Epithelial barrier and dendritic cell function in the intestinal mucosa
Verstege, M.I.
Chapter 4.

Dendritic cell populations in colon and mesenteric lymph nodes of patients with Crohn’s disease

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Abstract

Dendritic cells (DCs) are key cells in innate and adaptive immune responses that determine the pathophysiology of Crohn's disease. Intestinal DCs migrate from the mucosa into mesenteric lymph nodes (MLNs). A number of different markers are described to define the DC populations. In this study we have identified the phenotype and localisation of intestinal and MLN DCs in patients with Crohn's disease and non-IBD patients based on these markers. We used immunohistochemistry to demonstrate that all markers (s-100, CD83, DC-SIGN, BDCA1-4, and CD1a) showed a different staining pattern varying from localisation in T-cell areas of lymph follicles around blood vessels or single cells in the lamina propria and in the MLN in the medullary cords and in the subcapsular sinuses around blood vessels and in the T-cell areas. In conclusion, all different DC markers give variable staining patterns so there is no marker for the DC.
Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gut leading to Crohn's disease (CD) or ulcerative colitis (UC). The pathogenesis of these diseases is not well understood, but evidence is increasing that dendritic cells (DCs) play an important role in the induction and maintenance of chronic inflammation.\(^1\) DCs of CD patients seem to have an intrinsic abnormal responsiveness to antigens from the lumen of the gut. Mutations in receptors and/or signal transduction molecules may cause altered recognition of antigens such as NOD2 mutations.\(^3\)\(^-\)\(^6\) However, it is not yet known what DC populations are present in inflamed and control colon and mesenteric lymph nodes (MLNs).

For characterisation of human DCs, a series of markers have been used. In peripheral blood, five distinct subsets of DCs have been identified (see table 1). In addition, myeloid and plasmacytoid DCs can be distinguished (see table 1). Baumgart et al. demonstrated that, in blood of IBD patients during flare-ups of the disease, immature DCs of both myeloid and plasmacytoid origins are reduced, probably because these cells migrate to the gut.\(^19\)

<table>
<thead>
<tr>
<th>Blood DCs</th>
<th>subsets</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid</td>
<td>CD11c(^-)</td>
<td>CD11b/CD1c</td>
</tr>
<tr>
<td></td>
<td>CD11c(^+)</td>
<td>CD16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BDCA3</td>
</tr>
<tr>
<td>Plasmacytoid</td>
<td>CD11c(^-)</td>
<td>CD123/ BDCA2/ BDCA4</td>
</tr>
<tr>
<td>Stem cell</td>
<td></td>
<td>CD34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue DCs</th>
<th>subsets</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid</td>
<td>Langerhans cells</td>
<td>langerin/ CD1a/ s-100</td>
</tr>
<tr>
<td></td>
<td>dermal/ tissue/ interstitial DCs</td>
<td>immature DCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mature DCs</td>
</tr>
<tr>
<td>Plasmacytoid</td>
<td></td>
<td>CD123/ BDCA2/ BDCA4</td>
</tr>
</tbody>
</table>

**Table 1.** Markers that can be used for the characterisation of DC populations in blood and tissue.
In tissues, three major human DC populations are distinguished, i.e. two myeloid-derived DC populations and one plasmacytoid DC population. Table 2 lists the characteristics of the different DC populations in peripheral tissues \(^7,9,11,16,18,20-24\).

In the present study we have determined which DC subpopulations in human colon and MLN can be distinguished when these different markers are used. In addition, we speculate which of these populations may be involved in the pathogenesis of CD. As far as we know, we have performed the first \textit{in situ} analysis of human intestinal DCs and revealed that \textit{in vivo} populations in tissues differ from the widely used monocyte-derived DCs generated \textit{in vitro} \(^25\). Therefore, it is important for a better understanding of the pathophysiology of CD to characterise DC populations in colon and draining lymph nodes \textit{in situ}. On the basis of the \textit{in situ} analysis of DC subpopulations, we can determine which populations are of interest for future molecular characterisation. These DC populations may be potential targets for future therapy.

