Co-stimulatory blockade in xenotransplantation

Pig embryonic pancreatic tissue as a source for transplantation in diabetes: transient treatment with anti-LFA1, anti-CD48 and FTY720 enables long term graft maintenance in mice with only mild ongoing immunosuppression

Running head of the title: Co-stimulatory blockade in xenotransplantation

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Submitted 26 January 2009 and 15 April 2009 accepted.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Objective: Defining an optimal co-stimulatory blockade based immune suppression protocol enabling engraftment and functional development of E42 pig embryonic pancreatic tissue in mice.

Research Design and Methods: Considering that anti-CD40L was found to be thrombotic in humans we sought to test alternative co-stimulatory blockade agents already in clinical use, including CTLA4-Ig, anti-LFA1 and anti-CD48. These agents were tested in conjunction with T cell debulking by anti-CD4 and anti-CD8 antibodies, or with conventional immunosuppressive drugs. Engraftment and functional development of E42 pig pancreatic tissue was monitored by immunohistology and by measuring pig insulin blood levels.

Results: Fetal pig pancreatic tissue harvested at E42, or even as early as at E28, was fiercely rejected in C57BL/6 mice and in Lewis rats. A novel immune suppression comprised of anti-LFA1, anti-CD48, and FTY720 afforded optimal growth and functional development. Cessation of treatment with anti-LFA1 and anti-CD48 at 3 months post transplant did not lead to graft rejection, and graft maintenance could be achieved for more than 8 months with twice weekly low dose FTY720 treatment. These grafts exhibited normal morphology and were functional, as revealed by the high pig insulin blood levels in the transplanted mice, and by the ability of the recipients to resist alloxan induced diabetes.

Conclusions: This novel protocol, comprised of agents that simulate those approved for clinical use, offer an attractive approach for embryonic xenogeneic transplantation. Further studies in non-human primates (NHP) are warranted.
The potential use of embryonic tissues as a novel source for transplantation, which might be less immunogenic transplantable tissue, has been advocated over the years. During the past decade, several studies have suggested that the relative reduced expression of MHC molecules or adhesion molecules, as well as the lack of antigen presenting cells or endothelial cells in embryonic tissues, are likely associated with reduced immunogenicity (1-5). Furthermore, it has been more recently argued that early embryonic porcine precursor tissues could completely evade the immune system upon implantation into recipient rats or non-human primates (NHP) (6-9). We previously attempted to define an optimal window for transplantation of pig embryonic pancreatic tissue, based on the risk of teratoma, growth potential and immunogenicity (10; 11). Assessment of the first two parameters was performed following implantation of embryonic tissues harvested at different gestational time points under the renal capsule of xenogenic NOD-SCID mice that lack a functional adaptive immune system. To evaluate the relative immunogenicity of the different tissues, the recipient mice were infused with human peripheral blood mononuclear cells (PBMC) and homing and destruction of the embryonic tissues by human T cells or macrophages was analyzed. Based on this study, pig embryonic pancreatic tissue harvested at embryonic gestational age 42 (E42) was selected as the tissue of choice for transplantation. This choice was based, in particular, on the marked growth potential exhibited by E42 tissue, compared to E28 tissue, while no significant difference in their immunogenicity could be detected. Nevertheless, all early embryonic tissues, including tissue harvested at E28, were fiercely rejected when transplanted into immune competent mice in the complete absence of some form of immune suppression.

While our findings were in sharp contrast to the studies of Hammerman et al. (6; 9), who showed engraftment and normalization of glucose levels in rats transplanted with E28-29 pig pancreas, it is possible that this discrepancy was due to a difference between rats (used in Hammerman study), and mice (used in our study) in the strength of their rejection response. Therefore, in the present study, we further tested the potential of E28 pig tissue to evade the immune system in rats. As we previously reported in the mouse model, we observed fierce rejection of the implanted tissue, similar to that exhibited upon transplantation of E42 pancreas. These findings suggest that implantation of embryonic pig pancreas from any gestational stage will likely require some form of immune suppression.

Previous transplantation data suggest that co-stimulatory blockade does not interfere with pancreatic function. Unlike some of the conventional immunosuppressive drugs, such as Rapamycin, co-stimulatory blockade seems preferable for porcine embryonic pancreas transplantation. One approach, recently demonstrated in the NHP model and also in our previous mouse study, has demonstrated the impressive role of a protocol combining CTLA4-Ig and anti-CD40L (11-13). However, the recent observation that anti-CD40L mAb treatment is often associated with lethal thrombotic complications (14), suggests that the use of other co-stimulatory agents already tested in clinical trials may be preferable. In the present study, we indeed demonstrate that combining anti-LFA1 and anti-CD48 could markedly enhance engraftment and development of E42 pancreatic tissue in immune competent mice.

