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Original Article

Localized cytotoxic T cell–associated antigen 4 and antioxidant islet encapsulation alters macrophage signaling and induces regulatory and anergic T cells to enhance allograft survival

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ABSTRACT

The loss of functional β-cell mass is a hallmark of type 1 diabetes. Islet transplantation represents a promising alternative approach, but immune-mediated graft destruction remains a major challenge. We sought to use islet encapsulation technologies to improve graft survival and function without systemic immunosuppression. We hypothesized islet encapsulation with nanothin coatings consisting of tannic acid (TA), an antioxidant; poly(*N*-vinylpyrrolidone) (PVPON), a biocompatible polymer; and cytotoxic T cell–associated antigen 4 immunoglobulin (CTLA-4-Ig), an inhibitory immune receptor, will elicit localized immunosuppression to prolong islet allograft function and suppress effector T cell responses. In the absence of systemic immunosuppression, we demonstrated (PVPON/TA/CTLA-4-Ig)-encapsulated NOD.*Rag* islet grafts maintain function significantly longer than control IgG-containing (PVPON/TA/IgG) and nonencapsulated controls after transplantation into diabetic C57BL/6 mice. This protection coincided with diminished proinflammatory macrophage responses mediated by signal transducer and activator of transcription 1 signaling, decreased proinflammatory T cell effector responses, and CTLA-4-Ig-specific concomitant increases in anergic $CD4^+$ T cells and regulatory T cells. Our results provide evidence that conjugation of CTLA-4-Ig to (PVPON/TA) coatings can suppress T cell activation, enhance regulatory T cell populations, prolong islet allograft survival, and induce localized immunosuppression after transplantation.

Introduction

After autoimmune destruction of insulin-producing β cells, patients with type 1 diabetes (T1D) require exogenous insulin to restore euglycemia. The absence of tightly controlled glucose metabolism can increase risk for kidney failure, neuropathies, cardiovascular disease, and hypoglycemic unawareness events.[1] Transplantation of donor islets has shown clinical promise in restoring insulin-independence and reducing the risk of hypoglycemic events; however, graft longevity remains a challenge.[2] To suppress islet graft rejection, systemic immunosuppression is used but

Abbreviations: ANOVA, analysis of variance; APC, antigen-presenting cell; CTLA-4, cytotoxic T cell–associated antigen 4; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JAK1, janus kinase 1; NO, nitric oxide; ns, nonsignificant; PTP, protein tyrosine phosphatase; PVPON, poly(*N*-vinylpyrrolidone); STAT1, signal transducer and activator of transcription 1; STZ, streptozotocin; SOCS, suppressor of cytokine signaling; T1D, type 1 diabetes; TA, tannic acid; TNF, tumor necrosis factor; Treg, regulatory T cell; DAPI, 4′,6-diamidino-2-phenylindole; MHC, major histocompatibility complex.

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can compromise graft function and increase pathogenic infections.[3] Novel approaches of immunoprotection are needed to improve these clinical outcomes.

One mechanism contributing to inflammatory immune responses is the production of free radicals including superoxide, nitric oxide, and hydrogen peroxide. These molecules can activate immune populations but can be toxic at high concentrations and induce oxidative stress.[4] β Cells are sensitive to free radical–mediated because they contain reduced levels of endogenous antioxidants.[5,6] To fortify antioxidant defenses in β cells, we generated a layer-by-layer islet encapsulation material containing tannic acid (TA), which is a potent antioxidant, and poly(-*N*-vinylpyrrolidone) (PVPON), which is a nontoxic polymer that is biologically inert and, therefore, will not illicit an immune response.[7,8]

We previously established that (PVPON/TA) layers significantly reduce hydrogen peroxide, superoxide, proinflammatory immune responses, and decrease T cell migration in vitro.[9,10] These effects were not observed with PVPON alone. We also observed significant delays in islet graft destruction in non-obese diabetic (NOD) mice after encapsulation with (PVPON/TA) layers[7,9]; however, only ~50% of encapsulated islet recipients maintained lasting euglycemia. Therefore, decreasing innate immune responses cannot fully protect long-term graft function, and additional approaches to inhibit the adaptive immune system are needed.

One strategy for T cell suppression is upregulating cytotoxic T cell–associated antigen 4 (CTLA-4), an inhibitory receptor on regulatory $CD4^+$ T cells (Tregs). CTLA-4 binds CD80/86 on antigen-presenting cells (APCs), preventing CD28:CD80/86 interactions required for T cell activation.[11] To exploit this regulatory mechanism, we used recombinant CTLA-4-Ig. This fusion protein competitively binds CD80/86 similarly to CTLA-4 expressed on Tregs.[12] Variants of CTLA-4-Ig, abatacept and belatacept, are Food and Drug Administration approved for use in arthritis[13] and kidney transplantation,[14] with trials in islet transplantation (NCT00468403; NCT00501709). However, these studies use systemic administration of CTLA-4-Ig that may induce global immunosuppression. By adding CTLA-4-Ig to our (PVPON/TA) coatings, we hypothesized that it will enhance graft survival by inhibiting local proinflammatory APC and effector T cell responses.

Our data demonstrated that (PVPON/TA/CTLA-4-Ig)-encapsulated allogeneic islet grafts maintain function significantly longer than control IgG (PVPON/TA/IgG)-encapsulated and nonencapsulated islets after transplantation into hyperglycemic C57BL/6 mice. (PVPON/TA/CTLA-4-Ig)-encapsulation induced a reduction in proinflammatory macrophage and effector T cell responses. Contrary to (PVPON/TA/IgG) alone, the addition of CTLA-4-Ig significantly increased $FoxP3^+$ Treg and anergic $CD4^+$ T cells. Collectively, adding CTLA-4-Ig onto (PVPON/TA) layers enhanced localized immunosuppression of islet allograft responses compared with (PVPON/TA/IgG) alone and may be efficacious in future human translational studies.

Materials and methods

Mice

NOD.*Rag* (Strain #3729), C57BL/6 (Strain #664), and C57BL/6.Cg-Tg(Ins1-EGFP)1Hara/J (MIP-GFP)[15] (Strain #6864) mice were purchased from The Jackson Laboratory and housed on 12-hour light/dark cycle under specific pathogen-free conditions. Male and female C57BL/6, NOD.*Rag*, and MIP-GFP mice aged between 8 and 12 weeks were used in all experiments in accordance with UAB IACUC-approved protocols.

Islet isolation and encapsulation

NOD.*Rag* or MIP-GFP islets were isolated by Clyzme (VitaCyte) perfusion through the common bile duct, enzymatic and mechanical pancreas digestion at 37 °C for 17 minutes, and islet purification by gradient separation from acinar tissue. Islets were handpicked, cultured overnight, and encapsulated with (PVPON/TA) multilayers as previously described.[16] The addition of 0.5 mg/mL recombinant CTLA-4-Ig (BioXCell; BE0099) denoted (PVPON/TA/CTLA-4-Ig) or control IgG1 (BioXCell; BE0096) denoted (PVPON/TA/IgG) was adsorbed on the (PVPON/TA)-coated islets for 10 minutes and washed. The antibodies are bound to TA through hydrogen bonding, which is stable at the physiologic conditions.[16] PVPON (molecular weight [MW] = 1 300 000 g/mol) and TA (MW = 1,700 g/mol) were purchased from Fisher.

Induction of diabetes, islet transplantation, intraperitoneal glucose tolerance test, and AZD1480 treatment

Male and female C57BL/6 mice were rendered diabetic with 1 dose of streptozotocin (STZ; 190 mg/kg intraperitoneally; Sigma-Aldrich). Blood glucose values of ≥300 mg/dL were considered diabetic condition. Mice were transplanted with 250 nonencapsulated, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated NOD.*Rag* islets under the kidney capsule as described.[16,17] STZ-treated, nontransplanted recipients served as diabetic controls and untreated C57BL/6 mice were euglycemic controls. Mock transplants were performed on euglycemic mice as surgical controls. Intraperitoneal glucose tolerance test was performed after a 6-hour fast by injecting 2 g/kg of a 20% glucose solution intraperitoneally, followed by blood glucose measurements at 0, 5, 15, 30, 60, 90, and 120 minutes. AZD1480 was resuspended in 0.5% hypromellose/0.1% Tween 80 in H_2O. Recipient mice were given daily AZD1480 (25 μg/kg) intraperitoneal injections for 14 days, beginning a day before transplantation.

Allogeneic islet coculture assay

Nonencapsulated, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated NOD.*Rag* islets (100) were cultured with 4×10^6 allogeneic C57BL/6 splenocytes. No stimulation or 2.5 μg/mL of concanavalin A (Sigma) were used as negative and positive controls, respectively. AZD1480 (2 μM) was added to nonencapsulated islet cocultures. Cells and supernatants were collected after 48 hours for flow cytometry and enzyme-linked immunosorbent assay (ELISA). For western blotting, 400 islets were cultured with 1.6×10^7 allogeneic splenocytes for lysates.

