Dendritic Cells and Toll-Like Receptors 2 and 4 in the Ileum of Crohn's Disease Patients

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Abstract We investigated myeloid-dendritic cell (DC) marker and Toll-like receptor (TLR)-2 and 4 distributions in ileal samples from Crohn's disease (CD) patients ($n = 14$) and controls ($n = 13$). In controls, no TLR-2^+ cells were observed, and higher numbers of TLR-4^+ and DC-SIGN$^+$ cells ($P < 0.01$) were detected in ileal samples when compared versus colonic tissues. In non-inflamed CD ileum, TLR-4^+ and DC-SGN$^+$ cells were depleted from superficial areas of the villus, and a significant CD1a$^+$ cell infiltration ($P < 0.01$) was observed when compared to ileal controls and non-inflamed colonic CD samples. In inflamed CD ileum, DC-SIGN$^+$, CD1a$^+$, TLR-4^+ and few TLR-4^+DC-SIGN$^+$ cells were detected as well as CD83 depletion. No correlation between TLR-2 and DC markers was detected in CD samples. A unique distribution of myeloid-DC markers characterized the CD ileum. Also, the presence of significant amounts of ileal CD1a$^+$ cells may provide a relevant DC-mediated mechanism for antigen recognition in the pathogenesis of CD.

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Introduction

It is generally accepted that Crohn's disease (CD) is an inflammatory bowel disorder with environmental, immunological and genetic factors contributing to the pathogenesis [1]. Moreover, CD has been characterized by an abnormal dendritic cell (DC) distribution that may result in a deficient regulatory and/or excessive T-cell response when the immune system is challenged and activated [2–7]. DCs are antigen-presenting cells that operate as sentinels taking up antigens from peripheral tissues [8]. Then, they migrate to lymphoid organs where they induce T-cell activation. DCs are difficult to study in vitro because their isolation provokes changes in gene expression that mimic those that occur during inflammation [9]. In contrast, peripheral DCs can be studied in their physiological environment by immunohistochemical analysis [4].

Toll-like receptors (TLR) constitute a family of pattern-recognition receptors that detect conserved molecular products of microorganisms [10, 11]. TLR-2 and 4, which recognize specific bacterial antigens, have been associated with the pathogenesis of CD [12–14]. Specific mutations of TLR-4 have been observed in some patients with CD, which may result in an abnormal innate host defense toward gram-negative bacteria [12, 13, 15]. However, such TLR-4 mutations have not been detected in other populations with this disorder [16, 17]. Also, a TLR-2-dependent pro-inflammatory mechanism was recognized in peripheral blood monocytes from another group of patients [14]. In addition, CD involves increased intestinal epithelial permeability [18–20] which may allow abnormal penetration

of enteric bacterial antigens into the mucosa and a subsequent activation of innate pro-inflammatory mechanisms mediated in part by the up-regulated expression of TLR-2 and/or 4 [6, 21, 22].

To our knowledge, scant information is available regarding DCs in the ileum with CD [2]. Therefore, it is important to conduct a detailed analysis of their distribution and phenotype in this location. In this study, ileal CD samples were analyzed and compared to colonic tissues from CD patients and controls. We investigate the distribution of human myeloid-related DC markers such as CD1a, CD83 and DC-SIGN [23–25]. CD1a is not only a DC marker, but also a transmembrane receptor for self or foreign lipid antigens that act to bind T-cell receptors inducing their subsequent activation [23]. CD83 is a maturation marker that in conjunction with DC-SIGN has been used to characterize DCs in colonic specimens from CD patients [3, 4]. In addition, a correlation between the distribution of these DC markers with TLR-2 and 4 was assessed. We suggest that the expression of these TLR by some ileal DC and/or the presence of $CD1a^{+}$DC may provide evidence for the existence of different mechanisms that may initiate a host defense against bacterial antigens and conduct the pathogenesis of ileitis in CD.

Materials and methods

Patients and tissue specimens

Archival paraffin-embedded gut specimens were obtained from the University of Chile Clinical Hospital and Clínica Las Condes. Distal ileal resections from adult CD patients (2 male, 6 female; mean age 46.3 ± SEM of 4.2 years) operated on as emergency cases were used. No patient was under medical treatment, avoiding the effect of drugs that may interfere with intestinal DC distribution [2, 4]. In addition, colonic tissues from CD patients (two male, four female; 37.8 ± 6.5 years; one cecum, one ascending colon, two transverse and two descending colon specimens) were obtained to further compare small and large gut segments. The diagnosis was made by pathologists on the basis of clinical, bacteriological, radiological, endoscopic and histopathological criteria. Indications for bowel resection were the presence of intestinal perforation, fistula, or stenosis with clinical signs of obstruction. A total of 13 paraffin blocks were obtained with available small intestinal tissue, excluding Peyeŕs patches because of their small number observed in these samples. In five cases two blocks originated from the same patient. For the large intestine, one block per patient was obtained and histologically normal lymphoid follicles were avoided due to the scant amounts detected [4]. Tissues were classified as non-inflamed or inflamed according to a standard scoring protocol for CD histopathology that has been previously described [4, 26]. Non-inflamed controls with normal histology from distal ileal (four male, two female; mean age 51.2 ± 8.0 years) and colonic (five male, two female; 65.8 ± 3.3 years; one cecum, one ascending colon, one transverse colon, one descending colon and three sigmoid specimens) samples were obtained, respectively. Also, indications for resection were adenocarcinoma of the colon, intestinal bleeding, ischemia or obstruction. Serial sections from control and CD tissues were obtained through the complete intestinal wall and processed. The study was approved by the Ethics Committees of the University of Chile Clinical Hospital and Clínica Las Condes.

