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Similar Responses of Intestinal T-cells From Untreated Children and Adults With Celiac Disease to Deamidated Gluten Epitopes

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Abbreviations: TG2 (transglutaminase 2), TCL (T-cell line), TCC (T-cell clone), PT-gliadin (pepsin-trypsin digest of gliadin), CT-gluten (chymotrypsin digest of gluten)

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Abstract

Background & Aims: Celiac disease is a chronic small intestinal inflammatory disorder mediated by an immune response to gluten peptides in genetically susceptible individuals. Celiac disease is often diagnosed in early childhood, but some patients receive a diagnosis late in life. It is uncertain whether pediatric celiac disease is distinct from adult celiac disease. It has been proposed that gluten-reactive T cells in children recognize deamidated and native gluten epitopes, whereas T cells from adults only recognize deamidated gluten peptides. We studied the repertoire of gluten epitopes recognized by T cells from children and adults.

Methods: We examined T-cell responses against gluten by generating T-cell lines and T-cell clones from intestinal biopsies of adults and children and tested proliferative response to various gluten peptides. We analyzed T cells from 14 children (2–5 years old) at high risk for celiac disease who were followed for celiac disease development. We also analyzed T cells from 6 adults (26–55 years old) with untreated celiac disease. All children and adults were positive for HLA-DQ2.5. Biopsies were incubated with gluten digested with chymotrypsin (modified or unmodified by the enzyme transglutaminase 2) or the peptic-tryptic digest of gliadin (in native and deamidated forms) before T-cell collection.

Results: Levels of T-cell responses were higher to deamidated gluten than to native gluten in children and adults. T cells from children and adults each reacted to multiple gluten epitopes. Several T-cell clones were cross-reactive—especially clones that recognized epitopes from γ- and ω-gliadin. About half of the generated T-cell clones from children and adults reacted to unknown epitopes.

Conclusions: T-cell responses to different gluten peptides appear to be similar between adults and children at the time of diagnosis of celiac disease.

Keywords: autoimmunity, CD4+ T cell, TG2, PreventCD study

Introduction

Celiac disease is a chronic small intestinal inflammatory disease driven by gluten proteins of wheat, barley and rye[1]. The classical form of celiac disease presents with chronic diarrhea, malabsorption and failure to thrive. This condition was long considered a childhood disease[2]. Today, however, the diagnosis is frequently made in adults who typically have vaguer symptoms such as iron deficiency anemia, chronic fatigue, and recurrent abdominal pain.

Celiac disease is a multifactorial, polygenic disease, in which the HLA locus is by far the most important genetic risk factor[3]. Most celiac disease patients express the HLA class II allotype HLA-DQ2.5, and the remaining patients express either HLA-DQ8 or HLA-DQ2.2[4]. Notably, CD4+ gluten-reactive T cells recognize gluten peptides presented by the disease-associated HLA molecules. Most importantly, such cells can be isolated from the small intestine of celiac disease patients, but not from healthy controls[5, 6], suggesting that intestinal gluten-reactive T cells play an important role in the pathogenesis of celiac disease. Gluten epitopes recognized by CD4+ T cells of celiac disease patients expressing HLA-DQ2.5 are fairly well characterized[7], however, most previous studies examined T cells from adults. T cells of adult celiac disease patients preferentially recognize deamidated gluten peptides that are modified by the enzyme transglutaminase 2 (TG2).[8, 9] Moreover, it was suggested that there is a hierarchy of epitopes with a few

immunodominant peptides recognized by T cells from most patients, such as two epitopes from α-gliadin, the DQ2.5-glia-α1 and the DQ2.5-glia-α2.[7,10, 11]

As most studies were performed using biopsies from adult patients, it is unclear whether gluten-reactive T cells recognize a different set of peptides in children. The limited knowledge about T-cell repertoire in children is mostly due to the small amount of tissue that can be obtained because of ethical and technical reasons. We are aware of only two articles, one from 2002 by Vader and colleagues[12] and the other from 2015 by Hardy and co-workers[13], that compared T-cell repertoire in children and adults. Vader and colleagues proposed that T-cell reactivity towards gluten in children is broad, with preferential recognition of native gluten epitopes. They suggested that immune response against deamidated peptides develops later, and due to its higher affinity and efficiency leads to a focused immune response against deamidated gluten peptides in adults. In contrast to these findings, the study by Hardy and colleagues described similar T-cell responses in children and adults, although the median age of the participating children was considerably higher in this latter study, thus they have had the disease for a longer time.

Prevention of celiac disease is desirable, and it was suggested that by introducing gluten into children`s diet at the age of 4-6 months, one could lower the risk of development of celiac disease[14]. A European multicenter study, PreventCD (www.preventcd.com), recruited 1000 children at high

genetic risk for disease development and randomized 944 of them to receive 100 mg gluten or 100 mg lactose between the age of 4 and 6 months to test for primary prevention and to explore the underlying genetic and immunological mechanisms of celiac disease[15, 16]. Blood samples were taken from the participating children at 6, 9, 12, 18, 24, 36 months of age and then annually to measure IgA antibodies against TG2 and gliadin. Children with elevated levels of antibodies on two occasions and/or with clinical suspicion of celiac disease were offered to undergo gastroscopy and small-bowel biopsy[15]. Here we report the characterization of reactivity against gluten by T cells isolated from biopsies taken during the PreventCD study, and contrast the findings with generation of T cells from untreated adult patients using the same laboratory protocol.

Materials and methods

Subjects

The children recruited to this study were participants of PreventCD, a European multicenter cohort on the development of celiac disease (www.preventcd.com). In the PreventCD project, children with at least one first-degree relative affected by celiac disease and were genotyped to carry the disease-associated DQ2 or DQ8 HLA molecules were invited to participate shortly after birth[15]. Enrolled children were followed prospectively, and in cases of suspected celiac disease, based on monitoring of disease-specific antibodies and/or symptoms, the (parents of) the children were offered diagnostic small intestinal biopsies. In the current study, biopsies from eleven children from Hungary and three children from Spain, all subsequently proven to have celiac disease, were used to assess T-cell reactivity to gluten. In addition, biopsies from 6 adult Norwegian patients were obtained in order to compare T-cell reactivity with that of newly diagnosed adults. The biopsies of adult patients were obtained during endoscopic investigations as a part of routine diagnostic work-up. Detailed information about the participants is shown in Supplemental Table 1 and 2. Of note, all participants express HLA-DQ2.5. The regional committees for medical research ethics in Hungary and Spain approved the protocols for participating children and the regional committee for medical research ethics in Norway approved the protocol for the participating adults. The patients and/or their parents gave written consent before participation in the study.

Gluten and peptide antigens

Gluten antigens were used either without or with treatment by TG2; the former types we denote as native forms and the latter types we denote as deamidated forms. The peptic-tryptic digest of gliadin (PT gliadin), in both native and deamidated forms (PT and PT-TG2, respectively), were gifts from professor Frits Koning (Leiden University Medical Centre, Leiden, The Netherlands)[17]. These antigens were used for the initial stimulation of biopsies of all patients and for testing reactivity of T-cell lines. In addition, we included gluten digested with chymotrypsin (CT gluten)[18], also in native and deamidated forms for initial stimulation of some biopsies and for testing of reactivity of all the generated T cells. To obtain CT gluten, wheat flour was washed repeatedly in water-saturated n-butanol, centrifuged at 200 g for 5 min between washings and the supernatant was discarded. The resulting wheat gluten fraction was then dissolved in 70% ethanol and stirred overnight at room temperature, followed by centrifugation at 400 g for 5 min. and precipitation of the gliadin proteins with 1,5 w/v% NaCl solution. The precipitate was then dissolved in 0,01 M CH_3COOH and stored at 4°C until enzymatic digestion. For the enzymatic digestion, gliadin was dissolved in 0,01 M NH_4HCO_3 with 2 M urea, and 1:200 chymotrypsin was added after 2 hrs stirring at 37°C. The enzymatic digestion was run overnight and terminated by heat inactivation at 98°C for 5 min. CT gluten was dialyzed against 0,01 M NH_4HCO_3 and stored at room temperature after lyophilization. Peptides were purchased from GLS Biochem (Shanghai, China), Research

Genetics (Huntsville, AL), Pepscan (Lelystad, The Netherlands), EZ Biolabs (Carmel, IN) or were synthesized in house. Peptide sequences are shown in Supplemental Table 3.

