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Received 3 Jan 2014 | Accepted 2 May 2014 | Published 9 Jun 2014

DOI: 10.1038/ncomms5041

OPEN

Restricted VH/VL usage and limited mutations in gluten-specific IgA of coeliac disease lesion plasma cells

Øyvind Steinsbø¹, Carole J. Henry Dunand², Min Huang², Luka Mesin¹, Marlene Salgado-Ferrer², Knut E.A. Lundin^{1,3}, Jørgen Jahnsen⁴, Patrick C. Wilson² & Ludvig M. Sollid¹

Coeliac disease (CD), an enteropathy caused by cereal gluten ingestion, is characterized by CD4⁺ T cells recognizing deamidated gluten and by antibodies reactive to gluten or the self-antigen transglutaminase 2 (TG2). TG2-specific immunoglobulin A (IgA) of plasma cells (PCs) from CD lesions have limited somatic hypermutation (SHM). Here we report that gluten-specific IgA of lesion-resident PCs share this feature. Monoclonal antibodies were expression cloned from single PCs of patients either isolated from cultures with reactivity to complex deamidated gluten antigen or by sorting with gluten peptide tetramers. Typically, the antibodies bind gluten peptides related to T-cell epitopes and many have higher reactivity to deamidated peptides. There is restricted VH and VL combination and usage among the antibodies. Limited SHM suggests that a common factor governs the mutation level in PCs producing TG2- and gluten-specific IgA. The antibodies have potential use for diagnosis of CD and for detection of gluten.

¹Centre for Immune Regulation and Department of Immunology, University of Oslo and Oslo University Hospital-Rikshospitalet, 0372 Oslo, Norway.

²Department of Medicine, Section of Rheumatology, Committee on Immunology, The Knapp Center for Lupus and Immunology Research, University of Chicago, Chicago, Illinois 60637, USA. ³Department of Gastroenterology, Oslo University Hospital-Rikshospitalet, 0372 Oslo, Norway. ⁴Division of Medicine, Department of Gastroenterology, Akershus University Hospital, 1478 Lørenskog, Norway. Correspondence and requests for materials should be addressed to L.M.S. (email: l.m.sollid@medisin.uio.no).

Celiac disease (CD) is a chronic T-cell-mediated inflammatory enteropathy resulting from an inappropriate immune response to dietary gluten proteins (consisting of gliadin and glutenin components) of wheat, barley and rye. The disorder is controlled by gluten, as elimination of gluten from the diet leads to disease remission and resolution of the small intestinal disease lesion, whereas gluten provocation leads to reoccurrence of disease and pathology¹. Distinct features of the disease are cell-mediated immunity to gluten and highly specific humoral responses to both gluten and the self-antigen transglutaminase 2 (TG2)². Leukocyte infiltrations in the CD lesion reflect these immunological features; gluten-reactive CD4⁺ T cells recognizing deamidated gluten peptides in the context of the disease associated HLA-DQ2 and HLA-DQ8 molecules³ as well as plasma cells (PCs) secreting gluten-specific or TG2-specific immunoglobulin A (IgA) and IgM antibodies are found in the intestinal lamina propria of CD patients^{4,5}.

Antibodies to gluten and TG2 have become increasingly important for the diagnostic workup of CD^{6,7}. In children, histological assessment of biopsies in many cases is no longer considered mandatory due to high diagnostic accuracy of serologic assays⁷. A similar diagnostic pathway has also been suggested for adults⁸. Serologic testing is informative when subjects are consuming a gluten-containing diet. Antibodies both to gluten⁹ and TG2 (ref. 10) disappear within months after introduction of a gluten-free diet. The parallel fluctuation of antibodies to gluten and TG2 in serum and at the plasma cell level in the intestine¹¹ in response to dietary gluten suggests that the production of these antibodies is regulated in a coordinated way.

The epitopes recognized by gluten-reactive CD4⁺ T cells of CD patients are well characterized. Typically, the T-cell epitopes harbour

glutamate residues, which have been introduced by TG2-mediated deamidation of certain glutamine residues¹². Less is known about gluten B-cell epitopes. Some epitopes have been characterized by studying polyclonal serum antibody reactivity to synthetic peptides of gliadin proteins¹³ and by bacterial cell-displayed peptide libraries¹⁴. Deamidation is also relevant for the B-cell epitopes, and serum antibody reactivity is higher to deamidated than the corresponding native (non-deamidated) peptides^{13,15}.

Gliadin B-cell epitopes appear to be situated in proximity and/or to overlap with gliadin T-cell epitopes^{13,15}. Furthermore, serum IgA antibodies to TG2, and likely also to deamidated gliadin, only occur in subjects who are HLA-DQ2 or HLA-DQ8 (ref. 16). The HLA dependence of the antibody production and the colocalization of T-cell epitopes and B-cell epitopes suggest that the antibody response to gluten in CD is T cell dependent. Similarly, the HLA dependence of TG2 antibodies suggests T-cell involvement for their formation. Thus, it was surprising that the TG2 IgA expression cloned from CD lesions displayed limited somatic hypermutation (SHM)⁵.

In order to shed further light on the humoral immunity in CD, we have characterized the gluten-specific IgA antibody response of CD that happens in parallel to the autoantibody response to TG2. We have pursued two complementary approaches to identify gliadin-specific IgA⁺ PCs from small intestinal biopsies of subjects with untreated CD (UCD). In one, we isolated gliadin-specific PCs by culturing single PCs and screening the culture supernatants for IgA reactivity to complex gliadin antigen in an epitope unbiased fashion. In another, we analysed and sorted single IgA⁺ PCs with complexes of fluorescent streptavidin and biotinylated synthetic gliadin peptides by flow cytometry. We generated a panel of human monoclonal antibodies (hmAbs)

