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ORIGINAL ARTICLE

Islet allografts expressing a PD-L1 and IDO fusion protein evade immune rejection and reverse preexisting diabetes in immunocompetent mice without systemic immunosuppression

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Abstract

Allogeneic islet transplantation is a promising experimental therapy for poorly controlled diabetes. Despite pharmacological immunosuppression, long-term islet engraftment remains elusive. Here, we designed a synthetic fusion transgene coupling PD-L1 and indoleamine dioxygenase [hereafter PIDO] whose constitutive expression prevents immune destruction of genetically engineered islet allograft transplanted in immunocompetent mice. PIDO expressing murine islets maintain robust dynamic insulin secretion in vitro and when transplanted in allogeneic hyperglycemic murine recipients reverse pre-existing streptozotocin-induced and autoimmune diabetes in the absence of pharmacological immunosuppression for more than 50 and 8 weeks, respectively, and is dependent on host CD4 competence. Additionally, PIDO expression in allografts preserves endocrine functional viability of islets and promotes a localized tolerogenic milieu characterized by the suppression of host CD8 T cell and phagocyte recruitment and accumulation of FOXP3$^+$ Tregs. Furthermore, in the canine model of xenogeneic islet transplantation, muscle implanted PIDO-expressing porcine islets displayed physiological glucose-responsive insulin secretion competency in euglycemic recipient for up to 20 weeks. In conclusion, the PIDO transgenic technology enables host CD4$^+$ T cell-modulated immune evasiveness and long-term functional viability of islet allo- and xenografts in immune-competent recipients without the need for pharmacological immune suppression and would allow for improved outcomes for tissue transplantation.

KEYWORDS
diabetes, IDO, islets, PD-L1, tolerance, transplantation

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GSIS, glucose-stimulated insulin secretion; GTT, glucose tolerance test; HLA, human leukocyte antigen; IDO, indoleamine dioxygenase; KYN, Kynurenine; NOD, non-obese diabetic; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PIDO, fusion protein of PD-L1 and IDO; STZ, streptozotocin; T1D, type-1 diabetes.

1 | INTRODUCTION

Chronic systemic immunosuppression is currently the only clinical strategy and standard therapy to prevent tissue and organ allograft rejection from genetically non-identical donors.[1] Despite significant improvements in posttransplant immunosuppressive therapy, long-term pharmacological inhibition of host immune response still causes serious adverse effects such as opportunistic infections, cardiac and renal toxicity, and increased risk of malignancies.[2] Compounded with a severe shortage of cadaveric donor-derived cell/tissue and HLA mismatch, the side effects of chronic immunosuppression present a major obstacle in the broad adaptation of cell and organ transplant as a therapy for the treatment of several end-stage human diseases.[1,3–5] These obstacles have significantly impeded the development of clinical islet transplantation as a promising therapy for the treatment of type-1 diabetes (T1D).[6–8] The majority of islet allograft recipients lose graft function and, therefore, insulin independence within 3–5 years.[9] Furthermore, the major risks associated with chronic immunosuppression prevent from meeting the clinical objective of benefit in T1D and other pathologies where transplantation could be a lifesaving treatment option. Thus, novel tolerance induction approaches that specifically address the destructive alloimmune response by precluding or mitigating chronic immune suppression are critically needed.[10]

Our understanding of cancer immune evasiveness may provide insights that would inform translational strategies that can address the transplant immunology conundrum. Programmed death-1 (PD-1) and its ligand (PD-L1) constitute a potent cancer-enabling immune suppressive dyad that has emerged as a primary target for effective and durable cancer immunotherapy.[11] A member of the CD28/B7 superfamily, PD-1 interacts with two physiological ligands PD-L1 and PD-L2.[12] While PD-1 is expressed on resting and activated T cells and other immune cells,[12] PD-L1 is constitutively expressed by a wide variety of immune cells and can also be expressed by non-immune cells including pancreatic islets.[13,14] PD-1/PD-L1 binding leads to a blockade in T cell effector function and induces Foxp3-lineage regulatory CD4 T cells (Tregs) and thus, forms an important immune checkpoint pathway that modulates innate, adaptive, and regulatory immune responses and also plays a critical role in the maintenance of immune homeostasis and tolerance.[15,16] Combined with the observation that PD-1 or PD-L1 deficient non-obese diabetic (NOD) mice develop accelerated T1D,[17] these findings suggest a key role for the PD-1/PD-L1 pathway in the induction and/or maintenance of pancreatic immune homeostasis and tolerance.

Akin to PD-L1, indoleamine 2,3-dioxygenase (IDO) expression has also demonstrated similar immune suppressive properties. First identified in maternal-fetal tolerance, IDO contributes in the prevention of allogeneic fetal rejection by maternal T cells.[18] IDO is an intracellular heme-containing enzyme that mediates oxidative catabolism of Tryptophan (Trp). Cells expressing IDO catabolize Trp and generate bioactive catabolite kynurenine (Kyn). Kynurenines and depleted Trp suppress innate and adaptive immunity by several mechanisms including apoptosis, suppression of function, and/or blockade of cell cycle entry of TCR-activated T cells, and by activation of resting Tregs to promote tolerogenic responses to inflammatory signals.[19] While IDO expression is mostly induced by inflammatory stimuli, recent reports have also shown that subsets of human myeloid dendritic cells (DCs) and cancer cells might constitutively express IDO to suppress allogeneic T cell immune responses.[20–22] Taken together, these reports propose a compelling rationale for the clinical investigation of the tolerogenic capabilities of PD-L1 and IDO, although, very limited data exist about the role of these proteins/pathways in the context of transplantation immunotherapy. A few recent reports have explored the ectopic expression of PD-L1 in the skin, cardiac, liver, and islet[23,24] and IDO in the lung[25] and islet[26,27] transplant tolerance. However, most of these studies have focused on either blockade of the PD-1:PD-L1 axis, co-transplantation of accessory cells engineered to express PD-L1 or IDO, and tissue or biomaterial engineered to transiently or stably express one of these proteins. While these approaches showed some efficacy,[27,28] most lacked evidence of longevity and, therefore, faced hurdles to clinical translation.

Here, we engineered and generated a chimeric fusion transgene: PIDO, where PD-L1 was cloned N-terminus and linked to intracellular IDO at C-terminus. Allogeneic pancreatic islets were engineered to express and positionally display PD-L1 on the cell surface and bioactive IDO in the cytoplasm. We have observed that transplanting PIDO-engineered allogeneic islets reversed hyperglycemia in NOD mice without requiring any additional pharmaceutical immunosuppression. Our data demonstrate, for the first time to our knowledge, that induction of tolerogenic/immune suppressive pathways can reverse preexisting diabetes in an autoimmune setting. We also observed that recipient CD4 competence is required for PIDO-mediated immune evasion of islet allografts. Our data strongly suggest that PIDO expression shifts host immune response from inflammatory to tolerogenic at the site of transplantation. Finally, we show that PIDO-engineered porcine islet xenografts are protected from immune rejection in immune-competent murine and canine models. Cumulatively, these data suggest that PIDO genetic engineering of cells and tissue can be used as a novel immunotherapeutic for tissue-restricted immune evasion and prevention of allograft rejection.

2 | METHODS

For an expanded description of methods, please see *Detailed Description of Materials and Methods* in the Supplemental Information.

3 | RESULTS

3.1 | Synthetic PIDO fusion protein retains structural and functional characteristics of constituent PD-L1 and IDO domains and does not alter pancreatic islet function

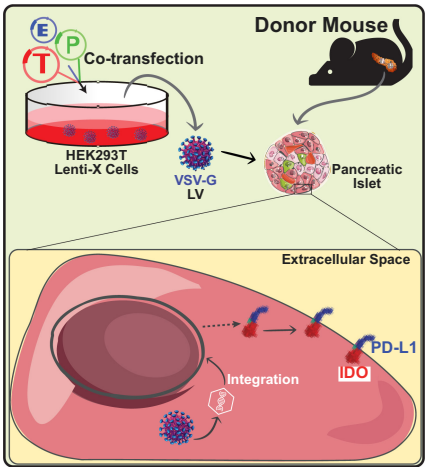
To combine PD-L1 and IDO, we created a synthetic gene containing the coding sequences of full-length mouse PD-L1 and human IDO1 proteins separated by a 3X GGGS linker. This synthetic gene

