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Novel Characterization of MHC Class II–Negative Population of Resident Corneal Langerhans Cell–Type Dendritic Cells

Pedram Hamrah, Qiang Zhang, Ying Liu, and M. Reza Dana

PURPOSE. The presence of antigen-presenting cells (APCs) such as Langerhans cells (LCs), an epithelial form of dendritic cells (DCs), in corneal tissue is critical for generation of immune responses, including graft rejection and herpetic keratitis. The purpose of this study was to characterize the distribution and major histocompatibility complex (MHC) antigen expression of corneal LCs.

METHODS. Normal and inflamed corneas were excised from BALB/c mice, and immunofluorescence staining for CD11c, CD11b, CD45, CD80 (B7.1), CD86 (B7.2), CD3, and MHC class II (Ia) was performed by confocal microscopy on wholemount corneal epithelium.

RESULTS. CD11c+ MHC class II–positive LCs were found in the limbus and corneal periphery, but not in the center of the normal cornea. These cells were CD45 positive, exhibiting bone marrow derivation, and CD3 and CD11b negative, confirming a DC lineage. Additionally, these cells were CD80 and CD86 negative, reflecting an immature phenotype. In the central and paracentral areas, however, significant numbers of CD11c+ LCs were detected that expressed no MHC class II. It is important to note that although the density of the LCs declined from the limbus toward the center of the cornea, they were always present. In the inflamed cornea, the expression of MHC class II and costimulatory molecules CD80 and CD86 was significantly enhanced, and present in all parts of the cornea, in contrast to the normal cornea.

CONCLUSIONS. The present study demonstrates for the first time the phenotype and distribution of MHC class II–negative LCs in the murine corneal epithelium. In the inflamed cornea, the LCs become activated as reflected by expression of B7 costimulatory markers. These changes in activation markers may provide additional information for devising novel immunomodulatory strategies.

Langerhans cells (LCs) were originally described by Paul Langerhans in 1868 in the skin epidermis, but their function and origin remained obscure for more than a century. It has now been established that LCs are a population of dendritic cells (DCs) that mediate antigen presentation. The current dogma holds that these cells represent a discrete leukocyte population of specialized or “professional” antigen-presenting cells (APCs) with an extraordinary capacity for initiating T-lymphocyte responses. The cardinal properties of APCs include their ability to take up, process, and present antigen; migrate selectively through tissues; and stimulate and direct T-lymphocyte–dependent responses. As such, LCs are the critical sentinel cells of the immune system in epithelial tissues that perform immune surveillance.

In the cornea, the presence of histologically similar atypical “onkeratinocytes” was noted in 1867. 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 thought to be 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 (Ia) molecules in the corneal epithelium and in the epidermis of the skin. MHC antigens have been implicated as important components in both the generation and expression of the immune response. Over the past several decades, the search for corneal APCs, largely reliant on their MHC class II expression, has led to the opinion that the normal central cornea is essentially devoid of APCs, regardless of species or strain, although rare MHC class II–positive cells have been described occasionally. Despite the purported absence of these cells in the normal cornea, it is generally acknowledged that a number of corneal stimuli (e.g., infection, trauma) can induce LC migration into the cornea from the limbus.

There are important biological implications for the contention that the normal cornea is devoid of a DC population. For example, the putative absence of a normal endowment of LCs in the central cornea has led many investigators to propose that the priming of recipient T cells in corneal transplantation relies practically exclusively on migration of host APCs into the cornea, where they can process antigens and then present processed peptides to naive T cells in lymphoid organs through the indirect pathway of sensitization. This is in contrast to other forms of solid organ transplantation, such as heart or kidney, where the donor tissue contains significant numbers of APC: passenger leukocytes that can directly sensitize host T cells by the concomitant expression of donor MHC and procured antigens. In addition to their role in transplantation, the number of class II–positive LCs present in the central areas of the cornea also correlates with the promotion of inflammatory responses in herpes simplex keratitis and the resultant degree of damage to the cornea.

