Epifluorescence Intravital Microscopy of Murine Corneal Dendritic Cells

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PURPOSE. Dendritic cells (DCs) are antigen-presenting cells vital for initiating immune responses. In this study the authors examined the in vivo migratory capability of resident corneal DCs to various stimuli.

METHODS. The authors used mice expressing enhanced yellow fluorescent protein (eYFP) under control of the CD11c promoter to visualize corneal DCs. To assess the distribution and mobility of DCs, normal corneas were imaged in vivo and ex vivo with fluorescence microscopy. Intravital microscopy was used to examine the responses of resident central and peripheral corneal DCs to silver nitrate injury, lipopolysaccharide, microspheres, and tumor necrosis factor (TNF- α). In some experiments, TNF- α injection was used to first induce centripetal migration of DCs to the central cornea, which was subsequently reinjected with microspheres.

RESULTS. In normal corneas, DCs were sparsely distributed centrally and were denser in the periphery, with epithelial-level DCs extending into the epithelium. Videomicroscopy showed that though cell processes were in continuous movement, cells generally did not migrate. Within the first 6 hours after stimulation, neither central nor peripheral corneal DCs exhibited significant lateral migration, but central corneal DCs assumed extreme morphologic changes. An increased number of DCs in the TNF- α -stimulated central cornea were responsive to subsequent microsphere injection by adopting a migratory behavior, but not with increased speed.

CONCLUSIONS. In vivo imaging reveals minimal lateral migration of corneal DCs after various stimuli. In contrast, DCs within the central cornea after initial TNF- α injection are more likely to respond to a secondary insult with lateral migration. (*Invest Ophthalmol Vis Sci.* 2010;51:2101–2108) DOI:10.1167/iovs.08-2213

Dendritic cells (DCs) make up a heterogeneous group of potent antigen-presenting cells (APCs) that function primarily to capture, process, and present antigens to T lymphocytes and thus are important for initiating adaptive immune responses. Typically, the acquisition of foreign antigens occurs in peripheral tissues, but optimal encounter with T cells requires that DCs traffic out of peripheral tissue to draining lymph nodes by way of lymphatic vessels.^{1,2} Much of what is

known about DC trafficking patterns into and out of tissue, comes from studies of the skin.^{3,4} Less is known about the DCs that populate the cornea.

The cornea was considered to be an immunoprivileged site, in part because of its lack of blood and lymphatic vessels. Early attempts to identify APCs in the cornea, based primarily on major histocompatibility complex (MHC) class II staining of ex vivo tissue, found most of these cells in the corneal periphery, whereas low to negligible numbers were observed in the central region.^{5–7} This paucity of resident corneal APCs has been considered a critical factor of corneal immunity; in particular, it is a major contributor to the success of allotransplantation.^{8,9} The presence of resident corneal APCs in the central cornea can influence the outcome of infections.^{10,11} It has long been known that DCs can be induced to migrate centripetally into the central cornea by injection of latex beads or TNF- α .^{12,13}

More recent studies, however, have reported that some APCs, both DCs and macrophages, do reside in the normal central cornea. A number of groups have used panels of antibodies to cell surface markers, such as CD45, CD11c, CD11b, and F4/80, in addition to MHC class II on ex vivo cornea preparations to identify DCs in both central corneal epithelium (Langerhans cells) and stroma, whereas macrophages have been found to occupy only the stroma.^{14–18}

The emigration of resident corneal DCs from cornea had been investigated in a study by Liu et al.¹⁹ in which GFP⁺ bone marrow-derived cells were identified in the host lymph node after transplantation of a GFP⁺ donor button into a non-GFP host. A more recent study by Ward et al.²⁰ described the dynamics of resident corneal epithelial DC (Langerhans cell [LC]) movement and migration in response to thermal injury. These authors performed videomicroscopy on corneas in organ culture from eGFP bone marrow chimeric mice and reported an increase in the extension and retraction of processes (a process they termed dSEARCH [dendrite surveillance extension and retraction cycling habitude]) and amoeba-like lateral movement. However, whether corneal cells in culture behave similarly to cells in vivo is unknown.

