# SUPPLEMENTAL MATERIAL

## Immunomodulatory Cell Therapy Using αGalCer-Pulsed Dendritic Cells Ameliorates Heart Failure in a Murine Dilated Cardiomyopathy Model

Masataka Ikeda MD, PhD*; Tomomi Ide MD, PhD*; Shouji Matsushima MD, PhD; Soichiro Ikeda MD, PhD; Kosuke Okabe MD, PhD; Akihito Ishikita MD; Tomonori Tadokoro MD, PhD; Masashi Sada MD; Ko Abe MD; Midori Sato, BEng; Akiko Hanada, Associate Degree; Shinobu Arai PhD; Kisho Ohtani MD, PhD; Atsushi Nonami MD, PhD; Shinichi Mizuno MD, PhD; Sachio Morimoto PhD; Shinichiro Motohashi MD, PhD; Koichi Akashi MD, PhD; Masaru Taniguchi MD, PhD; Hiroyuki Tsutsui MD, PhD

*To whom correspondence should be addressed: Masataka Ikeda and Tomomi Ide

Department of Cardiovascular Medicine, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan

3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Phone: +81-92-642-5360, Fax: +81-92-642-5374

Email: ikeda-m@cardiol.med.kyushu-u.ac.jp (M.I.) and tomomi_i@cardiol.med.kyushu-u.ac.jp (T.I.)

# SUPPLEMENTAL METHODS

## Study design

The objectives of this study were to (1) examine the responses to αGCDC treatment, (2) test the therapeutic efficacy of αGCDCs for HF in mouse DCM models, and (3) investigate the molecular basis underlying this therapy. To address the first objective, we examined dose-dependency and time-course of αGCDC treatment in BALB/c mice. To address the second objective, we evaluated the survival of DCM mice following treatment with CTRL-DCs and αGCDCs using a murine DCM model.[29, 30] All eligible DCM mice were genotyped and randomly assigned to PBS, CTRL-DC, and αGCDC treatment groups. To address the third objective, we assigned DCM mice as described above and obtained the myocardium from each animal on day 4 after treatment.

## Preparation of αGCDCs from human peripheral blood

Eligible volunteers, who provided written informed consent, underwent peripheral blood leukapheresis (Spectra Optia, TERUMOBCT, Tokyo, Japan), after which peripheral blood mononuclear cells (PBMCs) were collected and further separated by density gradient centrifugation using Ficoll-Paque PREMIUM (17544202, GE Healthcare Life Sciences, Amersham Place, Buckinghamshire, UK). PBMCs were washed thrice and re-suspended in AIM-V containing albumin extracted from self-plasma with 800 units/mL of human granulocyte macrophage colony-stimulating factor (GM-CSF; PMC-GM, Primmune Inc, Kobe, Japan) and 100 units/mL of recombinant IL-2 (Imunace, Shionogi, Osaka, Japan). After 6-day culture, the cells were pulsed with 100 ng/mL of αGalCer (Wako, Osaka, Japan

or Funakoshi, Tokyo, Japan). After pulsation with αGalCer for 24 h, the cells were collected as αGCDCs and administered to mice within 24 h after collection.

**Echocardiography**

Echocardiographic data were obtained as previously described.[48, 49] Briefly, two-dimensional targeted M-mode images were obtained from the short axis view at the papillary muscle level using a Vevo 2100 ultrasonography system (Visual Sonics, Toronto, Canada) under light anesthesia with 1–2% isoflurane. LVEF was calculated using the following formula that was pre-programmed in the system: LVEF = [(diastolic LV volume − systolic LV volume) / diastolic LV volume) × 100], where LV volume = [(7.0 / (2.4 + LV diameter)] × LV diameter$^3$. The mean wall thickness was measured as the mean value of the thicknesses of interventricular septum and LV posterior wall. After the measurements, the mice were euthanized with an overdose of sodium phenobarbital. The study design was fully comprehended by two investigators (M.I. and A.H.) but was blinded to other investigators (S.I., K.O., A.I., T.T., M. Sada., and K.A.) who injected PBS, CTRL-DCs, and αGCDCs and obtained echocardiographic measurements.

**Western blotting**

Western blotting was performed as previously described.[50, 51] Myocardial tissue and cells were homogenized in a radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), 1 mM NaF, and 0.1 mM $Na_3VO_4$. Equal amounts of proteins (10–20 μg per lane) were separated using sodium dodecyl sulphate-polyacrylamide gel

electrophoresis and then electrophoretically transferred to a nitrocellulose membrane using a Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After blocking for 1 h with skimmed milk in Tris-buffered saline with 1% Tween 20, the membrane was incubated with the indicated primary antibodies at 4°C overnight, followed by the incubation with appropriate secondary antibodies at room temperature for 1 h. Primary antibodies from Cell Signaling Technology (Danvers, MA, USA) against the following proteins were used: Stat1 (#14995), pStat1 (#7649), Stat6 (#5397), pStat6 (#56554), Stat3 (#30835), pStat3 (#9145), Smad2 (#5539), pSmad2 (#18338), Smad3 (#9523), and pSmad3 (#9520). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control.

**Reverse transcription quantitative PCR (RT-qPCR)**

Total RNA was isolated from tissues stored in RNA later (Sigma-Aldrich, St. Louis, MO, USA) or cultured cells using a RNeasy Mini kit (Qiagen, Valencia, CA, USA) and converted to cDNA using a ReverTra Ace kit (Toyobo, Tokyo, Japan) as described previously.[52, 53] The relative expression of each target gene was measured via RT-qPCR from cDNA, which was equivalent to 10 ng RNA, by using 12 pmol of each primer in a 25-μL reaction volume, utilizing the THUNDERBIRD SYBR qPCR Mix (Toyobo, Tokyo, Japan). The reactions were run on an AB7500 Fast Real-Time PCR system (Life Technologies, Rockville, MD, USA) in accordance with the cycle program recommended in the manufacturer's instructions for THUNDERBIRD SYBR qPCR Mix. The *18S* ribosomal RNA gene was used as an internal control. The forward (F) and reverse (R) primer sequences are as follows: (Mouse) *Rps18*: F 5′-

TTCTGGCCAACGGTCTAGACAAC-3′, R 5′-CCAGTGGTCTTGGTGTGCTGA-3′; *Vα14Jα18*: F 5′-CTAAGCACAGCACGCTGCACA-3′, R 5′-CAAAATGCAGCCTCCTAAG-3′; *Ifng*: F 5′-CGGCACAGTCATTGAAAGCCTA-3′, R 5′-GTTGCTGATGGCCTGATTGTC-3′; *Il10*: F 5′-GCCAGAGCCACATGCTCCTA-3′, R 5′-GATAAGGCTTGGCAACCCAAGTAA-3′; *Il4*: F 5′-ACGGAGATGGATGTGCCAAAC-3′, R 5′-AGCACCTTGGAAGCCCTACAGA-3′; *Nppb*: F 5′-AGCTGCTTTGGGCACAAGATAGA-3′, R 5′-CCAGGCAGAGTCAGAAACTGGAG-3′; *Tgfb*: F 5′-GTGTGGAGCAACATGTGGAACTCTA-3′, R 5′-CGCTGAATCGAAAGCCCTGTA-3′; *Ctgf* (*Ccn2*): F 5′-TGCAGACTGGAGAAGCAGAG-3′, R 5′-CGATTTTAGGTGTCCGGATG-3′; *Col1a*: F 5′-GACTGGCAACCTCAAGAAGG-3’, R 5′-GACTGTCTTGCCCCAAGTTC-3′; *Col3a*: F 5′-CTGTAACATGGAAACTGGGGAAA-3′, R 5′-CCATAGCTGAACTGAAAACCACC-3′; *Angpt1*: F 5′-GATCTTACACGGTGCCGATT-3′, R 5′-TGATTTTGTCCCGCAGTGTA-3′; *Vegfa*: F 5′-ACATTGGCTCACTTCCAGAAACAC-3′, R 5′-TGGTTGGAACCGGCATCTTTA-3′; *Smad7*: F 5′-CTGCTGTGCAAAGTGTTCAGG-3′, R 5′-CCATTGGGTATCTGGAGTAAGGA-3′; (Rat) *Rps18*: F 5′-AAGTTTCAGCACATCCTGCGAGTA-3′, R 5′-TTGGTGAGGTCAATGTCTGCTTTC-3′; *Ctgf* (*Ccn2*): F 5′-CATGGTCAGGCCCTGTGAA-3′, R 5′-CACAGAACTTAGCCCGGTAGGTC-3′; *Col1a1*: F 5′-GACATGTTCAGCTTTGTGGACCTC-3′, R 5′-GGGACCCTTAGGCCATTGTGTA-3′; *Col3a1*: F 5′-

TTTGGCACAGCAGTCCAATGTA-3′, R 5′-GACAGATCCCGAGTCGCAGA-3′; *Angpt1*: F 5′-ACCGTGAGGATGGAAGCCTAGA-3′, R 5′-ATGAACTCGTTCCCAAGCCAATA-3′; and *Smad7*: F 5′-TGCTGTGCAAAGTGTTCAGGTG-3′, R 5′-CCATCGGGTATCTGGAGTAAGGA-3′.

