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IXA Honorary Member Lecture, 2017: The Long and Winding Road to Tolerance

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Abstract

The last 15 years or so have seen exciting progress in xenotransplantation, with porcine organ grafts surviving months or even years in non-human primates. These advances reflect the application of new scientific knowledge, improved immunosuppressive agents and genetic engineering. The field has recently enjoyed a renaissance of interest and hope, largely due to the exponential increase in our capacity to genetically engineer porcine source animals. However, immune responses to xenografts are very powerful and widespread clinical application of xenotransplantation will depend on the ability to suppress these immune responses while preserving the capacity to protect both the recipient and the graft from infectious microorganisms. Our work over the last three decades has aimed to engineer the immune system of the recipient in a manner that achieves specific tolerance to the xenogeneic donor while preserving otherwise normal immune function. Important proofs of principle have been obtained, first in rodents, and later in human immune systems in “humanized mice” and finally in non-human primates, demonstrating the capacity and potential synergy of mixed xenogeneic chimerism and xenogeneic thymic transplantation in tolerizing multiple arms of the immune system. Considering the fact that clinical tolerance has recently been achieved for allografts and the even greater importance of avoiding excessive immunosuppression for xenografts, it is my belief that it is both possible and imperative that we likewise achieve xenograft tolerance. I expect this to be accomplished through the availability of targeted approaches to recipient immune conditioning, understanding of immunological mechanisms of tolerance, advanced knowledge of physiological incompatibilities, and the availability of inbred miniature swine with optimized use of genetic engineering.

Keywords

Tolerance; T cell; B cell; Natural killer cell; Macrophage; Mixed chimerism; Thymus; CD47; Natural antibody; Human; Pig; Rat; Miniature swine; Mouse; Xenotransplantation; Humanized mouse

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Introduction

We have witnessed encouraging progress toward clinical application of organ and cellular of xenotransplantation over the last 40 years. Recognition of the role of natural antibodies in causing hyperacute rejection (HAR) led to studies of plasma absorption by perfusion through xenogeneic organs in the 1980's that prolonged the survival of implanted organs from minutes to hours[1,2]. The development of the first transgenic pigs expressing human complement regulatory proteins led to further advancement, permitting organ survivals of days to weeks in the 1990's[3–5]. The seminal discovery of α1,3Gal as the major antigenic target of human natural antibodies binding to porcine endothelial cells[6–9], combined with the development of mammalian nuclear transfer and cloning technologies, permitted the generation of pigs lacking a functional α1,3Gal transferase (αGalT) gene and hence lacking the target of these natural antibodies, in the early 2000's[10–12]. This breakthrough, combined with advances in immunosuppression, permitted further prolongation of porcine organ xenograft survival in non-human primates for weeks to several months[13–19]. The αGalT KO was usually combined with one or more transgenes encoding a human complement regulatory protein in these studies. The newer immunosuppressive regimens, independent of the αGalT KO advance, also permitted porcine islet xenografts to survive for more than 6 months in non-human primates (NHPs) in this period[20–22]. These immunosuppressive regimens typically included costimulatory blockade and rapamycin. However, the T cell immune response to islet xenografts has not been fully suppressed by these multi-drug regimens, resulting in their eventual rejection. While encapsulation of xenogeneic islets has shown promise, permanent graft survival has not been achieved with this strategy alone[23,24]. Thus, tolerance is likely to be needed to overcome the powerful T cell-mediated immune responses to xenografts while preserving adequate recipient immune function.

The 2010's have seen further exciting advances, such as heterotopic cardiac xenograft survival for greater than a year in baboons when αGalT KO, human CD46 and thrombomodulin transgenic pig hearts were transplanted with a costimulation blockade-based immunosuppressive regimen[25]. However, antibody-mediated rejection developed quickly when the dosage of anti-CD40 antibody was reduced. Considerable optimism has been attached to the field of xenotransplantation in recent years, partly because of these advances and in large part due to advances in genetic engineering and gene editing technologies that are rapidly being applied to pigs used in NHP xenotransplantation studies, as we heard at this meeting. Indeed, one of the challenges now facing us is the need to dissect the role of each new genetic modification in improving xenograft survival, as each one will need to be justified if it is to be used in a clinically-approved source animal.

