

Original Article

Characterization of lymphoid follicles with red ring signs as first manifestation of early Crohn's disease by conventional histopathology and confocal laser endomicroscopy

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Abstract: Background and aims: Clinical observations suggest that the lymphoid follicles (LFs) may play a crucial role in the pathogenesis of inflammatory bowel disease (IBD), especially in Crohn's disease (CD) as the site of initial mucosal inflammation. The aim of this study was to compare the morphology of LFs in CD, ulcerative colitis (UC) and control patients using confocal laser endomicroscopy (CLE) in correlation to histological and immunohistochemical findings of biopsies. Methods: 79 patients with IBD (46 with CD, 32 with UC and 1 patient with indeterminate colitis) and 67 controls patients were enrolled prospectively in this study. Median age was 32.5 years (range 19-65) and 37.4 years (range 20-65 years) respectively. To analyze the LFs, standardized images from the terminal ileum and the colon were taken using white-light video endoscopes. Additionally, CLE was performed to analyze subsurface structure of LFs. Targeted biopsies of LFs were analyzed using haematoxylin and eosin stain and immunohistochemistry. Results: LFs were seen in all parts of the lower GI tract, but mostly in the terminal ileum and cecum. Endoscopy in 15 out of 17 patients with the first manifestation of CD showed LFs surrounded by red ring (so-called red ring sign, RRS). Histologically, LFs with RRS showed hypervascularization at the base of the LFs associated with numerous CD15-positive granulocytes. Similar features were not seen in LFs without RRS and in the control group. In some LFs with RRS early aphthous ulcers were seen. Using CLE, RRS showed abolished normal crypt architecture, crypt distortion, increased cellular infiltrate within the lamina propria, and dilated vessels. Conclusion: LFs with RRS probably represent an early sign of aphthous ulcers in early CD and, thus, may be considered as early markers of first manifestation and flares in CD.

Keywords: Endomicroscopy, CLE, red ring sign, Crohn's disease, lymphoid hyperplasia, inflammatory bowel disease, ulcerative colitis, lymphoid follicle, pathology

Introduction

Inflammatory bowel disease (IBD) is characterised by an abnormal immunological response to environmental antigens, especially the enteric bacterial flora within the gut lumen, in genetically susceptible persons [1, 2]. Antigenic invasion may be facilitated by decreased integrity of the mucosal barrier due to increased permeability, disruption, and ulceration of the surface epithelium in the course of the disease [3-5]. Antigen presentation and involvement of the

acquired immune system takes place within the lymphoid tissue in the gut immune system [6]. The lymphoid follicles (LFs) are distributed throughout the intestine and consist of a number of B-cell follicles with intervening T cells. A single layer of surface epithelial cells covers each follicle forming a dome between the surrounding villi [7, 8].

The follicle-associated epithelium (FAE) provides a route of entry for antigens and microorganisms. Specialized epithelial M cells of the FAE

deliver samples of antigens from the lumen directly to intraepithelial lymphoid cells and to organized lymphoid tissue, also playing a key role in the invasion of certain bacteria and viruses [9].

LFs have always been thought to be the main portal of entry for potential pathogens, and it has been suggested that aphthous ulceration in Crohn disease (CD) originates in FAE over the LFs [4, 10, 11]. The pathogenesis of the ulcerating lesions in IBD was the subject of several studies, trying to provide a better insight into the pathogenesis of the disease [6, 12, 13].

The aim of this study was the analysis of LFs with irregular vessel and epithelial pattern, comparing the morphology of LFs in IBD and control patients using confocal laser endomicroscopy (CLE) in correlation to histological results of targeted LFs biopsies, immunological findings and medical history.

Material and methods

Patients: Inclusion and exclusion criteria

Consecutive patients with and without IBD (CD and ulcerative colitis/UC) and control patients who underwent ileo-colonoscopy for other purposes were prospectively enrolled during the period 2006 to 2009. The diagnoses were made by correlation of patient's medical history, results of endoscopy and histopathology. The study protocol was reviewed and approved by the local Ethics Committee of the University of Erlangen, Germany (N°4032), and was conducted according to the declaration of Helsinki. Informed consent was obtained from all patients participating in the study.

Patients were included if they met the following inclusion criteria: age above 18 years, ability to provide written informed consent, known IBD (CD or UC) or non-IBD patients (surveillance, abdominal pain).

