A TCRα framework–centered codon shapes a biased T cell repertoire through direct MHC and CDR3β interactions

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Selection of biased T cell receptor (TCR) repertoires across individuals is seen in both infectious diseases and autoimmunity, but the underlying molecular basis leading to these shared repertoires remains unclear. Celiac disease (CD) occurs primarily in HLA-DQ2.5+ individuals and is characterized by a CD4+ T cell response against gluten epitopes dominated by DQ2.5-glia-α1a and DQ2.5-glia-α2. The DQ2.5-glia-α2 response recruits a highly biased TCR repertoire composed of TRAV26-1 paired with TRBV7-2 harboring a semipublic CDR3β loop. We aimed to unravel the molecular basis for this signature. By variable gene segment exchange, directed mutagenesis, and cellular T cell activation studies, we found that TRBV7-3 can substitute for TRBV7-2, as both can contain the canonical CDR3β loop. Furthermore, we identified a pivotal germline-encoded MHC recognition motif centered on framework residue Y40 in TRAV26-1 engaging both DQB1*02 and the canonical CDR3β. This allowed prediction of expanded DQ2.5-glia-α2-reactive TCR repertoires, which were confirmed by single-cell sorting and TCR sequencing from CD patient samples. Our data refine our understanding of how HLA-dependent biased TCR repertoires are selected in the periphery due to germline-encoded residues.

Introduction

The central role of the T cell receptor (TCR) in initiation and orchestration of adaptive immune responses underlies a long-standing focus on understanding the rules governing TCR-MHC recognition. Structural studies have revealed that the TCR engages peptide-MHC (pMHC) in a conserved binding topology where the germline-encoded CDR1 and CDR2 loops generally contact the MHC helices and the somatic, flexible CDR3 loops primarily contact the peptide (1). Moreover, biased and public TCR responses have been reported for infections, and malignant and autoimmune disorders (2). In general, the molecular basis for selection of biased TCR repertoires is not well understood, but has been suggested to involve a combination of preferences in the somatic rearrangement machinery (2) and germline-encoded features that influence thymic selection (3). A recent study showed a strong trans association between variation in the MHC locus and TCR variable (V) gene usage, further suggesting a codependent expression pattern (4). However, it is still unclear if and how certain TCR V genes are predisposed to interact with MHC and underlie the existence of biased repertoires.

To address these questions, we studied celiac disease (CD), a human T cell–mediated chronic inflammatory disorder with autoimmune features affecting the small intestine and which recruits biased TCR repertoires. CD is strongly HLA dependent, where 95% of CD patients express the HLA class II molecule HLA-DQ2.5 (DQA1*05/DQB1*02), while the remaining express HLA-DQ8 (DQA1*03/DQB1*03:02) or HLA-DQ2.2 (DQA1*02:01/DQB1*02) (5). There are 2 immunodominant HLA-DQ2.5–restricted T cell
epitopes derived from wheat α-gliadin, DQ2.5-glia-α1a (PFPQPELPY) and DQ2.5-glia-α2 (PQPELPYPQ), both found within a naturally processed 33mer peptide fragment (6, 7). In addition, responses to DQ2.5-glia-α1 and DQ2.5-glia-α2 derived from α-gliadin are commonly observed (8). Notably, efficient T cell recognition of the gluten-derived peptides requires posttranslational deamidation (glutamine to glutamate) by the enzyme transglutaminase 2 (9). In particular, the DQ2.5:DQ2.5-glia-α2 response recruits a dominant and semipublic TCR repertoire characterized by TRAV26-I and TRBV7-2, coupled with a canonical CDR3β loop (ASSxRxTDTQY) (10, 11). Central in this loop is a conserved non-germline-encoded R in position 5 (pSR) that makes important contacts with DQ2.5-glia-α2 (12). Although TRAV4 combined with TRBV20-1 or TRBV29-1 appears to be overrepresented in the DQ2.5:DQ2.5-glia-α1a response, TRBV7-2 is also used in some patients (10, 12).

To gain insight into the molecular details characterizing the dominant T cell responses in CD and the selection of biased repertoires, we first analyzed TCR fine-specificity. In the case of the signature DQ2.5-glia-α2 specificity, we extended the molecular characterization to map the contribution of germline-encoded residues. We identified a single residue, Y40 of TRAV26-1, to be of critical importance for T cell activity. Importantly, from our in vitro analyses we could prospectively identify TRAV and TRBV usage in the TCR repertoire. We show that germline-encoded TCR-MHC interactions underpin the recruited chronic and pathogenic TCR repertoire in CD, alluding to why TCR-biased germline repertoires are so frequently seen in HLA-associated diseases.

## Results

**Generation of α-gliadin–specific T cell clones and TCR cloning.** The dominating HLA-DQ2.5–restricted CD4+ T cell response in CD patients is directed towards a proteolytically resistant α-gliadin 33mer peptide harboring the 3 physically coupled epitopes, DQ2.5-glia-α1a, DQ2.5-glia-α1b, and DQ2.5-glia-α2. In particular, the response towards DQ2.5-glia-α2 exhibits a prominent TCR V gene bias with public features (11). To better understand the underlying molecular details governing this response, we here studied 2 representative T cell clones (TCCs) derived from gut biopsies of 2 CD patients. The DQ2.5:DQ2.5-glia-α2–specific TCC364 uses the signature V gene pair TRAV26-I/TRBV7-2 in combination with the canonical CDR3β and has been described previously (10). The DQ2.5:DQ2.5-glia-α1a–specific TCR of TCC380 was cloned in this study and uses TRAV9-2/TRBV7-2. Upon restimulation, both TCCs responded specifically to their cognate minimal epitopes presented as exogenously loaded peptides on HLA-DQ2.5+ EBV-B cells, with TCC380 responding at a lower peptide concentration compared with TCC364 (Figure 1, A and B). Notably, the proliferative response of TCC364 increased markedly to the epitope in the form of the 33mer peptide, underscoring the potency of this naturally occurring and highly immunogenic multivalent peptide (Figure 1B) (6).

