INFLAMMATORY BOWEL DISEASE

Increased number of mature dendritic cells in Crohn's disease: evidence for a chemokine mediated retention mechanism

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Background and aims: Activation of T cells by dendritic cells (DC) is thought to play a pivotal role in induction and maintenance of Crohn's disease. Detailed analyses however concerning the phenotype and maturation of DC as well as the mechanisms underlying their recruitment are still lacking for Crohn's disease.

Methods: Different myeloid and plasmacytoid DC subsets were characterised by immunohistochemistry. Expression of the so-called "lymphoid" chemokines CCL19, CCL20, and CCL21 was determined by real time reverse transcription-polymerase chain reaction in Crohn's disease and normal controls. Furthermore, expression of CCL19, CCL20, and CCL21 as well as their receptors CCR6 (for CCL20) and CCR7 (for CCL19 and CCL21) was characterised by immunohistochemistry and, in addition, their cellular localisation was determined by double immunofluorescence investigations.

Results: Colonic tissue affected by Crohn's disease was characterised by an increased number of mature myeloid DC forming clusters with proliferating T cells. In keeping with their advanced maturation, DC possess the chemokine receptor CCR7. Increased expression of the CCR7 ligands CCL19 by DC themselves as well as CCL21 by reticular cells and lymphatic vessels was observed in Crohn's disease, thereby causing the matured DC to be trapped at the site of inflammation.

Conclusion: Our results demonstrate that autocrine and paracrine actions of lymphoid chemokines in Crohn's disease may lead to increased numbers of mature DC away from their usual migration to lymphoid organs and result in the development of a tertiary lymphatic tissue within the bowel wall maintaining the autoimmune inflammation in Crohn's disease.

Immune alterations initiating and maintaining Crohn's disease (CD) as a chronic inflammatory disorder are still poorly understood.¹ Apart from genetic susceptibility, alterations of the innate immune system as well as modulation of T cell responses have been found to play an important role in the pathogenesis of CD. Antigen dependent activation of Th1 lymphocytes is now believed to represent the major mechanism in the pathogenesis of CD.^{2–3} Although many cells can function as antigen presenting cells, only dendritic cells (DC) can trigger naive T cells. Thus alterations in intestinal DC function probably could contribute to the poorly understood dysregulated immune responses of CD.^{4–6} However, little is known about the different DC subsets in the human intestine, and detailed investigations concerning the phenotype and function of DC in CD are still lacking.

For an intact immune response, the cross talk between resident and immigrating cells within a given tissue is critically dependent on cytokines.^{7–9} Chemokines are a family of cytokines characterised by the ability to induce migration of different cell types through the action on specific receptors. The chemokines CCL21 (also known as SLC or exodus 2) and CCL19 (also known as ELC or MIP-3β) are involved in the organisation of normal lymphoid tissue during development.^{10–12} CCL21 is expressed by activated lymphatic endothelial cells and by stromal cells in the T cell areas of secondary lymphoid organs, whereas CCL19 has been shown to be expressed by mature myeloid DC. CCL19 and CCL21 are structurally related chemokines and share the common receptor CCR7,¹³ which is expressed by mature DC, B-cells, and naive and memory T cells.^{11–13–14} For CCR7 deficient mice and paucity of lymph node T cell (plt) mice, which lack expression for CCL19 and CCL21, severe alterations of

secondary lymphoid organs have been shown, specially defective migration of DC and T cells into T zones.^{10–15–17} Furthermore, recent studies have demonstrated that lymphoid neogenesis as well as autoimmunity is strongly associated with CCL19 and CCL21 expression.^{7–18}

In the present study, we investigated the phenotype, maturation state, and distribution of the different DC subsets in CD. Furthermore, we explored the hypothesis of whether the autoimmune reaction of CD could be sustained by aberrant lymphoid chemokine expression favouring cell trafficking and interaction between DC and lymphocytes within the bowel wall. Our results showed that expression of CCL19 and CCL21 was induced in tissues affected by CD, leading to ongoing recruitment and/or retention as well as interaction of mature DC and T cells.

