FAP Delineates Heterogeneous and Functionally Divergent Stromal Cells in Immune-Excluded Breast Tumors

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Abstract

Cancer-associated fibroblasts (CAFs) are generally associated with poor clinical outcome. CAFs support tumor growth in a variety of ways and can suppress antitumor immunity and response to immunotherapy. However, a precise understanding of CAF contributions to tumor growth and therapeutic response is lacking. Discrepancies in this field of study may stem from heterogeneity in the composition and function of fibroblasts in the tumor microenvironment. Furthermore, it remains unclear whether CAFs directly interact with and suppress T cells. Here, mouse and human breast tumors were used to examine stromal cells expressing fibroblast activation protein (FAP), a surface marker for CAFs. Two discrete populations of FAP+ mesenchymal cells were identified on the basis of podoplanin (PDPN) expression: a FAP+PDPN+ population of CAFs and a FAP+PDPN− population of cancer-associated pericytes (CAPs). Although both subsets expressed extracellular matrix molecules, the CAF transcriptome was enriched in genes associated with TGFβ signaling and fibrosis compared with CAPs. In addition, CAFs were enriched at the outer edge of the tumor, in close contact with T cells, whereas CAPs were localized around vessels. Finally, FAP+PDPN+ CAFs suppressed the proliferation of T cells in a nitric oxide–dependent manner, whereas FAP+PDPN− pericytes were not immunosuppressive. Collectively, these findings demonstrate that breast tumors contain multiple populations of FAP-expressing stromal cells of dichotomous function, phenotype, and location. Cancer Immunol Res; 6(12): 1472–85. ©2018 AACR.

Introduction

Cancer-associated fibroblasts (CAFs) are the predominant nonhematopoietic stromal cell type in tumors, and their abundance often correlates with poor prognosis (1–4). Several roles have been ascribed to CAFs, including production of tumor mitogenic factors, deposition of extracellular matrix (ECM), stimulation of angiogenesis (5–7), and even immune cell trafficking and activation (5). Targeting fibroblast activation protein (FAP) to eradicate CAFs has demonstrated that a reduction in fibroblasts decreased collagen content and tumor burden (8–12), improving the effectiveness of immunotherapy (13, 14). Similarly, abrogation of CXCL12 produced by FAP+ cells synergized with PD-L1 blockade to control the growth of pancreatic cancer in mice (13). Whether CAFs suppress the function of tumor-infiltrating T cells through direct or indirect mechanisms remains unclear.

One of the factors that have limited our understanding of how CAFs modulate antitumor immunity is a lack of specific markers to identify CAFs. FAP, for example, is expressed by some tumor-infiltrating immune cells (14, 15) and is also expressed in lymph nodes (LN; refs. 16, 17). Similarly, expression of alpha-smooth muscle actin (αSMA), another putative fibroblast-specific marker (18–20), is detected in other stromal cells. Another limitation is represented by a dearth of techniques to freshly isolate low-abundance stromal cells with high...
viability and reproducibility from tumors. Furthermore, the potential heterogeneity within CAFs adds complexity to the issue of fibroblast identification (21, 22).

We developed a protocol to isolate mouse and human stromal cells from tumors, which enabled the identification of two FAP+stromal subsets differentiated by the expression of the glycoprotein podoplanin (PDPN). FAP+PDPN+ cells expressed canonical fibroblast genes and exhibited some resemblance to fibroblasts of LNs, including the ability to construct a reticulonetwork of fibers and secrete chemokines (23, 24), which engenders interactions of tumor-infiltrating lymphocytes and stromal cells. The PDPN+ subset of FAP+ cells was identified as cancer-associated pericytes (PDPN+CAPs), as confirmed by their localization around vasculature. Functionally, PDPN+CAPs suppressed T-cell proliferation through nitric oxide (NO) production, whereas PDPN+CAPs had no effect on T-cell proliferation.

Taked together, our study highlights heterogeneity within FAP+ tumor mesenchymal cells, identifying nitric oxide as a stromal mediator of immunosuppression.

Materials and Methods

Mice and tumor models

Sex-matched BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. Mice were maintained under specific pathogen-free conditions in accordance with institutional guidelines and guidelines of the U.S. National Institutes of Health and were used at 5 to 7 weeks of age. B16, EL4, 4T1, A20, MC38, and CT26 WT cells were obtained from American Type Culture Collection. Pan02 cells were obtained from the NCI Developmental Therapeutics Program repository. The 4T07 cells were obtained from Dr. J. Lieberman (Harvard University, Cambridge, MA). Cells were maintained in Dulbecco’s Modified Eagle Medium (B16, MC38, and EL4) or RPMI (4T1, A20, Pan02, CT26, and 4T07) supplemented with 10% FBS and penicillin/streptomycin and were not used for more than 10 passages after they were obtained in 2012. All cells were mycoplasma free but were not authenticated in the past year.

