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Transepithelial Pathogen Uptake into the Small Intestinal Lamina Propria

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The lamina propria that underlies and stabilizes the gut lining epithelium is densely populated with strategically located mononuclear phagocytes. Collectively, these lamina propria macrophages and dendritic cells (DC) are believed to be crucial for tissue homeostasis as well as the innate and adaptive host defense. Lamina propria DC were recently shown to gain direct access to the intestinal lumen by virtue of epithelium-penetrating dendrites. However, the role of these structures in pathogen uptake remains under debate. In this study, we report that entry of a noninvasive model pathogen (Aspergillus fumigatus conidia) into the murine small intestinal lamina propria persists in the absence of either transepithelial dendrites or lamina propria DC and macrophages. Our results suggest the existence of multiple pathogen entry pathways and point at the importance of villus M cells in the uptake of gut lumen Ags. Interestingly, transepithelial dendrites seem altogether absent from the small intestine of BALB/c mice suggesting that the function of lamina propria DC extensions resides in their potential selectivity for luminal Ags, rather than in general uptake or gut homeostasis. *The Journal of Immunology, 2006, 176: 2465–2469.*
CD11c<sup>+</sup>CX<sub>c</sub>CXCR<sub>1</sub> DCs (lpDC) and CD11c<sup>+</sup>CX<sub>c</sub>CXCR<sub>1</sub>- macrophages (lpMb). Together with the observation that transepithelial lpDC extensions seem absent from the small intestine of BALB/c mice, these findings argue against a general role of transepithelial dendrites in the maintenance of intestinal homeostasis, but suggest the existence of multiple pathogen entry routes.

Materials and Methods

Mice

This study involved the use of CD11c-diphertheria toxin receptor (DTR) transgenic mice (B6.FVB-Tg(Ifgax-DTR/GFP)S7Lau/J) (9) and CX<sub>c</sub>CXCR<sub>1</sub>GFP mice (10). C57BL/6 and BALB/c mice carrying the CX<sub>c</sub>CXCR<sub>1</sub>GFP allele were backcrossed >10 generations on the respective background. All mice were maintained under specific pathogen-free conditions and handled under protocols approved by The Weizmann Institute Animal Care Committee according to international guidelines.

Transformation of Aspergillus fumigatus and conidia isolation

A. fumigatus conidia (strain CBS 144.89) were provided by J.-P. Latgé (Aspergillus Unit, Pasteur Institute, Paris, France). Transformation was performed by electroporation using inflated spores. Briefly, conidia were incubated in yeast extract glucose 1% medium, plated in minimum medium supplemented with 200 μg/ml hygromycin B (Invitrogen Life Technologies) and incubated at 37°C. After centrifugation, spores were resuspended in yeast extract glucose 1% HEPES 20 mM (pH 8), and incubated for 1 h at 30°C. After centrifugation, spores were redispersed in electroporation buffer (10 mM Tris (pH 7.5), 270 mM sucrose, 1 mM lithium acetate). Plasmids (pAN7-1, pGPd-DsRed) harboring a hyg<sup>B</sup> resistance gene and DsRed gene, respectively (11) (provided by L. Mikkelsen, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark) were added and electroporation was carried out according the following conditions: 1 kV, 400 μF, in a 0.2-mm cuvette. Transformed spores were incubated for 15 min on ice in 1 ml of yeast extract glucose 1% medium, plated in minimum medium supplemented with 200 μg/ml hygromycin B (Invitrogen Life Technologies) and incubated at room temperature. For monosporic isolation, A. fumigatus spores were plated in sterilized water/Tween 0.1% at a final concentration of 10<sup>7</sup> conidia/ml and 2 × 10<sup>5</sup> S. typhimurium bacteria/ml. Bacteria were used in their late logarithmic phase of growth, by diluting a colony in Luria broth containing 0.3 M NaCl and incubating overnight at 37°C.

