Coating Human Pancreatic Islets With $CD4^{+}CD25^{high}CD127^{-}$ Regulatory T Cells as a Novel Approach for the Local Immunoprotection

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Objectives: To develop a novel approach for local immunoprotection using $CD4^{+}CD25^{high}CD127^{-}$ T regulatory cells (Tregs) attached to the surface of the islets before transplantation.

Background: Tregs expanded ex vivo can control allo and autoreactivity, therefore, Treg-based therapy may offer more effective protection for transplanted islets from immunologic attack than currently used immunosuppression. Local application of Tregs can make such therapy more clinically feasible and efficient.

Methods: Human islets were isolated and coated with allogeneic ex vivo expanded Tregs using biotin-poly(ethylene glycol)-N-hydroxysuccinimide ester (biotin-PEG-NHS) and streptavidin as binding molecules.

Results: Coating pancreatic islets with Tregs did not affect islet viability (>90% fluorescein diacetate/propidium iodide) or the insulin secretion profile in dynamic islet perifusion assays. After in vitro incubation with allogeneic T effector cells, Treg-coated islets revealed preserved function with higher insulin secretion compared with controls-native islets, coated islets with T effector cells or when Tregs were added to the culture, but not attached to islets ($P < 0.05$). In addition, the Enzyme-linked immunosorbent spot (ELISPOT) assay revealed suppression of interferon (IFN)-γ secretion, when T effector cells were challenged with Treg-coated islets comparing to controls (99 ± 7 vs 151 ± 8 dots, respectively; $P < 0.01$).

Conclusions: We demonstrated, for the first time, the ability to bind immune regulatory cells to target cells with preservation of their viability and function and protective activity against immune attack. If successfully tested in an animal model, local delivery of immunoprotective Tregs on the surface of transplanted pancreatic islets may be an alternative or improvement to the currently used immunosuppression.

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Pancreatic islet transplantation offers advantages of a minimally invasive procedure in comparison to the pancreas transplantation for brittle persons with type 1 diabetes with hypoglycemia unawareness. However, effectiveness of this therapy is severely compromised by gradual islet mass loss after transplantation.[1,2] Because the islet loss is substantially higher after islet allotransplantation in persons with type 1 diabetes than after autotransplantation due to chronic pancreatitis, it seems that recipient related allo- and autoreactivity in combination with immunosuppression ineffectiveness and toxicity are dominant factors responsible for the failure of the procedure in persons with type 1 diabetes. Recently, it has been demonstrated that the use of stronger immunosuppression than proposed in the Edmonton protocol might improve results to some extent.[3,4] Furthermore, recent studies indicate that the fate of islet grafts are predetermined by the level of autoreactive T cells before transplantation regardless of the type of immunosuppression.[5,6] The same conclusion was found on the basis of results from a large number of patients from Edmonton (B. Roep, oral communication at Levine Symposium, 2011). Moreover, the data indicate, that even the strongest currently available multiagent immunosuppression (including cyclophosphamide and antithymocyte globulin) applied in autologous stem cell transplantation in persons with recently diagnosed type 1 diabetes was not able to control autoimmunity in those patients who had high activity of anti-glutamic acid decarboxylase and anti-Insulinoma Antigen 2 autoreative CD8 T cells before transplant[7] (B. Roep, oral communication at Levine Symposium, 2011). In this light, it seems to be obvious that previous trials, which tested effectiveness of single drug immunosuppression, must have failed to prevent progression of type 1 diabetes in patients with new onset of the disease.

Therefore, looking for a new approach, we found regulatory T cells (Tregs) as a potential remedy. It was shown previously that Tregs can control allo- and autoimmunity and can be used successfully for the adoptive transfer in many animal models.[8–12] However, only recent advances in technology allowed for effective isolation and expansion of Tregs ex vivo and first clinical applications. Preliminary results in bone marrow transplant patients to prevent or treat Graft Versus Host Disease (GVHD) are very encouraging.[13,14] However, currently, Tregs have only been applied systemically, which requires application of very high number of cells. Systemic infusion of Tregs continues to have two known obstacles: (1) the large number of cells is still challenging to obtain, and (2) bears unknown risk of generalized, systemic immunologic unresponsiveness, opportunistic infections, or neoplasm.

Therefore in our study we proposed a new approach, where Tregs are isolated from peripheral blood, expanded ex vivo and then still in vitro attached to freshly isolated islets before transplantation. In the next step, Treg coated islets would be infused into the patients, so Tregs would localize and play an active role in the creation of an immunosuppressive environment at the engraftment site and local lymph nodes (once detached from islets), which are considered as the optimal locations for immunologic graft protection. In this way, the effect would be focused locally thus the immune modulation will be stronger and thus safer and more precise. Our approach is in contrast to

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systemic infusion of Tregs, where they are dispersed in the lymphatic system and need chemokine trafficking to find the graft site. Our goal also is not to create mechanical barrier as in microencapsulation approach, because limited fluid and molecule exchange between islet and environment must eventually, in our opinion, compromise islet graft function.

In the current study, we tested in vitro feasibility of our approach, whether Tregs can be effectively attached to the surface of the islets without compromising cell viability and function and whether those Tregs may protect islets from immunologic destruction.

METHODS

Cell Preparation

Pancreatic islets were isolated from the human cadaveric pancreata in the University of Chicago current Good Manufacture Practices cellular isolation facility according to the Ricordi modified method.[15,16] Liberase HI (Roche, Indianapolis, IN) was used for the organ digestion and COBE 2991 cell processor (CaridianBCT, Inc., Lakewood, CO) for islet separation with the continuous density gradient. Islet yield and purity were assessed after dithizone staining (Sigma Chemical Co., St. Louis, MO; 2 mg/mL).

