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# The antigen presenting cell in the human cornea – functional and morphological evaluation

**Dissertation**

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**Für meine Eltern**

**Contents:**

## 1. Introduction

The cornea is the transparent, with tear fluid moistened, frontal part of the eye that covers the iris, pupil and anterior chamber, providing most of an eye's optical refraction. The corneal tissue consists of five basic layers: the epithelium, Bowman's lamella (anterior limiting membrane), the stroma, Descemet's membrane (posterior limiting membrane) and the endothelium.

Today, corneal transplantation (keratoplasty) is the most common form of tissue allotransplantation. The corneal graft rejection rate is low, therefore systemic immunosuppression is not routinely used and human leukocyte antigen (HLA) matching is rarely performed, except for high-risk cases. Graft failure occures mostly due to immune rejection. Thus, corneal transplants enjoys a considerable degree of immune privilege and immune rejection remains the leading barrier of long-term corneal graft survival. The cornea is a site devoid of a vascular network but the immune privilege of corneal allografts is not only based on the presence of an avascular graft bed. Furthermore, low rates of donor-specific Langerhans cells (LCs), Fas ligand expression, expression of complement regulatory proteins and immunmodulatory effects of the humoral components of the aqueous humour and the immune modulation (Anterior chamber associated immune deviation = ACAID) are involved in this complex reaction. For general purposes, high-risk corneal transplantation refers to inhospitable qualities of the recipient, such as inflammatory neovascularization or ocular surface inflammation which leads to a high risk of rejection for a graft. Immunology has long been focused on antigens and lymphocytes, but the mere presence of these two parties does not always lead to immunity. A third party, the antigenpresenting cell system (APCs) is the initiator and modulator of the immune response (1).

APCs capture and process antigens for presentation to T-lymphocytes, and produce signals required for the proliferation and differentiation of lymphocytes. In general, APCs can be classified into professional (B cells, macrophages, dendritic cells and Langerhans cells) that are able to activate and induce clonal expansion of both naive and memory T-cells and nonprofessional types (mesenchymal cells, endothelial cells) that are competent to stimulate memory T-cells (1). Professional APCs are so

called because they generally have a high constitutive expression of major histocompatibility complex (MHC) class II antigen, as well as costimulatory molecules. APCs include a heterogeneous family of cells that are able to uptake and process both exogenous and endogenous antigens into 10-20-amino-acid peptides, and load them on to MHC molecules. They traffic into the lymph node where they present the foreign antigens in combination with MHC Class II molecules to the T helper cell T cell receptors. The most capable APCs in most tissues, mainly in skin, are dendritic cells (DCs) and LCs. In fact, these cells are also known to serve as the professional APCs of the cornea and ocular surface (2-6).

In immune-privileged sites, like the cornea, the process of immune activation is regulated by interfering with the maturation of the DCs and by altering the indigenous APCs toward the induction of tolerance. The primary function of dendritic cells is to capture and present protein antigens to naive T-lymphocytes located in the cortex of the lymph nodes.

One milestone was the detection of conspicious dendritically shaped cells in the human epidermis by Paul Langerhans 1868 and these cells were named after him. He found them by using gold cloride impregnation technique, a method for nerve cell identification. This is why they were believed to be of nervous origin at that time (7). However, another decade past until LCs were definitively recognized as a member of the DC system. However, it took another 12 years until LCs were definitively recognized as a member of the DC system. Gerold Schuler and Ralph Steinman mainly contributed to this milestone by several observations in 1985. Schuler and Steinman recognized that Langerhans cells are a special form of DCs, and that they occur in distinct states of maturation (i.e. the ‘Langerhans cell paradigm’) that are characterized by reciprocally expressed antigen processing and T-cell stimulatory capacities (8, 9). The 1985 breakthrough was important but not the end of LC research.

By the time electronmicroscopical analysis were possible tennis racket-like Birbeck granules were found in LCs and so these cells were associated with the immune system. There is strong evidence that Birbeck granules, in particular their main molecular constituent, i.e. the Langerin molecule, a mannose-specific C-type lectin, play a role in the endocytosis pathway (10). The Birbeck granules became a marker for Langerhans cells, especially for those in situ and in an immature state (7). LCs stem from bone-marrow cells of the myeloid lineage. Their precursors populate the

epidermis during fetal and early postnatal life where they gain their immunological function. They start expressing molecules like MHC class II and Langerin/CD207 (7). LCs are specialized to stimulate resting T cells and so they induce primary T cell immune responses. Thus, LCs play an important role in contact hypersensitivity, transplant rejection and other immunological processes.

Corneal LCs, similar to skin LCs, are bone-marrow-derived cells that represent the professional APCs of the ocular surface. Constitutive expression of major histocompatibility complex (MHC) class II antigens is a characteristic feature of DCs (including LCs) in the corneal epithelium. MHC antigens are important components in both the generation and expression of immune response. They are cell surface proteins classified as class I (also termed human leucocytic antigen [HLA] A, B and C), found on all nucleated cells and class II (termed HLA, DP, DQ and DR), found on all APCs. MHC molecules are the sine qua non of T cell induced immunity. Clinically, there is a strong association between HLA and certain systemic and ocular diseases. Hamrah et al. demonstrated the phenotype and distribution of MHC class II negative LCs in the murine corneal epithelium. They showed that LCs became activated in inflamed cornea by upregulation of MHC Class II antigen and costimulatory molecules like CD80 and CD86 (4). Furthermore they found that there are at least three bone marrow-derived subsets in the normal corneal stroma in addition to the corneal LCs (3). The markers on APCs vary in their expression of surface markers (11). During the last years new surface markers were established to characterize APCs especially in the human skin in more detail: Langerin (CD207), DC-SIGN (CD209) or DC-LAMP (CD208).

After activation, DCs and LCs must still undergo further changes for migrating to lymph nodes. This involves down-regulation of E-cadherin (12) and an upregulation of adhesion molecules, such as alpha-6-beta-1-Integrin (13) and CD44 (14) and the induction of matrix metalloproteinase-9 (15). Another important role to achieve migration capabilities is the de novo expression of CCR7 (chemotactic cytokines Receptor 7) (16). Both CCR7 ligands, CCL19 (also known as macrophage inflammatory protein 3-, MIP-3), and CCL21 (also known as secondary lymphoid tissue chemokine, SLC), are expressed in the T-cell zones of secondary lymphoid organs. Therefore, CCR7-mediated DCs migration, guided by CCL19 and CCL21, results in accumulation of mature DCs in the afferent lymphatics and the T-cell areas of draining lymph nodes. Studies in CCR7-deficient mice have revealed a marked

defect in DC migration to lymph nodes and impaired primary immune (17, 18).
DCs and LCs are the most important cells concerning immunogenicity of the cornea and are important for the faith of a corneal graft. Our aim was to characterize human corneal dendritic cells including Langerhans cells in freshly isolated corneas as well as in long-term cultured ones by using immunofluorescent staining methods with new cell surface markers, which are already approved in human skin (publication 1). Cryosections and epithelial sheets were obtained and novel cell surface markers like Langerin (CD207), CD1a and DC-SIGN (CD209) were applied to determine the cell type and distinguish between epithelial Langerhans cells and interstitial type dendritic cells. DC-LAMP (CD208) served as marker for the maturation state of DCs. We were interested how long DCs and LCs were detectable in corneas cultured for different time periods in comparison to fresh corneas. We further wanted to know, if these cells migrate during culture into the culture medium and if so, how fast this emigration of APCs does occur. The rejection rate of corneal transplants is very small, and less APCs remain in the donor cornea. We therefore investigated in a second work (publication 2) whether the migration of DCs, LCs and macrophages from cultured human corneas into organ culture medium can be accelerate by the addition of chemotactically active substances.

I contribute to these two manuscripts as a first author and was actively involved in all processes concerning literature review, establishing material and methods, performing observations, statistical analyses and writing.

## 2. Publications

1. Invest Ophthalmol Vis Sci. 2007 Oct;48(10):4459-67.

Characterization of antigen-presenting cells in fresh and cultured human corneas using novel dendritic cell markers.

*Mayer WJ, Irschick UM, Moser P, Wurm M, Huemer HP, Romani N, Irschick EU.*

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2. Curr Eye Res. 2010 Feb;35(2):176-83.