FACS analysis

Fluorochrome-labeled mAbs were purchased from BD Pharmingen or eBioscience and used according to the manufacturer’s protocols. CX<sub>c</sub>CXCR<sub>1</sub> staining was performed as previously described using a fractalkine (FKN)-Fc fusion protein (provided by Millennium Biotherapeutics) (10). Cells were analyzed on a FACSCalibur cytometer (BD Biosciences) using CellQest software (BD Biosciences).

Staining and microscopy of the small intestinal mucosa

After pathogen or mock challenge, ligated small intestinal loops were opened at their end, intensively flushed with PBS, opened by longitudinal incision, and rinsed again. For M cell staining, the cut tissue was fixed in 4% paraformaldehyde for 1 h on ice, incubated with biotin-conjugated anti-Ulex europaeus agglutinin (UEA-1; Vector Laboratories) at 20 μg/ml for 2 h on ice followed by staining with streptavidin-allophycocyanin. Living tissues were imaged with a Zeiss Axioskop II fluorescence microscope for three-color imaging. Image acquisition was conducted with Simple PCI software.

Results

The murine small intestinal lamina propria harbors two distinct mononuclear phagocyte populations

To characterize the mononuclear phagocyte content of the murine small intestinal lamina propria, we isolated tissue samples from the small bowel, prepared single-cell suspensions, and analyzed them by flow cytometry. Taking advantage of a mouse strain that harbors a targeted replacement of the gene encoding the chemokine receptor CX<sub>c</sub>CXCR<sub>1</sub> by a reporter gene encoding GFP (10), we previously showed that the murine intestine harbors a distinct population of CD11c<sup>+</sup>CD11b<sup>+</sup> DC that expresses CX<sub>c</sub>CXCR<sub>1</sub> (4). Flow cytometric analysis of unfractioned small intestinal single-cell suspensions revealed the presence of additional CD11c<sup>+</sup> mononuclear phagocytes that lack CX<sub>c</sub>CXCR<sub>1</sub> expression (Fig. 1). The two CD11c<sup>+</sup> populations differ with respect to maturation and activation markers, such as CD80, MHC class II, and the integrin αEβ7 (CD103), recently reported for rat intestinal DC (13). The classical macrophage marker F4/80 (14) did not consistently discriminate between the two populations (data not shown).

Pathogen uptake assay

Mice were sacrificed and 2- to 3-cm sections spanning the small intestinal terminal ileum were cut out, ligated at both ends, and placed in a petri dish. Pathogens were injected with a syringe into the loop and incubated for 10 min at 37°C in RPMI 1640. The loop was subsequently opened at both ends and extensively washed with PBS. This study involved two pathogens: DsRed-transformed A. fumigatus conidia and Salmonella typhimurium strain CS93 (provided by A. Porgador, Ben Gurion University, Beer Sheva, Israel). Conidia were added at a final concentration of 10<sup>7</sup> conidia/ml and 2 × 10<sup>5</sup> S. typhimurium bacteria/ml. Bacteria were used in their late logarithmic phase of growth, by diluting a colony in Luria broth containing 0.3 M NaCl and incubating overnight at 37°C.

FIGURE 1. Phenotypic dichotomy of lamina propria mononuclear phagocytes. Isolated small intestinal lamina propria cells of Rag<sup>−/−</sup>CX<sub>c</sub>CXCR<sub>1</sub>GFP<sup>+</sup> mice were stained with Abs for CD11c and one of the indicated surface markers. CX<sub>c</sub>CXCR<sub>1</sub> negative (Gate R1), open histograms: CD11c<sup>+</sup> CX<sub>c</sub>CXCR<sub>1</sub>GFP<sup>+</sup> (lpDC) (Gate R2).
CD11c and CX3CR1 expression (16, 17) (L. Landsman, unpublished observation). Accordingly, we propose to refer to the CD11c⁺CD11b⁺CX3CR1⁺ population as lpDC and the CD11c⁺CD11b⁻CX3CR1⁻ cells as lpMφ. Proximal and distal sections of the small intestine vary considerably in the microbial load. However, we did not observe significant differences between lamina propria cells isolated from the duodenum/jejunum and terminal ileum, for the markers analyzed (data not shown).

