RESEARCH ARTICLE

Extensive in vitro gastrointestinal digestion markedly reduces the immune-toxicity of *Triticum monococcum* wheat: Implication for celiac disease

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Scope: The ancient diploid *Triticum monococcum* is of special interest as a candidate low-toxic wheat species for celiac disease patients. Here, we investigated how an in vitro gastro-intestinal digestion, affected the immune toxic properties of gliadin from diploid compared to hexaploid wheat.

Methods and results: Gliadins from *Triticum monococcum*, and *Triticum aestivum* cultivars were digested using either a partial proteolysis with pepsin-chymotrypsin, or an extensive degradation that used gastrointestinal enzymes including the brush border membrane enzymes. The immune stimulatory properties of the digested samples were investigated on T-cell lines and jejunal biopsies from celiac disease patients. The T-cell response profile to the *Triticum monococcum* gliadin was comparable to that obtained with *Triticum aestivum* gliadin after the partial pepsin-chymotrypsin digestion. In contrast, the extensive gastrointestinal hydrolysis drastically reduced the immune stimulatory properties of *Triticum monococcum* gliadin. MS-based analysis showed that several *Triticum monococcum* peptides, including known T-cell epitopes, were degraded during the gastrointestinal treatment, whereas many of *Triticum aestivum* gliadin survived the gastrointestinal digestion.

Conclusion: The pattern of *Triticum monococcum* gliadin proteins is sufficiently different from those of common hexaploid wheat to determine a lower toxicity in celiac disease patients following in vitro simulation of human digestion.

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1 Introduction

Celiac disease (CD) is an immune-mediated systemic disorder triggered by the ingestion of gliadin fraction of wheat gluten in genetically predisposed individuals [1]. The strong genetic association of CD with HLA-DQ2/8 has emphasized

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Abbreviations: ACN, acetonitrile; **CD**, celiac disease; **BBM**, brush-border membrane; **FA**, formic acid; **GD-BBM**, gastroduodenal-brush-border membrane; **HPLC**, high perform liquid chromatography; **iTCL**, gliadin-specific T-cell line; **LC-MS/MS**, liquid Chromatography-Tandem Mass spectrometry; **PC**, pepsin-chymotrypsin; **PT**, pepsin-trypsin; **tTGase**, tissue transglutaminase; **TA**, *triticum aestivum*; **TFA**, trifluoroacetic acid; **TM**, *triticum monococcum*

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the pathogenic role of gliadin-specific adaptive immunity mediated by HLA-DQ2/8-resctricted CD4+ T lymphocytes [2]. These T lymphocytes specifically recognize gliadin peptides survived to gastrointestinal digestion and deamidated [Gln to Glu modification) by the tissue-transglutaminase (tTGase) [1]. In addition, recent studies indicate that specific gliadin peptides are able to activate the cells involved in innate immunity, such as macrophages, dendritic cells, and cytotoxic intraepithelial lymphocytes, or to induce a direct toxic effect on enterocytes [3, 4].

To date, a strictly gluten-free diet represents the only medical treatment for CD patients. Extensive research is currently aimed at discovering and breeding wheat cultivars that are potentially tolerated by most CD patients, while conserving reasonable baking aptitude. Diploid *Triticum monococcum* (TM) wheat species are among the most promising suitable candidates. Because of a reduced number of stimulatory epitopes of T-cell lines [5] and of the lack of a D-genome encoding the immunodominant 33-mer fragment [6] TM is expected to be less toxic for CD patients [7–9]. Conversely, trials on the safety assessment of TM strongly advise against its use in CD diets [10–12]. Our previous work [13] reported that two selected TM cultivars, Monlis and ID331, induced adaptive immunity with a magnitude comparable to polyploid wheat, even though ID331 was likely less effective at inducing CD because of its inability to activate the innate immune pathways.

Notably, the experimental design of the great majority of studies assessing the cereal toxicity for CD patients, including TM, consists of in vitro and ex vivo functional assays in which gliadin digested by pepsin-trypsin (PT), pepsin-chymotrypsin (PC), or chymotrypsin alone, have been used as stimulating triggers. However, in identifying new cereals with low or no immune toxicity for CD patients, we cannot overlook their susceptibility to digestion in the gastrointestinal track, including a final degradation step by endo-/exo-peptidases from the small intestinal brush border membrane (BBM) [14]. It has been reported that the resistance to gastrointestinal digestion is an important constrain in determining the immune stimulatory and toxicity properties of gliadin peptides [15].

Here, we used an in vitro static gastrointestinal digestion model to compare the digestibility of gliadin from TM (ID331 and Monlis) and from control *Triticum aestivum* (TA) grains as well as to assess the impact of this process on immunogenicity and toxicity in CD patients. After sequential digestion with gastric, duodenal, and BBM enzymes, we evaluated the immunogenic properties of the resistant peptides using intestinal T-cell lines and organ cultures of jejunal biopsies from CD patients. The panel of TM gliadin peptides that survived simulate digestion was characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2 Materials and methods

Wheat flours TM varieties ID331 and Monlis, and control TA were provided by CRA-QCE. Gliadin proteins were extracted from whole flour according to Mamone et al. [16]. Pig jejunum was collected at local slaughterhouse that meets the EU ethical standards for animal care. BBM vesicles were isolated from pig jejunum according to Moe et al. [17]. Solvents, chemicals, and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1 HPLC analysis

RP-HPLC was performed using a semipreparative C18 reverse-phase column (5 μ, 10 × 250 mm, 300A) (Phenomenex, Bologna, Italy) with a flow rate of 3 mL/min on a HP1100 system (Palo Alto, CA). Solvent A was 0.1% trifluoroacetic acid (TFA) v/v in water; solvent B was 0.1% TFA in acetonitrile (ACN). The column was equilibrated at 25% solvent B. Separation of the peptides was effected with a gradient of 25–55% solvent B over 100 min. The column effluent was monitored at 220 nm. The chromatographic separation was performed at 55°C, using a thermostatic column holder. Protein concentration was determined by the Modified-Lowry-Kit (Sigma-Aldrich).

2.2 Chymotrypsin digestion

HPLC gliadin fractions were dissolved in 100 mM ammonium bicarbonate, pH 8.2, and incubated with chymotrypsin (1:50 enzyme to protein, w/w ratio) over night at 37°C. Peptide mixtures were then stored at –80°C until LC-MS/MS analysis was performed.

2.3 Pepsin-chymotrypsin digestion

Whole gliadin fraction was dissolved in 5% formic acid (FA), pH ~2, and incubated at 37°C with pepsin (1:50 enzyme/substrate ratio) for 2 h. The sample was then dried and chymotrypsin was added at an enzyme/substrate ratio of 1:50 in 100 mM ammonium bicarbonate (pH 7.8). After 4 h at 37°C the reaction was stopped by heating for 5 min, and the sample was dried and washed twice with deionized water. Samples were stored at –80°C until further analysis.

