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ORIGINAL ARTICLE

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Alginate-microencapsulation of human stem cell–derived β cells with CXCL12 prolongs their survival and function in immunocompetent mice without systemic immunosuppression

David A. Alagpulinsa[1] | Jenny J. L. Cao[1,2] | Riley K. Driscoll[1,3] | Ruxandra F. Sîrbulescu[1] | Madeline F. E. Penson[1] | Marinko Sremac[1] | Elise N. Engquist[4] | Timothy A. Brauns[1] | James F. Markmann[5] | Douglas A. Melton[4] | Mark C. Poznansky[1]

[1]Vaccine and Immunotherapy Center, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

[2]Department of Pathology and School of Clinical Medicine, University of Cambridge, Cambridge, UK

[3]Department of Microbiology and Molecular Biology, Brigham Young University, Provo, Utah

[4]Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Harvard University, Cambridge, Massachusetts

[5]Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Correspondence
Mark C. Poznansky
Email: mpoznansky@mgh.harvard.edu

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Pancreatic β-cell replacement by islet transplantation for the treatment of type 1 diabetes (T1D) is currently limited by donor tissue scarcity and the requirement for lifelong immunosuppression. The advent of in vitro differentiation protocols for generating functional β-like cells from human pluripotent stem cells, also referred to as SC-β cells, could eliminate these obstacles. To avoid the need for immunosuppression, alginate-microencapsulation is widely investigated as a safe path to β-cell replacement. Nonetheless, inflammatory foreign body responses leading to pericapsular fibrotic overgrowth often causes microencapsulated islet-cell death and graft failure. Here we used a novel approach to evade the pericapsular fibrotic response to alginate-microencapsulated SC-β cells; an immunomodulatory chemokine, CXCL12, was incorporated into clinical grade sodium alginate to microencapsulate SC-β cells. CXCL12 enhanced glucose-stimulated insulin secretion activity of SC-β cells and induced expression of genes associated with β-cell function in vitro. SC-β cells co-encapsulated with CXCL12 showed enhanced insulin secretion in diabetic mice and accelerated the normalization of hyperglycemia. Additionally, SC-β cells co-encapsulated with CXCL12 evaded the pericapsular fibrotic response, resulting in long-term functional competence and glycemic correction (>150 days) without systemic immunosuppression in immunocompetent C57BL/6 mice. These findings lay the groundwork for further preclinical translation of this approach into large animal models of T1D.

KEYWORDS
basic (laboratory) research/science, diabetes: type 1, endocrinology/diabetology, fibrosis, immune regulation, immunosuppression/immune modulation, insulin/C-peptide, islet transplantation, islets of Langerhans, translational research/science

1 | INTRODUCTION

Type 1 diabetes (T1D) results from autoimmune destruction of insulin-producing pancreatic β cells. Logically, replacement of lost β cells with equally functional, insulin-secreting cells would be a cure for T1D. As proof-of-principle, replacement therapy using deceased donor islets has been demonstrated to restore normoglycemia and insulin independence in patients.[1] However, the scarcity of donor

Abbreviations: AUC, area under the curve; FBR, foreign body response; GSIS, glucose-stimulated insulin secretion; hESC, human embryonic stem cell; hiPSC, human induced-pluripotent stem cell; IPGTT, intraperitoneal glucose tolerance test; PGLA, poly-lactic-co-glycolytic acid; SC-β cells, stem cell-derived beta cells; STZ, streptozotocin; T1D, type 1 diabetes.

islets, variability in their isolation and quality, and the requirement for lifelong systemic immunosuppression has impeded wide-scale application of this treatment.

The Melton laboratory recently developed a novel in vitro differentiation protocol for efficient production of glucose-responsive insulin-secreting β-like cells from human embryonic and induced pluripotent stem cells (hESCs/hiPSCs).[2,3] Although stem-cell derived β cells (SC-βs) may not express abnormal antigens and therefore be less immunogenic for replacement therapy, especially in the autologous context,[4] autologous SC-β cells could be subject to autoimmune destruction in patients with T1D.[5] Moreover, unlike allogeneic SC-β cells, which could be made readily available on the shelf, manufacturing autologous SC-β cells would require optimization of differentiation conditions for each patient-specific–derived iPSC. This comes at a considerable cost and an extra load of clinical product development.[6] Essentially, strategies that provide immunoprotection while promoting functionality and safety of SC-β cells are needed for clinical translation.

To avoid the need for immunosuppression, alginate-microencapsulation of pancreatic islets has been widely investigated as a safe delivery vehicle for β-cell replacement.[7] However, clinical translation of this approach has been hampered by inflammatory foreign body responses (FBRs) leading to pericapsular fibrotic overgrowth that causes islet-cell death and early graft failure.[7] The fibrotic overgrowth, characterized by macrophages and myofibroblasts, forms a physical barrier that precludes islet interaction with the surrounding microenvironment, including transport of oxygen, other nutrients, and blood glucose sensing.[8-10] Various immunomodulatory strategies and chemical modification of alginate have been investigated to mitigate the fibrotic FBR to microencapsulated islet-implants.[11,12] However, none has resulted in successful clinical translation.

The chemokine stromal cell–derived factor-1α, known as CXCL12, has been shown to have immunoregulatory, anti-inflammatory, and wound-healing effects. Of interest, endogenous CXCL12 signaling promotes β-cell development and survival, and immunoprotects pancreatic islets to prevent autoimmune diabetes (reviewed in ref. 13). As proof-of-concept, we previously demonstrated that murine alloislets engineered to overexpress CXCL12[14] or porcine xenoislets co-microencapsulated with CXCL12 in alginate[15] are immunoprotected and function long-term to restore normoglycemia in immunocompetent mice without immunosuppression. Here we harness our previous work to achieve long-term replacement therapy with a potentially unlimited source of human β cells without immunosuppression. This includes an in vitro differentiation protocol for scalable production of glucose-responsive insulin-secreting β-like cells,[2,3] and CXCL12-releasing alginate microcapsules that create a local immunoprotective microenvironment.[15] We demonstrate enhanced immunoisolation and long-term functional competence of SC-β cells co-encapsulated with CXCL12, resulting in prolonged glycemic correction (>150 days) in immunocompetent C57BL/6 diabetic mice without systemic immunosuppression.

2 | MATERIALS AND METHODS

2.1 | Derivation and culture of SC-β cells

SC-β cell clusters were differentiated from the human embryonic stem cell line HUES8 per the previously published protocol,[2] with the following modifications—days 7-8: as published + 10 μmol/L Y27632; days 9-11: as published + 5 ng/mL Activin A + 10 μmol/L Y27632; and days 21-35: as published (Fig. 2C,D, 3B, 4A-H, 5A-C) or S3 basal media (Fig. 2A,B, 3A). (ref. 2). The cell clusters were cultured in spinner flasks (Corning, Inc., Corning, NY) on a stir platform rotating at 1.17 Hz in a humidified, 37°C and 5% CO_2 cell culture incubator.

