Title: Impact of Prevascularization on Immunological Environment and Early Engraftment in Subcutaneous Islet Transplantation

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K.I. performed the experiments, analyzed the data, and drafted the manuscript. T.A. obtained the grant, designed the study protocol, participated in the research, analyzed the data, and wrote the draft. N.F. designed the study protocol, participated in the research, analyzed the data, and wrote the draft. S.T., K.Y., N.E., A.I., H.S., H.F., and T.M. performed the experiments. K.N. drafted the manuscript. E.H. supervised the study and revised the manuscript.

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Abbreviations:

bFGF, basic fibroblast growth factors

CBA, cytometric bead array

ECM, extracellular matrix

FBS, fetal bovine serum

HE, hematoxylin and eosin

IPGTT, intraperitoneal glucose tolerance test

MPO, myeloperoxidase

PBS, phosphate-buffered saline

SPECT, single-photon emission computed tomography

STZ, streptozotocin

TNF, tumor necrosis factor

Abstract

Background: The utilization of islet-like cells derived from pluripotent stem cells may resolve the scarcity of islet transplantation donors. The subcutaneous space is a promising transplantation site because of its capacity for graft observation and removal, thereby ensuring safety. To guarantee subcutaneous islet transplantation, physicians should ensure ample blood supply. Numerous methodologies, including prevascularization, have been investigated to augment blood flow, but the optimal approach remains undetermined.

Methods: From C57BL/6 mice, 500 syngeneic islets were transplanted into the prevascularized subcutaneous site of recipient mice by implanting agarose rods with basic fibroblast growth factor at 1 and 2 weeks. Before transplantation, the blood glucose levels, cell infiltration, and cytokine levels at the transplant site were evaluated. Furthermore, we examined the impact of the extracellular matrix capsule on graft function and the inflammatory response.

Results: Compared with the 1-week group, the 2-week group exhibited improved glycemic control, indicating that longer prevascularization enhanced transplant success. Flow cytometry analysis detected immune cells, such as neutrophils and macrophages, in the extracellular matrix capsules, while cytometric bead array analysis indicated the release of inflammatory and proinflammatory cytokines. Treatment with anti-TNF and anti-IL-6R antibodies in the 1-week group improved graft survival, similar to the 2-week group.

Conclusions: In early prevascularization before subcutaneous transplantation, neutrophil and macrophage accumulation prevented early engraftment owing to inflammatory cytokine production.

INTRODUCTION

Cellular transplantation supplements or replaces lost organ and cell functions via minimally invasive methods.[1] Allogeneic pancreatic islet transplantation, the most typical cellular transplantation, is being pursued as a β-cell replacement therapy for type 1 diabetes mellitus with severe hypoglycemia unawareness.[2] Many countries recognize islet transplantation for its excellent therapeutic effect, that is, consistent glucose-responsive insulin secretion.[3,4] However, the necessary islet cells are difficult to secure because of donor shortage; the transplanted cells may also be damaged because of the activation of the complement and blood coagulation systems and innate immune response during intrahepatic portal vein transplantation,[5,6] which remains the gold standard for clinical islet transplantation.[7] Donor shortage might be solved by utilizing islet-like cells derived from embryonic stem cells/induced pluripotent stem cells. In this case, the subcutaneous region is a promising transplantation site because it enables graft observation and removal, thereby ensuring safety, and the transplantation of a large number of cells without adverse effects associated with intraportal transplantation using minimally invasive methods. Hence, subcutaneous transplantation has been selected for a clinical trial of stem cell–derived pancreatic endoderm cells conducted in recent years,[8] and further development is expected in the future.

Adequate blood flow is essential for successful islet engraftment[9]; however, blood supply in the subcutaneous space is limited. Various strategies to enhance blood flow have been explored, including the creation of a prevascularized site through the placement of a vascular access catheter,[10] use of scaffolds to promote

angiogenesis,[11,12] development of transferrable microvascular meshes,[13] incorporation of a device-free islet viability matrix,[14] use of macroencapsulation devices,[8] formation of vascularized β-cell spheroid tissue through a three-dimensional layer-by-layer cell-coating technique,[15] and delivery of exogenous growth factors via biomaterials.[16] Despite these approaches, the optimal method for subcutaneous transplantation remains undetermined.

Despite advances in subcutaneous implantation techniques, more transplanted islets are still needed to normalize blood glucose levels, and further advances in subcutaneous implantation methods are required. Currently, a clinically applicable method has not yet been established, and although prevascularization may be the most clinically applicable method, the mechanism for its success remains unclear. Subcutaneous transplantation and vascularization are very similar in terms of foreign body reactions. In the early stages of the reaction, foreign body reactions produce inflammatory cytokines. In the later stages, the inflammatory response subsides.[17] The effect of the subcutaneously induced reaction on the transplanted cells has not been sufficiently investigated. Devising new approaches by scientifically elucidating biological reactions elicited during implantation site creation or device implantation may be useful. This study aimed to evaluate the vascularization environment of the transplantation site before subcutaneous islet transplantation.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6–8 weeks old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and Japan SLC Inc. (Shizuoka, Japan). All experimental protocols conformed to the Institutional Animal Care protocols approved by Kyoto University (C21-63). The animals were housed under standard conditions and provided free access to food and water, except during fasting.

Pancreatic islet isolation

Pancreatic islets were isolated as previously described.[18] C57BL/6 donor mice were anesthetized by isoflurane inhalation and then secured by clamping the distal common bile duct. Hanks' balanced salt solution containing 0.15 mg/mL concentration of collagenase P (Roche Diagnostics, Indianapolis, IN, USA) was injected via cannulation of the common bile duct to distend the pancreas. Subsequently, the pancreas was excised and incubated at 37°C for 18 min. Next, we washed the dissociated tissue using Hank's balanced salt solution and purified it via discontinuous density gradient centrifugation (1.110, 1.103, 1.096, and 1.070 g/mL). Each islet layer was collected and washed by centrifugation. The isolated islets were cultured (37°C, 5% CO_2, and 95% air-humidified atmosphere) overnight before transplantation into RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific).

Diabetes mellitus induction and diagnosis in the recipients

Diabetes mellitus was induced by a single intravenous injection of streptozotocin (STZ, 150 mg/kg; Wako, Japan) 4–6 days before transplantation. Blood samples were collected via the tail vein and blood glucose levels were measured using an Accu-Chek blood glucose monitor (Roche Diagnostics K.K., Tokyo, Japan). Nonfasting blood glucose levels of ≥400 mg/dL on two consecutive measurements indicated diabetes.

Preparation of a basic fibroblast growth factor (bFGF) device

Briefly, in line with a previous study,[16] a rod-shaped agarose scaffold (4-mm diameter, 2.5-cm length) incorporating bFGF and heparin was prepared as follows. A 4.5% agarose solution was prepared by adding 450 mg of agarose (Seakem GTG agarose; Cambrex Bio Science Rockland, Inc., Rockland, ME) to 10 mL of double-distilled water, followed by autoclaving for dissolution and sterilization. The agarose solution was collected in a tube (inner diameter: 4 mm) and kept on ice to induce gelation. The agarose gel was then cut into 1.7 cm-long rods, frozen at −20°C overnight, and freeze-dried for 24 h under reduced pressure.

Islet transplantation and treatment protocol

We evenly dropped 50 μg of bFGF (Kaken Pharmaceutical Co., Tokyo, Japan) and 25 μg of heparin (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) onto the freeze-dried agarose rod. This device, which contained bFGF and heparin, was stored at 4°C overnight before use.

Recipient mice were divided into three groups: control, 1-week implantation (1w), and 2-week implantation (2w). In the control group, a total of 500 islets from C57BL/6 mice were divided into 250 islets each and transplanted into the left and right dorsal sites of recipient C57BL/6 mice. In the 1w and 2w groups, 1 and 2 weeks before transplantation, respectively, an agarose rod treated with bFGF and heparin was implanted into each of the recipient mice's two dorsal subcutaneous sites to induce neovascularization. After removing the agarose rods, we transplanted a total of 500 islets from C57BL/6 mice, split between the left and right neovascularized dorsal pockets of recipient mice with STZ-induced diabetes. After transplantation, nonfasting blood glucose levels were monitored thrice per week until euglycemia was achieved. Blood glucose levels below 200 mg/dL for two consecutive days indicated euglycemia.

Intraperitoneal glucose tolerance test (IPGTT)

Three months after transplantation, the recipient mice underwent IPGTT to evaluate islet graft function. Naïve nondiabetic mice were used as controls. After 14 h of fasting, the mice were intraperitoneally injected with a glucose solution (2 g glucose/kg body weight). Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min after glucose loading.