There were same exclusion criteria for both groups: inability to provide written informed consent, pregnancy or breast-feeding, severe uncontrolled coagulopathy, impaired renal function, known allergy to fluorescein, systemic infections e.g. pneumonia or sepsis, gastrointestinal mediated allergy, any kind of carcinoma (for the many patients were examined in the setting

of screening for colorectal carcinoma).

Clinical classification

According to the Montreal classification, CD patients were classified as follows [14]: 1. Age at diagnosis - <16 years, 17 to 40 years or >40 years. 2. The disease location (terminal ileal, colonic, ileocolic, upper gastrointestinal). 3. The pattern of disease (inflammatory, fistulating, or stricturing). UC was classified as follows: 1. Ulcerative proctitis - inflammation is limited to the rectum (proximal extent of inflammation is distal to the rectosigmoid junction). 2. Left sided UC (distal UC) - inflammation limited to a proportion of the colorectum up to the splenic flexure. 3. Extensive UC (pancolitis) - involvement extends beyond the splenic flexure.

Endoscopic study procedures

For bowel preparation all of the patients received as laxative polyethylenglycol-based medication (Kleanprep®) [15]. During the examination the patients received upon requirement sedation with Midazolam and Pethidin or disoprivane.

The LFs were macroscopically evaluated using white light magnification video endoscopy (Pentax Zoom EC 3830 and Pentax EC 3840). Approximately 15 endoscopic images per patient in connection with histological results and medical history were used to analyse the LFs. To clarify mucosal changes we used Kudo classification of pit pattern analysis [16, 17].

Confocal laser endomicroscopy (CLE, EG-3870 CIX, Pentax, Tokyo, Japan) was performed in 15 patients (eight with LFs with red ring sign/RRS in terminal ileum or cecum, seven patients undergoing surveillance colonoscopy with no pathological findings or LF`s without RRS) to compare the findings with histopathology. Furthermore, patients with LFs with RRS underwent follow-up colonoscopy after 8 weeks.

For CLE, 5ml 10% Fluorescein was administered intravenously. Targeted confocal images were obtained from RRS (n=15) and their surrounding mucosa and from LFs without RRS (n=20) and afterwards targeted biopsies were taken. Image data were collected and evaluated by two endoscopists, specialised in gastroenterology, blinded to histopathological findings.

Histology and immunohistochemistry

Biopsies taken from LFs have been fixed in formalin and embedded in paraffin. Sections were stained with standard hematoxylin and eosin stain. In addition, immunohistochemistry (IHC) was performed on fresh-cut 5 µm sections using a polymer Kit purchased from Zytomed systems (Zytomed systems Ltd., Berlin, Germany), and the following antibodies: CD15 (1:50, anti-human CD15 mouse monoclonal Ab, clone C3D-1, DakoCytomation, Hamburg, Germany), CD34 (1:1000, monoclonal, clone QBEND10, Immunotech, Marseille, France) and Keratin cocktail (1:200, clone KL1, Immunotech).

Statistical analysis

Statistical analysis was performed by Medistat medical statistic GmbH, Kronshagen/ Germany using SPSS and BiAS for Windows (SPSS, Chicago, IL, USA). The distribution of the LFs between the groups of the patients was tested using the Kolmogorov-Smirnov-Test. Kruskal-Wallis test was used to prove the distribution of LF between the subgroups. All the statistic tests and correlations were adjusted using Bonferroni and Holm methods [18].

Results

The mean age of the 146 patients who agreed to participate in this trial and signed informed consent was 32.5 yrs (range 19-65 yrs). 79 patients had a known or suspected IBD (46 CD, 32 UC and 1 with indeterminate colitis). The mean age was 30.2 yrs (range, 19-50 yrs) and 34.5 yrs (range 22-62 yrs) for CD and UC patients respectively. CD was known in 31 patients and first diagnosed in 15 patients. In the UC group the disease was known in 28 and first diagnosed in 4 patients. The patient with indeterminate colitis was 25 years old. Approximately the same number of consecutive patients (67), age-matched to IBD group (mean age 37.4, range 20-65 years), who agreed to participate in this study and were undergoing ileo-colonoscopy was included as control group (surveillance, abdominal pain), as was recommended by biometrics. The characteristics of the patients in terms of medication intake, disease duration, activity and involvement pattern in both CD and UC groups are presented in the **Tables 1** and **2**.

Distribution and macroscopic appearance of

lymphoid follicles with and without red ring sign

LFs were seen in all parts of the lower GI tract, but mostly in the terminal ileum and the cecum. The LFs presented themselves mostly flat or slightly elevated, 1-2mm in size (range 0.5-3mm).

