SUPPLEMENTAL DIGITAL CONTENT (SDC)

Materials and Methods

S1. Generation of Cardiac Cell Sheets

iPSCs were cultured in AK02 or AK03 medium (Ajinomoto, Tokyo, Japan) on an LN511E8-coated (Nippi, Japan) dish as previously described.9,10 Briefly, iPSCs (3 × 10^3 cells) were re-suspended in 70-µL aliquots of differentiation medium (DM; StemPro-34; Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine (Invitrogen), 150 μg/mL transferrin (Roche, Basel, Swiss), 50 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 0.05 mg/mL 1-thioglycerol (Sigma-Aldrich), 10 μM Y-27632 (Rho-associated coiled-coil forming kinase inhibitor; Wako Pure Chemical, Osaka, Japan), 0.5% BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, Franklin Lakes, NJ), and 2 ng/mL recombinant human bone morphogenetic protein 4 (BMP4; R&D Systems, Minneapolis, MN) and were cultured in 96-well Corning Costar Ultra-Low attachment multiwell plates (Sigma-Aldrich). On day 1, additional 70 µL of DM containing 18 ng/mL BMP4, 10 ng/mL recombinant human fibroblast growth factor basic (bFGF; R&D Systems), and 12
ng/mL recombinant human activin A (R&D Systems) were added to each well. On day 3, the aggregates were enzymatically digested to single cells, and $1 \times 10^4$ cells were re-suspended in 100-µL aliquots of DM containing 10 ng/mL recombinant human vascular endothelial growth factor (VEGF; R&D Systems), 1 µM IWP-3 (ReproCELL, Kanagawa, Japan), 5.4 µM SB431542 (Sigma-Aldrich), and 0.6 µM dorsomorphin (Sigma-Aldrich) and cultured in 96-well Corning Costar Ultra-Low attachment multiwell plates (Sigma-Aldrich). On day 7, individual cell aggregates were transferred to 24-well Corning Costar Ultra-Low attachment multiwell plates (Sigma-Aldrich; 10 aggregates per well) in DM containing 10 ng/mL VEGF (R&D Systems) and 5 ng/mL bFGF (R&D Systems). From day 0 to 10, the cells were incubated at 37°C under hypoxic atmosphere (5% O₂) with 5% CO₂ in a HERA cell CO₂ incubator (Thermo Fisher Scientific, Waltham, MA). On day 10, the aggregates were enzymatically digested to single cells, and $3.3 \times 10^6$ cells were seeded in 6-well UpCell dishes (temperature-responsive dishes; CellSeed, Tokyo, Japan) and incubated at 37°C under normoxic atmosphere with 5% CO₂. Two days later, the dish was incubated at 20 to 25°C for spontaneous
detachment of cynomolgus macaque iPSC-derived cardiac sheets from the dish.
**S2. 11[C]-Acetate-Positron-emission tomography (PET) imaging**

PET imaging was performed with the Headtome-V PET scanner (Shimadzu Corp.). After 5-min transmission scanning with a 68Ge–68Ga rod source for attenuation correction, 107 ± 10 MBq of 11[C]-acetate were administered as a bolus via the superficial vein of the upper limb, and a 15-min dynamic PET acquisition was initiated (6 × 10 s, 6 × 20 s, 12 × 30 s, and 6 × 60 s). One twenty minutes post completion of emission acquisition, dobutamine was administered with two stepwise increases in the infusion rate (10 μg/kg/min for 2 min and 20 μg/kg/min for 20 min). The second injection of 11[C]-acetate (175 ± 41 MBq) was initiated 7 min after the initiation of dobutamine infusion, and stress dynamic PET imaging data were acquired for 15 min (6 × 10 s, 6 × 20 s, 12 × 30 s, and 6 × 60 s) thereafter.

PET image data were analyzed using PMOD Cardiac Modeling. Anatomic images were reconstructed using static acquisition and reoriented according to the heart axis. After reconstruction, the dynamic images were reoriented. On the short-axis slices, regions of interest (ROIs) were manually drawn on the LV wall
from the apex through the base.

