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Regulatory T cell modulation of cytokine and cellular networks in corneal graft rejection

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Abstract

Purpose of review—Corneal allografts placed in vascularized or inflamed host beds are at increased risk of graft rejection due to the preponderance of activated immune cells in the host bed. Regulatory T cells (Tregs) are master regulators of the adaptive immune response and play a key role in the induction of immune tolerance. The aim of this review is to discuss mechanisms through which Tregs mediate tolerance in corneal transplantation and the novel therapeutic approaches that target Tregs to promote transplant survival.

Recent findings—The inflammatory environment of high-risk allografts not only promotes activation of effector T cells and their infiltration to graft site, but also impairs Treg immunomodulatory function. Recent studies have shown that expansion of Tregs and enhancing their modulatory function significantly improve graft survival.

Summary—As our understanding of the cellular and molecular pathways in corneal transplantation has deepened, novel therapeutic strategies have been developed to improve allograft survival. In this review, we discuss therapeutic approaches that focus on Tregs to promote corneal allograft survival.

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Conflict of Interest

Maryam Tahvildari, Takenori Inomata, Afsaneh Amouzegar, and Reza Dana declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

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Keywords

corneal transplantation; immune tolerance; regulatory T cells; antigen presenting cells; plasticity of regulatory T cells; alloantigen specific graft acceptance; graft rejection

Introduction

Allogeneic corneal transplants performed on inflamed and vascularized host beds are rejected at high rates despite maximum immunosuppressive therapy. Studies on immunobiology of corneal transplantation has provided us with a deeper understanding of cytokine and cellular networks involving antigen presenting cells (APCs), IFNγ producing effector T cells (Teffs) and Tregs, the interaction of which would lead to either transplant rejection or tolerance. Eexpedited allosensitization of the host by donor and recipient APCs in a vascularized graft primes Teffs, which are then recruited to the graft site, where they exert their effector function through production of inflammatory cytokines such as IFNγ [1, 2]. $CD4^+CD25^+Foxp3^+$ Tregs, which comprise 5–10% of $CD4^+$ T cells in lymphoid organs, have been shown to play an important role in the induction of allospecific tolerance through inhibition of T cell sensitization and effector functions [3]. Unlike broad-acting immunosuppressive regimens, Tregs display antigen-specific and long-lasting effects [4]. Parallel with our growing knowledge of Tregs, several Treg-based therapies have been implemented in skin, cardiac, kidney and islet cell transplantation with a focus on expanding the population of Tregs through perioperative transfer to graft recipients [5–7].

In this review, we discuss mechanisms through which Tregs regulate adaptive immune responses in corneal transplantation, with a focus on their immunoregulatory cytokine production, cell-cell interaction, and homing to draining lymph nodes (LNs). We also discuss stability and plasticity of Treg function within the inflammatory environment (i.e. Treg plasticity), and lastly, introduce therapeutic strategies that focus on expansion of Tregs and enhancement of their function to improve graft outcomes.

Immunoregulatory role of Tregs in corneal transplantation

Tregs are phenotypically characterized by their expression of CD4, CD25, and the lineage-specific transcription factor, forkhead box protein 3 (Foxp3) [8, 9]. Foxp3 plays an indispensable role in thymic development and differentiation of Tregs in the periphery [10], and its sustained expression is essential for maintenance of Treg suppressive function [11]. The central role of Foxp3 was mainly established by early studies showing the effect of ectopic expression of Foxp3 on inducing a suppressive phenotype in naive $CD4^+$ T cells, and development of profound immunodysregulation in Foxp3-deficient mice [10, 8, 11]. Tregs exert their suppressive function through a variety of mechanisms, such as expression of cell surface molecules, secretion of inhibitory cytokines, modulation of the antigen presenting capacity of antigen presenting cells (APCs), metabolic competition with target cells and cell-contact cytolysis [12]. In addition, expression of CC-chemokine receptor-7 (CCR7) on Tregs is shown to be important in both Treg homing to lymphoid tissues and their suppressive function [13].

