

Correspondence

Reply

To the Editor:

We recently devised a method to examine IgG₄⁺ B cells in blood and applied this to study patients with IgG₄-related disease (IgG₄-RD) with the implication of a noninvasive test.^{1,2} In our cohort of patients who all met the Boston criteria for IgG₄-RD, IgG₄⁺ memory B-cell numbers were increased,² as well as circulating plasma blasts, CD21^{low} B cells, T_H2 cells, and regulatory T (Treg) cells that were reported previously in other cohorts.^{3,4} Together, these abnormalities were regarded as a specific

“lymphocyte signature,” and we devised a combined score that could discriminate patients from controls with 93.3% sensitivity and 87.8% specificity.²

Akiyama brings up an important issue regarding active germinal centre in affected tissue and involvement of follicular helper T(fh) cells in IgG₄-RD.⁵ The accumulation of IgG₄-expressing B cells and plasma cells is thought to result from enhanced T-cell-dependent germinal center responses in lymphoid and nonlymphoid tissue of affected patients. Indeed, of 16 previously reported² and 3 newly included patients, 17 (89%) had B-cell follicles in the affected tissue (Fig 1, A; see Table E1 and Fig E1 in this article's Online Repository at www.

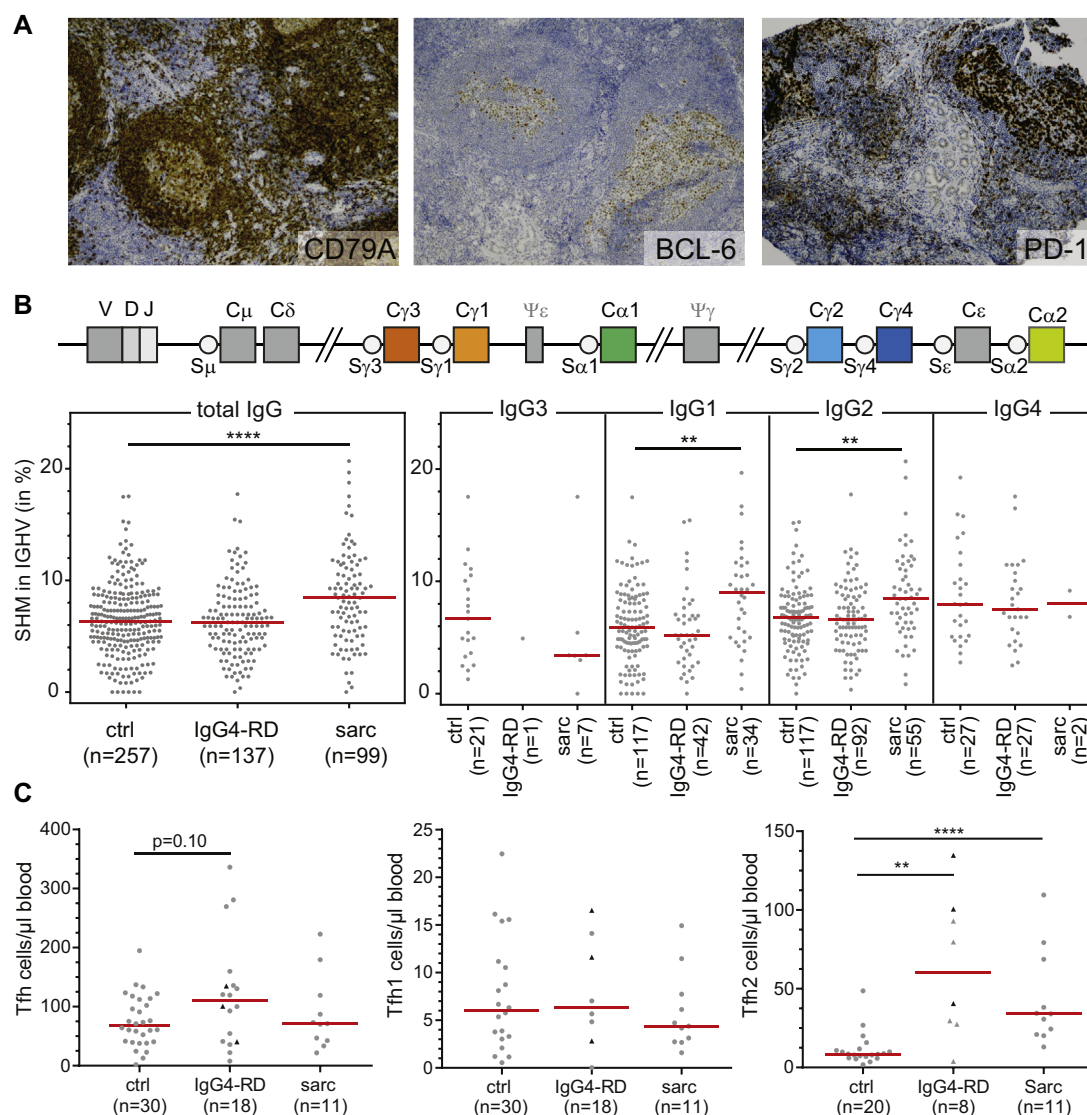


FIG 1. B-cell follicles, SHMs, and Tfh-cell subsets in patients with IgG₄-RD. **A**, Immunohistochemical analysis of orbital tissue from patient 7 reveals the presence of CD79A-positive B-cell follicles containing Bcl-6 positive, PD-1+ Tfh cells. **B**, SHMs in IgG transcripts and IgG subclasses of adult controls (ctrl), patients with IgG₄-RD, and patients with sarcoidosis (sarc).⁵ **C**, Number of Tfh, Tfh1, and Tfh2 cells in blood of controls, patients with IgG₄-RD, and patients with sarcoidosis. Black triangles denote newly included patients with IgG₄-RD. Statistics: Mann-Whitney U test, ** $P < .01$, **** $P < .0001$.