The soluble TCRs show binding characteristics previously seen for microbially derived TCRs. To elucidate the intrinsic pMHC binding properties of the TCRs 364 and 380, we first expressed and purified both TCRs in a single-chain TCR (scTCR) format containing stabilizing mutations (P50TRAV, L50TRBV, F103TRBV, and P2linker) (13, 14). We then performed surface plasmon resonance (SPR) studies employing recombinant, soluble TCRs and pMHCs. scTCR s380 bound specifically to DQ2.5:DQ2.5-glia-α1a with an average $K_d$ of 17 μM (Figure 1C, Supplemental Figure 1, and Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.95193DS1). Similarly, scTCR s364 bound specifically to DQ2.5:DQ2.5-glia-α2 with an average affinity of 38 μM (Figure 1D, Supplemental Figure 1, and Supplemental Table 1). Neither scTCR s380 nor s364 reacted with DQ2.5:CLIP2 (Figure 1, C and D). To gain insight into the TCR fine-specificity, we extended the SPR binding experiments to a panel of deamidated gliadin-pMHCs: HLA-DQ2.5 in complex with DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, and DQ2.5-glia-ω2 (Figure 1, E and F). While scTCR s380 showed a weak, concentration-dependent binding to the 3 gluten-pMHC complexes tested, scTCR s364 bound weakly to the highly similar DQ2.5-glia-ω2 only (Figure 1, G and H). Thus, the affinity of both TCRs are in the 1–100 μM range typically observed for microbially derived peptides (15, 16), as well as for other gluten-pMHC complexes (12, 17, 18), and reflected the EC50 values obtained in the T cell activation assays using the originating TCCs (Figure 1, A and B).

### 3D interactions do not predict the functional T cell reactivity

To investigate how the intrinsic binding characteristics of soluble molecules translate to the same TCR’s ability to function as membrane-
bound receptors, we performed complementary cellular assays using HLA-DQ2.5+ EBV-B cells loaded with 12mer peptides as antigen-presenting cells (APCs) and retrovirally generated BW 380 and 364 T cells (Supplemental Figure 2, A and B). The BW 380 T cell was stimulated by both deamidated DQ2.5-glia-α1a and DQ2.5-glia-ω1 peptides, but not with DQ2.5-glia-α2 or DQ2.5-glia-ω2 peptides or native counterparts (Figure 2A). Thus, despite the specific, yet weak binding across epitopes observed in SPR, only the response against the highly similar DQ2.5-glia-ω1 translated into functional activation of the BW 380 T cell. In contrast, the BW 364 T cell exclusively responded to deamidated DQ2.5-glia-α2, even though a weak binding to DQ2.5-glia-ω2 was observed in SPR (Figure 2B). The same was seen when using the longer, naturally occurring 33mer α-gliadin and ω-gliadin 17/19mer peptides (Figure 2, C and D). Both native and deamidated 33mer α-gliadin peptide stimulated the T cells, but clearly favored the deamidated version.

To further study TCR fine-specificity, we engineered murine A20 B cells to express equal amounts of HLA-DQ2.5 harboring covalently attached peptide, thereby largely bypassing effects of differences in peptide off-rates (Supplemental Figure 2C). We constructed WT peptides, as well as versions of DQ2.5-glia-α1a and DQ2.5-glia-α2 to resemble the highly similar DQ2.5-glia-ω1 and DQ2.5-glia-ω2 epitopes. When assessed for their ability to activate the BW 380 T cells, DQ2.5-glia-α1a pL7Q showed greatly reduced potency, while mutation DQ2.5-glia-α1a pY9F slightly increased responses compared with the WT peptide (Figure 2E). Similarly, pQ9W mutation of DQ2.5-glia-α2 increased the response of the BW 364 T cells exclusively responded to deamidated DQ2.5-glia-α2, even though a weak binding to DQ2.5-glia-ω2 was observed in SPR (Figure 2B). The same was seen when using the longer, naturally occurring 33mer α-gliadin and ω-gliadin 17/19mer peptides (Figure 2, C and D). Both native and deamidated 33mer α-gliadin peptide stimulated the T cells, but clearly favored the deamidated version.

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TRBV7.3 can substitute for TRBV7.2, while TRAV26-1 cannot be exchanged with its closest homolog TRAV26-2. While the DQ2.5:DQ2.5-glia-α2 TCR repertoire is characterized by a highly biased TRAV and TRBV gene segment usage (TRAV26-1/TRBV7-2), such a bias is much less prominent in the DQ2.5:DQ2.5-glia-α1a-reactive repertoire. To study the contribution of germline-encoded residues to pMHC recognition, we chose to focus on the DQ2.5:DQ2.5-glia-α2 response and we used TCR 364 as a representative clone. We were intrigued by the fact that neither TRAV26-2 nor TRBV7-3, the closest homologs of TRAV26-1 and TRBV7-2, respectively,
had appeared in the sequencing of the DQ2.5:DPQ2.5-glia-a2 TCR repertoire (10–12, 21). To understand this, we generated TCR variants where either TRAV26-1 or TRBV7-2 were exchanged with their closest homologs, while keeping the CDR3α and CDR3β as well as the J gene segments of TCR 364 unchanged (Table 1). We then introduced these modified TCRs into BW T cells (Supplemental Figure 3, A and B). When assessed for their ability to respond to the 33mer peptide, we observed a small decrease in reactivity when TRBV7-2 was exchanged with TRBV7-3 (Figure 3, A and B). In contrast, the exchange of TRAV26-1 with TRAV26-2 completely abolished activation of the T cells (Figure 3, A and B). As the CDR3 loops of the WT and the TCR 26 mutants were identical, the observed difference in DQ2.5:DPQ2.5-glia-a2 reactivity most likely arose from differences in germline-encoded V gene residues.