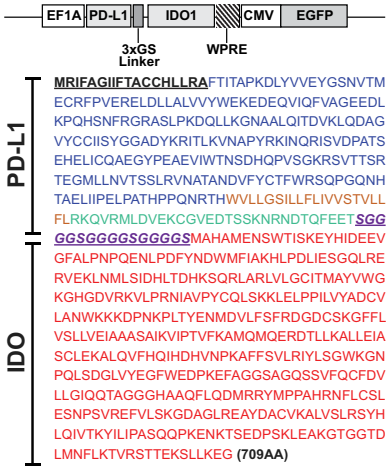
was subcloned in-frame with the PD-L1 membrane localization signal in the pLV-EXP/CMV-EGFP lentiviral vector. Final PIDO cDNA encodes a single polypeptide chain of 708 amino acids with a predicted non-glycosylated MW of ~80 kDa (Figure 1B). The in-silico 3D structure of PIDO was predicted and constructed using I-TASSER and webserver Phyre2[29,30] (Figure 1C). The expression vector was packaged in lentiviral particles. Next, we engineered A375 human melanoma cells or C57BL6/J mouse islets by transduction with PIDO-expressing lentiviral particles. The expression, sub-cellular localization, and biological activity of PIDO fusion protein were verified by immunofluorescence staining, flow cytometry, western blot, and enzyme-linked immunosorbent assay (ELISA). We detected robust expression of PD-L1, IDO, and PIDO fusion protein in mouse islets (fluorescent reporter proteins, Figure 1D). To investigate the cellular localization of chimeric PIDO protein, we assessed the surface PD-L1 expression of PIDO protein in dispersed islet cells by flow cytometry. Our data show that almost twice as many PIDO-expressing mouse islet cells displayed surface PD-L1 (aka CD274) expression compared to only PD-L1 expressing islet cells (65% vs 24%), suggesting that PIDO fusion protein is permissive to higher cell surface density afforded by PD-L1 ectopic expression on its own (Figure 1E). Denaturing immunoblotting performed on IDO or PIDO expressing mouse or pig islets showed that the fusion protein was robustly expressed and migrated at a combined molecular weight of ~90 kDa (Figure 1F). Our data also show that when normalized for input protein, the abundance of the fusion protein was significantly higher compared to IDO expressed alone or co-expressed with PD-L1 (Figure S1). Together, these data suggest that PIDO-engineered islets display PD-L1 on the membrane and express IDO in the cytoplasm tethered to the C-terminus of the cytoplasmic tail of PD-L1 as depicted in the schematic in Figure 1G. The functional activity of IDO can be assessed by the detection of extracellular kynurenine arising from the catalysis of culture media sourced Tryptophan. As shown in Figure 1H, kynurenine levels increased significantly in conditioned media of both IDO and PIDO expressing islets comparable to media of IFNγ-treated mesenchymal stromal cells (positive control). Interestingly, mouse islets dual transduced to express PD-L1 and IDO simultaneously but independently, displayed lower IDO activity as shown by significantly lower kynurenine levels in conditioned media of these islets. This suggests that the effect of co-expression of PD-L1 and IDO is not functionally equivalent to that of PIDO fusion protein supportive of gain-of-function features of PIDO.

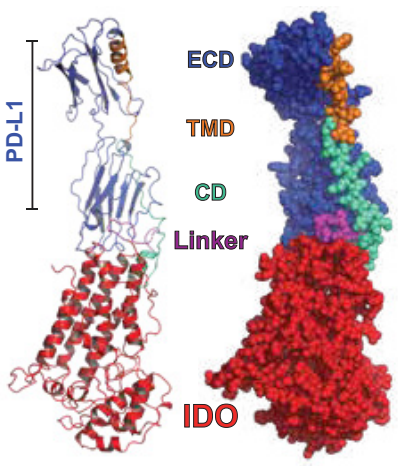
Islet β-cells are known to augment surface expression of PD-L1 during the development of insulitis.[31] While this could be a defense mechanism of β-cells against autoreactive T cells, such induction in PD-L1 expression may also initiate stress pathways in β-cells. In the same line of thought, IDO is not naturally expressed in islets and the functional impact of IDO-driven tryptophan depletion and KYN production on β-cell function are undefined. To understand the effect of ectopic PD-L1 surface expression and constitutive IDO catabolic functionality, we cultured islet expressing PD-L1, IDO, or PIDO for 48 hours and performed glucose-stimulated insulin secretion (GSIS) assay. The GSIS data showed no difference in insulin secretion as a function of transgene expression (Figure 1I). Together, these data show that the PIDO fusion protein expresses robustly on the cell surface, its constituent IDO enzyme retains catalytic activity and constitutive expression of PIDO does not interfere with islet GSIS.

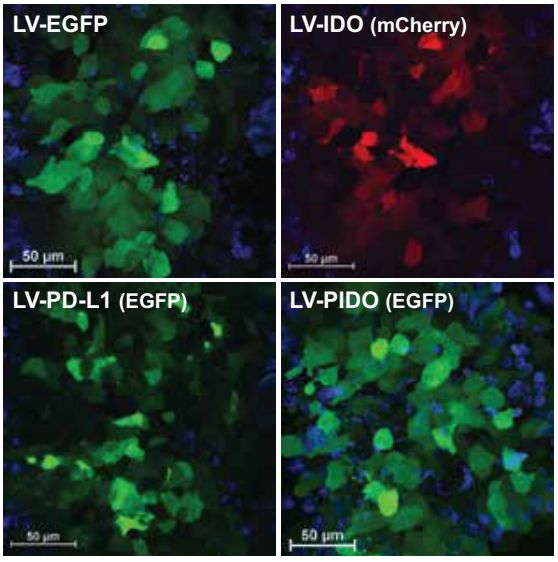
3.2 | PIDO-engineered islet allografts reverse hyperglycemia in diabetic mice

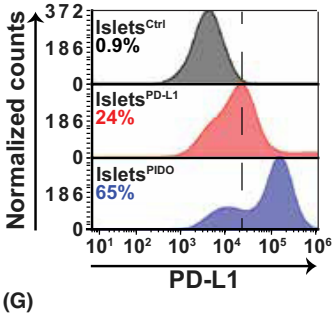
To determine the potential of PIDO-engineered allogeneic islets for transplantation in diabetic mice, we transplanted ~400 hand-picked and size-matched (islet size range, visually determined) lentivirus-transduced C57BL/6 islets under the left kidney capsule of BALB/c mice previously rendered diabetic by streptozotocin (STZ) to deplete endogenous islets (Figure 2A). Islet dose sufficiency was determined in both C57BL6 and BALB/c diabetic recipient mice (Figure S2A). Three mice (transplanted with islets transduced with control LV) died spontaneously, one at 12 weeks and two others at 24 weeks. At 20 weeks posttransplantation, the $PIDO^+$ islet allografts were detected under the kidney capsule and stained positive for insulin (Figure 2B). To understand whether the expression of PD-L1 or IDO alone or their co-expression is sufficient to reverse diabetes in mice, we transduced C57BL/6 islets with PD-L1, IDO, or co-transduced with PD-L1 and IDO together. Data in Figure 2C show that islets expressing PD-L1/IDO alone or together failed to reverse preexisting hyperglycemic diabetes in mice. While allograft recipients transplanted with islets co-expressing PD-L1 and IDO showed some initial recovery (~3 weeks posttransplantation), these never achieved normoglycemia and ~5 weeks posttransplantation, the initial glycemic improvement was lost leading to relapse of hyperglycemia in PD-L1 + IDO expressing islet recipients. This observation further strengthens the notion that the gain of function activity of PIDO fusion protein is distinct from the sum of PD-L1 and IDO. Next, we tracked the blood glucose of mice with preexisting STZ-induced diabetes and transplanted them with control or PIDO-expressing islets. In $PIDO^+$ islet transplanted mice, the blood glucose dropped to <200 mg/dl within 3 weeks (Figure 2D) and became completely normoglycemic by 10 weeks (no difference from healthy, non-transplanted mice). These $PIDO^+$ allograft recipients remained normoglycemic for the entire duration of the study with a blood glucose concentration of 87 ± 7 mg/dl (fasting) or 109 ± 12 mg/dl (random-fed, Figure 2D, Figure S2B,C). We performed a glucose tolerance test (GTT) 2 and 10 weeks posttransplantation. Mice transplanted with $PIDO^+$ islets demonstrated improved glucose tolerance as early as 2 weeks posttransplantation compared to control islet transplanted mice (Figure 2E). For the 50 weeks observation period, only $PIDO^+$ islet transplanted mice achieved and maintained normoglycemic blood glucose levels, whereas the mice transplanted with control islet allografts did not show any glycemic recovery. Serum collected from all groups of mice at 2 and 10 weeks posttransplantation were assayed for insulin. Figure 2F

