Research Article

On the immune response to barley in celiac disease: Biased and public T-cell receptor usage to a barley unique and immunodominant gluten epitope

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Celiac disease (CeD) is driven by CD4+ T-cell responses to dietary gluten proteins of wheat, barley, and rye when deamidated gluten epitopes are presented by certain disease-associated HLA-DQ allotypes. About 90% of the CeD patients express HLA-DQ2.5. In such patients, five gluten epitopes dominate the anti-gluten T-cell response; two epitopes unique to wheat, two epitopes present in wheat, barley, and rye and one epitope unique to barley. Despite presence of barley in commonly consumed food and beverages and hence being a prominent source of gluten, knowledge about T-cell responses elicited by barley in CeD is scarce. Therefore, in this study, we explored T-cell response toward the barley unique epitope DQ2.5-hor-3 (PIPEQPQPY) by undertaking HLA-DQ:gluten peptide tetramer staining, single-cell T-cell receptor (TCR) αβ sequencing, T-cell cloning, and T-cell proliferation studies. We demonstrate that majority of the CeD patients generate T-cell response to DQ2.5-hor-3, and this response is characterized by clonal expansion, preferential TCR V-gene usage and public TCR features thus echoing findings previously made for wheat gluten epitopes. The knowledge that biased and public TCRs underpin the T-cell response to all the immunodominant gluten epitopes in CeD suggests that such T cells are promising diagnostic and therapeutic targets.

Keywords: barley · CD4+ T cells · celiac disease · DQ2.5-hor-3 · single-cell T-cell receptor sequencing

Introduction

Celiac disease (CeD) is a chronic intestinal disorder mediated by disease specific and harmful immune responses to dietary gluten proteins [1]. Strict adherence to the gluten-free diet (GFD) is the only known treatment of the condition. The disease has a strong genetic basis with HLA being the chief genetic determinant. The majority of CeD patients express HLA-DQ2.5 and the remaining minority express either HLA-DQ8 or HLA-DQ2.2. The disease-associated HLA molecules present deamidated gluten peptides to CD4+ T cells, and these T cells orchestrate an immune response resulting in CeD [1].
Traditionally and strictly speaking, the term “gluten” is specific to wheat; however, nowadays gluten is a commonly used term for seed storage proteins of grains of wheat, rye, barley, and oats. In wheat, gluten proteins comprise gliadins (monomeric; α, γ, and ω subtypes) and glutenins (polymeric; LMW and HMW subtypes) [2]. In barley, the gluten proteins consist of B-, γ-, C-, and D-hordeins whereas in rye the gluten proteins comprise of γ-, ω-, and HMW-secalins [2]. Upon consumption of wheat, barley, and rye, the immune system is exposed to an array of immunogenic gluten peptides. Generally, T-cell responses to few gluten peptides of the many thousand peptides generated from breakdown of complex gluten proteomes dominate the anti-gluten T-cell immune response [3, 4]. The same dominance in T-cell response to certain peptides is observed across patients, and these gluten epitopes are hence regarded as immunodominant epitopes [5].

In HLA-DQ2.5 expressing CeD patients, five immunodominant gluten epitopes have been identified [3], namely DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-α1, DQ2.5-glia-ω2, and DQ2.5-hor-3. The amino acid sequences of DQ2.5-glia-α1a and DQ2.5-glia-α2 are unique to wheat whereas as DQ2.5-glia-ω1 and DQ2.5-glia-ω2 are not only found in wheat but also in barley (termed DQ2.5-hor-1 and DQ2.5-hor-2) and rye (termed DQ2.5-sec-1 and DQ2.5-sec-2) [3, 5]. Importantly, the epitope DQ2.5-hor-3 is unique to barley.

Upon gluten challenge of CeD patients in remission, increased number of gluten-specific T cells appear in peripheral blood after 6 days and their presence can be monitored by IFN-γ ELISPOT [6] or by HLA-DQ:gluten peptide tetramer staining [7]. Gluten challenge performed with different cereals leads to mobilization of distinct populations of T cells depending on their antigen specificity. While DQ2.5-glia-α1a- and DQ2.5-glia-α2-specific T cells dominate the response after wheat challenge, DQ2.5-glia-ω1- and DQ2.5-glia-ω2-specific T cells are equally dominant in the responses after wheat, rye, or barley challenge [3, 8]. T cells specific for DQ2.5-hor-3 dominate the response after barley challenge [3, 8] reflecting that this epitope is unique to barley and that T cells specific to this epitope are raised as a consequence of barley exposure.