Generation of T-cell lines

One or two biopsies from each patient were transported on ice in RPMI-1640 to our laboratory to generate T-cell lines (TCLs). Biopsies were processed within 24 hours from sampling. Upon arrival, biopsies were incubated in PBS containing 1 mM DTT twice for 5 minutes to remove mucus, then rinsed and incubated in 5 ml PBS supplemented with 0,75 mM EDTA for an hour with gentle rotation to remove epithelial cells. Subsequently, biopsies were transferred to a 24-well plate in 1,5 ml RPMI-1640 supplemented with 200 U/ml penicillin and 100 μg/ml streptomycin, 10% human serum, and 20 μg/ml each of native and deamidated gluten, or for the last three children, only 20 μg/ml of native gluten. When only one biopsy was received, native- and deamidated PT-gliadin was used for initial stimulation; when two biopsies were received from the same patient, the second biopsy was incubated with native- and deamidated CT-gluten. On day 3 and 6, another 1 ml medium with 20 U/ml IL-2 and 5 ng/ml IL-15 was added. Cells were collected on day 9-11, and were restimulated in 1,5 ml culture medium (RPMI-1640 with 10% human serum, 200U/ml penicillin, and 100 μg/ml streptomycin) with addition of 1 million/ml irradiated allogeneic peripheral blood mononuclear cells (PBMC), 20 U/ml IL-2 and 5 ng/ml IL-15. Cells were split up to day 6 if

necessary, followed by testing against native and deamidated CT-gluten and various peptides at day 9-10.

Generation of T-cell clones

T-cell clones (TCCs) were generated from lines with limiting dilution using Terasaki plates that were placed in a moist chamber inside a humidified incubator at 37°C with 5% CO_2. In each well of the Terasaki plates, 0.3-1 T cells were plated in 20 μl culture medium, supplemented with 1 million/ml irradiated (50 Gy) PBMC, 1 μg/ml PHA, 10 U/ml IL-2, and 1 ng/ml IL-15. After 10 days, growing TCCs were harvested and restimulated, followed by testing for reactivity against native and deamidated CT-gluten 9-11 days after restimulation.

T-cell proliferation assay

TCLs and TCCs were resuspended in 50 μl culture medium at a concentration of 10^6 cells/ml and cultured in the presence of 50.000 cells from an irradiated (75 Gy) B-cell line homozygous for DQ2.5 together with various gluten antigens. Gluten was added at a concentration of 100 μg/ml and peptides at 10 μM if not stated otherwise. T-cell proliferation was evaluated after 72 hrs stimulation by the uptake of [3H]thymidine (1 μCi/well (0.037 MBq/well), which was added to the wells 24 h before harvesting. Cells were harvested with an automated harvester (Mach III; TomTec, Hamden, CT, USA), and the incorporated radioactivity was measured by liquid scintillation counting (Wallac MicroBeta TriLux 1450; PerkinElmer, Wellesley, MA, USA) as

count-per-minutes (cpm). Each concentration of antigen was studied in duplicates or triplicates. Stimulation index (SI) was calculated by dividing the mean proliferation count for a given antigen with the mean proliferation count for T and B cells co-cultured without antigen.

Results

T-cell lines display reactivity to many gluten epitopes, both in children and adults

TCLs were generated from 11 children and all adult patients by incubating biopsies with 20 μg/ml each of native and deamidated pepsin-trypsin-treated (PT) gliadin followed by the addition of IL-2 and IL-15 after 3 days to ensure T-cell growth. When two biopsies were received from a patient, the second biopsy was incubated with 20 μg/ml of both native and deamidated chymotrypsin-treated (CT) gluten, similarly followed by the addition of IL-2 and IL-15. Biopsies of the remaining 3 children were initially incubated with only native PT gliadin or CT-gluten (see later). Upon harvesting, TCL were tested against native and deamidated PT gliadin, CT gluten and deamidated peptides. TCL with a stimulation index above 2 were scored as gluten reactive. One TCL from an adult and one from a child were considered not to be gluten-reactive. Of note, the biopsy of the child was frozen upon arrival, what likely explains the lack of reactivity. We observed that T-cell reactivity was usually lower in response to native gluten compared to deamidated gluten in both children and adults. T-cell reactivity was generally similar or lower towards deamidated PT-gliadin than deamidated CT-gluten. Overall, TCLs displayed reactivity to epitopes derived from α-, γ-, and ω-gliadin (Figure 1). In order to be able to give a more detailed assessment of T-cell reactivity against gluten, we generated altogether 78 gluten-reactive T-cell

clones (TCC) from six of the eleven children and 36 gluten-reactive TCCs from the four adult patients.

No T-cell clones recognize preferentially native gluten peptides

TCCs were tested against native- and deamidated CT-gluten and a panel of deamidated synthetic peptides representing the most common α-, γ-, and ω-gliadin epitopes. Some TCCs were tested further against additional synthetic peptides (see Supplemental Table 3 for the list of peptides). The majority of the TCCs, both from children and adults, proliferated better to deamidated than native gluten antigens. None of the TCCs proliferated more vigorously in response to native gluten compared to deamidated gluten antigens. Nine out of 78 TCCs from the children, but none of 36 TCCs from the adults responded equally well to native and deamidated gluten antigen. However, all four of the children-derived TCCs for which we were able to assign epitope specificity, responded better to deamidated than native peptide. Specifically, one DQ2.5-glia-γ1-reactive TCC from a child (TCC 856.1.11) showed similar dose-response curve to native and deamidated gluten, but when tested with synthetic peptides, required double concentration of native peptide compared to the deamidated in order to achieve half maximal response (Figure 2A). Three other TCCs from another child, all reactive against DQ2.5-glia-γ4b (TCC 827.1.20, TCC 827.1.23 and TCC 827.174), recognized native and deamidated gluten similarly, but needed 10 times less deamidated peptide for half maximal proliferation (IC50 value) compared to the corresponding native peptide (Figure 2B). Upon T-cell receptor (TCR) sequencing, two of

these three clones, TCC 827.1.23 and TCC 827.1.74, proved to be sister clones expressing the same TCR (data not shown). We found similar reactivity against native and deamidated gluten in further 5 TCCs from a third child (Figure 3), but the specificity of these clones is unknown, so comparative experiments with native vs. deamidated synthetic peptides could not be undertaken. It is thus uncertain whether these clones are truly deamidation-independent. It is also uncertain whether some of these TCCs are also sister clones.

To further examine reactivity against native gliadin, we generated TCLs from 3 additional children whose biopsies were initially incubated with 20 μg/ml native, but not deamidated gluten. Interestingly, these lines had somewhat lower reactivity upon testing (SI 3 – 5.5) compared to lines generated from biopsies incubated with both native and deamidated gluten (SI 5 - 21, mean 9.7). We attempted to generate TCCs from two of these TCLs and obtained three TCCs that were reactive against gluten; all three had a preference for recognition of deamidated CT-gluten (data not shown). We were not able to assign epitope specificity for any of these three TCCs, but one was cross-reactive to the 33mer peptide from α-gliadin with a proliferative response of about 50% compared to deamidated CT-gluten.

Taken together, the lion's share of TCCs from both children and adults recognized deamidated gluten better than native gluten antigen, while a few

TCCs from children but not from adults recognized native and deamidated gluten antigen equally well.

Broad epitope reactivity of T-cell clones in both children and adults

T cells in both children and adults demonstrated reactivity to a wide array of gluten peptides also on a clonal level. We considered a TCCs specific for a given peptide if it showed higher or equal than 75% proliferation (i.e. cpm) in response to 10 μM of the peptide compared to 100 μg/ml deamidated CT-gluten. Proliferative response below 75% of response to gluten was considered as cross-reactivity with unknown specificity. Surprisingly, we were unable to determine the epitope specificity in about half of the clones in both children and adults, as they responded to deamidated/native gluten, but not to any of the peptides in the test panel. The results are presented in Figure 4 and Table 1 and 2. While only about 10% of the clones were found to be reactive to α-gliadin derived peptides, almost all lines responded to the 33mer peptide from α-gliadin indicating that the immunodominance of α-gliadin epitopes observed in TCL are not correlated with the prevalence of α-gliadin reactive clones in TCCs cultured from the same lines. Interestingly, many TCCs recognized peptides from γ- and ω-gliadin.