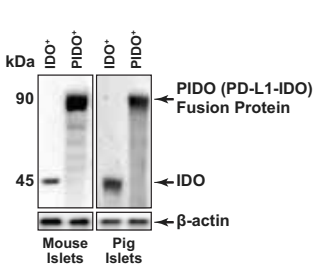


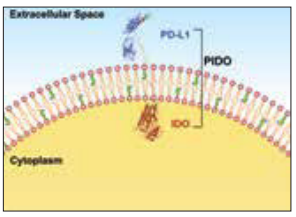


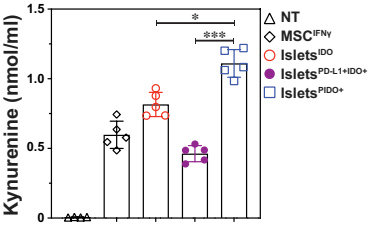


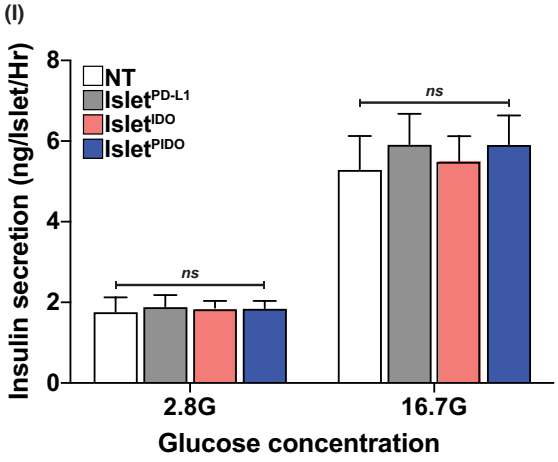












shows that the PIDO$^+$ islet transplantation groups had detectable insulin at 2 weeks (0.64 ± 0.38 ng/ml) and by 10 weeks, the insulin levels were comparable to normoglycemic, non-transplanted mice (0.9 ± 0.17 ng/ml).

Finally, we sought to test the tolerogenic potential of PIDO expression on the survival of allogeneic islets in NOD mice. Control or PIDO$^+$ allogeneic (C57BL/6J) islets were transplanted into diabetic female NOD mice. Transplanted mice were followed for 8 weeks. As shown in Figure 3,

FIGURE 1 Chimeric fusion protein PIDO engineered pancreatic islets. (A) Schematic of lentiviral transduction of pancreatic islets for PIDO expression. (B) Schematic presentation of PIDO construct and chimeric PIDO protein sequence. (C) Predicted 3D structure of PIDO fusion protein. (D) Lentivirus transduction efficiency of A375 human melanoma cells was detected by expression of indicated fluorescent reporter for PD-L1 (green), IDO (red), or PIDO fusion protein (green) compared to EGFP, DNA was counterstained with DAPI (blue). C57BL/6 mouse islets were transduced by lentiviruses expressing PD-L1, IDO or PIDO followed by enzymatic dispersion and analysis by (E) flow cytometry to measure PD-L1 expression, (F) Western blot (representative of three) of extracts from PIDO-engineered mouse or pig islets using an anti-IDO antibody to assess IDO/PIDO expression, and (G) schematic presentation of predicted subcellular localization of PIDO protein with PD-L1 being displayed on the membrane while IDO tethered to the cytoplasmic tail of PD-L1 and expressed in the cytoplasm. (H) Kynurenine ELISA to detect IDO catalytic activity ($n = 4$). (I) Mouse islets were transduced to express PD-L1, IDO, or PIDO and were compared to unmodified islets after 48 h of in vitro culture prior to glucose-stimulated insulin secretion assay. Data show secreted insulin (ng)/islet/hr at low (2.8G) and high (16.7G) glucose concentration. Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

control islet recipients showed a variable and transient improvement in blood glucose, but eventually rejected their grafts in a mean survival time of 8 days ($n = 4$). In contrast, PIDO$^+$ islet-recipients ($n = 5$) showed glycemic improvement within a week and remained normoglycemic for the duration of the study (8 weeks), and proved effective in reversing preexisting autoimmune diabetes (Figure 3B,C). Relapse incidence rate was 100% in control islet recipient group vs 20% in PIDO$^+$ islet-recipient group (blood glucose >250 mg/dl). All recipients were presumed non-diabetic for the ease of data visualization (Figure 3C). Cumulatively, these data demonstrate that constitutive PIDO expression enables islet allografts to evade alloimmune response and consequently, reverse preexisting diabetes (chemically induced or autoimmune) secondary to endogenous islet deficiency in immunocompetent mice. In addition, these data also support the hypothesis that PIDO fusion protein possess biochemical and functional characteristics distinct from than combined effect of PD-L1 and IDO proteins individually.

3.3 | PIDO-induced graft immune evasion does not lead to acquired immunologic tolerance to allogeneic islets

Reversal of preexisting diabetes in PIDO$^+$ islet allografts in BALB/c or NOD recipients is consistent with immune evasion. To test the possibility that acquired immune tolerance by BALB/c recipients contributes to the sustained survival of C57BL/6 islet allografts, we destroyed/removed PIDO$^+$ islet allografts in BALB/c recipients either by STZ treatment or nephrectomy. Thereafter, we retransplanted the previously cured BALB/c animals—now rendered diabetic anew—with naïve C57BL/6 islets (Figure 4). We injected the first set of BALB/c mice ($n = 5$) with a second dose of STZ to destroy β-cells and thus, the majority of PIDO$^+$ C57BL/6 islet allografts 20 weeks posttransplantation. All recipients developed hyperglycemia within 2 weeks (Figure 4B,C). Two weeks following the destruction of primary PIDO$^+$ C57BL/6 islet allografts and re-induction of diabetes, these BALB/c mice were transplanted with a second set of naïve C57BL/6 islets under the capsule of the contralateral kidney. The naïve allografts effected only a partial and transient recovery (Figure 4B) and quickly (within 3 weeks) led to the development of hyperglycemia indicating loss of naïve allografts. Streptozotocin is an efficient DNA alkylating agent that destroys islet β-cells via GLUT2 glucose transporter. While it is broadly used to establish mouse diabetes, its efficacy in pancreas and kidney capsule may not be equivalent or comparable due to inherent differences in vascularization of these two different tissues. We hypothesized that the partial and transient glycemic recovery by naïve islets could be attributed to the suboptimal effect of STZ on primary allograft islets under the kidney capsule and, therefore, tested the existence of acquired tolerance using an independent metric. In the second set of allograft recipients ($n = 5$), we removed the host kidney containing PIDO$^+$ islets. These recipients developed hyperglycemia swiftly (within 1 week). Two weeks post-nephrectomy, we transplanted these BALB/c mice with naïve C57BL/6 islet allografts under a contralateral kidney capsule. All recipients became hyperglycemic within 1 week (Figure 4C). These data demonstrate that in the absence of PIDO expression, secondary naïve C57BL/6 allografts failed at reversing diabetes suggesting that the originally cured BALB/c recipients did not acquire immunologic tolerance to allogeneic C57BL/6 islets and that the allograft immune evasion achieved by PIDO-expressing islets is not mediated through acquired memory immune tolerance mechanism(s) but rather through immune evasion.

3.4 | PIDO-mediated immune evasiveness requires host CD4 T cell competence

It is well established that alloreactive tissue rejection immune response is primarily CD8$^+$ T cell-mediated[32] and allo-tolerance is mediated by host CD4$^+$ T cells with Treg competency.[33,34] To determine if host tolerogenic cells mediating immune evasive effects of PIDO are indeed CD4$^+$ T cells, we tested the therapeutic efficacy of PIDO$^+$ islet allografts in CD4^{m1Mak} (CD4$^{-/-}$) recipients that had been rendered diabetic following STZ treatment (Figure 5A). The PIDO$^+$ islet allografts faced a rapid rejection in CD4$^{-/-}$ mice (Figure 5B). This data informs that host CD4$^+$ T cells are necessary for immune evasion induced by PIDO-engineered islet cells.

