Characterization of Antigen-Presenting Cells in Fresh and Cultured Human Corneas Using Novel Dendritic Cell Markers

Wolfgang J. Mayer,[1,2] *Ulrike M. Irschick,*[1] *Patrizia Moser,*[3] *Martin Wurm,*[4] *Hartwig P. Huemer,*[5] *Nikolaus Romani,*[6] *and Eveline U. Irschick*[1]

Purpose. Adult healthy human corneas bear a distinctive number of antigen-presenting cells (APCs) important for the fate of a graft. The purpose of this study was to differentiate between Langerhans cells (LCs) and other dendritic cells (DCs) and between mature and immature APCs in fresh and cultured human corneas using specific markers.

Methods. Immunofluorescence double staining was performed for Langerin/CD207, CD1a, DC-SIGN/CD209, DC-LAMP/CD208, CD45, CD11c, CD11b and HLA-DR.

Results. Langerin$^+$/CD1a$^+$/HLA-DR$^+$ LCs (approximately 100 cells/mm^2 in fresh corneas) were found in the limbal and peripheral regions of corneal epithelium and the anterior stroma up to 83 days of culture. All these cells coexpressed CD45 and CD11c. DC-SIGN$^+$/CD45$^+$ DCs (approximately 150 cells/mm^2 in fresh corneas) were detected mainly peripherally and in the anterior stroma, even in long-term cultured corneas. Most of these cells were HLA-DR$^-$. Few mature DCs (DC-LAMP$^+$/HLA-DR$^+$) were found in fresh and cultured corneas. Macrophages (CD11c$^-$/CD11b$^+$) were seen in the peripheral, paracentral, and even central regions of the posterior stroma.

Conclusions. This is the first demonstration that human corneas harbor populations of Langerin$^+$/CD1a$^+$/HLA-DR$^+$ LCs and DC-SIGN$^+$ DCs in a distribution pattern similar to that in the skin. Few APCs are in a mature state (DC-LAMP$^+$). Given the reduced but not complete depletion of APCs during organ culture, these grafts still bear a potential risk for rejection. (*Invest Ophthalmol Vis Sci.* 2007;48:4459–4467) DOI:10.1167/iovs.06-1184

The most capable antigen-presenting cells (APCs) in several tissues, primarily skin, are dendritic cells (DCs), including Langerhans cells (LCs). Besides macrophages, these cells function as professional APCs of the cornea and ocular surface.[1–5] They serve as immune "sentinels" against a foreign world. With the use of standard markers, different maturation stages of DC subsets are detectable at various corneal sites. These DCs have the capacity to determine the outcome of immunity or tolerance within this organ.[6]

From the [2]Department of Ophthalmology, Ludwig-Maximilians-University Munich, Munich, Germany; and the Departments of [1]Ophthalmology, [3]Pathology, [4]General and Transplant Surgery, [5]Hygiene and Social Medicine, and [6]Dermatology and Venereology, Innsbruck Medical University, Innsbruck, Austria.

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Corresponding author: Wolfgang J. Mayer, Department of Ophthalmology, Ludwig-Maximilians-University Munich, Mathildenstr. 8, D-80366 München, Germany; wolfgang.j.mayer@med.uni-muenchen.de.

Through electron microscopy, tennis racket-like Birbeck granules were found in LCs. Strong evidence indicates that Birbeck granules, particularly their main molecular constituent—the Langerin molecule, a mannose-specific C-type lectin—plays a role in the endocytosis pathway.[7] Birbeck granules become reliable markers for Langerhans cells, especially for immature cells.[8]

In human skin, LCs constitute approximately 2% to 4% of all epidermal cells.[9] They are the epidermal variant of DCs and are uniformly distributed (approximately 700 cells/mm^2). LCs stem from bone marrow cells of the myeloid lineage. Their precursors populate the epidermis during fetal and early postnatal life, when they gain their immunologic function. They start expressing molecules such as major histocompatibility complex (MHC) class II, Langerin/CD207, and DEC-205/CD205.[8] LCs are specialized to stimulate resting T cells and to induce primary T-cell immune responses. Thus, LCs play important roles in contact hypersensitivity, transplant rejection, and other immunologic processes.

Corneal LCs, similar to skin LCs, are bone-marrow-derived cells that represent the professional APCs of the ocular surface. It had been thought that constitutive expression of MHC class II antigens is a characteristic feature of DCs (including LCs) in the corneal epithelium. Under nonpathologic circumstances, LCs are the only cells that constitutively express MHC class II molecules in the corneal epithelium.[10] MHC antigens are important components in the generation and the maintenance of immune responses. However, a recent study by Hamrah et al.[3] demonstrated that an MHC class II$^-$ LC population exists in the murine corneal epithelium. The authors showed that LCs became activated in inflamed corneas by the upregulation of MHC class II antigens and costimulatory molecules such as CD80 and CD86.[3] Furthermore, they found at least three bone-marrow-derived subsets of DCs in the normal corneal stroma in addition to the corneal LCs.[2] Thus, APCs vary in their expression of surface markers.[6,11]

Langerin/CD207 is selectively expressed by LCs,[7,12] CD1a on cortical thymocytes, dendritic cells, and LCs, especially in skin. LCs express comparatively high levels of CD1a and Langerin, which imparts a unique functional role to these cells in initiating immune responses to microbial pathogens.[13] DC-SIGN/CD209 is a type 2 transmembrane protein that also contains a mannose-binding (C-type lectin) domain. It is not expressed on LCs[14–16] but is expressed on dermal DCs.[17] CD208/DC-LAMP is a member of the lysosome-associated membrane glycoprotein (LAMP) family.[18] DC-LAMP is specifically expressed by mature dendritic cells located in the T-cell areas of lymphoid tissues, which are known as interdigitating dendritic cells.[18–20] Mature skin LCs also express DC-LAMP.[17] A direct correlation between the expression of DC-LAMP and that of other maturation markers, such as CD86, has been established in skin.[18]

DCs and LCs are the most important cells regulating immunogenicity of the cornea and are important for the reliability of a corneal graft. The aim of this study was to characterize

human corneal dendritic cells, including LCs, in freshly isolated corneas and in long-term cultured corneas by using immunofluorescence staining with specific markers applied to human skin but hitherto not yet to the human cornea. Langerin, DC-SIGN, and CD1a were used to determine cell type and to distinguish between epidermal–epithelial Langerhans cells and dermal–interstitial dendritic cells. Costaining with antibodies against CD45, CD11c, and CD11b was performed to validate and extend our observations. Additionally, we screened the corneas for macrophages (i.e., $CD11c^-/CD11b^+$ cells). Furthermore, DC-LAMP served as a marker for the maturation state of dendritic cells. We examined the horizontal and vertical DC distribution in comparison with human skin and were interested in determining how long DCs and LCs were detectable in corneas cultured for different time periods compared with fresh corneas. We also investigated whether the routinely performed decontamination step for preparing donor corneas had an influence on DCs and LCs, and we compared the densities of LCs and DCs in young and elder corneas.

