Standing out of the crowd
Doorenspleet, M.E.

Citation for published version (APA):

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B CELLS IN IGG4-RELATED DISEASE


Immunoglobulin G4+ clones identified by next-generation sequencing dominate the B cell receptor repertoire in immunoglobulin G4 associated cholangitis

Hepatology 2013;57:2390–2398
**Background** The Immunoglobulin G4 (IgG4)-associated cholangitis (IAC) is a manifestation of the recently discovered idiopathic IgG4-related disease. The majority of patients have elevated serum IgG4 levels and/or IgG4-positive B-cell and plasma cell infiltrates in the affected tissue. We hypothesized that clonally expanded, class-switched IgG4⁺ B cells and plasma cells could be causal to these poorly understood phenomena.

**Methods** In a prospective cohort of six consecutive IAC patients, six healthy controls, and six disease controls, we used a novel next-generation sequencing approach to screen the B-cell receptor (BCR) repertoires, in blood as well as in affected tissue, for IgG4⁺ clones. A full repertoire analysis of the BCR heavy chain was performed using GS-FLX/454 and customized bioinformatics algorithms (> 10,000 sequences/sample; clones with a frequency ≥ 0.5% were considered dominant).

**Results** We found that the most dominant clones within the IgG⁺ BCR heavy repertoire of the peripheral blood at baseline were IgG4⁺ only in IAC patients. In all IAC patients, but none of the controls, IgG4⁺ BCR clones were among the 10 most dominant BCR clones of any immunoglobulin isotype (IgA, IgD, IgM, and IgG) in blood. The BCR repertoires of the duodenal papilla comprised the same dominant IgG4⁺ clones as the paired peripheral blood samples. In all IAC patients, after 4 and 8 weeks of corticosteroid therapy the contribution of these IgG4⁺ clones to the IgG⁺ repertoire as well as to total BCR repertoire was marginalized, mirroring sharp declines in serum IgG4 titers and regression of clinical symptoms.

**Conclusion** The novel finding of highly abundant IgG4⁺ BCR clones in blood and tissue of patients with active IAC, which disappear upon corticosteroid treatment, suggests that specific B cell responses are pivotal to the pathogenesis of IAC.

**INTRODUCTION**

Immunoglobulin G4 (IgG4)-related disease (IgG4-RD) is a common denominator for incompletely understood organ abnormalities associated with IgG4⁺ B-cell and plasma cell infiltrates and/or elevated serum IgG4 titers. Although the list of possibly affected organs in IgG4-RD is expanding, the pancreas in the form of autoimmune pancreatitis and biliary tree in the form of IgG4-associated cholangitis (IAC, also known as IgG4-related (sclerosing) cholangitis) are thus far the most frequently involved localizations. The diagnosis of IgG4-RD is currently made by exclusion of other causes and organ-specific diagnostic criteria, of which the histology, imaging, serology, other organ involvement, and response to steroid therapy (HISORT) criteria that were originally developed for the diagnosis of autoimmune
pancreatitis are arguably the most generally applied. Nevertheless, diagnosing IgG4-related disease of the biliary tree is challenging, because this rare disease often mimics malignancies of the bile ducts or pancreatic head as well as primary sclerosing cholangitis (PSC) and forms of secondary sclerosing cholangitis, mainly in terms of symptoms and imaging. The number of IAC patients diagnosed after histological evaluation of surgical specimens, either obtained in diagnostic procedures or during extended resections for suspected malignancies, is far from negligible.

IgG4-RD derived its name from the elevated serum levels of IgG4 that were found in the original cohorts. Although markedly elevated serum IgG4 levels (> 4 times the upper limit of normal) were reported to be highly specific for IgG4-RD, the use of serum IgG4 as a biomarker is limited due to its low sensitivity and limited specificity when it is only mildly elevated. It was reported that approximately 10% to 20% of autoimmune pancreatitis patients do not have elevated serum IgG4 levels, and similar percentages were suggested for IAC cohorts. Furthermore, IgG4 levels were reported to be elevated in substantial percentages of patients with PSC and patients with pancreatoco biliary malignancies, further fueling the discussion on the use of serum IgG4 levels as a biomarker of IgG4-related disease. Nevertheless, the majority of patients suffering from IgG4-related disease have elevated IgG4 serum levels or infiltrating IgG4+ plasma cells in the affected tissue. Whereas in normal individuals IgG4 is the least abundant IgG, it may surpass IgG1, IgG2, and IgG3 and become the major IgG subtype of all serum IgG levels in IgG4-RD. Although it has been a topic of speculation, the origin of this serum IgG4 and the processes leading to the tissue infiltration by IgG4+ B cells and plasma cells remain elusive. In theory, elevated IgG4 serum levels might be caused by antigen-driven immune responses. If so, these responses would be characterized by clonally expanded, class-switched B cells and plasma cells. If these clones could indeed be found, this would provide insight into the etiology of this disease and could eventually lead to the identification of potential causal antigens stimulating IgG4 production.

