Therapeutic approaches for induction of tolerance and immune quiescence in corneal allotransplantation

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Abstract

The cornea is the most commonly transplanted tissue in the body. Corneal grafts in low-risk recipients enjoy high success rates, yet over 50% of high-risk grafts (with inflamed and vascularized host beds) are rejected. As our understanding of the cellular and molecular pathways that mediate rejection has deepened, a number of novel therapeutic strategies have been unveiled. This manuscript reviews therapeutic approaches to promote corneal transplant survival through targeting (i) corneal lymphangiogenesis and hemangiogenesis, (ii) antigen presenting cells, (iii) effector and regulatory T cells, and (iv) mesenchymal stem cells.

Keywords

Corneal transplantation; Graft rejection; Allotolerance; Regulatory T cells; Antigen presenting cells; Corneal hemangiogenesis; Corneal lymphangiogenesis

INTRODUCTION

Corneal transplantation is the most common form of tissue transplantation performed worldwide. The cornea, due to its lack of blood and lymphatic vessels and the scarcity of resident immune cells, is regarded an immune privileged tissue, and thereby offers a highly favorable environment for allograft acceptance (1, 2). Resident antigen presenting cells (APCs) in the cornea normally stay in an inactive state; these immature cells are integral to induction of tolerance against alloantigens (3). The immune privileged status of cornea is reflected in high rates of graft survival in uninflamed and avascular host beds (4). Indeed, success rates of corneal grafts in these low-risk graft recipients are estimated to be 90% at 1 year and 55% at 15 years (5, 6). However, the tolerogenic milieu of cornea is abrogated in

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inflamed and vascularized host beds, in which failure rates exceed 50% despite maximal immunosuppressive therapy (7).

Immunosuppressive medications, in particular corticosteroids remain the primary therapeutic strategy for prevention of allograft rejection. However, their use is associated with numerous side effects. The prolonged use of steroids is associated with serious side effects including cataract, glaucoma and opportunistic infections (8). Over the past three decades, multiple studies have explored the cellular mechanisms underlying immune-mediated corneal graft rejection, with the aim of developing targeted therapies that could dampen the immune response towards the allograft without compromising the integrity of the immune system. The purpose of this review is to summarize the results of these studies, and to propose strategies with potential to prevent alloantigen-specific immune rejection in high-risk human corneal transplantation.

HEMANGIOGENESIS AND LYMPHANGIOGENESIS

The blood and lymphatic vascular systems play critical roles in both delivering oxygen and nutrients to tissues, as well as by draining redundant fluid and enabling the immune system to respond to foreign antigens. Hemangiogenesis describes the growth of new blood vessels, and can be either physiological (as in the case of wound healing) or pathological (as with neoplastic or chronic inflammatory diseases). Lymphangiogenesis is the formation of new lymphatic vessels, and can similarly be either physiological or pathological. The correlation between host bed vascularity and corneal allograft rejection has been recognized for several decades (9). In contrast, the critical role of the lymphatic system in mediating allosensitization to ocular antigens was established more recently, when in 2001, Yamagami et al. demonstrated that the removal of ipsilateral draining cervical lymph nodes prevented the rejection of murine orthotopic high-risk corneal allografts (10). This observation supported the paradigm that the alloimmune response driving corneal allograft rejection consists of two phases – a sensitization phase and an effector phase. During allosensitization, lymphatic vessels form the conduit by which antigen presenting cells (APCs) are trafficked from the graft site to the regional draining lymphoid tissues, where they present donor antigen to naïve host T cells (4). During the effector phase, blood vessels permit the transport of alloreactive T cells across the chemotactic gradients from the draining lymphoid tissues to the graft site (11). Accordingly, the suppression of either the sensitization arm of the response (via lymphatic vessels), or the effector arm (via blood vessels), offer feasible therapeutic approaches to promote allograft survival.

Different therapeutic methods for direct or indirect occlusion of corneal vessels have been evaluated, including the use of laser treatment and fine needle diathermy prior to corneal grafting (12, 13). Recently, studies have focused on the use of agents that inhibit Vascular Endothelial Growth Factor (VEGF). The family of VEGF ligands and receptors are crucial regulators of both angiogenesis and lymphangiogenesis. The most important molecule that orchestrates blood vessel morphogenesis is VEGF-A, which binds to the receptors VEGFR-1 and VEGFR-2 (14). Specifically, ligation of VEGFR-2 is the principal mechanism that stimulates endothelial cell differentiation, proliferation and sprouting (14). Echoing the critical function of VEGF-A in the growth of blood vessels, VEGF-C has been

shown to be essential for developmental lymphangiogenesis (15). Both VEGF-C and VEGF-D are activating ligands for the receptor VEGFR-3 (16). Despite the discrete functions of the VEGF family that have been described here, there is in fact considerable promiscuity. For example, in both physiological and pathological settings, proteolytically processed VEGF-C and VEGF-D can promote angiogenesis through stimulation of VEGFR-2 (17, 18). Likewise, VEGF-A has been demonstrated to induce lymphatic vessel formation (19, 20). In addition to direct effects, there are notable indirect consequences of VEGF interactions on the formation of blood and lymphatic vessels. For example, VEGF-A has been shown to stimulate both lymphangiogenesis and angiogenesis in inflammatory neovascularization via recruitment of macrophages (21).