Pan02 passages after they were obtained in 2012. All cells were pathogen-free conditions in accordance with institutional instructions.

Human samples

Human breast tissue samples for flow cytometry analysis were obtained from The MT Group. Primary human CAFs from breast carcinomas (CAF06) were obtained from Vitro-Biopharma and maintained in low-serum MSC-GroTM media as per company’s instructions.

Tissue digestion for stromal cell isolation

Tumors were minced into fine pieces (approximately 1 mm3) using scissors and razor blades, transferred into 15 mL conical tubes containing 2 mL of digestion buffer [RPMI (Gibco), 2% FBS, 0.2 mg/ml Collagenase P (Roche), 0.2 mg/ml Dispase (Gibco), and 0.1 mg/ml DNase I (Roche)], and placed into a water bath at 37°C. The tubes were vortexed every 5 minutes for 15 minutes, the tissue pieces were allowed to settle for 5 minutes, and then the supernatant containing freed cells was collected and quenched at 4°C in 50 mL conical tubes containing 20 mL of cold flow cytometry buffer (PBS, 2% FBS, and 2 mmol/L EDTA). Next, 2 mL of fresh digestion buffer was added to the remaining tumor fragments, and tubes were incubated at 37°C for another 20 minutes, vortexing every 5 minutes, prior to collecting the freed cells and adding them to the previously collected fractions on ice. These 20 minute digestion cycles were repeated for a total of 5 to 6 times, with progressively more forceful agitation methods (vortexing, pipetting 1 mL up and down using large orifice tips, then mixing with uncut 1 mL tips), until no tumor fragments larger than 1 mm remained. The collection tube containing the digested fractions in flow cytometry buffer was kept on ice until the digestion was complete, and then the contents was filtered through 70-μm mesh, centrifuged (1500 rpm, 10 minutes, 4°C), and the cells were counted. Pellets were resuspended in flow cytometry buffer or medium and subjected to immunostaining or in vitro assays. Skin fibroblasts were isolated from the flank of naive, age-matched mice using the same procedure indicated above for tumors, but with a higher (5×) concentration of collagenase P in the digestion buffer. FRCs were isolated from skin-draining LNs as previously described (25).

Antibodies and reagents

For flow cytometry and imaging studies with murine tissues, the following antibodies were purchased from BioLegend: anti-CD45 (30-F11), anti-CD31 (390), anti-PDPN (8.1.1), anti-smooth muscle actin (1A4), anti-Thy1 (53-2.1), anti-CD140a (APAS), anti-CD140b (APBS), anti-CD110 (429), anti-CD4 (RM4.5), and anti-CD8 (RM2206). The anti-FAP used for flow cytometry analysis was provided by Ellen Pure and has been validated (anti-mouse FAP 73.3; ref. 8). For immunofluorescence analysis, the Roche anti-FAP 28fH1 clone was used (26). Functional-grade purified anti-CD3e (145-2C11) was obtained from BD Biosciences, and anti-CD28 (37.51) was from BioLegend. Antibodies used to stain human samples include viability dye (1L0119; Life Technologies), anti-PDPN (clone NZ1.3; eBioscience), anti-Thy1 (clone 5E10; BD Biosciences), anti-CD4 (clone A161A1; BioLegend), and anti-CD8 (clone HIT8a; BioLegend). For PBMC stimulation, anti-human CD3 (clone OKT3; eBioscience) and anti-human CD28 (clone CD28.2; eBioscience) were used. L-NMMA (NG-normomethyl L-arginine) was purchased from Calbiochem and CFSE (carboxyfluorescein diacetate succinimidyl ester) was from Invitrogen.

Microscopic analysis

Isolated tissues were fixed for 2 to 4 hours at room temperature in 4% paraformaldehyde (PFA) in PBS and then transferred to 30% sucrose to saturate overnight. Tissues were embedded in optimal cutting temperature medium (Sakura Finetek) and frozen for storage at −80°C. Next, 10- to 20-μm thick sections were cut on a cryostat, immunostained, and imaged with a Leica SP5X laser-scanning confocal microscope. Alternatively, fixed tumors were serially cut in 100-μm thick sections, imaged by multiphoton microscopy and assembled using Imaris software (Bitplane) to
enable digital reconstruction. Final analysis was performed using Volocity image analysis software (PerkinElmer).

Flow cytometry analysis
Tumor single-cell suspensions were resuspended in flow cytometry buffer (PBS, 2% FBS, and 2 mmol/L EDTA), blocked with Fc block, and then stained with different fluorochrome-conjugated antibodies for 15 minutes on ice. Samples were run on a BD flow cytometry Aria II and analyzed with FlowJo.

