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CONCISE REPORT

Increased IgG4 responses to multiple food and animal antigens indicate a polyclonal expansion and differentiation of pre-existing B cells in IgG4-related disease

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ABSTRACT

Background IgG4-related disease (IgG4-RD) is a systemic fibroinflammatory condition, characterised by an elevated serum IgG4 concentration and abundant IgG4-positive plasma cells in the involved organs. An important question is whether the elevated IgG4 response is causal or a reflection of immune-regulatory mechanisms of the disease.

Objectives To investigate if the IgG4 response in IgG4-RD represents a generalised polyclonal amplification by examining the response to common environmental antigens.

Methods Serum from 24 patients with IgG4-RD (14 treatment-naive, 10 treatment-experienced), 9 patients with primary sclerosing cholangitis and an elevated serum IgG4 (PSC-high IgG4), and 18 healthy controls were tested against egg white and yolk, milk, banana, cat, peanut, rice and wheat antigens by radioimmunoassay.

Results We demonstrated an elevated polyclonal IgG4 response to multiple antigens in patients with IgG4-RD and in PSC-high IgG4, compared with healthy controls. There was a strong correlation between serum IgG4 and antigen-specific responses. Responses to antigens were higher in treatment-naive compared with treatment-experienced patients with IgG4-RD. Serum electrophoresis and immunofixation demonstrated polyclonality.

Conclusions This is the first study to show enhanced levels of polyclonal IgG4 to multiple antigens in IgG4-RD. This supports that elevated IgG4 levels reflect an aberrant immunological regulation of the overall IgG4 response, but does not exclude that causality of disease could be antigen-driven.

INTRODUCTION

IgG4-related disease (IgG4-RD) is a multisystem fibroinflammatory condition, characterised by the development of mass lesions with similar histopathological findings in the involved organs.[1] Histological characteristics include an infiltrate of lymphocytes and plasma cells, a storiform pattern of fibrosis, obliterative phlebitis and variable presence of eosinophils. An elevated serum IgG4 and abundant accumulation of IgG4-positive plasma cells in affected tissues is frequently seen. An increase in circulating plasmablasts and IgG4+ B cells have also been demonstrated.[2 3] Autoimmune pancreatitis (AIP) and IgG4-related cholangitis (IRC) were the first described manifestations of the disease.[4]

An immune-mediated pathogenesis has been suggested in IgG4-RD, supported by a human leucocyte antigen type II association, presence of autoantibodies and elevated levels of IgG4 and a dramatic response to corticosteroid therapy.[5 6] Antibodies against a range of autoantigens have been proposed including antinuclear antigens, lactoferrin, carbonic anhydrase II and IV, pancreatic secretory inhibitor and trypsinogens.[7 8] However, none has been consistently found in the disease, and the fact that they are of the IgG1 and not IgG4 subclass makes their overall significance unclear. A role for *Helicobacter pylori* plasminogen-binding peptide, through a process of antibody cross-reactivity with ubiquitin-protein ligase E3 component n-recognin 2 (molecular mimicry) in genetically predisposed individuals, has been suggested in AIP.[9] Furthermore, next-generation sequencing of whole blood in patients with IRC highlighted highly abundant IgG4-positive clones in the B cell repertoire, suggesting that specific B cell responses are pivotal to disease pathogenesis.[10]

Our alternative hypothesis is that the elevated IgG4 may not be (primarily) triggered by specific (auto)-antigens, but be an indirect consequence of the expansion of pre-existing IgG4-switched B cells as being responsible for IgG4-RD. In this case, one would expect to find a more generalised and (compared with an antibody response derived from long-lived, bone-marrow-resident plasma cells) a more transient increase in IgG4 antibodies against different antigens that are known to elicit an IgG4 antibody response in the general population. We tested this hypothesis by investigating the level and persistence of the IgG4 response to a variety of known IgG4-inducing non-infectious environmental antigens. We analysed patients with IgG4-RD, patients with primary sclerosing cholangitis (PSC) and elevated IgG4 (a subset of patients with PSC who have an elevated serum IgG4 level but no histological or radiological evidence of IgG4-RD), and healthy controls in a UK cohort.

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METHODS

Detailed description of patient inclusion criteria and methodology can be found in the online supplementary repository. Antigen-specific IgG4 responses to egg, milk, peanut, banana, rice, wheat and cat were quantified using a previously developed radioimmunoassay.[11]

RESULTS

Characteristics of the cohort

Demographics, clinical characteristics and serum immunoglobulin values of patients and controls are shown in the online supplementary table S1. Most patients with IgG4-RD (83%) had pancreatic (AIP) and/or biliary (IRC) involvement, with 71% having other systemic organ involvement. The concentration of serum total IgG, IgG4, IgE and, to a lesser extent IgG1, was higher in patients with IgG4-RD versus healthy controls, as well as in the patients with PSC-high IgG4 versus healthy controls (see online supplementary figure S1).

