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

Mochamad Ali Sobirin1,2, Shintaro Kinugawa1, Masashige Takahashi1, Arata Fukushima1, Tsuneaki Homma1, Taisuke Ono1, Kagami Hirabayashi1, Tadashi Suga1, Putri Azalia1, Shingo Takada1, Masaru Taniguchi3, Toshinori Nakayama4, Naoki Ishimori1, Kazuya Iwabuchi5, and Hiroyuki Tsutsui1

1Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo Faculty of Medicine, 2Diponegoro University, Semarang, Indonesia, 3RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan, 4Department of Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan (T.N.), and 5Division of Immunobiology, Kitasato University School of Medicine, Kanagawa, Japan.

Running title: Natural Killer T Cells in Heart Failure

Subject codes:
[110] Congestive
[115] Remodeling
[147] Growth factors/cytokines
[148] Heart failure - basic studies

Address correspondence to:
Dr. Shintaro Kinugawa
Department of Cardiovascular Medicine
Hokkaido University Graduate School of Medicine
Kita-15, Nishi-7
Kita-ku, Sapporo 060-8638
Japan
Fax: +81-11-706-7874
Tel: +81-11-706-6973
tuckahoe@med.hokudai.ac.jp

In July 2012, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.2 days.
ABSTRACT

**Rationale:** Chronic inflammation in the myocardium is involved in the development of left ventricular (LV) remodeling and failure after myocardial infarction (MI). Invariant natural killer T (iNKT) cells have been shown to produce inflammatory cytokines and orchestrate tissue inflammation. However, no previous studies have determined the pathophysiological role of iNKT cells in post-MI LV remodeling.

**Objective:** The purpose of this study was to examine whether the activation of iNKT cells might affect the development of LV remodeling and failure.

**Methods and Results:** After creation of MI, mice received the injection of either α-galactosylceramide (αGC; n=27), the activator of iNKT cells, or phosphate-buffered saline (PBS; n=31) 1 and 4 days after surgery, and were followed during 28 days. Survival rate was significantly higher in MI+αGC than MI+PBS (59% vs 32%, P<0.05). LV cavity dilatation and dysfunction were significantly attenuated in MI+αGC, despite comparable infarct size, accompanied by a decrease in myocardocyte hypertrophy, interstitial fibrosis, and apoptosis. The infiltration of iNKT cells were increased during early phase in non-infarcted LV from MI and αGC further enhanced them. It also enhanced LV interleukin (IL)-10 gene expression at 7 days, which persisted until 28 days. Anti IL-10 receptor antibody abrogated these protective effects of αGC on MI remodeling. The administration of αGC into iNKT cell-deficient Jα18-/- mice had no such effects, suggesting that αGC was a specific activator of iNKT cells.

**Conclusions:** iNKT cells play a protective role against post-MI LV remodeling and failure through the enhanced expression of cardioprotective cytokines such as IL-10.

**Keywords:**
Invariant natural killer T cells, myocardial infarction, inflammation, heart failure, cytokines.

**Non-standard Abbreviations:**
αGC α-galactosylceramide
HF heart failure
IL Interleukin
IFN-γ interferon-γ
iNKT invariant natural killer T
LV left ventricle
MI myocardial infarction
INTRODUCTION

Myocardial infarction (MI) leads to the development of heart failure (HF), which is the major cause of death in post-MI patients. The changes in left ventricular (LV) geometry, such as cavity dilatation associated with myocyte hypertrophy and interstitial fibrosis, referred to as remodeling, contribute to the development of depressed cardiac function in HF after MI.\textsuperscript{1} It has been reported that monocytes and lymphocytes are infiltrated in non-infarcted area as well as infarcted area of LV after MI.\textsuperscript{2,3} Chemokines, monocyte chemoattractant protein-1 (MCP-1) and regulated on activation normally T cell expressed and secreted (RANTES), are essential factors in the recruitment and activation of monocyte and lymphocyte. These chemokines are also increased in non-infarcted LV after MI and contribute to local inflammation through the release of inflammatory cytokines including tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\textsuperscript{2,4} Targeted deletion of CC chemokine receptor 2 or anti-MCP-1 gene therapy has been shown to attenuate LV remodeling after MI.\textsuperscript{2,5} Thus, chronic tissue inflammation plays an important role in LV remodeling process.

