

# Direct cloning and tetramer staining to measure the frequency of intestinal gluten-reactive T cells in celiac disease

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Knowledge of the frequency of disease-driving CD4<sup>+</sup> T cells in lesions of chronic human inflammatory diseases is limited. In celiac disease (CD), intestinal gluten-reactive CD4<sup>+</sup> T cells, which recognize gluten peptides only in the context of the disease-associated HLA-DQ molecules, are key pathogenic players. By cloning CD4<sup>+</sup> T cells directly from intestinal biopsies of CD patients, we found that 0.5–1.8% of CD4<sup>+</sup> T cells were gluten reactive. About half of the gluten-reactive T cells were specific for either the immunodominant DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 epitopes, suggesting that direct visualization of gluten-specific T cells could be possible. Assessed by flow cytometry, tetramer-positive T cells were present in 10/10 untreated CD patients with a frequency of 0.1–1.2% of CD4<sup>+</sup> T cells. Gluten-specific T cells were also detectable in most treated CD patients (7/10). Moreover, the frequency of gluten-specific T cells correlated with the degree of histological damage in the gut mucosa as scored by Marsh-grading, and also with serum IgA anti-transglutaminase 2 antibody levels. Tetramer staining of gluten-reactive T cells in biopsy material is a useful tool for future studies of such cells in CD and could also potentially serve as a diagnostic supplement in selected cases.

**Keywords:** Celiac disease · Frequency · Gluten-specific T cells · Tetramer



Additional supporting information may be found in the online version of this article at the publisher's web-site

## Introduction

Celiac disease (CD) is a chronic inflammatory disease of the small intestine triggered by gluten proteins from wheat, barley, and rye

[1]. The only available treatment for CD is a lifelong gluten-free diet. CD is a multifactorial, polygenic disease, and the HLA locus is by far the single most important genetic risk factor [2]. The great majority of CD patients express the HLA class II molecule

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HLA-DQ2.5 and the remaining patients either express HLA-DQ8 or HLA-DQ2.2 [3]. CD4<sup>+</sup> gluten-reactive T cells, recognizing gluten peptides in the context of the disease-associated HLA molecules [4–6] can be isolated only from the small intestine of CD patients, but not from that of healthy controls [7]. These findings strongly suggest that gluten-reactive T cells are key players in the pathogenesis of CD. Gluten epitopes recognized by CD4<sup>+</sup> T cells of CD patients expressing DQ2.5 have been well characterized [8]. Many of these T cells recognize either the  $\alpha$  gliadin epitope DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 [9, 10]. Importantly, deamidation of specific glutamine residues to glutamate by the enzyme transglutaminase 2 (TG2) increases the T-cell stimulatory capacity of most gluten epitopes [10–12].

CD provides a unique opportunity to study CD4<sup>+</sup> T cells recognizing the disease-driving antigen (gluten) at the site of inflammation. The development of MHC-peptide multimers has greatly facilitated the detection of antigen-specific T cells [13]. Gluten-specific T cells can be visualized in the peripheral blood of CD patients undergoing a short gluten challenge by using DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers [14]. However, isolation of gluten-reactive T cells from the intestinal lesion still requires long term in vitro culturing. It is becoming increasingly evident that the phenotype of CD4<sup>+</sup> T cells is plastic [15]. Studying freshly isolated T cells will therefore be important to accurately characterize these T cells.

The frequency of antigen-specific CD4<sup>+</sup> T cells at the site of inflammation in human immune-mediated diseases has not been thoroughly investigated. Given our knowledge of the disease-driving antigen (gluten) and the MHC restriction of the CD4<sup>+</sup> cells, CD provides a unique opportunity to address this issue.

Here, we aimed to assess the frequency of gluten-reactive T cells in the small intestinal lesion of CD patients and to examine their relation to the histological damage.

## Results

### Assessing the frequency of gluten-reactive T cells by direct cloning of CD4<sup>+</sup> T cells

To assess the frequency of gluten-reactive T cells, CD4<sup>+</sup> T cells were isolated from single cell suspensions prepared from small intestinal biopsies of three treated and two untreated CD patients (UCDs) expressing DQ2.5, and cloned directly by limiting dilution. Notably, cloning was done in the absence of gluten peptides using only mitogen, feeder cells, and cytokines. Pilot experiments suggested that pooling of T-cell clones during culturing led to an underestimation of the frequency of gluten-reactive T cells (data not shown), and we, therefore, cultured the generated T-cell clones individually. T cells were first plated in Terasaki plates at 1–2 cells/well. On average, T cells grew in 35–50% of wells and were transferred to 96-well plates for restimulation with allogeneic PBMC, PHA, and cytokines (data not shown). The majority (90–95%) of the transferred T cells proliferated well after restimulation. Subsequently, all T-cell clones were individually

screened against a chymotrypsin digest of gluten, treated with TG2, in the presence of DQ2.5-expressing APCs or autologous APCs (Fig. 1A). We isolated a total of 16 gluten-reactive T-cell clones from a total of 1728 restimulated/growing CD4<sup>+</sup> T cells, resulting in a frequency of 0.5–1.8% for the individual patients (Table 1).

### Specificity of the gluten-reactive T-cell clones generated

All gluten-reactive T-cell clones were retested (Fig. 1B) and examined for their response to known DQ2.5-restricted gluten epitopes using peptides consisting of 11–33 amino acids (Supporting Information Table 1). We found that 14/16 of the generated T-cell clones were reactive to one or more peptides in a panel of common DQ2.5-restricted gluten epitopes (Table 1). Approximately half of the gluten-reactive T-cell clones (9/16) recognized either the DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 epitope. Two of the gluten-reactive T-cell clones displayed high cross-reactivity toward DQ2.5-restricted gluten epitopes found in both  $\gamma$ - and  $\alpha$ -gliadin proteins. Alignment of these epitopes is shown in Supporting Information Table 2. Together, these data suggested that the frequency of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 specific T cells could be sufficiently high to enable direct visualization of these cells in biopsy material.

