Syngeneic Mesenchymal Stem Cells Reduce Immune Rejection After Induced Pluripotent Stem Cell-Derived Allogeneic Cardiomyocyte Transplantation

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Avoiding immune rejection after allogeneic induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) transplantation is a concern. However, mesenchymal stem cells (MSCs) can suppress immune rejection. To determine whether MSC co-transplantation can reduce immune rejection after allogeneic iPSC-CM transplantation, the latter cell type, harbouring a luciferase transgene, was subcutaneously transplanted alone or together with syngeneic MSCs into BALB/c mice. Bioluminescence imaging revealed that MSC co-transplantation significantly improved graft survival (day 7: iPSC-CMs alone 34 ± 5%; iPSC-CMs with MSCs, 61 ± 7%; P = 0.008). MSC co-transplantation increased CD4⁺CD25⁺FOXP3⁺ regulatory T cell numbers, apoptotic CD8-positive T cells, and IL-10 and TGF-beta expression at the implantation site. Analysis using a regulatory T cell depletion model indicated that enhanced regulatory T cell populations in the iPSC-CM with MSC group partially contributed to the extended iPSC-CM survival. Further, MSCs affected activated lymphocytes directly through cell–cell contact, which reduced the CD8/CD4 ratio, the proportion of Th1-positive cells among CD4-positive cells, and the secretion of several inflammation-related cytokines. Syngeneic MSC co-transplantation might thus control allogeneic iPSC-CM rejection by mediating immune tolerance via regulatory T cells and cell–cell contact with activated lymphocytes; this approach has promise for cardiomyogenesis-based therapy using allogeneic iPSC-CMs for severe heart failure.

Heart failure is still associated with a high mortality rate worldwide, despite significant advances in medical treatment and technology. Therefore, it is critical to apply new concepts for the development of novel therapeutic alternatives. In the last decade, stem cell therapy using bone marrow progenitor, cardiac, and somatic stem cells has been studied in clinical settings. Unfortunately, however, these treatments have limited efficacy, likely because they depend primarily on paracrine effects induced by the transplanted cells, and thus the recovery of cardiomyocyte numbers is not sufficient. Recently, induced pluripotent stem cell (iPSC)-derived cardiomyocytes (iPSC-CMs) were proposed as a cell source for cardiomyogenesis-based therapy, which could be an alternative candidate treatment to several previous stem cell applications to achieve the benefit of increasing the number of functioning cardiomyocytes, as well as paracrine effects. Although regenerative therapy using allogeneic iPSCs has more merit in terms of safety, cost, and preparation, compared to the use of autologous iPSCs, allogeneic cell transplantation is significantly hampered by immune rejection. Several immunosuppressive agents that can result in renal failure, infectious disease, and malignancy are required for allogeneic cell transplantation, even though this approach might not have the potential to provide the same therapeutic efficacy as organ transplantation.

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transplantation. Therefore, methods other than immunosuppressive agents should be studied to increase transplanted cell survival without the aforementioned side effects.

Bone marrow-derived mesenchymal stem cells (MSCs) were reported to have profound immunomodulatory properties by changing cell numbers and functions in the immune system. In fact, MSC-based therapy was shown to have a significant therapeutic effect on a variety of human diseases including multiple sclerosis, inflammatory bowel disease, autoimmune encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, and graft versus host disease. However, the detailed mechanism underlying the immunosuppressive effect of MSCs has not been fully uncovered. In this study, we demonstrated that the co-transplantation of syngeneic MSCs enhances the survival of allogeneic transplanted iPSC-CMs through the induction of regulatory T cells (Tregs) and cell–cell contact in mice.

