Original paper

Evaluation of spatial PD1 and PD-L1 expression in inflammatory bowel disease samples – a pilot study

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Alterations of PD1/PD-L1 pathway may be associated with an excessive inflammatory response in the intestinal wall in inflammatory bowel diseases (IBD). To evaluate the expression of PD-1 and PD-L1 in 4 compartments of intestinal wall (mucosa, submucosa, muscularis propria and lymphatic follicles), high-resolution immunohistochemically stained slides were obtained from formalin-fixed paraffin-embedded samples of 10 Crohn's disease (CD), 9 ulcerative colitis (UC) and 10 unaffected individuals cases. The levels of expression were quantified using the QuPath software.

PD-1 was detected in lymphatic follicles in affected and unaffected tissue samples and in inflammatory infiltration in IBD. There was no difference between groups neither in PD-1 overall expression nor in individual compartments, with the exception of the mucosal expression. It was higher in the mucosa of CD patients comparing to controls, however this difference was marginal ($p = 0.0461$). PD-L1 was expressed in endothelium and mesenteric nervous plexi, consistently in each group. There were no significant differences in PD-L1 immunoreactivity in context of histologic compartment nor clinical diagnosis. The results suggest that PD-1 and PD-L1 expression in intestinal tissue is heterogeneous in the analysed groups, thus it may be dependent on individual characteristics.

Key words: PD1, PDL1, IBD, ulcerative colitis, Crohn's disease.

Introduction

Inflammatory bowel diseases (IBD) are complex, disabling disorders, characterized by chronic relapsing inflammation of intestinal wall. They constitute world-wide health problem with continually increasing incidence [1].

There are two major subtypes of IBD: ulcerative colitis (UC) and Crohn's disease (CD), that share common clinical symptoms of abdominal pain, diarrhea, rectal bleeding and fever but can be classified based on some distinguishing features. CD can involve any region of gastrointestinal tract and is located intermittently, most commonly in the terminal ileum or the perianal region. The inflammation is transmural and often causes fistulas, strictures and perforations. In contrast, UC is characterized by continuous inflammation which affects colon and extends proximally. The inflammation is mostly limited to the mu-

cosal and sometimes submucosal layer where crypt abscess and ulceration could be observed [1, 2].

The etiology of IBDs remains unclear and is considered multifactorial. Recent studies have revealed that genetic background, environmental factors, intestinal microbiota and immune system are involved in the pathogenesis of IBD [3]. It has been revealed that aberrant intestinal inflammatory response observed in IBDs, includes diverse aspects of both, innate and adaptive immunity. An imbalanced activity of different T cell subpopulations is considered as one of the crucial elements of these alterations. In particular, CD has been believed a Th1-mediated disease, where UC was thought to be driven mainly by Th2 activation. However, there is growing evidence describing the role of other Th cell subpopulations, such as Th17 and regulatory T cells [4, 5].

The activation of T cells is modulated by several regulatory mechanisms. Programmed cell death protein 1 (PD1, also known as PDCD1 or CD279) is an inhibitory receptor expressed by T cells. By interactions with its ligands: programmed cell death ligand 1 (PDL1, also known as CD274 or B7 H1) and programmed cell death ligand 2 (PDL2, also known as CD273 or B7 DC), PD1 counters positive signaling from T-cell receptors (TCRs), reducing the activation and function of T cells [6].

It has been shown that alterations of PD1-pathway may contribute to the breakdown of tolerance and are associated with human autoimmune diseases, such as diabetes I [7], multiple sclerosis [8] and rheumatoid arthritis [9]. It is also suggested that the aberrant function of PD1 or its ligands may be associated with an excessive inflammatory response in the intestinal mucosa, observed in IBDs. However, the data regarding the role of PD-1 pathway in IBDs are limited and the mechanism of these associations is unclear [10].

In this study, we aimed to determine the spatial expression of PD1 and PD-L1 in individual compartments of intestinal wall in patients with CD and UC comparing to unaffected individuals using digital analysis software and verify the role of PD-1 pathway in the pathogenesis of IBD.

Material and methods

The study was performed in the conformity of the Declaration of Helsinki for Human Experimentation and received approval by the Local Bioethics Committee of the Medical University of Warsaw. As all data were anonymized, informed consent was not necessary.

Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from the archive of the Department of Pathology, Professor Witold Orlowski Public Clinical Hospital. All tissue samples were collected from surgical resections. The study included 9 samples from ulcerative colitis, 10 from Crohn's disease and 10 unaffected samples. Histologically normal portions of ileum (n = 5) and colon (n = 5) were obtained from uninvolved areas from bowel resections of 10 patients with adenocarcinoma of colon. The tissue with UC was collected from total or partial colectomies from 9 patients with the intractable disease. The CD tissue was obtained from surgical resection of ileitis (n = 8) and colitis (n = 2). All the patients with UC and CD were in the severe stage of disease and underwent the surgery due to the complications of the disease, most complications were due to persistent mucosal bleeding leading to anemia.

