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Animal models of high-risk corneal transplantation: A comprehensive review

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ABSTRACT

Over the past century, corneal transplantation has become the most commonly performed allogeneic solid tissue transplantation. Although more than 80% of the corneal transplantations have favorable outcomes, immune-mediated rejection continues to be the major cause of failure in well over 50% of graft recipients that have inflamed and vascularized host beds. Over the past two decades, the progress in our understanding of the immunological pathways that mediate graft rejection has aided in the development of novel therapeutic strategies. In order to successfully test the efficacy of these interventions, it is essential to model the immunological processes occurring as a consequence of corneal transplantation. Herein, we have comprehensively reviewed the established animal models used for replicating the immunopathological processes causing graft rejection in high-risk corneal transplantation settings. We have also discussed the practical and technical differences, as well as biological and immunological variations in different animal models.

1. Introduction

Since its inception by Eduard Zirm in 1905, penetrating keratoplasty has progressed to become the most common form of solid tissue transplantation (Moffatt et al., 2005). In 2018, nearly 50,000 corneal transplants were performed in the United States, using intermediate preserved corneal tissue (Eye Bank Association of America, 2019). The success rates of corneal transplantation in first time, low risk allograft recipients is over 80%, without histocompatibility matching or use of systemic prophylactic immunosuppressive therapies (Cornea Donor Study Investigator Group, 2008). The high success rates in the low-risk setting is attributed to the immunological privilege of the cornea. However, a vascularized or an inflamed recipient bed, due to pre-existing local or systemic inflammatory processes, i.e., allergy, trauma, or a previously rejected graft, loses its immunological privilege, thereby increasing graft rejection rates to well over 50% (The Collaborative Corneal Transplantation Studies Research Group, 1992).

The Collaborative Corneal Transplantation Study (CCTS) defines "high risk" transplantation as a cornea with two or more quadrants of vascularization or one in which a graft has been previously been rejected (The Collaborative Corneal Transplantation Studies Research Group, 1992). The etiological multiplicity of the underlying corneal pathologies that lead to graft failure depend upon the type and severity of the immune response. The neovascularization of the tissue further augments the immune response kinetics by providing a passage for the transportation of alloantigens from the ocular surface to the peripheral lymphoid organs, and subsequent migration of sensitized effector immune cells back into the allograft.

In this review, we comprehensively evaluate the established animal models of high-risk corneal transplantation. (summarized in Table 1, Figure 1) Furthermore, we have provided a thorough comparative analysis of the biological variations and immunological processes that promote allograft rejection in these models, making them indispensable in development of potential therapies for promoting graft survival in high-risk corneal transplantations.

2. Allergen induced models

Over the past 20 years, human studies have highlighted the immunopathological role of atopy in corneal graft rejection (Thomas et al., 2011). Magone et al. were the first to model induction of immunological response by application of short ragweed pollen (SRW) as a topical

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allergen in SWR/J mice, inducing dense conjunctival infiltration with polymorphonuclear leukocytes, macrophages, $CD4^+$ T lymphocytes, and high levels of ragweed-specific serum IgG1 and IgE (Magone et al., 1998). Currently, SRW pollen, or ovalbumin (OVA) are used in the majority of allergic models. The sensitized mice are re-challenged either intraperitoneally or intranasally to develop specific allergic phenotypes i.e. allergic conjunctivitis, allergic airway hyper reactivity etc. (Groneberg et al., 2003). The donor cornea is grafted onto a pre-sensitized recipient bed after a specific time period that varies from model to model. In some models, a sterile injury is added to the recipient bed as a secondary source of inflammation. The high heterogeneity of allergic phenotypes caused by SRW or OVA application in different strains of mice, makes these models disadvantageous compared to other models.

2.1. Allergic conjunctivitis (AC) models

Beauregard et al. developed the first allergic experimental model to study high-risk corneal transplantation in BALB/c mice by inducing conjunctivitis by application of SRW pollen (Beauregard et al., 2005). Mice were sensitized by footpad injection of 50 μg SRW pollen in 5 mg hydrated double sulfate salt of aluminum (alum) on day 0, and subsequently challenged with repeated topical application of 1.5 mg SRW in 10 μl PBS in the eye once daily from days 10–16. The donor corneal buttons from naïve C57BL/6 mice were subsequently transplanted onto the allergen-challenged recipient beds. Sensitized recipients rejected 100% of mismatched corneal allografts, with a mean survival time (MST) of 13 days, and mean rejection time (MRT) of 17 ± 8 days. In comparison, non-atopic hosts demonstrated 50% corneal allograft rejection rates with MST of 54 days and MRT of 36 ± 9 days. The authors reported 100% rejection in atopic BALB/c hosts of MHC-matched corneal allografts from B10.D2 mice with MST of 21 days and MRT of 23 ± 9 days. In comparison, non-atopic BALB/c recipients rejected 62.5% of allografts with MST of 37 days and MRT of 32 ± 9 days. However, no rejection was seen in atopic BALB/c mice when they were transplanted with syngeneic BALB/c corneal grafts. The authors attributed the higher rejection rate in pre-sensitized atopic recipients to both $CD4^+$ Th1 mediated delayed-type hypersensitivity (DTH) response with high IFNγ levels, as well as $CD4^+$Th2 mediated eosinophilic infiltration with high levels of IL-4 and IL-5. Reyes et al. combined Beauregard's AC model with induction of corneal neovascularization with 11–0 sutures into the central cornea two weeks prior to the transplantation to delineate the role of primary lymphoproliferative responses in the rejection process (Reyes et al., 2013).