MATERIAL AND METHODS

Tissue samples

Colon tissues were obtained from patients with CD who underwent partial bowel resection. CD was active in all patients, as confirmed by clinical features, laboratory parameters, as well as macroscopic and histopathological alterations. All patients underwent surgical intervention due to failure of medical treatment. Mean age of the group was 35.2 (13.5) years (n = 12). Control samples were obtained from patients (n = 10, with a mean age of 54.8 (15.3) years) who underwent resection for non-inflammatory gut disorders

Abbreviations: CD, Crohn's disease; DC, dendritic cells; NIGD, non-inflammatory gut disorder; RT-PCR, reverse transcription-polymerase chain reaction; Cy2, indocarbocyanine 2; Cy3, indocarbocyanine 3; CARD15, caspase activating and recruitment domain 15

Table 1 Antibodies used for immunohistochemistry and immunofluorescence

Antibody	Dilution	Fixation	Vendor
CD1a	1:50	F	LabVision, Fremont, CA, USA
CD3	1:50	F	Novocastra, Newcastle, UK
CD11c	1:50	Hope	Dako, Hamburg, Germany
CD14	1:20	F	NeoMarkers, Fremont, CA, USA
CD79a	1:50	F	Dako
CD83	1:20	F	Novocastra
CD123	1:20	F	eBioscience, San Diego, CA, USA
CCR6	1:20	Hope	R&D Systems, Heidelberg, Germany
CCR7	1:20	Hope	R&D Systems
CCL19	1:20	F	R&D Systems
CCL20	1:20	F	R&D Systems
CCL21	1:50	F	R&D Systems
BDCA-2	1:10	Hope	Miltenyi-Biotec, Bergisch-Gladbach, Germany
Fascin	1:100	F	Dako
D2-40	No	F	DCS-Diagnostics, Hamburg, Germany
IgG1-control	1:50	F	Dako
IgG2a-control	1:50	F	Dako
IgG2b-control	1:50	F	Dako
Rabbit IgG-control	1:50	F	Acris, Hiddenhausen, Germany
Goat IgG-control	1:50	F	Acris

(NIGD, not inflammatory affected colonic tissue obtained from diverticular disease or colon cancer) or diverticulitis. Prior to the resection procedure, eight of the 12 patients with CD were treated with corticosteroids.

Immunohistochemistry

The antibodies used in the study and their optimal working dilutions are listed in table 1. For immunostaining with "cryotype" antibodies, which cannot be made visible on formalin fixed tissue, HOPE fixed tissue slides were used which do not require antigen retrieval, as recommended for formalin fixed tissue.¹⁹ Sections were immunostained by applying the biotin-streptavidin-peroxidase method.

Immunostaining of formalin fixed paraffin embedded tissue sections for CCL19, CCL20, CCL21, and CD123 required application of the tyramide signal amplification system method (NEN, Boston, Massachusetts, USA), as described previously.⁹ In control reactions, irrelevant primary antibody was applied or primary antibodies were omitted.

Double immunofluorescence staining was performed on HOPE fixed tissue slides. Sections were stained for one hour with unconjugated primary antibody followed by incubation with indocarbocyanine 2 (Cy2) conjugated goat antimouse or goat antirabbit F(ab) fragments (both Dianova, Hamburg, Germany) at a saturating concentration for 60 minutes. For first step immunofluorescence staining of CCL19, CCL20, and CCL21, we adopted the TSA-Kit (NEN) using FITC conjugated tyramide for fluorescence amplification. Sections were washed and incubated with the second antibody for 60 minutes at room temperature followed by incubation with indocarbocyanine 3 (Cy3) conjugated goat antimouse or goat

antirabbit F(ab) fragments (both Dianova) for 60 minutes at room temperature.

