Archivum Immunologiae et Therapiae Experimentalis (2018) 66:355–364
https://doi.org/10.1007/s00005-018-0517-7

REVIEW

Transplant Tolerance: Current Insights and Strategies for Long-Term Survival of Xenografts

Lu Liu[1,2] · Chen He[3] · Jintao Liu[2] · Zhiwu Lv[2] · Ganlu Wang[2] · Hanchao Gao[1] · Yifan Dai[4] · David K. C. Cooper[5] · Zhiming Cai[1] · Lisha Mou[1]

Received: 15 March 2018 / Accepted: 18 June 2018 / Published online: 10 July 2018

Abstract

Xenotransplantation is an attractive solution to the problem of allograft shortage. However, transplants across discordant species barriers are subject to vigorous immunologic and pathobiologic hurdles, some of which might be overcome with the induction of immunologic tolerance. Several strategies have been designed to induce tolerance to a xenograft at both the central (including induction of mixed chimerism and thymic transplantation) and peripheral (including adoptive transfer of regulatory cells and blocking T cell costimulation) levels. Currently, xenograft tolerance has been well-established in rodent models, but these protocols have not yet achieved similar success in nonhuman primates. This review will discuss the major barriers that impede the establishment of immunological tolerance across xenogeneic barriers and the potential solution to these challenges, and provide a perspective on the future of the development of novel tolerance-inducing strategies.

Keywords Costimulation blockade · Immunological tolerance · Mixed chimerism · Thymic transplantation · Xenotransplantation

Abbreviations

APCs	Antigen-presenting cells
BMT	Bone marrow transplantation
DCs	Dendritic cells
GTKO	Alpha 1,3-galactosyltransferase knockout
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
SIRP-α	Signal regulatory protein alpha
TCR	T cell receptor
Tregs	T regulatory cells

✉ Lisha Mou
molly__molly@163.com

1 Shenzhen Xenotransplantation Medical Engineering Research and Development Center, Institute of Translational Medicine, Shenzhen Second People's Hospital, First Affiliated Hospital of Shenzhen University, Shenzhen University School of Medicine, Shenzhen University Health Science Center, Shenzhen 518035, Guangdong, China

2 Department of Gastroenterology, Center For Digestive Diseases, People's Hospital of Baoan District, The 8th people's Hospital of Shenzhen, Shenzhen 518101, Guangdong, China

3 Department of Ophthalmology, Shenzhen Second People's Hospital, First Affiliated Hospital of Shenzhen University, Shenzhen 518035, Guangdong, China

4 Jiangsu Key Laboratory of Xenotransplantation, Nanjing Medical University, Nanjing 210029, Jiangsu, China

5 Xenotransplantation Program/Department of Surgery, The University of Alabama at Birmingham, Birmingham, AL 35233, USA

Introduction

The severe shortage of allogeneic organ donors currently limits the number of transplants performed clinically. This supply–demand disparity could be solved by the ability to transplant organs from other species (xenografts) into human (Ekser et al. 2015; Griesemer et al. 2014). In view of the ethical issues and impracticalities associated with the use of nonhuman primates, interest has focused on other species, in particular the pig, as the most suitable organ-source species for humans. In addition to organ size and physiologic similarity to humans, the ability to rapidly breed pigs makes them particularly amenable to genetic modifications that could improve their ability to function as organ donors to humans. However, organ transplantation across discordant species barriers is subject to vigorous immunologic rejection (Griesemer et al. 2014).

The successful production of genetically engineered pigs, such as homozygous deletion of the gene encoding α1,3-galactosyltransferase and/or transgenic expression human complement regulatory proteins, has made it possible to avoid hyperacute rejection (Cooper et al. 2016). However, these genetically engineered xenografts can still be vigorously rejected as a result of the immune response, e.g., by antibodies to "non-Gal" antigens (Ezzelarab et al. 2006; Puga Yung et al. 2009; Samstein and Platt 2001). Therefore, continuing immunosuppressive therapy is required to prevent delayed rejection of the xenograft, which may lead to troublesome side-effects and/or complications in the recipients, including the increased risk of infection as well as direct drug-associated organ toxicity.

A possible solution for discordant xenografting is to achieve immunological tolerance, a state in which a vascularized xenograft would survive and function normally without features of severe rejections in the absence of intensive immunosuppressive therapy (Griesemer et al. 2014; Samstein and Platt 2001; Yamada et al. 2017). At present, anti-non-Gal antibodies contribute significantly to rejection of α1,3-galactosyltransferase knockout (GTKO) transplants in pig-to-primate xenotransplantation by activation of complement and interactions with a variety of effector cells, including macrophages and monocytes through Fcγ receptors and complement receptors (Ezzelarab et al. 2006; Puga Yung et al. 2009). However, antibody-mediated organ rejection is usually unresponsive to conventional anti-rejection therapy. If immunological tolerance could be attained, deletion of anti-non-Gal antibodies by absorption or removal of non-Gal antigens by genetic engineering, and suppression of the host immune responses by intensive immunosuppressive therapy would not be required.

This review will describe progress in our understanding of mechanisms of tolerance induction and the development of strategies to overcome immunological barriers by inducing tolerance.

Mechanisms of Tolerance

The induction of tolerance may involve several mechanisms that can be classified as "central" or "peripheral" (Singh and Schwartz 2006). Central tolerance involves deletional mechanisms analogous to self-tolerance and can be induced in experimental animals by creating mixed chimeras, using bone marrow transplantation (BMT), or by thymic transplantation. Peripheral tolerance has generally been ascribed to anergy and/or active immunoregulation, and can be induced by immune cells such as T regulatory cells (Tregs), or by targeted inhibition and/or depletion of lymphocytes with polyclonal or monoclonal antibodies (mAb). These mechanisms may act alone or together to achieve tolerance.

Central Tolerance

Central tolerance is established in the thymus and bone marrow where T cells and B cells develop and mature, respectively. Immature B cells expressing only surface IgM molecules undergo negative selection by recognizing self-molecules present in the bone marrow. These self-reacting B cells are eliminated by undergoing apoptosis (clonal deletion) or are rescued by further gene rearrangements (receptor editing), replacing the self-reactive antigens with new ones (Jacquemin et al. 2001). In the thymus, immature lymphocytes with a high affinity for self-peptide/major histocompatibility complex (MHC), receive signals that trigger apoptosis and die before they complete their maturation. This process, referred as to negative selection, is the major mechanism of T cell central tolerance. It is generally recognized that central tolerance provides the most robust long-lasting state of unresponsiveness (Hogquist et al. 2005; Sprent and Kishimoto 2001).