2.2 | Alginate microencapsulation of SC-β cell clusters ± CXCL12

The production of alginate microcapsules was carried out using the BUCHI encapsulator (Encapsulator B-395 Pro, BÜCHI Labortechnik AG, Switzerland) in a production unit that was set up in a type II class A2 biologic safety cabinet. A 1.6% w/v sodium alginate solution was prepared by dissolving endotoxin-free ultra-pure low-viscosity mannuronate sodium alginate (PRONOVA UP LVM, NOVAMATRIX, Sandvika, Norway) in 150 mmol/L NaCl solution, stirred overnight at 4°C, and filtered through a 0.8/0.2-μm sterile syringe filter (PALL Life Sciences, Ann Arbor, MI). Pelleted SC-β cell clusters were mixed in the 1.6% w/v sodium alginate solution to form a homogeneous suspension with a density of ~2000 SC-β cell clusters per 1.0 mL. To incorporate CXCL12 in the microcapsules, the 1.6% alginate solution was first thoroughly mixed with recombinant CXCL12-α (PeproTech) at concentrations of 0.2 μg/mL and 2.0 μg/mL before the addition of the SC-β cell clusters. We chose to use the slightly higher concentration of CXCL12 at 2.0 μg/mL above previously used 1.0 μg/mL because this was not toxic to SC-β cell function in vitro and resembled physiologically relevant concentrations of CXCL12 that we had previously demonstrated to be present in bone marrow stroma.[14-16] The homogeneous mixture was loaded into a 60-mL Luer-lock syringe (Thermo Fisher Scientific, Waltham, MA) and attached to an air-dripping nozzle system of the BUCHI Encapsulator B-395 Pro apparatus. The mixture was pumped through a 400-μm nozzle into a reaction chamber containing 100 mmol/L $CaCl_2$ cross-linking solution being stirred at 100 mBar. Microcapsules were produced with the following encapsulator settings: 1013 Hz encapsulator internal vibration frequency, 2.05 mBar air pressure and 1.8 mL/min syringe pump rate. The alginate microcapsule droplets were crosslinked in the $CaCl_2$ solution for 5 min, washed with CMRL culture medium, and subsequently cultured in the same medium and used for implantation or in vitro assays 24 hours later.

2.3 | Glucose-stimulated insulin secretion (GSIS) assays

Unencapsulated SC-β cell clusters and the respective alginate-microencapsulated clusters were cultured overnight in culture medium supplemented with 2 mmol/L glucose, washed with Krebs buffer (128 mmol/L

NaCl, 5 mmol/L KCl, 2.7 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 1 mmol/L Na_2HPO4, 1.2 mmol/L KH_2PO4, 5 mmol/L $NaHCO_3$, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% bovine serum albumin [BSA] in deionized water, pH 7.4), and incubated for 2 hours in Krebs buffer supplemented with 2 mmol/L glucose. The cell clusters or microcapsules were then subjected to 30-minute incubations in 2 mmol/L and 20 mmol/L glucose in Krebs buffer and the supernatants collected. After glucose stimulations, the cells were pelleted and lysed with radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Thermo Fisher Scientific). The concentration of human C-peptide in the supernatants was determined using a human C-peptide enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), whereas total protein in cell lysates was determined using a BCA test kit (Thermo Fisher Scientific, Waltham, MA). The C-peptide secreted was normalized to the total protein of the corresponding cell lysate or the number of microencapsulated SC-β cell clusters. GSIS indices were calculated by dividing the amount of C-peptide secreted following incubation in high glucose (20 mmol/L) by that following incubation with low glucose (2 mmol/L).

2.4 | Animal studies

2.4.1 | Animals

Animal studies were carried out following the Public Health Service Policy on Human Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital. Female C57BL/6 mice (6-weeks-old) were purchased from the Jackson Laboratories (Bar Harbor, ME) and maintained in a temperature-, humidity-, and light-controlled room (12-hour light/dark cycles) with free access to water and standard chow diet, unless specified. Animals were housed for at least 2 weeks to acclimatize after purchase before use.

2.4.2 | Induction of diabetes and pretreatment of mice with disrupted cell clusters

Mice were injected intraperitoneally with a single dose of 250-mg/kg-body weight of streptozotocin (STZ) freshly dissolved in 114 mmol/L sodium citrate (pH 4.5). Mice typically developed hyperglycemia within 3-5 days. Animals were considered diabetic for inclusion if they had at least 2 consecutive plasma glucose readings of ≥400 mg/dL. To prime mice from an innate immune response perspective, SC-β cell clusters were lysed via 5 cycles of repeated freeze-thaw in liquid nitrogen and a 37°C water bath, respectively. Lysates from ~1×10^6 cells were injected intraperitoneally into diabetic mice 5 days before transplantation.

2.4.3 | Transplantation, glucose monitoring, intraperitoneal glucose tolerance tests (IPGTTs), and serum C-peptide measurement

Anesthesia was induced in animals by subcutaneous injection of a cocktail comprising ketamine and xylazine (80 mg/kg and 10 mg/kg body, respectively). The abdomen was shaved and sterilized with povidone iodine solution (USP 10%), an ~1.0-cm incision was made along the midline abdominal skin, and the peritoneal lining was exposed by blunt dissection. While the peritoneal wall was grasped with forceps, an ~1.0-cm incision was made along the linea alba. Through the incision, the microcapsules containing 400 SC-β cell clusters (taking the fraction of empty microcapsules into account which was 30% ± 4.5%), were implanted into the peritoneal cavity using a sterile spatula. The inner abdominal lining was sealed with 2 Ethilon 5-0 nylon sutures (Ethicon, LLC) and the outer wall with 3 sutures and wiped with povidone iodine.

Nonrandom plasma glucose levels in blood from tail-vein prick were monitored regularly (8:00-10:00 AM) using a glucometer (Accu-Chek Performa Glucometer).

IPGTTs were preceded by a 6-hour fast with access to water. Mice were injected intraperitoneally with 2-g/kg body weight of glucose. Their plasma glucose levels in tail-vein-prick blood were determined before glucose administration and 15, 30, 60, 90, and 120 minutes after glucose administration.

To determine serum C-peptide levels, mice were fasted as described for IPGTTs and blood (100-200 μL) was obtained under anesthesia via retro-orbital bleed. The levels of human C-peptide and murine C-peptide were determined with their respective ELISA kits per the manufacturers' protocols (R&D Systems and ALPCO, respectively).