Histological study

Before transplantation, the skin and subcutaneous tissue around the agarose rod were

dissected, fixed in 10% formalin neutral buffer solution, embedded in paraffin, and stained with hematoxylin and eosin (HE) according to conventional protocols. Sections were incubated overnight at 4°C with anti-CD31 antibodies (1:100 dilution, clone: D8V9E; Cell Signaling Technology), followed by incubation for 40 min with a biotinylated secondary antibody diluted in phosphate-buffered saline (PBS) at a 1:300 ratio. The cells were then washed with PBS. For 50 min, avidin–biotin–peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, CA, USA) was diluted in bovine serum albumin at 1:100. After washing with PBS, we conducted a coloring reaction using diaminobenzidine and counterstained the nuclei with hematoxylin. Islets transplanted into the extracellular matrix (ECM) capsules were examined for morphology through staining with HE as well as immunohistochemistry using insulin antibody or glucagon antibody (Cell Signal Technology).

Islet graft evaluation using the ^{111}In-exendin-4 probe

We also synthesized ^{111}Indium-labeled exendin-4 derivative, {[Lys12(111In-BnDTPA-Ahx)]exendin-4} (^{111}In-exendin-4), as previously described.[19,20] We injected ^{111}In-exendin-4 (3.0 MBq/mouse) via the tail vein and performed single-photon emission computed tomography/computed tomography (SPECT/CT) scanning using Triumph Lab PET12/SPECT4/CT (TriFoil Imaging Inc., Chatsworth, CA, USA), as previously described.[19,20] The cumulative sum of SPECT signals was analyzed using Amira software, version 5.6.0 (FEI Visualization Sciences Group, Düsseldorf, Germany).

Moreover, the transplanted islet graft signals were determined by comparing them with the outline of the subcutaneous regions on CT images.

Flow cytometry

Before transplantation, we analyzed the cells in the capsules around the agarose rods. After removing the agarose rods, the capsules were carefully excised, cut, and minced into small fragments. Subsequently, these fragments were passed through a 100-μm nylon mesh using a sterile syringe plunger and then centrifuged for 5 min at 1500 rpm at 4°C. The red blood cell lysis buffer Hybri-Max™ (Sigma-Aldrich) was mixed with the resulting suspension. After washing with FBS Stain Buffer (FACS Buffer, BD Biosciences), cells from which red blood cells were removed were first blocked with anti-mouse CD16/32 antibody (clone:93, BioLegend) for 20 min at 4°C, followed by staining with LIVE/DEAD®Fixable Dead Cell Stains (Thermo Fisher Scientific) to discriminate dead cells and then stained with antibodies for surface markers, namely CD11b (clone: M1/70, BV510, BioLegend), Ly6g (clone:1A8, PE, BioLegend), Ly6C (clone: AL-21, BD Biosciences), F4/80 (clone: BM8, PE-Cyanine7, Invitrogen), CD86 (clone: GL-1, BV421, BioLegend), and CD206 (clone: MR5D3, AF647, BD) for 20 min at 4°C. After washing with FACS Buffer, the cells were resuspended and run on a BD FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo Software (Tree Star).

Cytometric bead array (CBA)

Inflammatory and proinflammatory cytokines in the capsules of agarose rods and plasma were analyzed using CBA according to the manufacturer's protocol. As previously mentioned, these capsules were carefully excised and cut into small fragments before transplantation. Proteins from these fragments were extracted using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The supernatant of the protein extraction was collected and stored at −80°C before use. The levels of interleukin (IL)-6, IL-10, monocyte chemotactic protein (MCP) 1, tumor necrosis factor (TNF), and IL-12p70 were measured using FACS BD Accuri™ C6 Plus (BD Biosciences) and analyzed using FCAP Array™ software (BD Biosciences).

Islet transplantation with anti-TNF antibody and anti-IL-6 receptor antibody

In addition to the three groups described above, transplantation was performed using anti-cytokine therapy. In one group consisting of diabetic mice, islets were transplanted with an anti-TNF antibody (etanercept; Pfizer Inc., USA). Etanercept (5 mg/kg) was intraperitoneally administered to recipient mice on days 0, 3, 7, and 10 to mimic the current clinical dosing schedule introduced by Hering et al.[21] In the other group, pancreatic islets were transplanted with the anti-IL-6 receptor antibody MR16-1 (kindly provided by Dr. Tadamitsu Kishimoto and Chugai Pharmaceutical Co., Ltd.). MR16-1 (500 μg) was administered to recipient mice with diabetes intraperitoneally on day 0 as

previously described.[22]

Statistical analysis

Quantitative data are expressed as mean ± standard deviation. Statistical data were analyzed using Student's *t* test or Welch's *t* test. Graft survival was compared between the different experimental groups using the Kaplan–Meier method. Statistical significance was set at $P < 0.05$. All statistical calculations were performed using JMP Pro 14.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Assessment of macroscopic and microscopic findings of subcutaneous space implanted with agarose rods with bFGF

We initially assessed the effects of angiogenesis on the agarose device absorbed with bFGF in the dorsal subcutaneous space of wild-type mice with STZ-induced diabetes. Figure 1 illustrates the macroscopic and microscopic images of untreated mice as the intact control group and mice at 2 weeks after implantation of the agarose device.

As shown in Figure 1A, the subcutaneous space of an intact mouse had few conspicuous blood vessels. Conversely, in the dorsal subcutaneous space of mice implanted with an agarose device, a neovascular network spreading into the subcutaneous space and a capsule-like structure ("ECM capsule") surrounding the

agarose device were observed (Figure 1B, 1C).

Histological analysis revealed CD31-positive blood vessels around the ECM capsule in both groups indwelled with agarose for 1 and 2 weeks (1w and 2w groups, respectively), but in the 2w group, these blood vessels infiltrated into the ECM capsule. The 1w group had more myeloperoxidase (MPO)-positive neutrophils accumulating in the agarose-related area of the ECM capsule than the 2w group. This group also contained more MPO-positive neutrophils outside the ECM capsule. Both groups contained F4/80-positive macrophages.

Transplant results after prevascularization using agarose rods in both groups

Next, we examined the effect of ECM capsule on blood glucose level normalization during subcutaneous islet transplantation (Figure 2A). Islets transplanted into an untreated subcutaneous space did not contribute to euglycemia; however, those transplanted into the ECM capsule eventually resulted in decreased blood glucose levels and euglycemia. On day 60 after islet transplantation, euglycemia occurred in 66.7% of the recipients in the 1w group (four out of six mice) and 83.3% of those in the 2w group (five out of six mice). The number of days required to achieve euglycemia after transplantation was 40.8 days for the 1w group and 12 days for the 2w group; thus, the 2w group had a significantly shorter time required for glycemic normalization (Figure 2B, 2C). After transplanting islets into the ECM capsule containing agarose for a 2-week period, blood glucose levels remained stable for >90 days. Notably, upon graft removal,

an immediate increase in blood glucose levels was observed (Figure 2D). Immunostaining of the retrieved grafts revealed the presence of insulin- and glucagon-positive cells (Figure 2E), confirming graft-dependent euglycemia.

Assessment for graft function

Islet graft function in control and recipient mice was evaluated using IPGTT 60 days after islet transplantation into the subcutaneous ECM capsule. After subcutaneous islet transplantation, the blood glucose curves of the 1w and 2w groups showed IPGTT profiles similar to those of the control group (Figure 3A), and the areas under the curves showed no significant differences between the control and subcutaneous groups (Figure 3B, 3 mice/group). Therefore, the graft preserved glucose responsiveness and insulin secretory capacity in the subcutaneous space.

Noninvasive imaging of pancreatic β-cells transplanted into the subcutaneous space

Figure 4 shows typical SPECT/CT fusion images of mice treated with ^{111}In-exendin-4 to detect pancreatic β-cells. Notably, radiopharmaceutical accumulation was observed in the pancreas of normal mice (Figure 4A) but not in the pancreas of mice with normalized blood glucose levels at 100 days after islet transplantation into the subcutaneous space following STZ-induced diabetes (Figure 4B). The ECM capsule encompassing the transplanted islets was removed according to the SPECT/CT fusion

image, leading to an increase in blood glucose levels (data not shown). One week after transplant site removal, we attempted to visualize pancreatic β-cells via SPECT/CT with ^{111}In-exendin-4 and found no radiopharmaceuticals in the subcutaneous space (Figure 4C).

Inflammatory cell and cytokines in the capsules around the agarose rods

The transplant site environment was examined to determine factors that might influence transplant outcomes. To analyze the immune response caused by the agarose rod absorbed with bFGF in the subcutaneous space, we collected the ECM capsules of the 1w and 2w groups and quantified the types of inflammatory cells infiltrating the ECM capsules through flow cytometric analysis and the inflammatory cytokines through CBA.

We analyzed the neutrophils and macrophages involved in the innate immune system using the panel design shown in Figure 5A. The 2w group had decreased neutrophil levels (similar to the histological analysis, Figure 5B) and increased macrophage levels within the ECM capsule (Figure 1D) compared with those in the 1w group. Subsequently, we analyzed the subsets of macrophages—M1 and M2. M1 macrophages produce proinflammatory cytokines and possibly mediate secondary inflammatory responses, cytotoxicity, and tissue damage, whereas M2 macrophages produce cytokines and growth factors that promote anti-inflammatory, immune suppressive responses, and tissue healing in the host. The numbers of M1 and M2 macrophages increased in the 2w group compared with those in the 1w group (Figure

5C), although the difference was not significant.

