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B lymphocytes contribute to stromal reaction in pancreatic ductal adenocarcinoma

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a prominent stromal reaction that has been variably implicated in both tumor growth and tumor suppression. B-lymphocytes have been recently implicated in PDAC progression but their contribution to the characteristic stromal desmoplasia has never been assessed before. In the present work, we aimed to verify whether B-lymphocytes contribute to stromal cell activation in PDAC. $CD19^+$ B-lymphocytes purified from peripheral blood of patients with PDAC were cultivated in the presence of human pancreatic fibroblasts and cancer-associated fibroblasts. Released pro-fibrotic soluble factors and collagen production were assessed by ELISA and Luminex assays. Quantitative RT-PCR was used to assess fibroblast activation in the presence of B cells. The expression of selected pro-fibrotic and inflammatory molecules was confirmed on PDAC tissue sections by multi-color immunofluorescence studies. We herein demonstrate that B-cells from PDAC patients (i) produce the pro-fibrotic molecule PDGF-B and stimulate collagen production by fibroblasts; (ii) express enzymes implicated in extracellular matrix remodeling including LOXL2; and (iii) produce the chemotactic factors CCL-4, CCL-5, and CCL-11. In addition we demonstrate that circulating plasmablasts are expanded in the peripheral blood of patients with PDAC, stimulate collagen production by fibroblasts, and infiltrate pancreatic lesions. Our results indicate that PDAC is characterized by perturbations of the B-cell compartment with expansion of B-lymphocyte subsets that directly contribute to the stromal reaction observed at disease site. These findings provide an additional rationale for modulating B-cell activity in patients with pancreatic cancer.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid tumors and the fourth leading cause of cancer-related mortality worldwide.[1] Escape from immune-surveillance, resistance to standard chemotherapies and early metastatic potential are considered the main reasons for this dismal prognosis .[1]

In apparent contrast with this aggressiveness, the majority of the tumor volume in PDAC is not made of malignant cells, but of a desmoplastic reaction consisting of cancer-associated fibroblasts (CAF) and immune cells .[2,3] Pancreatic CAF are believed to originate from different cellular sources, including pancreatic stellate cells, mesenchymal stem cells (MSC) resident fibroblasts, and epithelial cells .[4,5] Although CAF have been classically associated with tumor growth, immune suppression, and metastatic dissemination, recent evidences have challenged these tumor-promoting properties and showed more aggressive PDAC behavior in CAF deprived mouse models.[2–8] These findings suggest a complex network of signals between PDAC and CAF that is not uniformly stimulatory or inhibitory, and possibly support the existence of different tumor-promoting and tumor-suppressing populations of CAF. Indeed, a previously unappreciated heterogeneity in PDAC fibroblasts has been recently observed, with tumor-promoting subsets of CAF expressing variable combinations of activation and mesenchymal stem cell markers such as the secreted protein acidic and rich in cysteine (*SPARC*), the encoding fibroblast activation protein (*FAP), COL1A1, COL1A2*, and *COL3A1* collagen genes, CD73, and CD90.[9,10] In the rapidly evolving field of PDAC, understanding of the molecular mechanisms driving CAF differentiation and activation could lead to the identification of novel mechanistic insights as well as to innovative therapeutic targets.

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Based on our recent findings about B cells promoting tissue fibrosis in IgG4-related type-1 autoimmune pancreatitis (AIP), we here hypothesize that B lymphocytes might contribute to the prominent stromal reaction observed in PDAC by promoting CAF activation, collagen secretion, and extracellular matrix (ECM) remodeling.[11,12] This previously overlooked hypothesis stems from multiple clinical and pathological analogies existing between type-1 AIP and PDAC. Both conditions, in fact, present with tumefactive lesions of the pancreatic gland, and display common histological features that typically complicate the differential diagnosis, such as a dense stromal reaction and an abundant lymphoplasmocytic infiltrate rich in IgG4+ plasma cells .[13–17]

Results

B lymphocytes from patients with pancreatic adenocarcinoma secrete soluble factors that stimulate collagen production by pancreatic MSC and CAF

To demonstrate pro-fibrotic properties of B lymphocytes in PDAC, we set up co-cultures of human pancreatic fibroblasts with $CD19^{+}$ B cells isolated from the peripheral blood of five patients with PDAC, and performed transcriptomic studies of cultured fibroblasts. In particular, human pancreatic MSC and B cells from PDAC patients were first used to model *in vitro* some of the possible interactions occurring between these two cell types *in vivo*. B cells purified from the peripheral blood of five sex- and age-matched healthy donors were used as controls. Up-regulation of selected genes was assessed, based on our previous findings showing activation of *epithelial-to-mesenchymal transition* trascriptomic pathways in fibroblast co-cultured with B cells from patients with IgG4-related AIP.[12] As shown in Figure 1a, expression of *COL1A1, COL1A2*, and *COL3A1* collagen genes, and α-Smooth Muscle Actin (*ACTA2*) in pancreatic MSC was significantly up-regulated in the presence of B cells. Accordingly, collagen production in the co-cultures of MSC with B cells from PDAC patients was significantly increased compared to MSC cultured alone (Figure 1b). The expression of *COL1A1, COL1A2*, and *COL3A1* genes, but not of *ACTA2*, was also significantly increased in fibroblasts co-cultured with B cells from healthy donors (Figure 1).

To assess whether contact between B cells and fibroblasts is required for collagen production, co-culture experiments were repeated using a semipermeable membrane to separate these two cell types. As shown in Figure 1, co-culture with and without separation using semipermeable membranes (transwells) both led to a significant increase in collagen secretion compared to the incubation of fibroblasts without B cells.