**Results**

**Differentiation of cardiomyocytes derived from iPSCs.** Undifferentiated murine iPSCs expressed OCT4, NANO, and LIN28, and using a previously reported protocol, they were differentiated into cardiomyocytes that expressed cardiomyocyte marker genes such as ANP-1, MYH7B, 1STLI, NNX2.5, MYH6, and TNNT2, with decreased OCT4, NANO, and LIN28 expression (Fig. 1a,b). Nearly all embryoid bodies exhibited self-beating at day 16 and the cells were positive for troponin T and alpha-actinin, indicating that they were iPSC-CMs (Fig. 1c, Movie SI). In vitro bioluminescence imaging (BLI) analysis of iPSC-CMs showed a linear relationship between cell number and signal (p = 1.000, P < 0.001), validating the use of this technique for the quantification of luciferase-expressing iPSC-CMs (Fig. 1d).

**Co-transplantation of syngeneic MSCs enhances the survival of transplanted allogeneic iPSC-CMs.** We first subcutaneously transplanted allogeneic iPSC-CM sheets alone (iPSC-CM alone) or allogeneic iPSC-CM sheets with syngeneic MSCs (iPSC-CM with MSC) (Fig. 1e–g). The survival of transplanted luciferase-expressing iPSC-CMs was monitored by serial BLI over time (Fig. 2a). The transplanted iPSC-CM sheets in mice treated with tacrolimus (Prograf; Astellas Pharma) survived for more than 2 weeks (Fig. SI). In the iPSC-CM alone group, cell survival was significantly decreased compared to that in the iPSC-CM with MSC group after day 4 (day 7: iPSC-CM alone, 34 ± 4%, iPSC-CM with MSC, 61 ± 7%, P = 0.008; day 10: iPSC-CM alone, 23 ± 5%, iPSC-CM with MSC, 47 ± 8%, P = 0.010; day 12: iPSC-CM alone, 7 ± 2%, iPSC-CM with MSC, 33 ± 8%, P = 0.004; day 14: iPSC-CM alone, 0 ± 0%, iPSC-CM with MSC, 18 ± 6%, P = 0.020), indicating that cell rejection occurred in the iPSC-CM alone group, whereas co-transplantation with MSCs suppressed allogeneic immune rejection (Fig. 2b).

Next, the T cell receptor repertoire was analysed using the spleens of mice administered iPSC-CMs alone or iPSC-CMs with MSCs on day 4 and 7 after sheet implantation, as well as those of a normal BALB/c mouse treated with a sham operation as a control. At day 4, in control, iPSC-CM alone, and iPSC-CM with MSC groups, no specific proliferated T cells were observed; however, these were identified in both iPSC-CM alone and iPSC-CM with MSC groups at day 7 (Fig. SII). These findings indicated that cell rejection might be one reason for the disappearance of the transplanted cells in both groups and that acquired immune rejection might function systemically after day 7 even when syngeneic MSCs are co-transplanted, but is suppressed locally in the presence of these cells.

**Co-transplantation with syngeneic MSCs suppresses the allogeneic immune reaction through Treg induction.** The expression of TGFβ1, IL2, IL10, FOXP3, and ITGAE transcripts in the iPSC-CM with MSC group was higher than that in the iPSC-CM alone group at the site of iPSC-CM sheet transplantation for 2 weeks after sheet transplantation (Fig. 2c). Immunohistochemistry revealed that the number of CD4 + CD25 + FOXP3 + cells in the iPSC-CM with MSC group (25 ± 2 cells/section) was significantly higher than that in the iPSC-CM alone group (0 ± 0 cells/section; P = 0.006) at the site of transplantation on day 7 (Fig. 2d). Immunohistochemistry also revealed that the number of CD8 + Annexin V + cells in mice administered iPSC-CM with MSCs (13 ± 2 cells/section) was significantly higher than that in animals treated with iPSC-CMs alone (1 ± 1 cells/section; P = 0.020) at the site of transplantation on day 7 (Fig. 2e). Thus, co-transplanted MSCs induced CD4 + CD25 + FOXP3 + Tregs by secreting IL-2 and TGF-beta, as well as CD8 + T cell apoptosis, in the transplanted area, both of which might suppress immune reaction against the allogeneic iPSC-CM sheets.

