IMMUNOLOGY

Immunotherapy via PD-L1–presenting biomaterials leads to long-term islet graft survival

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Antibody-mediated immune checkpoint blockade is a transformative immunotherapy for cancer. These same mechanisms can be repurposed for the control of destructive alloreactive immune responses in the transplantation setting. Here, we implement a synthetic biomaterial platform for the local delivery of a chimeric streptavidin/ligand delivery represents a more attractive approach for therapeutic use. PD-L1 can have potent effects in reinvigorating immunological responses to cancer cells (8, 9). The PD-1/PD-L1 axis plays a central role in regulating alloimmune responses in the transplantation setting. Experimental models of fully mismatched cardiac allografts have demonstrated a need for an intact PD-1/PD-L1 interaction (10), whereby blockade of PD-1 leads to exacerbated rejection times. Investigation into the functional significance of this pathway in less allogeneic models (single antigen mismatch) in PD-1 knockout mice, however, demonstrates that PD-L1 plays a critical role in the induction and maintenance of transplant tolerance through an additional binding partner B7-1 (11). Thus, although targeting either receptor or ligand can provide inhibitory signals to immune cells, PD-L1 ligand delivery represents a more attractive approach for therapeutic delivery because (i) it can regulate immune responses in both lymphoid and nonlymphoid organs (12), (ii) it has a unique role in promoting self-tolerance, and (iii) it can signal through other receptors, in particular, B7-1 [same binding family as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) receptor] which controls peripheral homeostasis of regulatory T (Treg) cells (13).

Within the context of T1D, the PD-1/PD-L1 axis is pivotal to autoimmunity, as seen by abnormalities in PD-L1 presentation in human patients with T1D (14) and disease halting in transgenic models expressing PD-L1 (15). Strategies aimed at using PD-L1 to control disease progression and diabetes reversal have ranged from genetically modifying islets to express PD-L1 (16) to improve graft survival in allogeneic transplantation models (17) to the delivery of antigen-presenting cells genetically or chemically modified to overexpress PD-L1 (18). However, these approaches face barriers to clinical translation or rely on clinically relevant pharmacological interventions that lack evidence of longevity, limiting the long-term effect of the therapeutic strategy.

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INTRODUCTION

Pharmacological inhibition of immune responses via chronic systemic immunosuppression is currently the major clinically accepted strategy to prevent rejection of grafts from donors with different genotype backgrounds (i.e., allografts) (1). Whereas major advancements have been made in improving postoperative immunosuppression regimens, the need for long-term administration of these pharmacological agents is still associated with serious side effects, including infections, malignancies, and cardiac and kidney toxicity (2, 3). One promising cell therapy that has suffered from the requisite of chronic immunosuppression is clinical islet transplantation. Transplantation of allogeneic pancreatic islets has the potential to restore insulin production, improve glycemic control, and reduce complications in subjects with type 1 diabetes (T1D) (4, 5). Despite major improvements in immunosuppression regimens, most transplant recipients lose insulin independence by ~3 years after transplantation (5). Moreover, in the context of the patient population most affected by this pathology (i.e., children and young adults), the need for chronic immunosuppression does not meet the clinical objective of benefit without major risk (6), rendering this therapy available only to a subset of patients with refractory hypoglycemia. Thus, novel therapeutic strategies that address the challenge of immune rejection in the absence of chronic immunosuppression are urgently needed.

Immune checkpoint blockade has emerged in the last decade as a powerful approach to control pathogenic immune responses. One of the most effective and durable immunotherapies currently in clinical use involves the programmed cell death-1 (PD-1) pathway (7). In oncology settings, blockade of the receptor PD-1 or its ligand PD-L1 can have potend effects in reinvigorating immunological responses to cancer cells (8, 9). The PD-1/PD-L1 axis plays a central role in regulating alloimmune responses in the transplantation setting. Experimental models of fully mismatched cardiac allografts have demonstrated a need for an intact PD-1/PD-L1 interaction (10), whereby blockade of PD-1 leads to exacerbated rejection times. Investigation into the functional significance of this pathway in less allogeneic models (single antigen mismatch) in PD-1 knockout mice, however, demonstrates that PD-L1 plays a critical role in the induction and maintenance of transplant tolerance through an additional binding partner B7-1 (11). Thus, although targeting either receptor or ligand can provide inhibitory signals to immune cells, PD-L1 ligand delivery represents a more attractive approach for therapeutic delivery because (i) it can regulate immune responses in both lymphoid and nonlymphoid organs (12), (ii) it has a unique role in promoting self-tolerance, and (iii) it can signal through other receptors, in particular, B7-1 [same binding family as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) receptor] which controls peripheral homeostasis of regulatory T (Treg) cells (13).

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Delivery of systemic forms of checkpoint inhibitors, such as PD-L1, can improve transplant outcomes (10, 11, 17). However, systemic
delivery of these proteins has been associated with nonspecific immune responses and high rates of immune-related toxicity (7). Here, we sought to implement our previously described synthetic microgel platform (19) to positionally display streptavidin/PD-L1 (SA-PD-L1) on the surface of biotinylated poly(ethylene glycol) (PEG) microgels to regulate alloimmune responses to pancreatic islets within the graft microenvironment. We establish that a biomaterial approach for the controlled delivery of PD-L1 within the graft, as a localized means of immune regulation, can achieve sustained survival of allogeneic islet grafts. Analyses of the immune engineered graft demonstrated an increase in the regulatory cell population over time, followed by an increase in the CD4+ T anergic population over time, followed by an increase in the CD4+ T anergic population over time, followed by an increase in the CD4+ T anergic population over time, followed by an increase in the CD4+ T anergic cell population, which allowed long-term survival of islet allografts in the absence of chronic systemic immunosuppression.

RESULTS

Biotinylated synthetic microgels efficiently capture SA-PD-L1

To capture and present SA-PD-L1, we conjugated a PEG-biotin linker to a four-armed PEG macromer with maleimide groups at each terminus (PEG-4MAL). The biotinylated PEG-4MAL was rendered into droplets and cross-linked into hydrogel spheres (microgels) with a nondegradable linker using a microfluidic device, as previously described (19). Microgels (195 ± 11.8 μm in diameter) exhibited homogeneous biotin presentation as seen by confocal imaging (Fig. 1A, left). We focused on generating microgels with similar size as transplanted islets (150 to 200 μm in diameter) to facilitate mixing with islets and ensure appropriate surface densities of PD-L1. Orthogonal projections of SA-PD-L1 immobilized onto the biotinylated microgels demonstrated uniform presentation of the ligand on the surface of the microgels (Fig. 1A, right). Furthermore, quantification of surface-bound SA–Alexa Fluor 488 (AF488) intensity demonstrated tight control over the density of captured SA versus microgel size (Fig. 1B). These results demonstrate control of microgel size, conjugation of biotin to microgels, and surface presentation of SA-PD-L1 enabled by the PEG-4MAL platform.