Antigen-specific responses

We analysed the IgG4 response to proteins from egg, milk, peanut, banana, rice, wheat and cat. In line with our hypothesis, the response to egg (p=0.004), milk (p=0.04), peanut (p=0.0003), cat dander and serum (p=0.012), rice and wheat (0.006) antigens was found to be higher in patients with IgG4-RD than in healthy controls, and the response to egg (p=0.03), cat dander and serum (p=0.04), and rice and wheat (p=0.01) antigens was higher in patients with PSC-high IgG4 than in healthy controls (figure 1).

We further examined the correlation of serum immunoglobulin levels with antigen-specific responses. In patients with IgG4-RD, there was a positive correlation between serum IgG4 levels and IgG4 responses to banana (Rank 0.38, 95% CI −0.002 to 0.67, p=0.045), peanut (Rank 0.49, 95% CI 0.14 to 0.74, p=0.007), cat (Rank 0.61, 95% CI 0.29 to 0.80, p=0.0006), rice and wheat (Rank 0.38, 95% CI −0.002 to 0.67, p=0.045) antigens (see online supplementary figure S2). By contrast, total serum IgE levels did not correlate with antigen-specific responses (data not shown).

Treatment-naive and treatment-experienced patients with IgG4-RD

We also tested the differences in immunoglobulin levels and IgG4-specific antigen responses in 14 treatment-naive and 10 treatment-experienced patients with IgG4-RD receiving corticosteroid therapy. Levels of antibodies to banana (p=0.001), egg (p=0.039), peanut (p=0.003) and cat (p=0.006) antigens were lower in treatment-experienced compared with treatment-naive patients (figure 2), as were serum total IgG (p=0.017) and IgG4 (p=0.001) (see online supplementary figure S3).

Serum electrophoresis

We analysed serum electrophoresis data for evidence of monoclonality in patients and controls, but no monoclonal bands in the polyclonal gamma region were observed (see online supplementary figure S4). All individuals showed polyclonality; 11/14 (78.6%) patients with IgG4-RD, 3/9 (33.3%) PSC-high patients with IgG4, and 0/18 healthy controls showed hypergammaglobulinaemia. Further immunofixation analysis of patient samples with a diffuse increase in immunoglobulins, using IgG, IgA, IgM, kappa and lambda light-chain antisera, confirmed no monoclonal bands in the gamma region (see online supplementary figure S4).

DISCUSSION

Our data show that IgG4-RD is associated with an elevated IgG4 response to diverse antigens. Therefore, elevated levels of IgG4 in patients with IgG4-RD may be the result primarily of a polyclonal expansion of many IgG4 B cells irrespective of their specificity. This would imply that factors other than antigen induce an expansion and differentiation of IgG4 B cells, but does not exclude the possibility that a single antigen (self or exogenous) serves as the initial trigger of disease. Antigen-independent involvement of many IgG4-switched B cells implies differential selection via signals that uniquely act on IgG4 B cells. We have recently demonstrated differences in phenotype between IgG1 and IgG4 B cells,[12] and future studies may reveal traits that uniquely link IgG4 B cells to proliferation in inflamed or fibrotic tissue.[13] The IgG4-switched B cells may be selectively triggered to expand and differentiate into plasma cells. This may involve interleukin (IL)-21 driving proliferation and expansion of IgG4-switched cells and the upregulation of activation-induced cytidine deaminase, B-lymphocyte-induced maturation protein 1 and Xbox protein 1, all of which have been shown to be upregulated in patients with various organ manifestations of IgG4-RD.[14 15] Alternatively, signals from the inflamed/fibrotic tissue, including cytokines such as IL-4/IL-13 or IL-10 could selectively stimulate B cells to proliferate into IgG4 plasma cells, either directly or via Th2 and T regulatory cells.[16 17] Such an environment may be actively sustained via signals from IgG4 B cells themselves, possibly involving IL10.[3 18] Furthermore, since IgG4 responses are associated with chronic stimulation, this B cell subset may be more susceptible to expansion due to continued exposure to antigen(s), and therefore there does not need to be a functional link between antibody specificity and potential autoantigen. Importantly, these scenarios are not mutually exclusive. However, it remains unclear if the elevated IgG4 reflects a causal event or epiphenomena. Further research is necessary to determine if the large amounts of IgG4 plasma cells are predominantly the result of disease-associated class switch or disease-associated expansion and terminal differentiation of IgG4 B cells.

The fall in IgG4-specific antigen responses after corticosteroids may be explained by suppressed proliferation of expanded IgG4-switched B cells or suppressed differentiation into IgG4 plasmablasts. Corticosteroids interfere with the production of cytokines critical for T cell proliferation and interaction, and the binding of interleukins to B cells suppressing proliferation and antibody production. The steroid effect highlights the rapid turnover of the IgG4-producing plasma cells, as opposed to production by long-lived plasma cells in the bone marrow.