Invariant natural killer T (iNKT) cells are innate-like T lymphocyte population co-expressing NK markers and an \(\alpha\beta\) T cell receptor that recognize glycolipid antigens. They can rapidly and robustly produce a mixture of T-helper type 1 (T\(_{h1}\)) and T\(_{h2}\) cytokines, such as TNF-\(\alpha\), interferon-\(\gamma\) (IFN-\(\gamma\)), interleukin (IL)-10, and IL-4, and also a vast array of chemokines in shaping subsequent adaptive immune response.\textsuperscript{6} Thus, iNKT cells can function as a bridge between the innate and adaptive immune systems, and orchestrate tissue inflammation. Indeed, we have shown that iNKT cells activate vascular wall inflammation in atherogenesis and adipose tissue inflammation in obesity-induced glucose intolerance.\textsuperscript{7,8} On the other hand, iNKT cells play a protective role against autoimmune and inflammatory diseases such as type 1 diabetes,\textsuperscript{9,10} allergic encephalomyelitis,\textsuperscript{9,11} and rheumatoid arthritis.\textsuperscript{12} These findings suggest that iNKT cells may have bidirectional effects on tissue
inflammation. However, no previous studies have examined the changes of iNKT cells and their pathophysiological role in LV remodeling and failure after MI.

Therefore, the purpose of the present study was to determine whether iNKT cells might affect the development of LV remodeling and failure after MI. We demonstrated that the activation of iNKT cells by α-galactosylceramide (αGC), a specific activator for iNKT cells, attenuated the development of LV remodeling and failure after MI in mice. The enhanced gene expression of IL-10 might be involved in these beneficial effects of iNKT cells on this disease process.

METHODS

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. 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-polymerase chain reaction (qRT-PCR) measurements.

**qRT-PCR.**
Quantitative PCR for Vα14Jα18 (a specific marker of iNKT cells) was performed, as described previously.

**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 αGC (0.1μg/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 αGC was chosen based on the previous study of its efficacy. Thus, the experiment was performed in the following 4 groups of mice; sham+PBS (n=10), sham+αGC (n=10), MI+PBS (n=31), and MI+αGC (n=27).
**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/amylene hydrate (avertin; 2.5% wt/vol, 8 µL/g ip), as described previously.¹⁴

**Myocardial histopathology, infarct size, myocardial apoptosis, and matrix metalloproteinase (MMP) zymography.**

Myocyte cross-sectional area, collagen volume fraction, infarct size, myocardial apoptosis, and zymographic MMPs levels were determined as described previously.¹⁴, ¹⁵

**Isolation of cardiac mononuclear cell and flow cytometry.**

Cardiac mononuclear cells from 3 mice were isolated, pooled, and subjected to flow cytometric analysis as previously described.⁷, ¹⁶

**qRT-PCR.**

Quantitative PCR for Vα14Jα18, CD11c (a marker of M1 macrophages), arginase-1 (a marker of M2 macrophages), MCP-1, RANTES, IFN-γ, IL-4, IL-6, TNF-α, and IL-10 was performed, as described previously.⁸

**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-α, IFN-γ, 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 αGC-treated post-MI hearts.**

MI mice were divided into the following 3 groups; MI+αGC (n=18), MI+anti-IL-10 receptor antibody (n=12), and MI+αGC+anti-IL-10 receptor antibody (n=19). αGC was administered identically as in Experiment 2. Anti-IL-10 receptor antibody (500µg/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. Four weeks after surgery, echocardiographic and hemodynamics measurement were performed. Separate groups of mice were used in MI+αGC group in Experiment 2.

Experiment 4: Specificity of αGC for NKT cells.
Vα14+ NKT cell-deficient Jα18−/− (Jα18 KO) mice were provided from Dr. M. Taniguchi (RIKEN, Yokohama, Japan) and backcrossed 10 times to C57BL/6J. Sham and MI mice were created in male Jα18 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; KO+sham+PBS, KO+sham+αGC, KO+MI+PBS, and KO+MI+αGC. One week after surgery, all mice (n=9 for each group) were sacrificed, and used for immunohistochemistry (n=3 for each group), and for qRT-PCR (n=6 for each group). These analyses were performed as described in Experiment 2.

Statistical analysis.
Data were expressed as means ± 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.

RESULTS

Experiment 1: Time-dependent changes of iNKT cell receptors in post-MI hearts.
The quantification of iNKT cells by Vα14/Jα18 gene expression demonstrated that iNKT cell infiltration into the non-infarcted LV was significantly enhanced at 7 days (1.7±0.2 fold changes from baseline, P<0.05 vs. baseline) after MI and returned to baseline at 14 and 28 days after MI (1.0±0.2 and 1.1±0.1 fold changes from baseline, respectively). In the infarcted LV, its gene expression was significantly elevated 7 days and remained elevated 28 days after MI (data not shown).

