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Biological variability of human intraepithelial lymphocytes throughout the human gastrointestinal tract in health and coeliac disease

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Abstract

Background: Intraepithelial lymphocytes are the first line of defence of the human intestinal immune system. Besides, their composition is altered on patients with coeliac disease (CD), so they are considered as biomarkers with utility on their diagnose and/or monitoring. Our aim is to address their variability through the human gastrointestinal tract in health and characterized them in further depth in the coeliac duodenum.

Methods: Intraepithelial lymphocytes were isolated from human gastric, duodenal, ileal and colonic biopsies, then stained with specific antibodies and acquired by flow cytometry.

Results: Our results confirmed that the profile of Intraepithelial lymphocytes change through the length of the human gastrointestinal tract. Besides and given the central role that Interleukin-15 (IL-15) elicits on CD pathogenesis; we also assessed the expression of its receptor revealing that there was virtually no functional IL-15 receptor on duodenal Intraepithelial lymphocytes. Nevertheless and contrary to our expectations, the active IL-15 receptor was not increased either on Intraepithelial lymphocytes from CD patients.

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Conclusions: IL-15 might require additional stimulus to activate intraepithelial lymphocytes. These findings may provide novel tools to aid on a CD diagnosis and/or monitoring, at the time that provide the bases to perform functional studies in order of getting a deeper insight in the specific function that Intraepithelial lymphocytes elicit on CD pathogenesis.

KEYWORDS
coeliac disease, IL-15Rα, intraepithelial lymphocytes

1 | INTRODUCTION

The human gastrointestinal tract's first line of defence is made of intraepithelial lymphocytes (IELs). These specialized immune cells reside within the epithelial layer, acting as sentinels of the mucosal barrier. This heterogeneous group is predominantly composed of T-cells, with a unique enrichment of TCRγδ^{+} cells.[1]

Their strategic localization allows for immediate responses to antigens encountered in the gut lumen. This positioning is crucial for IELs to play a role in both oral tolerance and mucosal barrier integrity, ultimately contributing to immune homeostasis.[2] Maintaining a balance between inhibitory and activating pathways is critical for regulating the cytotoxic activity of these rapidly responding immune cells, thereby optimizing their potential for immune surveillance.[3] In chronic intestinal inflammatory diseases as Coeliac Disease (CD) and Inflammatory Bowel Disease, this balance appears disrupted, with IELs potentially contributing to epithelial damage.[4,5]

CD stands out as a model for understanding gut-related disorders as it is an autoimmune condition triggered by gluten ingestion in genetically predisposed individuals.[6,7] Currently, the only treatment for CD is a life-long, strict gluten-free diet (GFD). However, not all the patients properly respond to the GFD[8] and, besides, villus atrophy persists in over 50% of the patients.[9]

As for CD pathogenesis, Interleukin-15 (IL-15) upregulation is a hallmark of the disease. Besides, CD patients on a GFD maintain high levels of IL-15 expression on the epithelium, being produced in association with IL-15 Receptor α (IL-15Rα). IL-15 can be trans or cis presented binding to IL-2Rβ/γc.[10,11] Moreover, in response to IL-15, NKG2D is increased on the surface of IELs from CD patients. The ligand of NKG2D, MICA, is also upregulated on epithelial cells of CD patients therefore inducing intestinal epithelial cell death along with the apoptotic pathway.[12] Furthermore, IELs are increased in the CD mucosa and display a cytotoxic phenotype. Thus, TCRαβ^{+}CD8αβ^{+} IELs[13] drive the inflammatory mechanisms which are mediated by Fas ligand, perforin, granzyme B and NKG2D, contributing small villous atrophy development.[14,15]

In addition to changes on their activation status, CD patients display differences in the relative contribution of both TCRγδ^{+} and NK-like cells. Hence, changes on IELs distribution have been recognized as a useful tool on CD diagnosis.[16,17] Moreover, IELs adapt to the different locations within the gut since the environment and properties of the intestinal immune system systematically change through its length.[18] The small intestine harbours a higher proportion of memory IELs, experienced in responding quickly to familiar antigens, while the large intestine relies more on naïve IELs, which can adapt to novel antigens encountered less frequently.[19]

Hence, we hereby have studied the phenotype and activity of IELs throughout the human gastrointestinal tract, with particular emphasis on the activation profile of duodenal IEL in the CD setting. Thus, we aimed to get a deeper understanding of their role in maintaining gut homeostasis and the development of gut disease, as this could then be harnessed to develop therapies that modulate immune responses and restore a healthy gut environment.

2 | MATERIALS AND METHODS

2.1 | Patients and biological samples

Biopsies were taken during normal course of a gastroscopy or colonoscopy at the gastroenterology service at both Hospital Clínico Universitario and Hospital Universitario Río Hortega, from Valladolid (Spain). In all cases, samples were obtained following written informed consent after ethical approval from the local ethics committee from Valladolid Este (Valladolid, Spain) (PI19-1352, 25/04/2019). To ensure patient confidentiality, all samples were assigned unique identifiers that do not reveal any personal information using the coding system for later analysis.

Paired gastric samples from 10 controls (70% women, 59 ± 12 years) without any inflammatory disease were collected from the body, incisure and antrum of the stomach.

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FIGURE 1 Identification of Intraepithelial lymphocytes. Total Intraepithelial lymphocytes (IELs) were identified within the cells that passed the PeacoQC test, as singlet viable $CD45^{+}$ cells with low granularity (SSC). Total IEL were further divided based on the expression of CD3 and TCRγδ into NK-like cells ($CD3^{-}$ $TCR\gamma\delta^{-}$), classical T-cells ($CD3^{+}TCR\gamma\delta^{-}$) and TCRγδ T-cells ($CD3^{+}TCR\gamma\delta^{+}$). NK-like cells were further divided based on the expression of CD7 while both T-cell subsets were categorized based on the expression of CD4 and CD8. Results are representative of one single experiments.