2.4.4 | Microcapsule recovery, microscopy, and immunohistochemistry

The recovery of microcapsules and downstream analysis was carried out as described in Supplemental Methods.

2.5 | Statistical analysis

The number of animals per group was predetermined based on preliminary studies (n = 5). Data are presented as the mean ± standard error of the mean (SEM) from at least 3 biologic replicates (different batches of SC-β cell clusters), unless stated otherwise. Statistical analysis was done using GraphPad Prism (GraphPad Software 7.02, Inc., La Jolla, CA). Differences among groups or treatments were assessed by one-way analysis of variance (ANOVA) with post hoc Bonferroni test to identify the significance of differences among means at $P < .05$.

3 | RESULTS

3.1 | Characterization of SC-β cell clusters and alginate microcapsules

The differentiation (Figure 1A) produced SC-β cell clusters of 200-300 μm in diameter (Figure 1B). The sizes of the alginate microcapsules were 650 ± 50 μm in diameter (Figure 1C). Most microcapsules contained a single SC-β cell cluster; a minority (~30%)

of microcapsules were blank (containing 0 clusters) and ~10% of microcapsules contained 2 or more clusters. By intracellular immunostaining flow cytometry (Figure 1D) and immunofluorescent confocal microscopy (Figure 1E), 36.43 ± 2.71% of the cells in the SC-β cell clusters were C-peptide$^+$/NKX6.1$^+$, indicating differentiation into β cells. Each cluster contained 5000-9000 individual cells as measured using a hemocytometer after physical disaggregation of the cluster into a single cell suspension. The release characteristics of CXCL12 from the alginate microcapsules in vitro and in vivo were described previously.[15]

3.2 | CXCL12 enhances glucose-stimulated insulin secretion of SC-β cells

Twenty-four-hour treatment of unencapsulated SC-β cell clusters with 0.2 μg/mL CXCL12 enhanced C-peptide (insulin surrogate) secretion upon glucose stimulation compared with untreated cells, although this difference was not statistically significant ($P = .310$ compared with control treatment; Figure 2A). This resulted in a GSIS index of 3.20 ± 0.58 compared with 1.80 ± 0.17 for control vehicle treatment ($P = .075$ vs control treatment; Figure 2B). On the other hand, a higher concentration of CXCL12 (2.0 μg/mL) caused a slight but not significant decrease in C-peptide secretion upon glucose stimulation, causing a GSIS index of 1.65 ± 0.36 ($P > .999$ vs control treatment; Figure 2B). CXCL12 did not cause a noticeable effect on C-peptide secretion under low glucose (2 mmol/L) conditions compared with control. Decreasing the concentration of CXCL12 to 0.02 μg/mL significantly enhanced GSIS of SC-β cells compared with control treatment ($P = .0009$; Figure S1A). However, we did not to pursue this concentration in subsequent studies of microencapsulation and transplantation in diabetic mice as we previously showed that low concentrations of CXCL12 do not induce an immunoprotective environment that enables long-term function of transplanted alginate microencapsulated alloislets or xenoislets.[15,16]

Alginate-microencapsulation ± CXCL12 did not affect GSIS of SC-β cells. Thus, the C-peptide secreted by unencapsulated SC-β cells ± CXCL12 under low- and high-glucose conditions compared with the corresponding microencapsulated SC-β cells ± CXCL12 under the same glucose conditions were statistically indistinguishable (Figure 2C-D). The amount of C-peptide secreted per unencapsulated cell cluster upon glucose stimulation was 20 pM vs 22 pM for the corresponding alginate-encapsulated cluster (Figure 2C). The resulting GSIS index of microencapsulated SC-β cells without CXCL12 was 2.00 ± 0.34 compared with 2.14 ± 0.22 for the corresponding unencapsulated SC-β cells without CXCL12 treatment (Figure 2D). Like its effect on the unencapsulated SC-β cells, CXCL12 enhanced the GSIS response of microencapsulated SC-β cells in a dose-dependent manner; microencapsulation of SC-β cells with 0.2 and 2.0 μg/mL CXCL12 yielded GSIS indices of 3.13 ± 0.34 and 1.64 ± 0.43, respectively (Figure 2D).

Because enhanced β-cell function is often associated with improved survival, we assessed the effect of CXCL12 on genes associated with β-cell function and survival. The mRNA transcript levels of *PCSK1*, *TCF7L2*, *WNT5A*, *PDX1*, and *GCK* increased following 24-hour exposure of SC-β cell clusters to CXCL12 (Figure S1B, $P \leq .05$). Furthermore, low but not high doses of CXCL12 significantly protected SC-β cell clusters against cytokine-induced apoptosis (Figure S1C; $P < .05$ vs control treatment).