Peripheral Tolerance

Although central tolerance mechanisms are efficient, they cannot eliminate all self-reactive lymphocytes, in part because not all self-antigens are expressed at the primary sites of lymphocyte development. Therefore, peripheral tolerance mechanisms come into play, and these induce lymphocytes that first encounter their cognate self-antigen outside the primary site of development to become tolerant (Mueller 2010; Xing and Hogquist 2012). Deletion of activated T cells, T cell anergy, and active regulation can occur in the periphery. Deletion of self-reactive lymphocytes in the periphery is achieved though apoptotic cell death. Anergy results from the recognition of antigens in the absence of costimulatory signals, or in the presence of coinhibitory signals. In addition to these "passive" mechanisms, self-reactive lymphocytes can be suppressed by regulatory T cells, a dominant control mechanism.

Strategies for Inducing Tolerance of Xenografts

Thymic Transplantation

T cell-mediated immune responses to a xenograft are thought to be stronger than to an allograft (Bühler and Cooper 2005). As the origin of T cell immunity, the thymus has the unique potential to establish donor-specific tolerance by inducing (or "tricking") the recipient's immune system into treating donor antigens as

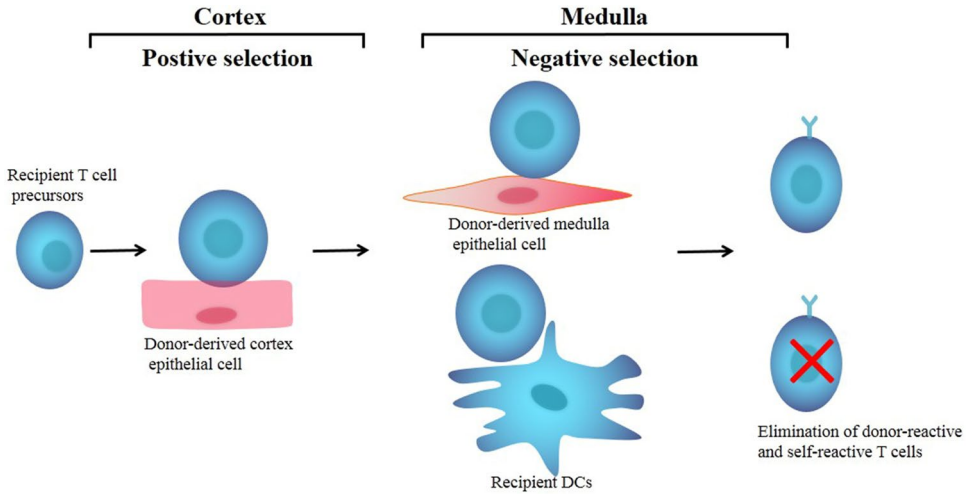

Fig. 1 Induction of T cell tolerance by thymic transplantation. Recipient T cell precursors entered the donor thymus, and migrated through the cortex where they underwent positive selection by interacting with the epithelial cell. They then migrated to the medulla, where any donor-reactive or recipient-reactive T cells could undergo apoptosis by recognizing donor- and recipient-antigens present in donor epithelial cells or recipient dendritic cells (DCs) in the medulla (negative selection). Donor cells were shown in red; recipient cells were shown in blue

self-antigens (Hogquist et al. 2005; Sprent and Kishimoto 2001) (Fig. 1).

Donor-specific xenograft tolerance can be induced in thymectomized, T cell-depleted mice by grafting with pig thymus (Zhao et al. 1996, 1997a). In this pig-to-mouse model, functional mouse $CD4^+$ T cells repopulated the periphery of the recipient and were tolerant to xenogeneic donor antigens, as indicated by specific unresponsiveness to donor antigens in mixed lymphocyte reactions and long-term acceptance of swine skin grafts. Using MHC knockout and various T cell receptor (TCR) transgenic mice, Zhao et al. (1997b) revealed that both porcine and mouse MHC mediated negative selection of mouse T cells in xenogeneic thymus grafts, resulting in specific tolerance to both donor and recipients. Based on these observations, elegant studies by Sykes's group confirmed the feasibility of inducing human T cell tolerance to porcine xenoantigens by thymic transplantation in mice with a pre-established human immune system (Habiro et al. 2009; Kalscheuer et al. 2014). Spectratyping analysis revealed that the human T cells developing in porcine thymus grafts were functional and similar to those generated in human thymus grafts (Nikolic et al. 1999). Porcine thymus grafts also supported the development of human Tregs which contributed to the donor-specific unresponsiveness (Kalscheuer et al. 2014). However, these thymus-grafted athymic or thymectomized recipients frequently developed a wasting syndrome of autoimmune origin (Kalscheuer et al. 2014; Zhao et al. 2003). Tregs developed normally in these models, but they failed to mediate normal regulation of host-reactive T cells. This defect was partly reversible by injecting recipient-type thymic epithelial cells into the donor thymus at the time of grafting (Zhao et al. 2003). Inclusion of recipient thymic epithelial cells in xenogeneic thymus grafts promoted positive selection of recipient-specific Tregs and negative selection of tissue antigen-specific effector T cells (Griesemer et al. 2014).

Recently, progress has been made towards the induction of tolerance in the highly disparate pig-to-nonhuman primate model (Fudaba et al. 2008). Implanting vascularized donor-specific thymus under the donor kidney capsule (as a composite "thymokidney") from GTKO pigs enabled swine kidney grafts to survive for up to 83 days with normal function in baboons (Yamada and Scalea 2012). These kidney grafts showed no signs of cellular infiltration or deposition of IgG, and no grafts were lost due to rejection (Yamada et al. 2005). In contrast, baboons receiving a kidney alone had unstable renal function due to severe rejection with the persistence of anti-donor cytotoxic T cell reactivity (Yamada and Scalea 2012; Yamada et al. 2005). Therefore, grafting donor thymus tissue may offer an approach for achieving discordant xenograft tolerance and reconstitution of host cellular immunity.

