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Gliadin-reactive T cells in Italian children from preventCD cohort at high risk of celiac disease

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Keywords
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Abstract

Background: Newborns at high risk of celiac disease (CD) were recruited in Italy in the context of the PreventCD study and closely monitored for CD, from 4 months up to a mean age of 8 years at follow-up. The aim of our study was to investigate intestinal T-cell reactivity to gliadin at the first clinical and/or serological signs of CD.
Methods: Gliadin-reactive T-cell lines were generated from intestinal biopsies of 19 HLA-DQ2-or HLA-DQ8-positive children. At biopsy, 11 children had a diagnosis of acute CD, two of potential CD, and six were non-celiac controls. Immune reactivity was evaluated against gliadin and known immunogenic peptides from α-, γ-, or ω-gliadins. The role of deamidation by transglutaminase (tTG) in determining the immunogenicity of gliadin was also investigated.
Results: Most of the children with CD (either acute or potential) had an inflammatory response to gliadin. Notably, signs of T-cell reactivity to gliadin were also found in some non-celiac subjects, in which IFN-γ responses occurred mainly when regulatory IL-10 and TGF-β cytokines were blocked. Interestingly, PreventCD children reacted to gliadin peptides found active in adult CD patients, and tTG deamidation markedly enhanced gliadin recognition.
Conclusions: T cells reactive to gliadin can be detected in the intestine of children at high risk of developing CD, in some cases also in the presence of a normal mucosa and negative CD-associated antibodies. Furthermore, children at a very early stage of CD recognize the same gliadin epitopes that are active in adult CD patients. Tissue transglutaminase strongly enhances gluten T-cell immunogenicity in early CD.

The prevalence of celiac disease (CD) is 1–3% in the general population and 5–15% among first-degree relatives of patients (1). HLA-DQ genes predispose to CD, as most patients are HLA-DQ2 or HLA-DQ8 positive. However, less than 5% of DQ2/DQ8-positive individuals in the general population develop the disease, and several other risk genes have been identified (2–4). HLA molecules are involved in CD pathogenesis as gluten, especially after being deamidated by tissue transglutaminase (tTG), bind to HLA-DQ2 or HLA-DQ8 and activate intestinal T lymphocytes, prevalently $CD4^+$ T cells (5–7).

Celiac disease affects subjects of all ages and clinical presentations differ (8, 9). Young CD patients usually present gastrointestinal symptoms, namely diarrhea, malabsorption, and abdominal pain, while adults often present such extra-intestinal manifestations as anemia or osteoporosis (8, 9). It is not known whether the differences in the time of disease onset, clinical manifestations, and responsiveness to dietary therapy are consequences of diverse intestinal immunity to gluten. Furthermore, most studies of the gluten-induced inflammatory cascade were performed in adults, and very little is known about the antigluten immune response in childhood CD (5, 6, 10–12). Indeed, three immunodominant peptides (DQ2.5-glia-α-1/2, DQ2.5-glia-ω-1/2, and DQ2.5-glia-γ-1) have been found to account for more than 95% of the T-cell response to gluten in adult celiacs (13–16). A very recent study demonstrated a profile of immunodominant epitopes in pediatric CD not dissimilar from that found in adults and confirmed that deamidation greatly increases T-cell responses (17). Besides a

strong Th1 response to gluten, which mediates mucosal damage (18, 19), anti-inflammatory T cells have been described in CD gut mucosa (20–23). Such regulatory T cells may be involved in promoting CD remission after a gluten-free diet and in preventing progression to mucosal damage.

Prevent Celiac Disease (http://www.preventcd.com) is an international project sponsored by the European Union's sixth Framework Programme. Children enrolled in the study had at least one-first-degree relative affected by CD, were HLA-DQ2 and/or HLA-DQ8 positive, and were followed for CD development since the age of 4 months (24). During follow-up, children with CD serological markers and/or persistent symptoms underwent duodenal biopsy. The biopsies obtained gave us the opportunity to investigate the specific intestinal reactivity to the causative antigen, the effect of tTG modification on gluten antigenicity, and the possible role of immune regulatory pathways in controlling the adverse T-cell response in a very early phase of CD development.

Methods

Patients and study protocol

Between 2007 and 2013, at the Pediatrics Section, Department of Translational Medicine (DISMET), University of Naples "Federico II," we enrolled a cohort of 170 Italian newborns of the PreventCD study (controlled trial number ISRCTN74582487). Parents/guardians gave their written informed consent. Children were typed for CD-associated genes using the Eu-Gen Risk test (Eurospital, Trieste, Italy). HLA-DQ2- or HLA-DQ8-positive children were monitored from 4 months of age up to 8.3 years (range 6.4–9.9 years) by clinical and serological evaluations (anti-tTG2-IgA and antigliadin-AGA antibodies) performed by Phadia-Thermo Fisher Scientific (Freiburg, Germany). Duodenal biopsy was performed in case of symptoms suggestive of CD and/or persistently positive serological tests (tTG2-IgA >7 U/ml; AGA >17 U/ml). CD was diagnosed according to ESPGHAN guidelines (25). The clinical and histological characteristics of the children who underwent duodenal biopsies are summarized in Table 1. The study protocol was approved by the Ethics Committee of the University of Naples "Federico II" (prot. No. 191/06).

