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Progress towards Xenogenic Tolerance

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Abstract

Purpose of review: To describe the most recent progress towards tolerance in xenotransplantation.

Recent findings: Mixed chimerism and thymic transplantation have been used to promote tolerance in xenotransplantation models. Intra-bone bone marrow transplantation is a recent advance for mixed chimerism, that promotes longer lasting chimerism and early graft function of subsequent organ transplantation. The hybrid thymus, an advancement to the vascularized thymokidney and vascularized thymic lobe, is being developed to allow for both donor and recipient T-cell selection in the chimeric thymus, encouraging tolerance to self and donor while maintaining appropriate immune function. Regulatory T cells show promise to promote tolerance by suppressing effector T cells and by supporting mixed chimerism. Monoclonal antibodies such as anti-CD2 may promote tolerance through suppression of CD2+ effector and memory T cells while Tregs, which express lower numbers of CD2, are relatively spared and might be used to promote tolerance.

Summary: These findings contribute major advances to tolerance in xenotransplantation. A combination of many of these mechanisms will likely be needed to have long term tolerance maintained without the use of immunosuppression.

Keywords

xenotransplantation; tolerance; chimerism; regulatory T cells; anti-CD2

Introduction

Xenotransplantation has been considered one of the most promising paths towards the amelioration of the chronic organ shortage. High doses of chronic immunosuppression, with its many toxicities, has been required to maintain xenografts long term in animal models. Tolerance induction, through mechanisms such as mixed chimerism and thymic transplantation, has been explored as a strategy to overcome xenogeneic immune barriers.

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The desirable mechanism of tolerance should promote non-destruction of the donor tissue by the immune system, while maintaining normal host immune function, in the absence of immunosuppression.¹ Here we review recent reports for promoting tolerance in xenotransplantation and new strategies, such as regulatory T cells and anti-CD2 antibodies, that are being investigated to promote tolerance across xenogeneic barriers.

Advances in Transgenic Donor Pigs

The genetically modified GalT-knockout (GalT-KO) donor pig is the current preferred species for xenotransplantation to nonhuman primates (NHP) and ultimately humans. Many xenoantigens have been identified, such as CMAH, β 4GALNT2, A3GAL2, that may also need to be eliminated to decrease organ damage from natural antibodies (nAb).^{2,3,4,5} With the advent of CRISPR/Cas9 genome editing, there is potential for more rapid development of knock-out and knock-in transgenic animals for preclinical testing.⁶ The Tector group developed GGTA1/CMAH double-knockout swine and GGTA1/CMAH/b4GalNT2 triple-knockout swine and demonstrated that the resultant triple-knockout pig cells had decreased binding of nAb from humans, baboons and macaques, although baboons and macaques displayed increased binding of nAb to GGTA1/CMAH double-knockout cells relative to GGTA1 knockout alone.⁴ Adams et al.⁷ published the first study describing the decreased binding of nAb in Rhesus macaques to cells from CRISPR-Cas9-modified GGTA1/B4GALNT2 KO pigs, although there was detectible antibody binding by complement-dependent cytotoxicity (CDC) and flow (FCM) crossmatch. Kidneys from these pigs were transplanted to 6 Rhesus with the least reactive cross matches. Of these grafts, 3 experienced early rejection of 6 days or less, which was IgM mediated. The final 3 had survival of 35, 100 and 435 days, respectively. The longest surviving graft had late IgG mediated rejection, demonstrating that antibody mediated rejection (AMR) was still the mechanism of failure. A triple knockout GGTA1/CMAH/B4GALNT2 KO pig (TKO) was studied in parallel. TKO peripheral blood mononuclear cells (PBMCs) and serum from over 800 patients on the kidney transplant waitlist underwent CDC and FCM crossmatches.⁵ Fifty-nine percent of unsensitized patients were found to have a negative crossmatch (positive was considered a mean fluorescence intensity of over 2000 for IgG only) and 39% were negative for both IgM and IgG.⁵ It was also found that some antibodies to human leukocyte antigen (HLA) in sensitized patients cross react with swine leukocyte antigen (SLA), which is currently being considered as a target for genetic modification, deletion or replacement.^{5,8}

Another method to decrease the immune response to xenogeneic organs is to add protective human proteins to the cell surface. Recently, transgenic expression of hPD-L1 (human programmed death-1 ligand/ CD274) was induced by Buermann et al.⁹ for its role in self-tolerance. Binding of PD-1 on T cells by its ligand PD-L1 suppresses autoreactive T cells through inhibitory signaling, as seen by the reduction in the ability of peripheral blood mononuclear cells (PBMCs) from hPD-L1 transgenic type pigs, compared to wild type, to stimulate the proliferation of hCD4+ T cells.⁹ Fibroblasts expressing hPD-L1 also had significantly lower cytotoxicity compared to wild type. It was also observed that hPD-L1 levels were upregulated by human cytokines, which may provide a window for regulation.⁹