Duodenal samples were also collected from nonCD controls, including 81 who did not display any type of inflammation, so they were considered as healthy controls (HC) (79% women, 40 ± 15 years) and 30 controls with nonCD unspecific inflammation so they were considered as nonCD inflamed controls (iC) (77% female, 41 ± 14 years). A total of 59 patients with CD were included (78% female, 39 ± 15 years). All of them had positive compatible genetics, serology Immunoglobulin A anti-Transglutaminase 2 and duodenal lesion at diagnosis. Of them, 26 had been following a GFD for over a year, had negative serology and no mucosal damage (Marsh Score 0), so they were considered as asymptomatic CD (aCD) (81% female, 39 ± 19 years). In addition, 33 CD patients had active disease (24% female, 39 ± 13 years) defined by positive serology and mucosal damage at the time of endoscopy. They were further stratified, based on the Marsh score, into 22 patients with severe CD (sCD) (Marsh Score of 3, 73% female, 38 ± 14 years) and 11 patients with mild disease (mCD) (Marsh score of 1–2, 82% female, 41 ± 11 years). Last, paired ileal and colonic samples were collected for 19 noninflamed controls (26% female, 71 ± 15 years) with not known autoimmune disease or malignancy. All samples were obtained in the context of a routinary endoscopy or colonoscopy for disease diagnose or monitoring.

2.2 | Biopsy processing and antibody labelling

IELs were obtained as previously described.[20] Briefly, biopsies were collected on RPMI 1640 (Gibco, cat # 11875093) and cryopreserved on Frozen Medium-Fetal Calf Serum, (Gibco, cat # 10500064) with 10% Dimethyl Sulfoxide (MP Biomedicals, cat # 190186) at −80°C until used. After thawing, biopsies were incubated twice with IEL Isolation Medium: Hank's Balanced Salt Solution (Gibco, cat # 24020117), 1 mM dithiothreitol (Sigma-Aldrich, cat # 43816) and 0.5 mM Ethylenediaminetetraacetic acid disodium salt dihydrate (Invitrogen, cat # 11568896) at 250 rpm at 37°C during 30 min.

Extracellular staining was performed on the IELs isolated using viability dye Near-IR (Invitrogen, cat # 10154363) and blocking the unspecific unions with Fc-block (BD Pharmingen, cat # 564220). Table S1 shows the specificity, clone, fluorochrome and source of the antibodies used. In all cases, cells were further washed in fluorescent activated cell sorting staining buffer (Dulbecco's Phosphate Buffered Saline (Cytiva, cat # SH30028.02) containing 1 mM Ethylenediaminetetraacetic acid disodium salt dihydrate and 0.02% sodium azide (Sigma-Aldrich, cat # S2002-25G)) and further fixed with Fixing Medium (Phosphate-buffered saline (Lonza, cat # 17-516F) with 2% Buffered Formalin (Protocol, cat # 032–059)) for 10 min at 4°C. Cell were then washed in FACS buffer before they were acquired (within 48 h) on a flow cytometer (Gallios Beckman Coulter).

FCS files were analysed using OMIQ software. Peaco QC algorithm was used to clean the data prior to follow the gating strategy. IELs were selected as SS-A low and $CD45^{+}$ cells. Then, singlet viable cells are identified and divided based on the Tγδ and CD3 expression on three groups: Tγδ ($T\gamma\delta^{+}CD3^{+}$), T cells ($T\gamma\delta^{-}CD3^{+}$) and NK-like cells ($T\gamma\delta^{-}CD3^{-}$). Within Tγδ and T cells, four more subsets were defined based on CD4 and CD8 expression,

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while within the NK like cells were further defined based on CD7 expression (Figure 1). The percentage of cells expressing NKG2D, IL-15Rα and IL-2Rβ was also measured on each immune subset using the fluorescence minus one method to set the positivity limits.

2.3 | Statistical analysis

Data normality was assessed using the Kolmogorov–Smirnov test. Nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed using GraphPad Prism version 8.4.3 for Windows (GraphPad Software, Boston, Massachusetts, US). Significance level was set at *p*-values <.05 (*$p<.05$; ** $p<.01$; *** $p<.001$; **** $p<.0001$). Figures were assembled using Inkscape version 1.3.2 (www.inkscape.org).

3 | RESULTS

3.1 | IEL distribution along the human gut

The proportion of the different IEL subsets throughout the human healthy or noninflamed gastrointestinal tract was determined as shown in Figure 1 using the OMIQ software from Dotmatics (© OMIQ, Inc. 2024, www.omiq.ai). TCRγδ$^+$ T-cells were particularly enriched in the human colon referred to the small bowel (duodenum or ileum) or the gastric mucosa (body, antrum or incisura) (Figure 2A), with a particular depletion of the CD4$^+$CD8$^+$ subset (Figure 2B). Conversely, classical or TCRγδ$^-$ T-cells were decreased in the colon referred to the other parts of the human gastrointestinal tract (Figure 2C) with no major differences in their subset composition through the human gut (Figure 2D). Last, NK-like cell density was