In the normal colonic mucosa of control patients, LFs were rarely seen (**Figure 1A**) or showed LFs of the flat type (**Figure 1B**). In contrast, LFs with RRS were macroscopically different due to their pale center (**Figure 1C**).

Interestingly, 15 out of 17 patients with early CD (including 2 patients from the control group, where CD could be diagnosed histologically for the first time) showed endoscopically different patterns of the LFs, being surrounded by red ring (RRS). The LFs with RRS were present mostly in the terminal ileum, but also in the cecum; they were seen as clusters of 1-2 mm round or oval slightly raised spots, each with a pale center and an erythematous border, with a normal vascular pattern of the intervening mucosa between lesions (**Figure 1C**).

Follow-up colonoscopies for the evaluation of mucosal healing were performed in the 8th week after start or change of the therapy in accordance with previous studies [19-21]. On control colonoscopies (using WLE and CLE), the LFs with RRS were present almost only in CD group. They indicated beginning aphthous ulcers in medical treatment non-responders (**Figures 1D, 2A** and **2B**). In patients responding to medical therapy LF with RRS partly disappeared. The characteristics of patients with RRS are presented in **Tables 3-4**. In all of the patients with RRS-positive LFs, the disease had an inflammatory pattern (according to Montreal classification).

One patient with newly diagnosed extensive UC (initially suspect to have an infectious colitis) showed LFs with RRS in colon ascendens, similar to those in CD. Although histology of biopsy specimens suggested UC, the possibility of CD could not be definitely ruled out in this case. Two further sigmoidoscopies in this patient did not reveal chronic inflammation. The cortisone therapy was stopped because of steroid-refractory course. In this case, mucosal healing could be achieved at week 8 after starting a therapy with anti-TNF alpha antibodies.

LF with RRS in early Crohn disease

Table 1. Characteristic of the patients in terms of medication intake and disease activity in CD group

CD	Number of patients	Age at diagnosis (years)			Disease activity CDAI score			The extent of the disease			Duration of disease in months (mean duration and range)	Duration of therapy in months (mean duration and range)
		<16	17 to 40	>40	Remission < 150	mild to moderate	severe > 450	ileal	colonic	ileo-colonic		
Without medication	13		12	1	2	3	8	7	1	5	10 (1-18)	6 (2-12)
5-ASA	10		1	9	5	2	3	1	8	1	24 (3-42)	20 (4-36)
Immunsuppressive therapy (cortisone, azathioprine, 6-MP)	23		18	5	12	19	1	4	4	15	12 (1-22)	6 (2-18)
Anti-TNF a	1	-	1	-	-	1	-	-	-	1	5	5

Table 1A. Characteristic of the patients in terms of medication intake and disease activity in CD group

CD	Number of patients	Age at diagnosis (years)			The pattern of the disease			The extent of the disease		
		<16	17 to 40	>40	inflammatory	fistulating	stricturing	ileal	colonic	ileo-colonic
Without medication	13	-	12	1	10	2	1	7	1	5
5-ASA	10	-	1	9	8	3	1	1	8	1
Immunsuppressive therapy (cortisone, azathioprine, 6-MP)	23	-	18	5	17	15	1	4	4	15
Anti-TNF a	1	-	1	-	-	1	-	-	-	1

LF with RRS in early Crohn disease

Table 2. Characteristic of the patients in terms of medication intake and disease activity in UC group

UC	Number of patients	Disease activity based on CAI score		The extent of the disease			Duration of disease in months (mean duration and range)	Duration of therapy in months (mean duration and range)
		< 4: remission	≥ 4: active disease	ulcerative proctitis	left sided	extensive UC		
Without medication	11	1	10	4	5	3	8 (1-24)	6 (1-15)
5-ASA	11	8	3	4	4	3	36 (2- 56)	32 (4-38)
Immunosuppressive therapy (cortisone, azathioprine, 6-MP)	10	6	4	2	3	5	12 (2-46)	8 (2-36)
Anti-TNF a	1	-	1	-	-	1	6	1

Table 3. Characteristic of the patients with the presence of LFs with RRS in CD and control group.

LFs with RRS	Number of patients	Follow-up week 8 after start or change of the therapy	The extent of the CD			Duration of disease in months (mean duration and range)	Disease activity based on CDAI score		
			ileal	colonic	Ileo-colonic		remission < 150	mild to moderate	severe > 450
CD known	4	2/ aphthous ulcers 2/ mucosal healing	3	-	1	6 (2-20)	-	3	1
CD suspected and histologically confirmed	9	8/aphthous ulcers 1/mucosal healing	7	-	2	8 (4-12)	-	7	4
Control group, histological confirmation of CD	2	1/ aphthous ulcers 1/mucosal healing	2	-	-	2 (1-4)			

Table 4. Characteristic of the patients with the presence of LFs with RRS in UC and IC group.