Confocal fluorescence images were obtained on a Leica TCS (Leica Microsystems, Heidelberg, Germany) confocal system mounted on an Olympus BX50 WI microscope (Tokyo, Japan). Possible cross talk between FITC or Cy2, and Cy3, which could give rise to false positive colocalisation of different signals, was avoided by careful selection of the imaging conditions.

Real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Sequences of primers used in the study are listed in table 2. Primers and probes (Operon-Qiagen, Hilden, Germany) were designed using the Primer-3 online primer design program (<http://www-genome.wi.mit.edu>). Optimal conditions for all primers were established by amplifying cDNA samples from human tonsil or lymph node.

Total cellular mRNA was extracted with the RNeasy Mini Kit (Qiagen). RNA integrity and quantity was assessed using the Agilent Bio-analyzer 2100 (Agilent Technologies, Waldbronn, Germany). RT with random hexamer primers was performed with the Omniscript RT Kit (Qiagen). Quantification of CCL19, CCL20, CCL21, and β -actin mRNA expression was performed on an iCycler iQ real time detection system (Bio-Rad, Hercules, California, USA) using the HotStar TaqDNA polymerase kit (Qiagen), as described previously.^{8,9} Expression of CCL19, CCL20, and CCL21 was normalised to β -actin expression to compensate for different sample capacities. Results derived from the PCR standard curve are given in attomoles per μ g total cellular RNA. cDNA

Table 2 Polymerase chain reaction (PCR) primers used for quantitative reverse transcription-PCR analysis of chemokines

Gene	Primer sequence	Product size (bp)
CCL19	5'-CCAGCCTCACATCACTCACACCTGC-3' 5'-TGTGGTGAACACTACAGCAGGCACCC-3'	324
CCL20	5'-CTGTACCAAGAGTTTGCTCC-3' 5'-GCACAATATATTTACCCCAAG-3'	193
CCL21	5'-AACCAAGCTTAGGCTGCTCCATCCCA-3' 5'-TATGGCCCTTAGGGGTCTGTGACCG-3'	249
β -Actin	5'-CTACAATGAGCTGCGTGTGGC-3' 5'-CAGGTCCAGACGCAGGATGGC-3'	270

Figure 1 Quantification of fascin (A) and CD83 (B) expressing dendritic cells (DC). Colonic tissue affected by Crohn's disease (CD) demonstrated a significantly increased number of fascin (A) and CD83 positive mature DC (B) compared with non-inflamed colonic tissue affected by CD (CD-RR), diverticulitis, and normal controls (non-inflammatory gut disorder (NIGD)). At least five fields in each tissue were counted for each specimen at a magnification of $\times 400$.

from a human lymph node was used as a positive control template for each primer pair. Negative controls with water instead of cDNA were always included.

Statistics

Evaluation of immunoreactivity was performed on sections stained for Fascin and CD83. Because of the heterogeneous distribution of DC, quantification was performed with a method first used for estimation of new blood vessel formation.⁷ This method allows quantification of cells in hot spots, defined in this study as areas containing the highest density of positive cells. Accordingly, five hot spots per section were counted with an eyepiece graticule at $400\times$ magnification. Data were analysed with a statistical package (Systat version 5.1; Systat Inc, Evanston, USA). Statistical comparisons were performed by applying the unpaired *t* test. Two sided *p* values lower than 0.05 were considered statistically significant.

RESULTS

Distribution pattern of DC subsets

For estimation of the total number of DC, immunostaining for fascin was performed on normal colon tissue as well as on colon affected by active CD. Our results demonstrated a significantly increased number of DC in CD versus NIGD controls ($p < 0.001$) (fig 1A). To estimate the number of immature myeloid DC, additional immunohistochemical investigations for CD1a were performed. Immature CD1a positive DC could not be detected in either CD or controls. In order to estimate the number of mature myeloid DC,

