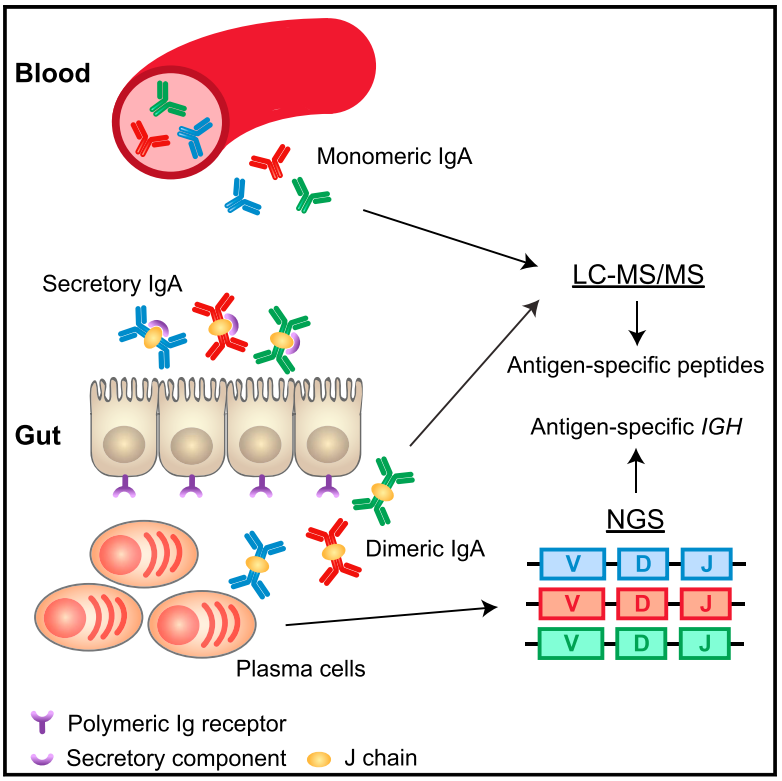
Article

Cell Reports

Strong Clonal Relatedness between Serum and Gut IgA despite Different Plasma Cell Origins

Graphical Abstract



Highlights

- Proteomics can be applied to map V-gene preferences of specific antibody responses
- The same CDR-H3 sequences are found in antigen-specific serum and gut IgA
- Celiac-disease-related serum IgA is not primarily derived from gut plasma cells
- Serum IgG shows a low degree of clonal relatedness to gut plasma cells

Authors

Rasmus Iversen, Omri Snir, Maria Stensland, ..., Knut E.A. Lundin, Gustavo A. de Souza, Ludvig M. Sollid

Correspondence

l.m.sollid@medisin.uio.no

In Brief

The relationship between mucosal antibody responses and antibodies in blood is not clearly understood. Iversen et al. use proteomics to characterize antibodies in serum and gut biopsy specimens obtained from celiac disease patients. Serum and gut IgA are derived from the same B cell clones but produced by different plasma cells.

Accession Numbers

PXD007047

Iversen et al., 2017, Cell Reports *20*, 2357–2367
September 5, 2017
http://dx.doi.org/10.1016/j.celrep.2017.08.036

Cell Reports
Article

Strong Clonal Relatedness between Serum and Gut IgA despite Different Plasma Cell Origins

Rasmus Iversen,[1] Omri Snir,[1] Maria Stensland,[1,2] José E. Kroll,[3] Øyvind Steinsbø,[1] Ilma R. Korponay-Szabó,[4] Knut E.A. Lundin,[1,5,6] Gustavo A. de Souza,[1,2,3] and Ludvig M. Sollid[1,6,7,*]

[1]Centre for Immune Regulation and Department of Immunology, University of Oslo and Oslo University Hospital-Rikshospitalet, NO-0372 Oslo, Norway
[2]Proteomics Core Facility, Oslo University Hospital-Rikshospitalet, NO-0372 Oslo, Norway
[3]Brain Institute, Federal University of Rio Grande do Norte, RN 59056-450 Natal, Brazil
[4]Celiac Disease Center, Heim Pál Children's Hospital, HU-1089 Budapest, Hungary
[5]Department of Gastroenterology, Oslo University Hospital-Rikshospitalet, NO-0372 Oslo, Norway
[6]KG Jebsen Coeliac Disease Research Centre, University of Oslo, NO-0372 Oslo, Norway
[7]Lead Contact
*Correspondence: l.m.sollid@medisin.uio.no
http://dx.doi.org/10.1016/j.celrep.2017.08.036

SUMMARY

Mucosal antigens induce generation of lamina propria plasma cells (PCs) that secrete dimeric immunoglobulin A (IgA) destined for transport across the epithelium. In addition, blood contains monomeric IgA. To study the relationship between mucosal and systemic antibody responses, we took advantage of celiac disease patient samples for isolation of gut PCs as well as serum IgA and IgG reactive with a gluten-derived peptide or the autoantigen transglutaminase 2. Proteomic analysis of serum IgA revealed antigen-specific V-gene preferences, which matched those found in gut PCs. Further, gut PC CDR-H3 sequences were abundant in serum IgA but also detectable in serum IgG. Our data indicate that the same B cell clones that give rise to gut PCs also contribute to the serum antibody pool. However, serum IgA antibodies had a molecular composition distinct from that of IgA antibodies secreted in the gut, suggesting that individual B cell clones give rise to different PC populations.

INTRODUCTION

Immunoglobulin A (IgA) is the antibody isotype that is produced in greatest quantities in the body. The majority of IgA molecules are secreted from the vast population of plasma cells (PCs) lining the entire gastrointestinal tract. These cells produce dimeric IgA in which two IgA monomers are covalently linked by the joining (J) chain. Dimeric IgA secreted in the lamina propria is transported across the epithelium via the polymeric immunoglobulin receptor and released into the gut lumen together with a fragment of the receptor known as the secretory component. These secretory IgA antibodies bind and regulate the intestinal microbiota and protect the epithelial barrier from pathogens (Macpherson et al., 2008).

Differentiation of B cells into IgA-producing PCs may be the result of either T cell-dependent or T cell-independent activation (Pabst et al., 2016; Spencer and Sollid, 2016). Studies carried out in mice suggest that much of the IgA generated against gut commensal bacteria does not rely on classical T-B collaboration and that such antibodies often bind multiple bacterial strains with low affinity (Macpherson et al., 2000; Bergqvist et al., 2006; Slack et al., 2012). However, T cell-dependent IgA responses resulting in high-affinity, antigen-specific antibodies can be induced in mice by oral immunization (Lycke et al., 1987). Importantly, in humans, the majority of antibody-producing cells in the gut appear to be specific (Benckert et al., 2011). A prominent example of a human condition characterized by sizeable populations of antigen-specific gut PCs is celiac disease. This gluten-sensitive enteropathy is associated with marked changes in the tissue architecture of the upper small bowel and infiltration of immune cells, including large numbers of PCs, in the mucosa (Stamnaes and Sollid, 2015). The immune reactions that lead to formation of the celiac disease lesion are orchestrated by $CD4^+$ T cells, which recognize certain gluten peptides in the context of disease-associated HLA molecules. However, gluten peptides only become T cell antigens after modification by the enzyme transglutaminase 2 (TG2) through a process known as deamidation, whereby glutamine residues are converted to glutamic acid (Molberg et al., 1998; van de Wal et al., 1998). $CD4^+$ T cells recognizing deamidated gluten can provide activation signals not only to cognate, gluten-specific B cells but also to self-reactive, TG2-specific B cells, which present gluten peptides on their surface upon internalization of TG2-gluten-B-cell receptor (BCR) complexes (Iversen et al., 2015; Stamnaes et al., 2015; Stamnaes and Sollid, 2015). Hence, both gluten-specific and TG2-specific PCs can readily be detected in intestinal biopsy specimens obtained from celiac patients (Marzari et al., 2001; Di Niro et al., 2012; Steinsbø et al., 2014). Interestingly, the latter specificity appears to dominate the response, and it was shown that, on average, 10% of IgA-producing cells in the lesion of untreated celiac disease patients are TG2 reactive (Di Niro et al., 2012).

In addition to the IgA antibodies that are transported across epithelial barriers, the body also contains a pool of serum IgA.

Unlike the IgA molecules that are secreted at mucosal surfaces, serum IgA is monomeric rather than dimeric and believed to be produced primarily by bone marrow PCs, although different production sites are possible (Kutteh et al., 1982). Serum IgA and IgG antibodies against deamidated gluten and TG2 are also present in celiac disease, and because of their high disease specificity, such antibodies have become important diagnostic markers (Leffler and Schuppan, 2010). How these antibodies relate to gut PCs of the same specificities is not known, although it has been suggested that TG2-specific serum IgA ends up in the circulation as a result of spillover from the gut production (Marzari et al., 2001; Sollid and Lundin, 2009).

Analysis of the V-region repertoire in lymphocyte populations comprising hundreds or thousands of clones has become possible with the development of next-generation sequencing (NGS) methods. Further, combining sequencing data with analysis of isolated antibodies by liquid chromatography-tandem mass spectrometry (LC-MS/MS) has proven to be a powerful tool to establish connections between individual B cell clones and secreted antibodies (Cheung et al., 2012; Lavinder et al., 2014). Here, we apply the approach to investigate the relationship between gut PCs and antibodies in serum or biopsy secretions using TG2 or deamidated gluten peptide (DGP) to isolate specific cells and antibodies from celiac patient samples. In addition, LC-MS/MS can be applied independently to map preferences for individual V-gene segments in specific antibody pools (Al Kindi et al., 2016). Hence, by using purified celiac antibodies, we demonstrate that important characteristics of polyclonal antibody responses can be obtained without the need to interrogate PC populations.

RESULTS

Antibody Responses against Celiac Antigens Display Isotype-Dependent Differences

In agreement with the well-established use of celiac antibodies in diagnostic tests, we found serum IgA and IgG antibodies targeting TG2 or DGP (peptide PLQPEQPFP) to be good markers for untreated celiac disease and readily detectable by ELISA (Figure 1A). For the IgA antibodies, it was evident that the reactivity against TG2 was higher than the reactivity against DGP, whereas this was not the case for the IgG antibodies. When comparing the IgA and IgG responses against each of the antigens, we found a much stronger correlation for the anti-DGP antibodies than for the anti-TG2 antibodies (Figure 1B). This difference might relate to the different nature of the antigens, as TG2 is a tissue-bound protein autoantigen, and DGP is a soluble peptide. Hence, DGP may distribute to different lymphoid structures in a concentration-dependent manner, whereas TG2 availability will be dictated by the expression levels in individual tissues.

In agreement with previous observations, ELISPOT detection of antibody-secreting cells in duodenal biopsy specimens from celiac disease patients revealed that the number of TG2-specific IgA PCs was higher than the number of DGP-specific cells (Figure 1C) (Steinsbø et al., 2014). There was no correlation between the numbers of antigen-specific cells and the corresponding serum IgA levels (Figure 1D), as recently reported for the anti-TG2 response (Di Niro et al., 2016). However, the ratio between the numbers of TG2- and DGP-specific cells reflected the ratio between the levels of the IgA antibodies in serum. That is, for each patient, the observed dominance of anti-TG2 over anti-DGP IgA was equal in serum and gut samples. These findings suggest that the serum IgA responses are tightly connected to PC generation in the gut, although the gut PCs might not be the direct source of serum antibodies. As expected, the total number of IgG-secreting cells in the gut was much lower than the number of IgA-secreting cells (Brandtzaeg and Johansen, 2005), and only a few cases of antigen-specific IgG cells could be detected (Figure 1C). Thus, it appears that the cells secreting celiac IgG antibodies are not primarily generated in the gut.

Serum Anti-TG2 Antibodies Primarily Target Epitopes Known from Gut PCs

The antibody response against TG2 was previously characterized by generation of monoclonal antibodies (mAbs) from TG2-specific gut PCs of celiac disease patients (Di Niro et al., 2012). The majority of these mAbs could be placed into three main epitope groups (epitopes 1–3), which reflected antibody *IGHV* gene segment usage (Iversen et al., 2013). Notably, epitope 1, which is recognized by antibodies using *IGHV5–51*, was targeted by 30 out of 57 mAbs. Epitope mapping studies have revealed that the epitopes are partly overlapping and located in the N-terminal part of the TG2 protein (Figure S2A) (Iversen et al., 2014; Chen et al., 2015). In order to test whether the N-terminal domain of TG2 is also important for binding of serum antibodies in celiac disease, we purified antibodies from patient serum samples (Figure S1A–S1C) and assessed the IgA reactivity against TG2 variants with either the N-terminal domain replaced by the N-terminal domain of TG3 or the two C-terminal domains missing (Figure 2A). Replacement of the N-terminal domain reduced the reactivity of patient serum IgA dramatically, whereas the two C-terminal domains could be removed without great loss of reactivity. Thus, as previously found for anti-TG2 mAbs generated from gut PCs, IgA antibodies in serum primarily target epitopes in the N-terminal part of TG2.

