

Aberrant plasmacytoid dendritic cell distribution and function in patients with Crohn's disease and ulcerative colitis

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are two debilitating, progressive, systemic inflammatory illnesses targeting mainly the gastrointestinal tract in genetically susceptible individuals [1]. Dendritic cells (DC) are thought to be involved critically in the breakdown of immunological tolerance towards indigenous microbiota and the resulting inflammatory T cell polarization and activation known to cause these two disorders [2]. To date, research on DC in human inflammatory bowel disease (IBD)

Summary

Dendritic cell (DC) function is believed to be of critical importance for the pathogenesis of inflammatory bowel disease (IBD). To date, most research in animal models and the few human data available is restricted to myeloid DC, while plasmacytoid DC (pDC) capable of controlling both innate and adaptive immune responses have not yet been investigated systematically in human Crohn's disease (CD) or ulcerative colitis (UC). CD11c⁻, CD303⁺/CD304⁺ and CD123⁺ pDC from peripheral blood ($n = 90$), mucosal tissue ($n = 28$) or mesenteric lymph nodes ($n = 40$) (MLNs) of patients with UC and CD or controls were purified and cultured. Thereafter, pDC were enumerated, phenotyped and cytokine secretion measured by flow cytometry (FACS), immunohistochemistry and/or cytometric bead array, respectively. Interferon (IFN)- α secretion following cytosine phosphatidyl guanine (CpG) A oligodeoxynucleotide (ODN) 2216 (5'-GGGGGACGATCGTCGGGGG-3') stimulation was assessed by enzyme-linked immunosorbent assay (ELISA). We found a significantly higher frequency of pDC in the inflamed colonic mucosa and MLN of IBD patients. Moreover, the fraction of CD40 and CD86 expressing cultured peripheral blood pDC was significantly higher in flaring UC and CD patients and their secretion of tumour necrosis factor (TNF)- α , interleukin (IL)-6 and IL-8 were increased significantly compared with controls. In contrast, the IFN- α secretion of peripheral blood pDC isolated from flaring IBD, particularly in UC patients, was reduced significantly compared with controls. Our data suggest an aberrant distribution and function of pDC in IBD, contrary to their generally implicated role as inducers of tolerance. We speculate that the impaired IFN- α secretion may relate to the hypothesized defect in innate immunity in IBD and could also impact upon the generation of regulatory T cells (T_{reg}).

Keywords: antigen presentation, Crohn's disease, dendritic cells, ulcerative colitis

is limited to the investigation of myeloid DC (mDC), monocyte generated DC (moDC) and animal work with DC derived from rodent models or *in vitro* systems, while plasmacytoid DC (pDC) have not yet been studied systematically in human IBD [3]. However, based on the available literature, pDC are thought to be of key importance for the regulation of tolerance, considered to be inducers of regulatory T cells and thus key to our understanding of the aetiology and pathophysiology of IBD [4–6]. Our group has recently demonstrated a striking correlation between pDC and disease activity [7]. Here, we present the first

systematic analysis of primary human pDC from IBD patients.

Materials and methods

Patients and tissues

Peripheral blood was obtained from patients with UC ($n = 39$) or CD ($n = 37$) seen at our Inflammatory Bowel Disease Center. Control samples ($n = 14$) were obtained from healthy volunteers or blood bank leucocyte filters [for the interferon (IFN)- α experiments]. For tissue investigations, mucosal tissue ($n = 28$) or mesenteric lymph nodes (MLNs) ($n = 40$) from IBD patients or patients undergoing surgery for non-inflammatory (diverticulosis or irritable bowel syndrome) conditions were collected. Samples were analysed exclusively from carefully selected patients off steroids, biologics, immunomodulators or immunosuppressants. The study protocol was approved by Charité's institutional review board. All patients and volunteers gave informed consent to the study. Two validated instruments, the modified Truelove Witts Severity Index (MTWSI) for UC and the Harvey Bradshaw Severity Index (HBSI) for CD were used to assess disease activity [8,9]. UC patients who scored ≥ 10 on the MTWSI and CD patients who scored ≥ 7 on the HBSI were categorized to have active disease [flare-up (FU)]; all others as remission (RM). Table 1 summarizes the demographic data.

Purification of pDC from peripheral blood

Peripheral blood mononuclear cells (PBMC) were obtained from whole blood as described previously [7]. pDC were isolated and purified by magnetic cell separation from PBMCs using CD304 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as published previously [7]. Viability and purity of the isolated pDC population was checked by fluorescence activated cell sorter (FACS) and samples with less than 95% pDC discarded.

