Pathways for the Recognition of the Intestinal Microbiota

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The specific role of most of these newly discovered DC subsets in antigen sampling and presentation is unknown and it remains therefore unclear whether they work synergistically, as alternatives or have distinct functions in the recognition of the intestinal microbiota. The focus of effort in this field will be to understand how these diverse DC subsets cooperate in regulating the complex homeostasis and host defense in the different intestinal immune compartments. The intestinal lamina propria contains a DC network that serves as a newly discovered gateway for the uptake and transport of the intestinal microbiota. Specialized vesicular structures at the end of transepithelial dendrites serve as 'luminal sensors' for the mucosal immune system. Characterization of the surface components of these luminal sensors may aid in developing strategies to prevent bacterial and viral pathogen entry. We propose that the lamina propria and M cell-dependent antigen sampling systems are associated with specific DC subsets. The discovery of functionally defined subsets of dendritic cells associated with distinct mechanisms responsible for the uptake of antigens across the intestinal barrier opens the path for strategies for targeting them specifically in the development of vaccines or treatment approaches for inflammatory bowel diseases. For these exciting possibilities to progress into practical applications, it needs to be established whether distinct DC subsets are associated with multiple pathways or if their function is linked to specific innate or adaptive immune responses. Defining DC function in the intestine will be pivotal in finally progressing our understanding on how the mucosal immune system makes the distinction between commensal microbiota, pathogens and self antigens.

Key words: dendritic cells; chemokine receptors; intestinal microbiota; antigen sampling

INTRODUCTION

The intestine is monitored by a highly adaptable host defense system in which dendritic cells (DCs), macrophages and epithelial cells together monitor the microbial environment and coordinate immune responses to danger signals (1). Mucosal DCs are the critical antigen presenting cells responsible for priming of naïve T lymphocytes triggering the induction of adaptive immune responses to control tissue inflammation and to maintain immune tolerance (2, 3). The gastrointestinal mucosa is in constant interaction with the luminal microenvironment which contains commensal microbiota as well as a variety of bacterial, viral and protozoan pathogens. A single layer of intestinal epithelial cells (IEC) covers the 260-300m² of mucosal surface of the digestive tract. In this highly antigenic environment the mucosal immune system must maintain tolerance to commensal bacteria, food and self antigens while initiating defensive responses to pathogens to prevent uncontrolled infection of the host. By sampling pathogens and bacteria DCs and macrophages play an

important role in the immune defense of the gut (4–7). Interactions of DCs with antigen-specific T lymphocytes triggers the induction of adaptive immune responses and is also critical for the control of tissue inflammation and the maintenance of immune tolerance (3, 8–11). Evidence from numerous mouse models of inflammatory bowel disease (IBD) indicates that dysregulation of antigen recognition and processing of intestinal microbiota is a common disease mechanism (12). However, the cellular properties of mucosal DCs that determine uptake and presentation of antigens in the intestinal microenvironment are not well understood.

Recent studies have demonstrated that distinct macrophage and DC subsets may utilize diverse mechanisms to sample antigen in the intestine. Besides acquiring antigen in the lamina propria upon intestinal epithelial barrier breakdown, intestinal DCs located in intraepithelial pockets below the M cells may detect incoming pathogens and phagocytose them constitutively (13). Intestinal villous M cells may provide an additional system for passing intestinal microbiota to intestinal DCs (14). Furthermore, intestinal DCs appear to transport captured proteins from the gut lumen and fragments of epithelial cells themselves (15).

Recently, evidence was presented that intestinal DCs

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Fig. 1. Pathways for antigen acquisition by DCs in the intestine. Lamina propria DCs are derived from blood monocytes and undergo differentiation to immature DCs. DCs in the intestine can obtain information about the intestinal microbiota by different mechanisms which involve direct phagocytosis of antigens and the crosstalk with intestinal epithelial cells. DCs may present antigens either in the lamina propria, Peyer's patches, or mesenteric lymph nodes. Failure in the recognition of intestinal commensal or pathogenic bacteria may lead to the dysregulation of pathways governing T cell activation leading to intestinal inflammation (IEC: intestinal epithelial cells).

can also monitor the contents of the intestinal lumen by entering it directly or by extending dendrites through the intestinal epithelium, although the molecular basis of this mechanism remains to be elucidated (16, 17). In recent years it has become apparent that a diversity of DC lineages with distinct characteristics contribute to innate and adaptive immune responses in the intestine. While it has become increasingly evident that several mechanisms are involved in the translocation of antigens across the intestinal barrier, it is clear that mucosal DCs play a key role in the transport and processing of intestinal antigens for presentation in Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) (Fig. 1). We now present evidence that distinct DC subsets are associated with these pathways as we begin to unravel their function in specific innate or adaptive immune responses.

