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HLA-DQ Molecules as Affinity Matrix for Identification of Gluten T Cell Epitopes

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Even though MHC class II is a dominant susceptibility factor for many diseases, culprit T cell epitopes presented by disease-associated MHC molecules remain largely elusive. T cells of celiac disease lesions recognize cereal gluten epitopes presented by the disease-associated HLA molecules DQ2.5, DQ2.2, or DQ8. Employing celiac disease and complex gluten Ag digests as a model, we tested the feasibility of using DQ2.5 and DQ2.2 as an affinity matrix for identification of disease-relevant T cell epitopes. Known gluten T cell epitope peptides were enriched by DQ2.5, whereas a different set of peptides was enriched by DQ2.2. Of 86 DQ2.2-enriched peptides, four core sequences dominated. One of these core sequences is a previously known epitope and two others are novel epitopes. The study provides insight into the selection of gluten epitopes by DQ2.2. Furthermore, the approach presented is relevant for epitope identification in other MHC class II–associated disorders. The Journal of Immunology, 2014, 193: 4497–4506.

Many immune-mediated diseases, such as type 1 diabetes, rheumatoid arthritis, multiple sclerosis, primary sclerosing cholangitis, and celiac disease (CD), display strong associations to particular HLA or MHC class II variants (1). There is a strong suspicion that the MHC association in such diseases could be caused by presentation of certain antigenic peptides to pathogenic CD4+ T cells (2, 3). With the exception of CD, which is a chronic inflammatory disease of the small intestine caused by CD4+ T cell–mediated immune hyperreactivity to wheat gluten and related proteins of barley and rye (4, 5), the causative antigenic peptides for most of these diseases remain elusive. Genome-wide association studies, reporting that for many diseases the genetic effects of MHC are much stronger than any of the non-MHC genes (6), have refueled the quest for understanding the mechanisms behind MHC/disease associations. Given the meager progress that have been made on the molecular basis of HLA associations with disease during the last 40 y, new approaches are clearly needed. One potential avenue could be to harness the disease-associated MHC molecules themselves and use them as affinity matrix to identify disease-relevant T cell epitopes. CD offers an opportunity to explore this approach, as distinct epitopes of the causative Ag gluten are recognized by lesion-resident CD4+ T cells in the context of disease-associated HLA-DQ molecules (7, 8). Given the extreme complexity of gluten, the model of CD would not be much different from a situation when mixtures of multiple Ags would be used. Wheat gluten consists of hundreds of distinct protein components that are grouped into entities of gliadins (α- , γ-, and ω-gliadins) and glutenins (low– and high–molecular mass glutenins) (9), and a proteolytic digest of gluten therefore contains many thousand unique peptides. If the CD-associated MHC class II molecules would bind and enrich from complex gluten peptide mixtures peptides that are recognized by T cells from the disease lesion, this approach would bear promise for a wider application.

About 90% of CD patients express HLA-DQ2.5 (DQA1*05/DQB1*02) whereas the remaining patients express HLA-DQ8 (DQA1*03/DQB1*03:02) or HLA-DQ2.2 (DQA1*02:01/B1*02:02) (10, 11). The gluten T cell epitopes presented by DQ2.5 are fairly well characterized (12). Less is known about the gluten epitopes presented by DQ8 or DQ2.2, and only one DQ2.2 T cell epitope is known (7, 12). Typically the gluten peptides that are recognized by T cells of CD patients are posttranslationally modified (13, 14). The enzyme transglutaminase 2 (TG2) converts certain residues in gluten peptides from glutamine to glutamate and this deamidation process improves the ability of DQ2.5, DQ2.2, and DQ8 to bind and present the peptides to T cells (15–17).