2.4 In vitro simulated gastrointestinal digestion

Gastric-duodenal-brush-border membrane hydrolysis (GD-BBM) was carried out as described by Shan et al. [15]. Briefly, gliadin was dissolved in 0.01 M chloride acid and incubated with pepsin (1:100, enzyme/substrate w/w ratio) for 30 min. The pH was then increased to 7.0 with phosphate buffer and incubated with a mixture of trypsin (1:100), chymotrypsin (1:100), elastase (1:100), and carboxypeptidase A (1:500) for 1 h at 37°C. The sample was twofold diluted with 0.1 M sodium phosphate buffer pH 7.2, supplemented with 100 mU BBM/100 mg peptides, and incubated at 37°C up to 6 h. Samples were stored at –80°C until further analysis.

2.5 Immunogenicity evaluation

PC and GD-BBM gliadin digests were deamidated by tTGase (Sigma-Aldrich) [15]. Gliadin-specific T-cell lines (iTCLs) were generated from duodenal biopsies of six DQ2+ CD patients (mean age 15.5, range 4–34), two of whom were on a gluten-free diet. All the information regarding the demographic, clinic, and ethical committee's approval to perform the study, as well as the procedure to generate gliadin-reactive iTCLs have been described in details in previous studies [18,19]. The specificity of the iTCLs was evaluated by assaying their reactivity to a panel of immunogenic gliadin peptides previously described [19]; an oligoclonal pattern of gliadin epitopes recognition was determined (Supporting Information Table 1). The reactivity of iTCLs to the PC and GD-BBM gliadin digests was assayed based on the detection of IFN-γ production using a sandwich ELISA [19].

2.6 Organ culture and immunohistochemistry

Jejunal biopsy were taken from seven treated adult CD patients on a gluten-free diet for at least two years with normal histology and negative serology (anti-endomysium). Biopsy were cultured as described [20]. Patients were enrolled at S.G. Moscati Hospital of Avellino, Italy (CE 06/09) and gave their full informed consent to the study.

After 24 h of culture with 500 μg/mL PC or GD-BBM gliadin, the biopsy specimens were embedded in optimal cutting temperature compound, snap-frozen in liquid nitrogen, and prepared for immunohistochemical staining. Acetone-fixed sections (5 μ) were individually tested with anti-CD25 (1:25) and anti-CD80 (1:25) mAbs BD Pharmingen (Milan, Italy) and stained according to the alkaline phosphatase/anti-alkaline phosphatase method. In experiments to detect activated T-cells (CD3+CD25+), immunofluorescence with confocal microscopy (Leica TCS-SP, Heidelberg, Germany) was used. Acetone-fixed sections were incubated with a mixture of mouse mAbs anti-CD25 (1:25) and rabbit polyclonal anti-CD3 (1:100) (Dako, Milan, Italy), followed by a horse anti-mouse FITC conjugate (1:200) (Vector, Peterborough, UK) and a swine anti-rabbit TRITC conjugate (1:300). Finally, sections were counterstained with ToPro-3 (Molecular Probes, Milan, Italy). The number of cells expressing CD25 and CD80 in the lamina propria was evaluated within a total area of 1 mm^2 of lamina propria. The expression of CD3+CD25+ cells was calculated as a percentage of CD3+ lamina propria lymphocytes.

2.7 LC-MS/MS analysis

Nanoflow LC-ESI MS/MS analysis was carried out using an Ultimate 3000 HPLC (Dionex, Sunnydale, CA, USA) coupled to a Q-STAR mass spectrometer (Applied BioSystems, Framingham, USA). The eluents were (A) 5% ACN in 0.1% FA and (B) 80% ACN in 0.08% FA. The peptides were loaded into a C_{18} loading cartridge (LC Packings, USA) and separated with a C_{18} PepMap100 column (15 cm length, 75 μm id, and 300 Å) (LC Packings), using a linear gradient of 5–60% B over 70 min at a constant flow rate of 300 nL/min. Experiments were performed in the information-dependent acquisition mode. Precursor ions were selected using the following MS to MS/MS switch criteria: ions greater than *m/z* 300, charge states 2–4, intensity exceeding 15 counts and ion tolerance of 50.0 mmu, and former target ions were excluded for 30 s. CID was used to fragment multiple charged ions, and nitrogen was used as the collision gas.

The MS/MS raw spectra files were used to generate text files in mascot generic file format, and submitted to Batch-tag (Protein Prospector), search engine (http://prospector.ucsf.edu). Chymotryptic peptides of fractionated HPLC gliadin proteins were identified using the following criteria: *databases*, NCBI; *taxonomy*, Triticum; *type of search*, MS/MS ion search; *enzyme*, chymotrypsin; *max cleaved missed*: 1; *fixed modifications*, none selected; *variable modifications*, pyro-Glu formation at N-terminal Gln; *mass values*, monoisotopic; *precursor charge range*: 2-3-4; *parent tolerance*, 0.08 Da; *ms/ms tolerance*, 0.1 Da; *min score protein*, 22; *min score peptide*, 20.

GD-BBM resistant peptides produced by digestion of fractionated HPLC gliadin, were identified using the same search criteria, with the exception that "*no enzyme*" was indicated. In addition, "*taxonomy*" search was restricted to those NCBI protein general indices that had been previously identified in each corresponding HPLC fraction. Output peptide identifications were validated by manual inspection of MS/MS spectra. Peptide entries were considered identified when the measured molecular weight corresponded to the expected value and a sequence of at least three consecutive *b*- or *y*-*ions* occurred in the spectrum.

2.8 Statistical analysis

Statistical analysis was performed using Student 2-tailed *t* test. A p value < 0.05 was considered statistically significant.

3 Results

The immune stimulatory properties of TA and TM cultivars were compared on gliadin-derived peptides arising from two different in vitro proteolytic strategies (Fig. 1 left panel). The first provided for the hydrolysis of gliadins with PC digestion, according to a typical CD experimental design [1,3]. This model was compared with an alternative protocol that mimics the in vivo gastrointestinal digestive process (GD-BBM) [15].