Antibodies and reagents

Polyclonal biotinylated goat anti-mouse IgG, polyclonal biotinylated rabbit anti-goat IgG, normal mouse IgG isotype control, normal human serum protein, antibody diluting buffer, alkaline phosphatase-conjugated streptavidin, HRP-conjugated streptavidin, fast red substrate system, faramount aqueous mounting medium and Mayer's hematoxylin were obtained from DakoCytomation (Mississauga, Canada). Monoclonal mouse anti-human TLR-2 (sc-21760), specific polyclonal goat anti-human CD1a (sc-7092), DC-SIGN (sc-11038) and TLR-4 (sc-8694) IgG, FITC-conjugated rabbit anti-goat IgG and PE-CY5-conjugated goat anti-mouse IgG adsorbed with human IgG, mounting media for fluorescence and normal goat IgG isotype control were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-human DC-SIGN (MAB-161) and TLR-4 (MAB-1478) IgG were obtained from R&D Systems (Minneapolis, MN). Monoclonal mouse anti-human CD83 (MCA-1582) IgG and DAB substrate kit for HRP were obtained from Serotec Ltd. (Kidlington, Oxford, UK) and Vector Laboratories (Burlingame, CA), respectively.

Immunohistochemistry

Sections were immunostained using standard protocols [4, 27]. Samples were treated with 10% H_2O_2 to block endogenous peroxidase activity, then with 0.05% trypsin to unmask the antigenicity, followed by incubations with universal blocking solution (Lab Vision Corporation, Fremont, CA) and specific F_c-receptor blocking with normal human serum (70 μg/ml). Subsequently, overnight incubation at 4°C was performed with the following primary antibodies: goat anti-human CD1a (18 μg/ml), mouse anti-human CD83

(18 μg/ml), goat anti-human DC-SIGN (16 μg/ml), mouse anti-human TLR-2 (9 μg/ml) or goat anti-human TLR-4 (9 μg/ml). Then, sections were treated with biotinylated goat anti-mouse IgG or biotinylated rabbit anti-goat IgG, incubated with alkaline phosphatase-conjugated streptavidin or horseradish peroxidase-conjugated streptavidin and stained with fast red or DAB substrates for single staining. Positive controls were performed with additional tissue sections. As a negative control, sections were stained with either an isotype-matched antibody of inappropriate specificity or by omitting the primary antibody. Tissue sections were examined avoiding the intravascular compartments. Stained cells were quantified using a magnification of 200× in five different fields/section, excluding unspecific staining and structures lacking a nucleus. Each field included similar proportions of lamina propria (LP) and submucosa, outside PP or lymphoid follicles. In inflamed CD tissues, quantifications were performed in areas infiltrated by clusters of inflammatory cells.

Immunofluorescence

Additional sections were processed as described above and incubated overnight at 4°C with the following pair of antibodies: mouse anti-human DC-SIGN + goat anti-human TLR-4 or mouse anti-human TLR-4 + goat anti-human CD1a (18 μg/ml each one). Then, sections were incubated in the dark with FITC-conjugated rabbit anti-goat IgG for 90 min followed by washes with PBS and another incubation with PE-CY5-conjugated goat anti-mouse IgG for 90 min. Sections were washed, mounted in media for fluorescence and analyzed under a Carl Zeiss confocal LSM510 laser scanning microscope using a standard setting that avoided the overlap of false-positive images.

Statistical analysis

Statistical analysis was performed with Graph Pad (Graph Pad Software, Inc., San Diego, CA) using unpaired *t*-test and ANOVA for simple and multiple comparisons, respectively. Data are presented as means ± standard error (SEM).

Results

Immunohistochemistry for DC-SIGN in control samples showed stained cells distributed throughout the LP and submucosa (Fig. 1a and b). In non-inflamed CD tissues, DC-SIGN$^+$ cells were mostly located in basal areas of the LP and submucosa (Fig. 1c), while they were depleted from superficial areas of the villus (Fig. 1d). Also, DC-SIGN$^+$ cells were observed in the muscular compartment mainly in non-inflamed CD samples (data not shown). In inflamed CD samples, DC-SIGN$^+$ cells were observed in deeper areas of ulcers (Fig. 1e), which were lesions mainly characterized by polymorphonuclear and mononuclear/macrophage infiltrate. Moreover, DC-SIGN$^+$ cells were located in non-ulcerated areas infiltrated by clusters of inflammatory cells and also in the muscular compartment (Fig. 1f and g, respectively). When colonic samples were examined, similar results to those described for the ileum were obtained. However, DC-SIGN$^+$ cells were more frequently detected in the mucosa from the ileum in control samples rather than in the mucosa from colonic controls (Table 1). Also, a significant increase in DC-SIGN$^+$ cells was detected in inflamed areas of the colon when compared to non-inflamed CD and control colonic samples (Table 1).

When CD83 immunostaining was performed, cells with similar morphology to DC-SIGN$^+$ cells were detected in the submucosa, but not in the LP or muscular compartment from ileal control and non-inflamed CD samples (Fig. 2a and b, respectively). In inflamed ileal CD tissues, CD83$^+$ cells were detected in the muscular layer (Fig. 2e), but not in ulcers or areas infiltrated by clusters of inflammatory cells (Fig. 2c and d, respectively). Moreover, when additional sections from inflamed ileal CD samples were immunostained without complete F_c blocking, numerous CD83 false-positive monocytes were detected in ulcers and areas infiltrated by clusters of inflammatory cells (Fig. 2d, insert). Also, no differences in CD83 distribution were detected in ileal versus colonic samples, but a significant reduction of CD83$^+$ cells was identified in both ileal and colonic inflamed areas when compared to controls and non-inflamed CD mucosa (Table 1).

CD1a immunostaining in ileal control samples showed CD1a$^+$ cells in the LP and in the submucosa, but not in the muscular compartment (Fig. 3a and b). In non-inflamed ileal CD samples, an increase of CD1a$^+$ cells was observed in the LP, submucosa, and muscular layer (Fig. 3c, d and e, respectively), which was significant when compared to controls (Table 1). In inflamed ileal CD tissues, CD1a$^+$ cells were observed in either deep areas of ulcers or areas infiltrated by clusters of inflammatory cells (Fig. 3f and g, respectively). Similarly, a high infiltration of CD1a$^+$ cells was detected in inflamed and non-inflamed colonic CD samples (Table 1). Also, significant differences were detected when ileal and colonic non-inflamed CD groups were compared (Table 1).