Flynn et al. developed an AC model by pre-sensitizing recipient A/J mice by either intraperitoneal injection of 200 μg SRW pollen in aluminum hydroxide on days 0, 7 and 14, followed by topical treatment with eye drops containing SRW on days 8 and 15 [sensitized and challenged group ($Sens^+Chall^+$)], or by intraperitoneal injection of 200 μg

Table 1
Summary of established high-risk corneal transplantation animal models.

Animal models of high-risk corneal transplantation	Studies performed	Species used	Donor	Recipient	Rejection Rate
ALLERGEN INDUCED MODELS					
Allergic Conjunctivitis model	Beauregard (2005)	Mice	C57BL/6	(SRW+) BALB/c	100%
			B10.D2	(SRW+) BALB/c	100%
	Flynn (2007)	Mice	C57BL/6	(Sens + Chall+) A/J	100%
	Niederkorn (2010)	Mice	C57BL/6	(SRW+) BALB/c	–
				(Ova+) BALB/c	–
	Reyes (2013)	Mice	C57BL/6	(SRW+) BALB/c	100%
Allergic Airway Hyperreactivity model	Niederkorn (2009)	Mice	C57BL/6	(Ova+) BALB/c	90%
				(SRW+) BALB/c	–
MODELS RELYING ON THE ABROGATION OF ANTERIOR CHAMBER ASSOCIATED IMMUNE DEVIATION (ACAID)					
Splenectomy	Niederkorn (1996)	Mice	LC + NZB	Sp- CB6F1	92%
			LC- NZB (Fully Allo)	Sp- CB6F1	100%
			LC- NZB (Minor H)	Sp- CB6F1	91%
	Yamagami (2001)	Mice	C57BL/6	Sp-BALB/c	88%
Low-dose cyclophosphamide	Cunnusamy (2010)	Mice	C57BL/6	BALB/c	80%
Depletion of γδ cells	Skelskey (2001)	Mice	NZB	BALB/c	75%
Depletion of iNKT cells	Sonoda (1999)	Mice	BALB/c	Jα281 KO	100%
NEOVASCULARIZATION MODEL					
Corneal suture model	Williams (1985)	Rats	DA	Fisher 344	75%
	Sano (1995)	Mice	C57BL/6	BALB/c	96.70%
	Ksander (1996)	Mice	BALB/c	C57BL/6	100%
	Liu (2002)	Mice	C57BL/6	BALB/c	–
	Amescua (2008)	Mice	BALB/c	C57BL/6	100%
	Niederkorn (2009)	Mice	C57BL/6	BALB/c	100%
	Dietrich (2010)	Mice	C57BL/6	BALB/c	–
Alkali Burn model	Ling (2009)	Rats	Fisher 344	Lewis	100%
ANTERIOR SYNECHIAE MODEL	Yamagami (1999)	Mice	C57BL/6	BALB/c	86%
LC MODEL	Callanan (1988)	Rats	Wistar-Furth (WF)	Lewis (LEW)	96%
	He, 1996	Mice	NZB	CB6F1	80%
CORNEAL RE-TRANSPLANTATION MODELS					
Antigen Specific T-Cell Induction Model	Vitova (2013)	Mice	B10.BR (H-2k)	B10.BR	100%
SLIP Model	Paunicka (2015)	Mice	C3H/Hej (H-2k), A/J (H-2a) [first] C57BL/6 [second]	BALB/c (H-2d)	100%
KNOCKOUT MODELS					
PD-L1 KO	Shen (2007)	Mice	BALB/c	PD-L1−/− C57BL/6	100%
DAF1 KO	Esposito (2010)	Mice	Daf1−/−	Daf1+/+	100%
			Daf1+/+	Daf1−/−	70%
IFN-γ KO	Cunnusamy (2013)	Mice	C57BL/6	IFNγ−/− BALB/c	90%
ANTIBODY MODELS					
Anti-PDL-1	Shen (2007)	Mice	C57BL/6	BALB/c	93%
Anti-CD25	Hori (2010)	Mice	C57BL/6	BALB/c	100%
Anti-GITRL	Hori (2010)	Mice	C57BL/6	BALB/c	100%
Anti-IFN-γ	Cunnusamy (2010)	Mice	C57BL/6	BALB/c	90%
Anti-IL-17	Cunnusamy (2010)	Mice	C57BL/6	BALB/c	90%

SRW pollen with aluminum hydroxide on days 0, 7,14 and topical treatment with PBS on days 8 and 15 [sensitized group ($Sens^{+}Chall^{-}$)] (Flynn et al., 2007). After 60 days, 73% rejection rate was seen in naïve A/J mice with an MST of 36 days. However, all the grafts were rejected in $Sens^{+}Chall^{+}$ mice with a significantly lower MST of 16 days. $Sens^{+}Chall^{-}$ mice rejected grafts at a comparable rate (71%) to naïve mice and an MST of 32 days. The authors also reported comparable frequencies of infiltrating $CD4^{+}$ T cells, $CD8^{+}$ T cells, and macrophages in the graft tissues among different groups. However, high frequencies of eosinophils were observed in grafts of $Sens^{+}Chall^{+}$ mice and very few eosinophils were seen in rejected grafts in $Sens^{+}Chall^{-}$ mice. A higher expression of MHC-II was recorded in the $CD11b^{+}$ cells in the cornea of $Sens^{+}Chall^{+}$ allograft recipients, which led to the activation of effector T cells in the draining lymph nodes (dLNs) and resulted to extensive injury to the graft tissue.

Niederkorn and colleagues used the AC model to study the reversibility of atopy associated immunopathological processes before allograft transplantation (Niederkorn et al., 2010). After terminating the exposure to allergens for 30 days, atopic or naïve BALB/c mice were transplanted with donor orthotopic corneal grafts from naïve C57BL/6 mice. The risk of allograft rejection decreased significantly after terminating the SRW exposure, and the ocular surface milieu of the recipient animals returned to an immune homeostatic state, thus confirming that there was only a transient effect of atopy in graft rejection.