3.5 | PIDO expression protects allograft viability and function and modulates host immune response at the transplantation site

The immunomodulatory effects of PIDO and consequent protection of functional viability of islet allografts were tested in an allogeneic islet transplantation setting. Control or PIDO lentivirus transduced

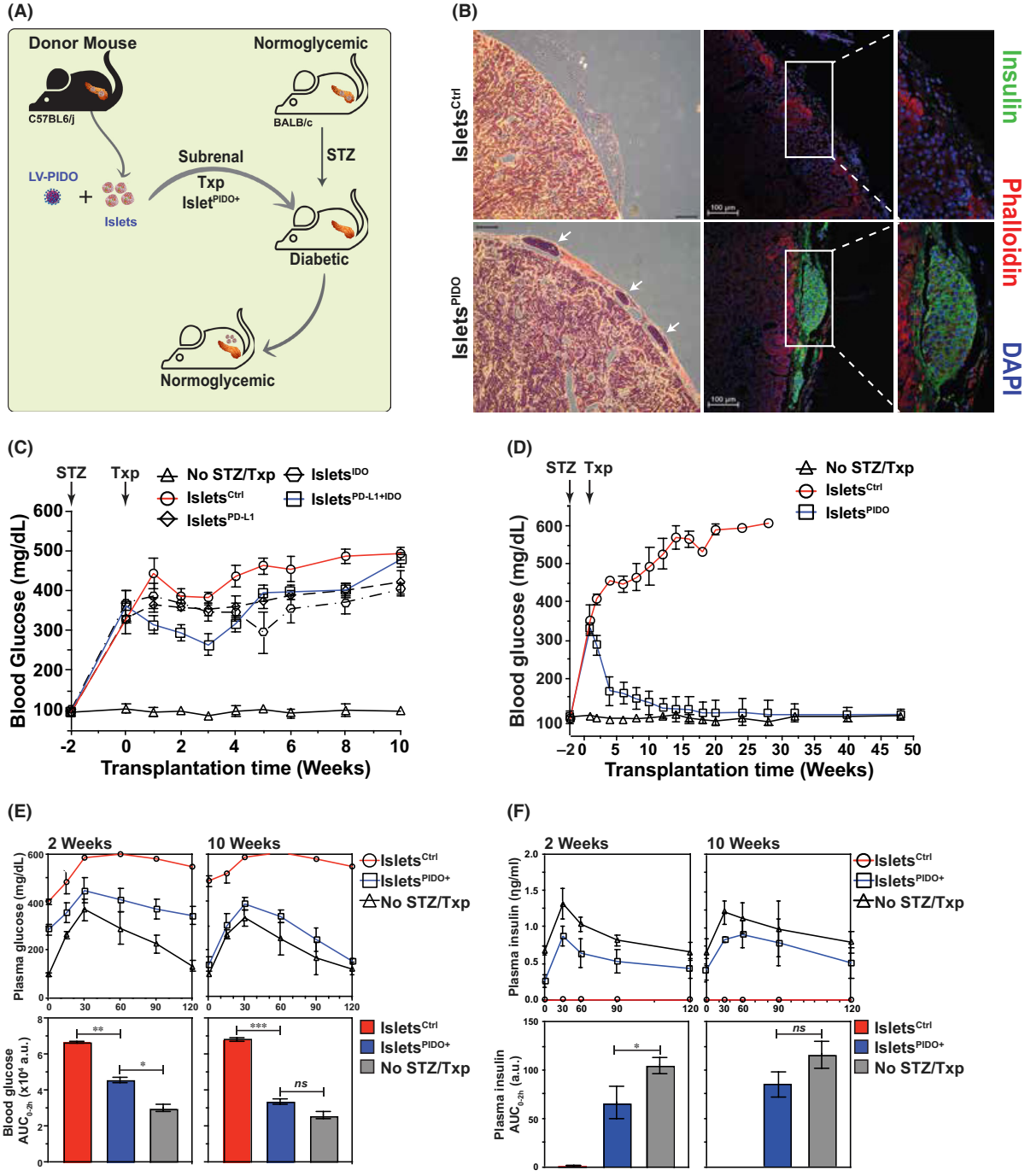


FIGURE 2 PIDO engineered allogeneic islets reverse pre-existing chemically induced diabetes in mice. (A) Schematic of diabetes induction with streptozotocin (STZ) and transplantation of PIDO expressing allogeneic C57BL/6 islets in BALB/c recipients. (B) Representative sections of transplanted islet allografts under the kidney capsule (bright field, left, magnification 4×) or stained for insulin (green), actin with phalloidin (red), and DNA counterstained with DAPI (blue); original magnification ×20. (C) Blood glucose measurements before and after STZ treatment and after transplantation with engineered allogeneic islets. Five groups were studied: Mice without a transplant (No STZ, No Txp; $n = 3$; dotted line), diabetic mice transplanted with control islets (STZ, EGFP Txp; ~400 islets; $n = 4$; red), diabetic mice transplanted with islets expressing PD-L1 (STZ, PD-L1 Txp; ~400 islets; $n = 5$; diamond symbols, broken line), diabetic mice transplanted with islets expressing IDO (STZ, IDO Txp; ~400 islets; $n = 5$; hexagon symbols, broken line) and diabetic mice transplanted with islets co-expressing PD-L1 and IDO (STZ, PD-L1 + IDO Txp; 400 islets; $n = 5$; blue). (D) Blood glucose measurements before and after STZ treatment and after transplantation with engineered allogeneic islets. Three groups were studied: Mice without a transplant (No STZ, No Txp; $n = 3$; dotted line), diabetic mice transplanted with control islets (STZ, EGFP Txp; 400 islets; $n = 4$; red), diabetic mice transplanted with PIDO expressing islets (STZ, PIDO Txp; 400 islets; $n = 5$; blue). (E) Glucose tolerance test (GTT) 2 weeks and 10 weeks after Txp. Three groups were studied: No STZ, No Txp; ($n = 3$; 2 Wk and 10 Wk, black), control Txp ($n = 5$; 2 Wk and 10 Wk, red) and PIDO Txp ($n = 5$; 2 Wk and 10 Wk, blue). Lower panel: Area under the curve (AUC) quantification of GTT data. (F) In vivo GSIS secretion 2 and 10 weeks after transplantation for No STZ, No Txp; ($n = 3$; 2Wk and 10 Wk, black), control Txp ($n = 4$; 2 Wk and 10 Wk, red) and PIDO Txp ($n = 5$; 2 Wk and 10 Wk, blue). Data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