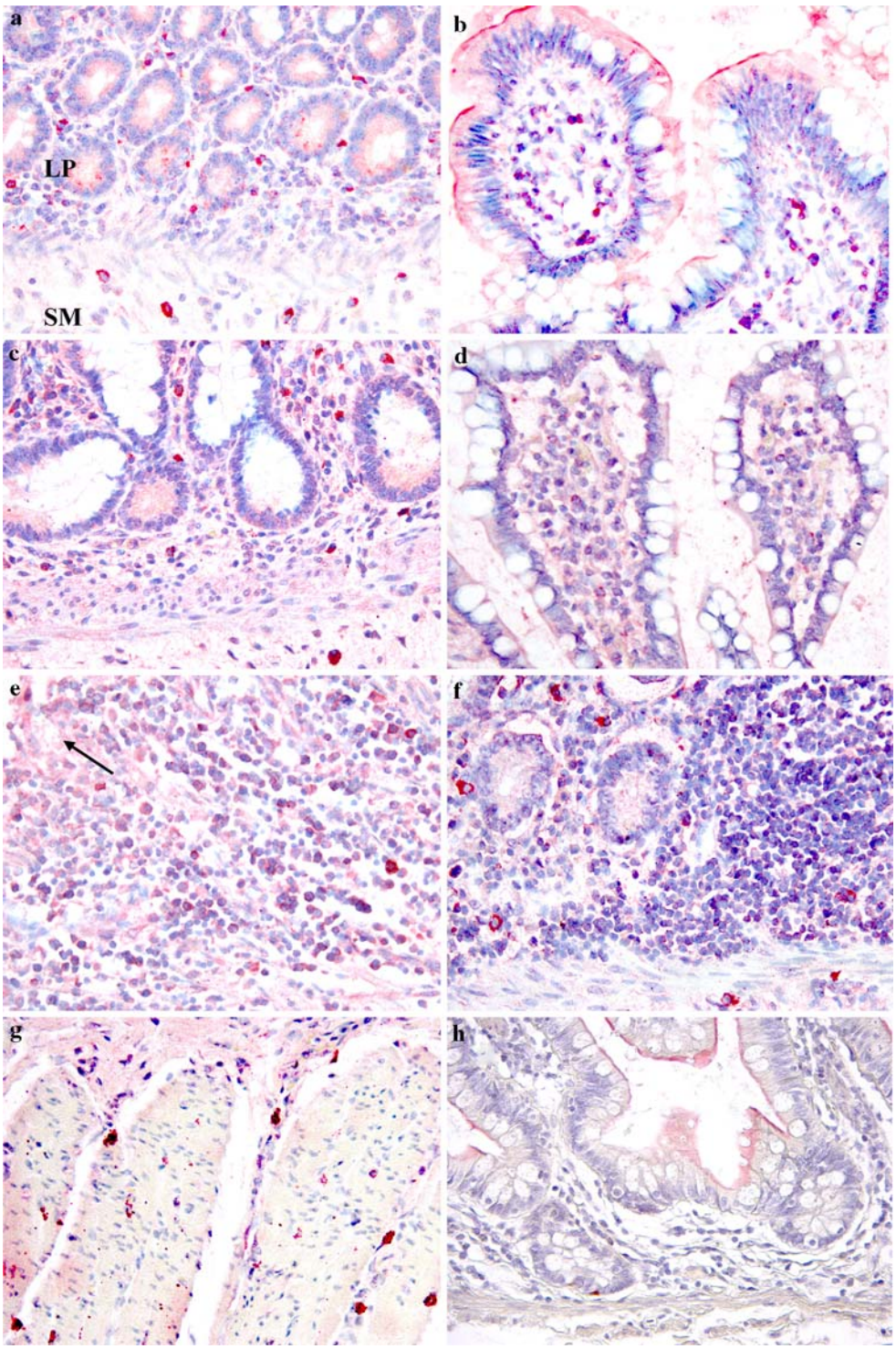

Fig. 1 Immunohistochemistry for DC-SIGN (red precipitate) performed in ileal sections. Control tissues: (**a**) and (**b**). Non-inflamed Crohn's disease (CD) samples: (**c**) and (**d**). Lamina propria (LP) and submucosa (SM): (**a**) and (**c**); villus: (**b**) and (**d**). Inflamed CD: (**e**) (ulcer with an arrow indicating the location of the intestinal lumen), (**f**) (infiltrated area by a cluster of inflammatory cells) and (**g**) (muscular compartment). (**h**): negative immunostainig control. Representative photomicrographs (magnifications 200×)

TLR-2 immunostaining showed no TLR-2$^+$ cells in ileal control and non-inflamed CD samples (Fig. 4a). When inflamed ileal CD tissues were studied, no TLR-2$^+$ cells were detected in ulcers as well as areas infiltrated by clusters of inflammatory cells or in the muscular compartment (Fig. 4c, d and e, respectively). However,

Table 1 Quantifications: cell number/field

Markers	*Ileum*		
	Controls	Crohn's disease	
		Non-Inflamed	Inflamed
CD1a	13.4 ± 5.1	142.2 ± 19.0***[a]	57.0 ± 8.1
CD83	18.9 ± 3.2	13.8 ± 3.1	0.2 ± 0.2**
DC-SIGN	47.8 ± 4.9[a]	38.7 ± 7.5	56.7 ± 5.9
TLR-4	124.9 ± 20.0[a]	79.7 ± 10.2[a]	68.4 ± 12.2
	Colon		
CD1a	12.3 ± 3.1	49.9 ± 4.1*	47.3 ± 5.3*
CD83	13.0 ± 2.2	11.1 ± 2.6	0.4 ± 0.3**
DC-SIGN	25.8 ± 2.8	26.1 ± 3.0	44.8 ± 5.2**
TLR-4	13.7 ± 1.7	14.2 ± 2.3	49.9 ± 14.6**

*$P < 0.01$ vs controls;
**$P < 0.05$ vs controls and non-inflamed Crohn's disease;
***$P < 0.01$ vs controls and inflamed Crohn's disease; and
[a]$P < 0.01$ vs value from the corresponding colonic group (n = 3–4 samples/group)

