Published in final edited form as:

Am J Transplant. 2013 February ; 13(2): 312–319. doi:10.1111/j.1600-6143.2012.04341.x.

Belatacept and Sirolimus Prolong Nonhuman Primate Islet Allograft Survival: adverse consequences of concomitant alefacept therapy

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Abstract

Calcineurin inhibitors (CNI) and steroids are known to promote insulin resistance, and their avoidance after islet transplantation is preferred from a metabolic standpoint. Belatacept, a B7-specific mediator of costimulation blockade (CoB), is clinically indicated as a CNI alternative in renal transplantation, and we have endeavored to develop a clinically translatable, belatacept-based regimen that could obviate the need for both CNIs and steroids. Based on the known synergy between CoB and mTOR inhibition, we studied rhesus monkeys undergoing MHC-mismatched islet allotransplants treated with belatacept and the mTOR inhibitor, sirolimus. To extend prior work on CoB-resistant rejection, some animals also received CD2 blockade with alefacept (LFA3-Ig). Nine rhesus macaques were rendered diabetic with streptozotocin and underwent islet allotransplantation. All received belatacept and sirolimus; six also received alefacept. Belatacept and sirolimus significantly prolonged rejection-free graft survival (median 225 days compared to 8 days in controls receiving basiliximab and sirolimus; $p=0.022$). The addition of alefacept provided no additional survival benefit, but was associated with *Cytomegalovirus* reactivation in 4/6 animals. No recipients produced donor-specific alloantibodies. The combination of belatacept and sirolimus successfully prevents islet allograft survival in rhesus monkeys, but induction with alefacept provides no survival benefit and increases the risk of viral reactivation.

Keywords

Belatacept; Alefacept; Costimulation Blockade; Islet transplantation

Introduction

While significant progress has been made towards development of a safe and effective immunosuppressive regimen for islet allotransplantation, current regimens remain variably dependent on the use of calcineurin inhibitors (CNI) and steroids, both of which promote insulin resistance (1–4), or the use of lymphocyte depleting biologic therapies (5, 6). Following from promising results in phase 3 trials in kidney transplantation (7–9), regulatory approval has been achieved for belatacept, a B7-specific mediator of

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Disclosures

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

costimulation blockade (CoB). Belatacept thus provides a new, non-depleting, metabolically well-tolerated agent that is clearly attractive for use in clinical islet transplantation.

The optimal use of belatacept remains to be established. In particular, CoB-resistant rejection has been seen in kidney transplant trials (7–9); and while these have been easily managed in kidney transplantation, they are anticipated to be prohibitive in islet transplantation given the limited capacity to monitor intrahepatic islets for burgeoning rejection. Thus, means of deploying CoB-based therapy and preventing CoB-resistant rejection are required prior to clinical use of belatacept in clinical islet transplantation.

We have shown previously that the combination of cytotoxic T lymphocyte associated antigen 4-immunoglobulin (CTLA4-Ig, a lower affinity form of belatacept) and oral sirolimus (a suboptimal delivery method for sirolimus in nonhuman primates; NHPs) fail to prevent *renal* allograft rejection in NHPs, but that addition of the CD2-specific fusion protein alefacept (LFA3-Ig) significantly prolongs survival (10). Our group also has shown that the higher affinity belatacept and oral sirolimus prolongs *islet* survival, but fails to induce durable allograft acceptance (11). In the experience reported herein, we have attempted to optimize this CoB approach by using the higher affinity belatacept in combination with intramuscular rather than oral sirolimus so as to combine optimal CoB with mTOR inhibition delivered in a fashion that achieves optimal and clinically relevant levels. To this optimized combination we have added alefacept, positing that this could enhance the effect of a belatacept-based regimen as it had the CTLA4-Ig-based regimen. Alefacept is known to target CoB-resistant alloreactive effector memory T cells, particularly those that are CD2^{hi} and CD28⁻ (12, 13). Alefacept also has been shown to significantly reduce circulating memory and effector T cell populations (14, 15) and significantly improve clinical plaque psoriasis (14).

In this study and the concurrently submitted renal transplant study by Lo, et al. (manuscript accepted), we have tested the regimen of belatacept and intramuscular sirolimus with and without alefacept, hypothesizing that, consistent with our previous findings, the combined regimen would control alloreactive effector T cell-mediated CoB-resistant rejection and be well tolerated. However, we find that while belatacept and sirolimus alone, when optimally delivered, significantly prolonged islet and kidney allograft survival, the addition of alefacept induction resulted a significantly increased risk for viral reactivation without improvements in rejection-free survival.