additional investigations were performed for the mature myeloid DC marker CD83. A significantly increased number of CD83 expressing DC in CD versus NIGD controls was established ($p < 0.001$) (fig 1B). Comparing the total number of fascin expressing DC with CD83 positive DC, approximately 70–80% of DC represented mature myeloid DC. No difference in the percentage of mature myeloid DC could be found when comparing colon affected by CD and controls. Further investigation of tissue samples from macroscopically uninvolved mucosa compared with inflamed colonic mucosa from the same patient revealed a significant increase in fascin ($p < 0.05$; fig 1) and CD83 positive DC ($p < 0.05$). Significant increased expression was also found compared with colonic tissue affected by diverticulitis for fascin ($p < 0.05$, fig 1) and CD83 positive DC ($p < 0.05$).

Additional investigations were performed to analyse the distribution of DC subsets in NIGD controls versus active CD. In control bowels, fascin positive (fig 2A, B) as well as CD83 positive DC (fig 2C, D) were mostly restricted to the T cell zone of occasionally observed Peyer's patches, which were located at the transition zone between the lamina propria and submucosa. Beneath the surface epithelium of normal colon, very few fascin positive or CD83 positive DC were observed, clustering with only a few T cells (fig 2B, D). The clusters were small and did not contain more than 3–5 DC.

In contrast, immunostaining for fascin of large bowel affected by CD demonstrated an increased number of DC within the entire bowel wall. Numerous DC were predominately found within extended inflammatory T cell clusters (fig 2E). DC/T cell clusters were found to be randomly distributed within the lamina propria and submucosa (fig 2F). Most of the observed DC within the T cell clusters demonstrated expression for CD83 (fig 2G). Smaller clusters were seen within the lamina propria (fig 2H). Most mature DC/T cell clusters were located in the vicinity of lymphatic vessels (fig 2K), as demonstrated by immunostaining for the lymphatic endothelial cell marker D2-40 (not shown)²⁰ or adjacent to granulomas (fig 2I). Occasionally, DC/T cell clusters were identified within lymphatic vessels resembling the previously described lymphatic organ neogenesis observed in CD.²¹

There was no significant difference observed in the amount of BDCA-2/CD123 positive fascin coexpressing plasmacytoid DC in CD versus controls. In NIGD colonic tissue and in CD, the mucosa as well as the submucosa, muscularis propria, and subserosal tissue contained only a few randomly distributed BDCA-2/CD123/fascin positive plasmacytoid DC (fig 2L, M). Plasmacytoid DC were found primarily outside Peyer's patches and did not colocalise with T cell clusters.

Detection of CCL19, CCL20, and CCL21 in CD

To explore the differential expression of CCL19, CCL20, and CCL21, real time quantitative RT-PCR was performed. In the total RNA isolated from tissue affected by active CD, statistically significant increased expression of CCL19 (fig 3; $p < 0.001$) and CCL21 (fig 3; $p < 0.001$) mRNA was determined in comparison with normal controls. In contrast, expression of CCL20 mRNA was found to be only slightly increased in CD compared with controls and did not prove to be statistically significant (fig 3; $p = 0.197$).

Expression of CCL19, CCL20, and CCL21 was then evaluated by immunohistochemistry and double immunofluorescence investigations on paraffin sections of normal colonic tissue and large bowel affected by CD. Sections of tonsil or lymph node were stained to determine the specificity of immunostaining.

In normal controls, immunostaining for CCL19 was found in a few DC within the T zone of Peyer's patches. Expression of CCL20 was found to be strictly confined to mucosal

