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# Localized immune tolerance from FasL-functionalized PLG scaffolds

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## Abstract

Intraportal allogeneic islet transplantation has been demonstrated as a potential therapy for type 1 diabetes (T1D). The placement of islets into the liver and chronic immunosuppression to control rejection are two major limitations of islet transplantation. We hypothesize that localized immunomodulation with a novel form of FasL chimeric with streptavidin, SA-FasL, can provide protection and long-term function of islets at an extrahepatic site in the absence of chronic immunosuppression. Allogeneic islets modified with biotin and engineered to transiently display SA-FasL on their surface showed sustained survival following transplantation on microporous scaffolds into the peritoneal fat in combination with a short course (15 days) of rapamycin treatment. The challenges with modifying islets for clinical translation motivated the modification of scaffolds with SA-FasL as an off-the-shelf product. Poly(lactide-co-glycolide) (PLG) was conjugated with biotin and fabricated into particles and subsequently formed into microporous scaffolds to allow for rapid and efficient conjugation with SA-FasL. Biotinylated particles and scaffolds efficiently bound SA-FasL and induced apoptosis in cells expressing Fas receptor (FasR). Scaffolds functionalized with SA-FasL were subsequently seeded with allogeneic islets

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**Conflict of Interest:** H.S., E.S.Y., A.J.G., and H.Z. are inventors on a pending patent on PEG engineered with SA-FasL and H.S. and E.S.Y. hold equity in FasCure Therapeutics, which has an option to license the SA-FasL technology from the University of Louisville.

and transplanted into the peritoneal fat under the short-course of rapamycin treatment. Scaffolds modified with SA-FasL had robust engraftment of the transplanted islets that restored normoglycemia for 200 days. Transplantation without rapamycin or without SA-FasL did not support long-term survival and function. This work demonstrates that scaffolds functionalized with SA-FasL support allogeneic islet engraftment and long-term survival and function in an extrahepatic site in the absence of chronic immunosuppression with significant potential for clinical translation.

## Introduction

Exogenous insulin treatment is the standard of care for type 1 diabetic (T1D) patients. However, daily insulin treatment negatively affects the quality of life and is often ineffective in preventing recurrent hyperglycemic episodes with consequent development of micro- and macro-angiopathic lesions and the development and progression of chronic complications [1]. Allogeneic islet transplantation has proven effective in improving metabolic control/quality of life and in preventing severe hypoglycemia in patients with T1D [2-6]. However, broad application of clinical allogeneic islet transplantation is limited by the chronic use of immunosuppression to control immune rejection and its sequelae [5], and transplantation into the liver, which compromises immediate post-graft islet engraftment as well as long-term survival [7].

T cells are the major culprit of both T1D and allogeneic islet graft rejection by recognizing and responding to beta cell autoantigens and allogeneic major and minor histocompatibility antigens [8-12]. T cells upregulate Fas receptor and become sensitive to FasL-mediated apoptosis [13]. Apoptosis induced by FasL interaction with Fas on immune cells plays an important role in immune homeostasis and tolerance to self-antigens [14-17], and can be applied to address allogeneic rejection [18]. FasL may also play an active role in the generation of immunoregulatory cells [19] and their function [20-23]. Several subsets of immunoregulatory cells were shown to be more resistant to Fas/FasL-mediated apoptosis, and some of which use FasL as a death molecule to eliminate antigen-reactive T cells [20-25]. We have recently demonstrated localized tolerance achieved by the direct display of SA-FasL on allogeneic islets transplanted under the kidney capsule was mediated by the elimination of alloreactive T effector cells and induction/expansion of $CD4^+CD25^+FoxP3^+$ Treg cells that maintained tolerance [26]. The critical role apoptosis plays in immune tolerance (central and peripheral) combined with the potential of SA-FasL in apoptosis and generation/function of immunoregulatory cells make this an attractive molecule to modulate alloreactive immune responses for tolerance induction to allogeneic islet grafts.

The development of an extrahepatic site lacking the short and long-term complications of intraportal transplantation will significantly contribute to wide-spread application of clinical islet transplantation. For intraportal islet transplantation, islets are infused into the portal vein allowing immediate exposure of the graft to the blood, which initiates instant blood-mediated inflammatory reactions (IBMIR [27]). IBMIR is responsible for 50-80% of early loss of transplanted islets and involves the binding of tissue factor expressed on islets with platelets, resulting in their activation [7, 27]. Activated platelets trigger coagulation and

complement cascades, leading to granulocyte and monocyte infiltration into the islet graft and their ultimate destruction [7, 27, 28]. Intraportal transplantation also suffers from long-term complications, including hepatic steatosis and β-cell apoptosis/non-function due to the inherent hyperglycemic environment and relatively higher concentrations of drug metabolites in the liver [29]. Porous scaffolds have demonstrated the capacity to modulate the local environment in order to promote engraftment and long-term function [30-34]. Islets can readily be seeded into the pores, while the porosity supports rapid cell infiltration for integration with the host tissue. Microporous scaffolds composed of the biocompatible, biodegradable, FDA approved copolymer of lactide and glycolide (poly(lactide-co-glycolide) (PLG), creates and maintains a space for transplanted islets while enabling control of their distribution and density within the scaffold [35, 36]. The high porosity enables nutrient diffusion, rapid host tissue infiltration and islet revascularization. These scaffolds have been decorated with extracellular matrix proteins from the basement membrane (e.g., collagen IV), which results in a significant increase in islet function post transplantation [35, 37].

In this report, we describe the attachment of SA-FasL to microporous PLG scaffolds to achieve chronic immunosuppression-free, long-term survival of allogeneic pancreatic islets transplanted into an extrahepatic site, i.e. the epididymal fat pad. The omentum has emerged as a leading candidate in human clinical trials due to its thin, highly vascularized membrane and portal draining that recreates the physiological effects of insulin in the liver [38, 39]. In mice, the epididymal fat pad has many similar features to the omentum, and we have developed scaffolds to support engraftment of transplanted islets at this site. Initial studies employed an established model in which islets are modified with SA-FasL, with subsequent transplantation onto a microporous scaffold. Subsequently, we developed procedures for modifying microporous PLG scaffolds with biotin for subsequent immobilization of SA-FasL, and characterized the binding and functionality of the immobilized protein. These studies employed islets isolated from BALB/c mice and transplanted into the epididymal fat pad of C57BL/6 mice, a fully mismatched transplantation model. The function of the transplanted islets was monitored by blood glucose levels as well as an intraperitoneal glucose tolerance test. Collectively, these studies address two major issues with clinical islet transplantation; development of an extrahepatic site for islet engraftment and overcoming immune rejection without of the use of chronic immunosuppression.

## Materials and methods

### Materials

Poly(lactide-co-glycolide) (75:25) (PLG) (approx. 80,000 g/mol) with a single carboxylic acid end-group and an inherent viscosity of 0.76 dL/g was purchased from Lakeshore Biomaterials (Birmingham, AL). Poly(ethylene-alt-maleic anhydride) (PEMA) was purchased from Polyscience, Inc. (Warrington, PA). EZ link Amine-PEG2-Biotin was purchased from Fisher Scientific. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), dichloromethane (DCM), dimethylsulfoxide (DMSO), and all other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless noted otherwise.