Tregs and T effector cells (Teffs) were isolated according to our previously described protocol.[13,17] In brief, peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from volunteer blood donors by Ficoll/Uropoline gradient centrifugation. Then, negative immunomagnetic sorting (StemCell Technologies, Canada) was applied for $CD4^+$ T cells (96%–99% purity) separation. Subsequently, $CD4^+$ T cells were stained with the following cocktail of monoclonal antibodies, all purchased from BD Biosciences (San Jose, CA, USA) (fluorochrome and clone in the brackets): anti-CD3 (PacificBlue or PE-Cy7, UCHT1), anti-CD4 (APC, RPA-T4), anti-CD8 (PerCP, SK1), anti-CD19 (PerCP, 4G7), anti-CD14 (PerCP, MφP9), anti-CD16 (PerCP-Cy5.5, 3G8), anti-CD25 (FITC, M-A251), anti-CD127 (PE, hIL-7R-M21), and 7-AAD Via-probe. The cells were sorted with BD Biosciences FACSAria cell sorter (BD Biosciences, San Jose, CA, USA) to the following phenotype of Tregs: $CD3^+CD4^+$ $CD25^{high}CD127^-$doublet$^-$ lineage$^-$dead$^-$and Teff cells : $CD3^+CD4^+CD25^-CD127^+$doublet$^-$ lineage$^-$dead$^-$. Next Tregs were in vitro expanded according to the previously described procedure.[17] In brief, Tregs were cultured in RPMI 1640 medium supplemented with glutamine (Gibco, Carlsbad, CA, USA), 10% human inactivated AB serum and interleukin 2 (1×10^4 U/mL, Aldesleukin, Chiron) with CD3/CD28 beads (T cell expander, Invitrogen, San Diego, CA) in a 1:1 ratio. The cells were passaged every day for 14 days. At the day +7, additional beads were added to equalize 1:1 bead/Tregs ratio. After expansion but before coating, a quality check of expanded Tregs was performed. To pass the test, Tregs had to maintain the sorted Tregs phenotype with FoxP3 level at least 80% and they had to suppress IFNγ secretion from autologous Teff cells.[17]

Teff cells were cultured in the same conditions as Tregs (RPMI 1640 medium, glutamine, 10% human inactivated AB serum, interleukin 2, and CD3/CD28 beads in a 1:1 ratio). For the experiments in vitro Teff cells were mixed with PBMC from the same donor in ratio 1:1 (Teff-PBMC).

Islet Coating

Islets and Tregs were washed with Hank's Balanced Salt Solution (HBSS) 3 times. Then, Tregs and islets were incubated separately in biotinylated poly(ethylene glycol)-N-hydroxysuccinimide (biotin-PEG-NHS) solution, (0.25 mg/mL for 2×10^6 cells and 0.45 mg/mL/400 islets) for 10 min at 37°C on the shaker. Subsequently, islets and Tregs were washed 3 times with HBSS. Then, Tregs were incubated with streptavidin (0.1 mg/mL) for 7 min at 37°C on the shaker, washed 3 times with HBSS and transferred to Petrie dish with the islets for 30-min final coating at 37°C on the shaker. Scheme of the coating protocol is presented in the Figure 1. The same procedure was applied for islets coated with allogeneic Teff-PBMC.

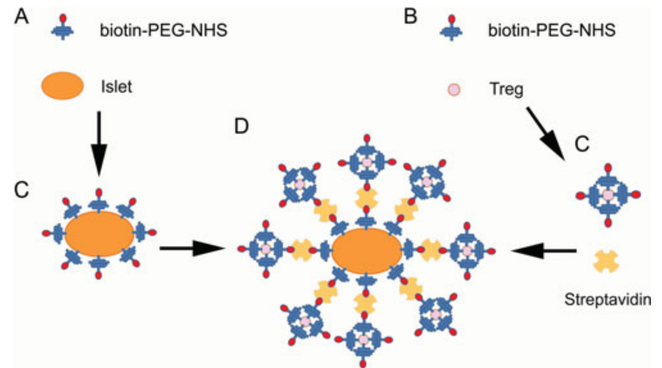

FIGURE 1. Scheme of the coating procedure. First biotin-PEG-NHS was attached to the surface of the islets (A) and Tregs (B), separately. Then, streptavidin was added to Treg suspension (C). Subsequently, modified islets and Tregs were mixed together to attach Tregs to the islet surface via NHS-PEG-biotin-streptavidin-biotin-PEG-NHS bonds (D).

Assessment of the Coating Efficiency

To visualize the coating effectiveness, Treg were stained with Hoechst dye (Invitrogen, San Diego, CA) before coating. In brief, 5×10^6 Tregs were washed twice with phosphate-buffered saline (PBS) and incubated in Hoechst solution (2 μg/mL) for 30 min at room temperature. Next, cells were washed thrice with HBSS and subjected to the coating procedure as described earlier. Coating effectiveness and stability was assessed under the fluorescence microscope (Nikon, Tokyo, Japan) immediately and 1 day after the coating. Additional images were taken using an Olympus IX80 DSU confocal microscope (New York, NY) with SlideBook software (Minneapolis, MN). Three-dimensional reconstruction of a stack of optical images was carried out using Imaris (Biplane, Saint Paul, MN). T cell coordinates were determined using manual cell mapping with Stereo Investigator (MicroBrightField, Wiliston, VT) software. Cutting plane reconstruction was performed by Mathematica (Wolfram Reserch, Champaign, IL).

Assessment of Cell Viability After the Coating

To assess the viability of the cells after modification, Treg coated islets were stained with propidium iodide (PI, Sigma, St Luis, MO) and fluorescein diacetate (FDA; Sigma, St Luis, MO), which is the current standard viability test before clinical islets transplantation. Treg coated islets were placed on Petri dish and suspended in 787 μL of phosphate-buffered saline. Then, PI and FDA were added (151 μL and 19 μL from stock solutions, respectively) and mixed. Subsequently viability was assessed. Living cells actively converted the nonfluorescent FDA into the fluorescein emitting green fluorescence; whereas, dead cells were permeable for PI and exhibit red fluorescence. Therefore, cells with green fluorescence (fluorescein positive) were considered viable, whereas, red fluorescence (PI positive) was a sign of cell death.

In addition, viability of the Tregs stained with Hoechst attached to the islets was confirmed with PI staining. After coating, islets with bound Tregs were incubated in PBS with 14.34 μM PI and transferred to glass bottom dish for imaging.

Assessment: Whether Coating Procedure Affected the Function of the Islets

To assess, whether the coating affects the islet function, naked islets (100 islets/mL/well), Treg coated islets (100 islets/mL/well),

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and Teff-PBMC coated islets (100 islets/mL/well) were cultured in CMRL 1066 (Cellgro,) with FBS (10%), L-glutamine and penicillin and streptomycin (100 U/mL and 100 μg/mL respectively; Sigma Aldrich, Germany) for 12 hours and then subjected to the dynamic perifusion assay.

