Characterization of Langerin-Expressing Dendritic Cell Subsets in the Normal Cornea

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PURPOSE. In addition to Langerhans cells (LCs), other dendritic cells (CD11c⁺) have recently been shown to express Langerin (c-type lectin). In skin, (non-LC) Langerin⁺ dendritic cells initiate adaptive immunity. However, whether such dendritic cells (DC) reside in the cornea, an immune-privileged tissue, is unknown.

METHODS. Normal C57BL/6 corneas were harvested for qRT-PCR analyses of Langerin expression in the epithelium versus stroma. Immunohistochemistry for Langerin was also performed. Single-cell preparations of epithelium versus stroma were FACS analyzed for CD11c, CD11b, and CD103 expression. Fluorescence microscopy of corneas from muLangerin-eGFP mice (in which all CD11c⁺ Langerin⁺ cells express eGFP), huLangerin-DTA mice (only LCs are constitutively deleted), and huLangerin-Cre eYFP-flox (only LCs express eYFP) was performed.

RESULTS. qRT-PCR, immunohistochemistry, and FACS analysis identified CD11c⁺ Langerin⁺ cells in the epithelium and stroma. Similarly, corneas of muLangerin-eGFP mice contained eGFP⁺ cells in the epithelium and stroma. However, FACS analysis indicated phenotypically differing CD11c⁺ Langerin⁺ populations in the epithelium (CD11b^{low}CD103^{low}) versus stroma (CD11b⁺CD103^{low}). Additionally, corneas from huLangerin-DTA mice were devoid of Langerin⁺ cells in the epithelium but were detectable in the stroma. In corneas from huLangerin-Cre eYFP-flox, eYFP⁺ cells were detectable in the epithelium but not in the stroma.

CONCLUSIONS. The normal corneal epithelium is endowed with CD11c⁺ Langerin⁺ cells that are LCs, whereas the stroma is endowed with a separate population of (non-LC) Langerin⁺ DCs. These findings should henceforth facilitate the examination of Langerin-expressing DC subsets in the immunopathogenesis of conditions such as keratoconjunctivitis sicca, allergic keratoconjunctivitis, and corneal allograft rejection. (*Invest Ophthalmol Vis Sci.* 2011;52:4598–4604) DOI:10.1167/iovs.10-6741

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Langerin is a c-type lectin expressed by specific dendritic cell (DC) populations, and it recognizes glycosylated patterns on pathogens such as mycobacteria. It was initially thought that Langerin is expressed only by Langerhans cells (LCs), a unique population of DCs originally identified in the epidermis that have Birbeck granules.^{1,2} Recently, however, other DC (CD11c⁺) populations distinct from LCs were also found to express Langerin.^{3–7} Such (non-LC) Langerin⁺ DCs include Langerin⁺ DCs in the dermis,^{3–5} a subset of CD8a⁺ DCs in lymphoid tissues,^{6,7} and distinct populations in tissues of the lung, gut, kidney, and liver.⁸ Collectively, (non-LC) Langerin⁺ DCs are mostly CD11b^{low}CD103⁺, albeit CD11b⁺CD103^{low} have also been described.^{8,9} Furthermore, (non-LC) Langerin⁺ DCs (often referred to elsewhere as CD103⁺ DCs) have recently become recognized for their proficiency at antigen cross-presentation through major histocompatibility complex (MHC) class I molecules and, thus, are thought to be critical in host defense against viral infection.¹⁰ In the skin, (non-LC) dermal Langerin⁺ DCs differ from LCs in their capacity to prime specific T-cell subsets.^{11,12} In addition, (non-LC) dermal Langerin⁺ DCs are crucial in mounting contact hypersensitivity (CHS), whereas LCs in the epidermis have been shown to play a tolerogenic role and thereby to counteract (non-LC) Langerin⁺ DCs in CHS.^{13–15} Thus, it is now well recognized that LCs and (non-LC) Langerin⁺ DCs are crucial in both mounting and regulating immunologic processes.

