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Antigen-Presenting Cell Candidates for HIV-1 Transmission in Human Distal Colonic Mucosa Defined by CD207 Dendritic Cells and CD209 Macrophages

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Abstract

A common route for HIV-1 infection is sexual transmission across colorectal mucosa, which is thought to be 10–2,000 times more vulnerable to infection than that of the female genital tract. Mucosal surfaces are the first line of defense against many pathogens but the antigen-presenting cells (APCs), key regulators of innate immunity and determinants of adaptive immunity, are not well defined in these target tissues. Using immunohistochemistry, dendritic cells expressing Langerin ($CD207^+$), a lectin known to bind and internalize HIV-1, were detected in the periphery of colonic glands and sparsely scattered in the submucosa similarly in colorectal mucosa. This cell type, well known in skin, has generally not been reported in colonic/rectal mucosa. Unexpectedly, the largest APC population observed was a macrophage-like population expressing the well-characterized tissue macrophage markers CD68 and CD163. Confocal microscopy of these cells revealed colocalization of CD209 (DC-SIGN), a presumed dendritic cell marker believed to facilitate HIV-1 transmission, but not other dendritic cell markers. These results show evidence of the unconfirmed presence of Langerhans cells in colorectal mucosa and a predominance of macrophage-like APCs that express CD209 (DC-SIGN). These findings define potential target cells in the pathogenesis of HIV-1 transmission, which may have key implications for the study of early transmission events in normal colorectal mucosa, as well as other infectious diseases and primary immune diseases involving the gut.

Introduction

Mucosal innate immune cells comprise a diverse group of cells that plays important roles in the induction and regulation of immune responses at mucosal surfaces. The most important innate immune cells are the professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. A key DC function is antigen presentation to T lymphocytes, whereas macrophages are more important for direct effector functions such as microbe recognition and killing.[1–3] In peripheral tissues, these cell types are among the first to sense invading pathogens through pattern recognition receptors (PRRs) that recognize conserved molecular motifs of microbes. DC and macrophage functions vary in different tissues due to differences in expression of PRRs and antigen exposure,[2] which in turn direct the induction of local and adaptive immune responses, both of which are thought critical to successful prevention of HIV mucosal infection.

APCs in the skin, mouth, small intestine, and the female genitourinary tract have been well defined.[1,2,4–7] However, thorough characterization in the mucosa of the distal colon and rectum mucosa, the most vulnerable sexually exposed mucosa, is lacking. To date, APCs in the lower gut mucosa are poorly understood and are primarily derived from studies of inflammatory bowel disease (IBD) and animal models.[6] Given that the sigmoid colon and rectal mucosae are an epidemiologically significant portal of entry for sexually transmitted pathogens, identifying the population of APCs in these anatomical regions is vital to developing prevention strategies for various pathogens, including HIV and other sexually transmitted infections.

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Rectal mucosa is the primary route of HIV-1 infection in men who have sex with men and an underappreciated (and possibly predominant) contributor to heterosexual infections. This tissue is exquisitely vulnerable to transmission, with 10- to 2,000-fold relative risk per sexual act compared to vaginal-cervical exposure, which is further increased when coexisting infections are present.[8–11] Moreover, rectal mucosal tissue constitutes a key anatomical reservoir for established HIV-1 infection,[12,13] is the first compartment for $CD4^+$ T cell depletion in new infections (regardless of the route of exposure), and is rich in virus-permissive immune cells, including DCs, macrophages, and activated $CD4^+$ T cells.[14,15]

Within mucosal tissues, DCs may be among the first cells to encounter HIV-1 and have been hypothesized to play a crucial role in initial virus dissemination by transporting HIV-1 to $CD4^+$ T lymphocytes in regional lymph nodes.[14,16,17] Moreover, DCs and monocyte/macrophages are a key determinant of the activation state of $CD4^+$ T lymphocytes in mucosal tissues. Thus mucosal innate immune function has critical implications for infectious diseases as well as autoimmunity and even systemic immune regulation. Therefore, detailed evaluation of APCs of this tissue site in normal subjects is important to establish baseline populations for comparison with altered states. This article describes a detailed evaluation of APCs using frozen tissue from two regions of this important, HIV-exposed tissue compartment (rectum and sigmoid colon) in normal subjects, using immunohistochemistry (IHC) and confocal microscopy.

Materials and Methods

Human normal sigmoid colon and rectal mucosal tissue

Gastrointestinal mucosal biopsies were freshly acquired from the sigmoid colon and rectum as previously described.[18] Briefly, healthy HIV-seronegative participants without evidence of rectal sexually transmitted disease (confirmed) or chronic gastrointestinal disorders were recruited to undergo flexible sigmoidoscopy. The study was approved both by the USC and UCLA Offices of the Human Research Protection Program Institutional Review Board and all subjects provided written informed consent. Using large-cup endoscopic biopsy forceps (Microvasive Radial Jaw #1589, outside diameter 3.3 mm), two to three biopsies (approximately 1 mm^3) were acquired endoscopically first from a location 5–8 cm from the anal verge ("rectal") and then another two or three biopsies from 25–30 cm ("sigmoid"). In the endoscopy suite, biopsy specimens were immediately embedded in OCT medium (Ames Co., Elkhart, IN) and snap-frozen in liquid nitrogen.