Materials and Methods

Diabetes induction, confirmation and treatment

All animal experiments and procedures were performed in compliance with the guidelines of the Emory University Institutional Animal Care and Use Committee, and the Guide for the Care and use of Laboratory Animals (Eighth Edition, 2011). Rhesus macaques weighing 3–4kg were trained to undergo earsticks to obtain measurements of blood glucose. Once trained, macaques were rendered diabetic using streptozotocin (1250 mg/m² IV; Zanosar, Teva Parenteral Medicines, Irvine, CA) approximately four weeks prior to transplantation. Prior to transplantation all animals underwent intravenous glucose tolerance test (IVGTT), in which 500mg/kg of dextrose was injected and blood glucose and C-peptide levels were measured at baseline and 10, 30, 60 and 90 minutes. Diabetes was confirmed by elevated fasting blood glucoses and the absence of C-peptide following glucose stimulation. Once diabetic, blood glucose measurements were taken twice daily and animals were treated with NPH (Novolin; Novo Nordisk, Princeton, NJ) and glargine (Lantus; Sanofi-Aventis, Bridgewater, NJ) insulin to maintain normoglycemia.

Donor pancreatectomy and islet isolation

One day prior to transplantation, donor rhesus macaques weighing 10–20kg underwent pancreatectomy via midline laparotomy following terminal exsanguination. Pancreata were infused with Collagenase/Neutral protease (950 Wunsch units and 63 units, respectively; Serva, Heidelberg, Germany) and digested in a Ricordi Chamber (Biorep Technologies, Miami FL). The digested islets were purified on a Euroficol gradient (Mediatech, Manassas, VA) and Cobe 2991 blood cell processor (CaridianBCT, Lakewood, CO). The final product was counted according to islet size, expressed as islet equivalents (IEq), cultured overnight, and suspended in Transplant Media (Mediatech) for transplantation.

Islet transplantation and immunosuppression regimens

Diabetic recipients underwent major histocompatibility complex-mismatched islet allotransplantation via a small midline laparotomy and cannulation of a mesenteric vein. A mean of 15,416 ($\pm$ 4,577) IEq were transplanted per kilogram of body weight. Animals were treated with belatacept (20mg/kg IV on day 0, 3, 7, 14, 21, 28 then twice monthly through day 180) and sirolimus (intramuscular twice daily through day 120 to achieve trough levels of 5–15 ng/mL) or belatacept and sirolimus with alefacept induction (1.0mg/kg IV on day 0, 3, 7, then weekly through day 56). Three animals that received basiliximab and sirolimus were used as historic controls(16). The primary endpoint was rejection-free survival, with rejection defined as two consecutive daily fasting blood glucoses greater than 130mg/dL.

Flow cytometric analysis

Blood was obtained at baseline and weekly after transplantation from recipients for laboratory and flow cytometric analysis. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and stained with CD3 PacBlue, CD4 PerCp-Cy5.5, CD8 PerCP, CD28 PE-Cy7, CD95 FITC, and CD2 PE (BD Biosciences, San Jose, CA). Naïve ($T_{Naïve}$), central memory (T_{CM}) and effector memory (T_{EM}) $CD3^+CD4^+$ T cells were defined as $CD28^+CD95^-$, $CD28^+CD95^+$ and $CD28^-CD95^+$, respectively.

Alloantibody assay

PBMC from transplant recipients were isolated and blocked with mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Following the addition of serum from the paired donor, donor PBMC were incubated with FITC-labeled mouse anti-rhesus IgG (clone 1B3, NHP Reagent Resource, Boston, MA). Cells were stained with CD3 and CD20 and analyzed by flow cytometry. The presence of donor-specific alloantibody was confirmed by a two-fold increase in anti-rhesus IgG at the time of rejection over pre-transplant levels.

Polymerase chain reaction (PCR) for *Cytomegalovirus*

DNA was extracted from whole blood on a QIAcube (QIAGEN, Valencia, CA) using the QIAamp DNA Blood Mini Kit (QIAGEN). Extracted DNA was analyzed by quantitative real-time polymerase chain reaction (PCR) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using TaqMan Universal PCR Master Mix (Applied Biosystems). The results were compared against a standard curve generated by simultaneously running known serial concentrations of plasmid containing the *Cytomegalovirus* (CMV) target sequence. Positive results were expressed in copies/mL. Transplant recipients with viremia exceeding 10,000 copies/mL were treated with ganciclovir (6mg/kg intramuscular twice daily) until complete resolution of viremia.