The diagnosis of ulcerative colitis or Crohn's disease has previously been made on the basis of conventional clinical, endoscopic, and histopathological criteria. The histopathological assessment of resected tissues was made in the Department of Pathology, Professor Witold Orlowski Public Clinical Hospital.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue slides were cut on a sliding microtome into 3 μm thick sections. The staining was performed with the Dako REAL Envision Detection system, according to the manufacturer's instructions. After deparaffinization and dehydration with xylene and alcohol, antigen retrieval was performed with Dako Target Retrieval Solution Citrate pH 9. Endogenous peroxidase was blocked for 5 minutes. Slides were incubated 30 minutes at room temperature with primary antibodies, as follows: anti-PD1 (Anti-PD-1-Antibody; Rabbit mAB; dilution 1:200; company: Cell Signaling; order number: #86163; antibody validation by isotype control and system control) and anti-PD-L1 (Anti-PD-L1-Antibody; Rabbit mAB; dilution 1:200; company: Cell Signaling; order number: #13684; antibody validation by iso-type control and system control). Following 30 min of incubation with the Dako REAL EnVision/HRP detection system, DAB (Dako Real DAB+Chromogen, K5007) was applied for about 10 min and then removed by rinsing with distilled water. Slides were counterstained with hematoxylin. Human tonsil tissue served as a positive control for each staining. For negative controls, slides were incubated with DAKO Antibody Diluent (DAKO, S0809) in replacement of primary antibodies.

Image data acquisition

All slides were scanned using the Hamamatsu NanoZoomer slide scanner at 40× magnification, with a resolution of 0.228 μm/pixel.

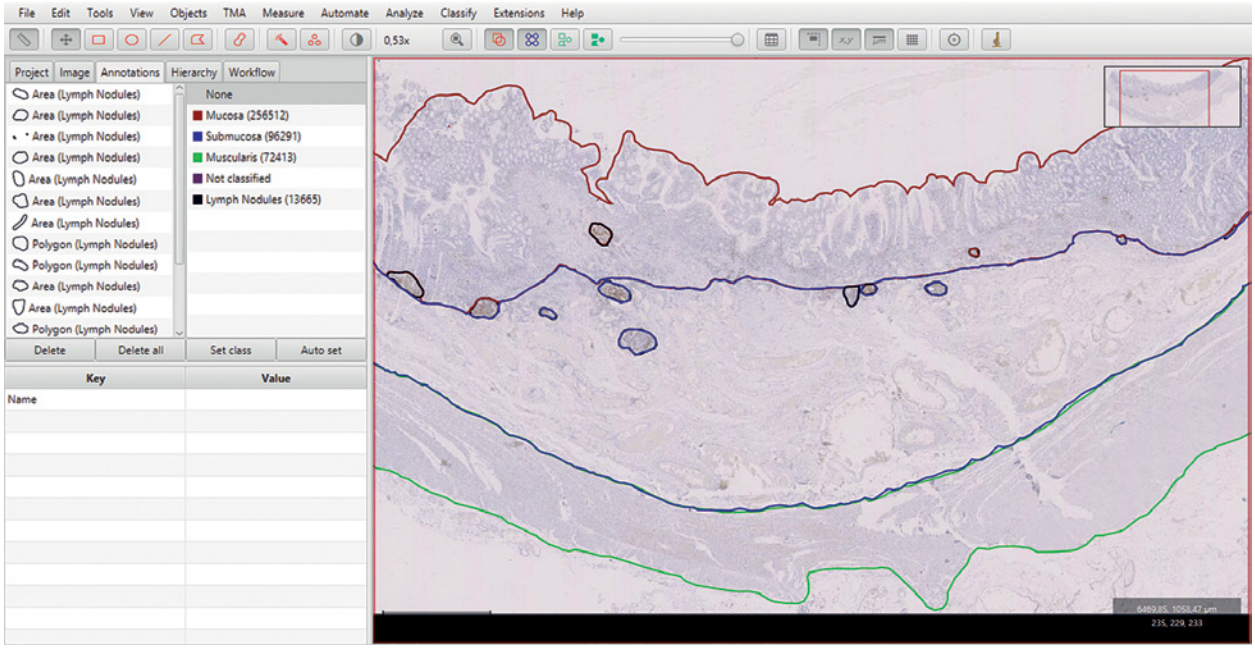

Fig. 1. Illustration of digital image analysis in QuPath software. For each sample, region of mucosa, submucosa and muscularis were separated by manual drawing, based on morphological characteristics of each layer of intestinal wall. Regions of lymphatic follicles were distinguished as a separate region for analysis

Digital image analysis

An assessment of PD-1 and PDL-1 immunoreactivity in each sample was performed using QuPath 0.1.2 open source software for digital pathology image analysis [11]. For each image, areas of mucosa, submucosa and muscularis propria were separated by manually drawing (Fig. 1). Regions of lymphatic follicles were selected separately for individual analysis. The percentage of positive cells and staining intensity scores were evaluated using positive cell detection algorithm in QuPath. For cell detection the following parameters were set: nucleus background radius – 8 μm; sigma – 1,5 μm; minimum area – 10 μm^2; maximum area – 400 μm^2; cell expansion – 5 μm; maximal background intensity: 2. As PD-1 and PD-L1 expression is rather cytoplasmic and/or membranous rather than nuclear, the score compartment was set as "Cytoplasm". The following intensity threshold parameters were applied: "Threshold 1+": 0,2; "Threshold 2+": 0,4, "Threshold 3+: 0,6". For each sample, H-score was calculated using the following formula: $1 \times (\textit{percentage of } 1+\textit{cells}) + 2 \times (\textit{percentage of } 2+\textit{cells}) + 3 \times (\textit{percentage of } 3+ \textit{ cells})$ [11, 12].