As a first step to test the above-outlined approach, we used DQ2.5 as an affinity matrix to see which peptides this HLA molecule enrich from a complex digest of gluten Ag. Soluble and biotinylated DQ molecules were incubated with digests of gluten treated with TG2. HLA molecules were immobilized with streptavidin beads, and HLA-bound peptides were eluted and identified by mass spectrometry (MS). We found that the gluten peptides enriched by DQ2.5 were highly dominated by known T cell epitopes. To further explore the potential of the method, we next used DQ2.2 as the affinity matrix for the same gluten digests. Notably, different peptides were enriched by DQ2.5 and DQ2.2. Of 86 DQ2.2-binding gluten peptides, four core sequences were found to dominate. One of these core sequences is a previously known epitope (7). Two of the other core sequences were found to represent novel T cell epitopes by testing T cells of the intestinal CD lesions. The present study

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The online version of this article contains supplemental material.

Abbreviations used in this article: CD, celiac disease; LC, liquid chromatography; LC-MS/MS, LC–tandem MS; LMW, low-m.w.; MS, mass spectrometry; MS/MS, tandem MS; PTCEC, pepsin, trypsin, chymotrypsin, elastase, and carboxypeptidase A; TCC, T cell clone; TCL, T cell line; TG2, transglutaminase 2.

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provides novel insight into the selection of gluten epitopes by DQ2.2. Furthermore, we demonstrate that MHC class II molecules can serve as an affinity matrix for efficient identification of disease-relevant T cell epitopes. The approach is potentially applicable to other diseases and systems where there is a need to define MHC class II-restricted T cell epitopes.

Materials and Methods

Peptides

Most synthetic peptides were purchased from GL Biochem (Shanghai, China). Some peptides were prepared in-house by solid-phase peptide synthesis on a robotic system (Syro, MultiSynTech, Bochum, Germany) using Fmoc-/O-butyryl on-resin. The following peptides were used for this study (epitope, amino acid sequence for native/deamidated peptides, respectively): DQ2.5-glia-γ1, pyroglu-POQPOQSFQPQRPQF EQPQPFSQPQPQRPQFERP; DQ2.5-glia-γ2, G1QPQPQQPAQLGHQGQPQPAQL; DQ2.5-glia-γ3, FQPQPQYQFPQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQEQPQYQYPQ
spectrometer (Ultraflex II; Bruker Daltonics) or on an ion trap mass spectrometer (HCT Ultra ETD II; Bruker Daltonics) coupled to a nano-LC (Easy-nLC; Thermo Scientific, former from Proxeon, Odense, Denmark). For nano-LC–ion trap analysis, peptides were separated on an analytical column (100 × 0.075 mm packed with 120 Å, C18 3-μm particles) (BioSphere, Proxeon/Thermo Scientific, Odense, Denmark). A linear gradient of 5–35% solvent B in 60 min was applied with a flow rate of 300 nl/min (solvent A, 0.1% formic acid; solvent B, 90% acetonitrile/0.1% formic acid). Peptides from gliadin fractions with or without HLA enrichment were separated on the Dionex U3000 capillary/nano-HPLC system (Dionex, Sunnyvale, CA), which was directly interfaced with a Thermo Fisher Q Exactive mass spectrometer. The HLA-enriched peptides and the PTCEC gliadin fractions were analyzed on Acclaim PepMap RSLC columns (Dionex) (75 μm inner diameter packed with 100 Å, C18, 2-μm particles) of 15 or 50 cm length, respectively. A linear gradient of 5–50% solvent B over 45 min was applied with a flow rate of 300 nl/min for the HLA-enriched peptides. For the PTCEC gliadin digests a linear gradient of 5–50% solvent B over 240 min at 200 nl/min was applied. The Q Exactive mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur 2.2 software. Single MS full-scan in the Orbitrap (300–1750 m/z, 70,000 resolution at m/z 200, AGC target 1e6, maximum injection time 20 ms) were followed by 10 data-dependent MS/MS scans in the Orbitrap after accumulation of 1e6 ions in the C-trap or an injection time of 120 ms (fixed injection time method) at 35,000 resolution (23) (isolation width 2.0 or 3.0 m/z, underfill ratio 0.1%, dynamic exclusion 20 or 45 s) at 25 or 30% normalized collision energy.

