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Anti-CD40-based Costimulation Blockade Enhances Neonatal Porcine Islet Survival in Nonhuman Primates

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Abstract

The widespread clinical implementation of allogeneal transplantation as therapy for type 1 diabetes has been hindered by the lack of suitable islet donors. Pig-to-human islet xenotransplantation is one strategy with potential to alleviate this shortage. Long-term survival of porcine islets has been achieved using antibodies targeting CD154; however, this approach lacks translational potential secondary to associated thromboembolic sequelae. As an alternative strategy targeting the CD40/CD154 T cell activation pathway, we evaluate the ability of a chimeric anti-CD40 monoclonal antibody (Chi220) to protect islet xenografts. Neonatal porcine islets (~50,000 IEQ/kg) were transplanted intraportally into surgically-induced diabetic macaques. Immunosuppression consisted of induction therapy with Chi220 and anti-IL-2 receptor (basiliximab), and maintenance therapy with sirolimus and belatacept (a high-affinity CTLA-4Ig variant). Chi220 effectively promoted xenoislet engraftment and survival; five of six treated recipients achieved insulin-independent normoglycemia (mean length of graft survival 90.8 days, maximum survival of 203 days). No thromboembolic phenomena were noted. CD40 represents a promising alternative to CD154 as a therapeutic target in xenogeneic transplantation; other potentially translatable anti-CD40 antibodies warrant further investigation in non-human primate models.

Keywords
Xenotransplantation; Costimulation Blockade; Type 1 Diabetes

Introduction

With the advent of the Edmonton Protocol, allogeneal transplantation as a therapy for certain patients with type 1 diabetes mellitus (T1DM) became a clinical reality (1, 2). Today, the scarcity of transplantable human islets remains one of the major obstacles to widespread therapeutic application. Currently in the United States, there are about 750,000 patients with T1DM who could benefit from β cell replacement; in stark contrast, the number of suitable
deceased human pancreas donors averages about 3,000 per year (3). Xenogeneic islets represent an untapped resource with potential to alleviate this severe shortage.

The rationale for use of islets from porcine sources is well defined. Porcine insulin has been used to treat diabetics for decades; in fact, porcine and human islets respond to glucose levels in the same physiologic range. Moreover, pigs breed rapidly and produce large litters, creating a virtually limitless and on-demand supply of available islets (4). Finally, pigs can be genetically modified in ways that allow transplanted islets to evade or modulate the host immune system, prolonging their survival (5, 6). Despite these advantages, the clinical utility of porcine islet xenotransplantation has been hindered by ethical concerns, fear of zoonosis transmission, and most importantly, the development of a safe yet effective strategy to overcome the potent xenospecific immune response (4, 7, 8).

Unlike conventional immunosuppressive therapies such as steroids and calcineurin inhibitors (9, 10), costimulation blockade-based therapies minimize systemic toxicity and generalized attenuation of protective immunity while specifically targeting the recipient response to donor antigen. Engraftment and long-term survival of porcine islets in a diabetic nonhuman primate (NHP) model was successfully achieved in two landmark proof-of-concept studies (11, 12) using a costimulation blockade-based strategy targeting CD154; however, the association of anti-CD154 therapy with thromboembolic phenomena (13), an effect thought to occur by CD40-independent mechanisms (14), has stifled its clinical development. While enthusiasm for CD154-specific antibodies has predictably waned, the CD40/CD154 interaction remains a promising target. CD40-directed therapy might avoid CD154-associated thrombotic sequelae while bestowing comparable xenograft protection.

Various anti-CD40 antibodies have shown the ability to prolong kidney allograft survival in NHP models (15-18). We have previously demonstrated that Chi220, a rationally developed chimeric mouse anti-human CD40 monoclonal antibody, extended kidney and islet allograft survival in rhesus macaques (15, 19). To date, all reports of successful adult or neonatal porcine islet transplantation into primates have included the use of CD154-specific agents (6, 11, 12, 20, 21). In this study, we evaluate the ability of a Chi220-based, anti-CD154-free immunosuppressive regimen to protect neonatal porcine islet (NPI) xenografts in a preclinical NHP model.