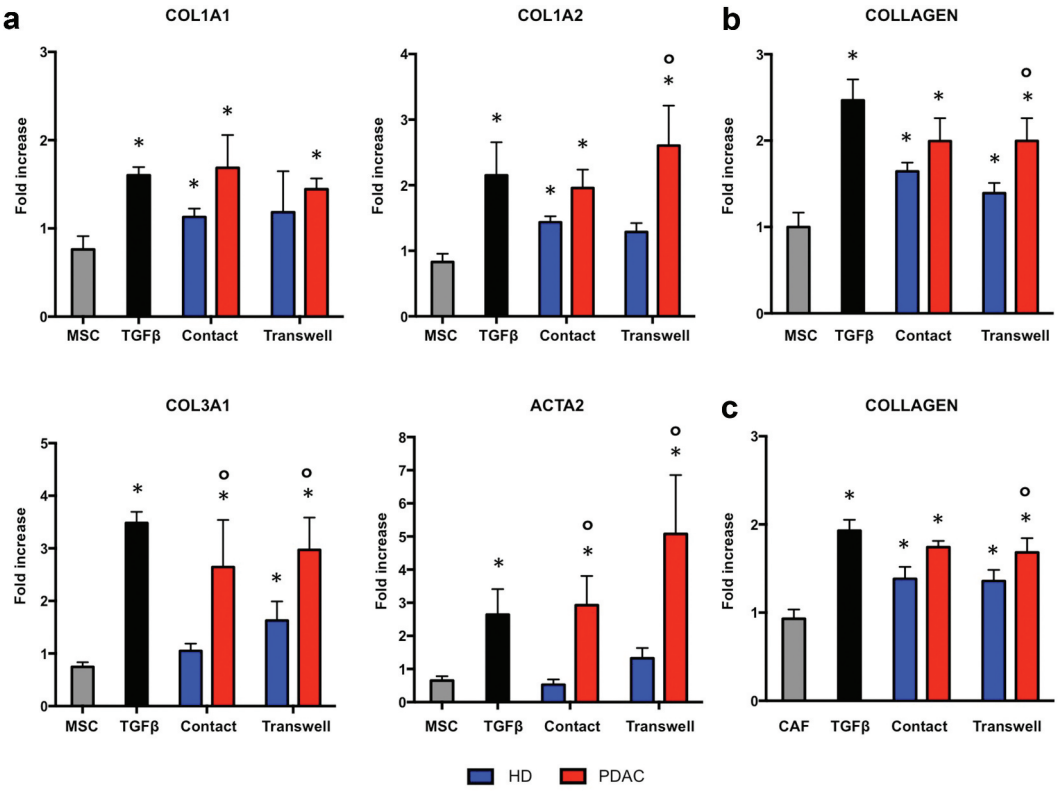

Figure 1. B lymphocytes from patients with PDAC secrete soluble factors that induce collagen production by human pancreatic fibroblasts. (a) Quantitative PCR showing fold change expression of COL1A1, COL1A2, COL3A1, and ACTA2 genes in pancreatic fibroblasts after 3 days co-culture with B lymphocytes from patients with PDAC and from healthy donors. (b) Fold change in collagen concentration in the supernatant of different experimental conditions using MSC. (c) Fold change in collagen concentration in the supernatant of different experimental conditions using CAF co-cultured in contact with B cells from healthy individuals or PDAC patients. Abbreviations: human pancreatic mesenchymal stem cells (MSC); human cancer-associated fibroblasts (CAF); FBL treated with TGFβ1 20 ng/ml (TGFβ); FBL co-cultured with B cells from healthy controls (blue bars); FBL co-cultured with B cells from patients with PDAC (red bars) in the presence (Transwell) or absence (Contact) of semipermeable membranes. * = $p < .05$ for the comparison with fibroblasts alone; ° = $p < .05$ for the comparison with co-cultures in the presence of B cells from healthy controls. Results are expressed as mean ± SD of five experiments performed in replicates. Comparisons between pairs of conditions were performed using Mann-Whitney U-test.

Collagen production in the co-cultures of fibroblasts with B cells from PDAC patients was significantly higher compared to co-cultures of fibroblasts with B cells from healthy donors. Similar results were obtained using CAF isolated from 5 PDAC patients co-cultured with B cells from healthy individuals and from patients with PDAC, suggesting that phenotypic differences between MSC and CAF (reported in Supplementary Figure 1) were not responsible for the different reactions observed with B-cells from healthy individuals and PDAC patients (Figure 1c). Taken together, these findings demonstrate that $CD19^{+}$ B lymphocytes from patients with PDAC and, to lesser extent, from healthy individuals induce collagen production by pancreatic MSC and CAF through the secretion of soluble factors.

Platelet-derived growth factor-B is increased in the co-cultures of human fibroblasts with B lymphocytes from patients with pancreatic adenocarcinoma

To identify pro-fibrotic soluble mediators secreted by B lymphocytes in the co-culture system, we quantitated the level of cytokines and chemokines implicated in tissue fibrosis using a Luminex-based approach. As shown in Figure 2a, the concentrations of platelet-derived growth factor-B (PDGF-B) was significantly higher when MSC were challenged with B cells compared to MSC alone. The effect of the co-incubation was specific, since the concentration of other pro-fibrotic molecules such as IL-1α, IL-4, IL-5, IL-10, IL-13, IFNγ, TNFα, and TGFβ did not differ between MSC alone and MSC co-cultured with B cells from either PDAC patients or healthy donors. The chemokines CCL-4 (MIP1β), CCL-5 (RANTES), and CCL-11 (EOTAXIN) were also significantly increased in the presence of B cells from PDAC patients and from healthy subjects. In particular, CCL-4 and CCL-5 production was induced by the presence of B lymphocytes while low levels of PDGF-B and CCL-11 were constitutively secreted by untreated MSC. The levels of PDGF-B, CCL-4, CCL-5, and CCL-11 were similar in co-cultures of MSC with B cells from PDAC patients and from healthy donors, and in co-cultures in which B cells and MSC were separated or not by semipermeable membranes (Figure 2b).

These results implicate PDGF-B as a mediator of the fibrotic action exerted by B lymphocytes but do not clarify whether PDGF-B is primarily secreted by B cells, by fibroblasts upon B-cell mediated activation, or by both cells. These results also suggest that MSC stimulated by B cells or perhaps B cells themselves, secrete chemokines that might contribute to the recruitment of inflammatory cells *in vivo*.