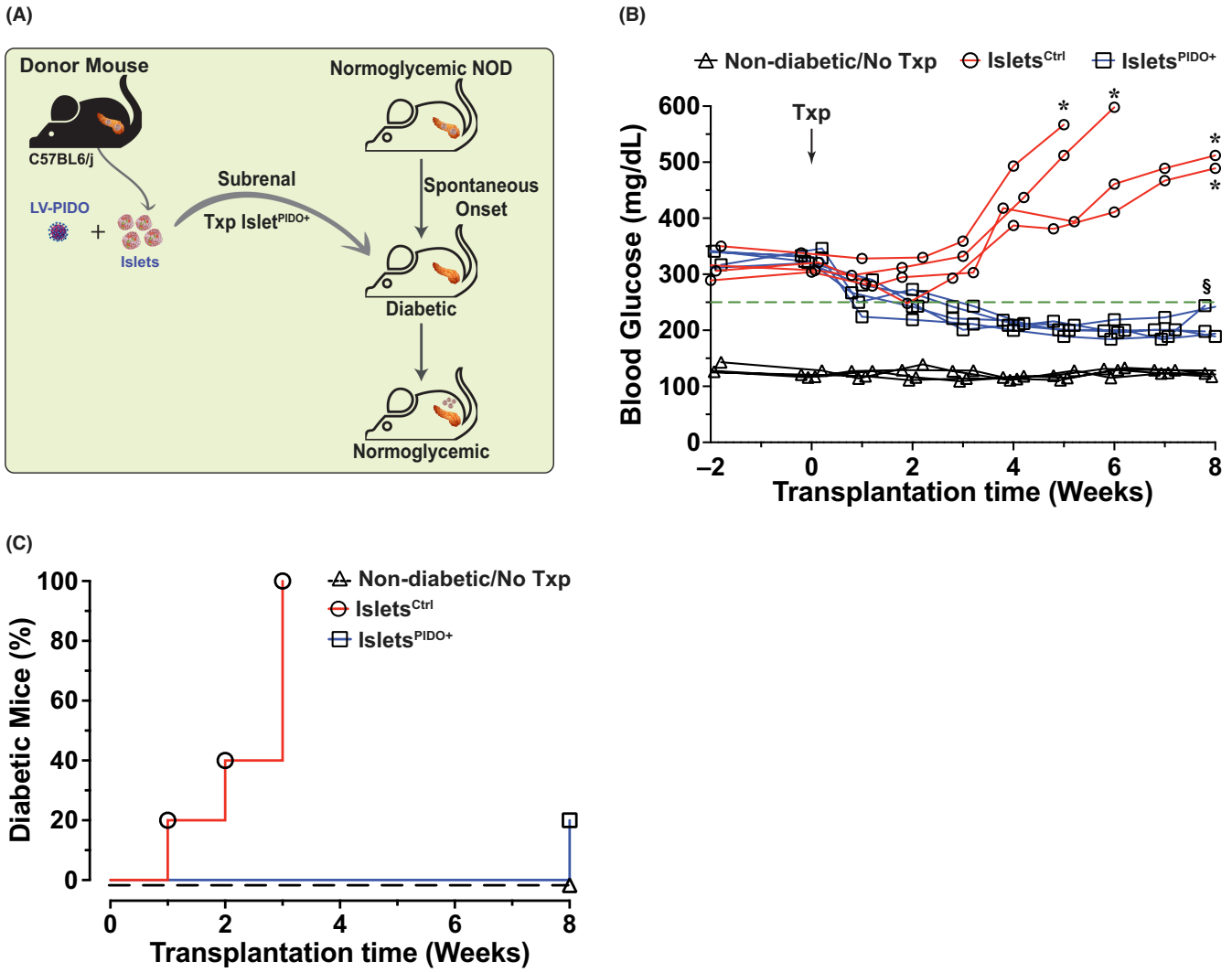


FIGURE 3 PIDO engineered islet allografts improve hyperglycemia in diabetic NOD mice. (A) Schematic of transplantation of PIDO expressing allogeneic C57BL/6 islets in diabetic NOD recipients. (B) Fed blood glucose measurements in NOD mice after transplantation with naïve or engineered allogeneic (C57BL/6J) islets. Three groups were studied: normoglycemic mice without a transplant (Non-diabetic/No Txp; $n = 4$; black), diabetic mice transplanted with control islets (EGFP Txp; 400 islets; $n = 4$; red), diabetic mice transplanted with PIDO expressing islets (PIDO Txp; 400 islets; $n = 5$; blue). Animals dying of diabetes complications (hypoinsulinemia) or with relapsing diabetes were censored from the analysis at the observed time of death/relapse and are marked on the plot with an * and § respectively. (C) Stairstep graph shows diabetes relapse incidence in PIDO$^+$ allogeneic islet-transplanted vs naïve allogeneic islet-transplanted NOD mice. Diabetes relapse (blood glucose >250 mg/dl) was used as the terminal event.

islets were mixed with Matrigel™ nutrient matrix and the plugs containing islets were transplanted subcutaneously in immunocompetent non-diabetic BALB/c mice. Matrigel plugs were explanted at 15 days posttransplant and cells contained therein were analyzed by flow cytometry (Figure 6A,B). Recipients of control islets acutely rejected all allografts. Compared to PIDO-expressing islet allograft plugs, very few insulin or glucagon positive events were detected in control islet-containing Matrigel plugs (Figure 6C). Additionally, we detected cell surface presentation of PD-L1 in a significant fraction of surviving insulin (66%) or glucagon (21%) positive cells (Figure S3).

Our data (Figure 4B,C) have unequivocally shown that the immunomodulatory effects of PIDO are localized. To understand the local effects further, we analyzed immune cell populations (CD45$^+$) harvested from Matrigel plugs (the gating strategy for flow cytometric analysis is depicted in Figure S4), with a particular focus on cytotoxic and regulatory T cells as targets of PIDO-mediated immunomodulation. While CD4$^+$ cell numbers were comparable between control or PIDO$^+$ islets, the magnitude of the effect was markedly different on infiltrating CD8$^+$ cells with substantial suppression of infiltrating cells in the PIDO group (Figure 6D).

Next, we examined infiltrating macrophages. We observed a highly specific and significant decrease in numbers of both F4/80$^+$ and CD11b$^+$ phagocytic macrophages in the plugs explanted from mice receiving PIDO-engineered islets compared with that from control islet recipients (Figure 6E).

Finally, we interrogated whether PIDO expression influenced the infiltration of Tregs. Our data (Figure 6F) show that the PIDO$^+$ islet

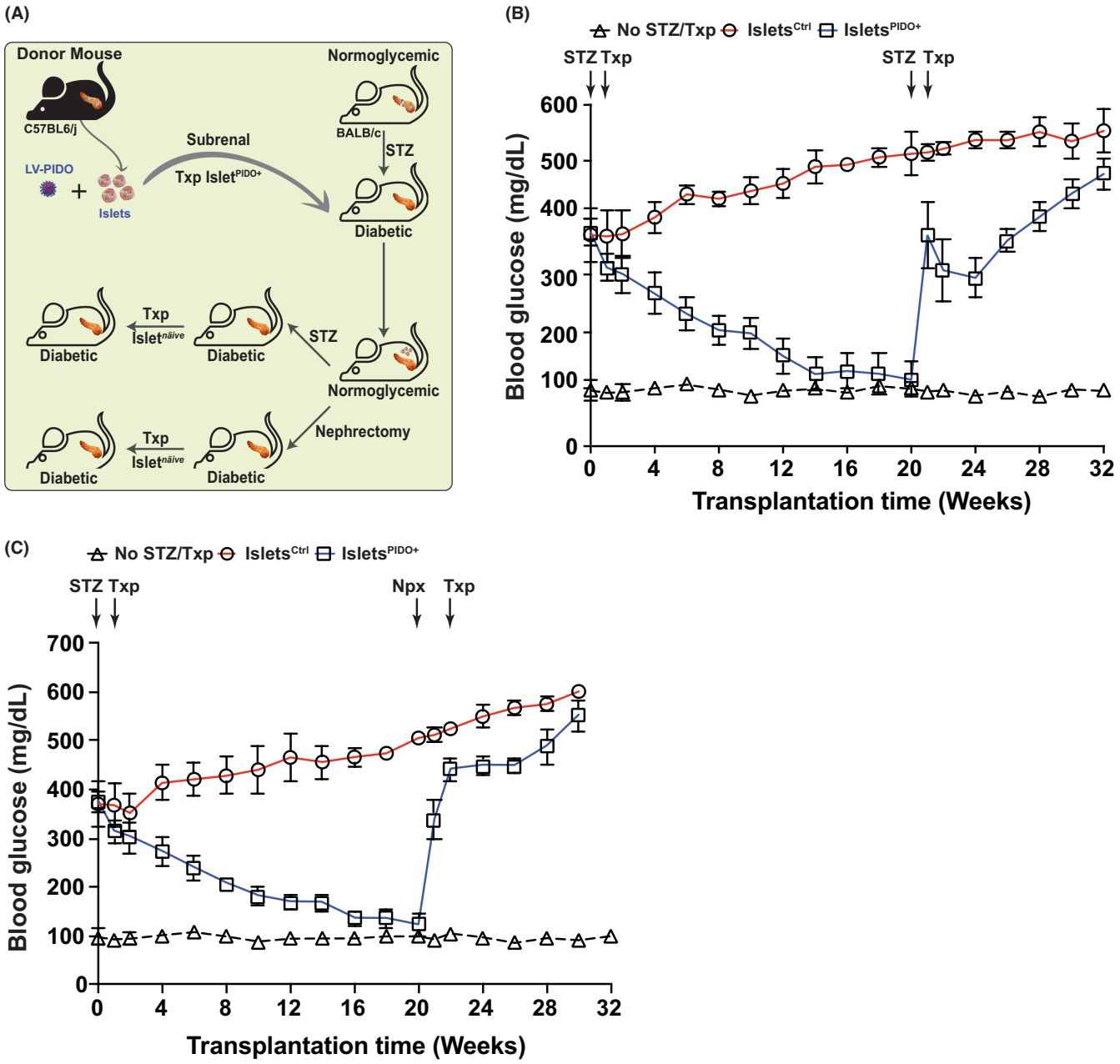


FIGURE 4 PIDO priming of the immune system does not confer acquired immune tolerance against naïve allogeneic islets. (A) Schematic of diabetes induction with streptozotocin (STZ) and transplantation of PIDO expressing allogeneic C57BL/6 islets in BALB/c recipients, rechallenge by STZ or nephrectomy of the transplanted mice and second subrenal transplantation in contralateral kidney. (B) Blood glucose measurements before and after STZ treatment, after transplantation with engineered allogeneic islets, after rechallenge with STZ and after second transplantation with naïve allogeneic islets. Three groups were studied: mice without a transplant (No STZ, No Txp; $n = 3$; dotted line), diabetic mice transplanted with control islets (STZ, EGFP Txp; 400 islets; $n = 4$; red), and diabetic mice transplanted with PIDO expressing islets (STZ, PD-L1 Txp; 400 islets; $n = 5$; blue) (C) Blood glucose measurements before and after STZ treatment, after transplantation with engineered allogeneic islets, after rechallenge with nephrectomy and after second transplantation with naïve allogeneic islets. Three groups were studied: mice without a transplant (No STZ, No Txp; $n = 3$; dotted line), diabetic mice transplanted with control islets (STZ, EGFP Txp; 400 islets; $n = 5$; red), diabetic mice transplanted with PIDO expressing islets (STZ, PIDO Txp; 400 islets; $n = 5$; blue). Data are presented as mean ± SD.

recipient group contained a significantly higher number of Treg cells compared to the control group (average 3200 vs 280 cells/preparation). Taken together, these results demonstrate that PIDO expression in islet allograft protects graft viability and function, suppresses cytotoxic CD8, and phagocyte tropism and induces a local FOXP3$^+$ Treg-rich milieu.