Flow cytometry and immunofluorescence staining

The entire islet graft region was excised, dispersed into single cells, and stained with fluorochrome-conjugated antibodies (Supplementary Table S1) as described.[7] Cells were collected by Attune NxT Flow Cytometer (ThermoFisher) with 300 000 events/sample and analyzed using the gating strategy outlined in Supplementary Figure S1 with FlowJo (10.0.8r1) software. Detection of CTLA-4-Ig through immunofluorescence was performed on encapsulated islets in chamber slides (TissueTek) as described.[7]

Western blots

Cell lysates (20 μg) were separated on an SDS-PAGE gel, transferred to nitrocellulose membranes, and blocked as described.[18] Membranes were incubated with primary antibodies (Supplementary Table S1) followed by fluorochrome-conjugated secondary antibodies (1:20,000; Li-Cor), imaged on the Li-Cor Odyssey, quantitated with Image Studio software (version 5.2.5), and normalized to β-actin.

ELISAs and Griess assay

Interferon (IFN) gamma, interleukin (IL)-10, and IL-2 were measured using ELISA antibody pairs as described.[19] Tumor necrosis factor (TNF), CCL5, and CXCL10 production were measured using DuoSet ELISA Kits (R&D Systems) per manufacturer instructions. NO_2^- production was measured by the Griess assay as described.[18] Plates were read on a

BioTek Synergy2 microplate reader and analyzed using the Gen5 v.1.10 software (BioTek).

Statistical analysis

Data were analyzed using GraphPad Prism (version 9). Results were expressed as mean ± SD. Graft survival was assessed using the Kaplan-Meier test for cumulative survival, and the log-rank test was used to compare survival curves. Normality was assessed using a Shapiro-Wilk test. If normality was achieved, determination of the difference between mean ± SD for each experimental group was assessed using 1-way or 2-way analysis of variance (ANOVA) with Tukey multiple comparisons test where appropriate. If normality was not achieved, a nonparametric Kruskal-Wallis test was used to assess differences between the groups. In all tests, $P < .05$ was considered significant.

Results

Transplantation of (PVPON/TA/CTLA-4-Ig)-encapsulated islets delays alloimmune-mediated graft failure

We previously demonstrated that (PVPON/TA) encapsulation can maintain long-term islet allograft survival in ~50% of recipients in the absence of immunosuppression.[7] Given the adaptability of (PVPON/TA) layers,[20] we modified our material to enhance T cell suppression and to further delay islet allograft rejection by conjugating CTLA-4-Ig onto the exterior of (PVPON/TA). The CTLA-4-Ig interaction with TA occurs through hydrogen bonding between the phenolic groups of TA and amine groups of CTLA-4-Ig. These hydrogen bonds are stable as reported earlier.[21,22] Immunofluorescence demonstrated that CTLA-4-Ig was present on (PVPON/TA/CTLA-4-Ig) layers and visible around the outer edge of the islet (Fig. 1A) but absent in control IgG (PVPON/TA/IgG). We transplanted 250 nonencapsulated, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated allogeneic NOD.*Rag* islets into STZ-treated diabetic C57BL/6 mice and monitored ad libitum blood glucose. Compared with (PVPON/TA/IgG)-encapsulated grafts, the addition of CTLA-4-Ig significantly delayed islet allograft rejection (Fig. 1B), with 80% of (PVPON/TA/CTLA-4-Ig)-encapsulated islet recipients maintaining long-term graft function without immunosuppression. Two weeks posttransplant, we performed an intraperitoneal glucose tolerance test on euglycemic islet graft recipients (Fig. 1C). All encapsulated grafts responded similarly to nonencapsulated controls (Fig. 1D); however after 2 months, although all nonencapsulated grafts had already failed, glucose responsiveness was maintained in (PVPON/TA/IgG)-encapsulated and (PVPON/TA/CTLA-4-Ig)-encapsulated recipients (Fig. 1E, F). Given the enhanced protective effect of (PVPON/TA/CTLA-4-Ig) encapsulation on islet allograft survival, we wanted to determine the cellular and molecular mechanisms of protection.

Encapsulated islet allografts reduce innate immune cell infiltration and p-STAT1$^+$ macrophages

Because proinflammatory myeloid cells are involved in early graft failure,[23,24] we investigated whether encapsulation affected innate immune responses. We performed flow cytometry at 7 and 14 days posttransplant and observed significant reductions in the number of CD11b$^+$ myeloid cells at day 7 and day 14 and in the number of CD11c$^+$ dendritic cells (DCs) at day 14 in (PVPON/TA/CTLA-4-Ig)-encapsulated islet grafts compared with those in the nonencapsulated recipients (Fig. 2A-C). No significant changes were observed in the number of F4/80$^+$ macrophages between the groups (data not shown). Because immune trafficking is regulated by STAT1 activation,[25,26] we examined STAT1 phosphorylation as a potential mechanism of inhibition with our coatings. The analysis of grafts on day 14 posttransplant for p-STAT1 (Ser727)$^+$ expression within all CD11b$^+$ cells revealed a significant reduction in the number of p-STAT1$^+$ myeloid cells within (PVPON/TA/CTLA-4-Ig)-encapsulated islet grafts (Fig. 2D); however, the frequency was unchanged. The number and frequency of p-STAT1$^+$ cells within total infiltrating F4/80$^+$ macrophages, CD11c$^+$ DCs, CD4$^+$ T cells, or CD8$^+$ T cells were unchanged at day 14 (Supplementary Fig. S2A-D). Although major histocompatibility complex (MHC)-II expression within the F4/80$^+$ macrophage population at day 14 was not decreased (Fig. 2E), we did observe a reduction in TNF expression (Fig. 2F) within (PVPON/TA/CTLA-4-Ig)-encapsulated grafts compared with that in nonencapsulated controls. CD86 expression within total CD11c$^+$ DCs was significantly reduced in both encapsulation groups at day 14 (Fig. 2G). Finally, the number of CD86$^+$ cells within total CD11b$^+$ myeloid cells were not decreased in any of the transplant groups (Supplementary Fig. S2E), but (PVPON/TA/CTLA-4-Ig)-encapsulated islet grafts did exhibit a further reduction in TNF$^+$ CD11b$^+$ myeloid cells compared with (PVPON/TA/IgG)-encapsulated islets (Supplementary Fig. S2F).

To define the importance of STAT1 in mediating islet rejection, we treated nonencapsulated islet allotransplant recipients with AZD1480, a JAK1/JAK2 inhibitor,[27] to block STAT1 phosphorylation. Compared with mice treated with vehicle control, mice treated with AZD1480 exhibited a significant delay in islet allograft rejection with ~75% maintaining allograft survival beyond 30 days and ~50% maintaining long-term graft survival beyond 100 days (Fig. 2H, I). These data demonstrate that STAT1-dependent responses contribute to islet allograft destruction and inhibition of this pathway may elicit immunoprotection driven by (PVPON/TA/CTLA-4-Ig) coatings.

(PVPON/TA/CTLA-4-Ig)-encapsulation decreases nitrite, proinflammatory cytokine/chemokine synthesis, and macrophage STAT1 activation in vitro

Next, we wanted to assess whether the addition of CTLA-4-Ig to (PVPON/TA) coatings further impaired proinflammatory innate immune responses and STAT1 activation. We performed in vitro islet coculture experiments using concanavalin A (ConA)—a positive control for immune cell activation—in nonencapsulated, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated NOD.*Rag* islets cultured with allogeneic C57BL/6 splenocytes in the presence or absence of AZD1480. After 48 hours, we analyzed JAK1 and STAT1 activation by western blotting. Both coatings reduced p-JAK1 (Y1034/1035), total JAK1, p-STAT1 (Y701), and total STAT1 (Fig. 3A-E) compared with nonencapsulated islets. Adding AZD1480 mimicked these reductions in JAK1 and STAT1 signaling. The analysis of CD11b$^+$ myeloid cell, CD11c$^+$ DC, CD4$^+$ T cell, and CD8$^+$ T cell populations displayed no differences in the frequency of p-STAT1 (Ser727) expression between islet cocultures (Supplementary Fig. S3A-D), similar to in vivo observations (Supplementary Fig. S2). However, stimulation with nonencapsulated islets and AZD1480, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated islets significantly reduced the frequency of p-STAT1 (Ser727)$^+$ F4/80$^+$ macrophages compared with nonencapsulated controls (Fig. 3F, G).

Macrophages analyzed from allogeneic cultures with either (PVPON/TA/IgG)-encapsulated or (PVPON/TA/CTLA-4-Ig)-encapsulated islets displayed significantly reduced frequencies of CD86 and MHC-II expression (Supplementary Fig. S3E, F) compared with nonencapsulated islet controls. Reduced frequencies of total CD11b$^+$ myeloid cells and CD11c$^+$ DCs expressing CD86 (Supplementary Fig. S3G, H) were also observed suggesting that encapsulated islets, irrespective of the addition of CTLA-4-Ig, can suppress proinflammatory innate immune cell activation in vitro. Moreover, inhibition of JAK1/JAK2 with AZD1480 reduced CD86 and MHC-II expression (Supplementary Fig. S3E-H), indicating that STAT1 activation is a key pathway for the upregulation of these cell surface molecules.