Database search

A Triticum aestivum database was created by extracting entries (n = 4722) from the UniProt Knowledge database (European Bioinformatics Institute;
accessed September 2012). The LC-MS/MS data were searched against this database using the Mascot search engine. In all Mascot searches, the digestion enzyme specificity was set as none and pyro-glu (N-term Q), deamidation (NQ), and oxidation (M) were selected as variable modifications. For analysis of Q Exactive data, the mass error tolerance for MS scans was set as 10 ppm for MS and 0.05 Da for MS/MS. The data were analyzed using Proteome Discoverer 1.3. Only peptides with a Mascot ion score of p < 0.01 were considered for the HLA-enriched peptides whereas peptides with a Mascot ion score of p < 0.05 were considered in the PTCEC gliadin fractions.

**Competitive peptide binding assay**

Peptide binding was measured in a competitive peptide binding assay as described previously (24). The source of the DQ2.2 molecules was the EBV-transformed B cell line 9050 MOU (lysate of 2 × 10^7 cells/well). IC50s were established by measuring the inhibitory effect of binding of the CD4+ isolation kit (Invitrogen). Cloning was performed by limiting dilution. TCCs were also generated from peripheral blood by tetramer staining and sorting, of two CD patients, CD555 and CD627, as previously reported (27). CD4+ T cells that stained positive with any of the three DQ2.2 tetramers, DQ2.2:DQ2.2-glia-1, DQ2.2:DQ2.2-glut-L1, were sorted. These cells were cloned by limiting dilution and expanded without antigenic stimulation. The obtained TCCs were tested in a proliferative T cell assay using irradiated lymphoblastoid B cell lines as APCs. The following cell lines were used: 9050 MOU (DQA1*02:01/DQB1*02:02; DQ2.2), 9076 T7526 (DQA1*03:02 /DQB1*03:03; DQ3.2), 9102 ARBO (DQA1*03:03/ DQB1*02:02; DQ2.3), 9052 DBB (DQA1*02:01/DQB1*03:03; DQ2.2), and CD114 (DQA1*05:01/DQB1*02:01; DQ2.5).

**Results**

DQ2.5 molecules as affinity matrix for gluten peptides

The method was established using recombinant soluble DQ2.5 molecules. Biotinylated DQ2.5 molecules, treated with thrombin to facilitate exchange of ligands by peptide-linker cleavage, were incubated with gluten peptide mixtures of increasing complexity in the presence of HLA-DM. DQ2.5 peptide complexes were immobilized by paramagnetic streptavidin beads and unbound peptides were washed away. Bound peptides were eluted and their sequence was determined by MS (Fig. 1). We first tested for selection by DQ2.5 using synthetic peptides harboring native and deamidated epitopes of DQ2.5-glia-γ1 and DQ2.5-glia-γ2. Additionally, the effect of HLA-DM on the ligand exchange was investigated. Equimolar amounts of the four gliadin peptides were incubated together with biotinylated DQ2.5 molecules, both in the presence or absence of soluble HLA-DM. HLA-DM is a catalyst for loading of high-affinity binding to MHC class II molecules (28). Peptides enriched by DQ2.5 were then analyzed by MS (LC-MS/MS). The results demonstrate that the deamidated versions of the DQ2.5-glia-γ1 and DQ2.5-glia-γ2 epitope peptides were preferentially enriched and identified. The ratio of deamidated to native peptides significantly increased in the presence of HLA-DM. In the absence of HLA-DM, 7- to 8-fold higher amounts of the prebound DQ2.5-glia-α1 peptide was identified, and consequently fewer incoming DQ2.5-glia-γ1 and DQ2.5-glia-γ2 epitope peptides were identified. Based on these results, HLA-DM was included in all experiments. The DQ2.5 molecule was applied to the same peptide mixture of native and deamidated DQ2.5-glia-γ1 and DQ2.5-glia-γ2 epitope peptides, and the enriched peptides were analyzed by MALDI-TOF MS. Fig. 2B shows the mass spectrum of the peptide mixture before (upper panel) and after (lower panel) enrichment with DQ2.5. Only the deamidated DQ2.5-glia-γ1 and DQ2.5-glia-γ2 epitope peptides were enriched.