Strategies to block the sensitization arm of the alloimmune response with antilymphangiogenic interventions have successfully promoted corneal graft survival (22, 23). Dietrich et al. selectively inhibited lymphangiogenesis using anti-VEGFR-3 antibodies or anti-integrin α5 small molecules, and found a substantial reduction in murine corneal allograft rejection in the treatment groups despite the presence of pre-existing blood vessels in the host bed (22). Administration of soluble VEGFR-2 has been shown to inhibit lymphangiogenesis, but not angiogenesis in response to corneal suture injury, with a concomitant decrease in allograft rejection in a high-risk murine model of corneal transplantation (23). Notably, Albuquerque et al. identified the existence of an endogenous spliced variant of soluble VEGFR-2 that is secreted by corneal epithelial and stromal cells, and demonstrated the essential role it plays in maintaining corneal alymphaticity (23).

Strategies to block the action of VEGF-A have been shown to inhibit the ingress of both blood and lymphatic vessels following keratoplasty (24–27). Cursiefen et al. conducted normal risk allogeneic and syngeneic corneal allografts in a murine model, and demonstrated that early postoperative neutralization of VEGF-A with VEGF-trap significantly reduced both angiogenesis and lymphangiogenesis, and promoted long-term graft survival (24). Bachmann et al. corroborated this finding in a high-risk murine model of corneal transplantation (25). Bevacizumab is a monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A. In a study of high-risk murine corneal transplantation, Dastjerdi et al. demonstrated that subconjunctival bevacizumab both inhibited postoperative neovascularization and promoted graft survival (26). Noting that strategies targeting both angiogenesis and lymphangiogenesis had been successful in promoting graft survival, Dohlman et al. conducted a study comparing adjunctive therapy of VEGF-trap, anti-VEGF-C and sVEGFR-3 in a murine model of high-risk transplantation (28). In this study, the authors reported that although all strategies improved graft survival, VEGF-trap was significantly more effective in promoting graft survival compared to anti-VEGF-C and sVEGFR-3. The investigators emphasized that although each of the three agents exerted an effect on both angiogenesis and lymphangiogenesis, VEGF-trap was relatively more effective at limiting angiogenesis, and anti-VEGF-C and sVEGFR-3 were more effective at limiting lymphangiogenesis (28). Notably, VEGF-trap was the most effective therapy at reducing CD3^{+} T cell infiltration of the corneal graft, the principal cellular mediators of allograft rejection. This observation is intriguing, since alloprimed T cells themselves have been shown to release VEGF-A and to promote vascular endothelial cell proliferation in

corneal transplantation, suggesting a positive feedback mechanism between VEGF-A expression and T cell recruitment (29, 30).

It is important to emphasize that the processes of angiogenesis and lymphangiogenesis are usually deeply entwined, and modulating the function of a particular ligand commonly affects both phenomena. As further evidence of this, Chung et al. have demonstrated in a murine model that the implantation of pellets containing VEGFR-3-specific ligands results in not only lymphangiogenesis, but also robust hemangiogenesis with blood vessels that express VEGFR-3 (31). Moreover, treatment with VEGFR-3-specific ligands prompted the recruitment of VEGF-A-secreting macrophages. Clinical studies of anti-VEGF therapy in corneal transplantation are limited. In a case-control series of 122 patients undergoing high-risk corneal transplantation, Bhatti et al. reported that bevacizumab administered either subconjunctivally or topically resulted in significantly lower corneal neovascularisation post-operatively; however, they did not investigate graft survival (32). In a prospective, consecutive, interventional case series of 50 eyes of 50 patients, Dekaris et al. treated high-risk penetrating keratoplasty cases with subconjunctival bevacizumab post-operatively combined with topical bevacizumab (33). The investigators reported a decrease in corneal neovascularization in forty-two treated eyes (84%), with thirty-five (70%) of the high-risk grafts remaining clear over three years of follow-up. Fasciani et al. considered the potential role of bevacizumab as a preconditioning therapy, and conducted a prospective interventional case-control series of 27 eyes of 27 patients undergoing high-risk corneal transplantation (34). The case group of 14 eyes received a cycle of three subconjunctival and/or intrastromal injections of bevacizumab prior to transplantation. The investigators described no corneal graft rejection in the case group over two-years of follow-up, contrasting with rejection in 6 of 13 eyes in the control group. In an interventional case series of 14 eyes of 14 patients undergoing high-risk corneal transplantation, Vassileva and Hergeldzhieva treated patients with subconjunctival, perilimbal, and/or intrastromal bevacizumab at the end of surgery and/or at follow-up visits (35). The authors reported decreased corneal neovascularization in eleven patients (79%) in response to treatment, with twelve grafts (86%) remaining transparent for the observation period (on average 7 months) despite their high-risk status. These preliminary reports are encouraging, yet there is a need for more substantive clinical evidence supporting the use of anti-VEGF therapeutics in corneal transplantation.

