Supplemental Materials and Methods

Materials

The Sekisui Chemical Co. (Osaka, Japan) kindly donated SEK-1005 (C_{46}H_{70}N_{8}O_{13}). Streptozotocin, the penicillin-streptomycin mixed solution, and Blocking One reagent were purchased from Nacalai Tesque (Kyoto, Japan). Hoechst 33258 nuclear stain was purchased from Dojindo Laboratories (Kumamoto, Japan); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); collagenase from Nitta Gelatin (Osaka, Japan); SeaKem GTG agarose from Cambrex Bio Science Rockland, Inc. (Rockland, ME); mitomycin C from Kyowa Hakko Kirin Co., Ltd; MORPHOSAVE from Ventana Medical Systems, Inc. (Tucson, AZ); and ACK buffer from Sigma-Aldrich (St. Louis, MO). Nunc 96-Well Polystyrene Round Bottom Microwell Plates were purchased from Thermo Fisher Scientific (Roskilde, Denmark). SeaKem GTG agarose, human umbilical vein endothelial cells (HUVECs), and basal medium (EBM-2) containing supplements and growth factors (growth medium; EGM-2) were obtained from Lonza (Basel, Switzerland). Percoll was obtained from GE Healthcare (Buckinghamshire, UK), and Fixable Viability Dye was purchased from Affymetrix.
Table S1 presents the antibodies used in this study. Alexa 488-labeled goat antiguinea pig IgG, Alexa 594-labeled goat antiguinea pig IgG, Hanks’ balanced salt solution (HBSS), CFSE fluorescent dye, acetoxyethyl ester of calcein, and RPMI 1640 were purchased from Life Technologies (Carlsbad, CA).

RNeasy plus kits were obtained from Qiagen Japan (Tokyo, Japan). High-Capacity cDNA Reverse Transcription Kits, and StepOnePlus were purchased from Applied Biosystems (Foster City, CA). Enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay were purchased from Shibayagi Co., Ltd. (Gunma, Japan). The formalin solution, Tween-20, the hemoglobin assay kit, and Triton X-100 were purchased from Wako Pure Chemical Industries (Osaka, Japan). ELISA kits for the rat TGF-β1 assay (TGF-β1 Emax Immuno Assay System) were purchased from Promega Co. (Madison, WI). TGF-β1 was obtained from Pepro Tech (London, United Kingdom); Tissue-Tek O.C.T. compound from Sakura Finetek USA, Inc. (Torrance, CA); and FITC-tomato lectin from Vector Laboratories, Inc. (Burlingame, CA).

The glucose sensor (GLUCOCARD MyDIA) was purchased from Arkray, Inc. (Kyoto, Japan). A heated microscope stage was purchased from Carl Zeiss.
(Jena, Germany). Cytofix/cytoperm, Perm Wash, and the FACS Canto II were purchased from Becton Dickinson and Company (Durham, NJ). The SH800 Cell Sorter was obtained from Sony Co. (Tokyo, Japan).

Islet Transplantation

Animals

We used 8- to 10-week-old male ACI/NSIc rats (RT-1\(^a\)) as islet transplant recipients, and 7- to 8-week-old male Fisher 344 rats (F344 /NSIc, RT-1\(^{1v}\)) as donors. All animals were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). All animal experiments were performed following the guidelines of the Kyoto University Animal Care Committee.

Preparation of the agarose-SEK rod

We prepared a rod-shaped agarose scaffold (diameter, 4 mm; length, 25 mm) as previously described.\(^1\) Briefly, a solution of 4.5% (w/v) agarose in distilled deionized water was poured into a polystyrene tube, and incubated on ice to induce gelation. Next, the agarose gel was cut into rods that were frozen at \(-30^\circ\text{C}\) overnight, and then freeze-dried for 24 hours under reduced pressure. The
freeze-dried agarose rods were evenly coated with SEK solution (10 µg or 100 µg SEK in 50 µL ethanol), and the ethanol could volatilize for 4 hours. Subsequently, the rods were coated with 150 µL saline. For control experiments, we coated freeze-dried agarose gel rods with 150 µL saline, without SEK. The agarose gel rods with SEK (agarose-SEK rods) and the control rods were stored at 4°C until use.