Next, we quantified six inflammatory cytokines in the ECM capsules using CBA (Figure 6, 6 mice/group). At both 1 and 2 weeks after implanting agarose rods with bFGF into the subcutaneous space to create the islet transplantation site, we found higher levels of four cytokines, except IL-12p70 and IL-10, in the plasma collected from the systemic blood sample than in the ECM capsules. One of them was MCP-1, a CC chemokine that has a strong mobilization effect on monocytes and macrophages. The concentration remained extremely high after 2 weeks, indicating continuous cell induction. Local TNF and IL-6 levels were higher in the 1w group than in the 2w group ($P < 0.05$). Differences in local cytokine levels may explain the differences in the number of days required to achieve euglycemia when isolated islets were transplanted into ECM capsules.

Anti-TNF antibody and anti-IL-6 receptor antibody

Locally high cytokine levels may directly or indirectly interfere with transplanted islets. We used anti-TNF and anti-IL-6 receptor antibody preparations to inhibit the effects of TNF and IL-6. Figure 7A shows the therapeutic schedule of the implants and medications before and after subcutaneous islet transplantation.

Regarding the blood glucose transition of the etanercept and MR16-1 groups (Figure 7B, six mice/group), 83.3% of each group showed euglycemia within 60 days after islet transplantation. The Kaplan–Meier curve indicated that euglycemia was significantly

faster in both groups than in the 1w group (1w vs. etanercept, P = 0.027; 1w vs. MR16-1, P = 0.026; log-rank test; Figure 7C), with 26.0 and 14.4 days required in the etanercept and MR16-1 groups, respectively, and 40.8 days in the 1w group (1w vs. etanercept, P = 0.0125; 1w vs. MR16-1, P = 0.0016; Figure 7D).

DISCUSSION

This study focused on the prevascularization technique, which reportedly enables long-term survival in drug-induced diabetic rats following subcutaneous transplantation of allogeneic islets without the need for immunosuppressive drugs.[16] We aimed to assess the outcomes of the transplantation and the local microenvironment at the transplantation site following prevascularization, prior to conducting subcutaneous islet transplantation. Compared with islet transplantation without prevascularization, islet transplantation into the prevascularized ECM capsule resulted in a marked decrease in blood glucose levels and a higher proportion of recipients achieving euglycemia. Notably, the 2w (prevascularization) group exhibited better transplant outcomes than the 1w group. Through a series of experiments, we analyzed the factors contributing to this discrepancy to elucidate the key success factors of subcutaneous islet transplantation. Intriguingly, the 2w group had fewer neutrophils but more macrophages in the ECM capsule than the 1w group. However, the levels of inflammatory cytokines, including TNF- and IL-6, were higher in the 1w group than in the 2w group. With the use of anti-TNF antibodies and anti-IL-6 receptor antibodies, the transplantation outcomes

significantly improved, even after just 1 week of prevascularization. These results strongly suggest that the inflammatory cytokines induced by prevascularization impede the functionality of transplanted islets. These novel findings highlight that controlling inflammation at the subcutaneous site is necessary for successful subcutaneous islet transplantation.

As reported in a previous study[23] and the present study, islet transplantation into a subcutaneous site without prevascularization cannot normalize blood glucose levels in recipients with diabetes. Various methods have been proposed to create subcutaneous implantation sites that allow hypoglycemia with transplanted islets. More recently, methods with a view toward clinical application have been reported. Pepper et al. described the subcutaneous transplantation of islets into a prevascularized site created by inserting biomedical-grade vascular access nylon catheters subcutaneously.[10] This simple method utilizes the foreign body reaction elicited by the catheter in place for 1 month to achieve angiogenesis and collagen growth and is similar in concept to our method. This catheter implantation led to local elevation of proinflammatory cytokines 1 week after implantation, suggesting that a marked inflammatory response favors rather than hinders neovascularization. Our results are in line with their assertion; however, they did not prove that the inflammatory response associated with neovascularization itself prevents islet engraftment and that blocking inflammatory cytokines promotes islet engraftment.

The mechanism by which the inflammatory and immunological responses elicited during vascular induction affect the immunological rejection of subsequently transplanted islets

is an interesting research topic. Our method using agarose with bFGF enabled allogeneic islet transplantation to be viable without the need for immunosuppressive drugs in three rat strain combinations.[16] Implantation of an agarose rod with bFGF increased the proportion of regulatory T (Treg) cells within CD4 cells at the subcutaneous site. This increase persisted even after allogeneic islet transplantation. Furthermore, bFGF-implanted agarose rods elevated chemotaxis-related gene expression levels and Treg cell generation at the subcutaneous site.[24] Although Tregs may be essential in circumventing immune rejection in subcutaneous islet transplantation. In our preliminary study, transplantation from BALB/c to C57BL/6 mice did not cause long-term engraftment without immunosuppressive drugs (data not shown). The establishment of local immune tolerance using this method remains to be studied; nonetheless, it is a promising approach. Yasunami et al. reported that C57BL/6 mice maintained euglycemia for more than 1 year after receiving BALB/c islet transplants without immunosuppressive drug therapy. This successful outcome was achieved when inguinal subcutaneous white adipose tissue (ISWAT) served as the transplantation site[25] and underwent bFGF pretreatment. In the ISWAT, mesenchymal stem cells exhibited a marked increase and played a key role in transforming growth factor-β production.[26] Given that immune responses vary based on the transplant site,[27] the immune response might differ depending on the specific subcutaneous tissue location or type of adipose tissue,[28] thereby broadening the potential applications of subcutaneous transplantation.

Macroscopic and microscopic analyses revealed the formation of a neovascular network

and an ECM capsule surrounding the agarose device. Histological examination confirmed the presence of CD31-positive blood vessels in the ECM capsule, with greater infiltration in the 2w group. Hence, a network of blood vessels was formed before transplantation, creating an environment that supplied oxygen and nutrients to the islets for transplantation. To elucidate the factors contributing to the time needed to achieve euglycemia after the implantation of bFGF-containing agarose rods for angiogenesis changes between 1 and 2 weeks following implantation, we need to understand how the foreign body response affects angiogenesis and the surrounding tissue. After foreign body placement, a provisional matrix forms immediately around the foreign body. Thereafter, acute inflammation occurs, consisting mostly of neutrophils with monocyte infiltration and differentiation. The subsequent chronic inflammatory phase is infiltrated by monocytes, which differentiate into macrophages, leading to neovascularization. Subsequently, fibroblasts proliferate and begin to produce ECM components such as collagen, forming a fibrous capsule.[13] In angiogenesis, an inflammatory response occurs, followed by monocyte infiltration, macrophage activation, and then angiogenesis at the tissue injury site.[29] Next, an initial angiogenesis by fibrin[30] and histamine is elicited,[31] followed by an initial angiogenic pulse caused by VEGF (released by platelets), maintained by hypoxic macrophages and hypoxic macrophages and fibroblasts.[32] In our study, MPO-positive neutrophils and F4/80-positive macrophages accumulated in the ECM capsule, reflecting a foreign body reaction. The successful engraftment of islets into the ECM capsule could be attributed to several factors. The neovascular network provided sufficient blood supply to support islet

survival and function. Considering the presence of macrophages and neutrophils in the ECM capsule, it may serve as a protective barrier against immune responses and inflammation. The use of subcutaneous foreign body reaction can promote not only angiogenesis but also collagen growth in the surrounding area, creating an environment conducive for the engraftment of transplanted cells.

One distinctive feature of our study was the utilization of SPECT/CT imaging with ^{111}In-exendin-4 for assessing islet grafts. To establish a causal relationship between the decline in the recipient's blood glucose levels and the action of the implanted graft, we must demonstrate graft viability during the period of reduced blood glucose levels. Currently, reliable techniques for detecting and evaluating the β-cell mass of islet grafts remain unavailable; thus, islet transplantation protocols cannot be optimized.[33] Our group previously demonstrated the efficacy of a probe called ^{111}In-exendin-4 in assessing the β-cell mass of islet grafts following intraportal islet transplantation.[13] By applying this approach to subcutaneous transplantation, we successfully detected islet grafts. ^{111}In-exendin-4 SPECT/CT imaging can help noninvasively visualize and evaluate subcutaneous islet grafts.

In the present study, the engraftment rate at 4 weeks following agarose implantation was not significantly different from that at 2 weeks after implantation (data not shown). The feasibility of transplanting fewer islets should be explored. However, we could not substantiate any such variance. Furthermore, we could not adequately assess the viability of islet transplantation at a less immunologically challenging site; thus, the optimal conditions should be further explored. Additionally, to examine the potential for

clinical application, it is necessary to evaluate the response in larger animal models and in humans, who exhibit distinct immune responses. In these studies, it is crucial to confirm the presence or absence of adverse events, such as bleeding, and to determine the optimal size of the prevascularized transplant site capable of ensuring adequate oxygen supply.

