Title

Tolerance Induction and Reversal of Diabetes in Mice Transplanted with Human Embryonic Stem Cell-Derived Pancreatic Endoderm

Journal

Cell Stem Cell, 16(2)

ISSN

1934-5909

Authors

Szot, Gregory L
Yadav, Mahesh
Lang, Jiena
et al.

Publication Date

2015-02-01

DOI

10.1016/j.stem.2014.12.001

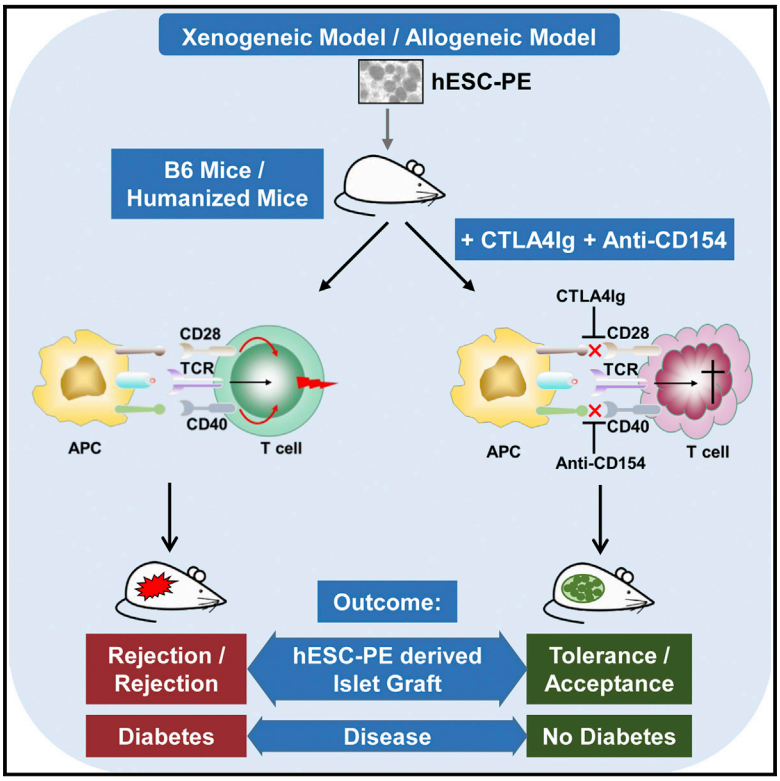
Peer reviewed

Article

Cell Stem Cell

Tolerance Induction and Reversal of Diabetes in Mice Transplanted with Human Embryonic-Stem-Cell-Derived Pancreatic Endoderm

Graphical Abstract



Authors

Gregory L. Szot, Mahesh Yadav, ..., Hélène Bour-Jordan, Jeffrey A. Bluestone

Correspondence

jeff.bluestone@ucsf.edu

In Brief

Szot et al. demonstrate that targeting T cell costimulatory pathways prevents rejection of xenogeneic human embryonic-stem-cell-derived pancreatic endoderm (hESC-PE) in mice and allogeneic hESC-PE in humanized mice. The approach enabled grafts to develop into islet-like structures capable of producing human insulin and maintaining normal blood glucose levels.

Highlights

- Costimulation blockade prevents rejection of xenogeneic hESC-derived islets
- Short-term treatment induces long-term tolerance to xenogeneic hESC-derived islets
- Tolerance induced by costimulation blockade is transferable independently of Tregs
- Costimulation blockade prevents rejection of allogeneic hESC islets by human PBMCs

Szot et al., 2015, Cell Stem Cell *16*, 1–10
February 5, 2015
http://dx.doi.org/10.1016/j.stem.2014.12.001

CellPress

Please cite this article in press as: Szot et al., Tolerance Induction and Reversal of Diabetes in Mice Transplanted with Human Embryonic-Stem-Cell-Derived Pancreatic Endoderm, Cell Stem Cell (2015), http://dx.doi.org/10.1016/j.stem.2014.12.001

Cell Stem Cell
Article

Tolerance Induction and Reversal of Diabetes in Mice Transplanted with Human Embryonic-Stem-Cell-Derived Pancreatic Endoderm

Gregory L. Szot,[1] Mahesh Yadav,[1] Jiena Lang,[1] Evert Kroon,[2] Justin Kerr,[2] Kuniko Kadoya,[2] Eugene P. Brandon,[2] Emmanuel E. Baetge,[2] Hélène Bour-Jordan,[1,3] and Jeffrey A. Bluestone[1,3,*]
[1]Diabetes Center, University of California, San Francisco, San Francisco, CA 94143, USA
[2]ViaCyte, Incorporated, San Diego, CA 92121, USA
[3]Co-senior author
*Correspondence: jeff.bluestone@ucsf.edu
http://dx.doi.org/10.1016/j.stem.2014.12.001

SUMMARY

Type 1 diabetes (T1D) is an autoimmune disease caused by T cell-mediated destruction of insulin-producing β cells in the islets of Langerhans. In most cases, reversal of disease would require strategies combining islet cell replacement with immunotherapy that are currently available only for the most severely affected patients. Here, we demonstrate that immunotherapies that target T cell costimulatory pathways block the rejection of xenogeneic human embryonic-stem-cell-derived pancreatic endoderm (hESC-PE) in mice. The therapy allowed for long-term development of hESC-PE into islet-like structures capable of producing human insulin and maintaining normoglycemia. Moreover, short-term costimulation blockade led to robust immune tolerance that could be transferred independently of regulatory T cells. Importantly, costimulation blockade prevented the rejection of allogeneic hESC-PE by human PBMCs in a humanized model in vivo. These results support the clinical development of hESC-derived therapy, combined with tolerogenic treatments, as a sustainable alternative strategy for patients with T1D.

INTRODUCTION

Type 1 diabetes (T1D) is a human autoimmune disease resulting from the destruction of insulin-producing β cells within the pancreatic islets of Langerhans by autoreactive T cells (Bluestone et al., 2010). Currently, the disease is managed by multiple daily injections of insulin that imperfectly control blood glucose levels in many diabetic patients, often leading to complications and a reduced quality of life (Kilpatrick et al., 2009). Any therapy for T1D patients with long-term disease will need to include islet replacement strategies. However, there are two major obstacles to making islet replacement therapies widely available, namely the lack of satisfactory immunotherapies in islet transplantation and the paucity of available donor organs.

During the last 15 years, islet cell transplantation has emerged as one of the most promising insulin replacement therapies for diabetic patients (Shapiro et al., 2000; Barton et al., 2012). The outcome of islet transplantation has steadily improved over time as the overall percentage of recipients who were insulin independent at 3 years posttransplantation increased from 27% in the 1999–2002 era to 44% in the 2007–2010 era (Barton et al., 2012; Bellin et al., 2012; Tiwari et al., 2012). Success rates for islet transplantation average 50%–70% insulin independence at 5 years in some transplant centers (Barton et al., 2012; Bellin et al., 2012; Shapiro and Ricordi, 2014). Nevertheless, current success rates suggest that available induction and maintenance immunosuppression regimens are unable to completely block islet cell loss in transplant recipients. In addition, continuous and indefinite immunosuppression can result in significant morbidity, including increased risks of cancer and infections, making islet transplantation a less than ideal option for most diabetic patients. Immunotherapies targeting T cell costimulation or adhesion pathways have been effective in inducing long-term tolerance in animal models of islet xenograft transplantation (Arefanian et al., 2010; Lenschow et al., 1992; Thompson et al., 2011) and have yielded promising results in small clinical trials in T1D patients (Posselt et al., 2010a, 2010b).

Recent progress in the capability to manufacture stem-cell-derived pancreatic endoderm cells that develop into fully functional β cells after in vivo transplantation has generated tremendous enthusiasm (Hebrok, 2012; Van Hoof et al., 2009). D'Amour et al. showed that in vitro culture conditions that mimicked embryonic pancreas development resulted in differentiation of human embryonic stem cells (hESCs) into definitive endoderm and subsequently into insulin-producing β-like cells (D'Amour et al., 2005, 2006). This multistep approach, corroborated by other groups (Eshpeter et al., 2008; Jiang et al., 2007; Rezania et al., 2012), showed that these cells released C peptide in response to a variety of stimuli but failed to respond to glucose stimulation. Recently, two groups showed that functional β cells that resemble adult mature β cells and produce insulin upon glucose stimulation could be generated in vitro using improved culture and differentiation protocols (Pagliuca et al., 2014; Rezania et al., 2014). Importantly, transplantation of hESC-derived pancreatic endoderm (hESC-PE) or more functional β cells into immunodeficient mice promoted its differentiation into glucose-responsive, single hormone-positive endocrine cells that produced insulin at levels similar to transplanted human islets upon glucose stimulation (Kroon et al., 2008) (Pagliuca et al., 2014; Rezania et al., 2014).