To further assess the overlap between epitopes targeted by serum antibodies and mAbs generated from gut PCs, we produced three typical mAbs targeting epitope 1, 2, or 3 by recombinant expression in 293 cells and assessed their ability to outcompete purified serum IgA in binding to TG2 (Figure 2B). Importantly, the mAbs showed a similar degree of competition between purified serum IgA and IgA secreted from PCs in gut biopsy specimens obtained from celiac disease patients, indicating that the distribution of reactivity among individual TG2 epitopes is the same for serum IgA and gut PCs (Figure 2B).

Whole serum samples rather than purified antibodies are typically used for studying TG2 reactivity. However, the use of whole serum samples results in underestimation of the contribution of epitope 1 specificity because of the serum content of fibronectin, which binds to TG2 with high affinity and competes with epitope-1-targeting antibodies for binding (Figures S1A–S2C) (Iversen et al., 2013; Cardoso et al., 2015). Although the epitope 1 reactivity was thus partly masked, we used whole serum samples to compare epitope targeting between adult celiac disease

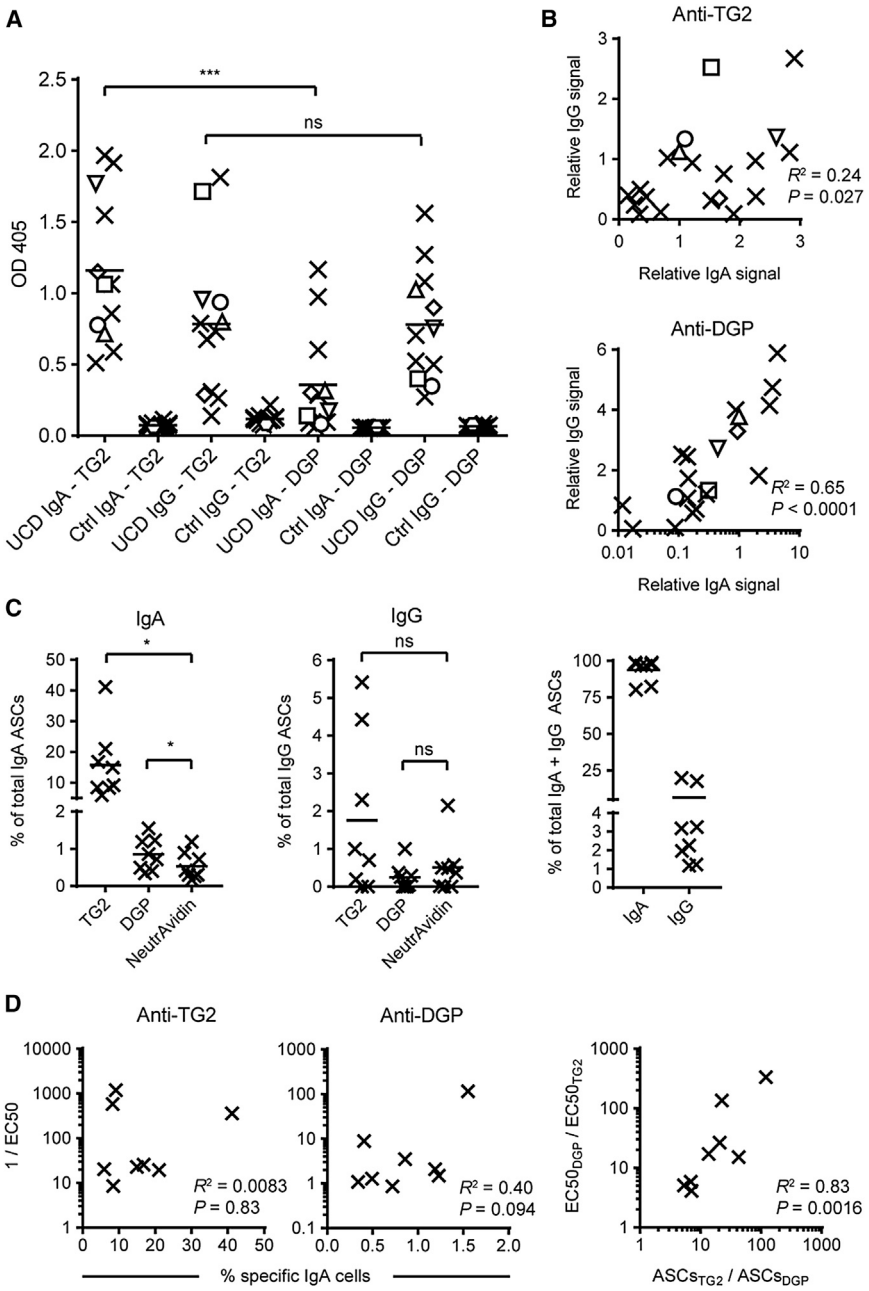


Figure 1. Detection of Antigen-Specific Serum Antibodies and Gut PCs

(A) Serum antibody reactivity against TG2 or DGP in patients with untreated celiac disease (UCD, n = 12) or control subjects (n = 12) as determined by ELISA. Sera were diluted 1:200 and added to biotinylated TG2 or biotinylated DGP (biotin-GSGSGS-PLQPEQPFP, harboring an immunodominant gliadin epitope) (Schwertz et al., 2004; Steinsbø et al., 2014) immobilized on streptavidin followed by detection of bound IgA or IgG using isotype-specific goat anti-human antibodies. The dynamic range of the assay was comparable for detection of the different antibodies. Open symbols indicate subjects from whom serum antibodies were purified for further characterization. The same symbols are used throughout this study.

(B) Correlation between the levels of serum IgA and IgG against each of the two antigens in celiac disease patients (n = 20). Reactivity was measured by ELISA, and the signals for each antigen are given relative to the IgA signal obtained with a reference serum sample (UCD1283).

(C) ELISPOT detection of total or antigen-specific antibody-secreting cells (ASCs) producing IgA or IgG in gut biopsy specimens obtained from celiac disease patients (n = 8). Cell suspensions were added to coated NeutrAvidin alone or NeutrAvidin associated with biotinylated TG2 or DGP as indicated. In the right panel, antigen specificity was not considered, and only the numbers of total IgA- or IgG-secreting cells were counted.

(D) Correlations between the number of gut ASCs making antigen-specific IgA and the level of antigen-specific IgA in serum. EC_{50} values represent the serum dilution, which gives half-maximal response in ELISA. The right panel shows the ratio between TG2- and DGP-specific gut ASCs (x axis) and the corresponding ratio between serum antibody levels (y axis). Horizontal lines indicate means, and differences between groups were analyzed using repeated-measures one-way ANOVA. *p < 0.05; ***p < 0.001.

patients, who are likely to have had an ongoing immune response for extended periods of time, and children with recently developed disease. The degree of competition between anti-TG2 mAbs and serum IgA was comparable in the two patient groups (Figure 2C). Hence, the same main epitopes are targeted very early in disease development and at later stages, in agreement with previous observations (Simon-Vecsei et al., 2012).

Serum Antibodies against Celiac Antigens Use the Same V-Gene Segments as Gut PCs

To further compare antigen-specific serum antibodies with the gut immune response, we purified the TG2-reactive fraction from either serum IgA or IgA secreted from gut biopsy PCs and analyzed TG2-binding and non-TG2-binding trypsinized fractions by LC-MS/MS. Peptides derived from antibody V-gene segments were identified using a database containing germline immunoglobulin sequences supplemented with peptides identified by de novo sequencing (Figure S3). De-novo-sequenced peptides were included in the database if their amino acid sequence contained one mismatch compared to any germline sequence. By allowing identification of such non-germline sequences, we were able to account for somatic hypermutation, leading to a substantial increase in the number of peptides that could be assigned to each V-gene segment (Table S1). Relative abundances of individual V-gene segments were then determined by summing up the peak intensities from the assigned peptides, allowing comparison of TG2-specific and non-TG2-specific

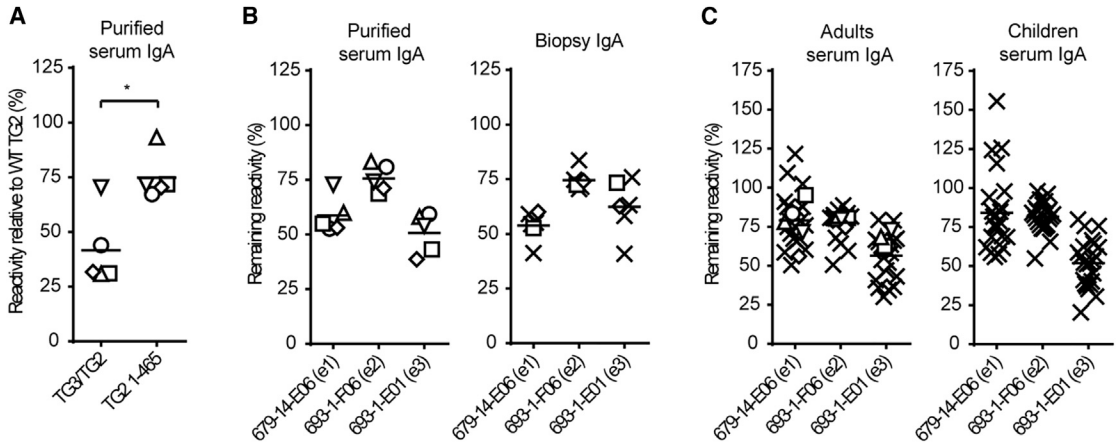


Figure 2. Epitope Targeting by Anti-TG2 Serum IgA

(A) Reactivity of total IgA purified from sera of celiac disease patients (n = 5) against different transglutaminase variants assessed by ELISA. In the TG3/TG2 protein, the N-terminal domain of TG2 was replaced with the N-terminal domain of TG3, whereas TG2 1–465 indicates a truncated TG2 variant lacking the two C-terminal domains. Reactivity levels are given relative to the signals obtained with wild-type (WT) TG2 and were compared using a paired t test. *$p < 0.05$.

(B) Ability of individual mAbs targeting epitope 1, 2, or 3 (e1–3) to outcompete binding of purified total serum IgA to TG2. Supernatants from cultured gut biopsy specimens obtained from celiac disease patients (n = 6) were included for comparison.

(C) Ability of the indicated mAbs to outcompete TG2-binding of IgA in whole sera of adult patients (n = 23) or children (n = 23) in whom celiac disease recently developed. Notably, the epitope 1 mAb shows reduced competitive capacity when using whole sera instead of purified serum IgA (compare B and C). Horizontal lines indicate means.

See also Figures S1 and S2.

antibodies. Ten V-gene segments were overrepresented in the TG2-binding IgA fraction in all five patients analyzed, and the same preferences were observed in serum and in biopsy secretions (Figure 3A; Table S2). Importantly, heavy and light chain V-gene segments found to be overrepresented among TG2-binding antibodies were in agreement with previous findings based on sequencing of the antibody genes in TG2-specific gut PCs (Di Niro et al., 2012; Snir et al., 2015; Roy et al., 2017), and the distribution of LC-MS intensity between different *IGHV* families reflected the distribution of sequences obtained from gut PCs of the same patient (Figure 3B). Hence, antigen-dependent V-gene preferences can be mapped efficiently by LC-MS/MS. *IGHV5-51* was the only *IGHV* segment that was consistently overrepresented among TG2-binding serum antibodies, reflecting a generally strong contribution of antibodies targeting epitope 1 to the anti-TG2 pool in celiac disease. To further confirm the connection between antigen-specific IgA in serum and gut PCs, we repeated the analysis using purified anti-DGP serum IgA. Also in this case, we identified an overrepresentation of V-gene segments previously found to be used by DGP-specific PCs, with the exception of *IGHV3-23*, which was not identified because of the lack of unique peptides mapping to this V-gene segment (Table S2) (Steinsbø et al., 2014). Collectively, these data show that both anti-TG2 and anti-DGP serum IgA antibodies are dominated by the same V-gene segments, which are preferentially used by the corresponding gut PC populations.

Sharing of CDR-H3 Sequences between Serum Antibodies and Gut PCs

In order to test whether antigen-specific serum antibodies are clonally related to gut PCs, we obtained CDR-H3 sequences from sorted TG2- or DGP-specific gut PCs and looked for matching amino acid sequences in affinity-purified antibody fractions from the same celiac disease patients using LC-MS/MS (Table 1; Figure S3). Antibody-derived peptide sequences matching CDR-H3 sequences of individual TG2-specific PCs could be detected both in serum IgA and in IgA secreted from gut biopsy PCs. Not all PC clonotypes were detected at the antibody level, but they were essentially the same sequences observed in serum and in biopsy secretions (Figure 4A; Table S3). Typically, most of the intensity was derived from a few prevalent clonotypes that often were represented by more than one peptide (Tables 1 and S3). Surprisingly, serum IgA and biopsy-derived IgA contained comparable levels of anti-TG2 CDR-H3 peptides (Figure 4B), suggesting that all gut PCs are also represented in the serum IgA pool. Further, our data imply that the entire anti-TG2 repertoire is contained within the small amount of gut tissue that was collected. In support of this notion, it was previously shown that individual biopsy specimens share a high number of clonotypes, indicating that relatively few clonally expanded founder cells make up the anti-TG2 repertoire in the gut (Di Niro et al., 2016). Anti-TG2 CDR-H3 peptides were clearly enriched in the TG2-binding fraction compared to the flow-through fraction collected during anti-TG2 affinity purification, confirming that the identified peptides indeed reflect TG2 specificity (Figure 4B). Likewise, enrichment of anti-DGP CDR-H3 peptides could be observed after affinity purification of anti-DGP serum IgA, although the low concentration of DGP-specific IgA in serum resulted in few detected peptides (Figure 4C; Table S4).