LFs with RRS	Number of patients	Follow-up week 8 after start or change of the therapy	The extent of the UC			Duration of disease in months (mean duration and range)	Disease activity based on CAI score	
			ulcerative proctitis	left sided	extensive UC		< 4: remission	≥ 4: active disease
IC known	1	1/mucosal healing	-	-	1	6	0	1

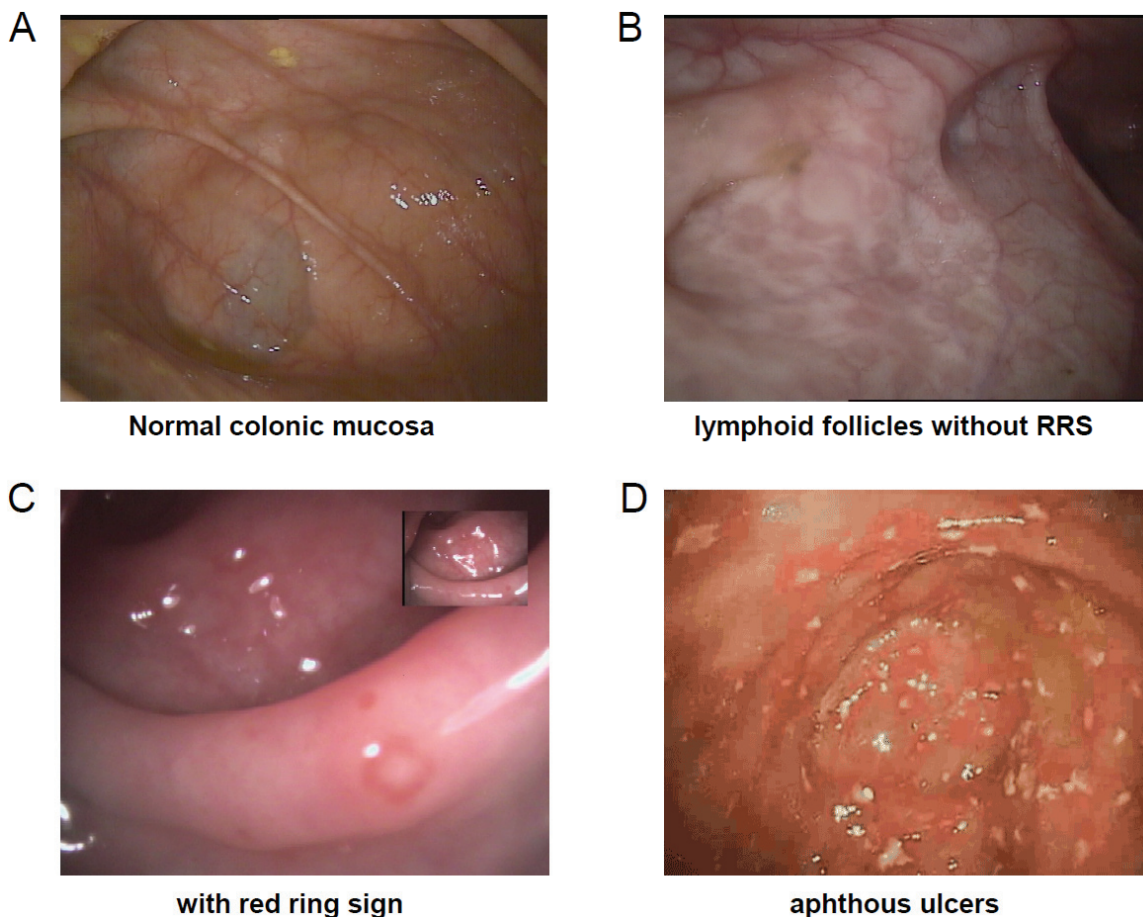


Figure 1. Endoscopic appearance of lymphoid follicles with red ring sign. A. Normal colonic mucosa (cecum); B. Lymphoid follicles (cecum); C. Lymphoid follicle with red ring sign in the terminal ileum in a patient with first diagnosis of Crohn's disease (terminal ileum); D. Development of aphthous ulcers from lymphoid follicles with red ring signs in a follow-up ileo-colonoscopy in the same patient with Crohn's disease (see 1C).

