


Phenotypic shift of small intestinal intra-epithelial type 1 innate lymphoid cells in celiac disease is associated with enhanced cytotoxic potential

M. Uhde,^{*1} X. Yu ,^{*1} A. Bunin,^{*}
C. Brauner,^{*} S. K. Lewis,^{*}
B. Lebowitz,^{*} S. Krishnareddy,^{*}
A. Alaedini,^{*†} B. Reizis,[‡] S. Ghosh,[§]
P. H. Green^{*} and G. Bhagat^{*¶}

^{*}Department of Medicine, Celiac Disease Center, Columbia University Irving Medical Center, Columbia University Irving Medical Center, [†]Institute of Human Nutrition, Columbia University Irving Medical Center, [‡]Department of Pathology, Department of Medicine, New York University Langone Medical Center, [§]Department of Microbiology and Immunology, College of Physicians and Surgeons, Columbia University, and [¶]Department of Pathology and Cell Biology, Columbia University Irving Medical Center, New York, NY.

Accepted for publication 27 December 2019
Correspondence: Govind Bhagat, Department of Pathology and Cell Biology, Columbia University Irving Medical Center, 630 W 168th Street, VC 14-228. New York, NY 10032. 212-342-1323, USA. E-mail: gb96@cumc.columbia.edu and Xuechen Yu, Department of Medicine, Celiac Disease Center, Columbia University Irving Medical Center, 701 W 168th Street, Suite 1204. New York, NY 10032. 212-304-5262, USA. E-mail: xy2314@cumc.columbia.edu

¹Joint first co-authors with equal contributions to the manuscript.

Summary

The small intestinal (SI) epithelium harbors a heterogeneous population of lymphocytes that mediate mucosal damage and repair in celiac disease (CD). The composition and roles of human proximal SI intra-epithelial innate lymphoid cells (ILCs), and their alterations in CD, are not well understood. We report that duodenal intra-epithelial ILCs predominantly consist of natural killer (NK)p44⁺CD127⁻ cytotoxic ILC1s and NKp44⁻CD127⁺ helper ILC1s, while ILC3s only represent a minor population. In patients with newly diagnosed or active CD (ACD) and refractory CD type 1 (RCD I), the frequency of SI NKp44⁺ ILCs is decreased, with restoration of NKp44⁺ ILC frequency observed in patients adhering to a gluten-free diet who show evidence of mucosal healing. Moreover, the frequency of SI NKp44⁻ ILCs is increased in ACD and RCD I patients and correlates with the severity of villous atrophy and epithelial damage, as assessed by serum levels of fatty acid binding protein 2 (FABP2). We show that the ILC alterations in CD represent a phenotypic shift of cytotoxic ILC1s rather than an increase in helper ILC1s or transdifferentiation of ILC1s to ILC3s, and activation-induced loss of NKp44 by cytotoxic ILC1s is associated with increased interferon (IFN)- γ expression and release of lytic granules. These findings suggest that intra-epithelial NKp44⁻CD127⁻ cytotoxic ILC1s may contribute to mucosal damage in CD.

Keywords: autoimmunity, cell surface molecules, human, inflammation, innate lymphoid cells

Introduction

Celiac disease (CD) is a common autoimmune disorder with intestinal and systemic manifestations triggered by consumption of wheat or related proteins in genetically predisposed individuals [1,2]. The activation of adaptive and innate immune responses by gliadin peptides results in small intestinal mucosal damage, and adherence to a gluten-free diet (GFD), the only effective available treatment, leads to a decrease in inflammation, recovery of

mucosal architecture and relief of symptoms [3]. A minority of patients (1–5%), however, have persistence or recurrence of gastrointestinal symptoms and villous atrophy despite adherence to a GFD for >1 year [4,5], and are deemed to have refractory celiac disease (RCD) [6].

The mechanisms underlying gliadin-mediated activation of the adaptive immune response are relatively well defined in CD, but the nature and regulation of the innate immune response within the epithelial compartment is not fully understood [7]. Intraepithelial lymphocytes (IELs)

constitute a diverse population, including thymus-derived T cells of the $\alpha\beta$ and $\gamma\delta$ lineage and subsets of bone marrow-derived innate lymphoid cells (ILCs) [8,9]. It has been shown that IELs, specifically cytotoxic T cell receptor (TCR)- $\alpha\beta^+$ T cells, which undergo natural killer (NK)-like reprogramming on interacting with epithelial stress-induced ligands, and IL-15, up-regulated upon exposure to toxic gliadin peptides, promote epithelial destruction [10,11]. Contributions of other types of lymphoid cells to the innate immune response in CD are suspected, but these are currently unclear [12–20]. Recent studies have implicated ILCs in the pathogenesis of a variety of intestinal inflammatory disorders [21]; however, data regarding the repertoire of human proximal small intestinal (SI) ILCs and their alterations in CD are limited [9,12–15,22].

To gain insight into the composition and dynamics of intra-epithelial ILCs in CD we analyzed the phenotypes of duodenal intra-epithelial ILCs in healthy adults, patients with active CD (ACD) and RCD type 1 (RCD I) and CD patients on GFD and performed correlative studies and *in-vitro* assays to assess potential cell functions.

Materials and methods

Biopsy collection and patient characteristics

Duodenal biopsies (four to five specimens) were obtained from adult CD patients, including 10 with biopsy-proven, newly diagnosed or active CD (ACD), 15 patients adherent to a GFD for greater than 6 months (0.73–28.81 years, median = 7.83 years), six patients with RCD I and 15 control subjects. ACD patients were on a gluten-containing diet, were seropositive and had villous atrophy (Marsh score >2) on histopathological examination. Celiac patients with persistent villous atrophy and with persistent or recurrent symptoms despite strict adherence to a GFD for >1 year were considered to have RCD. After excluding other possible causes of villous atrophy and symptoms, patients on a strict GFD and without a clonal population of immunophenotypically aberrant IELs were considered to have RCD type I for this study. Control subjects underwent upper endoscopy for gastrointestinal symptoms, but had normal duodenal mucosa without inflammation. Clinical data were obtained from the treating physicians. The biopsy specimens were collected after obtaining written informed consent from patients and the study was performed in accordance with the Declaration of Helsinki using a protocol approved by the institutional review board of Columbia University Irving Medical Center, New York.

Histopathological analysis

Formalin-fixed paraffin-embedded (FFPE) duodenal biopsies from patients and controls were stained with hematoxylin and eosin for morphological analysis.

Severity of villous atrophy was assessed using the modified Marsh–Oberhuber scoring system (0 = normal histology, <40 IEL/100 epithelial cells; 1 = normal histology, >40 IEL/100 epithelial cells; 2 = hyperplastic crypts, normal villi, >40 IEL/100 epithelial cells; 3a = hyperplastic crypts, partial villous atrophy, >40 IEL/100 epithelial cells; 3b = hyperplastic crypts, subtotal villous atrophy, >40 IEL/100 epithelial cells; and 3c = hyperplastic crypts, total villous atrophy, >40 IEL/100 epithelial cells) [23].

IEL isolation

IELs were extracted from biopsies as described previously, with minor modifications [24]. Freshly obtained duodenal biopsies were incubated in Hanks's balanced salt solution (HBSS) (Gibco, Gaithersburg, MD, USA) supplemented with 1 mM dithiothreitol (Sigma-Aldrich, St Louis, MO, USA), 1 mM ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich) and 1% (vol/vol) fetal calf serum (FBS) (Gibco), while shaking for 30 min at 37°C. The cell suspension was filtered through a 100 μ m cell strainer (Corning, Tewksbury, MA, USA) to separate tissue pieces. After centrifugation and resuspension of IELs in FBS containing 10% (vol/vol) dimethyl sulphoxide (DMSO) (Sigma-Aldrich), aliquots of 1×10^6 cells/mL were stored in liquid nitrogen.

