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CTLA4Ig Prevents Alloantibody Formation Following Nonhuman Primate Islet Transplantation Using the CD40-specific Antibody 3A8

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Abstract

Islet transplantation to treat type 1 diabetes has been limited in part by toxicities of current immunosuppression and recipient humoral sensitization. Blockade of the CD28/CD80/86 and CD40/CD154 pathways has shown promise to remedy both these limitations, but translation has been hampered by difficulties in translating CD154-directed therapies. Prior CD40-directed regimens have led to prolonged islet survival, but fail to prevent humoral allosensitization. We therefore evaluated the addition of CTLA4Ig to a CD40-blockade based regimen in nonhuman primate (NHP) alloislet transplantation. Diabetic rhesus macaques were transplanted allogeneic islets using the CD40-specific antibody 3A8, basiliximab induction, and sirolimus with or without CTLA4Ig maintenance therapy. Allograft survival was determined by fasting blood glucose levels and flow cytometric techniques were used to test for donor-specific antibody (DSA) formation. CTLA4Ig plus 3A8, basiliximab and sirolimus was well tolerated and induced long-term islet allograft survival. The addition of CTLA4Ig prevented DSA formation, but did not facilitate withdrawal of the 3A8-based regimen. Thus, CTLA4Ig combines with a CD40-specific regimen to prevent DSA formation in NHPs, and offers a potentially translatable calcineurin inhibitor-free protocol inclusive of a single investigational agent for use in clinical islet transplantation without relying upon CD154 blockade.

Keywords

Islet transplantation; nonhuman primate; immunosuppressive therapy; alloantibodies

Introduction

Advances in clinical islet transplantation have made it an attractive, albeit experimental, therapy available to a subset of type 1 diabetics with hypoglycemic unawareness (1). However, one major obstacle preventing the more widespread application of alloislet transplantation is the toxicity associated with current immunosuppression. Specifically, calcineurin inhibitors (CNI) and corticosteroids contribute to many long-term adverse

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effects experienced by transplant recipients. Although steroid avoidance has been shown to be feasible, CNIs associated with diabetes, hypertension and nephrotoxicity continue as standards in most clinical immunosuppressive protocols (1, 2). Furthermore, use of these agents is thought to hamper islet function and limit the already taxed islet mass transplanted using current islet procurement techniques.

The CD28/CD80/86 and CD40/CD154 costimulation pathways play important roles in allograft rejection. Inhibition of these pathways has been effective in promoting graft survival in experimental transplant models without the use of CNIs or steroids (3-7). Whereas in some animal models single pathway blockade alone has been sufficient for allograft protection (5, 6), simultaneous targeting of the CD28/CD80/86 and CD40/CD154 pathways has often synergistically prolonged graft survival (3, 4, 7). Unfortunately, clinical translation of this approach has been hampered by concerns that CD154-specific antibodies may promote thromboembolism (8). As such, alternative methods of blocking this pathway have been investigated, particularly blockade of the CD40 molecule (9, 10). Recent success targeting CD40 has sparked new interest in the translation of CD40-specific agents (11-13), as evidenced by upcoming clinical trials testing the fully human anti-CD40 mAb, 4D11. There are numerous CD40-specific agents under investigation at present that possess unique properties with regard to agonism, antagonisms, depletion potential and affinity, presumably varying based on epitope targeted and isotype. As such, the ideal agent remains to be defined.

Our group recently has shown that 3A8, a novel CD40-specific mAb can prolong allograft survival without cellular depletion in a nonhuman primate (NHP) model of alloislet transplantation (12). Despite its efficacy, 60% of islet recipients developed donor-specific antibodies (DSA), an increasingly recognized problem in clinical islet transplantation and a possible barrier for the broader use of this experimental therapy to treat type 1 diabetes (14). Based on prior reports that CD28/CD80/86 pathway blockade can attenuate alloantibody formation (5, 7, 15), the addition of CTLA4Ig to our 3A8-based regimen has potential to not only increase allograft survival, but also prevent the humoral sensitization observed in our previous study (12) while still avoiding the use of CNIs and steroids.