### DQ2.5-gluten tetramer staining of intestinal biopsy material

We next aimed to visualize gluten-reactive T cells directly from intestinal biopsies. For this purpose, we prepared single cell suspensions from small intestinal biopsies of 10 untreated DQ2.5-expressing CD patients, 10 treated DQ2.5-expressing CD patients and 10 control individuals. Single cell suspensions were then stained for surface markers and with a mixture of DQ2.5-glia- $\alpha$ 1a and DQ2.5- $\alpha$ 2 tetramers containing gliadin epitopes of 12 and 11 amino acids, respectively. We analyzed the cells by flow cytometry by using a strict gating and exclusion markers for non-T cells (Fig. 2A). Low background staining was seen with the DQ2.5-CLIP2 negative control tetramer (Fig. 2B). We were able to visualize a population of DQ2.5-glia- $\alpha$ 1a/DQ2.5-glia- $\alpha$ 2 positive CD4<sup>+</sup> T cells in all UCDs and most treated CD patients (TCDs) (Figs. 2B and 3). Among the T cells staining with the mix of the DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers, most cells were CD4<sup>+</sup>, supporting that tetramer staining was specific (Fig. 2B). To confirm the specificity of the staining, we sorted cells staining with the mix of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers by flow cytometry from five patients and generated T-cell clones from these cells. In four out of five patients, the majority of the T-cell clones obtained were specific for the DQ2.5-glia- $\alpha$ 1a or the DQ2.5-glia- $\alpha$ 2 tetramers after in vitro culturing (Supporting Information Table 3 and Fig. 2C). The last patient (CD1042) had a low frequency of DQ2.5-glia- $\alpha$ 1a/DQ2.5-glia- $\alpha$ 2 tetramer positive cells (0.2%) and only 3/7 of T-cell clones obtained were specific after

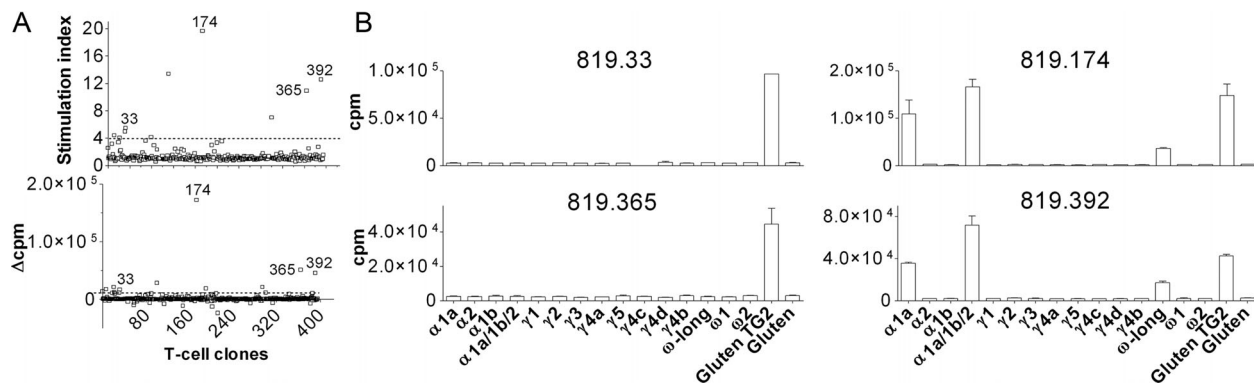


**Figure 1.** Direct cloning of CD4<sup>+</sup> T cells isolated from intestinal biopsies of the CD patient CD819 expressing DQ2.5. CD4<sup>+</sup> T cells were isolated from single cell suspension generated from small intestinal biopsies. (A) Single T-cell clones were generated and tested for reactivity toward a transglutaminase (TG2)-treated chymotrypsin digest of gluten and autologous APCs. Proliferation was measured using a <sup>3</sup>H-thymidine incorporation assay, measured in cpm and expressed as Δcpm (cpm after gluten stimulation—cpm after medium stimulation) or stimulation index (cpm after gluten stimulation/cpm after medium stimulation). (B) T-cell clones with a stimulation index above 4 and a Δcpm above 10<sup>4</sup> were considered gluten reactive (dotted line) and retested using a panel of peptides covering most of the known DQ2.5-gluten epitopes (see Supporting Information Table 1), and against a chymotrypsin digest of gluten in its native form or TG2-treated. Epitope names are abbreviated (i.e., DQ2.5-glia-α1a is abbreviated as α1a). Testing in (A) was done in single and testing in (B) in duplicates. Error bars show SEM. Data shown are from one experiment with cells from a single patient, representative of five patients tested (see Table 1).

culturing. None of the controls, including eight non-CD individuals and two non-DQ2.5 CD patients had a detectable population of DQ2.5-glia-α1a/DQ2.5-glia-α2 tetramer positive cells. A significantly lower frequency of DQ2.5-glia-α1a/DQ2.5-glia-α2 positive T cells was found in treated compared with UCDs. Overall, CD4<sup>+</sup> T cells staining with the DQ2.5-glia-α1a/DQ2.5-glia-α2 tetramers represented 0.1–1.2% of CD4<sup>+</sup> T cells in untreated and 0–0.3% of CD4<sup>+</sup> T cells in treated patients (Fig. 3 and Supporting Information Table 3).

