



<b>Title</b>	Activation of Natural Killer T Cells Ameliorates Postinfarct Cardiac Remodeling and Failure in Mice
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**Supplemental Material**

**The Activation of Natural Killer T Cells Ameliorates Post-Infarct Cardiac Remodeling and Failure in Mice**

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**Short title:** Natural killer T cells in heart failure

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## Detailed Methods

An expanded Methods section is available in the online Data Supplement at <http://circres.ahajournals.org>.

All procedures and animal care were approved by our institutional animal research committee and conformed to the animal care guideline for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

### Experiment 1: Time-dependent Changes of iNKT Cell Receptors in Post-MI Hearts

#### Animal Models

MI was created in male C57BL/6J mice, 6-8 weeks old and 20 to 25 g body weight, by ligating the left coronary artery as described previously.<sup>1</sup> Sham operation without ligating the coronary artery was also performed as control. MI mice were sacrificed and the hearts were excised at day 3, 7, 14 and 28 for quantitative reverse transcriptase (qRT)-PCR measurements.

#### Quantitative Reverse Transcriptase PCR

Total RNA was extracted from LV in sham mice and non-infarcted and infarcted LV from MI mice by using QuickGene-810 (FujiFilm, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). TaqMan quantitative PCR was performed with the 7300 real-time PCR system (Applied Biosystems) to amplify samples for V $\alpha$ 14J $\alpha$ 18 (a specific marker of iNKT cells).<sup>2</sup> This transcript was normalized to GAPDH. The primer was purchased from Applied Biosystems.

### Experiment 2: Effects of iNKT Cell Activation on Post-MI Hearts

#### Animal Models

Sham and MI mice were created in male C57BL/6J as described in Experiment 1. Each group of mice was randomly divided into 2 groups; either  $\alpha$ -galactosylceramide ( $\alpha$ GC; 0.1  $\mu$ g/g body weight; Funakoshi Company, Ltd., Tokyo, Japan), the activator of iNKT cells, or phosphate-buffered saline (PBS) was administered via intraperitoneal injection 1 and 4 days after surgery. The concentration of  $\alpha$ GC was chosen based on the previous study of its efficacy.<sup>2</sup> Thus, the experiment was performed in the following 4 groups of mice; sham+PBS (n=10), sham+ $\alpha$ GC (n=10), MI+PBS (n=31), and MI+ $\alpha$ GC (n=27).

Four weeks after surgery, echocardiographic studies and the hemodynamics measurement were performed. After collecting blood samples, mice were sacrificed and organ weight was measured. These measurements were performed in all survived mice (n=10 for sham+PBS, n=10 for sham+ $\alpha$ GC, n=10 for MI+PBS, and n=16 for MI+ $\alpha$ GC). The mice were further divided into 2 groups; for the histological analysis, including infarct size, myocyte cross-sectional area, collagen volume fraction, TUNEL staining (n=6 for each group), and for the quantitative reverse transcriptase PCR (n=4 for each group). Additional mice were also created for MMP zymography (n=5 for each group) and for

flow cytometry analysis (n=9 for each group).

A separate group of additional mice treated identically was created. One week after surgery, all mice (n=15 for each group) were sacrificed. These mice were used for immunohistochemistry (n=3 for each group), for the quantitative reverse transcriptase PCR (n=6 for each group), and for flow cytometry (n=9 for each group).

### **Survival**

The survival analysis was performed in all 4 groups of mice. During the study period, the cages were inspected daily for deceased animals. All deceased mice were examined for the presence of MI as well as pleural effusion and cardiac rupture.

### **Echocardiographic and Hemodynamic Measurements**

Echocardiographic and hemodynamic measurements were performed under light anesthesia with tribromoethanol/amyline hydrate (avertin; 2.5% wt/vol, 8  $\mu$ L/g ip) with known short duration of action and modest cardiodepressive effects. A two-dimensional parasternal short-axis view was obtained at the levels of the papillary muscles. In general, the best views obtained with the transducer lightly applied to the mid upper left anterior chest wall. The transducer was then gently moved cephalad or caudad and angulated until desirable images were obtained. After it had been ensured that the imaging was on the axis, two-dimensional targeted M-mode tracings were recorded at a paper speed of 50mm/s. A 1.4-Fr micromanometer-tipped catheter (Millar Instruments, Houston, Texas) was inserted into the right carotid artery and then advanced into the left ventricle (LV) to measure LV pressures.

