UNITED EUROPEAN GASTROENTEROLOGY ueg journal

Original Article

United European Gastroenterology Journal
2014, Vol. 2(4) 268–278

DOI: 10.1177/2050640614540154
ueg.sagepub.com
SAGE

Tetramer-visualized gluten-specific CD4+ T cells in blood as a potential diagnostic marker for coeliac disease without oral gluten challenge

Asbjørn Christophersen[1], Melinda Ráki[1], Elin Bergseng[1], Knut EA Lundin[1,2], Jørgen Jahnsen[3], Ludvig M Sollid[1] and Shuo-Wang Qiao[1]

Abstract

Background: Diagnosing coeliac disease (CD) can be challenging, despite highly specific autoantibodies and typical mucosal changes in the small intestine. The T-cell response to gluten is a hallmark of the disease that has been hitherto unexploited in clinical work-up.

Objectives: We aimed to develop a new method that directly visualizes and characterizes gluten-reactive CD4+ T cells in blood, independently of gluten challenge, and to explore its diagnostic potential.

Methods: We performed bead-enrichment of DQ2.5-glia-α1a and DQ2.5-glia-α2 tetramer+ cells in the blood of control individuals, treated (TCD) and untreated patients (UCD). We visualized these cells by flow cytometry, sorted them and cloned them. We assessed their specificity by antigen stimulation and re-staining with tetramers.

Results: We detected significantly more gliadin-tetramer+ CD4+ effector memory T cells (T_{EM}) in UCD and TCD patients, compared to controls. Significantly more gliadin-tetramer+ T_{EM} in the CD patients than in controls expressed the gut-homing marker integrin-β7.

Conclusion: Quantification of gut-homing, gluten-specific T_{EM} in peripheral blood, visualized with human leukocyte antigen (HLA) -tetramers, may be used to distinguish CD patients from healthy individuals. Easy access to gluten-reactive blood T cells from diseased and healthy individuals may lead to new insights on the disease-driving CD4+ T cells in CD.

Keywords

Blood test, celiac disease, diagnostic marker, diagnostics, gliadin, gluten, gluten reactivity, human leukocyte antigen, histology, immunology, T cells

Received: 19 February 2014; revised: 22 April 2014; accepted: 22 May 2014

Introduction

Coeliac disease (CD) is an inflammatory condition of the small intestine caused by intolerance to proline- and glutamine-rich cereal gluten proteins.[1] In wheat, gluten consists of several hundred distinct but similar proteins that can be classified into gliadins and glutenins.[2] CD is often detected after demonstration of autoantibodies specific for the enzyme transglutaminase 2 (TG2).[3] Children can be diagnosed with CD if the TG2 antibody titer is high, added by further laboratory tests[4]; however, in adults, duodenal biopsy and detection of typical histological changes remains a diagnostic premise.[5]

Despite clear diagnostic criteria,[5] diagnosing CD can be difficult and false negative tests are a problem. Autoantibodies can be present in tissues only, but not detectable in blood.[6] In other instances, the diagnosis cannot be made because of minor or no changes in the duodenal mucosa, despite elevated TG2 antibodies.[7] Some of these individuals will develop further histological changes and overt disease that can only be diagnosed if gastroduodenoscopy is repeated.[7]

The T-cell response to gluten is essential in the immunopathogenesis of CD.[8] CD4+ T cells that

[1]Centre for Immune Regulation and Department of Immunology, University of Oslo and Oslo University Hospital – Rikshospitalet, Oslo, Norway
[2]Department of Transplantation Medicine, Division of Cancer Medicine, Surgery and Transplantation, Oslo University Hospital – Rikshospitalet, Oslo, Norway
[3]Department of Gastroenterology, Oslo University Hospital – Ullevål, Oslo, Norway

Corresponding author:
Asbjørn Christophersen, Department of Immunology, PO Box 4956 Nydalen, 0424 Oslo, Norway.
Email: Asbjorn.Christophersen@rr-research.no

recognize distinct gluten epitopes in the context of the disease-associated human leukocyte antigen (HLA) molecules DQ2.5, DQ8 and DQ2.2 can be detected in the gut mucosa of CD patients, but not of healthy controls.[9,10] For HLA-DQ2.5, which is expressed by the great majority of CD patients, two epitopes of α-gliadin (DQ2.5-glia-α1a and DQ2.5-glia-α2) are among the immunodominant epitopes.[11]

Monitoring of the T-cell response to gluten has not been applied in the diagnostic work-up of CD patients. The clinical value of detecting gluten-reactive T cells in the gut of CD patients[9,12] is limited by the need of a gastroduodenoscopy. CD4+ T cells recognizing CD-relevant epitopes can also be detected in peripheral blood mononuclear cells (PBMC) using enzyme-linked immunospot- or tetramer-based assays; however, this has so far only been possible in treated CD patients (TCD) after a 3-day oral gluten-challenge.[13,14] Notably, gluten-specific T cells are not detectable above background in healthy controls, untreated CD patients (UCD) or TCD without gluten-challenge.

In the current study, we did bead-enrichment of gliadin-tetramer+ cells to increase the sensitivity for detection of gluten-reactive T cells. By doing so, we were able to track CD4+ T cells reactive to DQ2.5-glia-α1a and DQ2.5-glia-α2 of three T-cell subsets: naïve (T_N), central memory (T_{CM}) and effector memory (T_{EM}) cells. T_N are preimmune cells that can differentiate into memory T cells, if encountering a corresponding antigen. Memory T cells are categorized by homing markers and cytokine production into T_{EM} that act at the site of inflammation, and T_{CM} that can migrate to lymphoid tissues.[15] We found significantly more gliadin-tetramer+ CD4+ T_{EM} in the blood of CD patients than controls. These cells were gut-homing and were found in larger numbers in CD patients with severe duodenal changes, compared to CD patients with normal mucosa. The protocol gives easy access to gluten-reactive T cells from affected and healthy individuals. Further studies on these cells may extend our understanding of one of the key players in CD pathogenesis. Our findings demonstrated that this T-cell based, minimally invasive, ex-vivo assay has potential in the diagnosis of CD.

