Decreased number of colonic tuft cells in quiescent ulcerative colitis patients

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Background Colonic tuft cells are epithelial chemosensory cells involved in barrier integrity, modulation of inflammatory responses and gut homeostasis. Recent evidence indicates an involvement of tuft cells in ulcerative colitis pathogenesis, though mechanisms remain largely unknown.
Here, we quantified the colonic tuft cell population in patients with quiescent ulcerative colitis as compared to patients without identified colonic disease (controls).
Methods In this retrospective study, we obtained endoscopic colonic sigmoid biopsies from 14 patients with quiescent ulcerative colitis and from 17 controls. In a blinded central-reading design, we identified tuft cells by immunohistochemistry using a cyclooxygenase-1 antibody as a marker and performed a simple counting by visual inspection. Poisson regression was employed for statistics and results were adjusted for gender, age and smoking status.
Results Ulcerative colitis patients demonstrated a 55% reduced tuft cell count in colonic mucosa compared with the control group (95% confidence limit: range 31–71%, $P=0.0002$). Ulcerative colitis patients had a mean tuft cells count of 46 tuft cells/mm^2 (95% CI, 36–59), while controls demonstrated a mean of 104 tuft cells/mm^2 (95% CI, 79–136). No interactions of other covariates, such as age, smoking status, total duration of ulcerative colitis disease and duration of clinical remission prior to study inclusion were detected between ulcerative colitis patients and controls.
Conclusion Quiescent ulcerative colitis patients have a relatively low number of colonic tuft cells. Further studies are warranted to explore the potential involvement of tuft cells in ulcerative colitis pathogenesis. Eur J Gastroenterol Hepatol 33: 817–824

Introduction

Tuft cells were discovered more than 60 years ago, and yet their function and implication in human disease are still not understood [1]. They occupy a minor fraction of the epithelial lining of several tissues, such as the respiratory tract, ducts of the pancreas, testicular ducts and various parts of the gastrointestinal tract [2]. In adult mice, tuft cells constitute less than 1 % of the intestinal epithelial cells, where they apparently function as chemosensory cells, important for maintaining homeostasis, mucosal barrier integrity, and even orchestrating immunologic responses towards helminthic and protozoan infections [3–10].

A suspected involvement of tuft cells in various diseases is steadily attracting more attention by basic and clinical researchers. Especially, involvement in the pathogenesis of ulcerative colitis and colitis-associated cancer has been proposed [11,12].

Ulcerative colitis is an idiopathic chronic inflammatory bowel disease (IBD) characterized by latent quiescent periods exacerbated by sudden relapses of colonic mucosal inflammation with abdominal pain, increased stool frequency and bloody diarrhea [13]. The pathogenesis, including factors that trigger initiation and relapse of ulcerative colitis disease activity, is not fully understood. Interestingly, a growing body of preclinical evidence supports tuft cells to play a role in protection against certain enteric infections and inflammation, albeit investigations are still needed to show a causal and direct involvement of tuft cells in any human gastrointestinal disease [11]. So far, very few studies have focused on tuft cells in man. Based on animal studies, however, tuft cells are likely pivotal for sustaining colonic mucosal barrier integrity and alleviating inflammatory responses [7,9,11,14–16].

In terms of inflammation, tuft cells have recently been identified as the predominant colonic epithelial cell-derived source of interleukin 25 (IL-25), an important factor in the initiation of type 2 immune responses and recruitment of eosinophils [3,4,9,10,17,18]. Interestingly, IL-25 levels are lower in both serum and colonic mucosal

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biopsies from ulcerative colitis patients with active disease. Similar trends exist for patients with quiescent ulcerative colitis disease [19].

In this study, we test the hypothesis that patients with quiescent ulcerative colitis have an altered number of colonic tuft cells. By quantifying the colonic tuft cell populations, we compared the tuft cell counts in quiescent ulcerative colitis patients (i.e., clinical, endoscopic and histologic remission) to controls (without ulcerative colitis). Furthermore, we evaluated cyclooxygenase-1 (COX-1), cytokeratin 18 (CK18), hematopoietic prostaglandin D synthase (HPGDS) and doublecortin-like kinase 1 (Dclk-1) as potential tuft cells markers. The most complete staining of the tuft cell population was achieved with a COX-1 antibody and thus used for tuft cell identification.