Induction of Mixed Hematopoietic Cell Chimerism

Mixed hematopoietic chimerism is defined as the coexistence of donor and host hematopoietic cells in primary lymphoid organs after hematopoietic stem cell or BMT (Griesemer et al. 2009). It is one of best-studied approaches for establishing tolerance in organ transplantation. In a mixed chimera, both the recipient and donor bone marrow-derived antigen-presenting cells (APCs) take

residence in the recipient thymus and participate in intrathymic negative selection; T cells that express receptors reacting with either recipient or donor antigens are thereby deleted (Pilat and Wekerle 2010) (Fig. 2).

Mixed chimerism for the induction of xenogeneic tolerance has been investigated in several models. Early studies of xenogeneic BMT were performed in a closely related rodent model, in which sublethal pretreatment, including whole body irradiation and immunosuppressive therapy (anti-CD4 and anti-CD8 mAb) of the recipient was required, together with the infusion of large numbers of donor bone marrow cells (Anderson et al. 1996; Sharabi and Sachs 1989). In these rat-to-mouse chimeras, the mouse immune responses were tolerized, allowing prolonged donor-specific skin graft survival. Further studies indicated that tolerance was associated with the presence of rat bone marrow-derived APCs in the recipient thymus and deletion of donor-reactive T cell clones (Sharabi et al. 1990; Tomita et al. 1994). B cell responses in these rat-to-mouse chimeras were also tolerized, and chimeric animals lacked induced antibody responses even following secondary skin grafts (Ohdan et al. 2001). Studies using GTKO mice showed that preexisting xenoreactive antibody-producing B cells were rendered anergic early after BMT, but long-term B cell tolerance in these chimeras was mediated by clonal deletion and/or receptor editing (Nikolic et al. 1998).

Mixed chimerism across a disparate xenogeneic barrier has also been established in a pig-to-mouse transplant model, in which transgenic mice expressing three porcine cytokine transgenes were used (Abe et al. 2002a). Murine T cells developing in porcine hematopoietic chimeras were specifically unresponsive to porcine donors, as demonstrated by the acceptance of donor skin grafts and the lack of anti-donor mixed lymphocyte reactions or anti-donor IgG production.

However, mixed xenogeneic chimerism has been much more difficult to achieve in the pig-to-nonhuman primate model. Even with the infusion of GTKO bone marrow cells, the majority of cells were cleared from the blood within hours and peripheral chimerism was generally undetectable after 1 week (Tseng et al. 2004). Additionally, chimeric monkeys had no apparent B cell tolerance, as anti-pig IgM and IgG were found to be at pretransplant levels soon after transplantation (Sablinski et al. 1999).

Several in vivo studies demonstrated that, even in the absence of an adaptive immune response, macrophages caused immediate phagocytosis of xenogeneic bone marrow, contributing to rapid clearance of porcine bone marrow cells in both mice and nonhuman primates (Abe et al. 2002b; Basker et al. 2001). Development of strategies for overcoming macrophage-mediated rejection is likely to be crucial, since mixed chimerism does not seem to tolerize macrophages and continuous macrophage deletion is not clinically practical (Abe et al. 2002b). Signal regulatory protein (SIRP)-α is a critical immune inhibitory receptor on macrophages, and its interaction with CD47, a ligand for SIRP-α, prevents autologous phagocytosis (Vernon-Wilson et al. 2000). Studies indicated that interspecies incompatibility of CD47 and SIRP-α significantly contributed to phagocytosis of xenogeneic cells by human macrophages (Ide et al. 2007; Wang et al. 2007). Porcine cells expressing mouse or human CD47 exhibited significantly reduced phagocytosis by mouse or human macrophages, respectively. Tena et al. (2014) developed GTKO/human CD47 transgenic pigs, and found that overexpression of human CD47 markedly increased survival of porcine hematopoietic cells in mice. Recently, Tena et al. (2017) extended these studies to a pig-to-nonhuman primate model, and demonstrated that infusion of hematopoietic cells from GTKO/human CD47 pigs markedly prolonged the duration of transient chimerism in baboons,

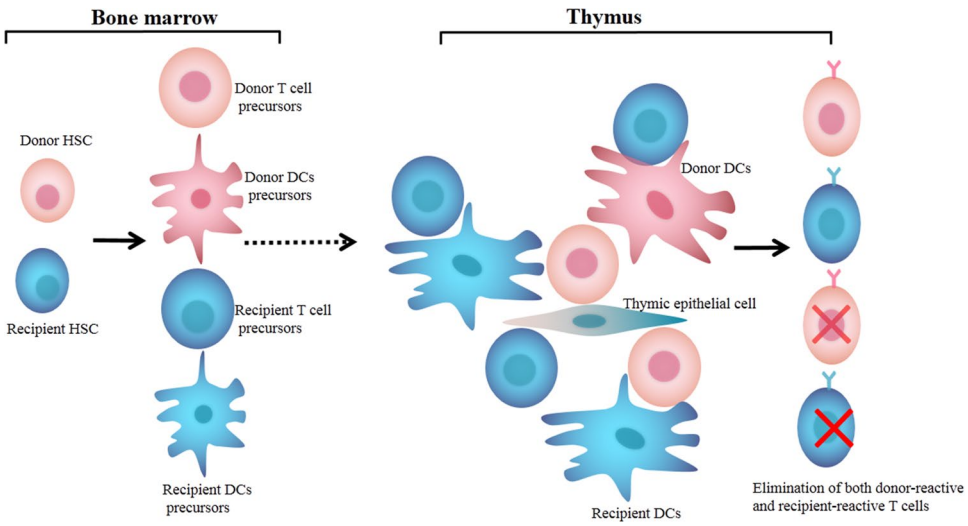

Fig. 2 Induction of T cell tolerance by mixed hematopoietic chimerism. After hematopoietic stem cell engrafted, they could produce T cell precursors and DC precursors in bone marrow, which then migrated to the thymus. In the thymus, donor-reactive or recipient-reactive T cells were deleted by negative selection through interacting with donor DCs as well as recipient epithelial cells and DCs. Donor cells were shown in red; recipient cells were shown in blue

as indicated by prolonged acceptance of swine skin grafts. These observations suggested that genetic manipulation of porcine donors to express human CD47 may provide a novel approach for improving donor hematopoietic engraftment in primates.