### **Myocardial Histopathology and Infarct Size**

After mice were sacrificed, the heart was excised and dissected into right ventricle and LV including septum. LV was cut into three transverse sections; apex, middle ring, and base. From the middle ring, 5- $\mu$ m sections were cut and stained with Masson's trichrome. Myocyte cross-sectional area and collagen volume fraction were determined by quantitative morphometry of tissue sections from the mid-LV as described previously.<sup>3</sup>

Infarct length was measured along the endocardial and epicardial surfaces in each of the cardiac sections, and the values from all specimens were summed. Infarct size (as a percentage) was calculated as total infarct circumference divided by total cardiac circumference.<sup>1</sup>

### **Myocardial Apoptosis**

To detect apoptosis, tissue sections from the mid-LV were stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (TaKaRa Shuzo Co. Ltd., Ohtsu, Japan). The number of TUNEL positive cardiac myocyte nuclei was counted, and the data were normalized per 10<sup>5</sup> total nuclei identified by hematoxylin-positive staining in the same sections. The proportion of apoptotic cells was counted in the non-infarcted LV.

### **MMP Zymography**

Zymographic MMP 2 and 9 levels in LV non-infarcted tissue was determined using gelatin zymography kit (Primary Cell Co., Ltd, Sapporo, Japan). The zymograms were digitized, and the size-fractionated bands, which indicated proteolytic levels, were measured by the integrated optical density in a rectangular region of interest.<sup>1</sup>

### **Isolation of Cardiac Mononuclear Cell and Flow Cytometry**

LV tissue was harvested, minced with a fine scissors, placed in 10 ml RPMI-1640 with 5% FBS, 1 mg/ml collagenase type IV and 100 U/ml DNase I, and shaken at 37 °C for 45 min. Tissue was then triturated through nylon mesh and centrifuged (1400 rpm, 5min, 4 °C). Red blood cells were lysed with Tris-NH<sub>4</sub>Cl solution. Cardiac mononuclear cells were isolated by density-gradient centrifugation with 33% Percoll™, as previously described.<sup>4</sup> Cardiac mononuclear cells from 3 mice were pooled, and subjected to flow cytometric analysis. All reagents were purchased from Sigma-Aldrich (St Louis, MO). Cardiac cell numbers were determined with Trypan blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The cells were incubated with 2.4G2 monoclonal antibody (mAb) to block non-specific binding of primary mAb and then reacted with Dimer X (CD1d:Ig recombinant fusion protein; BD Biosciences Pharmingen, San Diego, CA) loaded with  $\alpha$ GC, followed by detection with phycoerythrin (PE)-conjugated anti-mouse IgG1 mAb (BD) according to the manufacturer's protocol.<sup>5</sup> After washing, cells were stained with a combination of fluorescein isothiocyanate (FITC)-anti-TCR $\beta$  and PE-anti-mouse IgG1 (all from BD Biosciences). Stained cells were acquired with FACS Canto II flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA) and analyzed with FlowJo (Tommy Digital Biology, Tokyo, Japan). Propidium iodide (Sigma-Aldrich, St Louis, MO) positive cells were electronically gated as dead cells from the analysis.

### **RT-PCR**

RNA was extracted and cDNA was synthesized were described in Experiment 1. TaqMan quantitative PCR was performed with the 7300 real-time PCR system (Applied Biosystems) to amplify samples for V $\alpha$ 14J $\alpha$ 18, CD11c (a marker of M1 macrophages), arginase-1 (a marker of M2 macrophages), MCP-1, RANTES, interferon- $\gamma$  (IFN- $\gamma$ ), IL-4, IL-6, TNF- $\alpha$ , and IL-10 cDNA. These transcripts were normalized to GAPDH.

### **Immunohistochemistry**

LV sections were immunostained with antibody against mouse MAC3 (a macrophage marker), mouse CD3 (a T cell marker), or mouse myeloperoxidase (a leucocyte marker), followed by counter-staining with hematoxylin.

### **Plasma Cytokines Concentration**

Plasma IL-10, TNF- $\alpha$ , IFN- $\gamma$ , IL-6, and IL-4 levels were measured by commercially available ELISA kit (R&D systems, Inc.) in all groups.