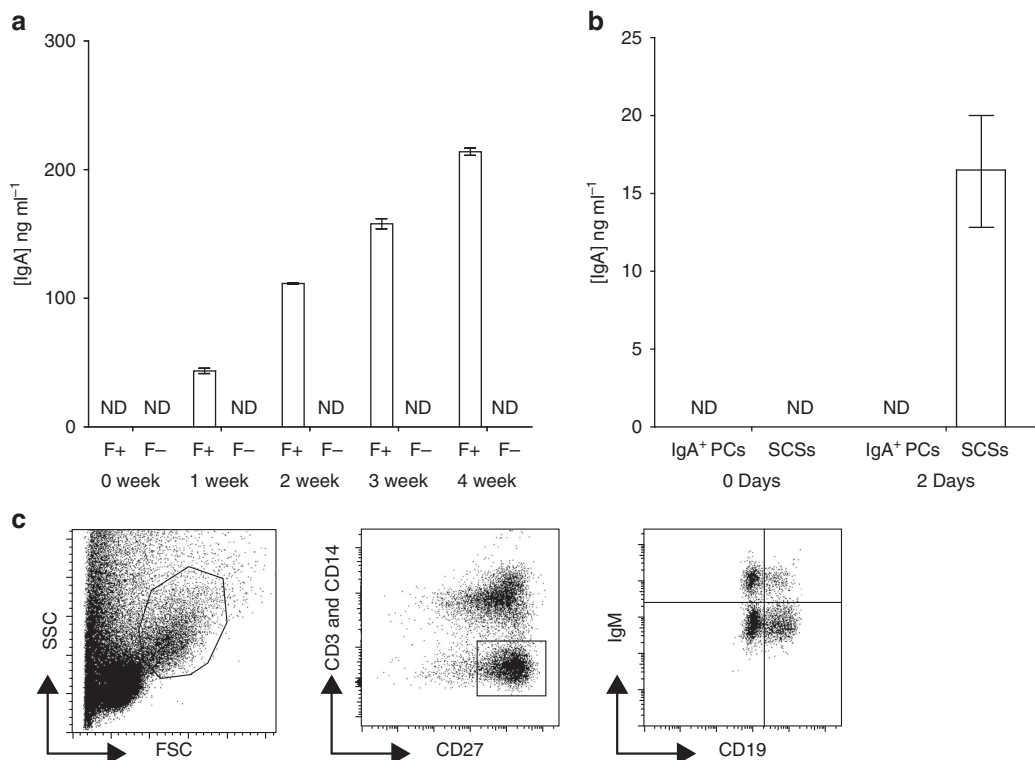


Figure 1 | Survival of intestinal PCs in culture. (a) Concentration of IgA in supernatants after 0, 1, 2, 3 and 4 weeks culture of SCSs (4–5000 cells ml⁻¹) grown with (F+) or without fibroblasts (F-). (b) Concentration of IgA after 0 and 2 days in supernatants from cultures of fibroblasts and PCs when PCs were added either as isolated IgA⁺ PCs (IgA⁺ PCs) or as part of SCSs (80–100 IgA⁺ PCs ml⁻¹). IgA was detectable only in cultures of PCs as part of SCSs. The bars represent mean values of three separate culture wells and error bars represent s.e.m. (ND denotes non-detectable). (c) Representative flow cytometry plots of SCSs after 4 weeks of co-culture with fibroblasts. Viable PCs identified as large (left), viable CD27⁺ CD3⁻ CD14⁻ (middle) cells as indicated by gating strategy. The PC population divides into IgM⁺ and IgM⁻ PCs, where the IgM⁻ are mainly IgA⁺ PCs (right).

from single PCs specific for gliadin and report that these antibodies have restricted VH and VL usage and limited SHM. These results give insights into the mechanisms of the production of gluten-specific and TG2-specific IgA antibodies in CD and suggest that the limited somatic mutation in both populations is controlled by similar factor(s).

Results

Intestinal PCs secreting IgA reactive with gliadin. *In vitro* culture was a critical step for isolation of PCs producing antibodies reactive with heat/acid-treated chymotrypsin-digested gliadin (hereafter termed CT-gliadin for short). Three conditions of PC cultures were compared. Single-cell suspensions (SCSs) from intestinal biopsies were generated, and PCs were cultured either as SCSs, as SCSs in co-culture with human intestinal fibroblasts or as PCs isolated by flow cytometry in co-culture with fibroblasts. The concentration of IgA in supernatants of cultures with SCSs together with fibroblasts increased with a constant rate over at least 4 weeks (Fig. 1a), suggesting that the majority of PCs survived in these cultures. We did not observe any increase of IgA in supernatants of cultures of SCSs alone (Fig. 1a) or flow cytometry sorted PCs cultured with fibroblasts (Fig. 1b). In co-cultures of SCSs and fibroblasts, we observed that intestinal PCs survived for weeks, and viable CD19⁺CD27⁺ PCs were detected by flow cytometry after 4 weeks of culture (Fig. 1c). PCs did not proliferate in these cultures, as tested in a BrdU incorporation assay. Based on these findings, the system of co-culturing SCSs and fibroblasts was selected for further experiments.

We aimed at analysing the specificity of antibodies produced at a single PC level. Based on the frequency of IgA⁺ PCs in SCSs that was determined by flow cytometry (mean 3.3%, range 1.0–5.3%, $n = 16$) (Supplementary Fig. 1a,b), we seeded 10–20 cells per well to obtain cultures with less than one IgA⁺ PC per well. Supernatants of single wells were screened for IgA reactivity to CT-gliadin and TG2. Supernatants containing IgA reactive to TG2 were three to four times more frequent than IgA reactive to CT-gliadin in cultures from subjects with UCD (Fig. 2a,b). Importantly, IgA reactivity to TG2 and CT-gliadin was not detected in supernatants from cultures of non-coeliac controls (Fig. 2c) or supernatants of cultures containing only fibroblasts (Fig. 2d). Thus, PCs secreting IgA reactive to CT-gliadin are present in small intestinal biopsies of subjects with UCD and are less frequent than PCs secreting TG2-reactive IgA.

Next, we wanted to clone and express the antibody genes of PCs from cultures in which we detected IgA reactivity to CT-gliadin in the supernatant. In the SCSs, the large PCs hugely outnumbered small resting B cells (Supplementary Fig. 1c,d). Conceivably, if one well of SCSs contained just one IgA⁺ PC, there was a high probability that this was the only cell expressing the IgA-encoding genes in that well. Where ELISA results suggested gliadin-specific PCs to be present (Fig. 2a), the cells were split into four PCR wells and processed individually to increase the likelihood of having only one PC in each well. Cells were then washed in phosphate-buffered saline (PBS) before snap freezing in DNase-containing buffer and subjected to expression cloning of antibodies in a human IgG1 format as previously described¹⁷. hmAbs were successfully produced from more than half of cultures processed. Of a total of 19 hmAbs produced, nine were reactive to CT-gliadin (Fig. 3a). These were tested for reactivity to bovine serum albumin (BSA) and CpG, of which one was found reactive to BSA (Fig. 3b). Thus, eight hmAbs were considered to be gliadin specific and included in further assays.

Intestinal IgA⁺ PCs stained with gliadin peptides in flow cytometry. We had previously succeeded in visualizing antigen-

specific IgA⁺ PCs by staining with biotinylated antigen bound to fluorescent streptavidin taking advantage of surface IgA and IgM expression of gut PCs^{5,18}. We used the same strategy to identify gliadin-specific IgA⁺ PCs by flow cytometry, using fluorescent streptavidin to form tetramer complexes with biotinylated synthetic peptides. Two different gliadin peptides with sequences targeted by serum IgA antibodies of CD patients, biotin-(GSGSGS)-PLQPEQFPF¹³ and biotin-(PEG)-LQLQFPFQPELPYPQPELPYPQPELPYPQFPF¹⁹, were used as antigen. The two peptides represent sequences of gliadin with Q to E transitions in positions targeted by TG2 (ref. 12). Importantly, both peptides are reported to bind antibodies from UCD patients, primarily in their deamidated versions¹⁵. PLQPEQFPF in particular has demonstrated high sensitivity and specificity for CD in serologic assays²⁰. This approach using synthetic peptides, introduced a restricted selection of gliadin-specific PCs compared with screening culture supernatants for IgA reactivity against CT-gliadin.

In flow cytometry, the mean percentage of IgA⁺ PCs stained with the PLQPEQFPF peptide tetramers was 1.0% (range 0.3–2.6%, $n = 10$) of total IgA⁺ PCs in SCSs generated from biopsies taken from UCD patients (Fig. 4a,b). In comparison, the mean percentage in non-CD disease controls was 0.2% (range 0–0.3%) (Fig. 4a,b). For IgA⁺ PCs stained with the deamidated 33-mer peptide tetramers, the mean percentage was 0.5% (range 0.4–0.9%, $n = 6$) for subjects with UCD and 0.1% in controls (range 0–0.3%, $n = 4$). Background levels were defined by staining IgA⁺ PCs with fluorescent streptavidin alone (mean 0.3%, range 0.2–0.4%, $n = 4$) (Fig. 4b).

