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Intraepithelial Lymphocytes and LAIR1 Expression in Celiac Disease

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Abstract: Background: The epithelium of intestinal mucosa is colonized by specialized immune cells known as intraepithelial lymphocytes (IELs). Celiac disease is a gluten-sensitive enteropathy and common immune-related inflammatory disease of the small intestine. **Objectives**: To characterize the phenotype of IELs and the lamina propria of the mucosa in small intestine control using immuno-oncology and immune-phenotype markers, and to test the most relevant marker in celiac disease. **Methods**: Immunohistochemical analysis of CD3, CD4, CD8, CD103 (ITGAE), Granzyme B, TCRβ, TCRδ, CD56 (NCAM), CD16, LAIR1 (CD305), PD-L1, PD1 (CD279), BTLA (CD272), TOX2, HVEM (TNFRSF14), CD163, HLA-DP-DQ, IL4I1, and FOXP3 was performed using conventional histological analysis; and deep learning (convolutional neural network) for LAIR1. **Results**: IELs exhibited a cytotoxic T-cell phenotype and were positive for CD3, CD8, CD103, TCRβ, and LAIR1. The lamina propria was rich in CD163, HLA-DP-DQ, BTLA, and LAIR1-positive cells corresponding to macrophages, and T and B-lymphocytes. In celiac disease, IELs and the lamina propria were LAIR1-positive. Finally, as a proof-of-concept artificial intelligence (AI) analysis, LAIR1 image patches were successfully classified between small intestine control, celiac disease, and reactive tonsils. **Conclusions**: IELs are LAIR1-positive, and LAIR1 appears to be a potentially relevant marker in celiac disease.

Keywords: small intestine; celiac disease; intraepithelial lymphocytes; lamina propria; immune-phenotype; immuno-oncology; LAIR1; artificial intelligence; deep learning; convolutional neural network

1. Introduction

1.1. Histology of the Small Intestine

The normal intestinal mucosa has defined characteristic features, and its architecture include the villi, crypts, lamina propria, and muscularis mucosae.

The villi exhibit a digitiform shape with a 3:1 ratio between the height of the villi and the depth of the glandular crypts. The glandular crypts comprise several cell subtypes, including epithelial, Paneth, goblet, and endocrine cells. Each of these cells has different functions.

Intestinal epithelial cells (IECs) line the surface of the intestine and are responsible for the digestion of aliments, absorption of nutrients, and protection from infection by creating a physical barrier and modulating the immune response [1]. Intestinal epithelial cells are sensitive to the nutrients in the diet [1]. Paneth cells secrete alfa defensins, which are broad-spectrum microbicides that control the gut microbiota and intestinal homeostasis. In H&E staining, Paneth cells display bright red cytoplasmic granules [2]. Goblet cells produce mucus and are intimately involved in the control of the mucosal immune system [3]. Goblet cells sample luminal antigens to initiate adaptive immune response. There are several subtypes of goblet cells, with different localization and gene expression [3]. There are several types of endocrine cells in the small intestine [4]: EC cells produce serotonin (5-HT)[5–7]; L cells, GLP-1, GLP-2, and peptide YY [8–10]; K cells, GIP and 5-HT [11,12]; I cells, cholecystokinin and 5-HT [13,14]; D cells, somatostatin [15,16]; G cells, gastrin [17–19]; N cells,

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neurotensin [20,21]; M cells, motilin [22,23]; and S cells, secretin [24,25]. The main functions of endocrine cells are gut motility, appetite control, insulin release, cell proliferation control, gastric acid motility, pancreatic enzyme secretion, and intestinal absorption [4].

The lamina propria is a thin layer of connective tissue located below the epithelial basement membrane. The lamina propria is rich in fibroblasts, myofibroblasts, vascular and lymphatic vessels, elastic fibers, smooth muscle fascicles, and immune cells, including lymphocytes, plasma cells, macrophages, eosinophils, and mast cells [26].

The muscularis mucosae is composed of a very thin layer of smooth muscular cells with motor activity, which are linked to mucosal absorption and secretion functions [27].

The submucosa contains blood and lymphatic vessels, and nerves of the parasympathetic system, including the submucous plexus, also known as Meissner's plexus [28]. The submucosal extracellular matrix is minimally immunogenic [28]. The muscularis propria [29] is composed of an inner circular and outer longitudinal layer, and within, Auerbach's (myenteric) plexus is located.

1.2. Instraepithelial Lymphocytes

Intraepithelial lymphocytes (IELs) are found in the epithelium of the skin, genitourinary tract, respiratory tract, and intestinal tract [30]. IELs are a first lines of the immune system against pathogens that have attacked the epithelial surface. The typical phenotype is of cytotoxic T-lymphocytes, being CD3-positive and CD8-positive [31]. The T-cell receptor (TCR) is both alphabeta ($\alpha\beta$) and gammadelta ($\gamma\delta$)-positive. The fact that some IELs present with self-reactive TCR suggests an extrathymic origin [30,32–36].

IELs are specialized immune cells that colonize the intestinal mucosa. While B and innate cell populations may also transit inside this compartment, T-lymphocytes comprise the majority of intestinal IELs. In the intestine, IELs represent one of the largest lymphocyte populations and contribute to epithelial homeostasis and barrier integrity, including tolerance, resistance, and tissue protection [37]. There are several subsets of IELs. However, all strains share common characteristics, including restricted TCR diversity, epithelium-adapted profile, innate-like properties, and cytotoxic potential [37]. Human IELs can recognize modified self-antigens using both natural killer (NK) receptors and foreign antigens using the TCR [31]. The main characteristics of IELs are as follows:

(1) IELs permanently reside in the epithelial tissue and do not recirculate because of the expression of CD103 [38,39] that binds to E-cadherin [39–41]. CD103 is also known as ITGAE (Integrin, Alpha E, and Human Mucosal Lymphocyte Antigen 1). E-cadherin is also known as Cadherin-1 (CDH1), and CD324.

(2) The epithelial environment of the mucosa is highly immunogenic, with constant activation, and tolerance that prevents tissue damage. Therefore, IELs express several T-cell coinhibitory molecules and NK inhibitory receptors [42,43] and downregulate TCR costimulatory molecules.

(3) In comparison to peripheral T-lymphocytes, the TCR diversity of IELs is limited [44,45], and specific to conserved microbial or dietary antigens [46].

(4) IELs have innate-like properties enabling rapid TCR-independent responses to stress signals [42,47].

(5) IELs have cytotoxic activity [47–50], and an alteration may be associated with several gastrointestinal diseases, such as celiac disease and inflammatory bowel disease (IBD) [50–54].

(6) IELs are stratified into natural IELs (nIELs) and peripherally induced IELs (pIELs) [55–58]. The nIELs are generated in the thymus and migrate to the intestine. In contrast, pIELs are derived from CD4-positive or CD8-positive T cells at inductive sites, such as gut-associated lymph nodes, in response to dietary and microbial antigens [31,37,55–61].

(7) IELs can be further subclassified according to their TCR subtype: (I) TCR$\gamma\delta$+ nIELs (tissue surveillance and repair), (II) TCR$\alpha\beta$+CD8$\alpha\alpha$+ nIELs (regulation), (III) TCR$\alpha\beta$+CD8$\alpha\beta$+ pIELs (effector memory, cytotoxicity), (IV) TCR$\alpha\beta$+CD4+ pIELs (regulation, cytotoxicity) [31,37]. Subtypes I and II may recognize self-antigens using their TCR, are present at birth, and are microbiota independent. On the other hand, subtypes III and IV may recognize microbial, viral,

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and dietary antigens using the TCRs, are absent at birth, increase with age, and are microbiota- and diet-dependent [31,37]. Of note, CD4+FOXP3+regulatory T lymphocytes (Tregs) can undergo CD4+CD8αα+ IEL differentiation in the intestinal epithelium [62,63].