Materials and methods

Study population

We included colonic sigmoid biopsies retrospectively from ulcerative colitis patients referred to endoscopy for disease management. The control group consisted of patients referred for a colonoscopy on nonspecific suspicion of colonic disease due to symptoms such as abdominal pain and altered stool pattern. Controls were deemed colon healthy based on normal findings by endoscopy and biopsy histology. Biopsies from 31 subjects (14 with quiescent ulcerative colitis and 17 controls) were included. All ulcerative colitis patients were in clinical, endoscopic and histologic remission. Clinical and endoscopic remission was defined by a total Mayo score ≤2 and no subscore >1 [20], and histologic remission by a Nancy histological index score = 0, Fig. 1 [21]. Most ulcerative colitis patients (86%, 12/14) had been in clinical remission for more than 3 months prior to study inclusion and the majority (86%, 12/14) had a Mayo endoscopic subscore of 0. Only two of the ulcerative colitis patients (14%, 2/14) had a Mayo endoscopic subscore of 1 in the rectum, and thus not at biopsy site. Controls had normal endoscopic and histological findings. The number of tuft cells was determined in a blinded fashion by simple visual counting of COX-1 immuno-positive cells in the epithelial lining of colonic biopsies from quiescent ulcerative colitis patients and controls, as shown in Fig. 2a. Patients were excluded if suffering from other acute or chronic gastrointestinal diseases; that is, colonic neoplasia or diverticulosis, celiac disease, dyspepsia, lactose intolerance and irritable bowel syndrome. Patients were also excluded if regularly treated with a nonsteroidal anti-inflammatory drug or nonselective COX-inhibitor, as COX-positive tuft cells might be affected. Other use of medication, including preventive treatment in ulcerative colitis patients, was allowed. Basic patient characteristics, including medications are listed in Table 1.

All endoscopies were performed and assessed by local physicians. To ensure nonbiased consistent disease activity assessment by Mayo endo subscore, all endoscopies from ulcerative colitis patients were recorded and evaluated with a blinded central reading by an external experienced gastroenterologist.

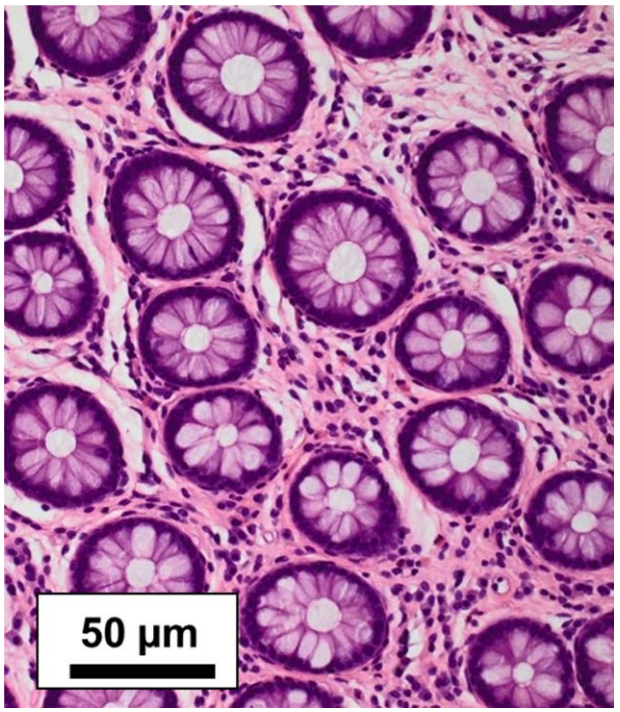

Fig. 1. H&E staining of colonic mucosal biopsy (×200 magnification). Representative histological features in included subject with quiescent ulcerative colitis. All ulcerative colitis patients were in histologic remission defined by a Nancy histological score of 0.

Biopsy collection and preparation

Biopsies were extracted from the sigmoid colon about 30 cm from the anal verge, on retraction of the endoscope, using standard biopsy forceps (Boston Scientific, Radical Jaw 4, outside diameter of 2.2 mm). Biopsies were fixed immediately in 4% paraformaldehyde and subsequently embedded in paraffin. Upon further preparation, biopsies were cut in 4 µm thick sections.

