SDC materials and methods

Materials and Methods

Materials

The following reagents were purchased from the indicated suppliers: Blocking One, trypsin, penicillin-streptomycin mixed solution, 2-mercaptethanol (2-ME), and streptozotocin (STZ) from Nacalai Tesuque (Kyoto, Japan); Hoechst 33258 nuclear stain from Dojindo Laboratories (Kumamoto, Japan); Alexa 488-labeled goat anti-guinea pig IgG, Hanks' balanced salt solution (HBSS), and RPMI 1640 from Invitrogen (Carlsbad, CA, USA); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, USA); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); enzyme-linked immunosorbent assay (ELISA) kits for the mouse insulin assay from Shibayagi Co., Ltd. (Gunma, Japan); polyclonal guinea pig anti-swine insulin from Dako (Glostrup, Denmark); formalin solution, Tween 20, and Triton X-100 from Wako Pure Chemical (Osaka, Japan); red blood cell lysing buffer from Sigma-Aldrich (St. Louis, MO, USA); goat affinity-purified antibody to rat IgG (Whole Molecule) from MP Biomedicals (Solon, OH, USA); biotin rat anti-mouse CD25, PE streptavidin, and FITC rat anti-mouse CD4 from BD Biosciences (San Jose, CA, USA); anti-PE MicroBeads from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany); mouse monoclonal anti-human Foxp3 from Abcam plc. (Cambridge, UK); Tissue-Tek® from Sakura Fine Technical Co., Ltd. (Tokyo, Japan); and collagenase from Nitta Gelatin (Osaka, Japan).

Rat anti-CD8 (3.155) and rat anti-CD24 (J11d) were kindly provided by Professor Shimon Sakaguchi (*20*).

Preparation of $CD4^+CD25^+$ regulatory T (Treg) cells and islet cells

The Kyoto University Animal Care Committee approved all animal experiments. $CD4^+CD25^+$ Treg cells were isolated from C57BL/6 mice (8-week-old male mice, Japan SLC, Inc., Shizuoka, Japan) using magnetic-activated cell sorting (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (*20*). Briefly, spleen and lymph node cell suspensions prepared from C57BL/6 mice and enriched for $CD4^+$ cells by panning; anti-rat IgG-coated dishes were used to remove $CD8^+$ or $CD24^+$ cells treated with rat anti-CD8 and rat anti-CD24 from the cell suspensions. These cells were treated with biotin rat anti-mouse CD25, PE streptavidin, and anti-PE MicroBeads, and sorted by MACS. The sorted cells were used immediately in the following experiments.

To examine the purity of MACS-sorted $CD4^+CD25^+$ Treg cells, the cells were treated with FITC rat anti-mouse CD4 and analyzed using a fluorescence-activated cell analyzer (Guava EasyCyte Mini; Millipore, Billerica, MA, USA).

Islets were isolated from BALB/c mice (7-week-old male mice, Japan SLC, Inc., Shizuoka, Japan) using the collagenase digestion method (*21*). Briefly, a collagenase solution (0.5 mg/mL in HBSS) was injected into each mouse pancreas through the bile duct. The pancreas was then collected from the mouse and incubated for 18 min at 37°C. After the digested tissue had dissociated into pieces, the tissue was washed with HBSS, and islets were isolated from the digestion reaction using Ficoll density gradient purification. Isolated islets were cultured for 2 days in RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin to remove cells damaged by the isolation procedure.

Islets were disassociated into single cells by incubation in 1 mL trypsin solution (500 μg/mL in HBSS) at 37°C for 2 min, followed by the addition of 1 mL of medium and pipetting with a Pasteur pipette to apply mechanical force. Resultant

single cells were resuspended in RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-ME.

Co-aggregate formation

Co-aggregates of Treg cells and islet cells were prepared on agarose hydrogel with small round-bottomed wells. A mold (Microtissues Inc., Providence, RI, USA) was used to create the hydrogel with 256 (16 × 16) wells, each 250 μm in diameter. Hot 2.5% agarose solution in PBS (50-90°C) was injected into the mold and chilled to form a gel on ice. The hydrogel was equilibrated in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-ME. Immediately after isolation, Treg cells were suspended in 100 μL RPMI 1640 medium at a density of 3000 cells per well. Cells that were obtained by the dissociation of 256 islets were suspended in 100 μL of RPMI 1640 medium with 10% FBS. These suspensions of Treg cells and islet cells were mixed together. The cell suspension (200 μL) was applied to the hydrogel to achieve a final concentration of approximately 3000 Treg cells and islet cells equivalent to one islet per well. Cells were cultured on the hydrogel for 4 days at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air.