Despite the importance of these cells, characterization of CD11c⁺ Langerin⁺ populations in the cornea, an immune-privileged tissue, is poorly defined. It is agreed that the normal cornea is endowed with a significant population of DCs,^{16–22} though their frequencies and anatomic location within the cornea are a subject of debate. DCs previously described in the stroma^{18,20} of normal cornea include CD11c⁺CD11b⁺ DCs²⁰ and a population of previously implicated plasmacytoid DCs.²¹ DCs have also been described in the epithelium of the normal cornea^{17–19}; they are CD11c⁺CD11b^{low}^{17,19} and are often collectively referred to as LCs. However, it has recently been shown that only a fraction of corneal epithelial DCs actually express Birbeck granules¹⁷ or Langerin.²² Furthermore, whether these are all LCs, or whether there are (non-LC) Langerin⁺ DCs in the cornea, is completely unknown.

In the present study, we found by qRT-PCR, immunohistochemistry, and FACS analysis that both the epithelium and the stroma of the normal murine cornea have CD11c⁺ Langerin⁺ populations. Furthermore, using genetically modified mice—including muLangerin-eGFP, huLangerin-DTA, and huLangerin-Cre YFP-flox mice¹³—previously established to study Langerin⁺ CD11c⁺ cells in the skin, we found that the CD11c⁺ Langerin⁺ population in the corneal epithelium consists mostly of LCs, whereas those in the corneal stroma were mostly (non-LC) Langerin⁺ DCs. Thus, such characterizations of CD11c⁺ Langerin⁺ subsets in the cornea will permit future work in addressing their role in the immunopathogenesis of

clinical conditions such as autoimmune dry eye, allergic conjunctivitis, and corneal transplant rejection.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice (age range, 8–12 weeks) purchased from Charles River Laboratories were housed in a specific pathogen-free environment. Genetically modified mice used were provided by Daniel H. Kaplan's laboratory at the University of Minnesota, and include muLangerin-eGFP, huLangerin-DTA, and huLangerin-Cre YFP-flox. All animals were treated according to guidelines of the ARVO Statement for the Use of Animals in Ophthalmic Research and the Public Health Policy on Humane Care and Use of Laboratory Animals (US Public Health Review).

Ex Vivo Separation of Corneal Epithelium from Stroma

Corneas were excised from immunologically naive mice using Vannas scissors. Remnant bulbar conjunctivae and iris tissues were removed from the excised cornea. Corneas were incubated in 20 mM EDTA (Sigma-Aldrich, St. Louis, MO) at 37°C for 45 minutes, and the epithelium was subsequently peeled from the stroma as an intact sheet.

Ex Vivo Separation of Epidermis from Dermis

Ears were excised from freshly euthanatized mice, and dorsal ear skin was separated from the ventral tissue. Cartilage was removed, and skin was floated in a 0.25% trypsin solution (Gibco) epidermis side up at 37°C for 1 to 2 hours. Epidermis was then peeled from the underlying dermis as an intact sheet.

Quantitative Real-time PCR

RNA was isolated with a purification kit (RNeasy Micro Kit; Qiagen, Valencia, CA) and reverse transcribed using a cDNA synthesis kit (Superscript III Kit; Invitrogen Life Technologies, Carlsbad, CA). Real-time PCR was performed using a PCR master mix (TaqMan Universal PCR Mastermix; Applied Biosystems, Foster City, CA) and preformulated primers for mouse Langerin (Mm00523545_m1; Applied Biosystems). The results were derived by the comparative threshold cycle method and normalized by GAPDH as an internal control.

Immunohistochemistry

Three penetrating incisions from the limbus to the central cornea were made to facilitate subsequent penetration of staining antibodies. Tissues were fixed at room temperature in 95% ethanol and subsequently washed. They were incubated at 1:100 with Fc-blocking antibody (clone 2.4G2; BD PharMingen, San Diego, CA) and then with PE-conjugated Langerin (clone eBioL31; eBiosciences, San Diego, CA) and FITC-conjugated I-A^b (clone AF6-120.1; BD PharMingen) at 1:100. Other tissues were stained in parallel with respective isotype controls. All tissues were stained at 4°C overnight in 1% BSA in the dark and subsequently washed thoroughly. Slides were prepared with DAPI mounting medium (Vector Laboratories, Burlingame, CA) and sealed for subsequent confocal analysis.