Active in vitro reduction of antigen presenting cells in human corneal grafts using different chemokines.

*Irschick UM*, Mayer WJ*, Kranebitter N, Romani N, Huemer HP, Irschick EU.*
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## 3. Summary

*German:*

*Einleitung:*

Antigenpräsentierende Zellen (APCs) spielen eine große Rolle für das Schicksal eines Hornhauttransplantates. In dieser Arbeit wurde die genaue Verteilung und Menge der APCs in der normalen frischen menschlichen Hornhaut untersucht. Dazu wurden neue Marker verwendet und mit bisher in der Hornhaut etablierten Markern verglichen. Die Abstoßungsrate von Hornhauttransplantaten ist umso geringer, je weniger APCs in der Spenderhornhaut verbleiben. Hier untersuchten wir auch, ob man die Migration von dendritischen Zellen (DCs), Langerhanszellen (LCs) und Makrophagen aus kultivierten Hornhäuten in ein Kulturmedium durch die Zugabe von chemotaktisch wirksamen Stoffen beschleunigen kann.

*Material/Methoden:*

Es wurden Gefrierschnitte sowie sogenannte „Epithelial Sheets“ angefertigt. Diese Präparate wurden anschließend mit einer Immunfluoreszenzdoppelfärbung in Dreistufentechnik gefärbt. Neue Marker (Langerin, CD1a, DC-SIGN und DC-LAMP)

wurden mit etablierten Markern verglichen. Humane Hautpräparate dienten als Positivkontrollen. Bei den Auswanderungsversuchen wurden die ins Kulturmedium migrierten Zellen nach 3, 7, 10, 14, 21, 28 und 35 Tagen gesammelt, und ein Zytospinpräparat angefertigt. Die Hornhäute wurden immer mit einer chemotaktischen Substanz inkubiert (CCL19, CCL21 oder C5a), Negativkontrollen nur mit Kulturmedium. Alle Präparate wurden mit einer Immunfluoreszenzfärbung in Dreistufentechnik gefärbt, entweder mit Langerin – HLA-DR (LCs), DC-SIGN – HLA-DR (DCs), oder mit CD14 – HLA-DR (Monozyten/Makrophagen). Diese Präparate wurden anschließend ausgezählt und die Menge der migrierten Zellen miteinander verglichen.

*Ergebnisse:*

LCs (Langerin+CD1a+CD11c+HLA-DR+CD45+) konnten in der frischen menschlichen Hornhaut im Epithel und subepithelial gefunden werden, vor allem in der Peripherie und im Limbusbereich. Auch parazentral konnten wir noch einige dieser Zellen finden, wohingegen im Zentrum keine Langerin+CD1a+ LCs mehr nachweisbar waren. Nur wenige dieser Zellen in der Peripherie waren DC-LAMP+, was sie als reife Zellen auszeichnet. Im Stroma fanden wir DC-SIGN+ DCs (meist HLA-DR- und CD11c-, alle CD45+).

Die meisten waren im anterioren Stroma der Peripherie und im Limbusbereich lokalisiert, aber auch parazentral und zentral konnten einige DC-SIGN+ DCs beobachtet werden. Wenige DCs in der Peripherie waren auch DC-LAMP+(reife DCs). Eine zweite Population DCs (DC-SIGN-CD11c+HLA-DR+) konnte im gesamten Stroma gefunden werden. Im posterioren Stroma konnten CD11c-CD11b+ Makrophagen und CD14+ Zellen lokalisiert werden.

In den Auswanderungsversuchen konnte gezeigt werden, dass sowohl LCs, als auch DCs und Makrophagen in Organkultur aus der Hornhaut in das Kulturmedium migrieren. Es konnte auch gezeigt werden, dass in der normalen frischen menschlichen Hornhaut CCR7+ Zellen lokalisiert sind. Hier konnte durch die Zugabe des CCR7-Liganden CCL19 eine vermehrte LCs Auswanderung in das Medium beobachtet werden. Auch DC-SIGN+ DCs konnten nach Zugabe von CCL19 oder CCL21 vermehrt im Medium gefunden werden, wohingegen eine Wirkung dieser Chemokine auf CD14+ Zellen nicht nachgewiesen werden konnte.

Danach konnte kein Unterschied mehr zu den Negativkontrollen festgestellt werden.

*Conclusio:*

In diesen Arbeiten konnte gezeigt werden, dass in der frischen menschlichen Hornhaut eine Vielzahl von APCs lokalisiert sind, welche einen Risikofaktor für eine Transplantatabstoßung darstellen. Es konnte außerdem gezeigt werden, dass es während der Organkultur zu einer Migration von APCs in das Kulturmedium kommt und diese Migration durch eine Vielzahl von Chemokinen geregelt wird.

*English:*

*Background:*

Antigen presenting cells (APCs) are responsible for the faith of corneal grafts. In this study we investigated the density and distribution of Langerhans cells (LCs), dendritic cells (DCs) and macrophages using novel markers. Furthermore we investigated the migration of APCs to the culture-medium during organ culture.

*Methods:*

Epithelial flat mounts and whole stromal cryosections were stained with immunofluorescence double staining triple layer technique. The following antibodies were used: Langerin/CD207, CD1a, DC-SIGN/CD209, CD11c, CD11b, CD45, DC-LAMP/CD208 and HLA-DR. Human skin was used for positive control. In the migration assay the cells were collected every 3, 7, 10, 14, 21, 28 and 35 days and a cytospin-slide was prepared. Corneas were incubated with an chemotactic substance (CCL19, CCL21 or C5a), negativ controls only with culture medium.

*Results:*

LCs (Langerin+CD1a+CD11c+HLA-DR+CD45+) were detected in the epithelium and subepithelial, mainly in the periphery and limbal part. Towards the corneal center a significant decrease of these cells was observed. Few LCs co-expressed DC-LAMP in the peripheral epithelium thus indicating mature LCs. In the stroma we found three populations of cells: DC-SIGN-positve DCs (most of them HLA-DR and CD11c-negative, all CD45+), most of them in the periphery, a few also paracentral in the central corneal part. Few of them in the peripheral stroma co-expressed DC-LAMP. The second population were DC-SIGN-negative, CD11c+CD45+HLA-DR+ DCs. We found a substantial number of these cells in the whole stroma. Furthermore macrophages (CD11c-CD11b+ / CD14+) were detectable. They were mainly located in the posterior peripheral stroma. We could also observe cells expressing the CCR7-antigen in the normal fresh human cornea. Furthermore, an increased migration rate

of LCs and DCs could be investigated by the addition of its ligands CCL19 and CCL21 to the culture-medium. No effect of these chemokines to CD14+ macrophages could be shown.

*Conclusion:*

These studies demonstrates the exact density and distribution of several APCs in the human cornea, in comparison to the human skin, using novel antibodies. Furthermore migration capabilities of APCs into the culture medium during organ culture incubation could be demonstrated and is modulated by a multitude of chemokines.

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

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**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]

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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).

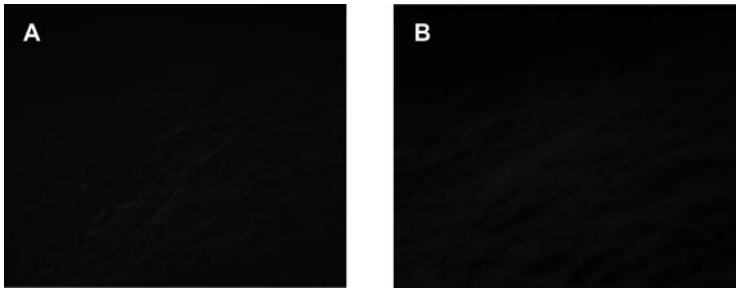


**FIGURE 1.** (**A**) Negative control for immunoglobulin class IgG1. (**B**) Negative control for immunoglobulin class IgG2b. Note that IgG2a is included in the double-staining procedure. (**A**, **B**) Magnification, ×200.

