Posttranslational Modification of Gluten Shapes TCR Usage in Celiac Disease

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

J Immunol 2011; 187:3064-3071; Prepublished online 17 August 2011;
doi: 10.4049/jimmunol.1101526
http://www.jimmunol.org/content/187/6/3064

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/08/18/jimmunol.1101526.DC1

References
This article cites 31 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/187/6/3064.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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, 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-α-II 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, semi-public 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 indicates 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: 3064–3071.
CD3/anti-CD28 beads (Invitrogen) for at least 7 d before mRNA isolation and TCR cloning.

Tetramerized recombinant HLA-DQ2 tethered with gluten peptides containing the T cell epitopes DQ2-α-1 (QLQPFPQPELPY, underlined 9mer core sequence), DQ2-α-2 (QPQELPFPQPQ), and the control peptide CLIP2 (MATMLPQALPMGAL) were generated as previously described (19, 20). Likewise, the DQ2-γ-2 tetramer was generated with the same protocol where the DQ2-γ-2 peptide QGHPQEP- PQLQ 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).

Single, Vβ6.7-positive (FTTC-OT145 from Endogen), DQ2-αⅱ tetramer-positive cells were sorted by a FACSaria sorter (BD) into 96-well PCR plates (Bio-Rad) with 10 μl ice-cold lysis reverse-transcription buffer containing 50 mM Tris, pH 8.2, 75 mM KCl, 0.5% Triton X-100, 2 mM deoxynucleobromide triphosphate 10 mM DTT, 800 nM TRBC_rev primer 5'-TTACCCACAGCTGATCGC-3', 800 nM TRAC_rev primer 5'-AGTCAGATTTGTTGCTCCAGGCC-3', 12 U RNasin (Promega), and 30 U SuperScript II reverse transcriptase (Invitrogen) in each well.

TCR cloning and sequencing

The TCR was cloned using a seminested PCR approach performed with Phusion DNA polymerase (Finzymes). mRNA of in vitro-cultured T cells was obtained using the NeuNex Mini kit (Qiagen) and TRA and TRB gene-specific first-strand cDNA was synthesized from mRNA with TRAC_rev and TRBC_rev primers and SuperScript II reverse transcriptase. After RNase H digestion (Rnasin Plus; Promega), the cDNA was precipitated with Pellet Paint (Novagen), and a poly-C-tail was added to the 3'-end with rTerminal transferase (Roche). The cDNA was reprecipitated, and the TRA and TRB genes were PCR amplified with forward poly-O-NotI primer (5'-ATA-GTCAGATTTGTTGCTCCAGGCC-3') and reverse TRAC_3rd_rev (5'-ATACCGGTGTCTCTGCTGCTTCTACACGGC-3') or TRBC_MluI_rev (5'-ATACCGGTGTAGATCTGCTTCTGTATGGC-3') primers for 35 cycles (98˚C for 10 s, 53˚C for 30 s, 72˚C for 15 s) per cycle. The PCR product was ligated into the pHG021-pHOx cloning vector and subjected to sequencing.

From sorted single cells, TCR-specific first-strand cDNA was synthesized from mRNA by insulating PCR product at 98˚C for 10 min followed by 10 min at 72˚C. Subsequently, 2 μl cDNA was used in the first PCR with 500 nM TRBV7-2 fwdA (5'-ATATGGCCGCGCCGCGCGGGGCGGGG-3') and reverse TRAC_MluI_rev (5'-ATACGCGGTGATATCTGCTTCTGTATGGC-3') primers in total 15 μl final volume. In the second PCR, 1 μl of the first PCR product was amplified with 500 nM TRBV7-2 fwdB (5'-ATATGGCCGCGCCGCGCGGGGCGGGG-3') and TRBC_MluI_rev primer in total 20 μl final volume. Both PCRs were performed for 8 cycles (98˚C for 20 s, 60˚C for 40 s, 72˚C for 40 s) followed by 32 (first PCR) or 22 (second PCR) cycles of 98˚C for 20 s, 55˚C for 40 s, 72˚C for 40 s, and a final elongation at 72˚C for 3 min. Six microlitres of the second PCR product was loaded onto a 1% agarose gel, and wells with expected bands at around 350 bp were subjected to sequencing. All sequences were analyzed with the international ImMunoGeneTics information system (IMGT/ V-QUEST online resource).