Methods

Nonhuman Primates: Recipient Care and Pancreatectomy

Captive bred adolescent rhesus macaques (Macaca mulatta) were used as xenograft recipients. As a mode of establishing diabetes, a duodenal-sparing pancreatectomy was performed 2-4 weeks before the intended xenotransplant, as previously described (22). Post-pancreatectomy care consisted of fasting and postprandial blood glucose measurements, pancreatic enzyme replacement, and nutritional support. All procedures with regard to primate care were performed in accordance with the “Guide for the Care and Use of Laboratory Animals (23),” and were approved by our institutional IACUC.
Procurement and Preparation of Neonatal Porcine Islets

One- to two-day-old Duroc or Large White crossbreed neonatal pigs of either sex (total body weight 1.5-2.0 kg, Swine Research and Technology Centre, University of Alberta) were used as pancreas donors. Pancreatectomies and islet isolations were performed at the Surgical-Medical Research Institute at the University of Alberta, Edmonton, using a previously described modified Korbutt technique (24). In brief, harvested pancreas tissue was first digested with 1.0 mg/mL collagenase (Type XI, Sigma). Free islets were then filtered and cultured in supplemented Ham's F10 medium (Gibco, Burlington, Ontario, Canada) for a period of 5 days prior to shipment to Emory’s Animal Cell & Tissue Laboratory (EACTL), where the islet suspensions were cultured overnight and then transplanted into NHPs on day 7 post-isolation.

Pre-transplant Characterization of Islets

On the day of transplantation, islets were assessed at EACTL for quantity by dithizone (Sigma-Aldrich), for viability by SYTOX® Green Fluorescent Nucleic Acid Stain (Invitrogen, Eugene, OR), for bacterial contamination by both Gram stain and culture and for in vitro function by static incubation assay as previously described (11). Functionality of islets was quantified using the glucose stimulation index (GSI), calculated by dividing the amount of insulin release at high glucose concentrations (20 mmol/L glucose) by that at low concentrations (2.8 mmol/L glucose). GSI values >1.0 are considered within normal limits.

Islet Transplantation

After receipt, overnight culture, washing and counting, the transplant preparations were resuspended in 20 mL of transplant media supplemented with 200 units of heparin and etanercept 3mg/kg (Enbrel; Amgen & Wyeth, Philadelphia, PA). Following mini-laparotomy, approximately 50,000 islet equivalents (IEQ)/kg of NPIs were transplanted intraportally into each of the NHP recipients via gravity drainage of the suspension into a mesocolic vein through a 22-gauge intravenous catheter.

Post-transplantation Monitoring of Xenoislet Function

Recipient fasting and postprandial blood glucose levels were monitored (Glucometer Elite; Bayer, Elkhart, IN) daily by ear-stick. Insulin (NPH, Ultralente; Eli Lilly, Indianapolis, IN) was administered twice daily to maintain fasting blood glucose (FBG) at <200 mg/dL. Intravenous glucose tolerance tests (IVGTTs) with a bolus of dextrose (500 mg/kg) were performed once before transplant, as well as at monthly intervals during the post-transplant period. Glucose levels were measured prior to the bolus, and then at 10, 30, 60, and 90-minute intervals.