A proteolytically stable α33-mer peptide, which harbors six copies of α-gliadin epitopes, is recognized by T cells isolated from the vast majority of DQ2.5-expressing CD patients (29). We thus

**FIGURE 3.** Size exclusion chromatography of PTCEC gliadin. PTCEC gliadin was fractionated by gel filtration using a Superdex peptide 10/300 GL column. Fractions marked from 1 to 18 were selected for the enrichment procedure. The peptides were measured at an absorbance of 214 nm. CV, column volume.
Table I. Identified DQ2.5-enriched peptides

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Sequence</th>
<th>Protein</th>
<th>T Cell epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1L Q L Q</td>
<td>PLPYPQPQLPYPQPQP</td>
<td>α</td>
<td>Glia-1a, 2b, 3c</td>
</tr>
<tr>
<td>2L Q L Q</td>
<td>PFPQPQLPYPQPQP</td>
<td>α</td>
<td>Glia-1a, 2b, 3c</td>
</tr>
<tr>
<td>3L Q L Q</td>
<td>PFPQPQLPYPQPQP</td>
<td>α</td>
<td>Glia-1a, 2b, 3c</td>
</tr>
<tr>
<td>4L Q L Q</td>
<td>PFPQPQLPYPQPQP</td>
<td>α</td>
<td>Glia-1a, 2b, 3c</td>
</tr>
<tr>
<td>5L Q L Q</td>
<td>PFPQPQLPYPQPQP</td>
<td>α</td>
<td>Glia-1a, 2b, 3c</td>
</tr>
<tr>
<td>6L Q L Q</td>
<td>PFPQPQLPYPQPQP</td>
<td>α</td>
<td>Glia-1a, 2b, 3c</td>
</tr>
</tbody>
</table>

Some few short peptides (5–7 mers) that derive from actin and heat shock proteins are not listed. Deamidated Q residues are given in bold type. The assignment of deamidation was done by the Mascot search engine. In some instances, deamidation was indicated for Q residues (in italics) that clearly violate the rules of TG2 substrate specificity (e.g., Q→E at another Q of the peptide). Erroneous deamidation assignment is particularly seen in Q-rich peptides that have repetitive motifs. The 9mer core region(s) of T cell epitopes, some that are overlapping with each other, are underlined.

Additionally, peptide 16 contains a DQ2.5-glutenin-glia-1–like epitope. Furthermore, we validated the method testing a chymotryptic digest of a single recombinantly expressed α2-gliadin protein (GenBank accession no. AJ133612) (30). The MALDI-TOF spectra of peptides eluted from DQ2.5 demonstrated that the α33-mer peptide was enriched also from this complex peptide mixture (Fig. 2D). Low amounts of a peptide with a mass of 2737.39 Da (VRVPVQLQPQPSPQPSQQPV) was also identified. However, this peptide contains an arginine residue giving good ionization and detection at lower amounts than other peptides. Also, note that this is not a known DQ2.5 T cell epitope. Taken together, the experiments suggest that the method can be used to identify peptides that have the highest binding affinity to DQ2.5 from a complex peptide mixture.