### 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

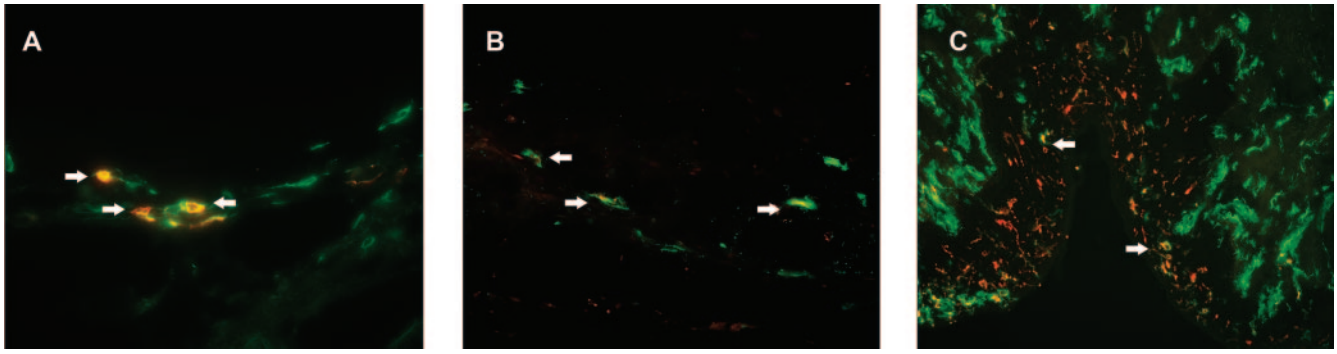


**FIGURE 2.** Langerin$^+$ cells in human corneas. (**A**) Fresh double-stained cornea with markers for Langerin (*red*) and HLA-DR (*green*). LCs are located in the epithelium and anterior stroma (*arrows*) and show expression of both markers. (**B**) Cultured cornea (56 days) stained with the same 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.

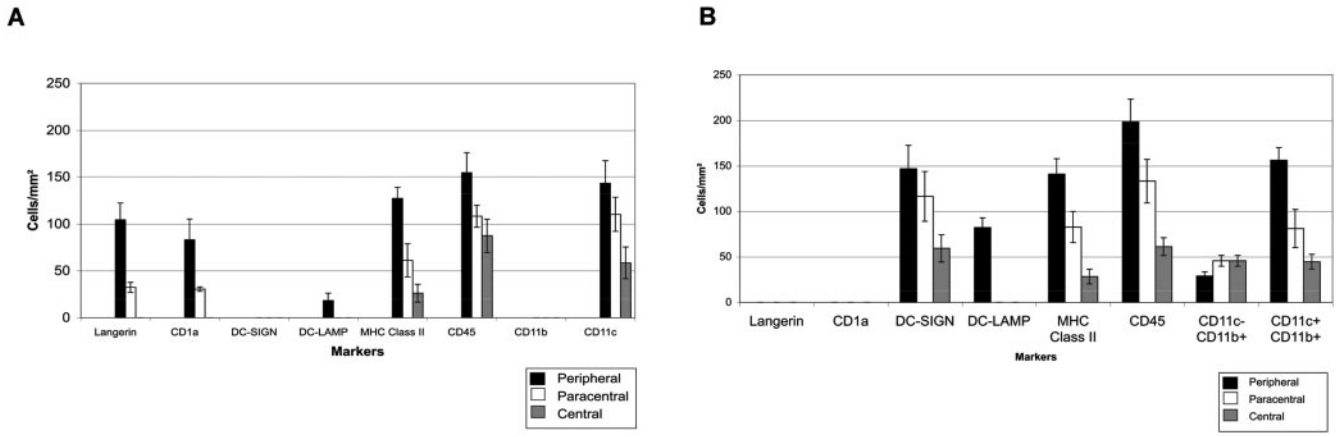


**FIGURE 3.** Densities of APCs (cells/mm$^2$) in fresh human corneas ($n$ = 9). APC densities in the peripheral, paracentral, and central areas of (**A**) epithelium and (**B**) stroma.

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.

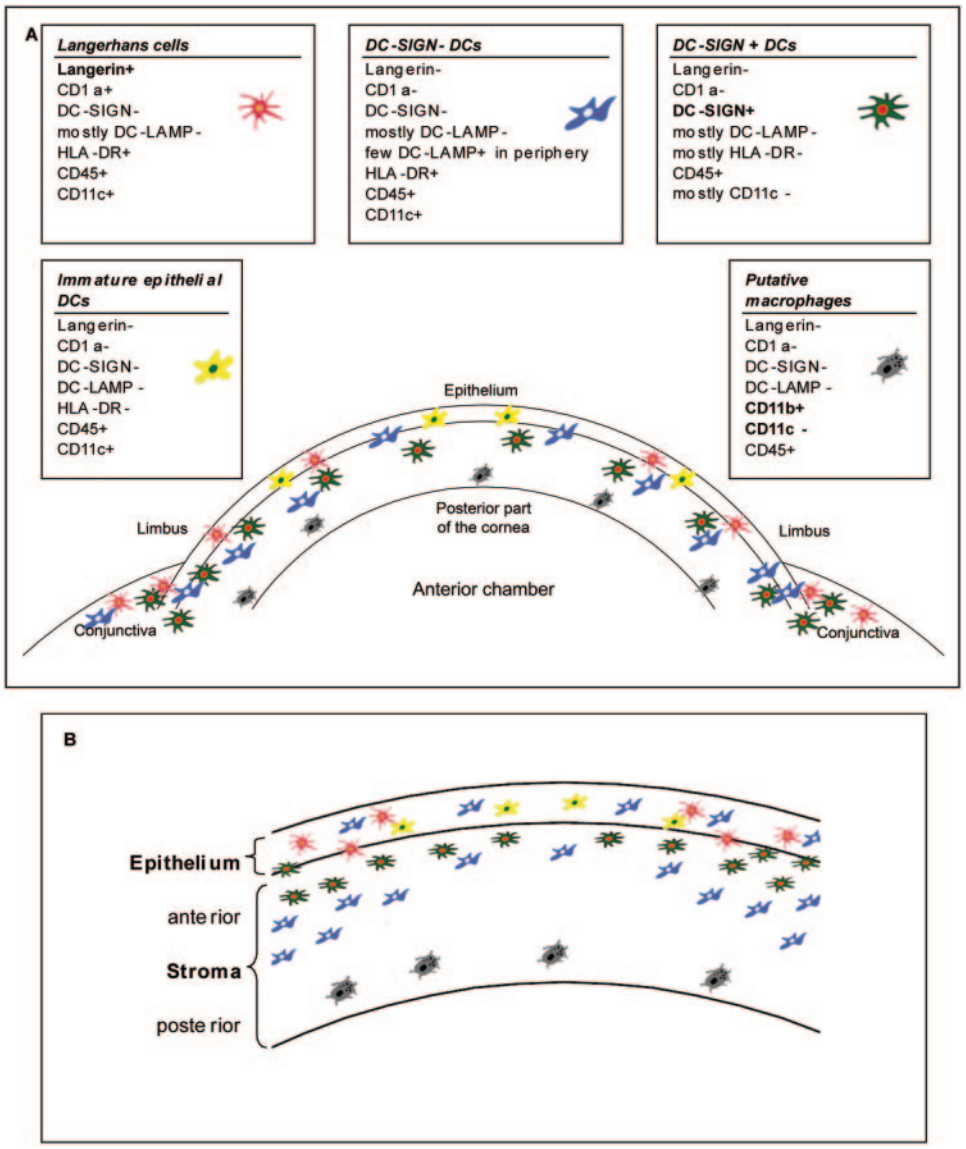


**FIGURE 4.** APCs within the whole fresh cornea. (**A**) Distribution of APCs within the central, paracentral, and peripheral regions of the human cornea. (**B**) Distribution of APCs within the corneal layers. All APCs were positive for CD45. Langerin, CD1a, and HLA-DR$^+$ (LCs) are located in the 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.