MATERIALS AND METHODS

Mice

Mice with a target deletion of CX₃CR1 or CCR6 with

green fluorescence protein (GFP) insertion were previously described (18, 19). This approach allowed the generation of a mutant CX₃CR1 or CCR6 locus and also the examination of the CX₃CR1 or CCR6 expression pattern and migration of cells that normally express CX₃CR1 or CCR6. All transgenic mice strains were bred and maintained in our specific pathogen-free animal facility according to institutional guidelines. All experiments were carried out with mice at 6 to 12 weeks of age according to protocols approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital and Harvard Medical School.

Isolation of PPs and small intestinal lamina propria DCs

PPs were crushed between frosted glass slides, washed twice in phosphate buffered Saline (PBS; Bio Whittaker™) with 1% bovine serum albumin (BSA) and 0.01% NaN₃ and then cells were stained with a cocktail of antibodies. Small intestines were inverted on

polyethylene tubes (outer diameter 2.08 mm, Becton Dickinson), washed with calcium and magnesium free PBS, and the mucus removed with 1 mM dithiothreitol (DTT; Sigma). The intestinal epithelium was eluted with 30 mM EDTA, followed by digestion of the tissue with 36 U/ml type IV collagenase (Sigma) and 150 μ g DNase I (Roche) in 5% FCS/DMEM for 90 min at 37°C in a 5% CO₂ humidified atmosphere. The digested tissue was passed through a Cell Strainer (40 μ m Nylon; BD Falcon™) and washed with Dulbecco's modified Eagle's medium (DMEM; Cellgro®).

Small intestinal lamina propria DCs are further enriched by performing an iodixanol (5,5'-(2-hydroxy-1-3 propanediyl)-bis(acetyl amino)) bis [N,N'-bis(2,3 dihydroxypropyl)- 2,4,6 triiodo 1,3 benzene-carboxamide]) density gradient centrifugation. The dilution of 10 ml of 30% iodixanol working solution with 21.4 ml diluents (0.8% NaCl, 5 mM EDTA, 10 mM Tricine-NaOH, pH 7.4) yields an isoosmotic 9.5% iodixanol solution ($\rho=1.055$ g/ml). Thirty percent iodixanol working solution has been previously prepared by mixing equal volumes of 60% iodixanol (OptiPrep®; Axis Shield, Oslo, Norway) with diluents (0.8% NaCl, 5 mM EDTA, 10 mM Tricine-NaOH, pH 7.4). Lamina propria leukocyte isolates are resuspended in 12 ml 9.5% iodixanol solution. Eight ml inactivated fetal calf serum (FCS; Biotechnics Research Inc.) is layered on top of this cell suspension, followed by centrifugation at 1700 g for 10 min at 20°C. DCs are harvested by transferring the FCS layer to 15 ml polypropylene conical tubes (Falcon). Mixing the transferred FCS with 10 ml DMEM (Cellgro®) dilutes the DC suspension. Lamina propria DCs are pelleted by centrifugation at 1700 g for 10 min at 5°C and washed in medium followed by centrifugation at 500 g for 7 min at 5°C.

HE staining

5 μ m cryostat sections are placed in haematoxylin for 5 min, rinsed with deionized water, and stain is developed with tap water. Sections are destained by dipping slides 8 to 12 times in acid ethanol and rinsing in tap and deionized water. Sections are stained with eosin for 45 sec, dehydrated and covered by using the xylene based PerMount (Fisher Scientific). Sections are analyzed using an Olympus AX 70 microscope.

Flow cytometry

Isolated mouse lamina propria DCs are incubated in 10% donkey serum (Santa Cruz) for 20 min at 4°C in FACS buffer (0.2% BSA, 0.1% sodium azide in PBS), and then stained with PE-conjugated antibodies (anti-

CD80, -CD86, -CD11c, -CD11b, -CD16, CD4, (all from PharMingen) and or biotin-conjugated antibodies (anti-CD11c, and -I-Ab (all from PharMingen) followed by Cy-Chrome™ conjugated streptavidin (from PharMingen). DC isolates are then analyzed on a FACSCalibur cytometer using CellQuest software (Becton Dickinson).

Confocal microscopy and 3D tissue reconstruction of living PPs

M cells or intestinal epithelial cells (IECs) of living mucus-free PPs or small intestines were stained with *Ulex europaeus* agglutinin (UEA) conjugated with Rhodamin (Vector Laboratories) or with wheat germ agglutinin (WGA) conjugated with Texas Red (Molecular Probes) at concentrations of 20 μ g/ml for 1 hour. Living intestinal tissues were imaged with a Bio-Rad Radiance 2000 confocal microscope using multi-tracking (line switching) for two-color imaging. Image acquisition was carried out with LaserSharp Scanning Software, and 3D reconstructions and cell volume analysis were completed with Volocity software.