A single germline-encoded TRAV residue is a crucial determinant for T cell responsiveness. We then constructed and tested a panel of TCR variants (TCR1–13) where TRAV26-1 or TRBV7-2 residues were exchanged with the corresponding TRAV26-2 or TRBV7-3 residues (all mutated residues are specified in Table 1, and Figure 4, A and B, and clone validation shown in Supplemental Figure 4, A and B). Mutations within TRAV26-1 resulted in more profound effects on T cell activation (Figure 4C and Supplemental Figure 4C) than the mutations within TRAV26-2, which were used. Representative responses of (A) BW 380 T cells and (B) BW 364 T cells are shown. (A and B) HLA-DQ2.5+ EBV-B cells were loaded with titrated amounts of 12mer peptides as indicated before addition of BW T cells. Both deamidated (E) and native (Q) peptides were used. Representative responses of (C) BW 380 T cells and (D) BW 364 T cells are shown. (C and D) HLA-DQ2.5+ EBV-B cells were loaded with titrated amounts of 33mer α-gliadin peptide (E or Q) containing both the DPQ2.5-glia-a1 and DPQ2.5-glia-a2 epitopes, or with 17mer/19mer α-gliadin peptide (E and Q, respectively) containing both the DPQ2.5-glia-a1 and DPQ2.5-glia-a2 epitopes. Representative IL-2 responses of (E) BW 380 T cells and (D) BW 364 T cells are shown. (E and F) Titrated amounts of A20 B cells expressing HLA-DQ2.5 with covalently linked peptide were used as antigen-presenting cells (APCs) to stimulate (E) BW 380 T cells and (D) BW 364 T cells. Wo = without T and A are controls with T cells or APCs with peptide alone. Error bars indicate ± SD of triplicates. (A–F) The experiment in each panel was performed 3 independent times. Figures were prepared using GraphPad Prism 7, and nonlinear regression analysis (3 parameters) was used to derive IL-2 concentrations from the standard curves.

### Table 1. Sequence alignment of TRAV26-1 and TRBV7-2 with their closest homologs

<table>
<thead>
<tr>
<th>V-gene(^a)</th>
<th>FR1</th>
<th>CDR1</th>
<th>FR2</th>
<th>CDR2</th>
<th>FR3</th>
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<tbody>
<tr>
<td>TRAV26-1</td>
<td>DAKTQ.PPSMBCAE GGRAALPCHNS</td>
<td>TSC.........TEY</td>
<td>VVYVRQKIH SQGQPQYIH</td>
<td>GLK.....NN</td>
<td>ETNE.....MLLHEIDR KSTTLILPHATL ROTAIVYC</td>
</tr>
<tr>
<td>TRAV26-2(^2)</td>
<td>DAKTQ.PNSMNEEPEPVI PCCHNS</td>
<td>TSC.........TEY</td>
<td>HVYRPLQ SQGQPQYIH</td>
<td>GLT.....SN</td>
<td>VNHR.....MLLHEIDR KSTTLILPHATL ROTAIVYC</td>
</tr>
<tr>
<td>TRBV7-2</td>
<td>GACVSQPSNKVTEK GKVDELRCPI</td>
<td>SGH.........TA</td>
<td>LVYVRQSL QGGLFELFY</td>
<td>FQG.....NSA</td>
<td>PDSGLPKns DISLAEETF GSVSTLTQQTQ EAIVYC</td>
</tr>
<tr>
<td>TRBV7-3(^3)</td>
<td>GACVSQPSNKVTEK GKVDELRCPI</td>
<td>SGH.........TA</td>
<td>LVYVRQSL QGGLFELFY</td>
<td>FQG.....TGA</td>
<td>AEDCGLPND RFAVNP EG SVSTK QRTQ EAIVYC</td>
</tr>
</tbody>
</table>

\(^a\)TRAV gene segment usage and numbering was defined by the IMGT Database. \(^\)Closest homolog gene fragments were identified using the IMGT database. TRAV26-2*01 and TRBV7-3*01 were chosen. Amino acids differing between TRAV26-1/TRAV26-2 and TRBV7-2/TRBV7-3, which were mutated in TCR 364, are illustrated with colors in the TRAV26-1/TRBV7-2 sequences corresponding to the different mutants in Figure 4 and in bold and underlined in the TRAV26-2/TRBV7-3 sequences.
in TRBV7-2 (Figure 4D and Supplemental Figure 4C). TCR3 that contained 6 framework (FR) 2 mutations was completely nonresponsive (Figure 4C). The second most pronounced effect was seen in TCR7, where residues of CDR1, CDR2, and FR3 were exchanged for those of TRAV26-2 (Figure 4C).

To dissect the individual effects of these regions, they were altered separately in TCR2, TCR4, and TCR5, respectively. Exchange of N36TTRAV and E37DTRAV within the CDR1α of TCR2 led to a modest decrease in T cell responsiveness, as did K58TTRAV and N64STRAV within the CDR3α of TCR4. The FR3α mutations of TCR5 had little impact. For the TRBV variants, only small changes in EC50 values relative to WT364 were observed, as could be expected from the exchange of TRAV7-2 with TRBV7-3 (Figure 3 and Figure 4D).

To map the structural basis for the observed effects of the mutations, we inspected the cocrystal structure of TCR S16 using TRAV26-1/TRBV7-2 and the canonical CDR3β bound to DQ2.5:DQ2.5-glia-α2 (12). Of the 6 FR2 residues mutated in TRAV26-1, residue Y40TRAV participates in an extensive network of polar interactions and directly interacts with both R70MHCβ and T115 TRBV of the canonical CDR3β. Both of these interactions are lost by the Y40H mutation in TCR3 (Figure 5A). Additional indirect effects on the H-bond network upon mutation, including the R70MHCβ-T113TRBV interaction cannot be excluded.

The reduced responsiveness of TCR2 and TCR4 could both be due to loss of direct interactions; a H-bond between N36TRAV of CDR1α and p2Q is lost by the N36T mutation of TCR2 (Figure 5B), and the K58TTRAV mutation within CDR2α of TCR4 abrogates the salt bridge between K58TRAV and D76MHCα (Figure 5C).

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Finally, to validate if the effect solely resided in Y40TRAV, we constructed the single Y40H mutant. In line with predictions, the BW 364 Y40H mutant was completely nonresponsive to pMHC (Figure 5D). Taken together, these results show that the germline sequence of TRBV7-2 does not contain features explaining its selection over TRBV7-3. In contrast, the single residue, Y40TRAV, which differs between TRAV26-1 and TRAV26-2, is crucial for dictating T cell responsiveness.

TRAV26-1/TRBV7-3 is used in the in vivo repertoire, whereas TRAV26-2 is not. Our data clearly show that TRBV7-2 and TRBV7-3 are interchangeable. Thus, we hypothesized that DQ2.5:DQ2.5-glia-α2-reactive T cells using TRBV7-3 with the canonical CDR3β paired with TRAV26-1, should be present in the repertoire of HLA-DQ2.5+ CD patients, and that TRAV26-2 should not be found paired with such TRBVs harboring the canonical CDR3β.

