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Technical Note

Combined RNAscope and immunohistochemistry staining on duodenal paraffin sections as a new tool to reveal cytolytic potential of intraepithelial lymphocytes

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ABSTRACT

Immunohistochemistry (IHC) is a consolidated technique for the identification of surface and cytoplasmic antigens in cells or tissue sections using specific antibodies, yet simultaneous detection of two markers on the same cell may be difficult to achieve. Here we develop a protocol to perform a double staining using RNAscope, a new in-situ hybridization (ISH) technology, to visualize perforin transcripts, and classical IHC to visualize either CD8 or TcRγδ positive intraepithelial lymphocytes (IELs) in small intestinal paraffin sections of celiac disease (CD) patients. This double assay will allow to investigate the cytotoxic properties of two subsets of IELs in different stages of CD, thus contributing to understand the events leading to tissue destruction and healing.

1. Introduction

In 2012 Fay Wang and colleagues described RNAscope, a new in situ hybridization (ISH) technology with high sensitivity and specificity. RNAscope allows detection of single mRNA transcripts of a target gene with a double-Z strategy that enables amplification of the target signal without background. Visualization can be performed by fluorescence microscopy, if fluorescently labeled probes are used, or by bright-field microscopy when using alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated probes (Wang et al., 2012). This technique may be combined with classical immunohistochemistry (IHC); however, few successful protocols have been developed on the same cell type in the small intestinal mucosa (Maidji et al., 2017; Xu et al., 2022).

IHC is a consolidated technique for the identification of surface and cytoplasmic antigens in cells or tissue sections using specific antibodies. The antigen-antibody binding can be detected with enzymatic reaction between an enzyme like HRP or AP and a chromogenic substrate. Signals can then be visualized by bright-field microscopy. Another approach is to use antibodies labeled with fluorescent molecules that can be visualized by fluorescence microscopy (Magaki et al., 2019). IHC can be performed on formalin-fixed paraffin embedded (FFPE) or fresh frozen tissue sections. It is widely used to study the abundance and/or phenotype of distinct cell types in tissues and useful for diagnosis and risk assessment in the context of different diseases.

One of the medical conditions in which the use of IHC has been crucial for diagnostic purposes is celiac disease (CD), an immune-mediated disorder triggered in genetically susceptible individuals by gluten ingestion. CD is typically characterized by a small intestinal enteropathy featuring increased intraepithelial lymphocytes (IELs) counts, crypts hyperplasia and villus atrophy. The recruitment of IELs is a key feature of the duodenal pathology of CD patients and involves two distinct populations: T cell receptor (TcR) αβ+CD8αβ+ and TcRγδ+ IELs. Activated TcRαβ+ IELs are thought to be responsible for mediating the killing of epithelial cells in an antigen independent fashion, leading to the mucosal damage observed in active CD (Abadie et al., 2012).

While disruption of the epithelial layer is the consequence of the cytolytic activation of TcRαβ+ IELs, the role of TcRγδ IELs is still unclear. The increase in TcRγδ IELs in the small intestinal epithelium is specific of CD, in fact it has not been described in other intestinal diseases, and it does not fully revert upon mucosal healing resulting from

Abbreviations: AMP, amplifiers; AP, alkaline phosphatase; CD, celiac disease; CD8, cluster differentiation 8; FFPE, formalin-fixed paraffin embedded; HRP, horseradish peroxidase; HybEZ oven, hybridization oven; IELs, intraepithelial lymphocytes; IHC, immunohistochemistry; ISH, in situ hybridization; NKRs, natural killer receptors; TcR, T cell receptor.

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gluten withdrawal (Mandile et al., 2022). Altogether these evidences suggest those cells play a key role in CD pathogenesis. Recently, it has been shown that Vδ1+TcRγδ+ IELs from healthy controls express high levels of the cytolytic molecule granzyme and more frequently degranulate compared to those from untreated CD patients, which instead produce IFN-γ (Mayassi et al., 2019), suggesting they may contribute to tissue destruction.

On another hand, in the context of full-blown CD, TcRαβ+ IELs express high levels of activating natural killer receptors (NKRs) including NKG2D and CD94+NKG2C+ (Setty et al., 2015) and display cytolytic molecules, including perforin (Bhagat et al., 2008), and granzyme. Their increased expression might be triggered by IL-15 and IL-21, two cytokines significantly increased in CD mucosa (Ciszewski et al., 2020).