Collagenase Digestion of Cornea and Skin

Tissues were digested in 2 mg/mL collagenase D (Roche, Indianapolis, IN) and 0.5 mg/mL DNase (Roche) for 2 to 3 hours at 37°C. Cell suspensions were triturated in 20 mM EDTA and then passed through a 70- μ m filter (BD Falcon; Becton-Dickinson, Franklin Lakes, NJ).

Flow Cytometry and Quantitation of Langerin+ DCs

Unfractionated or magnetically enriched CD45⁺ cells (clone 30F11.1; Miltenyi Biotec, Auburn, CA) obtained from normal skin or corneal

tissue digests were used here. Cells were incubated with Fc blocking antibody at 4°C in 0.5% BSA. They were subsequently labeled with PE-conjugated Langerin (clone eBioL31; eBiosciences), APC-conjugated CD103 (clone 2E7; eBiosciences), PE-Cy7-conjugated CD11c (clone HL3; BD PharMingen), and Alexa 488-conjugated CD11b (clone M1/70; BD PharMingen) for 30 minutes in 0.5% BSA at 4°C in the dark. Aliquots were made in parallel for respective staining with the appropriate isotype control. All samples were washed and reconstituted in 0.5% BSA. Samples received 500 ng/mL DAPI (Invitrogen) immediately before data acquisition with a flow cytometer (LSRII; Becton-Dickinson).

Absolute numbers of Langerin-expressing cells in the corneal epithelium versus the stroma were ascertained by trypan blue exclusion assay of corneal epithelium and stroma, respectively ($n = 3$ corneas). Enumeration of DC subsets was subsequently accomplished by extrapolation based on frequencies determined by FACS analysis; the experiment was repeated once.

RESULTS

Differential Expression of Langerin mRNA Levels Identified in Corneal Epithelium versus Stroma

We initially assayed normal cornea for Langerin mRNA expression using qRT-PCR to investigate the possible presence of Langerin+ DCs. Normal corneas were harvested from immunologically naive mice (C57BL/6), and the epithelium was separated from the subjacent stroma (Fig. 1A). We also assayed normal skin epidermis and dermis, respectively, as positive controls (Fig. 1B). Negative controls were served by using 3T3 fibroblasts because they do not express Langerin. Using this system, we found a nearly 30-fold increase of Langerin mRNA in the corneal epithelium compared with 3T3 fibroblasts (Fig. 1A). We also detected significant, albeit lower, Langerin mRNA expression in the corneal stroma.

Langerin+ Cells in the Corneal Epithelium Are Both Morphologically and Phenotypically Distinct from Those in the Corneal Stroma

Immunofluorescence labeling for confocal microscopy of corneal whole mount preparations was next performed to verify Langerin expression at the protein level and to obtain further information regarding location, morphology, and phenotype of Langerin+ cells in the cornea. Corroborating our mRNA results, Langerin+ cells were observed in both the corneal epithelium and the stroma (Fig. 2A). Interestingly, the epithelial Langerin+ population was distinct in several ways from the stromal population. First, most Langerin-labeled cells in the stroma were more rounded, in contrast to the long dendrite

FIGURE 1. Identification of Langerin+ mRNA expression in the corneal epithelium and subjacent stroma. (A) Normal corneas ($n = 20$) were collected from C57BL/6 mice, and epithelia were subsequently separated from stroma for analysis. 3T3 cells were used as a negative control because they do not express Langerin. (B) Normal skin ($n = 5$) was used as a positive control because it expressed Langerin in both the epidermis and the dermis. Shown are the results of two independent experiments.

FIGURE 2. Langerin⁺ cells are present in the corneal epithelium and the corneal stroma but differ morphologically and phenotypically. **(A)** Langerin⁺ cells in the epithelium are located in the far peripheral regions and morphologically have long dendrite extensions, whereas Langerin⁺ cells in the stroma can be found in the paracentral/central regions and are morphologically more rounded. Whole cornea, corneal epithelium, and stroma were immunostained for Langerin (red), and whole mount preparations were analyzed by confocal microscopy. Dotted lines demarcate the central cornea (~1-mm diameter), paracentral (~0.5-mm-wide ring around the central region), and peripheral cornea/limbus (~0.5-mm-wide ring around the paracentral region). Isotype antibody control for Langerin expression in the stroma is included. **(B)** A proportion of epithelial Langerin⁺ cells are devoid of MHC class II expression, whereas almost all stromal Langerin⁺ cells express some levels of MHC class II (I-A). Corneal epithelium and stroma were costained for MHC class II (green), and **(C)** micrographs were subsequently merged. (green lines) 100- μ m. (arrows) Langerin⁺ cells. Micrographs are representative of at least three independent experiments.