In the present study we show, using highly sensitive confocal microscopy techniques, that the normal uninfamed cornea is in fact endowed with a significant number of MHC class II–negative LC-type DCs that are in an immature state. In contrast, the inflamed cornea is characterized by activated LCs, reflected by the upregulated expression of class II MHC and costimulatory molecules, including CD80 (B7.1) and CD86 (B7.2).
Experimental Animals

Seven to 14-week-old male BALB/c and C57BL/6 mice (Taconic Farms, Germantown, NY) were used in these experiments. For the transplantation experiments BALB/c (H-2b) mice were used as recipients and C57BL/6 (H-2b) mice were used as donors. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cauterization of Corneal Surface

Two weeks before tissue procurement, animals were deeply anesthetized with an intraperitoneal injection of 3 to 4 mg ketamine and 0.1 mg xylazine and were placed under the operating microscope. Using the tip of a hand-held thermal cautery (Aaron Medical Industries Inc., St. Petersburg, FL), five light burns were applied to the central 50% of the cornea, as previously described. Immediately after surgery, erythromycin ophthalmic ointment was applied. Corneas were excised 1, 3, 7, and 14 days after cautery application and assessed in immunohistochemical studies. For analytical purposes, the cornea was divided in three different areas. The central area was defined as the area within 0.5 mm of the corneal center. The paracentral region was the area between 0.5 and 1.0 mm of the center. The periphery was defined as being within a 1.0- to 1.5-mm radial distance from the center. The limbus was defined as the intervening zone between the cornea and conjunctiva, slightly more than 1.5 mm radially from the corneal center.

Student’s t-test was used to compare the number of cells with specific surface markers in different portions of the cornea. P < 0.05 was considered significant.

Results

Phenotype, Distribution, and Density of LCs in the Normal Cornea

To characterize the LCs of the corneal epithelium, epithelial sheets were double stained with a combination of antibodies. CD45 staining (the panleukocyte marker) showed large numbers of dendrite-shaped cells throughout the corneal epithelium (Fig. 1A). The density of these cells decreased from the limbus toward the center. Double staining with MHC class II antibody showed expression of MHC class II only at the limbus and periphery of the cornea, whereas the CD45+ cells in the paracentral and central areas were all negative for MHC class II (Fig. 1B). Staining with isotype controls instead of primary antibodies showed no labeling (Fig. 1C). All CD45-labeled cells, both in the periphery and in the center of the cornea, had the

Methods

Transplantation

Orthotopic penetrating keratoplasty was performed as described previously. Briefly, the recipient cornea was marked with a trephine and excised with microscissors to a size of 1.5 mm. The donor cornea was excised with a 2.0-mm trephine (Storz Instrument Co., St. Louis, MO) and transplanted into the host corneal bed with eight interrupted 11-0 nylon sutures (Sharpoint, Vanguard, TX). Nongrafted donor buttons and transplanted corneas were excised at 2, 6, 14, and 24 hours after surgery and used in immunohistochemical studies.

Transmission Electron Microscopy

Freshly excised healthy BALB/c corneas were fixed in Karnovsky solution. After three washes in cacodylate buffer, corneas were postfixed for 1.5 hours in 1% osmium tetroxide in the same buffer. Corneas were washed with H2O, stained in aqueous 2% uranyl acetate, dehydrated, and embedded in Epon. Corneal sections were cut at 60 Å and viewed under a transmission electron microscope (model 410; Philips, Eindhoven, The Netherlands).

Immunohistochemical Studies

Normal, inflamed (cauterized), and transplanted corneas were excised from BALB/c mice. Corneal-conjunctival epithelium was obtained in whole sheets by a modification of a technique previously described. Briefly, freshly excised corneas were immersed in phosphate-buffered saline (PBS), containing 20 mM EDTA (Sigma Chemical Co., St. Louis, MO) at 37°C for 1 hour. The epithelium was separated from the underlying stroma with forceps and washed in PBS. Epithelial sheets were fixed in acetone for 15 minutes at room temperature (RT). To block nonspecific staining, epithelial sheets were incubated in 2% bovine serum albumin (BSA) diluted in PBS (PBS-BSA) for 15 minutes before addition of primary and secondary antibodies. Purified primary antibodies or isotype-matched control antibodies were applied to the samples for 2 hours, followed by a 60-minute incubation of a second FITC- or phycoerythrin (PE)-conjugated primary antibody or by incubation of the secondary antibodies for 60 minutes (all diluted for optimal concentrations in PBS-BSA). Epithelial sheets were further stained with secondary antibodies only, as additional controls. All staining procedures were performed at RT, and each step was followed by three thorough washings in PBS for 5 minutes each. Finally, epithelial sheets were covered with mounting medium (Vector, Burlingame, CA) and examined with a confocal microscope (Leica, Heidelberg, Germany). Epithelial sheets were examined at 160-fold and 400-fold magnifications using a ×10 eyepiece and ×16 or ×40 objective lens. Central, paracentral, and peripheral areas for each cornea were assessed separately. At least three different corneas were examined per each double-staining experiment. Five to eight different fields were analyzed for each specimen using a grid and the numbers averaged.

