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Review

Preventing T cell rejection of pig xenografts

Laura Higginbotham, Mandy L. Ford, Kenneth A. Newell, Andrew B. Adams*

Emory Transplant Center, Emory University School of Medicine, Atlanta, GA, USA

HIGHLIGHTS

- Genetic engineering advances have curtailed the antibody barrier in xenotransplantation.
- Most successful immunosuppressive strategies in xenotransplantation also involve T cell depletion.
- Use of T cell costimulatory blockade, specifically of the CD40-CD154 pathway, has resulted in long-term xenograft survival.
- Transgenic expression of coinhibitory molecules by porcine cells shows promise at decreasing the T cell response in vitro.
- Therapy with various immunomodulatory cells has shown potential at inhibiting T cell responses to xenografts in vitro.

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ABSTRACT

Xenotransplantation is a potential solution to the limited supply of donor organs. While early barriers to xenograft acceptance, such as hyperacute rejection, are now largely avoided through genetic engineering, the next frontier in successful xenograft survival will require prevention of T cell-mediated rejection. Most successful immunosuppressive regimens in xenotransplantation utilize T cell depletion with antibody therapy. Additionally, the use of T cell costimulatory blockade - specifically blockade of the CD40-CD154 pathway – shows promise with several reports of long-term xenograft survival. Additional therapies, such as transgenic expression of T cell coinhibitory molecules or transfer of immunomodulatory cells to promote tolerance, may be necessary to achieve reliable long-term xenograft acceptance. Further studies in pre-clinical models are essential in order to optimize these regimens prior to trials in patients.

Contents

Abbreviations: AMR, antibody-mediated rejection; APC, antigen-presenting cell; ATG, antithymocyte globulin; BM, bone marrow; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ECDI-SP, ethylene carbodiimide donor splenocytes; Gal, galactose-α1,3-galactose; GTKO, α1,3-galactosyltransferase gene-knockout; hDAF, human decay accelerating factor; IFN-γ, interferon-gamma; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed lymphocyte reactions; MSC, mesenchymal stromal cells; MST, median survival times; NHP, non-human primate; NK, natural killer; pAEC, porcine aortic endothelial cells; PBMC, peripheral blood mononuclear cells; PD-L1, programmed cell death ligand 1; POD, post-operative day; SLA, swine leukocyte antigen; TBM, thrombomodulin; TCR, T cell receptor; Treg, regulatory T cell.

* Corresponding author. Emory Transplant Center, Emory University School of Medicine, 101 Woodruff Circle, 5105 WMB, Atlanta, GA 30322, USA.
E-mail address: abadams@emory.edu (A.B. Adams).

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1. Introduction

Organ transplantation is the treatment of choice for most end-stage organ diseases, yet the largest barrier to wider clinical application is the shortage of available organs. In renal transplant, the median wait time for recipients is >4 years with an estimated fourteen patient deaths per day while awaiting a donor kidney [1]. Potential avenues for expanding the number of available organs include increased enrollment of deceased and living donors, bioengineering of donor organs, and xenotransplantation. In xenotransplantation, pigs are widely considered the optimal donor species due to similar anatomy and physiology between human and swine organs, high reproductive potential, and ethical tolerability. Recent advances in genetic editing technology are eliminating the prior hurdles that impeded xenotransplantation from becoming a clinical reality [2].

Traditionally, research aimed at prevention of xenograft rejection focused on B cells, complement cascade activation, and antibody-mediated rejection (AMR). Preformed antibodies bind xenoantigens such as galactose-α1,3-galactose (Gal) with subsequent activation of the complement and coagulation cascades which results in endothelial injury and hyperacute thrombosis of the xenograft. Genetic engineering advances have produced porcine xenografts capable of avoiding hyperacute rejection through deletion of the Gal-producing enzyme, α1,3-galactosyltransferase gene-knockouts (GTKO), or through expression of complement regulatory proteins. Transgenic heterotopic porcine hearts now survive 6 months in baboons [3–5], and xenoislet survival greater than 1 year is described in non-human primates (NHP) [3,6,7]. The authors recently reported survival >126 days in a pig-to-NHP kidney transplant model using GTKO/hDAF (human decay accelerating factor) pig kidneys [8] with current survival now over 10 months in one animal (>280 days) [unpublished results]. Cooper et al. also published >100 day survival with a multi-gene transgenic/GTKO porcine kidney transplanted into an NHP [9]. As the field continues to produce improvements in humoral rejection through genomic editing advancements, T cell rejection and strategies to overcome it will play an increasingly important role.

