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Article

Biased T Cell Receptor Usage Directed against Human Leukocyte Antigen DQ8-Restricted Gliadin Peptides Is Associated with Celiac Disease

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SUMMARY

Celiac disease is a human leukocyte antigen (HLA)-DQ2- and/or DQ8-associated T cell-mediated disorder that is induced by dietary gluten. Although it is established how gluten peptides bind HLA-DQ8 and HLA-DQ2, it is unclear how such peptide-HLA complexes are engaged by the T cell receptor (TCR), a recognition event that triggers disease pathology. We show that biased TCR usage (TRBV9*01) underpins the recognition of HLA-DQ8-α-I-gliadin. The structure of a prototypical TRBV9*01-TCR-HLA-DQ8-α-I-gliadin complex shows that the TCR docks centrally above HLA-DQ8-α-I-gliadin, in which all complementarity-determining region-β (CDRβ) loops interact with the gliadin peptide. Mutagenesis at the TRBV9*01-TCR-HLA-DQ8-α-I-gliadin interface provides an energetic basis for the Vβ bias. Moreover, CDR3 diversity accounts for TRBV9*01$^+$ TCRs exhibiting differing reactivities toward the gliadin epitopes at various deamidation states. Accordingly, biased TCR usage is an important factor in the pathogenesis of DQ8-mediated celiac disease.

INTRODUCTION

The adaptive immune response is regulated by human leukocyte antigen (HLA) molecules of the major histocompatibility complex (MHC) via interactions with the clonally distributed antigen (Ag)-specific αβ T cell receptor (TCR) expressed on T cells. HLA class II molecules, and in particular HLA-DQ8 that bears the β57 polymorphism, have long been associated with the development of diseases, including type 1 diabetes and celiac disease (Sollid et al., 1989; Spurkland et al., 1992; Todd et al., 1987). However, there are few examples in which the mechanism of HLA-associated disease is well understood.

Celiac disease is a common, T cell-mediated, T cell inflammatory disorder with autoimmune components, which is triggered by the ingestion of dietary wheat gluten or related proteins from rye and barley (Abadie et al., 2011; Jabri and Sollid, 2009). Celiac disease is predominantly limited to genetically predisposed individuals, specifically those who express HLA-DQ2 (A1*0501-B1*0201) and/or HLA-DQ8 (A1*0301-B1*0302) (Sollid, 2002). In celiac disease patients, 90%–95% express HLA-DQ2 molecules and HLA-DQ2$^-$ patients usually express HLA-DQ8 (Karell et al., 2003). In HLA-DQ2-associated celiac disease, intestinal T cell clones and lines are virtually all HLA-DQ2 restricted and recognize a range of peptides derived from each of the subfractions of wheat gluten and homologous sequences in barley hordeins and rye secalins (Anderson et al., 2000; Arentz-Hansen et al., 2000, 2002; Sjöström et al., 1998; Vader et al., 2003). Fine mapping of HLA-DQ2-restricted peptides has revealed a preference for glutamate at anchor positions P4 or P6, and sometimes P7, corresponding to glutamine residues susceptible to tissue transglutaminase (TG2) deamidation (Arentz-Hansen et al., 2000, 2002; Qiao et al., 2005; Vader et al., 2002b).

Despite HLA-DQ2 conveying a greater risk of celiac disease, gluten-reactive CD4$^+$ T cells recognizing certain HLA-DQ8-restricted determinants have been isolated from patients with celiac disease who possess both HLA-DQ2 and HLA-DQ8 and from patients who possess HLA-DQ8 but not HLA-DQ2 (Tollefsen et al., 2006; van de Wal et al., 1998a, 1998b, 1999). T cells specific for the DQ8-α-I-gliadin determinant (EGSFQPSQE) are commonly isolated from HLA-DQ2$^+$HLA-DQ8$^+$ celiac donors and this gluten determinant is often immunodominant

Table 1. The Variable Gene Usage of the HLA-DQ8-Gliadin or -Glutenin-Restricted TCRs

Clone	Restriction	TRAV[a]	TRAJ	CDR3α	TRBV	TRBD	TRBJ	CDR3β
SP3.4	DQ8-glia-α1	26-2*01	45*01	YYC ILRDGRGGADGLT FG	9*01	1*01	2-7*01	CAS SVAVSAGTYEQ YFG
L3-12	DQ8-glia-α1	26-2*01	54*01	YYC ILRDSRAQKLV FG	9*01	1*01	2-7*01	CAS SAGTSGEYEQ YFG
S13[b]	DQ8-glia-α1	26-2*01	49*01	YYC ILRDRSNQFY FG	9*01	1*01	2-5*01	CAS STTPGTGTETQ YFG
SP4.6	DQ8-glia-α1	38-2-DV8*01	31*01	YFC AYRSARGARLM FG	9*01	1*01	2-7*01	CAS SVAVSAGTYEQ YFG
S16[b]	DQ8-glia-α1	13-1*02	37*01/02	YFC AGGSSNTGKLI FG	4-2*01	-	2-2*01	CAS SQDIRNTGEL FFG
LS1.2	DQ8-glia-α1	17*01	44*01	YFC ATDFPGTASKLT FG	6-1*01	1*01	1-3*01	CAS SEALPGRSGNTI YFG
T316	DQ8/8.5-glia-α1[c]	8-3*01	36*01	YFC AVGETGANNLF FG	6-1*01	2*02	2-1*01	CAS SEARRYNEQ FGP
T15	DQ8/8.5-glia-γ1[d]	20*02	6*01	YLC AVQASGGSYIPT FG	9*01	1*01	2-3*01	CAS SNRGLGTDTQ YFG
S12[b]	DQ8-glut-H1	29-DV5*01	49*01	YFC AASAYPGNQFY FG	9*01	2*01	2-5*01	CAS SVYDGRGETQ YFG

Junctionally encoded residues are underlined. DQ8 epitope nomenclature according to Sollid et al. (2012). See also Figure S1 and Tables S1 and S2.
[a]TCR variable gene usage as defined in IMGT-V-QUEST Database (Brochet et al., 2008).
[b]The TRBV usage and CDR3β sequences of clones S12, S13, and S16 have been published previously (Hovhannisyan et al., 2008).
[c]DQ8/8.5-glia-α1 = T316 T cell clone recognizes α-I-gliadin presented by HLA-DQ8 or HLA-DQ8.5.
[d]DQ8/8.5-glia-γ1 = T15 T cell clone recognizes glia-γ1 presented by HLA-DQ8 or HLA-DQ8.5.

in HLA-DQ2$^-$HLA-DQ8$^+$ celiac disease (Henderson et al., 2007b; Tollefsen et al., 2006; van de Wal et al., 1998b). In contrast to the more absolute deamidation dependence and relative protease resistance of the immunodominant gluten peptides in HLA-DQ2-mediated disease, the immunodominant DQ8-α-I-gliadin determinant derives from a protease-sensitive region of α-gliadin, and a commonly recognized determinant from wheat high molecular glutenin does not require deamidation (Shan et al., 2002; Tye-Din and Anderson, 2008; van de Wal et al., 1999).

