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Improved Coating of Pancreatic Islets with Regulatory T cells (Tregs) to Create Local Immunosuppression by Using Biotin-Polyethylene glycol-Succinimidyl Valeric Acid Ester Molecule

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Category 2:

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Abstract

Background—We showed that T regulatory (Treg) cells can be attached to the surface of pancreatic islets providing local immunoprotection. Further optimization of the method can improve coating efficiency, which may prolong graft survival. In this study, we compared the effectiveness of two different molecules used for binding of the Tregs to the surface of pancreatic islets. Our aim was to increase the number of Treg cells attached to islets without compromising islets viability and function.

Methods—Cell surface of human Treg cells and pancreatic islets was modified using biotin-polyethylene glycol (-PEG-) -N-hydroxysuccinimide (biotin-PEG-NHS) or -succinimidyl valeric acid ester (biotin-PEG-SVA). Then, islets were incubated with streptavidin as islet/Treg cells binding molecule. Treg cells were stained with CellTracker™ CM-DiL dye and visualized using Laser Scanning Confocal Microscope. The number of Treg cells attached per islets surface area was analyzed by Imaris software. The effect of coating on islet functionality was determined using Glucose-Stimulated Insulin Response (GSIR) assay.

Results—Coating procedure with biotin-PEG-SVA allowed for attaching 40% more Treg cells per 1 μm^2 of islets surface. While viability was comparable, function of the islets after coating using biotin-PEG-SVA molecule was better preserved than with NHS molecule. GSIR was 62% higher for islets coated with biotin-PEG-SVA compared to biotin-PEG-NHS.

Conclusion—Coating of islets with Treg cells using biotin-PEG-SVA improves effectiveness with better preservation of the islet function. Improvement of the method of coating pancreatic islets with Treg cells, could further facilitate the effectiveness of this novel immunoprotective approach and translation into clinical settings.

INTRODUCTION

Transplantation of pancreatic islets has become a promising therapy for treatment of patients with type 1 Diabetes Mellitus (T1DM).¹ However, there are still limitations in this procedure to be accepted as a routine therapy. One of them is significant loss of islet mass related to allo- and autoimmunity.² Despite improvements in current immunosuppressive therapies, prolonged and stable long term islets graft function in many cases still cannot be accomplished.³

It has been shown that Tregs can be used as immunosuppressive agents for autoimmunity⁴⁻⁶ and in allogeneic transplantation.⁷⁻⁸ However, it was postulated that Tregs need to traffic through lymphatic system to the graft site in order to provide graft immune protection. This may not be challenging after intravenous Treg infusion. Therefore, we developed a novel approach, in which we conjugated isolated and expanded Treg cells to the surface of islets in order to enable Treg/graft proximity and create local immunosuppressive environment. We applied biotin-PEG-NHS as a molecule for cell surface modification and streptavidin as a binding molecule. Our previous study proved that such strategy was feasible, did not

compromise islets viability or functionality.⁹ Moreover, it was effective in protecting islets from immune cells attack in the *in vitro* settings.⁹

In this study, we tested biotin-PEG-SVA, alternative to previously used biotin-PEG-NHS for cell surface conjugation and coating of pancreatic islets with Treg cells, and investigated the efficiency compared to previous method. Both NHS and SVA can be used for conjugation with primary amines and for stable amide link formation. However, the SVA functional group is more stable in aqueous buffer medium due to its longer half-life (33.6 min.) than that of NHS (0.75 min). We hypothesized that coating of islets with Treg cells via biotin-PEG-SVA molecule could be more effective without compromising islets viability and functionality.

METHODS

Cells Preparation

Islets were isolated from pancreases of C57BL/6 mice (6–7 weeks, males, Charles River Laboratories, USA) by the collagenase digestion.¹⁶ The islets were cultured in CMRL 1066 (Mediatech, Manassas, USA) + 2% Fetal Bovine Serum (FBS, Thermo Scientific, South Logan, USA) and 10 U/ml heparin (App Pharmaceuticals, Lake Zurich, USA) overnight to remove or sediment cells that were damaged during isolation process.

Human Treg cells were isolated according to the protocol described elsewhere^{17–19}. Briefly, Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteer blood donors by a density gradient centrifugation. Treg cells were separated in two steps isolation. Firstly, negative immunomagnetic cell sorting was applied in order to achieve CD4⁺ T cell population; secondly these cells were sorted with BD FACSARIAIII cell sorter to the following phenotype of Treg cells:

CD3(+)CD4(+)CD25(high)CD127(–). Treg cells were cultured in RPMI 1640 medium (Mediatech, Manassas, USA) with 10% FBS and interleukin-2 (IL-2; Novartis, Emeryville, USA) (1000 U/ml) after stimulation with anti-CD3/anti-CD28 expansion beads (0.5 bead/cell) (Life Technologies, Oslo, Norway) for 7–10 days. Before coating procedure, beads were removed from Treg cells.