Importantly, IgA⁺ PCs stained with synthetic gliadin peptides and TG2 appeared as two separate populations in flow cytometry whereas double-positive cells were not detected (Fig. 4c). Also, the populations visualized with TG2 were larger than the populations stained with both the PLQPEQFPF and the deamidated 33-mer peptides (Fig. 4d). These findings are consistent with the ratio of the frequencies of IgA⁺ PCs reactive with TG2 versus those reactive with CT-gliadin observed in culture (Fig. 2b). The predominance of TG2⁺ PCs relative to the population stained with gliadin peptides was greater for the IgA⁺ PCs than for the IgM⁺ PCs (Fig. 4d). Based on these experiments, we conclude that there are two distinct populations of gliadin-specific and TG2-specific IgA⁺ PCs in the small intestinal lesions of UCD patients.

IgA⁺ PCs stained with fluorescent streptavidin in complex with either PLQPEQFPF or deamidated 33-mer were isolated as single cells by flow cytometry for further expression cloning. The hmAbs obtained were tested for reactivity to the peptide, which they were originally isolated with. Seventeen of 23 hmAbs from PCs isolated with the PLQPEQFPF peptide, and 13 of 16 hmAbs from IgA⁺ PCs isolated with the deamidated 33-mer peptide were reactive in ELISA to the peptide originally used in sorting. This indicates that the staining specificity of gluten peptide tetramers was ~75–80%.

Affinity of gliadin-specific hmAbs to deamidated and native gliadin.

The eight hmAbs that originated from *in vitro* PCs cultures reactive with CT-gliadin were tested in ELISA against the two synthetic peptides. Five reacted with PLQPEQFPF (Fig. 3c) and one reacted with deamidated 33-mer. Hence, six out of eight hmAbs were reactive to PLQPEQFPF and/or deamidated 33-mer. Since cloning of antibody genes from those cultures is unbiased with respect to specific epitope, this result suggests that the epitopes represented by PLQPEQFPF and the deamidated 33-mer are recognized by IgA of a significant part of gliadin-specific PCs.

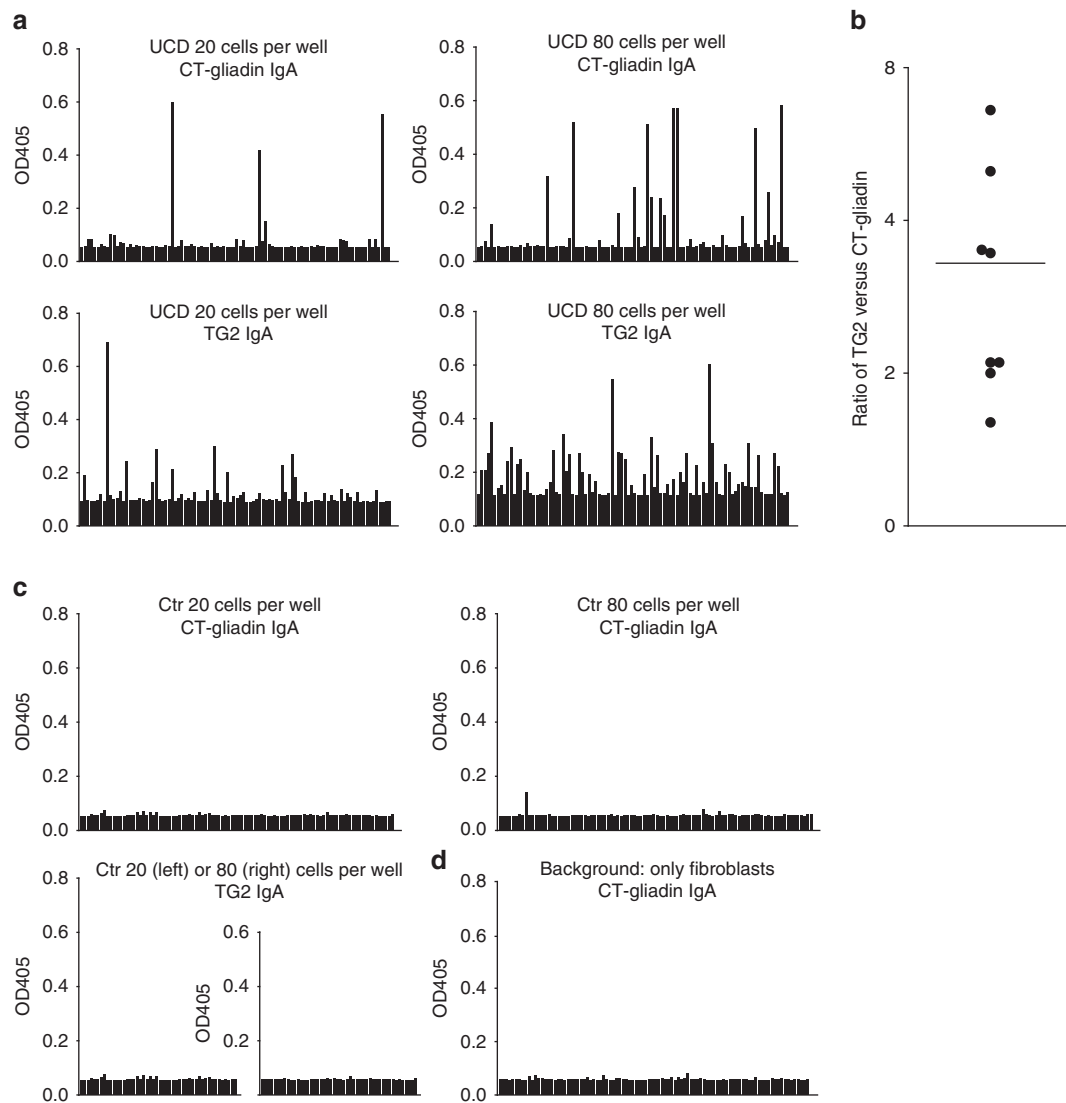


Figure 2 | Supernatant reactivity to CT-gliadin and TG2 by ELISA. IgA reactivity to antigen in representative cultures of SCSs from **(a)** subjects with UCD. **(b)** The ratio of culture supernatants with IgA reactivity to TG2 versus CT-gliadin, where the dots represent different subjects with UCD ($n=8$). Horizontal bar indicates mean value. **(c)** IgA reactivity to antigen in one representative of two tested non-CD controls (Ctr). **(d)** The background level was defined by signal in supernatants of cultures of fibroblasts only. In the headline are shown the number of cells of SCSs per culture well and antigen used as coating antigen in ELISA. Each column represents one well.

The specificity of all hmAbs reactive to one of the two synthetic peptides in ELISA was further characterized in AlphaLISA. In contrast to ELISA, where synthetic peptide was used as a coating antigen, the AlphaLISA format detected monovalent binding of hmAbs to soluble biotinylated synthetic peptide. Three of the hmAbs (UCD1002 1D03, UCD1143 1E01, UCD1130 4A04), originally from IgA⁺ PC sorted with tetramers of PLQPEQFPF and reactive to PLQPEQFPF in ELISA, were not reactive in AlphaLISA. One of the hmAbs (UCD1130 3B04) from IgA⁺ PC sorted with 33-mer and reactive to deamidated 33-mer in ELISA was not reactive in AlphaLISA. These four antibodies, not reactive in AlphaLISA, gave the lowest signals in the peptide-based ELISA and with signals that were lower than to the CT-gliadin antigen (Supplementary Fig. 2), suggesting that the synthetic peptides may not represent the complete epitope(s) of these four antibodies.