Here, we evaluated the effects of adding CTLA4Ig to our previous non-depleting anti-CD40-based protocol in the same rhesus macaque alloislet model (12). The addition of CTLA4Ig prevented DSA formation. However, although the addition of CTLA4Ig maintenance therapy was well tolerated and long-term islet allograft survival was observed, it did not facilitate withdrawal of the 3A8-based regimen, indicating that this approach does not induce tolerance and that CTLA4Ig requires adjuvant immunosuppressive agents to prevent the rejection of engrafted islets. These results support the translation of combined CD80/86 and CD40 blockade in an anti-CD154- and CNI-free regimen for use in clinical islet transplantation.

Materials and Methods

NHPs

This study was conducted according to the *Guide for the Care and Use of Laboratory Animals* and approved by Emory University's Institutional Animal Care and Use Committee. Captive bred rhesus macaques were used as recipients (3-5 kg) and donors (10-20 kg). Donor-recipient pairs were class I and/or class II mismatched by molecular MHC typing and exhibited alloreactivity in mixed lymphocyte cultures.

Donor pancreatectomy and islet isolation

Donor pancreatectomies were performed one day before transplantation. Via a midline laparotomy incision, the pancreas was mobilized, the aorta cannulated, and the animal exsanguinated. Cold slush was placed around the pancreas and the common bile and pancreatic ducts ligated. The remainder of the pancreas was dissected free and removed.

Islet isolation was achieved with minor modifications of the automated method for human islet isolation using Liberase (0.47-0.71 mg/ml; Roche, Indianapolis, IN). A four layer discontinuous Euroficoll gradient and Cobe 2991 blood cell processor were used for purification of islets.

Diabetes induction and islet transplantation

Diabetes was induced by streptozocin (Zanosar, Teva Pharmaceuticals, Irvine, CA). The first four recipients received 150 mg/kg intravenously, but because of streptozocin toxicity, the fifth was dosed according to body surface area (1600 mg/m²).

After overnight culture, samples of the final islet preparation were stained with dithizone, counted and expressed as islet equivalents (IEQ), and re-suspended in transplant media. Recipient abdomens were opened via a midline mini-laparotomy incision, a mesenteric colic vein cannulated with a 20-gauge catheter and the islet suspension infused into the liver.

Glucose management

Blood glucose was measured via earstick. Insulin NPH (Novolin; Novo Nordisk, Princeton, NJ) and glargine (Lantus; Sanofi-Aventis, Bridgewater, NJ) were administered to maintain fasting blood glucose (FBG) < 300 mg/dl in diabetic monkeys. Intravenous glucose tolerance tests (IVGTT) were performed pre-transplant to confirm diabetes and monthly post-transplant. One ml/kg of 50% dextrose was injected intravenously. Blood samples were taken for glucose and c-peptide measurements 0, 10, 30, 60 and 90 minutes after injection. Rejection was defined as FBG >150 mg/dl on two consecutive days.

Immunosuppression

Animals received CTLA4Ig, 3A8 (anti-CD40 mAb), basiliximab (anti-IL-2R mAb) and sirolimus. CTLA4Ig (20 mg/kg intravenously) was administered on post-operative days (POD) -2, 0, 2, 6, 13, 20 and indefinitely every two weeks thereafter. 3A8 was administered intravenously at 20 mg/kg on POD -2 and 0, 10 mg/kg on POD 2, 6 and 9, and 5 mg/kg on POD 13, 16, 20, 23, 27, 30. Basiliximab (0.3 mg/kg intravenously) was administered on POD 0 and 2. Sirolimus was given intramuscularly daily to achieve trough levels of 10-15 ng/ml until POD 60, and then decreased to achieve trough levels of 5-10 ng/ml until discontinuation on POD 134. Anti-viral prophylaxis consisting of oral valganciclovir (60 mg twice daily) was administered to all recipients while on immunosuppressive therapy. Bristol-Myers Squibb provided CTLA4Ig. The hybridoma producing 3A8 was obtained from the American Type Culture Collection (Manassas, VA) and antibody produced in vitro. Basiliximab (Simulect, Novartis, East Hanover, NJ), valganciclovir (Valcyte; Roche, Nutley, NJ) and sirolimus (Rapamune, Wyeth, New York, NY) were purchased from the Emory University Hospital Pharmacy.