Some genetic engineering approaches are aimed at making xenogeneic organs invisible to the immune system or resistant to immune attack. There are a few limitations to this approach that need to be considered. One is that the absence of porcine MHC antigens would likely render such xenografts susceptible to infection, as T lymphocytes would have no way of recognizing cells that lack MHC molecules required to present foreign peptides. Indeed, when considering xenotransplantation we must think about the impact of infection or viral reactivation on the xenograft itself and how the immune system will respond to it. It has been clearly demonstrated that porcine cytomegalovirus (CMV) reactivation on

xenografts results in widespread immune activation and early graft loss[26] and the lack of adaptive immunity to the porcine CMV is likely to be a contributing factor to these dramatic effects of its reactivation. Expression of inhibitory surface markers by the xenograft or secretion of soluble immunosuppressive proteins might have similar consequences. Furthermore, the absence of class I MHC could make xenografts more susceptible to natural killer (NK) cell-mediated rejection, a problem that could potentially be overcome by transgenic HLA-E molecules[27,28]. A third limitation is that invisibility to direct immune attack would not obviate indirect recognition of xenoantigens and associated effector mechanisms, including those mediated by *de novo* antibody formation against the donor.

Tolerance induction has an important role to play in combination with these advances. The xenograft tolerance approaches that I have focused on in this lecture include mixed chimerism induction and porcine thymic transplantation. As is discussed below, mixed chimerism has the potential to tolerize T cells, B cells and NK cells, while thymic transplantation effectively tolerizes T cells and possibly B cells in non-human primates. The above advances in immunosuppression for the induction phase and the use of genetically modified source pigs (see below) can be used to promote successful tolerance induction, which obviates the requirement for long-term immunosuppression while allowing normal immune function, and hence resistance to infectious microorganisms, to recover.

Lessons from mixed chimerism induction in rodent and humanized mouse xenograft models

A major requirement for the use of hematopoietic cell transplantation (HCT) for the purpose of inducing organ graft tolerance (in contrast to its more common use to treat a life-threatening malignancy) is that the preparative regimen for engraftment of donor hematopoietic cells must be nonmyeloablative. In other words, recipient hematopoietic cells must not be fully ablated by the conditioning regimen, so a failure or loss of donor engraftment will not be associated with hematopoietic failure. A second requirement is that the regimen must have relatively low toxicity and be free of the risk of graft-vs-host disease (GVHD). Mild/moderate GVHD is associated with the clinical benefit of improved graft-vs-tumor effects when HCT is carried out in the context of malignant disease, but is an unacceptable complication to introduce to an organ graft recipient who does not have a malignancy. Almost 30 years ago our group developed a regimen that met these criteria, in which mice were conditioned with monoclonal antibodies to deplete T cells and NK cells and also received low-dose total body irradiation and local thymic irradiation prior to administration of T cell depleted rat bone marrow[29]. Depletion of both NK cells and γδ T cells[30] in addition to conventional αβ T cells was found to be essential to permit engraftment of rat marrow. These requirements distinguished xenogeneic from allogeneic marrow transplantation, in which depletion of αβ T cells was sufficient to assure reliable engraftment with otherwise similar conditioning. In the allogeneic setting with this regimen, the addition of NK cell depletion had only a minimal effect[31]. Of note, while donor chimerism levels reached a peak and remained stable over time in the allogeneic model[32], chimerism very slowly declined over time in the xenogeneic model, despite persistent T cell, B cell and NK cell tolerance[29,33–36]. This phenomenon was shown to be due to a

competitive advantage enjoyed by recipient hematopoietic cells over xenogeneic cells[37], likely reflecting species specificity or selectivity of hematopoietic cytokines and perhaps adhesion molecules (see below). The resistance to xenogeneic hematopoietic engraftment in immunodeficient mice becomes more severe as the species disparity increases, resulting in considerable difficulty in achieving porcine hematopoiesis[38]. This barrier was partly overcome by the introduction of porcine hematopoietic cytokines into the murine recipients[39]. Collectively, these observations suggest that genetic modifications of pigs that enhance the ability of their hematopoietic cells to function in a human marrow microenvironment will be important for optimization of the mixed chimerism approach to promote xenograft tolerance.