Duodenal biopsy and immunohistochemical analysis

At gastro-duodenoscopy, at least five biopsies were taken between the bulb and distal duodenum in each patient. Three fragments were fixed in 10% formalin, paraffin-embedded, and stained with hematoxylin. The histological and morphometrical analysis was performed at the light microscope by experienced pathologists blind to serology results. One duodenal specimen was embedded in OCT compound and snap-frozen in liquid nitrogen for immunohistochemistry. These frozen biopsies were also investigated for the presence of extracellular deposits of anti-tTG2-IgA antibodies, as previously described (26). We evaluated the deposits in terms of the pattern and intensity of staining as follows: negative staining (absent), patchy staining (patchy), and homogeneous distribution

Table 1 Clinical and histological features of patients enrolled in the study

							Intra-epithelial cells*				
Patient	Age (months)	HLA-DR	HLA-DQ	AGA†	tTG2(IgA)†	Marsh‡	CD3	γδ	CD25	IgA deposit	Diagnosis
non-CD#1	18	DR3/DR7	DQ2.5/2.2	40.2	0.1	M1	36	2.3	5	Absent	Non-celiac
Non-CD#2	25	DR3/DR7	DQ2.5/2.2	7.1	0.7	M0	25	2.2	5	Patchy	Non-celiac
Non-CD#3	12	DR3/DR7	DQ2.5/2.2	12	0.1	M1	24	3	13	Absent	Non-celiac
Non-CD#4	21	DR3/DR5	DQ2.5/7	77.9	0.1	M0	13	0.3	13	Patchy	Non-celiac
Non-CD#5	38	DR7/X	DQ2.2/X	37	0.1	M0	8	1.2	2	Absent	Non-celiac
Non-CD#6	41	DR4/X	DQ8/X	20.9	0.5	M1	39	1.5	4	Absent	Non-celiac
potCD#1	22	DR5/DR7	DQ2.5/2.2	22.2	16.4	M1	20.5	4.7	10	Absent	Potential CD
potCD#2	31	DR5/DR7	DQ2.5/2.2	17	41	M1	45	15	4	Patchy	Potential CD
CD#1	28	DR7/DR7	DQ2.2/2.2	53.6	100	M3c	63	12.2	116	Present	Celiac
CD#2	46	DR3/DR7	DQ2.5/2.2	8.5	10.4	M3b	48	9.7	8	nd	Celiac
CD#3	11	DR3/DR5	DQ2.5/7	43.4	100	M3c	nd	nd	86	nd	Celiac
CD#4	47	DR3/DR7	DQ2.5/2.2	2.2	20.8	M3b/c	71	23	nd	Present	Celiac
CD#5	24	DR7/X	DQ2.2/X	100	100	M3b/c	nd	nd	nd	Present	Celiac
CD#6	27	DR3/X	DQ2.5/X	2.1	35	M3b	93	30	144	Present	Celiac
CD#7	51	DR3/DR7	DQ2.5/2.2	29.4	58.4	M3b/c	77	24	110	Patchy	Celiac
CD#8	36	DR5/DR7	DQ2.5/2.2	15.2	100	M3c	129	43	118	Present	Celiac
CD#9	78	DR3/DR5	DQ2.5/7	nd	31.8	M3a/b	57	22	38	Present	Celiac
CD#10	63	DR3/DR7	DQ2.5/2.2	nd	100	M3b	72	25	19	Present	Celiac
CD#11	69	DR3/X	DQ2.5/X	nd	100	M3c	137	45	92	Present	Celiac

*Number indicates the density of positive cells/mm^2 of epithelium.

†Serum level of AGA and anti-tTG2 antibodies are expressed as U per milliliter at the time of endoscopy.

‡Mucosal tissue damage was scored according to Marsh classification: Marsh 0: normal villous and intra-epithelial lymphocyte infiltration.

(present). Finally, one or two biopsies were collected in RPMI 1640 medium for T-cell line (TCL) generation, as reported below.

Antigens

The peptic-tryptic digest of gliadin (hereafter referred to as "gliadin") was provided by Dr F. Koning (Leiden University Medical Centre). Peptides (Table 2) were synthesized by automated continuous-flow solid phase according to the protocol described in Supporting Information. Gliadin and peptides were deamidated as reported in Supporting Information.