Cell enrichment and sorting
Single-cell suspensions prepared as described above were stained with biotinylated antibodies against CD45, CD31, CD49b, and Ter119, and negative cells were collected using the EasySep biotin selection kit according to the manufacturer’s instructions. After enrichment, cells were stained and sorted on a BD flow cytometry ARIA II (100-μm nozzle, 20 psi). Dead cells were excluded using propidium iodide (5 ng/mL). Purity was assessed every time by sorting some cells into PBS and immediately reanalyzing them.

Microarray analysis
Cells were sorted into TRizol, and RNA was prepared according to the guidelines of the Immunological Consortium. Isolated RNA was amplified and hybridized to the Affimex Mouse Gene 1.0 ST array. Normalized data were log2 transformed, row-centered, and clustered using the hierarchical clustering module of GenePattern and displayed using the heat map image module (www.genepattern.com).

Coculture of T cells and stromal cells
Freshly sorted 4T1 PDPN+ CAFs, 4T1 PDPN− CAPs, or LN FRCs were plated in 96-well plates (5 × 10^4 cells per well) and allowed to adhere overnight. The next day, CFSE-labeled splenocytes (1 × 10^6) were added to the plates, together with soluble anti-CD3 (0.25 μg/mL) and anti-CD28 (0.25 μg/mL), and cultures were analyzed by flow cytometry 24 to 72 hours later. For experiments with human cells, 2.5 × 10^5 crystal violet–labeled PBMCs from anonymous healthy donors were plated with 5 × 10^4 CAFs and activated with anti-CD3 (2 μg/mL) and anti-CD28 (1 μg/mL) for 72 hours.

Figure 1. Isolation of tumor mesenchymal stromal cells reveals heterogeneity within the FAP+ compartment. A, Schematic depicting tumor stromal cell digestion and preparation. B, Representative plot showing viability of single-cell suspensions after tissue digestion. C, Representative flow-cytometric profile of s.c. 4T1 tumor single-cell suspensions. The FAP+ PDPN+ (PDPN+ stroma, in red) and FAP+ PDPN− (PDPN− stroma, in green) populations are highlighted. D, Frequencies of FAP+ PDPN+ and FAP+ PDPN− cells in various subcutaneously implanted murine syngeneic tumors (percentage of total cellularity), and the ratio of FAP+ PDPN+ and FAP+ PDPN− cells in these tumors. N = 2–7 independent experiments per model. Data are expressed as mean ± standard deviation.
Figure 2. 
Transcriptomic analysis of FAP$^+$ cell populations. **A and B**, Representative gating strategy for 4T1 tumor stroma (**A**) and skin fibroblasts (**B**). **C**, Representative sort strategy and post-sort FACS analysis for PDPN$^+$ and PDPN$^-$ stromal subsets from 4T1 tumors. Numbers indicate frequencies. **D**, The top 100 differentially expressed genes between PDPN$^+$ and PDPN$^-$ stromal subsets were analyzed for pathway enrichment (KEGG) using the DAVIS database. **E and F**, Expression of fibroblast (**E**) and pericyte (**F**) canonical genes was determined in PDPN$^+$ and PDPN$^-$ stromal subsets and compared with skin fibroblasts and LN stromal cells. (Skin Fib, skin fibroblasts; Tum PDPN$^+$, tumor FAP$^+$ PDPN$^+$ stroma; LN FRCs, lymph node fibroblastic reticular cells; Tum PDPN$^-$, tumor FAP$^-$ PDPN$^-$ stroma; LN IAPs, lymph node integrin $a_7^+$ pericytes; LN LECs, lymph node lymphatic endothelial cells; LN BECs, lymph node blood endothelial cells.) Heat maps show hierarchical clustering for probes with EV$>120$ for at least one population. Values were normalized, row mean centered, and log$_2$ transformed.
Measurement of NO production

The concentration of NO in cell culture supernatants was determined using the Griess reagent system (Promega), according to the manufacturer’s instructions.

Statistical analysis

Two-tailed, unpaired Student t tests were used for statistical analyses, with the assumption of equal sample variance, with GraphPad Prism software. Differences with a P value of <0.05 were considered statistically significant.