Figure 2 Distribution of dendritic cell (DC) subsets in controls and in patients with Crohn's disease (CD). Immunohistochemistry for fascin (A, B, E, F), CD83 (C, D, G–K), and BDCA-2 (L, M). Within normal colonic tissue, fascin (A, $\times 100$) and CD83 (C, $\times 100$) expressing DC were mainly restricted to the T cell area of Peyer's patches. Only occasionally isolated fascin (B, $\times 400$) or CD83 positive (D, $\times 400$) DC were observed within the lamina propria. In contrast, in CD, numerous fascin (E, $\times 200$) and CD83 positive DC (G, $\times 200$) were observed within enlarged T cell aggregates. Numerous fascin positive DC were detected in association with T cells in the submucosa (F, $\times 400$). Numerous CD83 expressing mature myeloid DC were found within T cell clusters within the lamina propria (H, $\times 400$), in the T cell corona of epithelioid cell granulomas (I, $\times 200$), or adjacent to as well as within lymphatic vessels (K, $\times 400$). No significant difference in the number or distribution of BDCA-2 expressing plasmacytoid DC were observed in controls (L, $\times 200$) or in tissue affected by CD (M, $\times 200$).

epithelial cells of the crypts and surface (fig 4B). Strongest expression for CCL21 was observed by reticular stromal cells within the T cell zone of Peyer's patches as well as lymph vessels adjacent to Peyer's patches (fig 4D). Expression of CCL19, CCL20, or CCL21 could not be observed by reticular stromal cells or lymphatic endothelial cells within the mucosa or other parts of the bowel wall.

In contrast, in CD, an increased number of CCL19 expressing cells was observed within the enlarged T cell clusters (fig 4A). Cells expressing CCL19 proved to be mature myeloid DC showing coexpression for CD83, as determined

by double immunofluorescence staining (fig 5A). Similarly, increased numbers of CCL21 expressing dendritic shaped cells were observed within T cell aggregates (fig 4E, F; fig 5C), often in direct contact with fascin and CD83 positive DC (fig 5D). CCL21 expressing cells within these T cell clusters proved to be negative for DC markers (fascin, CD11c, and CD83; fig. 5D) as well as antigens related to T cells (CD3; fig 5C), B-cells (CD79a), or macrophages (CD14, latter data not shown). As CCL21 expressing cells demonstrated expression of vimentin (data not shown), they probably correspond to fibroblastic stromal cells which have already

Figure 3 Real time reverse transcription-polymerase chain reaction (RT-PCR) analysis of CCL19, CCL20, and CCL21 mRNA expression in lymph node (LN), Crohn's disease (CD), and normal colonic tissue (non-inflammatory gut disorder (NIGD)). Total RNA was analysed for CCL19, CCL20, and CCL21 mRNA expression by quantitative RT-PCR from tissue samples affected by CD ($n = 10$) and normal colonic bowel ($n = 7$). mRNA isolated from three normal lymph nodes served as controls. Real time PCR analysis of lymph node mRNA demonstrated abundant transcripts for CCL19 and CCL21 whereas only weak expression for CCL20 could be detected. Inflammatory affected colonic tissue of CD demonstrated significantly increased expression of CCL19 ($p < 0.001$) and CCL21 ($p < 0.001$) mRNA compared with normal controls (NIGD). In contrast, CCL20 did not prove to be significantly different between CD and controls ($p = 0.197$). Thus the increment in CCL19 and CCL21 mRNA expression without different regulation of CCL20 mRNA in CD mirrored the chemokine mRNA expression observed in lymph node mRNA.

Figure 4 Lymphoid chemokine and chemokine receptor expression in Crohn's disease (CD). Immunohistochemistry for CCL19, CCL20, CCL21, and CCR7 (A-I). Increased expression of CCL19 by numerous dendritic cells (DC) within the enlarged T cell aggregates in CD (A, $\times 200$). No difference was observed for expression of CCL20 by enterocytes in control (B, $\times 400$) or CD samples (C, $\times 400$). In control large intestine, CCL21 expression was restricted to lymphatic vessels and a few reticular stromal cells of the T zone of Peyer's patches (D, $\times 100$). In CD, CCL21 was expressed by numerous reticular cells within DC/T cell inflammatory aggregates (E, $\times 100$) as well as by numerous lymphatic vessels (F, $\times 200$). Numerous CCR7 expressing cells were seen within DC/T cell aggregates in CD (G, $\times 200$). Expression of CCR7 by granuloma histiocytes in CD (H, $\times 200$). Non-specific staining was excluded by negative controls, as shown for CCR7 (I, $\times 100$).

