Detailed Description of Materials and Methods

Study design

The objective of this study was to engineer and transplant insulin-producing islets into allogeneic recipient mice with preexisting diabetes to determine the translational potential of PIDO fusion protein expression in inducing functional immune tolerance to allogeneic islets. We used lentiviral delivery to genetically engineer islets derived from allogeneic or xenogeneic donors. We transplanted PIDO-engineered islets, and naïve islets into 15 and 9 STZ-treated diabetic mice, respectively. STZ-treated diabetic and nondiabetic mice without transplants served as transplantation controls. Mouse groups were assigned randomly, and the study was not blinded. Transplanted mice were monitored through blood glucose measurements and blood plasma collection and then euthanized for ex vivo analysis. Nephrectomy surgery was performed on $PIDO^+$ islet transplanted mice to confirm that transplanted islets were the source of glucose tolerance and nondiabetic blood glucose concentrations. Data collection was stopped at predetermined, arbitrary times. Mice that did not develop diabetes post-STZ administration and the mice that dies pre- or peri-transplant surgery were excluded from the study.

Enzymatic Activity of IDO1

Kynurenine levels were analyzed in conditioned media collected from mesenchymal stromal cells (positive control) or islets after 48-hr in vitro culture by Enzyme-linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions using Kynurenine ELISA kit (#F56401, LSBio, USA).

Glucose-stimulated insulin secretion

For static GSIS, approximately 50 size-matched islets (transduced to express PIDO or EGFP-control, in 48-well plates) were assessed in response to glucose challenge. Islets were washed

with KRB buffer, followed by pre-incubation in glucose-free KRB buffer for 30 min. Static insulin secretion was measured by incubating islets in media with basal (2.8mM or 2.8G) or stimulatory (16.7mM or 16.7G) glucose for 2 h each. The supernatant was collected for insulin assay. Intracellular insulin content was determined by harvesting islets, rinsing with PBS, resuspending in 300 µL acid ethanol and homogenizing by ultrasonic disruption of the cell membrane. Insulin was measured using a mouse insulin ELISA kit (#10-1247-01, Mercodia, Uppsala, Sweden) according to the manufacturer's protocol.

Immunocytochemical staining and imaging

Intact mouse islets transduced to express transgenes encoded by different lentiviruses (EGFP, PD-L1:EGFP, IDO:mCherry and PIDO:EGFP) were stained with nuclear counterstain Hoechst 33342 (Cat# H1399, ThermoFisher, USA). Formalin-fixed paraffin embedded kidney sections from recipient mice were either stained with hematoxylin-eosin (H&E) for visualization of islet microscopic anatomy or with anti-insulin antibody (1:1000; Immunostar, USA) and actin (Acti-Stain 555 Phalloidin, Cat # PHDH1) for detection of transplanted, insulin-positive islets by immunofluorescence (IF) microscopy and imaging. Nuclei were counterstained with ProLong™ Diamond Antifade Mountant (#P36970, ThermoFisher, USA). H&E images were acquired using a Zeiss AX10 inverted microscope equipped with a Zeiss Axiocam 305 color camera. IF images were acquired using a laser-scanning microscope (A1R; Nikon, USA).

Flow cytometry

Islets expressing PIDO fusion protein, PD-L1 or EGFP were dissociated into a single-cell suspension by gentle pipetting after washing in 2 mmol/l EDTA/PBS and incubating for 5 min at room temperature in Ca^{2+}-free PBS supplemented with 0.025% trypsin. Infiltrating immune cells were harvested from Matrigel™ plugs by digesting plugs in a dissociation solution of 0.1%

Collagenase type II, 0.8 U/mL Dispase, and 0.001% DNase. Digested plug suspension was filtered through a 70 μm strainer. Dispersed single cell preparations were stained with viability dye (Ghost Red 780, Cat# 13-0865, Tonbo Biosciences, USA) for 30 minutes followed by staining for specific antigens (please see supplementary data table 1). Gating schematic is show in supplementary figure 4.

Islet Isolation and Culture

Juvenile porcine islets were acquired from University of California, Irvine. The islets were isolated from the pancreata of 8- to 15-day-old, pre-weaned Yorkshire piglets and cultured according to previously described protocol(1). Mouse islets were isolated from male 12–16-week-old C57BL/6J (Jackson Laboratory, USA) as described previously(2).

Islets were cultured (37°C, 5% CO_2) in RPMI-1640 medium (Corning, USA) with 10% FBS (Gibco, USA) and 1% antibiotic-antimycotic (ThermoFisher, #15240096) for indicated duration or overnight before coculture with PSCs. Islet-PSC coculture was maintained in a 1:1 mix of complete RPMI and DMEM F-12 (RD^{mix}) media.

Lentiviral transduction of mouse and pig pancreatic islets

After assessment of islet viability with dithiazone, islets were cultured in RPMI medium overnight. Next day, islets were partially disrupted by mild enzymatic dissociation; briefly, islets were incubated for two minutes in prewarmed Accutase (2.5 ul/islet, StemCell technologies) and washed with Ca/Mg-free HBSS. Purified viruses were added to the islets in ultra-low attachment plate or dish (Costar, Corning) and were incubated with viral supernatant for 6 hours or overnight. For the default transduction condition a vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped cytomegalovirus-green fluorescent protein (CMV-GFP) vector was used at a multiplicity of infection (MOI) of 10, and transduction was performed in serum-free medium

supplemented with 0.1% bovine albumin, 1x Insulin-Transferrin-Selenium (ITS) (Sigma Aldrich) and 8 ug/ml polybrene. The transduction volume was kept uniform throughout all experiments, with a volume of 135.5 µl/cm2 of growth area of the well/dish the transduction took place in, and with a minimum of 50% of the transduction volume consisting of fresh medium. Islet were cultured in RPMI medium supplemented with 10% FBS for 48 hrs following which, transduction efficiency was evaluated prior to transplant.

