



## Concise Review: Immunological Properties of Ocular Surface and Importance of Limbal Stem Cells for Transplantation

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**Key Words.** Corneal transplantation • Limbal stem cells • Transplantation rejection • Major histocompatibility complex

### ABSTRACT

Cornea transplantation has been considered to be different from other solid organ transplantation because of the assumed immune-privileged state of the anterior chamber of the eye. Three major lines of thought regarding the molecular mechanisms of immune privilege in the eye are as follows: (a) anatomical, cellular, and molecular barriers in the eye; (b) anterior chamber-associated immune deviation; and (c) immunosuppressive microenvironment in the eye. However, cornea transplants suffer allograft rejection when breached by vascularization. In recent developments, cellular corneal transplantation from cultivated limbal epithelial cells has shown impressive advances as a future therapy. The limbal stem cell niche contains stem cells that promote proliferation and migration and have immunosuppressive mechanisms to protect them from immunological reactions. Limbal stem cells are also noted to display an enhanced expression of genes for the antiapoptotic proteins, a property that is imperative for the survival of transplanted tissues. Further investigation of the molecular mechanisms regulating the immune regulation of limbal stem cells is relevant in the clinical setting to promote the survival of whole corneal and limbal stem cell transplantation. *STEM CELLS TRANSLATIONAL MEDICINE* 2013;2:614–624

### INTRODUCTION

The cornea is the most frequently transplanted organ in the United Kingdom, with 3,061 transplantations, or 49.6 cases performed per million population in 2009–2010 [1]. This is the highest recorded number of transplantations, surpassing solid organ transplantation such as adult kidney (892), pancreas (200), cardiothoracic organ (272), and liver (679) cases during the same period.

Corneal transplantation is the definitive therapy for most causes of corneal blindness, which is the result of a range of pathophysiologies such as inherited disorders, infections, inflammations, perforations, trauma, and chemical injury. Importantly, transplantation is also performed to replace rejected or failed previous grafts in 20% of cases [2]. Approaches to corneal transplantation surgeries have evolved from whole cornea or partial thickness transplantation to single layer keratoplasty, with very promising outcomes [3, 4].

However, corneal transplantation is contraindicated in the treatment of limbal stem cell deficiency, which is an important cause of corneal blindness. Failure of grafts in this condition is due to the loss of host stem cells and inadequate self-renewing cells to replenish the surface of the

graft from the donor. In the case of limbal stem cell deficiency, cellular therapy such as ex vivo limbal epithelial transplantation has shown impressive advances as a future treatment in the past decade or so [5–9].

The cornea is located in the anterior part of the eye and acts as a protective barrier to the interior structures. Corneal transparency is important to allow transmission of light onto the retina to obtain clarity of vision. The cornea consists of three major layers, which are derived from different germ layers (Fig. 1). The epithelial layer of the cornea develops from ectoderm, whereas the stroma and endothelium are mesenchymal in origin. There are six to eight cell layers of the epithelium, which lies on the Bowman's membrane. The majority of the corneal thickness is contributed by the stromal layer, whereas the endothelial monolayer sits on the Descemet's membrane, making up the most posterior part of the cornea. The cornea enjoys beneficial physical properties that constantly keep it in a dehydrated state due to organization of tightly packed collagen lamellae and the presence of compact keratocyte networks. The endothelial layer is equipped by physiological tight junctions to maintain corneal functions.

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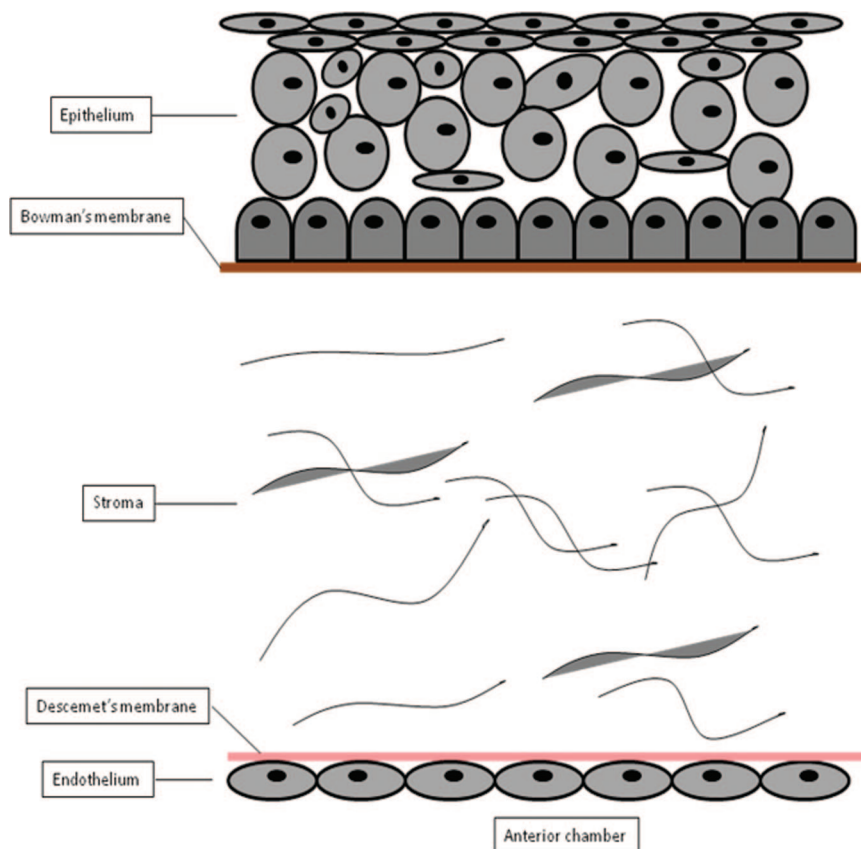
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Received October 28, 2012; accepted for publication March 25, 2013; first published online in *SCTM EXPRESS* July 1, 2013.