### Biotinylation of PLG and characterization

PLG (890 mg, 0.011 mmol) was added to a 20 mL glass scintillation vial and dissolved in 10 mL DMSO. The carboxyl end group of PLG was activated by first adding EDC (10.6 mg, 0.056 mmol) dissolved in 1 mL DMSO followed by NHS (6.4 mg, 0.056 mmol) dissolved in 1 mL DMSO, and the reaction was allowed to stir for 15 min. Amine-PEG2-Biotin (5 mg, 0.056 mmol) was dissolved in 1 mL DMSO and added dropwise to the stirring solution of PLG-NHS and the reaction was allowed to stir overnight. Excess biotinylation reagent was removed by extraction. The reaction mixture was diluted into 200 mL of DCM and washed 4 times with 150 mL of a saturated salt solution (brine). The organic layer was dried over anhydrous sodium sulfate, filtered, concentrated by rotary evaporation, precipitated into ice cold methanol, and *stored in vacuo* overnight to remove residual solvents. Functionalization was confirmed with $^{1}$H-NMR (DMSO-d6).

### Particle and scaffold fabrication

PLG microparticles were formed for scaffold fabrication as previously described [40]. Briefly, PLG was dissolved in 2.04 mL of DCM (6 wt%) and sonicated in 10 mL of a 1% solution of PEMA at 100% amplitude (Cole-Parmer, 130 W, 3 mm stepped tip). The emulsion was poured into 200 mL of 0.5% PEMA and the organic solvent was evaporated by stirring the emulsion overnight. The particles were recovered by washing four times with deionized water by centrifugation at 7000 x g for 15 min at 4 deg C. Particles were lyophilized for 48 hours and stored under vacuum. Biotin-PLG microparticles were similarly fabricated, however biotin-PLG conjugates were combined with unmodified PLG at a mass ratio of 3:1 (biotin-PLG:PLG) for a final concentration of 6 wt% in DCM.

Porous scaffolds were formed by mixing PLG particles with NaCl (250 μm< d < 425 μm) at a 1:30 ratio (PLG:NaCl). The mixture was pressed in a 5 mm KBr die using a Carver press at 1500 psi and foamed in $CO_2$ at 750 psi for 16 hours. Scaffolds were leached in water for 1 hour followed by a second wash for 30 minutes. Scaffolds were disinfected by soaking them in 70% ethanol and washed with deionized water.

### Particle characterization

The size and zeta potential of the particles was determined by dynamic light scattering (DLS) by mixing 10 μL of a 25 mg/mL particle solution into 990 μL of MilliQ water using a Malvern Zetasizer ZSP (Westborough, MA).

### Protein loading and quantification

Particles were incubated with various concentrations of fluorophore-labeled streptavidin at 1 mg particles/mL for 20 minutes at various concentrations. Unbound streptavidin was removed by washing the particles with PBS by centrifugation (7000 x g, 5 min, 4 deg. C). To quantify the amount of fluorophore binding to the particles, particles were dissolved in DMSO and fluorescence was quantified using a plate reader (Synergy 2 (BioTek)) at 578 nm excitation and 605 nm emission.

Scaffolds were incubated with fluorescent streptavidin by applying 10 μL of the SA solution (0-40 ng/μL) to both sides of the disc (a total of 20 μL) for 20 minutes. Unbound streptavidin

was removed by washing the scaffold three times with 1.5 mL of PBS. Scaffolds were dissolved in DMSO and fluorescence was quantified as described above.

### Apoptosis assay

Particles (1 mg) and scaffolds were incubated with SA-FasL (Particles: 1 mL, 400 ng/μL, scaffold: 20 μL, 0-50 ng/μL) and washed as described above. To assess the ability for FasL particles or scaffolds to induce apoptosis in vitro, 1 mg/mL particles or a single scaffold was added to a 96 well plate containing A20 cells (mouse B lymphoma) at a concentration of $1.5\times10^6$ cells/mL and incubated for 18 hours. Cells were removed from the plate, stained with annexin V and propidium iodide (Life Technologies), and analyzed via flow cytometry.

### Mice and recombinant proteins

C57BL/6 (*H-2*$^b$) and BALB/c (*H-2*$^d$) mice were purchased from Jackson Laboratory and bred in our specific pathogen-free animal facility at the University of Louisville using protocols approved by the Institutional Animal Care and Use Committee. Recombinant SA and SA-FasL proteins were made with the *Drosophila* DES expression system (Invitrogen) following standard protocols [18].

### Islet isolation and engineering with SA-FasL protein

BALB/c islets were harvested from 8 to 12-week-old donors under anesthesia. Donor pancreata were perfused with 3 mL of cold Liberase TL (Roche Diagnostics) then removed and incubated for 20 minutes at 37°C. Islets were isolated using a Ficoll gradient (Sigma-Aldrich). Islets were kept overnight in RPMI-1640 medium supplemented with penicillin/streptomycin (100 U/mL and 100 μg/mL) and 10% fetal bovine serum in an incubator at 37°C with 5% $CO_2$. Islets were transferred to a 14-mL round bottom tube and washed in PBS. Islets were then incubated in 5 μM EZ-Link™ Sulfo-NHS-LC-Biotin solution (Thermo Scientific) at 20°C for 30 minutes. After incubation, islets were washed twice in PBS to remove any unbound biotin. Then, islets were incubated in PBS containing SA-FasL protein (~200 ng SA-FasL/500-550 islets/200 μl PBS) at 20°C for 30 minutes. Islets were washed twice in PBS to remove any unbound protein before transplantation. Select biotin-PLG scaffolds were engineered by placing scaffolds to a round bottom tube and adding SA-FasL (0.5 or 2.5 μg /scaffold) diluted in PBS and incubating at 20°C for 30 minutes while rotating and shaking the tube every 10 minutes. Scaffolds were washed twice before being loaded with islets.

### Islet transplantation

C57BL/6 mice were chemically induced with diabetes by intravenous (i.v.) injection of streptozotocin (200 mg/kg). Mice were monitored by reading blood glucose where ≥ 250 mg/dL for two consecutive days was considered diabetic. Islets were loaded onto PLG scaffolds (2/mouse). Diabetic mice were given anesthesia and a small incision was made on the abdomen to allow scaffolds to be placed on epididymal fat pads. Adipose tissue was wrapped around scaffolds before being returned to the abdomen. Mice were then sutured. Select mice were administered rapamycin through i.p. injection of 0.2 mg/kg daily for 15

days starting the day of transplant. Mice were monitored for diabetes and those with ≥ 250 mg/dL blood glucose level for two consecutive days considered rejecting the islet graft.

### Intraperitoneal Glucose Tolerance Test

Mice were put in clean cages without food and allowed to fast for 6 hours. Mice were then injected with 25% glucose solution (2 g/kg body weight) and monitored for blood glucose levels before injection and at 10, 20, 30, 60, 90, and 120 minutes post glucose injection.

### Mixed Lymphocyte Reaction

Spleen and draining lymph nodes were harvested from mice after rejection of graft or at experimental end point (> 200 days) if mice remained euglycemic up to that point. Organs were processed into single cell suspensions using frosted slides. ACK lysis buffer (ThermoFisher Scientific) was added to spleen to lyse red blood cells. Splenocytes were panned, labelled with (5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester) (CFSE), and used as responders in a standard mixed lymphocyte reaction assay [41]. Stimulator cells were prepared from either naïve BALB/c (donor) or C3H ($3^{rd}$ party) mice, irradiated with 200 cGy, and cocultured with equal numbers of responder cells in 96-well plates ($0.1\times10^6$ cells/well). Cells were cultured in 200 μL DMEM supplemented with HEPES buffer, sodium pyruvate, penicillin/streptomycin, L-Glutamine (ThermoFisher Scientific), FBS, L-Arginine HCL, folic acid, L-Asparagine, 2-Mercaptoethanol (Sigma), and responder serum. Cells were harvested after four days of culture at 37°C and stained with Alexa 700-CD4 Ab, APC-Cy7- CD8 Ab, and 7AAD to separate dead cells (BD Pharmingen). Cells were collected using BD LSR II and analyzed using Diva software.