Neither transepithelial dendrites nor CD11c⁺ lamina propria cells are required for quantitative pathogen uptake

C57BL/6 CX3CR1⁺GFP mice allow the in situ detection of transepithelial dendrites in intact intestinal tissue (4). The latter allow strategically located lpDC to sense the content of the intestinal lumen. Supporting their role in immunosurveillance, lpDC extensions are restricted to the terminal ileum (4), a region characterized by increased bacterial load, a unique microflora as well as activated epithelium and lamina propria, suggesting constant exposure to bacterial stimuli (4, 18, 19). Furthermore, formation of transepithelial dendrites can be induced by challenge of intestinal villi with commensal and enteropathogenic bacteria (4). However, a direct role of lpDC and transepithelial dendrites in pathogen sampling, as originally suggested by Rescigno et al. (20) remains controversial (21, 22). Challenging the general accessibility of the lamina propria, Macpherson and Uhr (23) failed to detect bacteria-harboring lpDC after gavage with GFP-expressing Escherichia coli. In contrast, we reported that orally administered invasive S. typhimurium entered the lamina propria even in absence of transepithelial dendrites, while entry of a noninvasive Salmonella mutant to the lamina propria was CX3CR1-dependent (4). To directly study dendrite/pathogen interactions at the intestinal surface, we investigated the potential of the noninvasive fungal pathogen, Aspergillus fumigatus, to induce transepithelial dendrites and enter the lamina propria. Both Aspergillus hyphae and conidia have been reported to interact with DC involving receptors of the IL-1R/TLR superfamily and C-type lectins (24, 25). To visualize the pathogen, we generated red fluorescent conidia by transforming A. fumigatus with an expression vector encoding DsRed protein (11). Using an ex vivo-ligated loop system that allows controlled pathogen exposure of intact tissue, we challenged intestinal villi of C57BL/6 CX3CR1⁺GFP/+ mice with DsRed-expressing conidia. Aspergillus conidia readily induced the formation of transepithelial dendrites (Fig. 2B). Individual villi harbored extensions with previously reported globular endings (4) and extensions lacking the latter. Aspergillus conidia entered the CX3CR1⁺GFP/+ lamina propria, where they seemed largely confined to GFP-expressing cells in the tip of the villi. Furthermore, we found transepithelial dendrites and global structures to be associated with the conidia outside the epithelial cell layer (Fig. 2, C and D), suggesting their direct involvement in pathogen uptake. To further investigate this issue, we exposed conidia to villi of CX3CR1⁺GFP/+ mice that lack lpDC extensions (4). As shown in Fig. 2E, CX3CR1 deficiency did not impair pathogen entry as indicated by the abundance of noninvasive Aspergillus conidia in the lamina propria of CX3CR1⁻GFP/GFP mice.

Only a fraction of the CD11c⁺ mononuclear phagocytes in the intestinal lamina propria expresses CX3CR1 (Fig. 1) and is hence visible in CX3CR1⁺GFP mice. The above experiments thus do not exclude an involvement of CX3CR1-negative lamina propria CD11c⁺ cells (lpMφ) in pathogen uptake. Therefore, we investigated the uptake in a situation where all CD11c⁺ mononuclear phagocytes are missing from the lamina propria. To this end, we took advantage of an animal model that allows the diptheria toxin (DTx)-induced ablation of CD11c⁺ cells in the intact organism (9). To visualize lpDC, the DTR transgene was crossed on the CX3CR1⁺GFP background. Histological and flow cytometric analysis confirmed the rapid depletion of all CD11c⁺ MHC II⁺ cells from the small intestine of toxin-treated CX3CR1⁺GFP⁻/⁻ D-TX-DTR tg mice (Fig. 3). We then prepared ligated intestinal loops from DTx-treated mice and challenged them with Aspergillus conidia. As seen in Fig. 2F, conidia uptake by the villi persisted in the absence of CD11c⁺ lamina propria cells.