Studies of immune cell dynamics in live animals are technically challenging, but the eye is ideally suited for imaging. In contrast to internal organs and tissue, the eye does not have to be surgically exposed, permitting noninvasive repeated visualization of various ocular components with minimal physiologic perturbation. Given that immune cell behavior can be influenced by a multitude of physical and soluble factors from structures and cells in the local environment, we undertook a study to examine DC dynamics in the living mouse cornea using intravital microscopy. Phenotypic distinction between DCs and macrophages is not clear-cut and differs among species, but in the mouse, the cell surface marker CD11c (α_x integrin) is expressed specifically on DCs.^{21,22} We took advantage of transgenic mice expressing enhanced yellow fluorescent protein (eYFP) under the control of the promoter for CD11c (CD11c-eYFP⁺) to visualize DCs in the murine cor-

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nea.²³ We investigated DC distribution and movement in the normal cornea, performed correlative ex vivo immunophenotypic analysis of DCs with antibodies to CD11b and MHC II, and examined the response of DCs in the central and peripheral cornea in response to various stimuli.

MATERIALS AND METHODS

Experimental Animals

Mice expressing eYFP under the control of the CD11c promoter²³ were used (C57BL/6 background, 8–12 weeks old, bred in Oregon Health and Science University animal care facilities) to visualize DCs in intact cornea by fluorescence microscopy. Experimental protocols were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our institutional animal care and use committee.

Preparation of Corneal Wholemounts and Immunostaining

Eyes were enucleated and fixed whole in 4% paraformaldehyde overnight, after which corneas were excised from the rest of the globe and cut with eight radial relaxing incisions. Corneas were then incubated in DAPI (Sigma, St. Louis, MO) for 10 minutes at room temperature, mounted (Slow-fade; Molecular Probes Invitrogen, Eugene, OR) on glass slides, and coverslipped ($n = 17$ corneas). Immunostaining for CD11b or MHC II was performed on corneas that were separated from additional fixed eyes, similar to the method reported by Chinnery et al.²⁴ Briefly, after excision, corneas were washed in PBS, incubated in 20 mM prewarmed EDTA tetrasodium for 30 minutes at 37°C, and incubated with a 0.2% solution of Triton-X in PBS plus 1% bovine serum albumin for 20 minutes at room temperature. For labeling of CD11b, corneas were incubated overnight at 4°C with anti-mouse CD11b antibody (M1/70, 1:200, BD Biosciences, San Jose, CA). The following day, corneas were washed and incubated with Alexa Fluor 594-conjugated donkey anti-rat IgG (1:100; Molecular Probes Invitrogen) for 1 hour at room temperature. For labeling of MHC class II, corneas were incubated overnight at 4°C with anti-MHC class II (M5/114, 1:200; BD Biosciences). The following day, corneas were washed, incubated with biotinylated goat anti-rat IgG (1:100; BD Biosciences) for 1 hour at room temperature, washed again, and incubated in Alexa Fluor 594-streptavidin (Molecular Probes Invitrogen) for an additional hour at room temperature. All antibodies were diluted in PBS + 1% BSA for the staining step. All immunostained corneas were incubated in DAPI (Sigma) for 10 minutes at room temperature before mounting on glass slides in the presence of mounting medium (Slow-fade; Molecular Probes Invitrogen). At least four different corneas were examined per immunostaining experiment. In each experiment, three corneas served as negative controls in which primary antibodies were substituted with isotype IgG antibodies.

Examination of Corneal Wholemounts and Quantitative Analysis of DCs

Corneal wholemounts were examined with both widefield (DM5000B; Leica, Bannockburn, IL) and confocal (Fluoview FV1000; Olympus, Center Valley, PA) fluorescence microscopy. For widefield microscopy, the depth of field afforded by low- and intermediate-power magnification objectives allowed for simultaneous visualization of the entire thickness of the cornea. For confocal microscopy, image stacks were acquired of the entire corneal thickness (1- μ m z-step size). Cell quantification was performed on two regions of the cornea, the central and the peripheral, where other investigators have observed the lowest and highest APC density, respectively. The central area was defined as the area within 0.5 mm of the corneal center, and the peripheral cornea was defined as the area between 1.0 and 1.5 mm radial distance from the center. In each region, cells were counted with the aid of a

grid and were converted to express a density measurement where indicated.