### Statistical analysis

Flow data was tested for significance using a two tailed Welch's t-test. Graft survival was tested for significance using the log-rank test. P values less than 0.05 were considered significant. Survival curves, IPGTT, and flow graphs were created and analyzed using GraphPad Prism software.

## Results

### Transplantation of SA-FasL-engineered islets on microporous scaffolds

We investigated the transplantation of SA-FasL-engineered islets on microporous scaffolds implanted into the epididymal fat pad (Fig 1A). Initial studies employed syngeneic islets transplanted into streptozotocin-induced diabetic mice to determine the impact of the scaffolds and short-term rapamycin on the engraftment and function of the islets. Transplantation of the syngeneic islets led to the establishment of euglycemia within 10 days for all animals, and blood glucose levels fell below 200 mg/dL, which was used to define graft survival (Fig 1B). All animals with syngeneic islets engineered with SA-FasL had graft survival for the duration of the study (100 days). Subsequently, allogeneic islets engineered with SA-FasL were transplanted on microporous scaffolds. Unmodified islets transplanted on scaffolds with transient rapamycin underwent rejection, as indicated by increased blood glucose levels, by day 40 (Supplementary Fig. 1). Mice transplanted with allogeneic islets modified with SA-FasL and receiving transient rapamycin had graft survival that was

sustained for 200 days (Fig 1C), similar to the results with syngeneic islets. Rapamycin has been previously reported to synergize with SA-FasL presentation to prolong allograft survival [26], as either factor alone results in only short-term graft function. An intraperitoneal glucose tolerance test (IPGTT) study demonstrated that the normalization of blood glucose levels by the transplanted islets was similar to that observed with naïve mice (i.e., non-diabetic) (Fig 1D, E), which is consistent with previous reports of islets transplanted on scaffolds [36, 42].

We next assessed the immune competency of long-term islet graft recipients and if the observed graft survival is associated with systemic immune non-responsiveness against the donor alloantigens. T cell proliferative responses were analyzed from the spleens and draining lymph nodes of the long-term allogeneic islet graft survivors. The collected cells were labeled with CFSE and used against BALB/c donor and third party C3H stimulators in a standard ex vivo mixed lymphocyte reaction [41]. After 4 days of culture, the responses from CD8+ T cells indicated similar proliferative responses for the SA-FasL-engineered islets and an age-matched C57BL/6 control, with responses similar to both the donor and third-party stimulators (Fig 1F, G). Interestingly, $CD4^+$ T cell responses were greater for the SA-FasL-engineered islets relative to age-matched control (Fig H, I). This response was similar for both the donor and third-party stimulators. Representative dot plots for the donor and third-party stimulators are depicted in Fig 1J. These results demonstrate that recipients of long-surviving islet allografts are immunocompetent and show lack of systemic unresponsiveness to donor antigens, demonstrating localized nature of graft protection. Collectively, these studies demonstrate that the microporous scaffolds for transplantation of SA-FasL-engineered islets to an extrahepatic, extra-renal site provides for engraftment of the islets and protection from the immune response similar to previous reports performed with transplantation under the kidney capsule [26].

## Synthesis and characterization of biotin-PLG conjugates and particle formation

We subsequently investigated the modification of scaffolds with biotin to allow for immobilization of SA-FasL as a potential off-the-self product for immunomodulation. PLG was conjugated with a biotin linker and the biotin-PLG conjugates were subsequently formed into microspheres that were assembled into scaffolds. The carboxyl-terminal group of PLG was conjugated to the heterobifunctional linker $NH_2$-$PEG_2$-Biotin using carbodiimide chemistry and confirmed using $^1$H-NMR (Figs 2A, B). Biotin-PLG particles were prepared with a single emulsion-solvent evaporation procedure using the biotin-PLG conjugates. The particles displayed an average size of 860 ± 40 nm and a zeta potential of −16 ± 5.0 mV (Supplementary Fig 2).

The ability of biotin-PLG particles to bind streptavidin was quantified through the maximum loading and efficiency of fluorescently-tagged streptavidin (AF568-SA) (Fig 3A). The unmodified particles non-specifically bound a small amount of AF568-SA, however the presence of biotin significantly increased the amount of bound protein. The concentration of AF568-SA incubated with particles was varied from 100 to 800 ng AF568-SA per mg of particle, which resulted in loadings of 75 to 280 ng AF568-SA per mg of particle (Fig 3B). A dose of 40,000 ng AF568-SA per mg of particle was used as a saturating dose, which

produced a loading in excess of 8,000 ng AF568-SA per mg of particle. Although the amount loaded on the particle increased with greater concentration of AF568-SA, the loading did not increase linearly. The loading efficiency (defined as the amount of protein bound divided by the amount incubated) decreased from 75% to 35% as the concentration was increased from 100 to 800 ng AF568-SA per mg of particle (Fig 3C). At the maximal dose of 40,000 ng AF568-SA per mg of particle, the loading efficiency was 20% for the biotinylated particles and approached 0% for the non-biotinylated particles.

The bioactivity of immobilized SA-FasL was investigated next through their ability to induce apoptosis in the mouse B lymphoma cell line A20. Particles were incubated with 400 ng SA-FasL, which was selected based on the substantial difference in the binding to biotinylated and non-biotinylated particles (Fig 3B). The addition of particles modified with SA-FasL induced apoptosis in approximately 50% of the cells (Fig 3D). Biotinylated particles without SA-FasL did induce apoptosis at levels consistent with the negative control, which was approximately 1% of the cell population. The level of apoptosis induced by the particles was less than that obtained with soluble SA-FasL, which induced apoptosis in more than 90% of the cells for a dose of 10 ng.

## SA-FasL loading on biotin-PLG scaffolds

SA-FasL modified scaffolds were subsequently prepared by fabricating scaffolds from the biotinylated polymer with subsequent incubation with a solution containing SA-FasL (Fig 4A). Incubation of SA-AF568 at concentrations ranging from 100 to 800 ng SA-AF568 per mg of scaffold resulted in loadings that ranged from 70 to 620 ng SA per mg of scaffold, which was similar to particles at low concentrations (Fig 4B). The efficiency of loading was relatively constant across the range of concentrations (≈70%), which differed from what was observed with the particles that had a declining efficiency with increased concentration of SA-AF568 (Fig 4C). The increased efficiency may result from an increased surface area of the porous scaffold compared to the spherical particles. Unmodified scaffolds were used as a control and showed similar concentrations of non-specific binding as unmodified particles at higher protein concentrations.

SA-FasL-loaded scaffolds had a greater efficiency for inducing apoptosis of A20 cells relative to the particles (Fig 4D, E). The induction of apoptosis was concentration-dependent as 40 ng SA-FasL/mg scaffold was not significantly different than the control but 200 ng/mg and 400 ng/mg increased apoptosis (Annexin $V^+$) in more than 90% of the cells. These results demonstrate that SA-FasL could be functionalized to the surface of PLG scaffolds and effectively induce apoptosis in A20 cells.