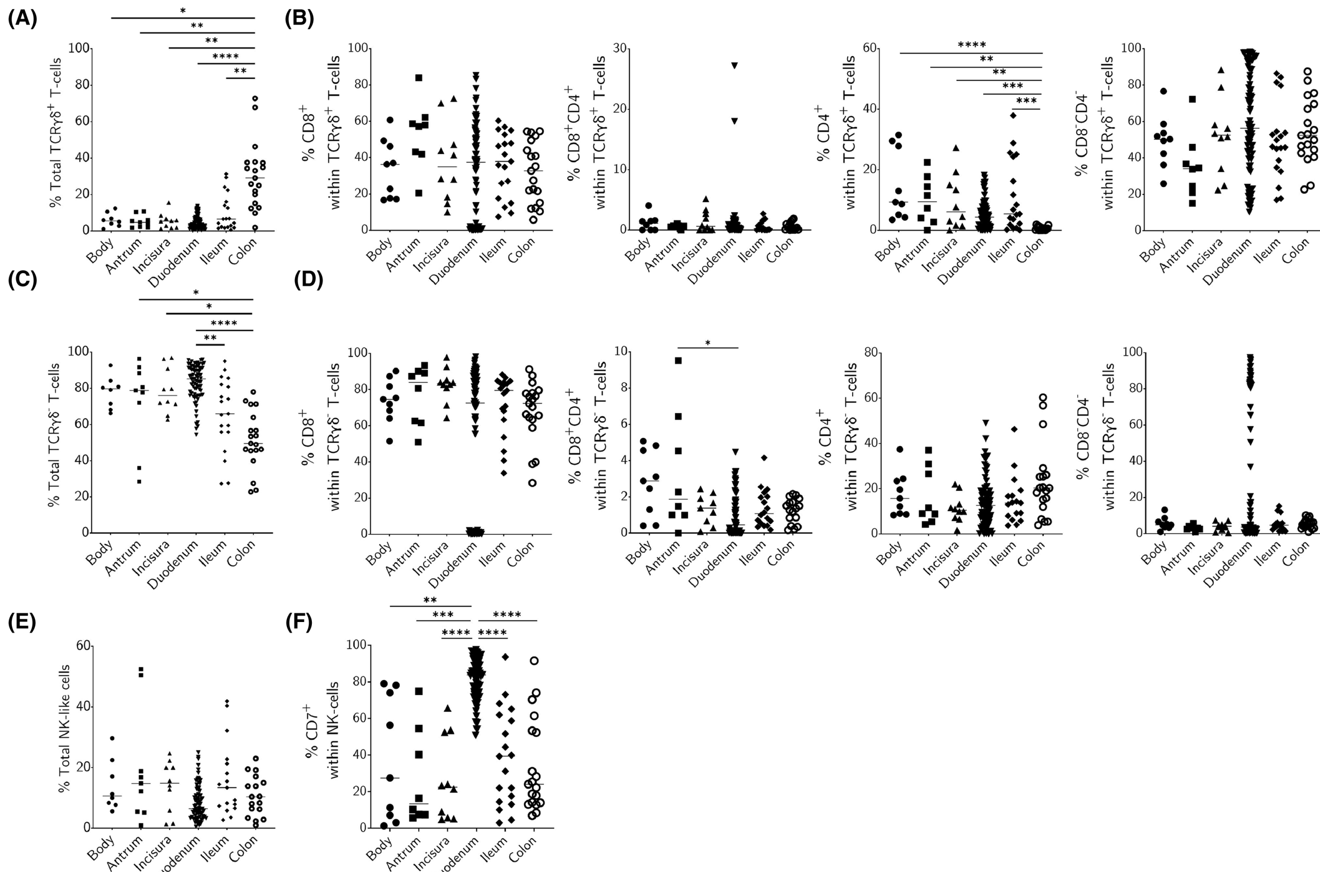

FIGURE 2 Intraepithelial lymphocytes through the human gut. Total intraepithelial lymphocytes (IELs), as well as their different subsets, were identified as in Figure 1. Their proportion was assessed in the human gastric mucosa (body, antrum and incisura), as well as in the small bowel (duodenum and ileum) and colon from controls. The percentage of total TCRγδ$^+$ T-cells is shown in (A), while their different subsets is displayed in (B). In a similar manner, the percentage of classical T-cells (CD3$^+$TCRγδ$^-$) though the human gut is shown in (C), with their different subsets in (D). Last, the percentage of total NK-like IEL, and their subsets through the human gut is respectively shown in (E) and (F). Data were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to non normal distribution (Kolmogorov–Smirnov test). *P*-values <.05 were considered significant (*$p<.05$; **$p<.01$; ***$p<.001$; ****$p<.0001$).

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not affected by the source of the tissue (Figure 2E), although the CD7$^+$ fraction was particularly enriched in the duodenum (Figure 2F).

3.2 | NKG2D is highly expressed on IEL throughout the human gut

Given the central role that NKG2D elicits on IEL function, we next assessed its relative expression though the human gut. When focused on Tγδ cells, NGK2D was virtually expressed on all cells (Figure 3A), with a smaller percentage of expression in the human duodenum (Figure 3B) which however not associated with any particular subset (Figure 3C).

NKG2D expression was also high on classical T-cells, but lower than on Tγδ cells (Figure 3D), with a particular increase on classical T-cells from the ileum (Figure 3E). Further analysis revealed that NKG2D was preferentially expressed on CD8$^+$ classical T-cells and, to a lesser extent, on the double negative T-cells, although its expression was specifically increased on double negative classical T-cells from the ileum (Figure 3F).

Last, NKG2D expression was also found on NK-like cells (Figure 3G) with no regional variation through the gut (Figure 3H), although its expression was increased on the ileal and colonic CD7$^+$ fraction and lowered on the CD7$^-$ duodenal population (Figure 3I).

3.3 | IL-15Rα is specifically decreased on duodenal IEL

Given the central role that IL-15 elicits orchestrating mucosal immune responses in the human gut, we also assessed the expression of its receptor on human IEL through the human gut. When focused on Tγδ cells (Figure 4A) and although IL-15Rα was constitutively expressed through the human gut, it was specifically decreased on the duodenum (Figure 4B), on both CD8$^+$ and CD8$^-$ Tγδ cells (Figure 4C). Of note, although IL-15Rα expression was lower on classical T-cells referred to the Tγδ compartment (Figure 4D), the same decreased expression was found in the duodenum (Figure 4E) again not being associated with any particular subset (Figure 4F). In a similar manner, IL-15Rα NK-like cells also expressed IL-15Rα (Figure 4G), although when consider their regional variability it was also identified a specific lower expression on the duodenum (Figure 4H) which however was not related to any particular subsets (Figure 4I).

