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Posttranslational Modification of Gluten Shapes TCR Usage in Celiac Disease

Shuo-Wang Qiao,* Melinda Ráki,†,1 Kristin S. Gunnarsen,‡,1 Geir-Åge Løset,‡ Knut E.A. Lundin,†,§ Inger Sandlie,‡ and Ludvig M. Sollid*†,‡

Posttranslational modification of Ag is a key element of the pathogenesis of celiac disease, and posttranslational modifications of Ags have been similarly implicated in several other autoimmune diseases (1–5). Celiac disease is a human inflammatory condition of the small intestine precipitated by the ingestion of cereal gluten proteins in wheat that consist of gliadin and glutenin subcomponents. The disease has a very strong HLA association, and T cells of celiac lesions recognize gluten epitopes presented by disease-associated HLA-DQ2 (DQA1*05:01/DQB1*02:01) or HLA-DQ8 (DQA1*03/DQB1*03:02) molecules (6, 7). These gluten-reactive CD4+ T cells appear to be essential for the development of the disease, and they can be readily cultured from intestinal lesions of diseased patients but not control subjects (6, 8). Over the years, a number of gluten T cell epitopes, each recognized by distinct T cell clones derived from celiac disease patients, have been identified (9–14). For most of these epitopes, a prototype DQ2-α-II–reactive TCR by analysis of TCR transfectants and soluble single-chain TCRs indicate that the deamidated residue in the DQ2-α-II peptide poses constraints on the TCR structure in which the conserved Arg residue is a critical element. The findings have implications for understanding T cell responses to posttranslationally modified Ags. The Journal of Immunology, 2011, 187: 000–000.

Posttranslational modification of Ag is implicated in several autoimmune diseases. In celiac disease, a cereal gluten-induced enteropathy with several autoimmune features, T cell recognition of the gluten Ag is heavily dependent on the posttranslational conversion of Gln to Glu residues. Evidence suggests that the enhanced recognition of deamidated gluten peptides results from improved peptide binding to the MHC and TCR interaction with the peptide–MHC complex. In this study, we report that there is a biased usage of TCR Vβ6.7 chain among TCRs reactive to the immunodominant DQ2-α-II gliadin epitope. We isolated Vβ6.7 and DQ2-αII tetramer-positive CD4+ T cells from peripheral blood of gluten-challenged celiac patients and sequenced the TCRs of a large number of single T cells. TCR sequence analysis revealed in vivo clonal expansion, convergent recombination, semipublic response, and the notable conservation of a non-germline-encoded Arg residue in the CDR3β loop. Functional testing of a prototype DQ2-α-II–reactive TCR by analysis of TCR transfectants and soluble single-chain TCRs indicate that the deamidated residue in the DQ2-α-II peptide poses constraints on the TCR structure in which the conserved Arg residue is a critical element. The findings have implications for understanding T cell responses to posttranslationally modified Ags. The Journal of Immunology, 2011, 187: 000–000.

Posttranslational modification of Ag is a biased usage of TCR Vβ6.7 chain among TCRs reactive to deamidated gliadin peptides at concentrations that are at least 100-fold less than concentrations required for similar T cell responses elicited by corresponding native peptides (9). This improved T cell recognition has been attributed to increased binding affinity of deamidated peptides to HLA-DQ2. Indeed, deamidation of the DQ2-α-II peptide introduces a negative charge in the residue residing in pocket 4 of the HLA-DQ2 peptide binding groove, a pocket where negatively charged residues are favored (16, 17). Consequently, the binding affinity of the DQ2-α-II peptide to HLA-DQ2 increases by up to 10-fold after deamidation (9). However, this is clearly less than the increase in T cell responses elicited by deamidation, suggesting that TCR recognition may also be involved in the improved recognition of the deamidated gluten peptides.

