

Transplantation of Allogeneic Islets into Vascularity-Rich Subcutaneous Pouches
Prepared from Hydrogels Carrying Angiogenic Factors

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2018

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Abbreviations

bFGF, basic fibroblast growth factor.

ES cells, embryonic stem cells.

ELISA, enzyme-linked immunosorbent assay.

FACS, fluorescence activated cell sorting.

FBS, fetal bovine serum.

FITC, fluorescein isothiocyanate.

H&E, hematoxylin and eosin.

HBSS, hank's balanced salt solution.

MHC, major histocompatibility complex.

IPGTT, intraperitoneal glucose tolerance test.

iPS cells, induced pluripotent stem cells.

Islet, islet of Langerhans.

PBS, phosphate buffered saline.

PFA, paraformaldehyde

RT-PCR, real-time polymerase chain reaction.

SEK, SEK-1005.

STZ, streptozotocin.

TGF- β 1, transforming growth factor- β 1.

Treg, regulatory T cell.

VEGF, vascular endothelial growth factor.

General Introduction

Type 1 diabetes mellitus is the disease to disorder glucose metabolism caused by the destruction of β cells, due to autoimmune reaction [1]. Crucial acute complications, such as hypoglycemic coma and diabetic ketoacidosis, often occur, because it is difficult for serious insulin dependent diabetic patients, including type 1 diabetic patients, to control blood glucose by self-injection of insulin. Long-term insufficient glycemic control will cause various chronic complications, diabetic nephropathy, diabetic retinopathy, and diabetic neuropathy [2]. Especially, the patients with serious diabetic nephropathy will need dialysis therapy. A fundamental medication of diabetes has been desired.

Organ transplantation is the medication to make a recovery by replacing the organ of end state with healthy one from another person. Organ transplantation could be the only treatment when an organ is failed. Cell and tissue transplantation have been promising as a medication of type 1 diabetes and other diseases, because it is expected that the invasive to patients and the immune reactions caused by host immune system will be smaller than organ transplantation.

Islets of Langerhans (islets) in pancreas is composed of β cell, α cell, and δ cell. β cell can secrete insulin, which is the sole hormone that can decrease blood glucose by ordering cells, such as muscle tissue, adipose tissue, and liver, to receive glucose. Pancreatic islet transplantation is a promising method as a fundamental therapy of type 1 diabetes. Since Edmonton protocol of steroid-free immunosuppression has been adapted in 2000, rate of islet engraftment significantly improved. Multiple islet transplantations for one patient, however, is essential [3]–[5]. In current islet transplantation, islets are isolated from pancreas of human donor in cardiac arrest. Pancreas from donor are digested

by enzyme. Islets are purified from digested tissue, and are infused into the liver via portal vein. Transplanted islet secretes insulin to blood, and enables severe glycemic control. Current clinical islet transplantation has several problems to overcome, such as shortage of islet donor, the life-long administration of the immunosuppressive drugs, and transplantation site.

In recent years, some efficient methods of induction to various cells, such as cardiomyocytes, foregut cells, and kidney cells, from pluripotent stem cells, e.g. embryonic stem (ES) cells and induced pluripotent stem (iPS) cells [6]–[9]. Several groups have successfully regenerated quasi-islets from ES/iPS cells and reported that transplantation of these quasi-islets in a murine diabetic model can normalize hyperglycemia [10],[11]. The shortage of islet donor will be solved in the near future.

In current clinical procedure, islets are transplanted into the liver. However, liver is an important organ and grafts cannot be removed, if there are any problems in grafts. Teratoma formation by residual undifferent ES/iPS cells and tumor formation by transformed cells arising in the process of differentiation are concerned [12]. When teratoma/tumor is formed in the liver, it cannot be removed surgically. In addition to that, intrahepatic islet transplantation has a risk of complications, such as hepatic hematoma and portal pressure increase, which can significantly hamper islet engraftment and liver function [13]. For these reasons, the liver is unsuitable transplantation site, and islet should be transplanted into safer, easier and minimal invasive transplantation site.

Extrahepatic islet transplantation, such as subcutaneous site [14], omental pouch [15]–[17], and intramuscular site [18], have been examined. Especially, subcutaneous sites have been promised, because they have large areas, and are easy to monitor and remove grafts. Subcutaneous sites, however, have low density of vasculature. Grafts

transplanted under the skin cannot survive, because of hypoalimentation and hypoxia [19],[20]. For the reason, vascular network should be constructed at subcutaneous site by using growth factors and/or scaffolds [14],[21]–[24]. Even if islets are transplanted at subcutaneous site, the immunosuppression will be needed.

Multihued methods have been studied for the islet engraftment without immunosuppression; the creation of a bioartificial pancreas [25]–[27] decreasing of islet immunogenicity[28]–[30]; transplantation into the immune privileged sites [31]–[33]; and co-transplantation of islets with immunosuppressive cells [34]–[38]. Although these methods look promising in animals, each has proven difficult to use in clinical transplantation. Especially, bioartificial pancreas is a promising method for islet transplantation without immunosuppression. Several types of bioartificial pancreas have been investigated, such as the diffusion chamber, hollow fiber unit, and microcapsule. In bioartificial pancreas, islets are embraced by semipermeable membrane. It can allow to permeate insulin, oxygen, and nutrients, but prevent from infiltrating immune cells. In microencapsulation of islets, clinical trials have been performed in xenotransplantation, pig islets to human peritoneal cavity [39]. However, the large volume and biofilm will hamper their clinical application in humans.

Graft rejection is the important problem to be overcome in cell and organ transplantation. Allogeneic transplantation is between same species, e.g. human to another human, and xenogeneic transplantation is between different species, e.g. pig to human. Generically, immune reactions in xenogeneic transplantation are stronger than in allogeneic transplantation. Due to the mismatch of major histocompatibility complex (MHC), that called human leukocyte antigen in humans, between a donor and a recipient, grafts from another person usually are attacked and excluded by host immune system

without immunosuppressive medication. For the reason, immunosuppressive drug is administrated before and after transplantation to prevent graft rejection in current clinical transplantation. However, the unspecific immunosuppression has serious risks of side effects, such as the infectious complications and the malignancy [40],[41]. Even if islet cells differentiated from each patient's own iPS cells are transplanted, it is necessary to administrate immunosuppressive drug, because type 1 diabetes is an autoimmune disease [42],[43].

Immune tolerance is a specifically unresponsive state to a particular antigen. In the case of transplantation, it is a state of immune unresponsiveness to the donor graft or antigen, but the host immune system can response to other antigen, unlike immunodeficiency [44]. Regulatory T (Treg) cells have a pivotal role of maintaining the immunological homeostasis and peripheral tolerance. Their depletion will cause autoimmune diseases [45]. Treg cells were also reported to assemble in the inflammatory sites and the location of injury, e.g. myocardial infarct [46]. There are naturally occurring Treg cells which are selected in the thymus and can suppress immune reactions to self-antigens in periphery [47],[48]. Additionally, when naive CD4⁺ T cells encounters antigens in the presence of TGF- β and IL-2, they can differentiate to 'induced' Treg cells *in vitro* [49]. Treg cells can regulate the activation of CD4⁺, CD8⁺ T cells [50]–[52], and B cells [53],[54]. They can also suppress the function of natural killer (NK) cells [55] and NKT cells [56], and dendritic cells [57]. Treg cells were reported to regulate immune reactions by the suppression of dendritic cells function with CTLA-4, the secretion of immunosuppressive cytokines, such as IL-10, TGF- β , and IL-35 [58]. In allogeneic organ or cell transplantation, induced Treg cells, which can regulate immune responses to foreign antigens, are promising [59],[60].

As described above, current islet transplantation has several problems; such as shortage of islet donor, the life-long administration of the immunosuppressive drugs, and transplantation site. There are some requirements for an ideal islet transplantation; 1) the unspecific immunosuppression should not be required before/after transplantation; 2) the islet graft should not be rejected for a long period; 3) the transplantation procedure should be minimally invasive as same degree as an insulin injection; 4) multiple islet transplantation is feasible, when necessary; 5) it should be easy to access the graft to remove and monitor, when necessary. Subcutaneous sites have been considered to be promising transplantation sites for islet transplantation, because they have the possibility to fulfill the requirements described above.

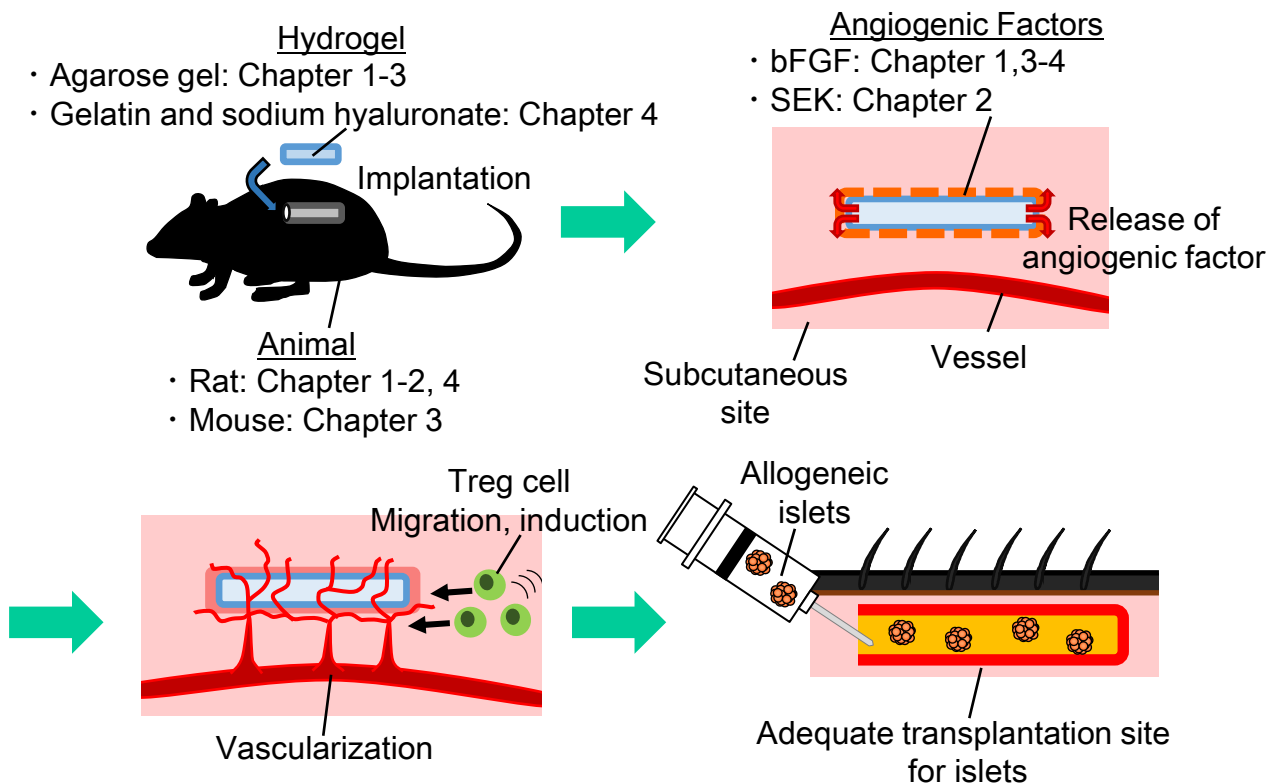


Figure 1. Schematic drawing of the methods for realizing islet transplantation without immunosuppression. The preparation of vascularity-rich immune tolerant pouches from the implantation of hydrogels carrying angiogenic factors at subcutaneous site, for islet transplantation without immunosuppressive drugs.

The aim of this thesis was the construction of adequate transplantation site for islet transplantation at subcutaneous site without immunosuppression. The author tried to induce vascularization and promote Treg cell induction and migration, in order to form vascularized immune tolerant site at subcutaneous site, using hydrogels carrying angiogenic factors (Figure 1).

The author focused angiogenic factors, such as SEK-1005 (SEK) known as a TGF- β inducer and an of wound healing [61],[62], and basic fibroblast growth factor (bFGF) which is used as a treatment for burn wound and skin ulcer [63],[64], and performed subcutaneous islet transplantation in some donor-recipient combinations of rat and mouse models. In order to prepare transplantation site under the skin, the author developed the hydrogel careers to deliver the factors at subcutaneous sites. The author used to agarose gel which won't adhere to the tissue in order to get the space for islet transplantation. The use of biodegradable materials, which are applied in clinical use, was also examined. The gelatin sheet was used as an adhesion prevention sheet. Sodium hyaluronate solution has been used for the purpose of accelerating the formation of rich blood vessels and the wound healing [65],[66]. Additionally, the immunological analysis was performed to reveal the mechanism of graft acceptance in present studies, focusing Treg cells.

Chapter 1 describes the subcutaneous islet transplantation by implantation of agarose rods loaded bFGF (agarose-bFGF) without immunosuppression. In this chapter, allogeneic islet transplantations were carried out in two donor-recipient combination, F344 rats (RT-1^{lv1})-ACI rats (RT-1^a, low responder) and Wister rats-Lewis rats (RT-1^l, high responder). Vascularized pockets were formed after 7 days by the implantation of agarose-bFGF in two dorsal subcutaneous sites in diabetic rats, and all of recipients

showed long-term survival of the allogeneic islet grafts transplanted into the vascularized pocket. In contrast, none of recipients that were transplanted donor islets at untreated subcutaneous sites demonstrated long-term normoglycemia.

The mechanisms underlying acceptance of the grafts also investigated by flow cytometry and gene expression analyses. The population of Treg cells was significantly increased at the granulomatous tissue formed by the implantation of agarose-bFGF for 7 days, compared with that formed by agarose rods without bFGF. Gene expression analyses revealed that the gene expressions related with the migration and induction of Treg cells were significantly increased at the granulomatous tissue formed by the implantation of agarose-bFGF for 7 days. In addition to that, the population of Treg cells was maintained aroslet grafts 30 days after transplantation.

This chapter indicates that immune tolerant environment formed by the implantation of agarose-bFGF for 7 days.

Chapter 2 describes the subcutaneous islet transplantation by implantation of agarose rods incorporating cyclic oligopeptide SEK-1005 (agarose-SEK) without immunosuppression, and the immunological analysis for the mechanism of graft acceptance. SEK is a more stable and smaller molecular than bFGF.

A minimally invasive method for islet transplantation without immunosuppression is needed to treat type 1 diabetes. In this chapter, vascularized pockets formed after 10 days by the implantation of agarose rods incorporating cyclic oligopeptide SEK-1005 in two dorsal subcutaneous sites in diabetic ACI rats, and 8 of 10 recipients showed long-term survival of the allogeneic islet grafts of F344 rats transplanted into the vascularized pocket.

The mechanisms of graft acceptance in this chapter also investigated by flow

cytometry and gene expression analyses. The population of Treg cells was significantly increased, and the ratio of CD8⁺ T cells over CD4⁺ T cells was significantly decreased at the granulomatous tissue formed by the implantation of agarose-SEK for 10 days, compared with that formed by agarose rods without SEK. Gene expression analyses revealed that the gene expressions related with the migration and induction of Treg cells were significantly increased at the granulomatous tissue formed by the implantation of agarose-SEK for 7 days. In addition to that, the population of Treg cells and the ratio of CD8⁺ T cells over CD4⁺ T cells were maintained around islet grafts 30 days after transplantation. These results are not competing with the previous report that SEK accelerates the production TGF- β , known to induce Treg cells, and skin ulcer of diabetes mouse [61],[62].

This chapter indicates that a subcutaneous site that is pre-vascularized using agarose-SEK may be useful for achieving successful allogeneic islet transplantation without immunosuppression, and the implantation of agarose-SEK had the equivalent efficacy as the agarose-bFGF.

Chapter 3 demonstrated the subcutaneous islet transplantation by implantation of agarose rods loaded bFGF and heparin (agarose rods with bFGF-heparin) without immunosuppression in mouse model. In this chapter, allogeneic islet transplantations were carried out in the donor-recipient combination, C57BL/6 (B6) mice- BALB/c mice. There are various genetically modified mice and antigens specific for mouse, compared with rat. The establishment of mouse model for this subcutaneous islet transplantation will enable to the more detailed immunological analysis about the mechanism of graft acceptance.

In order to establish prevascularized spaces, an agarose rod with bFGF-heparin

was implanted for 8 days into two subcutaneous sites at the groins of BALB/c mice. Four days after implantation, Diabetes was induced in the BALB/c mice by injecting streptozotocin (STZ). Eight of 10 recipient mice demonstrated long-term normoglycemia when 150 islets isolated from C57/BL6 mice were transplanted into the each prevascularized space. Few immune cells were observed in or around the long-term survived islet grafts.

These results indicated that by the implantation of agarose rods with bFGF-heparin enabled to engraft allogeneic islets without any immunosuppression in mouse model. The detailed immunological analysis about the mechanism of graft acceptance will be enabled by this establishment of subcutaneous islet transplantation in mouse model.

Chapter 4 demonstrated with the development of the device for subcutaneous islet transplantation constructed with the materials, which have been applied in clinical use. Agarose is has not yet been applied in human patients as a material for implantation into the body. In addition, agarose cannot be degraded and absorbed in the body. Therefore, it needs to be removed from the body. In this chapter, a bFGF-carrying device without these drawbacks was developed.

Allogeneic islet transplantations were carried out in the donor-recipient combination, F344 rats to ACI rats. A gelatin film was inserted as a spacer, and then the mixture of bFGF and sodium hyaluronate solution was injected at the two dorsal subcutaneous sites of STZ-induced diabetic ACI rats. 1500 islets isolated from F344 rats were transplanted into the each two pockets 7 days after implantation. Nine of 10 diabetic ACI rats with F344 islets into the prevascularized pockets demonstrated long-term normoglycemia without immunosuppressive medication. It was revealed that the functions

of islet grafts were maintained for a long time after transplantation by the analysis of plasma insulin levels and intraperitoneal glucose tolerance tests. Gelatin films were almost degraded in 67 days after implantation at subcutaneous site.

The removal procedure of hydrogel carrier is unnecessary and islet suspension can be transplanted by the injection. This chapter demonstrated the superior method because surgical invasion can be reduced.

In this thesis, the author could demonstrate that the implantation of hydrogels carrying angiogenic factors formed the vascularized immune tolerant pockets for islet transplantation at subcutaneous site, and the allogeneic islets transplanted into the pocket survived for a long period without immunosuppressive drug. The author could show a solution to the two problems of clinical islet transplantation, that is, the transplantation site and the administration of immunosuppressive drug.

The author is convinced that the achievement in this thesis will be valuable for insulin-dependent diabetes treatment.

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Chapter 1:

Long-term Functioning of Islet Allografts in Subcutaneous Space Formed with bFGF in Rat Models.

Introduction

Pancreatic islet transplantation has been expected to become a fundamental treatment for insulin-dependent diabetes mellitus. However, several requirements must be fulfilled for an ideal islet transplantation. First, the transplantation should be minimally invasive to the same degree as an insulin injection. Second, treatment should not require the administration of immunosuppressive agents after transplantation. Third, the islet graft should not be rejected for a long time. Fourth, it should be feasible to transplant islets multiple times, when necessary. Fifth, it should be easy to remove the graft when necessary. Subcutaneous sites have been considered promising for islet transplantation, because they can be transplanted with a minimally invasive method. It has been shown that blood glucose levels can be normalized for a long time by transplanting syngeneic islets into a subcutaneous site that was prepared in advance by inducing formation of a vascular network [1]–[3]. In recent years, it was demonstrated that allogeneic islets transplanted in subcutaneous tissue were accepted and could control blood glucose levels of chemically-induced diabetes for a long period without immunosuppressants [4]. Those results suggested that subcutaneous pancreatic islet transplantation might not require immunosuppression. Therefore, that islet transplantation approach was close to ideal, because it fulfills most of the above requirements.

Immune reactions against grafts depend on the animal species, the phenotype, and

the organ or tissue specificity [5]. In this study, it was examined whether long-term graft function could be realized in an allogeneic islet transplantation without immunosuppressive medications. Two donor-recipient combinations were studied: F344 rat donors (RT-1^{lv1}) with ACI rat recipients (RT-1^a) and Wistar rat donors with Lewis rat recipients (RT-1^l). Furthermore, immunological studies were carried out to gain some insight into the mechanisms underlying graft rejection and acceptance.

Materials and Methods

Materials

Streptozotocin, the penicillin-streptomycin mixed solution, Tween-20, Triton X-100, and Blocking One reagent were purchased from Nacalai Tesuque (Kyoto, Japan). FIBLAST Spray 500 (bFGF) were purchased from KAKEN PHARMACEUTICAL CO., LTD (Tokyo, Japan). Hoechst 33258 nuclear stain was purchased from Dojindo Laboratories (Kumamoto, Japan); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, USA); phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); collagenase from Nitta Gelatin (Osaka, Japan); MORPHOSAVE from Ventana Medical Systems, Inc. (Tucson, AZ, USA); and ACK buffer from Sigma-Aldrich (St. Louis, MO, USA). Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates were purchased from Thermo Fisher Scientific (Roskilde, Denmark). SeaKem GTG agarose were obtained from Lonza (Basel, Switzerland). Percoll was obtained from GE Healthcare (Buckinghamshire, UK), and Fixable Viability Dye was purchased from Affymetrix Inc. (Santa Clara, CA, USA).

Table 1 presents the antibodies used in this study. Alexa 488-labeled goat anti-guinea pig IgG, Alexa 594-labeled goat anti-guinea pig IgG, Hanks' balanced salt solution (HBSS), and RPMI 1640 were purchased from Life Technologies (Carlsbad, CA, USA).

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, USA). Enzyme-linked immunosorbent assay (ELISA) kits for the rat insulin assay were purchased from Shibayagi Co., Ltd. (Gunma, Japan). The formalin solution and the hemoglobin assay kit were purchased from Wako

Pure Chemical Industries (Osaka, Japan). ELISA kits for the rat TGF- β 1 assay (TGF- β 1 E_{max} Immuno Assay System) were purchased from Promega Co. (Madison, WI, USA). Tissue-Tek O.C.T. compound from Sakura Finetek USA, Inc. (Torrance, CA., USA); and FITC-tomato lectin from Vector Laboratories, Inc. (Burlingame, CA, USA).

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

Islet Transplantation

Animals

Two donor-recipient combinations were used: 8- to 12-week-old male ACI/NSlc rats (RT-1^a) and 8- to 12-week-old male Lewis/N Slc rats (RT-1^l) as islet transplant recipients; 7- to 9-week-old male Fisher 344 rats (F344 /NSlc, RT-1^{lv1}) and 7- to 9-week-old male Slc:Wistar rats 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-bFGF rod

We prepared a rod-shaped agarose scaffold (diameter, 4 mm; length, 25 mm) as previously described.[4] 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°C overnight, and then freeze-dried for 24 hours under reduced pressure. The bFGF solution were evenly dropped on to

the freeze-dried agarose rods (10 μ g or 50 μ g bFGF in 100 μ L buffer). For control experiments, 150 μ L saline was dropped freeze-dried agarose gel rods. The agarose gel rods with bFGF (agarose-bFGF rods) and the control rods without bFGF, were stored at 4°C until use.

Vascularization of subcutaneous tissue with the agarose-bFGF rod

To induce diabetes in the recipient rats, a single intraperitoneal injection of STZ in citrate buffer (pH 4.5) was administered at a dose of 60 mg/kg body weight. Rats with non-fasting blood glucose levels of >400 mg/dL for two consecutive days were considered diabetic. To induce vascularization in the STZ rats, an agarose-bFGF rod was implanted into the dorsal subcutaneous fat tissue (one rod in each of two sites) 7 days after agarose-bFGF rods implantation. At 7 days after implantation, these rods were removed, and tissue that contacted the agarose-bFGF rod was harvested for histological analysis. For semi-quantitative measurement of the vascularization level, the amount of hemoglobin in the subcutaneous tissue was determined as previously described [1]. Briefly, the harvested tissue was weighed, minced, and incubated for 48 hours at 4°C in 10 mL Gay's solution (0.75% NH_4Cl 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 and Wistar rats were isolated using the collagenase digestion method as previously described.[6] 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 two 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-bFGF rods, 1500 islets isolated from donor rats were transplanted into each of the two pre-vascularized dorsal pockets. Non-treated STZ-rats were also transplanted 1500 islets into each of the two dorsal sites. The wounds were closed by suturing. None of the recipient rats received any immunosuppressive drugs before or after islet transplantation. For determination of non-fasting blood glucose levels, blood samples were collected from the tail veins of the recipient rats daily for the first two weeks, and two or three times per week thereafter. Glucose levels were determined using a glucose sensor. The finding of two 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 in ACI recipients at between 50–55 days and between 100–105 days after islet transplantation. Normal non-diabetic ACI 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 ACI rats to determine the plasma insulin levels. For controls, blood samples were also collected from normal and STZ ACI 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 −80°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 [7]. 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 non-specific staining, and then washed with PBS. The sections were subsequently treated with a solution of polyclonal guinea pig anti-insulin antibody (1:200) in Blocking One reagent for one hour at room temperature. This was followed by a one-hour incubation at room temperature with fluorescently labeled secondary antibody—either Alexa 488-labeled goat anti-guinea pig IgG (1:500) or Alexa 594-labeled goat anti-guinea 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 one hour at room temperature to reduce autofluorescence [8].

Table 1. The antibodies used in this chapter.

Antigen	Clone	Host	Application	Dilution	Source
CD4	OX35	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, NJ, USA
CD8a	OX8	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD3e	G4.18	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD25	OX39	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
Granulocyte	RP-1	Mouse	Flowcytometry	1/200	Affymetrix Inc., Santa Clara, CA, USA
Macrophage	HIS36	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD11b/c	OX42	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD32	D34-485	Mouse	Flowcytometry	1/100	Becton, Dickinson and Company, Franklin Lakes NJ, USA
Foxp3	FJK-16s	Rat	Flowcytometry	1/100	Affymetrix Inc., Santa Clara, CA, USA
Insulin	polyclonal	Guinea pig	Immunohistology	1/200	Abcam, Cambridge, UK.

Intravenous 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-ACI rats and STZ-Lewis rats after 7 days of pre-vascularization, as well as into ACI recipient 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), 117 (n = 4), or 118 (n=1) days after islet transplantation, STZ-ACI rats with long-term functional F344 islet grafts were intraperitoneally injected with 10^7 F344 splenocytes isolated from islet donor F344 rats. The Wistar rat is an outbred stock. Splenocytes were isolated from islet donor at islet isolation and stored under frozen state. The Wistar splenocytes were given to the Lewis recipient of islets isolated from same donor at 100 (n=2) days after islet transplantation. 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 Pre-vascularized with Agarose-bFGF 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 [9]. 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 [10]. 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 (Sony Co., Tokyo, Japan). The sorted cells showed a cell purity of >96%, and were used in subsequent experiments.

TGF- β 1 concentrations in subcutaneous tissue implanted with agarose-bFGF rods

Wound exudate was sampled daily from the site of agarose-bFGF rod implantation. After centrifugation at $1000 \times g$, TGF- β 1 concentration was determined by ELISA.

Gene Expression Analyses of Pre-vascularized Subcutaneous Tissue

Microarray analysis

From subcutaneous tissue treated with agarose-bFGF 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. Specific gene groups comprising genes associated with Treg cells, the GO term cytokine, or the GO term chemokine were identified. A volcano plot was generated to visualize the normalized expression of all genes or specific gene groups. 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 were further compared. A heat map was generated to visualize the normalized expression of Treg cell-associated genes.

Statistical Analysis

Between-group comparisons were made using the Student's t -test. 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.

Results

Islet transplantation

Freeze-dried agarose rods were applied with basic fibroblast growth factor (bFGF; 50 µg/rod) to prepare agarose-bFGF rods. Rat diabetes models were created by injecting streptozotocin (STZ) into rats (STZ rats; Figure 1A). Under normal conditions, subcutaneous tissues of STZ rats displayed low levels of vascularization (Figure 1B, C). Agarose-bFGF rods were implanted into STZ rats in each of two dorsal subcutaneous sites. After 7 days, the formation of highly vascularized granulomatous tissue adjacent to the agarose-bFGF rods were observed (Figure 1D-G). The hemoglobin content of these tissues increased to 5.46 ± 1.70 mg/g (Figure 2A). Numerous endothelial cells, visualized with an intravenous injection of FITC-lectin, indicated that many blood vessels existed in the granulomatous tissue (Figure 2B, C). In subsequent experiments, the agarose-bFGF rods were removed on day 7, and islets were transplanted into the resulting subcutaneous spaces, surrounded by highly vascularized, granulomatous tissue (bFGF-spaces).

Two models of donor-recipient combinations were used for transplantations. In one model, islets from donor F344 rats (RT-1^{lv1}) were transplanted into recipient ACI rats (RT-1^a); in the other model, islets from donor Wistar rats were transplanted into recipient Lewis rats (RT-1^l). All recipient rats had STZ-induced diabetes (STZ-rats). After the agarose-bFGF rod treatments, a total of 1500 allogeneic islets were transplanted into each bFGF-space for a total of 3000 islets per recipient rat. No immunosuppressive medications were given to recipient STZ rats, before or after transplantation. Blood glucose levels of the 8 STZ-ACI rats and 5 STZ-Lewis rats are shown in Figure 3A and 3B, respectively. All recipients achieved normoglycemia more than 100 days after

transplantation. The full normalization of blood glucose levels took longer in STZ-Lewis rats than in STZ-ACI rats. This difference might have been due to the low quality of islets isolated from Wistar rats. In contrast, when islets were transplanted into untreated subcutaneous tissues, no recipients demonstrated normoglycemia (Figure 3C, D). Figure 3E and 3F show graft survival during 100 days after transplantation.