## SA-FasL scaffolds support allogeneic graft function without chronic immunosuppression

We subsequently investigated whether SA-FasL modified scaffolds could prevent allogeneic islet rejection similar to the SA-FasL-engineered islets, while also supporting engraftment and long-term function to maintain normoglycemia. Scaffolds engineered with SA-FasL were loaded with islets from BALB/c donors and transplanted into the peritoneal fat of diabetic C57BL/6 mice. Naïve islets mounted on SA-FasL-engineered PLG scaffolds along with transient rapamycin demonstrated graft survival for more than 200 days in more than

80% of the animals (Fig 5A), with one animal rejecting at day 30. Rapamycin without SA-FasL had a mean graft survival time of 23 ± 2 days (Fig 5A, B). Interestingly, when both islets and scaffolds were engineered with SA-FasL but did not receive the short course rapamycin treatment, one third of the mice established long-term tolerance while the rest rejected by day 50. For the transplantation of islets on SA-FasL modified scaffolds, normoglycemia was established within days of transplantation (Fig 5B). The blood glucose dynamics were similar between the SA-FasL-modified scaffolds and the unmodified scaffolds (with rapamycin) through day 20, at which point, the blood glucose levels began to rise for the unmodified scaffolds likely due to rejection of the islets. An IPGTT performed at day 200 demonstrated restoration of normoglycemia at a rate that was similar to naïve animals (Fig 5C, D).

## Discussion

This report investigated the combination of SA-FasL and biomaterial scaffolds as a means to create a translatable site that supported the engraftment and long-term function of allogeneic islets at an extrahepatic and extrarenal site. Immunoprivileged sites, such as the testes, anterior chamber of the eye, brain, and tumors, have the ability to suppress destructive immune responses by various mechanisms [43-46]. Importantly, FasL was initially discovered as one of the molecules that plays a critical role in immunoprivileged sites [14, 15]. Indeed, we have recently reported that SA-FasL-engineered allogeneic islets induced localized immune privilege when transplanted under the kidney capsule [26]. Therefore, FasL not only contributes to the regulatory mechanisms in naturally occurring immunoprivileged sites in the body, yet also can be used to create “induced” immunoprivileged sites [26]. Herein, we demonstrated that allogeneic SA-FasL-engineered islets engrafted and normalized blood glucose levels for more than 200 days under a short course of rapamycin treatment (0.2 mg/kg daily starting the day of transplantation for a total of 15 doses) with transplantation on PLG scaffold into an extrahepatic site, one that has translational potential [47].

Given that localized presentation of SA-FasL on islets supported long-term function on the scaffold, we subsequently investigated the immobilization of SA-FasL to the scaffold as a means to minimize the cellular manipulation prior to transplantation. Biotin decorated PLG particles have been reported for targeted drug delivery to cancer cells [48]. Manipulating the scaffold to present SA-FasL would eliminate the steps needed for direct islet engineering, and as such save time and overcome potential undesired effects associated with engineering process, such as cell fragmentation. Moreover, scaffolds engineered with SA-FasL can be manufactured as a potential off-the-shelf immunomodulatory product for islet transplantation, expediting clinical translation. Importantly, the presentation of SA-FasL from surfaces has previously been reported not to interfere with its apoptotic function [26, 49]. To create scaffolds modified with SA-FasL, we chemically modified the polymer in solution [48, 50-52], which was subsequently formed in to particles and then into scaffolds. Initial attempts to functionalize scaffolds involved conjugation of biotin to the surface of a pre-formed scaffold, which produced inconsistent modification with SA-FasL and thus the direct modification of the polymer was pursued based on prior reports indicating enhanced target binding [53]. Stable spherical particles could only be formed from mixtures of the

biotinylated and non-biotinylated polymer, as particles formed from only biotinylated polymer were unstable due to aggregation. The microspheres were employed to construct the scaffold using a gas foaming and particulate leaching process [54-56]. The scaffolds can readily be stored prior to islet seeding for transplantation; however, as a potential off-the-shelf product, further studies will be needed to test long-term storage of SA-FasL pre-engineered scaffolds for downstream applications.

The microporous structure of the scaffold enhanced protein loading and bioactivity relative to the particles. This observation likely results from the higher surface area of the scaffolds. Protein loading and efficiency were similar to other techniques like carbodiimide coupling to PLG particles [40, 57]. The presentation of SA-FasL from particles or scaffolds maintained the ability to induce apoptosis, although it was not as efficient as soluble SA-FasL, which may reflect the nature of *ex vivo* culture setting favoring better access of soluble SA-FasL to the Fas receptor on A20 cells. Further optimization of the PEG-biotin linker length may enable enhanced binding of SA-FasL to Fas by increasing the mobility of SA-FasL [58]. Interestingly, for concentrations between 40 and 400 ng/mg, the extent of binding was highly consistent within experiments, yet considerable variation in apoptosis was observed between experiments, suggesting a sensitivity to the protein loading or presentation within this range. Previous reports of surface modified apoptosis systems utilized surface anchored polymer chains with covalently linked anti-Fas antibodies but were only able to achieve up to 34% apoptosis in cells expressing Fas, whereas the method presented here achieved 92% apoptosis and *in vivo* protection of allogeneic islets [59]. This result may be due to the far greater surface density of protein (up to 150 ng/cm$^2$ vs 1.6 ng/cm$^2$), the reported robust apoptotic function of SA-FasL [60], and the positional display of this molecule on biotinylated scaffolds, preserving its function. These findings are consistent with our recent publication with polyethylene glycol (PEG) microparticles where we demonstrated a dose-dependent binding of SA-FasL to biotin presenting microparticles [61].

Importantly, microporous scaffolds functionalized with SA-FasL supported engraftment and function of the transplanted allogeneic islets that maintained normoglycemia for more than 200 days in the absence of chronic immunosuppression. Islets transplanted on unmodified PLG scaffolds were promptly rejected within 6-12 days after the rapamycin treatment ended, consistent with our previous results [26, 61]. The observed sustained survival and function of allogeneic islets without immunosuppression is consistent with our previously published data with islet directly engineered to transiently display SA-FasL on their surface, but not streptavidin or other apoptosis unrelated proteins used as controls [41]. Importantly, this data also consistent with our most recent study reporting sustained survival of allogeneic islets co-transplanted with biotinylated PEG microparticles presenting SA-FasL on their surface [61]. SA-FasL alone without rapamycin was able to delay rejection of all grafts with ~40% surviving for the 200-day observation period. This observation demonstrated that SA-FasL has the potential as monotherapy to achieve permanent graft survival, but complete efficacy may require further refinement of the protocol by increasing the dose of SA-FasL or duration of persistence. Our approach of functionalizing SA-FasL onto the surface of biotin-PLG scaffolds is an effective method to induce long-term function without chronic immunosuppression.

Long-term graft recipients generated T cell responses to both donor and third-party antigens at a similar magnitude to those of naïve mice. These findings demonstrate that immunomodulation with SA-FasL in context of PLG scaffolds does not result in immune incompetence of the graft recipients nor systemic tolerance to donor antigens. Lack of systemic unresponsiveness is expected given the localized nature of immunomodulation by SA-FasL and the demonstrated role of this molecule in physiological immune privilege [62]. These findings are further consistent with our published studies demonstrating that SA-FasL-engineered islets or PEG microgels presenting SA-FasL transplanted under the kidney capsule establish localized tolerance, which is maintained by $CD4^+CD25^+FoxP3^+$ T regulatory cells [41, 61]. PEG microgels modified with SA-FasL that were co-transplanted with islets had approximately 90% engraftment and return to normoglycemia with transplantation into the kidney capsule, which is widely used in mouse models yet is generally considered a non-translational site. Herein, we report that modification of the microporous scaffolds with FasL results in approximately 80% engraftment and long-term function of the transplanted islets in the epididymal fat pad, which models the omentum as a translational site for islet transplantation.