To assess the immunosuppressive effect of MSCs via the induction of Tregs, the Treg depletion model, in which an anti-CD25 antibody is administered to mice before the co-transplantation of iPSC-CMs and MSCs, was used. The survival of transplanted iPSC-CMs in the Treg depletion model increased compared to that with iPSC-CMs alone (day 7: iPSC-CM alone, 34 ± 5%, Treg depletion, 56 ± 9%, P = 0.049; day 10: iPSC-CM alone, 10 ± 2%, Treg depletion, 32 ± 8%, P = 0.025) and decreased compared to that in the iPSC-CM with MSC group (day 12: iPSC-CM with MSC, 33 ± 8%, Treg depletion, 12 ± 4%, P = 0.020; day 14: iPSC-CM with MSC, 18 ± 6%, Treg depletion, 1 ± 1%, P = 0.020; Fig. 2f). Based on immunohistochemistry, CD4 + CD25 + FOXP3 + cells or CD8 + AnnexinV+ cells were not detected at the transplant site in the Treg depletion model on day 7 after transplantation (Fig. 2g). These results indicated that the enhanced Treg population in the iPSC-CM with MSC group partially contributes to the extended survival of iPSC-CMs. Thus, mechanisms other than Tregs are required for the immunosuppressive effect of MSCs.

**Syngeneic MSCs affect activated lymphocytes through direct cell–cell contact.** To determine how MSCs exert their Treg-independent effect on iPSC-CM survival, we investigated the impact of syngeneic MSCs on activated lymphocytes using lymphocyte (lymph), lymphocytes + soluble factors from MSCs (lymph + SF), and lymphocytes + MSC (lymph + MSC) groups. First, the mitotic index, as a marker of the proliferation of activated lymphocytes, was analysed. There were no significant differences in mitotic indexes among the three groups (lymph: 1.2 ± 0.0, lymph + SF: 0.9 ± 0.1, lymph + MSC: 0.7 ± 0.0, ANOVA: P = 0.106; Fig. 3a). Second, the ratio of
CD8/CD4 lymphocytes as an effective T cell marker was analysed. The ratio in the lymph + SF group (0.24 ± 0.01) was lower than that in the lymph group (0.35 ± 0.00, P < 0.001), but was higher than that in the lymph + MSC group (0.06 ± 0.00, P < 0.001; ANOVA: P < 0.001; Fig. 3b). These results suggested that effective T cells are suppressed by MSCs but not by soluble factors. Third, the proportion of IFN-γ-positive cells among CD4-positive cells,
as a type 1 helper T (Th1) cell marker, was analysed. The proportion of IFN-γ-positive cells in the lymph group (4.7 ± 0.2%) was lower than that in the lymph + SF group (6.8 ± 0.1%, P < 0.001), but was significantly higher than that in the lymph + MSC group (0.9 ± 0.1%, P < 0.001; ANOVA: P < 0.001; Fig. 3c). These results suggested...
that the proportion of Th1 cells among CD4-positive cells were reduced by MSCs but not by soluble factors. Fourth, cytokine secretion in the culture medium was measured by enzyme-linked immunosorbent assay. The lymph + MSC group showed lower concentrations of GM-CSF (873 ± 291 pg/ml), IFN-g (248 ± 291 pg/ml), IL-2 (1708 ± 773 pg/ml), IL-3 (115 ± 82 pg/ml), and IL-13 (206 ± 3 pg/ml), compared to those in the lymph + SF group (GM-CSF: 2020 ± 277 pg/ml, P = 0.035; IFN-g: 1141 ± 286 pg/ml, P = 0.039; IL-2: 10021 ± 6 pg/ml, P = 0.001;
IL-3: 489 ± 76 pg/ml, *P* = 0.023; IL-13: 1652 ± 13 pg/ml, *P* < 0.001). Furthermore, the lymph + MSC group exhibited lower concentrations of IL-2, IL-4 (523 ± 272 pg/ml), and IL-13 compared to those in the lymph group (IL-2: 8826 ± 564 pg/ml, *P* = 0.002; IL-4: 2189 ± 557 pg/ml, *P* = 0.038; IL-13: 413 ± 18 pg/ml, *P* = 0.001; Fig. 3d). These results suggested that cytokine secretion was suppressed by MSCs but not by soluble factors. Thus, syngeneic MSCs reduced the CD8/CD4 ratio, the proportion of Th1 cells among CD4-positive cells, and the secretion of several cytokines, resulting in the suppression of effective T cell functions.

