Immunoprotection and Functional Improvement of Allogeneic Islets in Diabetic Mice, Using a Stable Indoleamine 2,3-Dioxygenase Producing Scaffold

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Background. We have previously shown that an immunomodulatory enzyme, indoleamine 2,3-dioxygenase (IDO) in dermal fibroblasts generates a tryptophan-deficient environment that selectively inhibits proliferation and induces apoptosis of bystander CD4+ and CD8+ T cells, but not pancreatic islets. Because these immune cells are involved in islet allograft rejection, we hypothesized that transplantation of islets embedded in a novel 3-dimensional composite scaffold within which stable IDO-expressing fibroblasts serve as source of local immunosuppression would lead to normoglycemia in a streptozotocin-induced diabetic mouse model. **Methods.** Islet grafts were prepared by embedding stable IDO-expressing fibroblasts and allogeneic islets into a protease-resistant composite scaffold. Islets function and survival were evaluated in vitro using immunohistochemistry. Allografts were transplanted under the kidney capsule of streptozotocin-induced diabetic mice; viability, function, and criteria for graft take were evaluated. Flow cytometry was performed to determine specific intragraft, draining lymph nodes and spleen T-cell population, and splenocytes alloantigen responsiveness of graft recipients. **Results.** The results of a series of in vitro experiments revealed that IDO-expressing fibroblasts do not compromise islet function or survival. The expression of IDO suppressed the proliferation of alloantigen-stimulated splenocytes. The in vivo experiments revealed that local IDO expression delivered by lentiviral vector prolonged islet allograft survival (51.0 ± 2.9 days) by increasing the population of FOXP3+ regulatory T cells at the graft site and graft-draining lymph nodes and preventing T-cell infiltration. **Conclusions.** This study shows that incorporation of islets within our novel matrix that is equipped with stable IDO-expressing fibroblasts prolongs allograft survival.

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Interest has been piqued in islet cell transplantation (ICT) as a treatment option for type 1 diabetes mellitus. Despite all the advances in ICT, several significant issues, including multiple donor requirements, side effects associated with immunosuppressive drugs, and maintaining long-term islet survival, limit the procedure and outcome of ICT.[1-5]

Pancreatic islets compose a small portion of the whole pancreas; the isolation process aims to remove the exocrine tissue through enzymatic digestion of the extracellular matrix (ECM) and mechanical separation followed by density gradient.[1] Islet isolation digests peripheral ECM and perinsular basement membrane, interrupting oxygen and nutrient delivery,[6,7] and compromises islet survival, reducing islet yield. Therefore, replacement of the ECM could be an attractive approach to improve the long-term survival of islets.

We previously developed a composite fibroblast-populated collagen matrix (FPCM) that improved islet viability and function and reduced the marginal islet mass needed to achieve euglycemia in streptozotocin (STZ)-induced diabetic mice.[8] Collagen is a surrogate for the natural ECM of islets while fibroblasts provide a favorable support by producing various growth and angiogenic factors and maintaining the structure.[7] However, the FPCM was prone to gradual biodegradation and contraction, negatively affecting the long-term survival and function of the islets.[9] Recently, we developed a novel bioengineered, cross-linked collagen-glycosaminoglycan matrix (CCM).[9,10] When populated by fibroblasts (FP-CCM),

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A.H.-T., R.B.J., and A.G. conceived and designed the experiments. A.H.-T., R.B.J., and M.K.M. performed the experiments. R.H. and A.H.-T. prepared the cross-linked collagen matrix. R.T.K. prepared the Lenti-IDO and Lenti-Vect fibroblasts. Y.Z. contributed to immunostaining. A.H.-T. analyzed the data. A.H.-T. and R.B.J. contributed to trouble shooting for the experiments. A.H.-T. wrote the manuscript. A.H.-T. and A.G. contributed to discussion. A.G. and A.H.-T. revised the final version of the manuscript.

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it provided enhanced mechanical strength and reduced contraction both in vitro and in vivo in a mouse model of syngeneic ICT.[10]

Despite their value in preventing islet allorejection, immunosuppressants can directly contribute to β-cell toxicity.[1,11,12] One option to avoid using systemic immunosuppressive regimens is to locally induce an immunosuppressive factor, indoleamine 2,3-dioxygenase (IDO), the first and rate-limiting enzyme in tryptophan catabolism pathway.[13] Indoleamine 2,3-dioxygenase generates a microenvironment, low in tryptophan and rich in kynurenine metabolites, that is selectively toxic to T-cells, whereas allogeneic islets remain intact.[13,14] Using the FPCM, we demonstrated that transient adenoviral transduction of IDO in fibroblasts prolongs the survival of allogeneic islets embedded within this scaffold up to 6 weeks.[15]

The purpose of this study was, therefore, to overcome 2 main difficulties that we encountered in our previous research: (i) the gradual biodegradation and contractibility of FPCM that could compromise long-term transplant outcome[8]; and (ii) the transient IDO expression in fibroblasts, limiting the period of graft survival.[15] Herein, we have applied: (i) a novel scaffold that offers an optimized biomimetic matrix with improved mechanical properties; and (ii) a recently constructed lentiviral vector bearing an IDO-expressing gene to generate more stable IDO expression in fibroblasts. Using these systems, we examined whether local stable IDO expression by FP-CCM prolongs islet allograft survival in a STZ-induced diabetic mouse model.