Purification of CD45⁺ mucosal cells

CD45⁺ cells were isolated from surgical specimens of mucosal tissue using magnetic separation with commercial kits (Miltenyi Biotec), as described previously [10]. Viability and purity of the isolated lymphocyte population were checked as described above for blood.

Analysis of pDC from MLNs

MLNs were homogenized and washed in magnetic affinity cell sorting (MACS) buffer. The cell suspension was filtered through a sterile gauze and passed through a 0.25-mm nylon mesh. Finally, cells were washed again and labelled with

CD123 and CD303 (BDCA-2) for further analysis as described above for blood.

Culture of pDC

pDC were cultured as described previously [7]. Briefly, 5×10^4 freshly isolated pDC were resuspended in 150 μ l RPMI-1640 plus L-glutamine medium with 1% penicillin/streptomycin, 1% sodium pyruvate (all GIBCO, New York, USA) and 10% human serum type AB (Cambrex, Charles City, VA, USA) supplemented with interleukin (IL)-3 at 10 ng/ml (R&D Systems, Wiesbaden, Germany) (to prevent apoptosis) and incubated for 21 h at 37°C and 5% CO₂.

Cytometric bead array (CBA)

Secretion of TNF- α , IL-6 and IL-8 in culture supernatants was measured by CBA analysis according to the manufacturer's guidelines (BD Biosciences, Heidelberg, Germany). After acquisition of sample data using FACS, the cytokine concentrations were calculated using the proprietary FCAP™ analysis software (BD Biosciences) [11].

Table 1. Demographic data of patients and controls for peripheral blood plasmacytoid dendritic cells (pDC) experiments.

	(n)	%	Mean	Min	Max
Number	76				
Ethnicity					
Caucasian	74	97.4			
Jewish	2	2.6			
Crohn's disease	37	41			
Male	7	18.9			
Female	30	81.1			
Age (years)			35	19	73
Disease duration (years)			10	0	41
Age at diagnosis (years)			26	10	63
Harvey Bradshaw Severity Index remission			1.5	0	5.5
Harvey Bradshaw Severity Index flare-up			8.2	7	18
Ulcerative colitis	39	51.3			
Male	17	45.6			
Female	22	56.4			
Age (years)			48	19	85
Disease duration (years)			12	0	39
Age at diagnosis (years)			36	14	65
Modified Truelove Witts Index remission			4.5	0	9
Modified Truelove Witts Index flare-up			11	10	13
Healthy controls	14				
Male	6				
Female	8				
Age (years)			30	26	37

IFN- α ELISA

To assess IFN- α secretion, pDC were isolated from peripheral blood from patients and healthy volunteers or fresh blood bank leucocyte filters (collected within the hour of blood donation). The cells were cultured as described above and stimulated with 3 μ g/ml cytosine phosphatidyl guanine (CpG) A [oligodeoxynucleotide (ODN) 2216] 5'-GGGGGA CGATCGTCGGGGGG-3' (TIB Molbiol, Berlin, Germany). Side-by-side experiments of volunteer- and filter-derived pDC did not find any differences regarding purity and IFN- α secretion (data on file). Supernatants were harvested and analysed with a human IFN- α ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The IFN- α concentration was calculated with KCjunior™ software (Wolf Laboratories, York, UK), version 1.41.4.

Antibodies

All monoclonal antibodies [CD40, CD45, CD83, CD86, CD123 and CD197 (CCR7)] were obtained as fluorochrome conjugates from BD Biosciences. CD303 was purchased from Miltenyi Biotech.

For immunofluorescence staining anti-CD3-phycoerythrin (PE) (Caltag, San Diego, CA, USA) and biotinylated anti-CD303 (Miltenyi Biotech) were used as primary antibodies. As a secondary antibody antibody-Alexa488 was used (Invitrogen, Darmstadt, Germany). To exclude unspecific binding, biotinylated mouse immunoglobulin (Ig)G1 (Caltag) was used as an isotype control.

Flowcytometric analysis

Three- or four-colour flow cytometric analysis (FACS) was used to identify and enumerate pDC as described previously [7,12].

Immunohistochemistry

MLNs were embedded in O.C.T. compound (Tissue-Tek, Zoeterwoude, Netherlands), frozen in liquid nitrogen and stored at -80°C . Specimens were sectioned at 7 μ m in a cryostat at -25°C on glass slides. Cryosections were circled using a Pap-Pen (Dako, Glostrup, Denmark) prior to staining. All incubation steps were performed in a moisture chamber at room temperature. To block unspecific protein binding of IgG molecules prior to the exposure of the primary antibodies, tissue sections were preincubated with blocking buffer. Slides were incubated for 1 h with the primary antibodies at a final concentration of 16.5 μ g/ml to 20 μ g/ml before washing them on a shaker 3 \times for 10 min. Fluorochrome-conjugated secondary antibodies, at a final concentration of 5 μ g/ml, were added for 1 h.

