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Xenotransplantation Tolerance: Applications for Recent Advances in Modified Swine

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Abstract

Purpose of review: To review the recent progress in xenotransplantation achieved through genetic engineering and discuss the potential of tolerance induction to overcome remaining barriers to extended xenograft survival.

Recent findings: The success of life-saving allotransplantation has created a demand for organ transplantation that cannot be met by the supply of human organs. Xenotransplantation is one possible solution that would allow for a nearly unlimited supply of organs. Recent genetic engineering of swine has decreased the reactivity of preformed antibodies to some, but not all, potential human recipients. Experiments using genetically-modified swine organs have now resulted in survival of life-supporting kidneys for over a year. However, the grafts show evidence of antibody-mediated rejection on histology, suggesting additional measures will be required for further extension of graft survival. Tolerance induction through mixed chimerism or thymic transplantation across xenogeneic barriers would be well-suited for patients with a positive crossmatch to genetically-modified swine or relatively negative crossmatches to genetically-modified swine, respectively.

Summary: This review highlights the current understanding of the immunologic processes in xenotransplantation, and describes the development and application of strategies designed to overcome them from the genetic modification of the source animal to the induction of tolerance to xenografts.

Keywords

xenotransplantation; tolerance; mixed chimerism; thymus transplant; genetically modified swine

Introduction

One of the most significant issues preventing the field of transplantation from reaching its full potential is the relative shortage of available donor organs. For xenotransplantation to become a clinical reality the intensity of antibody and cellular responses to xenografts must

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be addressed. Recent advances in genetically engineering the source animal to decrease the significance of preformed anti-swine antibodies has resulted in a negative crossmatch for a percentage of potential donors (1). Nonhuman primate (NHP) recipients of kidneys from genetically-modified (GM) swine have survived for 435 days, but have evidence of antibody-mediated rejection, thought to be induced post-transplant by helper T cell interactions with B cells (2-4). Thus, potential xenograft recipients can be classified into two categories: 1) those that would require modification of B and T cell responses (remaining preformed antibodies against GM swine) and: 2) those that would benefit from controlling T cell responses post-transplant (negative crossmatch against GM swine). We believe that induction of mixed chimerism and thymus transplantation, respectively, would allow for successful xenotransplantation for both patient populations.

Antibody responses:

In 1999 the US Department of Health and Human Services halted clinical NHP to human transplants due to concerns of disease transmission (5). Thereafter, swine became the most utilized source animal for preclinical xenotransplantation studies due to appropriate organ size, rapid breeding and ability to genetically modify the species (6, 7). Use of swine organs introduced the problem of preformed antibodies targeting swine antigens, such as α -galactose-1,3-galactose (Gal), a sugar displayed on swine cells (8). The vigorous natural antibody response led to hyperacute rejection of Gal-positive swine organs in Old World monkeys. The generation of GalT knockout (GalT-KO) animals in 2002 eliminated this particular antigen (9, 10). However, initial results of GalT-KO organ transplants revealed that other targets of natural antibodies remained. While not at levels that would result in hyperacute rejection, they can cause significant graft damage. Additional carbohydrate targets of non-Gal antibodies have been identified, including the antigens produced by β -1,4 N-acetylgalactosaminyl transferase 2 (β 4GALNT2) (11) and cytidine monophosphate-N-acetylneuraminic acid hydrolase (CMAH) (12). The production of swine null for β 4GALNT2 and CMAH significantly decreased the binding of antibodies in human sera to swine cells (1). However, significant numbers of patients possessed anti-swine antibodies, indicating that if xenotransplantation is to be a therapy for all potential transplant candidates, further genetic modifications or immune tolerance induction will be required. Nevertheless, even when the anti-swine antibody levels are low pre-transplant, early graft loss due to pre-formed IgM has occurred in macaques (2). Induced anti-swine antibodies that develop post-transplant also remain a significant obstacle. One of the first reports of GalT-KO alone renal transplantation had graft survival limited to 16 days due to the development of anti-swine IgG in the early post-transplant period (13). Although use of multiple knockout animals has resulted in survival of life-supporting swine kidneys in macaques for 435 days, antibody-mediated rejection (AMR) was seen on final histology (2, 4). The authors concluded that induced antibodies to the graft were elicited in the post-operative period despite the use of induction with anti-CD4 and anti-CD8 monoclonal antibodies (mAb), costimulation blockade with anti-CD154, and administration of mycophenolic acid and steroids. While there is evidence that adequate chemical immunosuppression can impede the development of induced antibodies (14), tolerance induction would be another approach to eliminate their development.