Antibodies

The primary antibodies (all from PharMingen, San Diego, CA), their specificity, and their respective control antibodies (all from PharMingen), are summarized in Table 1. Secondary antibodies were Cy5-conjugated goat anti-hamster IgG (PharMingen), rhodamine-conjugated goat anti-rat IgG, and FITC-conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

TABLE 1. Monoclonal Antibodies Used in Labeling

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<th>Primary Antibody (Clone)</th>
<th>Specificity</th>
<th>Control Antibody</th>
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<tr>
<td>IA4-FITC (39-10-8)</td>
<td>BALB/c mouse class II MHC antigen</td>
<td>Mouse IgG 3-FITC</td>
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<tr>
<td>IA3-FITC (AF6-120-1)</td>
<td>C57BL/6 mouse class II MHC antigen</td>
<td>Mouse IgG 2a-FITC</td>
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<td>CD11c (HL3)</td>
<td>Integrin DC/LC marker</td>
<td>Hamster IgG</td>
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<tr>
<td>CD11b-FITC (M1/70)</td>
<td>MAC-1, integrin monocyte-macrophage marker</td>
<td>Rat IgG 2b-FITC</td>
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<tr>
<td>CD45 (30-F11)</td>
<td>Panleukocyte marker</td>
<td>Rat IgG 2b</td>
</tr>
<tr>
<td>CD80-FITC (16-10A1)</td>
<td>B7.1 costimulatory molecule</td>
<td>Hamster IgG</td>
</tr>
<tr>
<td>CD86-PE (GL1)</td>
<td>B7.2 costimulatory molecule</td>
<td>Rat IgG 2a</td>
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<tr>
<td>CD3e-FITC (145-2C11)</td>
<td>T-lymphocyte marker</td>
<td>Hamster IgG</td>
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PE, phycoerythrin.
classic dendritic cell morphology of LCs, as shown at higher magnifications (Figs. 2A, 2B). The same cells, however, were all MHC class II negative (Fig. 2C) in paracentral and central areas of the cornea.

Double labeling of epithelial sheets with CD11c and B7 (CD80 or CD86) costimulatory molecules showed that all CD11c<sup>+</sup> cells in the normal uninflamed cornea were negative for both CD80 (Fig. 3) and CD86 (results not shown). Further analysis showed that all the dendritic-shaped cells were positive for CD11c, a marker for DCs, and negative for CD11b (monocyte marker) and CD3 (T cell marker), confirming their DC lineage.

The results on cell distribution and cell surface molecules in the normal cornea are summarized in Figure 4: CD45<sup>−</sup>CD11c<sup>+</sup> LCs decreased in density from the periphery of the cornea toward the center. Nearly one half of these cells were MHC class II positive in the periphery, but there was no expression of MHC class II in the central or paracentral areas. Regardless of localization of MHC class II expression, all samples were negative for CD11b, CD3, CD80c, and CD86.

In addition to the immunohistochemical studies, transmission electron microscopy (TEM) was performed to confirm presence of these bone marrow-derived cells in the cornea. TEM demonstrated the presence of numerous dendritic cells with long processes interdigitating among the corneal epithelial cells (Fig. 5A). A subset of these cells contained multilaminated Birbeck granules (Fig. 5B), a specific marker for LCs, suggesting that these cells represent an LC phenotype.