Serum IgG Exhibits Weaker Clonal Relatedness to Gut PCs than Serum IgA

Unlike purification of total serum IgA, purification of total serum IgG was associated with accumulation of low-affinity

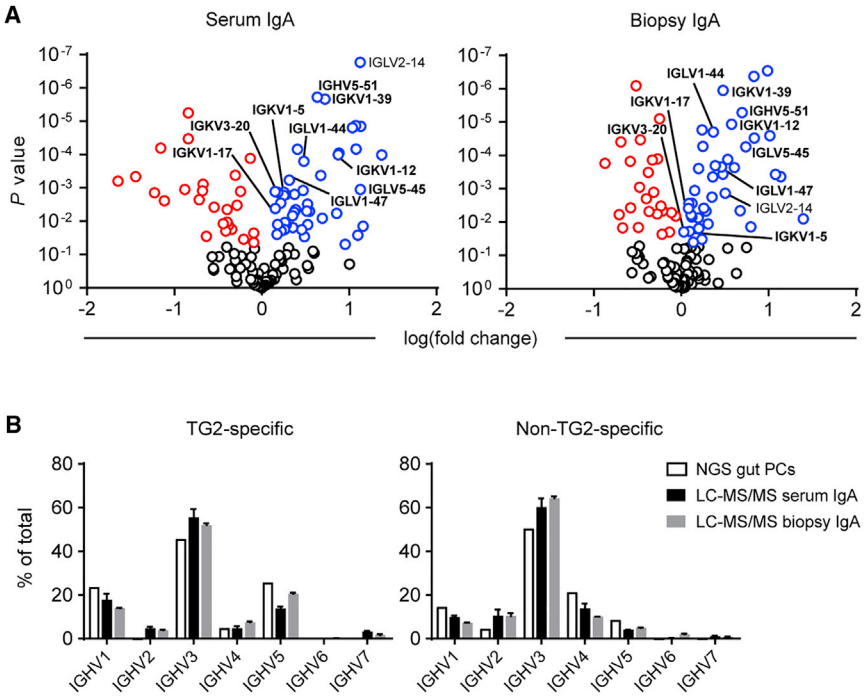


Figure 3. Bias in V-Gene Segments Used by Anti-TG2 IgA Antibodies

(A) Volcano plot showing differences in the use of V-gene segments between affinity-purified anti-TG2 IgA and non-TG2-specific IgA (flow-through fraction) obtained from serum or gut biopsies of a representative celiac disease patient (UCD1317). Functional V-gene segments were identified based on matching of MS/MS spectra to amino acid sequences obtained from http://www.imgt.org, resulting in detection of unique peptides for 80% of the database V-gene segments. The amounts of individual V-gene segments in each sample were quantified using MaxQuant software (Cox and Mann, 2008). V-gene segments significantly more abundant in the TG2-specific fraction are indicated with blue symbols, whereas V-gene segments of lower abundance are shown in red ($p < 0.05$). V-gene segments overrepresented among TG2-specific serum IgA in all five patients analyzed are indicated with their names, and names shown in bold indicate V-gene segments, which were previously found to be used by TG2-specific gut PCs (Di Niro et al., 2012; Snir et al., 2015; Roy et al., 2017).

(B) Distribution of antibodies and PCs among different *IGHV* families in UCD1317. The distribution of TG2-specific and non-TG2-specific gut PCs is based on the numbers of unique sequences obtained by NGS, whereas the distribution of affinity-purified anti-TG2 or flow-through IgA isolated from serum or gut biopsy specimens is based on *IGHV* peptide LC-MS intensities obtained as intensity-based absolute quantification (iBAQ) values in MaxQuant. Error bars represent SD based on triplicate LC-MS/MS runs. See also Figure S3 and Tables S1 and S2.

TG2-binding antibodies, which did not bind the antigen in whole serum and were not related to celiac disease (Figures S1A–S1C). Hence, the TG2-specific IgG fraction could not be isolated and analyzed after initial purification of total IgG in a way similar to that described above for the IgA antibodies. However, when TG2-binding antibodies were isolated from whole serum without initial separation into total IgA and IgG preparations, we were able to detect IgG-derived anti-TG2 CDR-H3 peptides in the fraction eluted from TG2 based on the sequence of constant-region amino acids following the CDR-H3 region (Figure 5A; Table S5). Thus, anti-TG2 CDR-H3 sequences obtained from gut PCs are represented among both serum IgA and serum IgG and are detectable after purification of TG2-binding molecules from whole serum samples. However, sequences shared with gut PCs seemed to be less frequent in anti-TG2 IgG than in anti-TG2 IgA (Figure 5B), and most of the detected anti-TG2 CDR-H3 peptides were different for the two antibody classes (Figure 5C). Thus, there exist serum IgA and IgG antibodies that are clonally related to gut PCs, but the connection is stronger for IgA than for IgG.

Serum and Gut Anti-TG2 IgA Display Different Molecular Compositions

To get an indication of the cellular origin of antigen-specific serum IgA in celiac disease, we assessed the distribution between monomeric and dimeric forms in TG2-specific IgA purified from celiac patient serum or gut biopsy secretions. The TG2-specific IgA fractions appeared similar to the total IgA preparations, with antibodies purified from serum being mostly monomeric and antibodies purified from biopsy specimens being dimeric (Figure 6A).

The relative abundances of individual proteins in a sample can be approximated from the peptide intensities of extracted-ion chromatograms (Figures 3B and S4A). Thus, to obtain further insight into the molecular composition of different IgA fractions, we quantified the levels of J chain and the distribution of IgA molecules between the IgA1 and IgA2 subclasses (Figure 6B). In agreement with the observed distributions between monomers and dimers, gut biopsy IgA contained higher levels of J chain than serum IgA. However, TG2-specific serum IgA seemed to be associated with more J chain than the non-TG2-specific serum fraction. As expected, both serum IgA and IgA secreted from duodenal biopsy specimens consisted mostly of IgA1 (Figure 6B) (Brandtzaeg and Johansen, 2005). Both in serum and in biopsy secretions, the TG2-specific fraction contained even lower levels of IgA2 than the non-TG2-specific fraction, but while IgA2 still made up an estimated 5%–10% of TG2-specific IgA in the gut, this subclass was virtually absent in the TG2-specific serum IgA fraction. Collectively, these data demonstrate that the TG2-specific IgA antibodies present in serum and gut biopsy secretions have different molecular compositions, suggesting that the majority of anti-TG2 serum antibodies are not produced by gut PCs, although some J-chain-containing IgA dimers generated in the gut might end up in the circulation, either by escaping transport across the epithelium or via retrotransport from the gut lumen mediated by binding of secretory IgA to CD71 on the apical surface of epithelial cells (Lebreton et al., 2012). In support of the latter mechanism, we did detect low

Table 1. Detection of CDR-H3 Sequences from Antigen-Specific Gut PCs in Affinity-Purified Serum or Gut IgA by LC-MS/MS

	NGS: Gut PCs				LC-MS/MS: Serum or Gut IgA[a]		
	No. of PCs	No. of Sequences	Unique CDR-H3 Sequences	Clonotypes[b]	CDR-H3 Peptides Detected	Clonotypes	Top Clonotype Intensity (%)[c]
Anti-TG2							
UCD1279	1,100	1,217	466	364	27/20	26/20	15/14
UCD1283	2,500	2,561	573	340	83	53	18
UCD1287	2,500	7,137	1,324	741	99	75	43
UCD1317	970	1,305	253	121	49/60	35/36	54/38
Anti-DGP							
UCD1283	1,300	274	140	134	7	4	73
UCD1287	550	753	164	132	5	3	67

LC-MS/MS, liquid chromatography-tandem mass spectrometry; NGS, next-generation sequencing; PCs, plasma cells.
See also Figure S3 and Tables S3 and S4.
[a]Numbers reflect CDR-H3 peptides identified in serum IgA. When two numbers are given, they indicate peptides identified in serum/biopsy supernatant.
[b]Sequences assigned to the same clonotype use the same *IGHV* and *IGHJ* gene segments and have ≥85% sequence identity in the CDR-H3 region (Tipton et al., 2015).
[c]The LC-MS intensity of the highest-intensity clonotype is given relative to the total LC-MS intensity of all identified CDR-H3 peptides in the sample.

amounts of the polymeric immunoglobulin receptor among serum antibodies eluted from TG2 by LC-MS/MS (Figure S4B). Notably, the IgA response against TG2 is skewed toward production of IgA1, and the PCs responsible for secretion of serum anti-TG2 IgA seem to be recruited almost exclusively from IgA1-expressing cells. Likewise, we observed a biased distribution of anti-TG2 serum IgG in favor of the IgG1 subclass (Figure S4C).

DISCUSSION

The relationship between mucosal and systemic immune responses is not well understood, and the origin of circulating antibodies against gut antigens has not previously been investigated thoroughly. In this study, we have characterized the antibody response against two antigens, TG2 and DGP, in celiac disease patients. Both responses are dependent on a gluten-containing diet and are associated with accumulation of antigen-specific PCs in the gut lesion. Strikingly, serum IgA antibodies targeted the same epitopes and used the same preferred V-gene segments as the gut PCs. Moreover, detection of antigen-specific CDR-H3 regions showed that serum antibodies and gut PCs are clonally related. Analogous to what has recently been observed for vaccine-elicited anti-flu antibodies (Lee et al., 2016) and anti-desmoglein autoantibodies (Chen et al., 2017), we found that relatively few clonotypes contributed most of the serum LC-MS intensity that could be ascribed to anti-TG2 CDR-H3 peptides. These clonotypes also represented some of the most abundant gut PCs, as judged by the number of sequenced transcripts, thus underscoring the tight connection between serum and gut IgA.

Despite the strong clonal relatedness between serum antibodies and gut PCs, we found anti-TG2 serum IgA to be mostly monomeric, whereas anti-TG2 antibodies secreted by gut PCs were J-chain-associated dimers and contained a larger fraction of the IgA2 subclass. Similar molecular differences have also been described for gluten-specific serum and gut IgA in celiac disease (Volta et al., 1990). Together with the lack of correlation between gut PC numbers and serum antibody levels, these findings suggest that gut PCs are not the direct source of the specific serum IgA antibodies.

Independent regulation of IgA responses in gut and serum has previously been observed in CD28-deficient mice (Gärdby et al., 2003). Our data are in agreement with this concept but at the same time strongly suggest that serum antibodies and gut PCs originate from the same B cell clones. In line with this model, IgA-expressing cells derived from mucosal immune responses are present in human blood and in bone marrow during steady-state conditions (Mei et al., 2009). Further, it was recently shown that antigen-specific, clonally related PCs are present in the bone marrow and gut after oral immunization of mice (Bemark et al., 2016; Lemke et al., 2016). Hence, together with previous studies, the data presented here contend that B cells activated in the gut immune system give rise to PCs that reside in the lamina propria and produce dimeric IgA for transport across the epithelium as well as an equivalent population of PCs that migrate to the bone marrow and secrete monomeric IgA into the circulation.

