Journal of Immunological Methods 505 (2022) 113276

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

Cell-type-specific gene expression profile by laser capture microdissection on mirror sections

Giuseppe Mazzarella [a,e], Giuseppe Iacomino [a], Pasquale De Luca [b], Salvatore Iaquinto [c], Fabiana Capuano [d], Riccardo Troncone [e], Vera Rotondi Aufiero [a,e,*]

[a] Institute of Food Sciences, CNR, Avellino, Italy
[b] Research Infrastructure for Marine Biological Resources Department, Stazione Zoologica Anton Dohrn, CAP80121 (NA) Villa Comunale, Italy
[c] Division of Gastroenterology, San Filippo Neri Hospital, Rome, Italy
[d] Gastroenterology Department, S. G. Moscati Hospital, Avellino, Italy
[e] Department of Translational Medical Science and E.L.F.I.D, University "Federico II" Napoli, Italy

ARTICLE INFO

Keywords:
Mirror sections
Laser capture microdissection
γδ + IELs
Celiac disease
Cell-specific gene expression profiles

ABSTRACT

Immuno-laser capture microdissection (Immuno-LCM) has been used to analyze cell-specific gene expression profiles. However, the usefulness of such a technique is frequently limited by RNA degradation. We, therefore, developed a rapid protocol of LCM on mirror sections, which allows for preserving RNA integrity. With such a procedure, we investigated cell-type-specific gene expression of γδ intraepithelial lymphocytes (IELs) in untreated celiac disease (CD).

An increase in TGF-β mRNA expression levels was observed in γδ + IELs compared to intestinal enterocytes (IEs), whereas anti-inflammatory IL-10 mRNA production from γδ + IELs was lower compared to IEs.

In untreated CD patients, the production of anti-inflammatory cytokines by γδ + IELs is suggestive of a regulatory function, thus playing a critical role in limiting inflammation. This work underscores the importance of LCM on mirror sections as a valuable tool to perform cell-type-specific molecular analysis in tissue.

1. Introduction

Laser capture microdissection (LCM) has allowed cell-type-specific molecular analysis of solid tissues, without contamination from surrounding cells, and provided new insights into both normal cellular biology and pathogenic mechanisms (Lawrie and Curran, 2005). By using such technology, we have shown that in celiac disease (CD), surface epithelium and lamina propria compartments, play a prominent role in determining innate and adaptive immunity, respectively (Iacomino et al., 2016, 2018). In contrast, the surface epithelium and lamina propria produce high levels of anti-inflammatory cytokines, suggesting that both compartments are involved in the immunoregulatory response.

CD is an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals, characterized by the activation of intraepithelial lymphocytes (IELs), a phenomenon that leads to the destruction of the intestinal epithelium. The majority of these cells are TCRαβ$^+$ CD8$^+$ and a significant proportion are TCR γδ$^+$ (Steenholt et al., 2017). In contrast to the deleterious role of CD8$^+$αβ IELs in CD mucosa, γδ$^+$ IELs, might play a prevalent regulatory role by secreting TGF-β (Bhagat et al., 2008).

In addition to the ability to isolate by LCM single cells or specific tissue regions by morphology, for molecular analysis, in recent years, the need to isolate subpopulations of cells that cannot be easily identified morphologically has become increasingly critical. Immunohistochemical staining of frozen sections could help to identify and isolate specific cell populations, even of identical morphology, based on their antigenic expression, allowing high selective microdissection (Fend et al., 1999). However, immunohistochemical staining protocols can often lead to RNA degradation (Tangrea et al., 2011).

Here, we described a custom protocol of LCM on mirror sections which allow precise isolation of specific cell population while preserving RNA quality. The mirror technique is previously used in colocalization studies (Kosaka et al., 1985). To investigate the possible colocation of

Abbreviations: LCM, laser capture microdissection; CD, celiac disease; IELs, intraepithelial lymphocytes; DEPC, diethylpyrocarbonate; IHC, immunohistochemistry; RT-qPCR, Real-Time Quantitative Reverse Transcription PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEs, intestinal enterocytes.