Slides were washed for 3 \times 10 min in washing buffer followed by water. Finally, slides were fixed with ethanol, mounted and examined with a confocal laser scanning microscope (LSM510; Zeiss, Jena, Germany). Confocal images were processed using Zeiss LSM Image Browser software.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (s.e.m.). Comparisons were performed by a Student's *t*-test with statistical significance accepted for $P < 0.05$.

Results

Increased frequency of pDC in the mucosa and MLNs

It is conceivable that circulating DCs in active IBD migrate to secondary lymphatic organs and sites of inflammation, as suggested by previous work by our group [7]. We decided to corroborate this hypothesis further by evaluating pDC in two other spaces – the colonic mucosa and MLNs.

Colonic mucosa. We found a significantly higher frequency of pDC in the inflamed mucosa of UC patients [$0.56 \pm 0.24\%$ of CD45+ lamina propria mononuclear cells (LPMC)] and CD patients ($0.19 \pm 0.04\%$ of CD45+ LPMC) compared with controls ($0.03 \pm 0.01\%$ of CD45+ LPMC) (Fig. 1a).

MLNs. The results from the mucosal space matched with the MLN. An increased frequency was found of pDC in MLNs of UC ($0.39 \pm 0.06\%$) and CD patients ($0.45 \pm 0.09\%$) compared with controls ($0.19 \pm 0.06\%$) (Fig. 1b). These data were corroborated by immunohistochemistry. Here we counted a higher number of pDC on immunohistochemical sections of both patients with UC (4.20 ± 0.77) and even more in those suffering from CD (8.34 ± 0.89) compared with non-IBD controls (3.67 ± 1.28) (Fig. 1c).

Distinct phenotype of peripheral blood pDC in CD and UC

Because we hypothesize that DC from IBD patients may induce and/or perpetuate inflammation, we investigated their native phenotype. We were particularly interested in their expression of co-stimulatory molecules. Expression of CD40 and CD86, two co-stimulatory molecules known to be up-regulated on mature, activated DC and critical for T cell activation was studied [13,14].

CD40. In UC, the fraction of CD40 expressing pDC was higher in UC patients in RM ($16.83 \pm 7.07\%$) and reached