Fifty-one TCCs of 8 patients were tested for reactivity against TG2. Twenty-eight of these TCCs turned out to have unknown specificity, but importantly none of the tested TCCs proliferated upon stimulation with TG2 alone (Figure 4 and 5 and data not shown).

Frequent T-cell cross-reactivity to several epitopes

We further observed that many TCCs proliferated upon stimulation with several peptides, especially those representing the DQ2.5-glia-ω1/2 epitopes and the DQ2.5-glia-γ4 epitopes (Figure 5A). TCCs recognizing DQ2.5- glia-γ1 were usually not cross-reactive (Figure 4, middle panel). TCCs recognizing α-gliadin were also rarely cross-reactive. We observed T-cell cross-reactivity to DQ2.5-glia-ω1 and the 33mer peptide from α-gliadin in two TCCs; one from an adult patient and another from a child (Figure 4 and 5). We did not find TCCs reactive to the DQ2.5-glia-γ2 and DQ2.5-glia-γ3 epitopes, only four TCCs that displayed a varying degree of cross-reactivity to these peptides (data not shown).

We sequenced the TCR of selected TCCs and found that many cross-reactive TCCs indeed expressed only one TCR, and thus were truly cross-reactive, such as TCC 1034.1.47, 1032.1.82, and 846.1.29 shown in Figure 5A. All but one TCC recognizing peptides representing DQ2.5-glia-ω epitopes were cross-reactive with peptides representing various DQ2.5-glia-γ epitopes. On the other hand, we obtained TCCs that were mono-specific to the DQ2.5-glia-γ4b or DQ2.5-glia-γ4c epitopes (Figure 5B). Interestingly, there is a substantial sequence homology between the DQ2.5-glia-γ4 and DQ2.5-glia-ω2 epitopes with amino acid identity at the critical position P2-P5 and P7. Alignment of the peptides tested with their registers for binding to HLA-DQ2.5 is shown in Supplemental Table 4.

Discussion

Here we demonstrate that young children and adults with untreated celiac disease have T-cell reactivity to a broad range of gluten epitopes at the time of diagnosis, and that T cells from children have no preferential recognition of peptides with native sequences.

The major strengths of this study is that it examines the T-cell responses to gluten in children prospectively followed for celiac disease development and diagnosed at the earliest possible time point, and that it compares the reactivity pattern of gluten-reactive TCLs and TCCs with those of adults generated by the same laboratory protocols and during the same time period. Children participating in the PreventCD study were monitored with frequent serological tests for early detection of celiac disease and 30-40% had no symptoms[16]. They were diagnosed much earlier than in a usual clinical setting, but they still had a chronic immunological reaction against gluten that had already resulted in elevated antibody levels against TG2. Biopsies used for culturing T cells were taken at the earliest time point which was ethically possible. Based on studies of this unique material, our finding of similar reactivity patterns of T cells from children and adults suggests that the observed differences do not relate to age of the patients or to clinical symptoms. Children participating in the PreventCD study were randomized to receive 100 mg gluten or lactose between the age of 4 and 6 months. We believe that the earlier gluten exposure did not influence the results, as the

T-cell repertoire was similar in children and adults and celiac disease development was similar among children with earlier gluten exposure and controls[16].

We are aware of two studies comparing T-cell responses against gluten in children and adults, reporting contradicting results[12 13]. The TCLs in our study were generated from a similar group of patients and with a similar method to that used by Vader et al.,[12], although in contrast to their results, we did not find a preferential recognition of native gluten sequences, and we never observed that T-cell response was abolished or diminished by deamidation of a peptide. Our findings are in accord with the recent report of Hardy and colleagues[13] who examined T-cell responses towards gluten in peripheral blood of treated patients after a short gluten challenge, although the median age of children participating in that study was 9 years (median age at diagnosis was 5.5 years), and thus they had the disease considerably longer than the children recruited to the PreventCD project.

We observed that the majority of TCCs from both children and adults preferentially recognized deamidated over native gluten antigen, while a few TCCs from children but not from adults recognized native and deamidated gluten antigen equally well. None of the TCCs for which we were able to assign the epitope specificity, showed better reactivity to native than deamidated gluten peptide.

Relevant to this study it was demonstrated in a humanized DQ8 mouse model of celiac disease that the native DQ8-glia-α1 epitope recruited a cross-reactive T-cell population and almost a fourth of T-cell hybridomas generated against the native peptide showed a heteroclitic (stronger) response to the deamidated epitope[19]. This observation gave rise to the hypothesis that the anti-gluten T-cell response in celiac disease starts with a response to native gluten peptides, which leads to tissue damage, causing release of intracellular TG2 and the generation of deamidated gluten peptides. Hence, on maturation of the T-cell response, reactivity to deamidated peptides should take over. A similar mechanism was proposed by Vader et al., based on their observation of predominant response to native peptides in DQ2.5 children with a focusing of the immune response towards deamidated epitopes in T-cell response of adults[12]. In our study of DQ2.5 children at the earliest possible time-point for monitoring, most TCCs responded much better to deamidated than native gluten. While equal recognition of native and deamidated gluten for some few TCCs of the children might be interpreted to represent a remnant of a primary response to native gluten, we believe that the general preferential recognition of deamidated over native gluten both in children and adults and the lack of better recognition of native over deamidated gluten should be given more weight in the interpretations. Hence, with the reservation that the investigated children already had acquired mature anti-gluten immune responses despite their early recruitment and that we have failed to record the initial reactions, our

observations do not provide support to the notion of an initial response to native gluten peptides in celiac disease.

As to whether the responses in vivo start to native or deamidated peptides, valuable insights come from studies of DQ2.5 and DQ2.2 celiac disease patients along with the mapping of which gluten epitopes the patients make responses to. DQ2.5 patients generate T-cell responses to gluten peptides that make kinetically stable complexes with DQ2.5 [20], and DQ2.2 patients generate T-cell responses to gluten peptides that make kinetically stable complexes with DQ2.2[21].Thus, gluten peptides that cannot make stable complexes with HLA molecules appear to be unable to mount in vivo responses in the patients. Deamidated gluten peptides bind more stably to DQ2 than their native counterparts[22] and the same is likely to be the case for DQ8. In this setting, the issue of heteroclitic responses shaping the final response is not relevant. These observations in our opinion support the notion that in vivo T-cell responses are generated to deamidated rather than native gluten.

We found reactivity to a broad range of gluten epitopes both in children and adults. Rather unexpectedly, many TCCs did not respond to defined epitopes despite being gluten-reactive, hence they appear to be specific for yet uncharacterized epitopes. Whether these clones recognize many different epitopes or only a few, remains unknown. Interestingly, Tye-Din and coworkers reported that T cells specific for the ω-gliadin/C-hordein sequence

PFPQPEQPFPW were particularly cross-reactive with other epitopes[23]. In agreement with this observation, we found cross-reactivity most frequently between DQ2.5-glia-ω1/2 epitopes and DQ2.5-glia-γ4 epitopes. Similarly to the above-mentioned study, we have found very little cross-reactivity of TCCs recognizing DQ2.5-glia-γ1 and α-gliadin epitopes.

The term immunodominance in T-cell reactivity to gluten epitopes has been used both to reflect that the majority of patients make response to a certain epitope, and also to indicate that a high proportion of T cells in a given patient recognize a particular epitope[24]. Studying polyclonal TCLs, the obtained information will be relevant to the former aspect. Information relevant to the latter aspect would, if addressing this issue with cultured T cells, ideally require generation of large panels TCCs. We found that most TCLs proliferated upon stimulation with peptides representing the DQ2.5-glia-α1/2 epitopes, which is in line with results of previous studies of treated patients [11, 25]. Of note, however, the TCCs reactive against these epitopes could be generated only in half of the patients, and DQ2.5-glia-α1/2 reactive TCCs represented only 10% of the TCCs. This is at variance with earlier reports studying treated patients[11], but in accord with results of Vader al al., who by investigating untreated children with an experimental protocol similar to the one used in the current study, found reactivity to the DQ2.5-glia-α1/2 epitopes in a limited proportion of TCCs[12]. While the different results obtained may relate to the culturing protocols used, it may also be that there is a difference between untreated and treated patients. While untreated patients

generally appear to make T-cell responses to the DQ2.5-glia-α1/2 epitopes, these patients also have many TCCs that respond to other epitopes and even to epitopes that are yet uncharacterized. The reactivity to a wide range of gluten epitopes could possibly be a feature of untreated disease and one could hypothesize that upon contraction of T-cell populations after elimination of gluten from the diet, the DQ2.5-glia-α1/2 reactive T cells remain at relatively higher frequency, and thereby are easier to detect. Further studies are required settle this issue.