MATERIALS AND METHODS

For detailed research plan and methods please see **SDC,** http://links.lww.com/TP/B130.

Ethics

This study has been approved by the University of British Columbia Animal Care Committee. All animals were maintained and undergone procedures in accordance with the principles of laboratory animal care, and the guidelines of the University of British Columbia Animal Care Committee. Six- to 8-week-old male C57BL/6 or Balb/c were used for this study (see **Materials and Methods, SDC,** http://links.lww.com/TP/B130).

Lentiviral Transduction of IDO Gene in Fibroblasts

Recombinant lentiviral vector carrying human IDO and blasticidin resistance gene was constructed and used to induce IDO expression in fibroblasts (see **Materials and Methods, SDC,** http://links.lww.com/TP/B130).

Preparation of CCM Composite Grafts

Three-dimensional CCM was prepared as previously described.[9,10] Murine islets were embedded within CCM either alone (ICCM) or cocultured with normal (B6-ICCM) or Lenti-IDO transduced C57BL/6 (I-ICCM) fibroblasts. A group of islets were cultured in 2-dimensional (2D) culture dish and another group of islets were embedded in the CCM with Lenti-Vect fibroblasts (V-ICCM). Each treatment group was prepared in triplicates within 48-well plates in a total volume of 100 μL. For in vitro studies, 50 islets per well were used; after preparation, mixtures were immediately transferred to a humidified incubator at 37°C in an atmosphere of 5% CO_2 for 1 hour for polymerization. The composite scaffolds were maintained in an incubator for up to 30 days.

The CCM is a liquid matrix that gets solidified within 15 minutes at 37°C and is injectable using a catheter. For in vivo studies, 500 islets per well were used; gels were freshly prepared within few hours before transplantation, and kept on ice after mixing with islets ± fibroblasts. A ratio of 100 fibroblasts per islet was considered for both in vitro and in vivo experiments.

Transplantation of Islet-Fibroblast Composite Grafts

Six- to 8-week-old male C57BL/6 recipient mice were rendered diabetic by a single intraperitoneal injection of 200 mg/kg STZ (Sigma, St. Louis, MO). Diabetes was defined as a minimum of 2 consecutive blood glucose (BG) levels of 20 mmol/L or higher.[8] Islet allografts were prepared as described above and transplanted under the kidney capsule of diabetic mice. An "Accu-Chek Compact Plus" (Roche Diagnostics, Indianapolis, IN) was used for BG measurement. Grafts were deemed functioning when BG levels decreased to less than 10 mmol/L.

Histology Assessments

For in vitro analyses, CCMs were digested at indicated time points using type I collagenase (1 mg/mL; Sigma), and islets were handpicked. Paraffin-embedded islet sections were double immunostained for insulin/glucagon or insulin/cleaved caspase-3. For in vivo studies, graft-bearing kidneys and -draining lymph nodes (dLNs) were retrieved at different time points. Paraffin-embedded sections were stained with hematoxylin-eosin (H&E) or double immunostained for insulin/CD3 or single stained for FOXP3 cells infiltration. Graft dLNs were stained for FOXP3+ cells infiltration (see **Materials and Methods, SDC,** http://links.lww.com/TP/B130).

Flow Cytometry Analyses

Splenocytes isolated from allograft-rejected diabetic mice were labeled with carboxyfluorescein succinimidyl ester and cocultured with Lenti-IDO fibroblasts and donor islets. After 72 hours, proliferation of splenocytes was evaluated in response to allostimulation using flow cytometry. For the mixed lymphocyte reaction, splenocytes isolated from graft recipients were labeled with carboxyfluorescein succinimidyl ester and cocultured with donor splenocytes. Proliferation of the responder cells was analyzed after 72 hours. Splenocytes, lymphocytes, and intragraft-infiltrated immune cells were subjected to flow cytometry for evaluation of cell surface and intracellular antigens (see **Materials and Methods, SDC,** http://links.lww.com/TP/B130).

Statistical Analyses

See Materials and Methods (**SDC,** http://links.lww.com/TP/B130).

RESULTS

Local IDO Expression Suppresses Proliferation of Diabetic Splenocytes Cocultured With Allogeneic Islets

We evaluated the suppressive effect of IDO on the proliferation of splenocytes isolated from STZ-induced diabetic C57BL/6 mice, when cocultured with allogeneic Balb/c islets for 72 hours (Figure 1A). Although splenocytes proliferated in response to allogeneic islets, local IDO expression delivered by lentiviral vector to fibroblasts (Lenti-IDO fibroblasts) reduced