Results

Tumor stroma heterogeneity within the FAP$^+$ compartment

In order to reproducibly isolate CAFs from primary tumors, we adapted a protocol that we had previously optimized to extract stromal cells from LNs (25). Using a combination of physical and enzymatic dissociation (Fig. 1A), stromal cells were released from transplanted murine syngeneic tumors, with viability greater than 90% (Fig. 1B). To examine the mesenchymal component of tumors, single-cell suspensions were stained with a FAP-specific antibody, together with a panel of antibodies against lineage markers to exclude hematopoietic cells (CD45), 4T1 tumor cells (CD49b; Supplementary Fig. S1A), and endothelial cells (CD31). The mesenchymal marker, Thy1 (27), and podoplanin (PDPN), a glycoprotein expressed by fibroblasts from a variety of tissues (28), were also evaluated. Mesenchymal tumor components were identified by high expression of FAP and Thy1, and the absence of hematopoietic, endothelial, and tumor cell markers (Fig. 1C). The FAP$^+$Thy1$^+$ mesenchymal cell compartment could be further subdivided on the basis of PDPN expression into FAP$^+$PDPN$^+$ and FAP$^+$PDPN$^-$ populations (Fig. 1C). Both the PDPN$^+$ and PDPN$^-$ fractions of FAP$^+$ stroma were present in tumors as soon as tumor nodules were palpable (~75 mm$^3$), and their frequency remained constant throughout tumor progression (Supplementary Fig. S1B and S1C). Additionally, both populations were observed in multiple syngeneic tumor models, including skin, breast, lung, and gastrointestinal carcinomas (Fig. 1D). FAP$^+$PDPN$^+$ and FAP$^+$PDPN$^-$ stromal cell populations were particularly abundant in tumors with dense ECM, such as 4T1, which is a poorly immunogenic murine tumor that closely resembles human triple-negative breast carcinoma (29, 30). Therefore, we focused on this model for further analyses.

PDPN expression distinguishes between CAFs and pericytes within FAP$^+$ stroma

To further characterize the FAP$^+$ mesenchymal cells, transcriptional profiling was performed on the two FAP$^+$ tumor stromal populations (Fig. 2A), using PDPN$^+$ fibroblasts from healthy skin as a comparison population for the tissue where the tumor was located (Fig. 2B). Stromal cells were extracted from established s.c. 4T1 tumors or healthy skin and sorted to greater than 95% purity (Fig. 2C) with good sample-to-sample reproducibility (Supplementary Fig. S1D). Following standards and quality control pipelines of the Immunological Genome Project Consortium (www.immunogen.org), transcriptomic profiles were generated for each subset.

Controlling for a false discovery rate of 5%, 489 genes were ≥2-fold differentially expressed between FAP$^+$PDPN$^+$ and FAP$^+$PDPN$^-$ stromal cells. Pathway enrichment analysis (KEGG) for the top 100 differentially expressed genes revealed that FAP$^+$PDPN$^+$ cells were characterized by expression of genes predictive of pathways associated with fibroblasts, including proteoglycan biology, WNT signaling, focal adhesions, and complement activation (Fig. 2D). On the other hand, FAP$^+$PDPN$^-$ stromal cells were enriched for pathways related to cAMP and cAMP signaling, as well as muscle contraction (Fig. 2D). Given that these pathways are associated with regulation of vascular tone (31), we hypothesized that FAP$^+$PDPN$^-$ stromal cells represent a fraction of FAP$^+$ pericytes, rather than fibroblasts, in the tumor microenvironment. To test this hypothesis, we compiled a list of fibroblast- and pericycle-specific genes based on several published papers (5, 7, 32, 33), and analyzed their expression in tumor stromal cells. This analysis revealed high expression of fibroblast markers in FAP$^+$PDPN$^+$ stromal cells, including Den, Lox, and Lof1 (Fig. 2E), as also depicted in skin fibroblasts and LN FRCs, underscoring their common lineage. FAP$^+$PDPN$^-$ stroma exhibited pronounced expression of genes previously attributed to pericytes, including Des, Rgs4, Rgs5, and the transcription factor Notch3 (refs. 32, 33; Fig. 2F). In addition, their expression pattern resembled that of primary pericytes from LNs (IAPs, integrin alpha7$^+$ pericytes; ref. 35), strengthening the argument that PDPN$^-$ tumor stromal cells represent a population of perivascular cells in tumors.

Further analysis of signature genes associated with different LN resident stromal cells also revealed similarities between PDPN$^+$ tumor stromal cells and LN FRCs, including expression of Fap, Acta2, and Cdh11, genes associated with activation and myofibroblast differentiation (Fig. 3A). On the other hand, PDPN$^-$ tumor stromal cells and LN IAPs shared expression of signature genes associated with pericytes (Fig. 3A). Examination of global relationships between PDPN$^+$ and PDPN$^-$ tumor stromal cells through principal component analysis reinforced their functional divergence, as depicted by their separation on principal components 1 and 2 (Fig. 3B). However, both subsets of FAP$^+$ tumor stromal cells were positioned closer to one another than to their LN counterparts (Fig. 3B), suggesting the existence of distinct transcriptomic programs in fibroblasts from different locations, likely dictated by tissue-specific environmental cues.