Insulin receptor substrate (IRS)-1 proteins have been demonstrated to play an important role in angiogenesis, with human endothelial cells expressing higher levels of IRS-1 proteins in proangiogenic relative to quiescent conditions (36). In a rat model, Al-Mahmood et al. have demonstrated the dose-dependent inhibition of corneal angiogenesis by aganirsen, an antisense oligonucleotide that inhibits IRS-1 mRNA expression (36). In the first phase III clinical study of a topical antiangiogenic agent for use at the ocular surface and cornea, Cursiefen et al. conducted a multicenter, double-masked, randomized, placebo-controlled study of 69 patients with keratitis-related progressive corneal neovascularization (37). The investigators reported a significant reduction in corneal neovascularization in those patients treated with aganirsen. However, the capacity of aganirsen to reduce corneal allograft rejection has not yet been evaluated.

Gene therapy may offer a further therapeutic modality to inhibit angiogenesis and prevent graft failure. Using a rabbit model, Murthy et al. transduced corneas with a lentivirus vector expressing endostatin (38). The investigators confirmed the presence of the unique fusion gene sequence by RT-PCR in the transduced corneas, and demonstrated reduced neovascularization and improved graft survival in the hosts that received these buttons. Parker et al. transduced rabbit corneas with lentivirus vectors expressing endostatin and angiostatin, and demonstrated significant suppression of neovascularization in the recipient animals relative to control (39). Furthermore, the treatment group exhibited decreased corneal opacity, edema and inflammatory infiltrates.

Angiogenesis and lymphangiogenesis are complex processes, which are contingent on a plethora of interactions between proangiogenic and antiangiogenic signals. The association between corneal neovascularisation and graft failure in penetrating keratoplasty has been confirmed in a meta-analysis of 19 studies including a total of 24,944 grafts (40). Accordingly, the requirement for a topical inhibitor of corneal angiogenesis has been declared by a panel of experts as an important, yet unmet medical need (41). As our understanding of the molecular mediators of angiogenesis and lymphangiogenesis deepens, so the spectrum of feasible therapeutics broadens. Anti-VEGF antibodies, trap proteins and receptor antagonists have all been shown to successfully limit allograft rejection in murine models of corneal transplantation (22, 25, 26, 28). Novel antiangiogenic molecules, such as those targeting insulin receptor substrate-1, may prove to be useful therapeutic tools in this setting (37). Furthermore, *ex vivo* gene therapy treatment has potential as an effective means of decreasing neovascularization and promoting graft survival (38, 39).

ANTIGEN PRESENTING CELLS

Antigen-presenting cells (APCs) including macrophages and dendritic cells (DCs) are the principal mediators of the adaptive immune response and play a sentinel role in development, maintenance and regulation of immune memory. It was previously believed that corneal stroma was devoid of immune cells, and that the immune privileged status of the cornea was resulting from its lack of passenger leukocytes (42). However, using mouse models Liu et al. demonstrated that in the cornea there are distinct populations of resident APCs that are negative for major histocompatibility complex (MHC) II but capable of expressing class II antigen after transplantation, and to migrate to draining lymph nodes of grafted hosts (43). Subsequently, several studies in mouse models established the fact that the corneal stroma harbors heterogeneous populations of bone marrow-derived APCs, including epithelial Langerhans cells (LCs) and dendritic cells (DCs) in the anterior stroma, and macrophages in the posterior stroma (44–47). In a study on human subjects undergoing corneal transplantation, Flynn et al. characterized the allo-reactive cells and cytokines in the aqueous humor during rejection and reported a significant increase in the population of CD45^{+}CD14^{+} macrophages in aqueous humor of grafts undergoing rejection (48). Strategies to extinguish immune cells from corneal buttons to enhance transplant survival have had equivocal results. Slegers et al. showed that depletion of macrophages in corneal allografts using administration of clodronate liposomes early after grafting improves allograft survival in a rat model (49). On the contrary, Zhang et al. used anti-CD45 monoclonal antibodies to deplete APCs in the murine corneal buttons (50) and showed that depletion of these cells in

the graft does not significantly promote allograft survival even in the high-risk setting, suggesting that while some donor APCs participate in host sensitization others could be involved in tolerance induction.