extensions displayed by positive cells in the corneal epithelium (Fig. 2A). In addition, epithelial Langerin⁺ cells were found only in the far peripheral regions and limbus of the cornea (Figs. 2A–C), whereas stromal Langerin⁺ cells could also be detected in central and paracentral corneal regions (Figs. 2A–C), albeit at lower densities than in peripheral and limbal regions. Last, double staining for Langerin and MHC class II (I-A) revealed that most Langerin⁺ cells in the stroma expressed MHC class II, at least at some level, whereas there clearly were some Langerin⁺ cells in the epithelium that showed no detectable levels of MHC class II (Figs. 2A–C).

Quantitation of Langerin⁺ DC Frequencies in Corneal Epithelium and Stroma

We next used flow cytometry to verify the DC lineage (CD11c⁺) of Langerin⁺ cells and to enumerate their frequencies in the normal cornea. Separated corneal epithelium and stroma collected from naive mice were collagenase digested into single-cell suspensions and triple stained with DAPI, anti-CD11c, and anti-Langerin antibodies. After the exclusion of

dead cells (DAPI⁺) and the exclusion of doublet and triplet cell clumps (Fig. 3A), we found that the frequency of Langerin⁺ CD11c⁺ cells was substantially less in the corneal epithelium (16%) than in the stroma (45%) (Fig. 3B). Nonetheless, mean fluorescence intensity of epithelial Langerin⁺ DCs (Fig. 3C) showed a statistically significant (approximately threefold) increase over the stromal population (3475.0 ± 423.5 vs. 1229.57 ± 14.4 , respectively). In addition, the quantitation of absolute numbers showed no statistically significant difference ($P = 0.06$) in the number of Langerin⁺ DCs in the epithelium (58.8 ± 13.2) compared with the stroma (28.38 ± 6.5) in the normal cornea.

Langerin⁺ DCs in the Normal Cornea Have a Unique Phenotype

Given that we were able to reliably assess corneal DCs through FACS analysis, we next aimed to use this method for discerning the potential presence of LCs and (non-LC) Langerin⁺ DCs in the normal cornea. We based our experiment on the knowledge that LCs in the epidermis are CD11b⁺CD103^{low},

FIGURE 3. Enumeration of Langerin+ DC frequencies in the corneal epithelium and stroma, respectively. (A) Flow cytometric gating scheme for enumeration of Langerin+ DC subsets in the cornea. Separated corneal tissues collected from naive C57BL/6 mice ($n = 20$) were collagenase digested into single-cell suspensions and triple stained with DAPI, CD207 antibody (Langerin), and CD11c antibody. Dead cells (DAPI+) were excluded, as were doublet and triplet cell clumps, by pulse width (FSc-W) measurements. (B) Frequencies of Langerin+ DCs in the corneal epithelium and stromal. The gate for CD207+ was established based on the isotype control (B), and a representative plot is included. (C) Corneal epithelial Langerin+ DCs have higher Langerin mean fluorescence intensity (MFI) than stromal Langerin+ DCs. CD11c+ Langerin+ cells were gated, and MFI of Langerin was measured. Data are representative of more than three independent experiments.

whereas (non-LC) Langerin+ DCs in the dermis are CD11b^{low}CD103⁺.^{3-5,8,9} Corneal epithelium and stroma were therefore harvested, as were the epidermis and dermis, and digested into single-cell suspensions. In addition, CD45⁺ cells were subsequently enriched by magnetic separation before immunofluorescence antibody staining. Respective CD45⁺ fractions were similarly assayed as a negative control (Fig. 4A). For flow cytometry analysis, DAPI+ dead cells and cell clumps were excluded, and CD11b and CD103 expression on CD11c+ Langerin+ gated cells was assessed.