Transgenic expression of hCD47 has been recently shown, in multiple studies, to mitigate macrophage-mediated stem cell destruction and support mixed chimerism in an attempt to promote tolerance.^{10,11,12} Macrophages, expressing SIRP- α , phagocytose cells found to be lacking a species-appropriate CD47, a marker of self. Human SIRP- α does not bind to porcine CD47, leading to the need for transgenic expression of CD47 on porcine cells to mitigate xenogeneic macrophage activation.^{13,14} Expression of hCD47 may be crucial for cellular transplants as demonstrated by Tena et al.¹⁰, where transplantation of porcine stem cells expressing hCD47 led to prolonged peripheral chimerism. Chimerism duration was additionally enhanced by intrabone injection of GalTKO-hCD47Tg bone marrow. Long-term chimeras had a decrease in, but not elimination of, the level anti-pig antibodies in the serum.¹²

Mixed Chimerism with Combined Organ/Bone Marrow Transplant (BMT)

Mixed chimerism through BMT has supported tolerance in allogenic, preclinical, and clinical models in the setting of even just transient mixed chimerism. In mice, persistent mixed chimerism has been achieved with the tolerance of MHC mismatched skin grafts, but this sustained chimerism has not been demonstrated in a NHP model.¹⁵ This is felt to be due to differences in ability to deplete T cells during preconditioning and an increased number of memory T cells seen in NHPs compared to lab bred mice.¹⁵ Over the past 10 years there have been successes in achieving tolerance to kidney grafts without chronic immunosuppression in NHP models and in humans using combined kidney/bone marrow transplant (CK/BMT), despite only inducing transient mixed chimerism of up to 2 months.^{16,17,18,19}

Induction of kidney tolerance with transient donor chimerism may be a phenomenon limited to the kidney in the allogeneic model. Tolerance to lungs, hearts, islets and livers is not induced by transient chimerism at the time of solid organ transplant.^{20,21} Thus, it is probable that durable xenogeneic mixed chimerism must be induced for tolerance to develop. In an attempt to prolong xenogenic chimerism, the Yamada group has studied the effect of injecting porcine bone marrow into the medullary bone (IBBMT) of baboon recipients to mitigate the loss of cells in the periphery by macrophages and natural non-Gal antibodies (non-Gal nAb) after injection and to permit better engraftment.^{12,22,23} The initial study in 2015 showed detectable, continuous macro-chimerism in the periphery, up to 13 days post IBBMT of GalT-KO bone marrow, with evidence of engraftment. Donor-specific hyporesponsiveness was seen beyond the time of loss of chimerism (to 64 days), without induction of elicited anti-donor antibodies. Pig kidneys transplanted after the induction of chimerism survived up to 60 days, although immunosuppression was continued so the effect on tolerance could not be determined.²² Most recently, Watanabe et al.¹² published the longest reported duration of chimerism in large animal xenotransplantation, lasting over 8 weeks. Bone marrow from five GalTKO/hCD47/hCD55Tg pigs, and from 2 transgenic pigs not expressing CD47, was grafted. Chimerism was detected by FCM in all recipients of CD47-expressing cells at 4 weeks, with four of the five baboons maintaining detectable (<1%) chimerism at 60 days.¹² Recipients of non-CD47 expressing marrow saw similar findings to initial studies, where chimerism was undetectable by around 3-4 weeks. Encouragingly, there was also a reduction in anti-pig non-Gal nAb levels in baboons

receiving hCD47+Tg IBBMT, but nAb were not eliminated. All 7 baboons received delayed porcine lung transplant and 2 of the 7, among those that had received hCD47/hCD55+/GalTKO IBBMT and synergistic lung grafts, maintained their lungs for 13-14 days post-transplant. Pathology revealed minimal antibody deposits, attributed to persistent preformed nAb.¹² The other recipients of hCD47Tg donors experienced early rejection and were found to only express hCD47 on alveolar epithelial cells but not on lung endothelium.¹²

Thymic Transplantation

Thymic transplant ideally supports thymopoiesis of both donor and recipient, resulting in T-cell tolerance of xenograft and self. Vascularized thymic grafts, such as the vascularized thymus lobe (VTL) or thymokidney (TK), have been used in studies of xenogeneic tolerance.^{25,26} Thymectomized NHP recipients in pig-to-baboon models of thymokidneys have survived over 6 months and demonstrated new populations of thymic emigrants, suggesting thymopoiesis, with donor-specific T-cell unresponsiveness.^{25,26,27,28} Despite this, there has not yet been success weaning immunosuppression completely without graft loss.