Polyreactivity was assessed by using cell lysate as well as a mixture of Jo-1, TG2, CpG and LPS as competing antigens. In either case, the binding of hmAbs to the synthetic peptides was

maintained (Fig. 5a). In contrast, the signal was blocked by CT-gliadin (Fig. 5a). We conclude that the hmAbs reactive to PLQPEQFPF or deamidated 33-mer are specific to gliadin and not polyreactive.

The reactivity of hmAbs to deamidated versus native gliadin was tested in a competitive AlphaLISA assay. Binding of hmAbs to biotinylated synthetic deamidated gliadin peptide (either PLQPEQFPF or the deamidated 33-mer) was tested against the homologous peptide in either deamidated or native (non-deamidated) versions. Gliadin-specific hmAbs divided into two groups, either only reactive to deamidated gliadin or reactive with both deamidated and native gliadin (Fig. 5b). None of the hmAbs had higher reactivity to native than to deamidated gliadin. Because some of the hmAbs were not reactive to the native gliadin peptide, this suggests that induction of gliadin-specific B cells *in vivo* likely happens in the presence of deamidated gliadin.

Gliadin-specific hmAbs bind gliadin peptides with shared sequences. We observed that some of the hmAbs were reactive

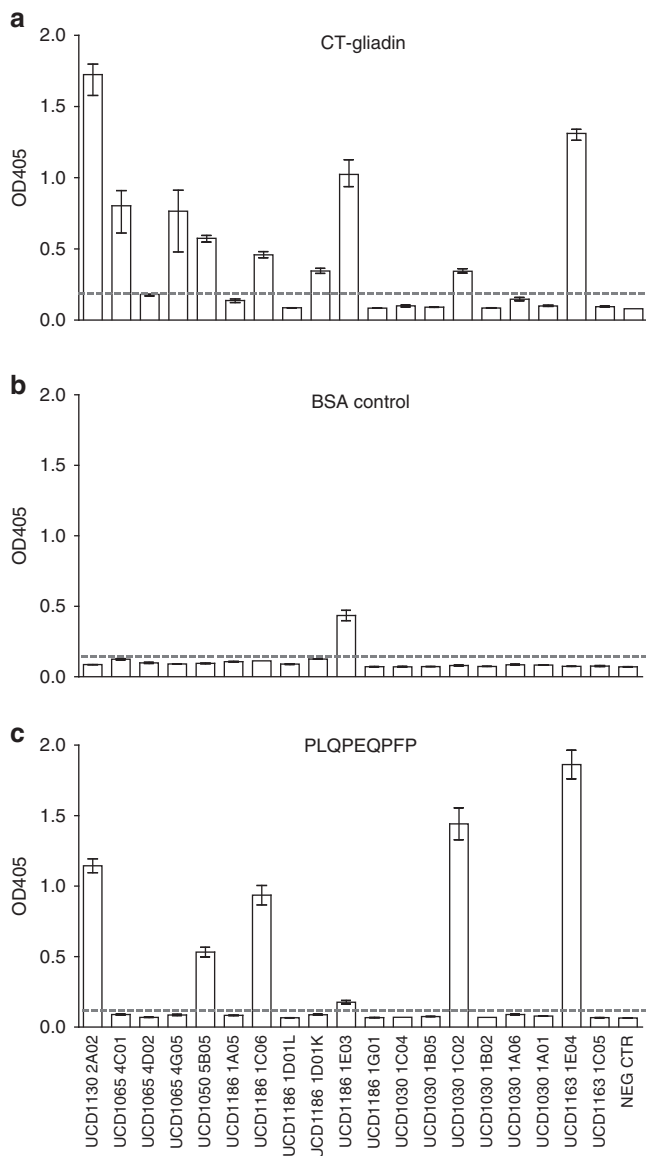


Figure 3 | ELISA reactivity of 19 hmAbs expression cloned from IgA⁺ PCs in culture. The antigens used for ELISA coating were CT-gliadin and PLQPEQFPF, and BSA for control as shown in headlines. Values are given as median and range of triplicates of hmAbs tested. The same hmAbs are tested for reactivity to all three antigens, and each hmAb is given the same position in each of the three graphs. The names of the hmAbs are depicted in **3c**. Horizontal bars denote cutoff defined by negative control hmAb (NEG CTR). **(a)** Nine of 19 hmAbs were reactive to CT-gliadin. **(b)** One of nine hmAbs reactive to CT-gliadin was also reactive to BSA. **(c)** Five of eight hmAbs reactive to CT-gliadin but not BSA were also reactive to PLQPEQFPF.

with both PLQPEQFPF and the deamidated 33-mer in AlphaLISA (Fig. 6a). Eight of the hmAbs originally from IgA⁺ PC sorted with tetramers of PLQPEQFPF were reactive to deamidated 33-mer (Supplementary Table 1). Of the hmAbs from IgA⁺ PC sorted with deamidated 33-mer, eleven were reactive to PLQPEQFPF (Supplementary Table 1). This suggests that at least some of the gliadin-specific PCs secrete antibodies that cross-react with different gliadin peptides. To test this further, we investigated whether a few selected synthetic gliadin peptides could compete with the antibody binding to biotinylated PLQPEQFPF. We tested the hmAbs (UCD1143 3B02 and

UCD1002 1B06) both of which were reactive with PLQPEQFPF but not PLQPQFPF (Supplementary Table 1). For both hmAbs, the signals were inhibited by peptides containing QPEQ or PEQP. Peptides without these sequences were non-inhibitory (Fig. 6b). Thus, the cross-reactivity of hmAbs to different gliadin peptides appears to be due to sharing of key sequences. Reactivity of many hmAbs to PLQPEQFPF and deamidated 33-mer, both harbouring the QPEXP (X = Q, L) and QFPF sequences, is consistent with this notion.

Binding to PLQPEQFPF of hmAbs is blocked by patient sera.

We wanted to see if binding of gliadin-specific hmAbs to synthetic gluten peptide was blocked by sera from patients with active CD. In a preliminary study, sera were incubated with biotinylated PLQPEQFPF before incubation with AlphaLISA beads conjugated with two hmAbs UCD1114 1F03 and UCD1143 3B02. Sera from patients with CD blocked the signal, while sera from control subjects did not (Fig. 7). This suggests that gliadin-specific antibodies from serum and IgA of gliadin-specific PCs in the lamina propria are specific to the same gliadin B-cell epitopes. This is also a promising result as a proof of concept that hmAbs derived from antigen-specific human B cells can be used to establish improved serologic assays.

Repertoire of gliadin-specific IgA⁺ PCs. The eight hmAbs from *in vitro*-cultured PCs with CT-gliadin were obtained from six subjects (UCD1050, 1; UCD1130, 1; UCD1065, 2; UCD1186, 2; UCD1163, 1; UCD1030, 1). The 30 hmAbs derived from single-sorted PCs using tetramerized synthetic peptides, originated from five different subjects (UCD1002, 7; UCD1079, 2; UCD1114, 2; UCD1130, 16; UCD1143, 3). Control IgA⁺ PCs, defined as PLQPEQFPF-negative, 33-mer-negative and TG2-negative (UCD1130, 55; UCD1143, 54) and TG2-specific PCs (UCD1030, 3; UCD1114, 4; UCD1010, 2), were isolated by flow cytometry and their antibody genes were cloned and sequenced. The variable regions of the hmAbs were analysed using tools of the IMGT webpage (www.imgt.org). Two combinations of VH/VL pairing were dominant (Fig. 8a); VH3-15/VK4-1 (seven hmAbs from five different subjects in total) and VH3-23/VL4-69 (15 hmAbs from seven different subjects in total). The VH3-23/VL4-69 combination was found in hmAbs from IgA⁺ PCs isolated with both CT-gliadin and synthetic peptides. In addition, VH3-23/VK3-11 was also found in hmAbs generated by both methods. Together, these three combinations made up ~75% of the panel of gliadin-specific hmAbs (Fig. 8a, Supplementary Table 1). We looked for similarity between heavy chain CDR3 sequences of the VH3-15/VK4-1 as well as the VH3-23/VL4-69 hmAbs. No obvious similarities could be found with variability in D- and J-gene segment usage and CDR3-length among the antibodies of each group (Supplementary Table 1).

