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Supplemental Information

Cardiomyocytes Derived from MHC-Homozygous Induced Pluripotent Stem Cells Exhibit Reduced Allogeneic Immunogenicity in MHC-Matched Non-human Primates

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Inventory of supplemental information

Figure S1. Cynomolgus Macaque iPSCs, iPSC-CMs and Transplanted iPSC-CM Rejection. Related to Figure 2, 4.

Figure S2. Rejection of Transplanted iPSC-CMs in the Heart. Related to Figures 3, 4.

Figure S3. Engraftment of Transplanted iPSC-CMs in the Heart. Related to Figures 3, 4.

Figure S4. Correlation of GFP Intensity with the Cell number and Positive Control for CD3 and CD4 Staining. Related to Figure 3B, 4

Movie S1. Beating cardiomyocytes derived from cynomolgus macaque iPSCs, related to Figure 2

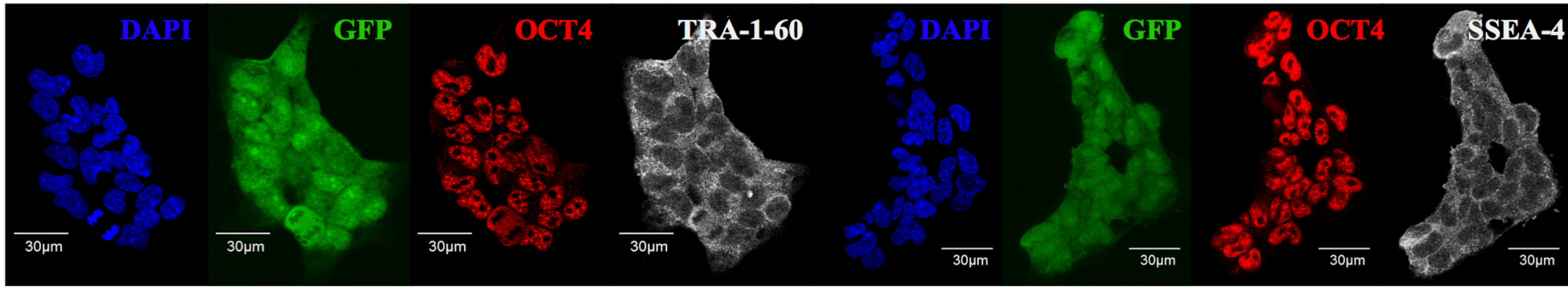
Movie S2. Beating iPSC-CM sheet, related to Figure 2

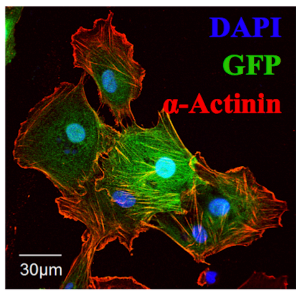
Table S1. MHC genotype, related to Figure 1

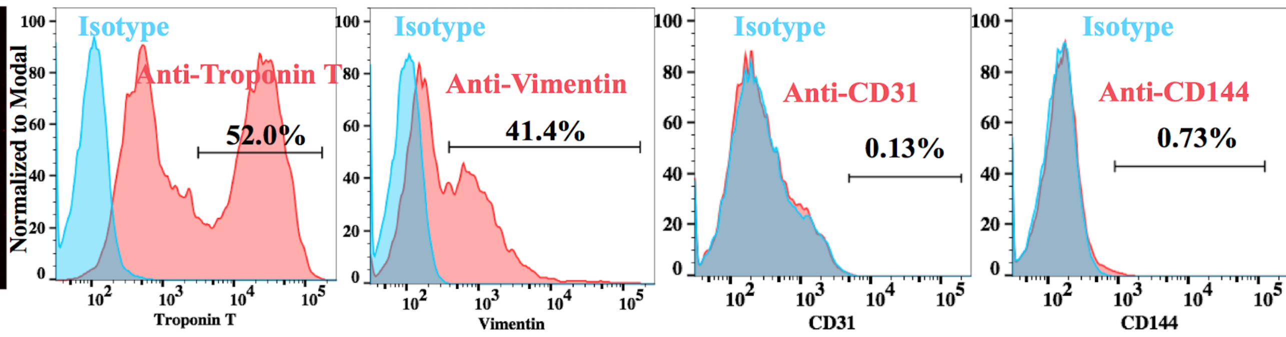
Table S2. Antibodies and Primers Used in This Study. Related to Figure 2, S1-4.