Porcine c-peptide (PCP) was measured from sera obtained at each IVGTT timepoint as well as from serial samples obtained throughout the post-transplant period, using the manufacturer’s protocol from Linco's radioimmune assay kit (Linco Research; St. Charles, MO) as previously described (11).
Experimental Groups, Immunosuppressive Regimens, and Animal Treatment Protocols

Three primates (Cohort 1) received immunosuppressive therapy beginning on the day of transplantation, which consisted of induction therapy with anti-IL-2 receptor antibody (basiliximab, 0.3 mg/kg iv, administered intraoperatively and on post-transplant day 2) and chimeric monoclonal anti-CD40 antibody (Chi220, 20 mg/kg iv, administered intraoperatively and then on post-transplant days 2, 6, and 14). In addition, these animals received ongoing maintenance therapy with belatacept (LEA29Y, a high-affinity CTLA-4g variant) and sirolimus. Belatacept (20 mg/kg i.v.) was administered intraoperatively and on post-transplant day (PTD) 2 and 6. Additional doses were given on PTD 14 and every 2 weeks thereafter until experimental endpoint. Sirolimus was given orally each day following transplant until experimental endpoint, and dosing adjusted weekly to obtain trough levels of 5-15 ng/mL. A second group of 3 primates (Cohort 2) received the same drugs, but additional doses of both belatacept and Chi220 were given 5 and 2 days prior to transplant. Three NPI recipients served as controls (Cohort 3); this group did not receive Chi220, but their immunosuppressive protocol was otherwise identical to Cohort 1.

Post-transplant recipient support consisted of three times daily replacement of pancreatic enzyme with pancrelipase enteric-coated microspheres (Creon20; Ortho-McNeil, Raritan, NJ), and daily administration of megestrol acetate (Megace; Bristol-Myers Squibb, Princeton, NJ). Megestrol was discontinued PTD 30. Viral prophylaxis consisted of 6 mg/kg of oral valganciclovir (Valcyte; Roche Nutley, N.J) daily. Animals developing rhesus cytomegalovirus (rhCMV) viremia were treated twice daily with intramuscular ganciclovir (Cytovene® injection- Roche Pharmaceuticals), 6 mg/kg/dose, until return of rhCMV viral load to normal and then for an additional two weeks.

The Chi220 and belatacept used in these experiments were provided by Bristol-Myers Squibb (Princeton, NJ). All other drugs were purchased from the Emory University Hospital Pharmacy.

Experimental Endpoints

Loss of islet function, failure of islet engraftment, or severe recipient illness were the experimental endpoints in this study. Loss of function (rejection) was defined as the need for resumption of exogenous insulin (determined by FBG >200 for two consecutive days) following a period of normoglycemia and insulin independence. Failure of engraftment, the inability to achieve insulin-independent normoglycemia for any period of time, was defined as four consecutive days after PTD 50 with FBGs >300mg/dL that were not associated with events that can cause hyperglycemia (i.e. infection). Animals judged to be severely ill, as determined by veterinary staff assessment of acuity and irreversibility of illness, animal distress, or weight loss (loss of 25% of body weight from pre-transplant baseline using nomogram to adjust for animal growth) were euthanized in accordance with IACUC guidelines.

Histologic and Immunohistochemical Evaluation

At time of recipient sacrifice, liver specimens were analyzed using standard hematoxylin and eosin histology. For immunohistochemical assessment, specific antigens in frozen tissue...
sections were labeled with primary antibody, then visualized using the Dako LSAB + labeled Streptavidin-Biotin kit (Dako; Carpenteria, Ca). The antigen-specific primary antibodies used in these immunohistochemical tests were for insulin (Fitzgerald; Concord, Ma), CD3 (Dako; Carpenteria, Ca), CD4 (BD; Franklin Lakes, NJ), CD8 (Dako; Carpenteria, Ca), CD20cy (Dako; Carpenteria, Ca), CD68 (Dako; Carpenteria, Ca), C4d (Quidel; San Diego, Ca) and neutrophil elastase (Dako; Carpenteria, Ca). In addition, all terminated recipients underwent necropsy by the Yerkes Veterinarian pathologist to assess for gross abnormalities.

Viral Reactivation and Xenosis Monitoring

Weekly monitoring for the presence of rhCMV and porcine endogenous retrovirus (PERV) in recipient blood samples was accomplished by real-time PCR using TaqMan chemistry and reagents from Applied Biosystems, as previously described (10). Briefly, reactions of 50 μL total volume contained 25 μL TaqMan Universal Master Mix, 7.5 μL genomic DNA isolated from whole blood and varying concentrations of primers and probe. Amplification and analyses were done on a model 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The details of our real-time PCR PERV assay, including primer sequences, have been previously published (11, 25, 26).

