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Advances on Non-CD4+Foxp3+ T regulatory cells: CD8+, Tr1, and double negative T regulatory cells in organ transplantation

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Abstract

The overwhelming body of research on T regulatory cells (Tregs) has focused on CD4+CD25+Foxp3+ T cells. However, recent years have witnessed a resurgence in interest in CD4-CD8+, CD4-CD8- (double negative; DN), and CD4+Foxp3- Tr1 Tregs and their role in controlling autoimmune diseases and in promoting the survival of organ allografts and xenografts. CD8+ and DN Tregs can arise spontaneously (natural Tregs) or can be induced *in situ*. Both CD8+ and DN Tregs have been shown to enhance the survival of organ allografts and xenografts. Additionally, both can suppress alloimmune responses by contact-dependent mechanisms by either inducing apoptosis or mediating direct cytolysis of effector T cells. CD8+, DN, and Tr1 Tregs can also act in a contact-independent manner by elaborating soluble immunosuppressive factors such TGF-β and IL-10. Applying CD8+, DN, and Tr1 Tregs for enhancing the survival of organ allografts and xenografts is still in its infancy but holds significant potential. Furthermore, there is a need for a more comprehensive understanding of how current immunosuppressive therapies applied to organ transplantations affect the wide array of Treg populations.

Introduction

Shortly after the role of T cells in protective immunity was recognized, Gershon and co-workers proposed that a subpopulation of T cells might be capable of suppressing the immune response. Accordingly, the term “suppressor T cell” was coined to describe T cells that were responsible for processes that were previously categorized as tolerance, antigen competition or feedback regulation (1). Initially, suppressor cells were defined largely by their *in vitro* functional properties. However, the suppressor cell concept quickly fell out of favor due to the absence of distinct surface markers or transcription factors unique to this cell population along with the inability of suppressor cell investigators to identify genes in the putative I-J region in the immune response region of the murine MHC implicated in suppressor cell development (2). As a result, suppressor cell research was largely abandoned for over a decade until it was resurrected by Sakaguchi et al. who provided compelling

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evidence for the presence of CD4+CD25+ suppressor cells. However, this time around suppressor cells wore the euphemistic moniker “T regulatory cells” (Tregs) (3). Since Sakaguchi's report, hundreds of publications have described and analyzed CD4+T regsand the overwhelming majority of these publications have focused on conventional CD4+CD25+Foxp3+ Tregs. Since numerous review articles have addressed the biology and immunologic properties of CD4+CD25+Foxp3+ Tregs, this review will focus on non-CD4+CD25+Foxp3+Tregs in the context of organ transplantation.

CD8+Tregs

CD8+ Treg phenotypes

The phenotype of Gershon's original suppressor cell was defined by its expression of the CD8 (Lyt-2) surface marker (4). However, in the 40 years since their original description, CD8+ Tregs have been categorized into several distinct phenotypes including: a) Qa-1/HLA-restricted Tregs (5-7); b) CD8+CD122+ Tregs (8); c) CD8+CD28- Tregs (9); d) CD8+Foxp3+ Tregs (10); e) CD8+CD103+ Tregs (11); f) CD8+LAG-3+Foxp3+CTLA-4+ Tregs (12); g) CD8+IL-10+CCR7+CD45RO+ Tregs (13); h) CD8+CD45RC^{low} Tregs (14); i) CD8+CD122+PD-1+ Tregs (15) and j) CD8+CD11c^{high} Tregs (16) (Table 1). CD8+ Tregs can arise within the thymus as naturally occurring CD8+ Tregs or they can be induced in peripheral tissues by a variety of maneuvers including donor-specific transfusion (17), injection of antigen into the anterior chamber (AC) of the eye (18), anti-idiotype immunization with allospecific T cells or MHC-derived peptides (19, 20), or by *in vivo* immunization combined with blockade of CD40 co-stimulatory molecules (21). Expression of the IL-2 receptor beta subunit, CD122, is crucial for CD8+ Treg development and function (7). Although CD122+ is also expressed on classical CD8+ memory T cells, expression of PD-1on CD8+CD122+ T cells is limited to Tregs and distinguishes Tregs from memory T cells (22). In addition to the surface markers mentioned above, CD8+ Tregs can also express CD44 and the natural killer (NK) cell inhibitory marker Ly49.