Among Treg cell surface-expressed molecules, the role of CD25 (IL-2 receptor-α chain), Cytotoxic T lymphocyte antigen-4 (CTLA-4), Lymphocyte activation gene 3 (LAG-3), and Programmed cell death protein-1 (PD-1) in mediating the suppressive activity of Tregs has been subject of ongoing research. Since Tregs are dependent on interleukin-2 (IL-2) for survival, they compete with effector T cells for this cytokine. This consumptive competition is dependent on constitutive expression of IL-2 receptor (CD25) by Tregs and is one of the mechanisms through which Tregs deprive effector T cells of IL-2 and lead to their apoptosis [14]. CTLA4, another cell-surface inhibitory molecule expressed by Tregs, modulate APC function by downregulating the surface expression of CD80 and CD86 through receptor trans-endocytosis, and thus inhibits effector T cell activation [15, 16]. Other mechanisms proposed for action of CTLA-4 are direct inhibition of activated responder T cells through interaction of CTLA-4 with CD80 and CD86 on the surface of T cells [17], and CTLA-4-mediated upregulation of lymphocyte function-associated antigen-1 (LFA-1) expression by Tregs, which enhances physical interaction of Tregs with target cells [18]. Studies on the role of CTLA-4 in Treg suppressor activity have yielded conflicting data [19]. While some in vitro reports show abrogated function of Tregs in CTLA-4 deficient mice and in humans treated with anti-CTLA antibody [15, 20], others indicate that Tregs maintain their suppressive function in vitro in the absence of CTLA-4 [21, 22]. LAG-3, a CD4 homolog transmembrane protein with higher affinity to MHC class II than its counterpart CD4, has been recognized both as a Treg-selective marker and a protein indispensable to optimal suppressive function of both naturally occurring and peripherally-induced Tregs [23]. Interaction of LAG-3 with MHC class II molecules at the Treg-APC synapse has been shown to inhibit maturation of APCs by suppressing their expression of co-stimulatory molecules (CD80/CD86) as well as indoleamine 2,3-dioxygenase (IDO) via CTLA-4 ligation, which render APCs tolerogenic and less effective in sensitizing T cells [24, 25]. Finally, expression of program death ligand-1 (PD-1) by Tregs is critical for maintaining Treg homeostasis, and this has been demonstrated in particular in studies using low dose IL-2 treatment for expansion of Tregs, where IL-2 treatment in the presence of PD-1 blockade failed to maintain Treg population and increased Treg apoptosis [26].

In addition to these cell surface-expressed molecules, immunoregulatory cytokines such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β) and IL-35 have been implicated in the suppressor activity of Tregs [27–30]. IL-10 derived from Tregs suppresses T helper immune responses through inhibiting the function of antigen presenting cells. Furthermore, IL-10 produced by other immune cells has been shown to augment the secretion of IL-10 by Tregs in a positive feedback loop [31]. Studies on mouse models of colitis have shown that expression of IL-10 receptor by Tregs is required for their phenotypic stability [32], but newer reports have challenged this notion by demonstrating that while IL-10R deficiency abrogates Tregs ability to suppress effector T cell responses, Foxp3 expression and thus functional stability of Tregs remain unaffected [31]. Stimulated Tregs secrete high levels of TGF-β1, levels of which are further enhanced upon CTLA-4 co-stimulation [33]. Studies have shown that in addition to producing this cytokine, Tregs express significant levels of TGF-β1 on their surface, which potentially mediates their cell-cell contact dependent immunosuppression [12, 33]. In addition to regulating differentiation, proliferation and function of effector T cells [34]. TGF-β promotes the generation and

function of Tregs by inducing Foxp3 expression [35]. It has been shown that Tregs derived from corneal graft acceptors express comparably higher levels of Foxp3, demonstrate increased expression of IL-10 and TGF-β and greater ability to suppress naive T-cell proliferation. Moreover, upon adoptive transfer to transplanted mice, these cells have the ability to protect against corneal allograft rejection [36]. IL-35 is an IL-12 family cytokine that has been shown to function in immune tolerance through conferring regulatory activity in naive T cells and suppressing T cell proliferation [29]. In addition, IL-35 is constitutively expressed at high levels in mouse Foxp3+ Tregs, and several studies have shown that IL-35 is required for maximal immunoregulatory activity of murine Tregs [37].

The cytolytic activity of Tregs against Teffs or APCs occurs mainly through granzyme (granzyme A in naturally occurring Tregs and granzyme B in peripherally induced Tregs) and perforin-dependent mechanisms [38, 39]. Though not all mechanisms involved in cytolytic activity of Tregs are understood, activated Tregs have also been shown to induce effector T cell apoptosis through tumor-necrosis-factor-related apoptosis-inducing ligand-death receptor-5 (TRAIL–DR5) and galectin-1 [40, 41].

Tissue factors also play important roles in generation and function of Tregs including their migration to the graft site. It has been demonstrated that constitutive expression of glucocorticoid induced tumor necrosis factor receptor family-related protein ligand (GITRL) by corneal cells is critical for recruitment of Tregs (expressing GITR) to the graft bed and subsequent improvement in corneal allograft survival [42]. In addition, it is shown that corneal endothelial cells are capable of converting T cells to $CD25^{hi}Foxp3^{+}$Tregs through cytotoxic T-lymphocyte antigen-2 alpha (CTLA-2a), a cysteine proteinase inhibitor, which also increases Treg production of TGF-β and enhances their regulatory function [43].