2.2. The allergic airway hyper-reactivity (AHR) model

Niederkorn and colleagues demonstrated the deleterious effect of allergic airway hyper-reactivity in corneal allograft rejection in recipient mice (Niederkorn et al., 2009). The authors induced AHR in BALB/c mice by i.p. injection of 10 μg of OVA in alum on days 0 and 14, and intranasally challenged with OVA on days 25–27. To induce AHR with SRW extract, BALB/c mice were immunized intraperitoneally with 40u μg of SRW mixed with alum on day 0 and day 7. On days 14, 15, 21 and 22, mice were rechallenged intranasally with 125 μg of SRW extract. AHR was assessed by methacholine-induced airflow obstruction in anaesthetized mice placed in a whole-body plethysmograph and the results were recorded as increased pauses in breathing. The cytospin analysis of Giemsa, Eosin and Hematoxylin stained bronchoalveolar lavage fluid (BALF) from mice with AHR displayed significant eosinophilia compared to naïve controls. BALF from the mice with AHR also had higher levels of IL-4, IL-5 and IL-13, typically associated with Th2 immune response. Corneal allografts transplanted to BALB/c mice with OVA and SRW-induced AHR were rejected in 90% recipients compared to a 50% rejection rate in the alum-treated control group. The inflammatory infiltrate from the grafts of AHR mice showed an inflammatory infiltrate predominantly consisting of mononuclear cells, with occasional neutrophils and no eosinophils. However, the authors reported no contrast in the extent of lymphoproliferation and DTH responses in the AHR group as compared to the control group. The recipient mice did not show clinical signs of allergic conjunctivitis or eosinophilic proliferation in either the rejected corneal grafts or the conjunctivae of the contralateral eyes. The authors speculated that the corneal allograft rejection was a result of Th2-type immune response on corneal allograft survival was a systemic effect and not due to local inflammatory changes in the corneal graft bed.

3. Models relying on the abrogation of Anterior Chamber Associated Immune Deviation (ACAID)

The absence of lymphatic-vascular channels between the cornea and lymphoid tissues provides a unique immune-privileged environment for the corneal allograft; however, the donor tissue related antigens may still elicit an immune response from the recipient. Intracameral inoculation of alloantigen in the anterior chamber of the eye elicits cytotoxic T lymphocyte (CTL) and humoral immune responses even if the delayed-type hypersensitivity (DTH) is systemically suppressed, resulting in a deviant systemic immune response known as the Anterior Chamber Associated Immune Deviation (ACAID) (Benson and Niederkorn, 1992). The ACAID immune response is a consequence of the parallel activity of primed $CD8^{+}$ cytotoxic T-cells, and the generation of non-complement fixing antibodies without the immunological involvement of $CD4^{+}$ Th1, Th2 cells, and B cells secreting complement-fixing antibodies (Skelsey et al., 2003). The immunological response arises as a consequence of intraocular F4/80-positive antigen-presenting cells (APCs) [maintained in an immature state by transforming growth factor

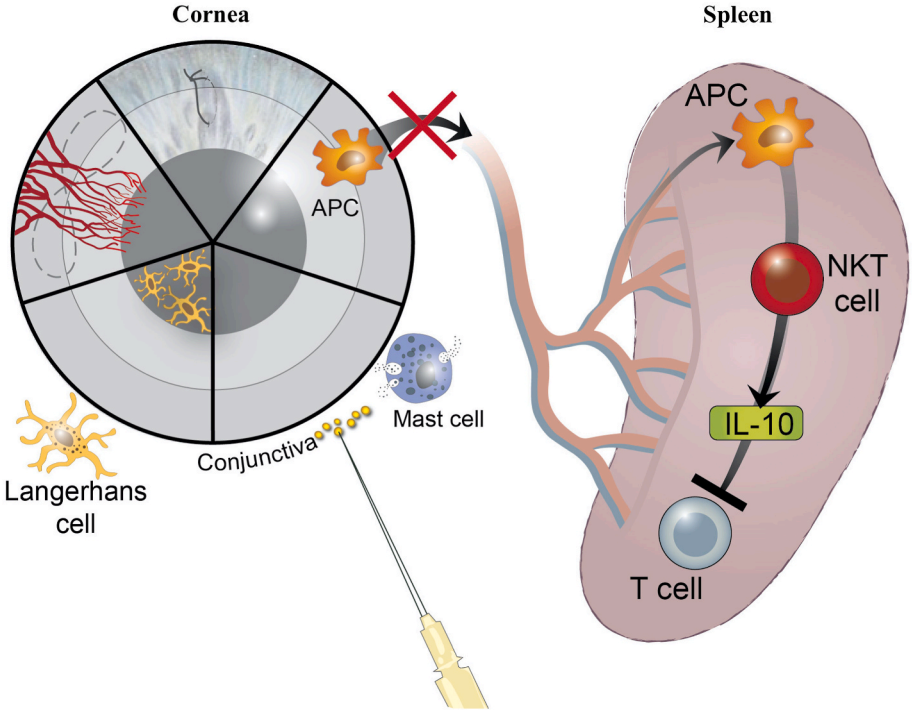

Fig. 1. Schematic illustration of major existing models of inducing high-risk recipient bed in corneal transplantation. The recipient graft bed is divided into five segments, each demonstrating a different procedure for generation of high-risk graft recipient bed (order clockwise): **(1)** Induction of neovascularization (NV) in the recipient bed by sutures (NV model); **(2)** Bringing iris vessels in physical contact with the cornea through anterior synechiae (Anterior synechiae model); **(3)** Abrogation of ACAID via splenectomy or depletion of NKT and γδ cells (ACAID model); **(4)** Pre-sensitization of recipient graft bed via application of allergens (Allergic model); and **(5)** Langerhans cell (LC) penetration into the graft bed by using the latex beads technique (LC model).