VH/VL usage and epitope specificity. We next compared VH/VL usage (Supplementary Table 1) with peptide reactivity pattern characterized in AlphaLISA. All VH3-23/VL4-69 hmAbs showed reactivity to both native and deamidated gliadin, demonstrated by inhibiting effect of both the native PLQPQFPF and the deamidated PLQPEQFPF. The VH3-15/VK4-1 hmAbs were specific to deamidated gliadin, as the native PLQPQFPF showed no inhibitory effect on the binding to PLQPEQFPF. VH3-23/VK3-11 hmAbs were the only antibodies with reactivity to deamidated 33-mer but not PLQPEQFPF. Two hmAbs had reactivity to CT-gliadin but neither to PLQPEQFPF nor to deamidated 33-mer. These two antibodies both used VH3-15/VK3-20. Taken together, this suggested that there is a correlation between VH/VL usage and epitope specificity of the gliadin-specific antibodies.

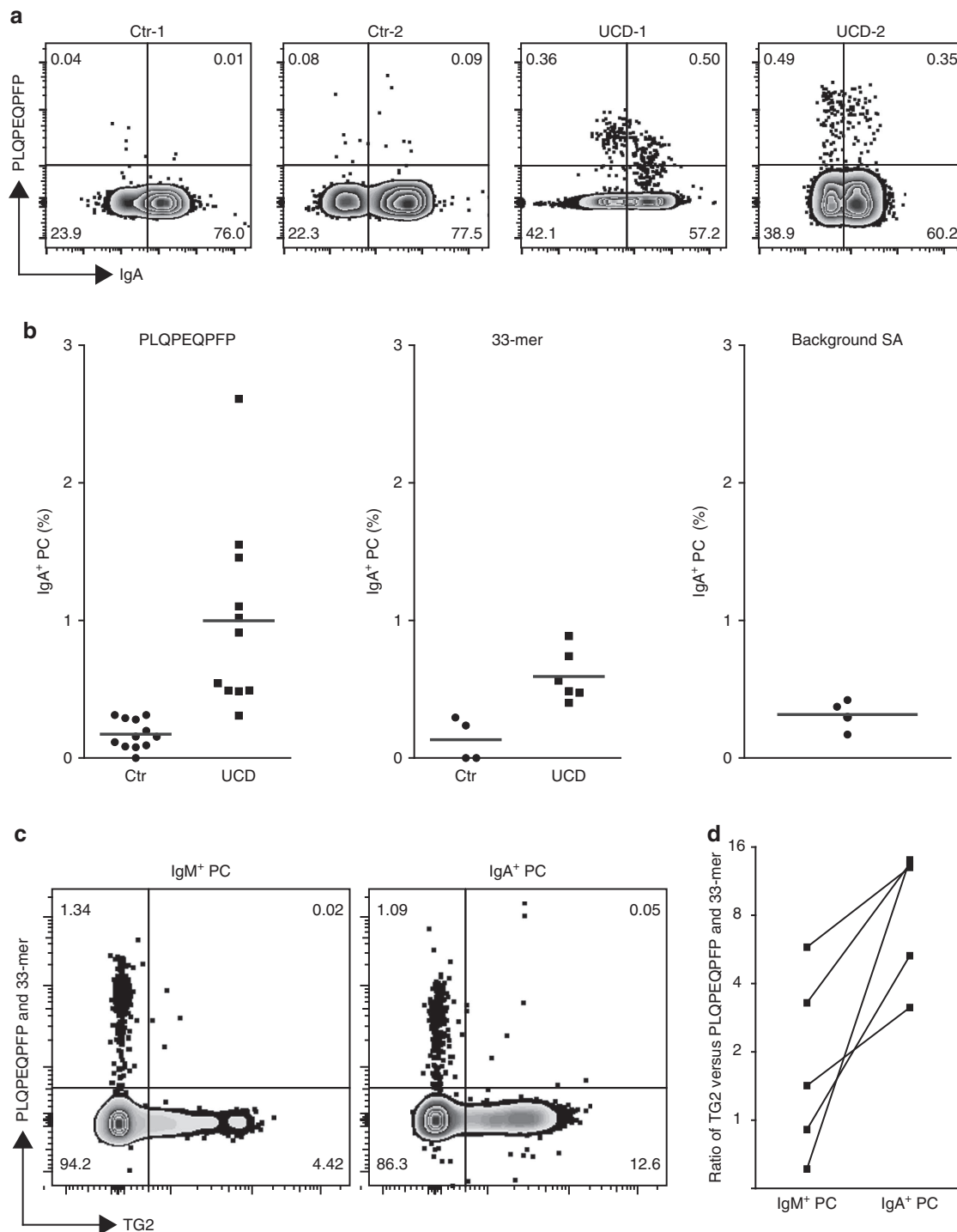


Figure 4 | Staining of intestinal PCs with tetramers of synthetic gluten peptides or TG2 in flow cytometry. (a) Representative plots of PCs from SCSs stained with APC-conjugated streptavidin in complex with biotinylated PLQPEQFPF peptide. The IgA-negative events are mainly IgM⁺ PCs. The four plots represent two control patients (Ctr) and two subjects with UCD. (b) Frequency of IgA⁺ PCs stained positive with peptide in percentage of total number of IgA⁺ PCs. The peptide used as antigen is given in headline. Each dot represents one subject, and mean values are indicated by horizontal bars. 'Background SA' represents cells stained with APC-streptavidin alone. (c) Representative plots of one subject with UCD of IgM⁺ PCs and IgA⁺ PCs from SCSs stained with biotinylated peptides in complex with APC-conjugated streptavidin and biotinylated TG2 in complex with PE-conjugated streptavidin. (d) Ratio of PCs stained positive with TG2 compared with synthetic gliadin peptides. IgM⁺ PCs and IgA⁺ PCs of the same sample are connected by lines.

SHM within gliadin-specific IgA⁺ PCs. The mutation rates in the hmAbs from PCs secreting IgA reactive with CT-gliadin (median 8.7, range 3–13, $n = 8$) as well as IgA⁺ PCs isolated with synthetic gliadin peptides (median 7.5, range 0–23, $n = 30$) were lower than in the control PCs (median 15, range 0–37, $n = 109$)

(Fig. 8b). TG2-specific IgA⁺ PCs were scarcely mutated as previously described⁵ (median 4, range 0–15, $n = 9$).