**RESEARCH DESIGN AND METHODS:**
**Animals:** Animals were maintained under conditions approved by the Institutional Animal Care and Use Committee at the Weizmann Institute of Science. NOD-SCID and C57BL mice, and nude and Lewis rats aged 8–12 wk (Weizmann Institute Animal Breeding Center, Rehovot, Israel) were used as hosts for the transplantation studies.

**Porcine Embryonic Pancreatic Tissue:** Pig embryos were obtained from the Lahav Institute of Animal Research (Kibbutz Lahav, Israel) as previously described (10; 11). Cold ischemia time until transplantation was routinely less than 2h. The study protocol was approved by the ethics committees at both the Weizmann Institute of Science and the Lahav Institute of Animal Research.

**Transplantation Procedure:** Implantation under the kidney capsule of mice. Implantation was performed as previously described (10; 11).

Implantation intra omentum of rats. Implantation was performed under general anesthesia (ketamine 60mg/kg and xylazine 7 mg/kg, i.p). Host omentum was exposed through a midline abdominal incision and 2 pancreata were deposited into sutured omental pockets.

**Induction of Diabetes:** Diabetes was induced by alloxan as previously described (11).

**Immune Suppression Protocols.** The immune-suppressive protocols included: T cell debulking with anti-CD4 and anti-CD8 antibodies (hybridomas GK1.5 and 53.6.72, BioExpress, West Lebanon, NH 03784, USA) administered intraperitoneally, on day -3 at a dosage of 300µg/mouse. The co-stimulatory blockade agents, 200µg/mouse mouse CTLA4-Ig fusion protein (lot number 20204, Chimerigen Laboratories), 250µg/mouse anti-CD48 (hybridoma HM48-1, provided by Dr. Hideo Yagita) and anti-LFA1 (hybridoma FD441.8, Bioexpress) antibodies were given intraperitoneally on days 0, 2, 4, and 6, and a single injection repeated bi-weekly until 3 months posttransplant. FTY720 was administered p.o daily from day -2 until day +3, and then bi-weekly at a dosage of 5mg/kg. Alternatively, 1.5mg/kg Rapamycin (Sirolimus, Wyeth Europa Ltd. UK) s.c, 1mg/kg FK506 (Prograf, Fujisawa Ltd. Ireland) s.c, or 40mg/kg mycophenolate mofetil [(MMF), CellCept, Roche Ltd. Germany] p.o, was administered daily until sacrifice. All drugs were administered according to the manufacturer instructions using doses previously shown to be effective in mice (15-17).

**Evaluation of Transplant Growth.** Grafted animals were sacrificed at 4-6 weeks after transplantation. Kidneys bearing the grafts were removed and fixed in 4% paraformaldehyde. Long (L) and short (W) axes of the grafts were measured, and transplant size was calculated by multiplying L×W.

**Histology and Immunohistochemistry:** Tissues were obtained from transplanted animals. Fixation, sectioning and staining were performed as in previous studies (10; 11; 18; 19). The following first antibodies were applied: guinea pig anti-rabbit insulin (Dako, Denmark), polyclonal rabbit anti-human CD3 (Dako), rat anti-mouse F4/80 antigen (Serotec, UK), mouse anti rat ED1 (Serotec), rabbit anti-human glucagon (Dako), mouse anti-human cytokeratin 20 (clone Ks 20.8; Dako), rabbit anti-cytokeratin (wide spectrum screening, Dako), monoclonal mouse anti-human cytokeratin, clone MNF116 (broad-spectrum cytokeratin) (Dako), rat anti mouse-CD31 (BD Pharmingen, UK), FITC conjugated anti-porcine CD31 (Serotec). The following secondary antibodies were used (purchased from Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, United States): Texas Red anti-mouse, Texas Red or Cy3 anti-rat, Texas Red or Cy2 anti-rabbit, biotinylated anti–guinea pig following
streptavidin aminomethylcoumarin, FITC anti-mouse IgM and Cy2 anti-mouse IgG.

ELISA Measurements of Pig Insulin: The porcine/human insulin kit (K6219, Dako), in which the primary pig anti-insulin antibody does not cross-react with mouse insulin, was used to follow pig insulin levels in the serum of transplanted mice according to the manufacturer's instructions.

Statistical Analysis: Differences between groups were evaluated by the Student's t-test. Data are expressed as mean ± SD and were considered statistically significant if p-values were ≤ 0.05.