In conclusion, in the early stage of prevascularization before subcutaneous transplantation, neutrophil and macrophage accumulation prevented early engraftment owing to the production of inflammatory cytokines. These findings provide valuable insights into the optimization of subcutaneous islet transplantation and potential therapeutic strategies to enhance graft survival and function.

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References

1. Parsons RF, Baquerizo A, Kirchner VA, et al. Challenges, highlights, and opportunities in cellular transplantation: A white paper of the current landscape. *Am J Transplant*. 2021;21:3225-3238. doi:10.1111/ajt.16740

2. Anazawa T, Okajima H, Masui T, Uemoto S. Current state and future evolution of pancreatic islet transplantation. *Ann Gastroenterol Surg*. 2019;3:34-42. doi:10.1002/ags3.12214

3. Berney T, Andres A, Bellin MD, et al. A worldwide survey of activities and practices in clinical islet of langerhans transplantation. *Transpl Int*. 2022;35:10507. doi:10.3389/ti.2022.10507

4. Rickels MR, Robertson RP. Pancreatic islet transplantation in humans: Recent progress and future directions. *Endocr Rev*. 2019;40:631-668. doi:10.1210/er.2018-00154

5. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: Implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci*. 2000;105:125-133.

6. Matsuoka N, Itoh T, Watarai H, et al. High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice. *J Clin Invest*. 2010;120:735-743. doi:10.1172/JCI41360

7. Verhoeff K, Marfil-Garza BA, Sandha G, et al. Outcomes following extrahepatic and intraportal pancreatic islet transplantation: A comparative cohort study.

Transplantation. 2022;106:2224-2231. doi:10.1097/TP.0000000000004180

8. Shapiro AMJ, Thompson D, Donner TW, et al. Insulin expression and C-peptide in type 1 diabetes subjects implanted with stem cell-derived pancreatic endoderm cells in an encapsulation device. *Cell Rep Med*. 2021;2:100466. doi:10.1016/j.xcrm.2021.100466

9. Li F, Lv Y, Li X, Yang Z, Guo T, Zhang J. A prevascularized sinus tract on the liver surface for islet transplantation. *Transplantation*. 2023;107:117-128. doi:10.1097/TP.0000000000004236

10. Pepper AR, Gala-Lopez B, Pawlick R, Merani S, Kin T, Shapiro AM. A prevascularized subcutaneous device-less site for islet and cellular transplantation. *Nat Biotechnol*. 2015;33:518-523. doi:10.1038/nbt.3211

11. Smink AM, Li S, Hertsig DT, et al. The efficacy of a prevascularized, retrievable poly(D,L,-lactide-co-ε-caprolactone) subcutaneous scaffold as transplantation site for pancreatic islets. *Transplantation*. 2017;101:e112-e119. doi:10.1097/TP.0000000000001663

12. Patikova A, Vojtiskova A, Fabryova E, et al. The optimal maturation of subcutaneous pouch can improve pancreatic islets engraftment in rat model. *Transplantation*. 2022;106:531-542. doi:10.1097/TP.0000000000003844

13. Song W, Chiu A, Wang LH, et al. Engineering transferrable microvascular meshes for subcutaneous islet transplantation. *Nat Commun*. 2019;10:4602. doi:10.1038/s41467-019-12373-5

14. Yu M, Agarwal D, Korutla L, et al. Islet transplantation in the subcutaneous

space achieves long-term euglycaemia in preclinical models of type 1 diabetes. *Nat Metab*. 2020;2:1013-1020. doi:10.1038/s42255-020-0269-7

15. Takaichi S, Tomimaru Y, Akagi T, et al. Three-dimensional vascularized β-cell spheroid tissue derived from human induced pluripotent stem cells for subcutaneous islet transplantation in a mouse model of type 1 diabetes. *Transplantation*. 2022;106:48-59. doi:10.1097/TP.0000000000003745

16. Luan NM, Iwata H. Long-term allogeneic islet graft survival in prevascularized subcutaneous sites without immunosuppressive treatment. *Am J Transplant*. 2014;14:1533-1542. doi:10.1111/ajt.12739

17. Geelhoed WJ, Moroni L, Rotmans JI. Utilizing the foreign body response to grow tissue engineered blood vessels in vivo. *J Cardiovasc Transl Res*. 2017;10:167-179. doi:10.1007/s12265-017-9731-7

18. Tada S, Anazawa T, Shindo T, et al. The MEK inhibitor trametinib suppresses major histocompatibility antigen-mismatched rejection following pancreatic islet transplantation. *Transplant Direct*. 2020;6:e591. doi:10.1097/TXD.0000000000001045

19. Botagarova A, Murakami T, Fujimoto H, et al. Noninvasive quantitative evaluation of viable islet grafts using. *FASEB J*. 2023;37:e22859. doi:10.1096/fj.202201787RR

20. Murakami T, Fujimoto H, Fujita N, Hamamatsu K, Matsumoto K, Inagaki N. Noninvasive evaluation of GPR119 agonist effects on β-cell mass in diabetic male mice using 111In-Exendin-4 SPECT/CT. *Endocrinology*. 2019;160:2959-2968. doi:10.1210/en.2019-00556

21. Hering B, Kandaswamy R, Ansite J, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA*. 2005;293:830-835.

22. Itoh T, Nitta T, Nishinakamura H, et al. HMGB1-mediated early loss of transplanted islets is prevented by anti-IL-6R antibody in mice. *Pancreas*. 2015;44:166-171. doi:10.1097/MPA.0000000000000188

23. Kawakami Y, Iwata H, Gu Y, et al. Modified subcutaneous tissue with neovascularization is useful as the site for pancreatic islet transplantation. *Cell Transplant*. 2000;9:729-732. doi:10.1177/096368970000900523

24. Kuwabara R, Hamaguchi M, Fukuda T, Sakaguchi S, Iwata H. Preparation of Immunotolerant space under the skin and transplantation of islets in the space. *Tissue Eng Part A*. 2019;25:183-192. doi:10.1089/ten.TEA.2018.0109

25. Yasunami Y, Nakafusa Y, Nitta N, et al. A novel subcutaneous site of islet transplantation superior to the liver. *Transplantation*. 2018;102:945-952. doi:10.1097/TP.0000000000002162

26. Nakafusa Y, Nitta N, Ishii K, et al. Acceptance of murine islet allografts without immunosuppression in inguinal subcutaneous white adipose tissue pretreated with bFGF. *Diabetes*. 2022;71:1721-1734. doi:10.2337/db21-0684

27. Cantarelli E, Citro A, Pellegrini S, et al. Transplant site influences the immune response after islet transplantation: Bone marrow versus liver. *Transplantation*. 2017;101:1046-1055. doi:10.1097/TP.0000000000001462

28. Xu K, Xie R, Lin X, et al. Brown adipose tissue: A potential site for islet transplantation. *Transplantation*. 2020;104:2059-2064.

doi:10.1097/TP.0000000000003322

29. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol*. 2008;20:86-100. doi:10.1016/j.smim.2007.11.004

30. Bootle-Wilbraham CA, Tazzyman S, Thompson WD, Stirk CM, Lewis CE. Fibrin fragment E stimulates the proliferation, migration and differentiation of human microvascular endothelial cells in vitro. *Angiogenesis*. 2001;4:269-275. doi:10.1023/a:1016076121918

31. Ghosh AK, Hirasawa N, Ohtsu H, Watanabe T, Ohuchi K. Defective angiogenesis in the inflammatory granulation tissue in histidine decarboxylase-deficient mice but not in mast cell-deficient mice. *J Exp Med*. 2002;195:973-982. doi:10.1084/jem.20011782

32. Luttikhuizen DT, Harmsen MC, Van Luyn MJ. Cellular and molecular dynamics in the foreign body reaction. *Tissue Eng*. 2006;12:1955-1970. doi:10.1089/ten.2006.12.1955

33. Murakami T, Fujimoto H, Inagaki N. Non-invasive beta-cell imaging: Visualization, quantification, and beyond. *Front Endocrinol (Lausanne)*. 2021;12:714348. doi:10.3389/fendo.2021.714348

Figure Legends

Figure 1 Macroscopic and microscopic images of the subcutaneous space in each mouse group.

Macroscopic images of the subcutaneous space in the untreated group (control) (A), 1w group (B), and 2w group (C). Microscopic images of the subcutaneous space (D); the upper, middle, and lower rows denote the control, 1w, and 2w groups, respectively.

