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*J Immunol* 2014; 193:4497-4506; Prepublished online 26 September 2014;
doi: 10.4049/jimmunol.1301466
http://www.jimmunol.org/content/193/9/4497

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**Supplementary Material**  [http://www.jimmunol.org/content/suppl/2014/09/26/jimmunol.1301466.DCSupplemental](http://www.jimmunol.org/content/suppl/2014/09/26/jimmunol.1301466.DCSupplemental)

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

Siri Dørum,*† Michael Bodd,‡,1 Lars-Egil Fallang,*† Elin Bergseng,* Asbjørn Christophersen,* Marie K. Johannesen,* Shuo-Wang Qiao,* Jorunn Stamnaes,* Gustavo A. de Souza,*† and Ludvig M. Sollid*‡

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 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) (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...
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 (Syroco/MultysynTech, Bochum, Germany) using Fmoc/O-Butyl chemistry. The following peptides were used for this study (epitope, amino acid sequence for native/deamidated peptides, respectively): DQ2.5-glia-yl, pyroglu-PQGPPQQFFPQGFRPEQGQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPEQGQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQ
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; European Molecular Biology Laboratory).

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**FIGURE 2.** Specific enrichment of peptides harboring deamidated DQ2.5-restricted T cell epitopes. Peptide mixtures (A–C) or a TG2-treated digest of a recombinant α-gliadin protein (D) were incubated with recombinant DQ2.5 molecules. DQ2.5–peptide complexes were isolated and bound peptides were analyzed by MS. MALDI-TOF mass spectra before (upper panels) and after enrichment by DQ2.5 (lower panels) are shown (B–D). (A) Equimolar amounts of native and deamidated derivatives of the DQ2.5-glia-γ1 and DQ2.5-glia-γ2 epitope peptides were mixed in the presence or absence of HLA-DM. After enrichment with DQ2.5, the eluates were analyzed by LC-MS/MS. The ratio of the deamidated/native peptides, calculated from their observed peptide intensity in the mass spectra, is shown. (B) MALDI-TOF mass spectra of native and deamidated variants of two epitope peptides, mixed in equimolar amounts, before and after enrichment by DQ2.5 are shown. (C) MALDI-TOF mass spectra of more complex peptide mixtures containing native and deamidated variants of six epitope peptides (equimolar amounts) or a TG2-treated chymotryptic digest of a recombinant α2-gliadin protein (D) are shown before and after enrichment with DQ2.5. Open symbols represent the native peptide variants, and filled symbols represent the deamidated variants.
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 2 transitions was set as 10 ppm. For analysis of Q Exactive data, the mass error tolerance for MS 1 transitions was set as 0.01.

The IC50s were established by measuring the inhibitory effect of binding of the CD4+ isolation kit (Invitrogen). Cloning was performed by limiting dilution and was facilitated 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–Q Exactive) and a representative example is shown in Fig. 2A. 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
Table I. Identified DQ2.5-enriched peptides

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>α-Gliadin</td>
<td>LPYPQPQLPYPQPQP</td>
</tr>
<tr>
<td>2</td>
<td>α-Gliadin</td>
<td>LPYPQPQLPYPQPQP</td>
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<tr>
<td>3</td>
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<td>6</td>
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<tr>
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<td>α-Gliadin</td>
<td>LPYPQPQLPYPQPQP</td>
</tr>
<tr>
<td>16</td>
<td>α-Gliadin</td>
<td>LPYPQPQLPYPQPQP</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, peptides 15–16 both harbor sequences highly similar to the DQ2.5-glia-1b, DQ2.5-glia-2, DQ2.5-glia-5, or the highly similar sequence PFPQPQLPF (Q→L in P7). Additionally, four of the peptides contain one or more copies of the DQ2.5-glia-γ4 epitope, the DQ2.5-glia-γ5 epitope, or a DQ2.5-glia-α2-like epitope (Y→F in P7). The γ-gliadin–derived peptides 15–16 both harbor sequences highly similar to the DQ2.5-glia-γ4 epitope (Q→L and Q→R at P5, respectively). Additionally, peptide 16 contains a DQ2.5-glia-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 included this peptide in our testing and exposed DQ2.5 molecules to a peptide mixture containing equimolar amounts of the α33-mer peptide and peptides containing the epitopes DQ2.5-glia-γ1, DQ2.5-glia-γ2, DQ2.5-glia-α2a/b, DQ2.5-glia-γ3, and DQ2.2-glut-L1, all in their native and deamidated versions. Fig. 2C depicts the MALDI-TOF mass spectrum of the original peptide mixture (upper panel) versus the mass spectrum of the DQ2.5-enriched peptides (lower panel). Only the deamidated α33-mer peptide was enriched. Note that the peptides in the peptide mixture ionize with different response factors in the mass spectrometer. The DQ2.5-glia-γ1 epitope peptide contains a positively charged arginine residue, and this peptide thus suppresses the MS signal of the other peptides.

