**TRANSPLANTATION OF XENOGENEIC ISLETS: ARE WE THERE YET?**

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**ABSTRACT**

Beta cell replacement therapy has been proposed as a novel therapy for the treatment of type 1 diabetes. The proof concept has been demonstrated with successful islet allotransplantation. Islet xenotransplantation has been proposed as an alternative, more reliable and infinite source of beta cells. The advantages of islet xenotransplantation are ability to transplant a well differentiated cell that is responsive to glucose and the potential for genetically modification which focuses the treatment on the donor rather than the recipient. The major hurdle remains overcoming the severe cellular rejection that affects xenografts. This review will focus on the major advances that have occurred with genetic modification and the successful therapeutic strategies that have been demonstrated in non-human primates. Novel approaches to overcome cell mediated rejection including biological agents that target selectively co-stimulation molecules, the development of local immunosuppression through genetic manipulation and encapsulation will be discussed. Overall there has been considerable progress in all these areas which eventually should lead to clinical trials.

**INTRODUCTION**

Pig insulin has been superseded by recombinant human insulin as replacement therapy for type 1 diabetes (T1D). Such therapy prevents acute keto-acidosis and associated fatalities. However, it exacts a heavy burden on lifestyle, does not alleviate all the "unwellness" of diabetic individuals and does not prevent serious long-term complications such as heart disease, renal failure, blindness and limb amputations. Insulin injections can also inadvertently result in hypoglycemic episodes with mild (e.g. blurred vision, tiredness) to extreme manifestations (coma and death). Even the advent of the insulin "pump" has not reduced severe hypoglycemic episodes (1). Pancreas or islet allotransplantation has been able to bypass these deleterious complications. However, pancreatic allograft transplantation requires major surgery and most patients only receive such when they also require a kidney

allograft. Islet allograft transplantation requires much less intervention, although long-term outcomes are not as good as other organ transplants. However, it has reduced hypoglycemic episodes and progression of long-term complications (2, 3). Both pancreas and islet allotransplantation suffer from a shortage of donor organs (indeed, <0.1% of T1D sufferers have had an islet transplant) and the need for continuous immunosuppression to prevent graft rejection. The large disparity between the number of organ donors and the numbers of recipients means that donations from human cadavers would never bridge the widening gap between numbers on the waiting list and those that would benefit from a transplant. Hence, there is strong rationale for a reproducible source of β-cell replacement such as islet xenotransplantation or stem cell transplants. For xenotransplantation the pig is the choice of species, as it is readily available, has a glucose physiology that is similar to humans and pig insulin has a long history of use in humans confirming its efficacy and predictability. It is also one of only a few large animal species whereby oocyte transgenesis and targeted disruption of genes have been achieved. This avenue to stable genetic modification means that for the first time it is possible to focus treatment strategies on the donor rather than the recipient by genetically tailoring pigs to overcome the hurdles associated with xenotransplantation as well as reduce the need for heavy immunosuppressive drug regimens.

This review will touch upon recent developments in improving graft survival, such as overcoming innate immune activation, which has been called the immediate blood mediated inflammatory reaction (IBMIR) and modulating acute cellular rejection. Immunoisolation, transfer of regulatory T cells and use of biologicals that specifically block T cell activation are just some of the strategies being entertained to overcome cellular rejection. Recent non-human primate studies are highlighted, as there is general consensus that pig islet tissue to non-human primates would be a preferable step before clinical trials. Most of the present data have been obtained in macaques and there is now much experience in the use of streptozotocin to induce diabetes in non-human primates(4). When compared to research in

solid organ xenotransplantation there is encouraging long-term functional data in pre-clinical models (summarized in Table 1) which suggests there has been substantial progress towards clinical trials in this area.

