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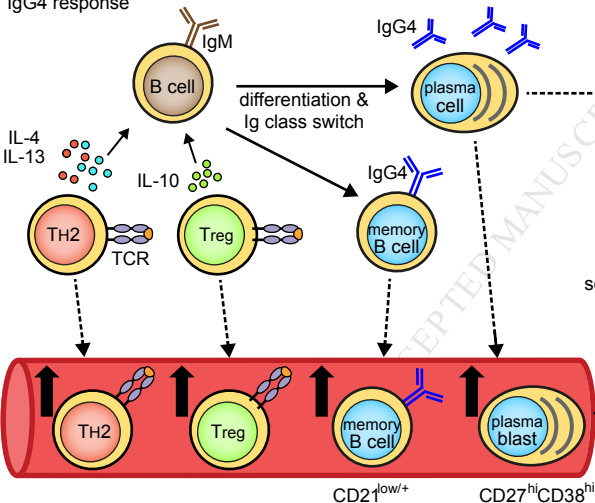
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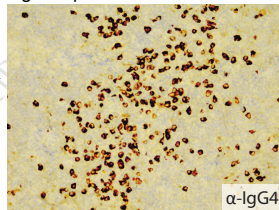
Cellular markers for dysregulated IgG4 responses in patients with IgG4-related disease



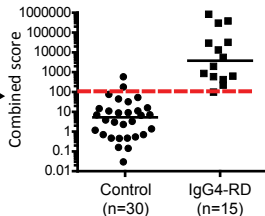
IgG4 response



IgG4+ plasma cells in tissue



$$\text{score} = \frac{\% \text{IgG2} \times (\% \text{IgG4})^2 \times \text{Th2} \times \text{Treg} \times \text{IgG4}}{\% \text{IgG1} \times \text{Th1} \times \text{IgG1}}$$



Increased cell numbers in blood of IgG4-RD patients

Expansion of blood IgG4+ Bcells, Th2 and Tregulatory cells in IgG4-related disease

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Abstract

Background IgG4-related disease (IgG4-RD) is a systemic fibro-inflammatory condition affecting various organs and has a diverse clinical presentation. Fibrosis and accumulation of IgG4⁺ plasma cells in tissue are hallmarks of the disease and IgG4-RD is associated with elevated IgG4 serum levels. However, disease pathogenesis is still unclear and these cellular and molecular parameters are neither sensitive nor specific for diagnosis of IgG4-RD.

Objective We here sought to develop a flowcytometric gating strategy to reliably identify blood IgG4⁺ B-cells to study their cellular and molecular characteristics and investigate their contribution in disease pathogenesis.

Methods Sixteen patients with histologically confirmed IgG4-RD, 11 patients with sarcoidosis and 30 healthy individuals were included for 11-color flowcytometric analysis of peripheral blood for IgG4-expressing B cells and T-helper (Th) subsets. In addition, detailed analysis of activation markers and chemokine receptors was performed on IgG4-expressing B cells and IgG4 transcripts were analyzed for somatic hypermutations.

Results Cellular and molecular analyses revealed increased numbers of blood IgG4⁺ memory B-cells in patients with IgG4-RD. These cells showed reduced expression of CD27 and CXCR5 and increased signs of antibody maturation. Furthermore, IgG4-RD patients, but not patients with sarcoidosis, had increased numbers of circulating plasma blasts and CD21^{low} B-cells, as well as Th2 and regulatory T-cells, indicating of a common disease pathogenesis in IgG4-RD.

Conclusion These results provide new insights into the dysregulated IgG4 response in patients with IgG4-RD. A specific “peripheral lymphocyte signature” observed in patients with IgG4-RD, could support diagnosis and treatment monitoring.

Key messages:

- IgG4⁺ memory B-cells can be reliably detected in human peripheral blood
- Patients with IgG4-RD have increased numbers of circulating IgG4⁺ memory B-cells. Molecular characteristics reveal that this is a result of an ongoing immune response in affected tissue
- The specific “peripheral lymphocyte signature” representing the combined changes in B- and T-cell subset numbers in patients with IgG4-RD might be exploited as a non-invasive marker for diagnosis and treatment monitoring.

Capsule summary

We here provide new insights into IgG4⁺ B-cells, and how their detection contributes to diagnosis and monitoring of treatment success in patients with IgG4-related disease.

Key words

IgG4; B cell; IgG4 related disease; T helper cell; regulatory T cell; plasma cell; flowcytometry; sarcoidosis; principal component analysis

Abbreviations

CSR:	class switch recombination
IgG4-RD:	IgG4 related disease
PCA:	principal component analysis
SHM:	somatic hypermutation
SLE:	systemic lupus erythematosus
TACI:	transmembrane activator and CAML interactor

Introduction

IgG4 related disease (IgG4-RD) is defined as a systemic fibro-inflammatory condition that can affect potentially any organ, but predominating the retroperitoneal space, thyroid, pancreas, salivary glands and orbital tissue (1, 2). The pathology is characterized by fibrosis and infiltration of IgG4-producing plasma cells of the affected organs (3, 4). As a result, a tumor-like swelling of the involved organ can occur, which causes organ dysfunction and, if left untreated, can lead to organ failure. Furthermore, AA amyloidosis can develop in IgG4-RD, emphasizing the need for prompt diagnosis and treatment of the disease (5). Previously, diseases with IgG4+ plasma cell infiltrates were defined predominantly by organ involvement, and only since 2012 these diverse manifestations have been recognized as one disease entity (3, 4).

The pathogenesis of IgG4-RD remains poorly understood. It is thought to be triggered by organ damage, resulting from e.g. a bacterial infection with molecular mimicry or from an underlying autoimmune process (1). In patients with pancreatic involvement, auto-antibodies directed against self-antigens have been observed (6, 7). Importantly, IgG4+ plasma cell infiltrates are not monoclonal (8), and serum IgG4 in patients is polyclonal with reactivity to multiple antigens (9). The function of IgG4 antibodies remains elusive, because these display weak or negligible binding to both C1q and Fcγ receptors (10, 11). Furthermore, IgG4 molecules have the exclusive ability to exchange Fab-arms, thus creating monovalent bispecific antibodies that can prevent the formation of immune complexes (12). Therefore, IgG4 has a presumed immune dampening effect, however its role in the pathogenesis of IgG4-RD is controversial (13).

Immunoglobulin class switching of B-cells to IgG4 is regulated by T helper 2 (Th2) cytokines and by IL-10, produced by regulatory T-cells (14, 15). Substantial evidence indicates that indeed Th2 cells and regulatory T-cells (Tregs) are involved in the

pathophysiology of IgG4-RD (16-18). Affected tissues express higher messenger RNA (mRNA) levels of the Th2 cytokines IL-4, IL-5 and IL-13 (16, 17). Furthermore, isolated circulating CD4⁺ T-cells from patients predominantly produce Th2 cytokines (18, 19). Similarly, IL-10 and tissue growth factor β (TGF- β) transcripts are increased in affected tissues (16), and patients with IgG4-related autoimmune pancreatitis have increased frequencies of circulating Tregs (20, 21). More recently, in affected tissue and blood of IgG4-RD patients clonal expansions of CD4 effector memory T(EM) cells have been identified, which potentially drive fibrosis and IgG4 production (22, 23).

Clinically, IgG4-RD manifestations can mimic those of many infectious, inflammatory and malignant disorders (24-30). Therefore, diagnosis of IgG4-RD can be challenging, often leading to a delay in start of proper treatment. The gold standard for diagnosis is histology (28, 31). Characteristic lymphoplasmacytic infiltrates, rich in IgG4 positive plasma cells, storiform fibrosis (cartwheel arrangement of fibroblasts) and obliterative phlebitis are seen in histological samples (28). An increase in serum IgG4 supports the diagnosis, but is found in only 50-70% of patients with histological proven IgG4-RD (32, 33). Recently, circulating plasma blasts have been postulated as a more reliable marker for the disease, irrelevant of IgG4 serum levels (34). However, the specificity of this marker is limited, because circulating plasma blasts are also increased in active infection, following vaccination and in other chronic diseases, such as systemic lupus erythematosus (SLE) (35-38).

Still the importance of B-cells in the pathophysiology of the disease is illustrated by recent observations demonstrating that B-cell depletion with rituximab is a promising therapy in IgG4-RD (39-42). As rituximab specifically binds CD20, it does not directly target IgG4⁺ plasma cells, which do not express CD20. Thus, the therapeutic effect could lie in depletion of memory B cells that are chronically stimulated to generate IgG4⁺ plasma cells. Furthermore, other chronically activated B cells might be involved in the disease through

antigen presentation and cytokine production, such as CD21^{low} B cells that are found to be expanded in diverse chronic inflammatory diseases (43).

Despite previously reported expansions of IgG4+ plasma cells, CD4 Tem, Th2 cells and Treg cells in affected tissue, little is known about the pathogenesis of IgG4-RD. Therefore, we developed a new flow cytometric approach, which enabled us to study IgG4-expressing B cells and their pathogenic contribution to the disease. Our insights into IgG4-expressing B cells in combination with abnormalities in B and T-cell subsets reveal abnormal systemic immune regulation, which are valuable for the improvement of diagnosis and treatment of IgG4-RD.

Methods

Patients.

Patients with IgG4-RD and patients with sarcoidosis were recruited following signed informed consent from the Immunology outpatient clinic at the Erasmus Medical Center Rotterdam and from the Rotterdam Eye Hospital, the Netherlands. All patients were >18 years and were diagnosed based on clinical, serological and histopathological findings. All IgG4-RD patients met the IgG4-RD diagnostic guidelines, including histological confirmation (28), and did not have a known history of an immunodeficiency or any autoimmune inflammatory disease other than IgG4-RD. All but two patients with sarcoidosis had tissue biopsy confirmed disease with typical presence of non-caseating granulomatous inflammation. In two patients the diagnosis was based on clinical presentation and supportive serological parameters (angiotensin converting enzyme and soluble interleukin-2 receptors) in combination with radiological imaging. Healthy controls were recruited from healthy individuals selected from department staff, and the control group was age- and gender-matched to the patient cohort. None of the healthy individuals showed signs of active inflammatory disease. This study was performed according to the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus MC (ethics approval numbers MEC-2014-476, MEC-2015-200 and MEC-2017-084).

Histopathology.

All patients with IgG4-RD were histologically diagnosed. The haematoxylin and eosin stainings were analyzed at the Department of Pathology of the Erasmus Medical Center 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, AZ, USA) with the

Ultraview Universal diaminobenzidine detection kit (Ventana). Mouse anti-human IgG (clone A57H, 1:200, Dako, Carpinteria, CA, USA) and mouse anti-human IgG4 (clone HP6025, 1:600, Invitrogen Zymed, Camarillo, CA, USA) were used for immunohistochemical staining and were applied to the sections for 32 min. The amount of IgG4⁺ plasma cells per high-power field (0.28 mm²) and the IgG4/IgG ratio were measured.

IgG serology.

IgG subclass serum levels were measured by immunonephelometry using a Siemens BN II nephelometer according to manufacturer guidelines. A possible prozone effect for IgG4 levels was excluded through dilution of serum samples until reliable values were obtained (44).

Flow cytometry of blood samples.

Patients and controls were included over a time period of three years. To ensure consistency in flowcytometry, standardized sample preparation, antibody staining and flowcytometer instrument settings were used (45). In short, absolute counts of CD3⁺ T-cells, CD19⁺ B-cells and CD16⁺/CD56⁺ NK-cells were obtained with a diagnostic lyse-no-wash protocol using commercial Trucount tubes (BD Biosciences, San Jose, Calif). For detailed 11-color flow cytometry, red blood cells were lysed with NH₄Cl prior to incubation of 1 million nucleated cells for 15 minutes at room temperature in a total volume of 100µL (antibodies listed in Repository Table E1). 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). Immunophenotypic definitions of lymphocyte and leukocyte subsets are listed in Repository Table E2.

Molecular analysis of immunoglobulin heavy chain (IGH) gene rearrangements.

RNA was isolated from post-Ficoll mononuclear cells with a GenElute mammalian RNA kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed to cDNA with random primers (Invitrogen Life technologies). *IGHV* gene rearrangements were amplified in a semi-nested multiplex PCR approach using 4 different L-VH-family forward primers (46) in combination with *IGHG4*-specific (5'GGGCATGATGGGCAYGGGGGACCATA; first round) and *IGHG*-consensus (5'CACGCTGCTGAGGGAGTAG; second round) reverse primers. PCR products were cloned into a pGEMT easy vector (Promega, Madison WI), amplified by colony PCR, and sequenced on an ABI Prism 3130XL (Applied Biosystems, Foster city, CA). The sequences were analyzed with the IMGT database (www.imgt.org) and BASELINE program (selection.med.yale.edu/baseline) (47, 48).