Over the years, structural and molecular aspects of the T-cell response toward immunodominant wheat gluten epitopes have been investigated [9–16]. Studies on usage of T-cell receptors (TCR) specific for these epitopes have demonstrated that the T-cell response toward immunodominant gliadin epitopes are characterized by biased TCR usage. Structural studies investigating the interaction between TCR expressing biased V-genes and HLA-bound gliadin peptide have further demonstrated that the conserved CDR3 residues and the germline residues of the biased V-gene play a crucial role in the interaction, hence providing insights into the molecular determinants underpinning the V-gene bias [10, 11, 13].

Barley is a prominent source of gluten in commonly consumed food and beverages, however, not much is known about the T-cell response solely generated by this cereal. Therefore, in this study, we have characterized the T-cell response in CeD patients toward the barley unique epitope DQ2.5-hor-3 by using HLA-DQ2.5:DQ2.5-hor-3 tetramer and single-cell TCR-αβ gene sequencing. We found that majority of the CeD patients possess T cells responsive to DQ2.5-hor-3. Most importantly, the DQ2.5-hor-3-specific CD4+ T cells also demonstrate clonal expansion, V-gene bias, and use of public TCRs. Taken together, this suggests that biased TCR usage underpins the response to all the immunodominant gluten epitopes whether from wheat or barley, which is of significance in understanding disease pathogenesis and will be of value in developing diagnostics and therapeutics strategies for CeD.

**Results**

**Majority of the CeD patients generate T-cell response toward DQ2.5-hor-3**

We identified populations of CD4+ T cells that stained with the HLA-DQ2.5:DQ2.5-hor-3 tetramer in blood and gut samples from both GFD-treated (TcCeD) and untreated CeD (UcCeD) patients (Fig. 1A–D). We calculated the percentage of CD4+ T cells that stained with the HLA-DQ2.5:DQ2.5-hor-3 tetramer to assess the frequency of DQ2.5-hor-3-specific CD4+ T cells in gut (Fig. 1A and C). We used HLA-DQ2.5:CLIP2 tetramer as negative control to exclude the nonspecific tetramer staining. Based on this analysis, we found that the DQ2.5-hor-3-specific CD4+ T cells were present at a higher frequency in UcCeD compared to that of TcCeD patients (p = 0.0005; Fig. 1C). We also calculated the number of effector memory and gut-homing CD4+ T cells that stained with the HLA-DQ2.5:DQ2.5-hor-3 tetramer to assess the frequency of DQ2.5-hor-3-specific CD4+ T cells in blood and found that these cells, as previously observed for other gluten epitopes, were present at a very low frequency (0.1 to 2.5 per million CD4+ T cells; Fig. 1B and D). Of note, the DQ2.5:DQ2.5-hor-3 tetramer defined a distinct population of CD4+ T cells in a T-cell line generated by hordein antigen stimulation of a gut biopsy from a CeD patient (Fig. 1E).

We generated DQ2.5-hor-3 reactive T-cell clones (TCCs) from the HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells from biopsy samples of three TCeD patients by the means of cloning by limited dilution and antigen-free expansion. These TCCs showed proliferative response only toward a peptide that harbored the DQ2.5-hor-3 epitope and not against peptides harboring other immunodominant gluten epitopes (Fig. 2A). Moreover, these TCCs stained only with the HLA-DQ2.5:DQ2.5-hor-3 tetramer and not with tetraters representing other immunodominant gluten epitopes (Fig. 2B). Further, by performing paired TCR-αβ sequencing, we confirmed that most of these TCCs are unique clones and not sister clones (Table 1). Taken together, the results of the HLA-DQ2.5:DQ2.5-hor-3 tetramer staining of CD4+ T cells in gut and blood as well as the T-cell proliferation assay and HLA-DQ2.5:DQ2.5-hor-3 tetramer re-staining of established TCCs confirm the notion that majority of the CeD patients make T-cell response to DQ2.5-hor-3 hence corroborating that this is an immunodominant gluten epitope.
Figure 1. DQ2.5-hor-3-specific CD4⁺ T cells CeD patients. (A) Representative plot showing the gating strategy used to isolate HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4⁺ T cells from single-cell suspension of the gut biopsies of an untreated CeD patient (CD1745). Live cells within the singlet lymphocyte population were gated to obtain HLA-DQ2.5:DQ2.5-hor-3 tetramer-binding CD4⁺ T cells that were CD3⁺, CD11c⁻, CD14⁻, CD15⁻, CD19⁻, CD56⁻, CD8⁺, HLA-DQ2.5:DQ2.5-hor-3 tetramer⁺, and HLA-DQ2.5:CLIP2 tetramer⁻. (B) Representative plot showing the gating strategy used to analyze the HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4⁺ T cells from peripheral blood of an untreated CeD patient (CD1745). PBMCs were stained with tetramers followed by bead enrichment of the tetramer-binding cells prior to staining with antibodies specific for cell surface markers. Live cells within the singlet lymphocyte population were gated to obtain HLA-DQ2.5:DQ2.5-hor-3 tetramer-binding effector-memory gut-homing CD4⁺ T cells that were CD3⁺, CD11c⁻, CD14⁻, CD15⁻, CD19⁻, CD56⁻, CD4⁺, CD45RA⁻, CD62L⁻, integrin β7⁺, and HLA-DQ2.5:DQ2.5-hor-3 tetramer⁺. (C) Frequency of HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4⁺ T cells that were isolated using the gating strategy depicted in (A) from nine untreated CeD patients (UCeD) and nine GFD-treated CeD patients (TCeD). The frequency was determined by calculating the percentage of CD4⁺ T cells staining with the HLA-DQ2.5:DQ2.5-hor-3 tetramer over total number of CD4⁺ T cells. The horizontal line represents the median value. The p-values were calculated with the Mann–Whitney U-test. (D) Frequency of HLA-DQ2.5:DQ2.5-hor-3 tetramer-binding effector-memory gut-homing CD4⁺ T cells using the gating strategy depicted in (C) from four UCeD patients and four TCeD patients. The frequency was obtained by dividing the number of HLA-DQ2.5:DQ2.5-hor-3 tetramer-binding effector-memory gut-homing CD4⁺ T cells by the total number of CD4⁺ T cells in the PBMC. The total number of CD4⁺ T cells in the PBMC was obtained by multiplying the frequency of CD4⁺ T cells in the PBMC sample taken prior to enrichment with the total PBMC count. (E) Gating strategy used to isolate HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4⁺ T cells from the T-cell line (TCL461) generated from a gut biopsy of a CeD patient (CD461) stimulated with hordein antigen. Live cells within the singlet lymphocyte population were gated to obtain HLA-DQ2.5:DQ2.5-hor-3 tetramer-binding CD4⁺ T cells that were CD3⁺, CD8⁻, CD4⁺, HLA-DQ2.5:DQ2.5-hor-3 tetramer⁺ and HLA-DQ2.5:CLIP2 tetramer⁻.
DQ2.5-hor-3 specific CD4$^+$ T cells demonstrate clonal expansion and V-gene bias