To this end, we tetramer-sorted CD4+ T cells from blood of 9 gluten-challenged CD patients followed by single-cell coupled sequencing of TCR and TCRβ genes (Louise Fremgaard Risnes, unpublished observations). Notably, these cells were sorted using a pool of HLA-DQ2.5:gluten tetramers consisting of 4 epitopes. After filtering the sequence data for TRAV26-1 paired with the canonical CDR3β found in DQ2.5:DQ2.5-glia-α2-reactive T cells only, we found that TRBV7.3 was in fact used, but 5 times less frequently than TRBV7.2 (Table 2). Interestingly, this difference corresponds well with the relative expression of TRBV7-2 and TRBV7-3 as reported in the naive repertoire (22, 23). Both the canonical CDR3β and the highly similar loop were formed by recombination of TRBV7-2 or TRBV7-3 with TRBJ2-3. Taken together, these results show that analysis of TCR V gene usage in CD patients sup-
A critical dependency on Y40\textsuperscript{TRAV} for binding to HLA-DQ2.2, which is highly similar to HLA-DQ2.5, would strongly indicate that the trio of amino acids engages HLA-DQ2.2 in a similar manner as to HLA-DQ2.5. With respect to the DQ2.5-glia-\(\alpha\)-2 epitope presentation, HLA-DQ2.2 and HLA-DQ2.5 have been shown to be functionally identical, with the exception of kinetically segregating the T cell response due to pMHC stability differences (24). To experimentally test this, we used cells expressing HLA-DQ2.2 with covalently linked peptide. Indeed, the Y40H mutation completely abrogated recognition of HLA-DQ2.2 peptide (Figure 5E). Taking these results together, we suggest that this trio of amino acids (Y38\textsuperscript{TRAV}, Y40\textsuperscript{TRAV}, and H55\textsuperscript{TRAV}) constitutes a recognition motif shared by multiple DQ2.5:DQ2.5-glia-\(\alpha\)-2–reactive T cells using TRBV7-2/7-3 with the canonical CDR3\(\beta\). Indeed, 7 such clonotypes were found that paired with other TRAVs than TRAV26-1; one used TRAV5 and one used TRAV39, while the others used different gene segments (Table 2, Supplemental Tables 2 and 3). Interestingly, all except 1 of the 7 also contained the Y38\textsuperscript{TRAV} characteristic of the trio of amino acids in TRAV26-1 (Supplemental Table 2).

Table 2. TRAV and TRBV7 pairing in CD patients

<table>
<thead>
<tr>
<th>Chain pairing</th>
<th>No. of clonotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total TRAV26-1/ TRBV(x)</td>
<td>82</td>
</tr>
<tr>
<td>TRAV26-1/ TRBV7-2 (^a)</td>
<td>52</td>
</tr>
<tr>
<td>TRAV26-1/ TRBV7-2</td>
<td>39</td>
</tr>
<tr>
<td>TRAV26-1/ TRBV7-2</td>
<td>6</td>
</tr>
<tr>
<td>TRAV26-1/ TRBV7-3 (^b)</td>
<td>9</td>
</tr>
<tr>
<td>TRAV26-1/ TRBV7-3</td>
<td>8</td>
</tr>
<tr>
<td>TRAV26-1/ TRBV7-3</td>
<td>0</td>
</tr>
<tr>
<td>Total TRAV26-2/ TRBV(x)</td>
<td>6</td>
</tr>
<tr>
<td>TRAV26-2/ TRBV7-2</td>
<td>0</td>
</tr>
<tr>
<td>TRAV26-2/ TRBV7-3</td>
<td>0</td>
</tr>
<tr>
<td>Total TRAV(x)/ TRBV7-2/ TRBV7-3</td>
<td>0</td>
</tr>
</tbody>
</table>

Sequencing of CD4\(^+\) T cells from blood of 9 HLA-DQ2.5\(^+\) CD patients after gluten challenge identified 536 unique clonotypes. \(^a\)TRAV26-1/ TRBV7-2 or TRAV26-2/ TRBV7-3 irrespective of CDR3 sequence. \(^b\)Sequences from \(^a\) were filtered on the canonical (can) CDR3\(\beta\) that is restricted to DQ2.5-glia-\(\alpha\)-2–reactive T cells.

A critical dependency on Y40\textsuperscript{TRAV} for binding to HLA-DQ2.2, which is highly similar to HLA-DQ2.5, would strongly indicate that the trio of amino acids engages HLA-DQ2.2 in a similar manner as to HLA-DQ2.5. With respect to the DQ2.5-glia-\(\alpha\)-2 epitope presentation, HLA-DQ2.2 and HLA-DQ2.5 have been shown to be functionally identical, with the exception of kinetically segregating the T cell response due to pMHC stability differences (24). To experimentally test this, we used cells expressing HLA-DQ2.2 with covalently linked peptide. Indeed, the Y40H mutation completely abrogated recognition of HLA-DQ2.2 peptide (Figure 5E). Taking these results together, we suggest that this trio of amino acids (Y38\textsuperscript{TRAV}, Y40\textsuperscript{TRAV}, and H55\textsuperscript{TRAV}) constitutes a recognition motif shared by multiple DQ2.5:DQ2.5-glia-\(\alpha\)-2–reactive TCRs. Importantly, this MHC recognition motif is engaged by virtue of also involving the public CDR3\(\beta\), thereby influencing chain pairing.

Discussion

The existence of biased TCR repertoires with preferential usage of certain TRAV and TRBV gene segments has emerged as a common phenomenon in adaptive immunity and has been described in the T cell response against gluten epitopes presented on both HLA-DQ2.5 and HLA-DQ8 in CD (10–12, 21, 25). To broaden our understanding of the molecular basis for the selection of biased TCR repertoires, we undertook a combination of affinity, directed mutagenesis, and cellular activation studies, complemented with structural analysis.

