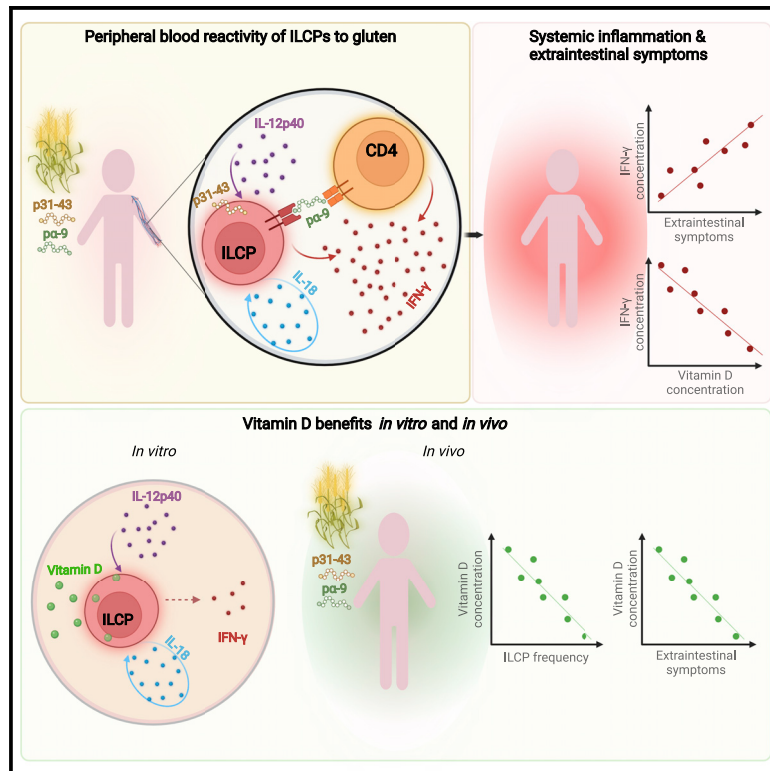


# Gliadin-reactive vitamin D-sensitive proinflammatory ILCPs are enriched in celiac patients

## Graphical abstract



## Authors

Giuseppe Ercolano, Alex Moretti, Maryline Falquet, ..., Silvia Salvatore, Camilla Jandus, Sara Trabanelli

## Correspondence

sara.trabanelli@gmail.com

## In brief

Ercolano et al. illustrate how gliadin peptide-driven ILCP activation contributes to the peripheral, extraintestinal inflammation characterizing celiac patients. ILCP activation is dampened by vitamin D, which is low in patients experiencing severe systemic inflammation and showing more extraintestinal symptoms.

## Highlights

- ILCPs are enriched in the peripheral blood of celiac patients
- ILCPs react to gliadin peptides by producing cytokines and stimulating T cells
- ILCP production of IFN- $\gamma$  can be inhibited by vitamin D
- High IFN- $\gamma$  and low vitamin D correlate with more extraintestinal symptoms



## Article

# Gliadin-reactive vitamin D-sensitive proinflammatory ILCs are enriched in celiac patients

Giuseppe Ercolano,<sup>1,5,8</sup> Alex Moretti,<sup>2,6,8</sup> Maryline Falquet,<sup>1,7</sup> Tania Wyss,<sup>1</sup> Ngoc Lan Tran,<sup>3</sup> Isis Senoner,<sup>3</sup> Maddalena Marinoni,<sup>2,4</sup> Massimo Agosti,<sup>2</sup> Silvia Salvatore,<sup>2</sup> Camilla Jandus,<sup>1,7,9</sup> and Sara Trabanelli<sup>1,7,9,10,\*</sup>

<sup>1</sup>Departement of Oncology, UNIL-CHUV, Ludwig Institute for Cancer Research, University of Lausanne, 1066 Epalinges, Switzerland

<sup>2</sup>Pediatric Department, Hospital "F. Del Ponte," University of Insubria, 21100 Varese, Italy

<sup>3</sup>Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1206 Geneva, Switzerland

<sup>4</sup>Pediatric Onco-haematological Day Hospital, Hospital "F. Del Ponte", ASST-Settelaghi, 21100 Varese, Italy

<sup>5</sup>Present address: Department of Pharmacy, University of Naples Federico II, 80131 Naples, Italy

<sup>6</sup>Present address: Tettamanti Research Center, University of Milano-Bicocca, Departments of Pediatric, San Gerardo Hospital, 20900 Monza, Italy

<sup>7</sup>Present address: Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1206 Geneva, Switzerland and Ludwig Institute for Cancer Research, Lausanne branch, 1066 Epalinges, Switzerland

<sup>8</sup>These authors contributed equally

<sup>9</sup>These authors contributed equally

<sup>10</sup>Lead contact

\*Correspondence: [sara.trabanelli@gmail.com](mailto:sara.trabanelli@gmail.com)

<https://doi.org/10.1016/j.celrep.2022.110956>

## SUMMARY

Celiac disease (CD) is a multisystem disease in which different organs may be affected. We investigate whether circulating innate lymphoid cells (ILCs) contribute to the CD peripheral inflammatory status. We find that the CD cytokine profile is characterized by high concentrations of IL-12p40, IL-18, and IFN- $\gamma$ , paralleled by an expansion of ILC precursors (ILCPs). In the presence of the gliadin peptides p31–43 and p $\alpha$ -9, ILCPs from CD patients increase transglutaminase 2 (TG2) expression, produce IL-18 and IFN- $\gamma$ , and stimulate CD4<sup>+</sup> T lymphocytes. IFN- $\gamma$  is also produced upon stimulation with IL-12p40 and IL-18 and is inhibited by the addition of vitamin D. Low levels of blood vitamin D correlate with high IFN- $\gamma$  and ILCP presence and mark the CD population mostly affected by extraintestinal symptoms. Dietary vitamin D supplementation appears to be an interesting therapeutic approach to dampen ILCP-mediated IFN- $\gamma$  production.

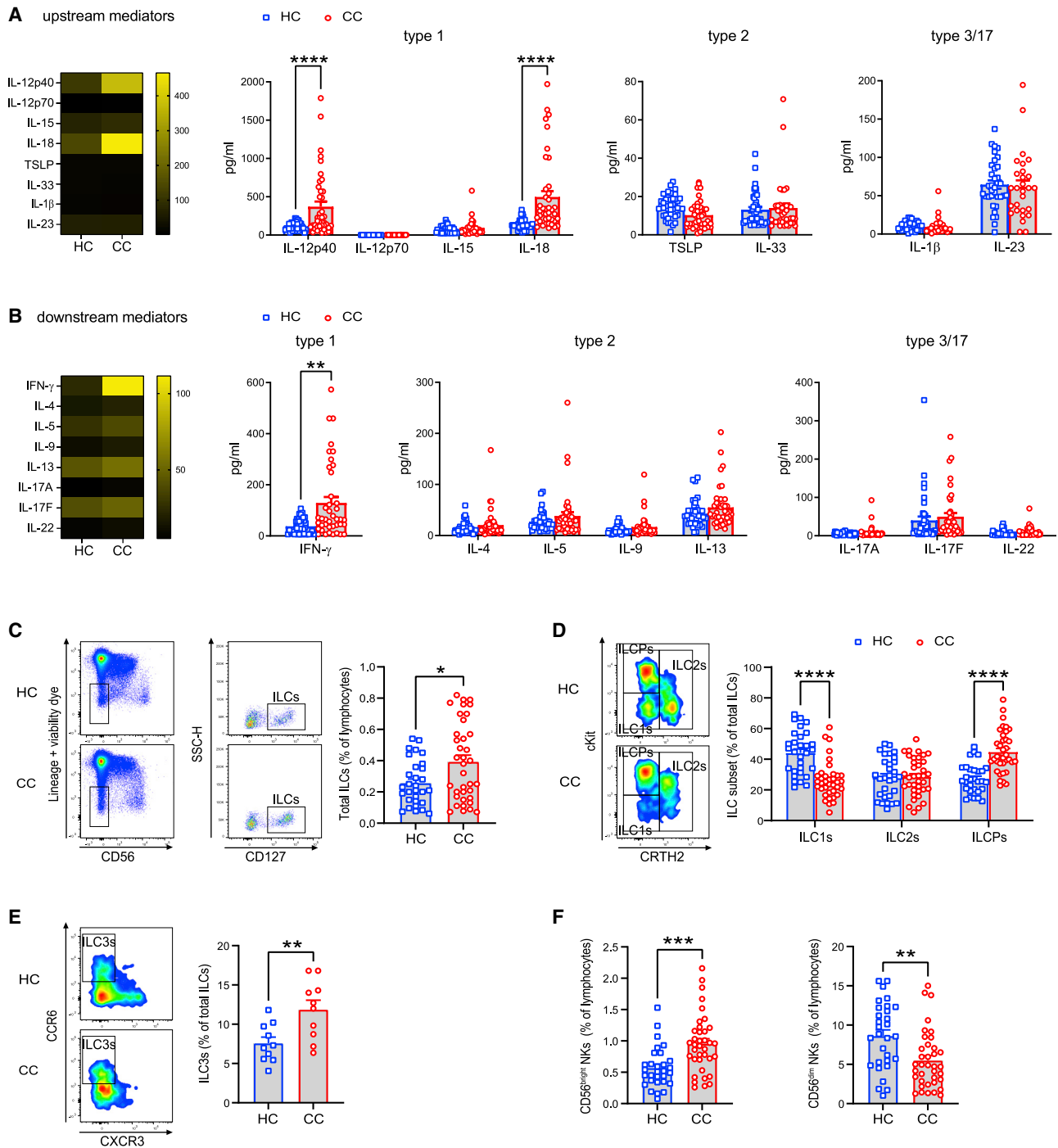
## INTRODUCTION

Innate lymphoid cells (ILCs) are recently identified innate lymphocytes that play a central role in controlling inflammation and tissue homeostasis at body barriers (McKenzie et al., 2014; Artis and Spits, 2015; Sonnenberg and Artis, 2015). In contrast to T and B cells, ILCs do not express rearranged antigen-specific receptors but can be rapidly activated by signals released by different cell types, intestinal bacteria, and dietary product (Brestoff et al., 2015; Halim et al., 2014; Trabanelli et al., 2017; Qiu et al., 2012; Konya et al., 2018). Cytotoxic ILCs are represented by natural killer (NK) cells (Vivier et al., 2018) that are classified as CD56<sup>bright</sup> and CD56<sup>dim</sup> NKs (Cooper et al., 2001). Helper ILCs are regulated at the transcriptional level by Tbx21 (T-bet), GATA-3, and Ror $\gamma$ t for ILC1s, ILC2s, and ILC3s, respectively. Analogous to the equivalent adaptive lymphocytes' populations ILC1s are characterized by production of IFN- $\gamma$ , ILC2s of IL-5 and IL-13, and ILC3s of IL-17A and/or IL-22 (Spits et al., 2013; Artis and Spits, 2015). Whereas ILC1s and ILC2s can be also found in the circulation of human healthy

individuals, ILC3s are poorly represented in peripheral blood, where they are, instead, included in a major population of ILC precursors (ILCPs). ILCPs can give rise to the three ILC subsets as well as to NK cells (Di Santo et al., 2017; Lim and Di Santo, 2019; Lim et al., 2017; Bar-Ephraim et al., 2019).