Evidence for polyclonality in IgG4-RD is supported by the diversity of antigen specificity, and by serum electrophoresis and immunofixation analysis no monoclonal bands in the polyclonal gamma region was observed (see online supplementary figure S4). However, this is complicated by the ability of the bispecific IgG4 antibody to undergo Fab-arm exchange (known to take place in vivo[12]), which limits the potential to detect an oligoclonal response where multiple clones have comparable abundance. In model systems using chimeric antibodies, Fab arm exchange can affect the electrophoretic mobility of IgG4, although if >50% of the IgG4 would be monoclonal then >25% would still remain monoclonal after Fab-arm exchange. Recently, oligoclonal somatically hypermutated plasmablast populations were demonstrated by next-generation sequencing in patients with active IgG4-RD.[19]

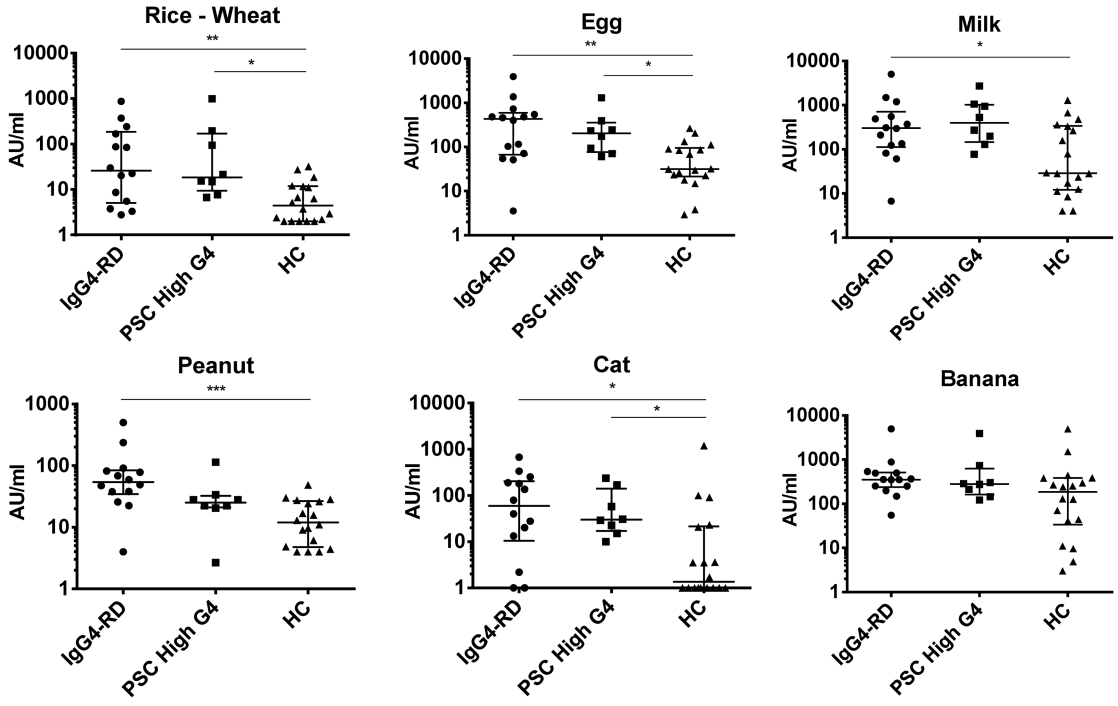

Figure 1 The dot plots show IgG4 antigen-specific responses in treatment-naive patients with IgG4-RD, patients with PSC-high IgG4, and healthy controls. X-axis labels as shown in the figure . Antigens were rice and wheat, egg, milk, peanut, cat dander and serum, and banana. On the Y-axis is IgG4-specific antigen response, log 10 scale, in arbitrary units per mL. Error bars represent median and IQR; p values: *p<0.05, **p<0.005, ***p<0.001. HC, healthy controls; IgG4-RD, IgG4-related disease; PSC, primary sclerosing cholangitis.