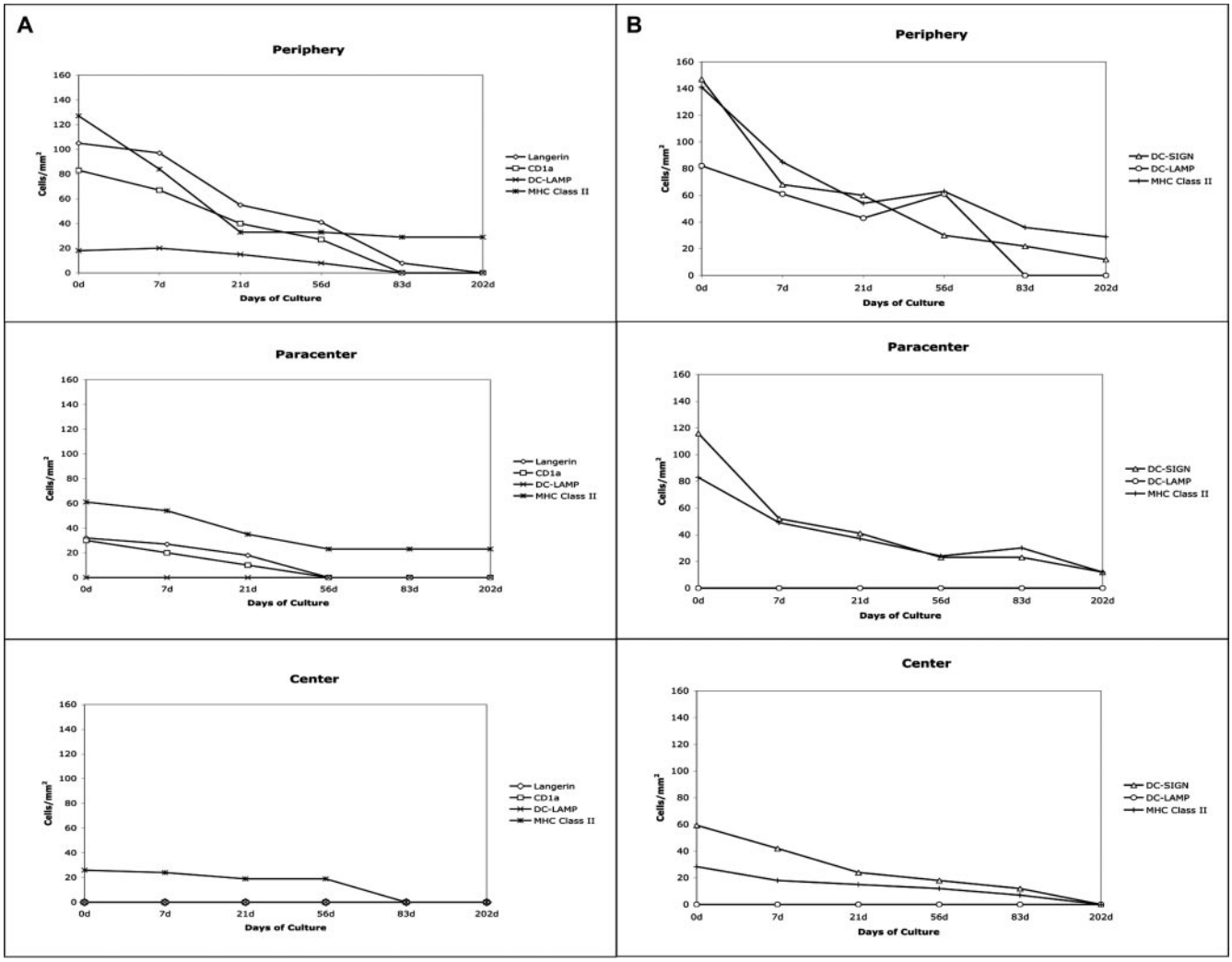


**FIGURE 5.** Kinetics of LC/DC reduction (cells/mm$^2$) during long-term culture. LC/DC densities in the peripheral, paracentral, and central area of the human corneal epithelium (**A**) and stroma (**B**).

## 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

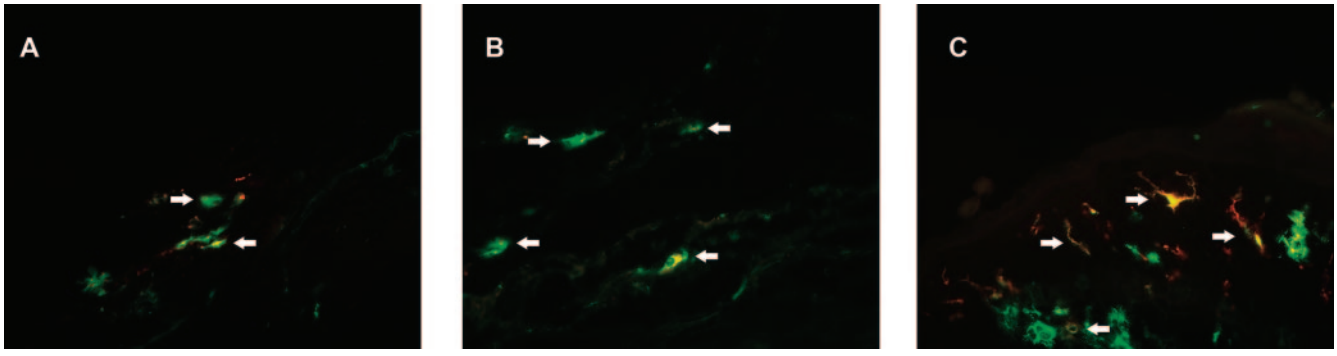


**FIGURE 6.** CD1a staining of human corneas. (**A**) Fresh double-stained cornea with markers for CD1a (*red*) and HLA-DR (*green*). LCs are located in the epithelium and anterior stroma (*arrows*), primarily in the periphery, and show expression of both markers. (**B**) Cultured cornea (culture period, 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**).

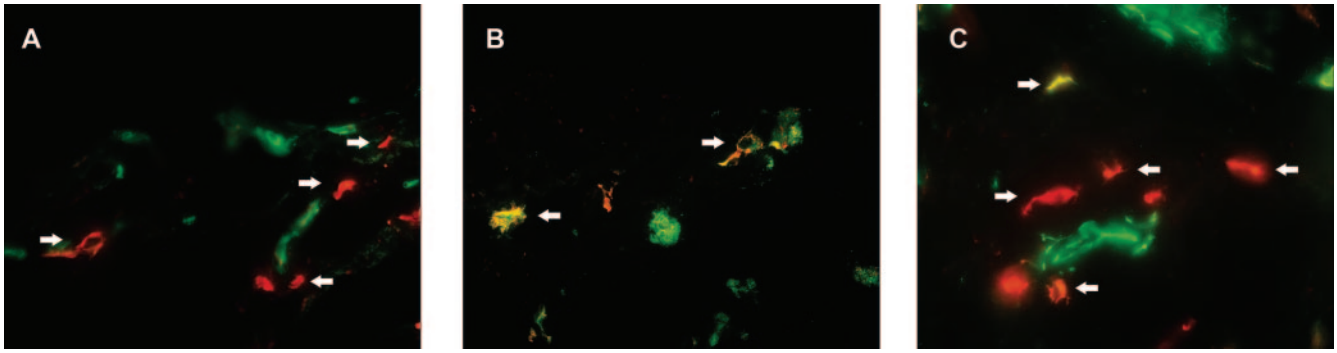


**FIGURE 7.** DC-SIGN–stained human corneas. (**A**) Fresh double-stained cornea immunolabeled for DCs. DC-SIGN (*red*) and HLA-DR (*green*). DCs are located in the stroma (*arrows*). Most of them were seen in the periphery; few were near the center (**B**). Double staining with HLA-DR shows that only 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

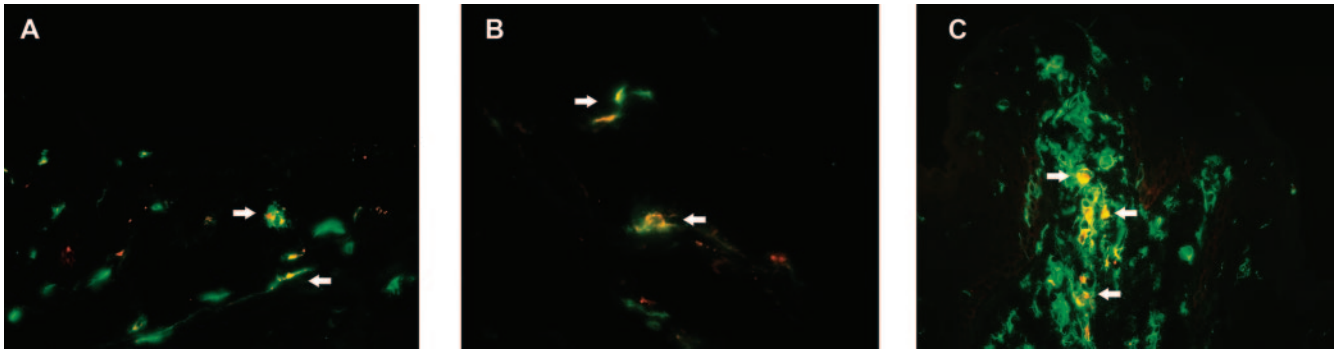


**FIGURE 8.** DC-LAMP–stained human corneas. (**A**) Fresh double-stained cornea with markers for DC-LAMP (*red)* and HLA-DR (*green*). Mature DCs are located in the peripheral stroma (*arrows*) and show expression of both markers. (**B**) Cultured cornea (7 days) stained with the same markers (*arrows*). 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.