Materials and Methods

Preparation and Culture of Human Corneas

This research was approved by the Department of Ophthalmology at Innsbruck Medical University and adhered to the tenets of the Declaration of Helsinki. Human corneas were primarily enucleated for transplantation purposes. Excluded corneas were used for our study. After enucleation, the eyes were dissected under the guidelines for Austrian eye banks. In total, 47 corneas were available from 24 male and 23 female donors ranging in age from 20 to 96 years (median, 63 years). Nine fresh corneas were used. The other corneas were cultured and grouped according to the duration of culture (9 corneas up to day 21, 29 corneas from day 22 to day 1076).

Preparation was performed as recently described.[21] In brief, bulbi were cleared from remainders of the conjunctiva and eye muscles. They were washed for 5 minutes with floating tap water, decontaminated with povidone iodine (Polyvidon-Jod 0.5%; Betaisodona; Mundipharma, Limburg/Lahn, Germany) for 2 minutes, discolored with sodium-thiosulfate (0.1%) for 1 minute (Gatt-Koller, Absam, Austria), and rinsed with PBS (PAA Laboratories, Pasching, Austria). To evaluate the influence of the decontamination procedure on the LCs and DCs, five corneas were used without decontamination. Further processing occurred under sterile conditions. The isolated corneas were washed with PBS and placed into sterile tubes (Sarstedt, Nümbrecht, Germany) containing 25 mL culture medium RPMI 1640 (with 2.0 g/L $NaHCO_3$, without phenol red and without L-glutamine [Biochrome AG, Berlin, Germany]) supplemented with 100 U/mL penicillin G, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Gibco/Invitrogen Corp., Grand Island NY), 2 mM L-glutamine (Low Endotoxine; Biochrome), 25 mM HEPES-buffer (Biochrome), and 5% fetal calf serum (Gibco/Invitrogen). With the use of a long-term culture medium, it is possible to culture human corneas at 31°C for long periods by renewing the medium every week. Cell viability after long-term culture was monitored with trypan blue (Sigma Chemical, St. Louis, MO) staining technique. Even after the longest observation period (more than 1000 days), epithelial cells were still viable.

Cryosections were obtained using a microtome (CM-3050; Leica-Microsystems, Wetzlar, Germany). Deep-frozen tissue was embedded in OCT compound medium (Tissue-Tek; Sanova, Vienna, Austria). Before cryosectioning, the human corneas were divided into central parts (diameter, 3 mm) and peripheral areas (adjacent to the central parts reaching the limbus). Human skin samples served as positive controls. Sections (8 μm) were cut at −26°C, allowed to dry at room temperature for 1 hour, and stored at −20°C.

Fresh corneas were used to obtain epithelial flat mounts and whole stromal cryosections. They were immersed in PBS containing 20 mM EDTA (Sigma-Aldrich) at 37°C for 1 hour. The epithelium was separated from the stroma with small tweezers and was washed again in PBS. Epithelial sheets were put on slides and fixed in acetone for 15 minutes at room temperature. Consecutive cryosections (10-μm thickness) were obtained from the whole corneal stroma.

Immunoreagents

The following unconjugated primary mouse antibodies were used at the indicated final concentrations: anti–CD207/Langerin (clone DCGM4, IgG1, 2 μg/mL; Beckman Coulter, Fullerton, CA), anti–CD1a (clone HI149, IgG1, 3.125 μg/mL; BD Biosciences, Franklin Lakes, NJ), anti–CD209/DC-SIGN (clone DCN46, IgG2b, 5 μg/mL; BD Biosciences), anti–CD208/DC-LAMP (clone 104.G4, IgG1, 2 μg/mL; Beckman Coulter), and anti–CD11c (clone B-ly6, IgG1, 10 μg/mL; BD Biosciences). As tertiary mouse antibodies, the following were used in an immunofluorescent double-staining technique: FITC-conjugated anti–HLA-DR (clone L243, IgG2a, 2.5 μg/mL; BD Biosciences), FITC-anti–CD45 (clone HI30, IgG1, 10 μg/mL; BD Biosciences), FITC-anti–CD1a (clone HI149, IgG1, 10 μg/mL; BD Biosciences), FITC-anti–CD11c (clone BU15, IgG1, 5 μg/mL; Serotec Ltd., Kidlington, Oxford, UK), FITC-anti–CD11b (clone ICRF44, IgG1, 5 μg/mL; Serotec), Alexa-488-conjugated anti–CD208/DC-LAMP (clone 104G4, IgG1, 50 μg/mL; Dendritics, Dardilly, France), FITC-anti–CD86 (clone 2331, IgG1, 4 μg/mL; BD Biosciences), and FITC-anti–CD80 (clone L307.4, IgG1, 8 μg/mL; BD Biosciences).

Immunofluorescent Double Labeling in Triple-Layer Technique

A biotin-streptavidin–based triple-layer technique was used. Sections and sheets were first incubated with primary unconjugated antibodies (all diluted in PBS/1% bovine serum albumin, 30 minutes, 37°C). After thorough washing (5 minutes in PBS/1% BSA), the biotinylated secondary antibody (sheep anti–mouse immunoglobulin, dilution of 1:100; Amersham Pharmacia Biotech, Buckinghamshire, UK) was applied (30 minutes, 37°C). In the third step, Texas Red-conjugated streptavidin was applied (Amersham; 1:100, 30 minutes, 37°C). Then a blocking reagent (mouse γ-globulin, final concentration 100 μg/mL; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to avoid binding of the subsequent FITC-conjugated antibody to residual free binding sites of the preceding anti–mouse immunoglobulin antibody. Finally, the FITC-conjugated antibody was applied (30 minutes, 37°C), and the tissue sections were covered with fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

An optical microscope (DMLB; Leica) and a digital microscope (DM6000B; Leica) were used for analysis and to obtain digital pictures. Because of the low density of LCs and DCs in the cornea, especially in long-term cultured corneas, it was necessary to evaluate a large number of corneal sections. Each section was inspected over its entire length using total magnifications of ×200, ×400, and ×630 (DM6000B; Leica) and ×100 and ×400 (DMLB; Leica), and the expression of all used markers was recorded.