Statistical analysis

Frequencies and absolute cell numbers were assumed a non-Gaussian distribution. All results are expressed as median values with interquartile range if applicable. Results were analyzed using the non-parametric Mann-Whitney *U* test. Linear regression was used to study the strength of association between cell-subsets with Spearman *r* to measure significance. All *P*-values are two-tailed and were considered statistically significant if values were lower than 0.05. Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA). Principal component analysis (PCA) was performed with Infinicyt software (Cytognos, Salamanca, Spain). Specificity and sensitivity were calculated with SPSS software (IBM SPSS statistics 21.0, Armonk, NY)

Results

Patient characteristics.

A total of 16 patients with IgG4-RD were included with a mean age of 56 years (range 18-79 yrs) and a male:female ratio of 2:1 (Table I). All patients were confirmed to have IgG4-RD based on the Boston consensus, with typical histopathologic characteristics and the presence of IgG4-producing plasma cells in affected tissue (Fig 1). Twelve out of 16 patients had increased serum IgG4 levels (mean 5.06g/L; range 0.27-25.25g/L). Four of these patients had an additional increase in serum IgG1 or IgG2 levels, and one patient with normal serum IgG4 had increased levels of IgG1 and IgG3 (see Repository Table E3). Six patients showed signs of active disease based on clinics and increased CRP (C-reactive protein) and/or ESR (erythrocyte sedimentation rate) (Table I).

The majority of IgG4-RD patients were treatment naive (no prior treatment), three patients had received immunomodulatory medication in the past (medication had been stopped for at least 6 months prior to inclusion) and only two patients received low dose prednisone (Table I). Most patients had normal counts of blood leukocytes and lymphocytes (see Repository Table E3). Increased lymphocytes resulted from high T-cell counts, often in combination with high B- and/or NK cell numbers. One of the two patients treated with prednisone had decreased numbers of B- and T-cells. In the analysis of the distribution of IgG subclasses within the total IgG memory B-cell compartment we analyzed material from 15 of the 16 patients.

Of the eleven patients with sarcoidosis, one patient used plaquenil. All other patients were therapy naive (see Repository Table E4). All patients had normal leukocyte counts, although some patients had slightly decreased or slightly increased T-cell or B-cell counts. One patient had an increased IgG4 serum level accompanied with an increased IgG1 serum level. One patient had a slightly decreased IgG4 serum level and from one patient IgG serum

levels were not determined. Five patients showed signs of active disease based on CRP and/or ESR (see Repository Table E4).

Immunophenotypical analysis of IgG4-expressing B-cells.

The hallmark of IgG4-RD is the accumulation of IgG4-producing plasma cells in affected tissue. To study whether patients had systemic abnormalities in IgG4-expressing B cells, we developed a reliable flow cytometric gating strategy to distinguish cells expressing one of the four IgG subclasses (Fig 2, A). Within total CD19⁺ B cells, CD27⁺CD38^{hi} plasma blasts were electronically gated and studied for expression of IgG1, 2, 3 and 4. However, surface Ig levels were too low to detect. Therefore, we next focused our attention on CD38^{dim}IgM-IgD⁻ memory B cells. These contained presumed memory B cell subsets that expressed either IgG1, IgG2 or IgG3, and within the triple negative fraction, a sizeable IgG4-expressing subset could be identified (Fig 2, A).

To confirm the activated nature and memory phenotype of all IgG⁺ B-cell subsets we studied the expression of B7 family members CD80 and CD86 and the TNF receptor superfamily member transmembrane activator and CAML interactor (TACI). All four IgG-subclass expressing B-cell subsets showed higher expression of the activation markers than naive B-cells (Fig 2, B), fitting with an activated memory B-cell phenotype.

Increased CD21^{low} B-cells and plasma blasts, but reduced IgM+IgD⁺ memory B cells in patients with IgG4-RD.

To study systemic abnormalities in IgG4-expressing B cells, we performed extensive immunophenotyping of B-cells in 16 patients with IgG4-RD and compared these with 30 age-matched healthy controls (Fig 3, A). The median numbers of transitional and naive mature B cells were not different between patients and controls. Of the six major memory B

cell subsets, only the IgM+IgD⁺ memory B cells were significantly affected in patients (reduced; $P<0.05$) (Fig 3, B). In contrast, the numbers of CD21^{low} B-cells (CD19+CD38^{dim}CD21^{low}), and plasma blasts (CD19+CD38+CD27⁺) were significantly higher in patients with IgG4-RD (both $P<0.05$).

Patients with IgG4-RD have increased numbers of IgG4⁺ memory B-cells.

Despite the normal total numbers of IgG⁺ memory B-cells, patients with IgG4-RD had significantly higher numbers of IgG4⁺ memory B-cells than controls ($P<0.01$) (Fig 3, C). In addition, patients had reduced numbers of memory B cells expressing IgG1 ($P<0.05$), whereas IgG2 and IgG3 expressing B-cell numbers were similar to controls (see Repository Fig E1, A). When analyzed as fraction of total IgG-expressing memory B cells, the decrease in IgG1 and increase in IgG4 in patients with IgG4-RD became more apparent (both $P<0.0001$). Furthermore, IgG4-RD patients showed a significant increase in the fraction of IgG memory B cells expressing IgG2 ($P<0.01$). To study if there was a direct relation with serum IgG4 levels, we performed linear regression analyses with the percentages and the absolute numbers of IgG4⁺ memory B-cells (see Repository Fig E1, B). These did not reveal significant correlations, indicating that the memory B cells and serum IgG4 levels were not directly related.

Since both IgG4-expressing B cells and CD21^{low} B cells were significantly increased in IgG4-RD patients, we studied if these were related and investigated CD21^{low} B cells expressing IgG4 (see Repository Fig E1, C). Indeed, patients carried significantly more CD21^{low}IgG4⁺ B cells (see Repository Fig E1, D) and these numbers were directly correlated to the total number of CD21^{low} B cells ($P<0.01$) (see Repository Fig E1, E).

Cellular and molecular analysis of IgG4-expressing B-cells in patients with IgG4-RD.

To analyze the nature of the IgG4-expressing B-cell expansion in patients with IgG4-RD, we first studied the expression of CD27 (Fig 4, A). In healthy controls, the frequencies of cells that expressed CD27 were highest within the IgG2 and IgG4 subsets, followed by IgG1 and IgG3 (Fig 4, B). Thus, CD27 positivity was higher in cells utilizing IgG subclasses encoded by the downstream genes within the *IGH* locus (*Cγ2* and *Cγ4*) (Fig 4, C). In patients with IgG4-RD, significantly fewer IgG4⁺ B-cells expressed CD27 than IgG4⁺ B-cells from healthy individuals.

To study whether the expanded IgG4-expressing B cells could be normally involved in immune responses, we analyzed the expression levels of cytokine receptors and chemokine receptors involved in germinal center responses. IL21 receptor (IL21R) and IL4R as well as C-C chemokine receptor type 7 (CCR7) expression levels were similar between IgG4⁺ memory B-cells from healthy individuals and from patients with IgG4-RD (Fig 4, D). In contrast, C-X-C receptor type 5 (CXCR5) expression was significantly lower on IgG4⁺ memory B-cells from patients.

To investigate the nature of the expanded IgG4⁺ memory B-cells in the pathogenesis of IgG4-RD, we studied somatic hypermutations (SHM) in the *IGHV* regions (Fig 4, E). Such mutations are molecular signs of B-cell responses and affinity maturation (49). In agreement with previous studies, SHM frequencies in IgG4 transcripts of healthy adults were higher than in the more proximal-encoded IgG1 and IgG2 (see Repository Fig E2, A) (50, 51). The SHM levels in IgG4 transcripts of patients with IgG4-RD (7.89%) were similarly high as in healthy adults (7.98%; $P=0.75$) (Fig 4, F). To study if the mutations were driven by selection for antigen binding, we analyzed the selection for replacement mutations in the complementarity determining regions (CDR) with the Bayesian estimation of Antigen-driven SElectIoN program BASELINE (48). IgG4 transcripts from healthy controls did not show more replacement mutations than expected by random chance in the CDR regions, i.e.

absence of positive selection (Fig 4, G). In contrast, CDR regions of IgG4-RD patients did show positive selection for replacement mutations. Both groups showed normal negative selection for replacement mutations in framework (FR) regions that compose the structure of the variable domain. Thus, IgG4+ B-cells in patients with IgG4-RD show increased selection for replacement mutations in CDR regions. Patients and controls displayed high diversity in sequence and length of the CDR3 region, which is encoded by the junction of the V, D and J genes. Still, patients showed significantly shorter IGH-CDR3 regions in IgG4 transcripts than healthy individuals (see Fig Repository E2, B). Furthermore, *IGHV* usage seemed to differ between groups with increased usage of IGHV5-51 in patients with IgG4-RD and reduced IGHV3-39 usage (see Repository Fig E2, C), although this did not reach statistical significance ($P=0.08$).

Increased numbers of Th2 cells and Tregs in IgG4-RD.

The impaired expression levels of CXCR5 and absence of positive selection of SHM in IgG4+ B cells are suggestive of abnormal immune responses in patients with IgG4-RD. IgG4 responses are mediated by IL-4 and IL-13, produced by Th2 cells, and IL-10, produced by Tregs. Therefore, we performed additional studies into Th subsets in our patients (Fig 5, A). Flowcytometric immunophenotyping of their blood T cells demonstrated normal overall numbers of naive, central memory and effector memory CD4 T cells (see Repository Fig E3, A). Still, patients with IgG4-RD had significantly more circulating Th2 cells (CD45RA-CCR6-CXCR3-CCR4+; $P<0.0001$) and Tregs (CD45RA-CD127-CD25+; $P<0.001$) than healthy individuals (Fig 5, B). Th1 cells seemed slightly lower, but this was not significant and numbers of follicular helper T cells (Tfh) and Th17 were not different. Linear regression showed a direct correlation between the numbers of IgG4+ memory B-cells and Th2 cells ($P<0.01$), but not for Tregs ($P=0.24$; Fig 5, C). CD21^{low} IgG4+ B cell numbers were similarly

correlated with Th2 cells ($P<0.05$) and not with Treg numbers ($P=0.49$; see Repository Fig E3, A). IgG4 serum levels in patients did not correlate with Th2 nor Treg cell numbers (see Repository Fig E3, B). In contrast to the changes in Th subsets, patients with IgG4-RD had no differences in numbers of CD4⁺CD45RA⁺CCR7⁻ TemRA cells, CD4⁺CD45RA⁻CCR7⁻ TemRO cells, nor CD27⁻ TemRO cells (CD4 CTL) (see Repository Fig E4, A and B). Finally, naive CD8 T-cell numbers were decreased in IgG4-RD patients, in absence of effector memory expansions (see Repository Fig E4, C).

Skewing of Th subsets towards Th2 is also observed in allergies and frequently accompanied by eosinophilia (52). However, we did not find increased numbers of eosinophils (SSC^{high}CD45⁺CD16^{dim}CD81⁺) in patients with IgG4-RD (see Repository Fig E5), nor any differences in the other granulocyte subsets (neutrophils and basophils). In contrast, the numbers of plasmacytoid dendritic cells (SSC^{low}CD45^{dim}HLADR⁺CD123⁺), which are potent drivers of Th1 responses (53), were significantly lower in IgG4-RD patients. Thus, the increased numbers of IgG4-expressing B cells in IgG4-RD patients are accompanied by systemic reduction in Th1 immunity and increased Th2 and Treg cells.

IgG4⁺ memory B-cells and other lymphocyte subsets as a diagnostic tool for IgG4-RD.

Despite clinical indications and high serum IgG4 levels, diagnosis of IgG4-RD requires analysis of a tissue biopsy. Obtaining a biopsy can be difficult, is invasive, sample error sensitive and might not always be sufficiently discriminative from other immune-proliferative and auto-inflammatory diseases. A specific marker in blood could be a valuable means to overcome these diagnostic challenges. However, the differences in B-cell and T-cell subsets we identified do not provide a clear discrimination between patients and controls. Therefore, we explored if a combination of measurements could be used as a disease marker. First, we performed a principal component analysis (PCA) on the absolute cell numbers of all B and T-

cell subsets from healthy individuals and patients with IgG4-RD and visualized this with the automated population separation (APS) tool of the Infinicyt program (54). In this analysis we also included a group of patients with treatment naive sarcoidosis, another fibro-inflammatory disease. IgG4-RD resembles many immune proliferative diseases, yet patients with sarcoidosis did not show typical expansions of IgG4⁺ memory B cells, Tregs and Th2 cells (see Repository Fig E6). Of the first 5 principle components, component 1 (PC1) separated patients with IgG4-RD from patients with sarcoidosis and from healthy controls. (Fig 6, A). This analysis indicates the usefulness to combine multiple parameters for optimal discrimination between IgG4-RD patients and controls and potentially from other auto-inflammatory conditions with tissue fibrosis. Therefore, we continued to develop a score based on the B- and T-cell subsets that were most significantly different between IgG4-RD patients and both controls and patients with sarcoidosis (Fig 6, B). Application of this scoring method to the 56 samples in our study resulted in 50 samples being correctly assigned as either non-IgG4-RD or IgG4-RD (Fig 6, B). The calculated sensitivity for this score was 93.3% (95% confidence interval, 68.05% - 99.83%) and the calculated specificity was 87.8% (95% CI, 73.80% - 95.92%). Thus, IgG4-RD patients carry changes in their blood B- and T-cell subsets that together reflect a unique “lymphocyte signature”.

