

B lymphocytes directly contribute to tissue fibrosis in patients with IgG₄-related disease



Emanuel Della-Torre, MD,^{a,b,c} Elena Rigamonti, PhD,^a Cory Perugino, DO,^{b,d} Simona Baghai-Sain, PhD,^e Na Sun, PhD,^b Naoki Kaneko, DDS, PhD,^{b,f} Takashi Maehara, DDS, PhD,^{b,f} Lucrezia Rovati, MD,^{a,b} Maurilio Ponzoni, MD,^{a,g} Raffaella Milani, MD,^h Marco Lanzillotta, MD,^a Vinay Mahajan, MBBS, PhD,^b Hamid Mattoo, PhD,ⁱ Ivan Molineris, PhD,^e Vikram Deshpande, MD,^j John H. Stone, MD,^d Massimo Falconi, MD,^{a,k} Angelo A. Manfredi, MD,^{a,c*} and Shiv Pillai, MBBS, PhD^{b*}
Milan, Italy, Boston and Cambridge, Mass, and Fukuoka, Japan

Background: IgG₄-related disease (IgG₄-RD) is a fibroinflammatory condition marked by rapid clinical improvement after selective depletion of B lymphocytes with rituximab. This feature suggests that B cells might participate in fibrogenesis and wound healing.

Objective: In the present work we aimed to demonstrate that B lymphocytes contribute directly to tissue fibrosis in patients with IgG₄-RD.

Methods: Total circulating CD19⁺ B lymphocytes, naïve B cells, memory B cells, or plasmablasts from patients with IgG₄-RD were cultivated with human fibroblasts. Profibrotic soluble factors and collagen production in cocultures were assessed by using ELISAs and Luminex assays. RNA sequencing and quantitative RT-PCR were used to assess fibroblast activation in the presence of B cells, as well as induction of profibrotic pathways in B-cell subsets. Relevant profibrotic and

inflammatory molecules were confirmed *in vitro* by using functional experiments and on IgG₄-RD tissue sections by using multicolor immunofluorescence studies.

Results: B cells from patients with IgG₄-RD (1) produced the profibrotic molecule platelet-derived growth factor B and stimulated collagen production by fibroblasts; (2) expressed enzymes implicated in extracellular matrix remodeling, such as lysyl oxidase homolog 2; (3) produced the chemotactic factors CCL4, CCL5, and CCL11; and (4) induced production of these same chemokines by activated fibroblasts. Plasmablasts expressed sets of genes implicated in fibroblast activation and proliferation and therefore represent cells with intrinsic profibrotic properties.

Conclusion: We have demonstrated that B cells contribute directly to tissue fibrosis in patients with IgG₄-RD. These unanticipated profibrotic properties of B lymphocytes, particularly plasmablasts, might be relevant for fibrogenesis in patients with other fibroinflammatory disorders and for wound-healing processes in physiologic conditions. (J Allergy Clin Immunol 2020;145:968-81.)

Key words: IgG₄, IgG₄-related disease, B cells, plasmablasts, fibrosis, fibroblasts, rituximab, lysyl oxidase homolog 2, platelet-derived growth factor

From ^aUniversità Vita-Salute San Raffaele, ^bthe Pathology Unit, ^cthe Immunohematology and Transfusion Medicine Unit, and ^dthe Division of Pancreatic Surgery, IRCCS San Raffaele Scientific Institute, Milan; ^ethe Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Harvard Medical School, Boston; ^fthe Ragon Institute of MGH, MIT, and Harvard, Massachusetts General Hospital, Harvard Medical School, Boston; ^gthe Unit of Immunology, Rheumatology, Allergy, and Rare Diseases, IRCCS San Raffaele Scientific Institute, Milan; ^hthe Section of Oral and Maxillofacial Oncology, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University, Fukuoka; ⁱthe Department of Pathology, Massachusetts General Hospital, Boston; ^jCenter for Translational Genomics and Bioinformatics, IRCCS San Raffaele Scientific Institute, Milan; and ^kImmunology and Inflammation Therapeutic Area, Sanofi, Cambridge.

*These authors contributed equally to this work.

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Corresponding author: Emanuel Della-Torre, MD, Università Vita-Salute San Raffaele, IRCCS San Raffaele Scientific Institute, via Olgettina 60, 20132, Milan, Italy. E-mail: dellatorre.emmanuel@hsr.it.

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Fibrosis is a reversible physiologic phenomenon of tissue scarring occurring during wound healing and the resolution of inflammation. When dysregulated, fibrosis can become irreversible and lead to tissue distortion and organ damage, thereby representing a major negative prognostic determinant across a host of different chronic inflammatory disorders.¹ In general, tissue fibrosis arises from deposition of collagenous proteins synthesized by activated fibroblasts, but the complex network of profibrotic signals that sustains fibroblast activation remains only partially elucidated. In particular, although a variety of cell types and molecular pathways have been causally associated with fibroblast activation, these events appear tightly integrated, interdependent, and probably redundant.² All these aspects complicate the identification of their relative contribution to fibrogenesis. This is the main reason why there are few, if any, treatment strategies available to reverse the course of most fibrotic disorders.³

Unlike most other fibrotic diseases, IgG₄-related disease (IgG₄-RD), a fibroinflammatory condition characterized by mass-forming lesions and lymphoplasmacytic infiltrates with an

Abbreviations used

ACTA2:	α -Smooth muscle actin gene
AIP:	Autoimmune pancreatitis
CD:	Castleman disease
Ct:	Cycle threshold
CTL:	Cytotoxic T lymphocyte
FDR:	False discovery rate
FITC:	Fluorescein isothiocyanate
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
IgG ₄ -RD:	IgG ₄ -related disease
IMDM:	Iscove modified Dulbecco medium
LOXL2:	Lysyl oxidase homolog 2
MEM:	Minimum Essential Medium
MM:	Multiple myeloma
PDGF:	Platelet-derived growth factor
SMA:	Smooth muscle actin
SS:	Sjögren syndrome

abundance of IgG₄⁺ plasma cells, promptly responds to B-cell depletion with rituximab.⁴ This disorder provides the ideal clinical condition to explore the contribution of B cells to fibrosis because B-cell depletion in patients with this disease leads to rapid shrinkage of the typical fibrous lesions and provides durable clinical improvement in most patients.⁵ In addition, rituximab attenuates serologic biomarkers of fibrosis and reverses myofibroblast activation in affected organs, suggesting that fibrogenesis in patients with IgG₄-RD might be halted by selectively targeting the B-cell compartment.⁶

Despite this indirect evidence supporting the possible involvement of B lymphocytes in tissue fibrosis,⁷⁻¹³ most pathogenic studies on IgG₄-RD conducted thus far have focused on putative fibrogenic T lymphocytes, namely CD4⁺ cytotoxic T lymphocytes (CTLs), T_H2 cells, and macrophages. Although B cells have been assumed to contribute to this disease indirectly, either by antigen presentation to potentially pathogenic T_H cells or by virtue of autoantibody generation, no direct profibrotic role for B cells in this condition has been hitherto postulated.¹⁴⁻¹⁹

In the present work we hypothesize a direct contribution of B lymphocytes to fibrogenesis in patients with IgG₄-RD that extends beyond their established roles as antibody-producing and antigen-presenting cells. By cocultivating circulating B cells from patients with IgG₄-RD with human fibroblasts, we demonstrate that B lymphocytes are capable of orchestrating tissue fibrosis through both direct and indirect mechanisms, namely (1) secretion of soluble profibrotic factors that induce collagen production by activated fibroblasts, (2) secretion of chemotactic signals for profibrotic populations of T lymphocytes and macrophages, (3) production of collagenous proteins; and (4) organization of the extracellular matrix. In addition, by profiling the transcriptome of circulating B-cell subpopulations in patients with IgG₄-RD, we have identified previously unknown profibrotic properties of circulating plasmablasts that might contribute to the pathogenesis of this condition, as well as that of other chronic fibrosing disorders.

METHODS

Patients

This study was approved by the Institutional Review Board of San Raffaele Scientific Institute (Milan, Italy) and of Massachusetts General Hospital

(Boston, Mass). Informed written consent was obtained from all patients with IgG₄-RD referred to the rheumatology clinics of both hospitals and enrolled in the study. Samples from 20 patients with IgG₄-RD autoimmune pancreatitis (AIP) were used (organ involvement and patients' demographics are listed in Table E1 in this article's Online Repository at www.jacionline.org). Patients with IgG₄-RD were compared with 5 healthy control subjects. Tissue sections from 5 patients with IgG₄-related sialadenitis, 5 patients with IgG₄-related AIP, and 5 patients with Sjögren syndrome (SS) obtained from the Massachusetts General Hospital and the Kyushu University Hospital Department of Oral and Maxillofacial Surgery, Fukuoka, Japan, were also studied.

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 in this article's Online Repository at www.jacionline.org.

Coculture experiments

Coculture experiments were carried out with B cells and B-cell subsets from patients with IgG₄-related AIP and primary human pancreatic or skin fibroblasts. Polycarbonate semipermeable membranes (Nunc Dominique Dutscher, Brumath, France) were used to identify soluble factors or contact-mediated profibrotic mechanisms. Soluble collagen concentrations in the supernatants of fibroblast/CD19⁺ B-cell cocultures were evaluated with the Sircol collagen assay (Biocolor Interchim, Montluçon, France). Soluble collagen concentrations in the supernatants of fibroblast/B-cell subsets cocultures was evaluated by using the Pro-collagen I α 1 ELISA Kit (R&D Systems, Minneapolis, Minn). Cytokines and chemokines present in the supernatants of fibroblast/CD19⁺ B-cell cocultures were measured with the Bio-Plex Pro Human Cytokine Grp I Panel 27-plex assay (Bio-Rad Laboratories, Hercules, Calif). A detailed description of the methods used for the establishment of primary human pancreatic fibroblast cell lines and cocultures with total B cells and B-cell subsets is provided in the [Methods](#) section in this article's Online Repository (see [Figs E1-E3](#)).

Gene expression analysis

For quantitative real-time PCR analysis, total RNA was extracted from cultured fibroblasts by using the ReliaPrep RNA Cell Miniprep System (Promega, Madison, Wis) and reverse transcribed with random hexameric primers. Real-time quantitative PCR for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *COL1A1*, *COL1A2*, *COL3A1*, *ACTA2*, and *CD19* genes was performed with the KAPA SYBR FAST qPCR Master Mix (2 $\times$) Kit (Bio-Rad Laboratories) using a Roche LightCycler (Roche Molecular Diagnostics, Pleasanton, Calif). For RNA-sequencing analysis on B cells and fibroblasts, cells were directly harvested in RLT buffer from Qiagen (Hilden, Germany) containing 1% 2-mercaptoethanol (Pierce/Thermo Scientific, Waltham, Mass), and RNA was isolated with the RNeasy Plus Micro Kit (Qiagen). Libraries were prepared with Superscript II Reverse Transcriptase (Thermo Fisher Scientific) and sequenced by using the Illumina NextSeq sequencer (Illumina, San Diego, Calif). Differential gene expression analysis was performed with the DESeq2 Analyzer. Detailed methodology is included in the [Methods](#) section in this article's Online Repository.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software 6.0 (GraphPad Software, La Jolla, Calif). Normal distribution of continuous variables was assessed with the D'Agostino and Pearson omnibus normality test. Nonnormally distributed variables were compared by using the Mann-Whitney *U* test. Unpaired *t* tests were used to perform comparisons between 2 groups. A *P* value of less than .05 was considered significant. Continuous variables are expressed as means $\pm$ SDs, unless otherwise specified.

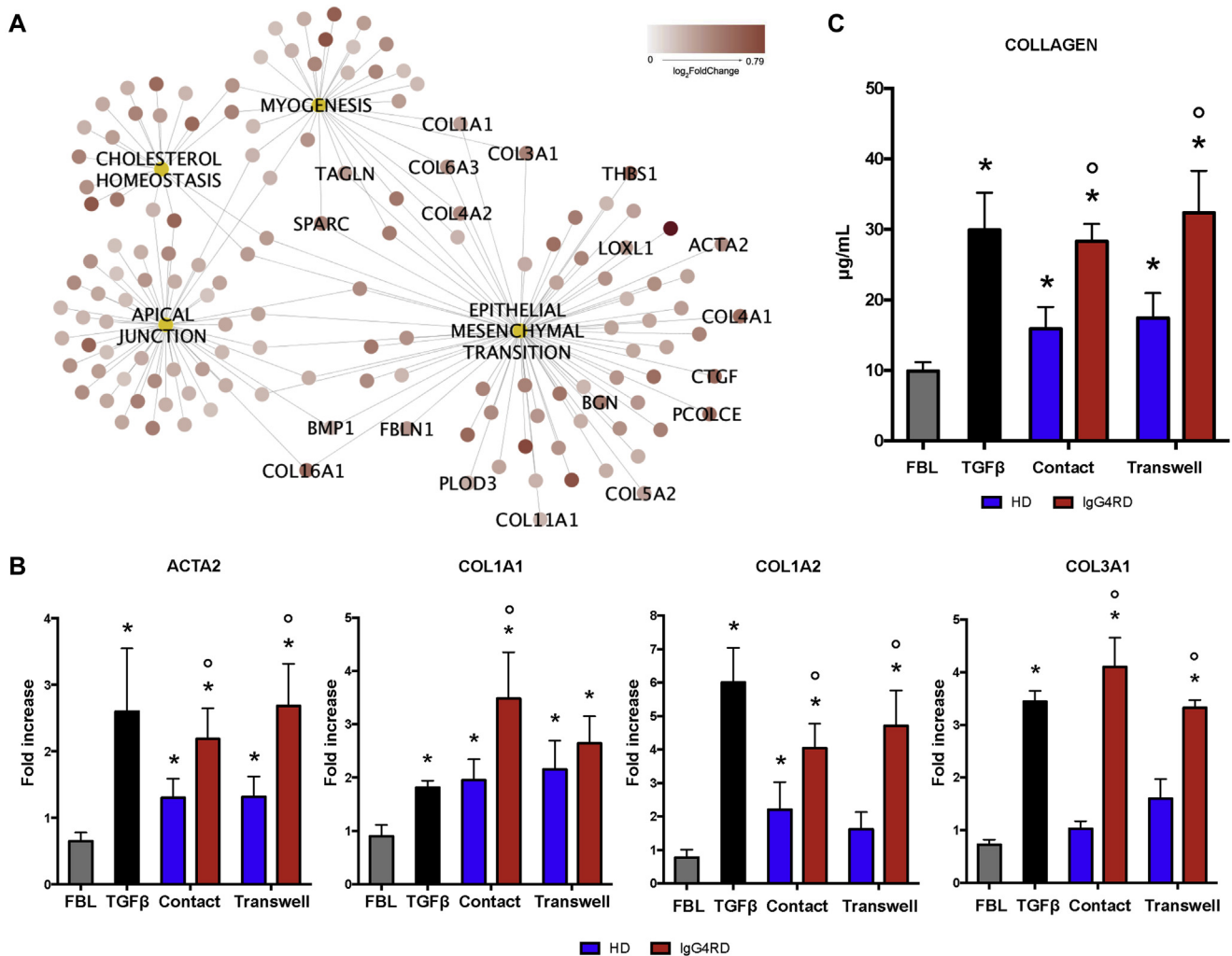


FIG 1. B lymphocytes drive profibrotic gene expression and collagen production by human pancreatic fibroblasts by means of soluble factors. **A**, Top results of gene set enrichment analysis (GSEA) performed on log₂ fold changes between gene expression of fibroblasts cocultured with B cells from patients with IgG₄-related AIP in the absence of semipermeable membranes and of control fibroblasts at 72 hours (see the Methods section in this article's Online Repository for details). The network graph shows the top-scoring enriched gene sets (false discovery rate [FDR] q-value < .01) and their associated leading-edge genes (those responsible of the core enrichment of the gene set). Nodes representing genes are colored according to their log₂ fold change, and genes considered of particular interest are labeled. **B**, Quantitative PCR showing fold change expression of ACTA2, COL1A1, COL1A2, and COL3A1 genes in pancreatic fibroblasts after 3 days of coculture with B lymphocytes from patients with IgG₄-related AIP (n = 5) and healthy donors (n = 5). **C**, Collagen concentration in supernatants of different experimental conditions. Human pancreatic fibroblasts (FBL), human pancreatic fibroblasts treated with 20 ng/mL TGF-β1 (TGFβ), and human pancreatic fibroblasts cocultured with B cells from healthy control subjects (blue bars) and human pancreatic fibroblasts cocultured with B cells from patients with IgG₄-RD (red bars) in the presence (Transwell) or absence (Contact) of semipermeable membranes are shown. *P < .05 for comparison with fibroblasts alone; *P < .05 for comparison with cocultures in the presence of B cells from healthy control subjects. Results are expressed as means ± SDs of 5 experiments. HD, Healthy donors.