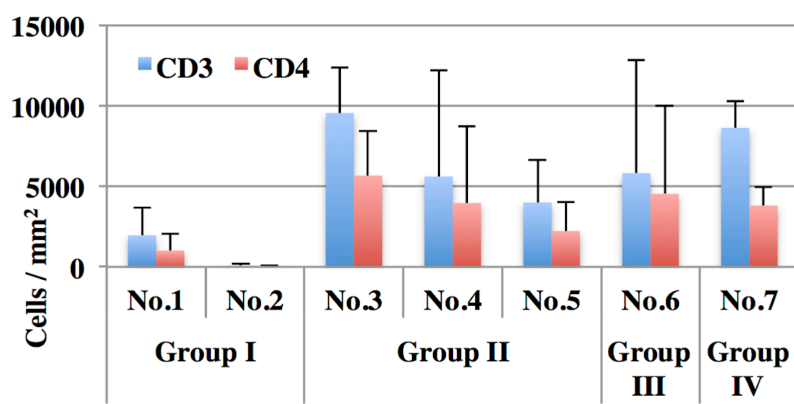
Supplemental experimental procedure

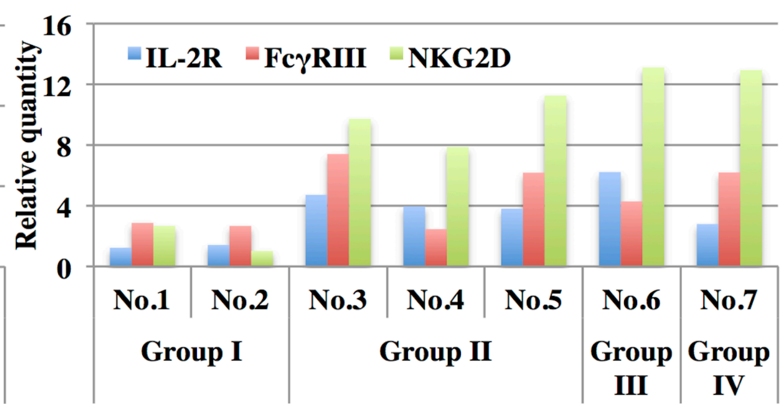
Figure S1

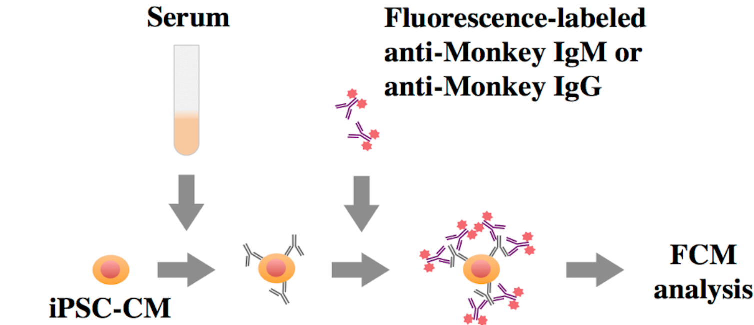


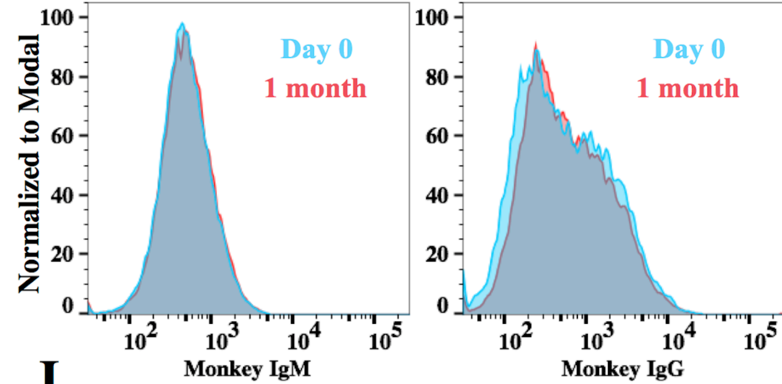