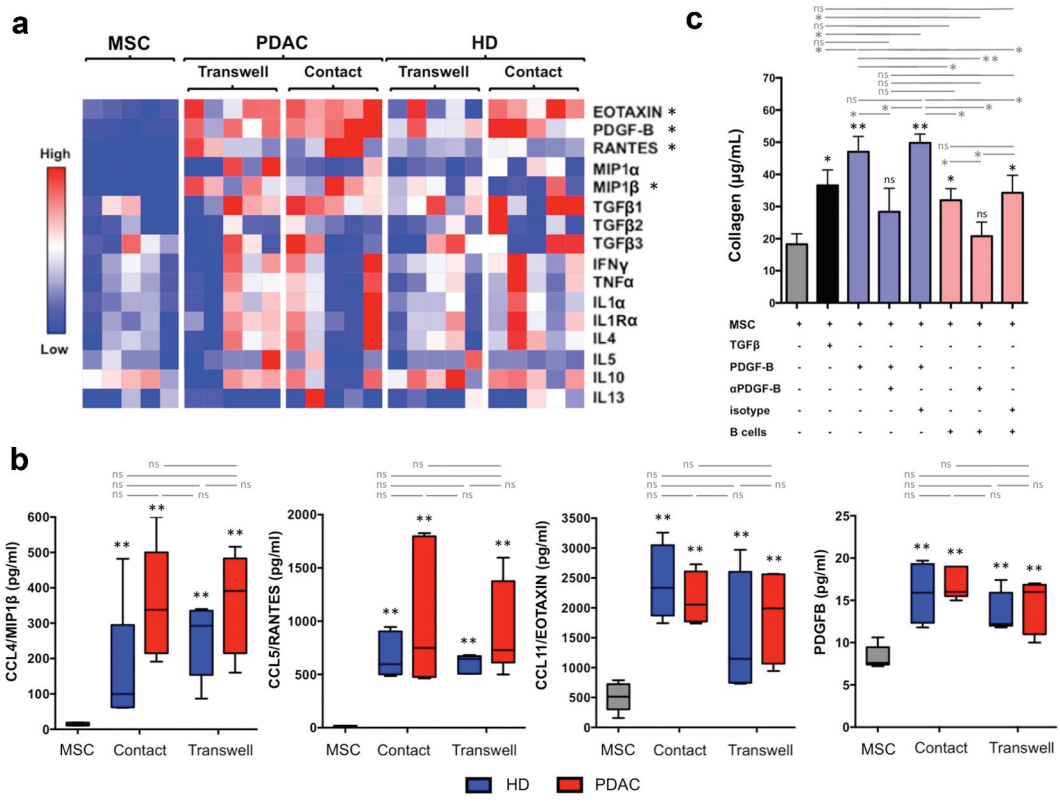

Figure 2. PDGF-B, CCL-4, CCL-5, and CCL-11 are increased in the co-cultures of pancreatic MSC with B lymphocytes. (a) Quantification of different cytokines and chemokines by Luminex analysis after 3 days of culture. (b) CCL-4, CCL-5, CCL-11, and PDGF-B secretion is increased in the supernatant of the co-cultures compared to control pancreatic MSC. (c) PDGF-B inhibition decreases collagen production by pancreatic MSC induced by B-cell from PDAC patients. Abbreviations: human pancreatic mesenchymal stem cells (MSC); MSC stimulated with 20 ng/ml of recombinant human TGF-β1 (TGFβ); MSC co-cultured with B cells from healthy controls (n = 5) (blue bars); MSC co-cultured with B cells from patients with PDAC (n = 5) (red bars), in the presence (Transwell) or absence (Contact) of semipermeable membranes. MSC stimulated with 10 ng/ml of recombinant human PDGF-B (PDGF-B); MSC incubated with 0.5 µg/ml of anti-PDGF-B antibody two hours prior to stimulation with 10 ng/ml of recombinant human PDGF-B (αPDGF-B); MSC incubated with 0.5 µg/ml of IgG isotype antibody two hours prior to stimulation with 10 ng/ml of recombinant human PDGF-B (Isotype); co-cultures with B cells from patients with PDAC (B cells). Results are expressed as mean ± SD of five experiments performed in replicates. ns = $p > .05$; * = $p < .05$; ** = $p < .01$. Black asterisks and "ns" refer to the comparison of each condition with MSC alone. Grey asterisks and "ns" refer to pairwise comparison between other conditions. Pairwise comparison between conditions was performed using Mann-Whitney U-test.

PDGF-B mediates B-lymphocyte dependent collagen production by human fibroblasts

In order to assess the biological relevance of PDGF-B in our model, we used an anti-PDGF-B antibody or an isotype control to interfere with collagen production. As shown in Figure 2c, the addition of anti-PDGF-B antibody, but not of irrelevant IgG of the same isotype, significantly reduced collagen secretion induced by B cells from patients with PDAC. Indeed, B lymphocytes might activate MSC either through the production of PDGF-B or by inducing the secretion of PDGF-B by activated fibroblasts, or by both mechanisms, thus perpetuating a paracrine/autocrine pro-fibrotic loop. Of note, B cells from PDAC patients induced significantly higher amounts of collagen in co-culture with pancreatic MSC compared to B cells from healthy individuals even if levels of PDGF-B in the supernatant were similar in the two conditions (Figure 1). Thus factors other than PDGF-B might also contribute to the fibrotic effect of B cells from PDAC patients.