Methods

Subjects and ethical aspects

As detailed in Table 1, 20 UCD, 18 TCD and 16 control individuals acceded to the study. They were genomically HLA-typed and we report only the commonly CD-associated HLA-types (DQ2.5 = DQA1*05 and DQB1*02; DQ2.2 = DQA1*02:01 and DQB1*02; and DQ8 = DQA1*03 and DQB1*03:02). We obtained blood from 14 DQ2.5+ controls through the blood bank at Oslo University Hospital, Norway. These individuals were anonymous. We have no data on their diet, biomarkers or clinical state; but diagnosed CD is an exclusion criterion for blood donation. All other participants were patients who, after giving informed written consent, donated additional blood for research purposes in conjunction to duodenal biopsies and routine clinical follow-up at the Oslo University Hospital. Patients were diagnosed according to statements from the American Gastroenterological Association (AGA).[5] Each TCD had been on a gluten-free diet (GFD) for 3 months or more. The study was approved by the regional ethics committee (S-97201).

Tetramers

Soluble, biotinylated DQ2.5 (DQA1*05:01, DQB1*02:01) molecules covalently linked with the gluten-derived T-cell epitopes DQ2.5-glia-α1a (QLQPFPQPELPY, with underlined 9mer core sequence) or DQ2.5-glia-α2 (PQPELPYPQPE) were multimerized on phycoerythrin (PE)-labeled streptavidin (Invitrogen) or allophycocyanin (APC)-labeled streptavidin (ProZyme).[16] Cells were incubated with the tetramers (300 µl; 10 µg/ml each) at room temperature, for 40 minutes.

Cell enrichment

We obtained 50–100 ml of citrated full blood, or 60 ml of citrated buffy coat produced from 450 ml of full blood, from each participant. We isolated the PBMC by density gradient centrifugation (Lymphoprep; Axis-Shield), and further handled in a buffer containing phosphate-buffered saline (PBS), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% human serum.

We followed an established protocol for enrichment of tetramer-positive cells.[17] Briefly, PBMC were counted and incubated with FcR blocking reagent (Miltenyi Biotec) before the PE- and APC-conjugated tetramers were added. The cells were washed and a small fraction removed for later staining as a 'pre-enriched sample' before we added the anti-PE- and anti-APC microbeads (Miltenyi Biotec). We then washed the cells, re-counted them and passed them over a magnetized column (MS or LS column, Miltenyi Biotec). We collected the cells that did not bind the column as 'depleted cells'.

Flow cytometry

The enriched cells were eluted and all samples were stained 20 minutes on ice at a volume of 25 µl. The following antibodies were used: CLA-FITC, Integrin-β7-PE, CD62L-PerCP/Cy5.5, CD14-Pacific blue,

Table 1. Characteristics of participants

Participant	Category[a]	HLA-type	Anti-TG2 < 5 U/mL[b]	Marsh-score	EM/N-ratio α1a	EM/N-ratio α2
P1	Control	DQ2.5	ND	ND	0.0	0.2
P2	Control	DQ2.5	ND	ND	6.8[d]	
P3	Control	DQ2.5	ND	ND	0.1	0.1
P4	Control	DQ2.5	ND	ND	0.1[d]	
P5	Control	DQ2.5	ND	ND	0.1	ND
P6	Control	DQ2.5	ND	ND	0.5	0.6
P7	Control	DQ2.5	ND	ND	0.0	0.0
P8	Control	DQ2.5	ND	ND	0.7	0.1
P9	Control	DQ2.5	ND	ND	0.1	0.2
P10	Control	DQ2.5	ND	ND	0.1	0.4
P11	Control	DQ2.5/DQ8	<1.0	1	0.4	0.0
P12	Control	DQ2.5/DQ8	<1.0	1	0.3	0.0
P13	Control	DQ2.5	ND	ND	0.3[d]	
P14	Control	DQ2.5	ND	ND	0.2[d]	
P15	Control	DQ2.5	ND	ND	0.4[d]	
P16	Control	DQ2.5	ND	ND	0.4[d]	
P17	UCD	DQ2.5/DQ8	10.6	3B	21.0	4.1
P18	UCD	DQ2.5	16.3	3B	14.5	4.9
P19	UCD	DQ2.5	10.0	3A	6.8	1.0
P20	UCD	DQ2.5/DQ8	>120	3B	13.2	4.6
P21	UCD	DQ2.5	12.5	3A/B	18.8	1.7
P22	UCD	DQ2.5	48.0	3A	55.0	40.0
P23	UCD	DQ2.5	67.0	3B/C	7.3	4.7
P24	UCD	DQ2.5	3.3	3A	ND	7.3
P25	UCD	DQ2.5	ND	3A	ND	12.8
P26	UCD	DQ2.5	ND	2	22.6	49.5
P27	UCD	DQ2.5	16.4	3A	2.3	1.7
P28	UCD	DQ2.5	5.4	3B-C[c]	0.4	0.4
P29	UCD	DQ2.5	3.8	3C	1.8	2.0
P30	UCD	DQ2.5/DQ8	4.8	3B	1.9	1.8
P31	UCD	DQ2.5	11.0	3B	13.7	12.2
P32	UCD	DQ2.5	35.7	2	33.0	11.7
P33	UCD	DQ2.5	5.7	3A	28.5	16.3
P34	UCD	DQ2.5	3.1	3B	7.5	8.8
P35	UCD	DQ2.5	2.2	3A	7.6	14.4
P36	TCD	DQ2.5	3.3	3B	1.5	3.0
P37	TCD	DQ2.5	1.2	0	0.7	0.4
P38	TCD	DQ2.5	1.1	0	2.0	0.8
P39	TCD	DQ2.5	2.1	2	15.9	19.3
P40	TCD	DQ2.5	ND	3A	52.0	22.8
P41	TCD	DQ2.5	<1.0	ND	5.3	4.0
P42	TCD	DQ2.5	<1.0	2	2.1	10.7
P43	TCD	DQ2.5/DQ8	<1.0	3A	9.8	36.3
P44	TCD	DQ2.5	<1.0	0	9.0	4.7
P45	TCD	DQ2.5	<1.0	0	9.5	0.8
P46	TCD	DQ2.5	ND	2	1.2	6.5
P47	TCD	DQ2.5	<1.0	0	2.0	0.8
P48	TCD	DQ2.5	ND	2	1.9	2.6
P49	TCD	DQ2.5	<1.0	0	1.7[d]	
P50	TCD	DQ2.5	1.4	ND	0.3[d]	
P51	TCD	DQ2.5	<1.0	0	9.9[d]	
P52	TCD	DQ2.5	<1.0	2	2.8[d]	
P53	TCD	DQ8	1.1	3A	0.0	0.4
P54	UCD	DQ2.2	<1.0	3A/B	0.0	0.1

[a]Patients P1-P10 and P13-P16 were anonymous blood bank donors.
[b]UCD were usually referred to gastroduodenoscopy with IgA anti-TG2 above cut-off. These values refer to the repeated sample at time of endoscopy.
[c]The mucosal changes were only asserted in the bulbus duodeni, in this participant.
[d]The gliadin-tetramer-staining was combined on one fluorochrome.
EM/N-ratio: the ratio between gliadin-tetramer+ CD4+ effector memory and naïve T cells; HLA: human leukocyte antigen; IgA: immunoglobulin A; ND: not done; P: participant; TCD: treated coeliac disease; UCD: untreated coeliac disease.