Results

Anti-CD40-based, Anti-CD154-free immunosuppression enhances neonatal porcine islet engraftment and survival

To determine whether anti-CD40-based, anti-CD154-free costimulation blockade could protect xenogeneic islets, nine diabetic macaques divided into 3 cohorts underwent NPI transplantation (Table 1). Five of six Chi220-treated recipients (cohorts 1 and 2), but only 1 of 3 controls (cohort 3) experienced NPI engraftment with resultant normoglycemia and insulin independence (Figure 1). Because of incomplete islet maturation and differentiation, transplanted NPIs require a 2-8 week latent period before realizing complete function (27). Therefore, exogenous insulin administration was required for initial glucose homeostasis following transplantation. For Chi220 recipients achieving euglycemia, mean rejection-free graft survival time was 90.8 days, with a maximum graft survival of 203 days (Table 2).

Two of three cohort 1 animals treated with Chi220 induction starting on the day of transplant achieved insulin independence and prolonged graft survival. Though one recipient experienced rejection, the other was euthanized secondary to excessive weight loss with a functioning xenograft. The third cohort 1 recipient failed to engraft despite a relative decrease in insulin requirement post-transplant.

In an attempt to improve on the engraftment rate of our cohort 1 recipients, we modified the induction regimen of recipients in cohort 2. In this group, therapy was begun 5 days prior to transplantation in order to obtain higher drug concentrations in secondary lymphoid tissues at the time of transplant, potentially improving naïve T cell priming therein. All recipients in cohort 2 achieved insulin-independent normoglycemia. One recipient was euthanized for loss of graft function, while two were euthanized for a combination of weight loss and
failure to thrive despite persistent normoglycemia. Average length of graft survival was better in cohort 2 (106 days) than in cohort 1 (63 days).

To directly assess the requirement for blockade of the CD40/CD154 pathway, cohort 3 received a Chi220-free immunosuppressive regimen. Two of three recipients experienced failure of engraftment and were sacrificed on PTDs 33 and 26 with undetectable porcine c-peptide (data not shown). One recipient did achieve normoglycemia; he was sacrificed following loss of graft function on PTD 71.

A similar pattern of weight loss affected all animals in this study, resulting in early experimental termination in several cohort 1 and 2 recipients. All recipients experienced rapid, significant weight loss following pancreatectomy, with relative weight stabilization following islet transplantation (Figure 2).

**Measurement of porcine c-peptide confirms xenograft function following transplant**

Prior to transplant, diabetes was confirmed by hyperglycemia in response to glucose challenge (Figure 3) and the complete absence of stimulated c-peptide production (data not shown). All recipients had detectable porcine c-peptide (PCP) immediately following transplant, both in fasting serial serum samples (Figure 4a) and in response to IVGTTs performed at regular intervals (Figure 4b). PCP levels correlated well with xenograft function; recipients euthanized because of illness (REp9, ROf9, RIu9) rather than rejection (RZy9, RYp9) demonstrated significant PCP production for the duration of their survival. Interestingly, the single recipient experiencing failure of engraftment (RDp9) had detectable PCP despite persistently high FBGs, suggesting a subtherapeutic or partially engrafted xenoislet mass.