Antibodies and flow cytometry

IELs were incubated on ice with conjugated antibodies in phosphate-buffered saline (PBS) containing 3% FBS for 30 min. Antibodies specific for human CD19 (HIB19), CD34 (581), CD45 (HI30), TCR- α/β (IP26), TCR- γ/δ (B1), CD107 α (1D4B), CD117 (104D2), CD122 (TU27), CD127 (A019D5), chemoattractant receptor-homologous molecule expressed on T helper type 2 (Th2) cells (CRTH2) (BM16) and NKp44 (P44-8) were from Biolegend (San Diego, CA, USA); anti-CD3 (OKT3 and UCHT1), CD14 (TUK4) and granzyme B (GB11) were from Invitrogen (Carlsbad, CA, USA); anti-CD56 (B159) and CD123 (9F5) were from BD Biosciences (Franklin Lakes NJ, USA); anti-CD8 α (RPA-T8), CD103 (Ber-ACT8), human leukocyte antigen D-related (HLA-DR) (LN3), NKp44 (44.189), interleukin (IL)-17A (eBio64DEC17), interferon (IFN)- γ (4S.B3), GATA binding protein 3 (GATA 3) (TWAJ), T-bet (eBio4B10), Eomesodermin (Eomes) (WD1928) and retinoic acid-related orphan receptor gamma t (ROR γ t) (AFKJS-9) were from eBioscience (San Diego, CA, USA); anti-CD16 (3G8) was from BD Pharmingen (San Jose, CA, USA); and anti-NKG2D (REA797) was from Miltenyi Biotec (Bergisch Gladbach, Germany).

Fluorochrome-labeled antibodies directed against human CD3, CD14, CD16, CD19, CD34, CD45, TCR- α/β , TCR/ δ , NKp44, CD123 and CD103 were used to identify CD45 $^+$ lineage-negative (Lin $^-$) intraepithelial ILCs. Fluorescence minus one (FMO) controls were used for all experiments. Cells were acquired on a BD LSR

Fortessa cell analyzer (BD Bioscience), and results were analyzed with FlowJo software, version 9.9 (Treestar, Ashland, OR, USA).

Analysis of ILC cytokine and transcription factor expression

IELs were stimulated with phorbol myristate acetate and ionomycin (PMA/IO) (1:500; Biolegend) for 3 h at 37°C in RPMI complete medium (CM), with the following composition: RPMI-1640 medium supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin, 25 mM HEPES, 1% (vol/vol) non-essential amino acids (NEAA), 1% (vol/vol) L-glutamine and 100 µM 2-mercaptoethanol (all from GIBCO) in the presence of brefeldin A (eBioscience). The cells were fixed and permeabilized with the forkhead box protein 3 (FoxP3)/transcription factor staining buffer kit (eBioscience), according to the manufacturer's protocol. After gating on ILCs, the intracellular expression of IFN-γ and IL-17A and transcription factors GATA 3, T-bet, Eomes and RORγT was analyzed.

In-vitro assessment of ILC NKp44 expression stability and cell viability

IELs were stimulated with PMA/IO for 0.5, 1, 2 and 3 h at 37°C in CM. After gating on ILC subpopulations, percentages of NKp44⁺CD103⁺ ILC1s were recorded at all time-points. The percentage of NKp44⁺ and NKp44⁺CD103⁺ apoptotic cells was assessed by staining with annexin V (Biolegend), according to the manufacturer's protocol. The rate of apoptosis was calculated as the ratio of annexin V⁺CD103⁺ ILCs at the point of isolation (time 0) to time-points 1, 2, 3 and 4, respectively. Additionally, CD127 expression was assessed at all time-points.

Serum fatty acid-binding protein 2 (FABP2) analysis

Serum was obtained on the same day as the duodenal biopsy specimens, and levels of FABP2 were determined by

enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN, USA), as previously described [25].

Statistical analysis

Difference in mean patient age in the different disease categories was evaluated by the Kruskal–Wallis one-way analysis of variance. Distribution of the modified Marsh scores was assessed by the χ^2 test. Difference in the intracellular expression of IFN-γ and IL-17A and cell surface expression of CD107a before and after *in-vitro* PMA/IO stimulation in control ILCs, as well as differences in the frequencies of NKp44⁺ and NKp44⁺ ILC1s in controls, ACD, GFD and RCD I samples were assessed by the Mann–Whitney *U*-test. Correlation between serum levels of FABP2 and the frequency of NKp44⁺ and NKp44⁺ ILC1s was analyzed using Spearman's rho. Difference in the frequency and mean fluorescence intensity (MFI) of IFN-γ-producing NKp44⁺ ILC1s in control and ACD samples after PMA/IO stimulation was evaluated by the Wilcoxon matched-pairs signed-rank test. All *P*-values were two-sided, and differences were considered statistically significant when *P* < 0.05. All data were analyzed with GraphPad Prism version 6 software (GraphPad, San Diego, CA, USA).

Results

Patient and biopsy characteristics

Clinical characteristics and biopsy histopathology of all subjects included in this study are described in Table 1. All celiac patients were confirmed to have celiac disease based on prior characteristic histopathological features and positive serology [anti-tissue transglutaminase (anti-tTG) and/or anti-endomysial antibody]. Controls lacked villous atrophy and had negative celiac serology. The mean ages of patients in different CD groups varied, in line with previous observations. RCD I was diagnosed more frequently in older individuals [26,27] and 17%

Table 1. Patient and biopsy characteristics

		Controls (<i>n</i> = 15) %	Newly diagnosed celiac disease (ACD) <i>n</i> = 10	Celiac disease patients on a gluten-free diet (GFD) <i>n</i> = 15	Refractory celiac disease I patients (RCD I) <i>n</i> = 6	<i>P</i> -values
Age (years)	Age, mean	57	34	53	60	0.0326*
	Age, standard error	20	14	15	23	
	Age range	20–80	20–68	23–78	33–88	
Sex	Female	7	6	10	3	n.s.†
	Male	8	4	5	3	
Seropositive	Seropositive	0	10	2	1	< 0.0001†
Marsh score	0	14	0	7	0	
	1	1	0	4	0	
	2	0	0	1	0	
	3a–c	0	10	3	6	

*Kruskal–Wallis test. † χ^2 test; n.s. = not significant.

were seropositive for anti-tTG immunoglobulin (Ig)A antibodies. Biopsies from CD patients showed differences in the distribution of Marsh scores or severity of villous atrophy in the different disease groups. All ACD and RCD I patients had villous atrophy and the majority of GFD patients had normalized villous architecture [28].

Phenotypic characterization of normal duodenal intraepithelial ILCs

After exclusion of T cells, B cells, monocytes and classical NK cells, Lin[−]CD45⁺ cells were analyzed for the expression of NKp44 and CD103 in duodenal IEL fractions. In healthy controls, more than 95% of the Lin[−]CD45⁺ cells were CD103⁺ and comprised two subsets, NKp44⁺ and NKp44[−] (Fig. 1a). We analyzed the expression of transcription factors (T-bet, RORγt, GATA3 and Eomes) and a panel of markers (CD127, CD117, HLA-DR, CD56, NKG2D, CD8α, CD122 and CRTH2) to further delineate the nature of the NKp44⁺ and NKp44[−] cells. The majority of NKp44⁺ cells expressed T-bet (Fig. 1b, upper panel) and lacked CD127 expression, with a subset expressing CD56, a profile consistent with cytotoxic intra-epithelial ILC1s (ieILC1s) (76–84%), also known as NK-like ILCs [29–31]. The NKp44⁺ population included a minor component of RORγt⁺ cells that expressed CD127, CD117 and HLA-DR, a profile indicative of ILC3s (4.29%) [21,32]. The majority of NKp44[−] cells also expressed T-bet (Fig. 1b, lower panel), with major subsets expressing CD127 and CD8α, consistent with helper ILC1s (11.6%) [33,34]. No distinct population of CRTH2⁺ cells was detected, confirming an absence of ILC2s within the epithelium.