The mechanisms of T cell tolerance induction in the rat→mouse model were found to be similar to those involved in the allogeneic non-myeloablative bone marrow transplant model upon which it was based, with a major role for central deletion of newly-developing donor-reactive thymocytes[33], which correlated with the appearance of rat MHC class II+ antigen-presenting cells in the recipient thymus[40]. This tolerance was observed both in vitro and in vivo, with marked and specific prolongation of donor rat skin grafts[29].

We subsequently developed a robust humanized mouse model that exhibited spontaneous porcine islet and skin xenograft rejection by implanting human fetal thymus tissue and infusing human CD34+ cells intravenously to immunodeficient mice[41, 41a]. Using immunodeficient mice that were genetically engineered to express porcine hematopoietic cytokines[39], mixed xenogeneic chimerism was successfully induced when porcine and human hematopoietic cells were infused and human fetal thymic tissue was implanted. Specific tolerance of the human T cells to porcine donor antigens was demonstrated in association with the presence of porcine antigen-presenting cells (APCs) in the human thymus grafts[42].

In addition to T cell tolerance, our early studies demonstrated disappearance of pre-existing natural IgM antibodies against the rat donor following induction of mixed xenogeneic chimerism in mice[35,43–45]. Since passive transfer studies showed that these mouse Nabs could inhibit rat marrow engraftment[46], these observations were consistent with the possibility that mouse Nabs bound to the rat hematopoietic cells were adsorbed from the circulation and that the Nabs were responsible for the gradual loss of chimerism. However, the absence of measurable anti-rat Nabs persisted as the chimerism declined[34] and studies in immunodeficient mice demonstrated a similar decline in rat chimerism in the absence of natural antibodies[37]. These data suggested that tolerance of Nab-forming B cells may have developed and persisted despite the decline of rat chimerism, which likely reflected “out-competition” by the recipient (mouse) hematopoietic cells. Indeed, when αGalT-deficient mice, which resemble humans in producing Nab against αGal[47], became available, we used Elispot assays to demonstrate that mixed chimerism in both the wild-type to knockout mouse[48,49] and xenogeneic rat to αGal knockout mouse model[50] was associated with true tolerance of Nab-forming B cells to the donor. Although most of these anti-αGal Nabs were of the IgM class, pre-immunization of the αGalT knockout recipients with αGal+ rabbit erythrocytes induced IgG anti-αGal antibodies in these mice. While increased doses of bone marrow were required to overcome this additional anti-αGal antibody barrier to

engraftment[51], tolerance of anti-αGal antibody-forming cells was again achieved once the cells engrafted. In this rat→ αGalT knockout mouse model, induction of mixed chimerism protected primarily vascularized rat xenografts (hearts) from all forms of rejection, including hyperacute and acute vascular rejection, which occurred in conditioned control animals[50] in which natural xenoantibodies are increased due to loss of T cell regulation[52]. They were also protected from cell-mediated and chronic rejection[50]. Together, these encouraging studies demonstrate the potential of mixed chimerism to tolerize donor-reactive T cells robustly, also preventing induced antibody responses and tolerizing T cell-independent Nab-producing B cells. One advantage of this approach over genetic modification of pigs to eliminate targets of natural antibodies is that all specificities of Nab-forming B cells are tolerized by mixed chimerism, obviating the need to know what these specificities are and thus the need to progressively knock out more and more porcine genes as additional targets are identified. The latter approach poses both potential risks to the health of the porcine source animals as well as the risk that new specificities will be revealed as existing carbohydrate targets are removed from these pigs. Thus, mixed chimerism may be the optimal way to overcome the natural antibody barrier to xenografts.