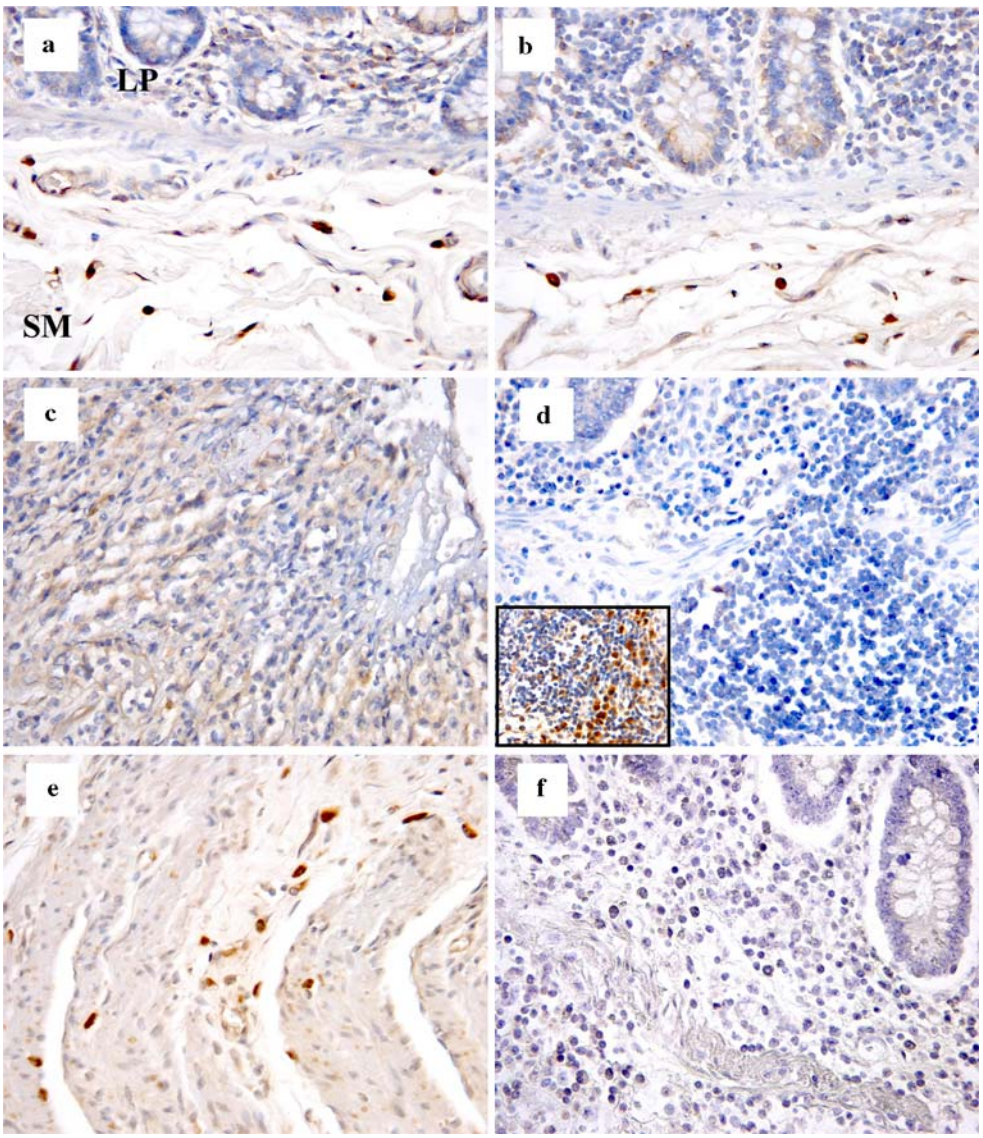

Fig. 2 Immunohistochemistry for CD83 (brown precipitate) assayed in ileal sections. Controls and non-inflamed Crohn's disease (CD) samples: (**a**) and (**b**), respectively. Lamina propria (LP) and submucosa (SM). Inflamed CD: (**c**) (ulcer), (**d**) (infiltrated area by a cluster of inflammatory cells; inset: false-positive monocytes) and (**e**) (SM and muscular compartment). (**f**): negative immunostaining control. Representative photomicrographs (magnifications 200×)