With the use of a software-controlled scanning grid (PicED Cora; Jomesa Messsysteme GmbH, Munich, Germany), it was possible to enumerate the cells in the different areas of the cornea. Six to eight different fields were analyzed for each specimen, and the numbers were averaged. For analytical purposes, the cornea was divided into different areas. The central area measured 3 mm in diameter. The paracentral region constituted the area between 3 and 4 mm around the center, and the peripheral rim reached from the paracenter to the limbus. Results were compared with observations found in human skin. Human foreskin served as a positive control because this tissue bears a high density of Langerhans and dermal dendritic cells. The following mouse antibodies were used as negative controls: IgG1 (clone DAK-G01, IgG1κ; DAKO A/S, Glostrup, Denmark), IgG2b (clone DAK-G09, IgG2bκ; DAKO), FITC-conjugated IgG2a (clone RPC5, IgG2a; Hölzel Diagnostika, Köln, Germany), and FITC-conjugated IgG1 (clone MOPC-21, IgG1; BD Biosciences). We did not find any unspecific binding of control immunoglobulins (Fig. 1).

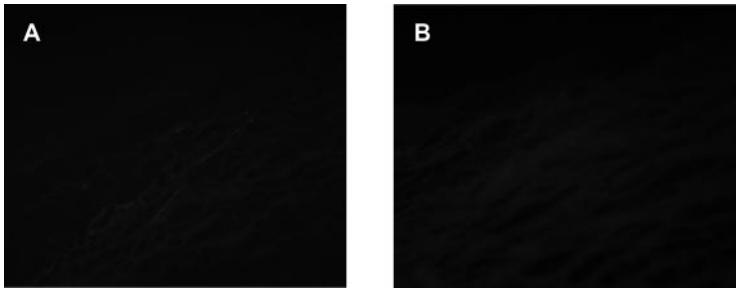

FIGURE 1. (**A**) Negative control for immunoglobulin class IgG1. (**B**) Negative control for immunoglobulin class IgG2b. Note that IgG2a is included in the double-staining procedure. (**A**, **B**) Magnification, ×200.

Statistical Analysis

Student's *t*-test was used to compare the number of positively labeled epithelial and stromal cells in different areas of fresh and cultured corneas. $P < 0.05$ was considered significant.

RESULTS

Expression of CD207/Langerin

Langerin discriminates between LCs and other DCs and thereby specifically identifies LCs. Corneal LCs (Langerin$^+$ cells) were primarily found in fresh corneas (Figs. 2, 3). Most of them were large cells and showed classic dendritic morphology with long dendrites. Their localization was mainly in the peripheral epithelium of the cornea, but they were also found within the limbus (Fig. 4). Toward the center of the cornea, the number of LCs strongly decreased. Few Langerin$^+$ LCs were found in the paracentral area of the cornea (diameter, 3–4 mm). No Langerin$^+$ cells were found in the centers of any of the tested corneas (Figs. 3–5).

Double staining with MHC class II antibody showed strong coexpression in all Langerin$^+$ LCs (Fig. 2A). In addition, all the Langerin$^+$ cells coexpressed CD45 (indicating bone-marrow derivation) and CD11c (indicating DC lineage). In the peripheral part of the cornea, few were positive for DC-LAMP; thus, they represented mature LCs.

Double labeling of human epidermis with Langerin and CD1a showed that virtually all Langerin$^+$ cells were CD1a$^+$.[12,17] This was also seen in fresh corneas.

Langerin$^+$ LCs were also found in long-term cultured corneas. The longer the culture, the fewer Langerin$^+$ LCs developed. We detected LCs consistently in corneas cultured up to 56 days and in some cultured for up to 3 months (Figs. 2B, 5). After approximately 80 days of culture, Langerin$^+$ cells were no longer found. This reduction in Langerin$^+$ LCs was statistically significant ($P < 0.05$) during the first 21 days of culture.

Expression of CD1a

We found CD1a$^+$ cells in fresh corneas in the limbal and peripheral regions (Fig. 6A). These cells were never found in central areas of all examined corneas, and few were located in the paracentral region (Figs. 3, 4). CD1a$^+$ cells were also detectable in cultured corneas until approximately 80 days (Figs. 5, 6B). In contrast to Langerin, CD1a was expressed at lower levels of fluorescence intensity in fresh and in long-term cultured corneas (Figs. 2A, 2B). As in Langerin$^+$ cells, CD1a$^+$ cells were statistically significantly reduced during the first 21 days of culture ($P < 0.05$; Fig. 5A). Double-labeling experiments revealed that most, if not all, LCs coexpressed Langerin and CD1a. Langerin$^+$ and CD1a$^+$ cells were double stained with antibodies against HLA-DR, CD45, and CD11c, and there was virtually 100% overlap. Few CD1a$^+$ cells in the peripheral epithelium were also positive for DC-LAMP and thus qualified as mature LCs.

Expression of CD209/DC-SIGN

In the skin, DC-SIGN is expressed on dermal DCs but not on LCs. In the cornea, DC-SIGN$^+$ cells were found only in the stroma, never in the epithelium (Fig. 7A). This observation is comparable to that in the skin, where DC-SIGN$^+$ cells are also found exclusively in the dermis (Fig. 7C). All DC-SIGN$^+$ coexpressed CD45, but only some (approximately 12%) coexpressed CD11c. Their density in the stroma was statistically significantly higher than Langerin$^+$ cells in the epithelium ($P < 0.05$; Figs. 3, 5).