Assessment: Whether Tregs Maintain Their Immunomodulatory Function and Protect Coated Islets From Destruction by Alloreactive Teffs

Long (72 h) cultures were prepared to assess, if Tregs attached to the surface of the islets protects them from allogeneic Teff cells. The clinical scenario, where Tregs would be isolated from Type I diabetic islet recipients was mimics by ensuring the Tregs were host origin, so autologous to Teffs but allogeneic to islets. Thus, Treg coated islets (100 islets/mL/well) were cultured with allogeneic Teff-PBMC (1.5 mln cells/mL/well) in CMRL 1066 (Cellgro,) with Fetal Bovine Serum (FBS) (10%), L-glutamine and penicillin and streptomycin (100 U/mL and 100 μg/mL respectively; Sigma Aldrich, Germany). In parallel, controls were prepared (1) naked islets only (100 islets/mL/well); (2) naked islets (100 islets/mL/well) cocultured with Teff-PBMC (1.5 mln cells/mL/well); (3) naked islets (100 islets/mL/well) cocultured with Teff-PBMC (1.5 mln cells/mL/well) and Tregs (1.5 mln cells/mL/well) not attached to the islets but freely floating in the medium, (4) Teff-PBMC coated islets (100 islets/mL/well) exposed to free Teff-PBMC in the medium (1.5 mln cells/mL/well).

Subsequently, dynamic perfusion assay was performed for islets from each of above groups separately to assess islet function and the degree of islet destruction.

Dynamic Perifusion Assay

Dynamic perifusion assay allows assessment of islet function based on insulin secretion in response to changing concentrations of glucose during the perifusion. Thirty coated or naked islets from groups described earlier were placed separately in the flow-through perifusion chamber (Millipore Corp.) of the perifusion machine PERI-04 (Biorep Technologies). Fluid was driven through the chamber at a rate of 1 mL/min and was collected downstream in 96-well plates. Islets were perifused for 60 min at 37°C, with a modified Krebs–Ringer bicarbonate buffer, containing Bovine Serum Albumin (0.5% w/v) and a basal low glucose concentration (3.3 mM, at pH 7.4). Subsequently, the concentration of the glucose changed to high-16.7 mM for 20 min, followed again by (low-3.3 mm) buffer for 20 min. Finally, the islets were perifused with KCl (33 mm) in Krebs–Ringer bicarbonate solution for the last 10 min to release the remaining amount of insulin. Insulin concentration was assessed in all samples with enzyme-linked immunosorbent assay (ELISA) according the manufacturer instruction. Samples were collected every 1 minute of the stimulation and insulin was measured in the samples from selected time points, which are depicted on the x-axis. Results were expressed as *Stimulation Index* values at several time points and plotted as a line on the figure. *Stimulation Index* was defined as the ratio of insulin released at a certain time point to the lowest basal level of insulin. Thus, use of *Stimulation Index* eliminates bias resulted from unequal islet size and different beta-cell mass within single islets.

To correlate degree of islet destruction by Teff-PBMC activity, IFN-γ secretion was measured with ELISPOT method.

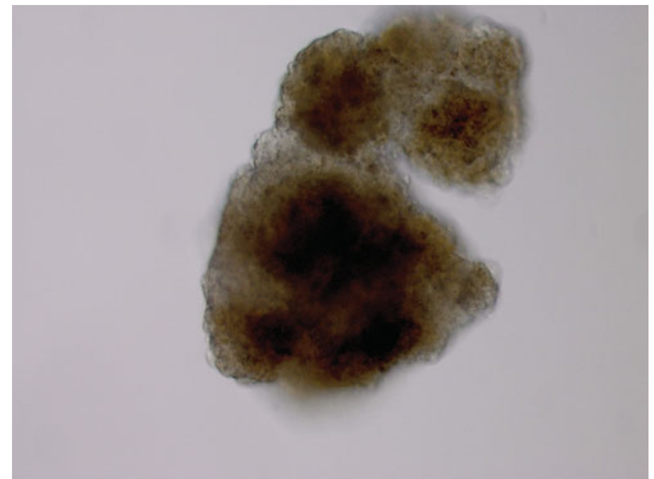
Measurement of IFN-γ Secretion by Allogeneic Teff-PBMC

Naked and coated islets were cocultured for 12 h with Teff-PBMC in the same groups described earlier. In additional, positive control group anti-CD3 antibody was added to stimulate Teff PBMC to IFN-γ secretion. Amount of IFN-γ in the media was measured using ELISPOT according to the manufacturer instructions. Each experiment was made in triplicates, n = 5, data are presented as mean number of spots ± SE.

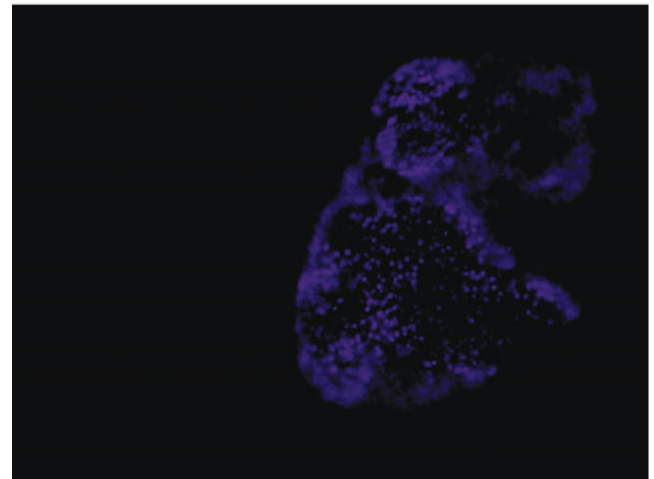
RESULTS

Tregs Were Effectively Attached to the Surface of the Pancreatic Islets Through NHS-PEG-Biotin-Streptavidin-Biotin-PEG-NHS Bonds

Tregs attached to the islets stained with Hoechst before coating procedure were not seen in light microscopy but exhibited homogeneous fluorescence in the ultraviolet light in contrast to uncoated islets (Fig 2). Additional confocal images confirmed effectiveness of the coating, the surface of the islets was covered with Tregs (Fig 3).



(A)



(B)

FIGURE 2. Tregs are effectively attached to the islets. Light and ultraviolet fluorescence microscopy imaging. Pictures present micrographs of the Treg coated islet: (A) in light microscopy, which is unable to visualize cells attached to the islet surface. Therefore, before coating, Tregs were stained with Hoechst and visualized in ultraviolet light due to blue fluorescence. It allowed to observe Tregs in blue on the surface of the islets after the coating (B).