In order to test the hypothesis of IgG4+ clones in IgG4-RD, we prospectively included material from a cohort of IAC patients. Using a novel next-generation sequencing technology, the B-cell receptor (BCR) heavy chain repertoire in IAC patients was screened, allowing us to fingerprint individual clones. In order to investigate whether the BCR repertoire in the peripheral blood mirrors the repertoire present in the affected tissue, we assessed the BCR repertoires in paired tissue samples. Lastly, we followed the BCR repertoire of IAC patients over the course of corticosteroid remission induction therapy to observe the effect of the currently preferred intervention in patients with this rare disorder.
**MATERIALS AND METHODS**

*Patients*

We prospectively included six patients clinically diagnosed with IAC and meeting the HISORt criteria as published and adapted for IAC (Table 1)\(^{1,14,15}\). Four patients were included during the symptomatic episode that led to the diagnosis of IAC, with the biliary tract as the primary site of inflammation. One patient was included suffering from relapsing IAC (disease duration 4 years) under maintenance dose corticosteroids (budesonide, 6 mg / day). One patient was included while suffering from the complications of liver cirrhosis due to an inadequately controlled IAC and was at the time of inclusion under prolonged immunosuppression (prednisolone, 5 mg / day plus azathioprine 100 mg / day). None of the IAC patients showed signs of malignant disease (hematological, pancreaticobiliary, or other) observed to date (mean follow-up 16 months, range 8 - 20 months). From all newly diagnosed patients, peripheral blood was drawn before the start of treatment with prednisolone (median 40 mg/day, range 20 - 40 mg / day). After 4 and 8 weeks, additional blood samples were collected. Two patients underwent endoscopic retrograde cholangio pancreatography for stent replacement, which allowed the collection of a duodenal papilla biopsy (ampulla of Vater), and paired peripheral blood. Patients included in the PSC control group were selected based on a diagnosis of PSC compliant with the current European Association for the Study of the Liver guidelines\(^{16}\) and including a history of colitis; one patient with elevated serum IgG4 underwent additional short-term prednisolone treatment without biochemical response. Patients included in the malignancy control group had a histologically proven hepatobiliary malignancy (pancreatic cancer or bile duct cancer) (Table 2). An extended overview of clinical characteristics of all analyzed patients is provided in Table S2. Anonymous healthy individuals were age- and sex-matched to the IAC patient group. The study was performed according to the Declaration of Helsinki and was approved by the local medical ethical committee of the Academic Medical Center (METC10/007). All patients provided written informed consent prior to inclusion in the study.

*Peripheral blood and papilla biopsy sampling*

Peripheral blood was collected and stored using PAXGene Blood RNA tubes according to the manufacturer's instructions (catalog #762165, PreAnalytiX, Breda, The Netherlands). Isolation of total RNA was performed using the PAXGene Blood RNA isolation kit (catalog #762174, Qiagen, Venlo, The Netherlands). Biopsies of the duodenal papilla (ampulla of Vater) during endoscopic retrograde cholangio pancreatography were immediately preserved in RNALater reagent (Qiagen) and stored at −80°C until use. Total RNA was isolated using polytron homogenizer in the presence of STAT60 reagent as described\(^{17}\). cDNA was synthesized with 250 ng total RNA input using Superscript III RT (Invitrogen Life
Technologies, Carlsbad, CA).