(8) CD8αα+ is an indication of intestinal IELs. Conventional CD8+ T cells express the CD8αβ heterodimer that is a TCR coreceptor, and enhance the TCR-MHC-I interactions during antigen presentation. Most IELs express CD8αα homodimer that decreases TCR sensitivity and prevents IEL hyperactivation via the mechanism of CD8αα homodimer interaction with thymus leukemia (TL) antigen [64], which is expressed by intestinal epithelial cells. Therefore, TL expression plays a critical role in maintaining IEL effector functions. TL deficiency is associated with colitis in a genetic model of inflammatory bowel disease [65].

(9) IELs contribute to the pathogenesis of chronic intestinal inflammatory disease. Inflammatory bowel disease (IBD) includes Crohn disease and ulcerative colitis. Dysregulated intestinal immune response to microbiota is a cause of IBD [66,67]. In IBD, IELs could play a regulatory role [65–72]. A preserved villous architecture and increased IELs characterize microscopic colitis [73–76]. Celiac disease is an autoimmune disease triggered by dietary gliadin and is characterized by villous atrophy, crypt hyperplasia, and chronic inflammation of the lamina propria [77–80]. In celiac disease, there are increased CD8αβ+ pIELs and TCRγδ+ nIELs [31]. IELs can undergo neoplastic transformation into Enteropathy-associated T-cell lymphoma, a rare complication in patients with celiac disease who are unresponsive to gluten-free diet and treatment [81–84].

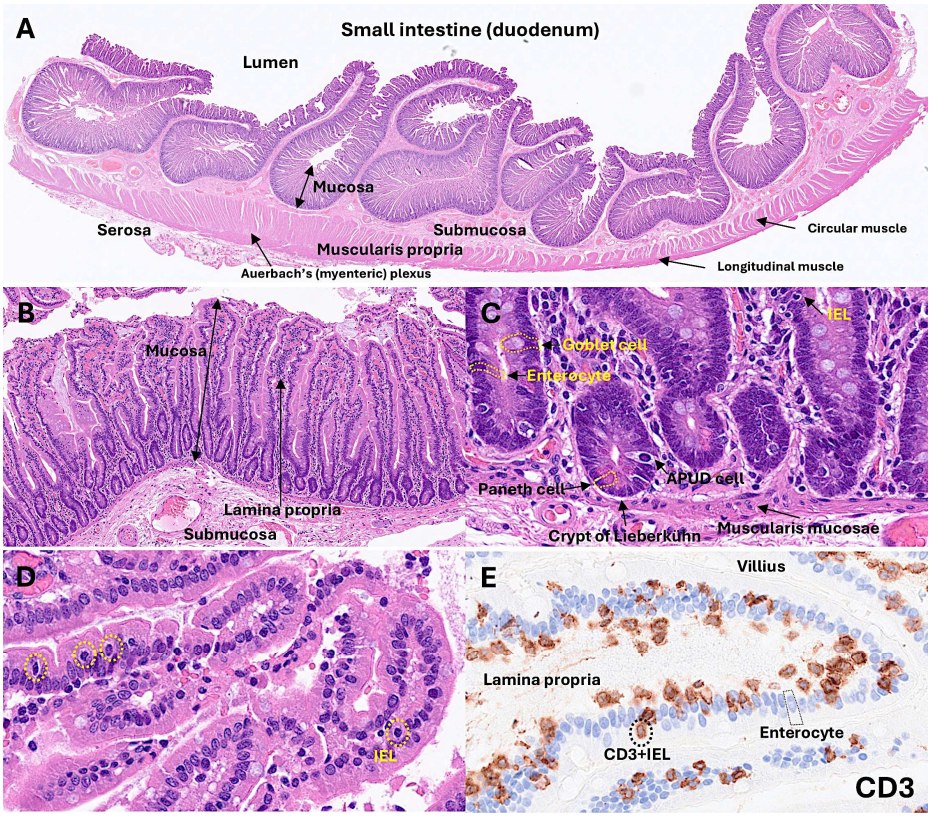

Figure 1. Histology of small intestine.

1.3. Celiac Disease

Celiac disease is a common immune-related disease, with a prevalence of approximately 1% in most populations [85]. The incidence of celiac disease has increased in recent years; the reason is

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unknown, but it may be related to environmental factors that are associated with the loss of tolerance to dietary gluten [85].

The pathogenesis of celiac disease is multifactorial. The pathogenesis includes a genomic background with the presence of several genetic factors [86], such as the close association with HLA-DR3-DQ2 and/or DR4-Dq8 gene locus, which is highly present in celiac disease patients [87]. Other gene loci have also been identified using genome-wide association studies (GWAS), which are related to metabolism and immune system, such as 3p21.31 (*CCR3* and *CCR2*), 4p27 (*KIAA1109*, *ADAD1*, *IL2*, and *IL21*), 6q15 (*BACH2*), 6q25.3 (*TAGAP*), 1q24.3 (*FASLG*, *TNFSF18*, and *TNFSF4*), 6q22.31 (*NKAIN2*), 10p15.1 (*PFKFB3* and *PRKCQ*), and 17q21.32 (*HOXB9*) [88]. Genome-wide gene expression studies have also highlighted similar biomarkers, including *APOC3*, *CYP3A4*, *OCLN*, *MAD2L1*, *MKI67*, *CXCL11*, and *IL17A* [89].

Celiac disease is characterized by an abnormal mucosal immune response to gliadin fractions, resulting in the infiltration of the lamina propria and epithelium by chronic inflammatory cells and villous atrophy [90]. Regarding adaptive immune response, key factors are peptide 56-89 (α-gliadin) that is resistant to gastrointestinal peptidases [91,92], tissue transglutaminase, and gliadin-reactive T cells. In active and gluten-sensitive celiac disease, the number of intraepithelial lymphocytes increases, express interferon gamma and IL-10 [93]. The gammadelta T-cell receptor ($\gamma\delta$TCR) is also found to be increased in intraepithelial lymphocytes [94] in addition to the common alfa-beta T-cell receptor ($\alpha\beta$TCR); in case of refractive celiac disease, intraepithelial lymphocytes may have an aberrant phenotype and restricted gene rearrangement [95,96]. In the serum of patients with celiac disease, several antibodies are found, including anti-gliadin antibodies (anti-AGA), anti-deamidated gliadin peptide antibodies (anti-DGP); anti-transglutaminase 2 antibodies (anti-TG2), anti-R1-type reticulin antibodies (anti-ARA), and anti-endomysial antibodies (anti-EMA) [97]. Gluten peptides also activate innate immune responses such as IL-15, intraepithelial lymphocytes, type 1 interferon (gamma), macrophages, monocytes, dendritic cells, and dysbiosis [98]. A summary of the pathogenesis of celiac disease has been presented in our previous publications [77,78].

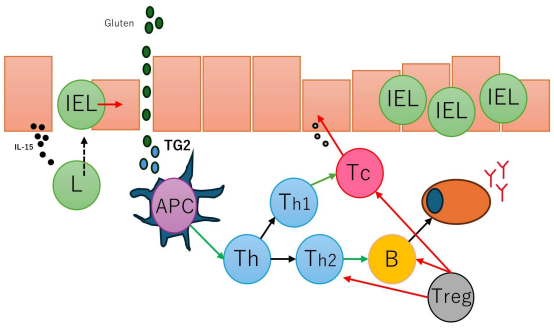

Figure 2. Simplified pathogenesis of celiac disease. The pathogenesis of celiac disease is multifactorial and involves several factors, including dietary gluten (gliadin), genetics (HLA-DQ2/8 and other metabolic and immune-related non-HLA regions), environmental (dysbiosis, smoking), and immune factors. The immune factors include gluten-specific T-cell responses, autoantibody generation, cytokine generation, cytotoxic transformation of IELs, and innate immune activation with epithelial stress [77].

