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REVIEW

High-risk corneal allografts: A therapeutic challenge

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Abstract

Corneal transplantation is the most common surgical procedure amongst solid organ transplants with a high survival rate of 86% at 1-year post-grafting. This high success rate has been attributed to the immune privilege of the eye. However, mechanisms originally thought to promote immune privilege, such as the lack of antigen presenting cells and vessels in the cornea, are challenged by recent studies. Nevertheless, the immunological and physiological features of the cornea promoting a relatively weak alloimmune response is likely responsible for the high survival rate in "low-risk" settings. Furthermore, although corneal graft survival in "low-risk" recipients is favourable, the prognosis in "high-risk" recipients for corneal graft is poor. In "high-risk" grafts, the process of indirect allorecognition is accelerated by the enhanced innate and adaptive immune responses due to pre-existing inflammation and neovascularization of the host bed. This leads to the irreversible rejection of the allograft and ultimately graft failure. Many therapeutic measures are being tested in pre-clinical and clinical studies to counter the immunological challenge of "high-risk" recipients. Despite the prevailing dogma, recent data suggest that tissue matching together with use of systemic immunosuppression may increase the likelihood of graft acceptance in "high-risk" recipients. However, immunosuppressive drugs are accompanied with intolerance/side effects and toxicity, and therefore, novel cell-based therapies are in development which target host immune cells and restore immune homeostasis without significant side effect of treatment. In addition, developments in regenerative medicine

may be able to solve both important short comings of allotransplantation: (1) graft rejection and ultimate graft failure; and (2) the lack of suitable donor corneas. The advances in technology and research indicate that wider therapeutic choices for patients may be available to address the worldwide problem of corneal blindness in both "low-risk" and "high-risk" hosts.

Key words: "High-risk" grafts; Graft rejection; Systemic immunosuppression; Cell-based immunomodulation; Keratoprosthesis; Collagen-based hydrogels

Core tip: Corneal grafts enjoy a high acceptance rate when performed in "low-risk" host graft beds. This is associated with a relatively weak alloimmune response. However, in "high-risk" hosts where the immunologically quiescent homeostatic environment of the cornea is compromised prior to graft procedure, heightened immune responses significantly increase the risk of graft rejection. Clinical approaches such as tissue matching and long-term immunosuppression could be beneficial in preventing graft rejection especially in "high-risk" settings. In addition, promotion of transplant tolerance by cell-based therapies and use of corneal "substitutes" such as collagen-based hydrogels are promising alternatives for "high-risk" recipients.

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INTRODUCTION

Corneal transplantation is the most common and successful form of solid organ transplantation[1]. It is considered the primary treatment to restore vision to patients with corneal blindness - a leading cause of blindness worldwide[1]. In the year 2014-2015, 3520 cases of corneal transplantation were performed in the United Kingdom compared to 2069 cases of kidney and 842 liver transplantations[2]. The corneal graft survival rate is 86% at 1-year for penetrating keratoplasty (PK), despite the fact that corneal grafts are rarely tissue matched for histocompatibility leukocyte antigens (HLA) and systemic immunosuppressant medications are not routinely used[3]. However, the 15-year graft acceptance declines to 55%, which is similar to survival rates in other forms of solid organ transplantation[3,4]. More importantly, corneal grafts performed in "high-risk" recipients have a much reduced acceptance rate with a 5-year survival of 54.2% compared to 91.3% in recipient eyes that have not been overtly inflamed. The "high-risk" recipients were defined by the Collaborative Corneal Transplantation Studies Research Group as two or more quadrants of the cornea vascularized or a previous graft had been rejected[5,6]. Unfortunately, any previous inflammatory response in the ocular surface such as corneal infectious diseases (*e.g.,* herpetic simplex keratitis or trachoma), severe trauma, alkali burn and previously failed graft place the host cornea at risk of corneal neovascularization[7,8]. Furthermore, "high-risk" recipients not only experience higher graft failure rate but also present with more frequent acute rejection episodes compared to "low-risk" grafts[7].

It is worth emphasizing here the difference between corneal graft failure and corneal graft rejection. In brief, clinical corneal graft failure is the irreversible loss of graft clarity, and rejection is one of the causes of corneal graft failure. However, the loss of graft clarity can be due to a number of reasons including infection, surgical trauma, glaucoma, aging as well as rejection, which is an exclusively immunological event. Graft rejection is moreover the most common cause of graft failure accounting for over 30% of cases[3,4]. The characteristic features of corneal graft rejection in which there is an immunological response against donor antigens are graft oedema, keratic precipitates on the endothelium of the transplanted graft and the presence of rejection lines [formed due to accumulation of inflammatory cells on corneal epithelium or endothelium (Khodadoust line)] together with the presence of inflammatory cells in the anterior chamber (AC) of the eye[9,10]. This review article focuses on the mechanism of corneal graft rejection revealed through experimental studies as well as current and potential treatments for corneal graft rejection.

EXPERIMENTAL CORNEAL ALLOGRAFT

The immunological responses mediating corneal graft rejection have been studied extensively using animal models, and especially in the well-established murine model of full-thickness orthotopic corneal transplantation. Similar to human corneal grafting, murine corneal allografts performed in an uninflamed graft bed, despite being mismatched for both major and minor histocompatibility complex antigens, half of the grafts failed, whereas in the inflamed "high-risk" graft bed, almost all of the grafts failed and with an increased tempo depending on the level of major histocompatibility complex (MHC)/non-MHC antigen mismatch[11,12].

The rejection mechanism of corneal allograft

Corneal allograft rejection represents a form of delayed-type hypersensitivity (DTH) response, predominantly mediated by allospecific CD4+ T cells. The response can affect one or more of the three cellular layers in the cornea (epithelium, stroma and endothelium)[13-15]. However, the endothelial layer is

the main target in PK with graft failure occurring when > 50% of the corneal endothelium is lost[16,17]. As the corneal endothelium possesses limited regenerative property and is the essential layer responsible for maintaining corneal deturgescence, alloimmune responses directed at the corneal endothelium eventually result in stromal and epithelial oedema and with irreversible corneal opacification[16].