(TGF-β_2)] capture the antigen at the ocular surface and subsequently migrate via the trabecular meshwork to the spleen, forming a camero-splenic axis (Streilein, 2003). Subsequently, splenic regulatory T-cells (Tregs) suppress the APC associated immune activation by preventing priming and generation of Th1 cells, and as a result inhibit the DTH response (Masli and Vega, 2011). Natural killer T (NKT) cells and splenic γδ T cells have been shown to play a critical role in the induction of ACAID (Skelsey et al., 2001). The established models are designed by abrogating any of the ACAID components by splenectomy, in vivo depletion of NKT cells or γδ T cells with monoclonal antibodies.

3.1. *Low-dose* cyclophosphamide *model*

Cyclophosphamide (CY) is a chemotherapeutic agent regularly used for cancer treatment (Emadi et al., 2009). When administered in high doses, the drug has an efficacious immunosuppressive effect. It is commonly prescribed for management of graft-versus-host disease after hematopoietic stem cell transplantation, and as prophylaxis to prevent solid organ transplantation rejection (Heberton et al., 2019; Paul et al., 2019). Paradoxically, low doses of CY augment the immune response to antigens (Rollinghoff et al., 1977). Lutsiak et al. also reported the inhibitory action of low-dose CY on Treg activity without producing global immunosuppression (Lutsiak et al., 2005).

Cunnusamy et al. developed a murine model to study the effect of generation and function of ACAID Tregs on survival of corneal allografts. ACAID Tregs were induced by injecting C57BL/6 spleen cells into the anterior chamber of BALB/c mice, followed by subcutaneous (s.c.) injection of spleen cells 7 days later. The induced Tregs were depleted by i.p. injection of low-dose cyclophosphamide (100 mg/injection) in C57BL/6 donor mice, one day prior to orthotopic corneal graft transplantation (Cunnusamy et al., 2010b). DTH responses to C57BL/6 alloantigens were evaluated by the conventional ear swelling assay. The BALB/c hosts treated with cyclophosphamide rejected 80% of allografts received from C57BL/6 donors with an MST of 28 days, whereas only 50% grafts were rejected in the control group and had an MST of 52 days.

3.2. Splenectomy model

Both, the spleen and the eye play active roles in ACAID induction. Splenectomized hosts fail to develop ACAID even when re-infused with cellular suspension derived from the entire spleen (Waldrep and Kaplan, 1983). The splenectomy model for ACAID abrogation was developed by Niederkorn and colleagues, to determine the role of activated B cells in modifying the immune response to antigens in the anterior chamber (Niederkorn and Mellon, 1996). CB6F1 mice were grafted with orthotopic corneal tissue from mismatched C3H (both major and minor histocompatibility loci mismatch) and NZB donors (minor histocompatibility loci mismatch). Langerhans Cells (LC) from the limbus were induced to migrate centripetally to the central corneal epithelium by the instillation of sterile latex beads 7 days before ACAID induction. ACAID was induced by priming in the AC with Ia^- and Ia^+ spleen cells, corneal endothelial cells, or epithelial cells from allograft donors before orthotopic transplantation. The role of ACAID in corneal allograft survival was confirmed by the permanent acceptance of NZB corneal grafts in 60% and 90% of CB6F1 hosts when AC was primed with Ia^- NZB spleen cells or NZB corneal endothelial cells, respectively. A significant effect was seen in splenectomized CB6F1 mice grafted with LC^+ NZB corneal grafts, which resulted in an increase in rejection frequency of LC^+ minor H disparate allografts. Moreover, splenectomy had a profound effect on the graft survival on the corneas from LC^- NZB mice, with only 29% rejection rate of LC^- NZB donor corneas in eusplenic CB6F1 hosts. However, a significantly higher (91%) rejection rate was observed in splenectomized hosts, with an MST of 31.7 ± 19.0 days in comparison to 55.3 ± 8.1 days in eusplenic hosts. The authors concluded that the disruption of the camero-splenic axis by splenectomy interfered with the induction of ACAID and thus resulted in a significant increase in the rate and incidence of rejection of minor H disparate corneal allografts.

Yamagami et al. evaluated the critical role of draining lymph nodes in corneal alloimmunization and graft rejection (Yamagami and Dana, 2001). The authors recorded 50% allograft rejection rate from C57BL/6 mice grafted onto BALB/c hosts with native cervical lymph nodes (CLNs) after 6 weeks of transplantation. However, upon removal of the CLNs in allograft recipients, none of the grafts were rejected. In order to eliminate the tolerance induced by oculosplenic axis, the graft survival in splenectomized hosts was compared with survival rates in CLN-deficient hosts as well. The authors reported 50% rejection rate of allografts in splenectomized hosts within two weeks after transplantation and 88% by five weeks. In contrast, only ~13% of allografts in splenectomized CLN-deficient hosts rejected the allografts, outlining the counter effect of lymphadenectomy against the deleterious effect of splenectomy on allograft survival. The DTH-type response against the allografts generated in the CLNs, which is reflected in prolonged graft survival in CLN-deficient mice. In addition, it has been shown that ACAID continued to be intact in CLN-deficient mice, thus eliciting the generation of tolerance in response to ocular antigens. The authors associated the lower rejection rate of allografts in CLN-deficient, splenectomized hosts to the interruption of the normal lymphatic drainage from the eye. The allosensitization due to failure in generation of donor-specific DTH, negates the effect of spleen dependent induction of tolerance in promoting graft survival, making the CLN^- splenectomized hosts less susceptible to any deleterious effect of splenectomy on transplant survival.