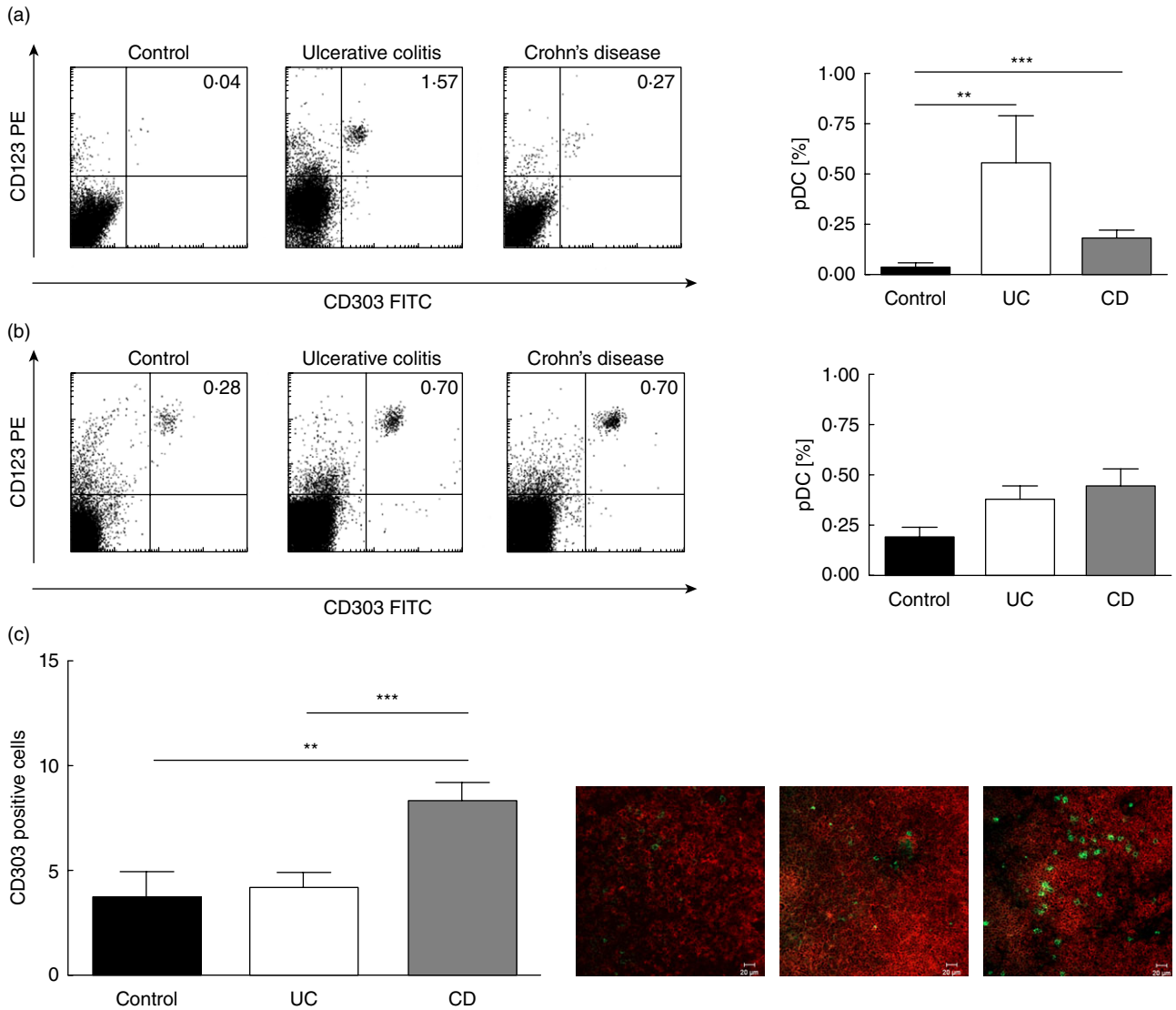


Fig. 1. Increased frequency of plasmacytoid dendritic cells (pDC) in mesenteric lymph nodes and colonic mucosa of flaring inflammatory bowel disease (IBD) patients. (a) Colonic mucosa: FACS plots from representative experiments. Quadrant thresholds were placed determined according to isotype controls. Bar graphs summarize data from all experiments with control mucosa ($n = 16$) or tissue from ulcerative colitis (UC) ($n = 6$) and Crohn's disease (CD) ($n = 6$) patients. (b) Mesenteric lymph node: FACS plots from representative experiments. Quadrant thresholds were placed determined according to isotype controls. Bar graphs summarize data from all experiments with control lymph node ($n = 6$) or tissue from UC ($n = 14$) and CD ($n = 20$) patients. (c) Mesenteric lymph node: sample immunofluorescence images from control, UC and CD. Asterisks denote statistical significance: ** $P < 0.01$; *** $P < 0.001$.

statistical significance in acute FUs ($15.16 \pm 0.58\%$, $P < 0.001$) compared with controls ($4.22 \pm 1.17\%$). In CD, the fraction of CD40 expressing pDC was comparable between controls and CD patients in RM ($3.67 \pm 2.38\%$). However, the fraction of CD40 expressing pDC was significantly higher in flaring patients ($11.60 \pm 3.4\%$) compared with controls ($P < 0.05$) (Fig. 2a).

CD86. In UC, the fraction of CD86 expressing pDC was comparable between controls ($21.78 \pm 4.48\%$) and UC patients in RM ($22.83 \pm 2.82\%$). However, the fraction

of CD86 expressing pDC was significantly higher in flaring patients ($69.13 \pm 2.87\%$) compared with controls ($P < 0.01$) and RM ($P < 0.001$). In CD, the fraction of CD86 expressing pDC was higher in patients in RM ($26.51 \pm 8.17\%$) and acute FUs ($41.48 \pm 11.68\%$) compared with controls (Fig. 2b).

CD83 and CD197 (CCR7). Overall, the fractions of CD83 and CD197 expressing pDC were very low and did not allow meaningful statistical comparisons (data not shown).

