

Identification of galectin-3 as an autoantigen in patients with IgG₄-related disease



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Background: The antigenic trigger that drives expansion of circulating plasmablasts and CD4⁺ cytotoxic T cells in patients with IgG₄-related disease (IgG₄-RD) is presently unknown. **Objective:** We sought to sequence immunoglobulin genes from single-cell clones of dominantly expanded plasmablasts and generate recombinant human mAbs to identify relevant antigens in patients with IgG₄-RD by using mass spectrometry. **Methods:** Paired heavy and light chain cDNAs from dominant plasmablast clones were expressed as mAbs and used to purify antigens by using immunoaffinity chromatography. Affinity-purified antigens were identified by using mass spectrometry and validated by means of ELISA. Plasma levels of the antigen of interest were also determined by using ELISA.

Results: mAbs expressed from the 2 dominant plasmablast clones of a patient with multiorgan IgG₄-RD stained human pancreatic tissue sections. Galectin-3 was identified as the antigen specifically recognized by both mAbs. Anti-galectin-3 autoantibody responses were predominantly of the IgG₄ isotype (28% of the IgG₄-RD cohort, $P = .0001$) and IgE isotype (11% of the IgG₄-RD cohort, $P = .009$). No significant responses were seen from the IgG₁, IgG₂, or IgG₃ isotypes. IgG₄ anti-galectin-3 autoantibodies correlated with increased plasma galectin-3 levels ($P = .001$), lymphadenopathy ($P = .04$), total IgG level increase ($P = .05$), and IgG₄ level increase ($P = .03$).

Conclusion: Affinity chromatography using patient-derived mAbs identifies relevant autoantigens in patients with IgG₄-RD. IgG₄ galectin-3 autoantibodies are present in a subset of patients with IgG₄-RD and correlate with galectin-3 plasma levels. The marked increases in levels of circulating IgG₄ and IgE observed clinically are, at least in part, caused by the development of IgG₄- and IgE-specific autoantibody responses. (J Allergy Clin Immunol 2019;143:736-45.)

Key words: IgG₄-related disease, plasmablast, autoantigen, galectin-3, autoantibody

IgG₄-related disease (IgG₄-RD) is a chronic immune-mediated disease of unclear cause. The most common clinical presentation involves painless enlargement of affected organs, raising concern for an underlying malignancy. The most frequent sites of disease are the major salivary glands (parotid and submandibular), lacrimal glands, pancreas, biliary tree, lung, kidneys, retroperitoneum, lymph nodes, and aorta.¹

Increased plasma levels of immunoglobulin are observed in the great majority of patients with IgG₄-RD, and IgG₄ is produced out of proportion to the levels of other IgG subclasses. Previous studies from our group have demonstrated a prominent expansion of circulating plasmablasts in patients with IgG₄-RD compared with healthy subjects. Plasmablasts in patients with IgG₄-RD display an oligoclonally restricted immunoglobulin repertoire in which 3 to 5 dominant clones typically account for the large majority of circulating plasmablasts.² Numbers of circulating plasmablasts correlate with IgG₄-RD disease activity, suggesting that the adaptive immune system is triggered during active disease.³ The tightly restricted repertoire of plasmablasts strongly supports the notion that IgG₄-RD is an antigen-driven disease. However, the precise mechanism through which activated B-cell populations contribute to the disease remains unclear.

The possible role of adaptive immunity in disease pathogenesis is further supported by the demonstration of large clonal expansions of CD4⁺ cytotoxic T lymphocytes (CTLs) but not of other T-cell subsets in affected tissues of patients with IgG₄-RD.^{4,5} These T cells express granzymes and perforin and secrete cytokines, such as IL-1 β , IFN- γ , and TGF- β , which have been linked to fibrosis. In addition, our studies have recently demonstrated a paucity of IL-4-expressing GATA-3⁺ T_H2 cells in patients with IgG₄-RD but a marked expansion of IL-4-secreting basic leucine zipper ATF-like transcription factor-positive follicular helper T cells in tertiary lymphoid organs and lymph nodes of patients with IgG₄-RD.⁶ The question of which antigens drive the striking clonal expansion of B and T cells remains unanswered.

Most previous investigations of antigens associated with IgG₄-RD have been performed in the context of type 1 autoimmune

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Abbreviations used

CTL: Cytotoxic T cell
IgG₄-RD: IgG₄-related disease
IgG₄-RD-RI: IgG₄-RD Responder Index
IPF: Idiopathic pulmonary fibrosis

pancreatitis or IgG₄-related cholangitis, which are now recognized as 2 cardinal manifestations of IgG₄-RD. By using whole or pooled serum as a primary antibody source, these studies have identified lactoferrin, carbonic anhydrases, pancreatic secretory trypsin inhibitor, amylase α 2A, the ubiquitin-protein ligase E3 component n-recogin 2, trypsinogen, and annexin A11 as autoantigens in patients with autoimmune pancreatitis and cholangitis.⁷⁻¹³ However, because nearly all of these studies were conducted before recognition of IgG₄-RD as a distinct disease, patients with type 2 autoimmune pancreatitis, a distinctly different condition from type 1 autoimmune pancreatitis, which is not part of the IgG₄-RD spectrum, were included. These studies were also limited to a small subset of manifestations of what is now regarded as a systemic disease.

A more recent investigation, which was conducted in patients with multiorgan IgG₄-RD, identified the nuclear protein prohibitin as a potential autoantigen in a high frequency of patients compared with control subjects, but this finding has not yet been validated in any other study cohort, and its pathogenic implications have not been elucidated.¹⁴ Finally, a study of the transfer of patient-derived antibodies into neonatal rodents suggests that antibodies in patients with IgG₄-RD might be of pathogenic significance,¹⁵ yet the true significance of IgG₄ and other antibodies in disease pathogenesis remains unclear.

Our group has shown that B-cell depletion mediates prompt clinical improvement¹⁶ and also leads to a corresponding decrease in numbers of CD4⁺ CTLs.⁵ Therefore one possibility with regard to pathophysiology is that dominantly expanded B cells maintain or present some unidentified antigens to expanded CD4⁺ CTLs in tissues of patients with IgG₄-RD. These dominant B cells can also present antigens to relevant follicular helper T cells in patients with disease, thereby driving germinal center responses and IgG₄ class-switching. Detection of an autoantibody at some level in a given subject does not necessarily imply that the most actively expanded B cells in that subject are the source of this antibody. Understanding disease pathogenesis would be greatly facilitated through identification of antigens recognized by expanded lymphocyte clones that dominate during active disease.

We describe here an approach involving the sequencing of immunoglobulin genes from dominant plasmablast clones from a patient with IgG₄-RD. We generated recombinant patient-derived human mAbs and used them to identify disease-relevant antigens using immunoaffinity chromatography and mass spectrometry.

METHODS

Patients

All studies were approved by the institutional review board of the Massachusetts General Hospital, and informed written consent was obtained from all patients before sample collection. One hundred twenty-one patients with IgG₄-RD were recruited between the dates of January 10, 2012, and February 6, 2017. Information regarding demographics, treatment, disease

activity, laboratory parameters, and disease manifestations for these patients is presented in Tables E1 and E2 in this article's Online Repository at www.jacionline.org. Disease activity was quantified by using the IgG₄-RD Responder Index (IgG₄-RD-RI).¹⁷ An IgG₄-RD-RI score of 1 or greater was considered active disease.

Patients with IgG₄-RD were compared with 45 patients with idiopathic pulmonary fibrosis (IPF) and 50 healthy donors. Plasma samples from patients with IPF were obtained through the Massachusetts General Hospital Division of Pulmonary & Critical Care Medicine's Biorepository of Interstitial Lung Diseases.

Healthy donor plasma was obtained from the Partners HealthCare Biobank, an institution-wide repository of samples from consented subjects. This database was filtered to exclude patients with autoimmune disease, chronic infection, and malignancy to identify age- and sex-matched healthy donors. Medical records were reviewed in detail to ensure appropriate "healthy" status without immunologic confounding factors.

Blood processing and storage

Blood from patients with IgG₄-RD was collected in ACD tubes and transported immediately to the laboratory for cell isolation. PBMCs were isolated by means of Ficoll-Paque density gradient centrifugation. Plasma was placed in aliquots and stored at -80°C for subsequent use. Unused PBMCs were suspended in FBS containing 10% dimethyl sulfoxide and cryopreserved in liquid nitrogen.