During the surgical procedure, trauma to corneal tissues induces local production of cytokines and chemokines such as interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-10 and CXCL2 which initially peaks at day 3-5 post graft procedure[18]. Meanwhile, infiltration of innate immune cells occurs into the cornea including dendritic cells (DC), macrophages, natural killer (NK) cells and neutrophils[19]. A unique feature of corneal allograft compared to other forms of solid organ transplantation is that the rejection response is mediated almost exclusively through the indirect pathway as the healthy central donor cornea possesses low numbers of antigen presenting cell (APC). Therefore, the activation of naïve T cells occurs predominantly through host APC newly recruited from the bone marrow and presenting donor antigenic peptides, including HLA antigens to host naïve T cells. In contrast, the direct pathway involves the direct recognition of alloantigen on donor origin APC which have migrated from the graft tissue to the local draining lymph nodes (DLN), by host naïve T cells[20,21]. Newly recruited bone marrow APC after processing antigens from the corneal allograft then migrate *via* lymphatic vessels to the DLN where they activate naïve T cells and mediate immune rejection against corneal graft.

Corneal allograft rejection is predominantly mediated through CD4+ Th1 cells that secrete cytokines IFN-γ, tumour necrosis factor (TNF)-α and IL-2[14,22]. In the rejected graft, abundant neutrophils, macrophages and CD4+ T cells are present[23]. Furthermore, studies have suggested that CD4+ T cells may function directly as effector cells mediating graft rejection as adoptive transfer of allogeneic CD4+ T cells to beige nude mice (impaired T cell production, but do produce macrophages) resulted in graft rejection even when macrophages were depleted[24]. Although *in vitro* experiments showed the ability of allo-specific CD4+ T cells to induce apoptosis of corneal endothelial and epithelial cells, investigations of the involvement of perforin or Fas-induced apoptosis by CD4+ T cells have eliminated both mechanisms[24]. In addition, allografts deficient in Fas-ligand (FasL or CD95L) demonstrated 100% rejection, further indicating that mechanisms other than Fas-FasL were used by CD4+ T cells in mediating graft rejection while FasL expressed in the cornea was more likely to promote immune privilege[25]. Nevertheless, prolonged exposure to proinflammatory Th1 type cytokines IFN-γ, TNF-α and IL-1 was shown to induce apoptosis of corneal endothelium and upregulation of inducible nitric oxide synthase, the latter generating nitric oxide which causes direct cytotoxicity to endothelial cells[26]. In addition, inhibition of inducible nitric oxide synthase showed protection against cytokine-mediated corneal tissue damage as well as prolonged allograft survival when administered systemically[26,27]. However, studies investigating the role of Th17 cells in mediating corneal allograft rejection have shown controversial results. While some studies showed that IL-17 demonstrated pathological effect during early corneal allograft rejection[28], recent findings have suggested that Th17 cells are involved in promoting allograft acceptance in the early post graft stages followed by a Th1 dominant response mediating graft rejection[29,30]. Interestingly, further investigation also indicated that enhanced expression of IL-17 at a late stage (> 45 d) post corneal allograft impaired graft survival. Late stage anti-IL-17 treatment not only reversed corneal opacity but also reduced the level of neovascularization[30]. Strikingly, IL-17 knockout mice that received anti-IFN-γ treatment failed to reveal any significant difference in graft survival compared to wild type mice. This indicates that mechanisms other than Th1 and Th17 cells were involved, which may be due to the redundancy of the immune system promoting an alternative and exaggerated Th2 response capable of mediating graft damage[29,31].

Is the success of unmatched corneal allografts due to immune privilege?

The relatively high acceptance of corneal allografts compared to other forms of solid organ transplantation has been largely ascribed to the immune privilege of the eye[32,33]. Immune privilege was a term coined by Sir Peter Medawar in the 1940s where skin allografts placed in the AC of the eye evaded immunological rejection but only if the graft was not invaded by blood vessels[34]. Extensive study of this phenomenon ascribed immune privilege especially in the context of corneal allograft to: (1) the reduced expression of MHC class Ⅰ molecules in corneal tissue and the lack of constitutive MHC class Ⅱ expression; (2) the absence of both blood and lymphatic vessels in the cornea; (3) the lack of "passenger leukocytes" in the cornea; (4) presence of immunoregulatory molecules in the AC and on corneal cells; and (5) anterior chamber-associated immune deviation (ACAID) induced post corneal allograft[32,33]. However, recent studies have shown that the corneal tissue possesses a population of MHCⅡ+ leukocytes with increased numbers towards the peripheral cornea[20,35-40]. Furthermore, corneal neovascularization rapidly develops post corneal grafting; within 1 wk, both blood and lymphatic vessels are already invading the donor cornea thus providing access of immune cells to the cornea as well as increasing homing of APC to the DLN. Furthermore, vessels persist regardless of the fate of the graft (Figure 1)[11]. This means that unmatched corneal allografts

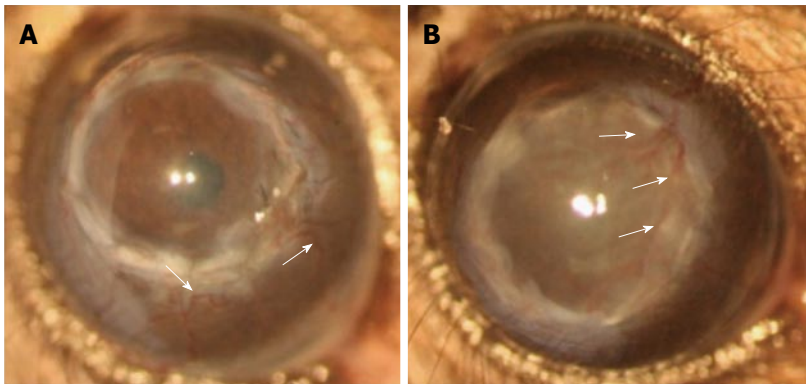

Figure 1 Corneal allografts in C57BL/6 mice. (A) Accepted and (B) rejected corneal allografts (Balb/c donor) in C57BL/6 mice demonstrating invasion of blood vessels (arrows); the rejected graft shows more blood vessels invading the donor graft.

are accepted in 50% cases indefinitely despite the presence of blood and lymphatic vessels and infiltration of host immune cells.