Assessment of Lymphoid follicles using confocal endomicroscopy

To better evaluate LFs with RRS during ongoing endoscopy, we performed CLE. Both techniques, probe based CLE and CLE were assessed and showed similar results. The endoscopic setting was as follows: we have analysed the surrounding area of the LF by scanning for quadrants and the LF itself. Both the optical quadrant biopsies from LFs surrounding tissue and normal LF surrounding tissue exhibited no signs of inflammation. Vessel density, leakage and crypt architecture were similar in both groups of LFs.

CLE of the LFs with RRS demonstrated abolished normal crypt architecture within the LFs, crypt distortion, increased number of dilated

vessels with fluorescense leakage in some cases and increased cellular infiltrate within the lamina propria (**Figures 2A-D**). In contrast, LFs without RRS exhibited normal crypt architecture and vessels; cellular infiltrate could not be observed. These data shows that signs of inflammation were restricted to the LFs with RRS and were not seen in LFs without RRS or in the surrounding tissue.

Lymphoid follicles: Histopathologic findings

In accordance with CLE, histological evaluation of LFs in control patients illustrated always normal ileo-colonic epithelium without any signs of inflammation (**Figures 3A and 3B** images on left side). **Figure 3C** shows histological images with use of immunostaining with antibodies to Kera-