Immunohistochemical staining and identification of colonic tuft cells

For immunofluorescence staining, paraffin colon sections were dewaxed, rehydrated and blocked in blocking buffer (2% BSA and PBS) and incubated overnight at 4°C. Antibodies for COX-1, CK18, HPGDS and two antibodies for Dclk-1 were used. Primary antibodies were detected with AlexaFluor-conjugated secondary antibodies, Table 2. Tissue sections were mounted using ProLong Gold Antifade Mountant with DAPI (P36931, Thermo Fisher Scientific) and analyzed using an IX71 Olympus microscope and XM10 Olympus camera. For quantification of tuft cells numbers, tissue was treated as described above and incubated with anti-COX-1 overnight at 4°C followed by incubation with biotinylated secondary antibodies diluted 1:200 in blocking buffer. After washing, endogenous peroxidase was blocked with 3% H_2O_2 in PBS, and sections were incubated with vectastain reagents (Vectastain ABC Kit PK 4000, Vector laboratories Inc., California, USA) and stained with 3'-Diaminobenzidine solution (Cat. No. 4170, Kementec Diagnostics, Denmark) and counterstained with Mayer's Haematoxylin (Ampliqon, Denmark).To identify a suitable tuft cell marker for the quantification studies, we applied double-labeling immunohistochemistry on human colon sections and compared three antibodies specific for tuft cell marker proteins; anti-COX-1, HPGDS and CK18

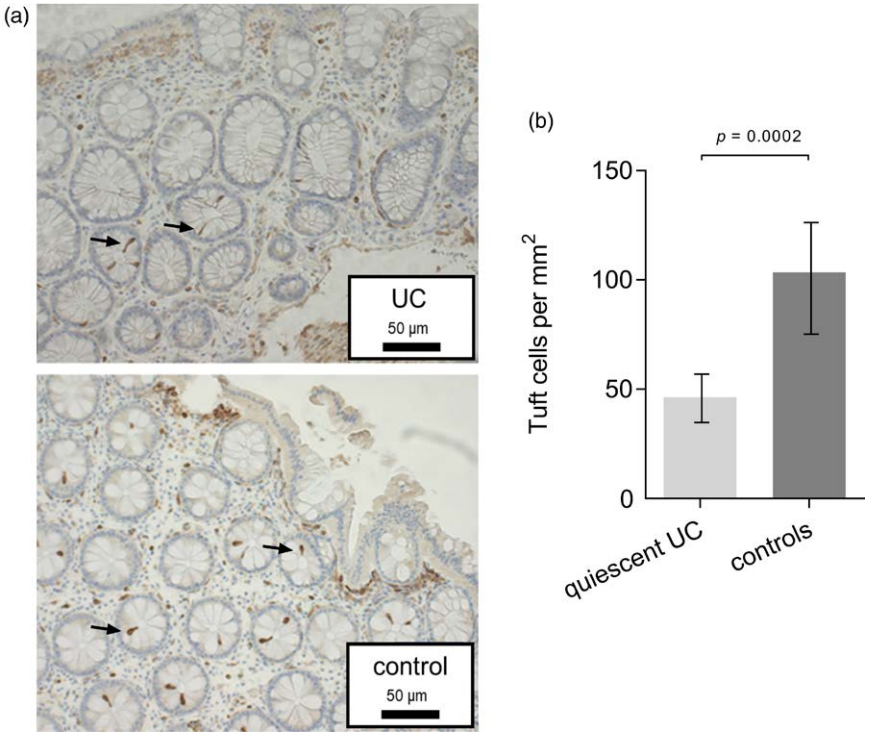

Fig. 2. Tuft cells in human sigmoid colon. (a) Examples of immunohistochemical staining of colonic tuft cells with cyclooxygenase-1 specific antibody in a patient with quiescent ulcerative colitis and a control patient. Enlargement × 200. Arrows indicate tuft cells. (b) Colonic mucosal tuft cell numbers per square millimeter in 14 patients with quiescent ulcerative colitis compared to 17 controls. Data presented as mean tuft cells/mm^2. The *P* value is based on Poisson regression with covariate adjustment.