One of the potential deficiencies leading to suboptimal tolerance and immune function of the host may be the limited ability of swine thymic cells to select for human T-cell receptor (TCR). In the study by Nauman et al.²⁹ it was seen that in a transgenic humanized mouse model, there was inefficient positive selection of cells expressing human restricted TCR (MART1 TCR, a well characterized human HLA-A2 restricted TCR) in a grafted pig thymus. Such a deficiency for positive selection for human antigens in a transplanted porcine thymus would lead to a deficient production of self-reactive Tregs in the xenogeneic thymus. Thymic epithelial cells (TECs) are the mediators of positive and negative selection in the thymus and are thought to be critical for development of thymic-derived Tregs³⁰. Therefore, the Sykes lab has studied the potential to create a recipient/swine hybrid thymus to provide self/donor-specific T-cell deletion and production of natural self/donor-reactive Tregs.^{31,32,33} By injecting recipient TECs into porcine thymic grafts in a fetal pig-nude mice model, Fudaba³⁴ found that autoimmunity was suppressed, and regulatory cell function was enhanced. These studies were then extended into humanized and primate models. The hybrid human/pig thymus contains both human and swine TEC for selection, thereby promoting tolerance to donor antigen while maintaining appropriate response to third party and minimizing autoimmunity.³¹ This was first described in a humanized model by Maharlooe³¹ using human thymic stromal cells injected into swine thymus, which was found to support human thymopoiesis in humanized mice. In the NHP model, Sekijima et al.³² introduced NHP TECs into hybrid thymic grafts from donor pigs and reported persistence of NHP TECs in miniature swine thymic lobes after 3 weeks.³²

Due to the lack of TECs in an adult thymus due to atrophy, induced pluripotent stem cells (PSC) for the generation of human TECs are being investigated to populate a hybrid thymus.³³ In vitro, it was possible to differentiate human PSC-derived thymic epithelial progenitors, which persisted in swine thymic grafts, and tolerance is currently being assessed for PSC specific antigens. The use of a hybrid thymus containing donor TECs will ideally provide both positive and negative selection of tissue specific antigens, development of pig and self-reactive Tregs and enable both self- and xenotolerance.

Regulatory T cells

Regulatory T cells are thought to have significant potential for inducing tolerance in a xenotransplantation model due to their ability to be expanded *ex-vivo* for therapeutic infusion and the ability to generate donor specific Tregs.³⁵ Our group has recently published the expansion of Tregs in the Mauritian Cynomolgus macaque to augment regulation of anti-donor immune responses.³⁶ Four protocols were examined in which Tregs expanded up to 3000-fold and demonstrated suppression of anti-CD2CD3CD8 bead-stimulated autologous PBMCs. Tregs maintained FoxP3 expression and successful cell growth up to 2 months in culture. These cells were also able to be cryopreserved, then thawed, with successful re-expansion and continued suppressive activity. This has implications for use in expanding and banking large numbers of Tregs that can be used for xenotransplantation and ultimately in deceased donor induction protocols.³⁶

Tregs also have been shown to play a role in promoting prolonged chimerism in allogenic BMT recipients in a Cynomolgus macaque model.³⁷ Chimerism was demonstrated up to 335 days and over 90% of donor T cells were found to be new thymic emigrants in 2 of the 5 animals receiving Treg + BMT. A delayed allogenic kidney transplant was tolerated for over 290 days without immunosuppression in one of these animals, compared to a maximum of 4 weeks in those receiving BMT alone, suggesting that Tregs play a role in promoting chimerism and immunomodulation of allogeneic responses.³⁷

Recently, Jin et al.³⁸ studied ex-vivo human Tregs expanded with xenoantigen stimulation (Xeno-Treg) and their effect on porcine neonatal islet cell clusters (NICC) in NOD-SCID IL-2 γ -/- mice. After adoptive transfer of human PBMC and infusion of ex-vivo expanded Xeno-Tregs or polyclonal Tregs (Poly-Tregs), NICC engraftment and survival was determined. In mice reconstituted with Xeno- or Poly-Treg and PBMC at a ratio of 1:10, it was found that over 75% of NICC xenografts survived over 84 days in the Xeno-Treg group vs 48 days in the Poly-Treg group, suggesting Xeno-Tregs may have greater ability to suppress xenogeneic cellular rejection.³⁸ In NHP, our group has reported that polyclonal Tregs administered at the time of GalTKO/hCD47Tg stem cells prolong the duration of donor chimerism. In these animals, we tested the potential for tolerance development by transplanting donor or donor-matched skin grafts without any maintained immunosuppression. Recipients of Tregs demonstrated prolonged skin graft survival over those that received stem cell transplants alone.³⁹ Studies to determine if xeno-specific Tregs are more potent than polyclonal Tregs in the primate model are ongoing.