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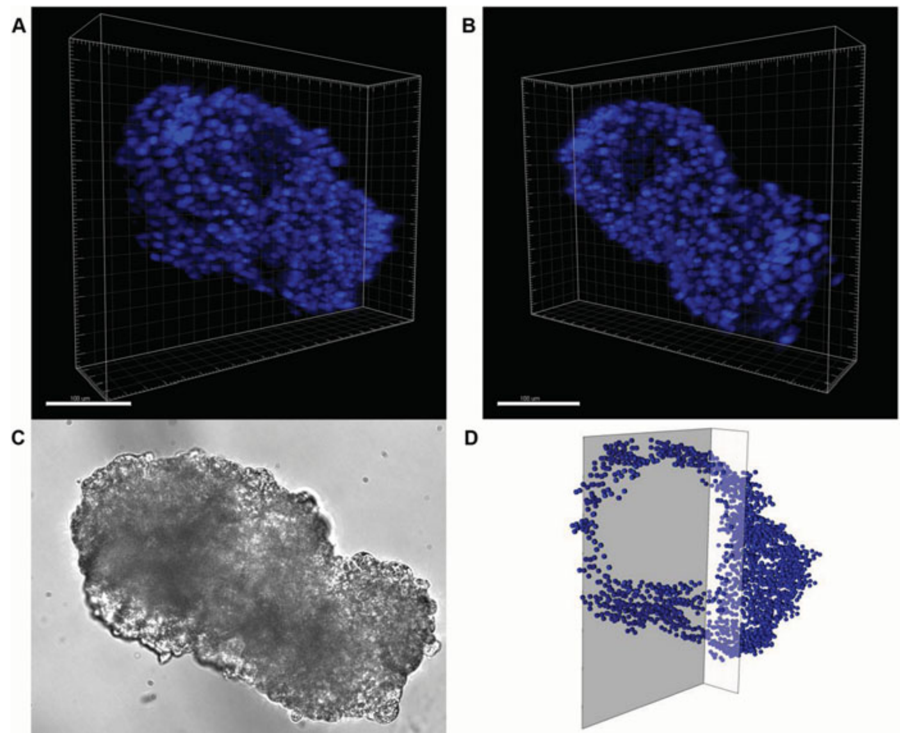

FIGURE 3. Tregs are effectively attached to the islets. Confocal microscopy imaging. Series present visualization of islet coating with T cells in confocal microscopy after 1-day culture. **A,** Three-dimensional view of a human islet coated with T cells (nuclei stained with Hoechst shown in blue) reconstructed from a stack of 18 optical images with 5-μm increment. **B,** Alternative viewing angle of the islet shown in A. **C,** Bright field image of the human islet. **D,** Cutting plane view reconstructed from T cell coordinates exposing the coating layer of T cells surrounding the unstained islet cells.

Coating Islets With Tregs Using Biotin-PEG-NHS and Streptavidin as Biding Molecules did not Affect Cell Viability

Freshly coated islets exhibited strong green fluorescence after staining with FDA and PI. Only a minimal number of PI positive (dead) cells were found also after 1-day culture so overall viability was high (>90%), and met criteria for clinical transplantation (Fig 4A). Separately, PI staining of the islets coated with Hoechst labeled Tregs excluded a substantial rate of the cell death after 1-day culture (Fig 4B).

Coating With Tregs did not Affect the Islet Function

Twelve hours after coating, the perifusion dynamic assay was performed to assess the effect of the coating procedure on islet function. The assay showed comparable profiles and amplitudes of insulin secretion curves by Treg or Teffs coated islets and naked islets, index of insulin secretion did not differ statistically (NS); (Fig 5).

Function of the Treg coated islets was better preserved after 72 h of coculture with allogeneic Teff-PBMC cells comparing to uncoated islets and other groups exposed to Teffs-PBMC (Fig 5; $P < 0.05$). Therefore, Tregs attached to the islets better protected them from immune destruction driven by allogeneic leukocytes compared to unattached Tregs or when none or other cells were attached to islets.

First, we confirmed that coating did not affect islet function even after 72-h in vitro culture. Uncoated islets and Treg-coated islets incubated alone exhibited similar profile and amplitude of insulin secretion in response to glucose and KCl (NS; Fig 6). However, in the presence of allogeneic PBMC enriched with T effector cells naked islets lost their ability to release insulin. Native islets challenged for 72 h with allogeneic Teff-PBMC did not respond to the high glucose and KCl—a flat, horizontal orange line on Figure 6. Similar results were observed for islets coated with allogenic Teff-PBMC or when uncoated islets were cocultured with free, unattached Tregs and exposed to Teff-PBMC (Fig 6). In contrast, Treg coated islets exposed to allogeneic Teff-PBMC for 72 h demonstrated well-preserved response to the high glucose and KCl solution with insulin secretion although slightly lower then uncoated islets unexposed to Teff-PBMC ($P < 0.05$; Fig 6).

To confirm that the islet function was compromised as a result of immunologic attack by allogeneic Teff-PBMC, we analyzed INF-γ secretion by Teff-PBMC as a marker for enhanced immunologic activity.

Treg Coated Islets Were Less Immunogenic

Naked islets induced secretion of IFN-γ by allogeneic Teff-PBMC. Similar intensity of IFN-γ secretion was observed when Teff-PBMCs were incubated with islets coated with Teff-PBMC. Addition of free, unattached Tregs to the naked islets coculture with Teff-PBMC significantly decreased secretion of the cytokine ($P < 0.05$). However, the lowest secretion of IFN-γ was observed, when Teff-PBMCs were exposed to Treg coated islets—99 $\pm$ 7 versus 151 $\pm$ 8 dots for naked islets cocultures ($P < 0.01$; Fig 7).