Celiac disease (CD) is a chronic immune-mediated enteropathy triggered by exposure to dietary gluten in genetically predisposed individuals (Ludvigsson et al., 2014; Sollid, 2000). Gluten intake results in small-intestinal mucosal damage and villous atrophy mediated by the activation of both adaptive and innate immune responses (Sollid, 2000; Abadie et al., 2011; Marafini et al., 2015, 2016; Uhde et al., 2020). In active CD, TNF- $\alpha$  and IFN- $\gamma$  producing ILCs are enriched in the lamina propria of patients and contribute to intestinal atrophy in mice (Marafini et al., 2015). Moreover, it has been shown that ILC1s with cytotoxic properties are increased in the small-intestinal mucosa of CD patients, where they lose the expression of NKp44 and might contribute to the epithelial destruction, since their frequency correlates with FABP2, a marker of epithelial cell damage (Uhde et al., 2020).





**Figure 1. IL-12p40, IL-18, IFN- $\gamma$ , and ILCPs are enriched in the PB of CC**

(A and B) Cytokine quantification in the sera of HC (n = 40) and CC (n = 41). Heatmaps show a summary of the differences between HC and CC serum concentrations of the different cytokines. (A) Histograms represent the quantification of upstream cytokines of type 1 (e.g., IL-12p40, IL-12p70, IL-15, and IL-18), type 2 (e.g., TSLP, IL-33), and type 3/17 (IL-1 $\beta$ , IL-23) immunity in the sera of HC and CC. Mann-Whitney tests were applied for the analyses. (B) Histograms represent the quantification of type 1 (e.g., IFN- $\gamma$ ), type 2 (e.g., IL-4, IL-5, IL-9, and IL-13), and type 3/17 (e.g., IL-17A, IL-17F, and IL-22) cytokines in the sera of HC and CC. A Mann-Whitney test was applied for the analysis.

(C) Representative examples of flow cytometry analysis of innate lymphoid cells (ILCs; Lineage<sup>-</sup>CD56<sup>-</sup>CD127<sup>+</sup>) in healthy children (HC) and celiac children (CC) peripheral blood (PB). HC were sex- and age-matched with CC. Histograms represent the frequencies of total ILCs in HC (n = 30) and CC (n = 37) among the total lymphocytes. A Mann-Whitney test was applied for the analysis.

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However, in addition to the classical intestinal symptoms, CD includes a variety of extraintestinal manifestations (e.g., affecting the cardiovascular, respiratory, and neurological systems) and can be associated with other autoimmune diseases, suggesting an immune involvement not restricted to the gut (Ludvigsson et al., 2014; Garcia-Quintanilla and Miranzo-Navarro, 2016; Campagna et al., 2017; Therrien et al., 2020). Therefore, a better understanding of circulating immune cells able to remain in the periphery or to migrate into distant organs is crucial to explain the multifaceted presentation of CD.

Although gluten-specific IFN- $\gamma$ -producing CD4<sup>+</sup> T lymphocytes were found in the peripheral blood of CD patients, where they may sustain peripheral inflammation (Raki et al., 2007; Anderson et al., 2005; Cook et al., 2017), no data are available about the presence and the function of peripheral ILCs in CD.

Here, we conducted an extensive phenotypical and functional analysis of circulating ILCs in pediatric CD patients and non-celiac controls to unravel their possible involvement in peripheral inflammation and to test the possibility to reverse/inhibit their proinflammatory behavior.

## RESULTS

### CD patients are characterized by a type 1 peripheral inflammation associated with an ILC1 decrease and an ILCP increase

To characterize the dominant type of systemic inflammation in the cohort of CD patients, the main cytokines characterizing type 1, type 2, or type 3/17 inflammation were quantified in the sera of celiac children (CC) and age- and sex-matched healthy control children (HC). We tested the sera for the presence of cytokines able to stimulate (upstream mediators) or to be secreted by (downstream mediators) ILCs. As shown in Figure 1A, among the cytokines known to stimulate ILCs, we found differences in type 1 inflammatory cytokines (e.g., IL-12p40, IL-12p70, IL-15, and IL-18), with higher levels of IL-12p40 and IL-18 in the circulation of CC. In line with this result, among the cytokines produced by ILCs, we found that IFN- $\gamma$  was upregulated in the sera of CC (Figure 1B). On the basis of these results and a previous report about the enrichment of ILC1s in the intestine of CD patients (Uhde et al., 2020), we expected to find an ILC1 enrichment also in the peripheral blood (PB) of CC. Surprisingly, analyzing ILC frequencies with the gating strategy shown in Figure S1, we found that, although the frequency of total ILCs was higher in CC compared with HC (Figure 1C), proportionally, ILC1s were reduced, whereas ILCPs were increased (Figure 1D). Moreover, despite that ILC3s are representing a rare population in circulation, we found that ILC3s were also upregulated in CC (Figure 1E), in line with what was observed in other intestinal

inflammatory disorders (Mazzurana et al., 2021; Creyns et al., 2020; Marafini et al., 2016). To complete the characterization of the innate lymphocyte distribution, we also analyzed the frequency of CD56<sup>bright</sup> and CD56<sup>dim</sup> NKs. In contrast to other groups that analyzed NKs as a whole population of CD3<sup>-</sup>CD56<sup>+</sup> lymphocytes (Dunne et al., 2013; Cseh et al., 2011; Marafini et al., 2016), we found that CD56<sup>bright</sup> NKs were increased, whereas CD56<sup>dim</sup> NKs were decreased, in CC compared with HC (Figure 1F). These data suggest that, even if a type 1 inflammation is present in the PB of CC, the target and the effector of this inflammation, among the helper ILCs, are mainly the ILCPs.

### Circulating ILCPs express TG2 that is active in CD patients

To ascertain any putative contribution of ILCPs to the pathogenesis of the disease, we mined our mRNA-seq data obtained by sorting the different subsets of helper and cytotoxic innate lymphoid cells, i.e., ILCs and NKs (Ercolano et al., 2020). We determined which genes previously reported to be relevant to CD were differentially expressed among ILCs and NKs (Figure S2) (Hardy and Tyé-Din, 2016). As shown in Figure 2A, pairwise comparisons of ILC and NK subsets indicated that ILCPs were the cells overexpressing the majority of genes involved in CD. One gene was significantly upregulated in ILCPs compared with the other ILC or NK subsets analyzed. As shown by the heatmap (Figure 2B), this gene is of particular importance for CD development, since it encodes for transglutaminase 2 (TG2, TGM2). Indeed, in CD, the most immunogenic peptides consist in TG2-deamidated  $\alpha$ - and  $\gamma$ -gliadins, which, after deamidation, anchor more strongly in the pocket of the peptide-binding groove of the HLA molecules (Vader et al., 2002; Arentz-Hansen et al., 2000; Qiao et al., 2005) and activate adaptive immune responses (Sollid, 2000). Since TG2 is expressed in the intestinal epithelium and it has been described to be involved in the establishment of CD (Arentz-Hansen et al., 2000), we compared the TG2 level of expression found in the ILCPs of our samples with the one reported in the intestinal epithelium and in other innate immune cells. To do so, we compared our mRNA-seq data with mRNA-seq data available in the EMBL-EBI Expression Atlas. As shown in Figure 2C, the expression of TG2 in ILCPs was the highest among circulating innate immune cells and the closest to the expression in the intestinal epithelial cells, suggesting that peripheral ILCPs might play an active role in CD.