Table 1. Study population characteristics

	Ulcerative colitis	Controls
Total number	14	17
Males/females	9/5	9/8
Mean age, years (range)	39 (23–75)	46 (20–68)
Smoking habit		
Active/nonsmoker	0/14	0/17
History of maximum disease extent		
Proctitis	3	N/A
Left sided colitis	4	N/A
Pancolitis	7	N/A
Disease duration, mean months (range)	124 (3–324)	N/A
Remission duration, mean months (range)	14 (2–61)	N/A
Medication		
No treatment	3	5
5-ASA	5	0
Anti-TNFα	2	0
Azathioprine	3	0
Antiepileptic	1	0
Antihistamine	0	2
Inhaler (β2-agonist, steroid)	0	2
Contraception	0	1
Proton pump inhibitor	0	1
Statins	0	2
Thiazides	0	2
Thyroid hormone	0	2
Vitamin/iron-supplements	1	2

Baseline values for patients with quiescent ulcerative colitis and controls. 5-ASA, 5-aminosalicylic acid; N/A, non-applicable; TNFα, tumor necrosis factor alpha.

staining, Fig. 3. Furthermore, we also tested two antibodies specific for the murine Dclk-1 (ab37994 and ab31704, Abcam) on the murine and human colon.

Counting of tuft cells

Images were recorded using a Zeiss Axioplan 2 plus microscope (Jena, Germany) fitted with a Photometrics CoolSNAP camera (Tucson, Arizona, USA). Colonic mucosal tuft cells were counted independently in each biopsy by the first three authors, all blinded to clinical diagnosis. A mean value from three counts was employed. Biopsy sizes were calculated, and data presented as mean counts of tuft cells per square millimeter mucosa (tuft cells/mm^2) with standard deviation (±SD). The analysis was performed using Image-Pro 9.1 software.

Statistical analysis

Statistical analysis of tuft cell counts was compared between groups using Poisson regression with a log of the biopsy size as an off-set and adjustment for covariates: age, sex and smoking status. Robust standard errors were used to allow for a possible overdispersion. In the sensitivity analysis, we also tested whether the group effect depended on sex, age or smoking status by including interaction terms. Additionally, a separate analysis was performed on ulcerative colitis patients only to determine the influence of total disease duration and duration of clinical remission prior to study inclusion.

Ethical considerations

This study was approved by the scientific ethical committee of Copenhagen (H-18000856) and the Danish Data Protection Agency (P-2019-313).

Results

Immunohistochemical staining and identification of colonic tuft cells

As shown in Fig. 3, anti-COX-1 displayed a near-complete overlap with anti-CK18 in solitaire, epithelial cells

Table 2. Summary of the antibodies used

Peptide target	Manufacturer/cat. no.	Host	Immunohistochemistry dilutions
COX-1	Santa Cruz Biotechnology/sc-1752	Goat	1:100
Cytokeratin-18	Cayman/160013	Mouse	1:25
Hematopoietic prostaglandin D synthase	Progen/61028	Rabbit	1:100
Doublecortin-like kinase 1	Abcam/ab37994	Rabbit	1:25
Doublecortin-like kinase 1	Abcam/ab31704	Rabbit	1:700
Alexa fluor 488 anti-Goat IgG	Thermo Fischer Scientific/A11055	Donkey	1:200
Alexa fluor 568 anti-Rabbit IgG	Thermo Fischer Scientific/A10042	Donkey	1:200
Alexa fluor 568 anti-mouse IgG	Thermo Fischer Scientific/A10037	Donkey	1:200
Alexa fluor 488 anti-Rabbit IgG	Thermo Fischer Scientific/A21206	Donkey	1:200
Goat Biotinylated anti-rabbit IgG	Vector Labs/BA-1000	Goat	1:200

Antibodies used and their specifications.
COX-1, cyclooxygenase-1.