RESULTS

Implantation of E28 versus E42 pig pancreatic tissue in immune competent mice and rats in the absence of immune suppression. To determine whether early embryonic pig pancreatic tissues can evade the immune system when transplanted into rodents, E28 and E42 pig tissues were implanted under the renal capsule in different strains of mice and rats. The enhanced growth potential of E42 pig pancreatic tissue, compared to that exhibited by E28 tissue, was demonstrated upon implantation in NOD-SCID mice by insulin secretion, by implant size and by histological documentation of ducts and islets (Fig.1a).

However, when transplanted under the kidney capsule of C57BL immune-competent mice without any immune-suppression, all early embryonic tissues tested, including tissue harvested at E28 and E42, were rejected. Thus, 4 weeks after transplantation, only fibrosis and massive infiltration in the site of implantation in the C57BL mice could be detected (6 out of 6 recipients in each group) (Fig.1b).

Considering that our findings in the mouse model were in sharp contrast to the results of Hammerman et al., who suggested that E28 pancreas can evade the immune response in rats (6; 9), we attempted to repeat these transplantation experiments in rats, using the same strain of recipients, namely immune-competent Lewis rats, and the same site of implantation, namely the omentum. As a control for the growth capacity of the implanted tissue we used immune deficient ‘Nude’ rats.

As can be seen in Fig.1c, following transplantation of two E28 pancreata into the omentum of Nude rats, normal growth and development was attained with grafts containing pancreatic elements without leukocyte infiltration in the pancreas parenchyma. Thus, at all time points tested, pig pancreatic epithelial cells, stained specifically by anti-pig cytokeratin 20, were present in the growing grafts (Fig.2a). As previously demonstrated in the mouse model, within 5-15 days post-transplant, most of the pancreatic tissue included pancreatic ducts. Within 2 to 5 months after transplantation, the grafts were composed mostly of endocrine pancreatic elements, and in 7 out of 8 Nude rats, insulin and glucagon producing cells arranged in islet structures or scattered as single cells in adipose tissue were clearly visible (Fig.2c).

In sharp contrast, rapid rejection of the E28 pancreas was documented following implantation into immune competent Lewis rats. Histological examination of the grafts at all time points, between 5 days and 5 months post transplant, revealed no pancreatic elements at the site of implantation, similar to our observations in the mouse model (Fig.1). Starting 5 days after transplantation, the grafts were heavily infiltrated, and long term follow-up revealed large areas of fibrosis and necrosis. Specific staining for CD3+ lymphocytes and macrophages, 2 weeks after transplantation, revealed massive infiltration of both cell types in the omentum of the transplanted Lewis rats (Fig.2e,f). Moreover, there were no cells stained for cytokeratin 20, nor could any insulin or glucagon producing
cells be detected in any of the rats transplanted (0 out of 10) (Fig.2b,d).

Accordingly, during the entire follow-up period low, but significant, blood levels of pig insulin were detected by specific ELISA in the Nude recipients, but not in the immune competent recipients. Thus, 3 months after transplantation, the blood level of pig insulin in the Nude rats was 1.048±0.887 µIU/ml (n=6), whereas in all the six Lewis rats tested it was below the detection threshold (less than 0.5 µIU/ml).

Although substantial pig insulin blood levels were recorded in the Nude rat recipients, they were significantly lower compared to those exhibited in SCID mice (Fig.1a). This could be caused in part by the different implantation site used for transplantation, namely omentum Vs renal capsule, respectively.

**Engraftment and functional growth in immune competent mice following implantation of E42 pig pancreatic tissue in conjunction with immune suppression.**

Considering that E42 embryonic pig tissue exhibits a more robust growth potential, and that E28 tissue, similarly to E42 tissue, is vigorously rejected in immune competent mice or rats, further studies defining optimal minimally toxic immune suppression protocols were warranted. Very recently, we demonstrated the curative potential of the E42 tissue in the NHP model, demonstrating its ability to induce complete insulin independence in STZ treated animals (Hecht et al., submitted). To demonstrate this proof of concept, we used a rather intensive immune-suppression protocol based on conditioning with a short treatment with ATG and Rituxan, followed by maintenance with RAD (a derivative of Rapamycin), CTLA4-Ig and FTY720. Clearly, while this protocol demonstrates the proof of concept for the curative potential of E42 pig pancreatic tissue, further reduction of its toxicity, if possible, would bring us closer to considering such transplants in human diabetic patients. One potential approach to reduce the toxicity of the protocol could entail replacing RAD and FTY720 with co-stimulatory blockade agents.