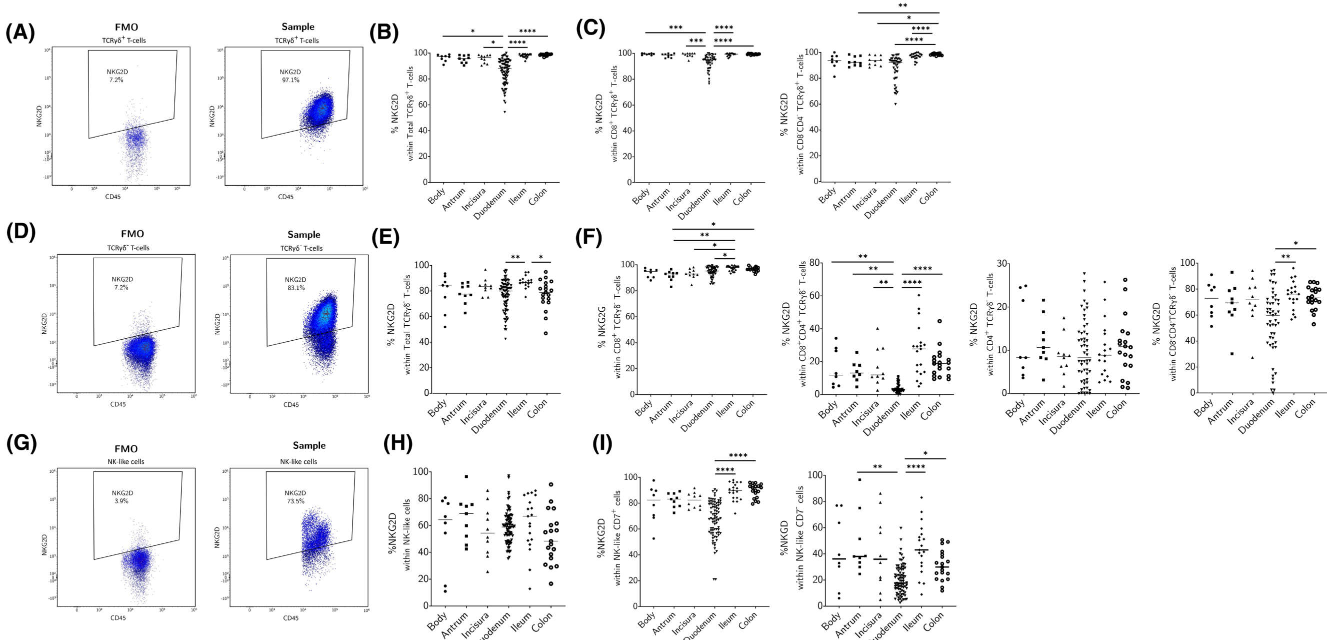

FIGURE 3 NKG2D expression on human Intraepithelial lymphocytes. Human intraepithelial lymphocytes were identified as in Figure 1. (A) NKG2D expression was determined on intraepithelial TCRγδ$^+$ T-cells referred to their fluorescence minus one (FMO) control. (B) its expression was assessed on total TCRγδ$^+$ T-cells through the human intestine, as well as on (C) their CD4$^-$CD8$^+$ and CD4$^-$CD8$^-$ subsets. (D) NKG2D was also assessed on TCRγδ$^-$ T-cells (E) through the human gut and, within then, (F) in the different subsets based on the expression of CD4 and CD8. (G) Last, NKG2D was determined on total NK-like cells through the human gut, (H) both within total cells and (I) within their CD7$^+$ and CD7$^-$ subsets. Data were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to non normal distribution (Kolmogorov–Smirnov test). *P*-values <.05 were considered significant (**p* <.05; ***p* <.01; ****p* <.001; *****p* <.0001).

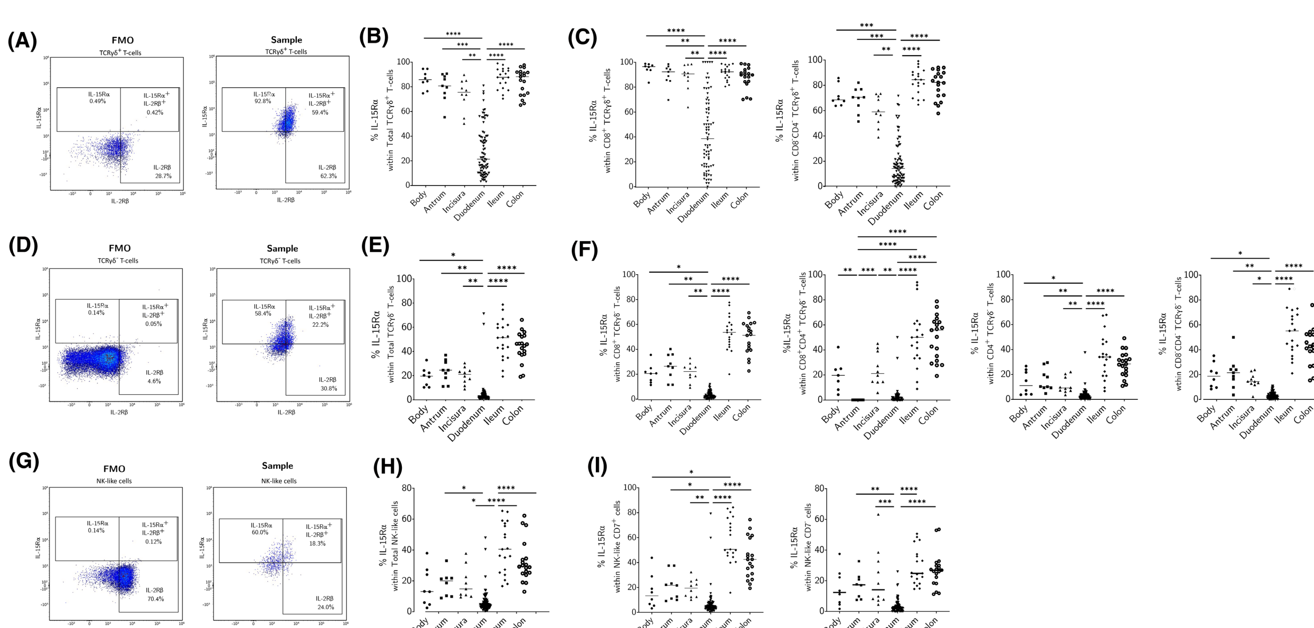

FIGURE 4 Expression of IL-15Rα on intraepithelial lymphocytes through the human gut. Human intraepithelial lymphocytes were identified as in Figure 1. (A) IL-15Rα expression was determined on intraepithelial TCRγδ^{+} T-cells referred to their fluorescence minus one (FMO) control. (B) its expression was assessed on total TCRγδ^{+} T-cells though the human intestine, as well as on (C) its CD4^{+}CD8^{+} and CD4^{-}CD8^{-} subsets. (D) IL-15Rα was also assessed on TCRγδ^{-} T-cells (E) through the human gut and, within then (F) in their different subsets based on the expression of CD4 and CD8. (G) Last, IL-15Rα was determined on total NK-like cells, as well as through the human gut, (H) both as total cells and (I) within their CD7^{+} and CD7^{-}. Data were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to non normal distribution (Kolmogorov–Smirnov test). *P*-values <.05 were considered significant (*p <.05; **p <.01; ***p <.001; ****p <.0001).