### Frequency of gluten-specific T cells and histological damage

Next, we wished to examine whether there is increasing damage in the small intestinal mucosa with increasing frequency of T cells staining positive with DQ2.5-glia-α1a/DQ2.5-glia-α2 tetramers. The degree of histological damage was determined according to the Marsh-Oberhuber classification [16] and related to the frequency of gluten-specific T cells (Fig. 4). Indeed, the frequency

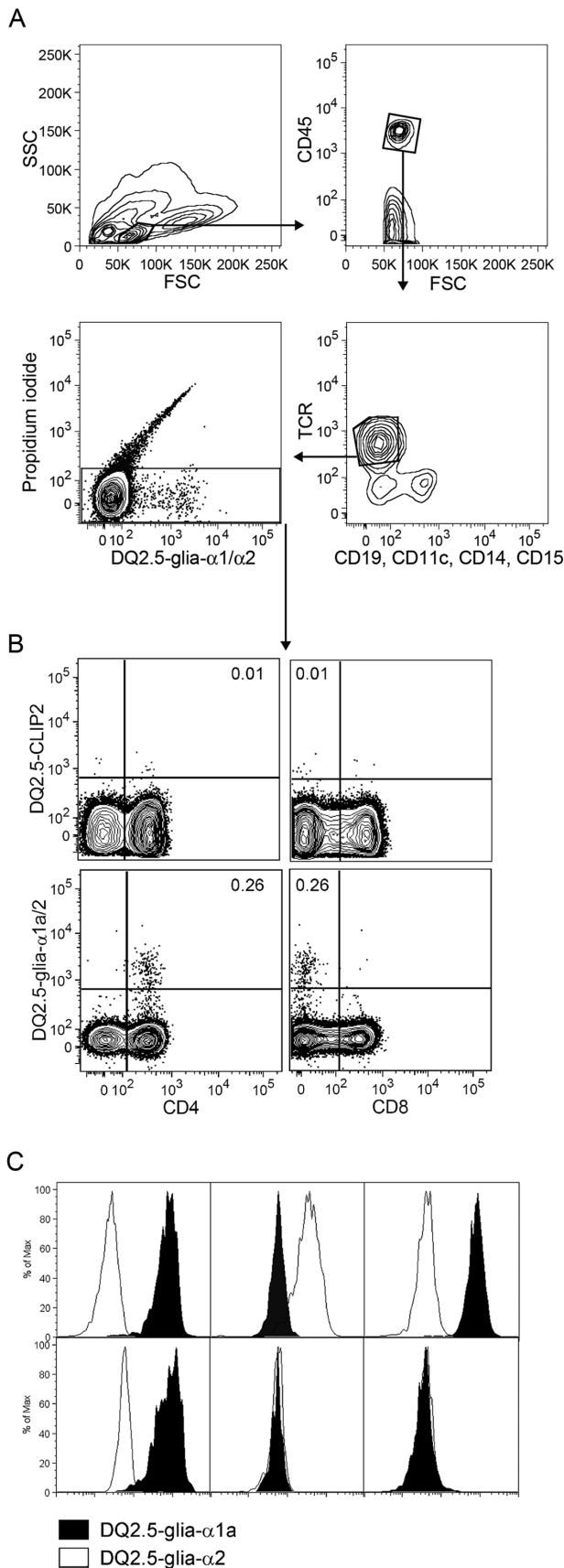
**Table 1.** Frequency and specificity of gluten-reactive T cells isolated from intestinal biopsies of five DQ2.5-expressing CD patients<sup>a)</sup>

| Patient | HLA type    | Clinical status   | Histology | Frequency among plated CD4 <sup>+</sup> T cells | Specificity of T-cell clones <sup>b)</sup>                                                                                                                                                                                                              |
|---------|-------------|-------------------|-----------|-------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| CD746   | DQ2.5/DQ8   | TCD <sup>c)</sup> | Marsh 1   | 1/192                                           | DQ2.5-glia-γ-5                                                                                                                                                                                                                                          |
| CD819   | DQ2.5/DQ2.2 | TCD               | Marsh 3A  | 4/384                                           | Unknown<br>Unknown<br>DQ2.5-glia-α1/ω-long<br>DQ2.5-glia-α1/ω-long<br>DQ2.5-glia-α1/ω1<br>DQ2.5-glia-ω2/α2<br>DQ2.5-glia-α2<br>DQ2.5-glia-γ4d<br>DQ2.5-glia-α2<br>DQ2.5-glia-α2<br>DQ2.5-glia-γ1<br>Long γ-glia peptide<br>DQ2.5-glia-α2/ω2/γ4a/γ4b/γ4c |
| CD820   | DQ2.5       | TCD               | Marsh 3A  | 7/384                                           | DQ2.5-glia-α2<br>DQ2.5-glia-α2<br>DQ2.5-glia-γ1<br>Long γ-glia peptide<br>DQ2.5-glia-α2/ω2/γ4a/γ4b/γ4c                                                                                                                                                  |
| CD1041  | DQ2.5/DQ2.2 | UCD               | Marsh 3B  | 2/384                                           | DQ2.5-glia-α2<br>DQ2.5-glia-α2                                                                                                                                                                                                                          |
| CD1042  | DQ2.5       | UCD               | Marsh 3A  | 2/384                                           | DQ2.5-glia-ω2/γ4b/γ4c/γ4d                                                                                                                                                                                                                               |

<sup>a)</sup>CD4<sup>+</sup> T cells were isolated from small intestinal biopsies of CD patients and CD4<sup>+</sup> T-cell clones were generated. Single T-cell clones were screened for reactivity against chymotrypsin digested gluten treated with transglutaminase 2 (see Fig. 1). Gluten-reactive T-cell clones were subsequently retested for reactivity against a panel of previously described DQ2.5-restricted gluten epitopes (see Supporting Information Table 1).