CD19-Pacific blue, CD56-Pacific blue, CD11c-V450, CD4-APC-H7 (all from BD Biosciences); as well as CD45RA-PE-Cy7 and CD3-eFluor605 (both from eBioscience). We washed and analyzed the cells on a LSR II (BD Biosciences) or sorted on a FACS Aria I cell sorter (BD Biosciences). The entire enriched samples were run through, in order to enumerate all tetramer-positive cells.

Cells binding the DQ2.5-glia-α1a- or the DQ2.5-glia-α2-tetramer were identified as relevant if they were: CD3+, CD11c-, CD14-, CD19-, CD56- and CD4+. Relevant gliadin-tetramer+ cells were sub-divided into and sorted as CD45RA+/CD62L+ cells (T_N), CD45RA-/CD62L- cells (T_{EM}) and CD45RA-/CD62L+ cells (T_{CM}) (see Figure 1(a) and Figure 1(b)).[18] Integrin-β7 staining was performed in four controls and four TCD; and cutaneous leucocyte-associated antigen (CLA) staining in one TCD (see Figure 4).

We calculated the frequencies of gliadin-tetramer+ cells as follows:

$$\left(\frac{\text{Gliadin-tetramer positive cells in the enriched sample}}{\text{Total number of CD4-positive T cells}}\right) \times 1 \text{ million}$$

The total number of CD4+ T cells was calculated by multiplying the fraction of CD4+ T cells stained in the pre-enriched sample with the total number of counted PBMC. We used FlowJo software (Tree Star) for analysis of flow data.

Culturing and screening of sorted cells

The sorted cells were cloned by limited dilution and expanded without antigens, as previously described.[19]

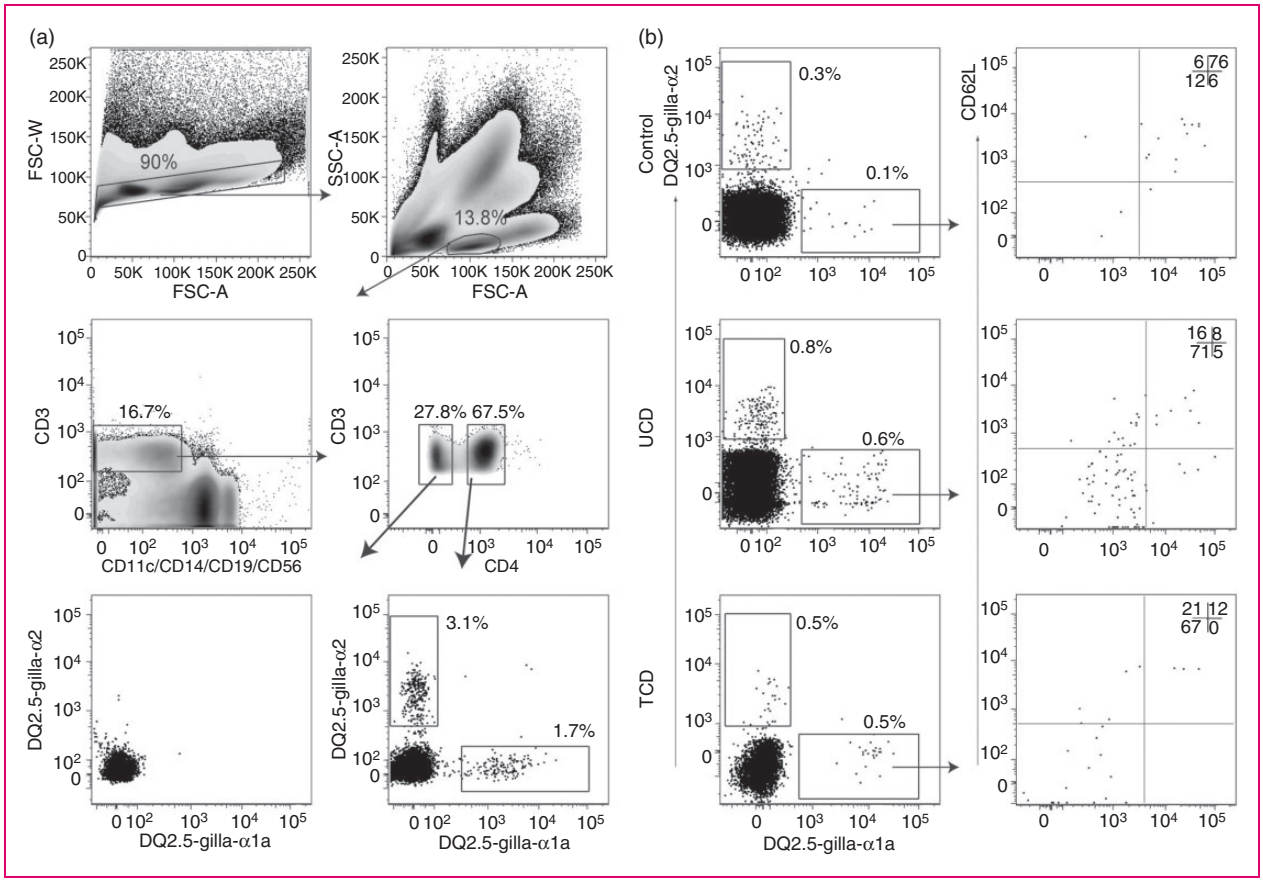

Figure 1. Gating strategy. Flow cytometric density plots and dot plots illustrating the gating strategy for relevant gliadin-tetramer+ cells. Percentages of gated cells within each plot are shown. (a) Gating was done on single cells → lymphocytes → CD3+ cells → CD4+ cells. There were few gliadin-tetramer+ cells among CD3+ CD4- cells (lower left plot); whereas there were distinct populations of CD3+ CD4+ gliadin-tetramer+ cells (lower right plot), here shown in a TCD patient. (b) Dot plots in the left panels show CD4+ T cells in bead-enriched samples from a control individual, UCD and TCD patients binding the two different gliadin-tetramers. Tetramer+ CD4+ T cells were subdivided by the results of CD62L- and CD45RA-staining into effector memory (double negative), naïve (double positive) and central memory (CD62L+ and CD45RA-) T cells.
FSC-W: Forward scatter width; FSC-A: Forward scatter areal; SSC-A: Side scatter areal; TCD: Treated coeliac disease; UCD: Untreated coeliac disease.