<table>
<thead>
<tr>
<th>DC marker</th>
<th>Synonym</th>
<th>Cellular expression</th>
<th>Known or proposed function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td></td>
<td>Thymocytes, DCs (including Langerhans cells)</td>
<td>Recognition of non-protein lipid antigen, ligand for some gamma-delta T cells</td>
<td>(21)</td>
</tr>
<tr>
<td>BDCA1 CD1c</td>
<td>Thrombomodulin</td>
<td>Thymocytes, subsets of B cells, myeloid DCs</td>
<td>Recognition of non-protein lipid antigen, ligand for some gamma-delta T cells</td>
<td>(21)</td>
</tr>
<tr>
<td>BDCA2 CD303</td>
<td>Plasmacytoid DCs</td>
<td>Internalisation of antigen for presentation to T cells</td>
<td></td>
<td>(8, 24)</td>
</tr>
<tr>
<td>BDCA3 CD141</td>
<td>Thrombomodulin</td>
<td>Myeloid DCs</td>
<td>Activation of protein C</td>
<td>(22)</td>
</tr>
<tr>
<td>BDCA4 CD304</td>
<td>Neuropilin-1</td>
<td>Plasmacytoid DCs, endothelial cells</td>
<td>Neuronal receptor, co-receptor for vascular endothelial growth factor A</td>
<td>(9)</td>
</tr>
<tr>
<td>CD83</td>
<td></td>
<td>Mature DCs</td>
<td>Co-stimulatory molecule</td>
<td>(18)</td>
</tr>
<tr>
<td>CD209</td>
<td>DC-SIGN</td>
<td>DCs, alveolar and decidual macrophages</td>
<td>Extravasation (ICAM-2), recognition of PAMPs, involved in T cell activation (ICAM-3)</td>
<td>(11, 20)</td>
</tr>
<tr>
<td>s-100</td>
<td></td>
<td>Several nerve cell types, melanocytes, Langerhans cells, DCs</td>
<td>Calcium-binding protein</td>
<td>(7, 16, 23)</td>
</tr>
</tbody>
</table>

\textbf{Table 2.} Cellular expression and known or proposed function of DCs present in tissues.
Materials and methods

Patients and tissue samples

Colon and MLNs were obtained with informed consent from patients with CD and non-IBD-related disorders (diverticulitis, polyposis coli, or colon carcinoma) by surgical resection. Non-diseased colonic mucosa samples were obtained from patients with colon cancer taken at least 7 cm from the tumour. MLNs that were devoid of cancer metastasis were also obtained from these patients (CD; n=7) and non-IBD-related disorders (n=3). Age range of the CD patients (n=9) was 26–41 years (mean age: 36 years), whereas the age range of patients with non-IBD-related disorders (n=11) was 34–84 years (mean age: 57 years). Prior to the resection procedure, six of the nine CD patients were treated with corticosteroids. After resection, colonic mucosa and MLNs were immediately snap frozen in liquid nitrogen and stored at −80°C until cryostat sectioning. Alternatively, samples were fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin.

Immunohistochemistry

Frozen sections

BDCA-1-4 staining

Serial cryostat sections were cut on a Cryo-Star HM560 (Microm; Walldorf, Germany) and transferred to aminopropyltriethoxysilane (APES)-coated glass slides (StarFrost; Knittel, Klinipath, Duiven, The Netherlands), dried, and stored at −80°C until further processing. Tissue sections were defrosted at room temperature, dried, and fixed in ice-cold acetone for 10 minutes at room temperature. After fixation, tissue sections were rinsed with PBS (pH 7.8), placed in a semi-automatic stainer (Sequenza; Shandon, Breda, The Netherlands), and incubated with normal goat serum (CLB; Amsterdam, The Netherlands) to block non-specific staining for 10 minutes. Sections were then incubated with primary antibody for 60 minutes at room temperature. Mouse anti-human monoclonal antibodies (Miltenyi Biotec; Bergisch Gladbach, Germany) directed against BDCA1 (AD5-8E7), BDCA2 (AC144), BDCA3 (AD5-14H12), and BDCA4 (AD5-17F6) were used. All primary antibodies were
diluted in PBS containing 1% blocking reagent (10,961,760; Roche Diagnostics GmbH, Mannheim, Germany) to block endogenous avidin and biotin activity. Following incubation with primary antibody, sections were rinsed with PBS for 5 minutes and incubated with biotinylated goat anti-mouse antiserum (Biogenics; Klinipath) for 30 minutes at room temperature. Next, sections were rinsed with PBS and incubated with streptavidin alkaline phosphatase (ss-AP; Biogenics, Klinipath) for 30 minutes at room temperature. Sections were then rinsed with PBS containing Tris buffer (0.2 mol/liter, pH 8.5) and incubated with New Fuchsin (Chroma; Kongen, Germany) substrate (containing levamisole to block endogenous AP enzyme activity) for 20 minutes at room temperature. Sections were counterstained with Gill's haematoxylin, rinsed with distilled water, and mounted in Vecta Mount (Vector Laboratories; Burlingame, CA). Control staining was performed by substituting primary antibody with an isotypic control monoclonal antibody.