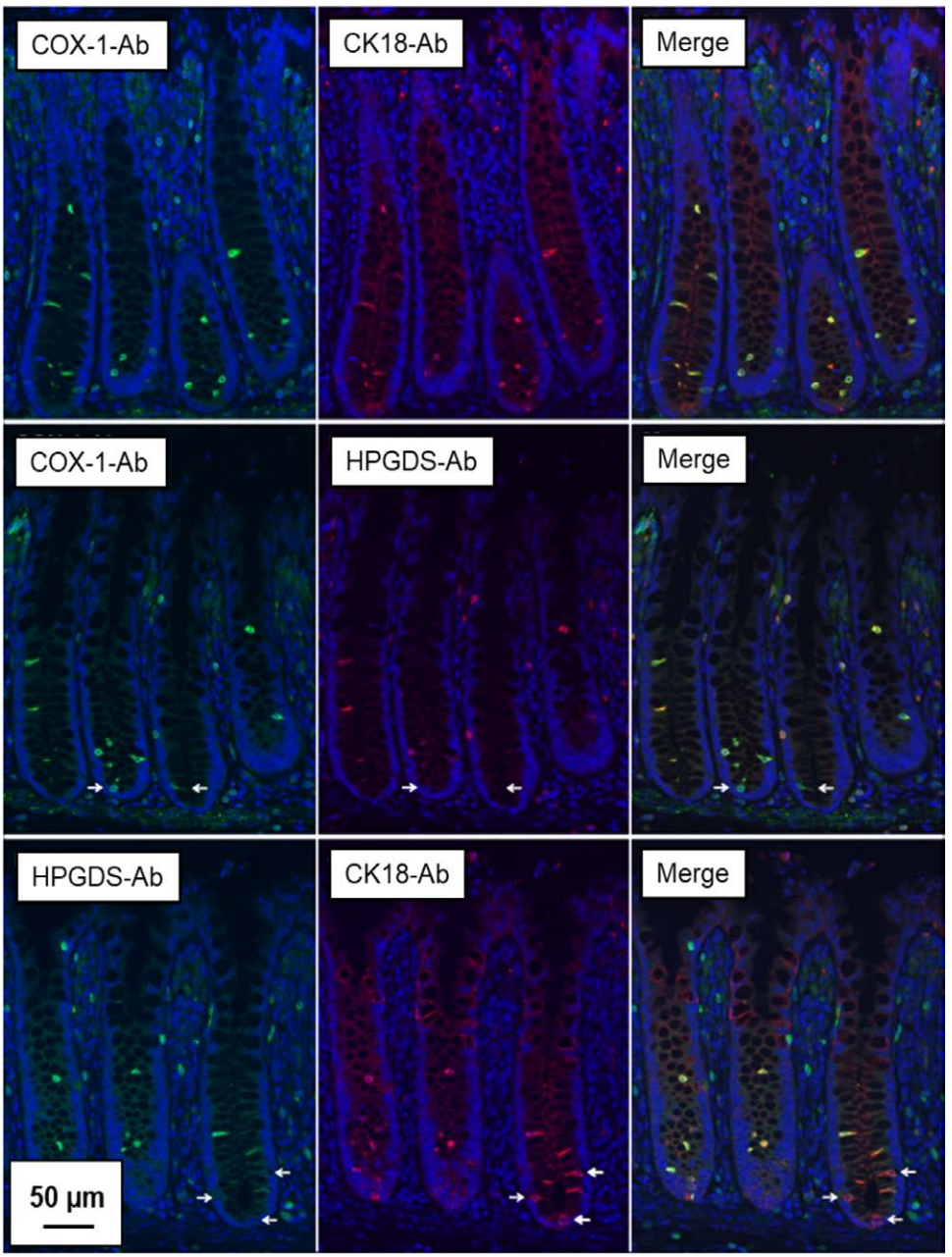

Fig. 3. Double labelling immunofluorescence staining of tuft cell marker proteins in human colon. Representative fluorescence microscopy images of human colonic tissue sections co-stained with cyclooxygenase-1 (COX-1), hematopoietic prostaglandin D synthase (HPGDS) and cytokeratin 18 (CK18) antibodies demonstrating a high degree of overlap. Arrows indicate cryptal tuft cells stained with either COX-1 or CK18 but lacking HPGDS staining. Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Bar = 50 µm.

of the human colonic crypts. Similarly, anti-COX-1 and anti-HPGDS showed overlap in most epithelial cells; however, a few COX-1-positive cells at the base of the crypts appeared devoid of HPGDS staining. Anti-CK18 and anti-HPGDS also displayed a large degree of overlap, and again, some CK18-positive epithelial cells at the base of the crypts appeared to lack anti-HPGDS staining. While both Dclk-1 antibodies gave positive epithelial staining in murine colon sections (data not shown), only ab37994 reacted on human colon sections. Meanwhile, we failed to obtain convincing immunolabelling compared to COX-1, HPGDS and CK18 antibodies, and Dclk-1 was therefore not used in the subsequent studies, Fig. 4. Taken together, these results show that anti-COX-1 overlap with other tuft cell marker protein antibodies; anti-HPGDS and anti-CK18 in most cases in the human colon sections and indicate that anti-COX-1 gave a more complete staining of the total tuft cell population.