1.4. LAIR1

Leukocyte-associated immunoglobulin like receptor 1 (LAIR1), also known as CD305, is an immune-inhibitory receptor found on mature hematopoietic cells, particularly on immune cells such as mononuclear cells, natural killer cells, and T and B-lymphocytes [99]. The gene is located in the 19q13.4 region and is known as the leukocyte receptor cluster, which contains several genes that encode leukocyte receptors of the immunoglobulin superfamily. The structure of LAIR1 is shown in Figure 3. It is a type I glycoprotein comprising 287 ammino acids belonging to the family IR [100]. In

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myeloid leukemia, LAIR1 induces cell death, inhibits cytokine release and the activation of the NFKB pathway.

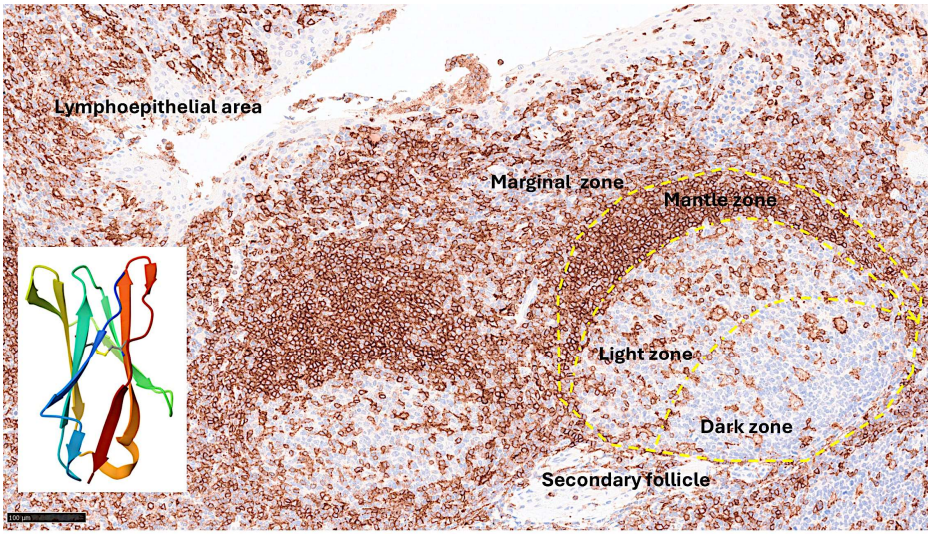

Figure 3. LAIR1 expression in reactive tonsils and crystal structure of human LAIR1. LAIR1 is expressed in both follicles and interfollicular areas of reactive tonsils. In the secondary follicles, the pattern is characteristic of macrophage/dendritic cells in the germinal center, and of naïve B-lymphocytes in the mantle zone. In the interfollicular area, the pattern is compatible with dendritic cells, including in the lymphoepithelial area. Occasional intraepithelial lymphocytes are also positive. The crystal structure of human LAIR1 in the C2 space group is also shown; experimental data using the X-ray diffraction method (website: https://www.rcsb.org/structure/3RP1; accessed on March 25, 2025).

1.5. Aim of the Study

This study aimed to analyze the phenotype of intraepithelial lymphocytes (IELs) and the lamina propria in the small intestine, including LAIR1; and to confirm the LAIR1 expression in celiac disease.

2. Materials and Methods

2.1. Patients and Sample

This study used several biopsies of small intestine control (n = 18) and 16 cases of celiac disease (number of biopsies n = 57). The celiac cases were selected from the Department of Pathology of Hospital Clinic Barcelona, Spain, as described in our previous publications [77,78]. The cases had been diagnosed as celiac disease following the conventional diagnosis, with clinical criteria, positive celiac serology, and histological criteria, including the presence of increased intraepithelial lymphocytes with crypt hyperplasia (Marsh type 2) or villous atrophy (Marsh type 3). The detailed data are presented in Appendix A, Table A1.

The study was conducted according to the principles of the Declaration of Helsinki for human experimentation. This study was approved by the Ethical Committee of Tokai University (IRB14R-080 and IRB20-156).

2.2. Immunohistochemistry

Several immunohistochemical markers were analyzed in the tissue samples using a Leica Bond Max automated stainer according to the manufacturer's instructions. The primary antibodies that were used were the following: CD3 (clone LN10, Leica Biosystems, Leica K.K., Tokyo, Japan), CD4 (4B12, Leica), CD8 (4B11, Leica), CD103 (EP206, Leica), granzyme B (11F1, Leica), TCRβ (TRBC1/TCRβ constant region 1 (E6Z3S) Rabbit mAb #79485, Cell Signaling Technology K.K., Tokyo,

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Japan), TCRδ (TRDC/TCRδ (E2E9T) XP® Rabbit mAb #55750, Cell Signaling), CD56 (CD56-504-L-CE, Leica), CD16 (CD16-L-CE, Leica), LAIR1 (CD305, JAVI82A, created by Giovanna Roncador, Spanish National Cancer Research Center (CNIO)), PD-L1 (73-10, Leica), PD1 (CD279, NAT105, CNIO), BTLA (CD272, FLO67B, CNIO), TOX2 (TOM924D, CNIO), HVEM (TNFRSF14, ab47677), CD163 (CD163-L-CE, Leica), HLA-DP-DQ (JS76, CNIO), IL4I1 (BALI265E,543H,573B, CNIO), and FOXP3 (236A, CNIO). The primary antibody details are presented in Table 1.

Table 1. Details of primary antibodies.

Antibody	Target/pathway	Source	Details
CD3	T-lymphocytes	Leica	Mouse monoclonal, clone LN10, IgG1, C-terminal region
CD4	Helper T-lymphocytes (+antigen presenting cells)	Leica	Mouse monoclonal, clone 4B12, IgG1, external domain
CD8	Cytotoxic T-lymphocytes	Leica	Mouse monoclonal, clone 4B11, IgG2b, alpha chain cytoplasmic portion
CD103	Alpha E integrin & human mucosal lymphocyte antigen 1 (ITGAE), intraepithelial T lymphocytes, FOXP3+Tregs, CD4+ and CD8+Tcells, dendritic cells, and mast cells in mucosal tissues. Interacts with E-cadherin (epithelial cells)	Leica	Rabbit monoclonal, clone EP206, IgG, residues of human CD103/ITGAE protein
Granzyme B	Lytic granules of cytotoxic-T lymphocytes (CTL) and in natural killer (NK) cells	Leica	Mouse monoclonal, clone 11F1, IgG2a, N-terminus of the mature granzyme B molecule
TCRβ	T-cell receptor	CST	Rabbit IgG, residues near the amino terminus of human TRBC1/TCRβ constant region 1 protein
TCRδ	T-cell receptor	CST	Rabbit IgG, total TRDC/TCRδ protein
CD56 (NCAM)	Neurons, astrocytes, Schwann cells, NK cells and a subset of activated T-lymphocytes	Leica	Mouse monoclonal, clone CD564, IgG2b, extracellular domain
CD16	NK cells, granulocytes, activated macrophages and subset T cells (TCRαβ and TCRγδ)	Leica	Mouse monoclonal, clone 2H7, IgG2a, external domain (both transmembrane and GPI-linked forms)
LAIR1 (CD305)	Co-inhibitory receptor	CNIO	Rat monoclonal, clone JAVI82A, IgG2a, k
PD-L1	Immune suppression and inhibition of T-cell activity	Leica	Rabbit IgG, clone 73-10, C-terminal domain
PD1 (CD279)	Co-inhibitory receptor	CNIO	Mouse monoclonal, clone NAT105, IgG1
BTLA (CD272)	Co-inhibitory receptor	CNIO	Mouse monoclonal, clone FLO67B, IgG1
TOX2	Transcription factor, maturation of NK cells and differentiation of T follicular helper (TFH) cells	CNIO	Rat monoclonal, clone TOM924D, IgG2a