With knowledge of the αGal ligand of these Nab-forming B cells, we were able to probe the recipient B cell repertoire for the presence of anti-αGal B cells at various stages in the tolerance process, permitting elucidation of the mechanisms of B cell tolerance in mixed chimeras. These studies revealed that initial, early tolerance was due to anergy of αGal-binding B cells, whereas the longer-term B cell tolerance was associated with deletion of these cells from the repertoire[49,50,53]. While the early anergy was reversible by transfer of anti-αGal-forming cells to a non-αGal-containing environment, the later deletional tolerance, as expected, could not be reversed by removal of B cells from the αGal-containing environment[53]. Further mechanistic studies of the early tolerance induction in this model revealed a role for complement receptors (CR1/2) expressed on non-hematopoietic cells in promoting both anti-αGal Nab formation following immunization and tolerance induction via mixed chimerism[54], possibly implicating follicular dendritic cells[55]. Unpublished studies (P. Bardwell, I. Shimizu, V. Levesque, H.-W. Li and M. Sykes, 2003–2012) revealed that complement itself, probably at the level of local cell-cell interactions, was required and that circulating IgM did not play a requisite role for this B cell tolerance. Of note, all of the same components required to tolerize αGal-producing B cells via mixed chimerism were also needed to maximize the anti-αGal response in immunized, non-tolerant mice. These observations suggest the hypothesis that complement fixation on the surface of anti-αGal surface Ig-bearing cells creates an immune complex that interacts with complement receptors on FDCs that normally activates anti-αGal-producing B cells. In the presence of mixed chimerism (*i.e.* with an αGal+ cell involved in the interaction), tolerization of the αGal-binding B cells occurs instead. If correct, this mechanism would be somewhat surprising, as our studies using αGal-binding fluorochrome conjugates to identify and sort anti-αGal-producing B cells revealed that these are largely B1b-like, but CD11b−, B cells in the spleen[56].

Precursors of these antibody-producing cells are more classical CD11b+ B1b cells in the peritoneal cavity, which can produce anti-αGal upon TLR stimulation and, following antigenic exposure *in vivo*, migrate to the spleen, where they become antibody-secreting

cells[56,57]. Although the existence of B1 B cell subset in humans is controversial[58–62], the use of αGal conjugates to identify and sort anti-αGal-producing cells in humans and baboons revealed several parallel phenotypic features to those in the mouse model, including a largely splenic location for IgM-secreting cells and expression of CD11b on many of them[63].

Importantly, recent studies in the pig to humanized mouse (H.W. Li and M. Sykes, unpublished data)[64] and pig to baboon model (H. Watanabe, K. Yamada et al, unpublished data) are consistent with the prediction that mixed chimerism can tolerize anti-pig xenoantibodies, suggesting that the immmunobiology of Nab production and tolerization will be translatable from the rat→mouse model to the large animal transplant arena.

As discussed above, NK cells pose a greater barrier to xenogeneic than to allogeneic hematopoietic cell engraftment. Once the xenogeneic hematopoietic cells engraft, however, the recipient NK cells are tolerized to the xenogeneic donor in the rat→mouse model, by a mechanism that results in global NK cell unresponsiveness[36]. This global NK cell unresponsiveness, which persists even in the presence of very low levels of rat chimerism, contrasts with the donor-specific NK cell unresponsiveness that is observed among recipient NK cells in mixed allogeneic chimeras[65]. These observations provide novel insights into the mechanism of tolerance of NK cells, for which a “licensing” model has been proposed to explain the presence of a self-binding inhibitory receptor on all functioning cytotoxic NK cells in mice and humans[66,67]. In the licensing model, developing NK cells do not mature functionally until they encounter an inhibitory self-ligand, ensuring that every functional NK cell has a receptor that inhibits killing of normal autologous cells. In an alternative model, developing or mature NK cells lose their cytolytic capability upon repeated encounter with cells that lack an inhibitory ligand. Our contrasting results regarding recipient NK cells in mixed xenogeneic vs allogeneic chimerism may be most easily interpreted as supporting the latter model as follows: Our observations (and those involving class I-deficient mixed chimeras[68]) reveal a “dominant” form of tolerance, whereby partial chimerism, even at low levels, makes the entire NK cell repertoire unresponsive to that donor. This implies that all cells (both donor and recipient) encountered by an NK cell under homeostatic conditions must express an inhibitory ligand in order for that NK cell to function normally. In contrast, a licensing mechanism would imply that recognition of an inhibitory ligand on either donor OR host cells would be sufficient to allow functional maturation of an NK cell, which would result in an NK cell repertoire in which each cell would not necessarily be unresponsive to both donor and host cells, but only to one or the other. NK cell inhibitory receptors are clonally distributed on individual NK cells and each NK cell has at least one inhibitory receptor recognizing a “self” MHC ligand[66,67]. We know that many inhibitory NK cell receptors in mice, mostly in the Ly49 family, cross-react on multiple allogeneic MHC haplotypes. Thus, only a subset of recipient NK cells in a mixed allogeneic chimera will fail to find an inhibitory MHC ligand on each recipient AND donor cell; consequently most NK cells will be functional and only those lacking an inhibitory ligand on both types of cells will be non-functional, resulting in specific tolerance to the donor and recipient, with otherwise normal function, as observed[65]. However, NK cell receptors, for the most part, have been found not to interact with xenogeneic MHC ligands[28,69–75]. Thus, the presence of even a small population of xenogeneic donor cells, as in rat→mouse mixed xenogeneic chimeras,