* Corresponding author at: Immuno-Morphology Lab, Institute of Food Sciences, National Council Research, 83100, Avellino, Italy.
E-mail address: vera.rotondiaufiero@isa.cnr.it (V. Rotondi Aufiero).

https://doi.org/10.1016/j.jim.2022.113276
Received 21 December 2021; Received in revised form 29 March 2022; Accepted 20 April 2022
Available online 25 April 2022

two antigens in the same cell, paired surfaces of neighbouring sections immunostained with different antigens were compared.

In our case, mirror sections were used to allow precise comparison of the same cells on the two consecutive sections. Using this protocol, we isolated γδ + IELs from jejunum, obtained from untreated CD patients, and examined their suppressive function by analyzing the expression of anti-inflammatory cytokines, such as IL10 and TGF-β, by real-time quantitative reverse transcription PCR (RT-qPCR).

2. Materials and methods

2.1. Patients

Jejunal biopsies, taken from 10 untreated CD patients (n.3 male and n.7 female; median age 39 years; range 18–45 years) were snap-frozen in liquid nitrogen. The diagnosis was based on typical mucosal lesions with crypt cell hyperplasia and total villous atrophy. All untreated CD patients were positive for serum anti endomysial antibodies. Jejunal biopsy samples were also obtained from 10 treated CD patients (n.1 male and n.9 female patients; median age 48 years; range 19–56 years), who were in clinical and histological remission, and negative for anti-endomysial antibodies.

All patients gave written informed consent. The study was approved by the ethics committee of San G. Moscati Hospital (Avellino, Italy, n°CECN/809) and conformed with the provisions of the Declaration of Helsinki.

2.2. Immuno-LCM

Freshly frozen jejunal mucosa samples were mounted on the cryostat stage (Leica CM1850; Leica Microsystems, Wetzlar, Germany) set at −25 °C, equilibrated for 20 min.

Ten criostatic sections of 8 μm were mounted on an RNase-free membrane slide (PEN-membrane, 1 mm glass, Carl Zeiss MicroImaging, Munich, Germany).

The slide was immunostained for γδ + T cells, as previously reported (Maglio et al., 2017).

Subsequently, the section was processed for LCM. Briefly, the section was fixed in ice-cold EtOH 70% for 2 min. Rehydration in ice-cold diethylpyrocarbonate (DEPC) water was followed by haematoxylin staining before dehydration in graded ethanol for 60 s and xylene for 2 min. Slides were air-dried for 3 min and processed for LCM by the Leica LMD 6000 microdissector.

1000 γδ + IELs and 1000 intestinal enterocytes (IEs), from each untreated and treated CD patient, respectively, were microdissected in at least 30 min for each LCM session.

2.3. LCM on mirror sections

Freshly frozen jejunal mucosa samples were mounted on the cryostat stage (Leica CM1850; Leica Microsystems, Wetzlar, Germany) set at −25 °C, equilibrated for 20 min. Ten criostatic sections of 8 μm were mounted on each slide. The mirror sections were obtained in two consecutive sections, mounted on two different glass slides. In particular, the first section, mounted on a typical glass slide, was immunostained for γδ + T cells (Fig. 1, upper panel), as previously reported (Maglio et al., 2017). The consecutive section was mounted upsidedown on an RNase-free membrane slide (PEN-membrane, 1 mm glass, Carl Zeiss MicroImaging, Munich, Germany) so that the same tissue cut surface was exposed like the first one, and processed for LCM as described in the 2.2 section (Fig. 1, lower panel).

By comparing the IHC-processed sections with the mirror sections collected on the PEN-membrane slide, we microdissected about 1000 γδ + IELs and 1000 intestinal enterocytes (IEs), from each untreated and treated CD patient, respectively.

Importantly, the first section was used as a reference to identify the location of γδ + T cells on the mirror section, from which were then isolated by LCM (see Schematic workflow).