**Vascularization of subcutaneous tissue with the agarose-SEK rod**

To induce diabetes in the recipient ACI rats, we administered a single intraperitoneal injection of STZ in citrate buffer (pH 4.5) at a dose of 60 mg/kg body weight. Rats with blood glucose levels of >400 mg/dL for 2 consecutive days were considered diabetic. To induce vascularization in the STZ rats, we implanted an agarose-SEK rod into the dorsal subcutaneous fat tissue (1 rod in each of 2 sites). At 10 days after implantation, these rods were removed, and tissue that contacted the agarose-SEK rod was harvested for histological analysis. For semiquantitative measurement of the vascularization level, we determined the amount of hemoglobin in the subcutaneous tissue as previously described.² Briefly, the harvested tissue was weighed, minced, and incubated for 48 hours at
4°C in 10 mL Gay’s solution (0.75% NH₄Cl in 17 mM Tris-HCl buffer solution, pH 7.6) with gentle periodical agitation to extract the hemoglobin. Hemoglobin concentrations were determined using a hemoglobin assay kit.

*Islet isolation*

Islets from donor F344 rats were isolated using the collagenase digestion method as previously described. Briefly, the pancreas was inflated by injection of collagenase solution (0.5 mg/mL in HBSS) through the portal vein. The pancreas was then removed, and digested in collagenase solution for 19 min at 37°C. Islets were isolated from the digestion reaction using the Ficoll/Conray density gradient purification method. The islets were then cultured for 2 days in RPMI 1640 (supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin) before transplantation.

*Islet transplantation*

Upon removal of the implanted agarose-SEK rods (or control agarose rods without SEK), 1500 islets isolated from F344 rats were transplanted into each of the 2 prevascularized dorsal pockets. The wounds were closed by
sutting. None of the recipient rats received any immunosuppressive drugs during the experiments. For determination of nonfasting blood glucose levels, blood samples were collected from the tail veins of the recipient rats daily for the first 2 weeks, and 2 or 3 times per week thereafter. Glucose levels were determined using a glucose sensor. The finding of 2 consecutive blood glucose measurements exceeding 250 mg/dL was considered to indicate graft rejection.

**Intraperitoneal glucose tolerance test (IPGTT)**

Recipient rats underwent an IPGTT to evaluate islet graft function at between 50–55 days and between 100–105 days after islet transplantation. Normal nondiabetic rats were also evaluated as controls. After 16 hours of fasting, the rats were intraperitoneally administered a glucose solution (1 g glucose/kg body weight). Blood samples were collected 0, 15, 30, 60, 90, and 120 min after glucose loading, and a glucose sensor was used to measure blood glucose levels.

**Plasma insulin analysis**

At 40 and 90 days after islet transplantation, blood was collected from the tail veins of recipient rats to determine the plasma insulin levels. For controls,
blood samples were also collected from normal and STZ rats without islet grafts. All blood samples were kept on ice, and then centrifuged at 4000 rpm for 15 min at 4°C. Plasma was collected and stored at −30°C until determination of the plasma insulin concentration by ELISA.

**Histological examination of islet grafts**

On predetermined days, subcutaneous tissues containing the islet grafts were collected and fixed in 4% paraformaldehyde (PFA) in PBS. Thin 4 µm tissue sections were prepared using standard methods, and stained using hematoxylin and eosin (H&E) or immunofluorescently stained for insulin. Briefly, for immunofluorescent staining, the sections were permeabilized with 0.2% Triton X-100 solution in PBS for 15 min at room temperature. Next, the samples were incubated for one hour with Blocking One reagent to block nonspecific staining, and then washed with PBS. The sections were subsequently treated with a solution of polyclonal guinea pig antiinsulin antibody (1:200) in Blocking One reagent for 1 hour at room temperature. This was followed by a 1 hour incubation at room temperature with fluorescently labeled secondary antibody—either Alexa 488-labeled goat antiguinea pig IgG (1:500) or Alexa 594-labeled goat antiguinea
pig IgG (1:500)—in Blocking One reagent. The sections were then washed with PBS containing 0.05% Tween-20, and the cell nuclei were counterstained using Hoechst 33258. Finally, the sections were treated with 1 mM CuSO₄ solution in 50 mM ammonium acetate (pH 5.0) for 1 hour at room temperature to reduce autofluorescence.⁵