We observed that CDR-H3 sequences obtained from TG2-specific gut PCs were represented both in the IgA fraction and in the IgG fraction of TG2-binding serum antibodies. In agreement with this finding, sharing of V-region sequences between TG2-specific gut PCs and IgG memory cells was previously demonstrated in celiac disease (Snir et al., 2015). Additionally, clonally related IgA and IgG PCs could be detected in orally immunized mice (Bemark et al., 2016). Thus, single B cells can give rise to both IgA- and IgG-expressing cells. However, compared to IgA antibodies, anti-TG2 IgG displayed a lower frequency of CDR-H3 sequences that were shared with gut PCs. One explanation for this observation could be that IgA- and IgG-expressing cells, despite being clonally related, acquire different V-region mutations and therefore tend to differ in their CDR-H3 sequences, as recently shown for B cell clonotypes

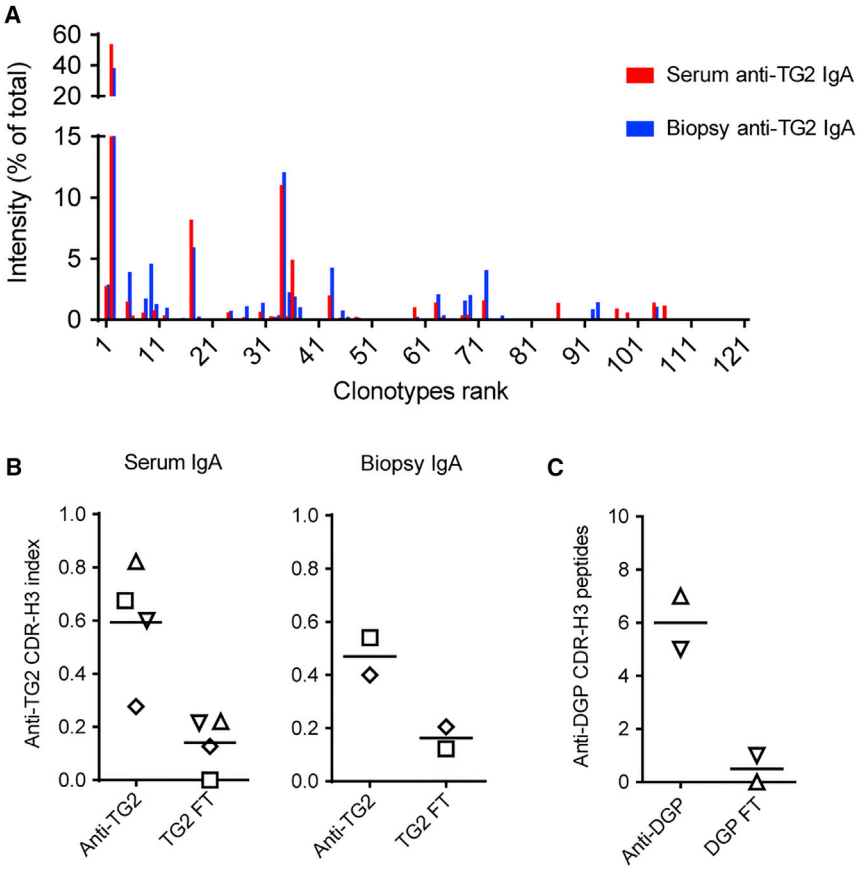


Figure 4. Sharing of CDR-H3 Sequences between Purified IgA Fractions and Gut PCs

(A) Matching between peptide sequences obtained from affinity-purified anti-TG2 IgA and CDR-H3 sequences of TG2-specific IgA gut PCs. Antibodies were isolated from serum or gut biopsy specimens, and peptides containing all or part of a CDR-H3 region were identified by LC-MS/MS based on matching to a database of CDR-H3 sequences obtained from TG2-specific gut PCs of the same celiac disease patient (UCD1317) by NGS. Sequences from PCs were grouped into clonotypes, which were ranked from 1 to 121 according to decreasing numbers of sequenced transcripts (i.e., clonotype 1 was associated with the highest number of immunoglobulin mRNA molecules). Bars show the summed LC-MS intensities of peptides matching individual clonotypes as a percentage of the total anti-TG2 CDR-H3 peptide intensity of each sample. Hence, each bar represents detection of a particular clonotype at the antibody level. The data are representative of four analyzed patients (summarized in Table 1).

(B) Quantification of the amount of antibodies containing CDR-H3 regions matching sequences of TG2-specific gut PCs in affinity-purified anti-TG2 antibodies or the TG2 flow-through (FT) fraction of celiac disease patient samples. TG2-binding and non-TG2-binding fractions were isolated from purified total IgA of sera or gut biopsy specimens. The normalized level of anti-TG2 CDR-H3 regions in each sample (the anti-TG2 CDR-H3 index) was determined by dividing the number of identified peptides containing TG2-specific CDR-H3 sequences with the number of all CDR-H3-containing peptides that could be identified by de novo peptide sequencing.

(C) Detection of peptides matching CDR-H3 sequences obtained from DGP-specific gut PCs in anti-DGP or DGP FT IgA isolated from purified total serum IgA of two celiac disease patients. Because of the low concentration of DGP-specific IgA, CDR-H3 indices could not be calculated, as was done for the TG2-specific antibodies. Horizontal lines indicate means.

See also Figure S3 and Tables S3 and S4.

comprising cells of different immunoglobulin classes (Horns et al., 2016). Alternatively, many of the TG2-specific IgG PCs are generated from a separate population of B cell clones, possibly at other anatomical locations. Such a distinction might explain why anti-TG2 IgG is generally a poorer diagnostic marker than anti-TG2 IgA (Leffler and Schuppan, 2010).

Immune reactions involving gluten might occur at sites distant from the gut, as exemplified by the conditions dermatitis herpetiformis and gluten ataxia, which can be considered extraintestinal manifestations of celiac disease affecting the skin and brain, respectively. Relevant to these clinical observations, gluten-derived peptides can be detected in urine following gluten consumption, demonstrating that antigenic peptides are systemically distributed (Moreno et al., 2017). The observed tight connection between serum levels of anti-DGP IgA and IgG might reflect the presence of gluten peptides both in the gut and in the circulation, as mucosal immune responses primarily give rise to IgA production while systemic responses are dominated by IgG (Mei et al., 2009). Thus, the IgA response could be initiated in Peyer's patches or mesenteric lymph nodes, whereas the spleen might also contribute to IgG induction. The anti-TG2 response, on the other hand, did not show the same strong correlation between IgA and IgG levels. Although this response is also dependent on availability of gluten peptides, it should also reflect the TG2 expression and activity levels, which may vary between different tissues. Thus, if anti-TG2 IgA and IgG mainly originate from different B cell populations at different anatomical sites, the local levels of available, active TG2 enzyme might control the relative contribution of the two isotypes to the serum antibody pool.

In conclusion, by using a combination of NGS and LC-MS/MS, we have shown that there is a close relationship between PCs in the gut and serum IgA antibodies against two different antigens in celiac disease patients. Although the gut PCs do not seem to produce the antibodies present in serum, a high level of shared CDR-H3 sequences implies that serum IgA and gut PCs are clonally related. Gut PC CDR-H3 sequences could also be detected in serum IgG, indicating that B cell clones activated in the mucosal immune system contribute to the serum IgG pool. In addition, we demonstrate the use of LC-MS/MS alone to obtain characteristics of affinity-purified antibodies, including V-gene preferences and subclass distributions. This analysis can be useful for assessing the composition of antibody responses against various antigens and requires only a serum sample.

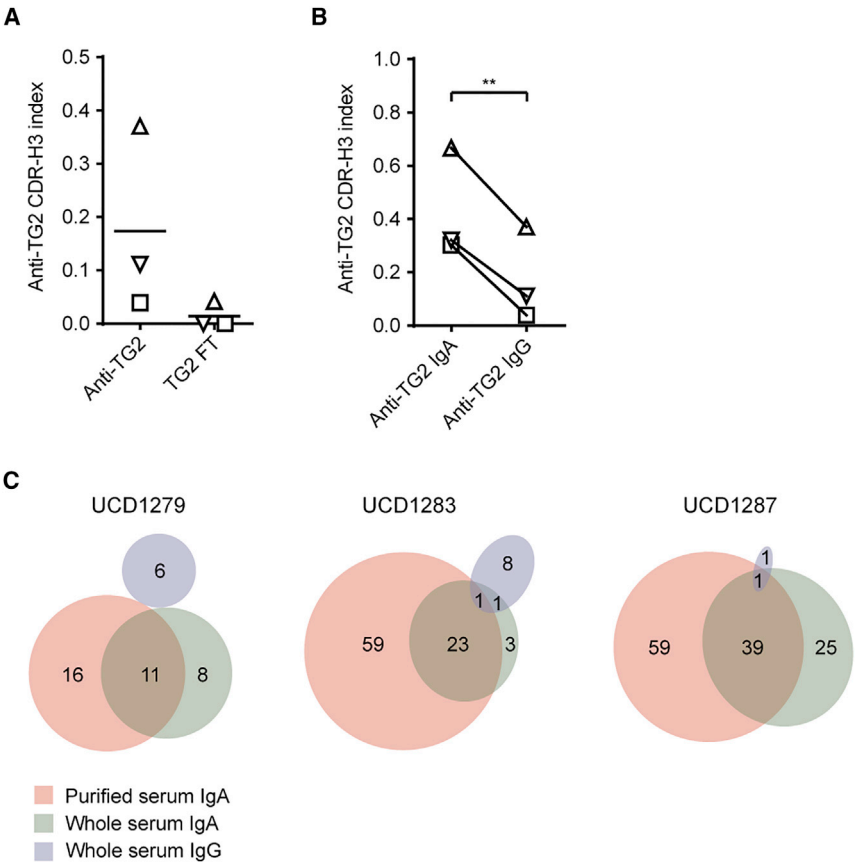


Figure 5. Detection of PC CDR-H3 Sequences in Anti-TG2 IgA and IgG Isolated from Whole Sera

(A) Quantification of the amount of antibodies with CDR-H3 regions matching sequences of TG2-specific gut PCs among affinity-purified anti-TG2 or TG2 flow-through (FT) IgG obtained from whole sera of three celiac disease patients. The anti-TG2 CDR-H3 levels are determined as CDR-H3 indices as shown in Figure 4B. Horizontal lines indicate means.

(B) Comparison of the level of anti-TG2 CDR-H3 peptides derived from IgA or IgG after isolation of TG2-binding antibodies from whole serum samples. Connecting lines indicate detection of IgA- and IgG-derived CDR-H3 peptides in the same sample. The anti-TG2 CDR-H3 levels were compared using a paired t test. $^{**}p < 0.01$.

(C) Venn diagrams showing overlap between anti-TG2 CDR-H3 peptides detected in affinity-purified anti-TG2 IgA or IgG obtained from whole serum or from purified total serum IgA of three celiac disease patients. Numbers indicate identified peptides containing CDR-H3 regions matching sequences obtained from TG2-specific gut PCs of the same patient. Area-proportional ellipses were generated using euler*APE* (Micallef and Rodgers, 2014).

See also Figure S1 and Table S5.

EXPERIMENTAL PROCEDURES

Human Samples

Blood and/or duodenal biopsy specimens were collected from 32 adult celiac disease patients and 12 control subjects who had given their informed consent. Ethical approval was given by the Regional Ethics Committee of South-Eastern Norway (project 2010/2472), and the diagnosis of celiac disease was given according to the recent guidelines of the British Society of Gastroenterology (Ludvigsson et al., 2014). In addition, 23 serum samples of young children (median age 2.8 years, range 1.9–5 years) with newly diagnosed celiac disease were included from the Hungarian participants in the PreventCD project (http://www.preventceliacdisease.com), a prospective FP6 European multicenter study following at-risk infants from celiac families after randomized gluten introduction at 4 or 6 months of age. Blood was collected upon informed consent from the parents 7 times until 3 years of age and then annually. Ethical approval was given by the ethics committees of all participating centers, including that of Heim Pál Children's Hospital (Budapest, Hungary). Unless otherwise stated, the analyzed samples were from adult celiac disease patients. Biopsy specimens (6–12 from each patient) were disrupted by incubation with 1 mg/mL collagenase (Sigma) in 2% (v/v) fetal calf serum (FCS)/PBS under constant rotation for 1 hr at 37°C. The digested sample was homogenized with a syringe and filtered through a 40-μm cell strainer. The obtained single-cell suspension was either used directly or cultured for 2–4 weeks in 10% (v/v) FCS/RPMI-1640 together with human intestinal fibroblasts as previously described (Steinsbø et al., 2014) prior to staining and fluorescence-activated cell sorting (FACS) of PCs. Supernatants from cultured biopsy specimens were collected for isolation of secreted antibodies.

Recombinant Proteins

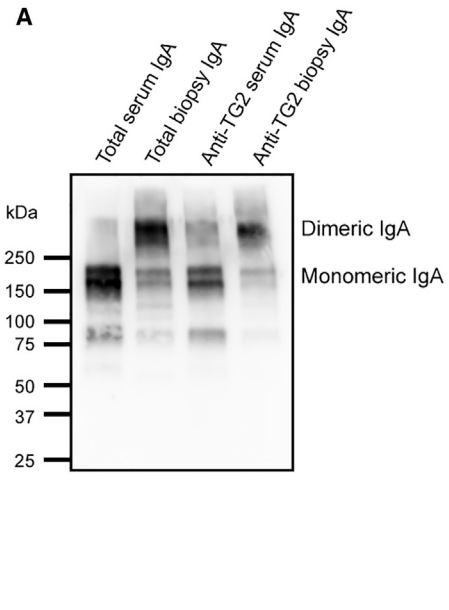
Recombinant human TG2 (Phadia) and TG3 (Zedira) produced in insect cells were used to assess antibody binding to the wild-type proteins by direct coating in ELISA as described in Supplemental Experimental Procedures. Proteins produced in *Escherichia coli* were used for comparison of wild-type TG2 and variants without the two C-terminal domains (residues 1–465) (Iversen et al., 2013) or with the N-terminal domain replaced by the N-terminal domain of TG3 (residues 1–136 from TG3 and residues 141–687 from TG2) (Cardoso et al., 2017). For attachment to streptavidin or NeutrAvidin, we used TG2 produced in *E. coli* with an N-terminal BirA biotinylation sequence (Hnida et al., 2016). Site-specific biotinylation was carried out with the BirA biotin-protein ligase (Avidity) according to the instructions provided by the manufacturer. Anti-TG2 mAbs targeting different epitopes were expressed as human IgG1 molecules in HEK293-F cells and purified on protein G (GE Healthcare) as previously described (Chen et al., 2015). The Jo-1 antigen was obtained from Phadia.