**Flow cytometric analysis of invariant NKT cells**

Murine spleens were homogenized in FBS buffer (553656, BD Biosciences, CA, USA), and homogenates were filtered using a Falcon Cell Strainer (352360, Corning Inc., Corning, NY, USA). After centrifuging at 200 × *g* for 5 min, 1 × BD Pharm Lyse B (555899, BD Pharm Lyse, BD Biosciences, CA, USA) was added to the lysates, and samples were incubated at 37 ℃ for 3 min. After incubation, FBS buffer was added to a total volume of 20 mL, and the samples were centrifuged at 200 × *g* for 5 min. The pellet was suspended in 2 mL FBS buffer and filtered using a Falcon Cell Strainer (352350, Corning Inc.). FBS buffer was added to a total volume of 20 mL and centrifuged at 200 × *g* for 5 min again. After cell counting, $3 \times 10^6$ cells were suspended in 100 μL FBS buffer and blocked with 1 μg antibody against CD16/CD32 (553141, BD) at 4 ℃ for 15 min. After washing cells twice with 1 mL FBS buffer, cells were labeled with 10 μL α-GalCer-loaded CD1d-tetramer (prepared as follows) and anti-CD3 antibody (BD, #561826) at room temperature for 30 min. After washing three times with 1 mL FBS buffer, cells were again suspended in 100 μL FBS buffer. Then, 5 μL of 7-AAD (7AAD, Immunostep, Salamanca, Spain) was added and incubated for 5 min immediately before the measurement. Next, 300 μL of FBS buffer was added, and flow cytometry was performed using BD FACSVerse (BD). Data obtained from the measurements were analyzed using

FlowJo as follows. First, 7-AAD-positive cells were removed as dead cells and lymphocytes were gated based on the 7-AAD-negative cells. Then, doublets were removed, and cells were gated by APC (CD3) and PE (CD1d tetramer). CD3-positive cells were defined as T cells, and CD3/CD1d-tetramer double positive cells were defined as invariant NKT cells. α-GalCer-loaded CD1d-tetramer was prepared using the following: 10 μL of CD1d tetramer solution (TS-MCD-1 [BML, Aichi, Japan]; 200 μg/mL in 0.5% Tween + 0.9% NaCl) plus 0.5 μL α-GalCer solution (G0509 [Tokyo Chemical Industry, Tokyo, Japan]; 1 mg/mL dissolved in pyridine [Q0034, Tokyo Chemical Industry, Tokyo, Japan]) incubated overnight at room temperature. α-GalCer-unloaded CD1d-tetramer and hamster IgG1κ-APC (553974, BD) were used as isotype controls, respectively.

**Plasma cytokine measurements**

Plasma levels of IFNγ, IL-4, and IL-10 were measured using the Quantikine ELISA Kit (IFNγ, MIF00; IL-4, M4000B; IL-10, M1000B, R&D systems, MN, USA) in accordance with the manufacturer's instructions.

**Histological analysis**

Interstitial fibrosis based on Picro-Sirius Red staining was measured as previously described.[54] Images were obtained using a BZ-X800 (KEYENCE, Osaka, Japan), and the collagen volume fraction (fibrosis/total myocardium) of each animal was measured from the tiling image of whole section at the apex using the ImageJ software (NIH, Bethesda, MD, USA). Capillary dense area was measured as follows: the 3 μm-thick sections cut from LV tissue embedded in paraffin were deparaffinized and subjected to antigen-retrieval

using a standard autoclave protocol in 10 mM citrate buffer (pH 6.0). After blocking the myocardium with carbo-free blocking solution (SP-5040) for 30 min, endothelial cells were stained with tomato lectin (10 μg/mL in PBS, DL-1177, Vector Laboratories, CA, USA). After washing with TBS-T, sections were fixed with mounting agent (H-1800, Vector Laboratories, CA, USA). Images were obtained using the BZ-X800 and the capillary density was defined as the ratio of the capillary area per total myocardium that was analyzed using ImageJ software.

**Microarray**

Microarray analysis was performed using the Low Input Quick Amp Labeling Kit and SurePrint G3 Mouse Gene Expression 8×60K v2 (Agilent, CA, USA). RNA samples were quantified on a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was confirmed with an Experion System (Bio-Rad Laboratories, Hercules, CA). For each group, four samples containing equal amounts of RNA, which passed the quality check, were mixed. The cRNA was amplified, labeled, and hybridized to a 60K Agilent 60-mer oligomicroarray, according to the manufacturer's instructions. All hybridized microarray slides were scanned using an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Raw signal intensities and Flags for each probe were calculated from hybridization intensities (gProcessedSignal) and spot information (glsSaturated, etc.), according to the procedures recommended by Agilent (Flag criteria on Gene Spring Software. Absent (A): "Feature is not positive and significant" and "Feature is not above background." Marginal (M): "Feature is not Uniform," "Feature is Saturated."

and "Feature is a population outlier." Present (P): Others). Raw signal intensities of two samples were $\log_2$-transformed and normalized based on a quantile algorithm with "preprocessCore" library package in the Bioconductor software.[55, 56] We selected probes that called "P" flags for at least one sample. To identify up- or down-regulated genes, we calculated Z-scores[57] and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe for comparisons between the control and the experiment sample. Finally, we established criteria for regulatory genes as follows: (upregulated genes) Z-score $\geq 2.0$ and ratio $\geq 1.5$-fold, (downregulated genes) Z-score $\leq -2.0$ and ratio $\leq 0.66$.

**K-means clustering and GO analysis**

Using the dataset obtained from the microarray, we performed K-means clustering utilizing the MeV program as described previously.[58] Briefly, we excluded the low intensity genes and selected 28,381 genes. Next, we categorized 10 clusters by calculating their distances 50 times using the Pearson correlation coefficient, and then analyzed cluster-2 (3,278 genes) and cluster-9 (1,899 genes). These clusters contained genes which exhibited changes in expression in the DCM+PBS group compared to the levels in the WT+PBS group. In cluster-2, we selected 277 genes that were upregulated in the DCM+PBS group > 1.5-fold compared to levels in the WT+PBS group but downregulated in the DCM+αGCDC group. We selected 117 genes that were downregulated in the DCM+PBS group > 1.5-fold, as compared to the WT+PBS group, but upregulated in the DCM+αGCDC group in cluster-9. These genes were then subjected to GO analysis via Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.8) bioinformatics resources

(http://david.abcc.ncifcrf.gov/) and demonstrated as GOTERM_BP_FAT (BP=Biological Process) after analysis.

**Cell culture**

Primary cultures of neonatal rat ventricular cardiomyocytes and fibroblasts were prepared from the ventricles of neonatal Sprague-Dawley (SD) rats as described previously.[59, 60] Briefly, neonatal rats were euthanized with an overdose of isoflurane, following which the hearts were rapidly excised. After digesting the myocardial tissues with trypsin and collagenase type 2, the cells were suspended in DMEM (Sigma-Aldrich, D6046) containing 10% FBS (Sigma-Aldrich, F7524, lot. BCBW9572) and 1% penicillin/streptomycin (P/S; Nacalai Tesque, 26253-84). Cells were plated twice in 100-mm culture dishes for 70 min each to reduce the number of non-myocytes. Nonadherent cells ($2.5 \times 10^5$/mL) were plated in culture dishes (Primaria, Corning Inc., NY, USA) for each experiment using cardiomyocytes. Adherent cells were passaged twice, and passaged cells (P2) were used as fibroblasts. Cells were maintained in DMEM containing 10% FBS and P/S, at 37 °C and humidified air at 5% $CO_2$.