Tregs ability to home to lymphoid tissues is also an important factor in their regulatory function. Tregs are found in two principal locations, lymphoid tissues, where they inhibit effector T cell priming through interaction with APCs, and peripheral tissues, where they suppress Teff functions [44–46]. It is shown that Tregs co-localize with APCs in the paracortical region of draining LNs. These Treg cells express high levels of L-selectin (CD62L) and CC-chemokine receptor-7 (CCR7) that regulate Treg extravasation across the high endothelial venules and homing to the lymphoid tissues [45, 47–49]. Using a murine model of corneal transplantation, it has been shown that Treg expression of CCR7 is most critical in regulating both their homing and suppressive function. Moreover, ex vivo conditioning of Tregs with CCL21 leads to significant upregulation of Treg expression of LN homing receptors, including CCR7, and these CCR7-amplified Tregs migrate more efficiently to the reactive LN of corneal allograft recipients to promote graft survival [13].

Antigen-specific peripherally-derived vs. thymus-derived Tregs

Foxp3 expressing Tregs maintain immune tolerance and homeostasis in various environments. Increasing evidence indicates that there are several distinctive Treg subtypes, each playing different roles in immune regulation [50]. Understanding the natural involvement and precise function of Treg subsets in allotolerance is essential for effective development of Treg-based strategies in corneal transplantation. $Foxp3^{+}$ Treg population can

be divided into thymus-derived Tregs (tTregs) and peripherally derived Tregs (pTregs) that differentiate locally from conventional $CD4^+$T cells (Tconv) [51]. Below, we discuss the distinct roles of these two main Tregs subtypes in allotolerance.

Several studies have shown that both tTregs and pTregs share common characteristics including secretion of immunosuppressive cytokines such as IL-10 and TGF-β; however, pTreg-specific markers are yet ambiguous and complete differentiation between tTregs and pTregs is difficult. Helios (Ilzf2) is a transcription factor that was previously reported to be specific for tTregs; later studies reported that it is also expressed in pTreg [52]. Current evidence suggests that differential expression of the surface marker neuropilin-1 (NRP1) on tTreg and pTreg cells would better characterize the two Treg cell subsets, as it is shown that higher levels of NRP1 on tTreg cells are correlated with expression of Helios [53, 54]. In a recent study from our group, the phenotypes and functions of tTregs and pTregs from allograft recipients were investigated using a mouse model of corneal transplantation. It was shown that 70-80% of total $Foxp3^+$ Tregs in the draining LNs of graft recipients were $NRP1^{hi}$tTregs, whereas 20–30% of the total $Foxp3^+$ Tregs were $NRP1^{low}$pTregs [55]. Similar distribution of $NRP1^{hi}$ and $NRP1^{low}$ Tregs were previously reported for tTregs and pTregs in the secondary lymphoid tissues [53, 54].

While tTregs and pTregs act in concert to promote tolerance, alloantigen-specific pTregs play an important role in local immune regulation and are induced locally by various triggers such as changes in the microenvironment [56]. Our group has previously reported that in high-risk corneal transplantation where recipient bed is inflamed and vascularized, Foxp3 expression and secretion of immunosuppressive cytokines such as IL-10 and TGF-β are decreased in pTregs [55]. When corneal transplants are grafted to recipients with dry eye disease (an inflammatory microenvironment), pTregs are more susceptible to conversion to IL-17^+/IFNγ^+ effector T cells (exTreg) [57]. These results suggest that pTregs function preferentially in the local mucosal interface, which is characterized by inflammation in cases of high-risk corneal transplantation and dry eye. These data also indicate that the pathologic conversion of Tregs and their impaired function contribute to the loss of corneal immune privilege and allograft rejection. Furthermore, our studies [55] have shown that pTregs isolated from low-risk graft recipients demonstrate superior protective function against graft rejection upon adoptive transfer to high-risk transplanted mice compared to tTregs, signifying the role of pTregs in induction of antigen-specific peripheral tolerance [58, 56, 59]. To date, only few studies have investigated the functions of different Treg subsets in corneal transplantation, which hence warrant further investigation.

