



# Phenotypic Analysis of Disease-Relevant T Cells in Dermatitis Herpetiformis

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## TO THE EDITOR

Gluten-specific CD4<sup>+</sup> T cells are central players in the pathogenesis of celiac disease (CeD), an inflammatory disease driven by exposure to dietary gluten proteins. Patients with CeD are treated with a lifelong gluten-free diet. When gluten is reintroduced to the diet of patients in remission, there is a wave of activated (CD38<sup>+</sup>) gluten-specific CD4<sup>+</sup> T cells in the blood that peaks on days 6–8 after the first gluten exposure. This increase in the frequency of activated gluten-specific CD4<sup>+</sup> effector-memory T cells (T<sub>EM</sub>) in the blood can be detected by IFN- $\gamma$  enzyme-linked immunospot assay (Anderson et al., 2000; Tye-Din et al., 2010) or by HLA-DQ:gluten tetramers (Ráki et al., 2007; Sarna et al., 2018b; Zühlke et al., 2019). The gluten-specific CD4<sup>+</sup> T cells in the blood that increase in frequency also alter their phenotype to that of gluten-specific CD4<sup>+</sup> T cells in the gut mucosa of untreated CeD (Christophersen et al., 2021, 2019). Parallel with the increase of gluten-specific CD4<sup>+</sup> T cells in the blood, there is an increase in the frequency of gut-homing and activated CD8<sup>+</sup> and  $\gamma\delta$  T cells (Christophersen et al., 2021; Han et al., 2013; Risnes et al., 2021).

Although dermatitis herpetiformis (DH) is considered an extraintestinal manifestation of CeD, little is known about the gluten-induced T-cell responses in patients with DH. Recently, Kallioikoski et al. (2020) performed IFN- $\gamma$  enzyme-linked immunospot assay on PBMCs from patients with DH and CeD on day 6 after a 3-day gluten challenge as part of an oral challenge for a period of up to 1 year. Only 47% of the patients with DH displayed reactivity to deamidated gluten, leading to the conclusion that early IFN- $\gamma$  response to

selected gluten peptides does not predict clinical relapse in patients with DH on long-term gluten provocation. The study was approved by the Finnish Regional Ethics Committee of Tampere University Hospital (Tampere, Finland), and all patients gave written informed consent.

Using cryopreserved PBMCs that were available from 7 of the 19 patients from the original challenge study (Mansikka et al., 2019), in this study, we aimed to identify the phenotypic markers of disease-relevant T cells in patients with DH by performing multiparametric flow cytometric analysis of CD4<sup>+</sup>, CD8<sup>+</sup>, and  $\gamma\delta$  T cells. We sought to identify gluten-reactive T cells by use of HLA-DQ2.5:gluten tetramers (termed HLA-tetramers in the remaining part of this paper) representing a mixture of the immunodominant DQ2.5-glia- $\alpha$ 1a, DQ2.5-glia- $\alpha$ 2, DQ2.5-glia- $\omega$ 1, DQ2.5-glia- $\omega$ 2, and DQ2.5-hor3 epitopes (Tye-Din et al., 2010). We stained the T cells for CD45RA and CD62L as well for gut-homing marker integrin  $\beta$ 7, cutaneous lymphocyte antigen (CLA), and CCR4 (for further details, see Supplementary Materials and Methods).

We detected CD4<sup>+</sup> T<sub>EM</sub> (CD45RA–CD62L<sup>–</sup>) cells that bound HLA tetramers in five of the seven patients investigated, but only two patients displayed a clear increase in the frequency of such cells on day 6 (Figure 1a and b). The number of cells retrieved from the cryopreserved PBMC samples ranged from 0.35 to 3.9 million cells (median = 1.1) with a viability of 78–97% (median = 93%). Ideally, more cells should have been analyzed. Although the tetramer enrichment method is highly sensitive and distinguishes patients with CeD from HLA-

matched controls, precise cell number estimates require higher numbers (>10 million) of PBMCs, especially for the baseline samples because the method detects low-frequency cells (typically one cell per million CD4<sup>+</sup> T cells) (Sarna et al., 2018a).