B lymphocytes and fibroblasts infiltrating pancreatic adenocarcinoma express PDGF-B, CCL-4, CCL-5, and CCL-11

To determine whether B cells or pancreatic fibroblasts represent the cellular source of PDGF-B, CCL-4, CCL-5, and CCL-11 in our co-culture system and to confirm the relevance of these molecules to the characteristic stromal reaction observed in PDAC, we performed multicolor immunofluorescence and quantitative analysis on tissue sections of 5 PDAC patients. Infiltrating $CD19^+$ B cell was either organized in tertiary lymphoid structures or spread throughout the tissue (Figure 3a,b). Quantification analysis showed that $CD19^+$ B lymphocytes expressing either PDGF-B, CCL-4, CCL-5, or CCL-11 represented on average 20.9% (range 10.2–21.0), 12.2% (range 9.2–15.4), 9.2% (range 5.4–16.4), and 24.1% (range 19.1–31.6), of total PDGF-B, CCL-4, CCL-5, CCL-11 expressing cells, respectively (Figure 3c,d). α-SMA^+ fibroblasts expressing either PDGF-B, CCL-4, CCL-5, or CCL-11 represented on average 4.9% (range 4.3–9.5), 5.0% (range 3.4–7.1), 3.1% (range 0.9–5.1), and 57.9% (range 47.5–62.4) of total PDGF-B, CCL-4, CCL-5, and CCL-11 expressing cells, respectively (Figure 3c,d). These findings indicate that the pro-fibrotic molecule PDGF-B

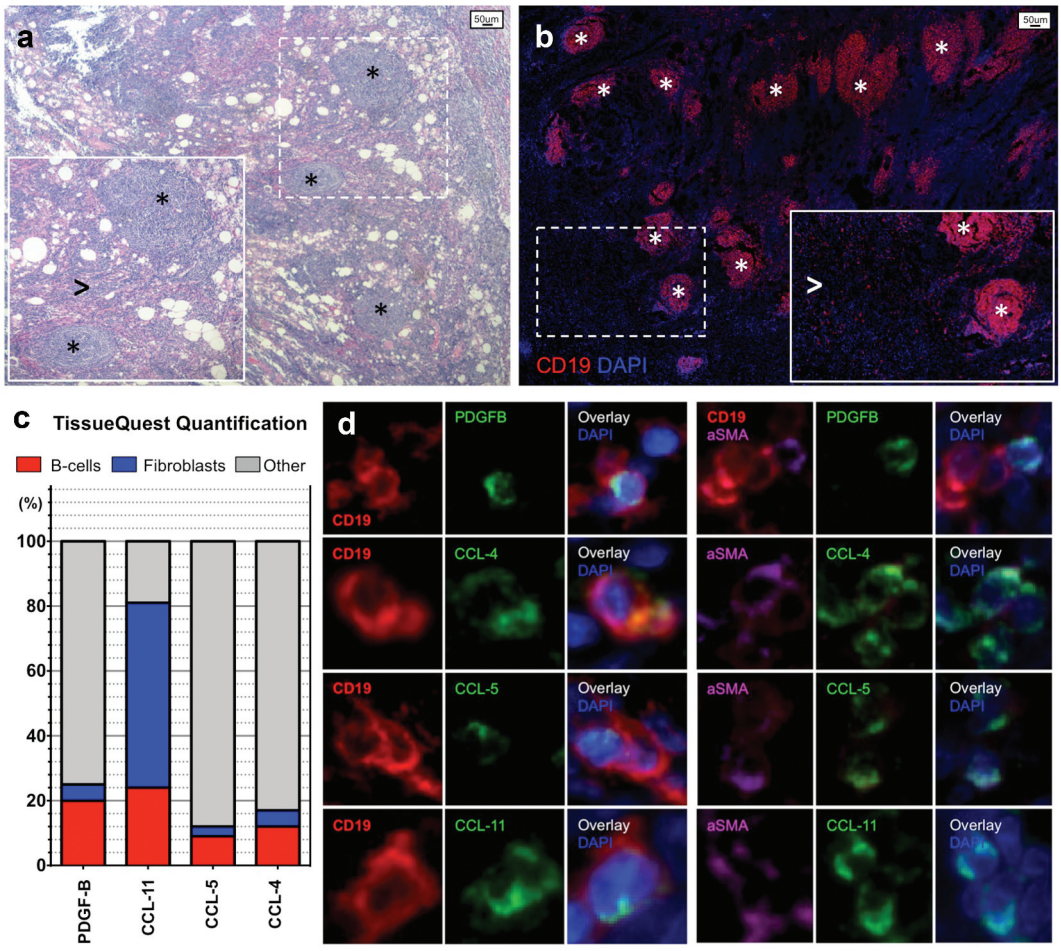

Figure 3. B-lymphocytes and fibroblasts infiltrating pancreatic adenocarcinoma express PDGF-B, CCL-4, CCL-5, and CCL-11. (a) Hematoxylin/Eosin stain and (b) immunofluorescence staining of a representative tissue section of PDAC showing an abundant infiltrate of B lymphocytes organized as either multiple lymphoid structures (asterisks) or spread within the neoplastic lesion and tumor stroma (arrowheads). Inserts (high magnification); CD19+ cells (red); DAPI stain (blue). (c) Quantification of double-positive cells for CD19+ (red bars) or αSMA+ (blue bars) and PDGF-B, CCL-11, CCL-5, or CCL-4 in PDAC expressed in percentage of total PDGF-B, CCL-11, CCL-5, or CCL-4 positive cells (i.e. CD19+ PDGF-B+ cells/PDGF-B+ cells). Bar length represents the average quantification of n = 5 tissue slides. (D) Immunofluorescence staining of CD19 + B cells (red) and αSMA+ fibroblasts for CCL-4, CCL-5, CCL-11, and PDGF-B expression (all green) in representative tissue samples from patients with PDAC. Cell nuclei have been stained with DAPI stain (blue).

and the inflammatory chemokines CCL-4, CCL-5, and CCL-11 are produced by both B lymphocytes and myofibroblasts infiltrating PDAC lesions *in vivo*. In particular, while B-lymphocytes seem capable of expressing relevant amounts of all four cytokine and chemokines *in vivo*, fibroblasts primarily express large amounts of CCL-11. These results also suggest that B lymphocytes represent a source of PDGF-B, CCL-4, CCL-5, and CCL-11 in the co-culture studies described above, and mirror the relatively minor contribution of fibroblasts to the secretion of CCL-4 and CCL-5 (Figure 2b). Indeed, transcriptomic analysis performed on circulating B-cells from PDAC patients and on pancreatic MSC from the co-cultures revealed that B-lymphocytes constitutively express PDGF-B and that they significantly up-regulate PDGF-B expression by co-cultured fibroblasts compared to fibroblasts alone (Supplementary Figure 2).