We next performed high-throughput sequencing of rearranged TCR-α and TCR-β genes of the HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4$^+$ T cells isolated from gut biopsies of CeD patients. We processed the sequencing data and only cells with productive paired TCR-αβ chains were analyzed. Cells of single subjects expressing paired identical nucleotide TCR-αβ chains were categorized as a single clonotype. From one TCeD and nine UCeD patients, we isolated 325 cells that were categorized into 179 clonotypes (Table 2).

Upon analysis of clonal distribution in five patients where we had obtained data from at least 20 cells, we observed that clonotypes with clonal size ≥ 2 cells dominated the T-cell response (Fig. 3). More specifically, in these five CeD patients, expanded clonotypes accounted for 30–80% of the TCR repertoire indicating that clonally expanded T cells dominated the T-cell response.

We then investigated V-gene usage in the DQ2.5-hor-3 specific T cells obtained from ten CeD patients (Figs. 4–6). Upon...
Table 1. TCRs of the DQ2.5-hor-3 specific TCCs

| TCC       | TRAV | TRAJ | CDR3α         | TRBV | TRBJ | CDR3β         |
|-----------|------|------|---------------|------|------|---------------|
| TCC1468.1 | TRAV8-3 | TRAJ45 | AVEAGSGGGGADGLT | TRBV20-1 | TRBJ2-1 | SASRTSGRAGDEQF |
| TCC1468.2 | TRAV35 | TRAJ40 | AGQDYKYI       | TRBV20-1 | TRBJ2-3 | SASRTGCGTDTQY  |
| TCC1468.3 | TRAV22 | TRAJ23 | AVIYQGGKLI     | TRBV2   | TRBJ2-7 | NO RESULTS    |
| TCC1468.4 | TRAV3  | TRAJ21 | AVBVYFNFKY     | TRBV4-2 | TRBJ2-1 | ASSQEGLADSYNQF |
| TCC1468.5 | TRAV4  | TRAJ32 | CLVGDMS#GATKLI | TRBV20-1 | TRBJ2-1 | CSASRTSNGDEQF |
| TCC1468.6 | TRAV8-3 | TRAJ45 | AVESSWKGLQ     | TRBV20-1 | TRBJ2-3 | SAARTSNGDEQF  |
| TCC1468.7 | TRAV3  | TRAJ21 | AVRVEYNFNKFY   | TRBV20-1 | TRBJ2-1 | SASRTSNGDEQF  |
| TCC1468.8 | TRAV4  | TRAJ32 | CLVGDSM#GATNKLIF | TRBV20-1 | TRBJ2-1 | CSASRTSNGDEQF |
| TCC1468.9 | TRAV12-2 | TRAJ9 | AVGGEFKTI      | TRBV6-1 | TRBJ2-7 | ASSEVGSPEEQY  |
| TCC1468.10 | TRAV19/2 | TRAV16 | AVRGYGNNRLA|ALENQGGKLI | TRBV20-1 | TRBJ2-3 | SAARTSNGDEQF |
| TCC2072.1 | TRAV8-3 | TRAJ34 | AVESSWGKLQ     | TRBV20-1 | TRBJ2-3 | SAARTSNGDEQF  |
| TCC2076.1 | TRAV14/DV4 | TRAJ56 | AMSLTGANSKLT   | TRBV4-2 | TRBJ2-3 | ASSHIAGAGTDQY |