We next characterized the binding affinity of SA-PD-L1 to biotin via a biotin–4-fluorescein (B4F) competitive binding assay (Fig. 1C), which can detect SA concentrations in the pM range (20). By quenching B4F intensity in a titration assay with different concentrations of (i) commercial SA, (ii) recombinant SA without PD-L1 (SA), and (iii) the full chimeric SA-PD-L1, we estimated their dissociation constants (Kd) using a one-step binding reaction model (Fig. 1C) (20). Quenching of the fluorescent signal is maximal when all four binding sites of the SA molecule are occupied (4:1 ratio of B4F to SA). Thus, when the fluorescence intensity of the sample is half of the unbound B4F, Kd can be estimated as

$$K_d = [S]_{\text{total}} - [B4F]^2$$

where [B4F]o is the concentration of total B4F and [S]total is the total protein monomer concentration. The Kd values for all proteins, estimated from nonlinear regression of binding curves, ranged from 2.2 × 10^-10 to 5.4 × 10^-10 M. This is 10^4-fold higher than the reported Kd of SA for biotin (∼10^-14 M) (20). This reduced binding affinity has been observed in other SA variants and could be attributed to the fluorescein group position over the biotin-binding site (20). Notably, the Kd for SA-PD-L1 is equivalent to that of the SA control.

Fig. 1. Microfluidic flow–generated biotinylated microgels allow controllable capturing of SA-modified proteins. (A) Transmission and orthogonal projection images of biotinylated microgels and SA-PD-L1, respectively; microgels were incubated in SA—AF488 showing binding to microgels (green), SA-PD-L1 was immobilized on the biotinylated microgels and stained with anti–PD-L1 antibody followed by secondary antibody staining. Scale bars, 200 μm. (B) Microgels have an average size of 195 μm, CV (coefficient of variation) of ~6%, and homogeneous biotinylation as seen by labeling with SA—AF488. (C) Fluorescence intensity values and binding curve fits (lines) for biotin–4-fluorescein (B4F) competition assay showing equivalent binding behavior among SA-PD-L1 (blue), SA (red), and commercial SA (black). (D) and (E) Microgels can efficiently capture SA-PD-L1 as seen by fluorescent intensity measurements of SA-PD-L1 labeled with AF488. MFI, mean fluorescence intensity; a.u., arbitrary units.
FoxP3 expression in naïve CD4+CD25− cells and for the development β IL-2 is required for TGF−β−dependent induction of CD25 and βCD4+CD25+ FoxP3+ induced T regs (iT regs) by flow cytometric analysis of the characteristic immunosuppressive properties of CD4+CD25+ Tregs. (23). Thus, the synergistic effects of IL-2 and TGF-β are needed for naïve CD4+CD25− T cells to develop the phenotype of CD4+CD25+ Tregs, in vitro, and this group was used as a positive control. The addition of TGF-β (2 ng/ml) alone led to the development of Tregs in vitro compared to IL-2 controls (19.8% ± 2.7 versus 5.0% ± 0.4; P < 0.005). The effect of TGF-β was enhanced when IL-2 was added to the coculture (36.3 ± 3.8 versus 19.8% ± 2.7; P < 0.0018). Notably, soluble SA-PD-L1−IL-2 alone induced T cell conversion into FoxP3+ Tregs to levels comparable to the TGF-β positive control (19.8% versus 26.4%, P = 0.26). The addition of TGF-β to the soluble SA-PD-L1 + IL-2 coculture did not further augment Treg cell conversion (26% versus 24%, P = 0.93). Induction of Tregs by PD-L1 is mediated by antagonizing the Akt signaling pathway. A decrease in phosphorylation of Akt, mTOR, and S6 has been observed in CD4 naïve cells with increasing quantities of PD-L1−presenting beads (21).

To assess whether SA-PD-L1 retains its immunosuppressive role in addition to its capacity to induced Tregs, we cocultured splenocytes from 4C mice [C57BL/6 host transgenic engineered to express a T cell receptor (TCR) recognizing BALB/c major histocompatibility complex (MHC) I-Ad] with irradiated BALB/c splenocytes in a mixed lymphocyte reaction (MLR) (fig. S2B). Activation of splenocytes, measured by 3H-thymidine incorporation, was 10-fold higher compared to unstimulated 4C only controls. In contrast, the presence of soluble SA-PD-L1 in the culture led to a 57% reduction in the proliferation of activated splenocytes when compared to controls (P < 0.0014). Collectively, these results demonstrate that SA-PD-L1 is capable of efficiently inducing Tregs in vitro and suppressing immune activation.

**SA-PD-L1-microgels do not alter pancreatic islet cell function**

β cells in the pancreas express PD-L1 as a defense mechanism during the insulin process (24). Therefore, the presence of microgels displaying PD-L1 could potentially lead to the activation of metabolic stress pathways in β cells. To investigate the impact of SA-PD-L1 exposure on β cell health and function, we cultured islets (i) free or (ii) mixed with SA-PD-L1−presenting microgels for 48 hours. No difference was observed in mitochondrial metabolic activity, glucose-stimulated insulin release (GSIR), viability, or expression of cytokines involved in stress activation of apoptotic pathways (Fig. 2, A to D) (25). Furthermore, immunohistochemical staining demonstrated insulin- and glucagon-positive cells in both the free and coculture groups (fig. S3A).

We next transplanted streptozotocin (STZ)−induced diabetic mice (n = 6) with a dose of syngeneic islets and microgels [600 islet equivalent (IEQ), 1 IEQ: 2 microgels] in a single epididymal fat pad (EFP) to assess whether the biomaterials interfere with islet engraftment. The microgel platform was tested in the EFP site as opposed to the kidney capsule, for a more clinically relevant transplant site (26). Nonfasting blood glucose levels demonstrated equivalent reversal rates between controls (n = 3) and islets transplanted with SA-PD-L1-microgels [mean survival time (MST), 3.0 ± 0.6 versus 4.5 ± 2.3; P = 0.44; Fig. 2E]. Furthermore, removal of the EFP containing the graft in a cohort of SA-PD-L1-microgel−transplanted animals led to hyperglycemia, demonstrating that diabetes reversal was due to islet transplantation (Fig. 2E, graft removal). The potency of the transplanted mass was comparable to that of age-matched naïve animals, as seen by no statistical difference in the area under the curve for glucose clearance between the two groups (973 ± 402 versus 1380 ± 264 µg/liter-min, P = 0.14) for an intraperitoneal blood glucose tolerance test (IPGTT) (Fig. 2F, inset). Immunostaining of grafts after engraftment showed an intact islet mass with blood vessel infiltration (fig. S3, B and C). Notably, varying degrees of mononuclear cell accumulation were observed around the microgels, but no fibrotic lesions were detected (fig. S3B). Furthermore, transplanted islets stained positive for insulin and glucagon (Fig. 2G).