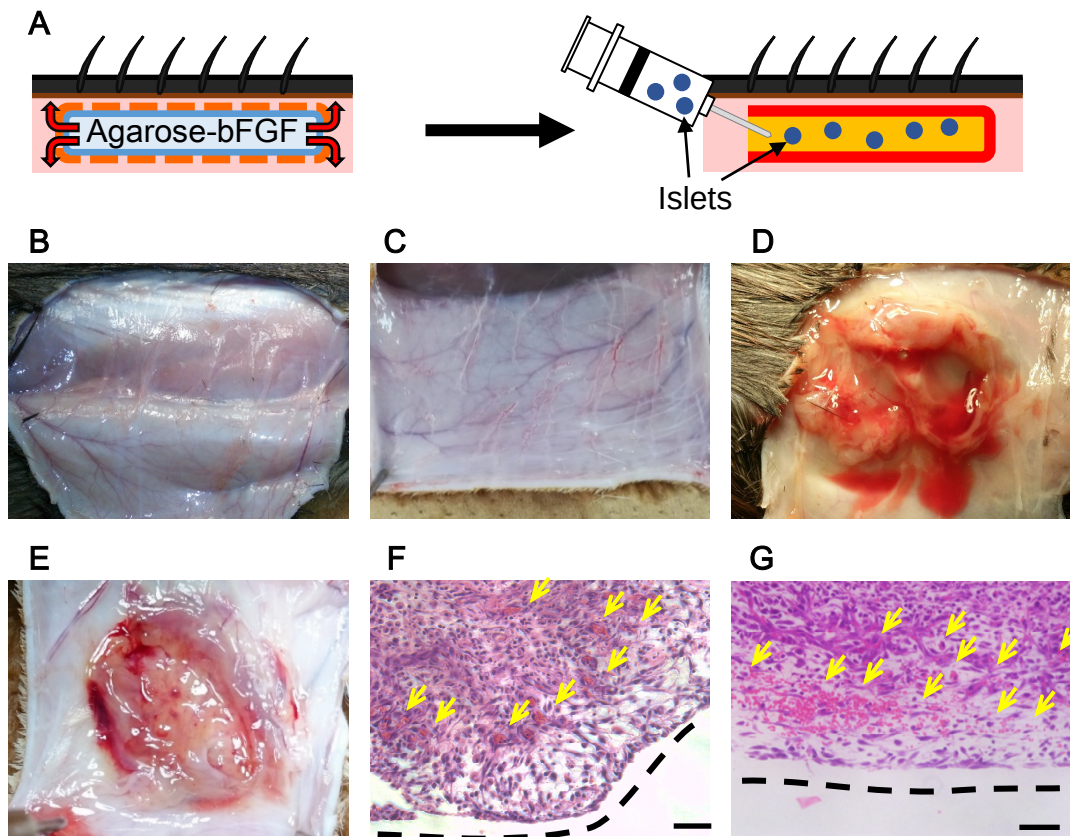


Figure 1. Preparation of a subcutaneous islet transplantation site. (A) Schematic drawing of the methods for (*left*) inducing the formation of a subcutaneous space surrounded by highly vascularized granulomatous tissue and (*right*) transplanting islets after removing the agarose rod. (B, C) Microscopic images show untreated subcutaneous sites in STZ-ACI rat (B), and in STZ-Lewis rats (C). (D, E) Microscopic images show prevascularized subcutaneous sites in a STZ-ACI rat (D), and a STZ-Lewis rat (E). (F, G) H&E staining of a section of subcutaneous tissue from a STZ-ACI rat (F), and in STZ-Lewis rats (G); the tissue was pre-vascularized by implanting an agarose rod with 50 µg bFGF for 7 days. Yellow arrows indicate vasculature.

Islet graft function was characterized in ACI recipients by determining plasma insulin levels and performing intraperitoneal glucose tolerance tests (IPGTTs; Figure 4A-C). The plasma insulin levels of the recipients were higher than those of untreated STZ rats, at 40 days and 90 days after transplantation (Figure 4A). Those were almost same levels of normal rats. Large differences were observed in the areas under the blood glucose curves (AUCs) of IPGTTs between untreated STZ rats and recipient STZ rats implanted with F344 islets at 50-55 days and 100-105 days after transplantation (Figure 4B, C).

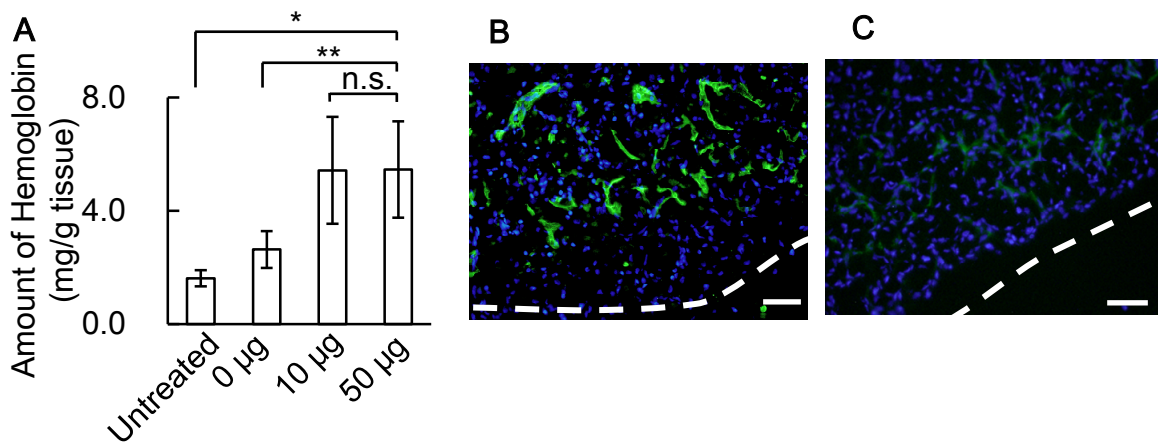


Figure 2. Vasculature of subcutaneous islet transplantation site. (A) The hemoglobin contents of untreated tissue (n=6) or granulomatous tissues prepared by the implantation for 7 days of agarose rods carrying the indicated amounts of bFGF (0 µg, n=3; 10 µg, n=7; 50 µg, n=7). Data are the mean $\pm$ SD. The statistical significance of between-group differences was determined by one-way ANOVA. *P=0.0004, **P=0.0444. (B) The endothelial layer in the granulomatous tissue of a STZ-ACI rat (B), and a STZ-Lewis rat (C), visualized with an intravenous FITC-lectin infusion, visualized with an intravenous FITC-lectin infusion.

The islet grafts on the left dorsal sites of ACI and Lewis recipients were retrieved at 159 days and 100 days after transplantation, respectively, for histochemical examinations. The islet grafts were clearly visible in hematoxylin and eosin (H&E) stained slices (Figure 5A, B). Most islets were incorporated into the connective tissue. Few lymphocytes were found in and around the islet grafts. Many insulin-positive cells were observed in immunofluorescently stained slices (Figure 5C, D). The vessels in islet grafts that were connected to host vessels of the recipients were visualized with an intravenous infusion of a FITC-lectin solution (Figure 5C).

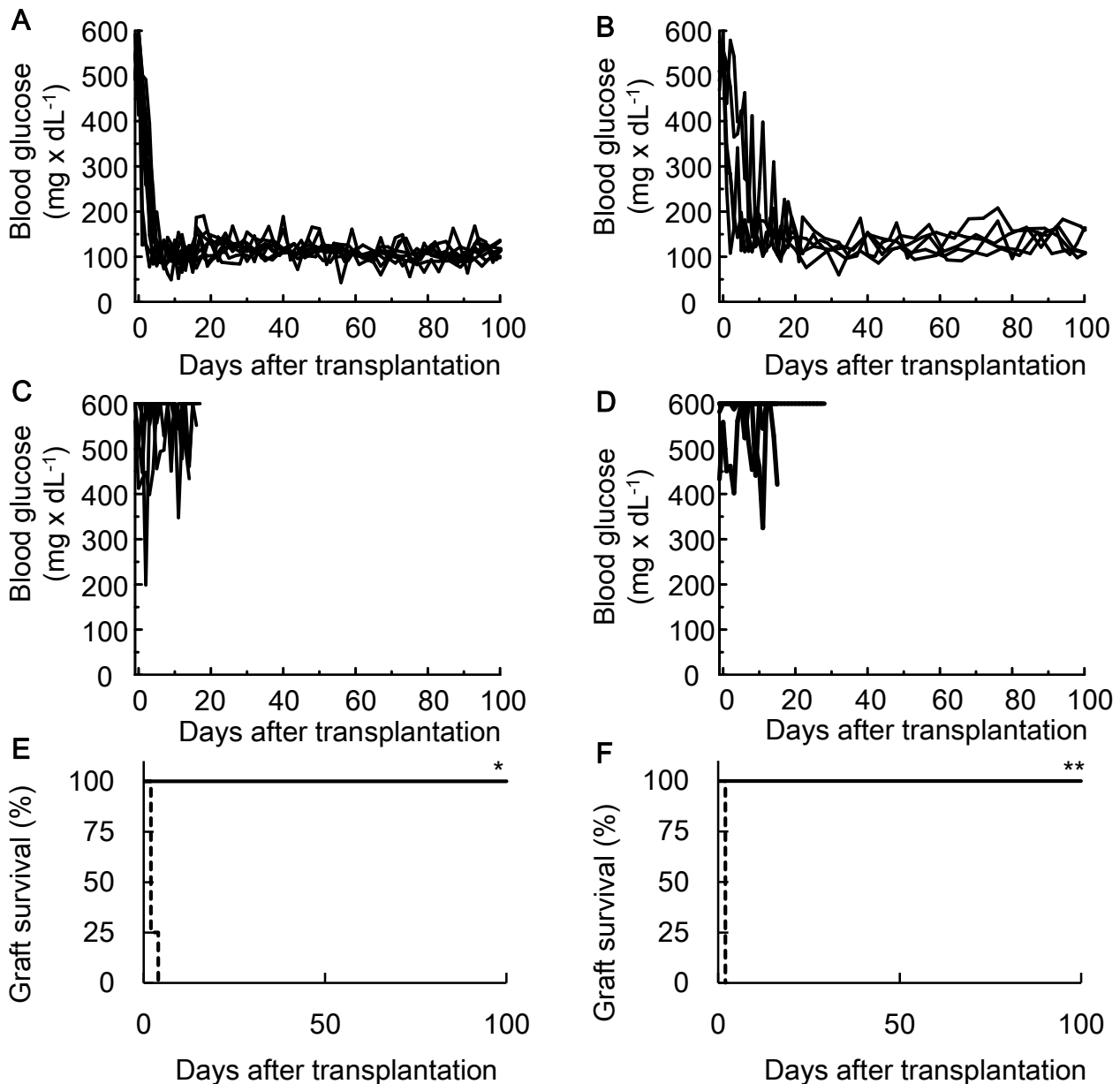


Figure 3. Normalization of blood glucose levels after islet transplantation into subcutaneous bFGF-spaces. Rats received no immunosuppressive drugs, before nor after islet transplantation. Blood glucose levels were measured after transplanting 1500 islets subcutaneously. (A) STZ-ACI rats transplanted with F344 islets in each of two bFGF-spaces (n=8); (B) STZ-Lewis rats transplanted with Wistar islets in each of two bFGF-spaces (n=5); (C) STZ-ACI rats transplanted with F344 islets in each of two dorsal untreated sites (n=5); (D) STZ-Lewis rats transplanted Wistar islets in each of two dorsal untreated sites (n=5). (E) Kaplan-Meier plots of graft survival for ACI rat recipients. *P = 0.0002; (F) Kaplan-Meier plots of graft survival for Lewis rat recipients. **P = 0.0005. (E, F) Solid lines show survival of islets transplanted into the bFGF-spaces; dotted lines show survival of islets transplanted into untreated subcutaneous sites.

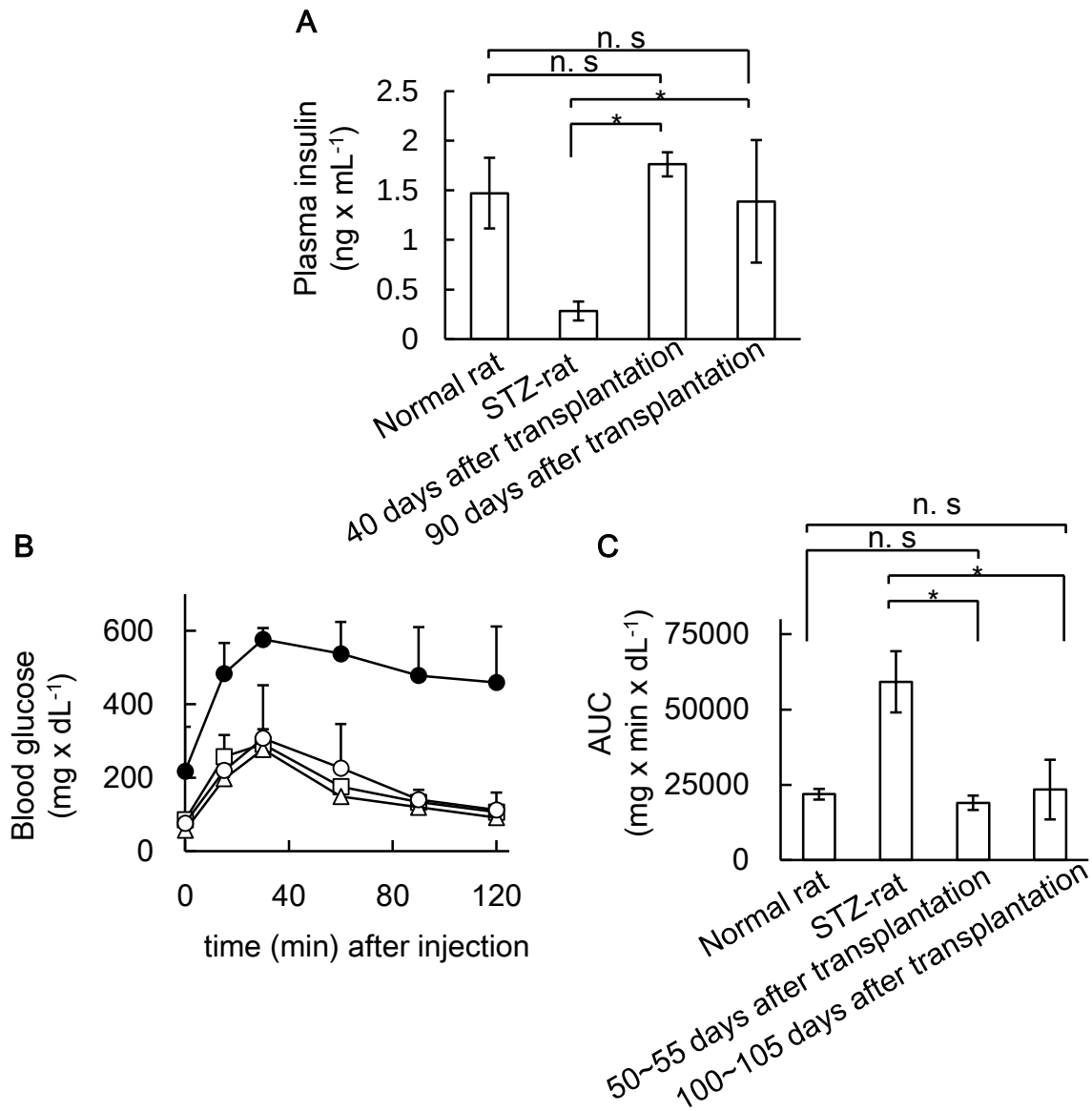


Figure 4. Functions and morphologies of islet grafts. (A) Plasma insulin levels in untreated healthy (normal) ACI rats (n=7), untreated diabetic (STZ-ACI) rats (n=7), and diabetic (STZ-ACI) rats treated with 3000 F344 islets transplanted into bFGF-spaces and tested at 40 days (n=4) and 90 days (n=4) after transplantation. (B) Results from intraperitoneal glucose tolerance tests (IPGTT) conducted in normal ACI rats (open squares, n=7), STZ-ACI rats (filled circles, n=7), and STZ-ACI rats treated with 3000 F344 islets transplanted into bFGF-spaces and tested at 50–55 days (open triangles, n=3) or at 100–105 days (open circles, n=3) after transplantation. (C) Areas under the IPGTT curves (AUCs). Data are the mean $\pm$ SD. The statistical significance of between-group differences was determined by one-way ANOVA. *P < 0.0001.

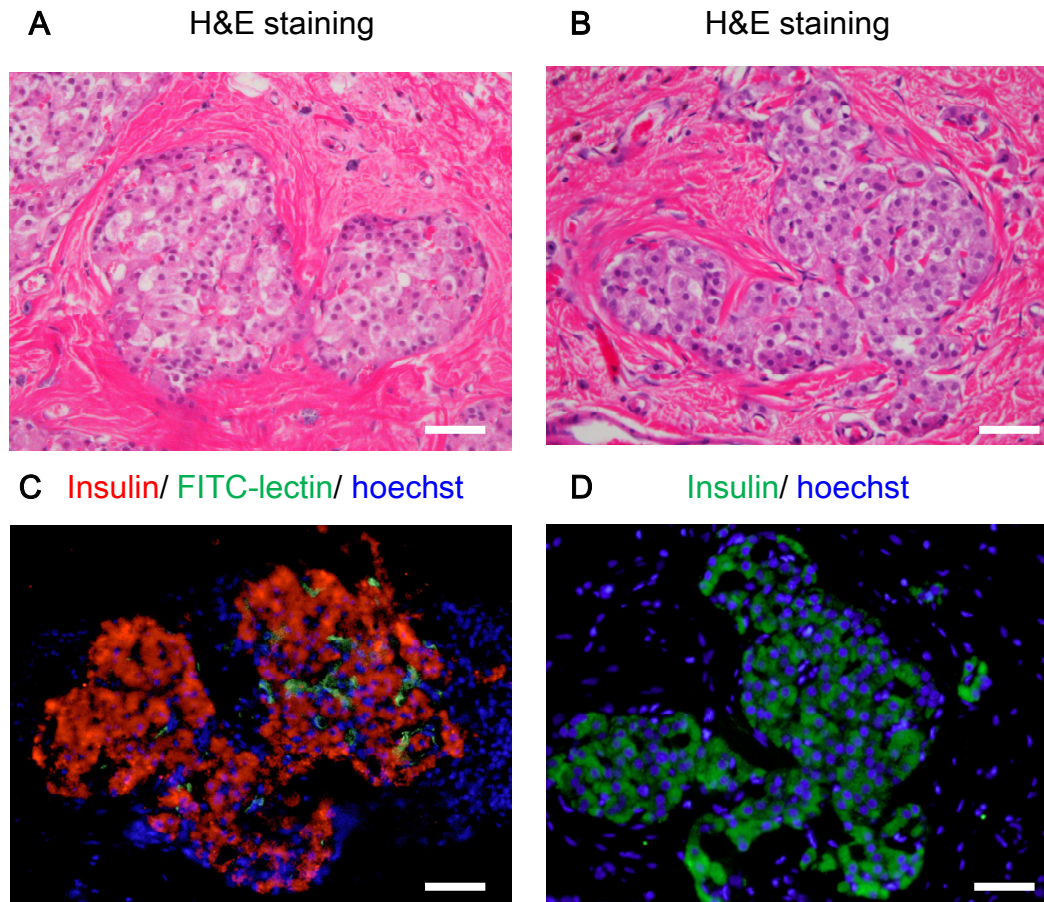


Figure 5. Morphologies of islet grafts. (A) H&E staining of graft Wistar islets retrieved from STZ Lewis rats at 100 days after transplantation. (B) Immunofluorescence staining of F344 islets in a graft retrieved 30 days after transplantation shows insulin (red) and endothelial cells (green); the intravenous infusion of FITC-lectin allowed examination of functional vasculature. (C) Immunofluorescence staining of Wistar islets in a graft retrieved 100 days after transplantation shows insulin (green). Scale bars: 50 μ m.

Immune characteristics of vascularized granulomatous tissue, before and after islet transplantation

Immune cells that infiltrated the granulomatous tissue before islet transplantation were isolated and analyzed with fluorescence-activated cell sorting (FACS; Figure 6-8). The percentage of Foxp3⁺ cells among all CD4⁺ T cells (Treg%) increased in the presence of agarose- bFGF rods, reaching 25% at 2 days after implantation. The Treg% was maintained at levels >24% from 7 to 14 days after agarose-bFGF rod implantation (Figure 6A). In contrast, the Treg% was <15%, when granulomatous tissue was prepared by implantation of a bFGF-free agarose rod. Moreover, in all STZ rats that received agarose rods, with or without bFGF, less than 10% Treg cells were detected in the draining lymph nodes, axillary lymph nodes, and spleen on any day (Figure 6B, 7A, and B). When Treg% in lymph nodes and spleen was compared, no difference was observed. Thus, the agarose-bFGF rods evoked regional, but not systemic, modifications in the immunological environment. The CD8⁺ T/CD4⁺ T ratio was also determined. After agarose rods with/without bFGF were implanted, the CD8⁺ T/CD4⁺ T ratios of both groups increased from ~35% to 50%–70% during the observation period (Figure 8A). However, the CD8⁺ T/CD4⁺ T ratios slightly decreased, from 37% to 28%, in the draining lymph nodes (Figure 8B). Implantation of agarose-bFGF rods did not produce a change in the CD8⁺ T/CD4⁺ T ratios in the axillary lymph nodes or spleens of STZ rats (Figure 7C, D). TGF- β 1, which is reported to play a role in inducing Treg cells [11], was maintained at higher concentrations in the tissue fluids of bFGF-spaces than in spaces prepared by implantation of bFGF-free agarose rods (Figure 8C).

The immunological characteristics of the islet graft environment played a pivotal role in the long-term survival of the graft and in its success in glycemic control. Immune

cells that were in the graft and surrounding connective tissues were analyzed at 5 and 30 days after islet transplantation. Around the islet grafts, >20% Treg cells was maintained for 30 days after transplantation (Figure 9A). In contrast, <10% Treg cells was observed in draining lymph nodes, in axillary lymph nodes, and in the spleen (Figure 9B, 10A, and B). Around the islet grafts, CD8⁺ T/CD4⁺ T ratios rose to slightly higher levels than observed pre-transplantation, but later, these ratios decreased to preoperative levels (Figure 9C). CD8⁺ T/CD4⁺ T ratios were <50% in draining lymph nodes, in axillary lymph nodes, and in the spleen, before and after islet transplantation (Figure 9D, 10C, and D).

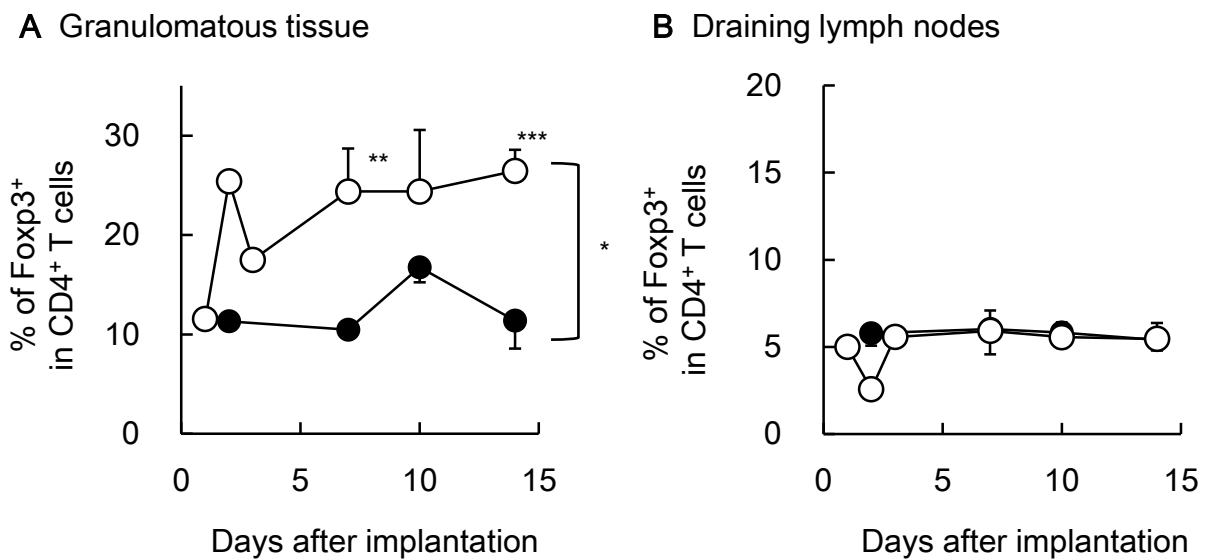


Figure 6. Recruitment of immune cells in granulomatous tissues formed at subcutaneous sites and in draining lymph nodes. Cells were measured for 14 days following implantation of agarose rods with or without bFGF. (A, B) The percentages of Foxp3⁺ cells (Treg cells) were evaluated in (A) CD4⁺ T cells isolated from granulomatous tissue and (B) CD4⁺ T cells isolated from the draining lymph nodes. Open circles: STZ-ACI rats treated with agarose-bFGF rods (days 1-3, n=1; day 7, n=3; days 10 and 14, n=4); filled circles: STZ-ACI rats treated with agarose rods without bFGF (days 2 and 7: n=3; days 10 and 14: n=4). Data are the mean $\pm$ SEM. * $P < 0.001$ (Two-way ANOVA), ** $P = 0.00345$, *** $P = 0.0126$.

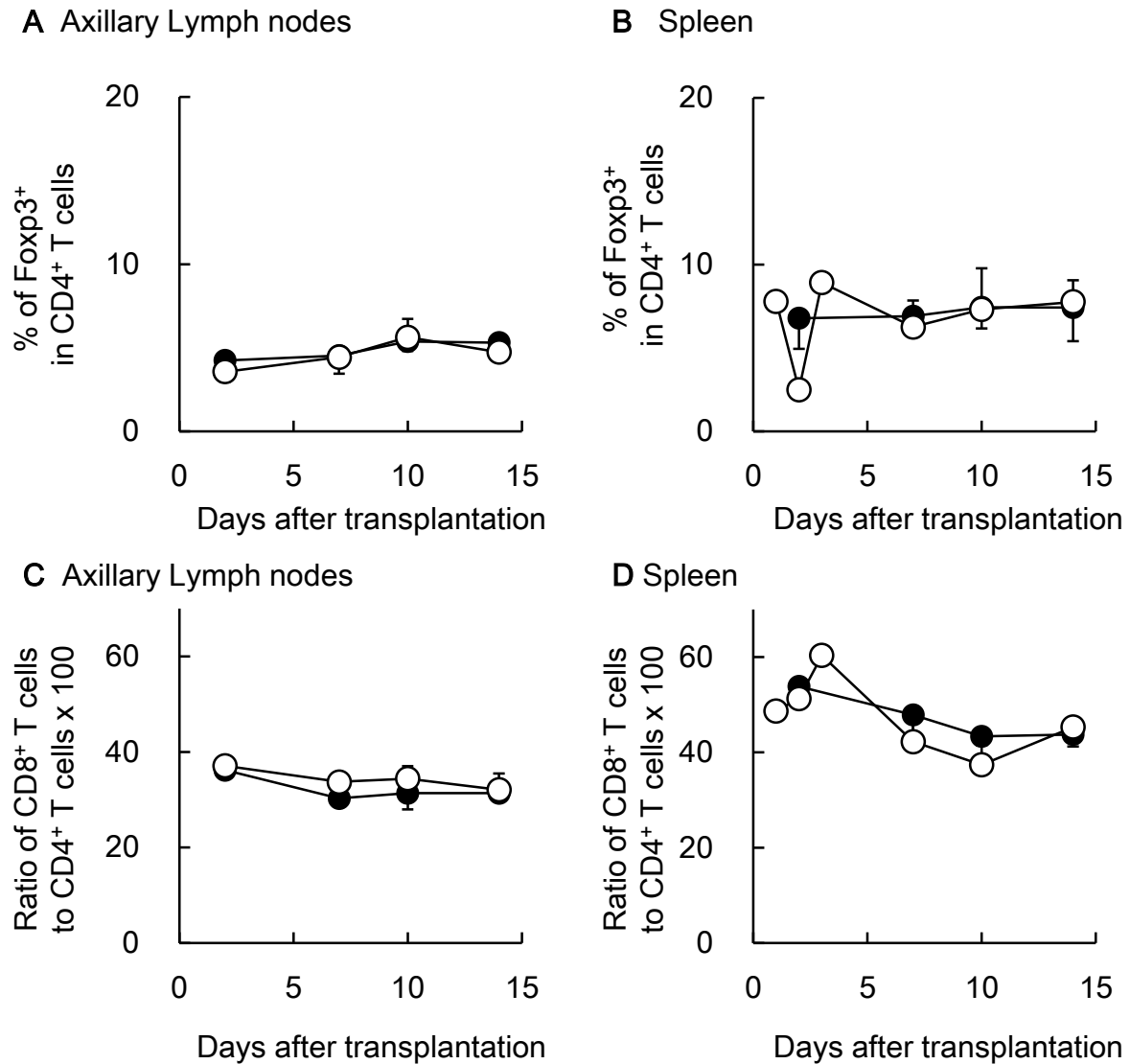


Figure 7. Recruitment of immune cells in axillary lymph nodes and spleen for 14 days following implantation of agarose rods with or without bFGF. (A, B) The percentages of Foxp3⁺ cells (Treg cells) were evaluated in CD4⁺ T cells isolated from (a) axillary lymph nodes and (B) spleen in the days after agarose rod implantation. (C, D) The ratios of CD8⁺ T cells (cytotoxic T cells) to CD4⁺ T cells isolated from (C) axillary lymph nodes and (D) spleen in the days after agarose rod implantation. (A-D) Open circles: STZ-ACI rats treated with agarose-bFGF rods (days 1-3: n=1; days 7 and 10: n=3; and day 14: n=4); filled circles: STZ-ACI rats treated with agarose rods without bFGF (days 2, 7, and 10: n=3; day 14: n=4). Data are the mean $\pm$ SEM.