Cells Surface Modification and Coating Procedure

Islets and Treg cells were washed 3 times with Hank's Balanced Salt Solution 1× (HBSS; Mediatech, Manassas, USA) supplemented with 11 mM glucose (Sigma Aldrich, St Louis, USA). 100 islets and 50×10⁶ splenocytes were incubated in either biotinylated biotin-PEG-NHS (MW=5000, Nanocs, New York, USA) solution or its derivative biotin-PEG-SVA (MW=5000, Laysan Bio Inc., Nanocs, Arab, USA) at the concentrations 1 mg/ml and 0.6 mg/ml for Treg cells and islets, respectively and incubated for 15 minutes. After incubation, cells were washed 3 times with HBSS 1× with 11 mM glucose separately. To islets 1 mg/ml of streptavidin (Sigma Aldrich, St Louis, USA) was added and they were incubated additional 15 minutes followed by 3 more washes with HBSS 1× with 11 mM glucose. Subsequently, islets were transferred to Treg cell suspension in CMRL1066 + 2% FBS on petri dish and incubated overnight in 37°C, 5% CO₂ gently swirling on the mixer.

Visualization of islets coated with Treg cells

To visualize Treg cells attached to the islets, before surface modification and coating procedures, they were stained with Cell Tracker™ DiL dye (Life Technologies, Eugene, USA) (50 µg/ml final concentration) and incubated for 10 min, then washed 3 times with HBSS 1x with 11 mM glucose. Islets coated with Treg cells were visualized using the Olympus FV1000 Laser Scanning Confocal Microscope (Olympus, Tokyo, Japan).

Assessment of the Coating Efficiency using NHS- or SVA-PEG-biotin

Three-dimensional (3D) reconstruction of confocal images and quantification of coating efficiency were performed using Imaris (South Windsor, CT, USA). A stack of confocal images (7 µm inclement) was loaded onto Imaris and 3D reconstructed with 3 µm inclement in order to reduce z-axis blurring. Noise was reduced with background subtraction with a filter width of 84 µm. Voxel intensities were smoothed using a Gaussian filter with a band of 1 µm. The islet surface area was determined by thresholding of islet voxels, with ~2 µm set as the smallest surface feature. DiI stained Treg cells were identified using the spots function as a sphere structure 7 µm in diameter. Treg cells-attached to islets and non-attached Treg cells (i.e. floating cells) were distinguished by a distance between the islet surface (captured by reflection) and Treg cells with a threshold of 5 µm. Efficiency of the coating using NHS- or SVA-PEG-biotin was examined and expressed as the ratio of Treg cells attached to islets to ½ of the islet surface area.

Assessment of the islets functionality after coating procedure – Glucose-Stimulated Insulin Response (GSIR)

Insulin Response tests were performed after coating procedure described above. Naked (control) or Treg cells coated islets were exposed to high (28 mM) and then to low (2.8 mM) glucose in Krebs-Ringer buffer (KRB) solution for 1 h at 37°C. The solutions of each glucose concentration were collected, concentration of released by islets insulin in each was determined by Enzyme-Linked Immunosorbent Assay (ELISA) using Mercodia Insulin ELISA kit (Mercodia AB, Uppsala, Sweden).

RESULTS

Islets can be effectively coated with Treg cells using alternative molecule: SVA-PEG-biotin

Tregs were stained with Cell Tracker™ DiL dye before coating procedure in order visualizes their attachment to islets surface. Detectable fluorescence under red light confirmed the presence of Treg on islets surface using either NHS- (Fig 1a) or biotin-PEG-SVA (Fig 1b) molecule for surface modification, indicating that coating with new SVA molecule was feasible. Red fluorescence intensity was stronger on images of coated islets when biotin-PEG-SVA was applied comparing to biotin-PEG-NHS molecule, demonstrating that more Treg cells were attached to islets surface.

Comparison of the Coating Efficiency using NHS- or SVA-PEG-biotin

In order to compare the coating efficiency between two molecules, confocal images of coated islets were re-constructed in 3-D and analyzed in Imaris software by assessing

number of spots assigned to red fluorescence coming from DiI-stained Treg cells per islet surface area captured by reflection using 488 nm excitation (Fig 2C). Analysis of confocal images by Imaris confirmed coating of islets with Treg cells using biotin-PEG-SVA is more efficient than with biotin-PEG-NHS. Based on Imaris analysis, the number of Treg cells attached to $1 \mu\text{m}^2$ of islets surface was 40% higher when biotin-PEG-SVA was used for coating, compared to the case when biotin-PEG-NHS molecule was used (Fig 2D) ($p < 0.05$).