**Microgels enhance SA-PD-L1 retention in vivo**

A major challenge in protein delivery is the relatively short half-life encountered in vivo, leading to unreliable efficacy or the requirement of frequent dosing, which exacerbates costs and can have clinically undesirable outcomes. We previously showed that immunomodulatory protein delivery via microgels improves retention of SA-FasL, an apoptotic modulator, in the kidney capsule (19). Although the biomaterial platform is similar, various features of the SA-PD-L1 protein, such as the sequence, molecular weight, and three-dimensional structure, are different from SA-FasL, which can affect its bioavailability and turnover kinetics. We conjugated a near-infrared fluorescent dye to SA-PD-L1 and immobilized the labeled protein on the surface of biotin−presenting microgels. These microgels were then transplanted in the EFP of nondiabetic BALB/c mice. Albino mice were used to avoid the high attenuations of the fluorescent signal observed with
highly pigmented skin backgrounds, such as C57BL/6 mice (27).
Free protein and protein immobilized on the surface of microgels were delivered to the EFPs of mice via a degradable PEG hydrogel containing vascular endothelial growth factor (VEGF) (28), creating two defined pockets of fluorescence signal as observed clearly at day 0 time point (Fig. 3A).

In vivo fluorescence signal was measured at predetermined time points via an in vivo imaging system (IVIS). The data of signal intensity for the free protein and SA-PD-L1-microgel were fitted to a single exponential decay fit curve (Fig. 3B, solid lines). By day 1, the signal in the free protein group had decreased 63% compared to 44% in the microgel group \((P < 0.0001)\). Furthermore, by day 10, fluorescence readings for the free protein group were reduced to background levels, whereas \(~30\%\) of the original signal was still present for the SA-PD-L1-microgel group. Calculated half-lives from single exponential decay fit showed no difference between the
SA-PD-L1-microgel group compared to free protein controls (0.77 versus 0.65 days, \( P = 0.58 \); Fig. 3B). However, area under the curve measurement demonstrated improved retention for immobilized SA-PD-L1 compared to free protein (7.5 ± 0.33 versus 3.4 ± 0.22, \( P < 0.0001 \)). Notably, fluorescence readings for the SA-PD-L1-microgel group remained stable from day 10 to 21 (\( P = 0.65 \)). This result was confirmed by performing readings on EFPs after explantation (day 25), which demonstrated comparable values to those obtained right before EFP removal (\( P = 0.15 \)). Immobilized SA-PD-L1 is retained at the transplant site by its presentation on the surface of the microgels and not by the PEG sealant gel used to keep microgels/islets on-site, as free protein delivered with a similar PEG sealant is quickly cleared from the site. The mechanism by which SA-PD-L1 is cleared from the site remains unknown but likely involves unbinding of the SA-PD-L1 from the biotinylated microgels due to endogenous biotin and/or degradation of the SA-PD-L1 protein but not degradation of the microgels, as these are present at the transplant site >100 days after transplantation (fig. S5). Overall, these data establish that the immobilization of the SA-PD-L1 protein on the surface of the microgel platform leads to improved retention of the immunomodulatory ligand in the transplant microenvironment.

**Long-term allogeneic graft function achieved via localized presentation of SA-PD-L1**

To assess whether the presentation of SA-PD-L1 on microgels protects islet allografts from immune rejection, we transplanted 1200 IEQ BALB/c islets mixed with microgels (1 IEQ: 2 microgels, 600 IEQ per EFP) into both EFPs of diabetic allogeneic C57BL/6 recipients (Fig. 4A). A subset of animals received a short, low-dose course of rapamycin (RAPA; 0.2 mg/kg daily for 15 doses starting the day of transplantation) to aid in alleviating the immune pressure on the graft during the early posttransplantation period. All graft recipients achieved normoglycemia within 3 days after transplantation (Fig. 4B and fig. S4, A to H). Control animals receiving microgels without protein and no immunosuppression (control) had a MST of 13 ± 3 days after transplantation (Fig. 4C and fig. S4A, green line). Animals that received free SA-PD-L1 + RAPA (delivered locally to the graft during transplantation) had a MST of 20 ± 5 days after transplantation (Fig. 4C and fig. S4C, magenta line). Subjects receiving SA-PD-L1-presenting microgels only (no RAPA) had a MST of 26 ± 7, with 22% (2 of 9) of the animals remaining normoglycemic for >50 days (Fig. 4C and fig. S4D, blue line). However, this protective effect was not durable as these animals rejected after 2 months after transplantation. The large majority (12 of 14) of the animals that received control microgels under the umbrella of low-dose RAPA regimen (control + RAPA) rejected by day 19. Two (of 14) control + RAPA animals did not reject the allograft within the observation window (>90 days); although graft function was confirmed by the return to hyperglycemia upon graft removal and total pancreatectomy insulin levels (Fig. 4C and fig. S4, B and I, black line). Whereas the dose of RAPA administered in this study is considerably lower than reported dosages for full allograft protection (29), long-term graft function attributed to short-term RAPA administration is not unusual and has been reported in a subset of animals (29, 30). Nevertheless, graft rejection in >85% of the animals in the control + RAPA cohort demonstrates that the dose of RAPA administered herein did not facilitate long-term function. Furthermore, to demonstrate that the SA used to capture and present SA-PD-L1 on microgels does not offer immune protection, we transplanted animals with recombinant SA construct (without the PD-L1 portion) immobilized on the surface of biotinylated microgels under the umbrella of RAPA (Fig. 4C and fig. S4E, orange line). This cohort of animals rejected the graft with survival time (28 days ±17) comparable to control microgels + RAPA (\( P = 0.3 \)). In addition, 6 of 7 SA-microgel + RAPA animals transplanted rejected the graft within 50 days after transplant, one animal that had not rejected the graft by day 52 was subjected to a survival graft removal, which demonstrated a prompt return to hyperglycemic levels. Furthermore, total insulin content of all SA samples analyzed was significantly lower than that of naive nondiabetic animal and comparable to STZ controls (\( P < 0.001 \); fig. S4I). Thus, this censored observation was treated as having not rejected the graft within the time window analyzed (100 days).