Growing T-cell clones (TCC) were tested both in a T-cell proliferation assay and by re-staining with gliadin-tetramers. We analyzed the tetramer-stained cells on a FACS Calibur (BD Biosciences) (Supplementary Figure 1). Cells showing a clear shift in staining-intensity with the DQ2.5-glia-α1a–tetramer compared to the DQ2.5-glia-α2-tetramer and the unstained control were identified as specific for the DQ2.5-glia-α1a-peptide, and vice versa.

We used a well-established protocol for antigen-dependent T-cell proliferation.[19] Briefly, we used DQ2.5 homozygous Epstein-Barr virus (EBV)-transformed cells (IHW #9023) presenting the DQ2.5-glia-α1a-epitope peptide (QLQPFPQPELPY, underlined 9mer core sequence) or a peptide containing the DQ2.5-glia-α2-epitope (PQPELPYPQPQL) (both from Research Genetics). The final peptide concentration was 10 µM. We assessed T-cell proliferation by thymidine incorporation.[19] The TCC that dispalyed a stimulation index (SI) above three, calculated by dividing counts per minute (cpm) after antigen stimulation with cpm after medium stimulation, were identified as peptide-specific.

Statistical analysis

We used the GraphPad Prism 5 software for statistical analysis and the Mann-Whitney U test to calculate statistical significance.

Results

Visualizing gluten-specific T cells in peripheral blood

Motivated by a protocol that can detect rare epitope-specific naïve CD4+ T cells by tetramer-staining and

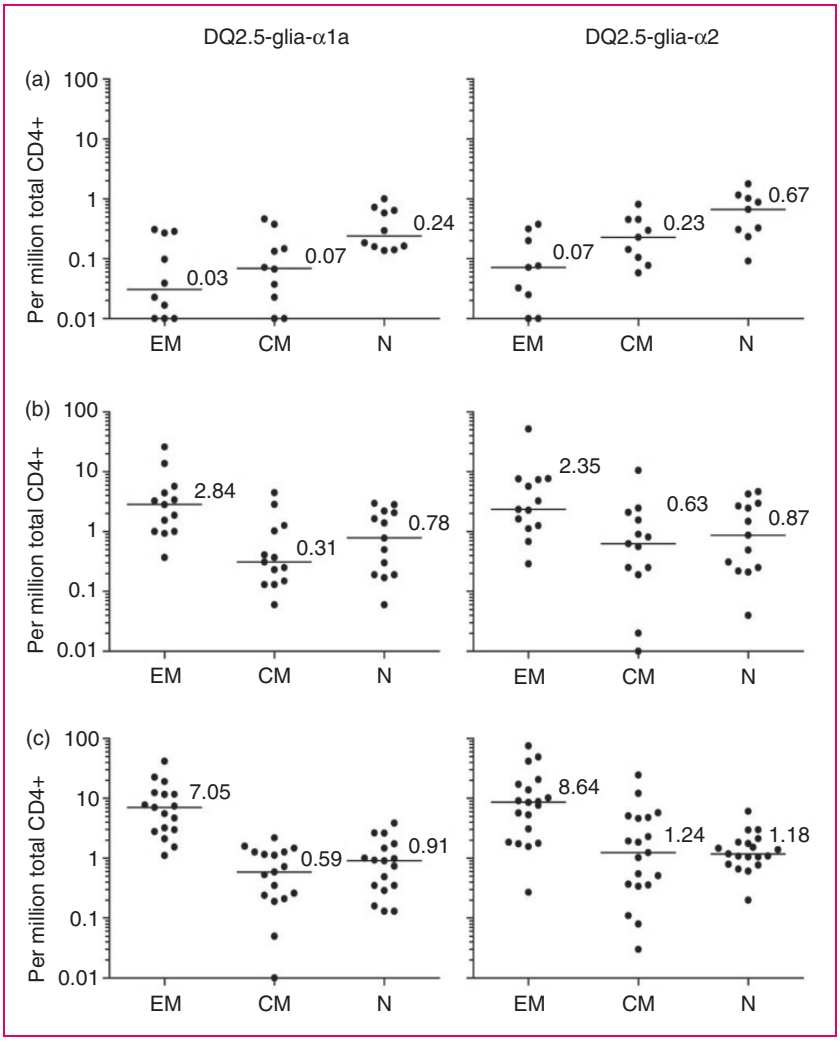

Figure 2. Frequency of CD4+ gliadin-tetramer+ T cells. Number of EM, CM and N T cells binding the DQ2.5-glia-α1a-tetramer (left) and the DQ2.5-glia-α2-tetramer (right) per million total CD4+ T cells. Each participant is indicated by a closed circle. The median frequency is denoted with numbers in (a) controls, (b) TCD patients and (c) UCD patients. Frequencies below <0.01 per million are placed on the *x*-axis for visualization purposes.
CM: Central memory; EM: effector memory; N: naïve T cells; TCD: treated coeliac disease patients; UCD: untreated coeliac disease patients.

bead-enrichment,[17] we aimed to identify CD4+ T cells that are reactive to the two dominant gluten-epitopes, DQ2.5-glia-α1a and DQ2.5-glia-α2, in blood from DQ2.5+ controls, UCD and TCD (Table 1) without oral gluten challenge. We used strict gating for identification of gliadin-tetramer+ CD4+ T cells (Figure 1(a)) and subpopulations of these cells (Figure 1(b)).