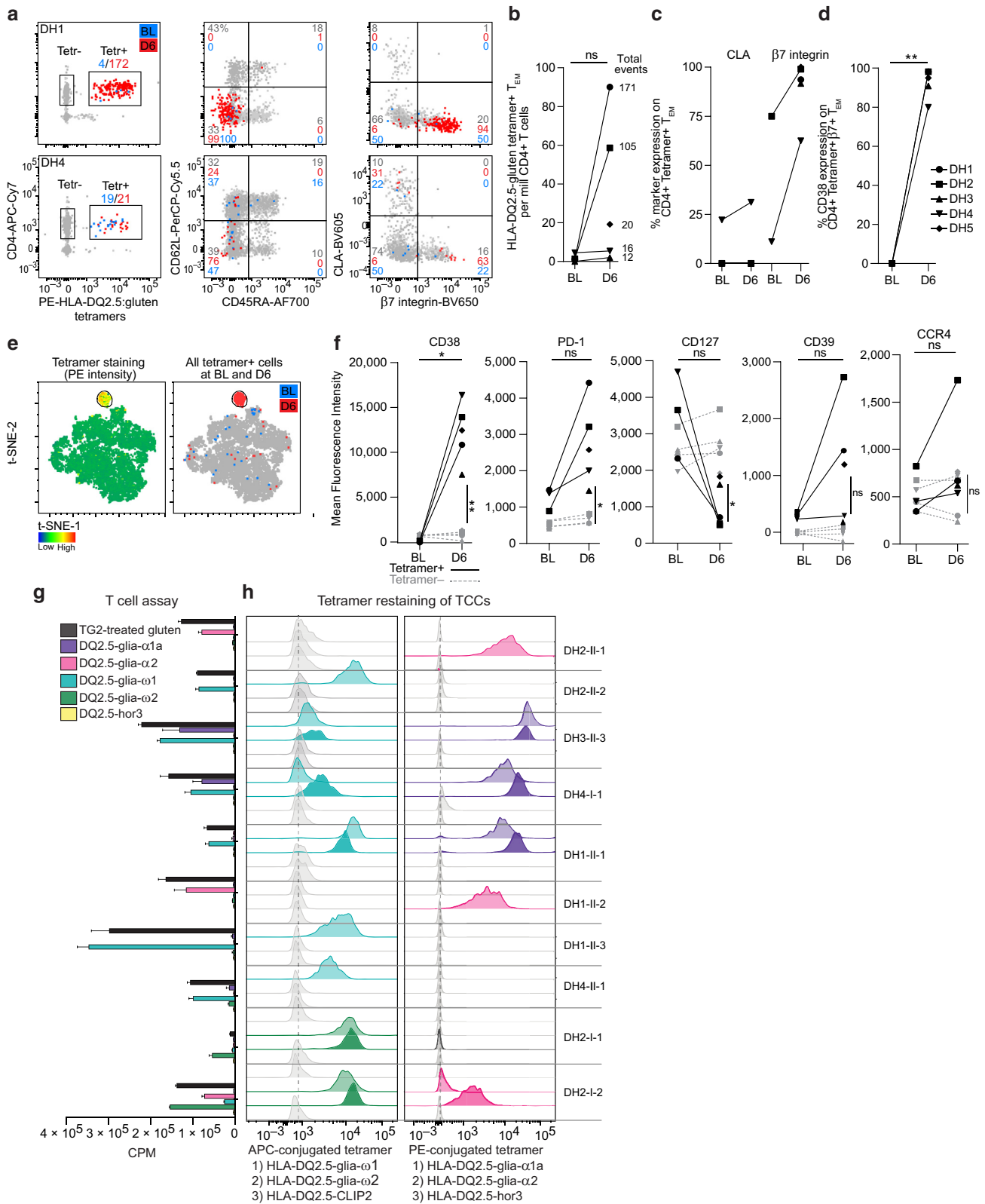
The majority of the HLA-tetramer-positive T<sub>EM</sub> cells expressed integrin  $\beta$ 7, suggesting that these are gut-homing cells. Curiously, 31% of the HLA-tetramer-positive T<sub>EM</sub> cells in one of the patients (DH4) expressed the skin-homing marker CLA (Figure 1a and c). The CLA<sup>+</sup> HLA-tetramer-positive T cells of this one patient also expressed high levels of CCR4. Whether additional patients with DH also have skin-homing gluten-specific CD4<sup>+</sup> T cells in addition to gut-homing gluten-specific CD4<sup>+</sup> T cells cannot be concluded from this single observation. More studies are warranted. Despite variation in the increase of numbers of HLA-tetramer-positive T<sub>EM</sub> cells, in all the five patients, we observed a uniform increase in CD38 expression in HLA-tetramer-positive T<sub>EM</sub> cells after gluten challenge (Figure 1d). We further showed that all the HLA-tetramer-positive T<sub>EM</sub> cells clustered distinctly from the HLA-tetramer-negative cells (Figure 1e). In line with previous observations of patients with CeD (Christophersen et al., 2021, 2019), the HLA-tetramer-positive T<sub>EM</sub> cells on gluten challenge upregulated CD38 and PD-1 and downregulated CD127 (Figure 1f).