CD8+ Treg mechanisms of suppression

CD8+ Treg suppression can be mediated by either contact-dependent (23) or contact-independent mechanisms (24) (Table 2). Contact-dependent suppression by CD8+ Tregs involves the direct killing of CD4+ T effector cells by perforin-mediated cytolysis (25, 26) or FasL-induced apoptosis (16). In addition to suppressing effector T cells, CD8+Foxp3+ Tregs are capable of inducing the *de novo* generation of CD4+Foxp3+ Tregs by a process that is contact-dependent and requires the production of soluble TGF-β (27), and is reminiscent of a phenomenon that has been previously described as “infectious tolerance”. When CD8+ Tregs are in contact with CD4+ T cells suppression is supported by IFN-γ, indoleamine 2,3 dioxygenase (IDO), and fibroleukin-2 (24). However, IDO can also mediate suppression when CD8+ Tregs are not in direct contact with CD4+ effector T cells by inhibiting T cell proliferation (24). CD8+ Tregs can also suppress immune effector responses by elaborating a variety of soluble factors such as TGF-β and IL-10, which inhibit T cell activation and proliferation (28, 29).

Recent investigations on CD8+ Tregs have demonstrated the importance of the non-classical MHC Ib molecules Qa-1 (mouse) and HLA-E (human) in the induction of CD8+ Tregs (7). Engagement of Qa-1-Qdm peptides expressed on CD4+ Th1 cells with its receptor, NKG2A/CD94 on CD8+ T cells leads to the generation of CD8+ Tregs (7). Qa-1-restriced CD8+ Tregs are believe to arise in the thymus and play a central role in the maintenance of self-tolerance (7). They can also be induced in the periphery by T cell vaccination (30-32) or by introducing alloantigens into the anterior chamber of the eye (33, 34).

CD8+ Tregsand transplantation

The majority of studies on Tregs have focused on CD4+CD25+ Tregs and it has been assumed by many that CD8+ Tregs were minor players in the maintenance of self-tolerance and in the promotion of allograft survival. However, recent evidence suggests that CD8+CD122+ naturally occurring Tregs play a crucial role in immune homeostasis and contribute to allograft survival (15, 22, 35-37) (Table 3). In fact, there is compelling evidence that CD8+ Tregs are more potent than conventional CD4+CD25+ Tregs in suppressing pancreatic islet allograft rejection (35). In one study, CD8+CD122+ Tregs underwent swifter expansion, produced more IL-10, and suppressed T cell proliferation *in vitro* more effectively than their CD4+CD25+ Treg counterparts (35). Importantly, adoptively transferred CD8+CD122+ Tregs were able to prolong the survival of pancreatic islet allografts, while similar adoptive transfers of CD4+CD25+ Tregs were unable to prolong allograft survival (35). CD8+CD28- Tregs can promote the generation of tolerogenic dendritic cells (DCs) and are expanded in heart allograft recipients (38). A mouse study of allogenic stem cell transplantation revealed that in addition to the conventional role of CD4+ Foxp3+ Tregs in this model, CD8+Foxp3+ Tregs were able to prevent graft versus host disease (GVHD) mortality (39).

CD8+ Tregs may also be one of the key elements sustaining immune privilege in the eye. Nominal antigens or alloantigens introduced into the anterior chamber (AC) of the eye elicit the generation of antigen-specific CD8+Tregs through a process called anterior chamber-associated immune deviation (ACAID) (40). Interestingly, two populations of Tregs are induced following AC injection of alloantigens, CD4+ Tregs and CD8+ Tregs (18). The CD8+ Tregs that are generated in the spleen after AC injection of antigen suppress both ocular and systemic immune responses by elaborating TGF-β, IL-10, and IFN-γ (40, 41). Additionally, CD8+ Tregs are also induced *in situ* by cells lining the AC of the eye. Corneal endothelial cells express membrane-bound TGFβ2, which directly inhibits the activation of effector CD8+ T cells *in situ* and also induces the conversion of effector CD8+ T cells to Tregs (42) that elaborate TGFβ1. The pigmented epithelial cells of the iris also induce the generation of CD8+ Tregs by B7-1/B7-2 interaction with CTLA-4 on effector T cells (43, 44). It bears noting that injection of donor-specific alloantigenic cells into the anterior chamber of the eye produces ACAID and dramatically enhances corneal allograft survival (45, 46).