Different VH genes are associated with various levels of SHM²¹. The mutation rate in gliadin-specific IgA with VH3-23 was significantly lower than the number of mutations observed in

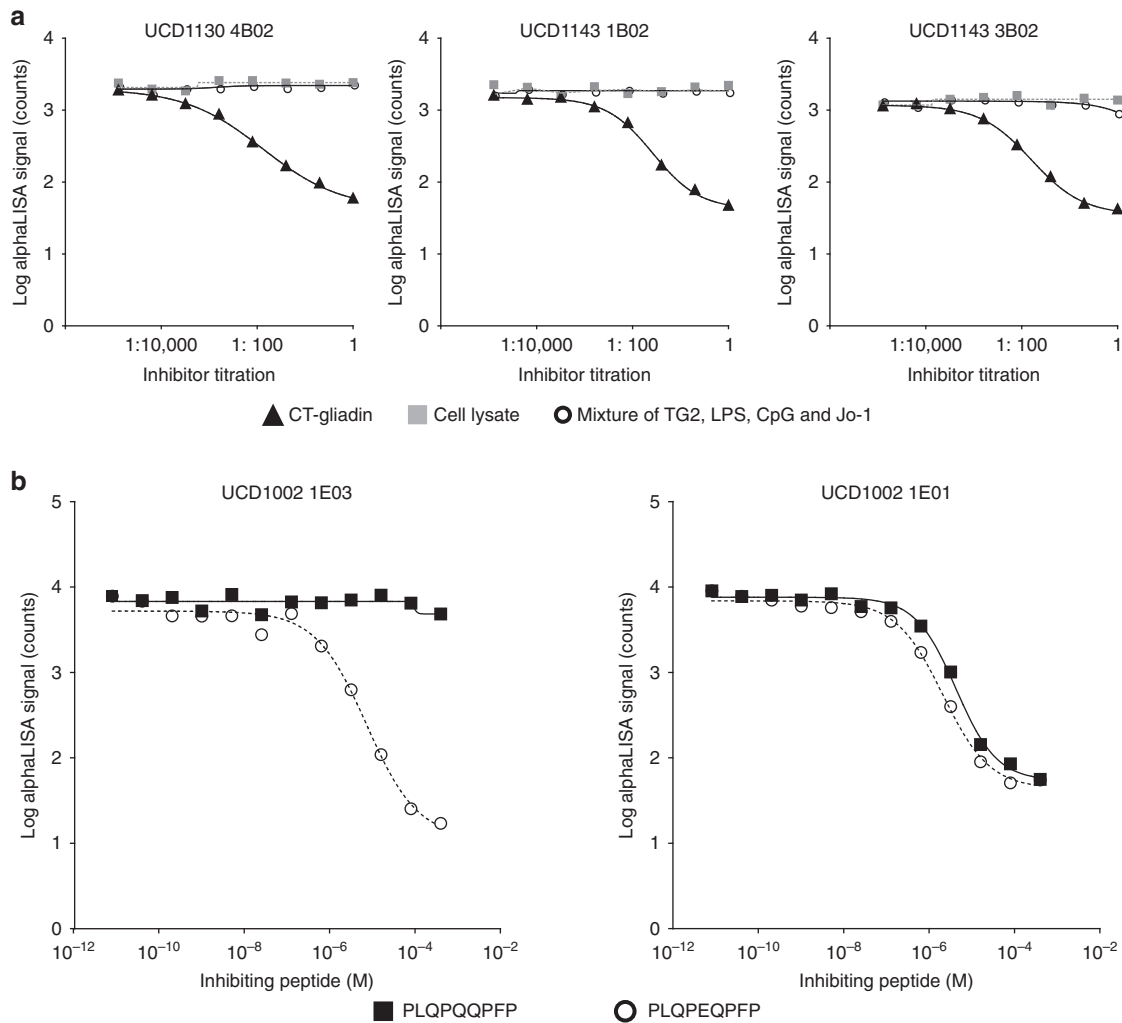


Figure 5 | AlphaLISA characterization of gliadin-reactive hmAbs. (a) Antibody reactivity to constant concentration of biotinylated PLQPEQFPF in the presence of competing antigens. The starting concentrations of competing antigens are CT-gliadin $10 \mu\text{g ml}^{-1}$, cell lysate $1 \text{ ml cells ml}^{-1}$, TG2 $200 \mu\text{g ml}^{-1}$, LPS $250 \mu\text{g ml}^{-1}$, CpG $100 \mu\text{g ml}^{-1}$ and Jo-1 $200 \mu\text{g ml}^{-1}$. Only CT-gliadin blocked the signal, while cell lysate or a mixture of TG2, LPS, CpG and Jo-1 did not. (b) Reactivity to deamidated gliadin versus native gliadin. Representative plots of two hmAbs binding to constant concentration of biotinylated PLQPEQFPF in competition with various concentrations native PLQPQQPFP or the deamidated PLQPEQFPF was measured. The concentrations of competitive peptide are depicted on x axis.

VH3-23 from control population (Fig. 8c). We conclude that the low mutation rate in gliadin-specific IgA is not a result of selected VH usage.

Discussion

We previously reported that IgA antibodies of TG2-specific PCs found in coeliac lesions have limited SHM⁵. In the present study, we have characterized gluten (gliadin)-reactive antibodies expression cloned from IgA⁺ PCs isolated from small intestinal biopsies. We found that there is also a limited mutation rate in these antibodies, demonstrating an unexpected common feature of the IgA antibody responses to gluten and TG2 in CD.

The number of mutations in antibody genes relates to the type of B-cell response elicited²². T-cell-independent B-cell responses have no or little SHM with little restriction of VH usage as often seen in T cell-dependent responses²³. T-cell-dependent B-cell responses that develop in germinal centers typically result in highly mutated antibodies²⁴. GC reactions may also happen without the involvement of T cells, but then with low level of

SHM²⁵. Low level of SHM is also seen in T-cell-dependent extrafollicular responses²⁶ and in germinal center responses of short duration²⁷.

For the TG2-specific IgA antibodies, the low mutation rate was suggested to relate to enzymatic activity of B-cell receptor bound TG2 (ref. 5) or to the early termination of autoreactive B cells in GCs or self-antigen driving an extrafollicular response, similar to what has been reported in other systems^{22,28,29}. None of these mechanisms should apply to IgA antibodies of gluten-specific PCs, and one could therefore expect that these PCs should have similar levels of SHM compared with control PCs. This is not what we observed. The mutation rate in IgA of gluten-specific PCs was low and similar to TG2-specific PCs. This suggests that the factor(s) causing the limited SHM in IgA of TG2-specific PCs is likely involved in both antibody responses.

The parallel fluctuation of antibodies to gluten and TG2 in response to dietary gluten¹⁰ suggests that the production of these antibodies is regulated in a coordinated way. In keeping with this notion, it has been suggested that gluten-specific T cells could provide help to TG2-specific B cells by means of complexes gluten-TG2 acting as hapten-carrier complexes^{30,31}. This model