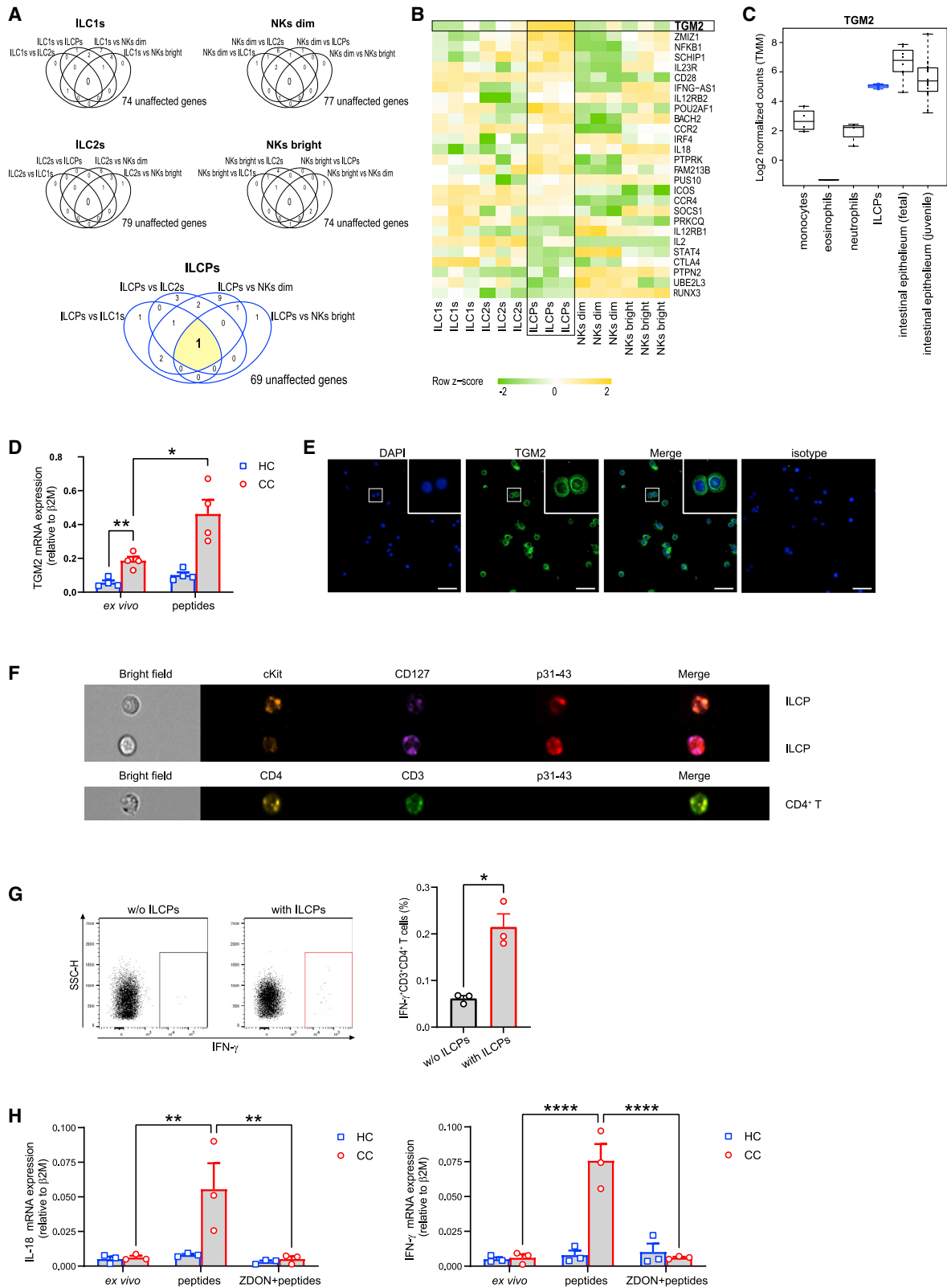
To verify whether TG2 was expressed and functional in ILCPs, we purified the ILCPs from HC and CC and evaluated their TG2 expression *ex vivo* and upon stimulation with the p31–43 gliadin peptide, followed by the p $\alpha$ -9 gliadin peptide (p57–68), a combination known to upregulate TG2 and activate T cells specifically

(D) Representative examples of flow cytometry analysis of ILC subsets in HC and CC. Histograms represent the relative frequencies of ILC subsets among total ILCs in HC (n = 30) and CC (n = 37). ILC subsets are defined as follows: ILC1s: CRTH2<sup>-</sup>cKit<sup>+</sup>, ILC2s: CRTH2<sup>+</sup>cKit<sup>+/+</sup>, ILCPs: CRTH2<sup>-</sup>cKit<sup>+</sup>. ILC subset distribution was analyzed with a two-way ANOVA.

(E) Representative examples of flow cytometry analysis of ILC3s in HC and CC. Histograms represent the relative frequencies of ILC3s among total ILCs in HC (n = 10) and CC (n = 10). ILC3s are defined as Lin<sup>-</sup>CD127<sup>+</sup>CXCR3<sup>-</sup>CCR6<sup>+</sup> lymphocytes. An unpaired t test was used for the analysis.

(F) CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cell frequencies in HC (n = 30) and CC (n = 37). A Mann-Whitney test was applied for the analysis.

Data are presented as bar graphs, with each bar representing the mean  $\pm$  S.E.M. of the data distribution, and the underlying individual data points are also displayed. \*\*, \*\*\*, and \*\*\*\* represent significant comparisons with p < 0.01, p < 0.005, and p < 0.001, respectively.



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in CD biopsy specimens (Maiuri et al., 2005). As shown in Figure 2D, *ex vivo* ILCPs from CC expressed a higher level of TG2 compared with HC and further upregulated its expression upon peptide stimulation, suggesting that ILCPs from CC are sensitive to gliadin peptide stimulation. Moreover, CC ILCPs expressed TG2 at the protein level (Figure 2E) and were able to bind (Figure 2F, top) and internalize (Figure 2F, bottom) the p31–43 peptide, Cy5 conjugated, differently from the CD4<sup>+</sup> T cells of the same patient, that are considered the adaptive counterpart of ILCs (Cella et al., 2014). In addition, CC ILCPs could act as antigen-presenting cells (APCs) and trigger CD4<sup>+</sup> T cell production of IFN- $\gamma$  in response to the gliadin peptides (Figure 2G).

To further understand whether the ILCPs from CC, in response to gliadin peptides, could produce the cytokines involved in the peripheral inflammation, we stimulated ILCPs from CC and HC with the p31–43 gliadin peptide followed by the p $\alpha$ -9 gliadin peptide (p57–68) in the absence or the presence of ZDON, a selective TG2 inhibitor (Schaertl et al., 2010). As shown in Figure 2H, only the ILCPs from CC could produce more IL-18 and IFN- $\gamma$  in the presence of the gliadin peptides compared with the baseline (i.e., *ex vivo* cytokine production without stimulation). This production was inhibited by the addition of ZDON, demonstrating that TG2 is functional in CC ILCPs and that its activity is involved in the induction/maintenance of the peripheral inflammation.

#### ILCPs from CC downregulate CCR6 and NKp46

Since gluten-specific CD4<sup>+</sup> T lymphocytes have a particular phenotype in CD (du Pre et al., 2011; Raki et al., 2007), we investigated whether the phenotype of CC ILCPs was distinct from that of HC ILCPs. As shown in Figure 3A, differently from gluten-specific CD4<sup>+</sup> T lymphocytes, ILCPs of CC did not downregulate CD45RA and CD62L and did not upregulate  $\beta$ 7 and CD38. This suggests that, despite their ability to respond to gluten by upregulating TG2, IL-18, and IFN- $\gamma$  (see Figures 2D and 2H), the ILCP phenotype is modulated differently from the one of their adaptive counterparts. To understand why ILCPs were increased in the PB of CC and not recruited to the intestine to support the local inflammation and the mucosal damage, we analyzed the expression of gut-homing receptors. As shown in Figure 3B, ILCPs from CC did not upregulate any of the gut-homing receptors that we tested; rather, they downregulated CCR6,

suggesting that they are unlikely to be involved in the intestinal inflammation. However, circulating ILC1s were also unlikely to be recruited to the gut via gut-homing receptors (Figure S3A), suggesting that the ILCP increase was not a consequence of the ILC1 recruitment to the gut. Furthermore, we screened the sera for the presence of ILC subset-specific chemokines. As shown in Figure 3C, among the chemokines that we tested, RANTES was highly present in the CC sera compared with HC. Interestingly, according to our mRNA-seq analysis, ILCPs differ from ILC1s for the expression of CCR3, one of the receptors for RANTES (Figure 3D), suggesting that they might be preferentially recruited/retained in the PB through RANTES-CCR3 binding. Besides recruitment/retention in the periphery, we speculated that the increase of ILCPs in peripheral blood could also be explained by their proliferation in response to gluten peptides. To test this hypothesis, we evaluated the transcript levels of Ki-67 as an index of proliferation in ILCPs from HC and CC *ex vivo* and upon peptides' stimulation. As shown in Figure 3E, only the ILCPs from CC were able to upregulate Ki-67 in response to the gluten peptides, suggesting that their increase could, at least in part, be explained by their enhanced proliferation.

Finally, we wanted to test whether *ex vivo* ILCPs from CC showed a more activated phenotype, since *in vivo* they are constantly stimulated by type 1 inflammatory cytokines. Our analysis showed that the expression of markers known to be specific either for gluten-specific CD4<sup>+</sup> T cells or for activated ILCs did not differ between CC and HC ILCPs (Figure S3B), with the exception of NKp46, which was downregulated in CC ILCPs (Figure 3F).

#### IFN- $\gamma$ secretion by ILCPs is stimulated by IL-12p40 and IL-18

Although we did not observe differences in T-bet expression between the ILCPs of HC and CC (Figure S4), we wanted to investigate whether, upon IL-12p40 and IL-18 stimulation (i.e., the cytokines characterizing the CD peripheral environment; see Figure 1A), the ILCPs from CC could produce more IFN- $\gamma$  than the ones of HC. As shown in Figure 4A, upon stimulation, the ILCPs from HC and CC exhibited similar gene expression levels of IFN- $\gamma$ . Therefore, to better study the impact of IL-12p40 and IL-18 without being limited in ILCPs, we purified ILCPs from

#### Figure 2. TG2 is preferentially expressed by human ILCPs and is active in CC ILCPs

(A) Venn diagrams of genes relevant for CD (Hardy and Tye-Din, 2016) differentially expressed between one innate lymphoid cell subset versus (vs) all the others (average of n = 3).

(B) Heatmap of the genes differentially expressed by ILCPs versus the other innate lymphoid cell subsets (n = 3). Statistical analysis was performed using multiple t tests.

(C) Comparison of TGM2 expression among ILCPs and innate circulating cells or intestinal cells.

(D) Quantification by qPCR of the TGM2 transcript normalized on  $\beta$ 2M transcript in *ex vivo* ILCPs and in ILCPs treated with the gliadin peptides (n = 4 different HC and CC). Data were analyzed with a two-way ANOVA.