RESULTS

B lymphocytes from patients with IgG₄-RD release soluble signals that increase collagen production by human fibroblasts

To assess a direct role of B lymphocytes in fibroblast activation and collagen secretion, we set up cocultures of primary human fibroblasts with CD19⁺ B cells isolated from the peripheral blood of 5 patients with IgG₄-RD and performed transcriptomic analyses on RNA extracted from cultured fibroblasts. We used

primary human pancreatic fibroblasts and B cells from patients with IgG₄-RD with pancreatic involvement to reproduce *in vitro* some of the possible interactions occurring between these 2 cell types in patients with IgG₄-related AIP, the most well-characterized manifestation of IgG₄-RD. Clinical features of the patient cohort are reported in Table E1. B cells purified from the peripheral blood of 5 sex- and age-matched healthy donors were used as controls. As shown in Fig 1, A, B cells from patients with IgG₄-related AIP induced the activation of profibrotic

pathways in cocultured fibroblasts, with a significant upregulation of genes involved in epithelial-to-mesenchymal transition, myogenesis, and formation of apical junctions compared with control fibroblasts, as assessed by using transcriptomic analysis on fibroblast RNA (see also Fig E4 and Table E2 in this article's Online Repository at www.jacionline.org).

We confirmed activation of profibrotic pathways by performing quantitative PCR for α -smooth muscle actin (*ACTA2*), *COL1A1*, *COL1A2*, and *COL3A1* collagen genes (Fig 1, B). Expression of *ACTA2*, *COL1A1*, and *COL1A2*, but not of *COL3A1*, was also significantly increased in fibroblasts cocultured with B cells from healthy donors, although to a significantly lower level than in fibroblasts cocultured with B cells from patients with IgG₄-RD (Fig 1, B). Accordingly, collagen production in cocultures of fibroblasts with B cells from patients with IgG₄-RD was significantly increased compared with both fibroblasts cultured alone and fibroblasts cultured with B cells from healthy donors (Fig 1, C).

To evaluate whether the increased collagen secretion observed in the presence of B cells was due to soluble or contact-mediated factors, we performed cocultures with and without semipermeable membranes (transwells) to, respectively, avoid or allow physical interactions between B cells and fibroblasts. As shown in Fig 1, B and C, profibrotic gene expression and collagen production by pancreatic fibroblasts did not differ between cocultures performed in the presence or absence of transwells, both being significantly increased compared with control fibroblasts, regardless of any physical interaction. Similar results were obtained by using human skin fibroblasts (Fig 2, D).

These findings demonstrate that CD19⁺ B lymphocytes from patients with IgG₄-RD and, to a lesser extent, from healthy subjects are capable of driving myofibroblast differentiation and collagen production through secretion of soluble factors rather than physical interactions.

Expression of the profibrotic factor platelet-derived growth factor B, and the inflammatory chemokines CCL4, CCL5, and CCL11 are increased in cocultures of human fibroblasts with B lymphocytes

To identify soluble profibrotic molecules secreted by B lymphocytes, we collected supernatants of cocultures of B cells from either healthy control subjects or patients with IgG₄-RD and pancreatic fibroblasts. We then quantitated cytokines and chemokines known to be implicated in fibrosis and wound healing using a Luminex-based approach. As shown in Fig 2, A, the presence of B cells from patients with IgG₄-RD and healthy subjects both led to significantly increased concentrations of platelet-derived growth factor (PDGF) B, CCL4 (macrophage inflammatory protein 1 β), CCL5 (RANTES), and CCL11 (eotaxin) compared with fibroblasts alone. In particular, CCL4 and CCL5 production was induced by the presence of B lymphocytes, whereas low levels of PDGF-B and CCL11 were constitutively secreted by untreated fibroblasts. No significant difference was observed in concentrations of PDGF-B, CCL4, CCL5, and CCL11 between cocultures of fibroblasts with B cells from patients with IgG₄-RD and from healthy donors or between cocultures with and without semipermeable membranes (Fig 2, A-C). Levels of other profibrotic molecules, such as IL-1 α , IL-4, IL-5, IL-10, IL-13, IFN- γ , TNF- α , and TGF- β , did not differ between fibroblasts alone and

fibroblasts cocultured with B cells from either patients with IgG₄-RD or healthy donors.

These results indicate that B lymphocytes either secrete the profibrotic molecule PDGF-B, stimulate activated fibroblasts to produce it, or both. These results also demonstrate that fibroblasts stimulated by B cells or perhaps B cells themselves secrete chemokines that might contribute to the recruitment of other inflammatory cell types *in vivo*.

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

PDGF-B is known to contribute to tissue fibrosis by stimulating fibroblast activation in several ways, including cell proliferation and stimulation of collagen production.^{20,21} To assess the biological relevance of PDGF-B in our model, we performed cocultures of skin fibroblasts and B cells from patients with IgG₄-RD and used blocking anti-PDGF-B antibody or isotype control to interfere with collagen production. As shown in Fig 2, D, PDGF-B blockade attenuated the fibrotic effect induced by B cells from patients with IgG₄-RD, whereas nonimmune IgG of the same isotype did not inhibit collagen production induced by the same B lymphocytes. These results support the notion that B lymphocytes might contribute to activation of fibroblasts into a profibrotic state, as observed in B cell/fibroblast cocultures, either through production of PDGF-B or by inducing secretion of PDGF-B by activated fibroblasts. It is also possible that both mechanisms occur together, thus perpetuating autocrine and paracrine profibrotic loops.

Of note, despite showing similar levels of PDGF-B in the supernatant, cocultures of fibroblasts with B cells from patients with IgG₄-RD yielded significantly greater amounts of collagen compared with cocultures of fibroblasts with B cells from healthy subjects (Fig 1, B and C). This suggests that factors other than PDGF-B might also contribute to the increased fibrotic effect of B cells from patients with IgG₄-RD.

B lymphocytes and fibroblasts infiltrating IgG₄-RD-affected organs express PDGF-B, CCL4, CCL5, and CCL11

To localize the cellular source of PDGF-B, CCL4, CCL5, and CCL11 in our coculture system, whether B cells or pancreatic fibroblasts, and to confirm the relevance of these molecules to the pathogenesis of IgG₄-RD, we performed multicolor immunofluorescence and quantitative analysis on tissue sections of patients with IgG₄-related AIP. Healthy pancreatic sections were used as controls. As shown in Fig 3, A, the CD19⁺ B-cell infiltrate in patients with IgG₄-related AIP was localized in the proximity of pancreatic ducts, and its distribution corresponded to areas of dense fibrosis observed in sequential tissue slides (Fig 3, B). Tertiary lymphoid structures rich in CD19⁺ cells were also observed. Few scattered CD19⁺ B cells were detected in healthy pancreatic tissue, with no organized lymphoid structures (see Fig E5 in this article's Online Repository at www.jacionline.org).

CD19⁺ B lymphocytes expressing either PDGF-B, CCL4, CCL5, or CCL11 represented on average 29.2% (range, 23.1% to 35.2%), 30.9% (range, 25.3% to 39.1%), 12.7% (range, 10.2% to 15.4%), and 16.9% (range, 15.1% to 20.4%) of total PDGF-B-, CCL4-, CCL5-, and CCL11-expressing cells, respectively (Fig 3, C-E). α -smooth muscle actin (SMA)⁺ fibroblasts expressing either PDGF-B, CCL4, CCL5, or CCL11 represented

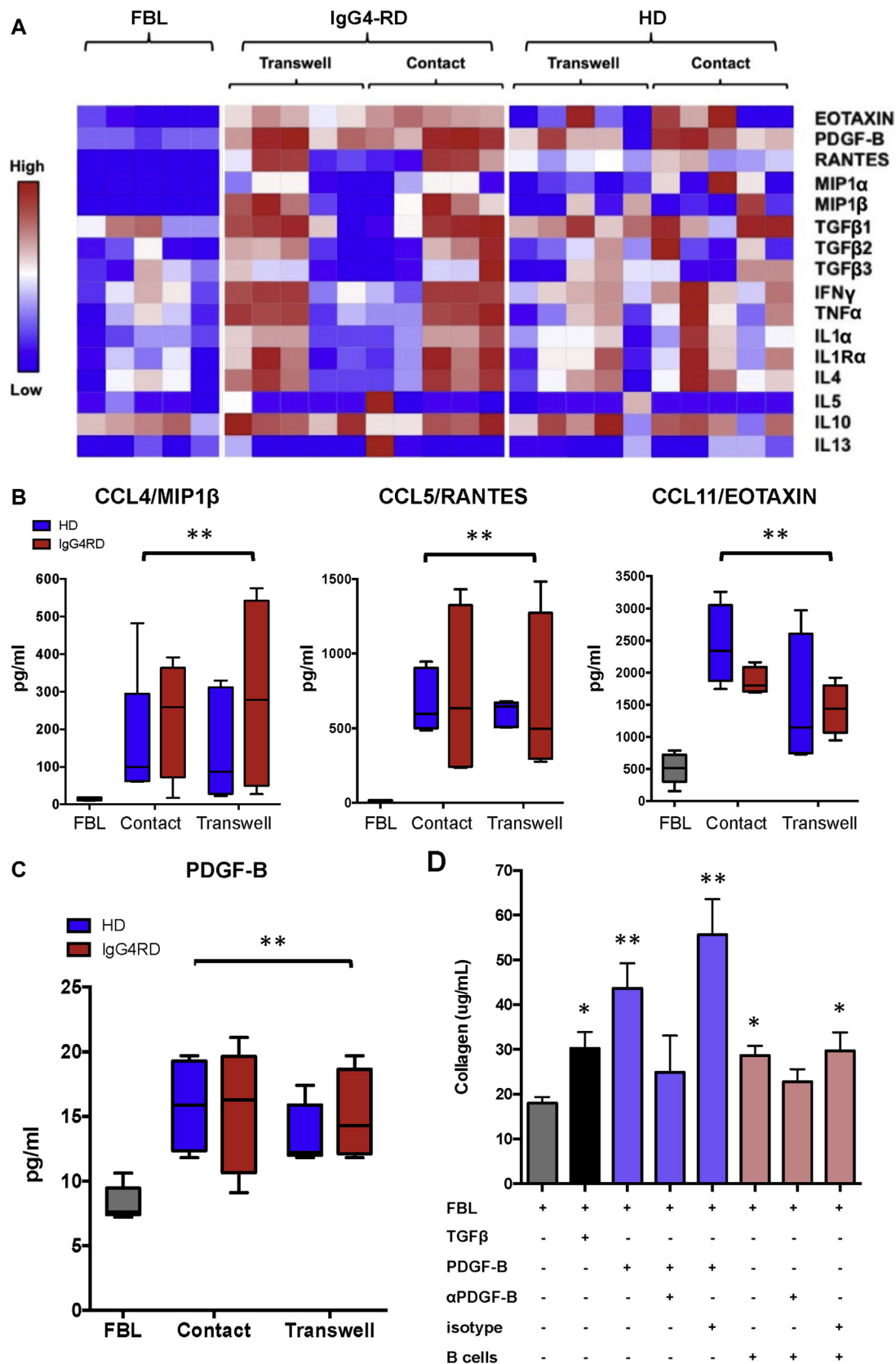


FIG 2. PDGF-B levels are increased in cocultures of fibroblasts with B lymphocytes together with CCL4, CCL5, and CCL11, and PDGF-B mediates collagen production. **A**, Quantification of different cytokines and chemokines by means of Luminex analysis after 3 days of culture with a specific focus on those significantly increased in coculture supernatants compared with control fibroblasts. **B**, CCL4, CCL5, and CCL11. **C**, PDGF-B.

on average 22.2% (range, 21.3% to 25.2%), 8.3% (range, 5.3% to 12.1%), 4.5% (range, 1.2% to 5.4%), and 19.6% (range, 14.1% to 21.2%) of total PDGF-B-, CCL4-, CCL5-, and CCL11- expressing cells, respectively (Fig 3, C, and see Fig E6 in this article's Online Repository at www.jacionline.org). α -SMA⁺ myofibroblasts and CD19⁺ B lymphocytes were observed in close proximity in tissue from patients with IgG-related AIP tissues (see Fig E6), suggesting that interactions between these 2 types of cells are likely to occur at disease sites.

These findings indicate that the profibrotic molecule PDGF-B and the inflammatory chemokines CCL4, CCL5, and CCL11 are produced by both B lymphocytes and myofibroblasts infiltrating IgG₄-RD lesions *in vivo*. Of note, only a fraction of total CD19⁺ cells and α -SMA⁺ cells expressed PDGF-B, CCL4, CCL5, or CCL11, suggesting the existence of pathogenic subsets of B lymphocytes and myofibroblasts capable of driving inflammation within affected tissues (see Fig E7, A, in this article's Online Repository at www.jacionline.org). Our results also indicate that other cell types known to abundantly infiltrate tissues of patients with IgG₄-RD likely contribute to production of these mediators, such as macrophages, eosinophils, and T cells. Finally, these results support the notion that both B lymphocytes and pancreatic fibroblasts represent a source of PDGF-B, CCL4, CCL5, and CCL11 in the coculture studies described above.