While most studies have focused on CD4+CD25+Foxp3+ Tregs in promoting allograft survival, there is a sizeable body of evidence that CD8+ Tregs can also enhance the survival of skin (21, 47), heart (17, 21, 24, 38, 48), pancreatic islet (21, 35), and kidney (49)

allografts and heart (21) and corneal (20) xenografts, and hematopoietic allografts (39) (Table 3).

TCRαβ+ CD4-CD8- Double Negative Tregs (DN Treg)

DN Treg phenotypes

In 1989, Strober and colleagues (50) described and cloned a population of spleen cells that did not express either CD4 or CD8, but could suppress T cell proliferation *in vitro*. The $CD4^-$, $CD8^-$ T cell population was termed “double negative” (DN) T cells. DN T cells express the αβ T cell receptor (TCR), yet do not express CD4, CD8, or NK cell markers and represent 1% to 5% of the αβ T cell receptor positive T cells in mice and humans (51, 52). Although no specific marker has been identified for DN Tregs, a lack of Foxp3 expression and patterns of surface markers have been reported (53) (Table 1). The origin of DN T cells is still unclear. Some studies suggest that DN αβ TCR+ T cells can be derived from CD8+ T cells (54) while others have shown that CD4+ T cells can convert to DN Tregs both *in vitro* and *in vivo* (55). Cloned DN Tregs from TCR transgenic mice are identified as $CD25+CD30+CD28^{low}$ (51), while DN Tregs arising from CD4+ precursors are CD28+CD25+CD44+CD69+ (55). Isolated human DN Tregs are $CD27+CD28^{low}CD25-$ (56). Subsequent studies reported that DN Tregs displayed antigen specific suppressive activity both *in vitro* and *in vivo* (51). DN Tregs not only suppress CD8+ T cell responses, but also inhibit CD4+ T cells (57), B cells (58), NK cells (59), and dendritic cells (DC) (60).

DN Treg mechanisms of suppression and impact on transplantation

DN Tregs have a unique capacity to acquire the entire MHC-alloantigen complex from APCs' cell membrane via a cell-contact-dependent interaction with the TCR on the DN Tregs via a process termed “trogocytosis” and to express the captured MHC-alloantigen complex on their cell surface (61). The effector CD8+ T cell binds to the captured MHC-alloantigen complex that is now presented on the DN Treg, which culminates in the transmission of a death signal to the CD8+ T cells (61-63). Accordingly, suppression by DN Tregs is antigen-specific (Figure 1).

There is evidence that the death signal delivered by the DN Tregs to the CD8+ T cells in mice is through Fas/FasL (63) while other studies suggest a role for perforin-mediated cytolysis (55) (Table 2). The suppression of CD8+ T cells by DN T cells via recognition of alloantigens and the killing of CD8+ effector T cells is reminiscent of the “veto cell” concept that was proposed 35 years ago (64). Adoptive transfer of DN Tregs following a cardiac allograft in mice prolonged graft survival as well as augmented the Foxp3+ Treg population (65). There is also evidence that murine DN Tregs regulate immune responses at the level of antigen presenting dendritic cells (DC). Gao and co-workers (60) demonstrated that murine DN Tregs expressed high levels of CTLA-4 and down-regulated costimulatory molecules CD80 and CD86 on antigen-presenting mature DCs. Moreover, DN Tregs killed syngeneic antigen-loaded DCs or allogeneic DCs through a Fas-FasL pathway.

DN Tregs can also suppress B cell and NK cell responses. Ma and co-workers found that adoptively transferred DN Tregs prolonged rat heart xenograft survival in mice and induced B cell apoptosis via a perforin-dependent process (58). As might be expected, anti-donor

IgM and IgG antibody titers were significantly diminished in recipients of adoptively transferred DN Tregs (58). In a model of murine allogenic bone marrow transfer, DN Tregs supported graft survival through suppressing NK cells by perforin and Fas-FasL dependent pathways (59).

In contrast to murine DN Tregs, human DN Tregs do not kill effector T cells (66). Although cell-cell contact is required for human DN Treg to function, the suppressive activity of human DN Tregs is not Fas/FasL or perforin-mediated. Suppression by DN Treg is reversible and the function of previously suppressed effector T cells can be restored once DN Tregs are removed. Soluble factors such as IL-10 and TGF- β are not involved in suppression mediated by DN Tregs (66). In a study of 40 human patients who received a hematopoietic stem cell allograft, the percent of DN Tregs in the peripheral blood was inversely correlated with risk of graft rejection (67).