In vivo experiments in mice have increased our understanding of the development and functions of DCs and macrophage subsets (51, 52). Below we summarize important findings regarding APC biology in the context of corneal transplantation. These studies are mainly carried out in rodent models. Relevant human studies in each category are also included in each section.

APC maturation and migration

While the periphery of the cornea contains both mature and immature immune cells, the central cornea is populated exclusively with highly immature and precursor-type APCs. Some of these cells are immunoregulatory, and serve to maintain a quiescent environment in homeostatic conditions. However, the microenvironment of an inflamed host bed (i.e. in high-risk transplantation), induces maturation of these APCs, and overturns the natural tendency of the eye to preserve immune privilege (53). After corneal allograft transplantation in inflamed host beds, the majority of resident APCs undergo maturation by acquiring high expression levels of MHC class II antigens and co-stimulatory molecules (CD80/CD86 and CD40) (54). These donor-derived APCs migrate to host cervical lymph nodes and activate host T cells via the direct and indirect pathways of allosensitization (44, 53, 55, 56).

The trafficking of corneal APCs to draining lymph nodes is critical in triggering immune responses. The lymphatic system serves as the sensitization arm of the immune response by enabling efficient trafficking of APCs to regional lymph nodes. Several studies on murine models have investigated the role of APC trafficking to draining lymph nodes in corneal transplantation. Jin et al. demonstrated that during an inflammatory response, APCs express chemokine receptor 7 (CCR7) on their cell surface, which interact with CCL21 and facilitate their migration from the cornea to draining lymph nodes via the lymphatics (57). Expression of chemokines is central to the recruitment of inflammatory cells to the graft site, and modulation of chemokine action has been shown to prevent graft rejection. In 2007, Hamrah et al. demonstrated that targeting specific chemokine pathways significantly promotes the survival of corneal allografts, and have proposed that the selective deletion or suppression of CCR1 may be a useful therapeutic strategy in promoting corneal graft survival (58). Pillai et al, have also examined the expression of 11 chemokines following corneal allotransplantation (59). The authors demonstrate that gene delivery of viral macrophage inflammatory protein II (vMIP II), a broad-acting chemokine antagonist, via a non-viral vector promotes corneal allograft survival. In another study, Hajrasouliha et al. showed that APC maturation is associated with upregulation of cell surface receptors of VEGF-C (i.e., VEGFR-2 and R-3), which render APCs more responsive to the VEGF-C gradient induced by inflammation. Further, they demonstrated that APC trafficking could be successfully blocked by anti-VEGF-C therapy (60). In a recent study by Hua et al., authors showed that CCR7 ligands, CCL19 and CCL21 are expressed at significantly higher levels in the draining lymph nodes of high-risk allograft recipients with inflamed graft beds (61). This is

correlated with an increased migration of mature APCs, which is abolished by neutralizing CCL19 or CCL21. These data suggest that graft site inflammation increases the expression of CCR7 ligands in the draining lymph nodes, which promote homing of mature APCs and thus allorejection. The authors concluded that the graft site microenvironment plays a critical role in alloimmunity by determining APC trafficking through the CCR7-CCL19/21 axis (61). Endothelial cell-expressed selectins mediate leukocyte tethering and rolling, a prerequisite for subsequent firm adhesion and migration of effector cells into tissue. In a recent study by Dohlman et al., the authors show that E-selectin mediates APC trafficking to lymphoid tissue, and that blockade of E-selectin improves long-term graft survival (62).

Among the family of chemokine receptors are 'decoy receptors' that serve as scavenging receptors. Originally defined by their ability to ligate chemokines in a non-signaling fashion, these decoy receptors include chemokine receptor 6 (D6), which is capable of scavenging more than 12 chemokines (mostly agonists of inflammatory CC chemokine receptors from CCR1 to CCR5) (63). Thus, in contrast to conventional chemokine receptors (in which chemokine ligation induces leukocyte recruitment in inflammation) ligation via D6 leads to targeted chemokine degradation and consequent reduction of their bioavailability. Consistent with this, the absence of D6 expression has been associated with uncontrolled and sustained inflammation, leading to the theory that expression of D6 by lymphatic endothelial cells plays a crucial role in mediating resolution of inflammation (64). Hajrasouliha et al. have demonstrated that D6 chemokine receptor expression by APCs has a critical function in mediating allograft rejection through its regulation of APC biology and consequently alloreactive T-cell responses (64). Using human corneal grafts, Lapp et al. designed an endothelial blood–eye barrier model, in which they targeted monocyte chemotaxis and showed that using inhibitors of chemokine receptors, such as CCR2 and CCR5 significantly attenuated recruitment of monocytes in vitro (65), proposing the potential application of this approach to promote corneal allograft survival. These studies altogether highlight the important role of APC maturation and migration to the draining lymph nodes in T cell sensitization and graft rejection, and provide evidence for potential therapeutic strategies in clinical studies of human corneal allografting.