Plasmablasts from patients with IgG₄-RD induce collagen secretion by human fibroblasts, produce collagenous proteins, and express extracellular matrix remodeling enzymes

A major finding of our previous experiments was the increased collagen production observed in cocultures of fibroblasts with B lymphocytes from healthy subjects compared with control fibroblasts (Fig 1, B and C). Yet despite similar levels of PDGF-B in supernatants of the 2 culture conditions, collagen production induced by B cells from healthy donors was significantly lower compared with that induced by B cells from patients with IgG₄-related AIP (Fig 1, B and C). This result was further confirmed by the significant upregulation of profibrotic pathways in pancreatic fibroblasts cultured with B cells from patients with IgG₄-RD compared with similar fibroblasts cultured with B cells from healthy subjects, suggesting the existence of fibrogenic B lymphocytes in the peripheral blood of patients with active untreated IgG₄-RD (Fig 4, A, and see Fig E4 and Table E3 in this article's Online Repository at www.jacionline.org). Indeed, we know from our previous studies that, in contrast to healthy subjects, active IgG₄-RD is characterized by an oligoclonal expansion of circulating plasmablasts and that circulating plasmablasts

dramatically decrease during disease remission.^{22,23} Plasmablast expansions in patients with IgG₄-RD also correlate with serologic biomarkers of collagen deposition, indicating that these activated B cells might contribute to disease pathogenesis and tissue fibrosis.^{6,24-26}

To address the profibrotic properties of B-cell subsets in patients with IgG₄-RD, we set up cocultures of skin fibroblasts with flow cytometrically sorted naive B cells, memory B cells, and circulating plasmablasts from 5 patients with active untreated IgG₄-related AIP. As shown in Fig 4, B, fibroblasts cultured with circulating plasmablasts produced significantly more collagen compared with control fibroblasts. Naive or memory B cells were significantly less proficient at eliciting collagen production (Fig 4, B).

To better characterize the profibrotic properties of circulating plasmablasts more thoroughly, we performed whole-transcriptome analyses of the 3 different B-cell subsets. Compared with naive and memory B cells, circulating plasmablasts showed significant upregulation of genes implicated in positive regulation of fibroblast proliferation, such as the collagen genes *COL1A1* and *COL1A2*, the insulin-like growth factor 1 gene, and the lysyl oxidase homolog 2 (*LOXL2*) and gremlin-1 (*GREM1*) genes (Fig 4, C). Collagen expression by circulating plasmablasts was significantly greater than that of naive and memory B cells, as assessed by using flow cytometry (Fig 4, D). Whole-transcriptome analysis of fibroblasts cultured with naive B cells, memory B cells, or plasmablasts also revealed that CCL11 and PDGF-B expression was significantly increased by all 3 B-lymphocyte subpopulations compared with control fibroblasts. Conversely, fibroblast expression of CCL4 and CCL5 was induced by naive B cells and circulating plasmablasts but not by memory B cells (see Fig E7, B).

Taken together, these results indicate that plasmablasts possess profibrotic properties that enable them to activate fibroblasts and contribute to organization of the extracellular matrix. Furthermore, plasmablasts directly express collagenous proteins and stimulate fibroblasts to produce inflammatory chemokines.

LOXL2 is abundantly expressed by plasma cells infiltrating IgG₄-RD tissues

LOXL2 is a member of a group of enzymes known as the lysyl oxidase family that control tissue stiffness and elasticity by catalyzing the covalent cross-linking of both collagen and elastin fibers.^{3,27} Different members of the LOX family are upregulated in human fibrotic disorders, and overexpression of LOXL2 has been implicated in the progression of experimental fibrosis.^{1-3,28-30} Indeed, stiffening of the extracellular matrix is

Primary human pancreatic fibroblasts (FBL), human pancreatic fibroblasts stimulated with 20 ng/mL recombinant human TGF- β 1 (TGF β), and human pancreatic fibroblasts cocultured with B cells from healthy control subjects (n = 5, blue bars) and human pancreatic fibroblasts cocultured with B cells from patients with IgG₄-RD (n = 5, red bars) in the presence (Transwell) or absence (Contact) of semipermeable membranes are shown. Results are expressed as means $\pm$ SDs. D, PDGF-B inhibition decreases B cell-induced collagen production by primary human skin fibroblasts. Primary human skin fibroblasts (FBL), human pancreatic fibroblasts stimulated with 20 ng/mL recombinant human TGF- β 1 (TGF β), human pancreatic fibroblasts stimulated with 10 ng/mL recombinant human PDGF-B (PDGF-B), human pancreatic fibroblasts incubated with 0.5 μ g/mL anti-PDGF-B antibody 2 hours before stimulation with 10 ng/mL recombinant human PDGF-B (α PDGF-B), human pancreatic fibroblasts incubated with 0.5 μ g/mL IgG isotype antibody 2 hours before stimulation with 10 ng/mL recombinant human PDGF-B (isotype), and cocultures with B cells from patients with IgG₄-RD (B cells) are shown.

*P < .05. Results are expressed as means $\pm$ SDs of 5 experiments. HD, Healthy donors.

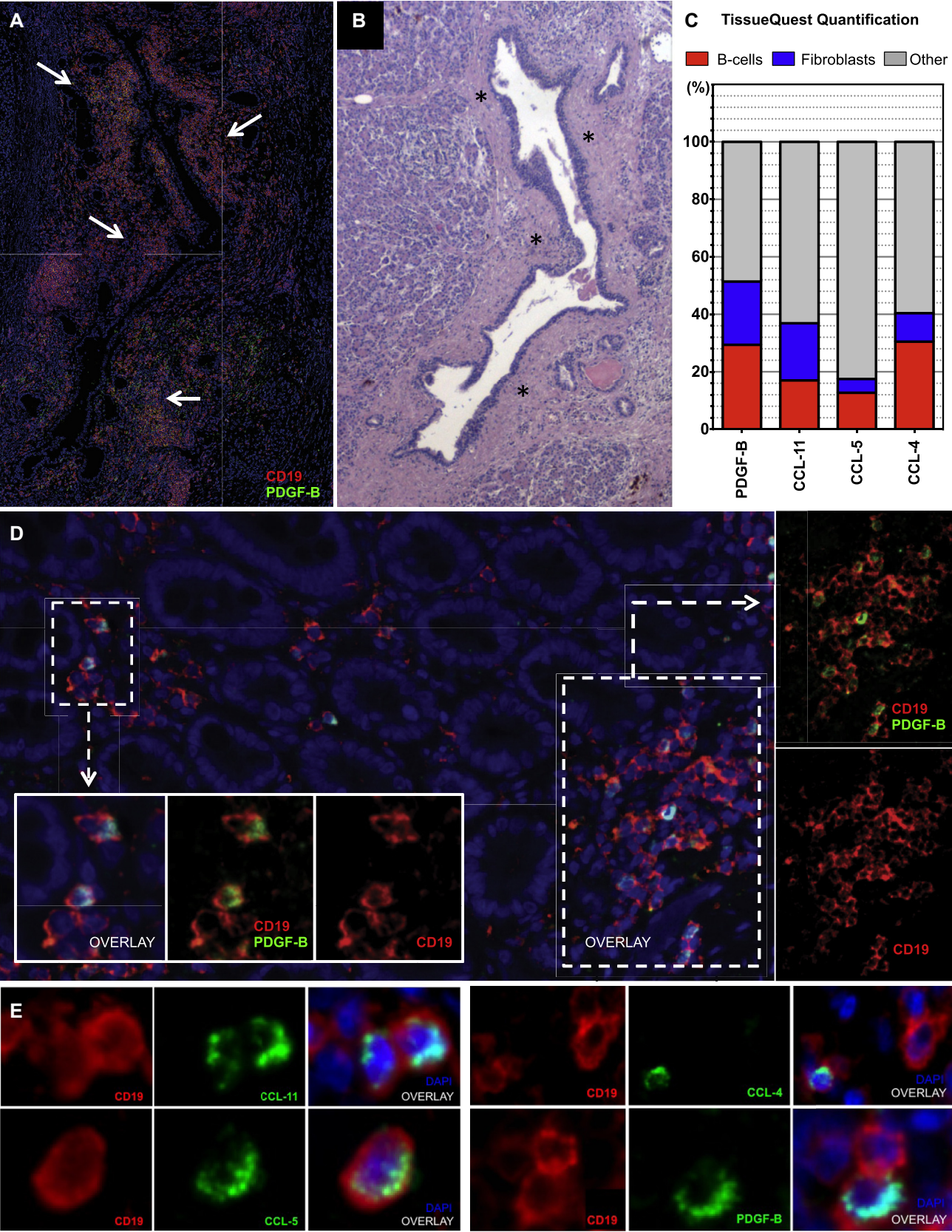


FIG 3. B lymphocytes and fibroblasts infiltrating patients with IgG₄-related AIP express PDGF-B, CCL4, CCL5, and CCL11. **A**, Immunofluorescence staining of an IgG₄-related AIP tissue showing PDGF-B-expressing B cells (*arrows*) surrounding the pancreatic ducts (CD19 [red], PDGF-B [green], and

emerging as a major driver of full myofibroblast differentiation, and mechanoreceptors are now considered to be as important as soluble factors in promoting fibrogenesis.^{1-3,31}

To confirm LOXL2 expression by plasmablasts infiltrating patients with IgG₄-RD, we performed multicolor immunofluorescence and quantitative analyses on salivary glands from patients with IgG₄-RD. As shown in Fig 5, up to 30% of LOXL2⁺ cells expressed CD19 and did not express CD20, representing plasmablasts or plasma cells (Fig 5, A and B). LOXL2 expression by B cells infiltrating IgG₄-RD lesions was observed in a proportion of IgG₄⁺ and IgG₁⁺ plasmablasts, suggesting that, at least in patients with IgG₄-RD, class-switched B cells bear the capability of modulating extracellular matrix stiffness (Fig 5, B and C). CD19 and IgG₄ double-positive cells infiltrating tissue from patients with IgG₄-RD also expressed the activation-induced cytidine deaminase, a master regulator of immunoglobulin gene recombination, further supporting ongoing processes of class-switch and somatic hypermutation.³²

To assess the relevance of LOXL2 to the pathogenesis of IgG₄-RD, we performed multicolor immunofluorescence and quantitative analyses on salivary glands from patients with SS. SS was used as a control condition because of the chronic lymphoplasmacytic inflammation in the absence of significant tissue fibrosis when compared with IgG₄-RD. LOXL2 was expressed at a significantly greater frequency in IgG₄-related sialadenitis than in SS, suggesting an important role of this enzyme in the pathophysiology of IgG₄-RD (Fig 5, D and E). In particular, the absolute number of LOXL2⁺ cells in patients with IgG₄-RD and SS was 1406 ± 436 and 240 ± 43 cells/mm², respectively, representing on average $12.1\% \pm 2.6\%$ and $3.7\% \pm 0.9\%$ of total cells in the tissue ($P < .05$ for both comparisons; Fig 5, E). CD19⁺ and IgG₄⁺ cell counts were also increased in patients with IgG₄-related sialadenitis compared with those with SS, with IgG₄⁺ B cells accounting for $48.4\% \pm 7.7\%$ of total CD19⁺ B lymphocytes in patients with IgG₄-RD and for $20.1\% \pm 9.1\%$ in patients with SS ($P < .05$; see Fig E8, A, in this article's Online Repository at www.jacionline.org). Likewise, LOXL2⁺CD19⁺CD20⁻ plasmablasts were significantly more abundant in patients with IgG₄-related sialadenitis than in patients with SS, accounting for $17.8\% \pm 12.5\%$ and $4.3\% \pm 2.4\%$ of total tissue-infiltrating LOXL2⁺ cells, respectively (Fig 5, B and D, and see Fig E8, A). These results demonstrate that plasmablasts represent a sizable proportion of LOXL2-expressing cells in IgG₄-RD lesions, thus reinforcing the pathogenic relevance of B lymphocytes for the development of tissue fibrosis in this condition.

Finally, to assess whether LOXL2 expression by plasmablasts/plasma cells represents a feature unique to patients with IgG₄-RD or is common to physiologic and pathologic plasmablasts, we performed immunohistochemistry and immunofluorescence studies on human tonsils from otherwise healthy subjects, on bone marrow smears from patients with multiple myeloma (MM),

and on lymph nodes from patients with Castleman disease (CD). In fact, in analogy with IgG₄-RD, MM and CD represent hematologic disorders characterized by expansions of IgG-secreting B lymphocytes. As shown in Fig E8, B-D, LOXL2 was expressed by a proportion of plasma cells residing in tonsils with reactive follicular hyperplasia, in the bone marrow of patients with MM, and in lymph nodes of patients with CD, indicating that plasmablasts/plasma cells might display a previously overlooked profibrotic machinery both in physiologic and pathologic conditions. Of note, LOXL2⁺CD19⁺CD20⁻ plasmablasts were more abundant in patients with IgG₄-related sialadenitis than in patients with SS and CD, accounting for $17.9\% \pm 12.5\%$, $4.4\% \pm 2.4\%$, and $8.9\% \pm 10.6\%$ of total tissue-infiltrating LOXL2⁺ cells, respectively (see Fig E8, D).

DISCUSSION

The pathogenicity of B lymphocytes in patients with immune-mediated disorders has been traditionally attributed to the production of autoantibodies and activation of autoreactive T cells through antigen presentation. Accordingly, the B cell-depleting agent rituximab has found to be a successful application both in conditions caused by pathogenic autoantibodies, such as autoimmune thrombotic thrombocytopenic purpura and hemolytic anemia, as well as in diseases presumably driven by autoreactive T lymphocytes, such as progressive multiple sclerosis and rheumatoid arthritis.³³⁻³⁷

Rituximab is also highly effective in patients with IgG₄-RD, a primarily fibrotic disorder in which the relevance of autoantibodies and T cells to fibrosis is still being explored.^{5,38} Our recent studies have implicated antibodies to galectin-3 in a subset of patients with IgG₄-RD, and others have identified autoantibodies to a recombinant truncated form of laminin 511, but definitive proof of a pathogenic role of these antibodies has not yet been demonstrated.^{39,40} Similarly, our studies have revealed expansions of CD4⁺ CTLs in patients with IgG₄-RD, as well as secretion of profibrotic cytokines and potential induction of apoptosis by these cells, but conclusive evidence that they drive fibrosis has not yet been obtained.¹⁴⁻¹⁶ Indeed, the fact that B-cell depletion has been shown to correlate with rapid improvement of tissue fibrosis in affected organs of patients with IgG₄-RD raises the hypothesis that B lymphocytes might be involved in fibrogenesis through mechanisms unrelated to either production of autoantibodies or activation of fibrogenic T cells.⁶ Despite the prominent plasma cell infiltrate found in IgG₄-RD lesions, however, the possibility that B cells could directly drive collagen deposition has not been investigated formally. The precise mechanisms through which rituximab leads to prompt clinical effects remain elusive.

