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Letter to the Editor

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A *TRAV26-1*-encoded recognition motif focuses the biased T cell response in celiac disease

Biased antigen-specific TCR repertoires are frequently observed in autoimmunity and infection. The T cell response against the immunodominant gluten epitope DQ2.5-glia-α2 presented on the MHC variant HLA-DQ2.5 in celiac disease (CeD) is characterized by overrepresentation and preferential pairing of V gene segments *TRAV26-1* and *TRBV7-2* and a canonical CDR3β loop sequence [1]. The molecular features driving this biased semi-public response are not well understood.

We have previously identified a framework-resident recognition motif centered on Y40$^{TCR\alpha}$ encoded by *TRAV26-1*, which is crucial for the response to DQ2.5-glia-α2 of the prototypic TCR 364 [2]. The co-crystal structure of the similar TCR S16 bound to peptide:MHC (pMHC) shows that not only Y40$^{TCR\alpha}$, but also Y38$^{TCR\alpha}$ and H55$^{TCR\alpha}$ contact particular residues of the MHC β-chain as well as the canonical CDR3β loop [3].

Both TCR 364 and TCR S16 are prototypic DQ2.5-glia-α2-specific TCRs, but use different *TRAJ* gene segments (*TRAJ43*01* and *TRAJ32*01*) and thus differ in their CDR3α loops which may affect how they react to pMHC (Fig. 1A). To establish the role of Y40$^{TCR\alpha}$ in TCR S16, we reconstructed the TCR on BW T cells as WT and Y40H$^{TCR\alpha}$ variants (Supporting Information Fig. S1) and assessed T cell activation by pMHC-expressing A20 B cells as APC. In line with our previous observations from the BW 364 T cells, residue Y40$^{TCR\alpha}$ was indeed crucial for activation of the BW S16 T cells (Fig. 1B and C), suggesting the Y40$^{TCR\alpha}$-centered recognition motif is likely a general feature of these prototypic TCRs.

To assess whether alternative *TRAV* germline segments can form a similar Y40$^{TCR\alpha}$-centered recognition motif and substitute for *TRAV26-1*, we re-analyzed with an improved processing pipeline a TCR sequence data set [2] (Supporting Information Table S1) generated by single-cell paired αβ-chain sequencing of gliadin-specific CD4$^+$ effector memory T cells sorted from CeD patients using a blend of four HLA-DQ2.5:gluten tetramers. We filtered out clones with the canonical CDR3β loop typical for DQ2.5-glia-α2-reactive TCRs and identified two private clonotypes using *TRAV* gene segments other than *TRAV26-1*. We chose the clonotype using *TRAV5*, as this gene segment is slightly overrepresented in the gluten-reactive repertoire compared to the naïve repertoire [4] (Supporting Information Table S1). Furthermore, *TRAV5* is the only other human germline that contains residues Y38$^{TCR\alpha}$ and Y40$^{TCR\alpha}$ (Supporting Information Fig. S2).

To determine the contribution of Y40$^{TCR\alpha}$ in a non-prototypic TCR, we reconstructed the *TRAV5*-encoded private clonotype on BW T cells (herein denoted TCR AV5/BW AV5 T cells) (Supporting Information Fig. S1 and Table S2). We confirmed DQ2.5-glia-α2-specificity using plate-bound recombinant pMHC complexes identical to the ones used for tetramer sorting (Fig. 1D) as well as peptide-loaded HLA-DQ2.5$^+$ Raji B lymphoma cells (Fig. 1E). The BW AV5 T cells reacted specifically and with sensitivity similar to the prototypic TCRs, but exhibited slightly elevated baseline signaling in coculture with the Raji cells independent of peptide (Supporting Information Fig. S3). Unlike T cells expressing the prototypic TCRs, BW AV5 T cell activation was not affected by introduction of the Y40H$^{TCR\alpha}$ mutation (Fig. 1F). Thus, the *TRAV5*-encoded TCR appears to use a different recognition mode largely independent of Y40$^{TCR\alpha}$. However, both the prototypic TCR 364 and TCR AV5 depend on contacting residue R70$^{MHC\beta}$ (Fig. 2A), which is a central residue in most TCR:pMHC interactions [5]. Unique TCRs employing such distinct yet convergent binding modes to the same pMHC have previously been described for the response to HLA-DQ8 presented gliadin epitopes in celiac disease [6].