Colonic tuft cell population in quiescent ulcerative colitis

Biopsies from ulcerative colitis patients and controls yielded counts of 46 ± 7 tuft cells/mm^2 (range 36–59) and 104 ± 21 tuft cells/mm^2 (range 79–136), respectively, Fig. 2b. With covariate adjustment quiescent ulcerative colitis patients demonstrated a highly significant 55% lower number of colonic tuft cells as compared to controls (95% CI, 31–71%, $P = 0.0002$).

Statistical evaluation of interaction and covariance

No statistically significant associations between groups and other covariates were detected, indicating a constant group effect across values of other covariates. For ulcerative colitis patients, we found no statistically significant effects of total disease duration ($P = 0.64$) nor of duration of clinical remission prior to study inclusion ($P = 0.095$).

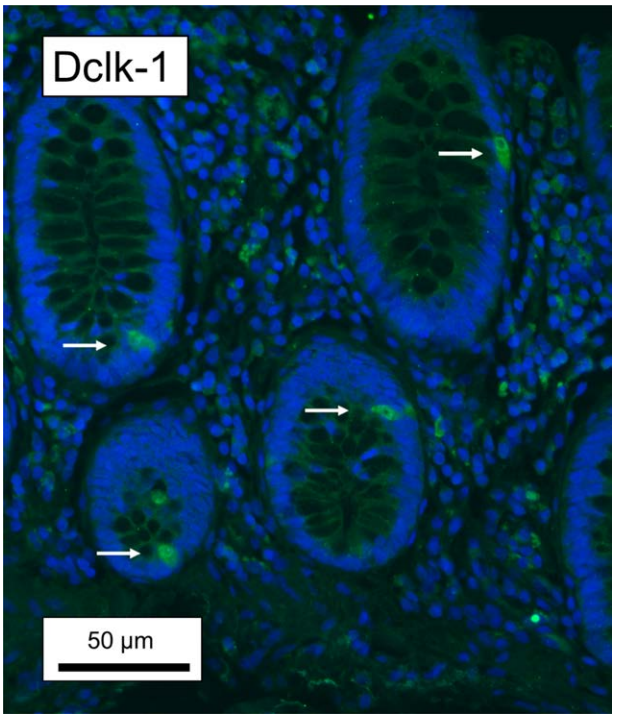

Fig. 4. Representative fluorescence microscopy images of human colonic tissue sections co-stained with doublecortin-like kinase 1 (Dclk-1, ab37994). Arrows indicate staining of epithelial cells. Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Bar = 50 µm.

Discussion

Animal studies indicate that tuft cells are involved in sustaining mucosal integrity and barrier function, mediating inflammatory processes, as well as the postinflammatory mucosal healing [3,4,11,14–16]. Whether similar associations and mechanisms exist in man is largely unknown.

The present observational retrospective study provides data which shows that ulcerative colitis patients in clinical, endoscopic and histologic remission (quiescent disease stage) have a relative reduced number of colonic tuft cells. A prospective expanded study in ulcerative colitis patients with an active disease state is needed in order to further explore and test the involvement of tuft cells in ulcerative colitis pathophysiology.

Investigation of tuft cells in man has been scarce, and the mechanisms behind the observed lowered number of tuft cells in quiescent ulcerative colitis remain unknown. A genetic predisposition, an increased apoptosis of tuft cells and a reduced positive feedback circuit of proliferation from progenitor tuft cells and stem cells have all been hypothesized, but none of them confirmed [11]. Interestingly, one study suggests that number of tuft cells increase in the human stomach during inflammation, hyperplasia and metaplasia [22]. Other studies find an increased number of tuft cells in patients with diarrhea-predominant irritable bowel syndrome [23], while a loss of tuft cells in the duodenum is observed in pediatric patients with severe duodenitis [24]. Accordingly, there is a need for additional studies to examine the association and potential direct and indirect involvement of tuft cells in specific gastrointestinal diseases in active and silent phases.

Detection of human colonic tuft cells

In this study, we identified human colonic tuft cells with COX-1 antibodies, which has proven to be a valid marker for human colonic tuft cells [25,26]. Further, we compared the anti-COX-1 immunolabeling pattern with that of tuft cells markers such as HPGDS, CK18 and Dclk-1. COX-1 immunolabeling displayed substantial overlap with that obtained using specific antibodies against HPDGS and CK18, Fig. 3. In contrast, we failed to obtain convincing immunolabeling for Dclk-1 using two commercially available antibodies; ab31704 failed completely (data not shown), while ab37994 yielded an unconvincing result, Fig. 4.