In contrast to all these groups, significantly delayed rejection times were observed in the SA-PD-L1-microgels + RAPA cohort (SA-PD-L1-microgel + RAPA), with ~60% (7 of 12) recipients exhibiting long-term (>100 days) function compared to the control + RAPA group (\( P = 0.0038 \); Fig. 4, B and C, and fig. S4F, red line). Of the SA-PD-L1-microgel + RAPA grafts that failed, 80% (4 of 5) did so at later time points compared to controls + RAPA (MST, 45 ± 12 versus 19 ± 17 days; \( P = 0.015 \)). Notably, presentation of SA-PD-L1 on microgels was required for long-term graft function as delivery of free SA-PD-L1 (Fig. 4C, purple line) did not improve graft retention over controls. Assessment of graft function using IPGTT at 30 days after transplantation demonstrated equivalent glucose clearing responses in the SA-PD-L1-microgel + RAPA cohort compared to age-matched naive animals (6900 ± 3952 versus 10,000 ± 2345, \( P = 0.63 \) μg/liter-min; Fig. 4D). Control animals under the same RAPA protocol (control + RAPA) had significantly delayed kinetics, with 4 of 5 control animals unable to reach normoglycemia within 120 min after bolus injection (19,150 ± 9386 versus 6920 ± 3953 μg/liter-min, \( P = 0.01 \)). Immunohistological assessment...
of the graft demonstrated insulin staining in animals receiving SA-PD-L1-microgel + RAPA (Fig. 4E), whereas no intact insulin-positive clusters were found in the control + RAPA group (fig. S5A). Evaluation of the immunological response by immunostaining demonstrated the presence of cells staining positive for FoxP3 and CD3 in the SA-PD-L1-microgel + RAPA group but negative staining in grafts which received control + RAPA (Fig. 4F and see isotype control staining in fig. S5A). An ex vivo MLR test to examine recipient’s systemic response to self (C57/B6) and BALB/c antigens demonstrated similar activation profiles in CD4+ and CD8+ T cells from animals that received control, SA-, or SA-PD-L1-microgels under the umbrella of RAPA. Furthermore, this response was comparable to naïve and control diabetic animals (fig. S4, J and K). These results demonstrate that delivery of SA-PD-L1-presenting microgels + RAPA favorably modulates immunological responses to allogeneic islet transplants and that, in combination with a short RAPA intervention, this treatment significantly improves long-term graft survival and function without eliciting systemic immunosuppression.

Host tissue responses to the implanted microgels were assessed by histological staining at different transplantation time points (day 30 after transplantation for control + RAPA group, >100 days after transplantation, SA-PD-L1-microgels + RAPA). No gross differences in cellular infiltration and collagen matrix deposition were observed between control microgels and SA-PD-L1-presenting microgels (fig. S5B), suggesting that the foreign body response to the material was not modulated by SA-PD-L1. Furthermore, no differences in foreign body responses were observed between the SA-PD-L1-microgel groups with or without RAPA. Together, with the lack of any systemic side effects from RAPA administration (no significant weight loss was observed), this evidence suggests that the differences in rejection rates in our study cannot be attributed to RAPA effects on foreign body response. In addition, heavy cellular infiltration was evident in rejected grafts in mice treated with SA-PD-L1-microgels + RAPA (fig. S5C, left) compared to minimal cell infiltration in functional grafts in this cohort (fig. S5C, right).

Implantation of SA-PD-L1–presenting microgels in mice did not elicit acute toxicity as determined by normal histopathological
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analysis of the liver, kidney, lungs, and spleen tissues and normal levels of serum transaminases at >50 days after transplantation (fig. S6, A and B). SA has been used for therapeutic protein delivery both in preclinical (31) and clinical studies (32) without significant adverse effects. Furthermore, the SA–PD-L1 synthetic gene encodes for the extracellular domain of mouse PD-L1 (16), mitigating concerns of immunogenicity due to interspecies differences. Nevertheless, the delivery of bacterial proteins can lead to antigenicity and subsequently reduced bioavailability. Dot blots to test the immunogenicity of the recombinant proteins were performed on sera of transplanted animals obtained before exposure and at 3 and 12 weeks after transplantation (fig. S6, D to I). Approximately, 15% (3 of 20) naïve mice tested positive for antibodies against commercial SA, 10% (2 of 20) positive for antibodies against recombinant SA, and 1 of 20 (5%) positive for antibodies against SA–PD-L1 antibodies before transplantation. At 3 weeks after transplantation, there was no difference between the control group (biotinylated microgels) and SA–PD-L1–presenting microgel group for the percentage of animals positive for antibodies against commercial SA (P = 0.41) or recombinant SA (P > 0.99). However, 60% (6 of 10) of animals receiving SA–PD-L1-microgels tested positive for antibodies against the chimeric protein at 3 weeks after transplantation compared to 11% (1 of 9) in the control group (P = 0.03). Antibody testing on these same sets of animals at 12 weeks after transplantation showed no reactivity against SA–PD-L1 (fig. S6, H and I). Although these results show an antigenic response to SA–PD-L1, the generated antibodies are transient and do not appear to affect the immunomodulatory efficacy of SA–PD-L1–presenting microgels in sustaining long-term survival of allogeneic islet grafts. SA–PD-L1–presenting microgels promote a tolerogenic microenvironment

Gene expression analysis of graft tissue containing allogeneic islets cotransplanted with SA–PD-L1–presenting microgels was performed to evaluate the effect of delivery of this immunomodulatory biomat erial on the local immune environment after transplantation. A marginal mass (400 IEQ) of allogeneic islets was cotransplanted with either SA–PD-L1–presenting or control-microgels in the EFP of nondiabetic mice (Fig. 5A). At 7 days post-transplantation, graft tissue was processed and evaluated using a gene array for adaptive immune function. Gene profiling identified enrichment in the expression of Ccr4, Cdk2, Egr3, Foxp3, Jun, Pdc–l, and Prf1 in the SA–PD-L1-microgel group compared to the group that received control-microgels (Fig. 5B). These transcriptional regulators play important roles in T cell homeostasis by controlling proliferation, anergy, and immunosuppressive capacity of tolerogenic T cell subsets (33, 34). High levels of expression were observed for Foxp3 (>6 fold) and Pdc–l (the gene encoding PD–1) (>3 fold) in the SA–PD-L1-microgel group compared to control. CD4+Foxp3+ cells expressing high levels of PD-1 have been shown to have higher proliferative and immunosuppressive capacities than those with lower expression (35). In addition, expression of regulators of adaptive immunity, such as Egr3 (36), and critical chemokines that control effector Treg function (37) and dendritic cell (DC)–Treg (38) interactions, such as Ccr4, demonstrate a potent effect of local delivery of SA–PD-L1–presenting microgels on recruiting and controlling immunosuppressive phenotypes in the local graft microenvironment.