**Experimental protocols for cell analysis**

To analyze the expression of Smad2/3 and fibrotic genes in primary cultured fibroblasts (P2), cells were treated with IFNγ (400-20, PeproTech, Cranbury, NJ, USA), IL-4 (504-RL-025, R&D systems, Minneapolis, MN, USA), or IL-10 (400-19, PeproTech, Cranbury, NJ, USA) for 24 h. Rat TGF-β (10 ng/mL, 80116-RNAH, Sino Biological, Beijing, China) treatment was started at 16 h after exchanging medium containing IFNγ without bovine

serum and run for 24 h. To analyze *Angpt1* expression in primary cultured cardiomyocytes, the cells were treated with IFNγ, IL-4, and IL-10 for 24 h at 16 h after exchanging the medium without bovine serum.

**Transfection of siRNA**

Rat Stat1 knockdown was performed via siRNA transfection using Lipofectamine RNAiMAX (Thermo Fisher Scientific) as previously described.[61, 62] The siRNA used against rat *Stat1* was purchased from Thermo Fisher Scientific (s129045).

# Figure S1

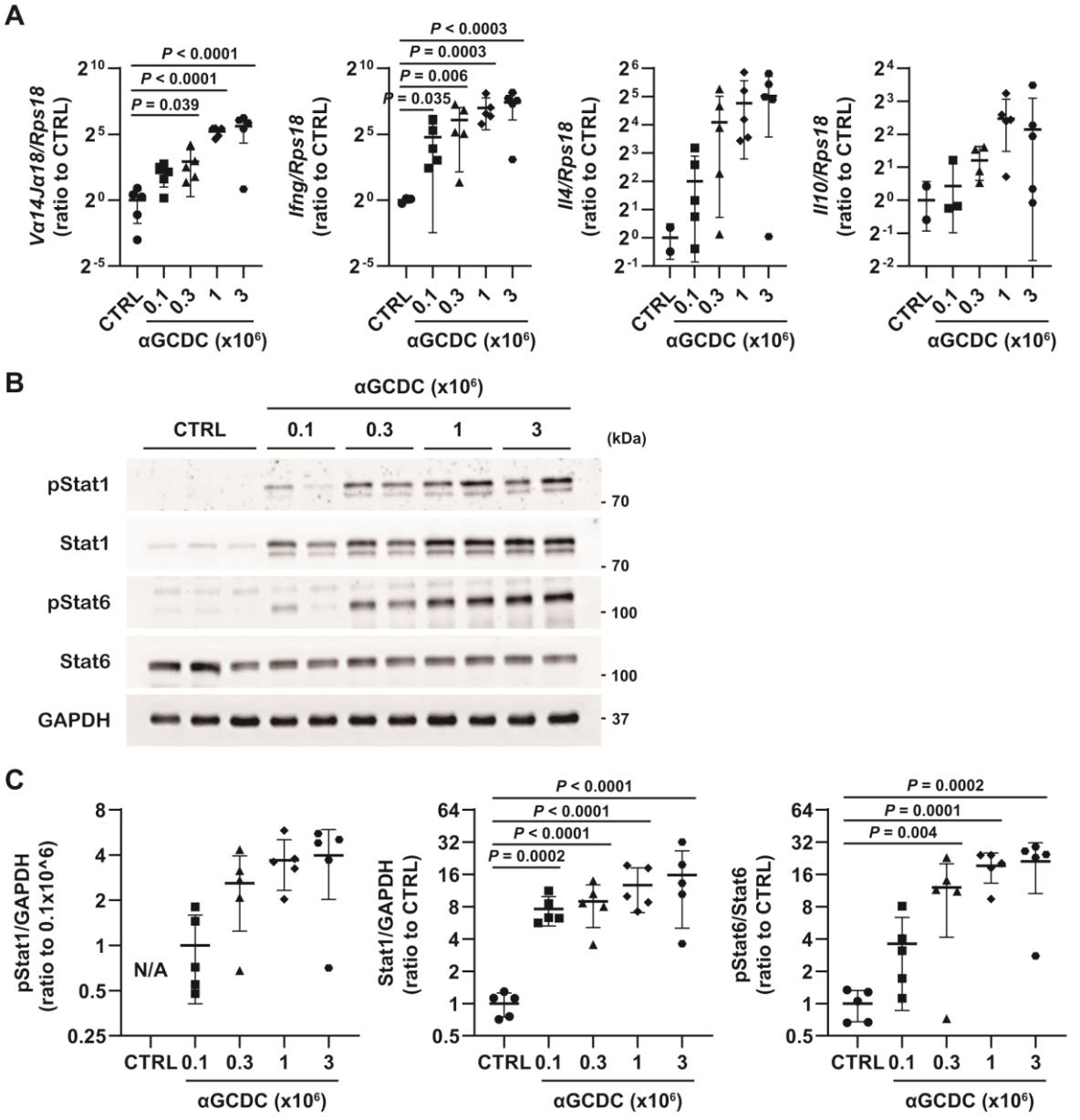



**Figure S1. Dose-dependent responses to αGalCer-pulsed dendritic cells (αGCDCs) in the myocardium on day 4 after treatment in BALB/c mice.** (**A**) Gene expression of invariant TCR *(Vα14Jα18)*, *Ifng, Il4*, and *Il10* in response to αGCDC treatment (n = 5), measured using RT-qPCR. (**B**) Phosphorylation of Stat1 and Stat6 in response to αGCDC treatment. (**C**) Quantification of western blot results shown in panel **B** (n = 5). GAPDH was used as a loading control. Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's test.