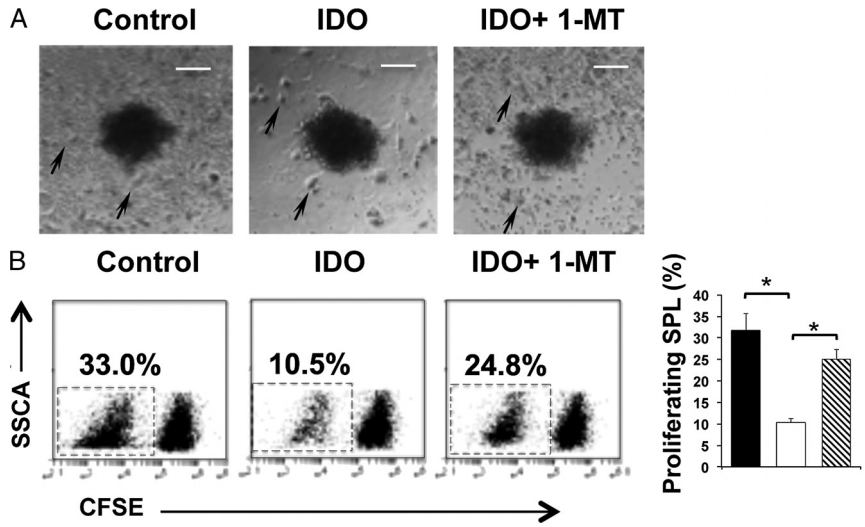

FIGURE 1. IDO-expressing fibroblasts suppress the proliferation of alloantigen-stimulated mouse splenocytes. A, Photomicrographs of diabetic C57BL/6 splenocytes after 72 hour coculture with allogeneic (Balb/c) islets in the presence or absence of syngeneic Lenti-IDO fibroblasts. Size bars = 50 μm. Arrows indicate cocultured fibroblasts. B, Proliferation rates of carboxyfluorescein succinimidyl ester-stained splenocytes stimulated with allogeneic islets for 72 hour in the presence or absence of IDO-expressing fibroblasts and measured by flow cytometry. 1-MT was used as an inhibitor of IDO activity. Lenti-IDO–transduced fibroblasts suppressed splenocyte proliferation and this effect was reversed by addition of 1-MT, confirming the suppressive effects of IDO expression. Arrows indicate fibroblasts in culture. C, Presentation of data as mean ± SD. *Statistically significant difference between indicated groups ($P < 0.001$; n = 3). Solid bar: nontransduced IDO fibroblasts coculture, open bar: Lenti-IDO fibroblasts coculture, hashed bar: Lenti-IDO fibroblasts coculture in the presence of 1-MT. SPL, splenocytes.

the proliferation of stimulated splenocytes. The suppressive effect of IDO was reversed in the presence of 1-methyl tryptophan (1-MT), a competitive IDO inhibitor. Flow cytometric analysis (Figure 1B) showed about 67% change in the proliferation of splenocytes when cocultured with allogeneic islets in the presence of IDO-producing fibroblasts (10.3% ± 0.9%) compared to that of control (31.%7 ± 3.8%)(<0.001, n = 3). The suppression of proliferation was due to IDO activity as confirmed by considerable reversal of proliferation in the presence of 1-MT (25.0% ± 2.3%) ($P < 0.001$, n = 3).

Cross-Linked Collagen Matrix Improves Viability and Function of Cultured Islets

We first confirmed viability and IDO expression of Lenti-IDO fibroblasts when incorporated within the CCM (**Figure S1 and Materials and Methods, SDC,** http://links.lww.com/TP/B130). Next, murine islets were embedded within CCM either alone or cocultured with nontransduced or Lenti-IDO fibroblasts for up to 30 days. A group of islets were cultured in 2D culture dish as a control group. As another control for the potential toxicity of lentiviral vector to embedded islets, we also included another group in which islets were embedded within the CCM cocultured with fibroblasts transduced with mock lentiviral vector (Lenti-Vect). Histological analysis revealed that embedding islets within CCM in the presence or absence of Lenti-IDO fibroblasts did not affect insulin and glucagon expression. Embedding islets within the CCM was associated with higher β-to-α cell ratio than 2D cultured islets on days 15 and 30 after culture (Figure 2A and B). Furthermore, CCM improved viability of the islets compared to that of 2D culture as shown by higher cleaved caspase-3 expression in the latter on days 15 and 30 after culture (Figure 2A and C). These data showed that the viability of islets when cultured alone or cocultured with Lenti-IDO fibroblasts within CCM was not different. Moreover, the lentiviral vector itself appears to be harmless to the function and viability of islets because Lenti-Vect fibroblasts did not noticeably affect insulin, glucagon or caspase-3 expression in islet cells compared to those of normal fibroblasts.

Local IDO Expression Delivered by Lentiviral Vector Improves Islet Transplantation Outcome

To investigate the immunomodulatory effects of IDO, composite islet grafts were prepared by embedding Balb/c islets within CCM populated with either Lenti-IDO, Lenti-Vect, or nontransduced control fibroblasts. Another control group received islets only. Islet grafts were transplanted into STZ-diabetic allogeneic (C57BL/6) recipients. Figure 3A and B shows BG levels of Lenti-Vect- and Lenti-IDO graft-recipients during the study. The Kaplan-Meier survival curve showed prolongation of graft survival in IDO-expressing graft recipients (51.0 ± 2.9 days, $P < 0.001$, n = 5) (Figure 3C). In contrast, control grafts were rejected within 2 weeks after transplantation. The mean survival duration of Lenti-Vect FP-CCM (12.0 ± 1.1 days) was not different from those of islet-CCM grafts with nontransduced fibroblasts (12.6 ± 1.0 days) or no fibroblast (11.6 ± 1.5 days), and islet-alone grafts with no matrix (11.0 ± 1.0 days).