Statistical analysis

All statistical analyses were performed with GraphPad Prism Software. The number of cases (above 9) in each group met the criteria to reach the statistical significance level, given the he statistic level at $\alpha = 0.05$, the power of the test of 0.8 and the effect size of 1.5 for the tests applied for statistical analysis. The normal distribution of the data was confirmed using the Shapiro-Wilk test. Parametric values are expressed as mean ±standard deviation (SD), non-parametric values are presented as median + IQR. Statistical comparisons of parametric distributed data were performed using one-way ANOVA with Dunnett multiple comparison tests. Non-parametric distributed data were assessed using the Kruskal-Wallis test followed by Dunn's test. $p < 0.05$ was considered as statistically significant.

Results

Microscopic examination revealed predominant PD-1 expression in the lymphatic follicles in affected and unaffected tissue samples

The expression of PD-1 was mainly observed in the lymphatic cells within the area of lymphatic follicles in either affected or unaffected tissue samples in ileum and colon (Fig. 2). In CD and UC samples, positive reactions were present in the inflammatory infiltration, predominantly in the mucosal and submucosal layers.

The expression of PD-1 tends to be higher in mucosa and submucosa than in muscularis propria

To examine the difference in PD-1 expression between individual compartments of intestinal wall, we compared the H-score in mucosa, submuco-

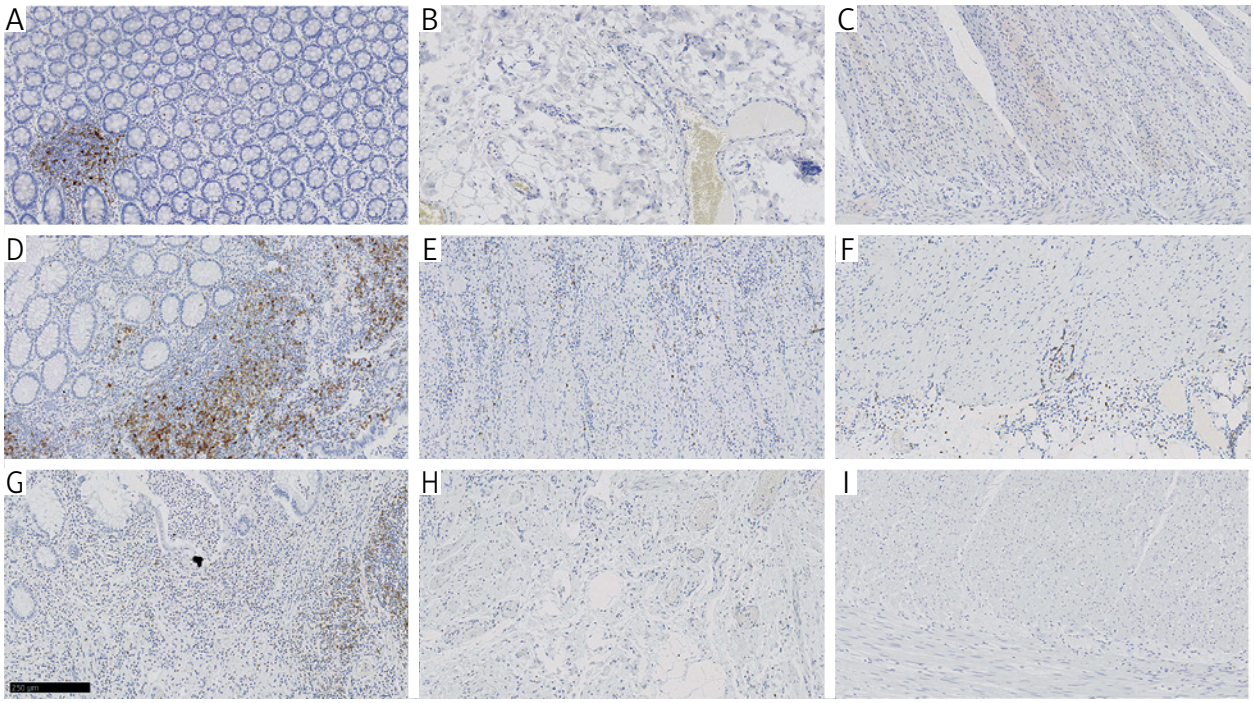

Fig. 2. Images of programmed death protein 1 (PD-1) immunohistochemical staining in: unaffected colon tissue – muscosa (A), submucosa (B), muscularis (C); Crohn's disease in colon – mucosa (D), submucosa (E), muscularis (F) and ulcerative colitis samples – mucosa (G), submucosa (H), muscularis (I). Scale bar 250 μm, original magnification 10x, images obtained from digitalized slides with NDP.view2 software (Hamamatsu)

sa and muscularis propria in CD, UC and normal controls, separately for colon and ileum (Fig. 3). In the ileum, the difference between individual layers was not significant. In the colon samples we observed the trend for higher expression in mucosa and submucosa that was more pronounced in IBD samples, reaching statistical significance in the UC group (comparison between mucosa and muscularis, p = 0.0450, median H-score mucosa: 1.593, IQR: 0.8375-4.678; median H-score muscularis: 0.02181, IQR: 0.01105-0.7463).