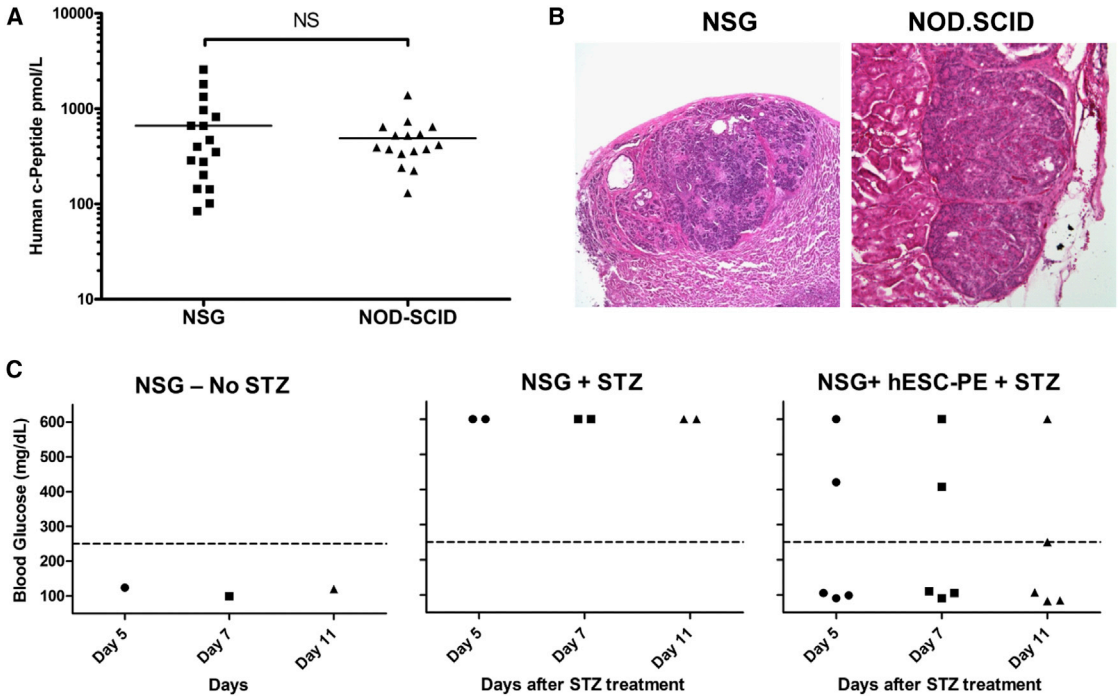


Figure 1. Human ESC-Derived Pancreatic Endoderm Differentiates into Fully Functional Insulin-Producing Cells in Immunodeficient Mice

(A) Human ESCs were differentiated into pancreatic endoderm (hESC-PE) in vitro and transplanted under the kidney capsule into immunodeficient NOD.SCID mice (n = 16) or NOD.SCID.IL-2Rgamma^{null} (NSG) mice (n = 20). Human C peptide levels were measured in the serum after glucose or arginine stimulation at least 80 days postengraftment and did not significantly differ between NOD-SCID and NSG recipients (t test, $p > 0.05$).

(B) Indicated recipients of hESC-PE were euthanized around 100 days posttransplantation and histological analysis was performed by H&E staining of hESC-PE grafts.

(C) NSG mice were left untreated (NSG controls, n = 1, left panel), treated with STZ (n = 2, middle panel), or implanted with hESC-PE and treated with STZ >90 days after implantation (n = 5, right panel). BG levels were measured at indicated time points after STZ treatment.

See also Figure S1.

In this study, we show that hESC-PE transplanted into immunocompetent mice is rejected. Costimulation blockade prevented the rejection of the hESC-PE and led to regulatory T cell-independent immune tolerance. Costimulation blockade further prevented human peripheral blood nonnuclear cells (PBMCs) from rapidly destroying allogeneic hESC-derived insulin-producing cells, resulting in their ability to regulate blood glucose in the absence of endogenous islets. These data show that despite their immunogenicity, hESC-PE is a self-replenishing source of cells that could potentially represent an attractive option for patients with T1D in the context of tolerogenic immunotherapy.

RESULTS

hESC-Derived Pancreatic Endoderm Generates Functional Insulin-Producing Cells In Vivo Regardless of the Presence of Innate Lymphoid Cell Elements

hESCs could be directed to differentiate into hESC-PE in vitro, which efficiently gave rise to functional islet-like structures in vivo after their implantation into immunodeficient SCID-Bg and nude mice on nonautoimmune backgrounds (Kroon et al., 2008). Innate lymphoid cell (ILC) populations (ILC1, ILC2, and ILC3) have been implicated in both inflammatory responses and tissue repair (Hazenberg and Spits, 2014). One subset, natural killer (NK) cells, has been shown to influence the in vivo growth or differentiation of ESCs and ESC-derived cells (Dressel et al., 2010; Frenzel et al., 2009; Ma et al., 2010; Nussbaum et al., 2007). Thus, we determined if the presence or absence of ILCs would alter the differentiation of hESC-derived endocrine progenitors after implantation. Human ESC-PEs were transplanted into immunodeficient NOD.SCID mice (which contain ILCs) or NOD.SCID.IL-2Rgamma^{null} (NSG) mice (deficient in ILCs). Ninety days postengraftment, high levels of human C peptide were detected in NSG mice upon glucose challenge (Figure 1A). Histological examination showed islet-like clusters of cells in the graft surrounded by connective tissue (Figure 1B) producing endocrine hormones, including insulin, somatostatin, and glucagon in the graft (data not shown). There were no differences in the generation of functional islet-like structures in *B6-TCR*$\alpha^{-/-}$, *B6-RAG*$^{-/-}$, NOD-SCID, and NSG mice (Figures 1 and S1 available online).

To examine graft function, we treated NSG mice transplanted with hESC-PE with streptozotocin (STZ), which specifically destroys mouse, but not human, β cells (Hosokawa et al., 2001), for over 90 days. hESC-PE grafts effectively regulated blood glucose (BG) independently of endogenous insulin-secreting β cells in most STZ-treated NSG mice (Figure 1C) and did so to a similar extent in NOD-SCID mice (Figure 2B). Thus, differentiation of fully functional islet-like clusters in vivo was not affected by NK or other ILC subsets.

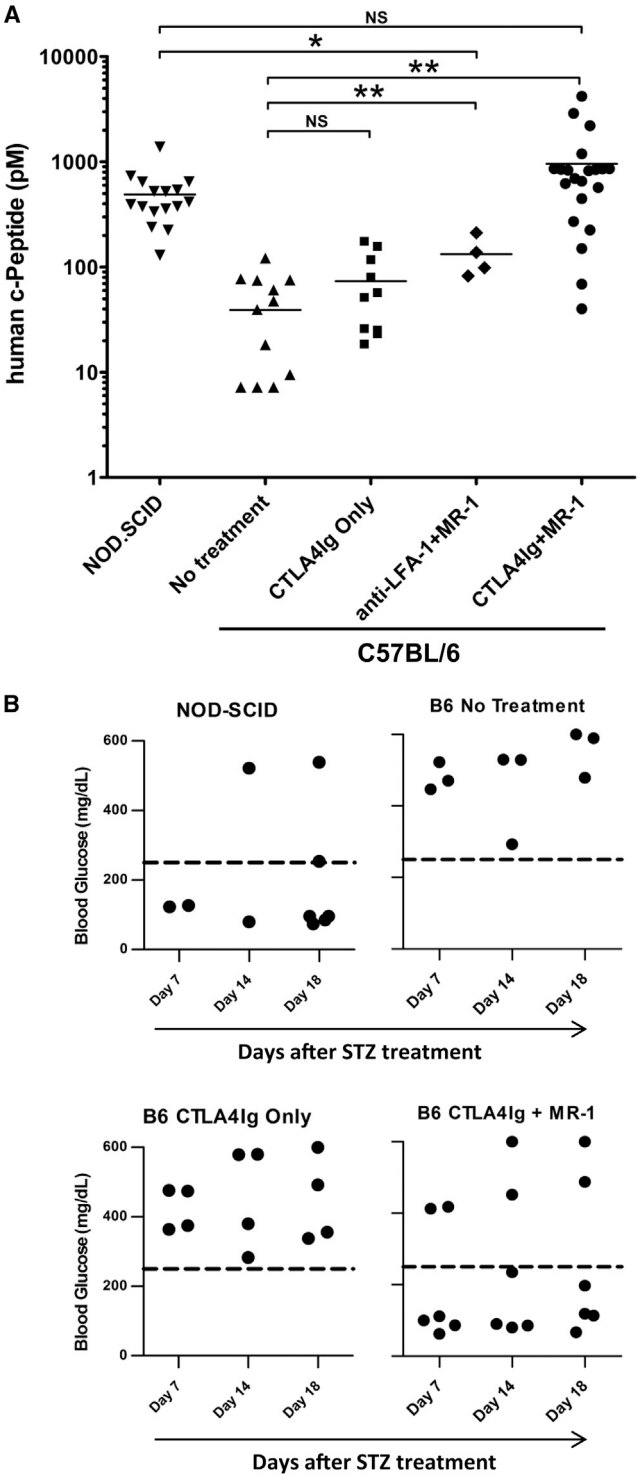


Figure 2. Differential Effectiveness of Costimulation Blockade Targeting Distinct Pathways in Preventing Rejection of hESC-PE

(A) Immunodeficient NOD-SCID controls (n = 16) and immunocompetent B6 mice were transplanted with hESC-PE on day 0 and were left untreated (n = 14) or treated with CTLA4Ig only (n = 10), anti-LFA-1 + MR-1 (n = 4), or CTLA4Ig + MR-1 (n = 21). Human C peptide levels were analyzed after >80 days.