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1066-5099/2013/\$20.00/0

<http://dx.doi.org/10.5966/sctm.2012-0143>



**Figure 1.** Diagrammatic representation of human cornea showing the three main layers: epithelium, stroma, and endothelium. The Bowman's membrane is an acellular layer that lies in the anterior stroma, just beneath the basement membrane of the epithelium. The Descemet's membrane is the basement membrane of the endothelium.

The most significant physiological changes during an immunological rejection occur in the endothelium, where there is rapid loss of cell density. The endothelium of the cornea is in a nonreplicative state after birth, making the loss of donor cells irreversible. In addition, the endothelium loses cellularity in an age-dependent manner at the rate of 0.56% cell loss per year [10]. This results in corneal decompensation and subsequent graft failure.

The cornea is devoid of blood vessels and is assumed to be protective of immune rejection of transplanted grafts, a condition termed corneal immune privilege [11]. Its avascularity implies a lack of angiogenic factors or the possibility that it may secrete antiangiogenic factors. Vascularization evokes an immune response and has implications for graft allojection. In addition, the absence of corneal lymphatics prevents the channeling of antigen-presenting cells to the regional lymph nodes, thus not allowing alloantigen-specific T cells to be activated. Activated T cells travel to the graft bed and initiate the crucial process of graft rejection. However, the relative ease of topical steroid application on the cornea and the immune tolerance in the anterior chamber also contribute to the relative success rate of corneal transplantation. On the other hand, the conjunctiva, which is the neighboring structure and continuous with the corneal epithelium, is highly vascularized and often invades the cornea in pathological conditions, resulting in a debilitating vision due to a process called corneal neovascularization [12].

Innate immune responses of the ocular surface are nonspecific but important as the front lines to defend against corneal

infections and toxins. The precorneal tear film serves as a physiological barrier to protect the eye. Among other substances, tears contain mucin, lactoferrin, and lysozyme [13]. Mucin has a gel property, to lubricate and become a chemical barrier, whereas lactoferrin and lysozyme have antimicrobial activities [14]. Other components of the ocular innate immune system include immunoglobulin (Ig)-A in the tears and inflammatory cytokines, such as interleukin (IL)-1 $\alpha$ , IL-6, IL-8, and tumor necrosis factor (TNF)- $\alpha$ . The ability of cells to recognize pathogen-associated molecular patterns is through the expression of several Toll-like receptors (TLRs) [15].

Adaptive immune responses are much more pathogen-specific than their innate immune counterparts. These pathways are cell-mediated responses, which are usually slower acting and more efficient but can cause damage to surrounding tissue, resulting in damage to the vision.

#### REJECTION IN CORNEAL (WHOLE TISSUE) TRANSPLANTATION

Even though the cornea is the most frequently transplanted organ, the immunology of corneal transplantation and rejection has not been fully studied. For the most part, outcomes for whole cornea transplantation (penetrating keratoplasty) are dependent on the indications for surgery. Common indications for penetrating keratoplasty are keratoconus, corneal dystrophies, corneal opacities, and post-cataract surgeries. Comparisons of the outcome of corneal transplantation are difficult because of

the nonuniformity of data on surgical indications, patient demographics, type of grafts, survival analysis, and range of follow-up. In general, uncomplicated low-risk grafts give the highest survival rate with topical steroidal immune suppression. Based on surgical indications, the Australian Corneal Graft Registry reported that keratoconus has the highest 10-year survival rate (89%), compared with Fuch's corneal dystrophy (73%), nonherpetic corneal opacity (70%), herpetic corneal opacity (60%), pseudophakic and aphakic corneal edema (40%), and regrafting (30%) [2].

A breakdown of survival analysis shows that the success rate of allogeneic penetrating keratoplasty for correcting visual acuity was highest in the first year of follow-up: it was 87% in the first year and was reduced to 52% after 10 years among Asians [16]. Generally, failed grafts are due to irreversible immunological rejection (34%), whereas corneal endothelial cell failure due to glaucoma (24%) and infection (14%) are common causes of failed grafts [2]. A similar trend was observed in a study of second grafts; in the majority of cases, immune rejection was the main cause of graft failure, although recurrence of disease and endothelial decompensation were also important causes [17]. Clinically, other associated factors that are implicated include the degree of prevascularization of the recipient bed, size of donor corneal button, position of the donor corneal button in the recipient bed, preservation methods for the donor corneal button, choice of immunosuppression, and immune status of the recipient.

Generally, the immune response to alloantigens is polarized in two directions: (a) activation of T helper type 1 (Th1) effector cells, which results in production of interferon (IFN)- $\gamma$ , introduction of CD4+ as a surface determinant, and mediation of delayed immune response; and (b) activation of the T helper type 2 (Th2) pathways, which may produce cytokines that cross-regulate Th1 factors and inhibit their mediator functions. IFN- $\gamma$  is an important proinflammatory cytokine in the rejection of major histocompatibility complex (MHC)-matched corneal allografts. It has been shown that manipulating the alloantigenic response from Th1 to Th2 pathways by blocking IFN- $\gamma$  production in MHC-matched allografts significantly reduces the graft rejection rate [18].