Taken together, there is unimpaired entry of our particulate model pathogen into the lamina propria of CX3CR1-deficient mice and mice depleted of both lpDC and lpMφ. This suggests that transepithelial dendrites do not significantly contribute to quantitative gut content sampling, which is likely to proceed via alternative routes. Kiyono and coworkers (7) recently reported the existence of intestinal villus M cells that like Peyers patch M cells can be identified on the basis of their display of (1-2)-fucose detected by UEA-1. Confirming these results, we could visualize numerous UEA-1⁺ cells on villi of the terminal ileum of C57BL/6

**FIGURE 2.** Pathogen entry into small intestinal lamina propria. Fluorescent microscopic analysis of DsRed-transduced Aspergillus conidia challenged villi of C57BL/6;CX3CR1⁺GFP mice (green, lpDC; red, conidia; diameter 2-3 μm); original magnification, ×40. A, Unchallenged CX3CR1⁺GFP/+ villi; B-D, Aspergillus conidia-challenged CX3CR1⁺GFP/+ villi (note conidia in luminal globular structure (※ in D); E, CX3CR1⁺GFP/+ C57BL/6 mice; and F, DTx-treated, lpDC and lpMφ-depleted CD11c-DTR: CX3CR1⁺GFP/+ C57BL/6 mouse. G, UEA-1⁺ M cells in the villus epithelium; H and H', colocalization of Aspergillus conidia with UEA-1⁺ M cells on villi of CX3CR1⁺GFP/+ mice.
CX3CR1GFP mice (Fig. 2G). Furthermore, analysis of pathogen-challenged ligated loops revealed conidia clusters in the epidermal layer that coincided with the M cell staining (Fig. 2, H and H'). These results support the notion that intestinal villus M cells are a major entry route for particulate pathogens to the lamina propria.

**Transepithelial dendrite formation of CX3CR1+ lpDC is mouse strain dependent**

Absence of lpDC extensions in CX3CR1-deficient mice (CX3CR1GFP/GFP) (4) indicates a critical role of the CX3CR1 chemokine receptor and its membrane-tethered ligand CXCL1 (FKN) in the penetration of the epithelial barrier. To test the general validity of this observation, we investigated the distribution of transepithelial extensions in another inbred mouse strain. Surprisingly, heterozygous mutant CX3CR1GFP/+ BALB/c mice lacked transepithelial dendrites in the terminal ileum both in steady state and after exposure to pathogens (Aspergillus conidia, Salmonella) (Fig. 4 and data not shown). GFP+ lpDC of CX3CR1GFP/+ BALB/c mice express surface CX3CR1 (data not shown). Furthermore, transepithelial dendrites are formed in F1 hybrid CX3CR1GFP/+ mice that inherited their wild-type CX3CR1 allele from the BALB/c and the mutant, CX3CR1GFP allele from the C57BL/6 parent (Fig. 4). The BALB/c allele thus encodes a functioning CX3CR1 receptor capable of promoting dendrite formation. Absence of transepithelial dendrites in BALB/c mice is thus a recessive phenotype not linked to the CX3CR1 locus but events upstream or downstream of CX3CR1 action.

The impact of the genetic background on the existence of transepithelial dendrites raised the question of whether their absence in C57BL/6 CX3CR1GFP/GFP mice is indeed due to lack of the CX3CR1 receptor, as we previously proposed (4). Alternatively, the phenotype of the C57BL/6 CX3CR1-deficient mice could result from homozygosity of the chromosomal region (chromosome no. 9) flanking the CX3CR1GFP allele, which is derived from 129svs mice (10). To this end, we tested whether transepithelial dendrites are present in mice homozygous for the respective chromosome or its subregion, i.e., F1 hybrid mice resulting from the following crosses: CX3CR1GFP/GFP B6 × 129sv and CX3CR1GFP/GFP B6 × C57BL/6 (CCR2 locus is adjacent to the CX3CR1 locus and CCR2 mutant mice were generated using the same 129svs ES cells as the CX3CR1 mutation (10, 26)). In both hybrid strains, we observed transepithelial dendrites upon bacteria challenge (data not shown) confirming our original interpretation that the absence of transepithelial dendrites in C57BL/6 CX3CR1GFP/GFP mice is due to their lack of the chemokine receptor CX3CR1. However, absence of lpDC extensions in CX3CR1GFP/+ BALB/c mice suggests control of their formation by multiple genetic factors.