DN Tregs have been shown to enhance the survival of skin (51, 55, 57), pancreatic islet (55, 68), and heart allografts (65, 69, 70) and heart xenografts (58, 71) and hematopoietic stem cell allografts (59, 67) (Table 3).

Type 1 Regulatory T Cells (Tr1)

Tr1 phenotypes

Two categories of CD4+ Tregs have been described: conventional CD4+CD25+Foxp3+Tregs (72) and type 1 Tregs (Tr1 cells) (73). Tr1 cells are found in both humans and mice and are characterized by their copious secretion of IL-10 and their lack of Foxp3 expression. Human and mouse Tr1 clones co-express CD49b and lymphocyte activation gene 3 (LAG-3), which distinguishes Tr1 cells from other CD4+ T cells including Th1, Th2, Th17, and Foxp3+ Tregs (74) (Table 1). Tr1 cells are induced *in vivo* following chronic antigenic stimulation in the presence of IL-10 (75) or *in vitro* by activating naïve T cells through their TCR in the presence or IL-10 alone or IL-10 in combination with immunosuppressive drugs such as dexamethasone or Rapamycin (73, 76, 77).

A growing body of evidence indicates that IL-27 produced by tolerogenic DCs is a crucial differentiation factor for the development of IL-10-producing Tr1 cells in mice (78-80) and humans (81). However, Jin and co-workers have recently shown that IL-6 can induce the differentiation of murine IL-10-producing Tr1-like Tregs from naïve CD4+ T cells in the absence of IL-27 suggesting that IL-6 produced in inflammatory conditions might serve as a feed-back mechanism for generating Tr1 cells that dampen inflammation and restore homeostasis (82).

Tr1 mechanisms of suppression and impact on transplantation

Tr1 cells mediate immune suppression by secreting IL-10 and by killing antigen presenting cells via a perforin/granzyme-dependent process that requires MHC class I recognition (83) (Table 2). Tr1 also suppress Th17 cells in murine model of colitis by an IL-10-dependent process (84).

The first indication that Tr1 cells might contribute to allograft tolerance came from severe combined immunodeficiency (SCID) patients who developed long-term tolerance to stem cell allografts and expressed cells with Tr1-like properties (85). Subsequent studies showed that Tr1 cells were associated with the induction of mixed chimerism in patients receiving hematopoietic stem cell transplants (86). Investigations in murine allograft models have demonstrated a role for Tr1 cells in tolerance in pancreatic islet transplantation (76, 87). In humans, Tr1 cells can also contribute to transplantation tolerance as Tr1 cells have been detected in patients who spontaneously developed tolerance to kidney or liver allografts (88) and also occur in mice treated with IL-10 in combination with Rapamycin a means of establishing tolerance for pancreatic islet transplants (76). In human subjects, Tr1 cells have been associated with tolerance in kidney (88), pancreatic islet (89), and liver (88) allografts (Table 3).

Effect of Immunosuppressive Agents on Treg Generation and Function

Immunosuppressive drugs such as Cyclosporine A (CsA) and Rapamycin (RPM) have made organ transplantation a feasible and effective therapeutic option for treating end stage organ failure. By contrast, the clinical application of Tregs in promoting the long-term survival of organ transplants is gaining traction but much remains to be improved before it becomes a reality. It is important to address the impact of immunosuppressive drugs in the context of various organ transplantations since they may have varied effects depending on dose, combination treatments, or the type of organ affected. A key issue is whether conventional anti-rejection agents can be combined with Tregs for promoting graft acceptance or if immunosuppressive drugs have untoward effects on Treg function. CsA and RPM have been used successfully to prevent transplant rejection. Although both CsA and RPM target IL-2, they have remarkably different effects on the generation and function of CD4+CD25+Foxp3+ Tregs. CsA inhibits IL-2 transcription and synthesis while RPM acts downstream by blocking T cell responses to IL-2. Using a murine heart allograft model Coenen and co-workers found that CsA treatment resulted in a sharp reduction in peripheral CD4+CD25+Foxp3+ Tregs while RPM treatment did not reduce the generation of these Tregs (90). Moreover, RPM, but not CsA, induces *de novo* generation of CD4+CD25+Foxp3+ Tregs which potentiate murine skin allograft survival in an alloantigen-specific manner (91). Similar findings were seen in another skin allograft model where RPM treatment supported Tregs and CsA antagonized Treg expansion (92).