In fresh and in cultured corneas, there was a pronounced accumulation of DC-SIGN$^+$ cells in the limbal and peripheral regions (Figs. 3, 5, 7A, 7B). They were predominantly localized in the anterior corneal stroma and were distributed more toward the center than were Langerin$^+$ cells (Figs. 3, 4). We found a statistically significant reduction during the first 21 days of culture ($P < 0.05$). Most DC-SIGN$^+$ cells did not

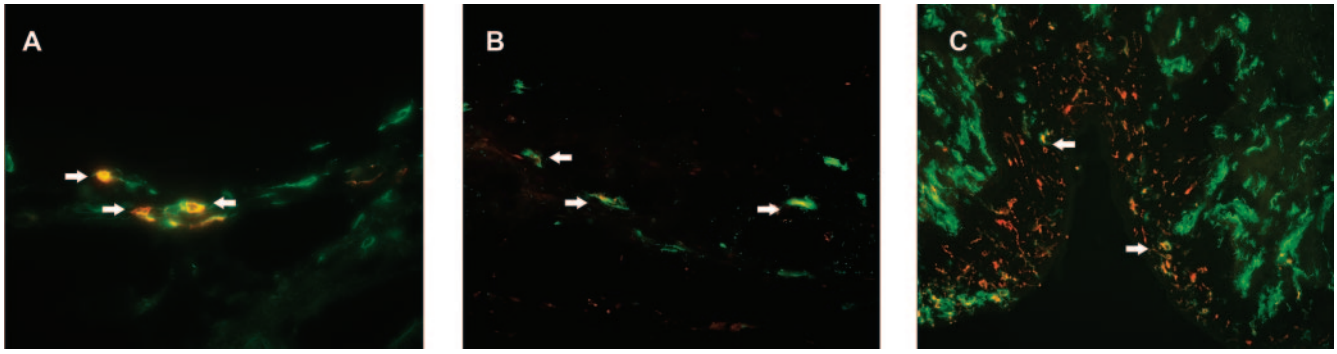

FIGURE 2. Langerin$^+$ cells in human corneas. (**A**) Fresh double-stained cornea with markers for Langerin (*red*) and HLA-DR (*green*). LCs are located in the epithelium and anterior stroma (*arrows*) and show expression of both markers. (**B**) Cultured cornea (56 days) stained with the same markers (*arrows*). (**C**) Human skin, which served as a positive control. LCs are located in the epidermis (*arrows*). Magnifications, (**A**) ×630, (**B**) ×400, (**C**) ×200.

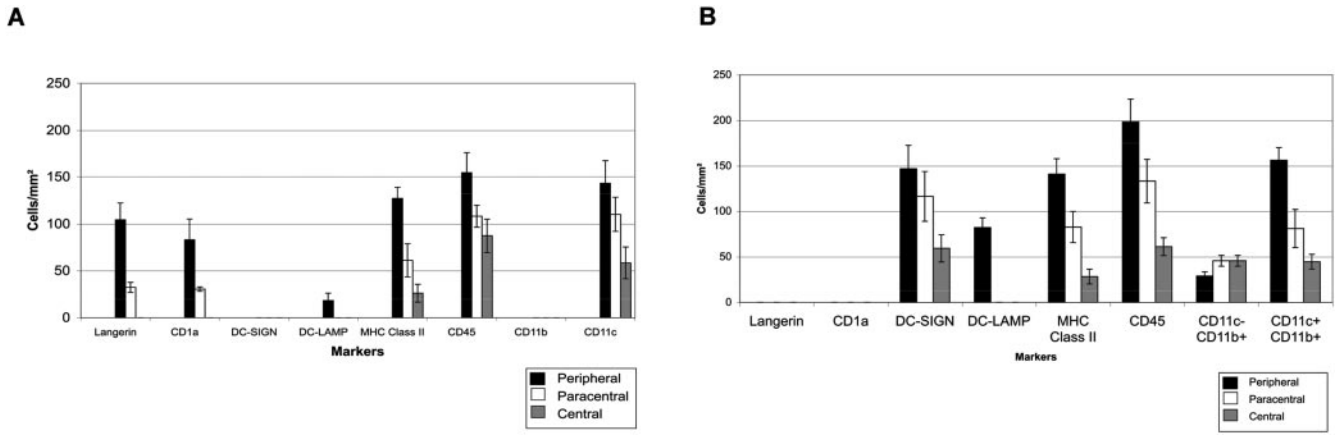

FIGURE 3. Densities of APCs (cells/mm^2) in fresh human corneas (n = 9). APC densities in the peripheral, paracentral, and central areas of (**A**) epithelium and (**B**) stroma.

express MHC class II; in those that did, the expression was below the threshold of detection (Fig. 4). Inversely, we observed substantial numbers of DC-SIGN$^-$ but MHC class II$^+$ cells in the periphery and in the central parts of the stroma. Few DC-SIGN$^+$ cells (approximately 6%) coexpressed DC-LAMP in the peripheral stroma, thus indicating mature DCs.