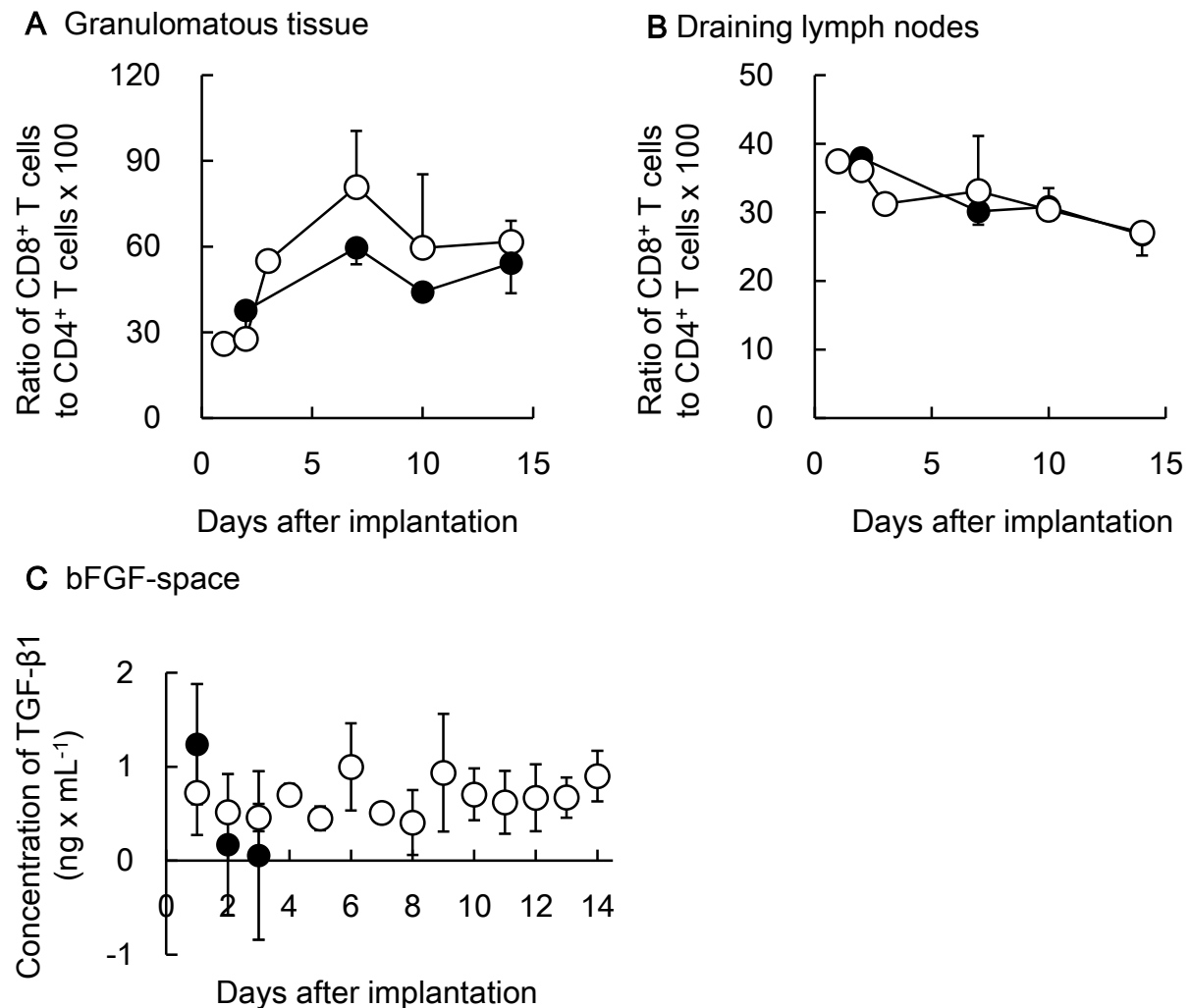


Figure 8. Recruitment of immune cells in granulomatous tissues formed at subcutaneous sites and in draining lymph nodes. Cells were measured for 14 days following implantation of agarose rods with or without bFGF. (A, B) The ratios of CD8⁺ T cells (cytotoxic T cells) to CD4⁺ T cells isolated from (A) granulomatous tissue and (B) the draining lymph nodes. (A, B) Open circles: STZ-ACI rats treated with agarose-bFGF rods (days 1-3, n=1; day 7, n=3; days 10 and 14, n=4); filled circles: STZ-ACI rats treated with agarose rods without bFGF (days 2 and 7: n=3; days 10 and 14: n=4). Data are the mean $\pm$ SEM. (C) TGF-β1 concentrations in the exudate surrounding implanted agarose rods. Open circles: STZ-ACI rats treated with agarose-bFGF rods (n=3); filled circles: STZ-ACI rats treated with agarose rods without bFGF (n=3). Data are the mean $\pm$ SD.

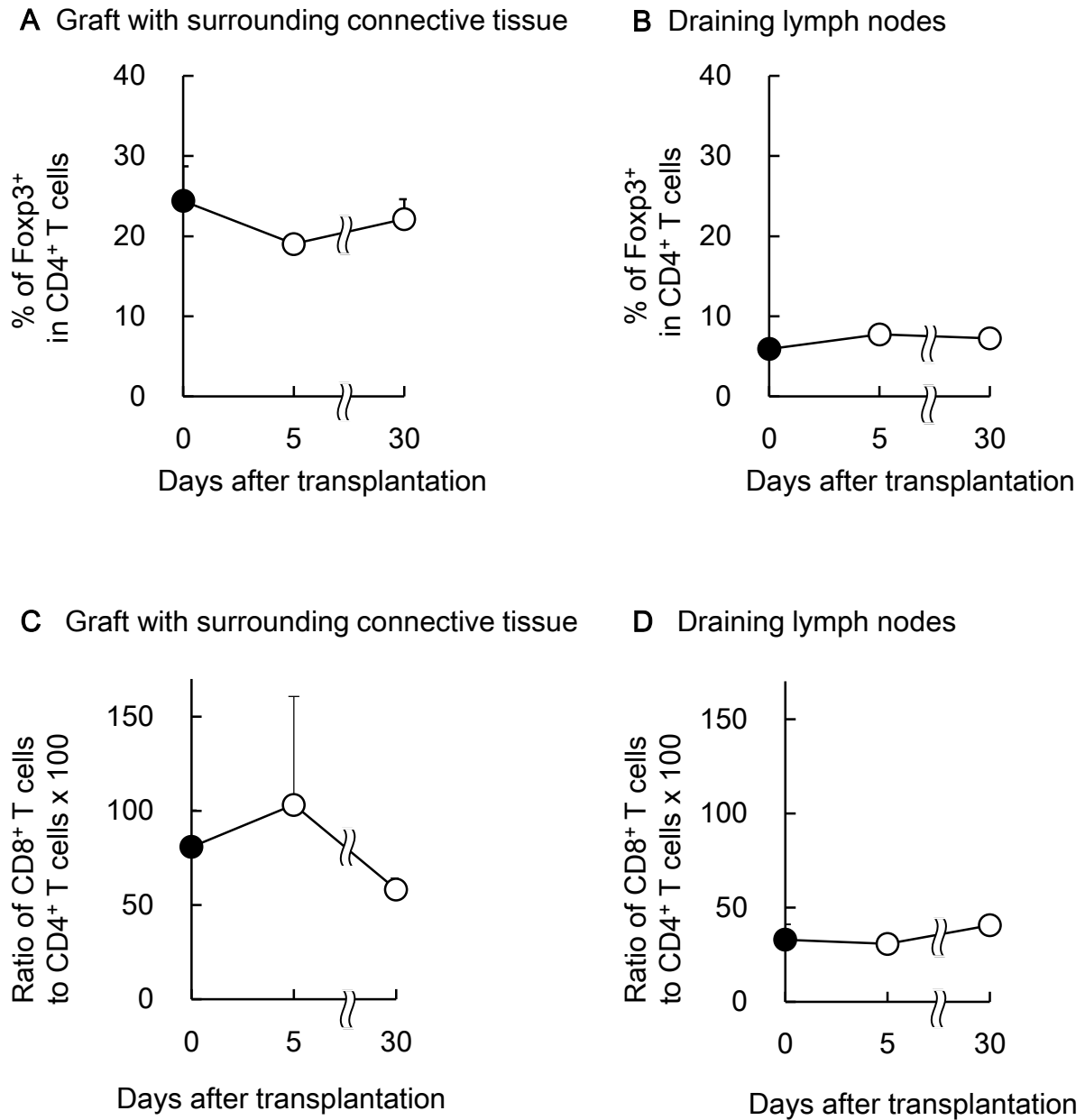


Figure 9. Recruitment of immune cells in STZ-ACI recipient rats measured at 5 and 30 days after F344 islet transplantation. (A, B) The percentages of Foxp3⁺ cells (Treg cells) were evaluated in (A) CD4⁺ T cells isolated from subcutaneous tissue containing the islet graft and (B) CD4⁺ T cells isolated from the draining lymph nodes. (C, D) The ratios of CD8⁺ T cells (cytotoxic T cells) to CD4⁺ T cells isolated from (C) subcutaneous tissue containing the islet graft and (D) the draining lymph nodes. Filled circles: values measured before islet transplantation (see Fig. 5). Open circles: values measured at 5 (n=3) and 30 (n=4) days after islet transplantation. Data are the mean $\pm$ SEM.

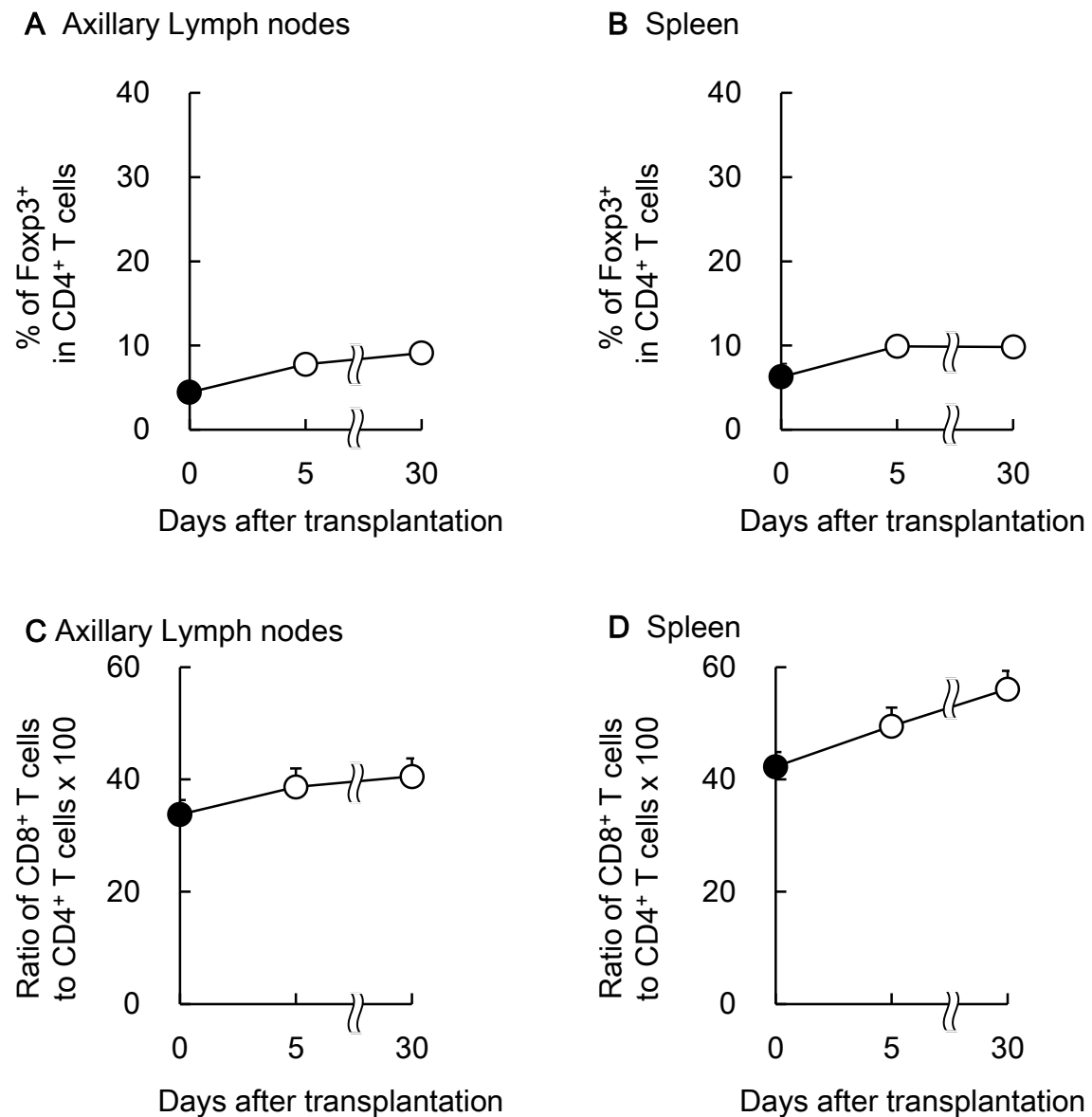


Figure 10. Recruitment of immune cells in axillary lymph nodes and spleens of STZ-ACI recipient rats, 5 and 30 days after islet transplantation. (A, B) The percentages of Foxp3⁺ cells (Treg cells) were evaluated in CD4⁺ T cells isolated from (a) axillary lymph nodes and (B) spleen. (C, D) The ratios of CD8⁺ T cells to CD4⁺ T cells isolated from (C) axillary lymph nodes and (D) spleen. Filled circles: values measured before islet transplantation (see Fig. 6). Open circles: values measured at 5 (n=3) and 30 (n=4) days after islet transplantation. Data are the mean $\pm$ SEM.

Gene expression in granulomatous tissue

Gene expression were also analyzed in granulomatous tissue formed after implanting agarose rods, with or without bFGF (Figure 11, 12). First the gene group defined in gene ontology (GO) terms as “chemokines and cytokines” was examined. The expression of those genes, excluding the Treg cell-associated genes, was significantly reduced in the bFGF group compared to the bFGF-free group (Figure 11). In contrast, expression levels of Treg cell-associated chemokines, *cxcl12* and the *tgf* signaling-associated gene (*grem1*), increased significantly in the bFGF group compared to the bFGF-free group (Figure 12A, B). The expression levels of *grem1* might play an important role in recruiting or inducing Treg cells [12]. These data suggested that the agarose-bFGF rod implantation stimulated the expression of some chemokines, cytokines, and receptors associated with chemotaxis and induction of Treg cells. Gene expression analyses also suggested that Treg cells contributed to the long-term acceptance of allogeneic islet grafts.

Effect of donor splenocyte injections on graft survival

The STZ-ACI rats with stable F344 islet grafts received an intraperitoneal injection of F344 rat splenocytes at 30 days, 117 days, and 118 days after transplantation. Blood glucose levels tended to increase a few days after splenocyte injections (Figure 13A). Two STZ-Lewis rats with Wistar islet grafts also received splenocytes from the donor Wistar rats at 100 days (Figure 13B). Again, blood glucose levels tended to increase. Among STZ-ACI rats that received splenocytes 30 days after transplantation, the immunological characteristics of the connective tissue in the graft environment were analyzed, before and after the splenocyte injection. The Treg% levels in and around islet

grafts were $23.4 \pm 5.2\%$ before donor-antigen injection, and they slightly decreased to $20.7 \pm 3.1\%$ at 5 days after the injection of splenocytes (Figure 13C). In contrast, the ratio of CD8⁺ to CD4⁺ T cells increased from $59.6 \pm 8.2\%$ to $131.8 \pm 41.8\%$ at 5 days after the injection (Figure 13D).

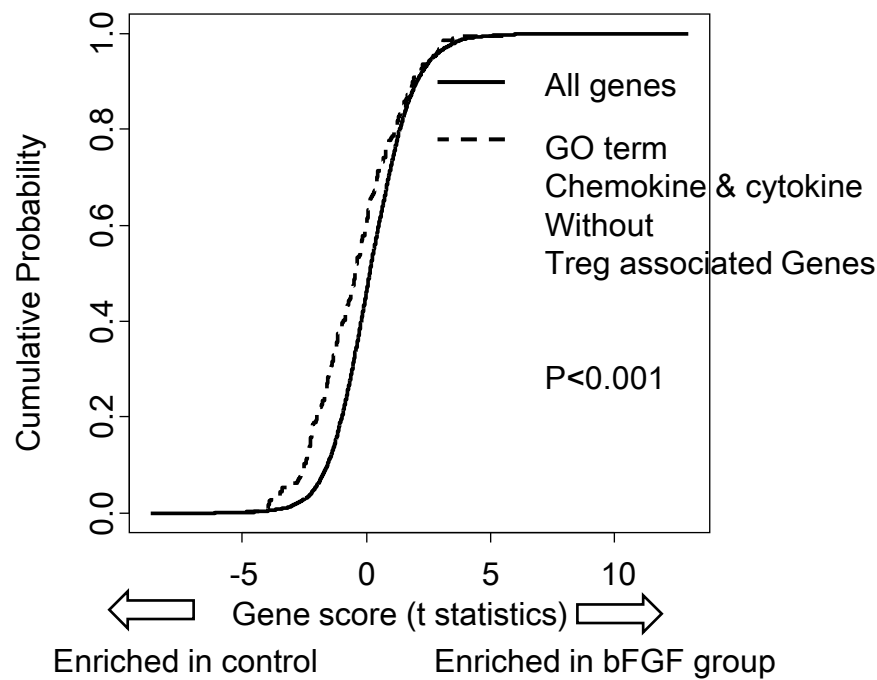


Figure 11. Gene expression changes in granulomatous tissue formed by implanting agarose rods with or without bFGF. Whole gene expression was analyzed in granulomatous tissue newly formed at 7 days after STZ-ACI rats were implanted with rods with or without (control) bFGF (n=3, each group). Gene scores (t statistics) are expressed on a cumulative frequency graph. The solid line indicates the cumulative frequency of the expression of all genes. The broken line indicates the cumulative frequency of expression of genes in a specific group; i.e., the group of genes identified with the GO terms “cytokines” and “chemokines”, after removing those associated with Treg cells. The significance of the difference in gene scores between the specific gene group and all genes (reference) was determined with the Student’s *t*-test.

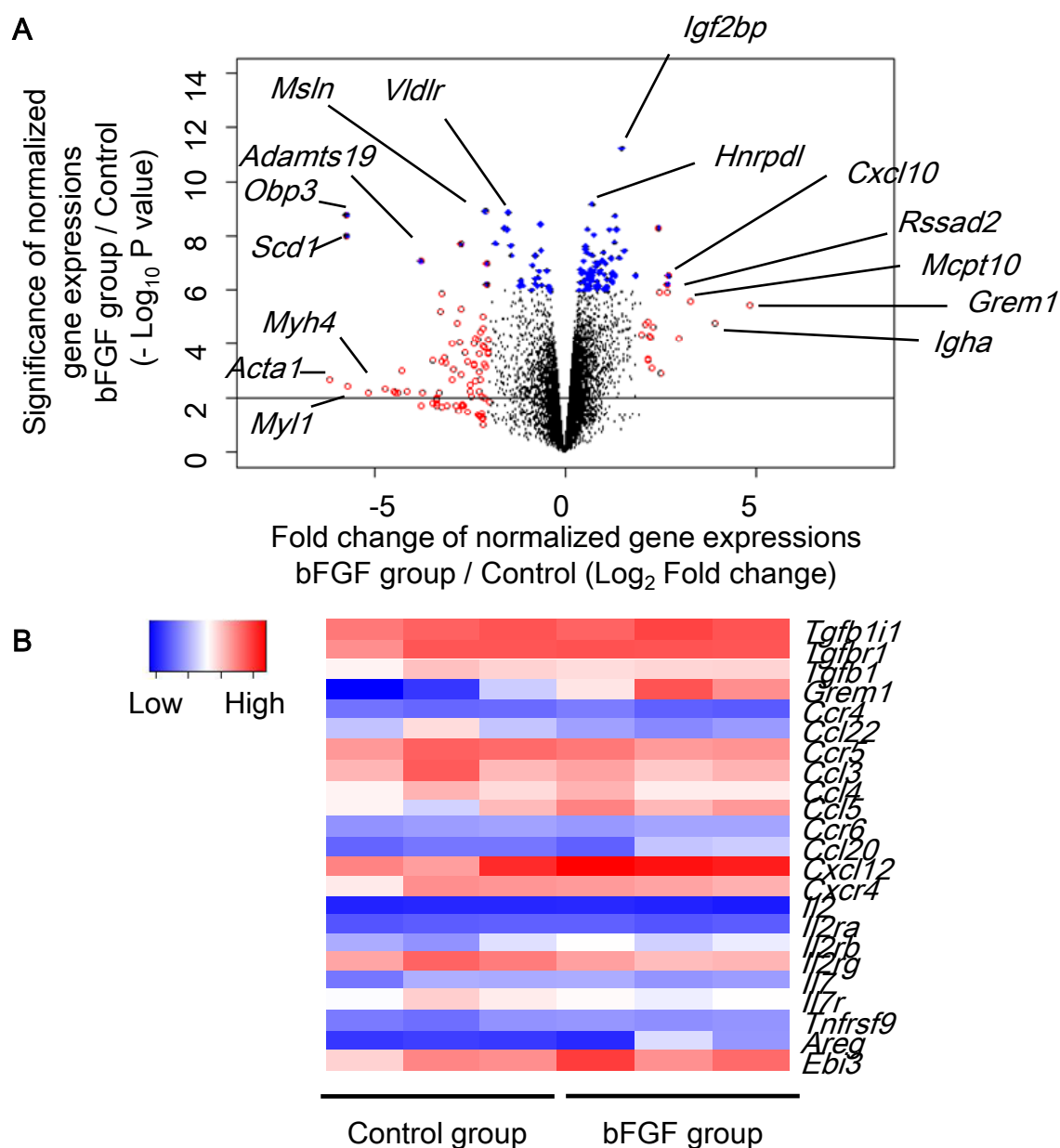


Figure 12. Gene expression changes in granulomatous tissue formed by implanting agarose rods with or without bFGF. Whole gene expression was analyzed in granulomatous tissue newly formed at 7 days after STZ-ACI rats were implanted with rods with or without (control) bFGF ($n=3$, each group). (A) Volcano plot shows the fold-changes in mRNA levels (dots). The x-axis shows the $\log_2$ of the fold-changes in gene expression observed in granulomatous tissues from bFGF-spaces relative to their expression in granulomatous tissues prepared by implantation of bFGF-free agarose rods (control). The y-axis indicates the $-\log_{10}$ of the P-values. Some representative gene names are shown. Red circles: the 100 genes that showed the greatest fold-changes. Blue filled circles: the 100 genes with the most significant P-values. (B) Heat map represents the expression of Treg-associated genes in bFGF-spaces and spaces prepared by implantation of bFGF-free agarose rods (control).

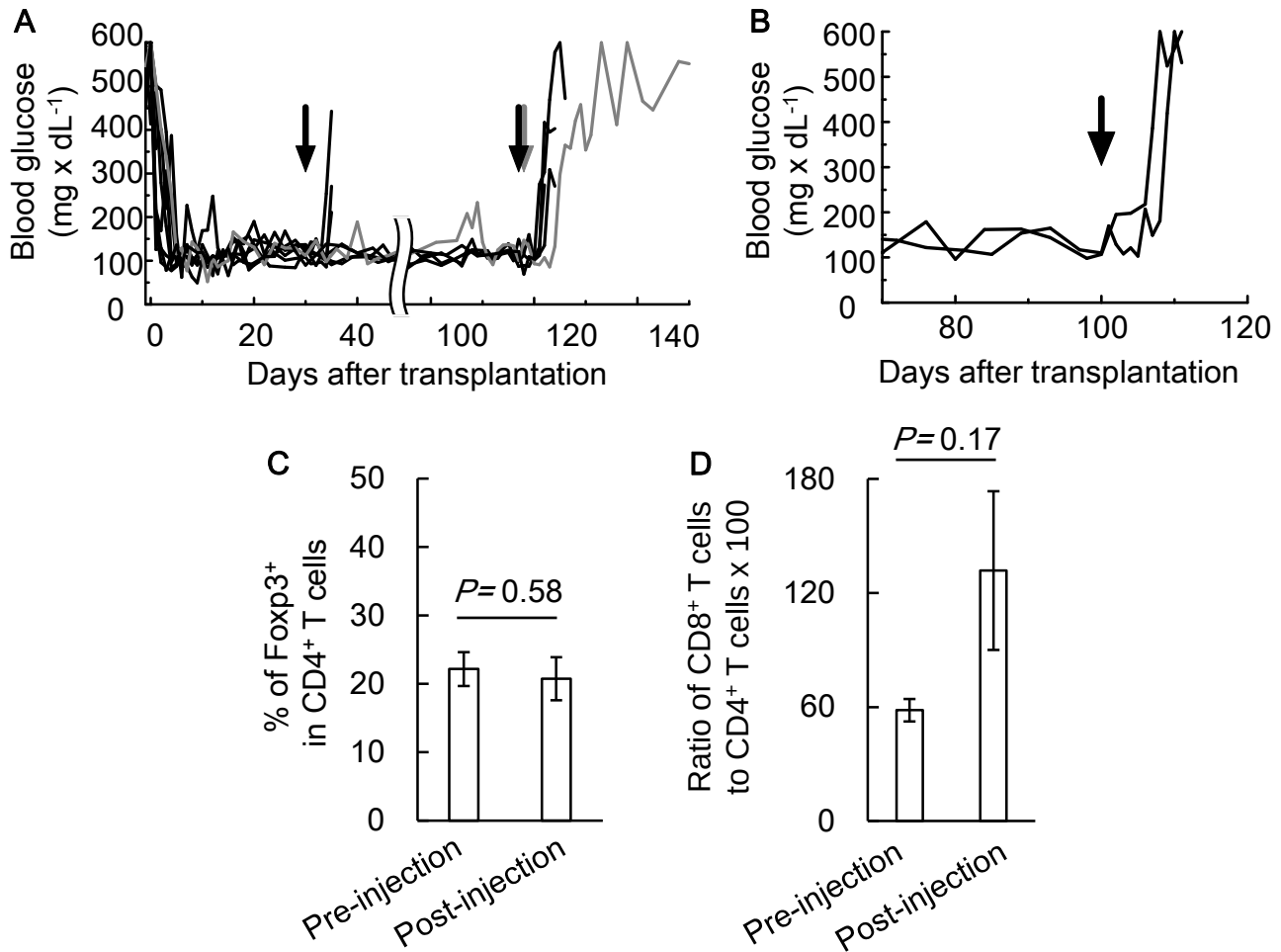


Figure 13. Rejection of established subcutaneous allogeneic islet grafts caused by infusing splenocytes from islet donors into the peritoneal cavities of recipient rats.

(A, B) Blood glucose levels before and after (A) 10^7 splenocytes of donor rats (F344) were injected (arrows) into the corresponding STZ-ACI recipient rats at 30 ($n=3$), 117 ($n=4$), or 118 ($n=1$) days after transplantation or (B) 10^7 splenocytes of donor (Wistar) rats were injected (arrows) into the STZ-Lewis recipient rats at 100 days after transplantation ($n=2$). The Wistar rat is an outbred stock. Splenocytes were isolated from donors at islet isolation and stored frozen. Splenocytes and islets isolated from same donor were given to recipients. (C) Percentage of Foxp3⁺ cells (Treg cells) in CD4⁺ T cells isolated from subcutaneous tissue containing the functioning islet graft of STZ-ACI recipient rats at 30 days after islet transplantation ($n=3$), and those isolated from subcutaneous tissue 5 days after 10^7 F344 splenocytes injection ($n=3$). (D) Ratios of CD8⁺ T cells to CD4⁺ T cells isolated from subcutaneous tissue containing the functioning islet graft of STZ-ACI recipient rats at 30 days after islet transplantation ($n=3$), and those isolated from subcutaneous tissue 5 days after 10^7 F344 splenocytes injection ($n=3$). (C, D) Post-injection measurements were taken just after blood glucose levels rose above 200 mg/dL. Data are the mean $\pm$ SEM.

Discussion

Various methods have been investigated for transplanting islets without immunosuppressive medications, including: 1) transplanting into immune privileged (or tolerant) sites; 2) creating a bioartificial pancreas; 3) reducing islet immunogenicity; and 4) transplanting islets plus suppressive immune cells. Although these methods looked promising in small animals, each has proven difficult to apply in humans. For example, immune privileged sites, such as the cerebral ventricle, the anterior eye chamber, and the testicle [13]–[15], were difficult to apply clinically, because they were too small for islet transplantation. Alternatively, a bioartificial pancreas or islets encapsulated in a semipermeable membrane [7] were limited in clinical application in humans, due to the large size or volume of the devices. Other methods investigated involved modulating islet immunogenicity, such as UV light irradiation or culturing at low temperatures [16]–[18]. To my knowledge, none of those methods were successful in clinical applications. Others have reported promising results by transplanting coaggregates that comprised islets and immunosuppressive cells, such as Treg cells [19],[20], Sertoli cells [21],[22], and mesenchymal stem cells [23]. However, it is difficult to prepare coaggregates of human islet cells and Treg cells in large quantities for use in human patients.

This approach to protecting islet grafts from the host immune system was to transplant islets into a subcutaneous site formed by implanting agarose rods applied with bFGF. It was found that the agarose-bFGF rod evoked regional, but not systemic immunological reactions. This feature was suggested by the increase observed in the percentage of Foxp3⁺ (Treg) cells among CD4⁺ T cells in granulomatous tissue, but not in the lymph nodes or spleen. Finally, the allogeneic islet grafts survived in subcutaneous

bFGF-spaces for long periods, in the absence of immunosuppressive medications.

As shown in Figure 13, islet grafts that had functioned for long periods of time were rejected, when donor lymphocytes were injected into the peritoneal cavities of recipients. This fact was interesting from an immunological standpoint, but also from a safety standpoint, which is important for clinical islet transplantations. Islet shortage is considered an obstacle that must be overcome, but this problem will likely be resolved in upcoming years. Several groups have successfully regenerated quasi-islets from human pluripotent stem cells (ES/iPS cells). They reported that transplantation of quasi-islets in a murine diabetes model could normalize hyperglycemia [24],[25]. However, it is important to ensure the safety of transplantation. The major safety concerns with those methods are teratoma formation by residual ES/iPS cells and tumor formation by transformed cells, which might arise in the process of differentiation [26]. When a teratoma/tumor forms in a subcutaneous site, it can be readily removed surgically, due to the easy access in subcutaneous tissue. Moreover, residual malignant cells can be killed by administering antigen prepared from the original ES/iPS cells. Thus, cell therapy safety can be increased by using functional cells derived from ES/iPS cells.

In this chapter, it was analyzed T cells that had infiltrated into the granulomatous tissue to gain some information about the immunological environment. This chapter suggested that Treg cells played a pivotal role in graft acceptance. However, in addition to Treg cells, various other cells, including regulatory B cells [27],[28] and M2 macrophages [29],[30], have been known to suppress immune reactions. Notably, immunological reactions to grafts highly depend on the response phenotype, the type of organ or tissue, and the animal species involved. Future studies should conduct islet transplantations in larger animal models, such as pigs and macaque monkeys, and perform

more detailed immunological analyses in small animal models.

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Chapter 2:

Long-term Functioning of Allogeneic Islets in Subcutaneous Tissue Pretreated with a Novel Cyclic Peptide without Immunosuppressive Medication.

Introduction

One method of treating insulin-dependent diabetic patients is the transplantation of islets of Langerhans (islets), whereby a suspension of islets isolated from a donor pancreas is infused into a recipient liver through the portal vein [1]. However, intrahepatic islet transplantation carries a risk of complications, such as hepatic hematoma and portal pressure increase, which can significantly impair liver function and islet graft survival [2]. Thus, recipients must take immunosuppressive medications to protect the islet graft against immune reactions. Other limitations of intrahepatic islet transplantation include the invasiveness of the transplantation procedure, the risk that islet transplantation might impair an important organ, the side effects of the required immunosuppressive drugs, and a shortage of islets.