These results demonstrate that duodenal intra-epithelial CD103⁺ ILCs can be divided into NKp44⁺ and NKp44[−] ILCs, with NKp44⁺ cells mainly constituting cytotoxic ILC1s and NKp44[−] cells largely comprising helper ILC1s.

Cytokine profiles and functional attributes of duodenal intraepithelial ILCs

Since ILC1s and ILC3s were identified as the intra-epithelial ILC subsets, we evaluated expression of intracellular IFN-γ and IL-17A, the main effector cytokines of ILC1s and ILC3s [29,35,36], and assessed granzyme B expression as well as the degranulation of cytotoxic granules upon stimulation. As expected, we observed a significant increase in IFN-γ expressing ILCs compared with unstimulated controls (Fig. 2a). Based on CD127 expression and in line with previous studies, we confirmed IFN-γ production by both CD127[−] (cytotoxic ILC1) and CD127⁺ (non-cytotoxic ILC1 and ILC3) subsets (Fig. 2b) [29]. Consistent with the identification of ILC3s among intra-epithelial ILCs, we detected IL-17A expression that was significantly elevated following stimulation compared with unstimulated controls (Fig. 2a). The median percentage of ILCs expressing IFN-γ was threefold higher than cells expressing IL-17A,

underlining the predominance of T-bet⁺ group 1 ILCs. We observed significantly increased expression of CD107a (Fig. 2a,b), a marker of release of lytic granules, and up-regulation of granzyme B (Fig. 2b) in only CD127[−] ILCs, confirming the cytotoxic potential of this subset.

Activation-induced NKp44 loss in cytotoxic ILC1s

We observed a loss of NKp44 expression after PMA/IO stimulation in CD103⁺ ILCs (Fig. 3a). To investigate if the increase in NKp44[−] ILCs was due to an expansion of helper ILC1s, transdifferentiation of NKp44⁺ ILC1s to ILC3s or the consequence of a phenotypic transition, i.e. loss of NKp44 expression by cytotoxic ILC1s, we stimulated IELs isolated from healthy adults with PMA/IO, and assessed the percentage of CD103⁺NKp44⁺ ILCs as well as their rate of apoptosis over time. The frequency of NKp44⁺ ILCs decreased upon stimulation, without a significant change in the apoptotic rate (Fig. 3b,c). Activation of ILCs also led to increased CD107a and granzyme B expression, as shown previously (Fig. 2b). The percentage of CD127⁺ ILCs did not change upon stimulation (Fig. 3d), arguing against transdifferentiation of NKp44⁺CD127[−] cytotoxic ILC1s to other CD127⁺ ILC subsets.

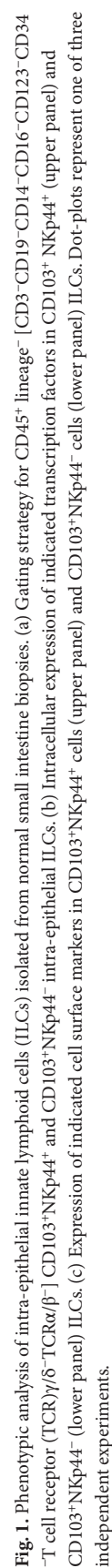
Composition of duodenal intraepithelial ILCs is altered in CD

While TCR⁺ IELs accumulate during the active state of CD, the percentage of CD7^(bright)⁺ intracellular CD3ε⁺/surfaceCD3[−] lymphocytes, which are now known to represent ILCs, has been reported to decrease in pediatric and adult patients [37,38]. Hence, we determined if there was a change in the relative abundance of NKp44[−] and NKp44⁺ ILCs in patients with ACD, RCD I and GFD. The frequency of NKp44⁺ ILCs was significantly decreased in ACD, RCD I and GFD patients, while the frequency of NKp44[−] ILCs in all disease categories showed a significant increase compared to controls (Fig. 4a,b).

Of note, the NKp44[−] ILC1 frequency was significantly lower in GFD patients compared to ACD patients. RCD I patients, despite being on GFD, had significantly fewer NKp44⁺ ILC1s than patients with uncomplicated CD on GFD (Fig. 4b). Although the frequency of NKp44⁺ ILC1s in GFD patients as a group did not differ significantly from ACD patients (Fig. 4b), an inverse correlative trend of NKp44⁺ ILC1 frequency was noted when GFD patients were stratified by the Marsh score (see section below and Supporting information, Fig. S1).

ILC alterations in CD correlate with the severity of mucosal damage and are associated with enhanced IFN-γ expression

Next, we sought to analyze the relationship between the frequencies of NKp44⁺ and NKp44[−] ILCs and severity