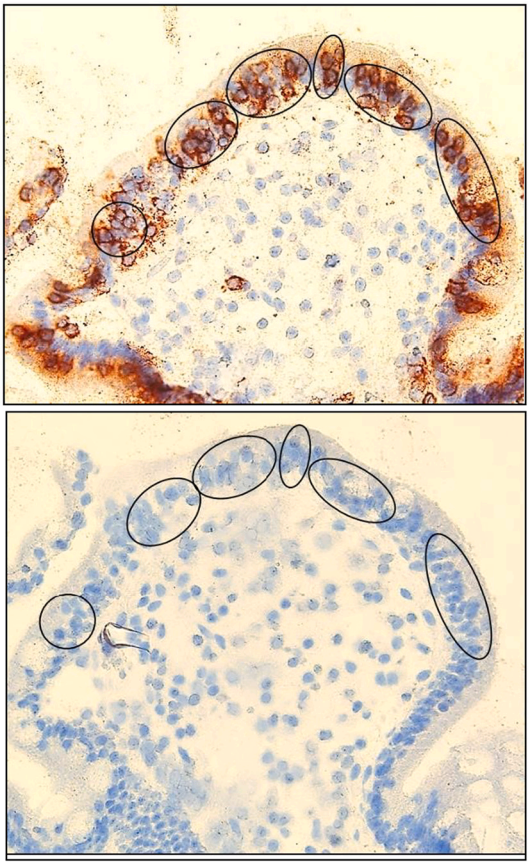

Fig. 1. Mirror surfaces of the adjoining sections were obtained from a jejunal biopsy of an untreated CD patient. Upper panel, positive γδ T cells selected on the section surface (reddish-brown spot in black circles). Lower panel, complementary parts of the same area containing γδ T cells on the mirror surface of the non-immunolabeled adjacent section (black circles), which will be isolated by LCM. Original magnification: X 200

Using the same protocol, an equal number of enterocytes, from the jejunum of treated CD patients, were collected as non-inflamed controls. Notably, the first slide, processed for immunohistochemistry (IHC) to identify CD3+ IELs (Fig. 2 A), was used as a reference to identify the location of T lymphocytes on the mirror section. The surface of the intestinal epithelium is mostly composed of enterocytes and T cells, therefore on the mirror section, we isolated enterocytes by microdissecting the epithelial surface without CD3+ cells (Fig. 2 B).

2.4. RNA extraction

Microdissected samples were treated with 4 U RNasin RNase inhibitor (Promega, Italy), and total RNA was obtained using the PicoPure RNA isolation kit according to the manufacturer's protocol (Arcturus, Life Technologies). RNA was eluted in 12 μl DEPC water.

2.5. RNA quantitation and integrity quality analysis

Following RNA extraction, RNA was quantified by a fluorimetric approach on a Qubit® ver. 3 instrument (Thermo Fisher Scientific, Italy); evaluation of RNA sample integrity (RIN) was assessed by the Bioanalyzer 2100 Expert system (Agilent Technologies), according to the manufacturer's instructions.

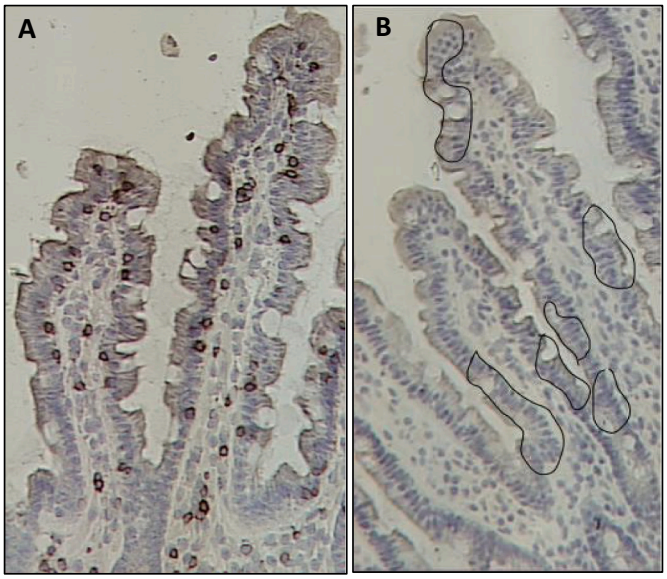

Fig. 2. The Mirror technique is useful for identifying labelled CD3 T cells (A, reddish-brown spots) and determining their location in the adjoining section, allowing to isolate epithelial areas without CD3+ T cells (B, black circles). The adjoining sections were obtained from the jejunum of treated CD patients. Original magnification: X 200. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Reverse transcription

Isolated RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) with random hexamer primers according to the manufacturer's instructions.