Indeed, the effectiveness of anti-CD40L has been recently demonstrated in the context of pig islet transplantation in the NHP model (12; 13). However, in initial clinical attempts in renal transplantation this agent was found to be associated with lethal thrombotic complications (14). Thus, we searched for alternative agents that have been given to patients without such toxicities including anti-LFA1 and anti-CD48, which are already used safely in patients with psoriasis (20; 21). These antibodies were tested in combination with CTLA4-Ig, in the presence or absence of T cell debulking mAbs (anti-CD4 and anti-CD8) or FTY720.

As can be seen in Table 1a, a protocol combining all these agents led to 100% (8/8) engraftment of E42 pancreas in C57BL mice. Histological examination of the grafts in the first days post transplantation revealed significant differences between untreated and treated mice (Fig.3). Thus, in the absence of any immune suppression, CD3+ lymphocytes heavily infiltrated the grafts 3 days after transplantation, leading to complete rejection by 2 weeks post transplantation, at which time only massive CD3+ lymphocyte and macrophage infiltrates were evident and no insulin or cytokeratin expressing cells could be detected.

In contrast, mice that were treated with the maximum immune suppression protocol of T cell debulking, FTY720, CTLA4-Ig, anti-LFA1 and anti-CD48, exhibited at 2 weeks post transplantation, large pancreatic grafts with insulin and cytokeratin expressing cells, and with minimal infiltration of CD3+ lymphocytes or macrophages.

Moreover, 2 months after E42 pancreas transplantation in mice treated with the maximal protocol, the grafts contained all
pancreatic elements and were functional, as indicated by post transplant blood levels of pig insulin (15.3 ± 14.2 µIU/ml) similar to the level exhibited in NOD-SCID control mice (9.7 ± 11 µIU/ml) (n=16).

Interestingly, CTLA4-Ig or T cell debulking could be removed from the maximal immune suppression protocol without loss of engraftment or graft functionality (5/5 and 7/7 engrafted respectively), while FTY720 was found to be essential to the engraftment sustaining effect of CTLA4-Ig, anti-LFA1 and anti-CD48 (1/7 engrafted). Based on these studies, a novel reduced intensity protocol associated with 100% graft acceptance and with functional grafts, has been defined. This protocol comprises anti-CD48, anti-LFA1 and FTY720 (4/4 engrafted). Replacement of FTY720 with CTLA4-Ig in this protocol led to marked rejection (0/6 engrafted).

Mice implanted under immune suppression with anti-CD48, anti-LFA1 and FTY720 exhibited robust implants at 2 months post transplant. Thus, as can be seen in Fig.4, large islets with insulin and glucagon secreting cells were clearly detected by immunohistological staining (Fig.4c). All the blood vessels in the pig pancreatic implant were of mouse origin, without evidence of pig endothelial cells (Fig.4d). The grafts exhibited minimal CD3⁺ lymphocytes and macrophage infiltration in the periphery (Fig4.a,b); however, the stained cells did not penetrate the pancreatic parenchyma. Moreover, we could not detect significant levels of IgG and IgM antibody deposits inside the pancreatic epithelium, despite minimal presence of bound immunoglobulin in the surrounding tissue, which was lower compared to that exhibited in the host kidney glomeruli which served as a positive control (Fig.4e).

Further analysis of the relative role of anti-LFA1 and anti-CD48 showed that both are critical for achieving 100% success rate. As can be seen in Table 1b, addition of anti-CD48 alone to FTY720, similarly to CTLA4-Ig along with FTY720, could not prevent rejection, and 8-10 weeks after transplantation all the grafts were completely rejected. Interestingly, the use of FTY720 with anti-LFA1 alone led to 50% engraftment. Thus, it seems that anti-LFA1 is more effective than anti CD48 or CTLA4-Ig when added to FTY720. Furthermore, in each of the FTY720 based protocols, addition of CTLA4-Ig to either anti-LFA1 (3/4 engrafted) or anti-CD48 (3/4 engrafted) appeared to be less effective compared to the combined use of anti-LFA1 and anti-CD48 (11/11 engrafted). The advantage of the anti-LFA1 and anti-CD48 combination is statistically significant as reflected by the pig insulin blood levels in the serum of transplanted mice 10 weeks after transplantation (Fig.5): 37.1 ± 34.8 µIU/ml, compared to 5.5 ± 5.7 µIU/ml (p<0.05) and 6.9 ± 6.8 µIU/ml (p<0.05) found upon addition of CTLA4-Ig to anti-LFA1 or to anti-CD48, respectively.