### **Experiment 3: Effects of IL-10 Neutralization on $\alpha$ GC-Treated Post-MI Hearts**

MI mice were divided into the following 3 groups of mice; MI+ $\alpha$ GC (n=18), MI+anti-IL-10 receptor antibody (n=12), and MI+ $\alpha$ GC+anti-IL-10 receptor antibody (n=19).  $\alpha$ GC was administered identically as in Experiment 2. Anti-IL-10 receptor antibody (500 $\mu$ g/mouse, BD Pharmingen, San Diego, CA) was administered via intraperitoneal injection 1, 4, and 14 days after surgery. The concentration of anti-IL-10 receptor antibody was chosen based on the previous study of its efficacy.<sup>6</sup> Four weeks after surgery, echocardiographic and hemodynamics measurement were performed as described in Experiment 2. Separate set of mice from Experiment 2 was used in MI+ $\alpha$ GC group.

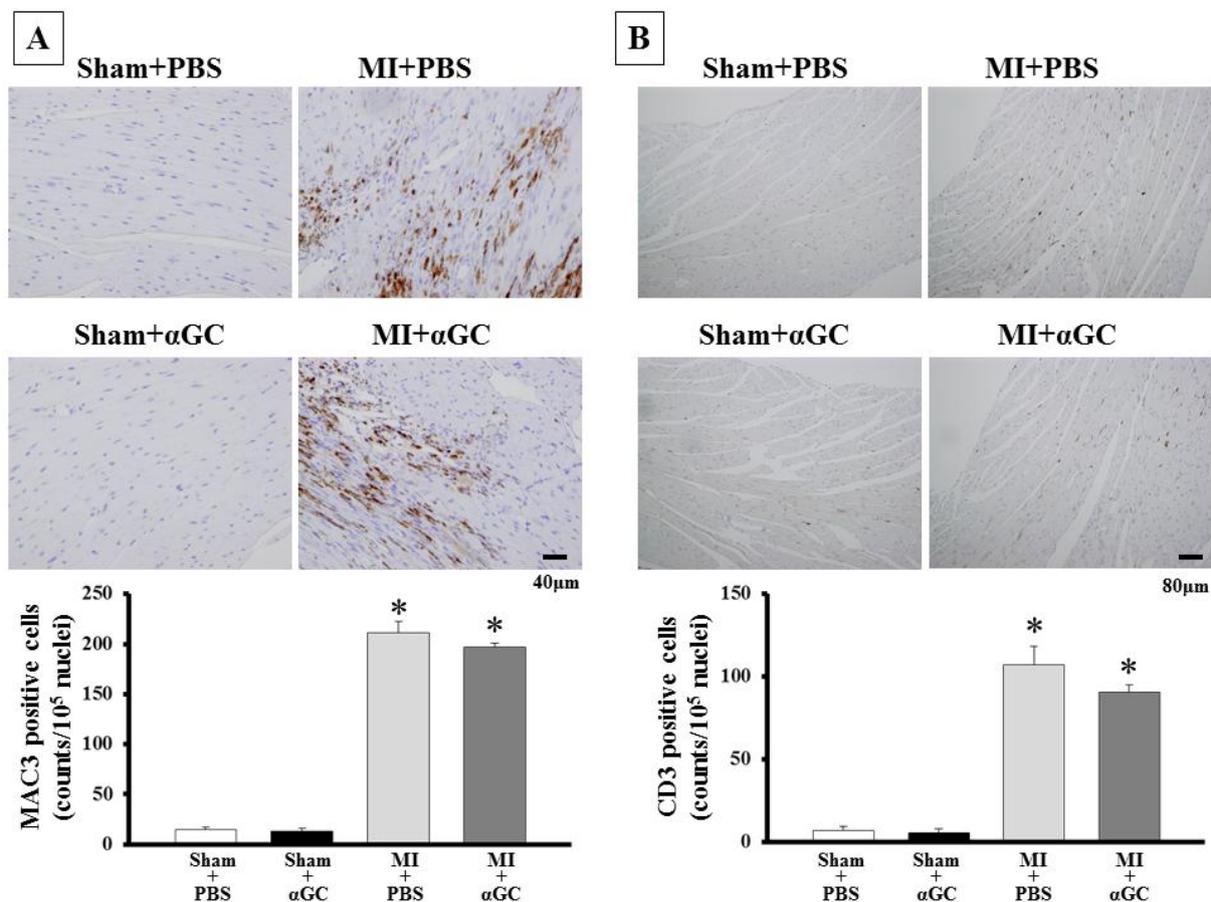
### **Experiment 4: Specificity of $\alpha$ GC for NKT Cells**