will result in a failure of the majority of recipient NK cells to function normally, resulting in global unresponsiveness, as we have observed[36]. Instead, the licensing model would permit those NK cells with an inhibitory ligand for "self" but not for xenogeneic ligands to function, in contrast to our actual observations.

We used the humanized mouse model described above, in which mixed xenogeneic (porcine and human) chimerism can be induced in pig cytokine transgenic immunodeficient mice, to assess the ability of pig chimerism to tolerize human NK cells. Since NK cells require IL-15 to develop and mouse IL-15 does not bind to the human IL-15 receptor, it was necessary to administer human cytokines to these mixed xenogeneic chimeras to induce human NK cell development. In such animals, we observed that, while human NK cells developing in non-chimeric mice were able to kill and produce cytokines in response to porcine lymphoblasts, those developing in mixed xenogeneic chimeras lacked cytolytic activity against porcine lymphoblasts. In some such animals, the lack of pig-specific killing was associated with a failure to kill the human class I MHC-deficient target K562 (*i.e.* global hyporesponsiveness), whereas in others the tolerance was donor-specific and associated with significant ability to lyse K562 targets[76]. These results are consistent with the observation that many human killer inhibitory receptors (KIRs) do not interact with porcine MHC molecules, whereas a few receptors do[77]. On balance, however, our data suggest that introduction of a human inhibitory ligand such as HLA-E, which binds to the NKG2A/CD94 molecule expressed on the vast majority of human NK cells, could be beneficial in assuring normal function of human NK cells in mixed xenogeneic chimeras. Moreover, given the prominent role of NK cells in resisting engraftment of xenogeneic hematopoietic cells[30], the transgenic expression of such a ligand on porcine hematopoietic cells could do much to enhance the achievement of mixed xenogeneic chimerism in human recipients.

Translating mixed chimerism to the large animal pig-to-primate model

The studies in pig-human chimeras in immunodeficient mice and in rat→mouse mixed chimeras that are summarized above indicate that induction of mixed xenogeneic chimerism has the potential to tolerize not only T cells, but also NK cells and Nab-producing B cells without requiring knowledge of their xenogeneic ligands. However, induction of mixed xenogeneic chimerism has been extremely challenging in pig-to-primate models, despite the extensive use of T and NK cell-depleting and antibody-adsorbing conditioning regimens[78–81]. Exploration of key adhesion interactions for hematopoiesis revealed largely effective interactions between porcine integrins and human ligands, suggesting that major limitations were not imposed by physiologic incompatibilities in hematopoietic cell homing and adhesion[82–86]. A longer-term competitive advantage enjoyed by host compared to xenogeneic hematopoietic cells, as discussed above in the context of rat to mouse xenotransplantation, would not explain the very rapid disappearance of high doses of infused pig hematopoietic cells. A more satisfying explanation for the rapid disappearance of porcine hematopoietic cells comes from observations in both the pig-to-mouse[87] and pig-to-baboon[88] models indicating that macrophages rapidly clear xenogeneic hematopoietic cells. Furthermore, antibody-independent complement activation also promotes clearance of xenogeneic cells[89]. The latter barrier may be overcome by the transgenic expression of human complement regulatory proteins in existing porcine source animals, whereas the