3.4 | Intraepithelial lymphogram in the coeliac duodenum

Having described that IL-15Rα is specifically decreased on IEL from the human duodenum (Figure 4) and given the central role that this cytokine together with NKG2D elicit on CD immunopathogenesis,[21,22] we next studied duodenal IEL in the context of CD.

Total Tγδ cells were expanded in the IEL compartment from CD patients irrespectively of the mucosal status (Figure 5A), in agreement with previous observations.[17,23] Further analysis revealed that the double negative fraction was the most predominant in the duodenum, being such subset increased on CD patients irrespectively of the mucosal status (Figure 5B). Conversely, classical T-cells were diminished in the CD duodenum (Figure 5C), also revealing a trend to higher levels of the double negative fraction on CD patients (Figure 5D). Also, in agreement with previous observations,[16] NK-like cells were specifically decreased on CD patients with mucosal atrophy, either mild or severe, but not on patients with asymptomatic disease or non-CD controls (both inflamed and noninflamed) (Figure 5E). Last all CD patients (irrespectively of the mucosa status) had a lower proportion of CD7^{+} NK-like cells (Figure 5F).

3.5 | Increased expression of NKG2D on double positive and CD4^{+} classical T-cells from patients with severe CD

Given that NKG2D was expressed on all Tγδ cells (Figure 3), its expression remained virtually unchanged on this population (Figure 6A) and its subsets (Figure 6B) irrespectively of the duodenal mucosal status (Figure 6A,B). As for classical T-cells, NKG2D expression remained unchanged among the different conditions upon study (Figure 6C). However, its expression was expanded on double positive T-cells and, to a lower extent, on helper T-cells from patients with severe CD (Figure 6D). As for the NK-like compartment, NKG2D was decreased on patients with severe CD (Figure 6E), with its expression being higher on the CD7^{+} cells and lower on the CD7^{-} fraction (Figure 6F).

3.6 | Decreased IL-15Rα expression on duodenal TCRγδ^{+} T-cells from CD patients

Last, we also assessed IL-15Rα expression on duodenal IEL. Of note, its expression was decreased on Tγδ cells from all CD patients, irrespectively of their mucosal

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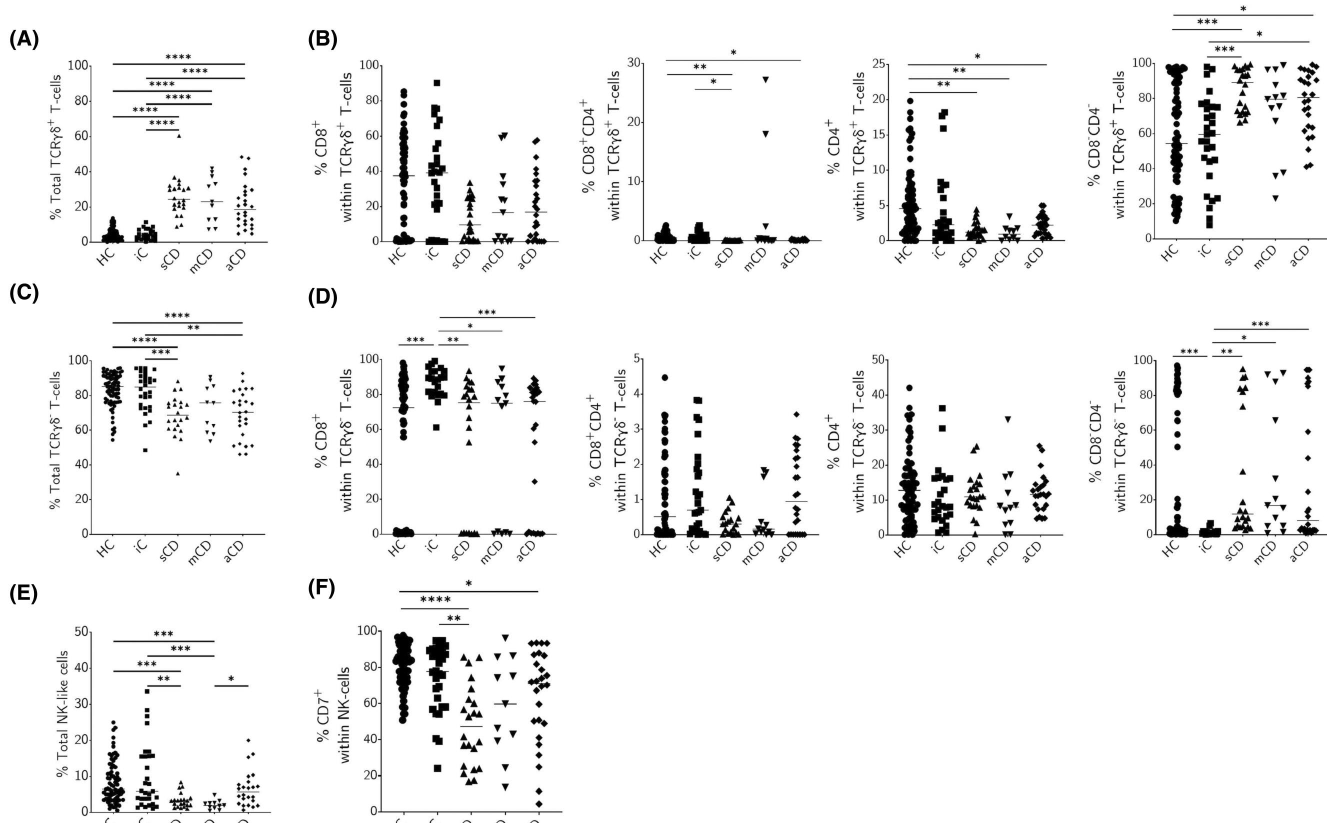