Generation of gliadin-specific T-cell lines and T-cell assays

Both the procedures used to process biopsies and to assess the T-cell lines specificity were agreed upon with the PreventCD study group, and reported in detail in Supporting Information. Briefly, biopsies were collected in RPMI, incubated in HBSS with DTT 1 mM for 5 min, and incubated in HBSS containing 0.75 mM EDTA for at least one hour. After removal of EDTA, biopsy samples were transferred in complete medium containing 20 μg/ml of both forms of gliadin and fed with 20 U/ml IL-2 and 5 ng/ml IL-15 at days 3 and 6. Growing cells were collected on days 9–11 and stimulated with a cocktail containing irradiated allogeneic feeder cells, IL-2, IL-15, and 1 μg/ml phytohemagglutinin. Autologous or allogeneic EBV-transformed B cells were pulsed with gliadin (50 μg/ml) or gluten peptides (10 μM) in duplicate wells overnight at 37°C, before the addition of responder T cells. In the experiments with neutralizing monoclonal antibodies, T cells were pre-incubated with anti-IL-10R (10 μg/ml, clone 3F9) or anti-TGF- β (10 μg/ml, clone 1D11) before the addition of antigen-pulsed APC. IFN-γ production was measured in the culture supernatants 48 h later.

Positivity criteria and statistical analysis

The amount of IFN-γ (pg/ml) was normalized to 1×10^6 cells/ml, and the IFN-γ fold increase (FI) was calculated as follows: amount of IFN-γ in the presence of antigen/amount of IFN-γ in the absence of antigen. Each TCL was considered responsive to the gliadin/peptide when the FI was ≥3. Differences among stimulation conditions and the three groups of subjects were evaluated with the Mann–Whitney rank-sum test, and considered significant when $p < 0.05$.

Results

Clinical follow-up and histological data

During clinical follow-up, 19 children (median age 31 months, range 11–78 months) underwent endoscopy for suspected CD, 13 because of high anti-tTG2 antibody titers and the remaining six because of high AGA antibody titers and clinical symptoms suggestive of CD (Table 1). Eleven of the 13 children with anti-tTG2 antibodies had subtotal villous atrophy (Marsh stage M3a/c) and were diagnosed with CD and put on a gluten-free diet, while the other two had a normal villous and crypt architecture, were classified "potential CD," and fed a gluten-containing diet. The six subjects with negative anti-tTG2 and positive AGA titers had a normal mucosa (M0/M1) and were thus classified "non-CD subjects," according to the current CD diagnosis guidelines (25). Three of the latter six children had mild mucosal inflammation (Marsh1) with increased densities of intraepithelial $CD3^+$ cells. Interestingly, one of the non-CD children (non-CD#1) developed the disease during the 30 months after the first endoscopy.

T-cell reactivity to native and deamidated gliadin in children at the early celiac disease diagnosis

The antigen specificity of TCLs derived from mucosa explants of the children enrolled in this study was defined as an IFN-γ increment in response to gliadin of at least three times the background value obtained without antigenic stimulation. Based on this arbitrary criterion, we observed a significant response to native gliadin in the TCLs from four of 11 children

Table 2 Gliadin peptides assayed for recognition in PreventCD children

Peptide name	Sequence	Antigenic determinant	New name*	Reference
α-gliadins				
α-17mer	QLQPFPQP**Q**LPYPQPQP†	PFPQP**Q**LPY	DQ2.5-glia-α1a	(14)
		PQP**Q**LPYPQ	DQ2.5-glia-α2	
ω-gliadins				
ω-17mer	QP**Q**QPFPQP**Q**QPFPWQP	PFPQP**Q**QPF	DQ2.5-glia-ω1	(15)
		PQP**Q**QPFPW	DQ2.5-glia-ω2	
γ-gliadins				
DQ2-γ-I	P**Q**QPQQSFPQQ**Q**QPA	PQQSFPQQ**Q**	DQ2.5-glia-γ1	(27)
DQ2-γ-II	GIIQP**Q**QPAQL	IQPQQPAQL	DQ2.5-glia-γ2	(25)
γ-26mer	FLQP**Q**QPFP**Q**QP**Q**QPYP**Q**QP**Q**QPFPQ	**Q**QP**Q**QP(Y/F)PQ	DQ2.5-glia-γ3	(25)
		QQPQQPFPQ	DQ2.5-glia-γ4a	
		QQPFP**Q**QPQ	DQ2.5-glia-γ5	

*The new nomenclature refers to Sollid et al. Immunogenetics, 2012.
†In bold, the Q residue deamidated by tTG2.

with villous atrophy (Fig. 1a,b and Table S1). These four TCLs reacted well also to deamidated gliadin while the TCLs of another three of 11 subjects reacted to only deamidated gliadin.