In all but one control subjects, we identified relatively few gliadin T_{CM} or T_{EM} and a distinct population of gliadin-tetramer+ CD4+ T_N. In control subject P2, we found a large number of gliadin-tetramer+ CD4+ T_{EM}, similar to levels found in UCD patients. We suspected that subject P2 had CD that was undiagnosed; however, as this participant was an anonymous blood donor, we were unable to do any clinical examination. This subject and the other blood bank donors were all included in the group of control individuals. We also observed some gliadin-tetramer+ CD4+ T cells in non-HLA-DQ2.5 subjects (Supplementary figures 2(a) and 2(b)), similar to what has been observed with other HLA II tetramers.[20]

Validating the gluten specificity of tetramer+ T cells

Gliadin-tetramer+ CD4+ T_N, and in some cases also T_{EM} and T_{CM} from six controls, two UCD and five TCD were sorted, cloned by limiting dilution and cultured in an antigen-independent manner. The success rate of generating TCC from sorted T cells differed

Table 2. The specificity of TCC cultured from tetramer-sorted CD4+ T cells

Participant	Category	Cultured TCC (%)			Specific/proliferating TCC		
		EM	CM	N	EM	CM	N
P1	Control	ND	ND	27	ND	ND	24/30
P2	Control	ND	ND	32	ND	ND	20/24
P3	Control	ND	ND	41	ND	ND	30/32
P4	Control	ND	ND	31	ND	ND	13/13
P8	Control	33	25	62	0/4	2/6	3/29
P11	Control	0	ND	42	0/0	ND	2/8
P17	UCD	3	ND	11	4/4	ND	1/1
P18	UCD	23	ND	43	70/72	ND	8/13
P36	TCD	20	ND	32	7/8	ND	3/9
P37	TCD	33	ND	8	1/2	ND	2/2
P40	TCD	21[a]		50	35/39[a]		0/1
P42	TCD	16	17	52	7/15	0/3	0/17
P43	TCD	33	16	18	38/58	2/11	0/16

Table 2 shows the percentage of sorted EM, CM and N type T cells successfully expanded by the antigen-independent cloning and the number of specific TCC, as defined by specific re-staining, of the total number of growing TCC. In this table, CD4+ T cells binding the DQ2.5-glia-α1a- or the DQ2.5-glia-α2-tetramer are merged.

[a]T_{EM} and T_{CM} were sorted into one tube.

CM: Central memory; EM: effector memory; N: naïve T cells; ND: not done; P: participant; TCC: T-cell clones; TCD: treated coeliac disease; UCD: untreated coeliac disease.

substantially between the subjects. On average, we cultured growing TCC from one-fourth of sorted cells (Table 2). Each generated TCC was assayed for proliferative response to the DQ2.5-glia-α1a- and the DQ2.5-glia-α2-epitope. We found that 122/163 T_{EM}, 4/20 T_{CM} and 76/193 T_N clones responded to the epitope (with a SI $\geq$ 3) of the tetramer for which they originally were isolated. Gliadin-tetramer+ T_{CM} and T_{EM} cells from subject P40 were sorted together and 23/30 of these clones were specific in the T-cell assay (Supplementary Figure 2(a)). All TCCs that gave specific responses in T-cell assays had a clear and specific staining with the corresponding tetramer. Five T_{EM} and 30 T_N clones showed poor proliferation (SI < 3), despite clear tetramer staining. Twelve of the co-sorted T_{EM} and T_{CM} clones from subject P40 also held this feature.

The frequency of gluten-specific T cells in peripheral blood

The frequency of CD4+ DQ2.5-glia-α1a- and the DQ2.5-glia-α2-tetramer+ cells was similar among the three participant groups for T_N (respective median frequency 0.61 and 0.94 per million total CD4+ T cells) and T_{CM} (respective median frequency 0.29 and 0.49 per million CD4+ T cells) (Figure 2). In contrast, there was a significantly higher frequency of gliadin-tetramer+ CD4+ T_{EM} in UCD, as compared to controls ($p < 0.0001$ for both tetramers) and in TCD compared to controls ($p < 0.0001$ and $p = 0.0002$) (Table 1 and Figure 3(a)). The frequency of T_{EM} binding either gliadin-tetramer was <0.4 per million total CD4+ T cells in control subjects. In comparison, the corresponding frequencies were ≥1 in 18 out of 19 HLA-DQ2.5+ UCD and 11 out of 13 HLA-DQ2.5+ TCD.

The EM/N ratio in patients and controls

In order to get a simpler and more robust parameter for the T-cell response to gluten, we divided the number of T_{EM} by the number of T_N (termed the EM/N ratio). We found this parameter more useful, as it is independent of the number of total CD4+ T cells. For both epitopes studied, we observed significant differences in the EM/N ratio between controls (all with a ratio <1, except for subject P2) and UCD (all with a ratio >1, except for subject P24), and between controls and TCD (15 out of 17, with a ratio >1 for one or both of the tetramers), as seen in Table 1 and Figure 3(b)).

Gluten-specific T_{EM} versus duodenal changes

The histological appearance in the duodenal mucosa can be graded into normal mucosa (Marsh score 0), increased numbers of intraepithelial lymphocytes (Marsh score 1), hyperplastic lesion and crypt