Figure 2 Effect of the extracellular matrix (ECM) capsule on subcutaneous islet transplantation

(A) Blood glucose transition of the control, 1w, and 2w groups after islet transplantation into the ECM capsules created in the subcutaneous space. (B) Euglycemia rate and (C) number of days it took to achieve euglycemia after subcutaneous islet transplantation. Each group had 6 mice. (D) Long-term blood glucose levels following islet transplantation into a transplant site prepared through a 2-week prevascularization. Blood glucose levels remained stable until the graft was retrieved (arrow). (E) Histological analysis of islets transplanted into ECM capsules formed in the subcutaneous space.

Figure 3 Intraperitoneal glucose tolerance tests (IPGTT) to evaluate graft effect

On day 60, IPGTT was performed in all groups 60 days after transplantation. Blood

glucose levels were measured and compared among the three groups (A). The area under the curve (AUC) was calculated (B).

Figure 4 Imaging of pancreatic β-cells transplanted into the subcutaneous space

(A) Control. (B) Image of an individual pancreatic islet transplanted into a subcutaneous pocket. (C) Re-imaging 1 week after graft removal. Endogenous pancreatic β-cells of control mice were detected, whereas the pancreatic β-cells of STZ-treated mice were not (confirming islet destruction). Radiopharmaceutical accumulation in kidneys and lungs is seen.

Figure 5 Analysis for inflammatory cells in the extracellular matrix (ECM) capsules around the agarose rods before islet transplantation

(A) Panel design for the analysis of inflammatory cells by flow cytometry. Live CD11 cells at low–high levels were selected from CD11b/LiveDead staining for dead cell removal, followed by Ly6g/Ly6c expansion. Ly6g-/Ly6c+ cells served as the monocyte subset, and Ly6g+/Ly6c+ cells as the neutrophil subset. The Ly6g-/Ly6c- cells were expanded with F4/80/CD11b; the F4/80+/ CD11b+ cells served as the macrophage subset. Furthermore, CD86+ cells were designed as the M1 macrophage subset, and CD206+ cells as the M2 macrophage subset. (B) Percentage of macrophages, monocytes, and neutrophils among live CD11+ cells. (C) Percentage of M1 and M2

macrophages among live CD11+ cells. (D) Ratio of M2/M1 macrophages calculated from the percentage (C). Data are expressed as the mean ± SD, with 5 mice in the 1w group and 6 in the 2w group, and were compared with cells of the same type in the 1w and 2w groups by using an unpaired Student's *t* test (*$P < 0.05$)

Figure 6 Analysis for inflammatory cytokines in the extracellular matrix (ECM) capsules around the agarose rods before transplantation

Inflammatory cytokine levels were measured using cytometric bead array (A) in plasma from systemic blood samples collected from the inferior aorta and (B) in tissue lysates from the ECM capsule before islet transplantation. Blood and ECM capsule samples were collected 1 or 2 weeks after agarose implantation.

Data are expressed as the mean ± SD, n = 6, and were compared with cells of the same type in the 1w and 2w groups using an unpaired Student's *t* test (*$P < 0.05$).

Figure 7 Effect of anticytokine administration on islet transplantation into the subcutaneous extracellular matrix (ECM) capsule

(A) Therapeutic schedule of the administration of anti-TNF preparation (etanercept) or anti-IL-6 preparation (MR16-1) in islet-transplanted mice. Recipient mice had an ECM pocket that was created in the dorsal subcutaneous space by implanting an agarose rod 1 week prior. The etanercept group was treated with etanercept at days 0, 3, 7, and 10.

The MR16-1 group was treated beginning at day 0. (B) Blood glucose transition, (C) percentage of euglycemia, and (D) number of days it took to achieve euglycemia after subcutaneous islet transplantation into the ECM capsule created for 1 week. Mice were administrated either etanercept or MR16-1, whereas the control group was not administered either (*$P < 0.05$).

Figures

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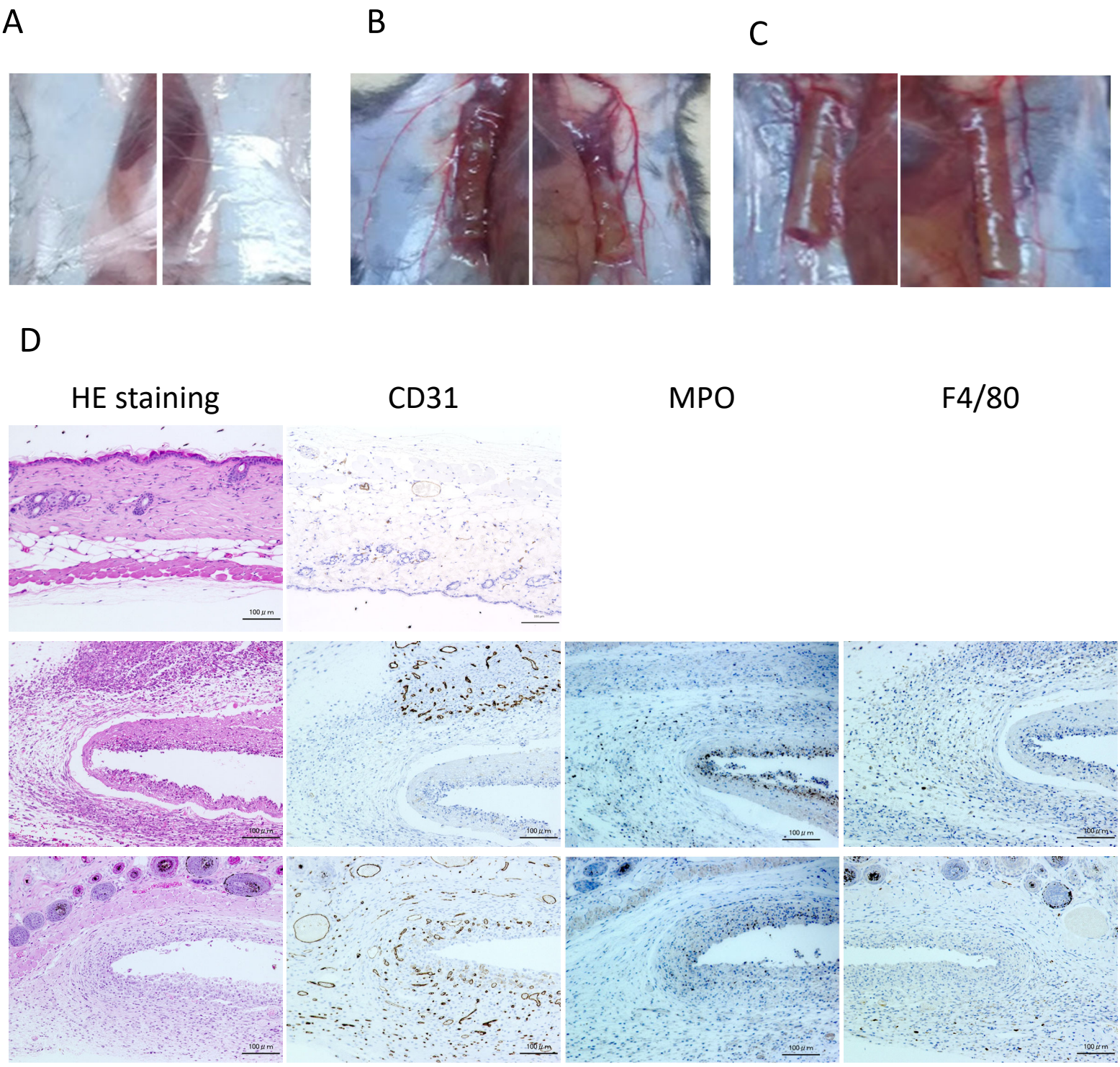