**DC-SIGN and CD83 staining**

Frozen sections were stained using a standard alkaline phosphatase protocol as described previously. In short, sections were incubated in 5% (v/v) normal goat serum (CLB) to block non-specific staining for 10 minutes. Sections were then stained with either anti-CD83 as primary MAb (Beckman Coulter; Mijdrecht, The Netherlands) or with FITC-labelled anti-DC-SIGN (obtained from Dr. Y. van Kooyk; Free University Medical Centre, Amsterdam, The Netherlands). Following incubation with primary antibody, sections were extensively rinsed in PBS and incubated with biotinylated goat anti-mouse IgG (Dako; Glostrup, Denmark). Next, FITC-labelled MAb sections were incubated with rabbit anti-FITC antibody (Dako) and goat anti-rabbit antibody (Dako). Subsequently, sections were incubated with streptavidin/biotin-conjugated alkaline phosphatase complex (ABC protocol; Dako). Colour was developed using as substrate naphthol AS-MX phosphate (0.3 mg/ml) plus New Fuchsin (0.1 mg/ml; Chroma) in 0.2 M Tris–HCl buffer, pH 8.0 (ABC protocol), and sections were counterstained with haematoxylin.
Paraffin sections

Paraffin sections were stretched and dried at 37°C overnight, deparaffinised in xylene, and rehydrated in alcohol series. To block endogenous peroxidase activity, sections were treated with 3% H₂O₂ in methanol for 20 minutes. Sections were subjected to heat-induced epitope retrieval for 10 min at 95°C. To block non-specific binding sites, sections were incubated with normal goat serum (5% in PBS) for 10 minutes. Sections were then incubated with primary antibodies CD1a (1:20; Dako) and s-100 (1:4000; Dako) diluted in PBS containing 5% BSA for 1 hour. After incubation with secondary antibodies (Dako), peroxidase activity was demonstrated by using 3,3-diaminobenzidine tetrachloride (Sigma; St Louis, MO). Finally, sections were counterstained with haematoxylin, dehydrated, and mounted in Pertex (Sigma–Aldrich; Steinheim, Germany).

All sections were examined in a double-blind manner by a pathologist.
## Results

### Myeloid DCs

Distribution patterns of myeloid DCs in colonic mucosa and MLNs of non-CD and CD patients using the DC markers BDCA1, BDCA3, s-100, DC-SIGN, CD83, and CD1a are summarised in Table 3.

<table>
<thead>
<tr>
<th>DC Marker</th>
<th>Localisation in colon in non-CD and CD patients</th>
<th>Presence in colon</th>
<th>Presence in MLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>absent</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BDCA1</td>
<td>very few cells scattered throughout LP, mantle zone of follicles</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>BDCA2</td>
<td>few cells in lymph follicles</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>BDCA3</td>
<td>scattered throughout the LP and around blood vessels</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BDCA4</td>
<td>high expression around blood vessels in LP</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD83(^1)</td>
<td>lymph follicles, scattered throughout LP and submucosa</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>CD209(^1)</td>
<td>scattered throughout mucosa</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>s-100</td>
<td>nerve vessels, DCs in lymph follicles, but not in the B cell follicles</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3. Localisation in colon and MLN of DC subtypes. \(^1\) Earlier published by te Velde et al. 2003.
**BDCA1 and BDCA3**

In colonic tissue, expression of BDCA1\(^+\) cells was mainly observed in association with lymph follicles. Only a few cells were scattered throughout the lamina propria (LP) (see figure 1a). In MLN, expression of BDCA1 was restricted to the mantle zone, and some subsets of cells were scattered throughout medullary sinuses (see figure 1b). BDCA3 expression was found in single cells in the LP of both non-CD and CD patients (see figure 1c). Moreover, BDCA3 was expressed around blood vessels in the LP, submucosa, and muscle layers of the colon. In MLN, BDCA3 was expressed around (sub)capsular and medullary sinuses, blood vessels, and lymph follicles accentuating the frontier between T- and B-cell areas (see figure 1d).

**s-100**

In MLNs, s-100\(^+\) cells were scattered throughout the cortical and paracortical T-cell areas, whereas cells located in B-cell areas did not express s-100 (see figure 2). S-100\(^+\) cells were also found in paracortical sinuses in MLNs of CD patients, whereas the paracortical sinuses of non-CD MLNs were completely devoid of s-100\(^+\) cells. The number of s-100\(^+\) cells was increased in MLNs of CD patients (see table 3). Lymph follicles in colonic tissue demonstrated a slight increase of s-100\(^+\) DCs in CD patients as compared with non-CD patients. S-100\(^+\) DCs were also absent in the B-cell areas of the follicles.