(B) Mice were treated with STZ >90 days after implantation and BG levels were measured. In the top left panel, two out of the six mice at day 18 were

hESC-PE Graft Rejection in Immunocompetent Mice Can Be Blocked by a Combination of CTLA4Ig and anti-CD40L mAbs

To directly test the potential rejection of hESC-PE by the adaptive immune system, we compared the engraftment of hESC-PE in immunocompetent C57BL/6 (B6) and immunodeficient (*B6.RAG*$^{-/-}$ or NOD.SCID) mice. After 90 days of engraftment, the amount of human C peptide detected after glucose challenge was significantly lower in B6 mice as compared to immunodeficient mice, indicating an active role of the adaptive immune system in the rejection of the hESC-PE grafts (Figure 2A).

In vivo blockade of costimulatory pathways has previously been shown to block rejection of allografts and xenografts in various transplantation settings (Salomon and Bluestone, 2001). Hence, we investigated the effect of various costimulatory blockade regimens on the rejection of hESC-PE in B6 mice. B6 mice were transplanted with hESC-PE, treated with different combinations of costimulation-blocking monoclonal antibodies (mAbs) for 2 weeks at the time of transplantation, and assessed for graft function after 90 days. Mice treated with a combination of CTLA4Ig and anti-CD40L mAbs (MR-1) demonstrated significantly improved graft function compared with untreated controls (Figure 2A). This combination of immunoregulatory proteins was the most effective at preserving graft function as compared to CTLA4Ig alone, MR-1 alone, and a combination of anti-LFA-1 + MR-1 (Figure 2A and data not shown). Indeed, human C peptide levels were not significantly different in B6 mice treated with CTLA4Ig + MR-1 as compared to immunodeficient NOD-SCID recipients. CTLA4Ig alone was only marginally effective, with graft function not significantly improved in B6 mice treated with CTLA4Ig alone compared to untreated B6 mice (Figure 2A). Treatment with anti-LFA-1 + MR-1 provided a certain level of protection for hESC-PE based on C peptide levels compared to untreated B6 mice (t test, $p = 0.001$), but graft function was significantly reduced as compared to NOD-SCID mive (t test, $p = 0.02$). Of note, treatment of B6 mice with a combination of rapamycin and anti-CD3 mAbs or FK506 completely failed to protect hESC-PE from immune rejection in B6 mice (data not shown). Upon treatment with STZ, only the CTLA4Ig + MR-1-treated group was able to regulate BG in the absence of endogenous murine islets, although normoglycemia was not maintained in all treated mice (Figure 2B and data not shown). Furthermore, nephrectomies were performed in a small group of STZ-treated, hESC-PE transplanted, CTLA4Ig + MR-1 treated mice to confirm that glycemic control was due the transplanted hESC-PE tissue (data not shown) and further show that transplanted tissue was effectively regulating BG.

Histological analysis of graft sections revealed the presence of intact graft structures surrounded by mouse connective tissues in all groups at day 3 (Figure 3). However, hESC-PE grafts were heavily infiltrated in B6 mice by day 14 posttransplantation, resulting in loss of structural integrity and failure to produce endocrine hormones (Figure 3 and data not shown). In

NOD.RAG$^{-/-}$ and were grouped with NOD-SCID mice since they are similar, i.e. T and B cell-deficient mice on the NOD background that can be used interchangeably in our studies.

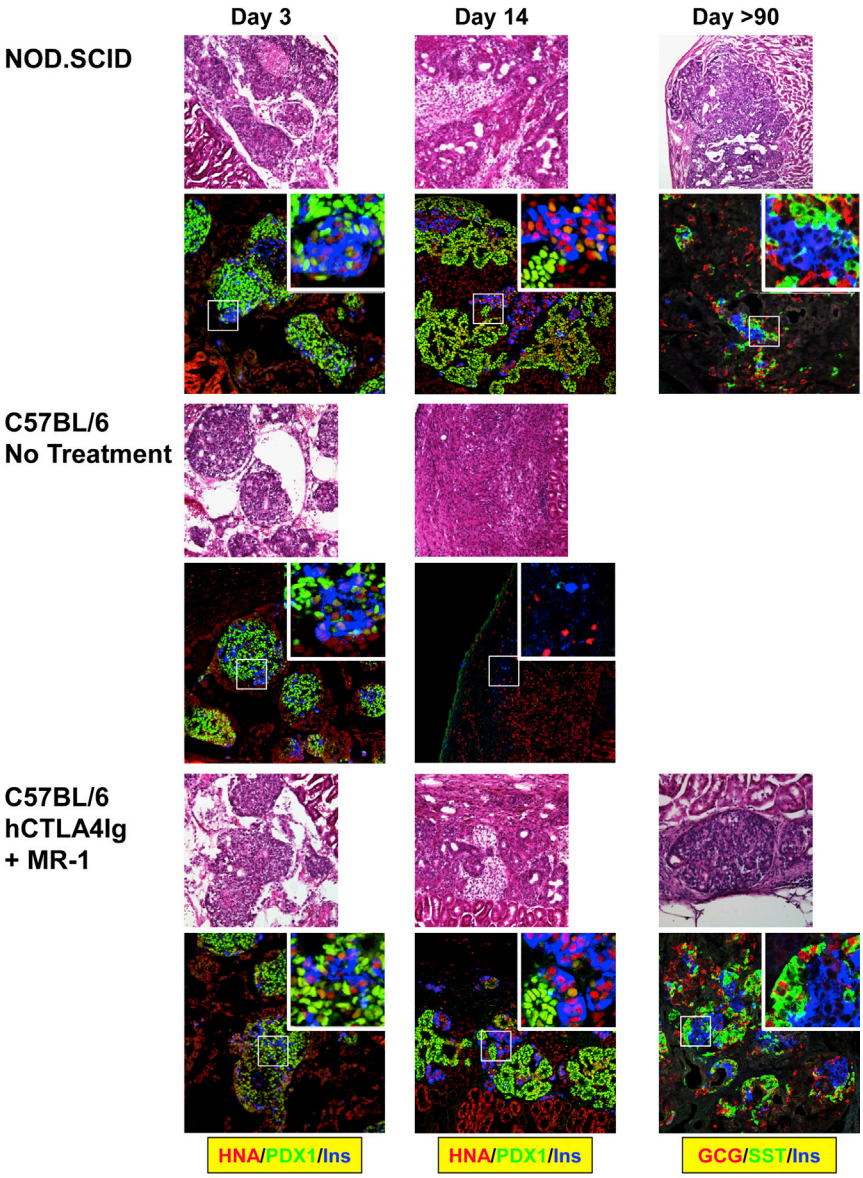


Figure 3. Histological Analysis of hESC-PE Tissue Transplanted with or without Costimulatory Blockade

NOD-SCID and B6 mice were transplanted with hESC-PE on day 0 and left untreated or treated with CTLA4Ig + MR-1 for 2 weeks. Recipients were euthanized at day 3, 14, or >90 posttransplantation and histological analysis was performed by H&E staining and immunofluorescence. Immunofluorescence staining was as follows. Days 3 and 14: red, Human Nuclear Antigen (HNA); green, Pancreatic and Duodenal Homeobox 1 (PDX1); blue, insulin (Ins); and day >90: red, glucagon (GCG); green, Somatostatin (SST); blue, insulin (Ins). Representative sections are shown. Immunofluorescence is shown as 10× pictures in which a higher magnification inset (40×) provides a detailed view of the area indicated by a white rectangle. See also Figure S2.

contrast, grafts in mice treated with the costimulation blockade regimen (CTLA4Ig + MR-1) maintained structural morphology for 90+ days. Immunofluorescence analyses (day 3) showed that hESC-PE grafts in all groups exhibited large clusters of human nuclear antigen (HNA)$^+$ PDX1$^+$ cells with occasional insulin staining. By day 14, most of the PDX1$^+$ cells had disappeared and only remnants of grafts were present in B6 mice, whereas clusters of PDX1$^+$ cells were readily detectable in control NOD-SCID mice and B6 mice treated with CTLA4Ig + MR-1 (Figures 3 and S2). At later time points (over 90 days), grafts in the immunodeficient and immunosuppressed groups exhibited large clusters of HNA$^+$ insulin$^+$ cells, consistent with glucose-stimulated C peptide levels (Figure 2A). Taken together, these results indicate that hESC-PE xenografts were rejected in B6 mice, but blocking the CD28 and CD40L-CD40 costimulatory pathways protected hESC-PE grafts from rejection.