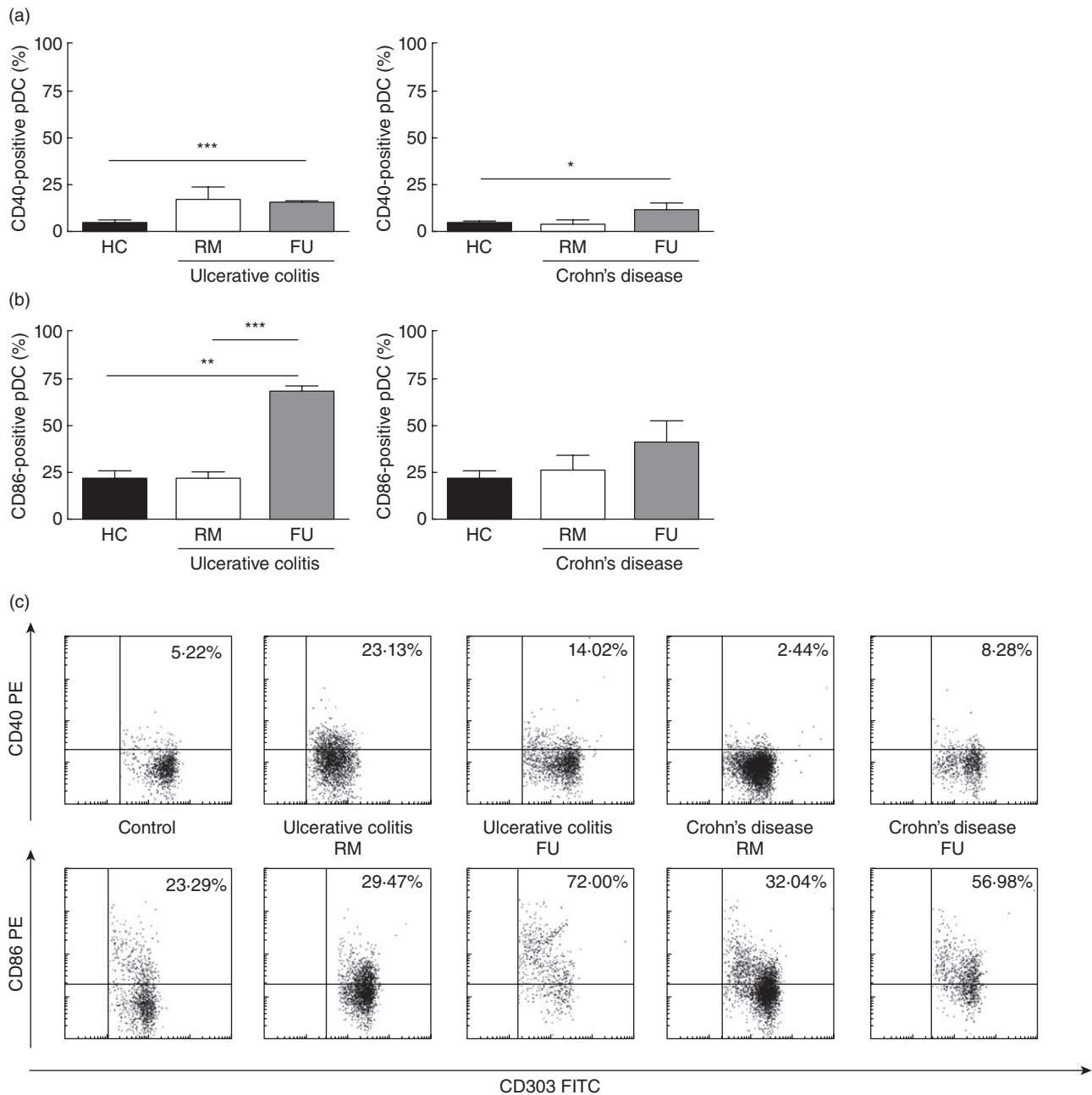


Fig. 2. Distinct phenotype of peripheral blood plasmacytoid dendritic cells (pDC) in Crohn's disease (CD) and ulcerative colitis. (a) CD40: increased fraction of CD40 expressing pDC in flaring UC [remission (RM) $n = 6$, flare-up (FU) $n = 3$] and CD (RM $n = 4$, FU $n = 3$) patients compared with controls ($n = 6$). (b) CD86: increased fraction of CD86 expressing pDC in flaring UC (RM $n = 3$, FU $n = 3$) and CD (RM $n = 4$, FU $n = 4$) patients compared with controls ($n = 5$). (c) FACS plots from representative experiments. Quadrant thresholds were placed determined according to isotype controls. Asterisks denote statistical significance: $**P < 0.01$; $***P < 0.001$.

Increased secretion of key inflammatory cytokines by peripheral blood pDC

Because our previous studies suggested not only migration but also an activated phenotype of DC in active IBD, we then studied functional aspects of pDC, such as the native

(unstimulated) secretion of key inflammatory cytokines such as TNF- α , IL-6 and IL-8.