Figure 5 Two colour immunofluorescence investigation of chemokine and chemokine receptors in Crohn's disease (CD) (A–I). CD83 positive mature dendritic cells (DC) (indocarbocyanine 3 (Cy3), red fluorescence) showed coexpression for CCL19 (A; indocarbocyanine 2 (Cy2), green fluorescence, $\times 600$ zoom). CCL21 expression (Cy2, green fluorescence) by D2-40 expression lymphatic endothelial cells (B; Cy3, red fluorescence, $\times 400$). CCL21 expressing reticular stromal cells (C, D; Cy2, green fluorescence) and CCL21 expressing lymphatic vessel (E, F; Cy2, green fluorescence) in the vicinity of CD3 positive T cells (C, F; Cy3, red fluorescence, $\times 600$) and CD83 positive DC (D, E; Cy3, red fluorescence, $\times 400$). CD83 positive DC (Cy2, green fluorescence) demonstrated strong expression of CCR7 (G; Cy3, red fluorescence, $\times 400$). In contrast, CCR6 (Cy3, red fluorescence) was only weakly expressed by CD83 positive DC (H; Cy2, green fluorescence, $\times 400$) in CD. Negative control with irrelevant idiotype antibodies demonstrated only background staining (I, $\times 400$).

been shown to represent the major source of CCL21 expressing cells within the lymph node paracortex. Furthermore, we found an increased number of lymphatic vessels showing strong expression for CCL21 throughout the entire bowel wall, as determined by double immunofluorescence investigation combining CCL21 with the lymphatic endothelial marker D2-40 (fig 5B). These strongly CCL21 positive lymphatic vessels were mostly observed adjacent to DC (fig 5E) and T cell clusters (fig 5F). In addition, clusters of T cell and DC were detected within or adjacent to lymphatic vessels demonstrating expression for CCL21 (fig 4F; fig 5E, F).

CCR7 expression in CD

As CCL19 and CCL21 are strong inducers of the migration of mature DC as well as naïve and memory T cells to the T cell zone of secondary lymphatic organs, we determined whether the appropriate receptor CCR7 could also be observed, with special emphasise on mature DC and T cells. Lymphocytes within the T cell aggregates demonstrated expression for CCR7, as determined by immunohistochemistry (fig 4G) and double immunofluorescence (not shown). In addition, we found numerous CD83 positive mature DC within the T cell clusters consistently expressing CCR7 on their surface (fig 5G). No differences concerning expression of CCR7 by

mature DC were observed in CD versus not inflammatory affected bowel, controls, or in diverticulitis. Interestingly, CCR7 expression was also observed by epithelioid histiocytes within the granulomas (fig 4H) which were occasionally found to develop within CCL21 expressing lymphatic vessels. CCR6 as the receptor for CCL20, which has been shown to be expressed by immature DC, could only occasionally and inconsistently be detected in CD83 positive DC showing weak coexpression (fig 5H).

DISCUSSION

During the past few years, advances have been made in the understanding of the pathogenesis of inflammatory bowel disease.^{2, 22–24} New insights have been gained into the function of caspase activating and recruitment domain 15 (CARD15)/NOD2, the first cloned susceptibility gene for CD. New data on CARD15/NOD2 function and nuclear factor κ B activation indicate that an inflammatory reaction of the intestinal mucosa as a response of the innate immune system may be necessary for maintenance of gut homeostasis. Data on CARD15/NOD2 expression suggest that macrophages and epithelial cells could be the site of a primary pathophysiological defect, and T cell activation by DC might be a secondary effect inducing chronification of the inflammation, perhaps as a backup mechanism to defective innate immunity. In addition to CARD15/NOD2, there are additional “innate” pathways by which commensal and pathogenic bacteria can directly interact with cells of the intestinal mucosa, such as toll-like receptors. Thus the innate immune system, with early responses to bacterial products as well as modulation of T cell responses by antigen presenting cells, represent important aspects in the pathophysiology of CD.