Table 2. Summary of TCR sequences generated by single-cell TCRαβ sequencing

| Patient ID | Number of cells | Number of clonotypes |
|------------|-----------------|----------------------|
| CD1945     | 38              | 15                   |
| CD1745     | 29              | 22                   |
| CD1998     | 13              | 10                   |
| CD5001     | 39              | 14                   |
| CD1930     | 31              | 23                   |
| CD1644     | 12              | 11                   |
| CD1468     | 130             | 55                   |
| CD1974     | 11              | 11                   |
| CD1997     | 12              | 8                    |
| CD1973     | 10              | 10                   |
| TOTAL      | 325             | 179                  |

analysis of TRAV:TRBV pairing in the DQ2.5-hor-3 specific TCR repertoire, we observed that TRAV26-1:TRBV29-1 was the most expressed TRAV:TRBV pair, found in 7% of the total clonotypes and 9% of the total cells generated from ten CeD patients (Fig. 4). The TRAV26-1:TRBV29-1 pair was expressed in seven out of ten CeD patients and clonotypes expressing TRAV26-1:TRBV29-1 were also clonally expanded in four CeD patients. Most interestingly, all the 13 unique clonotypes expressing TRAV26-1 were paired with only TRBV29-1 (two TCRs expressing dual TCRα chains excluded). Further, 12 of 13 of these unique clonotypes expressing TRAV26-1:TRBV29-1 also used TRAJ47, indicating a preference for the J gene segment as well. Similar analysis of TRAV-gene usage demonstrated that TRAV12-1 and TRAV26-1 were the most prevalently expressed TRAV genes, accounting for 8% and 7% of total clonotypes and 9% and 9% of total cells generated from ten CeD patients, respectively (Fig. 5). Both genes were expressed in seven of the ten CeD patients. Similar analysis of TRBV-gene usage demonstrated that TRBV20-1 and TRBV29-1 were the most prevalently expressed TRBV genes, accounting for 22% and 13% of the total clonotypes and 29% and 13% of the total cells generated from ten CeD patients, respectively (Fig. 6). TRBV29-1 was expressed in all the ten CeD patients while TRBV20-1 was expressed in seven of ten patients. It is noteworthy that TRBV20-1 and TRBV29-1...
Figure 4. TRAV:TRBV pairing in DQ2.5-hor-3-specific TCR repertoire. High-throughput TCR-αβ sequencing data of the HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells isolated from gut biopsies of ten CeD patients were analyzed. Only cells with productive paired TCR-αβ chains were analyzed and cells expressing paired identical nucleotide TCR-αβ chains in each patient were categorized as a clonotype. Frequency of clonotypes and cells expressing a TRAV:TRBV pair in each of the ten CeD patients and in all patients combined are shown in the heatmap. The total number of clonotypes and cells obtained from each patient are shown below each column of the heatmap.

are phylogenetically closely related TRBV genes. Collectively, they were preferentially expressed in all CeD patients and accounted for 34% and 42% of total clonotypes and cells, respectively. Taken together, the findings demonstrate that preferential V-gene usage underlies the T-cell response toward DQ2.5-hor-3 in CeD.