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 (VRVPVPQLQQPQPSQOPQEVPL) 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.
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 PFPFSQQQPV 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., PFPFSQPQPFSQQQPVP, DQ2.2-glut-L1-like epitopes underlined). Three other 9-mer peptide core sequences were also frequently represented: 31 peptides contained the core sequence QGSVPQOQQL, 9 peptides contained the QSQPQYSQPQQPI core sequence, and 6 peptides contained the core sequence QGISQVQPSQIQSQ (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, GGSVPQOQQL (DQ2.2-glia-α1) and QSQPQYQPSQIQSQ (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. We examined the response against the two novel epitopes by testing intestinal TCLs from a total of four different patients expressing DQ2.2, but not DQ8 or DQ2.5; CD627 (five TCLs from a total of four different biopsies), CD594 (one TCC), and CD555 (seven TCLs from four different biopsies, three shown in Fig. 4). Two TCLs recognized a DQ2.2-glia-α1 epitope–containing peptide (both from CD555; TCL 555.A.2.1 and TCL 555 A.1.3.S) whereas one TCL recognized the peptide representing the DQ2.2-glia-α2 epitope (TCL 1005.2). In comparison, the peptide representing the dominant DQ2.2-glut-L1 epitope was recognized by 7 of these 10 TCLs (Fig. 4). TCCs specific for the new epitopes and restricted by HLA-DQ2.2 were generated. Results from testing a DQ2.2-glia-α1–specific TCC (derived from TCL 555.A.1.3.S) and a DQ2.2-glia-α2–specific TCC (derived from TCL 1005.2) are shown in Fig. 5. Interestingly, recognition of the DQ2.2-glia-α1 epitope peptide was unaffected by treatment with TG2 (Fig. 5A). Furthermore, we generated HLA-DQ2.2 tetramers representing the DQ2.2-glut-L1, DQ2.2-glia-α1, and DQ2.2-glia-α2 epitopes. Staining of TCCs specific for DQ2.2-glut-L1 and

![Figure 4](http://www.jimmunol.org/)
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 TCCs 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 TCCs 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 TCCs 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 recently shown to be critical for stable binding of the DQ2.2-glut-L1 epitope (7). To investigate whether the serine residue in the DQ2.2-glia-α1 and DQ2.2-glia-α2 peptides was also located at the P3 position, we determined their binding registers by analyzing lysine-substituted analogs (15) (Fig. 8). For both sequences, amino acids in positions P4, P5, and P6 of the proposed binding core (QGSVQPQQL and QYSQPQPI, respectively) were substituted with lysine. A positively charged lysine will inhibit binding to DQ2.2 when located at positions P4 and P6, but will be tolerated at P5 as this side chain orients toward the TCR interface. As demonstrated for both DQ2.2-glia-α1 and DQ2.2-glia-α2 peptides in Fig. 8, the binding to DQ2.2 of the P4 and P6 lysine-substituted peptides were poor whereas the P5 lysine-substituted peptides bound well. The results thus establish that the serine residue is positioned at the P3 position in both epitopes.