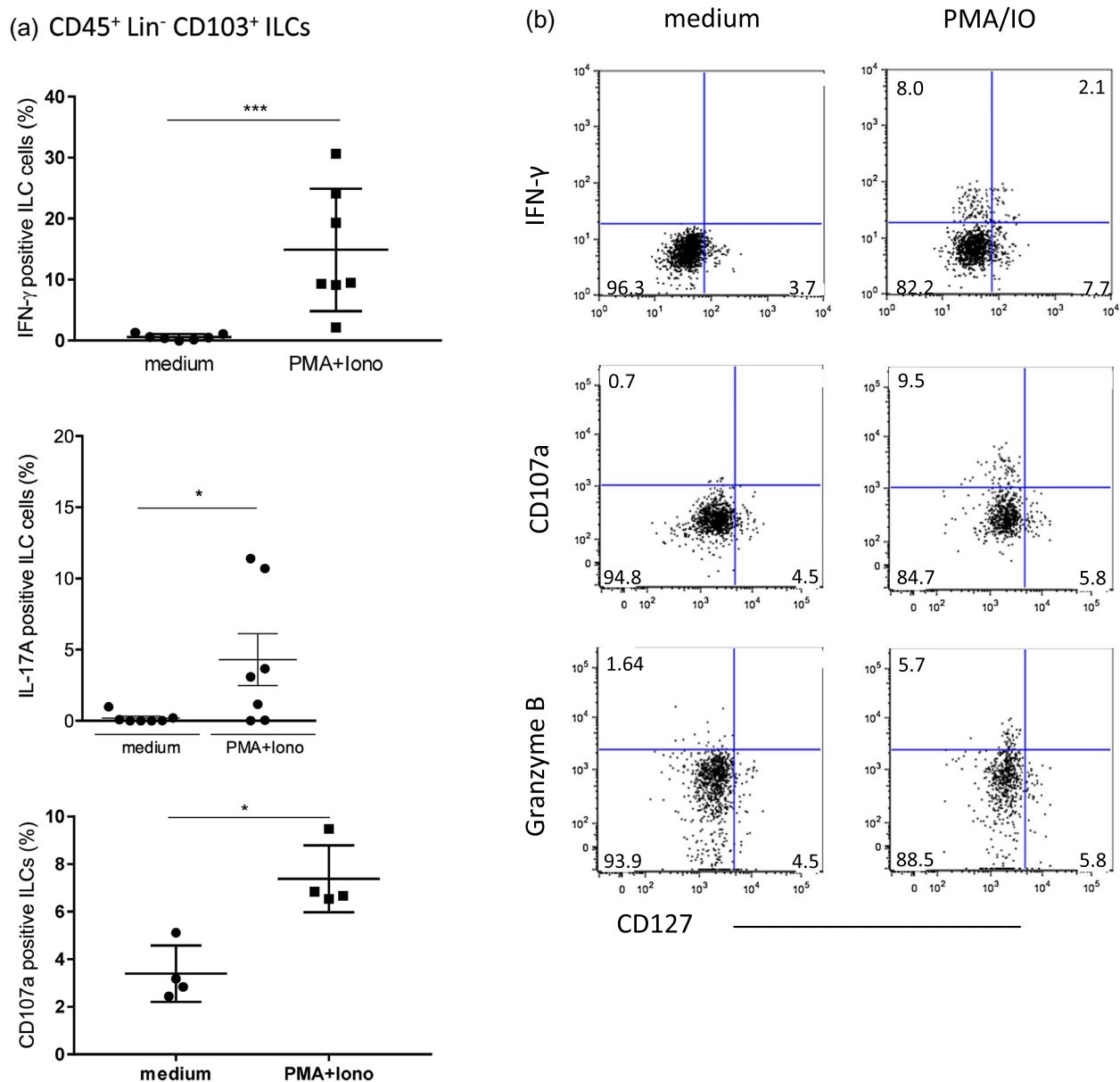


Fig. 2. Interferon (IFN)- γ , interleukin (IL)-17A, CD107a and granzyme B expression of total intraepithelial innate lymphoid cells (ILCs) after stimulation *in vitro*. (a) IFN- γ , IL-17A and CD107a-expressing ILCs. IFN- γ : medium = 0.57 ± 0.18 (n = 7), PMA/IO = 14.87 ± 3.80 (n = 7); IL-17A: medium = 0.18 ± 0.13 (n = 7), PMA/IO = 4.29 ± 1.82 (n = 7); CD107a: medium = 3.39 ± 0.59 (n = 4), PMA/IO = 7.38 ± 0.70 (n = 4) [mean percentage \pm standard error of the mean (SEM.)]. (b) Representative dot-plot of stimulated CD127⁺ and CD127⁻ ILCs. Dot-plots represent one of three independent experiments *** $P < 0.0001$; * $P < 0.05$.

of mucosal damage in CD patients. For this analysis, CD biopsies were stratified according to the modified Marsh score into three groups (Marsh scores 0, 1–2 and 3) [23]. The frequency of NKp44⁺ ILCs correlated inversely with the severity of villous atrophy being significantly higher in CD patients with Marsh 0 than Marsh 3 scores; conversely, the frequency of NKp44⁻ cells was significantly higher in patients with Marsh 3 than Marsh 0 scores (Fig. 5a). As the levels of FABP2,

a marker of epithelial cell damage and turnover, have been shown to correlate with the degree of villous atrophy in CD [39], we analyzed serum FABP2 levels in 15 CD patients (ACD, n = 7; GFD, n = 8) and three controls. Increasing FABP2 levels correlated with decreasing frequencies of NKp44⁺ and increasing frequencies of NKp44⁻ ILCs (Fig. 5b).

Lastly, to assess for any difference in the cytokine response of ILCs in CD, we compared intracellular IFN- γ

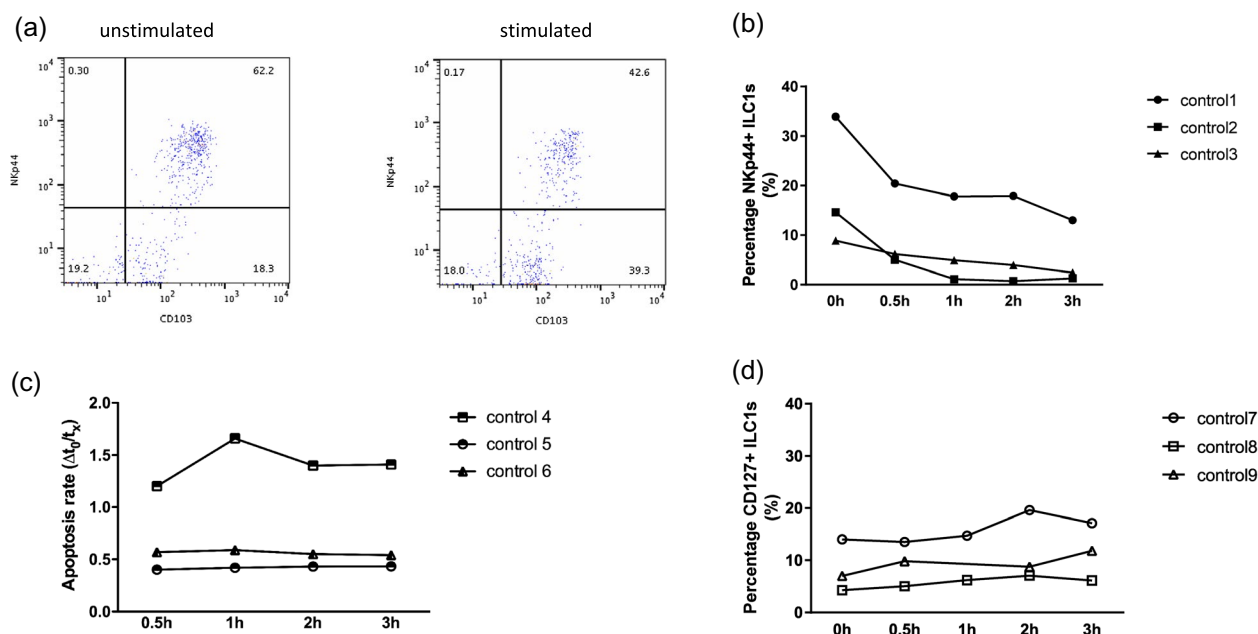


Fig. 3. Stimulation of control innate lymphoid cells (ILCs) with phorbol myristate acetate and ionomycin (PMA/IO) *in vitro*. (a) Down-regulation of NKp44 expression after PMA/IO stimulation in control ILCs. (b) Percentage of NKp44⁺ ILCs after 0.5, 1, 2 and 3 h stimulation. (c) Apoptotic rate is shown as the ratio of annexin V⁺ cells at time-point 0 (isolation) and at time-points 1–4. (d) Percentage of CD127⁺ ILCs after 0.5, 1, 2 and 3 h stimulation. Graphs represent three independent experiments.

expression in NKp44⁺ ILC1s from control and ACD patients. The frequency of IFN- γ -producing NKp44⁺ ILC1s was significantly higher in ACD patients. Moreover, the mean fluorescence intensity (MFI) of IFN- γ expression was significantly higher in NKp44⁺ ILC1s of ACD patients indicative of increased IFN- γ production (Fig. 5c).

Discussion

Investigations during recent years have led to the recognition of diverse types of ILCs with distinct functions [40]. The frequencies and phenotypes of ILC subsets differ between various anatomical sites, and the localization of specific ILCs in different compartments relates to their roles in immune and inflammatory responses [30,41]. Schmitz *et al.* showed that the composition of proximal intestinal intraepithelial ILCs changes with age, CD56⁺ conventional natural killer (cNK) cells being the dominant population at the fetal stage, with CD127⁺ cytotoxic ILCs and CD127⁺ helper ILCs predominating in children and adults, respectively [13]. In our analysis of healthy adult duodenal intraepithelial ILCs, CD103⁺ NKp44⁺ CD127⁺ IFN- γ -expressing cytotoxic ILC1s were the predominant subset, followed by NKp44⁺ CD127⁺ helper ILC1s and IL-17A-expressing ROR γ t⁺ ILC3s, while ILC2s were not identified. These findings are in line with those of Kramer *et al.*, who reported ILC1s to represent the dominant subset and a low frequency of ILC3s in the human duodenum [41].

A novel finding of our study was the loss of NKp44 expression by intra-epithelial cytotoxic ILC1s in CD. NKp44, a member of the natural cytotoxicity receptors (NCRs), was first identified in 1998 to be an activating receptor mediating non-major histocompatibility complex (MHC)-restricted cytotoxicity of NK cells [42]. It is a type I transmembrane glycoprotein with a single extracellular V-type Ig-like domain and a cytoplasmic tail containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) [43]. Upon ligand binding, NKp44 interacts with a dimer of immunoreceptor tyrosine-based activation motif (ITAM) of the adaptor DNAX-activating protein 12 (DAP12/KARAP/Tyrbp), via the positively charged lysine in the transmembrane region, to transmit an activating signal [42,44]. NKp44 signaling is important for potentiating NK cell cytotoxicity against tumor cells [45,46] and the recognition and lysis of virus-infected cells [47]. NKp44 expression has also been documented in other immune cells, including plasmacytoid dendritic cells [48], cytotoxic ILC1s [29] and a subset of ILC3s [49], potentially conferring NK-like signaling programs to these cells [50]. In the context of ILC1s, NKp44 expression has been used as a marker to distinguish cytotoxic intraepithelial ILC1s (ieILC1s) from classic helper ILC1s [29].