CTLA4Ig in Combination with MR-1 Induces Long-Term Immune Tolerance

We next determined whether the short course of immunosuppressive treatment led to long-term tolerance in B6 mice treated with CTLA4Ig + MR-1. To evaluate if tolerance was established, we utilized an experimental protocol in which splenocytes from B6 mice that received hESC-PE in the absence or presence of costimulation blockade (groups A and B, respectively) were adoptively transferred into *B6-RAG*$^{-/-}$ recipients of functional hESC-PE grafts in the absence of immunosuppressive treatment (groups C and D, respectively) (Figure S3). The endocrine function of cells differentiated from hESC-PE grafts in *B6-RAG*$^{-/-}$ recipients was demonstrated upon glucose challenge at day >150 prior to the adoptive transfer (Figure 4, day 0). *B6-RAG*$^{-/-}$ recipients bearing fully functional hESC-PE grafts received splenocytes from untreated B6 mice (group A) that had earlier rejected the same hESC-PE graft (Figure 4, group C). Not surprisingly, *B6-RAG*$^{-/-}$ recipients showed a dramatic reduction in C peptide levels after adoptive transfer of splenocytes from group A mice (Figure 4, group C). In fact, human C peptide levels became undetectable within 4 weeks after the adoptive transfer (Figure 4, group C, day 28). In contrast, when splenocytes from B6 mice treated with costimulation blockade at the time of transplantation (group B) were transferred into *B6-RAG*$^{-/-}$ recipients bearing fully functional hESC-PE grafts (Figure 4, group D), C peptide levels were maintained at similar levels after 4 weeks in most mice despite the absence of any immunosuppressive treatment in these *B6-RAG*$^{-/-}$ recipients (Figure 4, group D, day 28). Of note, C peptide levels increased in some of these mice between day 0 and day 28 due to the fact that the hESC-derived tissue develops further during this time in some mice,

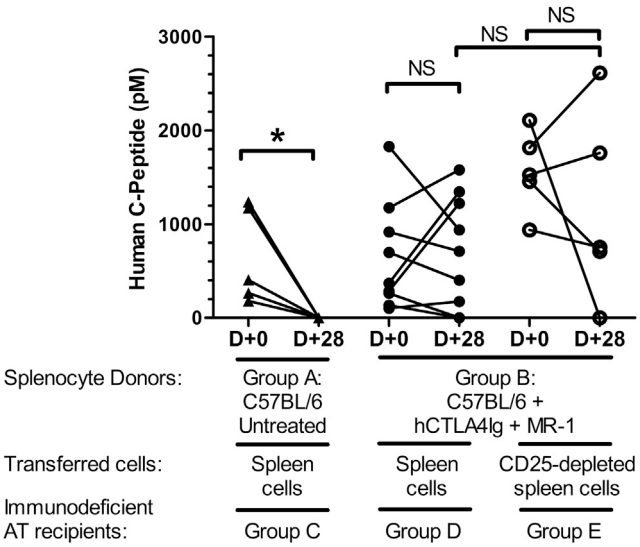


Figure 4. Costimulation Blockade Induces Long-Term Tolerance to hESC-PE in B6 Mice

Two groups of B6 mice (groups A and B) and three groups of immunodeficient, *B6.RAG*$^{-/-}$, or *B6.TCR*$\alpha^{-/-}$ mice (groups C–E) were transplanted with hESC-PE on day 0 (see Figure S3). B6 mice were left untreated (group A) or treated with CTLA4Ig + MR-1 for 2 weeks (group B). After >150 days, splenocytes were isolated from B6 mice in rejecting group A and accepting group B and adoptively transferred into groups C and D (respectively) of immunodeficient recipients that had been transplanted with the same hESC-PE on day 0. Group E received splenocytes from accepting group B that were depleted of Tregs prior to adoptive transfer (AT). Human C peptide levels were measured in immunodeficient recipients just before and 28 days after AT (D+0 and D+28). Results were similar between *B6.RAG*$^{-/-}$ or *B6.TCR*$\alpha^{-/-}$ recipients and were pooled. See also Figure S4.

although it is possible that there is some rejection in some individual animals in the context of a strong xenogeneic immune response (as in group C). The difference between groups C and D could not be attributed to differences in T cell numbers in the transferred splenocytes because there were similar percentages of CD3$^+$ T cells in the blood of mice in groups C and D after adoptive transfer (Figure S4). Thus, costimulation blockade not only prevented the rejection of hESC-PE xenografts but further induced a true state of immune tolerance in B6 mice. This tolerance could be adoptively transferred into graft-bearing immunodeficient recipients and confer protection from rejection in the absence of immunosuppression.

Tolerance to hESC-PE Grafts Does Not Require Regulatory T Cells

CD4$^+$CD25$^+$ regulatory T cells (Tregs) have been shown to play a crucial role in the maintenance of tolerance to self-antigens in homeostatic conditions and have been implicated in many models of transplantation tolerance. To determine if Tregs were involved in tolerance to hESC-PE grafts in B6 mice that were treated with costimulation blockade, we depleted CD25$^+$ cells from splenocytes of group B mice before their adoptive transfer into *B6-RAG*$^{-/-}$ recipients bearing fully functional hESC-PE grafts (Figure S3, group E). Treg depletion did not affect the outcome: four out of five *B6-RAG*$^{-/-}$ recipients of CD25-depleted splenocytes from tolerant B6 mice maintained relatively unchanged human C peptide levels 4 weeks after adoptive transfer (Figure 4, group E), similar to recipients of total splenocytes (i.e. those containing Tregs) from tolerant B6 mice (Figure 4, group D). Taken together, these results suggest that a short course of costimulation blockade induced a state of long-term immunological tolerance in B6 mice receiving hESC-PE grafts that is maintained independently of Tregs.

CTLA4Ig Combined with MR-1 Prevents the Rejection of hESC-PE Allografts by Human PBMCs

Next, the rejection of hESC-PE allografts in a humanized mouse model of transplantation was examined. NSG mice were transplanted with hESC-PE grafts and endocrine function was confirmed after at least 100 days when we measured human C peptide levels in the serum upon glucose stimulation (data not shown) and engaging in the adoptive transfer of human PBMCs from a healthy donor with or without costimulation blockade (CTLA4Ig + anti-human CD40L mAbs) (Figure S5). Five weeks after adoptive transfer, mice were treated with STZ and monitored for BG levels. As expected, BG levels rose rapidly in control NSG mice that received no hESC-PE graft, confirming that endogenous mouse β cells were destroyed by the STZ treatment (Figure 5A). BG levels increased by day 7 in four out of five STZ-treated mice bearing a functional hESC-PE graft that received human PBMCs alone (Figure 5B, left panel), indicating that hESC-PE-derived islet-like cells were destroyed by the adoptively transferred allogeneic PBMCs. In contrast, hESC-PE graft recipients that were treated with costimulation blockade at the time of adoptive transfer of human PBMCs remained euglycemic after STZ treatment (Figure 5B, right panel), indicating that hESC-derived islet-like cells were still present and functional. Immunofluorescence analyses of the grafts showed large clusters of HNA$^+$ insulin$^+$ cells in control NSG mice that received the hESC-PE graft but did not receive PBMCs (Figure 5C, left panel). In the PBMC alone group, hESC-PE grafts were infiltrated with large numbers of CD45$^+$ human leukocytes and had very few insulin$^+$ cell clusters (Figure 5C, middle panel). By comparison, insulin$^+$ cells were largely intact in the group that received PBMCs plus costimulation blockade and grafts completely lacked infiltration by CD45$^+$ human cells (Figure 5C, right panel). Thus, rejection of allogeneic hESC-derived islet-like cells by human PBMCs can be prevented by costimulation blockade in this humanized model.

