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T-cell-mediated immunological barriers to xenotransplantation

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Abstract

Xenotransplantation remains the most viable option for significant expansion of the donor organ pool in clinical transplantation. With the advent of nuclear transfer technologies, the production of transgenic swine has become a possibility. These animals have allowed transplant investigators to overcome humoral mechanisms of hyper-acute xenograft rejection in experimental pig-to-non-human primate models. However, other immunologic barriers preclude long-term acceptance of xenografts. This review article focuses on a major feature of xenogeneic rejection: xenogeneic T cell responses. Evidence obtained from both small and large animal models, particularly those using either islet cells or kidneys, have demonstrated that T cell responses play a major role in xenogeneic rejection, and that immunosuppression alone is likely incapable of completely suppressing these responses. Additionally, both the direct and indirect pathway of antigen presentation appear to be involved in these anti donor processes. Enhanced understanding of (i) CD47 and its role in transduced xeno-bone marrow (ii) CD39 and its role in coagulation dysregulation and (iii) thymic transplantation have provided us with encouraging results. Presently, experiments evaluating the possibility of xenogeneic tolerance are underway.

Keywords

cellular responses; T cell; tolerance; xenotransplantation

There is a growing disparity between the number of organs needed for transplantation and the number of organs available. The de novo generation of new organs using regenerative medicine or stem cell technologies has provided appealing potential solutions to the critical organ shortage. Several regenerative tissue technologies have shown encouraging results [1–3]. Recently, investigators found that by transplanting xenogeneic renal primordia which (i) are not susceptible to cellular and humoral rejection due of the absence of antigen-presenting cells (APCs) and (ii) are angiogenic, these tissues are able to grow, unabated in transplant recipients [1]. Unfortunately, the functionality of these regenerated tissues remains markedly limited. Tissue bioengineering has also provided a potential solution to the organ shortage. In an ex vivo model of vein-valve development, investigators have been successful in decellularizing human vein valves and reseeded them with allogeneic endothelial cells [2]. The reseeded valves were shown to express CD31, VE-Cadherin as well as vWF [2]. Regarding solid-organ tissue engineering, several groups have had success with decellularization of livers [3]. In one model, decellularized rat livers were successfully reseeded with hepatocytes and subsequently demonstrated approximately 90% cell engraftment. Additionally, rat hearts decellularized with detergents and reseeded with cardiac or endothelial cells led successfully to regeneration of partial myocardial function

(equivalent to about 2% of adult or 25% of 16-week fetal heart function) [4]. Although tissue-bioengineering technologies are exciting, in a recent review of the literature, authors summarized that the generation of complex tissues is still a distant milestone [5]. More recently, techniques for reprogramming adult tissues through gene transduction to produce induced pluripotent stem cells (iPS) have spawned interest in organ regeneration [6–8]. This is partly because this strategy minimizes the ethical issues associated with de novo generation of tissue. These approaches are innovative and may provide alternative sources of allogeneic organs in future, however, as with xenogeneic primordia transplantation, there are as of yet no reports demonstrating normal, life-supporting reprogrammed or regenerated solid organs in large animals.

Presently, xenotransplantation of solid organs remains the most viable option for large-scale expansion of the donor organ pool [9–12]. Given size, gestational cycle, and physiologic similarities, the miniature swine is considered the most promising candidate species for xenogeneic organ donation [9]. However, cells from outbred species of pigs constitutively express the sugar moiety alpha 1–3 galactosyltransferase (GalT) to which humans have preformed antibody. Thus, transplantation of non-manipulated swine tissue into primates (including humans) leads to hyperacute rejection [13–15]. With the advent of nuclear transfer technologies, we and others have successfully isolated and knocked out (KO) the gene responsible for production of Gal and subsequently bred a line of Gal knockout or GalT-KO pigs [16–18]. The development of GalT-KO pigs overcame hyper-acute rejection in non-human primates with low levels of cytotoxic non-Gal natural antibodies [19]. However, other immunologic barriers have prevented long-term acceptance of xenografts [12,20–24]. Among these barriers, this review article focus on the xenogeneic T-cell response.

T-cell responses across xenogeneic barriers

The development of GalTKO pigs opened the door for the future success of xenotransplantation. Since the inception of GalT-KO (and other transgenic) pigs, it has been debated as to whether xenogeneic T-cell responses can be successfully controlled using currently available immunosuppressive therapies alone [25].

Do human T cells recognize porcine antigens directly?