Lower frequencies of NKp44⁺ ILCs have previously been reported in other gastrointestinal diseases, including ileal lamina propria ILC3s in inflammatory bowel disease (IBD) and rectal mucosal ILC1s in non-celiac

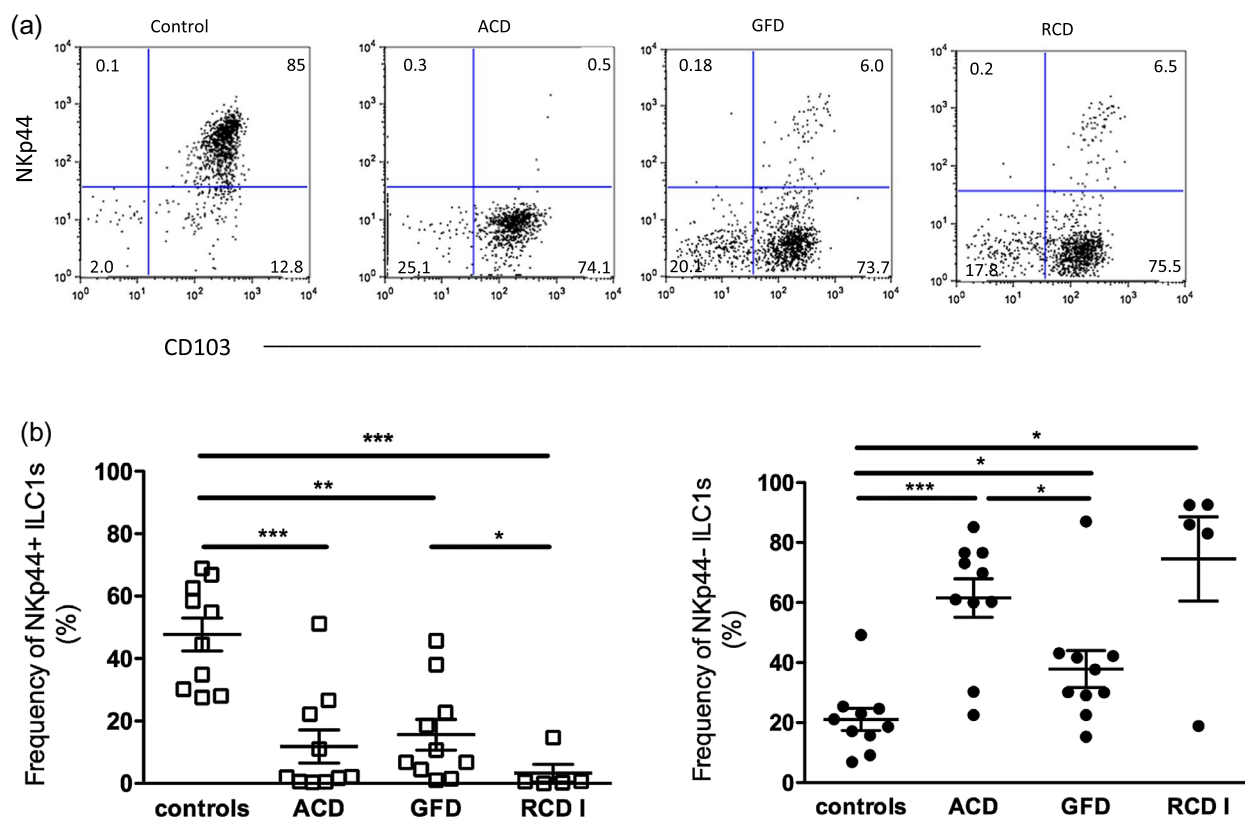


Fig. 4. Innate lymphoid cell (ILC) composition in controls and patients with celiac disease (CD). (a) Dot-plots represent one of five to 10 independent experiments. (b) The frequencies of NKp44⁺ and NKp44⁺ ILC1s in controls, patients with active CD (ACD), patients on a gluten-free diet (GFD) and patients with refractory CD type 1 (RCD I). Left: controls = 47.68 ± 5.25 ($n = 10$), ACD = 11.85 ± 5.31 ($n = 10$), GFD = 15.56 ± 4.92 ($n = 10$), RCD = 3.27 ± 2.86 ($n = 5$); right: controls = 21.07 ± 3.68 ($n = 10$), ACD = 61.55 ± 6.43 ($n = 10$), GFD = 37.87 ± 6.16 ($n = 10$), RCD I = 74.6 ± 14.05 ($n = 5$) [mean percentage \pm standard error of the mean (SEM.)]. *** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

wheat sensitivity (NCWS) [33,51], but the reason for attrition of NKp44⁺ ILCs has not been explored. We observed a marked decrease in NKp44⁺ ILCs in ACD (and RCD I). Additionally, *in-vitro* studies showed activation-induced loss of NKp44 expression on cytotoxic ILC1s. These findings suggest that the decrease in NKp44 expression is the consequence of an inflammation/activation-induced phenotypic shift of cytotoxic ILC1s to NKp44⁺ CD127⁺ ILCs rather than loss due to cell death, migration to another site, transdifferentiation into ILC3s or a relative expansion of helper NKp44⁺ CD127⁺ ILC1s. This observation is congruent with prior reports that did not identify any difference in the fraction of intraepithelial (or lamina propria) CD127⁺ ILCs between adult CD patients and healthy individuals [12,14]. The long-term fate of NKp44⁺ CD127⁺ ILCs is unknown. It is unclear whether CD127⁺ ILCs up-regulate NKp44 expression upon resolution of inflammation or if they are replaced by newly generated NKp44⁺ ILCs.

The precise mechanisms underlying the loss of NKp44 are not known. Up-regulation of indoleamine

2,3-dioxygenase (IDO) and prostaglandin E₂ (PGE₂) in CD [52–54] could potentially contribute, similarly to what has been observed in NK cells [55,56]. Of interest, Marafini *et al.* reported an absence of NKp44/NKp46 double-positive NK cells and NK T cells in the intestinal epithelia in ACD [57]. Functional exhaustion of NK cells during chronic infections and anti-tumor immune responses [58,59] is associated with downregulation of NKp44 among other antigens [60]. However, in contrast to NKp44⁺ CD127⁺ cytotoxic ILCs in CD, exhausted NK cells show diminished cytolytic activity and decreased expression of effector cytokines.