# Figure S2

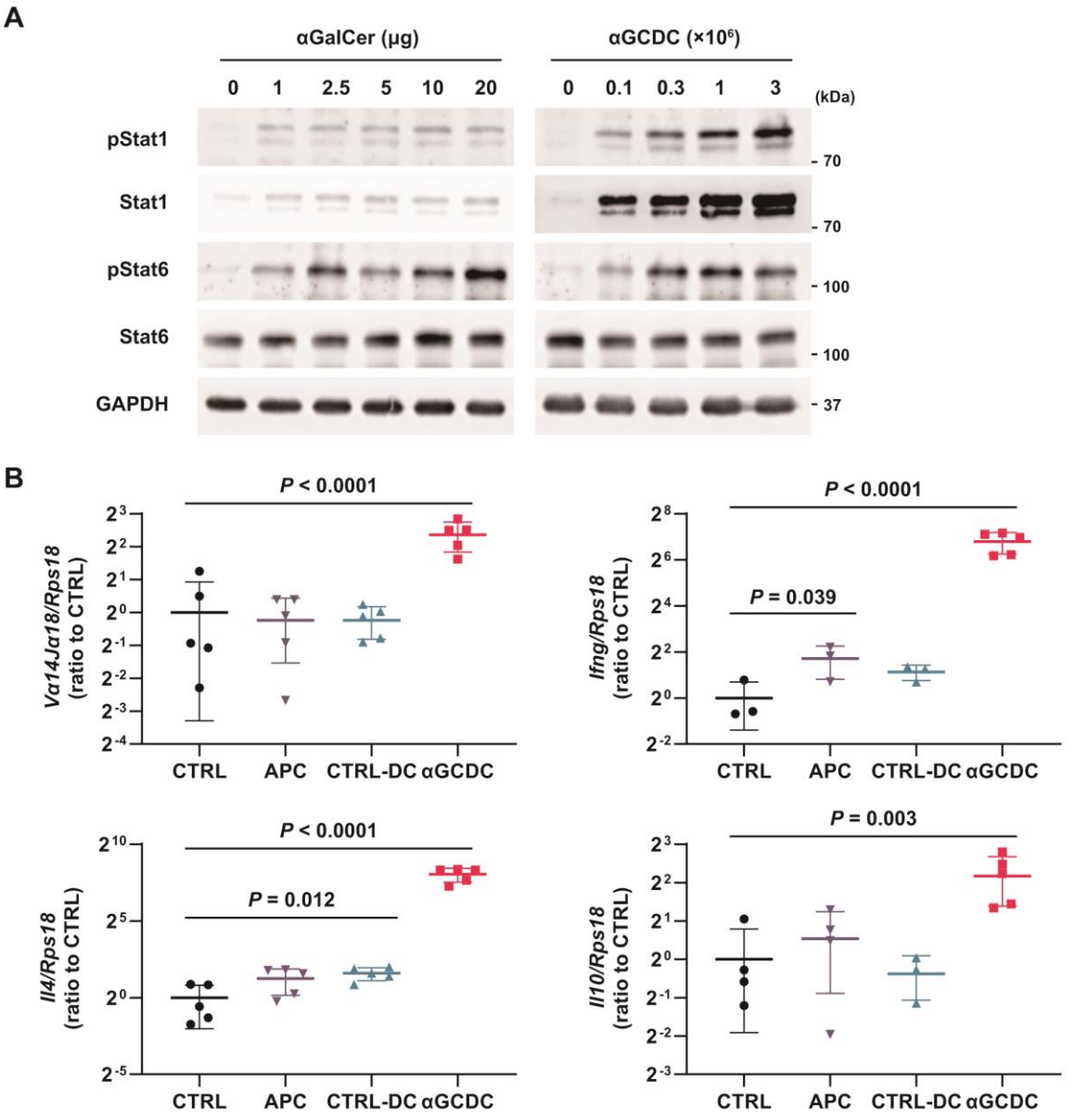



**Figure S2. Comparison of treatments with αGalCer-pulsed dendritic cells (αGCDCs), only αGalCer, isolated antigen-presenting cells (APCs), and control-dendritic cells without αGalCer (CTRL-DCs) in BALB/c mice.** (**A**) Phosphorylation of Stat1 and Stat6 in the myocardium on day 4 after αGalCer and αGCDC treatments. (**B**) Gene expression of *Vα14Jα18*, *Ifng*, *Il4*, and *Il10* in response to treatments with APCs, CTRL-DCs, and αGCDCs *in vivo*, measured using RT-qPCR (n = 3–5). Samples that could not be measured (not detected) were excluded. GAPDH was used as a loading control. Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's test.

# Figure S3

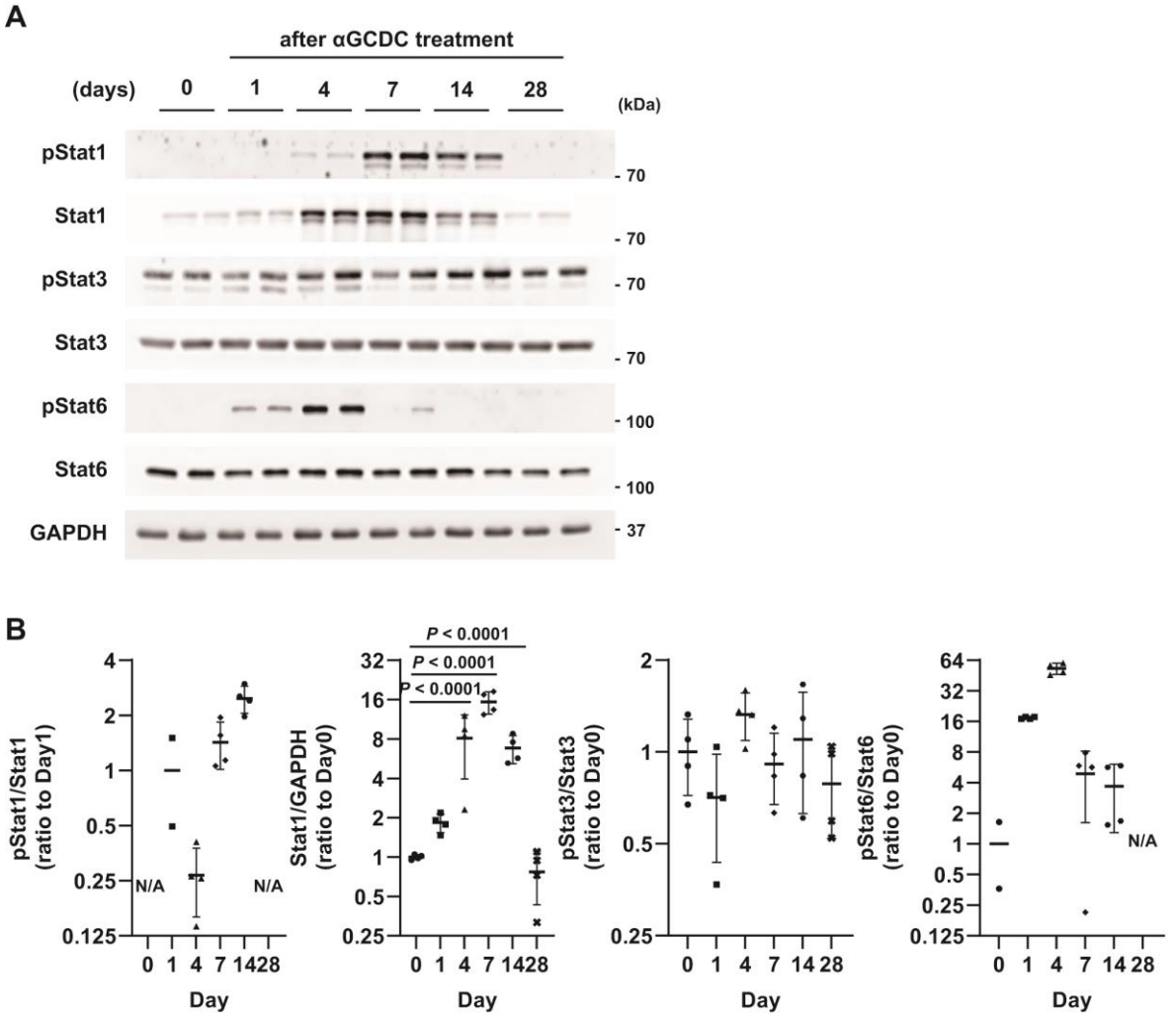



**Figure S3. Serial changes in Stat phosphorylation in the myocardium after αGalCer-pulsed dendritic cells (αGCDCs) treatment in BALB/c mice.** (**A**) Western blot for phosphorylation of Stat1, Stat3, and Stat6 until day 28 following αGCDC treatment. (**B**) Quantification of western blot results shown in panel **A** (n = 4). GAPDH was used as a loading control. Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's test.