We next investigated whether regulatory factors might mask the activation of gliadin-reactive T cells in patients unresponsive to both forms of gliadin. Interestingly, the TCLs of two patients (CD#1 and CD#4) had a positive IFN-γ response to deamidated gliadin when we neutralized the regulatory cytokines IL-10 and TGF-β. All patients with villous atrophy assayed (8/8) had a T-cell reactivity to tTG-deamidated gliadin, while five of eight reacted to native non-deamidated gliadin.

To evaluate in greater detail the reactivity in children at an early phase of CD, we investigated the recognition of peptides previously found to be immunogenic in HLA-DQ2.5 adult and infant CD patients (Table 2) (13–16). TCLs from four children reacting to native gliadin (CD#7, CD#8, CD#9, and CD#11) were assayed for recognition of both the wild-type and tTG-deaminated forms of five immunodominant peptides (Table 2). As shown in Fig. 1c, both forms of peptides DQ2.5-glia-α-1/2 (α-17mer), DQ2.5-glia-ω-1/2 (ω-17mer), and DQ2.5-glia-γ-1 (DQ2-γ-I) were well recognized. Furthermore, although reactivity to wild-type gliadin peptides was detected, the deamidated forms were by far the most active, which is in line with the IFN-γ response profile we observed to deamidated whole gliadin.

We also analyzed TCLs from another four of 11 children (CD#4, CD#5, CD#6, and CD#10) for the recognition of three