Immunosuppression by preventing the egress of T cells from lymphoid organs can be achieved by FTY720 (Fingolimod), a sphingosine-1-phosphate receptor agonist. In a murine corneal allograft model, graft survival was prolonged by topical treatment with CsA or FTY720, with an increase in CD4+ Tregs found in the FTY720 treated mice (93). A model utilizing adoptive transfer for allograft rejection found that either RPM or FTY720 treatment significantly enhanced conversion of CD4+CD25+Foxp3+ Tregs (94).

Histone deacetylase inhibitors (HDACs) may be a novel route for promoting Treg presence by preventing the conversion of Tregs into effector T cells through enhancing the access to Foxp3 within the chromatin. An HDAC, suberoylanilide hydroxamic acid (SAHA), synergized with low-doses of tacrolimus to prolong cardiac allograft survival in mice by

promoting expression of Treg molecules Foxp3 and CTLA-4 as well as increasing apoptosis of T effector cells (95). Furthermore, if the cardiac graft was introduced into Foxp3 deficient recipient mice, SAHA treatment was still able to marginally increase allograft survival suggesting that non-Foxp3 regulatory cells are also impacted by HDACs. Another HDAC, trichostatin A (TSA), in combination with donor-cell transfusion increased CD4+CD25+Foxp3+ Treg numbers and promoted mouse pancreatic islet graft survival (96).

There are still many clinical drugs that could potentially impact Treg numbers and functions. A high-throughput screen, flow cytometry based assay using Foxp3-GFP reporter mice evaluated the *in vitro* effects of 640 FDA-approved drugs and found that after 3 days in culture 75 drugs significantly increased Treg numbers (97). This study measured Foxp3+ T cell numbers, thus it warrants future investigations to determine if the same drugs, or a different combination of drugs, have similar impacts on non-Foxp3+ Tregs.

Few studies have been conducted with regards to the impact of immunosuppressive agents on the generation or function of non-CD4+CD25+Foxp3+ Tregs. *In vitro* studies using human peripheral blood mononuclear cells found that RPM caused an increase in the numbers of CD103+CD8+ alloreactive T cells with immunosuppressive properties, while CsA had no significant effect on the percentage of these cells and prednisolone diminished the numbers of these cells (98). CD8+CD28- Treg function was improved in rheumatoid arthritis patients that received TNF-α inhibitor therapy, while patients treated with methotrexate had no effects on the defective CD8+CD28- Treg activity normally found in these patients (99). There is a dearth of published reports on the effects of immunosuppressive agents on Tr1 Tregs and DN T regs activity in the context of allograft and xenograft survival. Thus, there are significant gaps in our knowledge about the effects of immunosuppressive agents and the non-CD4+CD25+Foxp3+ Tregs.

Conclusions

Since Sakaguchi's discovery of CD4+CD25+ Tregs, over 25,000 publications have dealt with the general topic of Tregs. The overwhelming majority of these publications have focused on the role of CD4+CD25+ Tregs. However, in recent years there has been a growing awareness of the importance of CD8+ Tregs, DN Tregs, and Tr1 cells in controlling autoimmune diseases and in enhancing allograft survival. The presence of multiple populations of Tregs is a reflection of the remarkable redundancy and plasticity of the immune system. The importance of CD4+CD25+Tregs for immune homeostasis is well-recognized. Deficiencies in Foxp3 expression invariably lead to lymphoproliferative and multi-organ autoimmune diseases in both humans and mice. It has been suggested that CD4+CD25+ Tregs are generated in response to the initial priming stage of the immune response and act to limit immune-mediated inflammation that inflicts damage to juxtaposed tissues in various organs. By contrast, CD8+ Tregs, and perhaps DN Tregs, are generated from previously activated T cells. In both cases, the Tregs act to suppress immune-mediated inflammation and restore immune homeostasis. In certain conditions, organ allografts and xenografts benefit from the development of these non-conventional Tregs. Harnessing CD4+CD25+, CD4-CD8+, DN Tregs, and Tr1 cells as a comprehensive means of enhancing

the survival of allografts and xenografts in patients at high risk of rejecting their grafts is an appealing goal that is still in its early stages of development.