**T cells mediate immunologic rejection of transplanted islets**

Immunohistochemical analysis of liver sections was performed at time of necropsy, and intrahepatic islets examined for evidence of immune cell infiltrates. Histologic appearance correlated well with graft function at experimental endpoint. In recipients exhibiting loss of graft function (RZy9, RYp9), remaining intrahepatic islets had minimal to no insulin positivity and were surrounded by a dense cellular infiltrate (Figure 5a); examination of liver specimens from recipients euthanized despite persistent normoglycemia (REp9, ROf9, RIu9) revealed abundant, strongly insulin positive intrahepatic islets with little to no cellular infiltrate (Figure 5b), suggesting ongoing xenograft survival and function. Characterization of the cellular infiltrate surrounding rejected islets revealed strong staining positivity for CD3+ T cells (both CD4+ and CD8+ subsets), but not for CD20+ B cells, complement C4d, (Figure 6) or neutrophils (data not shown). CD68+ macrophages were also present in the infiltrates to a lesser extent. These results suggest a largely T cell-mediated immunologic rejection as the cause of graft failure in these two recipients, consistent with previously reported results (11, 12). The single cohort 1 recipient experiencing failure of engraftment (RDp9) had intrahepatic islets with moderate insulin positivity despite a dense cellular infiltrate that stained strongly for T cells (data not shown).
As in our previous experience with Chi220 (15, 19), gross and histologic tissue analysis at necropsy revealed no evidence of thromboembolism or other pathologic phenomena contributing to experimental endpoint in any recipient.

**Chi220 transiently depletes B cells**

In order to investigate the effects of Chi220 on the various cells expressing CD40 (28), peripheral leukocyte populations were monitored by flow cytometry (Figure 6). As noted in previous publications (15, 19), anti-CD40 therapy with Chi220 resulted in an immediate and near complete depletion of peripheral CD20+ B cells. Absolute CD20+ counts began to rebound within 20 days following cessation of Chi220 therapy (in surviving recipients). Peripheral T cell populations were not similarly affected.

**PCR assays demonstrate reactivation of rhesus cytomegalovirus, absence of porcine endogenous retrovirus transmission in treated recipients**

The reactivation of latent viruses such as CMV is one potentially harmful effect of immunosuppressive therapy. We monitored each recipient for rhCMV (the rhesus CMV homolog) using real time PCR. All recipients developed detectable rhCMV levels following immunosuppression; however, with one exception (REp9), rhCMV levels returned below the limit of detection within 5 weeks after initiation of antiviral therapy with ganciclovir (data not shown).

Zoonosis transmission is a theoretical risk of pig-to-primate tissue transplantation. Using PCR, we monitored for porcine endogenous retrovirus (PERV) in recipient blood samples at regular intervals. No recipient had PERV detected at any point during the experiment (data not shown).

**Discussion**

The problems associated with anti-CD154 antibodies are well documented in various models of transplantation (12, 13, 29). CD154 expression on activated platelets is important for stabilization of arterial thrombi, and antibodies to CD154 appear to greatly increase the incidence of thrombus formation and embolic phenomena (30). While this dangerous adverse effect has for the time being halted any prospects of a clinical trial using anti-CD154-based immunotherapy, preclinical studies of xenoislet transplantation continue to employ various forms of an anti-CD154 antibody in a proof-of-concept role, as shown recently by van der Windt et al (6). All five published studies describing adult or neonatal porcine islet survival of two months or more in diabetic NHPs (excluding encapsulated islet studies) have employed anti-CD154 as a critical component of the immunosuppressive regimen (6, 11, 12, 20, 21). In contrast, published CD154-sparing regimens have had little success in prolonging xenoislet survival (31, 32); similarly, xenoislet studies directly comparing anti-CD154 containing and anti-CD154-free regimens have demonstrated significantly improved graft survival with anti-CD154 treatment (11, 12, 33).