The mechanism of rejection in cornea transplantation is a delayed type, T cell-mediated immune response, which is accelerated by the inflammatory process. In a rat corneal transplantation, CD4+ T cells had an important role in rejection, whereas the role of CD8+ T cells was not clearly shown. IL-2, IL-4, and IFN- $\gamma$  are regarded as important inflammatory markers for corneal rejection [19]. This finding is in agreement with an experimental sheep corneal transplantation model, where acute rejection is associated with graft neovascularization, infiltration of CD4+ and CD8+ T cells, and production of IFN- $\gamma$  and IL-2 in the graft [20]. But production of TNF- $\alpha$ , IL-4, and IL-10 was not evident, which the investigators hypothesized might be due to rapid T-cell death due to Fas-Fas ligand (FasL) interactions [21]. The expected result of this interaction would be induction of apoptosis in Fas bearing infiltrating leukocytes such as CD4+ T cells, CD8+ T cells, neutrophils, and macrophages [22]. The role of TNF- $\alpha$  in initiating, maintaining, and resolving inflammatory processes has been observed to show dynamic changes in allograft transplantation [23].

Corneal epithelium and stroma, both peripherally and centrally, contain dendritic cells (DCs), which undergo maturation by

expressing MHC class II and B7 costimulatory molecules during inflammation [24]. B7 costimulatory molecules provide potent stimulation for MHC/peptide-T cell receptor interaction to result in activation of T cells. B7 molecules are present as dimers on the surface of DCs or other forms of antigen-presenting cells (APCs).

A novel negative regulatory molecule has been described recently, which is a new member of the B7-CD28 superfamily, and it is referred to as programmed death-1 (PD-1) [25]. B7-H1 is a potential ligand for PD-1 [26]. B7-H1 has been found in corneal epithelium and endothelium and believed to have proapoptotic actions on T cells, thus prolonging the survival of corneal grafts [25].

#### CORNEA AS AN IMMUNE-PRIVILEGED ORGAN

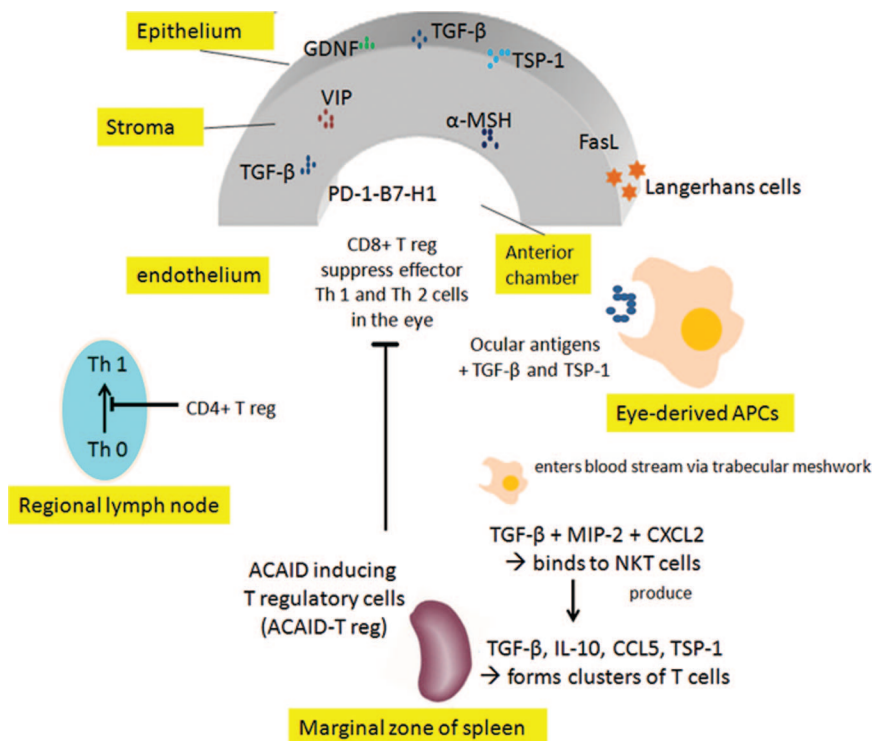
Cornea transplantation has been regarded differently from other organ transplants because of the immune-privileged nature of the anterior chamber of the eye and the lack of direct allorecognition. Medawar revolutionized the concept of immune privilege in the cornea in 1940s [27]. He observed how skin grafts transplanted in the brain and the anterior chamber of the eyes could remain intact for extended periods of time even in the absence of any vascularization [27]. Medawar suggested that the immune reaction is mediated by blood plasma or by cells transported in it. During corneal transplantation, vascularization causes the breakdown of the immunologically privileged status of the cornea, resulting in graft rejection. It was also suggested that the epithelium rather than the stroma plays a role in the immune reaction [28].

The immune privilege of the cornea depends on multiple mechanisms to prevent immune destruction of grafts by allogeneic responses. At present, three major lines of thought prevail regarding the molecular mechanisms of immune privilege in the eye: (a) anatomical, cellular, and molecular barriers in the eye; (b) anterior chamber-associated immune deviation (ACAID); and (c) immune suppressive microenvironment in the eye.

First, there is a direct absence of anatomical lymphatic drainage and a physiological blood-ocular barrier in the cornea that defines the immunological tolerance. Without lymphatic drainage, the antigens are unable to leave the grafts to the draining lymph nodes. The blood-ocular barrier effectively presents a physiological block for the immune cells to enter the tissues. These factors collectively act as physical barriers to stimulate immune response to grafts.

ACAID was first described as an aberrant immune response of the eye [29]. This refers to the phenomenon in which antigen-specific systemic immunological tolerance is induced to an antigen that has been introduced to the anterior chamber (Fig. 2). In this situation, antibody responses are preserved, whereas cellular responses such as delayed type hypersensitivity and cytotoxic T lymphocytes (CTLs) are suppressed. These include alloantigens involved in transplantation, soluble protein antigens, viral antigens, and tumor antigens [30].