FIGURE 5 Intraepithelial lymphocyte subsets in the coeliac duodenum. Human duodenal intraepithelial lymphocytes were identified as in Figure 1, and their proportion determined in the duodenum from Healthy controls (HC) and inflamed Controls (iC), as well as from patients with coeliac disease (CD) including severe (sCD), mild (mCD) and asymptomatic (aCD) disease. The percentage of total TCRγδ$^+$ is shown in (A), with the relative proportion of their subsets based on the expression of CD4 and CD8 is shown in (B). The same applies for TCRγδ$^-$ T-cells, both as (C) total cells and (D) their relative subsets; as well as for (E) total NK-like cells and (F) their subsets. Data were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to non normal distribution (Kolmogorov–Smirnov test). *P*-values <.05 were considered significant (**p* <.05; ***p* <.01; ****p* <.001; *****p* <.0001).

status, referred to nonCD controls, either inflamed or non-inflamed (Figure 7A), although these differences could not be attributable to any specific subset (Figure 7B). No major differences on IL-15Rα expression among the different conditions upon study were found on classical T-cells, either as total (Figure 7C) or when considered as subsets (Figure 7D) or within NK-like cells, again when considered as total (Figure 7E) or subsets (Figure 7F).

4 | DISCUSSION

Given that the properties of the immune system systematically change through the length of the human gastrointestinal tract,[18,24] we hereby have proved that the profile of total IEL also changes though its length, at the time that have also unveiled a lower expression of the IL-15 receptor on human duodenal IEL in health referred to other parts of the gastrointestinal tract, which was actually more evident on CD patients.

Hence, we hereby have demonstrated that the composition of the IEL compartment changes though the human gut, confirming a higher proportion of Tγδ cells on the colon[25] where the CD4$^+$ fraction was virtually absent on such compartment. Among all the studied tissues, we have also found that the duodenum was the most different compartment since classical T-cells and CD7$^+$ NK-like cells were expanded in such compartment (Figure 2). Besides, the expression of IL-15Rα was virtually absent on duodenal NK-like cells and classical T-cells, while its expression on Tγδ was much lower than in other areas of the human gut. Also, duodenal IELs seem also to display a lower activation status defined by a trend towards lower NKG2D expression on the Tγδ fraction. Together, that suggest that human duodenal IEL display a non pro-inflammatory phenotype in agreement with the regulatory functions attributed to this compartment.[18,19]

Our analysis of duodenal IELs in CD confirmed the established profile of increased Tγδ cells and decreased