## *Acknowledgments*

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ORIGINAL ARTICLE

# Active In Vitro Reduction of Antigen Presenting Cells in Human Corneal Grafts Using Different Chemokines

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## ABSTRACT

*Background:* For the fate of a graft the antigen presenting cells play an important role. Chemokines can lead to enhanced migration of these cells. We therefore investigated if fresh human corneas bear the chemokine receptor 7 (CCR7) and if its ligands can force the emigration of dendritic cells in an *in vitro* model.

*Methods:* We used human corneas excluded for transplantation and performed migration tests using chemokine ligands 19 (CCL19) and 21 (CCL21) or the complement factor 5a (C5a). Emigrated cells were collected up to 35 days and stained by immunofluorescent double labeling in triple layer technique with Langerin/CD207, DC-SIGN/CD209, CD14, and HLA-DR. In parallel, fresh and cultured human corneas were stained for CCR7.

*Results:* We found in fresh human corneas, as well as in long-term cultured ones, a low CCR7 expression that nearly diminished after 28 days. *In vitro* Langerhans cell emigration could be enhanced only by CCL19, whereas dendritic cells were strongly influenced by CCL19, CCL21, and C5a. HLA-DR$^+$ cells showed numerically the highest *in vitro* emigration rate. Macrophages/monocytes were not influenced by the used chemokines.

*Conclusions:* Although human corneas reduce their antigen presenting cells numbers during long-term culture, this effect could be significantly enhanced by using chemokines.

**KEYWORDS:** Antigen-presenting cells; C5a; CCL19; CCL21; CCR7; Cell migration; Chemokines; Dendritic cells; Human cornea

## INTRODUCTION

Even though keratoplasty is the most successful transplantation of human tissue, corneal graft failure is still mainly caused by immunological rejection. Therefore, an active reduction of antigen-presenting cells (APC) in human corneal graft would be of benefit.

Professional APC do serve as an important link between non-specific and specific immune defense mechanisms. Immune surveillance and defense are critically mediated by these cells, therefore, APC migration is essential in corneal immunology.[1,2]

These cells can lead to graft rejection either via the indirect (host APC dependent) pathway or via a direct donor APC dependent response (mainly in

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high risk host beds).[3] Huq et al. showed that a donor APC-dependent direct response is induced in corneal transplant hosts when the graft bed is inflamed, which determines the relevance of the graft microenvironment in controlling the pathway of allosensitization.[3]

Human corneal APC were thought to reside exclusively in the peripheral cornea.[4] Recent data of our study group confirmed that the whole human cornea is endowed with a characteristic population of antigen-presenting cells including Langerhans cells (LC) or other dendritic cells (DC), as shown by other authors mainly in the murine model.[5–8] We confirmed these data in human cornea using novel cell markers, such as Langerin/CD207 or DC-SIGN/CD209.[9]

Mature APC are highly capable of stimulating naive T-cells directly in the draining lymph nodes to cause an immune reaction.[10] Several chemokines are involved in APC trafficking to lymphatic tissue. Substances, such as the chemokine receptor 7 (CCR7) and its ligands CCL19 (macrophage inflammatory protein 3-beta, MIP-3-beta) or CCL21 (secondary lymphoid tissue chemokine, SLC), are involved in this process. Both chemokines are expressed in T-cell zones of secondary lymphoid organs.[11–14] By an inflammatory stimulus, DC are able to leave peripheral tissues. At this point, DC begin to mature and the expression of CCR7 increases,[15,16] which allows the DC to enter lymph vessels and to migrate to T cell areas in draining lymph nodes.[17] It was shown that a deficiency of CCR7 or its ligands, CCL19 and CCL21, affects DC migration into draining lymph nodes and abnormal lymph node function in peripheral tissues appears, suggesting that active blocking of CCR7 and CCL21 could potentially offer one such venue for inhibiting the induction of T cell-mediated immunity,[11,18]

However, neither the expression of CCR7 in human corneas nor the influence of this receptor and its ligands in human corneal APC migration has yet been investigated. Thus, the aim of this study was to investigate the presence of CCR7 on human corneal APC in fresh and cultured grafts and to observe the influence of various chemokines specific for CCR7 on APC behavior in an *in vitro* model.

## MATERIAL AND METHODS

### Human Donor Corneas—Preparation and Organ Culture

This study was conducted in accordance with the Declaration of Helsinki. Human bulbi were primarily enucleated for transplantation purposes. For transplantation, excluded corneas were used in our study, which were, for example, seropositive for infectious diseases or had a low endothelial cell count. Enucleated bulbi were dissected under the guidelines for Austrian eye banks. In total, 52 corneas of 26 donors were available, 16 from male and 10 from female donors, ranging in age from 49 to 86 years (median of 69 years).

Preparation was performed under sterile conditions as recently described.[9] In brief, dissected bulbi were washed for 5 min with floating tap water, decontaminated with povidone iodine (Polyvidon-Jod 0.5%; Betaisodona; Mundipharma, Limburg/Lahn, Germany). Bulbi were then trepanned (diameter of 14 mm) and then the limbal and scleral parts were removed using small scissors resulting in a final size of 10 to 11 mm of each cornea. Then the isolated corneas were washed with phosphate buffered saline (PBS).

### In Vitro Emigration Assay

Corneas were placed in pairs into a sterile 12-well-plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA), each cornea in a separate well. One cornea was used for the emigration test, the other one served as a negative control. Each well contained 3 ml of organ culture medium (RPMI 1640 with 2.0 g/L NaHCO3, without phenol-red and without L-glutamine; Biochrome, Berlin, Germany) supplemented with a mixture of 100 U/mL penicillin G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (Gibco/Invitrogen, Grand Island, NY, USA), 2 mM L-glutamine (low endotoxine; Biochrome, Berlin, Germany), 25 mM HEPES-buffer (Biochrome, Berlin, Germany), and 5% fetal calf serum (Gibco/Invitrogen). The incubation for the test series was performed at 37°C. The following chemotactical substances were used: recombinant human CCL19 (at a working concentration of 25 ng/ml), CCL21 (50 ng/ml), both from Peprotech, Rocky Hill, NJ, USA; and recombinant human C5a (5 ng/ml) from R&D Systems, Minneapolis, Minneapolis, USA.

On days 3, 7, 10, 14, 21, 28, and 35 the cornea pairs were placed into the next well under sterile conditions containing fresh culture medium with or without stimulating chemokines.

In addition, emigrated APC were collected. The nonadherent (dendritic) cells were isolated and resuspended in 1 ml PBS (Biochrome), while the adherent cells (macrophages/monocytes) were collected by washing the wells with cold PBS and trypsin/EDTA (0.05/0.02% trypsin/EDTA in HBSS, PAN-Biotech GmbH, Aidenbach, Germany). After a short period of cold incubation, the cells were manually scraped with a plastic cell scraper (Sarstedt, Newton, North Carolina, USA). The suspensions of the nonadherent and the adherent cells were spun down at 250 g (1200 rpm, Hettich, Rotanta, TRC) for 10 min and filtered through a 70 µm cell strainer

(Falcon, Becton Dickinson, Bedford, Massachusetts, USA) to separate the APCs from cell detritus.