The strikingly high levels of porcine insulin attained in the serum of implanted mice can likely be attributed to the molecular difference between pig and mouse insulin, associated with a reduced affinity of pig insulin to mouse insulin receptor. Thus, as we previously demonstrated when defining optimal gestation time window for transplantation (11), the relatively high pig insulin blood levels in transplanted mice is not associated with hypoglycemia or with an abnormal weight increase. Accordingly, correction of hyperglycemia in two different models of diabetes, induced by alloxan or by streptozotocin, requires higher pig insulin levels than those effective in humans (11). Interestingly, in line with the closer homology of pig and primate insulin (only one amino acid difference), ongoing experiments in the non-human primate model show that correction of hyperglycemia is attained by pig insulin blood levels more similar to those found in normal humans (around 10µIU/ml).
Since FTY720 is not approved for use in clinical transplantation, it was of interest to test whether it can be replaced with other conventional immunosuppressive drugs. As can be seen in Table 1c, neither FK506 (1 mg/kg), Rapamycin (1.5 mg/kg), nor MMF (40 mg/kg), administered on a daily basis (using doses shown by other studies to be effective in mice (15-17)) could substitute FTY720 and prevent rejection in conjunction with co-stimulatory blockade.

Cessation of immune suppression:
The immune-suppressive protocol of choice comprising anti-LFA1, anti-CD48 and FTY720 was further tested for graft acceptance and insulin levels during a follow-up period of more than 6 months, in the presence or absence of T cell debulking, prior to transplantation.

As can be seen in Fig.6a, similar and significant blood levels of pig insulin were attained during the entire follow-up period, in the presence or absence of induction with T cell debulking prior to transplantation. Thus, induction with anti-CD4 and anti-CD8 antibodies is not required. Furthermore, in these experiments, anti-LFA1 and anti-CD48 treatment was stopped 3 months after transplantation, and mice were maintained on a twice weekly dose of FTY720 alone. These mice remained positive for pig insulin for more than 8 months post transplantation.

In some mice (n=9), FTY720 treatment was also stopped at 5 months post transplant (Fig.6b). Four weeks after FTY720 cessation, pig insulin levels remained stable (40.1 ± 54 µIU/ml before withdrawal of FTY720 versus 38.9 ± 53.8 µIU/ml 4 weeks after withdrawal), although CD4⁺ and CD8⁺ cell levels in the peripheral blood were elevated from undetectable levels to 9.7 ± 6% and 10.6 ± 5.7% of CD4⁺ and CD8⁺ cells, respectively. However, after 2 additional weeks (i.e. 6 weeks after FTY720 discontinuation), pig insulin blood levels were drastically reduced (3.7 ± 6.8µIU/ml), and reached undetectable levels in seven of the nine mice tested.

A detailed analysis of the implant functionality in response to glucose challenge and its ability to maintain normoglycemia in different diabetes models has been described recently (11). In the present study the immune suppression of choice was selected based largely on histological evaluation and pig insulin blood levels which we found previously to be correlated with graft rejection or acceptance. However, the curative capacity of the pig embryonic implants, maintained long term under just a biweekly treatment with FTY720, was also verified in the two remaining mice by exposure to alloxan treatment at 6 months post transplant (alloxan selectively destroys rodent, but not human or porcine beta cells (22)). Moreover, the role of the implant in maintaining normoglycemia following alloxan treatment is further proven by removal of the implant which abruptly leads to marked elevation of glucose levels.

As can be seen in Fig.7, the mice indeed maintained normal glucose levels, in sharp contrast to control mice that did not receive a transplant. Removal of the implant by nephrectomy of the kidney harboring the pig implant was associated with loss of normoglycemia, confirming that maintenance of glucose levels following alloxan treatment was mediated by the implanted pig embryonic pancreatic tissue.

DISCUSSION

In the rat transplantation model, our data extends our previous observation in the mouse model, that embryonic pig pancreatic tissue harvested at different gestational time points, including E28 and E42, are fiercely rejected upon transplantation into immune competent recipients. Considering that E42 tissue exhibits more robust growth potential, the new data presented here further support the suggestion that E42 pancreatic pig tissue...
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is best suited for transplantation in diabetic recipients, at least in this rodent xenotransplantation model. Our results are in contrast to the previous suggestion of Rogers and Hammerman that E28 pancreatic tissue can evade the immune system in Lewis rats (6; 9). Our failure to find in these rats any detectable trace of pig pancreatic tissue cannot be due to growth deficiencies, as the same E28 tissue exhibited growth and development in immune deficient Nude rats.