The long term function in the absence of immunosuppression may result from localized Treg function. The sensitivity of Treg cells to FasL has previously been shown to be context dependent and regulated by various factors, including antigenic stimulation, proliferation, and cytokine milieu [63, 64]. Two lines of evidences from our own studies support this notion. First, we have demonstrated that Treg cells in NOD are preferentially more resistant to SA-FasL as compared with Teff cells [25]. Second, in collaboration with Askenasy and group, we have shown that sorted Treg cells modified to display SA-FasL on their surface have improved regulatory function and following adoptive trasnfer into prediabetic NOD prevented the development of diabetes [65, 66]. Importantly, SA-FasL-engineered Treg cells homed to the pancreas and regional LNs where they proliferated and induced apoptosis in Teff cells in situ via SA-FasL [66]. Our observations are also supported by a study demonstrating that cytokine-induced selective expression of FasL in the vasculature of human and mouse solid tumors results in preferential apoptosis in $CD8^+$ T effector cells, but not Treg cells because of their expression of higher levels of c-FLIP [67]. Treg cells were shown to use FasL as an effector molecule to eliminate Teff cells [68]. Also, Treg cells expressing FasL were shown to regulate hapten-specific $CD8^+$ T effector cell expansion through the elimination of Fas-expressing hapten-presenting DCs, resulting in the prevention of T-cell-mediated allergic immune responses in the skin [69].

A short course of rapamyicn was required for SA-FasL-induced tolerance. We have not extensively investigated the mechanistic synergy between SA-FasL and rapamycin, which we believe is complex and may operate on multiple levels. Rapamycin induces apoptosis via the mitochondria-mediated intrinsic pathway, while FasL apoptotic effect operates through extrinsic Fas-mediated pathway. As such, apoptosis mediated by these two pathways may ensure the physical elimination of pathogenic T cells. Furthermore, rapamycin has various other tolerogenic characteristics that involves generation of regulatory dendritic cells [70], T cell anergy [71], and most importantly generation and expansion of $CD4^+CD25^+FoxP3^+$ T regulatory cells [72]. We [67, 73, 74] and others [67, 69] have shown that Treg cells are resistant to FasL-mediated apoptosis under inflammatory conditions. Given that Treg cells

play a dominant role for the induced tolerance in our model as published previously [75, 76], we speculate that induction of apoptosis in Teff cells and generation and maintenance of Treg cells are the two potential regulatory steps targeted by SA-FasL and rapamycin for the observed synergy.

The positional display of SA-FasL on islets grafts or PLG scaffold did not negatively impact the long-term survival of islet grafts or their funciton. These observations are not in line with published studies implicating the expression of Fas by β cells in their destruction in autoimmunity setting. Unlike normal NOD female mice, which develop spontaneous diabetes within 30 weeks, NOD-*lpr/lpr* mice lacking a functional Fas do not develop diabetes [77]. Diabetes cannot be induced in NOD-*lpr/lpr* mice by adoptive transfer of diabetogenic T cells [77]. Also, transgenic expression of FasL in β cells resulted in accelerated diabetes caused by β cell suicide *via* Fas/FasL-induced apoptosis [78, 79]. It was shown that the expression of Fas on β cells is largely dependent on local levels of inflammatory cytokines. β cells in response to proinflammatory cytokines, such as IL-1β, TNF-α, and INF-γ, upregulate Fas on their surface and bocome senstive to FasL expressed on infiltrating lymphocytes [80, 81]. While an association with inflammatory cytokines has been proposed, the effector mechanisms that lead to β cell destruction are not well defined.

However, several lines of evidences have provided contradictory evidences against the role of Fas/FasL-mediated apoptosis in the destruction of β cells. Fas expression was not detectable on β cells from young NOD mice, but only on 1-5% of cells from older animals [82]. Treatment of NOD with an anti-FasL antibody did not prevent accelerated diabetes mediated by adoptive transfer of diabetogenic T cells [83]. Fas deficient neonatal pancreata of NOD-*lpr/lpr* mice, whihc do not develop spoantenous diabetes, underwent destruction when transplanted into diabetic NOD mice [83]. The resistance of NOD-*lpr/lpr* mice to diabetes was shown to be due to the presence of high numbers of double negative T cells expressing FasL and eliminating autoreactive T cells expressing Fas [84]. Furthermore, islets from young NOD mice are not sensitive to FasL-mediated killing unless precultured with IL-1β and IFN-γ to induce the expression of Fas [82]. Importantly, islet cells harvested from young NOD mice did not undergo apoptosis when cultured in the presence of anti-Fas Ab or FasL expressing transfectants [85]. Consistent with this observation are studies that islets of various species, including porcine, nonhuman primates, humans (Shirwan et al, unpublished observations), and rodents [76, 86], engineered to display SA-FasL on their surface did not negatively impact their viability or function both in vitro and in vivo. Extensive data in our recent publicaton [75] also demonstrate PEG microgels engineered with SA-FasL do not negatively impact islet viability and function when co-transplanted with naïve islets in an allogenic host. Taken together, these studies indicate that the precise role of Fas/FasL-mediated apoptosis in the destruction of β cells is ambiguous and may be model and condition dependent.

In conclusion, our studies demonstrate that PLG scaffolds support the engraftment and sustained survival of allogeneic islets directly engineered to transiently display SA-FasL on their surface in an extrahepatic and translational site in the absence of chronic immunosuppression. Importantly, scaffolds directly engineered with SA-FasL achieves a similar outcome as SA-FasL-engineered islets, demonstrating that immunoregulation

induced in this setting does not require T cells establishing simultaneous physical interaction with both the alloantigen and SA-FasL on the target tissue, consistent with our recent study with PEG microgels presenting SA-FasL [61]. This observation suggests that T cells activated in response to alloantigens are eliminated within graft microenvironment upon encounter with SA-FasL attached to the scaffold. Importantly, the modification of biomaterial with SA-FasL avoids the complications associated with direct engineering of islet grafts and provides a potential off-the-shelf immunomodulatory product that may have applicability to a multitude of applications involving cell transplantation.

## Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations. However, they can be provided upon request.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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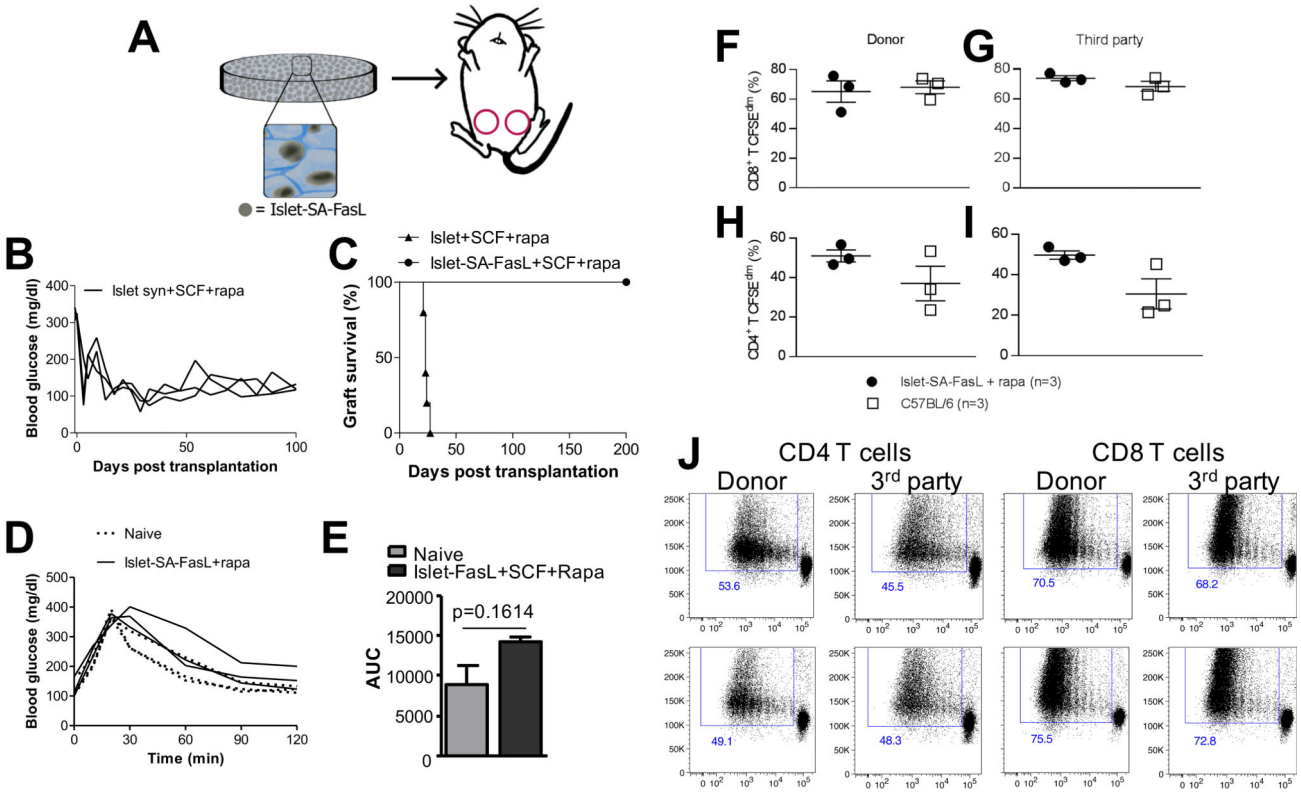


**Figure 1:**

SA-FasL engineered islets establish allogeneic tolerance when transplanted on PLG scaffolds. **(A)** Schematic showing biotinylated islets functionalized with SA-FasL are loaded on microporous PLG scaffolds and implanted in the epididymal fat pad of mice. **(B)** Individual tracing of blood glucose levels for syngeneic islets modified with SA-FasL and transplanted on scaffolds (SCF), with mice receiving a short course of rapamycin (n=3, mean survival time > 100 days). **(C)** Kaplan Meier analysis of allogeneic BALB/c islets transplanted under the short cover of rapamycin on unmodified PLG scaffolds in C57BL/6 recipients. Conditions include SA-FasL-engineered islets on scaffolds with rapamycin (n = 5, mean survival time >200 days), naïve islets on scaffolds with rapamycin (n = 5, mean survival time = 23 ± 2.19 days). **(D)** Intraperitoneal glucose tolerance test of long-term islet grafts compared to naïve C57BL/6 mice after fasting for 6 hours, followed by i.p. glucose injection. Blood glucose of mice was taken starting just before injection and at the indicated time points. **(E)** Area under curve for the intraperitoneal glucose tolerance test. The area for naïve mice compared to the transplanted mice is not significantly different (p=0.16). **(F-I)** T cell proliferative response from recipients of long-term (> 200 days) BALB/c SA-FasL-engineered islets mounted on unmodified PLG scaffolds plus rapamycin (n = 3) and naïve C57BL/6 as controls (n = 3). Responders were labeled with CFSE and used against BALB/c donor (F,H) and third party C3H (G,I) stimulators in a standard *ex vivo* mixed lymphocyte reaction. After 4 days of culture, cells were stained with antibodies against CD8 (F,G) and CD4 (H,I) molecules and incubated with 7AAD to gate out dead cells before flow cytometry analysis. (J) Representative dot plots of data presented in F, G, H and I. Bars represent mean

and SEM. Asterisks represent level of significance (*p<0.05, **p<0.01) found by using a two-tailed Welch's t-test.

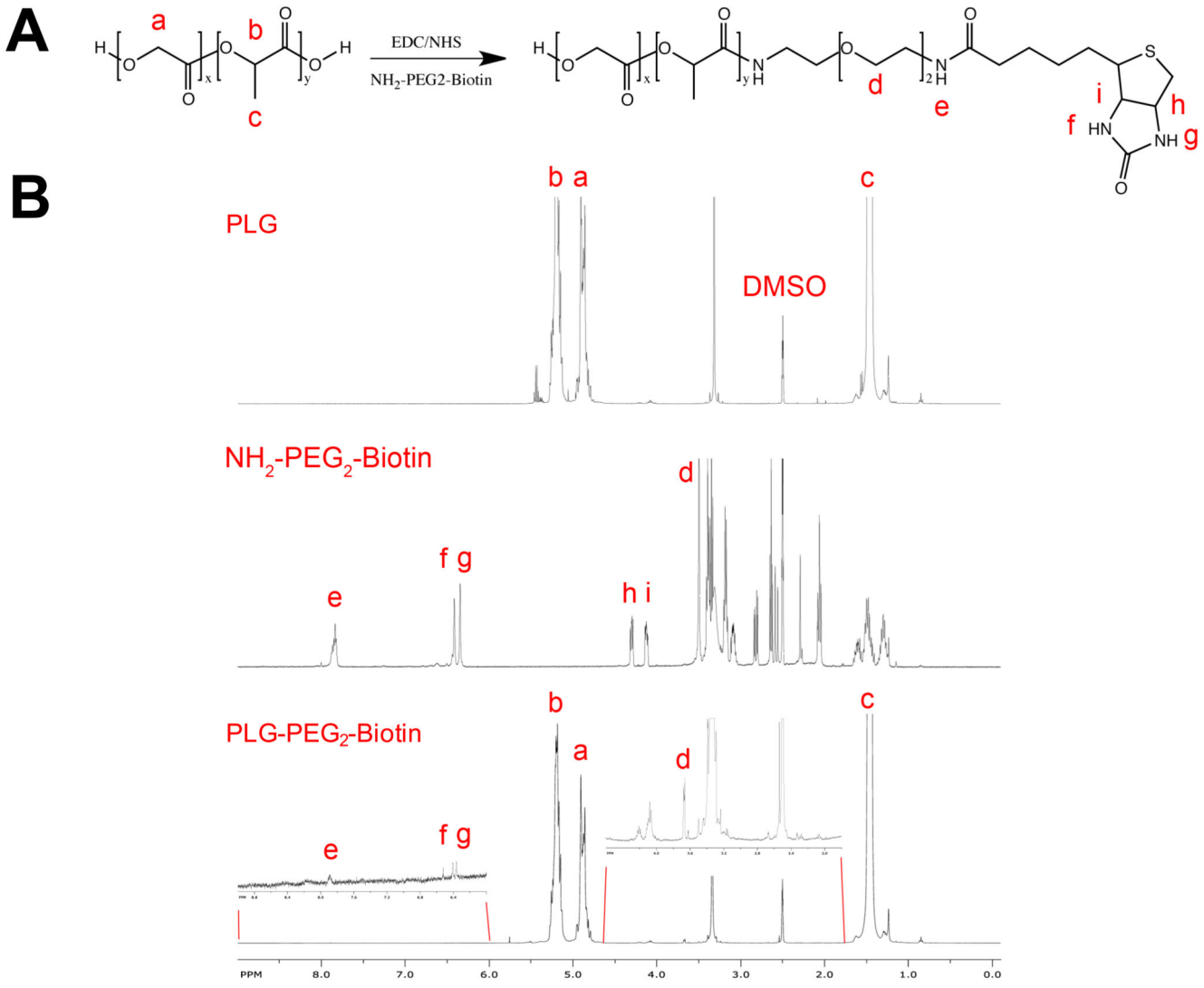


**Figure 2:**

Characterization of biotin-PLG polymer. **(A)** Conjugation of $NH_2$-$PEG_2$-biotin to PLG via carbodiimide chemistry, resulting in biotin-PLG. **(B)** $^1$H-NMR in $(D_3C)_2SO$ of PLG (top), biotin linker (middle), and biotin-PLG (bottom).

