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Plasmacytoid dendritic cells regulate host immune response to *Citrobacter rodentium* -induced colitis in colon-draining lymph nodes

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Abbreviations used in this article: cDC, conventional dendritic cell; coMLN, colon-draining MLN; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LN, lymph node; MLN, mesenteric LN; pDC, plasmacytoid dendritic cell

Abstract

Dendritic cells (DC) are first in line to sense invading microbes and to deliver signals to other immune cells. Plasmacytoid dendritic cells (pDC) produce high amounts of type I interferons (IFN) but also regulate immune responses. Using the *Clec4C* (BDCA2)-diphtheria toxin receptor (DTR) mouse model allowing conditional pDC-depletion, we identified an essential role for pDCs in regulating intestinal inflammation locally in the gut. In pDC-depleted mice, *C. rodentium* infection led to enhanced activation of conventional DCs and induction of IFNγ-producing Th1-cells in colon-draining lymph nodes, while induction of $Foxp3^+/CD25^+$ Treg and IL-17-producing Th17 cells was impaired. Concomitantly, $F4/80^+$ macrophages accumulated into the colon lamina propria in excess, levels of Il-1β and Tnf transcripts increased and $Foxp3^+$ Treg were fewer. Our results indicate that pDCs control inflammation in the gut during *C. rodentium* infection and that they have an important immune regulatory role in colon-draining lymph nodes.

Introduction

Citrobacter rodentium is an intestinal pathogen of mice, which due to its attaching and effacing behavior is often regarded analogous to human enteropathogenic and enterohemorrhagic *E. coli* (EPEC/EHEC) [1]. While in some inbred mouse strains and in many genetically modified mouse models with targeted defects in innate or adaptive immune defense it may cause severe colitis, in immunocompetent mice *C. rodentium*-induced colitis is mostly self-limiting and well tolerated [2] [3].

In the gut, dendritic cells (DC) recognize microbes and other antigens on luminal surfaces and within epithelial layer and lamina propria, and by migrating via afferent lymphatics, deliver them to gut-associated lymphoid tissues (GALTs). Therein DCs promote T-cell responses and are intimately involved in programming T-cell differentiation into effector T cells and/or regulatory Treg cells [4]. Plasmacytoid dendritic cells (pDCs) produce high amounts of type I interferons (i.e. IFN-α) [5] in response to viral nucleic acids but are also capable of many functions ascribed to cDCs, such as antigen presentation and induction of effector and regulatory T cells and promoting tolerance to self-antigens [6],[7]. Distinct from blood-borne pDCs and pDCs in other lymphoid tissues, GALT-associated pDCs appear to have adopted a unique phenotype with diminished IFN-α production [8],[9], yet able to promote Th17 and Treg differentiation [10]–[12]. Characterization of lymph nodes draining lymph [8],[13] and dendritic cells [14] from colon has enabled investigations into pDCs and their role in local immune responses to microbial and other challenges in the colon. Recently, we found that *Citrobacter rodentium* infection induced rapid activation of both cDCs and pDCs in colon-draining mesenteric lymph nodes (coMLNs) and that on transcriptome level, pDCs responded to infection by modifying their gene expression far more widely than cDCs [8]. This suggested that pDCs actively participate in immune response elicited by *Citrobacter* and perhaps, by other bacterial pathogens in the colon.

In the present study, we addressed the role of pDCs in immune response against *C. rodentium* infection using BDCA2-DTR mice, which allow selective and conditional depletion of pDCs [15]. Our results show that pDCs are essential in maintaining immune and epithelial cell homeostasis and barrier function locally in the gut during host response to *C. rodentium* infection.

Results and discussion

pDCs control bacterial invasion into colon mucosa

In pDC deficient mice, *C. rodentium* infection caused a severe colitis, which necessitated to end follow-up within 5-6 days from infection. This is consistent with lethal infection reported in another study [16], although the appearance of significant weight loss (Fig. 1A), lethargy and appearance of general indices of colitis (Supporting Information Fig. 1) appeared even earlier in the present study.