We tested about half of the generated TCCs for proliferative response against TG2 alone, but did not find reactivity. Human recombinant TG2 was present during initial stimulation of the biopsies in our study, albeit at a low concentration (up to 1 μg/ml), thus allowed for antigenic activation similar to gluten peptides. T-cell reactivity against TG2 is not described in the small intestine, but there are two studies that reported generation of TCLs from peripheral blood that are reactive against TG2 after repeated stimulation with the TG2 antigen[26, 27]. If TG2-specific memory T cells exist and are constantly present in celiac disease patients, one would expect a continuous production of antibodies against TG2, which does not fit with the dynamic change of antibody levels in serum in response to changes in gluten ingestion. Furthermore, TG2 is abundantly expressed in erythrocytes[28] and antigens expressed by erythrocytes cause effective T cell non-responsiveness by T cell deletion[29] or by induction of regulatory T cells[30]. It is therefore tempting to hypothesize that the TG2-reactive clones reported in the above-mentioned

studies are derived from low-affinity naïve T cells in peripheral blood that probably would not receive the necessary co-stimulatory signals to differentiate into memory cells *in vivo*.

In conclusion, T-cell responses against gluten are set from the earliest moment it is possible to assess and the same characteristics remain stable even if the diagnosis is made late in life.

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Figure legends

Figure 1. Gluten specific T-cell lines recognize a variety of epitopes both in adults and children.

Gluten-reactive T-cell lines were generated from biopsies from newly diagnosed children (A) and adults (B) and tested in triplicates against 100 μg/ml native (CT), and deamidated chymotrypsin-digested gluten (CT-TG2), native- (PT), and deamidated pepsin-trypsin-digested gliadin (PT-TG2) and 10 μM of synthetic gliadin peptides containing various DQ2.5-gliadin epitopes. The following peptides were tested: α-glia: the immunodominant 33mer peptide (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF), ω-glia: a 17mer containing the DQ2.5-glia-ω1 and DQ2.5-glia-ω2 epitopes (QPQQPFPQPEQPFPWQP), γ-glia: a 26mer peptide from γ-gliadin (EQPFPEQPEQPYPEQPEQPFPQP). Error bars show SEM.

Figure 2.

Few gluten-reactive T-cell clones (TCC) respond at the same extent to native- and deamidated gluten antigens. TCCs with at least 90% proliferation against 100 μg/ml native- compared to deamidated chymotrypsin-digested and TG2-treated gluten were tested with titration of gluten and peptide. (A) A single T-cell clone that react similarly to both to native (CT) and deamidated gluten (CT-TG2) and requires double concentration of the native DQ2.5-glia-γ1 epitope for half-maximal response compared to the

deamidated epitope. (B) Two TCCs from another patient proliferate similarly in response to native (CT) and deamidated gluten (CT-TG2), but respond more vigorously to the DQ2.5-glia-γ4b epitope sequence in the deamidated version than the native version.

Figure 3.

Five TCCs from a third child respond similarly to native (CT) and deamidated gluten (CT-TG2), but have unknown specificity.

Figure 4.

TCCs recognize a broad set of peptides both in children and adults with various patterns. From the upper panel: unknown-reactivity, true reactivity for the DQ2.5-γ1 epitope, and cross-reactivity for the DQ2.5-ω1 epitope and the immunodominant 33mer peptide from α-gliadin at about 50% proliferation in response to 10 μM peptide compared to 100 μg/ml gluten, but lack of "true" reactivity. CT: Chymotrypsin-digested gluten; CT-TG2: chymotrypsin digested and TG2-treated gluten, PT: pepsin-trypsin digested gliadin, PT-TG2: pepsin-trypsin digested and TG2-treated gliadin, α-glia: DQ2.5-α gliadin epitopes, ω-glia: DQ2.5-ω gliadin epitopes, γ-glia: DQ2.5-γ gliadin epitopes

Figure 5.

(A) Cross-reactivity is frequent, especially in TCCs recognizing peptides representing DQ2.5-glia-ω1/2 and DQ2.5-gliaγ-4 epitopes. One TCC from a

child showed cross-reactivity between peptides representing DQ2.5-glia-ω epitopes and the 33mer peptide from α-gliadin. (B) Some TCCs recognize peptides from γ-gliadin without cross-reactivity. Results from TCCs monospecific for the DQ2.5-glia-γ4c and DQ2.5-gliaγ4b epitopes are depicted.

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Author names in bold designate shared co-first authorship.

Table 1. Overview of the TCCs generated from children participating in the study, according to reactivity.
"True" reactivity is defined as at least 75% cpm compared to chymotrypsin-digested TG2-treated gluten (CT-TG2). In case of lower proliferative response, the clone is considered as having unknown reactivity with cross-reactivity.
The peptide that gave highest proliferation index is shown in T cell clones proliferating in response to several peptides.

Patient ID	Number of clones	α	γ (ω cross)*	ω (γ cross)*	Unknown 50-75%	Unknown 10-50%	Unknown < 10%	Other
827	35	3	13 (5)	0	0	4α + 1ω	14	
846	13	1	5 (0)	2 (2)	0	3γ	1	1 (peptide 2126##)
856	8	1	2 (0)	0	0	3γ	2	
1013	7	4	1 (0)	0	2γ	0	0	
1026	4	0	1 (0)	0	1γ	0	1	1 (DQ2.2 glutenin peptide, 2109)
1052	11	0	0	0	0	0	11	
1241#	2	0	0	0	1α	0	1	
108-162#	1	0	0	0	0	0	1	
Total	81	9	22 (5)	2 (2)	4	11	31	2

*The number of DQ2.5-glia-ω cross-reactive clones among the DQ2.5-glia-γ TCCs and the number of DQ2.5-glia-γ cross-reactive clones among the DQ2.5-glia-ω reactive TCCs are shown in parenthesis.
Biopsies were initially incubated only with native gluten.
Peptide 2126 contains DQ2.5-glia-ω1 epitope, but the clone was not reactive against two other peptides (denoted 2045 and 2046 in Supplemental table 3) containing the same epitope.

Table 2. Overview of the TCCs generated from adults participating in the study, according to specificity.
"True" reactivity is defined as at least 75% cpm compared to chymotrypsin-digested TG2-treated gluten (CT-TG2). In case of lower proliferative response, the clone is considered as having unknown reactivity with cross-reactivity.
The peptide that gave highest proliferation index is shown in T cell clones proliferating in response to several peptides.

Patient ID	Number of clones	α	γ (ω cross)*	ω (γ cross)*	Unknown 50-75%	Unknown 10-50%	Unknown < 10%	Other
1024	6	1	0	1 (0)	0	1γ	2	1 (peptide 2126##)
1025	1	1	0	0	0	0	0	
1032	13	0	1 (1)	0	1γ, 1ω	3α, 1γ	6	
1034	16	1	5 (2)	5 (5)	0	1γ	4	
Total	36	3	6 (3)	6 (5)	2	6	12	1

*The number of DQ2.5-glia-ω cross-reactive clones among the DQ2.5-glia-γ TCCs and the number of DQ2.5-glia-γ cross-reactive clones among the DQ2.5-glia-ω TCCs are shown in parenthesis.
Peptide 2126 contains DQ2.5-glia-ω1 epitope, but the clone was not reactive against two other peptides (denoted 2045 and 2046 in Supplemental table 3) containing the same epitope

Figure 1.