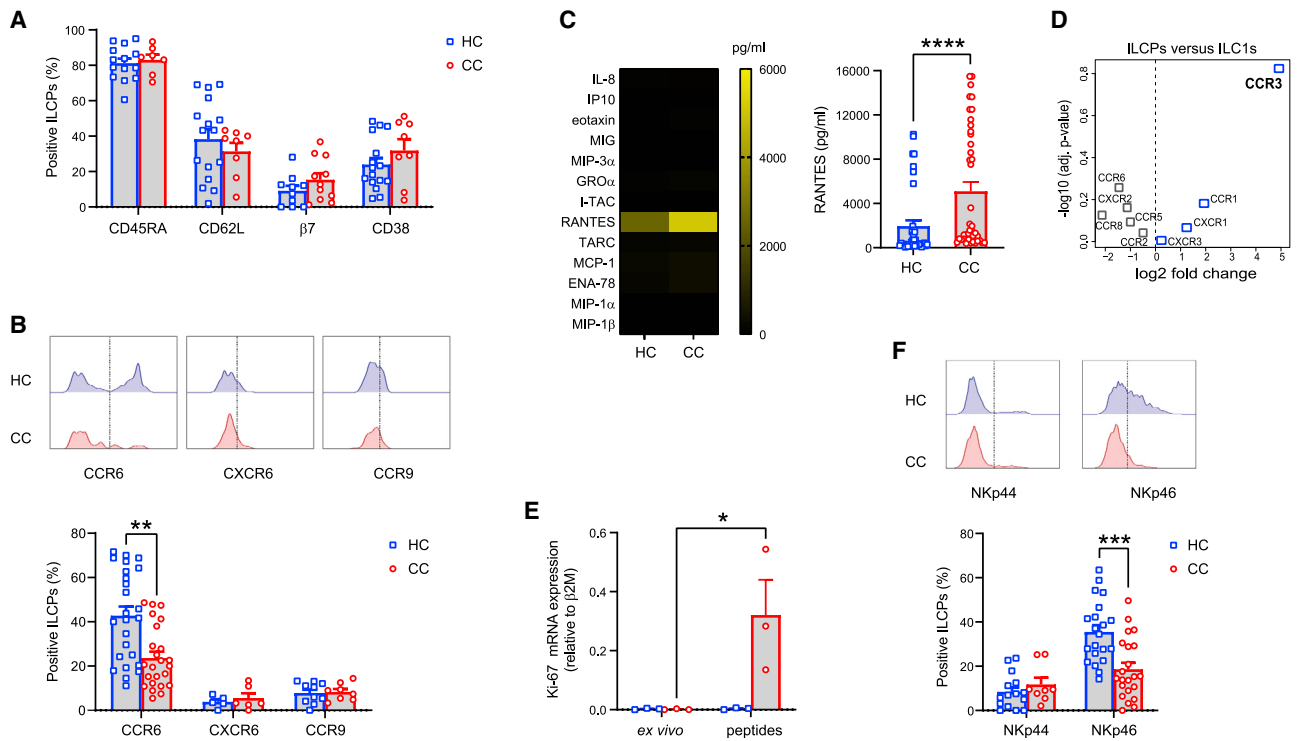
(E) Immunofluorescence of TG2 on ILCPs from CC. Scale bar 50,  $\mu$ m; insets, 4 $\times$  magnification.

(F) Representative image stream analysis of CC ILCPs and CD4<sup>+</sup> T lymphocytes incubated with the Cy5-conjugated p31–43 peptide. The merge shows the expression of markers specific for ILCPs (i.e., cKit and CD127) and for CD4<sup>+</sup> T lymphocytes (i.e., CD4 and CD3) and the ILCP-specific binding of the peptide.

(G) Representative examples of flow cytometry analysis of IFN- $\gamma$  production by CD3<sup>+</sup>CD4<sup>+</sup> T cells in the absence of ILCPs (w/o ILCPs) or in the presence of ILCPs (with ILCPs). Histograms represent the frequencies of IFN- $\gamma$ <sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells (n = 3 different CC). A paired t test was applied.

(H) Quantification by qPCR of the IL-18 and the IFN- $\gamma$  transcripts normalized on  $\beta$ 2M transcript in *ex vivo* ILCPs and in ILCPs treated with the gliadin peptides in the presence and the absence of the TG2 inhibitor ZDON (n = 3 different HC and CC). Data were analyzed with two-way ANOVAs.

Data are presented as bar graphs, with each bar representing the mean  $\pm$  S.E.M. of the data distribution, and the underlying individual data points are also displayed. \*, \*\*, and \*\*\*\* represent significant comparisons with p < 0.05, p < 0.01, and p < 0.001, respectively.



**Figure 3. ILCPs downregulate CCR6 and NKp46 in CC**

(A) Frequency of ILCPs positive for CD45RA, CD62L,  $\beta$ 7, and CD38 expression by flow cytometry in HC and CC. At least seven different donors were analyzed for each marker.

(B) Representative examples of flow cytometry analysis of the expression of CCR6, CXCR6, and CCR9. Histograms represent the frequency of positive ILCPs in HC and CC. At least five donors were analyzed for each marker. A Mann-Whitney test was used to analyze CCR6 expression.

(C) Heatmap shows the summary of the chemokine quantification in the sera of HC (n = 40) and CC (n = 41). Histograms represent the quantification of RANTES in the sera of HC and CC (Mann-Whitney test).

(D) Volcano plot of chemokine receptor gene expression by ILCPs versus ILC1s (average of n = 3). Statistical analysis was performed using unpaired t tests.

(E) Quantification by qPCR of the Ki-67 transcripts normalized on  $\beta$ 2M transcript in *ex vivo* ILCPs and in ILCPs treated with the gliadin peptides (n = 3 for both HC and CC). Data were analyzed with two-way ANOVAs.

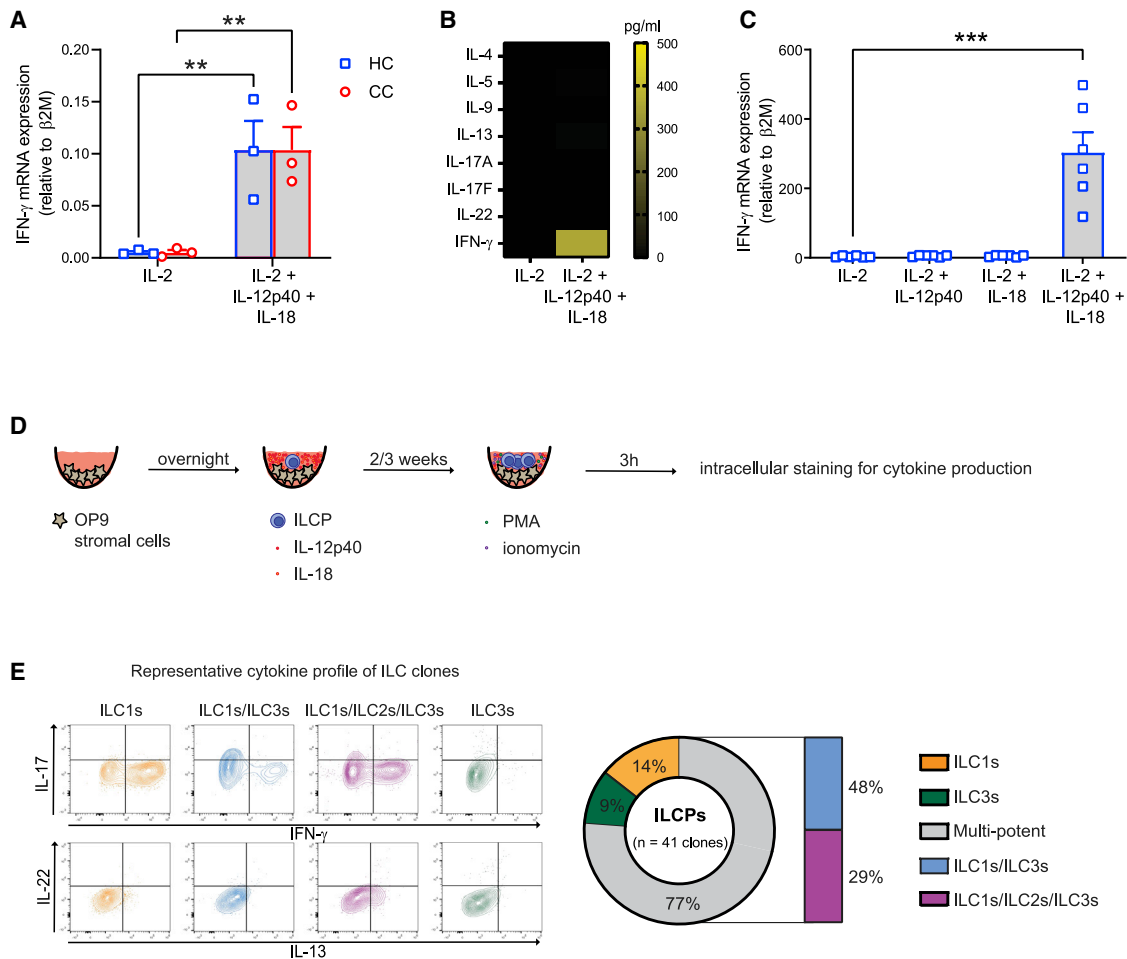
(F) Representative examples of flow cytometry analysis of the expression of NKp44 and NKp46. Histograms represent the frequency of positive ILCPs in HC and CC. At least eight donors were analyzed for each marker. Unpaired t tests were used for the analyses. Data are presented as bar graphs, with each bar representing the mean  $\pm$  S.E.M. of the data distribution, and the underlying individual data points are also displayed. \*, \*\*, \*\*\*, and \*\*\*\* represent significant comparisons with  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.005$ , and  $p < 0.001$ , respectively.

healthy adult volunteers and stimulated them with the cytokine combination or with IL-12p40 or IL-18 alone. As shown in Figures 4B and 4C, among the cytokines that we tested, ILCPs secreted only IFN- $\gamma$  and needed the synergistic stimulation by IL-12p40 and IL-18 to produce it. These data suggest that, in the CD context, ILCPs produce IFN- $\gamma$  not only in response to gliadin peptides but also to cytokine stimulation, thus sustaining the peripheral inflammation. To test whether ILCPs constantly exposed to IL-12p40 and IL-18 were able to secrete only IFN- $\gamma$  or could maintain their multipotency, we cultured ILCPs at single-cell level in the presence of IL-12p40 and IL-18 for 2–3 weeks and measured their cytokine production in response to PMA-ionomycin at the end of the culture (Figure 4D). As shown in Figure 4E, 14% of ILCPs differentiated into ILC1s, which secrete only IFN- $\gamma$ , and 9% into ILC3s, which secrete IL17A and/or IL-22. However, the majority of the cells (77%) remained multipotent and secreted different types of cytokines. In particular, 48% were characterized by the production of IL-17A and/or IL-22,

together with IFN- $\gamma$ , and 29% were also able to secrete IL-13. Thus, these data suggest that, despite the type 1 inflammatory environment, ILCPs remain pluripotent and therefore that their proinflammatory ability might be reverted.