**Linear amplification and next-generation sequencing**

The linear amplification used in this study was based on the protocol used for T cells and B cells in previous studies. In the first step of the protocol, a linear amplification of the complete immunoglobulin repertoire was performed using a primer set covering all functional $V_{\text{heavy}}$ genes of the BCR (Figure S4). The $V_{\text{heavy}}$-primers contained a primer B sequence required for Amplicon sequencing according to the 454 titanium protocol (version 2010; Roche Diagnostics, Mannheim, Germany). Amplified products were purified using AMPure XP SPRI-beads (catalog #A63881; Agencourt-Bioscience, Beverly, MA) in a template/bead ratio of 1:1. The cleaned product was used in a polymerase chain reaction using primer B as the forward primer and a generic primer specific for all functional $J_{\text{heavy}}$ genes containing primer A as the reverse primer. An additional primer set was designed for the amplification of all $C_{\text{heavy}}$ gene segments to analyze all possible immunoglobulin isotypes (primer sequences available on request). These primers all contained the primer A sequence and can therefore be used as a substitute for the $J_{\text{heavy}}$ primer. All amplified products encode the CDR3, a unique sequence that defines a unique clone. After amplification, samples were again purified using the AMPure beads and quantified via fluorospectrometry using the Quant-iT dsDNA HS Assay Kit (catalog #Q32851; Invitrogen Life Technologies). Samples were prepared for sequencing according to the manufacturer’s protocol for Amplicon Sequencing. Sequencing was performed on a Roche Genome Sequencer FLX using the Titanium platform. For each sample, at least 40,000 (bead-bound) immunoglobulin sequences were analyzed. The number of sequences reflects the amount of BCRs produced by that clone and can be used as a measure for dominance of that particular clone. Next-generation sequencing will visualize expanded B cells as a deviation in the repertoire because they carry the same BCR sequence. Moreover, plasma cells can be identified as these cells produce increased amounts of BCR messenger RNA, producing a comparable deviation in the repertoire. For clarity, we will use the term 'dominant clones' to denote unique BCR signals with a frequency $\geq 0.5\%$ within the repertoire.

**Bioinformatics and data analysis**

The bioinformatics pipeline used to obtain the BCR sequences has been described in detail and contains four modules: (1) MID-sorting, (2) identification of gene segments, (3) CDR3 detection, and (4) removal of artifacts. Immunoglobulin isotype homology was determined using the National Center for Biotechnology Information’s open-access web tool BLASTn (megablast algorithm) and reference sequences for the human Immunoglobulin heavy-chain constant regions, allowing a sequence homology $> 97\%$. Mutations in the
Immunoglobulin heavy-chain variable region were analyzed and characterized using IMGT’s V-quest (version 3.2.28)\(^2\).

**Statistical analysis**

Values are expressed either as the mean and standard deviation or as the median and interquartile range, depending on criteria for (non)parametric analysis. Comparisons between all three groups were performed using one-way analysis of variance (ANOVA) and Bonferroni post hoc test. Two sided P values of < 0.05 were considered statistically significant. Graphpad Prism version 5.1 was used to perform the analyses (Graphpad Prism, La Jolla, CA).

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Table 1 IAC patient characteristics

**RESULTS**

**IgG4\(^+\) clones dominate the IgG BCR repertoire of patients with IgG4-associated cholangitis, not of healthy controls or disease controls**

In order to test the hypothesis of IgG4\(^+\) clones in IgG4-RD, we used novel next-generation sequencing technology to screen the BCR\(_{heavy}\) chain repertoire in IAC patients and fingerprint individual clones. We prospectively included six patients meeting the HISORT criteria for IAC with or without concurrent autoimmune pancreatitis before and during immunosuppressive treatment (Table 1). Four patients were included upon diagnosis; the fifth was included during relapse on low-dose corticosteroid therapy. The sixth patient was not diagnosed until 5 years after the start of symptoms and was not included until 2 years after diagnosis, at which point this patient had already suffered from cirrhotic complications due to the initially uncontrolled disease. As control groups, we analyzed the blood of healthy age- and sex-matched controls (Hcs) (n = 6) and disease controls (DCs) for PSC (n = 3) and pancreaticobiliary malignancy (n = 3).