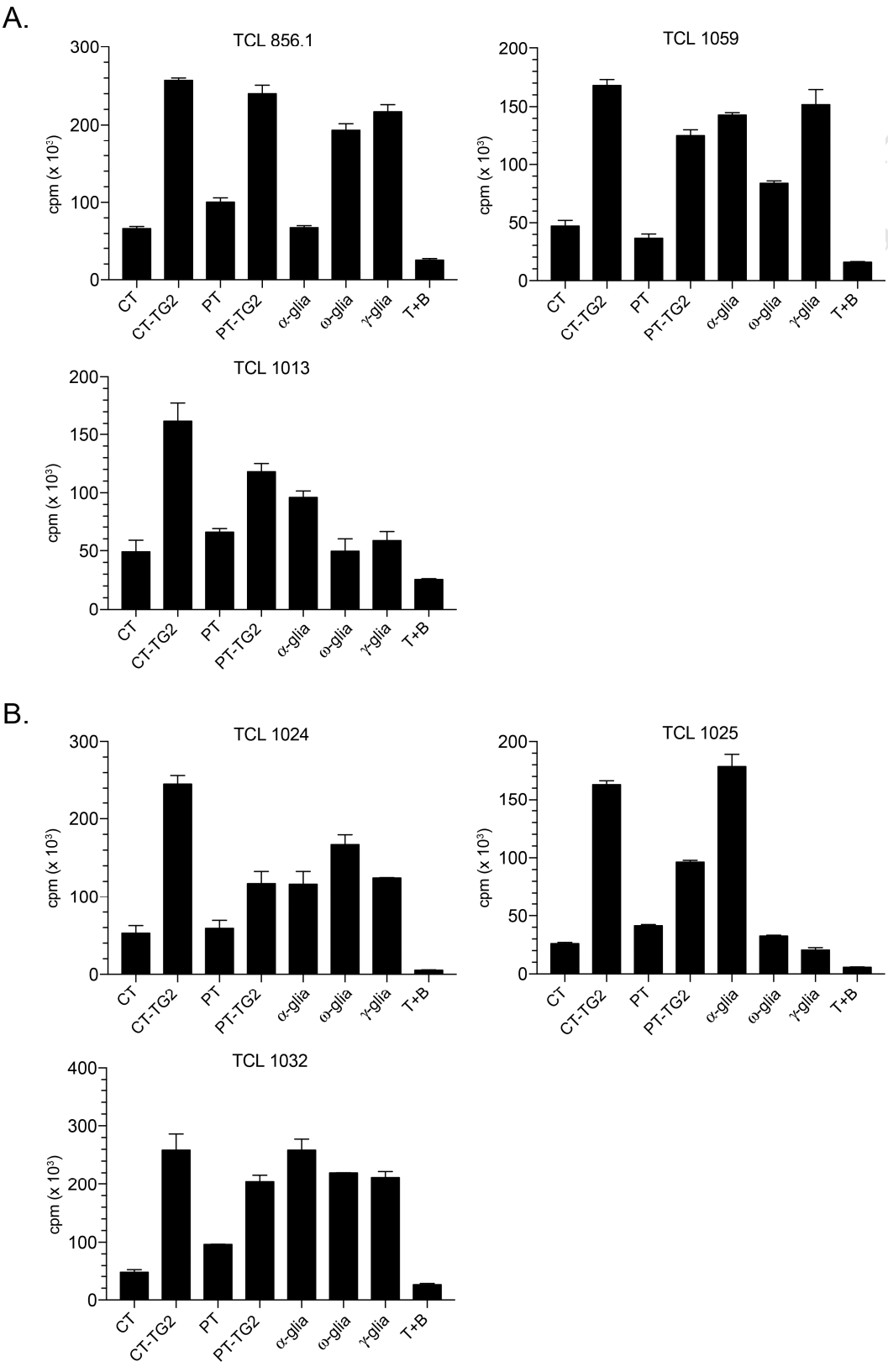

A.
TCL 856.1
TCL 1059
TCL 1013
B.
TCL 1024
TCL 1025
TCL 1032
cpm (x 10³)
CT
CT-TG2
PT
PT-TG2
α-glia
ω-glia
γ-glia
T+B

Figure 2.

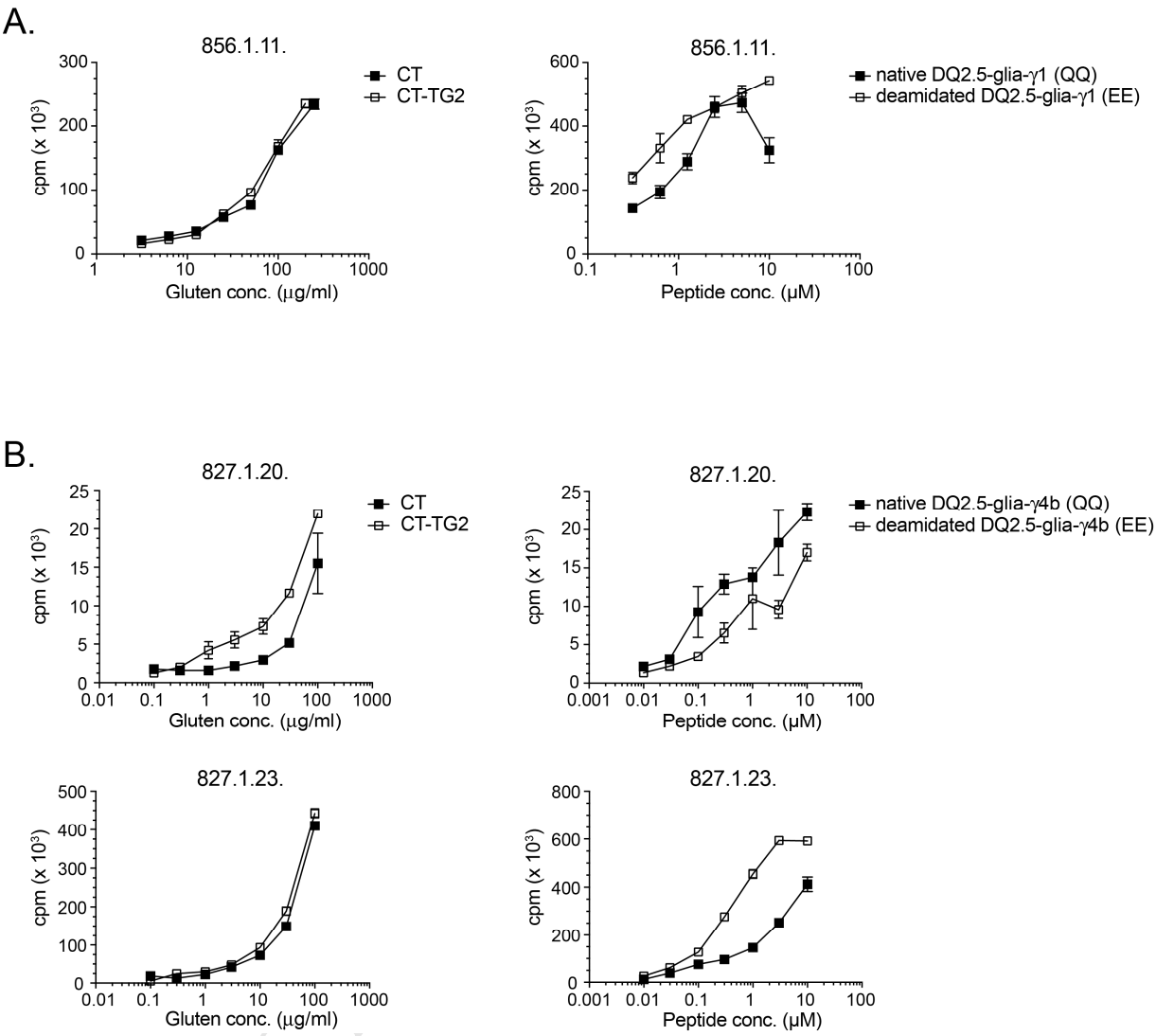

A.
856.1.11.
CT
CT-TG2
cpm (x 10³)
Gluten conc. (µg/ml)
856.1.11.
native DQ2.5-glia-γ1 (QQ)
deamidated DQ2.5-glia-γ1 (EE)
Peptide conc. (µM)
B.
827.1.20.
CT
CT-TG2
Gluten conc. (µg/ml)
827.1.20.
native DQ2.5-glia-γ4b (QQ)
deamidated DQ2.5-glia-γ4b (EE)
Peptide conc. (µM)
827.1.23.
Gluten conc. (µg/ml)
827.1.23.
Peptide conc. (µM)

Figure 3.