Anti-CD40 therapy represents an alternate strategy targeting the important CD40/CD154 T cell activation pathway. CD40 is constitutively expressed on various antigen-presenting cells (APCs) including B cells, macrophages, and dendritic cells. Engagement of the CD40
receptor by its ligand, CD154, results in augmentation of antigen presentation capability, upregulation of costimulatory and adhesive molecule expression, and increased production of proinflammatory cytokines (34). There is some compelling evidence that the critical immunosuppressive effect of CD154 blockade is inhibition of CD40 ligation, resulting in diminished APC activation and improved graft survival (35, 36). In contrast, the thromboembolic effects associated with anti-CD154 therapy appear to be primarily mediated via CD40-independent pathways; soluble CD154 expressed by platelets has a regulatory role in inflammation and thrombus stabilization not affected by CD40 deficiency (14, 30). Supporting these concepts, a chimeric monoclonal anti-CD40 antibody (ch5D12) was shown to effectively prolong renal allograft survival in NHPs, both as monotherapy and in combination with CD86 costimulation blockade and cyclosporine A, without causing adverse thrombotic events (16, 17). Similarly, Chi220 has been previously investigated by our group and found to significantly prolong both renal and islet allograft survival, an effect that was synergistic when Chi220 was combined with belatacept in the case of islet transplantation (15, 19). This study is the first to successfully apply an anti-CD40 immunosuppressive strategy in a NHP model of islet xenotransplantation.

The design of the immunosuppressive protocol in this experiment differs from our previously published NPI protocol (11) in the following ways: in this study (a) Chi220 was substituted for the humanized anti-CD154 antibody H106, and (b) some Chi220 recipients began therapy 5 days prior to transplant (cohort 2). Rates of engraftment were similar between the two regimens, with 5 of 6 Chi220 recipients achieving insulin-independent normoglycemia versus 6 of 7 H106 recipients. Glycemic “set points” were also similar in the two protocols; average fasting blood glucose for Chi220 recipients (calculated for period of insulin independence) ranged from 110 – 150 mg/dL, compared to 140 mg/dL for H106 recipients. In contrast, H106-based therapy conferred longer graft survival than Chi220 (see Table 2), though this disparity may be attributable to the construction of the antibodies themselves. While Chi220 is chimeric, H106 is humanized and therefore theoretically less immunogenic, a difference that could result in prolonged H106 serum half-life and therapeutic activity. Further obscuring the relative efficacy of Chi220, complications associated with pancreatectomy and rhCMV viral reactivation (discussed below) contributed to early experimental termination in 3 of our islet recipients before full duration of graft survival could be ascertained. Taking these potential confounders into account, Chi220 appears to be comparable with H106 in promoting islet xenograft acceptance and survival.

Results from our control group cast doubt on the absolute necessity for CD40/CD154 manipulation. Though Chi220 additively benefited graft survival and the overall engraftment rate of recipients treated with a Chi220-free regimen was poor, one control recipient did achieve insulin-independent normoglycemia, suggesting that neither CD40- nor CD154-targeted therapies may be required to prevent acute xenograft rejection.

To our knowledge, pre-transplant vs. day-of-transplant induction has never been directly compared in a xenotransplantation model. In our current study, earlier initiation of immunosuppressive therapy appeared to confer a slight improvement in graft survival, an advantage that may be attributable to differences in tissue drug levels at the time of immunologic challenge with xenoantigen. One of the main functions of induction therapy is
prevention of naïve T cell activation by recipient- and donor-derived APCs, which occurs in secondary lymphoid tissues such as lymph nodes and the spleen (37). Early initiation of induction immunosuppression may achieve higher therapeutic drug concentrations in these tissues at the time of transplant, resulting in enhanced costimulation blockade and therefore diminished T cell activation. More experimental subjects and direct measurement of tissue drug concentrations would be needed to establish the statistical significance of this finding.

Chi220 is thought to achieve its immunosuppressive effects through a combination of (a) activation-induced cell anergy or apoptosis resulting from a weak partial agonist effect of CD40 ligation and (b) antibody-dependent cellular cytotoxicity resulting in depletion of CD40-expressing B-cells and other APCs (19). Demonstrating that the therapeutic effects of Chi220 are not due to B cell depletion alone, Adams et al showed that animals treated with rituximab (an anti-CD20 antibody) instead of Chi220 rapidly rejected their islet allografts despite profound B cell depletion. In addition, graft survival following a short course of Chi220 often persisted long after B cell repopulation (both in Adams’ study and in the current study), implicating Chi220’s downstream, nondepletional immunoregulatory effects. It is likely that cellular depletion plays a crucial though incompletely understood role in promotion of xenoislet survival; to answer this question, our group is currently investigating non-depleting anti-CD40 therapies.