Statistics

Islet allograft survival was compared between groups using the log-rank (Mantel-Haenszel) test. A p value of <0.05 was considered to be statistically significant.

Results

Belatacept and sirolimus prolong islet allograft survival

Our group previously showed that the combination of CTLA4Ig, oral sirolimus, and alefacept provided prolonged renal allograft survival in NHPs (10). Since belatacept binds with higher affinity to the B7 molecules than CTLA4-Ig, and because oral administration of sirolimus results in markedly variable serum levels, we wanted to test the efficacy of belatacept and intramuscular sirolimus alone in preventing islet allograft rejection in nonhuman primates.

Three rhesus macaques were treated with belatacept and sirolimus after undergoing intraportal islet allotransplantation (Table 1). All three recipients achieved immediate insulin-independent normoglycemia with significantly prolonged allograft survival (median graft survival time of 225 days, range 172–245 days) compared to historic controls (16) receiving basiliximab and sirolimus therapy (median graft survival time of 8 days, range 8–10 days, $p=0.022$; Figure 1a). In addition, the combination of belatacept and sirolimus prevented the formation of alloantibody at the time of rejection (Figure 1b). Two of three recipients receiving belatacept and sirolimus rejected their allografts after cessation of all immunosuppression; the third recipient (RYo12) experienced rejection while on belatacept monotherapy (Figure 1c).

Addition of alefacept confers no additional survival benefit

Given that all three recipients rejected their grafts either at the cessation of belatacept therapy or on belatacept monotherapy, we concluded that this regimen failed to induce tolerance. There is evidence that treatment with belatacept incompletely controls the alloreactive CD28⁻ T cell response (12). Since these cells have been shown to be CD2 positive, we hypothesized that the addition of alefacept, an LFA3-Ig fusion protein, to the regimen of belatacept and sirolimus would effectively inhibit these CD8⁺CD2^{hi}CD28⁻ T cells and further promote tolerance.

Six rhesus macaques underwent intraportal islet transplantation and were treated with alefacept through post-operative day 56 along with belatacept and intramuscular sirolimus (Table 1). All six recipients achieved immediate insulin independent normoglycemia. One recipient was sacrificed with a functional graft at post-operative day 63 for weight loss and failure to thrive. The median graft survival time for the remaining five recipients in this cohort was 127 days (range 101–212 days), which was significantly shortened compared to the cohort receiving belatacept and sirolimus alone ($p=0.036$; Figure 2). Three recipients (RZw11, RVw11 and RRp12) experienced graft rejection following cessation of alefacept therapy but while still receiving belatacept and sirolimus. Rejection occurred on belatacept monotherapy or following cessation of all immunosuppression in the remaining two recipients (RDp12 and RAg12, respectively). As was seen with animals receiving belatacept and sirolimus alone, the regimen of alefacept, belatacept and sirolimus prevented the formation of alloantibody (data not shown).

The addition of alefacept increases frequency and severity of CMV reactivation

When compared to the cohort receiving belatacept and sirolimus alone, the cohort receiving alefacept had a significantly worse survival. We hypothesized that these disparate survival results could have resulted from incomplete CD2 blockade or from the consequences of over-immunosuppression, including viral reactivation.

Flow cytometric analysis of CD4⁺ and CD8⁺ T cells was performed to determine the extent of CD2 blockade by alefacept. CD2 MFI was markedly decreased on T_{Naive}, T_{CM} and T_{EM}

in recipients receiving alefacept (Figure 3a); this effect was not seen in animals receiving only belatacept and sirolimus (Figure 3b).

All islet allograft recipients were monitored for CMV reactivation given that selectively targeting T cells with alefacept could potentially alter protective immunity. All animals had undetectable levels of CMV prior to transplantation and initiation of immunosuppression. Four of six animals treated with alefacept had clinically significant CMV viremia (defined as viremia greater than 10,000 copies/mL), which necessitated intravenous antiviral therapy with ganciclovir. All four animals experienced significant viremia of greater than 40,000 copies/mL, and one recipient developed two episodes of viremia necessitating anti-viral therapy. However, none of the three animals that received belatacept and sirolimus alone had clinically significant CMV reactivation (Figure 4).

Discussion

These results, together with the concurrently submitted findings in an NHP kidney allograft model (Lo, et al., manuscript accepted), demonstrate that belatacept and optimally dosed sirolimus constitute a non-depleting, well-tolerated and effective regimen for the prolongation of islet and renal allograft survival, respectively, and prevent alloantibody production. As both belatacept and sirolimus are available for clinical use, investigations into the clinical utility of this combination are warranted. However, the results from both this and the Lo, et al. study clearly indicate that the mechanisms at play are immunosuppressive and not tolerogenic, and that additional immunosuppression added in the form of memory T cell elimination through alefacept exacts a cost in terms of protective immunity.