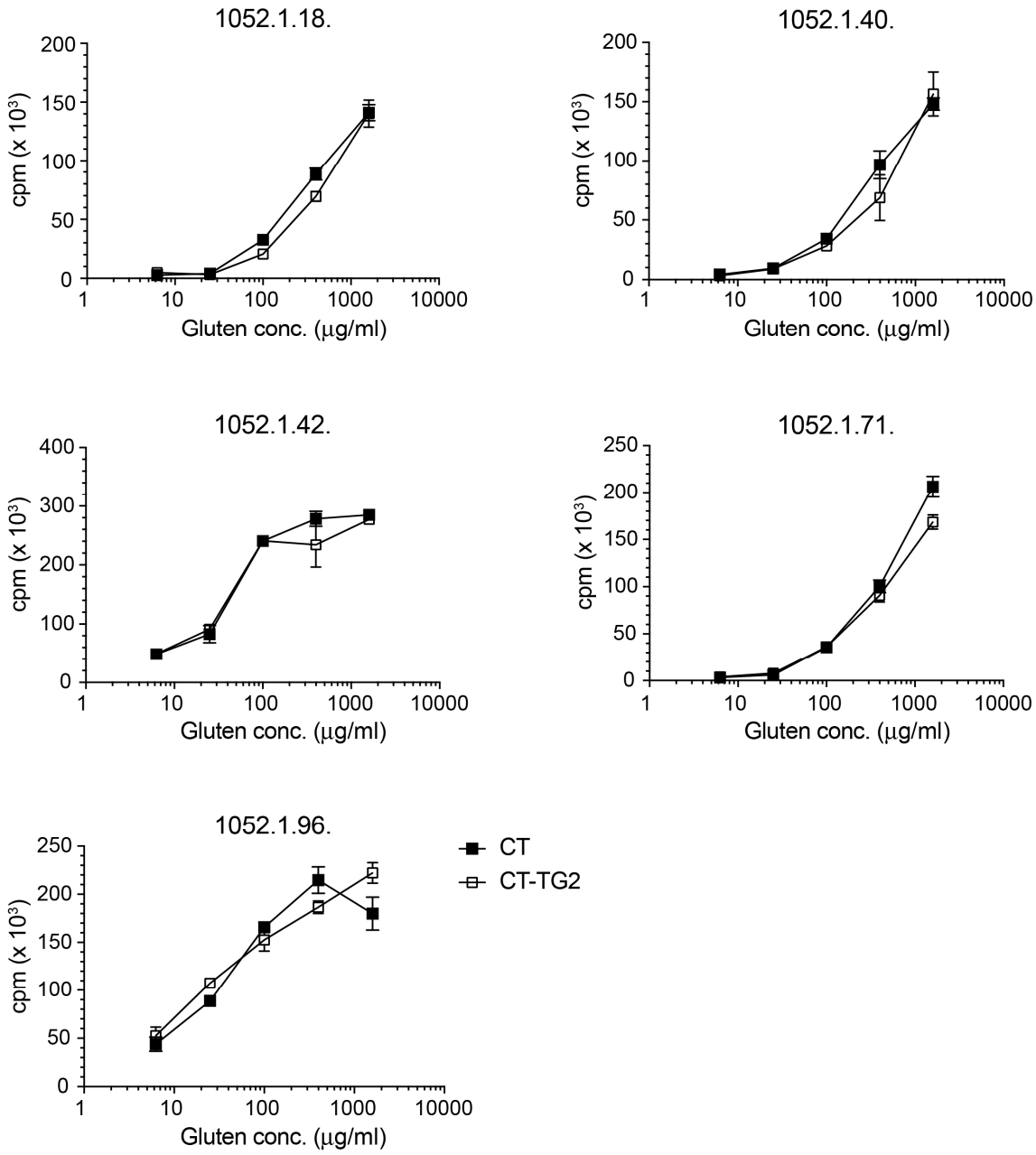

1052.1.18.
1052.1.40.
1052.1.42.
1052.1.71.
1052.1.96.
cpm (x 10³)
Gluten conc. (µg/ml)
CT
CT-TG2

Figure 4.

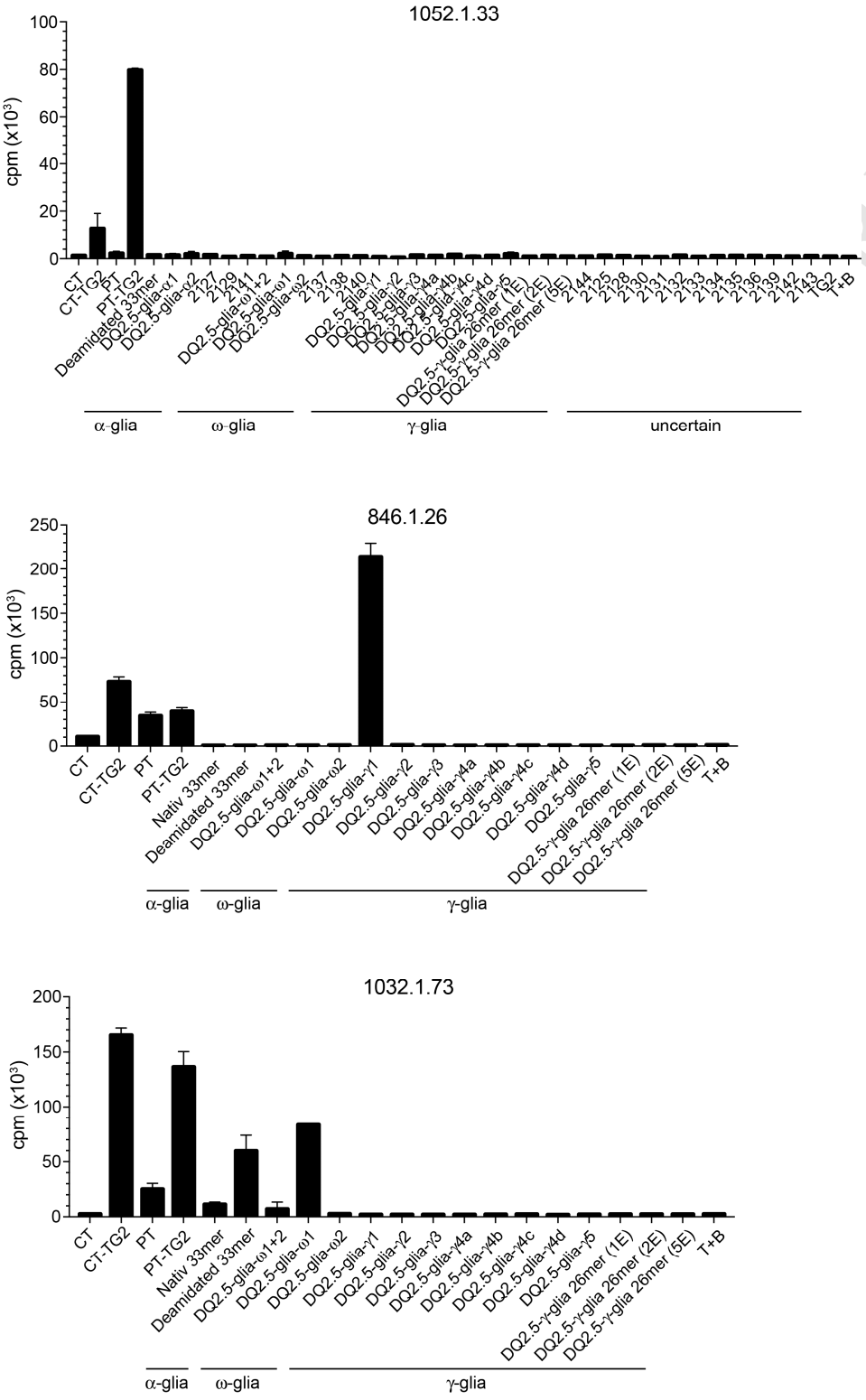
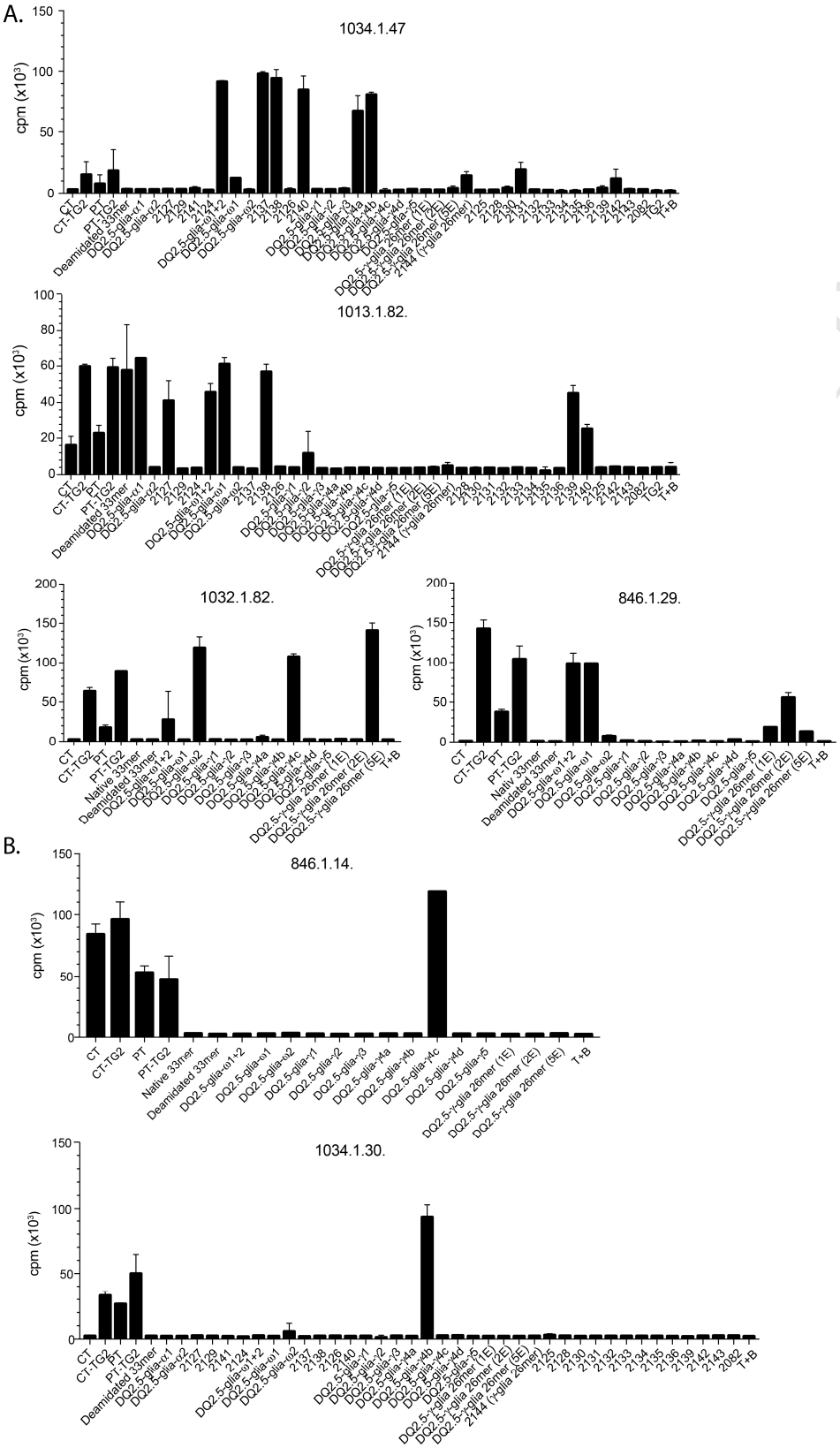