In addition to immunological factors, the failure of adhesive interactions and hematopoietic cytokines across discordant species barriers would impose severe limitations on the use of xenogeneic BMT in the induction of tolerance, as xenogeneic hematopoietic cells survival and function depended on the efficacy of those interactions (Gritsch et al. 1994; Sablinski et al. 1999; Simon et al. 1999; Yang et al. 1996). These barriers may potentially be overcome by genetic manipulation, as indicated by studies in which long-lasting chimerism was achieved in the very disparate pig-to-mouse combination by transgenic expression of porcine interleukin (IL)-3, granulocyte–macrophage colony-stimulating factor and stem cell factor in mice (Yang et al. 1996).

Adoptive Transfer of Regulatory Cells

Although most self-reactive cells are deleted centrally in the thymus, some manage to escape into the periphery. Tregs, displaying suppressive or regulatory properties, came to be recognized as an important way to promote the development of tolerance in the periphery (Mueller 2010; Sakaguchi et al. 2008) (Fig. 3). Several different types of regulatory cells have been discovered during the past two decades.

$CD4^+CD25^+FoxP3^+$Tregs

The best-studied Tregs are a subset of $CD4^+$ T cells characterized phenotypically by constitutive expression of the α chain of the IL-2 receptor, CD25, and the transcription factor Foxp3 (Hori et al. 2003). Initial studies by Hall et al. (1990) demonstrated that the transfer of $CD4^+$T cells from rats that had accepted heart allografts (following a short course of cyclosporine) into naive rats conferred donor-specific tolerance. Sakaguchi's group identified that $CD4^+CD25^+$ cells from naïve mice could prevent the rejection of fully mismatched skin allografts upon transfer into lymphopenic hosts (Sakaguchi et al. 1995).

In xenotransplantation, Porter et al. (2005) found that human $CD4^+CD25^+$Treg cells can dramatically suppress the proliferation, cytotoxicity, and cytokine production of $CD4^+CD25^-$ effector T cells induced in response to porcine cells. A series of in vitro studies demonstrated that $CD4^+CD25^+$Tregs can suppress the direct and indirect T cell xenogeneic responses to T cell, B cell, and macrophage activation (Fu et al. 2008; Nishimura et al. 2010; Singh et al. 2012; Sun et al. 2010). $CD4^+CD25^+$Tregs have also been shown to induce and maintain in vivo xenograft tolerance. A humanized mouse model was developed in which ex vivo expanded human Tregs had the capacity to inhibit the T cell-mediated rejection of porcine islet xenografts (Yi et al. 2012). However, a recent study in vivo demonstrated that adoptive transfer of autologous Tregs failed to maintenance survival of porcine islet in primates on its own and rejection was initiated as soon as the costimulation blockade ceased (Shin et al. 2016). Thus, the use of Tregs was insufficient

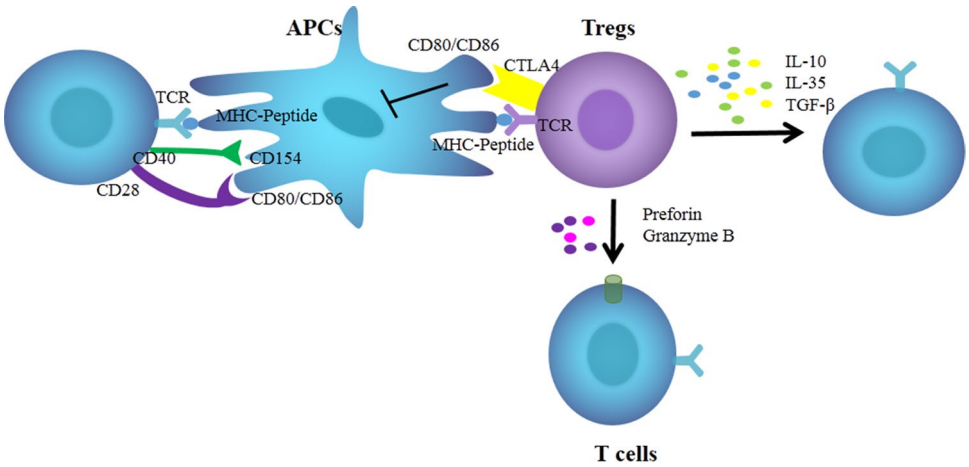

Fig. 3 Tolerance induction by Tregs. Tregs could utilize different mechanisms to promote the development of tolerance, including modulation of antigen-presenting cells (APCs) maturation and function, anti-inflammatory cytokine production and induction of T cell apoptosis. (1) Modulation of APCs maturation and function. Tregs downregulated costimulatory molecules (CD80 and CD86) on APCs. Additionally, interaction of CTLA4 on Tregs with its ligand CD80/CD86 on APCs delivered a negative signal for T cell activation. (2) Anti-inflammatory cytokine production. Tregs released IL-10, IL-35and TGF-β, directly inhibiting T cell activation. (3) Induction of T cell apoptosis. Tregs directly killed T cells through perforin- or granzyme B-dependent pathways

to induce and maintain xenogeneic tolerance by itself in primate.

$CD4^{-}CD8^{-}$ (Double-Negative) Tregs

Recent years have witnessed a resurgence in interest in $CD4^{-}CD8^{-}$ (double-negative) Tregs and their roles in controlling autoimmune diseases and in promoting the survival of organ allografts and xenografts (Ligocki and Niederkorn 2015; Lu and Cantor 2008). Studies in the rat-to-mouse model demonstrated that adoptive transfer of double-negative Tregs induced B cell apoptosis and suppressed the proliferation of both anti-donor-reactive $CD4^{+}$ and $CD8^{+}$ T cells, thus prolonging cardiac xenograft survival (Chen et al. 2003; Ma et al. 2008).

Mesenchymal Stem Cells

In addition to Tregs, mesenchymal stem cells (MSCs) also seem to be implicated in the maintenance of tolerance to xenografts (Li et al. 2014). Porcine MSCs have been shown in vitro to possess an immunoregulatory capacity by suppressing the activation and proliferation of human T cells and reducing levels of proinflammatory cytokines (Ezzelarab et al. 2011; Li et al. 2013). Furthermore, MSCs have shown promise in facilitating the induction of mixed chimerism. Eguchi et al. (2008) reported that cotransplantation of porcine bone marrow cells with donor-specific MSC improved short-term xenogeneic engraftment in nonobese diabetic/severe combined immunodeficiency mice.