DISCUSSION

Natural Treg cells are a subset of $CD4^+$ T cells originated from thymus which play a significant role in maintenance of peripheral tolerance. They are known to actively block immune responses, inflammation, and tissue destruction suppressing the functions of conventional $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, NK cells, antigen-presenting cell function and maturation, and B cell antibody production and affinity maturation.[18–20] Tregs also play an important role in inhibition of transplant rejection.[18] Previous studies revealed that graft survival positively correlates with the number of circulating Tregs,[21,22] and that the adoptive transfer of these cells promotes tolerance to allogeneic pancreatic islet grafts in animals.[23] Consequently, Tregs are excellent candidates to play major role in new protocols

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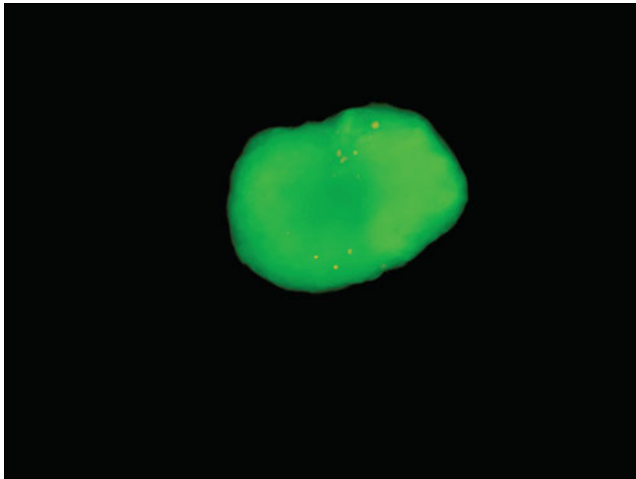
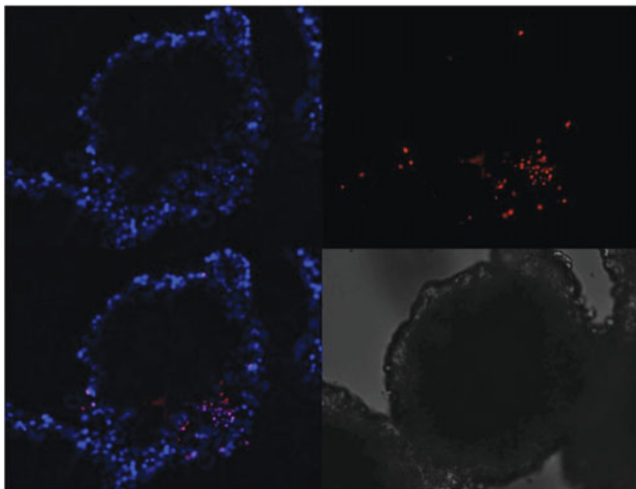


(A)



(B)

FIGURE 4. The coating procedure does not affect islet and Treg viability. **A,** Treg coated islets were stained with fluorescein diacetate and PI after 1-day culture in aim to assess cell viability. Cells positive for fluorescein (green fluorescence) are viable, whereas, cells positive for PI (red fluorescence) are dead. Viability of more than 90% of Treg coated islets was confirmed on the basis of green and red fluorescence. **B,** Confocal images of Tregs attached to the islet, stained with Hoechst before coating. After a day of culture cells were stained with PI to detect death. Upper left—blue Tregs are surrounding invisible islet, what confirms in addition, is effectiveness of the coating; Upper right—red fluorescence of dead cells stained with PI; Lower left—superimposed Hoeschst and PI stained cells; only few double positive (red and blue)—dead Tregs seen in pink color; and Right lower—the same islet seen in brightlight. Viability of the islets and Tregs was well.

involving transplant immunomodulation. An additional advantage, which is critical in persons with type 1 diabetes, is that Tregs are also able to control autoreactive destruction of transplanted islets. In our study, we tested the feasibility of a new approach for local immunoprotection, where regulatory T lymphocytes were bound in vitro with pancreatic islets. After separation from the peripheral blood and expansion ex vivo Tregs were bound in vitro to the sur-

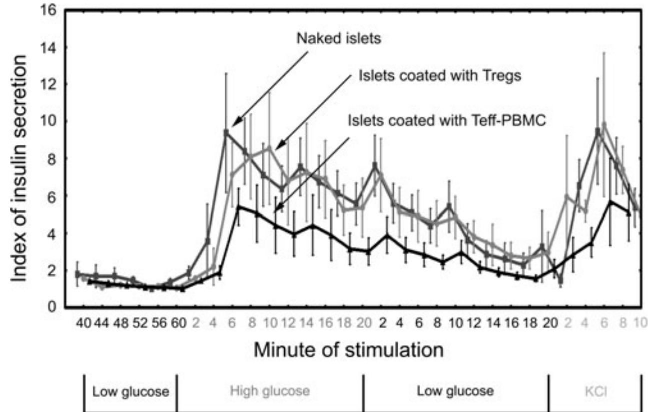


FIGURE 5. Coating with Tregs does not affect islets function. Diagram presents dynamic perifusion assay results and insulin secretion by naked islets, islets coated with Tregs and islets coated with Teff-PBMC 12 hours after coating. Insulin secretion is expressed as a *Stimulation Index* (SI) and presented as mean SI $\pm$ SE ($n = 5$). There was no statistical difference in insulin secretion based on SI between the groups (NS).

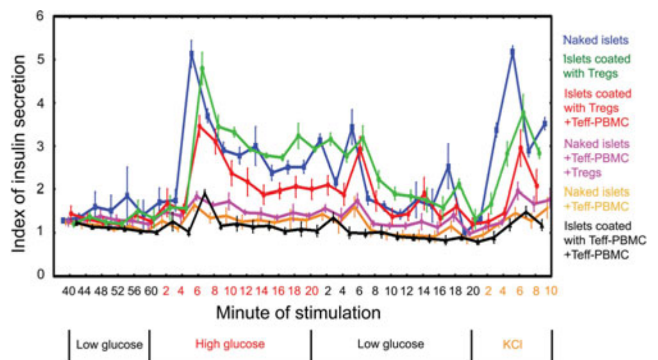


FIGURE 6. Function of the Treg coated islets was preserved despite exposure to allogeneic effector T cells. Diagram presents dynamic perifusion assay results presented as mean SI $\pm$ SE. Naked islets (blue line) and islets coated with Tregs (green line) as positive controls were not exposed to allogeneic Teff-PBMC. Both groups expressed comparable insulin response profile proving preserved islets function after 72-h culture. In the remaining conditions, islets were exposed to allogeneic Teff-PBMC for 72 h. Islets coated with Tregs and exposed to allogeneic Teff-PBMC (red line) expressed preserved insulin response in contrast to other groups-naked islets exposed to allogeneic Teff-PBMC (orange line), naked islets exposed to allogeneic Teff-PBMC in presence of free Tregs (pink line) or islets coated with Teffs-PBMC and exposed to allogeneic Teff-PBMC (black line; $P < 0.05$).