Discussion

We here demonstrate significant expansions of IgG4-expressing B cells in blood of patients with IgG4-RD. These changes were directly related to the previously described expansion of CD21^{low} B cells and are associated with expansions in plasma blasts, Th2 and Treg cells.

Increased numbers of circulating plasma blasts have been reported before in patients with IgG4-RD (8, 34). This is not a disease-specific effect as similar expansions have been observed in many chronic inflammatory diseases and in individuals with active infection or following vaccination (35-38). Thus, increased plasma blast numbers mostly reflect active humoral immune responses. Active inflammation is further supported by the increased numbers of CD21^{low} B cells in our patients. These cells have been reported to be increased in multiple states of inflammation (43, 55, 56) and are thought to result from prolonged B-cell activity due to chronic activation by self-antigens (57). CD21 functions as a co-receptor for the BCR, and downregulation of its expression makes the cells anergic for chronic stimuli (58). We now demonstrated that the expansion of CD21^{low} B cells is in part due to the expansion of IgG4⁺ population, which directly links it to the disease. The nature of chronic B-cell stimulation in IgG4-RD remains unclear. It is likely that auto-antigens are involved, as plasma blasts from patients with IgG4-RD produce immunoglobulins that react against human cell lysates (8).

Insights into IgG4-expressing B cells in patients with IgG4-RD are limited, most likely due to the lack of proper reagents to specifically detect and isolate these cells. To overcome this, we developed a flow cytometric approach to stain for IgG4-expressing B cells using newly available reagents and found that frequencies and absolute numbers of IgG4⁺ memory B-cells were increased in patients with IgG4-RD. However, in contrast to previous observations, these numbers were not correlated to IgG4 serum levels (59). Absence of correlation is most likely due to the fact that these cells were immunophenotypically memory

B-cells (60, 61) rather than IgG4-producing plasma cells. Previously we observed similar absence of correlation for IgE+ memory B-cell numbers and serum IgE levels in patients with atopic dermatitis (62).

On top of cell numbers, patients' IgG4+ memory B cells were phenotypically different from those of healthy controls. A lower frequency of cells expressed CD27, which is the conventional marker for memory B-cells (63, 64). Furthermore, expression levels of CXCR5 were lower in patients. These differences could originate from a different maturation pathway for IgG4+ memory B cells in patients. Generation of IgG+ memory B cells in humans is critically dependent on T-cell help (65). Yet, it is unclear if T-cell help is needed for sequential switching to IgG4 of memory B cells expressing other IgG subclasses. The reduced expression of CXCR5 on IgG4+ B-cells from patients could represent a reduced capacity for homing to B-cell zones in lymph nodes and would suggest that activation and differentiation of IgG4+ B-cells in patients occurs in peripheral tissue, rather than in lymphoid structures (66). Cytokines specifically inducing CSR to IgG4 have been found in IgG4-RD tissue biopsies (16). Moreover, expression of activation-induced cytidine deaminase (AID), the enzyme that triggers SHM and which is needed for CSR, was reported to be increased in tissue biopsies of IgG4-RD patients, confirming the ability of local CSR (15).

In addition to differences in IgG4+ memory B cells, we found systemic expansions of Th2 and Treg cells. Ig class switching to IgG4 is mediated by the cytokines IL4 and IL-10, which are predominantly produced by Th2 and Treg, respectively. This is referred to as "the modified T-helper 2 type response" (67), and has been reported in the context of the beneficial effects of immunotherapy in allergy, in which it favors CSR to IgG4 over IgE (68). Prolonged antigen exposure indeed leads to a serologic shift in the IgG4:IgG1 antibody ratio, as previously illustrated by natural immunization in bee keepers or during subcutaneous

immunotherapy with grass pollen (69, 70). Previous observations in tissue and in blood have also linked the modified Th2 type response to IgG4-RD (16-18, 20, 69). Our data confirms such a response, with the observation of increased numbers of Th2 cells and Tregs in IgG4-RD and a positive correlation between Th2 cells and IgG4⁺ B-cells. In contrast to earlier observations we did not find an increase in Tfh cells (71). Yet, this finding supports the hypothesis that differentiation towards IgG4 in IgG4-RD takes place in peripheral tissue, rather than in follicles in lymphoid structures.

Despite significant differences in numbers of B- and T-cell subsets between patients and controls in the presented study, none of these were sufficiently discriminative to be used as biomarker. This included plasma blasts, which had recently been proposed as a biomarker for IgG4-RD (34). Importantly, the numbers of plasma blasts in our patients (median 3.36 cells/ μ L) were in the same range as previously reported (median 4.70 cells/ μ L) (34), whereas plasma blast numbers were extremely low in controls of that study (0.10 cells/ μ L) (34). As plasma blasts in our study (median 1.70 cells/ μ L) were in the same range as reported by others, it is questionable if this subset is a reliable biomarker (72). Therefore, we propose to derive a biomarker based on the specific “lymphocyte signature” in peripheral blood of patients with IgG4-RD. Combined analysis of various subsets has an advantage since it does not rely on one biomarker, rather it reflects the pathophysiology of the disease. Since IgG4-RD can affect various organs, the clinical presentation is diverse, yet PCA of the peripheral lymphocyte compartment illustrates that the underlying immune response is clearly distinct. This is confirmed by the fact that combined analysis with PCA not only distinguishes healthy from disease, but can also discriminate between different fibro-inflammatory conditions (i.e. sarcoidosis from IgG4-RD). Examples of scoring systems in medical practice are ample and systems medicine has been proven of value to study complex diseases and generate predictive models, although some caution is warranted (73, 74).

441

442 In conclusion, we here demonstrate that patients with IgG4-RD present with increased
443 numbers of IgG4⁺ B-cells, CD21^{low} B-cells and plasma blasts in blood. Molecular
444 characteristics reveal that the latter expansion is a result of an ongoing immune response in
445 affected tissue. Furthermore, this is characterized by increased numbers of Th2 cells and
446 regulatory T-cells, known as the modified Th2 type response. This specific “peripheral
447 lymphocyte signature” in patients with IgG4-RD indicates that patients suffering from IgG4-
448 RD have a common disease pathogenesis and in the future, peripheral blood might be
449 exploited as a non-invasive tool to employ for diagnosis and treatment monitoring in patients
450 with IgG4-RD.

Author contributions

JJH, AFK, JAMvL, PMvH and MCvZ designed research. JJH and AFK performed research. JJH, AFK and MCvZ analyzed data. RMV and DP evaluated and included patients in the study. JJH, AFK and MCvZ wrote the paper, and all authors commented on the paper and approved the final version.

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References

1. Stone JH, Zen Y, Deshpande V. IgG4-related disease. *N Engl J Med*. 2012;366(6):539-51.
2. Kamisawa T, Zen Y, Pillai S, Stone JH. IgG4-related disease. *Lancet*. 2015;385(9976):1460-71.
3. Stone JH, Khosroshahi A, Deshpande V, Chan JK, Heathcote JG, Aalberse R, et al. Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations. *Arthritis and rheumatism*. 2012;64(10):3061-7.
4. Umehara H, Okazaki K, Masaki Y, Kawano M, Yamamoto M, Saeki T, et al. A novel clinical entity, IgG4-related disease (IgG4RD): general concept and details. *Modern rheumatology / the Japan Rheumatism Association*. 2012;22(1):1-14.
5. Karim F, Clahsen-van Groningen M, van Laar JA. AA Amyloidosis and IgG4-Related Disease. *N Engl J Med*. 2017;376(6):599-600.
6. Frulloni L, Lunardi C, Simone R, Dolcino M, Scattolini C, Falconi M, et al. Identification of a novel antibody associated with autoimmune pancreatitis. *N Engl J Med*. 2009;361(22):2135-42.
7. Okazaki K, Uchida K, Ohana M, Nakase H, Uose S, Inai M, et al. Autoimmune-related pancreatitis is associated with autoantibodies and a Th1/Th2-type cellular immune response. *Gastroenterology*. 2000;118(3):573-81.
8. Mattoo H, Mahajan VS, Della-Torre E, Sekigami Y, Carruthers M, Wallace ZS, et al. De novo oligoclonal expansions of circulating plasmablasts in active and relapsing IgG4-related disease. *J Allergy Clin Immunol*. 2014;134(3):679-87.
9. Culver EL, Vermeulen E, Makuch M, van Leeuwen A, Sadler R, Cargill T, et al. Increased IgG4 responses to multiple food and animal antigens indicate a polyclonal expansion and differentiation of pre-existing B cells in IgG4-related disease. *Ann Rheum Dis*. 2015;74(5):944-7.
10. Anderson CL, Abraham GN. Characterization of the Fc receptor for IgG on a human macrophage cell line, U937. *J Immunol*. 1980;125(6):2735-41.
11. Bruggemann M, Williams GT, Bindon CI, Clark MR, Walker MR, Jefferis R, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med*. 1987;166(5):1351-61.
12. Aalberse RC, Schuurman J. IgG4 breaking the rules. *Immunology*. 2002;105(1):9-19.
13. Mahajan VS, Mattoo H, Deshpande V, Pillai SS, Stone JH. IgG4-related disease. Annual review of pathology. 2014;9:315-47.
14. Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *Journal of immunology*. 1998;160(7):3555-61.
15. Tsuboi H, Matsuo N, Iizuka M, Tsuzuki S, Kondo Y, Tanaka A, et al. Analysis of IgG4 class switch-related molecules in IgG4-related disease. *Arthritis research & therapy*. 2012;14(4):R171.
16. Zen Y, Fujii T, Harada K, Kawano M, Yamada K, Takahira M, et al. Th2 and regulatory immune reactions are increased in immunoglobulin G4-related sclerosing pancreatitis and cholangitis. *Hepatology*. 2007;45(6):1538-46.
17. Miyake K, Moriyama M, Aizawa K, Nagano S, Inoue Y, Sadanaga A, et al. Peripheral CD4+ T cells showing a Th2 phenotype in a patient with Mikulicz's disease associated with lymphadenopathy and pleural effusion. *Modern rheumatology / the Japan Rheumatism Association*. 2008;18(1):86-90.
18. Mattoo H, Della-Torre E, Mahajan VS, Stone JH, Pillai S. Circulating Th2 memory cells in IgG4-related disease are restricted to a defined subset of subjects with atopy. *Allergy*. 2014;69(3):399-402.
19. Kanari H, Kagami S, Kashiwakuma D, Oya Y, Furuta S, Ikeda K, et al. Role of Th2 cells in IgG4-related lacrimal gland enlargement. *Int Arch Allergy Immunol*. 2010;152 Suppl 1:47-53.
20. Miyoshi H, Uchida K, Taniguchi T, Yazumi S, Matsushita M, Takaoka M, et al. Circulating naive and CD4+CD25high regulatory T cells in patients with autoimmune pancreatitis. *Pancreas*. 2008;36(2):133-40.