In the current study, we found a biased usage of TCR Vβ6.7 chain encoded by the TRBV7-2 gene segment among TCRs reactive to the DQ2-α-II gliadin epitope. Based on this knowledge, we performed single-cell TCR cloning of a large number of Vβ6.7-positive, DQ2-αII tetramer-positive CD4+ T cells sorted from peripheral blood of gluten-challenged celiac patients. Analysis of the TCR sequences revealed in vivo clonal expansion, convergent recombination, semipublic response, and the notable conservation of a non-germline-encoded Arg residue in the CDR3β loop. Sequencing and functional data support the notion that the deamidated residue in the DQ2-α-II peptide poses constraints on the TCR structure in which the conserved Arg residue is a critical element.

Materials and Methods

T cell culture, tetramer staining, and FACS sorting

Biological material was obtained from celiac disease patients according to protocols approved by the regional ethics committee, and subjects donating material gave written informed consent. Polyclonal T cell lines and T cell clones (TCCs) were established from intestinal biopsies of celiac disease patients as previously described (18). T cells were expanded with anti-
CD3/anti-CD28 beads (Invitrogen) for at least 7 d before mRNA isolation and TCR cloning. Tetramerized recombinant HLA-DQ2 tethered with gluten-tolerant peptides containing the T cell epitopes DQ2-α1 (QLQPFPQPELPY, underlined 9mer core sequence), DQ2-α-II (QPQELPYQPO), and the control peptide CLIP2 (MATPLMLQALPMLGAL) were generated as previously described (19, 20). Likewise, the DQ2-γ-γ tetramer was generated with the same protocol where the DQ2-γ-γ peptide QGIGHEEQ-PPG was covalently coupled to HLA-DQ2. Circulating tetramer-positive T cells in peripheral blood of celiac patients 6 d after a 3-d gluten challenge were stained according to a previously established protocol (21).

The murine T cell hybridoma line BW5147 was covalently coupled to HLA-DQ2. Circulating tetramer-positive T cells were sorted immediately after sorting by incubating the PCR plate at 42˚C for 50 min followed by a FACSAria sorter (BD) into 96-well PCR plates (Bio-Rad) with 10 µl/well polybrene and subjected to 3000 g centrifugation. All sequences were analyzed with the international ImMunoGeneTics information system (IMGT)/V-QUEST online interface between the Vβ and Jβ domains (27), resulting in the s.β109R, s.β109A, and s.β109E variants. Protein expression and purification reactions from bacteria transformed as described (26). Briefly, transformed E. coli Rosetta Blue cells were incubated from glycogen stocks into 100 ml 2xYT medium supplemented with 100 µg/ml ampicillin and 0.1 M glucose (YTAg medium) and incubated at 37˚C overnight. The cultures were reinoculated in 400 ml YTAg medium to an OD600nm of 0.025. At OD600nm of 0.6–0.8, the bacteria were pelleted and resuspended in 400 ml 2xYT medium supplemented with 0.5 mM/ml ampicillin, 0.1 M glucose, and 3% glycerol. The bacteria were pelleted and resuspended in 40 ml ice cold periplasmic extraction solution (50 mM Tris-Cl, pH 8) supplemented with 1 mg/ml lysozyme and 100 µg/ml RNaseA (Sigma-Aldrich) before incubation for 1 h at 4˚C with rotation. The mixture was then centrifuged, and the supernatant was collected as the soluble periplasmic fraction. After filtration through an 0.2µm sterile filter, the soluble periplasmic fractions were then purified by immobilized metal affinity chromatography spin columns (Qiagen) with a procedure modified from the manufacturer’s protocol such that the salt concentration was increased to 1 M NaCl and the pH was adjusted to 7. The immobilized metal affinity chromatography fractions were concentrated to 140 µl by Amicon Ultra-4 centrifugal filter devices (Millipore) and dialyzed against HBS-EP buffer. The DQ2.5 (patient CD114) homozygous EBV-transfected cells were incubated with native or deamidated gliadin 33mer peptides containing the DQ2-α-II epitope (33merQ, LQLQPFPQPELPYQPOQLPYQQLPYPQPQPF, 33merE, LQLQPFPQPELPYQPOQLPYQQLPYPQPQPF) and native or deamidated 14-mer DQ2-α-II peptides (GILQ-PQQLQPYQPPQ, GILQ-PQQLQPYQPPQ) at 37˚C 1–4 h before the addition of 25,000 TCR-transduced hybridoma cells. In some experiments, 10 µg/ml anti-HLA class II blocking Abs SPV-L3 (anti-DQ), B7/21 (anti-DR), or B8.11 (anti-DQ) was added. In anti-CD3 stimulation assays, wells were coated overnight with 0.2 to 5 µg/ml hamster anti-mouse CD3ε (Ab clone 145.2C11, a gift from B. Bogen, Oslo, Norway) and washed before the addition of 10,000 TCR-transduced hybridoma cells. In Troybody experiments, recombinant anti-HLA-DR Abs in which 33merQ or 33merE peptides were inserted into loops between β-strands in constant domains (25) were used as Ads, and 10,000 DQ2* monocyte-derived dendritic cells were used as APCs. The dendritic cells were cultured from positively selected monocytes (CD14 microbeads; Milenium Biotec) in the presence of 1000 U/ml GM-CSF and 500 U/ml IL-4 (both from R&D Systems) for 6 d. In some experiments, biotinylated recombinant soluble peptide–DQ2 complexes were immobilized onto streptavidin plates (Roche) and used to stimulate TCR-transduced hybridoma cells. The soluble DQ2 molecules contained either the native (QPQELPYQPOP) or the deamidated (QPQELPYQPOP) DQ2-α-II peptide covalently linked to the N terminus of the DQ2 β-chain spaced by a 6-aa (GGSGSGS) linker. Culture supernatant was assayed for murine IL-2 secretion in ELISA 18 h later.