# Figure S4

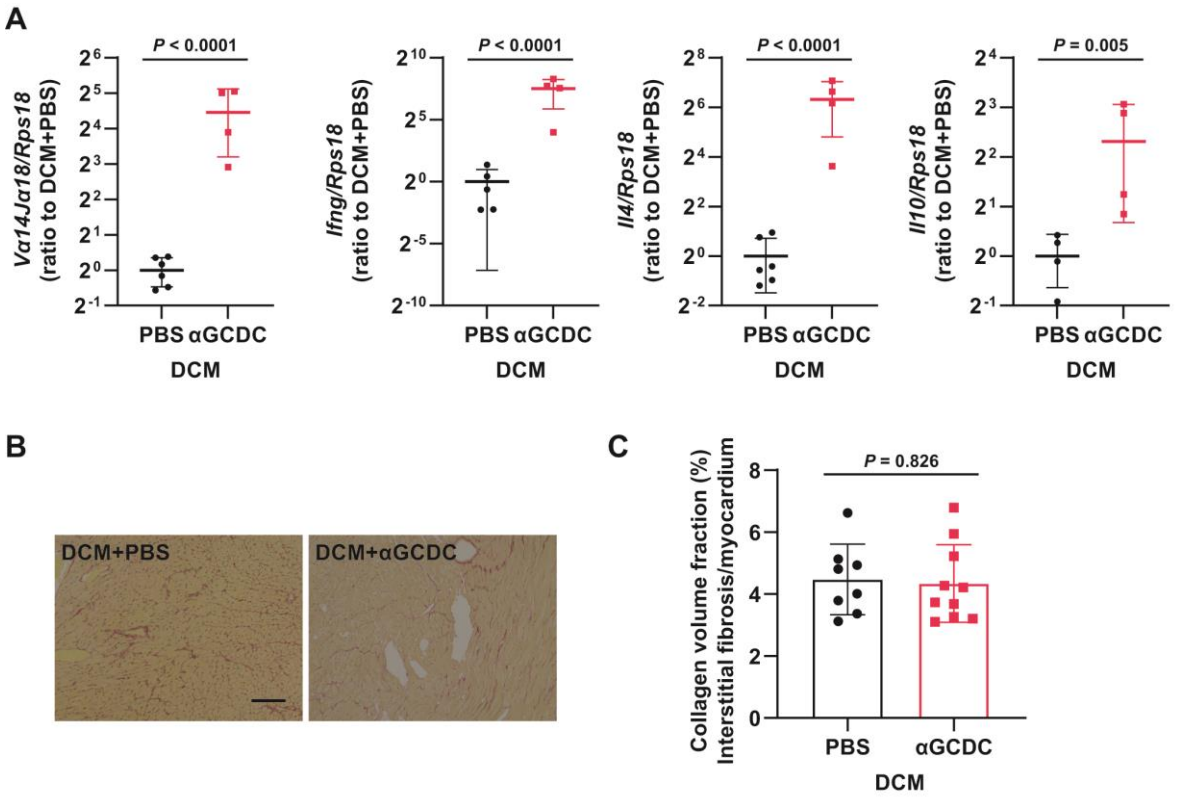



**Figure S4. Gene expression and fibrosis on day 4 after treatment with αGCDCs in dilated cardiomyopathy (DCM) mice.** (**A**) Gene expression of invariant TCR (*Vα14Jα18*), *Ifng*, *Il4*, and *Il10* in the myocardium of DCM mice on day 4 following αGCDC treatment, measured using RT-qPCR (n = 4–7). Samples that could not be measured (not detected) were excluded. (**B**) Representative images of Picro-Sirius Red-stained samples from DCM mice treated with PBS and αGCDCs. Scale bars indicate 100 μm. (**C**) Fibrotic area per myocardium in DCM mice treated with PBS (n = 8) and αGCDCs (n = 10). Data are presented as mean ± SD. Statistical significance was determined using Student's *t*-test**.**

# Figure S5

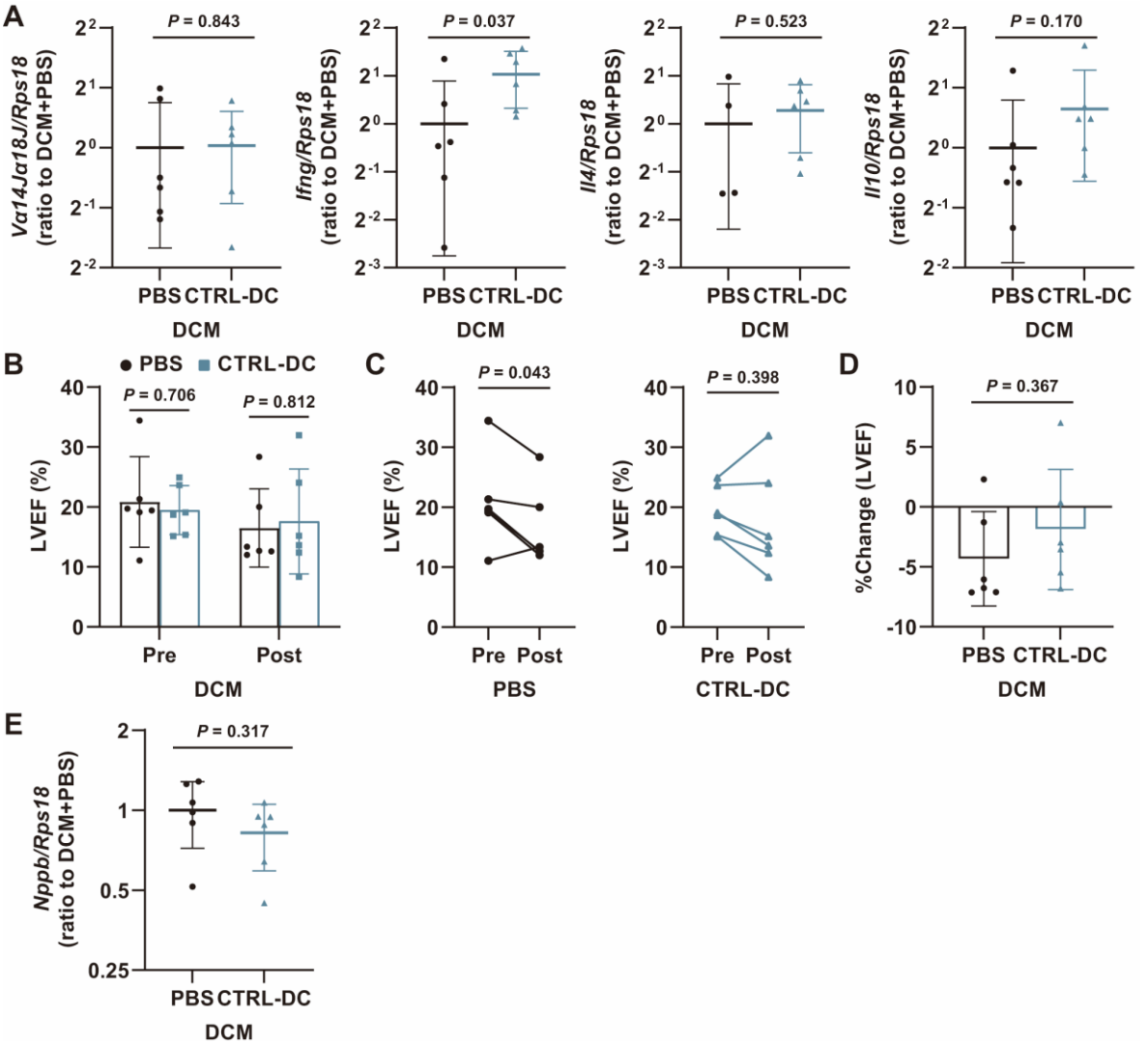



**Figure S5. Phenotypes and gene expression on day 4 after treatment with control-dendritic cells without αGalCer (CTRL-DCs) in dilated cardiomyopathy (DCM) mice.** (**A**) Gene expression of invariant TCR (*Vα14Jα18*), *Ifng*, *Il4*, and *Il10* in the myocardium of DCM mice on day 4 following CTRL-DC treatment, measured using RT-qPCR (n = 4–6). Undetectable samples were excluded. (**B**) Left ventricular ejection fraction (LVEF) in DCM mice treated with PBS and CTRL-DC (n = 6, each). (**C**) Individual changes in the LVEF of DCM mice treated with PBS and CTRL-DC (n = 6, each). (**D**) Percent change in the LVEF from the beginning to the end of the study (day 4) in mice treated with PBS and CTRL-DC (n = 6, each). (**E**) Gene expression of brain natriuretic peptide (BNP, *Nppb*) in the myocardium of DCM mice on day 4 after PBS and CTRL-DC treatment, measured using RT-qPCR (n = 6). Data are expressed as mean ± SD. Statistical significance was determined using paired (for panel **C**) and unpaired (other panels) Student's *t*-test.

# Figure S6

**A**

52145 genes

↓ Grouping genes by expression pattern (K-means)

10 clusters

↓ Genes changing 1.5-fold were picked.