To characterize the immune fingerprint of cells at the graft site in response to the SA–PD-L1–presenting microgels, islets (1200 IEQ) were codelivered with either SA–PD-L1–presenting microgels + RAPA or control-microgels + RAPA into diabetic C57BL/6 mice (Fig. 5A), as in our functional studies. At 3, 14, and >100 days after transplantation, the graft tissue was recovered and the stromal vascular fraction was isolated. Flow cytometry analysis was performed for lymphocyte and myeloid populations, and the cytometry data analysis tool (CATALYST) pipeline was used for high-dimensional visualization and analysis (39). For the lymphocyte population, samples were preprocessed using standard gating, and only data for singlets, live, and CD45+CD3+ population were used for analysis in CATALYST. To identify the main immune cell clusters based on the expression of lymphocyte markers (CD4, CD8, CD25, Foxp3, CD44, CD62L, Tim3, Lag3, KLRG-1, and PD-1), we implemented unsupervised flow clustering (FlowSOM) analysis from all samples (fig. S8). t-distributed stochastic neighbor embedding (tSNE) projections demonstrate several clusters segregated based on the expression of a combination of the aforementioned antigens for pooled data from naïve (n = 4), control-microgel (n = 3), and SA–PD-L1-microgel (n = 4) groups at day 3 (Fig. 5C). By manually merging these clusters, we identified eight major cell populations based on their expression of CD4 and CD8 and further broken down based on their expression of effector (CD44hiCD62Llo), central memory (CD44hiCD62Lhi), Treg (CD25+Foxp3), and exhaustion/nergy (Tim3, Lag3, KLRG-1, or PD-1) markers. Cells expressing neither CD4 nor CD8 were classified as other lymphoid cells. Distribution of these manually gated clusters shows a CD4-dominated response to microgel implantation biomat erial (control-microgels and SA–PD-L1-microgels) compared to naïve mice (Fig. 5D). Similarly, high-dimensional population analysis was performed at day 14 after transplantation for pooled data from naïve (n = 2), control-microgels (n = 4), and SA–PD-L1-microgels (n = 4). Comparable clustering was observed at this time point, with an elevated CD4 cell population in the grafts (Fig. 5, E and F).

In depth analysis of the infiltrating CD45+CD3+ cells revealed a sharp increase in the presence of CD4+ and CD8+ cells in transplanted mice compared to age-matched naïve animals (Fig. 5G). By day 14, however, there was a statistically significant decrease in the ratio of CD4:CD8 cells in the microgel-control group (P = 0.025; Fig. 5, G and H), whereas the numbers and ratio of CD4:CD8 remained elevated in the SA–PD-L1-microgel group (P = 0.11). Further inves tigation into individual cell populations revealed an increase in CD4 Treg (defined as CD45R0CD4+CD25+Foxp3+) in the SA–PD-L1-microgel group from day 3 to day 14 (P = 0.045; Fig. 5I), whereas this cell population remained low and constant in the microgel-control group (P = 0.59). The cell count for CD8+ cells expressing Foxp3 increased over time for both control-microgels + RAPA (P = 0.014) and SA–PD-L1–presenting microgels + RAPA (P = 0.004) (Fig. 5I). Concomitant with the CD4:CD8 ratio data, we found an increase in the total cell count of two clusters containing CD4+ cells expressing exhaustion/nergy markers, CD4+CD44+CD25+PD-1+Tim3+Lag3 (P = 0.032; Fig. 5K) and CD4+PD-1+Lag3+ (P = 0.048; Fig. 5L) in the SA–PD-L1-microgel group compared to control-microgel. tSNE visualization of a small subset of nonrejecting animals >100 days (n = 2 per group; fig. S7) for both control-microgels + RAPA and SA–PD-L1–presenting microgels + RAPA suggests different distributions in the CD4 and CD8 compartment of SA–PD-L1-microgel group (fig. S7A). Although no statistical analysis can be performed at this time point, the distribution of cells expressing regulatory markers follows similar patterns as those observed at earlier time points (fig. S7, B and C). Overall, the increase
in the presence of CD45^+CD3^-CD4^+ cells expressing exhaustion/anergic markers as well as the increase in regulatory cells over time in the SA-PD-L1–presenting microgels + RAPA grafts suggests that SA-PD-L1 delivered via the microgel platform modulates the local graft microenvironment by promoting a tolerogenic immune signature that results in improved graft protection.

To examine whether delivery of SA-PD-L1–presenting microgels modulates innate immune cells, we evaluated the myeloid compartment of infiltrating cells to the graft site. We first manually gated flow data on singlets, live cells, and CD45^+ cells. This specific cell population was subsequently analyzed using CATALYST. From the CD45^+ population, we used FlowSOM cluster identification to exclude CD11b^+CD11c^- cells (nonmyeloid), CD11b^+Ly6G^+ cells (granulocytes), and NK1.1^+ (natural killer cells) from the CD45^+ population. The resulting CD11b^+Ly6G^- and CD11c^- populations were analyzed via FlowSOM enabling us to identify the main mononuclear phagocyte...
populations based on the individual marker expression for Ly6C, MHC-II, CD11c, F4/80, CD86, CD206, and ICOS (inducible T cell co-stimulator), and these were visualized using tSNE (Fig. 6A and fig. S9). The FlowSOM-identified clusters were manually merged and annotated as DCs (Ly6C−MHCII+F4/80−CD11c+), macrophages (Ly6C−MHCII−F4/80+), monocytes (Ly6C−MHCII+/−F4/80−), a distinct myeloid population with a surface profile of CD11b+F4/80−CD11c+CD206hiCD86−MHCII+ (which has previously been referred to as scaffold associated macrophages (SAMs) (40), and other CD11b+ cells that express no identifiable pattern of markers based on the current panel. Distribution of the mononuclear phagocyte populations analyzed revealed a primarily monocyte/macrophage driven response at day 3 (Fig. 6B). Applying high-dimensional analysis and clustering algorithm to datasets obtained at day 14 revealed a similar monocyte/macrophage-driven response (Fig. 6, C and D).