In the present work, for the first time, we describe different mechanisms by which B lymphocytes can actively contribute to tissue fibrosis in patients with IgG₄-RD. In particular, we

4'-6-diamidino-2-phenylindole dihydrochloride [blue]). **B**, Sequential matched hematoxylin and eosin staining showing dense fibrosis (asterisks) likely generated as a consequence of the periductal inflammatory infiltrate described in Fig 3, A. **C**, Quantification of double-positive cells for CD19⁺ (red bars) or α -SMA⁺ (blue bars) and CCL4, CCL5, CCL11, or PDGF-B in patients with IgG₄-related AIP expressed as a percentage of total CCL4-, CCL5-, CCL11-, or PDGF-B-positive cells (ie, CD19⁺PDGF-B⁺ cells/PDGF-B⁺ cells). Bar length represents average quantification of 5 tissue slides. **D**, PDGF-B-expressing B lymphocytes infiltrating the subepithelial layer of the duodenum in a case of IgG₄-related AIP of the pancreatic head (low magnification); insets show high magnification. **E**, Immunofluorescence staining of CD19-positive (red); CCL4-, CCL5-, CCL11-, and PDGF-B-positive (all green); and 4'-6-diamidino-2-phenylindole dihydrochloride-positive (blue) cells in representative tissue samples from a patient with IgG₄-related AIP (high magnification).

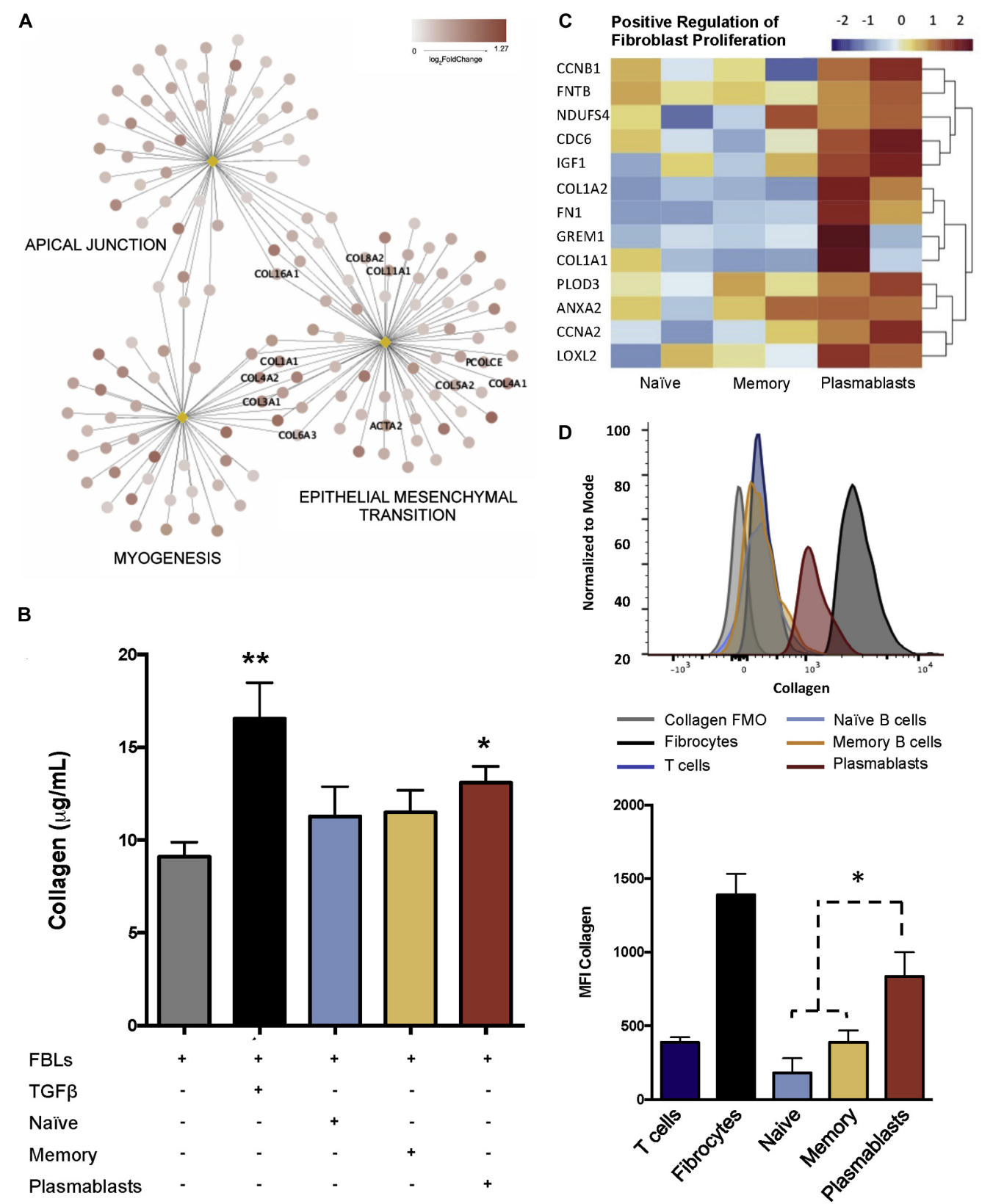


FIG 4. Plasmablasts from patients with IgG₄-RD induce collagen secretion by human fibroblasts, produce collagenous proteins, and express extracellular matrix remodeling enzymes. **A**, Top results of gene set enrichment analysis performed on log₂ fold changes of gene expression in fibroblasts cocultured with B cells from patients with IgG₄-related AIP and fibroblasts cocultured with B cells from healthy control subjects at 72 hours in the presence of semipermeable membranes (see the [Methods](#) section in this article's

demonstrate that B cells from patients with IgG₄-RD (1) stimulate collagen production by activated fibroblasts through soluble profibrotic signals, such as PDGF-B; (2) organize the extracellular matrix through specialized enzymes, such as lysyl oxidases; and (3) directly produce collagenous proteins. In addition, we demonstrate that B cells secrete the chemotactic factors CCL4, CCL5, and CCL11 and induce the production of these same chemokines by activated fibroblasts. Finally, we demonstrate that, among different B-cell subsets, plasmablasts display intrinsic fibrogenic properties because they express sets of genes implicated in fibroblast activation and proliferation.

Taken together, these novel findings add important pieces to the complex pathogenesis of IgG₄-RD and suggest a possible central role for B cells in the orchestration of fibrosis in patients with this condition. In particular, our results suggest a revised pathogenetic model of IgG₄-RD by which B lymphocytes exploit 5 major functions in addition to antibody secretion and to the presumed activation of pathogenic T lymphocytes: (1) they induce epithelial-to-mesenchymal transition of myofibroblast precursors and stimulate collagen production by activated fibroblasts, (2) they directly contribute to tissue fibrosis by secreting collagenous proteins, (3) they regulate extracellular matrix stiffness, (4) they secrete chemotactic factors for putative fibrogenic CD4⁺ CTLs and M2 macrophages, and (5) they stimulate fibroblasts to amplify the recruitment of inflammatory cells (Fig 6). Therefore depletion of B cells through strategies that target CD20 might interfere with all these processes, thereby rapidly halting fibroblast activation and collagen deposition.⁶

Another major finding of our study was the increased collagen production observed in cocultures of fibroblasts with B lymphocytes from healthy subjects compared with control fibroblasts. However, collagen production induced by B cells from healthy donors was significantly lower compared with that induced by B cells from patients with IgG₄-related AIP, even though we found similar levels of PDGF-B in supernatants of both cocultures. On the one hand, these results indicate that profibrotic mediators other than PDGF-B contribute to the increased collagen production observed in the presence of B lymphocytes from patients with IgG₄-RD. On the other hand, these data also suggest that normal B lymphocytes in the absence of disease possess some potential profibrotic properties that might contribute to normal wound healing, but activated B cells seen in a disease context are far more capable of contributing to the process of fibrosis.

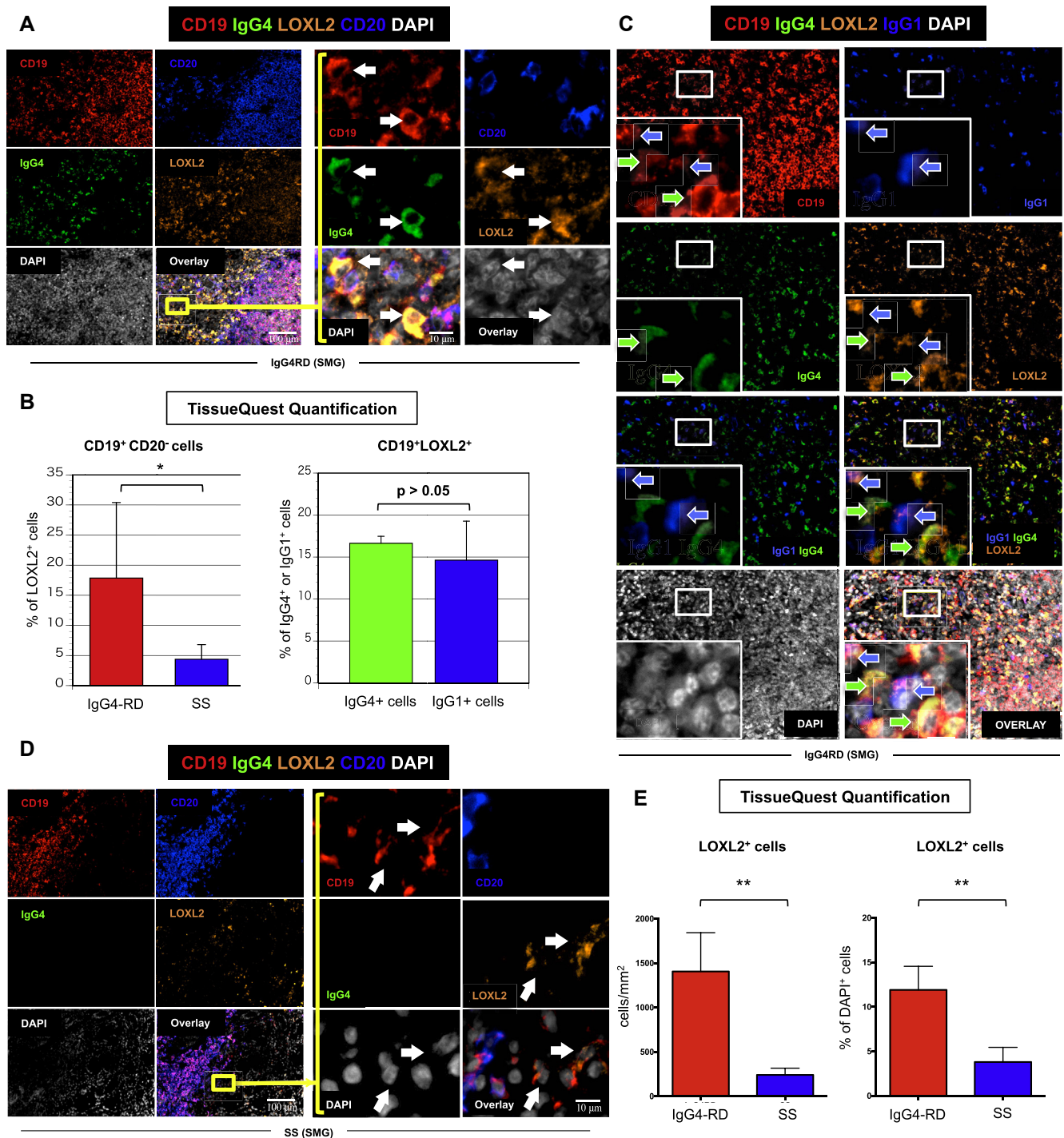
Indeed, from our previous studies, we know that a population of circulating plasmablasts is disproportionately expanded in patients with active IgG₄-RD compared with healthy subjects and patients with other inflammatory conditions, but they have not previously been causally linked to fibrotic phenomena.^{22,23}

Here we provide the first evidence that plasmablasts represent a B-cell subset with intrinsic fibrogenic potential because in contrast to both naive and memory B cells, they express higher levels of genes implicated in fibroblast activation, as well as in the pathogenesis of various human fibrotic disorders. Insulin-like growth factor 1, for instance, synergizes with PDGF-B to promote mesenchymal cell proliferation and has been linked to liver fibrosis and to stromal reaction in Grave orbitopathy.⁴¹⁻⁴³ Gremlin-1 is known for antagonizing bone morphogenetic protein and for upregulating fibroblast growth factors in different connective tissue and developmental disorders.^{44,45} LOXL2 has been causally associated with liver and pulmonary fibrosis, and clinical trials with LOXL2 inhibitors are ongoing for the treatment of various types of fibrotic diseases.^{27-31,46,47} In addition, LOXL2 and other members of the LOX family have been implicated in epithelial-to-mesenchymal transition in patients with cancer, thus contributing to acquisition of metastatic properties by neoplastic cells.^{48,49}

These profibrotic properties of expanded plasmablasts did not seem to depend on IgG class-switching or to be unique to IgG₄-RD. LOXL2 was expressed by IgG₄⁺ and IgG₁⁺ B cells in IgG₄-RD lesions, as well as by plasmablasts from patients with SS, CD, and MM, suggesting that a certain ability to regulate extracellular matrix stiffness represents a constitutive feature of some terminally differentiated antibody-producing cells. In this sense it is tempting to speculate that the abundant infiltrate of plasmablasts/plasma cells that characterizes IgG₄-RD and MM lesions is more likely to drive a fibrotic outcome compared with SS, in which the number of LOXL2⁺ B cells is far less prominent. Indeed, in contrast to SS, tissue fibrosis represents an established negative prognostic factor for end-stage organ damage in patients with IgG₄-RD and for overall survival in patients with MM.^{4,50}

Of note, although plasmablasts induced a significant increase of collagen secretion in cocultures compared with naive and memory B cells, the former were no more stimulatory than naive B cells in terms of PDGF-B, CCL4, CCL5, and CCL11 expression by fibroblasts, suggesting that plasmablasts stimulate fibroblast activation through mechanisms that differ from other B-cell subsets. The surprising observation that plasmablasts from patients with IgG₄-RD expressed type I collagen genes, for instance, raises the possibility that the increased collagen production observed in fibroblasts cocultured with plasmablasts (and B cells in general) was due at least in part to the direct secretion of collagenous proteins by B lymphocytes. Altogether, these results unveil unanticipated direct profibrotic properties of plasmablasts/plasma cells and indicate that the acquisition of these properties likely represents an intrinsic feature of plasmablasts or plasma cells that was not previously appreciated. This ability

Online Repository for details). The network has been realized with the same logic described for Fig 1, A, B. Collagen production is increased in cocultures with plasmablasts compared with control fibroblasts. Primary human skin fibroblasts (FBL), primary human skin fibroblasts stimulated with 20 ng/mL recombinant human TGF- β 1 (TGF β), and primary human skin fibroblasts cocultured with naive B cells, memory B cells, or circulating plasmablasts are shown. Results are presented as means $\pm$ SDs of 5 independent experiments. * $P < .05$. C, Heat map showing upregulation in circulating plasmablasts of genes significantly mapping to the Gene Ontology code "GO:0048146: positive regulation of fibroblasts proliferation." D, Flow cytometry showing increased collagen expression in circulating plasmablasts compared with naive and memory B cells. Circulating fibrocytes and CD3⁺ T cells are used as positive and negative controls, respectively. Overlaid histograms represent collagen expression in the different cell types in a representative patient. Histograms represent the mean fluorescence intensity (MFI) of intracellular collagen in different cell types. * $P < .05$. Results are expressed as means $\pm$ SDs of 5 experiments.