A network of polar interactions enhance the binding of the deamidated gliadin peptide DQ2-α-I-gliadin (PFPQPELPY) to HLA-DQ2 (Kim et al., 2004) and DQ8-α-I-gliadin (EGSFQPSQE) to HLA-DQ8 (referred to as DQ8-glia-α1) (Henderson et al., 2007b; Sollid et al., 2012). Recently it has been suggested that, because of the β57 Asp→Ala polymorphism in HLA-DQ8, TCRs bearing a negatively charged residue within the CDR3β loop are preferentially selected to interact with HLA-DQ8 complexed to native gluten peptides (Abadie et al., 2011; Hovhannisyan et al., 2008). The subsequent binding of deamidated gluten peptides to HLA-DQ8 generates a stronger T cell response and broadens the T cell repertoire to include TCRs that do not contain a negatively charged residue within the CDR3β loop. Presently, however, the nature of the αβ T cell repertoire directed against HLA-DQ8-restricted gliadin peptides is unclear, and moreover it is unknown how these TCRs, which are implicated in celiac disease pathology, engage the DQ8-glia-α1 complex. We show that biased TCR usage underpins DQ8-glia-α1 recognition and we provide a structural and energetic basis for this key interaction in HLA-DQ8-mediated celiac disease.

RESULTS

Biased TRBV9*01 Usage against DQ8-Glia-α1

To examine the T cell response in HLA-DQ8-mediated celiac disease, we previously isolated and expanded three DQ8-glia-α1-restricted T cell clones (SP3.4, SP4.6, LS1.2) from the PBMCs of two celiac disease patients (patients "SP" and "LS") (Henderson et al., 2007b; Mannering et al., 2005), all of which specifically bound the DQ8-glia-α1 tetramer (Figure S1 available online). In addition, three previously isolated DQ8-glia-α1-specific T cell clones (S13, L3-12, S16) from small intestinal biopsies of HLA-DQ2$^+$HLA-DQ8$^+$ patients (patients "S" and "L") and one (T316) from a small intestinal biopsy of a patient (patient "T") expressing the HLA-DQ8 transdimer (A*0501-B*0302) were characterized (Kooy-Winkelaar et al., 2011).

These seven DQ8-glia-α1-restricted T cell clones were analyzed for their αβ TCR gene usage, CDR3 sequences, and dose response by means of DQ8-glia-α1 deamidated peptide analogs (Table 1; Figure 1). Clone LS1.2 used TRAV17*01-TRBV6-1*01 pairing (Table 1). The T cell clones S16 and T316 used TRAV13-1*02-TRBV4-2*01 and TRAV8-3*01-TRBV6-1*01, respectively. The two clones isolated from patient SP, SP3.4 and SP4.6, shared the same TCR β chain, TRBV9*01, with an alternative TCR α chain pairing of TRAV26-2*01 and TRAV38-2-DV8*01, respectively (Table 1). Further, the T cell clones S13 and L3-12 were TRAV26-2*01-TRBV9*01$^+$ (Table 1). Notably, the three clones (SP3.4, S13, and L3-12) characterized that exhibit identical TRAV26-2-TRBV9*01 usage were derived from three celiac disease patients (two adults, one child) who are not related to each other. We also characterized the TRAV-TRBV usage of two other previously isolated T cell clones, S12 and T15 (Kooy-Winkelaar et al., 2011). S12 is specific for DQ8-glut-H1, whereas T15 cross-reacts with glia-γ1 presented by either HLA-DQ8 or HLA-DQ8.5 (Kooy-Winkelaar et al., 2011; van de Wal et al., 1999). Although both the T15 and S12 T cell clones exhibited different TRAV gene usage, they both used the TRBV9*01 gene (Table 1). Although no sequence homology was observed between the CDR3 loops of the nine DQ8-restricted clones, the presence of a non-germline-encoded Arg residue in the CDR3α loop of the TRBV9*01$^+$ DQ8-glia-α1-specific TCRs was notable (Table 1).

The public TRBV9*01 usage in six independent HLA-DQ8-restricted T cell clones, specific for α1-gliadin (or other gliadin determinants), suggested that biased TCR usage was an important factor underpinning this response. To investigate this, we tested the expression of TRBV9*01 on CD4$^+$ T cells present in peripheral blood of HLA-DQ8$^+$ and HLA-DQ8$^-$ individuals and compared this to the expression by T cells isolated from biopsies

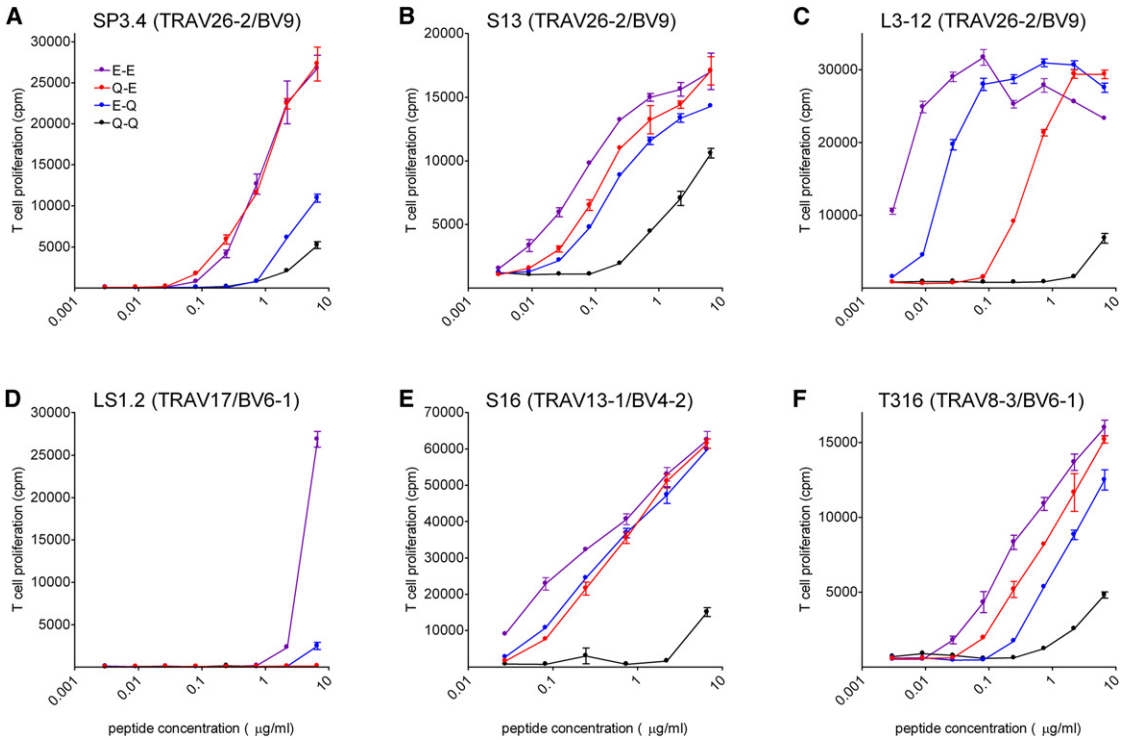

Figure 1. Deamidation Dependence of DQ8-Glia-α1-Restricted TCRs

Response of the T cell clones SP3.4 (A), S13 (B), L3-12 (C), LS1.2 (D), S16 (E), and T316 (F) to a concentration range of four variants of the DQ8-glia-α1 peptide: P1Q-P9Q, black line; P1E-P9Q, blue line; P1Q-P9E, red line; P1E-P9E, purple line. All measurements were performed in triplicate. Data are mean ± SEM.

of four celiac disease patients and one non-celiac disease patient. On average, 2% of peripheral blood $CD4^+$ T cells expressed TRBV9*01 (n = 9, range 0.8%–3.7%, no marked difference between HLA-$DQ8^+$ and HLA-$DQ8^-$ healthy controls) (Table S1). In contrast, elevated amounts of TRBV9*01 expression was observed in the four gluten-reactive T cell lines isolated from celiac disease patients (average 15.8%, range 6.8%–32.5%), whereas no such increase was observed in the T cell line from a non-celiac disease patient (0.9%) (Table S1).