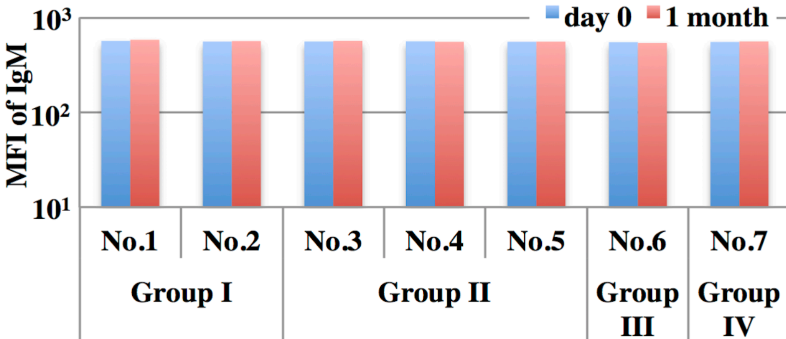












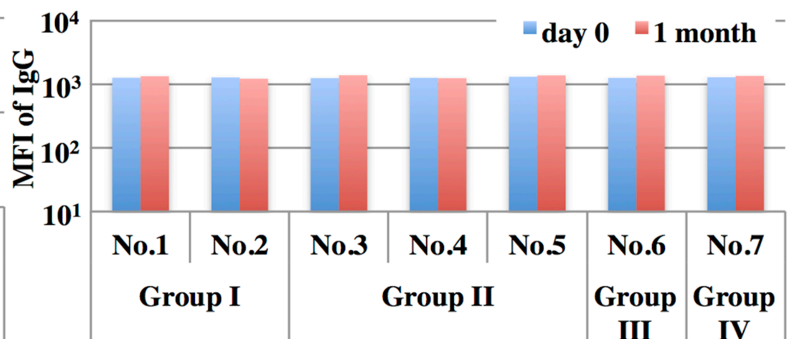
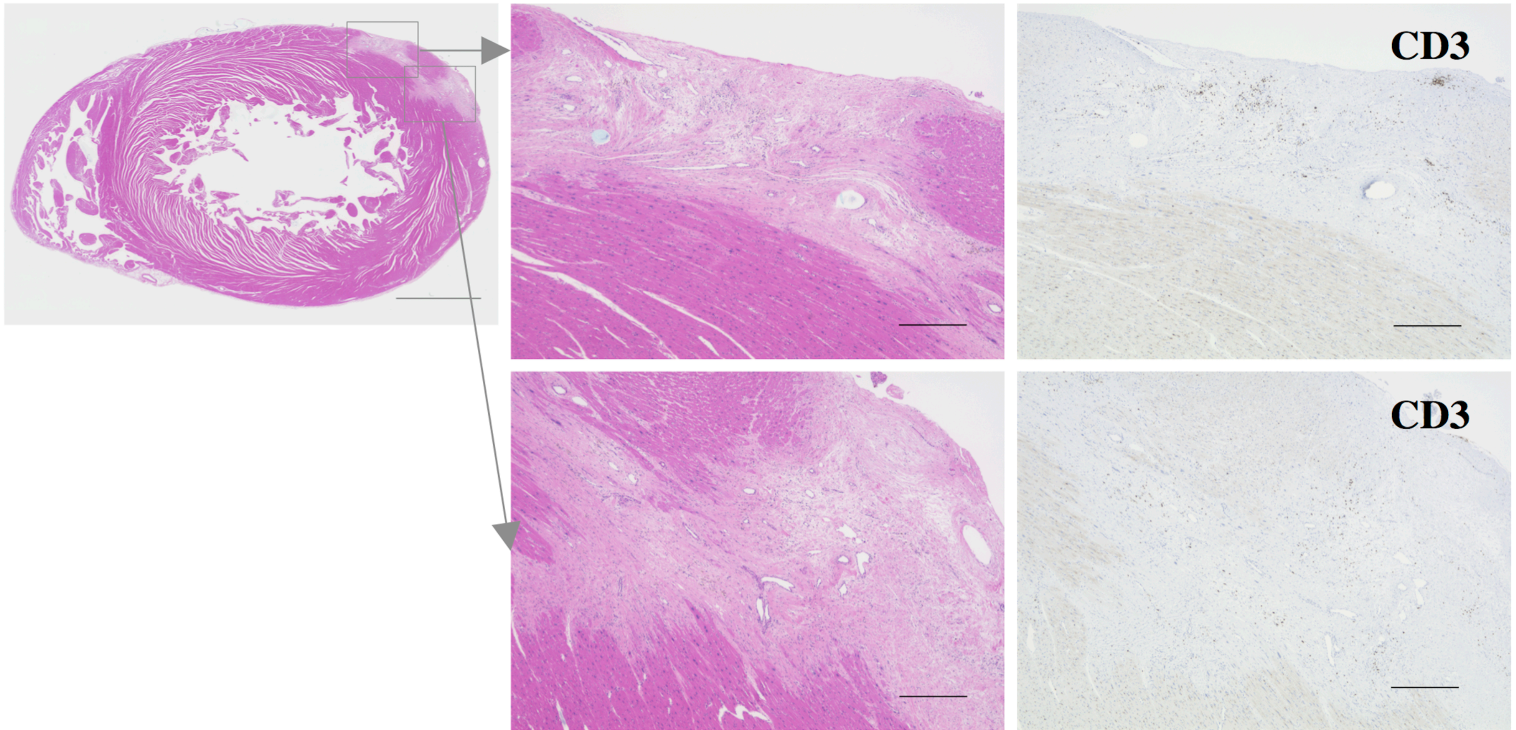
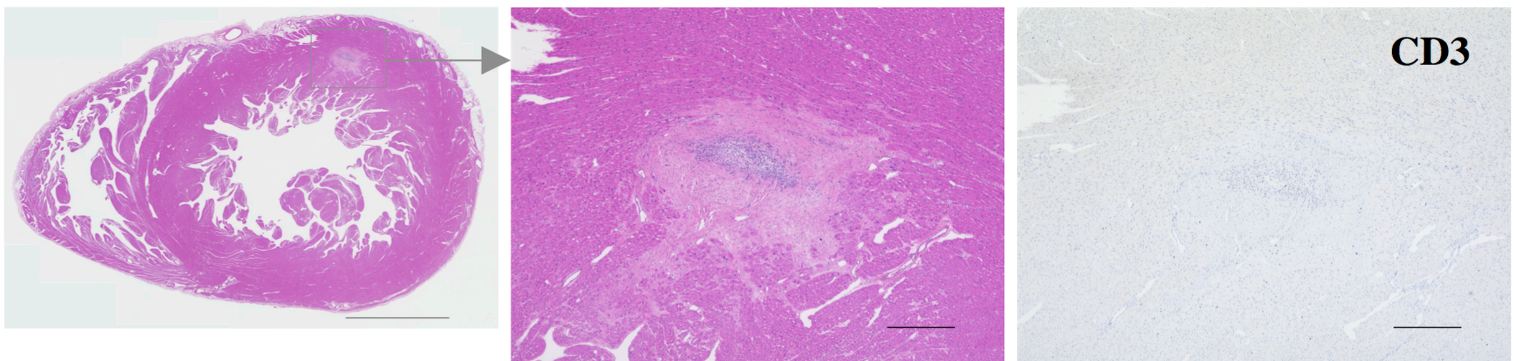