Local IDO Expression Preserved Insulin Expression and Reduced Immune Cell Infiltration

To examine histopathological changes in the islet allografts, graft-bearing kidneys and dLNs were harvested at different time points. Graft-bearing kidneys were subjected to H&E (**Figure S2, SDC,** http://links.lww.com/TP/B130) and double immunofluorescence staining for insulin and CD3. As IDO is known to increase the number of regulatory T cells (T-regs), we also investigated the infiltration of FOXP3+ cells into the grafts and dLNs at different time points. Immunostaining for insulin and CD3 revealed very few insulin-producing cells but extensive infiltration of T cells in Lenti-Vect grafts, confirming the data obtained

from H&E staining (**Figures 4A, S2A, and S2B, SDC,** http://links.lww.com/TP/B130). At 2 weeks (**Figures 4B, S2C, and S2D, SDC,** http://links.lww.com/TP/B130) and 7 weeks (**Figures 4C, S2E, and S2F, SDC,** http://links.lww.com/TP/B130) after transplantation, Lenti-IDO grafts had strong staining for insulin, indicating preservation of functional beta cell mass, and confirming the minimal immune cell infiltration at week 7 seen by H&E. Massive CD3+ T-cell infiltration into the IDO-expressing grafts started at week 8 after transplantation (**Figures 4D, S2G, and S2H, SDC,** http://links.lww.com/TP/B130).

Although the immunostaining showed very few FOXP3+ cells at the graft site and in dLNs of Lenti-Vect graft-recipients at week 2 after transplantation (Figure 4E and I, respectively), there were more infiltrating FOXP3+ cells in IDO-expressing grafts and dLNs at the same time point (Figures 4F and 4J). By week 7 after transplantation, the number of FOXP3+ cells decreased at both the graft site and dLNs (Figure 4G and K, respectively). By the time of graft rejection, the number of FOXP3+ cells in Lenti-IDO (week 8) group both at the graft site and dLNs (Figure 4H and L, respectively) was comparable to Lenti-Vect group