Vascularization causes an allograft to be deemed high risk and robs it of its immune-privileged status. Immunological rejections in high-risk vascularized allografts occur because of the increased level of chemokines and inflammations at the site of the grafts, attracting allospecific activated T cells to the site [11, 31, 32]. Chemokines are heparin-binding proinflammatory proteins that are responsible for directing leukocyte migration. Chemokines are categorized as CXC, CC, C, and CX<sub>3</sub>C based on the



**Figure 2.** Ocular immunity is provided by the physiological barrier of the precorneal tear film, induction of anterior chamber induced immune deviation (ACAID), and the immunosuppressive environment in the eye. Immunomodulatory molecules in the cornea consist of neuropeptides, cytokines, growth factors, and soluble immune peptides, which have anti-inflammatory, antiangiogenic, and proapoptotic activities that lead to death of allospecific T cells. Abbreviations: ACAID, anterior chamber induced immune deviation; APC, antigen-presenting cells; CCL5, C-C ligand-5; CXCL2, C-X-C ligand-2; FasL, Fas ligand; GDNF, glial-derived neurotrophic factor; IL-10, interleukin-10; MIP-2, macrophage inflammatory protein-2; NKT, natural killer T cells; PD-1-B7-H1, programmed death-1 ligand-B7-H1 binding; T reg, regulatory T cells; TGF-β, transforming growth factor-β; Th, T helper; TSP-1, thrombospondin-1; VIP, vasointestinal peptide.

cysteine residue at the protein terminal. CXC chemokines (e.g., CXC-ligand 1 [CXCL1]) play a major role in recruiting activated allospecific T cells, macrophages, and other potent inflammatory mediators to the graft site, causing immunogenic rejection [31].

The anterior chamber contains transforming growth factor-β (TGF-β) and thrombospondin-1 (TSP-1), and antigen-presenting cells, which capture the antigens and secrete these into the bloodstream through the trabecular meshwork and reach the marginal zone in the spleen. TGF-β stops proliferation of cells, induces differentiation, or promotes apoptosis, whereas TSP-1 has an inhibitory effect on angiogenesis. Furthermore, there is production of TGF-β, macrophage inflammatory protein-2, and CXCL2 in the spleen. These in turn attract and bind to natural killer (NK) T cells and stimulate the production of TGF-β, IL-10, TSP-1, and CC-ligand 5. In the spleen there is formation of clusters of T cells and subsequent induction of regulatory T cells, or ACAID-inducing-T regulatory cells. CD8+ T regulatory cells downregulate Th1 and Th2 effector functions in the eye, whereas CD4+ T regulatory cells inhibit differentiation of Th1 cells in the lymph node.

#### OCULAR GROWTH FACTORS, CYTOKINES, NEUROPEPTIDES, AND SOLUBLE IMMUNE FACTORS

Cytokine signaling and growth factor signaling are important factors that determine the functions of the cornea. In the limbal epithelium, there is a high expression of growth factors that have a role in normal human keratinocyte proliferation. They are members of the epidermal growth factor family, namely, transforming growth factor-α (TGF-α), amphiregulin, epiregulin, and hepatocyte-binding epidermal growth factor [33].

Human cornea epithelium has also been found to be protective against inflammation by production of glial cell-derived neurotrophic factor (GDNF), which suppresses IL-17-mediated in-

flammatory pathways [34]. It is also evident that limbal epithelial and stromal cells play a complementary role in limbal stem cell maintenance. GDNF, a member of the TGF-β superfamily, is abundant within the cornea stroma and is believed to mediate epithelial migration during corneal epithelial wound healing [35].

Cornea epithelial cells have been found to have receptors to GDNF (GFRα-1), which is involved in activation of several intracellular signaling pathways, including the focal adhesion kinase and mitogen-associated protein kinase pathways. Thus, as an inducer to these pathways, GDNF plays an important role in cell migration and corneal wound healing [36]. Neurotrophic growth factors (NGFs) are also important in the homeostasis of corneal epithelium and wound healing [37].

Cornea epithelial cells produce proallergic cytokines, including the thymic stromal lymphopoietin, which is believed to link innate and adaptive immune mechanism in ocular surface [15]. It also recognizes pathogens and becomes activated by cytokines through a family of TLRs [38] and by activating the nuclear factor-κB signaling pathways [15]. This signaling pathway is involved in many cellular processes, including immune response, cellular survival, proliferation, and differentiation (reviewed by Ghosh and Karin [39]).

The highly expressed chemokines observed in limbal epithelial cells include cytokines such as chemokine (C-X-C motif) ligands 1 (CXCL1), 2, 3, 10, and 11 [33]. CXC chemokines are subdivided into two categories, those with a specific amino acid sequence (or motif) of glutamic acid-leucine-arginine (ELR-positive) or those without an ELR motif (ELR-negative). ELR-positive CXC chemokines act through their chemokine receptor, CXCR2 [40], which is chemotactic for neutrophils.

CXCL1, CXCL2, and CXCL3 are expressed by macrophages, neutrophils, and epithelial cells and have neutrophil chemoattractant activity. CXCL1 elicits its effects by signaling through the chemokine receptors CXCR1 and CXCR2, whereas CXCL2 and



CXCL3 signal through the receptor CXCR2. They are involved in the processes of angiogenesis, inflammation, wound healing, and tumorigenesis [41, 42]. CXCL10 and CXCL11 are reactive to activated T and NK cells, which elicit their response through the chemokine receptor CXCR3. Additionally, CXCL10 is a potent inflammatory mediator that has been implicated in severe inflammatory diseases such as multiple sclerosis [43] and autoimmune thyroiditis [44].