**Immunoperoxidase staining of immune cells around the islet grafts**

Recovered subcutaneous tissue samples comprising islet grafts plus surrounding tissue were embedded in O.C.T. compound and snap frozen in liquid nitrogen. Thin 4 μm tissue sections were prepared using standard methods, and immunoperoxidase staining was performed to visualize insulin, CD4⁺ T cells, CD8⁺ T cells, and Foxp3⁺ cells. Tissue sections were fixed in iced acetone, soaked in MORPHOSAVE at room temperature for 15 min, and then washed with PBS. Next, the sections (except those used for visualizing CD4⁺ T cells) were soaked in 3% hydrogen peroxide in PBS at room temperature for 10 min, and then washed again with PBS. This was followed by overnight incubation at 4°C with primary antibodies against CD4⁺ T cells (1:50), against CD8⁺ T cells (1:50), or against Foxp3⁺ cells (1:50) diluted in PBS. For CD4⁺ T cell staining, the slides
were treated with 0.3% hydrogen peroxide in PBS at room temperature for 30 min, and then washed with PBS. At this point, all sections were incubated with Histofine Simple Stain at room temperature for 30 min, and then treated with Histofine Simple Stain DAB solution. Nuclei were counterstained using Mayer's hematoxylin solution.

**Intravital perfusion of FITC-lectin solution to visualize functional vasculature**

FITC-tomato lectin solution (200 μg in 1 mL saline, containing 1000 IU heparin) was intravenously perfused through the inferior vena cava into STZ rats after 10 days of prevascularization, as well as into ACI rats 30 days after transplantation with F344 islets. After 15 min of circulation, subcutaneous tissue with or without islet grafts was rapidly dissected, briefly washed twice with saline solution, fixed, and snap frozen in liquid nitrogen. Thin 4 μm tissue sections were prepared. Thin sections of the tissue with islet grafts were subjected to immunofluorescence staining to visualize insulin using standard methods.

*Effects of transplantation of F344 rat splenocytes on accepted islet grafts*
At 30 (n = 3) or 117 (n = 5) days after islet transplantation, STZ rats with long-term functional F344 islet grafts were intraperitoneally injected with $10^7$ F344 splenocytes isolated from islet donor F344 rats. Following splenocyte injection, blood glucose levels were determined daily for all rats to detect graft rejection. At 5 or 6 days after splenocyte injection, subcutaneous tissue including the islet grafts was recovered for histological examination.

Immunological Environment of Subcutaneous Tissue Prevascularized with Agarose-SEK Rods

**Preparation of tissue-infiltrated lymphocytes and of lymphocytes in regional lymph nodes**

Fresh rat tissue-infiltrated lymphocytes were obtained from rat subcutaneous tissue and separated using a previously described method.\(^6\) Briefly, subcutaneous tissue was excised and cut into small pieces. Cell suspensions were then filtered through a sieve, and cooled on ice for 45 min. Next, the supernatant was collected and purified by Percoll gradient centrifugation at $15,000 \times g$ for 20 min. Lymphocytes were also harvested from regional lymph nodes.\(^7\) Cell suspensions were obtained from homogenized lymph node tissue,
and filtered through a sieve. Contaminating red blood cells (RBCs) were lysed using ACK buffer.

**Flow cytometry analysis and cell sorting**

For intracellular staining, cells were fixed and permeabilized using Cytofix/cytoperm and Perm Wash (BD Pharmingen) following the manufacturer’s instructions. Flow cytometry was performed using the FACS Canto II (BD Biosciences), and cell sorting using the SH800. The sorted cells showed a cell purity of >96%, and were used in subsequent experiments.