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HVEM (TNFRSF14)	Ligand of BTLA	Abcam	Rabbit polyclonal, IgG, exact immunogen is proprietary information
CD163	M2-like macrophages	Leica	Mouse monoclonal, clone 10D6, IgG1, N-terminal region
HLA-DP-DQ	Antigen presentation by APC	CNIO	Mouse monoclonal, clone JS76, IgG2a
IL4I1	APC, T-cell inhibition	CNIO	Rat monoclonal, clone BALI265E,543H,573B, IgG2a
FOXP3	Regulatory T-lymphocytes (Tregs)	CNIO	Mouse monoclonal, clone 236A, IgG1

APC, antigen-presenting cells (B-lymphocytes, dendritic cells, and macrophages); CST, Cell Signaling Technology; Leica, Leica Biosystems K.K.; NK, natural killer cells; Spanish National Cancer Research Center (CNIO);.

Confocal microscopy was performed as previously described [101] using a Fluoview FV3000 confocal laser scanning microscope (Olympus K.K, Hachioji, Japan) with Alexa Fluor 488 and 594 and DAPI dyes.

2.3. Image Classification

Image classification based on LAIR1 immunohistochemical expression was performed using transfer learning and the ResNet18 deep learning model. The images were split into images patches of 224x224x3. The image patches were pooled into 3 different folders, and the data were split into 3 sets: training set (70%) for training the network, validation set (10%) for testing its performance during training, and test set (20%) used after training to assess how well the network performed on new data. Grad-CAM analysis was used as a method of explainable AI to visualize which areas of the input image were most important for the model prediction and image classification. All the methodology was performed as previously described in our previous publications [78,102,103].

3. Results

3.1. Immunophenotype of Intraepithelial Lymphocytes (IELs) in Intestinal Mucosa Control

IELs were defined as lymphoid cells within the epithelial layer of the mucosa. CD3 staining was used as a reference. In physiological conditions, IELs are characterized by T-cell phenotype that is positive for CD3 and a cytotoxic phenotype with CD8 expression. Occasionally, cytotoxic granules were identified by granzyme B staining. Most IELs were positive for CD103/ITGAE. Most IELs expressed TCRβ chains; therefore, expressed the TCR$\alpha\beta$ chains. Occasionally, CD56+IELs were found, as well as TCRδ chain-positive IELs (i.e., TCR$\gamma\delta$+IELs). All IELs were diffusely and strongly positive for LAIR1. Of note, occasional IELs were TOX2-positive. In the lamina propria, abundant CD163+macrophages/dendritic cells were found, which also expressed HLA-DP-DQ. CD4+cells were mainly found in the lamina propria; however, clusters attached below the epithelial basal membrane were found. BTLA+cells were found in the lamina propria, same result as we have previously described [77]. PD-L1 expression was limited to the lamina propria in a pattern compatible with APC (macrophages, dendritic cells). Regulatory T-lymphocytes were identified in the lamina propria using the FOXP3 marker. The characteristic images are shown in Figure 4-7. Figure 8 shows the confirmation that the IELs are CD3 and LAIR1 double-positive using confocal microscopy. Of note, LAIR1 staining revealed that many cells of the lamina propria were LAIR1-positive (Figures 4-8).

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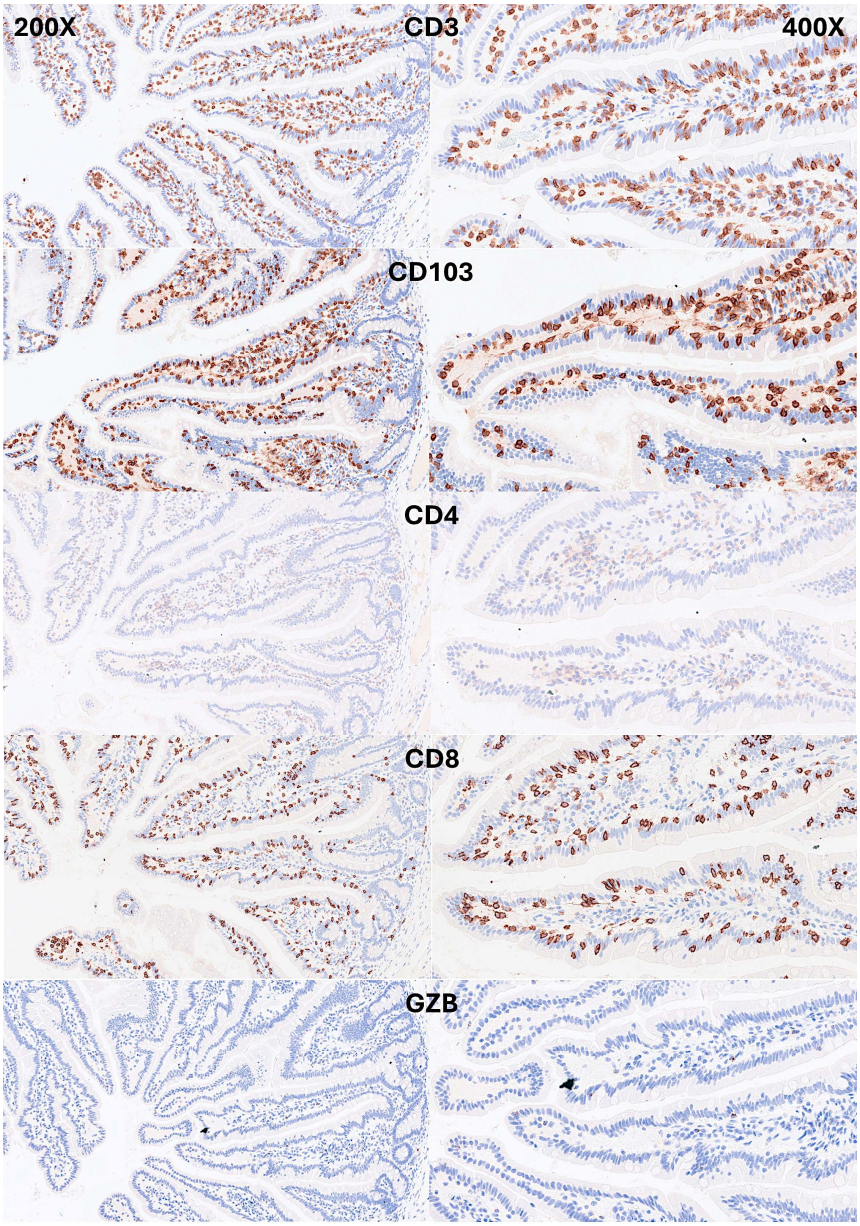

Figure 4. Immunophenotype characterization of IELs in intestinal mucosa control. Most IELs were CD3, CD103, and CD8-positive. CD4-positive cells were mainly found in the lamina propria.