Cytokines and chemokines present biphasic dynamic changes during the post-transplantation period in a rat model [45]. In the early phase, there was no significant difference in the elevated levels of the cytokines studied between the allograft and syngeneic grafts. However, these cytokines returned to pre-transplant levels after day 13 in the syngeneic group, whereas the levels of Th1 cytokines (IL-2, IL-12 p40, and IFN- $\gamma$ ), Th2 cytokines (IL-4, IL-6, IL-10, and IL-13), and anti-inflammatory/Th3 factors (TGF- $\beta$ 1/1 and IL-1RA) were increased in allografts [45].

The immunomodulatory factors also include cell surface molecules of the cornea and iris-ciliary body, which are the neuropeptides (i.e.,  $\alpha$ -melanocyte-stimulating hormone [ $\alpha$ -MSH], vasoactive intestinal peptide, and somatostatin). Somatostatin mediates the anti-inflammatory effect by the induction of regulatory T cells. These T cells produce TGF- $\beta$  and  $\alpha$ -MSH, and through  $\alpha$ -MSH production, regulatory T cells inhibit the proinflammatory action of IFN- $\gamma$  [46]. Together,  $\alpha$ -MSH and TGF- $\beta$  suppress macrophage activity and recruitment of neutrophils, thus exerting their anti-inflammatory effects.

In addition, the constitutive presence of tissue-associated CD95 ligand (FasL) molecules on ocular surfaces is an important aspect of ocular immune privilege [47]. The expression of FasL molecules preserves ocular immunity by suppressing the damage from inflammation, especially viral in nature [48] and in allogeneic rejections [21, 47]. In the latter, FasL interacts with Fas expressed on leukocytes, resulting in apoptosis induction. Collectively, the microenvironment in the eye provides not only factors that suppress the inflammation but also cells and molecules that are involved in immune regulation.

#### MAJOR HISTOCOMPATIBILITY COMPLEX EXPRESSION

The MHC gene complex presents antigens to T cells and determines the compatibility of donors for organ transplants. The human leukocyte antigen (HLA) system represents MHC, which encodes genes related to the immune system, and cell surface antigen-presenting proteins. MHC class II antigen presentation pathways are expressed by professional APCs, such as dendritic cells, macrophages, B cells, or epithelial Langerhans cells (LCs).

A critical factor influencing corneal graft rejection is the presence and the high density of such dendritic cells in the graft (donor APCs). Avoiding placement of a graft near the limbal area where the APCs are abundant is a logical surgical strategy in this instance. An approach to donor-specific immunosuppression by regulatory T cells to achieve graft tolerance may be taken to eliminate the risk of a violent rejection when donor and recipient dendritic cells coexist.

The most important antigen-presenting cells in the cornea are bone marrow-derived MHC class II<sup>+</sup> Langerhans cells, which are responsible for the sensitization of the host to alloantigens. Under normal conditions, LCs reside in the limbal area but may migrate to the cornea in response to inflammatory conditions [49]. Activation of alloreactive T cells is dependent on the anti-

gen-presenting cells for the host to recognize foreign antigens on the grafts.

There is uneven distribution of Langerhans cells, which express HLA class II molecules in the cornea; this could possibly be the reason behind the prolonged survival of these grafts [50]. LCs were originally thought to be exclusively abundant in the limbal and conjunctival region, but not at the central cornea [51, 52]. HLA class II expression in normal corneal stroma and endothelia is also lacking and is mostly confined to the limbal region of the epithelium [53]. Expression of class II is also absent without stimulation by inflammatory mediators [54]. However, recent findings demonstrated that a heterogeneous population of immature and precursor dendritic cells also reside in the central corneal region [50]. These cells, labeled MHC class II-negative, are believed to be progenitor or immature LCs. Although these immature cells are capable of antigen uptake and processing, they are incapable of furnishing naïve T cells with the requisite B7 costimulatory molecules and thus unable to activate T cells [55]. Active suppression of LC maturation could be an important mechanism of corneal immune privilege.

Not surprisingly, there is a low incidence of corneal graft rejection although HLA matching of donor and recipient is not normally performed, especially in low-risk grafts [56]. In a study performed by the Collaborative Corneal Transplantation Studies Group in high-risk corneal transplantation patients, HLA-A, -B, and -DR matching did not reduce the rate of graft failure [56]. However, a more recent finding showed a strong correlation between HLA mismatch and rate of graft rejection in low-risk as well as high-risk patients [57]. Although matching of HLA-A and -B did not significantly affect the rejection rate in the low-risk group, it certainly benefited the high-risk group. Donor HLA class I antigens become the target of host CD8<sup>+</sup> T lymphocytes when they are HLA-A and/or -B mismatched [57]. HLA-DR matching was also demonstrated to significantly reduce allograft rejection by 40% 3 years after surgery in the high-risk group [57]. The disadvantages of HLA matching include the costs and extended waiting time for suitable grafts. However, these need to be seriously weighed against the risk of failed grafts and further therapeutic complications, regrafting, and the overall impact on the pool of available donated cornea.

Despite encouraging results from MHC matching, grafts can still be rejected because of incompatibility in the minor MHC antigens. Th1 pathway and IFN- $\gamma$  production was shown to be the mechanism involved in rejection of MHC-matched corneal allografts where minor MHC antigens are presented to the host [18]. Minor MHC antigens are naturally occurring polypeptides processed and presented by MHC molecules, shown to increase risk of graft failure in grafts-versus-host disease. Minor MHC antigens induce activation of T cells and also play an important role in allograft rejection of solid organs following transplantations [58–60]. Minor H antigens are loaded to MHC molecules in corneal grafts allowing for indirect T-cell allorecognition [61]. Alloreactive T cells recognize allogeneic peptides indirectly when they are introduced to self-MHC class I and II molecules. On the other hand, direct allorecognition occurs when T cells recognize donor alloantigens directly, irrespective of the peptides associated with the MHC molecules.