Blocking T Cell Costimulation

Inhibition of costimulatory signals is critical for the establishment and maintenance of peripheral tolerance. Engagement of the TCR and the antigen without proper costimulatory signals may render T cells unresponsive to the antigen, thus inducing tolerance in the peripheral (Fig. 4).

Several costimulatory pathways have been identified, and the importance of the CD28–CD80/CD86 and CD40–CD154 pathways in the immune response to xenografts in rodents was first demonstrated by the combined administration of anti-CD154 mAb and CTLA4-Ig fusion protein (Elwood et al. 1998). The combined blockade of the CD28–CD80/CD86 and CD40–CD154 pathways markedly inhibited the cellular immune response and prolonged the survival of xenogeneic pig skin in mice. Lehnert et al. (2000) demonstrated that short-term inhibition of CD28–CD80/CD86 and CD40–CD154 costimulatory pathways could result in indefinite pancreatic islet survival in the concordant rat-to-mouse model. In a discordant pig-to-primate model, Bühler et al. (2000) reported that anti-CD154 mAb modified the humoral response to porcine hematopoietic cells in baboons pretreated with a nonmyeloablative regimen. Further studies indicated that B cell tolerance induced by CD154 blockade involved the indirect pathway of antigen presentation (Bühler et al. 2001). Recently, numerous studies have demonstrated that CD40–CD154 pathway blockade-based immunosuppression can successfully prevent both humoral and cellular adaptive immune responses in nonhuman primates receiving pig organs (Choi et al. 2015; Higginbotham et al. 2015; Thompson et al. 2012). However, rejection was initiated as soon as the costimulation blockade ceased, suggesting that blocking the costimulatory signal pathway alone seemed insufficient to maintain the long-term xenograft survival in these discordant transplantation settings.

Studies by Chong's group showed that peripheral tolerance induction and maintenance were susceptible to viral and bacterial infections (Chong and Alegre 2014; Wang et al. 2010). In contrast to thymic transplantation and mixed chimerism resulting in the physical elimination of donor-reactive T cells, both costimulation blockade and adoptive transfer of regulatory cells permitted such cells continued

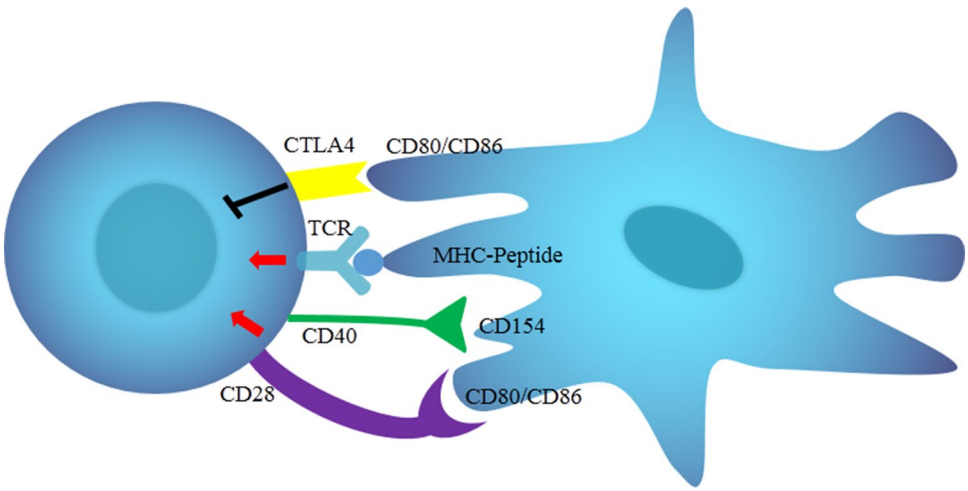

Fig. 4 Tolerance induction by blockade of costimulation. T cell activation required two signals. The first involved TCR triggering by MHC-peptide on APCs. The second signal (costimulation signal) was provided by interactions between costimulatory molecules expressed on APCs and T cell. Blocking costimulation signals with targeted mAb, rendered T cells unresponsive to the antigen or induced apoptosis

existence, and thus, had the inherent risk that changes in the immune environment of the recipient, such as infections or other inflammatory stimuli, could allow for the reactivation of immune responses against grafts. These results also suggested that long-term tolerance maintenance would require utilization of both central and peripheral mechanisms in a complementary manner.

Conclusion and Future Remarks

Considering vigorous immune rejection and the unacceptable side-effects of non-specific immunosuppressive regimen in achieving long-term xenograft survival, the induction of immunological tolerance is likely to be needed to bring xenotransplantation to clinical reality. Four strategies, as outlined at Table 1, are being pursued with the objective of achieving xenograft tolerance. Xenograft tolerance has been well-established in rodent models, but these protocols have not yet achieved similar success in nonhuman primates. The reasons for the difference in susceptibility to tolerance of xenograft between rodent and large animals remain speculative, but they are at least partly due to their differences in immune system development, activation, and response to challenges. For example, MHC class II molecules are notably absent from the vascular endothelium of rodents, but they are constitutively expressed in both pigs and humans, resulting in the direct and indirect activation of anti-porcine $CD4^+$ T cells in humans after pig-to-human xenotransplantation (Choo et al. 1997). Moreover, increasing evidence has demonstrated that the previous immunological exposures and resultant T cell memory can pose a formidable barrier to tolerance induction and maintenance in primates or humans (Adams et al. 2003; Sachs 2003). Laboratory mice bred in pathogen-free environment and studied at 4–8 weeks of age have a largely naive immune system, while primates or humans raised in a non-sterile environment are thereby likely to develop heterologous immunity. Donor-reactive memory T cells could rapidly react to infectious challenges which trigger reactivation of immune responses against grafts by cross-reactivity or bystander activation. In addition, compared with their naive T cell counterparts, memory T cells are resistance to conventional immunosuppression (Zhai et al. 2002). Thus, many tolerance strategies, such as regimens employing T cell costimulation which successfully target and control native T cell responses in mice, may fail when evaluated in primate models. These barriers that potentially impeded the translation of the work of rodent to large animal are outlined in Table 2.