Using this system, we observed that epidermal LCs are phenotypically distinct from those of CD11c+ Langerin+ gated cells in the corneal epithelium. We found that though epidermal LCs were uniformly CD11b⁺CD103^{low}, only a small fraction of CD11c+ Langerin+ gated cells in the corneal epithelium shared this phenotype. Rather, the majority of CD11c+ Langerin+ gated cells in the corneal epithelium were CD11b^{low}CD103^{low} (Fig. 4b). Similar comparisons of CD11c+ Langerin+ populations in skin dermis versus corneal stroma also demonstrated substantially different phenotypes. In normal dermis, we observed three distinct CD11c+ Langerin+ populations: a population of migrating LCs defined by CD11b⁺CD103^{low}; a population of (non-LC) Langerin+ DCs defined by CD11b^{low}CD103⁺; and a previously described population defined by CD11b^{low}CD103^{low} (Fig. 4c).⁹ In contrast,

the corneal stroma had only one population of CD11c+ Langerin+ gated cells, and these were uniformly CD11b⁺CD103^{low} (Fig. 4c). Thus, the aggregate data here left us unable to conclusively discern the potential presence of LCs versus (non-LC) Langerin+ DCs in the normal cornea based on the phenotypic expressions of CD11b and CD103.

LCs Are Restricted to the Corneal Epithelium and Are Distinct from (non-LC) Langerin+ DCs of the Corneal Stroma

Our subsequent efforts toward potentially discerning LCs from (non-LC) Langerin+ DCs in the normal cornea relied on the use of genetically altered mice previously established by Kaplan et al.¹³ for the study of CD11c+ Langerin+ subsets in the skin. Our investigation included the assessment of human (hu)Langerin-diphtheria toxin A (DTA) transgenic mice, which have a DTA sequence inserted into a bacterial artificial chromosome containing the human Langerin gene expressed specifically in LCs.¹³ Hence, only LCs express DTA and are consequently deleted, whereas (non-LC) Langerin+ DCs are spared.¹³ We therefore harvested normal corneas from these mice and evaluated whole mounts of separated epithelium and stroma with immunofluorescence microscopy. Strikingly, we could not detect any Langerin+ cells in the corneal epithelium; however,

FIGURE 4. Langerin⁺ DCs in the normal cornea have a unique CD11b and CD103 expression profile. CD45⁺ cells were magnetically enriched from single-cell preparations of normal epidermis, dermis, corneal epithelium, and stroma, respectively. (A) Representative plot from corneal epithelium of the CD45 fraction, which was similarly observed in all tissues assayed. (B, C) CD11c⁺ Langerin⁺ cells from respective CD45⁺ fractions were gated, and CD11b and CD103 expression profiles were ascertained. FACS plots are representative of more than three independent experiments.

Langerin⁺ cells were detectable in the stroma (Fig. 5A). This was also confirmed by qRT-PCR (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6741/-/DCSupplemental>). This suggested that CD11c⁺ Langerin⁺ cells in the corneal epithelium are uniformly LCs, whereas the significant population of CD11c⁺ Langerin⁺ cells in the stroma consists of (non-LC) Langerin⁺ DCs.

To verify these results and to potentially rule out the presence of LCs in the corneal stroma, we next assayed corneas from huLangerin-Cre eYFP-flox mice. Rather than lacking LCs, these transgenic mice have LCs that selectively express eYFP⁺; (non-LC) Langerin⁺ DCs do *not* express eYFP. Furthermore, we also assayed (mu)Langerin eGFP⁺ knock-in mice in which all Langerin⁺ cells express eGFP²³ and thus can be used to validate the use of such mouse models in assessing CD11c⁺ Langerin⁺ cells in the cornea (Fig. 5B). In muLangerin eGFP corneas, we observed eGFP⁺ cells in both the corneal epithelium and the stroma (Fig. 5B). This validates that genetic insertion of enhanced fluorescence proteins under the Langerin promoter can indeed be detected in the corneal epithelium and stroma, and it corroborates the presence of CD11c⁺ Langerin⁺ cells in the corneal epithelium and stroma observed here by qRT-PCR, immunohistochemistry, and FACS analysis. Interestingly, in normal corneas of huLangerin-Cre YFP-flox mice, we observed eYFP⁺ cells in the corneal epithelium but could not detect eYFP⁺ cells in the stroma (Fig. 5C). Thus, these data indicated that CD11c⁺ Langerin⁺ cells in the corneal epithelium are uniformly LCs and that stromal Langerin⁺ DCs are (non-LC) Langerin⁺ DCs.