3.3. Depletion of γδ cells

ACAID can be abolished by depleting γδ cells using anti-TCR δ-chain (GL3) antibody (Skelsey et al., 2001). Skelsey and colleagues primed BALB/c graft recipients with soluble protein antigen OVA or allogeneic NZB derived non-adherent spleen cells intracamerally on day 0. The animals were subsequently injected s.c. with OVA in PBS emulsified in complete Freund's adjuvant (CFA) or NZB derived spleen cells in Hank's Balanced Salt Solution (HBSS) emulsified in CFA on day 7. After one week an ear challenge was done by either OVA intradermal injection or irradiated NZB derived spleen cells. The mice injected with 200 μg GL3 on days −3, +4, and +11 to block the TCR δ-chain of γδ T cells failed to generate ACAID efferent suppressor cells and rejected their allografts in 75% of the recipient mice.

3.4. Depletion of iNKT cells

Sonoda et al. studied the role played by CD1d-reactive NKT cells in long-term survival of corneal allografts and associated deficiency of NKT cells in graft recipients with ACAID abrogation (Sonoda et al., 1999). The authors showed that all Jα281 knock-out graft recipients, deficient in NKT cells, rejected their allografts, while 50% of allografts survived in BALB/c mice with intact NKT cell population (Sonoda et al., 2002). Subsequently, anterior chambers of BALB/c and Jα281 KO mice were inoculated with B6 splenocytes on day 0 and day 7. The mice were then re-challenged in the ear pinnae with irradiated B6 splenocytes and ear swelling was measured after one day. A significantly suppressed DTH response was observed in the anterior chamber of BALB/c mice in comparison to Jα281 KO mice. The authors concluded that the inoculation induced allospecific Treg cells and subsequently ACAID in BALB/c mice but not in Jα281 KO mice, leading to high rejection rates in the latter.

4. Graft host bed neovascularization (NV) models

Over the past two decades, multiple studies have outlined the adverse role of neovessels in corneal graft rejection. The homeostatic upregulation in anti-angiogenic factors maintains corneal avascularity

after injury. However, pathological processes such as chronic inflammation and limbal stem cell deficiency overcome these native anti-angiogenic mechanisms, leading to the ingrowth of vessels into the corneal tissue and subsequent graft failure (Di Zazzo et al., 2017).

Corneal injury is a widely used approach to generate sterile inflammation to abolish the angiogenic and immunologic privileges of the cornea. Neovascularization is induced in vivo by placing corneal sutures. Lymphatic vessels and blood vessels form the afferent arm of alloimmune response and are responsible for abolishing cornea's immune privilege by acting as conduits for APCs from the graft bed to the regional dLNs. In the dLNs, APCs induce the activation and clonal expansion of alloantigen-specific effector T cells, which infiltrate the graft.

Sano et al. reported a 96.7% rejection rate in corneal allografts from C57BL/6 donors placed in neovascularized graft beds of BALB/c mice in comparison to 46.7% in the control group (Sano et al., 1995). Hosts with neovascularized recipient beds rejected their grafts within 2 weeks as compared to three to four weeks in the control group. The rejection of corneal allografts in high-risk eyes was attributed to the development of strong donor specific DTH, and the specificity of this immune response was directed solely at minor H antigens on the graft. William et al. developed an NV model by placing two 10–0 intracorneal nylon sutures in the rat cornea, or three interrupted 11–0 nylon sutures in the mouse cornea for three weeks prior to the transplantation procedure (Williams and Coster, 1985). All allografts, from inbred Dark Agouti (DA) grafted onto prevascularized beds of Fischer 344 rats became edematous by day 10, and eventually 76% grafts failed by week six. In comparison, the allografts from DA grafted onto avascular beds of Fischer 344 became edematous at a median of 12 days post-transplantation, with a rejection rate of 57%.

Ksander et al. grafted major and minor histo-incompatible corneas from C57BL/6 donors onto BALB/c mice with vascularized graft beds induced by penetrating sutures (Ksander et al., 1996). Corneal allografts in low-risk controls were accepted indefinitely in 50% recipients, however allografts placed on high-risk recipient eyes were rejected in all the recipients within two weeks after transplantation. $CD8^+$ cytotoxic T cells were not detected in the dLNs of mice with low-risk graft beds that either accepted or rejected their allografts. However, after two weeks of grafting, donor specific cytotoxic $CD8^+$ T cells were detected in the dLNs of mice with high-risk graft beds that rejected their allografts. The authors attributed the rejection of grafts placed in high-risk graft beds to the presence of donor-specific cytotoxic T cells.

Liu et al. induced neovascularization by three interrupted sutures in the central cornea of one eye of each recipient BALB/c mouse, which is also associated with significant lymphangiogenesis(Liu et al., 2002). Once all corneas developed extensive neovascularization after 2 weeks, all the sutures were removed and the neovascularized corneas then served as high-risk host beds for corneal transplants. The authors also applied six burns to the central 50% of the cornea using handheld thermal cautery to induce ingression of donor APCs into the central cornea, which were subsequently placed onto high risk host beds. All the C57BL/6 corneas from naïve mice grafted onto high risk BALB/c were swiftly and uniformly rejected by second week compared to 50% survival rate in controls.