face of the pancreatic islets using biotin-PEG-NHS and streptavidin molecules. Optimal conditions for the attachment of biotin-PEG-NHS molecule to the surface of the islets were established during our previous study, where Glucagon-like peptide-1 was bound to the surface of the islets.[24] Now, the same molecules were used for the first time to bind a living cell to islet. Therefore, we optimized the concentration of the same molecules for Tregs before coating (data not shown). To our knowledge, for the first time functional human cells (not cell line) are attached to another functional cell (or group of cells) for the pur-

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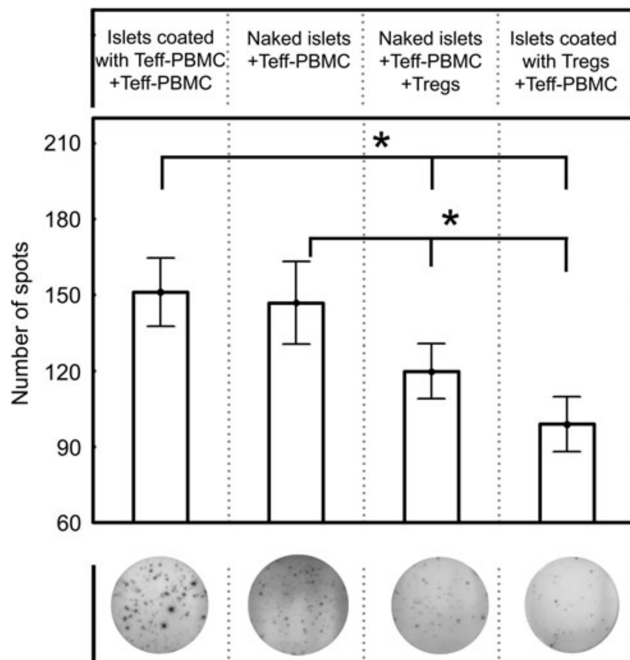

FIGURE 7. Treg coated islets induce lower IFN-γ secretion by allogeneic T effector and PBMC cells than naked islets. Diagram presents IFN-γ secretion by allogeneic Teff-PBMC exposed to naked and coated islets and expressed as number of spots in ELISPOT. The upper panel shows statistics. The lower panel presents photos of the test results. Differences, which were statistically significant ($P < 0.05$; ANOVA), are marked with "*". Although, addition of free, unattached Tregs to the naked islets coculture with Teff-PBMC significantly decreased secretion of the IFN-γ ($P < 0.05$). The strongest suppression of the Teff-PBMC; therefore, the lowest secretion of IFN-γ was observed, when Teff-PBMCs were exposed to Treg coated islets ($P < 0.05$).

pose of immunoprotection. Our results confirmed that viability and function of islets and Tregs was not compromised after the binding procedure. The profile and amplitude of insulin response to changing glucose concentration was the same for both Treg coated and naked islets.

In addition, we confirmed in vitro that Tregs attached to the surface of the islets can effectively prevent immunologic destruction driven by allogeneic effector T cells. Insulin response was much better preserved for Treg coated islets than naked islets and islets coated with other cells from the same individual, when challenged with allogeneic Teff-PBMC. Moreover, the level of INF-γ secreted by Teff-PBMC exposed to Treg coated islets was lower, than observed for naked islets and islets coated with other cell type. Interestingly, the protective effect of Tregs attached to the islets was stronger than unattached Treg cells, which previously was shown as a very efficient immunosuppressive approach.[8–12] The immunoprotective effect seems to be related to specific properties of the Tregs attached to the islets rather then to mechanical separation of islets and Teff-PBMC. The immunoprotective effect was not seen, when Teffs-PBMC cells from the same individual as Tregs were used for the coating. Because Tregs do not proliferate and do not form impermeable layer around the islets, it is unlikely that they can prevent from cytokine or antibody related islet destruction only by mechanical separation. Nevertheless, now we are testing the effect of the binding other type of cells to the islets limiting immune cell interactions, using K562-line cells without MHC molecules.

Historically, all attempts to create local immunoprotection around the islets were conceptually focused on construction of a mechanical barrier between the engrafted islets and host immune system. However, so far, all known encapsulation methods have failed to produce a clinically successful system. The most commonly used material-alginate was found to slow down the insulin diffusion,[25] diminish acute insulin secretion response of the islets,[26] cause oxygen and nutrient deprivation and induce a nonspecific foreign body reaction finally leading to the islet loss.[27,28] To improve biocompatibility, encapsulation of the islets with living cells was also tested (rat islets with porcine chondrocyte) but eventually also failed.[29] Recently, human islets were coated with HEK293 cells (human endoderm kidney cell line) for microencapsulation and immunoseparation. However, those cells overgrew the islet leading to compromised communication of islets with the local environment, islet central necrosis and slightly diminished insulin secretion.[30,31] Therefore, in our approach, we did not aim for immunoseparation at all, because it would likely compromise fluid and molecule exchange. We believe that recipient Tregs are optimal cells to be attached to islets to create an immunoprotective microenviroment around them. First of all, Tregs are active suppressor cells, which not only prevent alloantigen recognition, but also actively affect the function of most of cells involved in immunologic response. The immune inhibition includes the autoreactive response what is critical for islet transplantation success in persons with type 1 diabetes. In addition, as long as Tregs are attached to the islets, they may protect them passively; covering the surface of the graft, they increase islet immunocompatibility, as they are host origin. They do not proliferate, so there is no risk that they will overgrow limiting fluid exchange between islets and environment, like tested cell line.[30,31]

Bonds between the islets and Tregs are covalent so are relatively strong. Binding molecules are anchored on proteins of islet cell matrix and Treg cell membrane. However, because of protein turn over, which is shorter on the Treg cell membrane than on the islet, the islets and Treg will detach in vivo after the transplantation over some period of time. It may be also caused by variety of enzymes in vivo. We do not consider islet and Treg detachment as a potential failure, because the role of the Tregs is to protect the transplant in the place of the engraftment by their immunosuppressive activity, not physically separate islets from the environment, such as in microcapsules. We speculate that even after the detachment, Tregs would stay in the region of the graft due to chemokines secreted by host effector cells attacking the graft. Tregs may also go to the local lymph nodes and play tolerogenic role. In our approach, Tregs were attached to the islets to focus the Tregs at the graft site much more efficiently than after systemic infusion when Tregs are dispersed through the body and then traffick guided mainly by chemokine receptors. Therefore, we did not test stability of the bonds in vitro for an extended period of time.