<sup>b)</sup>For T-cell clones reactive against several peptides, all peptides are listed in order of the magnitude of the response.

<sup>c)</sup>TCD: treated celiac disease patient; UCD: untreated celiac disease patient.



of tetramer positive T cells correlated with both Marsh grade and serum IgA anti-TG2 antibody levels. As expected, based on earlier studies [17–19], Marsh grade also correlated with anti-TG2 antibody levels.

## Discussion

The frequency of disease-driving CD4<sup>+</sup> T cells in the active site of a chronic inflammation in humans has not been thoroughly investigated. Intestinal DQ-restricted gluten-reactive CD4<sup>+</sup> T cells are essential in the pathogenesis of CD, which provides a unique model to address this issue. By using two complementary methods we were able to assess the frequency of gluten-reactive T cells in the lesion of CD.

The first method we used was direct cloning of CD4<sup>+</sup> T cells. One to two CD4<sup>+</sup> T cells per well were stimulated on Terasaki plates, but since there was expansion in only 35–50% of the wells, there is over 70% chance that the proliferating cells are clonal. Of note, no antigen stimulation was done ex vivo to ensure similar growth conditions for all T-cell clones independent of gluten specificity. The read-out of this assay is proliferation of T-cell clones after stimulation with a TG2-treated wheat gluten digest and DQ2.5-expressing APCs or autologous APCs. We have chosen a cut-off at 10,000 cpm and SI (stimulation index)  $\geq 4$ , which in our experience should give a high sensitivity for detection of gluten-specific T-cell clones. All patients expressed DQ2.5, but one of the patients examined with this method also expressed DQ8 (CD746) and two patients expressed also DQ2.2 (CD819 and CD1041). These latter HLA molecules are also associated with CD and can present gluten epitopes to CD4<sup>+</sup> T cells [4, 20–22]. While the T cells from CD819 were screened with autologous APCs, those of CD746 and 1041 were only screened with DQ2.5-expressing APCs. Hence, in CD746 and 1041, DQ8- or DQ2.2-restricted (respectively) gluten-reactive T cells could

**Figure 2.** Staining of gluten-specific CD4<sup>+</sup> T cells in duodenal biopsies. (A) Gating strategy used to visualize CD4<sup>+</sup> T cells in single cell suspension from small intestinal biopsies of CD patients. T cells were identified on the basis of forward scatter and side scatter, CD45 expression, TCR/CD3 expression and the lack of CD19, CD11c, CD14, and CD15. Only live cells (not staining for propidium iodide) were analyzed. (B) Direct visualization of gluten-specific CD4<sup>+</sup> T cells in single cell suspension from small intestinal biopsies of a CD patient (CD1032). Single cell suspension was stained with a mixture of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers or DQ2.5-CLIP tetramer (negative control) and analyzed by flow-cytometry. Data shown are from one patient representative of a total of 10 untreated CD patients and seven treated CD patients analyzed (see Supporting Information Table 3 for details on individual patients). (C) Retesting of the sorted T cells from the same patient (CD1032). Cells staining with the gluten epitope tetramers were sorted, expanded, and retested in flow cytometry by staining with the DQ2.5-glia- $\alpha$ 1a/DQ2.5-glia- $\alpha$ 2 tetramers. DQ2.5-glia- $\alpha$ 1a tetramers were used as negative control staining for DQ2.5-glia- $\alpha$ 2-reactive T cells and vice versa. Each panel shows staining of a single clone. In this patient, four out of six sorted clones were gluten-specific upon retesting, three of which were DQ2.5-glia- $\alpha$ 1 specific, and one was DQ2.5-glia- $\alpha$ 2 specific. Data shown are from one patient representative of five untreated CD patients tested.

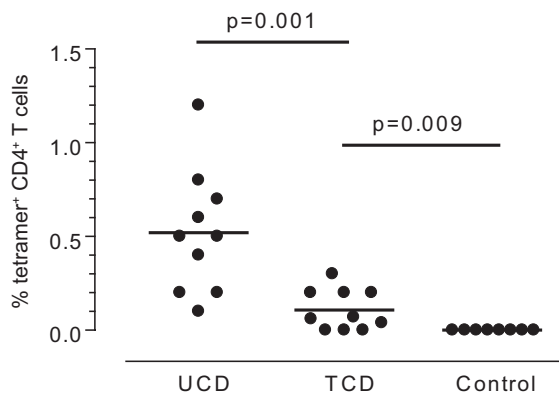


**Figure 3.** Frequency of CD4<sup>+</sup> T cells from intestinal biopsies stained with a mixture of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers. Single-cell suspension was generated from biopsies of UCD, TCD, or control subjects. Each symbol represents an individual patient and bars represent the means. Statistical analysis was done using Mann-Whitney test.

remain undetected and thus the frequency of gluten-reactive T cells may have been underestimated. Nevertheless, we believe to have obtained a good estimate of the frequency of gluten-reactive T cells among CD4<sup>+</sup> T cells in these patients, with a range of 0.5–1.8%. We further noticed that many of these T cells (over 50%) recognized the DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 epitopes, which are known to be dominant DQ2.5-restricted gluten epitopes [9, 10]. Based on this observation, we envisaged that it should be possible to visualize such cells directly in biopsy material by staining with DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 tetramers.