Figure S2





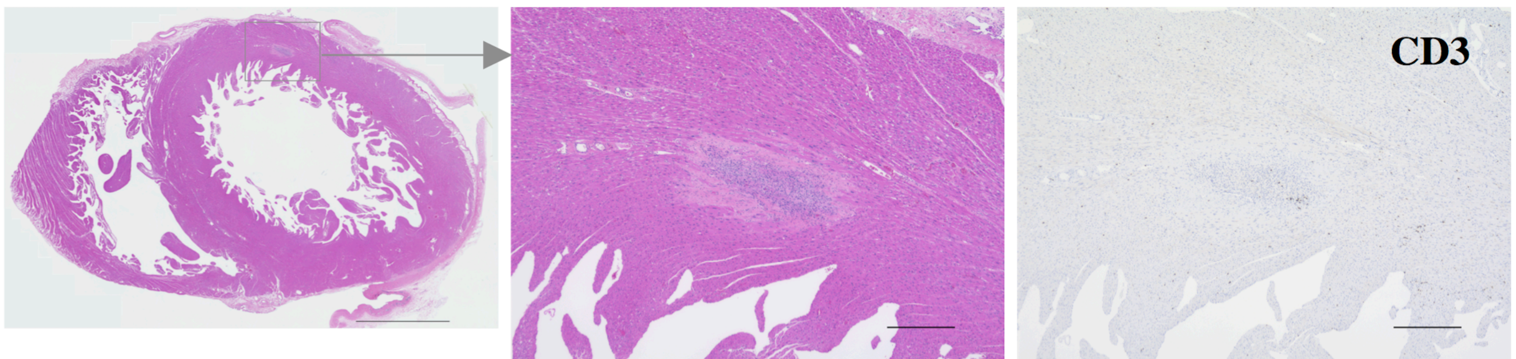
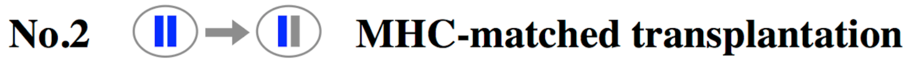
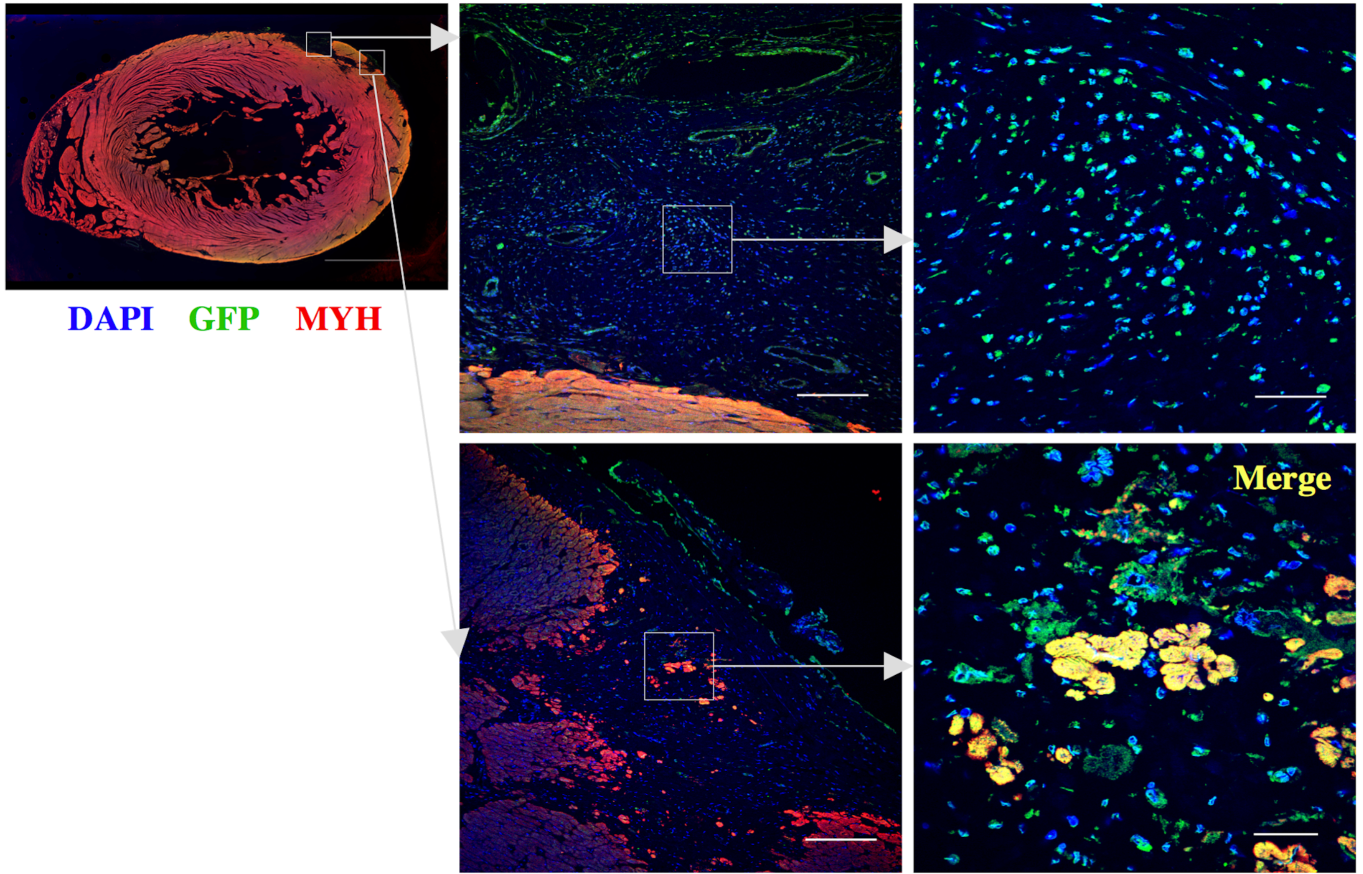
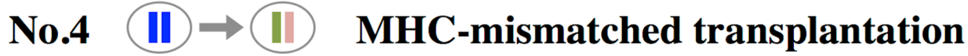
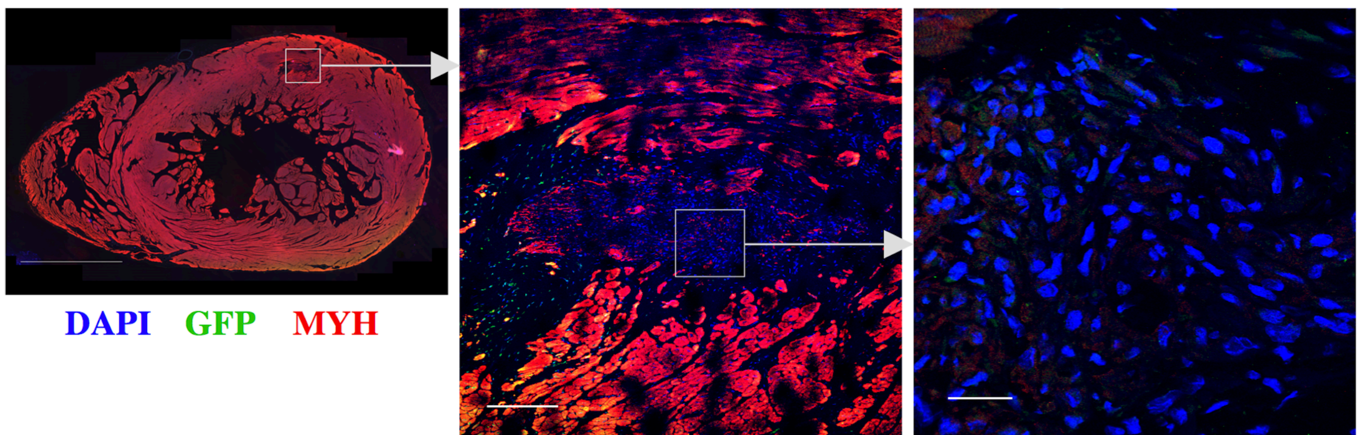