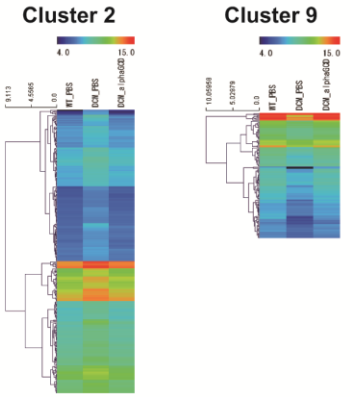
Cluster2:

277 genes, upregulated in DCM+PBS
suppressed in DCM+αGCDC

Cluster9:

117 genes, downregulated in DCM+PBS
increased in DCM+αGCDC

**B**




**C**

| Cluster2: GO Term | Count | % | P value |
|---|---|---|---|
| Fat cell differentiation | 4 | 9.1 | <0.001 |
| Cellular response to fibroblast growth factor stimulus | 3 | 6.8 | 0.002 |
| Positive regulation of transcription from RNA polymerase II promoter | 8 | 18.2 | 0.007 |
| Cellular response to transforming growth factor beta stimulus | 3 | 6.8 | 0.008 |
| Response to mechanical stimulus | 3 | 6.8 | 0.009 |
| Positive regulation of endothelial cell proliferation | 3 | 6.8 | 0.010 |
| Positive regulation of osteoblast differentiation | 3 | 6.8 | 0.010 |
| Endothelial cell chemotaxis | 2 | 4.5 | 0.022 |

| Cluster9: GO term | Count | % | P value |
|---|---|---|---|
| Morphogenesis of an epithelium | 4 | 3.7 | <0.001 |
| Vasculogenesis | 5 | 4.6 | <0.001 |
| Fatty acid metabolic process | 6 | 5.6 | 0.001 |
| Positive regulation of phagocytosis | 4 | 3.7 | 0.002 |
| Cellular response to tumor necrosis factor | 5 | 4.6 | 0.002 |
| Response to lipopolysaccharide | 6 | 5.6 | 0.003 |
| Innate immune response | 8 | 7.4 | 0.004 |
| Inflammatory response | 7 | 6.5 | 0.008 |

**Figure S6. Gene ontology (GO) analysis based on microarray data obtained from dilated cardiomyopathy (DCM) mouse hearts 4 days after α-galactosylceramide-pulsed dendritic cell (αGCDC) treatment.** (**A**) Algorithm of GO analysis using datasets obtained from the microarray. Expression of genes in cluster 2 was downregulated in DCM mice and restored by αGCDC treatment. Expression of genes in cluster 9 was upregulated in DCM mice and suppressed via αGCDC treatment. (**B**) Heatmaps of cluster 2 and cluster 9. (**C**) Top GO terms in cluster 2 and cluster 9.

# Figure S7

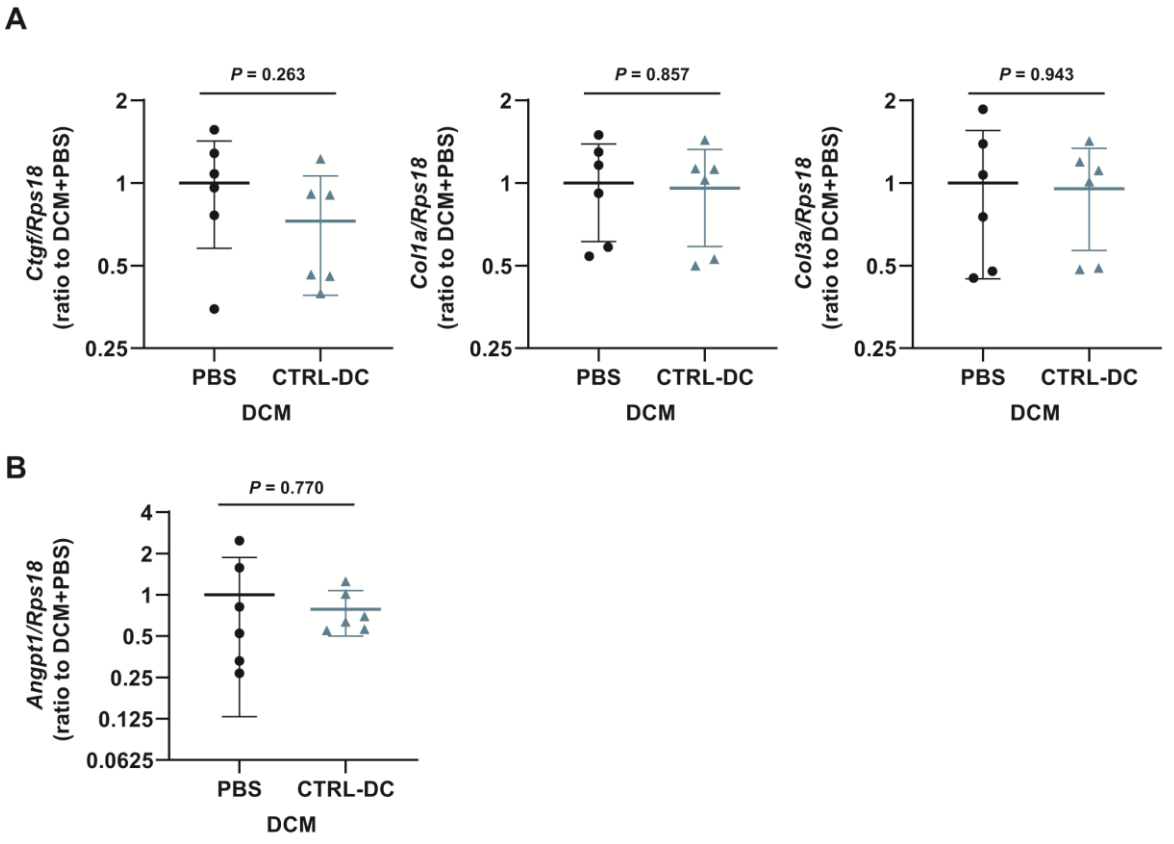



**Figure S7. Expression of fibrotic genes and *Angpt1* on day 4 after treatment with control-dendritic cells without αGalCer (CTRL-DCs) in dilated cardiomyopathy (DCM) mice.** (**A**) Expression of fibrotic genes such as *Ctgf*, *Col1a*, and *Col3a* in the myocardium of DCM mice following treatment with CTRL-DCs on day 4 (n = 6). (**B**) Gene expression of angiopoietin-1 (*Angpt1*) in the myocardium of DCM mice following treatment with CTRL-DCs on day 4 (n = 6). Data are expressed as mean ± SD. Statistical significance was determined using Student's *t*-test.