***Immediate Blood Mediated Inflammatory Reaction (IBMIR) as a cause of early islet destruction.***

Clinical islet transplantation exposes donor islets to the recipient's blood. An estimated 50% of islets are lost as a result of an innate immune-thrombotic response called IBMIR (5, 6). IBMIR is characterized by an initial activation of the coagulation and complement systems with rapid activation and binding of platelets and the recruitment and infiltration of leukocytes. This causes an entrapment of islets within thrombus and disruption and destruction of islet morphology (7, 8). The molecular events that initiate and link both the coagulation and inflammatory responses in IBMIR are poorly understood. TF a potent activator of the extrinsic pathway has been identified on islets and blocking TF with an inhibitory monoclonal antibody or inhibiting its expression prevents the response in vitro (9, 10). However, isolated islets also express collagen which is not normally exposed to blood and can promote thrombosis via the intrinsic pathway. Whilst thrombin is an important molecule in both the initiation and propagation phase of coagulation it is also a critical molecule for the recruitment of inflammatory cells. Thrombin promotes the activation of monocytes, neutrophils and platelets. Amongst other things it causes neutrophils to up regulate PSGL-1, the ligand for p-selectin, which in turn is up regulated on platelets. Although IBMIR has been described in islet allotransplantation it is likely to be a greater problem following xenotransplantation, as there are several incompatibilities between pig regulatory molecules and human thrombotic factors (11). In addition complement activation occurs early after transplantation, which is an alternate pathway for platelet activation, an essential step in clot formation(12). This cross talk between thrombosis and inflammation

leads to further amplification of the response. Once activated by thrombin, endothelial cells, monocytes and platelets all secrete soluble tissue factor, which in turn leads to greater thrombin production and an ongoing inflammatory response (13).

Other important initiators of IBMIR are preformed antibody and complement activation. Whilst adult islets express low levels of the oligosaccharide galactose α1-3 galactose (αGal), neonatal pig islet cell clusters have high levels of αGal expression (14). Humans and old world monkeys have high titres of anti-αGal antibodies that bind immediately to NICC following transplantation leading to complement activation via the classical pathway. However, in vitro studies in the absence of antibody showed that complement activation occurred when pig islets were exposed to human plasma, most likely via the alternate pathway. The activated complement products C3a and C5a lead to the further recruitment of neutrophils and monocytes and the C5b-9 complex leads to cell lysis (15).

There are three broad strategies for the prevention of IBMIR. These can be divided into treatment of the recipient, modulation of the islet and genetic modification. In clinical islet allotransplantation heparin has been used to prevent thrombosis. However, whether it is beneficial or improves outcomes has been difficult to prove. rhAPC has been shown to be of benefit in rodent models (16) and the addition of an anti-platelet agent been shown to be synergistic with rhAPC in an ex vivo model of human IBMIR (17). Other strategies shown to be of benefit are thrombin inhibitors such as megalotran (8) and complement inhibitors (18). The problem with these approaches is that they all involve treating the recipient with systemic therapy, which places them at risk of infection (from complement inhibition) or bleeding. Already bleeding is a significant complication and limits substantially the level of anti-coagulation given to patients (19, 20). An alternative strategy is to treat the islet, thereby limiting the

systemic treatment administered at the time of transplantation. Treatment options have focused on reduction of TF expression, reduction of their inflammatory state and protection from thrombosis. TF reduction can be obtained by using siRNA to suppress TF production and has been shown to reduce IBMIR in vitro (10) and nicotinamide has been used to pretreat mouse islets prior to transplantation and resulted in improved immediate islet survival (21). Another strategy is to coat islets with heparin (22). Not only does this have the potential to prevent thrombosis it also provides an anchor for VEGF-A which has been shown to promote re-endothelialisation in vitro. However all these strategies require substantial manipulation of the donor islets prior to transplantation. Hence genetically modifying islets to avoid innate immune attack has been an attractive option and shown to provide enhanced survival in NHP models [refs].

***Cell mechanisms of islet xenograft rejection.***

If the islet xenograft survives after IBMIR, it will be subjected to cellular xenograft rejection. T cell-mediated cellular rejection is strong and currently is the major impediment to clinical trails. In rodent models where this has been studied in detail, CD4+ T cells are the predominant cell type involved (23-27), with large numbers of activated CD4+ T cells infiltrating the rejecting pig islet xenografts (26, 27). The central role of CD4+ T cells in rejection of porcine islet xenografts has been confirmed by studies in SCID mice, where reconstitution with as few as $2x10^5$ CD4+ T cells was sufficient to induce rapid islet xenograft rejection of fetal pig pancreas grafts. Once activated CD4+ T cell-initiated activation and accumulation of macrophages and natural killer cells within the rejecting grafts, via an interferon-γ mediated mechanism (28). This central role for CD4+ T cells is likely to be true in humans. Humanized mouse models where porcine islet recipient immunodeficent mice are reconstituted with human PBMC, CD4+ T cells or even stem cells resulted in islet xenograft rejection within 2 to 4 weeks.