Glucose-Stimulated Insulin Secretion in Coated with Treg cells Islets

Next, we investigated the influence of this new binding molecule and improved coating on islet function via glucose dependent insulin secretion in a static incubation assay. Even though stimulation index value measured for islets coated using biotin-PEG-SVA was 1.58 ± 0.2 and was slightly lower than unmodified control islets (1.8 ± 0.22), this was still higher than the group of islets coated using biotin-PEG-NHS (1.02 ± 0.02) (Fig 3).

These results suggested that coating of islets with Treg cells using biotin-PEG-SVA was not only more efficient, but also resulted in better islets function compared to previously used biotin-PEG-NHS molecule.

DISCUSSION

One of the major obstacles in transplantation of pancreatic islets for treatment of T1D is significant loss of islet cells mass post-transplant due to auto- and allo-immune reactions.² Therefore, Treg cells therapy can be potentially used to prevent such loss. We developed new approach to protect transplanted islets from destruction by immune cells at the graft site by conjugating *in vitro* isolated and expanded Treg cells onto islet surface and proved in the *in vitro* model that such approach is effective.⁸

Here, we suggest that we can further optimize and improve the method by replacing the previous molecule – biotin-PEG-NHS used for cells surface modification and can achieved covalent binding between islets and Treg cells with more reactive and stable derivative – biotin-PEG-SVA.

Images of coated islets with Treg cells proved that coating with biotin-PEG-SVA is more feasible when compared with biotin-PEG-NHS. (Fig. 1a and 1b). When biotin-PEG-SVA was used for coating, intensity of red fluorescence due to the presence of Tregs was higher compared to the images obtained with biotin-PEG-NHS molecule (Fig. 1a and 1b). This suggested that higher number of Treg cells could be immobilized onto single islet surface when biotin-PEG-SVA was replaced with biotin-PEG-NHS for conjugation. This hypothesis was further confirmed by confocal image analysis and Imaris software, where exact number of Treg cells attached per islet surface area was calculated. (Fig. 2C) The results demonstrated that 40% more Treg cells per $1 \mu\text{m}^2$ of islets surface via coating with biotin-PEG-SVA could be achieved in comparison with biotin-PEG-NHS conjugation (Fig. 2D). Furthermore, slightly better insulin secretion capability was measured in response to altered glucose concentration for the group of islets coated with SVA conjugate (Fig. 3).

In summary, further optimization of the method for coating of pancreatic islets with Treg cells allowed for higher number of Treg cell binding per islets surface with better islets functionality. Based on a previous *in vivo* study, the ratio of Treg cells to conventional T cells should be between 1:1 and 1:2.¹⁰ The optimization carried out here would be significant for transplantation stages, as substantial number of Treg cells would be required to protect implanted tissue from immune attack, which would be beneficial to test the efficiency in the *in vivo*, and subsequently in a clinical setting.

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Figure 1. Confocal laser scanning images of islets coated with Treg cells

(a) Coating with biotin-PEG-NHS, **(b)** Coating with biotin-PEG-SVA. At the top- images in bright field and at the bottom- images from green fluorescence. Prior to coating procedure, Treg cells were stained with red CellTracker DiL dye in order to visualize their attachment to surface of islets. Using both: biotin-PEG-NHS and biotin-PEG-SVA as binding molecules coating of islets with Treg cells was found to be feasible.

Figure 2. Comparison of coating efficiency using biotin-PEG-NHS and biotin-PEG-SVA (a) Coating with biotin-PEG-NHS. (b) Coating with biotin-PEG-SVA **A:** Representative views of confocal images of islets coated with Treg cells. Treg cells (DiI staining) are shown in red. Islet cells were captured by reflection using a combination of a 488 nm excitation filter and a 650 nm emission filter and pseudo-colored in green. **B:** 3D reconstruction of islet coated with Treg cells. **C:** Quantification of coating efficiency. Treg cells that attached to islet surface are marked with pink dots, and Treg cells floating in medium around islets are marked as blue dots. **D:** Comparison of coating efficiency. Results are expressed as mean $\pm$ SEM; n=3.

Figure 3. Assessment of insulin release in coated with Treg cells islets

Stimulation index (ratio of insulin secreted at 28 mM glucose to the amount of insulin secreted at and 2.8 mM) in control islets, coated islets using biotin-PEG-SVA and coated islets using biotin-PEG-NHS. The amounts of insulin were determined by ELISA. Results are expressed as mean $\pm$ SEM; n=3.