As a starting point, we mapped the intrinsic binding properties of 2 prototypic, patient-derived TCRs against the dominant \(\alpha\)-gliadin epitopes, DQ2.5-glia-\(\alpha\)-1 and DQ2.5-glia-\(\alpha\)-2. There was a surprising discrepancy between specificity at the soluble protein level and the corresponding functional T cell reactivity. While TCR 364 preferentially bound DQ2.5-glia-\(\alpha\)-2 in SPR, a weak reactivity was observed towards DQ2.5-glia-\(\alpha\)-2. Stilt, the BW 364 T cells responded solely to DQ2.5-glia-\(\alpha\)-2 in cellular assays. In contrast, the BW 380 T cells responded to both DQ2.5-glia-\(\alpha\)-1 and DQ2.5-glia-\(\alpha\)-1, even though DQ2.5-glia-\(\alpha\)-1 was only 1 of a set of gluten peptides with weak binding to TCR 380 in SPR. Consistent with this, a substantial fraction of DQ2.5:DQ2.5-glia-\(\alpha\)-1–sorted TCCs from CD patients cross-react
with DQ2.5:DQ2.5-glia-o1 (Shiva Dahal-Koirala, unpublished observations). To our surprise, pL7Q and pY9F mutations of DQ2.5-glia-a1a to resemble DQ2.5-glia-o1 resulted in greatly reduced and increased T cell activation, respectively. A previous alanine scan showed a complete dependence on p7, whereas all other positions resulted in clone-dependent effects, even though the TCRs presumably do not directly interact with p7L (12). We speculate that the presence of a hydrophobic amino acid in p9 anchors the peptide more efficiently to the p9 pocket. Thus, recognition of DQ2.5-glia-o1 by TCR 380 seems to be due to a compensatory mechanism. Similarly, mutations of p5 and p7 completely abrogated activation of the BW 364 T cells in support of previous observations (12), whereas mutation of p9 to a hydrophobic anchor residue increased responses, albeit not compensating for the negative effects of the p5 and p7 mutations. Based on our data, TCR 380 may have been selected by either of the DQ2.5-glia-a1a or DQ2.5-glia-o1 epitopes in vivo.

TRAV26-1 paired with TRBV7-2 is characteristic of the DQ2.5:DQ2.5-glia-a2 response, and the closest homologs, TRAV26-2 and TRBV7-3, had not been observed using biased or unbiased approaches (10–12, 21). A combination of structural and mutagenesis data had suggested that a germline imprint of TRAV26-1 was important for recognition of HLA-DQ2.5, as mutation of Y38, Y40, and L57 in TRAV26-1 markedly affected affinity in SPR (12). Our data clearly show that SPR is not suitable to predict functional outcome. Thus, we here took on a cellular approach to map germline contributions. Exchange of TRAV26-1 with TRAV26-2 abolished T cell activation in our studies, presumably through a combination of direct disruption of MHC (R70H) and TCRβ interactions (T115TRBV of the canonical CDR3β) mediated by the FR2 residue Y40. Y38 and L57 are both present in TRAV26-2, and therefore cannot explain the preference for TRAV26-1 over TRAV26-2 (12). In contrast to the strong dependence on TRAV26-1, exchange of TRBV7-2 with TRBV7-3 only weakly affected pMHC recognition. Dissection of the contribution of residues differing between TRBV7-2/7-3 revealed only minor effects, in line with the small molecular imprint of TRBV7-2 on MHC, where P66TRBV is the only germline-encoded residue to interact with MHC through 2 H-bonds (12).

Based on these findings, we hypothesized that large-scale single-cell sequencing of the DQ2.5:DQ2.5-glia-a2 TCR repertoire would reveal the presence of T cells using TRBV7-3 and that TRAV26-2 would be absent. We did indeed find TRBV7-3 to be present, and the TRAV26-1/TRBV7-3 T cells formed the canonical CDR3β. Furthermore, the difference in the representation of TRBV7-2 versus TRBV7-3 mirrored the abundance of these gene segments in the naive repertoire (22, 23). Whether this is a general feature of antigen-expanded T cell populations remains unclear, but a previous study found that antigen-driven expansion of CD4+ T cells was reflected by the frequency of naive progenitors (26). Moreover, we found TRAV26-2 to pair with neither TRBV7-2 nor TRBV7-3. Interestingly, use of TRBV7-2/7-3 and the canonical CDR3β is almost always coupled with TRAV26-1, whereas use of TRBV7-2 without the canonical CDR3β show no pairing bias with TRAV26-1 or any other particular TRAVs (11). This indicates that the preferential pairing is not due to features in the Vα/Vβ interphase, but rather influenced by the presence of the canonical CDR3β, consistent with the direct CDR3β T115 interaction with Y40 of TRAV26-1 and the H-bond network further extending to T115TRBV and R70H. We propose that the crucial interactions TRAV26-1 Y40 makes with HLA-DQ2 and the canonical CDR3β is the driver for the selection of this biased repertoire.