In murine IL-2 ELISAs, wells were coated with 2 µl per well of mouse IL-2 (clone JES6-5H4; Pharmingen) and detected with 1 µg/ml biotin rat anti-mouse IL-2 (clone JES6-1A12; Pharmingen). The sensitivity of the assay was at least 4 pg/ml.

Expression and purification of soluble single-chain TCR 364.14 variants

The TCR Vα and Vβ amino acid sequences from TCC364.1.0.14 were fused by a synthetic linker to make single-chain TCR (scTCR). Codon-optimized synthetic DNA encoding wild-type or mutant scTCR sequences (GenScript) were cloned into the pEPCF vector (26) between the NcoI and NotI sites and transformed into Escherichia coli Rosetta Blue cells (Novagen). A LbH103F (amino acid number assigned according to IMGT V-gene alignment) mutation was introduced to stabilize the hydrophobic interface between the Vα and Vβ domains (27), resulting in the s.β109R, s.β109A, and s.β109E variants. Protein expression and purification reactions from bacteria transformed as described (26). Briefly, transformed E. coli Rosetta Blue cells were incubated from glycogen stocks into 100 ml 2xYT medium supplemented with 100 µg/ml ampicillin and 0.1 M glucose (YTAg medium) and incubated at 37˚C overnight. The cultures were reincubated in 400 ml YTAg medium to an OD600nm of 0.025. At OD600nm of 0.6–0.8, the bacteria were pelleted and resuspended in 400 ml 2xYT medium supplemented with 0.5 mM/ml ampicillin, 0.1 M glucose, and 3% glycerol. The bacteria were pelleted and resuspended in 40 ml ice cold periplasmic extraction solution (50 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8) supplemented with 1 mg/ml lysozyme and 100 µg/ml RNaseA (Sigma-Aldrich) before incubation for 1 h at 4˚C with rotation. The mixture was then centrifuged, and the supernatant was collected as the soluble periplasmic fraction. After filtration through an 0.2µm sterile filter, the soluble periplasmic fractions were then purified by immobilized metal affinity chromatography spin columns (Qiagen) with a procedure modified from the manufacturer’s protocol such that the salt concentration was increased to 1 M NaCl and the pH was adjusted to 7. The immobilized metal affinity chromatography fractions were concentrated to 140 µl by Amicon Ultra-4 centrifugal filter devices (Millipore) and dialyzed against HBS-EP buffer (GE Healthcare) supplemented with 0.5 M NaCl. Protein concentrations were determined by absorbance at 280 nm on a NanoDrop ND-1000 apparatus (Thermo Scientific) and found to be 12.6 mg/ml for wt.β109R, 16.5 mg/ml for s.β109R, 27 mg/ml for s.β109A, 5.7 mg/ml for s.β109K, and 4.5 mg/ml for s.β109E. Size-exclusion chromatography on a Superdex 200 10/300 GL column was performed on an automated AKTA 900 chromatography system (GE Healthcare). The monomeric fractions were pooled, concentrated to equal volumes, and protein concentrations were determined as described earlier.