Immunohistochemistry of dendritic cells, macrophages/monocytes in normal sigmoid colon, and rectal mucosa

Cryostat sections (4 μm each) were acetone fixed and blocked with normal horse serum and then sequentially incubated with the indicated murine antihuman antibodies, biotinylated horse antimouse IgG and an avidin/biotin-peroxidase. Tissues were counterstained with hematoxylin (VWR, Radnor, PA) for light microscopy (Olympus BX51 microscope, Central Valley, PA, equipped with an Olympus America camera). The following mouse antihuman antibodies were used: CD1a (clone NA1/34-HLK; AbD Serotec, Raleigh, NC), CD1b (clone SN13 K5-1B8; eBiosciences, San Diego, CA), CD1c (clone M241; Ancell, Bayport, MN), CD207 (clone DCGM4; Immunotech SAS, Beckman Coulter, Marseille, France), CD14 (clone RPA-MI; Zymed Laboratories, South San Francisco, CA), CD68 (clone EBM11; DAKO, Carpinteria, CA), CD163 (clone EDHu-1; AbD Serotec, Raleigh, NC), and CD209 (clone DCN46; BD Pharmingen, San Diego, CA).

Enumeration of immune cells

To identify and quantify immune cells in sigmoid and rectal tissue, immunostained cells in epithelium and lamina propria were enumerated and reported per mm^2 for each area. Slides were scanned using an Aperio ScanScope XT (Aperio Technologies, Inc., Vista, CA) and images were analyzed using ImageScope Aperio software. On each slide, three separate, nonoverlapping areas were circumscribed for counting; each area encompassed 10 colonic/rectal glands; cells were counted at 40×. Cells were categorized as positive if a nucleus was surrounded by brown staining for the particular cell marker in all three areas for each tissue section and normalized to cells/mm^2. For statistical analyses, averages of total cell counts (per mm^2) in three areas were sampled (Student's *t*-test).

Two-color immunofluorescence staining of frozen cryostat sections

Indirect immunofluorescence was performed by serially incubating cryostat-prepared tissue sections with mouse antihuman monoclonal antibodies (mAbs) to CD1a, CD207, CD209, CD68, and CD163 of different isotypes followed by incubation with isotype-specific fluorochrome- (Alexa 488 and Alexa 568, Molecular Probes, Eugene, OR) labeled goat antimouse Ig antibodies. Controls included were isotype-matched control mouse antibodies as well as specific mouse antibodies with a mismatched secondary antibody. Slides were mounted with ProLong Gold antifade reagent with a nuclear marker DAPI (Molecular Probes, Eugene, OR). Images were obtained using confocal laser microscopy.

Confocal microscopy

Frozen cryostat sections were examined using a Leica-TCS-SP5 MP inverted confocal laser-scanning and two-photon laser microscope (Heidelberg, Germany) fitted with DPSS-diode (561 nm), argon (488 nm), and two photon laser (Spectra-Physics Millenia X 532 diode pump laser and Tsunami picosecond Ti-sapphire laser) at the Craniofacial Center for Molecular Biology at USC. Sections were illuminated with 488/568 nm for green/red and DAPI for nuclear label. Images of specimens with Alexa 488, Alexa 568 were recorded sequentially through the spectral emission filters set from 500 to 550 nm for Alexa 488, 580 to 700 nm for Alexa 568, and 410 to 490 nm for DAPI. Pairs of single images were superimposed for colocalization analysis. All micrographs were compiled in serial Z-stack images 0.1–1.0 μm apart. All images were analyzed and normalized using LAS AF Lite software, Leica Microsystems (Heidelberg, Germany). Percentage double positive cells was assessed in three high-power fields in at least three sections.