Retroviral transcription and hybridoma functional assays

The murine T cell hybridoma line BW5147 was a kind gift from Dr. Malissen (Centre d’Immunologie de Marseille-Luminy, Marseille, France). The cell line was transfected with human CD4 cDNA in the pORF9-hCD4 plasmid (Invitrogen), cloned, sublicloned, and a stable human CD4-expressing clone was selected for further proviral TCR transduction.

The TCR Vα and Vβ sequences of an in vitro-cultured DQ2-αⅱ-reactive CD4⁺ TCC (TC336.1.0.14) was fused with murine TCR constant chains, and the chimeric TCR α and β chains were linked with a P2A peptide. Codon-optimized synthetic DNA encoding wild-type or mutant TCRα-P2A-TCRβ sequences (GenScript) were cloned into the pMIG-II retroviral plasmid (a generous gift from Dr. Vignali, Denver, CO) (24) by using EcoRI and NotI sites, transformed into SPhi298, and a non-TRBV7-2 TCR derived from an HLA-DQ8–restricted TCC generated in our laboratory was a gift from Dr. Edwin Bogen, Oslo, Norway) and washed before the addition of 10,000 TCR-transduced hybridoma cells. In some experiments, 10 μg/ml anti–HLA class II blocking Abs SPV-L3 (anti-DQ), B7/21 (anti-DP), or B8.11 (anti-DR) was added. In anti-CD3 stimulation assays, wells were coated overnight with 0.2 to 5 μg/ml hamster anti-mouse CD3e Ab (clone 145.2C11, a gift from B. Bogen, Oslo, Norway) and washed before the addition of 25,000 TCR-transduced hybridoma cells. In some experiments, 10 μg/ml anti–HLA class II blocking Abs were used throughout the experiments.

In Ag stimulation assays, 50,000 DQ2.2 (H69/0950) or DQ2.5 (patient CD114) homozygous EBV-transformed cells were incubated with native or deaminated glutamin 33mer peptides containing the DQ2-α-ⅱ epitope (33merQ, LQLQPFPQQLPFPQLPFPQLPFPQLPFPQPF; 33merE, LQLQPFPQQLPFPQLPFPQLPFPQLPFPQPF) and native or deaminated 14-mer DQ2-α-ⅱ peptides (sH6-QQQPQPCQPCQPQ; sH6-EQPQPCQPCQPCQPQ) at 37˚C for 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 in which 33merQ or 33merE peptides were inserted into loops between β-strands in constant domains (25) were used as Abs, and 10,000 DQ2⁺ monocyte-derived dendritic cells were used as APCs. The dendritic cells were cultured from positively selected monocytes (CD14 microbeads; Milenium Biotech) 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 (PQPQLPYPQPYQ) or the deamidated (QPQPELPQPYQ) 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 some experiments, recombinant anti–HLA-DP Abs in which 33merQ or 33merE peptides were inserted into loops between β-strands in constant domains (25) were used as Abs, and 10,000 DQ2⁺ monocyte-derived dendritic cells were used as APCs. The dendritic cells were cultured from positively selected monocytes (CD14 microbeads; Milenium Biotech) 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 (PQPQLPYPQPYQ) or the deamidated (QPQPELPQPYQ) 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 Ag stimulation assays, 50,000 DQ2.2 (H69/0950) or DQ2.5 (patient CD114) homozygous EBV-transformed cells were incubated with native or deaminated glutamin 33mer peptides containing the DQ2-α-ⅱ epitope (33merQ, LQLQPFPQQLPFPQLPFPQLPFPQPF; 33merE, LQLQPFPQQLPFPQLPFPQLPFPQPF) and native or deaminated 14-mer DQ2-α-ⅱ peptides (sH6-QQQPQPCQPCQPQ; sH6-EQPQPCQPCQPCQPQ) at 37˚C for 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 were used throughout the experiments.

