Phenotypic Analysis of Disease-Relevant T Cells in Dermatitis Herpetiformis

TO THE EDITOR

Gluten-specific CD4+ 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+) gluten-specific CD4+ 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+ effector-memory T cells (TEM) in the blood can be detected by IFN-γ enzyme-linked immunospot assay (Anderson et al., 2000; Tye-Din et al., 2010) or by HLA-DQ:gluten tetramers (Räki et al., 2007; Sarna et al., 2018b; Zühlke et al., 2019). The gluten-specific CD4+ T cells in the blood that increase in frequency also alter their phenotype to that of gluten-specific CD4+ T cells in the gut mucosa of untreated CeD patients (Christophersen et al., 2021, 2019). Parallel with the increase of gluten-specific CD4+ T cells in the blood, there is an increase in the frequency of gut-homing and activated CD8+ and γδ 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, Kalliokoski et al. (2020) performed IFN-γ 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-γ 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+, CD8+, and γδ 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-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2, 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 β7, cutaneous lymphocyte antigen (CLA), and CCR4 (for further details, see Supplementary Materials and Methods).

We detected CD4+ TEM (CD45RA−CD62L−) 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+ T cells) (Sarna et al., 2018a).

The majority of the HLA-tetramer–positive TEM cells expressed integrin β7, suggesting that these are gut-homing cells. Curiously, 31% of the HLA-tetramer–positive TEM cells in one of the patients (DH4) expressed the skin-homing marker CLA (Figure 1a and c). The CLA+ 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+ T cells in addition to gut-homing gluten-specific CD4+ T cells cannot be concluded from this single observation. More studies are warranted. Despite variation in the increase of numbers of HLA-tetramer–positive TEM cells, in all the five patients, we observed a uniform increase in CD38 expression in HLA-tetramer–positive TEM cells after gluten challenge (Figure 1d). We further showed that all the HLA-tetramer–positive TEM 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 TEM 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+ HLA-tetramer–positive TEM 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; TEM, 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 TEM per million CD4+ T cells of patients with DH (n = 5) before and after challenge. (c) Frequency of HLA-tetramer–positive TEM cells expressing integrin β7 and CLA before and after challenge for samples with >5 cells. (d) CD38 expression on integrin β7+ HLA-tetramer–positive TEM 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 TEM 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
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 γδ 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+ γδ 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 γδ 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+ γδ 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 β7+ 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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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...
SUPPLEMENTARY MATERIALS AND METHODS

HLA-DQ2.5:gluten tetramer staining and restaining

Monomeric HLA-DQ2.5:gluten molecules were multimerized on R-phycocerythrin (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-z1a, DQ2.5:glia-z2, DQ2.5:glia-w1, DQ2.5:glia-w2, and DQ2.5:hor3 (10 μg/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 μg/ml human IgG (Sigma-Aldrich) for Fc blocking, Live/dead (Violet) marker, and 0.25 μg/ml 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-Alexa Fluor 488 (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 β7-BV650 (clone FIB504, BD Bioscience), CD127-BV711 (clone A019D5, BioLegend), PD-1-PE-CF594 (clone EH12.1, BD Bioscience), CD39-PE-Cy7 (clone A11, BioLegend), CD38-APC (clone HB-7, BioLegend), CD45RA-Alexa Fluor 700 (clone H100, BioLegend), and CD4-APC-Cy7 (clone SK3, BioLegend). We included a dump channel consisting of CD11c-V450 (clone 2C7, BD Bioscience), CD19-Pacific Blue (clone MEM-188, BioLegend), CD38-APC (clone HB-7, BioLegend), and CD39-PE-Cy7 (clone A1, BioLegend). In this study, we included a dump channel consisting of CD62L-Pacific Blue (clone DREG-56, BioLegend), CD11c-V450, and CD14-Pacific Blue.

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 β7-BV650 (clone FIB504, BD Bioscience), CD27-BV711 (clone M-T271, BioLegend), γδTCR-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-Pacific Blue, the DQ2.5-glia-w1-epitope (PQQPFPQPEQPFP), the DQ2.5-glia-w2-epitope (FPQPEQPFPWQP), and the DQ2.5-hor3a-epitope (PEQPEQPYPQPQL) (all three from GenScript Biotech, Piscataway, NJ), the DQ2.5-glia-z2-epitope peptide (QLQFPQPQPQLYP, underlined 9-mer core sequence) (GenScript Biotech, Piscataway, NJ), the DQ2.5-glia-z2-epitope peptide (QLQFPQPQPQLYP, underlined 9-mer core sequence) (GenScript Biotech, Piscataway, NJ), and the DQ2.5-glia-z2-epitope peptide (PQQPFPQPEQPQP, the DQ2.5-glia-z2-epitope (FQQPPFPWPQP), and the DQ2.5-hor3a-epitope (PEQPI-PEQQPYPQPQ) (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 μg) and peptides (10 μM) 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, ³H-thymidine (Hartman Analytics, Braunschweig, Germany) (1 μCi/ well [0.037 MBq/well]) was added to each well. After 16 hours, the radioactivity of ³H-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.

T-cell proliferation assay

Proliferative response of TCCs to transglutaminase 2–deamidated CT-gluten and five immunodominant epitopes, namely DQ2.5:glia-z1a-epitope peptide (QLQFPQPQPQLYP, underlined 9-mer core sequence) (GenScript Biotech, Piscataway, NJ), the DQ2.5-glia-z2-epitope peptide (QPQPQPQPQP, the DQ2.5-glia-z2-epitope (FPQPEQPFPWQP), and the DQ2.5-hor3a-epitope (PEQPI-PEQQPYPQPQ) (all three from GenScript Biotech).
Supplementary Table S1. Clinical Parameters and T-Cell Measurements of Study Subjects

<table>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
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<td>GFD before challenge (mo)</td>
<td>8</td>
<td>24</td>
<td>20</td>
<td>40</td>
<td>22</td>
<td>5</td>
<td>22</td>
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<tr>
<td>Relapse (mo)</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td>4</td>
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<tr>
<td>Skin rash at relapse</td>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
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<td>no</td>
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<tr>
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<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>Skin IgA at relapse</td>
<td>neg</td>
<td>pos</td>
<td>pos</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>pos</td>
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<tr>
<td>Serum TG2 at BL (≥3.0 AU/ml)</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
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<tr>
<td>Serum TG2 at relapse</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>neg</td>
<td>neg</td>
<td>54.2</td>
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<td>Serum TG3 at BL (≥30 AU/ml)</td>
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<td>3</td>
<td>3</td>
<td>&lt;2.3</td>
<td>4</td>
<td>40</td>
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<tr>
<td>Serum TG3 at relapse</td>
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<td>&gt;189</td>
<td>&gt;189</td>
<td>63</td>
<td>7</td>
<td>24</td>
<td>89</td>
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<tr>
<td>Vh:CrD ratio at BL</td>
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<td>2.1</td>
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<td>2.7</td>
<td>2.2</td>
<td>2.8</td>
<td>2.2</td>
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<td>Vh:CrD ratio at relapse</td>
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<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
<td>1.3</td>
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<tr>
<td>Events of HLA-tetramer- positive TEM cells (BL)</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>105</td>
<td>12</td>
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<td>20</td>
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<td>n.d.</td>
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<td>104.3</td>
<td>81.1</td>
<td>2.2</td>
<td>—</td>
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<td>5.8</td>
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<td>Fold change CD103+CD38+ γδ T cells (D6/BL)</td>
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<td>5.1</td>
<td>2.6</td>
<td>1</td>
<td>—</td>
<td>94.5</td>
<td>1.9</td>
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</table>

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