LC-MS/MS Sample Preparation

Antigen-specific antibodies were purified from serum or supernatants of cultured gut biopsies as described in Supplemental Experimental Procedures. The eluate and flow-through fractions were dried under vacuum followed by resuspension in 50 μL 50 mM NH_4HCO_3, 8 M urea. The antibodies were reduced with 10 mM DTT for 1 hr at 60°C followed by alkylation with 30 mM iodoacetamide for 1 hr at room temperature. The samples were then diluted with 150 μL 50 mM NH_4HCO_3 before overnight digestion with Sequencing Grade Trypsin (Promega) at 37°C, using a trypsin/antibody ratio of 1:50 (w/w). The reaction was quenched by addition of trifluoracetic acid to a final concentration of 1% (v/v). Peptides were cleaned prior to LC injection by the STAGE-TIP method (Rappsilber et al., 2003) using a C18 resin disk (3M Empore). Sample analyses were based on duplicate or triplicate runs using an Easy nLC1000 nano-LC system connected to a quadrupole Orbitrap (Q Exactive) mass spectrometer (ThermoElectron) equipped with a nanoelectrospray ion source (EasySpray/Thermo) as detailed in Supplemental Experimental Procedures.

Database Generation

In order to increase identification coverage of antibody V-gene segments, we used a de novo approach to include mutations in the reference germline database, which contained amino acid sequences of all human V-gene segments



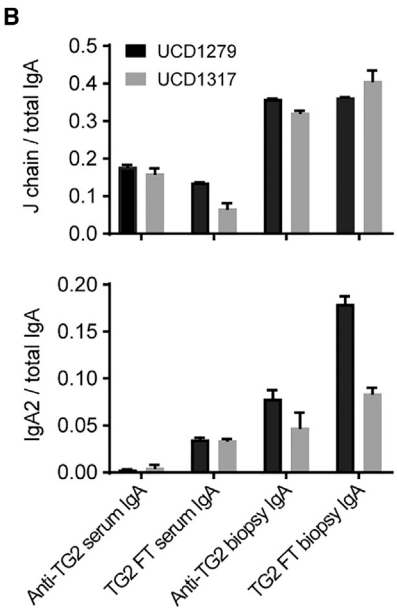


Figure 6. Composition of IgA Fractions Obtained from Sera or Gut Biopsy Specimens

(A) Western blot showing detection of monomeric and dimeric forms of purified total or TG2-specific IgA obtained from serum or gut biopsies of a celiac disease patient (UCD1317). The appearance of anti-TG2 serum IgA is representative of five analyzed patients.

(B) LC-MS/MS quantification of the amount of J chain and IgA2 subclass in the TG2-specific or TG2 flow-through (FT) fractions of total IgA in sera or gut biopsy specimens obtained from two celiac disease patients. Quantification is based on iBAQ values obtained with MaxQuant and is given relative to the summed iBAQ values of the IgA1 and IgA2 constant regions in each sample. The method overestimates the amount of J chain somewhat, as a dimeric IgA molecule consists of four IgA heavy chains and one J chain, giving a maximum J chain/IgA molar ratio of 0.25. Error bars represent SD based on duplicate (UCD1279) or triplicate (UCD1317) LC-MS/MS runs.

See also Figure S4.

obtained from the International ImMunoGeneTics Information System (IMGT) database (Lefranc et al., 1999). By using the germline database for proper sequence alignment, peptides carrying single amino acid substitutions were identified and added to the database as detailed in Supplemental Experimental Procedures.

Identification of CDR-H3 Peptides

For identification of CDR-H3 sequences obtained by NGS at the protein level, a database of complete tryptic peptides containing CDR-H3 sequences was built for each patient by adding the last six J-segment amino acids and the beginning of the constant region (ASTK for IgG and ASPTSPK for IgA) to the antibody sequences. Matching peptides from antigen-specific and non-specific antibody fractions were then identified by LC-MS/MS. In addition to CDR-H3 amino acids, all analyzed CDR-H3 peptides contained the conserved J-segment and constant-region sequences, and some peptides might therefore have been too long for detection. However, many CDR-H3 regions contained internal tryptic cleavage sites, resulting in shorter peptides harboring only part of the CDR-H3 region (see Tables S3, S4, and S5 for the complete list of identified CDR-H3 peptides). In order to normalize the number of database CDR-H3 peptides that could be detected in a sample, we used de novo sequencing to identify peptides harboring CDR-H3 regions of any specificity, characterized as amino acid sequences ending in ASTK (IgG) or ASPTSPK (IgA).

Sequencing of Antigen-Specific PCs

Antigen-specific IgA PCs in gut biopsy specimens were FACS sorted and their heavy chain V regions sequenced using Illumina MiSeq as detailed in Supplemental Experimental Procedures. *IGHV*, *IGHJ*, and CDR-H3 were assigned using IMGT, and clonal relatedness between sequences was established based on matching *IGHV* and *IGHJ* gene segments, identical CDR-H3 length, and $\geq$85% sequence identity throughout the CDR-H3 region (Tipton et al., 2015).

ELISPOT

MultiScreen 96-well filter plates (Millipore) were coated with 5 μg/mL NeutrAvidin (Thermo) or goat anti-human Ig (Southern Biotech) in PBS. The wells were washed with PBS and blocked with 1% (w/v) BSA/PBS before biotinylated TG2 (5 μg/mL) or biotinylated DGP (100 nM) was added to coated NeutrAvidin. After washing with PBS, various dilutions of gut biopsy single-cell suspensions were added to the wells in 10% (v/v) FCS/RPMI and incubated overnight at 37°C in a CO_2 incubator. The cell suspensions were discarded, and the wells were washed with PBS and PBS containing 0.1% (v/v) Tween 20 (PBST). Bound IgA or IgG was detected by incubation with alkaline-phosphatase-conjugated goat anti-human IgA or goat anti-human IgG in 1% (w/v) BSA/PBST followed by washing and addition of BCIP/NBT substrate (Bio-Rad). Spots were counted using an ImmunoSpot analyzer (Cellular Technology Limited).

Western Blotting

Purified TG2-specific or total IgA samples were boiled in non-reducing SDS-PAGE sample buffer and separated on a 4%–20% TGX gel (Bio-Rad). Proteins were blotted onto nitrocellulose, and the membrane was blocked in 2% (w/v) skim milk/PBS. IgA was detected using rabbit anti-human IgA (Dako) followed by horseradish-peroxidase-conjugated goat anti-rabbit IgG (Southern Biotech).

Statistics

Data are presented as mean ± SD and analyzed using GraphPad Prism software, unless otherwise stated. Differences between groups were analyzed using one-way ANOVA or Student's t test, and scatterplots were analyzed by Pearson correlation analysis. A p value of <0.05 was considered significant.

ACCESSION NUMBERS

The accession numbers for the raw mass spectrometry data and antibody sequences used for creating search databases are MassIVE: MSV000081328 (http://massive.ucsd.edu) and ProteomeXchange: PXD007047.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.036.

AUTHOR CONTRIBUTIONS

Conceptualization, R.I. and L.M.S.; Methodology, R.I., O.S., Ø.S., and G.A.d.S.; Software, J.E.K.; Investigation, R.I., O.S., M.S., Ø.S., and G.A.d.S.; Resources, K.E.A.L. and I.R.K.-S.; Writing – Original Draft, R.I. and L.M.S.;

Writing – Review & Editing, R.I., O.S., I.R.K.-S., G.A.d.S., and L.M.S.; Supervision, G.A.d.S. and L.M.S.; Funding Acquisition, I.R.K.-S. and L.M.S.

ACKNOWLEDGMENTS

We thank Astrid Tutturen and Bjørg Simonsen for technical assistance. This work was supported by grants from the Research Council of Norway through its Centre of Excellence funding scheme (project number 179573/V40), the South-Eastern Norway Regional Health Authority (projects 2011050, 2014045, and 2016113), the European Commission (projects ERC-2010-AdG-268541 and FP6-2005-FOOD-4B-36383), Stiftelsen KG Jebsen (SKGH-MED-017), and the Hungarian National Research Fund (OTKA K101788).

Received: May 19, 2017
Revised: July 14, 2017
Accepted: August 7, 2017
Published: September 5, 2017

REFERENCES

Al Kindi, M.A., Colella, A.D., Chataway, T.K., Jackson, M.W., Wang, J.J., and Gordon, T.P. (2016). Secreted autoantibody repertoires in Sjögren's syndrome and systemic lupus erythematosus: a proteomic approach. Autoimmun. Rev. *15*, 405–410.

Bemark, M., Hazanov, H., Strömberg, A., Komban, R., Holmqvist, J., Köster, S., Mattsson, J., Sikora, P., Mehr, R., and Lycke, N.Y. (2016). Limited clonal relatedness between gut IgA plasma cells and memory B cells after oral immunization. Nat. Commun. *7*, 12698.

Benckert, J., Schmolka, N., Kreschel, C., Zoller, M.J., Sturm, A., Wiedenmann, B., and Wardemann, H. (2011). The majority of intestinal IgA+ and IgG+ plasmablasts in the human gut are antigen-specific. J. Clin. Invest. *121*, 1946–1955.

Bergqvist, P., Gärdby, E., Stensson, A., Bemark, M., and Lycke, N.Y. (2006). Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. J. Immunol. *177*, 7772–7783.

Brandtzaeg, P., and Johansen, F.E. (2005). Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. Immunol. Rev. *206*, 32–63.

Cardoso, I., Stamnaes, J., Andersen, J.T., Melino, G., Iversen, R., and Sollid, L.M. (2015). Transglutaminase 2 interactions with extracellular matrix proteins as probed with celiac disease autoantibodies. FEBS J. *282*, 2063–2075.

Cardoso, I., Østerlund, E.C., Stamnaes, J., Iversen, R., Andersen, J.T., Jørgensen, T.J., and Sollid, L.M. (2017). Dissecting the interaction between transglutaminase 2 and fibronectin. Amino Acids *49*, 489–500.

Chen, X., Hnida, K., Graewert, M.A., Andersen, J.T., Iversen, R., Tuukkanen, A., Svergun, D., and Sollid, L.M. (2015). Structural basis for antigen recognition by transglutaminase 2-specific autoantibodies in celiac disease. J. Biol. Chem. *290*, 21365–21375.

Chen, J., Zheng, Q., Hammers, C.M., Ellebrecht, C.T., Mukherjee, E.M., Tang, H.Y., Lin, C., Yuan, H., Pan, M., Langenhan, J., et al. (2017). Proteomic analysis of pemphigus autoantibodies indicates a larger, more diverse, and more dynamic repertoire than determined by B cell genetics. Cell Rep. *18*, 237–247.

Cheung, W.C., Beausoleil, S.A., Zhang, X., Sato, S., Schieferl, S.M., Wieler, J.S., Beaudet, J.G., Ramenani, R.K., Popova, L., Comb, M.J., et al. (2012). A proteomics approach for the identification and cloning of monoclonal antibodies from serum. Nat. Biotechnol. *30*, 447–452.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. *26*, 1367–1372.

Di Niro, R., Mesin, L., Zheng, N.Y., Stamnaes, J., Morrissey, M., Lee, J.H., Huang, M., Iversen, R., du Pré, M.F., Qiao, S.W., et al. (2012). High abundance of plasma cells secreting transglutaminase 2-specific IgA autoantibodies with limited somatic hypermutation in celiac disease intestinal lesions. Nat. Med. *18*, 441–445.

Di Niro, R., Snir, O., Kaukinen, K., Yaari, G., Lundin, K.E., Gupta, N.T., Kleinstein, S.H., Cols, M., Cerutti, A., Mäki, M., et al. (2016). Responsive population dynamics and wide seeding into the duodenal lamina propria of transglutaminase-2-specific plasma cells in celiac disease. Mucosal Immunol. *9*, 254–264.

Gärdby, E., Wrammert, J., Schön, K., Ekman, L., Leanderson, T., and Lycke, N. (2003). Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant. J. Immunol. *170*, 55–63.

Hnida, K., Stamnaes, J., du Pré, M.F., Mysling, S., Jørgensen, T.J., Sollid, L.M., and Iversen, R. (2016). Epitope-dependent functional effects of celiac disease autoantibodies on transglutaminase 2. J. Biol. Chem. *291*, 25542–25552.

Horns, F., Vollmers, C., Croote, D., Mackey, S.F., Swan, G.E., Dekker, C.L., Davis, M.M., and Quake, S.R. (2016). Lineage tracing of human B cells reveals the in vivo landscape of human antibody class switching. eLife *5*, e16578.

Iversen, R., Di Niro, R., Stamnaes, J., Lundin, K.E., Wilson, P.C., and Sollid, L.M. (2013). Transglutaminase 2-specific autoantibodies in celiac disease target clustered, N-terminal epitopes not displayed on the surface of cells. J. Immunol. *190*, 5981–5991.