PD-1 overall expression does not differ between groups, whereas the expression in mucosal layer may be increased in CD

To determine if mucosal, submucosal and muscularis expression of PD-1 varies substantially between CD, UC and unaffected tissue, we compared H-scores from each individual compartment and the overall expression in each group. As microscopic observations revealed the expression is mainly visible in lymphatic follicles, we decided to distinguish the follicles as individual groups, as shown in the Fig. 4.

As shown in Fig. 5 the expression in ileum did not differ between groups, neither for whole sections, nor for individual compartments. Similarly, there was no difference in the overall colon PD-1 expression and the expression in individual compartments, with the exception of mucosal layer that was higher in CD samples compared to the control group (p = 0.0461; median H-score unaffected tissue: 8.390, IQR: 3.267-1.499; median H-score CD: 5.349, IQR: 4.848-5.851). However, the difference might be associated with the small number of samples and the use of multiple comparisons in statistical analysis.

PD-L1 is expressed predominantly in endothelium and mesenteric nervous plexi and the expression pattern is similar in affected and unaffected tissue

A weak expression of PD-L1 was found mainly in endothelial cells in mucosal and submucosal layers either in ileum and colon samples. Positive reactions were also observed among myenteric nervous plexi. To confirm the specificity of PD-L1 for Cajal cells, we used GIST samples, stained as described below, in which neoplastic cells revealed a high positive reaction. The expression pattern was similar in UC, CD and unaffected tissue samples (Fig. 6).

PD-L1 expression does not differ between mucosa, submucosa and muscularis propria of ileum and colon wall

We examined the divergence in the expression of PD-L1 between mucosa, submucosa and muscu-

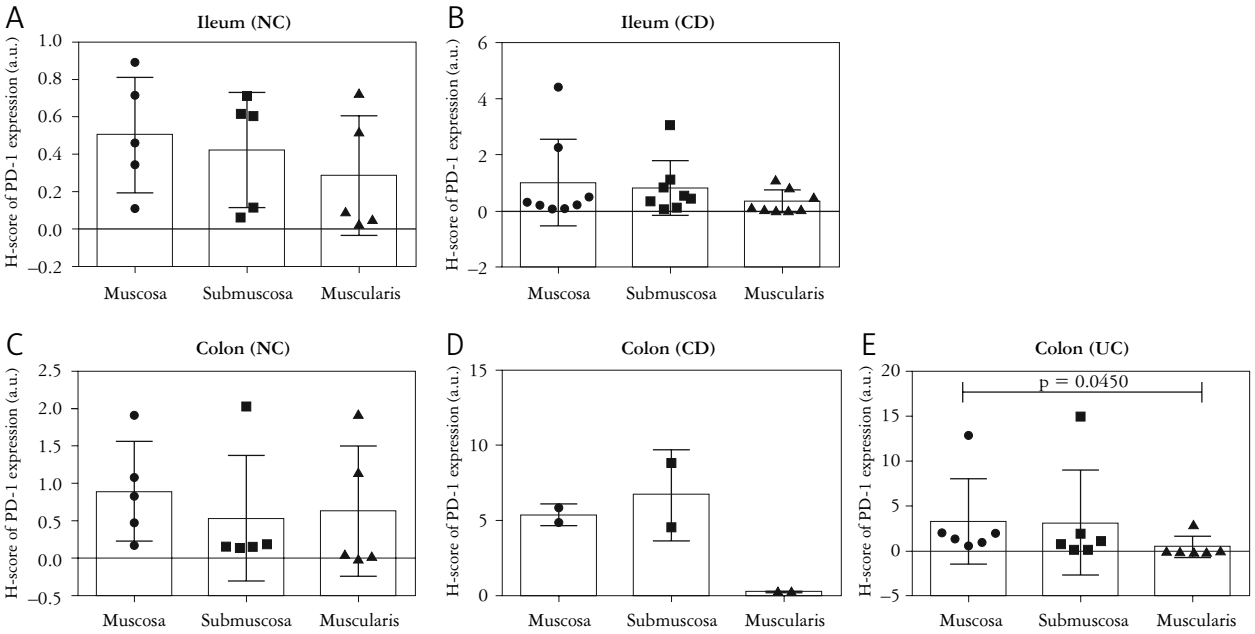

Fig. 3. Expression of PD-1 in mucosa, submucosa and muscularis. A) Control group (NC) – ileum; B) CD samples – ileum; C) Control group (NC) – colon; D) CD samples – colon; E) UC samples colon. Microscopic preparations after immunohistochemical staining with anti-PD-1 antibody were scanned and the H-score was determined for each sample with QuPath software. Data are presented as median ± range. Statistically significant differences were determined using the Kruskal-Wallis test followed by the Dunn's test