Between different species, the MHC is likely more disparate than those of allogeneic donor–recipient pairs; thus, it was initially thought that the xenogeneic T-cell response might be less severe than what is seen across allogeneic barriers [26–28]. Allotransplantation revealed two pathways for antigenic presentation: the *direct* and *indirect* pathways. In the direct pathway, recipient T cells respond to major histocompatibility complex (MHC) molecules displayed by donor APCs. The indirect pathway involves recipient T-cell stimulation via donor peptide presentation (on recipient class II molecules) by recipient APCs. Recently, a novel “semi-direct” pathway of antigen presentation has been described [29,30]. In the semi-direct pathway, recipient dendritic cells acquire intact MHC-antigen complexes from donor dendritic and endothelial cells, and then present antigens via direct antigen presentation to alloreactive T cells. Because these same dendritic cells can simultaneously present antigen indirectly, these cells are capable of stimulating not only CD4⁺ T cells but also CD 8⁺ T cells as well [29].

Early investigations into the T-cell response utilized the human anti-mouse model. Moses et al. [27] have demonstrated that there was a defective human CD4 T-cell-murine MHC class II molecule interaction, effectively eliminating T-cell activation by the direct pathway. These results encouraged researchers to propose that once the humoral mechanisms of rejection were overcome, cell-mediated rejection would be relatively a minor obstacle.

However, in studies carried out in the early 1990s, the authors' laboratory and others showed that the direct pathway of activation did exist in the pig-to-human model [21,31]. We have demonstrated that (i) human T cells responded to xeno-MHC antigens (Ags) in a mixed lymphocyte reaction (MLR) at least as well as they did to allo-MHC Ags and appeared to share similar requirements of either stimulator APC (direct pathway) or responder APC (indirect pathway) derivation; and (ii) the majority of the primary human anti-pig xeno-response was directed toward porcine MHC class II Ags and involved interaction with the human CD4 accessory molecule. In addition, we generated T-cell clones from human anti-swine MLR cultures using MHC homozygous and recombinant haplotypes of MGH-miniature swine. We observed clear evidence for anti-xenogeneic MHC Ags and cross-reactivity between haplotypes consistent with known sequence similarities between DR beta-chains. Our results indicated that the human anti-porcine T-cell response was similar in strength and specificity to an allogeneic response and that the T-cell receptor (TCR) repertoire, accessory molecule interactions and cytokine production required for both direct and indirect pathways of recognition in the human anti-porcine MHC class II responses were functionally intact [21]. Regarding MHC class I responses, it is also possible for recipient T cells to develop MHC class I restricted anti-xenogeneic cellular responses after exposure to exogenous donor antigen (e.g., soluble proteins) through antigen cross-priming, again highlighting the importance of T-cell responses in xenogeneic rejection [32]. Additionally, it has been demonstrated that the ability of porcine alveolar lavage cells to stimulate a human anti-pig response can be inhibited by anti-HLA-DR antibody indicating indirect antigen presentation [33]. The anti-pig response was also inhibited by anti-SLA-DR antibody, indicating that the direct pathway is also involved in the anti-xenograft response. Interestingly, the immunogenicity of porcine cells in vitro was dependent on the percentage of dendritic cells in the stimulatory population and the xenogeneic response, particularly the indirect response, was stronger than the allogeneic controls.

Xenogeneic T-cell-mediated killing has also been investigated in vitro. We have previously reported that (i) porcine primed human T cells demonstrated high levels of cytotoxic T cell (CTL) killing in vitro; (ii) this CTL killing was blocked by the addition of anti-CD8 antibodies, indicating that the CTL responses appear to be MHC class I dependent; and (iii) T cell help, such as IL-2 stimulation or CD4 T-cell stimulation, is required for NK-mediated direct cellular killing [34,35]. Cell populations responsible for xenograft induced human anti-pig cytotoxicity were investigated using SV40-T large antigen transfected endothelial cells in an annexin V binding model [36]. The authors found that more than 60% of the xenogeneic human anti-pig cytotoxic response could be attributed to CD4+ cells and that <20% of the anti-donor response was due to CD8+ cells. The same group has also reported that the anti-porcine lytic response was inhibited using anti-Fas-L antibody. Genetic studies confirmed that the FasL gene was expressed in primed human PBMC, suggesting that the Fas/FasL-mediated apoptosis is one of the possible underlying mechanisms in the human anti-pig cytotoxic response.

Do T cells play an important role in xenogeneic cellular rejection in vivo?

Although in vitro data indicate strong xenogeneic T cell responses, the role of T cells in xenogeneic rejection, especially in large animal cardiac xenograft models, remains controversial.