Fig. 1

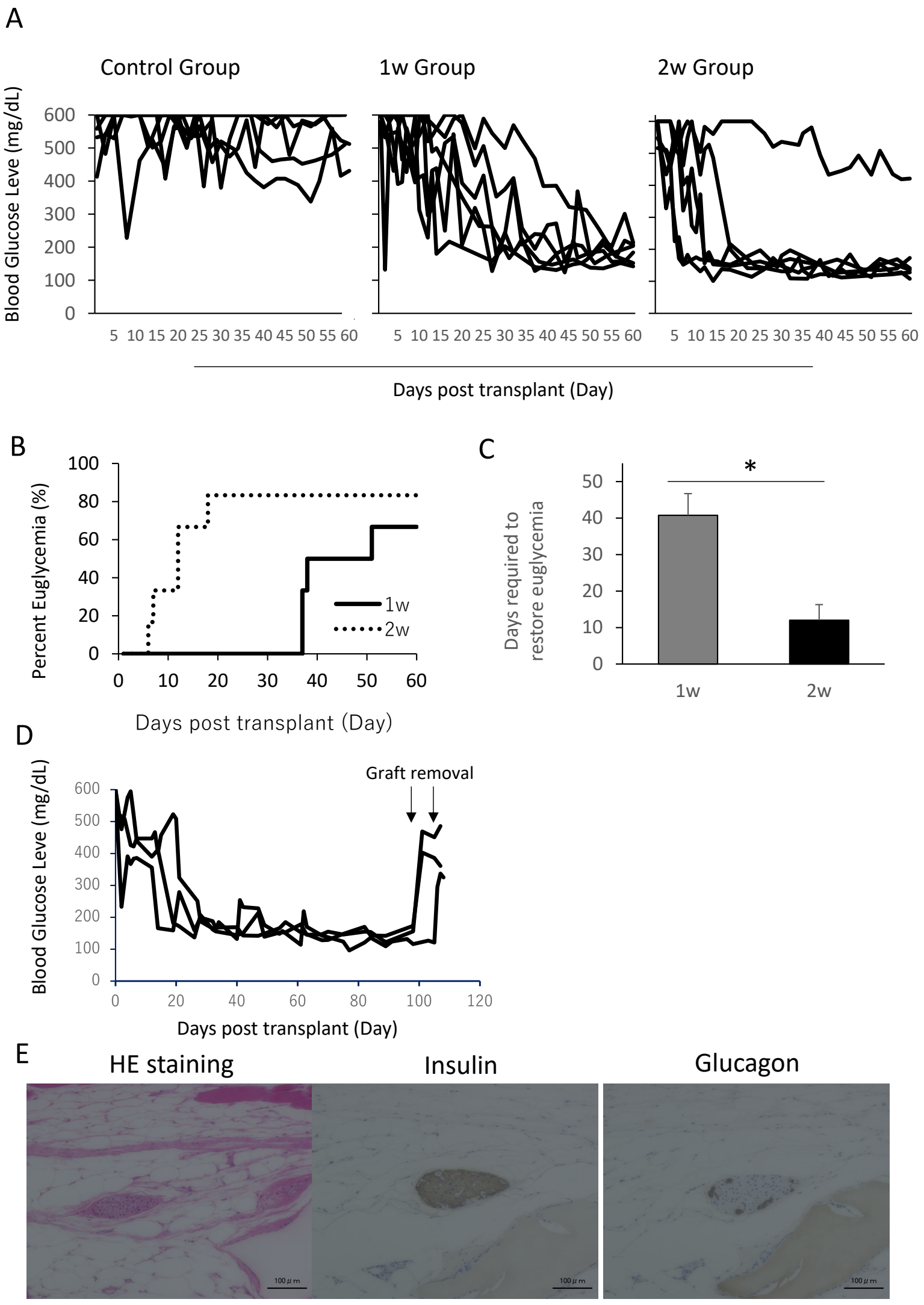
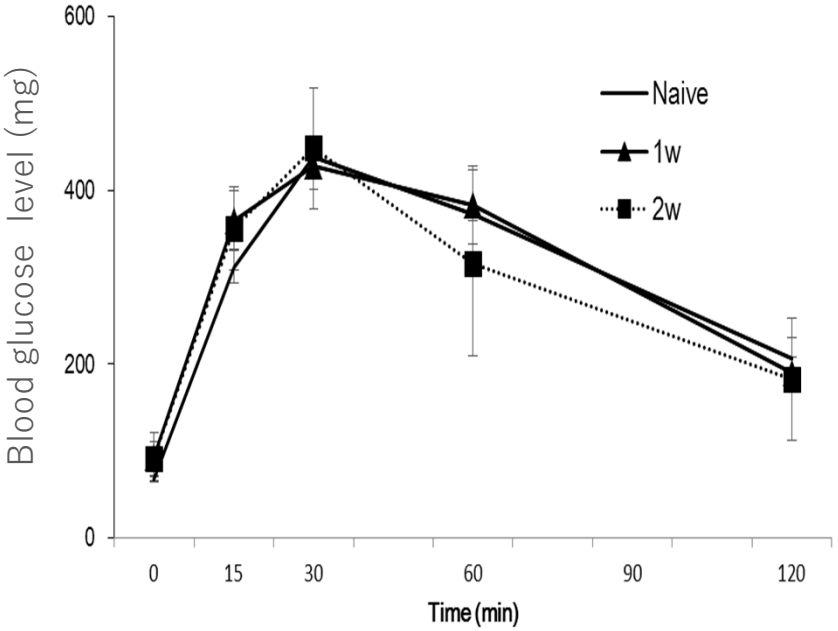

A
Control Group
1w Group
2w Group
Blood Glucose Leve (mg/dL)
600
500
400
300
200
100
0
5 10 15 20 25 30 35 40 45 50 55 60
Days post transplant (Day)
B
Percent Euglycemia (%)
100
80
60
40
20
0
1w
2w
0 10 20 30 40 50 60
Days post transplant (Day)
C
Days required to restore euglycemia
*
50
40
30
20
10
0
1w
2w
D
Blood Glucose Leve (mg/dL)
Graft removal
600
500
400
300
200
100
0
0 20 40 60 80 100 120
Days post transplant (Day)
E
HE staining
Insulin
Glucagon
100 μm
100 μm
100 μm

Fig. 2

A


600
400
200
0
Blood glucose level (mg)
Naive
1w
2w
0
15
30
60
90
120
Time (min)

B

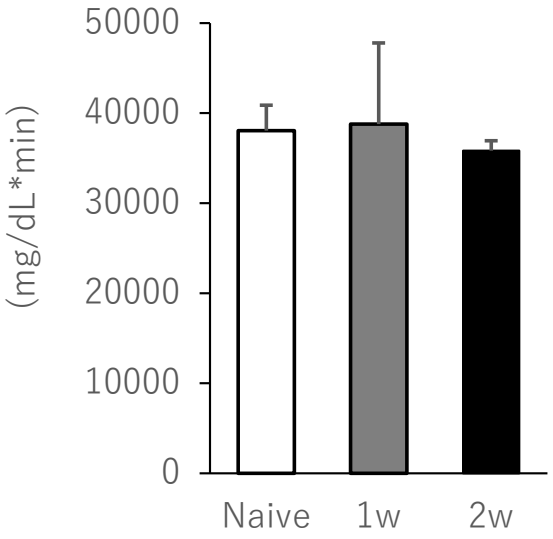
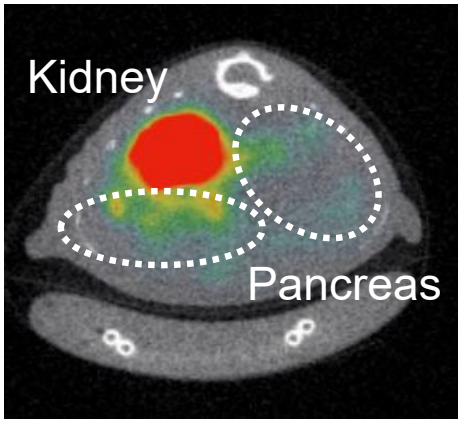