In contrast to immune privilege, which describes the local acceptance of grafts within the eye, ACAID is a systemic immune response. ACAID is an unusual suppression of the systemic immune system whereby alloantigen placed in the AC of the eye elicits a regulatory response in the spleen, which upon further exposure suppresses the immune response to the alloantigen (*e.g.*, skin graft), and prevents graft rejection[41]. This phenomenon has been shown to be mediated through CD8+ T regulatory cells (Treg) generated in the spleen[33]. It was believed that ACAID is induced not only when alloantigen is inoculated into the AC but also post corneal allograft due to shedding of alloantigenic materials from graft endothelial cells[42]. However, growing evidence suggested that Treg induced after corneal allograft show a phenotype of CD4+CD25+Foxp3+ whereas effector Treg in ACAID is CD8+ Treg[13,43,44]. Furthermore, blockade of CD8+ T cells only abrogated ACAID but with no effect on corneal allograft survival while blockade of IL-17A which reportedly impaired allograft induced Treg suppressive function also reduced corneal graft survival, but did not alter the induction of ACAID[43,45].

It is clear therefore that most of the proposed mechanisms to explain the phenomenon of immune privilege have proven not to be true. Instead, the prolonged acceptance in "low-risk" corneal allograft compared to other solid organ transplants may simply be due to the effect of an overall weak indirect alloimmune response as a result of the low levels of alloantigen acting together with local and systemic regulatory mechanisms. First, the insufficient strength of the alloimmune response in the initial stages of allosensitization is likely due to the limited number of donor derived passenger leukocytes particularly in the central cornea, and low expression of histocompatibility antigens. In addition, while other forms of solid organ transplants are rich in vascular networks and donor passenger leukocytes undergo both acute (direct pathway) and chronic (indirect pathway) rejection[46], corneal allograft rejection is predominantly mediated through the indirect pathway[47-50]. In the healthy cornea, the majority of MHC Ⅱ + cells are CD11b+ and CD11c+ cells distributed at the peripheral cornea whereas the central cornea which is used as donor cornea during corneal allograft procedure was believed to be devoid of MHC Ⅱ + cells but contains a population of MHC class Ⅱ negative immature DC and Langerhans cells[20,36-39]. Recently, studies using CD11c-eGFP mice have shown that a reduced number of MHC Ⅱ +CD11c+ cells are present in the central cornea and exclusively located in the corneal epithelial basal layer beneath which a layer of MHC Ⅱ +CD11b+ cells were also observed[40]. However, the expression level of MHC class Ⅱ molecules on these cells was found to be at a relatively low level indicating that these cells together with MHC class Ⅱ negative DC and Langerhans cells are more likely to promote immune tolerance rather than immunity[40]. We reported that in a "low-risk" setting, there was no evidence of donor leukocyte migration to the DLN[20]. Therefore, corneal allograft rejection in "low-risk" setting is exclusively mediated by indirect allorecognition. The lack of both blood and lymphatic vessels in initial stages post graft may delay the infiltration of host innate immune cells including APC, thus becoming a limiting factor for initiating a sufficient rejection response before the development of an established vessel network. Second, while new vessels invade the graft, other regulatory mechanisms including the induction of Treg come into play. It was found that rather than changes in frequency, the expression level of Foxp3 was significantly higher in the DLN of accepted allografts compare to either rejected or syngeneic grafts[44]. Moreover, adoptive transfer of Treg has been shown to promote corneal graft survival[51], associated with production of IFN-γ and IL-17A[45,52]. It was shown that IL-17A is required for the effective suppressive function of Treg in promoting allograft survival and unusually supports a protective role for Th17 cells during corneal allograft rejection[45]. Interestingly, IFN-γ was required for generation of Treg

under fully MHC and minor histocompatibility antigen mismatched condition, whereas IFN-γ inhibited the generation of allospecific Treg when only MHC or minor histocompatibility antigen was mismatched[52]. These somewhat puzzling findings suggest that possibly the balance between Th1, Th17 and Treg responses largely dictates the outcome of the graft. Consequently, when an effective peripheral tolerance response fails to be induced, the default balance favours a Th1 response and as such, promotes allograft rejection.

Lastly, the physiological milieu of the cornea and the anterior segment of the eye possess many immunoregulatory molecules that protect the cornea from immune mediated attack. For instance, FasL is expressed extensively in ocular compartments including all three cellular layers of the cornea[53,54]. Several studies have reported that FasL expressed in the eye is responsible for inducing apoptosis of infiltrating Fas-bearing leukocytes, especially lymphocytes. Furthermore, its expression in particular on corneal endothelial cells plays an important role in corneal allograft survival, since donor corneas lacking FasL in the endothelium and stroma but not epithelium were rejected vigorously compared to normal FasL expressing donor corneas[25,53,55,56]. Moreover, the interaction of Fas-FasL induced apoptotic cell death was shown to be an important mechanism in the induction of immunological tolerance to antigens injected into the AC, as in the absence of apoptotic cell death, immune tolerance failed to be elicited[55]. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is also capable of inducing apoptosis of various tumour cells and its functional expression was demonstrated in corneal tissue[57]. Overexpression of TRAIL in donor corneal tissue has been shown to significantly delay graft rejection, accompanied by an increased number of apoptotic cells in the graft[58]. However, other groups in attempts to establish a correlation between TRAIL expression and allograft survival have not found an effect[13].

Programmed death ligand-1 (PD-L1 or B7-H1) is another molecule with similar functions to FasL and TRAIL by promoting apoptosis of infiltrating PD-1 positive CD4 and CD8 T lymphocytes[59]. PD-L1 belongs to the B7 superfamily providing costimulatory signals to T cells and is constitutively expressed in both murine and human corneal tissues[59-61]. Its blockade or deficiency is associated with increased corneal graft rejection whereas strong ligation between PD-L1 and PD-1 revealed prolonged allograft survival[59-62].