**Treg suppression assays**

From the treated subcutaneous tissue of ACI/NSIc rats, we harvested CD25+CD4+ T cells, a Treg cell-rich fraction, for use as suppressor T cells. CD8+ T cells from ACI/NSIc rats were labeled with the fluorescent dye CFSE (5 μM), and used as responder T cells. We cultured a total of 2.5 × 10^4 responder T cells without CD25+ CD4+ regulatory T (Treg) cells or with Treg cells at cell ratios ranging from 1:1 to 1:8. These cells were cultured in 96-well polystyrene round bottom microwell plates for 4.5 days at 37°C under 5% CO₂, in the presence of
antigen-presenting cells (5 × 10⁴) treated with mitomycin C (0.1 mg/mL) and soluble anti-CD3 (1 μg/mL). Cultured cells were stained with a Fixable Viability Dye, and evaluated by flow cytometry.

**iTreg induction assay**

In the presence of antigen-presenting cells (5 × 10⁴) treated with mitomycin C (0.1 mg/mL) and soluble anti-CD3 (1 μg/mL), we cultured a total of 2.5 × 10⁴ naïve CD4⁺ T cells. These cells were cultured in 100 μL complete RPMI (RPMI 1640, 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin) plus TGF-β1 (5 μg/mL), and soluble anti-CD3 (1 μg/mL) in 96-well polystyrene round bottom microwell plates for 4.5 days at 37°C under 5% CO₂. Cultured cells were stained with Fixable Viability Dye, anti-CD4 antibody, anti-CD3 antibody, and anti-Foxp3 antibody, and were evaluated by flow cytometry analysis.

**TGF-β1 concentrations in subcutaneous tissue implanted with agarose-SEK rods**

Wound exudate was sampled daily from the site of agarose-SEK rod implantation. After centrifugation at 1000 × g, TGF-β1 concentration was
Gene Expression Analyses of Prevascularized Subcutaneous Tissue

**Real-time reverse-transcription polymerase chain reaction (RT-PCR)**

The expressions of several genes were analyzed by real-time quantitative RT-PCR based on real-time detection of accumulated fluorescence using the TaqMan system. Total RNA was extracted from subcutaneous tissue using the RNeasy Plus Kit. We then synthesized cDNA by reverse transcription using the High Capacity cDNA Reverse Transcription Kit. Quantitative RT-PCR was performed using StepOnePlus, and the results were analyzed using StepOne Software. Normalization was performed using a VIC-labeled probe for HPRT1 (Applied Biosystems).

**Microarray analysis**

From subcutaneous tissue treated with agarose-SEK rods (n = 3) or control agarose rods (n = 3), total RNA was extracted using the RNeasy Plus Kit. Then biotinylated antisense RNA was prepared by in vitro amplification using the Express Kit Assay (Affymetrix). Microarray analyses were performed by Kurabo.
Industries Ltd. (Tokyo, Japan). Briefly, biotinylated antisense RNA was hybridized to Affymetrix GeneChip Rat Genome 230 2.0 arrays. The acquired microarray data were uploaded to the National Center for Biotechnology Information Gene Expression Omnibus (GEO). The data were preprocessed with RMA (Robust Multichip Average) normalization. We identified specific gene groups comprising genes associated with Treg cells, the GO term cytokine, or the GO term chemokine. A volcano plot was generated to visualize the normalized expression of all genes or specific gene groups. We further compared the gene score (t statistics) of the specific groups of genes associated with the GO terms cytokine and chemokine (but not the gene group associated with Treg cells) with the total gene score as the reference. We generated a heat map to visualize the normalized expression of Treg cell-associated genes.