DISCUSSION

In this study, we found that transplantation of hESC-PE in immunodeficient animals resulted in the generation of fully functional β-like cells irrespective of the background strain and the presence or absence of ILCs. Progenitor cells transplanted into immunocompetent C57BL/6 mice failed to produce detectable amounts of human C peptide but costimulation blockade prevented the rejection of these hESC-PE xenografts. Human insulin secretion was preserved most effectively with CTLA4Ig + anti-CD40L mAb combination therapy with islet-like structures persisting over 100 days in mice receiving a short course at the time of transplantation. Importantly, this costimulation blockade regimen led to a state of immune tolerance that could be transferred to secondary recipients and resulted in preservation of

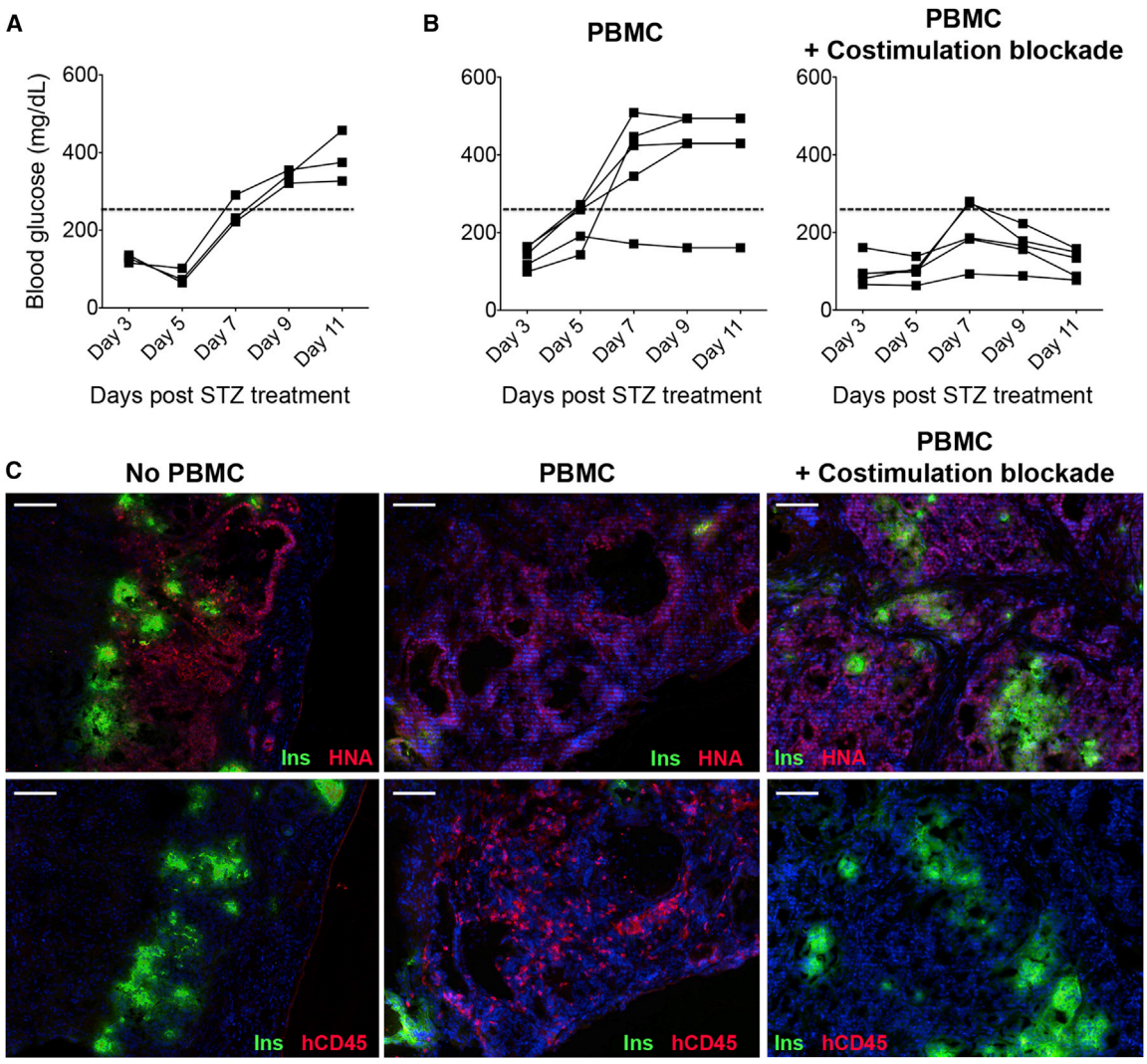


Figure 5. Costimulation Blockade Prevents the Rejection of Allogeneic hESC-Derived Insulin-Producing Cells by Human PBMCs in a Humanized Model In Vivo

(A and B) NSG negative controls did not receive hESC-PE or PBMCs (A). Other NSG mice were transplanted with hESC-PE on day 0 and adoptively transferred with 15×10^6 human PBMCs after >150 days (B). Recipients were either left untreated or treated with CTLA4Ig + anti-human CD40L mAbs on days 7, 9, 12, and 14 after AT (see Figure S5). Five weeks later, NSG mice were treated with STZ and BG levels were measured (n = 5 in each group).

(C) Recipients all received hESC-PE plus indicated cells/treatment for each group. Recipients were euthanized at day 11 after STZ treatment and an immunofluorescence analysis was performed. Top panels: red, Human Nuclear Factor (HNA); green, insulin. Bottom panels: red, human CD45; green, insulin. Representative sections are shown.

human islet-like function in the absence of immunosuppression. Moreover, the transfer of immune tolerance did not require the presence of Tregs, suggesting clonal elimination of the potentially reactive T cells. Finally, we showed that human PBMC-mediated destruction of allogeneic hESC-derived insulin-producing cells was blocked using costimulation blockade and preserved the ability of the hESC-derived insulin-producing cells to effectively regulate BG.

Despite initial hope that ESCs would experience an immune-privileged status, their differentiated derivatives have been shown to elicit potent immune responses in allogeneic and xenogeneic transplantation settings. However, susceptibility to immune rejection and the immune cells involved appear to differ between distinct types of stem cell products (de Almeida et al., 2013). Moreover, other innate lymphoid populations (ILC1, ILC2, and ILC3) have been recently identified and implicated in both inflammatory responses and tissue repair (Hazenberg and Spits, 2014), which might alter the in vivo differentiation process as well as immune rejection, thought this has not been studied as of yet. We observed that T and B cell-sufficient B6 mice, but not T cell-deficient *B6-TCR*$\alpha^{-/-}$ mice or T and B cell-deficient NOD-SCID, *B6-RAG*$^{-/-}$, or NSG mice, promptly rejected xenogeneic hESC-PE grafts generated from hESCs in vitro, consistent with previous reports that xenogeneic hESC products were rejected in immunocompetent animals (Deuse et al., 2011a; Pearl et al., 2011; Swijnenburg et al., 2008). Moreover, the finding that

differentiation of hESC-PE into fully functional islet-like cells was unaffected by the presence or absence of NK cells and ILCs suggests that the adaptive immune system is central to hESC-PE rejection and that therapeutic strategies should be focused on this arm of the immunity.

Immunogenicity of hESC-PE grafts was not limited to the xenogeneic setting because the tissue was rapidly rejected by allogeneic human PBMCs in a humanized model in vivo. The kinetics of rejection were comparable to those observed after transplantation of allogeneic adult islet grafts in a similar humanized model (Wu et al., 2013), suggesting that hESC-derived pancreatic tissues are fully immunogenic, similar to observations with murine ESCs products in allogeneic settings (Nussbaum et al., 2007; Pearl et al., 2011; Swijnenburg et al., 2008). Of note, earlier studies had concluded that hESCs benefited from an immune-privileged status in vitro and in vivo (Drukker et al., 2006; Li et al., 2004). In fact, Drukker et al. reported that allogeneic hESCs and their derivatives were not rejected in "Trimera" humanized mice generated by adoptive transfer of human PBMCs into irradiated recipient mice that were reconstituted with bone marrow cells from immunodeficient mice (Drukker et al., 2006). However, Rong et al. (2014) recently showed that allogeneic hESC-derived teratomas, as well as fibroblast and cardiomyocyte derivatives, were promptly rejected in humanized mice established by the engraftment of human fetal thymus and $CD34^+$ fetal liver cells into NSG mice. These discrepancies may be due to the robustness of the direct versus indirect pathways of allorecognition in distinct humanized models combined with the nature of the transplanted hESC derivatives, notably their ability to function as nonprofessional antigen-presenting cells (Shultz et al., 2012). For example, antigen-specific activation of T cells has been more difficult to achieve than allogeneic or xenogeneic responses in humanized mice reconstituted with mature lymphoid cells, possibly due to limited engraftment of human APCs. Thus, *allogeneic* responses to hESC products in vivo may vary depending on the types of hESC derivatives and humanized mouse models, which has implications for future in vivo studies of hESCs and their differentiated progeny in allogeneic settings.

Murine ESC derivatives require minimal immunosuppression for the prevention of rejection in allogeneic settings as compared to fully differentiated tissues, suggesting that they may be less immunogenic, even after differentiation (Lui et al., 2010; Magliocca et al., 2006; Robertson et al., 2007). This is important in regards to hESC-based islet cell replacement therapy because heavy immunosuppression may be perceived as a strong impediment to their clinical application in T1D. Indeed, benefits from this therapy must outweigh adverse side effects of immunosuppression as in the debate surrounding islet transplantation. Moreover, ethical considerations regarding hESC products have generated interest in other sources of islet-like cell progenitors that preclude the use of human embryos (Hebrok, 2012). The generation of "induced pluripotent stem cells" (iPSCs) by somatic cell reprogramming brought the promise of patient-specific syngeneic sources of cells that could be differentiated into any given lineage, and iPSCs that generate insulin-producing cells were successfully derived from T1D patients (Maehr et al., 2009; Thatava et al., 2013). However, iPSC-derived cells can display genetic, epigenetic, and transcriptional abnormalities that may make them immunogenic in syngeneic recipients (Holditch et al., 2014). Furthermore, ongoing autoimmunity in T1D patients will likely result in similar immunogenicity. Indeed, memory autoreactive T cells alter graft survival of pancreas or islet allografts in T1D recipients even when alloreactive responses were effectively controlled (Vendrame et al., 2010). Thus, stem-cell-based islet cell replacement approaches will likely require immunotherapy to hinder autoimmune responses, making the use of allogeneic hESCs together with immunosuppressive or tolerogenic treatments that control both alloreactivity and autoreactivity a good option for T1D patients.