**Discussion**

The major finding of this study was that the proliferation of specific T cells and adaptive immune rejection after allogeneic iPSC-CM transplantation occur despite the presence of syngeneic MSCs (Fig. 4). However, co-transplanted syngeneic MSCs were found to induce CD4 + CD25 + FOXP3 + Tregs by secreting IL-2 and TGF-beta, as well as CD8 + T cell apoptosis, in peripheral tissues. In addition, syngeneic MSCs reduced the CD8/CD4 ratio, the proportion of Th1 cells among CD4-positive cells, and the secretion of several cytokines through cell–cell contact. Accordingly, the subcutaneous engraftment of iPSC-CM sheets was enhanced by these mechanisms.

More than 90% of iPSCs differentiated into cardiomyocytes using this differentiation induction protocol, described in previous experiments. The immunogenicity of undifferentiated iPSCs has been discussed, and several reports indicate that immunogenicity is reduced in the cells derived from iPSCs. However, the reduction of immunogenicity varies depending on the cell type. It is unclear whether co-transplanted MSCs suppress the immunogenicity of iPSCs or iPSC-CMs, but challenges with respect to immunoreactions remain even if allogeneic immune rejection could be resolved.

It was reported that MSCs have well-defined immunomodulatory properties including the suppression of activated immune cells such as T cells, B cells, dendritic cells, natural killer cells, and macrophages. Many studies have suggested that MSCs and immune cells have established two-way regulatory mechanisms; thus, the activation of MSC immunomodulatory properties requires the presence of derived proinflammatory cytokines from immune cells. Similarly, as a result of this activation, factors secreted by MSCs also regulate the immune response. Syngeneic MSCs exert this immunosuppressive effect via the intravenous administration of allogeneic cells, although co-transplantation with syngeneic MSCs into the peripheral tissue was reported to suppress allogeneic immune rejection, supporting the results of this study. Co-transplanted MSCs only act locally at the transplant site avoiding non-specificity. In addition, MSCs were reported to produce several pro-survival trophic factors that might play an important role in enhanced neighbour cell survival. It was also suggested...
that MSCs, which were co-transplanted with iPSC-CMs, might have a positive effect on cardiomyocyte matura-
tion and enhance their therapeutic effects for ischemic heart disease49. In addition, Treg cells not only have an
immunosuppressive effect but also the ability to induce the proliferation of cardiomyocytes and endothelial cells,
which might facilitate myocardial regenerative therapy60–62. Interestingly, previous studies have reported that
MSCs express and secrete PD-1 and that Treg cells proliferate through PD1 signalling, suggesting an increase in
the number of Treg cells after induction by MSCs43,44.

Interestingly, the present study demonstrated that the fraction of activated lymphocytes was affected by cell–
cell contact between MSCs and lymphocytes. It was reported that MSCs inhibit the proliferation of CD4-positive
and CD8-positive T cells45. Engela et al. demonstrated that MSCs can control CD8-positive T cells by inhibiting
their proliferation, supporting the results of this study.46 Unfortunately, we could not clarify the mechanism
underlying the reduction in CD8-positive T cell and Th1 cell fractions, although previous reports have suggested
the involvement of the immune checkpoint, wherein this ligand, appearing on the surface of MSCs, binds the
inhibitory receptor of T cells to suppress T cell immunoreactions45,47. Among immune checkpoint molecules in
T cells, only TIM3 is expressed at higher levels on CD8-positive T cells than on CD4-positive T cells, suggesting
that it might play an important role in the immunosuppressive effect of cell–cell contact between MSCs and lympho-
cytes in this study48. In addition, Galactin-9, which is the ligand of TIM3, is expressed on the surface of MSCs
and induces Th1 cell apoptosis by binding TIM3 on Th1 cells, leading to a reduction in CD8-positive cells49,50.
Therefore, the immune checkpoint molecule TIM3 might be a major mechanism underlying the decrease in
CD8-positive cells via cell–cell contact.