1052.1.33
cpm (x10³)
100
80
60
40
20
0
CT
CT-TG2
PT
PT-TG2
Deamidated 33mer
DQ2.5-glia-α1
DQ2.5-glia-α2
2127
2129
2141
DQ2.5-glia-ω1+2
DQ2.5-glia-ω1
DQ2.5-glia-ω2
2137
2138
2140
DQ2.5-glia-γ1
DQ2.5-glia-γ2
DQ2.5-glia-γ3
DQ2.5-glia-γ4a
DQ2.5-glia-γ4b
DQ2.5-glia-γ4c
DQ2.5-glia-γ4d
DQ2.5-glia-γ5
DQ2.5-γ-glia 26mer (1E)
DQ2.5-γ-glia 26mer (2E)
DQ2.5-γ-glia 26mer (5E)
2144
2125
2128
2130
2131
2132
2133
2134
2135
2136
2139
2142
2143
TG2
T+B
α-glia
ω-glia
γ-glia
uncertain
846.1.26
cpm (x10³)
250
200
150
100
50
0
CT
CT-TG2
PT
PT-TG2
Nativ 33mer
Deamidated 33mer
DQ2.5-glia-ω1+2
DQ2.5-glia-ω1
DQ2.5-glia-ω2
DQ2.5-glia-γ1
DQ2.5-glia-γ2
DQ2.5-glia-γ3
DQ2.5-glia-γ4a
DQ2.5-glia-γ4b
DQ2.5-glia-γ4c
DQ2.5-glia-γ4d
DQ2.5-glia-γ5
DQ2.5-γ-glia 26mer (1E)
DQ2.5-γ-glia 26mer (2E)
DQ2.5-γ-glia 26mer (5E)
T+B
α-glia
ω-glia
γ-glia
1032.1.73
cpm (x10³)
200
150
100
50
0
CT
CT-TG2
PT
PT-TG2
Nativ 33mer
Deamidated 33mer
DQ2.5-glia-ω1+2
DQ2.5-glia-ω1
DQ2.5-glia-ω2
DQ2.5-glia-γ1
DQ2.5-glia-γ2
DQ2.5-glia-γ3
DQ2.5-glia-γ4a
DQ2.5-glia-γ4b
DQ2.5-glia-γ4c
DQ2.5-glia-γ4d
DQ2.5-glia-γ5
DQ2.5-γ-glia 26mer (1E)
DQ2.5-γ-glia 26mer (2E)
DQ2.5-γ-glia 26mer (5E)
T+B
α-glia
ω-glia
γ-glia

Figure 5.



Supplemental Table 1. Characteristics of the children included from the PreventCD study

Patient (M/F)	HLA type*	1st positive serology (U/ml)**	Age 1st positive serology	2nd positive serology (U/ml)**	Age 2nd positive serology	Age at biopsy (years)	Histology (Marsh)	Symptoms	Gluten or Placebo	T cells
771 (M)	DQ2.5/DQ2.5	TG2 25.9 AGA 7.3 EMA 1:20	1.9	TG2 >100 AGA 19.8 EMA 1:80	2.2	2.2	3B	No	Gluten	1 TCL
792 (F)	DQ2.5/DQ2.5	TG2 20.6 AGA 3.3 EMA 1:10	2.7	TG2 n.d. AGA n.d. EMA 1:10	2.8	2.8	3A	Mild GI symptoms	Gluten	1 TCL
827 (F)	DQ2.5/DQ7	TG2 >100 AGA 54.4 EMA 1>:640	2.8	n.d.		2.8	3C	No	Gluten	2 TCLs 35 TCCs
846 (F)	DQ2.5/X	TG2 >100 AGA 17 EMA 1:10	3.0	TG2 n.d. AGA n.d. EMA 1:20	3.3	3.3	3B	No	Placebo	2 TCLs 13 TCCs
856 (F)	DQ2.5/DQ2.2	TG2 >100 AGA 97 EMA 1:320	2.0	n.d.		2.2	3B	No	Gluten	2 TCLs 8 TCCs
899 (M)	DQ2.5/DQ2.5	TG2 47.6 AGA 6.7 EMA 1:40	2.0	TG2 47.1 AGA 7.1 EMA 1:10	2.2	2.2	3A	No	Placebo	2 TCLs
1013 (M)	DQ2.5/X	TG2 52.6 AGA 20.3 EMA 1:40	3.0	TG2 58.9 AGA 26.9 EMA 1:80	3.1	3.1	3C	No	Gluten	Frozen at arrival 1 TCL 7 TCCs
1014 (F)	DQ2.5/X	TG2 15.1 AGA 6.9 EMA 1:20	2.0	TG2 >100 AGA 17 EMA1:320	2.3	2.3	3A	No	Placebo	Frozen at arrival Not specific
1026 (F)	DQ2.5/DQ2.2	TG2 >100 AGA >100 EMA n.d.	2.0	TG2 >100 AGA > 100 EMA n.d.	2.1	2.1	3C	Anorexia, abdominal pain	Gluten	1 TCL 4 TCCs
1052 (F)	DQ2.5/DQ2.2	TG2 >100 AGA >100 EMA n.d.	2.3	n.d.		2.4	3C	Diarrhea, meteorism, anorexia	Gluten	1 TCL 11 TCCs
1059 (F)	DQ2.5/DQ2.2	TG2 38 AGA 86 EMA n.d.	3.6	TG2 > 100 AGA > 100 EMA n.d.	3.9	3.9	3B	Diarrhea, meteorism, anorexia	Gluten	1TCL
1241# (F)	DQ2.5/DQ2.5	TG2 48.5 AGA 10.6	3.1	TG2 54.2 AGA 6.7	3.3	3.3	3B	No	Gluten	2 TCLs 2 TCCs