Analysis of cell counts in the myeloid response revealed sustained counts of CD45+CD11b+ cells in the graft that did not change between days 3 and 14 for the SA-PD-L1-microgel group (P = 0.59; Fig. 6E), whereas this cell population decreased from day 3 to day 14 in the control-microgel group (P = 0.04). Elevated counts of F4/80+ cells were observed in the SA-PD-L1-microgel group between days 3 and 14 (P = 0.015; Fig. 6F), whereas this cell population remained low in the control-microgel group (P = 0.28). As the M1/M2 macrophage axis can influence tolerance induction, we further characterized the CD45+CD11b+F4/80+ population for their expression of CD86− (associated with an M1 phenotype) and CD206+ (associated with an M2 phenotype). At day 3, we observed no expression of CD86 or CD206 for F4/80+ cells in the graft, but by day 14, we observed an increase in the F4/80−CD206− cell population in the graft; however, no differences between the control-microgel and SA-PD-L1-microgel groups were detected (Fig. 6G). A population identified as SAMs was also present within the transplant microenvironment; however, no differences were observed at the time points evaluated (Fig. 6H).

DISCUSSION

Despite strong evidence supporting checkpoint inhibitors as targeted therapeutics to abrogate transplant rejection, its clinical implementation...
is challenging as the delivery of potent immunosuppressive agents can dampen or reactive immune responses causing life-threatening side effects (41). In this study, we sought to circumvent the toxicity of systemic immunotherapy delivery by implementing previously described synthetic microgels for the immobilization and targeted local delivery of PD-L1. This microgel platform has previously been used for controlled surface protein presentation of FasL (19). While FasL is a potent homeostatic mediator of activation-induced cell death (33), its potential therapeutic translation may be limited by the broad effects of FasL as an immunomodulator of inflammation (34) and, possibly, activation of innate immune responses (35). PD-L1 delivery represents a more targeted approach for mediating immunoprotection of islet allografts in autoimmune diabetes. Here, we delivered biomaterial-localized PD-L1 as an off-the-shelf immunomodulation strategy to prevent graft rejection and present evidence of an immunoprotective local response to the implanted synthetic immunotherapy.

Core SA–containing chimeric proteins take advantage of the high affinity of the SA-biotin system to capture and present the immunomodulatory protein on biomaterials with high bioactivity. The use of biotinylated microgels for controlled surface PD-L1 presentation at the transplantation site in the EFP improved retention times (~20 days after transplantation) compared to those previously observed (~6 days) for SA-FasL microgel under the kidney capsule (19). This difference in retention time could be due to different clearance rates of the EFP versus the kidney capsule or the implementation of a sealant degradable hydrogel in the EFP model, which has been shown to degrade within 2 to 4 weeks after transplantation when applied in vivo (28, 36). Likewise, changes in SA binding affinity between the PD-L1 and FasL chimeric proteins may affect its release from our biotinylated synthetic platform. Further studies are necessary to determine how different transplant sites influence the kinetic of clearance of ligand proteins.

The potential of the PD-L1 pathway to achieve long-term transplant tolerance was investigated by codelivering SA-PD-L1–presenting microgels with pancreatic islets in an allograft islet transplant model. The addition of a low-dose RAPA regimen for the first 2 weeks after transplantation together with SA-PD-L1–presenting microgels improved long-term engraftment, with ~60% of the animals remaining normoglycemic for 100 days. These results are consistent with previous studies in which systemic PD-L1 protein delivery alone did not prevent fully MHC mismatched heart allograft rejection, but the addition of an immunosuppressant (i.e., cyclosporine A or RAPA) substantially enhanced survival compared to free protein or immunosuppressant monotherapy alone (37). Notably, the functional outcomes presented here were achieved with a single dose of the immunomodulating ligand via the synthetic biomaterial during the transplantation procedure. This resulted in a protein dose reduction of 800-fold compared to concentrations implemented in similar studies using systemic PD-L1 proteins to achieve islet transplant tolerance (38). Thus, the synthetic biomaterial approach not only provides an off-the-shelf approach that can work with multiple ligands (i.e., SA-FasL, and SA-PD-L1) but also may reduce off-target effects by significantly reducing protein requirements.

Consistent with the role of PD-L1 as a master regulator of T effector function (39) and induction of Tregs (21, 22), delivery of SA-PD-L1–presenting microgels led to an increase in the expression of genes encoding regulatory factors such as FoxP3, Jun, Egr3, Ccr4, and PD-1, which are critical for the establishment of tolerance (21, 22, 40, 42, 43). Characterization of immune cell phenotypes at the graft site via high-dimensional analysis of multiparametric flow cytometric data revealed an increased CD4 response to the SA-PD-L1–presenting microgels over time. Concomitant to this response was an increase in the presence of CD4+CD25+FoxP3+ regulatory cells, followed by a decrease in the presence of cells characterized as anergic based on their expression of T cell exhaustion markers such as PD-1, Tim3, and Lag3. These results are consistent with the observation that increased levels of PD-L1 lead to enhanced recruitment of Tregs (18) and the expression of “stop signal” molecules (44). Changes in the myeloid population were less pronounced but indicated an increase in the presence of macrophages as a response to the implanted immunomodulatory biomaterial. While the PD-L1 effect on T cells has been well established, its role in regulating innate immunity remains unclear. Yet, recent reports negatively correlate signaling through the PD-1/PD-L1 axis with the phagocytic potential of macrophages (45). Although not investigated here, the increase in the number of F4/80+ cells in the SA-PD-L1-microgel group suggests a potential role for these cells in the therapeutic benefits observed. Future studies may reveal unique aspects of biomaterial delivered PD-L1 on the innate cellular infiltrate and their effects on allogeneic tolerance induction.

Tolerance induction has been hypothesized to require a reduction in the clonal size of the effector population for alloantigens to a level that can be controlled through anergy and/or immunoregulation over time (46). Our findings of anergic and tolerogenic cells at higher levels in the SA-PD-L1-microgel group early on following transplantation is in agreement with this hypothesis. Furthermore, analysis of a subset of recipients with functioning allografts at >100 days after transplantation suggests that this behavior might persist over the life of the graft. Overall, this study provides evidence that the SA-PD-L1-microgel platform induces local tolerance to islet allografts maintained by a delicate balance between Tregs and T effectors.

There are notable limitations to our study. The reason why some recipients only had a partial benefit from the therapeutic, and the mechanism by which Tregs are generated (i.e., recruitment or induction) remains to be elucidated. As an off-the-shelf therapy, the synthetic microgels can be implemented with multiple immobilized ligands to specifically modulate components of the immune response. Future studies will investigate combining this therapy with antibodies that block positive costimulatory pathways (i.e., anti-CD154) (38) or the codelivery of broad-spectrum immunomodulatory ligands such as FasL or CTLA-4 (19, 47), which can potentiate tolerance in autoreactive T cells via nonredundant signaling pathways.