This discrepancy between our results and those of Hammerman et al. are intriguing. However, it should be noted that in the latter study pig insulin blood levels were not monitored to establish the role of the pig implant in attaining normoglycemia and, therefore, it is conceivable that autologous recovery of the rat islets might have occurred as suggested in other recent studies (23; 24).

While unable to evade the immune system in our models, early embryonic pig or human tissues were shown to be less immunogenic compared to their adult counterparts (1; 2; 10; 11; 18; 19; 25). Thus, it was important to define a minimally toxic immunosuppressive protocol for transplantation of embryonic pig tissues.

In our initial attempt to demonstrate the proof of concept in the NHP model, we chose a conventional protocol comprising induction with ATG, and Rituxan, followed by maintenance with CTLA4-Ig, RAD, and FTY720. This relatively intensive immune suppression enabled us to demonstrate that, indeed, implanted E42 pig pancreatic tissue can grow, differentiate and induce complete insulin independence in diabetic NHP (Hecht et al. Submitted). However, further reduction of this immune suppression protocol is desirable before applying this approach for studies in diabetic primates or humans.

Our previous experience in mice, similar to results in NHP in the context of islet transplantation (11-13) demonstrated that co-stimulatory blockade using CTLA4-Ig and anti-CD40L can prevent E42 pancreatic graft rejection. However, anti-CD40L was shown to be associated with thrombotic complications in patients (14).

To eliminate this agent in the present study, we combined elements from the primate protocol, such as FTY720 and Rapamycin, with alternative co-stimulatory blockade agents such as anti-LFA1 and anti-CD48 (the murine homologue of human CD58 (LFA3) (26; 27)). Previous studies demonstrated that each agent could be used in strategies aimed at controlling GVHD or graft rejection following allogeneic BMT or solid organ transplantation in rodents (28-31).

Efalizumab (humanized anti LFA1) and Alefacept (human LFA3 fusion protein) are new immune-suppressive agents approved by the FDA in 2003 for the treatment of psoriasis (FDA application no. BLA 125075, and BL 125036). Both are currently in phase II/III clinical trials in the transplantation setting (32; 33).

Our results, comparing different combinations of anti-LFA1 and anti-CD48, suggest that optimal engraftment and development of E42 pig pancreatic tissue could be attained upon immune suppression with a protocol comprised of anti-LFA1 and anti-CD48, in combination with low doses of FTY720.

Substitution of FTY720 by other conventional immune-suppressive drugs, including Rapamycin, MMF and FK506, failed to overcome rejection in our model. This special attribute of FTY720 might be associated with its unique mechanism of action, affecting trafficking of lymphocytes. Thus, while all other agents do not have additive activity to that of co-stimulatory blockade agents (i.e. suppressing proliferation or activation of T cells), FTY720 might add an additional function which might be critical for survival of embryonic grafts. However, further studies with these agents using different dosing are still required to establish
whether this finding reflects a true mechanistic difference.

Collectively, immune suppression with anti-LFA1 and anti-CD48, in conjunction with low dose FTY720, might offer unique advantages in NHP and in humans, being free of the thrombotic side effects of anti-CD40L, the toxicity of ATG and Rituxan, and the proven deleterious effects of Rapamycin on pancreatic graft function and insulin secretion (34; 35). Our ability to prevent rejection of E42 pig pancreas in the mouse model for more than 6 months in C57BL/6 recipients and to attain secreted pig insulin blood levels, similar to the levels secreted from grafts that were transplanted in SCID mice, is indeed very encouraging.

Furthermore, the treatment with anti-LFA1 and anti-CD48 mAbs could be stopped at 11 weeks after transplantation, and the grafts will remain functional upon minimal maintenance with FTY720, administered twice weekly. However, cessation of this low dose FTY720 maintenance led to complete rejection within 1 month, suggesting that immune tolerance had not been attained.

In renal transplantation FTY720 was tested together with a reduced dose of cyclosporine A (CSA) and was found to be associated with macular edema cases and lower creatinine clearance. Thus, when compared to a protocol comprised of MMF, in conjunction with CSA, the latter was found to be safer and FTY720 was not licensed for use in this setting (36). However, in the present study we found that the use of FTY720 as a single agent could be very effective if administered in conjunction with a transient treatment with co-stimulatory blockade agents. Thus, our results suggest a new application as a single agent and not together with a nephrotoxic agent such as CSA. Indeed, the use of FTY720 as a single agent was found to be free of renal toxicity in a recent Phase2b randomized trial in patients with multiple sclerosis (37) and a further Phase 3 trial is currently underway. Considering that several conventional immunosuppressive drugs including FK506 and MMF could not replace FTY720 in our model, the latter represents an important potential single agent modality for the implantation of E42 pig pancreatic tissue in humans and, therefore, further studies in the NHP model are warranted.