		EMA 1:40		EMA 1:40						
108-098# (F)	DQ2.5/DQ8	TG2 25 AGA 1.6 EMA 1:20	5.0	TG2 12 AGA 1.8 EMA 1:20	5.1	5.1	3B	No	Placebo	2 TCLs
108-162# (F)	DQ2.5/DQ2.2	TG2 5.5 AGA 3.8 EMA 1:2.5	3.8	TG2 5.9 AGA 1.7 EMA 1:2.5	4.4	4.7	2-3A	No	Placebo	3 TCLs 1 TCC

* Samples were genotyped for HLA-DQ-alleles, DQA1*05, DQA1*0201, DQB1*02 and DQB1*0302, encoding HLA-DQ2 and -DQ8.
** IgA antibodies were measured at Phadia against TG2 and gliadin (AGA) using the following cutoff values: IgA anti-TG2 6 U/ml; IgA AGA 17 U/ml, EMA was measured in the local center.
# Biopsies were initially incubated only with native gluten
n.d. not done
(M) male, (F) female, EMA anti-endomysium antibodies, TG2 anti-transglutaminase 2 antibodies, TCL T-cell line, TCC T-cell clone

Supplemental Table 2. Characteristics of adult participants

Patient	HLA type	Serology, TG2 (U/ml)*	Age at biopsy (years)	Histology (local)	Symptoms	T cells
1015 (M)	DQ2.5/DQ8	>100	55	3C	Abdominal discomfort, iron deficiency, low B12	2 TCLs
1024 (F)	DQ2.5	20	29	3B	Abdominal pain, diarrhea, meteorism	1 TCL 6 TCCs
1025 (F)	DQ2.5/DQ2.2	14.1	35	3B	Fatigue	1TCL 1 TCC
1032 (F)	DQ2.5/DQ2.2	29	39	3B	Diarrhea, meteorism, iron deficiency	1 TCL 13 TCCs
1033 (F)	DQ2.5	2.5	48	3A	Meteorism, dyspepsia	1 TCL Not specific
1034 (F)	DQ2.5	>120	26	3B	Abdominal discomfort, meteorism, iron deficiency	1 TCL 16 TCCs

* IgA antibodies were measured at Oslo University Hospital against TG2 using cutoff value of 6 U/ml for IgA anti-TG2
(M) male, (F) female, TG2 anti-transglutaminase 2 antibodies, TCL T-cell line, TCC T-cell clone

Supplemental table 3.

List of peptides used for testing TCC-reactivity. Some sequences are from reference 23, by Tye-Din et al.

Peptide nr.	Old epitope name of the predicted/ defined gluten epitopes	Source	New nomenclature	Peptide sequence (core bold)
1501	33mer (native)			LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF
1502	33mer			LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF
1269	αI		DQ2.5-glia-α1a	QLQ**PFPQPELPY**
1313	αII		DQ2.5-glia-α2	**PQPELPYPQ**PQL
2127	αI	W08, Tye-Din et al	DQ2.5-glia-α1a	LQPFPQPELPYSQPQ
2129	αI	W14, Tye-Din et al		QQQFIQPEQPFPQQPQQTYP
2141	αII	W18, Tye-Din et al	DQ2.5-glia-α2	PYPQPELPYPQPQPFRP
2124	αII		DQ2.5-glia-α2	NPQAQGSVQPQQLPQF
2045	ω1&2			QPQQPFPQPEQPFPWQP
2046	ω1		DQ2.5-glia-ω1	PQQ**PFPQPEQPF**P
2047	ω2		DQ2.5-glia-ω2	F**PQPEQPFPW**QP
2137	ω1&2	B01, Tye-Din et al		PFPQPEQPFPWQPQQPFPQ
2138	ω1	B02, Tye-Din et al	DQ2.5-glia-ω1	GQQPFPQPEQPFPLQG
2126	ω1	W05, Tye-Din et al	DQ2.5-glia-ω1	QQQPFPQPEQPFSQQP
2140	Hα2/Sα2, Hα9/Sα9 (ω-I)	R02, Tye-Din et al		GQQPFPQPEQPFPQSG
1213	γI		DQ2.5-glia-γ1	PEQ**PQQSFPEQE**RP
1206	γI (native)		DQ2.5-glia-γ1	YQQLPQPQQPQQSFPQQQRPF
1298	γII		DQ2.5-glia-γ2	GI**IQPEQPAQL**
1584	γIII		DQ2.5-glia-γ3	FP**EQPEQPYPE**Q
1396	γIV		DQ2.5-glia-γ4a	F**SQPEQEFPQ**PQ
1935	γVIIc		DQ2.5-glia-γ4b	F**PQPEQEFPQ**PQ
1380	γVIIc (native)		DQ2.5-glia-γ4b	PQQPFPQPQQQFPQPQQPQQ
1653	γVII		DQ2.5-glia-γ4c	T**EQPEQPFPQ**P
1936	γVIIb		DQ2.5-glia-γ4d	PF**PQPEQPFCE**QPQR
1571	γVI		DQ2.5-glia-γ5	P**EQPFPEQPE**Q
1988	γ26mer (2E)			QQPFPEQPEQPYPQQPQQPFPQP
1989	γ 26-mer (1E)			QQPFPQQPEQPYPQQPQQPFPQP
1994	γ26mer (5E)			EQPFPEQPEQPYPEQPEQPFPQP
2144	γ 26-mer			FLQPEQPFPEQPEQPYPEQPEQPFPQ
2125	PFPQPQQPI, PQPQQPIPV	W04, Tye-Din et al		PQQPFPEQPIPVQPQ
2128	Not mentioned	W12, Tye-Din et al		CKVFLQQQCSPVAMPQRLAR
2130	Not mentioned	W15 LMW, Tye-Din et al		SHIPGLERPWQQQPLPPQQT

2131	QQPQQPFPL	W20, Tye-Din et al		LQQPIPEQPEQPFPLQP
2132	Not mentioned	W21 HMW, Tye-Din et al		GQQGYYPISPQQSGQGQQP
2133	Not mentioned	W22 HMW, Tye-Din et al		LQPGQGQPGYYPTSPQQIGQ
2134	Not mentioned	W24 HMW, Tye-Din et al		PGQGQSGYYPTSPQQSGQKQ
2135	Not mentioned	W29 HMW, Tye-Din et al		GQGQSGYYPTSPQQSGQEAT
2136	Not mentioned	W36, Tye-Din et al		PAQYEVIRSLVLRTLPNM
2139	PQPQQPIPQ	R01, Tye-Din et al		PQQPFPQPEQPIPQ
2142	Not mentioned	W19, Tye-Din et al		PFPQPQQPFPWQPEQPFPQ
2143	Not mentioned	W09, Tye-Din et al		PFPQPQPFLPELPYPQPQ
2082				SHQQQPFPQQPYPQQPYPS
2109	Glut-17 epitope			Ac-QQPPFSEQEQPVLPQ

Supplemental Table 4.

Alignment of peptides tested representing the DQ2.5-glia-ω□and DQ2.5-glia-γ□□epitopes. The numbers in the top line denote the 1 to 9 positions of the core register for binding of the epitopes to HLA-DQ2.5. The bold E letters represent glutamate residues that have been converted from glutamine by deamidation.

Epitope				**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**				
DQ2.5-glia-ω1	P	Q	Q	P	F	P	Q	P	**E**	Q	P	F	P			
DQ2.5-glia-ω2			F	P	Q	P	**E**	Q	P	F	P	W	Q	P		
DQ2.5-glia-γ4a			F	S	Q	P	**E**	Q	**E**	F	P	Q	P	Q		
DQ2.5-glia-γ4b			F	P	Q	P	**E**	Q	**E**	F	P	Q	P	Q		
DQ2.5-glia-γ4c			T	**E**	Q	P	**E**	Q	P	F	P	Q	P			
DQ2.5-glia-γ4d		P	F	P	Q	P	**E**	Q	P	F	C	**E**	Q	P	Q	R