Cytospins of these cells were performed (220 g/1100 rpm for 10 min and then 1500 g/3000 rpm for 1 min, Hettich, Rotanta, TRC). Cells were then put onto slides (Superfrost Plus, Menzel Braunschweig, Germany). Cells on slides were fixed for 10 min in pure aceton (Scharlau Chemie, Barcelona, Spain) at room temperature and stored at -20°C until staining.

Cytospin-slides were stained by immunofluorescent double labeling in triple-layer technique with different antibodies listed and described below.

Stained cells were counted with an optical microscope (DMLB, Leica, Leica Systems, Wetzlar, Germany) using a software-controlled scanning grid (PicED Cora; Jomesa Messsysteme GmbH, Munich, Germany). Each slide was completely inspected using magnifications of ×100 and ×400 and the numbers of cells were averaged.

Student's *t*-test was used to compare the number of positively labeled migrated antigen presenting cells of the epithelium and stroma of cultured corneas. $P < 0.05$ was considered as statistically significant.

## CCR7

To investigate if CCR7-positive cells can be found in human corneas, we prepared cryosections from fresh (not cultured) corneas, as well as from cultured corneas (up to 28 days) using a microtome (CR-3050, Leica-Microsystems, Wetzlar, Germany). Deep frozen tissue was embedded in optimum cutting temperature (OCT) compound medium (Tissue-Tek, Sanova, Vienna, Austria). Human skin and human tonsil samples served as positive controls. Sections were cut into 8 µm thin sheets at -26°C, allowed to dry at room temperature for 1 hr, and stored at -20°C until staining.

### Antibodies

The following unconjugated primary mouse antibodies were used at the indicated final concentrations: anti–CD207/Langerin (clone DCGM4, IgG1, 4 µg/mL; Beckman Coulter, Fullerton, CA, USA), anti–CD209/DC-SIGN (clone DCN46, IgG2b, 5 µg/mL; BD Biosciences, San Diego, CA, USA), anti-CD14 (clone M5E2, IgG2a, 5 µg/mL; BD Biosciences), and hCCR-7 (clone 150503, IgG2a, 15 µg/mL; R&D Systems, Minneapolis, MN, USA). As there is only low-level expression of CCR7 on LC and DC in the normal human cornea, we used tyramide amplification to boost the visualization of staining, according to the manufacturer's instructions (PerkinElmer Life Sciences, Boston, Massachusetts).

A FITC-conjugated anti-HLA-DR antibody mouse antibody FITC-anti–HLA-DR (clone L243, IgG2a, 2.5 µg/mL; BD Biosciences, Franklin Lakes, NY, USA) was used in immunofluorescent double-staining experiments.

Used isotype specific negative controls were as follows: mouse IgG1 (clone DAK G01, DAKO, Glostrup, Denmark), mouse IgG2b (clone DAK G09, DAKO), mouse IgG2a (DAK G05, DAKO) and FITC-conjugated mouse IgG2a (clone RPC 5, Hölzel Diagnostika, Cologne, Germany).

### Immunofluorescent Double Labeling in Triple-Layer Technique

Staining procedures were performed as recently described.[9] Briefly, cells on slides were first incubated with primary unconjugated antibodies (all diluted in PBS/1% bovine serum albumin for 30 min at 37°C). After 5 min of washing in PBS/1% BSA, the biotinylated secondary antibody (sheep anti-mouse immunoglobulin, dilution 1:100; Amersham Pharmacia Biotech, Buckinghamshire, UK) was applied for 30 min at 37°C. Afterwards, the slides were incubated with Texas Red-conjugated streptavidin (Amersham Pharmacia Biotech, Buckinghamshire, UK; dilution 1:100, for 30 min at 37°C). Then a mouse gamma-globulin blocking reagent at a final concentration of 100 µg/mL (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) 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 mouse anti-human HLA-DR antibody was incubated for 30 min at 37°C and the tissue sections were covered with fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, California, USA).

# RESULTS

### Expression of CCR7

Important for all experiments was to know if fresh human corneal APC always express CCR7, as CCL19 and CCL21 are ligands to this receptor. We found that CCR7 in the fresh (non-cultured) human cornea was only expressed to a slight amount. We, therefore, used the tyramid signal amplification system (TSA-System, PerkinElmer Inc., Newton, New Jersey, USA) to boost the immunofluorescent signal. A human palatine tonsil and human skin were used as positive controls. In the human skin, $CCR7^+$ cells were seen in the basal cell layer of the epidermis and the dermis (Figure 1B).

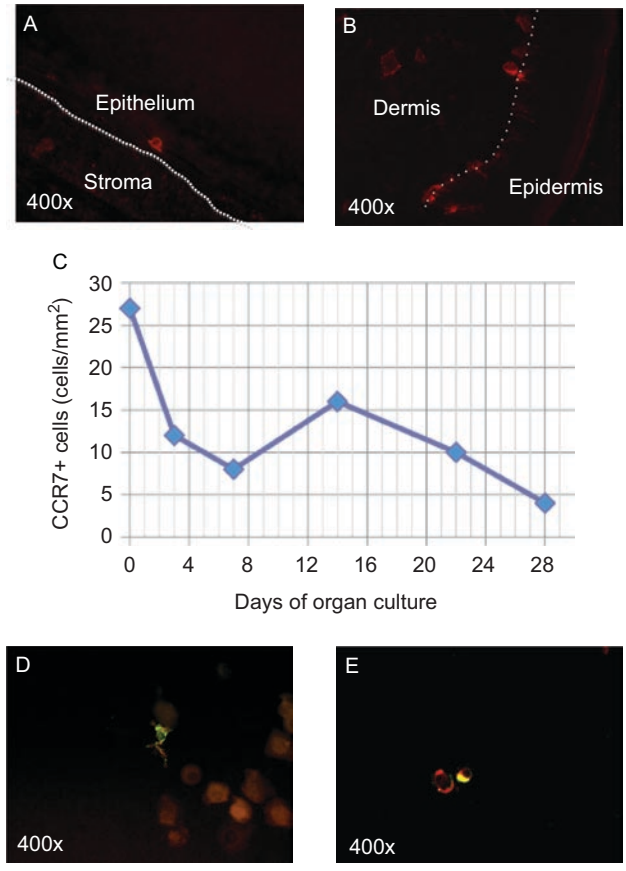


FIGURE 1 Immunofluorescent staining of LC/DC. (A) CCR7$^+$ cell of the fresh human cornea. Note that this cell is located subepithelially in the peripheral area. Magnification, ×400. (B) CCR7$^+$ cells in human skin as positive control. These cells are located between dermis and epidermis (dotted line). Magnification, ×400. (C) Alteration of CCR7+ cells during organ culture (cells/mm$^2$;). Although in total very few, most CCR7+ cells were found in fresh corneas, following a decrease during long-term culture. On day 14, a relative increase was observed and nearly diminished on day 28 of culture. (D) *In vitro* emigrated LC from corneas cultured for 10 days (Cytospin). Langerin$^+$ (red)/HLA-DR$^+$ (green) emigrated LC surrounded by epithelial cells. Magnification, ×400. (E) *In vitro* emigrated DC from corneas cultured for 7 days (Cytospin). The left cell shows only DC-SIGN$^+$ (red) staining, whereas the right cell is also HLA-DR$^+$ (yellow). Magnification, ×400.

Generally, CCR7 positive cells were only found to a slight amount. Most CCR7+ cells were observed in fresh human corneas, located mainly in the peripheral and limbal regions of the epithelium and the anterior stroma (Figure 1A). During the first days of organ culture, the number of CCR7 positive cells strongly decreased. On day 14, we observed a relative increase to about the half of the CCR7+ cells of a fresh cornea. Then again these cells decreased and after 28 days of culture they nearly disappeared (Figure 1C).

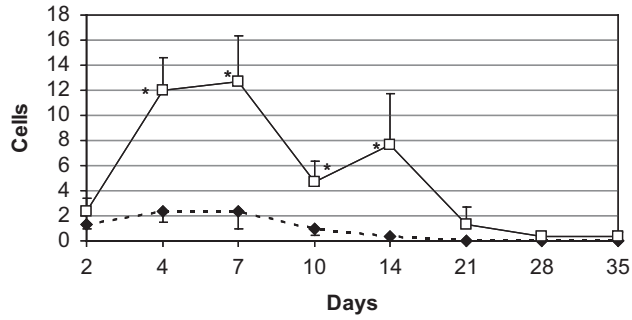


FIGURE 2 Influence on *in vitro* LC (Langerin$^+$/HLA-DR$^+$) migration using CCL19. This chemokine, in a concentration of 25 ng/ml (□), leads to a statistically significant (*) *in vitro* migration of LC up to 14 days compared to the negative controls (◆); $n = 6$. Error bars indicate the mean and standard devation (SD) of the experiments.