Similar to the allogeneic response, T cells in xenotransplantation are activated through direct and indirect pathways. During direct activation, recipient (NHP or human) T cell receptors (TCR) bind swine leukocyte antigen (SLA) class I and class II on porcine antigen-presenting cells (APCs). Porcine APCs are either passenger dendritic cells or endothelial cells that constitutively express CD80/86 (as opposed to human APCs) [10,11]. This interaction results in T cell-mediated cytotoxicity directed against the xenograft vascular endothelium. The indirect pathway of T cell activation involves NHP/human recipient T cells recognizing porcine donor peptide presented on NHP/human recipient major histocompatibility complex (MHC) class II. This leads to $CD4^+$ T cell stimulation, B cell activation, de-novo antibody production, and humoral xenograft rejection. Finally, activated T cells produce cytokines that prime the innate immune system, including macrophages and natural killer (NK) cells, a process which ultimately leads to xenograft dysfunction [11–13]. While the direct xenogeneic and allogeneic responses appear to be equivalent [14], the indirect xenogeneic response is stronger than its allogeneic counterpart [15]. Strategies that target T cell activation are an important component of any regimen that achieves successful long-term xenograft survival.

2. Costimulation blockade to prevent xenogeneic T cell responses

T cell activation requires binding of the TCR to an MHC-peptide complex on the APC as well as a second costimulatory signal. The development of fusion proteins or antibodies that block these second signals is an established strategy to prevent both allogeneic [16] and xenogeneic T cell responses. One of the first studies to utilize the fusion protein CTLA4-Ig (abatacept), which impedes CD28-CD80/86 interactions, was a xenogeneic human-to-mouse islet model where treatment with CTLA4-Ig significantly prolonged islet survival [17]. More recently, xenotransplant studies have focused on antibodies targeting the CD40-CD154 pathway [3,18]. Anti-human CD154 monoclonal antibody (anti-CD154 mAb) remains the most commonly used agent in xenokidney models with reported survival times of 4–83 days in life-sustaining models [3,19–21] (Table 1). Despite promising survival data, anti-CD154 mAb is limited in its potential for clinical translation due to thromboembolic concerns. As an alternative approach, our group and others have studied an antibody directed at CD40 [22–24].

Iwase et al. recently published comparisons of three regimens in pig-to-baboon heterotopic heart transplant: (i) anti-CD154 mAb, (ii) CTLA4-Ig (abatacept), and (iii) anti-CD40 mAb plus belatacept (high-affinity CTLA4-Ig). Both anti-CD154-and anti-CD40-containing regimens effectively controlled the adaptive immune response to xenoantigen, while CD28 blockade alone (abatacept) failed to adequately control the anti-porcine response [25].

Our group also recently compared CD40-CD154 blockade and CD28-CD80/86 blockade using a recombinant anti-CD154 antibody (5c8; NIH Nonhuman Primate Reagent Resource, Boston, MA, USA) and belatacept in a pig-to-primate renal transplant model [8]. Costimulation blockade with anti-CD154 more effectively prolonged survival than belatacept [8] (Table 1). Taken together, these studies suggest that currently available CD28-blocking reagents are insufficient to prevent T cell activation in xenotransplant (Table 1). This may be due to decreased binding ability of belatacept to porcine costimulatory molecules CD80/86 [26]. Moving forward, it appears that blockade of the CD40-CD154 pathway is a critical component of an immunosuppressive regimen aimed at controlling the xenogeneic T cell response.

Table 1
. Costimulation blockade and T cell depletion in large animal models of xenotransplantation. Pig-to-NHP models with successful long-term survival are summarized from the literature for renal, islet, and heterotopic heart xenotransplantation. Results are grouped by costimulation blockade reagent. Costimulation blockade of the CD40-CD154 pathway is more successful than CD28-CD80/86 blockade. In particular, anti-CD154 (5c8) yields the most impressive results in both renal and cardiac models. T cell depletion using ATG is most common with maximum survival of 136 days in life-sustaining models, i.e. renal transplantation. Anti-CD4 and anti-CD8 depleting antibodies have produced exciting results with ongoing survival >280 days in a renal model. Results of anti-CD3 rIT are less successful with survival of 23 days despite combination therapy with costimulation blockade.