Intravital Videomicroscopy

Intravital widefield epifluorescence videomicroscopy was performed on the intact corneas of mice anesthetized by inhalation (2% isoflurane in O₂). Animals were kept warm on a heating pad, and core body temperature was monitored. Gel (Vidisc; Dr. Mann Pharma, Berlin, Germany) was applied to the cornea as an aqueous contact medium for the water-immersion objective lens and as a means to maintain ocular surface moisture. Head movement during the imaging session was limited by gentle physical restraint with a flexible metal head clamp.

Corneas were visualized with a modified microscope (DM-LFS; Leica, Wetzlar, Germany) connected to a cooled three-chip color camera (DEI-750CE; Optronics, Goleta, CA). The central cornea was defined as the area within 0.5 mm from the pupil center. Because cell movement is too slow to be appreciated in real time, serial video images of the same corneal location were captured. Image intervals ranged from 3 frames/min (conventional time-lapse to detect “quick” movement) to 1 frame/h (modified time-lapse to detect “slow” movement) as indicated. A similar modified time-lapse imaging system has been described by Vishwanath et al.²⁵ in which the slow movement of Langerhans cells could be monitored by overlaying sets of images taken 24 hours apart. In our adaptation of this modified time-lapse protocol, animals were anesthetized once per hour for corneas to be imaged in the same location as the previous hour, and the images were overlaid offline. For imaging of the central cornea, the injury or injection sites could be directly visualized by the investigator at the early time points (1–6 hours). The disruptions to the cornea (e.g., because of needle injury from injections), though sometimes subtle, could be detected and were used as the “stable points” to align each corneal image on the screen at each hour and for additional image registration offline using stabilization software. For imaging of the peripheral cornea, the limbal vessels and approximate clock hour of the corneal region were used to help locate the same area at each time point. In vivo imaging of this part of the eye presented a slight technical challenge over central corneal imaging because the murine limbus is situated virtually at the equator of the eye and is normally obscured by the lids. In addition, the limited mouse head and eye manipulation we could achieve in our set-up and steep corneal curvature required that we compile maximal projection images from image stacks to encompass the entire tissue depth in the peripheral cornea.

These various imaging protocols were used to detect DC probing and lateral migration. Video sequences were recorded (Premiere; Adobe Systems, Inc., San Jose, CA). The raw video recordings contained residual movement, such as that from breathing or from the eye itself. These motion artifacts were reduced offline (Image ProPlus; Media Cybernetics, Inc., Silver Spring, MD). Masked observers tracked cells in two dimensions from time-lapse videos. ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) was used to record XY coordinates of randomly selected cells in each frame of aligned videos and was used to calculate cell speeds.

Experimental Protocol

Imaging experiments were conducted on normal unmanipulated corneas and on corneas stimulated in the central region (within 1-mm diameter over pupil center) by one of the following: silver nitrate burn injury (Graefes-Graham-Field, Atlanta, GA); Alexa Fluor 594-conjugated *Escherichia coli* LPS (intrastromal injection, 10 μ g/1 μ L; Molecular Probes); red fluorescent microspheres (FluoSphere [Molecular Probes]; 1 μ L intrastromal injection; 1- μ m diameter, ex/em 580/605); or recombinant murine TNF- α (1 ng/1 μ L intrastromal injection; R&D Systems, Minneapolis, MN) to induce DC maturation and migration. These stimuli were chosen to represent a range of different conditions. The chemical burn is large in area and damages primarily the epithelium. The introduction of LPS was selected to mimic microbial danger,

whereas the microspheres were injected to examine the response to particulates rather than a soluble factor such as LPS. Although TNF- α has not been shown to directly affect migration, it may indirectly lead to migration by inducing maturation. For each of these conditions, five corneas were imaged centrally by conventional time-lapse and five by modified time-lapse. Additional corneas were imaged in the periphery by conventional (five corneas) or modified time-lapse (five corneas). In separate experiments, corneas were first injected intrastromally with TNF- α to induce centripetal migration of DCs to the central cornea and then were injected 24 hours later with LPS ($n = 5$) or beads ($n = 5$).