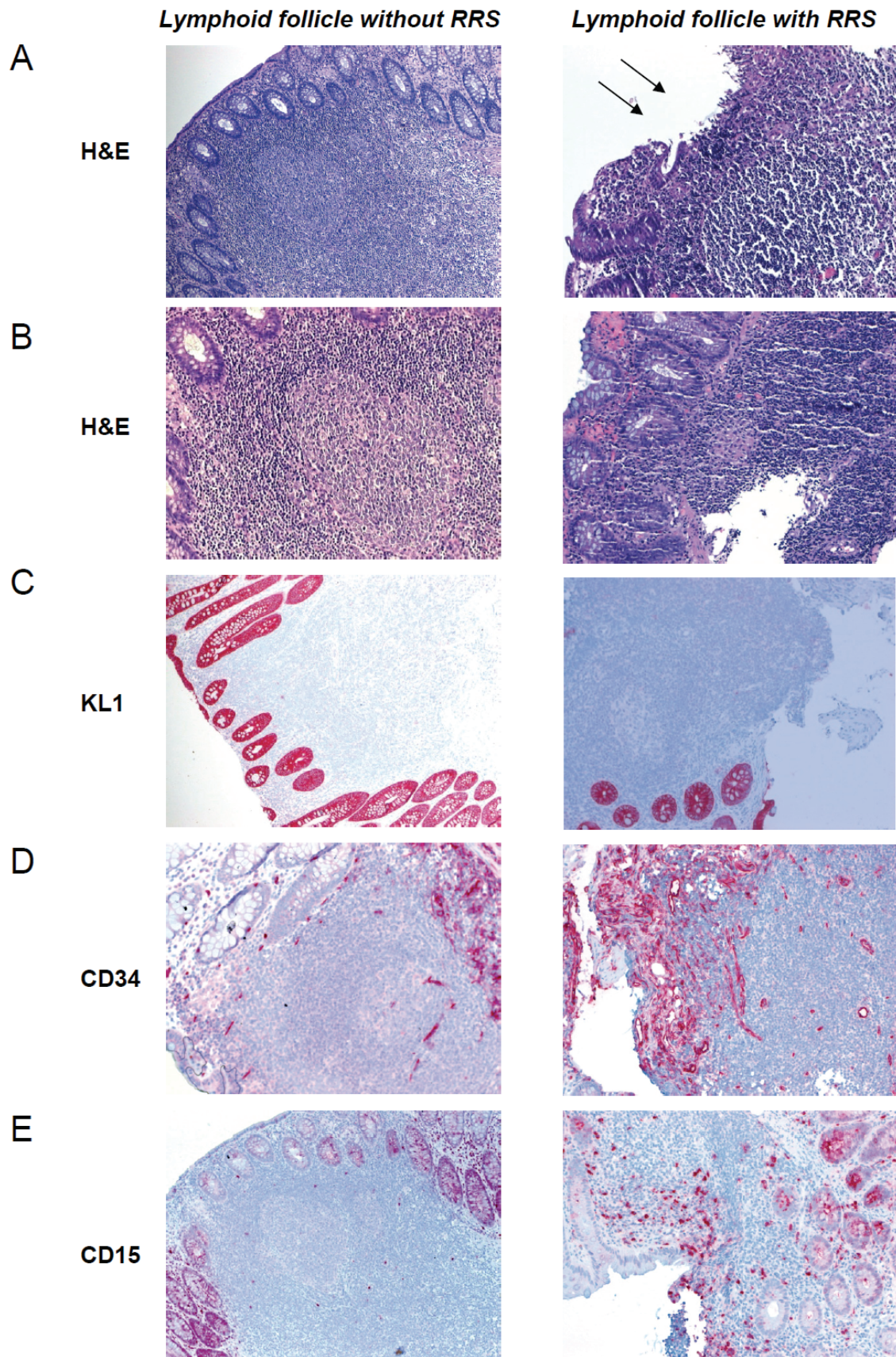


Figure 2. Microscopic analysis of hyperplastic lymphoid follicles in normal control (left column) and in patients with red ring sign (right). A. Hematoxylin and eosin (H&E) staining of hyperplastic lymphoid follicle. No epithelial defect is seen in normal control. In late state, lymphoid follicles with red ring sign show small epithelial defects as a sign of beginning aphthous changes (arrows, right picture). H&E stain, Original magnification x100; B. Higher magnification of control specimen showed activated follicle with germinal center. Lymphoid follicles with red ring in which no epithelial defect was observed may occasionally contain small non-caseating epithelioid granuloma (right). H&E stain, Original magnification x200; C. Keratin immunostaining (KL1): A normal lymphoid follicle is shown on the left side. Right: The deep epithelial defect with development of aphthous ulcer in hyperplastic lymphoid follicle with red ring is demonstrated. Original magnification x100; D. CD34 immunostaining: prominent small-caliber blood vessel may be seen at the base of lymphoid follicle without red ring sign (left picture), note absence of prominent vessels on the side of the LF. Immunohistochemistry in a patient with RRS demonstrates dilated blood vessels and an increase in the number of blood vessels both on sides and at the base of the LF (right picture). Original magnification x200; E. CD15 immunostaining: LF with RRS showed numerous infiltrating granulocytes (right) compared to scarce or absent granulocytes within normal control LF on the left. Original magnification x100.

tin (KL1) in LFs with intact mucosa (left side), and of LF with RRS in the late stage with beginning aphthous ulcer (right side).

Because RRS appeared to be a macroscopic sign of either hyperaemia or inflammation, we focused on analyzing influx of inflammatory cells and changes in blood perfusion characterized by numbers of vessels and vessel architecture (**Figures 3C-E**). Importantly, there was a marked difference comparing blood vessel patterns in LF's with and without RRS using CD34 IHC. In LFs without RRS, prominent vessel architecture was seen at the base of LF (**Figure 3D**, left image). No vessels were observed on the side of the LF. In contrast, IHC in patients with RRS demonstrates dilated blood vessels, and an increase in the number of blood vessels, both surrounding and at the base of the LF (**Figure 3B**, right image). IHC staining with antibodies to CD15 exhibited a marked infiltrate of granulocytes within the lymphoid tissue in RRS-positive LFs, which was not seen in LFs without RRS (**Figure 3E**).

Taken together, histology of LFs in control patients illustrated normal ileo-colonic epithelium without any signs of inflammation. The LFs with RRS showed epithelial defect in the late state. Histological findings correlated with those of CLE.

In the early stage two major criteria may differentiate LFs without RRS from those with RRS, namely, hypervascularisation surrounding and underneath the LF, and increased inflammatory activity above the LF indicated by presence of CD15 positive granulocytes (**Figure 3**). Both criteria were also detected by endomicroscopy with a high accuracy. Furthermore, crypt distortion was also seen at the surface of LFs with

RRS using CLE and histology, as a sign of chronic inflammation. In some cases epithelial erosions were observed using CLE and were all confirmed by histopathology. Interestingly, two cases of CD showed minute epithelioid cell granulomas within the hyperplastic LFs.

Discussion

This study describes and compares the appearance and morphology of LFs in the terminal ileum and colon, especially cecum in patients with IBD as well as controls. We could demonstrate that LFs with different epithelial and vessel pattern were seen almost only in CD and hence assume that LFs with red rings around them (RRS) might predict early CD; therefore these were examined in detail, using IHC and CLE.

The nature and morphology of the LFs has been determined by correlating results of white light magnification endoscopy, CLE and histopathology, demonstrating that the lesions, interpreted as LFs, belong to lymphoid tissue and were not mucosal elevations of other origin [22].