Rejection of islet xenografts in this study appeared to be T cell-mediated. Immunohistologic assessments in our previous xenoislet studies revealed a similar pattern (11, 20), suggesting that T cells are the most important mediators of rejection whether CD40 or CD154 is the therapeutic target. Both strategies appear to protect islet xenografts by common immunoregulatory mechanisms; or, conversely, both CD154 and CD40-specific therapies inadequately block immune activation pathways as yet unrecognized.

In NHP transplant models, weight status is one indicator of overall recipient well-being and protocol safety. Though immunosuppressive toxicity and viral reactivation can both contribute to weight loss, malnutrition resulting from the mode of establishing diabetes most significantly impacted weight in the current study. Pancreatectomy is associated with loss of exocrine as well as endocrine function, resulting in malnutrition that is in some cases refractory to oral replacement of pancreatic enzymes. Furthermore, performing multiple major survival surgeries on each recipient inflicts additional physiologic stress that can contribute to failure to thrive. In order to minimize the confounding effects of pancreatectomy, we have transitioned to a chemically-induced diabetic model using streptozocin (STZ).

Reactivation of latent viruses such as rhCMV can result from attenuation of protective immunity associated with immunosuppressive therapy. RhCMV viremia can cause significant recipient morbidity, and in previous studies was suspected to have contributed to xenograft rejection (11, 20). In the current study, one recipient's persistent viremia may have contributed to weight loss and eventual need for euthanasia, but in the majority of recipients CMV status was not clinically significant. This is likely due to improved viral prophylaxis and treatment protocols, but may also reflect a preservation of protective immunity comparable to anti-CD154 containing regimens.
One concept applicable to both CD40- and CD154-specific therapeutic strategies is that different versions of the same antibody can have vastly different effects in vivo. Previously described anti-CD40 antibodies include Chi220, the chimeric antibody ch5D12 (16, 17) and most recently the fully-human 4D11 (18), which now appears poised for clinical development (38). These antibodies differ in their method of fabrication, B cell depletional capability and incidence of anti-drug antibody formation; though difficult to compare directly, further differences in binding affinity and antigen specificity may exist. Our group continues to investigate the effectiveness of new anti-CD40 clones. As recently developed techniques for antibody engineering such as variable-domain resurfacing and complementarity-determining region grafting continue to improve, new anti-CD40s with promising translational potential will likely be developed.

In summary, we have demonstrated the ability of the chimeric anti-CD40 monoclonal antibody Chi220 to promote NPI engraftment, survival, and long-term function in NHPs. Chi220 is a safe and effective alternative to anti-CD154-based therapy as a component of a costimulation blockade-based, potentially translatable immunosuppressive regimen. This is the first published study to demonstrate the efficacy of a CD40-based therapy in an islet xenotransplantation model. More extensive investigation of alternative anti-CD40 antibodies with improved clinical potential is warranted.

Acknowledgments

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Figure 1. Anti-CD40 therapy facilitates xenoislet engraftment and function
Induction therapy with Chi220 resulted in prolonged insulin-independent normoglycemia in xenoislet recipients. Reason for experimental termination is indicated by the superscript following each ID. (a) Cohort 1, animals that received immunosuppression starting on the day of transplant. (b) Cohort 2, animals that received immunosuppression starting 5 days prior to transplant. (c) Cohort 3, animals that received a Chi220-free immunosuppressive regimen starting on day of transplant. Daily total insulin requirement is shown in red; daily FBG is shown in blue.