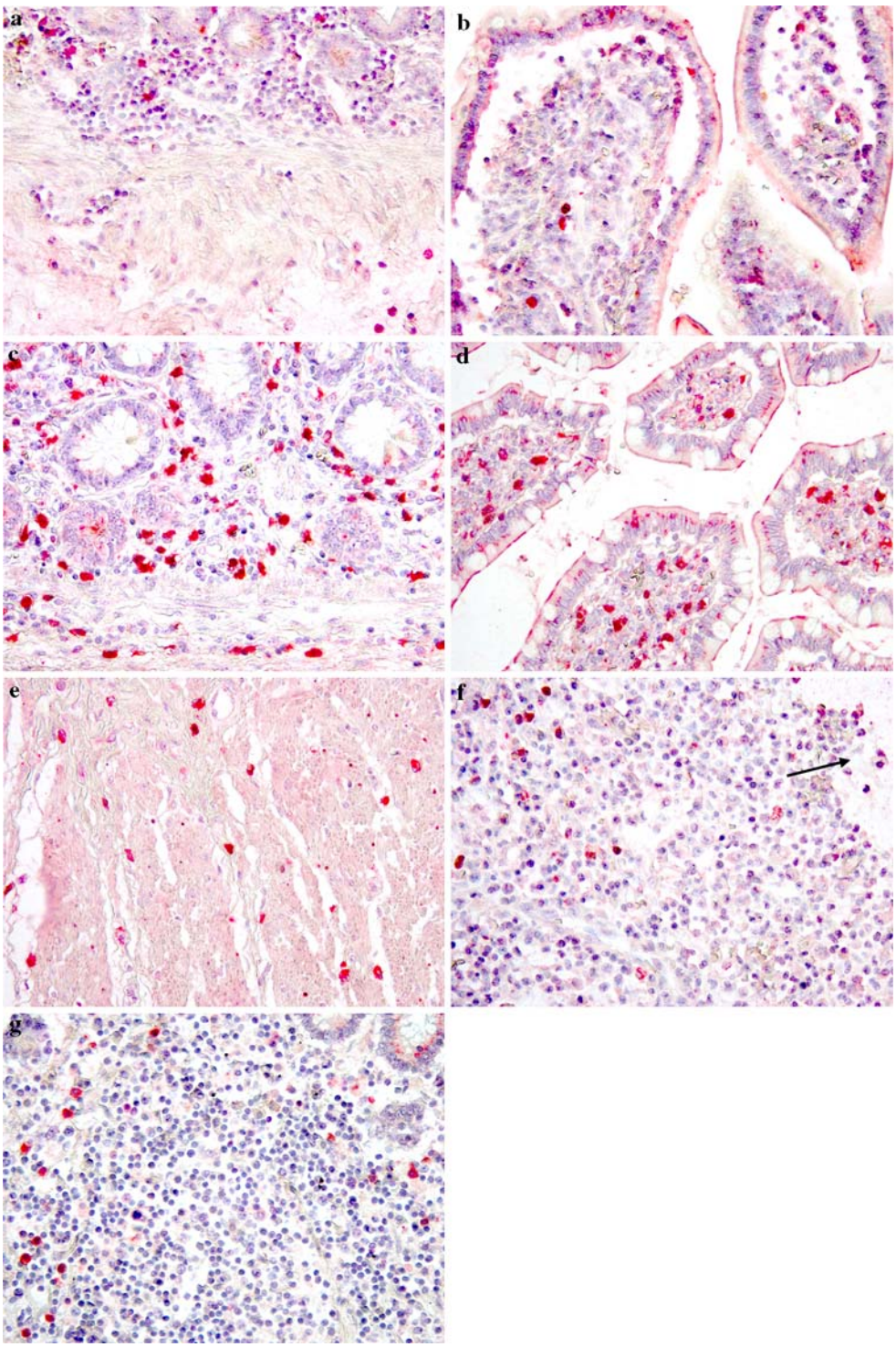

Fig. 3 CD1a immunohistochemistry (red precipitate) performed in ileal sections. Controls: (**a**) and (**b**). Non-inflamed Crohn's disease (CD): (**c**), (**d**) and (**e**) (muscular layer). Lamina propria (LP) and submucosa (SM): (**a**) and (**c**); villus: (**b**) and (**d**). Inflamed CD: (**f**) (ulcer with an arrow showing the location of the intestinal lumen) and (**g**) (area infiltrated by a cluster of inflammatory cells). Representative photomicrographs (magnifications 200×)

TLR-2$^+$ cells were detected in the serosal layer of these tissues (Fig. 4f). Also, TLR-2$^+$ cells were detected in the connective tissue of mildly inflamed ileal CD samples (Fig. 4b). In both cases, TLR-2$^+$ cells were morphologically characterized as polymorphonuclear cells and monocytes (insert, Fig. 4b). Similar differences