Figure S3









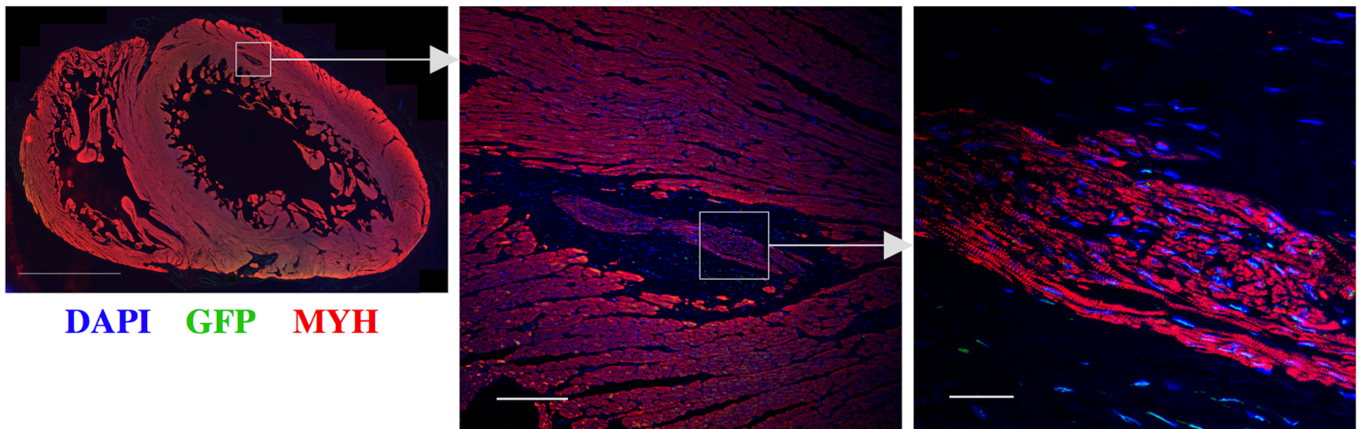


Figure S4

A

Green

Red

Blue

1-layer part of the sheet 2-layer part of the sheet Vessels: Background

Fluorescence intensity

140
120
100
80
60
40
20
0

$R^2 = 0.83383$

56 ± 15

112 ± 10

1-layer 2-layer

B

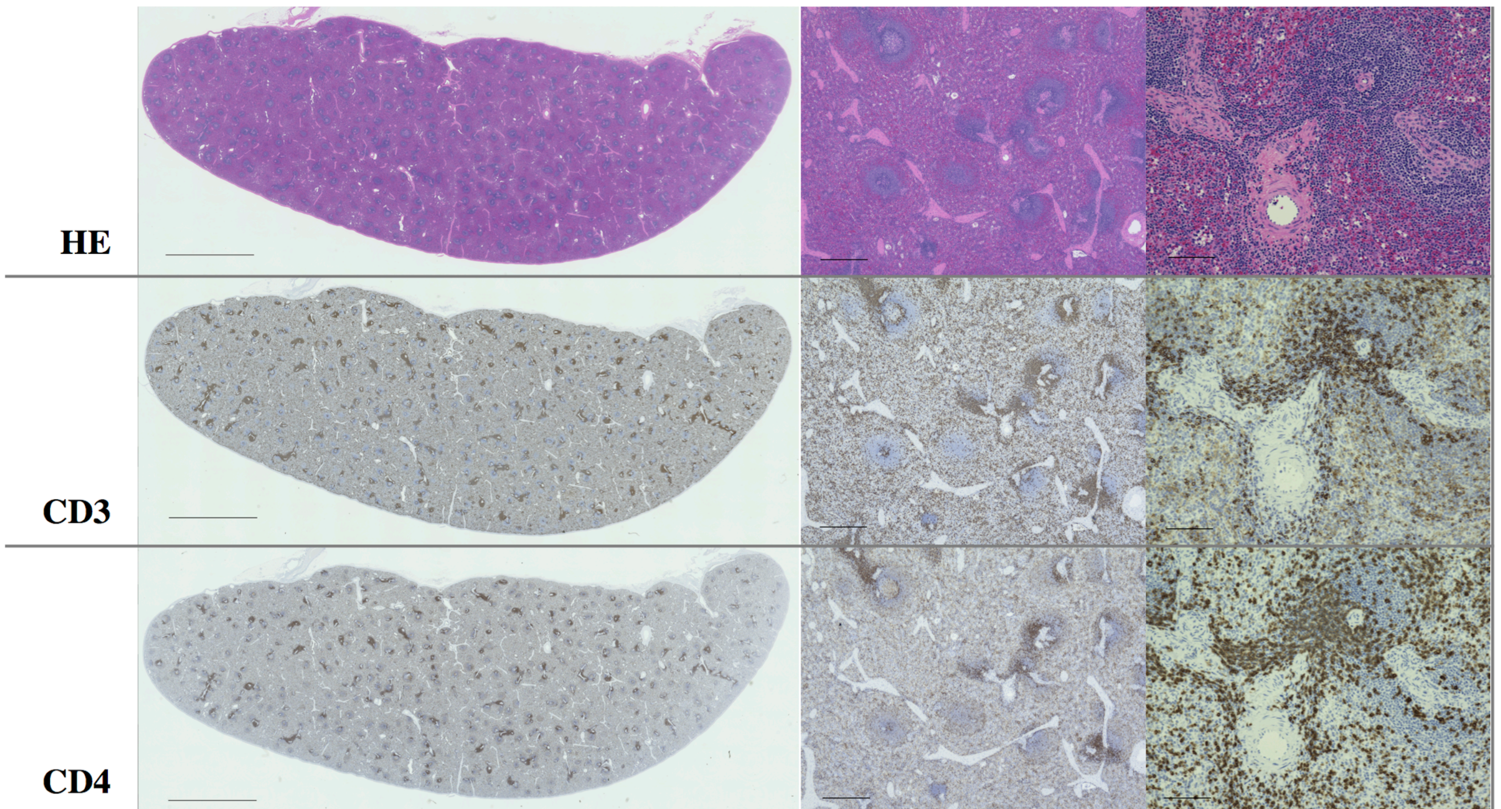


Table S1

	Group	iPSC		Group I				Group II						Group III		Group IV	
	No. : symbol			No. 1 :		No. 2 :		No. 3 :		No. 4 :		No. 5 :		No. 6 :		No. 7 :	
	Haplotype	HT1 near-homo		HT1 hetero				HT1 negative						HT1 hetero			
	Drug			TAC, MMF, PSL				TAC, MMF, PSL						TAC		–	
MHC class I	Mafa-A1	A1*052:02	A1*052:02	A1*052:02	A1*052:02	A1*052:02	A1*008:02	A1*089:03	A1*089:02	A1*089:03	A1*089:03	A1*089:03	A1*098:02	A1*052:02	A1*089:03	A1*052:02	A1*052:02
							A1*086:02										
	Mafa-A2-A8	A4*01:04	A4*01:04	A4*01:04	A4*01:04	A4*01:04		A2*05:50	A2*05:56	A2*05:50	A2*05:50	A2*05:50	A2*24:02	A4*01:04	A2*05:50	A4*01:04	A4*01:04
			A8*01:01		A8*01:01			A3*13:03:01	A3*13:03:01	A3*13:03:01	A3*13:03:01	A3*13:03:01	A8*01:01		A3*13:03:01		
	Mafa-B	B*095:01	B*095:01	B*095:01	B*099:01	B*095:01	B*056:02:01	B*104:03	B*137:03	B*104:03	B*159:01	B*091:02	B*160:01	B*095:01	B*099:01	B*095:01	B*095:01
		B*033:02	B*033:02	B*033:02	B*108:01	B*033:02	B*017:01	B*144:03N		B*144:03N	B*007:01:01	B*068:08	B*007:01:02	B*033:02	B*108:01	B*033:02	B*033:02
		B*098:10	B*098:10	B*098:10	B*045:05	B*098:10	B*157:01	B*057:04		B*057:04	B*158:01		B*070:05	B*098:10	B*045:05	B*098:10	B*098:10
					B*098:11		B*050:08	B*060:02		B*060:02			B*115:04:02		B*098:09		
							B*060:02	B*046:01:02		B*046:01:02					B*158:01		
							B*116:01	B*050:08		B*050:08					B*085:01		
								B*114:02		B*114:02							
								B*072:01		B*072:01							