The analysis of inducible nitric oxide synthase (iNOS), a known gene target of STAT1,[28] revealed that cultures containing (PVPON/TA/IgG)-encapsulated or (PVPON/TA/CTLA-4-Ig)-encapsulated islets reduced iNOS expression (Fig. 4A, B). In addition, iNOS was decreased when allogeneic C57BL/6 splenocytes were stimulated with nonencapsulated islets in the presence of AZD1480 (Fig. 4A, B). We also performed a Griess

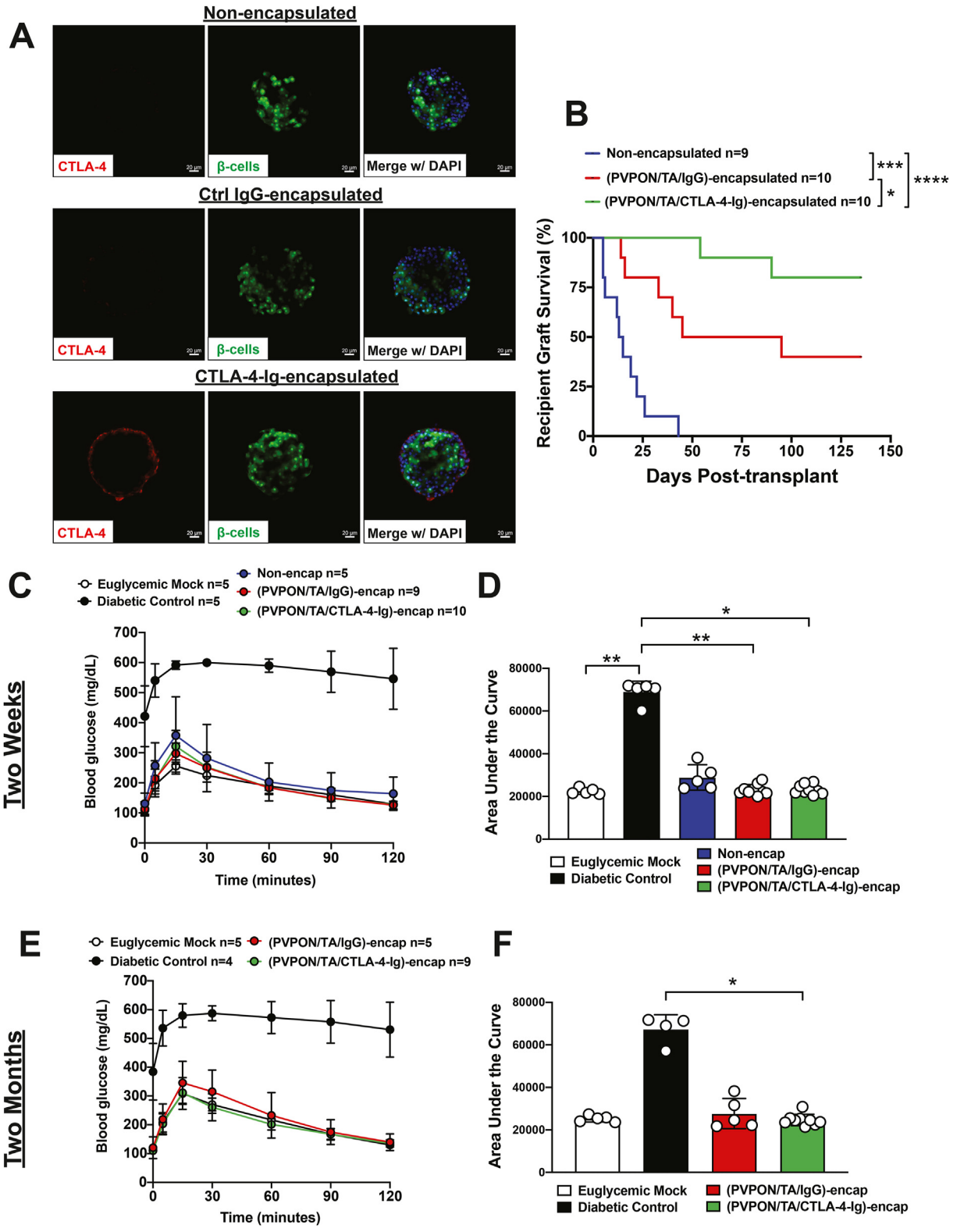

Figure 1. Transplantation of (PVPON/TA/CTLA-4-Ig)-encapsulated islets delays alloimmune-mediated graft failure. (A) Confocal microscopy immunofluorescence imaging of intact nonencapuslated, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated MIP-GFP islets for CTLA-4 (red), GFP^{+} β-cells (green), and DAPI (blue). (B) Kaplan-Meier log-rank test of STZ-treated C57BL/6 recipients, maintaining islet graft function based on blood glucose readings (<300 mg/dL) after transplantation with 250 non encapsulated, (PVPON/TA/IgG)-encapsulated, or (PVPON/TA/CTLA-4-Ig)-encapsulated allogeneic NOD.*Rag* islets under the kidney capsule (n = 9-10 per group). (C) Intraperitoneal glucose tolerance test (IPGTT) on transplanted recipients at 2 weeks posttransplant (n = 5-10). (D) Two-way ANOVA with multiple comparisons for area under the curve of the 2-week IPGTT assay. (E) IPGTT on transplanted recipients at 2 months posttransplant (n = 4-9). (F) Two-way ANOVA with multiple comparisons for area under the curve of the 2-month IPGTT assay. Data represents 4 independent experiments. $^{*}P < .05$; $^{***}P < .001$; $^{****}P < .0001$. ANOVA, analysis of variance; CTLA-4-Ig, cytotoxic T cell–associated antigen 4 immunoglobulin; DAPI, 4′,6-diamidino-2-phenylindole; PVPON, poly(*N*-vinylpyrrolidone); STZ, streptozotocin; TA, tannic acid.