The ability of co-stimulatory blockade protocols to induce durable immune tolerance in allogeneic and xenogeneic solid organ transplantation is highly controversial. A few studies have claimed permanent acceptance and donor specific tolerance after short treatment with co-stimulatory blockade agents (28; 30; 38; 39) while, more commonly, this protocol was shown to induce prolonged engraftment without establishment of tolerance (40-49).

This discrepancy could be attributed, in part, to strain or species differences investigated in the different studies reported, as well as to the different assays used to define rejection (31; 50).

The novel combination of anti-LFA1, anti CD48 and FTY720, introduced here, while short of inducing durable acceptance of the grafts, could likely serve as a reasonable starting point for new tolerance induction strategies, without relying on anti-CD40L. This platform is especially attractive, considering that clinical grade agents for neutralizing LFA1 and CD48 in humans are available.

Further studies using this protocol in the NHP model for E42 pancreatic tissue transplantation is expected to lead to the development of a safer transplantation protocol in patients with type 1 diabetes.

ACKNOWLEDGMENTS

This work was supported in part by Tissera, Inc. to Y.R and by NIH R01 HL56067, R01 34495, R01 HL63452, P01 AI056299 to B.B.
Disclosure of Potential Conflicts of Interest: Y.R. is a scientific consultant and holds equity with Tissera, Inc. which supported this work. Y.R. holds the Henry H. Drake Professional Chair in Immunology.
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when long-term acceptance is induced by CTLA4Ig/anti-CD40L. *Transplantation* 83:1259-1267, 2007


FIGURE LEGENDS

**Figure 1:** Comparison between E42 and E28 pancreas in growth potential, function and rejection patterns. (A) The difference in size and insulin secretion of the E42 and E28 pancreas transplanted under the kidney capsule of NOD-SCID mice (black (E42) and white (E28) bars, respectively). The graft size was determined 6 weeks following transplantation. (B) Histological (H&E) evaluation of E42 and E28 pancreatic grafts 1 month after transplantation under the kidney capsule of NOD-SCID or C57BL mice. (C) Histological (H&E) evaluation of E28 pancreatic grafts 10 days after transplantation in the omentum of Nude or Lewis rats.

**Figure 2:** Immuno-staining of E28 pig pancreatic tissue implanted in the omentum of nude and Lewis rats. Pig pancreatic epithelial cells, 15 days after transplantation in (a) nude or (b) Lewis rats, visualized using cytokeratin 20 (reddish color) specific staining. Nuclei are stained with yellow Hoechst. Pig endocrine cells, 4 months after transplantation in (c) Nude or (d) Lewis rats, visualized using insulin (blue) and glucagon (green) specific staining. Infiltration of the omentum in transplanted Lewis rats 15 days after transplantation is visualized using (e) CD3 for detection T lymphocytes, and (f) F4/80 for detection of macrophages.

**Figure 3:** Histological examination of E42 pig pancreatic tissue 2 weeks after transplantation under the renal capsule of C57BL (A) with and (B) without immune-suppression with co-stimulatory blockade (anti-LFA1, anti-CD48, and CTLA4-Ig), FTY720, and T cell debulking. Slides were stained for pig insulin, cytokeratin (MNF116), CD3+ lymphocytes and macrophages (F4/80), as indicated.

**Figure 4:** Histological examination of E42 pig pancreatic tissue 2-10 months after transplantation under the renal capsule of C57BL treated with the co-stimulatory blockade protocol (anti-LFA1, anti-CD48, and FTY720). Slides were stained for (a) CD3+ lymphocytes; (b) macrophages (F4/80); (c) insulin (blue) and glucagon (green), (d) mouse blood vessels (CD31, red) and pig blood vessels (CD31, green); and (e) cytokeratin (broad spectrum, red) and IgM and IgG deposits (green). The inset in (d) demonstrates positive staining for pig endothelial cells (green) in E42 pancreas. The inset in (e) demonstrates positive normal staining for IgM and IgG deposits in the glomeruli of the kidney. The data is representative of the experiment shown in Table 1 (n=11).

**Figure 5:** Porcine insulin blood levels 10 weeks after transplantation of E42 pancreas into immunosuppressed C57BL/6 mice. Recipients were treated with different combinations of co-stimulatory blockade agents (anti-LFA1, anti-CD48, and CTLA4-Ig) and FTY720, with or without T cell debulking. Data is presented as average ± SEM. * P<0.05.