## Enhanced *In Vitro* Migration of APC Using Different Chemokines

In this study we investigated the evasion of APC (LC, DC, macrophages, and HLA-DR$^+$ cells) of human corneas during organ culture without any stimulus and compared this to the recruitment effect of various chemokines. Comparable to fresh human corneas also all migrated LC expressed Langerin and HLA-DR (Figure 1D), whereas not all DC expressed HLA-DR in double staining experiments (Figure 1E).

We sometimes observed a loss of APC by a partial ablation of the corneal epithelium in the first days of organ culture, following agglutination of corneal epithelium, which implicated cell-death (loss of APC). This cell loss happened possibly because of the obligatory decontamination step when preparing the corneas. It is known that the surface will be covered by new epithelium within a few days of organ culture. But for our studies, if epithelial ablation was encountered in cytospin specimens (spot of high density) results were not included in the evalutation.

### *In Vitro Migration of Langerhans Cells*

Addition of CCL19 increased the *in vitro* migration of Langerin$^+$ LC up to six-fold in all of the examined corneas. First effect was already seen after 4 days of organ culture, and a maximum of the migration peak was achieved between days 4 and 7. The enhanced *in vitro* emigration was statistically significant ($p < 0.05$) during the first two weeks compared to the negative controls (Figure 2). No effect was seen using CCL21 or the anaphylatoxin C5a (data not shown).

### *In Vitro Migration of Dendritic Cells*

The chemokine CCL19 lead to a two- to six-fold increased *in vitro* migration during the first 21 days in

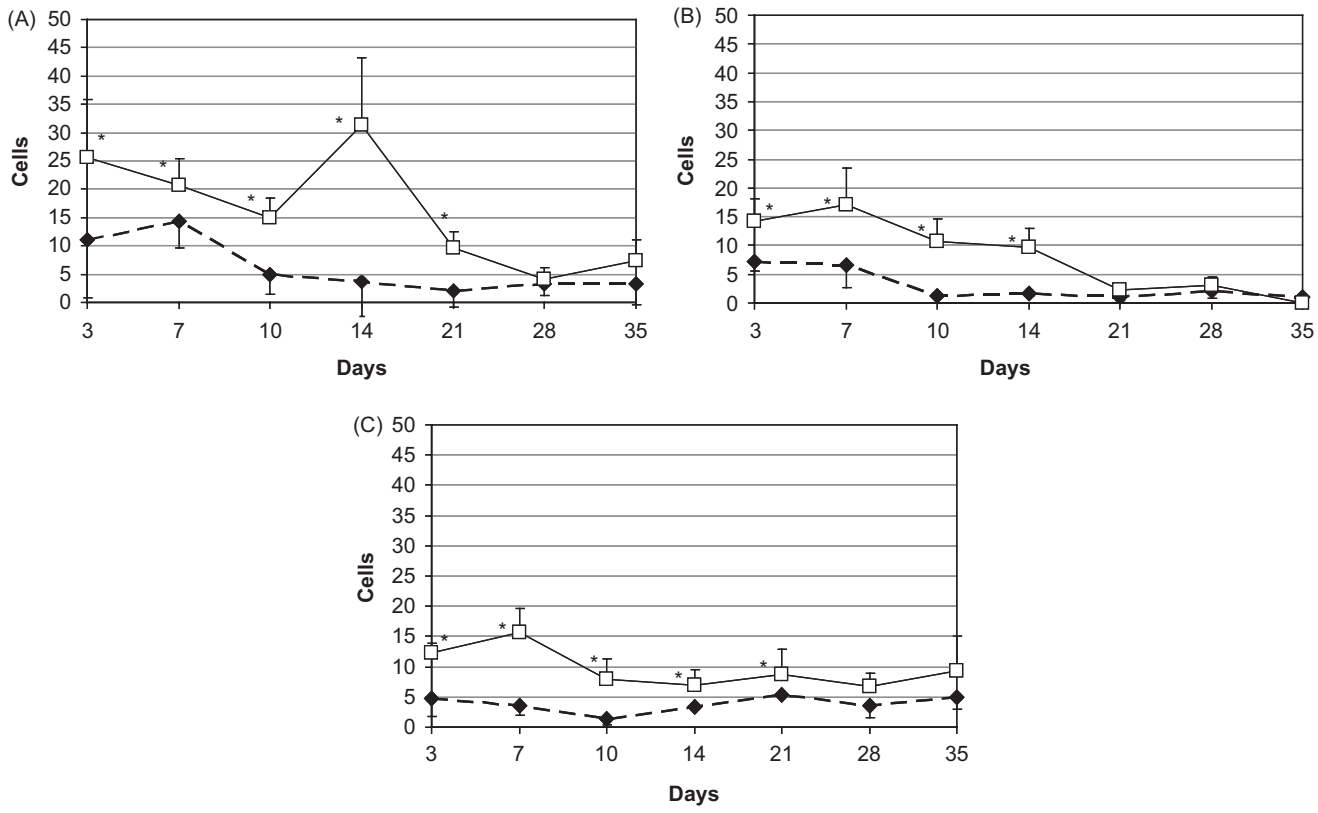


FIGURE 3 Influence on *in vitro* DC (DC-SIGN⁺) migration using different chemokines (□). (A) CCL19 (25 ng/ml) $n = 6$; (B) CCL21 (50 ng/ml) $n = 8$; (C) C5a (5 ng/ml) $n = 6$. Note that there was a statistically significant difference ($p < 0.05$) observed on the marked days (*) compared to the negative controls (◆).



all observed corneas. On day 14, we found the highest *in vitro* migration of DC-SIGN⁺ DC which was six-fold higher compared to the negative controls (Figure 3A). The difference of active *in vitro* migration using CCL19 during the first three weeks was statistically significant ($p < 0.05$).

Using CCL21 we also saw an enhancement of *in vitro* migrating cells during the first two weeks, which was in the range of two- to five-fold compared to the negative controls without a chemotactic stimulus (Figure 3B). The difference was statistically significant during the first two weeks ($p < 0.05$).

Using the anaphylatoxin C5a we also could enhance the *in vitro* migration of DC out of the cornea but not more than two-fold. During 21 days of observation the chemokine-driven *in vitro* emigration was statistically significant ($p < 0.05$) compared to the negative controls (Figure 3C). The numbers of emigrated cells were lower as compared to cultures containing CCL19 and CCL21.

### In Vitro *Migration of Macrophages/Monocytes*

None of the used attracting factors could influence the *in vitro* migration of macrophages/monocytes (CD14+/HLA-DR+ cells) at any time of cell harvesting. In general we only saw small numbers of *in vitro* migrating CD14+ cells during the whole observation period (data not shown).

### In Vitro *Migration of HLA-DR⁺ Cells*

We stained all into the culture medium emigrated cells not only for Langerin or DC-SIGN but also for HLA-DR to indicate the activated/mature status of the APC. Whereas all LC expressed HLA-DR, only some of the DC-SIGN⁺ cells and not all of the macrophages/monocytes (CD14⁺) did express HLA-DR. But there were even other cells, e.g., CD11c⁺, CD11b⁺, or CD45⁺, etc., which bear HLA-DR on their cell surface. This was already shown in a previous work and by other authors.[2,9]

Stimulation with CCL19 led to a strong peak of *in vitro* emigrating HLA-DR⁺ cells on day 7 (more than three-fold) and was still more than two-fold on day 10 (Figure 4A). The difference of chemokine-driven *in vitro* emigration compared to the negative controls was statistically significant at these days ($p < 0.05$).