V $\alpha$ 14<sup>+</sup> NKT cell-deficient J $\alpha$ 18<sup>-/-</sup> (J $\alpha$ 18 KO) mice were provided from Dr. M. Taniguchi (RIKEN, Yokohama, Japan) and backcrossed 10 times to C57BL/6J.<sup>7</sup> Sham and MI mice were created in male J $\alpha$ 18 KO mice as described in Experiment 1. Each group of mice was treated identically to Experiment 2. Thus, the experiment was performed in the following 4 groups of mice; KO+sham+PBS, KO+sham+ $\alpha$ GC, KO+MI+PBS, and KO+MI+ $\alpha$ GC. One week after surgery, all mice (n=9 for each group) were sacrificed, and used for immunohistochemistry (n=3 for each group), and for the quantitative reverse transcriptase PCR (n=6 for each group). These analyses were performed as described in Experiment 2.

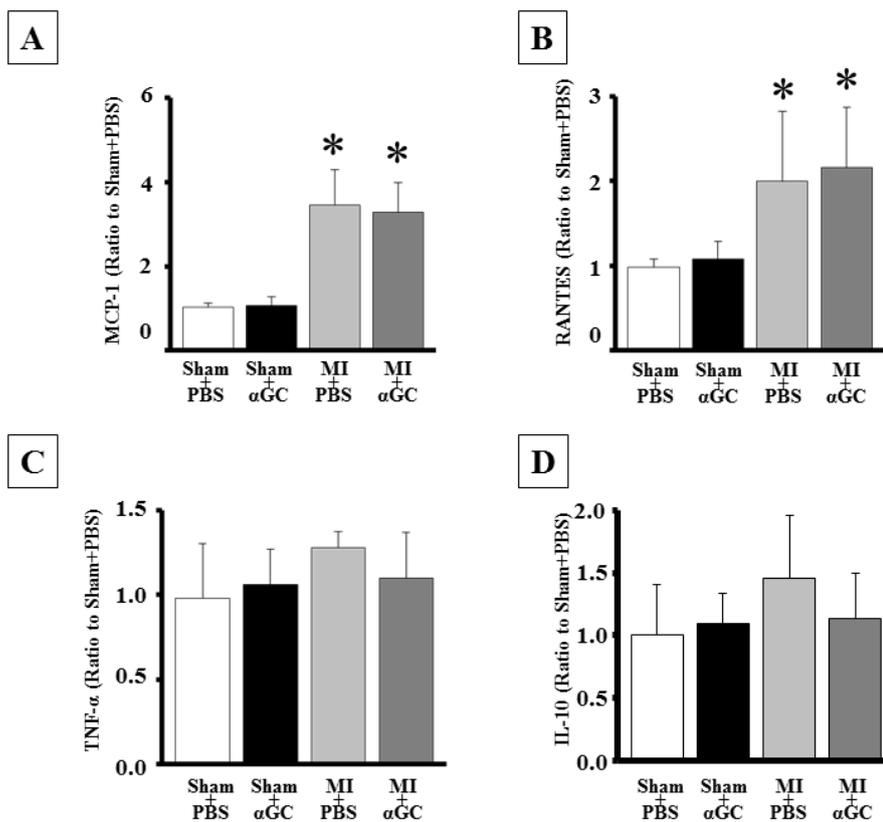
### **Statistical Analysis**

Data were expressed as means  $\pm$  SE. Survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the log-rank test. A between-group comparison of means was performed by 1-way ANOVA, followed by t test. The Bonferroni correction was applied for multiple comparisons of means.  $P < 0.05$  was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors had read and agreed to the manuscript as written.



**Supplemental Figure 1.** Representative photomicrographs of LV cross-sections stained with (A, upper panel) anti MAC-3 and (B, upper panel) anti CD3 in KO+Sham+PBS, KO+Sham+αGC, KO+MI+PBS and KO+MI+αGC. Summary data of the numbers of (A, lower panel) MAC-3 and (B, lower panel) CD3 positive cells in the LV (n=4-8 for each). Data are means±SE. \* $P < 0.05$  vs. Sham+PBS.



**Supplemental Figure 2.** Quantitative analysis of gene expression of MCP-1 (A), RANTES (B), TNF- $\alpha$  (C), and IL-10 (D) in the non-infarcted LV from KO mice at day 7 after surgery. Gene expression was normalized to GAPDH and depicted as the ratio to Sham+PBS. Data are expressed as means  $\pm$  SE. \* $P$ <0.05 vs. Sham+PBS.

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