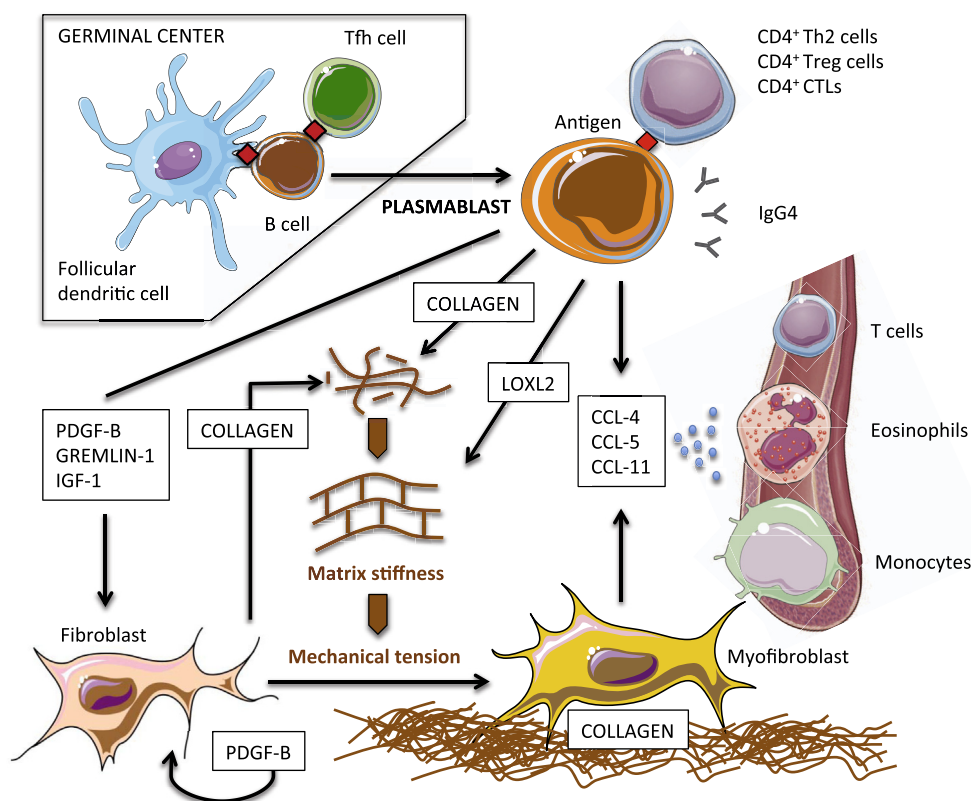


FIG 6. Revised pathogenetic model of IgG₄-RD pathogenesis: B-cell contribution to tissue fibrosis. After germinal center reaction, antigen-experienced naive B cells differentiate into class-switched plasmablasts and migrate into inflamed sites, where they contribute to tissue fibrosis in different ways. Plasmablasts initiate fibroblast differentiation into myofibroblasts through soluble factors that activate transcriptional programs implicated in mesenchymal transition. Plasmablasts also stimulate fibroblasts to secrete collagen and PDGF-B, thus amplifying fibroblast activation in an autocrine/paracrine loop. Plasmablasts directly contribute to tissue fibrosis by secreting collagenous proteins. Plasmablasts regulate extracellular matrix stiffness by secreting enzymes responsible for the cross-linking of collagen molecules, such as LOXL2. Increased extracellular matrix stiffness leads to activation of mechanoreceptors on fibroblasts, thus fostering their full differentiation into myofibroblasts. Plasmablasts attract inflammatory cells with additional fibrotic potential, such as CD4⁺ CTLs, eosinophils, and M2 macrophages, by secreting CCL4, CCL5, and CCL11 and by stimulating fibroblasts to produce these same chemokines. The pathogenic relevance of IgG₄ antibodies and of T-cell populations presumably sustained by cognate antigen-specific plasmablasts remains to be defined. IGF-1, Insulin-like growth factor 1; Tfh, follicular helper T; Treg, regulatory T.

to secrete profibrotic molecules is likely acquired at a discrete stage of terminal antigen-driven B-cell differentiation, and this subset of LOXL2-secreting plasmablasts/plasma cells is enriched in the tissues of certain fibrotic diseases.

Finally, we have demonstrated both *in vitro* and in affected tissues that B cells could potentially contribute to orchestrating the inflammatory infiltrate in patients with IgG₄-RD by directly secreting the chemotactic factors CCL4, CCL5, and CCL11 for cell types with fibrogenic properties typically found in IgG₄-RD lesions, such as T lymphocytes, macrophages, and eosinophils. B cells also stimulate fibroblast secretion of these chemokines, thus amplifying the recruitment of inflammatory cells at sites of disease. In particular, CCL4 is a chemokine involved in monocyte recruitment and activation by engaging the chemokine receptor CCR5⁵¹; CCL5 attracts T cells, eosinophils, and basophils by interacting with CCR1, CCR3, and CCR5⁵²; and CCL11 selectively recruits eosinophils through CCR2, CCR3, and CCR5.⁵³ Interestingly, CCL4, CCL5, and CCL11 secreted by both B lymphocytes and fibroblasts might also recruit CD4⁺ CTLs, a population of

CD4⁺ T lymphocytes with cytotoxic properties that is increasingly considered central to IgG₄-RD pathogenesis.^{15,16} CD4⁺ CTLs, in fact, express high levels of CCR5, and their decrease observed after rituximab treatment might be due to depletion of B cells responsible for their recruitment at disease sites.¹⁶

Our study is not the first to address a potential role of B cells in disease-related fibrogenesis. In fact, regulatory B cells are known to produce the profibrotic cytokines TGF- β and IL-10, but their contribution to tissue fibrosis has never been assessed in specific disease settings.⁷ Putative autoreactive B-cell clones are presumed to produce anti-PDGF receptor and anti-matrix metalloproteinase 1 autoantibodies in patients with systemic sclerosis, but the relevance *in vivo* of these antibodies is yet to be confirmed.^{8,13} More recently, 2 different studies on chronic lymphocytic leukemia and systemic sclerosis have specifically investigated the role of B lymphocytes in activating stromal cells. Ding et al¹¹ demonstrated that B cells from patients with chronic lymphocytic leukemia produce PDGF and that conditioned medium obtained from these cells activates marrow stromal cells through

PDGF receptor activation. Francois et al¹² showed that B cells from healthy subjects induce collagen production by skin fibroblasts from patients with scleroderma through contact-mediated mechanisms that depend on TGF- β production. In this sense our work not only confirms the production of PDGF by activated B lymphocytes, as well as the profibrotic properties of B cells from healthy subjects, but also unveils and extensively characterizes a fully functional profibrotic machinery in B lymphocyte that makes them an unexpected professional key player in wound-healing processes and fibrogenesis. Whether the prominent plasma cell infiltrate that is typically observed in IgG₄-RD lesions is the major cell type responsible for the characteristic tissue fibrosis seen remains difficult to conclude in the absence of animal models. Yet the results of our study and the prompt response of IgG₄-RD lesions to B-cell depletion with rituximab strongly support this possibility.

Our approaches have both potential strengths and weaknesses. Major potential strengths are (1) the adoption of 2 different human mesenchymal stromal cells (pancreatic and dermal fibroblasts) for coculture experiments, (2) the use of B lymphocytes from both healthy donors and patients with active untreated IgG₄-RD, and (3) the unbiased approach adopted to identify relevant profibrotic and immunomodulatory molecules in our system. In fact, pancreatic fibroblasts might have allowed us to recapitulate some of the possible interactions occurring between fibroblasts and B cells in patients with AIP, the prototypical clinical-pathologic scenario of IgG₄-RD. On the other hand, whole-transcriptome analysis represented the most powerful instrument to broadly detect changes in fibroblast gene expression induced by B lymphocytes, as well as profibrotic pathways in B-cell subsets, before focusing on specific molecules of interest. In addition, it is noteworthy that all the reported experiments were performed on human samples from 3 referral centers for IgG₄-RD, thus ensuring accurate patient selection, definitive diagnosis, and robust confirmation of our results on different study cohorts.

The major limitation of our work is that we only provide correlative *in vitro* and histologic data because reliable animal models of IgG₄-RD suitable for confirmatory pathogenic *in vivo* studies have not yet been developed. Indeed, IgG₄-RD represents a rare condition, and immunopathologic pathways amenable to mouse modeling remain unknown. Also, given that our work focused on IgG₄-RD, we limited our tissue analysis on IgG₄ class-switched B lymphocytes, but we cannot exclude that profibrotic properties of B cells are shared by class-switched plasmablasts in general.

In conclusion, our work provides novel insights into the pathophysiology of IgG₄-RD and unveils unexpected profibrotic properties of B lymphocytes in human subjects over and beyond the prevalent view of B cells functioning primarily as antigen-presenting cells and providers of surviving signals to putative pathogenic T cells. Because it is likely that the mechanisms described here are not unique to IgG₄-RD, selective targeting of profibrotic B-lymphocyte subpopulations and/or profibrotic mediators, such as PDGF-B and LOXL2, might offer alternative therapeutic approaches for IgG₄-RD and for other fibrosing conditions characterized by B-cell activation and lymphoplasmacytic infiltration.

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Key messages

- B cells from patients with IgG₄-RD produce profibrotic cytokines, chemokines, and enzymes implicated in extracellular matrix remodeling.
- Plasmablasts represent B-cells with intrinsic pro-fibrotic properties since they express sets of genes implicated in fibroblast activation and proliferation.
- The discovery that B cells contribute directly to tissue fibrosis in IgG₄-RD might be relevant for fibrogenesis in other fibro-inflammatory disorders and for wound healing processes in physiological conditions.

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METHODS

Study population

PBMCs were obtained from 20 patients with IgG₄-RD referred to the rheumatology clinics of San Raffaele Scientific Institute (Milan, Italy) and Massachusetts General Hospital (Boston, Massachusetts) between September 2015 and September 2017 (Table E1). All patients with IgG₄-RD had pancreatic involvement, either as their unique manifestation or as part of a multiorgan disease. Five sex- and age-matched healthy donors were included as experimental control subjects. Blood samples for the studies performed were drawn during active disease at the time of diagnosis before the institution of immunosuppressive treatment. Disease activity was defined according to the IgG₄-RD Responder Index, and active disease was considered for values of greater than 3.^{E1,E2}

The following tissue biopsy specimens from the pathology units of Massachusetts General Hospital, Kyushu University Hospital, and San Raffaele Hospital were used for immunohistochemistry and immunofluorescence studies: submandibular glands from patients with IgG₄-RD (n = 5), pancreatic glands from patients with IgG₄-RD (n = 5), submandibular glands from patients with SS (n = 5), inguinal lymph nodes and cervical lymph nodes from patients with CD (n = 3), bone marrow from patients with MM (n = 3), and tonsils from patients undergoing tonsillectomy (n = 1).

For immunofluorescence staining of healthy pancreatic tissue, we used disease-free margin pancreatic tissue from 5 patients who underwent tumor resection. IgG₄-RD was diagnosed according to the "Consensus statement on the pathology of IgG₄-related disease."^{E3} Patients with isolated pancreatic involvement who did not undergo pancreatic resection were given a diagnosis of "definite" IgG₄-RD according to the "International consensus diagnostic criteria for autoimmune pancreatitis."^{E4} SS was diagnosed as described based on objective evidence of salivary gland involvement, subjective xerostomia, decreased salivary flow rate, abnormal findings on parotid sialography, and focal lymphocytic infiltrates in labial salivary glands.^{E5}

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 Committee of the San Raffaele Scientific Institute and Massachusetts General Hospital.

Isolation of B cells from peripheral blood

PBMCs were freshly isolated from patients with IgG₄-RD and healthy blood donors by using Ficoll-Paque centrifugation on the same day of the blood draw, as described in standard protocols. For fibroblast/CD19⁺ B-cell cocultures, CD19⁺ B cells were selected by means of negative sorting with the EasySep Human B Cell Enrichment Kit (STEMCELL Technologies, Grenoble, France). The purity of B-cell isolation was determined with fluorescence-activated cell sorting analysis by using the following antibodies from BioLegend (San Diego, Calif): anti-human CD19–Pacific Blue (clone HIB19), anti-human CD3–phycoerythrin (clone OKT3), and anti-human CD14–fluorescein isothiocyanate (FITC; clone HCD14). The yield of isolated B cells was as follows: 99% CD3[−]CD14[−]CD19⁺ B cells, 0.02% CD3⁺CD14[−]CD19[−] T cells, and 0.06% CD3[−]CD14⁺CD19[−] monocytes. For fibroblast/B-cell subset cocultures and RNA-sequencing studies, naive B cells, memory B cells, and plasmablasts were stained as described below, analyzed by using fluorescence-activated cell sorting, and sorted. The recovery rate of each B-cell subset (purity) determined on the cell sorter was 98%. Viability of isolated B cells and B-cell subpopulations was assessed with an Automated Cell NucleoCounter NC-200 (ChemoMetec, Lillerød, Denmark) and ranged between 96% and 99%. Isolated CD19⁺ B cells and B-cell subpopulations were immediately seeded on preplated fibroblasts, as described below.

Flow cytometry and cell sorting

To preserve cell viability, freshly isolated PBMCs were stained with relevant cell-surface markers in Iscove modified Dulbecco medium (IMDM), resuspended at approximately 20 million cells/mL in HBSS (Gibco) plus

10 mmol/L glucose, and sorted on a BD FACSARIA II (BD Biosciences, San Jose, Calif). Cell viability for gating purposes was assessed by using the LIVE/DEAD Fixable Blue Dead Cell Stain kit (Thermo Fisher, Cambridge, Mass), according to the manufacturer's instructions. Except where indicated, all antibodies were procured from BioLegend. The following mAbs were used for surface staining: anti-human CD3–Alexa Fluor 700 (clone UCHT1), CD19–allophycocyanin/Cy7 (clone SJ25C1), anti-human CD20–BUV395 (clone 2H7; BD Biosciences), anti-human CD27–BV510 (clone O323), anti-human CD38–phycoerythrin/Cy7 (clone HIT2), and anti-human IgD–BV785 (clone IA6-2). For intracellular staining of type I collagen–FITC (Rockland Immunochemicals, Limerick, Pa) on B cells, cells were fixed and permeabilized with the BD Cytotfix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions, and then stained in permeabilization buffer at 4°C for 30 minutes.

For coculture experiments, 5×10^3 naive B cells, memory B cells, and plasmablasts were readily sorted in 1.5-mL tubes containing 300 μ L of culture medium (IMDM supplemented with 10% FBS, 1% L-glutamine, penicillin, and streptomycin; all from Thermo Fisher) and seeded in 96-well plates on preplated fibroblasts, as described below. For RNA extraction from B-cell subsets, 1×10^3 cells were directly sorted in RLT buffer from Qiagen containing 1% 2-mercaptoethanol (Pierce/Thermo Scientific). Gating strategies for flow cytometric analysis and cell sorting of naive B cells, memory B cells, plasmablasts, and fibrocytes are depicted in Fig E1.