Selective T-cell Depletion and Co-stimulation Blockade

Monoclonal antibody therapy has proven to be promising in modulating the immune system. An anti-CD40 based immunosuppression regimen is used in many protocols, and the use of anti-CD154 (CD40's ligand, a costimulatory molecule on activated T cells) is being investigated. Using anti-CD154 along with selective T-cell depletion, Kim et al.⁴⁰ demonstrated significant progress in survival of pig-to-rhesus macaque life-sustaining kidney transplant to over 1 year. Using pan-T-cell depletion along with peri-transplant anti-CD154, selective mAb depletion of CD4+ and CD8+ T cells were trialed. There was

significantly improved survival (over 400 days vs 6 days) with selective depletion of CD4⁺ T cells compared to CD8⁺, and biopsy results were normal until late, when chronic antibody rejection prevailed. This led the authors to hypothesize that CD4⁺ T cells, over CD8⁺, may play a bigger role in rejection and that sustained xenograft survival is dependent on selective CD4⁺ depletion.⁴⁰

The mechanism of anti-CD154 monoclonal antibody blockade alone was recently investigated in an intrahepatic murine allogenic islet transplantation model, where it was found that short term anti-CD154 mAb treatment may induce Treg-mediated tolerance.⁴¹ Diabetic mice that underwent intrahepatic-islet-transplant with short term CD154 blockade were found to have normoglycemia for over 100 days and islets were intact and positive for insulin over 250 days, compared to controls. As a secondary challenge to see if anti-CD154 induced immune tolerance, allo-islets and third-party islet grafts, respectively, were transplanted under the kidney capsules of two groups. Nephrectomy at 14 and 34 days showed survival of second-party islets at all time points, but third-party was uniformly rejected at 14 days. Tregs were consistently found in the peri-graft sites in the surviving second-party islets, which likely contributed to protection of the xenograft from rejection. The authors surmised that anti-CD154 mAb may induce Treg-mediated tolerance in this model.⁴¹ A second study by this group found similar results with survival of porcine islets over 200 days in diabetic mice who were treated with short term anti-CD154. A high ratio of Tregs were again found in the perigraft site, but not in secondary lymphoid organs, suggesting Treg migration to the graft.⁴²

Expression of CD2 is upregulated on activated and memory T cells compared to naive and resting T cells.⁴³ CD2 interacts with LFA-3 on antigen-presenting cells (APCs) and promotes T-cell activation.⁴⁴ Siplizumab, a species specific, humanized mAb to CD2 and its rat parent BTI-322 have immunomodulatory properties such as T-cell depletion, costimulatory blockade (CoB) via anti-CD2-LFA-3, and may potentially spare, or even enhance, the properties of donor-specific regulatory T cells.^{43,45,46} In an investigational chimpanzee study, these antibodies were found to result in rapid depletion of CD2, CD3, CD4, CD8 lymphocytes and NK cells.⁴⁷ This depletion was transient, with reconstitution occurring more rapidly in the group receiving BTI-322 (7-21 days vs 21-35 days). Compared to controls, animals treated with low dose siplizumab showed hyporesponsiveness in mixed lymphocyte reaction (MLR) and significant decrease in NK cell lysis response. This was thought to be due to CD2-LFA3 CoB inhibiting T-cell-APC interaction, prohibiting T-cell proliferation and inducing hyporesponsiveness to alloantigens. The authors propose that anti-CD2-LFA3 CoB along with selective depletion of CD2 expressing T cells may present a mechanism for decreased activation for memory T cells, which are typically difficult to control with immunosuppression alone.⁴⁷ While Tregs and naive T cells were shown to express lower levels of CD2 than effector T cells, the resting Treg subset consistently had the lowest expression of CD2 and T memory effector cells had the highest level.^{43,48} Siplizumab in MLR resulted in a significant reduction in proliferating and memory CD4⁺ and CD8⁺ T-cell numbers, overall increase in CD4⁺ and CD8⁺ naive T cells, and an increased percentage of Tregs, supporting the hypothesis that siplizumab's effect is targeted to activated T cells. Tregs also demonstrated relative expansion during reconstitution after CD2 deletion.^{43,48} This is promising data suggesting that siplizumab

may have success in immunomodulatory regimens, promoting tolerance in xenotransplantation, as it facilitates Treg expansion and selectively depletes effector T cells. As an alternative strategy to systemic CD2 depletion, genetic engineering to deliver anti-CD2 antibodies in the local xenograft environment is also being investigated. Pig islets that have been engineered to secrete anti-CD2 antibodies decrease the local human T-cell infiltration without altering the systemic T-cell number.⁴⁹ Recently, pigs whose cells have been engineered to secrete an anti-human CD2 antibody have been produced.⁵⁰ It is possible that these animals may control the local immune response with minimal effect on the recipient's overall immune system.