Dietrich et al. evaluated the role of lymphatic vessels compared to blood vessels in allograft rejection (Dietrich et al., 2010). To differentiate the mechanisms, the corneas from naïve C57BL/6 mice were transplanted onto normal, inflamed avascular, prehemvascularized only, or prehemvascularized and prelymphvascularized recipient BALB/c mice. To differentiate the implications of hemangiogenesis and lymphangiogenesis on graft survival, the two processes were selectively inhibited by a VEGF cytokine Trap (VEGF-TrapR1R2) in the recipient before corneal transplantation (Cursiefen et al., 2004). The results showed that grafts placed into low-inflamed avascular recipient beds exhibited survival rates comparable to that seen in normal-risk transplantation. The lymphangiogenesis was abolished using systemic integrin α5β1 blockade with small molecule inhibitor-JSM6427, and observed that it led to partial inhibition of the effector immune response and survival of allografts in ~50% of the hosts (Dietrich et al., 2007). On the contrary, heme- and lymph-vascularization on the recipient graft bed caused allograft failure in all the recipients, showing the deleterious effect of lymphvascular invasion on the survival of corneal allografts.

Amescusa et al. outlined the role of chemokine (C-X-C motif) ligand 1 (CXCL1/KC) in graft rejection. The authors detected an increase in the levels of CXCL1 in pre-vascularized recipient beds, and implicated it in the subsequent increase in production of potent T-cell chemo-attractants namely, CXCL9/Mig and CXCL10 (Amescua et al., 2008). The high-risk C57BL/6 mice with vascularized host beds all rejected the allografts from BALB/c mice between days 10–13 after transplantation, whereas 66% of grafts placed in non-vascularized host beds remained clear, and graft rejection started by day 15 with a failure rate of 84% by day 30. The authors correlated these observations with elevations in levels of CXCL1/KC as early as 72 h post-surgery in vascularized high-risk corneal allograft recipients, which were not detected in non-vascularized hosts. The authors further confirmed this by locally injecting recombinant CXCL1/KC to low risk graft recipients at the time of transplantation, demonstrating a sharp increase in graft failure rates (100% by day 50) in comparison with control low-risk recipients (25% by day 70). Furthermore, neutralization of CXCL-1/KC production in high-risk vascularized graft recipients with KC antiserum before and after transplantation increased graft survival rates to 50% by day 30.

Ling and colleagues induced inflammation and neovascularization in the host corneal bed using high concentration alkaline (1M NaOH) solution (Ling et al., 2009). The penetrating keratoplasty was performed in the host rats at different time points after induction of alkali burns. The authors reported a 100% rejection rate in all the host rats by two weeks post transplantation; and a significantly shorter MST of grafts transplanted on day three (4.67 ± 1.03 days) and twelve (5.00 ± 0.63 days) post burn induction, compared to the grafts transplanted at five (9.50 ± 1.05 days), six (9.83 ± 0.75 days), and eight (10 ± 0.89 days) weeks. The authors associated the higher rate of survival at later time points to regression of blood vessels by third week and lymphatic neovessels by five weeks after induction.

5. Anterior synechiae model

Yamagami et al. developed a high-risk corneal transplantation model to investigate the local and systemic immunologic changes induced by anterior synechiae (AS) formation. The authors induced anterior synechiae (AS+) formation by placement of three out of the twelve interrupted sutures through the iris and the cornea in three quadrants during transplantation of C57BL/6 donor corneas onto BALB/c recipient mice (Yamagami and Tsuru, 1999). The AS + mice had mild vascular invasion in the corneal tissue surrounding the iris synechiae. In the AS− recipients, 50% of the corneal grafts remained transparent 3 weeks post-surgery. However, 81% of the corneas in the AS + group had graft opacity scores higher than 2. At eight weeks post-transplantation, graft recipients had opacity scores of 3 or more in 54% of cases in the AS− group versus 86% in the AS + group. The authors further demonstrated that a similar expression pattern of corneal Th1 cytokine expression pattern in the AS + group to that observed in the rejected AS- group and related the higher rate of rejection to upregulated cytotoxic T lymphocyte activity rather than a DTH response.

6. Langerhans Cells (LCs) model

Langerhans cells (LCs) are bone marrow-derived class I-positive, B7-positive primary APCs in the eye, responsible for activating T lymphocytes to initiate effector immune responses (Steinman, 1991). Donor-derived Ia^+ LCs residing in allografts are essential regulators of immunogenicity and are a hurdle to successful organ transplantation. In homeostatic conditions, the central corneal epithelium is devoid of LCs

(Hamrah et al., 2003). However, various stimuli including electrocautery, suturing and instillation of sterile latex beads can induce the centripetal migration of peripheral LCs into the central cornea, which induces allospecific immune responses (Austyn et al., 1988; Liu et al., 2002). MHC-peptide complexes are expressed 10 to 100 times higher on LCs than other APCs, and a single LC can stimulate up to 3000 T cells (Banchereau and Steinman, 1998).

Callanan et al. induced LC migration into the central part of cornea grafts in the Lewis (LEW) rats by using the latex beads technique (Callanan et al., 1988). Polystyrene beads were instilled over shallow incisions in the donor epithelium seven days prior to corneal grafting. The incision in the donor epithelium rapidly healed, but the latex beads were phagocytized in the epithelium, causing LC migration into the central cornea after four days. These latex-treated corneal allografts infiltrated with donor-derived LC showed 96% rejection rates as compared to 55% rejection rates experienced by LC-free allografts. He et al. reported a dramatic increase in the immunogenicity and consequently a high rejection rate (80%) of corneal allografts due to the presence of donor-derived LC cells (from NZB mice) in CB6F1 recipients (He and Niederkorn, 1996).

Host-derived LCs process the antigen via the less efficient indirect pathway of allosensitization (Liu et al., 1993). Ross et al. studied the effect of keratoplasty in inducing the centripetal migration of host LCs from the recipient bed onto the corneal graft (Ross et al., 1991). Grafts pretreated with latex beads were infiltrated by donor-derived Langerhans cells, and were rejected by 59% of the naïve minor H-compatible recipients. In comparison, untreated corneal grafts from LEW rats underwent rejection only in 26% of the naïve F344 hosts despite significant infiltration of graft with host-derived Langerhans cells.