In summary, depending on the stage of disease, both IELs populations may exert cytolytic properties. To investigate the cytolytic potential of distinct IELs subsets, we aimed at establishing a method to visualize perforin expression, one of the key cytolytic molecules, in both TcRαβ+CD8αβ+ and TcRγδ+ IELs. Since classical IHC with more than one marker is often troubled by the availability of two or more antibodies obtained in different species for simultaneous detection to avoid cross-reactivity (Tan et al., 2020; Gown, 2016), and by the difficulty to easily distinguish cells positive for two markers, we decided to develop a combined IHC-RNAscope protocol to visualize two markers (perforin and one between CD8 or TcRγδ) on the same cell in the small intestinal biopsies of CD patients.

2. Materials and methods

2.1. Patients

We enrolled eight CD patients (6 females and 2 males; mean age: 10 years, range: 2–18) at the Department of Pediatrics, University Federico II, Naples, Italy. All enrolled patients underwent gastrointestinal endoscopy for suspected CD, based on increased levels of circulating CD-specific antibodies and duodenal biopsies were obtained to confirm diagnosis. The study was approved by the local Ethical Committee of the University Federico II (C.E. n. 179/19). A written informed consent was obtained from all patients and their parents. Since non-CD individuals display a low density of IELs, only biopsies from CD patients were studied to set-up the experimental protocol. Duodenal biopsies (four per patient) were collected in 0,9% saline solution in the endoscopy room, then fixed in 10% formalin for at least 24 h, dehydrated using ethanol, treated with xylene, embedded in paraffin and stored at room temperature.

2.2. Combined RNAscope and immunohistochemistry protocol

Classic IHC performed to highlight CD8+ and TcRγδ+ IELs was combined to perforin staining using the RNAscope 2.5 HD Reagent Kit-RED. The RNAscope kit and probes were purchased from Advanced Cell Diagnostics, Inc., California. The whole protocol can be carried out over three days (Fig. 1). It is crucial to ensure that tissue sections are entirely covered by reagents at each step.

2.2.1. Tissue preparation – Day 1

5 μm FFPE duodenal sections were cut using a microtome (Leica RM2125RT; Leica Microsystems, Nussloch GmbH Germany) and were incubated at 37° in a water-bath before layering them on glasses pretreated with 3-Triethoxysilyl-propylamine to prevent tissue folding. The slides were baked at 60° in a dry oven for 1 h, and deparaffinized according to manufacturer instructions.

2.2.2. Peroxidase blocking – Day 1

To block endogenous peroxidase activity, deparaffinized sections were incubated in Hydrogen Peroxide solution, provided in the kit according to the protocol.

2.2.3. Antigen retrieval – Day 1

Antigen retrieval treatment allows exposure of antigenic sites and is required for FFPE-sections. Sections were incubated for 30 min in boiling (>99°) target retrieval solution (1×) provided by the kit, rinsed with distilled water, and transferred in 100% alcohol for 3 min. To perform antigen retrieval treatment, we used a beaker covered with a aluminum foil to avoid solution evaporation and placed it on a hot plate. Sections were allowed to dry for a few minutes before being outlined with Pap Pen that creates a hydrophobic barrier around the tissue. The slides were left at room temperature until the next day.

2.2.4. Exposure of antigenic sites – Day 2

In order to better expose antigenic sites, sections were incubated for 15 min with Protease Plus, provided by the kit, at 40° in the slide hybridization oven (HybEZ oven; Advanced Cell Diagnostics, Inc., California) using a slide rack and the humidity control tray, provided by the same company (Advanced Cell Diagnostics, Inc., California). The slides were then rinsed 3 times with distilled water.