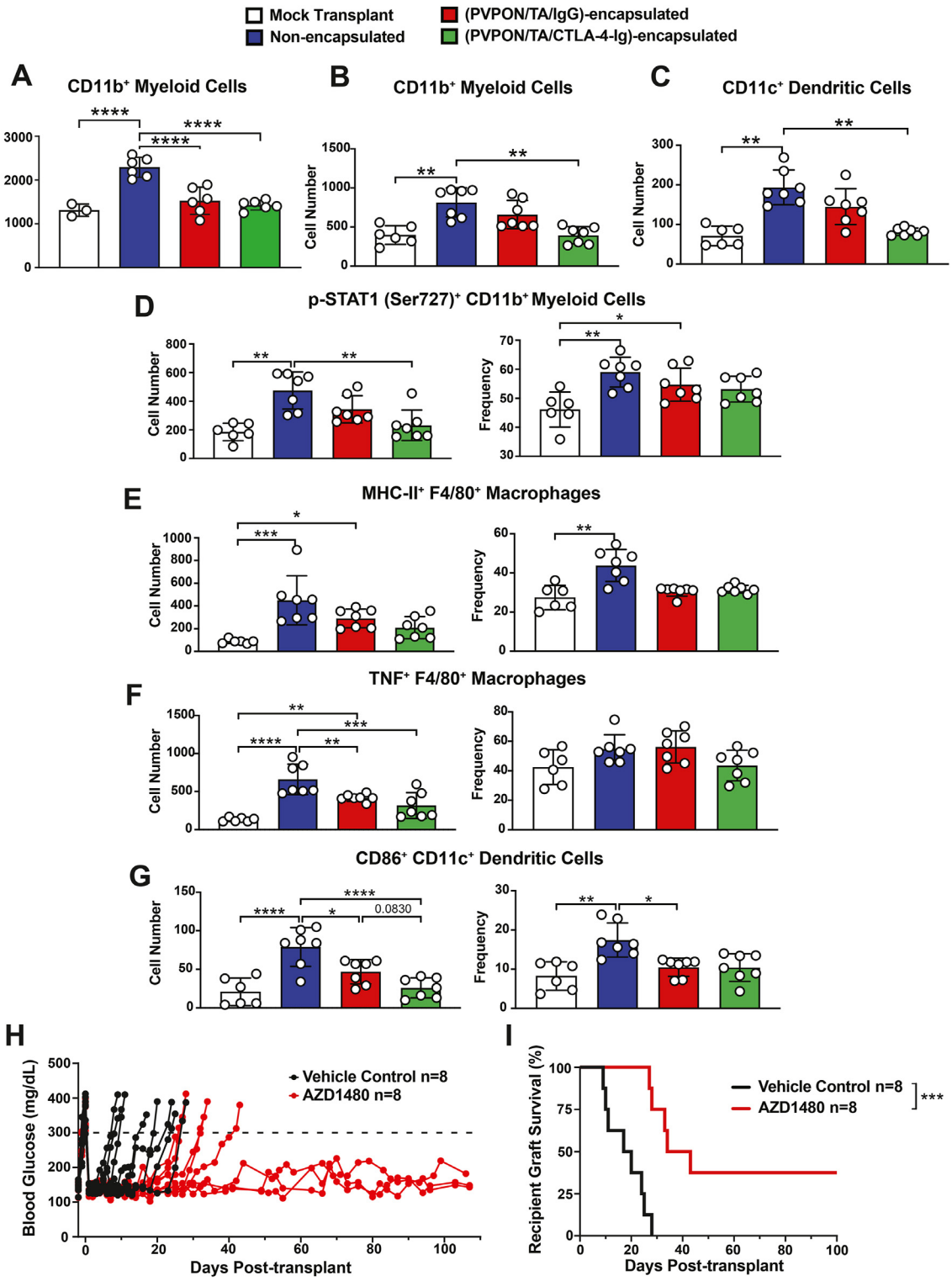

Figure 2. Encapsulated islet allografts reduce innate immune cell infiltration and p-STAT1^{+} macrophages. Immunophenotyping of islet allografts of NOD.*Rag* islets collected 7 or 14 days posttransplant into STZ-treated C57BL/6 mice were analyzed by flow cytometry. Quantification of number of CD11b^{+} myeloid cells on day 7 (A) and day 14 (B) and CD11c^{+} DCs on day 14 (C). After gating on CD11b^{+} myeloid cells, F4/80^{+} macrophages, or CD11c^{+} DCs within the day 14 samples, quantification of the number and frequency of p-STAT1 (Ser727)$^{+}$ CD11b^{+} myeloid cells (D), MHC-II^{+} F4/80^{+} macrophages (E), TNF^{+} F4/80^{+} macrophages (F), and CD86^{+} CD11c^{+} DCs (G) (n = 7). (H) Blood glucose values after daily intraperitoneal injection of 25 μg/kg bodyweight of AZD1480 or equal volume of vehicle control and transplantation of 250 allogeneic NOD.*Rag* islets into STZ-treated diabetic C57BL/6 mice (n = 8). (I) Kaplan-Meier log-rank test of STZ-treated C57BL/6 recipients maintaining islet graft function based on blood glucose readings (<300 mg/dL) after transplantation with 250 allogeneic NOD.*Rag* islets with or without AZD1480 administration (n = 8). Data represents at least 3 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$; ns = not significant. DC, dendritic cell; MHC, major histocompatibility complex; PVPON, poly(*N*-vinylpyrrolidone); STAT1, signal transducer and activator of transcription 1; STZ, streptozotocin; TNF, tumor necrosis factor.

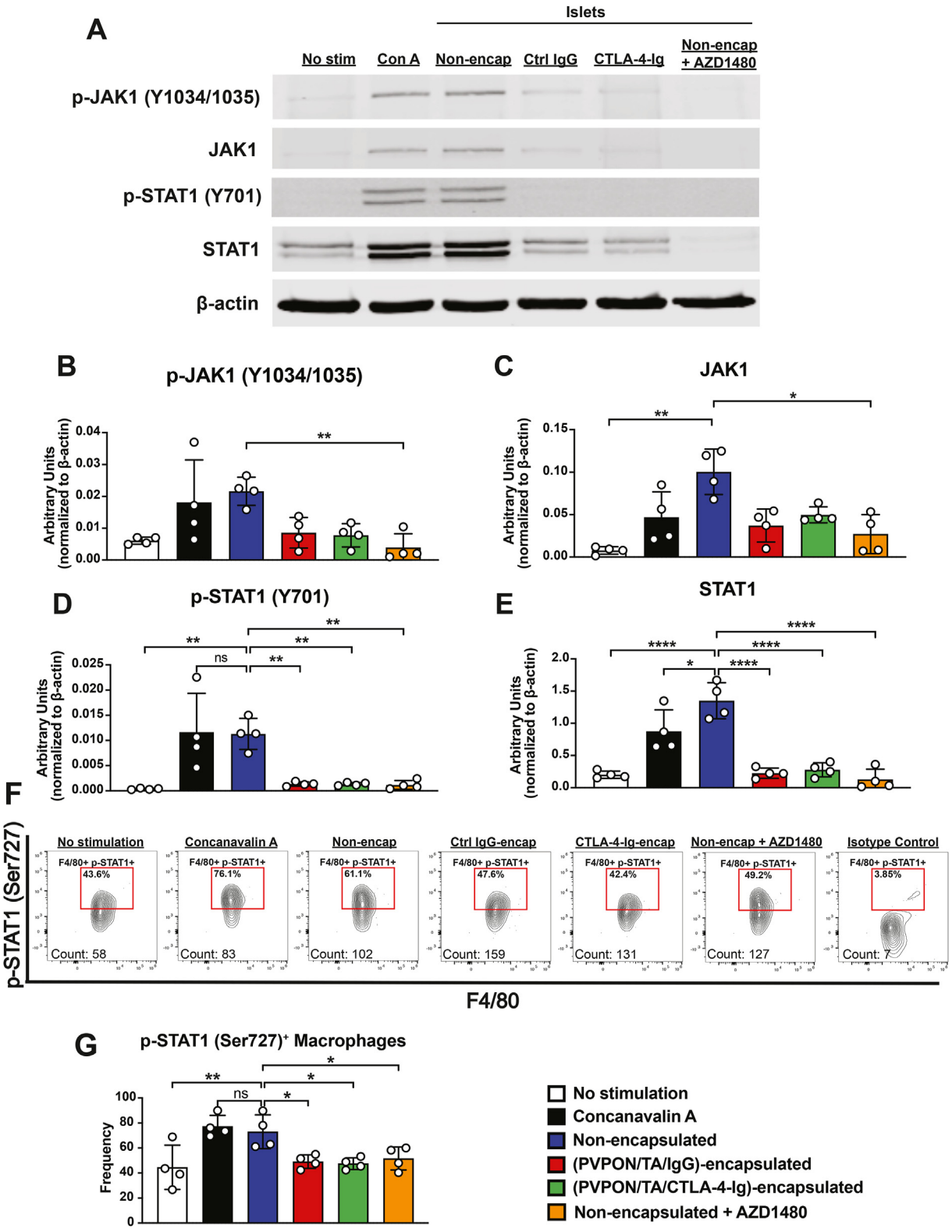

Figure 3. Islet encapsulation suppresses macrophage STAT1 activation. Allogeneic islet coculture assays were performed and whole cell lysates or cells collected after 48 hours for Western blot and flow cytometry analysis, respectively. (A) Representative blot for p-JAK1 (Y1034/1035), total JAK1, p-STAT1 (Y701), and total STAT1. The densitometry analysis for p-JAK1 (Y1034/1035) (B), total JAK1 (C), p-STAT1 (Y701) (D), and total STAT1 (E) normalized to β-actin (n = 4). Representative flow cytometry plots (F) and quantification of F4/80$^+$ macrophages expressing p-STAT1 (Ser727)$^+$ (G) from islet coculture assays (n = 4), analyzed by Kruskal-Wallis test with Dunn multiple comparisons. Data represents 4 independent experiments. *P < .05; **P < .01; ***P < .001; ****P < .0001t. JAK1, janus kinase 1; ns, nonsignificant; STAT1, signal transducer and activator of transcription 1.