First, we screened for IgG\(^+\) clones within the total BCR repertoire in peripheral blood to
evaluate the presence of IgG4+ BCR clones in this IgG+ compartment. In each patient and control, we sequenced 10,000 BCRs yielding 3,955 clones (range, 1,954 - 7,277; similar amounts of clones were found in HCs/DCs). Equal numbers of IgG+ clones were recovered in all three groups (compared with HCs [100%]: 116.0% in IAC and 120.8% in DCs; p = 0.20) (Figure 1A). Of the IgG subset, the most dominant clones (as a percentage of the total repertoire) were IgG4+ in IAC, which was not the case in HCs or DCs (Figure 1B). This is reflected in the rank of the most dominant IgG4+ clone (1st in IAC versus 88th in HCs (p < 0.005) and 65th in DCs (p < 0.005), Figure 1C). Moreover, in IAC, 9.4% of the IgG+ clones expressed IgG4 (compared with 0.13% in HCs (p < 0.005) and 0.15% in DCs (p < 0.005), Figure 1D), and these IgG4+ clones together occupied 30.1% of the IgG+ BCRs (1.4% in HCs and 1.2% in DCs, Figure 1E). Even when regarding the full IgA, IgD, IgM, and IgG repertoire, IgG4+ clones are among the top 10 most expanded clones in IAC (Figure S1A), which is not the case in HCs or DCs (Figure S1B). Comparison of dominant IgG4+ clones in IAC revealed distinctly different rearrangements between patients, with V-region mutations in 9.0% (mean, SD 1.3%) of the bases, the majority of which were nonsilent (Table S1 and Figure S2). Collectively, these data show that IgG4+ B-cell clones are present as dominant clones in IAC, but not in HCs
or DCs. These IgG4+ clones are not only dominant but are also present in greater numbers, together occupying a larger part of the repertoire in IAC. Mutational analysis of dominant IgG4+ clones showed multiple nonsilent mutations, suggesting affinity maturation.

**Dominant IgG4+ clones that are found in peripheral blood are also dominantly present in the affected tissue**

Dominant IgG4+ clones are present in the peripheral blood of IAC patients, suggesting a role in the pathogenesis of the disease. One would expect to recover these clones in inflamed tissue as well. To this end, we investigated whether dominant IgG4+ clones in peripheral blood represent the cellular infiltrate in inflamed tissue. In two patients suffering from intermittent cholestasis, duodenal papilla biopsies were collected during stent replacement (together with paired peripheral blood). In both patients, dominant IgG4+ clones were recovered in the BCR repertoire of the biopsy material (Figure 2A / D). In line with peripheral blood, the rank of the highest IgG4+ clone is again 1st when selecting the IgG+ repertoire only. Comparing the retrieved IgG+ clones, a strong overlap was present between the clones found in blood and inflamed tissue (Figure 2B / C / E), mainly consisting of IgG4+ clones suggestive

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**Figure 2** SCR clones overlap between peripheral blood and duodenal papilla tissue in IAC patients. (A) Contribution of individual clones to the IgG+ BCR repertoire in peripheral blood taken during biopsy sampling (-6 weeks) as well as 6 weeks later before the start of therapy (baseline), and affected tissue. The scatterplot shows clonal abundance as a percentage of the IgG+ BCR repertoire (each dot represents an individual clone). IgG4+ clones are marked in color; identical colors in both compartments represent identical clones. (B) Dot plot showing the frequency of overlapping clones in tissue (papilla biopsy) and peripheral blood at the time of biopsy sampling (-6 weeks). IgG4+ clones are color marked. (C) Dot plot showing the frequency of overlapping clones in tissue and peripheral blood after clearing the infection (baseline). IgG4+ clones are color marked. (D) The contribution of individual clones to the IgG+ BCR repertoire in affected tissue (biopsy) and paired peripheral blood (blood). (E) Dot plot showing the frequency of overlapping clones in tissue and peripheral blood.
of specific enrichment of the infiltrating cells with these IgG4+ BCR clones (Figure S3A / B). Collectively, IgG4+ clones were detectable in inflamed tissue, and these clones showed marked overlap with those in peripheral blood. This suggests that these IgG4+ clones have a role in the pathogenesis of the disease, rather than being an epiphenomenon.

The disappearance of dominant IgG4+ BCR clones in blood reflects the effects of immunosuppressive therapy in IAC