2.7. RT-qPCR analysis

Relative levels of IL-10 and TGF-β cDNA were quantified by SYBR green (PowerSYBR Green PCR Master Mix, Applied Biosystems)-based qRT-PCR assays on an ABI PRISM 7000 SDS instrument (Applied Biosystems). PCR was performed in triplicate in a final volume of 35 μl containing 2 μl of diluted cDNA. Serial dilutions of cDNA containing a known amount of each transcript were included in each quantitative PCR run to generate a standard curve. To assess the specificity of the amplification products, a melting curve analysis was performed. In preliminary experiments, the optimal dilution of cDNA for obtaining a PCR product within the linear phase of the amplification was determined. We included only study samples in which both genes and housekeeping genes had a sigmoid-shaped curve between Ct values of 15 and 36. Samples that did not meet the RNA quality and quantity requirements were excluded from the study. Primers, designed using the Primer Express Software (Applied Biosystems) according to ABI recommendations or derived from the literature, were synthesized by Sigma-Aldrich. Oligonucleotides were predicted to (a) generate amplicons of approximately 50–150 bps from specific National Center for Biotechnology Information (NCBI) reference sequences, and (b) span exons, and were blasted through NCBI GenBank to ensure a lack of homology to other known human cDNA sequences. Cycling conditions for PCR have been described elsewhere (Iacomino et al., 2016, 2018). Gene expression levels were normalized to the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primers sequences are presented in Table 1.

2.8. Agarose gel electrophoresis

After qRT-PCR amplification, amplicon size was determined using 2% agarose gel electrophoresis containing ethidium bromide (Sigma-Aldrich) (Fig. 4). Images were acquired by a GelDoc 2000 imaging system (BioRAD, Italy).

Table 1
Primers used in the qRT-PCR analysis.

Gene	Accession number	Oligonucleotide sequences (5′➔ 3′) forward primer reverse primer
IL-10	AY029171.1	GCTGGAGGACTTTAAGGGTTACCT CTTGATGTCTGGGTCTTGGTTCT
TGF-β1	NM_000660	CAAGGGCTACCATGCCAACT AGGGCCAGGACCTTGCTG
GAPDH	NM_002046.2	ATGACATCAAGAAGGTGGTG CATACCAGGAAATGAGCTTG

2.9. Data and statistical analysis

Relative transcript expression was calculated by the ΔΔCT method using Data Assist Software v3.01 (Applied Biosystems). Statistical analyses were performed with GraphPad Prism (GraphPad Software, CA, USA). Differences between groups were compared using paired two-tailed Student's *t*-tests. Statistical significance was achieved when $p < 0.05$. The results are reported as the normalized mean expression.

3. Results

3.1. Improved RNA recovery by LCM on mirror sections

RNA extracted from Immuno-LCM samples was strongly degraded (Fig. 3, lane 1; RIN = 1.4) compared to that obtained by samples processed by the mirror sections technique (Fig. 3, lane 2; RIN = 7.7).

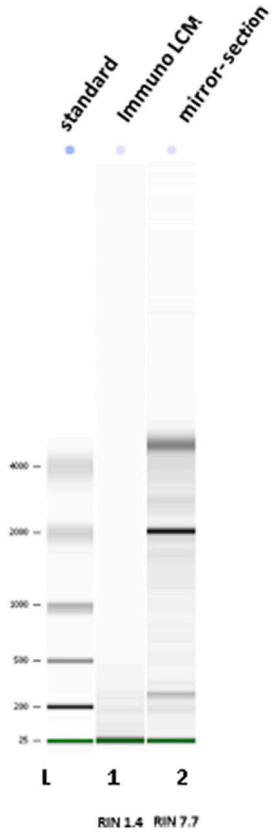

Fig. 3. Quality assessment of RNA extracted from LCM samples performed on an Agilent 2100 Bioanalyzer. Immuno-LCM sample, lane 1; mirror-section sample, lane 2; L, ladder.

The isolation of a specific cell population by LCM on mirror sections, without direct IHC processing, greatly improved the RNA integrity. LCM on mirror sections generates samples highly enriched for specific cell types with RNA of sufficient quality for downstream molecular analyses.