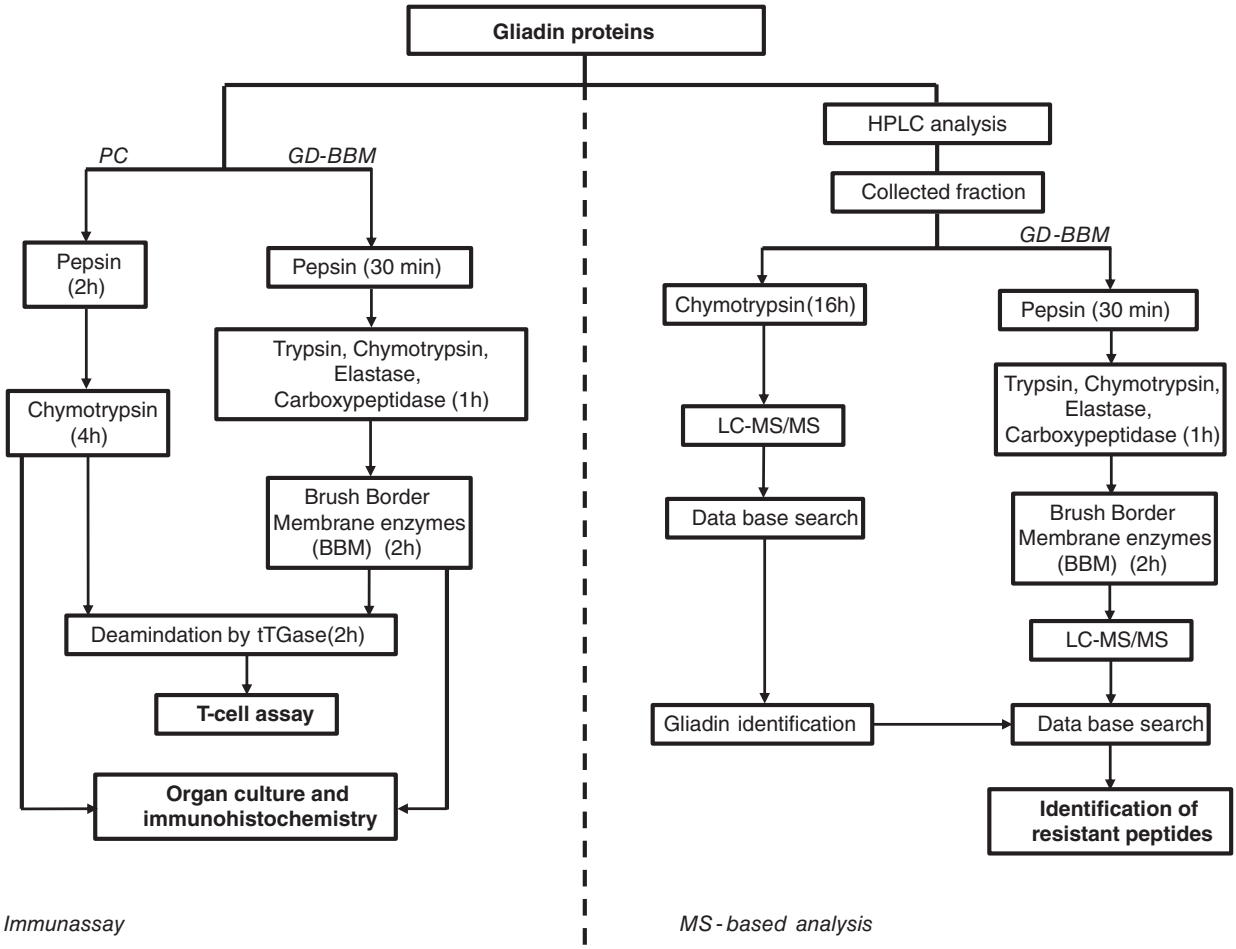

Figure 1. Schematic workflow diagram of the immunoassay and MS-based analysis of gliadins proteins from TA and TM wheats. (Left panel) Proteins were hydrolyzed according a partial (PC) or extensive (GD-BBM) digestion procedures. The digested samples were further analyzed for immune-toxicity on intestinal T cells and jejunal biopsy from CD patients. (Right panel) Proteins were fractionated by HPLC and digested by chymotrypsin alone or by sequential GD-BBM enzymes and analyzed by LC-MS/MS. The chymotryptic gliadin digests were first identified by a database search engine, then the GD-BBM resistant peptides were characterized limiting the search of the database to the accession numbers of previously identified gliadins.

3.1 Effect of GD-BBM proteolytic digestion on intestinal T-cell reactivity to gliadins

To evaluate whether and to what extent the digestion process affected the immunological activity of gliadin, we assessed the ability of the peptide mixtures from both partial (PC) and extensive (GD-BBM) hydrolysis of gliadins to activate CD4+ T-cell lines generated from the intestine of six DQ2+ celiac individuals. iTCLs were raised against deamidated PC gliadin of the hexaploid wheat. The fine specificity profile, which was previously analyzed [19], indicated that these polyclonal T-cell cultures recognized the most relevant gluten epitopes derived from all classes of gliadins (Supporting Information Table 1) [12, 13].

The GD-BBM digestion reduced the immunostimulatory activity of gliadins, evaluated by IFN-γ production, either from TA or from both TM cultivars, when compared to the corresponding PC gliadin digests (Supporting Information Fig. 1). Comparing the stimulating activity of gliadin digests obtained from the different proteolysis approaches among them, no statistically significant differences emerged among the PC-digests of both the TM cultivars and TA (Fig. 2A), confirming our previous study [13]. In contrast, the extensive hydrolysis of gliadin by GD-BBM enzymes markedly reduced the immunogenicity of both TM lines, whereas the activity of TA gliadins was only slightly affected (Fig. 2A). We next plotted the IFN-γ production as the response to the GD-BBM-digests of either TA or TM gliadins, obtained in all six iTCLs tested, as a percentage of IFN-γ response observed to partially digested (PC) gliadins. As shown in Fig. 2B, the GD-BBM hydrolysis significantly reduced the immunogenicity of both ID331 and Monlis gliadin digests. More specifically, the T-cell stimulation obtained in response to GD-BBM monococcum gliadins was 26% (range 4–47) and 19.5%, (range 0–49),