Iversen, R., Mysling, S., Hnida, K., Jørgensen, T.J., and Sollid, L.M. (2014). Activity-regulating structural changes and autoantibody epitopes in transglutaminase 2 assessed by hydrogen/deuterium exchange. Proc. Natl. Acad. Sci. USA *111*, 17146–17151.

Iversen, R., Fleur du Pré, M., Di Niro, R., and Sollid, L.M. (2015). Igs as substrates for transglutaminase 2: implications for autoantibody production in celiac disease. J. Immunol. *195*, 5159–5168.

Kutteh, W.H., Prince, S.J., and Mestecky, J. (1982). Tissue origins of human polymeric and monomeric IgA. J. Immunol. *128*, 990–995.

Lavinder, J.J., Wine, Y., Giesecke, C., Ippolito, G.C., Horton, A.P., Lungu, O.I., Hoi, K.H., DeKosky, B.J., Murrin, E.M., Wirth, M.M., et al. (2014). Identification and characterization of the constituent human serum antibodies elicited by vaccination. Proc. Natl. Acad. Sci. USA *111*, 2259–2264.

Lebreton, C., Menard, S., Abed, J., Moura, I.C., Coppo, R., Dugave, C., Monteiro, R.C., Fricot, A., Traore, M.G., Griffin, M., et al. (2012). Interactions among secretory immunoglobulin A, CD71, and transglutaminase-2 affect permeability of intestinal epithelial cells to gliadin peptides. Gastroenterology *143*, 698–707.

Lee, J., Boutz, D.R., Chromikova, V., Joyce, M.G., Vollmers, C., Leung, K., Horton, A.P., DeKosky, B.J., Lee, C.H., Lavinder, J.J., et al. (2016). Molecular-level analysis of the serum antibody repertoire in young adults before and after seasonal influenza vaccination. Nat. Med. *22*, 1456–1464.

Leffler, D.A., and Schuppan, D. (2010). Update on serologic testing in celiac disease. Am. J. Gastroenterol. *105*, 2520–2524.

Lefranc, M.P., Giudicelli, V., Ginestoux, C., Bodmer, J., Müller, W., Bontrop, R., Lemaitre, M., Malik, A., Barbié, V., and Chaume, D. (1999). IMGT, the international ImMunoGeneTics database. Nucleic Acids Res. *27*, 209–212.

Lemke, A., Kraft, M., Roth, K., Riedel, R., Lammerding, D., and Hauser, A.E. (2016). Long-lived plasma cells are generated in mucosal immune responses and contribute to the bone marrow plasma cell pool in mice. Mucosal Immunol. *9*, 83–97.

Ludvigsson, J.F., Bai, J.C., Biagi, F., Card, T.R., Ciacci, C., Ciclitira, P.J., Green, P.H., Hadjivassiliou, M., Holdoway, A., van Heel, D.A., et al.; BSG Coeliac Disease Guidelines Development Group; British Society of Gastroenterology (2014). Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. Gut *63*, 1210–1228.

Lycke, N., Eriksen, L., and Holmgren, J. (1987). Protection against cholera toxin after oral immunization is thymus-dependent and associated with intestinal production of neutralizing IgA antitoxin. Scand. J. Immunol. *25*, 413–419.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of

intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222–2226.

Macpherson, A.J., McCoy, K.D., Johansen, F.E., and Brandtzaeg, P. (2008). The immune geography of IgA induction and function. Mucosal Immunol. *1*, 11–22.

Marzari, R., Sblattero, D., Florian, F., Tongiorgi, E., Not, T., Tommasini, A., Ventura, A., and Bradbury, A. (2001). Molecular dissection of the tissue transglutaminase autoantibody response in celiac disease. J. Immunol. *166*, 4170–4176.

Mei, H.E., Yoshida, T., Sime, W., Hiepe, F., Thiele, K., Manz, R.A., Radbruch, A., and Dörner, T. (2009). Blood-borne human plasma cells in steady state are derived from mucosal immune responses. Blood *113*, 2461–2469.

Micallef, L., and Rodgers, P. (2014). eulerAPE: drawing area-proportional 3-Venn diagrams using ellipses. PLoS One *9*, e101717.

Molberg, O., Mcadam, S.N., Körner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Norén, O., Roepstorff, P., et al. (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat. Med. *4*, 713–717.

Moreno, M.L., Cebolla, Á., Muñoz-Suano, A., Carrillo-Carrion, C., Comino, I., Pizarro, Á., León, F., Rodríguez-Herrera, A., and Sousa, C. (2017). Detection of gluten immunogenic peptides in the urine of patients with coeliac disease reveals transgressions in the gluten-free diet and incomplete mucosal healing. Gut *66*, 250–257.

Pabst, O., Cerovic, V., and Hornef, M. (2016). Secretory IgA in the coordination of establishment and maintenance of the microbiota. Trends Immunol. *37*, 287–296.

Rappsilber, J., Ishihama, Y., and Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. *75*, 663–670.

Roy, B., Neumann, R.S., Snir, O., Iversen, R., Sandve, G.K., Lundin, K.E.A., and Sollid, L.M. (2017). High-throughput single-cell analysis of B cell receptor usage among autoantigen-specific plasma cells in celiac disease. J. Immunol. *199*, 782–791.

Schwertz, E., Kahlenberg, F., Sack, U., Richter, T., Stern, M., Conrad, K., Zimmer, K.P., and Mothes, T. (2004). Serologic assay based on gliadin-related nonapeptides as a highly sensitive and specific diagnostic aid in celiac disease. Clin. Chem. *50*, 2370–2375.

Simon-Vecsei, Z., Király, R., Bagossi, P., Tóth, B., Dahlbom, I., Caja, S., Csosz, É., Lindfors, K., Sblattero, D., Nemes, É., et al. (2012). A single conformational transglutaminase 2 epitope contributed by three domains is critical for celiac antibody binding and effects. Proc. Natl. Acad. Sci. USA *109*, 431–436.

Slack, E., Balmer, M.L., Fritz, J.H., and Hapfelmeier, S. (2012). Functional flexibility of intestinal IgA - broadening the fine line. Front. Immunol. *3*, 100.

Snir, O., Mesin, L., Gidoni, M., Lundin, K.E., Yaari, G., and Sollid, L.M. (2015). Analysis of celiac disease autoreactive gut plasma cells and their corresponding memory compartment in peripheral blood using high-throughput sequencing. J. Immunol. *194*, 5703–5712.

Sollid, L.M., and Lundin, K.E. (2009). Diagnosis and treatment of celiac disease. Mucosal Immunol. *2*, 3–7.

Spencer, J., and Sollid, L.M. (2016). The human intestinal B-cell response. Mucosal Immunol. *9*, 1113–1124.

Stamnaes, J., and Sollid, L.M. (2015). Celiac disease: autoimmunity in response to food antigen. Semin. Immunol. *27*, 343–352.

Stamnaes, J., Iversen, R., du Pré, M.F., Chen, X., and Sollid, L.M. (2015). Enhanced B-cell receptor recognition of the autoantigen transglutaminase 2 by efficient catalytic self-multimerization. PLoS One *10*, e0134922.

Steinsbø, Ø., Henry Dunand, C.J., Huang, M., Mesin, L., Salgado-Ferrer, M., Lundin, K.E., Jahnsen, J., Wilson, P.C., and Sollid, L.M. (2014). Restricted VH/VL usage and limited mutations in gluten-specific IgA of coeliac disease lesion plasma cells. Nat. Commun. *5*, 4041.

Tipton, C.M., Fucile, C.F., Darce, J., Chida, A., Ichikawa, T., Gregoretti, I., Schieferl, S., Hom, J., Jenks, S., Feldman, R.J., et al. (2015). Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus. Nat. Immunol. *16*, 755–765.

van de Wal, Y., Kooy, Y., van Veelen, P., Peña, S., Mearin, L., Papadopoulos, G., and Koning, F. (1998). Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. J. Immunol. *161*, 1585–1588.

Volta, U., Molinaro, N., Fratangelo, D., and Bianchi, F.B. (1990). IgA subclass antibodies to gliadin in serum and intestinal juice of patients with coeliac disease. Clin. Exp. Immunol. *80*, 192–195.

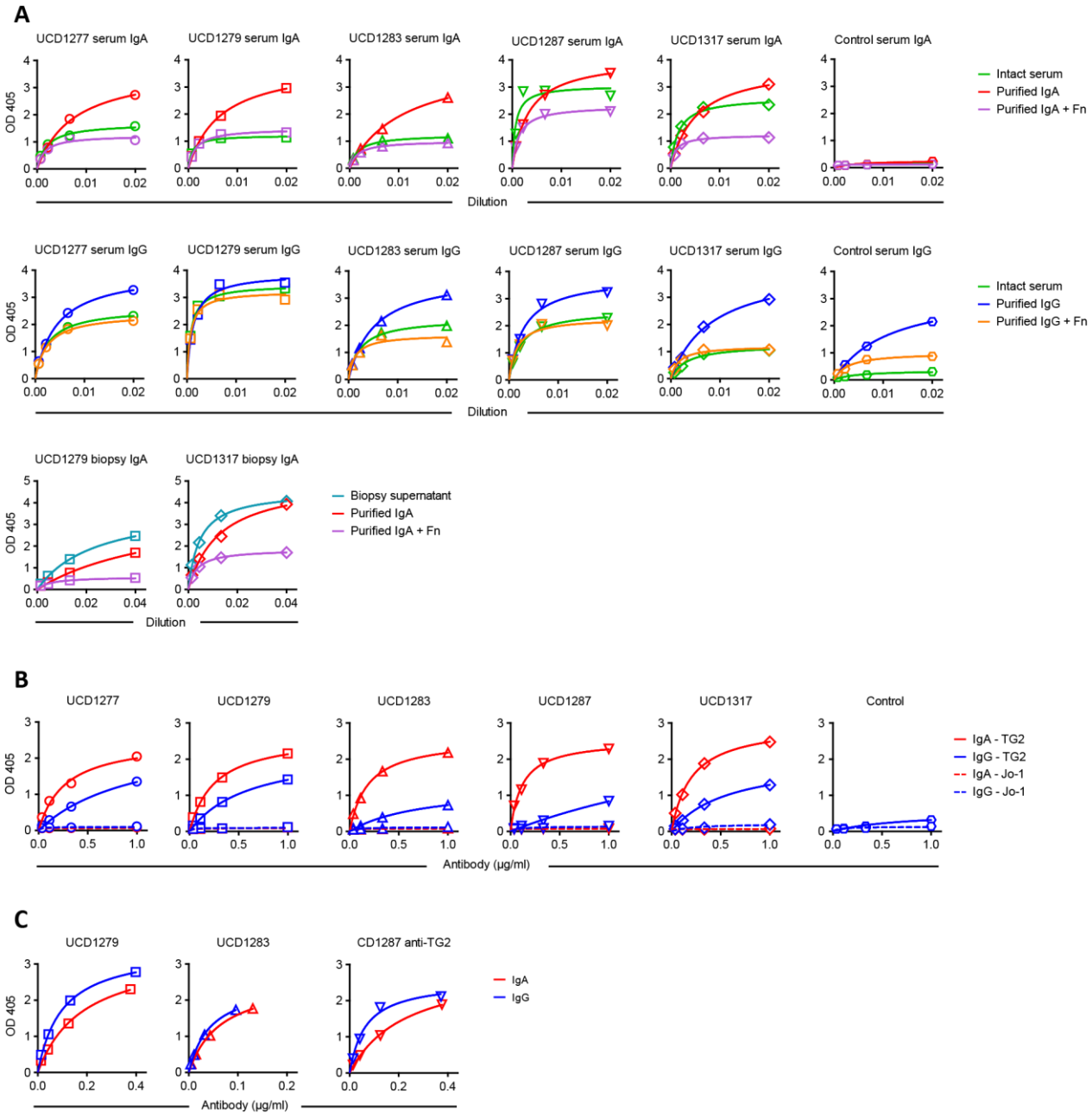
Cell Reports, Volume *20*

Supplemental Information

Strong Clonal Relatedness between Serum and Gut IgA despite Different Plasma Cell Origins

Rasmus Iversen, Omri Snir, Maria Stensland, José E. Kroll, Øyvind Steinsbø, Ilma R. Korponay-Szabó, Knut E.A. Lundin, Gustavo A. de Souza, and Ludvig M. Sollid