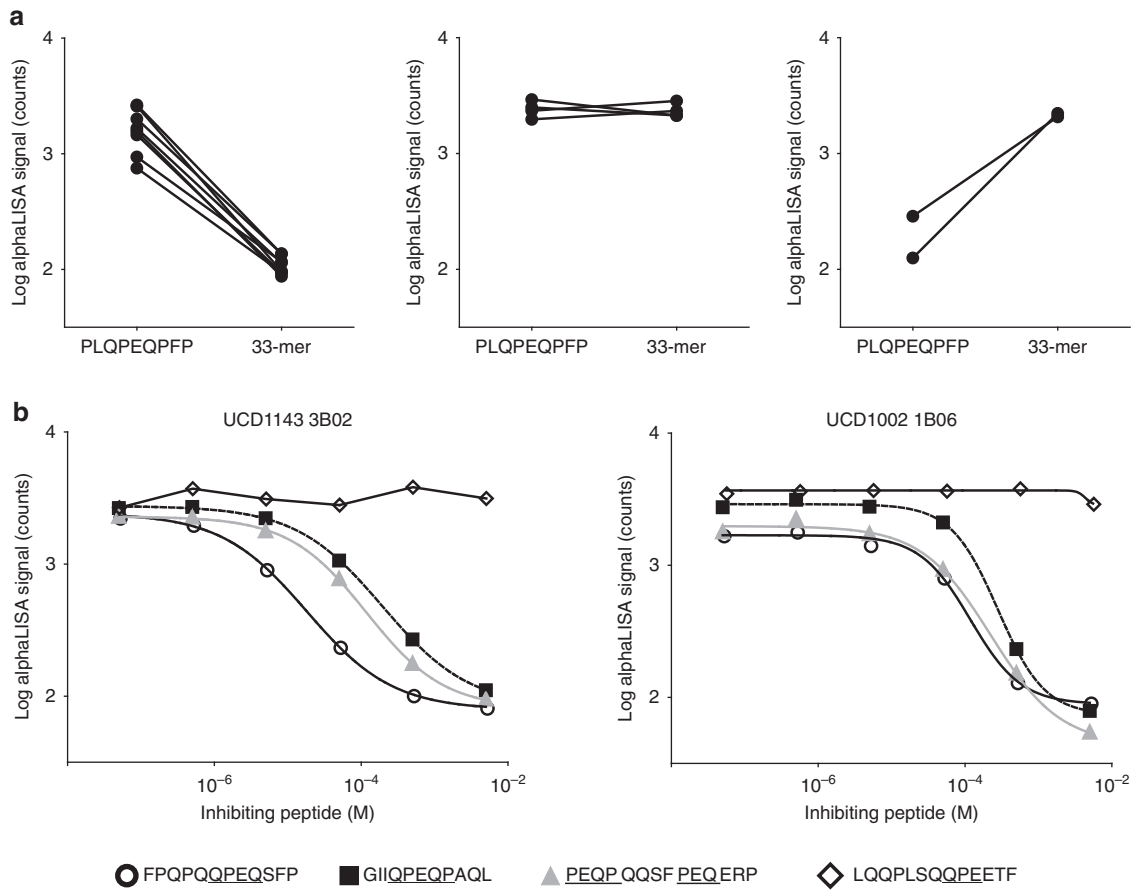


Figure 6 | Epitopes of gliadin-specific hmAbs. (a) Reactivity of hmAbs to PLQPEQFPF and 33-mer. Reactivity of each hmAb is connected by line. Antibodies are grouped according to their reactivity pattern with reactivity to only one of the two peptides (left, right) or cross-reactive with both peptides (middle). (b) Reactivity of two representative hmAbs to constant concentration of biotinylated PLQPEQFPF in competition with four different competitive synthetic gliadin peptides. The amino-acid sequences of competitive peptides are shown and the concentrations of competitive peptides are depicted on x axis. Amino-acid sequences shared with the PLQPEQFPF selecting peptide are underlined.

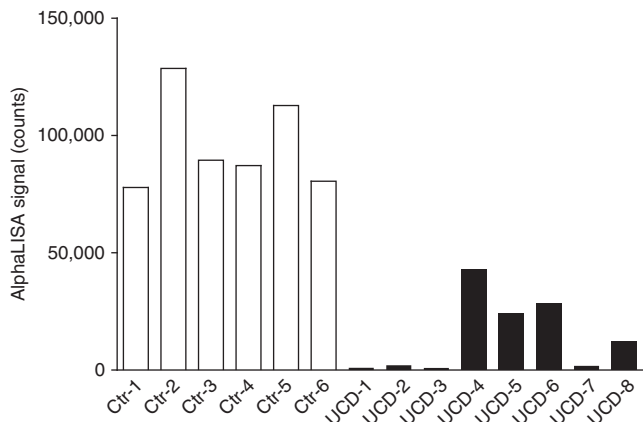


Figure 7 | AlphaLISA anti-PLQPEQFPF immunoglobulin inhibition assay. Reactivity of hmAbs UCD1114 1F03 and UCD1143 3B02 to constant concentration of biotinylated PLQPEQFPF in the presence of sera from either UCD or control subjects (Ctr). The signal was blocked by sera from UCD patients but not from control subjects.

would explain why the TG2 antibodies are produced upon gluten exposure, and it would also explain why only individuals who express HLA-DQ2 or HLA-DQ8 make these antibodies¹⁶. Gluten-specific T cells obviously also could provide help to

gluten-specific B cells. As gluten-specific T cells preferentially recognize deamidated gluten peptides³², B cells with surface immunoglobulin that bind and internalize deamidated gluten peptides would be better situated to receive T-cell help. Common sequences of the two gliadin peptides used to isolate IgA⁺ PCs, are found in or adjacent to many T-cell epitopes in gluten proteins. The QPEXP (X=Q, L) sequence is found in the DQ2.5-glia- α 1a/b, DQ2.5-glia- α 2, DQ2.5-glia- γ 2, DQ2.5-glia- γ 3, DQ2.5-glia- γ 4c/d, DQ2.5-glia- ω 1, DQ2.5-glia- ω 2, DQ2.5-hor-1, DQ2.5-hor-2, DQ2.5-sec-1, DQ2.5-sec-2 and DQ8-glia- γ 1a/b epitopes whereas the PFPF sequence is found in the DQ2.5-glia- γ 4c, DQ2.5-glia- γ 5, DQ2.5-glia- ω 2, DQ2.5-hor-2, DQ2.5-sec-2 and DQ8-glia- γ 1a epitopes³². The low mutation rate in TG2- and gluten-specific PCs compared with other intestinal PCs, suggests that a common factor distinguishing the TG2- and gluten-specific PCs from most other gut PCs could be implicated. Based on the model referred above, both gluten and gluten-reactive T cells could be such common factors.

Gluten is a protein antigen, which in contrast to most antigens of the gut has no bacterial or viral origin. Lack of strong concomitant innate signals along with B-cell receptor triggering could result in extrafollicular response, in accordance with a study of the B-cell response to a T cell-dependent antigen, where immunization without adjuvant was insufficient for GC formation³³. Analysis of mutation rate of antibodies to other dietary proteins would shed light on this and should be examined in the future.