Experiment 2: Effects of iNKT cell activation on post-MI hearts.
By using flow cytometric analysis, iNKT cells were detected in LV from all groups of mice (Figure 1A). αGC injection increased iNKT cells infiltration into the non-infarcted LV both in
sham+αGC and MI+αGC mice after 7 days (Figure 1A). Moreover, it remained enhanced at 28 days in MI+αGC (Figure 1A).

Quantitative RT-PCR also demonstrated that gene expression of Vα14/Jα18, a marker of iNKT cell infiltration, was significantly elevated in the non-infarcted LV from sham+αGC and MI+αGC mice after 7 days (Figure 1B). Interestingly, it remained significantly increased at 28 days only in MI+αGC (Figure 1B).

**Survival.**

There were no deaths in sham-operated groups. The survival rate during 28 days was significantly higher in MI+αGC compared with MI+PBS mice (59 % versus 32 %; \( P<0.05 \); Figure 2A). Thirteen MI+PBS (42%) and 8 MI+αGC (30%) mice died of LV rupture (\( P=\text{NS} \)).

**Echocardiography and hemodynamics.**

The echocardiographic and hemodynamic data from 4 groups of survived mice at 28 days are shown in Figure 2B and Table 1. There were no significant differences in either echocardiographic or hemodynamic parameters between sham+PBS and sham+αGC mice. LV diameters were significantly greater, and LV fractional shortening was significantly lower in MI mice than sham mice. These changes were ameliorated by the treatment of MI mice with αGC. There were no significant differences in heart rate or aortic blood pressure among groups. LV end-diastolic pressure (LVEDP) was significantly increased, and LV +dP/dt and LV –dP/dt were significantly decreased in MI compared to sham, which was ameliorated by the treatment of MI mice with αGC.

**Organ weights, infarct size, and histology.**

There were no significant differences in heart weight/body weight and lung weight/body weight between sham+PBS and sham+αGC mice (Table 1). In agreement with LVEDP, heart weight/body weight and lung weight/body weight were increased in MI mice, and these increases were significantly attenuated in MI+αGC (Table 1).

Infarct size measured by the morphometric analysis was comparable (56±2% versus 55±1%; \( P=\text{NS} \)) between MI+PBS (n=6) and MI+αGC (n=6) groups (Table 1).

Histomorphometric analysis of noninfarcted LV sections showed that myocyte cross-sectional area was increased in MI+PBS compared to sham mice, and was significantly attenuated in MI+αGC (Figure 3A). Collagen volume fraction was also increased in MI+PBS compared to sham mice, and was significantly attenuated in MI+αGC (Figure 3A).
There were rare TUNEL-positive nuclei in both sham and sham+αGC mice. The number of TUNEL-positive myocytes in the noninfarcted LV was increased in MI+PBS, and was significantly decreased in MI+αGC (Figure 3B).

*Myocardial MMP activity.*

Representative gelatin zymography of the non-infarcted LV tissue at day 7 from 4 groups of mice was shown in Figure 4A. There were no zymographic MMP-2 and 9 levels in the sham+PBS and sham+αGC. Zymographic MMP-2 level was significantly increased in MI+PBS mice compared to sham mice at day 7. αGC injection significantly decreased it after MI (Figure 4B). Zymographic MMP-9 level was also increased in MI+PBS mice compared to sham mice at day 7, which, however, was not affected by αGC (Figure 4C).

Zymographic MMP-2 level was increased in MI+PBS mice also at day 28, and αGC injection tended to decrease it (3.7±1.1 vs 2.1±0.8 in ratio to sham, P=0.08).

*Inflammatory and cytokine gene expression.*

Immunohistochemical stainings for MAC3 and CD3 were increased in MI+PBS compared to Sham+PBS, and were further increased by αGC at day 7 (Figure 5). MPO positive cells were not detected in the LV tissue from either group of mice (data not shown).

CD11c (a marker of M1 macrophage) and arginase 1 (a marker of M2 macrophage) gene expressions were significantly increased in non-infarcted LV from MI+PBS compared to sham+PBS at day 7 (Figure 6A and B). αGC significantly increased their expressions in both sham and MI animals at day 7. Arginase 1, but not CD11c, was increased in non-infarcted LV from MI+PBS and MI+αGC at day 28. There was no significant difference in arginase 1 between these 2 groups. MCP-1 and RANTES gene expressions were increased in non-infarcted LV from MI+PBS compared to sham+PBS at day 7 (Figure 6C and D). αGC significantly increased their expressions in both sham and MI animals at day 7. In contrast, there was no significant difference in their expressions among all groups at day 28.