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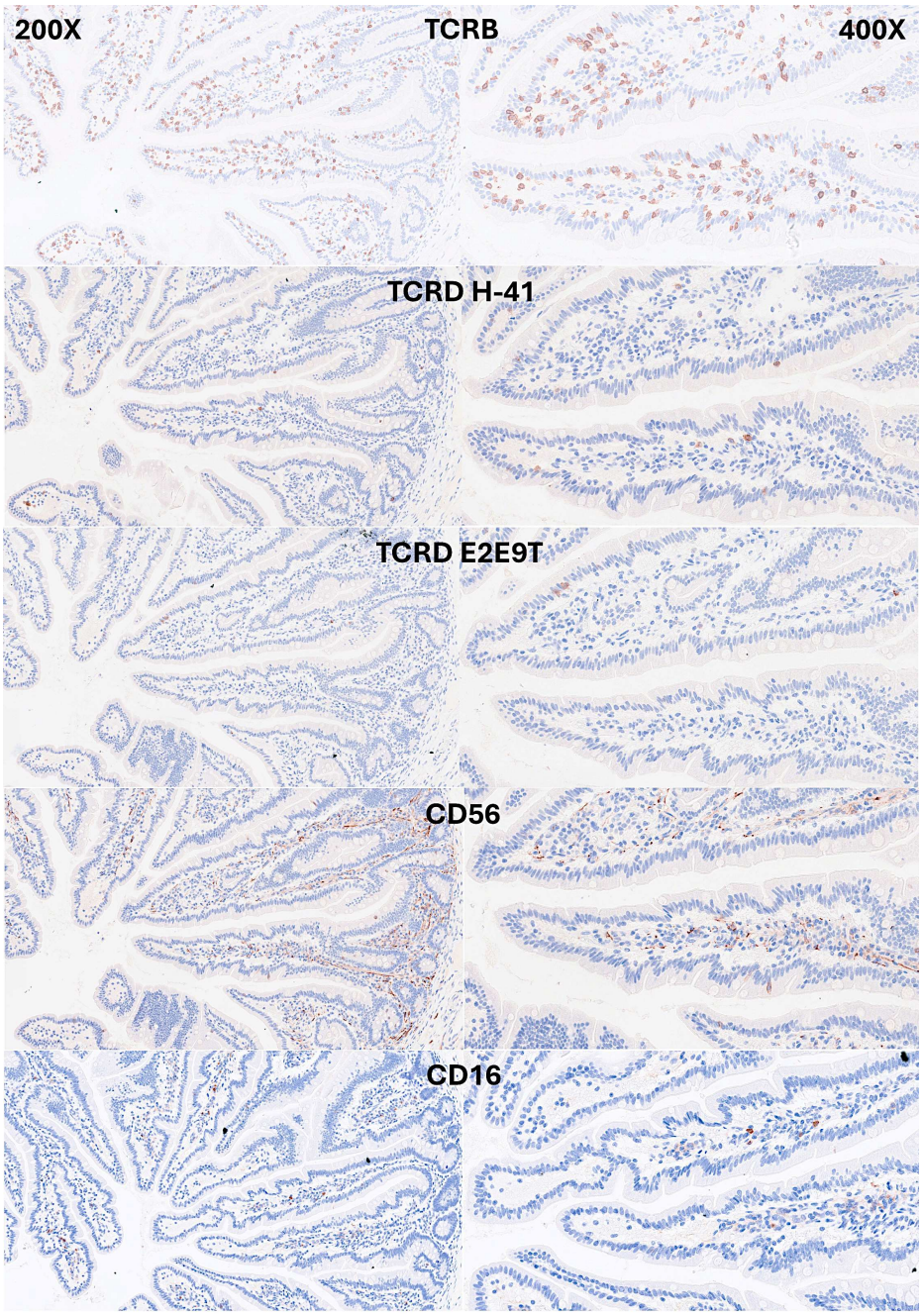

Figure 5. Immunophenotype characterization of IELs in intestinal mucosa control. Most IELs expressed TCRβ chains; therefore, expressed the TCRαβ chains. Occasionally, CD56+IELs were found, as well as TCRδ chain-positive IELs (i.e., TCRγδ+IELs).

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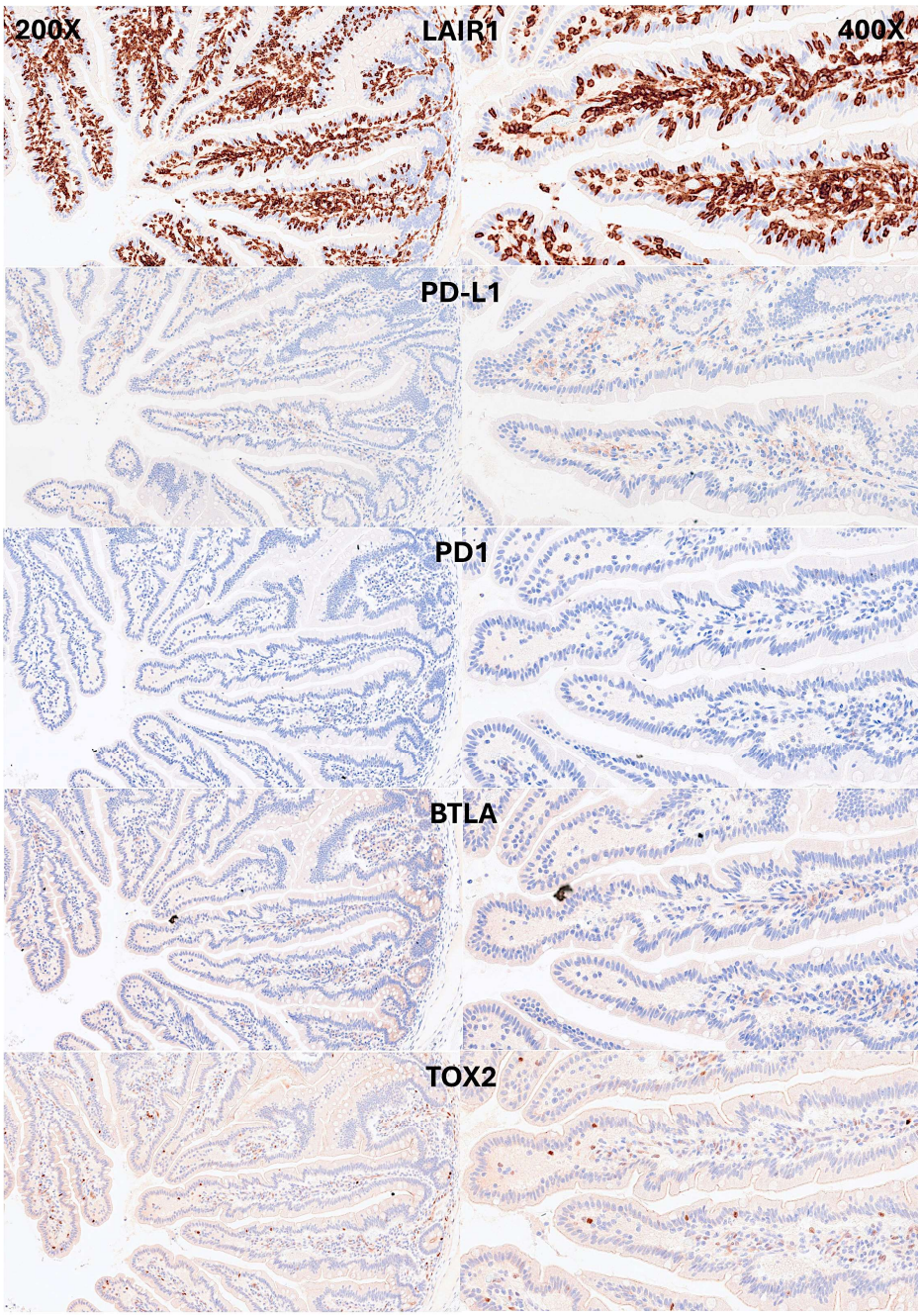

Figure 6. Immunophenotype characterization of IELs in the intestinal mucosa control. IELs were diffusely and strongly positive for LAIR1. LAIR1 also marked the inflammatory infiltrate of the lamina propria. PD-L1 and BTLA expression was limited in the lamina propria. Occasional PD1-positive cells were identified. TOX2+IELs were occasionally found.