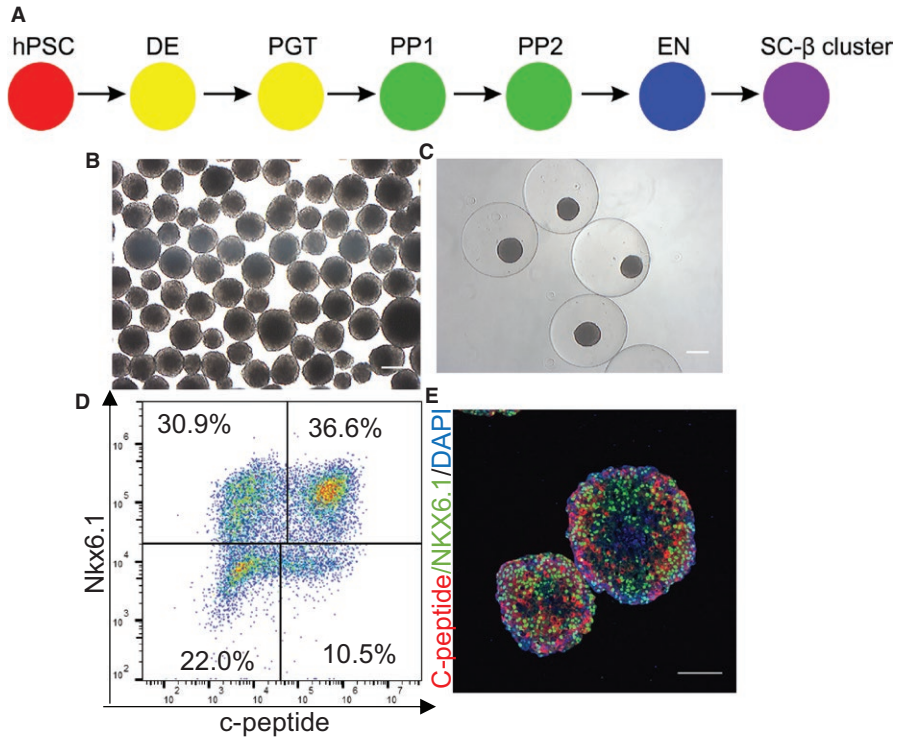

FIGURE 1 Characterization of SC-β cell clusters and alginate encapsulation. A, Schematic of directed differentiation of hPSCs into of SC-β cells (hPSC, human pluripotent stem cells; DE, definitive endoderm cells; PGT, primitive gut tube cells; PP1, early pancreatic progenitor cells; PP2, later pancreatic progenitor cells; EN, endocrine progenitor cells; SC-β cells, stem cell-derived β cells). B, Representative light microscopic images unencapsulated SC-β cell clusters. Scale bar, 200 μm. C, Representative light microscopic images of SC-β cell clusters in alginate microcapsules. Scale bar: 200 μm. D, Representative flow cytometry staining of dispersed of SC-β cell clusters for β-cell markers, C-peptide, and NKX6.1. E, Representative immunofluorescent staining of SC-β cell clusters for β-cell markers, C-peptide(red), and NKX6.1 (green). Scale bar: (C) = 100 μm, (E) = 200 μm

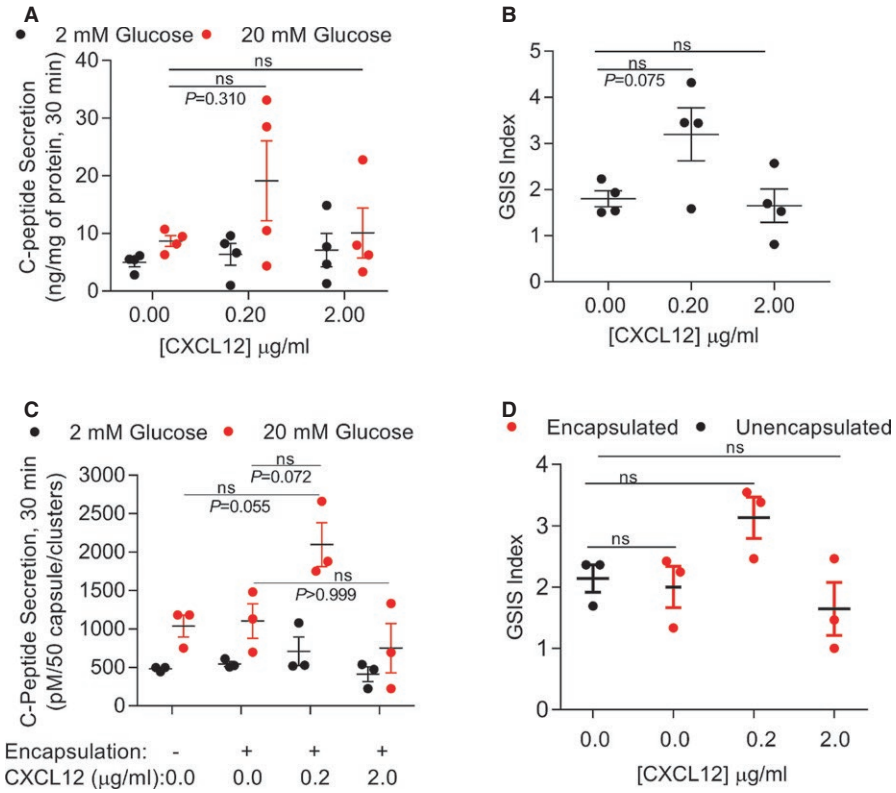

FIGURE 2 CXCL12 enhances GSIS of SC-β cells. A, B, Unencapsulated SC-β cell clusters were incubated ± CXCL12 for 24 hours and subjected to low and high glucose incubations in Krebs buffer. The amounts of C-peptide secreted into the supernatants were determined using C-peptide ELISA kit and the total protein in cell lysates determined with a BCA kit (n = 4, biologic replicates). A, The amount of C-peptide secreted was normalized to the total protein. B, The amount of C-peptide secreted under high glucose stimulation was divided by that under low glucose stimulation to obtain the GSIS index. C, D, SC-β cell clusters were co-encapsulated ± CXCL12 in alginate, and 24 hours later the encapsulated cells and their corresponding unencapsulated SC-β cell clusters were incubated with low and high glucose in Krebs buffer. The amount of C-peptide secreted into the supernatant was determined as in A and B (n = 3, biologic replicates). C, The amount of C-peptide secreted was normalized to the number of SC-β cell clusters. D, The GSIS index was calculated as in (B). Differences between treatments as indicated in graphs were assessed by ANOVA with post hoc Bonferroni test to identify the significance of differences among means at $^*P < .05$

3.3 | SC-β cells co-encapsulated with CXCL12 show enhanced insulin secretion in vivo, accelerating normalization of hyperglycemia in diabetic mice

We observed accelerated and significant reduction of hyperglycemia in immunocompetent diabetic mice implanted with SC-β cell clusters co-encapsulated with 0.2 μg/mL CXCL12 compared to those implanted with microcapsules without CXCL12 and those implanted with microcapsules incorporating 0 μg/mL or 2.0 μg/mL CXCL12 ($P < .05$; Figure 3A). All mice implanted with microcapsules incorporating 0.2 μg/mL CXCL12 became normoglycemic (<250 mg/dL plasma glucose levels) within 72 hours after transplantation, whereas mice implanted with microencapsulated SC-β cell clusters without CXCL12 or with 2.0 μg/mL CXCL12 remained hyperglycemic (>250 mg/mL plasma glucose levels) after 5 days post transplantation. Commensurate with the accelerated reduction of hyperglycemia, mice transplanted with microencapsulated SC-β cell clusters incorporating 0.2 μg/mL CXCL12 exhibited moderately higher basal and glucose-stimulated serum human C-peptide levels even at day 6 posttransplantation compared with mice transplanted with microcapsules without or with 2.0 μg/mL CXCL12 ($P = .287$; Figure 3B).