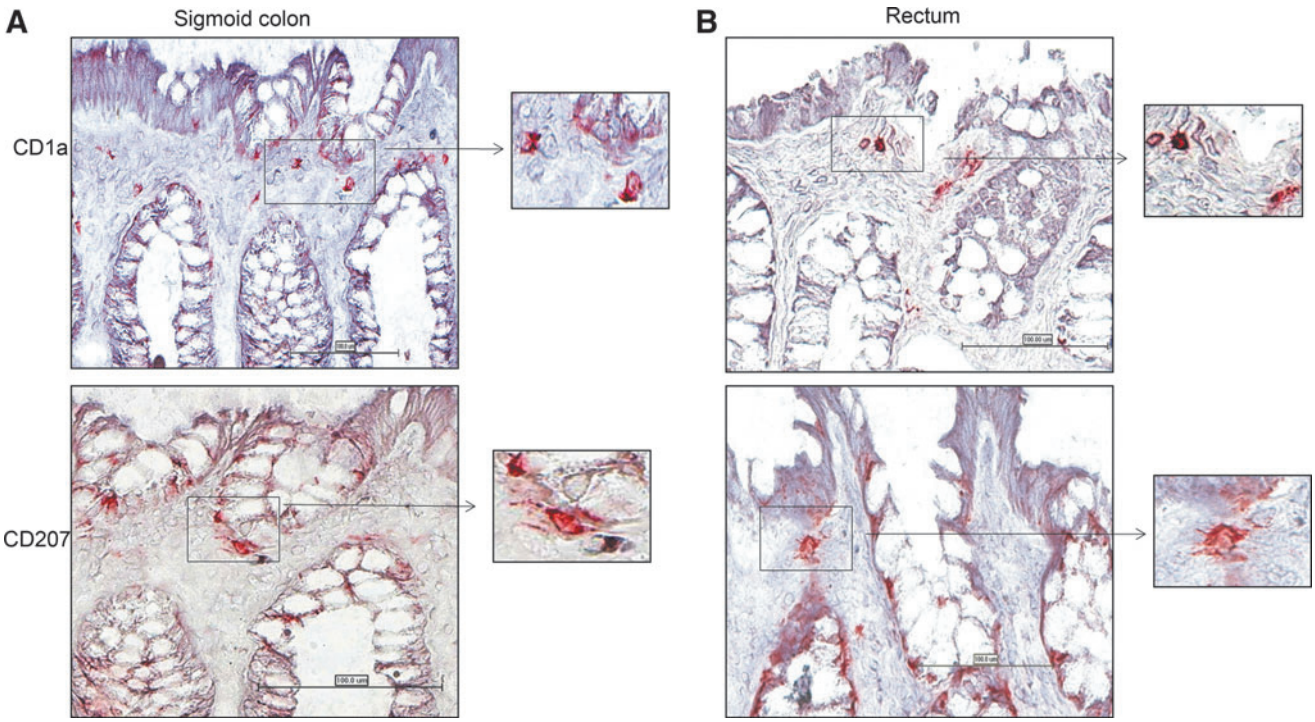

FIG. 1. Identification of Langerin$^+$ dendritic cells (DCs) in normal human sigmoid colon and rectum. Normal human sigmoid colon **(A)** and rectum **(B)** were labeled with monoclonal antibodies (mAbs) for CD207 (Langerin) and CD1a and visualized using the immunoperoxidase method. CD207 and CD1a were mostly located surrounding colon/rectal glands intraepithelial in between goblet cells and in the basement membrane. Inset: Langerhans cell displaying characteristic dendritic morphology. Original magnification 400×. Bar represents 100 μm. Images are representative of five different subjects.

Results

Identification and characterization of DCs expressing CD207 and CD1 in normal human sigmoid colonic and rectal mucosa

Cells with dendritic morphology were detected in both mucosal tissue sites in all subject's samples (Fig. 1). These DCs were predominantly located intraepithelially (between goblet cells). The dendritic morphology and presence of these characteristic markers (CD1a/CD207) suggest these could be Langerhans cells (LCs). Cells expressing other DC markers such as CD1b and CD1c were found in the periphery of colonic/rectal glands (Fig. 2). By contrast, evaluation for markers of mature DCs such as CD83 and CD208 (DC-LAMP) identified very few cells expressing these markers in either sigmoid colon or rectum as previously reported in the skin.

Distribution and morphology of cells expressing macrophage markers in human sigmoid colon and rectum

Cells expressing the characteristic monocyte/macrophage markers CD14, CD68, and CD163 were distributed from the lamina propria to the muscularis mucosae and were similarly located in both the sigmoid colon and rectum in all subject's samples (Fig. 3). These macrophage-like cells were distinct from DCs (cells expressing CD207 and CD1) and were found in different areas (IHC and CF) (Fig. 1). However, these macrophage-like cells did appear to have a distribution

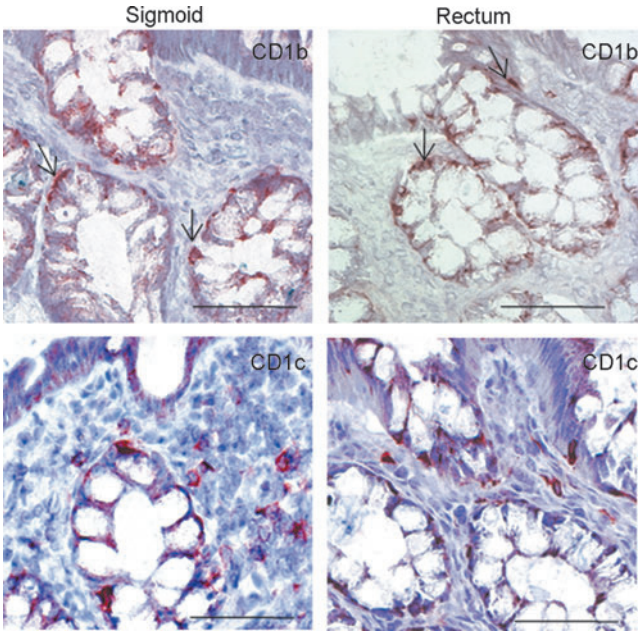

FIG. 2. Identification of CD1 dendritic cell markers in normal human sigmoid colon and rectum. Normal human sigmoid colon and rectum were labeled with mAbs for CD1b and CD1c and visualized using the immunoperoxidase method. CD1b and CD1c were located below simple columnar epithelium and interdispersed around colon/rectal glands in the lamina propria. Original magnification 400×. Bar represents 100 μm. Images are representative of five different subjects.