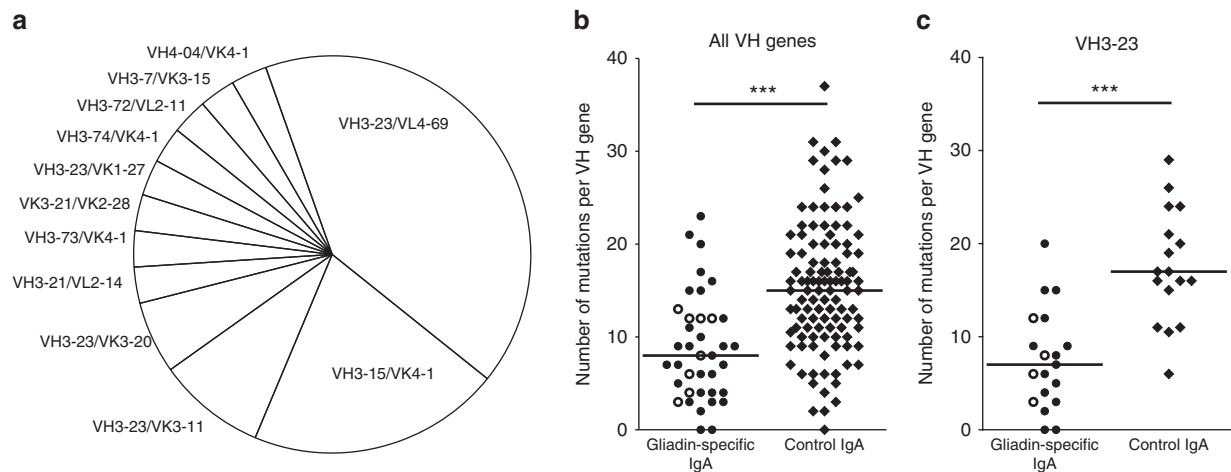


Figure 8 | VH and VL usage and SHMs. (a) VH/VL usage of 38 gliadin-specific hmAbs from PCs either isolated by culture or by flow sorting. (b) Frequencies of VH region somatic mutations (y axis) per sequence in the populations indicated on x axis. 'Gliadin-specific IgA' represents hmAbs from PCs sequestered after *in vitro* culture (open circles) and IgA⁺ PCs isolated with synthetic gliadin peptides (black dots). 'Control IgA' represents sequences derived from intestinal IgA⁺ PCs sorted as TG2-negative and PLQPEQFP/33-mer-negative from two subjects with untreated CD (UCD1130 and UCD1143). Each point represents a single IgA sequence. (c) Comparison of mutations in VH3-23 genes. Horizontal bars indicate median values and *P*-values were obtained by Student's *t*-test. NS > 0.05. ****P* < 0.001.

Extrafollicular responses typically give rise to short-lived PCs and rapid decline in serum antibodies after the immune response in addition to low SHM³⁴. TG2-specific PCs were rarely detected in small intestine of CD subjects on a gluten-free diet⁵, and levels of serum IgG and IgA antibodies to both gluten and TG2 typically fall below detection level months after the patient commence a gluten-free diet¹⁰. These clinical observations, in addition to our observations of limited SHM in gluten-specific and TG2-specific IgA⁺ PCs, argue for an extrafollicular origin of gluten and TG2-specific antibodies in CD.

T cells influence extrafollicular development of B cells. It has been reported that T cells located at the T-B border in lymph nodes, phenotypically different from GC T cells, are necessary for B-cell priming to form extrafollicular antibody responses³⁵. Sustained CD40 signalling during B-cell and T-cell interaction has been demonstrated to induce a plasma cell fate rather than GC B-cell development³⁶, and administration of a CD40 agonistic antibody was shown to ablate GC reaction and induced a pattern of extrafollicular B-cell differentiation³⁷.

Not only the quality, but also the degree of T-cell help could be a relevant factor. Excessive numbers of follicular helper T cells as seen in several mouse models, like the 'san' mutation in the RNA binding protein Roquin³⁸ and PD-1 deficiency³⁹, drive survival of GC B cells with a low degree of mutation and affinity and cause increased autoreactivity possibly due to a lowered threshold for GC B-cell selection⁴⁰. Interestingly, gluten-reactive T cells of CD patients produce large amounts of interferon- γ ⁴¹ and in mice, excess of interferon- γ has been demonstrated to promote accumulation of both follicular helper T cells and GC B cells and to trigger autoimmunity⁴². It may thus be that TG2- and gluten-reactive B cells in such a setting may develop in GC and not extrafollicularly.

Characterization of the specificity of antibodies produced by PCs reactive with gluten should give valuable insights into the pathogenesis in CD. We thus characterized the panel of hmAbs by AlphaLISA assays. First, we found that some antibodies are specific to deamidated gliadin peptides and do not recognize non-deamidated counterparts. Inevitably, gliadin-specific B cells must have encountered deamidated gliadin. Second, we found that gliadin-specific antibodies frequently cross-react with different gliadin peptides. Accordingly, gliadin-specific B cells may take up

and display several different T-cell epitopes. This may be beneficial if a B cell receives help from several different T-cell clones, as recently described⁴³.

In addition to the limited SHM, we observed a restricted VH/VL usage in the panel of gluten-specific antibodies. Limited VH usage is a sign of T-cell-dependent responses²³. Notably, the same VH/VL pairings appear in different subjects. This phenomenon is similarly seen in TG2-specific antibodies where VH restriction is observed in different patients⁵. The VH usage of TG2-specific antibodies was recently demonstrated to correlate with epitope specificity⁴⁴. We observed a similar phenomenon for the gliadin-specific hmAbs. Antibodies specific to deamidated variants of gluten peptides seemed to have different VH/VL usage than antibodies with reactivity to both the deamidated and native variants. Such VH/VL restriction is by no means unique to CD and has been reported in antibodies specific for influenza⁴⁵ and HIV⁴⁶ antigens. For HIV antibodies, there has even been reported an allelic preference for antibody usage⁴⁶. A deeper analysis may reveal similar allelic preferences of the antibody responses to TG2 and gluten in CD. Restricted VH/VL usage and limited SHM in antibodies as observed in CD would favour influence of VH and/or VL polymorphisms in shaping the antibody response. This could speak for the involvement of VH and VL genes as susceptibility loci in CD, even though these genes have not been indicated by recent genome-wide association studies in humans⁴⁷. Notably in mice, experiments with congenic strains demonstrated that the antibody response to gliadin is chiefly controlled by the MHC and the immunoglobulin heavy chain loci⁴⁸.

Interestingly, the hmAbs generated can be used to develop diagnostic assays detecting patient serum antibodies reactive with disease-relevant gluten epitopes. Given that the antibodies are derived from PCs of the CD lesion, inhibition assays reflecting the specificity of the hmAbs may potentially perform better than assays used today. The hmAbs could potentially also be used for detection of gluten contamination in gluten-free food.

In summary, our findings of restricted mutation rates in both IgA antibody responses to gluten and TG2 in CD are surprising. We suggest that a common factor important for gluten-specific and TG2-specific B cells, possibly gluten antigen or gluten-specific T cells, is responsible for causing low mutation rates in

streptavidin donor bead solution was diluted 1:200 in AlphaLISA Immunoassay buffer and 15 μ l added per well. The plate was read after 30 min incubation at RT in the dark.

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Acknowledgements

This work is supported by the Research Council of Norway, the South-Eastern Norway Regional Health Authority, the European Research Council (ERC-2010-Ad-268541), the United European Gastroenterology Research Prize (to L.M.S.) and by the National Institutes of Health/National Institute of Allergy and Infectious Diseases (U19 AI082724-01, R01 AI76585-03, and HHSN266200500026C) to (P.C.W.). We thank Marie K. Johannesen and Bjørn Simonsen for excellent technical assistance and staff at the Flow Cytometry Core Facility and the Endocopy Unit at the Oslo University Hospital-Rikshospitalet for kind help. We thank Omri Snir and Mats Bemark for critical reading of the manuscript.

Author contributions

L.M.S. conceived the project, L.M.S. and P.C.W. managed the project, Ø.S., C.J.H.D., M.H., L.M. and M.S.-F. did the experiments, K.E.A.L. and J.J. provided biological material, L.M.S. and Ø.S. wrote the manuscript with input from co-authors.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: Ø.S., L.M.S., C.J.H.D. and P.W. have submitted a patent application covering gliadin-specific monoclonal antibodies. The remaining authors declare no competing financial interests.

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How to cite this article: Steinsbø, Ø. *et al.* Restricted VH/VL usage and limited mutations in gluten-specific IgA of coeliac disease lesion plasma cells. *Nat. Commun.* 5:4041 doi: 10.1038/ncomms5041 (2014).



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