**In Vitro Examination of Effects of SEK on Endothelial Cells**

HUVECs were maintained at 37°C under 5% CO₂ in EBM-2 containing supplements and growth factors (growth medium; EGM-2). To assess the production of TGF-β1 and VEGF in cell culture or during tube formation, we assayed the conditioned medium after 1 hour of HUVEC culture. The tube
formation assay was performed using 3D collagen matrix as described previously. HUVECs were mixed with collagen solution (2.5 mg/mL type I collagen, 1× M199, 26.2 mM NaHCO3, 0.7 mM L-glutamine) at a density of 2 × 10^6 cells/mL. The cell–collagen mixture was then added to a 96-well half-area plate. After 30 min of incubation at 37°C under 5% CO2, the cells were treated with EBM-2 containing various concentrations of SEK for 24 hours. Next, the cells were washed with HBSS, and acetoxymethyl ester of calcein was added to a final concentration of 2.5 μM. After 30 min, tube formation was observed under a fluorescent microscope, and tube length was analyzed using NIH ImageJ software.

**Statistical Analysis**

Between-group comparisons were made using the Student’s t test. Comparisons among 3 or more groups were performed using 1-way analysis of variance (ANOVA) followed by posthoc analysis using the Tukey honestly significant difference (HSD) method. A log-rank test was used to compare graft survival between experimental and control groups. A p value of <0.05 was considered statistically significant.


FIGURE S1: The chemical structure of SEK.

FIGURE S2: Effects of agarose-SEK rod implantation on subcutaneous tissue. (A–C) Macroscopic images of the subcutaneous tissue of streptozotocin (STZ)-induced diabetic ACI rats with no treatment (A); at 10 days after implantation of agarose rods without SEK (B); and at 10 days after implantation of agarose-SEK rods (C). Scale bar: 10 mm. (D) Schematic illustration of agarose-SEK rod implantation. H&E staining of thin sections of subcutaneous tissue. (E, F) Histology of subcutaneous tissue at 10 days after implantation of agarose rods without SEK. (G, H) Histology of subcutaneous tissue after 10 days of prevascularization with agarose-SEK rods. Scale bars: (E, G) 200 μm, (F, H) 50 μm. Dotted lines indicate the interface between agarose rods and subcutaneous tissue. (I) Functional vasculature is visualized through intravenous infusion of a FITC-lectin solution. Scale bar, 100 μm.
FIGURE S3: Transplantation of $10^7$ splenocytes from F344 rats (islet donors) into the peritoneal cavity of recipient rats at 117 days (red arrow) after islet transplantation resulted in the rejection of established subcutaneous allogeneic islet grafts (A-C). Tissue containing islet grafts was removed and processed at 6 days after splenocyte injection. (A) Effects of splenocyte injection on islet graft function in terms of blood glucose levels. (B, C) Subcutaneous tissue containing islet grafts stained with H&E and immunofluorescently stained for insulin. Most of the insulin-positive cells disappeared after splenocyte injection. Scale bar: 100 µm. (D) Immunoperoxidase staining of CD4$^+$ T cells, Foxp3$^+$ cells, insulin-positive cells, and CD8$^+$ T cells in thin sections of subcutaneous tissue from the allogeneic islet graft at 5 days after infusion of $10^7$ F344 splenocytes into the intraperitoneal cavity at 30 days after graft transplantation. Scale bar: 100 µm.
FIGURE S4: Percentages of Foxp3+ cells among CD4+ T cells (Treg cells), and the ratios of CD8+ T cells to CD4+ T cells in the axillary lymph nodes and in the spleen are plotted against the days after agarose rod implantation. (A–D) Percentages of Foxp3+ cells among CD4+ T cells in axillary lymph nodes (A) and in the spleen (C). Ratios of CD8+ T cells to CD4+ T cells in axillary lymph nodes (B) and in the spleen (D). Values are for streptozotocin (STZ)-induced diabetic ACI rats treated with agarose-SEK rods (open circles), and with agarose rods without SEK (closed circles). For both groups, day 2, n = 3; days 7, 10, and 14, n = 4. Data are shown as the mean ± SEM.
FIGURE S5: Whole gene expression analysis of granulomatous tissue formed at 7 days after implantation of agarose rods with or without SEK (n = 3 for each). (A) Gene scores (t statistics) presented in a cumulative frequency graph. The black line indicates the cumulative frequency of the expressions of all genes. The red line indicates the cumulative frequency of expressions of genes in a specific group ie, genes identified with the GO terms cytokines and chemokines and that are not associated with Treg cells. The Student’s t test was used to determine the difference between the gene score of a specific gene group and the gene score for all genes (as the reference). (B) Volcano plot showing the normalized expressions of 239 specific genes, ie, those identified using the GO terms cytokines and chemokines.
FIGURE S6: Percentages of Foxp3+ cells among CD4+ T cells (Treg cells) and ratios of CD8+ T cells to CD4+ T cells are plotted against the days after islet transplantation. (A) Representative flow cytometry images for tissue containing an islet graft, with analyses gated for CD3e+ CD4+ T cells. (B, C, E, G) The percentages of Foxp3+ cells among CD4+ T cells (Treg cells) in tissue containing islet grafts (B), draining lymph nodes (C), axillary lymph nodes (E), and spleen (G) are plotted against the number of days after islet transplantation. (D, F, H) The ratios of CD8+ T cells to CD4+ T cells in draining lymph nodes (D), axillary lymph nodes (F), and the spleen (H) are plotted against the number of days after islet transplantation. Values are shown for tissue formed before islet transplantation (closed circles; n = 3), and after islet transplantation (open circles; n = 3). Data are shown as the mean ± SEM.
FIGURE S7: Immunoperoxidase staining for CD4⁺ T cells, Foxp3⁺ cells (Treg cells), insulin-positive cells, and CD8⁺ T cells in thin sections of subcutaneous tissue with allogeneic islet grafts. F344 islets were transplanted into granulomatous tissue of streptozotocin (STZ)-induced diabetic ACI rats after implantation of agarose-SEK rods without immunosuppressive therapy. (A) Tissue isolated at 5 days after islet transplantation. (B) Tissue isolated at 30 days after islet transplantation (B). Scale bars: 100 µm, 40 µm (magnified insets). Yellow arrows indicate Foxp3⁺ cells or CD8⁺ cells.
Figure. S8