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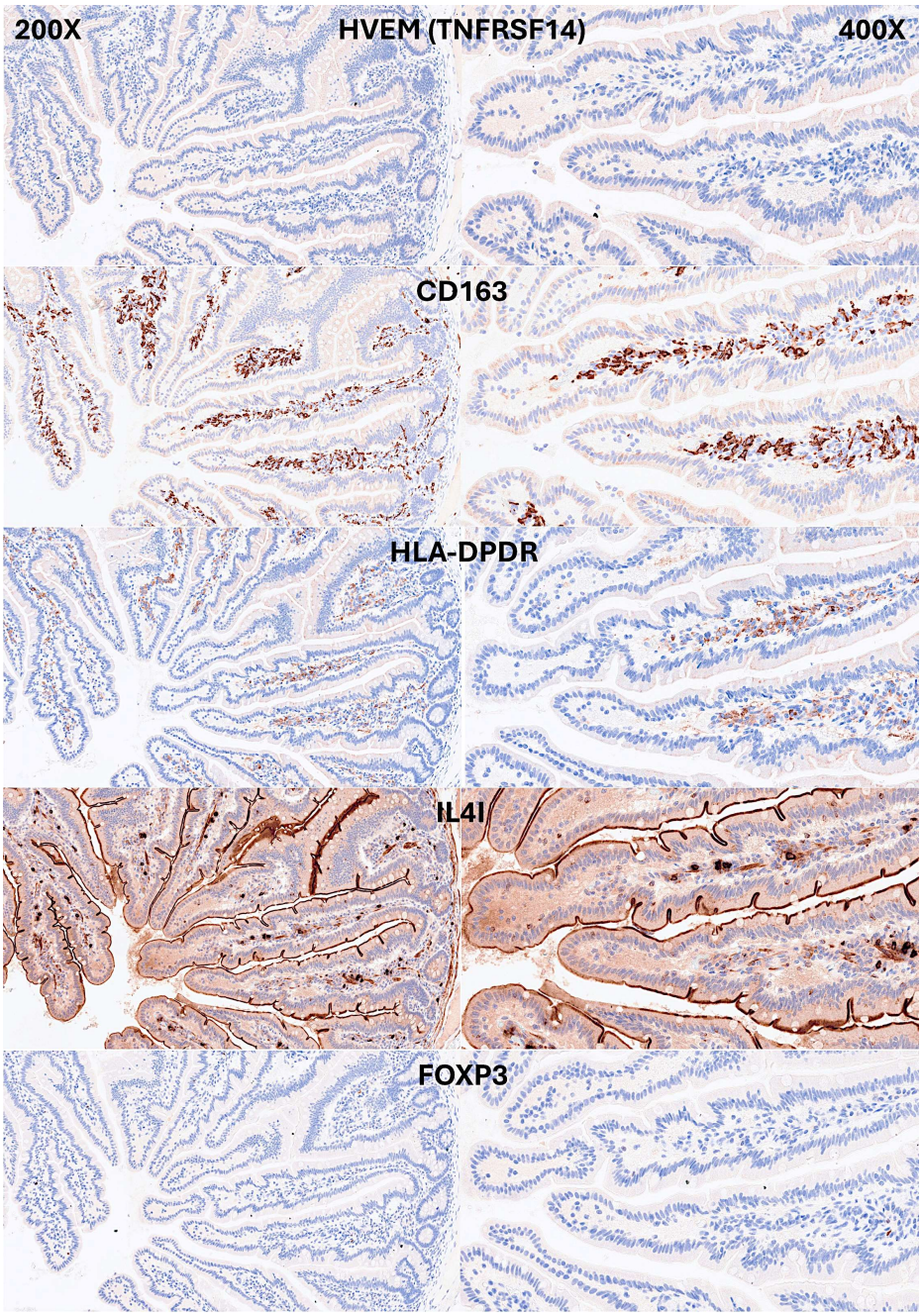

Figure 7. Immunophenotype characterization of IELs in intestinal mucosa control. CD163 and HLA-DPDR identified antigen-presenting cells (APCs), mainly macrophages and dendritic cells in the lamina propria. Few FOXP3+Tregs were identified in the lamina propria.

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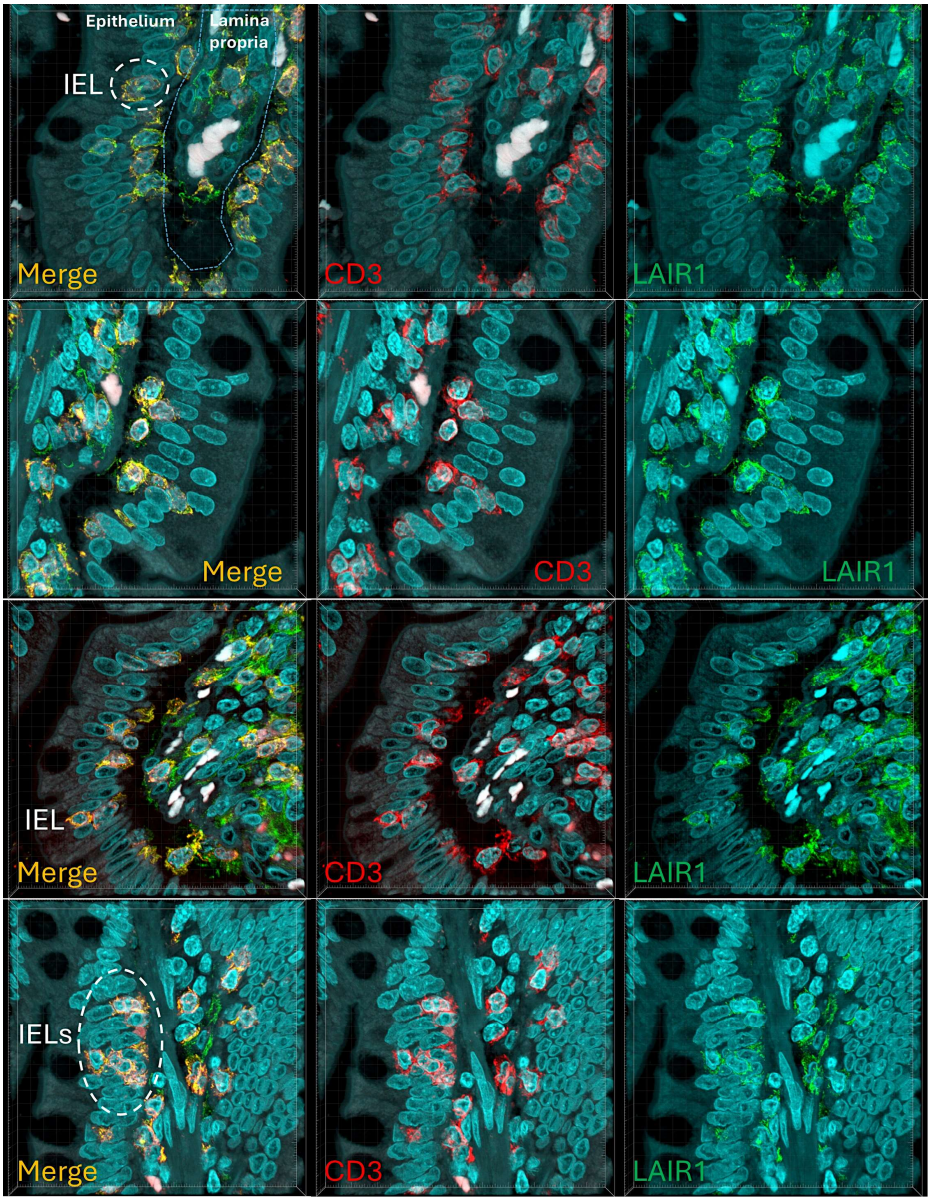

Figure 8. Confocal microscopy showing double immunofluorescence between CD3 (red) and LAIR1(green) in the small intestine control. The IELs were double CD3 and LAIR1-positive.