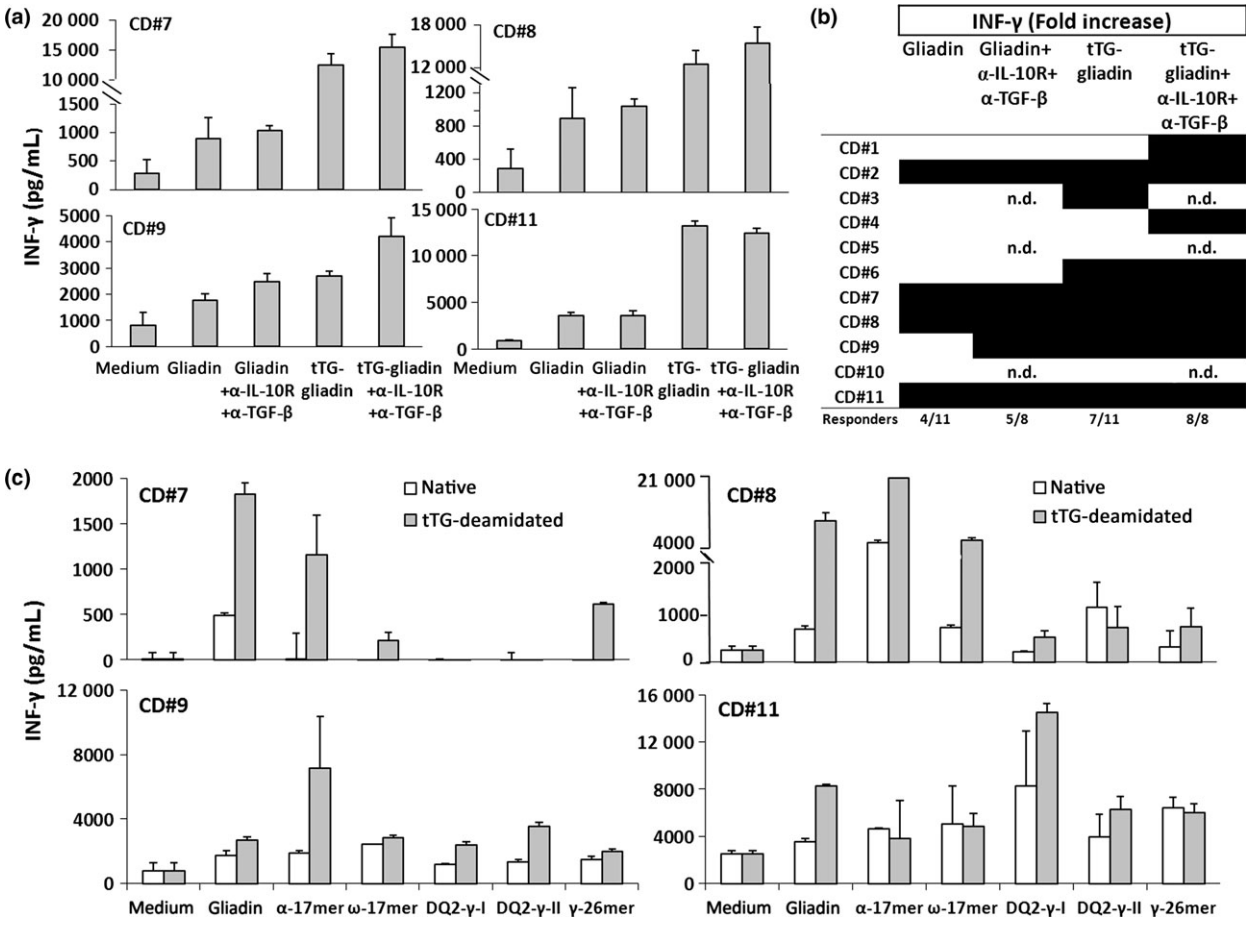

Figure 1 Responsivity to gliadin in T-cell lines from small intestinal mucosa of PreventCD children with a diagnosis of acute celiac disease. Polyclonal intestinal T-cell lines (TCLs) were generated from biopsies of 11 children with a diagnosis of celiac disease (high anti-tTG2 antibody-IgA titers and villous atrophy). Immune reactivity was assessed against both whole gliadin enzymatic digest (panels a and b) and known immunogenic peptides from α-, γ-, or ω-gliadins (panel c). Both gliadin (50 μg/ml) and peptides (10 μM) were assayed in both native and tTG-deamidated form. Autologous lymphoblastoid B cells served as antigen-presenting cells, and IFN-γ production was evaluated by ELISA in cellular supernatant after 48 h of incubation. Monoclonal antibodies neutralizing IL-10R and TGF-β were used (10 μg/ml) at the experimental points to assess regulatory mechanisms. (a) IFN-γ production in TCLs from four representative children responsive to gliadin. Results are shown as the mean ± s.d. of triplicate IFN-γ measurements. (b) Frequency of positive INF-γ responses obtained in TCLs from the 11 children with a diagnosis of full-blown CD. Black areas indicate a positive response (FI = fold increase equal to or more than 3). (c) Recognition profile of immunogenic gliadin peptides (in native and in tTG-deamidated form). Results are shown as mean ± s.d. of triplicate wells and are representative of four children. n.d.: not done.

of the five most immunogenic peptides (α-17mer, ω-17mer, and DQ2-γ-I) in their deamidated version. Similar to the findings shown in Fig. 1c, the profile of peptide recognition was heterogeneous. The T cells of patients CD#6 and CD#10 reacted to a single epitope, that is, α-17mer and DQ2-γ-I, respectively, while the remaining two (CD#4 and CD#5) did not respond to any of these three peptides (Fig. S1).

Finally, we compared the T-cell response to these three peptides observed in PreventCD children with full-blown CD, with those previously found in untreated adult CD patients (15). The frequency of α-17mer recognition was 50% in both cohorts. The homologue peptide from ω-gliadin (ω-17mer) was more frequent in the PreventCD children than in adults (62% and 36%, respectively), whereas DQ2-γ-1 was less active in children than in adult CD patients (25% and 36%, respectively).

Gliadin-specific TCLs can be expanded from the intestine of PreventCD children with normal mucosa

Eight of 19 children who met the criteria for gastroduodenoscopy had a normal duodenal mucosa architecture (Marsh grade 0/1). Of these children, two had positive anti-tTG2 antibodies (potential CD) and six had positive anti-gliadin antibodies and symptoms suggestive of CD, but were negative for anti-tTG2 antibodies (non-celiac controls) (see Table 1). Gliadin-specific T cells were obtained from one of two potential CD subjects (potCD#2; Fig. 2a and Table S1). This patient was reactive to deamidated gliadin and produced an amount of IFN-γ comparable to that observed in children with full-blown CD (Fig. 1a), which increased under the effect of anti-IL-10R and anti-TGF-β monoclonal antibodies. Furthermore, this TCL retained its gluten specificity after several