# Figure S8

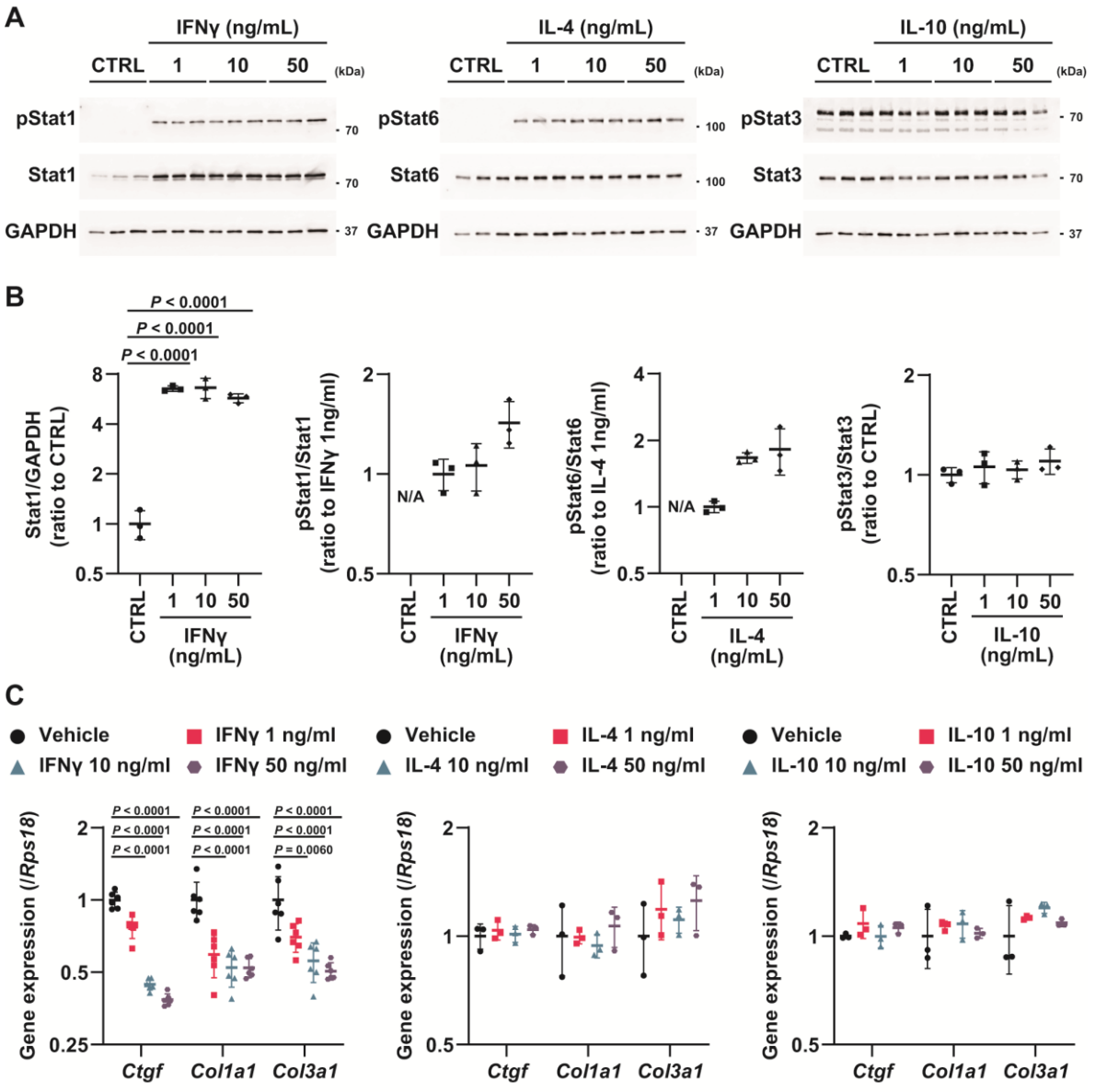



**Figure S8. Effect of cytokine treatment on fibrotic gene expression and Stat phosphorylation in primary cardiac fibroblasts.** (**A**) Western blot for the phosphorylation of Stat1, Stat6, and Stat3 in response to IFNγ, IL-4, and IL-10 treatment, respectively, in primary cardiac fibroblasts. (**B**) Quantification of western blotting results shown in panel **A**. (**C**) Fibrotic genes such as *Ctgf*, *Col1a1*, and *Col3a1* in response to IFNγ, IL-4, or IL-10 in primary cardiac fibroblasts (n = 3–6). GAPDH was used as a loading control. Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's test.

# Figure S9

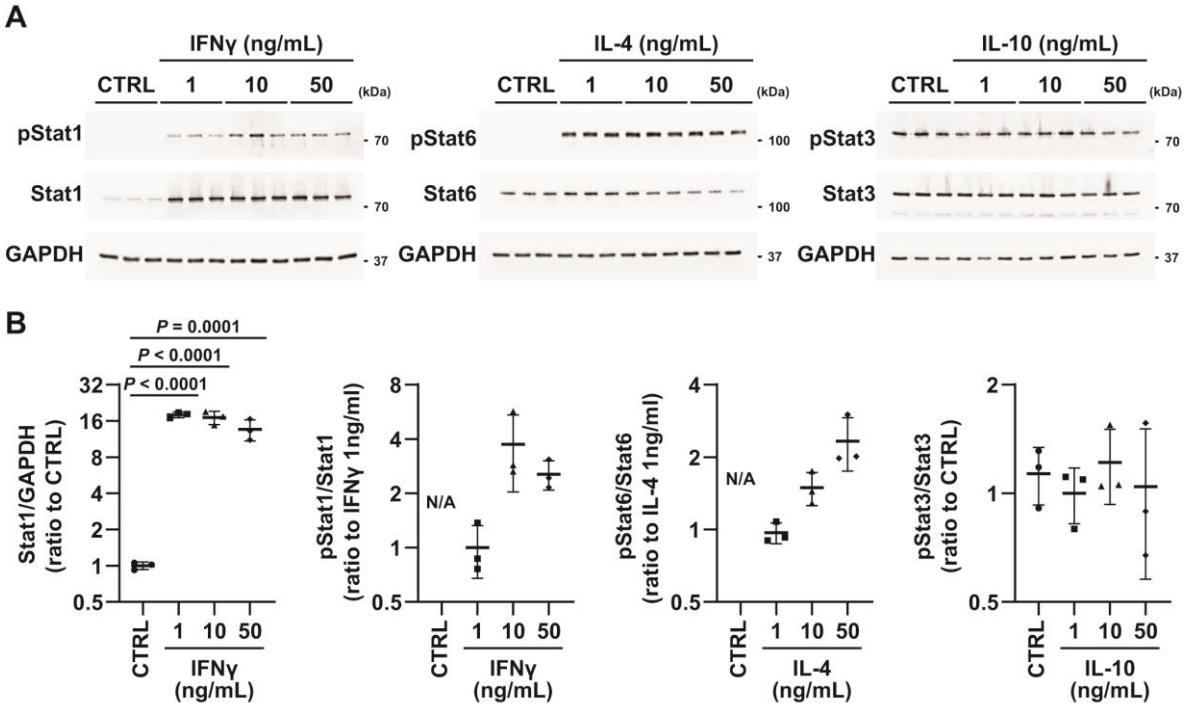



**Figure S9. Effect of cytokine treatment on Stat phosphorylation in primary cultured cardiomyocytes.** (**A**) Western blot for phosphorylation of Stat1, Stat6, and Stat3 in response to IFNγ, IL-4, and IL-10 treatment respectively, in primary cultured cardiomyocytes. (**B**) Quantification of western blot results shown in panel **A**. GAPDH was used as a loading control. Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's test.

# Figure S10

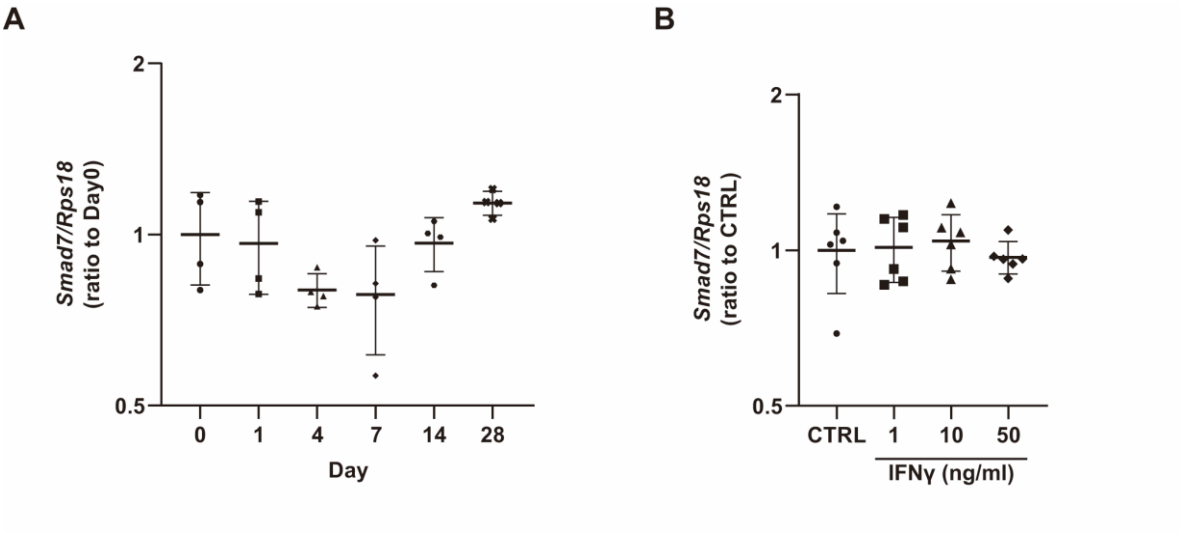



**Figure S10. Smad7 expression in response to αGalCer-pulsed dendritic cell (αGCDC) treatment in the myocardium and IFNγ treatment in fibroblasts.** (**A**) Gene expression of *Smad7* in the myocardium of BALB/c mice until day 28 after single treatment with αGCDCs (n = 4). The expression was measured using RT-qPCR (each point). (**B**) Expression of *Smad7* in response to IFNγ treatment of primary cardiac fibroblasts (n = 6). Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's test.