**DC-SIGN\(^+\) and CD83\(^+\) cells**

It has been previously demonstrated that DC-SIGN\(^+\) cells are scattered throughout the mucosa, and that the CD83\(^+\) population is present in aggregated lymphoid nodules and as single cells in the LP\(^{25}\). In addition, we demonstrate in this present study that, in MLN, expression of DC-SIGN is present in the periphery of medullary cords, which is populated by macrophages and plasma cells. CD83\(^+\) cells were found in paracortical zones populated by T cells (see figure 3). There were no differences between CD and non-CD patients.


**CD1a cells**

Both colonic tissues and MLNs of non-CD and CD patients were completely negative for CD1a expression (data not shown).

**Plasmacytoid DCs**

Plasmacytoid DCs are characterised by the expression of BDCA2 and BDCA4. BDCA2 expression of a few cells was found in lymph follicles in colon, whereas BDCA2⁺ cells were absent in mucosa of both non-CD and CD patients (see figure 4). In contrast, expression of BDCA4 was strong in endothelial cells in LP of both non-CD and CD patients. In MLN, expression of both BDCA2 and BDCA4 was closely associated with high endothelial venules (HEVs).
Figure 1. Photographs of the immunohistochemical determination of the expression of the myeloid DC markers BDCA1 (a,b) and BDCA3 (c,d) in colonic tissue (a,c) and MLNs (b,d) from CD patients. Bar = 100 μm. The inserts in b and d indicate the localisation of the staining in MLN.

Figure 2. Photographs of the immunohistochemical localisation of s-100 expression in colon (a,c) and MLN (b,d) of non-CD (a,b) and CD patients (c,d). Bar = 100 μm.
Figure 3. Photographs of the immunohistochemical localisation of DC-SIGN (a) and CD83 (b) expression in the MLN of CD patients. Bar = 100 μm. The inserts indicate the localisation of staining in MLN.

Figure 4. Photographs of the immunohistochemical localisation of expression of the plasmacytoid DC markers BDCA2 (a,b) and BDCA4 (c,d) in colonic tissue (a,c) and MLNs (b,d) of CD patients. Bar = 100 μm. The insert in a indicates the presence of BDCA2⁺ cells in follicles in the wall of the colon. Bar = 200 μm. The inserts in b and d indicate the localisation of staining in MLN.
Discussion

During an immune response, DCs traffic from peripheral tissues into draining lymph nodes through lymphatic vessels. However, it is not yet known which DC populations are present in the colon wall and which DC populations migrate from colonic tissue into the MLNs. In humans, these DC populations may play an important role in the pathogenesis of CD. Therefore, we investigated subtypes and their localisation of DCs in colon and MLN. We demonstrate here that three different subpopulations of myeloid DCs populate the mucosa of the colon and MLN of non-CD and CD patients. These populations consist of the following: (1) immature DCs or macrophages that express DC-SIGN, (2) mature DCs that express s-100 or CD83, and (3) mature DCs that express BDCA3. BDCA1 and CD1a expressing DCs were virtually absent in colon as well as MLNs. Immature DCs and macrophages are mainly localised at antigen-capturing sites such as the mucosa and medullary cords, whereas mature DCs are present where antigen is presented, including the T-cell areas in colonic lymph follicles and MLNs.

In general, lymph nodes contain three types of DCs, i.e., interdigitating DCs (IDCs), follicular DCs, and plasmacytoid DCs. IDCs are like Langerhans cells characterised by the expression of s-100, which is a member of the family of calcium-binding proteins. Based on the localisation of markers, we demonstrated that s-100+ and also CD83+ cells present in the MLN are most likely IDCs, which are mature DCs from myeloid origin and capable of presenting antigens to naive T cells in the paracortical areas. Moreover, in colonic tissue, s-100 as well as CD83 demonstrated a similar expression pattern in lymph aggregates, suggesting that they are involved in similar functional processes. In contrast, s-100 expression in cervical lymph nodes was absent in lymph follicles. Furthermore, only subcapsular sinuses of CD patients and not of non-CD patients harbour s-100+ DCs, indicating that in CD patients there is an increased influx of mature DCs of MLNs via subcapsular sinuses into the T-cell areas. An increased number of s-100+ DCs was also found in extrafollicular T-cell areas in hyperplastic palatine tonsils of patients with tonsillitis, in several autoimmune diseases such as juvenile rheumatoid arthritis and in primary biliary cirrhosis confirming a role for s-100+ cells in
inflammatory processes. Moreover, s-100 is expressed by nerve cells and cancer cells and is used as a marker in cases of melanoma, nerve tissue cancer, and several carcinomas.