In Ag stimulation assays, 50,000 DQ2.2 (H69/0950) or DQ2.5 (patient CD114) homozygous EBV-transformed cells were incubated with native or deaminated glutamin 33mer peptides containing the DQ2-α-ⅱ epitope (33merQ, LQLQPFPQQLPFPQLPFPQLPFPQPF; 33merE, LQLQPFPQQLPFPQLPFPQLPFPQPF) and native or deaminated 14-mer DQ2-α-ⅱ peptides (sH6-QQQPQPCQPCQPQ; sH6-EQPQPCQPCQPCQPQ) at 37˚C for 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 were used throughout the experiments.
travidin was immobilized by amine coupling to ~4500 resonance units (RU) followed by capture of ~3500 RU biotinylated DQ2-αII or DQ2-CLIP2. Samples of s.β109R and s.β109A at concentrations from 0.32 to 5 μM were then injected over each surface, as well as a negative reference cell with Neutravidin only, at a flow rate of 30 μl/min at 25°C. In all experiments, data were zero-adjusted and the Neutravidin reference cell value subtracted.

Results

Overusage of Vβ6.7 (TRBV7-2) in DQ2-αII–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, as well as a negative reference cell with Neutravidin only, at a flow rate of 30 μl/min at 25°C. In all experiments, data were zero-adjusted and the Neutravidin reference cell value subtracted.

To probe how frequently Vβ6.7 was used by Ag-specific T cells in vitro, we performed high-throughput sequencing in vitro expanded polyclonal lines, T cell lines cultured from celiac lesions were co-stained with various DQ2-tetramers and in vitro-expanded polyclonal lines, T cell lines cultured from celiac lesions were costained with various DQ2-tetramers and influenza antigens. The percentage of cells reactive to either the DQ2-αII tetramer was 31% (patient CD757) or 36% of all CD4+ T cells (patient CD823) sequences obtained for each of the two patients (Fig. 2). In comparison, the TCR repertoire in the TRBV7-2 sequences we obtained from both ex vivo DQ2-aI and DQ2-al tetramer-positive single-cell sequencing and in vitro-cultured

FIGURE 1. Overusage of TRBV7-2 encoding the Vβ6.7 chain in DQ2-aII tetramer-positive cells analyzed directly ex vivo. Peripheral blood cells from treated celiac disease patients after gluten challenge were stained with Vβ6.7 Ab and DQ2 tetramers containing an irrelevant control peptide (CLIP2) or the DQ2-αI or DQ2-αII gliadin epitopes. A, CD4-gated T cells are shown. In a representative patient, ~5% of tetramer-negative cells were Vβ6.7-positive compared with 16% among DQ2-αII-positive and 50% among DQ2-αI-positive cells. B, The percentage of cells staining positive for Vβ6.7 of all CD4+ T cells and of DQ2-αI or DQ2-αII tetramer-positive cells in nine celiac patients is shown. The p values were calculated with Wilcoxon rank test.
DQ2-α-II–reactive TCCs, we observed several examples of public response in which TCR CDR3\( \beta \) sequences from different patients displayed identical amino acid sequences (Fig. 4 A–E).

The fact that several CDR3\( \beta \) 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. 4 A,4 C,4 E) 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. 4 B,4 F), 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\( \beta \)6.7-CDR3\( \beta \) 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\( \beta \) loops in V\( \beta \)6.7-positive DQ2-α-II tetramer-binding T cells. The vast majority (78%) of the CDR3\( \beta \) 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\( \beta \) loop from V\( \beta \)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\( \beta \) 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).