Several studies using different commercially available Dclk-1 antibodies have confirmed them as excellent markers for intestinal tuft cells in mice, including colonic mucosa [5,16,25,27]. Meanwhile, except for one study by Aigbologa *et al.*, [23] none of these markers have been claimed to be reliable markers for human colonic tuft cells [25], thus corroborating our finding with 2 Abcam Dclk-1 antibodies as unreliable markers for tuft cells in the human colonic mucosa, Fig. 4. Looking carefully at the figures in the study by Aigbologa *et al.*, we can conclude that their data are not convincing for the rabbit Dclk-1 polyclonal antibody as a reliable marker for human colonic tuft cells. Contrary, the COX-1 antibody from Santa Cruz has

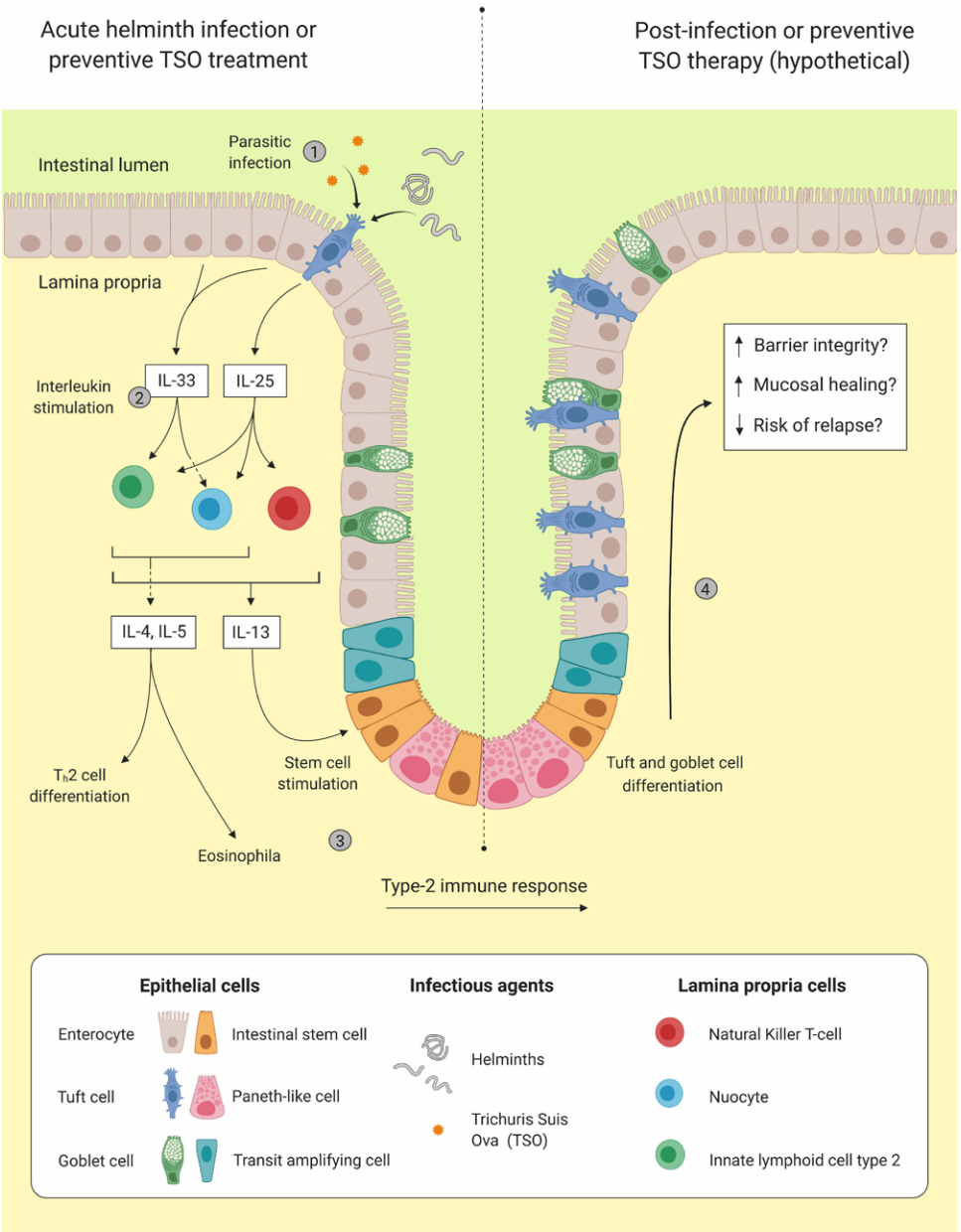

Fig. 5. Presentation of the potential beneficial effects of an infection with helminths or preventive *Trichuris suis ova* (TSO) treatment in patients with ulcerative colitis. As data on human tuft cells are rare, this theory is obviously based on limited amount of evidence. Left side shows colonic mucosa of ulcerative colitis patient in quiescent disease stage before infection/preventive TSO therapy. (1) Upon helminths/TSO exposure tuft cells increase secretion of interleukin (IL)-25, while enterocytes release IL-33. (2) Both IL-25 and IL-33 stimulate lamina propria immune cells such as, type-2 innate lymphoid cells, natural killer T-cells and nuocytes to produce and release type-2 inflammatory cytokines (e.g. IL-4, IL-5 and IL-13). (3) A type-2 immune response is initiated including differentiation of T helper type 2 cells (T_h2), recruitment of eosinophils and stimulation of intestinal stem cells. Right side shows potential beneficial effects of infection on colonic mucosa of ulcerative colitis patient. (4) Stimulation of intestinal stem cells leading to tuft cells and goblet cell differentiation and hyperplasia, which normalize and strengthen mucosa.

turned out to be an excellent marker for human tuft cells, also in the colonic mucosa [25,26], supporting our use of this antibody as a marker for human colonic tuft cells.

Pro-biotic Trichuris suis ova treatment of ulcerative colitis with tuft cell implications

Our finding of a lowered number of tuft cells and the observed lower levels of IL-25 in circulating blood and colonic mucosal biopsies by Su *et al.*, [19] may indicate an underlying failed immunosuppressive action eliciting part of the ulcerative colitis symptoms. Ulcerative colitis is less common in developing countries and genetic factors are not solely responsible for these differences [28,29]. Additional explanations are related to higher rates of gastrointestinal infections with parasitic agents [30]. As such, ingestion of pro-biotic *Trichuris suis* ovae (pig whipworm eggs, TSO) has been observed to be associated with increased numbers of IL-25 secreting intestinal tuft cells in mice, and may therefore be an interim treatment [4]. Furthermore, several studies have tested the effect on gut immunity provoked by parasitic worms (e.g. *T. suis*) as therapies for IBD in mouse models and in humans [31–36]. So far, the majority of clinical studies with *T. suis*