To formally demonstrate that TRBV9*01 expression correlates with gliadin reactivity in HLA-$DQ8^+$ celiac disease patients, we isolated TRBV9*01^+ and TRBV9*01^- T cells from a gluten-reactive T cell line derived from a biopsy of a celiac disease patient by fluorescence activated cell sorting. Cells were sorted either 1 cell per well or 100 cells per well, expanded, and subsequently tested for reactivity against a deamidated gliadin preparation (Glia-TG2) and the deamidated DQ8-glia-α1 peptide (Table S2). Although the results clearly demonstrate that gliadin reactivity is found in both the TRBV9*01^- and TRBV9*01^+ fractions, there was a strong selection for reactivity with the DQ8-glia-α1 peptide in the TRBV9*01^+ cell fraction, as indicated by the fact that 11 out of 12 gliadin-reactive T cell clones and 9 out of 9 T cell lines were found to specifically react with the DQ8-glia-α1 peptide (Table S2). In contrast, in the TRBV9*01^- cell fraction, 3 out of 10 T cell clones and 5 out of 7 T cell lines responded to gliadin but only one of the cell lines and none of the clones responded to the DQ8-glia-α1 peptide. Similar results were found with a gliadin-reactive T cell line from a second celiac disease patient (not shown). Thus, TRBV9*01 expression is elevated in gluten-reactive T cell lines derived from biopsies of celiac disease patients and correlates with reactivity toward the DQ8-glia-α1 peptide. Accordingly, biased TRBV9*01 usage appears to be a prominent feature of the HLA-DQ8-restricted response to the α-I-gliadin determinant.

Deamidation Dependence

To compare the DQ8-glia-α1-specific T cell clones and determine their dependence on the deamidation of the Q at the p1 and/or p9 positions, three TRAV26-2*01^+-TRBV9*01^+ T cell clones and three TRAV26-2*01^--TRBV9*01^- T cell clones were tested against a concentration range of four variants of the α-I-gliadin peptide with a Q and/or E at position p1 and p9 (Figure 1). We observed striking differences in sensitivity of the T cell clones with an at least 100-fold difference between clone LS1.2 and L3-12 (the least and most sensitive, respectively). Both the TRAV26-2*01-TRBV9*01-positive and -negative T cell clones displayed unique reactivity patterns, but in all cases stronger responses were observed against peptides in which either one or both of the Q residues at P1 and P9 were replaced by an E. Strikingly, the SP3.4 and L3-12 clones displayed differential reactivity toward the deamidated peptides. Namely, whereas clone SP3.4 preferred the P1Q-P9E peptide to the P1E-P9Q variant, clone L3-12 displays the opposite pattern and responded more strongly to the P1E-P9Q peptide. In contrast, the third TRAV26-2*01-TRBV9*01^+ T cell clone, S13, did not display a strong preference for either the P1Q-P9E or P1E-P9Q variant (Figure 1). Thus, the three TRAV26-2*01^+-TRBV9*01^+ T cell clones displayed unique reactivity patterns and are differentially affected

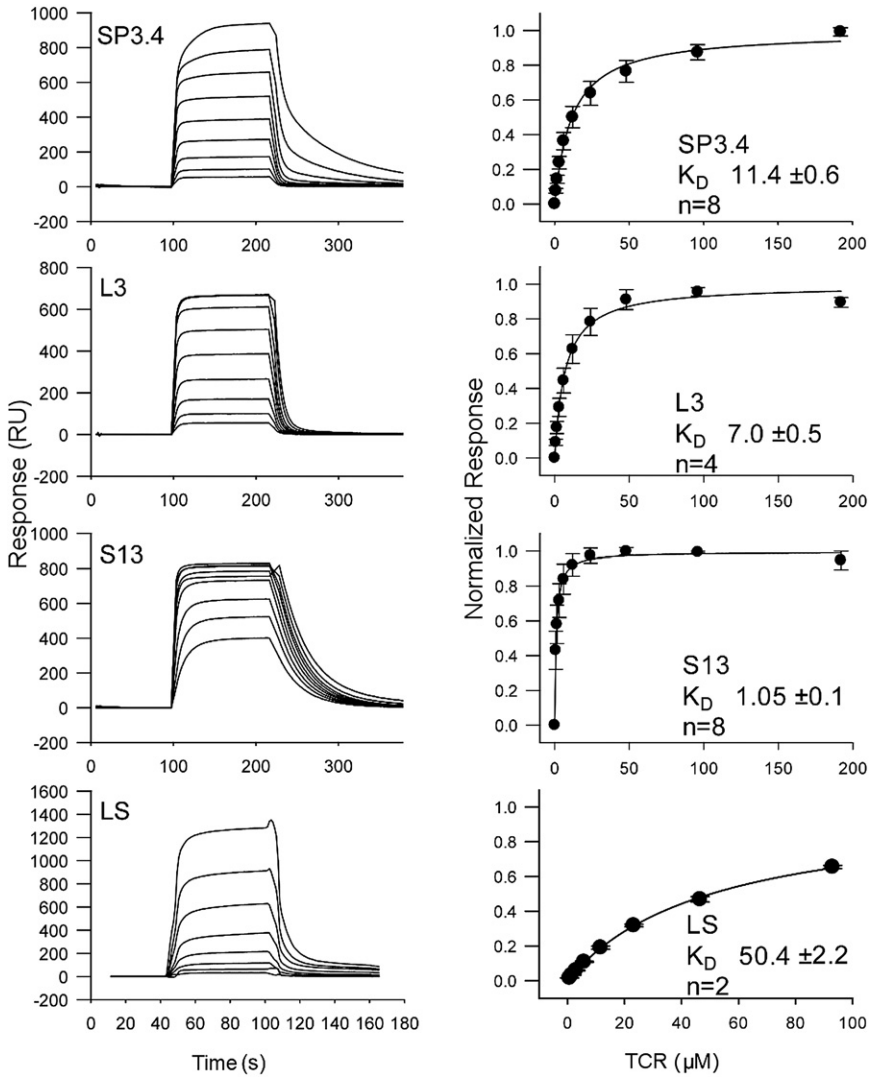

Figure 2. Affinity Data for the DQ8-Glia-α1-Restricted TCRs

Binding analysis of TCRs SP3.4, L3-12, S13, and LS1.2 to DQ8-glia-α1 via surface plasmon resonance. Concentration series of each TCR was passed over surface immobilized DQ8-glia-α1. Right column: Measured response curves of single dilution series for each TCR. Left column: Curve fits for TCR-DQ8-glia-α1 K_D determination with single ligand binding model. Data from multiple (n =) measurements was combined after normalizing each equilibrium response curve against the calculated response maximum. Data are mean ± standard deviation.

by deamidation of the Q at p1 and p9. Because the TRAV-TRBV usage is conserved between these three clones, it indicates that CDR3 sequence variability underpins the differing degrees of deamidation dependence of these T cell clones.