Establishment of primary human pancreatic and skin fibroblast cell lines

Primary cultures of human pancreatic fibroblasts were established from the digest remaining after isolation of islet cells from human healthy pancreas, as previously described.^{E6} In particular, pancreata were obtained from heart-beating cadaveric multiorgan donors through the North Italian Transplant Organization. The dense fraction recovered in the pellet and normally discarded was processed for fibroblast isolation. After 2 washes in PBS, the equivalent of a 1-mL packed pellet was resuspended in Minimum Essential Medium (MEM) with 10% FBS, plated in one T75 tissue culture–treated flask (Costar), and grown at 37°C in a humidified incubator at 5% CO₂. After 24 hours, nonadherent material was removed, and fresh medium was added to the cells. Medium was changed every 3 days. Cells were expanded in α -MEM with 10% FBS, 1% L-glutamine, and 10 ng/mL β fibroblast growth factor. When 80% confluency was reached, cells were trypsinized and stored in α -MEM 10% dimethyl sulfoxide in liquid nitrogen until use. In all experiments pancreatic fibroblasts were used until passage 10. Human skin fibroblasts were purchased from Lonza (NHDF-Ad-Der Fibroblasts FGM-2, cryopreserved ampule; Lonza, Basel, Switzerland). Pancreatic and skin fibroblasts were used for coculture experiments with total B lymphocytes to demonstrate that the profibrotic effect mediated by B cells was unrelated to the type of fibroblast used. Once it was shown that B lymphocytes induced similar levels of collagen production in coculture with either pancreatic or skin fibroblasts, we only used skin fibroblasts for coculture experiments with B-cell subsets because they were commercially available and did not require time-consuming procedures to be isolated from human pancreas.

Coculture experiments

Coculture experiments were carried out with B cells from patients with IgG₄-RD and pancreatic involvement and primary human pancreatic fibroblasts to reproduce *in vitro* some of the possible interactions between these 2 cell types central to the pathogenesis of IgG₄-related AIP. B cells and fibroblasts were cocultured in IMDM 10% FBS 1% penicillin/streptomycin for a total of 3 days.

For fibroblast/CD19⁺ B-cell cocultures (Fig E2), 5×10^4 fibroblasts were seeded in 24-well plates either alone or in the presence of CD19⁺ B cells (3×10^5 or 1.5×10^5 cells; fibroblast/B-cell ratio, 1:6 or 1:3). For transwell experiments, CD19⁺ B cells (3×10^5 or 1.5×10^5 cells) and fibroblasts (5×10^4 cells) were seeded in the upper and lower chambers, respectively,

of a 0.4- μ m polycarbonate semipermeable membrane (Nunc Dominique Dutscher). B cells were also seeded alone to check their viability along the experiment. Between 70% and 88% of B cells were alive at day 3 when cultured alone ($n = 15$ independent experiments), whereas 80% to 95% were alive after coculture with fibroblasts ($n = 15$ independent experiments). Sixty-seven percent to 73% of fibroblasts were alive at day 3 regardless of the presence of B cells in cocultures ($n = 20$ independent experiments). For fibroblast stimulation, cells were incubated with 20 ng/mL recombinant human TGF- β 1 (R&D Systems, Lille, France) or 10 ng/mL recombinant human PDGF-B (R&D Systems). For PDGF-B inhibition experiments, fibroblasts were preincubated with 0.5 μ g/mL human PDGF-B antibody (clone AF-220-FP) or isotype IgG control (all from R&D Systems) for 2 hours before either PDGF-B stimulation or addition of CD19⁺ B cells.

For fibroblast/B-cell subset co-cultures (Fig E3), fibroblasts (5×10^3 cells) were seeded in 96-well plates either alone or in the presence of naive B cells (5×10^3 cells), memory B cells (5×10^3 cells), or plasmablasts (5×10^3 cells). B-cell subsets were also seeded alone to check their viability along the experiment. Viability of fibroblasts, CD19⁺ B cells, naive B cells, memory B cells, and plasmablasts was assessed after 3 days of culture by using the NucleoCounter NC-200 (ChemoMetec) as well as by using flow cytometry with the LIVE/DEAD Fixable Blue Dead Cell Stain kit (Thermo Fisher), according to the manufacturer's instructions. Vital B cells and fibroblasts excluded the LIVE/DEAD dye. Fibroblast proliferation was assessed after 3 days of culture by using flow cytometry with an optimized concentration of the anti-Ki67-allophycocyanin antibody (clone Ki67; BioLegend), using a fibroblast gate set through forward-angle and side-angle light-scatter characteristics.

Measurement of soluble collagen and cytokine levels

Soluble collagen concentrations in supernatants of fibroblast/CD19⁺ B-cell cocultures were evaluated by using the Sircol collagen assay (Biocolor Interchim), according to the manufacturer's instructions, with minor modifications. Briefly, 100 μ L of supernatant was mixed with 1 mL of Sircol dye reagent for 30 minutes. After centrifugation, pellets were washed with Acid-Salt Wash Reagent and finally dissolved in 1 mL of Sircol alkali reagent. Relative absorbance was measured at 540 nm.

Soluble collagen concentrations in supernatants of fibroblast/B-cell subpopulation cocultures were evaluated by using the Pro-collagen I α 1 ELISA Kit (R&D Systems) and a standard curve according to the manufacturer's protocol. Cytokines and chemokines present in supernatants of fibroblast/CD19⁺ B-cell cocultures were measured with the Bio-Plex Pro Human Cytokine Grp I Panel 27-plex assay, according to the manufacturer's instruction, with the Bio-Plex 200 Suspension Array System (Bio-Rad Laboratories).

Quantitative real-time PCR analysis

Total RNA was extracted from cultured fibroblasts by using the ReliaPrep RNA Cell Miniprep System (Promega), according to the manufacturer's recommendations. Total RNA (1 μ g) was reverse transcribed with random hexameric primers and MultiScribe reverse transcriptase (Applied Biosystems), according to the manufacturer's instructions. Real-time quantitative PCR was performed in a total volume of 15 μ L by using the KAPA SYBR FAST qPCR Master Mix (2 $\times$) Kit (Bio-Rad Laboratories). After an initial incubation at 95°C for 3 minutes, samples were subjected to 40 rounds of amplification for 10 seconds at 95°C, 20 seconds at 60°C, and 1 second at 72°C by using a Roche LightCycler (Roche Molecular Diagnostics). Melting-curve analysis was performed to assess the specificity of PCR products. The primers used were as follows: human *GAPDH* forward (5'-GGTGAAGTCCGAGTCAACGGA-3') and reverse (5'-GAGGGATCTCGCTCCTGGAAGA-3'); human *COL1A1* forward (5'-CACACGTCTCGGTCAATGGTA-3') and reverse (5'-CGGCTCCTGCTCCTCTTAG-3'); human *COL1A2* forward (5'-AGCAGGTCTTGGAAACCTT-3') and reverse (5'-GAAAAGGAGTTGGACTTGGC-3'); human *COL3A1* forward (5'-ATATTTGGCA

TGGTTCTGGC-3') and reverse (5'-TGGCTACTTCTCGCTCTGCT-3'); human *ACTA2* forward (5'-GATGGCCACTGCCGATCCT-3') and reverse (5'-ACAGGTCTCTGGCAGCGG-3'); and human *CD19* forward (5'-CTCCACACTCTGGCTGTCT-3') and reverse (5'-CAGCAGCCAGTGC-CATAGTA-3'). Cycle threshold (Ct) values of greater than 35 were considered negative. Data points were analyzed in triplicate. Fold quantification of a specific gene was calculated by using the comparative Ct method normalized for the housekeeping gene *GAPDH*, according to the following equation:

$$\text{Fold} = 2^{-(\Delta\Delta C_t)}$$

Fibroblast RNA contamination by B cells was minimized by means of repeated washing of the plated fibroblasts with PBS before harvesting. Further possible contamination was excluded by means of negative qPCR for the *CD19* gene on RNA extracted from fibroblasts.

RNA-sequencing analysis

For RNA extraction, cells were directly sorted in RLT buffer from Qiagen containing 1% 2-mercaptoethanol (Pierce/Thermo Scientific). RNA was isolated with the RNeasy Plus Micro kit (Qiagen). Libraries were prepared with SmartSeq v2 protocol Superscript II Reverse Transcriptase (Thermo Fisher Scientific).^{E7} Libraries were quantified by using the Quant-iT DNA Assay Kit at high sensitivity (Thermo Fisher Scientific) before tagmentation. Libraries were then quantified by using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific) with a Qubit 3.0 Fluorometer and sequenced with the NextSeq 500/550 High Output v2 kit (75 cycles) on the Illumina NextSeq sequencer.

Raw sequencing reads (FASTQ) were mapped to the human reference genome (GRCh38) with STAR aligner (version 2.5.3a),^{E8} and expression was quantified at the gene level with featureCounts^{E9} by using GENCODE basic gene annotation (version 26). Differential gene expression analysis was performed with DESeq2,^{E10} and model fitting was made, taking into account as covariates the different fibroblast donors and the method of coculture of fibroblasts with B cells.

For the first differential expression analysis, where the contrast called was between fibroblasts cocultured with B cells from patients with IgG₄-RD in the absence of semipermeable membranes and fibroblasts alone, raw counts were prefiltered to consider as expressed only those genes with at least 10 counts in at least half of the samples ($n = 6$) to exclude some very low-count genes otherwise retained by using DESeq2 independent filtering. Fibroblast RNA contamination by B cells in general was minimized by repeated washing of the plated fibroblasts with PBS before harvesting and further ruled out by excluding samples from the bioinformatics analysis that contained detectable *CD19*, *CD20*, or immunoglobulin *IGH* gene counts.

Gene set enrichment analysis^{E11} was carried out on preranked gene log₂ fold changes from DESeq2 by using the hallmark gene sets from the Molecular Signatures Database (MSigDB) Collections of the Broad Institute^{E12} and a gene set enriched with FDR q-values of less than 0.01 considered. The FDR was calculated by comparing actual data with 1000 Monte Carlo simulations. The normalized enrichment score was used to compute the density of modified genes in the data set with the random expectancies normalized by the number of genes found in a given gene cluster to take into account the size of the cluster. The network graphs were generated with Cytoscape 3.6^{E13} by showing top-scoring enriched gene sets selected from the hallmark collection of the MSigDB.^{E12}

Immunofluorescence and immunohistochemistry

For immunofluorescence studies, pancreatic, submandibular, and lymph node tissue samples were fixed in formalin, embedded in paraffin, and sectioned. Specimens were incubated with anti-human CD19 (clone 2E2B6B10; Abcam, Cambridge, Mass), CD20 (clone: L26; Novus Biologicals, Centennial, Colo), α -SMA (clone E184; Abcam), PDGF-B (clone MM0014-5F66; Abcam), CCL5/RANTES (clone MAB278-SP; R&D Systems), CCL4/macrophage inflammatory protein 1 β (clone EP494Y; Abcam), CCL11/eotaxin (clone MAB320-SP; R&D Systems), IgG₁ (clone

OAAF05805; Aviva Systems Biology, San Diego, Calif), IgG₄ (clone ab109493; Abcam), and LOXL2 (clone 8B2; LifeSpan Biosciences, Seattle, Wash) antibodies followed by incubation with secondary antibody using a SuperPicTure Polymer Detection Kit (Invitrogen, Carlsbad, Calif) and an Opal 3-Plex Kit (fluorescein, Cy3, Cy5, and Alexa Flour 750; PerkinElmer, Waltham, Mass). The samples were mounted with ProLong antifade-containing 4'-6-diamidino-2-phenylindole dihydrochloride (Invitrogen). Images were acquired with the TissueFAXS platform (TissueGnostics, Vienna, Austria).

For quantitative analysis, the entire area of the tissue involved by the lymphoplasmacytic infiltrate was acquired as digital grayscale images in 4 channels with filter settings for FITC, Cy3, and Cy5 in addition to 4'-6-diamidino-2-phenylindole dihydrochloride. Cells of a given phenotype were identified and quantitated with TissueQuest software (TissueGnostics), with cutoff values determined relative to the negative controls. This microscopy-based multicolor tissue cytometry software permits multicolor analysis of single cells within tissue sections similar to flow cytometry. The principle of the method and the algorithms used have been described in detail elsewhere.^{E14}

For immunohistochemistry studies, EDTA whole bone marrow blood samples were prepared by means of smearing on a slide, air-dried, and incubated with anti-human LOXL2 antibody (clone LS-A9350; LifeSpan Biosciences) at 1:100 dilution.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software 6.0 (GraphPad Software, La Jolla, Calif). Normal distribution of continuous variables was assessed with the D'Agostino & Pearson omnibus normality test. Nonnormally distributed variables were compared by using the Mann-Whitney *U* test. Unpaired *t* tests were used to perform comparisons between 2 groups. A *P* value of less than .05 was considered significant. Continuous variables are expressed as means $\pm$ SDs, unless otherwise specified.

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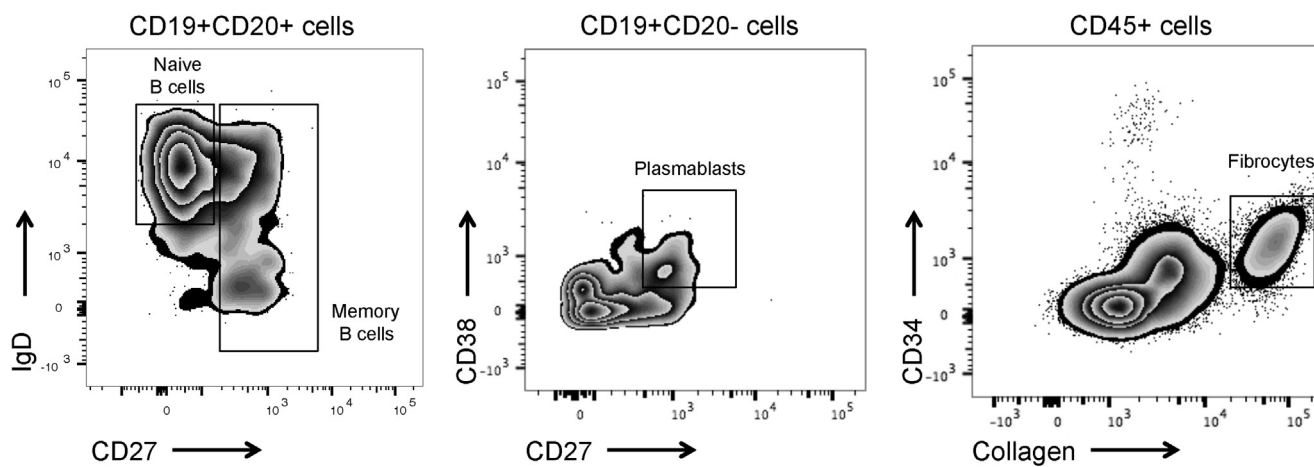


FIG E1. Gating strategy for flow cytometric analysis and cell sorting of naive B cells, memory B cells, circulating plasmablasts, and circulating fibrocytes.