Circulating plasmablasts are expanded in patients with pancreatic adenocarcinoma and induce collagen production by human fibroblasts

As shown in Figure 1, B lymphocytes from both PDAC patients and healthy subjects were found to increase collagen production in co-cultures with pancreatic MSC but collagen concentration was higher in the presence of B cells from PDAC patients than from healthy subjects. Similar results were obtained by using B cells from patients with IgG4-RD AIP and were attributed to previously unanticipated pro-fibrotic properties of circulating plasmablasts.[12] Indeed, plasmablasts, the precursors of tissue resident plasma cells, are oligoclonally expanded in the blood of patients with IgG4-RD AIP and activate fibroblasts in co-culture experiments.[12,18-20] We therefore hypothesized the existence of fibrogenic B-lymphocyte subpopulations in PDAC as well and compared the levels of circulating plasmablasts, naive and memory B cells in 20 patients with resectable pancreatic cancer (mean age 66; range 56–75; male to female ratio = 12:8) and 20 age- and sex-matched healthy controls by flow cytometry. As shown in Figure 4a,b, circulating plasmablasts, but not naïve and memory B cells, were significantly higher in patients with PDAC than in healthy individuals both as absolute numbers (Figure 4b) and as percentage of total $CD19^+$ cells (Supplementary Figure 3a).

To assess the pro-fibrotic properties of circulating plasmablasts in the setting of PDAC, we set up co-cultures of pancreatic MSC with naïve B cells, memory B cells, and plasmablasts from PDAC patients isolated by sorting via flow-cytometry. Addition of circulating plasmablasts resulted in the production of significantly more collagen compared to that produced constitutively by MSC alone, or by fibroblasts challenged with naïve or with memory B cells (Figure 4c).

LOXL2 is expressed by plasmablasts/plasma cells in pancreatic adenocarcinoma

As we previously reported, plasmablasts represent a subset of B lymphocytes with intrinsic fibrotic properties because, in contrast to naïve and memory B cells, in addition to PDGF-B, they express a set of genes implicated in *positive regulation of fibroblast proliferation* such as *COL1A1* and *COL1A2* collagen genes, insulin-like growth factor-1 (*IGF-1*), lysyl oxidase homolog 2 (*LOXL2*), and gremlin-1 (*GREM-1*).[8] In particular, LOXL2, a member of the lysyl oxidases family, controls ECM stiffness by crosslinking collagen fibers and supports the pathologic stromal microenvironment in various solid tumors and fibrotic diseases.[21–29] To demonstrate LOXL2 expression by B-lymphocytes infiltrating PDAC we performed multicolor immunofluorescence studies and found that $CD19^+$ cells accounted for up to 12.6% of total LOXL2 expressing cells in PDAC (average 8.9%; range 7.3%–12.6%) (Supplementary Figure 3b). LOXL2 expression by class-switched plasmablasts or plasma cells was further confirmed by additional immunofluorescence for CD19 and IgG4 double-positive cells, a subset of B lymphocytes that has been reported in PDAC biopsies[9] (Figure 4d). Of note, plasmablasts from PDAC patients also expressed PDGF-B on RNA-seq analysis, suggesting multiple intrinsic pro-fibrotic properties of this antigen experienced class-switched B-cell subpopulation (Supplementary Figure 1b)

Discussion

The disproportion between stromal elements and the less prevalent neoplastic epithelial cells represents a hallmark pathological feature of PDAC but the contribution of this characteristic desmoplastic reaction to cancer progression remains lively debated.[2] While stromal cells have been classically thought to favor tumor growth and dissemination, recent evidence have shown that CAF can also hamper PDAC development by restraining angiogenetic phenomena[2-8,30–33] In addition, CAF seem to exert diverse effects on pancreatic cancer progression – either inhibitory or stimulatory – depending on their relative proportion to neoplastic cells.[34] Accordingly, although selective blockade of signals associated with CAF activation improves survival in animal models, interference with these signals has not proven as effective when translated into clinical trials on pancreatic and non-pancreatic cancers.[2,7,30] All together, these findings indicate that, besides being tightly integrated and probably redundant, tumor-stroma interactions are not uniformly suppressive or supportive. Understanding the biology of the stromal compartment in PDAC is, therefore, key to intercept relevant pathways implicated in tumor spreading and immune-surveillance.

In the present work we describe for the first time that B lymphocytes actively contribute to fibrogenesis in PDAC and introduce a novel player involved in CAF differentiation and activation. In addition, we demonstrate that circulating plasmablasts with fibrogenic properties are expanded in the peripheral blood of patients with PDAC and in neoplastic lesions, reflecting unexpected perturbations of the B-cell compartment in pancreatic cancer. Indeed, B lymphocytes have been already shown to infiltrate neoplastic lesions and pre-malignant pancreatic intraepithelial neoplasia (PanIN), but the role of tumor-infiltrating B cells (TIL-Bs) in PDAC did not receive much attention until recent .[35–38] B cells infiltrating PDAC, in fact, have been traditionally assumed to bear a regulatory phenotype and to support malignant cells growth by hampering antitumor immunity in analogy with other solid tumors.[35–39] Compelling evidence for the involvement of TIL-Bs in pancreatic tumorigenesis came only recently, when B-cells were shown to contribute to PDAC progression through diverse mechanisms,