3.2. Multicolor Analysis of LAIR1 and Other Immune Markers

Quadruple and triple immunofluorescence analyses were performed using confocal microscopy. The combinations that also included nuclear staining were as follows: PD1 (Cyan), CD163 (green), and LAIR1 (red); and CD4 (green), CD8 (cyan), and LAIR1 (red).

The results showed that in the human mucosa, CD4- and probably CD8-positive cells were positive for LAIR1. PD1-positive cells also expressed LAIR1. In the interfollicular area and/or lamina propria, CD163-positive cells (M2-like macrophages) were partially positive for LAIR1 (Appendix B, Figure A1).

3.3. Analysis of LAIR1 Expression in Celiac Disease

The expression of LAIR1 was evaluated in the celiac disease biopsies. LAIR1 was expressed in the IELs, and the distribution of LAIR1+IELs was heterogeneous within and between biopsies. The

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expression of LAIR1 in the lamina propria was also variable ranging from low (1/16, 6.25%), intermediate (6/16, 37.5%), and high (9/16, 56.25%).

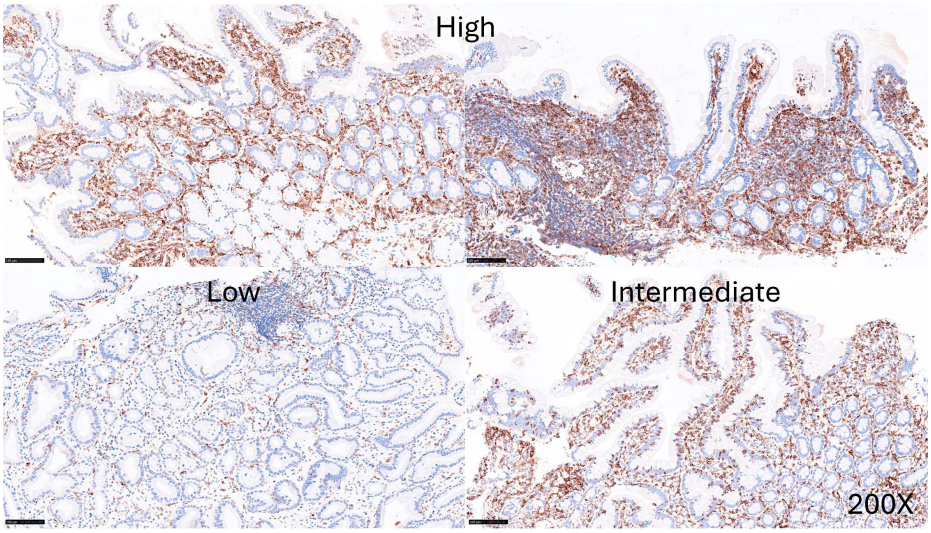

Figure 9. LAIR1 expression in celiac disease. The expression of LAIR1 in the lamina propria was also variable ranging from low (1/16, 6.25%), intermediate (6/16, 37.5%), and high (9/16, 56.25%).

3.4. Image Classification of Celiac Disease, Small Intestine Control, and Reactive Tonsil Control Based on LAIR1 Immunohistochemical Expression

Images of LAIR1 protein expression analyzed by immunohistochemistry in celiac disease, small intestine control, and reactive tonsils were used as input data in a ResNet18 model.

The ResNet18 model comprises 18 layers, including convolutional layers and residual blocks. The series included 11,367 image patches of celiac disease, 11,630 patches of small intestine control, and 8147 patches of reactive tonsil control. The image patches were pooled into 3 different folders, and the data were split into 3 sets: training set (70%) for training the network, validation set (10%) for testing its performance during training, and test set (20%) used after training to assess how well the network performed on new data.

After 5 epochs in the training progress, the validation accuracy was 99.5%. After image patch classification using the test (holdout) series, the accuracy was 99.6%. The confusion matrix shows the distribution of image patches, including correctly classified and misclassified patches. The Grad-CAM technique was used to understand why the deep learning network made its classification decisions in incorrectly classified cases.

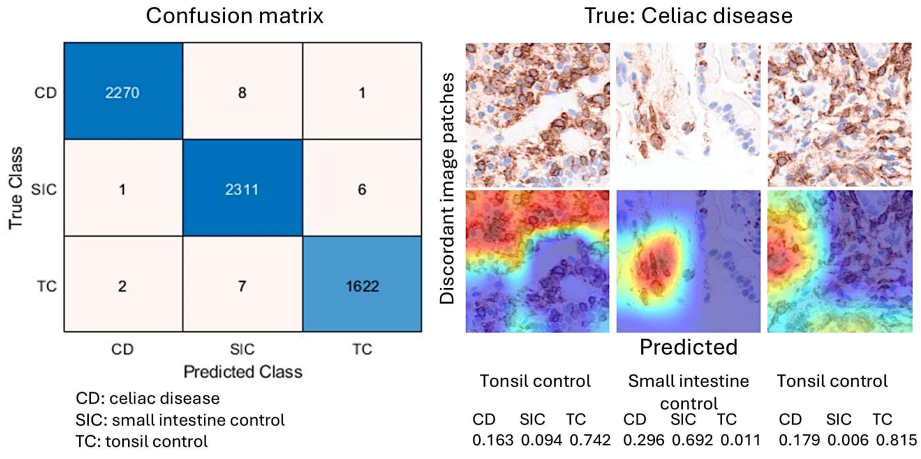

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Figure 10. Image classification using LAIR1 image patches. A ResNet18 model was created to classify histochemical images of celiac disease (CD), small intestine control (SIC), and reactive tonsil control (TC). The image classification results in the test set are summarized in a confusion matrix.

4. Discussion

Celiac disease is a gluten-sensitive enteropathy and common immune-mediated inflammatory condition of the small intestine caused by sensitivity to dietary gluten and related proteins in genetically predisposed individuals [104]. In western countries, celiac disease is estimated to affect approximately 1% of the population. Celiac disease clinically presents heterogeneous; therefore, it continues to be underestimated [104].

There are several phenotypes of celiac disease. Symptomatic disease includes classic and nonclassic celiac diseases. The classic celiac disease is a gluten-sensitive enteropathy characterized by diarrhea, malabsorption signs and symptoms, villous atrophy, and resolution upon withdrawal from gluten-containing foods [79]. Nonclassic celiac disease is also known as “atypical” and the patients lack the classic symptoms of malabsorption and only present with minor gastrointestinal complaints. However, duodenal biopsies show villous atrophy, the production of celiac autoantibodies such as antitissue transglutaminase, and extraintestinal manifestations are present [105,106].

The other phenotypes are subclinical or asymptomatic, potential, latent, and refractory celiac disease. Refractory celiac disease is defined the persistence of clinical symptoms and villous atrophy despite a gluten-free diet. Of note, failure to improve on a gluten-free diet is mostly due to non-compliance. However, in few cases, a pure refractory condition is found: refractory celiac disease type 1 (normal population of IELs), the semi-malignant inflammatory condition (refractory type 2; aberrant immunophenotype and T-cell receptor clonality analysis of IELs), transformation to enteropathy-associated T-cell lymphoma (EATL), collagenous sprue, or alternative diagnosis of autoimmune enteropathy [107–109].