It has become clear that stable xenograft tolerance in primate could not be established using a single tolerance-inducing protocol. Tolerance induction and maintenance of xenografts may require utilization of both central and peripheral mechanisms in a complementary manner. A recent study has

Table 1 Tolerance-inducing approaches

Approach	Mechanism and method
Thymic transplantation	Implantation of donor thymus tissue in thymetozied, T cell-depleted recipient, to achieve life-long intrathymic deletion of donor-reactive T cell T cell/lymphocyte depletion: thymic irradiation/total body irradiation; antithymocyte globulin, anti-CD4 mAb, anti-CD8 mAb, anti-Thy1.2 mAb, anti-NK1.1 mAb Thymectomy
Mixed chimerism[a]	Infusion of donor bone marrow into myoablated/immune-conditioned recipient, to produce co-existence of donor and recipient cells T cell/lymphocyte depletion: thymic irradiation/total body irradiation; antithymocyte globulin, anti-CD4 mAb, anti-CD8 mAb, anti-Thy1.2 mAb, anti-NK1.1 mAb Adoptive Treg transfer Costimulatory blockade: anti-CD40L mAb, CTLA-4 Ig
Adoptive transfer of regulatory cells	Tregs: Infusion of expanded Tregs, to modulate APCs maturation and function by down-regulating costimulatory and adhesion molecules, produce suppressive cytokines IL-10, TGF-β, and IL-35 that inhibited T cell activation, and promote energy and cell death, convert effector T cells to a regulatory phenotype Double-negative Tregs: Inducing B cell apoptosis and suppressing the proliferation of donor-reactive T cells Mesenchymal stromal cells: Inhibition of T cell activation and proliferation, potentially due to production of IL-10, NO, and IDO, and suppression of IFN-γ and IL-17
Costimulatory blockade	Blocking proper costimulation signals to render effector T cells unresponsive to donor antigen or inducing apoptosis CTLA-4 Ig, blockade of CD28:CD80/60 costimulatory pathway Anti-CD40L mAb, blockade of CD40:CD154 costimulatory pathway

[a]Combing costimulatory blockade or/and Tregs infusion with bone marrow transplantation not only reduced the toxicity of conditioning necessary to the engraftment of bone marrow cells, but also suppressed the activation or function of T cells in a cell–cell contact or contact-independent manner, which prolonged xenograft survival

Table 2 Barriers potentially impeded the translation of the work in rodent models to primate or human

Barriers	Key findings
Interspecies molecular incompatibilities	The lack of adhesive interactions and hematopoietic cytokines necessary to survival and function of implanted bone marrow cells The failure of costimulatory molecules to function across discordant species barriers, for example, incompatibility between porcine CD47 and human SIRP-α
Heterologous immunity	T cell memory resulting from previous immunological exposure rapidly react to infectious challenges which triggers reactivation of immune responses against grafts by TCR cross-reactivity or non-specific bystander activation
Lack of effective immunosuppressive regimen	Complete T cell depletion not easily achievable in primates partly due to memory T cells resistant to conventional immunosuppression
Others	The differential expression patterns of MHC class II antigens

shown that combined with anti-154 mAb, adoptive transfer of Tregs markedly prolonged allogneic chimerism in primate (Duran-Struuck et al. 2017). In addition to deletion of intrathymic donor-reactive T cells via central mechanisms, preexisting mature donor-reactive T cells were tolerized through anergy by blocking CD40–CD154 signal pathway and through regulation by infusion of antigen-specific Tregs in this protocol. Similar attempts, combining some of tolerance-inducing approaches, might improve the short-term xenogeneic tolerance in primate. Finally, other pathobiologic barriers, such as coagulation dysfunction and CD47-SIRP-α incompatibilities, need to be overcome by genetic manipulations of porcine donor before T and/or B cell tolerance will be able to be achieved.

Acknowledgements This work was supported by National Key R&D Program of China (2017YFC1103704), National Natural Science Foundation of China (31500639), Shenzhen Foundation of Science and Technology (JCYJ20170307094549868, JCYJ20160427191926655, JCYJ20160427192552523, JCYJ20160229204849975 and GCZX2015043017281705), Fund for High Level Medical Discipline Construction of Shenzhen (2016031638), Sanming Project of Medicine in Shenzhen (SZSM201412020), Shenzhen Foundation of Health and Family Planning Commission (SZXJ2017021), Shenzhen Baoan District Science and Technology Bureau (2018JD124), Clinical Doctor-Basic Scientist Combination Foundation of Shenzhen Secondary people's Hospital and Key Laboratory Project of Shenzhen Second People's Hospital.

Compliance with Ethical Standards

Conflict of interest There are no conflicts of interest.

References

Abe M, Qi J, Sykes M et al (2002a) Mixed chimerism induces donor-specific T cell tolerance across a highly disparate xenogeneic barrier. Blood 99:3823–3829

Abe M, Cheng J, Qi J et al (2002b) Elimination of porcine hemopoietic cells by macrophages in mice. J Immunol 168:621–628

Adams AB, Pearson TC, Larsen CP (2003) Heterologous immunity: an overlooked barrier to tolerance. Immunol Rev 196:147–160

Anderson G, Moore NC, Owen JJ et al (1996) Cellular interactions in thymocyte development. Annu Rev Immunol 14:73–99

Basker M, Alwayn IP, Buhler L et al (2001) Clearance of mobilized porcine peripheral blood progenitor cells is delayed by depletion of the phagocytic reticuloendothelial system in baboons. Transplantation 72:1278–1285

Bühler LH, Cooper DK (2005) How strong is the T cell response in the pig-to-primate model? Xenotransplantation 12:85–87

Bühler LH, Awwad M, Basker M et al (2000) High-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade in baboons prevents an induced anti-pig humoral response. Transplantation 69:2296–2304

Bühler LH, Alwayn IP, Basker M et al (2001) CD40–CD154 pathway blockade requires host macrophages to induce humoral unresponsiveness to pig hematopoietic cells in baboons. Transplantation 72:1759–1768

Chen W, Ford MS, Young KJ et al (2003) Role of double-negative regulatory T cells in long-term cardiac xenograft survival. J Immunol 170:1846–1853