Increased expression of stress related genes in the colon and haptoglobin levels in serum (Supporting Information Fig. 2) suggested impaired barrier function of the gut. Fluorescence *in situ* hybridization of bacterial DNA in sections of colon revealed that in pDC-deficient mice, bacteria associated more firmly to colon epithelium, and some bacteria had invaded in between individual epithelial cells (Fig. 1B,C). This suggested that via some mechanism, pDC participate in controlling barrier function and bacterial invasion in epithelium.

Macrophages accumulate in excess in colon of pDC-depleted mice while FoxP3^{+} T cells decrease

Since pDC-deficiency impaired gut barrier function and increased bacterial invasion, we evaluated if pDC-depletion associated with qualitative or quantitative changes in infiltration of immune cells or colon cytokine levels. Lack of pDCs favored expression of proinflammatory cytokines interleukin-1β (*Il-1β*) and tumor necrosis factor alpha (*Tnfα*), while transforming growth factor beta (*Tgfβ*) expression was significantly decreased (Fig. 2A). Thus, absence of pDCs amplified proinflammatory cytokine response to infection. This was associated with increased infiltration of F4/80 (Fig. 2A, B) and CD11b (Supporting Information Fig. 3) positive cells in colon lamina propria of pDC-depleted mice.

In light of the increased infiltration of apparently proinflammatory macrophages in lamina propria and possible immune-regulatory effects of pDC, we evaluated numbers of T cells and especially of Tregs in colon. While no differences in CD8 cells (data not shown) or in total numbers of CD4 cells in colon were seen, the proportion of Tregs among CD4 cells was significantly lower in pDC-deficient mice (Fig. 2D-F). This suggests that Tregs regulate proinflammatory activity of tissue-infiltrating macrophages. Although local responses to the pathogen in the mucosa are of importance [3], these may also affect epithelial integrity. Interestingly, TLR-4 deficient mice do not succumb to *C. rodentium* infection despite reduced immune cell infiltration in the intestinal mucosa [17].

Lack of pDCs associates with cDC activation and impairs Treg activation in coMLN

According to earlier studies [8] *C. rodentium* infection activates both pDCs and cDCs in colon-draining lymph nodes (coMLN). The potential anti-inflammatory and counter-regulatory roles of gut-associated pDCs prompted us to determine the activation status of conventional CD11c^{+}/MHCII^{+} DC (cDC). In pDC-depleted mice, both CD80 and CD86 were significantly upregulated on cDCs (Fig. 3A, B) suggesting that pDCs counteract pathways towards full activation of cDCs in coMLN. Because cDCs are likely to present *C. rodentium* –derived antigens to antigen-specific T cells in coMLN, the activation status of cDC in coMLN is likely to affect the balance between effective antimicrobial and regulatory immunity.

In GALT, pDCs may be particularly apt [9] to support regulatory T cells. We therefore evaluated the effect of pDC depletion on induction of Treg cells. In pDC-depleted mice, FoxP3^{+}/CD25^{+} Treg cells were significantly decreased (Fig. 3C) suggesting that pDCs may be required for normal activation of Treg cells in coMLN. IL-17A -producing CD4^{+} T cells were also fewer in pDC-depleted mice (Fig. 3D), indicating reduced Th17 activity. Reduced TGFβ expression in the colon, which associates with activation of Treg and Th17 cells and Treg functionality [11], may reflect this. In contrast, IFNγ-

producing $CD4^+$ cells were significantly increased in BDCA2-DTR mice (7,20%±0,820) compared to WT mice (4,76%±0,55) (Fig. 3E) indicative of enhanced Th1 response to *C. rodentium* infection. IFNγ-producing $CD8^+$ T cells were also moderately increased although not statistically significantly (Fig. 3F). Thus, in absence of pDCs, Treg are not induced normally, and effector T-cell response to *C. rodentium* infection is biased towards IFNγ-production, consistent with macrophage influx and proinflammatory cytokine production in lamina propria. Increased expression of co-stimulatory ligands CD80 and CD86 in cDCs of pDC-depleted mice suggests that pDC suppress Th1- and augment Foxp3 Treg-response by modifying the activity of conventional DC. However, pDC can dampen autoimmune T-cell responses also via cognate interactions [18], and thus potential effects of pDC on T cells through direct cell-to-cell contacts cannot be excluded.