### Vitamin D is crucial to inhibit IFN- $\gamma$ production and peripheral inflammation and to limit extraintestinal manifestations

Similar to what has recently been found in another cohort of CC, most of the CC enrolled in our cohort were reported to be vitamin D deficient (Deora et al., 2017; Saggese et al., 2018), with low blood levels of vitamin D characterizing 75.6% of them (Figure 5A). To understand if low vitamin D could contribute to the peripheral inflammation (together with high IL-12p40 and IL-18, Figure S5A), we correlated vitamin D concentration with IFN- $\gamma$  concentration and ILCP frequency. As shown in Figure 5B, we found that the concentration of vitamin D in PB inversely correlated with the IFN- $\gamma$  concentration and with the ILCP frequency.



**Figure 4. ILCPs produce IFN- $\gamma$  in response to IL-12p40 and IL-18 but remain multipotent**

(A) Quantification by qPCR of the IFN- $\gamma$  transcript normalized on  $\beta$ 2M transcript in ILCPs cultured with IL-2 or with IL-2 plus IL-12p40 and IL-18 (n = 3 different HC and CC). Data were analyzed with a two-way ANOVA.

(B) Heatmap shows the summary of the cytokines produced by ILCPs of healthy adult donors cultured with IL-2 alone or in the presence of IL-12p40 and IL-18.

(C) Histograms show the IFN- $\gamma$  quantification in the supernatant of ILCPs cultured with IL-2 alone or in the presence of IL-12p40 and/or IL-18 (n = 6). Statistical analysis was performed with a Kruskal-Wallis test.

(D) Schema representing the ILCP culture at single-cell level. Single-cell-sorted ILCPs were cultured on OP9 stromal cells in the presence of IL-12p40 and IL-18 for 14–18 days and stimulated with PMA-ionomycin 3 h before cytokine analysis.

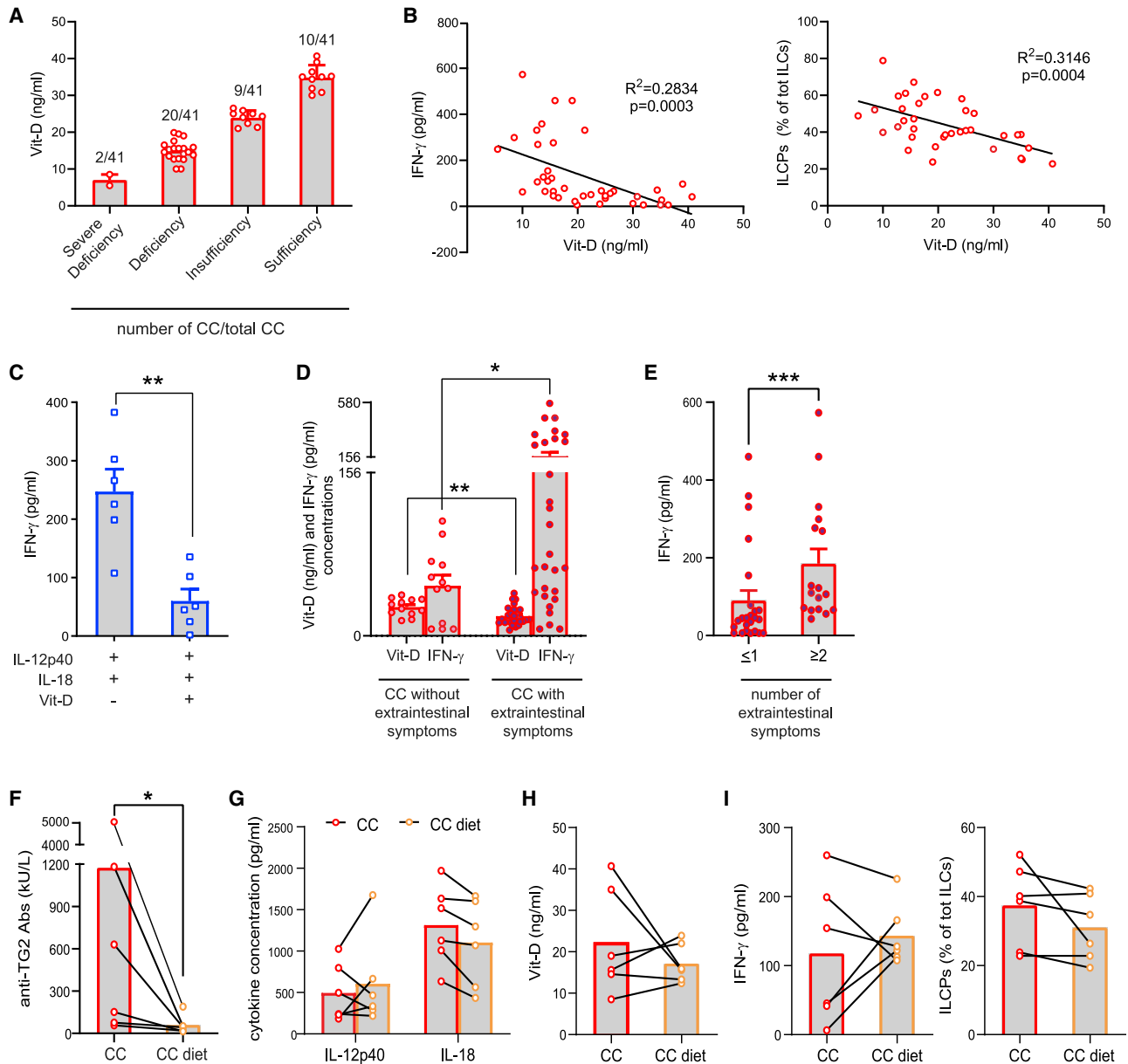
(E) Representative examples of flow cytometry analysis of the intracellular production of IL-17, IFN- $\gamma$ , IL-22, and IL-13. Presence of a cytokine was scored when more than 5% of positive cells were detected. The pie chart depicts all possible combinations of cytokine production detected. Data summarize nine independent experiments with one donor each.

Data are presented as bar graphs, with each bar representing the mean  $\pm$  S.E.M. of the data distribution, and the underlying individual data points are also displayed. \*\* and \*\*\* represent significant comparisons with p < 0.01 and p < 0.005, respectively.

Moreover, the *in vitro* addition of vitamin D, a molecule known for its anti-inflammatory properties (Cyprian et al., 2019), to ILCPs stimulated with IL-12p40 and IL-18, strongly inhibited their IFN- $\gamma$  production (Figure 5C). The presented data suggest that vitamin D deficiency can contribute to an ILCP uncontrolled IFN- $\gamma$  production. To investigate whether the balance between vitamin D and IFN- $\gamma$  could be related to the clinical manifestations of the CC, we divided our cohort into children showing extraintestinal symptoms (e.g., headache, muscle or joint pain, fatigue, irritability) and children without extraintestinal symptoms. As shown in Figure 5D, our results show that children suffering

from extraintestinal symptoms had lower vitamin D and higher IFN- $\gamma$  compared with children without extraintestinal symptoms. Furthermore, CC showing two or more extraintestinal symptoms had higher circulating IFN- $\gamma$  concentration than CC showing only one or no symptoms (Figure 5E). On the contrary, growth failure was not associated with IFN- $\gamma$  concentrations (Figure S5B). We then wondered whether the gluten-free diet was sufficient, in a short time, to revert the proinflammatory peripheral status of CC. Therefore, we tested the available paired sera of CC patients at diagnosis and upon a 6-month gluten-free diet. As expected, in the absence of the gluten peptides the anti-TG2 antibodies





**Figure 5. Vitamin D correlation with IFN- $\gamma$ , ILCPs, and extraintestinal symptoms**

(A–D) Analysis of the CC cohort at diagnosis (n = 41). (A) Distribution of the CC cohort at diagnosis according to the vitamin D concentration measured in the blood (severe deficiency <10 ng/mL, deficiency <20 ng/mL, insufficiency 20–29 ng/mL, sufficiency  $\geq$ 30 ng/mL) (Saggese et al., 2018). (B) Correlations between the concentration of circulating vitamin D and the concentration of circulating IFN- $\gamma$  or the frequency of circulating ILCPs. (C) IFN- $\gamma$  production by ILCPs of healthy donors stimulated with IL-12p40 and IL-18 in the absence and the presence of vitamin D. Statistical analysis was performed using a paired t test. (D) Comparison between the concentration of vitamin D and IFN- $\gamma$  in CC without extraintestinal symptoms (n = 12) and in CC suffering from extraintestinal symptoms (n = 29). Mann-Whitney tests were used for the analysis.

(E) IFN- $\gamma$  concentration of CC suffering from one/less than one (n = 24) or two/more than two (n = 17) extraintestinal symptoms. A Mann-Whitney test was used for the analysis.