DQ2.5-hor-3 specific CD4+ T cells express public TCRs

Upon analysis of the TCR-αβ sequences, we found usage of identical amino acid sequences for the TCR-α or/and TCR-β chain/s across different CeD patients suggesting the presence of public TCRs specific to DQ2.5-hor-3. More specifically, we found three cases of identical paired TCR-αβ chains (Table 3), nine identical TCR-α chains (Table 4), and six identical TCR-β chains (Table 5) that were shared across at least two CeD patients. In majority of the cases, the identical CDR3 amino acid sequences of these TCRs across CeD patients were encoded by different nucleotides, representing a phenomenon called convergent recombination [17]. Corresponding to the preferential V-gene usage, TRAV26-1:TRBV29-1, the most prevalently expressed TRAV:TRBV pair in the DQ2.5-hor-3-specific T cells was expressed in two of the three public TCRαβ paired sequences. Similarly, TCR-α sequences expressing TRAV26-1 (3 of 9), TRAV12-1 (2 of 9), and TCRβ sequences expressing TRBV29-1 (2 of 6) and TRBV20-1 (2 of 6) were the most prominently shared public TCR sequences. Notably, all public TCR-α sequences expressing TRAV26-1 also utilized TRAJ47. Of note, the T cells expressing the public TCR-α or/and TCR-β sequences were not only present in the DQ2.5-hor-3 specific T-cell repertoire of multiple CeD patients, but such T cells were found to be clonally expanded in several donors. Further, one public TCR-α and one public TCR-β sequence were also observed among the five unique TCRs obtained from a T-cell line generated by stimulating the biopsy with deamidated barley antigen. Most interestingly, three of the six public TCR-β sequences were also expressed by the TCCs that were reactive with the DQ2.5-hor-3 epitopes as demonstrated by both T-cell proliferation assay and HLA-DQ2.5:DQ2.5-hor-3 tetramer staining. Taken together, the findings demonstrate that DQ2.5-hor-3-specific T-cells display use of public TCRs.

Discussion

The classic feeding trials conducted by Dicke in the 1950s established that wheat and rye are harmful to CeD patients [18]. Dicke
Figure 5. TRAV-gene usage in DQ2.5-hor-3-specific TCR repertoire. Cells and clonotypes were defined as reported in legend of Figure 4. Frequency of clonotypes and cells expressing a TRAV gene in each of the ten CeD patients and in all patients combined are shown in the heatmap. The total number of clonotypes and cells obtained from each patient are shown below each column of the heatmap.

did not investigate barley, but later Baker and Read in the 1970s investigated barley toxicity and concluded that barley is also harmful for CeD patients [19]. Subsequent studies also confirmed that barley and barley-derived food and beverages are harmful to CeD patients [20, 21]. It is now universally accepted that wheat, barley, and rye should be excluded from CeD patients’ diet. Since CD4+ T-cell response toward dietary gluten is the key element of CeD pathogenesis, it is important to investigate the...
T-cell responses toward epitopes derived from these CeD driving cereals.

A more recent comprehensive study on mapping of T-cell epitopes in gluten in CeD was conducted by performing IFN-γ ELISPOT on the peripheral blood cells after gluten challenge [3]. Based on this work the authors concluded that the five epitopes DQ2.5-glia-α1, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2, DQ2.5-hor-3 are immunodominant gluten epitopes in CeD patients expressing HLA-DQ2.5. Recently, it was also reported that the panel of HLA-DQ:gluten tetramers presenting these five immunodominant gluten epitopes can be used in a flow cytometry based test to diagnose CeD patients from controls even in absence of gluten consumption [22]. Since this HLA-DQ2.5:gluten tetramer-based test is performed in blood and does not require the patient to be in gluten-containing diet, it has a potential to replace the current diagnostic approach that is based on histological evidence and which requires patients to be on gluten-containing diet at the time of work-up. The disease-specific signal of the HLA-DQ2.5:gluten tetramer test [22] reinforces the notion that T cells are central in the pathogenesis of CeD. Hence, studies of

Figure 6. TRBV-gene usage in DQ2.5-hor-3-specific TCR repertoire patient. Cells and clonotypes were defined as reported in legend of Figure 4. Frequency of clonotypes and cells expressing TRBV gene in each of the ten CeD patients and in all patients combined are shown the heatmap. The total number of clonotypes and cells obtained from each patient are shown below each column of the heatmap.
the T-cell response toward DQ2.5-hor-3 as an immunodominant gluten epitope are relevant and important.

The specificities of the T-cell response generated in CeD patients in remission when challenged with gluten is dependent on the grain (wheat, barley, or rye) used for gluten challenge [3, 8]. The DQ2.5-glia-α1- and DQ2.5-glia-α2 specific T cells dominate the immune response only after oral wheat challenge, while the T-cell response toward DQ2.5-glia-α1 and DQ2.5-glia-α2 is equally dominant after oral wheat, rye, or barley challenge [3, 8]. This goes in line with the observation that the nine-amino acid core sequence of DQ2.5-glia-α1, epitope derived from wheat is identical to the epitopes derived from barley (DQ2.5-hor-1) and rye (DQ2.5-sec-1) [3, 5]. Similarly, DQ2.5-glia-α2, which is derived from wheat, is almost identical (P9 different) to the epitopes derived from barley (DQ2.5-hor-2) and rye (DQ2.5-sec-2) [3, 5]. This suggests that consumption of any of the three cereals (wheat, rye, or barley) could result in mobilization of T cells specific to these identical or highly homologous epitopes. By contrast, the sequence of the DQ2.5-hor-3 epitope is unique to barley and it is not present in wheat or rye. Correspondingly, and unlike the other barley epitopes DQ2.5-hor-1 and DQ2.5-hor-2, T-cell responses toward DQ2.5-hor-3 are observed only after barley challenge [3, 8]. Therefore, studies of DQ2.5-hor-3 reactive T cells provide an opportunity to explore the nature of T-cell responses solely influenced by barley exposure in CeD.