Next, we sought to fine-map the recognition motif of the prototypic TCR 364 by generating BW T cell variants with mutations of residues predicted to participate in the interaction network centered on Y40$^{TCR\alpha}$ guided by the TCR S16 structure [3] (Fig. 2B, Supporting Information Fig. S1). We found that single mutations of Y38A$^{TCR\alpha}$ and H55A$^{TCR\alpha}$, as well as T115A$^{TCR\beta}$ abrogated activation of BW 364 T cells (Fig. 2C and D). T115$^{TCR\beta}$ does not directly contact the pMHC, but rather seems important for the CDR3β loop to be recruited or stabilized through the *TRAV26-1*-encoded framework residues [3]. Thus, the recognition motif identified in the prototypic TCRs does not exclusively rely on residue

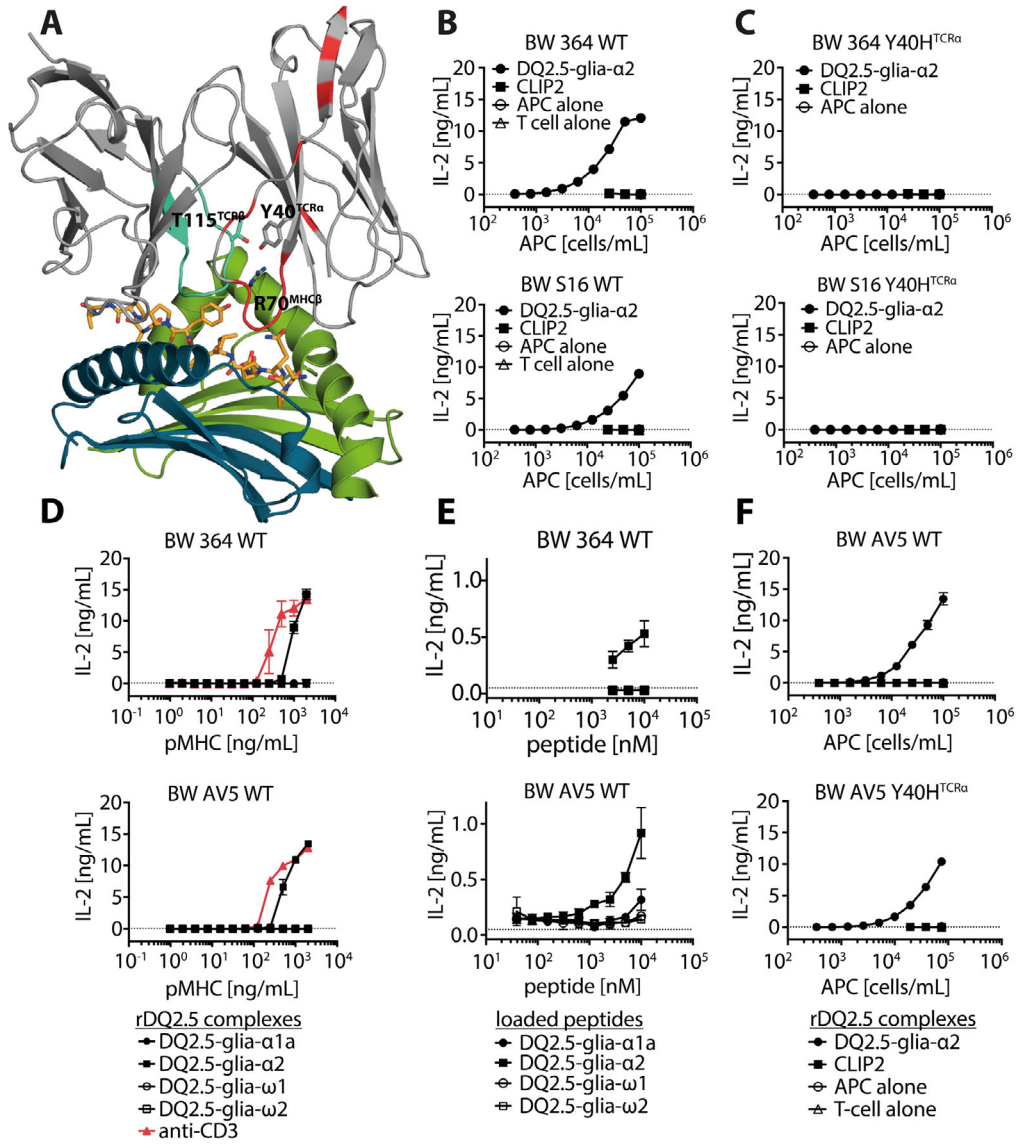

Figure 1. The Y40$^{TCR\alpha}$-centered recognition motif. Co-crystal structure of TCR S16 (grey) bound to HLA-DQ2.5 (blue, green) with bound DQ2.5-glia-α2 (orange). The prototypic CDR3β loop (cyan) and the sequence differences in the TCR α-chain between TCRs 364 and S16 (red) are highlighted (A). The IL-2 response of BW T cells expressing variants of TCRs 364, S16, and AV5 to pMHC-expressing A20 B cells (B, C, F), plate-bound pMHC (D), or peptide pulsed Raji cells (E) was assayed by ELISA. A20 B cells expressing HLA-DQ2.5:CLIP2, APC alone and T cells alone were used as negative controls (B, C, F). Anti-CD3ε antibody was used as a positive control (D). Data is shown for representative experiments ($n = 2$). Error bars indicate mean ± SD of triplicates (B, C, E, F) or duplicates (D) and dotted lines indicate detection limits.