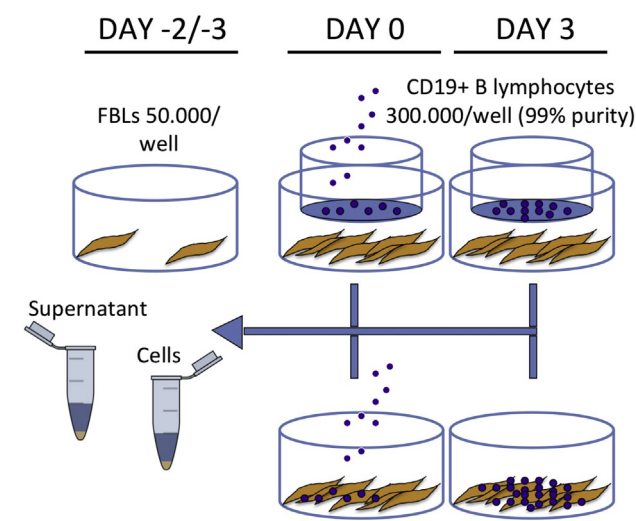


FIG E2. Schematic cartoon of primary human fibroblast (*FBL*) cocultures with total CD19⁺ B cells.

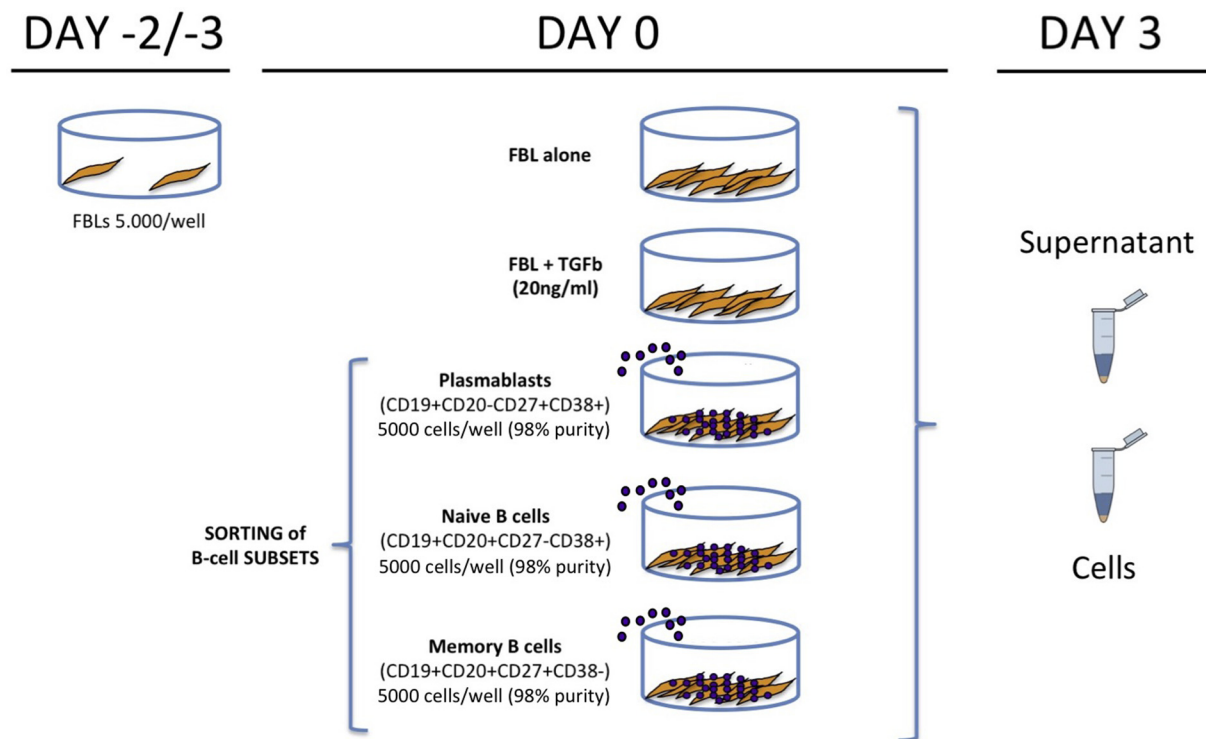


FIG E3. Schematic cartoon of primary human fibroblasts co-cultures with B cell subpopulations.

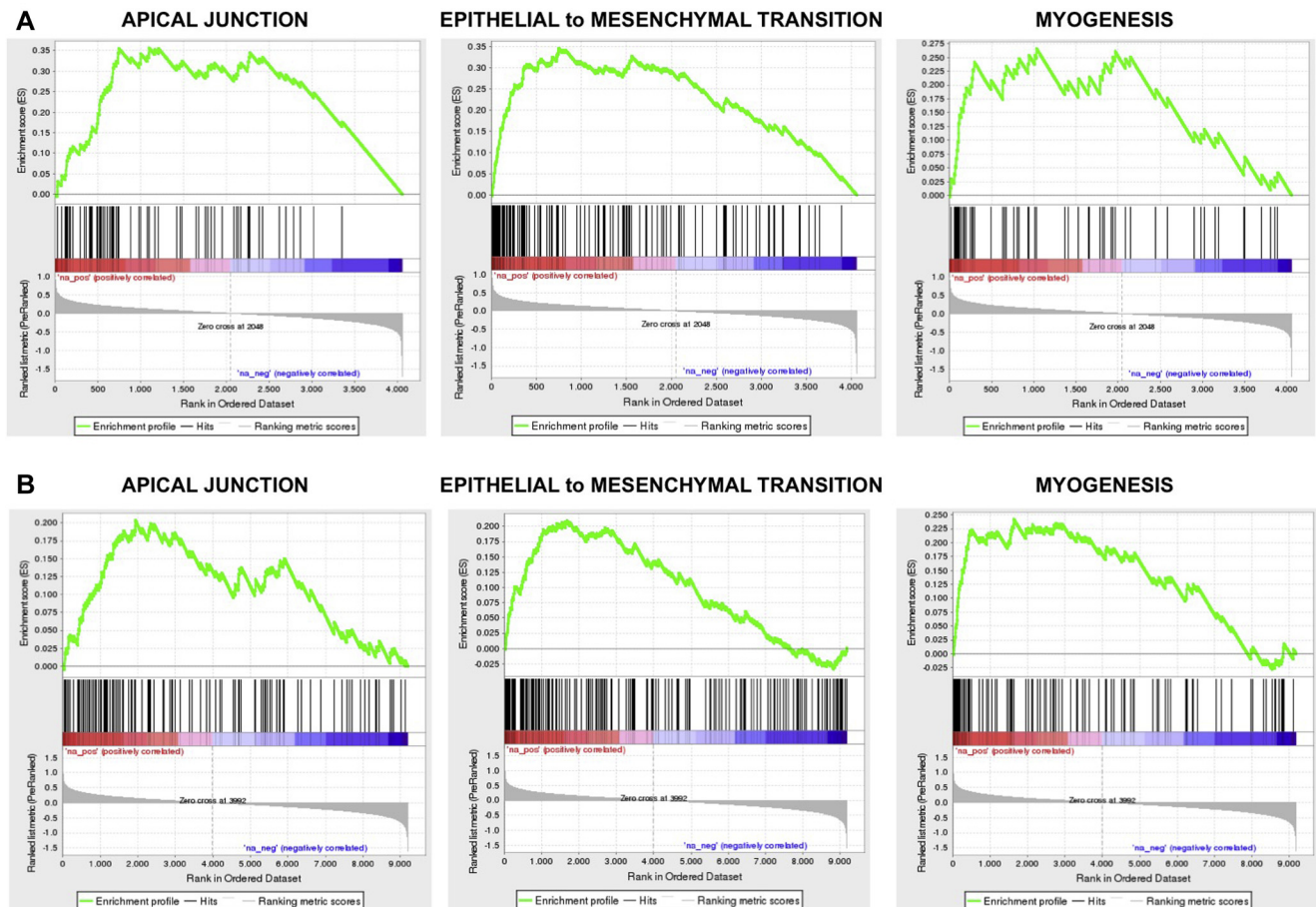
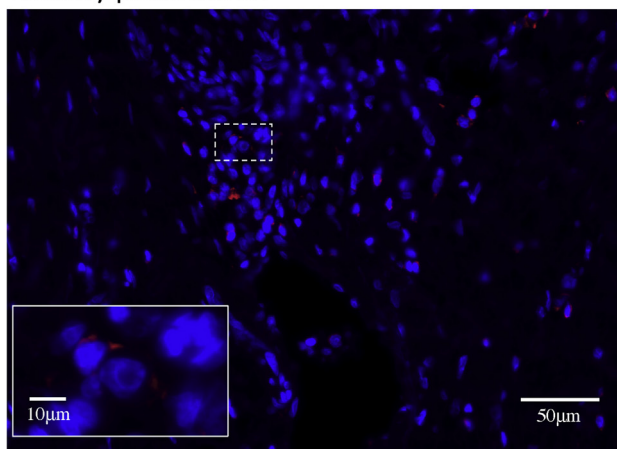


FIG E4. Transcriptional changes in the human pancreatic fibroblast transcriptome after coculture with CD19⁺ B lymphocytes from patients with IgG₄-related AIP and healthy donors. **A**, Gene set enrichment analysis (GSEA) performed on log₂ fold changes between gene expression of fibroblasts cocultured with B cells from patients with IgG₄-related AIP and control fibroblasts at 72 hours. The GSEA enrichment plot for the top 3 significant enriched gene sets are as follows: apical junction, epithelial-to-mesenchymal transition, and myogenesis. The *green curve* represents the running sum of the weighted enrichment score for the 3 gene sets: apical junction (normalized enrichment score [NES] = 3.64; FDR q-value = 0.0), epithelial-to-mesenchymal transition (NES = 4.59; FDR q-value = 0.0), and myogenesis (NES = 2.38; FDR q-value = 0.0027). **B**, GSEA performed on log₂ fold changes between gene expression of fibroblasts cocultured with B cells from patients with IgG₄-related AIP and human pancreatic fibroblasts cocultured with CD19⁺ B lymphocytes from healthy control subjects at 72 hours. The GSEA enrichment plot shows the top 3 significant enriched gene sets as follows: apical junction, epithelial-to-mesenchymal transition, and myogenesis. The *green curve* represents the running sum of the weighted enrichment score for the 3 gene sets: apical junction (NES = 2.61; FDR q-value = 2.17E-04), epithelial-to-mesenchymal transition (NES = 3.34; FDR q-value = 0.0), and myogenesis (NES = 3.03; FDR q-value = 0.0).

Healthy pancreas #1



Healthy pancreas #2

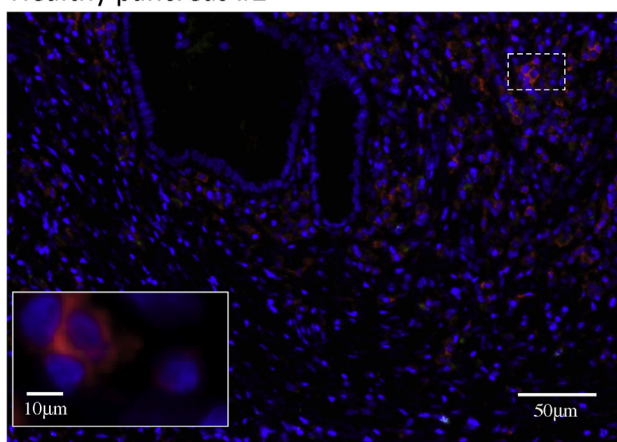


FIG E5. Rare CD19⁺ B lymphocytes are found in healthy pancreatic tissue. B cells in healthy pancreatic tissue were observed rarely and either isolated or aggregated in nests of 3 to 4 cells. Immunofluorescence staining of CD19-positive (red) and 4'-6-diamidino-2-phenylindole dihydrochloride-positive (blue) cells in 2 representative healthy pancreatic tissue samples from 2 different patients (low magnification); *insets* show high magnification.

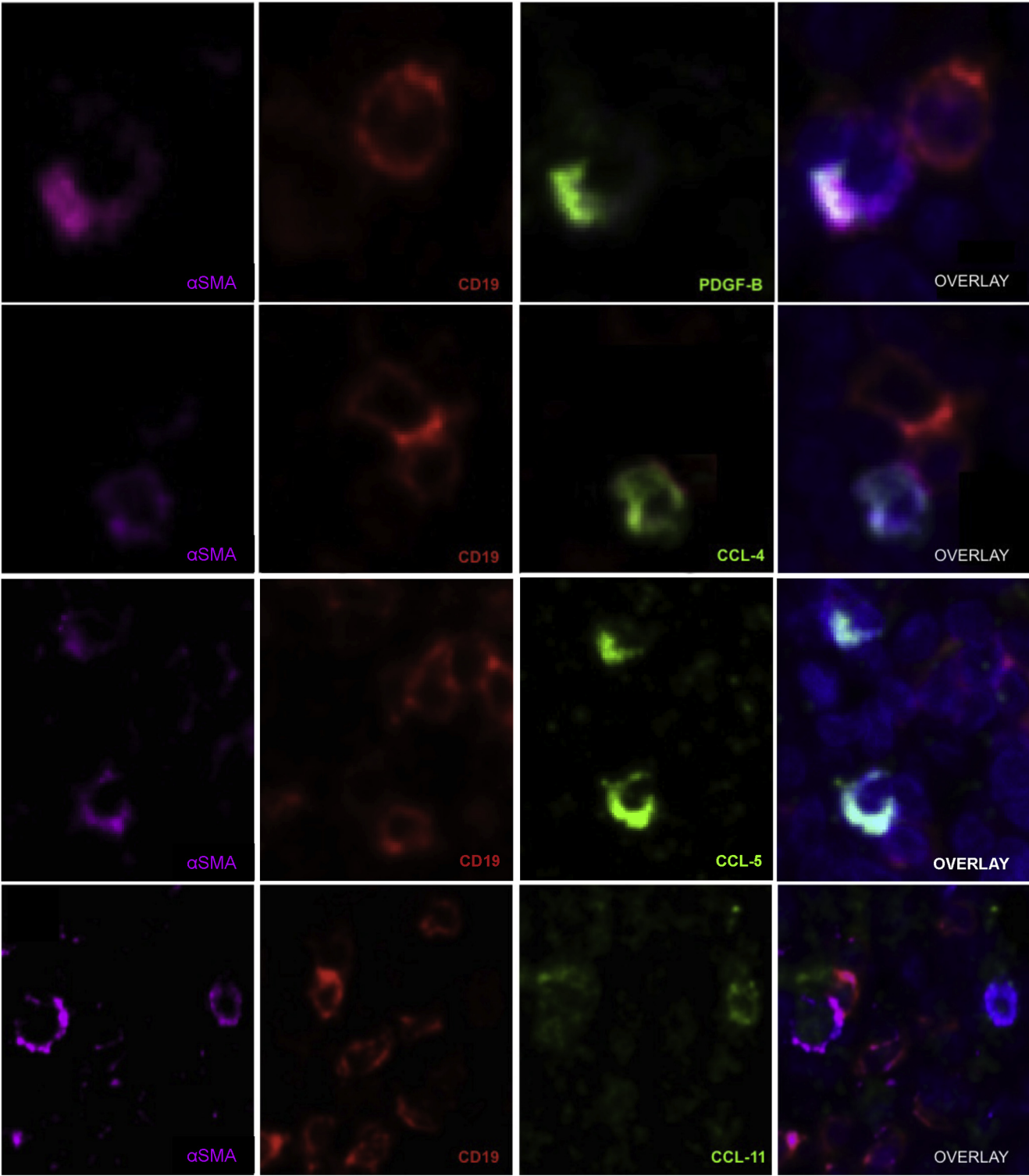


FIG E6. Fibroblasts infiltrating patients with IgG₄-related AIP express PDGF-B, CCL4, CCL5, and CCL11 and are found in close proximity to CD19⁺ B lymphocytes. Immunofluorescence staining of CD19-positive (red); α-SMA-positive (magenta); PDGF-B-, CCL4-, CCL5-, and CCL11-positive (all green); and 4'-6-diamidino-2-phenylindole dihydrochloride-positive (blue) cells in representative tissue samples from a patient with IgG₄-related AIP is shown. In some areas close proximity of CD19⁺ B cells and α-SMA⁺ myofibroblasts is observed, possibly leading to cytokine and chemokine production by myofibroblasts.