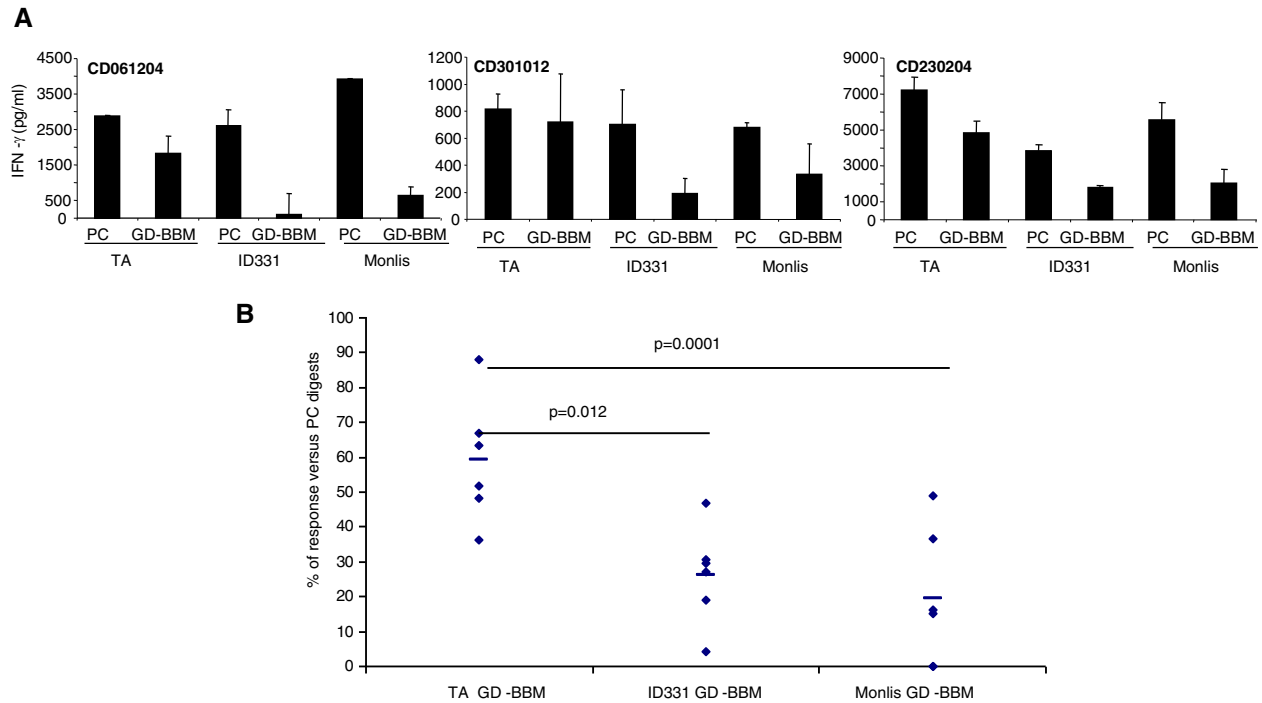

Figure 2. Marked reduction of specific T-cell response to monococcum gliadin after the GD-BBM digestion. The intestinal T cells highly reactive to TA gliadin were derived from six CD patients. T cells were stimulated with PC or GD-BBM proteolytic gliadin digests of either TA or TM (ID331 and Monlis) wheats, and IFN-γ production was thereafter evaluated by ELISA in cell supernatants. (A) IFN-γ responses to the indicated gliadin digests obtained from three different CD-patient T-cell lines are reported. Results are shown as mean of duplicates plus SD, and are representative of three separate experiments. (B) IFN-γ responses from all six T-cell lines to the extensive GD-BBM gliadin digests are shown. Results are plotted as the response rate compared to the corresponding PC digest, calculated as follows: (IFN-γ versus GD-BBM digests/IFN-γ versus PC digests) ×100.

respectively, for ID331 and Monlis, significantly lower than the immune stimulation obtained with GD-BBM gliadin digest from TA (mean 59% range 36–88; $p = 0.00008$). These results suggest that GD-BBM digestion has a stronger impact on the immunogenicity of TM gliadins, most likely due a greater susceptibility to hydrolysis of the harmful TM prolamin sequences.

3.2 Intestinal mucosa activation by GD-BBM gliadin digests

We next evaluated how PC and GD-BBM digestion models modified the capability of gliadins to induce an inflammatory immune activation in organ culture of small intestine. Because of the low number of the mucosal explants available from treated CD patients, these analysis were limited to the ID331 and TA samples. The number of CD25+ cells observed in the cultured CD mucosa was significantly increased after stimulation with either PC-gliadin of TA or ID331 (Mean ± SD: 110 ± 13, and 89 ± 33, respectively), compared to baseline (24 ±17, $p < 0.0001$; $p < 0.001$, respectively) (Fig. 3A). A high CD25+ expression, comparable to that induced by PC gliadin, was obtained in mucosal explants cultured with GD-BBM gliadin of TA (98 ± 14, $p < 0.003$). In contrast, the GD-BBM gliadin of ID331 resulted in a lower number of CD25+ cells (47 ± 28) compared to that elicited by either PC or GD-BBM gliadin of TA ($p = 0.005$ and $p = 0.003$, respectively), and by PC gliadin of ID331 itself ($p < 0.05$) (Fig. 3A).

Further evidence of reduced mucosal inflammation by gliadin after GD-BBM treatment was provided by monitoring the expression of the activation marker CD80. The number of CD80+ mononuclear cells increased in the lamina propria following the addition of PC gliadin of either TA (78 ± 25) or ID331 (73 ± 25), compared to baseline value (20.2 ± 14,3, $p < 0.0001$) (Fig. 3B). Instead, the GD-BBM gliadin of ID331 induced a CD80+ expression (45 ± 30) markedly reduced compared to the level observed with GD-BBM gliadin of TA (74 ± 25, $p < 0.01$) (Fig. 3B).

We next investigated whether this reduced activity specifically affected the mucosal T-cell response by costaining experiments with anti-CD3 and anti-CD25 mAbs. The number of activated CD3+CD25+ T cells was increased in biopsy specimens cultured with PC gliadin of TA (10.4 ± 2.7) and ID331 (7.7 ± 2.0), than in those cultured in medium alone (2.1 ± 0.5) (Fig. 3C, D). Similarly to the CD3 infiltration and CD80 immunohistochemistry findings, the GD-BBM gliadin of ID331 resulted in a marked reduction of T cells expressing