Morphologic examination of cytoskeletal organization in FAP$^+$ stroma from 4T1 tumors also highlighted distinct features of the...
two subsets. Specifically, when plated directly after sorting, PDNP⁺ stromal cells possessed numerous protrusions, with cytoskeletal structures emblematic of myofibroblasts, such as stress fibers and superstructure focal adhesions (i.e., >8 μm; Supplementary Fig. S1E). In vivo, FAP⁺ PDNP⁺ stromal cells also presented a fibroblast-like shape, with elongated cell bodies (Fig. 3C). Conversely, PDNP⁺ tumor stromal cells cultured in vitro exhibited membrane ruffles (Supplementary Fig. S1F), characteristic of contractile cells. Additionally, confocal microscopic analysis of 4T1 cryosections revealed perivascular localization of FAP⁺ PDNP⁺ stromal cells, as well as expression of integrin α7, a marker of LN pericytes (hereafter referred to as PDNP⁺ PDPN⁺ pericytes; Supplementary Fig. S2A). These two populations of FAP⁺ mesenchymal cells were also observed in primary human breast carcinomas (Fig. 3F; Supplementary Fig. S1G). Similar to murine tumor stromal cells, both of these FAP⁺ populations in human tumors also expressed αSMA and αSMA-C (Supplementary Fig. S1H). The subset expressing both FAP and αSMA was restricted to PDNP⁺ FAP⁺αSMA⁺ cells (hereafter referred to as PDNP⁺ CAFs) but also by PDNP⁺ CD140b⁻ ITGα7⁺ pericytes (hereafter referred to as PDNP⁺ CAPs) in both mouse and human tumors.

**Distinct contributions of CAFs and CAPs to the matrix architecture of tumors**

The discovery that only a subset of FAP⁺ mesenchymal cells possessed features of fibroblasts prompted us to conduct a deeper analysis of the fibrotic potential of FAP⁺ cells in the tumor microenvironment. Analysis across multiple stromal cells from tumors and healthy tissues revealed a fibrotic potential in PDNP⁺ CAFs, as depicted by expression of many collagen species, including type I collagen and collagen III, expression of other fibrillar collagen genes (collagen V), and fibril-associated collagens (collagen XIV), including CD140b (Fig. 3G) and CD44 (Supplementary Fig. S1H). Together, these results demonstrate that FAP is expressed not only by PDNP⁺ CD140b⁻ αSMA⁺ CAFs (hereafter referred to as PDNP⁺ CAFs) but also by PDNP⁺ CD140b⁻ ITGα7⁺ pericytes (hereafter referred to as PDNP⁺ CAPs), revealing a unique subset of fibroblast/ECM interactions that are absent in other fibroblasts and pericytes.

Expression of ECM genes in PDNP⁺ CAFs was not merely a result of their fibroblastic nature. Genes associated with fibroblast activation and fibrosis were enriched in PDNP⁺ CAFs as compared with PDNP⁻ CAPs but also with other fibroblast populations, suggesting that the inflammatory environment of tumors promotes expression of fibrosis-related genes (Fig. 4A and B).

Given that TGFβ drives fibrosis in various pathologic conditions, and TGFβ signaling in CAFs has been associated with metastatic dissemination (33) and poor response to immunotherapy (36, 37), it is conceivable that increased fibrotic potential of fibroblasts in tumors may stem from increased TGFβ signaling. Indeed, expression of an array of TGFβ-responsive genes was significantly higher in PDNP⁺ CAFs, as compared with PDNP⁻ CAFs from the same tumor, fibroblasts from skin, and LN FRCs (Fig. 4C and D). Additionally, both CAFs and CAPs exhibited increased expression of TGFβ isoforms relative to nontumor fibroblasts (Fig. 4E and F). These data suggest that high TGFβ concentrations in the tumor microenvironment and increased TGFβ signaling in PDNP⁺ CAFs may prompt ECM deposition. Indeed, PDNP⁺ CAFs were associated with a reticular network of matrix fibers (Fig. 4G). The fact that PDNP⁺ CAFs express TGFβ isoforms and some of TGFβ response genes suggests TGFβ signaling is active in pericytes as well, perhaps modulating their association with endothelial cells and vessel permeability (38).

Together, these data suggest that all FAP⁺ stromal cells express an array of ECM and ECM remodeling genes and possess fibrotic potential. However, of the two FAP⁺ stromal populations identified here, PDNP⁺ CAFs likely dominate in shaping the matrix architecture surrounding tumors in response to profibrotic signals such as TGFβ.

**T cells interact closely with CAFs in tumor periphery**

The localization of T cells in tumors is influenced by the matrix architecture (39). Therefore, we sought to determine whether T cells interact with PDNP⁺ CAFs. To this end, cryopreserved, subcutaneously grown 4T1 tumors were serially sectioned into thick (100 μm) slices, stained, and imaged by multiphoton microscopy, and individual z-planes were stitched together to create a single 3D reconstructed image (Fig. 5A and B). Collagen structures were visualized with second harmonic generation, PDNP⁺ CAFs were detected with an antibody against PDNP, and immune cells were visualized with CD45 staining.