Islet shortage was initially considered a daunting challenge, but this problem will likely be resolved in upcoming years, as several groups have successfully regenerated quasi-islets from human pluripotent stem cells (ES/iPS cells). Transplantation of these quasi-islets in a murine diabetic model can reportedly normalize hyperglycemia [3],[4]. Tremendous research efforts have been dedicated to overcoming the remaining problems. To address the invasiveness of intrahepatic transplantation, subcutaneous transplantation has been investigated. Syngeneic islets have been successfully subcutaneously

transplanted in rat and mouse models [5]–[7]. The use of a subcutaneous transplantation site facilitates islet transplantation and removal under local anesthesia. Additionally, in a diabetic rat model, allogeneic islets transplanted in a subcutaneous site prevascularized with agarose-fibroblast growth factor-2 (bFGF) can survive and normalize blood glucose levels for a long period without immunosuppressive therapy [8].

The cyclic oligopeptide SEK-1005 (SEK) (its molecular structure are shown in Figure 1) was isolated from *Streptomyces nobilis* and appears to be a new type of antiinflammatory agent [9],[10].

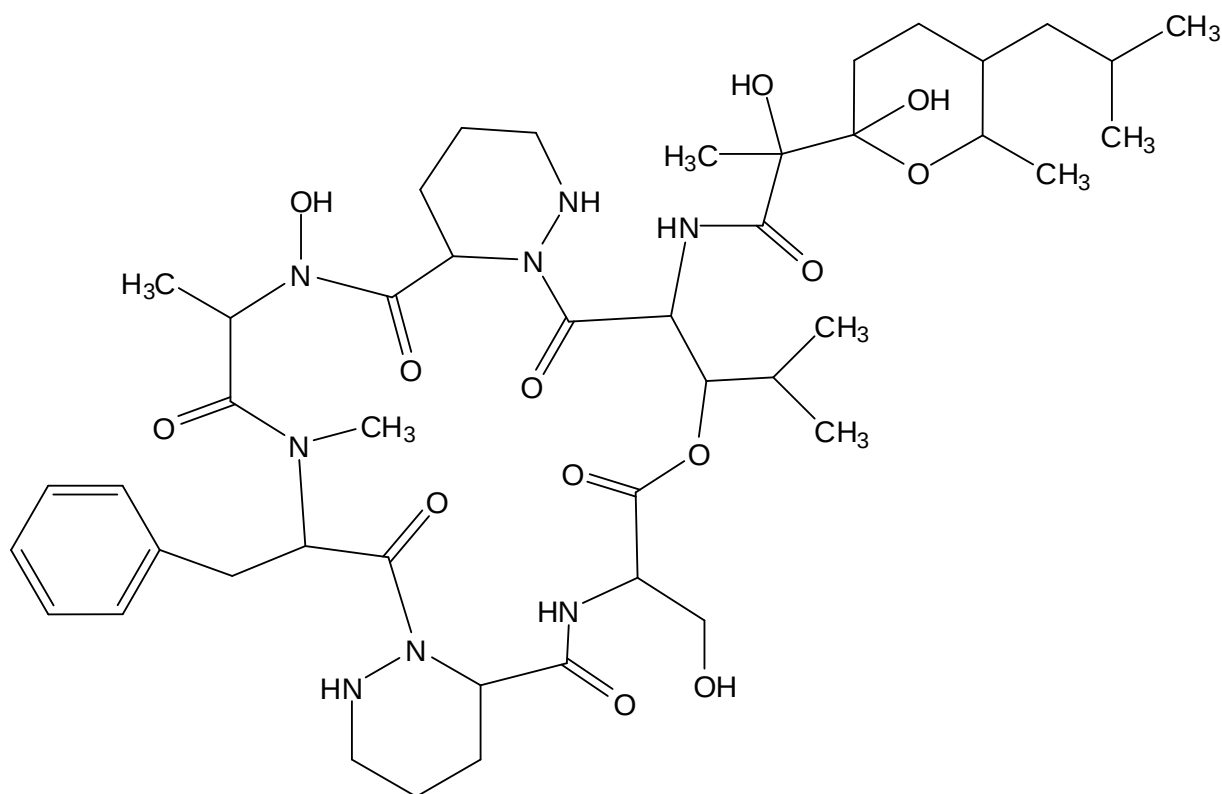


Figure 1. The chemical structure of SEK.

Hydroxy-N-[2-hydroxy-1-oxo-2-tetrahydro-2-hydroxy-6-methyl-5-(2-methylpropyl)-2H-pyran-2-yl-propyl]-Leu-Pip(hexahydro-3-pyridazinecarbonyl)-N-hydroxy-Ala-N-methyl-Phe-Pip-,rho.-lactone.

SEK can reportedly accelerate healing of full-thickness wounds, and acts as a potent inducer of transforming growth factor- β 1 (TGF- β 1) [11]. Based on these findings, it was hypothesized that SEK could potentially help create a richly vascularized space for islet transplantation in subcutaneous tissue. In the present study, the author investigated the use of SEK to create a subcutaneous site surrounded by highly vascularized granulomatous tissue for islet transplantation in diabetic rats, and examined allogeneic islet graft survival in this setting without immunosuppressive treatment.

Materials and Methods

Materials

The Sekisui Chemical Co. (Osaka, Japan) kindly donated SEK-1005 ($C_{45}H_{70}N_8O_{13}$). Streptozotocin, the penicillin-streptomycin mixed solution, Tween-20, Triton X-100, and Blocking One reagent were purchased from Nacalai Tesuque (Kyoto, Japan). Hoechst 33258 nuclear stain was purchased from Dojindo Laboratories (Kumamoto, Japan); fetal bovine serum (FBS) from Equitech-Bio, Inc. (Kerrville, TX, USA); 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, USA); mitomycin C from Kyowa Hakko Kirin Co., Ltd; MORPHOSAVE from Ventana Medical Systems, Inc. (Tucson, AZ, USA); and ACK buffer from Sigma-Aldrich (St. Louis, MO, USA). 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 Inc. (Santa Clara, CA, USA).

Table. 1 presents the antibodies used in this study. Alexa 488 Anti-Guinea pig IgG, Alexa 594 Anti-Guinea pig IgG, Hanks' balanced salt solution (HBSS), CFSE fluorescent dye, acetoxymethyl ester of calcein, and RPMI 1640 were purchased from Life Technologies (Carlsbad, CA, USA).

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 and the hemoglobin assay kit were purchased from Wako Pure Chemical Industries (Osaka, Japan). ELISA kits for the rat TGF- β 1 assay (TGF- β 1 E_{max} Immuno Assay System) were purchased from Promega Co. (Madison, WI, USA). TGF- β 1 was obtained from Pepro Tech (London, UK); Tissue-Tek O.C.T. compound from Sakura Finetek USA, Inc. (Torrance, CA, USA); and FITC-tomato lectin from Vector Laboratories, Inc. (Burlingame, CA, USA).

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, USA). The SH800 Cell Sorter was obtained from Sony Co. (Tokyo, Japan). Histofine® Simple Stain™ MAX PO (MULTI) and Histofine® Simple Stain DAB solution were obtained from NICHIREI BIOSCIENCES INC. (Tokyo, Japan).

Table 1. The antibodies used in this chapter.

Antigen	Clone	Host	Application	Dilution	Source
CD4	W3/25	Mouse	Immunohistology	1/50	Abcam, Cambridge.
CD4	OX35	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, NJ, USA
CD8a	OX8	Mouse	Immunohistology	1/50	Novus Biologicals LLC., Littleton, CO., USA.
CD8a	OX8	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD3e	G4.18	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD25	OX39	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
Granulocyte	RP-1	Mouse	Flowcytometry	1/200	Affymetrix Inc., Santa Clara, CA, USA
Macrophage	HIS36	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD11b/c	OX42	Mouse	Flowcytometry	1/200	Becton, Dickinson and Company, Franklin Lakes NJ, USA
CD32	D34-485	Mouse	Flowcytometry	1/100	Becton, Dickinson and Company, Franklin Lakes NJ, USA
Foxp3	polyclonal	Rabbit	Immunohistology	1/50	Novus Biologicals LLC., Littleton, CO., USA.
Foxp3	FJK-16s	Rat	Flowcytometry	1/100	Affymetrix Inc., Santa Clara, CA, USA
Insulin	polyclonal	Guinea pig	Immunohistology	1/200	Abcam, Cambridge.
Insulin	C27C9	Rabbit	Immunohistology	1/100	Cell Signaling Technology, Beverly, MA, USA
TGF- β 1, 2, 3	1D11	Mouse	Neutralizing		R&D Systems, Minneapolis, MN, USA

Islet Transplantation

Animals

8- to 10-week-old male ACI/NSIc rats (RT-1^a) as islet transplant recipients were used, and 7- to 8-week-old male Fisher 344 rats (F344 /NSIc, RT-1^{lv1}) were used 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

Rod-shaped agarose scaffolds (diameter, 4 mm; length, 25 mm) were prepared as previously described [8]. 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°C overnight, and then freeze-dried for 24 hours under reduced pressure. The freeze-dried agarose rods were evenly loaded 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 loaded with 150 μL saline. For control experiments, freeze-dried agarose gel rods were loaded 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, they were 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 [5]. 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 [12]. 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 suturing. 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 [13]. 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 [14].

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 the 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 [15]. 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 [16]. 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, CD25⁺CD4⁺ T cells, a Treg cell-rich fraction, were harvested for use as suppressor T cells. CD8⁺ T cells from ACI/NSIc rats were labeled with the fluorescent dye CFSE (5 μ M), and used as responder T cells. A total of 2.5×10^4 responder T cells were cultured 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 (5×10^4) treated with mitomycin C (0.1 mg/mL) and soluble anti-CD3 (1 μ g/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 (5×10^4) treated with mitomycin C (0.1 mg/mL) and soluble anti-CD3 (1 μ g/mL), we cultured a total of 2.5×10^4 naïve CD4⁺ T cells. These cells were cultured in 100 μ L complete RPMI (RPMI 1640, 10% FBS, 50 U/mL penicillin, and 50 μ g/mL streptomycin) plus TGF- β 1 (5 μ g/mL), and soluble anti-CD3 (1 μ g/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- β 1 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 $1000 \times g$, TGF- β 1 concentration was determined by ELISA.

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. Then, cDNA was synthesized 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. Specific gene groups were identified 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. The author 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. A heat map was generated 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, the conditioned medium after 1 hour of HUVEC culture was assayed. The tube formation assay was performed using 3D collagen matrix as described previously [17]. 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.

Results

Preparation of Highly Vascularized Granulomatous Tissue in Subcutaneous Tissue Using Agarose-SEK Rods

To induce vascularization in subcutaneous tissue, SEK was delivered using a freeze-dried agarose rod. The agarose-SEK rods were implanted in each of 2 dorsal subcutaneous sites in streptozotocin (STZ)-induced diabetic ACI rats (STZ ACI rats). Normally, only a low level of vascularization is present in the subcutaneous tissue of an STZ ACI rat (Figure 2B). Implantation of agarose rods without SEK did not seem to increase vascularization (Figure 2C, E, F). In contrast, at 10 days after the implantation of agarose-SEK rods into STZ ACI rats, we observed highly vascularized granulomatous tissue adjacent to the agarose rods (Figure 2D, G, H). The agarose-SEK rods were easily removed, without tissue adhesion, creating subcutaneous spaces surrounded by highly vascularized granulomatous tissue (SEK spaces; Figure 2A). To visualize the functional vasculature in the granulomatous tissue, we performed intravenous perfusion of an FITC-lectin solution, which revealed numerous lectin-positive circles, indicating blood vessels in the granulomatous tissue (Figure 2I). Evaluated blood vessel induction was also semi-quantitated by determining the hemoglobin content of the tissue [5]. These data showed that tissue hemoglobin content depended on the amount of SEK loaded into the agarose rod (Figure 2J). Following implantation of a rod containing 100 μ g SEK, tissue hemoglobin content significantly increased from 1.63 ± 0.434 mg/g in untreated tissue to 8.07 ± 2.08 mg/g in treated tissue. Thus, for the subsequent in vivo experiments, agarose-SEK rods prepared with 100 μ g SEK/rod were used.

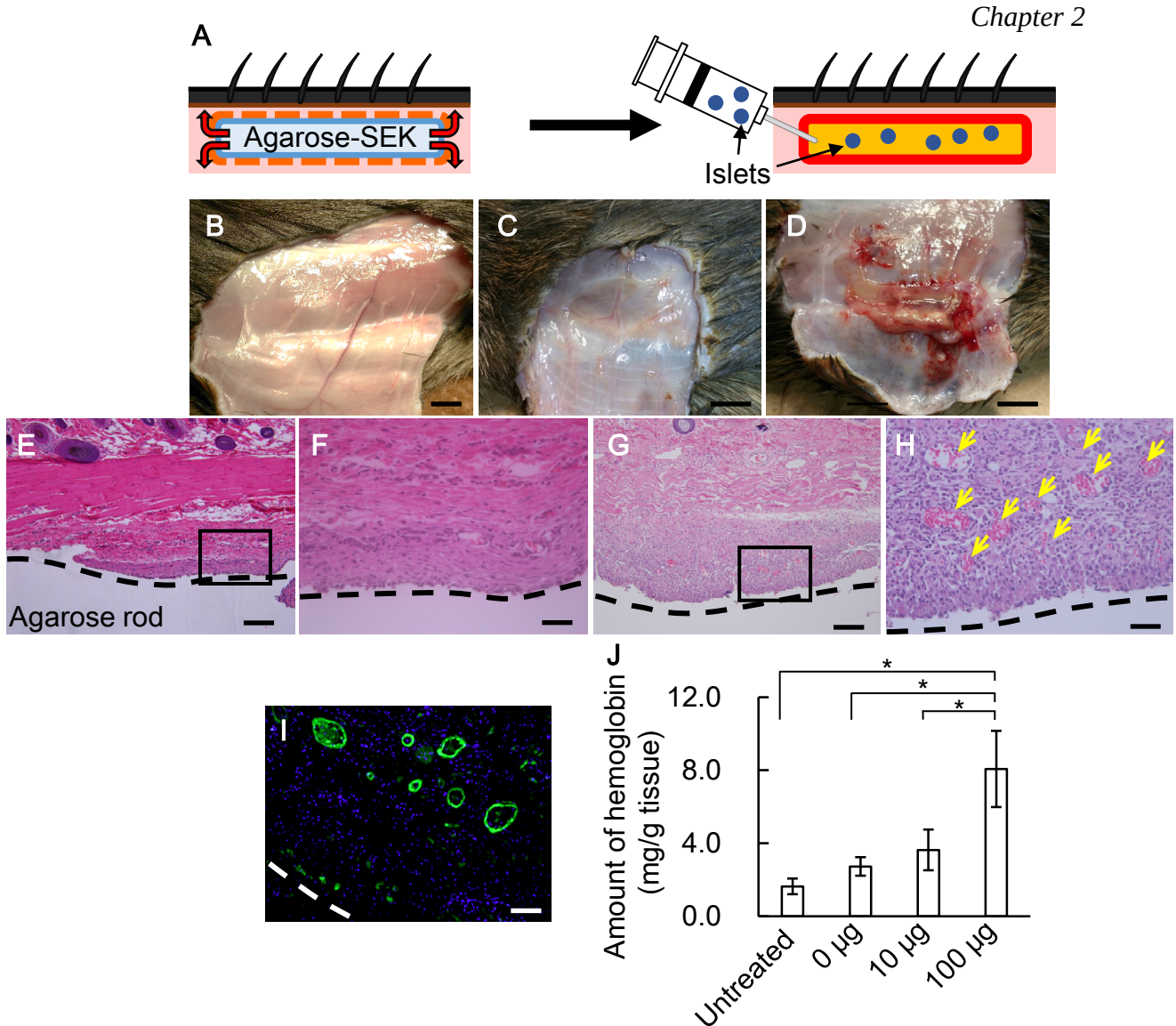


Figure 2. Preparation of a subcutaneous islet transplantation site enriched in blood vessels. (A) Schematic drawing of the methods of inducing subcutaneous blood vessel formation, and of islet transplantation. Macroscopic images of the subcutaneous tissue of streptozotocin (STZ)-induced diabetic ACI rats with no treatment (B); at 10 days after implantation of agarose rods without SEK (C); and at 10 days after implantation of agarose-SEK rods (D). Scale bars: 10 mm. (E, F) H&E staining of tissue after implantation of an agarose rod without SEK, and (G, H) H&E staining of tissue pre-vascularized by implantation of an agarose rod containing 100 µg SEK for 10 days. Yellow arrows indicate vessels. (I) Endothelial layer of granulomatous tissue visualized by FITC-lectin infusion. (J) Hemoglobin content of tissue from untreated STZ ACI rats ($n = 3$), and from STZ ACI rats with implantation of agarose rods without SEK ($n = 3$), with 10 µg SEK ($n = 3$), and with 100 µg SEK ($n = 5$) for 10 days. Data are shown as the mean $\pm$ SD. $*P < 0.01$, Scale bars: (E, G) 200 µm, (F, H, I) 50 µm. Experimental results of untreated STZ ACI rats are composed the part of experimental results presented from chapter 1.

Allogeneic Islet Transplantation

Male ACI/NSIc rats (RT-1^a; ACT rats) were used as recipients of islet transplantation, and male F344/NSIc rats (RT-1^{lv1}; F433 rats) were used as islet donors. In this donor–recipient combination, islets that are transplanted into the liver via the portal vein are rejected by the host (ACI rat) immune system [8]. Diabetes was induced in the ACI rats (n = 10) by STZ injection. To prepare SEK spaces, agarose-SEK rods were implanted on each dorsal side of STZ ACI rats (1 rod per site) for 10 days. 1500 F344 islets were transplanted into each SEK space, totaling 3000 islets in each recipient rat. The recipient STZ ACI rats received no immunosuppressive medications before or after transplantation.

Figure 3A shows the blood glucose levels of the 10 recipient rats. Among these 10 rats, 2 did not exhibit normoglycemia beyond 30 days after transplantation, while 7 showed normoglycemia for over 100 days after transplantation. One recipient was sacrificed on day 60 for histological analysis. In contrast, when islets were transplanted into subcutaneous spaces formed by the implantation of agarose rods without SEK, none of the 5 recipients exhibited long-term normoglycemia (Figure 3B). In these rats, the grafts may have deteriorated due to low oxygen tension.[18]–[20] Figure 3C shows graft survival plotted against days after transplantation.

The recipients' mean plasma insulin levels were 1.62 ± 0.61 ng/mL at 40 days posttransplantation, and 1.48 ± 0.14 ng/mL at 90 days posttransplantation (Figure 4A). Mean plasma insulin levels were 1.27 ± 0.03 ng/mL in normal ACI rats and 0.35 ± 0.07 ng/mL in STZ ACI rats. While plasma insulin levels were slightly higher in STZ ACI recipients compared to in normal rats, this difference was not significant. IPGTT was performed in normal rats and STZ ACI rats, and in recipient rats at between 50–55 days

and between 100–105 days posttransplantation (Figure 4B). The area under the curve (AUC) from IPGTT data did not significantly differ between normal rats and recipient rats with islets in SEK space (Figure 4C).

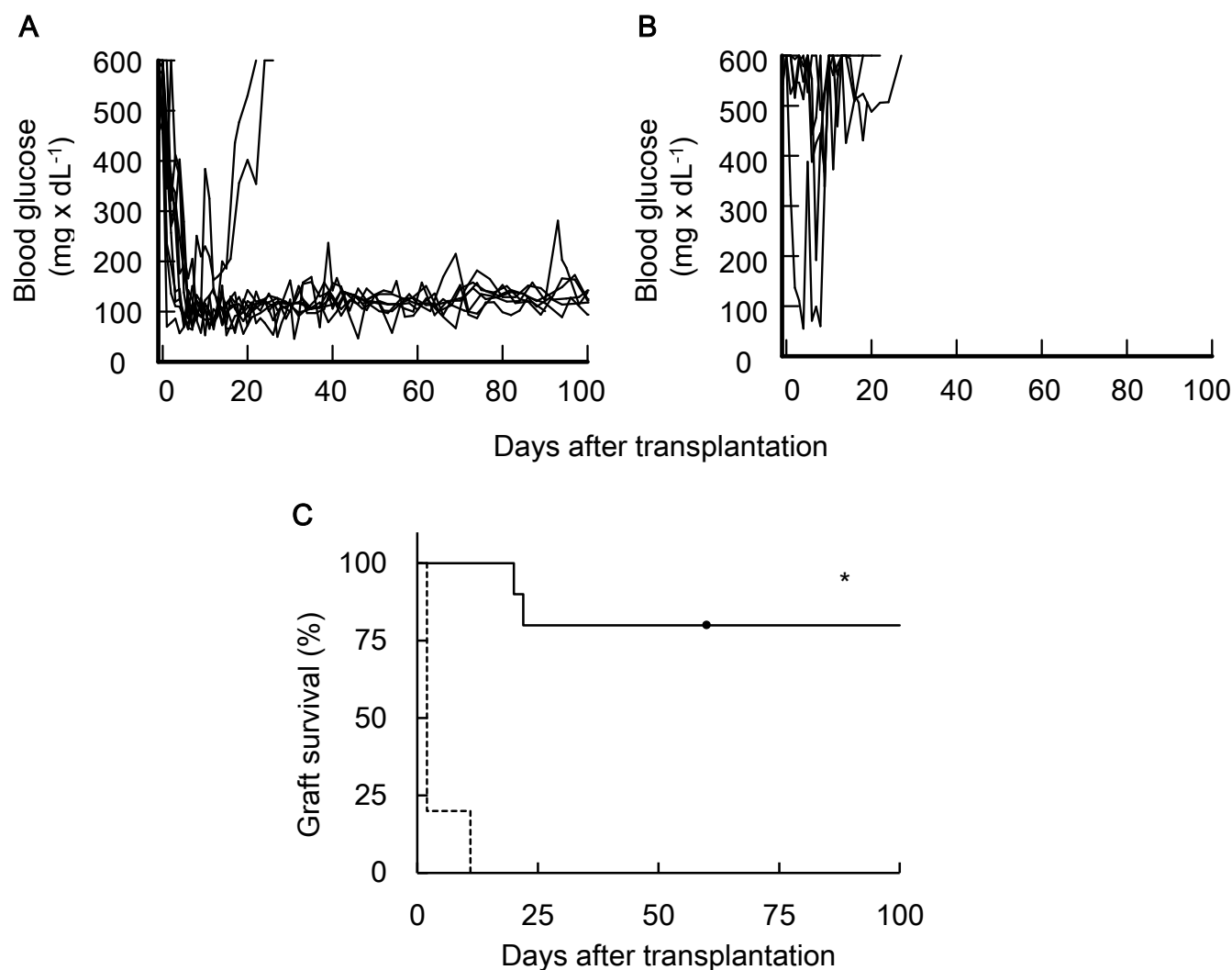


Figure 3. The effects of allogeneic islet transplantation on chemically-induced diabetic (STZ ACI) rats. (A) Blood glucose levels of STZ ACI rats after transplantation of 3000 F344 islets into SEK spaces without immunosuppressive drugs. (B) Blood glucose levels of STZ ACI rats after transplantation of 3000 F344 islets into subcutaneous site prepared by implantation of agarose rods without SEK. (C) Kaplan-Meier plots of graft survival. Solid line: Islets transplanted into SEK spaces. Closed circle indicates the sacrifice of a rat on day 60 for histological analyses. Dotted line: Islets transplanted into subcutaneous sites prepared by implantation of agarose rods without SEK. * $P < 0.01$.

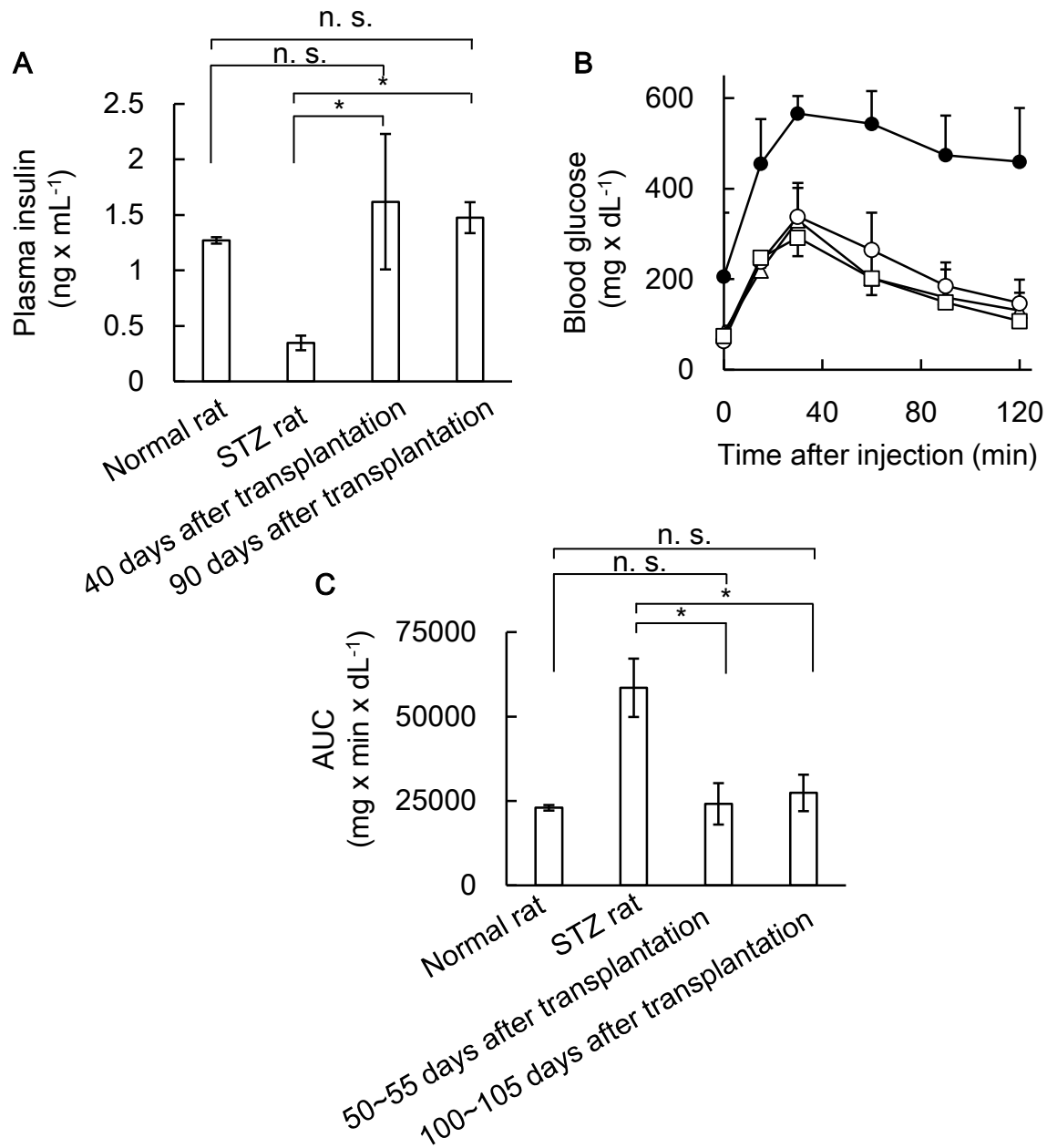


Figure 4. Functions of islet grafts into the SEK-spaces. (A) Plasma insulin levels in normal ACI rats ($n = 4$), STZ ACI rats ($n = 4$), and STZ ACI rats with 3000 F344 islets transplanted into tissue pretreated with agarose-SEK rods at 40 days ($n = 4$) and 90 days ($n = 4$) post-transplantation. Data are shown as the mean $\pm$ SD. $*P < 0.01$. (B) Results of intraperitoneal glucose tolerance test (IPGTT) in normal ACI rats (open squares, $n = 4$), STZ ACI rats (closed circles, $n = 4$), and STZ ACI rats with 3000 F344 islets transplanted into tissue pretreated with agarose-SEK rods at 50–55 days (open triangles, $n = 4$) or 100–105 days (open circles, $n = 4$) after islet transplantation. (C) Area under the curve for the IPGTT. Data are shown as the mean $\pm$ SD. $*P < 0.01$. Experimental results of normal rats and STZ rats are composed the part of experimental results presented from chapter 1.

All recipients received 2 grafts, 1 each on the left and right dorsal sites. At 106 days posttransplantation, the left islet graft of each recipient was retrieved for histological examination. Blood glucose levels were maintained by the remaining right islet graft (Figure 14A). Microscopic images of typical sections are shown in Figure 5A, and B. The islets were clearly visible in the H&E stained slices (Figure 5A), and many insulin-positive cells were observed in the slices immunofluorescently stained with anti-insulin

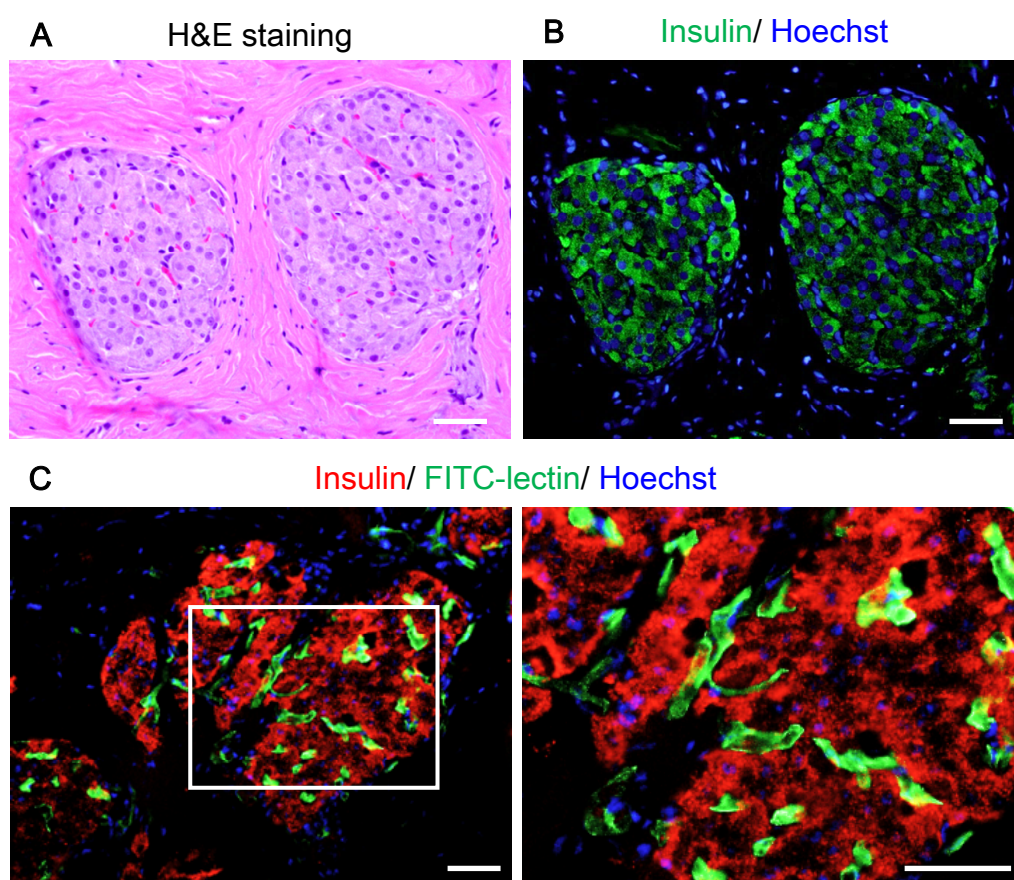


Figure 5. Histological analysis of islet grafts at subcutaneous site. (A, B) Histological analysis of islet grafts that functioned for over 106 days. Tissue was retrieved 106 days after transplantation. (A) H&E staining, and (B) immunofluorescence staining for insulin (green), and nuclear staining with Hoechst (blue). Scale bar: 50 μ m. (C) Intravenous infusion of FITC-lectin to examine the functional vasculature of islet grafts at 43 days post-transplantation. Immunofluorescent staining of insulin (red), staining of endothelial cells with FITC-lectin (green), and nuclear staining with Hoechst (blue). Scale bar: 50 μ m.

antibodies (Figure 5B, C). Few lymphocytes infiltrating into and around the islet grafts were observed. To assess the connection of vessels in the islet grafts to the recipient's own vessels, intravenous infusion of a FITC-lectin solution was performed. Functioning vessels within the islet grafts were clearly visible as indicated by FITC-lectin stained cells (Figure 5C).