Flow cytometry and cell sorting

Fresh PBMCs were washed and quantified by using an automated cell counter. Cells were stained for flow cytometric identification at a concentration of 20 million cells/mL by using the following antibody panel: anti-human CD19-Pacific Blue (clone HIB19), anti-human CD27-allophycocyanin (clone O323), and anti-human CD38-fluorescein isothiocyanate (clone HIT2). All antibodies were procured from BioLegend (San Diego, Calif). Conjugated antibodies were incubated at 4°C and protected from light to minimize photodegradation. Flow cytometry was performed on a BD LSR II (BD Biosciences, San Jose, Calif), and the FCS files were analyzed by using FlowJo software (version 10; TreeStar, Ashland, Ore). Plasmablasts were identified as CD19⁺CD27⁺CD38^{Hi} cells, as previously described.³ Plasmablasts were sorted with a FACSAria 3 sorter in both bulk and single-cell format. For single-cell plasmablast sorting, cells were collected in 96-well PCR plates (VWR, Radnor, Pa) containing 4 μL of cell lysis buffer (0.5 $\times$ PBS containing 10 mmol/L dithiothreitol) and 8 U of RNasin (Promega, Madison, Wis), and plates were stored at -80°C to preserve RNA integrity. Intracellular flow cytometry was also performed on pancreatic cancer cell lines (PaCa2, AsPC-1, SU.86.86, SW-1990, and HPAF-II). For these experiments, we fixed freshly harvested cells with BD Cytofix/Cytoperm solution (catalog no. 554722; BD Biosciences, San Jose, Calif) and then permeabilized fixed cells with 0.01% Tween 20. Subsequently, cells were stained with biotinylated patient-derived mAbs (see the "Immunofluorescence" section below) at a concentration of 10 $\mu\text{g}/\text{mL}$. Cells were washed twice with PBS, followed by secondary antibody incubation with Alexa Fluor 488-Streptavidin (catalog no. S32354; Invitrogen, Carlsbad, Calif). An IgG₁ lambda isotype control and secondary antibody alone were used as controls to confirm adequate washing and determine positive binding, respectively. Cells were immediately analyzed by using flow cytometry.

Immunoglobulin heavy chain repertoire analysis

Next-generation sequencing analysis of the B-cell receptor IGH repertoire was performed by using the ImmunoSeq platform (Adaptive Biotechnologies, Seattle, Wash). Assembly of the VDJ region in the rearranged IGH sequences was performed with the IMGT V-Quest server and VDJ solver. Bulk repertoire analysis was used to identify the dominantly expanded clones based on the frequency of sequencing reads of the recombined heavy chain sequence. Plasmablasts were sorted as single cells, and RNA was extracted from each cell, followed by RT-PCR and Sanger sequencing of expressed

immunoglobulin heavy and light chain genes, as described by Tiller et al,¹⁸ with minor primer modifications, as previously described.² The light chain sequence corresponding to the wells of the dominant heavy chains were identified. The paired heavy and light chain PCR products from single plasmablasts were cloned into expression vectors, as previously described.¹⁸

mAb expression

Transient transfection was performed with a polyethylenimine/DNA ratio of 3:1 into 293F suspension cells in 2-L volume flasks (500 mL of medium at 1.2 million cells per milliliter). Both the heavy chain- and light chain-containing plasmids were transfected simultaneously. Cells were incubated for 5 days at 37°C, with 5% CO₂ in an oscillating incubator before harvesting. Immunoglobulin was purified from the supernatant with Pierce protein A/G Agarose beads (Pierce, Rockford, Ill). Antibody purity was confirmed by using SDS-PAGE. Final antibody concentrations were quantified with a bicinchoninic acid–based colorimetric assay.

Immunofluorescence

Patient-derived mAbs were biotinylated with an EZ-Link Sulfo-NHS-SS-biotinylation Kit (Thermo Scientific, Waltham, Mass). We procured healthy pancreatic tissue immediately postoperatively from the Massachusetts General Hospital Pathology Department. Tissue was deemed “healthy” on histologic grounds because it was taken from the disease-free margin of a patient undergoing a tumor resection. Surgical specimens were immediately snap-frozen in isopentane cooled to –80°C for storage. Frozen sections were fixed in precooled acetone and rehydrated in PBS. A 30-minute incubation in 1% Sudan Black and 70% ethanol was used to quench autofluorescence. Slides were rinsed with water, blocked, and gently permeabilized with 5% BSA plus 0.01% Tween 20 for 60 minutes. Endogenous biotin was blocked by incubating with avidin for 15 minutes. Primary antibodies (biotinylated patient-derived mAbs, a positive control biotinylated anti-actin [Thermo Fisher Scientific, ACTN05(C4)], and a negative control biotinylated anti-CD57) were incubated for 2 hours. All antibodies were incubated at the same concentration, as determined by using 2 independent bicinchoninic acid assays. Slides were washed with PBS 0.01%–Tween 20 before secondary antibody incubation with Alexa Fluor 488–Streptavidin (catalog no. S32354; Invitrogen) for 60 minutes at room temperature and protected from light in a humidifier. Slides were washed and counterstained with a fluorescent-antiblastic mountant containing 4′-6-diamidino-2-phenylindole dihydrochloride (catalog no. P36931; ProLong Gold; Thermo Fisher Scientific). Images were captured on a TissueFAXS microscope (TissueGnostics, Vienna, Austria). A similar protocol was used for cytospun human pancreatic epithelial cancer cells (PaCa2), which were kindly provided by Dr Cyril Benes (Massachusetts General Hospital).

For intracellular staining, we fixed freshly harvested cells (pancreatic and nonpancreatic cancer cell lines) with BD Cytofix/Cytoperm solution (catalog no. 554722) and then permeabilized fixed cells by washing twice with a BD Perm/Wash buffer (catalog no. 554732). Subsequently, cells were stained with patient-derived mAbs or an IgG₁ isotype control at a concentration of 10 µg/mL and incubated. Cells were washed twice with PBS, followed by secondary antibody incubation (catalog no. 560951; mouse anti-Human IgG1 PE; BD Biosciences), washed twice, and immediately analyzed by using flow cytometry.

Immunoaffinity purification and mass spectrometry

Purified mAbs from 2 separate clones and an isotype control were bound separately to protein A/G Sepharose beads and then covalently crosslinked with disuccinimidyl suberate (catalog no. 26147; Pierce Crosslink Immuno-precipitation Kit; Thermo Fisher Scientific). PaCa2 cell lysate was prepared with 1% NP-40 in 20 mmol/L Tris and 150 mmol/L NaCl, pH 7.8. EDTA-free cOmplete protease inhibitor cocktail (no. 11697498001; Sigma-Aldrich, St Louis, Mo) was added to the lysis buffer to prevent proteolysis of potential antigenic targets.

After standard centrifugation steps and preclearing on protein A/G Sepharose beads, the lysate was incubated with the cross-linked mAb/protein

A/G beads, washed extensively, and eluted in 50 µL of glycine-HCl, pH 2.8. Eluted fractions were collected in 1 mol/L Tris to neutralize the elution buffer. The eluted proteins were reduced with dithiothreitol and alkylated with iodoacetamide and digested with Lys-C and trypsin.¹⁹ Generated peptides from the individual samples were derivatized with 10-plex tandem mass tag reagents (Thermo Fisher), and protein concentration differences between the samples were determined by using multiplexed quantitative proteomics with synchronous-precursor-selection MS3 technology on an Orbitrap Fusion Mass Spectrometer (Thermo Fisher), essentially as described previously, but omitting the basic pH reversed-phase liquid chromatography fractionation caused by the protein samples’ low complexity.²⁰

ELISA

Alternating rows of Nunc Medisorb 96-well plates were coated overnight at 4°C with recombinant galectin-3 (no. 450-38; PeproTech, Rocky Hills, NJ) at a concentration of 5 µg/mL in PBS. Intervening rows were used as negative control wells for each subject tested and coated with only 1% BSA during the blocking step. Each subject was tested in triplicate with corresponding triplicate BSA-only wells without galectin-3. BSA-only wells were used to subtract nonspecific protein-protein and protein-well interactions. Plates were blocked overnight with 1% BSA plus 0.01% Tween in PBS. Stored plasma was thawed, diluted 1:100 in blocking buffer, and incubated overnight at 4°C. Respective peroxidase-labeled anti-human IgG subclass antibody was diluted 1:1000 in blocking buffer and incubated for 2 hours at 21°C. Secondary antibodies used included the mouse anti-human IgG₁ hinge heavy chain (no. 99774; Abcam, Cambridge, United Kingdom), mouse anti-human IgG₂ Fc (no. 99779; Abcam), mouse anti-human IgG₃ (hinge region; no. MCA516G; Bio-Rad Laboratories, Hercules, Calif), mouse anti-human IgG₄ pFc’ (no. 99817; Abcam), and goat anti-human IgE (NB7459; Novus Biologicals, Littleton, Colo). All IgG subclass secondary antibodies were diluted at 1:1,000 in blocking buffer, whereas the anti-IgE secondary antibody was diluted at 1:10,000 in blocking buffer. After 3 washes with PBS plus 0.01% Tween, 100 µL of OPD substrate at a concentration of 0.4 mg/mL was incubated at 21°C for 30 to 60 minutes, and the reaction was quenched with 100 µL of 2N sulfuric acid. Absorbance was quantified on a Magellan plate reader at 492 nm with a reference range of 650 nm. Triplicate values were averaged, and negative control average was subtracted from each subject. If any individual well deviated by more than 50% from its respective partners, that well was not included in calculating the average absorbance.