To identify which epitope the gluten-specific T cells in patients with DH were reactive to, we sorted CD4<sup>+</sup> HLA-tetramer-positive T<sub>EM</sub> cells to generate T-cell clones and successfully established 10 T-cell clones from four patients. All the 10 T-cell clones displayed proliferative response against deamidated gluten and to at least one of the four immunodominant epitopes of wheat gluten that were represented in the HLA-tetramer cocktail used during sorting (Figure 1g). We also verified the HLA-tetramer binding of these T-

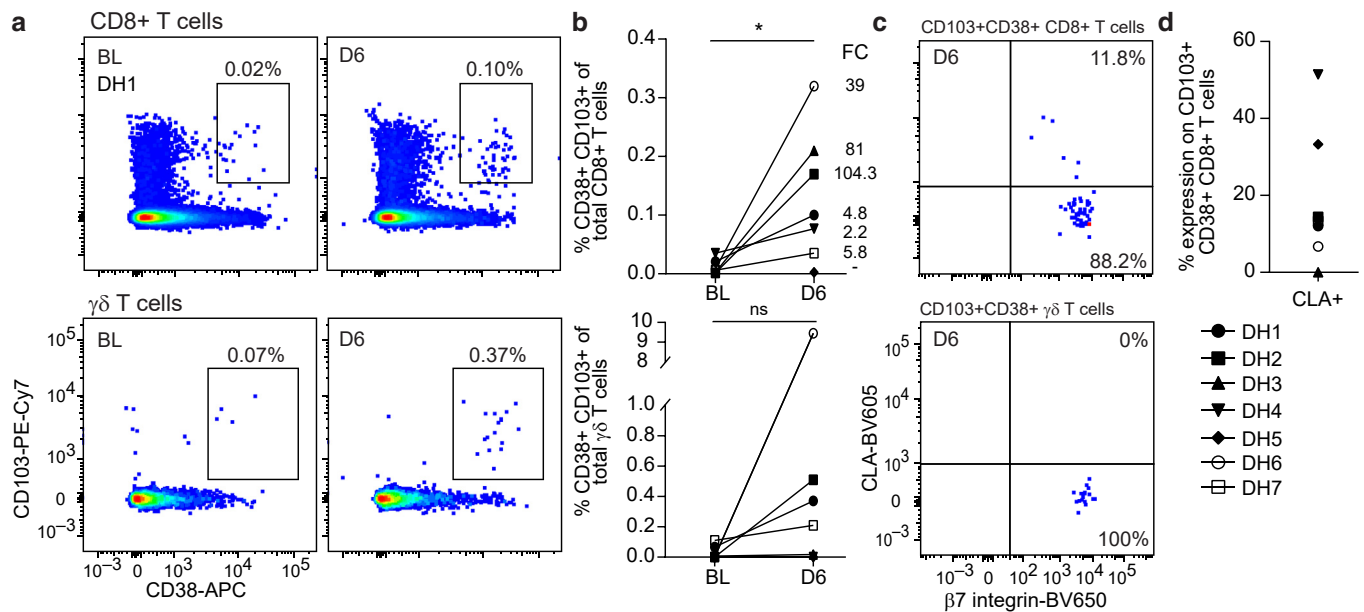
Abbreviations: CeD, celiac disease; CLA, cutaneous lymphocyte antigen; DH, dermatitis herpetiformis; T<sub>EM</sub>, effector-memory T cell