Therefore, we determined whether rejection of hESC-PE could be prevented by costimulation blockade regimens that have been among the most successful in islet transplantation, notably those targeting two primary pathways of costimulation, CD28 and CD40L. Indeed, blockade of CD28 and CD40L pathways prolonged islet xenograft and allograft survival and induced long-term tolerance in rodent models, and it even showed promising results in nonhuman primates (Benda et al., 2002; Kenyon et al., 1999; Lenschow et al., 1992; Levisetti et al., 1997). Importantly, we recently showed that calcineurin inhibitor-free immunosuppressive protocols based on CTLA4Ig therapy gave promising results in T1D recipients of islet allografts (Posselt et al., 2010a). Here, we found that short-term costimulatory blockade using CTLA4Ig + MR-1 resulted in long-term survival of fully functional hESC-derived insulin-producing cells. MR-1 was more effective in combination with CTLA4Ig than anti-LFA-1 mAbs, and CTLA4Ig alone only marginally improved xenogeneic hESC graft survival. This is a notable report of successful engraftment of xenogeneic hESC-derived islet-like cells in immunocompetent animals. CTLA4Ig/anti-CD40L/anti-LFA-1 tritherapy was shown to allow indefinite survival of hESCs in the immune-privileged environment of the testis (Grinnemo et al., 2008) but also in immunocompetent sites (Ljung et al., 2013; Pearl et al., 2011). Monotherapy failed to provide sustained protection from rejection, in agreement with our data on the marginal effect of CTLA4Ig alone on hESC-PE. Of note, costimulation blockade was found to be more effective at preventing rejection of xenogeneic hESCs than standard immunosuppressive regimens were (Huber et al., 2013; Swijnenburg et al., 2008). This is consistent with our data that showed no effect of anti-CD3 mAbs + rapamycin or FK-506 + rapamycin on rejection of hESC-PE (data not shown) and with reports that FK-506 + rapamycin interferes with engraftment and islet regeneration (Chatenoud, 2008).

Importantly, we observed that treatment with CTLA4Ig + MR-1 successfully prevented the rejection of allogeneic hESC-PE in a humanized mouse model. This is a noteworthy report of short-term costimulatory blockade preventing the rejection of fully functional allogeneic hESC-derived insulin-producing cells by human PBMCs in vivo, demonstrating the potential therapeutic value of this approach for T1D patients because these hESC-derived cells effectively controlled BG levels in the absence of endogenous islets. However, as mentioned above, it will be necessary to control autoimmune responses in addition to allogeneic responses for hESC-based therapy to become a viable therapeutic option for large numbers of T1D patients. Protection of hESC-PE allografts was associated with reduced leukocyte numbers and infiltration in the graft, in agreement with reduced infiltration by human T cells in knockin hESC-derived cells

constitutively expressing CTLA4Ig and PD-L1 (Rong et al., 2014), as well as with clinical data showing that lymphocyte-depleting induction therapy promotes long-term insulin independence in TID patients after islet transplantation (Bellin et al., 2012). Of note, clinical trials using MR-1 were halted early on due to thromboembolic events, but new reagents targeting the CD40/CD40L pathway have shown promising results in experimental models without thromboembolic complications (Adams et al., 2005; Kanmaz et al., 2004; Thompson et al., 2011). Finally, Rong et al. recently showed that knockin hESC derivatives that constitutively expressed CTLA4Ig and PD-L1 were protected from rejection after transplantation into humanized mice (Rong et al., 2014), suggesting that this costimulation blockade combination is worth pursuing for clinical purposes as well.

Our study showed that a short course of treatment with CTLA4Ig + MR-1 not only prolonged hESC-PE graft survival but also resulted in the establishment of immune tolerance. This is in contrast with the report that expression of CTLA4Ig and PD-L1 on knockin hESC derivatives could achieve immune protection of transplanted cells without inducing immune tolerance (Rong et al., 2014). This discrepancy could be due to differences in the type of hESC-derived tissue and targeted pathways. We found that depletion of $CD4^+CD25^+$ Tregs did not affect the transfer of tolerance, suggesting that Tregs were dispensable for the maintenance of tolerance to hESC-derived islet-like cells. This was unexpected because Tregs have been implicated in many experimental models of transplantation tolerance (Tang et al., 2012). However, Tregs may play a more prominent role in early posttransplantation in induction of tolerance than their role in maintenance of long-term tolerance, at which point other regulatory mechanisms or cell populations may become dominant. Thus, the lack of a need for Tregs in the transfer of tolerance in our study does not preclude a role of Tregs in induction of the tolerant state to hESC-PE by costimulatory blockade. Previous reports have shown that Tregs were locally enriched in murine models that achieved acceptance of undifferentiated hESCs, including after costimulation blockade, but the potential role of Tregs in transplantation of differentiated hESC-derived tissues that are more immunogenic has not been elucidated (Deuse et al., 2011b; Grinnemo et al., 2008; Ljung et al., 2013). Tregs were also enriched and even required in mouse models of spontaneous acceptance of allogeneic mESC-derived tissues (Lui et al., 2010; Robertson et al., 2007). Other immunomodulatory mechanisms may be involved as well. For example, long-term survival of hESC endothelial cells (hESC-ECs after treatment with CTLA4Ig + anti-LFA-1 was associated with upregulation of T cell immunoglobulin and mucin domain 3 (Tim-3) and PD-1 (Huber et al., 2013; Swijnenburg et al., 2008), two inhibitory receptors implicated in transplantation tolerance (Fife and Bluestone, 2008). In addition to reduced T cell infiltration, acceptance of CTLA4Ig/PD-L1 knockin hESC derivatives was associated with increased intragraft levels of immunosuppressive cytokines TGFβ and IL-10 (Rong et al., 2014).

Lastly, these results may have implications for preclinical development of human cell therapies. For the purposes of investigating allometric dosing relationships, product scaling, and clinical safety/toxicity concerns, traditional preclinical drug development involves testing in multiple species, including larger nonrodent species, such as rabbits, dogs, or nonhuman primates. However, this has not been possible for human cell therapies because cells are rejected in immunocompetent animals; therefore testing can only be performed in genetically immunocompromised animals that are limited to rodents. If the reagents used in the present study were found to be protective and tolerogenic in larger animals, preclinical testing of candidate human cell therapies in such species might be possible. This could represent a substantial breakthrough in preclinical testing of human cell therapies.

In conclusion, our study showed that a short course of costimulation blockade led to long-term survival and immune tolerance to hESC-PE xenografts and prevented the rejection of allogeneic hESC-derived insulin-producing cells in a humanized model in vivo. Moreover, this short course of immunotherapy did not interfere with the development and differentiation of hESC prepancreatic endoderm into functioning islet-like PE. Thus, transplantation of allogeneic hESC-derived pancreatic progenitors in the context of tolerogenic immunotherapy is an option worth pursuing for islet cell replacement in T1D.

EXPERIMENTAL PROCEDURES

Mice

NOD.SCID and NSG mice were bred in house. B6, *B6.RAG*$^{-/-}$, and *B6.TCR*$\alpha^{-/-}$ mice were purchased from Jackson Laboratories. All mice were housed in a pathogen-free facility at the University of California, San Francisco. All experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were approved by the UCSF Institutional Animal Care and Use Committee.