Conclusion

Tolerance is likely the key to successful long-term survival of xenografts. It is unlikely that a single mechanism will induce tolerance across xenogenic barriers. With new technologies, such as CRISPR/Cas9, we have new abilities to genetically engineer animals whose organs that are inherently more tolerated in their recipient model. Promoting tolerance through Treg infusion or monoclonal antibodies, such as anti-CD2, may provide regulatable mechanisms that could be pulled “off the shelf” and may require less exhaustive conditioning than thymic transplantation or BMT for chimerism, though employing multiple strategies may ultimately bring the most success.

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Abbreviations:

Gal	α -galactose-1,3-galactose
GalT, GGTA1	α -1,3-galactosyltransferase
AMR	antibody-mediated rejection
APC	antigen-presenting cells
β4GALNT2	β -1,4 N-acetylgalactosaminyl transferase 2
BMT	bone marrow transplant
CDC	complement dependent cytotoxicity
CK/BMT	combined kidney/bone marrow transplant
CMAH	cytidine monophosphate-N-acetylneuraminic acid hydrolase
CoB	costimulatory blockade

FCM	flow cytometry
GalT-KO	GalT-knockout
GM	genetically modified
HLA	human leukocyte antigen
hCD47	human CD47
hPD-L1	human programmed death ligand
IBBMT	intrabone bone marrow transplant
MHC	major histocompatibility complex
nAb	natural antibodies
NeuGc	N-glycolylneuraminic acid
NHP	nonhuman primate
NICC	neonatal islet cell clusters
NK	natural killer
PBMCs	peripheral blood mononuclear cells
PSC	pluripotent stem cells
SIRP-α	signal-regulatory protein alpha
SLA	swine leukocyte antigen
TCR	T-cell receptor
TEC	thymic epithelial cell
TK	thymokidney
Tg	transgenic
VTL	vascularized thymic lobe

References:

1. Sachs DH. Transplant tolerance: bench to bedside--26th annual Samuel Jason Mixter Lecture. Arch Surg. 2011;146(5):501–505. doi:10.1001/archsurg.2011.101 [PubMed: 21576601]
2. Byrne GW, Stalboerger PG, Du Z, et al. Identification of new carbohydrate and membrane protein antigens in cardiac xenotransplantation. Transplantation. 2011;91(3):287–92. [PubMed: 21119562]
3. Burlak C, Bern M, Brito A, et al. N-linked glycan profiling of GGTA1/CMAH knockout pigs identifies new potential carbohydrate xenoantigens. Xenotransplantation. 2013;20(5):347.
4. Estrada JL, Martens G, Li P, et al. Evaluation of human and non-human primate antibody binding to pig cells lacking GGTA1/CMAH/ β 4GalNT2 genes. Xenotransplantation. 2015;22(3):194–202. [PubMed: 25728481]