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**Figure 1. Analysis of CD4+ T cells in response to 3-day gluten challenge.** (a) Staining of CD4+ T cells with HLA-DQ2.5:gluten tetramers. HLA-tetramer-positive cells at BL are in blue, and those on D6 are in red. HLA-tetramer-negative cells are in gray. (b) Frequency estimates of HLA-tetramer-positive T<sub>EM</sub> per million CD4+ T cells of patients with DH (n = 5) before and after challenge. (c) Frequency of HLA-tetramer-positive T<sub>EM</sub> cells expressing integrin  $\beta 7$  and CLA before and after challenge for samples with >5 cells. (d) CD38 expression on integrin  $\beta 7$ + HLA-tetramer-positive T<sub>EM</sub> cells. (e) t-SNE plots of HLA-tetramer-positive cells (n = 366) and HLA-tetramer-negative cells (n = 9,750) from five patients. (f) Mean fluorescence intensity of HLA-tetramer-positive T<sub>EM</sub> cells for different markers. (g) Reactivity of TCCs (n = 10) against gluten and five gluten peptides represented in HLA-DQ2.5:gluten tetramer mixture. (h) TCCs restained with five



**Figure 2. CD8+ and  $\gamma\delta$  T cell responses to 3-day gluten challenge.** (a) Representative flow plot of one patient depicting CD103+CD38+ CD8+ T cells (top) and CD103+CD38+  $\gamma\delta$  T cells (bottom) before (BL) and after (D6) gluten challenge. (b) Frequency of CD103+CD38+ cells among all CD8+ T cells (top) and all  $\gamma\delta$  T cells (bottom) for all patients ( $n = 7$ ). The FC in the frequency of cells from D6 to BL is shown for CD8+ T cells. (c) Representative flow plot of one patient plot depicting coexpression of integrin  $\beta 7$  and CLA on CD8+ and  $\gamma\delta$  T cells. (d) Percentage of CD103+CD38+CD8+ T cells expressing integrin  $\beta 7$  and CLA plotted for all patients.  $P$ -values are obtained with paired  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . APC, allophycocyanin; BL, baseline; CLA, cutaneous lymphocyte antigen; D6, day 6; DH, dermatitis herpetiformis; FC, fold change; ns, not significant; PE, phycoerythrin.

cell clones by restaining them with individual HLA-tetramers used for analysis and sorting (Figure 1h). The results indicate that epitope specificities of gluten-specific CD4+ T cells of patients with DH are similar to those of regular patients with CeD. Clinical parameters and T-cell measurements of the study subjects are summarized in Supplementary Table S1.

Furthermore, we investigated the response of CD8+ T cells and  $\gamma\delta$  T cells. For this purpose, we used the depleted PBMCs after enrichment for HLA-tetramer-binding cells. Similar to the flow panel used for CD4+ T cells, we stained for CLA and CCR4 to investigate for potential skin homing. After gluten challenge, we observed an increase of CD103+CD38+ CD8+ T cells in six of seven patients ( $P = 0.02$ ) and an increase of CD103+CD38+  $\gamma\delta$  T cells in four of seven patients (Figure 2a and b). Moreover, the increase in the frequency of CD8+ T cells as fold change ranged from 2.2 to 104. Interestingly, patient DH5 had the lowest frequency of CD103+CD38+

CD8+ T cells on day 6, and this was the only patient who continued for the entire 12-months challenge period. CLA was expressed by a substantial proportion of total CD8+ (9–76%, median = 32%) and  $\gamma\delta$  T cells (13–63%, median = 36%). Also among CD103+CD38+ CD8+ T cells, some expressed CLA (range = 0–51%, median = 13%) (Figure 2c and d). None of the CD103+CD38+  $\gamma\delta$  T cells expressed the CLA skin-homing marker. Whether any of the activated CD8+ T cells found in the blood on day 6 after gluten challenge home to skin and exert effector functions there remains to be proven.