We investigated the T-cell response to DQ2.5-hor-3 using HLA-DQ2.5:DQ2.5-hor-3 tetramers and single-cell TCR-αβ gene sequencing. By performing HLA-DQ2.5:DQ2.5-hor-3 tetramer staining of CD4+ T cells in gut-biopsy and PBMC samples as well as by undertaking T-cell proliferation assay and HLA-DQ:DQ2.5-hor-3 tetramer re-staining of established TCCs, we show that majority of the CeD patients make T-cell response to DQ2.5-hor-3 and hence that this is truly an immunodominant epitope. We also performed high-throughput sequencing of rearranged paired TCR-α and TCR-β genes of the HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells isolated from gut biopsies of CeD patients to investigate the TCR repertoire. We found that clonally expanded T cells dominate the T-cell response to DQ2.5-hor-3. Further, we observed that the TRAV26-1:TRBV29-1 is the most preferred TRAV:TRBV pair in the DQ2.5-hor-3-specific TCR repertoire and most notably, TRAV26-1 exclusively pairs with TRBV29-1. In addition, TCRs expressing TRAV26-1:TRBV29-1 almost exclusively use TRAJ47. Further, TRAV26-1 was also among the two most used TRAV genes. The biased expression of TRBV20-1 and TRBV29-1 was very prominent as these two phylogenetically close TRBV genes were expressed in almost half of the DQ2.5-hor-3-specific T cells. Most importantly, we found usage of identical TCR-α or/and TCR-β amino acid sequences across different CeD patients indicating the presence of public TCRs specific to DQ2.5-hor-3. Taken together, we conclude that majority of the CeD patients generate T-cell response to DQ2.5-hor-3, and that this response is characterized by clonal expansion, preferential TCR V-gene usage and public TCR features.

Previous studies have shown that T cells specific to immunodominant gluten epitopes in HLA-DQ2.5 mediated CeD
| Nr | TRAV CDR3α (AA) | Patient ID | Nr of T cells/ TCC name | CDR3α (NT) |
|----|-----------------|------------|-------------------------|------------|
| 1  | AV26-1, IVRASEYGNKLV_AJ47 | CD1745     | 1                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
|    |                  | CD1973     | 1                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
|    |                  | CD5001     | 4                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
| 2  | AV12-1, VVNPAGGAGSYGNKLV_AJ52 | CD1930     | 1                        | GTGGTGAACTCATGCTATGGAAAGCTGACA |
|    |                  | CD1644     | 1                        | GTGGTGAACTCATGCTATGGAAAGCTGACA |
|    |                  | CD1468     | 1                        | GTGGTGAACTCATGCTATGGAAAGCTGACA |
| 3  | AV19, ALSEAGYGNKL_VNKL_AJ32 | CD461 (TCL) | 5                        | GCTCTGAGAGCTACGATGCTACAAACAAACTGATC |
|    |                  | CD1644     | 1                        | GCTCTGAGAGCTACGATGCTACAAACAAACTGATC |
|    |                  | CD1974     | 1                        | GCTCTGAGAGCTACGATGCTACAAACAAACTGATC |
| 4  | AV16, ALEGGA_TNKL_AJ32 | CD1998     | 3                        | GCTCTAGAAGCTTGCTACAAACAAACTGATC |
|    |                  | CD1930     | 1                        | GCTCTAGAAGCTTGCTACAAACAAACTGATC |
|    |                  | CD1745     | 1                        | GCTCTAGAAGCTTGCTACAAACAAACTGATC |
| 5  | AV26-1, IVRAGEYGNKLV_AJ47 | CD1745     | 1                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
|    |                  | CD1930     | 1                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
|    |                  | CD1468     | 7                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
| 6  | AV14/DV4, AMSLTGANSKTL_AJ56 | CD1468     | 2                        | GCAATGATTTAAGCTTGAAGACCAATAGTAACTGACA |
|    |                  | CD2076     | TCC2076.1                | GCAATGATTTAAGCTTGAAGACCAATAGTAACTGACA |
| 7  | AV26-1, IVRPSEYGNKLV_AJ47 | CD1930     | 1                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
|    |                  | CD1468     | 1                        | ATCGTCAGAGCTTCGGAATATGGAACAAACTGTC |
| 8  | AV5, AESITGGGNKL_AJ10 | CD1998     | 1                        | GCAAGAGATATTAAAGGGAGAGGAACAAACTCACC |
|    |                  | CD1930     | 2                        | GCAAGAGATATTAAAGGGAGAGGAACAAACTCACC |
| 9  | AV12-1, VVNMAGGAGSYGNKL_AJ52 | CD1644     | 1                        | GTGGTGAAACATGCTGCTACGATGCTACGGAAGCTGACA |
|    |                  | CD1745     | 2                        | GTGGTGAAACATGCTGCTACGATGCTACGGAAGCTGACA |