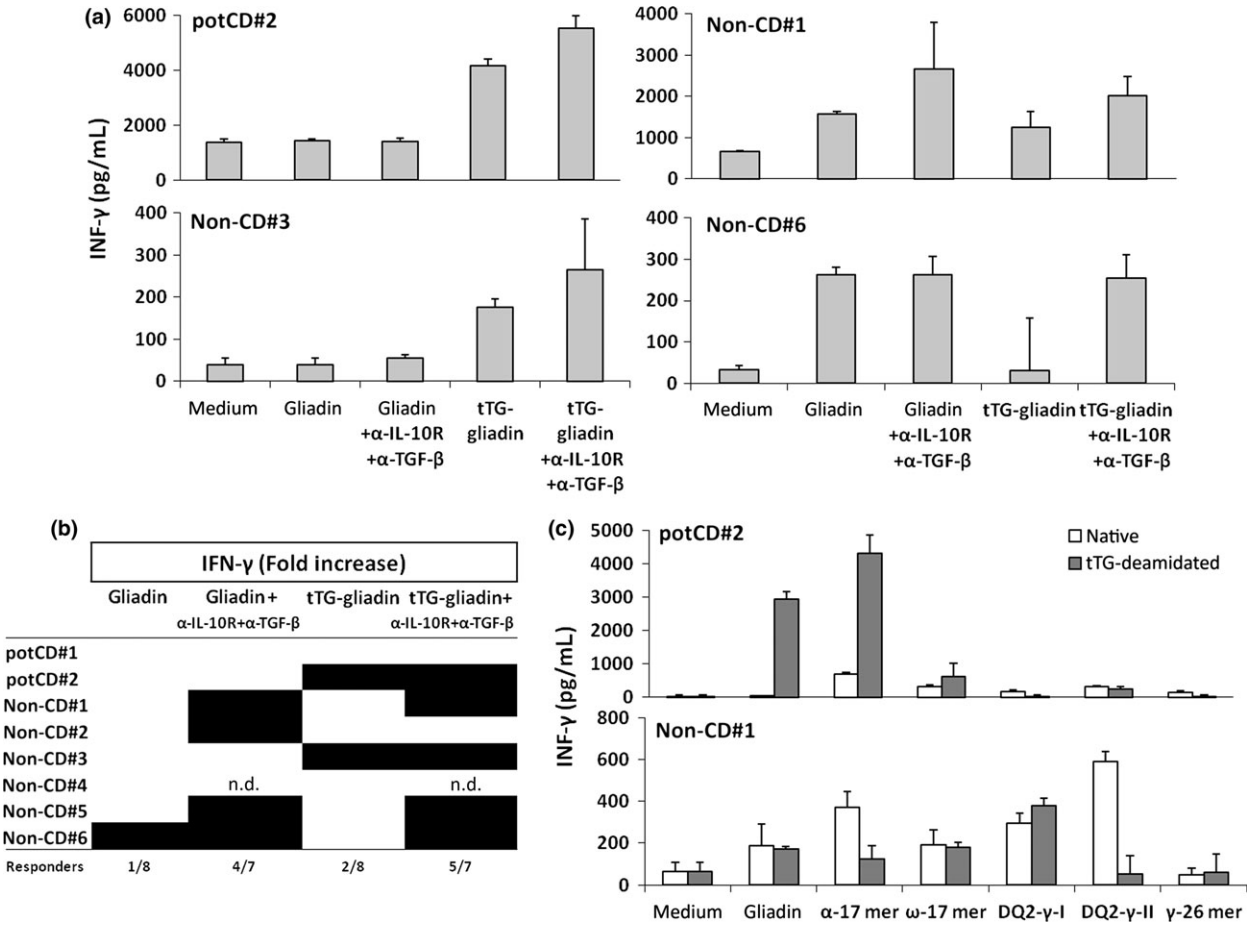

Figure 2 Responsivity to gliadin in intestinal T-cell lines from PreventCD children with a normal intestinal mucosa. Polyclonal intestinal T-cell lines were generated from two PreventCD children with potential CD (potCD) and six children without celiac disease (non-CD). T cells were assayed for recognition of both native and tTG-deamidated gliadin/peptides, as reported in Fig. 1. (a) IFN-γ production against gliadin obtained in TCLs from one representative potCD and three non-CD children responsive to gliadin. Results are shown as mean ± s.d. of triplicate wells. (b) Frequency of positive responses (black areas, FI ≥3) observed under the different stimulatory conditions, as indicated. Fold increase was calculated as described in Fig. 1. (c) Recognition of known gliadin peptides assayed in both native and tTG-deamidated form (10 μM). Representative specificity experiments conducted in a child with potential CD (potCD#2) and in a non-celiac control (non-CD#1) are illustrated. The latter child developed villous atrophy 30 months after the first endoscopy. n.d.: not done.

in vitro stimulations, as evaluated by both IFN-γ production (Fig. 2a,b and Table S1) and cell proliferation (data not shown).

Of the six children non-celiac at the first endoscopy (Table 1), two (non-CD#6 and non-CD#3) had a positive response to native and deamidated gliadin, respectively. When we blocked regulatory pathways, with specific antibodies neutralizing the regulatory cytokines (20), the frequency of responder children increased to wild-type gliadin and/or tTG-modified gliadin (non-CD#1, non-CD#2, and non-CD#5) (Fig. 2a,b and Table S1). These results suggest the presence in the intestinal mucosa of children at a high CD risk, of modulatory pathways that downregulate, or even mask, the gluten-reactive T cells (20, 21, 23, 27). Notably, one of the PreventCD children with a negative biopsy at the first endoscopy but with a moderate T-cell reactivity to both native and deamidated gliadin (non-CD#1) had a high tTG2-IgA titer (100U/ml) and villous atrophy (M3b/c) 30 months after the first biopsy. The presence of a gliadin-specific T-cell response in children with a histologically normal gut mucosa was confirmed by the recognition of immunogenic gluten peptides. As shown in Fig. 2c, the potCD#2 TCLs reacted well to α-17mer, and also to ω-17mer and DQ2-γ-II, albeit to a lesser extent. Moreover, gluten peptides were recognized also in the TCL from non-CD#1. Specifically, this child responded to peptides derived from all three gliadin families (α-17mer, ω-17mer, DQ2-γ-I, and DQ2-γ-II), and mainly in the non-deamidated form. Unfortunately, given the low number of cells recovered, we were unable to screen all the TCLs and both the native and deamidated epitopes. Despite this limitation, the three most active peptides, (α-17mer, ω-17mer, DQ2-γ-I), were efficiently recognized in three children with normal biopsies analyzed (Fig. S1) (13, 15, 17).