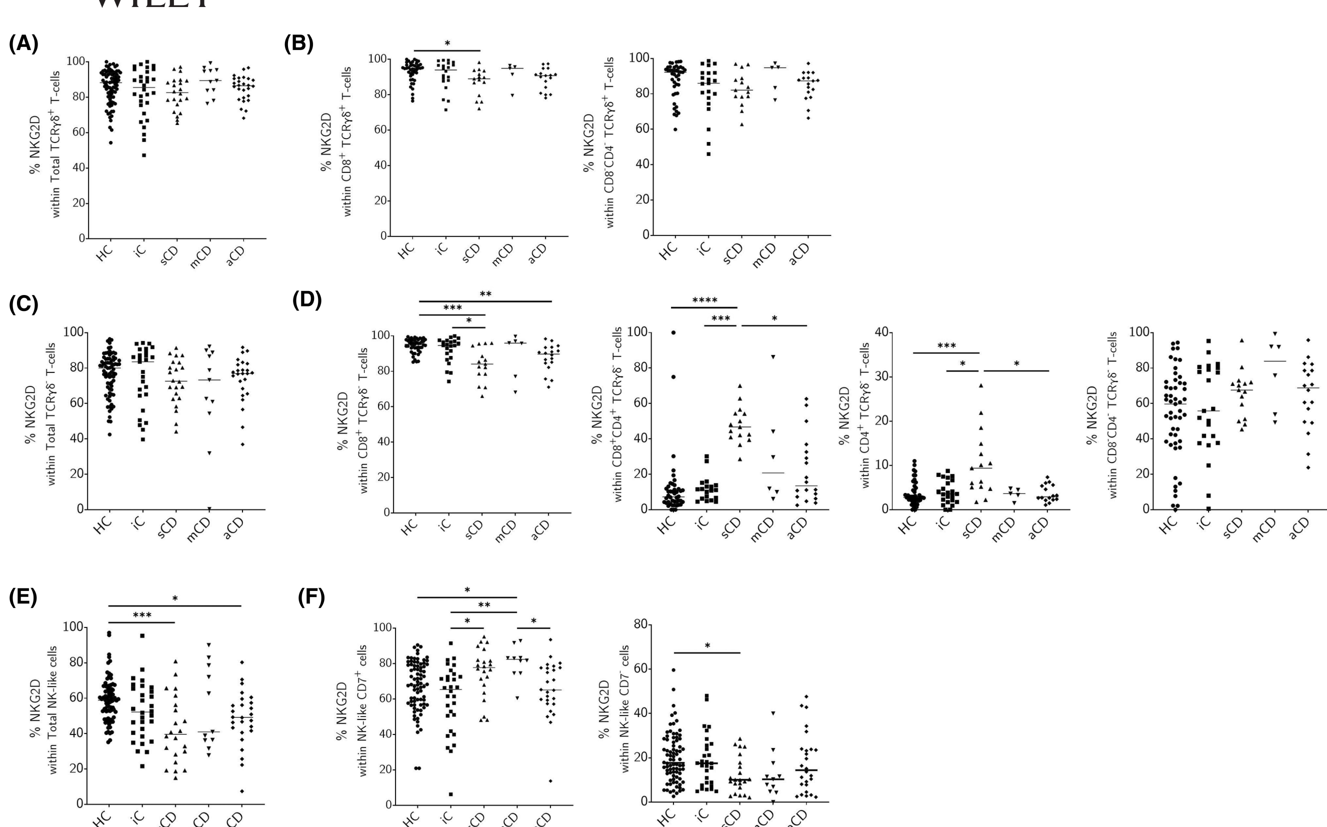

FIGURE 6 NKG2D expression in the human duodenum. Human duodenal intraepithelial lymphocytes were identified as in Figure 1 in the duodenum from Healthy controls (HC) and inflamed Controls (iC) as well as from patients with coeliac disease (CD) including severe (sCD), mild (mCD) and asymptomatic (aCD) disease. Within them, the expression of NKG2D was determined on (A) TCRγδ$^+$ T-cells and (B) their CD4$^-$CD8$^+$ and CD8$^-$CD4$^-$ subsets, as well as on (C) TCRγδ$^-$ T-cells and (D) their CD4/CD8 subsets, together with the expression on (E) total NK-like cells and (F) their CD7$^{+/-}$ compartment. Data were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to non normal distribution (Kolmogorov–Smirnov test). *P*-values <.05 were considered significant (**p* <.05; ***p* <.01; ****p* <.001; *****p* <.0001).

NK-like cells irrespectively of the mucosal status, consistent with previous studies.[23,26] While γδ T cells are enriched in the duodenum of CD patients, their abundance is notably higher in the healthy colon, suggesting distinct functions in these locations. In the colon, Tγδ cells might primarily contribute to antimicrobial defence and can exhibit antitumorigenic properties while on the duodenum tissue repair and oral tolerance might be the main function of this population.[27] However, specific subsets, characterized by V gene usage, can promote IL-17 production and tumorigenesis on the colon. On the duodenum, gluten exposure in CD induces the recruitment and expansion of Interferon-γ producing Vγ3$^+$ on the IELs at the expense of protective Vγ4$^+$ IELs.[28] These highlight the dynamic nature of the IEL Tγδ cell population.

As for the mechanisms mediating mucosal damage on CD patients, NKG2D mediated cytotoxicity plays a central role. The interaction between NKG2D and its ligand, the nonclassical MHC class I molecule MICA on intestinal epithelial cells, triggers apoptosis, contributing to mucosal damage.[14] This mechanism is supported by the expression of MICA in the duodenum of CD patients[29] and our findings of elevated NKG2D expression on CD4$^+$ fraction of T cells (either single positive or double positive) and CD7$^+$ NK-like cells in patients with active disease, which was also more prominent on those with severe disease. Importantly, CD7$^+$ IELs represent a potential source of NKG2D-expressing cells contributing to epithelial damage. Recent studies have expanded our understanding of this process by demonstrating the direct cytotoxic activity of certain Tγδ cell subsets against enterocytes through recognition of nonclassical MHC molecules.[30] Moreover, the association between NKG2D ligand expression and cellular stress in the inflamed mucosa[31] reinforces the role of this pathway in CD. These results are therefore in agreement with murine models where it has been proved that mucosal recovery following a GFD can reduce NKG2D expression in the mucosa.[32]

Given the central role that IL-15 elicits triggering immune responses in the gastrointestinal tract,[33] something which is particularly true on CD as these patients display a lower immune response threshold towards this cytokine,[34]

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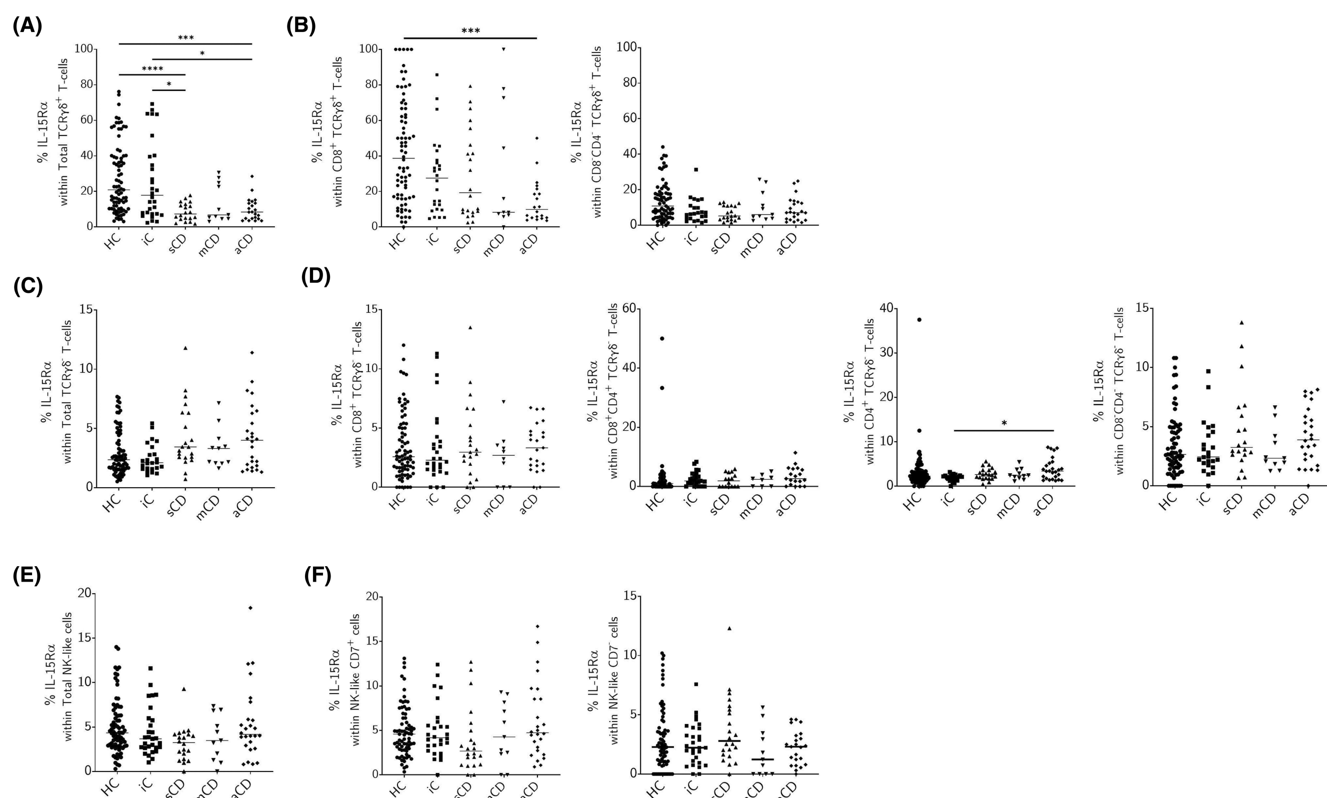