Although further studies with a higher number of patients and cells are warranted, our study suggests that patients with DH have gluten-induced T-cell responses with similar characteristics to regular patients with CeD. Furthermore, CD38 expression in gluten-specific integrin  $\beta 7$ + CD4+ T cells and CD103+CD38+ CD8+ T cells are promising markers to predict clinical relapse on a short gluten challenge in patients with DH.

#### Data availability statement

The data are not publicly available owing to Finnish legislation concerning patient-related data.

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#### CONFLICT OF INTEREST

The authors state no conflict of interest.

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HLA-DQ2.5:gluten tetramers and HLA-DQ2.5-CLIP2-negative control tetramer. Darker color indicates cells treated with PKI dasatinib.  $P$ -values were obtained with paired  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . BL, baseline; CLA, cutaneous lymphocyte antigen; D6, day 6; DH, dermatitis herpetiformis; ns, not significant; PKI, protein kinase inhibitor; TCC, T-cell clone;  $T_{EM}$ , effector-memory cell; t-SNE, t-distributed stochastic neighbor embedding.

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#### AUTHOR CONTRIBUTIONS

Conceptualization: LFR, LMS, KL, TS; Formal Analysis: LFR, MC, SDK, LMS; Funding Acquisition: LMS; Investigation: LFR, MC, EMag, SDK; Methodology: LFR, MC, EMag, SDK; Resources: LMS, TS; Supervision: LFR, SDK, LMS; Visualization: LFR, MC, SDK; Writing – Original Draft Preparation: LFR, SDK, LMS; Writing – Review and Editing: LFR, MC, EMag, EK, KH, EMan, KL, TS, SDK, LMS

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#### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org/](http://www.jidonline.org/), and at <https://doi.org/10.1016/j.jid.2022.07.007>

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## Distinct Radiation Responses in Virus-Positive and Virus-Negative Merkel Cell Carcinoma

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#### TO THE EDITOR

Merkel cell carcinoma (MCC) is an aggressive skin neuroendocrine cancer (Ahmed et al., 2021). Nonviral MCC (MCCN) is caused by excessive exposure to UVR, whereas polyomavirus-associated MCC (MCCP) is caused by clonal integration of Merkel cell polyomavirus (Feng et al., 2008). MCCP tumors constitutively express the viral large T and small T antigens (Ahmed et al.,

2021). *TP53* is frequently wild type (WT) in MCCP and often altered in MCCN (Knepper et al., 2019).

Despite their distinct etiologies, MCCP and MCCN tumors have similar clinical features and treatment strategies (Schrama et al., 2011). MCC is managed by surgical excision followed by adjuvant radiation therapy (Bichakjian et al., 2018). Radiation therapy alone leads to survival rates similar to those of surgery (Patel et al.,

2018). Radiation sensitivity has been shown to be associated with viral status in head and neck squamous cell carcinoma (Rieckmann et al., 2013).

We assessed the sensitivity to ionizing radiation (IR) in the p53-mutant MCCN cell lines, MCC13 and MCC26, and the p53 WT MCCP cell lines, MKL-1 and WaGa (Houben et al., 2013). We considered MCC26 to be p53 mutant because it does not express p53 protein and because the DepMap portal reports damaging mutations in exon 3 of *TP53* in this cell line (<https://depmap.org/portal/>). Cells were treated with increasing doses of IR, and cell viability was measured 3 days later. MCCP cells had a significantly greater

Abbreviations: IR, ionizing radiation; MCC, Merkel cell carcinoma; MCCN, nonviral Merkel cell carcinoma; MCCP, polyomavirus-associated Merkel cell carcinoma; WT, wild type

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## SUPPLEMENTARY MATERIALS AND METHODS