As a next step, we plan to test effectiveness of our technique in an animal model. Tregs will be isolated from transplant recipients before the procedure and ex vivo expanded. If it is successful, translation of the proposed approach into a clinical setting is feasible. Currently, we are able to isolate enough Tregs from 500 mL of patient peripheral blood and expand them in vitro for clinical application. It was shown that Tregs isolated from blood of persons with type 1 diabetes are as immunosuppressive as Tregs isolated from healthy individuals[32] (also our unpublished data). We have initiated the first clinical study with systemic use of Tregs in prediabetic children in Poland and a similar study is recruiting patients in San Francisco (personal communications). Other centers in Europe have started using Tregs in kidney

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transplant recipients (M. Battaglia, oral communication at Levine Symposium, 2011).

The Biotin-PEG-NHS molecule used for binding the cells together has been found to be nontoxic and nonimmunogenic and may promote islet survival in vivo limiting ischemia reperfusion injury.[31] PEG has an established history in wide range of approved clinical applications as a material that can be implanted into the human body. Another advantage of this kind of polymer is that it allows development of a uniformly thin coating despite islet populations that are heterogeneous in size and does not affect the islet function and viability.[24] Furthermore, as we confirmed in our previous studies the thickness of this coating can be strictly controlled by the procedure conditions,[24] which is important in clinical studies requiring standardized techniques.

Concluding, we are proposing a novel approach for a local immunoprotection, which combines natural immunosuppressive capacity of Treg cells with bioengineering technology. Our data suggest that coating of pancreatic islets with Tregs may prevent immune-mediated rejection and does not affect the cell viability and function. Local delivery of immunosuppressive Tregs on the surface of pancreatic islets to the engraftment site is a clinically feasible approach. If further animal studies confirm its effectiveness and safety of the approach, it may be utilized for improvement of islet transplantation.

REFERENCES

1. Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med.* 2006; 355(13):1318–1330.
2. Kobayashi N, Okitsu T, Lakey JR, et al. The current situation in human pancreatic islet transplantation: problems and prospects. *J Artif Organs.* 2004;7(1):1–8.
3. Koh A, Imes S, Kin T, et al. Supplemental islet infusions restore insulin independence after graft dysfunction in islet transplant recipients. *Transplantation.* 2010;89:361–365.
4. Hering B, Bellin M, Barton F. Induction immunosuppression with T-Cell depleting antibodies facilitates long-term insulin independence after islet allotransplantation in type I diabetes. *Am J Transplant.* 2010;10(suppl 4):141–142.
5. Huurman VA, Hilbrands R, Pinkse GG, et al. Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. *PLoS One.* 2008; 3(6):e2435.
6. Hilbrands R, Huurman VA, Gillard P, et al. Differences in baseline lymphocyte count and autoreactivity are associated with differences in outcome of islet cell transplantation in type 1 diabetic patients. *Diabetes.* 2009;58:2267–2276.
7. Voltarelli JC, Couri CE, Stracieri AB, et al. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *JAMA.* 2007;297(14):1568–1576.
8. Kim JI, O'connor MR, Duff PE, et al. Generation of adaptive regulatory T cells by alloantigen is required for some but not all transplant tolerance protocols. *Transplantation*. 2011;91(7):707–713.
9. Cao J, Li L, Chen C, et al. Influence of mouse genetic engineering regulatory T cells infusion on post-allogeneic bone marrow transplantation acute graft-versus-host disease in mice. *Zhonghua Xue Ye Xue Za Zhi.* 2011;32(2):83–88.
10. Feng G, Wood KJ, Bushell A. Regulatory T cell enrichment by IFN-γ conditioning. *Methods Mol Biol*. 2011;677:281–301.
11. Karim M, Feng G, Wood KJ, Bushell A. $CD25^+CD4^+$ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation. *Blood*. 2005;105(12):4871–4817.
12. Feng G, Wood KJ, Bushell A. Interferon-gamma conditioning ex vivo generates $CD25^+CD62L^+Foxp3^+$ regulatory T cells that prevent allograft rejection: potential avenues for cellular therapy. *Transplantation*. 2008;86(4):578–589.
13. Trzonkowski P, Bieniaszewska M, Juścińska J, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded $CD4^+CD25^+CD127$–T regulatory cells. *Clin Immunol.* 2009;133(1):22–26.
14. Brunstein CG, Miller J, Cao Q, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. 2011;117(3):1061–1070.
15. Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. *Diabetes*. 1989;38(suppl 1):140–142.
16. Matsumoto S, Okitsu T, Iwanaga Y, et al. Successful islet transplantation from nonheartbeating donor pancreata using modified Ricordi islet isolation method. *Transplantation.* 2006;82(4):460–465.
17. Marek N, Bieniaszewska M, Krzystyniak A, et al. The time is crucial for ex-vivo expansion of T regulatory cells for the therapy. *Cell Transplant.* [published online ahead of print April 1, 2011]. DOI: 10.3727/096368911 × 566217.
18. Wood KJ, Sakaguchi S. Regulatory T: cells in transplantation tolerance. *Nat Rev Immonol.* 2003;3:199–210.
19. Huang Y, Shan J, Zhang C, et al. Peripheral blood T regulatory cell counts may not predict transplant rejection. *BMC Immunol.* 2010;11:40.
20. Trzonkowski P, Szmit E, Myśliwska J, et al. $CD4^+CD25^+$ T regulatory cells inhibit cytotoxic activity of T $CD8^+$ and NK lymphocytes in the direct cell-to-cell interaction. *Clin Immunol.* 2004;112(3):258–267.
21. Demirkiran A, Kok A, Kwekkeboom J, et al. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. *Liver Transpl.* 2006;12:277–284.
22. Segundo DS, Ruiz JC, Izquierdo M, et al. Calcineurin inhibitors, but not rapamycin, reduce percentages of $CD4^+CD25^+Foxp3^+$ regulatory T cells in renal transplant recipients. *Transplantation.* 2006;82:550–557.
23. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells. *Blood.* 2005;105:4743–4748.
24. Kizilel S, Scavone A, Liu X, et al. Encapsulation of pancreatic islets within nano-thin functional polyethylene glycol coatings for enhanced insulin secretion. *Tissue Eng Part A.* 2010;16(7):2217–2228.
25. De Vos P, Vegter D, Strubbe JH, et al. Impaired glucose tolerance in recipients of an intraperitoneally implanted microencapsulated islet allograft is caused by the slow diffusion of insulin through the peritoneal membrane. *Transplant Proc.* 1997;29(1-2):756–757.
26. Trivedi N, Keegan M, Steil GM, et al. Islets in alginate macrobeads reverse diabetes despite minimal acute insulin secretory responses. *Transplantation.* 2001;71(2):203–211.
27. De Vos P, De Haan BJ, Wolters GH, et al. Improved biocompatibility but limited graft survival after purification of alginate for microencapsulation of pancreatic islets. *Diabetologia*. 1997;40(3):262–270.
28. Cole DR, Waterfall M, Mcintyre M, et al. Microencapsulated islet grafts in the BB/E rat: a possible role for cytokines in graft failure. *Diabetologia*. 1992;35(3):231–237.
29. Pollok JM, Lorenzen M, Kolln PA, et al. In vitro function of islets of Langerhans encapsulated with a membrane of porcine chondrocytes for immunoisolation. *Dig Surg.* 2001;18:204–210.
30. Teramura Y, Iwata H. Islet encapsulation with living cells for improvement of biocompatibility. *Biomaterials.* 2009;30(12):2270–2275.
31. Teramura Y, Iwata H. Surface modification of islets with PEG-lipid for improvement of graft survival in intraportal transplantation. *Transplantation.* 2009;88(5):624–630.
32. Putnam AL, Brusko TM, Lee MR, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes*. 2009;58(3):652–662.