Experimental stimulation of human corneal epithelial cells with recombinant human IFN- $\gamma$  demonstrated increased expression of MHC class II antigens [62, 63] and significant ability to stimulate allogeneic lymphocytes [63]. Another study revealed

**Table 1.** Percentage of human telomerase-immortalized corneal epithelial cells expressing HLA-A,B,C, HLA-DR, HLA-DP, and HLA-DQ antigens stimulated by interferon- $\gamma$  at 10 ng/ml for 3 days analyzed by flow cytometry

HLAs	Unstimulated (%)	Average stimulated (%) (n = 2)	Standard deviation ( $\pm$ )
HLA-A,B,C	0.5	95.1	0.71
HLA-DR	0.6	91.75	1.06
HLA-DQ	0.4	32.35	7.42
HLA-DP	0.6	73.06	3.8

Abbreviation: HLA, human leukocyte antigen.

that corneal epithelium and endothelium express MHC class II antigens when stimulated by IFN- $\gamma$ , whereas IFN- $\gamma$  had no effect on the expression of MHC class I antigens [64].

A preliminary study in our laboratory using human telomerase-immortalized corneal epithelial cells [65–67] showed that HLA-A,B,C, HLA-DR, HLA-DQ, and HLA-DP antigens were significantly upregulated following IFN- $\gamma$  stimulation at a concentration of 10 ng/ml for 3 days (Table 1) compared with unstimulated cells.

#### LIMBAL STEM CELL TRANSPLANTATION

Replacement of donor cells by the host-derived cells is a special feature of corneal transplantation that takes place 1–2 years after surgery [68]. In most cases, all types of donor cells (epithelial, stromal keratocytes, and endothelial layers) were observed to be taken over by the host cells [69]. This is explained by the presence of a potentially proliferative and self-renewal capacity of the corneal stem cells (reviewed in [70, 71]). In addition, cellular therapies such as ex vivo corneal epithelial transplantation show impressive potential as a future therapy [7, 8, 72]. Endothelial cell transplantations have also shown promising results in animal studies [73–76].

The X, Y, Z hypothesis [77] has emphasized our understanding of the limbal stem cell maintenance. It clarifies the epithelial cell renewal to maintain its homeostasis. The hypothesis states that aged or damaged central corneal epithelial cells (through exfoliation, trauma or disease) are continuously replenished through centripetal migration of limbal stem cells. The balance between cell proliferation and cell loss is directed by the equation  $X + Y = Z$ , where X represents basal corneal epithelial cell movement toward the surface, Y represents movement of peripheral epithelial cells toward the central cornea, and Z represents desquamation of the surface epithelium (Fig. 3). Since then, Majo et al. have challenged the hypothesis by proposing that opposing dynamic factors confronting on the limbus result in limbal stem cells being confined, but not exclusively, to the limbal region [78]. Despite this controversy, evidence that corneal limbal stem cells reside in the basal layer of the limbus has been well documented [79–83]. When comparing the proliferative potential of limbal versus central corneal cells, limbal cells showed greater growth rates than corneal cells when they were serially transplanted [84, 85].

Cells from the limbal region respond efficiently to cornea wound, whereby they have been shown to migrate centripetally [78]. Furthermore, removal of the corneal limbus diminishes the capacity of cornea to sustain itself [83, 86]. Supporting evidence also comes from the fact that there is an absence of major corneal epithelial differentiation markers (keratins K3 and K12) in limbal basal cells. The exclusive location of slow-cycling and label

retaining cells in the limbal basal layer was also observed in mouse limbal epithelium [87].

The location of the limbal stem cells has been identified under the surface of palisades of Vogt, now termed the limbal epithelial crypts. These are papilla-like columns, richly vascularized and highly innervated. They extend radially into the conjunctiva or circumferentially at the periphery of the cornea [88]. It has been estimated that up to six limbal epithelial crypts may be present in the human cornea. The unique structure of the column and the undulating junctions between limbal epithelium and stroma also protect the cells from shear forces.

Limbal stem cells, irrespective of the states they are in (quiescent state, apoptotic, dividing, or differentiated), are dependent on the interaction between the cell and its microenvironment [81]. Thus, the underlying stroma and local blood supply make up the limbal stem cell niche, containing factors that determine the stem cell fate. Melanin pigments, which cause dark discoloration in these cells, offer protection against possible insults of ultraviolet light and the generation of reactive oxygen species. The presence of limbal blood vessels close to the underlying stroma also provides nourishment to the cells. The limbal stroma is also highly innervated. These characteristics make the limbal region an ideal protective microenvironment and fulfill the requirements of a stem cell niche (Fig. 3).

The respective role of nerves and blood vessels in the niche remains to be fully defined. Evidence is accumulating that suggests the presence of stromal mesenchymal cells as part of the stem cell niche, possessing immunological privilege [89, 90].