21. Kusuda T, Uchida K, Miyoshi H, Koyabu M, Satoi S, Takaoka M, et al. Involvement of inducible costimulator- and interleukin 10-positive regulatory T cells in the development of IgG4-related autoimmune pancreatitis. *Pancreas*. 2011;40(7):1120-30.
22. Maehara T, Mattoo H, Ohta M, Mahajan VS, Moriyama M, Yamauchi M, et al. Lesional CD4+ IFN-gamma+ cytotoxic T lymphocytes in IgG4-related dacryoadenitis and sialoadenitis. *Ann Rheum Dis*. 2016.
23. Mattoo H, Mahajan VS, Maehara T, Deshpande V, Della-Torre E, Wallace ZS, et al. Clonal expansion of CD4(+) cytotoxic T lymphocytes in patients with IgG4-related disease. *J Allergy Clin Immunol*. 2016;138(3):825-38.
24. Zen Y, Fujii T, Sato Y, Masuda S, Nakanuma Y. Pathological classification of hepatic inflammatory pseudotumor with respect to IgG4-related disease. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2007;20(8):884-94.
25. Kuo TT, Chen TC, Lee LY. Sclerosing angiomatoid nodular transformation of the spleen (SANT): clinicopathological study of 10 cases with or without abdominal disseminated calcifying fibrous tumors, and the presence of a significant number of IgG4+ plasma cells. *Pathology international*. 2009;59(12):844-50.
26. Narula N, Vasudev M, Marshall JK. IgG(4)-related sclerosing disease: a novel mimic of inflammatory bowel disease. *Digestive diseases and sciences*. 2010;55(11):3047-51.
27. Strehl JD, Hartmann A, Agaimy A. Numerous IgG4-positive plasma cells are ubiquitous in diverse localised non-specific chronic inflammatory conditions and need to be distinguished from IgG4-related systemic disorders. *Journal of clinical pathology*. 2011;64(3):237-43.
28. Deshpande V, Zen Y, Chan JK, Yi EE, Sato Y, Yoshino T, et al. Consensus statement on the pathology of IgG4-related disease. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2012;25(9):1181-92.
29. Karim F, Loeffen J, Bramer W, Westenberg L, Verdijk R, van Hagen M, et al. IgG4-related disease: a systematic review of this unrecognized disease in pediatrics. *Pediatr Rheumatol Online J*. 2016;14(1):18.
30. Karim AF, Verdijk RM, Guenoun J, van Hagen PM, van Laar JA. An inflammatory condition with different faces: immunoglobulin G4-related disease. *Neth J Med*. 2016;74(3):110-5.
31. Stone JH, Brito-Zeron P, Bosch X, Ramos-Casals M. Diagnostic Approach to the Complexity of IgG4-Related Disease. *Mayo Clinic proceedings*. 2015;90(7):927-39.
32. Ngwa TN, Law R, Murray D, Chari ST. Serum immunoglobulin G4 level is a poor predictor of immunoglobulin G4-related disease. *Pancreas*. 2014;43(5):704-7.
33. Sah RP, Chari ST. Serologic issues in IgG4-related systemic disease and autoimmune pancreatitis. *Current opinion in rheumatology*. 2011;23(1):108-13.
34. Wallace ZS, Mattoo H, Carruthers M, Mahajan VS, Della Torre E, Lee H, et al. Plasmablasts as a biomarker for IgG4-related disease, independent of serum IgG4 concentrations. *Annals of the rheumatic diseases*. 2015;74(1):190-5.
35. Blanchard-Rohner G, Pulickal AS, Jol-van der Zijde CM, Snape MD, Pollard AJ. Appearance of peripheral blood plasma cells and memory B cells in a primary and secondary immune response in humans. *Blood*. 2009;114(24):4998-5002.
36. Kelly DF, Snape MD, Perrett KP, Clutterbuck EA, Lewis S, Blanchard Rohner G, et al. Plasma and memory B-cell kinetics in infants following a primary schedule of CRM 197-conjugated serogroup C meningococcal polysaccharide vaccine. *Immunology*. 2009;127(1):134-43.
37. Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D, et al. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *J Exp Med*. 2004;199(11):1577-84.
38. Jacobi AM, Odendahl M, Reiter K, Bruns A, Burmester GR, Radbruch A, et al. Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2003;48(5):1332-42.

39. Khosroshahi A, Bloch DB, Deshpande V, Stone JH. Rituximab therapy leads to rapid decline of serum IgG4 levels and prompt clinical improvement in IgG4-related systemic disease. *Arthritis Rheum.* 2010;62(6):1755-62.
40. Khosroshahi A, Carruthers MN, Deshpande V, Unizony S, Bloch DB, Stone JH. Rituximab for the treatment of IgG4-related disease: lessons from 10 consecutive patients. *Medicine (Baltimore).* 2012;91(1):57-66.
41. Carruthers MN, Topazian MD, Khosroshahi A, Witzig TE, Wallace ZS, Hart PA, et al. Rituximab for IgG4-related disease: a prospective, open-label trial. *Ann Rheum Dis.* 2015;74(6):1171-7.
42. Karim F, Paridaens D, Westenberg LE, Guenoun J, Verdijk RM, van Hagen PM, et al. Infliximab for IgG4-Related Orbital Disease. *Ophthal Plast Reconstr Surg.* 2016.
43. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21^{low} B cell population in the peripheral blood of patients with SLE. *Clin Immunol.* 2004;113(2):161-71.
44. Khosroshahi A, Cheryk LA, Carruthers MN, Edwards JA, Bloch DB, Stone JH. Brief Report: spuriously low serum IgG4 concentrations caused by the prozone phenomenon in patients with IgG4-related disease. *Arthritis & rheumatology.* 2014;66(1):213-7.
45. Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, Bottcher S, Ritgen M, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia.* 2012;26(9):1986-2010.
46. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *Journal of immunological methods.* 2008;329(1-2):112-24.
47. Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, et al. IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res.* 2009;37(Database issue):D1006-12.
48. Yaari G, Uduman M, Kleinstein SH. Quantifying selection in high-throughput Immunoglobulin sequencing data sets. *Nucleic Acids Res.* 2012;40(17):e134.
49. McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. *Nat Rev Immunol.* 2012;12(1):24-34.
50. Jackson KJ, Wang Y, Collins AM. Human immunoglobulin classes and subclasses show variability in VDJ gene mutation levels. *Immunol Cell Biol.* 2014;92(8):729-33.
51. van Zelm MC. B cells take their time: sequential IgG class switching over the course of an immune response? *Immunol Cell Biol.* 2014;92(8):645-6.
52. Wynn TA. Type 2 cytokines: mechanisms and therapeutic strategies. *Nat Rev Immunol.* 2015;15(5):271-82.
53. Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat Immunol.* 2000;1(4):305-10.
54. Costa ES, Pedreira CE, Barrena S, Lecrevisse Q, Flores J, Quijano S, et al. Automated pattern-guided principal component analysis vs expert-based immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping. *Leukemia.* 2010;24(11):1927-33.
55. Warnatz K, Wehr C, Drager R, Schmidt S, Eibel H, Schlesier M, et al. Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia. *Immunobiology.* 2002;206(5):502-13.
56. Saadoun D, Terrier B, Bannock J, Vazquez T, Massad C, Kang I, et al. Expansion of autoreactive unresponsive CD21-/low B cells in Sjogren's syndrome-associated lymphoproliferation. *Arthritis Rheum.* 2013;65(4):1085-96.
57. Tedder TF, Inaoki M, Sato S. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity.* 1997;6(2):107-18.
58. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood.* 2010;115(24):5026-36.

59. Lighaam LC, Vermeulen E, Bleker T, Meijlink KJ, Aalberse RC, Barnes E, et al. Phenotypic differences between IgG4+ and IgG1+ B cells point to distinct regulation of the IgG4 response. *J Allergy Clin Immunol*. 2014;133(1):267-70 e1-6.
60. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood*. 2011;118(8):2150-8.
61. Good KL, Avery DT, Tangye SG. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol*. 2009;182(2):890-901.
62. Berkowska MA, Heeringa JJ, Hajdarbegovic E, van der Burg M, Thio HB, van Hagen PM, et al. Human IgE(+) B cells are derived from T cell-dependent and T cell-independent pathways. *J Allergy Clin Immunol*. 2014;134(3):688-97 e6.
63. Agematsu K, Nagumo H, Yang FC, Nakazawa T, Fukushima K, Ito S, et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol*. 1997;27(8):2073-9.
64. Tangye SG, Liu YJ, Aversa G, Phillips JH, de Vries JE. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J Exp Med*. 1998;188(9):1691-703.
65. van Zelm MC, Bartol SJ, Driessen GJ, Mascart F, Reisli I, Franco JL, et al. Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation. *J Allergy Clin Immunol*. 2014;134(1):135-44.
66. Legler DF, Loetscher M, Roos RS, Clark-Lewis I, Baggiolini M, Moser B. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med*. 1998;187(4):655-60.
67. Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. *Lancet*. 2001;357(9258):752-6.
68. Akdis CA, Akdis M. Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. *J Clin Invest*. 2014;124(11):4678-80.
69. Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy*. 2009;39(4):469-77.
70. Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *Journal of immunology*. 1983;130(2):722-6.
71. Akiyama M, Suzuki K, Yamaoka K, Yasuoka H, Takeshita M, Kaneko Y, et al. Number of Circulating Follicular Helper 2 T Cells Correlates With IgG4 and Interleukin-4 Levels and Plasmablast Numbers in IgG4-Related Disease. *Arthritis & rheumatology*. 2015;67(9):2476-81.
72. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol*. 2010;162(2):271-9.
73. Gomez-Cabrero D, Menche J, Cano I, Abugessaisa I, Huertas-Miguelanez M, Tenyi A, et al. Systems Medicine: from molecular features and models to the clinic in COPD. *J Transl Med*. 2014;12 Suppl 2:S4.
74. Fischer T, Brothers KB, Erdmann P, Langanke M. Clinical decision-making and secondary findings in systems medicine. *BMC Med Ethics*. 2016;17(1):32.

654 **Table (n=1)****Table 1. Characteristics of patients with IgG4-RD**

patient	gender	age (yr)	organs affected	medication at inclusion	time until diagnosis	treatment started after inclusion	CRP (<10mg/L)	ESR (20mm/h)
1	F	60	orbita	n.i.m.	14 years	prednisone, methotrexate, cyclosporine, infliximab	14	40
2	F	67	orbita	n.i.m.	8 years	dexamethasone	1.1	1
3	M	63	orbita, lymph node, lung, prostate	prednisone	3 years	prednisone, rituximab, methotrexate	0.9	5
4	M	54	orbita	prednisone	10 years	prednisone	1.9	9
5	F	53	orbita	n.i.m.	3 years	unknown	1.7	n.d.
6	M	60	orbita, lymph node	t.n.	5 years	prednisone, methotrexate	0.7	16
7	M	44	orbita	t.n.	12 years	prednisone	0.4	1
8	F	59	skin	t.n.	5 months	hydroxochloroquine	6.0	11
9	M	18	lymph node, lung, cerebra	t.n.	1 month	dexamethasone, azathioprine	50	107
10	F	62	pancreas, lymph node, lung	t.n.	3 months	prednisone	6	2.4
11	M	74	lymph node, salivary gland, lung	t.n.	5 months	prednisone, azathioprine	0.8	37
12	M	53	mesenterium	t.n.	20 years	prednisone, azathioprine, rituximab	84	119
13	M	62	thyroid gland	t.n.	1 year	prednisone	1.9	6
14	M	32	serous membrane	t.n.	3 months	prednisone	18.7	8
15	M	79	lymph node, pancreas	t.n.	1 year	-	6.4	90
16	M	60	bile duct, lymph node	t.n.	2 weeks	prednisone, methotrexate	4	0.7

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n.i.m., no immunomodulatory medication for at least 6 months prior to inclusion; t.n., treatment naïve; n.d., not determined

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Figures (n=6)

Figure 1. Representative hematoxylin and eosin (H&E) and IgG4 stainings of tissue sections of affected organs in patients with IgG4-RD. **A.** and **B.** Pericardial tissue of patient 15 demonstrating lymphoplasmacytoid fibrosis and obliterative phlebitis. **C.** and **D.** Orbital tissue of patient 6 with lymphoid infiltration. **E.** and **F.** Lung tissue of patient 10 with lymphoplasmacellular infiltrates. All images are 200X magnification.