The morphologic features of LFs with RRS in this study were characterized by macroscopic, endomicroscopic and histologic analysis. Typical features of RRS as assessed by endomicroscopy were the presence of dilated blood vessels, hypervascularisation, and extravasation of fluoresceine characterizing the vessel leakage, crypt distortion and central small epithelial lesions. Histological evaluation correlated well with these endomicroscopic findings.

Additionally, characteristic vessel patterns were observed by immunostaining. Increased vessels were seen above and underneath the LFs with

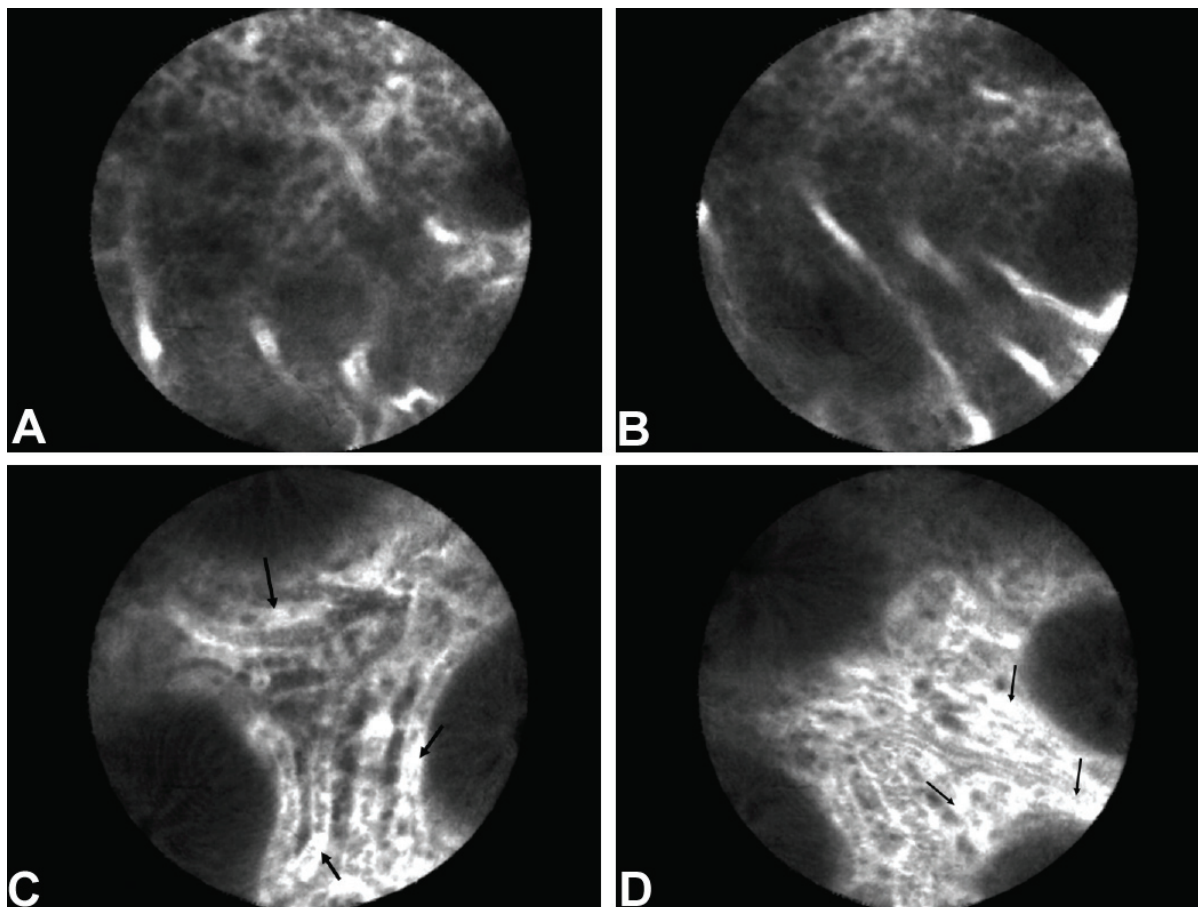


Figure 3. Features of the red ring sign in confocal laser endomicroscopy. A. Confocal laser endomicroscopy illustrates crypt distortion and increased cellular infiltrate within the lamina propria; B. Confocal laser endomicroscopy in a patient with red ring sign. The normal crypt architecture is completely abolished; C. Microvessels appear bright due to the uptake of fluorescein sodium on the surface of the lymphoid follicle with red ring sign. Note the increased number of blood vessels that are all dilated (arrows) and increased in number; D. The red ring sign follicle exhibits a distinguished increased number of blood vessels and fluorescein leaks demonstrated by extravasation of fluorescein around fragile vessels (arrows).