Mouse and dog transplants

Animal studies were performed in accordance with University of Wisconsin-Madison Institutional Animal Care and Use Committee under approved protocols.

Mice were randomly designated for STZ treatment and transplantation groups. Mouse number per group was selected to allow for statistical significance (n = 9 and 15). Surgical procedures and follow-up studies were performed by unblinded individuals. Male ~8-week-old BALB/c, C57BL6/j and CD4$^{-/-}$ (B6.129S2-*Cd4*^{tm1Mak}/J, Strain #002663) mice were purchased from The Jackson Laboratory, rendered diabetic with injection of STZ (45 mg/kg; R&D systems) for 5 days, with diabetes (>300 mg/dl) confirmed after 7 days. Spontaneously diabetic female NOD mice (~12-16 weeks old) with blood glucose levels >350 mg/dl were transplanted with islets harvested from euglycemic 8-week-old C57BL/6J donor mice. Anaesthetized mice were transplanted with ~400 hand-picked, mixed size islets (PIDO expressing or control transduced), or saline under the kidney capsule. Animals were monitored up to 50 weeks. Blood glucose was measured with a Contour Blood Glucose Monitoring System (Bayer). Glucose tolerance and in vivo GSIS assays were performed by fasting mice for 4 hours and injecting with glucose (2 g/kg). Serum hormones were quantified using ELISA kits for insulin (mouse #10-1247-01, porcine # 10-1200-01) and porcine C-peptide (#10-1256-01) following manufacturer's

instructions (Mercodia, Uppsala, Sweden). Twenty weeks after transplantation, transplant recipient mice were rechallenged either by second STZ injection or by live nephrectomy performed on 5 anaesthetized mice each group. For in vivo assay for evaluation of surviving islet cells or infiltrating immune cells, control or PIDO-engineered islets were mixed with liquid Matrigel (at 4ºC) and injected subcutaneously in recipient mice under isoflurane anesthesia.

An intact male beagle (10 kg) was used in this study. The dog was sedated and anesthetized using approved agents. Anesthesia was maintained by inhalation of isoflurane 0.75-1.75%) in oxygen. Carprofen (4.4 mg/kg; Rimadyl®, Zoetis, Parsippany, NJ) was given subcutaneously at the time of anesthesia and on the day after implantation of cells to provide analgesia. The skin overlying the epaxial musculature of the back was prepared for aseptic surgery by removal of hair and scrubbing with chlorhexidine from the 13th rib to the cranial limit of the ileal crest. A small (5 mm) stab incision was made in the skin 2 cm caudal to the 13th rib. An 18 ga 6-inch spinal needle (Becton Dickinson, Franklin Lakes, NJ) that was preloaded with porcine pancreatic islets (30,000 IEQ/kg; total volume of 2.0 ml) was inserted through the skin incision into the epaxial musculature for a distance of 10 cm. 0.5 ml of the islet suspension was instilled, and the needle was withdrawn in 1.5 cm increments allowing 4 total injections of the islet suspension, each 2.5 cm from the previous injection site. The needle was withdrawn from the site of insertion, and the skin was sealed with tissue glue (Vetbond Tissue Adhesive™, 3M, Minneapolis, MN). Glucose tolerance tests were performed starting 3 weeks after cell implantation and repeated at 3–5-week intervals for 28 weeks post-transplantation. An 18 ga intravenous catheter was placed in a cephalic vein. At time 0, sterile 50% glucose in water (500 mg/ml; total dose of 500 mg/kg) was given intravenously over 1-2 minutes. A 1 ml blood sample was removed prior to intravenous administration of glucose and 5, 10, 20, 60, 90, and 120

minutes after instillation of glucose. A drop of blood was tested for glucose concentration using a glucometer (AlphaTrak, Abbott, Chicago, IL), and the remainder of the blood was placed in a tube containing EDTA. Tubes were placed on ice until separation of plasma by cold centrifugation at 1100xg for 10 minutes (Sorvall, ThermoScientific, Waltham, MA). Plasma was stored at -80^0 C until tested for concentrations of C peptide.

Western Blot

Protein samples for western blotting were isolated from murine or porcine islets by homogenization with lysis buffer (#9803, CST, USA). The samples were boiled in laemmli buffer (#161-0737, BioRad, USA) for 5 minutes and were resolved on a 4-12% gradient SDS-PAGE gel and blotted to PVDF membrane. Following overnight incubation with primary antibody against IDO (1:1000; #86630, CST, USA) and beta-actin (1:1000, # NB600-503, Novus Biologicals, USA) and detection by HRP-conjugated IgG. Bands were visualized using an Azure 300 chemiluminescent imaging system (Azure Biosystems, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Unpaired, non-parametric t tests with Mann Whitney tests were used for datasets without assuming a normal distribution. $P < 0.05$ was considered statistically significant. Data are shown as means ± SEM unless otherwise noted. The sample size, n, indicates the total number of biological replicates.

References:

1. Vanderschelden R, Sathialingam M, Alexander M, Lakey JRT. Cost and Scalability Analysis of Porcine Islet Isolation for Islet Transplantation: Comparison of Juvenile, Neonatal and Adult Pigs. Cell Transplant 2019;28(7):967-972.
2. de Souza AH, Santos LRB, Roma LP, Bensellam M, Carpinelli AR, Jonas JC. NADPH oxidase-2 does not contribute to beta-cell glucotoxicity in cultured pancreatic islets from C57BL/6J mice. Mol Cell Endocrinol 2017;439:354-362.