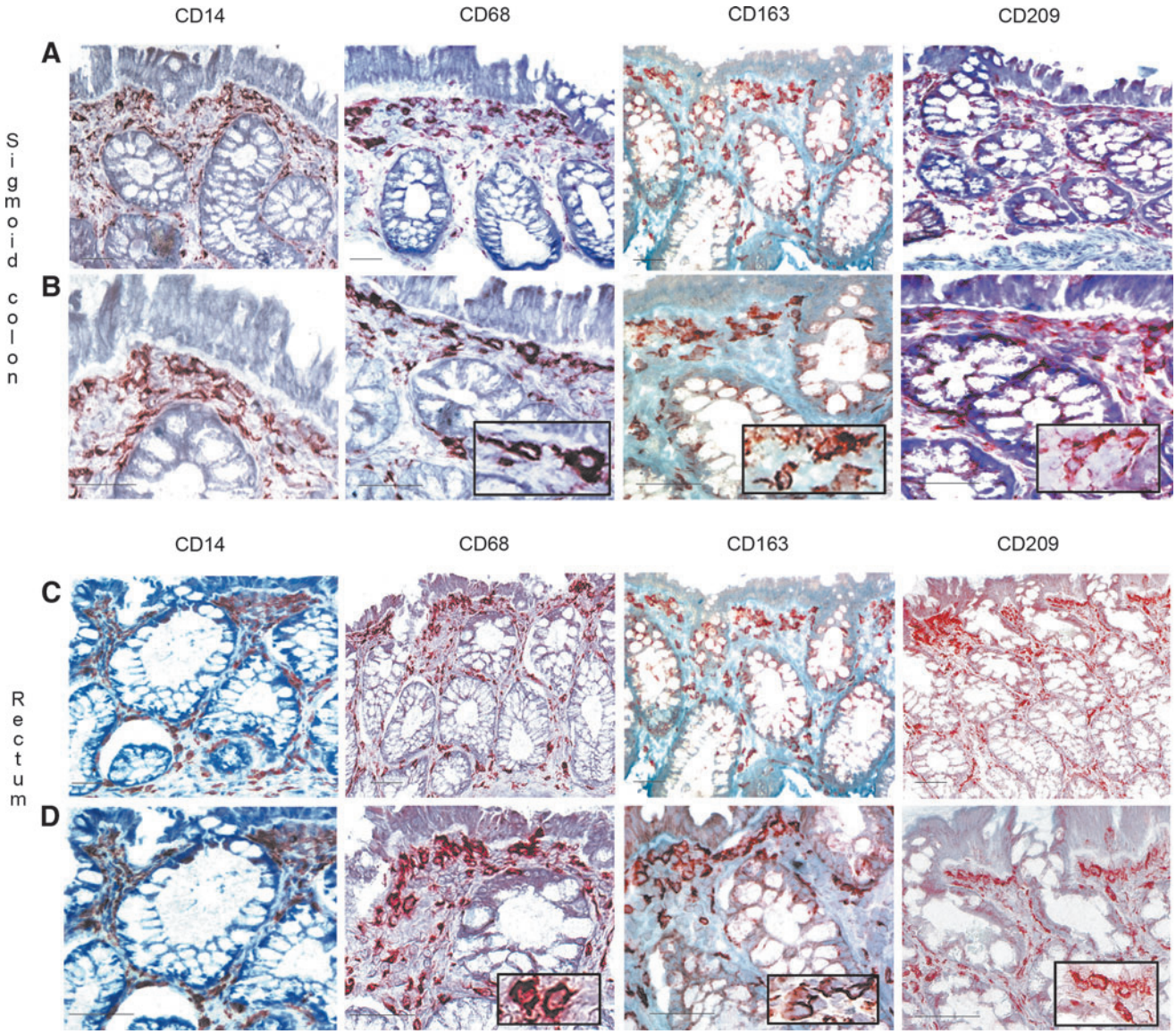

FIG. 3. Morphology and distribution of monocyte/macrophage cell markers in normal human sigmoid colon and rectum. Normal human sigmoid colon and rectum were labeled with mAbs for CD14, CD68, CD163, and CD209, and were visualized using the immunoperoxidase method. CD14, CD68, CD163, and CD209 were all found below simple columnar epithelium, mostly expressed throughout the lamina propria down to the muscularis mucosae layer, and in the lamina propria separating colon/rectal glands **(A,C)**. Images were taken from both tissues for each marker. The distribution was determined with an original magnification of ×200 **(A,C)** and the cell morphology is shown with a magnification of ×400 **(B,D)**. Monocyte/macrophage cells display dendritic cell morphology (insets). Bar = 50 μm. Pictures are representative of five different subjects.

similar to DC-SIGN (CD209)-expressing DCs (Fig. 3). The morphologies of these cells ranged from long and spindled to round and stellate (Fig. 3, inset).

Macrophages are more abundant than DCs in human sigmoid colon and rectum

In situ imaging analysis was used to enumerate cells expressing DC or monocyte/macrophage markers in freshly acquired colonic and rectal biopsies from five healthy volunteers without known gastrointestinal pathology. Ten colonic and 10 rectal glands were scanned per person (including epithelium and lamina propria above the muscularis mucosae, excluding lymphoid aggregates). Of all the characterized APCs quantified here, there were significantly greater numbers of macrophage-like cells (expressing CD14, CD163, CD68, and CD209) (875–1,487 cells/mm^2) in both rectum and sigmoid colon locations than dendritic cells expressing CD1 and CD207 (84–188 cells/mm^2), a consistent finding in all subjects ($p < 0.001$; Mann–Whitney rank sum test) (Fig. 4 and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid). The numbers of cells within each APC type were similar regardless of whether they were from the sigmoid colon or rectum. However, there were statistically significant differences between the number of DC cell subsets and monocyte/macrophage cell subsets at both sites ($p < 0.001$),