Affinity for the DQ8-Glia-α1 Epitope

Next, we expressed, refolded, and purified the SP3.4, L3-12, S13, and LS1.2 TCRs (data not shown). Native gel-shift analysis demonstrated that the SP3.4 TCR ligated to DQ8-glia-α1, yet did not bind to an irrelevant pMHC class II (pMHC-II) (data not shown). As determined by surface plasmon resonance (SPR) analysis, the steady-state affinity (K_D) of the SP3.4, L3-12, and S13 TCRs for DQ8-glia-α1 were 11.4 ± 0.6 μM, 7.0 ± 0.5 μM, and 1.0 ± 0.1 μM, respectively (Figure 2). However the affinity for the LS1.2 TCR interaction with DQ8-glia-α1 was weaker (50.4 ± 2.2 μM) (Figure 2). These data correlate with the much weaker reactivity of the LS1.2 clone in the gluten-specific proliferation assay compared to the other clones (Figure 1). We also determined the kinetic rate constants for the SP3.4 TCR-DQ8-glia-α1 interaction, which exhibited association (k_a) and dissociation (k_d) rate constants of 2.53 × 10^4 ± 0.29 × 10^4 M^{-1} s^{-1} and 0.224 ± 0.029 s^{-1}, respectively (Figure 2), values that fall within the range for typical TCR-pMHC interactions. However, the affinity values of the SP3.4 TCR, L3-12 TCR, and S13 TCR for DQ8-glia-α1 were higher than that typically observed for microbial and non-self TCR-pMHC-II (~30 μM) (Cole et al., 2007) and much higher than the low affinity for autoreactive TCR-pMHC complexes (~100–200 μM) (Bulek et al., 2012; Deng and Mariuzza, 2007). Indeed, the affinity of the SP3.4 TCR, L3-12 TCR, and S13 TCR toward TCR-DQ8-glia-α1 was more typical of the affinity of microbial TCR-pMHC-I complexes (~1–10 μM) (Clements et al., 2006; Cole et al., 2007; Godfrey et al., 2008).

Structural Overview of the SP3.4 TCR-DQ8-Glia-α1 Complex

To establish how the SP3.4 TCR interacts with DQ8-glia-α1, we solved the structure of the SP3.4 TCR-DQ8-glia-α1 complex at 3.2 Å resolution (Figures 3A and 3B; Table 2). Further, we determined the structure of the SP3.4 TCR in the nonliganded state at 3.2 Å resolution (Table 2). Together with the binary DQ8-glia-α1 complex previously determined (Henderson et al., 2007b), we were thus able to establish the extent of plasticity in the SP3.4 TCR-DQ8-glia-α1 interaction.

The SP3.4 TCR docks centrally above HLA-DQ8, at approximately 70° across the long axis of the Ag-binding cleft, where the Vα and Vβ domains of SP3.4 TCR sits above the β and α helix of HLA-DQ8, respectively (Figure 3C). The buried surface area (BSA) at the SP3.4 TCR-DQ8-glia-α1 interface is ~880 $Å^2$, a value at the lower end of the range for TCR-pMHC-II complexes (Table S3; Rudolph et al., 2006). Thus, the overall docking mode of the SP3.4 TCR-DQ8-glia-α1 complex is comparable to other TCR-pMHC structures determined to date (Burrows et al., 2010) and does not display the "nonstandard" docking modes that have generally been associated with autoimmune TCR-pMHC-II complexes (Deng and Mariuzza, 2007), which may be a consequence of the SP3.4 TCR

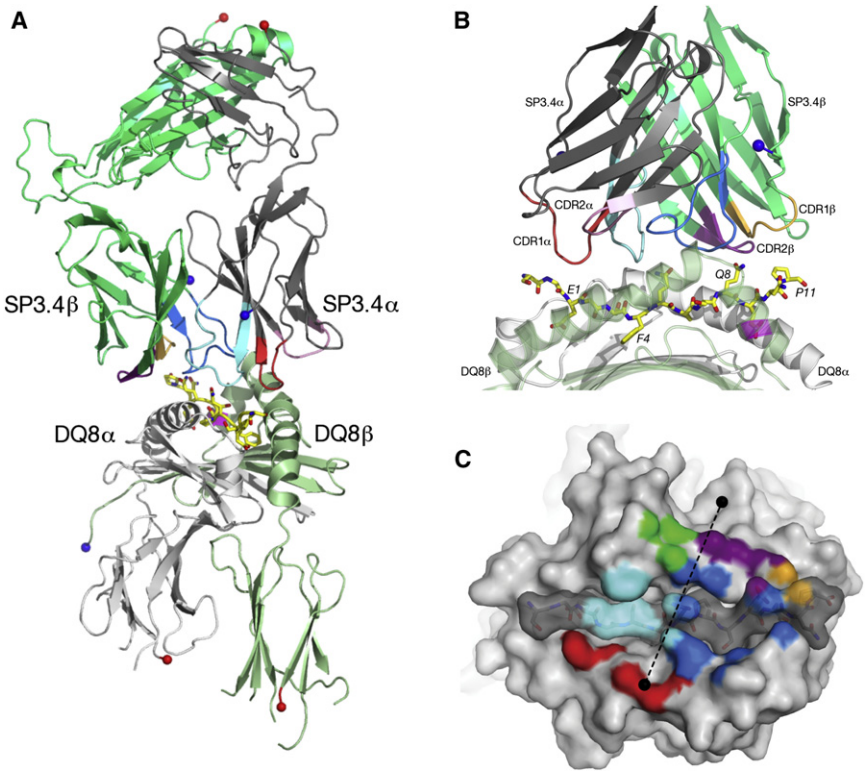

Figure 3. The SP3.4 TCR-DQ8-Glia-α1 Ternary Complex

(A and B) Structural overview (A) and close up (B) of the Ag-binding interface.

(C) The SP3.4 TCR-DQ8-glia-α1 footprint.

The β57 position is colored magenta. CDR1α, CDR2α, and CDR3α are colored red, pink, and cyan, respectively; CDR1β, CDR2β, and CDR3β colored orange, purple, and blue, respectively. The DQ8 α and β chains are colored light gray and green, respectively. The α-I-gliadin peptide is in yellow. The SP3.4 α and β chain framework regions are colored dark gray and lime green, respectively. N and C termini of SP3.4 and DQ8 are shown as blue and red spheres, respectively. See also Figure S3 and Table S3.

recognizing a non-self Ag restricted to HLA-DQ8. Further, the HLA-DQ8-restricted TCR does not reside over the β57 position of HLA-DQ8 (Figures 3B and 4).

To engage the DQ8-glia-α1, the CDR loops of the SP3.4 TCR show limited plasticity, with a movement in the CDR1α loop (main chain rmsd, 1.2 Å) and CDR3α loop (main chain rmsd for observed atoms, 2.0 Å), whereas all the CDRβ loops do not appreciably change conformation upon ligation (not shown). Further, the Ag-binding clefts of the nonliganded DQ8-glia-α1 (Henderson et al., 2007b) and DQ8-glia-α1 from the ternary complex structure overlay closely, with an rmsd of 0.35 Å, and there are only two side chains that move notably to accommodate the SP3.4 TCR. These are the side chains of Val67 and Arg70 from HLA-DQ8, which reorients to avoid steric clashes with Tyr114β and, consequently, forms interactions with CDR3α and the CDR3β loops of the SP3.4 TCR (Figure S3). Thus, in comparison to the plasticity of the CDR loops seen in many TCR-pMHC interactions, a relatively rigid interaction underpins the SP3.4 TCR-DQ8-glia-α1 complexation, although some changes were observed in the Cα domain that may relate to signaling (Beddoe et al., 2009; Ishizuka et al., 2008; Garcia et al., 1998; Tynan et al., 2007). The SP3.4 forms a number of van der Waals (vdw) interactions and ten hydrogen bonds with DQ8-glia-α1 (Table S3). The Vα and Vβ domains of the SP3.4 TCR contribute 43% and 57% to the BSA, respectively, at the DQ8-glia-α1 interface with the Vα and Vβ chains contacting both helices of HLA-DQ8. Within this interface, all six CDR loops contribute to the interaction, but to varying degrees (Table S3). The CDR1α and CDR2α loops of the SP3.4 TCR contribute 14% and 5% to the BSA, respectively, whereas the CDR1β and CDR2β loops contribute 6% and 11%, respectively. Notably, the CDR3α and CDR3β loops contribute 23% and 29% of the BSA, respectively, and thus the CDR3 loops dominate contacts, interacting with both the α-I-gliadin peptide and HLA-DQ8.