Pig islet xenograft rejection could be prevented by human T-cell depletion prior to transplantation, and islet xenografts harvested from T-cell-depleted humanized mice were functional and showed no cell infiltration. Collectively, these studies indicate that the pig islet xenograft rejection in humanized mice is largely T-cell-dependent (29-31). These rodent studies have been supported by studies in NHP where long term islet xenograft survival can be achieved by maintaining immunosuppression that is aimed predominantly at suppressing the T cell response (32, 33). The human T cell response to pig tissue is stronger that an allo-immune response because of the greater molecular incompatibility between the pig donor and the human recipient. Recognizing porcine antigens through both direct and indirect pathways can activate the human T-cell dependant xenoresponse. By evaluating MLR assays it has been shown that human T cells respond to pig-MHC antigens in a manner that is similar to their response to allogeneic-MHC antigens, with similar molecular interactions required for stimulator APC (direct pathway) or responder APC (indirect pathway). This human anti-pig xeno-response was directed toward porcine MHC class II antigens and involved an interaction pig and human CD4 accessory molecule (34, 35). However whilst the T cell-precursor frequency for direct pathway responses to pig APC was similar to that of allogeneic APC, the precursor frequency for the indirect response was far greater (36, 37) because of the greater molecular incompatibility between host and donor tissue (38, 39). The T cell mediated effector mechanisms involved in porcine islet xenograft destruction are extensive and include direct killing by T cells, as well as indirect T-cell-mediated mechanisms, including cytokine production (32, 40), recruitment and activation of other cytotoxic cells (such as macrophages and NK cells) (28, 41, 42), and providing help for B cells that produce xenoreactive antibodies (25, 39). There are qualitative as well as quantitative differences in the response. As well as the quantitative differences between the allo-immune and xeno-immune response (37, 42), there are important qualitative

differences. Rodent studies have shown that T-cell initiated xenograft rejection, was accompanied by a large accumulation of macrophages in the rejecting grafts (42, 43), and that CD4+ T cell-activated macrophages harvested from porcine islet recipient NOD-SCID mice with rejecting grafts were capable of both recognition and rejection of pancreatic islet xenografts when transferred (without T cells) to secondary NOD-SCID islet xenograft recipients (43). Because of the large molecular difference and a greater impact from IBMIR, innate immune activation has a greater impact on the T-cell initiated xenograft response. The end result is there is a greater requirement for systemic immunosuppression to prevent rejection, which currently makes it unsuitable for clinical application. To overcome this hurdle grafts must be protected from the immune response by a physical barrier such as islet encapsulation (44) or alternately they must be genetically modified to secrete local immunosuppression.

***Genetic modifications to promote survival***

A major advantage of xenotransplantation over allotransplantation is that it is possible to genetically modify the donor to promote engraftment and to protect or hide the xenograft from the immune response. The techniques for engineering the pig genome are becoming increasingly sophisticated and powerful. Recent advances include rapid targeted gene knockout using transcription activator-like effector nucleases (TALENs) (45) and efficient co-expression of multiple transgenes (46). Genetic modification has thus far been focused on attenuating IBMIR, innate immune cell activity and the T cell-mediated adaptive response.

*Anti-IBMIR strategies.* As described above IBMIR is characterised by activation of complement and coagulation, adherence of platelets, entrapment in clots, and

infiltration by neutrophils and monocytes (47). It is exacerbated in pig islet xenotransplantation by the binding of complement-fixing anti-αGal antibodies and compounded by molecular incompatibilities affecting the regulation of coagulation (48). Approaches to tackle IBMIR include deletion of the αGal xenoantigen and transgenic expression of human complement regulatory proteins (hCRPs) and anti-thrombotic/anti-inflammatory molecules.