Niederkorn et al. showed the stimulatory role played by IL-1, released after mild trauma as a potent stimulatory factor for inducing LC migration (Niederkorn et al. 2008). These findings were confirmed by inhibition of centripetal migration of host LCs into the corneal graft upon treatment with topical IL-1 receptor antagonist and reduction of graft rejection rates by 50% (Dana et al., 1997). Ray-Keil et al. recorded higher allograft survival rates in the recipient mice in which LC depletion was performed by hyperbaric treatment and ultraviolet radiation (UVR) (Ray-Keil and Chandler, 1986).

7. Corneal Re-transplantation models

7.1. Antigen specific T-Cell induction model

In 2013, Vitova and colleagues reported accelerated graft rejection associated with significant induction of effector and memory T cells in mice with previous corneal graft rejection with a single-antigen disparity (Vitova et al., 2013). The authors transplanted corneas from B10.BR (H-2^k), in which hen-egg lysozyme as a membrane-bound antigen is expressed as MHC Class I promoter, were transplanted to wild type B10.BR recipient mice. The mice which rejected the first graft had a 100% rejection on re-grafting, and a significantly shorter MST (5.9 ± 1.5 days) compared to first time acceptors, which had 39% rejection rate and an MST > 60 days. The rejector hosts showed strongly proliferative antigen specific T-cell responses in the draining lymph nodes post re-transplantation.

7.2. Sympathetic loss of immune privilege model (SLIP)

Paunicka and colleagues reported a threefold increase in rejection of second corneal transplants due to secretion of substance P from corneal nerve injury induced during the initial transplantation procedure(Paunicka et al., 2015). Substance P has known suppressive effect on regulatory T (Treg) cell function, which are critical for allograft survival. The corneas from C3H/Hej (H-2k), A/J (H-2a) or BALB/c donors were grafted to recipient beds of BALB/c (H-2d) mice. After eight weeks, allografts were removed and replaced with C57BL/6 corneal allografts. The authors reported 100% rejection of C57BL/6 grafts placed into previously grafted eyes underwent rejection and had an MST of seven days. Moreover, 100% of C57BL/6 grafts transplanted into eyes that previously had healthy BALB/c syngeneic grafts underwent rejection and had a comparatively longer MST of 15.5 days. By contrast, only 50% of C57BL/6 corneal allografts underwent rejection in hosts that had not received previous corneal grafts. The authors also reported the abolition of immune privilege in the contralateral eye and observed 90–100% allograft rejection with an MST of 16 days. This immunological phenomenon in which corneal nerve injury in one eye disrupts the immune privilege in the opposite eye is known as sympathetic loss of immune privilege.

8. Depletion/blockade models

8.1. IFN-γ depletion models

The depletion of Th1 derived IFN-γ exacerbates rejection of C57BL/6 allogeneic grafts in the BALB/c recipients due to the shift of the alloimmune T cell response from Th1 to Th2 phenotype. (Cunnusamy and Niederkorn, 2013). The authors reported a 90% rejection rate in C57BL/6 corneal allografts transplanted to BALB/c recipients treated with anti–IFN–γ with an MRT of 17.4 ± 12.5 days and an MST of 10 days compared to 50% rejection in control-treated BALB/c mice with a MRT of 36.3 ± 9.2 days and an median MST of 54 days. A comparable outcome was seen in C57BL/6 corneal allografts transplanted to IFN-γ Knockout (KO) BALB/c recipients, which rejected 90% of the graft with an MRT of 27.2 ± 8.2 days and an MST of 22 days.

8.2. IL-17 depletion models

Interleukin 17A (IL-17A) is a pro-inflammatory cytokine produced by a group of T helper cell 17 cells in response to their stimulation with IL-23. Cunnusamy et al. reported that the depletion of IL-17A in the BALB/c recipients led to inhibition of the Th1 or Th17 cell lineages and emergence of Th2 cell subset that exacerbated the allograft rejection (Cunnusamy et al., 2010a). The authors observed rejection in 90% C57BL/6 corneal allografts transplanted to BALB/c mice treated with monoclonal and polyclonal anti–IL-17A antibodies. The authors also reported an MRT of 26 ± 7.9 days and MST of 24 days in monoclonal antibody treated mice, and an MRT of 24.7 ± 12.8 days and MST of 22.5 days in polyclonal antibody treated group. In comparison, C57BL/6 corneal allografts underwent rejection in 50% of hosts treated with the isotype control IgG and had an MRT of 35.2 ± 8.0 days and an MST of 52 days.

8.3. DAF-1 depletion model

Decay Accelerating Factor (DAF), is an intrinsic complement inhibitor that prevents C3b/C5b deposition on self-cell surfaces and also plays a critical role in modulation of T cell responses(Edward Medof et al., 1984). An exaggerated immune response is seen in DAF deficient APCs or T cells, resulting in enhanced T cell proliferation, higher frequency of T effector cells and IFN-γ responses(Heeger et al., 2005).

Esposito and colleagues observed a marked increase in T cell infiltration in absence of Decay Accelerating Factor-1 (DAF-1) in either donor or recipient, leading to a rapid rate of graft rejection, as compared to DAF1+ tissue.(Esposito et al., 2010). The authors reported that 70% of the DAF1+ grafts transplanted into DAF1- recipients and 100% of the DAF1- grafts transplanted into DAF1+ recipients rejected, whereas only 30% rejection was observed when DAF + grafts were grafted onto DAF + recipients, by day 21. The spleen cells from the donor to recipient transplants showed enhanced $CD4^+$ and $CD8^+$ T cell responses to donor antigens when either recipient or donor tissue was DAF-. A significantly decreased expression of IL-10 and TGF-β was also seen in DAF- recipients, whereas DAF- donor corneas showed production of

complement-fixing IgG2a and IgG2b as compared to DAF1+ where no anti-HY antibodies were produced.