Transplantation of hESC-PE, Analysis of Graft Tissue, Adoptive Transfers, and In Vivo Treatments

Cell culture and differentiation protocols to generate hESC-PE were performed as described (D'Amour et al., 2006; Kroon et al., 2008; Schulz et al., 2012). For transplantation, 10–25 μl of cell aggregate slurry (representing 3–7 × 10^6 cells) was implanted below the kidney capsule with a PE-50 catheter (Szot et al., 2007). For histology, 5 μm sections of formalin-fixed (10%) and paraffin-embedded graft tissues were stained with hematoxylin and eosin. Immunofluorescence analyses were performed on 10 μm sections of frozen graft tissues as previously described (Kroon et al., 2008). To assess graft function >90 days after implantation, serum human C peptide produced in response to glucose administration was measured by performing glucose- or arginine-stimulated insulin secretion assays as previously described (Kroon et al., 2008). BG levels were measured with a Lifescan glucose meter (One Touch II; Lifescan). Mice with BG levels >250 mg/dl were considered diabetic. For adoptive transfers, single-cell suspensions were prepared from the spleen of B6 mice and depleted of $CD4^+CD25^+$ Tregs using anti-CD25 mAbs (7D4) and rabbit complement. Depending on experiments, 18 × 10^6 or 34 × 10^6 total or CD25-depleted spleen cells were injected i.v. in *B6.RAG*$^{-/-}$ or *TCR*$\alpha^{-/-}$ recipient mice. Results were similar with different cell numbers or immunodeficient strains (*B6.RAG*$^{-/-}$ or *TCR*$\alpha^{-/-}$) and pooled. For the humanized model, PBMCs were prepared from a healthy donor and 15 × 10^6 PBMCs were injected i.v. into NSG recipients >150 days after transplantation of hESC-PE. For immunosuppression, CTLA4Ig (Repligen) was administered at 500 μg/mouse on days 0, 2, 4, and 6 posttransplant in combination with 500 μg/mouse anti-CD154 mAbs (MR-1, UCSF Hybridoma Core) on days 0, 2, and 4. Anti-LFA-1 mAbs (gift from Ron Gill) were administered at 200 μg/mouse on days 0, 1, 7, and 14 in combination with 250 μg/mouse MR-1 on days −1 and 1 and twice a week for 4 weeks. In the humanized mouse model, mice received 20 mg/kg CTLA4Ig and 10 mg/kg anti-human CD40L mAbs (5C8) on days 7, 9, 12, and 14 after the adoptive transfer of human PBMCs. To destroy endogenous mouse islet cells, we treated mice with STZ via i.p. injection, using either a single dose of 8 mg/mouse or five doses of 50 mg/kg administered over 5 consecutive days. Additional details are available in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.12.001.

AUTHOR CONTRIBUTIONS

G.L.S. and E.K. designed experiments, performed experiments, and analyzed data; M.Y. designed experiments, performed experiments, analyzed data, and wrote the manuscript; J.L., J.K., and K.K. performed experiments; E.P.B. and E.E.B. designed experiments and analyzed data; H.B.J. and J.A.B. designed experiments, analyzed data, and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dorothy Fuentes for animal care and Matthias Hebrok and members of the Bluestone and Hebrok laboratories for discussions. We thank Holly Young and Laura Martinson for technical assistance in preparing hESC-PE for implant. This research was supported by CIRM grants RM1-01703 and DR1-01423. J.A.B. is a consultant for ViaCyte, Inc., and E.K., J.K., K.K., E.P.B., and E.E.B. are employed or formerly employed by, and are shareholders of, ViaCyte, Inc., which is developing ESC-based products.

Received: August 13, 2014
Revised: October 30, 2014
Accepted: December 2, 2014
Published: December 18, 2014

REFERENCES

Adams, A.B., Shirasugi, N., Jones, T.R., Durham, M.M., Strobert, E.A., Cowan, S., Rees, P., Hendrix, R., Price, K., Kenyon, N.S., et al. (2005). Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. J. Immunol. *174*, 542–550.

Arefanian, H., Tredget, E.B., Rajotte, R.V., Gill, R.G., Korbutt, G.S., and Rayat, G.R. (2010). Short-term administrations of a combination of anti-LFA-1 and anti-CD154 monoclonal antibodies induce tolerance to neonatal porcine islet xenografts in mice. Diabetes *59*, 958–966.

Barton, F.B., Rickels, M.R., Alejandro, R., Hering, B.J., Wease, S., Naziruddin, B., Oberholzer, J., Odorico, J.S., Garfinkel, M.R., Levy, M., et al. (2012). Improvement in outcomes of clinical islet transplantation: 1999-2010. Diabetes Care *35*, 1436–1445.

Bellin, M.D., Barton, F.B., Heitman, A., Harmon, J.V., Kandaswamy, R., Balamurugan, A.N., Sutherland, D.E., Alejandro, R., and Hering, B.J. (2012). Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. Am. J. Transplant. *12*, 1576–1583.

Benda, B., Ljunggren, H.G., Peach, R., Sandberg, J.O., and Korsgren, O. (2002). Co-stimulatory molecules in islet xenotransplantation: CTLA4Ig treatment in CD40 ligand-deficient mice. Cell Transplant. *11*, 715–720.

Bluestone, J.A., Herold, K., and Eisenbarth, G. (2010). Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature *464*, 1293–1300.

Chatenoud, L. (2008). Chemical immunosuppression in islet transplantation—friend or foe? N. Engl. J. Med. *358*, 1192–1193.

D'Amour, K.A., Agulnick, A.D., Eliazer, S., Kelly, O.G., Kroon, E., and Baetge, E.E. (2005). Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat. Biotechnol. *23*, 1534–1541.

D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K., and Baetge, E.E. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat. Biotechnol. *24*, 1392–1401.

de Almeida, P.E., Ransohoff, J.D., Nahid, A., and Wu, J.C. (2013). Immunogenicity of pluripotent stem cells and their derivatives. Circ. Res. *112*, 549–561.

Deuse, T., Seifert, M., Phillips, N., Fire, A., Tyan, D., Kay, M., Tsao, P.S., Hua, X., Velden, J., Eiermann, T., et al. (2011a). Human leukocyte antigen I knockdown human embryonic stem cells induce host ignorance and achieve prolonged xenogeneic survival. Circulation *124*, S3–S9.

Deuse, T., Seifert, M., Tyan, D., Tsao, P.S., Hua, X., Velden, J., Eiermann, T., Volk, H.D., Reichenspurner, H., Robbins, R.C., and Schrepfer, S. (2011b). Immunobiology of naïve and genetically modified HLA-class-I-knockdown human embryonic stem cells. J. Cell Sci. *124*, 3029–3037.

Dressel, R., Nolte, J., Elsner, L., Novota, P., Guan, K., Streckfuss-Bömeke, K., Hasenfuss, G., Jaenisch, R., and Engel, W. (2010). Pluripotent stem cells are highly susceptible targets for syngeneic, allogeneic, and xenogeneic natural killer cells. FASEB J. *24*, 2164–2177.

Drukker, M., Katchman, H., Katz, G., Even-Tov Friedman, S., Shezen, E., Hornstein, E., Mandelboim, O., Reisner, Y., and Benvenisty, N. (2006). Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. Stem Cells *24*, 221–229.

Eshpeter, A., Jiang, J., Au, M., Rajotte, R.V., Lu, K., Lebkowski, J.S., Majumdar, A.S., and Korbutt, G.S. (2008). In vivo characterization of transplanted human embryonic stem cell-derived pancreatic endocrine islet cells. Cell Prolif. *41*, 843–858.

Fife, B.T., and Bluestone, J.A. (2008). Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol. Rev. *224*, 166–182.

Frenzel, L.P., Abdullah, Z., Kriegeskorte, A.K., Dieterich, R., Lange, N., Busch, D.H., Krönke, M., Utermöhlen, O., Hescheler, J., and Sarić, T. (2009). Role of natural-killer group 2 member D ligands and intercellular adhesion molecule 1 in natural killer cell-mediated lysis of murine embryonic stem cells and embryonic stem cell-derived cardiomyocytes. Stem Cells *27*, 307–316.

Grinnemo, K.H., Genead, R., Kumagai-Braesch, M., Andersson, A., Danielsson, C., Månsson-Broberg, A., Dellgren, G., Strömberg, A.M., Ekberg, H., Hovatta, O., et al. (2008). Costimulation blockade induces tolerance to HESC transplanted to the testis and induces regulatory T-cells to HESC transplanted into the heart. Stem Cells *26*, 1850–1857.

Hazenberg, M.D., and Spits, H. (2014). Human innate lymphoid cells. Blood *124*, 700–709.

Hebrok, M. (2012). Generating β cells from stem cells-the story so far. Cold Spring Harb. Perspect. Med. *2*, a007674.

Holditch, S.J., Terzic, A., and Ikeda, Y. (2014). Concise review: pluripotent stem cell-based regenerative applications for failing β-cell function. Stem Cells Transl. Med. *3*, 653–661.

Hosokawa, M., Dolci, W., and Thorens, B. (2001). Differential sensitivity of GLUT1- and GLUT2-expressing beta cells to streptozotocin. Biochem. Biophys. Res. Commun. *289*, 1114–1117.

Huber, B.C., Ransohoff, J.D., Ransohoff, K.J., Riegler, J., Ebert, A., Kodo, K., Gong, Y., Sanchez-Freire, V., Dey, D., Kooreman, N.G., et al. (2013). Costimulation-adhesion blockade is superior to cyclosporine A and prednisone immunosuppressive therapy for preventing rejection of differentiated human embryonic stem cells following transplantation. Stem Cells *31*, 2354–2363.

Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G., and Majumdar, A.S. (2007). Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells *25*, 1940–1953.

Kanmaz, T., Fechner, J.J., Jr., Torrealba, J., Kim, H.T., Dong, Y., Oberley, T.D., Schultz, J.M., Bloom, D.D., Katayama, M., Dar, W., et al. (2004). Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. Transplantation *77*, 914–920.

Kenyon, N.S., Fernandez, L.A., Lehmann, R., Masetti, M., Ranuncoli, A., Chatzipetrou, M., Iaria, G., Han, D., Wagner, J.L., Ruiz, P., et al. (1999). Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. Diabetes *48*, 1473–1481.