MATERIALS AND METHODS

SA-PD-L1 fabrication

A synthetic gene was constructed as previously described (16). Briefly, the N terminus of the extracellular domain of mouse PD-L1 (68 to 728 base pairs; GI: AF233517.1) was linked to a modified form of core SA and a 6His tag for purification. This sequence was then subcloned into the pMT/Bip/V5-His A CuSO4-inducible expression vector (Invitrogen) for stable expression in Drosophila S2 cells following previously published protocols (48). SA-PD-L1 protein was purified using metal affinity chromatography and assessed for structure and purity using SDS–polyacrylamide gel electrophoresis and Western blots.
Microgel fabrication and tethering of SA-PD-L1

Microgels were fabricated as previously described (19). Briefly, maleimide-terminated four-arm poly(ethylene glycol) (PEG-4MAL) macromers (20 kDa; Laysan Bio) were reacted with 2.0 mM biotin-PEG-thiol (1 kDa; Nanocs) in phosphate-buffered saline (PBS). The precursor was dispersed into 200-μm droplets and subsequently cross-linked with mineral oil (Sigma-Aldrich) containing 2% SPAN80 (Sigma-Aldrich) and a 1:15 emulsion of dithiothreitol (30 mg/ml; Sigma-Aldrich) within a flow-focusing microfluidic device (49).

After fabrication, microgels were washed five times in PBS containing 1% fetal bovine serum (FBS) (Hyclone). To assess biotinylatation, 1000 microgels were incubated with SA—AP488 (Invitrogen) for 30 min in 100 μl of PBS and subsequently washed three times by centrifugation to remove unbound fluorescent SA. Aliquots (25 μl) in triplicate from five different microgel runs were placed in a 24-well plate with a glass bottom and imaged using a BioTek Cytation 3 Imaging Plate reader (BioTek). Cellular analysis modality was implemented after imaging to determine the size and fluorescence intensity of individual microgels.

SA-PD-L1 was labeled with AF488 NHS ester (Thermo Fisher Scientific), followed by removal of free dye by desalting three times in Zeba columns [7k MWCO (molecular weight cutoff), Thermo Fisher Scientific]. Free protein fluorescence intensity, as well as the fluorescence intensity of tethered SA-PD-L1 to microgels, was assessed via a BioTek Synergy H4 microplate reader (BioTek).

For tethering SA-PD-L1, microgels were washed with PBS and transferred to PBS-coated conical tubes. SA-PD-L1 was added at 1 μg per 1000 microgels, followed by incubation for 2 hours at a rotator at room temperature. Following incubation, microgels were pelleted, and the supernatant was removed for Western blot analysis; samples were rinsed twice with PBS to remove unbound protein. Western blot was performed on supernatant control samples containing 1 μg of protein or control samples at a similar concentration as the tethering samples. Western blot was analyzed by using a SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) as described (19). Labeled protein (1 μg per well) was analyzed by using a BioTek Synergy H4 microplate reader (BioTek).

In vitro cytocompatibility of SA-PD-L1–presenting microgels

Cellular analysis of protein or control samples at a similar concentration as the tethering samples was assessed via a BioTek Synergy H4 microplate reader (BioTek). Polyclonal rabbit anti-Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) was added to corresponding wells; 56 hours after starting cultures, fluorescence intensity of individual microgels.

In vitro functional assays

Recognition of soluble SA-PD-L1 was assessed via (i) T cell conversion assay and (ii) a proliferation assay. (i) Naïve CD4 cells were isolated from splenocytes of C57BL/6 mice using a BioLegend mouse naïve isolation kit. Cells were cocultured in 96-well plates coated with anti-CD3 (5 μg/ml; BD Bioscience, 553058; clone: 145-2C11) and soluble of anti-CD28 (1 μg/ml; BD Bioscience, 553295; clone: 37.51). Groups consisted of the following: (i) cells with TGF-β (2 ng/ml; R&D Systems, 240B002); (ii) cells with TGF-β and human IL-2 (20 U/ml; PeproTech, AF-200-02); (iii) cells with SA-PD-L1 (5 μg per well) and IL-2; (iv) cells with SA-PD-L1, TGF-β, and IL-2. After 3 days in culture, cells were stained for live markers (1:1000; Fixable LIVE/DEAD Violet; Thermo Fisher Scientific) and fluorescent dye–conjugated antibodies against CD4 (1:100; PE-Cy7; BD Bioscience, 563933; clone: GL1.5), CD25 (1:100; APC; BD Bioscience, 557192; clone: PC61), and FoxP3 (PE; 1 μl per tube; BD Bioscience, 560414; clone: MF23).

Proliferation assay was performed in an MLR on splenocytes harvested from 4C mice (C57BL/6 transgenic for a TCR recognizing the BALB/c MHC I-Ad) cocultured with irradiated splenocytes (2000 cGy) from BALB/c mice at a ratio of 1:8 4°C to irradiated BALB/c splenocytes. After 24 hours, coculture SA-PD-L1 (5 μg) was added to corresponding wells; 56 hours after starting cultures, [3H]-thymidine was added. Uptake of [3H]-thymidine was measured with a scintillation counter.

In vivo SA-PD-L1 tracking

SA-PD-L1 was labeled with Alexa Fluor 750 NHS Ester (Thermo Fisher Scientific) as described (19). Labeled protein (1 μg) was tethered to 1000 microgels. Microgels displaying SA-PD-L1 or free labeled protein were transplanted in the EFP of nondiabetic BALB/c recipients (1200 microgels per EFP, n = 4 SA-PD-L1–presenting microgels, n = 5 free protein). Signal intensity was monitored over 20 days using an IVIS SpectrumCT imaging system (PerkinElmer). Intensity measurements were normalized to day 0 values.

In vivo phenotyping of microgel microenvironment

Microgels (600) functionalized with SA-PD-L1 (1 μg per 1000 microgels) or control microgels were transplanted in the EFP of nondiabetic C57BL/6 with a dose of 200 IEQ BALB/c islets per EFP (n = 3 per group). Seven days after transplantation, EFPs were removed and RNA was isolated using an RNeasy Lipid Tissue Mini Kit (Qiagen). RNA quality was assessed via NanoDrop 2000 (Thermo Fisher Scientific). A gene array for T Cell Anergy & Immune Tolerance was performed on samples following the manufacturer’s instructions [RT2 Profiler polymerase chain reaction (PCR) Array Mouse T Cell Anergy & Immune Tolerance; Qiagen]. Qualitative real-time PCR was performed on genes of interest (table S1) using the High Capacity
cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Relative gene expression was quantified using TaqMan assays in QuantiStudio 6 flex (Life Technologies), normalized against β-actin. Relative expression was calculated by the ΔΔct method, fold over the control microgels.