Figure 2. Identification and immunophenotyping of IgG subclass expressing memory B-cells. **A.** Stepwise flowcytometric gating strategy to identify IgG1+, IgG2+, IgG3+ and IgG4+ memory B-cells. **B.** Expression of activation and memory markers on B-cell subsets. Shaded grey histograms represent naive B-cells, light blue represent IgG1+ memory B-cells and dark blue represent IgG4+ memory B-cells. Each dot represents one individual, red lines indicate medians. Statistical analysis between the groups was performed with the Mann Whitney U test. * $p < 0.05$

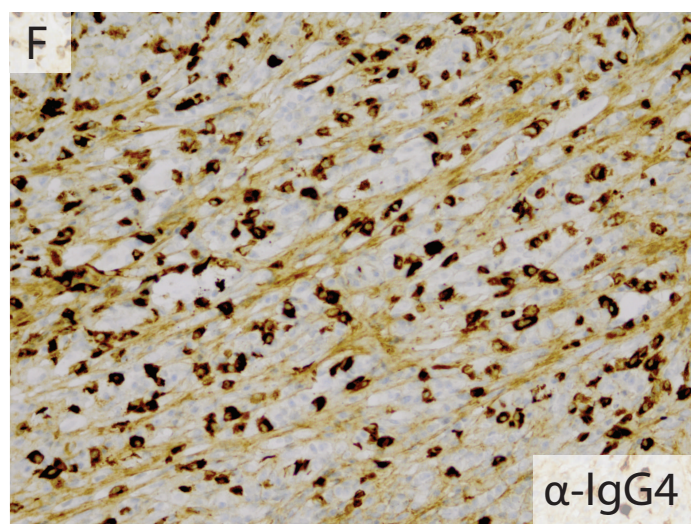
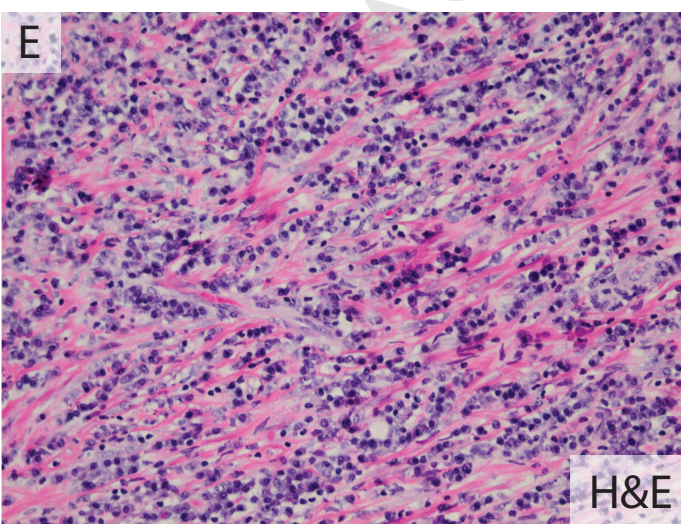
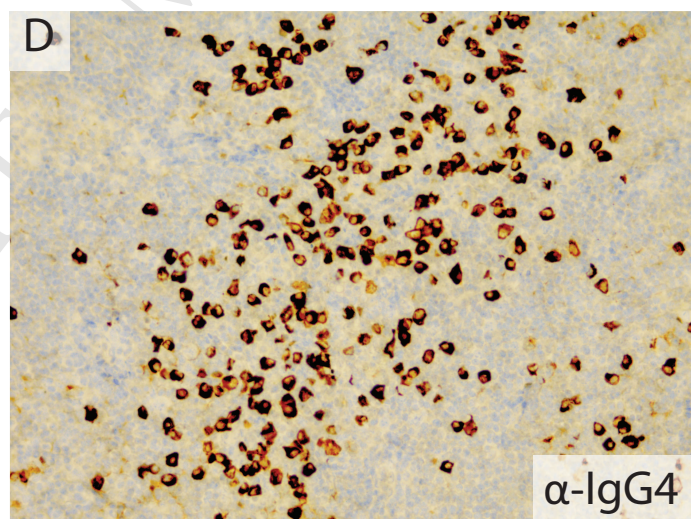
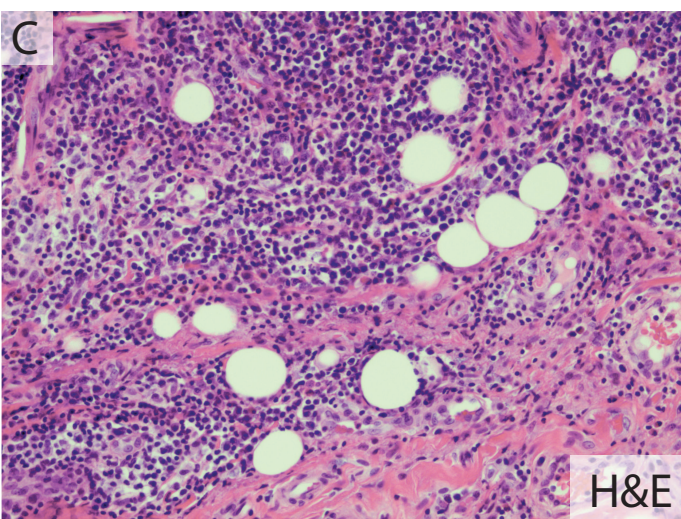
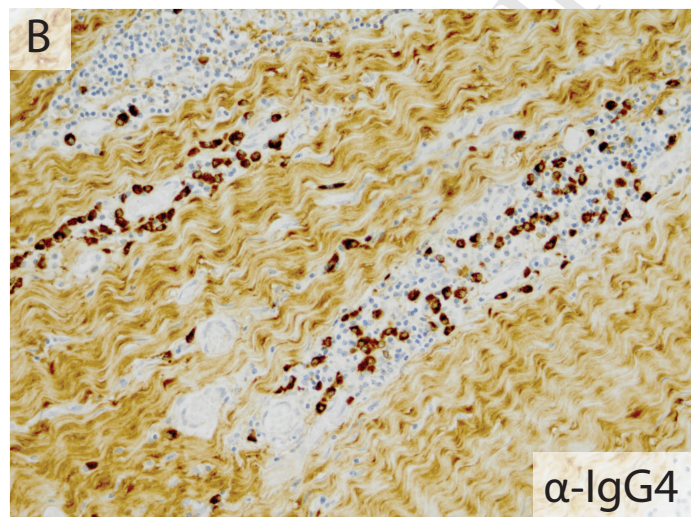
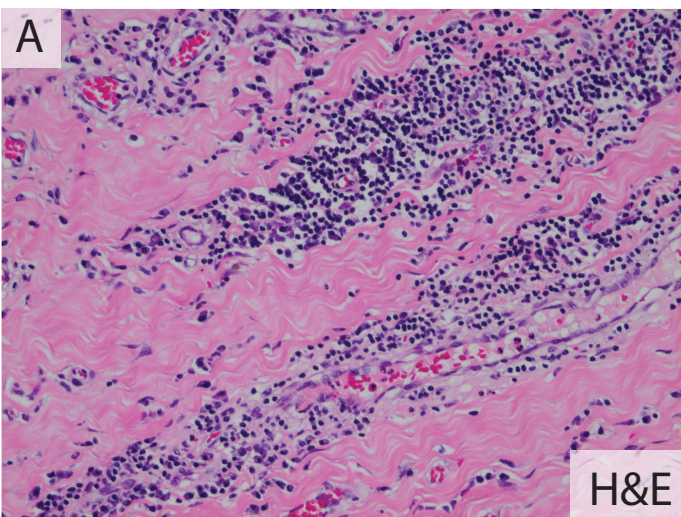
Figure 3. Composition of the blood B-cell compartment in healthy individuals and in patients with IgG4-RD. **A.** Model of peripheral B-cell maturation. **B.** Absolute numbers of naive and memory B-cell subsets and plasma cells. Columns indicate median values with interquartile range. **C.** Absolute numbers of IgG4+ memory B-cells. **D.** Distribution of IgG subclass memory B-cells within the total IgG memory B-cell compartment. **E.** Absolute numbers of CD21^{low}IgG4+ memory B-cells. Each dot represents one individual, red lines indicate medians. Statistical analysis between the groups was performed with the Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, **** $P < 0.0001$

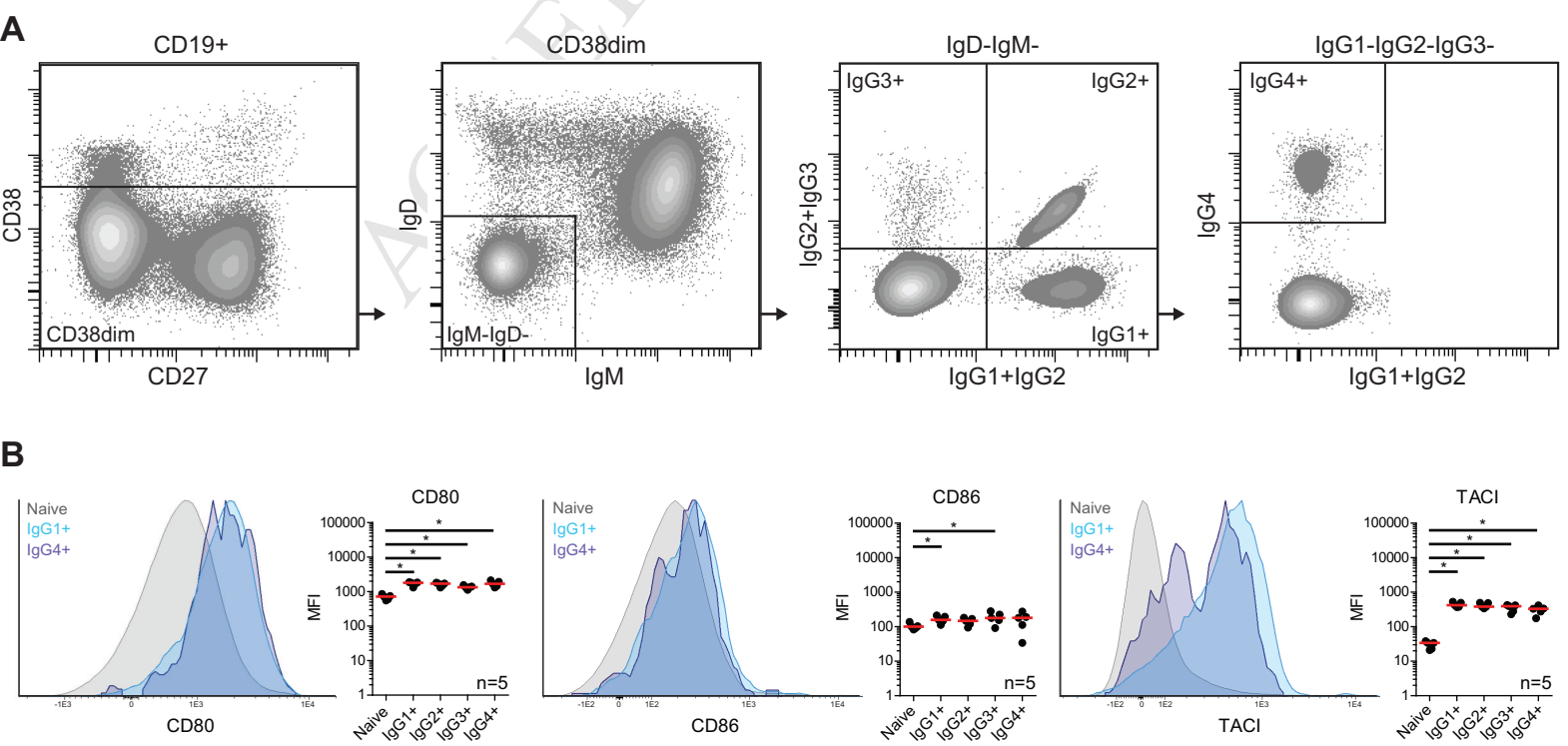
Figure 4. Molecular characteristics of IgG4+ B-cells from healthy individuals and patients with IgG4-RD. **A.** Representative flowcytometry plots of CD27 expression on total B cells and IgG4+ B-cells from a healthy individual and from a patient with IgG4-RD. **B.** Frequencies of IgG subclass memory B-cells expressing CD27. **C.** Expression of cytokine and chemokine receptors on IgG4+ B-cells. **D.** Schematic representation of the human *IGH* locus. **E.** Representation of the membrane B-cell receptor (BCR) with the variable domain of the Ig heavy chain (V, D and J) and Ig light chain (V and J). Asterisks in the RNA represent mutations in the genes encoding the proteins of the BCR. **F.** Frequency of somatic hypermutation (SHM) in rearranged *IGHV* gene. **G.** Selection for replacement mutations in the CDR (red) and FR (blue) regions. Solid lines represent patients; dotted lines represent healthy controls. A selection strength of more than 0 is indicative of positive selection (48).