Kilpatrick, E.S., Rigby, A.S., and Atkin, S.L. (2009). The Diabetes Control and Complications Trial: the gift that keeps giving. Nat. Rev. Endocrinol. *5*, 537–545.

Kroon, E., Martinson, L.A., Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., Cunningham, J., et al. (2008). Pancreatic endoderm derived from human embryonic stem cells generates

glucose-responsive insulin-secreting cells in vivo. Nat. Biotechnol. *26*, 443–452.

Lenschow, D.J., Zeng, Y., Thistlethwaite, J.R., Montag, A., Brady, W., Gibson, M.G., Linsley, P.S., and Bluestone, J.A. (1992). Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. Science *257*, 789–792.

Levisetti, M.G., Padrid, P.A., Szot, G.L., Mittal, N., Meehan, S.M., Wardrip, C.L., Gray, G.S., Bruce, D.S., Thistlethwaite, J.R., Jr., and Bluestone, J.A. (1997). Immunosuppressive effects of human CTLA4Ig in a non-human primate model of allogeneic pancreatic islet transplantation. J. Immunol. *159*, 5187–5191.

Li, L., Baroja, M.L., Majumdar, A., Chadwick, K., Rouleau, A., Gallacher, L., Ferber, I., Lebkowski, J., Martin, T., Madrenas, J., and Bhatia, M. (2004). Human embryonic stem cells possess immune-privileged properties. Stem Cells *22*, 448–456.

Ljung, K., Simonson, O.E., Felldin, U., Wärdell, E., Ibarra, C., Antonsson, L., Kumagai-Braesch, M., Hovatta, O., Lampela, R., Grinnemo, K.H., and Corbascio, M. (2013). Costimulation blockade induces foxp3(+) regulatory T cells to human embryonic stem cells. Biores. Open Access *2*, 455–458.

Lui, K.O., Boyd, A.S., Cobbold, S.P., Waldmann, H., and Fairchild, P.J. (2010). A role for regulatory T cells in acceptance of ESC-derived tissues transplanted across an major histocompatibility complex barrier. Stem Cells *28*, 1905–1914.

Ma, M., Ding, S., Lundqvist, A., San, H., Fang, F., Konoplyannikov, M., Berry, C., Beltran, L.E., Chen, G., Kovacic, J.C., and Boehm, M. (2010). Major histocompatibility complex-I expression on embryonic stem cell-derived vascular progenitor cells is critical for syngeneic transplant survival. Stem Cells *28*, 1465–1475.

Maehr, R., Chen, S., Snitow, M., Ludwig, T., Yagasaki, L., Goland, R., Leibel, R.L., and Melton, D.A. (2009). Generation of pluripotent stem cells from patients with type 1 diabetes. Proc. Natl. Acad. Sci. USA *106*, 15768–15773.

Magliocca, J.F., Held, I.K., and Odorico, J.S. (2006). Undifferentiated murine embryonic stem cells cannot induce portal tolerance but may possess immune privilege secondary to reduced major histocompatibility complex antigen expression. Stem Cells Dev. *15*, 707–717.

Nussbaum, J., Minami, E., Laflamme, M.A., Virag, J.A., Ware, C.B., Masino, A., Muskheli, V., Pabon, L., Reinecke, H., and Murry, C.E. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J. *21*, 1345–1357.

Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., and Melton, D.A. (2014). Generation of functional human pancreatic β cells in vitro. Cell *159*, 428–439.

Pearl, J.I., Lee, A.S., Leveson-Gower, D.B., Sun, N., Ghosh, Z., Lan, F., Ransohoff, J., Negrin, R.S., Davis, M.M., and Wu, J.C. (2011). Short-term immunosuppression promotes engraftment of embryonic and induced pluripotent stem cells. Cell Stem Cell *8*, 309–317.

Posselt, A.M., Bellin, M.D., Tavakol, M., Szot, G.L., Frassetto, L.A., Masharani, U., Kerlan, R.K., Fong, L., Vincenti, F.G., Hering, B.J., et al. (2010a). Islet transplantation in type 1 diabetics using an immunosuppressive protocol based on the anti-LFA-1 antibody efalizumab. Am. J. Transplant. *10*, 1870–1880.

Posselt, A.M., Szot, G.L., Frassetto, L.A., Masharani, U., Tavakol, M., Amin, R., McElroy, J., Ramos, M.D., Kerlan, R.K., Fong, L., et al. (2010b). Islet transplantation in type 1 diabetic patients using calcineurin inhibitor-free immunosuppressive protocols based on T-cell adhesion or costimulation blockade. Transplantation *90*, 1595–1601.

Rezania, A., Bruin, J.E., Riedel, M.J., Mojibian, M., Asadi, A., Xu, J., Gauvin, R., Narayan, K., Karanu, F., O'Neil, J.J., et al. (2012). Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes *61*, 2016–2029.

Rezania, A., Bruin, J.E., Arora, P., Rubin, A., Batushansky, I., Asadi, A., O'Dwyer, S., Quiskamp, N., Mojibian, M., Albrecht, T., et al. (2014). Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat. Biotechnol. *32*, 1121–1133.

Robertson, N.J., Brook, F.A., Gardner, R.L., Cobbold, S.P., Waldmann, H., and Fairchild, P.J. (2007). Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance. Proc. Natl. Acad. Sci. USA *104*, 20920–20925.

Rong, Z., Wang, M., Hu, Z., Stradner, M., Zhu, S., Kong, H., Yi, H., Goldrath, A., Yang, Y.G., Xu, Y., and Fu, X. (2014). An effective approach to prevent immune rejection of human ESC-derived allografts. Cell Stem Cell *14*, 121–130.

Salomon, B., and Bluestone, J.A. (2001). Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu. Rev. Immunol. *19*, 225–252.

Schulz, T.C., Young, H.Y., Agulnick, A.D., Babin, M.J., Baetge, E.E., Bang, A.G., Bhoumik, A., Cepa, I., Cesario, R.M., Haakmeester, C., et al. (2012). A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS ONE *7*, e37004.

Shapiro, A.M.J., and Ricordi, C. (2014). Long-term Outcomes after Islet Transplantation. In Textbook of Organ Transplantation, A. Kirk, ed. (Chichester, West Sussex, UK: John Wiley & Sons, Ltd), pp. 1314–1333.

Shapiro, A.M., Lakey, J.R., Ryan, E.A., Korbutt, G.S., Toth, E., Warnock, G.L., Kneteman, N.M., and Rajotte, R.V. (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N. Engl. J. Med. *343*, 230–238.

Shultz, L.D., Brehm, M.A., Garcia-Martinez, J.V., and Greiner, D.L. (2012). Humanized mice for immune system investigation: progress, promise and challenges. Nat. Rev. Immunol. *12*, 786–798.

Swijnenburg, R.J., Schrepfer, S., Govaert, J.A., Cao, F., Ransohoff, K., Sheikh, A.Y., Haddad, M., Connolly, A.J., Davis, M.M., Robbins, R.C., and Wu, J.C. (2008). Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. Proc. Natl. Acad. Sci. USA *105*, 12991–12996.

Szot, G.L., Koudria, P., and Bluestone, J.A. (2007). Transplantation of pancreatic islets into the kidney capsule of diabetic mice. J. Vis. Exp. *9*, 404.

Tang, Q., Bluestone, J.A., and Kang, S.M. (2012). CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. J. Mol. Cell Biol. *4*, 11–21.

Thatava, T., Kudva, Y.C., Edukulla, R., Squillace, K., De Lamo, J.G., Khan, Y.K., Sakuma, T., Ohmine, S., Terzic, A., and Ikeda, Y. (2013). Intrapatient variations in type 1 diabetes-specific iPS cell differentiation into insulin-producing cells. Mol. Ther. *21*, 228–239.

Thompson, P., Cardona, K., Russell, M., Badell, I.R., Shaffer, V., Korbutt, G., Rayat, G.R., Cano, J., Song, M., Jiang, W., et al. (2011). CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. Am. J. Transplant. *11*, 947–957.

Tiwari, J.L., Schneider, B., Barton, F., and Anderson, S.A. (2012). Islet cell transplantation in type 1 diabetes: an analysis of efficacy outcomes and considerations for trial designs. Am. J. Transplant. *12*, 1898–1907.

Van Hoof, D., D'Amour, K.A., and German, M.S. (2009). Derivation of insulin-producing cells from human embryonic stem cells. Stem Cell Res. (Amst.) *3*, 73–87.

Vendrame, F., Pileggi, A., Laughlin, E., Allende, G., Martin-Pagola, A., Molano, R.D., Diamantopoulos, S., Standifer, N., Geubtner, K., Falk, B.A., et al. (2010). Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells. Diabetes *59*, 947–957.

Wu, D.C., Hester, J., Nadig, S.N., Zhang, W., Trzonkowski, P., Gray, D., Hughes, S., Johnson, P., and Wood, K.J. (2013). Ex vivo expanded human regulatory T cells can prolong survival of a human islet allograft in a humanized mouse model. Transplantation *96*, 707–716.