The SP3.4 TCR exclusively interacts with the HLA-DQ8 α chain via vdw contacts, binding to a region of the α1 helix that spans from Phe58 to His68 (Figure 4A). The interactions are mediated principally by the CDR3α, CDR1β, and CDR2β loops, in which Phe58 sits underneath the CDR3α loop, with its aromatic ring packing against Arg110α. Arg66β, a framework residue adjacent to the CDR2β loop, packs against Thr61 and Leu60, while Tyr57β interacts with Ala64 and points toward His68, with the latter residue also contacting Leu37 of the CDR1β loop (Figure 4A).

The β1 helix of HLA-DQ8 sits underneath the CDR1α and CDR2α loops, but also mediates contacts with the CDR3α and CDR3β loops largely as a result of the long side chain of Arg70 that stretches across the Ag-binding cleft, and subsequently forms vdw interactions and H bonds with Gly109α and Thr113β of SP3.4 TCR. Thr36α and Tyr38α from the CDR1α loop and Leu57α from the CDR2α loop converge to form a hydrophobic focal point on the β1 helix, interacting with Ala73, Thr77, and His81 of HLA-DQ8 (Figure 4B). Accordingly, the SP3.4 TCR adopts a standard and central docking topology over HLA-DQ8.

Interactions with the α-I-Gliadin Peptide

Although the extent of contacts between the SP3.4 TCR and HLA-DQ8 is relatively small, the interaction with the α-I-gliadin peptide is, by comparison, more extensive, with the CDR3α loop and the CDR1β, CDR2β, and CDR3β loops contacting positions P1-Glu, P2-Gly, P3-Ser, P5-Gln, and P8-Gln of the peptide (Figure 4C). Of the ten H bonds present at the SP3.4 TCR-DQ8-glia-α1 interface, eight are mediated by peptide residues. This distribution of H bonds suggests that the largest energetic contribution to SP3.4 TCR-DQ8-glia-α1 binding is provided by polar interactions with the peptide. A key residue appears to be Arg110α of the CDR3α loop with its side chain oriented alongside the peptide and toward its N terminus. The guanidinium moiety of Arg110α packs tightly against Phe58α of HLA-DQ8 while also forming four H bonds to the backbone and side chains

Table 2. Data Collection and Refinement Statistics

Data Collection Statistics	SP3.4-DQ8-Glia-α1	SP3.4
Wavelength (Å)	0.95666	0.956591
Resolution limits (Å)	40–3.20 (3.31–3.20)[a]	50.0–3.20 (3.31–3.20)[a]
Space group	$P2_12_12_1$	$P3$
Cell dimensions (Å)	a = 111.71, b = 134.33, c = 140.30	a = 124.56, b = 124.56, c = 61.10, γ = 120°
Total number of observations	148,589	31,344
Number of unique observations	32,429	15,462
Data completeness (%)	91.0 (93.8)	87.2 (89.7)
R_{pim} (%)[b]	8.4 (26.1)	7.2 (38.9)
$\langle I/\sigma(I)\rangle$	9.1(2.6)	14.6 (2.2)
Multiplicity	4.6 (4.6)	2.0 (2.0)
Refinement statistics		
R factor (%)[c]	24.6	26.1
R free (%)[d]	28.5	30.8
Number of Atoms		
Protein and peptide	11,879	6,107
Other	42	46
Average B Factor (Å^2)		
Main chain atoms	43.8	85.3
Side chain atoms	42.6	84.4
Other atoms	52.7	102.6
Rmsd bond lengths (Å)	0.006	0.007
Rmsd bond angles (°)	0.975	1.049
Ramachandran favored (%)[e]	95.5	91.2
Ramachandran outliers (%)[e]	0.3	1.0

See also Figure S2.

[a]Values in parentheses refer to the highest resolution bin.

[b]$R_{pim} = \Sigma_h\,[1/(N-1)]^{1/2}\,\Sigma_i|I_i(h) - \langle I(h)\rangle| / \Sigma_h\Sigma_i I_i(h)$ where I is the observed intensity and $\langle I\rangle$ is the average intensity of multiple observations from symmetry-related reflections.

[c]$R_{factor} = (\Sigma\,||F_o| - |F_c||) / (\Sigma\,|F_o|)$ for all reflections except as indicated in footnote d.

[d]Calculated as for the R_{factor} (see footnote c) using 5% of reflections.

[e]Output from the MolProbity Server (Chen et al., 2010).

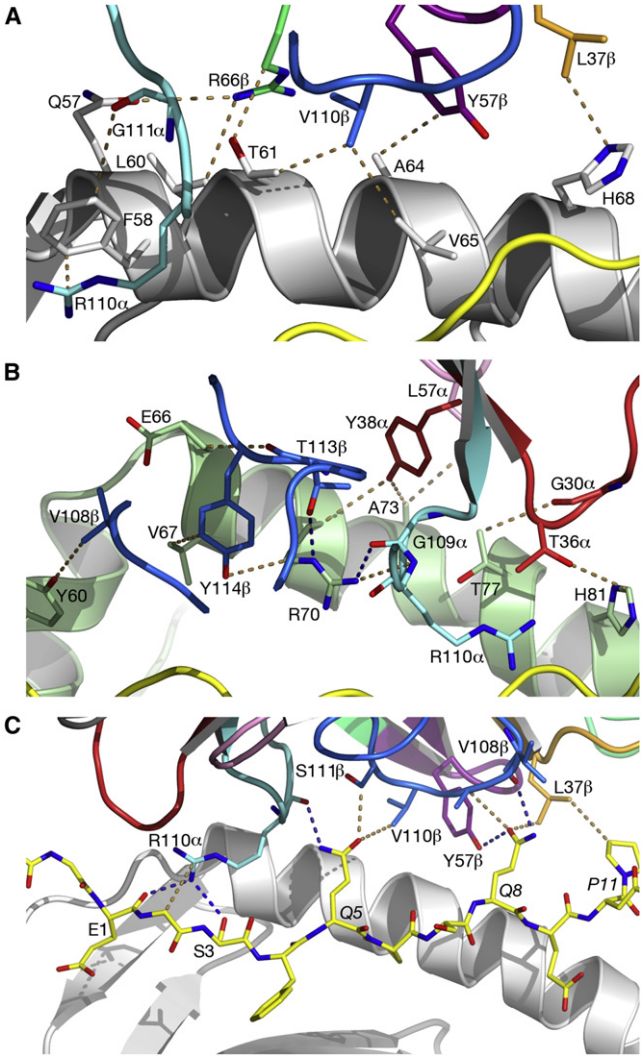

Figure 4. Interactions at the Interface of SP3.4 TCR-DQ8-Glia-α1

(A) DQ8-α chain-SP3.4 contacts.

(B) DQ8-β chain-SP3.4 contacts.

(C) α-I-gliadin peptide-SP3.4 contacts.

The TCR and pHLA are displayed in cartoon format, with key residues shown as sticks. The residue color scheme is as for Figure 3. vdw interactions are highlighted by beige dashes and hydrogen bonds by blue dashes. See also Figure S4 and Table S4.

of the α-I-gliadin peptide (at P1-Glu and P3-Ser) (Figures 4C and S4). In the unliganded structure, the CDR3α loop is disordered. However, upon ligation, its conformation, in particular Arg110α, is stabilized by an intrachain salt bridge to the adjacent Asp108α and by a H bond between Asp108α and the backbone of Arg110α.