RESULTS

Dendritic Cells in the Normal Quiescent Cornea

The eYFP expression of DCs in the normal corneas of these mice was readily visualized by standard epifluorescence *in vivo* microscopy such that even fine dendrites could be easily seen (Movie S1; all movies available at <http://www.iovs.org/cgi/content/full/51/4/2101/DC1>). Corneas from such mice had DCs that were sparsely distributed in the central cornea (49 ± 15 cells/mm² corneal tissue [average $\pm$ SD]) but increased in density toward the periphery (152 ± 64 cells/mm² corneal tissue) (Fig. 1). Morphologically, they ranged from those with cell bodies that were extremely thin and had fine, long dendrites to those that were oblong or round and had few processes (Figs. 1B, 1C). Confocal microscopy of *ex vivo* corneas showed DCs with slender bodies and fine dendrites to be located at the level of, or just beneath, the basal epithelial cell layer (Fig. 2). Fine processes emerging anteriorly from these cells and inserting between epithelial cells could also occasionally be seen (Figs. 2B, 2D). In contrast, similar processes extending in the anterior-posterior direction were not detected on cells situated more deeply in the corneal stroma. In addition, the DCs in the deeper corneal stroma had more substantial cell bodies that were round or oval.

Intravital time-lapse videomicroscopy of DCs in the unmanipulated central cornea over a 30-minute recording period revealed active probing of cell processes, such as ruffling of lamellipodia and extension and retraction of dendrites (Movie S2). On rare occasion (4/398 observed cells in 20 videos, $\sim 1\%$ of cells), a cell was seen to crawl slowly from one location to another in the cornea (speed 3.8 ± 2.3 μ m/min; see example in Movie S2). The migratory cells observed were oblong or amoeboid-like and did not have fine dendrites. However, not all cells with this morphology were migratory.

Immunostaining of corneas from CD11c-eYFP mice with antibody to CD11b revealed that coexpression of CD11b and CD11c was similar in the central cornea and in the peripheral cornea (Table 1, Fig. 3A). In contrast, immunostaining with antibody to MHC class II revealed exclusively MHC class II-negative cells in the central cornea and expression in only a portion of DCs in the periphery (Table 1, Fig. 3B).

Dendritic Cells in the Stimulated Cornea

In vivo microscopy showed that the number of DCs resident within the normal central cornea is substantial. Given the importance of DC trafficking to lymph nodes in the initiation of adaptive immune responses, we examined whether and how DCs begin to traffic out of the central cornea, which is devoid of lymphatic vessels. We expected that DCs, as tissue sentinels, would initiate a rapid response to injury and other insults. Thus, we first focused our imaging on the initial 6 hours after a given stimulus.

The central cornea was subjected to 1 of 4 stimuli and at first was imaged with time-lapse microscopy for up to 2 hours during the 6-hour time period after the stimulus (3 frames/min from 0–2 hours, 2–4 hours, or 4–6 hours). No significant

FIGURE 1. Widefield epifluorescence microscopy of normal flat-mounted corneas showing the distribution of DCs throughout the cornea under low magnification (A). Under intermediate magnification, the diverse morphology of DCs in the central cornea, where they are sparsely distributed (B), compared with the peripheral cornea, where they are more densely distributed (C), is evident.

migration was noted using this imaging protocol (see Movie S3 for example of injured cornea), so a modified time-lapse imaging protocol was applied (1 frame/h). Using this protocol, resident central corneal DCs appeared not to migrate away from or toward the stimulus. Instead, they remained sessile during the initial 6-hour period (Fig. 3).