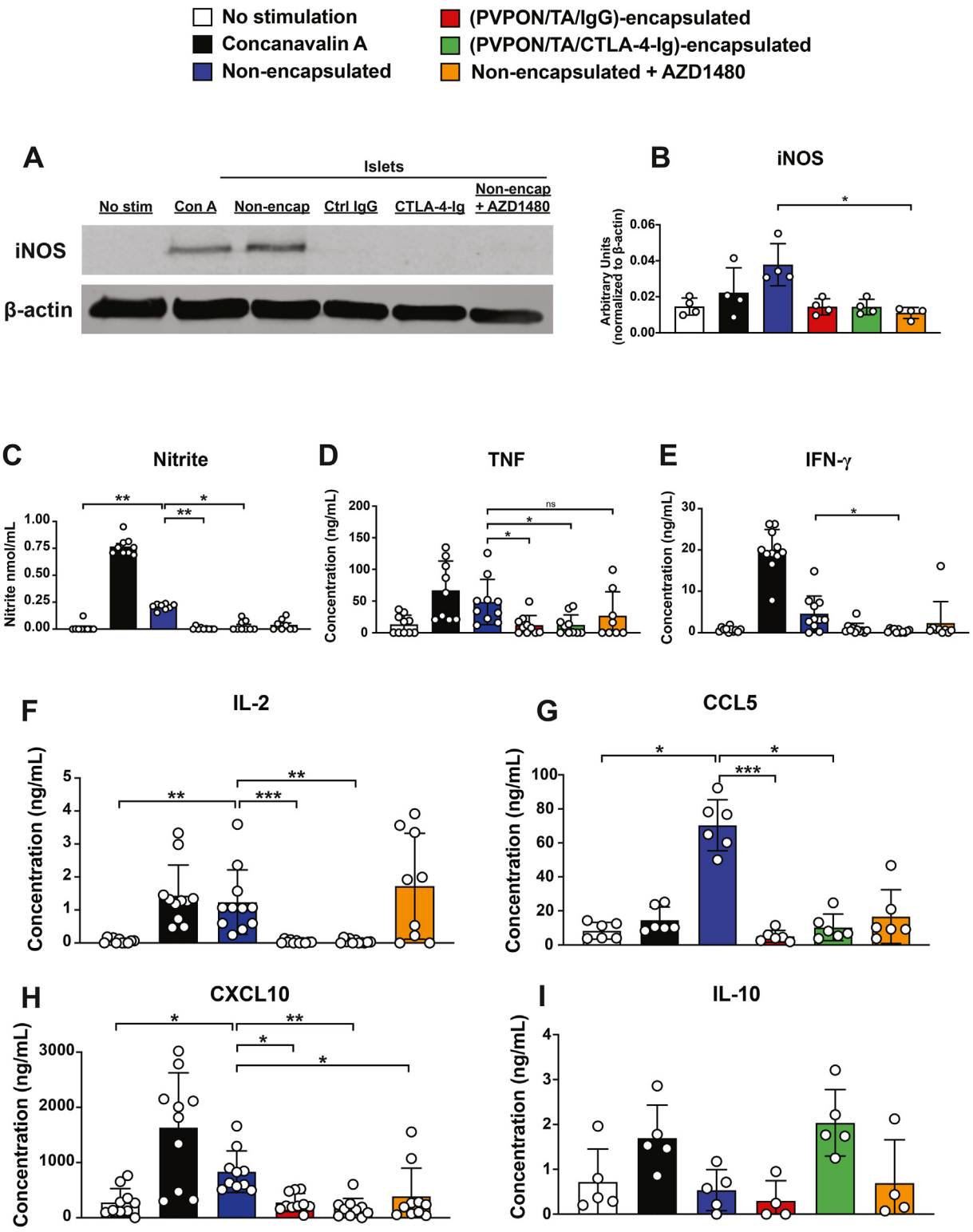

Figure 4. Islet encapsulation decreases nitrite and proinflammatory cytokine/chemokine synthesis from allogeneic islet coculture assays. Supernatants from allogeneic islet coculture assays were collected after 48 (A-F) and 72 hours (G) for western blotting, ELISA, and Griess assay. Representative blot (A) and densitometry analysis (B) for iNOS normalized to β-actin (n = 4). Griess assay for nitrite (C) and ELISA results for TNF (D), IFN gamma (E), IL-2 (F), CCL5 (G), CXCL10 (H), and IL-10 production (I) (n = 4-11), analyzed by Kruskal-Wallis test with Dunn multiple comparisons. Data represents 3 independent experiments. *$P < .05$; **$P < .01$; ****$P < .0001$. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; ns, nonsignificant; TNF, tumor necrosis factor.

assay to quantify nitrite levels, a downstream product of nitric oxide production.[29] (PVPON/TA/IgG) or (PVPON/TA/CTLA-4-Ig) coatings significantly reduced nitrite levels compared with nonencapsulated islets (Fig. 4C), demonstrating that TA functions as an antioxidant. AZD1480 treatment also decreased nitrite levels, corroborating previous reports that iNOS is regulated by STAT1.[28] Furthermore, we observed significant reductions in TNF (Fig. 4D), IFN gamma (Fig. 4E), and IL-2 (Fig. 4F) within (PVPON/TA/IgG)-encapsulated and (PVPON/TA/CTLA-4-Ig)-encapsulated islet cocultures compared with those in nonencapsulated controls.

However, AZD1480 treatment of C57BL/6 splenocytes stimulated with nonencapsulated islets displayed no significant differences in TNF (Fig. 4D), IFN gamma (Fig. 4E), or IL-2 (Fig. 4F) compared with that of nonencapsulated islets. This observation provides evidence that (PVPON/TA/IgG) or (PVPON/TA/CTLA-4-Ig) coatings can suppress additional pathways to induce cytokine synthesis, including redox-dependent signaling pathways (H_2O_2-mediated) and NF-κB. We have preliminary data showing that allogeneic C57BL/6 splenocytes exhibit a reduction in NF-κB p65 phosphorylation when stimulated with (PVPON/TA/IgG)-encapsulated or (PVPON/TA/CTLA-4-Ig)-encapsulated islets (data not shown). In addition, (PVPON/TA/IgG)-encapsulated islet cocultures demonstrated significant reductions in CCL5 and CXCL10 (Fig. 4G, H) compared with nonencapsulated controls. The addition of CTLA-4-Ig significantly reduced CCL5 similar to (PVPON/TA/IgG) and further reduced CXCL10 compared with nonencapsulated controls (Fig. 4H). Nonencapsulated islets treated with AZD1480 demonstrated some reductions in CCL5 and CXCL10 production as well, suggesting STAT1 signaling is involved in the production of these chemokines.[30] However, nonencapsulated islets display a higher level of CCL5 secretion at 48 hours than the ConA positive control. We believe this elevated level of chemokine production within the islet groups is due to differences in the kinetics of immune activation being analyzed. Excitingly, coculturing with (PVPON/TA/CTLA-4-Ig)-encapsulated islets displayed an increase in anti-inflammatory IL-10 secretion compared with both nonencapsulated and (PVPON/TA/IgG)-encapsulated islets (Fig. 4I), signifying that CTLA-4-Ig may be increasing immunosuppressive cytokine production.

(PVPON/TA/CTLA-4-Ig) encapsulation suppresses CD4$^+$ T cell activation and enhances regulatory T cell populations in vitro

The effects of encapsulation on in vitro allogeneic T cell responses were examined by flow cytometry. Within all CD4$^+$ T cells analyzed, stimulation with (PVPON/TA/IgG)-encapsulated and (PVPON/TA/CTLA-4-Ig)-encapsulated islets significantly reduced CD44$^+$ CD62L$^-$ effector CD4$^+$ T cell frequencies (Fig. 5A, B) and concomitantly increased CD44$^-$ CD62L$^+$ naïve CD4$^+$ T cell frequencies (Fig. 5A, C) compared with nonencapsulated controls. There was no change to the CD44$^+$ CD62L$^+$ central memory population (Fig. 5A, D). The analysis of CD25$^+$ and CD69$^+$ (Fig. 5E, F) CD4$^+$ T cell populations provided further evidence that T cell activation was suppressed by encapsulation.

Because CTLA-4-Ig induces an increase in FoxP3$^+$ Tregs,[31] we investigated whether (PVPON/TA/CTLA-4-Ig) layers would have similar effects. After gating on total CD4$^+$ T cells, (PVPON/TA/IgG) encapsulation did not alter Treg frequency or number; however, (PVPON/TA/CTLA-4-Ig)-encapsulated islets significantly increased the frequency of CD25$^+$ FoxP3$^+$ Treg cells (Fig. 5G, H). This population could explain the significant increase in IL-10 secretion observed within (PVPON/TA/CTLA-4-Ig)-encapsulated islet cocultures (Fig. 4I). Moreover, we investigated the induction of CD4$^+$ T cell anergy, another mechanism of immunosuppression elicited by CTLA-4-Ig treatment.[32] In the flow cytometry analysis, the frequency of CD73$^+$ and folate receptor (FR)4$^+$ anergic cells within all CD4$^+$ T cells increased after stimulation with (PVPON/TA/CTLA-4-Ig)-encapsulated islets (Fig. 5I); however, this population was small in all groups. Although STAT1 inhibition was effective in suppressing proinflammatory innate immune responses, the addition of AZD1480 did not affect effector T cell responses.

Encapsulated islets reduce CD8$^+$ T cell activation

Next, we investigated the effects on CD8$^+$ T cells with our in vitro allogeneic islet coculture assay. With all CD8$^+$ T cells analyzed, stimulation with (PVPON/TA/IgG)-encapsulated and (PVPON/TA/CTLA-4-Ig)-encapsulated islets significantly reduced frequencies of CD44$^+$ CD62L$^-$ effector CD8$^+$ T cells (Fig. 6A, B) and increased CD44$^-$ CD62L$^+$ naïve CD8$^+$ T cells (Fig. 6A, C) compared with that with nonencapsulated controls. No differences in CD44$^+$ CD62L$^+$ central memory CD8$^+$ T cells were detected (Fig. 6A, D). We also observed significant reductions in the frequencies of CD25$^+$ and CD69$^+$ CD8$^+$ T cells when stimulated with encapsulated islets or AZD1480 treatment (Fig. 6E, F) but no changes in perforin expression (Fig. 6G). Collectively, STAT1 inhibition can compromise in vitro T cell activation but not effector responses.

Encapsulated islets reduce T cell infiltration and effector responses.

To examine ex vivo T cell responses after islet allotransplants, flow cytometry was performed at day 14 posttransplant. We observed a significant decrease in the number of CD4$^+$ T cells and CD8$^+$ T cells (Fig. 7A, B) within (PVPON/TA/IgG)-encapsulated and (PVPON/TA/CTLA-4-Ig)-encapsulated allografts compared with that in nonencapsulated controls. Combined with our in vitro data demonstrating dampened chemokine production (Fig. 4), these data provide evidence that encapsulation of islets can suppress T cell infiltration. These data are supported by significant decreases in the number of CXCR3$^+$ CD4$^+$ T cells (Fig. 7C, D) and CXCR3$^+$ CD8$^+$ T cells (Fig. 7E). We did not observe many differences in the frequency of CXCR3$^+$ T cells between the groups. This could be due to an overall reduction in infiltrating T cells. Given the importance of STAT1 signaling in the generation of chemokines, particularly CXCL10 and CCL5,[33,34] decreased chemokine receptor–positive T cells may be due to STAT1 inhibition within macrophages (Figs. 2 and 4 and Supplementary Figs. S2 and S3). We also observed significant reductions in the number and frequency of IFN gamma–positive CD4$^+$ T cells (Fig. 7F) and a reduction in the number of perforin$^+$ CD8$^+$ T cells (Fig. 7G), demonstrating encapsulation of islets, especially with the addition of CTLA-4-Ig, can decrease T cell effector responses.