Recently, an association was found between expression of TRAV26-1 and DQB1*02:01 (β-chain of HLA-DQ2.5), whereas this was not the case with TRAV26-2 (4). We therefore hypothesize that the trio of amino acids, Y38, Y40, and H55 of TRAV26-1, constitute a germline-encoded recognition motif with specificity towards HLA-DQ2 (2). This same notion has been extensively investigated for TRBV-centric TCRs using Vβ8.2 (TRBV13-2), where a trio of amino acids in the CDR1β and CDR2β were suggested to represent an evolutionary signature of germline specificity, forming close to the same contacts with distinct MHC alleles (IA1, IA2, and IA3) irrespective of peptide and TRAV (27–31). As in our case, TRAV-centric germline codons have also been previously suggested for the Vα3-containing (TRAV9D-3/TRAV9-4) TCRs 2C and 42F3 specific for H2-Ld (32). It appears that amino acids in CDR1 and CDR2 can contribute to different MHC interaction modes influenced by the CDR3 regions (30). Our data further expand our understanding of the motifs involved and how the tuning of the recognition might occur, as we here report a TRAV FR residue (Y40) that coordinates the interaction network. The results explain the selection and expansion of the public T cell repertoire shared across patients. Of the 7 clonotypes we identified that harbored TRBV7-2/7-3 with the canonical CDR3β, but did not pair with TRAV26-1, two used TRAV5 and TRAV39 that both encode Y40. Y38 of the interaction trio is present in 6 out of 7 clonotypes, and it is not unlikely
Table 3. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAC_rv</td>
<td>5′-AGTCAGATTTGTGCTCCAGGCG-3′</td>
</tr>
<tr>
<td>TRBC_rv</td>
<td>5′-TTCAACCCACGACTAGCTCC-3′</td>
</tr>
<tr>
<td>poly-G-NotI_fw</td>
<td>5′-ATAGGCGCCGGGAGGGGGGGGGGG-3′</td>
</tr>
<tr>
<td>TRAC_MluI_rv</td>
<td>5′-ATACCGGTTCCTCAGCGATCAGGG-3′</td>
</tr>
<tr>
<td>TRBC_MluI_rv</td>
<td>5′-ATACCGGTATGACCTGTCGACG-3′</td>
</tr>
<tr>
<td>TRAV_HindII_rv</td>
<td>5′-CTGCTGGCAAAAAATACACGGCCGAGTCCTCCTG-3′</td>
</tr>
<tr>
<td>TRAV_NcoI_fw</td>
<td>5′-CAGGAGGACTCGGCCGTGTATTTTTGTGCCAGCAG-3′</td>
</tr>
<tr>
<td>TRBV_MluI_rv</td>
<td>5′-GGCGGATGCACTCCCTGACGGCTTAGGTAAAACAGTCA-3′</td>
</tr>
<tr>
<td>TRBV_NotI_rv</td>
<td>5′-TGACTGTTTTACCTAAGCCGTCAGGGAGTGCATCCGCC-3′</td>
</tr>
<tr>
<td>TRBV_L50P_fw</td>
<td>5′-GGCTTTCAGGAGGAGCTGCGGACCTTCTCCAGGATATT-3′</td>
</tr>
<tr>
<td>TRBV_L50P_rv</td>
<td>5′-ATATGCGGCCGCTGTGACCGTGAGCCTGG-3′</td>
</tr>
<tr>
<td>Linker_L2P_rv</td>
<td>5′-ATATACGCGTAGGAGCTGGAGTCTCCCAG-3′</td>
</tr>
<tr>
<td>TRBV_L103F_fw</td>
<td>5′-ATCCATGGCCGGAGATTCAGTGACCCAGATGG-3′</td>
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<tr>
<td>TRBV_L103F_rv</td>
<td>5′-ATACGCGTTCTCTCAGCTGGTACACGG-3′</td>
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<tr>
<td>TRBV_FW</td>
<td>5′-ATATGCGGCCGCGGGGGGGGGGGGGG-3′</td>
</tr>
<tr>
<td>TRBV_rv</td>
<td>5′-TTCACCCACCACGACTAGCTCC-3′</td>
</tr>
</tbody>
</table>

Methods

Isolation of T cells from CD patients and TCR cloning and expression. TCCs were established from intestinal biopsies of CD patients (CD364 and CD380) as previously described (36). Briefly, T cells were expanded with anti-CD3 and anti-CD28 beads (Invitrogen) for a minimum of 7 days prior to mRNA extraction and TCR cloning. mRNA was isolated from TCC380.E48 (herein denoted TCR 380) using an Absolutely RNA miniprep kit (Stratagene), followed by a seminested PCR approach. Briefly, first-strand cDNA synthesis was performed using gene-specific TRAC_rv and TRBC_rv primers (Table 3) and SuperScript II reverse transcriptase (Invitrogen). cDNA was precipitated using Nuclease (NEB), followed by poly dCTP-tailing of the cDNA at the 3′-end with rTerminal transferase (Roche) and reprecipitation. The Vα and Vβ were PCR amplified using Phusion HotStart DNA polymerase (Finnzymes) and the poly-G-NotI_FW primer and either the TRAC_MluI_rv or TRBC_MluI_rv primer (Table 3) annealing in the TCR constant domains. The PCR products were restriction enzyme (RE) digested and ligated into the RE-digested pFKPEN vector (37) and verified by sequencing. Genespecific primers were designed (TRAC_Ncol_FW and TRBV_HindII_rv for Vα; TRBC_MluI_rv for Vβ; Table 3) and the exact V domains were re-amplified, followed by ligation into RE-digested pFKPEN, generating the scTCR-WT380 (Vα-linker-Vβ) construct followed by c-Myc and His6 tags. To generate pFKPEN encoding the stabilized scTCR s380 (P50[TRAV], L50[TRBV], F103[TRBV], and P2[TRBV]), the scTCR-WT380 vector was used as template for QuikChange site-directed mutagenesis using TRAV_L50P_FW/TRAV_L50P_RV, Linker_L2P_FW/Linker_L2P_RV, and TRBV_L103F_FW/TRBV_L103_F_RV primer pairs (Table 3). TCR 364.1.0.14 (herein denoted 364) and scTCR s364 have been described before (10). All constructs were verified by sequencing.

Soluble periplasmic TCR expression and purification. For soluble periplasmic expression of TCR, cells were inoculated from glycerol stocks into 1 liter of LB-TAG medium (1× LB medium supplemented with 30 μg/ml tetracycline, 100 μg/ml ampicillin, and 0.1 M glucose) and incubated overnight at 37°C/220 rpm. After inoculation to an OD₆₀₀ of 0.025, the cultures were further grown until an OD₆₀₀ of 0.6–0.8. Cells were harvested by centrifugation (3,220 g for 10 minutes) and resuspended in 1× LB-TA. Notably, we abstained from IPTG induction of the LacPO promoter. After overnight incubation at 30°C/220 rpm, cells were pelleted at 3,220 g for 30 minutes and the resulting cell pellets were resuspended in 80 ml ice-cold periplasmic extraction solution (50 mM Tris-HCl, 20% sucrose, 1 mM EDTA) supplemented with 0.1 mg/ml RNase A and 1 mg/ml hen egg lysozyme and incubated 1 hour at 4°C with rotation. After centrifugation, the supernatant containing the periplasmic content was filtered (0.22 μm) before purification.
TCRs were purified by IMAC purification according to the manufacturer's protocol (HiTrap HP, GE Healthcare) followed by size-exclusion chromatography using either a HiLoad 26/600 Superdex 200 column or a Superdex 200 10/30 GL (both GE Healthcare). A final polishing step of the samples on a ResourceQ or Superdex 75 10/300 GL (both GE Healthcare) were performed when needed. Columns were run in PBS supplemented with 150 mM NaCl and protein concentration was determined using the MW and extinction coefficient of each individual protein. Purified samples were verified by analytical gel filtration using either a Superdex 200 10/300 GL column or a Superdex 200 PC3.2/30 column (both GE Healthcare).