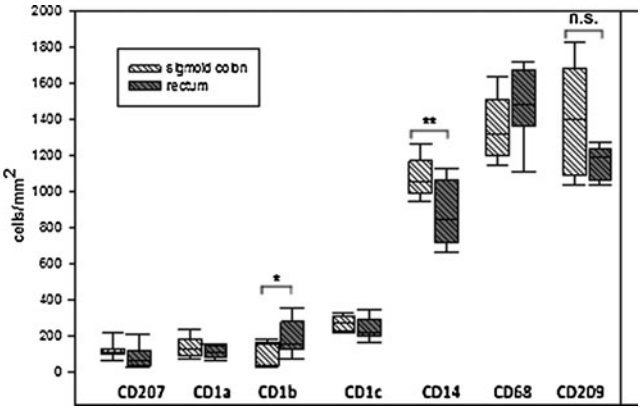

FIG. 4. DC and monocyte/macrophage enumeration in normal human sigmoid colon and rectum. Quantitative enumeration was performed at the single cell level in immunohistochemically stained sections by *in situ* analysis using aperio imaging software. $N = 5$. Maximum, minimum, and median values ± SEM are shown. $*p = 0.023$, $**p = 0.007$, n.s. = not significant.

Mann–Whitney rank sum test. There were no statistically significant differences between the two anatomical sites for most other markers, with the exception of CD1b (rectum > sigmoid: $p = 0.023$) and CD14 (sigmoid > rectum; $p = 0.007$; by Student's *t*-test) (Fig. 4). Thus the major population of innate immune cells in both the rectum and the sigmoid colon consists of macrophage-type cells expressing CD163, CD14, CD68, and CD209 that are present in nearly a 7-fold excess compared to DCs.

CD209 (DC-sign) is expressed on different cell subsets than DCs, and colocalizes with the macrophage cell markers CD68 and CD163, in human sigmoid colon and rectum

Based on the similar distribution of CD209 (DC-SIGN) and the macrophage markers CD68, CD163, and CD14, as determined by immunohistochemistry, we decided to examine the phenotype of $CD209^+$ cells in human sigmoid colon and rectum. Double immunofluorescence labeling of frozen tissue sections with antibodies to CD209 was performed and paired with DC markers (CD1a, CD1b, CD1c, and CD207) and macrophage markers (CD68 and CD163). Confocal microscopy analysis revealed that $CD209^+$ cells did not colocalize with conventional DC markers CD1a, CD1b, CD1c, and CD207 (Fig. 5). DC-SIGN expression (CD209)