Histology

Tissues were fixed in 10% formalin and processed in paraffin blocks for hematoxylin and eosin (H&E) staining and immunohistochemical analysis. Tissue sections were labeled with insulin-, CD3-, CD20- and C4d-specific primary antibodies, and then visualized using the

LSAB+ labeled Streptavidin-Biotin kit. All materials were obtained from Dako (Carpinteria, CA) except the anti-C4d antibody (American Research Products, Waltham, MA).

DSA detection

Donor lymphocytes (5×10^5 cells) were blocked with goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), mixed with recipient sera, washed twice, and incubated with FITC-labeled goat anti-monkey IgG (KPL, Gaithersburg, MD). Flow cytometry was used to determine anti-rhesus IgG mean fluorescence intensity (MFI) for each test serum for comparison to pre-transplant values and controls.

Statistical Analysis

The logrank (Mantel-Haenszel) test was used to compare survival between groups. A P value < 0.05 was considered statistically significant.

Results

CTLA4Ig plus anti-CD40-based therapy promoted alloislet survival

Based on previous successes using dual blockade of the CD28/CD80/86 and CD40/CD154 pathways to synergistically prolong allograft survival (3, 4, 7, 16, 17), we evaluated the addition of CTLA4Ig to a non-depleting anti-CD40-based regimen in a NHP islet transplantation model. Five recipients were treated with 3A8 and basiliximab induction, sirolimus, and CTLA4Ig maintenance therapy. These were compared to previously published controls lacking CTLA4Ig (12).

Diabetic monkeys were transplanted allogeneic islets ($>10,000$ IEQ/kg) from a single donor. All recipients experienced normoglycemia, insulin independence, and long-term graft survival for 142, 298, 284, >71 and 117 days after transplant (Figure 1A). The addition of CTLA4Ig did not facilitate discontinuation of the 3A8-based regimen by further prolonging allograft survival compared to historical controls treated with 3A8 plus basiliximab and sirolimus alone (median graft survival 213 vs. 202 days, $P = 0.7174$) (Table 1) (Figure 1B). Importantly, the addition of CTLA4Ig was not deleterious, as survival remained significantly prolonged when compared to basiliximab and sirolimus-treated controls (median graft survival 213 vs. 8 days, $P = 0.0046$).

IVGTTs were performed to further characterize the ability of recipients to respond to glucose challenge. Following diabetes induction, all animals experienced sustained hyperglycemia with no c-peptide production (< 0.2 ng/ml) at baseline and post-glucose challenge. After transplantation, recipients demonstrated normalization of their blood glucose curves and detectable c-peptide levels resembling pre-diabetes kinetics (Figure 1C).

Given the potential for our immunosuppressive regimen to disrupt the maintenance of protective immunity to latent viral infections and adversely affect islet engraftment or survival, we monitored peripheral blood levels of rhesus CMV. With the use of anti-viral prophylaxis, CMV levels were controlled below clinically relevant levels (data not shown). Histologic examination of recipient livers at necropsy was consistent with cellular rejection, characterized by islet destruction and dense focal lymphocytic infiltrates comprised of many CD3⁺ and few CD20⁺ cells without C4d staining (Figure 2). Recipient monkeys exhibited excellent weight retention and growth, and pathologic analysis of all islet recipients at necropsy was grossly and microscopically negative for any evidence of thromboembolism.

One animal was euthanized on POD 71 due to failure to thrive. This recipient was normoglycemic and insulin independent at the time of sacrifice. Histologically, viable islets

were identified in the liver free of lymphocytic infiltrates and strong insulin positivity (Figure 2). These findings support that this animal was not undergoing immunologic rejection of the islet allograft.

CTLA4Ig plus 3A8-based therapy did not alter peripheral lymphocyte populations

Examination of leukocyte lineages in CTLA4Ig/3A8-treated monkeys revealed no alteration in circulating lymphocyte counts. The absolute numbers of CD3⁺ and CD20⁺ cells in the peripheral blood did not significantly increase or decrease during the post-transplant period (Figure 3). This is in contrast to the cellular depletion seen in previous studies with other anti-CD40 mAbs (9, 11, 16). Analysis of CD4⁺ and CD8⁺ T cell subsets also showed no gross contraction or expansion of naïve or memory cell phenotypes (data not shown).