We next analyzed peptides enriched from a complex digest of wheat gliadin. The gliadin was digested with PTCEC to mimic the in vivo digestion process (29). To reduce the complexity of the digest, the PTCEC gliadin was fractionated by size exclusion chromatography (Fig. 3). The 18 fractions marked in the chromatogram were selected. Single gel filtration fractions were divided in four, treated with TG2, and analyzed either directly by LC-MS/MS or after enrichment by incubating with either the DQ2.5, the DQ2.2, or the DQ2.5 negative control molecule. A disulfide bond between the peptide and HLA-DQ2.5 prevents the release of the prebound peptide from the negative control DQ2.5 molecule. Enriched peptides were analyzed by LC-MS/MS analysis and subsequently identified by searching in a T. aestivum database. Results from fractions 6–10 were not included, as unspecific binding to the DQ2.5 negative control molecule was observed for these. Table I gives an overview of the identified DQ2.5 binding peptides. Altogether, 17 unique peptides were identified, and some of these were enriched from multiple fractions. Remarkably, the vast majority of these contained known gluten T cell epitopes, and often several overlapping epitopes were found within each peptide. The nine identified α-gliadin–derived peptides harbored each at least two of the DQ2.5-glutenin-α1a, DQ2.5-glutenin-α1b, or DQ2.5-glutenin-α2 epitopes. Peptides 1–4 are slightly shorter versions of the known immunogenic α33-mer peptide (30 and 32 mers) and similarly harbor six copies of the α-gliadin–derived epitopes. Five identified peptides derive from α-gliadin proteins, all of which (peptides 10–14) contain the DQ2.5-glutenin-α1 epitope (PFPQPQQPFPF) or the highly similar sequence PFPQPQQLFQ (Q→L in P7). Additionally, four of the peptides contain one or more copies of the DQ2.5-glutenin-γ4 epitope, the DQ2.5-glutenin-γ5 epitope, or a DQ2.5-glutenin-α2-like epitope (Y→F in P7). The γ-gliadin—derived peptides 15–16 both harbor sequences highly similar to the DQ2.5-glutenin-γ4 epitope (Q→L and Q→R at P5, respectively). Additionally, peptide 16 contains a DQ2.5-glutenin-α1–like epitope (F→Q in P9). The low-m.w. (LMW) glutenin-derived peptide 17 does not contain any known T cell epitopes. No peptides were enriched from the gel filtration fractions 13–18 that contain peptides of lower molecular mass.
DQ2.2 molecules as affinity matrix for gluten peptides

A higher number of individual peptides were enriched using DQ2.2 than DQ2.5 molecules. Altogether, 86 different peptides were identified from the gel filtration fractions (Supplemental Table I). Of these 86 peptides, 34 peptides harbored the 9-mer core sequence of the previously characterized DQ2.2-restricted epitope, DQ2.2-glut-L1 (PFSQQQQPV or highly similar sequences) (Table II). Peptides harboring this epitope were enriched from most of the fractions. For instance, the 38-residue-long peptide PFSQQQQPVIPQQPSFSQQQLPPFSQQQPP (DQ2.2-glut-L1 epitope underlined) was identified in fraction 1 whereas the short peptide QQP (DQ2.2-glut-L1 peptide) was identified in fractions 12–13 (Supplemental Table I). Some of the peptides contained two copies of the epitope or highly similar sequences (e.g., PQPSQPPQPFPFSQQOLP, DQ2.2-glut-L1–like epitope underlined). Three other 9-mer core peptide sequences were also frequently represented: 31 peptides contained the core sequence QQISPQPOQP, 9 peptides contained the core sequence QGSVQPPOQPQPI, and 6 peptides contained the core sequence QQISPQPOPOQP (Table II). The two former peptides are from α-gliadin whereas the latter is from LMW glutenin. The identified peptides represent good candidates for harboring yet unidentified epitopes recognized by T cells of CD patients.

Identification of novel T cell epitopes among DQ2.2-enriched gluten peptides

We wondered whether any of the other peptides enriched by DQ2.2 would represent T cell epitopes in addition to the already characterized DQ2.2-glut-L1 epitope. By testing T cells of the DQ2.2-expressing CD patient we indeed found responses, which following testing of TCC and HLA-binding analysis (see below), we concluded to be directed against two novel T cell epitopes, DQ2.2-glia-α1 and DQ2.2-glia-α2 (Table III). The two most frequently identified DQ2.2-enriched peptides (aside of the dominating DQ2.2-glut-L1 epitope–containing peptides) representing prototype core peptides 2 and 3 of Table II harbor these epitopes, that is, QGSVQPPOQL and QFPQYQQPOPPQI (DQ2.2-glia-α2). We were unable to demonstrate T cell responses to peptides representing prototype core peptides 4–6 (Table II) by testing six TCLs, although based on our testing it should not be excluded that they may contain T cell epitopes.