# Figure S11

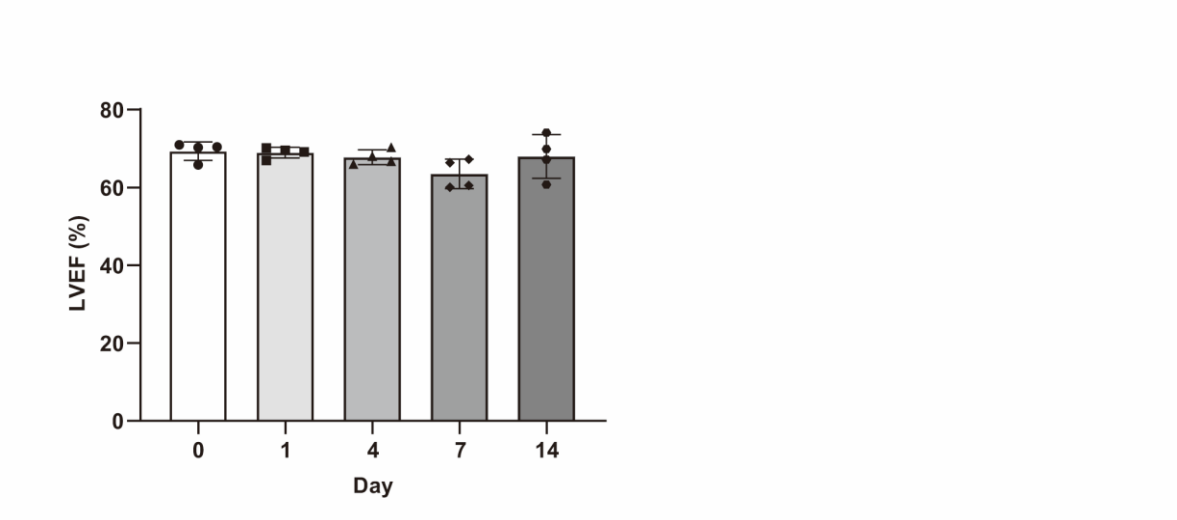



**Figure S11. Left ventricular ejection fraction (LVEF) for wild-type BALB/c mice in response to αGalCer-pulsed dendritic cell (αGCDC) treatment until day 14 (n = 4, each group).** Data are expressed as mean ± SD. Statistical significance was determined using Dunnett's *t*-test.

# Table S1

**Table S1. Heart rate (HR) and echocardiographic parameters of DCM mice treated with PBS, control-dendritic cells without α-galactosylceramide (CTRL-DCs), or α-galactosylceramide-pulsed dendritic cells (αGCDCs) at enrollment (5 weeks of age).**

| | DCM | | |
|---|---|---|---|
| Variables | PBS | CTRL-DC | αGCDC |
| n | 34 | 30 | 30 |
| HR, bpm | 474 ± 61 | 489 ± 39 | 499 ± 49 |
| LVEDD, mm | 5.6 ± 0.7 | 5.5 ± 0.6 | 5.4 ± 0.6 |
| LVESD, mm | 5.1 ± 0.8 | 5.0 ± 0.6 | 5.0 ± 0.6 |
| LVEDV, μL | 154 ± 42 | 148 ± 34 | 146 ± 35 |
| LVESV, μL | 127 ± 42 | 119 ± 35 | 121 ± 33 |
| LVEF, % | 19 ± 10 | 21 ± 7 | 17 ± 8 |
| FS, % | 9 ± 5 | 10 ± 3 | 8 ± 4 |

Data are presented as mean ± SD. None of the parameters showed significant differences among the three groups in the one way-ANOVA with a post-hoc test (Tukey). LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction; and FS, fractional shortening.

# Table S2

**Table S2. Heart rate (HR) and echocardiographic parameters of DCM mice treated with PBS, control-dendritic cells without α-galactosylceramide (CTRL-DCs), or α-galactosylceramide-pulsed dendritic cells (αGCDCs) at sacrifice (9 weeks of age).**

| | DCM | | |
|---|---|---|---|
| Variables | PBS | CTRL-DC | αGCDC |
| n | 9 | 10 | 19 |
| HR, bpm | 466 ± 86 | 464 ± 82 | 440 ± 77 |
| LVEDD, mm | 5.6 ± 0.4 | 5.8 ± 0.6 | 6.1 ± 0.5 |
| LVESD, mm | 5.3 ± 0.4 | 5.5 ± 0.7 | 5.5 ± 0.6 |
| LVEDV, μL | 157 ± 22 | 171 ± 41 | 185 ± 35 |
| LVESV, μL | 136 ± 26 | 151 ± 45 | 150 ± 38 |
| LVEF, % | 14 ± 6 | 13 ± 7 | 20 ± 9 |
| FS, % | 6 ± 3 | 6 ± 4 | 9 ± 5 |

Data are presented as mean ± SD. None of the parameters showed statistically significant differences among the three groups in the one way-ANOVA with a post-hoc test (Tukey). LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction; and FS, fractional shortening.

# Table S3

**Table S3. Heart rate (HR) and echocardiographic parameters of DCM mice treated with PBS or α-galactosylceramide-pulsed dendritic cells (αGCDCs) at pre- and post (day 4)-treatment.**

| | Pre-treatment | | Post-treatment | |
|---|---|---|---|---|
| Variables | PBS | αGCDC | PBS | αGCDC |
| n | 8 | 10 | 8 | 10 |
| HR, bpm | 502 ± 61 | 498 ± 75 | 460 ± 115 | 431 ± 84 |
| LVEDD, mm | 5.4 ± 0.4 | 5.4 ± 0.5 | 5.7 ± 0.6 | 5.5 ± 0.4 |
| LVESD, mm | 4.9 ± 0.5 | 5.1 ± 0.7 | 5.4 ± 0.7 | 4.9 ± 0.5 |
| LVEDV, μL | 140 ± 24 | 145 ± 30 | 159 ± 40 | 146 ± 24 |
| LVESV, μL | 115 ± 26 | 127 ± 37 | 141 ± 41 | 114 ± 27 |
| LVEF, % | 18 ± 8 | 14 ± 10 | 12 ± 8 | 22 ± 11* |
| FS, % | 8 ± 4 | 7 ± 5 | 5 ± 4 | 10 ± 6* |

Data are presented as mean ± SD. Statistical significance was determined using Student's *t*-test. Significant differences between the PBS and αGCDC groups at post-treatment (day 4) are indicated with *$P < 0.05$. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction; and FS, fractional shortening.

# Table S4

**Table S4. Heart rate (HR) and echocardiographic parameters of DCM mice treated with PBS or control-dendritic cells without α-galactosylceramide (CTRL-DCs) at pre- and post (day 4)-treatment.**

| | Pre-treatment | | Post-treatment | |
|---|---|---|---|---|
| Variables | PBS | CTRL-DC | PBS | CTRL-DC |
| n | 6 | 6 | 6 | 6 |
| HR, bpm | 543 ± 75 | 484 ± 24 | 487 ± 62 | 535 ± 82 |
| LVEDD, mm | 5.4 ± 0.6 | 5.7 ± 0.4 | 6.1 ± 0.5 | 5.5 ± 0.4 |
| LVESD, mm | 4.9 ± 0.6 | 5.2 ± 0.4 | 5.6 ± 0.6 | 5.1 ± 0.5 |
| LVEDV, μL | 145 ± 35 | 160 ± 22 | 185 ± 37 | 149 ± 23 |
| LVESV, μL | 116 ± 34 | 129 ± 22 | 156 ± 39 | 124 ± 30 |
| LVEF, % | 21 ± 8 | 20 ± 4 | 17 ± 7 | 18 ± 9 |
| FS, % | 10 ± 4 | 9 ± 2 | 8 ± 3 | 8 ± 4 |

Data are presented as mean ± SD. Significance was determined using Student's *t*-test. None of the parameters showed significant differences between the PBS and CTRL-DC groups at pre- and post (day 4)-treatment. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction; and FS, fractional shortening.