former obstacle has required additional genetic modifications of pigs. In particular, CD47 is an inhibitory ligand required for the inhibition of macrophage-mediated phagocytosis of hematopoietic cells[90]. The porcine CD47 molecule, however, does not transmit an inhibitory signal to human macrophages through the inhibitory receptor SIRPα, resulting in activation of human macrophages and engulfment of porcine hematopoietic cells[91,92]. Thus, introduction of a human CD47 transgene is likely to be needed to prevent the rapid destruction of porcine hematopoietic cells by human macrophages. Indeed, the introduction of a human CD47 molecule into the Sachs miniature swine line greatly prolonged the survival of porcine hematopoietic cells both in mice expressing a SIRPα allele that transmits an inhibitory signal from human CD47[93] and in non-human primates[94]. Prolonged porcine chimerism in baboons receiving hCD47 transgenic porcine hematopoietic cell transplantation was associated with remarkable prolongation of porcine skin xenograft survival[94]. In studies presented at this congress, polyclonal recipient regulatory T cells (Tregs) were shown to further prolong the survival of porcine xenogeneic skin grafts in recipients of hCD47 transgenic hematopoietic cells from the same donor 90 days earlier[95]. Thus, a human CD47 transgene is likely to play an important role in optimizing the survival, and hence the tolerance-inducing capacity, of porcine hematopoietic cell transplantation in humans. Indeed, marked prolongation of porcine hematopoietic cell chimerism has been observed in baboons receiving porcine bone marrow injected directly into their long bones (H.Watanabe, K. Yamada et al, unpublished data), building on the advance of intrabone injection, which prolongs porcine chimerism[96] likely in large part by evading the rapid rejection of xenogeneic cells by recipient reticuloendothelial macrophages. A surprising and unexpected effect of the human CD47 transgene in pigs may be a salutary effect on porcine lung xenograft survival in baboons[97].

Thymic transplantation for the induction of xenograft tolerance

In view of the early obstacles encountered to the induction of mixed xenogeneic (porcine) chimerism in non-human primate models as discussed above, we developed an alternative approach to achieving central T cell tolerance of highly disparate xenogeneic donors that involved transplantation of a porcine thymus to an immunocompetent, T cell-depleted and thymectomized recipient. These studies were initiated in mice, which demonstrated marked and specific unresponsiveness *in vitro* and prolongation of donor-specific skin graft survival[98,99]. The murine model permitted extensive studies of the mechanisms of tolerance and of immune function conferred by T cell reconstitution in a xenogeneic thymic graft. Intrathymic clonal deletion is a major mechanism tolerizing newly-developing thymocytes to the xenogeneic donor and the recipient[100,101]. Additional studies implicated Tregs developing in the porcine thymus graft in the suppression of residual mouse anti-pig responses[102,103]. Using T cell receptor (TCR) transgenic recipient mice of different MHC haplotypes and TCRs for which positively and negatively selecting murine MHC alleles had been identified previously, we were able to demonstrate that positive selection in a porcine thymus graft was mediated exclusively by the porcine thymic MHC, with no contribution from the murine hematopoietic cells, whereas negative selection was mediated by both the pig and the mouse MHC, consistent with the presence of class II MHC+ APCs from both species in the donor pig thymus grafts[100,104,105]. Remarkably, despite the lack of murine

MHC participation in positive selection and the complete MHC disparity of the porcine thymus and the murine recipient, these T cells were able to respond to immunization with protein antigens presented by murine MHC molecules and, most importantly to protect the mice from an opportunistic pathogen whose clearance was dependent on CD4+ T cells[106]. These results are interpreted as demonstrating that sufficient cross-reactivity for recognition of foreign antigens on recipient MHC can occur if a diverse T cell repertoire is selected in a xenogeneic thymic graft.

While mice whose T cells developed in a porcine thymus graft generally showed good health, about 10% of thymectomized, T cell-depleted immunocompetent mice receiving porcine thymic grafts eventually (after about 40 weeks) showed evidence of a multi-organ autoimmune disease mediated by murine CD4 T cells, despite negative selection of T cells recognizing murine antigens expressed on their own APCs[101]. This disease occurred more frequently in mice that congenitally lacked a thymus and therefore lacked preexisting Tregs[101]. Indeed, adoptive transfer studies revealed that the disease was due to both a failure to select Tregs that prevented autoimmunity and incomplete deletion of effector T cells that could cause disease[107]. To reconcile the observed deletion of T cells recognizing antigens presented by murine APCs with the multiorgan autoimmune disease, we hypothesize that autoimmunity reflects the inability of porcine thymic epithelial cells to produce mouse host-specific tissue-restricted antigens (TRAs), and hence to delete T cells with these specificities or to generate Tregs recognizing them, as occurs during normal thymic development[108–112].