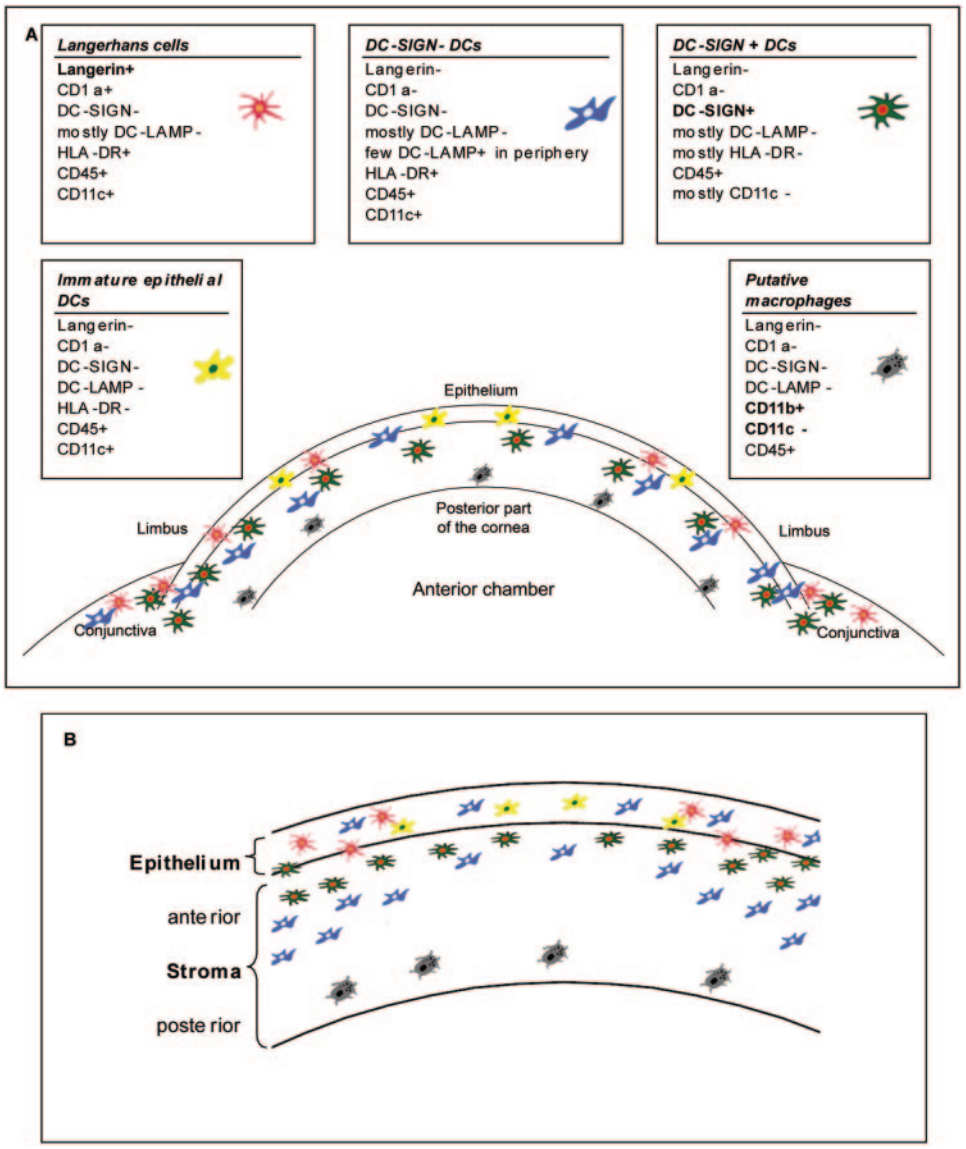

FIGURE 4. APCs within the whole fresh cornea. (**A**) Distribution of APCs within the central, paracentral, and peripheral regions of the human cornea. (**B**) Distribution of APCs within the corneal layers. All APCs were positive for CD45. Langerin, CD1a, and HLA-DR$^+$ (LCs) are located in the peripheral regions of the corneal epithelium. LCs were also positive for CD11c. HLA-DR$^-$/CD11c$^+$ cells (*yellow*) were present in the paracentral and central corneal epithelium. DC-SIGN$^+$/HLA-DR$^-$ cells (DCs) are located in the peripheral, paracentral, and even central anterior stroma. Few were positive for HLA-DR. They showed higher density than LCs. DC-SIGN$^+$ cells only partially overlapped with CD11c. Other HLA-DR–expressing cells (*blue*) were found in the whole cornea, primarily in the anterior stroma. DC-LAMP and HLA-DR$^+$ cells (mature DCs) are rare and are located only in the limbal and peripheral stroma/epithelium of the cornea. Macrophages (CD11c$^-$/CD11b$^+$) are located in the peripheral, paracentral, and central part of the posterior stroma.