DISCUSSION

Results of this study have led to the conclusion that in the normal cornea, epithelium is endowed with a resident population of LCs, whereas the underlying stromal layer is endowed with a separate population of resident (non-LC) Langerin⁺ DCs.

Initial evidence in our study to support this was based on the finding that Langerin expression levels in corneal epithelium are higher than in the corneal stroma. This is because in the skin, higher epidermal Langerin expression, as seen here,

can be attributed to the local population of LCs given that they are known to express higher levels of Langerin relative to (non-LC) Langerin⁺ DCs of the dermis.^{3-5,8,9} Although we found here that overall Langerin expression in the cornea was orders of magnitude lower than in the skin (as to be expected given the far fewer DC numbers in the cornea), Langerin mRNA expression in the corneal epithelium was significantly higher than in the stroma. Furthermore, this was consistent with the significantly increased mean fluorescence intensity of Langerin expression detected by FACS analysis in CD11c⁺ Langerin⁺ cells in the corneal epithelium compared with those in the stroma. Thus, taken together, these data pointed toward the possibility that higher epithelial expression of Langerin is indicative of LC presence in the corneal epithelium, whereas lower stromal expression is indicative of (non-LC) Langerin⁺ DC presence in the stroma.

Conclusive evidence to support this hypothesis came from our observations made here using various genetically modified mouse models previously established to study Langerin⁺ CD11c⁺ cells in the skin.^{13,23} This included our use of muLangerin-eGFP mice, in which both LCs and (non-LC) Langerin⁺ DCs express eGFP in the skin. In the normal corneas of these mice, we detected eGFP⁺ cells in both the corneal epithelium and the stroma, thereby corroborating the presence of Langerin⁺ cells in both layers of the cornea detected here by qRT-PCR, immunohistochemistry, and FACS analysis. In addition, we also assayed huLangerin-DTA mice in which LCs, but *not* (non-LC) Langerin⁺ DCs, are selectively deleted in the skin.¹³ Consistent with this, we observed that Langerin⁺ cells were detectable only in the corneal stroma of these mice, thus suggesting that Langerin⁺ cells in the epithelium are, by and large, LCs. Furthermore, by also assaying huLangerin-Cre YFP-flox mice, it was conclusive that in the normal cornea, LCs reside in the epithelial layer. This is because only LCs expressed eYFP in the skin of these mice. Thus, our observations that eYFP⁺ cells were detectable only in the corneal epithelium indicate that LCs reside in, and are restricted to, the epithelium. Collective results in conjunction with evidence obtained from muLangerin-eGFP, huLangerin-DTA, and huLangerin-Cre YFP-flox mice led us to conclude that CD11c⁺ Langerin⁺ cells in the corneal epithelium are primarily LCs,

FIGURE 5. LCs are restricted to the corneal epithelium and are distinct from (non-LC) Langerin+ DCs of the stroma. **(A)** Normal corneas from huLangerin-DTA mice are devoid of Langerin+ DCs in the epithelium, but not in the stroma. In these previously described mice, LCs are constitutively deleted, whereas (non-LC) Langerin+ DCs are not.¹³ Normal corneas from huLangerin-DTA mice were collected and separated for subsequent immunofluorescence staining with Langerin (red). **(B)** Corneas from muLangerin-eGFP mice corroborated the presence of Langerin+ DCs in both the corneal epithelium and the stroma. In muLangerin-eGFP mice, all Langerin+ DCs constitutively express eGFP (green).²³ Normal corneas were collected from muLangerin-eGFP mice, and epithelium and stroma were separated for subsequent whole mount preparations. **(C)** Normal corneas from huLangerin-Cre YFP-flox mice had eYFP+ cells in the epithelium (yellow) but not in the stroma. In huLangerin-Cre YFP-flox mice, LCs selectively expressed eYFP+, but (non-LC) Langerin+ DCs did not. Normal corneas from huLangerin-Cre YFP-flox mice were separated, and whole mounts were prepared for confocal analysis. (green lines) 100 μ m. Micrographs are representative of $n \geq 6$ corneas, and experiments were performed twice independently.

whereas those present in the corneal stroma are a separate population of primarily (non-LC) Langerin+ DCs.