3D reconstruction of these 4T1 tumors revealed an immune-excluded phenotype characterized by a dense fibroblast/ECM structure surrounding the tumor and the presence of most tumor-infiltrating leukocytes in this region, with fewer immune cells deeper in the tumor parenchyma (Fig. 5C and D). Preferential localization of tumor-infiltrating leukocytes in stroma- and collagen-rich regions was also observed in several other murine syngeneic tumor models (Supplementary Fig. S4A-S4C). Further analysis of these stromal regions in murine tumors revealed interactions between tumor-infiltrating T cells and PDNP⁺ CAFs and PDNP⁺ fibroblasts, with the majority of CD8⁺ T cells establishing direct contact with fibroblasts and surrounding matrix (Fig. 5E; Supplementary Fig. S5A-S5C).

Altogether, these findings suggest that dense ECM generated by cancer-associated stromal cells may directly influence the localization of tumor-infiltrating immune cells. Furthermore,
close interactions observed between PDPN+ CAFs and T cells raise the possibility that CAFs in peritumoral stromal-dense areas have also been observed in patients with cancer and has been suggested to curtail clinical benefits of immunotherapies (36, 37, 40). These findings raise the possibility that tumor cells co-opt tissue fibroblasts to generate a specialized architecture that collects tumor-infiltrating immune cells and impedes proper function of CD8+ T lymphocytes.

PDPN+ CAFs express a functional gene signature resembling that of immunosuppressive FRCs

The interactions observed between PDPN+ CAFs and lymphocytes in the tumor microenvironment resembled the FRC–immune cell interplay characteristic of lymphoid organs, prompting us to explore whether PDPN+ CAFs share some functional properties with LN FRCs. PDPN+ CAFs did not express Ccl9, Ccl21, Cxcl12, or Cxcl13, chemokines expressed by FRCs and involved in the recruitment of naïve lymphocytes (refs. 24, 41; Fig. 6A). On the other hand, PDPN+ CAFs and PDPN− CAFs were poised to support the influx of antigen-experienced T cells, as evidenced by their expression of the CXCR3 ligands Cxcl9, Cxcl10, and Cxcl11 (Fig. 6B), in addition to other immune-relevant chemokines (Supplementary Fig. S6). Genes implicated in the survival of naïve B and T cells, namely, Itg7 and Tnfsf13b, were also not expressed by PDPN+ CAFs, PDPN− CAFs, or skin fibroblasts (Fig. 6C). Thus, FRCs appear to be unique in their ability to support naïve lymphocytes.

On the other hand, PDPN+ CAFs expressed genes associated with the induction of an immunosuppressive microenvironment, including Tgfβ (as shown in Fig. 4E and F). One gene expressed in PDPN+ CAFs, Nos2 (Fig. 6D), was of particular interest given that NO is produced by FRCs in response to activated T cells and required for FRC-mediated suppression of these cells (42–44).
When examined directly ex vivo, Nos2 was expressed by PDPN⁺ CAFs, but was not detectable in resting FRCs, PDPN⁻/C0 CAPs, or skin fibroblasts (Fig. 6D). Nos2 expression in CAFs was validated at the protein level. Using confocal imaging, iNOS protein was detected in PDPN⁺ CAFs from cryopreserved s.c. 4T1 tumor tissues (Fig. 6E). Additionally, flow cytometry analysis of freshly isolated tumor stroma cells further demonstrated that expression of iNOS in FAP⁺ stromal cells was restricted to PDPN⁺ CAFs, as no iNOS protein was detected in PDPN⁻/C0 CAPs (Fig. 6F). Finally, CAFs produced NO when cocultured with activated splenocytes. This observation is in line with our previous data indicating that IFNγ and TNFα produced by activated splenocytes cause an upregulation of iNOS and NO in FRCs (35, 43). In contrast, NO was not detected in the supernatant of PDPN⁻ CAPs cultured in the same conditions, consistent with their lack of Nos2 RNA and iNOS protein (Fig. 6G). Together, these data support the hypothesis that interactions between PDPN⁺ CAFs and tumor-infiltrating lymphocytes in the inflammatory tumor microenvironment promotes NO production by PDPN⁺ CAFs and may lead to immunosuppression.