Y40$^{TCR\alpha}$ but also requires nearby residues in both TCR chains and the MHC β-chain.

To rationalize the observed differences in pMHC recognition between the prototypic TCRs S16 and 364, and the private TCR AV5, we generated a structural model of TCR AV5 bound to pMHC (Fig. 2E), assuming its overall docking orientation would closely resemble that of TCR S16. The S16 co-crystal structure and previous interaction studies where a Y40F mutation in a soluble TCR abrogated binding to pMHC [3], suggest a hydrogen bond network where residues Y38$^{TCR\alpha}$ and H55$^{TCR\alpha}$ interact with E69$^{MHC\beta}$, while Y40$^{TCR\alpha}$ interacts with the CDR3β loop residue T115$^{TCR\beta}$ and R70$^{MHC\beta}$ (Fig. 2E). Direct interaction between T115$^{TCR\beta}$ and R70$^{MHC\beta}$ seems impossible due to the large distance. TCR AV5 shares residues Y38$^{TCR\alpha}$ and Y40$^{TCR\alpha}$ with the prototypic TCRs, but has a Tyr residue in position 55$^{TCR\alpha}$ rather than a His. The model of TCR AV5 suggests slightly altered side chain conformations where T115$^{TCR\beta}$ may be in closer proximity to R70$^{MHC\beta}$ allowing for direct interaction with this residue without bridging via Y40$^{TCR\alpha}$. Thus, this model offers a structural hypothesis for the independence of Y40$^{TCR\alpha}$ observed in TCR AV5. It is also in line with the observation that an R70A$^{MHC\beta}$ mutation abrogated activation of both WT and Y40H$^{TCR\alpha}$ variants of TCR AV5.