FIGURE 7 Expression of IL-15Rα on duodenal intraepithelial lymphocytes. Human duodenal intraepithelial lymphocytes were identified as in Figure 1 in the duodenum from Healthy controls (HC) and inflamed Controls (iC), as well as from patients with coeliac disease (CD) including severe (sCD), mild (mCD) and asymptomatic (aCD) disease. Within them, the expression of IL-15Rα was determined on (A) TCRγδ$^+$ T-cells and (B) their CD4$^-$CD8$^+$ and CD8$^-$CD4$^-$ subsets, as well as on (C) TCRγδ$^-$ T-cells and (D) their different CD4/CD8 subsets, together with the expression on (E) total NK-like cells and (F) their CD7$^+$/$^-$ compartment. Data were analysed using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test due to non normal distribution (Kolmogorov–Smirnov test). *P*-values <.05 were considered significant (**p* <.05; ***p* <.01; ****p* <.001; *****p* <.0001).

we focused on the study of its receptor. IL-15R expression was studied for all IEL populations, in agreement with Dunne et al.[35] theory of two steps process to mediate gut-damage of IEL, T cells acquiring an aberrant NK like cytotoxic phenotype coupled with Tγδ IELs losing regulatory functions. As previously stated, our results have revealed that the expression of IL-15Rα was decreased on duodenal IEL. Given that IL-15 receptor shares its beta chain with the IL-2 receptor,[36] we also assessed the expression of the latter though the human gut revealing that although IL-2Rβ expression could be found on most IEL subsets through the gastrointestinal tract, it was also decreased in the duodenum (Figure S1) rendering such compartment, as opposed to the other sides of the human gastrointestinal tract, with no functional IL-15 receptor on classical T-cells and a very low expression on Tγδ cells (Figure S2). A lack of IL-15 or its receptor in the gut epithelium hinders the development of intestinal IELs.[37] While IL-2Rβ expression has been documented on IEL from some animal models,[38] its presence and function in human IELs require further investigation. The absence of an active IL-15 receptor on the human duodenum may therefore be a mechanism to mediate oral tolerance towards the nutrients and the commensals.[39]

IL-15 and IL-15Rα are synthesized as a complex within the endoplasmic reticulum and can subsequently be secreted as soluble molecules or expressed on the cell surface. The primary mechanism of IL-15 signalling in vivo involves trans-presentation to cells expressing the complementary IL-15 receptor subunits, IL-2Rβ/IL-15Rβ and γc.[40] While previous studies have suggested a role for cis-presentation and soluble IL-15Rα/IL-15 complexes in celiac disease,[34] our findings indicate a different scenario. We observed consistent IL-15Rα and functional IL-15R expression on the surface of IELs from both control and celiac disease patients (Figure 7; and S4 respectively), suggesting that elevated IL-15Rα/IL-15 production by IELs likely occurs in response to specific stimuli. Instead, our data suggest a model where enterocytes in CD exhibit increased production of IL-15Rα/IL-15, thereby enhancing the ability of IELs to amplify the cytokine signal. This hypothesis is supported by previous reports of elevated

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IL-15Rα mRNA levels in biopsies and lamina propria lymphocytes.[34] Furthermore, as IL-2Rβ expression was similar in IELs from control and celiac disease patients (Figure S3), our results indicate that the dysregulated response of IELs to IL-15 is primarily driven by increased cytokine production by enterocytes rather than alterations in trans-presentation mechanisms.

We are nevertheless aware that this is mainly a descriptive study. Therefore, further studies are needed to clarify the implications of the increased expression of NKG2D found on the $CD4^+$ fraction from duodenal classical T-cells and, more important, to unravel the function of IL-15 in the duodenum in health and its specific mechanism of action on the IEL from CD patients. Besides, given the central role that IL-15 elicits on its pathogenesis, future studies should address the implication of the absence of such functional receptor on classical IEL T-cells. However, a potential explanation relies in the fact that IL-15 may elicit its role in the CD duodenum by trans-presentation,[41] hence providing an explanation to why IEL increase NKG2D expression following IL-15 exposure.[42,43] Hence, further functional experiments should evaluate the impact of IL-15 stimulation on the IEL NKG2D upregulation, but also of the IL-15 receptor subunits, although recent evidence suggests that the role of other cytokines (including IL-7), on this mechanism, should not be disregarded.[44]

In summary, we hereby have performed a comprehensive characterization of the biological variability that the human IEL display through the length of the gastrointestinal tract, describing the presence of regional differences and providing further insights about the specific changes in the phenotype and function that IEL elicit on CD. These findings may therefore provide novel tools to aid on a CD diagnosis and/or monitoring, at the time that provide the bases to perform functional studies in order of getting a deeper insight in the specific function that IEL elicit on the CD pathogenesis.

AUTHOR CONTRIBUTIONS

Guarantor of article: David Bernardo: Author Contributions: Aida Fiz López: Investigation (lead), formal analysis, visualization and writing-Original Draft Preparation. Ángel De Prado: Investigation (supporting). Elisa Arribas-Rodríguez: Investigation (supporting). Francisco Javier García-Alonso: Data curation (equal) and resources (equal). Sandra Izquierdo: Data curation (equal) and resources (equal). Álvaro Martín: Resources (equal). José A. Garrote: Supervision (equal). Eduardo Arranz: Supervision (equal). Jesús Barrio: Resources (equal). Luis Fernández-Salazar: Conceptualization, resources (equal) and funding acquisition (equal). David Bernardo: Funding acquisition (equal), project administration and writing-Review& editing: All authors approved the final version of the article, including the authorship list.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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