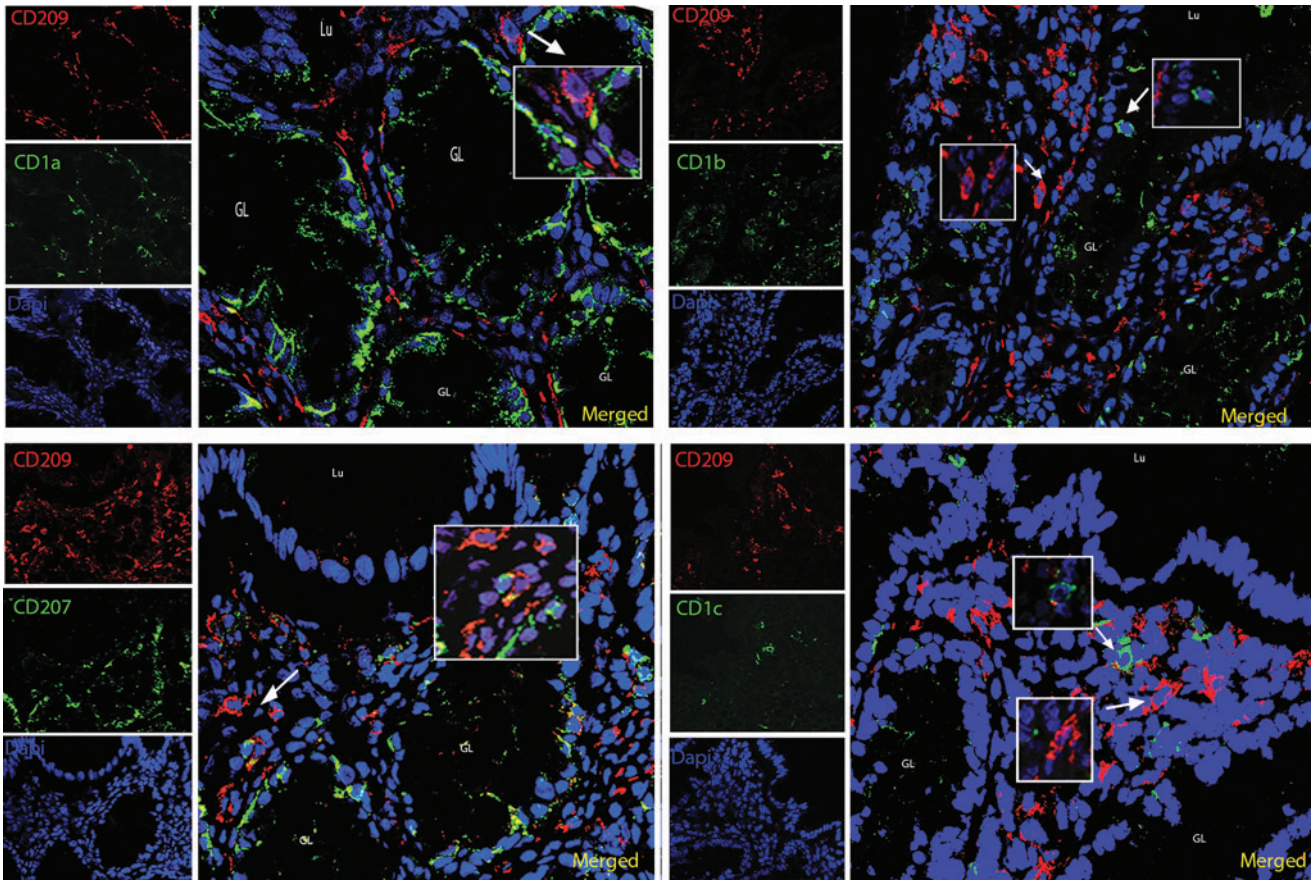

FIG. 5. $CD209^+$ (DC-SIGN) cells are expressed on different cells subsets than DC markers. Normal human rectum was labeled with mAbs for CD209, CD207, CD1a, CD1b, and CD1c and then visualized using confocal microscopy. $CD209^+$ (red images) did not colocolize with CD207 or CD1a (green images), markers for Langerhans cells. CD209 did not colocalize with the CD1 markers CD1b and CD1c. Nuclei were labeled with DAPI (blue images). Inset corresponds to a zoomed area marked by white arrows. Original magnification × 630, bars = 50 μm. Lu, lumen; Gl, gland.

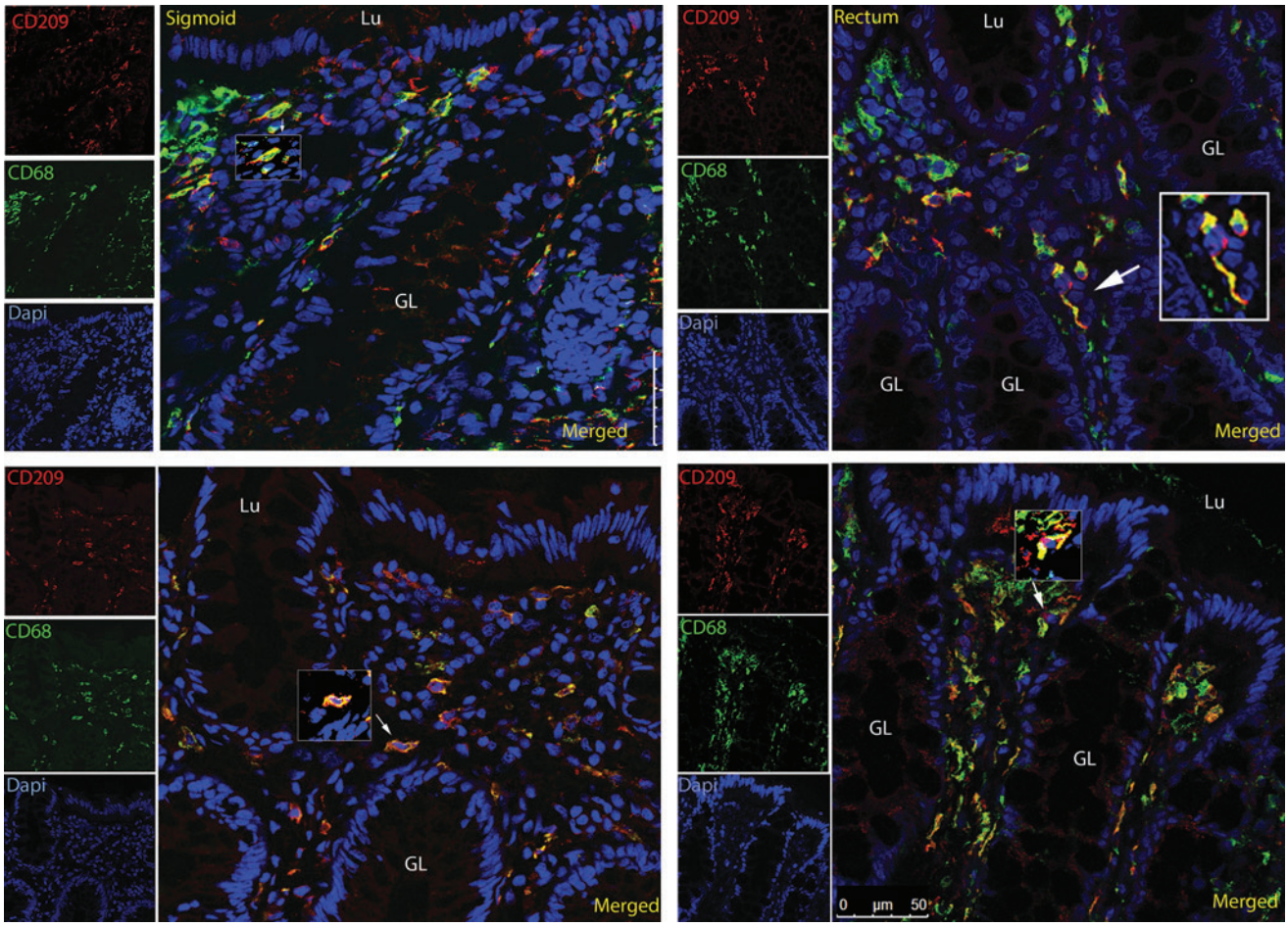

FIG. 6. CD209$^+$ (DC-sign) cells and CD68$^+$, CD163$^+$ monocyte/macrophage cell markers colocalize on the same cell subsets in normal sigmoid colon and rectum. Normal human sigmoid colon and rectum were labeled with mAbs for CD209, CD68, and CD163 and visualized using confocal microscopy. CD209 (red images) colocalized with CD68 and CD163 (green images) monocyte/macrophage cell markers. Nuclei were labeled with DAPI (blue images). Inset corresponds to magnified positive cells marked by white arrows. Original magnification × 630, bars = 50 μm. Lu, lumen; Gl, gland.