TRBV7-2 sequences in DQ2-α-II–reactive cells display a conserved Arg in positive 5 of the CDR3\( \beta \) loop. The CDR3\( \beta \) amino acid sequences of all sorted V\( \beta \)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\( \beta \) 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\( \beta \) amino acid sequences from DQ2-α-II–positive cells from CD823 were encoded by two different nucleotide sequences (superscripts a and b).
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 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 expressing the conserved Arg residue in a prototypic CDR3 loop, ASSI-RSTDTQY, and mutant 364.14 TCRs were retrovirally transduced into a murine hybridoma cell line, BW5147, which was devoid of endogenous TCR (23). Human CD4 was introduced into the hybridoma cells by stable transfection for coreceptor binding to HLA-DQ2. Despite similar levels of CD3ε and TCR surface expression in the wild-type and mutant transfectants (Supplemental Fig. 4), only hybridoma cells expressing the wild-type TCR were reactive to the DQ2-α-II ligand (Fig. 5A). None of the mutants in which the Arg residue in position 5 of the CDR3β (position 109 of the TCR β-chain according to IMGT V-gene alignment) was mutated to Ala (β109A), Lys (β109K), or Glu (β109E), responded to deamidated (Fig. 5A) or native peptides containing the gliadin DQ2-α-II epitope (Table II). The cell signaling machineries in the transfectants were intact as anti-CD3ε stimulation elicited similar levels of murine IL-2 secretion in both wild-type and mutants (Fig. 5B). Additional experiments confirmed that 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 CDR3β 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

![FIGURE 3. In vivo clonal expansion of Vβ6.7 and DQ2-α-II tetramer double-positive cells. The clonal distribution based on CDR3β nucleotide sequences of Vβ6.7 and DQ2-α-II tetramer double-positive cells from single-cell TRBV sequencing in four patients is shown. Patient ID and the total number of cells analyzed in each individual are shown in the center of each distribution scheme. Number of cells sharing the same CDR3β nucleotide sequence is shown in the slices.](http://www.jimmunol.org/)

![FIGURE 4. Convergent recombination and public responses in TRBV7-2 sequences from DQ2-α-II-reactive cells. Amino acid and nucleotide sequences of some DQ2-α-II-reactive TRBV7-2-CDR3β loops are grouped A–F according to shared CDR3β amino acid sequences. All sequences derived from blood were obtained with TRBV7-2–specific single-cell PCR, and sequences derived from duodenum were obtained from in vitro-generated DQ2-α-II-reactive TCCs. The conserved Arg residue and the corresponding encoding nucleotides are underlined. Column 3 shows the IDs of patients from whom the respective sequences were derived, and column 5 shows the number of cells found to express the given CDR3β nucleotide sequence. All sequences use TRBJ2-3*01 but different TRBD gene segments. The functional analysis was performed by the IMGT/V-QUEST online resource (22). Dots represent germline nucleotides that are removed during the VDJ recombination.](http://www.jimmunol.org/)
FIGURE 5. Arginine residue in position 5 of CDR3β is important for DQ2-α-II recognition by TCR 364.14. A, BW5147/β-2.1CD4 cells transfected with wild-type 364.14 TCR (solid circle), β109A (diamond), β109K (+) or β109E (×) mutant TCRs were tested in assays where deamidated 33mer peptide (33merE) containing gliadin DQ2-α-II epitope was presented by DQ2.5 APC. B, The same TCR transfectants were stimulated with plate-bound anti-CD3e Abs. Untransfected cells (*). C, Activation of wild-type 364.14 TCR transfectant by 1 μM 33merE peptide was blocked by 10 μg/ml anti-DQ Ab but not by anti-DP or anti-DR Abs. D, The wild-type 364.14 TCR transfectant was tested with either deamidated 33mer (solid circle), native 33mer (open circle), deamidated 14mer DQ2-α-II peptide (solid triangle), or native 14mer (open triangle). The arrows indicate peptide concentrations needed to elicit 18 pg/ml or native 33mer peptide concentrations needed to elicit 18 pg/ml mouse IL-2 secretion. None of the mutant transfectants were activated by native 33mer peptide. E, Two-log stronger activation of wild-type 364.14 TCR transfectant by anti–HLA-DP Abs (Troybodies) engineered to contain deamidated (solid circle) 33mer compared with those containing native 33mer (open circle). F, Two-log stronger activation of wild-type 364.14 TCR transfectant by streptavidin-immobilized biotinylated soluble deamidated DQ2-αII molecules (solid triangle) compared with native DQ2-αII (open triangle) molecules. For all panels, the experiments were repeated at least two times, and the error bars indicate SEM of each triplicate. The dotted lines indicate the detection limit of mouse IL-2 ELISA. WT, wild-type.