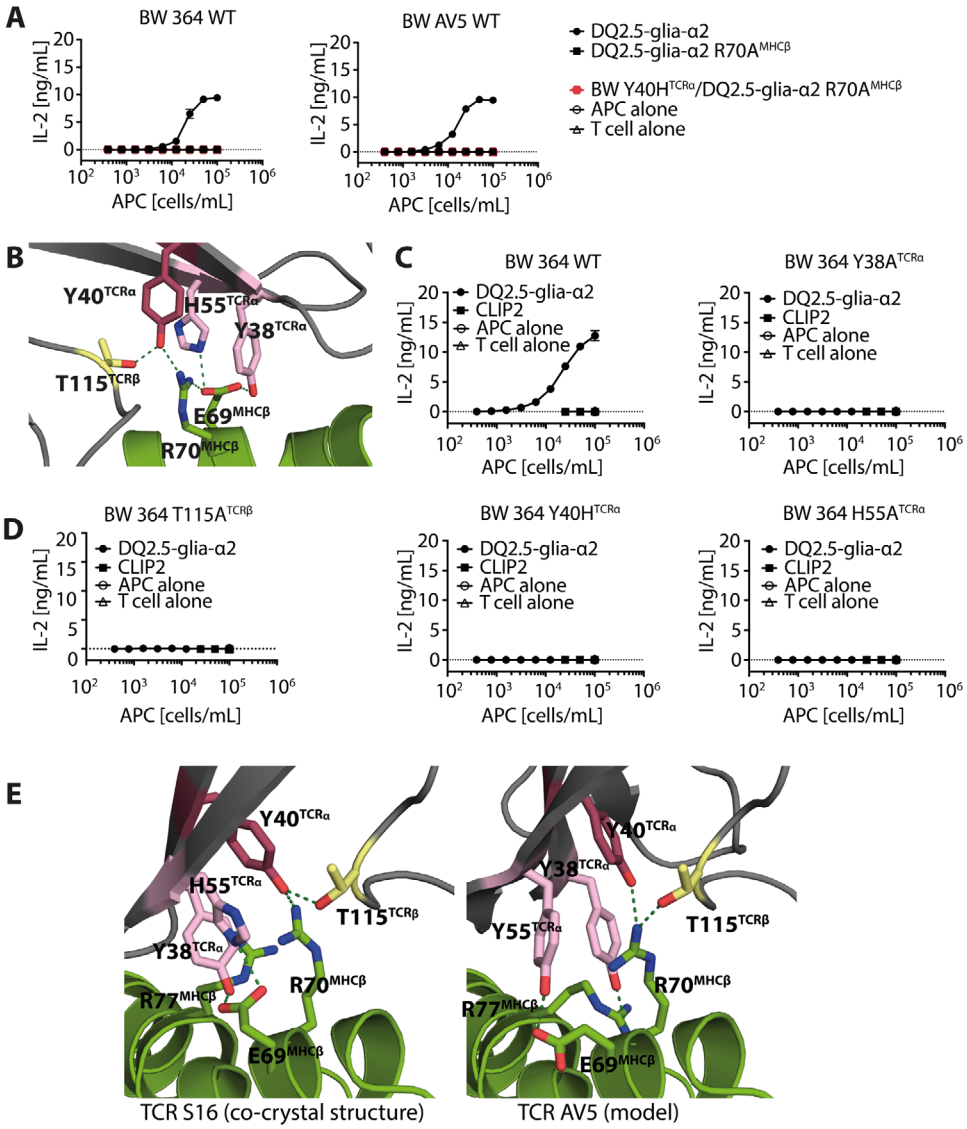

Figure 2. Fine-mapping the recognition motif used by TCR 364. The IL-2 response of BW T cells to pMHC-expressing A20 B cells was assayed by ELISA (A). Co-crystal structure of TCR S16 (grey) with HLA-DQ2.5:DQ2.5-glia-α2 (MHC β-chain: green). Interacting residues are labeled and represented as sticks (B). The IL-2 response of BW T cells expressing variants of TCR 364 to pMHC-expressing A20 B cells was measured in ELISA (C+D). A model of TCR AV5 bound to pMHC was generated using the TCRmodel webserver, superimposed with TCR S16, minimized into the Rosetta score function, and compared to the co-crystal structure of TCR S16 (E). Possible hydrogen bonds are visualized in green if the distance is ≤3.5 Å. Data is shown for representative experiments ($n = 2$), error bars indicate mean ± SD of triplicates (A, C, D).

The clonotype expressing a *TRAV5*-encoded TCR using the prototypic TCR β-chain was private, while the prototypic TCRs were semi-public and frequent. Since all three BW T cells tested were specifically and efficiently activated by HLA-DQ2.5:DQ2.5-glia-α2 *in vitro*, there is no obvious explanation for differential recruitment into the antigen-experienced repertoire. Clonotype frequencies in antigen-specific repertoires are linked to precursor frequencies. The frequency of *TRAV5* paired with *TRBV7-2* or *TRBV7-3* in the naïve repertoire differs across datasets and may be low [7, 8]. Prevalence of *TRAV26-1*, on the other hand, was found to be associated with usage of the HLA-DQ2.5 β-chain encoded by HLA-DQB1*02 [9]. Thus, differences in precursor frequencies may contribute to the biased repertoire.

In summary, we observed that the Y40$^{TCR\alpha}$-centered recognition motif is sensitive and likely unique to the prototypic TCRs using *TRAV26-1*. This recognition motif may well contribute to the formation of a biased gluten-specific T cell repertoire in CeD patients.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **CeD**: celiac disease · **pMHC**: peptide:major histocompatibility complex

Keywords: Celiac disease • pMHC recognition • Repertoire bias • TCR • *TRAV26-1*

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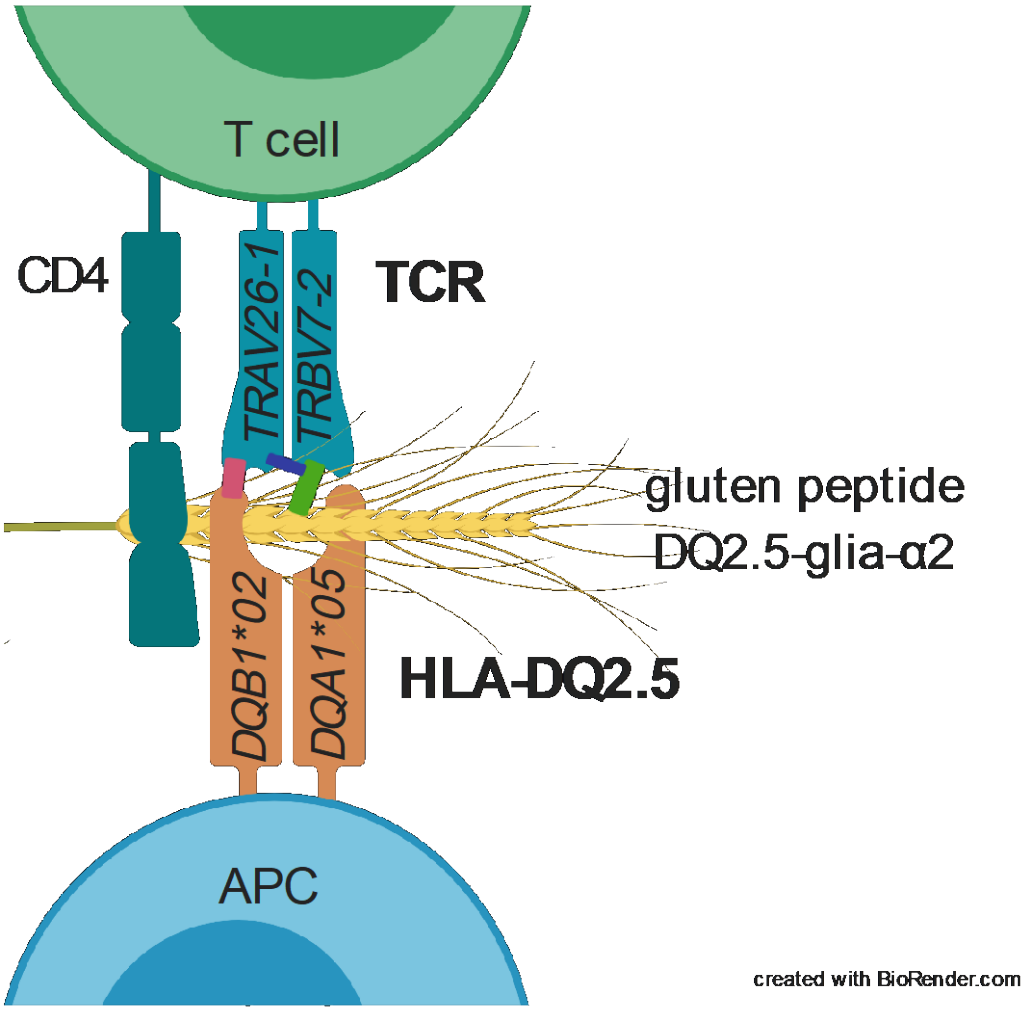
The detailed *Materials and methods* for Technical comments are available online in the Supporting information