Furthermore, there is an increasing body of evidence indicating that tunnelling nanotubes facilitate molecular
and subcellular structure exchange between neighbouring cells via the transfer of molecules and organelles such
as calcium ions, prions, viral and bacterial pathogens, small lysosomes, and mitochondria51–55. Notably, Matula et al.
reported the bidirectional exchange of cytoplasmic components between MSCs and T cells, which was found
to be mediated by tunnelling nanotubes56. These findings support the contention that MSCs can affect the cell
microenvironment through the transfer of cellular components to neighbouring T cells, in a manner that signi-
ficantly contributes to cell regulation. Tunnelling nanotubes might thus contribute to the transfer of apoptosis
signals to neighbouring CD8-positive cells and Th1 cells. Thus, MSCs might have multiple functions regarding
the immunosuppression of neighbour cells, and our results could shed light on the mechanism underlying the
immunosuppressive effect of MSCs.

There are many merits associated with the use of allogeneic MSCs, and not syngeneic MSCs, considering
clinical applications; allogeneic MSCs can be prepared well in advance, and are independent of the recipient's
condition including disease status and age57. However, allogeneic MSCs were found to be immunoprimed early
after implantation but gradually lost this phenotype58,59. Recently, methods to prolong the engraftment of allogene-
ic MSCs have also been reported and the further development of such methods might allow the long-term cell
engraftment of allogeneic MSCs60,61.

Therapeutic treatment using allogeneic cells might be accompanied by the problem of immune rejection10. To
avoid this, extracellular vesicles or cytokines secreted by the cells could be collected and used as a treatment62–64.
However, this study aimed to establish a treatment that can provide not only the paracrine effect from the
iPSC-CMs but can also increase the number of cardiomyocytes to enhance cardiac contraction. If the problem of
immune rejection is resolved, the transplanted cells could continue to live in the recipient, secreting extracellular
vesicles and cytokines and forming contracts with native cardiomyocytes, resulting in more effective treatment.

In conclusion, the co-transplantation of MSCs might control immune rejection against allogeneic iPSC-CMs
after in vivo transplantation through the Treg induction and direct cell–cell contact; thus, this comprises a promis-
ing strategy for cardiomyogenic therapy using allogeneic iPSCs for severe heart failure.

Materials and Methods

Animal care procedures were consistent with the “Guide for the Care and Use of Laboratory Animals” (National
Institutes of Health publication). Experimental protocols were approved by the Ethics Review Committee for
Animal Experimentation of Osaka University Graduate School of Medicine (reference no. 25-025-045).