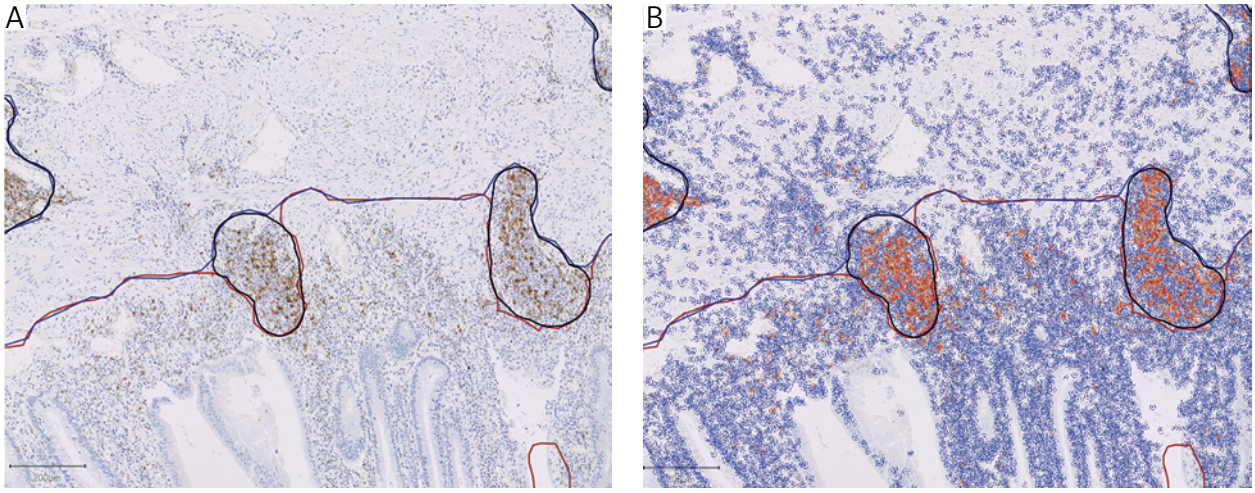

Fig. 4. Illustration of manual separation of lymphatic follicles in QuPath software (A) and positive cell detection (B). Scale bar 200 μm, original magnification 5×, images obtained from digitalized slides with NDP.view2 software (Hamamatsu)

laris propria layer in each study group. Although the results did not reach statistical significance, we observed a trend to express PD-L1 more prominently within muscularis propria layer in the colon samples (Fig. 7). This observation reflects the expression pattern of PD-L1 observed in the microscopic slides.

PD-L1 expression is not altered in samples from patients with IBD

To determine if PD-L1 expression in colon and ileum is altered in IBD, we compared the general, mucosal, submucosal and muscularis H-score in CD, UC and the control group. As for PD-1, we distinguished

lymph follicles as a separate analysis group, based on microscopic observations. In each study group, a similar expression level was observed as shown in Fig. 8. The comparison of expression in individual layers also revealed no difference between groups.

Discussion

As has been described, PD-1 (CD279) serves as a critical factor for maintaining the balance between immunity and self-tolerance. As a key regulatory receptor expressed on T-cells, B-cells and some myeloid cells, it counteracts the costimulatory signals during lymphocytes activation. Despite similarities in function, the spectrum of cells expressing PD-1 is broader compared to CTLA-4 – another major immunoregulatory transmembrane, that suggests the unique role of PD-1 in the regulation of immune response [13]. In the intestine, the highest expression of PD-1 was described in Payer's patches mostly in the light zone of germinal centers (GC), which is considered as the region of B-cell selection [14].

Studies on knockout mice with PD-1 deficiency (Pdcd1−/−) revealed developing organ-specific autoimmune diseases with the varied incidence among certain mice colonies [15], including SLE-like disease [16], diabetes [17], cardiomyopathy [18], hydronephrosis or gastritis [19]. Mechanisms responsible for the effects of PD-1 depletion include invigoration of T-cells, as well as the production of autoantibodies, that suggests an alteration of both T-cell and B-cells function [18, 19].

Assessment of PD-1/PDL-1 expression has become an important step routinely used in histopathological diagnostics of non-small cell lung cancer, urothelial carcinoma, and renal cell cancer. Despite several years of experience with PD-L1 as a predictive biomarker, the problem of interobserver variability remains an obstacle for pathologists due to the expression of PD-L1 in both neoplastic and non-neoplastic cells, marker heterogeneity and varying positivity thresholds [20, 21]. Therefore, digital scoring appears to be a promising method to make the expression assessment more robust and reliable [22]. Several reports confirmed that QuPath can serve as an effective tool for PD-1 and PD-L1 immunopositivity scoring, that could overcome the problem of variability between samples observed in tissue microarrays or small biopsies, as PD-1/PD-L1 axis is involved in dynamic immune reactions [23, 24]. Application of the method allowed us to obtain more objective and quantitative data on the spatial expression of targets.

In our cohort, PD-1 expression was not detected in unaffected ileum nor colon tissue, where the immunopositivity was present only in lymphatic follicles. It corresponds with the findings from the literature, which describe strong PD-1 expression in lymphoid