Graphical abstract



The semi-public T-cell response towards the gluten epitope DQ2.5-glia-α2 uses a prototypic TCR encoded by the germline segments *TRAV26-1* and *TRBV7-2*. Through mutagenesis experiments, we show that a *TRAV26-1*encoded recognition motif contacts the MHC β-chain and the TCR CDR3β loop underpinning this conserved T-cell response restricted to the prototypic TCRs.

Supplementary methods

Retroviral transduction of cells

The murine BW58 T-cell line hybridoma transduced with human CD4 and devoid of endogenous TCR (BW T cells, obtained from Bernard Malissen, Centre d'Immunologie de Marseille-Luminy, Marseille, France) [1] was retrovirally transduced as described before to express the different TCR variants [2]. TCR 364 was previously cloned from TCC364.1.0.14 and is described in [2]. BW 364 WT and BW 364 WT Y40H$^{TCR\alpha}$ T cells have been described in [2] and [3], respectively. The new BW T-cell lines were generated as described before [2],[3]. Briefly, synthetic DNA constructs for the TCRα and TCRβ V domains (Genscript, Table S2) fused to mouse constant domains were cloned into pMIG-II-TCRα-P2A-TCRβ retroviral plasmid (obtained from Dario Vignali, University of Pittsburgh, Pittsburgh, Pennsylvania, USA) [4] as EcoRI/XhoI fragments. The TCR α- and β-chains are connected by a P2A peptide linker. The pMIG-II-TCR plasmids were cotransfected (Lipofectamine 2000, Invitrogen) with the pCL-Eco plasmid into the GP2-293 packaging cell line (Clontech). Virus containing supernatant was harvested 48 h and 72 h after transfection and filtrated (0.45 μm) to remove cell debris. 50,000 BW T cells were infected with 1 mL virus-containing supernatant supplemented with 10 μg/mL polybrene by centrifugation at 3,100xg at 32°C for 90 minutes. The supernatant was removed and the BW T cells were cultured in RPMI with 10% FCS and Penicillin/Streptomycin. The A20 B-cell line (HLA-DQ2.5:DQ2.5-glia-α2 with a R70A$^{MHC\beta}$ mutation) was generated essentially as described for the BW cells and as described before [3]. Briefly, the murine A20 B-cell line was used as target cell line and the pAmpho plasmid was used for cotransfection with pMIG constructs containing the pMHC sequence and eGFP. All other A20 cell lines have been described before [2],[3].

Flow cytometry

The BW T cells were incubated with hamster anti-mouse TCR β-chain Alexa-647 mAb (1:20, clone H57/597, Life Technologies) and anti-mouse CD16/CD32 Fc block (BD Biosciences, 1:200) for 30 min in the dark at 4 °C and sorted using a FACSAria II (BD Biosiences) for high and even TCR expression. A20 cells were washed and directly sorted for eGFP expression. To control for functional and even surface expression of receptors, BW T cells were stained as before. A20 B cells were stained with anti-HLA-DQ2 mAb 2.12.E11 (5 μg/mL, Diatec) directly conjugated to PE. Data was acquired on an Attune NxT flow cytometer and data was analyzed using FlowJo software v10.