5. Martens GR, Reyes LM, Butler JR, Ladowski JM, Estrada JL, Sidner RA, et al. Humoral Reactivity of Renal Transplant-Waitlisted Patients to Cells From GGTA1/CMAH/B4GalNT2, and SLA Class I Knockout Pigs. *Transplantation*. 2017;101(4):e86–e92. [PubMed: 28114170]
6. Cowan PJ, Hawthorne WJ, Nottle MB. Xenogeneic transplantation and tolerance in the era of CRISPR-Cas9. *Curr Opin Organ Transplant*. 2019;24(1):5–11. [PubMed: 30480643] *Review paper discussing the advances in transgenic knock-outs and knock-ins being studied to promote tolerance, facilitated by the advent of CRISPR-Cas9 genome editing.
7. Adams AB, Kim SC, Martens GR, et al. Xenoantigen Deletion and Chemical Immunosuppression Can Prolong Renal Xenograft Survival. *Ann Surg*. 2018;268(4):564–573. [PubMed: 30048323] **First preclinical study done using xenoantigen deletion in donor pigs in a NHP model to promote survival over one year. Kidneys from GGTA1/b4GALNT2 double KO pigs functioned for up to 435 days in Rhesus monkeys.
8. Ladowski JM, Reyes LM, Martens GR, et al. Swine Leukocyte Antigen Class II Is a Xenoantigen. *Transplantation*. 2018;102(2):249–254. [PubMed: 28846555] *Used sera from patients on transplant wait list to test for IgG to class II SLA proteins expressed on mammalian cells. Found that patients with antibodies to class II HLA had higher binding to cells expressing class II SLA than SLA negative cells.
9. Buermann A, Petkov S, Petersen B, et al. Pigs expressing the human inhibitory ligand PD-L1 (CD 274) provide a new source of xenogeneic cells and tissues with low immunogenic properties. *Xenotransplantation*. 2018;25(5):e12387. [PubMed: 29446180] *Demonstrated that PBMC from hPD-L1+ transgenic pigs had reduced ability than PBMC from wild type pigs to stimulate proliferation of CD4+ T cells and that the expression of PD-L1 may be regulatable by treatment with human cytokines.
10. Tena AA, Sachs DH, Mallard C, et al. Prolonged Survival of Pig Skin on Baboons After Administration of Pig Cells Expressing Human CD47. *Transplantation*. 2017;101(2):316–321. [PubMed: 27232934]
11. Boettcher AN, Cunnick JE, Powell EJ, et al. Porcine signal regulatory protein alpha binds to human CD47 to inhibit phagocytosis: Implications for human hematopoietic stem cell transplantation into severe combined immunodeficient pigs. *Xenotransplantation*. 2019;26(2):e12466. [PubMed: 30311702]
12. Watanabe H, Ariyoshi Y, Pomposelli T, et al. Intra-bone bone marrow transplantation from hCD47 transgenic pigs to baboons prolongs chimerism to >60 days and promotes increased porcine lung transplant survival. *Xenotransplantation*. 2020;27(1):e12552. [PubMed: 31544995] **Using IBBMT, first study to demonstrate chimerism over 8 weeks in baboons receiving IBBMT and up to two week survival of subsequently transplanted lung grafts from hCD47Tg pigs.
13. Ide K, Wang H, Tahara H, et al. Role for CD47-SIRPalpha signaling in xenograft rejection by macrophages. *Proc Natl Acad Sci U S A*. 2007;104(12):5062–5066. [PubMed: 17360380]
14. Llore NP, Bruestle KA, Griesemer A. Xenotransplantation tolerance: applications for recent advances in modified swine. *Curr Opin Organ Transplant*. 2018;23(6):642–648. [PubMed: 30379724]
15. Sasaki H, Oura T, Spitzer TR, et al. Preclinical and clinical studies for transplant tolerance via the mixed chimerism approach. *Hum Immunol*. 2018;79(5):258–265. [PubMed: 29175110] *Review of the recent advances in pre-clinical and clinical of allograft tolerance induction by CKBMT.
16. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008;358(4):353–361. [PubMed: 18216355]
17. Andreola G, Chittenden M, Shaffer J, et al. Mechanisms of donor-specific tolerance in recipients of haploidentical combined bone marrow/kidney transplantation. *Am J Transplant*. 2011;11(6):1236–1247. [PubMed: 21645255]
18. Kawai T, Cosimi AB, Sachs DH. Preclinical and clinical studies on the induction of renal allograft tolerance through transient mixed chimerism. *Curr Opin Organ Transplant*. 2011;16(4):366–371. [PubMed: 21666482]
19. Leventhal J, Abecassis M, Miller J, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med*. 2012;4(124):124ra128.