The correlation of intraepithelial NKp44⁺ ILC frequency with increased severity of villous atrophy and increased serum levels of FABP2, a marker for enterocyte damage, as well as increased IFN- γ expression and enhanced cytotoxic potential, suggests roles of cytotoxic ILC1s in fostering inflammation and mediating epithelial destruction in CD. These findings support the superior cytotoxic activity of CD3⁺ IELs previously reported by Leon *et al.* [61]. A few studies have explored mechanisms of intraepithelial ILC

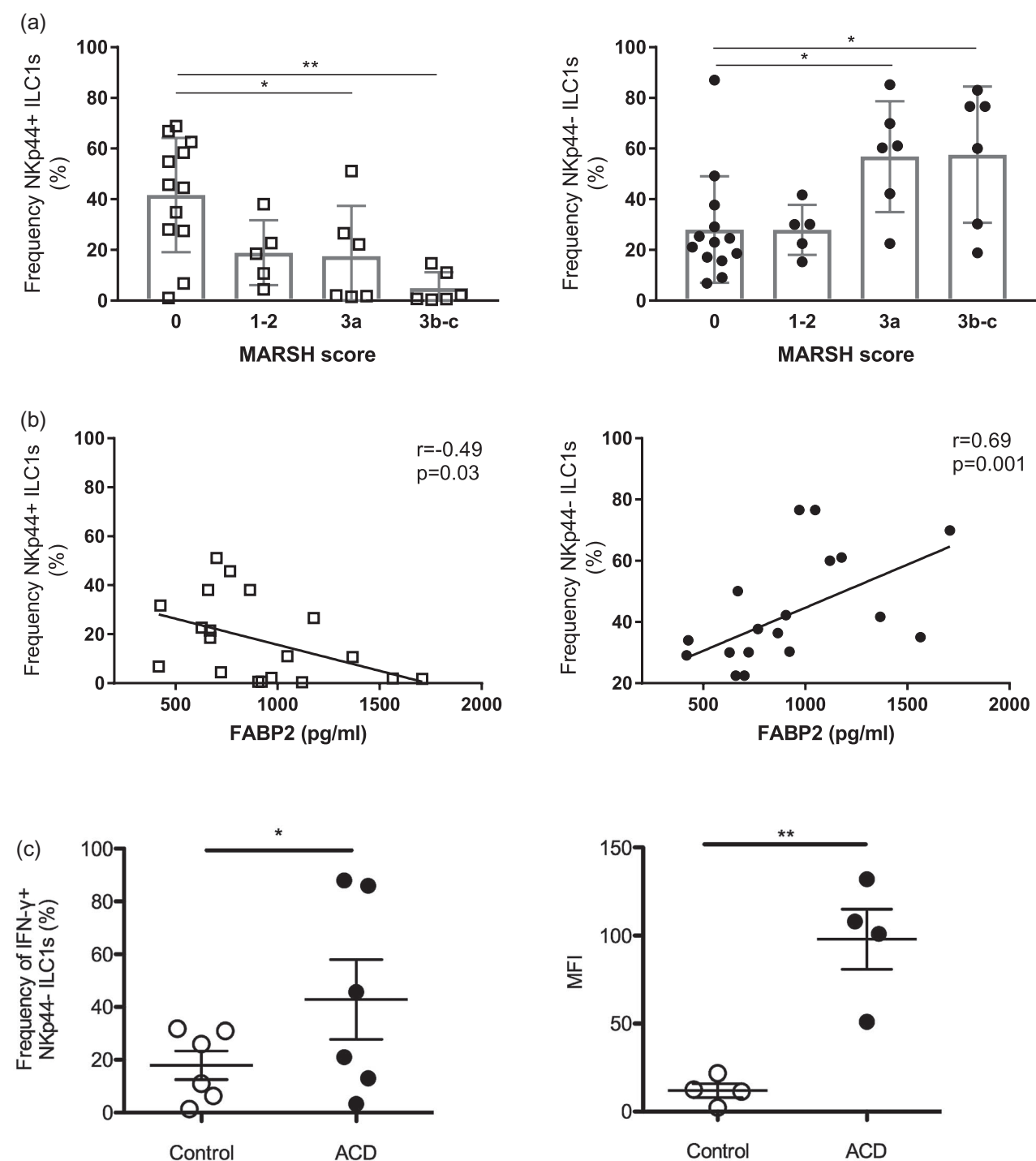


Fig. 5. Correlation between natural killer (NK)p44⁺ and NKp44⁻ innate lymphoid cell (ILC) frequencies and intestinal epithelial cell damage in celiac disease (CD) patients. (a) Frequency of NKp44⁺ and NKp44⁻ intraepithelial ILCs in CD patients, including patients with active CD (ACD) and patients on a gluten-free diet (GFD), and controls stratified for the degree of villous atrophy based on Marsh scores 0–3. Left: Marsh 0 = 41.68 ± 6.51 (n = 13), 1–2 = 18.88 ± 5.72 (n = 5), 3a = 17.55 ± 8.11 (n = 6), 3b–c = 4.95 ± 2.56 (n = 6); right: Marsh 0 = 28.04 ± 5.82 (n = 13), 1–2 = 27.92 ± 4.40 (n = 5); 3a = 56.85 ± 8.94 (n = 6), 3b–c = 57.57 ± 10.98 (n = 6) [mean percentage ± standard error of the mean (SEM.)]. (b) Correlation between serum fatty acid binding protein 2 (FABP2) levels and frequencies of NKp44⁺ and NKp44⁻ ILCs in CD patients (n = 15) and controls (n = 3). (c) Stimulation of control and active CD (ACD) NKp44⁻ CD103⁺ ILC1s with phorbol myristate acetate and ionomycin (PMA/IO) *in vitro*. Left: percentage of interferon (IFN)-γ producing NKp44⁻ CD103⁺ ILC1s. Control = 24.95 ± 4.82 (n = 6), ACD = 60.18 ± 16.29 (n = 6) (mean percentage ± s.e.m.); right: MFI of IFN-γ expression in NKp44⁻ CD103⁺ ILC1s. Control = 12.03 ± 3.98 (n = 4), ACD = 98 ± 17.01 (n = 4) (mean percentage ± SEM.). **P < 0.01.

activation in CD and suggested roles for factors derived from both innate and adaptive immune cells. While immunogenic gliadin peptides activate HLA-DQ2 or -DQ8 restricted T cells in CD, some gliadin peptides can induce an epithelial stress response resulting in the up-regulation of IL-15 [62–64], which has been shown to stimulate ILC1s in CD [12,13,15,29]. Other cytokines, e.g. TNF, IL-2 and IL-21, released by gliadin responsive lamina propria CD4⁺ T cells have also been reported to stimulate the proliferation of Lin[−] innate IELs, in addition to enhancing their survival [65]. Additionally, non-gluten proteins such as amylase-trypsininhibitors (ATIs) have been demonstrated to initiate an innate immune response by binding to Toll-like receptor (TLR)-4 on monocytes, macrophages and dendritic cells [66], but it is not known if they can activate ILCs. We found no evidence of ILC activation by gluten and non-gluten proteins (data not shown). Further studies are awaited to elucidate other relevant contact dependent and independent mechanisms of intraepithelial ILC activation in CD and other inflammatory disorders.

RCD, a rare complication of CD, is currently subdivided into two types, RCD I and RCD II, based on the absence or presence of a clonal population of immunophenotypically aberrant IELs [67]. RCD II is a neoplastic disorder, also referred to as cryptic enteropathy-associated T cell lymphoma or '*in-situ*' lymphoma [68] and the aberrant IELs have recently been shown to derive from CD127[−]CD122⁺ (IL-15R β) ILCs [12,15]. Acquisition of gain-of-function mutations in Janus kinase 1 (JAK1) and signal transducer and activator of transcription 3 (STAT3), which enhance response to IL-15, are thought to contribute to the clonal expansion of these ILCs [15]. The etiology of RCD I is unresolved and is probably multi-factorial. Up to a third of patients are seropositive for tissue transglutaminase antibodies [69–72], suggesting either inadvertent gluten intake or a protracted inflammatory or immune response to ingested gluten. An increased frequency of infections has also been documented in RCD I patients [73,74]. Although polyclonal expansions of intraepithelial CD3⁺ CD8⁺TCR⁺ T cells are well recognized, ILC alterations have not been previously examined in RCD I. We observed a similar ILC profile and phenotypic shift of cytotoxic ILC1s (NKp44 loss) in RCD I as in ACD, suggesting an analogous mucosal inflammatory milieu. Although we recommend further exploration of the biological consequences of NKp44 loss by ILCs in CD and RCD, this marker does not appear to be suitable for the evaluation of aberrant IELs in RCD II, due to its infrequent expression [12].

In summary, this study is the first, to our knowledge, to characterize duodenal intraepithelial ILC alteration in CD. We identified a phenotypic shift of cytotoxic ILCs associated with enhanced cytotoxic potential and increased IFN- γ

expression, which correlated with the severity of mucosal damage. It remains to be determined whether therapeutic targeting of specific ILC subsets can dampen mucosal inflammation and hasten the healing of SI mucosa in CD.