AA, amino acid; NT, nucleotide; TCL, T-cell line.
Table 5. Public TCRβ sequences in DQ2.5-hor-3 specific T cells

| Nr | TRBV.CDR3β(AA).TRBJ | Patient ID | Nr of T cells/TCC name | CDR3β (NT) |
|----|----------------------|------------|------------------------|------------|
| 1  | BV20-1_SASRTSGRAGDEQF_BJ2-1 | CD1930 | 6 | AGTGCTAGTCGGACTAGGGGAGGCAGGAGCAGTTTC |
| | | | 1 | AGTGCCAGCCGACTAGGGGAGGAGGAGGCAGGAGCAGTTTC |
| | | | 1 | AGTGCTAGTCGGACTAGGGGAGGCAGGAGCAGTTTC |
| | | | 2 | AGTGCCAGCCGACTAGGGGAGGAGGAGGCAGGAGCAGTTTC |
| | | | 3 | AGTGCTAGTCGGACTAGGGGAGGAGGAGGCAGGAGCAGTTTC |
| | | | 4 | AGTGCTAGTCGGACTAGGGGAGGAGGAGGCAGGAGCAGTTTC |
| | | | 5 | AGTGCTAGTCGGACTAGGGGAGGAGGAGGCAGGAGCAGTTTC |
| 2  | BV29-1_SVEGGNYGYT_BJ1-2 | CD1930 | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 2 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 3 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| 3  | BV20-1_SAARTSGRAGDEQF_BJ2-1 | CD1930 | 1 | AGTGCCAGCAGAGCTAGGGGAGGAGGAGGCAGGAGCAGTTTC |
| | | | 1 | AGTGCTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGTGCTGAGGGAGGAGCTATGGCTACACC |
| | | | 2 | AGTGCTGAGGGAGGAGCTATGGCTACACC |
| | | | 3 | AGTGCTGAGGGAGGAGCTATGGCTACACC |
| | | | 4 | AGTGCTGAGGGAGGAGCTATGGCTACACC |
| | | | 5 | AGTGCTGAGGGAGGAGCTATGGCTACACC |
| 4  | BV29-1_SVEGGSYEQY_BJ2-7 | CD1930 | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 2 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 3 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| 5  | BV4-2_ASSHIAGATITQY_BJ2-3 | CD1930 | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 2 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| 6  | BV11-1_ASSFGQGSTEAF_BJ1-1 | CD1930 | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |
| | | | 1 | AGCGTTGAGGGAGGAGCTATGGCTACACC |

AA, amino acid; NT, nucleotide; TCL, T-cell line.
(DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, and DQ2.5-glia-ω2) as well as in HLA-DQ8 mediated CeD (DQ8-glia-α1) unanimously express biased V-gene usage with or without public TCR motifs/sequences [9–15]. Here, we demonstrate that DQ2.5-hor-3-specific T cells also exhibit V-gene bias and expression of public TCR sequences. Given the presence of TCR bias in the T cells specific to the all the immunodominant gluten epitopes, these biased TCRs likely are particularly central in development of CeD. While gluten and certain HLA genes are key determining factors, the disease does not develop at a uniform age and there is variation in disease severity, hence suggesting contribution of some additional unknown factors to the onset/severity of the disease. Based on these observations, generation of the biased TCR repertoire specific for the immunodominant gluten epitopes and their frequency could be a potential determinant for disease development and severity. In addition, given the recent observation that gluten-specific T-cell clonotypes persist for decades [23], the public and biased TCRs can be a potential target for diagnosis and treatment of CeD.

Material and methods

Patient material

We obtained approximately eight to ten gut biopsies taken as part of a gastroduodenoscopy from both untreated and GFD-treated CeD patients. We also obtained 50–100 mL blood from both untreated and GFD-treated CeD patients. The gut biopsies and the blood samples were processed and cryopreserved as described previously [23]. The study was approved by Regional Committee for Medical and Health Research Ethics South-East Norway (project 2010/2720). The patients (Supporting Information Table S1) participating in the study signed written informed consent.