jacionline.org). In contrast, we did not identify cellular or molecular signs of enhanced B-cell activation in blood. Rather, IgG₄⁺ memory B cells showed a more anergic phenotype with significantly more of these cells lacking CD21 and CD27 expression, and having reduced expression levels of CXCR5, a chemokine receptor important for homing to B-cell follicles.² Moreover, transcripts of these IgG₄⁺ B cells did not show enhanced accumulation of somatic hypermutations (SHMs). Additional SHM analysis on transcripts amplified with an IgG consensus primer revealed no increased SHM in the other IgG subclasses either, which is in contrast to patients with sarcoidosis (Fig 1, B; see Fig E2 in this article's Online Repository at www.jacionline.org).⁶ Still, the frequency of IgG₂ transcripts was significantly increased (Fig E2), fitting with our flow cytometry data² and repeated immune responses.¹ This contrasted a previous study, in which increased SHMs were found in the FR3 and CDR3 regions of IgG₄ transcripts. It should be noted that FR3 and CDR3 form less than one-third of the variable domain and that mutations in the CDR3 are notoriously difficult to define, because this includes the junctional regions between *IGHV*, *IGHD*, and *IGHJ* genes with random nucleotide insertions and deletions from the V(D)J recombination process. Our analysis involved the strongly recommended approach for SHM analysis, which included the whole *IGHV* gene (>280 nt) including FR1, FR2, and FR3, and the CDR1 and CDR2.⁷ Thus, the patients in our original IgG₄-RD cohort do show signs of chronic B-cell responses in the affected tissue, whereas their circulating B cells have increased anergic properties.

Similar to Akiyama et al,⁸ we did not find a significant increase in absolute numbers of circulating Tfh cells in our original study,² and this remained nonsignificant on inclusion of our 3 new patients (Fig 1, C). The importance of Tfh subsetting is a relative new finding and was not yet exploited at the start of our original study. Given recent reports that underline the important role of Tfh subsets in IgG₄-RD,⁸ we here analyzed Tfh1 and Tfh2 numbers in more detail and found the latter to be significantly increased as compared with controls (Fig 1, C). However, circulating Tfh2 cell numbers were also significantly increased in patients with sarcoidosis, and are therefore less specific for IgG₄-RD as circulating IgG₄⁺ B cells, Treg cells, and T_H2 cells.