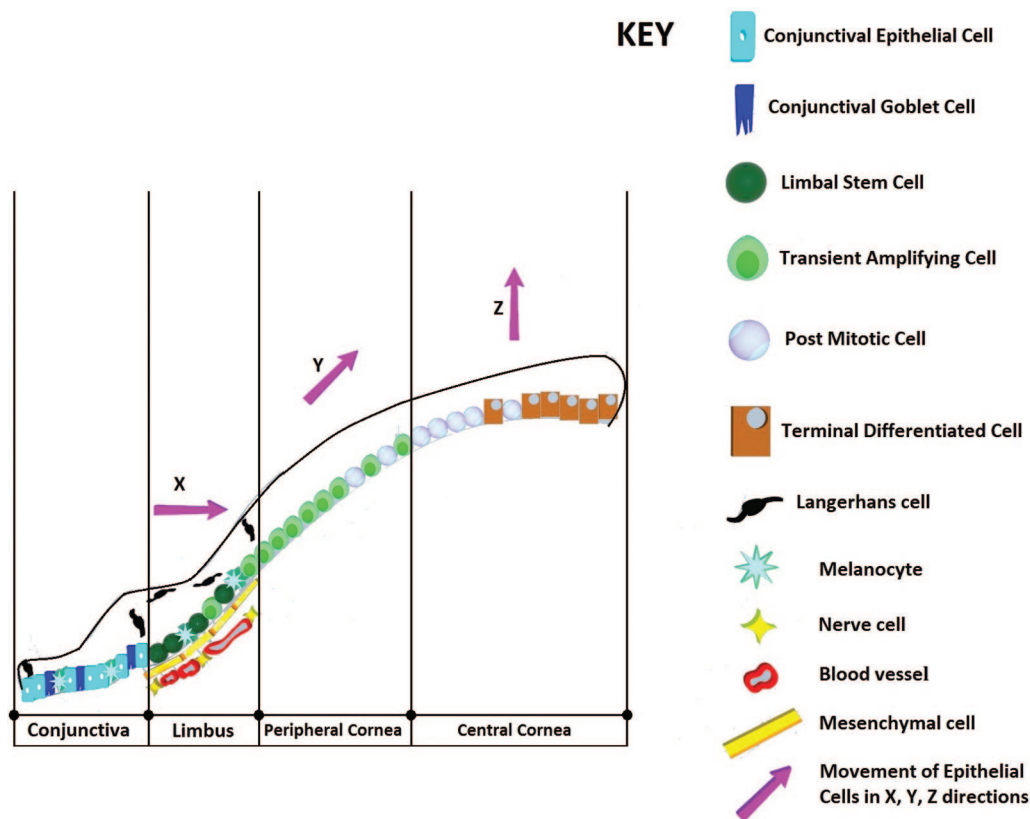
#### LIMBAL STEM CELLS AS A POTENTIAL THERAPEUTIC STRATEGY TO OVERCOME REJECTION

Tolerance toward allogeneic organ grafts represents one of the major aims of transplantation medicine. Stem cells are promising candidates for promoting donor-specific tolerance [91]. Adult stem cells have immune-suppressive mechanisms to protect them from immunological reactions and apoptosis, which are damaging to the survival of the transplanted tissues (reviewed in [92]).

In a study conducted in adult mice, a small population of limbal stem cells (LSCs) effectively inhibited lymphocyte proliferation and modulated cytokine production. LSCs were also noted to display an enhanced expression of genes for the antiapoptotic proteins such as induced myeloid leukemia cell differentiation protein (Mcl-1), B cell lymphoma-2 (Bcl-2), X-linked inhibitor of apoptosis protein (XIAP), and survivin. They were also more resistant to CTL-mediated or apoptotic cell death compared with other limbal cells that are necessary for tissue survival [93]. These regulatory properties are important to safeguard limbal stem cells from the damaging effects of inflammatory processes and cell toxicity in the anterior chamber of the eye.

#### ROLE OF LIMBAL AND CORNEAL STROMAL MESENCHYMAL STEM CELLS IN CELLULAR THERAPY

Corneal stroma makes up 90% of the corneal volume and consists of tightly packed lamella of collagen fibrils. The size of collagen fibrils and the organization of these densely packed structures contribute to corneal transparency. Corneal stroma is



**Figure 3.** Limbal stem cell niche and the X, Y, Z hypothesis of corneal epithelial maintenance.

populated by mesenchymal keratocytes, which are quiescent after birth but undergo differentiation in response to wound, trauma, inflammation, or infection [94], which suggests plasticity of the cells to adapt to their environment.

Mesenchymal stem cells are nonhemopoietic stem cells that have regenerative ability and can differentiate into cell lineages of mesenchymal origin (i.e., adipocytes, chondrocytes, and osteocytes). Bone marrow-derived mesenchymal stem cells have shown excellent anti-inflammatory and wound healing properties when introduced to corneal injury models in animal studies [95–100]. Bone-marrow-derived mesenchymal stem cells (MSCs) have been investigated in the repair of corneal damage [99], and the proposed mechanism is suppression of new vessel formation and inflammation post-transplantation.

Evidence shows that graft is populated by host stromal cells [68] after corneal transplantation, which suggests that corneal keratocytes have potential stem cell properties [89]. Immunological privilege of corneal stromal stem cells was supported by the absence of T cell-mediated immune rejection, when these stem cells were injected in mouse corneal stroma [101]. The proliferative capacity of corneal stroma stem cells and their immune privilege clearly play an important role in corneal stromal tissue engineering [102–104].

Limbal epithelium also possesses stromal cells, which are adherent to plastic, have fibroblastic morphology, and are able to differentiate into multiple lineages suggestive of mesenchymal stem cells [89, 90, 105]. These cells can be isolated using protocols as described previously [106]. Limbal stromal MSCs grown in tissue culture also demonstrated inhibition of T-cell proliferation, and this is believed to be mediated by soluble factor(s) such

as TGF- $\beta$  [105]. Furthermore, limbal stem cell isolation and culture conditions can be enriched with other limbal niche cells or corneal stromal stem cells [107–109] or by using a side population discrimination assay, as a future cell therapy [101].