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 (QPQQLPYPQPE) or equivalent amounts of biotinylated DQ2-αII.E molecules containing the deamidated epitope (QPQELPYPQPE), 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

The TCR interacts with the posttranslationally modified Glu residue of the DQ2-α-II epitope

Data thus far showed in vivo Ag-driven TCR selection of DQ2-α-II-reactive T cells in celiac patients that resulted in biased Vβ usage, in vivo clonal expansion, convergent recombination, semi-public response, and the notable conservation of an Arg residue at position 5 of CDR3β. We next asked which part of the 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.

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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT β109A β109K β109E</td>
</tr>
<tr>
<td>P Q P E L P Y Q P E</td>
<td>+++</td>
</tr>
<tr>
<td>−−−</td>
<td>D</td>
</tr>
<tr>
<td>−−−</td>
<td>Q</td>
</tr>
<tr>
<td>−−−</td>
<td>L P Y (+)</td>
</tr>
<tr>
<td>−−−</td>
<td>K</td>
</tr>
</tbody>
</table>

+++ , 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-II/IE44D 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 CDR3β loops of DQ2-α-II-reactive, VB6.7-positive TCRs. Similarly, a TCR displaying Lys that was introduced into this position by in vitro mutagenesis was not reactive to the DQ2-α-II ligand (Fig. 5A). This demonstrates the presence of additional structural constraints that may modify the charge-to-charge interaction between Arg in the CDR3β and Glu in the DQ2-α-II peptide.

In addition to the highly conserved Arg residue in position 5, the neighboring residues, which were often non-germline encoded, also exhibited certain degrees of conservation. The residue in position 4 of the canonical 11-aa CDR3 loop was most often Ile or Leu, whereas the position 6 C-terminally to the conserved Arg residue was most often occupied by Ser, His, Tyr, or Ala residues. Collectively, the canonical CDR3β loop sequence ASS(I/L)RS/H(Y/A)TD/TQY was present in 67% of the VB6.7-positive TCRs (96 of total 143 single cells) reactive to DQ2-α-II and represent more than 86% (96 of 111) of the CDR3β loops with a length of 11 aa. Thus, the positively charged Arg residue in the TCR is probably the primary residue interacting with the negatively charged Glu residue of the peptide, demonstrated by its highly conserved occurrence, with additional contributions from neighboring residues that showed clear nonrandom distribution, but nevertheless less conservation.

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-α-II 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 of 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:DK2-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 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

**FIGURE 6.** SPR analyses of scTCRs s.β109R and s.β109A with soluble DQ2-α-II and DQ2-CLIP2. Representative sensograms are shown. A–D. In all experiments, Neutravidin was immobilized to ∼4500 RU, followed by capture of ∼3500 RU biotinylated DQ2-α-II (A, B) or DQ2-CLIP2 (C, D) molecules. Soluble scTCR s.β109R (A, C) and mutant s.β109A (B, D) were then injected at various concentrations ranging from 0.32 to 5 μM at 25°C.
individuals). Altogether, the CDR3β sequence ASS(L/I)R(S/H/Y/A)TDTQY was observed in roughly two thirds of all sorted VPβ7.7 and DPβ2-αII 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 DPβ2-αII–reactive TCRs or analysis of the TCR repertoire of naive DPβ2-αII–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 (32) in rheumatoid arthritis, disulfide 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-gemline-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

We thank the celiac disease patients for donating biological material, Dr. Kjetil Taskén (Biotechnology Centre of Oslo, Oslo University) for access to the Biacore T100 instrument, and Marie Kongshaug Johannesen and Bjørn Simonsen for excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