The percentage of $Foxp3^+$ cells was examined in the co-aggregates at 4 days of culture. The co-aggregates were separated into single cells, which were then treated with mouse monoclonal anti-human Foxp3 and fluorescently labeled secondary antibodies, followed by analysis using a fluorescence-activated cell analyzer. Thin sections of the aggregates were also prepared for Foxp3 and insulin immunostaining. For this, the aggregates were immersed in a 4% formalin solution for 30 min at room temperature (RT), and subsequently embedded in Tissue-Tek® for freezing. The

frozen specimens were sliced into 6-μm-thick sections. The sections were treated with mouse monoclonal anti-human Foxp3 and polyclonal guinea pig anti-swine insulin antibodies for 1 h at RT, and were then incubated with fluorescently labeled secondary antibody at RT for 1 h. Finally, the sections were observed under a fluorescence microscope (BX51, Olympus Optical Co., Ltd., Tokyo, Japan).

In vitro glucose stimulation test

Fifty co-aggregates (each composed of 3000 Treg cells and islet cells equivalent to one islet) were exposed sequentially to solutions of 0.1 g/dL, 0.3 g/dL, and 0.1 g/dL glucose in Krebs-Ringer's buffer (KRB); they were incubated for 1 h at 37°C in each solution. The supernatants were collected, and we determined the insulin concentrations of each solution using ELISA according to the manufacturer's instructions. The glucose stimulation test was also performed using 50 unmodified islets as a reference.

Intraportal transplantation of co-aggregates

Eight-week-old male C57BL/6 mice were used as the co-aggregate transplant recipients. Diabetes was induced in C57BL/6 mice with a single intraperitoneal injection of STZ (120 mg/kg body weight in citrate buffer, pH 4.2) 1 week before transplantation. Mice were used as diabetic recipients when their plasma glucose levels exceeded 450 mg/dL in two consecutive measurements. The diabetic mice were anesthetized during surgery by mask inhalation of isoflurane using a specialized instrument (400 Anesthesia Unit, Univentor, Malta); the isoflurane concentration was 4.5% to 5.0% for induction and 2.0% for maintenance (airflow rate, 200 mL/min).

A suspension of 400 co-aggregates containing 1.2×10^6 Treg cells isolated from C57BL/6 mice and islet cells equivalent to 400 BALB/c mouse islets was infused into the portal vein and the co-aggregates plugged small vessels in the liver. As a control experiment, 400 unmodified BALB/c mouse islets were also infused through the portal vein. A total of 400 unmodified BALB/c mouse islets or 400 co-aggregates that contained 1.2×10^6 Treg cells isolated from C57BL/6 mice and islet cells equivalent to 400 BALB/c mouse islets were transplanted into each C57BL/6 mouse to ensure that the number of islet cells was equal in the grafts. Plasma glucose levels were monitored daily during the initial 30 post-transplantation days and subsequently monitored every 2 days. The graft survival periods were defined from the day of transplantation to the day that the first of two consecutive plasma glucose levels >250 mg/dL was recorded. Blood was collected from recipients and centrifuged to obtain plasma. We determined plasma insulin levels using ELISA according to the manufacturer's instructions. Recipients were subjected to intraperitoneal glucose tolerance tests (IPGTTs) 90 days post-transplantation to evaluate glucose tolerance.

Histochemical analysis

Some recipient mice were sacrificed at predetermined times after transplantation of the co-aggregates or unmodified islets. The livers were retrieved and immersed in 4% formalin solution for 2 days at RT. After the formalin solution was removed, the livers were dehydrated in alcohol solutions and embedded in paraffin. The livers were sliced into sections 4 μm in thickness. After deparaffinization, the sections were permeabilized with 0.2% Triton X-100 in PBS at RT for 15 min. The specimens were then treated with Blocking One for 1 h followed by washing with PBS. Sections were treated with 1% polyclonal guinea pig anti-

swine insulin in Blocking One for 1 h at RT. Sections were then incubated with fluorescently labeled secondary antibody (0.2% Alexa 488-labeled goat anti-guinea pig IgG in Blocking One) at RT for 1 h followed by washing with PBS containing 0.05% Tween-20. The stained specimens were observed under a fluorescence microscope (BX51, Olympus Optical Co., Ltd., Tokyo, Japan). The specimens were also stained with hematoxylin-eosin (HE) using a conventional staining protocol.

Statistical analysis

Data are shown as mean ± standard deviation (SD) from at least three independent samples. The data were compared using Student's *t*-test, the Tukey-Kramer honestly significant difference (HSD) test, or a log-rank test. All statistical calculations were performed using the software JMP (SAS Institute Inc., NC). A p value of <0.05 was considered statistically significant.