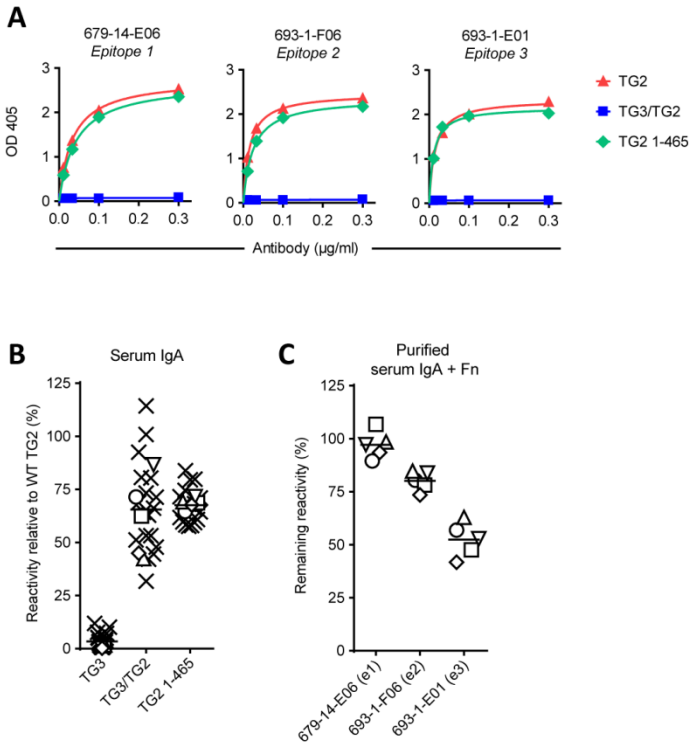
Figure S1. Assessment of purified anti-TG2 IgA or IgG reactivity by ELISA. Related to Figures 2 and 5



(A) Total IgA or IgG purified from celiac disease patient or control sera or from supernatants of cultured gut biopsies were incubated in TG2-coated microtiter plates in the presence or absence of fibronectin (Fn, 5 µg/ml in the highest concentration). Whole sera or culture supernatants were added in corresponding dilutions for comparison, and bound IgA or IgG was detected using isotype-specific goat anti-human antibodies. Supplementation of purified antibodies with Fn decreases their TG2 reactivity and make them mimic the behavior of whole sera, indicating that binding between TG2 and serum Fn blocks a substantial number of antibody binding sites. For the control subject, TG2 reactivity is present in the purified IgG fraction but not in whole serum or in purified IgA, indicating that purification of IgG alters the binding properties of some antibody molecules. The reason for this behavior of IgG is most likely

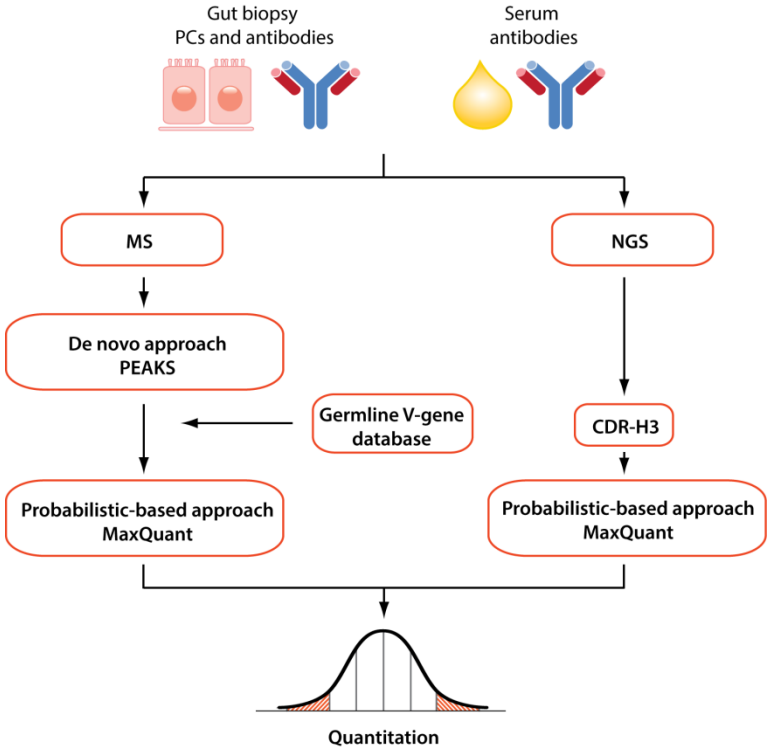
that exposure to low pH during purification alters the structure of some IgG molecules, allowing them to interact with new antigens (Dimitrov et al., 2013).
(B) Binding of affinity-purified anti-TG2 antibodies isolated from total serum IgA or IgG preparations. Histidyl tRNA synthetase (Jo-1) was included as an irrelevant antigen to test the specificity of the purified antibodies. In all cases, the purified IgG antibodies have lower apparent affinity than the IgA antibodies. Notably, TG2-binding IgG, but not IgA, could be isolated from non-celiac control serum, but these antibodies had very low affinity.
(C) TG2 binding of affinity-purified IgA and IgG isolated from whole sera without initial separation into total IgA and IgG fractions. When purifying the TG2-binding fraction from whole sera, there is no clear difference between IgA and IgG affinities.

Figure S2. Reactivity of antibodies with different regions of TG2 assessedby ELISA. Related to Figure 2



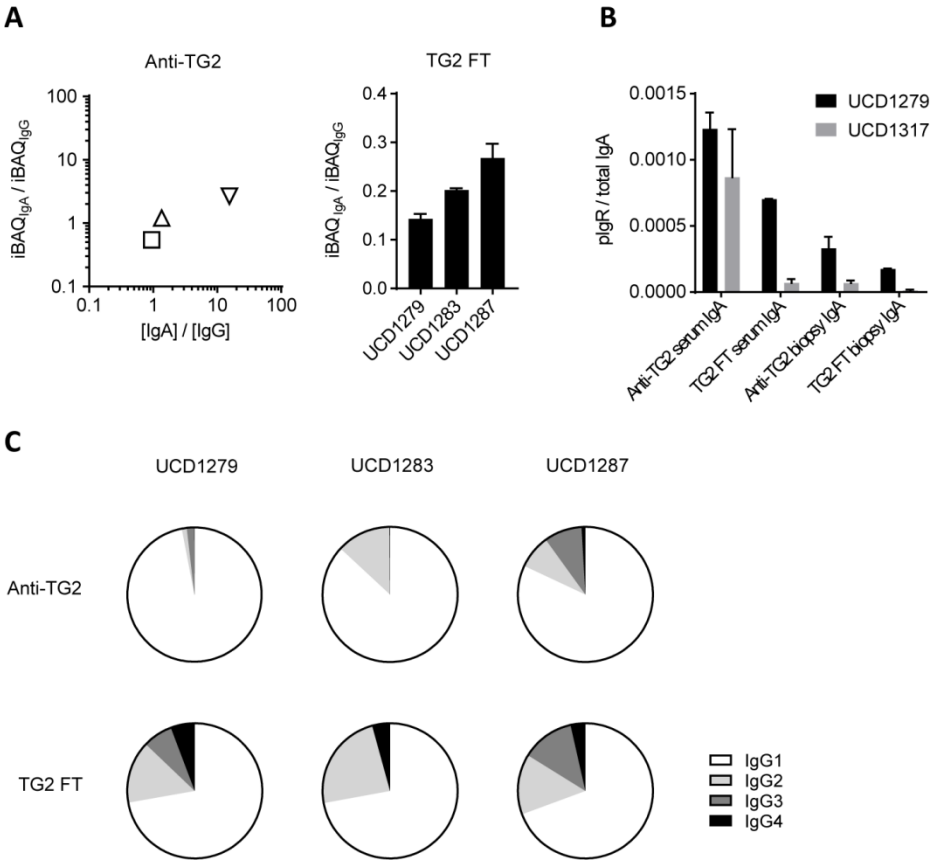
(A) Saturation binding curves for anti-TG2 mAbs targeting different epitopes to TG2 domain variants as determined by ELISA. All three mAbs lose reactivity when the N-terminal domain of TG2 is replaced with the N-terminal domain of TG3 (TG3/TG2) but show intact reactivity when the two C-terminal domains are removed (TG2 1-465). (B) Reactivity of IgA in whole serum samples from adult celiac disease patients (n = 23) with TG2 domain variants as shown for purified serum IgA in Figure 2A. No reactivity was observed against full-length TG3, indicating that binding observed with TG3/TG2 is mediated solely by TG2 epitopes located outside of the N-terminal domain. (C) Ability of the indicated anti-TG2 mAbs to compete with serum IgA for TG2 binding in the presence of added fibronectin (Fn, 1 µg/ml). Total serum IgA was purified from celiac disease patients (n = 5), and the reactivity is given relative to the signal obtained in the absence of mAbs but with added Fn. In the presence of Fn, addition of epitope-1 mAb does not have an effect on binding of serum IgA (compare with Figure 2B). Horizontal lines indicate means.

Figure S3. Overview of the quantitative proteomics approach and software used for antibody analysis. Related to Table 1 and Figures 3 and 4



Antigen-specific antibodies were either isolated from cultured gut biopsies or serum samples of celiac disease patients and analyzed by LC-MS/MS. Peptides derived from V-gene segments and CDR-H3 were identified by two separate procedures. Heavy and light chain V-gene peptides were identified using a database consisting of germline amino acid sequences combined with mutated peptides discovered by de novo MS sequencing. In order to identify CDR-H3 peptides, antigen-specific PCs were sorted from gut biopsies, and heavy chain V-region sequences were obtained by NGS.

Figure S4. Quantification of antibody class distributions by LC-MS/MS. Related to Figure 6



(A) The ratio between IgA and IgG antibodies was determined based on iBAQ values obtained with MaxQuant software in affinity-purified anti-TG2 or TG2 flow-through (FT) fractions obtained from whole sera of three celiac disease patients. For the TG2-binding fraction (left panel), the IgA:IgG ratio determined by LC-MS/MS (y-axis) was compared to the ratio obtained by measuring the IgA and IgG concentrations by ELISA (x-axis). At higher IgA:IgG ratios, the LC-MS/MS method appears to overestimate the contribution of IgG. The ratios obtained from the TG2 FT fractions (right panel) are within the expected range (Gonzalez-Quintela et al., 2008). Error bars indicate SD based on duplicate LC-MS/MS runs.

(B) Quantification of the amount of polymeric Ig receptor (pIgR) associated with the TG2-binding or TG2 FT fractions of purified total IgA from sera or gut biopsies of two celiac disease patients. The quantification is based on iBAQ values and is given relative to the summed iBAQ values of the IgA1 and IgA2 constant regions in each sample. This ratio should give an indication of the contribution of secretory IgA to each population. Error bars represent SD based on duplicate (UCD1279) or triplicate (UCD1317) LC-MS/MS runs.

(C) The distribution of IgG between individual subclasses based on iBAQ values measured in affinity-purified anti-TG2 and TG2 FT fractions obtained from whole sera of celiac disease patients. In all cases, the IgG1 subclass is overrepresented among TG2-specific antibodies compared to the TG2 FT fraction.

Table S1. Identification of Ig proteins in affinity-purified gut biopsy anti-TG2 and TG2 flow-through IgA of a representative celiac disease patient (UCD1317) by LC-MS/MS. Related to Figure 3

	Anti-TG2		TG2 flow-through	
	Germline only	Germline + de novo	Germline only	Germline + de novo
Protein groups identified[a]	121	147	116	144
Proteins per group (avg.)	2.72	2.35	2.91	2.36
Peptides per group (avg.)	4.03	41.4	3.55	48.2
Unique peptides per group (avg.)	1.73	5.57	1.82	6.75

[a] Each protein group comprises all proteins in the database that share the same identified peptides. The germline only database contains all Ig V-gene segments and constant regions available from http://www.imgt.org. Entries in this database were supplemented with peptide sequences harboring single-amino acid substitutions identified by de novo sequencing to obtain the germline + de novo database. Typically, individual alleles could not be distinguished and were placed in the same protein group, whereas different V-gene segments often could be separated. Due to the large degree of similarity between V-gene segments, many peptides were shared between groups, but each group is represented by at least one unique peptide.

Table S2. List of V-gene segments overrepresented among purified anti-TG2 or anti-DGP serum IgA of celiac disease patients. Related to Figure 3

	Anti-TG2[a]		Anti-DGP[a]
	5 of 5 patients	4 of 5 patients	2 of 2 patients
IGHV1-69		**X**	
IGHV3-7		X	**X**
IGHV3-15			**X**
IGHV3-30			X
IGHV3-43		**X**	
IGHV3-74			**X**
IGHV5-51	**X**		
IGKV1-5	**X**		
IGKV1-12	**X**		
IGKV1-16		**X**	
IGKV1-17	**X**		
IGKV1-27		**X**	
IGKV1-39	**X**		
IGKV3-20	**X**		
IGKV4-1			**X**
IGLV1-44	**X**		
IGLV1-47	**X**		
IGLV2-11		X	
IGLV2-14	X		
IGLV4-69			**X**
IGLV5-45	**X**		

[a] V-gene segments that were significantly more abundant in antigen-specific compared to non-specific serum IgA fractions across celiac disease patients are indicated. Symbols in bold indicate V-gene segments previously found to be used by TG2-specific (Di Niro et al., 2012; Snir et al., 2015; Roy et al., 2017) and DGP-specific (Steinsbø et al., 2014) gut PCs.