Costimulation blockade	T cell depletion	Pig genotype	Organ type	Survival (days)	First author
CD40-CD154 blockade					
anti-CD154 mAb	ATG	CD46	Islet	396	Van der Windt 2009 [7]
	ATG	GTKO	Kidney	83	Griesemer 2009 [20] Shimizu 2012 [21]
	anti-CD3 rIT	GTKO	Kidney	23	Nishimura 2011 [27]
Human-mouse chimeric anti-CD154 (5c8)	anti-CD4 (CD4R1) and anti-CD8 (M-T807R1) depleting antibodies	GTKO/hDAF	Kidney	>280	Higginbotham 2015 [unpublished, [8]]
	ATG	GTKO/CD46/TBM	Heterotopic heart	236	Mohiuddin 2014 [5]
anti-CD40 mAb (2C10R4)	ATG	GTKO/CD46/TBM	Heterotopic heart	>500	Mohiuddin 2014 [5]
	ATG	GTKO/multi-gene transgenic	Kidney	136	Iwase 2015 [9]
CD28-CD80/86 blockade					
High affinity CTLA4-Ig (belatacept)	anti-CD4 (CD4R1) and anti-CD8 (M-T807R1) depleting antibodies	GTKO/hDAF	Kidney	21	Higginbotham 2015 [8]
CTLA4-Ig (abatacept)	ATG	GTKO/CD46/CD55	Heterotopic heart	23	Iwase 2015 [25]

3. T cell depletion and clonal reduction

The clone size of the xenogeneic T cell response is estimated to be similar, if not larger, than the typical allogeneic response [14,15]. Given this challenging barrier, most successful immunosuppressive regimens include a method of T cell depletion such as mono- or polyclonal anti-T cell antibodies, chemotherapeutic agents such as cyclophosphamide, or radiation therapy (whole body or thymic) [3]. The most commonly used reagent is antithymocyte globulin (ATG), a polyclonal preparation that results in T cell levels <200/μL for approximately 5 weeks post-transplant [20] and extends survival to 2–3 months in some xenokidney models [3] (Table 1). More specific reagents such as anti-CD3 recombinant immunotoxin (anti-CD3 rIT) or anti-CD4 and anti-CD8 depleting antibodies have also shown success [8,27] (Table 1). Overall, immunosuppressive regimens that combine transient T cell depletion (using ATG or monoclonal antibodies) and anti-CD154 costimulation blockade have produced positive results across organ types [3]. Thus, it seems that clonal reduction with transient depletion is a critical component of controlling the T cell response in xenotransplantation.

4. Genetic engineering to evade T cell rejection of xenografts

Several reports have detailed genetic modification of porcine donors as a method to circumvent T cell rejection. Work in this area has primarily focused on the transgenic expression of coinhibitory molecules on porcine cells. Phelps et al. described the engineering of pigs expressing porcine cytotoxic T-lymphocyte-associated protein 4 immunoglobulin (pCTLA4-Ig) [28]. Subsequent *in vitro* work by the group demonstrated the ability of pig peripheral blood mononuclear cells (PBMC) transgenic for pCTLA4-Ig to directly inhibit human $CD4^+$ T cell responses when co-cultured compared to wildtype (WT) or GTKO pig PBMC [29]. While the results are promising, GTKO/pCTLA4-Ig pigs were plagued by infectious complications due to an immunocompromised state [28]. Another group recently published a description of transgenic pigs expressing human CTLA4-Ig (hCTLA4-Ig) in skin, heart, kidney, and corneal tissue. The pigs developed no infectious complications and appeared healthy. Prolonged skin xenograft survival was seen in pig-to-rat models compared to wildtype [30].