The P5-Gln and P8-Gln are the prominent upward-facing residues of the gliadin peptide, with the CDR3β loop being positioned in between these two residues and P8-Gln having to reorientate its side chain to accommodate the CDR3β loop. P5-Gln contacts the CDR3α and CDR3β loops of the SP3.4 TCR, forming a H bond with the main chain of Arg110α and vdw contacts with Ser 111β (Figure 4C). P8-Gln contacts the CDR1β, CDR2β, and the CDR3β loops and forms H bonds with the main chain of Val108β and side chain of Tyr57β, from the CDR3β and CDR2β loops, respectively, as well as vdw contacts with Ala109β and Leu37β from the CDR3β and CDR1β loops, respectively. Accordingly, the P5-Gln-X-X-P8-Gln sequence mediates many contacts with the SP3.4 TCR.

Peptide Library Scan

Given that the P5-Gln-X-X-P8-Gln motif appears to play a central role in the interaction with the SP3.4 TCR, the specificity of the SP3.4 T cell clone was analyzed for reactivity to a library of 1,363 native and in silico deamidated gliadin-derived 18-mers comprising 8,114 10-mer candidate determinants, via

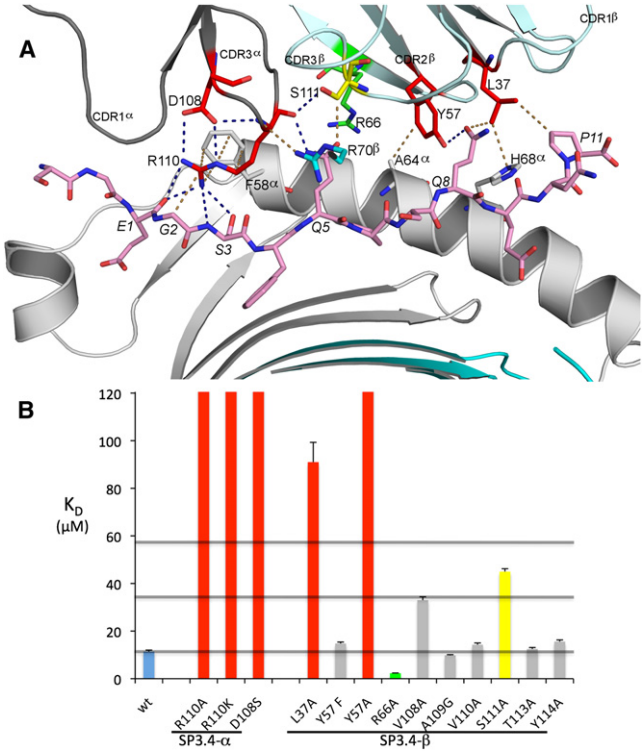

Figure 5. Energetic Basis of TRBV9*01 Bias

Energetic hot spots of the SP3.4-DQ8-glia-α1 interface.

(A) Cartoon representation of the SP3.4-DQ8-glia-α1 interface with the peptide and energetically important SP3.4 contact residues shown as sticks and colored according to the changed K_D of the mutant (red, K_D > 57 μM; yellow, K_D between 35 and 57 μM; green, K_D < 2.3 μM). The corresponding contact residues of DQ8 are shown in gray (α chain), cyan (β chain), and pink (peptide). Interactions between the highlighted residues are shown as dashes (blue, H bonds; beige, vdw contacts).

(B) Comparison of the measured equilibrium dissociation constants of SP3.4 mutants. Differences in K_D were classed as moderate (K_D between 35 and 57 μM, yellow bar) and marked (K_D > 57 μM [red] or K_D < 2.3 μM [green]). Horizontal lines show 1, 3, and 5 times multiples of the WT SP3.4 K_D.

Data are mean ± standard deviation. See also Figure S5.

an overnight interferon-γ (IFN-γ) ELISpot assay (Tye-Din et al., 2010). The SP3.4 T cell clone recognized a wide variety of deamidated and native gliadin peptides, all containing highly homologous sequences to the core P1-P9 of the α-I-gliadin peptide (Table S4; Figure 1). Indeed, peptides containing all permutations of P1-Gln or Glu and P9-Gln or Glu were among the peptides eliciting the strongest response. These data support the earlier T cell proliferation results indicating that the SP3.4 T cell clone recognizes both deamidated and native gliadin peptides in the context of HLA-DQ8 (Henderson et al., 2007b). Moreover, six of the top ten peptides possess the Gln-X-X-Gln motif, thereby highlighting the role this motif plays in SP3.4 TCR recognition of DQ8-glia-α1.

Energetic Basis of the TRBV9*01 Bias

Next, we examined the energetic basis underpinning the biased TRBV9*01 usage directed toward the DQ8-glia-α1 complex. Solvent-exposed SP3.4 TCR residues from the TCRβ chain, whose side chains interacted with DQ8-glia-α1 were selected for substitution to alanine. We also mutated Arg110α and Asp108α from the CDR3α loop, because Arg110α represented the most prominent DQ8-glia-α1 contact point on the Vα chain, and Asp108α appeared to stabilize the conformation of Arg110α. In total, 13 SP3.4 TCR amino acid substitutions were made and included CDR3α: Asp108αSer, Arg110αAla, Arg110αLys; CDR1β: Leu37βAla; CDR2β: Tyr57βPhe, Tyr57βAla; Vβ framework: Arg66βAla; CDR3β: Val108βAla, Ala109βGly, Val110βAla, Ser111βAla, Thr113βAla, Tyr114βAla. All of the mutant SP3.4 TCRs expressed and refolded with similar yield as to wild-type (WT) SP3.4 TCR and exhibited similar biophysical properties to the WT SP3.4 TCR (not shown). The impact of the mutants on the affinity of the SP3.4 TCR-DQ8-glia-α1 interaction were assessed by SPR analysis. The effects of the SP3.4 TCR mutants were grouped into three categories: no effect, a moderate effect (K_D between 35 and 57 μM), and a marked effect (K_D > 57 μM or K_D < 2.3 μM) (Figures 5 and S5).

Mutation of Arg110α to Ala or Lys abrogated DQ8-glia-α1 binding, thereby underscoring the key role this residue plays in contacting the α-I-gliadin determinant and HLA-DQ8. Further, the Arg110αLys mutant highlighted the importance of its guandanium group in forming multiple H bonding contacts with DQ8-glia-α1. Mutation of Asp108α also abrogated DQ8-glia-α1 binding, thereby highlighting the importance of the Asp108α-X-Arg110α sequence and associated conformation in enabling binding (Figures 5A and 5B). Of the nine positions mutated within the TCRβ chain, only two residues (Leu37β and Tyr57β) were critically important for the interaction; one residue (Ser111β) moderately impacted on the affinity of the interaction, and mutation of five residues (Val108β, Ala109β, Val110β, Thr113β, and Tyr114β) had no impact on the interaction with DQ8-glia-α1 (Figures 5A and 5B). The moderate impact of the Ser111βAla mutant could be attributed to the loss of a stabilizing contact with the CDR3α loop as opposed to a direct reduction in the number of contacts with the P5-Gln residue. Thus, despite the CDR3β loop contributing 29% BSA to the SP3.4 TCR-DQ8-glia-α1 interface, the amino acid side chains within the CDR3β loop of the SP3.4 TCR are not a critical factor in mediating binding with DQ8-glia-α1. This indicates that a diverse repertoire of CDR3β sequences would potentially be permissive to DQ8-glia-α1 binding.