20. Hull TD, Benichou G, Madsen JC. Why some organ allografts are tolerated better than others: new insights for an old question. *Curr Opin Organ Transplant*. 2019;24(1):49–57. [PubMed: 30516578] *Review of organ-specific differences in tolerance induction with emphasis on the tolerogenicity of organs with transient donor mixed chimerism.
21. Chaudhry S, Kato Y, Weiner J, et al. Transient Mixed Chimerism With Nonmyeloablative Conditioning Does Not Induce Liver Allograft Tolerance in Nonhuman Primates. Published online ahead of print, 4 6, 2020 Transplantation.*Study of tolerance induction using mixed chimerism with combined BMT. They did not find transient mixed chimerism to promote tolerance in NHP allogenic liver model, unlike in the CKBMT models. Rejection after withdrawal of immunosuppression was associated with an increase in CD8+ effector cells.
22. Tasaki M, Wamala I, Tena A, et al. High incidence of xenogenic bone marrow engraftment in pig-to-baboon intra-bone bone marrow transplantation. *Am J Transplant*. 2015;15(4):974–983. [PubMed: 25676635]
23. Yamada K, Ariyoshi Y, Pomposelli T, Takeuchi K. Intra-bone Bone Marrow Transplantation in Pig-to-Nonhuman Primates for the Induction of Tolerance Across Xenogeneic Barriers. *Methods Mol Biol*. 2020;2110:213–225. [PubMed: 32002911] *Publication of methodology to perform IBBMT in a pig to baboon model for use in clinical studies.
24. Watanabe H, Sahara H, Nomura S, et al. GalT-KO pig lungs are highly susceptible to acute vascular rejection in baboons, which may be mitigated by transgenic expression of hCD47 on porcine blood vessels. *Xenotransplantation*. 2018;25(5):e12391. [PubMed: 29527745] *Study demonstrating the first viable porcine lung grafts into baboons surviving over 7 days in patients with transgenic expression of hCD47 on graft endothelial cells.
25. Griesemer AD, Hirakata A, Shimizu A, et al. Results of gal knockout porcine thymokidney xenografts. *Am J Transplant*. 2009;9(12):2669–78. [PubMed: 19845583]
26. Yamada K, Ariyoshi Y, Pomposelli T, et al. Co-transplantation of Vascularized Thymic Graft with Kidney in Pig-to-Nonhuman Primates for the Induction of Tolerance Across Xenogeneic Barriers. *Methods Mol Biol*. 2020;2110:151–171. [PubMed: 32002908] **Important study demonstrating vascularized thymic grafts plus kidneys from GalT-KO pigs to baboons achieving over 6-month survival of life supporting kidneys. New populations of thymic emigrants were found and donor specific T-cell unresponsiveness.
27. Yamada K, Yazawa K, Shimizu A, et al. 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*. 2005;11(1):32–4. [PubMed: 15619627]
28. Rivard CJ, Tanabe T, Lanaspa MA, et al. Upregulation of CD80 on glomerular podocytes plays an important role in development of proteinuria following pig-to-baboon xeno-renal transplantation - an experimental study. *Transplant Int*. 2018;31:1164–1177.
29. Nauman G, Borsotti C, Danzl N, et al. Reduced positive selection of a human TCR in a swine thymus using a humanized mouse model for xenotolerance induction. *Xenotransplantation*. 2020;27(1):e12558. [PubMed: 31565822] **Study in humanized mouse model with porcine thymic grafts. Demonstrated inefficient positive selection of cells expressing human restricted TCR in the porcine thymus, setting the stage for the recent advances in development of the hybrid thymus.
30. Griesemer AD, Sorenson EC, Hardy MA. The role of the thymus in tolerance. *Transplantation*. 2010;90(5): 465–474. [PubMed: 20555306]
31. Maharlooei MK, Holzl M, Li H, et al. Generation of human/pig hybrid thymus to achieve immune tolerance to pig antigens with optimal immune function. Presented at: IXA 2017, 14th Congress 9 11, 2017.
32. Sekijima M, Sahara H, Shimizu A, et al. Preparation of hybrid porcine thymus containing non-human primate thymic epithelial cells in miniature swine. *Xenotransplantation*. 2019;26(6):e12543. [PubMed: 31293016]
33. Maharlooei MK, Danzl N, Gras-Pena R, et al. Use of human pluripotent stem cells to produce human/pig hybrid thymus grafts to achieve immune tolerance to pig antigens with optimal immune function [Abstract]. *Am J Transplant*. 2019; Presented at: IXA 2019, 15th Congress Oct. 12, 2019.*Data presented regarding ability to generate a hybrid thymus by injecting human thymic

stromal cells into swine thymus. Peripheral T cells of mice generated with the hybrid thymus were hyporesponsive to human TEC donor-derived dendritic cells.