**Distribution and Density of LCs in the Inflamed Cornea**

The cell surface phenotype of corneal LCs changed dramatically in corneal inflammation. CD45 and CD11c double stain-
ing with MHC class II showed that the density of central LCs increased after cautery application. Furthermore, in BALB/c corneas, a subset of LCs in the center and paracentral areas of the cornea uniformly expressed MHC class II by day 3 after cauteryization (Figs. 6A, 6B). The number of MHC class II-positive cells increased through 14 days after cautery. In addition, there was novel expression of CD80 and CD86 (B7 costimulatory molecules) by CD11c+ cells throughout the corneas, uniformly by day 3 and at later time points (Fig. 7). The expression of MHC class II and B7 by epithelial DCs, was first observed around the (central) cautery sites at day 3, whereas the paracentral areas between the cautery sites and the limbus remained MHC class II negative. By days 7 and 14 after cautery, these sites also contained class II- and B7-positive cells.

The results on cell distribution and cell surface molecules in inflamed corneas at day 14 after cautery are summarized in Figure 8: The number of leukocytic (CD45+) cells in the peripheral, paracentral, and central areas of the cornea increased by 100%, 43%, and 29%, respectively (P < 0.005, P < 0.09, P < 0.31, respectively) compared with the normal cornea. This increase consisted largely of a DC population as suggested by CD11c expression (Fig. 8). In addition to changes in cell number, cells detected in the central and paracentral areas also showed upregulated MHC class II expression in the inflamed cornea. Enhanced expression of MHC class II was particularly apparent in the paracentral and central areas of the cornea. Put another way, although in the uninflamed setting the absolute number of MHC class II+ cells was still higher in the periphery than in the center, the relative increase in class II expression was more marked in the center of the cornea, in that there was virtually no expression of class II by these cells in the uninflamed setting. In addition, inflammation was also associated with enhanced expression of the B7 costimulatory markers, CD80 and CD86, indicating the relative maturation of these cells and their greater potential capacity for sensitization.

Expression of MHC Class II by Donor Corneal Cells in Transplantation

The presence (or absence) of donor MHC class II antigen in the corneal graft has important implications for the mechanisms by which graft-recipient hosts become sensitized to transplanted antigens. To determine whether the enhanced detection of MHC class II-positive cells in grafts is (solely) due to influx of host leukocytes or also due to upregulated expression of resident donor LCs (as described earlier), we performed double staining against the DC marker CD11c and donor-type MHC of C57Bl/6 mice (Ia+) at different time points after transplantation into BALB/c (Ia+) mice. Staining for donor-derived MHC class II on nongrafted corneal buttons and at 2, 6, and 16 hours after corneal transplantation showed no expression of donor MHC class II; however, as early as 24 hours after corneal transplantation, novel expression of donor class II was detected (Fig. 9). These MHC class II-positive donor cells were CD11c and CD45 positive. An interesting observation at the early time points when no staining for donor MHC class II was detected was a centrifugal migration of MHC class II-negative CD45+CD11c+ cells toward the graft-host border (Fig. 9, arrows).

DISCUSSION

The present study indicates that resident MHC class II-negative LCs are present in the epithelium of the normal murine cornea. Previous studies in guinea pig, hamster, mouse, and human have established that MHC class II-positive LCs are present in the epithelium of the conjunctiva and peripheral cornea but are essentially absent from the central cornea. The results on cell distribution and cell surface molecules in inflamed corneas at day 14 after cautery are summarized in Figure 8: The number of leukocytic (CD45+) cells in the peripheral, paracentral, and central areas of the cornea increased by 100%, 43%, and 29%, respectively (P < 0.005, P < 0.09, P < 0.31, respectively) compared with the normal cornea. This increase consisted largely of a DC population as suggested by CD11c expression (Fig. 8). In addition to changes in cell number, cells detected in the central and paracentral areas also showed upregulated MHC class II expression in the inflamed cornea. Enhanced expression of MHC class II was particularly apparent in the paracentral and central areas of the cornea. Put another way, although in the uninflamed setting the absolute number of MHC class II+ cells was still higher in the periphery than in the center, the relative increase in class II expression was more marked in the center of the cornea, in that there was virtually no expression of class II by these cells in the uninflamed setting. In addition, inflammation was also associated with enhanced expression of the B7 costimulatory markers, CD80 and CD86, indicating the relative maturation of these cells and their greater potential capacity for sensitization.