Neonatal pig islets express significantly higher levels of αGal than adult pig islets (14). Not surprisingly, therefore, elimination of αGal by GalT gene knockout (GTKO) has a greater protective effect for neonatal than for adult pig islet xenografts. Neonatal GTKO xenografts showed improved engraftment and induced less intrahepatic inflammation in nonhuman primate recipients than wild type xenografts (49). In contrast, a small study in a similar model showed no survival benefit for adult GTKO versus wild type xenografts (50). The same study reported that transgenic expression of the hCRP CD46 on αGal-positive adult pig islet xenografts significantly prolonged survival (50). This appeared to be a post-IBMIR effect, suggesting potential synergy for the GTKO/hCRP combination. Transgenic expression of human regulators of thrombosis and inflammation such as CD39 and thrombomodulin has been achieved in pigs (51, 52). Although their efficacy against IBMIR in the pig-to-nonhuman primate model has not yet been reported, data from studies using CD39-transgenic mice are encouraging (53).

*Additional measures to control innate immunity.* There is evidence that human innate immune cells are hyper-reactive to pig cells (54). The mechanisms include failure of pig ligands to transmit signals to inhibitory ligands on human cells, in particular pig SLA I (the porcine equivalent of MHC class I) to NKG2A on human NK cells and pig CD47 to SIRPα on human macrophages (55). Human HLA-E transgenic pigs have been generated,

but expression was largely restricted to endothelium and staining of islets was negative (56). Expression of human CD47 protects porcine cells from human macrophages (57) but hCD47 transgenic pigs have not yet been reported.

*Blunting the T cell response.* As described elsewhere in this review, local immunosuppression is an approach in which the graft is engineered to secrete antibodies into the local environment to deplete T cells and/or block their co-stimulation. Transgenic pigs with islet-specific expression of LEA29Y (a high-affinity variant of CTLA4Ig) have been produced, and their islets have been shown to be more resistant to rejection than wild type islets in a humanized mouse model (58). An anti-CD2 transgene has also produced promising results in mouse models (59).

**Immunosuppression to overcome islet xenograft rejection.**

With successful control of IBMIR by genetic modification, T-cell rejection remains the biggest immunological hurdle. A clinically acceptable regimen against xenoresponses has not been attained. Drugs like tacrolimus, mycophenolate mofetil and rapamycin have been used successfully in islet allotransplantation, but their long-term off-target effects remain problematic. The latter is assumed to be much reduced with the use of biologicals. Anti-CD154 mAb has shown good success in non-human primates (50) but the thrombo-embolic complications associated precludes it from clinical use. Whether other costimulation/adhesion blockade (LEA29Y, LFA3Ig, anti-ICAM, a blocking anti-CD40(60, 61)) or anti-T-cell Ab (anti-CD25, anti-CD2) would be as effective are likely to be tested soon. New strategies are continually being tested experimentally. For example, reduced survival of immune cells by Bcl-2 antagonists have shown efficacy in prolonging islet allografts in mice(62). Another

example are drugs that target lymphocyte migration (beyond the bradycardia-prone FTY-720) (63).

Although the advent of immunosuppressive drugs that are not myelosuppressive have transformed the allotransplantation landscape, their effects are systemic. Hence susceptibility to cancer and serious infections is increased(64). In addition, some drugs (e.g. tacrolimus) are toxic to islets. To avoid these off-target effects, genetic modification of the graft to secrete immunosuppressive factors in situ would seem advantageous. Indeed, this has been achieved in a huSCID model using pig NICCs transgenic for LEA29Y (58) or transduced with anti-CD2 genes (65); the latter also showing that depletion of human T cells were localized to the graft site. There is emerging evidence that the islet (graft) site (beyond the local lymph node) may be a critical target. For example, expansion of T cells in islets would seem important during autoimmune insulitis (4) and CTLA4Ig-producing islet allografts protected themselves but not control grafts at the opposite pole of the same kidney (66). The expectation is that pig islets secreting immunosuppressive agents locally will avoid systemic side effects and allow systemic immunosuppressive protocols that are safe and suitable for clinical application.