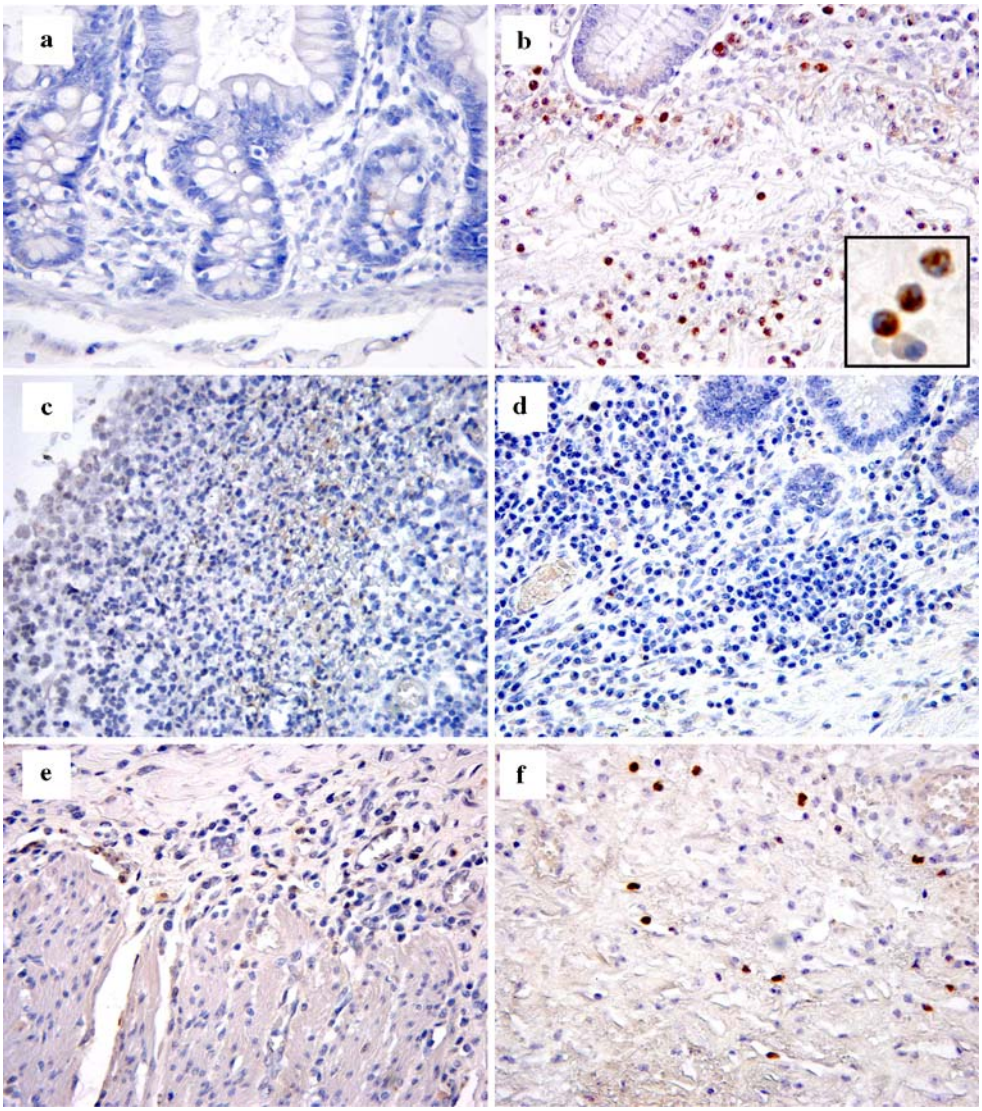

Fig. 4 TLR-2 immunohistochemistry (brown precipitate) performed in ileal sections. Controls: (**a**). Mildly-inflamed Crohn's disease (CD): (**b**) (insert ×400, stained polymorphonuclear cells). Inflamed CD: (**c**) (ulcer), (**d**) (area infiltrated by clusters of inflammatory cells), (**e**) (muscular compartment) and (**f**) (serosal layer). Representative photomicrographs (magnifications 200×)

between colonic CD tissues and controls were observed (data not shown).

TLR-4 immunohistochemistry in ileal control tissues showed stained cells in the submucosa and a large amount of them in both basal and superficial areas of the LP, including clusters of TLR-4$^+$ cells in apical areas of the intestinal epithelium (Fig. 5a and b). In non-inflamed ileal CD tissues, TLR-4$^+$ cells were frequently observed in the submucosa and basal areas of the LP as well as in deeper areas of the villus, but scant intraepithelial cells were detected (Fig. 5c and d). In inflamed ileal CD samples, TLR-4$^+$ cells were located in superficial areas of ulcers, areas infiltrated by clusters of inflammatory cells and in the muscular layer (Fig. 5e, f and g, respectively). Differences in TLR-4 distribution were observed between colonic and ileal controls and when colonic and ileal non-inflamed CD groups were compared (Table 1). Also, increased numbers of TLR-4$^+$ cells were observed in the inflamed colonic CD group when compared to the non-inflamed colonic CD group and colonic controls (Table 1). No intraepithelial clusters of TLR-4$^+$ cells were detected in the LP from all colonic samples.

The morphologic findings described above were complemented by two-color confocal laser scanning microscopy analysis for TLR-4 versus CD1a or DC-SIGN. Results for TLR-4 versus DC-SIGN in non-inflamed ileal CD samples showed a lack of co-localization, but a similar distribution of

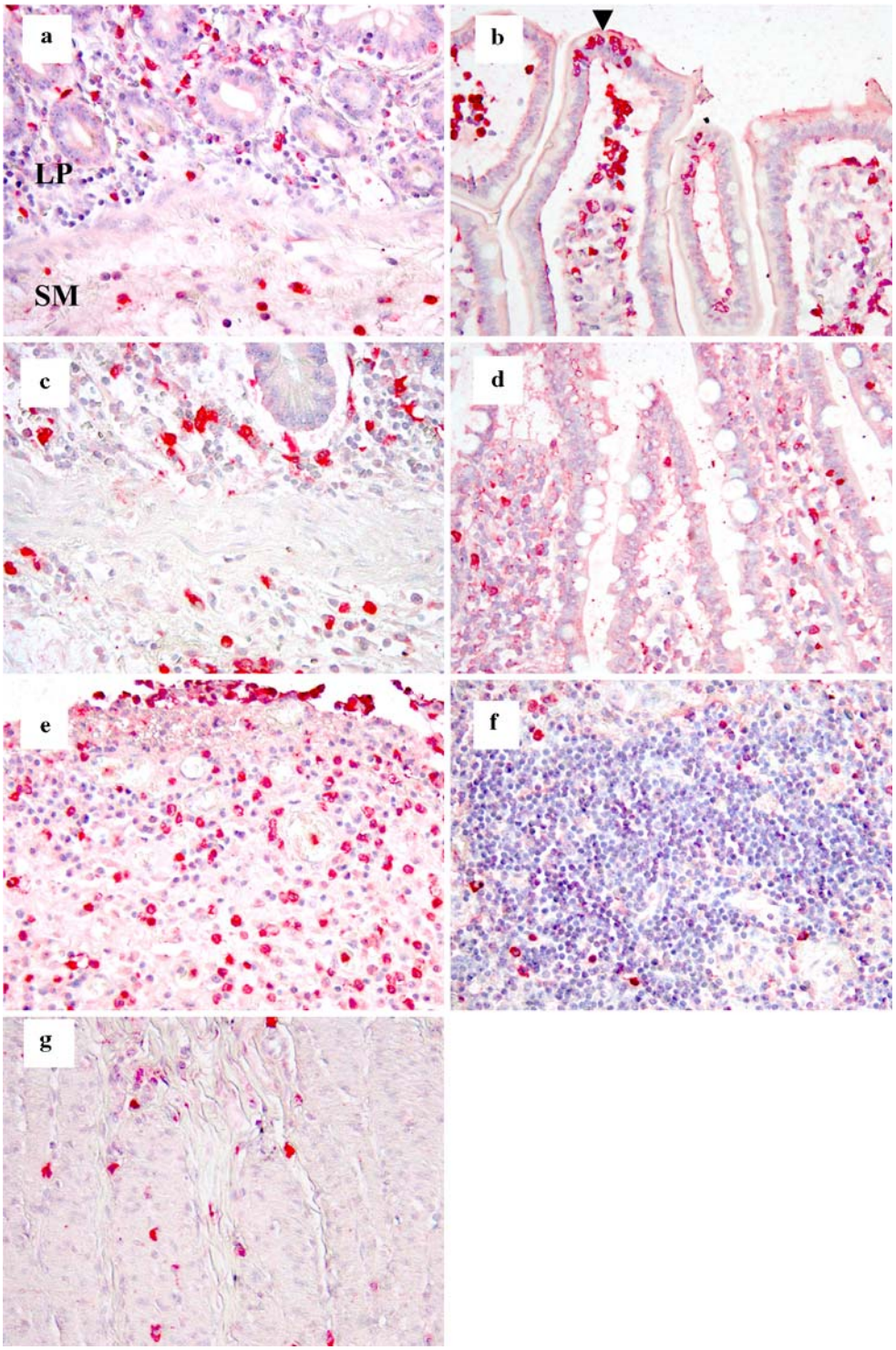

Fig. 5 TLR-4 immunohistochemistry (red precipitate) performed in ileal sections. Control tissues: (**a**) and (**b**) (epithelial clusters: ▶). Non-inflamed Crohn's disease (CD) samples: (**c**) and (**d**). Lamina propria (LP) and submucosa (SM): (**a**) and (**c**); villus: (**b**) and (**d**). Inflamed CD: (**e**) (ulcer), (**f**) (infiltrated area by a cluster of inflammatory cells) and (**g**) (muscular compartment). Representative photomicrographs (magnifications 200×)