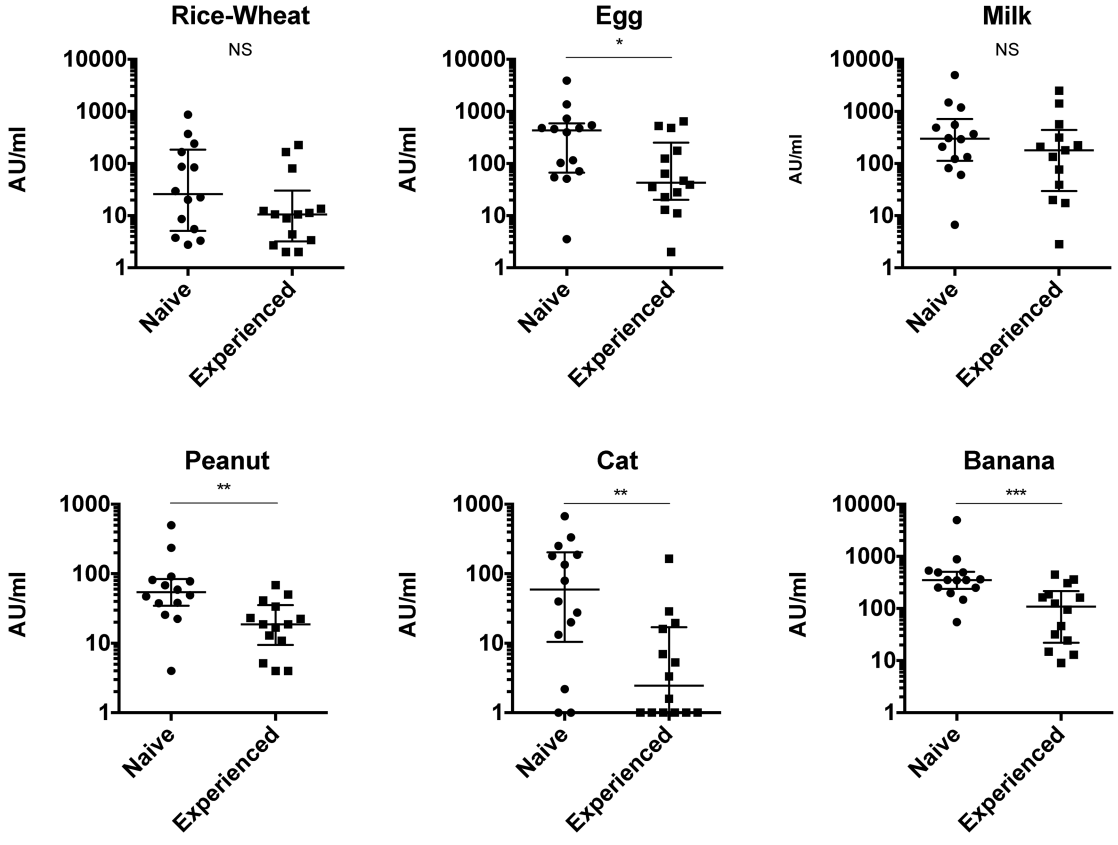

Figure 2 The dot plots show the IgG4 antigen-specific responses in treatment-naive and treatment-experienced (on corticosteroids) patients with IgG4-related disease (IgG4-RD). Antigens were rice and wheat, egg, milk, peanut, cat dander and serum, and banana. Units as in figure 1. Error bars represent median and IQR. Mann–Whitney p values: *p<0.05, **p<0.005, ***p<0.001.

Mechanisms responsible for driving IgG4-RD (eg, Th2 cytokines) may also increase IgE B cell expansion in certain individuals. In this instance, one may expect an increased predisposition to allergies in later years of life. However, the frequency of allergy/atopy was similar in patient and control groups (see online supplementary table S1). Furthermore, there was no significant difference between total and antigen-specific IgG4 responses in patient and control groups (not shown). However, serum IgE levels in patients with IgG4-RD with allergy/atopy was higher than in those without (p=0.0255) (see online supplementary figure S3); consistent with Della Torre *et al*[20] Therefore, it is plausible that elevated serum IgG4, resulting from polyclonal expansion of many IgG4 B cells irrespective of antigen specificity, may be linked to IgE B cell expansion in a subset of allergic/atopic individuals with IgG4-RD.

To summarise, this is the first study to show an enhanced polyclonal IgG4 response to multiple non-infectious environmental antigens in IgG4-RD. Multiple different antigen responses are higher in treatment-naive compared with treatment-experienced patients. The elevated IgG4 levels may reflect the aberrant immunological regulation of the overall IgG4 response in the disease.

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Contributors ELC diagnosed and consented patients, acquired serum samples, was involved in experimental design and analysis, drafted the original manuscript and the final version. EV was involved in experimental design, performed the RAI assay and was involved in analysis. MM was involved in experimental design and analysis, and edited the manuscript. AvL was involved in experimental design, RAI assay development and optimisation, and performed the assay. RS was involved in experimental design, measurement of serum immunoglobulins, electrophoresis and immunofixation, and edited the manuscript. TC was involved in patient data collection and analysis, and edited the manuscript. PK was involved in experimental critique and editing the manuscript. RCA was involved in study design and concept, developed the RAI assay, involved in analysis, and edited the manuscript. EB was involved in experimental critique and editing the manuscript. SMvH was involved in experimental critique and editing the final manuscript. TR was involved in design and concept of the study, analysis, editing the manuscript. All authors approved the final version of the manuscript.

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Competing interests None.

Ethics approval Oxfordshire REC.

Provenance and peer review Not commissioned; externally peer reviewed.

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Supplementary Data

Methods

Patient and control cohorts: definitions and recruitment

Patients and controls were recruited from the John Radcliffe Hospital in Oxford, a tertiary referral centre for IgG4-RD and PSC in the UK. Patients with IgG4-RD who were referred to Oxford from February 2005 to February 2013 and followed prospectively were recruited. Diagnosis of IgG4-RD was made using the HISORt criteria (since 2007), and the International Consensus Diagnostic Criteria (ICDC) (since 2011),[1, S1]. The Boston Consensus Histopathological Criteria for IgG4-RD (2012) was applied to those with biopsy and surgical resection specimens,[S2]. Patients with primary sclerosing cholangitis (PSC) referred to Oxford from January 2002 to February 2013 and followed prospectively were recruited. Diagnosis of PSC was made in accordance with the European Association for the Study of the Liver Guidelines in those with a cholestatic biochemical profile, characteristic cholangiography and/or consistent liver histology when secondary sclerosing cholangitis had been excluded,[S3]. Healthy controls (HC) with no history of autoimmune conditions were recruited. The European Academy of Allergy and Clinical Immunology classification was used to record a history of allergy or atopy,[S4]. All patients gave written informed consent. Ethical approval was obtained from the Research Ethics Committee, Oxfordshire (ref: 10/H0604/51).