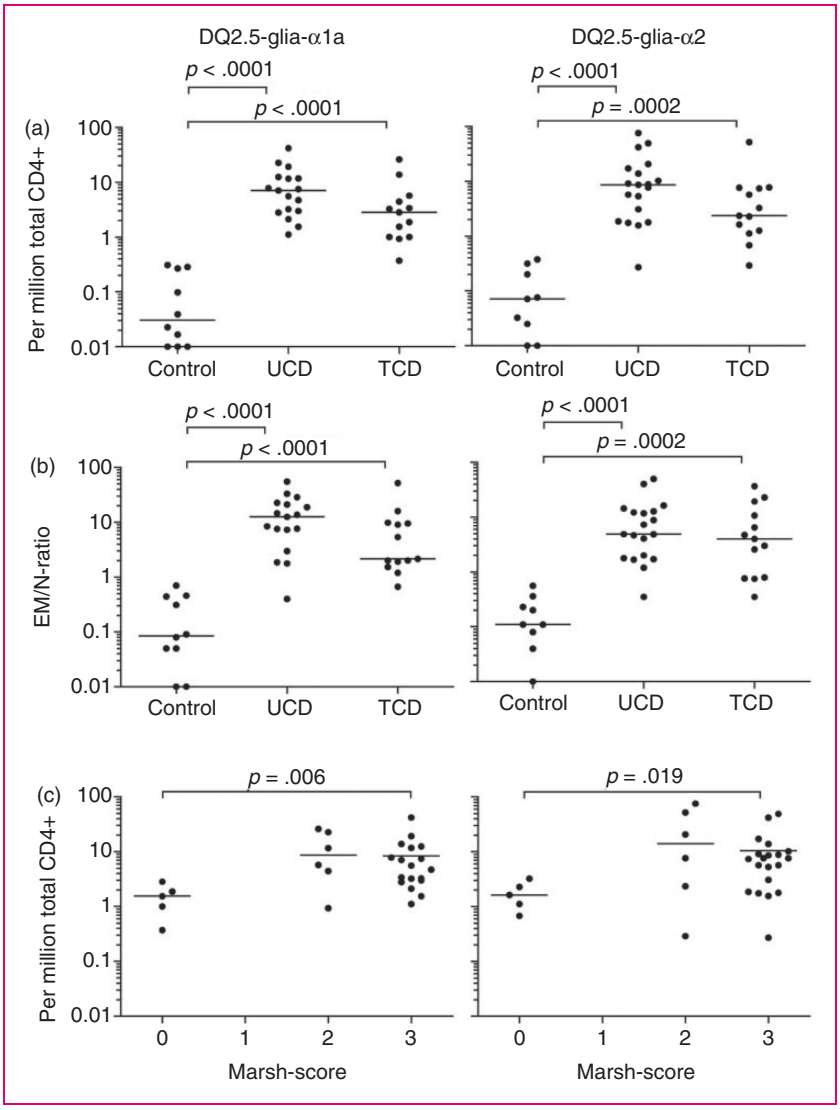

Figure 3. Significantly more gliadin-tetramer+ TEM in patients compared to controls. (a) Frequency of CD4+ TEM binding the DQ2.5-glia-α1-tetramer (left) and the DQ2.5-glia-α2-tetramer (right) among controls, UCD and TCD. (b) Ratio between gliadin-tetramer+ CD4+ TEM and naïve T cells (EM/N-ratio). (c) The prevalence of gliadin-tetramer+ CD4+ TEM was grouped by Marsh score in those participants where duodenal biopsies were obtained. Frequencies and ratios below <0.01 per million are placed on the *x*-axis for visualization purposes. Each frequency and ratio is indicated by a closed circle. *P* values were calculated with the Mann-Whitney U test.
N: naïve T cells; TCD: treated coeliac disease patients; TEM: Effector memory T cells; UCD: untreated coeliac disease patients.

hyperplasia (Marsh score 2), and variable degrees of villous blunting (Marsh score 3).[21,22] It is poorly understood how histological changes related to gluten ingestion develop, but gluten-specific CD4+ T cells are thought to play a crucial role.[9,10,12] We looked at whether the observed variations in the frequency of gliadin-tetramer+ CD4+ T_{EM} in peripheral blood correlated with the Marsh score of CD patients at the time of blood analysis (Figure 3(c)). We found a significantly higher frequency of DQ2.5-glia-α1a and DQ2.5-glia-α2 -specific CD4+ T_{EM} in participants with a Marsh score 3, compared to those with a Marsh score 0. Very few of our CD patients had Marsh score 2 and the frequency of gliadin-tetramer+ cells among these was variable.

Gut homing of gluten-specific T_{EM}

A few CD patients had either frequencies of gliadin-tetramer+ T_{EM} or an EM/N ratio similar to controls. To test whether patients and controls could be further distinguished, we analyzed CD4+ gliadin-tetramer positive versus negative cells in four TCD and four controls, for the gut-homing marker integrin-β7 in the

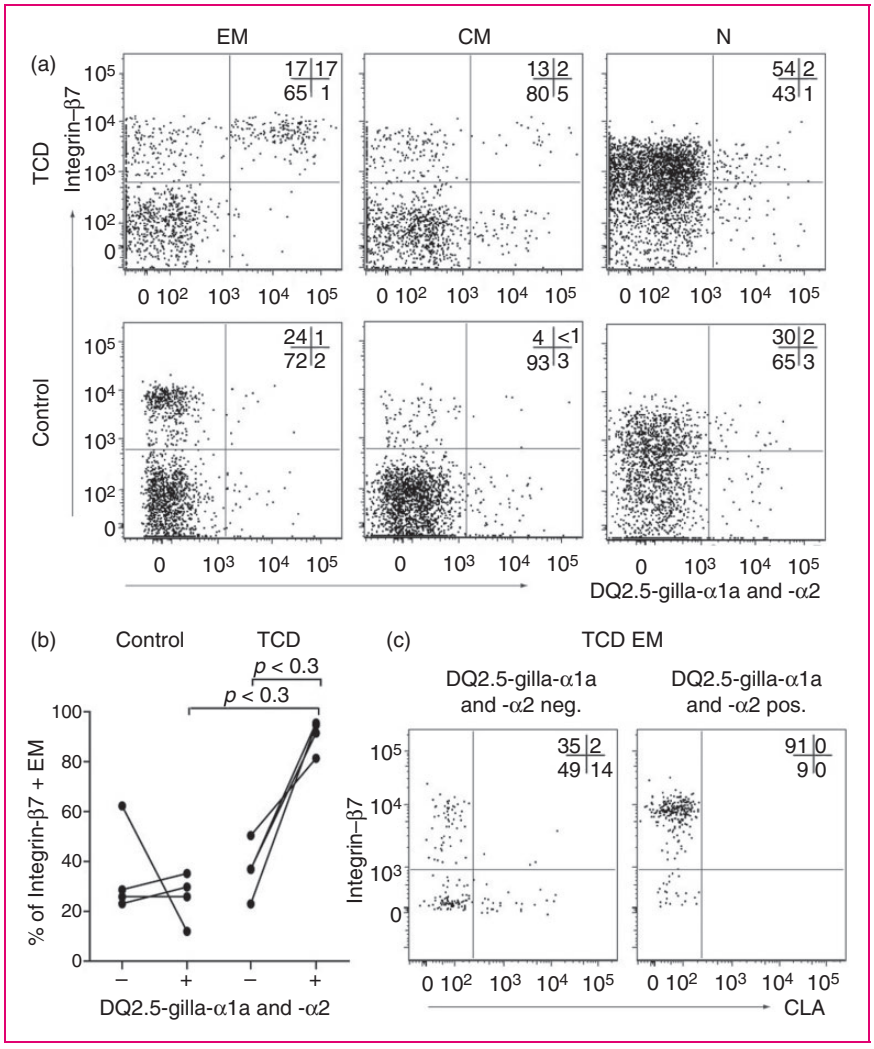

Figure 4. Gut-homing of T cells. (a) While gliadin-tetramer+ N T cells expressed integrin-β7 at intermediate levels and the CM T cells showed no clear staining, nearly all gliadin-tetramer+ EM T cells in TCD expressed integrin-β7. Gliadin-tetramer positive and negative EM T cells in controls expressed similar amounts of integrin-β7. The DQ2.5-glia-α1a- and DQ2.5-glia-α2-tetramer were combined on one fluorochrome, to increase the number of relevant cells. (b) The percent of integrin-β7 expression on tetramer-positive and tetramer-negative cells in four TCD and four controls. (c) Tetramer+ EM in one tested TCD did not express the skin-homing CLA. Percentages of gated cells within each plot are denoted by numbers.
CLA: cutaneous leukocyte-associated antigen; CM: central memory; EM: effector memory; N: naïve; TCD: treated coeliac disease patients.