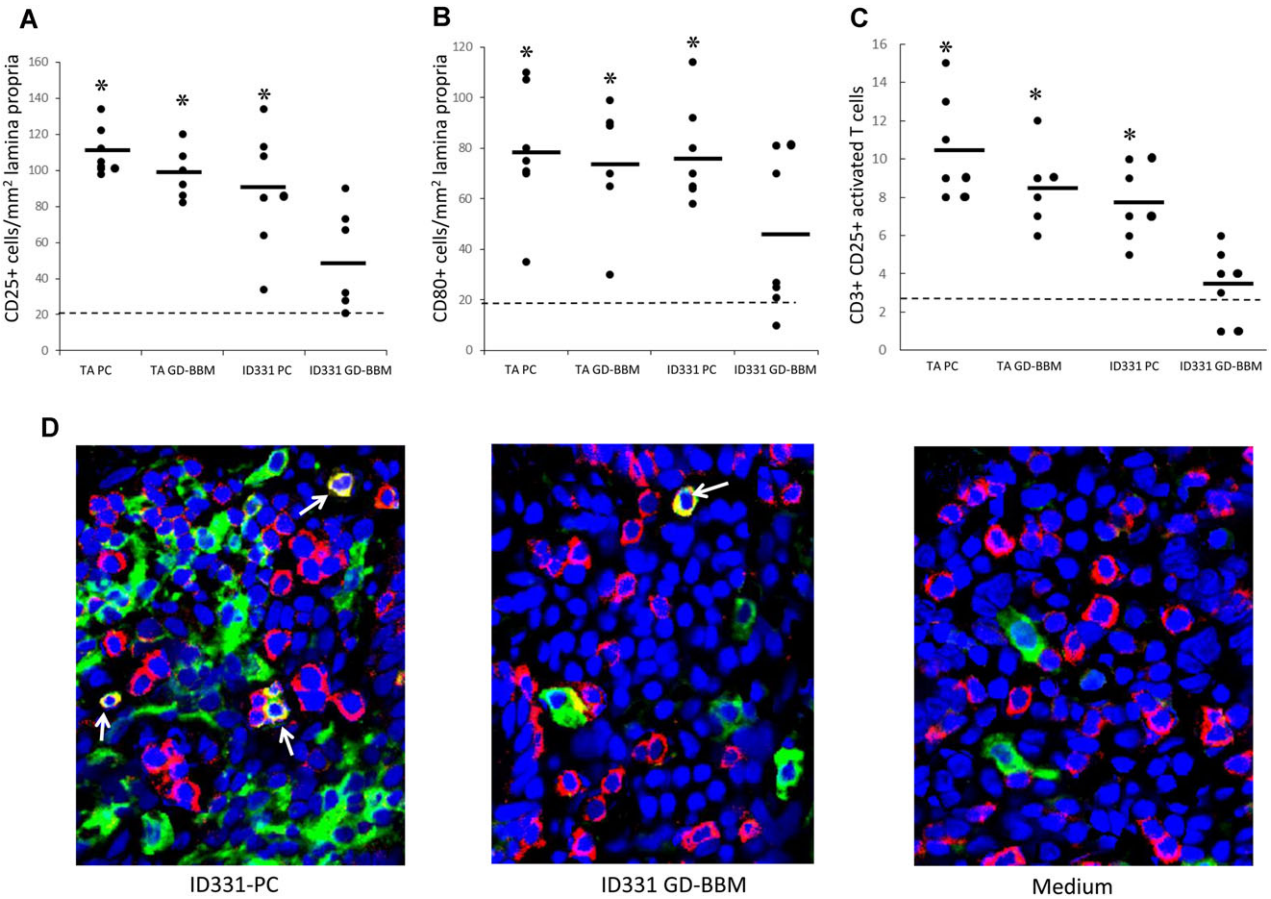

Figure 3. Immunohistochemical staining for CD25 (A), CD80 (B), and CD3CD25 (C) was performed in jejunal biopsies from treated CD patients in vitro challenged with PC or GD-BBM enzymatic digests of TA and TM *ID331* gliadins. The broken lines indicate the mean value of positive cells detected in biopsies cultured with medium alone (baseline). Dots represent single patients. Dashes indicate mean values. The * indicates a statistical significance of $p < 0.0001$, and has been calculated comparing each condition with the baseline value. (D) Immunofluorescence images of the duodenal mucosa from treated CD patients in vitro cultured with TM ID331 gliadin either partially digested with PC or with GD-BBM, or with medium alone. The slides report the staining of the lamina propria compartment CD3+ lymphocytes are colored in red, the CD25+ cells in green, and the merged CD3+CD25+ activated T cells in yellow (arrows). Original tissue magnification 40×.

CD25 (3.4 ± 1.9) (Fig. 3C, D), whilst the GD-BBM gliadin of TA induced an activation of T lymphocytes almost comparable to the level observed in response to PC gliadin digests (8.5 ± 2.0), (Fig. 3C).

3.3 Characterization of GD-BBM gliadin peptides

The expression of different gliadin epitopes among the TA, ID331, and Monlis wheat was determined by LC-MS/MS. The identification of GD-BBM gliadin peptides resulting from simulated digestion was complicated by the incompleteness of the database annotation and by the heterogeneity of the peptide mixture, generated with a not predictable specificity of hydrolysis. To restrain the research of peptides, a parallel MS-based identification of the HPLC-isolated protein fraction (Fig. 4), individually hydrolyzed by chymotrypsin, was carried out (Fig. 1, left panel). The MS-based identification of chymotryptic gliadin peptides is detailed in Supporting Information Material and Supporting Information Tables 2, 3, and 4.

HPLC chromatogram of ID331, Monlis, and TA gliadins (Fig. 4 panel A, B, and C, respectively) resolved the protein complexity into 14, 12, and 15 major peaks, respectively. Gliadin type proteins were classified into three subgroups based on their retention time (Rt): ω- (Rt 30–40 min), α/β- (Rt 40–60 min), and γ-gliadin (Rt. 60–90 min) [21]. With the exclusion of ω-gliadin, which was missing in Monlis, the protein profiles of the TM lines did not appreciably differ (Supporting Information Material; Supporting Information Tables 2 and 3). Through defining the profile of gliadin expression, MS-based analysis confirmed that TM cultivars actually exhibit the

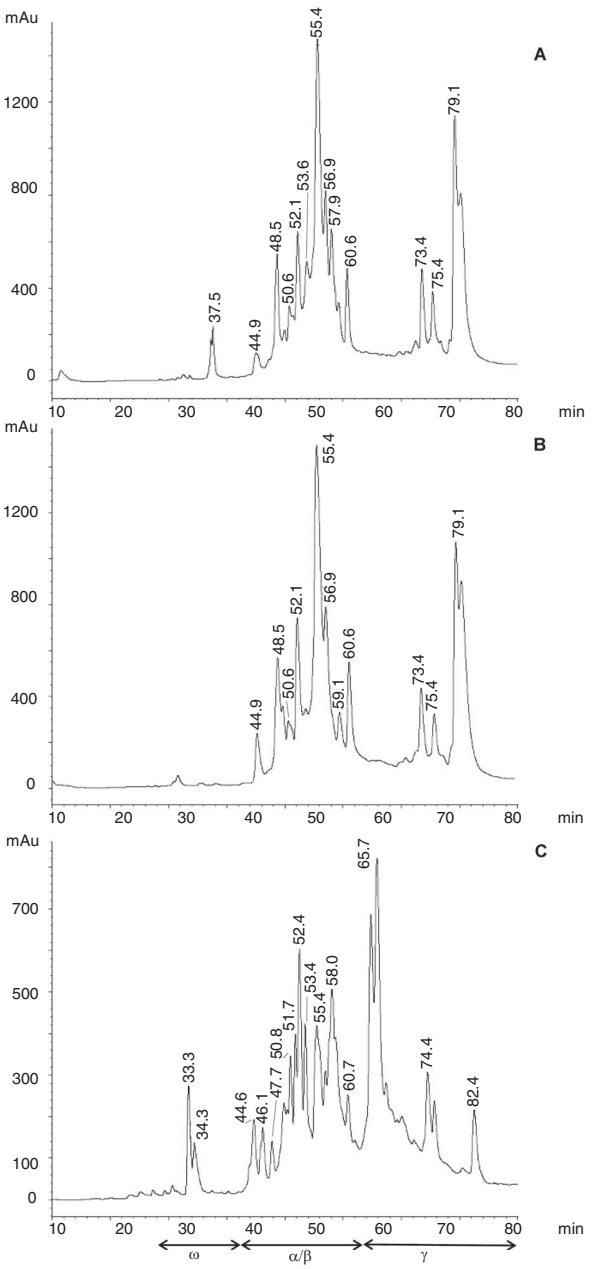

Figure 4. Comparison among HPLC chromatograms of gliadin proteins from TA and from TM cultivars. HPLC chromatograms of gliadin extracts from ID331 (panel A), Monlis (panel B), and TA (panel C). Gliadin types were classified in ω- (Rt 30–40 min), α- (Rt 40–60 min), and γ-gliadin (Rt. 60–90 min). Proteins were identified by MS-based analysis (see Supporting Information Tables II, III, IV).

potential to induce the CD syndrome, consistently with previous studies [10–13].