In vitro cardiomyogenic differentiation of murine iPSCs. Luciferase-miPSCs (959A2-1-6) generated
from C57BL/6 (B6) (CLEA) mouse embryonic fibroblasts were cultured in the absence of serum and feeder cells
using ESGRO Complete PLUS Clonal Grade Medium (Millipore, Burlington, MA, USA). Cardiomyogenic differ-
etiation of the iPSCs was performed as described, with modifications, followed by purification with glucose-free
medium supplemented with lactic acid52,53; iPSCs (3 × 10⁶) were resuspended in 100-μl aliquots of differentiation
medium [DM; Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque, Kyoto, Japan) containing
15% foetal bovine serum (FBS; Biofill, Melbourne, Victoria, Australia), 100 mmol/l non-essential amino acids
(NEAA; Invitrogen, Carlsbad, CA, USA), 2 mmol/l L-glutamine (Invitrogen), and 0.1 mmol/l 2-mercaptoethanol
(Invitrogen)], containing 0.2 mmol/l 6-bromoindirubin-3′-oxime (BIO; a glycerogen synthase kinase-3β) inhibitor
to activate the Wnt-signalling pathway; Calbiochem, San Diego, CA, USA), and cultured in 96-well Corning
Costar Ultra-Low attachment multiwell plates (MilliporeSigma, Burlington, MA) for 3 days. On day 3, an addi-
tional 100 μl of DM without BIO was added to each well. On day 5, individual embryoid bodies were transferred
to 100-mm gelatine-coated dishes (250 μl per dish). On days 6, 7, 10, 11, 14, and 15, the medium was exchanged for
serum-free Modified Eagle's Medium (MEM; Invitrogen) with insulin transferrin selenium X (Invitrogen). On
days 8, 9, 12, and 13, the medium was exchanged for glucose-free MEM (no glucose, no pyruvate, Invitrogen)
supplemented with 4 mmol/l lactic acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for
the purification of cardiomyocytes. On day 16, the contracting cell clusters were dissociated, seeded on thermo-
dresponsive dishes (5 × 10⁶ CMs/well; Upcell; CellSeed, Tokyo, Japan), and incubated at 37°C for 2 days. At this
time, they were transferred to 20°C until the cells detached spontaneously to form scaffold-free cell sheets. The protocol and purification process are illustrated in Fig. 1a.

**Immunocytotoxicity and analysis.** Dissociated single cells or harvested tissues around the transplant site were fixed with 4% paraformaldehyde and labelled with primary antibodies, which was followed by incubation with fluorescence-conjugated secondary antibodies, counterstaining with 4′, 6-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) or Hoechst33258 (Dojindo, Kumamoto, Japan), and finally analysis by confocal microscopy (FV1200, Olympus, Tokyo, Japan). The labelled cells were captured based on their fluorescence intensity. A list of the antibodies used can be found in Table SI.

**Quantitative reverse transcription PCR (RT-qPCR).** Total RNA from hiPSC-CMs cultured in vitro or from isolated tissue around the transplant site after cell sheet transplantation in vivo was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) or RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), respectively. RNA was reverse transcribed to cDNA using the SuperScript III reverse transcription kit (Thermo Fisher Scientific). RT-qPCR was performed using the Viia7 Real-Time PCR system (Thermo Fisher Scientific) in triplicate for each sample with TaqMan (Thermo Fisher Scientific) or SYBR green (Thermo Fisher Scientific) probes. The primers used for all PCR analyses can be found in Table SI. Expression levels were normalised to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Flow cytometry.** Cells were dissociated with 0.25% trypsin-EDTA (Thermo Fisher Scientific), fixed with CytoFix fixation buffer (Becton Dickinson, Franklin Lakes, NJ, USA) for 20 min, permeabilised with Perm/Wash buffer (Becton Dickinson) at 20°C for 10 min, and then incubated with primary antibody or isotype antibodies for 30 min. The labelled cells were washed with Perm/Wash buffer prior to incubation with the secondary antibody at room temperature for 30 min, and then assayed using a FACS Canto II (Becton Dickinson). A list of the antibodies used can be found in Table SI.