Plasticity of Tregs in high-risk corneal transplantation

$Foxp3^+$ Tregs maintain immune tolerance and homeostasis through homing to the target tissue in an antigen-specific manner and through maintaining their differentiated status in various microenvironments. However, several studies have shown that Tregs lose their expression of Foxp3 and immunosuppressive function and are reprogrammed to various helper T cells in the inflamed microenvironment [60, 61]. This reprogramming of Tregs to various helper T cells is believed to result from epigenetic or posttranslational modifications [62]. To develop new immunomodulatory therapies using regulatory T cells, it is necessary

to elucidate the plasticity of Tregs to stably deliver Tregs to the target organ. Constitutive expression of Foxp3, as well as cell surface expression of CD25 (IL-2Rα) and CTLA4 are all essential for maintaining phenotypical features of Tregs as discussed in detail above. Although these markers are expressed in a stable fashion in physiological conditions, they may be temporarily downregulated in certain conditions including inflammatory processes and infections [57, 55, 63, 64].

Low-risk corneal transplantation, where graft bed is non-inflamed and non-vascularized, is associated with a lower rejection rate due to the immune privileged status of the cornea; however, in high-risk corneal transplantation characterized by induced angiogenesis or inflammation, more than 50% of the allografts are rejected [65, 66]. In murine corneal transplantation, it has been shown that the adoptive transfer of Tregs from accepted corneal allografts induce allotolerance in high-risk corneal allograft recipients [36]; however, a proportion of these Tregs destabilize and lose Foxp3 expression under inflammatory conditions. Treg frequencies in corneal transplantation remains unchanged in the rejected grafts compared to the accepted grafts; however, Foxp3 is downregulated in Tregs (16). In addition, Tregs with upregulated Foxp3 show enhanced suppressive function upon adoptive transfer. Reduced Treg immunosuppressive function with downregulated Foxp3 and CTLA4 expression is observed in high-risk corneal transplantation with local microenvironmental changes such as inflammation and angiogenesis [55, 67].

It is essential to assess the plasticity and stability of Treg subtypes for stable therapeutic application of Tregs. Although Treg cell lineage appears stable in physiologic conditions [68], a proportion of Tregs destabilize upon inflammation, which is accompanied by Foxp3 downregulation and reduced immunosuppressive function. In addition, Tregs may convert to effector T cells (the so called, exTregs) [69]. Previous studies have reported that the adoptive transfer of exTregs can induce inflammation in animal models such as in experimental autoimmune encephalitis [70]. In a mouse model of high-risk corneal transplantation, our group has shown that pTreg frequencies and immunosuppressive function as well as their secretion of immunosuppressive cytokines such as IL-10 and TGF-β1 and CTLA-4 expression is diminished [55]. In addition, when dry eye is induced on the ocular surface of graft recipients as an inflammatory model, the $NRP1^{low}$pTregs have shown to be highly susceptible to environmental cues promoting their conversion to exTregs [57]. These exTregs acquire an effector memory T cell phenotype and produce IFNγ, thereby potentially contributing further to the development of autoimmunity. These data suggest that pTreg dysfunction and their pathological conversion to exTregs contribute to the loss of immune privilege and allograft rejection in inflammatory conditions such as dry eye and high-risk corneal transplantation.

Treg centered therapies in corneal transplantation

As discussed ealier, Tregs play a key role in the induction of allotolerance and compelling evidence suggests that corneal allograft–induced donor-specific Tregs are capable of suppressing the adaptive immune responses and enhancing allograft survival [7, 71–74]. However, as mentioned above, Tregs are susceptible to losing their immunosuppressive capability in the inflammatory environment of the high-risk host bed, as shown by their

diminished levels of Foxp3, immunoregulatory cytokine production and in vitro suppressive capacity [75]. Therefore, expanding the Treg pool and increasing their tolerogenic potential are attractive approaches for increasing transplant survival in graft recipients that are at a high risk for rejection.

In 1995, Sakaguchi et al. [9] showed that administration of $CD4^+CD25^+$ Tregs from naive mice prevented rejection of allogeneic skin grafts in T cell deficient nude. Since then, several studies have investigated the role of Tregs in transplantation tolerance and have assessed the efficacy of Treg therapies in experimental models of skin, kidney, islet cell and cardiac transplantations as well as hematopoietic stem cell transplantation (HSCT) [76–80]. More recently, a number of phase I clinical trials have used freshly isolated or ex vivo expanded Tregs in HSCT as well as umbilical cord and islet cell transplantations with promising results [81–84]. Below we discuss different approaches used in experimental models to achieve corneal allograft survival through expansion of Tregs and enhancing their immunosuppressive function.