Surface plasmon resonance binding assays

A Biacore T100 instrument was used together with a CM3 chip (GE Healthcare). All experiments were performed in HBS-EP buffer. Neu-
Results

Overusage of Vβ6.7 (TRBV7-2) in DQ2-α-II–reactive T cells

Systematic TCR sequencing of 40 in vitro-generated gluten-reactive TCCs derived from 14 celiac disease patients produced 29 unique TRBV sequences encoded by 14 different V-gene segments. Notably, one of the V-gene segments, TRBV7-2, was used in 10 of the 29 unique TRBV sequences. Closer examination revealed that 9 of the 10 TCCs that used this particular V-gene segment were reactive to either the DQ2-α-I or DQ2-α-II–reactive T cells (Table I). No other V-gene segment was overrepresented.

To probe how frequently Vβ6.7 was used by Ag-specific T cells in vitro-expanded polyclonal lines, T cell lines cultured from celiac lesions were co-cultured with various DQ2-tetramers and a Vβ6.7 (28) specific mAb, OT145. Although this Ab was previously reported to specifically bind Vβ-chain encoded by the TRBV7-2*01 allele only (29), we found that CD4+ T cells from both DQ2-α-I and DQ2-α-II–reactive T cells were Vβ6.7 positive (Fig. 1). We observed overusage of Vβ6.7 among DQ2-αII tetramer-positive T cells in several polyclonal lines (Supplemental Fig. 2), whereas few or none of the DQ2-αI or DQ2-αII tetramer-positive T cells used Vβ6.7.

Extended in vitro culture may introduce bias. Therefore, we looked at the Vβ6.7 usage in peripheral blood T cells of nine celiac patients where the T cells were directly stained with tetramers. Oral gluten challenge for 3 d of celiac disease patients in remission leads to reexpression of gluten-reactive T cells into the peripheral blood on day 6, and such cells can be detected by IFN-γ ELISPOT assay (30) or by HLA-DQ2 tetramer staining (21). We found that significantly more DQ2-αI tetramer-positive T cells were Vβ6.7 positive compared with all CD4+ T cells (p < 0.01, paired Wilcoxon rank test), whereas a slight increase for DQ2-αI tetramer-positive cells did not reach statistical significance (p = 0.10) (Fig. 1).

In vivo clonal expansion of DQ2-αI and DQ2-αII tetramer-positive cells

The knowledge of overusage of the TRBV7-2 gene segment among DQ2-αI– or DQ2-αII–reactive T cells allowed us to sequence TRBV gene segments from sorted single cells that were double positive for Vβ6.7 and either tetramer. We successfully sequenced TRBV genes of 23–108 sorted single cells from peripheral blood of each of the four gluten-challenged celiac disease patients (Fig. 2). All cells were Vβ6.7-positive and either DQ2-αI tetramer-positive, DQ2-αII tetramer-positive, or DQ2-αI–tetramer-negative. Within both DQ2-αI and DQ2-αII tetramer-positive cells, we found clonal dominance as a result of in vivo clonal expansion. This was particularly evident for the DQ2-αI tetramer-positive cells, in which 32 of 33 (patient CD757) or 36 of 37 (patient CD823) sequences obtained represented one clone in each of the two patients (Fig. 2). In comparison, the TCR repertoire in Vβ6.7-positive, DQ2-αII–tetramer-positive T cells was more diverse. Between 5 and 19 unique TRBV sequences were retrieved from each patient of which more than half of the cells expressed the two most frequently used sequences (Fig. 3), indicating a considerable clonal expansion in the DQ2-αII tetramer-positive cells as well. In contrast, we found no identical sequences among tetramer-negative cells.