Concluding remarks

While in wild-type mice, *C. rodentium* induces relatively mild and non-invasive infection of colonic mucosa, mice modified genetically may succumb to infection due to impaired host response and invasion of microbes in the body [19]–[21]. This study corroborates the finding of an earlier study [16] regarding the importance of pDC in controlling *C. rodentium* infection and related immune response and reveals hitherto unrecognized roles for pDC in controlling local host-response in the gut.

An increase in TNFα and IL-1β transcripts in colon and imaging of bacterial DNA by *in situ* hybridization and of inflammatory macrophages by immunohistochemistry suggested that pDCs moderate a facet of host-response augmenting accumulation of inflammatory macrophages and perhaps secondary to that, bacterial invasion in colon mucosa.

Although a Th1-response is necessary to clear *C. rodentium* infection [22], it may be necessary also to control Th1 activity. Otherwise, an aggravated proinflammatory response in colon mucosa impairs epithelial barrier function. Controlling Th1 activity appears as a mechanism by which pDC moderate antimicrobial host-response to avoid detrimental inflammation.

Colon-draining lymph nodes were identified relatively recently as lymph nodes receiving lymph and cells from the colon [8],[13],[14]. By focusing on these lymph nodes, we identified a regulatory role for pDCs in infectious colitis. Our findings emphasize the role of colon-draining lymph nodes as an inductive site for adaptive immune responses, and pDC as a type of DC essential for regulating local immune responses in colon.

Materials and methods

Mouse strains and C. rodentium infection

Experiments were performed using C57BL/6-Tg(CLEC4C-HBEGF) 956Cln/J mice, hereafter BDCA2-DTR mice (The Jackson Laboratory #014176) and wild-type littermates. Mice were used in the age of 6-8 weeks. Both male and female mice were used as no gender-specific differences were seen. Animals were bred and maintained under SPF conditions with water and food ad libitum. All experiments were approved by the National Animal Experiment Board of Finland (ESAVI/6608/04.10.07/2017). For pDC-depletion, 1000 ng of diphtheria toxin (DT) (Merck, Darmstadt, Germany) was injected in peritoneal cavity every second day. Where indicated, DT was injected to controls in the same dose.

Citrobacter rodentium (strain ICC168, originally from G. Frankel, Imperial College, London, and obtained from F. Powrie, University of Oxford, U.K.) was grown overnight in Luria broth medium with nalidixic acid (50 mg/L) and 10^9 CFU was inoculated orally in each mouse. Following infection, mice were monitored daily for weight and activity until day 5, after which they were killed due to severe (>20%) weight loss and loss of activity of the BDCA2-DTR mice.

Colon immunohistochemistry

To evaluate bacterial penetration into colon epithelium, colons were collected into Carnoy's fixative (60 % methanol, 30 % chloroform, 10 % acetic acid). Fixed colon samples were further prepared to 5µm paraffin sections and stained with *in situ* hybridization method. Paraffin was first removed from sections with xylene-ethanol processing. Alexa Fluor 647-conjugated EUB-338 probe (5'-GCTGCCTCCCGTAGGAGT-3', Invitrogen, USA) in Tris-HCl buffer (20 mM Tris-HCl, 0,9 M NaCl, 0,1 % SDS, 20 % formamide; pH 7,4) was used to label bacterial DNA. Mucin-2 was stained

with rabbit H-300 primary antibody (Santa Cruz Biotechnology, USA) and anti-Rabbit Alexa 488 secondary antibody (Abcam, USA). Actin was stained with 1 µg/ml phalloidin-tetramethylrhodamine (AAT Bioquest, USA). Sections were covered with ProLong Diamond Antifade Mountant including DAPI. Nikon Eclipse Ti2-E microscope was used to image the samples and images were analyzed with ImageJ software. Levels of bacterial DNA were quantified by counting single bacterial DNA per area.