Acknowledgements

We would like to thank Cyrus Garcia for help with sample processing and obtaining and organizing the clinical data. This study was made possible with a research grant from the Celiac Disease Center at Columbia University and a Bouncer Foundation grant.

Disclosures

The authors declare no conflicts of interest.

Author contributions

G. B. conceived the study idea; M. U., A. B. and G. B. designed the experiments. G. B., M. U., A. B., C. B., X. Y., P. G. and G. B. analyzed data. P. G., S. K., S. L. and B. L. provided control and celiac disease biopsy samples; A. A. provided digested wheat proteins and rice proteins for the stimulation studies. S. G., B. R., P. G. and G. B. supervised the study. X. Y. and G. B. drafted and wrote the manuscript; all authors provided critical comments and reviewed the manuscript.

References

- Green PH, Cellier C. Celiac disease. *N Engl J Med* 2007; **357**:1731–43.
- Lebwohl B, Sanders DS, Green PHR. Coeliac disease. *Lancet* 2018; **391**:70–81.
- Abadie V, Sollid LM, Barreiro LB, Jabri B. Integration of genetic and immunological insights into a model of celiac disease pathogenesis. *Ann Rev Immunol* 2011; **29**:493–525.
- Roshan B, Leffler DA, Jamma S *et al.* The incidence and clinical spectrum of refractory celiac disease in a North American referral center. *Am J Gastroenterol* 2011; **106**:923–8.
- van Wanrooij RLJ, Bouma G, Bontkes HJ *et al.* Outcome of referrals for non-responsive celiac disease in a tertiary center: low incidence of refractory celiac disease in the Netherlands. *Clin Transl Gastroen* 2017; **8**:e218.
- Ludvigsson JF, Bai JC, Biagi F *et al.* Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. *Gut* 2014; **63**:1210–28.
- Jabri B, Sollid LM. Tissue-specific immune responses. Tissue-mediated control of immunopathology in coeliac disease. *Nat Rev Immunol* 2009; **9**:858–70.
- McDonald BD, Jabri B, Bendelac A. Diverse developmental pathways of intestinal intra-epithelial lymphocytes. *Nat Rev Immunol* 2018; **18**:514–25.

- 9 Van Kaer L, Olivares-Villagomez D. Development, homeostasis, and functions of intestinal intra-epithelial lymphocytes. *J Immunol* 2018; **200**:2235–44.
- 10 Hue S, Mention JJ, Monteiro RC *et al.* A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* 2004; **21**:367–77.
- 11 Meresse B, Curran SA, Ciszewski C *et al.* Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med* 2006; **203**:1343–55.
- 12 Schmitz F, Tjon JM, Lai Y *et al.* Identification of a potential physiological precursor of aberrant cells in refractory coeliac disease type II. *Gut* 2013; **62**:509–19.
- 13 Schmitz F, Kooy-Winkelaar Y, Wiekmeijer AS *et al.* The composition and differentiation potential of the duodenal intra-epithelial innate lymphocyte compartment is altered in coeliac disease. *Gut* 2016; **65**:1269–78.
- 14 Marafini I, Monteleone I, Di Fusco D *et al.* TNF-alpha producing innate lymphoid cells (ILCs) are increased in active celiac disease and contribute to promote intestinal atrophy in mice. *PLOS ONE* 2015; **10**:e0126291.
- 15 Ettersperger J, Montcuquet N, Malamut G *et al.* Interleukin-15-dependent T-cell-like innate intra-epithelial lymphocytes develop in the intestine and transform into lymphomas in celiac disease. *Immunity* 2016; **45**:610–25.
- 16 Montalvillo E, Bernardo D, Martinez-Abad B *et al.* Increased intra-epithelial valpha24 invariant NKT cells in the celiac duodenum. *Nutrients* 2015; **7**:8960–76.
- 17 Grose RH, Cummins AG, Thompson FM. Deficiency of invariant natural killer T cells in coeliac disease. *Gut* 2007; **56**:790–5.
- 18 Marafini I, Monteleone I, Di Fusco D *et al.* Celiac disease-related inflammation is marked by reduction of Nkp44/Nkp46-double positive natural killer cells. *PLoS ONE* 2016; **11**:e0155103.
- 19 Jabri B, de Serre NP, Cellier C *et al.* Selective expansion of intra-epithelial lymphocytes expressing the HLA-E-specific natural killer receptor CD94 in celiac disease. *Gastroenterology* 2000; **118**:867–79.
- 20 Dunne MR, Elliott L, Hussey S *et al.* Persistent changes in circulating and intestinal gamma delta T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease. *PLOS ONE* 2013; **8**:e76008.
- 21 Geremia A, Arancibia-Carcamo CV. Innate lymphoid cells in intestinal inflammation. *Front Immunol* 2017; **8**:1296.
- 22 van Unen V, Li N, Molendijk I *et al.* Mass cytometry of the human mucosal immune system identifies tissue- and disease-associated immune subsets. *Immunity* 2016; **44**:1227–39.
- 23 Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; **11**:1185–94.
- 24 Mayassi T, Ladell K, Gudjonson H *et al.* Chronic inflammation permanently reshapes tissue-resident immunity in celiac disease. *Cell* 2019; **176**:967–81.e19.
- 25 Uhde M, Indart AC, Yu XCB *et al.* Markers of non-coeliac wheat sensitivity in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Gut* 2019; **68**:377–8.
- 26 Chander U, Leeman-Neill RJ, Bhagat G. Pathogenesis of enteropathy-associated T cell lymphoma. *Curr Hematol Malig Rep* 2018; **13**:308–17.
- 27 Malamut G, Afchain P, Verkarre V *et al.* Presentation and long-term follow-up of refractory celiac disease: comparison of type I with type II. *Gastroenterology* 2009; **136**:81–90.
- 28 Wahab PJ, Meijer JW, Mulder CJ. Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. *Am J Clin Pathol* 2002; **118**:459–63.
- 29 Fuchs A, Vermi W, Lee JS *et al.* Intra-epithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells. *Immunity* 2013; **38**:769–81.
- 30 Simoni Y, Fehlings M, Kloverpris HN *et al.* Human innate lymphoid cell subsets possess tissue-type based heterogeneity in phenotype and frequency. *Immunity* 2017; **46**:148–61.
- 31 Lim AI, Li Y, Lopez-Lastra S *et al.* Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell* 2017; **168**:1086–100.e10.
- 32 Hepworth MR, Fung TC, Masur SH *et al.* Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. *Science* 2015; **348**:1031–5.
- 33 Bernink JH, Peters CP, Munneke M *et al.* Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol* 2013; **14**:221–9.
- 34 Van Kaer L, Algood HM, Singh K *et al.* CD8alphaalpha(+) innate-type lymphocytes in the intestinal epithelium mediate mucosal immunity. *Immunity* 2014; **41**:451–64.
- 35 Artis D, Spits H. The biology of innate lymphoid cells. *Nature* 2015; **517**:293–301.
- 36 Sonnenberg GF, Artis D. Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. *Immunity* 2012; **37**:601–10.
- 37 Spencer J, MacDonald TT, Diss TC, Walker-Smith JA, Ciclitira PJ, Isaacson PG. Changes in intra-epithelial lymphocyte subpopulations in coeliac disease and enteropathy associated T cell lymphoma (malignant histiocytosis of the intestine). *Gut* 1989; **30**:339–46.
- 38 Camarero C, Eiras P, Asensio A *et al.* Intra-epithelial lymphocytes and coeliac disease: permanent changes in CD3⁺/CD7⁺ and T cell receptor gamma delta subsets studied by flow cytometry. *Acta Paediatr* 2000; **89**:285–90.
- 39 Adriaanse MPM, Tack GJ, Passos VL *et al.* Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. *Aliment Pharm Ther* 2013; **37**:482–90.
- 40 Vivier E, Artis D, Colonna M *et al.* Innate lymphoid cells: 10 years on. *Cell* 2018; **174**:1054–66.
- 41 Kramer B, Goeser F, Lutz P *et al.* Compartment-specific distribution of human intestinal innate lymphoid cells is altered