TNF- α . Cultured pDC from UC patients (UC RM 24.11 ± 2.40 versus UC FU 32.94 ± 27.27 pg/ml) (Fig. 3a) and CD (CD RM 30.54 ± 9.18 pg/ml versus CD FU 34.81 ± 9.33 pg/ml) secreted more TNF- α than controls

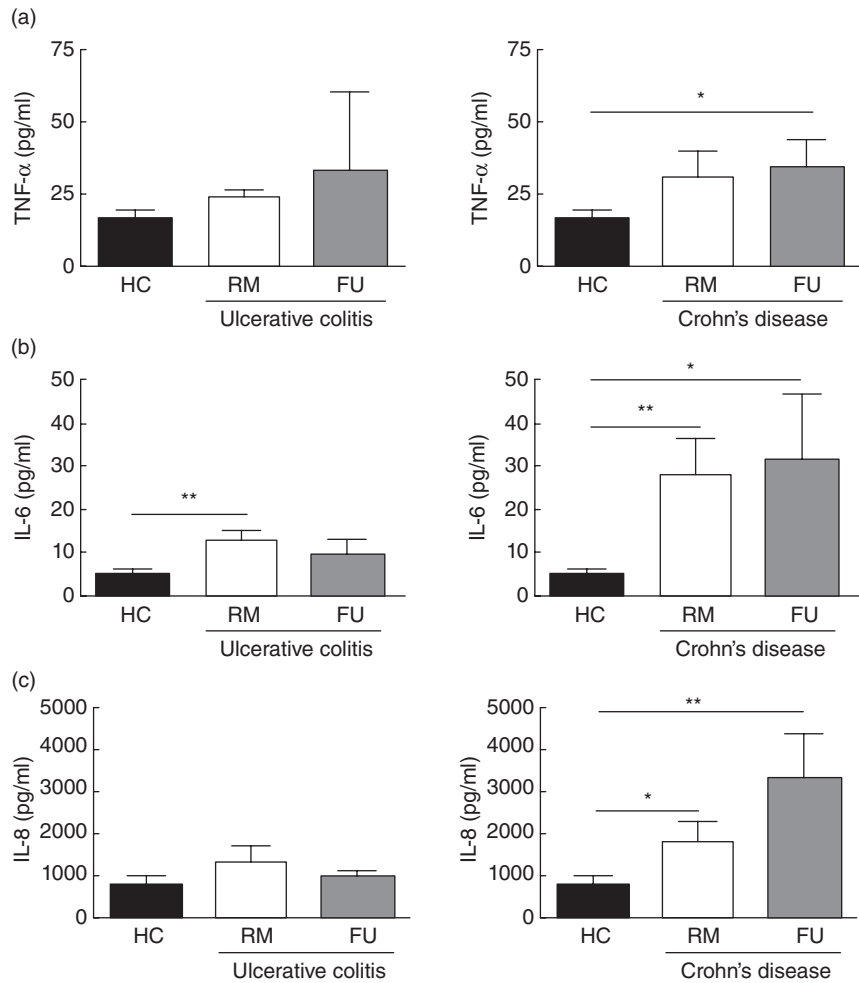


Fig. 3. Increased secretion of key inflammatory cytokines by peripheral blood plasmacytoid dendritic cells (pDC) from inflammatory bowel disease (IBD) patients. (a) Tumour necrosis factor (TNF)- α : cultured pDC from ulcerative colitis [remission (RM) $n = 7$, flare-up (FU) $n = 3$] and Crohn's disease (CD) (RM $n = 8$, FU $n = 10$) patients secrete more tumour necrosis factor (TNF)- α than healthy controls ($n = 14$). (b) Interleukin (IL)-6: cultured pDC from UC (RM $n = 7$, FU $n = 3$) and CD (RM $n = 8$, FU $n = 9$) patients secrete more IL-6 than healthy controls ($n = 13$). (c) IL-8: cultured pDC from UC (RM $n = 6$, FU $n = 3$) and CD (RM $n = 9$, FU $n = 9$) patients secrete more IL-8 than healthy controls ($n = 14$). Asterisk denotes statistical significance * $P < 0.05$; ** $P < 0.01$.

(15.90 ± 3.44 pg/ml). This effect was most apparent in flaring Crohn's patients, where it also reached statistical significance ($P < 0.05$).