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Abbreviations

ACAID	anterior chamber-associated immune deviation
APC	antigen presenting cell
CsA	Cyclosporine
DC	dendritic cell
DN	double negative
DTH	delayed-type hypersensitivity
HDAC	Histone deacetylase inhibitor
IDO	indoleamine 2,3 dioxygenase
MHC	major histocompatibility complex
NK cell	natural killer cell
RPM	Rapamycin
TCR	T cell receptor
Tregs	T regulatory cells

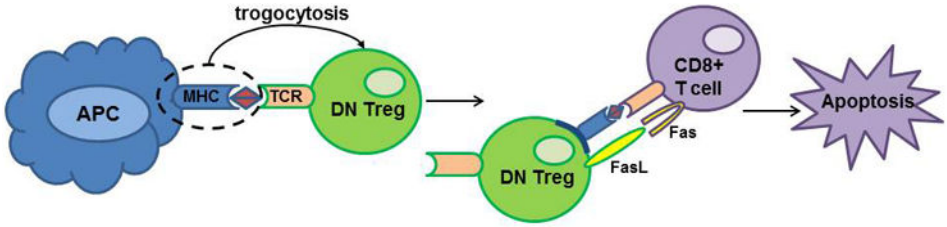

Figure 1. "Trogocytosis" as a mechanism for suppression mediated by double-negative T cells

MHC-alloantigen complex on antigen presenting cells (APC) engages the T cell receptor (TCR) on double-negative (DN) Tregs. This is followed by release of MHC/antigen complex by the APC and capture of the MHC/antigen complex by the DN Treg which integrates the complex into its cell membrane. The DN Tregs express this captured MHC/antigen complex on their cell surface. CD8+ T cells bearing the cognate TCR engage the MHC/antigen complex on the DN Tregs. DN Tregs then transmit an apoptotic signal via FasL on the DN Tregs through the Fas receptor on CD8+ T cells. The CD8+ effector T cells undergo apoptosis and are deleted.

Table 1

Phenotypic markers for non-CD4+CD25+T regs

Treg Population	Phenotypic Markers	Reference
CD8+ Tregs	Qa-1/HLA-restricted	(5-7)
	CD8+ CD122+	(8)
	CD8+CD28-	(9)
	CD8+Foxp3+	(10)
	CD8+CD103+	(11)
	CD8+LAG-3+ Foxp3+CTLA-4+	(12)
	CD8+ IL-10+CCR7+CD45RO+	(13)
	CD8+CD45RC^{low}	(14)
	CD8+CD122+PD-1+	(15)
	CD8+CD11c^{high}	(16)
CD4-CD8- DN Tregs	CD25+CD30+CD28^{low}	(51)
	CD25+CD28+CD44+CD69+	(55)
	CD25-CD27+CD28^{low}	(56)
CD4+Foxp3- Tr1 Tregs	CD49b+LAG-3+	(74)

Table 2

Mechanisms of suppression for non-CD4+CD25+T regs

Treg Population	Contact-Dependent	Contact-Independent	Other
CD8+ Tregs	Perforin	IDO	Promotes generation of CD4+CD25+ Tregs
	FasL/Fas	IFN-γ	
		Fibroleukin-2 (Fgl-2)	
		TGF-β	
DN Tregs	Perforin	None reported	Down-regulate costimulatory molecules on APCs
	FasL/Fas		
Tr1 Tregs	Perforin	IL-10	

Table 3

Treg Populations Associated with Allograft and Xenograft Tolerance

Graft	CD8+ Tregs	DN Tregs	Tr1 Tregs	References
Skin allograft	Yes	Yes	?	(21, 47, 51, 55, 57)
Heart allograft	Yes	Yes	?	(17, 21, 24, 38, 48, 65, 69, 70)
Pancreatic islet allograft	Yes	Yes	Yes	(21, 35, 55, 68, 76, 87, 89)
Kidney allograft	Yes	?	Yes	(49, 88)
Heart xenograft	Yes	Yes	?	(21, 58, 71)
Corneal xenograft	Yes	?	?	(20)
Hematopoietic stem cell allograft	Yes	Yes	Yes	(39, 59, 67, 86)