	Mafa-E	E-like5	E-like5	E-like5	E-like5	E-like5	E-like3	E-like3	E-like9	E-like3		E-like1	E-like2	E-like5	E-like3	E-like5	E-like5
		E-like11	E-like11	E-like11	E-like11	E-like11	E-like10	E-like10		E-like10				E-like11	E-like10	E-like11	E-like11
	Mafa-F	F-like4	F-like4	F-like4	F-like4	F-like4	F-like2	F-like1	F-like1	F-like1	F-like4	F-like1	F-like2	F-like4	F-like1	F-like4	F-like4
	Mafa-I	I*01:11	I*01:11	I*01:11	I*01:10:02	I*01:11	I*01:01:01	I*01:12:01	I*01:18:02	I*01:12:01		I*01:14	I*01:11	I*01:11	I*01:13:01	I*01:11	I*01:11
MHC class II	Mafa-DPA1	DPA1*02:05	DPA1*02:05	DPA1*02:05	DPA1*07:02	DPA1*02:05	DPA1*02:15:02	DPA1*02:15:02	DPA1*04:02	DPA1*04:02	DPA1*07:04	DPA1*02:15:02	DPA1*07:04	DPA1*02:05	DPA1*07:04	DPA1*02:05	DPA1*04:02
	Mafa-DPB1	DPB1*15:04	DPB1*15:04	DPB1*15:04	DPB1*19:03	DPB1*15:04	DPB1*10:01	DPB1*10:01	DPB1*03:04	DPB1*03:04	DPB1*21:01	DPB1*10:01	DPB1*21:01	DPB1*15:04	DPB1*21:01	DPB1*15:04	DPB1*03:04
	Mafa-DQA1	DQA1*01:07:01	DQA1*01:07:01	DQA1*01:07:01	DQA1*01:20	DQA1*01:07:01	DQA1*26:03	DQA1*24:11	DQA1*01:18	DQA1*26:03	DQA1*24:10	DQA1*26:03	DQA1*01:13	DQA1*01:07:01	DQA1*24:05	DQA1*01:07:01	DQA1*26:03
	Mafa-DQB1	DQB1*06:08	DQB1*06:08	DQB1*06:08	DQB1*06:25	DQB1*06:08	DQB1*18:07	DQB1*18:27	DQB1*06:35	DQB1*18:07	DQB1*18:26	DQB1*18:07	DQB1*06:17:02	DQB1*06:08	DQB1*18:05	DQB1*06:08	DQB1*18:07
	Mafa-DRB	DRB1*10:07	DRB1*10:07	DRB1*10:07	DRB1*10:06	DRB1*10:07	DRB*W1:08	DRB1*03:17:02	DRB*W54:01	DRB*W1:08	DRB3*04:02:01	DRB*W33:02	DRB1*03:18	DRB1*10:07	DRB*W33:02	DRB1*10:07	DRB*W36:01
		DRB1*03:21	DRB1*03:21	DRB1*03:21	DRB1*03:07	DRB1*03:21	DRB*W36:01	DRB1*10:05	DRB*W53:01	DRB*W36:01	DRB*W1:07	DRB*W36:01	DRB*W2:02	DRB1*03:21	DRB1*03:19	DRB1*03:21	DRB*W3:01
							DRB*W3:01			DRB*W3:01	DRB*W6:02		DRB*W1:04				DRB*W1:08
											DRB*W6:03:01						