Serum immunoglobulin measurements

Serum levels of IgG and subclasses (IgG1 and IgG4) were measured by nephelometry using a Siemens BNII analyser (Siemens, Surry, UK). Total IgE was measured by the Immunocap method using a Phadia 250 (Phadia, Milton Keynes, UK). Both sets of assays were carried out at the Department of Immunology, Churchill Hospital OUH Trust, Oxford, UK. The

normal range for serum immunoglobulin concentrations was determined by the institute reference values; IgG 6-16.2g/l, IgG1 3.2-10.2g/l, IgG2 1.2-6.6g/l, IgG3 0.2-1.9g/l, IgG4 0.1-1.3g/l and IgE 5-120KU/L. For this study, an elevated serum IgG4 was defined as ≥1.4g/l as used in the Mayo HISORt criteria for AIP[3] and an elevated IgE as >120KU/L as per institute reference. For IgE units are expressed as KU/L, equivalent to 1 IU/mL, and equivalent to 2.4ng/mL. Serum values for IgG subclasses were comparable at Oxford and Sanquin institutes. The prozone effect was avoided by testing samples at serial dilutions.

Inclusion criteria for the study

A total of 60 patients with IgG4-RD met diagnostic criteria, of whom 53 patients (88.3%) had an elevated serum IgG4. Serum from 24 patients (23 with elevated serum IgG4 and 1 with normal serum IgG4) was collected at disease presentation for the 14 treatment-naïve patients. For the treatment-experienced patients (n=10), serum was collected at 4-8 weeks after starting a high dose (30-40 mg prednisolone) of corticosteroids. The treatment-experienced group included 4 samples from patients also included in the naive group. No patient was on concomitant immunosuppression. A total of 220 patients met PSC criteria, of whom 33 patients (15%) had an elevated serum IgG4. Serum from 9 PSC patients with elevated serum IgG4 were collected at recruitment, with no concurrent corticosteroid or immunosuppressive medication, imaging evidence to confirm no extra-biliary involvement except inflammatory bowel disease, and no evidence on liver biopsy of IRC morphology. A total 50 healthy controls were recruited, of whom 2 controls (4%) had an elevated serum IgG4. Serum from 18 controls with normal serum IgG4 and no history of autoimmune disease, corticosteroid or immunosuppressive therapies were included.

IgG4 Radio Immunoassay (RIA)

The radio immunoassay (RIA) is a sensitive *in vitro* test to determine and quantify antigen-specific antibodies in serum. For the RIA, 250µl of a 2 mg/ml Sepharose suspension (antigen coupled to CNBr-activated Sepharose-4B (Amersham Pharmacia Biotech AB)) was washed (x3) then incubated with 50µl of 1:50 diluted patient or control serum plus 450µl of PBS with human serum albumin, Tween-20 (with mannoside in the case of banana to avoid measurement of non-specific IgG4 due to the presence of lectins in banana), overnight at RT,[9, S5]. The suspension was washed (x5) with PBS containing 0.005% Tween-20 (PBS-T) to remove unbound IgG4 antibodies and other irrelevant serum components, then 500µl of PBS with 1mg/ml sheep IgG and 3mg/ml human serum albumin was added to 50µl of monoclonal mouse-anti-human IgG4 (radiolabelled ^{125}I (Sanquin)) and left overnight at RT. After washing 5 times with PBS-T, bound radioactivity was measured. Bound radioactivity was converted to arbitrary units (AU) using a calibrator serum with IgG4 to grass pollen set at 1400 AU/ml; one AU is estimated to equal approximately 50 ng IgG4.

The antigens used were egg white and yolk, milk, cat dander and serum, banana, milk, rice and wheat (Sanquin Institute, Amsterdam). All foods were obtained locally, cat dander from HAL (Haarlem, The Netherlands), and cat serum from a local veterinarian. Antigen extracts (for example, egg white and yolk) were separately coupled to beads, but the beads were combined before testing with serum. The amounts of antigen extracts coupled to dry weight Sepharose are: egg white extract 200 mg coupled to 40 g Sepharose-4B; egg yolk extract 160 mg coupled to 20g Sepharose-4B; cat dander extract 100 mg coupled to 20g Sepharose-4B; cat serum ~~extract~~ 5.5 mg coupled to 0.8g Sepharose-4B; banana extract 81 mg coupled to 2g Sepharose-4B; milk extract 609 mg coupled to 10g Sepharose-4B; rice and wheat extracts 240 mg coupled to 6 g of Sepharose-4B. To compare results, signals were compared to a standard curve (a serially diluted reference serum incubated with 4mg/ml Dactylis Sepharose)

and expressed as arbitrary units. Mixtures of chimeric antibodies (anti-Bet v1 and anti-Feld 1) were used to exclude the risk of second-generation IgG4 binding.