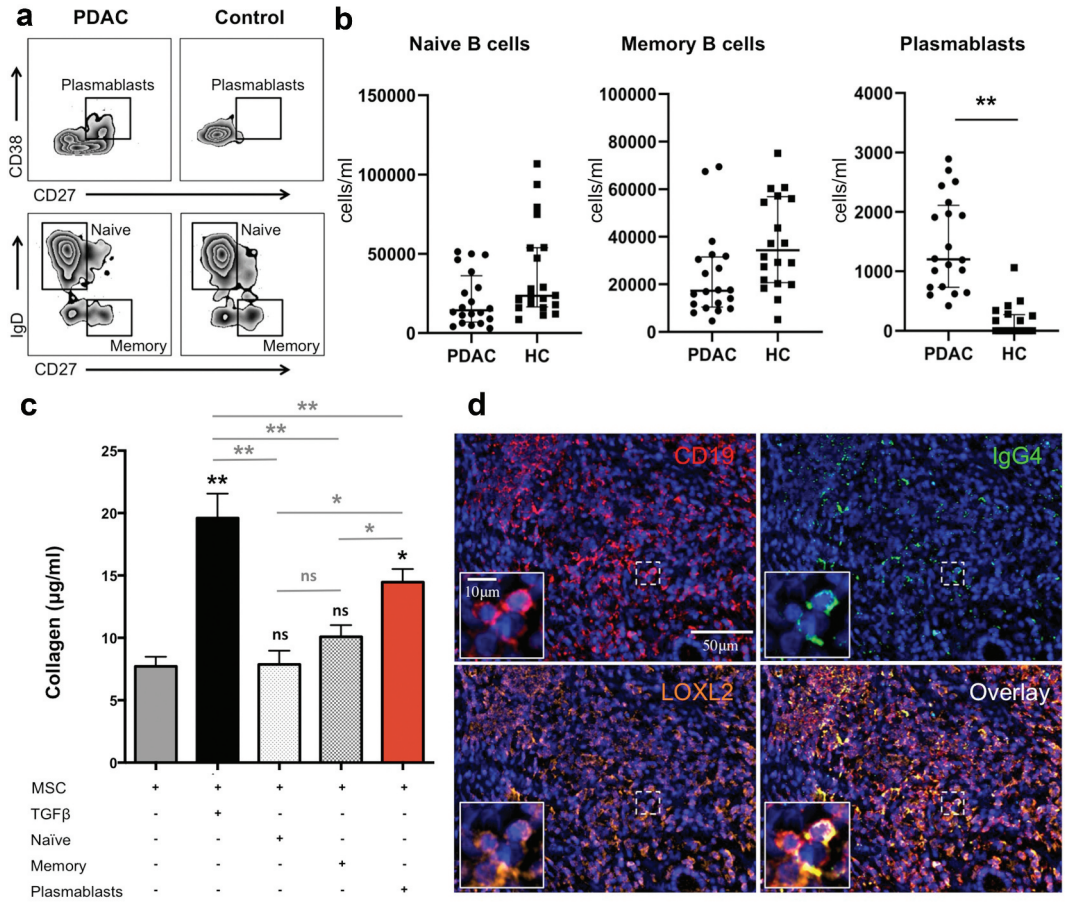

Figure 4. Pro-fibrotic role of plasmablasts/plasma cells in pancreatic adenocarcinoma. **(a)** Flow cytometric gating strategy used to identify circulating CD27+ CD38hi plasmablasts among CD19+ CD20- cells, IgD+CD27- naïve and IgD-CD27+ memory B cells among CD19+ CD20+ cells. (**b**) Absolute numbers of naive, memory B cells, and plasmablasts in peripheral blood of patients with PDAC (n = 20) and healthy control subjects (n = 20) (mean ± SD; ** = $p < .01$; comparison between groups was performed using Unpaired T-test). (**c**) Collagen production is increased in co-cultures with plasmablasts from patients with PDAC compared to control fibroblasts. **Abbreviations**: human pancreatic mesenchymal stem cells (MSC); MSC stimulated with 20 ng/ml of recombinant human TGF-β1 (TGFβ); MSC co-cultured with naive B cells, memory B cells, or circulating plasmablasts. Results are presented as mean ± SD of five independent experiments performed in replicates. ns = $p > .05$; * = $p < .05$; ** = $p < .01$. Black asterisks and "ns" refer to the comparison of each condition with MSC alone. Grey asterisks and "ns" refer to the pairwise comparison between other conditions. Pairwise comparison between conditions was performed using Mann-Whitney U-test. (**D**) LOXL2 expressing IgG4$^+$ B lymphocytes in a representative case of PDAC (inserts: high magnification).

including paracrine secretion of IL-35 – a proliferative stimulus for transformed epithelial cells – and suppression of cytotoxic T-cells. Accordingly, prevention of B-cell infiltration into the tumor by blocking the B-cell chemo attractant CXCL13, by inhibiting B-cell activity using a Bruton tyrosine kinase inhibitor, or by simple depletion of B cells, all reduced tumor progression in preclinical models of PDAC.[40–42]

By demonstrating a direct interaction with pancreatic MSC and CAF, our results suggest an additional and previously overlooked contribution of B lymphocytes to PDAC pathophysiology *via* direct orchestration of stromal activation. In particular, B lymphocytes (i) induced epithelial-to-mesenchymal transition of myofibroblast precursors; (ii) stimulated collagen production by pancreatic fibroblasts through PDGF-B; (iii) regulated extracellular matrix stiffness through specialized enzymes such as LOXL2; and (iv) secreted chemotactic factors for putative fibrogenic immune-cells (Figure 5). Of note, production of both PDGF-B and LOXL2 by malignant cells has been already demonstrated in PDAC lesions, while secretion of these molecules by TIL-Bs has never been observed before.[21–26,43,44] PDGF-B is a member of the PDGF family and contributes to tissue fibrosis by stimulating fibroblast proliferation and collagen production through its tyrosine kinases receptor.[45] Depending on its expression level, PDGF-B has been also found to regulate neoplastic cell proliferation, vascular sprouting, and metastatic potential in a variety of solid tumors.[43,44] LOXL2 is an enzyme that catalyzes the cross-linking of collagen components in the ECM, a process that controls the structure of the ECM, the tensile strength of collagen bundles, and the differentiation of quiescent fibroblasts into activated myofibroblasts.[24–29,46] LOXL2 has been also implicated in carcinogenesis and invasiveness of pancreatic cancer cells, and its inhibition has been shown to reduce tumor volume and metastases in preclinical studies.[21,22] In addition, increased LOX2 activity has been shown to promote resistance to gemcitabine, and its expression in PDAC biopsies has been associated with a poorer prognosis.[22] B cells also secreted CCL-4, CCL-5, and CCL-11, chemotactic factors for cells with fibrogenic properties that are typically found in PDAC lesions, such as T lymphocytes, macrophages, and eosinophils.[47–50] CCL-4 is a chemokine involved in monocyte recruitment and activation by engaging the chemokine receptor CCR5;[51] CCL-5 attracts T cells, eosinophils, and basophils, by interacting with CCR1, CCR3, and CCR5 ;[52] and CCL-11