RRS in contrast to hyperplastic LFs without RRS. The red ring develops as a consequence of hyperemia in these dilated mucosal blood vessels surrounding the LF while the pale central area of the red ring corresponds to the well-circumscribed lymphoid aggregate (LF) [23]. Also this finding correlated well with endoscopic observations.

Interestingly, granulocytes as sign of florid inflammatory activity were solely detected in those LFs with RRS. However, granulocytes were occasionally inconspicuous on H&E-stained slides, but they were readily identified by CD15 immunostaining. Furthermore, in some LFs with RRS focal superficial mucosal disruption centered over lymphoid aggregates was

seen. Consistent with these observations, the histopathology of aphthous ulcers in follow-up ileo-colonoscopies of the CD patients with LFs with RRS showed that the ulcers were overlying LFs. When lesions were endoscopically visible as ulcers, the histopathology showed a definite ulcer base, or more extensive area of mucosal loss covered with an exudate consisting of fibrin and granulocytes. These findings indicate that the sites of initial inflammation in CD might be the LFs, where the ulcerations originate from small erosions of the FAE overlying the LFs and that further aphthous ulcers may in part directly develop from LFs with RRS [7].

It has been hypothesized that, LFs surrounded by red ring, might be precursors for develop-

ment of the aphthous lesions in CD [24]. Fujimura et al observed that lymphoid hyperplasia and aphthous colitis might be early features of the primary event of CD [9]. The further colonoscopies in their patients showed progression to more advanced lesions over time. They could demonstrate that the aphthous lesions of CD are preceded by ultrastructural erosions 150-200 microns in size in the FAE of hyperaemic LFs [10]. Another study by Morson et al demonstrated that aphthous lesions occur at sites of accumulations of lymphocytes in the intestinal mucosa [26, 27].

Faigel and Hixson reported finding RRS and ulcerations in the sigmoid colon and rectum in patients whose colonic preparation was carried out applying sodium phosphate solution, suggesting that those features might have been caused by sodium phosphate directly [28, 29]. In our study we did not use sodium phosphate solutions for colon preparation, and RRS were seen mostly in the terminal ileum in CD patients.

Several cases have been reported in which the initial aphthous lesions eventually developed into typical longitudinal ulcers in CD [30, 31]. Chiba et al reported four cases of the RRS, where CD was first diagnosed [13]. In our study, the LFs could be further characterized by using different immunostaining and endomicroscopy. Our results demonstrate that histomorphologic aspects (as shown by histological/immunostaining and endomicroscopy) of LFs with and without RRS are different. In detail, LFs with RRS showed an activated germinal center, edema within the adjacent lamina propria, extravasation of red blood cells, and at the late state focal mucosal disruption with ulcers development; none of these features were seen in LFs without RRS. Furthermore, there was significant difference regarding vessel architecture, granulocyte infiltration and presence of non-caseous epithelioid cell granuloma within some of the LFs in CD.

In our study we did not observe the diffuse lymphoid hyperplasia in IBD patients in general; the expansion of lymphoid tissue depended on involvement pattern and state of the disease and medication (intake of the medication in **Table 1**). Neither polypoid LFs (as in cases of infectious or allergic colitis), nor flat-type LFs, typically seen in the cecum, were seen in the IBD

group (except for uninvolved cecum).

The LFs with RRS were seen at the beginning of the disease, and before acute relapsing. During the relapse, the acute inflammation precluded reliable assessment of the lymphoid tissue.

In summary, we could demonstrate that LFs surrounded by red ring (RRS) might be the first sign of development of aphthous ulcers of CD. To the best of our knowledge, this is the first study, using ileo-colonoscopy (including follow-up endoscopy), different immunostainings and CLE to study the spectrum of morphological features of LFs and their role in the pathogenesis of early IBD.

Abbreviations: Follicle-associated epithelium (FAE), gastrointestinal (GI), gut-associated lymphoid tissue (GALT), endoscopic visible field (VF), inflammatory bowel disease (IBD), immunohistochemistry (IHC), Crohn's disease (CD), lymphoid follicle(s) (LF), lymphoid follicle per visible field (LF/VF), Peyer's patches (PP), red ring sign (RRS), ulcerative colitis (UC).

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