Expression and purification of soluble pMHC

Recombinant, soluble HLA-DQ2.5 with the gliadin peptides containing the deamidated T-cell epitopes DQ2.5-glia-α1a (QLQPFPQPELPY, 9mer core sequence is underlined), DQ2.5-glia-α2

(PQPELPYPQPE), DQ2.5-glia-ω1 (QQPFPQPEQPFP), DQ2.5-glia-ω2 (FPQPEQPFPWQP), and DQ2.5-CLIP2 (MATPLLMQALPMGAL) linked to the N-terminus of the DQ2.5 β-chain were expressed in insect cells using a baculovirus expression vector system as previously described [5],[6]. Proteins were affinity purified using mAb 2.12.E11 specific for the HLA-DQ2 β-chain [7], before site specific biotinylation using BirA (Avidity).

T-cell activation assays

For T-cell activation assays we used recombinant soluble, biotinylated pMHC, peptide-loaded Raji human B lymphoma cells or mouse A20 B cells engineered to express HLA-DQ2.5 with covalently linked peptides. For assays using plate-bound pMHC molecules, U-shaped tissue culture 96-well plates (VWR) were coated with NeutrAvidin (5 µg/mL in PBS, Thermo Scientific) 50 µL/well or hamster anti-mouse CD3ε antibody (5 µg/mL in PBS, Thermo Scientific) 100 µL/well and incubated at 4°C/overnight. The plates were washed with PBS twice. Titrated amounts of recombinant, biotinylated pMHC were added to NeutrAvidin coated plates and incubated 1 h at room temperature. Plates were washed twice before addition of 100,000 BW T cells per well. For assays using peptide-loaded cells, 60,000 HLA-DQ2.5+ Raji cells were incubated in RPMI/10% FCS at 37°C for 3–4 h with titrated amounts of peptide before addition of 30,000 BW T cells. The following gliadin peptides were used DQ2.5-glia-α1a (QLQPFPQPELPY), DQ2.5-glia-α2 (PQPELPYPQPQL), DQ2.5-glia-ω1 (PQQPFPQPEQPFP), DQ2.5-glia-ω2 (FPQPEQPFPWQP). In assays using engineered mouse A20 B cells transduced with HLA-DQ2.5 with covalently linked peptide, titrated amounts of APCs were incubated with 30,000 BW T cells. As a positive control, BW T cells were incubated with Cell Stimulation Cocktail containing PMA and ionomycin (eBioscience, 1:500). In all assays, cells were incubated overnight at 37°C/5% CO_2. 25 µL culture supernatant were assayed for murine IL-2 secretion by ELISA (1:2 dilutions with PBS/2% FCS). Wells were coated with 50 µL of 2 µg/mL rat anti-mouse IL-2 (clone JES6-1A12, Pharmingen) and detected with 50 µL of 5 µg/mL biotin rat anti-mouse IL-2 (clone JES6-5H4, Pharmingen) followed by 80 µL of 1:2,000 diluted Streptavidin-ALP (Amersham). Plates were blocked with PBS/2% FCS and recombinant mouse IL-2 (Biolegend) was used as standard. Figures were prepared using GraphPad Prism 7. A four-parameter dose-response curve was used to derive IL-2 concentrations from a standard curve.

Structural TCR model

A homology model of the *TRAV5* TCR was generated using the TCRmodel webserver (https://tcrmodel.ibbr.umd.edu/) [8] using default settings without additional refinement of the CDR3 loops. The TCR model was then aligned to the S16 TCR in complex with HLA-DQ2.5:DQ2.5-glia-α2 (PDB ID: 4OZH). In order to remove steric clashes between the pMHC and the TCR

molecules, the structure was energy minimized in the Rosetta energy function [9],[10]. All structural figures were prepared using PyMOL v1.8.0.5.

Re-analysis of single-cell TCR sequencing dataset

The single-cell paired αβ TCR sequencing dataset [3] was re-analyzed using an improved pipeline for processing the raw TCR sequences using MiXCR [11] followed by in-house generated sequence analysis program for clonotype analysis.

Supplementary Tables

Table S1. TCR clonotype repertoire of gliadin-tetramer positive T cells.[1]

	Number of clonotypes[3]				
***TRAV*[2]**	***TRAV***	***TRAV* with *TRBV7-2/ TRBV7-3***	***TRAV* with *TRBV7-2/ TRBV7-3* + canonical CDR3β**	**Overrepresentation of *TRAV*[4]**	**Triad residues[5]**
TRAV26-1	104	85	67	8.1 (11.3σ)	**Y38, Y40, H55**
TRAV5	18	5	1	2.6 (7.9σ)	**Y38, Y40,** Y55
TRAV12-3	40	5	1	1.3 (0.8σ)	**Y38,** M40, S55

[1] Results from re-analysis of data set of TCRs carried by single gliadin-tetramer positive T cells [3]. The results correspond to those presented in Table 2 of [3]**.** The improved processing pipeline gave conclusive results for 586 unique clonotypes compared to the 536 unique clonotypes reported in [3]**.**

[2] *TRAV* gene segments in combination with *TRBV7-2* or *TRBV7-3* and the canonical CDR3β loop found at least once.