3.4 | SC-β cells co-encapsulated with high dose of CXCL12 function long-term, causing prolonged glycemic correction in C57BL/6 diabetic mice

In preliminary exploratory studies, we observed dense pericapsular fibrotic overgrowth on recovered microcapsules containing SC-β cell clusters without CXCL12 after 12 weeks of intraperitoneal implantation in immunocompetent C57BL/6 diabetic mice. In contrast, microcapsules that did not contain cells and those that contained SC-β cell clusters incorporating 2.0 μg/mL CXCL12 showed little to no pericapsular fibrotic overgrowth. This suggested that the fibrotic overgrowth was likely elicited by antigens/molecules shed by the encapsulated SC-β cell clusters. We, therefore, reasoned that prior intraperitoneal administration of disrupted SC-β cell clusters could

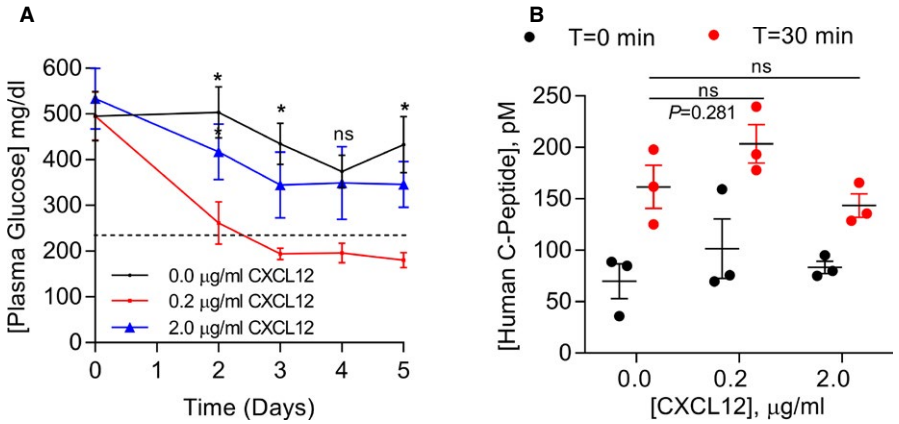

FIGURE 3 Microencapsulation of SC-β cells with CXCL12 enhances insulin secretion in vivo and accelerates the hyperglycemic reversal in diabetic mice. Streptozotocin-induced C57BL/6 diabetic mice were implanted with 400 microencapsulated SC-β cell clusters in the peritoneum (n = 3 per group). A, Blood plasma glucose levels of mice were monitored daily. B, Six days after implantation of encapsulated SC-β cell clusters, mice were fasted overnight and injected with 2 g/kg body weight with glucose. The serum levels of human C-peptide before glucose administration (T_0) and 30 minutes postinjection (T_{30}) were determined. Differences between treatments as indicated in graphs were assessed by ANOVA with post hoc Bonferroni test to identify the significance of differences among means at $^*P < .05$

activate peritoneal macrophages to elicit a stronger innate macrophage response upon subsequent intraperitoneal implantation of the encapsulated cells. To this end, immunocompetent C57BL/6 diabetic mice were administered intraperitoneal injections of repeated freeze-thaw disrupted SC-β cell clusters 5 days before implantation with the microencapsulated SC-β cell clusters. As expected, diabetic mice implanted with microcapsules that incorporated 0.2 μg/mL CXCL12 became normoglycemic (blood plasma glucose <250 mg/dL) 2 days after transplantation, whereas mice transplanted with microcapsules without CXCL12 or with 2.0 μg/mL CXCL12 only became normoglycemic after at least 7 days (Figures 4A-C and S2A-B). Three weeks after transplantation, all diabetic mice transplanted with microencapsulated SC-β cell clusters ± CXCL12 were tolerant of intraperitoneal glucose tolerance test (or IPGTT), with their blood glucose levels returning to basal levels within 90 minutes after glucose challenge. There was neither significant differences in peak glucose levels (Figure 4D) nor glucose area under the curve (AUC) (Figure S2C) between treatment groups. Furthermore, 6 weeks after transplantation, significant amounts of fasting human C-peptide (100-120 pM) were detected in all treated mice and no significant differences between treatment groups (Figure 4E). These findings established the functionality of the microencapsulated SC-β cells.

Based on the criterion for graft rejection or failure,[15,17] 80% of microcapsule implants without CXCL12 and 50% of those that incorporated 0.2 μg/mL CXCL12 were rejected or failed by 150 days after transplantation. On the other hand, 100% of microcapsule implants that incorporated 2.0 μg/mL CXCL12 survived and were functional at 150 days posttransplantation (Figure 4G; $P = .0132$, Mantel-Cox test and $P = .0042$, log-rank test for trend compared with 0.0 μg/mL CXCL12 control treatment). Average plasma glucose levels of mice bearing microcapsules that did not incorporate CXCL12 and those incorporating 0.2 μg/mL CXCL12 were 345 ± 30.1 mg/dL and 244 ± 10.5 mg/dL at 150 days after transplantation, respectively. On the other hand, average plasma glucose levels of those bearing microcapsules that incorporated 2.0 μg/mL CXCL12 were 198.0 ± 6.1 mg/mL (Figure S3B, $P = .006$ compared with 0.0 μg/mL CXCL12). Furthermore, only mice bearing microcapsules that incorporated 2.0 μg/mL CXCL12 could maintain glucose homeostasis in response to intraperitoneal bolus glucose challenge, similar to healthy control mice and in contrast to control STZ-treated diabetic mice at 150 days after implantation (Figure 4H). The glucose AUC for mice bearing microcapsules that incorporated 2.0 μg/mL CXCL12 was significantly lower compared with those bearing microcapsules without CXCL12 (Figure S2D, $P = .0013$). The fasting human C-peptide levels of mice bearing microcapsules that incorporated 2 μg/mL CXCL12 were also significantly higher compared with those bearing microcapsules without CXCL12 or with 0.2 μg/mL CXCL12 (Figure 4F, $P < .05$) 142 days after transplantation. This is in commensurate with the ability of these mice (bearing microcapsules that incorporated 2.0 μg/mL CXCL12) to maintain glucose homeostasis in response to glucose challenge unlike the other treatment groups. The levels of mouse C-peptide in all diabetic mice transplanted with microencapsulated SC-β cell clusters were below detection at 142 days after transplantation which was similar to control untreated STZ-induced diabetic mice (Figure S2E). In contrast, mouse C-peptide was clearly detectable in healthy untreated control mice (Figure S2E). These observations support the view that the STZ-treated diabetic mice depended on the implanted human cells to maintain glucose homeostasis.