Indeed, using a mixture of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers, we were able to visualize gluten-reactive T cells directly from single cell suspension of biopsy material. We observed a frequency for the individual patients ranging from 0 to 1.2%, compared with a range of 0.5–1.8% using direct cloning. This correlates well with our findings that approximately half of the DQ2.5-restricted gluten-specific T-cell clones were reactive to either the DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 epitopes. Furthermore, for two of the patients (CD1041 and CD1042), both direct cloning and tetramer staining was performed and the frequency estimates obtained were similar as 2/384 T-cell clones were gluten-reactive and 0.2% of CD4<sup>+</sup> T cells from the lamina propria stained with the mixture of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers.

We found that tetramer staining of biopsy material was specific as a distinct population of DQ2.5-glia- $\alpha$ 1a/DQ2.5-glia- $\alpha$ 2 staining T cells was found only in CD patients expressing DQ2.5 but not two CD patients not expressing DQ2.5 or in non-CD controls. Unfortunately, none of the tested non-CD controls expressed the DQ2.5 allele. Further supporting the specificity of the staining, retesting of sorted tetramer positive T cells after in vitro expansion confirmed that most of these cells (range from 3/7 to 8/8 cells) were truly DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 specific. As only a preliminary, less strict gating was set up prior to flow-cytometry sorting and as the purity after sorting may be variable (could not be tested

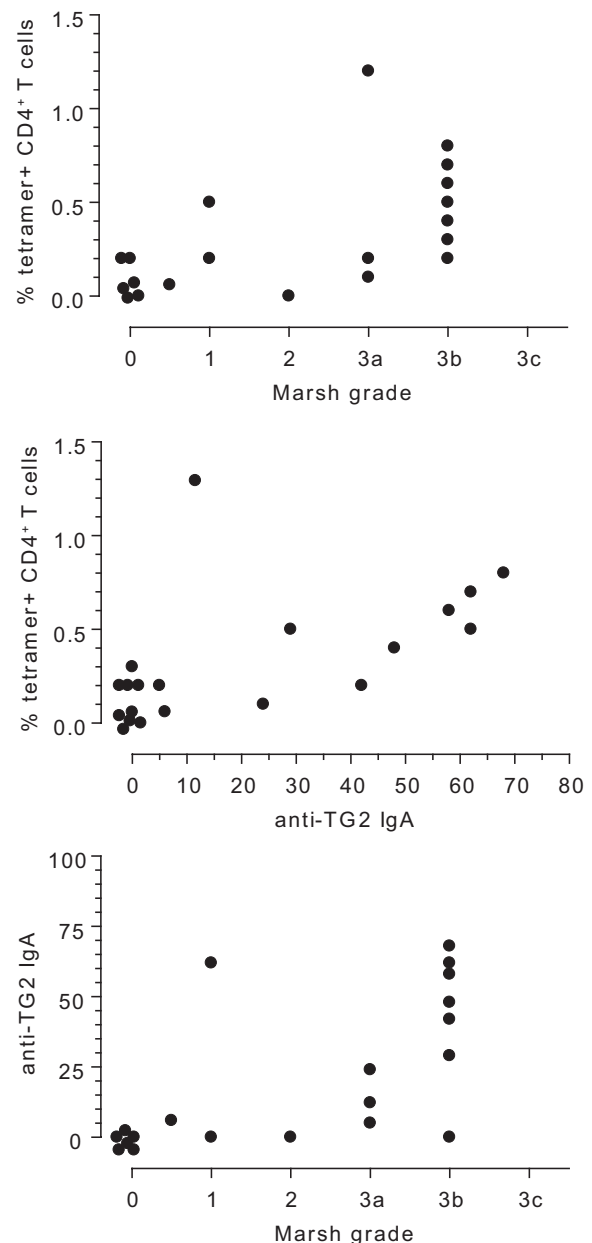


**Figure 4.** The relationship between frequency of gluten-specific CD4<sup>+</sup> T cells, histological damage, and titers of antitissue transglutaminase 2 (TG2) IgA in DQ2.5-expressing CD patients. The frequency of gluten-specific T cells correlated with (A) Marsh grade ( $p = 0.0007$ ) and (B) levels of anti-TG2 antibodies ( $p = 0.0003$ ), determined by Spearman rank correlation using the R software. (C) There is a significant correlation between Marsh grade and levels of anti-TG2 antibodies ( $p = 0.0003$ ).

given the low number of sorted cells), the specificity of the staining is probably even higher than the sorting and subsequent expansion would suggest.

The frequency of disease-driving CD4<sup>+</sup> T cells at the site of inflammation in chronic human inflammatory diseases remains poorly studied. We are aware of one study on Lyme arthritis, which demonstrated that CD4<sup>+</sup> T cells specific to an immunodominant epitope from *Borrelia burgdorferi* could be visualized in synovial fluid using MHC class II tetramers [23]. Interestingly, the

frequency of *Borrelia*-specific CD4<sup>+</sup> T cells was in the same range both in peripheral blood (0–0.1%) and in the target organ (synovial fluid, 0–3.1%) as gluten-specific T cells in CD. Intriguingly, the frequency of gluten-specific CD4<sup>+</sup> T cells in the CD lesion is five to tenfold lower than the frequency of intestinal TG2-specific plasma cells found in UCDs [24]. The correlation between intestinal gluten-specific T cells and anti-TG2 IgA levels suggest a link between gluten-specific T cells and anti-TG2-specific B cells, possibly via T-cell help from gluten-specific T cells to TG2-specific B cells [25].

Noteworthy, previous studies using duodenal organ cultures and overnight stimulation with gluten showed a frequency of activated CD4<sup>+</sup> T cells of over 10% (as evaluated by cytokine production or CD25 expression) in TCDs [26, 27]. We found that only a minority (less than 8%) of activated T cells (as assessed by CD25 expression) after 2 days of stimulation with a TG2-treated gluten digest was gluten-reactive after culture and retesting (data not shown). This suggests a prominent bystander activation of nongluten-reactive T cells and stresses the importance of being cautious when assessing the antigen specificity of cells activated in an organ culture system.