In vivo expansion of Tregs

Several protocols have been established for generation of Tregs from naive $CD4^+$ T cells in human and in mice, mainly through TCR activation (using anti-CD3/28 as co-stimuli) and culturing Tregs in the presence of IL-2. The addition of TGF- β is shown to significantly improve generation and stability of Tregs through induction of Fxop3 expression [85–87]. In a recent study, treatment of transplanted mice with TGF-β injected into the subconjunctival space was shown to induce Tregs and increase corneal allograft survival [88]. Administration of IL-2 is also shown to be successful in promoting transplant survival. IL-2 regulates the development and survival of Tregs [89–91] and maintains Treg suppressive function through enhanced Foxp3 expression and subsequent production of immunoregulatory molecules, IL-10, TGF β and IL-35 via STAT5 pathway [92]. Tregs constantly express CD25, the α subunit of the IL-2 receptor (IL-2Rα), which dramatically increases the affinity of IL-2 to bind its receptor [93]. Thus, low concentrations of IL-2 selectively activate Treg, whereas high doses will stimulate both Treg and Teffs [94]. In a recent study from our group, using a murine model of corneal transplantation, we demonstrated that systemic administration of low-dose IL-2 promotes Treg frequencies and function, reduces activation of T cells, and promotes long-term allograft survival. In addition, expanded Tregs from IL-2–treated hosts demonstrated alloantigen specific activity and were capable of inducing long-term allograft tolerance [63]. Combination of IL-2 and rapamycin (a macrolide immunosuppressive drug) has also been shown to be effective in in vivo induction of $Foxp3^+$ Tregs leading to reduction of inflammation at the site of graft early after transplantation in mice [95].

Systemic and local delivery of Tregs

Systemic injection of Tregs to allograft recipients is shown to promote corneal allograft survival [71]. In other reports, intravenous injection of allograft acceptor-derived Tregs or ex vivo-conditioned CCR7 high Tregs significantly reduced corneal allograft rejection rates [36, 13]. Despite these promising results, a major obstacle in translating adoptive transfer approaches into clinical treatments is the relatively low frequencies of Tregs in lymphoid

tissues requiring in vitro expansion of Tregs prior to adoptive transfer [96]. Prolonged in vitro expansion of Tregs itself has been shown to lead to loss of Foxp3 expression and decreased suppressive function [97]. Local delivery of naïve Tregs via subconjunctival injection to grafted baby rats is shown to improve corneal allograft survival [98]. In another study by our group (R. Dana, unpublished data), local delivery of freshly isolated Tregs into the subconjunctival space of graft recipients showed that the administered Tregs migrated to the ipsilateral cornea and draining LN where they suppressed APC maturation and Teff activation, increased expression of immunosuppressive cytokines and decreased infiltration of immune cells to the graft.

In vivo expansion of Tregs through induction of tolerogenic antigen presenting cells (tolAPCs)

APCs are principal mediators of the adaptive immune response. They are known to play an important role in transplant rejection and tolerance. Different populations of resident immature APCs have been characterized in the corneal stroma with an immature phenotype and regulatory function that maintain a non-inflammatory environment [99, 100]. These cells stay in a highly immature state under normal conditions, expressing very low levels of MHC-II and co-stimulatory molecules. Upon allogeneic corneal transplantation, these resident APCs quickly mature and leave the graft site to migrate to lymphoid tissues where they prime host T cells [101–106]. Between the two states of immaturity and maturity of APCs is a ‘maturation-resistant’ or ‘tolerogenic’ state. 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 [107–109]. Ex vivo manipulation of donor-type bone marrow-derived dendritic cells with immunomodulatory cytokines (IL-10, TGFβ1) renders them tolerogenic [110]. Our group has previously demonstrated that systemic transfer of these cells to corneal transplant recipients result in increased frequencies of Tregs and Foxp3 expression in draining LNs and significantly improves allograft survival. In another study we have also demonstrated 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 [111]. It has been previously shown that the beneficial effect of tolAPCs on corneal graft survival is mediated through expansion of CTLA-4 expressing Tregs [112]. This strategy is translatable to human corneal allografts and can potentially induce long-term graft acceptance without exposing the recipients to immunosuppressive therapies. 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 [113]. CD154 (or CD40 ligand) binds to its receptor CD40 on APCs, providing a co-stimulatory signal for T cell priming [114]. 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 [115].

Conclusions

In the physiologic state, healthy corneal tissue maintains an immune privileged status, indispensable for its clarity and normal function. Inflammation and subsequent vascularization compromise the ability of the cornea 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 regulatory T cells to expand their population, enhance or stabilize their function while preventing their conversion to exTreg is shown to be promising in experimental models of corneal grafting and have improved short-term outcomes in high-risk corneal transplantation. Further studies are required to assess safety and efficacy of these approaches in human corneal transplantation.

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