**T cell proliferation assay.** After labelling with CFSE (Cayman Chemical Company, Ann Arbor, MI, USA), splenic lymphocytes (5 × 10⁵ cells/well) from BALB/c mice were incubated on 24-well plates coated with an anti-mouse CD3 antibody (clone: 145-2C11) with the same number of MSCs from BALB/c mice (MUBMX-01001, Cyagen Biosciences Inc, Santa Clara, CA, U.S.) (lymp + MSC) and without MSCs (lymp) using 1000µl of RPMI 1640 containing 10% FBS. To assess the effects of MSC-secreted soluble factors, lymphocytes and MSCs were also co-cultured without direct cell–cell contact using transwell inserts (3.0-µm pore polycarbonate membrane; Corning Inc., Armonk, NY, USA) and the MSCs were removed before performing the assay (lymp + SF). An anti-mouse CD28 antibody (clone; 37.51, 100 ng/ml) was added to each well of the CD3-coated plate. After incubation for 72 h, the cells were dissociated with 0.25% trypsin-EDTA (Thermo Fisher Scientific) and stained with anti-mouse CD3 antibodies for 30 min, and then assayed using a FACS Canto II (Becton Dickinson). The mitotic index was provided as the sum of the mitotic events in all generations divided by the calculated number of original parent cells. A list of antibodies used can be found in Table SI.

**Bio-plex.** The concentration of proteins secreted from cultured cells was measured using the Bio-Plex suspension array system (23-plex; Bio-Rad Laboratories) according to the manufacturer's instructions. The culture media in the lym, lym + SF, and lym + MSC groups were used for this experiment.

**Cell sheet transplantation.** Adult male BALB/c mice (6–7 weeks old, 17–22 g) were generally anesthetised through the inhalation of isoflurane, and subjected to the subcutaneous transplantation of luciferase transgenic allogeneic iPSC-CM sheets with the intramuscular injection of 200µl of PBS alone (CM) or 5 × 10⁶ MSCs in 200µl of PBS (CM + MSC) at the site of iPSC-CM sheet transplant.

**Treg depletion model.** BALB/c mice were administered intraperitoneal injections of 0.5 mg of an anti-CD25 antibody (clone PC61) 3 days before and 4, 11, and 18 days after the implantation of iPSC-CM sheets. PC61 administration promoted the long-lasting depletion/neutralisation of CD25 expression (longer than 2 weeks) in native mice, as previously demonstrated⁷. A list of antibodies used can be found in Table SI.

**Tacrolimus administration.** An osmotic pump (ALZET Micro-Osmotic Pump model 1002; DURECT Corporation, Cupertino, CA, USA) filled with 1.5 mg/kg body weight of tacrolimus was subcutaneously implanted under the abdominal skin of the BALB/c mice to create an allogeneic model of immunosuppression. As one pump can continuously deliver the solution for 14 days, another new pump was added every 13 days to ensure the constant release of tacrolimus.

**In vivo imaging.** Bioluminescent images were acquired using an In Vivo Imaging System (Perkin Elmer, Waltham, MA, USA). Animals were anesthetised with 2% isoflurane gas in oxygen, and 150 mg/kg D-luciferin (Summit Pharmaceuticals International Corporation, Tokyo, Japan) was injected intraperitoneally. Images were acquired 10 min after injection at the peak of the bioluminescence signal. Images were quantified by drawing a region of interest over the transplanted region, with data expressed as photon flux (p/s).

**Statistical analysis.** Data are presented as the means with standard errors for continuous variables. Continuous variables were examined using the Student t-test. The one-way ANOVA test was used to compare values between more than two groups. When the one-way ANOVA test was significant, group differences were compared using the post hoc Tukey HSD test. Statistical analyses were performed using JMP® 13 (SAS Institute Inc., Cary, NC, USA). Statistical significance was defined as P < 0.05.
Data availability
The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Received: 19 July 2019; Accepted: 7 January 2020;
Published online: 12 March 2020

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Acknowledgements
We thank Seiko Eiraku and Akima Harada for their technical support. This research was supported by The Japan Agency for Medical Research and Development project (Research Center Network for Realization of Regenerative Medicine) under Grant Number 17hm0204003h0005.
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S.Y.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; S.M. and T.T.: conception and design, administrative support, data interpretation, manuscript writing; S.F.: conception and design; T.K., A.K., N.K., and Y.N.: collection of data; K.T.: conception and design, data interpretation; Y.S.: conception and design, administrative support, provision of study material, data interpretation, final approval of manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-58126-z.

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