The porcine thymic transplantation approach to tolerance has been extended to the humanized mouse model to provide proof-of-principle that human T cells can develop normally and are centrally tolerized to porcine xenoantigens in pig thymic grafts[113,114]. Both thymic and peripheral human T cells developing in a porcine thymus graft show specific unresponsiveness to the donor pig, with intact responses to third party pigs and allogeneic humans in mixed lymphocyte reactions (MLRs)[113,114]. These T cells also show unresponsiveness to the human hematopoietic stem cell (HSC) donor and the murine recipient in MLRs, reflecting the contribution of human donor APCs and murine APCs, both of which are detected in thymic xenografts[113,115], to negative selection. Importantly, donor-specific skin graft tolerance is observed for human T cells developing in a porcine thymus graft[114].

Based on results in the murine model, the thymic xenotransplantation approach to tolerance has been extended to the large animal pig-to-baboon species combination. Initial studies using porcine thymic fragments placed under the kidney capsule of the baboon demonstrated some T cell recovery, donor-specific hyporesponsiveness *in vitro* and prolongation of donor skin graft survival compared to controls. However, the amount of pig thymic tissue that was implanted and vascularized was quite limited[116]. In order to achieve more robust thymic function and, in view of the murine data cited above, expecting that donor-specific Tregs developing in a pig thymus would be needed to suppress pre-existing T cells not depleted by the conditioning regimen[102,103], subsequent studies utilized a primarily vascularized pig thymus, which had already shown efficacy in tolerance induction in an allogeneic pig kidney transplant model[117]. Thymi were transplanted either as part of a composite “thymokidney” graft prepared in the donor pig several months earlier by placing autologous thymic

fragments under the pig's kidney capsule or by direct vascular anastomosis of a pig thymic lobe in a baboon[16]. Both approaches led, for the first time, to long-term survival of GalT knockout pig kidneys in baboons[16,118]. Survival of animals receiving this treatment has been limited by thrombotic complications of anti-CD40L and by proteinuria due to a minimal change disease-like glomerulopathy, which can be avoided by using non-thrombogenic anti-CD40 and by administering rituximab and CTLA4Ig[119], respectively. Interestingly, work presented at this meeting suggests that transgenic expression of human CD47 on porcine glomeruli also appears to mitigate proteinuria due to this glomerulopathy[120]. We speculate that this additional, unexpected benefit of human CD47 expression on porcine source animals reflects a role for CD47-ligand interactions in maintaining glomerular function and integrity, underscoring the need for further studies on the biology of CD47 and its potential interaction with several different ligands in the context of xenotransplantation.

While baboons receiving porcine thymokidney grafts have died of unrelated causes before being completely removed from a low dose of MMF, they have shown evidence of de novo recipient (baboon) thymopoiesis in the porcine thymic graft[16], appearance of recent thymic emigrants in the periphery[121](H. Watanabe, K. Yamada et al, unpublished data) and donor-specific unresponsiveness in Elispot and MLR assays[121], as well as a decline in non-Gal natural antibodies (H.Watanabe, K.Yamada et al, unpublished data). While the latter may reflect absorption by the pig kidney, minimal IgM binding was detected on these xenografts, with no complement fixation or significant pathology. Thus, the results obtained with this model demonstrate the potential of composite thymus-kidney xenografts to induce tolerance in primates and are very encouraging with regard to the clinical applicability of this approach.

A few concerns about generating a human T cell repertoire in a xenogeneic porcine thymus have been touched upon above. These include the preferential recognition of microbial antigens on porcine MHC, which would be useful for protecting the graft but would not optimize protection against microbial pathogens infecting the host, as well as the failure to negatively select conventional T cells and positively select Tregs recognizing human TRAs. Indeed, studies in humanized mice have shown reduced responses to peptides presented by human APCs following immunization when the human T cells developed in a pig rather than a human thymus graft[114]. The approach we are using to overcome this problem involves creation of a "hybrid thymus", in which recipient thymic epithelial cells obtained either from thymectomy specimens or generated from stem cells are injected into the porcine thymic tissue. In studies presented at this IXA congress[122], we were able to successfully generate such hybrid thymi from post-natal thymus donors, and ongoing studies suggest that this approach indeed promotes tolerance to human TRAs among human T cells (M.Khosravi-Maharlooei, M.Sykes et al, unpublished data).