We found a significantly higher number of DQ2.5-glia- $\alpha$ 1a- or DQ2.5-glia- $\alpha$ 2-specific T cells as assessed by tetramer staining in untreated compared to TCDs (Fig. 3). Notwithstanding, among the subjects from whom T cells were cloned directly (Table 1), the treated patients had an apparent higher frequency of gluten-reactive T cells than the untreated patients. This might be due to the fact that several of these treated patients had profound histological damage or it could be due to chance, as only five patients were examined by direct cloning (no statistically significant difference).

We also observed that the frequency of gluten-reactive T cells as assessed by tetramer staining correlated with the grade of histological damage (Fig. 4A). This correlation may be secondary being the result of the different histological scores among untreated and treated patients. Alternatively, it may be primary if the healing of the mucosa — indicated by improving Marsh grade — is a consequence of the decreasing frequency of the pathogenic gluten-specific T cells. We find the latter explanation more likely.

We find it interesting that gluten-reactive T cells can be detected in the small intestine of TCDs after decades of gluten-free diet. The absence of the proliferation marker Ki-67 on CD4<sup>+</sup> T cells in the intestinal lesion of UCDs [28] suggests that longevity of gluten-reactive T cells or recruitment of such T cells from the circulation are more likely to explain their presence than local proliferation in the tissue. Intermittent antigen exposure, as by a poor compliance to a gluten-free diet or inadvertent consumption of gluten-containing food could possibly contribute to maintenance of memory T cells in peripheral tissues [29].

Recent findings in our laboratory suggest that the frequency of gluten-reactive CD4<sup>+</sup> memory T cells is in the range of 2–20/10<sup>6</sup> in peripheral blood of UCDs (Christophersen et al., unpublished data). The frequency of gluten-specific T cells is

thus several hundred-fold higher in the small intestine than in peripheral blood. This underscores the importance of searching for antigen-specific T cells at the site of inflammation itself.

In conclusion, we have been able to visualize gluten-reactive T cells directly in biopsy material without prior *in vitro* expansion and showed that their frequency correlates with the degree of histological damage of the gut lesion. Knowledge of the frequency of antigen-specific T cells in the setting of a chronic inflammation might be of interest for those studying chronic inflammatory diseases in which the search for the elusive disease-driving antigen-specific T cells is still ongoing.

## Materials and methods

### Subjects

CD patients in the study were diagnosed in accordance with the American Gastroenterology Association guidelines [30]. Genomic or serologic HLA typing was performed. Only the most common CD-associated HLA types of the patients are reported in this paper (DQ2.5, DQ8 and DQ2.2). Biopsies were taken as part of routine clinical follow-up or to investigate a suspected diagnosis of CD. Biopsies were also obtained from non-CD patients, referred to endoscopy for other reasons. The regional committee for medical research ethics had approved the relevant protocols, and patients gave written consent before participating.

### Antigens

As complex cereal antigen, we used gluten (prepared in house from wheat flour) digested with chymotrypsin and treated with TG2. The procedures for proteolytic digestion of gluten and treatment with TG2 have been described elsewhere [31]. Peptides were purchased from GLS Biochem (Shanghai, China), Research Genetics (Huntsville, AL, USA), Pepscan (Lelystad, The Netherlands), EZ Biolabs (Carmel, IN, USAs), or were synthesized in house. For sequences of the peptides, see Supporting Information Table 1.

### Generation of single cell suspension

Multiple biopsies (4–7) were sampled in ice-chilled RPMI-1640. Epithelial cells and intraepithelial lymphocytes were removed by incubation of biopsies in 2 mM EDTA/2% FCS (fetal calf serum)/PBS with continuous rotation for 1 hour at 37°C (this step was sometimes omitted). The remaining material was digested with collagenase (Sigma-Aldrich C-8051; 1 mg/mL) and DNase I (Sigma-Aldrich DN25; 50  $\mu$ g/mL) in 3% FCS/PBS for 1 hour in 37°C. Samples were homogenized by using a 1 mL pipette or a 1.2 mm syringe and then filtered through a 40  $\mu$ m cell strainer to remove cell aggregates.

## Direct cloning from biopsies

Single cell suspension was stained for CD4<sup>+</sup> T cells, which were sorted by flow cytometry. Cloning of these sorted T cells was done by limiting dilution as previously described [31]. The probability for clonality was over 70% for all T-cell clones tested based on Poisson distribution analysis. T cells were screened for reactivity to a TG2-treated chymotrypsin digest of gluten (in single in 96-well plates) using the irradiated DQ2.5-expressing lymphoblastoid B-cell line IHW9023 as APCs or autologous EBV-transformed B cells (for patients CD819 and 820). T-cell proliferation was assessed using thymidine incorporation assay as previously described [31]. T-cell clones with a stimulation index (cpm after antigen stimulation/cpm after medium stimulation) above 4 and a  $\Delta$ cpm (cpm after antigen stimulation – cpm after medium stimulation) above 10<sup>4</sup> were considered gluten reactive. Such T-cell clones were expanded, as previously described [31] and retested against a panel of known DQ2.5-restricted gluten epitopes and DQ2.5-expressing APCs.

