0.05$; ns, $P > 0.05$). Scale bar, 200 μm. Images are representative of multiple sections taken from three mice from each cohort.

no change in the proportion of tumoral CD8$^+$ T cells that were both OVA-specific and expressed the activation marker CD69, the cytotoxic molecule granzyme B, or produced interferon-γ (IFN-γ) in response to antigenic stimulation (fig. S5). Taken together, these findings are not consistent with immune suppression of OVA-specific CD8$^+$ T cells by FAP$^+$ stromal cells. Nevertheless, the absence of arrested growth of LL2/OVA tumors in Rag2-deficient mice depleted of FAP$^+$ cells confirms the immunological basis of this response (fig. S6).

Acute hypoxic necrosis secondary to ischemia caused by prothrombotic effects of IFN-γ and tumor necrosis factor–α (TNF-α) is an indirect immunological mechanism that may have been involved in the rapid cell death of LL2/OVA tumors depleted of FAP$^+$ cells (*25*, *26*). The presence of mRNA for TNF-α and IFN-γ in the LL2/OVA tumor and the higher amounts of mRNA for IFN-γ and two of its target genes—*IRF-1* (interferon regulatory factor-1) and *iNOS* (inducible nitric oxide synthase)—in LL2/OVA than in LL2 tumors supported this possibility (table S1). Accordingly, non-Tg and DTR Tg mice with established LL2/OVA tumors were given isotype control or neutralizing anti–TNF-α and anti–IFN-γ antibodies during the 48 hours of treatment with DTX, and tumors were then assessed. The impaired tumor growth and decreased recovery of viable tumor cells caused by depleting FAP$^+$ cells were largely reversed by anti–TNF-α/anti–IFN-γ treatment (Fig. 4, A and B). The hypoxia occurring in the LL2/OVA tumor after the loss of FAP$^+$ cells was also suppressed by anti–TNF-α/anti–IFN-γ treatment (Fig. 4C and fig. S7). Therefore, FAP$^+$ stromal cells either suppress the production of TNF-α and IFN-γ, or they attenuate cellular responses to these cytokines to protect the immunogenic tumor from cytokine-induced hypoxic necrosis. The relatively unchanged expression of these cytokines 48 hours after ablation of FAP$^+$ cells would favor the latter explanation (table S1). In addition, there was no marked change in the expression of four potentially immune-suppressive cytokines—transforming growth factor–β1, interleukin (IL)–4, IL-10, and IL-13—after depletion of FAP$^+$ cells (table S1), consistent with the absence of any changes in tumoral CD8$^+$ T cell phenotypes.

We determined whether FAP$^+$ stromal cells suppress immunological control of another subcutaneous tumor that was established with a cell line derived from a murine pancreatic ductal adenocarcinoma (PDA) arising in the KPC mouse (*27*). These cancer cells resemble human PDA in many respects, including their expression of oncogenic Kras^{G12D} and the tumor-associated antigen mesothelin, and both spontaneous and subcutaneous tumors contain FAP$^+$ stromal cells (fig. S8). When transplanted tumors became palpable, we immunized non-Tg and DTR Tg recipients with a mesothelin peptide. Nine days later, we treated mice with DTX and assessed tumors 48 hours later. Only when depletion of FAP$^+$ cells was combined with immunization did the PDA tumor acutely regress (fig. S9A). The immunological basis of this response was demonstrated by its absence in DTR Tg, Rag2-deficient mice that were similarly immunized and depleted of FAP$^+$ cells (fig. S9B).

The acute, hypoxic death of both cancer and stromal cells that is observed after FAP$^+$ cell ablation is mediated by TNF-α and IFN-γ. These cytokines have previously been shown to be involved in CD8$^+$ T cell–dependent killing of antigen-loss variant tumor cells (*28*) and the suppression of angiogenesis (*29*). The finding of a possible relation between FAP$^+$ cells and MSCs and fibrocytes, which promote wound healing, is reminiscent of the description of tumors as chronic, nonhealing wounds (*30*). Therefore, immune suppression by FAP$^+$ cells may be a developmentally programmed, tissue-protective function that, in the context of a tumor, is catastrophically inappropriate. Interfering with suppression by FAP$^+$ cells of cellular responses to these two cytokines may complement the current most effective form of cancer immunotherapy, the enhancement of lymphocyte activation by antibody to cytotoxic T lymphocyte antigen–4 (*31*).

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