population of cells obtained after tetramer bead enrichment (Figure 4(a) and Figure 4(b)). In TCD, significantly more gliadin-tetramer+ T_{EM} (80–95%), compared to gliadin-tetramer- cells, expressed integrin-β7. In contrast, integrin-β7 expression did not exceed background in the gliadin-tetramer+ T_{EM} of controls.

Integrin-β7 forms gut-homing dimers with α4- or α_E-subunits; and a skin-homing dimer with the α1 chain. Peripheral blood from one TCD was stained for the CLA. None of the gliadin-tetramer+ T_{EM} in this TCD expressed the skin-homing marker (Figure 4(c)), indicating that the observed integrin-β7 expression is associated with gut-homing rather than skin-homing.

Discussion

We here demonstrate that gluten-reactive T cells in peripheral blood can be characterized and enumerated directly ex vivo in TCD, UCD and controls without oral gluten challenge. Gut-homing gliadin-tetramer+ CD4+ T_{EM} were significantly more frequent in CD patients

than controls. These cells likely reflect an antigen-driven, CD-associated T-cell response, and there is a potential for using this parameter for the diagnostic work-up of CD.

All UCD and TCD had either a ratio of T_{EM}/T_N cells >1 or a frequency of T_{EM} specific for one or both of the epitopes DQ2.5-glia-α1a or DQ2.5-glia-α2 > 1 per million total CD4+ T cells. The only exception was TCD P50 (EM/N ratio 0.3). Still, the gliadin-tetramer+ T_{EM} of this treated patient expressed integrin-β7 significantly above background and thus, is distinguishable from the tetramer+ T_{EM} of controls. In comparison, all controls (except P2) had both the EM/N ratio <0.8 and the frequency of gliadin-tetramer+ $T_{EM} < 0.4$ per million total CD4+ T cells. We strongly suspect that the anonymous donor P2 had undiagnosed CD. The combination of integrin-β7+ T_{EM} percentage with the EM/N ratio seemed to give a good discrimination of patients versus controls. This notion must be corroborated in future studies.

GFD will often normalize diagnosis-dependent parameters like histology and disease-specific antibodies in CD patients.[23] Still, proliferating intraepithelial lymphocytes,[24] duodenal TG2-specific antibody-secreting cells[25] and duodenal gluten-specific T cells can be detected in TCD.[9] These data indicate a persistent immune response to gluten in many TCD. Our finding supports this notion, as gliadin-tetramer+ EM cells were detected in the blood of all included TCD, despite normal mucosa and negative antibody titers in many of them. The persistent T-cell response to gluten in TCD may be explained by long-lived memory T cells,[26] local IL-15 production[27] or sporadic exposure to small amounts of gluten antigen. Importantly, we are able to detect a persistent T-cell response in blood that can prove to be very helpful in diagnosing patients that are already on a GFD, without previously confirmed CD diagnosis.

Notably, we found a statistically significant difference in T_{EM} numbers between CD patients with Marsh score 0 and 3, despite the small group sizes and the issue of CD lesion patchiness.[28] Although gluten-specific T cells may not be directly responsible for the remodeling of the intestinal mucosa, they can drive inflammation through pro-inflammatory mediators that activate intraepithelial cytotoxic T lymphocytes.[29] As gliadin-tetramer+ T_{EM} cells in the blood of CD patients show high expression of gut-homing markers and use T-cell receptors typical of CD lesion-derived T cells,[30] they likely have intestinal origin and reflect the disease-driving gluten-reactive T-cell response in the lamina propria. Whether the latter is the case could be further tested in studies where the T-cell receptor sequences of intestinal and peripheral blood gliadin-tetramer+ cells are compared.

The rate of the proliferative response to peptide stimulation seemed to be phenotype-dependent. In general, the fraction of TCC proliferating after peptide stimulation was lower among T_{EM} from controls, T_N from CD patients and T_{CM} overall. Notably, we were not able to confirm gluten reactivity of the few gliadin-tetramer+ memory cells of our controls, except for two T_{CM} clones. One might speculate that tetramer+ T_{EM} from controls represent gluten-specific regulatory T cells or some other T-cell phenotypes that do not necessarily respond to antigen stimulation with proliferation. Overall, poor yield of specific tetramer-sorted blood cells could possibly be explained by tetramer-induced cell apoptosis[31] and by a certain degree of background staining.[32] Nevertheless, we believe that this protocol can be used in further studies on gluten-reactive T cells of CD patients and controls, give important insights into CD pathogenesis and accommodate a search for disease-specific T-cell receptors.[33]

CD patients can present with histological changes in the small intestine, but negative serology,[6] and vice versa.[7] This has led to an ongoing debate on the diagnostic criteria for CD.[7,34] A large number of individuals are also on a GFD without any confirmed CD diagnosis[35] reducing the sensitivity of currently available diagnostic tools substantially.

We report a significant difference in the number of gluten tetramer+ T_{EM} in UCD and TCD, and in the gut-homing of these cells in CD patients compared to controls without performing a gluten challenge. Flow cytometry is already routinely used in clinical work-up. The current protocol allows analysis with 50 mL of blood; and this volume may be further reduced, if gluten epitopes are combined. Tetramerized HLA-DQ8- and HLA-DQ2.2-restricted gluten epitopes should also be tested with the current approach, to include also these minor groups of CD patients. Moreover, HLA tetramer production may be optimized in an industrial setting, with production costs similar to other diagnostic assays. Thus, we will argue that this minimally invasive, ex-vivo method may find use in clinical practice, particularly in patients with vague diagnostic conditions, or in cases where a gastroduodenoscopy is inappropriate or undesirable, as well as in undiagnosed individuals adhering to a GFD.