To analyze immune cells in colon, frozen colon sections were stained with F4/80 (APC-conjugated, clone BM8, BioLegend) and CD4 (Alexa 488-conjugated, clone GK1.5, BioLegend), or with CD11b (FITC-conjugated, clone M1/70, BioLegend) and CD8 (APC-conjugated, clone 53-6.7, BioLegend). $CD4^{+}$ Tregs were analyzed by staining sections with CD4 (Alexa 488-conjugated, clone GK1.5, BioLegend) and FoxP3 (PE-conjugated, clone XMG1.2, BioLegend). Actin was stained and sections were covered and imaged as described above. F4/80 and CD11b expressions were quantified by measuring fluorescence signal per area.

RNA isolation and quantitative PCR

To analyze how pDC deficiency in bacterial infection effects cytokine and transcription factor levels, colons were collected into RNA later (Qiagen, Germantown, MD, USA). RNA was isolated from ascending part of the colons 5 days after *C. rodentium* treatment by PowerLyzer® UltraClean® Tissue & Cells RNA Isolation Kit (MoBio, Carlsbad, CA, USA). Genomic DNA was removed with DNase Max Kit (Qiagen). RNA concentrations were measured with NanoDrop (Thermo Fisher, Waltham, MA, USA). 1000 ng of RNA was used for First Strand cDNA synthesis, which was made applying Maxima Reverse Transcriptase and oligo-dT primers (Thermo Fisher). Produced cDNA was diluted to 1:10 in LightCycler® 480 SYBR Green I Master solution for qPCR analyzed with

LightCycler 480 (Roche, Basel, Switzerland). Primers are listed in Supporting Information Table 1. Ct values were normalized to β-actin and target gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

DC activation and lymphocyte subsets

In all experiments coMLN1 and coMLN2 were identified and cell isolation was performed as described in ref. 9. Isolated cells from these lymph nodes (C1 and C2) were pooled prior to staining. To monitor activation of cDCs in coMLNs, cells were stained with selected antibodies listed in Supplementary Table 1 and analyzed with NovoCyte flow cytometry (Acea, CA). Gating strategy of pDC identification and cDC activation is described in Supporting Information Fig 4.

T cell populations from coMLN1 and coMLN2 were stained with selected antibodies listed in Supporting Information Table 2. Transcription Factor Buffer Set (Cat#:562725; BD Biosciences) was used to perform intracellular staining. For IFNγ and IL-17 staining, 2×10^6 cells were stimulated for 4 h (37 °C) in DMEM 10% FCS, and Cell Activation mixture (Cat#:423304; BioLegend). Stained cells were analyzed with BD LSRFortessa™ (BD BioSciences). Demonstration of the gating strategy can be seen in Supporting Information Fig. 5.

Data analysis and statistics

FACS analyses were made by using FlowJo software v10 (TreeStar, Ashland, OR, USA) or NovoExpress software (Acea, CA). Statistical analyses were made using GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Two-tailed Student t test was used to test significance of numerical data. Image J software was used to analyze IHC sections.