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METHODS

Histopathology

All patients with IgG₄-RD were histologically diagnosed. The amount of IgG₄⁺ plasma cells per hpf (0.28 mm²) and the IgG₄/IgG ratio were measured (Table E1), and all patients met the Boston criteria for IgG₄-RD. The hematoxylin and eosin stainings were analyzed at the Department of Pathology of the Erasmus MC Rotterdam by a trained pathologist with experience in diagnosing IgG₄-RD. The deparaffinized formalin-fixed paraffin-embedded sections of the tissue (4 mm thick) were stained using a BenchMark automated immunostainer (Ventana, Tucson, Ariz) with the Ultraview Universal diaminobenzidine detection kit (Ventana). Mouse antihuman IgG (clone A57H, 1:200; Dako, Carpinteria, Calif) and mouse antihuman IgG₄ (clone HP6025, 1:600; Invitrogen Zymed, Camarillo, Calif) were used for immunohistochemical staining and were applied to the sections for 32 minutes. Additional stainings were performed with CD3 (2GV6, ready to use), CD79A (Sp18, ready to use), and Bcl-2 (Sp66, ready to use; all from Ventana) and Bcl-6 (G11GE/A8, 1:10) and PD-1 (Nat105, 1:50; both from Cell Marque, Rocklin, Calif).

Molecular analysis of IgG transcripts

RNA was isolated from post-Ficoll mononuclear cells of IgG₄-RD patients 5, 7, 8, 10, 11, and 12 with a GenElute mammalian RNA kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed to cDNA with random primers (Thermo Fischer, Waltham, Mass). Rearranged IgG transcripts were amplified in a multiplex PCR approach using 4 different *IGHV*-family leader forward primers in combination with an *IGHG*-consensus (5' CACGCTGCTGAGGGAGTAG) reverse primer.^{E1} PCR products were cloned into a pGEMT easy vector (Promega, Madison, Wis), amplified by colony PCR, and sequenced by Micromon facility of Monash University on an Applied Biosystems 3730s DNA Analyzer (Thermo Fisher). Obtained sequences were analyzed using the IMGT database (http://www.imgt.org/IMGT_vquest/vquest) to assign the *IGHV*, *IGHD*, and *IGHJ* genes and alleles, and to identify SHMs. Of each unique clone, the position and frequency of mutations were determined within the entire *IGHV* gene (FR1-CDR1-FR2-CDR2-FR3). SHMs were determined as variations on the best-matched V-gene and represented as the percentage of mutations of the total sequenced V-gene nucleotides. The IgG receptor subclasses were determined using the *IGH* reference sequence (NG_001019). All results of patients with

IgG₄-RD were compared with previously generated data sets of controls and of patients with sarcoidosis.^{E1,E2}

Flow cytometry

Stored PBMCs from controls, patients with IgG₄-RD (2, 3, 6, 9, 15-19), and patients with sarcoidosis were thawed and prepared for detailed flow cytometric analysis of Th and Tfh subsets. Two million PBMCs were incubated for 15 minutes at room temperature in a total volume of 100 µL with the following antibodies: CD4-BV510 (RPA-T4), CD25-BV421 (BC96), CD45RA-BV605 (HI100), CD127-APC (A019D5), CCR4-PE-Cy7 (L291H4), CCR6-PerCP-Cy5.5 (G034E3; all from Biolegend, San Diego, Calif), CD3-BV711 (UCHT1), CD8-APC-H7 (SK1), CCR7-PE-CF594 (150503), CXCR3-PE (1C6/CXCR3), and CXCR5-BB515 (RF8B2; all from BD Biosciences, San Jose, Calif). After preparation, cells were measured on a 4-laser LSRFortessa flow cytometer (BD Biosciences) using standardized settings. Data were analyzed with FACSDiva software V8.0 (BD Biosciences). Tfh cells were defined as CD3⁺CD4⁺CD45RA⁻CXCR5⁺, and within this subset the Tfh1 cells were defined as being CCR6⁻CXCR3⁺CCR4⁻ and Tfh2 cells as CCR6⁻CXCR3⁻CCR4⁺.