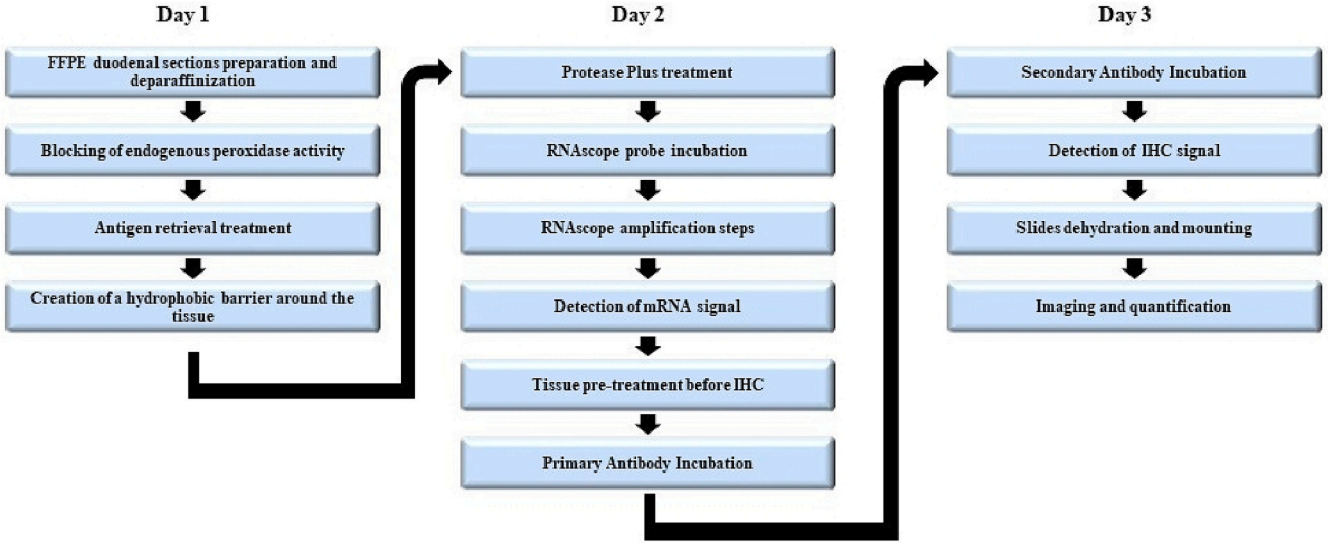

Fig. 1. Work-flow diagram for combined RNAscope and IHC. Flow chart including a step-by-step protocol for the RNAscope-IHC assay from day 1 to day 3.

2.2.5. RNAscope probe incubation – Day 2

The provided probes need to be pre-warmed before use. The kit included 3 probes: one specific for the detection of Perforin RNA plus a negative and positive control probes that targeted bacterial DapB gene and Peptidyl Prolyl isomerase B, a common housekeeping gene, respectively. The slides were incubated with each probe for 2 h at 40° in the HybEZ oven and washed twice with Wash Buffer provided in the kit for 2 min.

2.2.6. RNAscope amplification – Day 2

The probe signal was amplified using a multi-step process in which six amplifiers (AMP) were added to the sections sequentially. We followed manufacturer's instructions for all AMP steps but for AMP5. In fact, to have a better perforin signal, the slides were incubated with AMP5 for 1 h instead of 30 min.

2.2.7. RNA signal detection – Day 2

Sections were incubated with RED working solution for perforin signal detection according to manufacturer's instructions.

2.2.8. Blocking to avoid nonspecific binding – Day 2

Before proceeding with IHC, the slides were rinsed twice with TBS 0,15 M and incubated with 1% Bovine Serum Albumin (BSA, Sigma) in TBS 0,15 M for 15 min in the humidity control tray.

2.2.9. IHC primary antibody – Day 2

Once the solution had been removed, sections were washed twice with TBS 0,15 M and incubated overnight at room temperature with a primary antibody, either anti-TcRγδ (1:50, Santacruz Biotechnology, clone H-41) or anti-CD8 (1:50, Dako, clone C8/144B), in the humidity control tray.

2.2.10. IHC secondary antibody – Day 3

The slides were rinsed with TBS 0,15 M for 10 min and were incubated with the HRP-labeled Goat anti-Mouse secondary antibody (ImmunoReagents) for 45 min in the humidity control tray. The secondary antibody was the same for both primary antibodies. The last wash was carried out with TBS 0,05 M.

2.2.11. Detection – Day 3

DAB substrate kit (Vector Laboratories, Inc., Burlingame, CA) was used to detect the IHC signal. DAB substrate kit gave a brown signal, but a gray-black signal could be obtained if nickel solution was added. The substrate working solution was prepared according to manufacturer's instructions and was added to the sections. The detection time ranged from 2 to 10 min, and it could be determined by monitoring with a bright-field microscope. To stop the reaction, the slides were rinsed with tap water for 5 min.