50000
40000
30000
20000
10000
0
(mg/dL*min)
Naive
1w
2w

Fig. 3

A

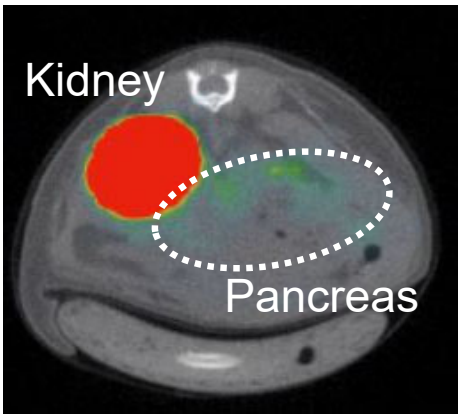
Abdomen Axial

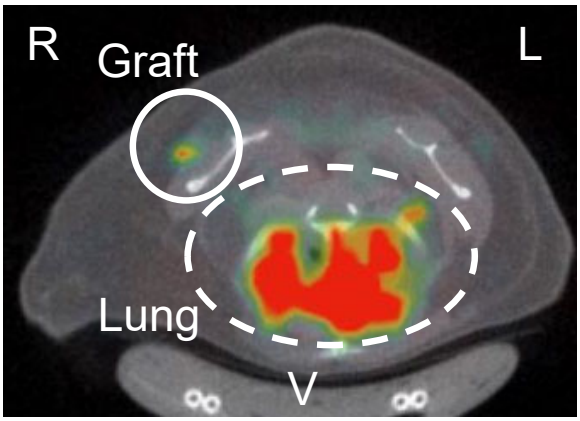


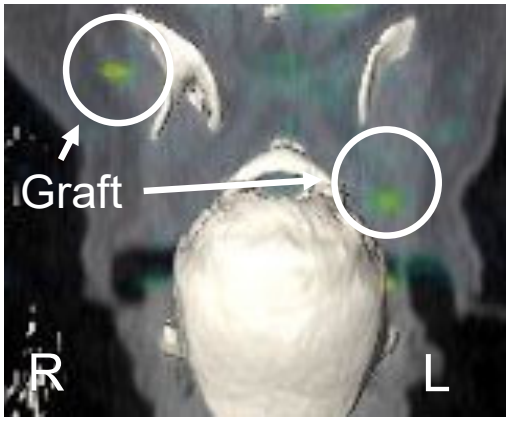
B

Abdomen Axial

Corona



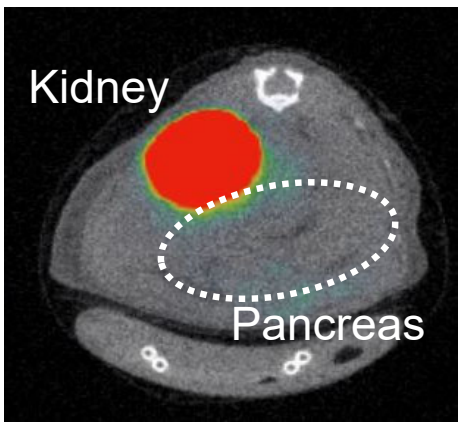


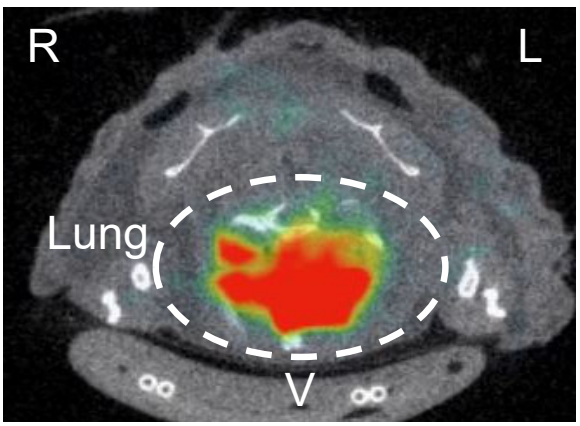


C

Abdomen Axial

Corona





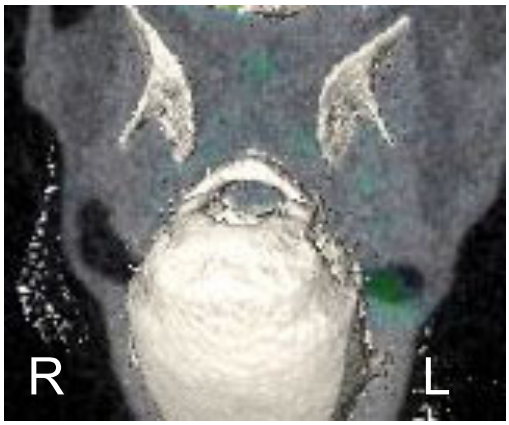
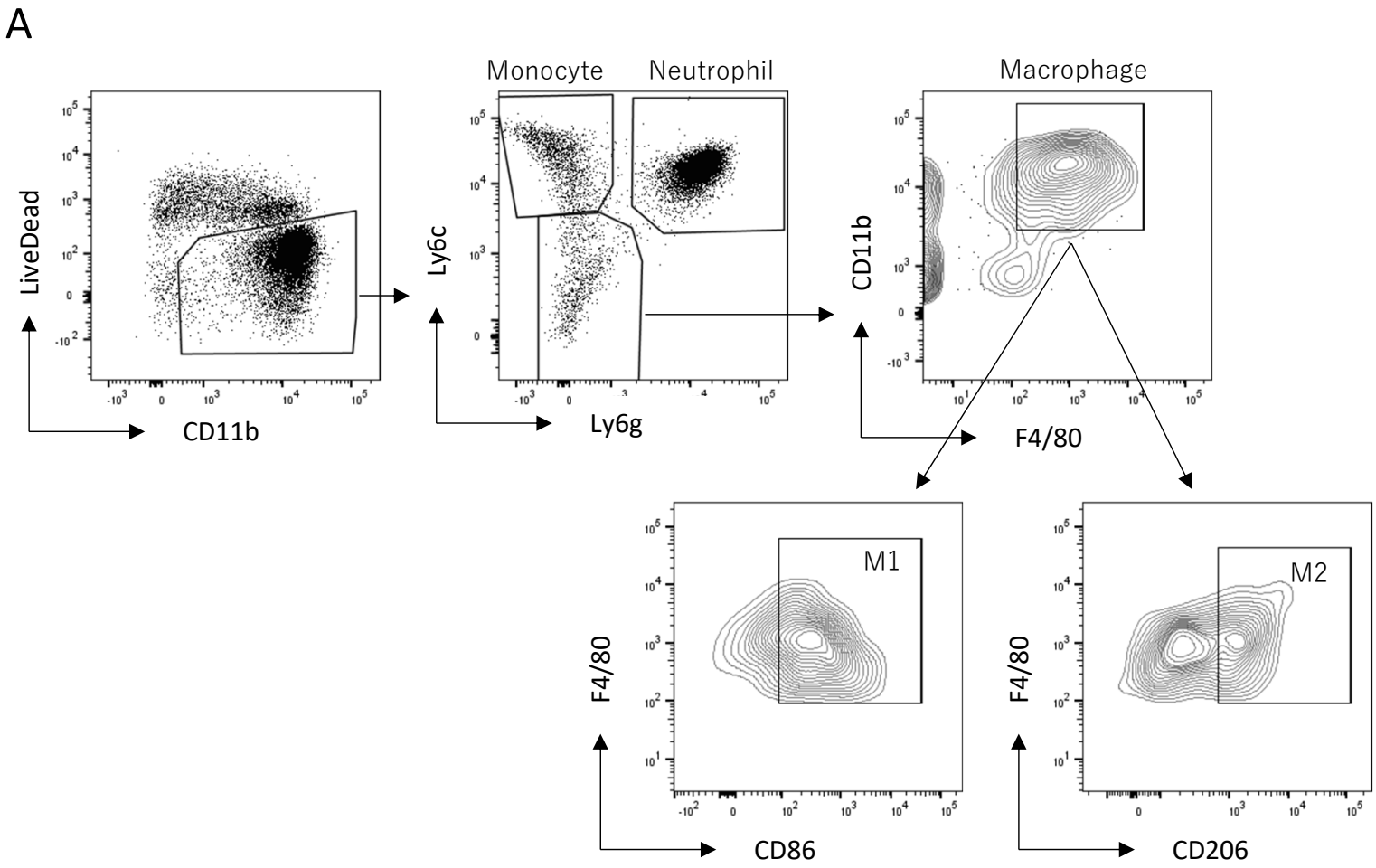
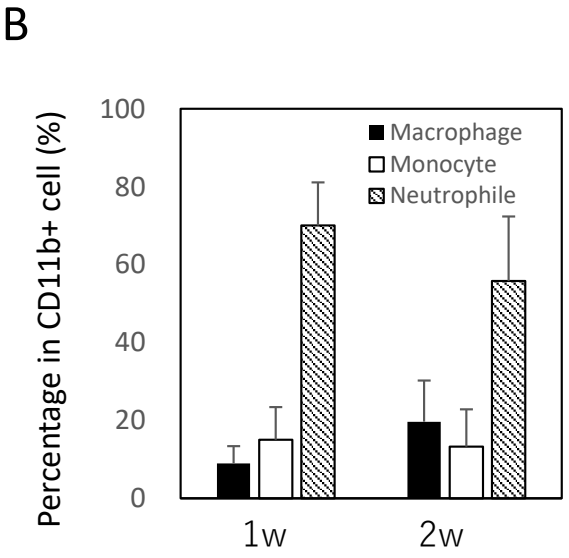
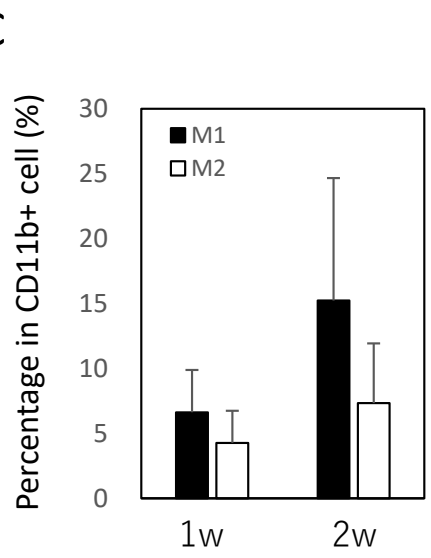


Fig. 4


A
Monocyte
Neutrophil
Macrophage
LiveDead
CD11b
Ly6c
Ly6g
CD11b
F4/80
M1
M2
F4/80
CD86
F4/80
CD206


B
Percentage in CD11b+ cell (%)
100
80
60
40
20
0
Macrophage
Monocyte
Neutrophile
1w
2w