Tetramer staining, surface antibody staining, and fluorescence-activated cell sorting

Tetramers of recombinant HLA-DQ2.5 covalently linked with APLMMQALPM (negative control for nonspecific binding) and DQ2.5-CLIP2 (ATPTMAAPYMPQ, underlined 9-mer core amino acid sequence) and DQ2.5-CLIP2 (ATPTMAAPYMPQ, underlined 9-mer core amino acid sequence) were produced using the protocol described before [24] and conjugated with PE-labeled streptavidin (Invitrogen) and allophycocyanin-labeled streptavidin (ProZyme), respectively. Tetramer staining was performed on single-cell suspension prepared from gut biopsies, PBMCs, TCLs, and TCCs. Cryopreserved single-cell suspensions of gut biopsies were thawed and stained with PE-conjugated HLA-DQ2.5:DQ2.5-hor-3 (10 µg/mL) and APC conjugated HLA-DQ2.5:DQ2.5-CLIP2 (10 µg/mL) for 30–45 min at room temperature for tetramer re-staining. Following tetramer staining, the cells were stained with the antibody mixtures reactive with cell surface markers for fluorescence-activated cell sorting (FACS). For single-cell suspension of gut biopsies, we sorted live, single HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells that were: CD3+, CD11c+, CD14+, CD15+, CD19+, CD56+, CD8+, CD4+, HLA-DQ2.5:DQ2.5-hor-3 tetramer+; and HLA-DQ2.5:DQ2.5-CLIP2 tetramer+. For PBMCs, we analyzed live, single HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells that were: CD3+, CD11c+, CD14+, CD15+, CD19+, CD56+, CD4+, CD45RA+, CD62L+, integrin β7+, and HLA-DQ2.5:DQ2.5-hor-3+. For TCLs and TCCs, we sorted live, single HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells that were: CD3+, CD8+, CD4+, HLA-DQ2.5:DQ2.5-hor-3+ and HLA-DQ2.5:DQ2.5-CLIP2+. The cells were analyzed with Fortessa (BD Biosciences) or Attune™ NxT (Thermo Fisher Scientific) instruments or sorted with FACS Aria II instrument (BD Biosciences) at the Flow Cytometry Core Facility (Oslo University Hospital). The flow cytometry data were analyzed with FlowJo software (FlowJo LLC). Cells were either sorted for single-cell TCR-αβ sequencing or for generation of TCCs by in vitro expansion. The following antibodies were used in the study: CD14-Pacific Blue (Biolegend), CD15-Pacific Blue (Biolegend), CD19-Pacific Blue (Biolegend), CD56-Pacific Blue (Biolegend), CD3-FITC (Biolegend) or CD3-Superbright 600 (eBioscience) or CD3- Brilliant Violet 510 (Biolegend), CD11c-Horizon V450 (BD Biosciences), CD4-APC-Cy7(Biolegend) or CD4-APC-H7 (BD Biosciences), CD62L-PerCP/Cy5.5 (BD Biosciences), CD45RA-PECy7 (eBioscience), integrin-β7-APC (Biolegend), and CD8-PerCP (eBioscience). LIVE/DEAD marker fixable violet stain (Thermo Fischer Invitrogen) was used to exclude the dead cells. Flow cytometry and cell sorting experiments were conducted in line with the published guideline [25].

Generation of T-cell clones (TCCs)

TCCs were established from sorted HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells by limited dilution cloning and antigen-free expansion according to previously described protocol [26]. In brief, HLA-DQ2.5:DQ2.5-hor-3 tetramer binding CD4+ T cells were sorted in tube containing a standard feeder mix. A
standard feeder mix contained allogenic irradiated PBMC (1 × 10^6 cells/mL) in a T-cell growth medium (RPMI-1640 (Gibco) with penicillin/streptomycin and 0.01 M 2-ME (M-6250, Sigma)) supplemented with 10% human serum, PHA (1 µg/mL), IL-2 (20 IU/mL), and IL-15 (1 ng/mL). The aliquot of the T-cell/feeder mixture was then seeded into wells in Terasaki plates (20 µL per well) and incubated for 7 days. Terasaki plates were screened by Anne Kjersti Uhlen, Agricultural University of Norway, Ås, Norway.

Single-cell TCR gene sequencing and processing of TCR gene sequences

We followed a previously established protocol for single-cell paired TCR-αβ sequencing using multiplex primers of TRAV and TRBV genes and nested PCR amplification [23, 28]. Sequencing was performed using Illumina MiSeq (250 bp PE) platform at the Norwegian Sequencing Centre (Oslo University Hospital). We processed raw sequences as described [23]. All the single-cell TCR-αβ sequencing raw data generated in this study are uploaded to the European Genome-phenome Archive (EGAS00001003674).

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Author contributions: S.D.-K. designed the study, collected patient material, performed experiments, analyzed the data, and wrote the manuscript. R.S.N. developed the bioinformatics tools for analyzing the sequencing data. K.E.L. and J.J. organized supply of CeD patient material. L.M.S. designed the study, analyzed data, and wrote the manuscript. All the authors reviewed and approved the manuscript.

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Abbreviations: CeD: celiac disease · GFD: gluten-free diet · TCC: T-cell clones · TCR: T-cell receptor

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