Another coinhibitory molecule, programmed cell death ligand 1 (PD-L1), also showed promise in manipulating T cell responses. Expression of human PD-L1 on porcine cells resulted in decreased T cell infiltrate when transfected pig cells were placed under the kidney capsule of rat recipients [31]. *In vitro* studies utilizing porcine B cells and porcine aortic endothelial cells (pAECs) overexpressing PD-L1 demonstrate their ability to suppress proliferation of human $CD4^+$ T cells while expanding regulatory T cell (Treg) populations. These studies also report increased interleukin (IL)-10 production after T cells were cultured in the presence of transgenic PD-L1 porcine cells [32,33].

In addition to transgenic expression of T cell coinhibitory molecules, other genetic modifications aimed at limiting anti-pig T cell responses include the downregulation of pig MHC. Hara et al. engineered pigs transgenic for a mutant human variant of the class II transactivator (CIITA-DN) gene that decreased expression of SLA class II on porcine cells. They reported reduction in SLA class II molecules of 40–50% on APCs and complete lack of expression on pAECs. Furthermore, mixed lymphocyte reactions (MLR) between human $CD4^+$ T cells and pAECs or porcine PBMC resulted in decreased T cell proliferation compared to wildtype [34]. In a pig-to-baboon artery patch model, GTKO/CD46/CIITA-DN grafts without immunosuppression still demonstrated a significant full-thickness lymphocytic infiltrate. When coupled with costimulation blockade, however, cellular infiltration was minimal or absent [35].

Finally, transgenic expression of human cytokines such as CCL17 and CCL22 on pig endothelial cells as a means of recruiting Tregs into the xenograft has been suggested [12], though no *in vitro* or *in vivo* data exists at this time to support its plausibility.

5. Induction of tolerance to pig xenografts

Recent studies of novel immunosuppression regimens have reported exciting results, however the ultimate goal of allo- and xenotransplantation is immunological tolerance. The development of xenograft tolerance is especially appealing given infectious complications associated with the rigorous immunosuppressive regimens currently required for xenotransplantation. Several promising strategies are reviewed below.

5.1. Mixed chimerism

Mixed chimerism achieves tolerance via central deletion, where dendritic cells from donor bone marrow migrate to recipient

Table 2
Induction of tolerance in xenotransplantation. Studies investigating tolerance induction are summarized with survival times when applicable. Thymic cotransplant has generated the most favorable results in NHP models with survival of 83 days. While cellular therapy is promising, only *in vitro* data exists currently.

Tolerance strategy	Model	Additional therapy	Survival (days)	First author
Mixed chimerism	WT pig-to-NHP kidney	ATG plasmapheresis	15	Sablinski 1997 [56]
Thymic cotransplant	GTKO pig-to-NHP kidney	ATG anti-CD154 mAb	83	Yamada 2005 [42]
Cellular therapy: MSC	*In vitro assays*	–	–	Kumar 2012 [46] Li 2014 [45]
Cellular therapy: ECDI-SP	Rat-to-mouse islet	anti-CD20 mAb	>100	Wang 2013 [49]
Cellular therapy: Tregs	*In vitro assays*	–	–	Lin 2009 [51] Porter 2007 [53] Singh 2012 [52] Wu 2008 [54]

thymus and participate in negative selection. Several researchers have achieved stable, mixed chimerism in rodents [36], but translation to large animal models is difficult. One unique challenge of chimerism in xenotransplant is species-specific hematopoietic factors. Studies show that host stem cells have a competitive advantage over donor cells, and long-term chimerism is lost [37]. Attempts in large animal pig-to-NHP models have met limited success [2] (Table 2). Infusion of GTKO porcine bone marrow (BM) cells into baboons failed to achieve long-term engraftment with only low-level chimerism in the blood and bone marrow within the first week [38]. However, decreased donor-specific T cell responsiveness was observed two months post-transplant [39]. Mixed chimerism is a powerful strategy to promote both T and B cell tolerance to donor tissue, but additional studies are needed to refine such strategies in xenotransplantation.