Serum Electrophoresis and Immunofixation

Serum electrophoresis was performed to check for evidence of monoclonality. This was carried out using a Sebia hydrasys focusing system (Sebia Cedex, France). Initial gel electrophoresis of samples was performed using the Sebia Hydragel 54 protein gel kit (Sebia, Cedex, France). Densitometry of electrophoresis gels was analysed using Phoresis (Sebia, Cedex, France). In patients where no monoclonality was detected, individuals were designated as hypogammaglobulinaemic, normal or hypergammaglobulinaemic, determined by the levels of serum IgG and the appearance of the beta/gamma region of densitometry plot. In samples with a diffuse increase in immunoglobulins, immunofixation analysis using IgG, IgA, IgM, kappa light chain and lambda light chain antisera was carried out (Sebia hydragel IF 2/4 kit (Sebia, Cedex, France)) as a higher sensitivity method to test for the presence of monoclonal bands in the polyclonal gamma region. This method has a lower limit of detection of approximately 0.2g/l of protein.

Statistical analysis

A Kruskal-Wallis test with multiple comparisons and an ANOVA using log-transformed data were used to compare IgG4-specific antigen responses in IgG4-RD patients, PSC patients and healthy controls. A two-tailed Mann Whitney was used to compare responses in treatment-naïve and treatment-experienced IgG4-RD patients. Statistics were calculated using Graphpad Prism v6.0. A P-value of <0.05 was considered statistically significant.

Supplementary References for methods section

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Supplementary Figures:

Figure S1: The dot plots show serum IgG, IgG1, IgG4 and IgE levels in treatment-naïve IgG4-RD patients, PSC high IgG4 patients and healthy controls. On the X-axis is IgG4-RD – IgG4-RD patients, PSC High G4 –PSC patients with elevated serum IgG4 levels, HC – healthy controls with normal serum IgG4. On the Y-axis is serum immunoglobulin levels, log 10 scale, IgG, IgG1 and IgG4 in grams per litre and IgE in recognised international units KU/L. Error bars represent median and interquartile range. Kruskal-Wallis test with multiple comparison p values * $p<0.05$, ** $p<0.005$, ***$p<0.001$.

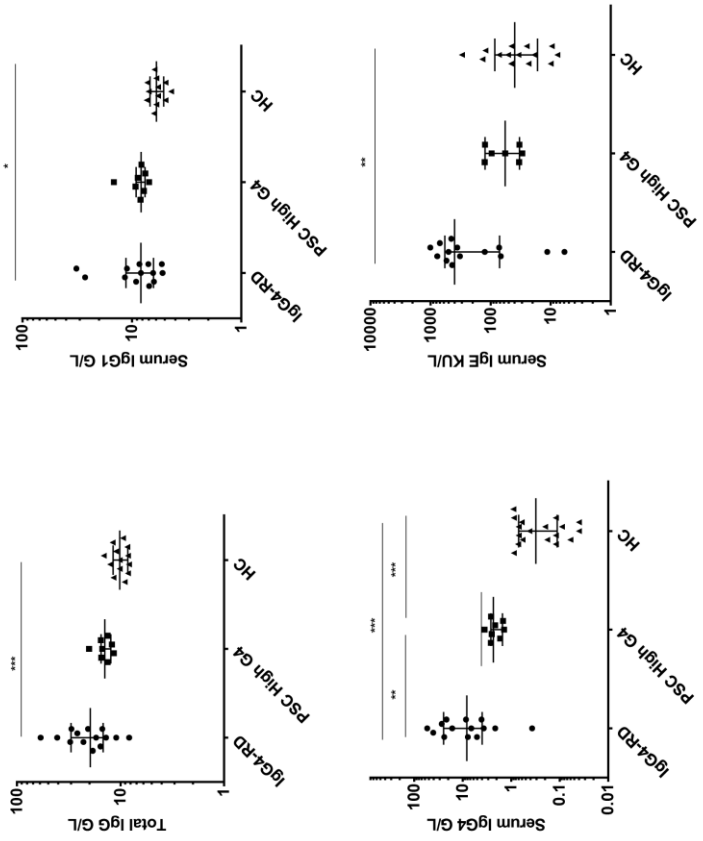


Figure S2: Correlation plots of serum IgG4 and IgG4-specific antigen responses in treatment-naïve and experienced IgG4-RD patients. Antigens were rice and wheat, egg, milk, peanut, cat dander and serum, banana. On the X-axis is serum IgG4, log 10 scale, in grams per litre. On the Y axis is IgG4 specific antigen response, log 10 scale, in arbitary units per ml. Spearman rank correlation R values and p values * $p<0.05$, ** $p<0.005$, ***$p<0.001$.