Choi HJ, Lee JJ, Kim DH et al (2015) Blockade of CD40–CD154 costimulatory pathway promotes long-term survival of full-thickness porcine corneal grafts in nonhuman primates: clinically applicable xenocorneal transplantation. Am J Transplant 15:628–641

Chong AS, Alegre ML (2014) Transplantation tolerance and its outcome during infections and inflammation. Immunol Rev 258:80–101

Choo JK, Seebach JD, Nickeleit V et al (1997) Species differences in the expression of major histocompatibility complex class II antigens on coronary artery endothelium: implications for cell-mediated xenoreactivity. Transplantation 64:1315–1322

Cooper DK, Ezzelarab MB, Hara H et al (2016) The pathobiology of pig-to-primate xenotransplantation: a historical review. Xenotransplantation 23:83–105

Duran-Struuck R, Sondermeijer HP, Bühler L et al (2017) Effect of ex vivo-expanded recipient regulatory T cells on hematopoietic chimerism and kidney allograft tolerance across MHC barriers in cynomolgus macaques. Transplantation 101:274–283

Eguchi H, Kuroiwa Y, Matsui A et al (2008) Intra-bone marrow cotransplantation of donor mesenchymal stem cells in pig-to-NOD/SCID mouse bone marrow transplantation facilitates short-term xenogeneic hematopoietic engraftment. Transplant Proc 40:574–577

Ekser B, Cooper DK, Tector AJ (2015) The need for xenotransplantation as a source of organs and cells for clinical transplantation. Int J Surg 23:199–204

Elwood ET, Larsen CP, Cho HR et al (1998) Prolonged acceptance of concordant and discordant xenografts with combined CD40 and CD28 pathway blockade. Transplantation 65:1422–1428

Ezzelarab M, Hara H, Busch J et al (2006) Antibodies directed to pig non-Gal antigens in naïve and sensitized baboons. Xenotransplantation 13:400–407

Ezzelarab M, Ezzelarab C, Wilhite T et al (2011) Genetically-modified pig mesenchymal stromal cells: xenoantigenicity and effect on human T-cell xenoresponses. Xenotransplantation 18:183–195

Fu Y, Yi S, Wu J et al (2008) In vitro suppression of xenoimmune-mediated macrophage activation by human $CD4^+$ $CD25^+$ regulatory T cells. Transplantation 86:865–874

Fudaba Y, Onoe T, Chittenden M et al (2008) Abnormal regulatory and effector T cell function predispose to autoimmunity following xenogeneic thymic transplantation. J Immunol 181:7649–7659

Griesemer A, Hirakata A, Shimizu A et al (2009) Results of gal-knockout porcine thymokidney xenografts. Am J Transplant 9:2669–2678

Griesemer A, Yamada K, Sykes M (2014) Xenotransplantation: immunological hurdles and progress toward tolerance. Immunol Rev 258:241–258

Gritsch HA, Glaser RM, Emery DW et al (1994) The importance of nonimmune factors in reconstitution by discordant xenogeneic hematopoietic cells. Transplantation 57:906–917

Habiro K, Sykes M, Yang YG (2009) Induction of human T-cell tolerance to pig xenoantigens via thymus transplantation in mice with an established human immune system. Am J Transplant 9:1324–1329

Hall BM, Pearce NW, Gurley KE et al (1990) Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the $CD4^+$ suppressor cell and its mechanisms of action. J Exp Med 171:141–157

Higginbotham L, Mathews D, Breeden CA et al (2015) Pre-transplant antibody screening and anti-CD154 costimulation blockade promote long-term xenograft survival in a pig-to-primate kidney transplant model. Xenotransplantation 22:221–230

Hogquist KA, Baldwin TA, Jameson SC (2005) Central tolerance: learning self-control in the thymus. Nat Rev Immunol 5:772–782

Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061

Ide K, Wang H, Tahara H et al (2007) Role for CD47-SIRPalpha signaling in xenograft rejection by macrophages. Proc Natl Acad Sci USA 104:5062–5066

Jacquemin MG, Vanzieleghem B, Saint-Remy JM (2001) Mechanisms of B-cell tolerance. Adv Exp Med Biol 489:99–108

Kalscheuer H, Onoe T, Dahmani A et al (2014) Xenograft tolerance and immune function of human T cells developing in pig thymus xenografts. J Immunol 192:3442–3450

Lehnert AM, Yi S, Burgess JS et al (2000) Pancreatic islet xenograft tolerance after short-term costimulation blockade is associated with increased $CD4^+$ T cell apoptosis but not immune deviation. Transplantation 69:1176–1185

Li J, Andreyev O, Chen M et al (2013) Human T cells upregulate CD69 after coculture with xenogeneic genetically-modified pig mesenchymal stromal cells. Cell Immunol 285:23–30

Li J, Ezzelarab MB, Ayares D et al (2014) The potential role of genetically-modified pig mesenchymal stromal cells in xenotransplantation. Stem Cell Rev 10:79–85

Ligocki AJ, Niederkorn JY (2015) Advances on non-$CD4^+$ $Foxp3^+$ T regulatory cells: $CD8^+$, type 1, and double negative T regulatory cells in organ transplantation. Transplantation 99:1553–1559

Lu L, Cantor H (2008) Generation and regulation of CD8(+) regulatory T cells. Cell Mol Immunol 5:401–406

Ma Y, He KM, Garcia B et al (2008) Adoptive transfer of double negative T regulatory cells induces B-cell death in vivo and alters rejection pattern of rat-to-mouse heart transplantation. Xenotransplantation 15:56–63

Mueller DL (2010) Mechanisms maintaining peripheral tolerance. Nat Immunol 11:21–27

Nikolic B, Lei H, Pearson DA et al (1998) Role of intrathymic rat class II^+ cells in maintaining deletional tolerance in xenogeneic rat → mouse bone marrow chimeras. Transplantation 65:1216–1224

Nikolic B, Gardner JP, Scadden DT et al (1999) Normal development in porcine thymus grafts and specific tolerance of human T cells to porcine donor MHC. J Immunol 162:3402–3407

Nishimura T, Onda M, Takao S (2010) $CD4^+$ $CD25^+$ regulatory T cells suppressed the indirect xenogeneic immune response mediated by porcine epithelial cell pulsed dendritic cells. Xenotransplantation 17:313–323