a failure of engraftment; b weight loss / failure to thrive; c rejection
Figure 2. Weight loss in islet recipients
Change in weight over time for individual recipients in cohort 1 (a) and cohort 2 (b) shown as percentage of weight on day of transplant. All recipients demonstrated significant rapid weight loss following pancreatectomy, with a decrease in the rate of weight loss following islet transplantation. Horizontal line represents weight at transplant. POD – Post-operative day.
Figure 3. Improved glucose tolerance following islet transplant

Representative graph. An intravenous glucose bolus was given immediately after time 0 and blood glucose levels measured at the intervals shown. Recipients had poor glycemic control following pancreatectomy that improved following xenoislet transplant. Pre-transplant IVGTT was performed one week after pancreatectomy and two weeks prior to transplant. Post-transplant IVGTT was performed approximately one month following islet transplantation.
Figure 4. Confirmation of xenograft function with measurement of porcine c-peptide
(a) Serial c-peptide measurement. Porcine c-peptide was measured in serum samples from fasting recipients taken at regular intervals. Values were pooled by cohort, averaged, and arranged by number of weeks following transplant. While all recipients had detectable c-peptide levels until experimental endpoint, measurements taken in animals following rejection approached the lower threshold of detection. (b) Stimulated c-peptide production, representative graph. Porcine c-peptide was measured at the time intervals shown following an intravenous glucose bolus. Each line represents a different post-transplant day (legend
inset) for a single animal with long-term graft function, ROF9. POD – post-operative day. IVGTT – intravenous glucose tolerance test.
Figure 5. Cellular infiltration and insulin staining in rejected vs. functional islet
Staining of intrahepatic islets with anti-insulin antibody (brown), performed on liver sections obtained at recipient necropsy. (a) Representative section from recipient experiencing loss of graft function (RZy9), showing dense cellular infiltrate with minimal insulin positivity. (b) Representative section from recipient euthanized in the setting of persistent normoglycemia (RIu9), showing minimal cellular infiltrate with strong insulin positivity.
Figure 6. Cellular infiltrate surrounding rejected islets consists mainly of T cells
Immunohistochemical assessment of intrahepatic islets from recipient experiencing acute rejection (RZy9 - left), compared with recipient undergoing euthanasia prior to loss of graft function (Rlu9 - right). Brown areas represent staining positivity. The rejected islet displays a significant cellular infiltrate staining strongly for CD3 (T cell marker) and moderately for CD68 (macrophage marker), with minimal CD20 and C4d staining (B cell and complement markers, respectively). The cellular infiltrate also stained strongly for both CD4 and CD8 T cell subsets, but did not stain for neutrophil elastase (data not shown).
Table 1

Recipient and islet characteristics

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<td>51,862</td>
<td>1.85</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>RU19</td>
<td>3.6</td>
<td>59,149</td>
<td>2.18</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>RZu9</td>
<td>4.4</td>
<td>46,019</td>
<td>1.10</td>
<td>73</td>
</tr>
</tbody>
</table>

IEQ/kg – Islet equivalents/kilogram; GSI – glucose stimulation index
**Table 2**

Xenoislet survival

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Graft survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIL-2R, sirolimus, belatacept, Chi220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cohort 1</td>
<td>3</td>
<td>(54), 47, 89</td>
</tr>
<tr>
<td>Cohort 2</td>
<td>3</td>
<td>56, 59, 203</td>
</tr>
<tr>
<td>αIL-2R, sirolimus, belatacept</td>
<td>3</td>
<td>(26), (33), 71</td>
</tr>
<tr>
<td>αIL-2R, sirolimus, belatacept, H106&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>(30), 76, 86, 147, 169, 194, 344</td>
</tr>
<tr>
<td>None&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
<td>(15), (15)</td>
</tr>
</tbody>
</table>

Parentheses ( ) denote survival of recipients experiencing failure of engraftment.

<sup>a</sup>Previously published cohort, presented for comparison (11). The data listed represent final graft survivals for all recipients, some of which had not reached experimental endpoints at the time of our previous publication.

<sup>b</sup>Previously published historical controls (11).