Complement regulatory proteins were found to be expressed by corneal tissues and in the AC, which protects the cornea from being the target of complement-fixing antibodies[63,64]. One such molecule strongly expressed in the corneal epithelium is decay-accelerating factor (DAF) which function is to inhibit complement deposition on the cell surface, thus preventing autologous complement activation[63,65]. Further studies have suggested that DAF shows regulatory properties towards the T cell response[66]. DAF deficiency on donor or recipient cornea accelerated graft rejection together with increased numbers of IFN-γ producing T cells, reduced levels of transforming growth factor (TGF)-β and IL-10[66]. Furthermore, NK cells attack cells that lack the expression of MHC class Ⅰ molecules and the poor expression of MHC class Ⅰ by corneal endothelial cells makes them prone to NK cells mediated tissue damage[13,67]. However, studies have shown that the AC contains NK cell inhibitory factors such as macrophage migration inhibitory factor and TGF-β, which prevent corneal endothelial cells becoming targets for NK cells[13,68,69]. Galectin-9 was demonstrated as another immunosuppressive molecule constitutively expressed on corneal tissues, which significantly promoted corneal allograft survival by inducing apoptosis of alloreactive T cells[70].

Many other immunoregulatory molecules present in the anterior segment of the eye have also been demonstrated to have potential in prolonging corneal allograft survival including alpha-melanocyte stimulating hormone, calcitonin gene-related peptide, vasointestinal peptide, somatostatin or indoleamine dioxygenase[71-73].

Elevated innate and adaptive immune responses in "high-risk" corneal allograft promote graft rejection

Although clinically and experimentally, there are many causes of a "high-risk" graft bed, a common denominator is an already activated immune system both systemically and locally (cornea and eye-DLN) providing a proinflammatory milieu unlike the situation in "low-risk" dormant recipients. In general, murine corneal allografts performed in "high-risk" recipients not only experience over 95% graft rejection rates compared to 50% in "low-risk" recipients, but in addition grafts are usually rejected rapidly, 2 wk post-surgery compared to 3-4 wk in uninflamed corneas[12]. As early as 24 h post corneal allograft, increased levels of chemokine mRNA expression including CCL2 and CXCL2 were observed in "high-risk" recipients compared to "low-risk" recipients[74]. No difference in the number of infiltrating leukocytes was observed between "high-risk" and "low-risk" recipients at day 1 suggesting the source of the early increased chemokine levels was from resident corneal cells[74]. Increased numbers of infiltrating macrophages and neutrophils in "high-risk" recipients were found at day 3 recruited by CCL2 and CXCL2 which leads to a dramatic increase in chemokine levels in the "high-risk" group at day 6 post graft with a broader spectrum of chemokines including CCL2-CCL5, CCL11, CXCL2 and to a lesser extent CXCL10[74]. Furthermore, the local proinflammatory environment in "high-risk" recipients post-surgery contains high levels of vascular adhesion molecules further increasing the recruitment of both innate immune cells and memory T cells to the cornea[75].

Accordingly, the increased levels of innate leukocytes especially macrophages and DC which serve as APC together with pre-existing vascularization significantly increases the number of APC reaching the DLN within a shorter period compared to "low-risk" recipients. In addition, although the presence of donor APC in the DLN as well as their ability to upregulate expression of MHC class Ⅱ post "high-risk" allograft were reported in several studies, it remains controversial whether direct pathway-activated allospecific T cells play a role in mediating corneal allograft rejection[76] or rather promotes tolerance to the allograft[77]. Depletion of leukocytes from donor corneas prior to "high-risk" corneal allograft as well as using $CCR7^{-/-}$ donor corneas failed to demonstrate a significant difference in allograft survival[77,78]. Thus, these studies indicate that the frequency of donor APC is unlikely to be sufficient to mediate significant acute graft rejection through direct antigen presentation during corneal allograft rejection. Therefore, it remains likely that the heightened innate immune responses leading to increased infiltration of host APC presenting alloantigen to host T cells is (indirect pathway) responsible for the increased rejection of "high-risk" grafts, as well as "low-risk" grafts as described in previous sections.

Neovascularization is the common feature that distinguishes "high-risk" and "low-risk" host graft beds. In "high-risk" corneal allografts, despite vascularization of the cornea prior to the graft procedure, further vascularization is also induced after grafting[79]. Lymphatic vessels in the cornea act as conduits for efferent migration of APC to DLN while blood vessels provide afferent access of inflammatory leukocytes to the cornea; infiltrating leukocytes then act as a further source of pro-angiogenic factors. Studies have shown that inhibition of either blood or lymphatic vessels was able to significantly prolong graft survival comparable to "low-risk" recipients suggesting that either disruption of efferent or afferent access of leukocytes can suppress alloimmune responses[80-82]. Furthermore, although the definition of "high-risk" recipients included corneas with two or more quadrants with evidence of vascularization, clinically the incidence of graft rejection has been shown to increase with increased levels of vascularization present prior to the corneal graft procedure[83], further suggesting that increased corneal vascularization shifted the balance towards immune rejection.

The adaptive immune response was also shown to be elevated in various ways among "high-risk" recipients. One of the consequences of an increased innate immune response is the increased number of APC with the ability to activate naïve T cells. Indeed, the DTH response in "high-risk" recipients was found significantly accelerated compared to "low-risk" recipients[12,47]. Furthermore, the allograft was rejected promptly if the recipient had been previously sensitized with a previous corneal graft or skin graft[84]. It was clearly shown that in "high-risk" recipients which previously experienced graft rejection, the effector/memory T cell response promoted accelerated rejection of regraft of the same donor origin[85]. It is also possible that memory T cells due to a previous infectious disease of the cornea such as herpes keratitis becomes activated by bystander mechanisms, when a subsequent corneal graft procedure is performed (Kuffova *et al*, in press). Thus, two types of increased adaptive immune responses are present in "high-risk" recipients to promote graft rejection, namely, enhanced activation of allospecific T cells as well as reactivation of memory T cells due to previous immune mediated conditions of the cornea such as infection or previous graft.