CTLA4Ig prevented DSA formation

Recipient monkey serum at rejection was tested for donor-specific IgG using a flow cytometry-based assay. Table 1 demonstrates that 60% of historical controls receiving the 3A8-based regimen alone had developed DSAs at rejection. In these recipients, alloantibodies had formed at the time of or following immunosuppression withdrawal (12). In contrast, all five animals treated with CTLA4Ig plus the 3A8-based regimen failed to generate an IgG antibody response against their donors (Figures 4A and 4B). We cannot exclude the possibility of late alloantibody formation in the one islet recipient euthanized prematurely without rejection.

Discussion

Islet transplantation is currently under investigation for the treatment of type I diabetics with hypoglycemic unawareness. One of the main obstacles to broaden its application, however, continues to be toxicities associated with current immunosuppression. Since the implementation of the Edmonton Protocol (1), overall reliance on steroids has decreased, but replacement with drugs such as CNIs remains problematic, particularly given the potential for CNIs to promote insulin resistance.

Costimulatory blockade offers the benefit of selectively targeting pathways required for optimal T-cell activation, while minimizing non-immune toxicities (18). As such, several notable studies have described prolongation of graft survival across several transplant models using CD40/CD154 costimulation blockade alone (6, 9, 11, 12) and in combination with blockade of the CD28/CD80/86 pathway (3, 4, 7). Specifically, combination CD80/86-blockade and anti-CD40 therapy has synergistically prolonged graft survival in murine and NHP models (16, 17). Costimulation blockade with CD40/CD154 pathway-specific agents has required simultaneous CD80/86-blockade to protect highly immunogenic neonatal porcine islet xenografts from rejection in NHPs (19). Furthermore, both CTLA4Ig and 3A8 were necessary to produce mixed chimerism in a rhesus bone marrow transplant model (13).

The inability of CD28/CD80/86 pathway blockade to improve allograft survival or permit withdrawal of the anti-CD40-based regimen in this study underscores the importance of evaluating therapeutics in multiple models. While in the chimerism model, engraftment was only observed when 3A8 was combined with CTLA4Ig, basiliximab and sirolimus (13), alloislet survival in our model was not improved with maintenance CD80/86 blockade. This result possibly reflects the relative immunogenicity of the two transplant types, in that bone marrow transplants despite MHC matching are highly immunogenic, likely due to the ubiquitous presence of donor antigen in peripheral blood, secondary lymph organs, and bone marrow. These allografts may represent a more challenging immune barrier than islet transplants confined to the liver. The synergy observed in the chimerism model between

CTLA4Ig and 3A8 suggests that this combination, though not synergistic in the NHP islet model, may be required in more immunogenic transplant settings, such as clinical islet transplantation.

The exact mechanisms responsible for islet allograft failure remain unclear. However in this and previous studies (5, 16, 19), cellular mechanisms have been observed to play a primary role in mediating rejection. Here, immunohistologic analysis of islets experiencing rejection demonstrated a predominant T cell infiltrate without C4d staining (Figure 2). Furthermore, in our experience a minority of islet recipients has manifested clinical and histologic rejection in the absence of DSA formation (12). These findings are supported by the persistence of recipient T cell alloreactivity in vitro as measured by mixed lymphocyte cultures throughout the post-transplant period (data not shown). Consequently, further investigation of the cellular processes responsible for alloislet rejection is warranted to guide the continued development of novel immunosuppressive regimens such as the one tested in this study.

Notably, the addition of CTLA4Ig did prevent the development of DSAs in all graft recipients, even at the time of rejection. This salutary influence of CTLA4Ig is critically important given the pervasive allosensitization observed in recent clinical islet transplantation trials (14). This broad alloantigen sensitization limits the ability of islet recipients to obtain future kidney, solid organ pancreas or islet transplants, and has been cited as a significant concern for the safety of patients enrolling in clinical islet trials. While the mechanisms underlying the inhibitory effect on alloantibody formation was beyond the scope of this preclinical in vivo NHP study, we hypothesize that the ability of CTLA4Ig to inhibit donor-reactive CD4⁺ T cell responses (20), thereby reducing the availability of CD4⁺ T cell help for B cell class switching, may be responsible. The observed impact of CD28/CD80/86 pathway blockade on the development of DSA provides rationale for continued research to translate these concepts into clinical trials, considering the limitations associated with DSA formation.