Table S2

A

Antibodies	Source	Dilution
For immunocytochemistry and immunohistochemistry		
SSEA-4	Biolegend, 330401	100
TRA-1-60	Biolegend, 330602	100
OCT4	Abcam, ab19857	100
α-Actinin	Sigma-Aldrich, A7811	100
GFP	Abcam, ab290	200
MYL (myosin light chain)	Abcam, ab2480	200
MYH (myosin heavy chain)	Abcam, ab185967	200
TNT (troponin T)	Abcam, ab45932	200
Vimentin	Abcam, ab8978	200
CD3	NICHIREI BIOSCIENCES INC., 413591	1
CD4	NICHIREI BIOSCIENCES INC., 413181	1
For flow cytometry		
TNT (troponin T)	Thermo, #MS-295	100
Vimentin	Abcam, ab8978	100
CD31	BD Biosciences, 555446	100
CD144	BD Biosciences, 561567	100
Monkey IgM	LSBio, LS-C61219	100
Monkey IgG	LSBio, LS-C56744	100

B

Gene name	Primers (5’-3’)
MHC-class I (monkey)	F: TCGTGCGGTTYGAYAGCGACG R: CCAGCAYCTCAGGGTGGCCTC
Oct4	F: GAGAACAATGAGAACCTTCAGGACA R: TTCTGGCGCCGGTTACAGAACCA
Nkx2.5	F: CCACCCACCCGTATTTATGTTT R: GGGGTCAACGCACTCTCTTT
MYH (myosin heavy chain)	F: CTGTACCAGAAGTCCTCCCTCAA R: TCTTGCCTCCTTTGCTTTTACC
TnT (troponin T)	F: TCTCCATCCTCTGCCTCACC R: CTGCTTCTTCCTGCTCCTCCT
IL-2R (interleukin-2 receptor)	F: TGAAGGGGTGCGATGGA R: AGTAGTGGGAGGCTTGGGAGA
NKG2D (natural-killer group 2, member D)	F: ATACAGCAAAGAGGACCAGGATTT R: AGTAGGTTGGGTGAGAGAATGGAG
FcγR III (immunoglobulin gamma Fc region receptor III)	F: TGGGTGTTCAAGGAGGAAGAA R: TGAGTGTGGCTTTTGGAATGTAG
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	F: GAAGGTGAAGGTCGGAGTC R: CATTGATGGCAACAATATCC

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Cynomolgus Macaque iPSCs, iPSC-CMs and Transplanted iPSC-CM Rejection. Related to Figure 2, 4.

(A) Undifferentiated cynomolgus macaque iPSCs continuously expressing GFP stained with anti-OCT4 (Alexa Fluor 546), anti-TRA-1-60, or anti-SSEA-4 antibodies (Alexa Fluor 647) and DAPI. Scale bar, 30 μm. (B) iPSC-CMs at day 10, stained with anti-α-actinin antibodies (Alexa Fluor 546) and DAPI. Scale bar, 30 μm. (C) The cells in the iPSC-CM sheet, stained with anti-troponin T, anti-vimentin, anti-CD31, or anti-CD144 antibodies or the isotype control, analyzed with flow cytometry. (D) Semi-quantitative scoring for the number of CD3 or CD4 positive cells per mm^2 in the graft 1 month after transplantation are shown as the means ± standard deviation for each animal. (randomly selected 10 views) (E) mRNA expression of FcγRIII, NKG2D, and IL-2R in the harvested iPSC-CM grafts 1 month after transplantation relative to those in normal subcutaneous tissue as analyzed by real-time PCR. The samples were harvested randomly from the graft (n = 3) and separately measured by triplicate experiments. The results are expressed as the means. (F) The schema for quantification of antibodies against iPSC-CMs (G) FCM analysis for anti-iPSC-CM IgM or IgG in the sera of No.1 macaque at day 0 and at 1 month after the transplantation. (H, I) Mean fluorescence intensity of iPSC-CMs, suspended in the serum of each macaque at day 0 and at 1 month after the transplantation, and subsequently stained with fluorescence labeled anti-monkey IgM (H) and IgG (I). Abbreviation: MFI, mean fluorescence intensity

Figure S2. Rejection of Transplanted iPSC-CMs in the Heart. Related to Figures 3, 4.

Hematoxylin-eosin (left) and CD3 staining of the heart (animal Nos. 2, 4, and 5) 2 months after iPSC-CM transplantation. Scale bars: 5 mm (left), 500 μm (middle and right).

Figure S3. Engraftment of Transplanted iPSC-CMs in the Heart. Related to Figures 3, 4.

The heart (animal Nos. 2, 4 and 5) 2 months after iPSC-CM transplantation stained by anti-GFP (Alexa Fluor 488) and anti-MYH antibodies (Alexa Fluor 546) and DAPI. Scale bars: 5 mm (left), 200 μm (middle), 30 μm (right).

Figure S4. Correlation of GFP Intensity with the Cell number and Positive Control for CD3 and CD4 Staining. Related to Figure 3B, 4.

(A) Observation of the cell sheet on day 0 of the transplantation was performed to confirm the correlation of GFP intensity with the cell number. Using Image J software, the image indicated in Figure 1E was split into 3 color files (Red, Blue, and Green). The intensities of green color in the solid circles within the 2-layer part of the cell sheet were about twice those in the dotted circles within the 1-layer and showed a high correlation (coefficient of determination: R = 0.83383). The result was expressed as the mean ± standard deviation of indicated 10 circles. The green color intensities were standardized by

subtracting the intensities of the vessels as the background. (B) Hematoxylin-eosin staining and CD3 or CD4 staining of cynomolgus macaque spleens as a positive control. Scale bars: 5 mm (left), 500 μm (middle), and 100 μm (right).

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Beating Cardiomyocytes Derived from Cynomolgus Macaque iPSCs. Related to Figure 2.

Beating cell aggregations of cynomolgus macaque iPSCs at day 10 of cardiomyogenic differentiation at room temperature.

Movie S2. Beating iPSC-CM Sheet. Related to Figure 2.

A beating cynomolgus macaque iPSC-CM sheet at day 12 of cardiomyogenic differentiation at room temperature.

SUPPLEMENTAL TABLE LEGENDS

Table S1. MHC genotypes. Related to Figure 1.

MHC genotypes of iPSCs and the seven cynomolgus macaque recipients used in this study. The alleles constituting the HT1 haplotype are indicated by blue characters.

Table S2. Antibodies and Primers Used in This Study. Related to Figure 2.