Statistical analysis

For ELISA analysis, 2 SDs greater than the healthy donor mean was used to define a positive antibody response. This value effectively excluded 96% of healthy donor subjects. Because this study is exploratory and hypothesis generating, the analysis of clinical correlations is descriptive. We used measures of means, medians, SDs, interquartile ranges, frequencies, and percentages to summarize the data. The Fisher exact test was used to compare the proportion of antibody responses between subjects with and without each organ manifestation, and the sample size was determined by availability of resources. Positive antibody responses were defined by an absorbance of at least 2 SDs greater than the mean of healthy donor antibody responses. For continuous nonparametric variables, such as IgG and IgG subclasses, a 2-tailed Mann-Whitney *U* test was used to compare groups. For all statistical analyses, a *P* value of less than .05 was considered significant. Statistical analysis was performed with GraphPad Prism software (version 7; GraphPad Software, La Jolla, Calif).

RESULTS

mAbs from dominant plasmablast clones from a patient with IgG₄-RD recognize a cytosolic pancreatic antigen

Circulating plasmablasts (CD19⁺CD27⁺CD38^{Hi}) were quantified in a treatment-naïve patient with IgG₄-RD with multiorgan

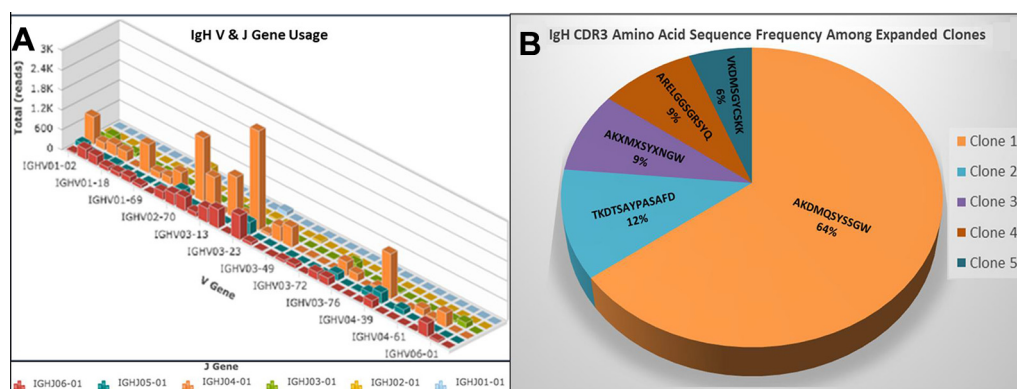


FIG 1. **A**, Bulk repertoire of sorted plasmablasts demonstrating IGH V gene use on the x-axis, IGH J gene use on the z-axis, and number of sequencing reads on the y-axis. Each bar represents a clonal family. **B**, Pie chart showing the frequency of dominantly expanded clones from single-cell sequencing. The specific CDR3 amino acid sequence and percentage of wells from the single-cell plate are displayed. The dominant clone, Clone 1, represented 22 of 34 sequenced individual cells.

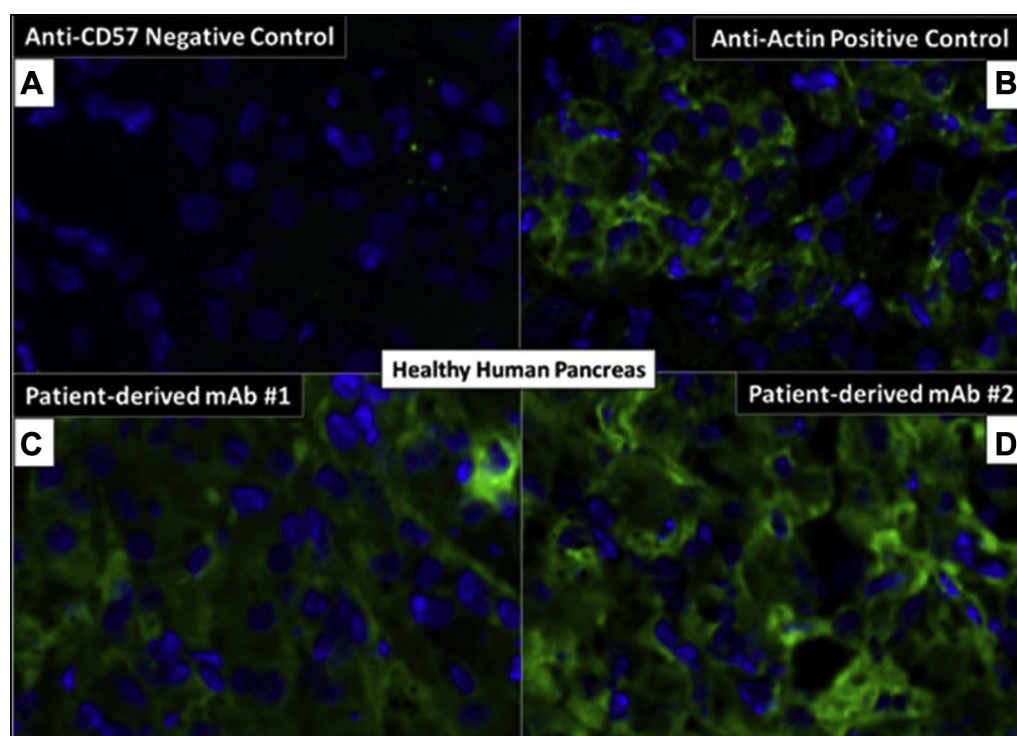


FIG 2. Immunofluorescence of pancreatic tissue. **A** and **B**, Negative (anti-CD57) and positive (anti-actin) control antibodies. **C** and **D**, Patient-derived mAbs from dominantly expanded plasmablasts demonstrating intense reactivity in a cytosolic pattern.

involvement, including the pancreas, bile ducts, lungs, salivary glands, and retroperitoneum. Plasmablasts represented approximately 10% of all CD19⁺ B cells in contrast to 1% to 3% observed in healthy donors (see Fig E1 in this article's Online Repository at www.jacionline.org). Next-generation sequencing of IgH genes revealed that the heavy chain repertoire was oligoclonal, with 3 to 5 clones representing the majority of sequence reads (Fig 1, A). These data suggested that an antigen-driven immune response contributed to the clonal expansion of B cells in this patient.

Paired heavy chain VDJ and light chain VJ sequences from individually sorted plasmablasts were identified through single-

cell sequencing. Clones were defined by identical heavy chain VDJ, light chain VJ, and predicted IGH CDR3 amino acid sequences. These clones are identified henceforth as clone 1, clone 2, and so on. Twenty-two of 34 individually sequenced plasmablasts were found to be identical, representing 64% of all wells from the single-cell sorted plate. The clonal frequency of individually sorted plasmablasts is depicted in Fig 1, B. The 2 clones with the highest frequency from single-cell sequencing were selected for mAb expression.

Because our index patient had a history of type 1 (IgG₄-related) autoimmune pancreatitis, we tested human pancreatic tissue for mAb reactivity using immunofluorescence.