We have shown that CTLA4Ig in combination with a non-depleting 3A8-based regimen markedly prolongs islet allograft survival in a NHP model and prevents DSA formation. Our results support the continued development of combined therapies involving CD80/86-blockade and CD40-specific agents as a strategy to obviate the requirement for CD154-specific agents, and offer a potentially translatable CNI-free regimen - one that requires only a single unapproved agent for testing in clinical islet transplantation.

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List of Abbreviations

CNI	Calcineurin inhibitor
DSA	Donor-specific antibody
FBG	Fasting blood glucose
H&E	Hematoxylin and eosin
IEQ	Islet equivalent

IVGTT	Intravenous glucose tolerance test
MFI	Mean fluorescence intensity
NHP	Nonhuman primate
POD	Post-operative day

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Figure 1. CTLA4Ig plus an anti-CD40-based regimen induced long-term islet allograft survival and restoration of glucose tolerance kinetics in diabetic rhesus macaques

Diabetic rhesus macaques were transplanted allogeneic islets and treated with CTLA4Ig plus 3A8 (anti-CD40 mAb), basiliximab and sirolimus ($n = 5$). Rejection was defined as FBG values >150 mg/dl on two consecutive days after transplant. (A) Representative FBG graph of islet recipients. Survival times in days of each recipient are listed. (B) Kaplan-Meier survival curves of current experimental group: CTLA4Ig plus 3A8, basiliximab and sirolimus (B/S) (white squares), and historical control groups: 3A8 and B/S (black triangles, $n = 5$), and B/S alone (white circles, $n = 3$) (12). (C) Islet recipient ability to achieve glycemic control in response to glucose challenge was tested pre-diabetes (white squares), post-diabetes (black triangles) and post-transplant (white circles). Representative blood glucose and c-peptide levels in one recipient measured prior to the administration of a glucose bolus (time 0) and 10, 30, 60 and 90 minutes later.

Figure 2. Islet allograft histology

Histologic liver sections harvested at necropsy are shown. Sections were stained by standard H&E and immunohistochemical methods for insulin, CD3, CD20 and C4d. Representative recipient liver sections of experimental group animals experiencing rejection and one recipient euthanized with a functioning graft due to failure to thrive. Scale bar: 100 μ m.

Figure 3. CTLA4Ig plus 3A8-based therapy did not alter peripheral lymphocyte populations
Peripheral leukocyte counts were serially monitored in all recipients (n = 5). Absolute numbers of CD3⁺ (black squares) and CD20⁺ (white circles) cells before and after transplant are depicted. Data represent mean $\pm$ SEM.

Figure 4. Addition of CTLA4Ig to an anti-CD40-based regimen prevented DSA formation

Donor lymphocytes were incubated with allograft recipient pre- and post-transplant serum at time of rejection followed by fluorescent anti-rhesus IgG to test for the presence of alloantibodies by flow cytometry. (A) Pre- and post-transplant anti-donor IgG levels in a known positive control and a representative islet recipient (4-184). (B) Anti-donor IgG MFI pre- and post-transplant for all islet recipients and a known positive control.

Table 1

Recipient Groups and Islet Allograft Survival

Treatment	Recipients (n)	IEQ/kg (mean $\pm$ SD)	Graft Survival (days)	DSA Formation
CTLA4Ig 3A8 Basiliximab Sirolimus	5	13,679 $\pm$ 1,953	142, 298, 284, >71 [*] , 117	0/5 (0%)
3A8 [†] Basiliximab Sirolimus	5	14,020 $\pm$ 3,179	155, 312, 208, 158, 202	3/5 (60%)
Basiliximab [†] Sirolimus	3	12,560 $\pm$ 1,492	8, 8, 10	Not Determined

IEQ/kg - Islet equivalents/kilogram

DSA - Donor-specific antibody

^{*}
- Failure to thrive

[†]
- Historical controls (12)