Immunological Properties of Granulomatous Tissue Formed by Implantation of Agarose-SEK Rods

Most allogeneic islet grafts survived when transplanted into the SEK spaces surrounded by granulomatous tissue induced by implantation of agarose-SEK rods. The immune cells that infiltrated the granulomatous tissue were isolated and analyzed by FACS. The percentage of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) was increased in the presence of agarose-SEK rods, reaching 18% at 2 days after implantation. This percentage continuously increased, reaching >24% at 14 days after agarose-SEK rod implantation (Figure 6A, B, lower panel). In contrast, this percentage was <15% following implantation of an agarose rod without SEK (Figure 6A, B, upper panel). On the other hand, the percentages of Treg cells in the draining and axial lymph nodes and in the spleen, did not differ between the rats that received agarose-SEK rods vs. agarose rods without SEK (Figure 6C, and Figure 7A, C). These findings indicate that the agarose-SEK rod evoked regional but not systemic immunological reactions.

The ratio of CD8⁺ T cells to CD4⁺ T cells was also determined. This ratio increased from ~35% to 50–60% following implantation of agarose rods without SEK (Figure 6D, E upper panel). In contrast, this ratio was <30% in the granulomatous tissue of STZ ACI rats with agarose-SEK rod implantation (Figure 6D, E, lower panel). With regards to the percentage of Treg cells among CD4⁺ T cells, the ratio of CD8⁺ T cells to

CD4⁺ T cells did not change (~28–30%) in the draining lymph nodes, the axial lymph nodes, or the spleens of STZ ACI rats following implantation of agarose-SEK rods (Figure 6F, and Figure 7B, D).

Next, the immunosuppressive activity of CD4⁺ CD25⁺ T cells (which are mostly Treg cells) [21] isolated from the granulomatous tissue formed following implantation of agarose-SEK rods was examined. The presence of tissue-infiltrating CD4⁺ CD25⁺ T cells suppressed the proliferation of CD8⁺ cells. The percentage of proliferated cells was 13.4% in the presence of CD4⁺ CD25⁺ T cells versus 42.4% in their absence (Figure 6G, H, I).

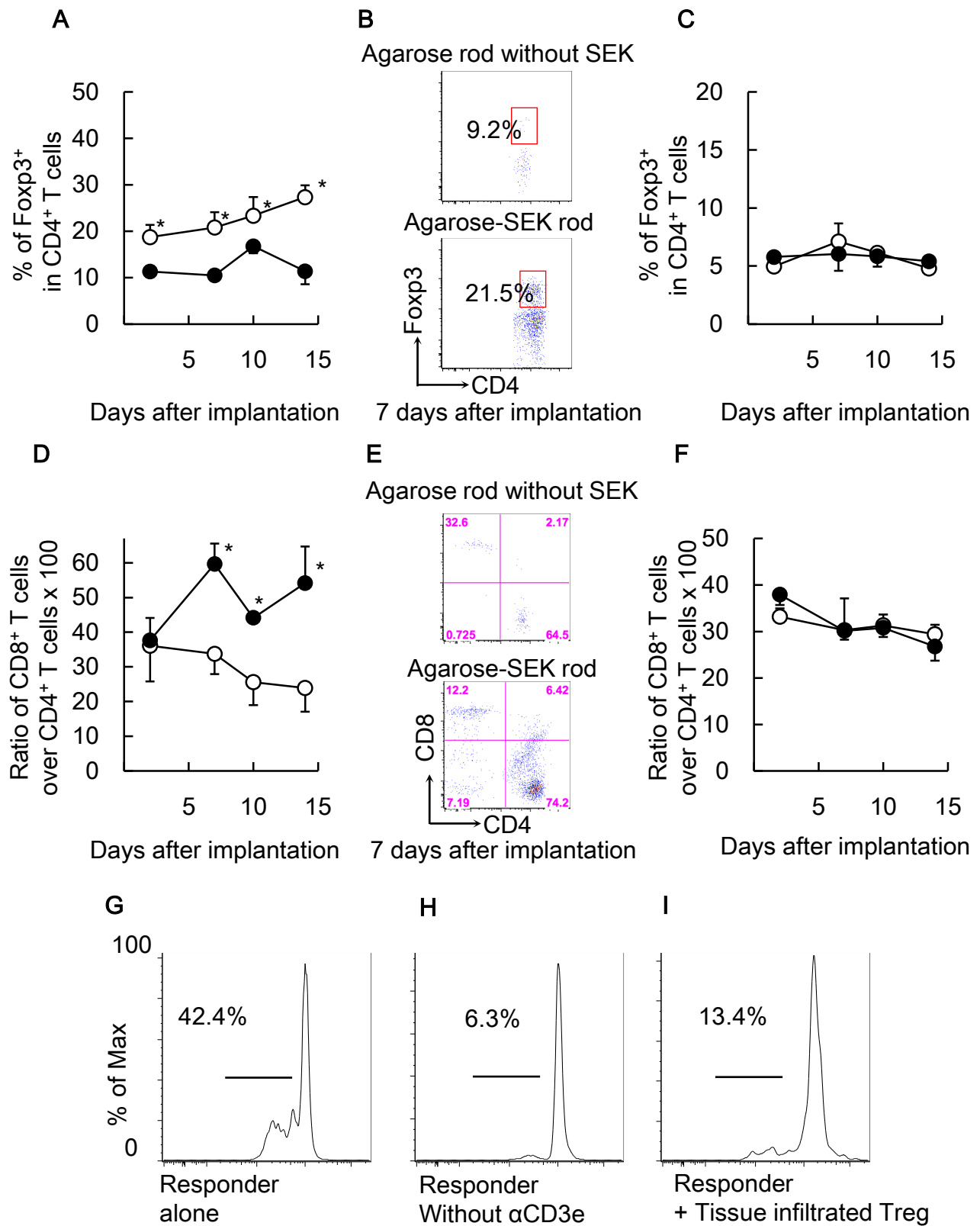


Figure 6. Profiles of immune cells in the granulomatous tissue formed at subcutaneous sites after treatment with agarose-SEK rods. (A) The percentage of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) in granulomatous tissue is plotted against the days after agarose rod implantation. Presented values are from tissue formed by implanted agarose-SEK rods (open circles; n = 3) and by implanted agarose rods without SEK (closed circles; n = 3). The statistical significance of between-group differences was determined by analyses of variance (ANOVA). Data are shown as the mean $\pm$ SEM. **P* < 0.05. (B) Representative flow cytometry images, from analyses gated for CD3e⁺ CD4⁺ T cells. (C) The percentage of Treg cells among CD4⁺ T cells in draining lymph nodes is plotted against the number of days after agarose rod implantation. Presented values are for draining lymph nodes in rats treated with agarose-SEK rods (open circles) and with agarose rods without SEK (closed circles). In both groups: day 2, n = 3; days 7, 10, and 14, n = 4. Data are shown as the mean $\pm$ SEM. Between-group differences were not statistically significant. (D) The ratio of CD8⁺ T cells to CD4⁺ T cells in granulomatous tissue is plotted against the number of days after agarose rod implantation. Presented values are for tissue from rats treated with agarose-SEK rods (open circles; n = 3), and with agarose rods without SEK (closed circles; n = 3). The statistical significance of between-group differences was determined by ANOVA. Data are shown as the mean $\pm$ SEM. **P* < 0.05. (E) Representative flow cytometry images from analyses gated for CD3e⁺ (n = 3). (F) The ratio of CD8⁺ T cells to CD4⁺ T cells in draining lymph nodes is plotted against the number of days after agarose rod implantation. Values are for draining lymph nodes in rats treated with agarose-SEK rods (closed circles), and with agarose rods without SEK (open circles). For both groups, day 2, n = 3; days 7, 10, and 14, n = 4. Data are shown as the mean $\pm$ SEM. Between-group differences were not statistically significant. (G, H, I) The effects of CD4⁺ CD25⁺ T cells (Treg-rich fraction) on CD8⁺ T-cell proliferation. CD4⁺ CD25⁺ T cells were isolated from granulomatous tissue formed around implanted agarose-SEK rods. Histograms of CFSE-stained cells are shown. Responder (CD8⁺) T cells were stained with CFSE (5 μ M) and cultured in the presence of mitomycin C-treated antigen-presenting cells in medium supplemented with anti-CD3e antibody (G), without anti-CD3e antibody (H), or with anti-CD3e antibody plus Treg cells (I). The numbers on the black lines indicate the percentages of proliferated responder T cells that showed decreased CFSE intensity. Each plot is representative of three independent experiments. In all panels, data for STZ ACI rats treated with agarose rods without SEK (filled circles) are reproduced from chapter 1.

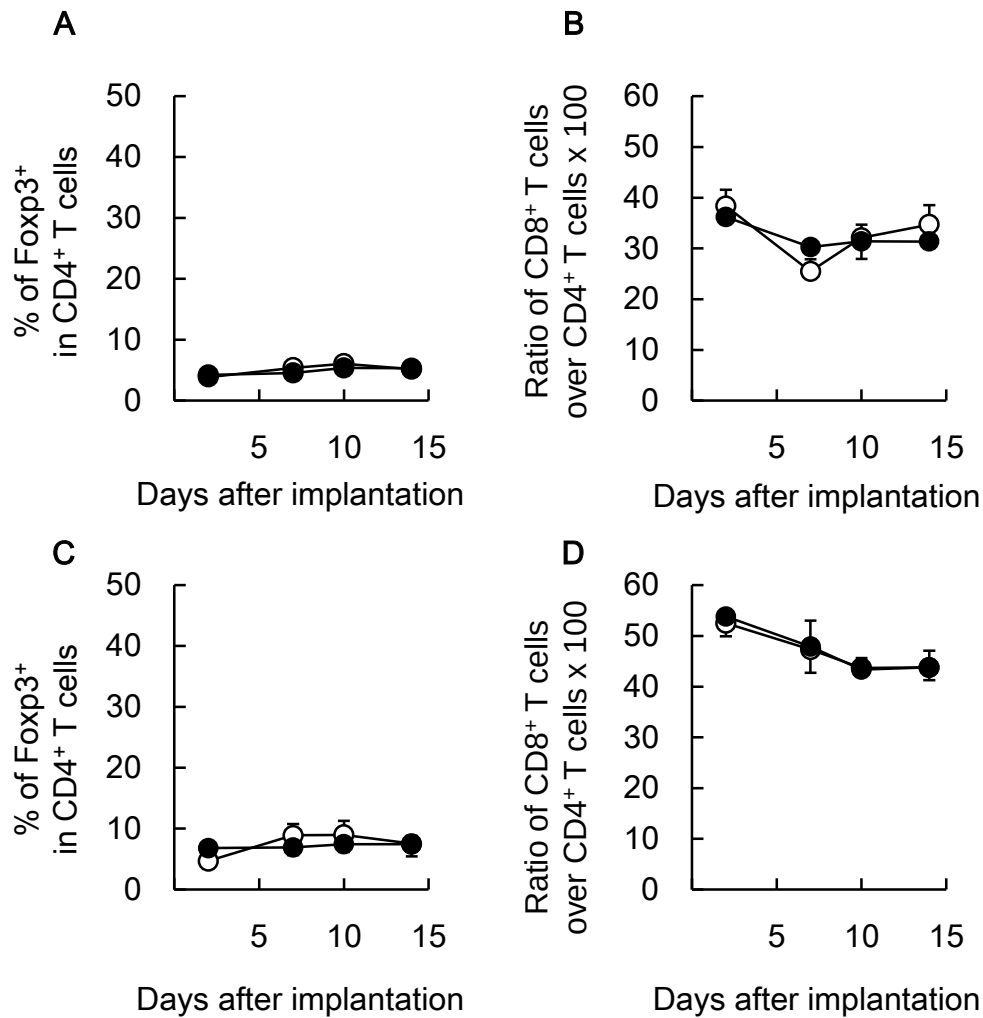


Figure 7. Percentages of Foxp3⁺ cells among CD4⁺ T cells (Treg cells), and the ratios of CD8⁺ T cells to CD4⁺ T cells in the axillary lymph nodes and in the spleen are plotted against the days after agarose rod implantation. (A–D) Percentages of Foxp3⁺ cells among CD4⁺ T cells in axillary lymph nodes (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 $\pm$ SEM. In all panels, data for STZ ACI rats treated with agarose rods without SEK (filled circles) are reproduced from chapter 1.

Gene Expression in Granulomatous Tissue Formed by Agarose-SEK Rod Implantation

Microarray analysis of mRNA isolated from granulomatous tissue formed by agarose-SEK rod implantation was performed to glean insights into the mechanisms supporting generation of the immune-tolerant environment. In Figure 8A, a volcano plot presents the results of whole gene expression analysis. The agarose-SEK rod group showed significantly increased expression levels of the *tgfb1* signaling-associated gene *grem1*, and of the granulocyte chemotaxis-associated genes *s100a8* and *s100a9*.

To elucidate the functional gene groups altered by SEK, an unbiased analysis was performed using GO terms. The gene scores of gene groups defined by GO terms were examined using empirical cumulative distribution function (ecdf) analysis. The results indicated that the scores of a gene group defined by the GO terms chemokines and cytokines (except for Treg cell-associated genes) significantly decreased in the agarose-SEK rod group compared to in the agarose rod without SEK group (Figure 9). In contrast, the expression levels/scores of 2 genes encoding the Treg cell-associated chemokines and cytokines *tgfr1* and *cxcl12* significantly increased in the agarose-SEK rod group compared to the SEK-free agarose rod group (Figure 8B). Time-course analysis revealed increased *tgfr1* expression levels at 7 and 10 days after implantation of agarose-SEK rods (Figure 8C). Overall, these data demonstrate that agarose-SEK rod implantation increased the expressions of some chemokines and cytokines, along with the expressions of receptors associated with chemotaxis and the Treg cell induction.

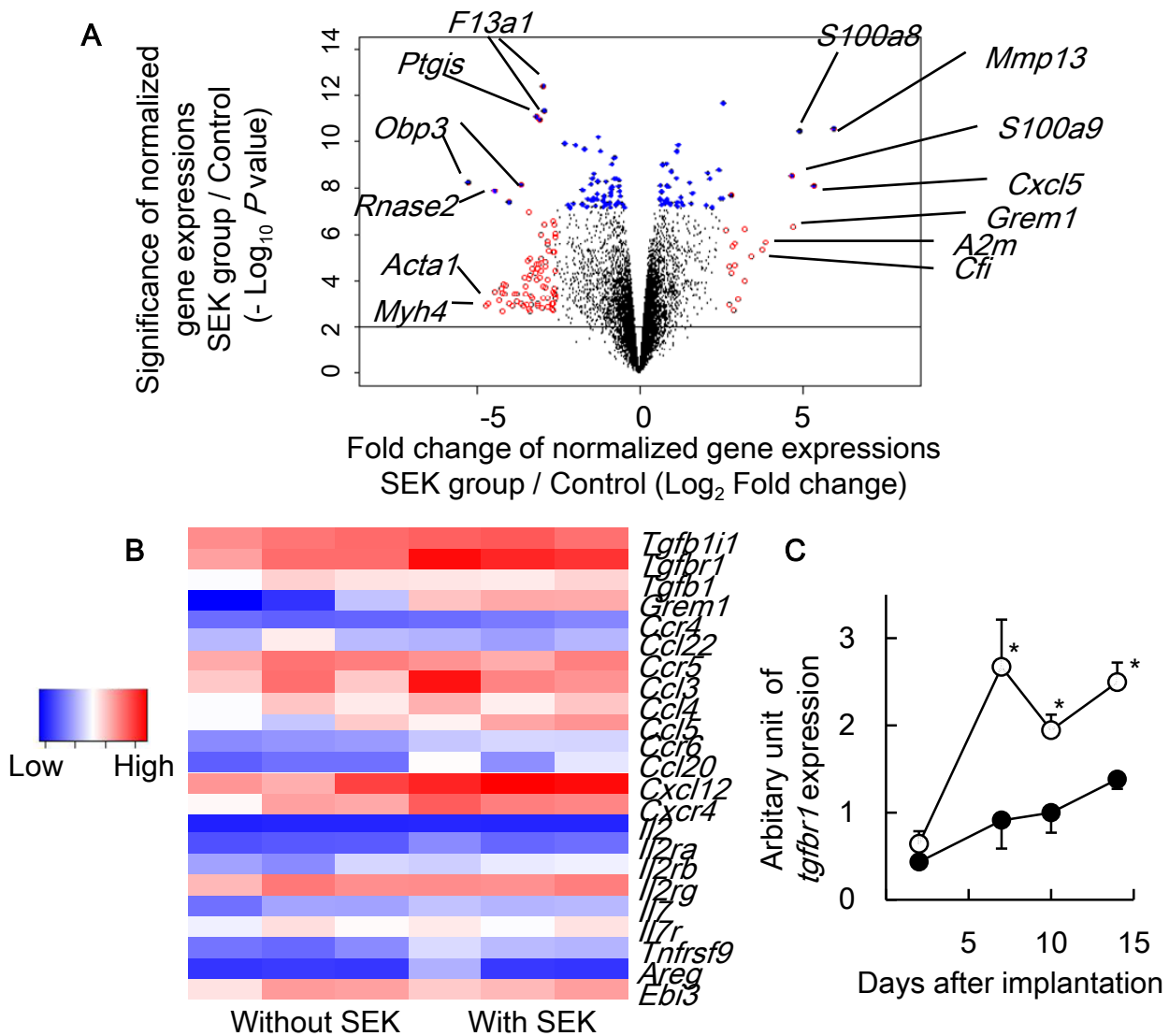


Figure 8. Whole gene expression analysis of formed granulomatous tissue. (A) Volcano plot in which each dot represents a single type of mRNA present at 7 days after implantation of agarose rods with or without SEK ($n = 3$ for each). The x-axis shows the $\log_2$ of the fold-change of gene expression in subcutaneous tissue implanted with agarose-SEK rods relative to their expression in tissue implanted with rods without SEK. The y-axis indicates the $-\log_{10}$ of the P value. Representative gene names are shown. Red circles indicate the 100 genes showing the greatest fold-change. Blue closed circles indicate the 100 genes with the most significant P values. (B) Heat-map representing the expressions of Treg cell-associated genes at 7 days after implantation of rods with or without SEK ($n = 3$ for each). (C) *Tgfb1* gene expression (in arbitrary units) is plotted against the number of days after agarose rod implantation. Open circles indicate the agarose-SEK group, and closed circles indicate the group without SEK. Statistical significance of between-group differences was determined by analyses of variance (ANOVA). Data are shown as the mean $\pm$ SEM. * $P < 0.01$. Data for STZ ACI rats treated with agarose rods without SEK (controls) are reproduced from chapter 1.

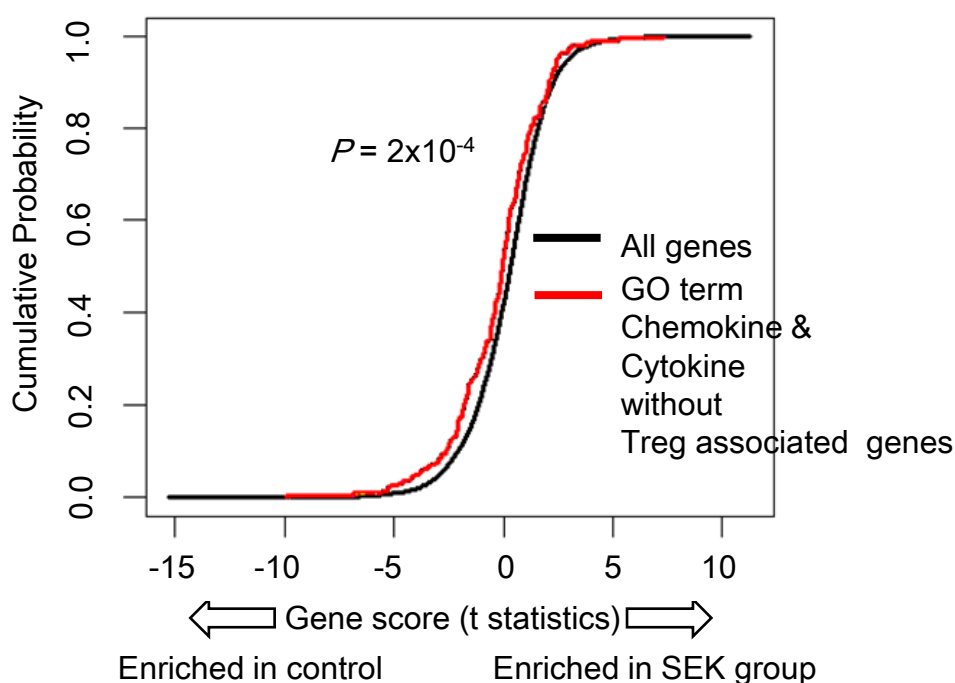


Figure 9. Whole gene expression analysis of granulomatous tissue formed at 7 days after implantation of agarose rods with or without SEK (n = 3 for each). (A) Gene scores (t statistics) presented in a cumulative frequency graph. The black line indicates the cumulative frequency of the expressions of 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). Data for STZ ACI rats treated with agarose rods without SEK (controls) are reproduced from chapter 1.

Immunological Environment around the Subcutaneous Islet Graft

The immunological environment around an islet graft plays a pivotal role in the graft's long term survival and its success in glycemic control. Using FACS, the immune cells were analyzed, isolated from subcutaneous tissue containing islet grafts at 5 and 30 days after islet transplantation. During the 30 days posttransplantation, the percentage of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) was maintained at >20% around the islet grafts (Figure 10A). In contrast, this percentage was <10% in draining lymph nodes, axillary lymph nodes, and the spleen (Figure 10B, and Figure 11A, C). The ratio of CD8⁺ T cells to CD4⁺ T cells were also examined. Around the islet graft and draining lymph nodes, this ratio remained at the pretransplantation value, which was as low as ~40% (Figure 10C, D). The ratio was about 60% in subcutaneous tissue formed by agarose rods without SEK, and it was 40–50% in the spleen (Figure 11D). Additionally, the percentage of granulocytes among total immune cells was maintained at >25% during the 30 days posttransplantation (Figure 10E). Overall, the immunological environment of subcutaneous tissue containing an allogeneic islet graft was the same as the environment before islet transplantation, ie, in the tissue 10 days after agarose-SEK rod implantation.

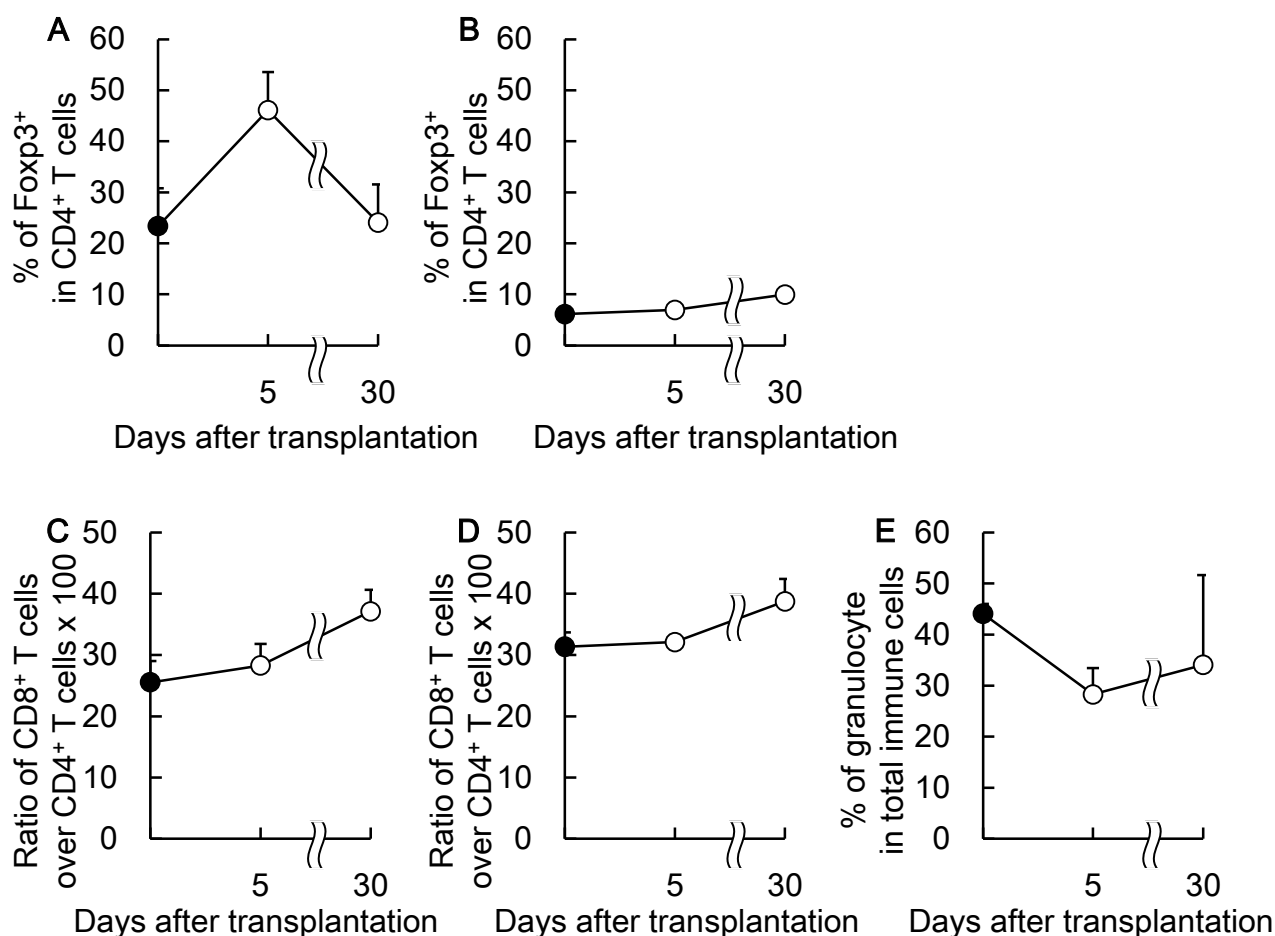


Figure 10. Immunological environment around the transplanted islet grafts. (A, B)

The percentage of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) in granulomatous tissue (A) and in draining lymph nodes (B) plotted against the days after 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 $\pm$ SEM. (C, D) The ratio of CD8⁺ T cells to CD4⁺ T cells plotted against the days after transplantation in granulomatous tissue (C) and in draining lymph nodes (D). 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 $\pm$ SEM. (E) The percentage of granulocytes among total immune cells in granulomatous tissue over time. Values are shown for tissue before islet transplantation (closed circles; $n = 3$), and for tissue containing islet grafts (open circles; $n = 3$). Data are shown as the mean $\pm$ SEM.

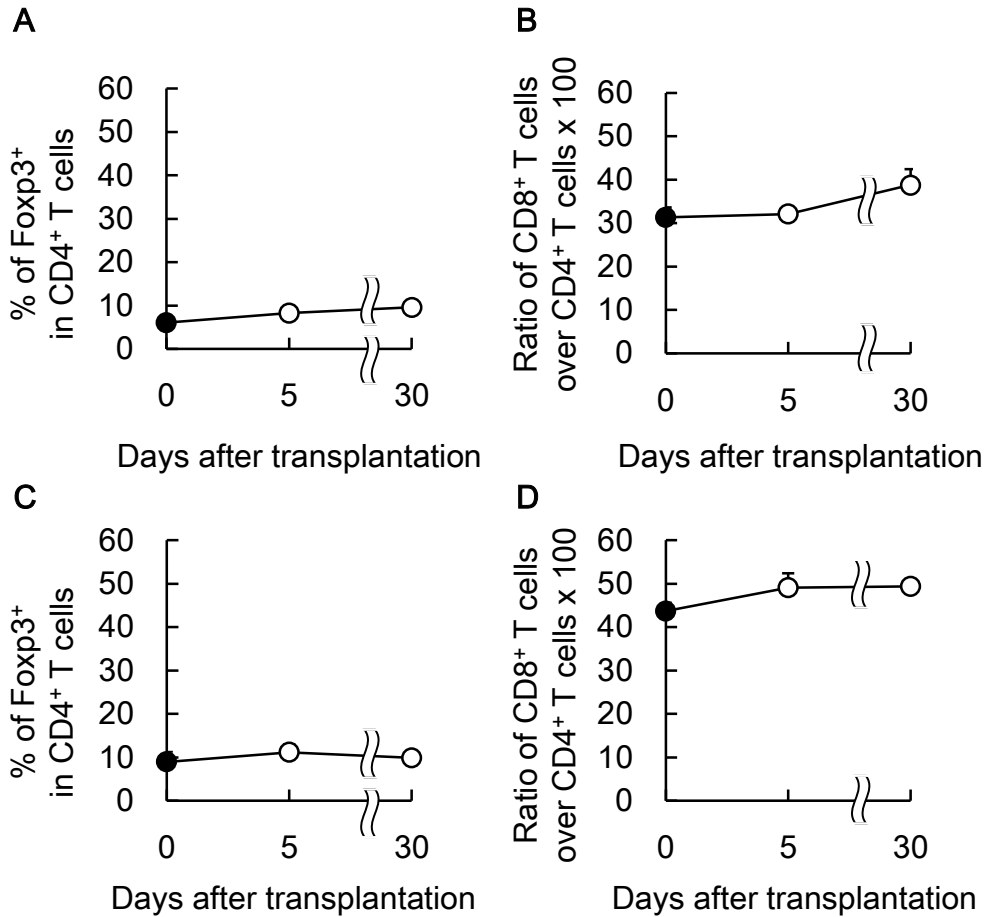


Figure 11. Percentages of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) and ratios of CD8⁺ T cells to CD4⁺ T cells are plotted against the days after islet transplantation. (A, C) The percentages of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) in axillary lymph nodes (A), and spleen (C) are plotted against the number of days after islet transplantation. (B, D) The ratios of CD8⁺ T cells to CD4⁺ T cells in axillary lymph nodes (B), and the spleen (D) 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 $\pm$ SEM.