## Preparation of DQ2.5 tetramers

Water-soluble DQ2.5 (DQA1\*05:01/DQB1\*02:01) molecules with peptides covalently tethered to the DQ $\beta$  chain were produced in a baculovirus expression system as described elsewhere [32]. DQ2.5 molecules with three different peptides were produced; two with deamidated T-cell epitopes of  $\alpha$ -gliadin, the DQ2.5-gliadin- $\alpha$ 1a (QLQPFPPQPELPY) and DQ2.5-gliadin- $\alpha$ 2 (PQPELPYPQPE) and one control tetramer with the CLIP2 peptide (MATPLLMQALPMGAL) [33] from invariant chain, which is a chaperone molecule during MHC class II synthesis and loading. Tetramers were made by conjugating the DQ2-peptide complexes with streptavidin-PE (Molecular Probes, Eugene, OR, USA).

## DQ2.5-tetramer staining in biopsy material

Single cell suspension (80  $\mu$ L) was incubated with 20  $\mu$ L FcR blocker (130–059–901, Miltenyi biotec, Bergisch Gladbach, Germany) for 20 min at 4°C and then with a mixture of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramers (10  $\mu$ g/mL of each) or negative control tetramer (DQ2.5-CLIP2; 10  $\mu$ g/mL) (all PE conjugated) for 30 min at 37°C in PBS with 2% FCS and 0.5  $\mu$ M EDTA. Thereafter, cells were cooled down on ice for a few minutes before staining for surface markers. We used the following antibodies for surface staining: anti-TCR (FITC, BD, Franklin Lakes, NJ, USA), anti-CD3 (FITC, Biolegend, San Diego, CA, USA) anti-CD4 (allophycocyanin-H7, BD), anti-CD45 (allophycocyanin, eBioscience, San Diego, CA, USA), anti-CD11c (Horizon V450, BD), anti-CD15 (Pacific blue, Biolegend), anti-CD14 (Pacific Blue, Biolegend), anti-CD19 (Pacific Blue, Biolegend), anti-CD8 (PE-Cy7, BD) and anti-CD3 (Brilliant Violet 570, Biolegend). Pacific Blue/Horizon V450 was used as dump channel. Propidium iodide (0.2  $\mu$ g/mL) was used to exclude dead cells. T cells

were identified on the basis of forward and side scatter and as CD45<sup>+</sup>/TCR<sup>+</sup> (or CD3<sup>+</sup>)/CD19, CD14, CD15, CD11c negative/propidium iodide negative cells. Cell sorting was performed on a FACSaria flow cytometer (BD) or data acquired on an LSR II flow cytometer (BD). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

## Statistical analysis

Statistical analysis was done using GraphPad Prism 4 software (San Diego, CA, USA) or R software (www.R-project.org) as indicated.

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**Abbreviations:** CD: celiac disease · TCD: treated CD patient · TG2: transglutaminase 2 · UCD: untreated CD patient

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## **Supporting Information for**

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**Direct cloning and tetramer staining to measure  
the frequency of intestinal gluten-reactive T cells  
in celiac disease**

# Supporting Information

**Supporting Information Table 1:** Overview of peptides representing gluten epitopes, which have been used in the study.

| Epitope(s)                   | Sequences                                                      |
|------------------------------|----------------------------------------------------------------|
| DQ2.5-glia- $\alpha$ 1a      | QLQPFPQPELPY                                                   |
| DQ2.5-glia- $\alpha$ 1b      | PQLPYQPPELPY                                                   |
| DQ2.5-glia- $\alpha$ 2       | PQPELPYPQPQL                                                   |
| DQ2.5-glia- $\alpha$ 1a/1b/2 | LQLQPF(PQPELPY) <sup>3</sup> PQPQPF                            |
| DQ2.5-glia- $\omega$ long    | QPQQPFPQPEQPFPWQP                                              |
| DQ2.5-glia- $\omega$ 1       | PQQPFPQPEQPFP                                                  |
| DQ2.5-glia- $\omega$ 2       | FPQPEQPFPWQP                                                   |
| DQ2.5-glia- $\gamma$ 1       | PEQPQQSFPEQERP or<br>YQQLPQPEQPQQSFPEQERPF or<br>PQQPQQSFPEQQR |
| DQ2.5-glia- $\gamma$ 2       | GIIQPEQPAQL                                                    |
| DQ2.5-glia- $\gamma$ 3       | FPEQPEQPYPEQ OR<br>FPQQPEQYPQQP                                |
| DQ2.5-glia- $\gamma$ 4a      | FSQPEQEFQPQ                                                    |
| DQ2.5-glia- $\gamma$ 4b      | FPQPEQEFQPQ                                                    |
| DQ2.5-glia- $\gamma$ 4c      | TEQPEQFPQP                                                     |
| DQ2.5-glia- $\gamma$ 4d      | PFPQPEQPFCEQPQR                                                |
| DQ2.5-glia- $\gamma$ 5       | PEQPFPEQPEQ                                                    |
| Long $\gamma$ -glia peptide  | QQPFPEQPEQYPQQPQQPFPQP                                         |

**Supporting Information Table 2:** Alignment of predicted binding cores to HLA-DQ2.5 of the stimulating peptides of 2 highly cross-reactive TCC.

|                          | TCC 1041.5 | TCC 1042.5 |
|--------------------------|------------|------------|
| DQ2.5-glia- $\alpha$ -2  | PQPELPYPQ  |            |
| DQ2.5-glia- $\gamma$ -4a | SQPEQEFPQ  |            |
| DQ2.5-glia- $\gamma$ -4b | PQPEQEFPQ  | PQPEQEFPQ  |
| DQ2.5-glia- $\gamma$ -4c | QQPEQPFPQ  | QQPEQPFPQ  |
| DQ2.5-glia- $\gamma$ -4d |            | PQPEQPFCQ  |
| DQ2.5-glia- $\omega$ -2  | PQPEQPFPW  | PQPEQPFPW  |