**Multiparametric flow cytometry and CATALYST analysis**
Biotinylated microgels (1200) conjugated with SA-PD-L1 (1 μg per 1000 microgels) or control microgels were transplanted in the EFPs of diabetic C57BL/6 with a dose of 600 IEQ BALB/c islets per EFP (n = 4 for control and n = 3 for SA-PD-L1). Animals were given RAPA (LC Laboratories) every day after transplantation for up to 15 days at 0.2 mg/kg. At 3 and 14 days after transplantation, EFPs were removed and processed with collagenase to obtain single cells. Samples for lymphocyte panel were stained according to manufacturer instructions for CD45 (BioLegend, 103108), CD3ε (BD Bioscience, 561416), CD4 (BioLegend, 100412), CD8a (BioLegend, 100372), CD25 (BioLegend, 102016), FoxP3 (BD Bioscience, 560408), CD44 (BD Bioscience, 5560568), CD62L (BioLegend, 104440), PD-1 (BD Bioscience, 562523), Tim3 (BD Bioscience, 742857), KLRG1 (BioLegend, 136419), Lag3 (BioLegend, 125227), zombie violet (BioLegend, 423113), PE Rat IgG2b, and κ Isotype Control (BioLegend, 400607). Samples for myeloid panel were stained for CD45 (BioLegend, 103146), CD11b (BioLegend, 101228), CD11c (BioLegend, 117349), Ly6G (BioLegend, 127633), Ly6C (BioLegend, 128041), MHCII (BioLegend, 107652), CD11c (BioLegend, 11734), F4/80 (BioLegend, 123108), CD86 (BioLegend, 105012), CD206 (BioLegend, 141720), NK1.1 (BioLegend, 108736), ICOS (BioLegend, 107706), PE Syrian Hamster IgG Isotype Ctrl Antibody (BioLegend, 402008), and zombie violet (BioLegend, 423113). Samples were treated with FcR (Fc receptor) true block and monocyte block reagents before staining (BioLegend, 103139, 426102). FMOs (fluorescence minus one) and isotype controls for intracellular staining were run with every collection. Absolute cell counts were calculated on the basis of precision count beads (BioLegend, 424902). FCS files were imported into FCSExpress; samples were gated for singlets by FSC-H/FSC-A, SSC-H versus SSC-A discrimination; and gating on stained cells.

**Dot blot for antibody generation**
A dot blot assay on serum samples were performed as follows: a nitrocellulose membrane was hydrated in 1% tris-buffered saline (TBS), and the target proteins (SA wild type, SA chimeric, SA-PD-L1, and mouse IgG) were bound to the membrane using a vacuum-driven Manifold I Spot Blot System (Schleicher & Schuell). The membrane was dried for 10 min, followed by blocking with protein-free blocker (Thermo Fisher Scientific). After washing three times with 1% TBS, diluted serum from 12 different animals (1:1000; 50 to 100 days after transplantation) were passed through the membrane. Following washing, antibodies against the target proteins were detected using an Alexa Fluor 680- or Alexa Fluor 488–labeled rabbit anti-mouse IgG (Thermo Fisher Scientific). The blot was imaged using a Licor Odyssey gel imager. Blot intensity was then quantified using the protein array analysis plug-in from ImageJ (http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-Image&artpage=6-6#outil_sommaire_5). Positive blots were determined to be five times the average intensity of background controls (2% bovine serum albumin).

**Islet transplantation**
BALB/c or C57BL/6 pancreatic islets were isolated using Liberase TFlex as a digestive enzyme (Roche Life Science) and purified by a Ficoll density gradient (Corning). For syngeneic transplantation, 600 IEQ with microgels were transplanted into a single EFP of STZ diabetic recipients (n = 6) and sealed with a PEG VEGF hydrogel (28), control subjects for syngeneic transplants refer to historical controls used for internal quality control of islet isolations. For allogeneic studies, naked BALB/c islets were cotransplanted either with unmodified microgels with no RAPA treatment (n = 5), free SA-PD-L1 with RAPA treatment (n = 6), unmodified microgels with RAPA treatment (n = 14), SA-PD-L1–presenting microgels with no RAPA treatment (n = 9), SA-presenting microgels with RAPA treatment (n = 7), and SA-PD-L1–presenting microgels with RAPA treatment (n = 12). STZ (Sigma-Aldrich)–induced diabetic C57BL/6, 175 mg/kg, were used as recipients if blood sugar is >350 mg/dl for three consecutive readings. Transplantation into the EFP was performed as previously described (19). Briefly, islets were transplanted in a ratio of 1 IEQ:2 microgel into the EFP of STZ-induced diabetic C57BL/6 mice, which was then sealed with an in situ cross-linked PEG hydrogel containing VEGF (28). For a subset of animals, RAPA (LC laboratories) was administered intraperitoneally for 15 days at 0.2 mg/kg starting the day of surgery. IPGTT was performed on day 30 after transplantation after 6 hours fasting using a glucose solution (2 g/kg; 25%).

For a group of recipients (five to seven mice per group), blood samples were collected at 50 to 70 days after transplantation for serum analysis. Blinded blood samples were deposited in serum separator tubes and sent to ANTECH Diagnostics for analysis. Full necropsies were also performed for evidence of any gross abnormalities after transplantation. Explanted kidneys, livers, lungs, and spleens were preserved in 10% neutral formalin solution (Sigma-Aldrich) and processed and stained with hematoxylin and eosin (H&E). Microscopy was performed using an optical microscope (Zeiss 510), and the images were taken using Axio Vision software. Images presented are representative of all samples collected (n = 10 mice per group).

MLR was performed on splenocytes from naïve, diabetic, and animals transplanted with control, SA-, or SA-PD-L1–presenting microgels that had received RAPA. Splenocytes were labeled with CellTrace Violet and used as responders against alloantigens. The cultures were harvested 4 days later and run on a BD Aria flow cytometer to assess the proliferation of CD4+ and CD8+ T cells by gating on stained cells. Diabetes reversal due to treatment and not spontaneous regeneration of β cell function was confirmed in a subset of long-term recipients by survival graft explants and total pancreatectomy. Insulin from the extracted pancreas was measured via a MERCODIA Insulin ELISA kit.

Explanted grafts were fixed in 10% formalin and embedded in paraffin blocks for immunohistochemical analysis. Slides were stained for insulin (1:100; Dako, A0564), glucagon (1:50; Abcam, ab10988),
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