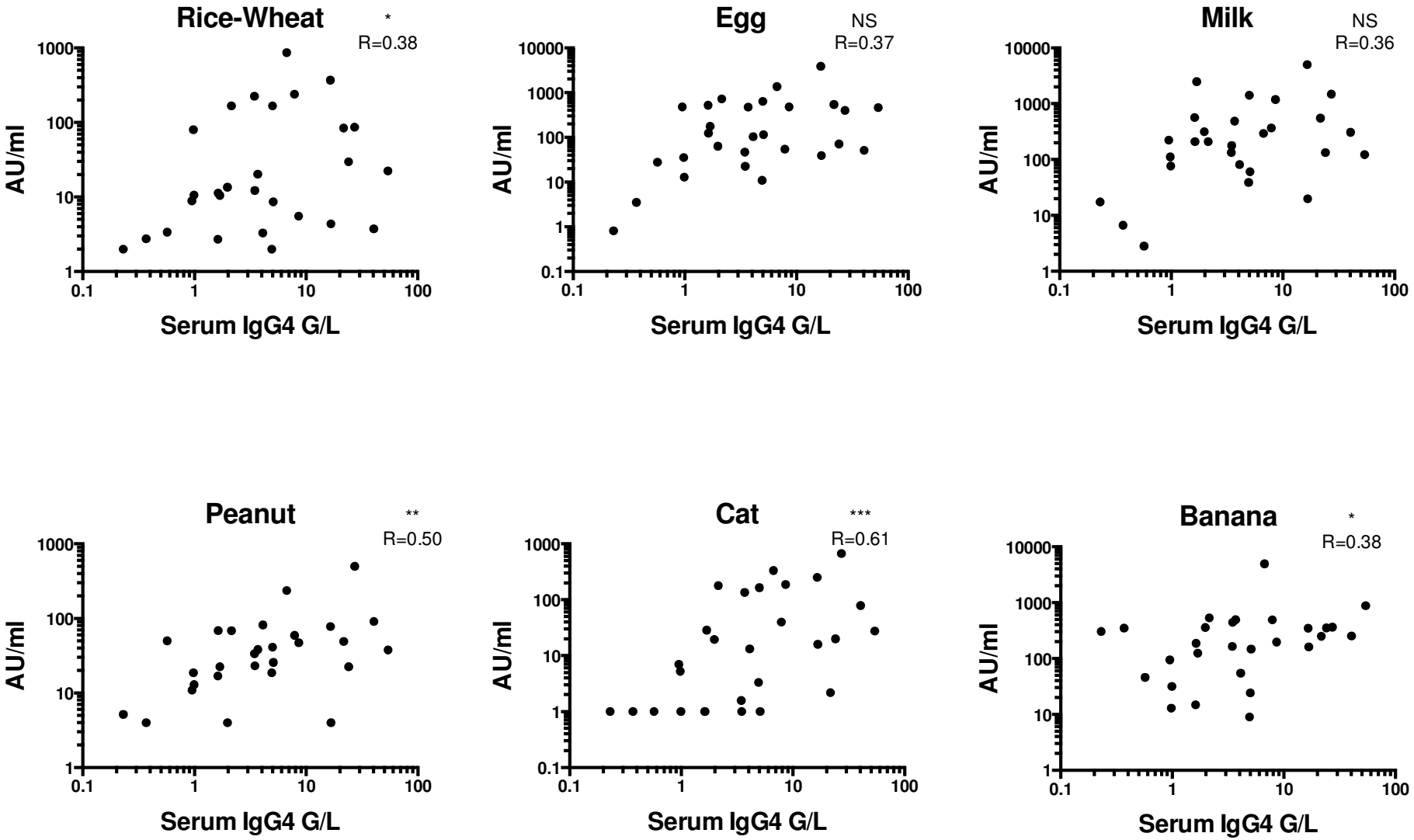


Figure S3: The dot plots show the serum IgG, IgG1, IgG4, IgE levels and IgG4/IgE ratio in treatment-naïve and experienced IgG4-RD patients. Units as in Figure S1. Error bars represent median and interquartile range. Lower right panel: IgE levels in IgG4-RD patients with and without atopy. Mann U Whitney p values * p<0.05, ** p<0.005, ***p<0.001.