Our initial experience with GalT-KO life-supporting renal transplantation, in the absence of a tolerance-inducing regimen, demonstrated a mono-nuclear cellular infiltrate in rejected organs, with organ survival reaching a maximum at 1 month [19], suggesting that overcoming acute T-cell-mediated rejection is of great importance in any attempts at pre-

clinical xenotransplantation, whether the attack is directed at the endothelium or at the parenchyma.

Much of the experimental work elucidating xenogeneic cellular responses has focused on experimental transplantation of islets. In these cellular transplant models, T-cell-mediated rejection has been well documented. As antibody responses did not seem to be largely associated with the rejection of xenogeneic islets, efforts to extend graft survival have focused on the anti-porcine cellular responses [37–41]. When fetal porcine islets were transplanted beneath the renal capsule of mice that are immunosuppressed with Cyclosporine A and Deoxyspergalin, infiltration with macrophages, T cells, and B cells was observed, but antibody deposition was not. These findings demonstrated that conventional/systemic immunosuppression for T-cell responses did not inhibit anti-porcine xenogeneic cellular responses [37]. With the addition of CTLA4 Ig or anti-CD40L, porcine xeno-islet survival was substantially increased [38]. Other groups showed that (i) CD4+ T cells are both necessary and sufficient for islet xenograft rejection [39] and (ii) simultaneous treatment with anti-LFA-1 plus either anti-CD154 or anti-CD45RB therapy could achieve indefinite xenograft function in the majority of recipients in a small animal model. These studies demonstrated that therapies targeting different pathways affecting T-cell function can have marked efficacy in inducing long-term xenograft survival and produce a prolonged state of host hyporeactivity in vivo.

Extrapolating on these small animal data indicating early successful immunosuppressive strategies, particularly costimulatory blockade, 12 cynomolgous macaques underwent intraportal adult porcine islet xenotransplantation and were followed for up to 6 months [42]. All animals became C-peptide positive and five animals enjoyed graft survival of >100 days [42]. This study investigated the use of various immunosuppressive regimens; all animals that received the authors' standard protocol in addition to everolimus and anti-CD154 had indefinite graft survival, suggesting that overcoming the anti-porcine T-cell response alone can lead to prolonged xenogeneic islet survival. These results are encouraging; however, it should be noted that despite graft function, peri-islet infiltration of CD4+, CD8+, as well as occasional CD20+ cells was observed [42]. Other groups also demonstrated islet survival using neonatal porcine islets in pig-to-non-human primate (NHP) models with extensive/long-lasting immunosuppression of T-cell responses including anti-CD40L [43].

In contrast to kidney or islet transplant models, the results of heterotopic heart transplant in pigs to baboons using hCD46 transgenic or GalT-KO donors revealed that these grafts had significantly less cellular infiltration than other models [44,45], which raises the question of whether xenogeneic T-cell responses can be successfully controlled using currently available immunosuppressive therapies alone [25]. However, increased immunosuppression, not anti-coagulation, extended cardiac xenograft survival in hCD46 transgenic porcine hearts [46], and CD3 positive T cells were clearly identified in GalT-KO porcine hearts that were treated with chronic immunosuppression [45]. Therefore, the question may be proposed: can only a small number of T cells initiate xenogeneic sequential cellular rejection, including recruitment of innate responses?

A small number of T cells initiate xenogeneic cellular rejection

Korsgren and colleagues have reported that when fetal porcine islet-like cell clusters were placed under the renal capsule of athymic (nude) or normal mice, and evaluated 6 days later, significant cell-mediated rejection occurred in the normal mice, but not in the nude mice [47]. In addition, it was observed that the majority of infiltrating cells were macrophages, and a small percentage (~15%) was lymphocytes. Using the same model, investigators then

attempted to exclude the possibility that destruction of the islet-like cells clusters was antibody mediated by performing the same experiment in Fc-receptor-depleted mice [48]. In this experiment, investigators again showed that the primary effector of graft destruction was the macrophage and that the graft was infiltrated with lymphocytes. To distill the contribution of T cells in xenogeneic rejection, normal mice and TCR-deficient mice underwent transplantation of islet-like clusters beneath the renal capsule. As in the prior studies, normal mice rejected their grafts. In contrast, the TCR-deficient animals did not, providing more concrete evidence that the xenogeneic rejection process exclusively requires cells bearing the TCR [49]. Taken together, these studies have shown that xenogeneic islet cell rejection is T-cell dependent and can be mediated by very small number of cells.