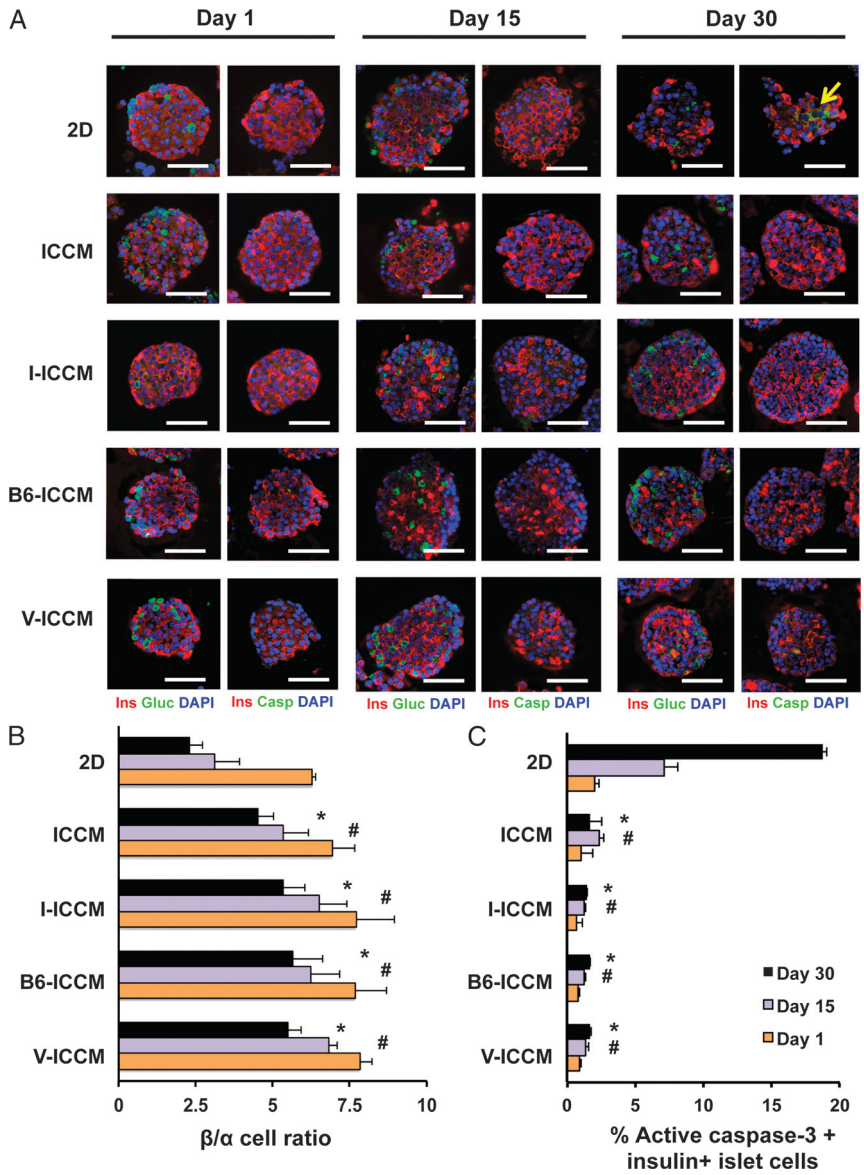

FIGURE 2. Lenti-IDO FP-CCM preserves islet insulin and glucagon expression while reducing caspase-3 expression in β cells in vitro. Islet grafts were harvested and fixed in paraformaldehyde on days 1, 15, and 30 after culture. A, Paraffin-embedded sections were subjected to double-immunofluorescence staining for insulin (red)/glucagon (green), and insulin (red)/cleaved caspase-3 (green). Size bar = 50 μm. Note the apparent increase in the number of caspase 3-positive cells over time in 2D cultured islets (yellow arrows) compared to other groups. Cross-linked matrix, local IDO expression or lentiviral vector did not negatively affect islet insulin/ glucagon expression. B, The β-to-α cell ratio was calculated by counting insulin- and glucagon-expressing cells in each islet, in a minimum of 20 islets per condition. Data are presented as mean ± SD. Note that islets embedded within CCM have higher β/α cell ratio compared to 2D cultured islets. C, Active caspase-3–positive β cells were counted in each islet (minimum of 20 islets per condition). Data are reported as the percentage of cleaved caspase-3+ insulin + islet cells (mean ± SD). Note that the number of caspase-3–expressing β cells is higher in 2D group compared with other conditions at each time point. *,#Significant difference versus 2D cultured islets ($P < 0.01$). 2D, regular 2-dimensional culture dish; ICCM, islets embedded alone in CCM; IICCM, islets embedded in Lenti-IDO fibroblast populated-CCM; B6-ICCM, islets embedded in normal C57BL/6 fibroblast populated CCM; V-ICCM, islets embedded in Lenti-Vect fibroblast populated-CCM; Ins, insulin; Gluc, glucagon; Casp, cleaved caspase-3.

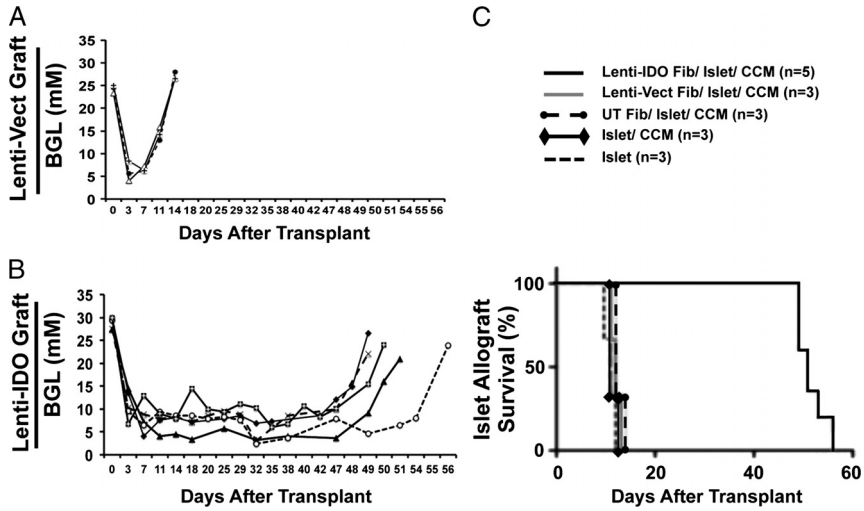

FIGURE 3. Islet graft survival after transplantation. Allogeneic islets were embedded within CCM populated with either IDO gene transduced, Mock vector transduced, or untreated C57BL/6 fibroblasts and transplanted under the kidney capsule of STZ-induced C57BL/6 diabetic mice. Some mice received islets alone embedded in CCM or untreated islets with no matrix. A, B, Blood glucose levels in recipients of Lenti-IDO transduced islet grafts and Lenti-Vect transduced islet grafts (control group), respectively. C, Kaplan-Meier islet allograft survival curve. Lenti-IDO Fib/Islet/ CCM, islets embedded in IDO gene-transduced fibroblast populated-CCM; Lenti-Vect Fib/Islet/ CCM; islets embedded in mock lentiviral vector-transduced fibroblast populated-CCM, UT Fib/Islet/CCM, islets embedded in untreated fibroblast populated-CCM; Islet/ CCM, islets embedded alone in CCM.

(week 2). These findings were confirmed by quantification of the number of FOXP3+ cells in the graft and dLNs (Figure 4M). The percentage of T-regs was not significantly different within spleens of graft recipients at indicated time points (**Figure S4, SDC,** http://links.lww.com/TP/B130).