#### METHODS TO PREVENT REJECTION

Strategies to reduce the rejection rate must take into consideration immunosuppressive reagents and gene therapy. The use of anti-inflammatory mediators is an additional strategy to prolong survival of grafts. Soluble TNF- $\alpha$  receptor type I in a topical instillation form has shown favorable results in reducing murine allogeneic reactions [110]. TNF- $\alpha$  is a potent proinflammatory cytokine in alloimmune response. It is a macrophage-derived cytokine responsible for expression of adhesion and costimulatory molecules, neutrophil activation, chemokine stimulation, and inducing the nuclear factor- $\kappa$ B signaling pathway.

More recently, administration of IL-1 receptor antagonist (IL-1Ra) has been shown to be superior to corticosteroid immune suppression in mice allogeneic grafts [111, 112]. IL-1 is involved in mediation of acute phase reactions, chemotaxis, stimulation of new vessels, and, most importantly, the migration and recruitment of LCs. IL-1Ra is produced in abundance at the apical epithelial region in fresh cornea [113]. This potent inflammatory cytokine is derived from monocytes, macrophages, and resident corneal cells and expressed at high levels during corneal transplantation immune response. IL-1Ra has the effect of neutralizing IL-1 by suppressing LC migration, thus prolonging survival of allografts. Interestingly, the success of experimental ex vivo transduction of IL-1Ra in rat cornea will be a new landmark in

corneal gene therapy to treat corneal inflammatory conditions and corneal transplantation [114].

NGF gene therapy in combination with a cytotoxic T lymphocyte antigen 4 immunoglobulin G (CTLA4Ig) has shown to prolong rodent corneal allografts survival by mediating its anti-inflammatory and antiapoptotic pathways [115]. This is not surprising since NGF and its receptors are abundantly present in a densely innervated organ like the cornea and are responsible for the wound healing process [38]. In other pharmacological developments, suppression of inflammation by IL-10 [116] and inhibition of intercellular adhesion molecule-1 or its ligand [117] are prospective potential therapies.

## CONCLUSION

A challenging aspect of limbal stem cells is the absence of specific markers to uniquely identify these stem cells and to distinguish them from other adult stem cells, or limbal stem cells derived from transient amplifying cells. This necessitates a characterization process involving several modalities, such as reverse transcription-polymerase chain reaction, immunochemistry, or cell cycling methods. A consistent isolation method for limbal stem cells should also be instigated, to include protocols to enrich for limbal stem cells, such as functional phenotypic assays like the side population assay.

Ex vivo expanded limbal epithelial transplantation has proven to be able to reconstruct the ocular surface in limbal stem cell-deficient eyes. However, this method needs to be further refined to include protocols to assess tissue viability, the quality of tissue constructs, and safety assessment. In order for limbal stem cells to be successfully translated into favorable clinical outcomes, a good manufacturing practice-compliant protocol would be a positive step toward achieving high-quality tissues to fulfill the requirements for clinical transplantation [118].

Currently, it is still unclear how restoration of the damaged ocular surface takes place after limbal stem cell transplantation. It is very unlikely that it is due to replacement of stem cell numbers alone. Studying the limbal stem cell fate in different etiologies of stem cell deficiency and the types of tissue transplantation would be a future direction to explain the process of cellular restoration. At present, there is no consensus on limbal stem cell fate in different types of transplantation [119–121] such as in penetrating keratoplasty, alone or in combination with limbal allograft transplantation, or in the case of ex vivo limbal stem cell transplantation. The problem for case-to-case comparison exists in multiple diagnosis of limbal stem cell deficiency, tissue transplantation types, methods for cell tracking to determine the cellular fate, and nonstandard cut off points for cellular analysis to take place.

Importantly, the time taken for normalization of corneal epithelia after transplantation without trace of donor cells would

change the type and duration for immunosuppression postsurgery [119]. The use of appropriate immune suppression and the duration of administration would ensure survival of the grafts without compromising the general well-being of the recipient.

Since cornea is the most frequently transplanted organ, the importance of understanding corneal immunology is of considerable interest. A major issue with whole cornea transplantation, allogeneic limbal tissues, or ex vivo limbal stem cell transplants remains their immunogenicity. Questions arise as to whether the ocular immune system is similar in all regions of the eye, and whether the immune privilege status of the anterior chamber applies to other ocular tissues. It would be interesting to explore immunogenicity of grafts placed on the cornea in an ocular reconstruction surgery where no breach of the anterior chamber happens at all.

Further investigation on the immunological factors in the cornea that allow tolerogenic potential of the organ to respond to ocular antigens is necessary to exploit corneal immune privilege to its full advantage. Identification of inflammatory molecules and apoptotic markers, identification of their signaling pathways, and identification of the role of HLA matching are some of the strategies to promote the survival of whole corneal and limbal stem cell transplantation. Furthermore, differential expressions of HLAs during the inflammation and rejection process may elucidate the role of antibodies alone or in combination with other soluble factors in donor antigen-specific immune suppression.

Molecular mechanisms regulating the immune plasticity of corneal stem cells could be relevant in the clinical setting and may explain the differences in the outcomes between murine and human systems in vitro and in vivo. Thus, understanding these mechanisms will contribute to the development of better therapeutic approaches for transplantation not only of the cornea but of other organs as well.

## ACKNOWLEDGMENTS

We thank Dr. Charles Osei-Bempong and Mazen Darwiche for assistance with the graphics.

## AUTHOR CONTRIBUTIONS

B.S.: conception and design, collection and/or assembly of data, manuscript writing; S. Ahmad: conception and design; A.M.: conception and design, final approval of manuscript; S. Ali: conception and design, collection and/or assembly of data, manuscript writing, final approval of manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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