FIGURE 4. Proliferative response of intestinal T cells of CD patients to the DQ2.2-glut-L1, DQ2.2-glia-α1, and DQ2.2-glia-α2 epitopes. Glutensensitive T cell lines from four different patients expressing DQ2.2, but not DQ2.5 or DQ8, were stimulated with DQ2.2-expressing APCs and DQ2.2-glut-L1 peptide (Ac-QQPSQFSEQFQVLPQ), DQ2.2-glia-α1 peptide (NPQAQGSVQPPOQPQ), and DQ2.2-glia-α2 peptide (PSQPOQYQQPQIPS) treated with TG2, a TG2-treated gluten digest (gluten TG2), or medium control. Proliferation was assessed by [3H]thymidine incorporation, measured in cpm and expressed as stimulation index (cpm without Ag/cpm with Ag). The dashed line marks the stimulation index of 3, which was considered the threshold for a positive response. Testing was repeated in duplicates, and error bars represent the SEM.
DQ2.2-glia-α2 with DQ2.2:DQ2.2-glut-L1 and DQ2.2:DQ2.2-glia-α2 tetramers are shown in Fig. 6. We obtained new peripheral blood samples of two CD patients, CD627 and CD555, who previously had given biopsy material that generated reactive TCLs. We stained PBMCs from these two patients with a pool of the three DQ2.2 tetramers and sorted by flow cytometry tetramer+ effector memory CD4+ T cells that were subsequently cloned and expanded in culture. Altogether 44 TCLs were tested for recognition of the three epitopes (Table IV). The great majority (n = 38) were responsive to the DQ2.2-glut-L1 epitope. Three were responsive to the DQ2.2-glia-α2 epitope, and none was responsive to the DQ2.2-glia-α1 epitope. This indicates that there is a hierarchy of the three epitopes, with the DQ2.2-glut-L1 being the dominant epitope.

We have previously found that the DQ2.2-glut-L1 epitope binds stably to DQ2.2 as tested in a T cell assay (7). We wanted to test whether this was the case for the two novel DQ2.2 epitopes as well. Only the DQ2.2-glia-α2 epitope could be tested, as we were unable to expand sufficient numbers of TCLs required to perform the experiment for the DQ2.2-glia-α1 epitope. As demonstrated in Fig. 7, APCs pulsed with the DQ2.2-glia-α2 epitope and then washed were able to present the epitope to T cells after 96 h incubation. Of note, the DQ2.2-glia-α2–specific TCLs of the DQ2.2-expressing patient CD1005 were barely responsive to the epitope-containing peptide when presented in the context of DQ2.5 (Fig. 7).

Unique feature of gluten epitopes binding to DQ2.2

A striking characteristic of all identified DQ2.2-enriched gluten peptides (except for 1 of 86 sequences) is the presence of a serine in the suggested peptide binding core sequence. Serine at P3 was present among the peptides identified in the pre-enriched mixture. This underscores the high degree of enrichment mediated by the DQ2.2 affinity matrix. Of the six peptides enriched by DQ2.5, five were present among the peptides identified in the pre-enriched mixture.

DQ2.2-glut-L1. To investigate whether the serine residue in the P3 position of the DQ2.2-glut-L1 epitope is critical for stable binding of the DQ2.2-glut-L1 epitope (7). To investigate whether the serine residue in the peptide binding core sequence. Serine at P3 was present among the peptides identified in the pre-enriched mixture. This underscores the high degree of enrichment mediated by the DQ2.2 affinity matrix. Of the six peptides enriched by DQ2.5, five were present among the peptides identified in the pre-enriched mixture.