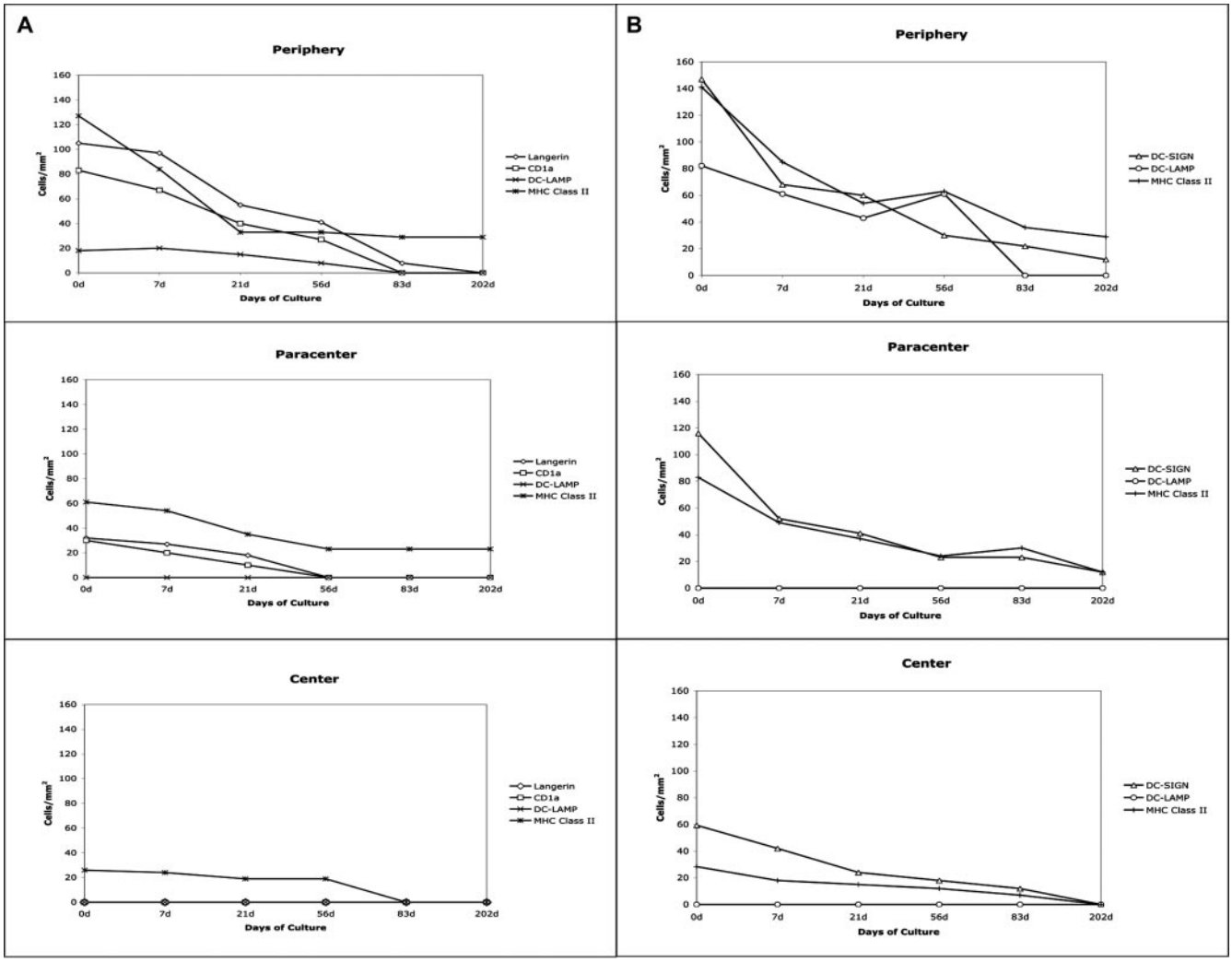

FIGURE 5. Kinetics of LC/DC reduction (cells/mm^2) during long-term culture. LC/DC densities in the peripheral, paracentral, and central area of the human corneal epithelium (**A**) and stroma (**B**).

Expression of CD208/DC-LAMP

As best illustrated in human skin, numbers of DC-LAMP– expressing DC are always much lower than numbers of MHC class II– expressing cells.[17] Similarly, few DC-LAMP$^+$ cells were found in fresh or in cultured corneas (Figs. 8A, 8B). DC-LAMP$^+$ cells were rare in the epithelium, where LCs reside (Figs. 3–5). Rather they were invariably Langerin$^+$ and thus qualified as scarce, mature LCs. In the stromal layer, DC-LAMP$^+$ cells were located only in the periphery, preferentially in the anterior stroma but never in the center. Costaining with CD45, HLA-DR, and CD11c showed that all DC-LAMP$^+$ cells were positive for

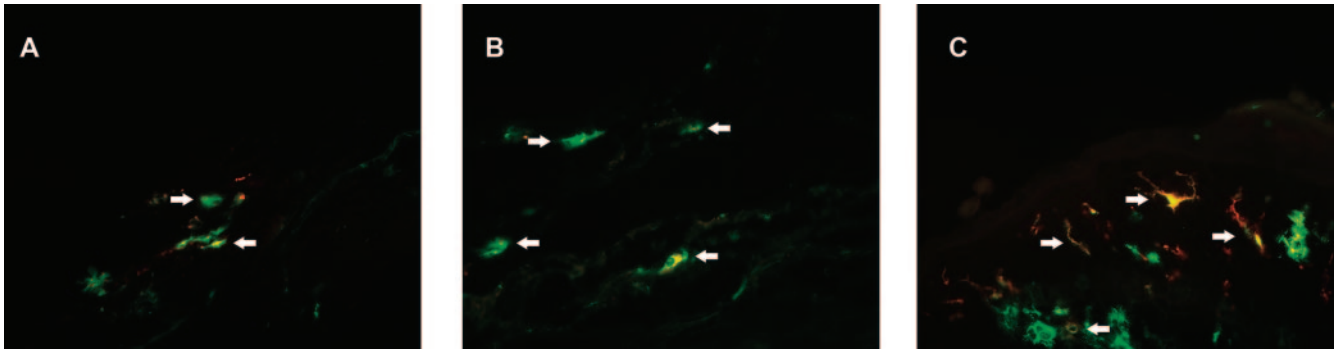

FIGURE 6. CD1a staining of human corneas. (**A**) Fresh double-stained cornea with markers for CD1a (*red*) and HLA-DR (*green*). LCs are located in the epithelium and anterior stroma (*arrows*), primarily in the periphery, and show expression of both markers. (**B**) Cultured cornea (culture period, 21 days) stained with the same markers (*arrows*). (**C**) Human skin as positive control. In skin, LCs are located in the epidermis (*arrows*). Magnification, ×400 (**A–C**).

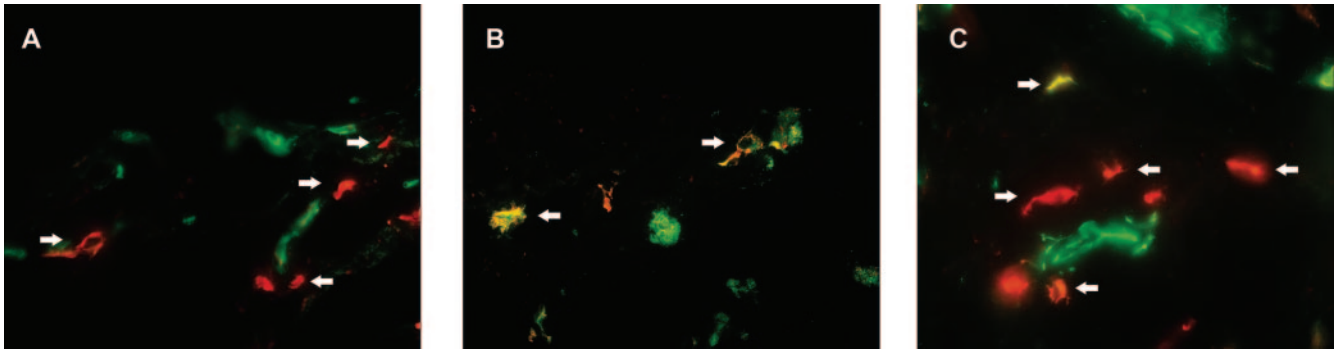

FIGURE 7. DC-SIGN–stained human corneas. (**A**) Fresh double-stained cornea immunolabeled for DCs. DC-SIGN (*red*) and HLA-DR (*green*). DCs are located in the stroma (*arrows*). Most of them were seen in the periphery; few were near the center (**B**). Double staining with HLA-DR shows that only some DCs express both markers. (**B**) Cultured cornea (285 days) stained with the same markers (*arrows*). (**C**) Human skin as positive control. Here DCs are only located in the dermis (*arrows*). Magnifications, ×630 (**A**, **C**), ×400 (**B**).