C
Percentage in CD11b+ cell (%)
30
25
20
15
10
5
0
M1
M2
1w
2w

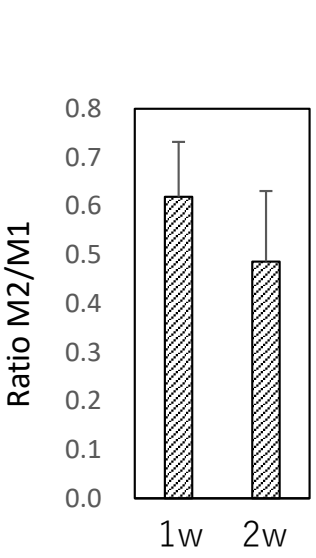

D
Ratio M2/M1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1w
2w

Fig. 5

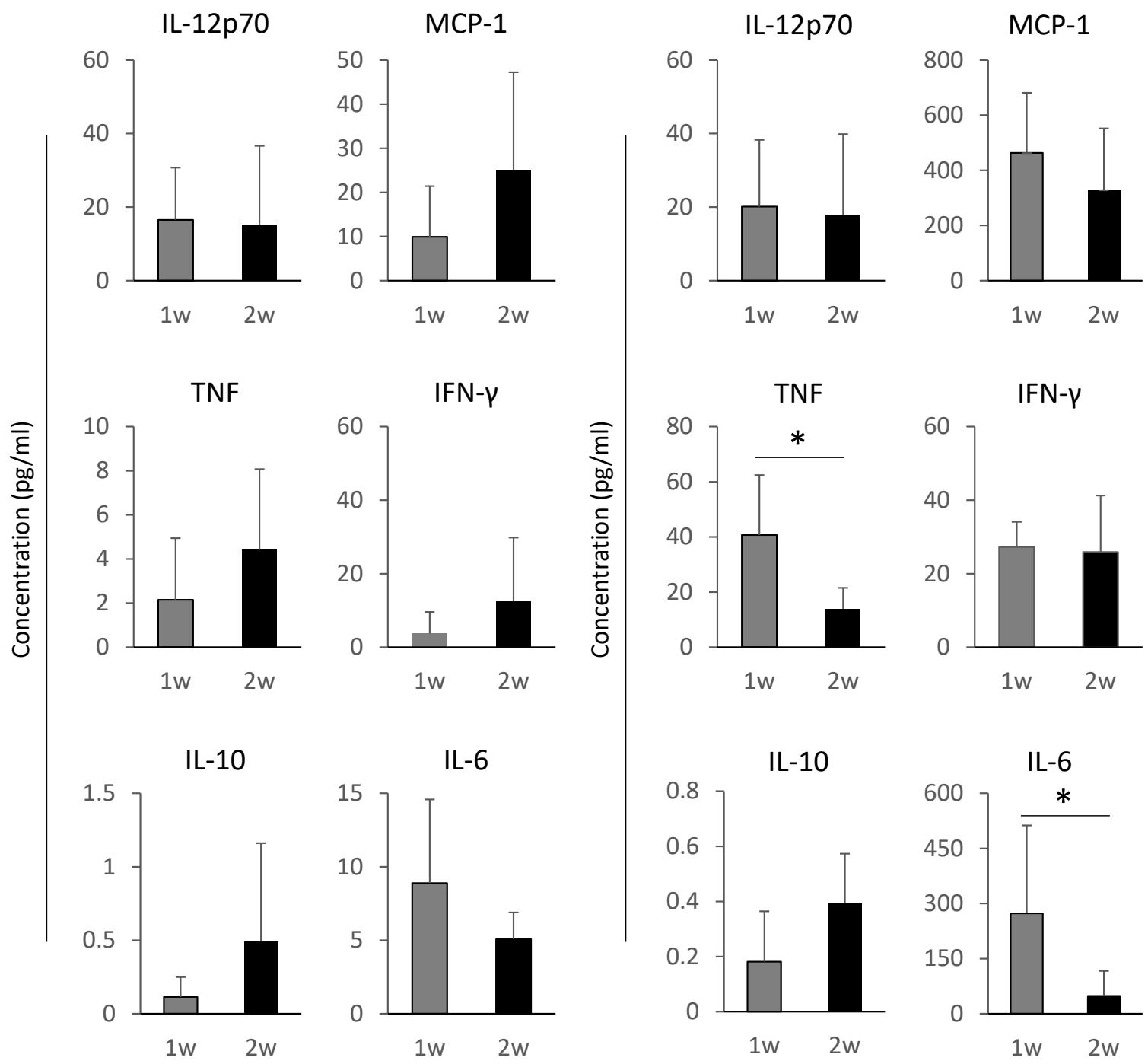

A
Systemic
B
Subcutaneous (Graft Site)
IL-12p70
MCP-1
TNF
IFN-γ
IL-10
IL-6
Concentration (pg/ml)
1w
2w
*

Fig. 6

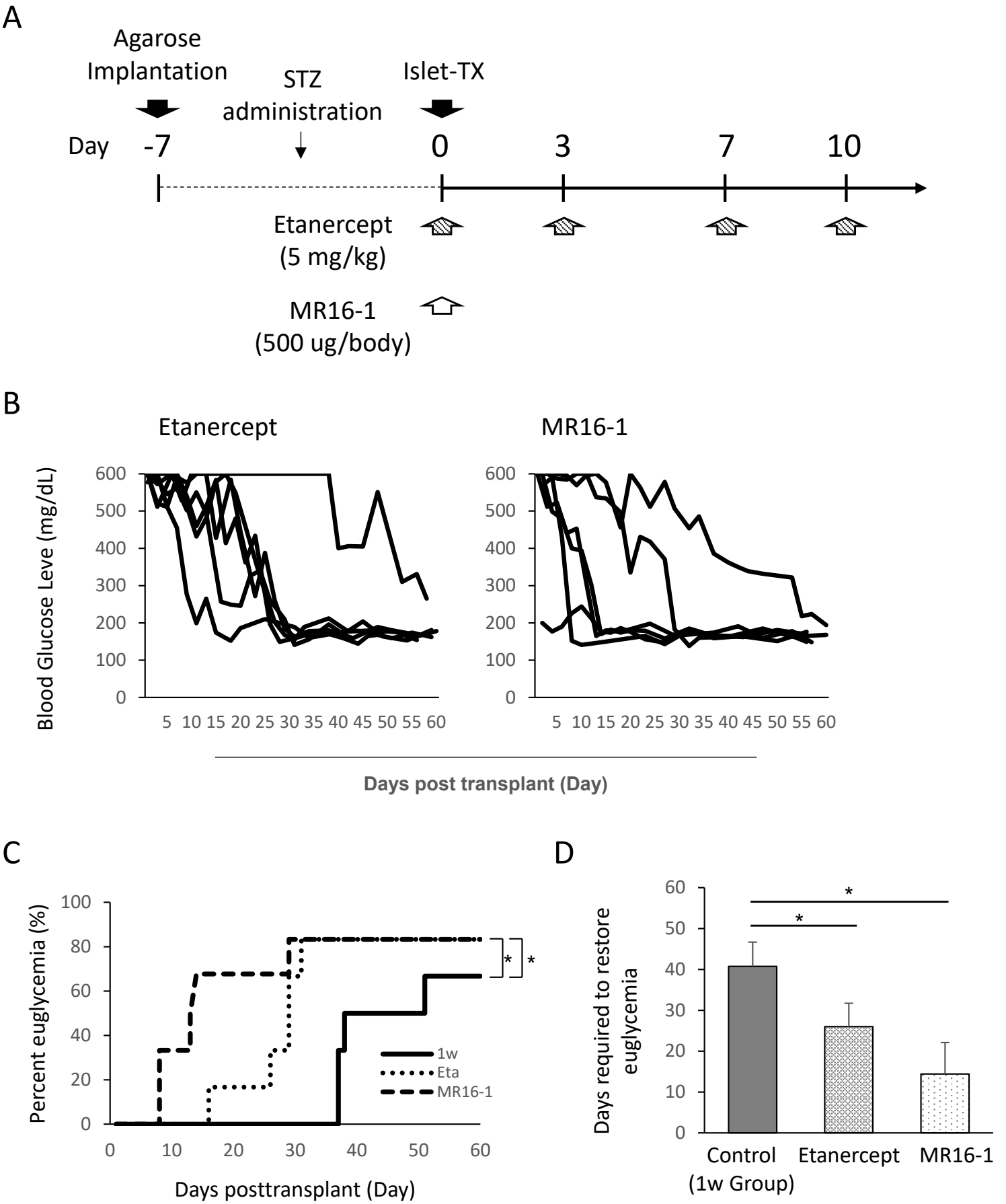

A
Agarose
Implantation
STZ
administration
Islet-TX
Day
-7
0
3
7
10
Etanercept
(5 mg/kg)
MR16-1
(500 ug/body)
B
Etanercept
MR16-1
Blood Glucose Leve (mg/dL)
600
500
400
300
200
100
0
5 10 15 20 25 30 35 40 45 50 55 60
Days post transplant (Day)
C
Percent euglycemia (%)
100
80
60
40
20
0
0 10 20 30 40 50 60
Days posttransplant (Day)
1w
Eta
MR16-1
*
*
D
Days required to restore euglycemia
60
50
40
30
20
10
0
*
*
Control
(1w Group)
Etanercept
MR16-1

Fig. 7