**Figure 6:** (a) Porcine insulin levels in the serum of C57BL mice transplanted with E42 pancreas and treated with co-stimulatory blockade agents (anti-LFA1, anti-CD48, ±CTLA4-Ig), FTY720, with debulking (white diamonds) or without debulking (white circles), at different time points after transplantation. Treatment with co-stimulatory antibodies was stopped at 3 months post transplant and graft maintenance was continued twice weekly only with FTY720. Insulin levels in the serum of NOD-SCID mice transplanted with E42 pancreas served as a positive control (black diamonds). The inset demonstrates average pig insulin levels in transplanted mice over a course of 6 months. No statistical difference could be found between the tested groups.
(b) Porcine insulin levels in the serum of C57BL mice transplanted with E42 pancreas and treated with co-stimulatory blockade agents with or without debulking, at different time points after FTY720 withdrawal. Data is presented as average ± SEM.

**Figure 7:** Long term engrafted mice (black diamonds) as opposed to non-engafted (grey diamonds) mice resist Alloxan challenge. At the indicated time points, the implanted mice were subjected to nephrectomy of the kidney harboring the implant, leading to prompt induction of hyperglycemia.

**Table 1:** Outcomes of E42 pig pancreatic tissue implantation in C57BL/6 mice under different immune suppression protocols.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Engraftment*</th>
<th>Porcine Insulin**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Tests of immune suppressive combinations to identify maximum effective protocol:</td>
<td></td>
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</tr>
<tr>
<td>Debulking***, FTY720, CTLA4-Ig, anti-LFA1, anti-CD48</td>
<td>8/8</td>
<td>5.3±4.3</td>
</tr>
<tr>
<td>Debulking, CTLA4-Ig, anti-LFA1, anti-CD48</td>
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<td>0.28±0.48</td>
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<tr>
<td>Debulking, FTY720, anti-LFA1, anti-CD48</td>
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<td>16.9±10.4</td>
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<tr>
<td>FTY720, CTLA4-Ig, anti-LFA1, anti-CD48</td>
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<td>27.9±29</td>
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<tr>
<td>FTY720, anti-LFA1, anti-CD48</td>
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<td>8.5±7.7</td>
</tr>
<tr>
<td>CTLA4-Ig, anti-LFA1, anti-CD48</td>
<td>0/6</td>
<td>0.1±0.2</td>
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<tr>
<td>B. The role of FTY720 with individual co-stimulatory blockade agents:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTY720, CTLA4-Ig</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>FTY720, anti-CD48</td>
<td>0/4</td>
<td>0</td>
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<tr>
<td>FTY720, anti-LFA1</td>
<td>2/4</td>
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<tr>
<td>FTY720, CTLA4-Ig, anti-CD48</td>
<td>3/4</td>
<td>3.3±5.1</td>
</tr>
<tr>
<td>FTY720, CTLA4-Ig, anti-LFA1</td>
<td>3/4</td>
<td>5.7±8.2</td>
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<tr>
<td>FTY720, anti-LFA1, anti-CD48</td>
<td>11/11</td>
<td>13.8±10</td>
</tr>
<tr>
<td>FTY720, CTLA4-Ig, anti-LFA1, anti-CD48</td>
<td>13/13</td>
<td>15.6±22</td>
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<tr>
<td>C. The effect of various conventional immunosuppressive drugs with anti-LFA1, anti-CD48:</td>
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<td></td>
</tr>
<tr>
<td>FK506, CTLA4-Ig, anti-LFA1, anti-CD48</td>
<td>0/3</td>
<td>2.2±3.5</td>
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<tr>
<td>Rapamycin, CTLA4-Ig, anti-LFA1, anti-CD48</td>
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<td>0.6±0.8</td>
</tr>
<tr>
<td>MMF, CTLA4-Ig, anti-LFA1, anti-CD48</td>
<td>0/4</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

*Mice were defined engrafted according to histology and/or porcine insulin in the serum. Mice were defined positive for porcine insulin if porcine insulin level in the serum of the mice 8 weeks post transplantation was ≥1uIU/ml.

**Porcine insulin in the serum of transplanted mice 8 weeks post transplantation. Data is presented as average ± SD

*** anti-CD4 + anti-CD8 mAbs on day -3
Fig. 1: Comparison between E42 and E28 pancreas in growth potential, function and rejection patterns.
Fig. 2: Immuno-staining of E28 pig pancreatic tissue implanted in the omentum of nude and Lewis rats.

Figure 3:
Figure 5:
Figure 6:

![Graph showing Pig insulin (µU/ml) over weeks after transplantation](image)

Figure 7:

![Graph showing Glucose (mg/dL) over days post alloxan treatment](image)