If the dominant IgG4+ clones were indeed pathogenic, it would be expected that they would regress or even disappear following successful therapeutic intervention. We thus compared BCR repertoires in IAC patients before and 4 and 8 weeks after initiation of their first immunosuppressive treatment episode. In patients treated with high-dose prednisolone, serum liver tests improved rapidly (Figure 3A). Simultaneously, corticosteroid therapy induced a specific decline of serum IgG4 levels, while total IgG serum levels on average remained nearly stable within or close to physiological levels (Figure 3B). In line, after 4 weeks of treatment, the contribution of IgG4+ clones to the total blood BCR repertoire already had become negligible. The IgG+ clones with an IgG4+ subtype fell from 9.2% at baseline to 0.3% and 0.2% after 4 and 8 weeks of therapy, respectively (Figure 4A / B). Consequently, the contribution of individual dominant IgG4+ clones to the BCR repertoire regressed; the most dominant IgG4+ clone in IAC patients dropped in rank from a median of 1st to 51st (p < 0.001) and 67th (p < 0.001) after 4 and 8 weeks, respectively (Figure 4C). Furthermore, corticosteroid therapy appears to have a more profound effect on the presence of dominant IgG4+ clones than on other clones in the BCR repertoire. While dominant IgG4+ clones are rapidly suppressed by corticosteroid use, the majority of the non-IgG4 B cell clones remained stable during 4 and 8 weeks of immunosuppressive therapy (median percentage of BCR clones recovered from the BCR repertoire after 4 and 8 weeks, 70.3% and 66.1%, respectively, Figure 4D). The notion that dominant IgG4+ clones can be found in patients with active IAC is also supported by observations in one patient who experienced a relapse.
of disease while using a maintenance dose of the enterotropic corticosteroid budesonide. In this patient, the repertoire was assessed at baseline and 4 and 8 weeks after the daily dose of budesonide was increased. Also in this patient, IgG4+ clones were present at the time of active relapsing disease and were suppressed by therapeutic intervention (Figure 4E). In summary, these findings show that dominant IgG4+ clones disappear specifically from the repertoire after successful therapy and support the notion that these cells might be pathogenic.

**DISCUSSION**

This study is the first to show a sensitive and fully quantitative repertoire analysis of B cells and plasma cells in peripheral blood as well as affected tissue in a prospective cohort of patients with active IAC. Our findings indicate that IgG4+ clones are abundantly present within the repertoire of IAC patients with active disease, in contrast to healthy or disease controls. The inflamed tissue was shown to contain the identical expanded IgG4+ clones, and these clones seem to have undergone affinity maturation, suggesting an antigen-driven immune response. A possible central role for IgG4+ cells is furthermore supported.
of these unique clones to the total repertoire. The exact role of IgG4+ cells in approximately 50% of cases and can therefore be regarded as a proper representation of the infiltrate in the biliary tract\textsuperscript{24,25}. Nevertheless, evidence indicates these cells have a role in the pathogenesis of this disease. First, IgG4+ cells in approximately 50% of cases and can therefore be regarded as a proper representation of the infiltrate in the biliary tract\textsuperscript{24,25}. Nevertheless, by the finding that IgG4+ clones in peripheral blood specifically disappear upon successful corticosteroid therapy, both in treatment-naive patients as well as after disease relapse.

To our knowledge, this is the first report to provide a full BCR repertoire perspective on patients with IgG4-RD. The use of a high-resolution next-generation sequencing-based method allows the qualitative reconstruction of the full BCR repertoire as defined by its single clones, uniquely identified by their variable CDR3 and including their isotype and IgG subclass, based on a representative random sample of BCR expressing cells in these patients, and couples this to a quantitative analysis that assesses the relative contribution of these unique clones to the total repertoire.

The exact role of IgG4+ B cells and plasma cells in IAC remains obscure, though circumstantial evidence indicates these cells have a role in the pathogenesis of this disease. First, IgG4+ B cells and plasma cells are present in the majority of the inflamed tissues in IgG4-RD. Second, germinal center-like structures were described in IgG4-RD. These germinal center-like structures are generally thought to depend on the presence of B cells as well as T cells for ectopic lymphoid organogenesis and can regulate T-cell activation\textsuperscript{22}. Finally, it has been shown in a small cohort study that targeting B cells with rituximab in IgG4-RD results in prompt clinical and serologic improvement\textsuperscript{23}. Our data add to this knowledge, showing that IgG4+ B cells and plasma cells in IAC are also present in peripheral blood. These clones in the blood showed nonsilent mutations and a high degree of overlap with the IgG4+, clonally expanded B cells and plasma cells in inflamed tissue. This could also be the case in other IgG4-RD manifestations. Moreover, the most abundant IgG4+ clones in blood specifically disappeared upon successful corticosteroid therapy already after 4 weeks.