RESULTS AND DISCUSSION

To characterize the origin and functional diversity of intestinal mononuclear phagocytes responsible for recognition of intestinal pathogens we have utilized GFP-reporter mice, which allow the characterization of myeloid derived macrophages and DC subsets and their antigen sampling and immune regulatory properties. Using 3D reconstructive confocal microscopy in living tissues and optimized intestinal DC isolation techniques, we found that the small intestinal lamina propria is colonized by myeloid derived CD11c⁺ DC populations which are distinguished by their expression levels of either CD11c or CX₃CR1. CX₃CR1⁺ CD11c⁺ DCs populate the entire lamina propria and can potentially sample antigens at the basolateral surfaces of intestinal epithelial cells in the jejunum, proximal ileum and the colon. In addition, intestinal DCs extend transepithelial dendrites into the terminal ileum in a process that requires CX₃CR1. The intestinal microbiota provides the essential stimuli for the development of CD11c⁺ DCs in the terminal ileum. The ability of intestinal macrophages and DCs to phagocytose and clear *Salmonella typhimurium* occurs only if CX₃CR1 is present. Our study thus assigns functional significance to the role of the CX₃CL1/CX₃CR1 ligand/receptor in the regulation of the major myeloid-derived intestinal DC subset responsible for antigen sampling and host defense in the intestinal lamina propria.

It has previously been shown that intestinal epithelial cells and endothelial cells in the lamina propria express the membrane-bound chemokine CX₃CL1/fractalkine (20, 21). In order to identify the cellular targets of IEC-derived fractalkine, we utilized mice in which one or both copies of the *cx3cr1* allele was replaced with a gene encoding GFP (18). In these mice, GFP expression is under the control of the CX₃CR1 promoter and, consequently, heterozygous mice (*cx3cr1*^{GFP/+}) express the receptor and GFP while homozygous mice (*cx3cr1*^{GFP/GFP}) express GFP but are CX₃CR1-deficient. GFP expression in these mice can be used to identify, isolate and characterize organ specific cell populations that normally express CX₃CR1. In earlier studies, we found GFP and CX₃CR1 expression in circulating monocytes and their progeny within tissues. No phenotypic or functional differences were detected in the peripheral lymphoid system between heterozygous and wild type mice (18), but *cx3cr1*^{GFP/GFP} animals were found to have compromised migration of CD16⁺ monocytes from blood into various tissues (22).

Examination of gut-associated tissues from *cx3cr1*^{GFP/+} and *cx3cr1*^{GFP/GFP} mice by confocal fluorescence microscopy revealed GFP-positive mononuclear cells in the lamina propria, PPs and MLNs. The isolated lamina propria leukocytes from *cx3cr1*^{GFP/+} mice represent different developmental stages of immature and mature DCs. The GFP⁺ cells in both homozygous and heterozygous animals expressed CD11c, CD11b, CD4 and CD16/32 (23). The lamina propria cell isolations also contained CD16⁺ macrophages which did not express CD11c or CX₃CR1/GFP.

CD11c⁺CD11b⁺ DCs from *cx3cr1*^{GFP/+} and *cx3cr1*^{GFP/GFP} mice can be divided into subsets in which the CX₃CR1 promoter is either activated or silent. CX₃CR1 deficiency did not influence the relative percentage of CD11c⁺ DCs with active CX₃CR1 promoter nor their expression levels of MHC class II, CD80, and CD86 which were similar to those observed in control C57BL/6 wild type mice (23).

CCR6 influences antigen recognition in the intestine by regulating the migration of CD11c⁺CD11b⁺ DC in response to CCL20 into the dome regions of the PPs (19, 24, 25). Analysis of isolated lamina propria DC populations revealed a subdivision of CD11c⁺ cells into subsets, which can be distinguished by their level of CD11c expression and co-expression of either CX₃CR1 or CCR6.

Analysis of living intestinal tissues of *ccr6*^{GFP/+} and *cx3cr1*^{GFP/+} mice revealed that CCR6 expression indeed characterizes the immune compartment of PPs. CCR6⁺

DCs were found to be enriched in the PPs while CX₃CR1⁺ DCs populated the entire lamina propria (LP), as well as PPs. CD11c⁺CCR6⁺ DCs are a distinct subset from CX₃CR1⁺ DCs, which lack expression of CCR6. CCR6⁺ B cells were also detected in the PPs but occurred rarely in the lamina propria.

In *ccr6*^{GFP/+} and *ccr6*^{GFP/GFP} mice, GFP positive DCs were not observed within the follicle associated epithelium (FAE) or the dome regions of PPs, which contained a number of GFP⁺ lymphocytes as determined by average cell volume. In contrast, CX₃CR1⁺ DCs were normally closely associated with the FAE. Together, these experiments demonstrate that DC subsets in the intestine can be distinguished from one another based on chemokine receptor expression. Our experiments indicate that CCR6⁺ DCs are associated with antigen recognition in PPs, while CX₃CR1⁺ DCs collect antigens from the lamina propria of the intestine and sample the intestinal microbiota through transepithelial dendrites (13, 23, 26). However, it is not clear whether one particular subset or both participate in the activation of pathogen-specific T cells following oral infection with invasive pathogens.

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