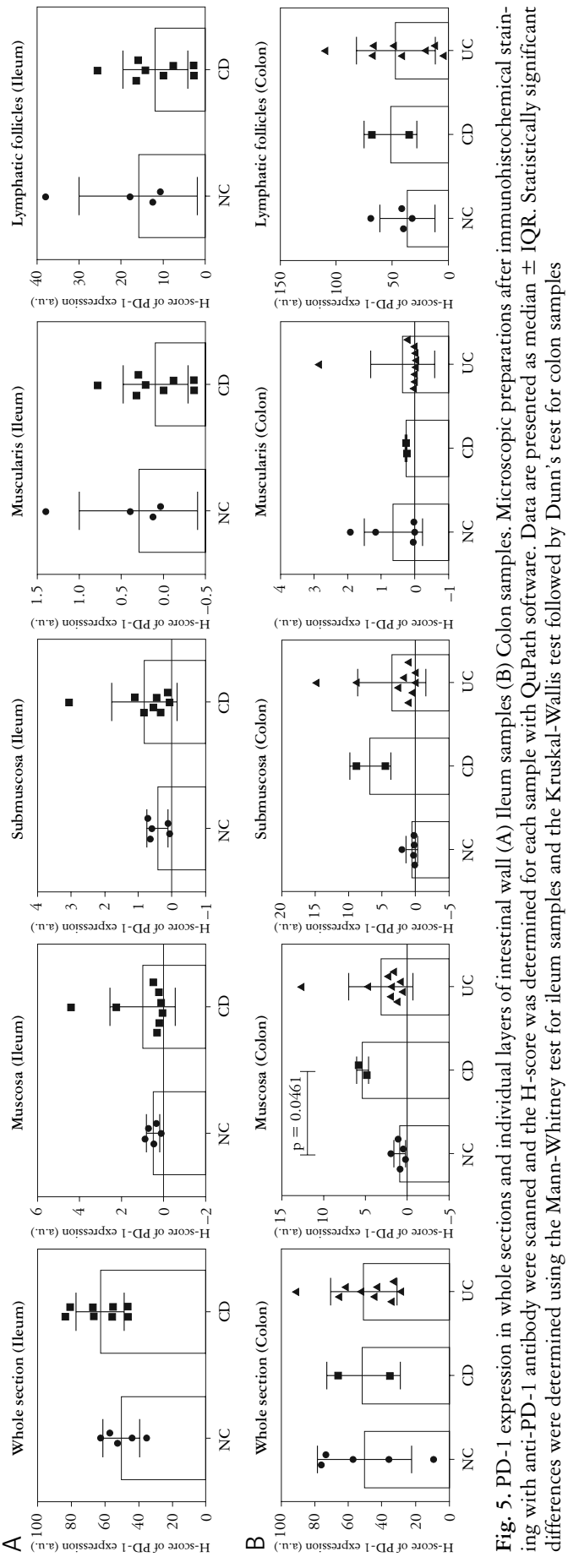

Fig. 5. PD-1 expression in whole sections and individual layers of intestinal wall (A) Ileum samples (B) Colon samples. Microscopic preparations after immunohistochemical staining with anti-PD-1 antibody were scanned and the H-score was determined for each sample with QuPath software. Data are presented as median ± IQR. Statistically significant differences were determined using the Mann-Whitney test for ileum samples and the Kruskal-Wallis test followed by Dunn's test for colon samples

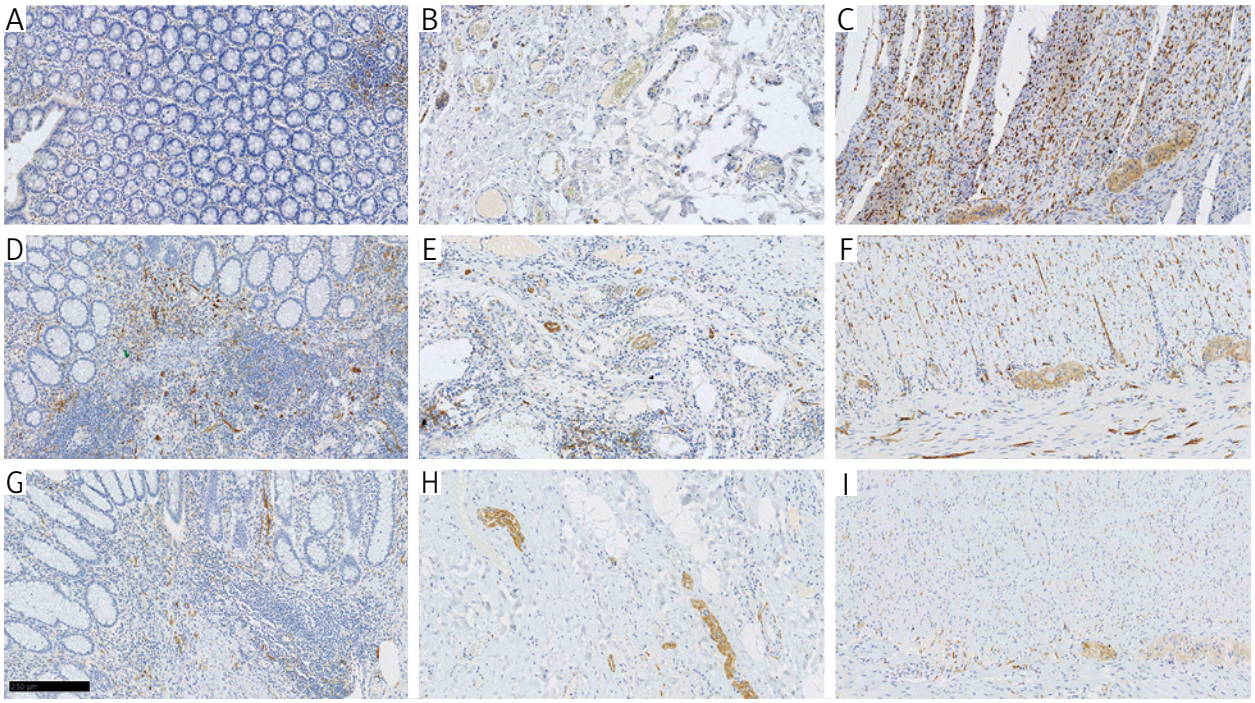

Fig. 6. Images of programmed death ligand 1 (PD-L1) immunohistochemical staining in: unaffected colon tissue – mucosa (A), submucosa (B), muscularis (C); Crohn's disease in colon – mucosa (D), submucosa (E), muscularis (F) and ulcerative colitis samples – mucosa (G), submucosa (H), muscularis (I). Scale bar 250 μm, original magnification 10×, images obtained from digitalized slides with NDP.view2 software (Hamamatsu)