Antibody Depletion:

An adjuvant therapy that could prevent antibody development after transplant would be depletion of antibody-producing cells directly. However, attempts to decrease natural antibody production this way have been only somewhat effective. Bortezomib, a proteasome inhibitor resulting in apoptosis of plasma cells, had no significant effect when serum anti-swine antibody levels (non-Gal and anti-Gal IgM or IgG) were measured in patients undergoing organ transplantation (15, 16). IdeS (imlifidase) is an endopeptidase derived from *Streptococcus pyogenes* that targets and cleaves human IgG (but not IgM). This agent has been administered to highly HLA-sensitized patients before kidney transplantation and found to eliminate or reduce donor-specific antibodies (17, 18). These results make IdeS a potential agent to reduce anti-swine antibodies in xenotransplantation, although the rebound of IgG antibodies in a few weeks and the occurrence of AMR due to IgM antibodies suggests caution in extrapolating the results to xenotransplantation. Nevertheless, it seems unlikely the issue of preformed antibodies will be eliminated for all patients by genetic modification or pharmacotherapy alone and that elimination of antibody responses through B-cell tolerance induction would be the most broadly-applicable strategy to prevent AMR (19).

T-cell responses:

In the early days of xenotransplantation, hopes were high that structural differences between the human T cell receptor (TCR) and swine leukocyte antigen (SLA) would prevent T cell activation via the direct pathway (20). However, SLA of both class I and class II were shown to be capable of activating a direct pathway response comparable to that of a fully MHC mismatched allogeneic transplant (21-23). Porcine endothelial cells have been identified as a key figure in direct pathway T cell activation. Not only do they mediate costimulation via human CD2 and CD28 (24), they have also been shown to constitutively express both class I and II molecules (25, 26). In order to mitigate direct pathway activation, donor swine have been genetically engineered to have low levels of class II antigen (27). Costimulatory molecules can also be modified by genetic alteration of CTLA4-Ig and PD-1 ligands (28-30), both serving as early inhibitors of direct T-cell activation.

While direct T-cell activation involves interaction of recipient T cells with donor MHC/peptide, the indirect pathway requires a recipient antigen-presenting cell to process and present donor-derived peptides to a recipient T cell. The presentation of donor swine peptides through the indirect pathway has been shown to activate recipient T cells and lead to xenograft rejection (21, 23). It appears that the porcine antigen may act as a more potent catalyst in xenotransplantation compared to the allogeneic setting. Xenotransplantation studies of solid organs with only moderate T-cell depletion underline these findings by showing that the primate recipients of porcine kidneys or hearts develop anti-swine IgG antibody (13, 31, 32). The finding of anti-swine IgG in the recipient is explained by indirect pathway activation resulting in the cognate CD4 T cell-B cell interaction, which in turn leads to IgG class switching and ultimately humoral xenograft rejection despite an intensive immunosuppressive regimen. Thus, T-cell and/or B-cell tolerance post-transplant would have a salutary effect on the production of anti-swine antibodies.

Tolerance Induction:

Current understanding of T and B cell activation suggests that systemic immunosuppression alone is insufficient to overcome immunologic barriers in xenotransplantation. The induction of immunological tolerance is a more favorable solution with the potential of long term graft acceptance, all while preventing the development of immunosuppression-related side effects. Xenotransplantation possesses unique advantages for tolerance induction: organ availability allows for advanced planning, scheduled pretransplant conditioning regimens that can be tailored to the patients' needs, as well as modifying and genetically engineering transplanted organs. Two strategies, the induction of mixed chimerism and thymic transplantation, emerged as promising strategies for developing tolerance to xenografts.

Mixed Chimerism – Development in Rodents: Initial attempts to induce mixed chimerism in a xenogeneic model were performed in via rat-to-mouse bone marrow transplants with a nonmyeloablative conditioning regimen (33). Several key principles were revealed in these studies. Chimerism in this model only developed if NK cells and $\gamma\delta$ T cells were depleted (34, 35). The dose of bone marrow was also shown to be a critical variable for chimerism to develop, likely due to depletion of a fraction of the marrow dose by preformed natural anti-donor antibodies (36-38). Critically for the induction of xenogeneic tolerance, B-cell responses, including T cell-independent IgM responses, were tolerated in chimeric mice and secondary skin grafts failed to elicit an antibody response (39, 40). Interestingly, peripheral chimerism was detected for 28 weeks and then gradually decreased to <1% (41), suggesting a key concept that recipient cells have a competitive advantage over donor cells in the recipient marrow microenvironment (42, 43), potentially due to the lack of sufficient stimulation by species-specific host cytokines and growth factors.

The Sykes group performed extensive studies on the mechanism of B-cell tolerance to Gal in xenogeneic mixed chimeras (44). GalT-KO mouse recipients were transplanted with wild-type bone marrow and anti-Gal antibody levels decreased significantly 2 weeks after. Given this result and *in vitro* assays showing undetectable levels of anti-Gal antibody-producing B cells, it was hypothesized that mixed chimerism allowed for anti-Gal producing B cells to be tolerated (45). In addition, these chimeric recipients accepted Gal+ hearts (46). In future studies these chimeras would prove to evade all forms of cardiac xenograft rejection (47).