intervention therapy has been explored in patients with Crohn's disease and only one in ulcerative colitis patients [34,35,37]. Summers *et al*., find that TSO treatment is both well tolerated and effective in reducing disease activity in ulcerative colitis [36,38]. If TSO therapy for ulcerative colitis is associated with elevation of tuft cell numbers, we speculate that tuft cells are involved in TSO mode of action and as such, an efficacy and predictive biomarker for disease activity. Figure 5 illustrates a hypothetical model on preventive TSO therapy.

Study limitations

As some ulcerative colitis patients were referred for only a sigmoidoscopy, we might have failed to detect polyps or other uncharacteristic noncontinuous activity in the right and transverse segments of the colon.

The retrospective character of this study and the relatively low number of patients and observations per patient are also obvious inherent limitations. Also, the visual counting of tuft cells, and lack of supplementing methods, also carry some limitations.

Furthermore, despite tuft cells being the only COX-1 positive cells of the epithelial lining, COX-1 positive immune cells from the lamina propria can migrate towards the epithelium, as is seen sometimes in ulcerative colitis. If this is the case, however, it would only make the difference in tuft cell numbers even more pronounced, and therefore not influence the conclusion.

Despite results indicating tuft cell involvement in ulcerative colitis, this study is still exploratory. However, the study outcome speaks clearly in favor of further exploring tuft cells in ulcerative colitis pathogenesis and maybe even therapy eventually. As such, patients with and without active disease and effects of TSO intervention on colonic tuft cell numbers in ulcerative colitis patients could be addressed.

Conclusion

Patients with quiescent ulcerative colitis demonstrated a relative reduced number of colonic mucosal tuft cells when compared to controls without ulcerative colitis in this relatively small, observational retrospective study. Accordingly, tuft cells might be involved in ulcerative colitis pathogenesis. Finally, tuft cells are potential novel diagnostic, predictive and prognostic ulcerative colitis disease biomarkers and ultimately maybe even a therapeutic target.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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