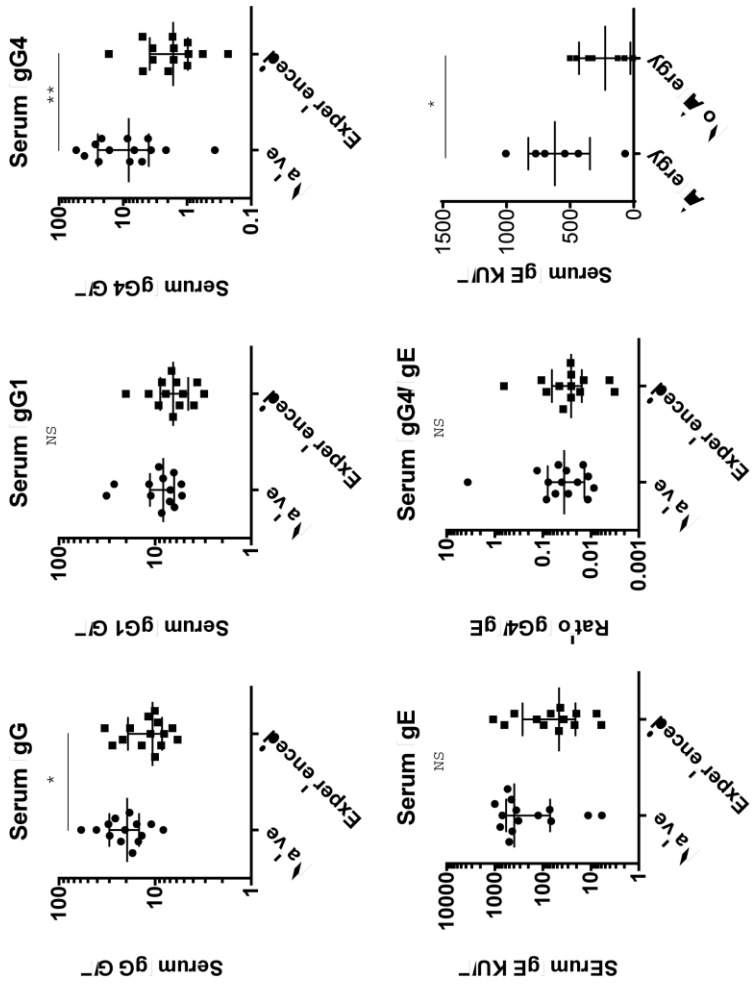
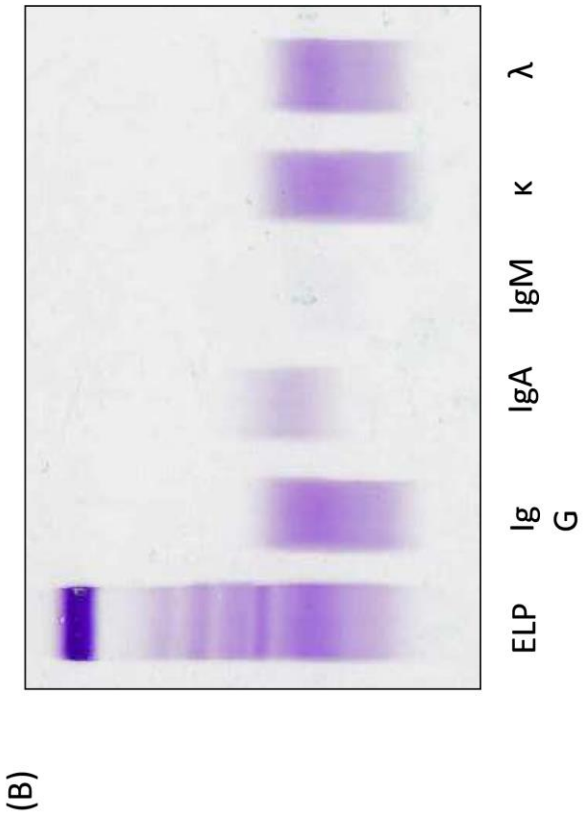


Figure S4: A. Representative serum protein electrophoresis gel in a patient with multiple myeloma (M), patient with IgG4-RD (G4) and a healthy control (HC). B. Representative negative serum protein immunofixation using antisera directed at heavy chains of IgG, IgA and IgM plus Kappa (κ) and Lambda (λ) light chain in an IgG4-RD patient who displayed hypergammaglobulinaemia. ELP is the original standard gel control.



Supplementary Table

Table S1: Demographics, characteristics and immunoglobulin values of the cohort.

	IgG4-RD patients	**PSC high IgG4**	**Healthy controls**	**P values (ANOVA)**	**IgG4-RD vs PSC (KW)**	**IgG4-RD vs HC (KW)**	**PSC vs HC (KW)**
Absolute number	24 14 treatment-naïve 10 treatment-experienced	8	18				
Age years median (range)	65 (32-84)	65 (35-76)	48.5 (24-67)	0.001	0.7085	0.0007	0.0905
Gender (male) %	87.5	62.5	55.6	0.608			
Organs involved AIP/IRC % Systemic OOI %	 83.3 70.8	 0 0	 0 0				
Allergy or atopy %	41.67	25.0	37.5	0.073			
Serum IgG G/L median (range)	19.55 (8.24-59.1)	14.15 (11.6-20.1)	10.22 (8.3-14.5)	<0.0001	0.0727	<0.0001	0.1054
Serum IgG1 G/L median (range)	8.27 (5.25-32.2)	8.26 (6.94-14.6)	5.98 (4.37-7.31)	0.0104	0.9512	0.0126	0.0622
Serum IgG4 G/L median (range)	8.22 (0.37-54.1)	2.33 (1.4-3.53)	0.31 (0.04-0.9)	<0.0001	0.0186	<0.0001	0.0002
Serum IgE G/L median (range)	397.5 (5.9-1006.0)	57.1 (29.6-125)	39.65 (7.75-300)	0.0025	0.1148	0.0019	0.5994

Abbreviations: IgG4-RD ‘IgG4-related disease’; PSC ‘primary sclerosing cholangitis’; AIP ‘autoimmune pancreatitis’; IRC ‘IgG4-related cholangitis’; OOI ‘other organ involvement’; KW ‘Kruskal-Wallis test with multiple comparisons’.