3.6 | PIDO-expressing porcine pancreatic islets are immune evasive in xenogeneic murine and canine recipients

While gene editing methods have raised hopes by improving tolerance to porcine xenograft donors,[35] immunosuppression has

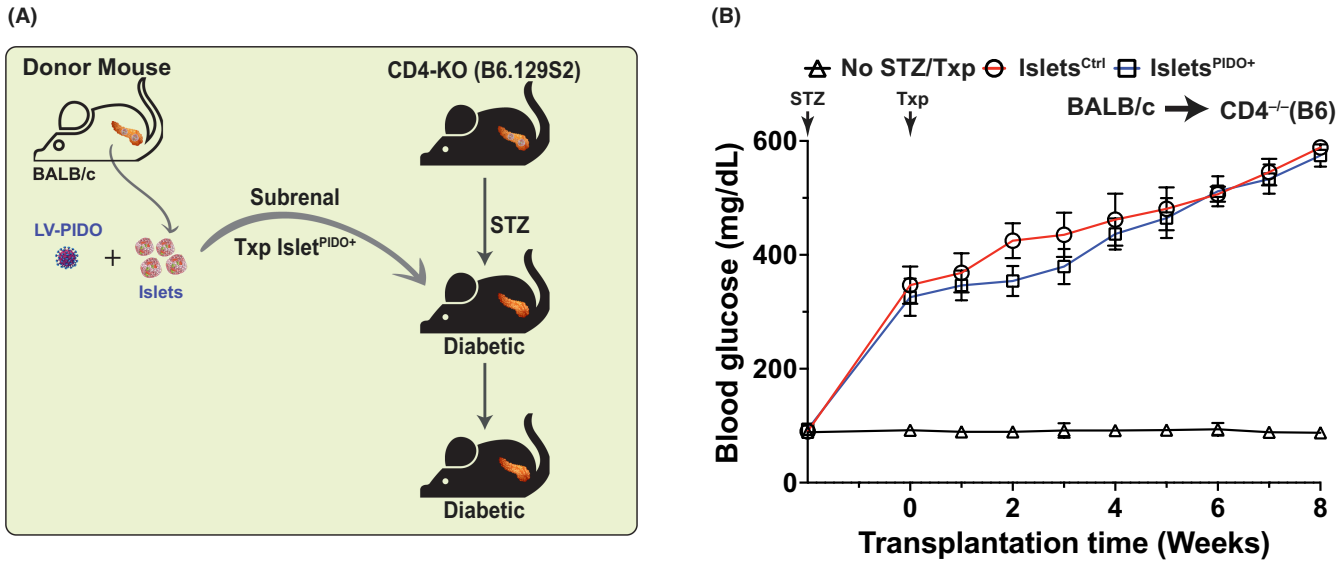


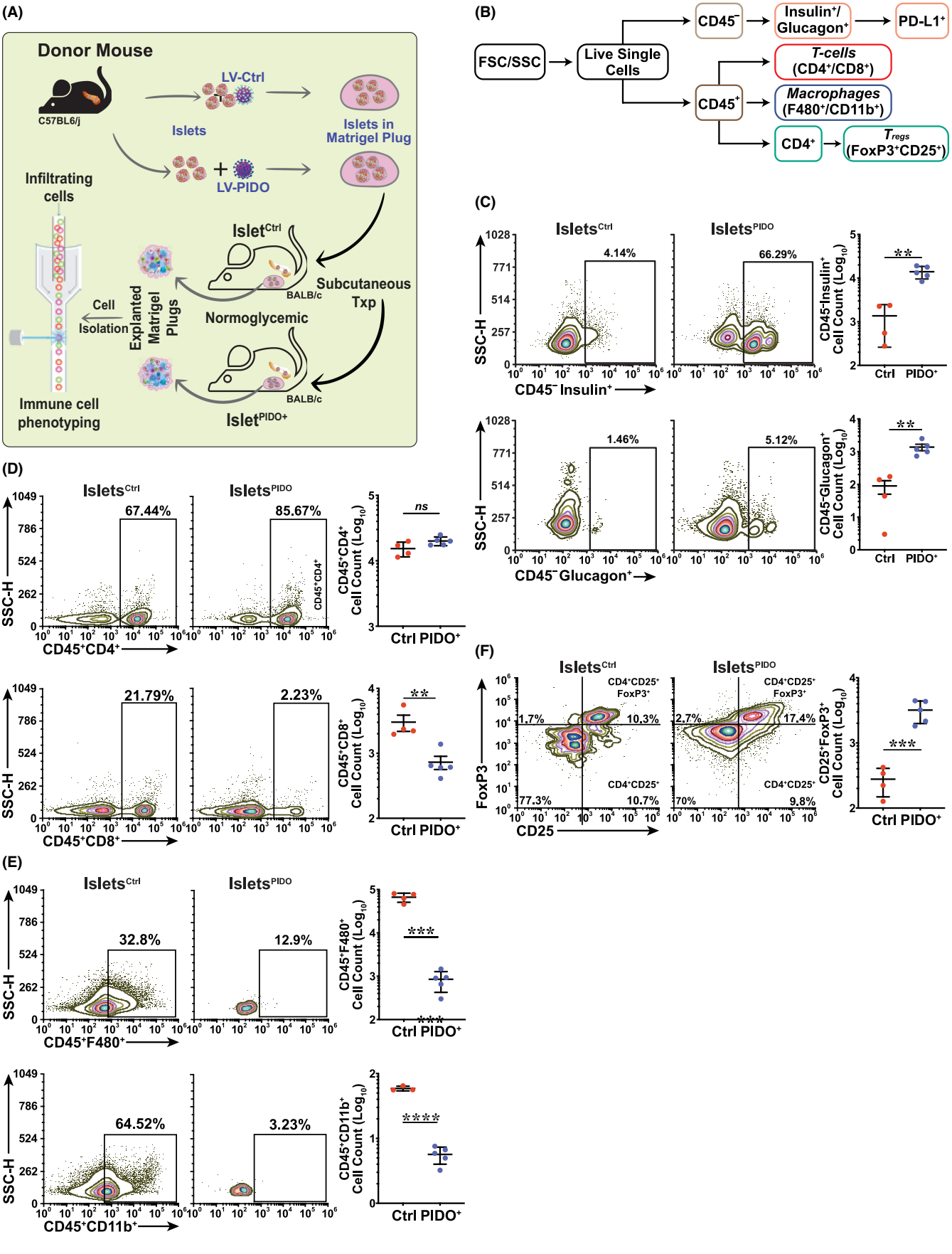
FIGURE 5 PIDO-promoted immune evasion of engineered islet allografts requires CD4 expression. (A) Schematic of PIDO expressing BALB/c allogeneic islet transplant in diabetic CD4-deficient mice. (B) Blood glucose measurements before and after STZ treatment and after transplantation with engineered allogeneic (BALB/c) islets in CD4-deficient mice. Three groups were studied: mice without a transplant (No STZ, No Txp; black line), control islets (STZ, EGFP; red), and with islets expressing PIDO (STZ, PIDO; blue). Data are presented as mean ± SEM.

remained a necessity to prevent immune rejection of islet xenografts in simians.[36,37] In our murine model of allotransplantation, the successful prolonged reversal of diabetes by PIDO$^+$ allografts prompted the testing of its possible competence in the induction of cross-species xenogeneic islet tolerance. To examine this, we designed two islet xenotransplant models: porcine-to-murine and porcine-to-canine (Figure 7A). In both models, in vitro matured juvenile porcine islets were engineered to express PIDO fusion protein and transplanted either under the kidney capsule (porcine-to-murine) or in epaxial muscle (porcine-to-canine). We detected porcine insulin in recipient immunocompetent hyperglycemic C57BL/6 mice up to 16 weeks posttransplantation (Figure 7B). While these data show that naïve pig islets are quickly rejected and only PIDO$^+$ islet xenografts survived and remained functional in immunocompetent diabetic mice, further inquiry in this model on the impact on clinical diabetes was not feasible due to distinct rodent insulin receptor that renders porcine insulin unable to regulate glucose homeostasis in mice and rats.[38]

To test PIDO-induced xenograft tolerance in a large animal model, we transplanted a normoglycemic, immunocompetent non-diabetic Beagle dog with PIDO-engineered porcine islets. A previous report has shown that naïve pig islets lost function (most probably due to immune rejection) quickly in diabetic canine recipients.[39] The hypothesis tested here is whether an ectopic muscle implant of PIDO$^+$ porcine islets would preserve glucose homeostasis (e.g., not lead to hypoglycemia) and display a physiologic response to a glucose challenge. The C-peptide (connecting peptide) is a short polypeptide that connects insulin's A- and B-chains in the proinsulin molecule. Cleaved during mature insulin production, C-peptide is secreted along with insulin and is thus, used as a marker of insulin secretion. In this proof-of-concept model, we measured porcine C-peptide in canine plasma which is feasible due to negligible porcine/canine interspecies C-peptide cross-reactivity. We performed an intravenous glucose tolerance test (ivGTT) to invoke a response in a euglycemic dog that otherwise would not recruit porcine islets due to the presence of fully competent pancreatic islet mass. We detected porcine C-peptide for 20 weeks in dog plasma in response to glucose stimulus (Figure 7C) which strongly suggests prolonged survival of porcine islet xenograft. We also observed a progressive decline in C-peptide response to GTT over time. This decline, however, cannot be attributed solely to loss of xenograft by immune rejection as the duration of detectable graft function extended well beyond the known period of immune rejection in canine recipients.[39]