PREVENTION OF ALLOGRAFT REJECTION

Tissue matching - controversies and justifications

Tissue matching is not routinely performed clinically for patients undergoing corneal transplantation due to its remarkable success rate in "low-risk" recipients[3,86,87]. However, the markedly poorer prognosis of "high-risk" grafts suggests this should be reconsidered, although, the controversy has not been resolved[6,7,88]. Some of the studies addressing this issue are reviewed below: In clinical practice, matching for HLA class Ⅰ antigens under "low-risk" and HLA class Ⅱ antigens under "high-risk" conditions have both been shown to significantly reduce the risk of rejection[89,90]. In a preclinical model, minor H antigen incompatibility has been shown to have higher rates of rejection even in "low-risk" grafts than MHC mismatches, and similarly, improvement in prognosis of "high-risk" grafts were demonstrated in a clinical study as well, when matched for minor H antigens[91,92]. Differences in donor-recipient blood groups may also contribute to graft rejection in "high-risk" recipients as ABO antigens are expressed in the corneal epithelium and endothelium[93]. ABO and Rh ± incompatibility were shown to have a significant influence on corneal allograft rejection in earlier clinical studies[6,94], but recently, no influence in allograft failure due to immune rejection was shown in a 5-year follow up clinical study in "low-risk" corneal transplants. However, conflicting results were reported in "high-risk" cases[93,95]. The major reasons for differences in success rates of allografts in humans are thought to be due to surgical techniques, competency of surgeons and properly distinguished risk factors associated with graft bed[96]. Furthermore, a recent review identified the lack of specificity and low sensitivity in tissue typing methods compromise the quality of HLA matching in different centres performing clinical studies[97].

A possible reason behind the high success rates of acceptance of corneal allograft in "low-risk" recipients without tissue matching is, regardless of the technical factors discussed above, the relative

weakness of the alloimmune response (as discussed above), which is relatively easily controlled with daily application of topical steroidal drops. This concept is supported by the observation that more frequent graft rejection "episodes" and eventual graft failure develop after topical steroids are discontinued in "low-risk" graft recipients (*e.g.,* after first year post corneal transplantation)[98-100].

The shortage of donor corneas worldwide, the high demand and the long wait time for the "right" donor match restricts the wider application of corneal grafts, while on some occasions, it has to be performed as an emergency procedure with high risk of failure[101,102]. As the immunological events behind the "high-risk" grafts lead inevitably to irreversible graft failure, a treatment protocol is currently being developed which will assess and compare the HLA matching along with longer wait time for the surgery, but may be associated with more favourable graft survival outcome especially in "high-risk" graft recipients[101].

Support for tissue matching comes from experimental studies using a "high-risk" regraft model, with single antigen disparity, in which antigen-specific memory T cell activation was directly correlated with accelerated graft rejection. Thus matching is advised to prevent risk of rejection by ensuring that a donor regraft has no or minimal concordance with the original graft[85].

Use of immunosuppressive agents

Generally, for "low-risk" patients, treatment with topical steroids will prevent rejection as indicated above. Daily application of steroid drops plays a major role in local control of the host immune system by preventing the invasion of IL-1 and IL-6 producing macrophages and subsequent initiation of adaptive T cell responses[103]. However, topical steroids alone are not sufficient in preventing rejection in "high-risk" recipients due to much stronger immune response generated by unfavourable microenvironment of the graft bed[103]. Though clinical studies have shown improvement of graft outcome by administering systemic (oral) steroids, steroid treatment alone is not advised in the long-term due to side effects[104-106]. Further studies have shown that use of systemic immunosuppressive therapy with either cyclosporine A (CsA) or mycophenolate mofetil (MMF) is successful in preventing corneal allograft rejection, but MMF has shown greater success than CsA[104,107-109]. Intraocular delivery of immunosuppressants has been shown to prevent "high-risk" graft rejection in rabbits while topical treatment did not show any significant effect[110,111].

Biologics, the novel immunosuppressive agents, comprised mainly of recombinant antibodies and fusion proteins, bind to receptors and block immune cells; similarly inhibitors of mediators of corneal inflammation and vascularization like IL-2 receptor (IL-2R), TNF-α, vascular endothelial growth factor (VEGF) and CCL2, all of which are involved in allograft rejection may be effective[112]. Local anti-VEGF treatment is a proficient strategy to reduce corneal angiogenesis and lymphangiogenesis and this may reduce the incidence of rejection especially in "high-risk" recipients[113-116]. Some biologics like anti-VEGF, anti-TNF-α or anti-IL-2R are already in use to inhibit "high-risk" graft rejection while potent blockers of TNF receptors are currently being evaluated in clinical trials[112].

Corneal allograft survival would be greatly improved if, in addition to tissue matching and topical steroids, an appropriate low dose immunosuppressant was also used[98]. However, alternative therapies should also be considered as discussed below.

PROMOTION OF IMMUNOLOGICAL TOLERANCE - CELL-BASED THERAPIES

Currently, cell-based therapies such as stem cells, tolerogenic DC or Treg are proposed as alternative treatments especially for "high-risk" corneal grafts and they function by promoting immune tolerance.

Stem cells

Stem cells are undifferentiated cells which give rise to two daughter cells comprising one self-renewing and one differentiating progenitor generated by asymmetric cell division[117]. Stem cells include embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and mesenchymal stem cells (MSC) and they have been investigated as a therapeutic strategy in promoting transplant tolerance[118] and in ocular surface reconstruction[119].

ESC and iPSC: The most fascinating breakthrough of the last decade is the generation of iPSC from adult somatic cells. This is a novel method of generating stem cell which ensures a continuous supply of self-renewing PSC. The process of reprogramming somatic cells *ex vivo* by transmitting the signalling cues through four well-defined transcription factors such as Oct3/4, Sox2, c-Myc, and Klf4 has opened the way for a wide range of clinical applications[120,121]. Like ESC, iPSC are also capable of trans-differentiating into cells of different lineages. Several *in vitro*, *in vivo* studies and even phase Ⅰ clinical trials were initiated using ESC and iPSC to treat sequelae of sight threatening intraocular inflammation or retinal degenerative diseases[122-126].