Although active migration was not seen during this time, resident central corneal DCs did exhibit morphologic changes. In the case of injury or injection with LPS or microspheres, cells appeared to be directed toward the injury or injection sites. Entire cell bodies (Figs. 4A–H) or cell projections (Fig. 5) were seen to orient toward the stimulus. This is in stark contrast to the normal morphology of DCs in this region of the cornea (Movies S1 and S2). With TNF- α injection (Figs. 4I–K), however, shape changes were not as dramatic as with the other tested stimuli. Injections with PBS were performed as a control for the injections of LPS, microspheres, and TNF- α (data not shown). Lateral migration was not detectable, and

FIGURE 2. Confocal microscopy of central (A, B) and peripheral (C, D) cornea shows details of the fine extensions of DCs (green) with typical dendriform morphology at the layer of the basal epithelium (all nuclei stained blue). These extensions are seen both en face (A, C) and in cross-section (B, D; epithelial side, top; endothelial side, bottom). Both en face and cross-sectional views are z-projections of the entire imaged cornea, not single slices at one plane of focus. Scale bar, 100 μ m.

although some extensions of cell processes were elicited, they were only noted at more than 5 hours after injection.

Like central corneal DCs, peripheral DCs, when evaluated with conventional time-lapse microscopy, did not migrate substantially in response to any of the stimuli applied: silver nitrate burn ($1.3 \pm 0.8 \mu\text{m}/\text{min}$), LPS ($2 \pm 1.1 \mu\text{m}/\text{min}$), microspheres ($1.6 \pm 1 \mu\text{m}/\text{min}$), TNF- α ($2.4 \pm 1.7 \mu\text{m}/\text{min}$; see Movie S4 of peripheral cornea after silver nitrate burn). Few cells were seen to actively migrate when peripheral corneas were imaged with modified time-lapse microscopy over a period of 6 hours and, when observed, did not necessarily appear to move with clear directionality toward the central cornea (Fig. 6). Interestingly, peripheral corneal DCs did not assume a hyperpolarized morphology such as that observed in central corneal DCs.

We did not observe significant immediate (within 6 h) DC migration after injection of either TNF- α or microspheres alone. However, the injection of TNF- α or microspheres into the central cornea has been shown by other investigators to lead to centripetal migration of DC at later times.^{12,13} TNF- α injection resulted in an increased number of visible DCs in the central cornea after 24 hours ($212 \pm 69 \text{ cells}/\text{mm}^2 \text{ cornea}$). Lateral migration of ($4.1 \pm 2.1 \mu\text{m}/\text{min}$) was measured in 3.6%

FIGURE 3. Immunohistochemistry of eYFP-CD11c corneas showing coexpression with CD11b or with MHC class II. Cells in the peripheral cornea stroma (A) were varied in their expression of CD11b (red) and CD11c (green). Cells in the peripheral cornea that coexpressed CD11c (green) and MHC class II (red; B) had typical dendriform morphology. Scale bar, 50 μ m.

of cells (10/276 observed cells in 8 videos). Although the precise origin of the cells visualized cannot be definitively determined using intravital microscopy alone, these cells would likely be composed of the few normal central corneal residents, cells newly immigrant from the conjunctiva, para-central, peripheral, or deep stromal cornea, and possibly cells newly expressing eYFP (i.e., CD11c) after TNF- α injection. When TNF- α -injected corneas were subsequently injected with microspheres in the central cornea, more (9/144 observed cells in 4 videos, 6.3%) exhibited lateral migration 2 hours after microsphere injection. Calculated lateral speeds of these migratory cells were slightly, but not significantly, faster ($5.7 \pm 1.9 \mu\text{m}/\text{min}$) than their speeds before microsphere injection. In contrast, when TNF- α -injected corneas were subsequently injected with phosphate-buffered saline in the central cornea, 3.9% of cells visualized (5/129 observed cells in 4 videos), which were composed of normal residents of the central cornea and newly immigrated cells or cells newly expressing CD11c, exhibited lateral migration speeds of $3.6 \pm 1.5 \mu\text{m}/\text{min}$.