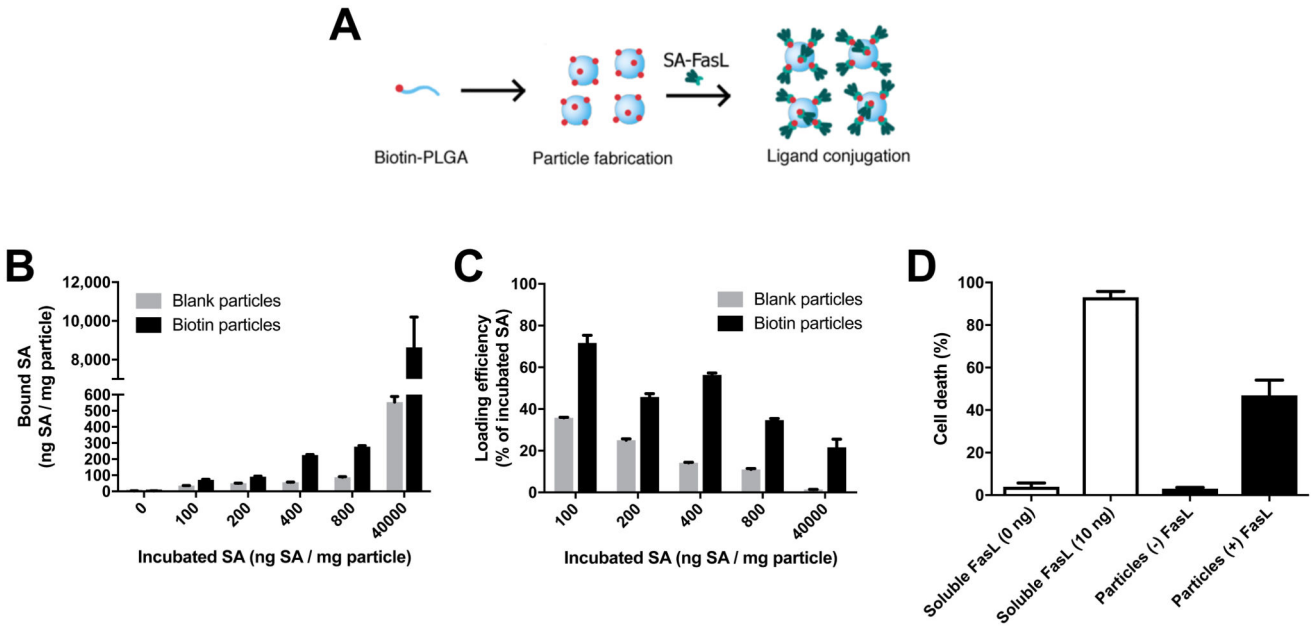


**Figure 3:**

Biotin-PLG particles can be efficiently conjugated with SA-FasL and induce apoptosis in mouse B lymphoma cell line. **(A)** Schematic of biotin-PLG particles being functionalized with SA-FasL. **(B)** The amount of fluorescently tagged SA bound to the particles was investigated by varying the protein concentration (0, 100, 200, 400, 800, and 40,000 ng SA/mL in 1 mL) incubated with 1 mg of biotin-PLG particles ("biotin particles"). As a control, 1 mg of unmodified PLG particles was incubated with the same SA concentrations ("blank particles"). After two spins and washes, particles were dissolved in DMSO and fluorescence was measured, n=3. **(C)** The loading efficiency was calculated by dividing the bound SA by the incubated SA, n=3. **(D)** Biotin-PLG particles functionalized with SA-FasL induce cell death in A20 cells. Particles were incubated with 400 ng / mL of SA-FasL in 1 mL with 1 mg of particles. After washing to remove unbound SA-FasL, particles were incubated with $1.5 \times 10^5$ A20 cells for 18 hours and cell death was analyzed via propidium iodide stain and flow cytometry (n=3). For comparison, soluble FasL was added to cells (0, 10 ng) and demonstrated the ability to induce cell death at low concentrations (n=3).