both sub-populations (Fig. 6a). In inflamed ileal CD samples few DC-SIGN^{+}TLR-4^{+} cells were detected (Fig. 6b). Additional serial sections from inflamed and non-inflamed ileal CD samples were used for CD1a and TLR-4 double immunofluorescence, but no co-localization was observed (data not shown).

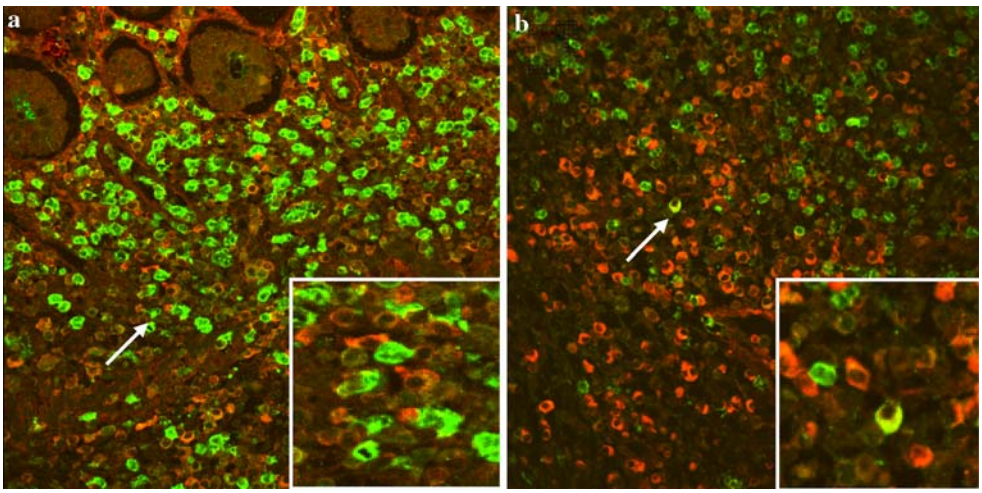

Fig. 6 Two-color immunofluorescence investigation of TLR-4 vs DC-SIGN in inflamed and non-inflamed ileal tissues from Crohn's disease samples. TLR-4$^+$ cells (FITC-conjugated antibody) are shown in green and DC-SIGN$^+$ dendritic cells (CY5-conjugated antibody) in red, respectively. (**a**): non-inflamed sample; insert: higher magnification from the area indicated by the arrow. (**b**): inflamed area of an ulcer; insert: higher magnification from the area indicated by the arrow showing co-localization of TLR-4 and DC-SIGN in yellow. Representative photomicrographs (200×)

Discussion

Human intestinal DC are heterogeneous in terms of surface marker expression representing myeloid or plasmacytoid lineages, different stages of maturation and function [2–7, 28, 29]. In this study, we examined ileal samples from CD patients showing the distribution of myeloid-related DC markers and their correlation with the expression of TLR-2 and 4 by tissue immunoanalysis. We observed a heterogeneous distribution of DC markers in the ileum, establishing differences with colonic samples, the presence or absence of disease and inflammation. Also, a high number of CD1a$^+$ cells were detected in the ileum from CD patients suggesting that CD1a may provide a new and relevant mechanism for antigen recognition by ileal DC during the pathogenesis of this chronic inflammatory disorder.

In this study we demonstrate that DC-SIGN and CD83 distribution in ileal controls and CD samples were similar to the results obtained with the control and CD colonic groups (Table 1). However, differences in the number of DC-SIGN$^+$ cells between the ileal and the colonic control groups were observed. Also, our results were similar to the ones obtained studying colonic samples from pediatric CD patients [4]. In both studies, DC-SIGN$^+$ and CD83$^+$ cells were frequently observed in the submucosa in controls and non-inflamed CD sections. In our study, when inflamed CD samples were analyzed, both DC markers were observed deep in the muscular layer as previously reported [4]. Also, DC-SIGN$^+$ but not CD83$^+$ cells were frequently detected in inflamed areas from both colonic and ileal CD samples (Table 1). In a different study, CD83$^+$DC were observed in inflamed CD areas [7]. However, we demonstrate that when a partial F_c blocking was performed, false CD83$^+$ monocytes were detected in inflamed CD areas (insert, Fig. 2d). Additionally, we observed that the LP from all controls and CD samples did not show CD83 immunostaining (Fig. 2). Immature CD11c$^+$DC with low levels of CD83 and CD86 expression have been isolated from the LP of colonic samples obtained from controls and adult CD patients [28]. Also, the LP in adult CD patients and controls, excluding lymphoid aggregates, was previously characterized by the presence of DC-SIGN$^+$CD83$^-$DC [3]. Conversely, CD83$^+$DC have been observed in the LP from controls and CD colonic tissues [3, 4, 7]. The immunohistochemical methods do not differentiate between high or low levels of CD83 expression. Therefore, it could not be established if the CD83$^+$ cells were low CD83$^+$ expressing immature DC, high CD83$^+$ semi-mature or mature DC. Only the absence of CD83 immunostaining is indicative of immaturity.