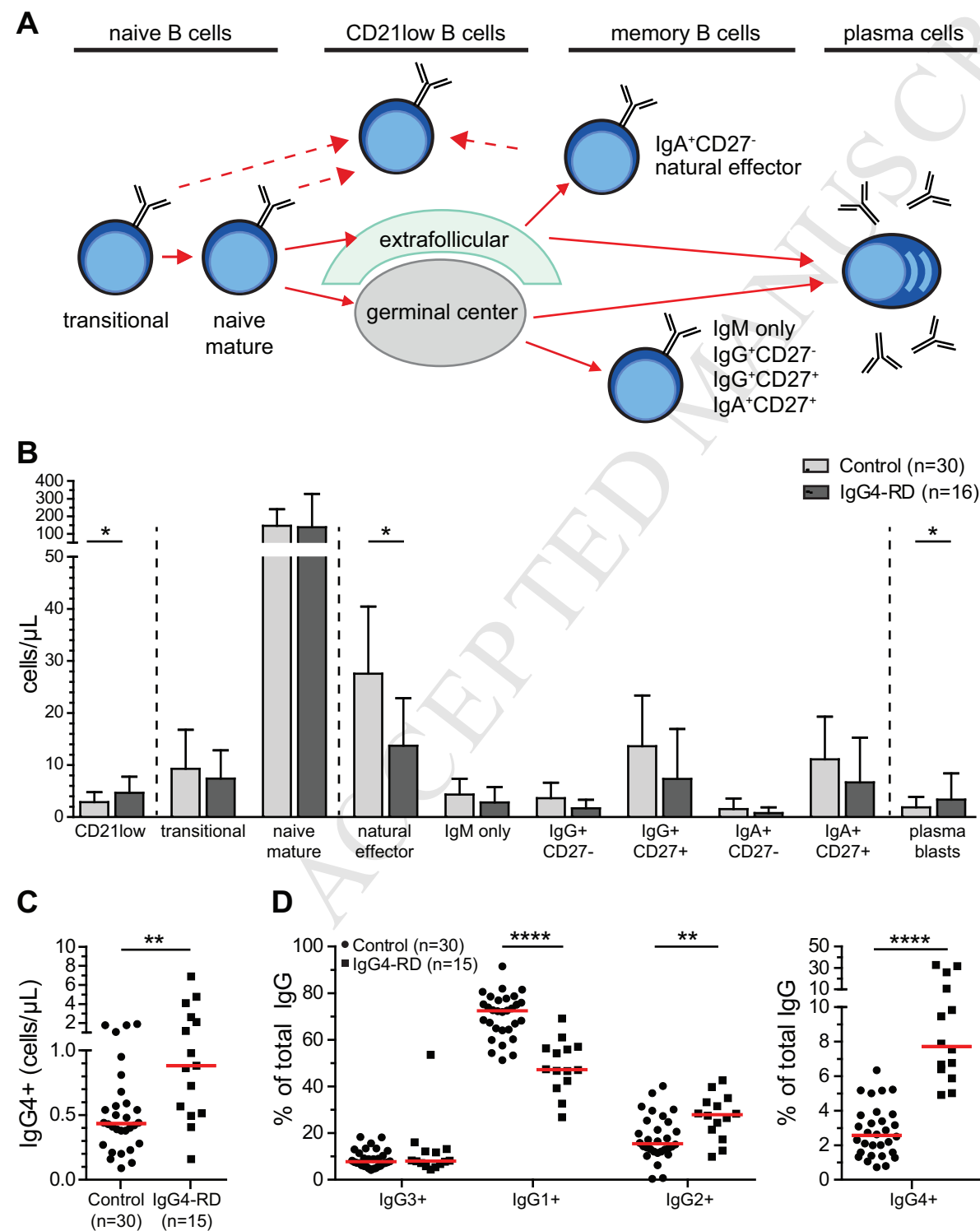
Figure 5. Blood T-helper subsets. **A.** Representative plots from flow cytometric analysis. **B.** Absolute numbers of T-helper 1 (Th1), T-helper 2 (Th2), follicular helper T-cells (Tfh) and regulatory T-cells (Treg). **C.** Regression analysis of Th2 cells and IgG4+ B-cells and of Treg cells and IgG4+ B-cells. Each dot represents one individual, red lines indicate medians. Statistical analysis between the groups was performed with the Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$. Correlation was calculated with Spearman R.

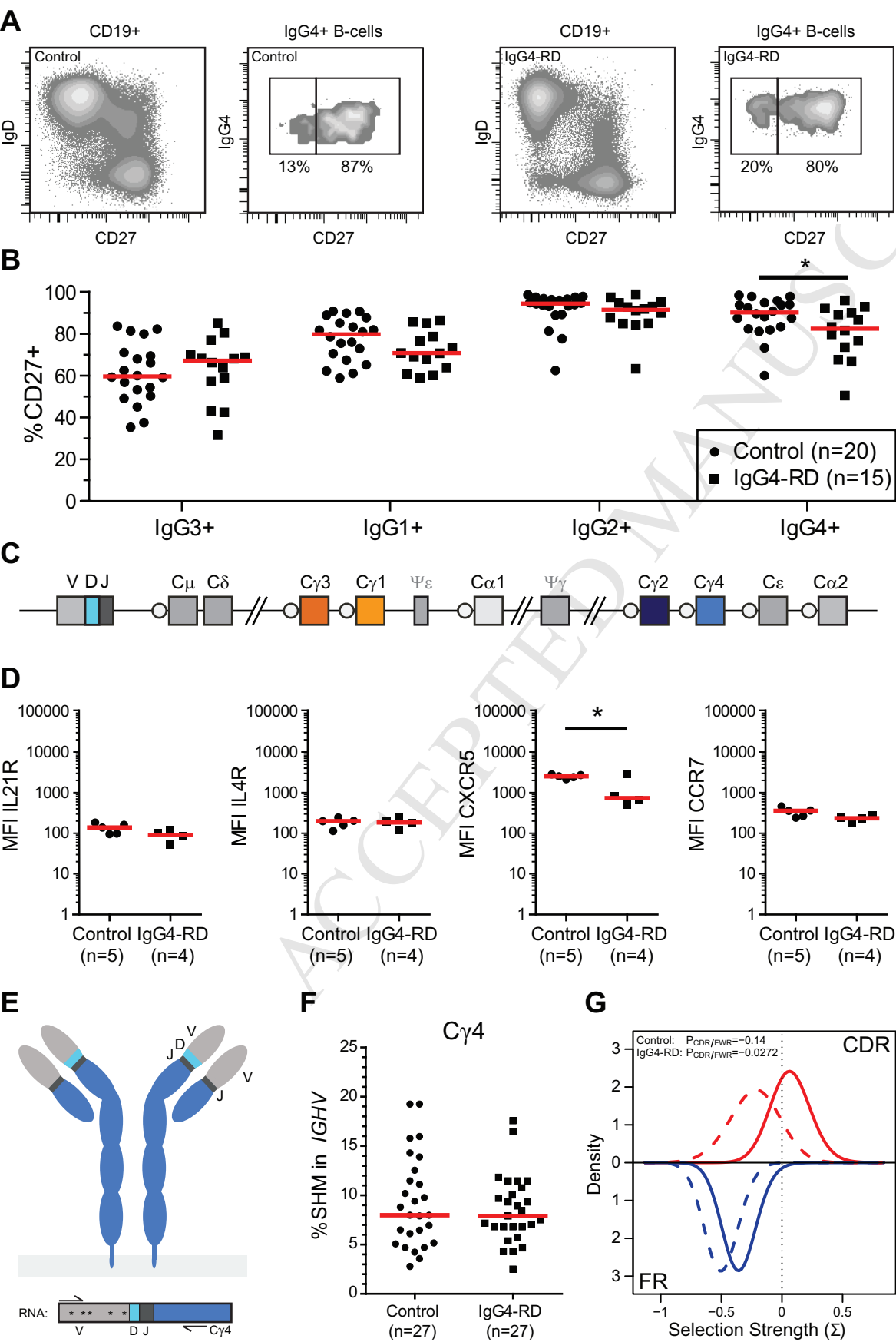
Figure 6. Multi-parameter analysis and clustering of individuals based on blood lymphocyte subsets. **A.** Principal component analysis of peripheral blood lymphocyte subsets from healthy individuals (green), patients with sarcoidosis (blue) and patients with IgG4-RD (red). Each individual is represented by one dot, with indication of group median (large dot) together with 1SD and 2SD. Following automated population separator (APS) based on the absolute counts of various subsets, PC1 and PC5 were selected to visualize separation

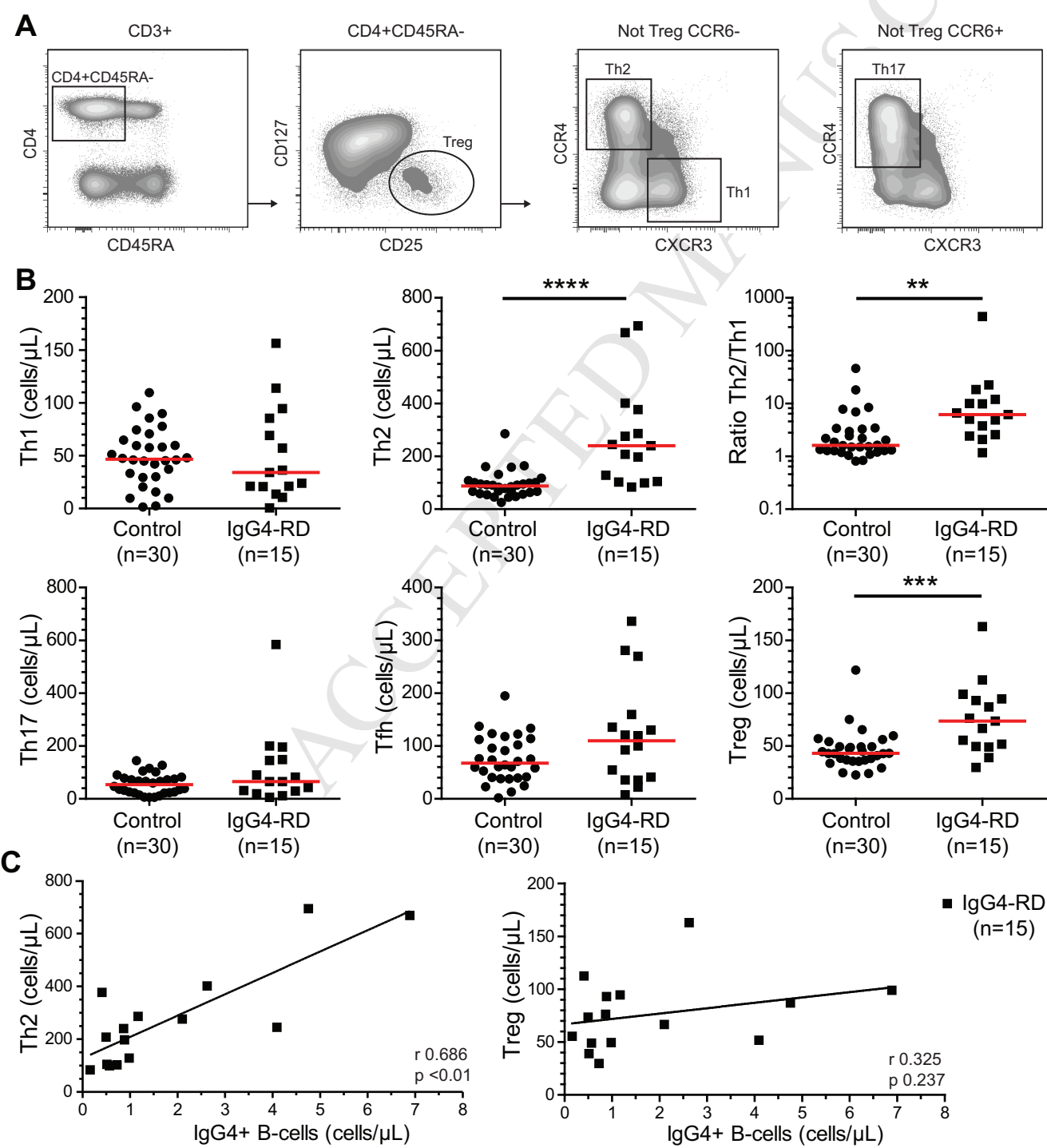
between healthy individuals, patients with sarcoidosis and patients with IgG4-RD. The contribution of the top 5 components of each PC are listed. **B.** Scoring of blood samples based on a formula composed of the subsets that were significantly different between healthy individuals and patients with IgG4-RD. The percentage of IgG2+ B-cells and IgG4+ B-cells within total IgG B-cells are multiplied by the absolute numbers of Th2, Treg and IgG4+ B-cells and divided by the percentage of IgG1+ B-cells within total IgG+ B-cells multiplied with the absolute numbers of Th1 and IgG1+ B-cells. Red dotted line indicates a score of 100 considered as cut-off value.