Antigen presentation and induction of adaptive immune response

Antigen presentation and T cell priming not only require binding of MHC molecules to T cell receptors, but are also dependent on co-stimulatory pathways, including the interaction between CD28 on T cells with B7 molecules (CD80, CD86) on APCs and ligation of CD40 on APCs with CD154 (CD40L) on the T cells. The interaction of CD40-CD154 activates both B7 and interleukin-12 (IL-12) expression by APCs, leading to differentiation of naïve T cells to Th1 cells. Blockade of the CD40-CD154 costimulatory pathway in murine corneal transplantation has been shown to inhibit Th1-mediated responses and suppress ocular chemokine gene expression and leukocytic infiltration into allografts (66, 67). In another study, treatment of graft recipient mice with recombinant cytotoxic T Lymphocyte antigen-4 (CTLA-4), which is a competitive inhibitor of CD28 and blocks the CD28/B7 interaction and inhibits T cell activation, has been shown to significantly prolong the survival of corneal allografts (68). Comer et al. were able to reproduce similar results showing that both protein- or gene-based administration of CTLA4-Ig prolongs allograft survival when treating either

the recipient or the donor tissue *ex vivo* before grafting (69). In another study Kagaya et al. showed that treatment of corneal graft recipients with anti-CD80 and anti-CD86 antibodies decreased rejection rates in corneal allografts in a murine model of corneal transplantation (70). Using a similar animal model, Watson et al. demonstrated that augmented ligation of the PD-1 negative costimulatory molecule with a dimeric PD-L1 Ig fusion protein inhibits in vitro activation of T cells and significantly prolongs corneal allograft survival (71). Members of the T cell immunoglobulin domain and mucin domain (TIM) protein family are expressed at the cell surface of APCs as well as T cells, and have emerged recently as important regulators of immune responses (72). Tan et al. have demonstrated that the anti-Tim-1 monoclonal antibody RMT-10 is effective in promoting corneal allograft survival in a high-risk murine model of corneal transplantation (73).

The expansion of our knowledge on the molecular pathways leading to T cell sensitization and graft rejection has yielded other novel treatment strategies that target APCs and their function. In a study by Yamada et al., the investigators demonstrated that the local application of N,N′-diacetyl-L-cystine dimethylester (NM2) to mice receiving corneal allografts improves the survival of MHC-disparate allografts (74). NM2 has been shown to reduce the intracellular glutathione content in APCs, which in turn downregulates Th1 responses (74). In a more recent study by our group, Hua et al. showed that treating hosts with a resolvin D1 (RvD1) analogue significantly reduces allosensitization as seen through decreased Th1 cell activation and IFNγ production and reduced T cell infiltration into the grafts (75). Resolvins are lipid mediators produced by leukocytes, endothelial and epithelial cells, macrophages, and lymphoid tissues. Among them, resolvin D1 is known for its potent anti-inflammatory actions. It reduces inflammatory and allergic immune responses, as well as APC maturation, migration and IL-12 production. Using the murine model, the investigators demonstrated that graft survival was significantly enhanced in RvD1a-treated hosts compared to vehicle-treated graft recipients, and that enhanced survival was accompanied by suppression of angiogenesis at the graft site (75). Thrombospondin (TSP)-1 is a matricellular glycoprotein with immunoregulatory properties, such as inhibition of APC function through downregulation of TNF-α and IL-12 expression, with concomitant upregulation of IL-10 expression, as well as a reduced capacity of APCs to sensitize and mount a T cell (Th1) immune response. Using a murine model of corneal transplantation, Saban et al. demonstrated that APC-derived TSP-1 inhibits T cell allosensitization, and consequently suppresses immune rejection (76). The investigators showed that TSP-1-null APCs have enhanced expression of MHC class II and B7 maturation markers relative to wild-type APCs in an inflammatory microenvironment, thereby implicating TSP-1 in the regulation of APC maturation. Future strategies aimed at upregulating TSP-1 expression by APCs may therefore be effective in promoting transplant survival.