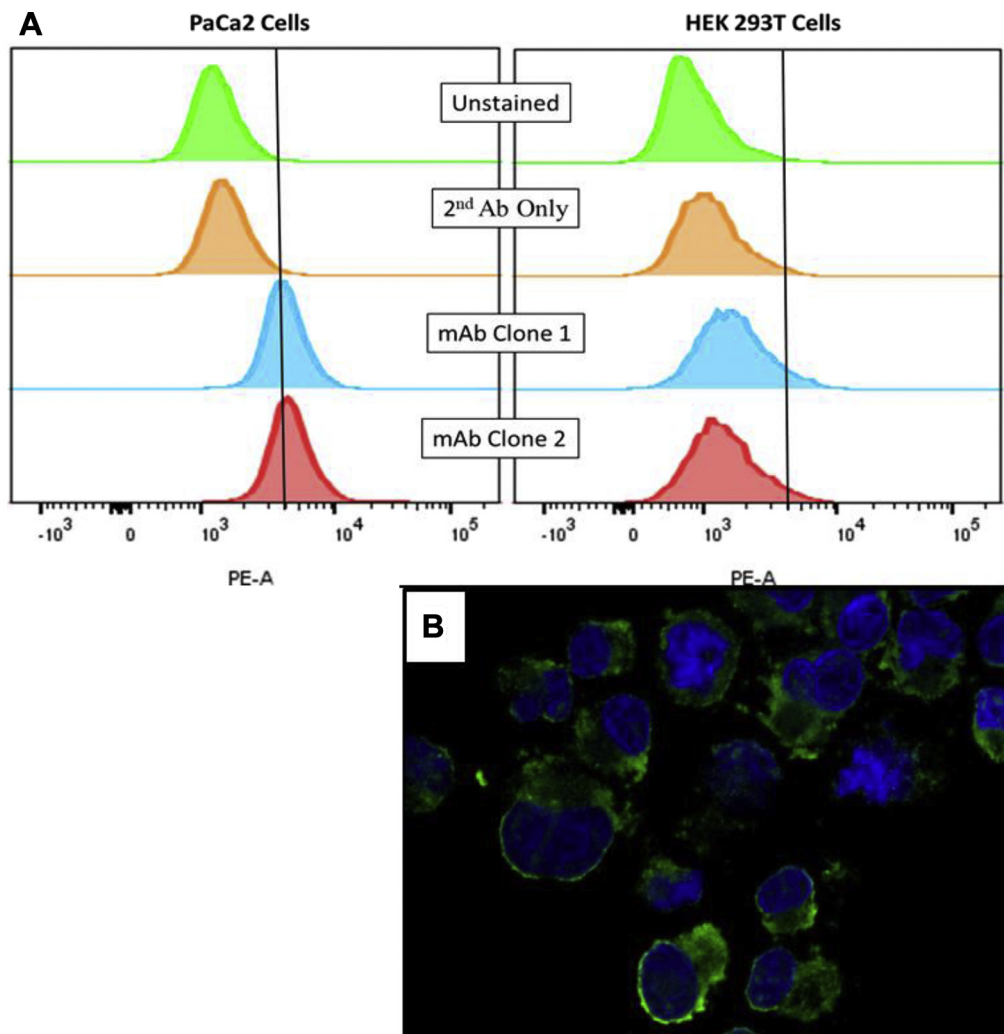


FIG 3. **A**, Intracellular flow cytometry revealed differential reactivity of mAbs with PaCa2 cells compared with control cells. Vertical cutoff lines were drawn to exclude unstained cells. No difference was observed in HEK293T cells. **B**, Confocal microscopy of PaCa2 cells stained with biotinylated mAb clone 1 demonstrating cytosolic and cell-surface staining patterns. *Ab*, Antibody.

Intense reactivity was observed in a cytosolic pattern for both mAbs (Fig 2).

To address whether the reactivity observed on pancreatic tissue was due to recognition of antigens expressed by pancreatic epithelial or stromal cells, we performed intracellular flow cytometry and immunofluorescence studies on pancreatic cancer cell lines and human primary skin fibroblasts. Both mAbs differentially recognized proteins expressed by the PaCa2 pancreatic cancer cell line compared with unstained cells (Fig 3, A) and displayed both a cytosolic and cell-surface staining pattern (Fig 3, B).

Affinity purification and mass spectrometry identifies galectin-3 as an autoantigen in patients with IgG₄-RD

PaCa2 cells were lysed to generate a protein source for immunoprecipitation to identify the antigen expressed by PaCa2 cells that was recognized by both patient-derived mAbs.

Both mAbs bound a protein of approximately 30 kDa, which was not recognized by the isotype control (see Fig E2 in this article's Online Repository at www.jacionline.org). Galectin-3 was then identified by using multiplexed quantitative mass spectrometry-based proteomics as the protein with the highest differential binding affinity for both mAbs (Fig 4).

To validate the finding of galectin-3 as an autoantigen in patients with IgG₄-RD, we quantified anti-galectin-3 antibodies using ELISA in a large and clinically diverse cohort of patients with IgG₄-RD. Forty-five patients with IPF were used as fibrotic disease control subjects, and 50 age-matched healthy donors were used to control for autoreactivity that can occur with aging.²¹ Because dominant plasmablast clones have been shown to express IgG₄² and IgG₄ is the most robustly increased IgG subclass observed in the plasma of patients with IgG₄-RD, we first measured IgG₄-specific anti-galectin-3 antibody responses. This revealed a reactive frequency of 28% among the IgG₄-RD cohort, with little to no reactivity in control groups (Fig 5).

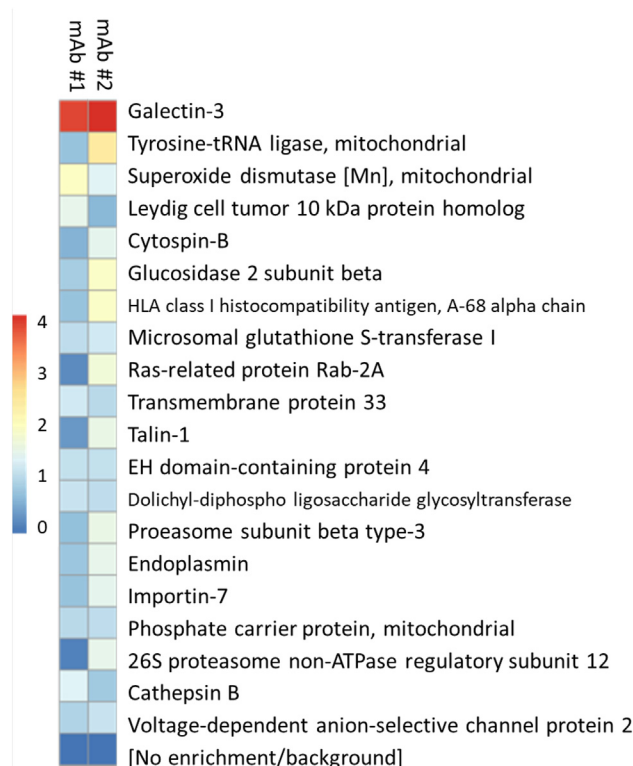


FIG 4. Heat map demonstrating differential protein binding of PaCa2 cell lysate by mAb clones 1 and 2. Scale reflects $\log_2$ fold change divided by isotype control. Background was determined by using an isotype control. Galectin-3 was identified as the antigen with the highest binding affinity for both mAb clones.