IL-6. Surprisingly, cultured pDC from UC patients in RM secreted more IL-6 (UC RM 12.90 ± 2.25 pg/ml *versus* UC FU 9.56 ± 3.53 pg/ml) than flaring patients and both more than healthy controls (4.88 ± 1.39 pg/ml) (Fig. 3b). In CD, however, both patients in RM and during FUs (CD RM 28.24 ± 8.56 pg/ml *versus* CD FU 31.75 ± 15.20 pg/ml) secreted significantly more IL-6, than controls ($P < 0.01$ and $P < 0.05$), respectively.

IL-8. As already observed for IL-6, cultured pDC from UC patients in RM secreted more IL-8 (UC RM 1312 ± 385.6 pg/ml *versus* UC FU 964.1 ± 143.7 pg/ml) than flaring patients and both more than healthy controls (740.7 ± 234 pg/ml). However, these differences did not reach statistical significance (Fig. 3c). In CD, both patients in RM and during FUs (CD RM 1777 ± 481.9 pg/ml *versus* CD FU 3301 ± 1039 pg/ml) secreted significantly more IL-8, than controls ($P < 0.05$ and $P < 0.01$), respectively.

Impaired secretion of IFN- α by pDC

Because native (unstimulated) pDC from both IBD patients and controls do not secrete any IFN- α (data not shown), we had to use a stimulant for these experiments. Cultured and CpG ODN 2216-stimulated pDC from UC patients (UC RM 154.9 ± 61.08 and UC FU 87.29 ± 83.51 ng/ml) secreted significantly less IFN- α than control pDC (585.6 ± 72.18 ng/ml, $P < 0.01$). In CD the significantly decreased secretion was restricted to flaring patients (CD RM 707.2 ± 117.9 ng/ml *versus* CD FU 195.9 ± 107.8 ng/ml, $P < 0.01$) (Fig. 4).

Discussion

To date, DC research in human IBD has focused mainly on mDC [10,15–19]. However, while the occasionally confusing classification of DC subsets in man and mice is still ongoing, there is largely agreement that mDC and pDC originate from different progenitors alongside different lineages and thus have distinct properties and functions within the immune system [20,21].

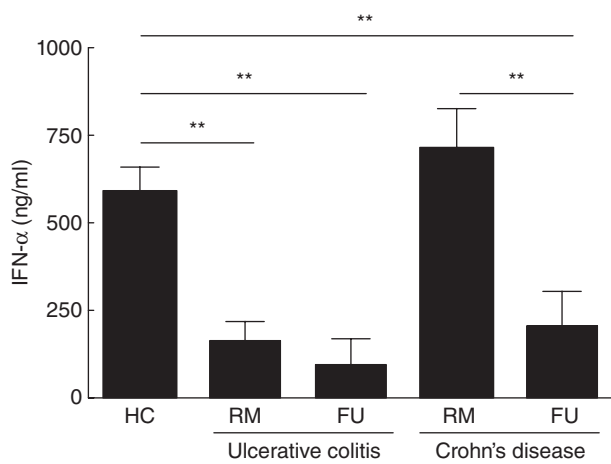


Fig. 4. Impaired secretion of interferon (IFN)- α by peripheral blood plasmacytoid dendritic cells (pDC) from inflammatory bowel disease (IBD) patients. Cytosine-phosphatidyl guanine (CpG) A oligodeoxynucleotide (ODN) 2216-stimulated pDC from flaring ulcerative colitis (UC) (RM $n = 6$, FU $n = 3$) and CD (RM $n = 6$, FU $n = 6$) patients secrete significantly less IFN- α than healthy controls ($n = 14$). Asterisks denote statistical significance: ** $P < 0.01$.

pDC develop in the bone marrow from a continuum of CD135⁺(Flt3⁺), CD117⁺(c-kit_{low}) lymphoid and common DC progenitors (CDP) and proceed through the putative committed pDC precursor and immature pDC (both still in the bone marrow) towards mature peripheral pDC [22]. The main function of pDC is their recognition of foreign (normally bacterial and viral) nucleic acids through their endosomal Toll-like receptors TLR-7 and TLR-9, resulting in the secretion of large amounts of type I IFNs, but also IBD typical inflammatory cytokines such as TNF- α and IL-12 (in animals) that recruit additional immune cells such as natural killer (NK) cells and thereby link the innate and adaptive immune responses [23,24]. Their frequency is rare, at approximately 0.1–2%, and renders their study in human disease somewhat difficult [7,25]. Therefore, it is perhaps not surprising that this is the first systematic analysis of their role in human CD and UC.

For this project we have exclusively analysed highly purified pDC from IBD patients in RM and acute FUs off immunomodulating, immunosuppressive or biological drugs to avoid any influence on their function, which we consider a major strength of our work. Some patients were on mesalamine, which is not known to alter DC function.