After quantification, RNA was reverse transcribed and analyzed by RT-qPCR assays for GAPDH.

Agarose gel electrophoresis of the amplification product (Fig. 4), exhibited a proper profile, as confirmed by the presence of a single band at 177 base pairs (bps).

3.2. γδ + IELs-specific anti-inflammatory cytokines profiles in untreated CD

We analyzed the mRNA expression of anti-inflammatory cytokines in γδ + IELs and IEs, isolated by LCM on mirror sections, in untreated and treated CD, respectively. We found increased mRNA levels of TGF-β in γδ + IELs compared to IEs ($p < 0.05$) (Fig. 5 A). In contrast, a higher gene expression ($p < 0.0001$) of IL-10 was observed in IEs compared with γδ + IELs (Fig. 5 B).

This finding suggests that in untreated CD, as previously shown in treated CD patients (Bhagat et al., 2008), γδ + IELs produce TGF-β, as an anti-inflammatory cytokine.

4. Discussion

LCM is an automated technique that enables the isolation of tissue compartments or specific cells from a mixed population under microscopic visualization. Several studies (Fassunke et al., 2004), have shown that immuno-LCM techniques facilitate the gene expression analysis associated with cell populations immunophenotypically identified from whole tissue sections, thereby improving the knowledge of many disease pathways.

However, the long exposure to aqueous solutions, required for conventional IHC, is likely to be deleterious to RNA. This is especially pronounced in frozen tissue, where the loss of RNA is probably mainly caused by the activity of endogenous Rnases (Fend et al., 1999).

As a result, IHC staining protocols for use with LCM require marked modification compared with conventional protocols.

Adaptation of IHC protocols by shortening times for stain and precipitating fixatives (such as ethanol or acetone), besides conventional protection against RNases, helped to improve RNA quality.

Although we have optimized the immuno-LCM protocols, reducing the time of exposure to the aqueous solution and using RNase inhibitors, RNA integrity has not been preserved, as showed by a RIN of 1.4.

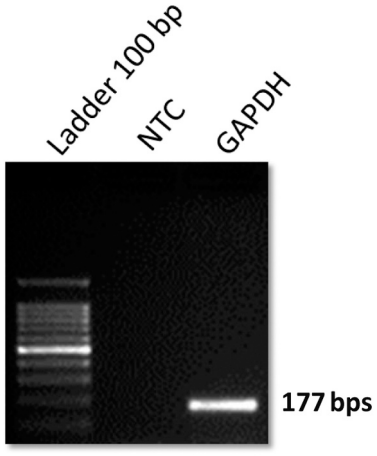

Fig. 4. Size of expected PCR products detectable in LCM sample (GAPDH). No products are detectable in the negative control (NTC). The experiment was representative of 3 performed with similar results.

Here, we reported an application of the mirror technique to LCM, by which one section, processed by immunohistochemistry for γδ + IELs, was used as a reference to identify the locations of such specific cell types on the mirror section, subjected to LCM.

Important to note that the mirror section was not subjected to long incubation times and dealing with aqueous solutions. A RIN value of typically >7.7 for RNA extracted from samples obtained by LCM on mirror sections was of sufficient quality for our downstream molecular applications.

In our experience, to recover a high-quality RNA for cytokine gene expression profiling, it is also mandatory that LCM should be performed within 30 min (Iacomino et al., 2018).

The massive infiltration of γδ + in the epithelial compartment of untreated CD patients, allowed us to isolate a large number of cells in 30 min, greatly increasing the quality and yield of RNA.

Of note, the RT-qPCR of the housekeeping gene (GAPDH), showed good Ct values (typically 22) and a single spike in the analysis of the melting curve performed from samples collected by LCM on mirror sections (data not shown).

Similarly, the single band detected after gel-electrophoresis of GAPDH amplicons, confirmed the good quality of resulting cDNA and the absence of non-specific amplification products.

With this protocol, we isolated γδ + T IELs and examined their suppressive function in untreated CD, by analyzing the expression of IL-10 and TGF-β down-regulatory cytokines.

By flow cytometric analysis and sorting of IELs, a high density of γδ + IELs that express intracellular TGF-β has been shown in treated CD patients, suggesting that such cells could play a prevalent regulatory role (Bhagat et al., 2008).