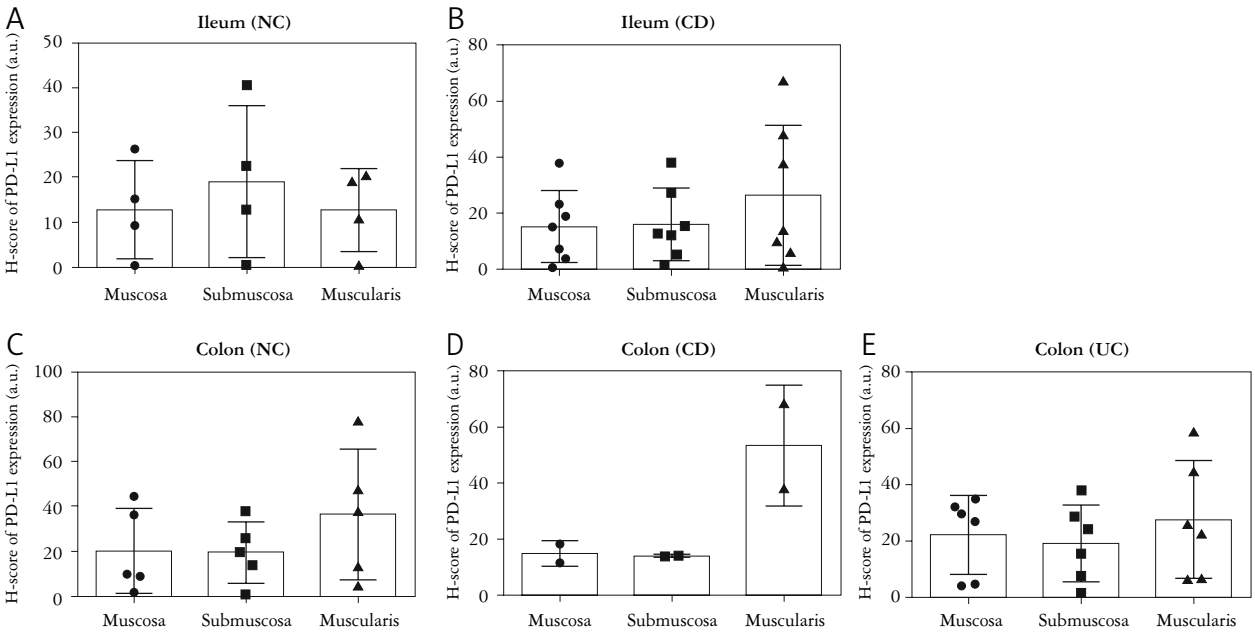

Fig. 7. Expression of PD-L1 in mucosa, submucosa and muscularis. A) Control group (NC) – ileum; B) CD samples – ileum; C) Control group (NC) – colon; D) CD samples – colon; E) UC samples colon. Microscopic preparations after immunohistochemical staining with anti-PD-L1 antibody were scanned and the H-score was determined for each sample with QuPath software. Data are presented as median + IQR. Statistically significant differences were determined using Ordinary one-way ANOVA with multiple comparisons followed by the Dunn's test

tissue, whereas do not detect PD-1 positivity either in glandular or epithelial intestinal cells [25]. Interestingly, we observed PD-1 immunopositivity also in immune cells within the inflammatory infiltration in CD and UC tissue samples. The expression of PD-1 by infiltrating cells may explain the higher positive reaction in mucosal layer in CD comparing to the control group, however the statistical significance

was marginal and further studies are needed to verify these observations.

It is worth noticing that only in UC the difference in PD-1 expression between mucosa and other compartments reached the level of significance. It might be associated with the fact that in UC only the mucosal layer is affected and therefore the inflammatory infiltrate is mainly observed in this part of intestinal wall, whereas in CD the inflammation is transmural, thus the immune cells are dispersed in each layer. However, due to a small number of analyzed cases, the significance of the observation remains limited.

In the human colon, PD-L1 has been revealed to be highly expressed within the peripheral nerves, whereas in endothelial and glandular cells the expression was not detected [26]. Our observations followed that pattern in unaffected controls as well as in IBD samples. Although the difference between individual parts of intestinal wall did not reach significance, we observed the tendency for higher expression of PD-L1 in muscularis layer, which reflects the presence of Cajal cells in this compartment.

There are some reports described deficiency of Cajal cells in small intestine of patients with CD and hypertrophy of those cells in colon tissue affected with UC [27, 28]. However, our results did not confirm those findings, since we have not seen an alteration in PD-L1 expression in muscularis layer in IBD comparing to the control group.