8.4. Treg depletion models

Hori et al. first demonstrated the significance of $CD4^+$ $CD25^+$ Tregs for corneal allograft survival (Hori et al., 2010). $CD4^+$ $CD25^+$ Tregs were depleted in BALB/c recipients by administering 0.5 mg anti-CD25 antibody to naïve BALB/c recipient mice 4 days before, and on the day of transplantation. The authors reported 100% allografts rejection in naïve BALB/c recipient mice within eight weeks of transplantation. Survival of allografts was significantly shorter in anti-CD25 antibody--treated mice than in control treated mice.

Similarly, Cunnusamy et al. also demonstrated the effect of Treg depletion on corneal allograft survival. BALB/c mice were treated with anti-CD25 antibody (250 μg/injection IP) or isotype control once before and once weekly after AC injection or corneal transplantation(Cunnusamy et al., 2010b). C57BL/6 corneal allografts transplanted to BALB/c recipients treated with anti-CD25 were rejected in 100% of hosts with an MST of 26 days, whereas only 50% of the allografts were rejected in hosts treated with the isotype control and had an MST of 52 days.

8.5. PD-L1 blockade models

Programmed Death-Ligand 1 (PD-L1) plays a critical role in down-regulating post-transplant immune response and promoting peripheral tolerance by binding to programmed cell death protein 1 (PD-1) expressed by T cells, thereby inhibiting their proliferation and cytokine secretion(Erickson and Leonard, 2002; Mazanet and Hughes, 2002). It has been shown that high level of PD-L1 is constitutively expressed on the corneal epithelial cells and is further up-regulated during inflammation(Shen et al., 2007). PD-L1 blockade via systemic administration of neutralizing anti-PD-L1 antibody to C57BL/6 recipients led to graft rejection in 93% recipients at 8 weeks post engraftment, compared to 50% in control treated recipient mice. The allografts placed in anti PD-L1 antibody treated recipients also exhibited an accelerated rejection starting by 2 weeks.

Shen et al. also reported rejection of all the corneal allografts in the PD-L1 KO recipients by 4 weeks post engraftment, whereas 21% survived in wild-type recipients after 8 weeks (Shen et al., 2007). Moreover, when PD-L1 KO mice were used as donors, the rejection of PD-L1-deficient grafts was also enhanced to 80% compared to 53% wild-type allografts by 8 weeks, thereby showing the preponderance of PD-L1deficient mice to corneal allograft rejection.

8.6. GITRL blockade model

The pathway between the glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and GITR ligand (GITRL) controls the regulatory T cells function. Hori et al. demonstrated that the infiltration of allografts with $CD4^+CD25^+FoxP3^+GITR^+$ Tregs depends on the expression of GITRL by the transplanted allografts. However, blockade of GITRL by treatment 0.2 mg anti-mouse GITRL antibody, three times a week for 8 weeks, led to depletion of Tregs, thus accelerating the destruction of corneal endothelial cells by T cells. The authors reported 100% rejection in GITRL antibody treated BALB/c recipients compared to 50% rejection in control IgG-treated recipients after 8 weeks. Moreover, the duration of allograft survival was significantly shortened in anti-GITRL murine antibody-treated mice than in control IgG-treated mice.

9. Conclusion

Corneal allograft rejection is a complex immunopathological process, involving delicate interactions between innate and adaptive immune cells and the lymphovascular system that regulate a balance between alloreactive and tolerogenic mechanisms. Our understanding of the immunological processes that cause corneal allograft rejection has steadily evolved over the past two decades, helping in the development of novel therapies that target the afferent and efferent arms of immunity at a molecular level, without undermining the integrity of the recipient's immune system. The currently established animal models are essential tools to elucidate the fundamental immunological mechanisms that govern corneal allograft rejection, especially in the high-risk setting, and for developing potential therapeutic modalities to improve long-term graft survival. Their indispensable role can be evidenced from the translational application of Bevacizumab, an anti-VEGF drug, previously studied by Dastjerdi et al. in a neovascularization model using BALB/c mice to the on-going clinical trials to prevent corneal graft rejection due to neovascularization (Dastjerdi et al., 2010; NCT01996826; NCT01072357). Previously, Vitova et al. showed the efficacy of mycophenolate mofetil and cyclosporine A in preventing corneal graft rejection in prevascularized murine model, which were later studied in multiple clinical trials (Birnbaum et al., 2009; Reinhard et al., 2005; Vitová et al., 2004). The current scope of research is expanding beyond allograft transplantation, allowing the researchers to develop novel corneal xenotransplantation models(Choi et al., 2015). Additionally, mesenchymal stem cell therapies in early stages of development have shown promising therapeutic potential in reducing graft rejection by limiting neovascularization as well as immune mediated rejection(Sahu et al., 2019; Treacy et al., 2014).

Despite the steady and significant progress in identifying efficacious interventional strategies using animal models of high-risk corneal transplantation, the application of immunomodulating therapeutics in improving the clinical outcomes of high-risk corneal transplantation in patients continues to be extremely limited. The variance in the genetic background of the animals is a major hurdle preventing the translation of progress from the bench to the clinics, which is evident from the disparity in the outcomes seen in different murine strains used to model high-risk corneal transplantation(Mills et al., 2000; Yamada and Wayne Streilein, 1998). In spite of developing a deep understanding of immunopathological mechanisms, very few randomized controlled trials have been conducted to test the efficacy of therapeutics developed using these animal models. Although, there are substantial obstacles in the translation of breakthroughs from animal models to clinical trials, the progress of in-vitro and in-vivo studies to clinics are imperative to the development of efficacious interventions to improve the outcomes of high-risk corneal transplantation.

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