2.2.12. Slides dehydration and mounting – Day 3

The slides were incubated at 60° in a dry oven for 15 min to dehydrate. Note that, dehydration cannot be performed in alcohol because the RED substrate is alcohol sensitive. After dehydration, slides were briefly put into xylene and mounted with EcoMount mounting media provided by the RNAscope kit.

2.3. Imaging

The double-stained slides were observed by an optical microscope (Axioskop, Zeiss, West Germany). Perforin staining was visualized as red dots in the cytoplasm of positive cells, while CD8+ and TcRγδ+ T cells as black rings around the nucleus.

2.4. Quantification

To count CD8+ and TcRγδ+ IELs a graduated scale was inserted into the microscope ocular and aligned to the basal membrane of duodenal epithelium. We evaluated the total number of CD8+ and TcRγδ+ IELs per millimeter of epithelium and, among those, the number of perforin positive cells to establish the percentage of double positive cells over the total number of each IELs subsets per millimeter of epithelium.

3. Results

To ensure RNAscope worked properly on human FFPE duodenal sections, we first standardized the protocol using the RNAscope assay to stain perforin alone, without combined IHC. RNAscope 2.5 HD Reagent Kit-RED for mRNA signal detection allows visualization of the target probes using Fast Red with alkaline phosphatase for chromogenic reaction. Perforin positive cells displayed small red dots within the cytoplasm (Fig. 2a). In our laboratory we routinely use DAB reaction for CD8+ and TcRγδ+ T cells detection, resulting in IELs visualized as brown rings around hematoxylin-stained blue nuclei (Fig. 2b and c). For a better contrast in the double staining, red was preferred for RNAscope.

An antigen retrieval treatment that allows to expose antigenic sites was required for both IHC and RNAscope protocols. During the early set-up stages this step was carried out twice, the first time during RNAscope tissue pretreatment step and the second before starting CD8 and TcRγδ incubation, however, duodenal mucosa was damaged by the double antigen retrieval. Thus, we tried to carry it out only during the RNAscope tissue pretreatment step with a great outcome and no tissue damage.

Another pitfall was to set up the amplification protocol. All the steps provided in the RNAscope protocol were followed as outlined by the manufacturer instruction, except for the 5th amplification step, which duration was increased from 30 min to 1 h, this improved perforin staining intensity.

The IHC protocol standardized in our laboratory was modified to extend the primary antibodies incubation time from 1 h and 30 min to overnight. To visualize CD8 and TcRγδ positive cells, we usually employ DAB as chromogenic substrate that gives a brown signal (Fig. 2b and c), however, to obtain a stronger contrast for double positive cells when staining along with red-labeled perforin, we added a nickel solution to the DAB, that resulted in a gray-black signal. On this basis, we preferred the combination perforin-red IELs-black, that provided a better contrast than red-perforin brown-IELs (Fig. 3) and decided not to contrast the nuclei with hematoxylin, since a three colors staining would worsen the visualization of double positive cells.

By improving the above-mentioned steps, we were able to fine-tune an optimal double RNAscope-IHC assay with a low-grade background and a clear and specific signal for simultaneous detection of perforin and T cells. The protocol for the double staining was carried out on FFPE duodenal biopsies from 8 CD patients obtaining a very clear staining.

4. Discussion and conclusions

We developed a protocol to perform a double staining using RNAscope to visualize mRNA transcripts and classical IHC to visualize proteins on human duodenal explants. This double staining allowed to visualize two markers on the same cell subset, namely perforin transcripts in CD8 and TcRγδ positive IELs.

RNAscope represents an evolution of ISH technique, that allows to detect even few copies of a target transcript by a double-Z strategy, that amplifies target signals without background. Because of its features, RNAscope is widely used for both research and diagnostics.