**Supporting Information Table 3:** Details on individuals used for validation of DQ2.5-glia- $\alpha$ 1a and DQ2.5-glia- $\alpha$ 2 tetramer staining of single cell suspension from intestinal biopsies<sup>a)</sup>.

| Patient CD | Clinical status | Frequency of tetramer+ CD4+ T cells | Histology at time of biopsy | TG2 IgA at time of biopsy | Time on GFD (years) | Specificity of sorted cells | HLA type                           |
|------------|-----------------|-------------------------------------|-----------------------------|---------------------------|---------------------|-----------------------------|------------------------------------|
| 712        | UCD             | 0.70%                               | Marsh 3B                    | 62                        | 0                   | ND                          | DQ2.5*                             |
| 838        | UCD             | 1.20%                               | Marsh 3A                    | 12                        | 0                   | 6/8                         | DQ2.5                              |
| 1031       | UCD             | 0.80%                               | Marsh 3B                    | 68                        | 0                   | 8/8                         | DQ2.5*                             |
| 1032       | UCD             | 0.50%                               | Marsh 3B                    | 29                        | 0                   | 4/6                         | DQ2.5/DQ2.2                        |
| 1041       | UCD             | 0.20%                               | Marsh 3B                    | 42                        | 0                   | ND                          | DQ2.5/DQ2.2                        |
| 1042       | UCD             | 0.20%                               | Marsh 3A                    | 5                         | 0                   | 3/7                         | DQ2.5                              |
| 1054       | UCD             | 0.10%                               | Marsh 3A                    | 24                        | 0                   | ND                          | DQ2.5/DQ2.2                        |
| 1079       | UCD             | 0.60%                               | Marsh 3B                    | 58                        | 0                   | ND                          | DQ2.5/DQ2.2                        |
| 1114       | UCD             | 0.50%                               | Marsh 1**                   | 62                        | 0                   | ND                          | DQ2.5                              |
| 1143       | UCD             | 0.40%                               | Marsh 3A-3C                 | 48                        | 0                   | 8/9                         | DQ2.5 <sup>+</sup>                 |
| 1065       | UCD             | UD                                  | Marsh 3C                    | 20                        | 0                   | ND                          | DQ8*                               |
| 364        | TCD             | UD                                  | Marsh 0                     | <1                        | 20                  | ND                          | DQ2.5*                             |
| 1056       | TCD             | 0.30%                               | Marsh 3B                    | <2                        | 24                  | ND                          | DQ2.5*                             |
| 1084       | TCD             | 0.06%                               | Marsh 0/1                   | 6                         | 17                  | ND                          | DQ2.5*                             |
| 1087       | TCD             | 0.07%                               | Marsh 0                     | <1                        | 10                  | ND                          | DQ2.5*                             |
| 1088       | TCD             | 0.20%                               | Marsh 0                     | <1                        | 15                  | ND                          | DQ2.5*                             |
| 1089       | TCD             | 0.04%                               | Marsh 0                     | <1                        | 39                  | ND                          | DQ2.5*                             |
| 1090       | TCD             | 0.20%                               | Marsh 1                     | <1                        | 43                  | ND                          | DQ2.5*                             |
| 1092       | TCD             | UD                                  | Marsh 0                     | <1                        | 41                  | ND                          | DQ2.5*                             |
| 1093       | TCD             | 0.20%                               | Marsh 0                     | <1                        | 10                  | ND                          | DQ2.5*                             |
| 1132       | TCD             | UD                                  | Marsh 2                     | <1                        | 2                   | ND                          | DQ2.5*                             |
| 1073       | TCD             | UD                                  | Marsh 0                     | <1                        | 24                  | ND                          | DQ2.2                              |
| 1128       | TCD             | UD                                  | Marsh 0                     | 6                         | 10                  | ND                          | DQ2 <sup>+</sup> /DQ8 <sup>-</sup> |
| 1082       | NCD             | UD                                  | Marsh 0                     | <1                        | 0                   | ND                          | DQ2 <sup>+</sup> /DQ8 <sup>-</sup> |
| 1083       | NCD             | UD                                  | Marsh 0                     | ND                        | 0                   | ND                          | DQ8*                               |
| 1070       | NCD             | UD                                  | Marsh 0                     | <1                        | 0                   | ND                          | DQ2-/DQ8-                          |
| 1133       | NCD             | UD                                  | Marsh 0                     | ND                        | 0                   | ND                          | DQ2.2                              |
| 1135       | NCD             | UD                                  | Marsh 0                     | ND                        | 0                   | ND                          | DQ8                                |
| 1136       | NCD             | UD                                  | Marsh 0                     | ND                        | 0                   | ND                          | DQ2-/DQ8-                          |
| 1137       | NCD             | UD                                  | Marsh 0                     | ND                        | 0                   | ND                          | DQ2-/DQ8-                          |

a) ND: not done; UD: undetectable; UCD: untreated celiac disease; TCD: treated celiac disease; NCD: non-celiac disease individual. The specificity of sorted cells refers to the number of DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 specific T-cell clones after sorting by flow-cytometry of DQ2.5-glia- $\alpha$ 1a or DQ2.5-glia- $\alpha$ 2 staining cells from single cell suspension (e.g., 4/6 indicates that 4 out of 6 clones generated are specific, see Figure 3). For some patients (marked with \*), HLA typing for was performed only for the risk alleles at the department of immunology and only the highest risk molecule was reported in the following order (DQ2.5/DQ8/DQ2.2).

\*\* This patient was considered untreated despite of Marsh 1 grade histology because of the high IgA anti-TG2 antibody levels and clinical signs and symptoms of fatigue, abdominal discomfort and iron deficiency anemia.