3.5 | Microencapsulation of SC-β cell clusters with alginate incorporating high-dose CXCL12 evades the fibrotic FBR C57BL/6 diabetic mice

We recovered the microcapsule implants 154 days after transplantation for microscopic and histologic analysis of the local foreign

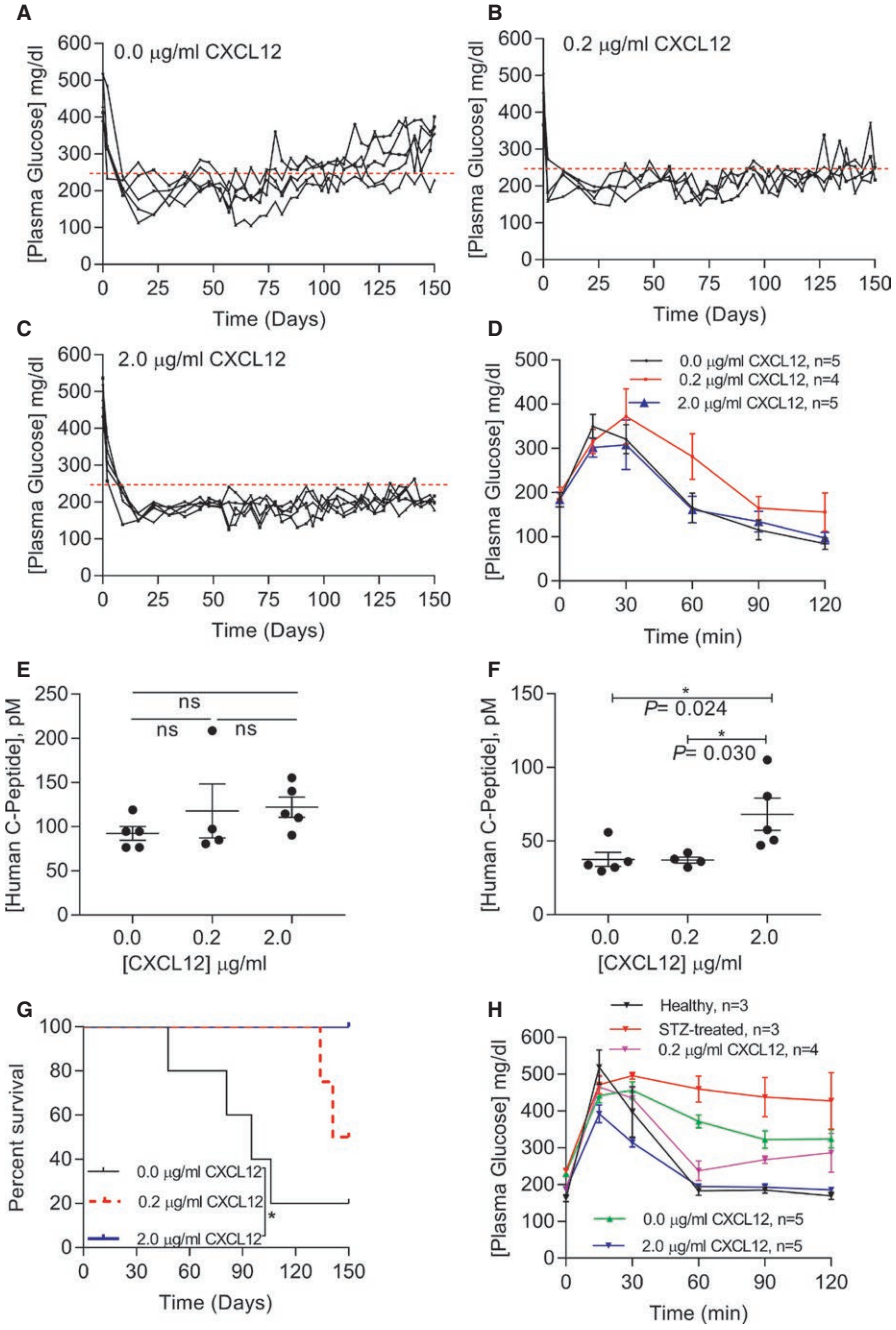

FIGURE 4 Co-encapsulation of SC-β cell clusters with CXCL12 in alginate enhances long-term glucose-responsiveness and glycemic correction in sensitized diabetic C57BL/6 mice. A-G, STZ-induced C57BL/6 diabetic mice were sensitized with disrupted SC-β cell clusters as described in Materials and Methods and implanted with 400 alginate-microencapsulated SC-β cell clusters into the peritoneal space (n = 5, except 0.2 μg/mL [n = 4]). Nonrandom plasma glucose levels were monitored at regular times ≥150 days. Mice were considered hyperglycemic if blood glucose readings exceeded ≥250 mg/dL on 2 consecutive occasions A-C, Plasma glucose readings for diabetic mice implanted with microcapsules as indicated. D, Intraperitoneal glucose tolerance test (IPGTT) 3 weeks after implantation. Mice were fasted for 6 hours with access to water and injected intraperitoneally with 2 g/kg of body weight of glucose and the plasma glucose levels in tail vein blood measured at the indicated time points. E, F, Fasting serum human C-peptide levels at weeks 6 and 20 posttransplantation, respectively. Mice were fasted as in B and blood drawn via retroorbital bleed under anesthesia for serum collection. The levels of human C-peptide in sera were determined using human C-peptide ELISA kit. Differences between treatments (indicated in graphs) were assessed by ANOVA with post hoc Bonferroni test to identify the significance of differences among means at $P < .05$. G, Kaplan-Meier survival curve for mice transplanted with indicated treatments. Rejection was defined as plasma glucose levels ≥250 mg/dL on 2 consecutive readings. Incidence of rejection in all groups of mice (0.0, 0.2, and 2.0 μg/mL CXCL12) was compared using the Mantel-Cox log-rank test. H, IPGTT 150 days after implantation. Mice were fasted and treated as described in B