The Sykes group went on to study chimerism and tolerance in more stringent models of discordant species barriers via porcine BM transplants in immunodeficient SCID mice, porcine hematopoietic cytokine expressing transgenic mice and humanized mice (48, 49). These recipients demonstrated low levels of porcine chimerism (in SCID mice) that was enhanced by the addition of porcine-specific growth factors IL-3 and GM-CSF (in transgenic mice), again indicating a need for species-specific growth factors for long-term chimerism to be achieved (42, 50, 51). In addition to porcine BM engraftment, porcine class II cells were detected in the host thymus and donor skin grafts were accepted in a background of donor unresponsiveness on *in vitro* assays (49, 52). Anti-swine IgG antibodies were absent following placement donor skin grafts (53). These results suggest that the successful induction of mixed chimerism would result in T-cell and B-cell tolerance

and optimal survival of xenogeneic transplants even in recipients with persistent anti-swine antibodies against the current genetically-modified swine.

Thymus Transplant – Development in Rodents: The idea behind a thymic transplant for the induction of tolerance is the aim of reprogramming donor T cells to accept porcine antigens as “self”. First studies in the 1990s demonstrated successful induction of tolerance after host thymectomy and T-cell depletion followed by donor thymic transplantation. The peripheral CD4⁺ T cells were able to develop in the transplanted donor thymic graft and go on to repopulate the recipient (54, 55). Positive selection of CD4⁺ T cells were shown to be dependent on donor swine MHC only (56), while negative selection of CD4⁺ T cells relies on both donor swine and host murine MHC, leading to a simultaneously donor and host tolerant T cell repertoire (57). Classic immunologic responses were preserved after tolerance induction, proven by adequate responsiveness to 3rd-party stimulators in MLR assays (58) as well as by *in vivo* skin graft studies (55, 58).

In order to prove swine thymus would support human thymopoiesis, fetal porcine thymus and human fetal liver were implanted in SCID mice (59). Maturing T cells were able to repopulate the periphery in these animals and were shown to have a human T-cell repertoire (60). *In vitro* studies showed that these thymocytes remained tolerant towards both donor and host, but reacted against 3rd-party and allogeneic stimulators. These principles of xenogeneic thymic transplantation, engraftment and subsequent thymopoiesis could later be reproduced in more advanced study models in humanized mice (61). However, in GalT-KO mice that produce anti-swine antibodies, the engraftment of swine thymus was reduced (62). When thymus engraftment was achieved, T cell-dependent production of antibodies was not detected. However, the production of antibodies by T cell-independent mechanisms was not eliminated (62). Together, these results indicate that porcine thymus transplants can support human thymopoiesis resulting in a normal T-cell repertoire and specific T-cell tolerance. Since B cells producing xenoantibodies in a T cell-independent manner are not tolerized, the application of this strategy may be more appropriate for recipients with a negative pretransplant crossmatch to genetically modified swine as it would help prevent the development of induced antibodies.

Mixed Chimerism — Nonhuman Primate Studies: The first studies transplanting Gal + swine bone marrow to baboons reaffirmed the barrier presented by high-levels of natural antibodies, as high-doses of swine stem cells were required to achieve detectable peripheral chimerism (63-65). If co-stimulatory blockade was included in the induction regimen, recipients of swine stem cells did not have an induced antibody response, but they also did not show decreases in anti-Gal antibody levels (66, 67).

Using bone marrow from GalT-KO swine, swine cells were detected by flow cytometry in the peripheral blood for up to 5 days (64). Peripheral microchimerism was detected two weeks after the transplant, suggesting low-level engraftment of swine progenitors or stem cells. Alterations in the induction protocol led to a lack of detectable peripheral chimerism, but swine progenitor cells were isolated from the recipient marrow at day 28, again indicating low-level engraftment (68). The failure to detect swine cells in the periphery may be due to the lack of CD47-SIRP α interactions between the swine cells and recipient

macrophages. CD47 acts as a marker-of-self to macrophages expressing SIRP α that engulf cells lacking the appropriate CD47 (69). Using stem cells from swine expressing human CD47 (hCD47), peripheral chimerism was enhanced over recipients of hCD47^{lo} stem cells. Donor skin grafts were placed 14-weeks after stem cell transplant without additional immunosuppression and recipients of hCD47^{hi} stem cells had skin graft survival prolonged up to 70 days (70). In an attempt to evade depletion by antibodies and macrophages that can occur in the blood after intravenous injection, intra-bone injection of GalT-KO swine stem cells has been reported. This resulted in prolonged detection of swine cells in the periphery for several weeks. No increase in anti-swine antibodies were observed, but neither were decreases in the levels of preformed anti-swine antibodies (71). Thus, while no increase in anti-swine responses is observed after induction of transient xenogeneic chimerism, elimination of the antibody response has not been observed. It is possible that for the antibody response to be eliminated, chimerism has to be prolonged over what has been achieved to date. For this to occur, further modifications of the source animal or induction protocol may be required. As was demonstrated in the rodent models, swine stem cells may require long-term administration porcine-specific growth factors, or need to be modified to respond appropriately to human growth factors.