Expression and purification of soluble pMHC. Recombinant HLA-DQ2.5 with the gluten-derived peptides containing the deamidated T cell epitopes DQ2.5-glia-α1a (QLOPFPQPFPY, underlined 9mer core sequence), DQ2.5-glia-α2 (POPELPQPQP), DQ2.5-glia-α1 (QPPOPEPQPP), DQ2.5-glia-α2 (FPPOPEPFPWQP), and DQ2.5-CLIP2 (MATPPLLMOALPMGAL) coupled to the N-terminus of the DQ2.5 β-chain via a thrombin-cleavable linker peptide was expressed in insect cells using a baculovirus expression vector system as previously described (33, 38). Soluble, recombinant pMHC was affinity purified using mAb 2.12.E11 specific for the HLA-DQ2 β-chain (39). After site-specific biotinylation using BirA (Avidi), pMHCs were purified using a Superdex 200 GL10/30 run in PBS.

SPR. SPR was performed on either a Biacore T100 (T200 sensitivity enhanced) or Biacore T200 (GE Healthcare) by immobilizing Neutravidin (10 μg/ml in acetate buffer pH 4.5) on a CM3 Series S sensor chip by amine coupling to 1,000 resonance units (RU). One hundred or 300 RU of pMHC was captured on the chip surface, followed by injections of dilution series of TCR using a single-cycle kinetics or multi-cycle kinetics method (data collection rate 10 Hz). For experiments determining kinetics, 4-fold dilutions from 50 μM or 3-fold dilutions from 55 μM were used, and for cross-reactivity experiments, 3-fold dilutions from 30 μM were used. All experiments were performed at 25°C with a flow rate of 30 μl/min and using 1× PBS supplemented with 150 mM NaCl and 0.05% surfactant P20. Glycine-HCl pH 2.7 was used to regenerate the sensor chip surface. All data were zero-adjusted and the Neutravidin reference flow cell value was subtracted before evaluation with T200 Evaluation Software, version 1.0 and RI set to constant. A 1:1 Langmuir binding model was used for determination of $K_D$. Figures were prepared using GraphPad Prism 7 and graphs were smoothed by averaging 8 neighbors.

Retroviral TCR transduction of hybridoma T cells and FACS sorting of transduced cells. The BW58 αβ human CD4+ T cell hybridoma line devoid of endogenous TCR (herein denoted BW T cells, obtained from Bernard Malissen, Centre d’Immunologie de Marseille-Luminy, Marseille, France) (40) was retrovirally transduced as described before to express TCR 380 and the mutant TCR 364 versions (10). Synthetic, codon-optimized DNA encoding the TCRα and TCRβ V domains (Genscript) fused to murine TCR constant domains was cloned into pMIG-II-TCRα-P2A-TCRβ-eGFP retroviral plasmid (obtained from Dario Vignali, University of Pittsburgh, Pittsburgh, Pennsylvania, USA) (41) between the EcoRI/BamHI site or BamHI/XhoI sites, respectively. The assembly connects the TCR α- and β-chains through a P2A peptide linker. The pMIG-II-TCR plasmids were cotransfected (Lipofectamine 2000, Invitrogen) together with the pCL-Eco plasmid into GP2-293 packaging cells (Clonetech). Virus supernatants were collected 48 and 72 hours after transfection, followed by centrifugation and filtration (0.45 μm) to remove cell debris. BW T (50,000 cells) were incubated with 1 ml virus-containing supernatant supplemented with 10 μg/ml polybrene and subjected to centrifugation (3,000 g) at 32°C for 90 minutes. Following removal of the supernatant, the BW T cells were cultured in RPMI with 10% FCS. To ensure equal TCR expression levels, cells were stained with hamster anti–mouse TCR α/β Alexa-647 mAb (1:20, clone H57/597, Life Technologies) and sorted using a FACSAria II (BD Biosciences) based on eGFP and α/β-PE or 1:20, clone H57/597, Life Technologies) and sorted using a FACSAria II (BD Biosciences) based on eGFP and α/β-PE or 3-fold dilutions from 55 μM were used, and for cross-reactivity experiments, 3-fold dilutions from 30 μM were used. All experiments were performed at 25°C with a flow rate of 30 μl/min and using 1× PBS supplemented with 150 mM NaCl and 0.05% surfactant P20. Glycine-HCl pH 2.7 was used to regenerate the sensor chip surface. All data were zero-adjusted and the Neutravidin reference flow cell value was subtracted before evaluation with T200 Evaluation Software, version 1.0 and RI set to constant. A 1:1 Langmuir binding model was used for determination of $K_D$. Figures were prepared using GraphPad Prism 7 and graphs were smoothed by averaging 8 neighbors.

Retroviral TCR transduction of A20 B cells and FACS sorting of transduced cells. The murine A20 B cell line was retrovirally transduced to express HLA-DQ2.5 or HLA-DQ2.2 with different covalently coupled peptides. Notably, the ectodomains were made to be identical to the soluble, recombinant molecules. Epitope mutants were generated by mutating single positions in the DQ2.5-glia-α1a or DQ2.5-glia-α2 constructs. Codon-optimized synthetic DNA (Genscript) encoding the different peptides coupled to the N-terminal end of the HLA-DQ2.5 β-chain (DQΒ1*02:01) through a peptide linker was cloned as BamHI/XhoI fragment into the pMIG-II-DQ2.5-eGFP retroviral plasmid already encoding the HLA-DQ2.5 α-chain (DQA1*05:01). HLA-DQ2.2 with DQ2.5-glia-α2 was constructed by exchanging DQA1*05:01 with DQA1*02:01 clones as a BglII/BamHI fragment (Genscript). After assembly, the HLA-DQ2.5/2.2 α- and β-chains are connected through a P2A peptide linker. The pMIG-II-eGFP-peptide-DQ2.5/2.2 plasmids and the pAmpho plasmid
were cotransfected into GP2-932 packaging cells as for the TCR transductions. A20 (50,000 cells) were incubated with 1.3 ml of virus-containing supernatant. After 5 days of culturing in RPMI/10% FCS, A20 cells expressing peptide-HLA-DQ2.5/2.2 were sorted (FACSria II) based on eGFP expression level. The A20 B cells were cultivated in RPMI/10% FCS. Data were analyzed using FlowJo software V10.