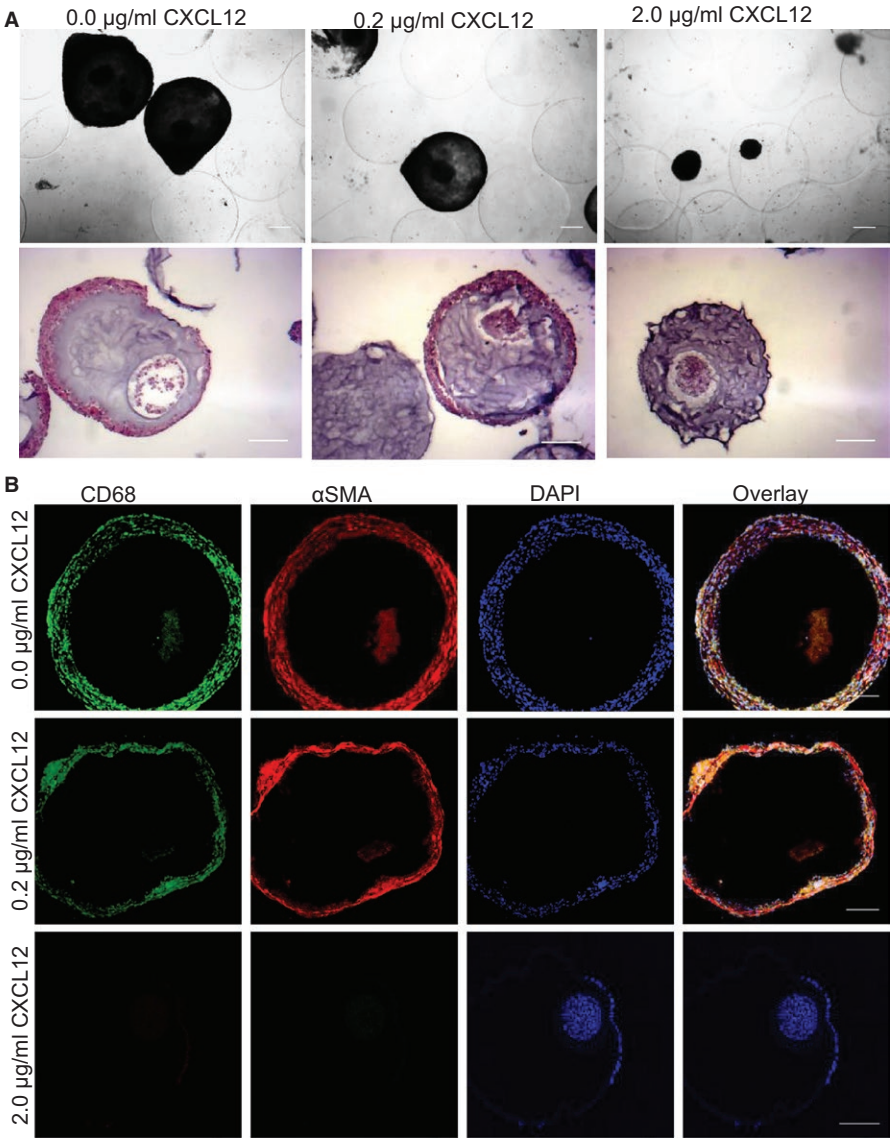

FIGURE 5 Co-encapsulation of SC-β cell clusters with CXCL12 prevents the foreign body fibrotic response and prolongs SC-β-cell viability in sensitized diabetic mice. A-C, STZ-induced diabetic C57BL/6 mice were sensitized to SC-β cell clusters as described in Materials and Methods and legend of Figure 4. A, Representative phase contrast microscopy images and H&E staining of microcapsules retrieved from mice 154 days after implantation. B, Representative images of immunofluorescence staining analysis of microcapsules retrieved from mice 154 days after implantation for markers of the fibrotic response including macrophages (CD68) and myofibroblasts (αSMA). C,D, Representative images of immunofluorescence staining analysis of microcapsules recovered from mice 154 days after implantation for β-cell markers including C-peptide, NKX6.1 and glucagon. Scale bar = 200 µm

body response (FBR). Phase contrast microscopy and hematoxylin and eosin (H&E) staining showed dense pericapsular cellular overgrowth on microcapsules that contained SC-β cell clusters without CXCL12, followed by those with 0.2 µg/mL CXCL12 (Figures 5A and S3B). In contrast, microcapsules that contained SC-β cell clusters with 2.0 µg/mL CXCL12 and microcapsules with or without CXCL12 that did not contain SC-β cell clusters were observed to have scanty to no pericapsular cellular overgrowth (Figures 5A). Immunofluorescence microscopy revealed that the pericapsular cellular overgrowths on the recovered microcapsules without and with 0.2 µg/mL CXCL12 are characterized by intense staining for macrophages (CD68) and myofibroblasts (alpha-smooth muscle actin; αSMA), which is reminiscent of the fibrotic response. Individual cells within the fibrotic cellular overgrowth were resolvable by nuclear 4',6-diamidino-2-phenylindole (DAPI) staining (Figure 5B). In contrast, microcapsules that incorporated 2.0 µg/mL CXCL12 showed little to no staining for both CD68 and αSMA. Compared to CD68 and αSMA, the pericapsular overgrowths stained less densely for $CD3^+$ T cells and $CD19^+$ B cells (Figure S3A). The immunofluorescence staining showed C-peptide–positive cells in microcapsules that incorporated 2.0 µg/mL CXCL12, which also stained positive for NKX6.1 (Figure 5D). These microcapsules containing the highest concentration of CXCL12 also stained positive for glucagon, which did not co-localize with C-peptide (Figure 5D). These findings indicate that not only were the SC-β cells viable, but they also maintained their differentiation state over the 154-day period in vivo in microcapsules that incorporated 2.0 µg/mL of the chemokine.

4 | DISCUSSION

Pancreatic β-cell replacement using SC-β cells represents a potential practical treatment for T1D. The fulfillment of this goal is ultimately contingent on achieving immunoprotection, long-term survival, and

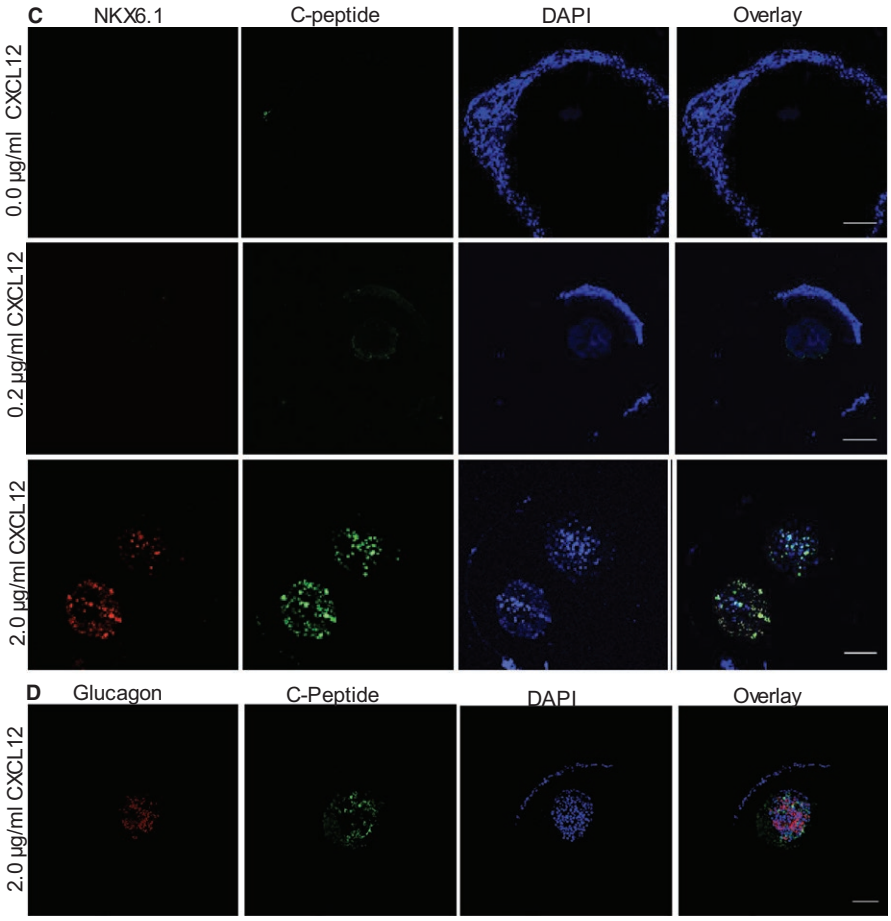

FIGURE 5 (Continued)