4 | DISCUSSION

Strategies for enabling pharmacopeia-free durable allogeneic islet immune evasion spanning the use of isolators to "blockade" gene engineering are the object of scrutiny.[40] Along this line of thought, knowledge gained from the cancer immunotherapy space focused on defeating immune evasion provides insights on how best to achieve allogeneic tissue tolerance. Indeed, malignancies often exploit several immunosuppressive pathways to evade the immune response. In particular, PD-1:PD-L1[41] and IDO[42] are two pathways implicated in such microenvironments and have been recognized as important immune checkpoints. This recognition has led to efforts focused on blocking these pathways as therapeutic targets in oncology, speaking to the biological potency of immunological escape


(A)
Donor Mouse
C57BL6/j
LV-Ctrl
Islets
Islets in Matrigel Plug
LV-PIDO
Infiltrating cells
Islet^Ctrl
BALB/c
Subcutaneous Txp
Cell Isolation
Explanted Matrigel Plugs
Normoglycemic
BALB/c
Immune cell phenotyping
Islet^PIDO+
(B)
FSC/SSC
Live Single Cells
CD45⁻
Insulin⁺/ Glucagon⁺
PD-L1⁺
T-cells (CD4⁺/CD8⁺)
CD45⁺
Macrophages (F480⁺/CD11b⁺)
CD4⁺
Tregs (FoxP3⁺CD25⁺)
(C)
Islets^Ctrl
Islets^PIDO
4.14%
66.29%
SSC-H
CD45⁻ Insulin⁺
CD45-Insulin⁺ Cell Count (Log10)
Ctrl PIDO⁺
1.46%
5.12%
CD45⁻ Glucagon⁺
CD45-Glucagon⁺ Cell Count (Log10)
(D)
67.44%
85.67%
ns
CD45⁺CD4⁺
CD45⁺CD4⁺ Cell Count (Log10)
21.79%
2.23%
CD45⁺CD8⁺
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(E)
32.8%
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CD45⁺F480⁺
CD45⁺F480⁺ Cell Count (Log10)
64.52%
3.23%
CD45⁺CD11b⁺
CD45⁺CD11b⁺ Cell Count (Log10)
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CD4⁺CD25⁺ FoxP3⁺
1.7%
10.3%
77.3%
10.7%
2.7%
17.4%
70%
9.8%
FoxP3
CD25
CD25⁺FoxP3⁺ Cell Count (Log10)

FIGURE 6 PIDO-expression on islet allografts modulates host immune response at the transplantation site. (A) Schematic of subcutaneous implantation of Matrigel™ plugs containing PIDO expressing allogeneic C57BL/6 islets in normoglycemic BALB/c recipients, plug removal at 2 weeks, cell isolation, and immune cell phenotyping. (B) Gating schema of flow cytometric analysis performed for phenotyping of transplanted Matrigel™ plug resident islet cells and infiltrating lymphomyeloid cells. (C) Flow cytometric analysis of $CD45^-$ insulin-expressing or glucagon-expressing islet cell frequencies among live cells and quantification of numbers of $insulin^+$ and $glucagon^+$ events in the cells isolated from Matrigel™ plug containing $Islet^{Ctrl}$ (red dots) and $Islet^{PIDO}$ (blue dots). Flow cytometric analysis of PD-L1 expression by $CD45^-$ insulin-expressing or glucagon-expressing islet cells is shown in Figure S3. (D) Flow cytometric analysis of T cell frequencies among $CD45^+$ cells among live cells and quantification of numbers of $CD4^+$ and $CD8^+$ events in the cells isolated from Matrigel™ plug containing $Islet^{Ctrl}$ (red dots) and $Islet^{PIDO}$ (blue dots). (E) Flow cytometric analysis of macrophage cell frequencies among $CD45^+$ cells among live cells and quantification of numbers of $F480^+$ and $CD11b^+$ events in the cells isolated from Matrigel™ plug containing $Islet^{Ctrl}$ (red dots) and $Islet^{PIDO}$ (blue dots). (F) Flow cytometric analysis of T_{reg} cell frequencies among $CD4^+$ cells among $CD45^+$ live cells and quantification of the numbers of $CD25^+FoxP3^+$ events in the cells isolated from Matrigel™ plug containing $Islet^{Ctrl}$ (red dots) and $Islet^{PIDO}$ (blue dots). Each symbol in quantification plots (C–F) indicates an individual recipient mouse; the results are presented as the mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, by Student's *t*-test (Mann–Whitney method). Data are pooled from or are representative of three (B) or four (C–E) independent experiments for $Islet^{Ctrl}$ (red dots) and four (B) or five (C–E) independent experiments for $Islet^{PIDO}$ (blue dots) with similar results.

for select malignant disorders with a high mutation burden. Indeed, a recent report on the utility of PD-L1 constitutive expression by human islet-like organoids as a means to evade xenorejection within 60 days in mice speaks to this line of inquiry.[27] In analogous work, PD-L1 engineered islets or microgel/biomaterial platform bypassed genetic modification of the graft cell/tissue[24,43] by transient expression of PD-L1, however, it failed to provide sustained protection of islets against alloreactive responses and needed contemporaneous additive pharmacological immunosuppression. These precedents served as an impetus to develop a novel approach that can provide specific, localized, and durable immune evasion while circumventing the need for immunosuppressive therapy. In this study, we present the concept of generating a novel chimeric recombinant fusion protein of PD-L1 and IDO. By harnessing the immune evasive potential of PD-1:PD-L1 and IDO pathways, we sought to modulate the alloreactive immune response against pancreatic islets allografts in murine recipients. These proteins have not been used together earlier: neither in tandem as a fusion protein nor simultaneously/independently as a tissue-restricted immune blockade therapeutic. The observations made here suggest that the tethering of IDO to the cytoplasmic tail of transmembrane PD-L1 apparently leads to salutary gain-of-function properties not otherwise deployed by contemporaneous expression of monomeric PD-L1 and IDO. Indeed, we observed that both immortalized cell lines and primary islet cells expressing PIDO displayed a higher density of PD-L1 on the surface and superior enzymatically active IDO in the cytoplasm. PIDO fusion protein not only retained the biological functionality of both constituent proteins but also gained enhanced steady state expression likely explaining the gain of function relative to individual parent proteins. We determined the efficacy of PIDO fusion protein in immunoprotection of allografts by engineering islets which, after transplantation, reversed preexisting diabetes and established sustained euglycemia for more than 50 weeks without requiring chronic immunosuppression in STZ-diabetic mice.

In agreement with some previous studies, we observed that stable expression of PD-L1 or IDO individually did not meaningfully improve graft survival. Interestingly, we also observed that stable co-expression of PD-L1 and IDO only delayed the immune rejection of islet allografts temporarily. These seminal observations reveal that while PD-L1 and IDO are potent, they are insufficient when expressed individually or in tandem in contrast to the novel gain of function of PIDO fusion protein.

Non-specific off-target effects are always a conceptual concern with ectopic, especially stable expression of immunomodulatory proteins. We did not observe any effect of PIDO expression on features of bona fide mature islet β-cells such as robust dynamic function or diabetes reversal upon transplantation. Similarly, the absence of meaningful cellular proliferation (data not shown) in homeostatic conditions in mature, terminally differentiated islet β-cells[44] remained uninfluenced by PIDO expression.