5.2. Thymic transplant

The thymus is critical in defining immunological self from non-self and represents an ideal environment for manipulation of the host immune system. Xenogeneic thymic transplant consists of effective T cell depletion and recipient thymectomy prior to thymic transfer. Exposure to donor pig thymus during T cell reconstitution results in central deletion of cells that strongly recognize porcine peptide-MHC complexes, thus educating the recipient's T cell repertoire to recognize pig antigen as self [2,40,41]. In large animal models, porcine thymus is transplanted either as composite "thymokidney" or as a separate vascularized thymic lobe. This strategy results in significant prolongation of porcine kidneys in NHP recipients [42] with investigators routinely reporting survival to 80 days [43] (Table 2). This novel strategy shows potential in pre-clinical models, however theoretical concerns exist including the possibility of autoimmunity from incomplete deletion of autoreactive T cells on porcine donor thymus.

5.3. Cellular therapy to control xenograft rejection

The delivery of tolerogenic donor or recipient cells, purified and/or modified *ex vivo*, is an increasingly-studied strategy to promote donor-specific T cell hyporesponsiveness. Numerous clinical trials, in transplantation and autoimmunity, are examining the safety and efficacy of these strategies. Mesenchymal stromal cells (MSC) are a subset of cells derived from blood, bone marrow, or adipose tissue that maintain the ability to differentiate into osteoblasts, chondroblasts, and adipocytes [44]. They are of particular interest in allogeneic and xenogeneic transplant for their unique anti-inflammatory and immunomodulatory properties. Cooper et al. described the generation of MSCs from porcine bone marrow and adipose tissue. They performed MLR utilizing GTKO pAECs and human PBMC in the setting of porcine MSCs (pMSCs) and showed that the presence of pMSCs significantly decreased anti-pig T cell proliferation [45–47].

Another promising strategy is the use of 1-ethyl-3-(3-diethylaminopropyl) carbodiimide (ECDI) to promote donor cell apoptosis prior to infusion; long-lasting tolerance is described after allogeneic cellular infusions in mice [48]. More recently, ECDI-treated splenocytes (ECDI-SP) were tested in rat-to-mouse islet xenotransplantation. Mice receiving ECDI-SP infusions survived longer than control mice, however tolerance was not achieved [49]. Moreover, recipient serum tested positive for the presence of anti-rat antibodies after ECDI-SP infusion, and immunofluorescent staining of islet xenografts was positive for C4d deposition demonstrating activation of the immune system rather than suppression. While researchers ultimately achieved survival >100 days with addition of B cell depletion to cellular therapy [49] (Table 2), more investigation is necessary using porcine ECDI-SPs in large animal models of xenotransplantation.

The ability of $CD4^+CD25^+FoxP3^+$ cells, or Tregs, to suppress auto- or allo-reactive T cells is well-described. Cooper et al. investigated the human anti-pig cellular response by MLR and showed that addition of human Tregs suppressed the xenogeneic T cell response [50,51]. Interestingly, more Tregs were required to suppress the xeno-response than were needed for suppression of the allo-response [50]. Other investigators have expanded Tregs *ex vivo* and used the Treg product to suppress NHP anti-pig T cell responses in a donor-specific manner [52,53]. Wu et al. cultured human Tregs using CD3 and CD28 beads, rapamycin, and IL-2. They showed equivalent suppressive capacity between fresh and expanded Tregs against $CD4^+CD25^-$ T cells targeting pig cells. Interestingly, however, different cytokine production profiles emerged between the two groups with T cells in the presence of fresh Tregs secreting interferon-gamma (IFN-γ), and T cells mixed with expanded Tregs generating IL-4 and IL-10 [54]. While *in vitro* studies are promising, we await data from large animal xenotransplant models to determine clinical translatability.

6. Conclusions

Recent advances in genetic engineering hold promise for the generation of pigs less susceptible to early antibody-mediated rejection and subsequent coagulation dysfunction. As humoral barriers are surmounted, control of the anti-pig T cell response will become increasingly critical to achievement of long-term xenograft survival. Any successful strategy will require reduction of the magnitude of anti-pig T cell response. Combining T cell depletion with costimulatory blockade, particularly of the CD40-CD154 pathway, shows the most potential. Finally, the addition of novel cellular therapies with immunomodulatory capacity may further enhance the likelihood of long-term survival in pre-clinical models.

Conflict of interest

None.

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Author contribution

L.H. and A.A. contributed to review of the literature and manuscript preparation. A.A., M.F., and K.N. contributed to final manuscript revisions and approval.

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