- in HIV patients under effective therapy. *PLOS Pathog* 2017; **13**:e1006373.
- 42 Vitale M, Bottino C, Sivori S *et al.* Nkp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med* 1998; **187**:2065–72.
 - 43 Cantoni C, Ponassi M, Biassoni R *et al.* The three-dimensional structure of the human NK cell receptor Nkp44, a triggering partner in natural cytotoxicity. *Structure* 2003; **11**:725–34.
 - 44 Kruse PH, Matta J, Ugolini S, Vivier E. Natural cytotoxicity receptors and their ligands. *Immunol Cell Biol* 2014; **92**:221–9.
 - 45 Parodi M, Favoreel H, Candiano G *et al.* Nkp44-NKp44 ligand interactions in the regulation of natural killer cells and other innate lymphoid cells in humans. *Front Immunol* 2019;**10**:719.
 - 46 Baychelier F, Sennepin A, Ermonval M, Dorgham K, Debre P, Vieillard V. Identification of a cellular ligand for the natural cytotoxicity receptor Nkp44. *Blood* 2013; **122**:2935–42.
 - 47 Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by Nkp44 but not by Nkp30. *Eur J Immunol* 2001; **31**:2680–9.
 - 48 Bonaccorsi I, Cantoni C, Carrega P *et al.* The immune inhibitory receptor LAIR-1 is highly expressed by plasmacytoid dendritic cells and acts complementary with Nkp44 to control IFN alpha production. *PLOS ONE* 2010; **5**:e15080.
 - 49 Hoorweg K, Peters CP, Cornelissen F *et al.* Functional differences between human Nkp44(–) and Nkp44(+) RORC+ innate lymphoid cells. *Front Immunol* 2012; **3**:72.
 - 50 Villanova F, Flutter B, Tosi I *et al.* Characterization of innate lymphoid cells in human skin and blood demonstrates increase of Nkp44+ILC3 in psoriasis. *J Invest Dermatol* 2014; **134**:984–91.
 - 51 Di Liberto D, Mansueto P, D'Alcamo A *et al.* Predominance of type 1 innate lymphoid cells in the rectal mucosa of patients with non-celiac wheat sensitivity: reversal after a wheat-free diet. *Clin Transl Gastroenterol* 2016; **7**:e178.
 - 52 Branski D, Karmeli F, Gross-Kieselstein E, Abrahamov A, Rachmilewitz D. Prostaglandins in small intestinal mucosa of children with celiac disease. *J Pediatr Gastroenterol Nutr* 1984; **3**:672–5.
 - 53 Lavo B, Knutson L, Loof L, Hallgren R. Gliadin challenge-induced jejunal prostaglandin E2 secretion in celiac disease. *Gastroenterology* 1990; **99**:703–7.
 - 54 Torres MI, Lopez-Casado MA, Lorite P, Rios A. Tryptophan metabolism and indoleamine 2,3-dioxygenase expression in coeliac disease. *Clin Exp Immunol* 2007; **148**:419–24.
 - 55 Pietra G, Manzini C, Rivara S *et al.* Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res* 2012; **72**:1407–15.
 - 56 Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008; **111**:1327–33.
 - 57 Marafini I, Monteleone I, Di Fusco D *et al.* Celiac disease-related inflammation is marked by reduction of Nkp44/Nkp46-double positive natural killer cells. *PLOS ONE* 2016; **11**:e0155103.
 - 58 Wherry EJ, Teichgraber V, Becker TC *et al.* Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 2003; **4**:225–34.
 - 59 Bi J, Tian Z. NK cell exhaustion. *Front Immunol* 2017; **8**:760.
 - 60 Kaufmann DE, Walker BD. Programmed death-1 as a factor in immune exhaustion and activation in HIV infection. *Curr Opin HIV AIDS* 2008; **3**:362–7.
 - 61 Leon F, Roldan E, Sanchez L, Camarero C, Bootello A, Roy G. Human small-intestinal epithelium contains functional natural killer lymphocytes. *Gastroenterology* 2003; **125**:345–56.
 - 62 Setty M, Discepolo V, Abadie V *et al.* Distinct and synergistic contributions of epithelial stress and adaptive immunity to functions of intra-epithelial killer cells and active celiac disease. *Gastroenterology* 2015; **149**:681–91.e10.
 - 63 Meresse B, Chen ZG, Ciszewski C *et al.* Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 2004; **21**:357–66.
 - 64 Hue S, Mention JJ, Monteiro RC *et al.* A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* 2004; **21**:367–77.
 - 65 Kooy-Winkelaar YMC, Bouwer D, Janssen GMC *et al.* CD4 T-cell cytokines synergize to induce proliferation of malignant and nonmalignant innate intra-epithelial lymphocytes. *Proc Natl Acad Sci USA* 2017; **114**:E980–9.
 - 66 Junker Y, Zeissig S, Kim SJ *et al.* Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *J Exp Med* 2012; **209**:2395–408.
 - 67 Daum S, Cellier C, Mulder CJ. Refractory coeliac disease. *Best Pract Res Clin Gastroenterol* 2005; **19**:413–24.
 - 68 Cellier C, Delabesse E, Helmer C *et al.* Refractory sprue, coeliac disease, and enteropathy-associated T-cell lymphoma. French Coeliac Disease Study Group. *Lancet* 2000; **356**:203–8.
 - 69 Malamut G, Meresse B, Cellier C, Cerf-Bensussan N. Refractory celiac disease: from bench to bedside. *Semin Immunopathol* 2012; **34**:601–13.
 - 70 Arguelles-Grande C, Brar P, Green PHR, Bhagat G. Immunohistochemical and T-cell receptor gene rearrangement analyses as predictors of morbidity and mortality in refractory celiac disease. *J Clin Gastroenterol* 2013; **47**:593–601.
 - 71 Ilus T, Kaukinen K, Virta LJ *et al.* Refractory coeliac disease in a country with a high prevalence of clinically-diagnosed coeliac disease. *Aliment Pharm Ther* 2014; **39**:418–25.
 - 72 Hollon JR, Cureton PA, Martin ML, Puppa ELL, Fasano A. Trace gluten contamination may play a role in mucosal and

clinical recovery in a subgroup of diet-adherent non-responsive celiac disease patients. *BMC Gastroenterol* 2013; **13**:40.

- 73 Meresse B, Malamut G, Cerf-Bensussan N. Celiac disease: an immunological jigsaw. *Immunity* 2012; **36**:907–19.
- 74 Perfetti V, Baldanti F, Lenti MV *et al.* Detection of active Epstein–Barr virus infection in duodenal mucosa of patients with refractory celiac disease. *Clin Gastroenterol Hepatol* 2016; **14**:1216–20.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Frequency of NKp44⁺ and NKp44[−] ILC1s in patients on a gluten-free diet. NKp44⁺ and NKp44[−] ILC1s in CD patients on a gluten-free diet (GFD) for at least 6 months stratified by degree of persistent villous atrophy compared with controls and ACD patients. Left: ACD = 11.85 ± 5.31 ($n = 10$), MARSH 3 = 15.02 ± 10.95 ($n = 3$), 1–2 = 18.88 ± 5.72 ($n = 5$), 0 = 28.96 ± 12.9 ($n = 5$), Controls = 47.68 ± 5.25 ($n = 10$); right: ACD = 61.55 ± 6.43 ($n = 10$), MARSH 3 = 41.97 ± 0.73 ($n = 3$), 1–2 = 27.92 ± 4.40 ($n = 5$), 0 = 36.52 ± 13.44 ($n = 5$), Controls = 21.07 ± 3.68 ($n = 10$) (Mean percentage \pm SEM) ** = $P < 0.01$.