In the present study, we were only able to collect duodenal Vater papilla tissue instead of the actual inflamed tissue of the bile ducts. However, recent studies have shown that the papilla can be infiltrated by IgG4+ cells in approximately 50% of cases and can therefore be regarded as a proper representation of the infiltrate in the biliary tract\textsuperscript{24,25}. Nevertheless,
further studies should focus on the inclusion of the primary inflamed tissue, not only of the biliary tract but also of other affected tissues given the known tendency of IgG4-RD to either simultaneously or consecutively cause symptoms in multiple different organ systems.

A gold standard for the detection of IAC is currently lacking, and diagnosis relies on the application of HISORt criteria. However, in clinical practice the discrimination between IAC, PSC, and pancreatico-biliary malignancy can often only be made by the careful weighing of all clinical data, including serological tests, imaging, histology, and even response to short-term corticosteroid treatment in selected patients.

The presence of substantial numbers of IgG4+ cells is not fully diagnostic, as IgG4+ cells can be seen in inflammatory infiltrates of other origin. These diagnostic dilemmas are underlined by the fact that several of the patients with an established PSC or pancreatico-biliary malignancy included in this study did have IgG4+ cells infiltrating the tissue, albeit in low numbers, or elevated serum IgG4 levels. To assure the validity of our findings in this study, we only included PSC and cancer patients with an unchallenged diagnosis. However, in clinical practice, it may be difficult to discriminate between, for example, a PSC patient who presents at age 52 years with elevated serum liver tests and a serum IgG4 concentration of 3.6 g/L but without colitis or other supporting signs, and a younger IAC patient who has the same IgG4 concentration but has no other manifestations.

It is tempting to speculate whether these clonally expanded and class-switched B cells and plasma cells might have the ability to recognize 'self'. Initial screens in patients with autoimmune pancreatitis have not been able to yield conclusive epitopes\textsuperscript{26}. Even though we did not find any homology between the CDR3 sequences of the IgG4+ clones, it is possible that they harbor reactivity against the same epitope. Currently, it is impossible to predict the antigenic specificity of the B-cell receptor by its amino acid sequence. A more comprehensive set-up using high-throughput and quantitative peptide screens together with next-generation sequencing-based BCR repertoire analysis might provide more insight. In this procedure, it might be necessary to screen also for glycosylated peptides, since most organs commonly affected by IgG4-RD produce large amounts of mucins and other heavily glycosylated proteins. The specific overlap of IgG4+ clones between tissue and blood indicates that these screens can be performed on the more easily accessible peripheral blood instead of the inflamed tissue. The identification of IgG4+ clones and their antigens will help to better understand the pathogenesis of IgG4-RD, ultimately providing more targeted therapies or even cure.

Our data provide a new vision for the selectivity of suppression of dominant IgG4+ clones
by high-dose corticosteroid therapy. While corticosteroids are generally regarded as nonspecific immunosuppressants, we showed that in our cohort, prednisolone therapy induced a specific suppression of dominant IgG4⁺ clones, while the majority of the BCR repertoire remained intact despite the high-dose therapy. Arguably, corticosteroid therapy may ultimately have less generic effects on the circulating B-cell repertoire than rituximab, which is currently investigated for its value in corticosteroid-resistant IgG4-RD patients and has the advantage that it only targets CD20⁺ cells, while leaving other cells of the immune system unharmed. It would be of interest to compare the effects of corticosteroids and rituximab on the BCR repertoire in IgG4-RD.

The consistent detection of dominant IgG4⁺ clones in IAC patients appears to be a specific feature of IAC and may open up new possibilities for the development of more sensitive and specific biomarkers for this group of IgG4-RD. Larger prospective cohorts are needed to verify the feasibility of using the presence of dominant IgG4⁺ clones in the peripheral blood IgG repertoire as a diagnostic marker for IgG4-RD.
REFERENCES


SUPPLEMENTARY FIGURES AND TABLES

**Figure S1** IgG4+ clones are dominant in blood of IgG4-associated cholangitis (IAC), but not in healthy (HC) or disease (DC) controls. (A) The contribution of individual clones to the total BCR repertoire in all individuals (including IgA+, IgD+, IgG+ and IgM+ clones). Scatterplot showing clonal abundance as percentage of the IgG+ BCR repertoire (each dot represents an individual clone). IgG4+ clones are marked in red. (B) The number of dominant clones (clonal frequency >0.5% of the total BCR repertoire) found in IAC, HC and DC.