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characteristic high expression of MHC class II by professional APC (including DCs and LCs) populations, on the one hand, coupled with the failure to routinely detect class II–positive cells in the uninflamed cornea on the other, has led to the conclusion that the cornea is devoid of resident APCs.9,12,19–29

Using an immunofluorescence double-staining technique applied to confocal microscopy, we observed two phenotypically distinct populations of leukocytes throughout the corneal epithelium: a MHC class II–positive population, located in the periphery of the cornea and the limbus, and a MHC class II–negative population, located in the central areas of the normal cornea. TEM studies confirmed the presence of dendritic-shaped cells in the central corneal epithelium; moreover, a subset of these cells contained Birbeck granules, identifying them as having an LC lineage. Given that these cells collectively exhibit dendritic morphology and, except for MHC class II expression, have identical expression (or absence of expression) of cell surface markers (CD45+; CD11b−, CD11c−, CD3−, CD80−, CD86−), we believe that they represent an LC-type DC population.48 The absence of CD80 or CD86 expression by these cells regardless of their location is characteristic of LCs in normal uninflamed tissues48,49 and defines these cells as being in an immature or precursor stage. The negative expression for CD3 and CD11b excludes the presence of T-cells (including Thy-1 positive dendrite-shaped T-cells), and monocytes-macrophages in this lineage.

The MHC class II–positive LCs in the periphery account for slightly more than half of the total resident LCs (CD11c−; CD45−) in the cornea. The MHC class II–positive LC density of 100 cells/mm² in the periphery correlates with results previously obtained by other groups.9,50 However, to our knowl-
edge, we are the first group to report the presence of MHC class II-negative LCs in the cornea. In 1964, when LCs were thought to be melanocytes, Segawa et al. described three different populations of LCs in the human cornea.31 One of these populations, the “nonpigmented dendritic cell” was found in “all” parts of the cornea, including the center of the epithelium. In retrospect, it is not clear whether Segawa et al. were looking at the same cells as are described in this study. In several studies, MHC class II-negative LCs have been described in the skin.52–55 Because CD1a is a highly reliable indicator for noncorneal LCs, those studies demonstrate that CD1a+ LCs may be class II negative in the skin epidermis.

The comparison between CD1a and MHC class II antigen expression is not possible in the corneal epithelium, however, because corneal LCs do not express the CD1a antigen, in contrast to their counterparts in the skin.51,56,57 Therefore, it may not be surprising that the novel MHC class II-negative LC population described herein was not detected previously. In addition, our studies are based on confocal microscopic evaluation of corneal epithelial sheets, which has the advantage of examining multiple layers over a broad surface area. In our experience, even with confocal microscopy, detection of LC populations in the cornea is very difficult in cross-sectional studies, because the transection of the LCs makes it difficult to evaluate these cells’ morphology. This may explain why some investigators have described occasional class II+ dendrite-like cells11,14,24,30–32 in the cross-sectioned cornea, but were unable to make any firm conclusions regarding their identity.

We found that in inflammation, the expression of MHC class II and B7 molecules (CD80 and CD86) is potently upregulated. This upregulation was observed in cataractous corneas uniformly at day 3 after cataractation: first near the cautery sites and later throughout the cornea. Although cells migrating into the cornea from the limbus also contribute to the increased density of MHC class II+ and B7+ cells, our data suggest that most of these cells, especially in the central and paracentral areas, are resident LCs. There are several lines of evidence to support this: First, although the total number of CD11c+ cells increased from 80/mm² in the normal cornea to 103/mm² in the inflamed cornea, the number of MHC class II+ cells increased from 0/mm² to 51/mm², suggesting that recruitment of cells into the corneal center was not entirely responsible for changes in MHC class II expression. Second, at early time points after cautery, MHC class II and B7 positive cells were first present around cautery sites in the corneal center, whereas the peripheral sites were still negative for these activation markers, suggesting that cells expressing these markers were not simply being recruited from the periphery. Third, in the transplantation experiments, we noted novel expression of donor-derived MHC Ia antigens among the graft’s CD11c+ cells 24 hours after transplantation, similarly suggesting that recruitment of cells from the (host) periphery could not entirely explain the upregulation of MHCs in the graft.8,32,37 Moreover, the negative expression of donor MHC antigens at early time points after transplantation rules out contamination as a cause of this expression. We also noted that the CD11c cells migrated centrifugally toward the graft-host interface before class II upregulation, suggesting that the environment at the graft-host border plays a major role in the upregulation of class II in these cells.