We next tested the cohort for galectin-3 reactivity by IgG₁, IgG₂, IgG₃, and IgE isotypes. IgE isotype anti-galectin-3 antibodies were also detected in these experiments (Fig 5), but there was little to no reactivity by other IgG subclasses (see Fig E3 in this article's Online Repository at www.jacionline.org). The presence of anti-galectin-3 antibodies of only IgG₄ and IgE isotypes is consistent with the clinical observation of predominant IgG₄ and IgE level increases in the blood of patients with IgG₄-RD compared with other IgG subclasses, which tend to be only modestly increased and at a lower overall frequency (Fig 6). These results support the notion that at least a portion of the IgG₄ and IgE expansion in the blood of patients with IgG₄-RD is due to the development of autoantibodies of these isotypes. One potential explanation for the observation of concordant IgG₄ and IgE isotype responses is the effect of certain cytokine combinations, such as IL-4 plus IL-10. This combination is reported to drive isotype switching to both IgG₄ and IgE, with some skewing toward IgG₄.²²

Overexpression of galectin-3 might contribute to anti-galectin-3 autoantibody responses

We next investigated whether the occurrence of IgG₄-specific anti-galectin-3 antibodies was associated with overexpression of this protein in accordance with the notion that accumulation of proteins within tumors of various types has been associated previously with development of autoantibodies directed against

the overexpressed protein.²³ In the context of anti-p53 antibodies, for example, this autoimmunization process has been demonstrated in a mouse model of Simian virus 40-induced malignancy in which p53 is stabilized by SV40 antigen and accumulates without any mutation or truncation to the wild-type form.²⁴

Recently, by using an unbiased quantitative proteomic approach, pancreata of patients with IgG₄-RD were compared with those of control subjects.²⁵ Galectin-3 was observed as one of the highest differentially expressed proteins in pancreata of patients with IgG₄-RD, which is present at a 13-fold higher amount compared with control subjects.²⁵ To support this idea, we quantified circulating galectin-3 levels in a subset of the IgG₄-RD cohort compared with control subjects. Using a cutoff value of 10.25 ng/mL based on systemic sclerosis literature, in which this cutoff value was an independent predictor of all-cause mortality,²⁶ we found that 15.5% of subjects in the IgG₄-RD cohort had increased plasma galectin-3 levels (Fig 7, A) in contrast to systemic sclerosis, in which 41% of patients have circulating levels greater than this value.²⁶ Based on the dispersion of plasma galectin-3 levels among the patient and healthy donor cohorts, we found this cutoff to be an appropriate delineation in which the increased IgG₄-RD subjects clearly differed from the less than 10.25 ng/mL set and only 1 of the healthy donors were at greater than this value. After segregating the IgG₄-RD cohort based on increased as opposed to normal plasma galectin-3 levels, IgG₄ anti-galectin-3 antibodies were detected in 64% of those with increased levels compared with only 23% in those with normal levels ($P = .0032$; Fig 7, B).

Finally, we analyzed the IgG₄-RD cohort for clinical correlations with the presence of IgG₄ anti-galectin-3 autoantibody responses as presented in Table E3 in this article's Online Repository at www.jacionline.org. Total IgG and IgG₄ levels correlated positively with the presence of this antibody response ($P = .05$ and $.03$, respectively), but there was no correlation with levels of any other IgG subclass or of IgE or IgM levels.

Given the reduced effector functions of the IgG₄ molecule compared with other IgG subclasses, we hypothesized that there would be no clinically apparent correlation between IgG₄ anti-galectin-3 autoantibodies and either systemic inflammation or complement consumption. Because hypergammaglobulinemia is known to cause red blood cell aggregation and increase the erythrocyte sedimentation rate (regardless of any actual acute-phase response), we used the C-reactive protein level as a more specific indicator of systemic inflammation in this analysis. There was no statistically significant correlation between C3 ($P = .18$), C4 ($P = .17$), or C-reactive protein ($P = .69$) levels and the presence of IgG₄ anti-galectin-3 autoantibodies.

We used the IgG₄-RD-RI score and extent of organ involvement as indicators of disease severity. By using these indices, we found trends toward a higher IgG₄-RD-RI score ($P = .13$), number of organs involved ($P = .10$), and multiorgan involvement ($P = .09$) in the IgG₄ anti-galectin-3 antibody group. We also found a statistically significant correlation between lymphadenopathy and a positive IgG₄ anti-galectin-3 antibody response ($P = .04$) but with no significant correlation with any other organ involvement. In summary, IgG₄ anti-galectin-3 autoantibody levels correlate with increased total IgG, IgG₄, and galectin-3 levels and lymphadenopathy, with a trend toward more extensive disease involvement.

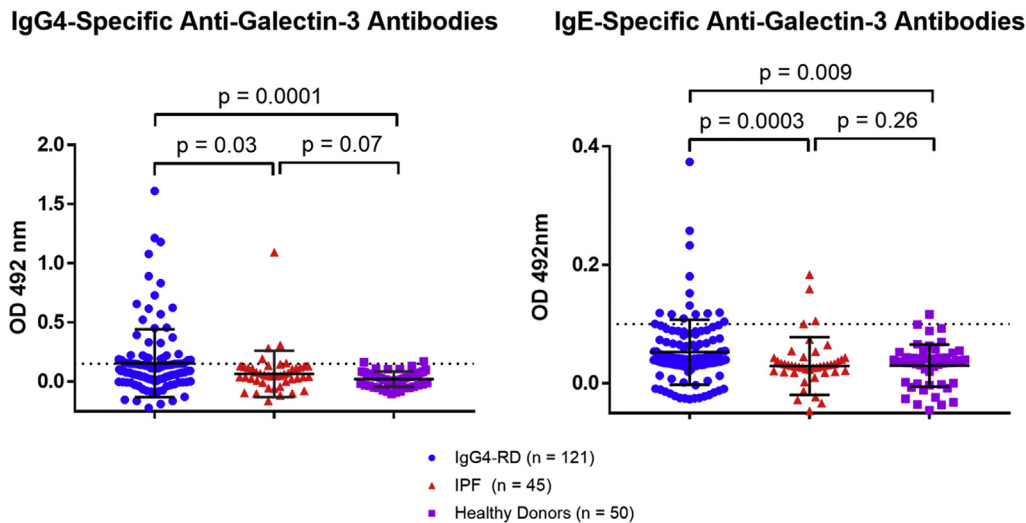


FIG 5. IgG₄ and IgE isotype-specific anti-galectin-3 antibodies were confirmed by means of ELISA with 34 and 12 of 121 patients with IgG₄-RD demonstrating IgG₄ and IgE antibody responses, respectively. *Dashed lines* represent 2 SDs greater than the healthy donor mean.

Immunoglobulin Isotype Elevation Among IgG₄-RD Cohort

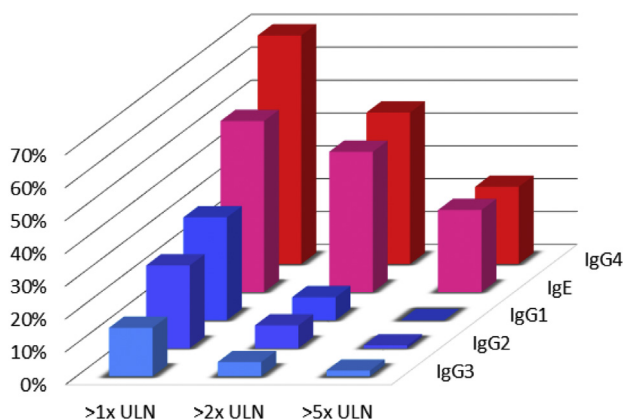


FIG 6. IgG subclass and IgE serum values of the IgG₄-RD cohort plotted as frequency of elevation among the cohort (*y*-axis), isotype (*z*-axis), and degree of elevation (*x*-axis). *ULN*, Upper limit of normal for the clinical reference laboratory value for each isotype.

IgG₄-RD response to rituximab is associated with a decrease in levels of anti-galectin 3 autoantibodies

In 3 subjects with positive anti-galectin-3 antibody responses who were treated subsequently with B-cell depletion therapy, we measured the kinetics of autoantibody responses longitudinally. If autoantibody responses do not decrease with B-cell depletion (which corresponds to plasmablast depletion), one might infer that the response is being driven by long-lived plasma cells. A clear decrease in autoantibody responses after B-cell depletion therapy was observed in all 3 subjects examined, which is consistent with production of a substantial proportion of autoantibodies by plasmablasts and short-lived plasma cells. This decrease in autoantibody response corresponded to an improvement in the subjects' disease activity (Fig 8). Subject 336 was treated with a single course of rituximab, after which both the IgG₄-RD-RI score and anti-galectin-3 antibody levels decreased

but never resolved fully as they had in subjects 365 and 876. Clinical remission was then maintained with the start of low-dose prednisone (as indicated by the black arrow in Fig 8). With further monitoring and time from B-cell depletion, anti-galectin-3 autoantibody levels increased during low-dose prednisone maintenance to reach the pre-rituximab response level, a kinetic that likely reflects a contribution from both short-lived plasmablasts and long-lived plasma cells to the anti-galectin-3 autoantibody response.

DISCUSSION

We used next-generation sequencing, single-cell cloning of dominantly expanded plasmablasts, mAb generation, antigen purification, and mass spectrometry as a strategy to identify disease-relevant autoantigens in a patient with active IgG₄-RD. In contrast to all previous studies examining autoantigens in patients with IgG₄-RD, we used an approach to identify the antigenic specificity of dominantly expanded plasmablasts. We report the presence of autoantibodies against galectin-3, which were predominantly of the IgG₄ isotype.