Enrichment obtained by the HLA affinity matrix

The efficacy of enrichment by the HLA affinity matrix was evaluated by comparing the peptides identified in the gliadin digest fractions offered to the HLA-DQ molecules with the peptides enriched by the HLA-DQ molecules. This pairwise comparison was done for all fractions. The results of fraction 5 are representative and are presented in detail. This fraction contained 1092 unique peptides as identified by LC-MS/MS analysis before loading on the HLA matrix. Six peptides were identified after enrichment by DQ2.5 (Table I), and 38 peptides were identified after enrichment by DQ2.2 (Supplemental Table I). Only 15 of the DQ2.2-enriched peptides were identified in the fraction before enrichment. The remaining 23 peptides were likely scarce in the gliadin digest and were therefore only identified after enrichment. In complex mixtures of peptides the low MS signals of scarce peptides are typically suppressed by more abundant peptides. 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 TCCs from two CD patients expressing DQ2.2 recognize the novel DQ2.2-glia-α1 and DQ2.2-glia-α2 epitopes. (A) Proliferation of TCC 555. A.1,3,5,1 to titrated amounts of TG2-treated (C) and native (E) DQ2.2-glia-α1 peptide (NPQAQGSVQPQQLPQF). (B) Proliferation of TCC 1005.2.2 to titrated amounts of the deamidated DQ2.2-glia-α2 peptide (QPOYSQPQPI) (Δ) or the native DQ2.2-glia-α2 peptide (POQPOYSQPQPI) (○). 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.

FIGURE 6. Tetramer staining of TCCs. Two gluten-specific TCCs (TCC 1005.2.2, DQ2.2-glia-α2–specific; TCC 1005.2.54, DQ2.2-glut-L1–specific) were stained by the HLA tetramers DQ2.2:DQ2.2-glia-α2 (filled) and DQ2.2:DQ2.2-glut-L1 (open).
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 Qx22 (7, 34). Notably, the 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 relates 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

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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 (QPQYSPEQPH) or DQ2.2-glia-α2 (QPPEL-PYPQPL) 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.

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

<table>
<thead>
<tr>
<th>Specificity</th>
<th>CE Patient</th>
<th>CD627</th>
<th>CD555</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ2.2-glia-α1</td>
<td>23</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>DQ2.2-glia-α2</td>
<td>10</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>DQ2.5</td>
<td>36</td>
<td>9</td>
<td>36</td>
</tr>
<tr>
<td>Other specificity</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total number of TCCs</td>
<td>35</td>
<td>9</td>
<td>35</td>
</tr>
</tbody>
</table>
exception that they carry serine at P3. This gives extra constraint for binding, reducing the repertoire of peptides available for binding. Differential proteolytic stability of DQ2.5 versus DQ2.2 epitopes could also be a relevant factor. The DQ2.2 epitope peptides DQ2.2-glut-L1 and DQ2.2-glia-α1, similar to the α33-mer peptide that harbors immunodominant DQ2.5 epitopes, were present in substantial amounts in the gliadin digest. In contrast to the α33-mer peptide, the DQ2.2-glut-L1– and DQ2.2-glia-α1–related peptides were observed in many length variants and often without an intact 9-mer core. Some peptides were truncated adjacent to a serine residue, possibly as the result of elastase cleavage, as this enzyme is reported to cleave after serine, alanine, glycine, and valine residues (36). Testing of TCL reactivity and staining with tetramers indicate that there is a hierarchy among the three DQ2.2 epitopes, with DQ2.2-glut-L1 being the epitope recognized by most T cells. Both the DQ2.2-glut-L1 epitope (7) and DQ2.2-glia-α2 epitope (Fig. 7) were proven to make kinetic stability complexes with HLA. For DQ2.5-restricted gluten epitopes of CD, this feature has been demonstrated to be a common denominator and thus most likely a critical factor for establishment of the T cell response toward the epitopes (16).

The outlined approach should be applicable for identifying epitopes underlying HLA association with disease. The method is relevant not only for autoimmune disease but for other systems of MHC class II–restricted epitopes in allergic and infectious diseases as well as for designing effective vaccination, be that against pathogens or against hyper-reactants. Ag-specific immunotherapy is an attractive approach for treatment of autoimmune diseases (37). A prerequisite for this therapy is the identification of pathogenic T cell epitopes. The peptide therapy currently under development for CD is based 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

We thank Hao Yu for help with testing of TCLs, Magnus Arntzen for bioinformatic advice, and Bana Jabri for careful reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References