Previous versions of this regimen employed CTLA4-Ig, a variant with much lower affinity for the B7 complex, and oral sirolimus(10). Improving the regimen by using belatacept and intramuscular sirolimus provides more robust CD28/B7 blockade and more consistent serum rapamycin levels, respectively. These improvements resulted in significantly prolonged islet allograft survival compared to control animals receiving basiliximab induction and sirolimus maintenance therapy. We also have tested the effects of belatacept alone in the prevention of rejection in both kidney and islet models of allotransplantation, and found it to be ineffective as a monotherapy for prophylaxis against rejection. Thus, it is likely that the addition of sirolimus provided a survival benefit in comparison. Although the belatacept dosing was not identical to previous studies, the need for a contemporaneous control group to make this point was not viewed as sufficiently compelling to warrant additional primate surgery.

Given that all three islet allograft recipients treated with belatacept and sirolimus experienced rejection either on belatacept monotherapy or immediately after cessation of immunosuppression, we conclude that this regimen does not confer durable allograft tolerance. One explanation for this lack of tolerance may be the incomplete control the alloreactive T cell response by belatacept. Our group has previously shown that CD8 effector memory T cells tend to be CD2⁺ and CD28⁻(12). This suggests that belatacept may be ineffective at inhibiting these effector cells that lose CD28 expression, a population of cells that are more likely to produce cytokine and cytotoxic effector molecules. Alefacept, a CD2-specific LFA3-Ig fusion protein, has been found to inhibit belatacept-resistant proliferation(12). Thus, we hypothesized that adding alefacept to the combination of belatacept and intramuscular sirolimus would further improve allograft survival, as we had previously shown when combined with CTLA4-Ig and oral sirolimus(10).

Combining alefacept with belatacept and sirolimus provided no additional survival benefit despite providing marked decrease in CD2 expression on all T cell subsets. This lack of

additional efficacy is likely due to the improvements made to the regimen: using a higher affinity variant of CTLA4-Ig and achieving more consistent and therapeutic serum rapamycin levels by administering sirolimus intramuscularly. This optimized regimen provided prolonged allograft survival without the need for additional immunosuppressive agents. However, it was unexpected to find that the addition of alefacept to this optimized regimen resulted in significantly worse allograft survival. This finding can likely be explained by alterations in protective immunity of immunosuppressed recipients. The addition of alefacept to belatacept and sirolimus resulted in compromised anti-viral protective immunity, which manifested as more frequent and severe CMV reactivation.

The relationship between viral infection and allospecific T cell responses has been well documented and likely contributes to allograft rejection. Heterologous immune responses, such as those to viruses, can act as a significant barrier to long-term tolerance and graft survival(17). A large percentage of virus-specific T cells have been shown to be cross-reactive to allo-HLA molecules(18), and allospecific CD8 memory T cells can be found in response to viral infections(19). These findings suggest that in this experiment viral reactivation resulting from altered protective immunity following the addition of alefacept may have potentiated an allospecific response resulting in worse islet allograft survival.

Another possible explanation for the worse survival seen following the addition of alefacept to belatacept and sirolimus is the direct effects of CMV infection on islet allograft survival. Infecting rats with CMV has been shown to accelerate islet graft failure compared to control animals, and this effect was not abrogated by infection control with ganciclovir(20). This suggests that CMV viremia, even if controlled by treatment with antiviral therapy, may be an independent risk factor for early islet allograft failure. A final consideration, addressed by Lo, et al., but not investigated in this set of experiments, is the depletion effect of alefacept on regulatory T cells (Lo, et al., manuscript submitted). Regardless, the addition of alefacept did not have the anticipated salutary effect.

In summary, these results and those concurrently published in an NHP renal allograft transplantation model show that the combination of belatacept and sirolimus significantly prolongs islet and renal allograft survival, respectively. This calcineurin inhibitor-free and steroid-free regimen is well tolerated and should be considered as a candidate therapy for investigation in clinical islet allotransplantation. While treatment with belatacept and sirolimus failed to induce tolerance, the resultant prolonged survival suggests that this regimen may have a role as maintenance therapy. Consistent with findings in human de novo kidney transplantation(21), we have also shown in both islet and kidney transplant models that combining alefacept to an optimized immunosuppression regimen provides no additional survival benefit. Establishing a safe and effective immunosuppression regimen for use in clinical islet transplantation is one of several issues hindering more widespread applicability of this treatment for patients with type 1 diabetes mellitus; however, these results provide evidence that progress is being made towards that goal.