**Evaluation of TCR and HLA-DQ2-peptide transductants by flow cytometry.** To assess TCR and CD4 expression levels on the BW T cell variants, 3 × 10^5 cells were stained with hamster anti–mouse TCR α/β Alexa-647 mAb (1:20, clone H57/597, Life Technologies), anti–human CD4-PerCP-Cy5.5 mAb (1:20, clone RPA-T4, eBioscience), or isotype control mAbs (1:20, catalog number MCA2357A647, AbD Serotec; 1:160, clone P3.6.2.8.1, eBioscience). For validation of pMHC expression level on the A20 B cell variants, 2 × 10^5 cells were stained with biotinylated mAb 2.12.E11 (clone RPA-T4, eBioscience), or isotype control mAbs (1:20, catalog number MCA2357A647, AbD Serotec; 1:160, clone P3.6.2.8.1, eBioscience). Samples were fixed using a 2% paraformaldehyde solution and acquired on a FACSAirul (BD) and analyzed by FlowJo software V10.

* T cell activation assays. For T cell activations assays using peptide-loaded APCs, 60,000 HLA-DQ2.5*1 EBV-transduced human B cells (CD114 derived from a DR3-DQ2.5 (DQA1*05:01/DQB1*02:01) homozygous CD patient) were incubated in RPMI/10% FCS at 37°C for 1–4 hours with titrated amounts of peptide (as indicated in the figures) before addition of 30,000 BW T cells. The following native (Q) or deamidated (E) gliadin peptides were used (epitopes are underlined, except in the long peptides that contain several overlapping epitopes): DQ2.5-glia-α1Q (QLQPFPQPQPQP), DQ2.5-glia-α1AE (QLQPFPQPQPQP), DQ2.5-glia-α2Q (QPQPQPQPQPQP), DQ2.5-glia-α2E (QPQPQPQPQPQP), DQ2.5-glia-ωE (FPQPQPQPQPQPQP), α33merQ (QLQPFPQPQPQPQPQP), α19merQ (PQPQPQPQPQP), and ω17merE (QPQPQPQPQPQPQPQP). In assays using TCC380 and TCC364, the 12mer DQ2.5-glia-α1AE and DQ2.5-glia-α2E peptides and the 33merE peptide were used. In T cell activation assays using murine A20 cells transduced with HLA-DQ2.5/2.2 covalently linked peptide, titrated amounts of APCs were incubated with 30,000 BW T cells as indicated in the figures. As a control, Cell Stimulation Cocktail containing PMA and ionomycin (eBioscience, 1:500) was added to wells containing BW T cells only. Culture supernatants (25 μl) were assayed for murine IL-2 secretion after overnight incubation by ELISA (Amersham). Plates were blocked with PBS–2% FCS and recombinant mouse IL-2 (Biolegend) was used as standard. T cell activation assays with thymidine incorporation as readout was performed essentially as described above, except for the following: APCs (CD114) were irradiated with 75 Gy before overnight incubation with titrated amounts of peptide at 37°C, before addition of 50,000 T cells (TCC380 and TCC364) in medium containing 10% heat-inactivated, pooled human serum. [3H]Thymidine (1 μCi/ml; see ref. 39) specific for the HLA-DQ2.5/2.2 β-chain (in-house biotinylated), followed by streptavidin Alexa-647 (1:200, Invitrogen). Nonspecific binding to FCγ receptors was blocked with anti–mouse–mouse CD16/32 (1:200, BD Pharmingen). Samples were fixed using a 2% paraformaldehyde solution and acquired on a FACSAirul (BD) and analyzed by FlowJo software V10.

**Analysis of single-cell TCR sequencing data.** The single-cell TCR sequencing data generated in a gluten challenge study (Louise Fremgaard Risnes, unpublished observations) was used for analysis of V gene usage and pairing in 9 CD patients. The cells were isolated using a pool of DQ2.5:DQ2.5-glia-α1, DQ2.5:DQ2.5-glia-α2, and DQ2.5:DQ2.5-glia-ω2 tetramers (10 μg/ml each), directly ex vivo from blood samples. The IMGT/HighV-QUEST online tool (imgt.org/HighV-QUEST/) was used for identifying V, D, and J genes and CDR3 junction sequences. T cells with at least 1 productive TRA and TRB pair (allowing dual on either TRA or TRB, maximum 3 chains) with dupcount greater than 100 were defined as valid cells. The valid cells with identical V and J gene (subgroup level) together with identical CDR3 region (allowing for 1 nucleotide mismatch) were defined as clonotypes. These unique clonotypes were used for comparative analysis of TRBV7-2, TRBV7-3, TRAV26-1, and TRAV26-2 pairing and usage in the entire TCR repertoire. All sequencing data are deposited in the NCBI Sequence Read Archive under accession number SRP102402.
Molecular interaction studies. Molecular structures of TCR and pMHC were prepared using PyMol v1.8.4.2 using the crystal structure of TCR S16 bound to DQ2.5:DQ2.5-gli-a2 (PDB: 4OZH) (12). Sequence alignments and residue nomenclature were assigned by use of the IMGT database (http://www.imgt.org). Modeling of the DQ2.5-glia-a2 and DQ2.5-glia-a2 epitopes was done using SPDV based on DQ2.5:DQ2.5-glia-a1 (PDB: 1S9V) and DQ2.5:DQ2.5-glia-a2 (PDB: 4OZH), respectively (12, 42). The 4OZH structure contains 2 asymmetric units. We used chains A, B, G, H, and J for structural analysis. Molecular visualization was done in PyMol.

Statistics. Statistical analyses were performed using GraphPad Prism 7.01 software. Values are expressed as mean and standard deviation or standard error of the mean as indicated in the figure legends. Nonlinear regression analysis (3 parameters) was used to derive IL-2 concentrations from the standard curves. Graphs were normalized and EC50 values calculated using nonlinear regression analysis (log[agonist] vs. normalized response).

Study approval. Biological material was obtained from CD patients according to protocols approved by the Regional Ethics Committee of South-Eastern Norway (2010/2720 and 2013/1237), and informed written consent was given by all subjects.

Author contributions
KSG and LSH contributed equally to the manuscript and are co–first authors. KSG, LSH, LFR, SDK, SWQ, and GÅL designed and performed experiments and analyzed data. RSN performed experiments. EB provided some of the recombinant, soluble pMHC. RF, TF, and MFP provided some of the BW or A20 transduced cells. BD contributed to structural analyses. KEAL provided biopsies from CD patients. SWQ, IS, LMS, and GÅL designed experiments, analyzed data, and supervised the study. KSG, LSH, IS and GÅL wrote the manuscript. All authors critically reviewed the manuscript.

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