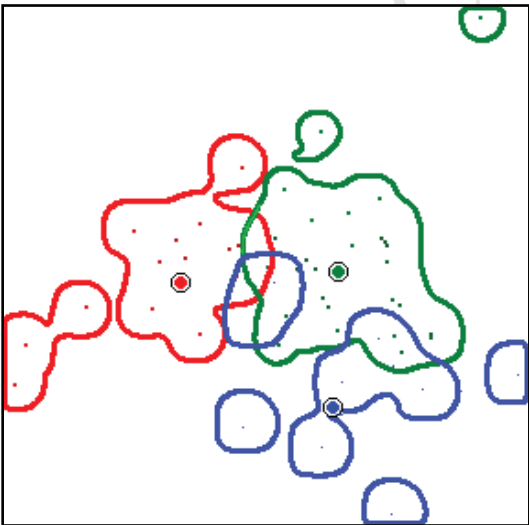








A



parameter	PC1 contribution (%)
% IgG4 in total IgG	50.74
Th2	19.95
Treg	11.84
% IgG1 in total IgG	8.27
% IgG2 in total IgG	6.75

parameter	PC5 contribution (%)
Treg	43.32
Th2	37.41
% IgG2 in total IgG	5.71
% IgG1 in total IgG	4.79
% IgG4 in total IgG	4.41

B

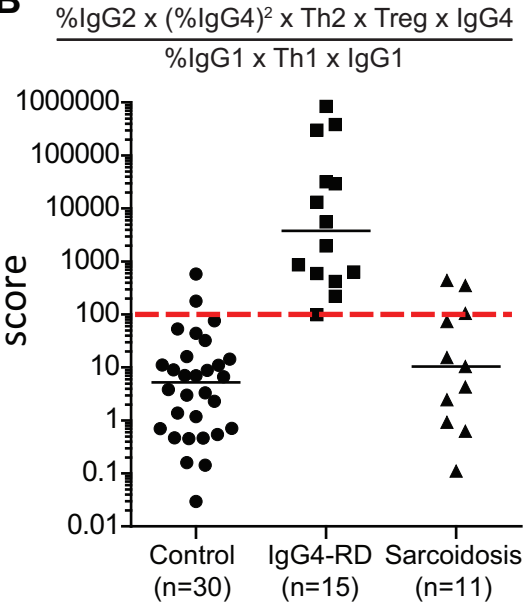


Table E1. Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Supplier
CD3	BV711	UCHT1	BD Biosciences, San Jose, CA
CD4	BV510	OKT4	BioLegend, San Diego, CA
CD8	APC-H7	SK1	BD Biosciences
CD16	PerCP-Cy5.5	3G8	BD Biosciences
CD19	PC7	J3-119	Beckman Coulter, Indianapolis, IN
CD19	BV785	SJ25C1	BioLegend
CD20	BV605	2H7	BioLegend
CD21	BV711	B-ly4	BD Biosciences
CD25	BV421	BC96	BioLegend
CD27	BV421	M-T271	BD Biosciences
CD38	APC-H7	HB7	BD Biosciences
CD45	OC515	GA90	Cytognos, Salamanca, Spain
CD45RA	BV605	HI100	BioLegend
CD45RO	FITC	UCHL1	Exbio, Vestec, Czech Republic
CD80	BV605	2D10	BioLegend
CD81	APC-H7	JS-81	BD Biosciences
CD86	PE-Cy7	IT2.2	BioLegend
CD123	PE-Cy7	6H6	eBioscience, San Diego, CA
CD124	PE-Cy7	G077F6	BioLegend
CD127	APC	A019D5	BioLegend
CD180	Biotin	RP/14	BioLegend
CD267	PE/Dazzle	1A1	BioLegend
CD360	PE-CF594	17A12	BD Biosciences
CCR4	PE-Cy7	L291H4	BioLegend
CCR6	PerCP-Cy5.5	G034E3	BioLegend
CCR7	PE	REA108	Miltenyi Biotec, Bergisch Gladbach, Germany
CXCR3	FITC	G025H7	BioLegend
CXCR5	APC	51505	R&D systems, Minneapolis, MN
HLADR	BV605	L243	BioLegend
IgA	FITC	IS11-8E10	Miltenyi Biotec
IgA	PE	IS11-8E10	Miltenyi Biotec
IgD	PerCP-Cy5.5	IA6-2	BioLegend
IgD	PE-CF594	IA6-2	BD Biosciences
IgE	FITC	Goat anti-human	Invitrogen, Waltham, MA
IgG	PE	IS11-3B2.2.3	Miltenyi Biotec
IgG1	PE	SAG1	Cytognos
IgG2	PE	SAG2	Cytognos
IgG2	FITC	SAG2	Cytognos
IgG3	FITC	SAG3	Cytognos
IgG4	APC	SAG4	Cytognos
IgM	BV510	MHM-88	BioLegend
Streptavidin	BV605	-	BioLegend

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin

Table E2. Immunophenotypic definitions of T- and B-cell subsets

CD3⁺ T-cell subset	Immunophenotype
Total CD8 ⁺ T-cell	CD3 ⁺ CD8 ⁺
CD8 ⁺ naive T-cell	CD3 ⁺ CD8 ⁺ CCR7 ⁺ CD45RO ⁻
CD8 ⁺ central memory T-cell	CD3 ⁺ CD8 ⁺ CCR7 ⁺ CD45RO ⁺
CD8 ⁺ effector memory RA T-cell	CD3 ⁺ CD8 ⁺ CCR7 ⁻ CD45RO ⁻
CD8 ⁺ effector memory RO T-cell	CD3 ⁺ CD8 ⁺ CCR7 ⁻ CD45RO ⁺
Total CD4 ⁺ T-cell	CD3 ⁺ CD4 ⁺
CD4 ⁺ naive T-cell	CD3 ⁺ CD4 ⁺ CCR7 ⁺ CD45RO ⁻
CD4 ⁺ central memory T-cell	CD3 ⁺ CD4 ⁺ CCR7 ⁺ CD45RO ⁺
CD4 ⁺ effector memory RA T-cell	CD3 ⁺ CD4 ⁺ CCR7 ⁻ CD45RO ⁻
CD4 ⁺ effector memory RO T-cell	CD3 ⁺ CD4 ⁺ CCR7 ⁻ CD45RO ⁺
CD4 ⁺ CTL cell	CD3 ⁺ CD4 ⁺ CCR7 ⁻ CD45RO ⁺ CD27 ⁻
T helper 1 cell	CD3 ⁺ CD4 ⁺ CD45RA ⁺ CCR6 ⁻ CCR4 ⁺ CXCR3 ⁺
T helper 2 cell	CD3 ⁺ CD4 ⁺ CD45RA ⁺ CCR6 ⁻ CCR4 ⁺ CXCR3 ⁻
T follicular helper cell	CD3 ⁺ CD4 ⁺ CD45RA ⁻ CXCR5 ⁺
Regulatory T-cell	CD3 ⁺ CD4 ⁺ CD45RA ⁻ CD127 ⁻ CD25 ⁺
CD19⁺ B-cell subset	Immunophenotype
transitional B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{high} CD27 ⁻
naive mature B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁺ /IgM ⁺ CD27 ⁻
IgM+IgD ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁺ IgM ⁺ CD27 ⁺
IgM-only memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgM ⁺ CD27 ⁺ IgD ⁻
IgA ⁺ CD27 ⁻ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgA ⁺ CD27 ⁻
IgA ⁺ CD27 ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgA ⁺ CD27 ⁺
IgG ⁺ CD27 ⁻ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgG ⁺ CD27 ⁻
IgG ⁺ CD27 ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgG ⁺ CD27 ⁺
IgG1 ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgG1 ⁺
IgG2 ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgG2 ⁺
IgG3 ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgG3 ⁺
IgG4 ⁺ memory B-cell	CD19 ⁺ CD21 ⁺ CD38 ^{dim} IgD ⁻ IgM ⁻ IgG4 ⁺
plasmablast	CD19 ⁺ CD38 ^{high} CD27 ⁺
CD21 ^{low} B-cell	CD19 ⁺ CD21 ^{low} CD38 ^{dim}
CD21 ^{low} IgG4 ⁺ B cell	CD19 ⁺ CD21 ^{low} CD38 ^{dim} IgD ⁻ IgM ⁻ IgG4 ⁺

Table E3. Basic immunological characteristics of patients with IgG4-RD

Patient	serum immunoglobulins (g/L)				blood leukocytes and subsets (cells/ μ l)				
	IgG1	IgG2	IgG3	IgG4	leukocytes	lymphocytes	T-cells	B-cells	NK-cells
1	8.61	6.8	0.4	2.12	6500	3480	2640	620	220
2	10.40	2.28	0.34	2.73	4500	2440	1930	400	70
3	5.24	2.36	0.38	5.96	7300	790	610	60	90
4	5.37	2.13	0.35	1.22	6200	1960	1640	200	120
5	4.59	3.16	0.62	0.27	12100	4470	3500	490	450
6	10.4	4.56	0.75	7.50	4800	1790	1480	110	180
7	6.49	2.49	0.32	1.85	7100	1650	1160	190	270
8	6.89	3.39	0.59	1.93	6500	2040	1510	270	220
9	25.1	5.12	0.96	12.8	5800	2360	1620	570	130
10	5.27	4.31	0.35	0.27	8800	2970	2140	430	370
11	10.4	2.93	0.32	5.75	7700	1280	1040	60	170
12	11.4	2.68	1.09	25.25	10200	4100	3300	280	440
13	6.85	4.66	0.23	2.76	5400	2280	1440	210	530
14	9.91	7.15	0.64	5.40	7600	1530	1130	110	260
15	19.30	2.27	2.28	1.13	7000	1710	1010	470	150
16	8.87	4.36	0.36	5.55	6800	1480	1150	60	160
normal range	4.9-11.4	1.5-6.4	0.2-1.1	0.08-1.4	3500-10000	1100-2500	700-1900	100-400	100-400

Subnormal values are depicted in italics, supranormal values in bold font

Table E4. Clinical and immunological characteristics of patients with Sarcoidosis

patient	gender	age (yr)	organs affected	medication at inclusion	CRP (mg/L)	ESR (mm/h)	immunoglobulins (g/L)				Leukocytes (cells/ μ L)	T-cells (cells/ μ L)	B-cell (cells/ μ L)
							IgG1	IgG2	IgG3	IgG4			
1	F	43	lung, eye, skin	n.i.m.	2.6	3	n.d.	n.d.	n.d.	n.d.	5000	430	180
2	F	33	lymph node, eye	n.i.m	12	22	5.56	3.51	0.31	<i>0.05</i>	4300	350	80
3	M	67	ocular	n.i.m	1.0	5	5.90	2.15	0.27	0.36	8100	2180	200
4	M	49	lung, lymph node	n.i.m	3.9	49	11.8	6.38	0.79	3.47	6300	980	450
5	M	59	lymph node	n.i.m	0.7	13	9.0	4.74	0.30	1.22	8800	760	330
6	M	44	lymph node, eye	n.i.m	0.6	7	9.32	2.60	<i>0.13</i>	0.82	3600	930	140
7	F	66	lymph node, skin	n.i.m	2.4	12	6.81	3.21	0.35	0.17	7200	940	570
8	F	86	lymph node, eye	n.i.m	27	12	6.56	2.27	0.31	0.28	5500	830	90
9	M	51	eye, lymph node, kidney	n.i.m	10	12	7.12	3.88	0.47	0.23	4100	430	260
10	M	52	eye, lung	plaquenil	0.3	3	7.18	2.74	0.34	0.51	3700	350	60
11	F	58	lung, eye, skin, joint	n.i.m	n.d.	n.d.	7.78	2.66	<i>0.11</i>	0.55	5700	1220	380
normal range					<10mg/L	20mm/h	4.9-11.4	1.5-6.4	0.2-1.1	0.08-1.4	3500-10000	700-1900	100-400

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n.i.m., no immunomodulatory medication, n.d., not determined; Subnormal values are depicted in italics, supranormal values in bold font.

Supplemental Figure Legends (n=6)

Figure E1. A. Absolute counts of IgG1+, IgG2+ and IgG3+ memory B-cells. Each dot represents one individual, red lines represent median values. Statistical analysis between the groups was performed with the Mann Whitney U test; *P<0.05. **B.** Correlation between IgG4 serum values and absolute numbers of IgG4+ memory B-cells and between percentages of IgG4+ B-cells within total IgG+ B-cells. Grey shaded area indicates the reference value for IgG4 serum values. Correlation was calculated with Spearman R. **C.** Representative plots of the flow cytometric analysis of IgG subclass expressing CD21^{low} B-cells **D.** Absolute counts of CD21^{low} IgG4+ B-cells. Each dot represents one individual, red lines represent median values. Statistical analysis between the groups was performed with the Mann Whitney U test; *P<0.05, **P<0.01. **E.** Correlation between CD21^{low} B-cells and IgG4+ CD21^{low} B-cells in healthy controls and in patients with IgG4-RD.

Figure E2. A. Frequencies of somatic hypermutations (SHM) in rearranged *IGHV* genes of IgG3+, IgG1+, IgG2+ and IgG4+ B-cells from healthy individuals. Each dot represents one sequence, red lines represent median values. Statistical analysis was performed with the Mann Whitney U test; *P<0.05, **P<0.01. **B.** IGH-CDR3 size of IgG4+ B-cells. Each dot represents one sequence, red lines represent median values. Statistical analysis was performed with the Mann Whitney U test; *P<0.05, **P<0.01. **C.** Frequencies of the *IGHV* gene usage in IgG4+ B-cells from healthy individuals and patients with IgG4-RD.