Data are presented as bar graphs, with each bar representing the mean  $\pm$  S.E.M. of the data distribution, and the underlying individual data points are also displayed. \*, \*\*, and \*\*\* represent significant comparisons with  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.005$ , respectively.

(F–I) Analysis of the paired samples of 6 CC at diagnosis and upon a 6-months gluten-free diet (CC diet). Longitudinal quantification of (F) anti-TG2 antibodies (analyzed by Wilcoxon test), (G) circulating IL-12p40 and IL-18 concentrations, (H) vitamin D blood level, and (I) circulating IFN- $\gamma$  concentration and ILCP frequency.

Data are presented as bar graphs, with each bar representing the mean of the data distribution, and the underlying individual data points are also displayed. \* represents significant comparisons with  $p < 0.05$ .

were reduced almost to normal levels (Figure 5F). However, no differences were found between the circulating concentrations of IL-12p40 and IL-18 before and after the 6-month gluten-free diet (Figure 5G). Importantly, the vitamin D levels were still below the lower limit of sufficiency (Figure 5H). In line with our *in vitro* and correlative patients' data, the sera IFN- $\gamma$  level and frequency of ILCPs remained unaltered after the 6-month gluten-free diet (Figure 5I). These data show that the peripheral inflammation persists even in the absence of the triggering gluten peptides and that it is associated with vitamin D deficiency, which in turn linked with the presence of extraintestinal symptoms. In addition, ILCP frequency was not restored upon a 6-month gluten-free diet, suggesting that the involvement of this cell population in sustaining the peripheral inflammation might persist long term after the beginning of the diet. Our data suggest that vitamin D supplements could facilitate the resolution of the peripheral inflammation.

## DISCUSSION

In this study, by combining the *ex vivo* analysis of primary samples from CC and *in vitro* assays, we have uncovered that circulating ILCPs contribute to the peripheral inflammation occurring in CD. Our data show that elevated circulating IL-12p40 and IL-18 levels, in combination with low vitamin D concentration, result in an increase of total ILCs and in particular of ILCPs, expressing TG2, able to bind and internalize the gliadin peptides to sustain inflammation by producing IL-18 and IFN- $\gamma$ . Differently from the intestine, we did not find an increase in ILC1s and in IL-15 and TNF- $\alpha$  (Di Sabatino et al., 2006; Maiuri et al., 2003; Uhde et al., 2020), supporting the fact that the players involved in the periphery are different from the intestinal ones.

The binding of the p31–43 peptide resulted in an upregulation of TG2 expression and IL-18 and IFN- $\gamma$  production in ILCPs of CC but not of HC. This result is in line with previous data showing that, upon p31–43 challenge, TG2 is upregulated in the CD intestinal biopsies but not in the ones of healthy controls (Maiuri et al., 2005). To trigger TG2 in ILCPs we adapted a protocol already published in which CD biopsies were stimulated with the p31–43 followed by the p $\alpha$ -9 gliadin peptide (Maiuri et al., 2005). However, since the p31–43 is able to promote innate but not adaptive immune responses (Calvanese et al., 2019), we cannot exclude that the p31–43 binding is already sufficient to trigger CC ILCPs. For instance, ILCPs activation could be p $\alpha$ -9 independent and the binding of p31–43 could activate TG2 to deamidate other gluten peptides (e.g., p $\alpha$ -2 and p $\alpha$ -9) to facilitate their anchoring in the HLA pocket of antigen-presenting cells (Vader et al., 2002; Arentz-Hansen et al., 2000; Qiao et al., 2005). In fact, ILCPs might even present the gliadin peptides after deamidation to drive antigen-specific T cell responses (von Burg et al., 2014; Hepworth et al., 2013).

We found increased IL-18 in the peripheral blood of CC, as previously reported (Lettesjo et al., 2005). In contrast, we could not confirm the normalization of circulating IL-18 upon gluten-free diet, likely related to our shorter period of patient monitoring (6 versus 10–36 months). Previously, the circulating levels of IL-18 were also correlated with the level of anti-TG2 antibodies and the degree of villous flattening (Lettesjo et al., 2005). In our cohort,

this correlation could not be done, since most of our CC did not need intestinal biopsies to diagnose CD, in accordance with the current pediatric guidelines (Hill and Holmes, 2008; Reilly et al., 2018). Although IL-18 is a potent inducer of IFN- $\gamma$  production (Koutoulaki et al., 2010), in our *in vitro* study, IL-18 alone was not sufficient to induce IFN- $\gamma$  production by ILCPs. In fact, ILCPs needed a second signal to trigger IFN- $\gamma$  production, provided by the IL-12p40. We observed higher levels of circulating IL-12p40 in patients with CD compared with matched controls. IL-12p40 is a component of the heterodimers IL-12p70 and IL-23, but it can also act in a monomeric or homodimeric form. IL-12p40 neutralization, indeed, results in IFN- $\gamma$  inhibition and IL-4 production (Cooper and Khader, 2007; Wang et al., 2015). Although in our experiments IL-12p40 alone did not trigger IFN- $\gamma$  production, it cannot be excluded that it can induce ILCP proliferation as described for CD4<sup>+</sup> T lymphocytes (Wang et al., 2015). Interestingly, neutralization of IL-18 and of IL-12p40 was proven to be effective in reducing intestinal inflammation as well as IFN- $\gamma$  production in inflammatory bowel disease models (Siegmund et al., 2001; Neurath et al., 1995), opening the possibility to consider such a therapy also in CD.

According to our results, ILCPs sustain IFN- $\gamma$ -mediated inflammation only in the periphery, since, by downregulating CCR6 and by expressing low levels of  $\beta$ 7 and CCR9, they are unlikely to be recruited to the gut. Moreover, the expression of CCR3 might facilitate their recruitment/retention in the bloodstream, where high concentrations of RANTES are found. CCR6 is not only a gut-homing receptor, but it is also a marker of cells producing IL-17A and IL-22 (Robert et al., 2017). Indeed CCR6<sup>+</sup> ILCs, expressing also NKp46, represent the innate source of IL-17A and IL-22 in both mice (Takatori et al., 2009; Sanos et al., 2009) and humans (Nagasawa et al., 2019), while CCR6<sup>-</sup> ILCs can upregulate T-bet and become IFN- $\gamma$ -producing ILCs (Klose et al., 2013). In humans, the transition from ILC3s to ILC1s is also dependent on T-bet upregulation, which is induced by IL-12 and results in IFN- $\gamma$  production (Bernink et al., 2015). However, in our setting, even though ILCPs downregulated CCR6 and NKp46, they did not upregulate T-bet and did not produce IFN- $\gamma$  in response to IL-12 only. This confirms that ILCPs are regulated differently from ILC3s and suggests that, in ILCPs, the IFN- $\gamma$  production is not dependent on IL-12-induced T-bet expression. Moreover, in CC, ILCPs maintained the expression of CD45RA and CD62L, a phenotype that was described to characterize resting ILCs (Bar-Ephraim et al., 2019), and did not upregulate markers previously described on gluten-specific CD4<sup>+</sup> T lymphocytes (du Pre et al., 2011; Raki et al., 2007). This argues for a distinct regulation of innate and adaptive immune cells in CD and also suggests that the ILCPs that we found in the circulation of CC are not terminally skewed toward a specific IFN- $\gamma$ -producing subset but maintain their pluripotency and could therefore be reverted more easily than subset-committed cells. In line with this observation, the addition of vitamin D was already sufficient to strongly dampen their pathologic IFN- $\gamma$  production. Since vitamin D deficiency is a hallmark of CD (Deora et al., 2017), our data suggest that, together with a gluten-free diet, benefits of vitamin D supplementation should be further explored in larger studies of CD, since targeting proinflammatory circulating ILCs could be highly beneficial and

relevant to reduce/suppress chronic peripheral inflammation that may induce clinical complications or other autoimmune diseases.

### Limitations of the study

The RNA-seq data used in this study that allowed us to identify TG2 as an important player for the ILCP functions were obtained using ILCPs from healthy donors. Future studies sequencing ILCs from celiac patients could provide more insights into the presence of other molecules that could be implicated in the innate cell involvement in this pathology. To prove that vitamin D supplementation should be adopted as therapy in all CD patients, clinical studies addressing this point should be performed.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.110956>.

### ACKNOWLEDGMENTS

We are thankful to the families who accepted their children participating in this study. This study was developed from insightful discussions with the recently deceased Prof. Luigi Maiuri. This work was supported by the SNSF Ambizione and PRIMA grants (PZ00P3\_161459 and PR00P3\_179727) and a fellowship from the Helmut Horten Foundation to C.J. and by the Bourse Pro-Femmes, University of Lausanne to S.T. G.E. was supported by Fondazione Umberto Veronesi. The graphical abstract was created with [BioRender.com](https://www.biorender.com).