FIGURE 5. Intestinal TCLs from two CD patients expressing DQ2.2 recognize the novel DQ2.2-glia-α1 (filled) and DQ2.2-glia-α2 epitopes. (A) Proliferation of TCC 555. A.1.3.S.1 to titrated amounts of TG2-treated (○) and native (□) DQ2.2-glia-α1 peptide (NPQAQPQVQPQLPQ). (B) Proliferation of TCC 1005.2.2 to titrated amounts of the deamidated DQ2.2-glia-α2 peptide (QPOYSQPQPI) (▲) or the native DQ2.2-glia-α2 peptide (QPOYSQPQVIQPI) (●). Proliferation was assessed by [3H]thymidine incorporation and is expressed in cpm. Testing was done in duplicates (A) or triplicates (B), and error bars represent the SEM.
One of these peptides was a truncated version of the immunodominant α33-mer peptide, an α32-mer lacking the C-terminal F residue. This peptide was detected in several of the early size exclusion fractions (i.e., containing peptides of higher molecular mass) before and after enrichment. In fractions of lower molecular mass, peptides harboring single DQ2.5 epitopes of α-gliadin were seldom observed. Alternatively, peptides representing the DQ2.2 epitopes DQ2.2-glut-L1 and DQ2.2-glia-α1 were abundantly present. However, their 9-mer epitope cores were often not intact, suggesting that these sequences could be susceptible to proteolysis.

Discussion

In this study, we present an approach for T cell epitope identification based on enrichment of MHC peptides from complex digests of Ag using soluble recombinant MHC class II molecules as affinity matrix. We used CD to develop and benchmark the method, as CD patients recognize certain gluten epitopes in the context of disease-associated HLA-DQ molecules. The two distinct HLA-DQ molecules, DQ2.5 and DQ2.2, which both are associated with the disease, enriched different sets of peptides from complex digest of gluten proteins. Nearly all peptides enriched by DQ2.5 were previously known T cell epitopes. More than 80 different gluten peptides were enriched by DQ2.2, and most of them were variants of four sequences. Three of these sequences were shown to be epitopes for T cells of DQ2.2-expressing CD patients. Thus, the method efficiently identifies gluten T cell epitopes in CD and allows the definition of the most important DQ2.2 epitopes.

It is increasingly acknowledged that MHC association and epitope selection integrates posttranslational modifications of culprit Ags (31). The approach we present encompasses the dimension of posttranslational modification and is founded on principles of in vivo selection of epitopes recognized by T cells in CD. First, the gluten Ag was digested with enzymes to mimic gastrointestinal digestion. Only peptides with proteolytic stability are expected to survive this digestion. Second, the digest was treated with TG2, which has been demonstrated to be an important step in the generation of gluten T cell epitopes (32). Finally, the TG2-treated peptides were allowed to bind to HLA-DQ molecules in the presence of HLA-DM, which facilitates peptide exchange and loading. When HLA-DM was present in the assay, a higher amount of the tested epitope peptides was enriched with a preference for the deamidated versions of the peptides. The combined effect of these three in vitro selection steps gave enrichment of peptides that likely were those that give activation and clonal expansion of T cells in vivo in CD patients. The results with DQ2.5 demonstrate that the immunodominant gluten epitopes are highly enriched, likely due to strong binding. The method may thus be less able to detect peptides that bind with lower affinity.

A similar methodology that was used to identify T cell epitopes presented by HLA-DR1 was recently described (33). In this study a system for cell-free Ag processing was established. Single recombinant Ags were incubated with DR1, and three distinct cathepsins were added for processing. Bound peptides were eluted off DR1, and candidate peptides were identified by MALDI-TOF MS. Our study demonstrates that the approach is functional also when MHC class II molecules are incubated with a very complex digest of Ag, and our comparison of peptides enriched by two different MHC class II molecules demonstrates that the method allows dissection of biologically important differences in binding specificity.