T cells play an important role in innate immunity

On the basis of the above-mentioned work, as well as the work of others, there has been recent interest in the response of the innate immune system to xenogeneic stimuli, specifically regarding the role of macrophages [47,50]. One way in which xenografts activate an immunologic response is through the absence of a required ligand for a host inhibitory receptor. CD47 is the ligand of an immune inhibitory receptor—signal-regulatory protein alpha (SIRP α). When this ligand is absent or incompatible, phagocytosis of the ligand-negative/incompatible foreign cells may be initiated [24,51,52]. Such incompatibility may lead to the rejection of xenogeneic islets. However, data in humanized mice prepared by transplantation of human fetal thymus/liver tissues and CD34(+) liver cells into immunodeficient mice demonstrated that T-cell responses play a critical role in xenogeneic islet rejection [53]. Adult pig islets were completely rejected by 4 weeks, and histology revealed diffuse intragraft infiltration by human T cells, macrophages, and B cells in the untreated humanized mice. In contrast, pig islet rejection was prevented by human T-cell depletion prior to islet xenotransplantation. Islet xenografts harvested from T-cell-depleted humanized mice were functional and showed no human cell infiltration. Absence of innate responses in T-cell-depleted mice indicated that pig islet rejection is largely T-cell-dependent.

Tolerance strategies and the importance of T-cell depletion

In an attempt to control xenogeneic anti-swine cellular reactions, many protocols have been developed to block various parts of the immune response. To suppress T-cell-mediated responses, which likely initiate a myriad of other cellular responses including B cell, NK cell and some of innate responses, the level of immunosuppression needed to prolong solid-organ xenograft survival is prohibitive and associated with significant morbidity and mortality. Given these factors, it is likely that the induction of tolerance across xenogeneic barriers will be necessary for successful xenotransplantation.

Mixed chimerism

One approach to tolerance is through donor chimerism. Hematopoietic stem cells obtained from miniature swine, GalT-KO miniature swine, or hDAF pigs have been transplanted into irradiated baboons [54,55]. Thus far, even with the use of GalT-KO bone marrow, the majority of the cells (>90%) are cleared from circulation within hours and peripheral chimerism is generally undetectable after 1 week [56]. These results suggested that especially in bone marrow models, an innate immune response, possibly mediated by macrophages, was responsible for the cell loss.

Macrophage depletion has been shown to enhance the engraftment of porcine hematopoietic cells in mice [57,58]. As described in the earlier section “T cell play an important role in Innate immunity”, one means of controlling macrophages may be through the exploitation of

the species-specific interaction of CD47 with signal-regulatory protein alpha (SIRPa) [24,51,52]. Recent experiments at the Transplantation Biology Research Center (TBRC) have been undertaken to evaluate the efficacy of human-CD47-transduced porcine GalT-KO bone marrow cells. Further studies in this model are ongoing to evaluate the efficacy of this method for inducing tolerance. Although it is too early to tell, the anticipated result is that these cells will be “protected” from host macrophages as they express host CD47 instead of being cleared by the reticuloendothelial system.

Thymic transplantation

Thymic transplantation is thought to lead to tolerance by a central or deletional mechanism. Recipient donor reactive T cells are depleted with anti-T-cell antibodies prior to thymic transplantation. Donor-derived lymphoid precursors then migrate to the donor thymus where alloreactive (or xenoreactive) T cells are deleted via negative selection. Recipient dendritic cells may also migrate to the donor thymus, allowing for negative selection for autoreactive T cells. T cells active against other antigens should mature normally, leading to donor-specific tolerance. Early studies in mice demonstrated that transplantation of fetal or neonatal pig thymic tissue to thymectomized mice produced tolerance of pig skin grafts [59,60]. Further studies showed that polyclonal, functional human T cells develop in swine thymic tissue and that these cells exhibit donor-specific unresponsiveness [61]. Studies in the authors' laboratory have shown the advantage of using prevascularized thymic tissue over non-vascularized tissue. During the period of neovascularization, a substantial loss of thymic graft architecture is observed [62], resulting in poorer thymic deletion of recipient xenoreactive T cells.

Our laboratory at the TBRC has developed two methods of transplanting vascularized thymic tissue; either by direct vascular anastomosis of the thymic blood supply [63], or as a thymokidney (prepared by injecting autologous thymic tissue beneath the renal capsule and allowing 2 months for neovascularization prior to transplantation) [64]. We have demonstrated functional thymopoiesis in transplanted thymic grafts and donor-specific tolerance induction across a full MHC-mismatch barrier in an allogeneic miniature swine model [65–67]. When we applied this strategy to GalT-KO pigs to baboons, our initial attempts resulted in markedly prolonged renal xenograft survival with normal renal function for up to 83 days [19]. Thymic grafts supported thymopoiesis with evidence of CD4+CD3-baboon cells adjacent to cytokeratin-positive thymic epithelial cells [68]. Additionally, donor-specific unresponsiveness with normal anti-third-party allogeneic responses in CTL assays was observed in the maintenance period, suggesting the baboon was on a path toward tolerance [19,68].