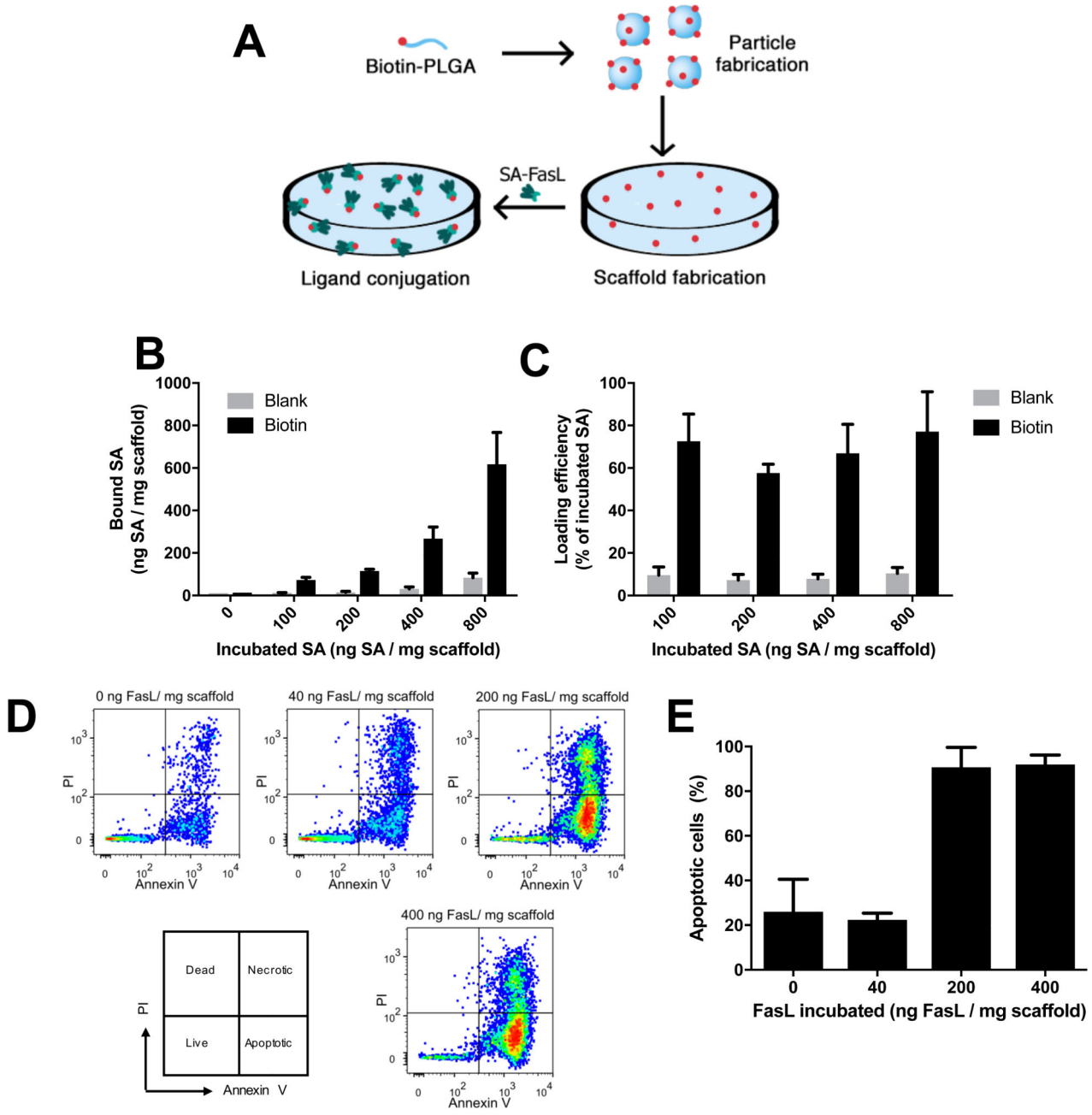


**Figure 4:**

Biotin-PLG scaffolds can be efficiently conjugated with SA-FasL and induce apoptosis in mouse B lymphoma cell line. **(A)** Schematic of biotin-PLG particles functionalization with SA-FasL. **(B)** The amount of fluorescently tagged SA bound to scaffolds was investigated by varying the protein concentration (0, 100, 200, 400, and 800 ng SA/20μL) incubated with 2.5 mg biotin-PLG scaffolds (“biotin”). As a control, unmodified PLG scaffolds were incubated with the same SA concentrations (“blank”). After two washes, scaffolds were dissolved in DMSO and fluorescence was measured, n=3. **(C)** The loading efficiency was calculated by dividing the bound SA by the incubated SA, n=3. **(D)** Scaffolds were

incubated with SA-FasL (0, 40, 200, and 400 ng / 20 μL). After washing to remove unbound SA-FasL, scaffolds were incubated with $1.5 \times 10^5$ A20 cells for 18 hours. Apoptotic and dead cells were analyzed via propidium iodide and Annexin V staining and flow cytometry (n=3). For comparison, 2.5 mg of soluble FasL was added to cells and induced apoptosis in the 98% of the cells (n=3). **(E)** Scaffolds required a minimum loading of 200 ng FasL / mg scaffold to induce apoptosis in the majority of A20 cells.

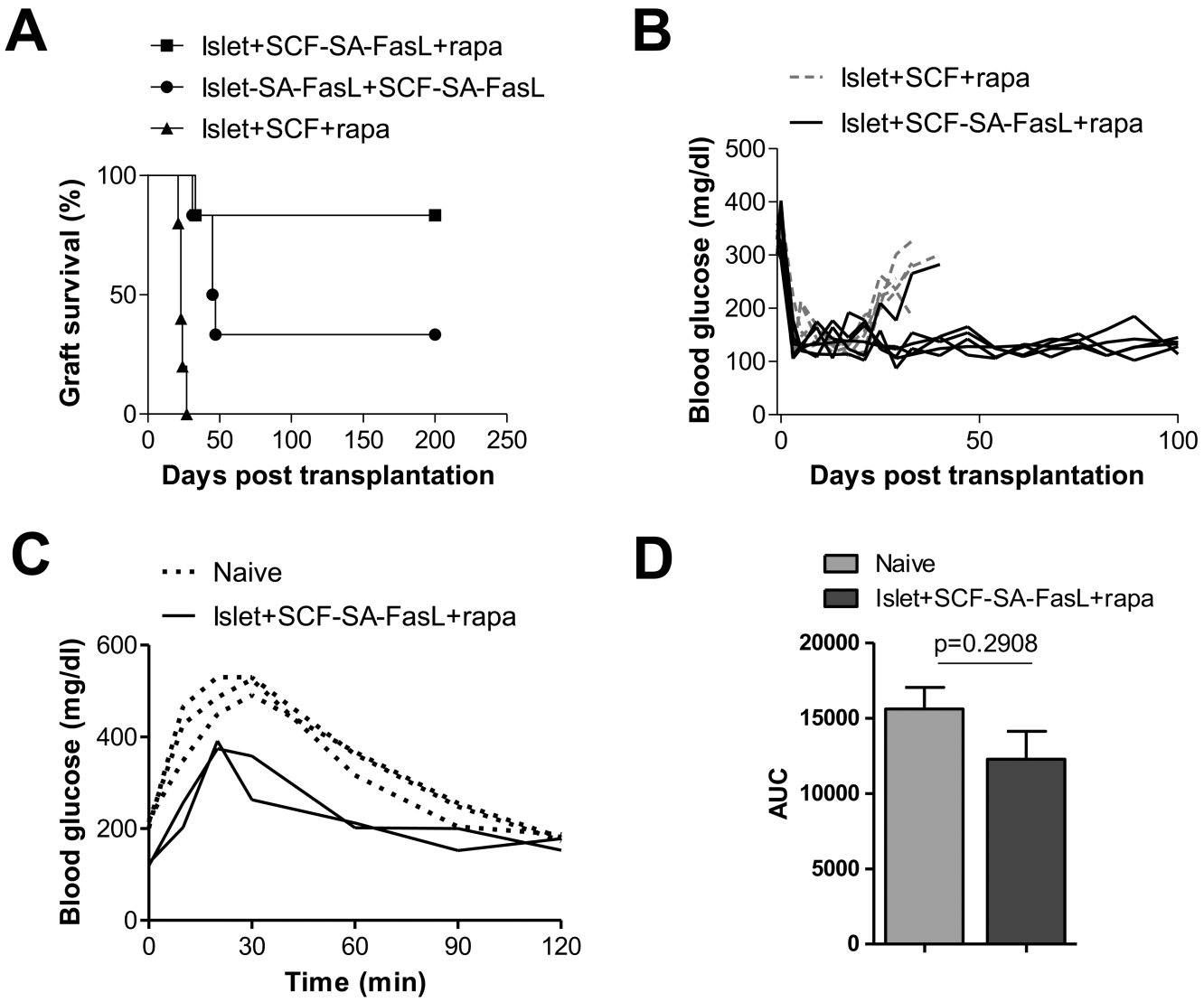


**Figure 5:**

Islets on scaffolds conjugated with SA-FasL demonstrate robust long-term tolerance. **(A)** Survival of allogeneic BALB/c islets mounted on PLG scaffolds and transplanted in the epididymal fat pad of chemically diabetic C57BL/6 recipients. Groups included naïve islets mounted on SA-FasL-engineered PLG scaffolds plus rapamycin (n = 6, MST > 200 days, P=0.0007 vs rapamycin alone), SA-FasL-engineered islets mounted on SA-FasL-engineered PLG scaffolds (n = 6, MST = 46 days, P = 0.0007 vs rapamycin alone), and naïve islets transplanted under a short cover of rapamycin (n = 5, MST = 23 ± 2.19 days). All mice received islets loaded on 2 PLG scaffolds. PLG scaffolds were engineered with 2.5 μg/scaffold, except 3 mice in the islet-FasL + PLG-SA-FasL that were transplanted with PLG scaffolds engineered with 0.5μg/scaffold and all 3 animals rejected their grafts. Rapamycin was given to the indicated groups through i.p. injection at 0.2 mg/kg daily for 15 days starting on the day of transplantation. Mice were monitored twice weekly for blood glucose levels. Those with > 250 mg/dL for two consecutive readings 24 hours apart were considered diabetic and rejecting the graft. Statistical analysis was performed using log-rank

test, **P<0.01, ***P<0.001. **(B)** Blood glucose readings of two groups of mice from (A). **(C)** Intraperitoneal glucose tolerance test (IPGTT) of long-term islet grafts compared to naïve C57BL/6 mice after fasting for 6 hours, followed by i.p. glucose injection. Blood glucose of mice was taken starting just before injection and at the indicated time points. **(D)** Area under the curve for **(C)**.