A higher magnitude of antigliadin T-cell response in the gut of children with villous atrophy

We next compared the antigliadin T-cell response in children at a high genetic risk of CD vs. those with full-blown CD. Fig. 3 shows the intensity of INF-γ responses elicited by gliadin under the different stimulating conditions, that is, wild-type vs the tTG-treated form, and with or without blockage of regulatory cytokines. IFN-γ production against deamidated gliadin was significantly higher in children with full-blown CD (filled

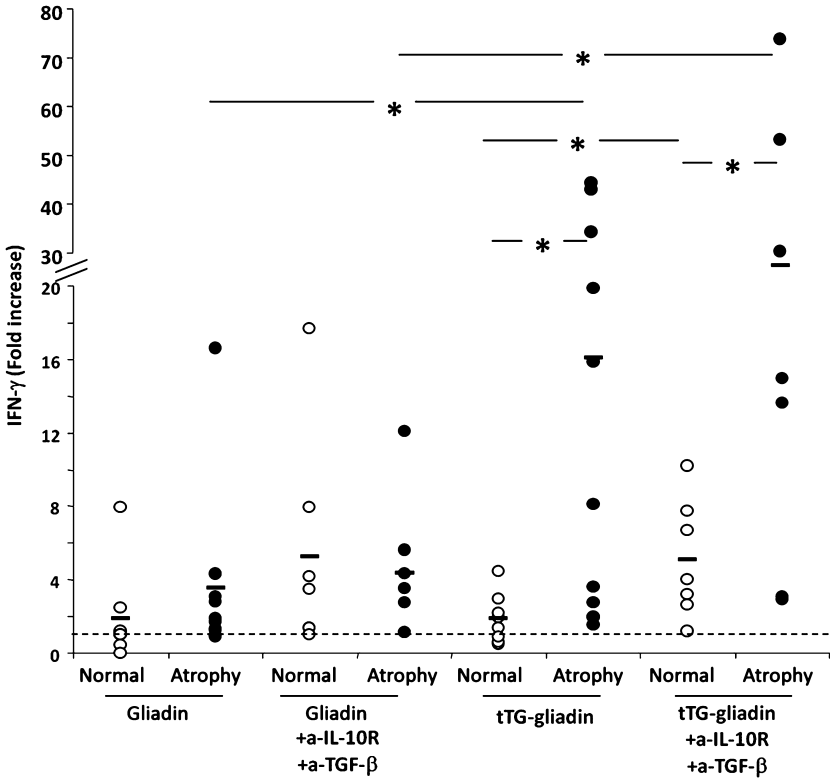

Figure 3 Comparative magnitude analysis of antigliadin T-cell responses between children with histologically normal vs. villous damaged gut mucosa. The gliadin-specific IFN-γ production detected in intestinal T-cell lines from each child of the PreventCD cohort is shown. The intensity of IFN-γ production in response to gliadin is shown as fold increase (FI, see Material and Methods for details). The IFN-γ responses from children with a normal mucosa (either potential CD or non-celiac healthy controls) are reported as open circles; IFN-γ responses from children with villous atrophy (full-blown CD) are reported as filled circles. Bars represent the median FI values. Statistical analysis was performed by Mann-Whitney rank-sum test *$p < 0.05$. Open circles represent the IFN-γ response observed in children with normal mucosa; filled circles represent IFN-γ response observed in children with villous atrophy.

circles) than in children with a normal mucosa (open circles) both in the presence and absence of anti-IL-10R- and anti-TGF-β-neutralizing antibodies ($p < 0.05$). By contrast, IFN-γ levels elicited by native gliadin did not differ significantly between the two cohorts in either of the experimental conditions. Interestingly, IFN-γ production was significantly higher in CD children in response to tTG-deamidated gliadin than in response to unmodified gliadin both in the absence and presence of blocking antibodies ($p < 0.05$), which confirms the higher stimulatory capacity of gliadin after post-translational modification of specific glutamines (10, 13–15, 28, 29).