Preliminary characterization of gliadin proteins was the basis for subsequent identification of the GD-BBM resistant gliadin peptides. Aliquots of HPLC fractions (Fig. 4) were digested according to the GD-BBM protocol (Fig. 1, right panel). To extend the identification of the resistant peptides, the LC-MS/MS of digested samples was separately processed by a database search engine, limiting the search to the accession number(s) of protein(s) that had been previously identified in each HPLC fraction. An overview of the epitopes within each identified gliadin proteins and after GD-BBM gliadin digestion is provided in Table 1 (ID331 and Monlis) and Table 2 (TA). T-cell stimulatory epitopes are named according to standard nomenclature as reviewed by Sollid et al. [22].

3.4 TM peptides resistant to GD-BBM

GD-BBM digestion dramatically affected the stability of epitopes within each gliadin, and 106 and 92 peptides were identified from ID331 and Monlis, respectively. TM cultivars shared the same GD-BBM patterns, if the ω-gliadin fragments occurring in ID331 are excepted. The GD-BBM peptides are listed in Supporting Information Table 5.

Several peptides were resistant to GD-BBM digestion, but few among them harbored T-cell stimulatory epitopes, as summarized in Table 1. ID331 ω-gliadin gi|294998449 (Rt 37.5) generated a peptide harboring the DQ2.5-glia-γ-5 epitope so called because also found in γ-gliadin.

Among the α-gliadin protein, no peptides carrying DQ2.5-glia-α3 and DQ8-glia-α1 epitopes were detected. Interestingly, DQ2.5-glia-α1a epitope fragments (PFPQPQL, QPFPQPQL, and QPFPQPQLP), were detected from hydrolysis of some α-gliadin expressing immunogenic peptide P56-68 (LQLQPFPQPQLPY). These fragments only partially conserved the DQ2.5-glia-α1 structure and have weaker immunogenicity compared to their parent P56-68, as previously demonstrated [23]. Hydrolysis of α-gliadin gi|217039694 (Rt 50.6), and gi|217039690 (ID331-peak4) yielded a stable peptide 81–110 (FPQPQPFPPQLPYPQPQPFPPQQPYPQPQT) (MH^+ 3511.5851), which does not include any known T-cell epitope. α-gliadin P31-55, which includes the P31-43 and P44-55 toxic sequences, were released from the digestion of α-gliadin gi|66393346 (Rt 55.4 and 59.1) [24]. Although as minor components, shortened forms arising from the action of endo- and amino-peptideases P31-43, P32-43, and P32-55, were also identified in hydrolyzed α-gliadin. GD-BBM hydrolysis of gi|217039705 and gi|217039703 and gi|66393346 gliadins also released α-peptide P31-55.

Some regions of γ-gliadins harbor T-cell stimulatory epitopes that survived the GD-BBM hydrolysis. Peptides carrying the DQ2-γ-I epitope were released from GD-BBM digestion of gi|217039731 (Rt 75.4) and gi|217039727 (Rt 75.4), whereas gliadin gi217039721 (Rt 75.4), gi|217039719, gi|217039715, and gi|217039717 (Rt 79.1) released peptides harboring the DQ2.5-glia-γ4c motif.

3.5 TA peptides resistant to GD-BBM

As expected, GD-BBM digestion of TA gliadin produced a higher number of peptides that retained the major harmful epitopes intact (Table 2), if compared to TM. LC-MS/MS

Table 1. T-cell epitopes and toxic peptides expression within the ID331 and Monlis cultivars before and after GD-BBM digestion

HPLC Rt[a)]	Protein	Gliadin type[b)]	Expected epitopes in identified gliadin[c)]	Epitope encrypted in peptide resistant to GD-BBM digestion[d)]
37.5*	ω-Gliadin	294998449	DQ2.5-glia-γ4c(1); DQ2.5-glia-γ5(3);	DQ2.5-glia-γ5(1);
44.2	α-Gliadin	217039690	-	-
48.5	α-Gliadin	217039694	-	-
50.6	α-Gliadin	217039705	DQ2.5-glia-α1a(1); glia-α-20(1); P31-43(1)	P31-43
50.6	α-Gliadin	217039684	-	
52.1	α-Gliadin	217039682	-	
52.1	α-Gliadin	66393328	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P44-55(1)	
53.6	α-Gliadin	217039697	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
55.4	α-Gliadin	66393346	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
56.9	α-Gliadin	66393328	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P44-55(1)	
57.9*	α-Gliadin	66393328	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P44-55(1)	
59.1	α-Gliadin	66393346	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
60.6	α-Gliadin	66393336	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P44-55 (1)	
73.4	γ-Gliadin	217039729	DQ2.5-glia-γ4c (2)	
73.4	γ-Gliadin	217039731	DQ2.5-glia-γ4c (2); DQ2.5-glia-γ1 (1)	DQ2.5-glia-γ1(1)
75.4	γ-Gliadin	217039727	DQ2.5-glia-γ4c (3); DQ2.5-glia-γ1 (1)	DQ2.5-glia-γ1(1)
75.4	γ-Gliadin	217039721	DQ2.5-glia-γ4c (2)	DQ2.5-glia-γ4c(1)
79.1	γ-Gliadin	217039719	DQ2.5-glia-γ4c (3)	DQ2.5-glia-γ4c(1)
79.1	γ-Gliadin	217039715	DQ2.5-glia-γ4c (5); DQ2.5-glia-γ2(1); DQ2.5-glia-γ5 (1)	DQ2.5-glia-γ4c(1);
79.1	γ-Gliadin	217039717	DQ2.5-glia-γ4c (4); DQ2.5-glia-γ5(1)	DQ2.5-glia-γ4c(1);

a) Rt of chromatogram peak as shown in Fig. 5A and B.
b) NCBI general index (GI) of protein previously identified (see Supporting Information Tables II and III).
c) Epitope toxic peptides expressed in identified in gliadin proteins.
d) Epitope and toxic peptides within peptides resistant to GD-BBM digestion. Nomenclatures are according to Sollid et al. [22] except toxic peptides P31–43 and P44–55. Number in brackets indicates the copy of epitope within protein.
e) Indicates HPLC peaks lacking in Monlis (4B).

analysis led to the identification of 150 GD-BBM resistant peptides (Supporting Information Table 6).