Our data indicate that PIDO-enabled immune evasion protects islet allografts from autoimmune destruction consequently reversing diabetes in NOD mice. Previous studies[45,46] have revealed that induced expansion or differentiation of Tregs prolongs survival in diabetic NOD mice. Since both PD-L1 and IDO pathways converge on Treg induction, we hypothesized that the impact of PIDO on allograft survival may be related to host CD4 T cell competency. Our observation that PIDO expression by allogeneic islets elicits an endogenous $CD4^+$ dependent immune evasive response is convergent with the central role of host-acquired T cell drivers of tolerance. However, acquired antibody-driven humoral reaction to allografted islets may occur despite PIDO expression, especially when porcine islet xenotransplantation is contemplated. Although, we can speculate that human ES or iPS-derived islet-like cells may be spared this fate considering the central role of PIDO interaction with host CD4 T cells (and not B-cells) in the mouse-to-mouse in vivo systems here used. Among various traditional and novel tolerogenic approaches to prevent graft rejection, allo-, and autoreactive T cell suppression via Treg cell therapy have shown feasibility, tolerability, and potential efficacy in transplantation settings.[47–50] However, these approaches (including Treg cell enhancing drugs and cell therapy antigen-specific Tregs) have demonstrated only modest and limited efficacy in T1DM and transplant rejection clinical settings.[51,52] Islet

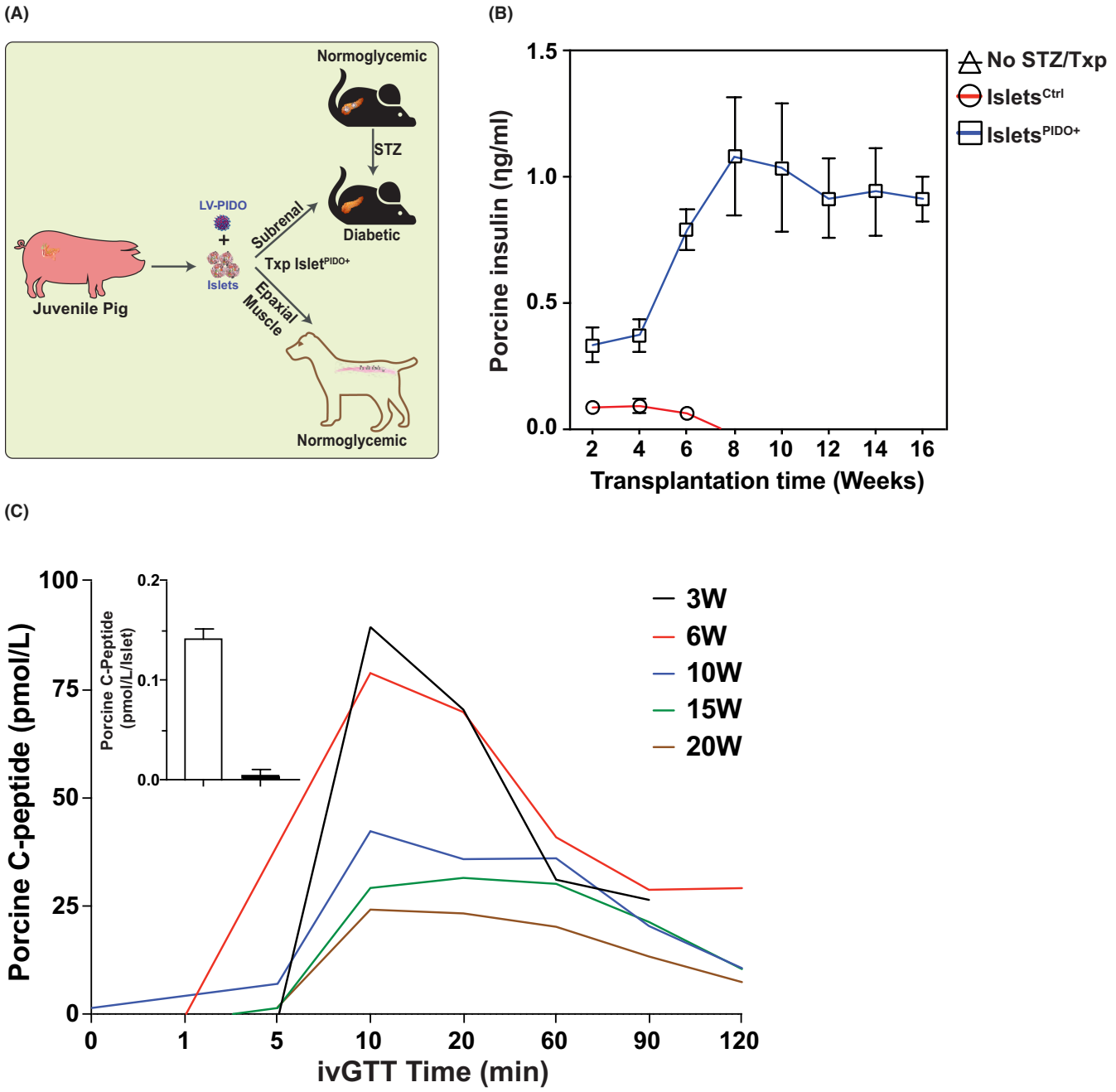

FIGURE 7 PIDO-engineered xenogeneic islets survive in immunocompetent murine and canine recipients. (A) Schematic of PIDO expressing porcine islet transplant in normoglycemic C57BL/6 mice and dog recipients. (B) Porcine insulin measurements in normoglycemic immunocompetent C57BL/6 mice after renal subcapsular transplantation with engineered pig islets. Three groups were studied: mice without a transplant (No Txp; $n = 3$; black), mice transplanted with control islets (EGFP Txp; 400 islets; $n = 4$; red) and mice transplanted with PIDO expressing islets (PIDO Txp; 400 islets; $n = 5$; blue). (C) Porcine C-peptide measurement after intravenous glucose tolerance test (GTT) in a normoglycemic beagle dog at 3, 6 10, 15, and 20 weeks posttransplantation in epaxial muscle.

restricted, constitutive PIDO expression and its attendant host CD4-dependent immune evasion may address the shortcomings of Treg adoptive cell therapy via its continuous in vivo solicitation of endogenous regulatory $CD4^+$ cells.

Utilizing two different models of rechallenge, we determined that long-term (20-weeks) stable but localized PIDO expression did not establish acquired memory tolerance as the host rejected naïve islet allografts competently and promptly (~3 weeks) after re-transplantation. These results highlight the immune evasive qualities conferred by PIDO require constitutive expression by islets and do not lead to acquired memory immune tolerance as part of the mechanism of action.

Sustained graft acceptance requires tolerogenic modulation by Treg cells.[53] Our data indicate that PIDO expression not only confers protection of viability and function of islet cells but also causes a significant shift in phenotypes of immune cells accumulated at

the site of transplantation. In our islet-containing Matrigel plug transplantation followed by flow cytometry analysis of islet and infiltrating cells, a significant decrease in pro-inflammatory immune cells ($CD8^+$, $F4/80^+$, and $CD11b^+$) and contrasting increase in Tregs ($CD25^+FoxP3^+$) is evident. These observations are consistent with previous reports[54,55] on the immunomodulatory role of PD-L1 and IDO in the induction of Tregs. While these data connect localized, sustained expression of PIDO in islets with tolerogenic induction in the transplantation microenvironment, additional studies will be necessary for further proof of the obligatory need for Treg induction to mediate PIDO-conferred immune evasion of allografts.

Long-term success in crossing xenogeneic immune barriers for tissue transplantation has remained challenging[36,38,39] although biomaterials offer a pathway toward porcine islet xenograft success.[56] We demonstrate the feasibility of transplanting $PIDO^+$ porcine islet xenografts in murine and canine immunocompetent recipients. Indeed, there is virtually no published precedent reporting a pharmacopeia-free survival of xenografts in immune-sufficient mammalian recipients. However, there are some important limitations to these xenograft survival experiments. Our results were obtained from normoglycemic recipients, hence, limiting our ability to comment on the functional competence of islet xenografts in reversing pathological hyperglycemia. Further, in an effort to glean as much information possible from as few experimental canines as possible, we studied porcine islet xenograft engraftment and function longitudinally in a singular canine recipient. Lastly, the murine model of diabetes we utilized speaks to drug-induced (STZ) islet insufficiency and secondary diabetes mellitus. The porcine to murine and canine recipients' data presented here demonstrate that PIDO can overcome interspecies immune barrier albeit bypassing hyper-acute humoral rejection of porcine tissue that is idiosyncratic to simian (and human) recipients. It remains to be tested whether PIDO expression overcomes hyperacute rejection or whether islets derived from gene-engineered pigs devoid of αGal and Neu5Gc xenoantigens are required.

Though this model system mirrors clinical diabetes in the human clinical settings of non-immune pancreatic insufficiency or pancreatectomy, it does not reflect the pathology of autoimmune islet destruction typically seen in T1D. However, our data derived from diabetic NOD mice suggests that PIDO enables immune evasion in the setting of autoimmune diabetes as well. In conclusion, our data suggest that PIDO cell engineering technology may allow for allogeneic and off-the-shelf islet-like tissue transplant—including xeno-sourced cells—as a standard therapy for the treatment of poorly controlled insulin-dependent diabetes.

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DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. A US Provisional Patent application for PIDO has been made prior to publication and is assigned to the Wisconsin Alumni Research Fund (WARF).

DATA AVAILABILITY STATEMENT

All data and material used in this study and analysis will be available upon request to corresponding author and may be subject to material transfer agreements (MTAs).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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