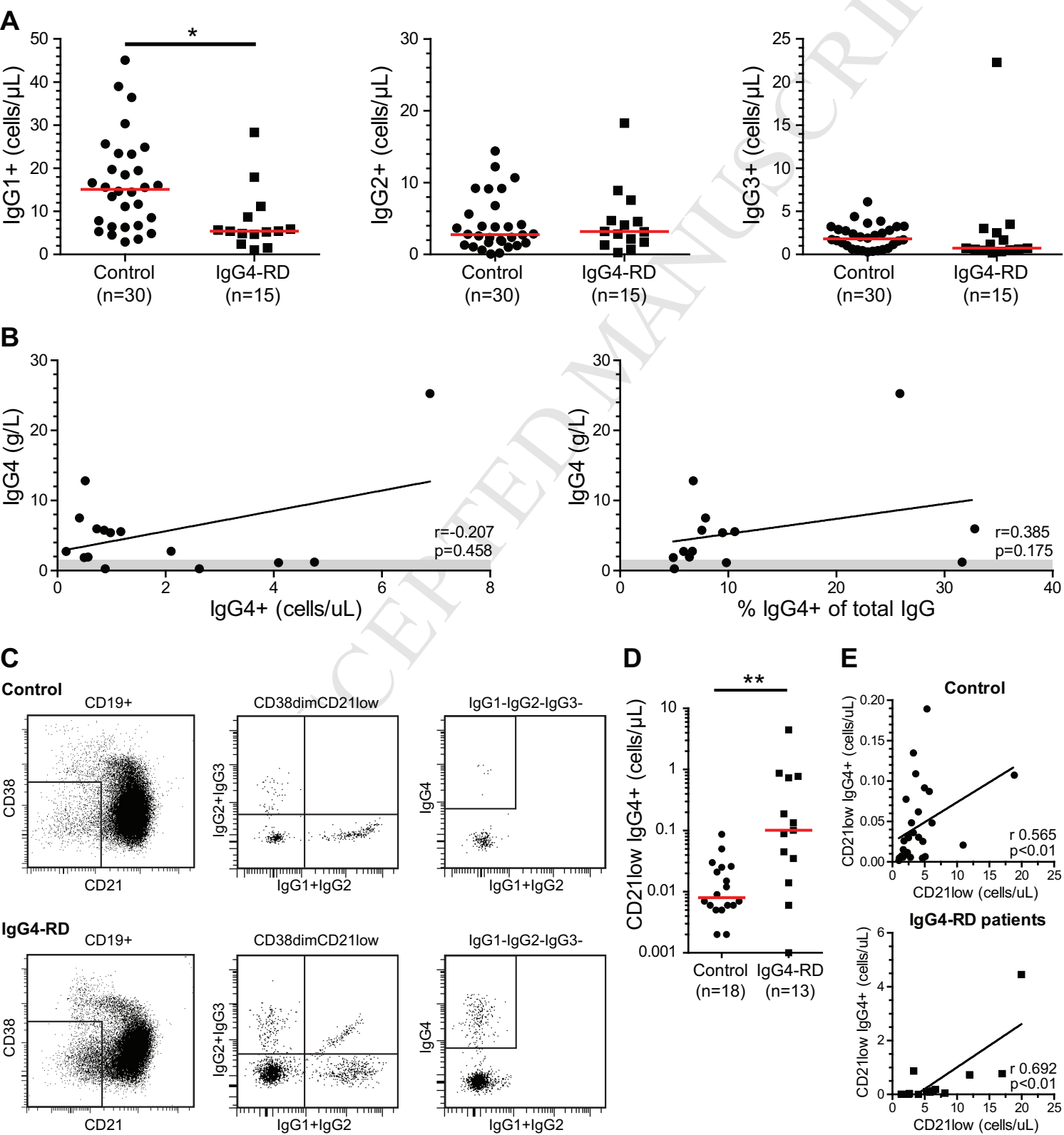
Figure E3. A. Correlation between CD21^{low} IgG4+ B-cells and Th2 cells and between CD21^{low} IgG4+ B-cells and Treg cells in patients with IgG4-RD. Correlation was calculated with Spearman R. **B.** Correlation between IgG4 serum values and absolute numbers of Th2

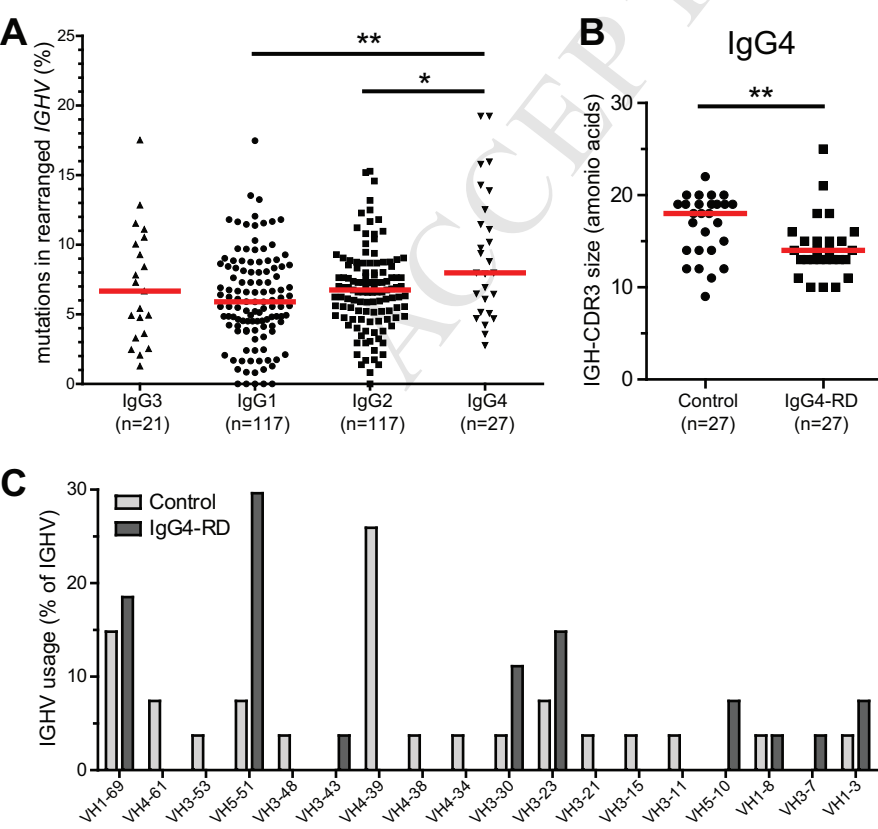
and of Treg cells. Grey shaded area indicates the reference value for IgG4 serum values. Correlation was calculated with Spearman R.

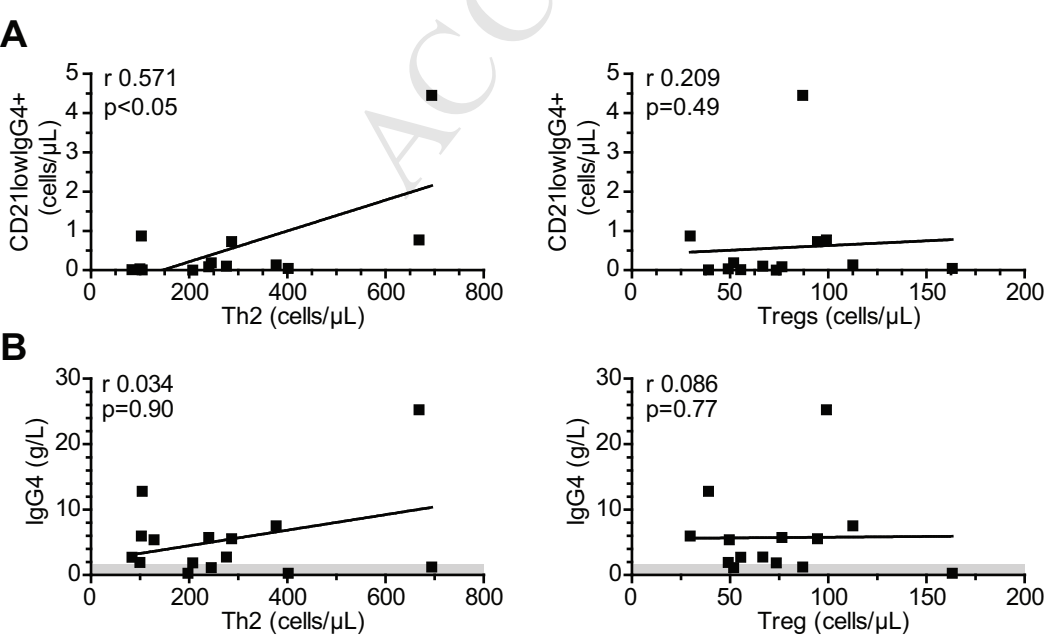
Figure E4. A. Representative plots of the flow cytometric analysis and absolute counts of CD4⁺ naive T-cells (CD4⁺ Tnaive), CD4⁺ central memory T-cells (CD4⁺ Tcm), CD4⁺ effector memory CD45RO⁺ T-cells (CD4⁺ TemRO) and CD4⁺ effector memory CD45RO⁻ T-cells (CD4⁺ TemRA). **B.** Absolute numbers of CD4⁺CD45RO⁻CCR7⁻CD27⁻ CTL cells **C.** Representative plots of the flow cytometric analysis and absolute counts of CD8⁺ Tnaive, CD8⁺ Tcm, CD8⁺ TemRO and CD8⁺ TemRA. Columns represent median values with interquartile range. Statistical analysis was performed with the Mann Whitney U test; **P<0.01.

Figure E5. A. Representative plots of the flow cytometric analysis of granulocyte subsets and plasmacytoid dendritic cells (DC) **B.** Absolute counts of neutrophils, basophils, eosinophils and plasmacytoid DC. Columns represent median values with interquartile range. Statistical analysis was performed with the Mann Whitney U test, *P<0.05.

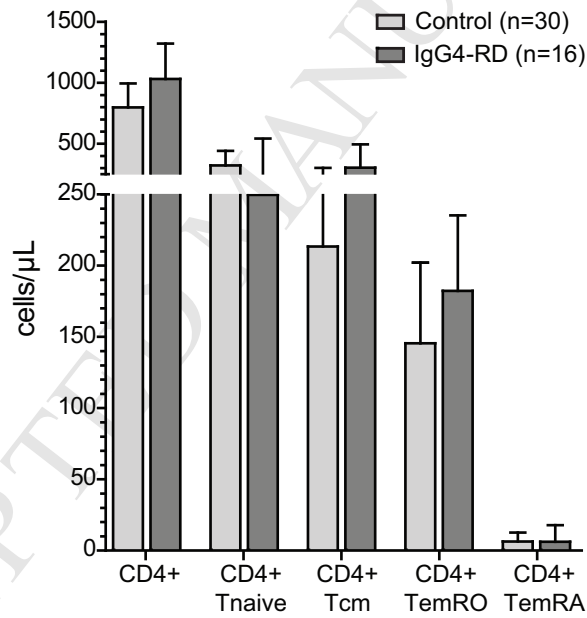
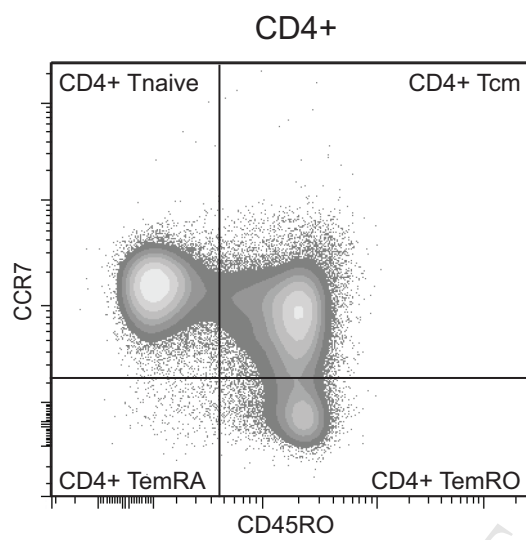
Figure E6. Percentage of IgG4⁺ memory B-cells within total IgG memory B-cells and absolute counts of Th2 and regulatory T-cells in patients with sarcoidosis. Each dot represents one individual, red lines represent median values.



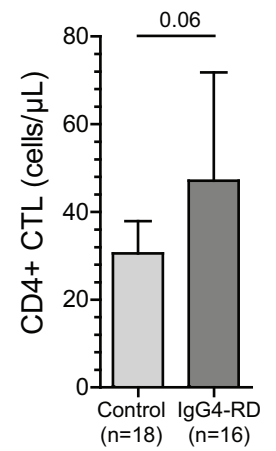




A



B



C