Regional oxidative metabolism was determined from the monoexponential function (k mono) fit to the linear portion of the semilogarithmic plot of the LV wall time activity curve. The start of the curve was visually defined corresponding to when the blood-pool activity had cleared (usually 4–5 min). Oxidative metabolic reserve was defined by change in the k mono with stress as follows: k mono during dobutamine infusion minus the k mono at baseline.¹
S3. Flow Cytometry

Cardiomyogenically differentiated iPSCs were dissociated with 0.25% trypsin-EDTA, fixed with CytoFix fixation buffer (BD) for 20 min, permeabilized with Perm/Wash buffer (BD) at room temperature for 10 min, and incubated with mouse anti-human troponin T antibody (Thermo Scientific), mouse anti-human TE-7 antibody (Merck Millipore, Bilerica, MA), mouse anti-human Tra1-60 antibody (BD HorizonTM), and mouse anti-human CD31 antibody (BD Pharmingen) for 30 min. The labeled cells were washed with Perm/Wash buffer prior to incubation with Alexa Fluor 647 goat anti-mouse IgG secondary antibody (Life technologies) and PE Goat anti-rabbit IgG secondary antibody (Merk Millipore, Bilerica) at room temperature for 30 min and were then assayed using a FACS Canto II (BD) followed by Flowjo software (Tree Star, Ashland, OR) analysis.
S4. Histology and Immunohistolabeling

Dissociated iPSC-derived CMs were cultured on 4-well Lab-TekII chamber slides (Thermo Scientific) and fixed with 4% paraformaldehyde. The cells were labeled by the following primary antibodies: mouse anti-GFP antibody (Abcam, Cambridge, United Kingdom), rabbit anti-alpha-actinin antibody (Sigma-Aldrich), and rabbit anti-alpha-sarcomeric actin (Sigma-Aldrich) and were then visualized by the following secondary antibodies: Alexa Fluor 647 donkey anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). The nuclei of the cells were stained with 4',6-diamidino-2 phenylindole dihydrochloride (DAPI) and then assessed by a confocal laser scanning microscopy FV1200 (Olympus). The harvested heart specimen with transplanted iPSC–cardiac tissue was fixed with 10% buffered formalin and embedded in paraffin. Five micrometer-thick serial paraffin-embedded sections were deparaffinized in xylene, dehydrated in graded ethanol mixtures, and processed for antigen retrieval by autoclaving in 0.01 M citrate buffer. The sections were immersed in methanol containing 3% hydrogen peroxide and then incubated with rabbit anti-CD3 antibody (clone SP7, ab116669,
Abcam), anti-CD31 antibody, and anti-GFP antibody for immunohistological examination. Subsequently, the sections were incubated with a biotinylated anti-rabbit IgG antibody (DAKO, Glostrup, Denmark), further incubated with a peroxidase-conjugated streptavidin (GE Healthcare, Little Chalfont, United Kingdom), and then visualized by biphenyl-3, 30, 4, 40-tetramin (DAB) solution (Wako Pure Chemical). Slides were scanned using Biorevo BZ-9000 (Keyence, Osaka, Japan).
S5. Reverse-Transcription and Quantitative PCR

Total RNA was extracted from cardiac tissue using the RNeasy RNA Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) and from peripheral blood using QIAamp RNA Blood Mini Kit (Qiagen) and was then reverse transcribed into cDNA using TaqMan reverse transcription reagents (Applied Biosystems). Expression profiles of the genes interleukin (IL)-2 receptor (Mf02789780_m1), interferon-gamma (IFN-γ; Mf02788577_m1), stromal cell-derived factor 1 (SDF-1; Mf02788131_m1), VEGF (Mf00900054_m1), hepatocyte growth factor (HGF; Hs00300159_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mf04392546_g1) were validated by quantitative real-time PCR using the ABI PRISM 7700 (Applied Biosystems) system. The average copy number of gene transcripts was normalized to that of GAPDH for each sample in duplicate experiments. Relative quantity of the expression was calculated and compared among each group.
REFERENCES

Figure S1

Serial changes in the blood concentration of tacrolimus.
Figure S2

A

[Image showing a section of tissue labeled with GFP+DAPI, a-sarcomeric actin+DAPI, and merged views.]