**Discussion**

Studies in the rat have established that lpDC sample enteric Ags and apoptotic epithelial cells in the lamina propria translocate to the mesenteric nodes and present their cargo to lymphocytes (27). The observation that lpDC can penetrate the epithelial tight junction barrier (8) thus led to speculations that besides Peyer’s patch-associated M cells, lpDC might be a major route of luminal Ag transport to lamina propria-draining lymph nodes. Such a scenario would be of critical importance for our understanding of immunosurveillance and pathogen invasion. In the present study, we focus on the most proximal step of this cascade of events, i.e., pathogen entry into the lamina propria. Using hosts that either lack small intestinal transepithelial dendrites (CX3CR1GFP/GFP mice; Ref. 4) or were depleted of lpDC (DTx-treated CD11c-DTR transgenic mice (9)) we show that entry of fungal conidia by in large tissue barrier (8) thus led to speculations that besides Peyer’s patch-associated M cells, lpDC might be a major route of luminal Ag transport to lamina propria-draining lymph nodes. Such a scenario would be of critical importance for our understanding of immunosurveillance and pathogen invasion. In the present study, we focus on the most proximal step of this cascade of events, i.e., pathogen entry into the lamina propria. Using hosts that either lack small intestinal transepithelial dendrites (CX3CR1GFP/GFP mice; Ref. 4) or were depleted of lpDC (DTx-treated CD11c-DTR transgenic mice (9)) we show that entry of fungal conidia by in large relies on an alternative uptake route. These results suggest that lpDC extensions are dispensable for quantitative sampling of luminal Ags, which are readily accessible to the lamina propria through intestinal villus M cells (7). The study of CX3CR1GFP/+ BALB/c mice revealed that lpDC of this inbred mouse strain seem unable to form transepithelial dendrites. BALB/c and C57BL/6 mice differ in the kinetics of postnatal formation of isolated lymphoid follicles and cryptopatches (27). In addition, Kennedy et al. (28) reported that C57BL/6 mice harbor a natural disruption of the secretory group II phospholipase A2 gene (sPLA2). Murine intestinal sPLA2 has bactericidal activity (29) and the observed high expression of sPLA2 in the intestine of BALB/c mice (28) might therefore affect the intestinal microflora. Furthermore, old BALB/c harbor more IgA+ B cells in their lamina propria than C57BL/6 mice and have elevated IgA titers in fecal extracts (30). However, it remains to be shown whether and how these facts are related to differences in transepithelial dendrite formation. Differential lpDC access to the intestinal lumen is likely to contribute to the well-established observation that inbred mouse strains vary significantly in their susceptibility to pathogen infection known to be under mono- or multigenic control (31).

Absence of transepithelial dendrites in CX3CR1GFP/GFP C57BL/6 and CX3CR1GFP/+ BALB/c mice impairs neither intestinal homeostasis, nor pathogen entry to the lamina propria. This
study thus seems to argue against the importance of these structures in intestinal pathogen uptake. However, IpDC extensions are likely decorated with DC-restricted pathogen receptors (for a recent review, see Ref. 32). Unlike the M cell route, the IpDC pathway might therefore allow for specific sensing and sampling of the intestinal lumen. Such a notion is also supported by our observation that some IpDC dendrites form upon epithelial penetration globular structures, i.e., an extended matrix for interaction with the gut content (4) (Fig. 2). The IpDC route could be an attractive target for the development of vaccination strategies. Furthermore, it could constitute a critical invasion path of pathogens targeting DC, such as HIV (33). However, a definitive answer on this topic will have to await experimental evidence showing IpDC-dependent entry of specific pathogens or Ag uptake by the transepithelial IpDC processes, migration of Ag-loaded IpDC to lymphoid organs, and activation of naive T cells.

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**Disclosures**

The authors have no financial conflict of interest.

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