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Conflict of Interest Disclosure

The authors declare no commercial or financial conflict of interest

Data availability statement

Data available on request from the authors

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Figures

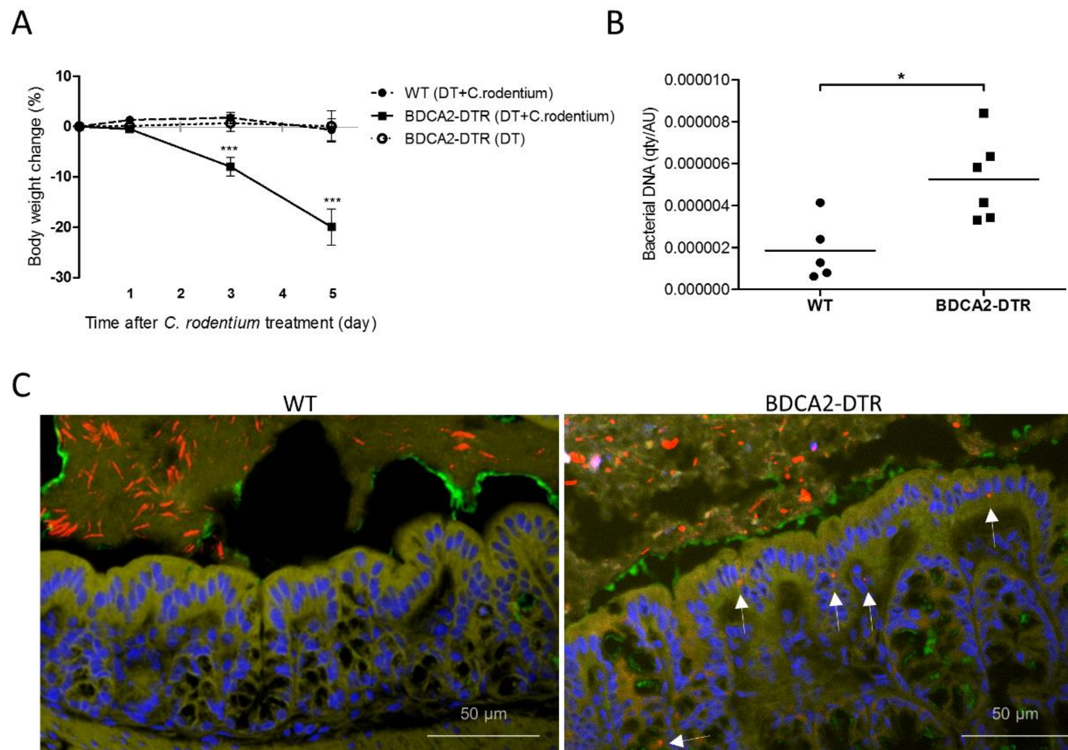

FIGURE 1. pDC-depletion leads to severe weight loss and impairs colon barrier function during *C. rodentium* infection. (A) Weight loss in mice of indicated groups following C. rodentium infection (n=8 mice/group). Data are represented as mean ± SEM. (B, C) Fluorescence *in situ* hybridization shows bacterial penetration (white arrows) into colon epithelium in pDC-depleted BDCA2-DTR mice (Red = bacterial DNA, Yellow = actin, Blue = nuclei, Green = mucin). Scale bar= 50µm and 20x magnification. Each data point represents one mouse and horizontal line depicts mean value. *p < 0,05, **p < 0,01, ****p < 0,0001 (unpaired two-tailed Student's t test). Data are from 3 (A) and 2 (B,C) independent experiments with n=2-3 mice per experimental group. In (B), bacterial DNA was quantitated from in situ-hybridization micrographs (see text).

FIGURE 2. Macrophages and macrophage-derived cytokines and regulatory FoxP3⁺ cells in the colon during *C.rodentium* infection. (A) qPCR analysis from the colon showed that proinflammatory cytokines Il-1β and Tnfα were increased in BDCA2-DTR mice while Tgfβ was decreased (mean + SD) (data was normalized to β-actin). (B, C) Immunohistochemical staining of frozen colon sections for F4/80 (Red = F4/80, Yellow = actin). Scale bar= 50µm and 20x magnification (D) Enumeration of CD4 cell numbers and (E) the proportion of FoxP3⁺ cells among CD4 cells in WT and BDCA2-DTR mice. (F) Representative staining for CD4 and FoxP3 (Grey = actin, Green = CD4, Red = FoxP3). Scale bar= 50µm and 20x magnification .Data are from 2 independent experiments with n=5-6 (A) and n=2-3 (B-D) mice per experimental group. Each data point represents one mouse and horizontal line depicts mean value. *p < 0,05 (unpaired two-tailed Student's t test).