Statistical analysis

SHM frequencies and both frequencies and absolute cell numbers were assumed a non-Gaussian distribution. Results were analyzed using the nonparametric Mann-Whitney *U* test. IgG subclass distributions were analyzed with the chi-square test. All *P* values are 2-tailed and were considered statistically significant if values were lower than .05. Statistical analysis was performed using GraphPad Prism software, version 7 (GraphPad Software, La Jolla, Calif).

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- E2. Kamphuis LS, van Zelm MC, Lam KH, Rimmelzwaan GF, Baarsma GS, Dik WA. Perigranuloma localization and abnormal maturation of B cells: emerging key players in sarcoidosis? *Am J Respir Crit Care Med* 2013;188:406-16.

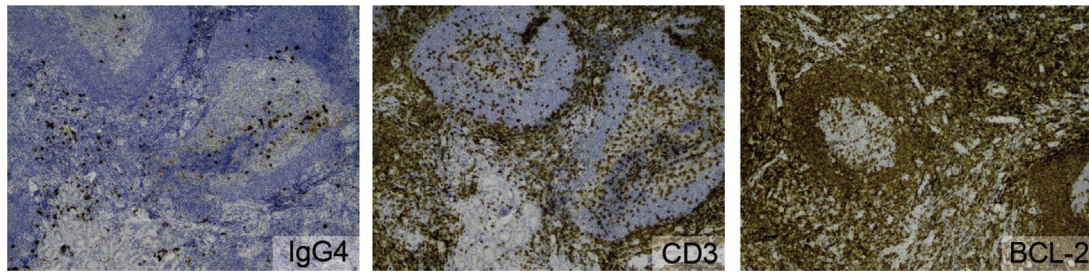


FIG E1. Additional immunohistochemical analysis of orbital tissue from IgG₄-RD patient 7 with anti-IgG₄, CD3, and Bcl-2.