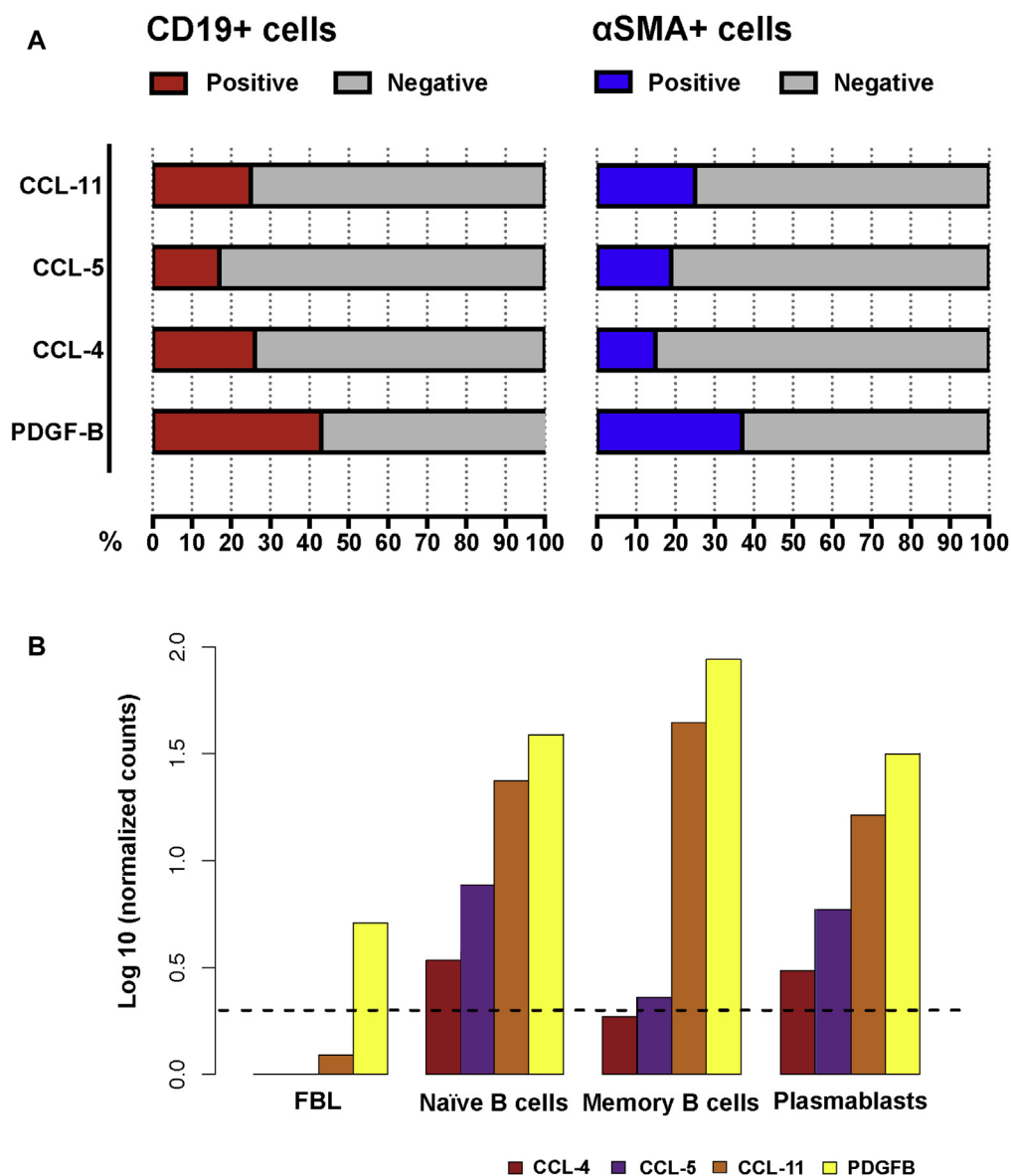


FIG E7. A, PDGF-B, CCL4, CCL5, and CCL11 are expressed by a subset of CD19⁺ B lymphocytes and α-SMA⁺ myofibroblasts. Quantification of double-positive cells for CD19⁺ or α-SMA⁺ and CCL4, CCL5, CCL11, or PDGF-B in patients with IgG₄-related AIP expressed in percentages of total CD19⁺ or α-SMA⁺ cells (ie, CD19⁺PDGF-B⁺ cells/CD19⁺ cells) is shown. *Bar length* represents the average quantification of 5 tissue slides. *Red and blue bars* indicate percentages of B cells and myofibroblasts, respectively, expressing CCL4, CCL5, CCL11, or PDGF-B. *Gray bars* indicate percentages of B cells and myofibroblasts not expressing these molecules. **B**, Expression levels of selected genes in primary human pancreatic fibroblasts. Pancreatic fibroblasts were either cultivated alone (*FBL*) or in the presence of naïve B cells, memory B cells, or plasmablasts from patients with IgG₄-related AIP, as outlined in Fig E2. CCL11 and PDGF-B expression was significantly increased by all 3 B-lymphocyte subpopulations compared with that in control fibroblasts. Fibroblast expression of CCL4 and CCL5 was induced by naïve B cells and circulating plasmablasts but not by memory B cells. The *y-axis* indicates normalized read counts after log₁₀ transformation, the *x-axis* shows different conditions, and the *dashed line* indicates the threshold to define whether a gene was expressed. *Bars* represent means of 5 different experiments. **P* < .05 in comparison with fibroblasts alone.

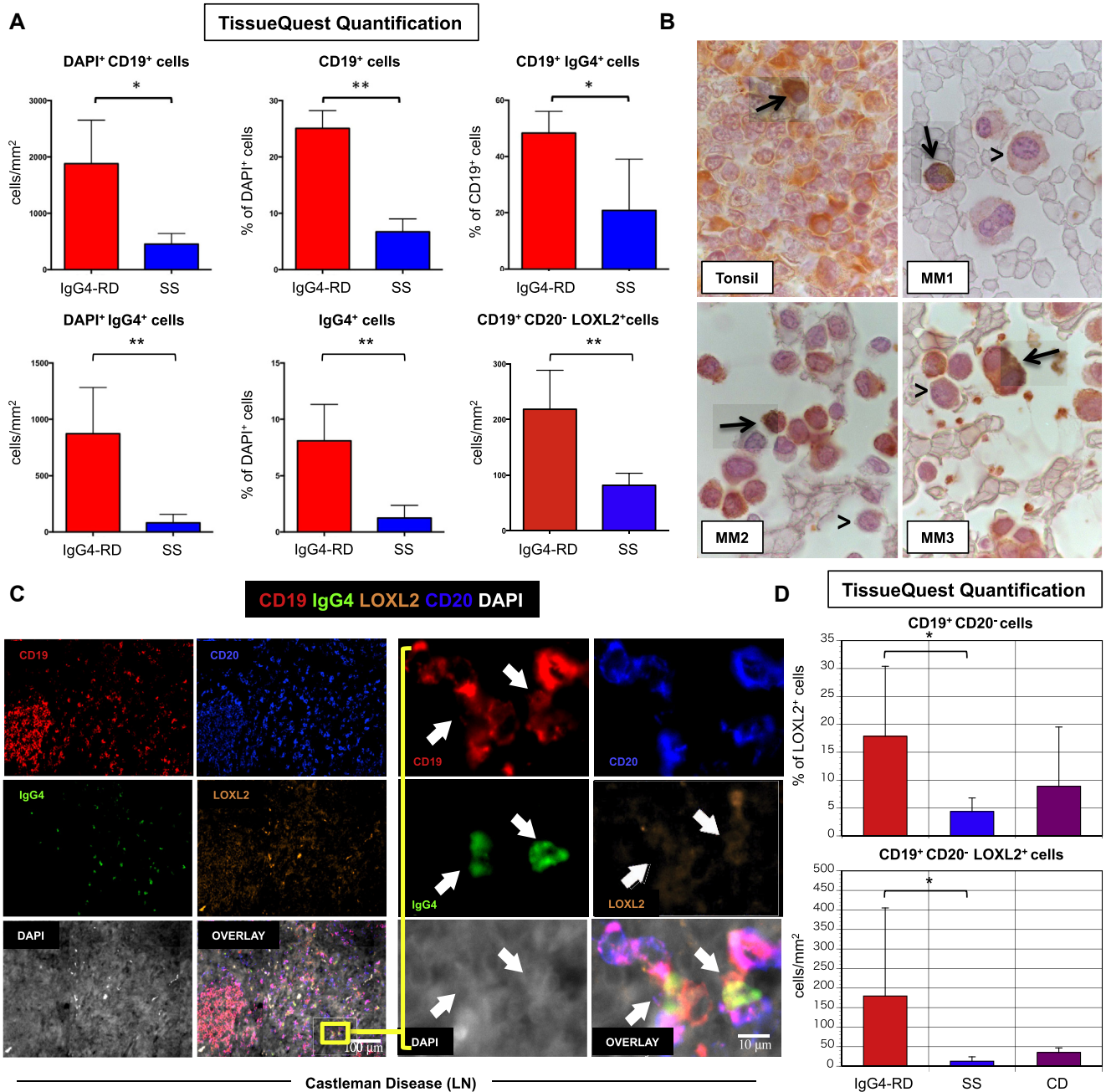


FIG E8. LOXL2 expression by plasmablasts in patients with SS, CD, and MM. **A**, Quantification of CD19⁺ and IgG4⁺ B lymphocytes and LOXL2-expressing plasmablasts in salivary glands from patients with IgG4-RD (red bars) compared with patients with SS (blue bars). * $P < .05$ and ** $P < .01$. Results are expressed as means $\pm$ SDs of 5 experiments. **B**, Immunohistochemistry on human tonsils from a healthy subject and bone marrow smears from 3 patients with MM showing LOXL2 expression by a proportion of plasmablasts/plasma cells (arrows). A proportion of plasmablasts/plasma cells do not express LOXL2 (arrowheads). **C**, Immunofluorescence staining of CD19 (red), IgG4 (green), LOXL2 (orange), CD20 (blue), and 4'-6-diamidino-2-phenylindole dihydrochloride (white) in the lymph nodes of a representative patient with CD (arrows; insets show high magnification). **D**, Quantification of LOXL2⁺ plasmablasts in salivary glands from patients with IgG4-RD (red bars) and patients with SS (blue bars) and in lymph nodes from patients with CD (purple bars) showing increased LOXL2-expressing plasmablasts in patients with IgG4-RD compared with patients with SS and CD (TissueQuest software). * $P < .05$ (mean $\pm$ SD of $n = 5$ experiments).

TABLE E1. Clinical, serologic, and immunologic features of the patient cohort

Patient no.	Age (y)	Sex	Referral center	Plasmablasts (cells/mL)	Serum IgG ₄ (mg/dL)	Organ involvement	IgG ₄ -RD RI
1	83	F	SRH	9.000	1.360	Pancreas, biliary tree	12
3	67	M	SRH	9400	720	Pancreas	6
6	70	M	SRH	3.560	221	Pancreas, lymph nodes	9
8	73	F	SRH	7.000	534	Pancreas, lymph nodes	12
9	53	M	SRH	9.300	973	Pancreas, lymph nodes	6
11	65	M	SRH	4.000	308	Pancreas	6
12	71	F	SRH	1.000	420	Pancreas, aorta	6
13	51	F	SRH	6.000	178	Pancreas, aorta	6
14	53	F	SRH	1.030	173	Pancreas, aorta	6
16	75	M	SRH	2.150	314	Pancreas, biliary tree	6
2	66	M	MGH	7.140	485	Pancreas, biliary tree, parotid glands, lymph nodes	15
4	70	M	MGH	5.400	343	Pancreas, lymph nodes	9
5	72	M	MGH	2500	368	Pancreas, lacrimal glands, lungs	6
7	47	F	MGH	1400	177	Pancreas, lacrimal glands	9
10	63	M	MGH	8100	156	Pancreas, submandibular glands	6
15	66	M	MGH	1.760	362	Pancreas	6
17	51	F	MGH	40.840	253	Pancreas	15
18	71	M	MGH	3.740	282	Pancreas, lymph nodes	6
19	67	M	MGH	2700	147	Pancreas, salivary glands	9
20	58	M	MGH	3450	201	Pancreas, retroperitoneal fibrosis, skin	9

Normal values: plasmablasts, 0-653^{E1}; serum IgG₄, <135 mg/dL; IgG₄-RD RI, 0-3.^{E2}

F, Female; IgG₄-RD RI, IgG₄-RD responder index; M, male; MGH, Massachusetts General Hospital; SRH, San Raffaele Hospital.

TABLE E2. Details of gene set enrichment analysis results shown in [Fig 1, A](#), and [Fig E4](#)

Gene set name	Normalized enrichment score	FDR q-value
EPITHELIAL_MESENCHYMAL_TRANSITION	4.59	0.0
APICAL_JUNCTION	3.64	0.0
CHOLESTEROL_HOMEOSTASIS	2.62	5.16E-04
MYOGENESIS	2.38	0.0027
MYC_TARGETS_V1	−4.84	0.0
MYC_TARGETS_V2	−2.43	0.0017
E2F_TARGETS	−2.24	0.0078
EPITHELIAL_MESENCHYMAL_TRANSITION	2.73	0.0

TABLE E3. Details of gene set enrichment analysis results shown in [Fig 4, A](#), and in [Fig E4](#)

Gene set name	Normalized enrichment score	FDR q-value
EPITHELIAL_MESENCHYMAL_TRANSITION	3.34	0.0
MYOGENESIS	3.03	0.0
APICAL_JUNCTION	2.61	2.17E-04
E2F_TARGETS	-4.74	0.0
MYC_TARGETS_V1	-4.03	0.0
G2M_CHECKPOINT	-3.56	0.0
TNFA_SIGNALING_VIA_NFKB	-2.87	0.0
INTERFERON_GAMMA_RESPONSE	-2.54	0.0011
MTORC1_SIGNALING	-2.53	9.48E-04
UNFOLDED_PROTEIN_RESPONSE	-2.51	8.12E-04
INTERFERON_ALPHA_RESPONSE	-2.40	0.0014
KRAS_SIGNALING_UP	-2.22	0.0054
INFLAMMATORY_RESPONSE	-2.13	0.0087