A) TGFβ1 in culture medium (pg x mL⁻¹)

B) VEGF in culture Medium (pg x mL⁻¹)

C) Control SEK VEGF

D) Tube length (% of control)

E) Concentration of TGFβ1 (ng x mL⁻¹)

TGFβ1 in culture medium

VEGF in culture Medium

Anti-TGF-β:

Concentration of TGFβ1

Days after implantation
FIGURE S8: The effects of SEK-1005 (SEK) on HUVEC production of TGF-β1 and VEGF, and tube formation. (A) HUVECs were incubated for 1 hour with or without 5 nM SEK, and then the concentrations of TGF-β1 in the culture medium were determined. Data are shown as the mean ± SEM of eight independent experiments. ***P < 0.001. (B) HUVECs were incubated for 1 hour with or without 5 nM SEK, and with or without TGF-β neutralizing antibodies (5 µg/mL), and then the VEGF concentrations in the culture medium were determined. Data are shown as the mean ± SEM of eight independent experiments. *P < 0.05, ***P < 0.001 for the indicated comparisons. (C) Effects of SEK on HUVEC tube formation. For 24 hours, HUVECs were incubated in the absence (control) or presence of SEK (50 nM) or VEGF (10 ng/mL), and tube formation was visualized using a fluorescence microscope. (D) Dose-dependent effects of SEK on tube formation (n = 12–16). ***P < 0.001 versus the value in control experiments without SEK and VEGF. (E) TGF-β1 concentrations in the exudate of granulomatous tissue formed over time after implantation of rods with or without SEK (n = 3 for each). Values are shown for tissue formed following implantation of agarose-SEK rods (open circles; n = 3), and of agarose rods without SEK (closed circles; n = 3).
**TABLE S1:** The antibodies used in this study.

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