Galectin-3 is a β -galactoside-binding lectin that lacks a signal peptide but can be secreted or adhere to the cell surface.²⁷ It has also been described in the nucleus and cytosol. Corresponding to its subcellular location, this protein has been noted to play a role in a wide variety of biological processes, including apoptosis, cell migration, inflammation, and fibrosis.²⁸ Macrophages are the primary cellular source of secreted galectin-3. Galectin-3 has been suggested to contribute to numerous fibroproliferative disease states, including pulmonary fibrosis, chronic kidney disease, congestive heart failure, and nonalcoholic steatohepatitis.²⁸ Galectin-3 knockout mice demonstrate a marked abrogation in the development of lung, kidney, liver, and heart fibrosis compared with their wild-type counterparts, suggesting a mechanistic contribution of galectin-3 to fibrogenesis.²⁹⁻³² Inhibition of galectin-3 was shown to mitigate the development of pulmonary fibrosis in the bleomycin mouse model,²⁹ and an inhaled galectin-3

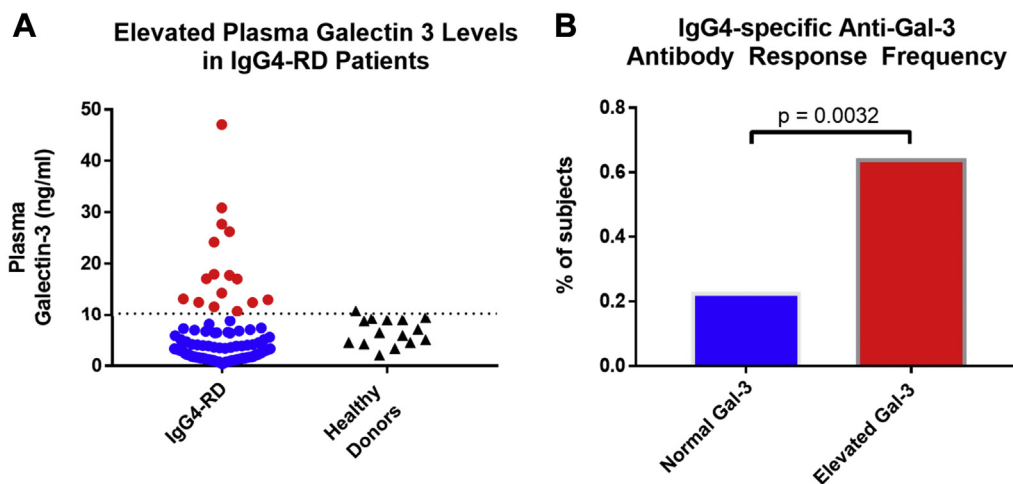


FIG 7. **A**, Circulating galectin-3 levels were quantified by means of ELISA, and 15.5% of the IgG₄-RD cohort were found to have increased levels compared with age-matched healthy donors. Subjects with indicated galectin-3 levels are indicated in red. **B**, Segregating the cohort by those with versus those without increased galectin-3 levels showed a nearly 3-fold enrichment in IgG₄ anti-galectin-3 autoantibody responses among the subset with increased circulating galectin-3 levels.

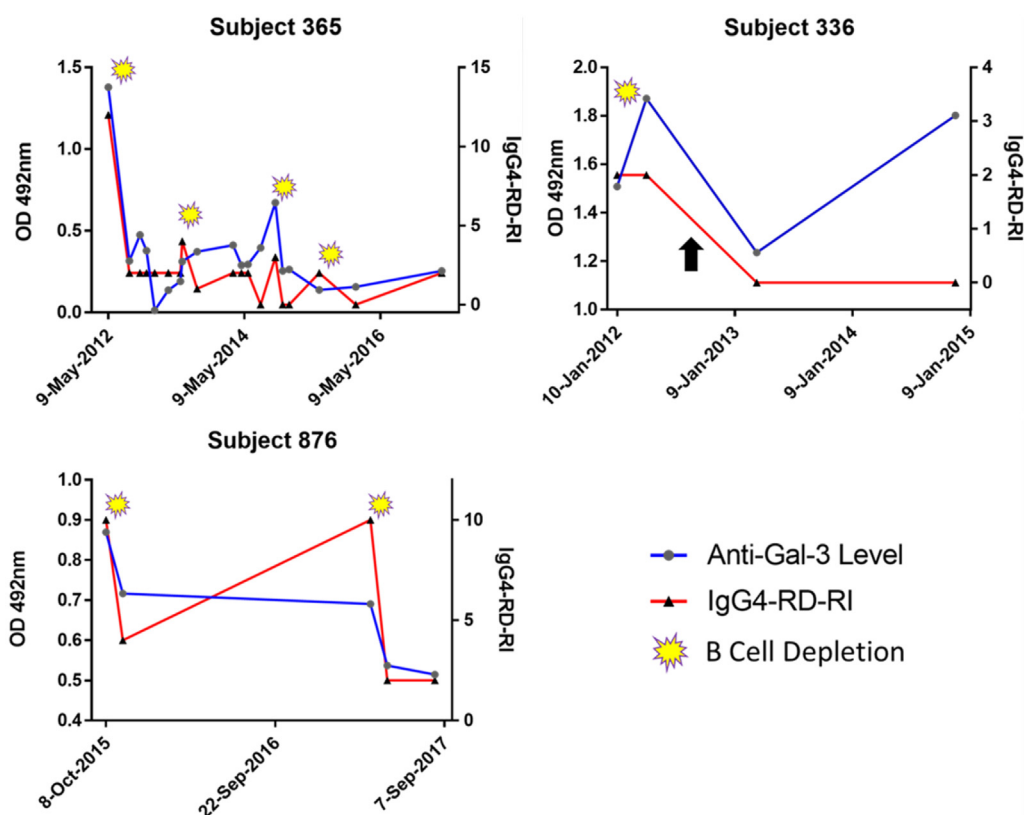


FIG 8. Three subjects with positive anti-galectin-3 antibody responses studied longitudinally by means of ELISA and plotted against IgG₄-RD-RI on the right y-axis. Yellow symbols indicate dates of B-cell depletion therapy with rituximab, after which anti-galectin-3 antibody titers consistently decreased. The black arrow in Subject 336 indicates initiation of low-dose prednisone that successfully maintained remission in this subject with a subsequent increase in anti-galectin-3 response.

inhibitor is currently being investigated for use in patients with IPF, with a phase IIa study recently completed.³³ In patients with systemic sclerosis, which is widely viewed as a prototypic human fibrotic disease, increased circulating galectin-3 levels

were shown to be the strongest predictor of all-cause and cardiovascular mortality after multivariate analysis.²⁶

Proteomic analysis of pancreatic tissue from patients with IgG₄-RD revealed that tissue galectin-3 was among the proteins

with the highest level of differential expression, with a 13-fold higher level in diseased compared with healthy pancreata.²⁵ Immunofluorescence-based analyses of tissues from patients with IgG₄-RD demonstrated that a diverse collection of both stromal and immune cells express galectin-3, including macrophages, dendritic cells, and myofibroblasts.²⁵ In addition, interstitial staining for galectin-3 is consistent with the fact that this protein has both secreted and intracellular forms. This work further characterized galectin-3 expression in many affected tissues in patients with IgG₄-RD, including the lymph nodes, lung, salivary gland, pancreas, kidney, aorta, retroperitoneum, and bile ducts.²⁵ Interestingly, diseased lymph nodes were found to have the greatest number of galectin-3-positive cells per high-power field compared with all other organs studied, and this was also approximately 8-fold greater than the number of positive cells observed in normal lymph nodes. These data, in conjunction with our observations that IgG₄ anti-galectin-3 autoantibodies correlate with both increased plasma levels of galectin-3 and lymph node involvement, support the notion that protein overexpression can sometimes contribute to autoantibody formation.

We tested our IgG₄-RD cohort for anti-galectin-3 autoantibody responses using plasma from each subject's initial clinical encounter as a means of ensuring the highest frequency of untreated and active patients. Thus it is not possible for us to determine which alteration occurs first, the overexpression of galectin-3 or the development of anti-galectin-3 autoantibodies. This answer must await further studies.