### HLA-DQ2.5:gluten tetramer staining and restaining

Monomeric HLA-DQ2.5:gluten molecules were multimerized on R-phycoerythrin (PE)-conjugated streptavidin (SA-PE S866, Invitrogen, Waltham, MA). Before tetramer staining, thawed and filtered PBMC samples were incubated at 37 °C for 10 minutes in a buffer containing 50 nM dasatinib (Sigma-Aldrich, St. Louis, MO). The samples were then directly stained with a mixture of five PE-conjugated HLA-DQ2.5:gluten tetramers representing the epitopes DQ2.5-glia- $\alpha$ 1a, DQ2.5-glia- $\alpha$ 2, DQ2.5-glia- $\omega$ 1, DQ2.5-glia- $\omega$ 2, and DQ2.5-hor3 (10  $\mu$ g/ml of each tetramer) for 45 minutes at room temperature. Tetramer-binding cells were further enriched using anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). After washing, we added 100  $\mu$ g/ml human IgG (Sigma-Aldrich) for Fc blocking, Live/dead (Violet) marker, and 0.25  $\mu$ g anti-PE (clone PE001) unconjugated antibody to the enriched samples for 10 minutes on ice before antibody staining. The samples were stained with a surface antibody mixture for 20 minutes on ice.

For tetramer restaining of T-cell clones (TCCs), we stained with three different mixtures of HLA-DQ2.5:gluten tetramers consisting of one PE-conjugated and one allophycocyanin (APC)-conjugated tetramer to determine the epitope specificity of each TCC (indicated in Figure 1g).

### Antibody panels and flow analyses

We designed the following 13-color flow panel for HLA-tetramer-stained samples used for cell sorting: CCR4-AlexaFluor488 (clone 205410, R&D System, Minneapolis, MN), CD62L-PerCP-Cy5.5 (clone DREG-56, BioLegend, San Diego, CA), CD3-BV510 (clone OKT3, BioLegend), CLA-BV605 (clone HECA-452, BD Bioscience, San Jose, CA), Integrin  $\beta$ 7-BV650 (clone FIB504, BD Bioscience), CD127-BV711 (clone A019D5, BioLegend),

PD-1-PE-CF594 (clone EH12.1, BD Bioscience), CD39-PE-Cy7 (clone A1, BioLegend), CD38-APC (clone HB-7, BioLegend), CD45RA-AlexaFluor700 (clone HI100, BioLegend), and CD4-APC-Cy7 (clone SK3, BioLegend). We included a dump channel consisting of CD11c-V450 (clone B-Ly6, BD Bioscience), CD14-Pacific Blue (clone M5E2, BD Bioscience), CD19-Pacific Blue (clone HIB19, BioLegend), and CD56-Pacific Blue (clone MEM-188, BioLegend).

The depleted PBMC samples after tetramer enrichment were stained with a 12-color flow panel with the following antibodies: CCR4-Alexa Fluor 488, CD8-PerCP (clone SK1, BioLegend), CD3-BV510, CLA-BV605, Integrin  $\beta$ 7-BV650 (clone FIB504, BD Bioscience), CD27-BV711 (clone M-T271, BioLegend),  $\gamma\delta$ TCR-PE (clone 5A6.E9, Invitrogen), CD103-PE-Cy7 (clone B-Ly7, eBioscience, San Diego, CA), CD38-APC (clone HB-7, BioLegend), CD45RA-Alexa Fluor 700, and CD39-APC-Fire750 (clone A1, BioLegend). In this study, we included a dump channel consisting of CD62L-PacificBlue (clone DREG-56, BioLegend), CD11c-V450, and CD14-Pacific Blue.

The flow analyses were performed on Aria III cell sorter (BD Biosciences) for the tetramer-stained samples and BD Fortessa for the analyses of CD8+ and  $\gamma\delta$  T cells, at the Flow Cytometry Core Facility at Oslo University Hospital (Oslo, Norway). Data analysis was done with FlowJo software (FlowJo LLC, Ashland, OR). The number of HLA-DQ2.5:gluten tetramer-positive effector-memory T cells was normalized to the number of million CD4+ T cells. Total PBMC was counted before enrichment, and the frequency of CD4+ T cells was analyzed in a pre-enriched sample.