34. Fudaba Y, Onoe T, Chittenden M, et al. Abnormal regulatory and effector T cell function predispose to autoimmunity following xenogeneic thymic transplantation. *J Immunol.* 2008;181:7649–7659. [PubMed: 19017953]
35. Ezzelarab MB. Regulatory T cells from allo- to xenotransplantation: Opportunities and challenges. *Xenotransplantation.* 2018;25(3):e12415. [PubMed: 29913039]
36. Alonso-Guallart P, Zitsman JS, Stern J, et al. Characterization, biology, and expansion of regulatory T cells in the Cynomolgus macaque for preclinical studies. *Am J Transplant.* 2019;19(8):2186–2198. [PubMed: 30768842] *Publication of a successful expansion and cryopreservation protocol for NHP Tregs to be used in preclinical studies. Tregs were found to be suppressive in vitro, both before cryopreservation and after thawing.
37. Duran-Struuck R, Sondermeijer HP, Bühler L, et al. Effect of Ex Vivo-Expanded Recipient Regulatory T Cells on Hematopoietic Chimerism and Kidney Allograft Tolerance Across MHC Barriers in Cynomolgus Macaques. *Transplantation.* 2018 5;102(5):e252. [PubMed: 29702539] *Study demonstrating prolonged chimerism, up to 335 days, after allogeneic BMT with cotransplantation of host Tregs in the NHP model.
38. Jin X, Hu M, Gong L, et al. Adoptive transfer of xenoantigen stimulated T cell receptor V β restricted human regulatory T cells prevents porcine islet xenograft rejection in humanized mice. *Mol Med Rep.* 2018;18(5):4457–4467. [PubMed: 30221725] **Study showing prolonged porcine NICC xenograft survival (over 84 days) with adoptive transfer of human PBMCs and xenoantigen-stimulated Tregs in humanized mouse model.
39. Stern J, Kofman S, Alonso-Guallart P, et al. Extended pig skin graft survival in baboons receiving Hcd27 Tg/Galt-KO pig PBSC plus host Treg infusion [Abstract]. *Am J Transplant.* Presented at: IXA 2017, 14th Congress 9 11, 2017.
40. Kim SC, Mathews DV, Breeden CP, et al. Long-term survival of pig-to-rhesus macaque renal xenografts is dependent on CD4 T cell depletion. *Am J Transplant.* 2019;19(8):2174–2185. [PubMed: 30821922] *Demonstrated that selective CD4+ T-cell depletion, but not CD8+ T cell depletion, resulted in long term survival of over 400 days for renal xenografts in a pig-to-rhesus model with recipients chosen for having low pre-transplant anti-pig antibody.
41. Lee SJ, Kim HJ, Byun NR, et al. Donor-Specific Regulatory T Cell-Mediated Immune Tolerance in an Intrahepatic Murine Allogeneic Islet Transplantation Model with Short-Term Anti-CD154 mAb Single Treatment. *Cell Transplant.* 2020;29:963689720913876.*In vivo study of intrahepatic murine allogeneic islet transplant model. Found that short term treatment with anti-CD154 mAb induced Treg mediated tolerance of islets for over 250 days and persisted with secondary transplantation of islets under the kidney capsule of the recipient.
42. Yoon IH, Chung H, Kim HJ, et al. Peri-graft porcine-specific CD4+ FoxP3+ regulatory T cells by CD40-CD154 blockade prevented the rejection of porcine islet graft in diabetic mice. *Xenotransplantation.* 2019;26(5):e12533. [PubMed: 31111577] **Survival of porcine islet grafts in diabetic mice over 200 days with short-term anti-CD154 mAb therapy. Also found a higher ratio of Tregs in the perigraft site, but not secondary lymphoid organs, suggesting migration of Tregs into the graft as the mechanism tolerance.
43. Podestà MA, Binder C, Sellberg F, et al. Siplizumab selectively depletes effector memory T cells and promotes a relative expansion of alloreactive regulatory T cells in vitro. *Am J Transplant.* 2020;20(1):88–100. [PubMed: 31319439] **Study to assess siplizumab's effect on Tregs compared to other T-cell subsets in MLRs. Found that it reduced effector memory T cells, which express higher CD2, and supported expansion of donor-reactive T regs, which have lower CD2 expression, providing a mechanism for promoting tolerance induction.
44. Springer TA, Dustin ML, Kishimoto TK, et al. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu Rev Immunol.* 1987;5:223–252. [PubMed: 3109455]
45. Latinne D, De La Parra B, Nizet Y, et al. An anti-CD2 mAb induces immunosuppression and hyporesponsiveness of CD2+ human T cells in vitro. *Int Immunol.* 1996; 8(7): 1113–1119. [PubMed: 8757956]

46. Xu Y, Kolber-Simonds D, Hope JA, et al. The anti-CD2 monoclonal antibody BTI-322 generates unresponsiveness by activation-associated T cell depletion. *Clin Exp Immunol*. 2004; 138(3): 476–483. [PubMed: 15544625]
47. Sellberg F, Berglund D, Binder C, et al. Pharmacokinetic and pharmacodynamic study of a clinically effective anti-CD2 monoclonal antibody. *Scand J Immunol*. 2020;91(1):e12839. [PubMed: 31630416]
48. Berglund E, Alonso-Guallart P, Danton M, et al. Safety and pharmacodynamics of anti-CD2 monoclonal antibody treatment in cynomolgus macaques - an experimental study. *Transpl Int*. 2020;33(1):98–107. [PubMed: 31523849] *Demonstrates the safety and pharmacodynamic profile of primate specific anti-CD2 in the NHP model, data that is necessary for use of this mAb in clinical trials. Authors found that memory T cells were depleted and that Tregs were relatively spared along with naïve T cells.
49. Brady JL, Sutherland RM, Hancock M, et al. Anti-CD2 producing pig xenografts effect localized depletion of human T cells in a huSCID model. *Xenotransplantation*. 2013;20(2): 100–109. [PubMed: 23442186]
50. Nottle MB, Salvaris EJ, Fisicaro N, et al. Targeted insertion of an anti-CD2 monoclonal antibody transgene into the GGTA1 locus in pigs using FokI-dCas9. *Sci Rep*. 2017;7(1): 8383. [PubMed: 28814758]

Key Points

- Advances in genome editing will facilitate transgenic pigs for xenotransplantation
- Intra-bone bone marrow transplant may prolong chimerism, supporting xenograft survival compared to infused stem cells alone
- The hybrid thymus, containing thymic epithelial cells from the recipient as well as donor, will allow for both positive (Treg) and negative (xenoreactive, autoreactive) selection in the thymus and allow selection of human TCR
- Treg infusion for tolerance induction has been shown in porcine islet models to have sustained survival of grafts and hyporesponsiveness to donor
- Anti-CD2 antibody has demonstrated its potential to suppress memory effector T cells, while also promoting expansion of Tregs