The pathophysiologic significance of anti-galectin-3 antibodies remains unknown. These antibodies could either reflect an attempt to attenuate the disease process or might actually be of causal significance. IgG₄ is known to bind poorly to activating Fc receptors and does not fix complement efficiently compared with other IgG subclasses. Consistent with the inability of IgG₄ to fix complement, we did not observe any significant increase in C-reactive protein levels or complement consumption in the subgroup with IgG₄ anti-galectin-3 autoantibody responses compared with those without. IgG₄ anti-galectin-3 antibodies could potentially reflect an attempt to mitigate the profibrotic function of galectin-3.³¹ These autoantibodies might sterically interfere with galectin-3-induced myofibroblast proliferation and collagen secretion, thereby tempering the fibrotic process.

In contrast, IgG₄ anti-galectin-3 antibodies could be of direct pathogenic significance in patients with IgG₄-RD. Spontaneous germinal center formation and accentuated autoreactivity have been observed recently in *Gal3*^{-/-} mice, suggesting that galectin-3 negatively regulates germinal center formation.³⁴ IgG₄ anti-galectin-3 autoantibodies might enhance inflammation and autoimmunity by interfering with the inhibitory role of galectin-3 in germinal center responses. Thus these anti-galectin-3 autoantibodies might accelerate plasmablast differentiation, immunoglobulin production, follicular helper T-cell expansion, and further autoantibody development. Activated B cells and plasmablasts that arise in germinal centers and infiltrate tissue sites are likely to be important for reactivation of CD4⁺ CTLs and might thus contribute to the inflammatory pathology observed in this disease.

In addition to IgG₄-specific autoantibody responses, we also observed IgE anti-galectin-3 responses in a subset of patients. Although the infiltrate of IgG₄-RD is not composed of a large fraction of mast cells or basophils, a mild-to-moderate eosinophilic component is often observed.³⁵ It is possible that IgE

anti-galectin-3 antibodies might be activating eosinophils through Fcε receptor binding. However, given the lack of a preponderant eosinophilic infiltrate in patients with this disease, and the relatively miniscule amount of IgE in the blood compared with IgG₄, it is unlikely that IgE autoantibodies play a large role in the pathophysiology of IgG₄-RD. Why they occur is likely an immunologic epiphenomenon possibly related to the fact that both IgG₄ and IgE class-switch recombination are driven at least in part by IL-4-secreting follicular helper T cells.

Approaching autoantigen discovery through the lens of dominantly expanded plasmablasts detected autoantibodies that correlate with lymphadenopathy. It is possible, but not yet proven, that the additional use of this approach might yield a diverse collection of autoantigens associated with IgG₄-RD. We speculate that expanding our identification of disease-relevant autoantigens might provide a tool to cluster patients by autoantibody specificity with corresponding organ manifestation, similar to what has been exemplified in patients with systemic lupus erythematosus.

By exploring the antigenic specificity of dominantly expanded plasmablasts, we have gained further insight into the oligoclonal restriction observed among plasmablasts in patients with this disease.² In the case of galectin-3 as an autoantigen, the association of autoantibodies with overexpression of this protein suggests that this specific immune response and the clonal expansion of plasmablasts in the index subject occurred secondary to rather than being the cause of the underlying disease. In the microenvironment of tumefactive lesions, as in patients with IgG₄-RD, similar to malignant tumors, many proteins can accumulate to pathologic levels. Therefore, over time and without treatment, IgG₄-RD might beget additional antigenic triggers, which could either amplify or attempt to quell the fibroinflammatory process, depending on the predominant autoantibody subclass response.

In summary, we have validated a multistep approach to antigen discovery using next-generation sequencing, single-cell cloning of dominantly expanded plasmablasts, unbiased antigen purification, and mass spectrometry. We have identified galectin-3, a protein that contributes to the pathophysiology of IgG₄-RD, as an antigenic target. Future work will expand on this approach by generating additional mAbs from dominantly expanded plasmablast clones as a means of examining the antigenic diversity of IgG₄-RD. The possibility that activated B-cell populations other than plasmablasts also comprise sources of relevant immunodominant antibodies in individual subjects must also be explored. These studies can then be extended to determine whether linear peptides within the same antigens are recognized in an appropriate MHC class II context by follicular helper T cells and CD4⁺ CTLs in patients with IgG₄-RD.

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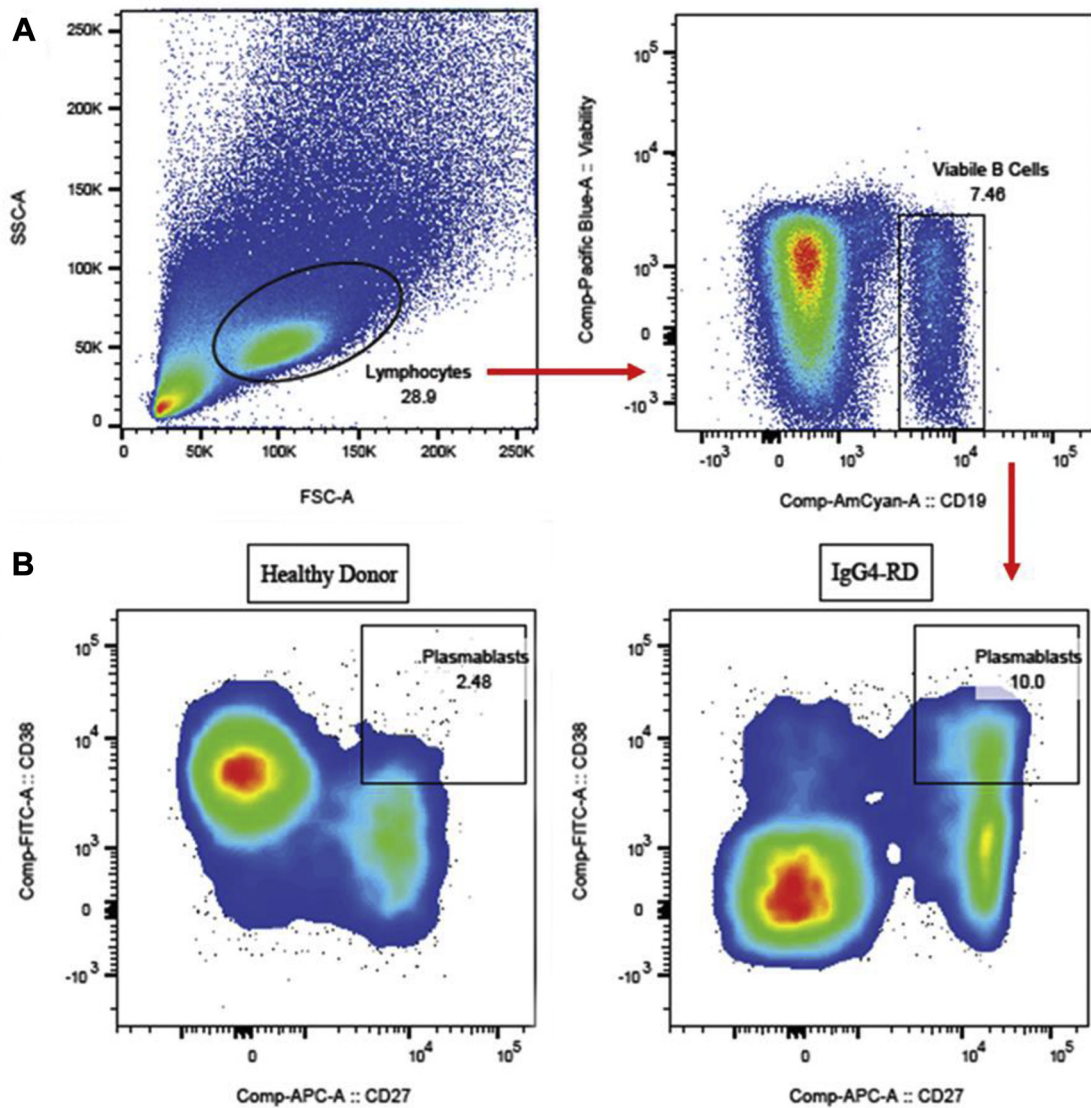


FIG E1. A, Gating strategy used to sort plasmablasts, as defined by $CD19^+CD27^{hi}CD38^{hi}$ expression. **B,** Representative healthy donor displaying no significant expansion of plasmablasts. *FSC*, Forward scatter; *SSC*, side scatter.