Our studies also revealed a number of distinctions among CD11c+ Langerin+ subsets in the cornea compared with those in the skin. In contrast to the skin, LCs make up only a very small fraction of DCs in the corneal epithelium, but the reasons for this are not clear. Furthermore, LCs in the normal skin can also be found subjacent to the epidermis, as seen here, whereas corneal LCs appear to be restricted to the epithelial layer. This is likely to be explained by differences in the anatomic location of lymphatics in these tissues. For example, skin LCs must mobilize downward into the dermis to access skin lymphatic channels, whereas lymphatic vessels are located laterally in the far peripheral regions of the cornea and limbus.

Another distinction revealed in our studies between CD11c+ Langerin+ subsets in the cornea and the skin are altered integrin expression profiles, as seen by assaying CD11b

and CD103. In contrast to epidermal LCs, which are known to be CD11b+CD103^{low},^{3-5,8,9} we observed that corneal epithelial LCs are primarily CD11b^{low}CD103^{low}. However, though the precise reasons for this distinction remain unclear, low to absent expression of CD11b appears to be a common feature among corneal epithelial DCs.^{17,19} Similarly, the phenotype of (non-LC) Langerin+ DCs in the corneal stroma (CD11b+CD103^{low}) though altered compared with those in the dermis (CD11b^{low}CD103⁺),^{3-5,8,9} is consistent with bone marrow-derived cells of the stroma, which are primarily CD11b⁺.^{16,17,19,20,24-26} Thus, there appears to be some tissue site-specific association with phenotypic integrin expression of CD11c+ Langerin+ subsets. Consistent with this notion, skin LCs mobilized to draining lymph nodes are known to upregulate CD11b+ expression and to downregulate E-cadherin expression.^{3-5,8,9}

Regarding our capacity to characterize CD11c+ Langerin+ subsets of the normal cornea, sensitive techniques such as FACS analysis certainly facilitated these studies because this fraction is small indeed. Interestingly, this is relatively consistent with CD11c+ Langerin+ subsets described elsewhere, such as the dermis and the tissues of the lung, liver, kidney, and gut, which stereotypically make up a very small fraction of bone marrow cells in these areas.^{3,4,5,8,9,27,28} Nonetheless, numerous independent reports have established their relevance in mounting adaptive immune responses. This is exemplified in the dermis, where (non-LC) Langerin+ DCs are required for developing CHS responses in mice,^{13,28} and in MHC class I cross-presentation^{9,10} against cutaneous HSV-1 infection.¹⁰ This is also exemplified in the gut, where (non-LC) Langerin+ DCs contribute to the immunopathogenesis of experimental colitis²⁸ and have been shown to be paramount in the transport of pathogenic *Salmonella* from the intestinal tract to the mesenteric lymph nodes.²⁷ Seminal studies such as these have highlighted the notion that absolute number is not indicative of the level of tissue DC contribution to immunologic responses.

In summary, we show here that the normal murine cornea is endowed with a population of LCs in the corneal epithelium and a separate population of Langerin+ DCs in the subjacent stroma; hence, divisions seen in the skin between epidermal LCs and dermal Langerin+ DCs appear to be relevant in the cornea as well. Why these populations differ phenotypically from those of the skin remains to be determined. Furthermore, whether LCs and Langerin+ DCs in the cornea have divergent roles in immunity versus tolerance, as described in the skin, remains to be seen. Nonetheless, results from this study should facilitate future work in understanding the specific roles of such DC populations in ocular immune conditions such as autoimmune dry eye, allergic conjunctivitis, and cornea transplantation.

References

1. Stingl G, Katz SI, Clement L, Green I, Shevach EM. Immunologic functions of Ia-bearing epidermal Langerhans cells. *J Immunol.* 1978;121:2005-2013.
2. Schuler G, Steinman RM. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med.* 1985;161:526-546.
3. Bursch LS, Wang L, Igyarto B, et al. Identification of a novel population of Langerin+ dendritic cells. *J Exp Med.* Dec 24. 2007;204:3147-3156.
4. Ginhoux F, Collin MP, Bogunovic M, et al. Blood-derived dermal Langerin+ dendritic cells survey the skin in the steady state. *J Exp Med.* 2007;204:3133-3146.
5. Poulin LF, Henri S, de Bovis B, Devillard E, Kissenpfennig A, Malissen B. The dermis contains Langerin+ dendritic cells that de-