Public response as a result of convergent recombination in Vβ6.7 and DQ2-αII tetramer double-positive cells

In the TRBV7-2 sequences we obtained from both ex vivo DQ2-αI and DQ2-αII tetramer-positive single-cell sequencing and in vitro-cultured...
DQ2-α-II–reactive TCCs, we observed several examples of public response in which TCR CDR3\(^b\) sequences from different patients displayed identical amino acid sequences (Fig. 4A–E).

The fact that several CDR3\(^b\) sequences obtained from single-cell sequencing were identical to amino acid sequences from well-characterized in vitro-generated DQ2-α-II–specific TCCs from duodenum tissue (Fig. 4A, 4C, 4E) strengthened the assumption that the cells sorted based on DQ2-α-II tetramer binding were indeed Ag specific. On the nucleotide level, identical amino acid sequences were found to be encoded by different recombination events, observed both between individuals and within the same individual (CD823) (Fig. 4B, 4F), implying that the public response observed was a result of convergent recombination supporting the notion of an Ag-driven selection process.

**Conserved Arg in V\(^b\)6.7–CDR3\(^b\) from DQ2-α-II tetramer-positive cells as a result of selection**

The high throughput of ex vivo single-cell sequencing generated a large body of sequence data that revealed interesting molecular features of the CDR3\(^b\) loops in V\(^b\)6.7–positive DQ2-α-II tetramer-binding T cells. The vast majority (78%) of the CDR3\(^b\) loops were of the same length of 11 aa, a fact that would have led to the assumption of oligoclonality if the cells had been subjected to CDR3 length analysis only. However, single-cell TCR sequencing revealed a number of different clones on the nucleotide level underscoring the importance of TCR sequencing.

The most striking finding from the single-cell TCR data was the conservation of an Arg residue in position 5 of the CDR3\(^b\) loop from V\(^b\)6.7–positive DQ2-α-II tetramer-binding T cells. Of the total 143 DQ2-α-II cells from which we obtained TRBV7-2 sequences by single-cell sequencing, 132 (92%) expressed CDR3\(^b\) loops with an Arg in position 5. On the clonal level, 35 of 41 (85%) unique TRBV7-2 sequences from DQ2-α-II tetramer-positive cells contained Arg in this position. In comparison, only 1 of 70 (1%) DQ2-α-I positive and 4 of 49 (8%) DQ2-α-II tetramer-negative cells showed this Arg signature (Fig. 2).

Examination of the nucleotide sequences revealed that 73% (30 of 41) of the Arg residues from DQ2-α-II cells were non-germline encoded (Supplemental Fig. 3). The fact that most of the Arg residues in our data set were non-germline encoded indicated that

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**FIGURE 2.** TRBV7-2 sequences in DQ2-α-II–reactive cells display a conserved Arg in positive 5 of the CDR3\(^b\) loop. The CDR3\(^b\) amino acid sequences of all sorted V\(^b\)6.7–positive peripheral blood single cells from four different patients (CD757, CD761, CD767, and CD823) and in vitro-cultured DQ2-α-I– and DQ2-α-II–reactive TCCs that use TRBV7-2 are summarized. Numbers to the right of the CDR3\(^b\) sequences denote the number of cells expressing the sequence in single-cell PCR data or the patient ID for in vitro-cultured TCCs. All patients are TRBV7-2*01/*01 homozygous except CD757 (*) who is TRBV7-2*02/*02 homozygous and CD761 (**) who is TRBV7-2*01/*02 heterozygous. TRBV7-2*01 sequences appear in black, and TRBV7-2*02 sequences appear in blue. The conserved Arg in position 5 appear in red. Two CDR3\(^b\) amino acid sequences from DQ2-α-II–positive cells from CD823 were encoded by two different nucleotide sequences (superscripts a and b).
The conserved Arg residue in position 5 of CDR3β is crucial for DQ2-α-II epitope recognition

To probe the functional importance of Arg in position 5 of CDR3β, we made transfectants with the TCR of the TCC TCC364.1.0.14 (i.e., TCR 364.14). The wild-type 364.14 TCR was retrovirally transduced into a murine hybridoma cell line, BW58, and mutant 364.14 TCRs were retrovirally transduced into the cognate Ag, the DQ2-α-II epitope (Fig. 4). The wild-type transfectant responded to Ag in the same way as the corresponding TCC from which the TCR sequence originated. This included DQ2 restriction (Fig. 5C), deamidation dependence (Fig. 5D), epitope specificity, as well as ability to recognize the DQ2-α-II peptide presented by the closely related DQ2.2 molecule.