Transplantation of (PVPON/TA/CTLA-4-Ig)-encapsulated islets enhances Tregs and anergic T cells and decreases effector T cells

To determine whether Treg and anergic CD4$^+$ T cells were increased ex vivo in (PVPON/TA/CTLA-4-Ig)-encapsulated islets, we performed flow cytometry at 14 days posttransplant. (PVPON/TA/CTLA-4-Ig)-encapsulated islet grafts contained increased numbers of FoxP3$^+$ Tregs (Fig. 8A, B) and anergic CD4$^+$ T cells (Fig. 8C, D) compared with nonencapsulated and (PVPON/TA/IgG)-encapsulated controls. The analysis of effector and central memory T cell populations revealed a significant reduction in the number of CD44$^+$ CD62L$^-$ CD4$^+$ effector T cells within both encapsulated groups compared with those in nonencapsulated islets (Fig. 8E). CTLA-4-Ig addition also significantly decreased the number of CD44$^+$ CD62L$^+$ central memory CD4$^+$ T cells compared with that of nonencapsulated controls (Fig. 8F). Identical effects on CD8$^+$ effector and central memory (Fig. 8G, H) populations were observed. These data suggest the addition of CTLA-4-Ig to (PVPON/TA) layers delay islet allograft rejection partly because of significant increases in Treg and anergic T cell differentiation and a concomitant decrease in effector T cell responses.

Discussion

Islet transplantation represents a promising alternative to restore euglycemia and prevent damaging hypoglycemic episodes in patients with T1D[2]; however, rejection represents a significant clinical hurdle. One promising alternative is encapsulation of isolated islets in biocompatible polymers. We previously demonstrated that (PVPON/TA)

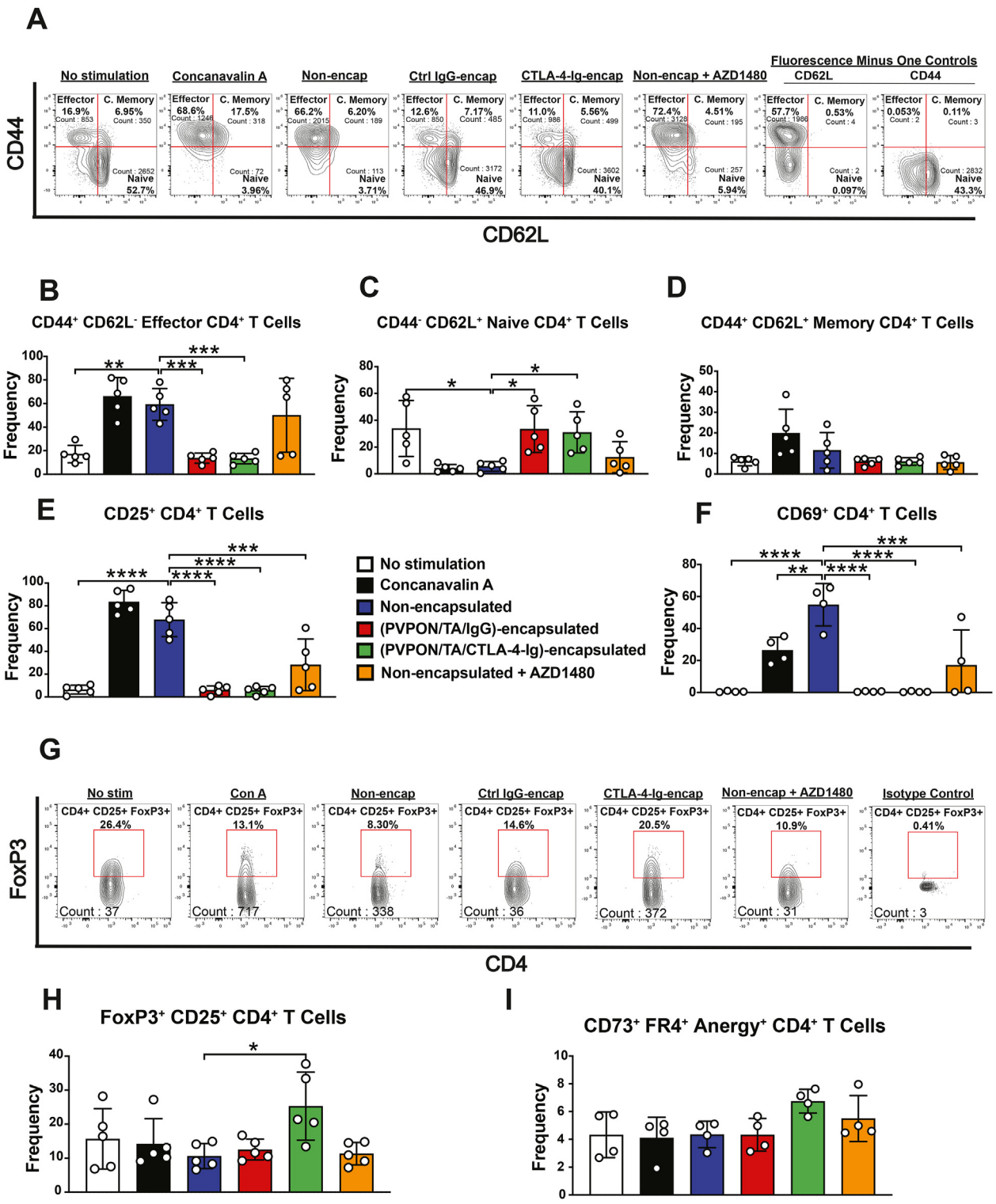

Figure 5. (PVPON/TA/CTLA-4-Ig) encapsulation suppresses $CD4^+$ T cell activation and enhances regulatory T cell populations in vitro. Allogeneic islet coculture assays were performed, and immune cells and supernatants were collected after 48 hours for flow cytometry. (A) Representative flow cytometry plot of $CD4^+$ T cells analyzed for expression of CD44 and CD62L. Quantification of $CD44^+$ $CD62L^-$ effector $CD4^+$ T cell (B), $CD44^-$ $CD62L^+$ naïve $CD4^+$ T cell (C), and $CD44^+$ $CD62L^+$ central memory $CD4^+$ T cell (D) frequencies (n = 5). Quantification of the frequency of $CD25^+$ (E) and $CD69^+$ (F) within $CD4^+$ T cell populations. Representative flow cytometry plots (G) and frequency of $CD4^+$ T cells (H) analyzed for expression of FoxP3. (I) Quantification of the frequency of $CD73^+$ $FR4^+$ expression within $CD4^+$ T cells (n = 4), analyzed by Kruskal-Wallis test with Dunn multiple comparisons. Data represents at least 4 independent experiments. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$. CTLA-4-Ig, cytotoxic T cell–associated antigen 4 immunoglobulin; PVPON, poly(*N*-vinylpyrrolidone); TA, tannic acid.