### AUTHOR CONTRIBUTIONS

G.E., A.M., M.F., N.L.T., I.S., and S.T. performed the experiments; A.M., M.M., M.A., and S.S. provided patients' samples and clinical data and critically revised the manuscript; T.W. analyzed the mRNA-seq data and critically revised the manuscript; G.E. and S.T. designed research, analyzed the exper-

iments; G.E., M.F., N.L.T., and I.S. critically revised the manuscript; C.J. and S.T. discussed the results and wrote the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 24, 2021

Revised: April 5, 2022

Accepted: May 23, 2022

Published: June 14, 2022

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
CD3	Biologend	clone: UCHT1; RRID: AB_2562046
CD4	Biologend	clone: RPA-T4; RRID: AB_314073 or clone: OKT4; RRID: AB_11218995
CD8	Immunotools	lot: 276276
CD14	Biologend	clone: HCD14; RRID: AB_830677
CD15	Biologend	clone: HI98; RRID: AB_314196
CD16	Biologend	clone: 3G8; RRID: AB_314206
CD19	Biologend	clone: HIB19; RRID: AB_314236
CD20	Biologend	clone: 2H7; RRID: AB_314252
CD33	Biologend	clone: HIM3-4; RRID: AB_314344
CD34	Biologend	clone: 561; RRID: AB_1731852
CD203c	Biologend	clone: NP4D6; RRID: AB_11218991
FcεRI	Biologend	clone: AER-37; RRID: AB_1227653
CD56	BD Biosciences	clone: NTAM16.2; RRID: AB_2744432
CD127	Biologend	clone: A019D5; RRID: AB_2563605
CRTH2	BD Biosciences; Biologend	clone: BM16; RRID: AB_2738244 and RRID: AB_2562467
cKit	Biologend	clone: 104D2; RRID: AB_2562025 and RRID: AB_2632949
β7	Biologend	clone: FIB504; RRID: AB_571970
CCR6	Biologend	clone: G034E3; RRID: AB_10916518 and RRID: AB_2562235
CCR9	Biologend	clone: L053E8; RRID: AB_2687387
CXCR6	Biologend	clone: K041E5; RRID: AB_2562514
CXCR3	Biologend	clone: G025H7; RRID: AB_10962908
CD45RA	Biologend	clone: HI100; RRID: AB_2561947
CD69	Biologend	clone: FN50; RRID: AB_2074956 and RRID: AB_2563158
CD137	Biologend	clone: 4B4-1; RRID: AB_2207742
HLA-DR	Biologend	clone: L243; RRID: AB_2562913
NKp44	Biologend	clone: P44-S; RRID: AB_2149432
NKp46	Biologend	clone: 9E2; RRID: AB_2561665
CD28	Biologend	clone: CD28.2; RRID: AB_528786
CD38	eBioscience	clone: HIT2; RRID: AB_10804040
CD39	Biologend	clone: A1; RRID: AB_2099950
CD62L	eBioscience	clone: DREG-56; RRID: AB_1582224
OX40	Biologend	clone: Ber-ACT35(ACT35); RRID: AB_10645478
KLRG1	eBioscience	clone: 13F12F2; RRID: AB_2572715
CD94	Biologend	clone: DX22; RRID: AB_314534
IFN-γ	Biologend	clone: 4S.B3; RRID: AB_2561398
IL-13	BD Biosciences	clone: JES10-5A2; RRID: AB_397225
IL-22	Biologend	clone: 2G12A41; RRID: AB_2571931
IL-17A	eBioscience	clone: eBio64DEC17; RRID: AB_1582222

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TG2	Abcam	CUB 7402; RRID: AB_2287299
<b>Biological samples</b>		
Patients' samples	Hospital "F. Del Ponte"	CD1-CD51
Healthy donors' samples	Hospital "F. Del Ponte"	HC1-HC40
<b>Chemicals, peptides, and recombinant proteins</b>		
p $\alpha$ -9	Protein and Tetramer Core Facility – UNIL/CHUV	57-68, QLQFPQPQLPY
p31-43	Protein and Tetramer Core Facility – UNIL/CHUV	LGQQQFPQPQPY
ZDON	Sigma	616467
IL-12p40	Sigma	Catalog: SRP3074
IL-18	R&D System	Catalog: 9124-IL-010/CF
IL-7	Peptotech	Catalog: 200-07
1 $\alpha$ ,25-Dihydroxyvitamin D3	Sigma	Catalog: D1530
<b>Critical commercial assays</b>		
Human Cytokine Panel 2 (13-plex)	Biologend	Catalog: 740102
Human T Helper Cytokine Panels Version 2 (12-plex)	Biologend	Catalog: 741028
Human Proinflammatory Chemokine Panel 1 (13-plex)	Biologend	Catalog: 740985
<b>Experimental models: Cell lines</b>		
OP9 stromal cells	kindly provided by Prof. H. Spits and Dr. B. Blum	
<b>Software and algorithms</b>		
FlowJo software (TreeStar)	BD Biosciences	
<b>Oligonucleotides</b>		
hTGM2 FW primer	Microsynth	5'-TCAACTGCAACGATGACCAGG-3'
hTGM2 RV primer	Microsynth	5'-TGTTCTGGTCATGGGCCGAG-3'
hIL-18 FW primer	Microsynth	5'-TCTTCATTGACCAAGGAAATCGG-3'
hIL-18 RV primer	Microsynth	5'-TCCGGGGTGCATTATCTCTAC-3'
hIFNG FW primer	Microsynth	5'-TCGGTAACTGACTTGAATGTCCA-3'
hIFNG RV primer	Microsynth	5'-TCGCTTCCCTGTTTTAGCTGC-3'
hMKI67 FW primer	Microsynth	5'-ACGCCTGGTTACTATCAAAGG-3'
hMKI67 RV primer	Microsynth	5'-CAGACCCATTTACTTGTGTTGGA-3'
$\beta$ 2M FW primer	Microsynth	5'-GAGGCTATCCAGCGTACTCC-3'
$\beta$ 2M RV primer	Microsynth	5'-CGGCAGGCATACTCATCTTTT-3'

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sara Trabanelli ([sara.trabanelli@gmail.com](mailto:sara.trabanelli@gmail.com)).

**Materials availability**

This study did not generate new unique reagents. Antibodies and cytokines were obtained from the commercial sources stated in the [Antibodies for flow cytometry](#) and [ILCP stimulation and single cell cultures](#) and [ILCP-CD4+ lymphocyte coculture](#) sections. Peptides were synthesized at the Protein and Tetramer Core Facility – UNIL/CHUV (Epalinges, Switzerland). OP9 stromal cells were kindly provided by Prof. H. Spits and Dr. B. Blum. ILCP clones were generated in the lab and immediately used.

**Data and code availability**

- RNA-seq data used in this paper have been deposited at the GEO the first time they were used ([Salome et al., 2019](#)).
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

## EXPERIMENTAL MODELS AND SUBJECT DETAILS

### Patients

Peripheral blood was withdrawn for diagnostic purpose from 91 children screened for anti-TG2 antibodies and HLA-DQ2- or -DQ8-positive haplotypes, because of suspected CD, based on clinical symptoms and family risk. Fifty-one children (M:F 16:35, median age 5.5 years; range, 2–17 years (see [Table S1](#) in the supplementary material for patients' details) were diagnosed as CD patients according to the guidelines of European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) ([Husby et al., 2012](#)). Forty children (M:F 13:27, median age 8 years; range, 2.8–16.9 years) with normal TG2 antibodies and no history or signs of inflammatory disorders were considered as healthy children (HC). From 6 children with celiac disease, blood was withdrawn also after a 6-month gluten-free diet (median age 6.3 years; range, 4–14.8 years). For functional tests, to compare the results obtained in the celiac cohort, blood was withdrawn from twelve children hospitalized for minor surgical interventions not linked with intestinal or extraintestinal inflammation (i.e., urogenital or orthopedic malformations).

The work has been carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from the parents of all the individuals and the study was performed according to the Ethical Committee of the Hospital F. Del Ponte, Varese, Italy (protocol number 0049364). Samples were used for diagnostic tests, to isolate peripheral blood mononuclear cells (PBMCs) and to collect serum.

## METHOD DETAILS

### PBMC and serum isolation

PBMCs were isolated by density gradient centrifugation, washed and immediately cryopreserved. Serum was also collected on the same sampling day after centrifugation of whole blood and immediately frozen. Of the 103 children, for *in vitro* and *ex vivo* assays, we selected samples exclusively according to cell viability (more than 80% living cells) and cell counts (more than  $2 \times 10^6$  living cells) upon thawing. When needed, cells were cultured in RPMI-1640 (Gibco) supplemented with 8% heat-inactivated, pooled human serum (HS), 1% penicillin/streptomycin, 1% L-Glutamine, 1% non-essential amino acids, 1% Na pyruvate, 1% Kanamycin 100x (all from Gibco) and 0.1% 2 $\beta$ -mercaptoethanol 500 mM (Sigma) at 37°C, in 5% CO<sub>2</sub>. PBMCs were also isolated from adult healthy donors to avoid limitation in cell numbers when performing the *in vitro* experiments (specified in the text) in which we were not comparing healthy with celiac conditions.

### Antibodies for flow cytometry

ILCs were identified as lineage negative (FITC-conjugated anti-CD3 (clone: UCHT1), anti-CD4 (clone: RPA-T4), anti-CD8 (lot: 276276, Immunotools), anti-CD14 (clone: HCD14), anti-CD15 (clone: HI98), anti-CD16 (clone: 3G8, Beckman Coulter), anti-CD19 (clone: HIB19), anti-CD20 (clone: 2H7), anti-CD33 (clone: HIM3-4), anti-CD34 (clone: 561), anti-CD203c (clone: NP4D6), anti-Fc $\epsilon$ RI (clone: AER-37) all from Biolegend), CD56 negative (BUV737-conjugated anti-CD56, clone: NTAM16.2, BD), CD127 positive (BV785-conjugated anti-CD127, clone: A019D5, Biolegend) lymphocytes. ILC1s, ILC2s, and ILCPs were discriminated using anti-CRTH2 (PE-CF594 or PerCP-Cy5.5, clone: BM16, BD Biosciences or Biolegend, respectively) and anti-cKit (CD117, BV605 or APC-Fire750, clone: 104D2, Biolegend).