IHC is a consolidated technique based on the antigen-antibody binding reaction, used for the identification of several markers in cells or tissue sections for both diagnostic and research purposes (Sukswai and Khoury, 2019). IHC is routinely used in our laboratory for diagnostic purposes, but also to investigate immune pathways involved in CD pathogenesis. Several protocols were standardized for the identification of pro-inflammatory cytokines, such as IL-15, IL-21, IL-17, (Borrelli

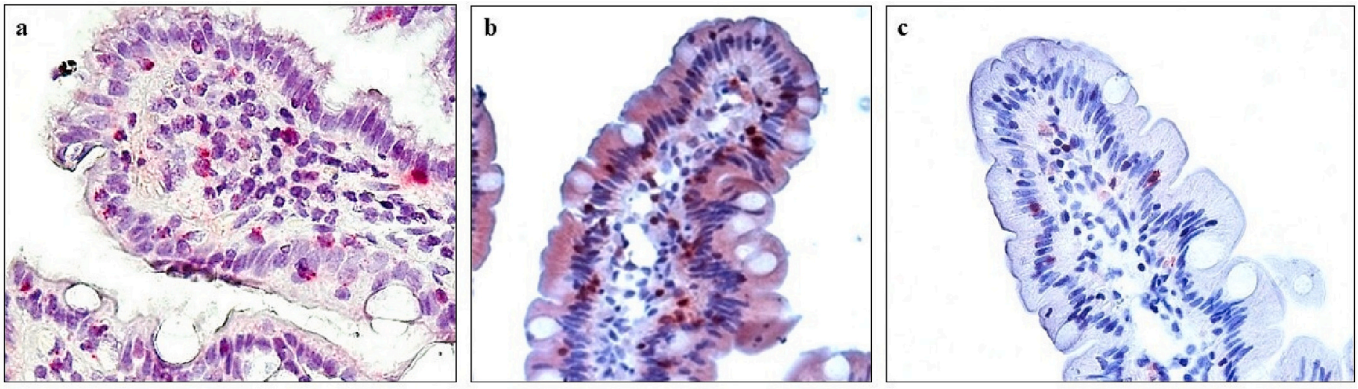

Fig. 2. Single staining of perforin and IELs in CD patients. These images represent duodenal mucosa of potential CD patients, that are characterized by positive CD serology but normal intestinal architecture. Perforin transcripts staining by RNAscope are visualized as small red dots within the cell cytoplasm and the nuclei are stained by hematoxylin. One representative image of 2 stained celiac patients. Each experiment was performed in technical triplicates (a). Classical IHC on FFPE sections for detection of CD8+ (b) and TcRγδ+ (c) IELs visualized as brown rings around hematoxylin-stained blue nuclei. Magnification 400×.

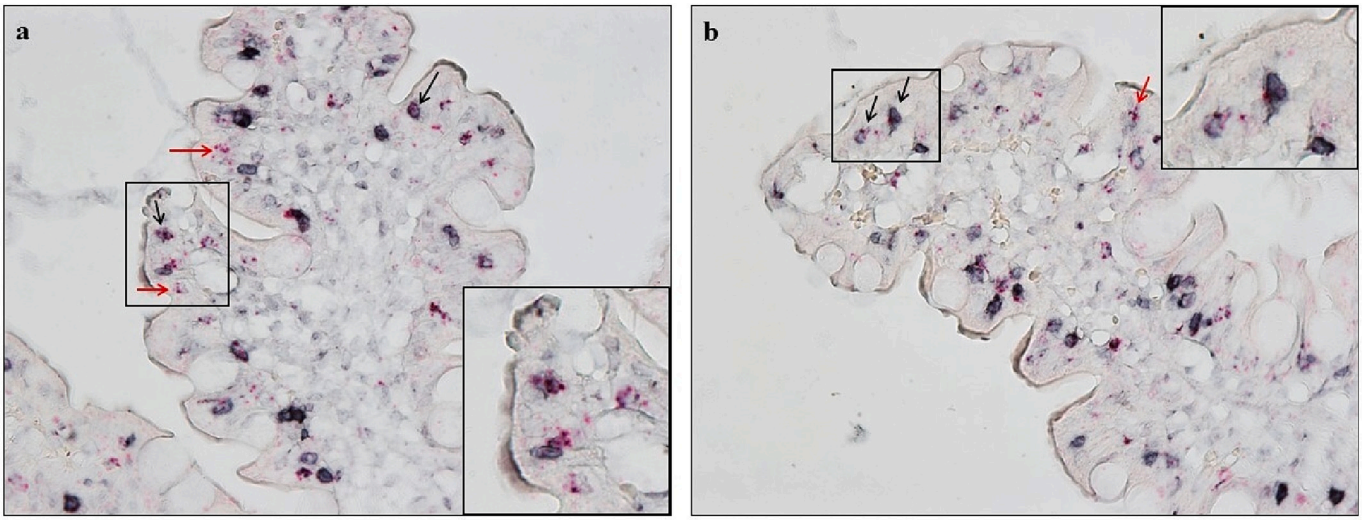

Fig. 3. Combined RNAscope and IHC to identify perforin positive IELs. Perforin positive TcRγδ+ (a) or CD8+ IELs (b) in the duodenal epithelium of potential CD patients are visualized as black round cells with red dots within the cytoplasm. In both images black arrows point at double positive cells and red arrows point at perforin single positive cells. Each image is from one celiac patient representative of 8 stained patients. Each experiment was performed in technical triplicates. Magnification 400×.