The cause of refractory disease is unknown, and the treatment has focused on immunosuppression. Traditional glucocorticoids such as intravenous hydrocortisone and oral prednisolone. Alternative immunosuppressant therapy includes azathioprine, 6-mercaptopurine, and thioguanine [107,110–113]. A monoclonal antibody therapy using anti-CD52 (alemtuzumab) was reported [114].

Badran YR et al. reported eight cases of immune checkpoint inhibitor-associated celiac disease, suggesting that the drugs disrupted the mechanism of gut immune homeostasis and tolerance mechanism [115]. In that study, immunohistochemical analysis of several markers were CD3, CD8, TCRγδ, PD1, CD68, and PD-L1, and quantification of IELs [115]. In our study, we analyzed several immuno-oncology markers in small intestine control, and later LAIR1 expression in celiac disease. Our findings showed that in the small intestine, LAIR1 expression is found in IELs but also in immune cells of the lamina propria. In celiac disease, LAIR1 was diffusely expressed.

LAIR1 belongs to the family of immune-inhibitory receptors and is expressed by mature hematopoietic cells, particularly in the immune cells of natural killer (NK) and T/B-lymphocytes [114]. Beyond the physiological function of immune homeostasis and immune tolerance, LAIR1 has been involved in several autoimmune and inflammatory conditions and neoplasia [99]: allergy [116], systemic lupus erythematosus [117], rheumatoid arthritis [118–120], graft rejection [121], breast carcinoma [122], glioma [123], solid tumors [124], hepatocellular carcinoma [125], among others.

We recently demonstrated the usefulness of using deep learning to analyze gene expression and classify images of celiac disease [77,78] and ulcerative colitis [102,103,126]. In this study, deep learning was used to classify LAIR1 image patches between celiac disease, small intestine control, and reactive tonsils. The proposed network managed to classify images with good performance. However, the aim was to conduct a proof-of-concept analysis, not to create a trained network for use in production or commercial applications. Narrow artificial intelligence is not ready to take over the job of pathology-trained medical doctors because histological biopsies obtained from endoscopic examinations may have other diseases. Of note, other research groups such as Denholm et al., Molder

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et al., Scheppach et al., and Schreiber et al. (among others), have successfully used deep learning in celiac disease as well [127–134].

5. Conclusions

This study used several immuno-oncology and immune-phenotype markers to characterize the intraepithelial lymphocytes and the lamina propria of small intestine control, and to confirm the expression of LAIR1 in celiac disease. This study also successfully performed a proof-of-concept deep learning histological analysis of LAIR1 between small intestine control, celiac disease, and reactive tonsils. Therefore, LAIR1 appears to be a potentially relevant marker in celiac disease.

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Author Contributions: N/A

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of TOKAI UNIVERSITY, SCHOOL OF MEDICINE (protocol code IRB14R-080 and IRB20-156).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are uploaded into Zenodo CERN OpenAIRE repository.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

IELs	Intraepithelial lymphocytes
EATL	Enteropathy-associated T-cell lymphoma
LAIR1	Leukocyte-associated immunoglobulin like receptor 1

Appendix A

Appendix A.1.

Table A1. Clinicopatholgical characteristics of celiac disease cases.

Age	Sex	Biopsy Location	Diagnosis	Marsh-Oberhuber Classification
70	Male	Duodenum	Celiac Disease	3a
62	Male	Pylorus/duodenum	Celiac Disease/Chronic gastritis	2
62	Male	Duodenum	Celiac Disease	2
78	Female	Duodenum	Celiac Disease	3b
59	Male	Duodenum	Celiac Disease	3a
44	Female	Duodenum	Celiac Disease	2
17	Female	Duodenum	Celiac Disease	3b
56	Female	Duodenum	Celiac Disease	3a

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54	Female	Duodenum	Celiac Disease	2
58	Female	Duodenum	Celiac Disease	3b
61	Female	Duodenum	Celiac Disease	3c
45	Male	Duodenum	Celiac Disease	3a
70	Female	Duodenum	Celiac Disease	2
40	Female	Duodenum	Celiac Disease	3a
61	Female	Duodenum	Celiac Disease	3c
44	Female	Duodenum	Celiac Disease	3a

Appendix B

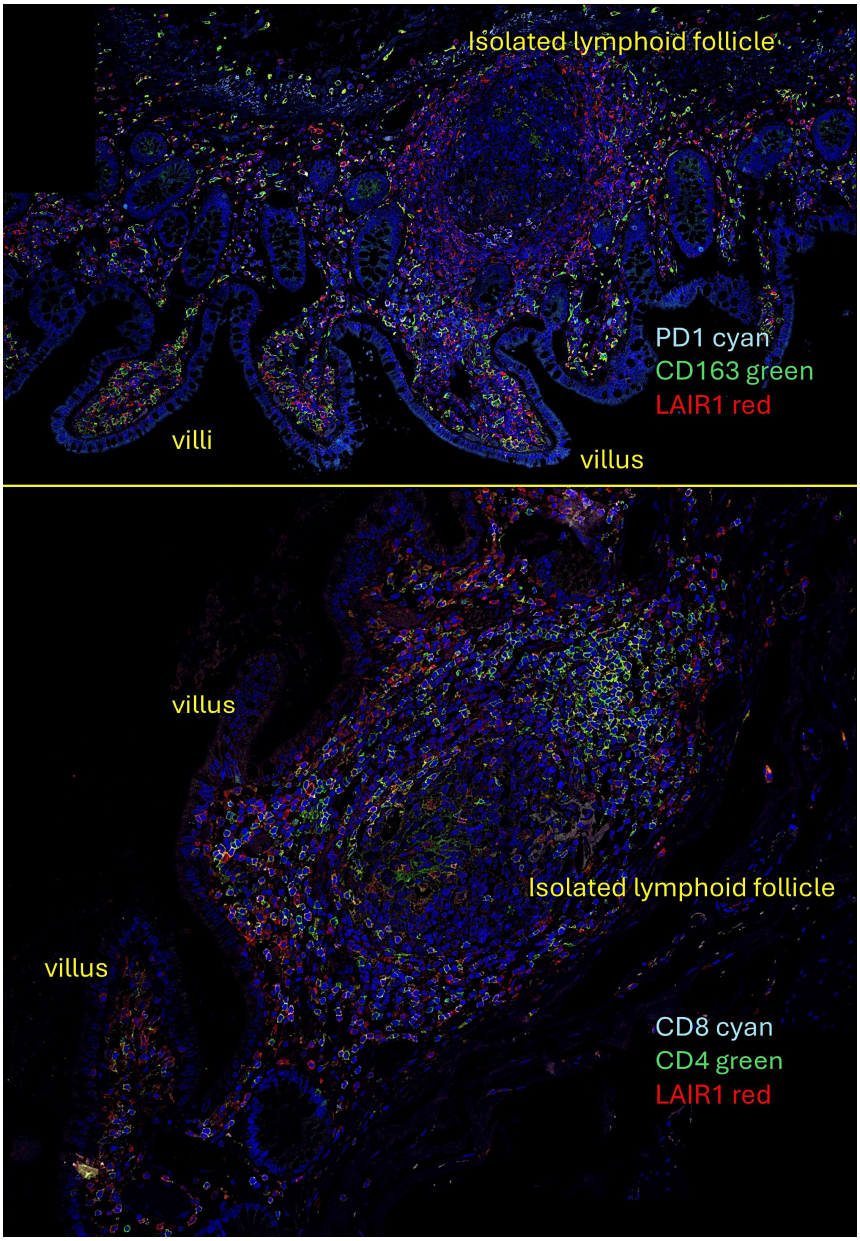

Figure A1. Multicolor immunofluorescence. Multicolor immunofluorescence of LAIR1 in relationship with other immune markers in human intestinal control.

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