The germline-encoded Leu37β and Tyr57β residues are positioned closely together, situated above HLA-DQ8 α chain, with Leu37β interacting with the His68 side chain and Tyr57β packing against the main chain of the α1 helix. Both of these residues form vdw contacts with P8-Gln of the α1-gliadin determinant, with Tyr57β also forming a H bond with P8-Gln, although the effect of the Tyr57βPhe mutant shows that this interaction is not energetically required for DQ8-glia-α1 ligation. Accordingly, residues from within the CDR3α loop, Leu37β from the CDR1β loop, and Tyr57β from the CDR2β loop are the "hot-spot" residues underpinning the SP3.4 TCR-DQ8-glia-α1 interaction providing a basis for the TRBV9*01 bias (Figures 5A and 5B).

DISCUSSION

Although it is established that $CD4^+$ T cells play a pivotal role in DQ8- and DQ2-mediated celiac disease, little was known

regarding the nature of the responding T cell repertoire and how the pathogenic TCRs interacted with the gliadin determinants complexed to the disease-associated HLA class II molecules. Previously it was shown that DQ8-native gliadin complexes select TCRs that harbor a negatively charged residue within the CDR3β loop, whereas the subsequent binding of deamidated gliadin determinants to HLA-DQ8 generates a stronger T cell response and diversifies the T cell repertoire (Hovhannisyan et al., 2008). We show that biased TCR usage underpins the adaptive immune response to the deamidated DQ8-glia-α1 peptide complexed to HLA-DQ8. Biased TCR usage is often linked to TCR recognition of atypical (featured or featureless) pMHC landscapes in viral immunity (Turner et al., 2006). However, biased Vβ6.7 (TRBV7-2*01) usage has also recently been described in the adaptive immune response to DQ2.5-glia-α2, although the structural basis for this biased gene usage remains unclear (Qiao et al., 2011). Our present findings extend this to HLA-DQ8 and provide a structural basis for the observed biased TCR usage in HLA-DQ8-associated celiac disease.

To address the basis of TRBV9*01 bias, we determined the structure of a prototypical TRBV9*01 TCR, SP3.4, in complex with DQ8-glia-α1, and undertook associated mutagenesis and affinity studies. The TRAV26-2-TRBV9*01-HLA-DQ8-glia-α1 structure did not exhibit an atypical binding mode and the central docking topology also precluded the SP3.4 TCR interacting with the β57 residue on HLA-DQ8, a polymorphic site located at the base of the C-terminal end of the Ag-binding cleft, which is linked to increased risk of autoimmune disease in humans and mice possessing the HLA-DQ8 and I-A^{g7} alleles, respectively. Indeed, consistent with a recent structural report on an autoreactive TCR-I-$A^{g7}$$HEL_{11-27}$ complex (Yoshida et al., 2010), the SP3.4 TCR did not interact with residues of the P9 pocket, the P9-Glu, or the β57 position.

Despite the TRBV bias, the non-germline-encoded CDR3 loops dominated the interactions with DQ8-glia-α1; however, the mutagenesis data indicated that the CDR3β loop was dispensable for the interaction, which is consistent with its lack of sequence conservation among DQ8-glia-α1-restricted TCRs. These observations resonate with CDR3β variability in a recently described antiviral response that exhibited a biased T cell repertoire (Day et al., 2011). Instead, the energetic hot spot within the TRBV9*01 chain resided with two residues only, Leu37β and Tyr57β, in the CDR1β and CDR2β loops, respectively. Recently, the structure of a TRBV9*01^+ TCR (termed TK3) complexed to a pMHC-I has been reported, permitting a broader perspective on the role of this CDR2β loop in MHC-restricted immunity (Gras et al., 2010). Namely, Tyr57β of the TK3 TCR solely contacts the virally encoded peptide, whereas the principal role of Tyr57β in SP3.4 TCR is to mediate contacts with HLA-DQ8. This clearly highlights the variable role identical germline-encoded regions can play in MHC immunity. Indeed, it was surprising that the TRBV bias is essentially limited to two residues forming essential specificity-governing contacts with HLA-DQ8, suggesting that the TRBV9*01 usage may relate to other factors, such as TRAV-TRAJ pairing, analogous to a recently described observation (Stadinski et al., 2011; Turner and Rossjohn, 2011).

Some of the DQ8-α1-glia-restricted TCRs displayed a lesser dependence on P1 and/or P9 deamidation, suggesting that higher-affinity TCRs provide additional stability to the TCR-pMHC-II complex, which ordinarily would be provided by the greater stability of the deamidated HLA-DQ8 complex. In contrast, the LS TCR possessed much weaker affinity for the DQ8-glia-α1 complex and was more sensitive to deamidation of the α1-gliadin determinant, thereby suggesting that TCR-pMHC-II affinity is linked to the absolute requirement for deamidation dependence on the interaction. This resonates with a recent observation whereby autoimmunity can be triggered by high-affinity TCRs binding to weak self-pMHC complexes (Yin et al., 2011). Interestingly, a differing extent of deamidation dependence was observed in the three biased TRAV26-2*01-TRBV9*01 TCRs, indicating that the differing CDR3 usage can modulate the fine specificity of the DQ8-α1-glia-mediated response, which is also consistent with TRBV9*01^+ TCRs being able to recognize other gliadin determinants. Moreover, the variable degree in which the T cell clones depend on deamidation at the P1 and/or P9 position may reflect the fact that deamidation of the two Q residues at the P1 and P9 position is variable, indicating that all four variants of the DQ8-glia-α1 peptide probably exist in vivo and elicit T cell responses.

For a full understanding of celiac disease pathogenesis, it will be necessary to unravel the interactions between the lamina propria and epithelial compartments (DePaolo et al., 2011). There is no doubt that the T cell response to gluten peptides bound to HLA-DQ2 and/or DQ8 plays a crucial role. Accordingly, we report a celiac disease-associated TCR-pMHC-II ternary complex and thereby provide insight into the structural requirements that govern TCR recognition of antigenic HLA-DQ8-restricted gliadin peptides. The mode of recognition by the SP3.4 TCR is likely to be shared by a number of TRBV9*01^+ TCRs implicated in HLA-DQ8-mediated celiac disease. Thus, it may be important to target such selected TCRs in immunotherapy.

EXPERIMENTAL PROCEDURES

Antigens

Gliadin (Sigma G3375) was incubated for 4 hr at 37°C in 10-fold excess with chymotrypsin (Sigma C3142) in ammonium bicarbonate (pH 8) and finally boiled for 15 min. Protein concentration was determined by the BCA method (Pierce, USA). Deamidation with guinea pig liver TG2 (Sigma T5398) was as described previously (Anderson et al., 2000, 2005).

Isolation of Gluten-Specific T Cell Lines and Clones from Intestinal Biopsies

Polyclonal gluten-specific T cell lines were isolated from the small intestine of celiac disease patients, as described (Vader et al., 2002b). T cell clones were isolated from HLA-DQ2 or HLA-DQ8 heterozygous patients, except clones T15 and T316, which were isolated from a rare patient that expresses neither HLA-DQ2 or HLA-DQ8 but does express the HLA-DQ8 transdimer (HLA-DQ8.5) (A*0501-B*0302) (Kooy-Winkelaar et al., 2011). The study was approved by the Medical Ethics Committees of the Free University Medical Center and the Leiden University Medical Center. Written informed consent was obtained from each subject before enrollment. TRBV9*01-positive cells in peripheral blood and T cell lines and clones isolated from small intestinal biopsies were stained with a FITC-labeled monoclonal antibody specific for TRVB9 (clone BL37.2; Beckman Coulter). Cells were either analyzed for expression of TRBV9*01 or sorted into TRBV9-positive and -negative cell fractions by fluorescence activated cell sorting. See Supplemental Experimental Procedures for more information.