For ILC phenotyping the following antibodies were used: anti- $\beta$ 7 (PE, clone: FIB504, Biolegend), anti-CCR6 (PE-Cy7 or BV650, clone: G034E3, Biolegend), anti-CCR9 (BV421, clone: L053E8, Biolegend), anti-CXCR6 (PE, clone: K041E5, Biolegend), anti-CXCR3 (PerCP-Cy5.5, clone: G025H7, Biolegend), anti-CD45RA (BV510, clone: HI100, Biolegend), anti-CD69 (PerCP-Cy5.5 or BV650, clone: FN50, Biolegend), anti-CD137 (41BB, Alexa Fluor 700, clone: 4B4-1, Biolegend), anti-HLA-DR (BV711, clone: L243, Biolegend), anti-NKp44 (APC, clone: P44-S, Biolegend), anti-NKp46 (PerCP-Cy5.5, clone: 9E2, Biolegend), anti-CD28 (Alexa Fluor 700, clone: CD28.2, Biolegend), anti-CD38 (Alexa Fluor 700, clone: HIT2, eBioscience), anti-CD39 (PE-Cy7, clone: A1, Biolegend), anti-CD62L (APC-eFluor780, clone: DREG-56, eBioscience), anti-OX40 (PE, clone: Ber-ACT35(ACT35), Biolegend) and anti-KLRG1 (PE, clone: 13F12F2, eBioscience). Dead cells were always excluded using a viability dye. Samples were acquired on an LSR SORP flow cytometer (BD<sup>TM</sup>) and data were analysed using FlowJo software (TreeStar). For immunophenotyping, each marker was analysed on the PBMCs of at least 5 different donors.

To sort ILCPs, FITC-conjugated anti-CD94 (clone: DX22, Biolegend) was added in the lineage to avoid any NK cell contamination.

For intracellular staining, BV421-conjugated anti-IFN- $\gamma$  (clone: 4S.B3, Biolegend), PE-conjugated anti-IL-13 (clone: JES10-5A2, BD Biosciences), PE-Cy7-conjugated anti-IL-22 (clone: 2G12A41, Biolegend) and APC-conjugated IL-17A (clone: eBio64DEC17, eBioscience) were used.

### Analysis of secreted cytokines

Sera from celiac and healthy children were analyzed using BioLegend's LEGENDplex<sup>TM</sup> bead-based immunoassays to quantify TSLP, IL-1 $\alpha$ , IL-1 $\beta$ , GM-CSF, IFN- $\alpha$ 2, IL-23, IL-12p40, IL-12p70, IL-15, IL-18, IL-11, IL-27, IL-33 (Human Cytokine Panel 2



(13-plex)), to quantify IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFN- $\gamma$  and TNF- $\alpha$  (Human T Helper Cytokine Panels Version 2 (12-plex)) and to quantify IL-8, IP-10, Eotaxin, TARC, MCP-1, RANTES, MIP-1 $\alpha$ , MIG, ENA-78, MIP-3 $\alpha$ , GRO $\alpha$ , I-TAC and MIP-1 $\beta$  (Human Proinflammatory Chemokine Panel 1 (13-plex)). The Human Th Cytokine Panel assays was also used to quantify cytokines in the supernatants of cultured bulk ILCPs. The analyses were performed according to the manufacturer's instructions.

### mRNA sequencing analysis

We sorted ILC1s, ILC2s, ILCPs and NKs from peripheral blood of 3 healthy donors, and subjected these cell subsets to mRNA sequencing (mRNAseq, see [methods](#) in (Salome et al., 2019)) to determine whether the expression of genes reviewed in Hardy et al. (Hardy and Tye-Din, 2016), differed among these cell subsets. Additionally, we compared the expression level of the tissue transglutaminase (TG2) in ILCs and NKs to the level in other circulating adaptive and innate immune cells or intestinal epithelial cells available in the EMBL-EBI Expression Atlas (<https://www.ebi.ac.uk/gxa/home>). We used the R package ExpressionAtlas (v.1.10.0) to obtain raw count mRNAseq data of human circulating immune cells (accession number E-MTAB-3827) and intestinal epithelial cells (accession number E-MTAB-5015). The raw counts of ILC and NK subsets, immune cells and epithelial cells were merged, normalized using the trimmed-mean of M variance normalization implemented in edgeR (v.3.24.3 (Robinson et al., 2010)) and converted to log<sub>2</sub> counts per million (cpm) using the voom function implemented in limma (v.3.38.3 (Ritchie et al., 2015)).

### Peptide preparation

Peptides p $\alpha$ -9 (57–68, QLQFPQPQLPY) and p31-43 (LGQQQFPFPQQPY) were synthesized at the Protein and Tetramer Core Facility – UNIL/CHUV (Epalinges, Switzerland) and used at 20  $\mu$ g/mL. Purity was determined by high-performance liquid chromatography purification. When indicated, p31-43 was used conjugated with Cy5 (at 2  $\mu$ g/mL) and cells were acquired using the Image Stream Technology. When indicated, to block TG2 activity, the transglutaminase 2 inhibitor ZDON (500 nM, Sigma) was added in the ILCP culture 1 h before adding the peptides.

### ILCP stimulation and single cell cultures

ILCPs were isolated by FACS on a FACS Aria III (BD) from CC and HC. To test their responsiveness to gliadin peptides, ILCPs were cultured for 3 hours with p31-43 and then with p $\alpha$ -9 for an over-night, as previously described for other cell types (Maiuri et al., 2005). In another setting, ILCPs were cultured with IL-12p40 (50 ng/mL, Sigma) and IL-18 for 48 h (50 ng/mL, R&D System). After incubation, the cells were harvested to test mRNA expression of selected genes by qPCR. In other experiments, ILCPs were seeded as single cell cultures in terasaki plates for 14 days. Briefly, OP9 stromal cells (kindly provided by Prof. H. Spits and Dr. B. Blum) were plated the day before (200 cells/well, in 10  $\mu$ L of RPMI 8% HS). The day after, ILCPs were plated on OP9 cells (0.5 cells/well, in 10  $\mu$ L of RPMI 8% HS, supplemented with 400 U/mL IL-2 (from Roche), 20 ng/mL IL-7 (from Peprotech), 100 ng/mL IL-12p40 (from Sigma) and 100 ng/mL IL-18 (from R&D System)) and cultured for 2 weeks adding medium after 7 days. At day 16–18, growing ILCPs were transferred in a 96-well plate. The day after they were stimulated with PMA-Ionomycin (10 ng/mL and 1  $\mu$ g/mL, respectively, from Sigma) in the presence of Brefeldin A (10  $\mu$ g/mL, Sigma) for 3 h in a 37°C incubator. Cells were fixed, permeabilized with 0.1% saponin (Sigma) and stained for cytokine production. Samples were acquired on an LSR SORP flow cytometer (BD™) and data were analysed using FlowJo software (TreeStar). For stimulation of bulk ILCPs, ILCPs from adults were isolated by FACS and directly incubated with 20 U/mL IL-2 in the presence of IL-12p40 and IL-18 alone or in combination (both at 50 ng/mL). When indicated 200 nM of vitamin D (1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub>, Sigma) was added. 1000 ILCPs were used per condition, incubated in 100  $\mu$ L for 48 h. Supernatants were harvested and analysed as previously described.

### ILCP-CD4<sup>+</sup> lymphocyte coculture

ILCPs and CD4<sup>+</sup> T cells were isolated by FACS from CC. ILCPs were cultured in RPMI 8% HS, supplemented with 200 U/mL IL-2 and 10 ng/mL IL-7 for a week. During that week, CD4<sup>+</sup> lymphocytes were kept in culture in RPMI 8% HS, supplemented with 100 U/mL IL-2. At the end of the week, ILCPs were cultured for 3 hours with the p31-43 peptide followed by over-night culture with the p $\alpha$ -9 peptide. Then, ILCPs were added (or not) 1:10 to the CD4<sup>+</sup> T cells and cocultured for a week. IFN- $\gamma$  production was evaluated by intracellular cytokine staining using: zombie green viability dye (Biolegend), BV605-conjugated anti-CD4 (clone: OKT4, Biolegend) and APC-Fire750-conjugated IFN- $\gamma$  (clone: 4S.B3, Biolegend).

### RNA purification and quantitative real-time PCR (qPCR)

mRNA was isolated from sorted ILCPs and reverse-transcribed as previously described (Mastelic-Gavillet et al., 2019). The qPCR was carried out in the Applied Biosystems 7900HT with specific primers (hTGM2 5'-TCAACTGCAACGATGACCAGG-3', 5'-TGTTCTGGTCATGGGCCGAG-3'; hIL-18 5'-TCTTCATTGACCAAGGAAATCGG-3', 5'-TCCGGGGTGCATTATCTCTAC-3'; hIFNG 5'-TCGGTAACTGACTTGAATGTCCA-3', 5'-TCGCTCCCTGTTTTAGCTGC-3'; hMKI67 5'-ACGCCTGGTTACTATCAAAGG-3', 5'-CAGACCAATTTACTTGTGGGA-3' using KAPA SYBR® FAST qPCR Kits (Roche). Samples were amplified simultaneously in triplicate and the Ct value for each experimental group was determined. The housekeeping gene  $\beta$ 2M (Beta-2-Microglobulin 5'-GAGGCTATCCAGCGTACTCC-3', 5'-CGGCAGGCATACTCATCTTTT-3') was used as an internal control to normalize the Ct values, using the  $2^{-\Delta Ct}$  formula.

### Immunofluorescence

Expanded ILCPs ( $4 \times 10^5$  cells/mL) were cytopsin on Superfrost slides (Thermo Fisher Scientific) and fixed 5 min with cold methanol. Slides were blocked and permeabilized for 30 min in PBS with 1% bovine serum albumin and 0.1% Triton X-100. Ab against human TG2 (CUB 7402, Abcam) was applied at 1:100 dilution and incubated overnight. After several washes with PBS, secondary anti-mouse (A21200, Invitrogen) Ab was used at 1:500 dilution for 1 h, together with DAPI (50  $\mu\text{g/mL}$ ) to counterstain cell nuclei. After several washes, the slides were mounted with FluoreGuard mounting medium (Scy Tek Laboratories) and kept at 4°C. The slides were imaged in the Bioimaging Facility of the University of Geneva using a Leica SP5 Axiocam system (Leica MicroImaging).

### QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using t tests or ANOVA test. Data are presented as bar graphs, with each bar representing the mean  $\pm$  S.E.M. of the data distribution and the underlying individual data points are also displayed. A p value less than 0.05 (two-tailed) was considered as statistically significant and labelled with \*. p values less than 0.01, 0.001 or 0.0001 were labelled respectively with \*\*, \*\*\* or \*\*\*\*.