Digestion of gi|508732623 (RT 33.3) released peptides harboring a DQ2.5-glia-ω1/2 epitope, and four and five copies of the DQ2.5-glia-γ4c and DQ2.5-glia-γ5 epitopes, respectively (Table 2). The DQ2.5-glia-γ5 and DQ2.5-glia-γ4c epitopes survived digestion of ω-gliadin 224747073 (Rt 34.3), whereas only DQ2.5-glia-γ5 (Rt 34.3) arising from ω-gliadin gi|22474071, was detected.

As previously demonstrated [15], immunogenic 33-mer peptide from α-gliadin gi|147883548 (Rt 52.4) was stable to digestion, along with less intense products due to N-terminus trimming. The 33-mer encloses six T-cell overlapping epitopes consisting of DQ2.5-glia-α1a, DQ2.5-glia-α2 (three copies), and DQ2.5-glia-α1b (two copies). Similar to 33-mer, a stable peptide of 26aa (LQLQPF-PQPQLPYPQPQLPYPQPQPF) was released by digestion of α-gliadin gi|2932402 (Rt 50.08) and contains four overlapping epitopes: DQ2.5-glia-α1a, DQ2.5-glia-α1b, and two copies of DQ2.5-glia-α2. Hydrolysis of α-gliadin gi|401787274 (Rt 51.7) yielded another 26-mer (LQLQPF-PQPQLPYPQPHLPYPQPQPF) carrying the DQ2.5-glia-α1a, and DQ2.5-glia-α2 epitopes. As observed for the TM, peptides including DQ2.5-glia-α3 and DQ8-glia-α1 epitopes were not detected in the digested samples. Released toxic peptide P31-55 eluted from GD-BBM digestion of gliadins in the chromatogram region (Fig. 5A and B) between Rt 55.4 and 60.7, (gi|1304264, gi|188530129, gi|188530133, gi|147883568).

TA γ-gliadins produced a high number of peptides resistant to GD-BBM hydrolysis. DQ2.5-glia-γ1, considered a major CD γ-epitope, was identified in a peptide from gi|478683577 (Rt 65.7). GD-BBM resistant fragments harboring the DQ2.5-glia-γ4c epitope were detected in gi|47868347 (Rt 65.7), gi|209971871 (Rt 65.7), 478683585 (Rt 74.4), and 209971883 (Rt82.4). The γ-peptides carrying a DQ2.5-glia-γ5 were in gi|478683577 and gi|209971871. Peptides from gi|209971847 and gi|209971883 hydrolysis also contained a copy of DQ2.5-glia-γ4a and DQ2.5-glia-γ3, respectively.

4 Discussion

In the present study, we aimed to determine the immune toxic properties of gliadin from two TM cultivars (ID331 and Monlis) in comparison to TA (Sagittario) and the impact of the in vitro simulated extensive gastrointestinal (GD-BBM) digestion process on the immune toxic activity in CD patients. Our findings unequivocally showed a great variability

Table 2. T-cell epitopes and toxic peptides expression within TA (*Sagittario*) cultivar before and after GD-BBM digestion

HPLC Rt[a)]	Protein	Gliadin type[b)]	Expected epitopes in identified gliadin[c)]	Epitope encrypted in peptide resistant to GD-BBM digestion[d)]
33.3	ω-Gliadin	508732623	DQ2.5-glia-ω1(1); DQ2.5-glia-ω2(1); DQ2.5-glia-γ4c (7); DQ2.5-glia-γ5(10)	DQ2.5-glia-ω1(1); DQ2.5-glia-γ4c(5); DQ2.5-glia-γ5(4)
34.3	ω-Gliadin	224747073	DQ2.5-glia-ω1(1); DQ2.5-glia-γ4c(2); DQ2.5-glia-γ5(2)	DQ2.5-glia-γ5(1); DQ2.5-glia-γ4c(1)
34.3	ω-Gliadin	224747071	DQ2.5-glia-γ5(2)	DQ2.5-glia-γ5(1)
44.6	α-Gliadin	383210737	DQ8-glia-α1 (1)	
46.1	α-Gliadin	383210749	DQ8-glia-α1 (1)	
47.7	α-Gliadin	383210754		
47.7	α-Gliadin	121094	P44-55(1)	
47.7	α-Gliadin	147883560	P44-55(1)	
50.8	α-Gliadin	421932498		
50.8	α-Gliadin	421932402	DQ2.5-glia-α1a (1); DQ2.5-glia-α1b(1); DQ2.5-glia-α2(2); DQ2.5-glia-α3; DQ8-glia-α1(1); P31-43(1); P44-55(1)	DQ2.5-glia-α1a(1); DQ2.5-glia-α1b(1); DQ2.5-glia-α2 (2)
51.7	α-Gliadin	401787284	DQ2.5-glia-α1a(1); DQ2.5-glia-α2(1); DQ2.5-glia-α3(1); DQ8-glia-α1(1)	DQ2.5-glia-α1a (1); DQ2.5-glia-α2(1);
51.7	α-Gliadin	401787274	DQ2.5-glia-α1a (1); DQ2.5-glia-α2 (1); DQ2.5-glia-α3(1); DQ8-glia-α1 (1)	DQ2.5-glia-α1a(1); DQ2.5-glia-α2(1);
52.4	α-Gliadin	147883548	DQ2.5-glia-α1a(1); DQ2.5-glia-α1b(3); DQ2.5-glia-α2(2); DQ2.5-glia-α3;DQ8-glia-α1 (1); P31-43(1); P44-55(1)	DQ2.5-glia-α1a (1); DQ2.5-glia-α1b(3); DQ2.5-glia-α2(2); P31-43(1); P44-55(1)
53.4	α-Gliadin	147883552	DQ2.5-glia-α1a(1); DQ2.5-glia-α2(1); DQ2.5-glia-α3(1); P31-43(1); 44–55(1)	P31-43(1);P44-55(1)
55.4	α-Gliadin	1304264	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1); P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
55.4	α-Gliadin	188530129	DQ2.5-glia-α3(1); P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
58.0	α-Gliadin	188530133	DQ2.5-glia-α1a(1); DQ2.5-glia-α3(1) P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
58.0	α-Gliadin	7209257	P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
60.7	α-Gliadin	147883568	DQ2.5-glia-α3(1); P31-43(1); P44-55(1)	P31-43(1); P44-55(1)
65.7	γ-Gliadin	478683577	DQ2.5-glia-γ1(1); DQ2.5-glia-γ4b(1); DQ2.5-glia-γ4c(8); DQ2.5-glia-γ5(3)	DQ2.5-glia-γ1(1); DQ2.5-glia-γ4c(3); DQ2.5-glia-γ5(1)
65.7	γ-Gliadin	209971847	DQ2.5-glia-γ1(1); DQ2.5-glia-γ2(1); DQ2.5-glia-γ4a(1); DQ2.5-glia-γ4c(1);	DQ2.5-glia-γ4a(1);
65.7	γ-Gliadin	209971871	DQ2.5-glia-γ1(1); DQ2.5-glia-γ2(1); DQ2.5-glia-γ4a(3); DQ2.5-glia-γ4c(7);	DQ2.5-glia-γ5(2); DQ2.5-glia-γ4c(3)
74.4	γ-Gliadin	478683585	DQ2.5-glia-γ1(1); DQ2.5-glia-γ2(1); DQ2.5-glia-γ4c (1); DQ2.5-glia-γ4b(1);	DQ2.5-glia-γ4c(1)
82.4	γ-Gliadin	209971883.	DQ2.5-glia-γ1(1); DQ2.5-glia-γ3 (1); DQ2.5-glia-γ4c(2);	DQ2.5-glia-γ3 (1);DQ2.5-glia-γ4c(1)