T cell activation and tolerance strongly depend on antigen presentation by DC. Antigen ingestion and processing are best performed by immature DC whereas T cell activation requires mature DC. Various models have been put forward to explain the dual role of DC in the stimulation of T cell immunity or induction of tolerance.^{6, 25–26} One model suggests that distinct DC subtypes specialise in activation of antigen specific T cells or in removing T cells from the repertoire.²⁷ Another model assumes that the outcome of DC/T cell interactions depends on the stage of DC maturation. In this model, immature DC were implicated as favouring the development of tolerance by induction of interleukin 10 producing CD4⁺CD25⁺ regulatory T cells, which in turn promote tolerance.²⁸ In contrast, fully mature DC have the ability to initiate T cell proliferation and effector function,^{25, 29} which can be observed in certain autoimmune diseases such as CD. Furthermore, it has been proposed that the same type of DC can be responsible for induction of either tolerance or immunity, depending on several factors, such as number of DC at the site of inflammation, their life span, and the nature and amount of costimulatory molecules they express. Thus increased numbers of mature DC will favour priming whereas low numbers of mature DC or immaturity of DC will lead to immune tolerance.

In the present study, we investigated the distribution and maturation of the different DC subtypes in CD. To determine total DC cell number in normal and colonic tissue affected by CD, we first performed immunostaining using a fascin antihuman specific antibody, which has been shown to detect mature and immature myeloid and plasmacytoid DC.³⁰ A more detailed subtype analysis was done using antibodies for plasmacytoid (BDCA-2⁺, CD123⁺) and myeloid (CD11c⁺) immature (CD1a⁺) or mature (CD83⁺) DC. Quantitative analysis demonstrated an increased number of DC in tissue affected by CD versus controls, non-inflamed mucosa of CD, or diverticulitis using the pan-DC marker fascin (fig 1A; fig 2A, B, E, F).

In additional investigations for ulcerative colitis, we observed an increased number of immature as well as mature DC compared with normal controls. The number of DC in heavily inflamed ulcerative colitis colonic tissue was comparable with numbers observed for CD. However, most cases affected by ulcerative colitis demonstrated weak to moderate inflammation, with lower numbers of DC (data not shown). This may be due to the different indications for surgical intervention in ulcerative colitis versus CD. Thus increased numbers of DC may also occur in inflammatory active ulcerative colitis.

Additional immunohistochemical investigations for the mature myeloid DC marker CD83 showed that approximately 80% of the total DC represented mature myeloid DC (fig 1B; fig 2C, D, G–K). No significant difference was found between the percentage of CD83 positive cells between colon affected by CD or normal controls. As these mature DC were found exclusively within proliferating T cell clusters, our results indicate ongoing antigen presentation by mature DC within colonic tissue affected by CD. This assumption is further supported by the finding that CD1a, which is well known as being expressed by immature myeloid DC as well as by Langerhans cells, could not be detected in control or colonic tissue affected by CD. Concerning the role of plasmacytoid DC, no significant differences in cell number or distribution were observed between normal controls and colonic bowel tissue affected by CD. In contrast with myeloid DC, plasmacytoid DC were randomly distributed within the lamina propria and submucosa and not associated with T cell clusters (fig 2L, M). Thus antigen presentation or T cell stimulation by plasmacytoid DC obviously does not play a significant role in CD affected colonic tissue.