Tolerogenic APCs

Tolerogenic APCs (tolAPCs) have been characterized by their ability to induce T cell tolerance through various mechanisms, including diminished antigen presentation, production of anti-inflammatory cytokines, and generation and expansion of regulatory T cells (77, 78). These maturation-resistant APCs are therefore thought to be potentially powerful tools for promoting transplant survival (79). A variety of pharmacological

inhibitors have been developed to generate tolAPCs from their undifferentiated precursors in order to achieve transplant tolerance, such as immunomodulatory cytokines, rapamycin, dexamethasone, and Vitamin D (77). Hattori et al. have shown that *ex vivo* manipulation of donor-type bone marrow-derived dendritic cells (BMDCs) with immunomodulatory cytokines (IL-10, TGFβ1) renders them tolerogenic (56). The investigators demonstrated that, when systemically transferred to corneal transplant recipients, these tolAPCs significantly improve allograft survival. Khan et al. have demonstrated that intravenous administration of DCs transduced with a lentiviral vector expressing CTLA4-KDEL (a fusion protein that prevents surface CD80/86 expression by retaining the co-stimulatory molecules within the endoplasmic reticulum) promotes corneal allograft survival (80). In a recent study on mice, we demonstrated that the donor cornea *itself* can be manipulated to generate tolAPCs (3). We showed that treatment of donor corneal buttons with IL-10 and TGF-β1 induces phenotypic and functional changes in tissue-resident APCs, rendering them tolerogenic and capable of suppressing allosensitization in high-risk allograft recipients that swiftly reject their corneal transplants (3). This strategy is important, as it is translatable to human corneal allografts and can potentially induce long-term graft acceptance without exposing the recipients to immunosuppressive therapies.

EFFECTOR AND REGULATORY T CELLS

Effector T cells

IFNγ-producing CD4+ Th1 cells are considered the principal mediators of corneal allograft rejection (81, 82). The mechanism through which Th1 cells mediate allograft rejection is still not yet fully understood; studies have shown that these cells induce corneal endothelial cell apoptosis *in vitro* (83, 84). Furthermore, high levels of Th1-type cytokines, IL-2 and IFNγ, are detected in corneas undergoing rejection (85). Studies performed in mouse and rat models of orthotropic corneal transplantation have shown that depletion of CD4+ T cells using anti-CD4 monoclonal antibodies significantly prolongs corneal allograft survival (86, 87). However, depletion of neither CD4+ T cells nor IFNγ completely prevents allograft rejection, suggesting the involvement of CD4-independent effector mechanisms in the rejection process (87–89). Alloprimed cytotoxic CD8+ T cells have been implicated in high-risk corneal allograft rejection as well, though evidence suggests that despite their activation, CD8+ T cells cannot induce rejection in the absence of proper co-stimulatory signals and are thus not essential for corneal allograft rejection (90, 91).

The role of Th17 cells in the pathogenesis of corneal allograft rejection has been controversial. It has been suggested that the presence of IL-17 in early timepoints after corneal transplantation is actually essential for allograft survival, which is supported by studies demonstrating that anti-IL-17A treatment or IL-17A depletion can accelerate the tempo of corneal allograft rejection early after transplantation (92–94). These observations have been attributed to both the emergence of a Th2 type immune response that mediates graft rejection upon IL-17 blockade, and the critical role of IL-17 in Treg-mediated immunosuppression (92–94). Interestingly, however, IL-17 seems to play a role in graft rejection in later stages after transplantation, and anti-IL-17A treatment has been shown to significantly reduce late-term corneal graft allorejection (93). Continuous treatment of

transplanted mice displaying early signs of graft rejection with anti-IL-17A antibody significantly suppresses graft opacity and vascularization and reverses late-term corneal allograft rejection (93).

Various strategies to improve corneal allograft survival have focused on reducing the migration of effector T cells to draining lymph nodes and to the site of graft. Th1 cells have been found to express PSGL-1 and glyco-CD43 ligands, which interact with P- and E-selectins expressed by vascular endothelial cells, respectively, and mediate the tethering, adhesion and subsequent tissue migration of activated T cells. Treatment of corneal allograft recipients with E-selectin neutralizing antibody results in a significant decrease in frequencies of graft infiltrating Th1 cells and leads to a modest improvement in corneal allograft survival in mice (62). Therapeutic modalities targeting sphingosine 1-phosphate receptor 1 (S1P1), the cell receptor involved in sequestration of lymphocytes in lymphoid tissues, have also yielded promising results. Topical and systemic treatment with S1P1 receptor both lead to retention of $CD4^+$ T cells in peripheral lymphoid tissues and decrease the frequencies of these cells in the blood (95, 96). Systemic combination therapy with S1P1 receptor agonist and cyclosporine/rapamycin, and topical combination therapy with S1P receptor agonist and cyclosporine have both been associated with prolonged corneal allograft survival in mice (95, 96). Thus, strategies that inhibit Th1 and Th17 cells, either through inducing apoptosis or via inhibiting their migration to graft site, can potentially induce long-term allograft acceptance.