Ohdan H, Swenson KG, Kitamura H et al (2001) Tolerization of Gal alpha 1,3 Gal-reactive B cells in pre-sensitized alpha 1,3-galactosyltransferase-deficient mice by nonmyeloablative induction of mixed chimerism. Xenotransplantation 8:227–238

Pilat N, Wekerle T (2010) Transplantation tolerance through mixed chimerism. Nat Rev Nephrol 6:594–605

Porter CM, Bloom ET et al (2005) Human $CD4^+$ $CD25^+$ regulatory T cells suppress anti-porcine xenogeneic responses. Am J Transplant 5:2052–2057

Puga Yung G, Schneider MK, Seebach JD (2009) Immune responses to alpha1,3galactosyltransferase knockout pigs. Curr Opin Organ Transplant 14:154–160

Sablinski T, Emery DW, Monroy R et al (1999) Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. Transplantation 67:972–977

Sachs DH (2003) Tolerance: of mice and men. J Clin Invest 111:1819–1821

Sakaguchi S, Sakaguchi N, Asano M et al (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155:1151–1164

Sakaguchi S, Yamaguchi T, Nomura T et al (2008) Regulatory T cells and immune tolerance. Cell 133:775–787

Samstein B, Platt JL (2001) Xenotransplantation and tolerance. Philos Trans R Soc Lond B Biol Sci 356:749–758

Sharabi Y, Sachs DH (1989) Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. J Exp Med 169:493–502

Sharabi Y, Aksentijevich I, Sundt TM IIIrd et al (1990) Specific tolerance induction across a xenogeneic barrier: production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. J Exp Med 172:195–202

Shin JS, Min BH, Kim JM et al (2016) Failure of transplantation tolerance induction by autologous regulatory T cells in the pig-to-non-human primate islet xenotransplantation model. Xenotransplantation 23:300–309

Simon AR, Warrens AN, Sykes M (1999) Efficacy of adhesive interactions in pig-to-human xenotransplantation. Immunol Today 20:323–330

Singh NJ, Schwartz RH (2006) Primer: mechanisms of immunologic tolerance. Nat Clin Pract Rheumatol 2:44–52

Singh AK, Seavey CN, Horvath KA et al (2012) Ex-vivo expanded baboon $CD4^+$ CD25 Hi Treg cells suppress baboon anti-pig T and B cell immune response. Xenotransplantation 19:102–111

Sprent J, Kishimoto H (2001) The thymus and central tolerance. Philos Trans R Soc Lond B Biol Sci 356:609–616

Sun L, Yi S, O'Connell PJ (2010) Foxp3 regulates human natural $CD4^+$ $CD25^+$ regulatory T-cell-mediated suppression of xenogeneic response. Xenotransplantation 17:121–130

Tena AA, Kurtz J, Leonard DA et al (2014) Transgenic expression of human CD47 markedly increases engraftment in a murine model of pig-to-human hematopoietic cell transplantation. Am J Transplant 14:2713–2722

Tena AA, Sachs DH, Mallard C et al (2017) Prolonged survival of pig skin on baboons after administration of pig cells expressing human CD47. Transplantation 101:316–321

Thompson P, Badell IR, Lowe M et al (2012) Alternative immunomodulatory strategies for xenotransplantation: CD40/154 pathway-sparing regimens promote xenograft survival. Am J Transplant 12:1765–1775

Tomita Y, Lee LA, Sykes M (1994) Engraftment of rat bone marrow and its role in negative selection of murine T cells in mice conditioned with a modified nonmyeloablative regimen. Xenotransplantation 1:109–117

Tseng YL, Dor FJ, Kuwaki K et al (2004) Bone marrow transplantation from α-1,3-galactosyltransferase gene-knockout pigs in baboons. Xenotransplantation 11:361–370

Vernon-Wilson EF, Kee WJ, Willis AC et al (2000) CD47 is a ligand for rat macrophage membrane signal regulatory protein SIRP (OX41) and human SIRPalpha 1. Eur J Immunol 30:2130–2137

Wang H, VerHalen J, Madariaga ML et al (2007) Attenuation of phagocytosis of xenogeneic cells by manipulating CD47. Blood 109:836–842

Wang T, Ahmed EB, Chen L et al (2010) Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model. Am J Transplant 10:1524–1533

Xing Y, Hogquist KA (2012) T-cell tolerance: central and peripheral. Cold Spring Harb Perspect Biol 4:a006957

Yamada K, Scalea J (2012) Thymic transplantation in pig-to-nonhuman primates for the induction of tolerance across xenogeneic barriers. Methods Mol Biol 885:191–212

Yamada K, Yazawa K, Shimizu A et al (2005) Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. Nat Med 11:32–34

Yamada K, Sykes M, Sachs DH (2017) Tolerance in xenotransplantation. Curr Opin Organ Transplant 22:522–528

Yang YG, Sergio JJ, Swenson K et al (1996) Donor-specific growth factors promote swine hematopoiesis in severe combined immune deficient mice. Xenotransplantation 3:92–101

Yi S, Ji M, Wu J et al (2012) Adoptive transfer with in vitro expanded human regulatory T cells protects against porcine islet xenograft rejection via interleukin-10 in humanized mice. Diabetes 61:1180–1191

Zhai Y, Meng L, Gao F et al (2002) Allograft rejection by primed/memory $CD8^+$ T cells is CD154 blockade resistant: therapeutic implications for sensitized transplant recipients. J Immunol 169:4667–4673

Zhao Y, Swenson K, Sergio JJ et al (1996) Skin graft tolerance across a discordant xenogeneic barrier. Nat Med 2:1211–1216

Zhao Y, Fishman JA, Sergio JJ et al (1997a) Immune restoration by fetal pig thymus grafts in T cell-depleted, thymectomized mice. J Immunology 158:1641–1649

Zhao Y, Sergio JJ, Swenson K et al (1997b) Positive and negative selection of functional mouse CD4 cells by porcine MHC in pig thymus grafts. J Immunol 159:2100–2107

Zhao Y, Rodriguez-Barbosa JI, Shimizu A et al (2003) Despite efficient intrathymic negative selection of host-reactive T cells, autoimmune disease may develop in porcine thymus-grafted athymic mice: evidence for failure of regulatory mechanisms suppressing autoimmunity. Transplantation 75:1832–1840