Several challenges remain in applying the vascularized thymic transplantation approach to xenograft tolerance induction in humans. One is that the need for host thymectomy adds another procedure to the preparation for xenotransplantation. However, since senescent baboons have not yet been used in our studies, the possibility that thymectomy may be unnecessary in older individuals, in whom the rate of thymopoiesis is very low, remains. Indeed, with the capacity of porcine thymic transplantation to generate human Tregs specific

for porcine xenoantigens, adequate suppression may be expected for the few xenoreactive T cells that emerge from a senescent human thymus over time and for those that escape depletion with the initial T cell-depleting conditioning regimen. For younger recipients with more robust thymic function, the combination of mixed chimerism induction and thymic xenotransplantation would avoid the need for thymectomy and could be ideal for several reasons. With durable mixed pig-human chimerism, both pig and human APCs would be present in the native human thymus and the porcine thymic xenograft, ensuring lifelong negative selection of thymocytes recognizing either pig or human antigens expressed on hematopoietic cells. Moreover, conventional T cells recognizing pig or human TRAs would be deleted in the relevant species' thymus and those escaping deletion due to development in the thymus of the opposite species would be adequately suppressed by TRA-specific Tregs developing in the other thymus. The mixed porcine chimerism would assure tolerance of natural antibodies recognizing unknown xenogeneic targets and NK cells would be tolerized as well, as is discussed above.

Conclusions

Thus, while the road to xenograft tolerance may be aptly described as having been long and winding thus far, the important proofs of principle that have been obtained both in humanized mouse models and in pig-to-baboon models of thymic and hematopoietic cell transplantation for tolerance induction encourage the pursuit of this important goal, which is likely to be critical for the widespread clinical success of xenotransplantation. Indeed, the ability of mixed chimerism to tolerize both adaptive and innate components of the immune response suggest a way of minimizing the number of genetic modifications of porcine source animals that will be needed to assure success. For example, while lung xenografts appear to be especially sensitive to early rejection in the presence of low levels of natural antibodies[97], the ability of mixed chimerism to tolerize Nab-producing B cells without knowing their specificity obviates the requirement to eliminate every possible carbohydrate target from porcine source animals. The human CD47 transgene appears to be particularly salutary, not only in having the predicted effect of prolonging porcine hematopoietic cell chimerism in primates, but also, somewhat unexpectedly, in improving the survival of porcine kidney and lung xenografts through mechanisms that remain to be dissected. Thus, porcine source animals lacking αGal and perhaps β4Gal[123,124] and expressing human CD47 as well as complement and hematopoietic cytokine receptors may be sufficient to permit the goal of long-term tolerance to be achieved. Data obtained in conventional pig-to-miniature pig transplantation[121] and in pig-to-primate xenotransplantation[125] suggest that the ideal source animal will be a miniature swine, as intrinsic growth properties of the graft seem likely to limit the success of transplantation from conventional pigs.

Xenotransplantation lends itself to tolerance induction more readily than allotransplantation from deceased human donors, as the ability to perform xenotransplantation electively permits the application of a tolerance protocol (e.g. mixed chimerism induction) in advance of the organ xenograft. It will thereby be possible to tolerize the immune system first, confirm that tolerance has been achieved and subsequently perform the organ transplant without immunosuppression. The availability of miniature swine that have been inbred for over 40 years[126] is a major asset for this purpose, as the HSC or thymic donor will tolerize

the recipient to the same porcine antigens as those expressed by the subsequent organ source animal. Our studies have shown that, while there may some degree of hyporesponviveness to the donor species overall (compared to allogeneic donors), complete and specific T cell tolerance is only achieved to xenogeneic animals of the same “strain” as that used to induce the tolerance[16,29,42,113,114,121]. Further advantages of using inbred animals in xenotransplantation include the consistency and standardization of source animals and the ability to combine genetic modifications at different loci without adding further genetic variability each time an animal is bred. Therefore, inbred miniature swine are uniquely suited to the promising approach of xenograft tolerance induction.

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Abbreviations

αGal	α1,3G
αGalT	α1,3Galactosyl transferase
APC	antigen-presenting cell
CMV	cytomegalovirus
GVHD	graft-versus-host disease
HAR	hyperacute rejection
HCT	hematopoietic cell transplantation
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IL-15	interleukin 15
MHC	major histocompatibility complex
NHP	non-human primate
NK cell	natural killer cells
TRA	tissue-restricted antigen

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