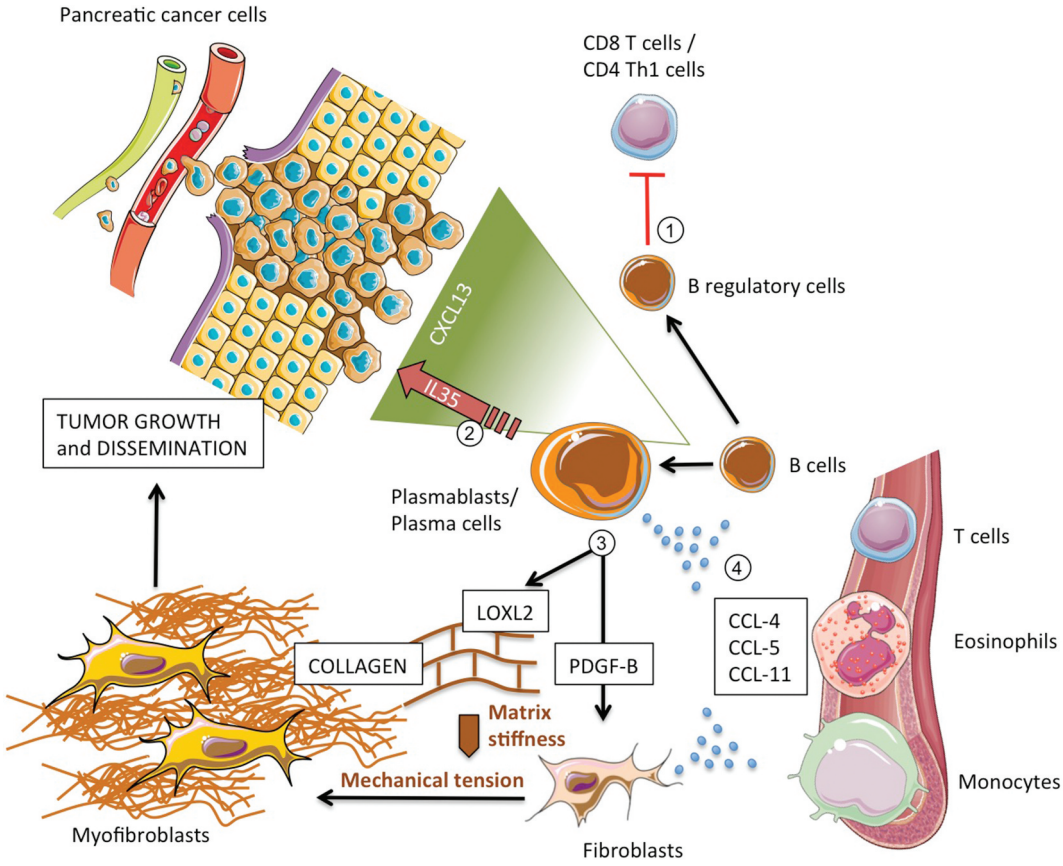

Figure 5. Contribution of B lymphocytes to pancreatic adenocarcinoma growth and dissemination. B cells infiltrate PDAC in response to the release of local chemokines, such as CXCL13, and contribute to tumorigenesis in different direct and indirect manners. Gunderson et al. showed that B lymphocytes with a regulatory phenotype (Bregs) can secrete immunosuppressive cytokines, including TGF-b and IL-10, which inhibit cytotoxic T cells responses (1).[31] Pylayeva-Gupta et al. demonstrated that TIL-B secretes IL35 that stimulates tumor cell proliferation and angiogenesis (2).[33] Here we demonstrate that plasmablasts/plasma cells can sustain tumor stroma activation through the secretion of proliferative stimuli, such as PDGFB, and the production of enzymes that regulate extracellular matrix stiffness, such as LOXL2. Increased extracellular matrix stiffness leads, in turn, to the activation of mechanoceptors on fibroblasts, thus fostering their full differentiation into myofibroblasts (3). Finally, B cells infiltrating PDAC seem capable to attract inflammatory cells with additional fibrotic potential such as Th2 cells, eosinophils, and M2 macrophages, by secreting CCL-4, CCL-5, and CCL-11, and by stimulating fibroblasts to produce these same chemokines (4).

selectively recruits eosinophils through CCR2, CCR3, and CCR5.[53] Moreover, according to our previous findings, B cells might stimulate fibroblasts secretion of these same chemokines, thus amplifying the recruitment of inflammatory cells at tumor site and further sustaining the desmoplastic reaction.[12] Finally, B cells from PDAC patients induced the expression of genes associated with a pro-tumorigenic stroma such as *COL1A1, COL1A2*, and *COL3A1* collagen genes. Indeed, overexpression of *COL1A1, COL1A2, COL3A1* – in addition to *SPARC* and *FAP* that we previously found overexpressed in pancreatic MSC exposed to B lymphocytes from patients with IgG4-RD AIP – has been identified by Moffitt and colleagues as a characteristic gene signature of "activated" tumor-promoting stroma in human PDAC.[9,12,54] If confirmed on further *in vivo* studies, these preliminary findings may suggest that B lymphocytes contribute to the complex interplay between CAFs and tumor cells by favoring the acquisition of pro-tumorigenic properties by pancreatic fibroblasts.