Thymic Transplant — Nonhuman Primate Studies: Thymus transplants from swine to baboons have been performed either as a vascularized thymus lobe or a composite “thymokidney” graft where autologous thymus tissue is autotransplanted under the renal capsule and allowed to engraft prior to transplantation of the composite graft (31, 72-75). When Gal-positive swine thymi and organs were transplanted, there was evidence for T-cell tolerance *in vitro*, but grafts were lost due to anti-Gal antibodies (72, 73). Thymus transplants from GalT-KO swine, which encounter lower levels of pre-formed anti-swine antibody, led to significantly prolonged survival of co-transplanted kidneys compared to recipients of swine kidneys alone (31). Recipients of thymus grafts had decreased levels of anti-swine antibodies, although anti-swine antibodies were not eliminated (31, 74). Importantly, the swine thymus supported baboon thymopoiesis with evidence for T-cell tolerance *in vitro* (74, 75). As in the rodent model, these data suggest that xenogeneic thymus transplantation can result in T-cell but not B-cell tolerance. However, T-cell tolerance may be sufficient to prevent the production of induced anti-swine antibodies and be useful if low-to-negative pre-transplant crossmatches are achieved with a given source animal.

Role of Tregs in Tolerance:

Tregs have been extensively studied in allotransplantation, and it is not surprising there is considerable interest in their use for xenotransplantation (25, 76). Some studies have suggested that Tregs play a role in mediating xenograft survival. In an NHP model of heart xenotransplantation, there was an increased percentage of Tregs in peripheral blood of long-term survivors and this percentage of Tregs decreased prior to rejection (77). In rodent models, the infusion of *ex vivo* expanded Tregs has prolonged survival of porcine islets (78, 79). Several groups have reported expansion of primate Tregs *in vitro* that suppress primate anti-swine T cell responses (80-82). Our group has investigated the ability of Tregs to enhance the level and duration of chimerism in allogeneic and xenogeneic models (83). We

have recently presented that *ex vivo* expanded polyclonal Tregs prolong the duration of swine peripheral chimerism in baboon recipients of GalT-KO/hCD46/hCD47 stem cells. Additionally, Treg recipients showed prolonged skin graft survivals compared to controls that received identical treatment without Treg administration (Am J Transplant. 2017;17(Suppl 3); and IXA2017 <https://doi.org/10.1111/xen.12328>). We are now investigating the potential for donor-specific Tregs to further prolong chimerism and swine skin graft survival.

Conclusion:

Significant progress has been made in extending xenograft survival through recent genetic modifications of the source animal. However, immunologic hurdles remain even for patients that can achieve a negative crossmatch to these GM organs. The induction of xenogeneic mixed chimerism would allow for xenotransplantation of swine organs for any recipient, while T-cell tolerance via thymic transplantation may be sufficient for patients with a negative crossmatch. Additional modifications of the donor stem cells to respond to host growth factors and the addition of Tregs to induction protocols are promising approaches to enhance donor chimerism and achieve tolerance to xenografts.

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Abbreviations:

Gal	α -galactose-1,3-galactose
GalT	α -1,3-galactosyltransferase
AMR	antibody-mediated rejection
CMAH	cytidine monophosphate-N-acetylneuraminic acid hydrolase
GalT-KO	GalT-knockout
GM	genetically modified
HLA	human leukocyte antigen
hCD47	human CD47
SLA	swine leukocyte antigen
MHC	major histocompatibility complex
SIRPα	signal-regulatory protein alpha

NeuGc	N-glycolylneuraminic acid
NHP	nonhuman primate
TCR	T cell receptor
β4GALNT2	β-1,4 N-acetylgalactosaminyl transferase 2

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* of specific interest

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Key points:

- Genetic modifications of swine have decreased the level of preformed antibodies to porcine organs.
- Strong immune responses to xenografts due to B cell and T cell responses remain significant barriers to prolonged xenograft survivals.
- Mixed xenogeneic chimerism can induce B cell and T cell tolerance in rodent models, but improved duration of chimerism in translational primate models is likely needed for tolerance to be induced.
- Thymus transplant can induce T cell tolerance in rodent models and has shown promise in translational models, but B cell tolerance is not induced by this strategy.
- Control of xenogeneic responses by Treg cellular therapy is a promising strategy to help facilitate tolerance to xenotransplants.