Funding

This work was supported by the Research Council of Norway and the Health Authority of South-Eastern Norway. Asbjørn Christophersen was supported by the University of Oslo (PhD fellowship). No funding source had any role in the design, conduct or reporting of this study.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank all the subjects whom donated biological material to this study, Marie K Johannesen and Louise F Risnes for technical assistance, Bjørg Simonsen for tetramer production and Yan Zhang for assisting with cell sorting.

References

1. Ludvigsson JF, Leffler DA, Bai JC, et al. The Oslo definitions for coeliac disease and related terms. *Gut* 2012; 62: 43–52.
2. Wieser H. Chemistry of gluten proteins. *Food Microbiol* 2007; 24: 115–119.
3. Green PH and Cellier C. Celiac disease. *N Engl J Med* 2007; 357: 1731–1743.
4. Husby S, Koletzko S, Korponay-Szabo IR, et al. European Society for Pediatric Gastroenterology, Hepatology and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 2012; 54: 136–160.
5. American Gastroenterological Association (AGA). Institute medical position statement on the diagnosis and management of celiac disease. *Gastroenterology* 2006; 131: 1977–1980.
6. Dickey W, Hughes DF and McMillan SA. Reliance on serum endomysial antibody testing underestimates the true prevalence of coeliac disease by one fifth. *Scand J Gastroenterol* 2000; 35: 181–183.
7. Kurppa K, Collin P, Viljamaa M, et al. Diagnosing mild enteropathy celiac disease: A randomized, controlled clinical study. *Gastroenterology* 2009; 136: 816–823.
8. Sollid LM. Coeliac disease: Dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002; 2: 647–655.
9. Lundin KE, Scott H, Hansen T, et al. Gliadin-specific, HLA-DQ (alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J Exp Med* 1993; 178: 187–196.
10. Molberg Ø, Kett K, Scott H, et al. Gliadin specific, HLA DQ2-restricted T cells are commonly found in small intestinal biopsies from coeliac disease patients, but not from controls. *Scand J Immunol* 1997; 46: 103–109.
11. Shan L, Molberg O, Parrot I, et al. Structural basis for gluten intolerance in celiac sprue. *Science* 2002; 297: 2275–2279.
12. Lundin KE, Scott H, Fausa O, et al. T cells from the small intestinal mucosa of a DR4, DQ7/DR4, DQ8 celiac disease patient preferentially recognize gliadin, when presented by DQ8. *Hum Immunol* 1994; 41: 285–291.
13. Anderson RP, Degano P, Godkin AJ, et al. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med* 2000; 6: 337–342.
14. Raki M, Fallang LE, Brottveit M, et al. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. *Proc Natl Acad Sci USA* 2007; 104: 2831–2836.
15. Pepper M and Jenkins MK. Origins of CD4(+) effector and central memory T cells. *Nat Immunol* 2011; 12: 467–471.
16. Quarsten H, McAdam SN, Jensen T, et al. Staining of celiac disease-relevant T cells by peptide-DQ2 multimers. *J Immunol* 2001; 167: 4861–4868.
17. Moon JJ, Chu HH, Pepper M, et al. Naive CD4(+) T-cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 2007; 27: 203–213.
18. Sallusto F, Lenig D, Forster R, et al. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; 401: 708–712.
19. Molberg Ø, McAdam SN, Lundin KE, et al. Studies of gliadin-specific T cells in celiac disease. *Methods Mol Med* 2000; 41: 105–124.
20. Kwok WW, Tan V, Gillette L, et al. Frequency of epitope-specific naive CD4+ T cells correlates with immunodominance in the human memory repertoire. *J Immunol* 2012; 188: 2537–2544.
21. Marsh MN and Crowe PT. Morphology of the mucosal lesion in gluten sensitivity. *Baillieres Clin Gastroenterol* 1995; 9: 273–293.
22. Oberhuber G, Granditsch G and Vogelsang H. The histopathology of coeliac disease: Time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; 11: 1185–1194.
23. Sulkanen S, Halttunen T, Laurila K, et al. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 1998; 115: 1322–1328.
24. Olaussen RW, Karlsson MR, Lundin KE, et al. Reduced chemokine receptor 9 on intraepithelial lymphocytes in celiac disease suggests persistent epithelial activation. *Gastroenterology* 2007; 132: 2371–2382.
25. Di Niro R, Mesin L, Zheng NY, et al. High abundance of plasma cells secreting transglutaminase 2-specific IgA autoantibodies with limited somatic hypermutation in celiac disease intestinal lesions. *Nat Med* 2012; 18: 441–445.
26. Pepper M, Linehan JL, Pagan AJ, et al. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nat Immunol* 2010; 11: 83–89.
27. Meresse B, Chen Z, Ciszewski C, et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 2004; 21: 357–366.
28. Pais WP, Duerksen DR, Pettigrew NM, et al. How many duodenal biopsy specimens are required to make a diagnosis of celiac disease? *Gastrointest Endosc* 2008; 67: 1082–1087.
29. Jabri B and Sollid LM. Tissue-mediated control of immunopathology in coeliac disease. *Nat Rev Immunol* 2009; 9: 858–870.
30. Qiao SW, Christophersen A, Lundin KE, et al. Biased usage and preferred pairing of alpha- and beta-chains of TCRs specific for an immunodominant gluten epitope in coeliac disease. *Int Immunol* 2014; 26: 13–19.
31. Knabel M, Franz TJ, Schiemann M, et al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med* 2002; 8: 631–637.

32. Geiger R, Duhen T, Lanzavecchia A, et al. Human naive and memory CD4+ T-cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. *J Exp Med* 2009; 206: 1525–1534.
33. Qiao SW, Raki M, Gunnarsen KS, et al. Post-translational modification of gluten shapes TCR usage in celiac disease. *J Immunol* 2011; 187: 3064–3071.
34. Kaukinen K, Maki M, Partanen J, et al. Celiac disease without villous atrophy: Revision of criteria called for. *Dig Dis Sci* 2001; 46: 879–887.
35. Rubio-Tapia A, Ludvigsson JF, Brantner TL, et al. The prevalence of celiac disease in the United States. *Am J Gastroenterol* 2012; 107: 1538–1544.