The present data suggest that local IDO expression delivered by lentiviral vector prevents islet allograft rejection via prevention of intragraft T-cell infiltration while increasing the number of FOXP3+ cells within the graft and graft-dLNs. The population of proliferating T cells was evaluated in graft, dLNs, and spleens of untreated (control) and IDO-expressing graft recipients at 2 weeks and 7 weeks after transplantation using flow cytometry (Figure 5A). Ki67 + CD4+, and Ki67 + CD8+ T-cells were much more abundant in control grafts (35.5% ± 3.7% and 25.2% ± 1.5%, respectively) compared with IDO-expressing grafts (12.0% ± 0.7% and 4.4% ± 1.97%, respectively) at 2 weeks after transplantation. The frequency of proliferating CD4+ and CD8+ in IDO-expressing grafts decreased by week 7 after transplantation (17.3% ± 0.9% and 10.8% ± 0.7% respectively). The data showed that Ki67 + CD4+ and Ki67 + CD8+ T cells were fewer in dLNs of IDO graft recipients compared with control group at 2 weeks after transplantation (9.0% ± 0.7% and 5.9% ± 0.7%, respectively for IDO group versus 10.8% ± 1.0% and 16% ± 0.9%, respectively for control group). The frequency of proliferating CD4+ T cells within dLNs of IDO graft recipients did not significantly change by week 7 after transplantation (12.8%± 1.1%). However, the frequency of proliferating CD8+ IDO graft recipients increased by week 7 after transplantation (8.8 ± 0.5%) when compared with week 2 (Figure 5A).

No difference was observed comparing the Ki67 + CD4+ T-cell population in the spleens of IDO graft recipients and control group (12.1% ± 1.9% vs. 14.7% ± 0.1%, respectively) at 2 weeks after transplantation. However, this value highly increased by week 7 in IDO graft recipients (18.75% ± 0.6%). The population of Ki67 + CD8+ T cells was similar in the spleens of untreated and IDO graft recipients at week 2 after transplantation (7.9% ± 0.2% vs 7.4% ± 0.2%, respectively) but slightly increased in IDO graft recipients by week 7 after transplantation (13.3% ± 0.5%) (Figure 5A).

To investigate possible mechanisms underlying the decline in IDO-mediated protection against rejection at 8 weeks after transplantation, IDO transgene mRNA expression was evaluated in nontransduced and Lenti-Vect grafts at 2 weeks and in Lenti-IDO grafts at different time points after transplantation (**Figure S3, SDC,** http://links.lww.com/TP/B130). Our data indicated that IDO mRNA was highly expressed in Lenti-IDO allografts at week 1 followed by a gradual decline through week 7; however, no IDO mRNA expression was detected in control allografts.

Impaired Allospecific T-Cell Proliferation in dLNs of Lenti-IDO Graft-Recipients

Next, to understand the effect of IDO transgene expression on T-cell priming capacity, we performed a mixed lymphocyte reaction. Splenocytes isolated from nontransduced (week 2) and Lenti-IDO–transduced graft recipients (weeks 2 and 7) were cocultured with responder cells, that is, splenocytes isolated from allogeneic donors (Figure 5B). Our data showed that both CD4+ and CD4– (i.e. CD8+) T cells isolated from spleens of IDO-expressing graft recipients revealed diminished responsiveness to allostimulation with BALB/c lymphocytes at week 2 after transplantation when compared with nontransduced graft recipients (control). This hyporesponsiveness was reverted by week 7 after transplantation (Figure 5B).

DISCUSSION

Re-establishment of the islet ECM is an attractive approach to improve islet survival and function while maintaining healthy islet morphology.[16-18] The present study shows that using a novel bioengineered scaffold along with local