DISCUSSION

In nonlymphoid tissue DCs act as sentinels that capture and then transport antigens to lymphoid organs, where they present antigen to naive T cells. In this study, we performed intravital imaging to examine the dynamic migratory abilities of corneal DCs to a variety of stimuli. We examined DCs within the central and peripheral cornea and particularly on their short-term (<6-hour) responses. In vivo imaging indicated that central corneal DCs did not migrate rapidly (within 6 hours) even to proximal threats and perturbations. However, dramatic morphologic changes were observed with some stimuli. It

TABLE 1. Coexpression of CD11c with CD11b or MHC Class II

	Central Cornea (%)	Peripheral Cornea (%)
CD11b ⁻ CD11c ⁺	49.1 $\pm$ 15.9	43.7 $\pm$ 8.5
CD11b ⁺ CD11c ⁻	29 $\pm$ 2.4	32.3 $\pm$ 6.5
CD11b ⁺ CD11c ⁺	21.9 $\pm$ 6	24 $\pm$ 7.4
MHCII ⁻ CD11c ⁺	100	59.3 $\pm$ 1.5
MHCII ⁺ CD11c ⁻	0	19.1 $\pm$ 3.7
MHCII ⁺ CD11c ⁺	0	21.6 $\pm$ 3.9

FIGURE 4. Resident central corneal DCs remained sessile during the immediate 6 hours after various stimuli. Serial photographs of the same corneal location were taken hourly from 1 hour to 6 hours. Here select images at 1, 3, or 6 hours after silver nitrate injury (A–C) or after intrastromal LPS injection with red fluorescent LPS (D–F), red fluorescent microspheres (G, H), or unlabeled TNF- α (I–K) are shown. The faint outline of the pupil can be seen in the background of some photographs, indicating the central corneal location of the stimuli and imaged DCs. For each case, arrows highlight several cells proximal to injury or injection sites that remained stationary over time. The boundary of the burn injury can be seen in the upper right (A–C). (D–F, I–K) Injection entry sites of the LPS or unlabeled TNF- α are indicated by asterisks. In the case of microsphere-injected corneas (seen as red fluorescence in the upper right corners of images), microspheres present in the tear film obscured visualization of the corneal cells at 1 hour, but by 3 and 6 hours they were almost completely cleared from the ocular surface, allowing improved visualization of DCs. Scale bar, 100 μ m.

appeared that cell body orientation and extension of processes were directed toward the stimulus, though this was not measured directly. In contrast, no notable morphologic changes were observed in peripheral DCs. When DCs that were first induced to migrate centripetally into the central cornea were again stimulated by microsphere application, more cells migrated, but not at faster speeds, than those injected with PBS after centripetal migration.

The distribution of corneal DCs, as shown here by *in vivo* and *ex vivo* examination, is in general agreement with *ex vivo* studies in mice using different techniques^{14–18} in that they are more numerous in peripheral than in central cornea and reside in both stroma and epithelium, as shown by confocal microscopy. The precise depth of DCs cannot be determined with the *in vivo* microscopy technique, but with confocal microscopy it was noted that the cells that reside at the basal epithelial layer had thinner bodies and longer dendrites than DCs in the anterior and posterior corneal stroma, which had more rounded, amoeboid-shaped bodies. Such morphologic differences may be used, only as a general indicator, of DCs in basal epithelial versus stromal layers. For example, Movie S2 shows one slowly migrating amoeboid-shaped cell among sessile dendriform cells. Efforts to alter our intravital microscopy system to allow for collection of z-depth information are under way so that cells at different corneal depths can be definitively distinguished from each other *in vivo*.