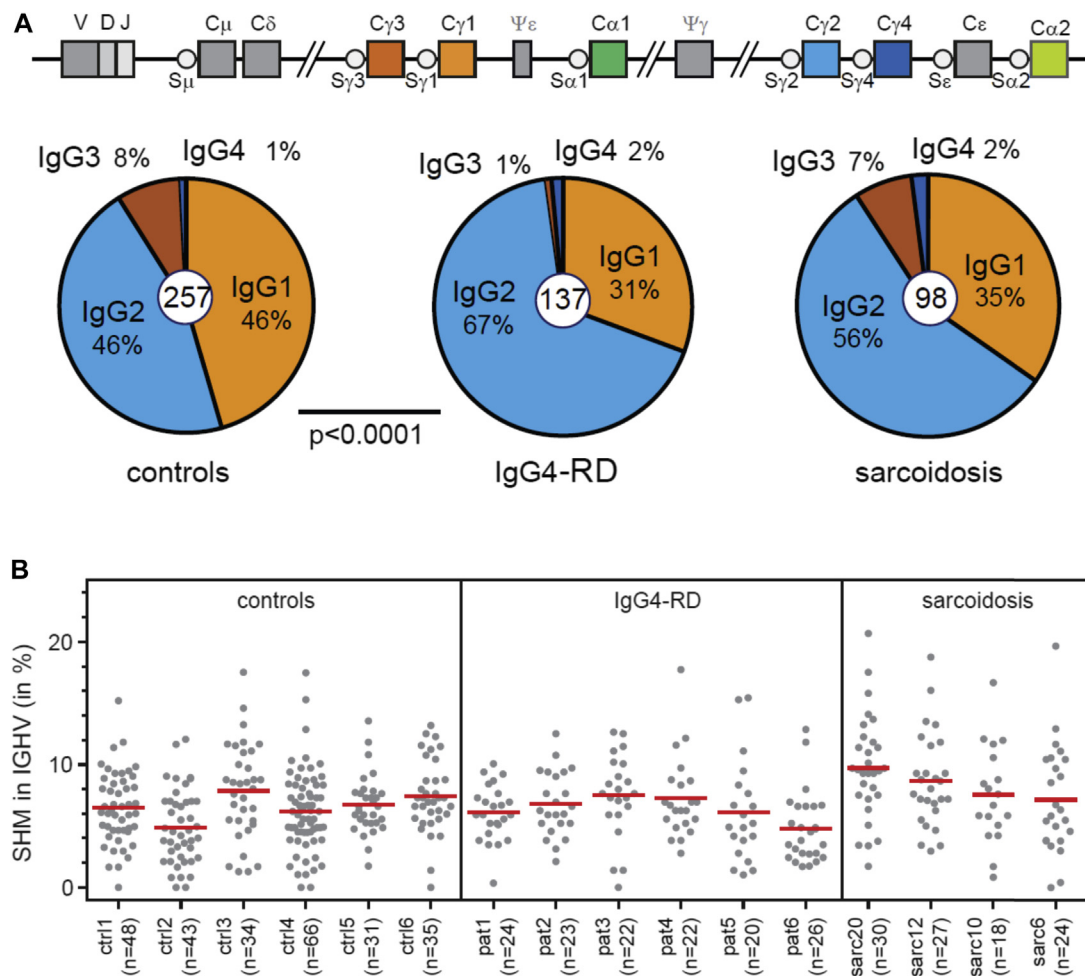


FIG E2. SHM analysis in IgG transcripts. **A**, IgG transcripts were amplified with an IgG consensus primer from 6 patients (pat) with IgG₄-RD and the relative composition of IgG subclasses was compared with previously generated data from controls (ctrl) and from patients with sarcoidosis (sarc).^{E1,E2} Statistics, χ^2 test. **B**, SHM levels in unique transcripts from 6 controls,^{E1} 6 patients with IgG₄-RD, and 4 patients with sarcoidosis.^{E2}

TABLE E1. Histological findings in patients with IgG₄-RD

Patient	Sex	Age (y)	Organs affected	IgG ₄ + PC/hpf (ratio IgG ₄ /IgG)	B-cell follicles	Medication at inclusion	Time until diagnosis	Serum IgG ₄ (g/L)
1	F	60	Orbita	149 (>0.4)	+	NIM	14 y	2.12
2	F	67	Orbita	>50 (>0.4)	+	NIM	8 y	2.73
3	M	63	Orbita, lymph node, lung, prostate	>100 (>0.4)	+	Prednisone	3 y	5.96
4	M	54	Orbita	156 (0.90)	+	Prednisone	10 y	1.22
5	F	53	Orbita	>64 (0.4)	+	NIM	3 y	0.27
6	M	60	Orbita, lymph node	411 (0.67)	+	TN	5 y	7.50
7	M	44	Orbita	110 (0.4)	+	TN	12 y	1.85
8	F	59	Skin	>135 (0.95)	—	TN	5 mo	1.93
9	M	18	Lung, lymph node, cerebra	150 (>0.4)	+	TN	1 mo	12.8
10	F	62	Pancreas, lymph node, lung	131 (0.72)	+	TN	3 mo	0.27
11	M	74	Lymph node, salivary gland, lung	138 (0.7)	+	TN	5 mo	5.75
12	M	53	Mesenterium	458 (0.5)	+	TN	20 y	25.25
13	M	62	Thyroid gland	330 (0.8)	+	TN	1 y	2.76
14	M	32	Serous membrane	136 (0.70)	+	TN	3 mo	5.40
15	M	79	Lymph node, pancreas	1223 (0.9)	+	TN	1 y	1.13
16	M	60	Bile duct, lymph node	205 (0.63)	ND	TN	2 wk	5.55
17	M	48	Mesenterium	120 (0.56)	+	TN	5 mo	3.01
18	M	60	Orbit, ENT	207 (0.85)	+	TN	6 y	15.02
19	M	74	Pancreas, orbit, lymph node	210 (0.8)	+	TN	3 y	8.82

IgG₄ serum levels above normal range are shown in boldface. Histology analysis was performed on the affected organ listed first. Patients 17 to 19 were newly included and not reported before.

ENT, Ear-nose-throat; F, female; M, male; ND, not determined; NIM, no immunomodulatory medication for at least 6 mo before inclusion; PC, plasma cells; TN, treatment naive.