- velop and function independently of epidermal Langerhans cells. *J Exp Med*. 2007;204:3119–3131.
6. Douillard P, Stoitzner P, Tripp CH, et al. Mouse lymphoid tissue contains distinct subsets of Langerin/CD207+ dendritic cells, only one of which represents epidermal-derived Langerhans cells. *J Invest Dermatol*. 2005;125:983–994.
 7. McLellan AD, Kapp M, Eggert A, et al. Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression. *Blood*. 2002;99:2084–2093.
 8. Ginhoux F, Liu K, Helft J, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med*. 2009;206:3115–3130.
 9. Henri S, Poulin LF, Tamoutounour S, et al. CD207+ D103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med*. 2010;207:189–206.
 10. Bedoui S, Whitney PG, Waithman J, et al. Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat Immunol*. 2009;10:488–495.
 11. Mathers AR, Janelins BM, Rubin JP, et al. Differential capability of human cutaneous dendritic cell subsets to initiate Th17 responses. *J Immunol*. 2009;182:921–933.
 12. Morelli AE, Rubin JP, Erdos G, et al. CD4+ T cell responses elicited by different subsets of human skin migratory dendritic cells. *J Immunol*. 2005;175:7905–7915.
 13. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity*. 2005;23:611–620.
 14. Igyarto BZ, Jenison MC, Dudda JC, et al. Langerhans cells suppress contact hypersensitivity responses via cognate CD4 interaction and Langerhans cell-derived IL-10. *J Immunol*. 2009;183:5085–5093.
 15. Wang L, Bursch LS, Kissenpfennig A, Malissen B, Jameson SC, Hogquist KA. Langerin expressing cells promote skin immune responses under defined conditions. *J Immunol*. 2008;180:4722–4727.
 16. Forrester JV, Xu H, Kuffová L, Dick AD, McMenamin PG. Dendritic cell physiology and function in the eye. *Immunol Rev*. 2010;234:282–304.
 17. Hamrah P, Zhang Q, Liu Y, Dana MR. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci*. 2002;43:639–646.
 18. Lee E, Rosenbaum JT, Planck SR. Epifluorescence intravital microscopy of murine corneal dendritic cells. *IOVS*. 2010;51:2101–2108.
 19. Knickelbein JE, Watkins SC, McMenamin PG, Hendricks RL. Stratification of antigen-presenting cells within the normal cornea. *Ophthalmol Eye Dis*. 2009;1:45–54.
 20. Hamrah P, Liu Y, Zhang Q, Dana MR. The corneal stroma is endowed with a significant number of resident dendritic cells. *Invest Ophthalmol Vis Sci*. 2003;44:581–589.
 21. Sosnova M, Bradl M, Forrester JV. CD34+ corneal stromal cells are bone marrow-derived and express hemopoietic stem cell markers. *Stem Cells*. 2005;23:507–515.
 22. Mayer WJ, Irschick UM, Moser P, et al. Characterization of antigen-presenting cells in fresh and cultured human corneas using novel dendritic cell markers. *Invest Ophthalmol Vis Sci*. 2007;48:4459–4467.
 23. Kissenpfennig A, Henri S, Dubois B, et al. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity*. 2005;22:643–654.
 24. Chinnery HR, Ruitenberg MJ, Plant GW, Pearlman E, Jung S, McMenamin PG. The chemokine receptor CX3CR1 mediates homing of MHC class II-positive cells to the normal mouse corneal epithelium. *Invest Ophthalmol Vis Sci*. 2007;48:1568–1574.
 25. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci*. 2002;43:2264–2271.
 26. Kuffová L, Netuková M, Duncan L, Porter A, Stockinger B, Forrester JV. Cross presentation of antigen on MHC class II via the draining lymph node after corneal transplantation in mice. *J Immunol*. 2008;180:1353–1361.
 27. Bogunovic M, Ginhoux F, Helft J, et al. Origin of the lamina propria dendritic cell network. *Immunity*. 2009;31:513–525.
 28. Edelson BT, Wumesh KC, Juang R, et al. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med*. 2010;207:823–836.