It is noteworthy to mention that as shown in Fig. 1, non-inflamed ileal CD samples were partially depleted of DC-SIGN$^+$ cells in the villus when compared to controls, while a high infiltration of CD1a$^+$ cells was detected throughout the tissue (Fig. 3). Similarly, increases in CD1a$^+$ cells were observed in non-inflamed colonic CD samples (Table 1). Differences between non-inflamed CD samples and controls may be the result of variations in recruitment of different inflammatory monocytes that could differentiate into DCs in the gut and/or represent changes in the phenotype of resident DCs. CD1a expression can be induced by pro-inflammatory cytokines and bacterial antigen stimuli [23], which are factors related to the pathogenesis of CD [1, 18].

In the ileal and colonic inflamed CD areas we also observed increased numbers of DC-SIGN$^+$ and CD1a$^+$ cells when compared to their respective controls, but only significant differences were reached for the colonic samples (Table 1).

DC orchestrates the function of the immune system by inducing tolerance or immune priming [8, 30]. Also, a loss of normal tolerance towards the commensal microbiota has been demonstrated in CD [29, 31, 32]. Thus, the abnormal ileal as well as the colonic DC marker distribution demonstrated in this CD study may reflect an immunological imbalance that can contribute to explain, in part, a lack of tolerance. Our results of DC marker distribution, based on a selected subgroup of CD patients that required resection, were not easy to compare with other studies due to differences in ages, disease distribution, genetic background, treatment, experimental strategies and DC markers performed.

TLR-2 and 4 are transmembrane receptors that recognize, in conjunction, a large variety of components from gram-positive and gram-negative bacteria [10, 11]. In our study, we investigate their distribution in CD tissue samples by immunhistochemistry. We observed that only cells from some ileal CD samples expressed TLR-2, but no TLR-2$^+$ cells were detected in control samples as shown in other reports [21, 22]. TLR-2 distribution in the ileum did not mimic the pattern of any of our DC markers, indicating that this receptor may not be present in these DC sub-populations. This result is in agreement with a previous characterization of TLR-2$^+$ cells performed in colonic CD samples demonstrating that they were CD68$^+$ macrophages [21]. However, in a subsequent study, a small (19%) and a higher percentage (approximately 50%) of isolated colonic CD11c$^+$DC from controls and CD patients expressed TLR-2, respectively [6]. Our immunohistochemical method using formalin-fixed samples is likely to be less sensitive than the immunoassays performed by flow cytometry or fluorescence [6]. Also, the use of different DC markers may help to explain the dissimilar outcomes between both studies. In addition, the absence of medical treatment in our patients and the diverse genetic background of this population may have implications in the mucosal immune system that might contribute to explain these differences.

When studying TLR-4, colonic CD samples did not shown immunostaining in the intestinal epithelium but high numbers of TLR-4$^+$ monocytes were observed in inflamed areas when compared to controls (Table 1). Similar results were obtained in another immunohistochemistry study [21]. However, TLR-4 has been detected by immunofluorescense in the intestinal epithelium from colonic CD samples [22]. Also, TLR-4 was observed in the apical membrane of the intestinal epithelium and in cells from the connective tissue in ileal CD samples, but not in control tissues. Conversely, we shown numerous TLR-4$^+$ cells located in the apical epithelium and connective tissue from ileal control samples (Fig. 5a and b). The normal small intestine has a lower load of commensal bacteria than the large intestine [33, 34], so the epithelium from the normal small intestine may not need to down-regulate the expression of TLR-4 like the normal colonic epithelium does to protect itself against a potential harmful pro-inflammatory response that can be induced by overwhelming amounts of commensal lipopolysaccharide present in the lumen [35, 36]. Furthermore, TLR-4 is required to maintain intestinal homeostasis [37, 38]. Then, the presence of TLR-4$^+$ cells in the normal small intestine may contribute to maintain this balance, while a reduction of TLR-4$^+$ cells in our CD samples may be a sign of disease. In addition, TLR-4 induces phagocytosis and translocation of bacteria in enterocytes [39]. Therefore, we propose that under normal conditions, bacterial antigens may gain access to the mucosa not just via M cells from the epithelium that cover the Peyer's patches [40, 41], but also across the TLR-4$^+$ cells located in the apical epithelium of conventional villus. A regulated access of bacterial antigens into the mucosa is an important step to develop tolerance towards the resident commensal bacteria [40, 41]. However, in CD, increased mucosal permeability may allow a free and deregulated access of antigens into the mucosa disrupting this potential TLR-4-dependant pathway in the villus. Also, this may contribute to the loss of tolerance towards commensal bacteria observed in this disease [27, 28].

Our results from the CD tissue samples showed that ileal DC-SIGN$^+$ and CD1a$^+$ cells were TLR-4$^-$, with the exception of a few DC-SIGN$^+$TLR-4$^+$ cells. Ileal TLR-4$^-$ and TLR-4$^+$ cells may interact via cytokine secretion or cellular contact [23], especially for the TLR-4$^+$ and DC-SIGN$^+$ cells that are closely distributed (Fig. 6b). TLR-4$^+$ cells in inflamed and non-inflamed ileal CD samples may represent diverse types of inflammatory cells, such as polymorphonuclear cells, macrophages or TLR-4$^+$CD11c$^+$DC [6, 21]. We avoided the use of CD11c as a myeloid-related DC marker to characterize the TLR-4$^+$ population in this tissue analysis because CD11c is commonly expressed by intestinal macrophages in CD [4, 42, 43]. Additionally, we cannot exclude the expression of other relevant pattern recognition receptors for bacterial antigens in intestinal DC [44, 45]. In fact, we observed a high prevalence of CD1a$^+$ cells in ileal CD samples as reported in another CD study, but not in ulcerative colitis [46, 47]. This molecule is not only a DC marker, but also a receptor for lipidic antigens capable of inducing T-cell activation [23, 48]. Therefore, CD1a may link DC with recognition of lipidic antigens from the enteric microflora, such as bacteria and other microbes, during the pathogenesis of ileitis in CD. However, further studies are required to demonstrate this hypothesis.

In conclusion, we demonstrated a unique distribution of DC markers in ileal samples from CD patients. Also, increased numbers of $CD1a^+$ cells provide evidence for the presence of a different DC-mediated mechanism for antigen recognition during the pathogenesis of chronic inflammation in CD.

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