The importance of the Arg residue in DQ2-α-II recognition was independently confirmed in a set of experiments using soluble recombinant TCRs. The variable domains of 364.14 TCR were expressed recombinantly as single-chain constructs either with the wild-type sequence (wt.β109R), a stabilized version of the wild type (s.β109R), or with mutants where the Arg residue was mutated to Ala (s.β109A), Lys (s.β109K), or Glu (s.β109E) residues. Initial surface plasmon resonance (SPR) binding experiments demonstrated poor functional yields of wt.β109R, and therefore, point mutations were introduced at the V domain interface to increase intrinsic molecular stability as described (27). The mutants s.β109K and s.β109E could not be produced in functional yields that allowed SPR measurements. A dramatic reduction in binding to DQ2-α-II was observed in the s.β109A mutant compared with s.β109R, which expressed the wild-type DQ3Rβ sequence (Fig. 6A, 6B). Neither s.β109R nor s.β109A bound the DQ2-CLIP2 molecule that displayed an irrelevant peptide, demonstrating the specificity of binding to DQ2-α-II (Fig. 6C, 6D).

Combined, consistent functional data from 364.14 TCR transfectants and binding data from soluble 364.14 TCRs showed that this trait had been subjected to selection, presumably driven by the cognate Ag, the DQ2-α-II peptide.
the conserved Arg in position 5 of the CDR3β loop was crucial for TCR recognition of the cognate peptide–MHC ligand DQ2:DQ2-α-II peptide complex. Mutation of this Arg residue to the small amino acid residue Ala, to a positively charged Lys residue, or to a negatively charged Glu residue had detrimental effects on 364.14 TCR recognition of the cognate peptide–MHC ligand DQ2-α-II complex was interacting with the Arg residue and thus participated in the selection of Vβ6.7 chains containing the signature Arg in the CDR3β loop.

Although peptide binding to DQ2 increased after deamidation, it alone could not account for the dramatic improvement in T cell recognition. In standard T cell assays as that shown in Fig. 5D, the peptide Ag was present during the entire assay time such that in theory, an apparent 10-fold lower binding affinity to HLA-DQ2 of the native gliadin α-II peptide (9) could be compensated by a 10-fold higher peptide concentration in T cell assay. However, as shown in Fig. 5D, a 2-log higher concentration of the native peptide was necessary to elicit T cell responses comparable to that observed with the deamidated peptide. This difference in recognition was confirmed when the native and deamidated gliadin epitopes were offered as part of Ab molecules (Troybodies) (25) that targeted cell surface receptors of the APC (Fig. 5E). The efficient presentation of the Troybody reagents allowed more accurate assessment of Ag recognition, and importantly the conversion of Gln to Glu that to some extent occurred in the workup of synthetic peptides could be excluded as a confounding factor. Finally, to remove HLA-DQ2 binding affinity differences in the assay, we engineered soluble recombinant HLA-DQ2 molecules in which the native and the deamidated DQ2-α-II epitope peptides were tethered covalently to the peptide binding groove via a 6-aa peptide linker. Biotinylated DQ2-αII.Q molecules, either containing a peptide with a native sequence (PQPQLPYPQPE) or equivalent amounts of biotinylated DQ2-αII.E molecules containing the deamidated epitope (PQPELPYPQPE), were immobilized on streptavidin plates and presented to hybridoma cells expressing the DQ2-α-II–reactive TCR. In this assay, where T cell recognition of titrated amounts of stable DQ2-αII complexes were tested, a near 2-log difference in T cell recognition elicited by the deamidated and native Ag was observed (Fig. 5F). This showed