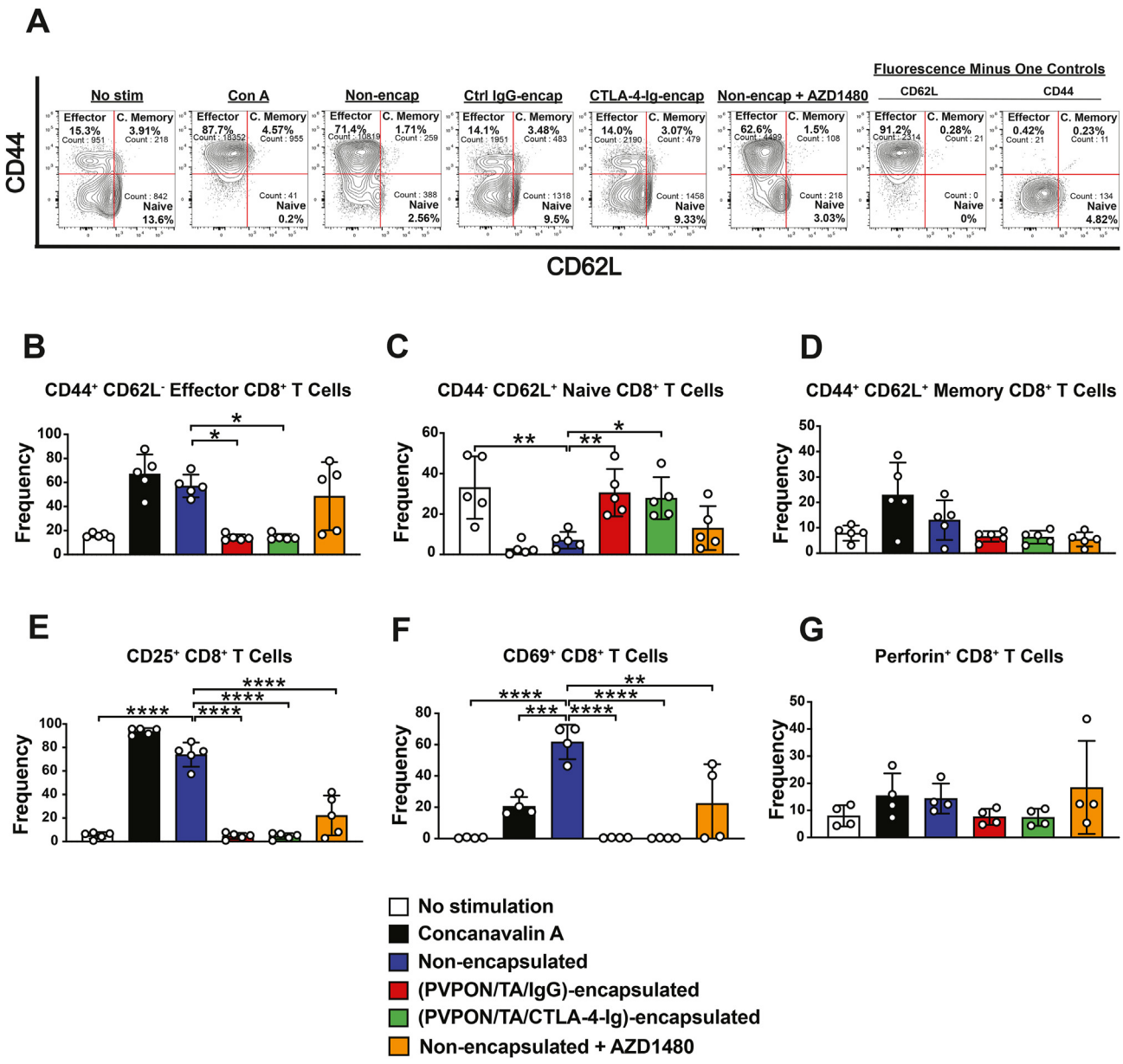

Figure 6. Encapsulated islets reduce CD8$^+$ T cell activation. Allogeneic islet coculture assays were performed, and immune cells were collected after 48 hours for the flow cytometry analysis. (A) Representative flow cytometry plot of CD8$^+$ T cells analyzed for expression of CD44 and CD62L. Quantification of CD44$^+$ CD62L$^-$ effector CD8$^+$ T cell (B), CD44$^-$ C62L$^+$ naïve CD8$^+$ T cell (C), and CD44$^+$ CD62L$^+$ central memory CD8$^+$ T cell (D) frequencies (n = 4-5), pregated on CD8$^+$ T cells. Quantification of the frequency of CD25$^+$ (E) CD69$^+$ (F), and perforin$^+$ (G) expression within CD8$^+$ T cell populations (n = 4-5). Analyzed by 2-way ANOVA with multiple comparisons or by Kruskal-Wallis test with Dunn multiple comparisons. Data represents at least 4 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. ANOVA, analysis of variance.

encapsulation of islets can skew macrophages toward an anti-inflammatory phenotype and delay immune rejection in NOD mice.[7,9,35,36] However, the specific cellular and molecular mechanism(s) of protection elicited by (PVPON/TA) encapsulation were unknown.

This study provides evidence (PVPON/TA) coatings dampen allogeneic immune responses partly owing to reducing STAT1 activation within F4/80$^+$ macrophages. A decrease in STAT1 signaling may explain enhanced anti-inflammatory M2 macrophages observed in (PVPON/TA)-encapsulated islet allografts.[7] The inhibition of STAT1 using AZD1480 was effective in delaying allograft rejection, highlighting the importance of this pathway in promoting immune rejection. Inhibition of STAT1 signaling can also influence T1D because AZD1480 treatment can delay T1D in NOD mice[27] and STAT1-deficient NOD mice are protected from T1D through reduced islet stress, nitric oxide, and CD4$^+$ T cell effector differentiation.[37] Because islet stress and apoptosis contribute to islet allograft loss,[38] the ability of (PVPON/TA) coatings to suppress local STAT1 activation may prolong graft survival.

Redox regulation of the STAT pathway involves mechanisms that activate and inhibit STAT-dependent signaling. A cysteine-based redox switch within JAK1 is inactivated by H_2O_2,[39] but H_2O_2 can bypass JAK1 to directly phosphorylate STAT1 through S-glutathionylation.[40] Increased H_2O_2 or NO can also activate STAT1 through effects on inhibitory protein tyrosine phosphatases (PTPs) and suppressors of cytokine signaling (SOCS) members[41] by inducing their degradation.[42,43] Given that (PVPON/TA)-encapsulated and (PVPON/TA/CTLA-4-Ig)-encapsulated islet cocultures demonstrated significant reductions in both p-STAT1 and nitrite, TA may suppress redox-dependent STAT1 signaling by direct or indirect effects on PTPs and SOCS. Future studies will examine whether (PVPON/TA/IgG) and (PVPON/TA/CTLA-4-Ig) coatings can modulate PTPs and/or SOCS to hinder STAT1 signaling.

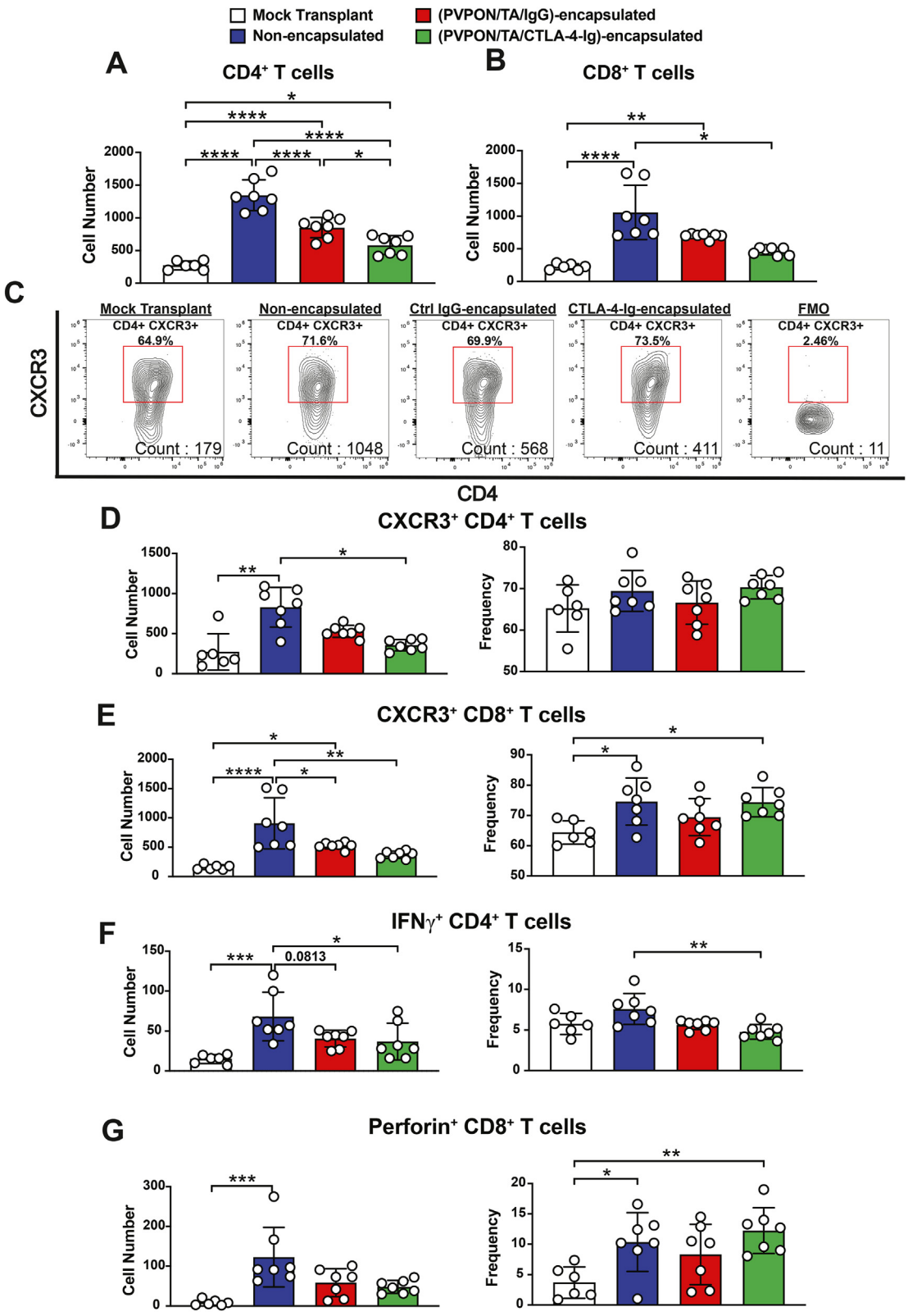

Figure 7. Encapsulated islets reduce T cell infiltration and effector responses. Islet allografts of NOD.*Rag* islets were collected 14 days posttransplant from STZ-treated C57BL/6 mice and analyzed by flow cytometry. Quantification of number of $CD4^+$ T cells (A) and $CD8^+$ T cells (B) (n = 6-7). Representative flow cytometry plots (C) and quantification of the number and frequency of $CD4^+$ (D) or $CD8^+$ T cells (E) expressing CXCR3 (n = 6-7). Quantification of the number and frequency of $CD4^+$ T cells expressing IFN gamma (F), and $CD8^+$ T cells expressing perforin (G) (n = 6-7), analyzed by 2-way ANOVA with multiple comparisons or by Kruskal-Wallis test with Dunn multiple comparisons. Data represents at least 4 independent experiments. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$. ANOVA, analysis of variance; IFN, interferon; STZ, streptozotocin.