Other strategies have been proposed to modulate the immune response and hence reduce the requirement for immunosuppression. Analogous to transfer of Treg cells (except possibly requiring less cells), co-transplantation of Sertoli cells, tolerogenic dendritic cells and mesenchymal stem cells have been reported with varying degrees of success and their mechanism of action remains unclear(67-69). An alternative strategy is immunoisolation. Although not exactly immunosuppression, immunoisolation of islets within capsules enveloped in semi-permeable membranes (so immunocytes cannot enter) or microbeads can reduce the level of immune attack. Alginate-encapsulated pig islets reversed diabetes for six months without immunosuppression in cynomolgus monkeys (70). Also, Living Cell

Technologies in New Zealand have established a biocertified designated pathogen free pig facility for transplanting pig tissues into humans and have generated encapsulated islets under GMP conditions. The quality control of islet viability and islet function has not been reported in detail.

**CONCLUSION.**

Over the past five years there has been a consistent improvement in outcomes of islet xenotransplantation in non-human primate models. Both adult and neonatal tissue have been shown to normalise blood glucose control over months. What was surprising was that this was achieved using islets that were unmodified [32, 33]. However immunosuppression protocols with anti-CD154 antibodies as their foundation were required and this will not be allowed for clinical trials. Recently islets lacking αGal and or expressing human complement regulatory proteins have been tried resulting in better outcomes and a reduction in immunosuppression. Using NICC from pigs expressing hCD46 and using an immunosuppressive protocol that included anti-CD154 blockade graft survival of 3 to 12 months was seen whereas wild type or αGal KO islets survived a maximum of 46 days (50). Using NICC from αGal KO pigs resulted in improved rates of normoglycemia, less transaminitis and better graft function in rhesus macaques (49). In vitro studies confirmed less antibody binding and complement activation suggesting that αGal KO NICC had better survival from IBMIR. Recently anti-CD154mAb was replaced with an anti-CD40mAb with good medium term graft survival (71). Although the results were not as robust it does suggest that a clinically acceptable clinical immunosuppressive protocol will be achievable However if islet xenotransplantation is be a viable alternative to insulin pump therapy the systemic immunosuppression burden needs to be reduced further. Hence, several research

groups are developing pigs whose islets secrete immuno-modulatory molecules and other groups are developing islet encapsulation strategies to protect islets from immune attack. Islet sequestered into an alginate sheet has been able to reverse diabetes for up to 6 months in non-human primates without immunosuppression (70). What is required to move this toward clinical trials is a successful combination of genetic modification to avoid the innate immune response and immunoisolation or encapsulation to reduce the requirement for immunosuppression. In isolation, each of these strategies have been shown to lead to a well functioning graft in an appropriate pre-clinical model albeit for a limited period of time. It is anticipated that the appropriate combination of these strategies will lead to a clinically viable therapy that is both effective and safe.

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Table 1. Pancreatic islet xenograft survival in pre-clinical models.

| **Graft Recipient** | **Donor Islet & Genetic Modification** | **Immunosuppression** | **Graft Survival** | **Ref** |
|---|---|---|---|---|
| cynomolgus macaques | Adult islets<br>Nil modifications | Basiliximab, FTY720, everolimus, anti-CD154 mAb, leflunomide | 68-158 days | 32 |
| rhesus macaques | NICC<br>Nil modifications | Basiliximab, belatacept, anti-CD154mAb, sirolimus | >140 days | 33 |
| Cynomolgus monkeys | Adult islets<br>CD46 Tg pigs | MMF, ATG, anti-CD154mab, asprin | 87-396 days | 50 |
| rhesus macaques | NICC<br>Gal-KO | Anti-CD154mAb, anti-LFA-1, MMF, belatacept | 50-249 days | 49 |
| rhesus macaques | NICC<br>Nil modifications | Anti-CD40mAb<br>Belatacept, sirolimus | 59 days (median) | 71 |
| cynomolgus monkeys | Adult Islets<br>Nil modifications | Macro-encapsulation, no immunosuppression | 140 – 196 days | 70 |