In the context of corneal reconstruction and repair, *in vitro* studies have shown the feasibility of differentiating ESC and iPSC into corneal epithelial, keratocytes and endothelial cells individually as an option to treat corneal scarring, stromal opacity and malfunctioned endothelial cells[127-130]. Furthermore, *ex vivo* transplantation of ESC derived cells onto partially de-epithelialized cornea led to regeneration of normal stratified layers of the corneal

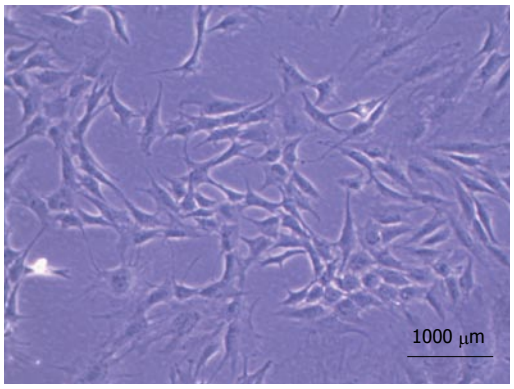

Figure 2 Spindle shaped morphology characteristic of multipotent mesenchymal stem cells. Figure shows passage 4 mesenchymal stem cells derived from the non-haematopoietic sub-population of bone marrow harvested from 6-8 wk old Balb/c mice.

epithelium[131]. iPSC are able to differentiate into limbal stem cells (LSC) *in vitro,* confirmed by expression of LSC markers ABCG-2 and p63α at both cellular and molecular levels[119]. The successful engraftment of a differentiated LSC-seeded scaffold demonstrated significant reconstruction of the ocular surface with functional re-epithelization, minimal corneal scars and corneal vascularization in an experimental model of alkali burn in rabbits[132]. Hence, PSC could potentially be used to replace damaged LSC which is a characteristic feature found in many "high-risk" ocular pathologies[119,132].

Though there is much to be explored, the therapeutic impact of PSC is remarkable. The advantages of PSC are they do not induce allogenecity and related immune rejection[126]. However, problems with insufficient supply of cells as well as the possibility of differentiating into the malignant cells still remain[133,134].

LSC: LSC play a vital role in maintaining corneal integrity and renewal of epithelial cells. The limbus, reservoir of LSC, is responsible for homeostasis of the corneal epithelium[135,136]. Damage to LSC occurs during severe burns, injury or infection to the ocular surface and results in a "high-risk" cornea with limbal stem cell deficiency (LSCD) features such as chronic inflammation, severe corneal vascularization, persistent epithelial defects, conjunctivalization of the cornea and increased risk of corneal perforation[137].

Autologous transplantation of limbal epithelial sheets is considered a long-term effective clinical solution for unilateral corneal stem cell deficiency; and for bilateral deficiency, LSC from deceased donors is a possible option but raises the problems of matching and increased chance of rejection[138-140]. In addition, autologous limbal transplantation was shown to be performed in a 2 step approach, with PK performed at a later date. However, the outcome of these procedures were not satisfactory in bilaterally deficient patients with severe ocular damage[139,140]. Nevertheless, a large clinical study reported that autologous LSC transplantation was effective even in "high-risk" patients post alkali burn or with previously failed corneal graft where the outcome was restoration of a stable ocular surface and vision[141].

Currently, LSC therapy is a promising strategy clinically to improve the chance of normalization of ocular surface and later acceptance of "high-risk" corneal grafts[142,143]. However, there are still considerable obstacles to overcome such as methods to isolate/prepare cells, expand the cells in culture and avoiding damaging cells due to the surgical procedure and immune reaction. As such, the procedure is limited to clinics that have a specialized laboratory for cell expansion, operating at a level conforming to guidelines for good manufacturing practice. A new simpler method that has been recently developed, termed simple limbal epithelial transplantation combines existing know-how but allows for the entire grafting procedure to be performed in the operating room[144].

MSC: MSC are multipotent stem cells mainly isolated from bone marrow amongst other sources[145-151] (Figure 2). These cells are being tested currently in repairing tissue defects by attenuating scar formation and in immunomodulation[152]. MSC have the capability of differentiating into cells of mesenchymal and non-mesenchymal origin induced by paracrine and autocrine signals according to the local microenvironment[153]. Several *in vitro* studies have shown MSC capable of reducing T cell immune responses by promoting the activation of Treg and production of IL-10, TGF-β, prostaglandin E2 and thrombospondin-1[154,155]. Likewise, *in vivo* studies of different solid organ transplantation models also suggested significant reduction of adaptive immune response and promotion of immune tolerance in the presence of MSC[156-159].

Initial studies demonstrated that MSC are promising candidates to treat corneal blindness by restoring corneal transparency in a congenital keratocyte dysfunction model[160] and differentiating into keratocytes in corneal stroma, thereby facilitating tissue repair[161]. Based on these studies, MSC therapy has been promoted in many acquired corneal disease and injury models. Recent studies have shown that systemic injection of MSC prolonged corneal allograft survival by homing into the inflamed graft site and DLN and suppressing APC function thus inhibiting allosensitization[162-165]. Local administration of MSC was also able to induce anti-inflammatory and anti-angiogenic effects and prevent LSCD in models of acute alkali burn[166,167].

Despite relative scarcity and difficulties with isolation and expansion, MSC are safer than PSC for treatment in pre-clinical studies as no adverse effects such as a tumour formation (teratoma), have so far been observed[168].

Immune cell therapy: Dendritic cells and T regulatory cells

DC possess both immunogenic and tolerogenic functions[169]. Activated mature immunogenic DC have been used in cancer immunotherapy for more than a decade and found to be efficacious. In this setting, DC are used as natural adjuvants carrying tumour specific peptides and induce antigen specific T cells in the DLN with subsequent tumour lysis[170,171]. DC based immunotherapy can also be used as vaccination to protect against tumours by promoting tumour antigen specific immunity and prevent cancer recurrence[172,173].