Acknowledgments

This study was supported by National Institute of Allergy and Infectious Diseases contract U19 AI051731 (CPL and ADK), the National Center for Research Resources P51 RR000165 (currently Office of Research Infrastructure Programs/OD R24 OD010976), and the Juvenile Diabetes Research Foundation. IRB and MCL were supported by the Robbins Fellowship in Islet Transplantation.

The authors would like to thank members of Yerkes National Primate Research Center and the Emory Transplant Center for their excellent technical assistance and support.

Abbreviations

CTLA4-Ig	Cytotoxic T lymphocyte associated antigen 4 – immunoglobulin
CMV	<i>Cytomegalovirus</i>
CNI	Calcineurin inhibitors
CoB	Costimulation blockade
FBG	Fasting blood glucose
IEq	Islet equivalents
IVGTT	Intravenous glucose tolerance test
LFA3-Ig	Leukocyte function associated antigen 3 – immunoglobulin
NHP	Nonhuman primate
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{Naive}	Naïve T cell

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Figure 1. Treatment with belatacept and sirolimus prolongs islet allograft survival and prevents donor specific alloantibody

The immunosuppression regimen of belatacept and intramuscular sirolimus (A) significantly prolongs islet allograft survival compared to historic controls that received basiliximab and sirolimus(16) ($p=0.022$) and (B) prevents the formation of donor specific alloantibody at the time of rejection as measured by anti-rhesus IgG. (C) Fasting blood glucose (FBG) graphs (solid line) for transplant recipients receiving belatacept and sirolimus reveal that two recipients (RMt12 and RCd13) rejected their grafts after all immunosuppression was withdrawn and one recipient (RYo12) rejected its graft after rapamycin levels waned (dashed line) but while still on belatacept therapy.

Figure 2. The addition of alefacept provides no survival benefit

(A) Adding alefacept to the regimen of belatacept and intramuscular sirolimus results in worse survival ($p=0.036$). (B) Fasting blood glucose (FBG) graphs (solid line) for transplant recipients receiving alefacept, belatacept and sirolimus show that three recipients (RZw11, RVw11 and RRRp12) rejected their grafts prior to cessation of sirolimus (dashed line), one recipient (RDp12) rejected its graft after rapamycin levels waned while still receiving belatacept, and one recipient (RAg12) rejected its graft following cessation of all immunosuppression.

Figure 3. Treatment with alefacept results in CD2 blockade

(A) Treatment with alefacept resulted in a marked decrease in CD2 expression as measured by mean fluorescence intensity (MFI) on CD4⁺ and CD8⁺ naïve (T_{Naive}), central memory (T_{CM}) and effector memory (T_{EM}) T cells. (B) This effect is not seen in animals treated with belatacept and sirolimus alone.

Figure 4. CMV reactivation may have contributed to worse graft survival in the cohort receiving alefacept

(A) Clinically significant *Cytomegalovirus* (CMV) reactivation (as measured by PCR and expressed as copies/mL) in the cohort receiving alefacept, belatacept and sirolimus may have contributed to worse survival compared to the transplant recipients receiving belatacept and sirolimus alone (B), which experienced no clinically significant viral reactivation.

Table 1

Treatment cohorts, transplant characteristics and survival

Recipient	Therapy	IEQ/kg	Graft Survival (days)	Comments
RMt12	Belatacept/sirolimus	10,295	225	Rejection
RYo12	Belatacept/sirolimus	18,035	172	Rejection
RCd13	Belatacept/sirolimus	10,683	245	Rejection
RQz6 *	Basiliximab/sirolimus	12,980	8	Rejection
RIb7 *	Basiliximab/sirolimus	10,903	8	Rejection
RMc11 *	Basiliximab/sirolimus	13,796	10	Rejection
RVv11	Alefacept/belatacept/sirolimus	14,466	>63	Failure to thrive
RZw11	Alefacept/belatacept/sirolimus	17,943	127	Rejection
RAg12	Alefacept/belatacept/sirolimus	22,540	212	Rejection
RVw11	Alefacept/belatacept/sirolimus	11,002	124	Rejection
RDp12	Alefacept/belatacept/sirolimus	20,150	142	Rejection
RRp12	Alefacept/belatacept/sirolimus	22,203	101	Rejection

* Historic control