DQ2.5 and DQ2.2 enriched distinct sets of gluten peptides. Strikingly, the two novel DQ2.2-restricted epitopes as well as the previously identified DQ2.2-restricted epitope, DQ2.2-glut-L1 (7), all carry serine residues at position P3. Additionally, all of the remaining DQ2.2-binding peptides we identified carry serine residues in their sequences, which could potentially be positioned at P3 when bound to the HLA molecule. None of the known DQ2.5 gluten epitopes carries serine at P3 (12). Serine at P3 thus seems to play a crucial role for the differential selection of gluten epitopes by DQ2.5 and DQ2.2. This selective binding likely depends on a hydrogen bonding network whose functionality is differentially influenced by tyrosine (DQ2.5) and phenylalanine (DQ2.2) at position DQ2.2-glia-α1 epitope was not dependent on TG2-mediated deamidation for T cell recognition. Such TG2 independence has rarely been seen for DQ2.5 epitopes but has been observed for DQ8 epitopes (35).

There is a huge difference in risk for CD between DQ2.5 and DQ2.2 (11). Our results support the notion that this correlates to quantitative differences in the amount of gluten peptides that can bind to DQ2.5 and DQ2.2. Gluten peptides that bind to DQ2.2 have sequences similar to DQ2.5-binding peptides, with the

Table IV. Reactivity of TCCs generated from two CD patients with HLA-DQ2.2 tetramers

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FIGURE 7. The DQ2.2-glia-α2 epitope shows sustained binding to DQ2.2. T cell proliferative responses of two TCCs stimulated with irradiated DQ2.2 (9050 MOU) or DQ2.5 (CD114) APCs loaded for 2 h with 40 μM DQ2.2-glia-α2 (QPOYSOPEQPH) or DQ2.2-glia-α2 (QPQEL-PYPOPQL) peptides, then washed and incubated for 0, 18, 48, or 96 h, followed by the addition of the TCCs. Proliferation was assessed by [3H]thymidine incorporation.
The upper detection limit of the assay is 200 inhibition of the binding of the indicator peptide EPRAPWIEQEGPEYW. IC50 is the peptide concentration required to give 50% inhibition of the binding of the indicator peptide EPRAPWIEQEGPEYW. The upper detection limit of the assay is 200 μM.

The intestinal T cell response to gliadin-α1 in adult celiac disease is focused on the immunodominant DQ2.5 epitopes (37). The results presented in the present study suggest that these epitopes will be effective in CD patients who express DQ2.5 but not DQ2.2. The identification of DQ2.2 epitopes provides the elements needed for peptide therapy in CD patients who express DQ2.2.

In summary, our results demonstrate a concordant identification of epitopes recognized by panels of T cells established from lesions of CD patients and those enriched by MHC class II molecules associated with the disease. This finding underscores that the method indeed identifies physiologically relevant T cell epitopes. The approach should be applicable to any antigenic system where there is a need for identification of MHC class II epitopes, such as autoimmune, allergic, or infectious diseases. As demonstrated, the method copes with huge peptide complexity, speaking to the fact that crude sources of proteolytically digested Ag could be applied. This will be important for applying the method in human MHC class II–associated diseases, that is, diseases where the culprit epitopes still are largely unknown.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


## Supplementary table 1: Identified DQ2.2 enriched peptides

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DQ2.2 gluten epitopes are underlined

*isobaric mass. Slight changes in sequence but same amino acid composition.

Some few short peptides (5-7mers) that derive from actin and heat shock proteins are not listed.

Deamidated Q residues are given in bold. The assignment of deamidation was done by the Mascot search engine. In some instances, deamidation was indicated for Q residues (in italics) that clearly violate the rules of TG2 substrate specificity (e.g. QXP) (Vader et al, 2002), and the true deamidation site is thus likely at another Q of the peptide. Erroneous deamidation assignment is particularly seen in Q rich peptides that have repetitive motifs.

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