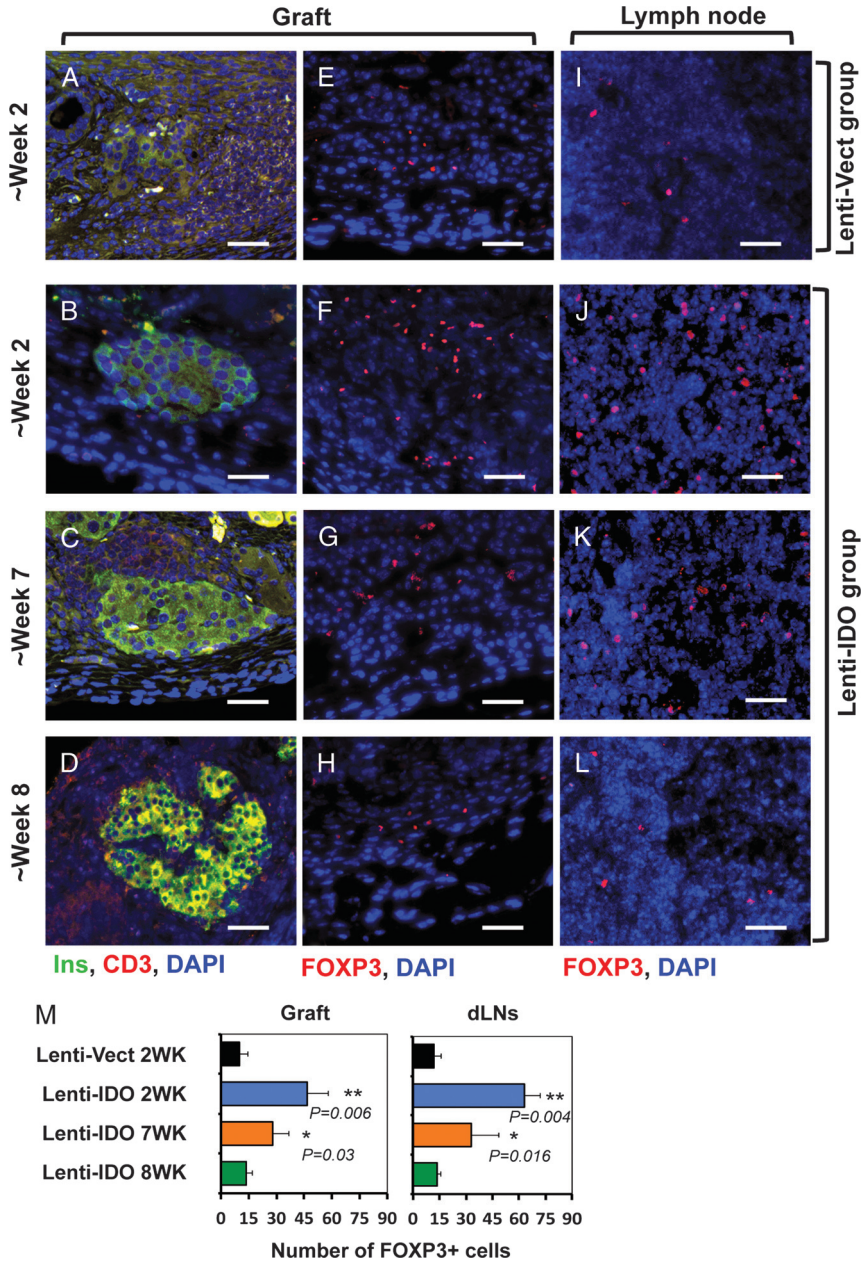

FIGURE 4. Histological analysis of grafts and dLNs. A-D, Insulin expression and T-cell infiltration in control (Lenti-Vect transduced) islet grafts at 2 weeks and IDO-expressing (Lenti-IDO transduced) islet grafts at 2, 7, and 8 weeks after transplantation, respectively. E-H, Intragraft FOXP3+ cells in control (week 2) and IDO-expressing grafts (weeks 2, 7, and 8) after transplantation, respectively. I-L, FOXP3+ cells infiltrating graft-dLNs of control graft recipients (week 2) and IDO-expressing graft recipients (weeks 2, 7, and 8) after transplantation. Note that T cell abundantly infiltrated control grafts around week 2 after transplantation, whereas the infiltration was delayed until week 7 in IDO-expressing grafts. (M) Quantification of intragraft FOPX3+ cells (n = 3, refers to the number of independent experiments). *,**Significant differences versus Lenti-Vect 2Wk. Data are presented as mean ± SD. Size bar = 20 μm. Lenti-Vect group, recipients of islets embedded in mock lentiviral vector-transduced fibroblast populated CCM; Lenti-IDO group, recipients of islets embedded in IDO gene-transduced fibroblast populated-CCM; Lenti-Vect 2Wk, Lenti-Vect graft at week 2; Lenti-IDO 2Wk, IDO-expressing graft at week 2; Lenti-IDO 7Wk, IDO-expressing graft at week 7; Lenti-IDO 8Wk, IDO-expressing graft at week 8.

IDO expression delivered by lentiviral vector prolongs islet allograft survival.

Various viral vectors have been used for gene transduction. The latest generation of adenoviral vectors represents high capacity, reduced inflammation, and leads to a prolonged gene expression. However, they are unable to induce lifelong transgene expression in vivo because the viral genome remains episomal in transduced cells.[19,20] In contrast, lentiviral vectors lead to efficient and more prolonged gene expression in proliferating and nonproliferating cells. Herein, we applied lentiviral gene transduction for local delivery of IDO at the graft site, hoping to generate a more stable nonrejectable islet allograft.[19,20] The immunostaining confirmed IDO expression in fibroblasts within the matrix while majority of fibroblasts remained viable.[9,10]

We found that the alloantigen specific proliferation of splenocytes was remarkably suppressed in the presence of Lenti-IDO fibroblast-mediated environment; this effect was