Discussion

In the context of the PreventCD study, we analyzed T-cell reactivity to gliadin in the gut of a cohort of children monitored for the onset of CD from 4 months of age. We found that the response to gliadin was mainly directed toward the deamidated form of gliadin in the intestinal mucosa of children with an early diagnosis of acute CD. We also demonstrate that PreventCD children with overt CD respond to the same repertoire of gluten peptides that are immunodominant in adult patients. Adaptive T-cell reactivity to gluten was detected also in the gut of children at a high genetic risk of CD, but with a normal mucosa and negative autoantibody serology. Our data also indicate the existence of immunoregulatory pathways, mediated by IL-10 and TGF-β, that could "prevent" the onset and/or progression of mucosal damage in high-risk subjects.

To identify gliadin-specific T cells, we generated short-term TCLs by expanding the lamina propria mononuclear cells of PreventCD children undergoing endoscopy because of symptoms suggestive of CD and/or of antigliadin/anti-tTG2 antibodies. In TCLs of children with early villous atrophy, deamidated gliadin clearly dominated over the native form, both in terms of frequency of responder subjects and of intensity of INF-γ production. The higher stimulatory capacity of deamidated gliadin was confirmed by the recognition of five gliadin peptides, including the main immunodominant epitopes (10). To our knowledge, only Vader and co-workers have previously investigated the reactivity to both native and deamidated gluten in the intestine of children affected by CD (12). Notwithstanding differences between the protocols used, their results were not dissimilar to ours.

Interestingly, we found a gliadin-specific T-cell response also in one of two children classified as potential CD, although at 6 years of follow-up, neither of the two children has developed villous atrophy. This finding is consistent with our recent finding of gliadin-specific T-cell responses that fulfill the positive criteria of the present study, in 5 of 14 children with potential CD randomly recruited at the Pediatric Section, University of Naples Federico II, during clinical practice (Gianfrani, Camarca, et al. unpublished data). Also our previous finding of enhanced IFN-γ mRNA expression at mucosal level in potential celiac children (Marsh1) (23) confirms activation of inflammatory pathways in these subjects (23). Taken together, the above findings indicate that gluten-reactive Th1 cells can be monitored in the intestinal mucosa in association with autoantibody positivity but absence of villous atrophy.

Surprisingly, we found signals of gluten-specific T-cell activation also in TCLs from our cohort of PreventCD children with a normal mucosa and negative autoantibodies, although the intensity of response to deamidated antigens was much lower than that in children with overt CD. Importantly, one of these children, diagnosed as non-celiac at the first endoscopy, developed villous atrophy 30 months later, which indicates the presence of proinflammatory T cells in the intestinal mucosa of this high-risk child long before the onset of mucosal damage. Interestingly, in a subgroup of non-CD high-risk children, we detected the response of intestinal T cells to gliadin (both native and tTG-modified) only when the regulatory T-cell pathways were blocked by antibodies neutralizing IL-10 and TGF-β. Furthermore, blockage of these two cytokines increased the IFN-γ production elicited by gliadin also in some children with atrophic mucosa.

IL-10- and TGF-β-producing regulatory T cells have already been reported by us and others in the intestinal mucosa of adults with villous atrophy (7, 20, 21) and in mouse models (27). These findings suggest that regulatory pathways may be activated in this at-risk children upon priming of the intestinal T-cell response, as secondary mechanisms to silence gluten-triggered inflammatory reactions. However, studies are required to determine whether regulatory T cells/pathways can contrast the inflammatory cascade elicited by gluten in normal mucosa, and so "prevent" the development of CD mucosal lesions in this peculiar group of individuals at a high genetic risk for CD.

Finally, we confirm that immunodominant gliadin peptides identified in adults are well recognized also by intestinal T cells from HLA-DQ2-positive children that had just developed villous atrophy. Results obtained with three of these peptides (DQ2.5-glia-α-1/2, DQ2.5-glia-ω-1/2, and DQ2.5-glia-γ-I) are in agreement with the pattern of immunodominant epitopes recently reported in the peripheral blood of children/adolescents after short *in vivo* gluten challenge (17). However, given the limited number of patients analyzed, we are unable to obtain information about peptide immunodominance in our cohort of subjects, and additional studies are required to determine whether the epitope repertoire differs between children with overt CD and those with a high CD risk.

In conclusion, we identified reactivity to gluten in the gut of children with early signs of villous atrophy, similar to that reported in adult CD patients in terms of both responses to whole gliadin and recognition of immunodominant peptides (13–16). Importantly, in the PreventCD children, we identified signs of inflammatory T-cell response to gluten also in normal mucosa, not only in those with anti-tTG2 antibodies but also in those with negative serology. Studies are required to determine whether the presence of gluten-specific T cells in the small intestinal mucosa predicts villous atrophy, and to identify the factors that, besides specific T-cell and B-cell responses, drive mucosal damage in at-risk subjects.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Recognition profile by PreventCD children of the most immunodominant gliadin T-cell epitopes.

Table S1. IFN-γ values (pg/ml) obtained in one representative experiment for each T cell lines from small intestinal mucosa of prevent CD children*

Appendix S1. Supplementary methods.