BDCA3$^+$ cells are likely to be IDCs, but because of their specific localisation at the border of lymph follicles and sinuses they seem to belong to a myeloid cell type other than s-100$^+$ and CD83$^+$ DCs. These cells may be capable of interacting with B and T cells to induce humoral responses. However, BDCA3$^+$ cells may be more involved in antigen-capturing processes because they are found in LP.

Follicular DCs are characterised by none of the described markers. Expression of BDCA1 in the mantle zone of B-cell follicles may be indicative of follicular DCs; however, expression is not restricted to DCs. B-cell subsets in the germinal centre and mantle zone of lymph nodes, marginal zone B cells in the spleen, and a subpopulation of B cells in the peripheral blood also express BDCA1, which belongs to a family of lipid antigen-presenting molecules. The observation that BDCA1$^+$ cells were smaller and without the characteristic morphology of DCs suggests that expression of BDCA1 in MLN is due to the presence of B cells rather than DCs.

DC-SIGN is a C-type lectin involved in sampling antigens by recognition of specific carbohydrate structures of autoantigens and foreign antigens and is therefore mainly expressed by immature DCs and macrophages. This is consistent with our earlier findings that DC-SIGN$^+$ cells are scattered throughout colonic mucosa where these cells can encounter antigens. DC-SIGN$^+$ cells were present in medullary cords of MLNs, but in contrast to cervical lymph nodes no expression was detected in the outer zone of the paracortex.

Although CD1a is mainly used as a marker for Langerhans cells in the skin, expression of this marker is not restricted to this cell type because subsets of interstitial and monocyte-derived DCs also express CD1a. However, CD1a was not detectable in MLNs and colonic tissue of both non-CD and CD patients. Moreover, subsets of DCs in the superficial lymph nodes were CD1a positive, whereas CD1a expression is absent in deeply
located lymph nodes including mesenteric, mediastinal, and para-aortic lymph nodes. Additionally, expression of CD1a does not occur in inguinal and liver lymph nodes.

Expression of plasmacytoid DC marker BDCA2 has been observed in close association with HEVs, indicating that tissue plasmacytoid DC precursors are blood-derived cells that enter MLN in the HEVs. In agreement with these results, Yoneyama et al. demonstrated that myeloid DCs migrate via lymph vessels into draining lymph nodes, whereas plasmacytoid DCs enter lymph nodes via the HEVs. BDCA2+ DCs seem to be mainly involved in antigen-capturing processes, and BDCA2 is a C-type lectin transmembrane glycoprotein that internalises antigens to present to T cells. BDCA2 expression was absent in colonic mucosa of both non-CD and CD patients, which coincides with the results of Middel et al. BDCA4 is a neuronal receptor of the class 3 semaphorin family and is also expressed by endothelial cells to function as a coreceptor for vascular endothelial growth factor A. This suggests that BDCA4 expression at HEVs and in the LP is related to endothelial cells instead of DCs.

In the current study we have shown that different DC markers give variable staining patterns so that there is no marker for DCs. These different ‘DC markers’ are present in different sites in the colon and MLN in CD patients, indicating that DCs are a heterogeneous group of antigen-presenting cells harbouring different functions. Our data indicate that colonic tissue and MLNs harbour at least three different myeloid DC populations. One population consists of immature DCs, which express DC-SIGN and is mainly located at antigen-capturing sites in the mucosa and medullary cords. A second population of DCs expresses BDCA3 and, similar to DC-SIGN+ DCs, is also present as single cells in the mucosa, suggesting that BDCA3+ DCs may be involved in antigen-capturing processes. In the MLN, their location around the lymph follicles suggests a role in the activation of B and T cells. The third DC population consists of mature DCs that express s-100 and CD83. Their location in the T-cell areas in both the colonic lymph follicles as well as the MLN suggests that they have the capacity to present antigen to T cells. DCs from plasmacytoid origin were hardly present in the colon and MLN. Although
BDCA4 expression was found around the blood vessels, its expression is probably on endothelial cells instead of DCs.

Future studies will involve the isolation of the described different DC populations for further investigation to utilise favourable results for the development of new therapeutic strategies in the treatment of CD.

Acknowledgements

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DC populations in colon and MLNs of patients with CD

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