Candidate molecules that are known to upregulate expression of costimulatory and MHC class II molecules and induce the maturation of DCs and LCs, include tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)-1.58–62 Previous studies by our group using the IL-1 receptor antagonist (IL-1ra) to suppress IL-1 function locally, showed that topical IL-1ra suppresses LC activities and ultimately promotes ocular immune privilege and corneal transplant survival.63,64 One way IL-1ra’s immune modulatory effect could be explained is its suppression of limbal (host) APC recruitment into the graft, which would result in decreased capacity for antigen processing. However, in view of our current data, we speculate that one additional mechanism by which anti-inflammatory agents may downmodulate corneal immunity is by suppressing the maturation of resident APCs in

**Figure 8.** Enumeration of multiple cell surface phenotypic markers, based on location, in the inflamed cornea. The data indicate that CD45–CD11c+ cells migrated into the cornea. Moreover, a significant number of cells upregulated expression of MHC class II and B7 costimulatory molecules after induction of inflammation. Mean cells per square millimeter ± SD from five to eight fields of at least three corneas per staining are compared.

**Figure 9.** MHC class II-negative corneal button shows upregulated donor-type MHC class II 24 hours after transplantation. Corneal transplantation was performed using C57BL/6 (Ia-) mice as donors and BALB/c (Ia+) mice as recipients. The central donor cornea, which was devoid of MHC class II (Ia+)-positive cells before transplantation, exhibited donor-derived MHC class II-positive cells (arrows) as early as 24 hours after transplantation. These cells were CD11c positive in double-stained corneas. Magnification, ×400.
the cornea. This hypothesis is supported by previous data from our laboratory in which we showed that intracorneal injection of LCs can promote generation of immunity to intracameral antigens, but that suppression of IL-1 activity (even in the presence of high numbers of intracorneal LCs) abrogates the capacity of LCs to promote immunity to ocular antigens.64 Hence, we propose that the absence (or generation) of immunity to corneal antigens cannot be explained simply by the numbers of APCs present in the cornea, but also must take into account these cells’ maturation state.

The described herein fit the phenotypic characteristics of progenitor or immature APCs. Progenitor and immature APCs (including DCs and LCs) in general have negligible to absent MHC class II and B7 costimulatory expression.65 Although immature LCs are highly capable of antigen uptake and processing, in contrast to mature LCs, they have a weak stimulatory capacity for activating T cells, due to their failure to provide naïve T cells with requisite costimulatory signals.66 However, exposure of these cells to the proper (proinflammatory) cytokine microenvironment promotes their maturation, during which these cells lose their capacity to process antigens and instead gain the capacity to stimulate T cells. Our data suggest that the proinflammatory milieu induced by cauteronation or transplantation of the cornea is associated with maturation of resident corneal LCs.

Little is known about the exact molecular mechanisms that regulate LC maturation in the cornea on the one hand, or those that retain large numbers of these LCs in an immature state on the other. It is known that prostaglandins E, and cytokines such as TGF-β and IL-10 have a profound capacity to suppress the stimulatory role of LCs and to downregulate MHC class II expression. Given that there is constitutive expression of a variety of immunosuppressive factors in the eye (including the cornea), it is attractive to propose that active suppression of LC maturation, with associated absence of MHC class II and costimulatory molecule expression, may in fact represent an important facet of ocular immune privilege.

The presence of an MHC class II-negative subpopulation of immature LCs in the cornea may have important implications for a wide range of immunoinflammatory responses in the anterior segment, including alloimmune, autoimmune, and innate immune responses. Further studies are needed, to determine the molecular mechanisms that regulate the maturation of these cells and their immunobiologic phenotype in stimulating (or tolerizing) T cells generated in response to ocular antigens.

Acknowledgments

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References