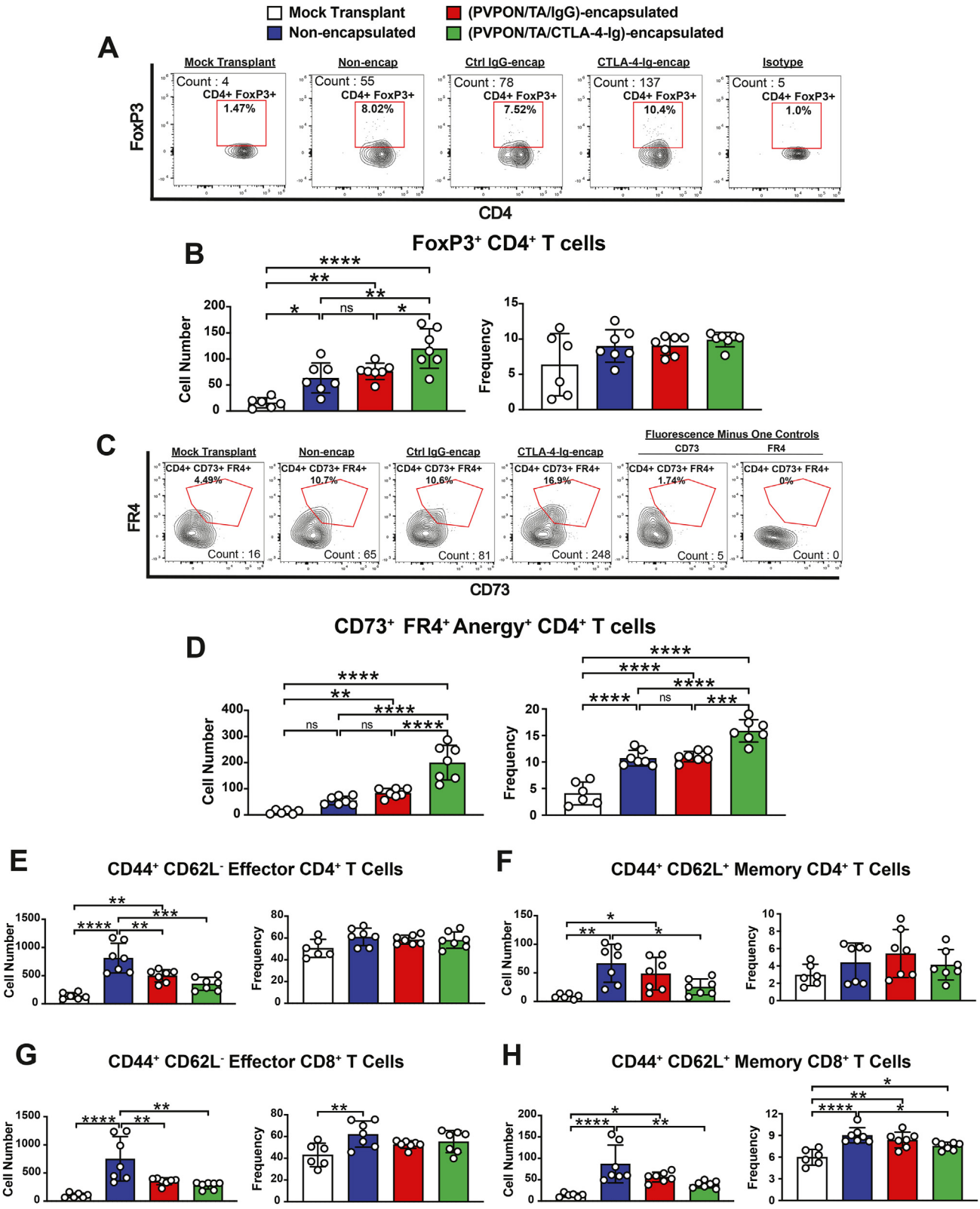

Figure 8. Transplantation of (PVPON/TA/CTLA-4-Ig)-encapsulated islets enhances Tregs, anergic T cells, and decreases effector T cells. Islet allografts of NOD.*Rag* islets were collected 14 days posttransplant from STZ-treated C57BL/6 mice and analyzed by flow cytometry. Representative flow cytometry plots (A) and quantification of the number and frequency of $CD4^+$ (B) T cells expressing FoxP3 (n = 6-7). Representative flow cytometry plots (C) and quantification of the number and frequency of anergic $CD4^+$ T cells (D) expressing CD73 and FR4 (n = 6-7). Within total $CD4^+$ or $CD8^+$ T cells, quantification of the number and frequency of $CD44^+$ $CD62L^-$ effector $CD4^+$ T cells (E), $CD44^+$ $CD62L^+$ central memory $CD4^+$ T cells (F), $CD44^+$ $CD62L^-$ effector $CD8^+$ T cells (G), and $CD44^+$ $CD62L^+$ central memory $CD8^+$ T cells (H) (n = 6-7), analyzed by 2-way ANOVA with multiple comparisons or by Kruskal-Wallis test with Dunn multiple comparisons. Data represents at least 4 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. ANOVA, analysis of variance; CTLA-4-Ig, cytotoxic T cell–associated antigen 4 immunoglobulin; ns, nonsignificant; PVPON, poly(*N*-vinylpyrrolidone); STZ, streptozotocin; TA, tannic acid.

Although (PVPON/TA) layers delayed islet graft rejection, only ~50% of encapsulated islet allograft recipients maintained long-term glycemic control.[7] To improve immunoprotection, we conjugated recombinant CTLA-4-Ig onto the outer surface of (PVPON/TA) layers. Transplantation of (PVPON/TA/CTLA-4-Ig)-encapsulated islet allografts maintained function significantly longer than nonencapsulated and (PVPON/TA/IgG)-encapsulated islets. The addition of CTLA-4-Ig induced an expansion of $CD25^+$ $FoxP3^+$ Tregs, anergic $CD4^+$ T cells, and enhanced IL-10 production.

Published studies suggest that lasting graft tolerance after transplantation requires both effector T cell anergy and functional Treg populations.[44] This can be accomplished by direct induction with regulatory DCs[45] or with CTLA-4-Ig. Although systemic administration of regulatory DCs[46] or CTLA-4-Ig[47] improved islet transplant outcomes, this approach may impair global T cell activation. Conjugating CTLA-4-Ig onto (PVPON/TA) coatings could allow for suppression of graft-specific responses without impairing systemic T cell responses. Our future studies will determine mechanisms of protection with (PVPON/TA/CTLA-4-Ig)-encapsulated islets including the enzyme, indoleamine 2,3-dioxygenase (IDO). Upregulation of IDO suppresses T cell responses through deprivation of tryptophan and Treg induction.[47] Systemic treatment with CTLA-4-Ig reportedly enhanced expression of this enzyme to delay islet transplant rejection.[47] In the future, we will investigate whether (PVPON/TA/CTLA-4-Ig) encapsulation increases IDO activity within APCs to induce Treg and anergic $CD4^+$ T cell expansion. In addition, it is unknown whether (PVPON/TA/CTLA-4-Ig) encapsulation materials have any effect on systemic immune function. However, we predict because CTLA-4-Ig is not given systemically and only present within our (PVPON/TA) coatings, we do not anticipate any adverse global immunosuppressive effects on polyclonal T cell responses. We will perform subsequent studies in recipient mice containing (PVPON/TA/CTLA-4-Ig)-encapsulated islets to further assess immune cell activation and function by performing a skin allograft or examine their ability to clear a nonlethal viral infection. Another long-standing issue regarding our (PVPON/TA/CTLA-4-Ig) coatings is their longevity in vivo with encapsulated islets. We have used numerous strategies to answer this question, such as standard immunohistochemical approaches and the use of fluorescent dyes for in vivo imaging, but these approaches have not allowed us to visualize the islet allografts long-term. Owing to the adaptability of our (PVPON/TA/CTLA-4-Ig) coatings, we will perform future studies to conjugate gold nanoparticles to visualize and confirm the integrity of these coatings by scanning electron microscopy.

(PVPON/TA/CTLA-4-Ig) encapsulation could improve the feasibility of using other sources of insulin-producing cells beyond deceased donor human islets. We demonstrated that (PVPON/TA)-encapsulated neonatal porcine islets (NPIs) suppressed xenogeneic proinflammatory innate immune responses but were unable to prevent xenograft rejection in NOD mice.[36] (PVPON/TA/CTLA-4-Ig) encapsulation of NPIs may induce anergic and Treg populations to improve xenograft survival. This approach could also protect stem cell-derived β cells from immune rejection, a potential unlimited source of insulin-producing cells for transplantation.[48] Regardless of the source of islets, (PVPON/TA/CTLA-4-Ig) encapsulation and the adaptability of these coatings to conjugate other immune inhibitors may delay immune-mediated rejection of transplanted β cells in patients with T1D.

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Author contributions

J.M.B. designed research studies, conducted experiments, acquired data, analyzed data, and wrote the manuscript. K.S.B. conducted experiments and acquired data. R.B. and C.A.F. designed research studies and wrote the manuscript. V.K. and E.K. designed research studies, conducted experiments, and wrote the manuscript. H.M.T. designed research studies, analyzed data, and wrote the manuscript. H.M.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Data availability

All data generated or analyzed during this study are included in the published article (and its online supplementary files). The (PVPON/TA) encapsulation and transplantation protocols for islets generated and analyzed during this study are available from the corresponding authors on reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2023.01.007.

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