In the current study, we found that γδ + IELs from untreated CD retained an increased expression of TGF-β compared to enterocytes. On the contrary, we observed an increased expression of IL-10 in enterocytes compared with γδ + IELs. This downregulatory cytokine, which is involved in local homeostasis, is produced not only by T cells and antigen-presenting cells but also by epithelial cells (Autschbach et al., 1998). Therefore, the overexpression of IL-10 by enterocytes in untreated CD could be related to the attempt of such cells to counterbalance active inflammation.

5. Conclusions

We conclude that specific cells can be identified and microdissected by the mirror section technique, preventing the critical problem of RNA degradation. This method extends the ability to isolate pure populations of immunotypically defined cells from a sea of similarly appearing cells and process for further analysis to determine the functional state of the cells.

Herein, by using this procedure we reported that γδ + IELs in untreated CD still retain anti-inflammatory activity, suggesting that such cells are actively trying to downregulate ongoing inflammation.

Further understanding of the biology and role of γδ + T cells in homeostasis and disease may shed light on potential novel therapies.

Founding sources

The study was supported by the Fondazione Celiachia FC Grant 2017_014.

CRediT authorship contribution statement

Giuseppe Mazzarella: Conceptualization, Supervision, Investigation, Formal analysis, Writing – review & editing. **Giuseppe Iacomino:** Formal analysis, Software, Validation, Supervision. **Pasquale De Luca:** Methodology. **Salvatore Iaquinto:** Resources. **Fabiana Capuano:** Resources. **Riccardo Troncone:** Supervision. **Vera Rotondi Aufiero:** Conceptualization, Investigation, Formal analysis, Writing – original

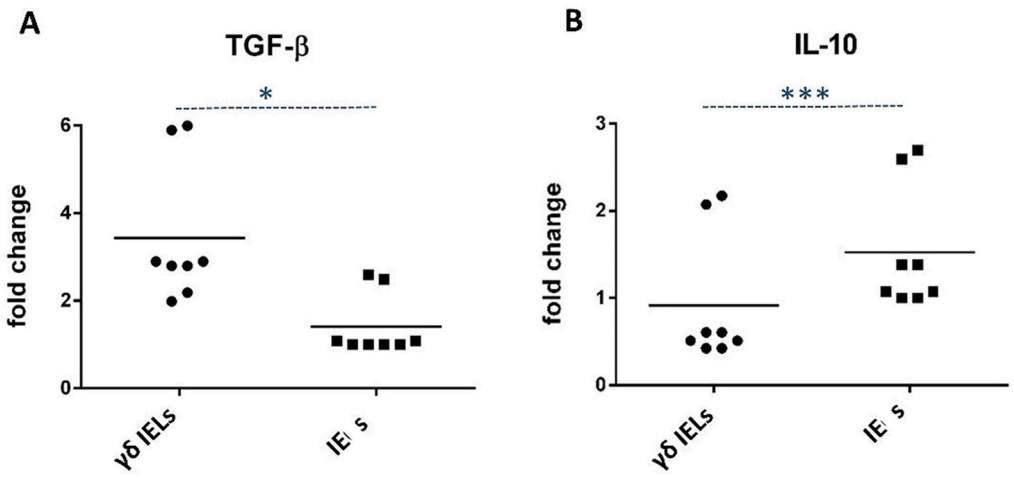

Fig. 5. TGF-β (A) and IL-10 (B) mRNA levels determined by RT-qPCR, in γδ + intraepithelial lymphocytes (IELs) and intestinal enterocytes (IEs) isolated by LCM from jejunal biopsies of untreated (n = 10) and treated CD (n = 10), respectively. Fold change (y-axis) represents mRNA expression levels normalized to the GAPDH mRNA level. Dots represent single patients. Dashes indicate the mean values. Dashed lines indicate significant intergroup (IELs *vs* IEs) differences, as indicated by the corresponding symbols. * = $p < 0.05$, and ** = $p < 0.001$.

draft, Methodology.

Declaration of Competing Interest

There are no conflicts of interest.