et al., 2016; Iacomino et al., 2016), as well as for surface markers used in diagnostics, such as CD3 and TcRγδ, for IELs counting (Tosco et al., 2015) and CD25 positive cells in the lamina propria compartment, representative of the inflammatory infiltration of the duodenal mucosa (Paparo et al., 2005).

Given our long-standing experience with the IHC technique, we intended to study the IELs cytotoxic properties in the context of CD by performing a double IHC-staining to visualize perforin positive IELs. However, sourcing primary antibodies obtained in different species and working on the same tissue can be problematic (Tan et al., 2020; Gown, 2016). Thus, to avoid cross-reactivity with non-target proteins and false-positive signals, we decided to combine two distinct techniques (RNAscope and IHC) for the detection of cytotoxic perforin-positive TcRγδ and CD8 T cells in duodenal epithelium of CD patients.

Other groups have combined RNAscope with IHC before, Grabinski and colleagues provided a protocol for combined RNAscope/IHC in rat brain tissue sections (Grabinski et al., 2015). They applied this technique to detect tau protein and βIII-tubulin in primary neuron cultures and to detect small proline-rich repeat 1a (Sprr1a) and tyrosine hydroxylase to visualize dopamine neurons on tissue sections. Their work showed that the combined approach is easily applicable in primary neurons and in tissue sections from rodent brain and can be used for gene-specific detection in a specific cell population or for simultaneous identification of gene and protein in the same cells.

To investigate the persistence of Parvovirus genomes in intestinal samples after acute infection, Xu M et al. used the double assay for the simultaneous identification of Human Parvovirus B19V nucleic acids and cellular markers, such as CD31, on intestinal sections providing a demonstration of B19V persistence in B lymphocytes of lymphoid follicles and in vascular endothelial cells of blood vessel (Xu et al., 2022).

On this basis, we developed a double RNAscope-IHC assay on paraffinized human duodenal mucosa samples from CD patients visualizing perforin transcripts by RNAscope and CD8 and TcRγδ IELs by IHC. After a setup stage, we were able to perform the double assay with excellent results, indeed the signal obtained in our duodenal sections was clear with a very low background. Moreover, the two distinct signals co-stained without covering each other and working with bright-field microscopy had the advantage of the signal being maintained over time, unlike fluorescence.

Nevertheless, there were some drawbacks in performing the RNAscope assay. In contrast to a double IHC approach, we are detecting perforin mRNA which does not have the same functional relevance as

detecting the protein. The RNAscope kit is expensive and has a short expiration date. The protocol was time-consuming and required skilled pathologists; moreover, the manufacturing company recommends using their oven, which has a considerable cost. Nevertheless, the recommended reagents' volumes were overestimated, in fact decreasing the recommended volumes allowed to perform more assays without compromising the results.

Despite these limitations, the established RNAscope-IHC assay will be useful to shed light on the cytolitic properties of distinct IELs subsets, which are key cells in the pathogenesis of CD. Moreover, the assay may be used to detect other markers that could be important in the context of CD, especially in the progression to full-blown disease.

In conclusion we developed a double staining by combining RNAscope and classical IHC that will allow to study cytotoxic properties of the two subgroups of IELs, TcRαβ+CD8αβ+ and TcRγδ+, in different stages of CD, providing an important tool to functionally characterize IELs in large cohort of CD patients, thus contributing to understand the events leading to tissue destruction and healing.

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CRediT authorship contribution statement

Antonella Marano: Investigation, Formal analysis, Writing – review & editing. **Riccardo Troncone:** Supervision. **Valentina Discepolo:** Conceptualization, Supervision, Writing – review & editing. **Mariantonia Maglio:** Conceptualization, Supervision, Investigation, Formal analysis, Writing – original draft.

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