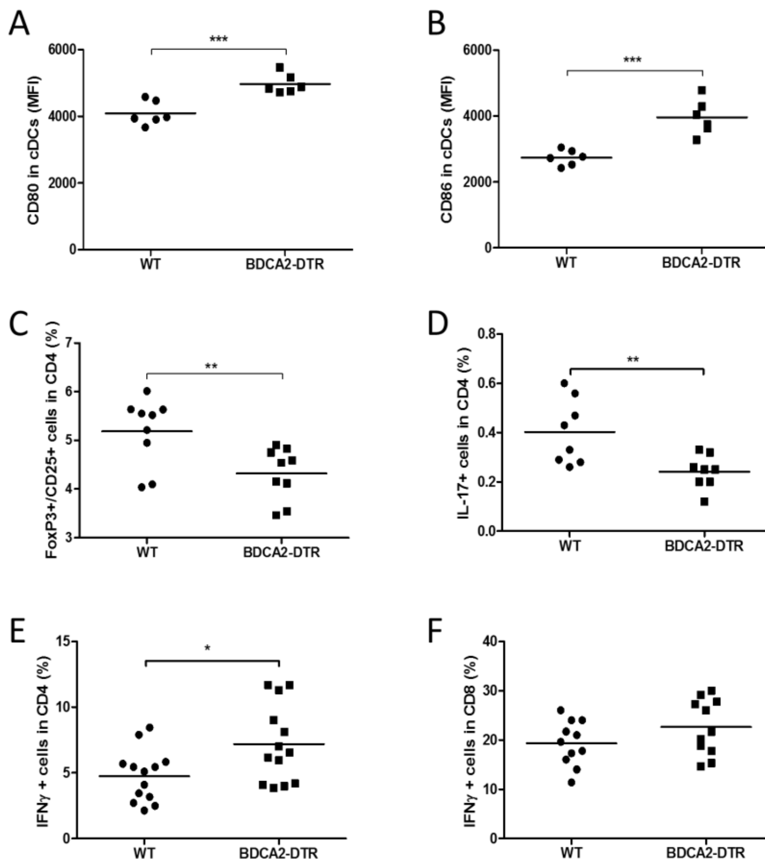

FIGURE 3. pDC depletion enhances cDC activation and alters the balance of T cell subsets in coMLN following *C. rodentium* infection. Expression of CD80 (A) and CD86 (B) on cDCs analyzed 3 days post infection (gated as $CD11c^{+}$/ $MHCII^{+}$ cells). (C) FoxP3+Treg cells were significantly decreased in pDC-depleted mice during *C. rodentium* infection. (D) Decreased IL-17 and (E) increased IFNγ producing CD4 cells in pDC deficient mice. (F) IFNγ production in CD8 cells. Data from 2 (A, B) or 3 (C-F) independent experiments with n=3-4 mice per experimental group were measured by flow cytometry. Each data point represents one mouse and horizontal line depicts mean value. *p < 0,05, **p < 0,01, ***p < 0,001 (unpaired two-tailed Student's t test).

Graphical abstract

pDCs regulate inflammatory responses in the gut during C. rodentium infection. (1) pDC-depletion leads to impaired barrier function of the colon epithelium, increases cytokine transcripts and decreases Foxp3+Treg cells in the gut. (2) In coMLN, pDC-deficiency allows activation of cDCs, decreases Treg and Th17 cells and promotes IFNγ-producing Th1 cells.