Using CCL21 the enhanced *in vitro* migration was in a two- to three-fold range compared to the non-stimulated corneas and was observed from days 3 to

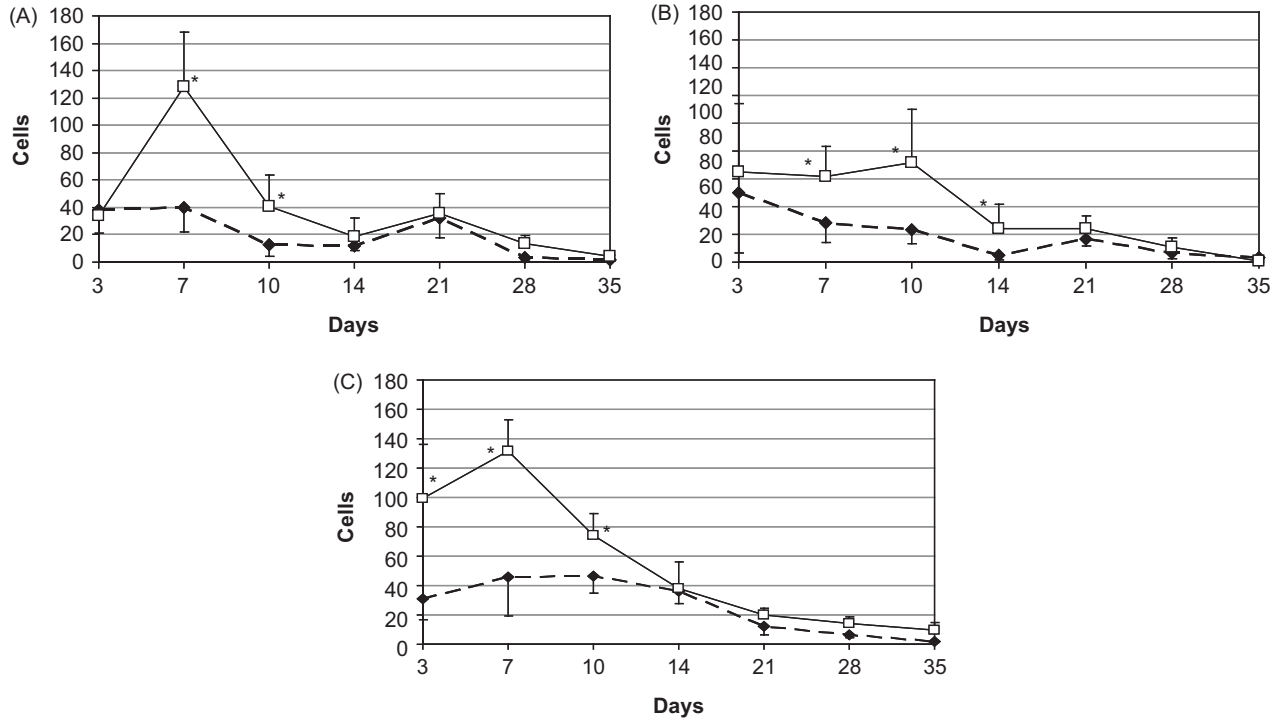


FIGURE 4 Enhancement of *in vitro* migrating HLA-DR+ cells using different chemokines (□). (A) CCL19 (25 ng/ml) $n = 12$; (B) CCL21 (50 ng/ml) $n = 18$; and (C) C5a (5 ng/ml) $n = 18$. Note that there was a statistically significant difference ($p < 0.05$) seen on the marked days (*) compared to the negative controls (◆).

14 (Figure 4B). This was statistically significant from 7 to 21 days ($p < 0.05$).

Also C5a led to a strong enhancement of *in vitro* emigrating cells, which was statistically significant already from 3 to 10 days ($p < 0.05$). A maximum up to a three-fold rate was reached with this chemokine (Figure 4C).

## DISCUSSION

The fate of a graft is dependent on one mainly part by the immune competent APC. Although a diminution in organ culture of these cells was observed,[9,19,20] it would be of benefit to actively reduce these cells. In a long-term follow up of cultured human corneas we found a markedly loss of APC during the first three weeks of culture.[9] Küchle et al. showed in a clinical study that corneal organ culture leads to a reduced risk for immune reactions after performing penetrating keratoplasty.[21] It is known that DC can be attracted by chemokines and that chemokine receptor 7 plays an important role in mediating APC trafficking.[11,22,23] Jin et al. showed in a mouse model that murine corneal APC expressing CCR7 interact with CCL21 to facilitate DC migration to the lymph nodes.[18] They found CCR7 expression only in inflamed murine corneas.

In contrast to this, our findings—performed in human corneas—showed that CCR7 could be observed even in non-inflamed corneas. We detected this receptor at a low expression level not only on *in vitro* emigrated cells but also in fresh human corneas in the sub-epithelial and stromal areas where LC or DC are residing. Although in a low number, CCR7+ cells were mainly found in fresh corneas. During organ culture, the amount of these cells strongly decreased. Interestingly, we observed a relative increase on day 14, possibly due to the influence of organ culture medium, i.e., fetal calf serum. From other human tissues, such as skin or tonsils, it is known that CCR7 is expressed at low level also without stimulation,[13] suggesting that CCR7 may not be the exclusive mediator of DC migration from the cornea to lymphoid tissues.

Chemo-attractant substances, such as CCL19 and CCL21, are the ligands for this receptor and lead to an emigration of DC and trafficking into lymph nodes. Using CCL19 and CCL21 we could indeed detect a significant difference in active *in vitro* migration of DC.

Jin et al. obtained similar results in a corneal mouse model.[18] Interestingly, we also found a significant increase of *in vitro* cell migration with CCL19 in LC, which was not reported for the mouse cornea. Analogous observations were already made in skin.[24]

Additionally, we found that CCL19 and CCL21 could not influence the *in vitro* migration of $CD14^+$ cells (i.e., macrophages/monocytes) in human corneas.

C5a is an anaphylatoxin that regulates chemotaxis and activation of neutrophils, eosinophils, basophils, and monocytes. Another important function of this complement factor is directing skin DC into lymphatic tissue. [25,26] In skin, only 5–10% of LC do express the C5a receptor (CD88) in contrast to dermal DC.[27] This is in accordance with our results that C5a could not stimulate $Langerin^+$ LC for *in vitro* migration, but could stimulate $DC\text{-}SIGN^+$ DC. Soederholm et al. found that C5a mediates cell recruitment mainly of immature DC (i.e., $HLA\text{-}DR^-$).[28] This is in accordance with our observations that most of the $DC\text{-}SIGN^+$ DC were HLA-DR.

Comparing the rate of *in vitro* cell migration using the three different chemokines we found the highest amount in the $HLA\text{-}DR^+$ cells, because this cell surface molecule is not only expressed on LC or DC but also on a wide range of APC. It is well known that the human cornea harbors a heterogeneous population of bone marrow derived cells which express the leukocyte common antigen, CD45.[2,6] We confirmed in our previous study that $HLA\text{-}DR^+$ APC are $CD45^+$.[9]

Interestingly, we found emigrated cells over the entire observation period of 35 days, whereas Ardjomand et al. detected $HLA\text{-}DR^+$ cells up to 14 days.[19] It must be considered that in our *in vitro* cornea model the organ culture medium (i.e., the used fetal calf serum) may have an effect on APC maturation/activation[19] and their recruitment, which is possibly associated with a stronger expression of different cell surface receptors such as the CCR7.

The number of *in vitro* migrated cells was lower than expected compared to previous results of our study group, where we investigated various APC in the human cornea during organ culture.[9] One possible reason could be the obligatory chemical decontamination step that often leads to a detachment of corneal epithelium with a loss of epithelial APC, but this would explain only a diminution of LC in the epithelium and not of stromal DC. Therefore, either a down-regulation of receptors or cell death during the culture could happen. The cell loss associated with mechanical collection at the various time points of observation may be another good reason why only few cells compared to previous findings in whole corneal flat mounts were seen.

There is no doubt that APC play a critical role in graft rejection. Ourselves and other have shown earlier that a long-term culture of human corneas leads to a decrease of residing APC. Nevertheless, it would be desirable to deplete or at least actively reduce these immunocompetent cells.

This study is meant to deliver another step for the understanding of immunological processes in the cornea. It is the first study which shows that in human corneas some APC bear receptors such as CCR7 suitable for the use of chemokine ligands like CCL19 or CCL21 as possible tools to significantly enhance the *in vitro* migration of APC out of the corneas.

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