Lists of (A) primary antibodies used for immunocytochemistry, immunohistochemistry, and flow cytometry and (B) primers for real-time PCR.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cardiomyogenic Differentiation Protocol

iPSCs (3×10^3) were resuspended in 70 µl aliquots differentiation medium (DM; StemPro-34 [Invitrogen] containing 2 mM L-glutamine [Invitrogen], 30 mg/ml transferrin [Roche], 5 mg/ml ascorbic acid [Sigma], and 0.05 mg/ml 1-thioglycerol [Sigma]) containing 10 µM Y-27632 (Rho-associated coiled-coil forming kinase inhibitor; Wako), 0.5% BD Matrigel Matrix Growth Factor Reduced (BD Biosciences), and 2 ng/ml recombinant human bone morphogenetic protein 4 (BMP4; R&D Systems) and cultured in 96-well Corning Costar Ultra-Low attachment multiwell plates (Sigma-Aldrich). On day 1, an additional 70 µl 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) was added to each well. On day 3, the aggregates were enzymatically digested to single cells, and 1×10^4 cells were resuspended in 100 µl aliquots DM containing 10 ng/ml recombinant human vascular endothelial growth factor (VEGF; R&D Systems), 1 µM IWP-3 (Wnt signaling inhibitor; Stemolecule), 5.4 µM SB431542 (transforming growth factor-β signaling inhibitor; Sigma-Aldrich), and 0.6 µM dorsomorphin (BMP signaling inhibitor; 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 in a hypoxic atmosphere (5% O_2) with 5% CO_2 using a HERAcell CO_2 incubator (Thermo Fisher Scientific). On day 10, the aggregates were enzymatically digested to single cells and 3.3×10^6 cells were seeded onto 6-well UpCell dishes (temperature-responsive dishes; CellSeed) and incubated at 37°C in a normoxic atmosphere with 5% CO_2. On day 12, the dish was incubated at room temperature, which caused the cells to detach spontaneously to form scaffold-free cynomolgus macaque iPSC-derived cardiomyocyte sheets.

Immunocytochemistry Analysis

Cynomolgus macaque iPSCs and iPSC-CMs were fixed with 4% paraformaldehyde and stained with the listed primary antibodies (Table S2A). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and observed with the confocal laser scanning microscope FV1200 (Olympus).

Flow Cytometry

iPSC-CMs were dissociated with 0.25% trypsin-EDTA and then fixed with CytoFix fixation buffer (BD Biosciences). The cells were stained with mouse anti-troponin T antibodies (Thermo) or anti-vimentin antibodies (Abcam) in Perm/Wash buffer (BD Biosciences) and visualized with Alexa Fluor 647 rabbit anti-mouse IgG (Invitrogen), or stained with phycoerythrin conjugated anti-CD31 antibodies (BD Biosciences) or Alexa Fluor 647 conjugated anti-CD144 antibodies (BD Biosciences).

The labeled cells were analyzed on a FACS Canto II (BD Biosciences). All data were analyzed in five independent experiments.

Immunohistochemistry Analysis

iPSC-CM sheets, harvested iPSC-CM grafts, and the hearts were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. The heart samples of animals 2, 4, and 5 were obtained by cutting the heart laterally at the point in which the iPSC-CMs had been injected and sectioning. After antigen retrieval by heating in ethylenediaminetetraacetic acid and endogenous peroxidase deactivation with 3% H_2O_2, the sections were stained with the listed antibodies (Table S2A) and horseradish peroxidase-labeled polymer (Nichirei Biosciences, Inc.), followed by 3,3′-diaminobenzidine (DAB) color development, or fluorescence labeled secondary antibodies, as appropriate.

Semi-quantitative scoring for the number of the CD3 or CD4 positive cells in the grafts

CD3 or CD4 positive cells in the grafts were counted on the DAB color stained sample. The number of DAB color positive cells were automatically counted with a microscope and its software (BZ-X700, KEYENCE, Osaka, Japan) from randomly selected 10 views in each sample. The result was expressed as the mean ± standard deviation for each animal. CD3 and CD4 staining was confirmed using cynomolgus macaque spleens as a positive control (Figure S4B).

Quantification of Antibodies against iPSC-CMs

iPSC-CMs were suspended in 10% diluted serum obtained from the macaques at day 0 or at 1 month after the transplantation. Subsequently, the cells were stained with a Texas Red-conjugated goat anti-monkey IgM antibody or a fluorescein isothiocyanate-conjugated goat anti-monkey IgG antibody (LSBio) (Figure S3). Fluorescence intensities were measured by a FACSCanto II instrument (BD Biosciences). Dead cells were excluded after staining with the LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen) according to the manufacturer's recommended protocol.

Semi-quantitative Polymerase Chain Reaction (PCR)

DNA-free total RNA was extracted with an RNeasy RNA isolation Kit (Qiagen), reverse-transcribed into cDNA using Omniscript reverse transcriptase (Qiagen), and analyzed by quantitative real-time PCR on an ABI PRISM 7700 thermocycler (Applied Biosystems) with SYBR Green dye (Applied Biosystems) using the listed primers (Table S2B). All data were analyzed by the relative standard curve method in three independent experiments in triplicate.

Genotyping of MHC

MHC genotyping was performed as previously described (Morizane et al., 2013). Briefly, total RNA was isolated from the

peripheral white blood cells using TRIzol (Invitrogen). cDNA was synthesized using ReverTra Ace (TOYOBO). The MHC class I gene primer pairs used for macaque genes were designed based on generic sequences in exons 2 to 4 that amplify all known class I genes. After RT-PCR amplification using high fidelity KOD FX polymerase (TOYOBO), pyrosequencing of the PCR products was carried out using the GS Junior system and amplicon sequencing protocol (Roche). MHC genotypes were assigned by comparing the sequences to known MHC allele sequences released from the Immuno Polymorphism Database.

Statistical Analysis

Data are reported as the means ± standard deviation and were compared using Student's t-tests. Differences with p-values of less than 0.05 were considered statistically significant. All statistical analyses were performed using JMP 11.0 (SAS Institute).