Our data show an increased frequency of IBD pDC in both the mucosal space and MLNs that, taken together by our previously reported drop in the peripheral circulation, suggest their migration to areas where they could potentially encounter their cognate antigens, e.g. foreign (bacterial) or foreign-thought nucleic acids [7]. Our data corroborate observations in murine adoptive transfer models that previously reported an accumulation of pDC in the inflamed mucosa [26] and demonstrated the CCR9-dependent

homing of pDC to the intestine under inflammatory conditions [27,28].

Interestingly, the frequency of CD40 and CD86, i.e. co-stimulatory molecule expressing unstimulated peripheral blood pDC from flaring IBD patients, is already higher than in controls. This activated phenotype suggests a premature activation of pDC that may contribute to the well-established overactivation of T cells in IBD.

This interpretation is supported further by an increased secretion of the inflammatory cytokines IL-6, IL-8 and TNF- α by pDC isolated from flaring IBD patients. In other words, the observed phenotypic differences are paralleled by functional consequences. An increased secretion of these prototypical inflammatory cytokines polarizes T cells towards a proinflammatory phenotype [T helper type 1 (Th1)] that contributes to the perpetuation of inflammation known to occur in IBD [29]. Approved and investigational drugs individually targeting TNF and IL-6 are available and clinically effective, emphasizing their importance in IBD [30,31]. Conversely, CpG ODN 2216-stimulated secretion of the pDC hallmark cytokine IFN- α is reduced significantly in flaring UC and CD patients. IFN- α links both adaptive and innate immunity and recent IBD research has pointed to defects in the innate immune response [32,33]. IFN- α protects from and ameliorates experimental colitis in animal models [34,35], and has been used successfully to treat human UC [36]. While the exact role of IFN- α in chronic (intestinal) inflammation remains unclear, it appears there is a dichotomy of effects mediated through different pathways. On one hand, IFN- α -TLR-9 engagement could activate the NF- κ B pathway [37], known to be critically important in IBD [2]. This mechanism has been demonstrated recently with pDC in lupus patients and contributes to the perpetuation of inflammation [38]. However, IFN- α has also been demonstrated to indirectly [39] and directly [40] induce regulatory T cells (T_{reg}) and thereby attenuate Th1 responses. Our research demonstrates a diminished IFN- α secretion of predominantly UC pDC in both RM and FUs. This provides a new explanation for the known lack of IFN- α in human UC and the reported mixed efficacy in preclinical models and patients with UC [41,42].

Chemokine receptor 7 (CCR7, CD197), which contributes to both immunity and tolerance, was essentially not expressed by unstimulated pDC [43]. The functional dichotomy of CCR7 is reflected by CCR7-deficient mice that exhibit a phenotype with inflammatory lymphoid structures of the gastrointestinal mucosa and in turn its requirement for proper T_{reg} function [44,45]. The low expression of CCR7 clearly does not appear to impact on homing of human pDC in human IBD shown by us in this study, and previously in animal models [26–28].

Two other studies have attempted to characterize pDC in human mucosal tissue and MLNs from IBD patients [46,47]. The first study was limited by critical methodological flaws. Here the authors claim that fascin would be a global marker

for human DC including pDC. In immunohistochemical studies using triple staining with fascin, CD123, CD303, they did not find an increased frequency of pDC in mucosal and lymph node samples of CD patients. Neither fascin nor CD123 are specific markers for human pDC [22]. However, the combined staining may have masked the epitope for proper CD303 binding and thus caused misleading results. As our data are not based exclusively on immunohistochemistry but flow cytometry of complete mucosal tissue, not simply sections, we are confident that they reflect the true situation in the human gut. Moreover, CD patients were not phenotyped according to the Montreal classification in that study, and their disease activity was not measured with validated instruments in this study [8,9,48]. This may have contributed to a failure in the proper detection of pDC.

The second study reported larger MLNs in CD and increased total number of DCs, and thus generally supports our findings [47]. However, their panel to identify pDC, CD11c⁻, CD123⁺ and major histocompatibility complex (MHC)-II⁺ does not identify pDC unequivocally in humans [22].

Taken together, these data suggest that pDC differ substantially in their distribution, phenotype and function in human IBD. Their animal model-derived role as inducers of oral tolerance and T_{reg} in inflammatory conditions of the intestine may be impaired in human inflammatory bowel disease [49,50]. Further research into additional molecular mechanisms appears warranted.

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Disclosure

None.

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