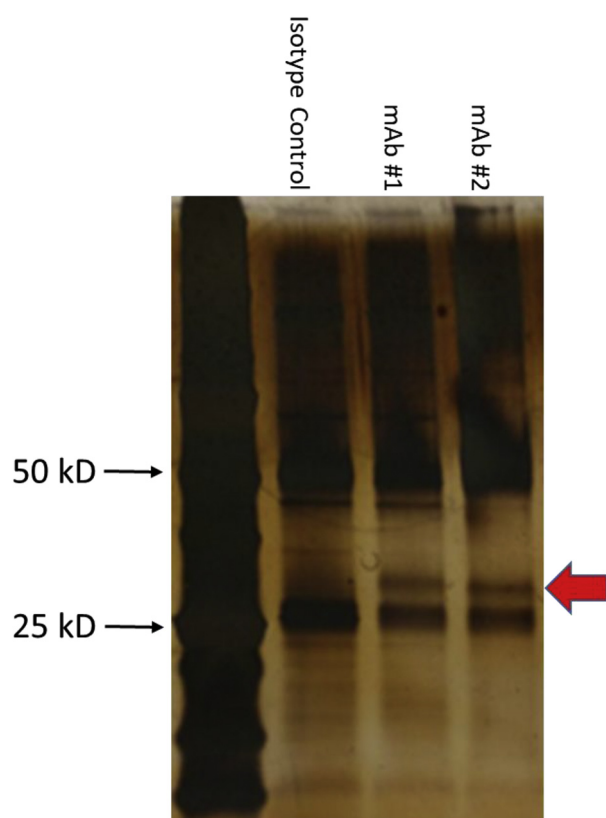


FIG E2. Silver-stained protein gel demonstrating an additional band at approximately 30 kDa compared with the isotype control in *lane 2*.

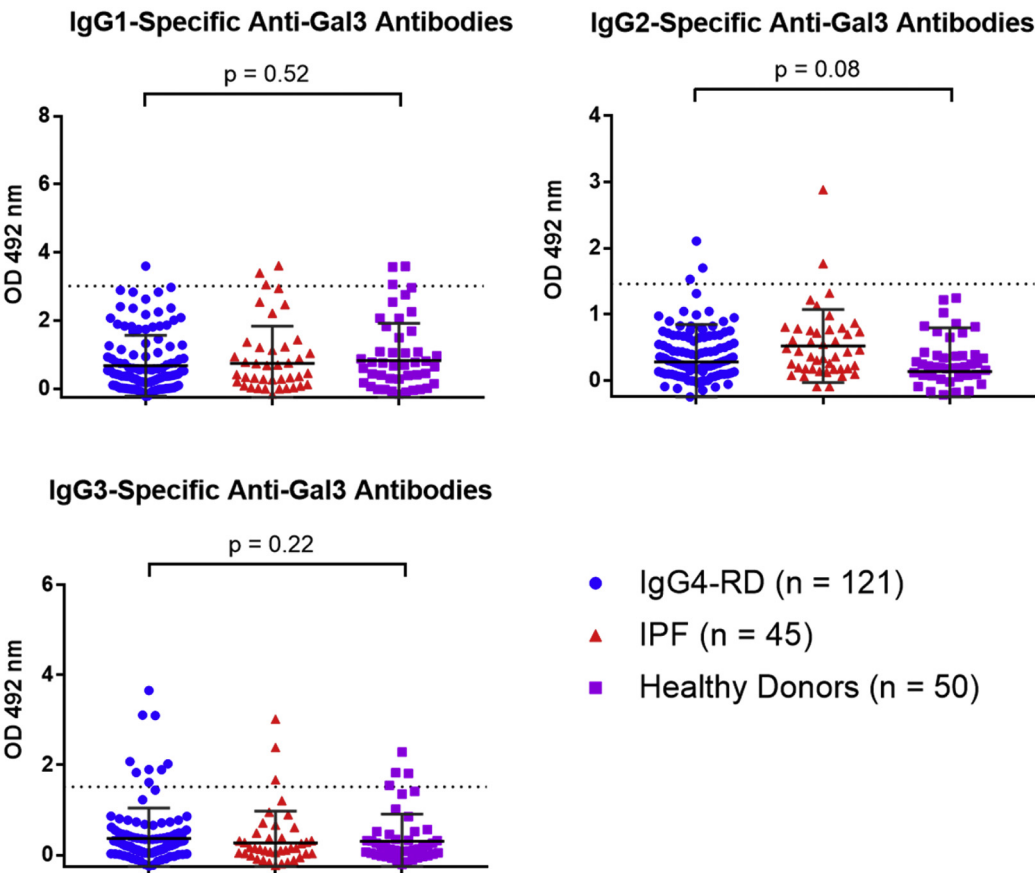


FIG E3. IgG₁, IgG₂, and IgG₃ isotype-specific galectin-3 antibody responses were measured by means of ELISA. Of these, only IgE anti-galectin-3 responses reached statistical significance compared with control groups.

TABLE E1. Demographic and clinical data

IgG₄-RD cohort (n = 121)	
Demographic data	
Age at onset (y [mean])	56
Age at diagnosis (y [mean])	59
Sex (% male)	66
Ethnicity (%)	
White	73
Asian	12
Hispanic	6
African American	5
Clinical data	
Clinically active (%)	83
IgG ₄ -RD-RI score (mean)	4.5
On treatment (%)	26
Supportive biopsy (%)	74
Organ distribution	
Multiorgan involvement (%)	79
No. of organs involved (mean)	3
Lymph nodes (%)	45
Submandibular gland (%)	45
Pancreas (%)	33
Parotid gland (%)	23
Lacrimal gland (%)	22
Lungs (%)	21
Retroperitoneum (%)	19
Kidney (%)	17
Bile ducts (%)	16

TABLE E2. Laboratory data

IgG₄-RD cohort (n = 121)	
Laboratory parameters	
Increased total IgG (%)	58
Increased serum IgG ₁ (%)	37
>2× upper limit of normal (%)	22
>5× upper limit of normal (%)	0
Increased serum IgG ₂ (%)	33
>2× upper limit of normal (%)	28
>5× upper limit of normal (%)	3
Increased serum IgG ₃ (%)	32
>2× upper limit of normal (%)	29
>5× upper limit of normal (%)	12
Increased serum IgG ₄ (%)	73
>2× upper limit of normal (%)	66
>5× upper limit of normal (%)	34
Increased serum IgE (%)	52
>2× upper limit of normal (%)	42
>5× upper limit of normal (%)	25
Decreased serum IgM (%)	41
Hypocomplementemia (%)	32
Increased C-reactive protein (%)	35
Increased erythrocyte sedimentation rate (%)	59

TABLE E3. Laboratory and disease activity correlations with plasma galectin-3 levels

Laboratory parameter, median (IQR)	IgG ₄ anti-galectin-3 positive	IgG ₄ anti-galectin-3 negative	P value
Total IgG (mg/dL)	1635 (1213-2342)	1400 (1080-1680)	.05
IgG ₁ (mg/dL)	894 (588-1080)	754 (525-1001)	.18
IgG ₂ (mg/dL)	525 (439-811)	508 (375-703)	.25
IgG ₃ (mg/dL)	69 (55-127)	76 (39-134)	.85
IgG ₄ (mg/dL)	360 (125-851)	201 (86-392)	.03
C3 (mg/dL)	106 (73-137)	115 (92-152)	.18
C4 (mg/dL)	20 (10-27)	23 (16-30)	.17
CRP (mg/L)	4.8 (1-13)	4.9 (1.8-11)	.69
IgE (IU/mL)	208 (87-678)	75 (24-448)	.16
IgM (mg/dL)	84 (41-114)	61 (41-126)	.69
Galectin-3 (ng/mL)	5.5 (1.9-12.3)	2.5 (1.3-3.9)	.001
Organ involvement (%)			
Lacrimal gland	24	24	1.00
Salivary gland	56	49	.55
Biliary	15	17	.79
Pancreas	35	29	.51
Kidney	18	18	1.00
Lung	24	20	.80
Retroperitoneum	18	20	1.00
Lymph node	59	38	.04
IgG ₄ -RD-RI (mean)	5.1	4.3	.13
No. of organs involved	4 (2-5)	3 (1-4)	.10
Multiorgan involvement	88%	72%	.09

CRP, C-reactive protein; IQR, interquartile range.