Quantification of murine DCs, especially within the central region, varies among studies, which may reflect differences in strains (e.g., BALB/c vs. C57BL/6), immunohistochemical reagents and protocols, cell quantification methodology, and other technical differences. For example, in contrast to findings from other investigators^{14,17,24} and from a previous study from our group, which used a different labeling technique,²⁶ we did not detect MHC II⁺ cells within the central cornea. This might have been because of a technical issue or difference with

the immunostaining protocol used. In addition, in this study, the number of DCs, as defined by CD11c expression and tagged by eYFP, was determined for the entire corneal thickness (i.e., cornea encompassing both epithelial and stromal layers), yet the numbers arrived here are fewer than those reported by Hamrah et al.¹⁸ when they examined only the epithelium. Some variability in eYFP intensity was noted among cells, even within the same cornea, and this variability did not appear to correlate with location in the cornea, morphology, or size.

High magnification three-dimensional corneal reconstructions revealed extensions from subepithelial DCs anteriorly into the epithelium. Such fine processes have also been described by Ward et al.²⁰ on bone marrow-derived cells and specifically on MHC II⁺ and CD11c⁺ cells by Meng et al.²⁶ Time-lapse intravital microscopy (Movie S2) revealed constant lateral extension and retraction of processes. Anterior-posterior movement of dendrites intercalating between the epithelial cells was not detectable with our system. Elegant four-dimensional imaging of DCs in the epithelium of the small bowel, however, has revealed that not only do cells extend processes across the epithelium and sample the gut lumen in the steady state, the number of extensions markedly increases after bacterial exposure.²⁷ Almost all DCs remained sessile in the cornea, but, on rare occasion, a cell was seen to migrate from one location to another. It is not possible with our current imaging system to determine which corneal layer was traversed by the migratory cells *in vivo*. Migratory bone marrow-derived cells in the normal *ex vivo* cornea have also been noted by Ward et al.,²⁰ though they did not determine whether the cells they observed were macrophages or DCs. Together, these behaviors suggest that corneal DCs carry out their sentinel function primarily by frequent antigen sampling of the local environment by largely sessile cells combined with active patrolling by few migrating cells.

FIGURE 5. Polarization of corneal DCs oriented toward the site of corneal stimulus. Shown are examples of cells with extremely elongated extensions that point toward a site of LPS injection at 1 hour (**A**) or 3 hours (**B**) after injection. Asterisks: injection entry sites. Scale bar, 100 μm .

Pathologic stimuli such as microbial products, inflammatory mediators, and other “danger” signals initiate a series of changes in DCs in peripheral tissue, including an increase in size, extension of processes, increased expression of MHC II and costimulatory molecules, secretion of cytokines and chemokines, and mobilization to draining lymph nodes.²⁸ Almost any stimulus or irritation to the central cornea results in a centripetal migration of DCs from the corneal periphery, limbus, or conjunctiva.^{12,13,29–31} Less is known about the egress of DCs from the central cornea to draining lymph nodes that would be expected with other peripheral tissue, such as with skin.

Much of what is known about the mechanisms of peripheral DC migration to lymph nodes comes from studies of the skin using models to produce local inflammation by applying pathologic agents or mechanical disruption of the tissue.^{3,32,33} The use of intravital microscopy has provided interesting information about the dynamics of DC and LC movement and migration, and our results presented here of corneal DC behavior bear similarities to that of skin DC behavior. Under steady state conditions, most skin LCs are sessile, for example over a 1 hour recording period, but rare, motile LCs are also observed over the same period.³² Treatment with reactive hapten or skin trauma induced by tape stripping results in both increased probing activity and lateral migration after approximately 24 hours.^{32,33} Kissenpfennig et al.³² have analyzed LC migration early (5 hours) after tape stripping and observed that most LC bodies and dendrites were sessile and that rare LCs

exhibited tethered motions. Lateral migration speeds of DCs, compared with those of other cells such as neutrophils, are complicated to measure; because DCs can adopt extremely varied and asymmetric shapes, it can be difficult to ascertain the center of the cell body to calculate cell displacement.