Both for the PD-1 and for PD-L1 expression analysis the levels of expressions were divergent within each study group. These observations suggest that the expression of PD-1 and its ligand may be heterogeneous between individuals and expression of PD-1 and it may be associated with multiple factors. The studies investigating the presence and immunological function of PD-1/PD-L1 in colitis seem to confirm these conclusions showing contradictory results. In the study of Kanai *et al*. [29] PD-1and PD-L1 mononuclear cells in the lamina propria were markedly increased in inflamed mucosa from patients with UC or CD compared with normal controls, as well as in colitic mice comparing to control. Nakazawa *et al*. [30] reported that intestinal epithelial cells from patients with IBD but not normal controls expressed PD-1 and PD-L1 protein on their surface. Recently Roosenboom *et al*. [31] described PD-1+ and PD-L1+ lymphocytes in the colonic mucosa of UC patients comparing to normal control and – interestingly – to CD group. The observations of PD-1 expression in UC and PD-1 inhibitor-associated colitis have also shown contrary results suggesting that the role of PD-1 pathway in IBD-pathogenesis could be more complex, than it has been expected [31, 32]. Chulkina *et al*. [33] made an attempt to summarize these observations, concluding that the relative con-

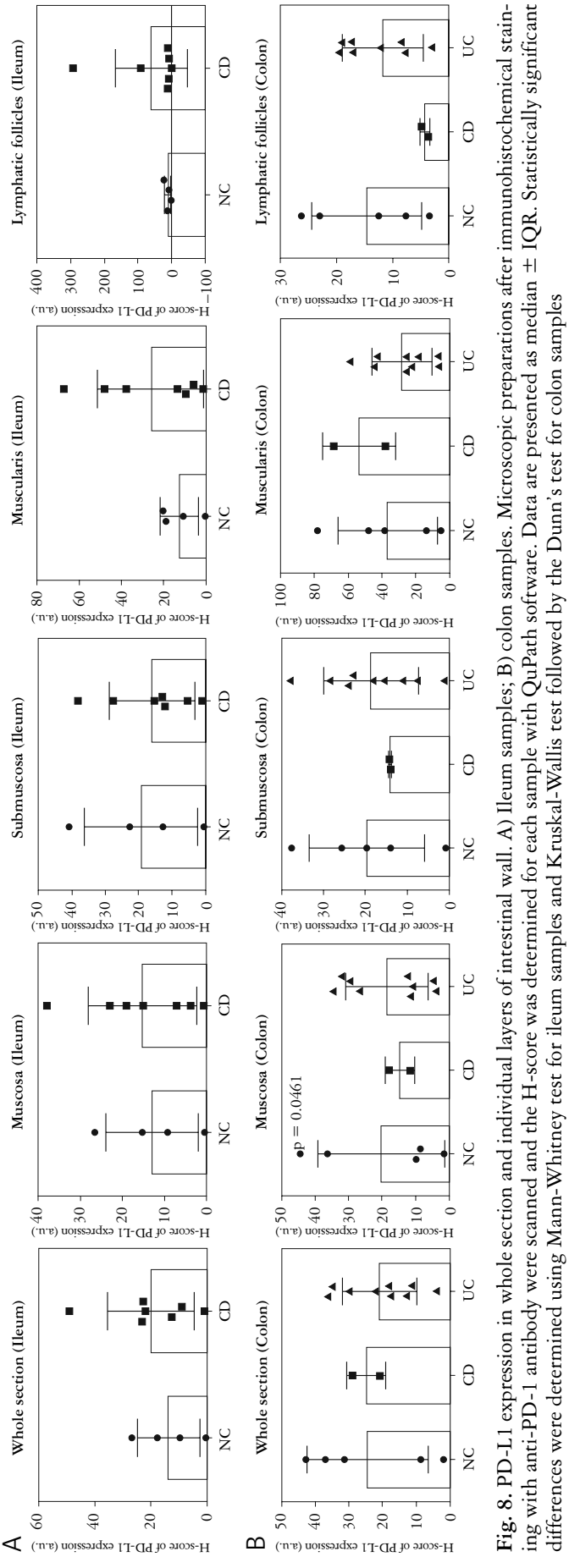

Fig. 8. PD-L1 expression in whole section and individual layers of intestinal wall. A) Ileum samples; B) colon samples. Microscopic preparations after immunohistochemical staining with anti-PD-1 antibody were scanned and the H-score was determined for each sample with QuPath software. Data are presented as median ± IQR. Statistically significant differences were determined using Mann-Whitney test for ileum samples and Kruskal-Wallis test followed by the Dunn's test for colon samples

tribution of the regulatory pathway to PD-L1 expression may depend on the cell type and type of stimuli and may vary depending on the type of pathogenesis. Thus verification of PD-L1 molecular regulatory mechanisms in specific tissue types in the particular pathological process is needed. Therefore, our report describing divergence in expression may support this hypothesis and suggests the immunohistochemical analysis should not be sufficient to measure these subtle mechanisms in the pathogenesis of IBD.

Despite more than two decades of extensive investigation, there is still a lot of uncertainties about the function of PD-1 and its ligands as a rheostat of immune responses. Understanding the delicate mechanism responsible for immunomodulation by PD-1 and its ligands may lead us to the appreciation of this precise signaling pathway as a potential target for therapy [34, 35].

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The authors declare no conflict of interest.

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