[3] The number of clonotypes using a particular *TRAV* gene segment as well as the number of clonotypes paired with *TRBV7-2*/*TRBV7-3* and additionally containing the canonical CDR3β loop.

[4] Overrepresentation was calculated as the ratio of relative frequency of *TRAV* gene usage in clonotypes in the tetramer-sorted repertoire [3] to the relative frequency in the naïve repertoire [13]. Deviations from means in the naïve distribution are given in parentheses as multiples of standard deviations σ.

[5] Amino acids in the triad positions $38^{TCR\alpha}$, $40^{TCR\alpha}$ and $55^{TCR\alpha}$ are listed. Residues identical with those encoded by *TRAV26-1* are highlighted in bold.

Table S2. Sequences of TCR α-chains and β-chains. TCRs 364 and S16 were reported previously [2],[14]. The *TRAV5* TCR was reconstructed using the variable domain sequence as stated in this table. Underlined sequence stretches are directly derived from the patient-derived sequence. The rest of the sequences was reconstructed with the germline. $Y40^{TCR\alpha}$ is highlighted in red. Sequence stretches encoded by *TRAV* and TRBV are marked in green and blue, respectively.

AV5 TCR α	GEDVEQSLFLSVREGDSSVINCTYTDSSSTYLYWYKQEPGAGLQLLTYIFSNMDMKQD QRLTVLLNKKDKHLSLRIADTQTGDSAIYFCAESPGPGKLIFGQGTTLQVKP
AV5 TCR β	GAGVSQSPSNKVTEKGKDVELRCDPISGHTALYWYRQSLGQGLEFLIYFQGNSAPDKSG LPSDRFFAERTGGSVSTLTIQRTQQEDSAVYLCASSLRSADTQYFGPGTRLTVL
364 α	DAKTTQPPSMDCAEGRAANLPCNHSTISGNEYVYWYRQIHSQGPQYIIHGLKNNETNE MASLIITEDRKSSTLILPHATLRDTAVYYCIVTNNNDMRFGAGTRLTVKP
364 β	GAGVSQSPSNKVTEKGKDVELRCDPISGHTALYWYRQSLGQGLEFLIYFQGNSAPDKSG LPSDRFSAERTGGSVSTLTIQRTQQEDSAVYLCASSIRSTDTQYFGPGTRLTVL
S16 α	DAKTTQPPSMDCAEGRAANLPCNHSTISGNEYVYWYRQIHSQGPQYIIHGLKNNETNE MASLIITEDRKSSTLILPHATLRDTAVYYCIVWGGATNKLIFGTGTLLAVQP
S16 β	GAGVSQSPSNKVTEKGKDVELRCDPISGHTALYWYRQSLGQGLEFLIYFQGNSAPDKSG LPSDRFSAERTGGSVSTLTIQRTQQEDSAVYLCASSVRSTDTQYFGPGTRLTVL