was predominantly found coexpressed on macrophage-type cells (CD68$^+$/CD163$^+$) and not on DC-type cells (CD1a,b,c$^+$/207$^+$) and were located periapically near the base of the colonic glands (Fig. 6). Not all CD209$^+$ cells were double positive. We find that approximately 70.3% of CD209$^+$ immune cells were double positive for CD209/CD68 and CD209/CD163.

Discussion

We demonstrate that the APC population in normal human sigmoid colon and rectum is composed of two phenotypic subpopulations. The first are immature dendritic cells that express CD1 and CD207, and the second are macrophages/monocytes that express CD14, CD68, CD163, and CD209 (Fig. 7). We conclude that CD209$^+$/CD68$^+$/CD14$^+$/CD163$^+$ cells are the most abundant innate immune cells at the colorectal mucosa, and may be an important component of the innate immune response at the mucosa.

Given the proposed role of CD207 (Langerin) in HIV-1 transmission[16] and the key role of CD207$^+$ and CD1a-expressing LCs in skin and various mucosal surfaces, we investigated the *in situ* distribution of CD207 and CD1a in normal human sigmoid colon and rectum (Supplementary Fig. S1). To our knowledge LCs, a myeloid DC subtype common in stratified epithelium,[19,20] have not been thoroughly examined in these microcompartments. LCs can be defined by the expression of the PRR Langerin,[21] a C-type lectin and CD1a, that presents lipid and glycolipid antigens to T lymphocytes.[22–25] LCs have been described in the skin, mucosa of the mouth, foreskin, and vagina,[26] but there is a paucity of direct evidence showing expression of LCs in rectal epithelial tissue.[16] We identify cells with typical dendritic cell morphology expressing CD1a and CD207 predominantly intraepithelially and surrounding the colonic/rectal glands but sparse in the lamina propria (LP) where they are seen directly below the epithelium (Fig. 1). There are about 84–188 cells/mm^2 (Fig. 4 and Supplementary Table S1), similar to the densities of LCs observed in human foreskin,[27] human penile explant tissue,[20,28] and vagina,[26] but far less than is seen in the epidermis of the skin.[29] Although sparse, the intraepithelial location of these CD1a$^+$ and CD207$^+$ cells is consistent with the notion that in some areas of the small intestine of mice dendritic cells form intraepithelial dendrites that extend into the

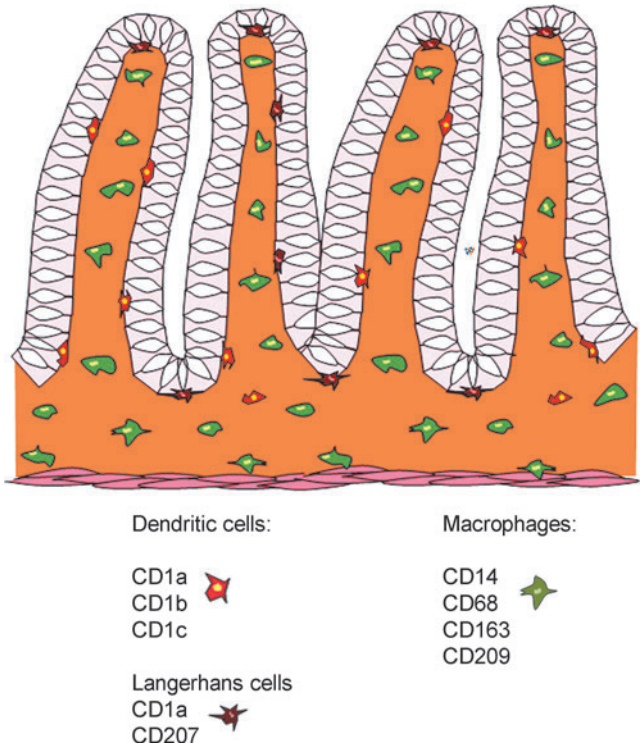

FIG. 7. DC and macrophage subsets as HIV targets in distal colonic and rectal mucosa. Antigen-presenting cells consists of at least two populations: a small population that expresses CD207 and CD1 and a major population of CD14, CD68, CD163, and CD209 positive cells. CD209 (DC-SIGN) and CD207 (Langerin) are pathogen recognition receptors important in HIV infection and transmission.

lumen for antigen sampling.[1] Further analysis of these $CD1^+$ and $CD207^+$ cells in the human colon and rectum could provide an indication of their function in relation to this specific location in the human colorectal mucosa. Langerin has been reported in cells that differ from LCs in mice[30–32] and in $Langerin^+$ monocyte-derived DCs from humans.[33,34] Although we believe these colorectal $CD1^+$ and $CD207^+$ cells to be LCs, it cannot be ruled out that the $Langerin^+$ cells expressed in the colorectal mucosa may differ from those found in the epidermis of other tissues.