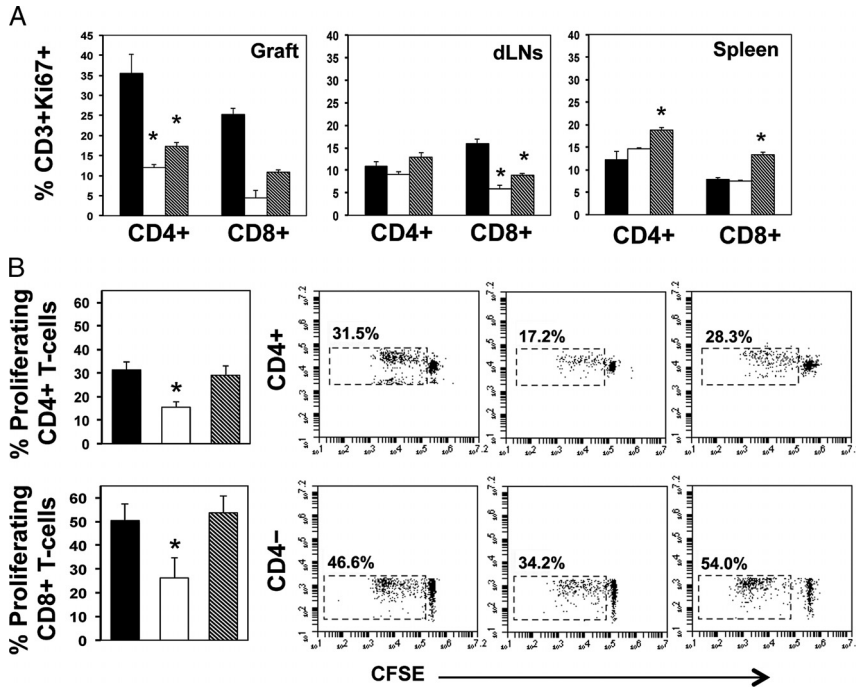

FIGURE 5. Flow cytometric analysis of spleens, dLNs, and graft-infiltrating immune cells. A, Frequency of Ki67 + CD4+ and Ki67 + CD4+ T cells in the grafts, spleens, and dLNs of recipients at indicated time points. B, Splenocytes from graft recipients (C57BL/6; H-2Kb) were stained with carboxyfluorescein succinimidyl ester and cultured with mitomycin C-treated donor lymphocytes (BALB/c) for 72 hours. Frequency of proliferating H-2Kb + CD3+ CD4+ and H2kb + CD3+ CD4– T cells (considered as CD8+ T cells) was measured at indicated time points. Data are shown as means ± SD. *,#Significant difference between indicated group versus control ($P < 0.001$ and $P < 0.05$, respectively; $n = 3$). Solid bar: recipients of islets embedded in untreated fibroblast populated CCM evaluated at 2 weeks after transplantation, Open bar: recipients of IDO-expressing graft evaluated at week 2 after transplantation, hashed bar: recipients of IDO-expressing graft evaluated at week 7 after transplantation.

partially restored by the addition of 1-MT, an IDO inhibitor, indicating that the observed suppressive effect was due to IDO activity.

Our findings further demonstrated that the FP-CCM serves as a suitable scaffold for the islets and when transduced with lentiviral vector showed relatively no toxicity for the islets. Immunostaining revealed that when embedded within the matrix, islets maintained insulin and glucagon expressions and were more viable compared to 2D cultured islets. Furthermore, embedding islets within CCM was associated with higher β/α cell ratio and less cleaved caspase-3 expression versus 2D cultured islets.

The histology revealed that the FP-CCM maintains islets with healthy structure and minimal cell infiltration and stronger insulin staining in IDO-expressing grafts for up to 7 weeks. The number of intragraft FOXP3+ T-regs in IDO-expressing grafts increased by 78% within the first 2 weeks after transplantation. Our data suggested that as long as there was sufficient number of FOXP3+ T-regs at the graft site, islet grafts survived and function normally while being protected from T-cell infiltration. Indoleamine 2,3-dioxygenase is shown to induce de novo differentiation of FOXP3+ T-regs from uncommitted CD4+ T cells while promoting effector function in mature FOXP3+ T-regs. The T-regs are also able to generate tolerogeneic dendritic cells (DCs) through the induction of IDO expression by CTLA-4 interactions and, in turn, tolerogeneic DCs can contribute to the spreading of immunosuppression via a phenomenon known as "infectious tolerance".[21,22] Indoleamine 2,3-dioxygenase establishes a tolerogeneic phenotype in DCs and expands the population of T-reg,[23] known as downstream mediators of IDO-mediated allograft protection.[13,17,24] Therefore, IDO activity links 2 arms of innate and adaptive immunity to create local immunosuppression and to encourage systemic tolerance by activating T-regs. Herein, we found that T-regs population in IDO-expressing grafts and dLNs was higher than those of controls at 2 weeks after transplantation. This observation was by reduced antigen-specific T-cell priming capacity in spleens of IDO-expressing raft recipients at the same time point. However, T-regs population gradually decreased in and dLNs by week 7, and reached to comparable levels with Lenti-Vect group by week 8 after transplantation. Evaluation of IDO transgene expression in harvested allografts suggested a gradual decline in IDO expression within Lenti-IDO transduced grafts. Considering these findings, one could suggest that such decline might be responsible for waning of graft protection due to inadequate number of T-regs.

The present study confirms that local intragraft IDO expression induced in bystander fibroblasts increases islet allograft survival and function. Our data for the first time revealed that the lentiviral transduction method for IDO gene delivery in bystander fibroblasts could prolong islet allograft survival. Having said that, the rejection of allografts after 7 weeks was surprising, as we anticipated Lenti-IDO transduced fibroblasts to generate more stable IDO production and protect transplanted allogeneic islet for much longer duration. Nevertheless, these studies raise the possibility that local IDO expression could be used as an adjunct with other low-dose immunosuppression for prevention of islet allograft rejection. Considering the potential benefits of IDO in cell replacement therapies,

further strategies should be evaluated to provide sufficient IDO expression or IDO-mediated FOXP3+ T-regs at the graft site to prevent allograft rejection.

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