Importance of T-cell depletion during the induction period for the induction of tolerance through chimerism or thymic transplantation

Both strategies described earlier potentially induce central tolerance. T-cell depletion (TCD) during the induction period is essential not only for mixed chimerism, but also for the development of in vitro donor-specific unresponsiveness using porcine fetal-thymic tissue transplantation in a pig-to-mouse model [69–71]. Mouse recipients in this study accepted skin grafts from the porcine thymic donors [59]. We recently investigated the levels of TCD that are required for prolongation of GalT-KO thymokidney survival in baboons [19,68,72]. Our findings suggest that maintenance of an “optimal number” of circulating T cells during the induction period is crucial to thymo-kidney xenograft survival [72]. A comparison of T-cell levels for the 14 days following thymokidney transplantation with various TCD regimens showed a correlation between the level of TCD at day 7 and graft survival. Animals that maintained T-cell levels below 200/ul in the first 7 days had stable renal function for longer than 40 days, despite T-cell recovery (>400 cells/ul) by three to 4 weeks.

In contrast, animals with transient TCD during the first 3 days demonstrated T-cell and B-cell sensitization and subsequent rejection. However, we also found that extensive immunosuppression led to lethal infection. One baboon had prolonged TCD (<50 cells/ul) for the first 14 days. It developed lethal baboon CMV and died at Day 28. The data suggest that an “optimal level of T-cell depletion” is required for xenogeneic thymokidney graft survival and avoidance of lethal infection. On the basis of these findings, we are currently controlling T-cell levels between 50 and 150 cells/ul, during the first-2 weeks post-transplant.

Purinergic signaling and CD39 in xenotransplantation T-cell tolerance induction

Robson and colleagues have defined and characterized the role of signaling by extracellular nucleotides and nucleoside derivatives in transplant rejection and have examined purinergic mechanisms of inflammation in transplantation [73]. One of the key players in these processes is CD39, an ectonucleoside triphosphate diphosphohydrolase that hydrolyzes extracellular ATP and ADP to AMP and is uniquely expressed at high levels by vascular endothelium and also on T-regulatory cells (Treg) [74–76]. Furthermore, CD39-associated molecules, including CD39-dependent ectonucleotidase and NTPDase1/CD39 have thromboregulatory functions that are both homeostatic and protective [75,77]. In addition, these pathways have extensive effects within the immune system that might be expected to also influence xenoreactivity and cellular rejection.

CD39 efficiently distinguishes resting and activated foxP3+ CD4+CD25+ Treg from other T cells in mouse and human/primate systems [78,79] and in pig T cells (Robson S., et al. Unpublished data) and is indicative of cellular suppressive potential. Hence, acquired expression of CD39 by transgenesis [80,81] promotes immune-suppressive pathways. Given species differences with low, intrinsic CD39 basal functional expression in porcine tissues [82–84], it might be predicted that transgenic pig cells (bone marrow, passenger leukocytes, or vascular cells) over-expressing CD39 may exert immunosuppressive effects locally. Certainly, the cardiac grafts from CD39 transgenic mice are resistant to humoral-type rejection with elicited antibodies to Gal [80]. Crucially, CD39 expression by vasculature and regulatory T-immune cells allows the integration of host responses by controlling both ATP-activatory and adenosine-mediated suppressive purinergic responses within the xenograft. CD39 and purinergic signaling pathways provide a potentially crucial bridge between vascular inflammation, thrombosis, and tolerance induction in xenotransplantation. Such integrated mechanisms are also possible in the setting of thymic xenotransplantation where “xeno-thymic-educated or deleted” T cells may ameliorate vascular injury of the graft.

Conclusion

Taken together, both in vivo and in vitro studies indicate that xenogeneic T-cell responses play a major role in cellular rejection. Inhibition of these responses is crucial because even a small number of xenoreactive T cells potentially initiate other cellular responses. Induction of donor-specific T-cell tolerance across xenogeneic barriers is not only essential to the success of xenotransplantation but also an attainable goal. In the next several years, tolerance strategies combined with genetic manipulation of donors through transgenic or knockout technologies could make xenogeneic organs more acceptable to patients in need of transplants.

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Abbreviations

APCs	antigen-presenting cells
CTL	cytotoxic T lympholysis
GalT	galactosyltransferase
iPS	induced pluripotent stem cells
KO	knocked out
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
TCD	T-cell depletion
TCR	T-cell receptor

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