The ability of DCs to process and present different antigens to T cells relies in part on the expression of the CD1 family glycoprotein receptors. Apart from CD1a, CD1b and CD1c are also capable of presenting various classes of lipid antigens and are involved in cross presentation of antigen, which is thought to boost $CD8^+$ T lymphocyte responses.[35,36] We find that these molecules have a similar distribution to CD1 and are mainly localized intraepithelially and within the lining of the basement membrane (Fig. 2). Previous studies describe DCs as the most abundant population in the human colonic lamina propria. DCs were found to be the major APC in the colonic and rectal isolated lymphoid follicles in humans and these HLA^+DR^+ DCs form a reticular framework throughout the lamina propria and beneath the basement membrane of the colonic crypts.[1] However, those studies analyzed surgically resected colonic mucosa from patients with cancer and other nonmalignant conditions.[37,38] Previous studies investigating the reactivity of lamina propria T cells to commensal bacteria determined, using flow cytometry, that the majority of DCs isolated from the lamina propria of human small intestine and colon were $CD1c^+$ DCs.[39,40] We show that freshly acquired, endoscopic biopsy tissues from healthy sigmoid colon and rectum have what appear to be LCs located in the epithelium and a second group of DCs comprised of either $CD1a^+/CD1b^+$ and $CD1c^+$ cells inside and around the glands.

Another key C-type lectin found on DCs besides Langerin (CD207) is DC-SIGN (CD209), which is expressed on non-Langerhans DCs in skin subepithelium. Our results show that a large population of cells express CD209, are directly below the mucosal epithelium, and are distributed throughout the lamina propria, between colon/rectal glands, extending to the muscularis mucosae (Fig. 3). However, consistent with prior reports that this molecule can be expressed on macrophages[41] we find that CD209 is coexpressed with the monocyte/macrophage markers CD14 (coreceptor for lipopolysaccharide), CD68 (tissue macrophage surface molecule that binds LDL), and CD163 (haptoglobin receptor involved in the transport of iron into cells), but not with DC markers CD1a, CD1b, CD1c, and CD207 as previously described in skin and the renal interstitium.[42] Krutzik *et al.* demonstrated that activation of Toll-like receptors causes rapid differentiation of distinct precursors of human peripheral blood monocytes into CD1b and CD209 (DC-SIGN) cells.[43] Montoya *et al.* further showed the ability of distinct innate immune cytokines to trigger macrophage functional programs such as phagocytosis, and the ability of interleukin (IL)-15 to induce the coexpression of CD209 and CD163 on macrophages and not CD1b (dendritic cell-specific marker).[44] These studies provide evidence that the dual host defense roles of the innate immune system are mediated by induction of two distinct phenotypic and functional populations.

DCs and macrophages are candidate cells for early transmission events in HIV pathogenesis and it is unclear which cell type(s) HIV-1 must reach to establish systemic infection. Knowing which specific DC and macrophage cell subtypes are present at the colorectal mucosa may have implications for the pathogenesis of HIV-1 transmission, for which the early events involving rectal transmission are poorly understood. Macrophages are susceptible to HIV-1 infection and are productively infected during chronic infection *in vivo*.[28,45] It has been suggested that these cells may contribute to HIV-1 entry in penile urethral and vaginal mucosal sites.[28] Furthermore, the cell surface molecule DC-SIGN expressed on DCs has been proposed to be important for binding and transporting HIV-1 from mucosa to regional lymph nodes,[14,16,17] although we find this molecule on macrophages and not DCs.

We speculate that the high density of mucosal macrophages (and not DCs) expressing CD209 may possibly contribute to the higher efficiency of rectal transmission, but additional studies are warranted in support of this notion. Although the DCs in sigmoid colonic and rectal mucosa do not express CD209, they express Langerin and may appear to be LCs, a myeloid DC subtype common in stratified epithelium[19,20] that is capable of uptake and degradation of HIV-1 in Birbeck granules[16,46] or facilitation of its spread.[47] It is thus unclear whether LCs might play a facilitating or inhibiting role in HIV-1 transmission rectally. Even though this study did not examine plasmacytoid DCs, it has been shown that

plasmacytoid DCs, which play an important role in HIV-1 immunopathogenesis, are recruited into mucosal surfaces including the colon during SIV infection.[48,49]

In agreement with our results McElrath *et al.* very recently reported the presence of $CD68^+$ macrophages expressing CCR5 more in the rectum compared to colon, using fluorescence microscopy and superimposed images to establish expression of both markers on the same cell.[50] Using confocal microscopy we better define a $CD209^+/CD163^+/CD68^+$ macrophage population in human colon and rectum that does not express characteristic DC markers CD1a, CD1b, CD1c, and CD207.

In summary, we characterized the distribution and phenotype of colorectal APCs, important innate immune cells for regulating immune responses at mucosal surfaces. We examined the expression in sigmoid colon and rectum of key pathogen recognition receptors involved in innate immune regulation and HIV-1 pathogenesis, finding that the dominant APCs are rectal macrophages expressing DC-SIGN, which are strategically positioned adjacent to the epithelium primed to interact with microorganisms and microbial products that have breached the epithelium. Identification and understanding of the resident mucosal innate immune system and the knowledge of specific cell candidates at the colonic/rectal mucosa are critical to informing rectal microbicide drug development, HIV-1 vaccine design, and the pathogenesis of other mucosa-associated infections and inflammatory bowel disease.

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Author Disclosure Statement

No competing financial interests exist.

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