CD45 and HLA-DR but that only some were positive for CD11c. DC-LAMP$^+$ mature DCs were only partially DC-SIGN$^+$, implying that the peripheral stroma harbors two sets of rare mature DCs, those that coexpress DC-SIGN (mostly CD11c$^-$) and those that do not coexpress DC-SIGN (CD11c$^+$). Given that multicolor immunofluorescence analyses could not be performed, these cells were not characterized further. In addition, costaining with CD86 showed that approximately two thirds of the stromal DC-LAMP$^+$ cells were also positive for CD86. In accordance with previous findings,[22–24] the expression level of CD80 was extremely low in human DCs; therefore, no comparisons could be made (data not shown).

DC-LAMP was detectable in cultured corneas for more than 60 days (Fig. 8B). Although the total number of DC-LAMP$^+$ cells decreased during long-term culture, some in situ maturation of DCs in culture still occurred, as reflected by the peak on day 56 (Fig. 5B).

Macrophages

We found cells expressing a phenotype compatible with macrophages (CD11c$^-$/CD11b$^+$) only in the posterior part of the corneal stroma. They were located in the peripheral, paracentral, and even in the central regions (Figs. 3, 4). No CD11c$^-$/CD11b$^+$ cells were found in the epithelium of the cornea. All macrophages were positive for CD45. They did not express Langerin, CD1a, DC-SIGN, or DC-LAMP, as deduced from the observation that none of these molecules was ever found in that location of the stroma.

Comparison of corneas from young and elder donors revealed no difference in the distribution of LCs and DCs. We also noted that the decontamination step with an iodine-containing solution did not change the distribution or density of the APCs of the human cornea (data not shown).

Discussion

Corneal grafts were initially thought to be almost devoid of donor-derived APCs.[5,25] The presence of corneal APCs was described more than 15 years ago.[10] Their exact characterization and localization, however, are still being discussed.[26,27] For instance, LCs were often defined on the mere basis of their epidermal/epithelial location. Therefore, we reinvestigated this issue with novel, dendritic cell subset-specific antibodies that were already extensively used in human skin.[17,28] It was possible, then, to identify and mutually discriminate between LCs by using Langerin or CD1a and interstitial DCs by using DC-SIGN. Given that Langerin and DC-SIGN are c-type lectins that may endow these cells with differential capacities to take up and respond to pathogens, this characterization of dendritic cell subsets is of biological relevance.[29] We were also able to distinguish between immature and mature DCs for the first time by using DC-LAMP.[18] Additionally, we compared these markers with the well-established markers for leukocytes in general (CD45) and for dendritic cells in particular (CD11c).

Langerhans Cells

Interestingly, we found corneal Langerin/MHC class II$^+$ LCs, especially in the limbal and peripheral epithelium and in the paracentral region of fresh corneas. Similarly, LCs coexpressing

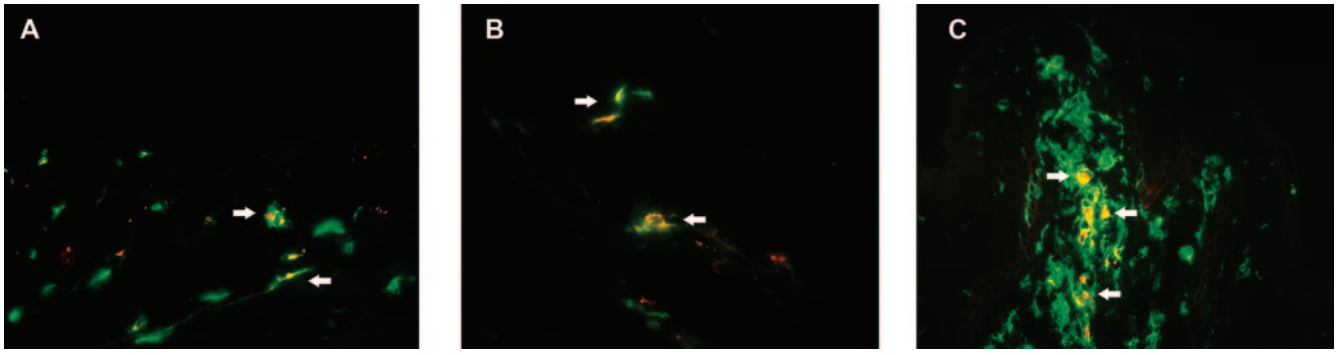

FIGURE 8. DC-LAMP–stained human corneas. (**A**) Fresh double-stained cornea with markers for DC-LAMP (*red)* and HLA-DR (*green*). Mature DCs are located in the peripheral stroma (*arrows*) and show expression of both markers. (**B**) Cultured cornea (7 days) stained with the same markers (*arrows*). In human skin (**C**), mature DCs are located primarily in the dermis (*arrows*). Magnifications, ×630 (**B**), ×400 (**A**, **C**).

CD1a and MHC class II in fresh corneas were also located in the peripheral and paracentral regions, as were Langerin$^+$ cells. Langerin$^+$ and CD1a$^+$ cells were not detectable in the centers of the corneas. Double-labeling experiments revealed that most, if not all, LCs coexpressed Langerin and CD1a, similar to the epidermis of skin.[17,28] In the skin, Langerin$^+$/MHC class II$^+$ and CD1a$^+$/MHC class II$^+$ cells (LCs) were found only in the epidermis,[17,28] which is comparable to our observation that these cells are located almost exclusively in the corneal epithelium. As expected, all Langerin and CD1a$^+$ cells coexpressed CD45, MHC class II, and CD11c.

Corneal LCs have been defined by their epithelial localization.[1,3,30] CD1a-expressing cells were not detected in earlier studies using different antibodies and staining techniques.[31–34] Expression of CD1a was mainly found in diseased corneas.[33] Our observations are in accordance with recent findings of Yamagami et al.,[35] who also found MHC class II$^+$ epithelial DCs mainly in the peripheral human cornea. Our observations that largely immature (DC-LAMP/CD86$^-$) MHC class II$^+$ dendritically shaped leukocytes in the peripheral cornea express Langerin and CD1a strongly support the notion that they are LCs. It remains to be determined to what extent Birbeck granules are present at the ultrastructural level.