Regulatory T cells

Regulatory T cells (Tregs) play a pivotal role in curbing the effector response to alloantigens, and Treg-based immunotherapies have emerged as promising therapeutic tools in promoting corneal allograft survival (97). Several mechanisms have been identified in Treg mediated immunosuppression. Tregs can outcompete effector T cells in interacting with APCs (through lymphocyte function associated antigen-1 [LFA-1]); downregulating CD80/CD86 expression by APCs (via CTLA-4), and destroying or inactivating effector T cells (through granzymes, perforins, IL-10, TGF-β, IL-35) (98, 99). Tregs derived from corneal graft acceptors have been shown to express comparably higher levels of Foxp3 (the transcription factor implicated in the suppressive function of Tregs), demonstrate greater potency in suppressing naïve T cell proliferation, and have the ability to grant protection against corneal allograft rejection upon adoptive transfer to transplanted mice (100). Hori et al. demonstrated that constitutive expression of glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand (GITRL) by corneal cells is critical for recruitment of $GITR^+$ Tregs to the graft bed and subsequent improvement in corneal allograft survival (101).

While adoptive transfer of naïve Tregs does not prevent graft rejection, a recent report by Hildebrand et al. demonstrated that subconjunctival injection of naïve Tregs to grafted baby rats improves corneal allograft survival (102). Different approaches to control allograft rejection through *in vivo* expansion of Tregs or transfer of *in vitro*-expanded Tregs have been studied. Xu et al. demonstrated that subconjunctival treatment of transplanted mice with TGF-β-induced Tregs promotes corneal allograft survival (103). *In vivo* expansion of

Tregs using a combination of systemic rapamycin and IL-2 has also been associated with reduced graft opacity scores and neovascularization early after transplantation (104). IL-2 maintains Treg suppressive function by improving Foxp3 and immunoregulatory cytokine expression (105, 106). In a more recent study from our group, intravenous injection of mice with low dose IL-2 alone proved effective in expanding $CD4^+$ $CD25^+$ Tregs and improving corneal allograft survival in the high-risk setting (107). Proper homing of Tregs to draining lymph nodes has also been implicated in their ability to suppress alloimmune responses. Adoptive transfer of CCL21-treated Tregs, which express higher levels of CCR7 and CD62L lymph node homing receptors has been associated with significantly enhanced corneal allograft survival in mice (108).

Apart from strategies to expand Treg population, other therapeutic modalities have been proposed, which alter the balance between Tregs and Th1/Th17 cells. Wang et al. demonstrated that systemic treatment of mice with all-trans retinoid acid (ATRA), a metabolite of vitamin A, in conjunction with TGF-β increases the frequencies and suppressive function of Tregs, skews the Th17-Treg balance towards Tregs, and subsequently improves corneal allograft survival (109). Systemic treatment with anti-CD154 monoclonal antibody has been found to prolong corneal graft survival by preferentially increasing Treg-associated anti-inflammatory cytokines and suppressing Th1 inflammatory immune response in mice (110). CD154 (or CD40 ligand) binds to its receptor CD40 on antigen-presenting cells (APC), providing a co-stimulatory signal for T cell priming (111). Anti-CD154 antibody immunosuppression has also been investigated in a pig-to-primate xenocorneal transplantation model, resulting in significantly reduced inflammatory cell infiltration and Th1-associated cytokine expression, and improved corneal allograft survival (112). In aggregate, these experimental animal studies can serve as a guide for designing novel Treg-based therapeutic strategies in patients at high risk of corneal allograft rejection.

MESENCHYMAL STEM CELLS

Bone marrow-derived mesenchymal stem cells (MSCs) are multipotent nonhematopoietic stem cells that interact with cells of both innate and adaptive immune systems to modulate the effector response (113). MSCs have been shown, *in vivo*, to migrate to injured tissue and limit the release of pro-inflammatory cytokines (113). Consequently, MSCs have attracted attention as a potential therapeutic tool in preventing corneal allograft rejection (114–117).