However, in contrast to their immunogenicity when activated, DC mainly maintain immune homeostasis by immune regulatory action against self-antigen specific T effector cells and so prevent autoimmunity[174]. This tolerogenic feature of DC presents them as a possible candidate for treatment in autoimmune disease and allograft rejection[175]. Phenotypically immature DC remain tolerogenic as they fail to deliver an adequate costimulatory signal required for specific T cell activation. These non-activated or partially activated T cells undergo optimally low proliferation, cell death, anergy or develop the phenotype of Treg[176,177]. *In vitro* manipulation of DC by exposing them to an antigen at a sub-optimal level or treating them with anti-inflammatory cytokines such as IL-10 and TGF-β leads to alternatively activated DC which are poor stimulators of the alloimmune response but promote immune tolerance[174,176]. The *in vitro* manipulated immature DC have been shown to impair CD4+ effector T cell induction and enrich CD4+CD25+Foxp3+ Treg by inducing hyporesponsiveness of the DC to the antigenic stimuli through toll-like receptors[178].

This phenomenon of inducing or restoring tolerance by DC therapy has been applied in transplantation models in an attempt to enhance allograft survival[179]. A number of pre-clinical studies on rodents and non-human primate transplantation models have shown long-term survival and function of allograft by administering *ex vivo* manipulated DC[175,177,180]. The efficacy of donor derived DC based therapy was tested in a pre-clinical "high-risk" corneal transplantation model and was reportedly effective by significant reduction in IFN-γ and increased production of Foxp3+ Treg[181,182].

Treg are crucial in maintaining self-tolerance and their absence leads to autoimmune diseases[183,184]. The *in vitro* generation, phenotype and immunosuppressive function of Treg have been reviewed in detail previously[185]. *In vitro* manipulated donor-derived CD8+Foxp3+ Treg were infused and found to induce CD4+CD25+Foxp3+ to provide donor specific tolerance to allografts and protect from aggressive host immune rejection in a fully mismatched skin graft murine model[186]. Similarly, production of Treg is critical for the survival of corneal allografts[44] (as discussed above) and interestingly, even the local administration of naïve Treg prolongs corneal allograft survival in infant rats[187].

DC and Treg are recognised as promising candidates for the clinical application of immunosuppressive therapy to promote corneal graft survival. It has been demonstrated that autologous DC are safe with no toxic or immunogenic effects[188,189] while graft versus host disease (GVHD) was not observed when allogeneic cells were used[173,190]. Instead, they were shown to inhibit GVHD after bone marrow transplantation in pre-clinical and clinical studies of leukemia[173,190]. Though already in clinical trials, efficient isolation without manipulation of their phenotype and function is still under development for potential application, especially in "high-risk" grafts.

ALTERNATIVES TO NORMAL CORNEAL TISSUE - ARTIFICIAL CORNEAS

The use of artificial corneas is an exciting option, which would overcome the problems with shortage of donors and frequent graft rejection in "high-risk" hosts[191,192]. Two approaches have been used to replace the damaged corneal tissue so far: (1) keratoprosthesis; and (2) bioengineered scaffolds that serve as templates for promoting corneal regeneration[193].

Keratoprosthesis

Keratoprostheses are synthetically generated corneas made of artificial materials which are not fully biocompatible and "only" provide central vision, yet are a viable option for patients who are at the end stage of severe corneal disease where grafting a donor cornea is almost certain to fail[194-196]. The Boston Keratoprosthesis (BKPro) is the most commonly used artificial cornea in clinical practice. Though the device is made of synthetic material, a donor cornea still has to be used as the carrier of the central optical device[197,198]. Patients with "high-risk" herpetic keratitis transplanted with BKPro were shown to have better outcomes than transplanted allografts only[199]. Nevertheless, several postoperative complications including keratolysis (corneal melt), tissue necrosis which may result in corneal perforation in both host and donor cornea, and retro-prosthetic membrane formation have been reported[197,200,201]. In addition, lack of bio-integration of the prosthesis seems to be the major reason for BKPro extrusion, instability and ultimate failure[195,197]. The other type of prosthesis known as the osteo-odonto-keratoprosthesis (OOKP) was designed with an autologous tooth that forms the frame for central transparent optical cylinder[196]. This is a complicated procedure, and an end stage choice for patients with severe dry eye disease. Retro-prosthetic membrane is not a significant complication in OOKP unlike BKPro[202] but, the osteo-dental lamina resorption is a specific problem of OOKP as it compromises integrity of the eye[202] while glaucoma and retinal detachment are the secondary complications of both types[203].

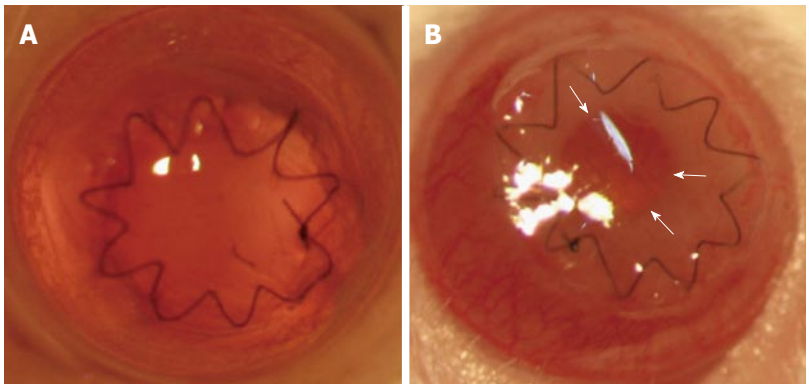

Figure 3 Clinical images of tissue engineered collagen-based hydrogels transplanted by full-thickness keratoplasty into naïve Balb/c mice at different time points post grafting. A: Clear hydrogel 1 d post transplantation; B: Hydrogel clarity is reduced 9 d post transplantation due to retro-hydrogel membrane formation (from periphery towards central cornea as indicated by arrows).

The persisting problem of stable integration of corneal implants with host and implant extrusion may be better addressed by developing tissue engineered biomimetic collagen-based corneal equivalents as discussed below.