PDPN⁺ CAFs restrain proliferation of activated T cells

To evaluate the functional consequences of NO produced by CAFs and the interactions between CAFs and lymphocytes in the tumor microenvironment, PDPN⁺ CAFs were isolated from 4T1 tumors and cocultured with splenocytes in vitro, in the presence of anti-CD3 and anti-CD28. The proliferation of both CD4⁺ and CD8⁺ T cells was significantly dampened in the presence of PDPN⁺ CAFs (Fig. 7A; Supplementary Fig. S7A), whereas early T-cell activation remained unperturbed, as indicated by increased expression of CD69 and CD25 and down-regulation of CD62L (Fig. 7B). This pattern is similar to what we and others previously reported for FRCs in contact with activated T cells (42–44). These findings were recapitulated with PDPN⁺ CAFs derived from human breast carcinomas. Briefly, human FAP⁺PDPN⁺ CAFs (Supplementary Fig. S7B)
Figure 6.
Expression of immunoregulatory molecules by tumor stromal cells. A–D, Mean expression values from independent replicates of CAFs, FRCs, skin fibroblasts, and CAPs for lymphoid chemokines (A), CXCR3 ligands (B), cytokines (C), and Nos2 (D). Dotted line indicates the cutoff for expression (EV = 120).
E, Cryopreserved 4T1 tumors were sectioned and stained with Dapi and with antibodies against iNOS and PDPN. N = 2 independent experiments.
F, Histogram indicating the expression of iNOS in freshly isolated PDPN⁺ CAFs (red) and PDPN⁻/C0 CAPs (green), compared with isotype control (gray). N = 2 independent experiments.
G, NO levels in supernatants of splenocytes alone (negative control) and PDPN⁺ CAFs or PDPN⁻ CAPs cultured with activated splenocytes. N = 5 independent experiments.
were cultured together with human PBMCs in the presence of anti-CD3, and T-cell proliferation was subsequently measured. Consistent with mouse CAFs, the proliferation of human CD4⁺ and CD8⁺ T cells was almost entirely abrogated in the presence of human PDPN⁺ CAFs (Fig. 7C; Supplementary Fig. S7C), suggesting a common immunosuppressive capability of PDPN⁺ CAFs across species.

To assess whether fibroblast-mediated immunosuppression was mediated by the release of NO from PDPN⁺ CAFs, we blocked NO production using L-NMMA, a cell-permeable inhibitor of NO synthase. Consistent with our hypothesis, addition of L-NMMA was sufficient to abate NO production by PDPN⁺ CAFs and rescue proliferation of T cells (Fig. 7D and E), pointing to a role for NO in restraining the expansion of activated T cells in vitro. L-NMMA had no effect on TGFβ expression observed in CAFs, TGFβ neutralization in the splenocyte-CAF coculture did not affect the suppressive potential of CAFs.

Figure 7. PDPN⁺ CAFs suppress the proliferation of activated T cells through the production of NO. A, CFSE profiles of CD4⁺ or CD8⁺ T cells from cultures of nonactivated splenocytes (Nonact spl) and splenocytes activated for 72 hours with anti-CD3/CD28 in the absence (Act spl) or presence of PDPN⁺ CAFs (Act spl + CAFs). Numbers indicate the percentage of divided cells. N = 5 independent experiments. B, Expression of CD25, CD69, and CD62L on T cells cultured as in A for 24 hours. Numbers indicate MFI. C, Proliferation profile of crystal violet-labeled CD8⁺ T cells from human PBMCs that were not activated (Nonact PBMCs), activated alone (Act PBMCs) or activated in the presence of human CAFs (Act PBMCs + huCAFs). Numbers indicate the percentage of divided cells. N = 4 independent experiments. D, NO levels produced by activated splenocytes (Act spl) cultured with L-NMMA (Act spl + L-NMMA) or with CAFs (Act spl + CAFs, Act spl + CAFs + L-NMMA). N = 3 independent experiments. E, CFSE profiles of CD4⁺ or CD8⁺ T cells from cultures of nonactivated splenocytes (Nonact spl) and splenocytes activated for 72 hours in the absence (Act spl) or presence of PDPN⁺ CAFs (Act spl + CAFs) or PDPN⁺/C0 CAFs (Act spl + CAPs) from the same 4T1 tumors. Numbers indicate the percentage of divided cells. N = 4 independent experiments.
Collectively, these data highlight the phenotypic, functional, and spatial heterogeneity in FAP+ tumor stromal cells, identifying two distinct subsets with characteristics of fibroblasts and pericytes (Supplementary Fig. S8). Additionally, this study suggests that the NO axis may represent one of the mechanisms governing CAF-mediated T-cell suppression in the tumor microenvironment.

Discussion
Stromal cells within the tumor microenvironment can promote cancer growth, progression, chemoresistance, and even immunosuppression (5, 45, 46); however, the precise composition and function of tumor stroma remain unclear. In this study, we identified two discrete populations of FAP+ mesenchymal cells in mouse and human breast tumors. FAP was expressed on the surface of CAFs and CAPs, whereas PDPN expression was restricted to CAPs. The breast tumor CAFs shared some functional attributes with LN FRCs, including production of reticular fibers supporting stromal–immune cell interactions and inhibition of T-cell proliferation through NO production. In contrast, CAPs did not produce NO and had no effect on T-cell proliferation. Although these results suggest that CAPs are not immunosuppressive, we cannot exclude that under certain conditions, they may influence the function of other immune cells in the tumor microenvironment.