Table S3. Anti-TG2 CDR-H3 peptides detected by LC-MS/MS in affinity-purified anti-TG2 or flow-through fractions isolated from IgA of serum or gut biopsy secretions. Related to Figure 4 and Table 1.
See Excel file

Table S4. Anti-DGP CDR-H3 peptides detected by LC-MS/MS in affinity-purified anti-DGP or flow-through fractions isolated from serum IgA. Related to Figure 4 and Table 1.
See Excel file

Table S5. Anti-TG2 CDR-H3 peptides detected by LC-MS/MS in affinity-purified anti-TG2 or flow-through fractions isolated from whole sera. Related to Figure 5.
See Excel file

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

ELISAs. Recombinant human proteins were coated in microtiter plates at 3 µg/ml in TBS. For comparison of TG2 and DGP reactivity, biotinylated TG2 (1 µg/ml) or biotinylated DGP (biotin-GSGSGS-PLQPEQPFP, obtained from GL Biochem, 50 nM) was attached to streptavidin-coated plates (Thermo) in TBS containing 0.1% (v/v) Tween 20 (TBST). For comparison of reactivity to different TG2 variants, the enzymes were associated with the irreversible inhibitor Ac-P(DON)LPF-NH_2 (Zedira) to ensure that they had equal and homogenous conformations prior to coating (Iversen et al., 2014). To this end, the TG2 variants were incubated with 0.5 mM of the inhibitor for 20 min at room temperature before the reaction was started by addition of $CaCl_2$ to a final concentration of 5 mM and further incubation for 30 min. Incubation with antibodies were carried out at 37°C in 3% (w/v) BSA/TBST followed by detection of bound IgA or IgG using alkaline phosphatase-conjugated goat anti-human IgA (Sigma) or goat anti-human IgG (Southern Biotech). To compare antibody reactivity against different TG2 variants or in the presence or absence of competitor, initial titration of all sera or purified antibodies was carried out, and a concentration falling within the linear range of the reference assay (using wild-type TG2 in the absence of competitor) was in each case picked for determination of relative binding levels based on changes in OD value. Competition between polyclonal antibodies and anti-TG2 mAbs was assessed by incubating coated TG2 with 10 µg/ml anti-TG2 IgG1 mAbs for 30 min at 37°C, before polyclonal antibody preparations were added without removing the mAbs, and incubation was continued for 1 h. The level of bound polyclonal IgA was subsequently detected as above. Concentrations of IgA and IgG in TG2-binding fractions purified from whole sera were determined by incubation with goat anti-human IgA (Sigma) or rabbit anti-human IgG (Dako), each coated at 5 µg/ml, and detection of bound antibodies using alkaline phosphatase-conjugated goat anti-human IgA or rabbit anti-human IgG (Abcam). Obtained signals were compared to a standard curve obtained using purified total serum IgA or IgG.

Antibody purification. Total IgG was purified from 20 ml of serum or plasma by diluting the sample in 20 mM NaP_i, pH 7.4 and passing it over a protein G column (GE Healthcare). Bound IgG was eluted with 0.1 M glycine-HCl, pH 2.5 and immediately neutralized with 1 M Tris-HCl, pH 9. IgA was purified from the protein G flow-through fraction using peptide M-agarose (Invivogen) followed by elution of bound IgA as described for purification of IgG. IgA secreted from duodenal biopsies was purified from culture supernatants in the same way. To isolate TG2-binding IgA and IgG, we mixed up to 10 ml of purified antibodies or 1 ml of whole serum diluted ten times in TBS with 400 µl 50% (v/v) streptavidin-agarose (Novagen) that had been pre-associated with 100 µg biotinylated TG2 in a Poly-Prep chromatography column (Bio-Rad), followed by incubation for 1 h under constant rotation at room temperature. A new column with fresh TG2 protein was prepared for each sample. The flow-through fraction was collected, and a small part to be analyzed by LC-MS/MS was desalted using a Zeba Spin column (Thermo). The column containing TG2-bound antibodies was centrifuged at 1700 rpm for 1 min and washed three times with 1 ml TBS and three times with 1 ml water followed by elution three times with 200 µl 20 mM HCl. The column was centrifuged between each washing and elution step to ensure proper clearing of liquid. A small part of the eluate to be used in binding studies was immediately neutralized with NaOH and diluted in TBS. DGP-binding antibodies were purified from the flow-through fraction of the anti-TG2 purification step in the same way as described above, using streptavidin-agarose pre-incubated with 10 µM biotinylated DGP.

LC-MS/MS. For liquid chromatography separation, we used an EasySpray column (C18, 2 µm beads, 100 Å, 75 µm inner diameter) (Thermo) capillary of 25 cm bed length and a flow rate of 0.3 µl/min. The applied solvent gradient was from 5% (v/v) B to 30% B over 60 min, followed by washing with 90% B for 20 min. Solvent A was aqueous 0.1% (v/v) formic acid, and solvent B was acetonitrile with 0.1 % formic acid. The column temperature was kept at 60°C. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 400 to 1200) were acquired in the Orbitrap with resolution R = 70,000 at m/z 200 (after accumulation to a target of 3,000,000 ions in the quadrupole). The method used allowed sequential isolation of the most intense multiply-charged ions, up to ten, depending on signal intensity, for fragmentation on the HCD cell using high-energy collision dissociation at a target value of 100,000 charges or maximum acquisition time of 100 ms. MS/MS scans were collected at 17,500 resolution at the Orbitrap cell. Target ions already selected for MS/MS were dynamically excluded for 30 s. General mass spectrometry conditions were: electrospray voltage, 2.1 kV; no sheath and auxiliary gas flow, heated capillary temperature of 250°C, normalized HCD collision energy 25%. Ion selection threshold was set to 5e4 counts. Isolation width of 3.0 Da was used.

De novo peptide sequencing. Peptide sequences were identified using PEAKS Studio (Zhang et al., 2012) with the following parameters: methionine oxidation as variable modification and cysteine carbamidomethylation as fixed modification, trypsin without proline restriction as enzyme option with three allowed miscleavages. Precursor and fragment ion error tolerances were set to 20 ppm and 0.05 Da respectively. The SPIDER (Sequence Tag Homology/Mutated Peptides) function in PEAKS was used to find mutations. Peptide confidence cutoff was set to FDR 1% and ALC (Average local confidence) 80% in order to only include peptides with high-quality MS spectra. We then developed an in-house Perl script which collected de novo-sequenced peptides from the PEAKS output and added them to the aligned protein entry in the germline database. Peptides were added N-terminally to the reference germline sequence in order to keep tryptic site structure. The modified germline database was then used for a regular, probabilistic-based peptide search, using MaxQuant (version 1.5.2.8)(Cox and Mann, 2008) for improved protein identification, sequence coverage and label-free quantitation. Methionine oxidation and cysteine carbamidomethylation were included as variable and fixed modifications, respectively. First search error window was set to 20 ppm and mains search error was set to 6 ppm at MS level. Trypsin without proline restriction was used as enzyme option with two allowed miscleavages. Minimal unique peptides were set to 1, and FDR allowed was 1% for peptide and protein identification. Generation of reversed sequences was selected to assign FDR rates. The levels of individual V-gene segments in antigen-specific and non-specific fractions were compared by *t*-test analysis using Perseus software (Tyanova et al., 2016).

Isolation of TG2- and DGP-reactive PCs and sequencing of heavy chain V regions. PCs reactive with TG2 or DGP were stained using multimers as previously described (Di Niro et al., 2012; Steinsbø et al., 2014; Snir et al., 2015). In short, biotinylated TG2 and DGP were multimerized using PE- and APC-labeled streptavidin, respectively, at a 4:1 molar ratio. Single-cell suspensions from duodenal biopsies were stained with the two multimers and the following cellular lineage markers: IgA-FITC (Southern Biotech), CD3- and CD14-Brilliant Violet 570 (BioLegend), CD19-pacific blue (BD Biosciences) and CD27-PE-Cy7 (eBioscience). PCs were defined as live, large CD14/CD3-negative and with high expression of CD27. IgA PCs binding either TG2 or DGP were sorted into 25 µl PBS supplemented with 10 mM DTT and 2 U/µl RNAsin (Promega) and kept at -70°C until cDNA synthesis. For cDNA synthesis, 13.5 µl of sorted PCs extracts were incubated with 1.4 µM indexed IGHJ-rev primer (see primer list below), 0.45% (v/v) NP-40, 0.5 µl 40 U/µl and DEPC-treated water in a total volume of 25 µl at 65°C for 5 min. Next, we added 10 µl 5x RT buffer, 3.5 µl 100 mM DTT, 4.3 µl 10 mM dNTP-Mix (Promega), 0.7 µl 40 U/µl RNAsin, 0.85 µl SuperScript III (Invitrogen) and 4.5 µl DEPC-treated water, and cDNA was synthesized using the following temperature steps: 42°C for 10 min, 25°C for 10 min, 50°C for 60 min and 94°C for 5 min followed by storage at −20°C. Second-strand cDNA was synthesized using AmpliTaq Gold polymerase (Applied Biosystems) with indexed IGHV1-6 framework region (FR) 2 (van Dongen et al., 2003). Six random nucleotides were included in IGHJ and IGHV1-6 primers and were used as unique molecular identifiers (UMIs). Second-strand synthesis was carried out using the temperature steps: 95°C for 7.5 min, 52°C for 2 min, and 72°C for 10 minutes. Double-stranded cDNA was further purified using AMPure XP (Beckman Coulter) at a 1:1 ratio according to the manufacturer's instructions. Next, the second part of the Illumina adapter was connected using R1 and R2 primers. PCR was performed using Qiagen Multiplex PCR at 95°C for 15 min, x25 (95°C for 30 seconds, 60°C for 45 seconds, 72°C for 90 seconds) and 72°C for 10 minutes. Primers were previously described (Snir et al., 2015). Final *IGHV* amplicon libraries were purified and concentrated using AMPure XP and further extracted from agarose gel. Paired-end sequencing of 2x300 bp was performed using Illumina MiSeq at the Norwegian Sequencing Centre in Oslo, Norway (http://www.sequencing.uio.no). Raw sequencing data was processed using pRESTO (Vander Heiden et al., 2014). Unique sequences for which at least two copies were present were further analyzed using IMGT and Immunoglobulin Analysis Tool (IgAT) software (Rogosch et al., 2012).

Primer sequences

PCR	Name	Sequence (5´-3´)
1st	IGHV1-FR2	GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN(Index)CTGGGTGCGACAGGCCCCTGGACAA
1st	IGHV2-FR2	GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN(Index)TGGATCCGTCAGCCCCCAGGGAAGG
1st	IGHV3-FR2	GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN(Index)GGTCCGCCAGGCTCCAGGGAA
1st	IGHV4-FR2	GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN(Index)TGGATCCGCCAGCCCCCAGGGAAGG
1st	IGHV5-FR2	GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN(Index)GGGTGCGCCAGATGCCCGGGAAAGG
1st	IGHV6-FR2	GGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNNN(Index)TGGATCAGGCAGTCCCCATCGAGAG
1st	IGHJ-Rev	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN(Index)CTTACCTGAGGAGACGGTGACC
2nd	R1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
2nd	R2	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTC

List of primers that were used for amplification of *IGHV*. IGHV1-FR2 – IGHV6-FR2 (forward) and IGHJ-Rev (reverse) were used for first PCR. Blue and red color indicate partial read (R)1 and partial R2 Illumina adapters, respectively. Six random nucleotides (NNNNNN) were used as unique molecular identifiers (UMIs), followed by 6 nucleotides indices and gene specific sequences. The remaining parts of Ilumina adapters were incorporated by using R1 and R2 primers in the second PCR.

SUPPLEMENTAL REFERENCES

Dimitrov, J.D., Planchais, C., Roumenina, L.T., Vassilev, T.L., Kaveri, S.V., and Lacroix-Desmazes, S. (2013). Antibody polyreactivity in health and disease: statu variabilis. J. Immunol. *191*, 993-999.

Gonzalez-Quintela, A., Alende, R., Gude, F., Campos, J., Rey, J., Meijide, L.M., Fernandez-Merino, C., and Vidal, C. (2008). Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. Clin. Exp. Immunol. *151*, 42-50.

Rogosch, T., Kerzel, S., Hoi, K.H., Zhang, Z., Maier, R.F., Ippolito, G.C., and Zemlin, M. (2012). Immunoglobulin analysis tool: a novel tool for the analysis of human and mouse heavy and light chain transcripts. Front. Immunol. *3*, 176.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., and Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat. Methods *13*, 731-740.

van Dongen, J.J., Langerak, A.W., Bruggemann, M., Evans, P.A., Hummel, M., Lavender, F.L., Delabesse, E., Davi, F., Schuuring, E., Garcia-Sanz, R., et al. (2003). Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia *17*, 2257-2317.

Vander Heiden, J.A., Yaari, G., Uduman, M., Stern, J.N., O'Connor, K.C., Hafler, D.A., Vigneault, F., and Kleinstein, S.H. (2014). pRESTO: a toolkit for processing high-throughput sequencing raw reads of lymphocyte receptor repertoires. Bioinformatics *30*, 1930-1932.

Zhang, J., Xin, L., Shan, B., Chen, W., Xie, M., Yuen, D., Zhang, W., Zhang, Z., Lajoie, G.A., and Ma, B. (2012). PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. Mol. Cell. Proteomics *11*, M111 010587.