Bioengineered corneal equivalents

Bioengineered equivalents of the corneal stromal extracellular matrix have also been tested clinically. These biosynthetic implants are based on chemically crosslinked collagen designed as regeneration templates[204-206].

Pre-clinical studies were performed in a murine full-thickness orthotopic corneal transplantation model using porcine collagen and recombinant human collagen (RHC) (Figure 3), the latter of which, by using fully biologically synthetic material, reduces the risk of transmission of disease across species as well as reducing the chance of inducing adaptive immune responses[207,208]. Studies show a strong local innate immune response associated with excessive fibrin production and deposition in the AC. This may represent an exaggerated tissue repair/wound healing response[207]. Interestingly, only minimal or no activation of APC or CD4+ and CD8+ T lymphocytes in eye-DLN as well as a minimal systemic humoral response was detected[204,207]. Thus, the main problem seems to be the generation of a retro-hydrogel membrane (Figure 3, arrows), which ultimately reduces the clarity of the graft. Surprisingly, neither an immune response to the hydrogel nor retro-hydrogel membrane formation was detected in a guinea pig model of PK[209]. Additionally, regeneration of endogenous corneal layers and functional corneal nerves were also determined in the collagen matrix[209]. Similar findings were demonstrated when the structurally reinforced collagen-based hydrogels were transplanted in a "high-risk" graft model of ocular alkali burn in rabbits[210]. Furthermore, additional advancements were made in the fabrication of biomimetic, acellular, corneal implants by incorporating biocompatible silica (Sio2) nanoparticle (NP) carriers for sustained release of anti-viral drugs such as acyclovir and LL-37 for use in "high-risk" grafts due to herpetic keratitis to prevent re-activation/re-infection of virus and this was supported by low viral copy numbers in *in vitro* experiments[211,212].

Hydrogel implants have also had their premiere in clinical medicine. A phase Ⅰ human clinical study using the biosynthetically designed corneal hydrogel substitutes made of RHC which were shown to mirror the natural cornea structurally, mechanistically and functionally by promoting active regeneration of endogenous corneal epithelial and stromal cells has been reported[213]. In addition, recent outcomes of the 4-year follow-up clinical study show high acceptance/adaptation of the hydrogel to the ocular surface with improved visual acuity and sensory nerve ingrowth[214]. A most recent clinical observation (case report) in three patients with severe corneal ulcers and recurrent erosions suggests that RHCⅢ hydrogels reinforced with phosphorylcholine polymer networks potentially withstand the "high-risk" environment (Figure 4) and is a safe and efficient alternative to donor corneal allografts in emergency situations where a corneal allograft is not available, as the corneal integrity can be well maintained in recipients[215].

Instead of fully *in vitro* generated hydrogel matrixes, decellularized corneas have also been tested in a clinical study[216]. This study showed promising clinical results in "high-risk" fungal keratitic patients where the implanted decellularized porcine corneas caused regression of corneal vascularization and improved corneal clarity. Although no safety problems were demonstrated, immunogenicity still could be a problem and so further studies addressing this issue may be required[216].

Thus, bioengineered collagen-based corneal equivalents have shown to be a promising alternative to keratoprosthesis. Though collagen hydrogels show promise in the clinic, this applies mainly to lamellar keratoplasty, which is a partial thickness replacement of damaged cornea, where host endothelium is intact. Thus, the complications observed in experimental

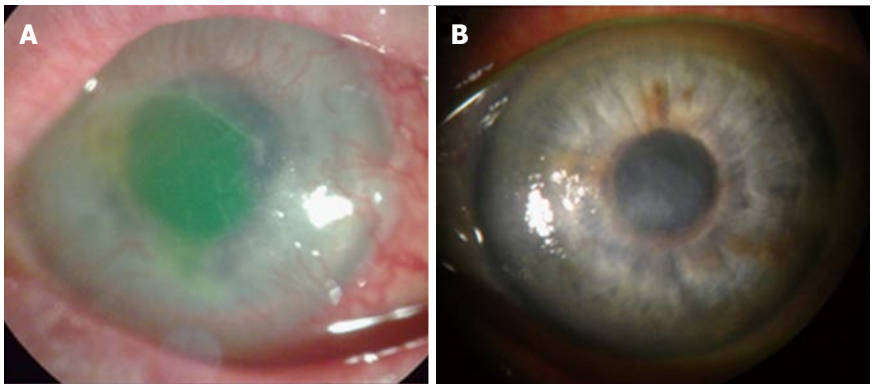

Figure 4 Clinical images of a "high-risk" cornea from a patient with ocular surface disease transplanted with a recombinant human collagen-based hydrogel. A: Corneal graft bed showing fluorescein stained epithelial erosion/ulcer (green/yellow staining) and vascularization before transplantation; B: Relatively clear cornea with clinically visible regression of peripheral corneal vessels 12 mo post-surgery[215].

models - fibrin deposition and retro-hydrogel membranes formation are eliminated as the integrity of the anterior segment microenvironment is preserved. For PK, the "holy grail" of full-thickness artificial cornea remains the ultimate aim of current research.

CONCLUSION AND FUTURE DIRECTIONS

In full-thickness corneal transplantation in "low-risk" settings - the balance between the strength of alloimmune response and regulatory mechanisms dictates the outcome of the graft, whereas in "high-risk" settings heightened innate and adaptive immune responses significantly tilt the balance to favour graft rejection. Though highly debated, tissue matching with long-term immunosuppression is recommended to reduce the rejection of "high-risk" grafts. Meanwhile, alternative approaches are being explored to avoid the side effects of prolonged use of systemic immunosuppressants. Such approaches including cell-based therapies and development of collagen-based corneal equivalents appear to be promising. Research continues to refine the available therapies for the betterment of the clinical outcomes. The recent surgical advances made in endothelial and stromal lamellar keratoplasty would be a potential realistic option to increase the success rates of some "high-risk" grafts. Manipulation of immunomodulatory molecules like TGF-β and IL-10 in the donor corneal layers by gene therapy might facilitate weakening the aggravated host immune response in "high-risk" grafts. The combined approach of cell or gene therapy along with allograft transplantation might render a better preventive measure for "high-risk" corneal graft rejection.

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