Histological examination was performed to investigate the location of immune cells in tissues with islet grafts. At 5 days posttransplantation, many lymphocytes were observed surrounding the islets (Figure 12A). In contrast, at 30 days posttransplantation, few lymphocytes were observed and Treg cells had infiltrated into the islets (Figure 12B). CD8⁺ T cells remained at the islet surfaces or in vessels ie, they did not infiltrate into the islets (Figure 12B). The locations of the Treg cells and CD8⁺ T cells suggested that the islet grafts were specifically protected from rejection.

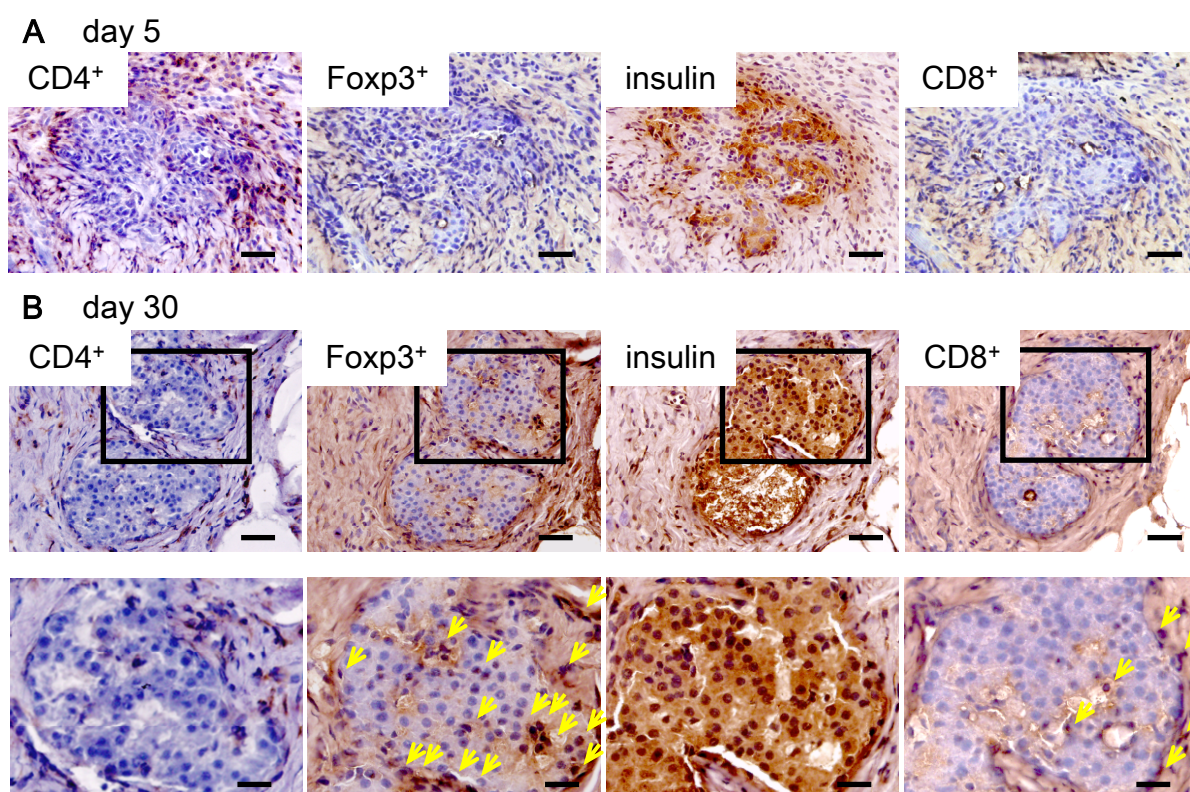


Figure 126. Immunoperoxidase staining for CD4⁺ T cells, Foxp3⁺ cells (Treg cells), insulin-positive cells, and CD8⁺ T cells in thin sections of subcutaneous tissue with allogeneic islet grafts. F344 islets were transplanted into granulomatous tissue of streptozotocin (STZ)-induced diabetic ACI rats after 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: 50 μ m, 20 μ m (magnified insets). Yellow arrows indicate Foxp3⁺ cells or CD8⁺ cells.

Effects of Donor Splenocyte Injection in Graft Recipients

STZ ACI rats that had functional F344 islet grafts for 30 days were next systematically sensitized by intraperitoneal injection of F344 rat splenocytes. Their blood glucose levels tended to increase for a few days after splenocyte injection (Figure 13A). It was also examined how donor-antigen injection impacted the proportion of immune cells around and within the islet grafts. The percentage of Treg cells infiltrating into and around the islet grafts was $24.1 \pm 1.2\%$ before donor-antigen injection, and decreased to $18.8 \pm 1.9\%$ at 5 days after antigen injection (Figure 13B). In contrast, the ratio of CD8⁺ T cells to CD4⁺ T cells was $37.1 \pm 6.5\%$ before donor-antigen injection and increased to $55.8 \pm 18.5\%$ at 5 days after injection (Figure 13C). Histological examination revealed that many CD8⁺ T cells infiltrated into the islet graft, although Treg cells were still found in the islets. Insulin-positive cells were disrupted (Figure 14D). The same phenomenon was observed when these experiments were performed in STZ ACI rats with stable functioning F344 islet grafts at 117 days (Figure 14A, B, and C).

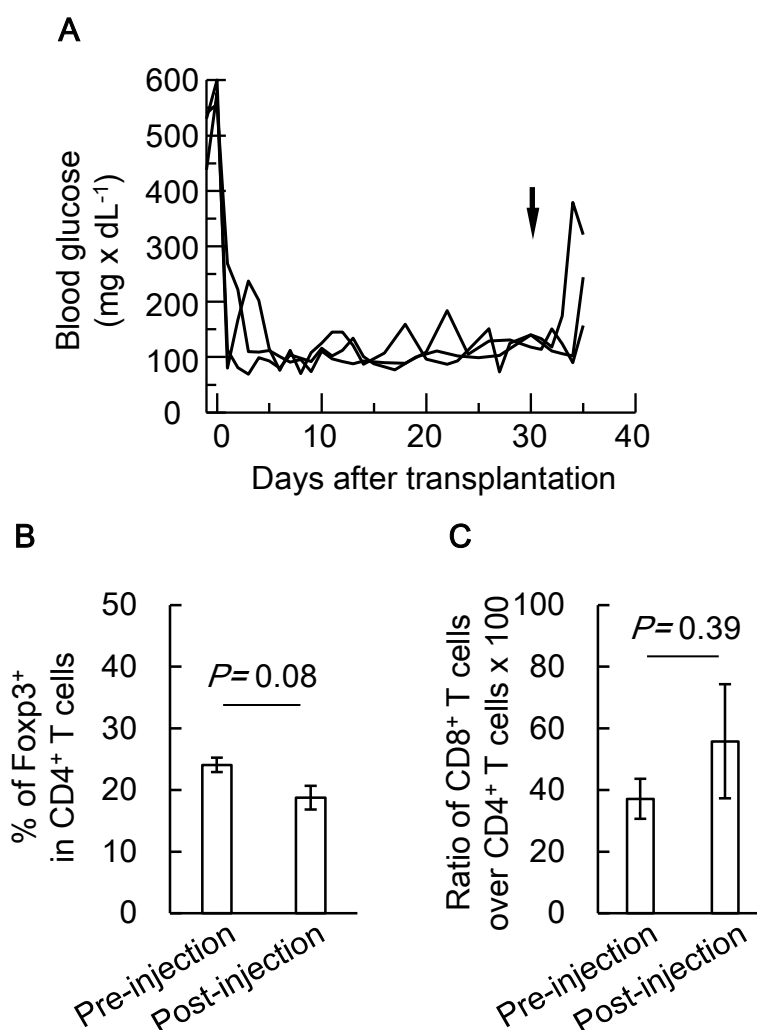


Figure 13. Rejection of established subcutaneous allogeneic islet grafts after injection of splenocytes from F344 rats (islet donors) into the peritoneal cavity of recipient ACI rats. All recipients carried two grafts, one each on the left and right dorsal sides. Before injection of splenocytes, one graft was removed for examination. A total of 10^7 cells from the spleen of a donor F344 rat were infused into the abdominal cavity of the recipient ACI rat at 30 days after graft transplantation ($n = 3$). (A) Effects of injection of 10^7 F344 splenocytes on islet graft function. Red arrow indicates time-point of splenocyte injection (day 30). (B, C) Effects of injection of 10^7 F344 splenocytes on the immunological environment around the islet graft. Tissue containing islet grafts was removed and analyzed at 5 days after splenocyte injection. (B) The percentage of Foxp3⁺ cells among CD4⁺ T cells (Treg cells) in tissue removed from rats with or without injection of donor splenocytes. Data are shown as the mean $\pm$ SEM. (C) The ratio of CD8⁺ T cells to CD4⁺ T cells in the tissue removed from rats with or without injection of donor splenocytes. Data are shown as the mean $\pm$ SEM.

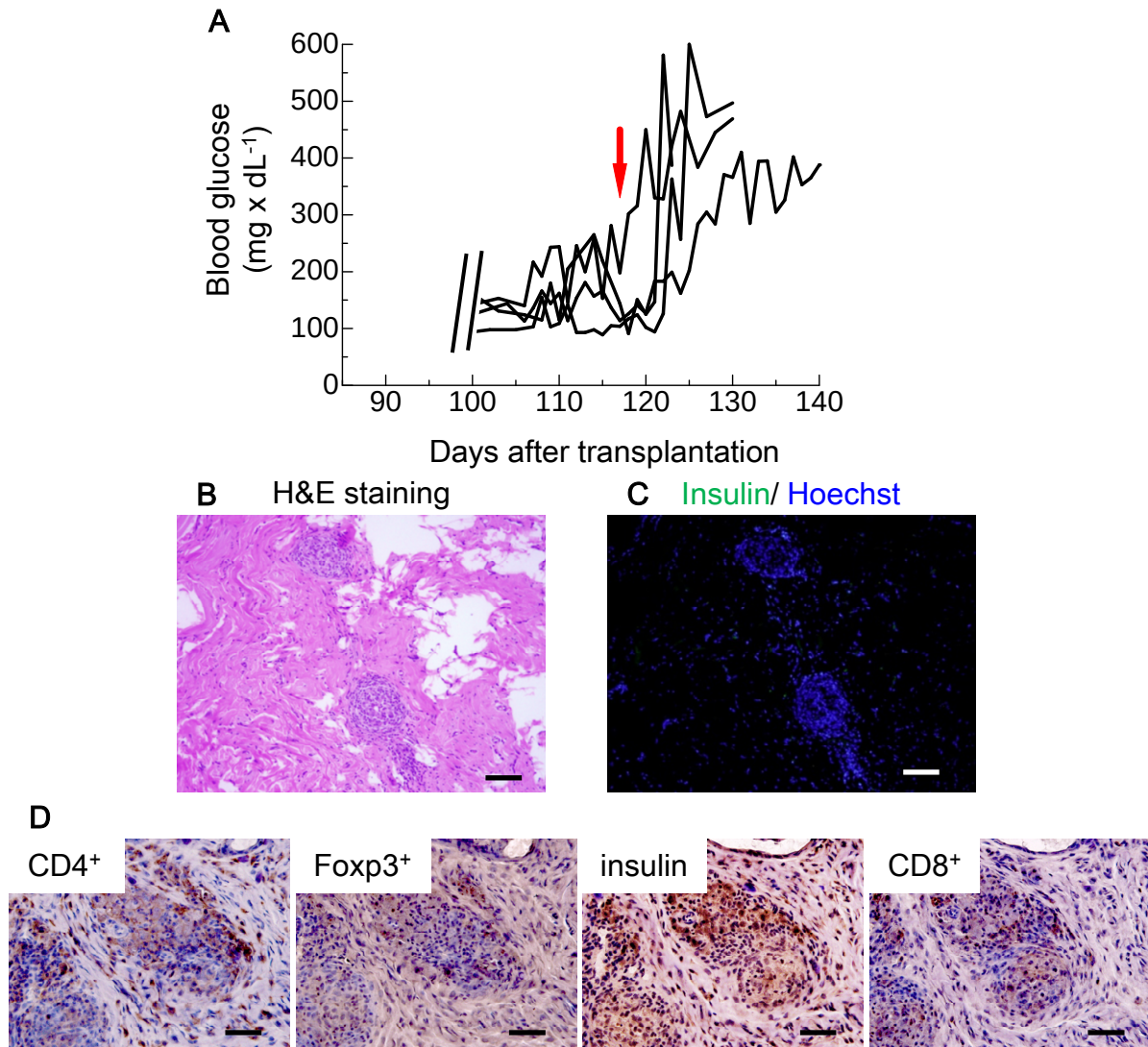


Figure 147. Transplantation of 10^7 splenocytes from F344 rats (islet donors) into the peritoneal cavity of recipient rats at 117 days (red arrow) after islet transplantation resulted in the rejection of established subcutaneous allogeneic islet grafts (A-C). Tissue containing islet grafts was removed 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 μ m. (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: 50 μ m.

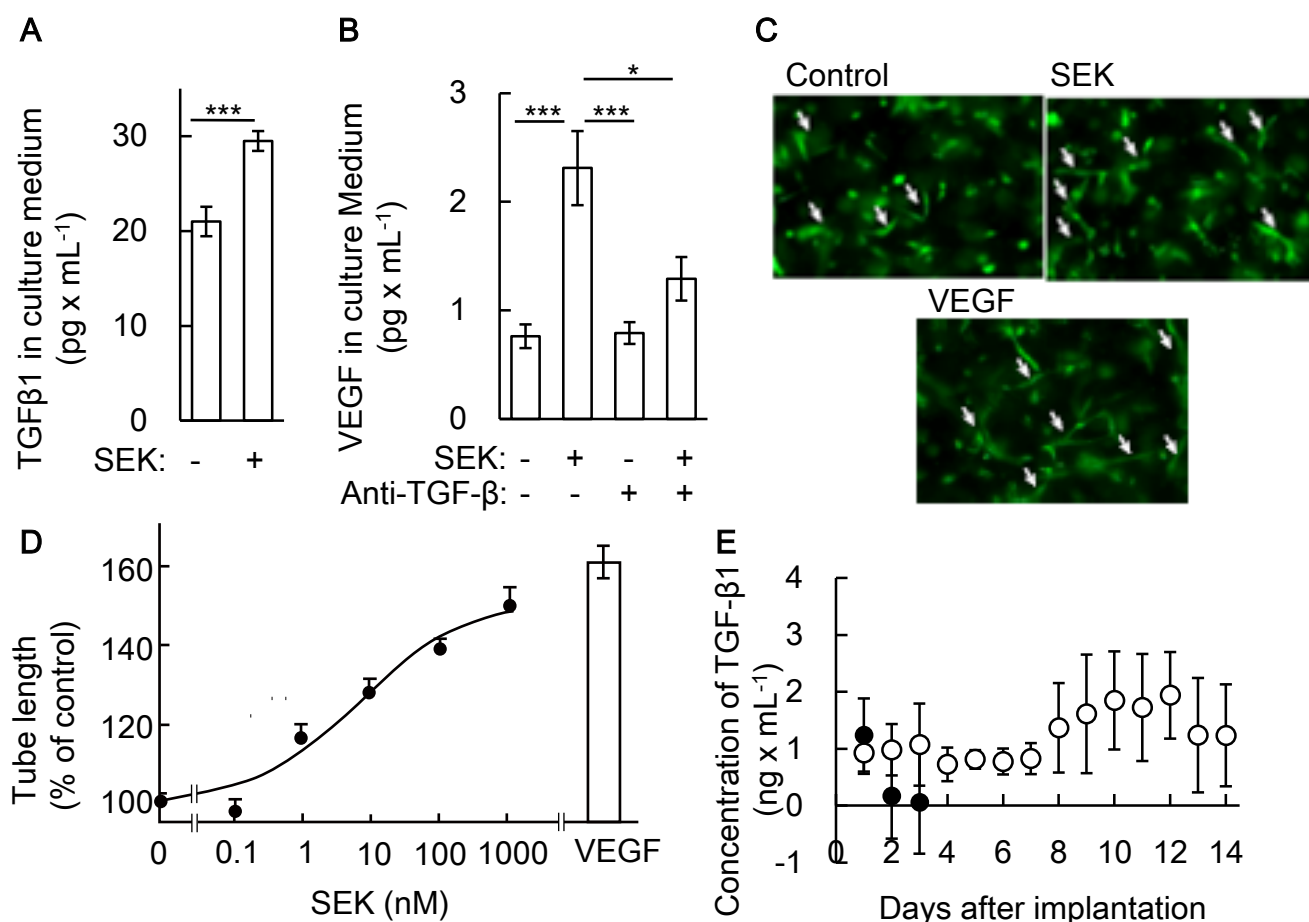


Figure 15. The effects of SEK on HUVEC production of TGF-β1 and VEGF, and tube formation. (A) HUVECs were incubated for 1 hour with or without 5 nM SEK, and then the concentrations of TGF-β1 in the culture medium were determined. Data are shown as the mean ± SEM of eight independent experiments. ***P < 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).

Discussion

Various methods have been investigated for enabling islet transplantation without immunosuppressive treatment—including transplantation into immune privileged sites [22]–[24]; creation of a bioartificial pancreas [13]; reduction of islet immunogenicity [25]–[27]; transplantation of islets plus immunosuppressive cells [28]–[32]; and treatment with antibodies [33]. While these methods have shown promise in animal models, each has proven difficult to use in humans.

In the presently investigated approach, a richly vascularized and immune tolerant environment was prepared via implantation of agarose-SEK rods in a subcutaneous site. *In vitro* studies of the effects of SEK on human umbilical vein endothelial cells (HUVECs) conducted preliminary to gain some insight into the mechanisms involved in the transformation of the tissue environment. Notably, SEK induced HUVECs to release TGF- β 1 and vascular endothelial growth factor (VEGF) into the culture medium (Figure 15A, B) and promoted HUVEC tube formation in collagen gel (Figure 15C, D). These effects in the presence of anti-TGF- β antibody were also examined. The results showed that SEK did not directly induce VEGF production, but rather stimulated HUVECs to release TGF- β 1, which in turn stimulated HUVECs to release VEGF (Figure 15B). TGF- β 1 can also induce Treg cells [34]. As mentioned above, the activated TGF- β 1 concentration in the exudate reached a maximum at 10 days after agarose-SEK rod implantation (Figure 15E). These findings suggested that SEK stimulates cells, including HUVECs, to release TGF- β 1, and that the released TGF- β 1 plays important roles in the formation of highly vascularized and immune tolerant granulomatous tissue.

The transplantation of allogeneic islets into a richly vascularized space formed in

subcutaneous tissue by implantation of agarose-bFGF were previously examined, showing that these islets could survive and control blood glucose levels for over 100 days [8]. Although bFGF and SEK have different origins and biological functions, they both promoted the same effects, ie, formation of the highly vascularized and immune tolerant environment, and the long-term survival of allogeneic islets in this space without any immunosuppressive medication.

In the present study, it was tentatively hypothesized that the immune tolerant environment was primarily initiated and maintained by an increased number of Treg cells. In fact, it was found that the percentage of Treg cells among $CD4^+$ T cells in the tissue increased to 24% at 10 days after implantation of agarose-SEK rods (Figure 6A). At 10 days after implantation, critical suppression of $CD8^+$ T cells were also observed, which can be involved in graft rejection. The mechanism and time-course of maintenance of this immune tolerant environment should be studied in detail. Here graft rejection following injection of donor-antigen were observed (Figure 13, 14), suggesting that maintenance of immune tolerance involves Treg cell density and activities. Further studies, such as investigations of Treg depletion, are required to clarify the immune states at the transplantation site.

Transplantation of quasiislets derived from a patient's own iPS cells could be a good method of treating autoimmune type 1 diabetic patients. However, immunosuppressive medications are still required to suppress autoimmune reactions against the patient's own islets, since autoimmune Type 1 diabetic patients retain islet reactive $CD4^+$ T cells as memory T cells. The present results demonstrate a method by which allogeneic islets, including allogeneic iPS-derived islets, could potentially be tolerated in patients without immunosuppressive medications.

Clinical and experimental studies of islet transplantation have examined various potential transplantation sites. The site requirements can be summarized as follows [35],[36]: must have a rich vascular supply; must be accessible via a minimally invasive procedure; and it must be easy to discern the islet graft's function and morphology. In the present study, agarose-SEK rod implantation led to the formation of an immune tolerant environment in the subcutaneous tissue, which was surrounded by vascularized connective tissue. The generated site meets most of the above-stated requirements; however, further studies are needed to determine whether the generated site is truly appropriate for clinical application. Notably, immunological reactions to grafts are highly species-dependent, and skin structure differs between humans and most animals, including rats, in terms of the musculus cutaneus. Such variations could result in differences in the formation of an immune tolerant environment. It is planned to conduct further studies of this method in larger animal models, such as pigs and macaque monkeys.

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Chapter 3:**Long-term Subcutaneous Allogeneic Islet Graft Survival in a Mouse Model Without Immunosuppressive Treatment.****Introduction**

Transplantation of islets of Langerhans is anticipated to be a radical treatment for patients with insulin-dependent diabetes mellitus. Currently, about 200 transplantations of islets per year were performed between 2011 and 2013 in the world [1]. In most clinical islet transplantation procedures, islets are transplanted into the liver by infusion through the portal vein, and immunosuppressive drugs are administered for the rest of the patient's life to prevent rejection of the islet graft [2],[3]. Islet transplantation into subcutaneous tissue has been studied as a less invasive way to transplant islets [4]–[7]. In rat models, the previous research demonstrated long term functioning of allogeneic islets transplanted into subcutaneous prevascularized pockets prepared using agarose rods containing bFGF and heparin even under no immunosuppressive medication [5]. Immunological analyses of the islet transplantation sites in rats were performed to garner insights into the mechanisms underlying graft acceptance in chapter 1-2. It appeared that regulatory T cells (Tregs) might play a pivotal role in protecting the islet graft from rejection. However, there are not very many commercially available antibodies that can be used to analyze immunological environment at the long-term acceptance of allogeneic islets, and it is difficult to obtain rats that are genetically modified to study rules of Treg cells in acceptance of allogeneic islets. Thus, it was difficult to analyze the engraftment mechanism in detail in the rat allograft models.

It is easier to work with mice as experimental animals, as there are more resources, such as relevant antibodies and mice with Treg-related genetic modifications [8]. In this chapter, the aim was to reproduce the previous results in a mouse model, that is, to perform an allogeneic mouse islet graft into a subcutaneous site that could function long-term even without immunosuppressive treatment. Towards this end, islets from C57BL/6 mice were transplanted into diabetic BALB/c mice.

Materials and Methods

Materials

Streptozotocin (STZ), a penicillin-streptomycin solution, Blocking One reagent, D-(+)-glucose, heparin sodium salt, paraformaldehyde (PFA), Tween-20, Triton X-100, and copper (II) sulfate anhydrous (CuSO_4) were purchased from Nacalai Tesuque (Kyoto, Japan). The hemoglobin assay kit, Tris (hydroxymethyl) aminomethane (Tris), ammonium acetate, and ammonium chloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Equitech-Bio, Inc. (Kerrville, TX, USA).

Collagenase P was obtained from Roche Diagnostics GmbH. (Mannheim, Germany). FicollTM PM400 (Ficoll) was obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). Conray was purchased from Daiichi Sankyo Co. Ltd. (Tokyo, Japan). Tissue-Tek O.C.T. compound was obtained from Sakura Finetek USA, Inc. (Torrance, CA, USA). SeaKem GTG agarose were obtained from Lonza (Basel, Switzerland). FIBLAST Spray 500 (bFGF) was purchased from Kaken Pharmaceutical Co, Ltd. (Tokyo, Japan). Hoechst 33258 nuclear stain was purchased from Dojindo Laboratories (Kumamoto, Japan).

Guinea pig anti-insulin antibody (ab6995) was purchased from Abcam (Cambridge, UK). Alexa 488-labeled goat anti-guinea pig IgG, Alexa 594-labeled goat anti-guinea pig IgG, Hanks' balanced salt solution (HBSS), and RPMI 1640 were purchased from Life Technologies (Carlsbad, CA, USA). FITC-tomato lectin was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). The glucose sensor

(GLUCOCARD MyDIA) was purchased from Arkray, Inc. (Kyoto, Japan).

Animals

A fully mismatched mouse model was used, 7- to 8-week-old male C57BL/6N (C57BL/6) mice (H-2Kb) as donors and 7- to 8-week-old male BALB/c mice (H-2Kd) as recipients for the allogeneic transplantation. All animals were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). All animal experiments were carried out in accordance with the guidelines of the Kyoto University Animal Care Committee.

Vascularization of subcutaneous tissue in response to the agarose rod with bFGF-heparin

A rod-shaped agarose scaffold (4-mm in diameter, 20-mm in length) was prepared as described previously [2]. Briefly, a 4.5% (w/v) agarose solution in distilled deionized water was placed in a polystyrene tube and kept on ice to induce gelation. The agarose gel was then cut into 20-mm rods that were frozen at -30°C overnight and then freeze-dried for 24 hours under reduced pressure. The bFGF solution (50 µg of bFGF and 25 µg of heparin in 100 µl of buffer) was applied evenly to the freeze-dried agarose rod. The agarose gel rods with bFGF-heparin (agarose-bFGF rods) were stored overnight at 4°C. For control experiments, 100 µl of saline without bFGF was applied to a freeze-dried agarose gel rod and stored overnight at 4°C until use (agarose-saline rods).

Vascularization of subcutaneous tissue in response to the agarose-bFGF rod

bFGF which is known to be a potent angiogenic factor [9] was employed to prepare prevascularized pockets. Heparin protects bFGF from denaturation and enzymatic

degradation and is also potent modulator of bFGF [10],[11]. Heparin was also added to a bFGF solution. A rod-shaped agarose (50074, Lonza) (4-mm in diameter, 20-mm in length) which is used as a carrier of bFGF and heparin was prepared as described previously [5]. Briefly, a 4.5% (w/v) agarose solution in distilled deionized water was placed in a polystyrene tube and kept on ice to induce gelation. The agarose gel was then cut into 20-mm rods that were frozen at -30°C overnight and then freeze-dried for 24 hours under reduced pressure. The bFGF solution (50 µg of bFGF and 25 µg of heparin in 100 µl of buffer) was applied evenly to the freeze-dried agarose rod. The agarose rods with bFGF-heparin were stored overnight at 4°C. For control experiments, 100 µl of saline without bFGF was applied to a freeze-dried agarose gel rod and stored overnight at 4°C until use (agarose-saline rods).

In chapter 1-2, agarose rod with bFGF-heparin were implanted into two dorsal subcutaneous tissue sites to induce vascularization [5]. A difficulty, however, was met to implant agarose rods in 20 mm long into dorsal subcutaneous tissue of a mouse because mouse body is smaller than a rat. Two agarose rods with bFGF-heparin (one rod into each of two sites) were implanted into the subcutaneous fat tissue in the groin of each mouse to induce vascularization. Four days after implantation of the agarose rods, diabetes was induced in recipient BALB/c mice with a single intraperitoneal injection of STZ at a dose of 160 mg/kg body weight. Mice with blood glucose levels >400 mg/dl for 2 consecutive days were considered diabetic. The agarose rods were removed 8 days after implantation. The tissue next to the agarose rod with bFGF-heparin was harvested for histological analysis. To measure the level of vascularization semi-quantitatively, the amount of hemoglobin in the subcutaneous tissue facing the agarose rod with bFGF-heparin was determined as described previously [7]. Briefly, the harvested tissue was minced and

incubated for 48 h at 4°C in 10 ml of Gay's solution (0.75% NH₄Cl in 17 mM Tris-HCl buffer solution, pH 7.6) with gentle periodic agitation to extract the hemoglobin. The amount of hemoglobin was determined using a hemoglobin assay kit.

Islet isolation

Islets were isolated from donor C57BL/6 mice by the collagenase digestion method. Briefly, the pancreas was inflated with a collagenase solution (0.15 mg/ml in HBSS) that was injected through the portal vein, collected from the mouse, and then digested for 18 min at 37°C in a collagenase solution. Islets were isolated from the digested pancreas by the Ficoll/Conray density gradient purification method and cultured for 2 days in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin before transplantation.

Islet transplantation

When the rods were removed 8 days after implantation, 150 islets were transplanted into each of the two pre-vascularized pockets in the groin. The wounds were closed by suturing. For control experiments, 150 C57BL/6 islets were transplanted into each of the two sites in the groins of STZ-BALB/c mice that were treated with agarose-saline rods, and into STZ-mice that did not have agarose rods implanted. None of the recipient mice received immunosuppressive drugs during the experiments. For determination of the non-fasting blood glucose levels, blood samples were collected from the tail veins of the recipients every day for the first 2 weeks after islet transplantation and 2 or 3 times a week thereafter. Glucose levels were determined using a glucose sensor. Graft rejection was defined as when two consecutive blood glucose measurements

exceeded 250 mg/dL.

Intraperitoneal glucose tolerance test (IPGTT)

IPGTTs were used to evaluate islet graft function in recipient BALB/c mice 100–120 days after islet transplantation. Non-diabetic mice and STZ-mice without islet grafts were used as controls. After 16 hours of fasting, the mice received a glucose solution intraperitoneally (1 g glucose/kg body weight). Blood samples were collected 0, 15, 30, 60, 90, and 120 min after glucose loading, and the glucose concentration of the blood samples was determined by GLUCOCARD MyDIA.