Our study highlighted some resemblance between CAFs and FRCs, including expression of FAP and PDPN, and myofibroblast-like characteristics. In LNs, FRCs construct a microarchitecture that facilitates interactions between immune cells, conduit function, vascular support, and the initiation of immune responses (23). Analysis of the CAF-associated matrix architecture across multiple mouse tumors illuminated a dense reticular framework, with prominent staining for ERTR7 and various collagens. Notably, tumor-infiltrating immune cells, particularly CD8+ T cells, were more closely associated with CAFs and their matrix fibers than with the epithelial compartment of the tumor. Why CD8 T cells accumulate in the CAF-rich peritumoral compartment rather than in the tumor bed remains unclear. It has been suggested that “immune-exclusion” derives from a matrix-based physical barrier limiting T-cell infiltration of the tumor bed (39, 47, 48). However, chemotactic gradients and matrix scaffolding may also account for this phenotype. Indeed, both CAFs and CAPs produced several chemokines, including Ccl9, Ccl10, and Ccl11, which could attract CXCR3+ T cells entering the tumor bed. Beyond organizing the peritumoral localization of immune cells, mouse and human CAFs exhibited immunosuppressive potential by inhibiting the proliferation of activated T cells. Bone marrow stroma and synovial fibroblasts possess immunosuppressive ability, and prostaglandin E2, TGFβ, and IL10 have been linked to this function (49–52). However, our study suggests that NO underlies the inhibitory effect of PDPN+ CAFs in mouse breast tumors.

Mechanistically, NO may alter T-cell function through protein modifications, via either s-nitrosylation or peroxynitrite activity (53). In the tumor microenvironment, NO produced by myeloid cells has been associated with formation of peroxynitrite, leading to nitration of the T-cell receptor and desensitization (54). NO released by PDPM+ CAFs may have similar repercussions in neighboring T cells. Additionally, nitration of chemokines, particularly CCL2, has been associated with trapping of tumor-specific T cells in peritumoral stroma. Thus, NO production by CAFs may also hinder T-cell infiltration through NO production (55). The capacity of CAFs to produce NO and suppress T-cell proliferation points to some resemblance between PDPM+ CAFs and FRCs; indeed, during inflammatory conditions, FRCs acquire immunosuppressive potential, dampening the expansion of the activated T-cell pool (42–44).

Despite these similarities, FRCs exhibited unique features compared with CAFs, such as expression of chemokines and growth factors that guide and nurture naïve lymphocytes, including CCR7 ligands and II7. In LNs, the drainage of tumor-derived material is sufficient to reprogram FRCs and downregulate these factors (56). Many of the transcriptional changes that occur in FRCs in tumordraining LNs were reflected in our analysis of PDPN+ CAFs. For example, we detected high expression of genes associated with fibroblast activation, such as Acta2, Thyl, and Pdpn, together with greater fibrotic potential, whereas Ccl19, Ccl21, Tnfα3b, and II7 were not expressed. Consistent with previous studies, our data support the idea that the tumor milieu programs the function of tissue-resident fibroblasts, converting at least some local fibroblasts into CAFs (57, 58).

Going forward, genetic and pharmacologic efforts to manipulate tumor stromal cells using FAP-based approaches will need to be carefully considered given that this molecule can be expressed by at least two mesenchymal cell populations with dissimilar functions. Given that pericytes have been associated with a variety of functions in the tumor microenvironment (59–64), the influence of CAPs on tumor progression and antitumor immunity remains to be determined. High dimensional analysis of stromal heterogeneity across different cancer types and treatments will be crucial for understanding the diverse functions played by cell populations within this compartment and how to target them for new therapeutic approaches.

Disclosure of Potential Conflicts of Interest
J. L. Astarta is a senior scientist at Spotlight Therapeutics. G. Dranoff is Global Head, Exploratory Immuno-oncology at Novartis. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
This study was supported by the NIH [5R01 DK074500-08, 2P01AI045757-15, and R21 CA182598-01 to S.J. Turley, R01 AI039246, P01 AI078979, and R37 AI054636 to M.C. Carroll, and T32 CA 070083-15 to V. Cremasco], the Barr Foundation (S.J. Turley), the American Cancer Society (S.J. Turley), the Cancer Research Institute (V. Cremasco), and the Science Foundation (S.J. Turley).

We thank Dr. Michael Brenner for the Cadherin-11 antibody and Dr. Jun Sonoda for critical reading of the manuscript.

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Received February 17, 2018; revised July 11, 2018; accepted September 17, 2018; published first September 28, 2018.

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