Supplementary Figures

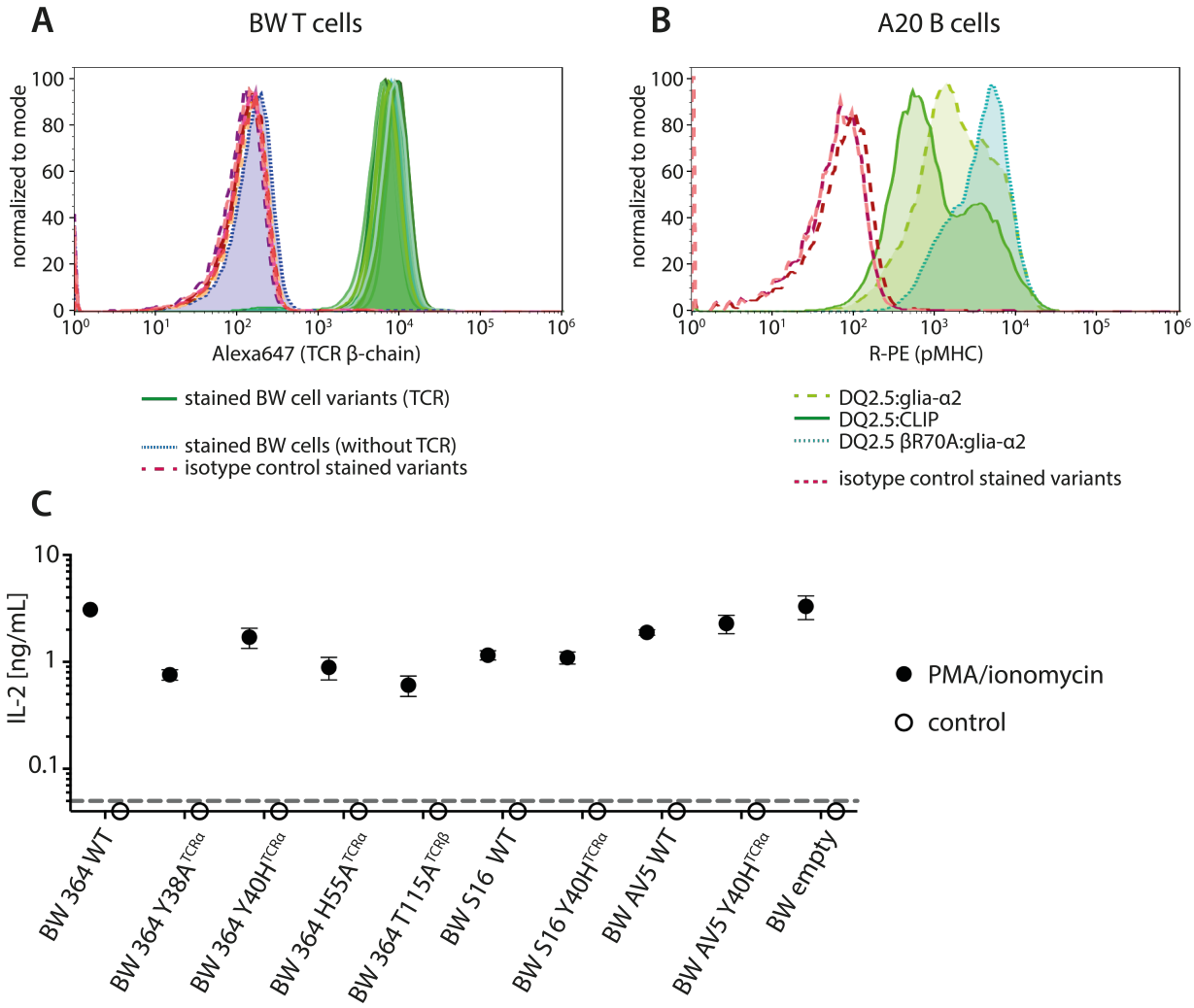


Figure S1. Validation of TCR and pMHC expression of engineered cells. All engineered BW T cells (green solid) and the untransduced BW cells without TCR (blue dotted) were stained with anti TCRβ-Alexa647 and analyzed by flow cytometry. Staining with an isotype control antibody was included as a control (red dashed) (A). All engineered A20 mouse B cells were stained with mAb 2.12.E11-biotin specific for the HLA-DQ2 β-chain and streptavidin-R-PE to assess functional expression of pMHC (green filled). Staining with an isotype control antibody was included as a control (red dotted) (B). Data is shown from a representative experiment (n=2). Figures were prepared with FlowJo v10 (A, B)**.** BW T cells were stimulated with PMA/ionomycin cell stimulation cocktail (eBiosciences). IL-2 secretion was determined by ELISA, by fitting a four-parameter dose-response curve to an IL-2 standard. Error bars indicate mean ± standard deviation of triplicates and dotted line indicates detection limit. Data is shown from a representative experiment (n=2) (C).

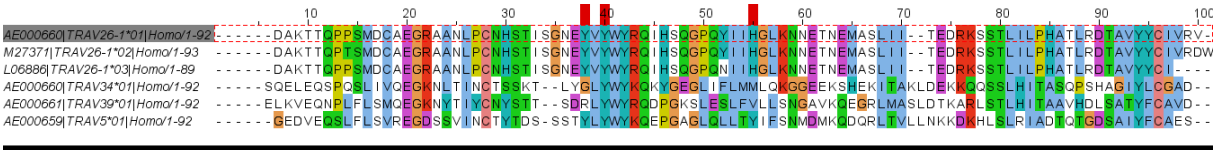


Figure S2. Alignment of TRAV gene segments. All human TRAV gene segments were downloaded from IMGT (http://www.imgt.org/) and aligned with ClustalO to identify Y38, Y40 and H55 (marked with red in numbers block). The six TRAV segments containing Tyr (Y) in the position corresponding to TRAV26-1 residue position 40 were extracted and are shown for comparison. JalView was used for analysis [15].

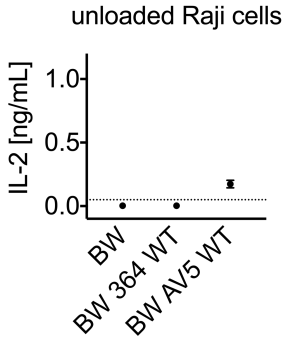


Fig. S3. Peptide-independent BW T-cell response to HLA-DQ2.5+ Raji cells: BW T cell variants and Raji B lymphoma cells were co-cultured overnight in absence of peptides. IL-2 secretion was measured by ELISA. Error bars indicate mean ± standard deviation of triplicates and dotted line indicates detection limit. Data is shown from a representative experiment (n=2).

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