Histological study of islet grafts

Subcutaneous tissues containing the islet grafts were collected 8 days after the agarose rod implantation, fixed in 4% paraformaldehyde (PFA) in Phosphate-buffered saline (PBS), and embedded in paraffin or O.C.T compound. Thin (4- μ m) tissue sections were prepared using standard methods [5]. The sections were stained using hematoxylin and eosin (H&E). Immunofluorescent staining was performed using an anti-insulin antibody. Briefly, the sections were permeabilized with 0.2% Triton X-100 solution in PBS at room temperature for 15 min. The samples were then incubated with Blocking One reagent for 1 hour to block non-specific staining, followed by washing with PBS. The sections were treated with a solution of polyclonal guinea pig anti-insulin antibody (1:200) in Blocking One reagent for 1 hour at room temperature. The sections were then incubated with fluorescently-labeled secondary antibody, namely Alexa 488-labeled goat anti-guinea pig IgG (1:500) or Alexa 594-labeled goat anti-guinea pig IgG (1:500) in Blocking One reagent at room temperature for 1 hour. This was followed by washing with

PBS containing 0.05% Tween-20. The cell nuclei was counterstained with 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 [12]. The images of H&E and immunofluorescent staining were taken with a BX51 microscope equipped with a DP72 CCD camera.

Intravenous infusion of FITC-lectin solution to visualize the functional vasculature in the islet grafts

Endothelial cells in an islet graft were stained by FITC-tomato lectin to see connection between recipient's and the islet graft blood vessels. FITC-tomato lectin solution (200 µg in 200 µL of saline containing 200 IU heparin) was infused through the inferior vena cava of recipient mice 43 days after transplantation. After 15 min of circulation, the subcutaneous tissue with the islet graft was dissected, washed briefly twice with saline solution, fixed with 4% PFA in PBS, embedded in O.C.T. compound, and snap-frozen in liquid nitrogen. Thin (4-µm) tissue sections were prepared. Immunofluorescent staining of the thin sections of the tissue with the islet grafts was performed to visualize insulin as described above.

Statistical analysis

Comparisons among 3 groups were performed using one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc test. A *P* value <0.05 was considered statistically significant.

Results

Pre-vascularization of BALB/c subcutaneous tissue by implantation of agarose rods with bFGF-heparin

Fig. 1A schematically shows the methods used to create subcutaneous pockets that were surrounded by highly vascularized granulomatous tissue and to transplant the islets after removing the agarose rod. The subcutaneous tissue of a diabetic BALB/c mouse induced by the injection of streptozotocin (STZ) (STZ- BALB/c mouse) usually shows a low level of vascularization (Fig. 1B-1 and B-2). Implantation of agarose-saline rods did not seem to increase vascularization (Fig. 1C-1 and C-2). In contrast, implantation of the agarose rod with bFGF-heparin, which contained 50 μ g of bFGF and 25 μ g of heparin per rod, for 8 days resulted in the formation of highly vascularized granulomatous tissue adjacent to the agarose rods with bFGF-heparin (Fig. 1D-1 and D-2). The agarose rods with bFGF-heparin were removed easily without tissue adhesion, resulting in subcutaneous pockets that were surrounded by highly vascularized tissue (bFGF-heparin spaces). The hemoglobin content of the tissue was determined in order to semi-quantitatively evaluate blood vessel induction. When agarose rods with bFGF-heparin were implanted, the hemoglobin content of the tissue significantly increased from 0.432 ± 0.222 mg/rod in untreated tissue to 1.70 ± 0.648 mg/rod in treated tissue (Fig. 1E).

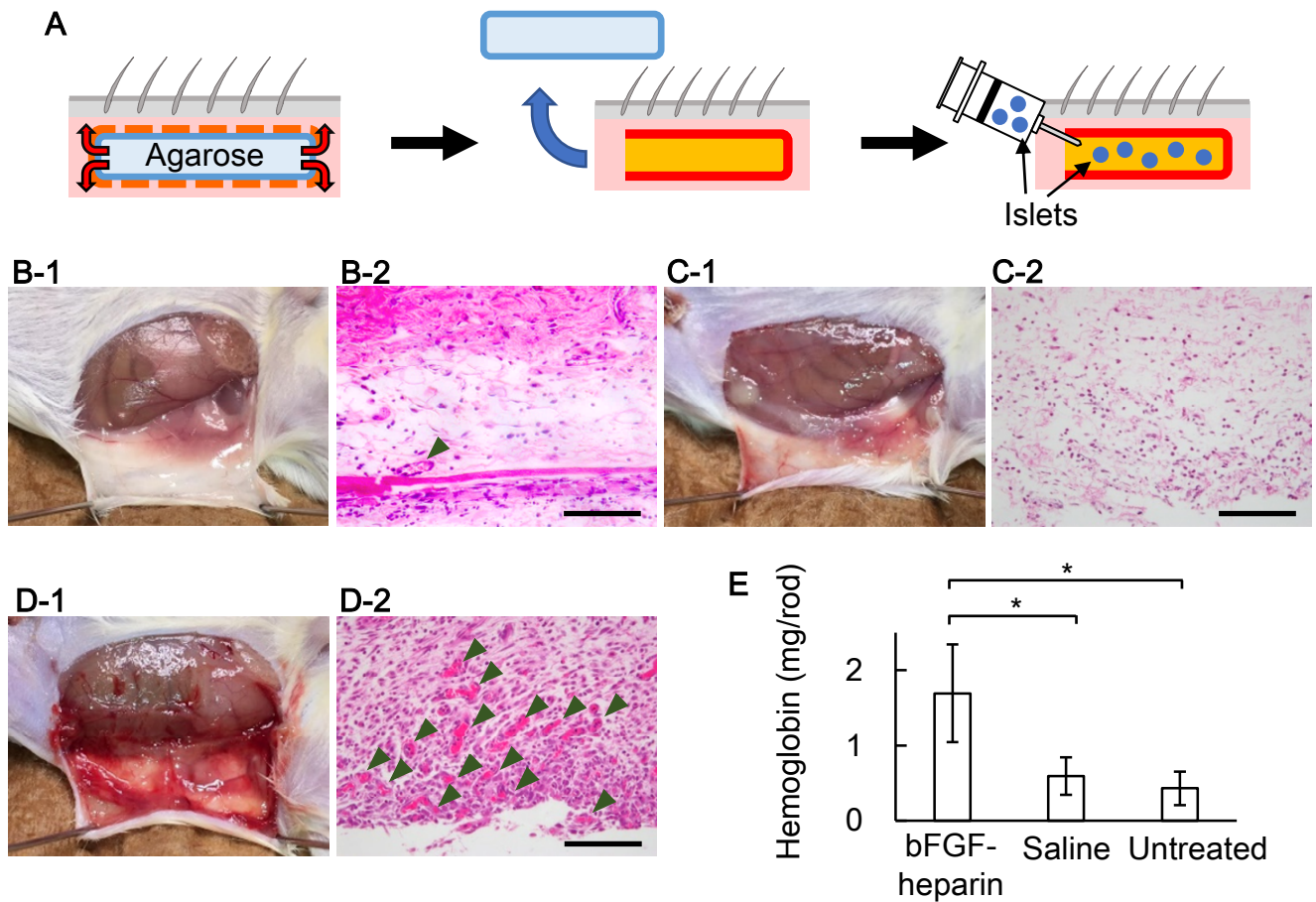


Figure 1. Prevascularization in subcutaneous tissue in response to the implantation of agarose rods containing basic fibroblast growth factor (bFGF)-heparin. (A) Schematic drawing of the method used to induce the formation of a subcutaneous space surrounded by highly vascularized granulomatous tissue. The islets were transplanted after removing the agarose rods. (B-1) Macroscopic image and (B-2) H&E staining of subcutaneous tissue of an untreated diabetic BALB/c mouse. (C-1) Macroscopic image and (C-2) H&E staining of subcutaneous tissue 8 days after implantation of an agarose rod with saline. (D-1) Macroscopic image and (D-2) H&E staining of subcutaneous tissue 8 days after implantation of an agarose rod with bFGF-heparin. Scale bars: 100 μ m. (E) Amount of hemoglobin in the subcutaneous tissue 8 days after implantation of an agarose rod with bFGF-heparin (n=7), with saline (n=6), and in untreated diabetic BALB/c mice (n=6). Data are reported as means $\pm$ SDs. Statistical analysis was performed using one-way ANOVA with Tukey-Kramer post hoc test ($p < 0.0001$; one-way ANOVA between the 3 groups, *, $p = 0.0010$, **, $p = 0.0003$).

Normalization of blood glucose levels in STZ-BALB/c mice by transplantation of C57BL/6 islets

Table 1 summarizes the graft survival periods and the transplantation conditions, such the donor and recipient mice combinations, the number of transplanted islets, and the agarose rod implantation time. BALB/c mice (H-2Kd) were used as donors and C57BL/6 mice (H-2Kb) as recipients as a model of a fully MHC-mismatched donor/recipient combination. Pre-vascularized pockets were prepared on both sides of the groin of BALB/c mice by implanting agarose-bFGF rods (one per site) for 8 days. Four days after implantation, diabetes was induced by a single injection of STZ (STZ-BALB/c). After removal of the agarose rods, a suspension of 150 C57BL/6 islets was infused into each bFGF-heparin space for a total of 300 islets for each recipient mouse. No immunosuppressive medications were given to the STZ-BALB/c mice before or after transplantation.

Table 1. Transplantation conditions and islet graft survival.

Donor mice	Recipient mice	Number of islets	The days of implantation devices	bFGF	heparin	n	Normoglycemia, days/ mouse	Mean survival time (means $\pm$ SDs)
C57BL/6	BALB/c	300	8	(+)	(+)	10	8, 14, 100, 100, 100, 100, 100, 100, 100, 100	82.2 $\pm$ 37.6
C57BL/6	BALB/c	300	8	(-)	(-)	5	0, 0, 0, 3, 4	1.4 $\pm$ 1.9
C57BL/6	BALB/c	300	0	(-)	(-)	5	0, 0, 0, 0, 0	0.0

The blood glucose levels of the 10 recipient mice are shown in Figure 2A. Of the 10 recipient mice, 2 rejected the grafts within 30 days after transplantation. Of the remaining 8 recipient mice, all showed normoglycemia for over 100 days after islet

transplantation, although the blood glucose levels of 2 of the 10 recipient mice showed transient high glucose levels on 20 to 40 days. In contrast, when islets were transplanted into subcutaneous tissue 8 days after the implantation of agarose-saline rods, none of the 5 recipient mice demonstrated long-term normoglycemia (Figure 2B). Similarly, when islets were transplanted into subcutaneous tissue without pre-treatment, none of the 5 recipient mice demonstrated long-term normoglycemia (Figure 2C).

Assessment of graft function > 100 days after transplantation

IPGTTs were performed 100–120 days post-transplantation (Fig. 2D). The area under the curve (AUC) was $1.81 \pm 0.34 \times 10^4$ min mg/dL for non-diabetic normal BALB/c mice, $2.09 \pm 0.72 \times 10^4$ min mg/dL for the mice with islet grafts in the subcutaneous spaces formed using agarose rods with bFGF-heparin, and $5.77 \pm 0.24 \times 10^4$ min mg/dL for diabetic mice without islet grafts. No difference was seen in the AUC values between the mice with islet grafts and the non-diabetic BALB/c mice, while significant difference was observed in the mice with and without islet graft ($p < 0.0001$; one-way ANOVA between the 3 groups, $p = 0.67$; non-diabetic BALB/c mice versus the mice with islet grafts, $p < 0.0001$ the mice with versus without islet grafts). These results suggest that islet grafts in a subcutaneous site can control blood glucose levels as well as in healthy mice.

Histological examination of islet grafts long after transplantation

An islet graft was retrieved from each mouse for histological examination 120 days post-transplantation. H&E staining of the thin sections is shown in Fig. 3A and B. The islets were clearly visible. Several blood vessels containing many erythrocytes were

seen in the islet grafts (Fig. 3B). When immunofluorescent staining was performed using anti-insulin antibodies, insulin-positive cells were clearly observed in the slices (Fig. 3C). To assess the connection of vessels in the islets grafts to the vascular system of the recipient mice at early transplantation days, we performed intravenous infusion of a FITC-lectin solution 43 days after transplantation. Vessels within the islet grafts were stained green by FITC-lectin (Fig. 3D). It indicates that blood flows into the islet grafts through the vessels. Vessels in the islet grafts were already connected to the vascular system of the recipient mice at 43 days after transplantation.

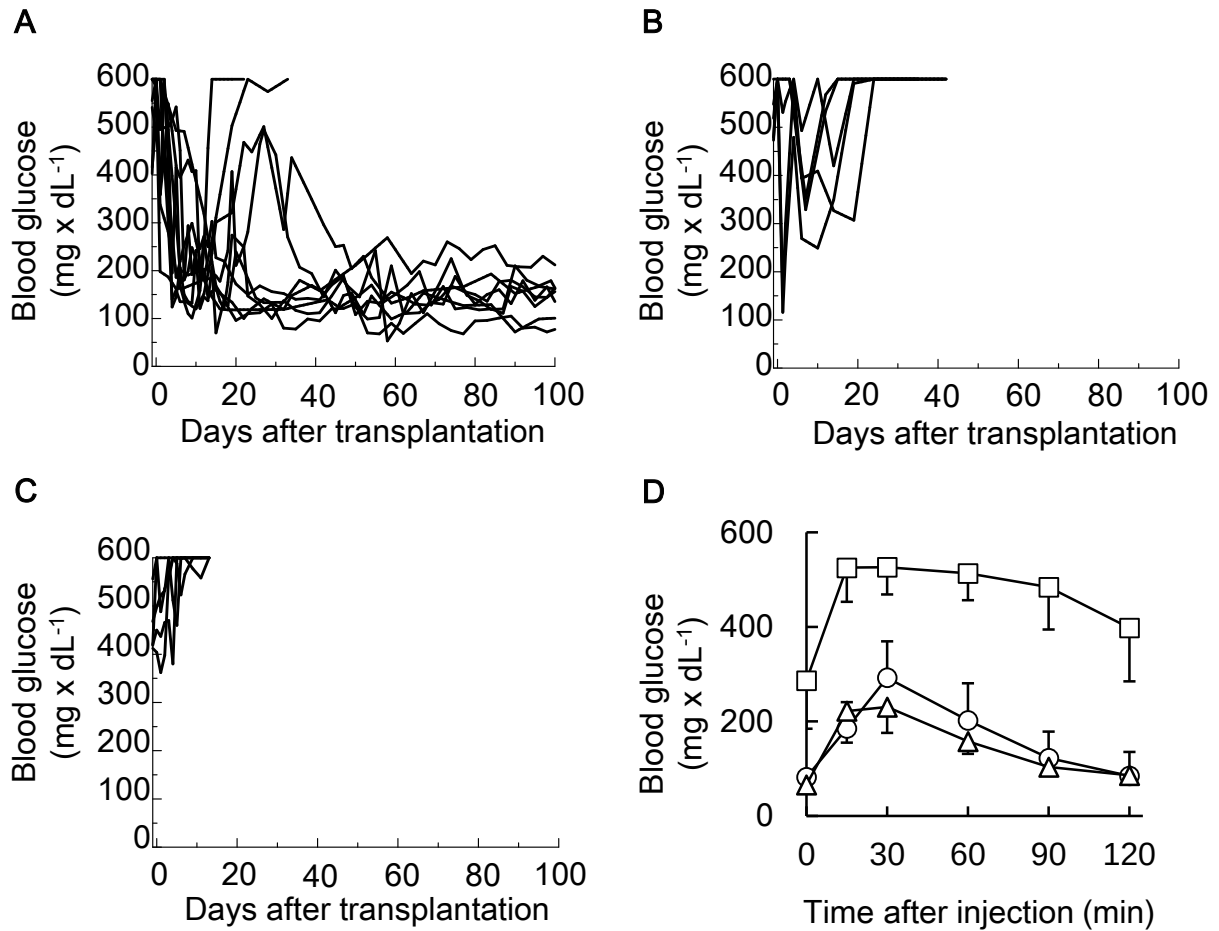


Figure 2. Transplantation of C57BL/6 islets into diabetic BALB/c mice. The blood glucose levels of STZ-BALB/c mice after transplantation of 300 C57BL/6 islets/mouse into the subcutaneous spaces formed by implantation of agarose rods with (A) bFGF-heparin ($n = 10$) or (B) saline ($n=5$). (C) 300 islets were transplanted into untreated subcutaneous sites ($n=5$). (D) The intraperitoneal glucose tolerance test (IPGTT) was performed in non-diabetic BALB/c mice (triangles, $n=4$), in recipient mice 100–120 days after the transplantation of 300 allogeneic islets (circles, $n=3$) and in diabetic BALB/c mice (square, $n=4$).

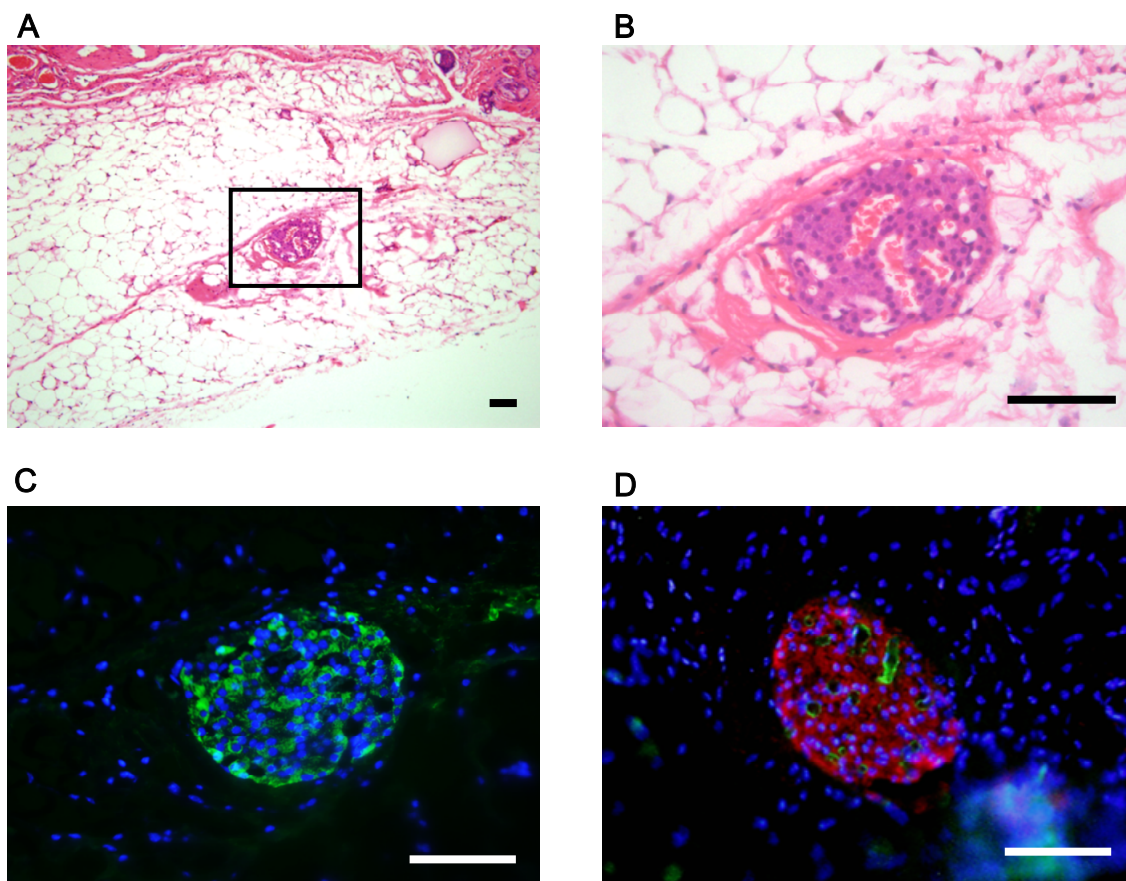


Figure 3. Histological analysis of islet allografts in mice after long-term transplantation. Subcutaneous tissue containing allogeneic islets was retrieved 120 days after transplantation. (A, B) H&E staining and (C) immunofluorescent staining of insulin (green), and nuclei (blue). (D) The tissue containing allogeneic islets was retrieved 43 days after transplantation, and FITC-lectin solution was infused intravenously to examine the functional vasculature in islet grafts. Immunofluorescent staining of insulin (red), FITC-lectin (green), and nuclei (blue). Scale bars: 100 μ m.

Discussion

In recent years, others have also used this donor-recipient mouse combination to study allogeneic islet transplantation. Cantarelli et al. reported that the median time to graft rejection was 8 ± 3.3 days for transplantation into the liver and 6 ± 0.5 days for transplantation into the bone marrow in the absence of immunosuppression [13]. When the recipient mice were treated with tacrolimus (FK-506) and mycophenolate mofetil from day -1 to 14 every day, the median time to graft rejection was prolonged to 12 ± 3 days for transplantation into the liver and 8 ± 2.2 days for transplantation into the bone marrow, respectively. They, however, could not achieve long-term islet graft survival after immunosuppressive treatment stopped. Hsu et al. also used this donor and recipient combination [14] and reported that the normoglycemic period was 10.8 ± 1.2 days for mice who were transplanted with 300 islets under the renal capsule and 18.9 ± 5.3 days for mice who were transplanted with 900 islets subcutaneously. Long-term islet graft survivals were achieved in both renal subcapsular and subcutaneous transplantation by full donor bone marrow chimerism. Khosravi-Maharlooei et al. reported that intraperitoneal injection of indoleamine 2,3-dioxygenase-expressing fibroblasts, termed IDO-fibroblasts, prolong graft survival period [15]. Indeed, 2 of 5 recipient mice demonstrated long-term graft survival of more than 100 days. In these experiments, allogeneic islet grafts between donor C57BL/6 mice and recipient STZ-BALB/c mice combination are rejected by the host when their immune systems are not modified. These facts indicate that the C57BL/6 donor and STZ-BALB/c recipient combination is appropriate for examining whether a method is suitable for prolonging allogeneic islet graft survival. In the studies by Hsu et al. and Khosravi-Maharlooei et al., long-term graft

survival of more than 100 days were reported. Although their methods are interesting, bone marrow chimerism or injection of genetically modified cells is not practical for human recipients of islet transplantation. In comparison, the method, that is, islet transplantation into a subcutaneous pocket which is prepared by implantation of an agarose rod with bFGF-heparin, is surgically and immunologically minimum invasive.

In this study, donor C57BL/6 mice and recipient STZ-BALB/c mice were employed and it was demonstrated that allogeneic islets transplanted into highly vascularized granulomatous tissue can survive long term even under no immunosuppressive therapy. In the chapter 1-2 using a rat model and this chapter using a mouse model, it was demonstrated that allogeneic islets that were transplanted into subcutaneous sites could function for more than 100 days without the use of immunosuppressive drugs. No conspicuously difference was seen in rat and mouse models. Mouse models are better suited for detailed immunological analysis of allogeneic islet graft acceptance in subcutaneous tissue, mainly due to the greater commercial availability of antibodies that can be used for immunophenotyping mouse cells. There are also BALB/c mice that express the human diphtheria toxin receptor-enhanced green fluorescent protein (DTR-eGFP) under the control of the Foxp3 promoter, which is required for the development and the function of Treg [8],[16]. Use of anti-CD25 antibodies, anti-CTLA-4 antibodies, and DERE (Depletion of Tregs) BALB/c mice will be helpful for investigating the mechanisms underlying the acceptance of allogeneic islet grafts in subcutaneous sites in greater detail.

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Chapter 4:**Bioabsorbable Device to Prepare Subcutaneous Pockets for Islet Transplantation.****Introduction**

Transplantation of islets of Langerhans is expected to be a fundamental therapy for insulin-dependent diabetes mellitus. Approximately 300 islet transplantations are performed annually worldwide, but it is not yet accepted as a general treatment. In order to promote islet transplant as a general treatment, three problems must be solved: securing a sufficient number of islets, avoiding the use of an immunosuppressive agent with strong side effects, and a minimally invasive method of islet transplantation needs to be established. In recent years, methods of islet differentiation from induced pluripotent stem (iPS)/embryonic stem (ES) cells have been reported by several groups [1],[2], addressing islet shortage. In addition, a method of transplanting islets under the skin was established and maintaining their functions for a long period of time, even without the use of immune suppressive agents [3]. It is thought that the remaining two problems could be solved by extending the previous chapters.

In order to apply this method to human patients, problems related to device safety must be solved. In this chapter, it was attempted to develop a device for creating a transplantation site using materials that guarantee safety. Gelatin sheets conventionally used as adhesion prevention materials and sodium hyaluronate, which is used as a viscosupplement for injections into the knee, are used as a material for device fabrication. In the previous chapters, it was necessary to cut the skin two times, at the time the device was implanted and at the time of removing it to create an islet transplantation site. As the

new device was made using only bioabsorbable materials, it is not necessary to remove the device, and only one skin incision is required to transplant a device, which it is expected to reduce surgical invasion.

Materials and Methods

Materials

FIBLAST Spray 500 (bFGF) and ARTZ 25 mg (1% sodium hyaluronate solution) were purchased from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Collagenase P and the gelatin sheet (Gelfilm) were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Pfizer, Inc. (Tokyo, Japan), respectively. Streptozotocin, the penicillin-streptomycin mixed solution, Tween-20, Triton X-100, and Blocking One reagent were purchased from Nacalai Tesuque (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), and Alexa 488-labeled goat anti-guinea pig IgG, Hank's balanced salt solution (HBSS), and RPMI 1640 were purchased from Life Technologies (Carlsbad, CA).

Guinea pig anti-insulin polyclonal antibody (ab7842) was obtained from Abcam (Cambridge, UK) and enzyme-linked immunosorbent assay (ELISA) kits for the rat insulin assay were purchased from Shibayagi Co., Ltd. (Gunma, Japan). The formalin solution and hemoglobin assay kit were purchased from Wako Pure Chemical Industries (Osaka, Japan). The glucose sensor (GLUCOCARD MyDIA) was purchased from Arkray, Inc. (Kyoto, Japan).

Animals

Seven to 9-week-old male Fisher 344 rats (F344 /NSIc, RT-1^{lv1}) and 8 to 15-week-old male ACI/NSIc rats (RT-1^a) were used as a model of a fully MHC-incompatible donor–

recipient combination. 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.

Islet Transplantation

Islets were isolated from donor F344 rats using the collagenase digestion method [4]. Briefly, the pancreas was inflated by injecting 8 mL collagenase solution (0.5 mg/mL in HBSS), and then harvested and digested at 37 °C. Islets were collected by the Ficoll/Conray density gradient method and cultured for 2 days in RPMI 1640 with 10% FBS before transplantation.

Diabetes was induced in ACI rats by a single intraperitoneal injection of STZ at a dose of 60 mg/kg body weight. After STZ injection, two gelatin sheets (width, 10 mm; length, 25 mm; thickness, 0.12 ± 0.0023 mm) were implanted into each of two dorsal subcutaneous sites in STZ-induced diabetic ACI rats (STZ-ACI rats) as a spacer, and a viscous sodium hyaluronate-bFGF mixture (50 µg bFGF solution in 50 µL buffer) was injected into the area surrounding the gelatin sheets (Fig. 1A). The gelatin sheets and surrounding tissue were retrieved 7, 37, or 67 days after gelatin sheet implantation to examine the induction of blood vessels and the fate of the gelatin sheets. Gelatin sheet harvested from subcutaneous sites was washed with DDW, freeze-dried for 24 hours under reduced pressure, and weighed.

A total of 1500 isolated islets were transplanted (around the gelatin sheet) into each of the two pre-vascularized dorsal pockets in the STZ-ACI rats 7 days after injection of a sodium hyaluronate-bFGF mixture. None of the recipients received immunosuppressive drugs before or after islet transplantation. Blood samples were

collected from the recipients' tail veins, and non-fasting blood glucose levels were determined using a glucose sensor. Graft rejection was defined as two consecutive blood glucose measurements > 250 mg/dL. The day of the first measurement was considered the day of rejection.

Plasma Insulin analysis and IPGTTs

Blood was collected from recipients to determine plasma insulin levels 30-40 and 60-90 days after islet transplantation. Blood samples were also collected from normal and STZ-ACI rats without islet grafts as controls. Plasma insulin concentrations were determined using ELISA kits for rat insulin. All blood samples were kept on ice and centrifuged at 4000 rpm for 15 min at 4°C. Plasma was collected and stored at -80°C until determination of the plasma insulin concentration by ELISA.

Recipient rats also underwent an intraperitoneal glucose tolerance test (IPGTT) 50–55 days and 100–120 days after islet transplantation. 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 glucose levels determined using a glucose sensor.

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- μ m tissue sections were prepared using standard methods and stained by hematoxylin and eosin (H&E) or for insulin immunofluorescence [5]. Briefly, for immunofluorescent staining, the sections were permeabilized with 0.2% Triton X-100 solution in PBS for 15 min at room

temperature. The samples were then incubated with Blocking One reagent for 1 hour to block non-specific staining and washed with PBS. Subsequently, the sections were treated with a polyclonal guinea pig anti-insulin antibody (1:200) in Blocking One reagent for 1 hour at room temperature, followed by a 1-hour incubation at room temperature with Alexa 488-labeled goat anti-guinea pig IgG (1:500) in Blocking One reagent. The sections were then washed with PBS containing 0.05% Tween-20 and cell nuclei counterstained with 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 [6].

Statistical Analysis

The Student's t-test was used for between-group comparisons. The log-rank test was used to compare graft survival between experimental and control groups. $P < 0.05$ was considered significant.

Results

Whereas vascularized tissue surrounded the gelatin sheet 7 days after implantation (Figure 1B, C), few vessels were observed in untreated skin tissue in STZ-ACI rats (Figure 1D, E). No tissue adhesion to the gelatin sheets was observed. The gelatin sheets gradually degraded under the skin during implantation. The weight of the gelatin sheets decreased from 0.049 ± 0.0033 g to 0.027 ± 0.0034 g 7 days after implantation (Figure 2A). Smaller fragments of gelation sheet were found 67 days after implantation compared to 37 days (Figure 2A, B, and C).

F344 rats (RT-1^{lv1}) and ACI rats (RT-1^a) were used as a model of a donor-recipient combination. Blood glucose levels in four recipients were normalized 1 week after islet transplantation, whereas six recipients took approximately 2 weeks to achieve fully normalized blood glucose levels. This may be due to low quality islets or a slow vascular connection between the islet graft and recipients. Eight of 10 recipients demonstrated normoglycemia > 100 days after islet transplantation. One recipient was sacrificed on day 90 for histological analysis (Figure 3A). In contrast, when islets were transplanted into untreated subcutaneous sites of STZ-ACI rats, no recipients demonstrated normoglycemia (Figure 3B). In these recipients, the islet grafts may have been destroyed by hypoxia [7]–[9]. Kaplan-Meier plots of graft survival over time are shown in Figure 3C.