The trafficking of resident central corneal DCs to the lymph node has thus far been inferred from static examination of ex vivo tissue in a corneal allotransplantation model.^{19,34} In this study we performed intravital microscopy on corneas that were stimulated in various ways to visualize the short-term (within 6 hours) response of resident central corneal DCs. We observed profound morphologic changes such as dendrite hyperelongation similar to that described by Ward et al.²⁰ in corneas that were exposed to an inflammatory cytokine. However, though they observed increased lateral movement of bone marrow-derived cells immediately after local laser injury or TNF- α exposure, we did not detect increased lateral migration after a silver nitrate injury or intrastromal injection of TNF- α using an imaging capture rate of either 3 frames/min for the initial hour (data not shown) or 1 frame/h (Fig. 3) for the

FIGURE 6. Peripheral corneal DC migration within 6 hours after TNF- α injection in the central cornea. Serial photographs of the same corneal location were taken hourly from 1 hour to 6 hours. Here select images at 1, 3, or 6 hours (**A–C**, respectively) are shown. The conjunctiva lies to the left and the central cornea lies to the right of the portion of peripheral cornea shown. Arrow: one cell appeared to migrate over time in a direction roughly parallel to that of the limbus (starting location indicated by asterisk). Scale bar, 100 μm .

initial 6 hours. One possible explanation for this difference in result may be related to differences in cell behavior in organ culture compared with their in vivo environment. A number of immunosuppressive factors are present in the aqueous humor and cornea, and Shen et al.³⁵ have recently demonstrated the importance of some of these factors in inhibiting DC maturation. The absence of immunosuppressive factors in organ culture may thus result in increased DC maturation. Given that maturation and lymph node-directed migration have been shown to occur in parallel, DCs may also develop enhanced migratory capability. In addition, Ward et al.²⁰ might have tracked the migration of macrophages that were not labeled in our mice. Tissue temperature is a critical parameter that can also affect cell migration.³⁶ Strict local control of temperature is necessary for extended imaging of cell migration, such as in lymph nodes or the gastrointestinal tract,^{27,37} but one group has performed imaging of the footpad without local temperature regulation.³⁸ Although rectal temperature was monitored during imaging sessions described here on the cornea, ocular surface temperature itself was not monitored. It is possible that deviations from physiological corneal temperature could account for the observed lack of corneal DC migration.

The importance of central corneal DCs in shaping immune responses has been clearly demonstrated by other investigators. DCs in the cornea are clearly critical factors in corneal transplantation or in the response to infection such as by herpes simplex virus or *Pseudomonas aeruginosa*.^{8–10,29} It was thus surprising to us that a more rapid migratory response did not follow the various corneal threats, even when the cells were proximal to the area of insult.

No DC migration was apparent up to 6 hours after stimulation, but, by 24 hours, the number of DCs had increased in the central cornea. This accumulation of cells could be composed of normal residents of the central cornea, cells that have immigrated into the central cornea from elsewhere, and cells newly expressing CD11c. It would be important to explore how and from where DCs arrive at the area of insult between 6 hours and 24 hours. Efforts to directly visualize lateral DC migration during this time period with either the conventional (1 frame/20 s) or modified (1 frame/h) time-lapse protocol have, thus far, been unsuccessful because of technical limitations; hence, other imaging parameters, specific for DCs, are being explored. The dynamics of DC migration in the cornea appears to be much slower than what we have previously observed for neutrophils (Douglas SB, et al. *IOVS* 2007;48:ARVO E-Abstract 4310) using the conventional time-lapse imaging protocol.

This study provides definitive evidence for the presence of DCs in the normal central cornea. Our study shows that the cell bodies of DCs resident in the central cornea are generally immobile, that their dendritic processes are regularly in motion in the steady state, and that these cells remain largely nonmobile immediately after corneal stimulation. Although it appears that DCs, which are generally considered the most potent APCs for naive T cells, are not quickly responsive by adopting a migratory behavior, there are other resident corneal APCs, such as macrophages, that may behave differently. It would be of interest to investigate in future studies how macrophage behavior in response to corneal trauma and insults compares with that of DCs reported here.

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