optimal function of SC-β cells while ensuring safety. Here we demonstrated substantial progress towards achieving these goals using clinically biocompatible elements—incorporation of an endogenous immunomodulatory chemokine (CXCL12) with clinical grade alginate to encapsulate SC-β cells. The resulting microencapsulated SC-β cells elicited little to no fibrotic FBRs and induced glycemic correction for >150 days in immunocompetent C57BL/6 diabetic mice without systemic immunosuppression. These core findings underscore the safety of our encapsulation strategy, and thus help mitigate development and safety risks related to clinical translation.

Previous studies have used alginate encapsulation to immunoisolate islets for transplantation. However, these studies involved either nonhuman β cells[14,15] or undifferentiated pancreatic endocrine cells.[11] Vegas et al employed chemically modified alginate in conjunction with relatively large-sized (1.5 mm) capsules that were cross-linked with barium to minimize the fibrotic response to achieve long-term glycemic control using SC-β cells without immunosuppression.[18] The clinical safety of the chemically modified alginate is yet to be established and approved for human use.[11] Furthermore, large-sized capsules are associated with impaired oxygen permeability and insulin secretion.[19] These issues may make clinical translation problematic.

Under normal physiologic conditions, CXCL12 is repressed in terminally differentiated mature islets and β cells,[20,21] although its cognate receptor, CXCR4, is the most abundantly expressed chemokine receptor in the mature islet and co-expresses predominantly with insulin in β cells.[22] This suggests that CXCL12/CXCR4 signaling is active in mature β cells. Indeed, CXCL12 has been shown to exert β-cell prosurvival effects in mature islets.[21,22] In this study, CXCL12 did not negatively impact the function of SC-β cells; instead CXCL12 induced expression of genes associated with β-cell function and enhanced GSIS at relatively low concentrations. In contrast to its effect on insulin secretion, a higher concentration of CXCL12 was more effective in ameliorating the fibrotic response to microencapsulated SC-β cells than the lower concentration. These differential effects of CXCL12 on function of SC-β cells and immunoprotection of microencapsulated SC-β cells are reconcilable with previous findings. For instance, in vitro studies suggest that low concentrations of CXCL12 can induce calcium influx as well as chemotaxis, whereas high concentrations can induce calcium influx but lack chemotactic response.[23-25] Furthermore, high concentrations of CXCL12 have been shown to create an immunosuppressive microenvironment such as in tumors[26] and in the bone marrow.[27]

We previously showed that co-encapsulation of mouse or porcine islets with CXCL12 leads to immunoprotection and long-term function without systemic immunosuppression.[14,15] CXCL12-induced immunoprotection was characterized by limited infiltration of effector T cells, macrophages, and fibroblasts and enhanced recruitment of $Foxp3^+$ regulatory T cells to the islet graft.[14,15] Macrophages have been identified as key mediators of the fibrotic

FBR to biomaterial implants[8,28,29]; their activation at the site of the implant leads to a cascade of events including recruitment and differentiation of fibroblasts to myofibroblasts responsible for final fibrosis of the implant.[8,28,30] Indeed, the fibrotic overgrowth in this study was characterized predominantly by macrophages (CD68) and myofibroblasts (αSMA), and relatively less $CD3^+$ T cells and $CD19^+$ B cells. Thevenot et al also showed that local delivery of CXCL12 into poly-lactic-co-glycolytic acid (PGLA) scaffolds led to a profound amelioration of the inflammatory response and prevented fibrosis of the implanted scaffolds.[31] This effect was also characterized by sparse recruitment of macrophages and reduced production of proinflammatory cytokines and chemokines at the site of the implant. Alginate microcapsules that did not contain cells elicited little to no fibrotic responses up to 150 days. This suggests that antigens or cytokines released by the encapsulated cells could elicit the fibrotic response, which is consistent with previous studies.[11,32] Although it would be interesting to evaluate the efficacy of this encapsulation approach in autoimmune diabetic nonobese diabetic mice, the C57BL/6 mouse produces strong fibrotic FBRs that are similar to those in humans.[33] The ability of our approach to evade the fibrotic FBR in this mouse model may thus predict success in clinical translation in the context of microencapsulation, where the FBR plays an important role in graft failure. However, the ability of CXCL12 to specifically protect SC-β cells from autoimmune destruction is yet to be determined and forms the basis of ongoing studies. Furthermore, the impact of a waning concentration of exogenous CXCL12 within microcapsules over time on the FBR and the durability of microencapsulated SC-β cells requires further investigation, especially considering that the fibrotic overgrowth appears to be elicited by the encapsulated cells in the absence of CXCL12.

In summary, this study demonstrates the capacity of human SC-β cells microencapsulated with clinically compatible elements, alginate and CXCL12, to evade the fibrotic FBR and induce long-term glycemic correction in an immunocompetent murine model of T1D without systemic immunosuppression. This study thus lays the groundwork for translation to more demanding and clinically relevant diabetic nonhuman primate models, with the ultimate goal of translating this approach to enable SC-β cell transplantation for individuals with T1D.

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AUTHOR CONTRIBUTIONS

D.A.A., D.A.M., T.B., and M.C.P. conceived the study. D.A.A., M.C.P., and D.A.M. designed the study. D.A.A., J.J.L.C., R.A.D., M.F.P., M.S., and E.N.E. acquired the data. D.A.A., J.J.L.C., and M.C.P. analyzed and interpreted the data. D.A.A. drafted the manuscript. R.F.S., J.J.L.C., T.B., J.F.M., M.C.P., and D.A.M. revised the manuscript. M.C.P. and D.A.M. supervised the study.

DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. D.A.M. is scientific founder of Semma Therapeutics and M.C.P. is scientific founder of Vicapsys, Inc. The other authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

All relevant data are available in the manuscript.

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SUPPORTING INFORMATION

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

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