Jia et al. have evaluated the immunomodulatory effects of MSCs in a rat model of corneal allograft rejection (114). In their study, MSCs (isolated and cultured from Wistar rats) were delivered intravenously following transplantation of a donor corneal button from Wistar rats to Lewis rat recipients. The investigators reported that treatment with MSCs prolonged graft survival, modulated the effector T cell response, and upregulated Tregs (114). Oh et al. shed light on the mechanism of action of human MSCs in promoting corneal allograft survival, when in a murine model, they demonstrated that the anti-inflammatory protein tumor necrosis factor-α stimulated gene/protein 6 (TSG-6) was essential for the suppression of inflammation (115). This study corroborated the prolongation of allograft survival by MSC therapy. The authors noted that most of the intravenously delivered human MSCs became trapped in the lungs, where they increased the expression of the TSG-6 gene. Importantly,

the therapeutic effect of MSCs was repealed when human MSCs with a knockdown of TSG-6 were used, and IV infusion of recombinant TSG-6 emulated the effects of MSC therapy (115). Omoto et al. further investigated the homing of systemically MSCs injected following corneal transplantation, by using MSCs derived from either wild-type BALB/c or GFP C57/BL/6 mice (116). The investigators reported that numerous GFP$^+$ MSCs were found in the transplanted cornea, ipsilateral conjunctiva and ipsilateral lymph nodes, but not in the contralateral tissues. Moreover, the study revealed a significant decrease in the frequencies of mature antigen presenting cells (MHC II$^+$CD11c$^+$) in the corneas and draining lymph nodes of MSC-injected allograft recipients. The apparent conflict in MSC homing between the two previously referenced studies (115, 116) may be due to the xeno-species barrier inhibiting MSC migration in the work by Oh et al., where human MSCs were used. Using an allogeneic rat model of corneal transplantation with Dark Agouti donors and Lewis recipients, O'Treacy et al. have evaluated the capacity of MSCs from three distinct sources (syngeneic Lewis, allogeneic Dark Agouti and third-party Wistar Furth rats) to prolong rat corneal allograft survival (118). The investigators showed that corneal allograft survival was significantly prolonged in those mice treated with allogeneic or third party MSCs, but not in untreated mice or those treated with syngeneic MSCs. In aggregate, these studies suggest that MSCs and specifically TSG-6 may be viable tools for promoting corneal allograft survival.

FUTURE DIRECTIONS

Animal keratoplasty models (particularly mouse and rat systems) have been critical for gaining insight into the cellular and molecular pathways that mediate allograft rejection. The onset of rejection in these models is determined based upon clinical scoring systems that assess graft opacity and corneal neovascularization. This subjective evaluation is inherently vulnerable to issues of reproducibility and interobserver variability. Recently, new tools (such as anterior segment spectral domain optic coherence tomography) have been introduced for defining graft rejection (119, 120). In the future, these tools may help researchers to improve readout reproducibility and reduce interobserver variability.

Despite the considerable progress made with murine models of corneal transplantation in identifying potential therapeutics, the goal of improving clinical outcomes by promoting allotolerance in humans remains elusive. This translational discrepancy between bench-side discoveries and clinical solutions is troubling, particularly given the failure rates seen in high-risk grafts and the manifest clinical need for effective therapies (7). One of the issues that animal keratoplasty models face concerns the genetic background of the system – we know that murine immunology is contingent on the strain used (121). An illustrative example of this phenomenon in corneal transplantation is the increased incidence and tempo of allograft rejection in C57BL/6 mice relative to BALB/c mice (122). Certainly, there are substantial challenges in moving from mouse models to clinical trials (123). Nevertheless, there is an exigent need for human in vitro and translational clinical studies to permit progress in this field. While to date no randomized controlled clinical studies have been conducted to ascertain the efficacy of tolerance induction in corneal transplantation, it is clear that this is the next frontier in the field given the immerse progress that has been made

toward a better understanding of the cellular and molecular bases of allotolerance and immune quiescence in the recent past.

CONCLUSION

Multiple cellular and molecular pathways have been identified in the immunopathology of corneal graft rejection, including corneal lymphangiogenesis and hemangiogenesis, antigen presentation, and induction of both antigen-specific effector and regulatory T cells. Despite the immune privileged status of the healthy cornea, inflammation and subsequent neovascularization compromise the cornea's ability to maintain its clarity upon allografting. Several immunomodulatory approaches have been developed based on our current knowledge of immune mechanisms underlying corneal allograft rejection. Targeting heme/lymphangiogenesis, inhibiting maturation and migration of APCs and inducing tolerogenic APCs, suppressing effector T cells and expanding regulatory T cells are successful examples of such approaches that have been investigated in various experimental models. Preliminary clinical studies using anti-VEGF therapies have shown promising results in reducing neovascularization in inflamed corneas and have improved short-term outcomes in high-risk grafts. Induction of tolerogenic APCs in the donor cornea is another potential therapeutic approach that can be achieved by pre-treatment of human donor corneas with immunomodulatory cytokines prior to transplantation without exposing graft recipients to toxic side effects of systemic immunosuppression. Finally, expanding regulatory T cells using IL-2 therapy and the use of MSCs are strategies that hold promise in promoting graft survival with the potential to be translated into human corneal allografting.

Acknowledgments

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