Another major finding of our study is the expansion of plasmablasts in the peripheral blood of patients with PDAC. Plasmablasts, identified as $CD19^{+}CD20^{-}CD27^{+}CD38^{+}$ cells by flow cytometry, may arise extra-follicular or in germinal centers after affinity maturation from $CD20^{+}$ naive precursors, enter the circulation and home to inflammatory niches or to the bone marrow, where they differentiate into antibody-secreting long-lived plasma cells.[18] Some plasmablasts may differentiate into short-lived plasma cells at the site of tissue reactivation, including the tumor milieu. Plasmablasts can circulate for prolonged periods in the setting of chronic antigenic stimulation or autoimmune diseases, but are generally infrequent in the peripheral blood of healthy individuals.[55] As opposite to naïve and memory B cells, circulating plasmablasts from patients with PDAC increased collagen production in co-culture with human fibroblasts, thus confirming the intrinsic pro-fibrotic properties of this B-cell population that we previously reported in another fibrotic disorder.[12] In this sense, it is tempting to speculate that the difference in collagen production observed in the co-cultures of fibroblasts with total B cells from PDAC patients and controls is likely due to the significant increase in circulating plasmablasts in patients with pancreatic cancer. Finally, in analogy with what observed in tissues affected by IgG4-related AIP, LOXL2 expressing class-switched plasmablasts/plasma cells were also found to infiltrate tumor lesions in PDAC, supporting their direct contribution to stromal activation at disease site. Whether plasmablasts also bear a regulatory phenotype, down-modulate antitumor

immune-response, and promote neoplastic cell proliferation remains to be elucidated.

Our study has both strengths and weaknesses. A major potential strength is the adoption of human samples for co-culture experiments. On the one hand, MSC and CAF may have allowed us to recapitulate some of the possible interactions occurring between mesenchymal cells and B cells *in vivo* in PDAC lesions. On the other hand, by using freshly isolated B lymphocytes from healthy donors and patients with PDAC we focused on direct pro-fibrotic interactions occurring between B cells and fibroblasts, and avoided confounding factors. However, despite we generated compelling evidence for the involvement of B cells in the stromal reaction associated with PDAC, we also recognize that our study is limited to *in vitro* and histological data and lacks *in vivo* validation. In addition, our co-culture system based on B cells and fibroblasts excludes the possibility of other cell types being relevant to the stromal reaction occurring at tissue level.

In conclusion, our work provides novel insights into the pathophysiology of the stromal reaction associated with PDAC by unveiling perturbations of the B cell compartment and unexpected pro-fibrotic properties of B lymphocytes in patients with pancreatic cancer. These findings indicate that B cells might contribute to PDAC progression over and beyond the prevalent view of TIL-Bs functioning primarily as immunosuppressive cells and promoters of epithelial cell transformation. In addition, our study paves the way for further researches aimed at clarifying the role of B cells in the complex interplay between stromal elements and tumor cells and in the differentiation of pancreatic fibroblasts into tumor-promoting or tumor-suppressing CAF. Although much work needs to be done to decipher tumor-stroma interactions in PDAC, our data reinforce the notion that future therapeutic strategies for PDAC should not be limited to neoplastic cells or the stromal compartment, but should rather combine agents that target the tumor, the stroma, and immune-regulatory mechanisms.

Materials and methods

Patients

B cells from 35 patients with PDAC and 25 sex- and age-matched healthy donors were studied. For *in vitro* experiments, peripheral blood mononuclear cells (PBMC) were obtained before surgery, chemotherapy, or radiotherapy from 20 patients with histologically proven resectable T3M0 PDAC referred to the Division of Pancreatic Surgery of San Raffaele Scientific Institute (Milan, Italy) between September 2015 and September 2019. Five sex-and age-matched healthy donors were included as controls. Pancreatic glands from n = 5 patients with PDAC from the Pathology unit of Massachusetts General Hospital were used for immunofluorescence studies. For flow-cytometry quantification of B cell subsets we used blood samples from 20 patients with resectable T3M0 PDAC and 20 age- and sex-matched healthy individuals.[56,57] All subjects enrolled provided written informed consent for the analyses performed. The study was conducted according to the Declaration of Helsinki and approved by the Ethical Committees of the San Raffaele Scientific Institute and Massachusetts General Hospital.

Flow cytometry and microscopy

A detailed description of the methods used for PBMC isolation, flow cytometry, cell sorting, immunohistochemistry, and immunofluorescence is provided in the Methods section of this article's Online Repository.

Co-culture experiments

Co-culture experiments were carried out with B cells and B-cell subsets from patients with PDAC, primary human pancreatic fibroblasts, and CAF. Polycarbonate semipermeable membranes (Nunc Dominique Dutscher, Brumath, France) were used to investigate soluble factors or contact-mediated pro-fibrotic mechanisms. Soluble collagen concentration in the supernatant of fibroblasts/CD19 + B cells co-cultures was evaluated using Sircol collagen assay (Biocolor Ltd. Interchim, Montluçon, France). Soluble collagen concentration in the supernatant of fibroblasts/B-cells subsets co-cultures was evaluated using the Pro-collagen Iα1 ELISA kit (R&D Systems Inc., Minneapolis, MN, USA). Cytokines and chemokines in the supernatant of fibroblasts/CD19 + B cells co-cultures were measured using the Bio-Plex Pro Human Cytokine Grp I Panel 27-plex assay (Bio-Rad, Hercules, CA, USA). A detailed description of the methods used for the establishment of primary human pancreatic fibroblasts lines and of co-cultures with total B cells and B-cell subsets is provided in the Methods section of this article's Online Repository.

Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from cultured fibroblasts using ReliaPrep™ RNA Cell Miniprep System (Promega) and reverse transcribed with random hexameric primers. Real-time quantitative PCR for human GAPDH, COL1A1, COLA1A2, COL3A1, ACTA2, and CD19 genes was performed with the KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Bio-Rad, Hercules, CA, USA) using a Roche LightCycler® (Roche Molecular Diagnostic, Pleasanton, CA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software 6.0 (La Jolla, CA, USA). Normal distribution of continuous variables was assessed with the D'Agostino & Pearson omnibus normality test. Non-normally distributed variables were compared using the Mann-Whitney U-test. Normally distributed variables were compared using Unpaired t-test. A p-value < 0.05 was considered significant. Continuous variables are expressed as mean ± standard deviation (SD), unless otherwise specified.

Disclosure statement

Nothing to report.

Disclosure

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