B

[Images showing MHC matched and mismatched transplants at 3M and 6M post-transplantation.]
Figure S2.
Macroscopic findings of transplanted iPSC-CM sheets on the heart and immunohistochemistry of biopsied grafts labeled by GFP, alpha-sarcomeric actin, and DAPI. Scale bars, 1000 µm × 3, 100 µm × 1. GFP staining of the harvested cardiac tissues at 3 and 6 months after iPSC-CM sheet transplantation. Scale bars, 100 µm.
Figure S3.

(A) Changes in Rest kmono

(B) Changes in Rest kmono differentiation

(C) Changes in Work Metabolic Index

Figure S3. The results of cardiac perfusion assessed by acetate PET/CT imaging.

(A–C) Comparison among all ‘s of the changes in resting k mono, k mono differentiation, and work metabolic index, as measured by acetate PET/CT imaging. N.S., not significant.
Figure S4

A

\[ \Delta \text{LVEDV} \]

Changes in LVEDV (mL)

Pre 0M 1M 2M 3M 4M 5M 6M

N.S. N.S. N.S. N.S. N.S. N.S. N.S.

MHC-mismatched (Drugs were continued) - solid line
MHC-mismatched (Drugs were discontinued) - dashed line

B

\[ \Delta \text{LVESV} \]

Changes in LVESV (mL)

Pre 0M 1M 2M 3M 4M 5M 6M

N.S. N.S. N.S. N.S. N.S. N.S. N.S.

MHC-mismatched (Drugs were continued) - solid line
MHC-mismatched (Drugs were discontinued) - dashed line

C

\[ \Delta \text{LVEF} \]

Changes in LVEF (%)

Pre 0M 1M 2M 3M 4M 5M 6M

N.S. N.S. N.S. N.S. N.S. N.S. N.S.

MHC-mismatched (Drugs were continued) - solid line
MHC-mismatched (Drugs were discontinued) - dashed line

D

\[ \text{VEGF} \]

RQ of VEGF expression in cardiac tissue

MHC-mismatched Tx (Drugs were continued) - left bar
MHC-mismatched Tx (Drugs were discontinued) - right bar

N.S.

E

\[ \text{SDF-1} \]

RQ of SDF-1 expression in cardiac tissue

MHC-mismatched Tx (Drugs were continued) - left bar
MHC-mismatched Tx (Drugs were discontinued) - right bar

N.S.

F

\[ \text{IL-2 receptor} \]

RQ of IL-2 receptor expression in cardiac tissue

MHC-mismatched Tx (Drugs were continued) - left bar
MHC-mismatched Tx (Drugs were discontinued) - right bar

N.S.

G

\[ \text{IFN-\gamma} \]

RQ of IFN-\gamma expression in cardiac tissue

MHC-mismatched Tx (Drugs were continued) - left bar
MHC-mismatched Tx (Drugs were discontinued) - right bar

N.S.
Figure S4. Comparing the changes in cardiac functions and gene expression of markers related to neovascularization and immune reaction between MHC-mismatched AI with continuing immunosuppressive therapy and discontinuing therapy at 3 months. (A–C) Changes in LVEDV/ESV and LVEF during the follow-up period, comparison between MHC-mismatched AI with continuing immunosuppressive therapy over time and with discontinuing immunosuppressive therapy at 3 months (n = 2).

(D–G) VEGF and SDF-1 expression in cardiac tissues at 6 months after iPSC-CM sheet transplantation, comparing between MHC-mismatched AI with continuing immunosuppressive therapy over time and discontinuing therapy at 3 months (n = 2).