Under normal conditions the immune system of the gut is characterised by a steady state condition of immune tolerance. It is believed that with immune tolerance, immature DC pick up antigen in the mucosa and travel to the T cell zone of regional mesocolic or mesenteric lymph nodes.⁴ During their migration, maturation of DC takes place with concomitant upregulation of CCR7 guiding migration of DC. Within the lymph node paracortex, DC drive activation of naïve T cells. As the normal gut mucosa is nearly devoid of naïve T cells, their activation is strictly confined to the T cell area of secondary lymphatic organs where the contact with matured DC takes place.³¹ However, our results demonstrated an increased number of mature CD83 positive myeloid DC within the inflamed mucosa of CD. These DC were always found in close association with proliferating T cells. Approximately 30% of the T cells within these clusters have been shown to represent naïve T cells.³² Thus the inflammatory conditions observed in CD mirror DC/T cell interactions normally occurring in regional lymph nodes and thus de novo established tertiary lymphatic tissue within the bowel wall, as suggested previously.²¹

What instigates the mechanisms behind the development of this tertiary lymphoid tissue showing increased numbers of T and B cell accessory cells such as DC and follicular dendritic cells with concomitant T and B cell areas? Our results support the concept that specific homing of CCR7 positive leucocytes to the bowel wall may contribute to the autoimmune reaction of CD. We showed significant increased expression of the lymphoid chemokines CCL19 and CCL21 in CD versus normal colonic tissue whereas expression of CCL20 was not differentially regulated (fig 3; fig 4A–F; fig 5A–F). These results strongly imply that significantly increased expression of CCL19 and CCL21 leads to a chemokine microenvironment normally observed in lymph nodes. These chemokines may favour homing and interaction of CCR7 expressing cells such as mature myeloid DC and naïve or memory T cells within the bowel wall

instead of within regional lymph nodes, as observed with immune tolerance (fig 4G, H; fig 5G). Our results demonstrating expression of CCL19 by DC as well as CCL21 by reticular stromal cells and lymphatic endothelial cells adjacent to inflammatory infiltrates within the bowel wall of CD are in accordance with the above mentioned chemokine mediated attraction pathways established in lymphoid tissue. Analogous to secondary lymphoid organs, T cells and antigen presenting cells colocalise in a chemokine dependant manner at the site of inflammation in CD, leading to the development of autoreactive T cells.^{33–36} CCL21 and CCR7 induced attraction of immune cells for targeting organs has recently been reported for various other autoimmune conditions.^{11 12 14 18 33 37 38 39}

Additional investigations have been performed for two other so-called lymphoid chemokines, SDF-1 and BLC. Investigation of mRNA as well as protein expression of both chemokines in CD versus controls and tissue derived from lymph nodes demonstrated increased expression in CD. These results support our suggestion that CD is characterised by a chemokine environment observed in lymphoid tissue.

Concerning the role of CCL20 in CD, Kaser and colleagues⁴⁰ recently found increased expression of CCL20 in tissue affected by CD, which is in contrast with our results. This fact may be explained by the different tissue specimens used for investigation of CCL20 expression. Kaser *et al* used biopsy specimens derived from the mucosa, the site demonstrating strongest expression of CCL20 by epithelial cells, as demonstrated by immunohistochemistry by us and Kaser *et al*. In contrast, tissue investigated by us was derived from the entire bowel wall. Thus increased local expression of CCL20 will probably play a role in CCR6 positive cell accumulation beneath the mucosa, as demonstrated by Kaser *et al*. However, our results did not demonstrate increased recruitment of CCR6 positive DC at the site of inflammation.

In conclusion, our results demonstrated an increased number of mature myeloid DC leading to activation and proliferation of T cells within the inflammatory affected bowel wall in CD. Mature DC as well as naïve and memory T cells within the DC/T cell clusters showed expression of the ligand CCR7 of the so-called lymphoid chemokines CCL19 and CCL21. Increased expression of these lymphoid chemokines by DC themselves as well as by reticular stromal cell and activated lymphatic vessels at the site of inflammation promote the DC/T cell interactions important for the priming and expansion of autoreactive T cells in CD. It remains to be clarified whether modulation of expression of lymphoid chemokines might represent an effective therapeutic approach for disrupting the inflammatory autoreactive process observed in CD.

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