Acknowledgements

We thank Mario de Falco, Claudio Pagliuca, Rosa Maria Bevilacqua, Clementina Carratù and Anna Capriolo (Gastroenterology and Digestive Endoscopy Service, San G. Moscati Hospital, Avellino, Italy) for their help in the collection of blood and mucosal samples. The authors are grateful to Clemente Meccariello and Vincenzo De Sena for their technical assistance.

References

Autschbach, F., Braunstein, J., Helmke, B., Zuna, I., Schurmann, G., Niemir, Z.I., Wallich, R., Otto, H.F., Meuer, S.C., 1998. In situ expression of interleukin-10 in non inflamed human gut and in inflammatory bowel disease. Am. J. Pathol. 153, 121–130.

Bhagat, G., Naiyer, A.J., Shah, J.G., Harper, J., Jabri, B., Wang, T.C., Green, P.H., Manavalan, J.S., 2008. Small intestinal CD8+TCR gammadelta+NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. J. Clin. Invest. 118 (1), 281–293. https://doi.org/10.1172/JCI30989. PMID: 18064301; PMCID: PMC2117760.

Fassunke, J., Majores, M., Ullmann, C., et al., 2004. *In situ*-RT and immunolaser microdissection for mRNA analysis of individual cells isolated from epilepsyassociated glioneuronal tumors. Lab. Investig. 84, 1520–1525. https://doi.org/10.1038/labinvest.3700165.

Fend, F., Emmert-Buck, M.R., Chuaqui, R., Cole, K., Lee, J., Liotta, L.A., Raffeld, M., 1999. Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. Am. J. Pathol. 154 (1), 61–66. https://doi.org/10.1016/S00029440(10)65251-0. PMID: 9916919; PMCID: PMC1853427.

Iacomino, G., Marano, A., Stillitano, I., et al., 2016. Celiac disease: role of intestinal compartments in the mucosal immune response. Mol.Cell. Biochem. 411, 341–349. https://doi.org/10.1007/s11010-015-2596-7.

Iacomino, G., Aufiero, V.R., Marena, P., Venezia, A., Troncone, R., Auricchio, S., Mazzarella, G., 2018. Laser capture microdissection as a tool to study the mucosal immune response in celiac disease. Methods Mol. Biol. 1723, 139–154. https://doi.org/10.1007/978-1-4939-7558-7_7 (PMID: 29344858).

Kosaka, T., Kosaka, K., Tateishi, K., Hamaoka, Y., Yanaihara, N., Wu, J.-Y., Hama, K., 1985. GABAergic neurons containing CCK-8-1ike and/or VIP-like immunoreactivities in the rat hippocampus and dentate gyms. J. Comp. Neurol. 239, 420–430.

Lawrie, L.C., Curran, S., 2005. Laser-capture microdissection and colorectal cancer proteomics. Methods Mol. Biol. 293, 245–253.

Maglio, M., Ziberna, F., Aitoro, R., Discepolo, V., Lania, G., Bassi, V., Miele, E., Not, T., Troncone, R., Auricchio, R., 2017. Intestinal production of anti-tissue transglutaminase 2 antibodies in patients with diagnosis other than celiac disease. Nutrients 9 (10), 1050. https://doi.org/10.3390/nu9101050. PMID: 28934109; PMCID: PMC5691667.

Steenholt, J.V., Nielsen, C., Baudewijn, L., Staal, A., Rasmussen, K.S., Sabir, H.J., Barington, T., Husby, S., Toft-Hansen, H., 2017. The composition of T cell subtypes in duodenal biopsies are altered in coeliac disease patients. PLoS One 12 (2), e0170270. https://doi.org/10.1371/journal.pone.0170270.

Tangrea, M.A., Mukherjee, S., Gao, B., Markey, S.P., Du, Q., Armani, M., Kreitman, M.S., Rosenberg, A.M., Wallis, B.S., Eberle, F.C., Duncan, F.C., Hanson, J.C., Chuaqui, R.F., Rodriguez-Canales, J., Emmert-Buck, M.R., 2011. Effect of immunohistochemistry on molecular analysis of tissue samples: implications for microdissection technologies. J. Histochem. Cytochem. 59 (6), 591–600. https://doi.org/10.1369/0022155411404704. Epub 2011 Mar 23. PMID: 21430260; PMCID: PMC3201191.