a) Rt of chromatogram peak as shown in Fig. 5A.
b) NCBI general index (GI) of protein previously identified by LC-MS/MS (see Supporting Information Table III).
c) Epitope toxic peptides expressed in identified in gliadin proteins.
d) Epitope and toxic peptides within peptides resistant to GD-BBM digestion. Nomenclatures are according to Sollid et al. 2012, [22] except toxic peptides P31–43 and P44–55. Number in brackets indicates the copies of epitope.

in the induction of an inflammatory response depending on the digestion protocol used for gliadin hydrolysis. PC digested gliadins of ID331 and Monlis showed high immunogenicity property on polyclonal CD intestinal T-cell cultures comparable to TA. Notably, the capability of activating the adaptive CD4+ T-cell mediated immune response of TM gliadin markedly decreased after an GD-BBM treatment. In contrast, the capability of TA to stimulate intestinal mucosa T cells was almost unaffected by GD-BBM degradation. The ex vivo organ culture experiments using normalized biopsies from treated CD patients further confirmed the substantial reduction of the immunotoxic potential of gliadin from ID331 after the GD-BBM digestion. In fact, following the incubation of CD mucosal explants with GD-BBM digests of ID331

gliadin, very few CD25+ and CD80+ cells, as well as activated CD3+CD25+ lymphocytes, were counted in the lamina propria, compared to the activated cells obtained in response to GD-BBM gliadin of TA.

MS-based analysis similarly demonstrated that T-cell epitopes are spread out across undigested TM gliadin. In particular, almost all TM α-gliadins harbor a copy of the DQ2.5-α-I epitope, which is recognized in the majority of CD patients [19, 25, 26]. PC digestion released TM gliadin peptides that are able to evoke an immune response in celiac T cells [22, 27] confirming the findings of previous studies from both our [13] and other groups [10–12] that no distinct differences were observed among TM and hexaploid wheat in inducing anti-gluten T-cell responses. Nevertheless, the in vivo gastrointestinal digestion is a more complex process than PC hydrolysis because several additional proteases are involved in the sequential proteolytic stages [14]. In particular, the use of proteases located on enterocyte microvilli (BBM) is a fundamental step in assessing intestinal stability because they contain a series of *endo*- and *exo*-peptidases, which have not negligible effects in gradually shortening peptides into di-, tri-, and oligopeptides as well as free amino acids [28, 29]. Because of similarities with human intestinal physiology [30], porcine BBM was used in this study. The resistance to the GD-BBM process is a prerequisite for wheat gliadin peptides to reach the lamina propria intact and exert their harmful action on the immune competent T cells [14]. To this purpose, the immunodominant capability of α-gliadin 33-mer peptide was discovered due to its stability to BBM enzymes [15]. In addition to 33-mer, we identified other GD-BBM stable TA α-peptides, such as LQLQPFPQPQLPYPQPQLPYPQPQPF (gi|2932402) and LQLQPFPQPQLPYPQPHLPYPQPQPF (gi|401787274), which carried overlapping DQ2.5 restricted T-cell epitopes (Table 2). The stability to gastrointestinal proteolytic enzymes of these TA α-gliadin fragments, as well as ω- and γ-peptides harboring immunogenic sequences, might yield a synergistic effect on the activation of proinflammatory T lymphocytes driving intestinal damage in CD patients.

The A-genome of TM gliadin does not express the 33-mer sequence but does express a shortened form (P56-68) homologue for the 13 N-terminal residues. The P56-68 includes only a copy of the DQ2.5-glia-α1a epitope (Table 1). Interestingly, the P56-68 sequence of TM gliadin was stable to PC digestion but was degraded by enzymes used in the GD-BBM digestive process. As a consequence, the GD-BBM treatment of TM gliadin results in the marked reduction of harmful property of the primary immunodominant T-cell epitope [23]. Notably, following GD-BBM, peptides harboring the DQ2.5-glia-α3 and DQ8-glia-α1 epitopes were not detected in either TA or TM gliadins (Tables 1 and 2).

TM γ-gliadins also contain a repetitive copy of the T-cell stimulatory DQ2.5-γ epitope. Our analysis has showed that, although GD-BBM digestion in part affected the stability of these epitopes, some regions carrying DQ2.5-glia-γ4c, DQ2.5-glia-γ3, DQ2.5-glia-γ4a, DQ2.5-glia-γ5, and DQ2.5-glia-γ1 remained resistant to proteases. The former is known as a major CD epitope derived from the γ-gliadin fraction. Camarca et al. [19, 27] found that some CD patients are more reactive to γ-gliadin epitopes than to α-gliadin-derived ones. Because of this large number of gliadin immune toxic sequences and of a well-documented wide variability of CD patient recognition patterns, we used stable CD4+ T-cell lines in our specificity assays covering the reactivity to the most immunogenic T-cell sequences of α-, ω-, and γ-gliadins (Supporting Information Table 1).

In conclusion, we demonstrated that gliadin proteins of TM are sufficiently different from those of common TA wheat so to determine a lower immune toxicity following in vitro simulation of human digestion. It has been found that the intensity of the T-cell response to gluten peptides is dependent on the HLA-DQ2 gene dosage of celiac patients [31], suggesting a quantitative (HLA/gluten epitopes) model to reach a pathogenic T-cell reaction. Therefore we hypothesize that a regular diet based on ancient TM that have a reduced amount of gluten immune toxic peptides may delay the onset of CD, particularly in at risk subjects such as first degree relatives of celiac patients carrying the CD-associated HLA alleles. In this context, we are currently performing experiments looking at the immune toxicity in celiac disease patients after a brief oral consumption of monococcum wheat bread.

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