Interstitial Dendritic Cells

This is the first description of interstitial DCs, distinct from LCs, in the cornea. They were characterized by their DC-SIGN expression and were located primarily in the peripheral and paracentral regions and even toward the central anterior stroma. We also found that few DC-SIGN$^+$ cells coexpressed HLA-DR, in contrast to DC-SIGN$^+$ cells of the human skin.[17] This may be a sign of reduced immune competence. Alternatively, these cells may be progenitor cells that do not yet express MHC class II, as shown for mouse Langerhans cells.[36] In the skin, DC-SIGN$^+$/MHC class II$^+$ cells were exclusively located in the dermis.[17] Geijtenbeek et al.[15] found human cutaneous DC-SIGN$^+$ DCs that were not mature or terminally differentiated, and DC-SIGN expression did not increase during maturation. This raises the possibility that DC-SIGN may be needed primarily by developing DCs not yet expressing optimal levels of MHC peptide and accessory molecules.[16] Alternatively, DC-SIGN$^+$ but MHC class II$^-$ cells may be macrophages, as described recently for macrophages of human lymph nodes.[37]

Other Antigen-Presenting Cells in the Cornea

The different combinations of antibodies in double-labeling experiments revealed two additional populations (or differentiation stages) of corneal DCs. In the epithelium, we found CD45$^+$ cells that were Langerin$^-$ or CD1a$^-$. They were located primarily in the center of the corneal epithelium, where little or no MHC class II or DC-LAMP expression was detected.

Location in the epithelium meant that they also lacked DC-SIGN. Therefore, we conclude that this additional population has the phenotype CD45$^+$/CD11c$^+$/MHC-II$^-$/DC-LAMP$^-$/Langerin$^-$/CD1a$^-$/DC-SIGN$^-$ (Fig. 4, yellow cells). One could speculate that these cells might be recently arrived LC progenitors that still lack MHC class II and Langerin expression.[36]

Another additional population (Fig. 4, blue cells) became evident mainly in the stroma. Numbers of MHC class II$^+$ and CD11c$^+$ cells exceeded markedly the numbers of DC-LAMP$^+$ mature DCs. Furthermore, in double-labeling experiments, these cells also lacked expression of DC-SIGN. From this one may infer a population with the phenotype CD45$^+$/CD11c$^+$/MHC class II$^+$/Langerin$^-$/CD1a$^-$/DC-SIGN$^-$. From the observation that in the peripheral parts of the stroma DC-LAMP$^+$ but DC-SIGN$^-$ cells occurred, one may conclude that some of these DCs were mature in this anatomic region of the cornea. We did not further characterize this population.

Finally, we found a CD11c$^-$/CD11b$^+$ population (presumably macrophage lineage) only in the posterior part of the stroma. This was in accordance with data from the mouse cornea.[2] They were located in the peripheral, paracentral, and even the central regions (Figs. 3, 4). No such cells were found in the corneal epithelium. All CD11c$^-$/CD11b$^+$ cells coexpressed CD45.

Maturation State of Dendritic Cells

DC-LAMP/CD208 is considered a standard maturation marker for human DCs, including skin DCs.[17,38] It is a stringent and discriminative marker because immature DCs do not express it at all, whereas they often express low levels of costimulatory molecules such as CD80 and CD86.[17,38] Moreover, DC-LAMP expression is better suited to determine the state of maturation by immunofluorescence on tissue sections than by assessing the different levels in MHC class II expression levels between immature (low levels; mainly intracellular) and mature (high levels; mainly cell surface bound) DCs. For the first time, we could unequivocally discriminate between mature and immature LCs/DCs.

Overall, we found few mature DCs (DC-LAMP$^+$/HLA-DR$^+$) in the fresh peripheral cornea, correlating with observations in the human skin.[17] This applied for the corneal epithelium (i.e., for LCs) and for the stroma (i.e., for interstitial/dermal type DCs). This observation is also consistent with the suggestion of Hamrah et al.[3] that the proinflammatory milieu induced by, for example, corneal allotransplantation is needed for the maturation of resident immature corneal LCs.[3]

The virtual absence of DC-LAMP expression in the central parts of the cornea supports and extends the conclusions of other reports[3] that DCs (CD11c$^+$ cells in the center of the cornea) are immunologically immature. In contrast to the murine cornea,[3] however, not all lack MHC class II expression, though they are devoid of maturation markers such as DC-LAMP and the costimulatory molecules CD80 and CD86.

Dendritic Cells in Cultured Human Corneas

Few observations refer to the density of LCs/DCs using long-term cultured human corneas. Most authors have used culture periods up to 14 days and monitored only HLA-DR expression of LCs/DCs. Our observations emphasize that DCs and LCs of the cornea persist longer in culture than had been assumed.[10,39–41] Even in the corneas cultured for the longest periods (1076 days), nearly all epithelial cells were viable. We found that the densities of LCs and DC-SIGN$^+$ DCs decreased with time in culture. Up to 56 days, it was always possible to verify LCs. Some of them were sporadically found, even after 3 months, which impressively demonstrates the longevity of LCs and confirms and extends observations from other human[42] and murine[43] experimental skin models. DC-SIGN$^+$ cells persist in the cornea even longer than LCs. They were detectable in corneas cultured up to 3 months and some sporadically up to 1 year. This observation is important for the allogenicity of a graft, even after long-term culture. Few DC-SIGN$^+$ cells of the long-term cultured corneas were HLA-DR$^+$. Mature DCs and LCs, identified by DC-LAMP, also decreased in cultured corneas, comparable with data from the skin. However, some were found after more than 60 days. It was interesting that although the total number of DC-LAMP$^+$ cells decreased during culture, some maturation of DCs still occurred for both maturation markers (HLA-DR and DC-LAMP) that peaked at day 56 (Fig. 5B). These results are in accordance with the observations

from Hamrah et al.,[2] who detected mature murine DCs using costimulatory markers (CD80, CD86). These mature cells might be functional during an immune reaction.

This is the first documentation of a long-term (more than 200 days) kinetic study of DCs in cornea culture. Given that a significant decrease of APCs was observed within the first 3 weeks of culture, one could assume that these corneas would be less immunogenic. This is in agreement with clinical observations by which increased graft survival was reported, when corneas were cultured for more than 7 days.[10,39–41] Because some APCs remained for a long time, the graft may still bear a potential risk for rejection. Most DCs stay in an immature state and may be capable of antigen uptake and processing. Eventually, in the inflammatory graft situation, they may mature and induce rejection.

In summary, our data suggest that culturing corneas for a certain time span rather than using fresh corneas and manipulating LCs and DCs for maturation and survival may increase the success rate of corneal transplantation.

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