TABLE II. Recognition of variants of the DQ2-α-II epitope by TCR transfectants expressing wild-type or mutant 364.14 TCR

<table>
<thead>
<tr>
<th>Peptide Ag</th>
<th>TCR Transfectant</th>
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<tr>
<td></td>
<td>WT β109A β109K β109E</td>
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<tr>
<td>P Q P E L P Y Q P E</td>
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<td>- - - D - L P Y</td>
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<td>- - - K - - -</td>
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| ++, strong T cell response; (+), weak T cell response at the highest Ag concentration only; -, no T cell response; and WT, wild-type.
clearly that the DQ2-α-II-reactive TCR was able to differentiate between the native Gln residue and the deamidated Glu residue despite that these residues were positioned in the P4 pocket and should not be solvent exposed.

Given the conspicuous presence of a positively charged Arg residue at the tip of the CDR3β loop, one can envision that this may be the TCR residue that “reads” the Glu residue of the DQ2-α-II complex through a charge-to-charge interaction. This notion was supported by a vigorous proliferative response elicited by the DQ2-α-IIIE4D peptide where the Glu residue in P4 was replaced by another negatively charged residue, Asp (Table II). To note, we never observed Lys, another positively charged residue, in position 5 of the other negatively charged residue, Asp (Table II). To note, we never observed Lys, another positively charged residue, in position 5 of the other negatively charged residue, Asp (Table II).

Discussion

Gluten-reactive CD4+ T cells from celiac disease patients represent a unique source of disease-relevant human T cells. The TCR repertoire of these cells is the result of natural Ag-driven selection and expansion in a human HLA-associated inflammatory disease. By use of highly specific MHC tetramers and sequencing of TCRs from a large number of single T cells, either derived from in vitro-generated TCCs or directly isolated from peripheral blood of celiac disease patients, we provide a comprehensive characterization of the TCR repertoire of T cells specific for immunodominant gluten epitopes. Our results provide novel insights into how CD4+ T cells that are centrally involved in the development of celiac disease recognize posttranslationally modified gluten Ags and reveal that a non-germline-encoded part of TCR is central in recognition of a posttranslationally modified residue.

Analysis of TRBV7-2 sequences obtained from single-cell sequencing of DQ2-αII tetramer-positive T cells revealed in vivo clonal expansion, convergent recombination, semipublic response, and the notable conservation of an Arg residue at position 5 of CDR3β. Importantly, the observations were made from direct sequencing of Ag-specific cells that have not been subjected to culture and thus any bias introduced by in vitro expansion. Moreover, similar results were found in all four celiac disease patients studied, and identical Vβ-chain amino acid sequences were found in direct sequencing of tetramer-sorted peripheral blood T cells and TCR sequencing in vitro-generated TCCs derived from intestinal biopsies of a different set of celiac disease patients.

The fact that most of the Arg residues in our data set are non-germline encoded indicates that this trait has been subjected to selection, presumably driven by the cognate Ag, the DQ2-α-II epitope. This epitope has undergone a posttranslational modification (i.e., a deamidation from Gln to Glu). Our data suggest that the in vivo-selected TCRs specifically sense this posttranslational modification at the P4 position of the epitope. The amino acid residue positioned in the P4 pocket should not be much solvent exposed so it is not obvious how this sensing takes place. The exact determination will require a crystal structure, but obtaining this is unfortunately not trivial. We, as well as others (31), have previously tried to solve the crystal structure of the DQ2:DQ2-α-II complex without success. Our functional data demonstrate that the Glu residue of the DQ2-α-II peptide can be replaced by Asp suggesting that the negative charge is important for TCR recognition. Further, our mutational analysis of a prototype TCR indicates that the Arg residue in the CDR3β loop is critical for the TCR recognition. It is hence conceivable that there is direct interaction between the negatively charged P4 Glu residue of the peptide and the positively charged Arg residue of the CDR3β loop in the TCRs.