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<td>CAHGGWLYDYW</td>
<td>4</td>
<td>10.0%</td>
<td>9.3% (72%)</td>
</tr>
<tr>
<td></td>
<td>1-18</td>
<td>CARDPYDGYSDGYSDYW</td>
<td>6</td>
<td>9.9%</td>
<td>8.2% (62%)</td>
</tr>
<tr>
<td></td>
<td>3-23</td>
<td>CAKELYYYFGMDVW</td>
<td>6</td>
<td>1.5%</td>
<td>7.4% (50%)</td>
</tr>
<tr>
<td></td>
<td>4-30-4</td>
<td>CARAPSSGVYLDYW</td>
<td>4</td>
<td>1.1%</td>
<td>6.5% (56%)</td>
</tr>
</tbody>
</table>

Table S1 The CDR3 amino acid sequence of dominant IgG4+ BCR clones shows no consistent homology. Characteristics are summarized of all individual IgG4+ dominant clones, including IGHH and IGHL usage, CDR3 amino acid sequence, the frequency of the clone as the percentage of the total IgG+ BCR repertoire, as well as the percentage of nucleotides mutated in the V-region and the percentage of mutation marked as non-silent mutations. The relatively high percentage of mutations and the fact that the majority of these mutations result in amino acid changes support the notion that these clones have undergone affinity maturation.
Figure S2 The IGHV and IGHJ usage of the full BCR repertoire is similar in IAC patients, disease controls and healthy controls. Bar chart showing the use of individual IGHV genes (A) and IGHJ genes (B) as percentage of all BCR clones.

Figure S3 IgG4+ clones are enriched in inflamed papilla tissue. To investigate the possibility that the observed overlap of dominant IgG4+ clones was due to contamination from peripheral blood, we re-analyzed the repertoires in tissue and blood. If contamination would explain the overlap between peripheral blood and tissue, the most frequent clones should be similar in both compartments. To this end, in both tissue and peripheral blood (IAC4 and IACS), we took the 25 most dominant clones from peripheral blood and subsequently determined their frequency within the tissue sample and vice versa (top 25 clones listed in Figure 3A-B). We observed that although some of the top 25 clones in blood could also be found in tissue, a substantial number of these were not among the most dominant clones in tissue or could not be retrieved at all. When dividing these clones in IgG4+ and IgG4-, we observed that IgG4+ clones are among the highest ranked clones, while clones with another isotype than IgG4 were only found as low-ranked clones or not found at all (Figure C, Mann-Whitney U test p < 0.0001 for IAC4 / IACS combined, p = 0.0003 in separate anal-
Figure S4 Graphical representation of the experimental procedures workflow. Samples were collected from peripheral blood or tissue and mRNA was isolated and cDNA was synthesized for downstream application. A linear amplification was performed, using a primer set covering all functional V_{heavy} genes. This product was then used either for the determination of the total BCR repertoire (V-CDR3-J amplification) or for the subtyping of individual clones (V-CDR3-C amplification). For the former, a PCR using primerB as a forward primer and a generic primer specific for all functional J_{heavy} genes containing the primerA as reverse primer was performed. For the latter, the Ig isotypes were determined using primerB as a forward primer, and primers specific for the IgA, IgD, IgM and IgG isotype as reverse primers. Sequencing was performed on both pools of sequences (both V-CDR3-J and V-CDR3-C) according to the manual for 454 amplicon sequencing on a genome sequencer FLX (using primerA and primeB sequences). Using custom-made bioinformatics algorithms, the frequencies of individual clones were determined based on their unique VDJ rearrangement and CDR3 sequence and matched with their isotype and subclass characteristics.
| IAC1 | 12.00 | 7.18 | 0.17 |
| IAC2 | 11.80 | 11.50 | 1.63 |
| IAC3 | 14.70 | 11.20 | 1.31 |
| IAC4 | 4.81 | 14.70 | 0.33 |
| IAC5 | 3.45 | 11.13 | 0.31 |
| IAC6 | 15.90 | 9.96 | 1.60 |
| DC1 | 13.47 | 16.90 | 0.21 |
| DC2 | 20.23 | 6.58 | 0.03 |
| DC3 | 0.07 | 9.64 | 0.01 |
| DC4 | 13.50 | 9.96 | 1.36 |

**Table S2**

A color code was used to mark the observations that could be subjective (light green) or are strongly suggestive (light orange) of any of the criteria of gut symptoms, and the identified gut symptoms were addressed in the text. The number of NLP-positive plasma cells was assessed by counting the maximal number of these cells per high power field (HPF).