DISCUSSANTS

K. Brayman (Charlottesville, VA):

Islet transplantation remains an attractive alternative to traditional insulin therapy for diabetic patients. However, the effectiveness of clinical islet transplantation therapy is limited by a number of factors, including gradual loss of islet function and loss of insulin independence after transplantation. This article presents a cotransplantation strategy using donor cells targeted with specific recipient–derived regulatory T–cells and, conceptually, this represents a major advance in the new era of regenerative medicine and cell replacement strategies. I have 3 questions for the authors.

First, regulatory T–cells have been demonstrated to control auto– and alloimmunity. Because type I diabetes is an established autoimmune disease, with inherent regulatory T–cell defects, do you have any evidence that Tregs from animal models of diabetes or diabetic humans can be expanded ex vivo and used in the manner outlined in your presentation? Second, regulatory T–cells can be subverted in certain circumstances, such as in the setting of inflammation, to become other T–cell subtypes, such as TH17 cells, which are proinflammatory. Do you have any evidence for the stability of the Treg phenotype in the islet-binding complex in the milieu of inflamma-

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tory cytokines, such as might be seen in the actual setting of in vivo transplantation?

Third, the Treg islet T–effector cell cocultures demonstrated effective immunoprotection. Could you hypothesize on the mechanism for protection?

Response From J. M. Millis:

There is evidence that Tregs can affect the autoimmune response in this situation; Tregs isolated from persons with type 1 diabetes blood showed robust immunosuppressive effect in vitro. Recently, a clinical trial has started to look at the ability of Treg therapy to rescue "fresh" persons with type 1 diabetes. As to your second question, we know that in the different Treg populations there are different levels of stability and that the specific subgroup of Tregs that we utilize in our in vitro analysis is the most stable. The first clinical studies with the same Treg therapy in GVHD patients confirm their immunosuppressive effects. How that stability translates to an in vivo model in combination with islets, is still yet to be determined. Regarding the mechanism of Treg activity, there are a variety of mechanisms involved in immunoprotection, some of which rely on cell–to–cell contact and others on cytokine secretion, such as interleukin-10. We believe the effect that we see in these studies is from the immunomodulation activities of the Tregs rather than a stereochemical effect of the cells bound to PEG or to the islet. The same effect is not seen when other cells are attached to islets, so we believe the effect is because of Treg modulation rather than a physical barrier, per se, around the islets.

DISCUSSANTS

A. Tzakis (Miami, FL):

Could you please comment on the purity of the preparation that is used for the coating of the islet cells, and whether the method is applicable for human use or if it is limited to laboratory use. Also, can the Tregs be derived from the donor rather than the recipient? Would you speculate on the use of donor Tregs for coating?

Response From J. M. Millis:

The islets are isolated just as they would be for clinical islet transplantation, with the same level of purity and viability, so we believe that the same method can be used for clinical islet transplantation. As to whether the Tregs from the donor rather than the recipient can be used, we felt that the advantage of getting the Tregs from the recipient is that immunomodulation is achieved through indirect antigen presentation rather than through an allogenic cell interaction.

DISCUSSANTS

A. Tzakis (Miami, FL):

I'm sorry, I meant the purity of the Treg preparation.

Response From J. M. Millis:

Both Tregs and islets have been used in clinical settings, albeit in separate studies, and the technology and materials used to attach Tregs to islets do not preclude clinical application of the approach. The use of recipient Tregs has the additional advantage that the recipient cells covering donor islets decrease the immunogeninicity of the graft in contrast to donor Tregs.

DISCUSSANTS

G. Warnock (Vancouver, British Columbia, Canada):

Your complex islets are quite a bit bigger than native islets. How do you think this may affect the ability to inject them so they remain in suspension? Also, what will be the duration of action of the Treg constituents when you perform in vivo studies? My final question pertains to the effect on the viability of the human islets. Do you see any membrane leakiness of the islets if you look at absolute insulin release? Do they have higher basal insulin release?

Response From J. M. Millis:

Yes, the complex of the islets is theoretically going to be larger, primarily because of the additional Treg cells that are surrounding the islet. But practically speaking, the actual PEG binding intermediary is one nanometer, making the PEG part very small. And we certainly can keep coated islets in suspension. The question is whether the additional size is going to cause problems, and whether we might have to space out some of the infusions versus the standard method, which is basically infusing all of the mass at once into the portal vein. Certainly, this is an area that we will look at as we move forward to clinical application. Your third question deals with the duration of the bind. We have to look at the bind in 2 parts. The PEG complex is bound to the islet and the Treg cell. We have found that the binding of the PEG to the islet is more stable than the binding of the PEG to the Treg cell, and that, in 3 days, about 30% of the binding has gone away in culture. It is certainly going to be different in vivo. Once a Treg drops off of its binding site in vivo, where there are presumably some chemokines that will actually keep the Treg attracted to that area or perhaps to the local lymph node, it will probably still be effective. We are focusing on the Treg in that area even once it drops off, expecting it to drop off at a reasonable time but expecting it to stay in that same area. As to viability, we have not seen any effect of this process on viability. The viability stains are the same as what we see in clinical islet transplantation, and what we look at perifusion. We see the same cycle with the perifusion studies as we do with normal naked islets, so we did not notice increased basal insulin release.

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