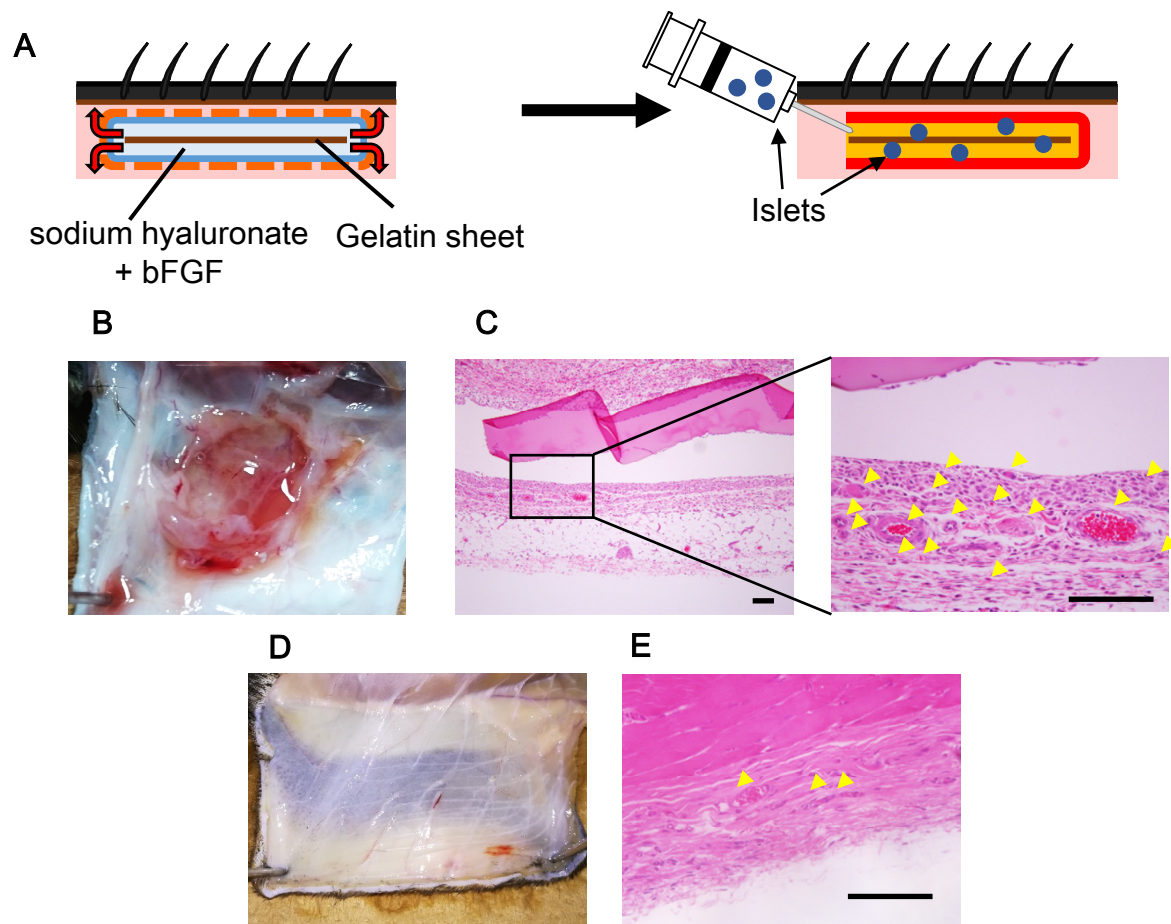


Figure 1. Preparation of a subcutaneous islet transplantation site. (A) Schematic drawing of the method used to induce a subcutaneous pocket surrounded by highly vascularized tissue (bFGF-pockets) and islet transplantation, (B) Macroscopic image of the subcutaneous site 7 days after implantation of a gelatin sheet and sodium hyaluronate + bFGF. (C) H&E staining of subcutaneous tissue prevascularized by implantation of a gelatin sheet and sodium hyaluronate + bFGF for 7 days. (D) Macroscopic image of the untreated subcutaneous site in a STZ-ACI rat. (E) H&E staining of the untreated subcutaneous site in a STZ-ACI rat. Yellow arrowheads indicate the vasculature. Scale bars: 100 μm .

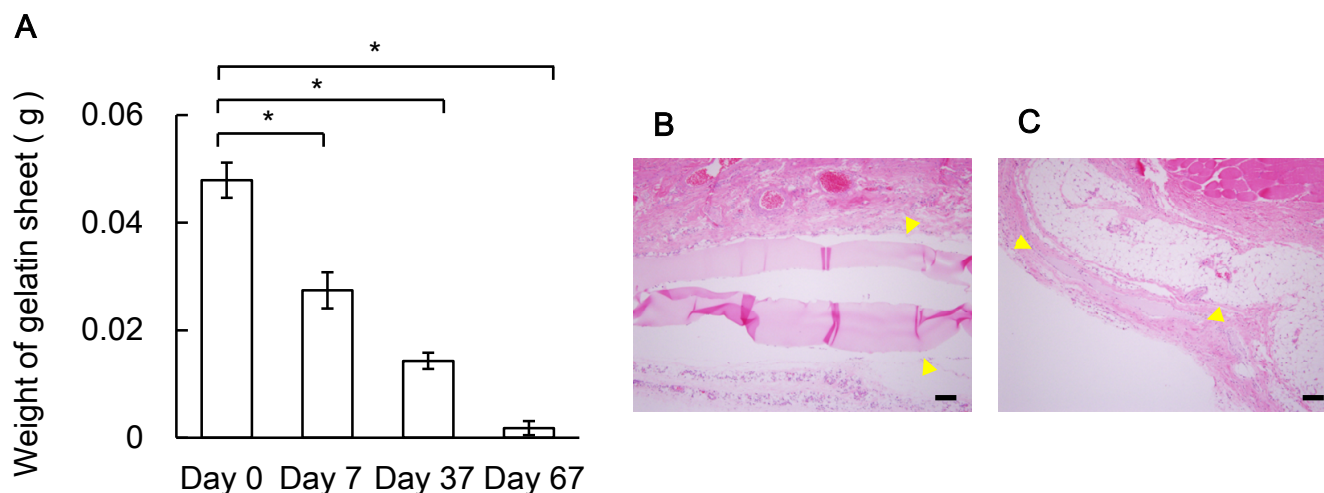


Figure 2. Gelatin sheets after implantation at subcutaneous site of STZ-ACI rats..

(A) The weights of remaining gelatin sheets retrieved the indicated days after implantation (n=3). *P < 0.001 (B) Remains of the gelatin sheet at 37 and (C) 67 days after implantation of a gelatin sheet and sodium hyaluronate + bFGF. Yellow arrows indicate gelatin sheet fragments. Scale bars: 100 μ m.

The function of islet grafts in recipients was characterized by plasma insulin levels and IPGTTs (Figure 3A-C). The mean plasma insulin level in recipients was 0.48 ± 0.11 ng/mL 30-40 days after transplantation and 0.69 ± 0.15 ng/mL 60-90 days after transplantation (Figure 4A). The mean plasma insulin level was 0.85 ± 0.25 ng/mL in normal ACI rats and 0.21 ± 0.12 ng/mL in STZ-ACI rats. Thirty to 40 days after transplantation, the plasma insulin levels in STZ-ACI recipients were approximately half that of normal ACI rats. During following several ten days, the plasma insulin levels increased and no significant difference was observed in the plasma insulin levels between STZ-ACI recipients and normal ACI rats (Figure 4C). IPGTTs were performed in normal rats, STZ-ACI rats, and STZ-ACI recipients 50–55 days and 100–120 days post-transplantation (Figure 4B). The areas under the curve (AUCs) for the IPGTT data did

not differ significantly between normal rats and recipient rats with islets in the vascularized pockets (Figure 4C).

All STZ-ACI recipients had two grafts, one each on the left and right dorsal sides. The right islet graft was retrieved 30 days after transplantation and the left graft 60 days after transplantation for histochemical analysis. The islet graft was clearly visible with H&E staining (Figure 5A, C), and insulin-positive cells were clearly observed by immunofluorescence (Figure 5B, D). Islet grafts were surrounded with the connective tissue. Few lymphocytes were observed around or infiltrated islet grafts. Thus, the islet grafts were protected from attack by the host immune system in the subcutaneous vascularized pocket, allowing it to survive under the skin for a long time without any immunosuppression. The STZ-ACI rats with stable F344 islet grafts received an intraperitoneal injection of F344 rat splenocytes 117 days after transplantation, which tended to increase blood glucose levels a few days later (Figure 5E).

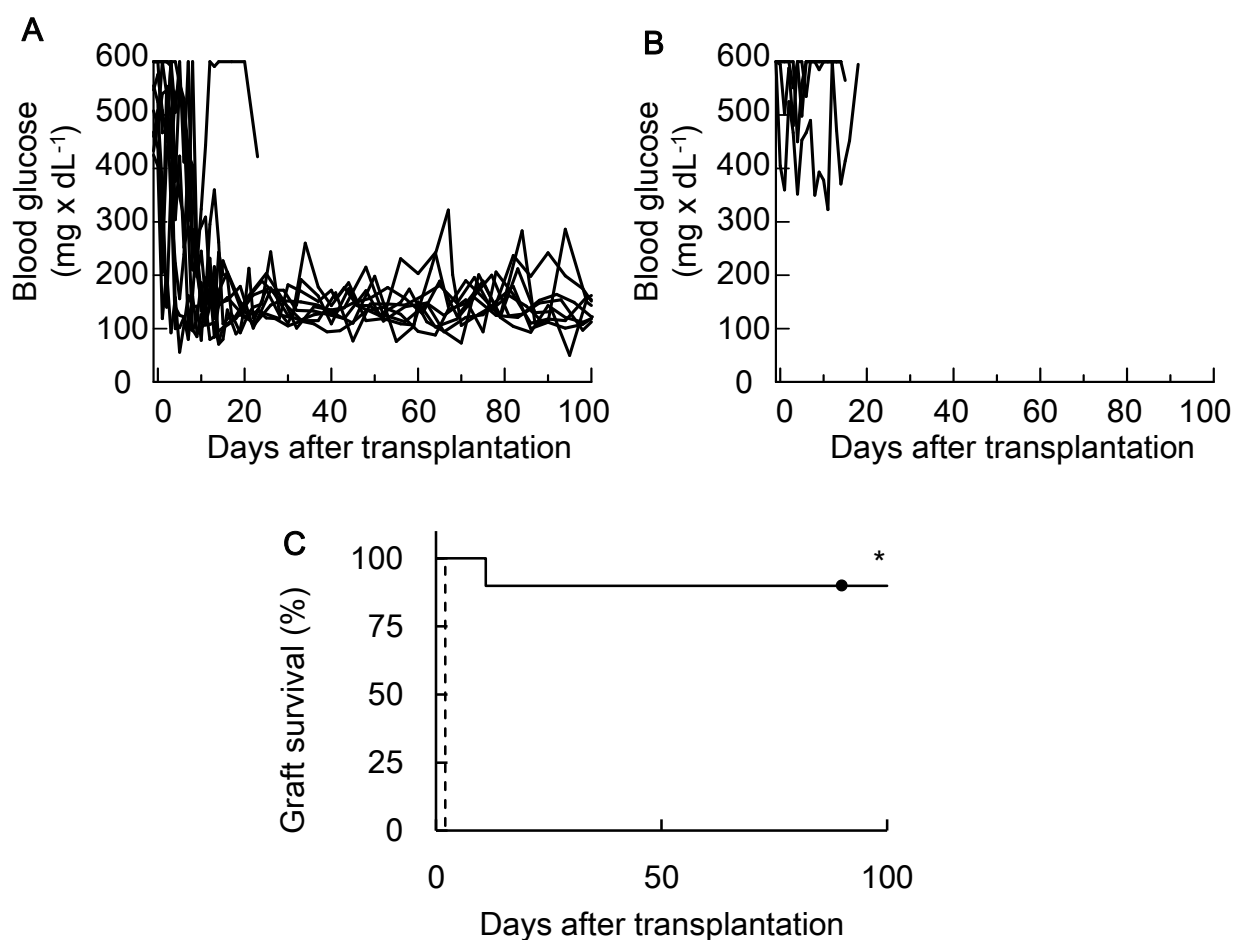


Figure 3. Normalization of blood glucose levels by transplantation of islets into subcutaneous bFGF-pockets of STZ-ACI rats without immunosuppressive drug treatment before or after islet transplantation. (A) Blood glucose levels in STZ-ACI rats after transplantation of 1500 F344 islets into each of two bFGF-pockets (n=10). (B) Blood glucose levels in STZ-ACI rats after transplantation of 1500 F344 islets into each of two dorsal untreated subcutaneous sites (n=4). (C) Kaplan-Meier plots of graft survival for ACI recipients. The solid line indicates islets transplanted into subcutaneous bFGF-pockets of STZ-ACI rats. Closed circle indicates the sacrifice of a rat on day 90 for histological analyses. The dotted line islets transplanted into untreated subcutaneous sites in STZ-ACI rats. *P = 0.0003.

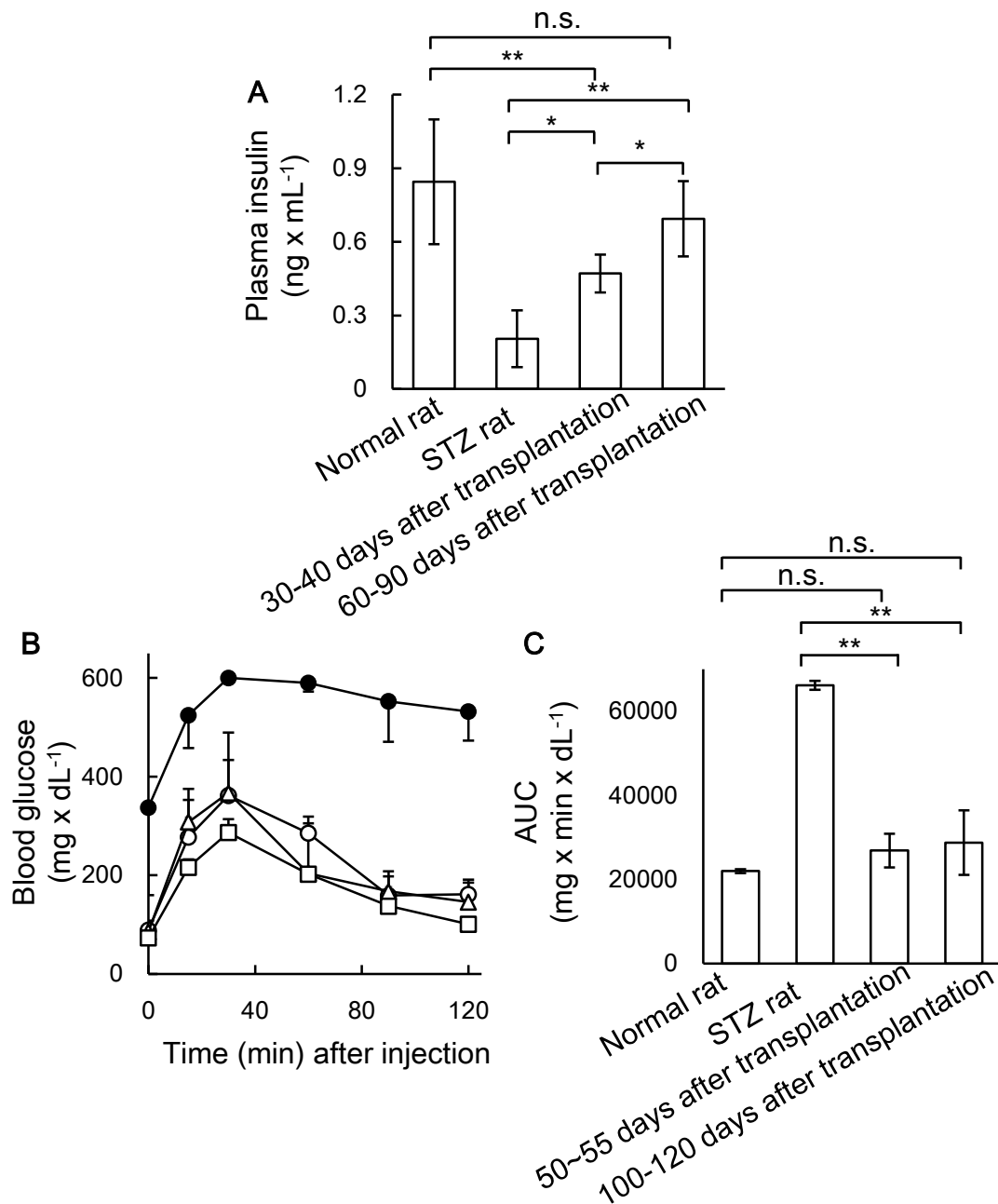


Figure 4. Functions of islet grafts. (A) Plasma insulin levels in normal ACI rats ($n=3$), STZ-ACI rats ($n=3$), and STZ-ACI rats with 3000 F344 islets transplanted into bFGF-pockets ~30-40 days ($n=6$) and ~60-90 days ($n=6$) after transplantation. (B) The intraperitoneal glucose tolerance tests (IPGTTs) were conducted in normal ACI rats (open squares, $n=3$), STZ-ACI rats (closed circles, $n=3$), and STZ-ACI rats with 3000 F344 islets in bFGF-spaces ~50-55 days (open triangles, $n=3$) or ~100-120 days (open circles, $n=3$) after islet transplantation. (C) Areas under the curves (AUCs) for the IPGTTs. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

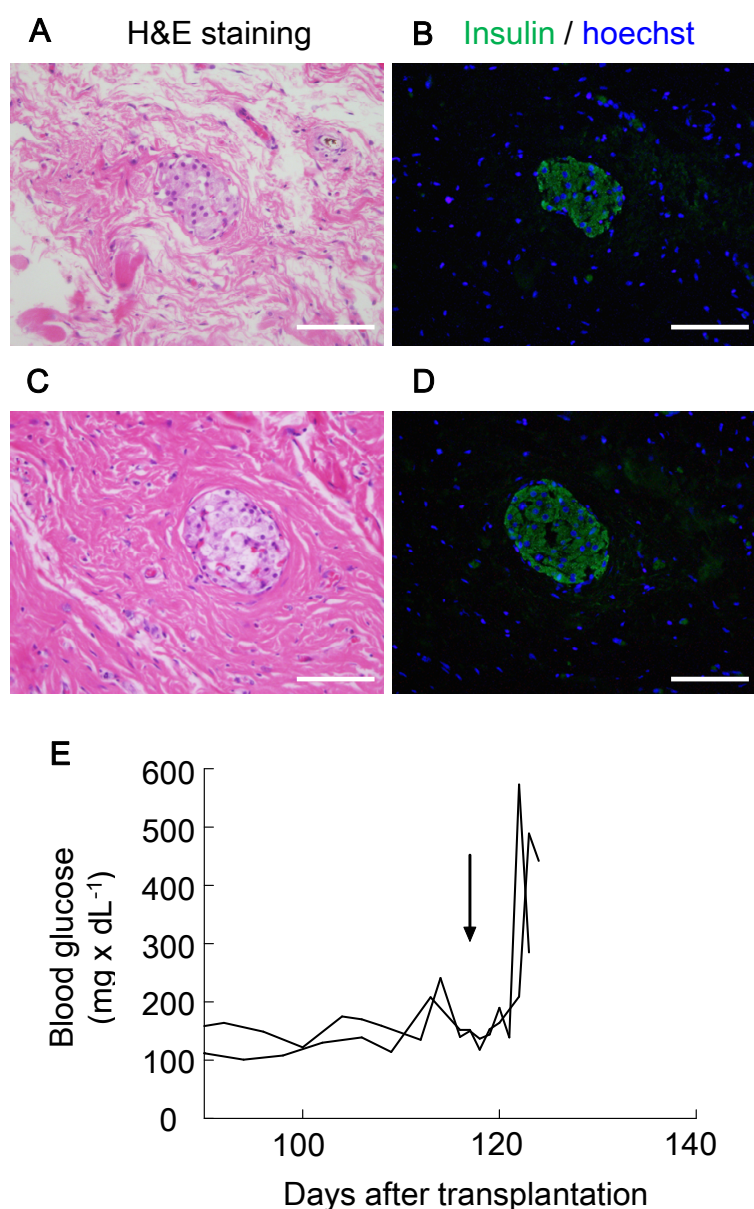


Figure 5. Morphologies of islet grafts and rejection of established subcutaneous allogeneic islet grafts caused by infusing splenocytes from islet donors into the peritoneal cavities of recipient rats. (A) H&E staining of islet graft retrieved from STZ-ACI rats 30 days after transplantation. (B) Immunofluorescent staining of insulin (green) in an islet graft retrieved from STZ-ACI rats 30 days after transplantation. (C) H&E staining of F344 islets retrieved from STZ-ACI rats 60 days after transplantation. (D) Immunofluorescent staining of insulin (green) in an islet graft retrieved 60 days after transplantation. Scale bars = 100 μ m. (E) Blood glucose levels before and after 10^7 splenocytes from donor rats (F344) were injected (arrows) into the corresponding STZ-ACI recipient rats 117 days after transplantation (n=2).

Discussion

A way to transplant islets have been studied that achieves minimum surgical invasion and prevents rejection of the islet grafts without using an immunosuppressant. In diabetic rodent allogeneic transplantation models, a subcutaneous pocket surrounded by vascular-rich tissue has been created by embedding an agarose gel carrying bFGF in chapter 1 or cyclic peptide SEK-1005 in chapter 2 subcutaneously. Allogeneic islets are then transplanted into the pocket and have been shown to survive without rejection or treatment with immunosuppressive agent. These islets normalize the blood glucose levels over a long period of time. However, there are still many problems that need to be solved for this method to be applicable in human patients.

Agarose is a polysaccharide isolated from seaweed and has traditionally been used as an edible. Therefore, toxicity is not expected to be a problem, but it has not yet been applied in human patients as a material for implantation into the body. In addition, agarose is not degraded and absorbed in the body, which is a disadvantage, as it needs to be removed from the body when it is no longer needed. In this chapter, a bFGF-carrying device without these drawbacks was developed.

The desired property of a bFGF-carrier complex is the prevention of bFGF flowing off in a short period of time when the bFGF-carrier complex is placed under the skin. It remains where it is placed for several days and gradually releases bFGF. Subcutaneous pockets surrounded by vascular-rich tissue into which islets can be transplanted are desirable, and the materials used to make the carrier should be absorbed and eliminated in the body. The gelatin sheet used in this chapter was used to prevent tissue adhesion and could be expected to form a subcutaneous pocket. The sodium hyaluronate-bFGF mixture

has been used to accelerate the formation of blood vessels and repair of tissues in the body [10]. By combining these two, a subcutaneous islet transplant site could be created. The sodium hyaluronate-bFGF mixture + gelatin sheet is a device that meets the above requirements.

When allogeneic pancreatic islets are transplanted into the subcutaneous pocket prepared by the sodium hyaluronate-bFGF mixture + gelatin sheet in diabetic rats, they are engrafted long-term without being rejected, normalizing the blood glucose levels. The results are almost equivalent to those of bFGF-agarose in chapter 1. When agarose was used, it was necessary to surgically remove the agarose rod at the time of islet transplantation. However, in the case of gelatin sheets, removal is not necessary because it is absorbed in vivo and a suspension of pancreatic islets injected.

Though a subcutaneous site is a promising site for islet transplantation [8],[11],[12], further studies are needed to determine whether the generated site is appropriate for clinical application. Notably, immunological reactions to grafts are highly species-dependent and skin structure differs between humans and most animals. Thus, islet transplantation using larger animal models, such as pigs and macaque monkeys, is needed in future studies.

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Summary

Chapter 1

Allogeneic islet transplantations were performed with two donor-recipient combinations. One combination was F344 rat donors (RT-1^{lv1}) and ACI rat recipients (RT-1^a, low responders); the other combination was Wister rat donors and Lewis rat recipients (RT-1^l, high responders).

Allogeneic islets were transplanted into subcutaneous spaces surrounded by highly vascularized granulomatous tissue. These spaces were formed by implanting agarose rods carrying basic fibroblast growth factor (bFGF; 50 µg bFGF/rod) into streptozotocin-induced diabetic rats for 7 days. No immunosuppressive medication was given before or after islet transplantation. In both donor-recipient combinations, the allogeneic islet grafts showed long-term survival (>100 days) in all recipients, evidenced by normalized non-fasting blood glucose levels.

The mechanisms underlying graft acceptance were investigated with flow cytometry and gene expression analyses. The agarose-bFGF rod caused formation of granulomatous tissue that contained regulatory T (Treg) cells. Treg cells suppressed immune reactions against the allogeneic islet grafts. These results indicated that this method of creating a pre-vascularized subcutaneous space with an agarose-bFGF rod may be useful for achieving successful allogeneic islet transplantation without a need for immunosuppression.

Chapter 2

There exists a need for a minimally invasive method of islet transplantation

without immunosuppressive drugs for the treatment of type 1 diabetes. In diabetic ACI rats, an agarose rod containing the cyclic oligopeptide SEK-1005 (agarose-SEK rod) was implanted at two dorsal subcutaneous sites. Then these rods were removed, and 1500 islets isolated from F344 rats were transplanted into each of the pockets.

Ten days after implantation of agarose-SEK rods, vascularized pockets were present. Non-fasting blood glucose levels confirmed long-term survival of the allogeneic islet grafts, without immunosuppressive therapy, in 8 of 10 recipients.

Flow cytometry and gene expression analyses were performed to investigate the mechanisms underlying graft acceptance. Agarose-SEK rod implantation led to the formation of granulomatous tissue containing regulatory T cells that suppressed immune reactions against the allogeneic islet grafts.

These results indicate that the use of an agarose-SEK rod to pre-vascularize a subcutaneous site may be a useful method for achieving successful allogeneic islet transplantation without immunosuppression.

Chapter 3

A great deal of effort has been devoted to developing a minimally invasive method for transplanting islets of Langerhans and to maintaining the islet grafts for a long period without immunosuppressive medication in order to treat type 1 diabetes.

An agarose rod containing basic fibroblast growth factor (bFGF) and heparin (bFGF-heparin) was implanted for 8 days into two subcutaneous sites in the groins of BALB/c mice to establish subcutaneous pockets. Diabetes was induced in the BALB/c mice by injecting streptozotocin 4 days after implantation. After the rods were removed, 150 islets that were isolated from C57BL/6 mice were transplanted into each of the two

pockets, total 300 islets/mouse.

Implantation of the agarose rods with bFGF-heparin for 8 days resulted in the formation of pockets that were surrounded by highly vascularized granulomatous tissue. Transplantation of C57BL/6 islets into the pockets normalized the blood glucose levels of diabetic BALB/c mice, and 8 of 10 recipient mice demonstrated >100 days of normoglycemia without immunosuppressive treatment. While, blood glucose levels of diabetic BALB/c mice in the control group, in which agarose rods without bFGF or heparin were used to prepare subcutaneous pockets, were transiently decreased by transplantation of C57BL/6 islets and reverted to over 500 mg/dl during 20 days observation.

In conclusion, the method to realize long term functioning of allogeneic islet grafts in the mouse model without immunosuppressive treatment were established. The author obtained a means, the mouse model, to analyze the immune phenomena in acceptance allogeneic islet graft in subcutaneous site.

Chapter 4

Small invasive transplantation of islets and long maintenance of the islet graft without immunosuppression has been studied for the treatment of type 1 diabetes. Clinically, subcutaneous pockets surrounding vascular-rich tissue are prepared for islet transplantation using a device made of the materials.

Here, gelatin sheets were implanted into two dorsal subcutaneous sites in diabetic ACI rats, and a mixture of bFGF and sodium hyaluronate solution was injected onto the gelatin sheets.

A total of 1500 islets isolated from F344 rats were transplanted into each of the pockets 7 days after injection of the bFGF mixture. Nine of 10 diabetic ACI rats with allogeneic islet graft demonstrated long-term normoglycemia without administration of immunosuppressant. Gelatin sheets almost disappeared 67 days after implantation.

Thus, subcutaneous immune-tolerant sites can be prepared using gelatin sheets and a sodium hyaluronate-bFGF mixture. Allogeneic islets transplanted into the sites can survive and control blood glucose levels for a long period, even without immunosuppression.

List of Publications

Chapter 1:

Kuwabara R, Hamaguchi M, Fukuda, Sakaguchi S, Iwata H. Long term functioning of islet allografts in subcutaneous space formed with bFGF in rat models. **Tissue Engineering: Part A**, *submitted*.

Chapter 2:

Kuwabara R, Hamaguchi M, Fukuda T, Sakai H, Inui M, Sakaguchi S, Iwata H (2018). Long-term Functioning of Allogeneic Islets in Subcutaneous Tissue Pretreated with a Novel Cyclic Peptide without Immunosuppressive Medication. **Transplantation**, 102(3):417–425.

Chapter 3:

Nishiyama T, Nishizawa Y, Kuwabara R, Iwata H. Long-term Subcutaneous Allogeneic Islet Graft Survival in a Mouse Model Without Immunosuppressive Treatment. **Islets**, *submitted*.

Chapter 4:

Kuwabara R, Iwata H. Bioabsorbable Device to Prepare Subcutaneous Pockets for Islet Transplantation, **Journal of Biomedical Materials Research: Part B - Applied Biomaterials**, *revision*.

Acknowledgements

The present research was carried out under the continuous guidance of Prof. Kazunari Akiyoshi, Graduate School of Engineering, Kyoto University. The author is very grateful to Prof. Akiyoshi for his patience, guidance, and helpful suggestions.

The author is also deeply indebted to Prof. Hiroo Iwata, Compass to Healthy Life Research Complex Program, RIKEN for his elaborated guidance, valuable discussion, detailed criticism, and encouragement for the completion of the present work.

The author would like to express the great thanks to Prof. Shimon Sakaguchi, Immunology Frontier Research Center, Osaka University, Dr. Masahide Hamaguchi, Dr. Takuya Fukuda, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Prof. Makoto Inui, Dr. Hiroki Sakai, Graduate School of Medicine, Yamaguchi University, for their valuable cooperation and useful suggestions.

The author would like to thank Dr. Yusuke Arima, Ms. Yoshiko Suzuki, Mr. Takaharu Nishiyama, Mr. Yuma Nishizawa, and other members in the laboratory of Prof. Iwata, Institute for Frontier Life and Medical Sciences, Kyoto University, for their kind help.

The author is very grateful to Japan Society for the Promotion of Science for making the present work possible by the financial support.

April, 2018

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