### Generation of TCCs

TCCs were generated by limiting dilution and antigen-free expansion. HLA-tetramer binding CD4+ T cells

were sorted in a tube containing feeder mixture, which contains irradiated PBMCs (1 million/ml, 60 Gy) of three healthy donors in 10% human serum/RPMI medium with penicillin/streptomycin, phytohemagglutinin (1  $\mu$ g/ml), IL-2 (20 IU/ml), and IL-15 (1 ng/ml). This T cell/feeder mixture suspension was cultured on Terasaki plates for 10 days. Subsequently, growing TCCs were transferred to 48-well plates containing 500  $\mu$ l of feeder mixture and cultured for additional 10 days.

### T-cell proliferation assay

Proliferative response of TCCs to transglutaminase 2-deamidated CT-gluten and five immunodominant epitopes, namely DQ2.5-glia- $\alpha$ 1a-epitope peptide (QLQPFQPELPY, underlined 9-mer core sequence) (GenScript Biotech, Piscataway, NJ), the DQ2.5-glia- $\alpha$ 2-epitope (PQPELPYPQPQL) (Research Genetics, Huntsville, AL), the DQ2.5-glia- $\omega$ 1 epitope (PQQPFQPEQPFQ), the DQ2.5-glia- $\omega$ 2 epitope (FPQPEQFPWQP), and the DQ2.5-hor3a epitope (PEQPIPEQPYPQPQP) (all three from GenScript Biotech), was measured in triplicates using thymidine incorporation assay. On the first day, antigen-presenting cells (Epstein-Barr virus-immortalized B-cell line from HLA-DQ2.5 homozygous patients with Crohn's disease) irradiated at 75 Gy were incubated with gluten (10  $\mu$ g) and epitopes (10  $\mu$ M) on 96-well plate overnight at 37 °C. On the second day, TCCs were added to the plate and again incubated overnight at 37 °C. On the third day,  $^3$ H-thymidine (Hartman Analytix, Braunschweig, Germany) (1  $\mu$ Ci/well [0.037 MBq/well]) was added to each well. After 16 hours, the radioactivity of  $^3$ H-thymidine taken up by proliferating cells was measured by liquid stimulation counting (Wallac MicroBeta TriLux 1450, PerkinElmer, Waltham, MA) as counts per minute. The experiments were performed twice, and a representative experiment is shown.

**Supplementary Table S1. Clinical Parameters and T-Cell Measurements of Study Subjects**

<b>Patients with DH</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>
GFD before challenge (mo)	8	24	20	40	22	5	22
Relapse (mo)	4	3	3	6	12	1	4
Skin rash at relapse	yes	yes	yes	yes	no	yes	no
Skin IgA at BL	neg	neg	neg	neg	neg	pos	neg
Skin IgA at relapse	neg	pos	pos	pos	neg	pos	neg
Serum TG2 at BL ( $\geq 3.0$ AU/ml)	neg	neg	neg	neg	neg	neg	neg
Serum TG2 at relapse	>100	>100	3.1	neg	neg	neg	54.2
Serum TG3 at BL ( $\geq 30$ AU/ml)	30	23	3	3	<2.3	4	40
Serum TG3 at relapse	>189	>189	>189	63	7	24	89
Vh:CrD ratio at BL	4.5	2.1	3.7	2.7	2.2	2.8	2.2
Vh:CrD ratio at relapse	1	0.4	0.8	0.7	0.8	1.3	1
Events of HLA-tetramer-positive T <sub>EM</sub> cells (BL)	4	8	0	9	n.d.	n.d.	n.d.
Events of HLA-tetramer-positive T <sub>EM</sub> cells (D6)	171	105	12	16	20	n.d.	n.d.
Fold change CD103+CD38+ CD8+ T cells (D6/BL)	4.8	104.3	81.1	2.2	—	39	5.8
Fold change CD103+CD38+ $\gamma\delta$ T cells (D6/BL)	5.6	5.1	2.6	1	—	94.5	1.9

Abbreviations: AU, Arbitrary unit; BL, baseline; CrD, crypt depth; D6, day 6; n.d., not determined; neg, negative; pos, positive; T<sub>EM</sub>, effector-memory T cell; TG2, transglutaminase 2; Vh, villus height.