T Cell Proliferation Assays

Proliferation assays were performed in triplicate in 150 ml IMDM supplemented with glutamine (Life Technologies, Invitrogen, Grand Island, NY) and 10% human serum in 96-well flat-bottom plates. In brief, APCs were loaded with antigen for 2 hr, after which 15,000 T cells were added. We used 30,000 mitomycin C-treated (Sigma) EBV-LCL cells (cell line BSM, HLA-DQ8$^+$: DQA1*0301; DQB1*0302) as APCs. Unless otherwise indicated, TG2-treated gliadin (Glia-TG2) was used at a final concentration of 450 μg/ml, and synthetic peptides were used at the concentrations indicated. After 48 hr at 37°C, cultures were pulsed with 0.5 mCi [^{3}H]thymidine and harvested 18 hr later.

Peptide Library Design

A comprehensive wheat gliadin 18-mer peptide library was designed to encompass all unique 10 amino acid sequences in 131 entries for *Triticum aestivum* (bread-making wheat) α-, β-, γ-, and ω-gliadins found by searching NCBI GenBank (October 2006). The 1,363-member library was synthesized in batches of 96 peptides as Pepsets (Pepscan, Netherlands). Peptides were initially dissolved in 50% aqueous acetonitrile (v:v) to 12.5 mM (~25 mg/ml). Rather than pretreat peptides with and without TG2, the peptide library was designed to encompass all unique 10-mers with and without deamidation based on in silico deamidation according to the motifs Gln-X_1-Pro-X_3 and Gln-X_1-X_2-Y_3, where X_1 and X_3 are any amino acids except proline, Pro is proline, X_2 is any amino acid, and Y_3 is any hydrophobic amino acid (Phe, Tyr, Trp, Ile, Leu, Val, Met) (Fleckenstein et al., 2002; Vader et al., 2002a). The peptide library was assessed by IFN-γ ELISpot assay at a concentration of 25 μg/ml.

ELISpot Assay

Overnight IFN-γ ELISpot assays (Mabtech; Sweden) with 96-well plates (MSIP-S45-10; Millipore) were performed by a modification to the manufacturer's instructions as previously described (Anderson et al., 2000, 2005). See Supplemental Experimental Procedures.

TCR Isolation and Sequence Analysis

Total RNA was extracted from the LS1.2, SP3.4, and SP4.6 T cell clones via TRIzol reagent (Invitrogen). cDNA clones encoding the TCR α and β chains were produced from the total RNA with the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) according to the manufacturer's instructions. Total RNA was isolated from L3-12, S13, S16, T316, T15, and S12 T cell clones employing the RNeasy Mini Kit (QIAGEN) and cDNA synthesized with superscript reverse transcriptase III (Invitrogen, USA) according to the manufacturer's instructions. See Supplemental Experimental Procedures.

Protein Expression and Purification

TCRs were expressed, refolded, and purified with an engineered disulfide linkage in the constant domains essentially as previously described (Boulter et al., 2003; Garboczi et al., 1996). The extracellular domains of the HLA-DQ8 α and β chains were coexpressed as soluble protein in High Five insect cells (*Trichoplusia ni* BTI-TN-5B1-4 cells; Invitrogen), via a baculovirus expression system as described previously (Henderson et al., 2007a). Purified DQ8-glia-α1 was mixed in a 1:1 ratio with purified SP3.4 TCR, and the resultant SP3.4-DQ8-glia-α1 complex was then purified by size exclusion chromatography (Superdex 200; GE Healthcare).

DQ8-Glia-α1 Tetramer Production and Staining

Purified DQ8-glia-α1 protein was biotinylated on the BirA peptide substrate tag incorporated on the C terminus of the β chain by means of the BirA enzyme (Beckett et al., 1999; O'callaghan et al., 1999). Tetramer reagents were generated by the addition of NeutrAvidin R-phycoerythrin (PE) conjugate (Invitrogen) to the biotinylated DQ8-glia-α1. T cell clones to be stained by the PE-DQ8-glia-α1 tetramer were washed and resuspended in culture medium (Iscove's modified Dulbecco's medium [IMDM] [Invitrogen] supplemented with 5% [v/v] pooled human serum [PHS], 2 mM glutamine [Glutamax, Invitrogen], 5 × 10^{-5} M 2-mercaptoethanol [Sigma-Aldrich], and 100 μM nonessential amino acids [Invitrogen]) in a final volume of 50 μl. PE-DQ8-glia-α1 tetramer (50 μg/ml final concentration) was added to the cells and staining performed in the dark at 37°C for 2 hr. Cells were washed in PBS containing 0.1% (w/v) BSA and stained for 20 min on ice, with fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (BD Bioscience). Cells were washed again and resuspended in PBS with 0.1% (w/v) BSA. Propidium iodide was added to the cells immediately prior to flow cytometric analysis. Cells were analyzed on a FACSAria instrument (BD Bioscience), and in each experiment, optimal compensation and gain settings were determined with unstained and single-stained samples.

Crystallography

The SP3.4 TCR and the SP3.4-DQ8-glia-α1 complex, in 10 mM Tris (pH 8), 150 mM NaCl, were concentrated to 10 mg/ml and 5 mg/ml, respectively. In both cases, crystallization was carried out by the hanging-drop vapor-diffusion method at 20°C. Crystals of the unliganded TCR were obtained in 3.8 M sodium formate, 0.1 M Tris (pH 7.5), 5% PEG 4k. The ternary complex was crystallized in 0.2 M sodium acetate, 0.1 M MES (pH 6.5), 17% PEG 8k. Data collection, processing, structure determination, refinement, and validation were conducted with standard crystallography software (see Supplemental Experimental Procedures; Table 2).

Surface Plasmon Resonance Measurement and Analysis

Equilibrium affinity constants of the TCR-DQ8-glia-α1 interactions were determined by surface plasmon resonance with a Biacore 3000 instrument. Biotinylated DQ8-glia-α1 was immobilized at different concentrations on two flow-cell surfaces of a Biacore CAPture sensor chip (600–800 RU and 1,500–1,700 RU, respectively) and biotinylated DR4-CLIP (600-800 RU) was used as a negative control. After two 1 min injections of 1 mM biotin, equilibrium affinities were determined at 25°C in HBS buffer (10 mM HEPES-HCl [pH 7.4], 150 mM NaCl, and 0.005% surfactant P20 supplied by the manufacturer). Decreasing concentrations of each TCR were passed over all four flow cells at 10 μl/min for 2 min. The maximum concentration for all dilution series was 192 μM, with the exception of the LS TCR, for which the set-up was altered because of low protein yields (injection for 1 min at a flow rate of 5 μl/min and a maximum concentration of 93 μM). The final response curves were obtained by subtracting the signal of the untreated flow cell from the signals of each sample cell, and equilibrium response curves of each dilution series were normalized against R_{max} to account for the differential surface loading. The equilibrium dissociation constant, K_D, was subsequently obtained by fitting the combined data with Sigmaplot12 (Systat software).

ACCESSION NUMBERS

The coordinates and structure factors for the SP3.4-DQ8-glia-α1 complex and unliganded SP3.4 structures have been deposited to Protein Data Bank Japan (PDBj) with entry codes 4GG6 and 4GG8, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.07.013.

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Nexpep Pty Ltd and R.P.A. is a shareholder in ImmusanT Inc. R.P.A. is Chief Scientific Officer of and J.A.T.-D. is a consultant to ImmusanT, Inc., a company developing a peptide-based therapy and diagnostic suitable for celiac disease.

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