In our data set, we observed semipublic TCR response (i.e., similar Vβ sequences were found to be dominant in different
individuals). Altogether, the CDR3β sequence ASS(I/L)R(S/H/Y/ A)TDQY was observed in roughly two thirds of all sorted VB6.7 and DQ2-a11 double-positive cells from four different patients. Public TCR response arises from either highly recurrent TCRs that are exported from the thymus to the periphery in relatively high frequency or as a result of high-avidity interaction with the peptide–MHC complex followed by in vivo expansion. Our data cannot differentiate the relative contribution of these two not mutually exclusive mechanisms. Affinity studies of a larger panel of prototypical versus non-prototypical DQ2-α-Ⅱ-reactive TCRs or analysis of the TCR repertoire of naïve DQ2-a1-Ⅱ-reactive T cells will give answers to this question.

In several human autoimmune diseases, increasing evidence suggests that T cells bypass tolerance by recognizing autoantigens that have been posttranslationally modified. This is observed in anticitrulline response (2) or glycosylation-dependent T cell response (3) in rheumatoid arthritis, disulphide bond formation in proinsulin in type 1 diabetes (5), and anticitrulline response in multiple sclerosis (3). In this article, we show that a posttranslationally modified residue in the antigenic peptide, the negatively charged Glu residue in P4, interacts with the TCR and consequently selects TCRs with a conserved Arg residue in a non-gemine-encoded part of the CDR3β loop. This represents an interesting example of in vivo TCR selection exerted by a posttranslationally modified antigenic peptide in a human disease. Future studies will reveal if posttranslationally modified Ags of other autoimmune diseases impose similar selection of particular TCR repertoires.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


The monoclonal antibody OT145 binds Vβ chains encoded by both TRBV7-2*01 and TRBV7-2*02 alleles. Healthy volunteers were genotyped for TRBV7-2*01 / *02 alleles using a custom Taqman SNP genotyping kit targeting the g251→a (*01 vs. *02) polymorphic site assayed on a StepOnePlus qPCR machine (ABI). Genomic DNA from non-T-cell sources was isolated from PBMC, frozen cryo-sections or EBV transformed B cell lines with DNeasy blood and tissue kit (QiaGen). Peripheral blood cells were subsequently stained with FITC conjugated OT145 and APC conjugated anti-human CD4 (Diatec). There is a clear CD4+ T cell population that stain OT145 in all three *02/*02 homozygous individuals tested.
Supplementary Figure 2

Over-usage of Vβ6.7 in DQ2-αII tetramer positive cells in in vitro expanded T cell lines.

Polyclonal T cell lines TCL496.1.2 (A), TCL.KT.CD.E3 (B&D) and TCL465.1.4 (C) were stained with Vβ6.7 and DQ2-γII (A), DQ2-αI (B) or DQ2-αII (C&D) tetramer. The percentage of Vβ6.7 positive cells among tetramer-positive cells is shown.
**Supplementary Figure 3**

Complete amino acid and nucleotide sequence alignment of all DQ2-α-II reactive TRBV7-2-CDR3β loops that contain conserved Arg in position 5. All sequences derived from blood were obtained with TRBV7-2 specific single cell PCR of Vβ6.7 and DQ2-all tetramer double positive cells, and all sequences derived from duodenum were sequenced from in vitro generated DQ2-α-II reactive T cell clones. The conserved Arg residue and the corresponding encoding nucleotides are in red, TRBV7-2*01 sequences are in black and TRBV7-2*02 sequences are in blue. Non-germline encoded amino acid residues are underlined.
Supplementary Figure 4

WT and mutant 364.14 TCR transfected hybridoma cells express similar levels of CD3ε and Vβ6.7. (A) BW58α-β-hCD4 cells not transfected with TCR (black) and cells retrovirally transduced with WT 364.14 TCR (red), β109A (green), β109K (blue) or β109E (yellow) mutant TCRs were stained with PE conjugated hamster-anti-mouse-CD3ε. (B) BW58α-β-hCD4 cells transfected with human non-TRBV7-2 TCR (TCR489: TRBV9, black line) and cells retrovirally transduced with WT 364.14 TCR, β109A, β109K or β109E mutant TCRs were stained with Vβ6.7 antibody (clone OT145) followed by secondary PE conjugated goat-anti-mouse-IgG.