IFN-γ, TNF-α, IL-6, and IL-10 gene expression levels were significantly increased in sham and MI mice by αGC at day 7 (Figure 6E-H). IL-10 gene expression alone significantly elevated up to 2.6 fold in the non-infarcted LV from MI+αGC mice at day 28 (Figure 6H). These time-dependent and αGC-mediated changes in IL-10 gene expression (Figure 6H) in the LV were matched with those in NKT cell infiltration (Figure 1B). IL-4 was not detected in either group.
**Plasma cytokines concentration.**

Plasma IL-10 level was similar among sham+PBS, sham+αGC, and MI+PBS groups (9.0±0.5 vs 9.8±2.3 vs 10.6±2.3 pg/ml). However, in parallel to IL-10 gene expression in the LV, it significantly increased up to 2-fold in MI+αGC (21.1±2.3 pg/ml) compared with sham and MI+PBS mice (P<0.05). Plasma IFN-γ levels were similar among 4 groups of mice (1.4±0.3 vs 1.7±0.3 vs 0.9±0.2 vs 1.0±0.2 pg/ml, P=NS). Plasma TNF-α, IL-6, and IL-4 levels were not detected in either group.

**Experiment 3: Effects of IL-10 neutralization on αGC-treated post-MI hearts survival.**

The survival rate during 28 days tended to be higher in MI+αGC than MI+anti-IL-10 receptor antibody and MI+αGC+anti-IL-10 receptor antibody (66.7 % versus 44.4 % and 42.1%, P=0.4).

**Echocardiography and hemodynamics.**

The echocardiographic and hemodynamic data from 3 groups of surviving mice are shown in Table 2. IL-10 receptor antibody injection significantly increased LV diameters, LVEDP, and decreased LV fractional shortening in αGC-treated MI mice. In contrast, there were no differences in these parameters between MI+anti-IL-10 receptor antibody and MI+αGC+anti-IL-10 receptor antibody. There was no significant difference in heart rate and aortic blood pressure among 3 groups.

**Organ weights and infarct size.**

In agreement with LVEDP, lung weight/body weight ratio was significantly increased in MI+αGC +anti-IL-10 receptor antibody compared to MI+αGC (Table 2). There were also no differences in these parameters between MI+anti-IL-10 receptor antibody and MI+αGC+anti-IL-10 receptor antibody.

Infarct size was comparable (56±2%, 54±2%, and 56±4%; P=NS) among MI+αGC (n=8), MI+anti-IL-10 antibody (n=8), and MI+αGC+anti-IL-10 receptor antibody (n=8) groups.

**Experiment 4: Specificity of αGC for iNKT cells.**

Immunohistochemical stainings for MAC3 and CD3 were increased in KO+MI+PBS compared to KO+Sham+PBS. In contrast to the results from WT (Figure 5), αGC did not alter them (Online Figure I). MPO positive cells were not detected in the LV tissue from either group of mice (data not shown). MCP-1 and RANTES were increased in KO+MI+PBS compared to KO+Sham+PBS, and were not affected by αGC (Online Figure IIA and B). There was no difference in TNF-α and IL-10 in the LV tissue from either group of mice (Online Figure IIC and D). These data
suggest that αGC did not directly activate other inflammatory cell, induce chemokines, and produce inflammatory cytokines.

DISCUSSION

The present study demonstrated that the activation of iNKT cells by αGC improved survival and ameliorated LV remodeling and failure after MI in mice, accompanied by the decreases in interstitial fibrosis, cardiomyocyte hypertrophy and apoptosis. Furthermore, the enhanced expression of IL-10 by αGC is involved in these effects. This is the first report to provide direct evidence for increased iNKT cells in MI and the inhibitory effects of their activation on the development of post-MI HF.

Chronic infiltration of inflammatory cells including iNKT cells in Post-MI heart.

In the setting of acute MI, the infiltration of inflammatory cells such as neutrophils, macrophages, and lymphocytes is a physiological repair process and beneficial removing dead cardiomyocytes and leading to the repair and scar formation of infarcted area. However, the chronic inflammatory response in the non-infarcted area causes the further myocardial damage and fibrosis, leading to the progressive impairment of cardiac function. We have previously demonstrated that anti-MCP-1 gene therapy improved survival and attenuated LV dilation and contractile dysfunction, which was associated with the decreases in macrophage infiltration and gene expression of myocardial inflammatory cytokines. Therefore, chronic myocardial inflammation plays a crucial role on LV remodeling and failure after MI. However, the precise role of various inflammatory cells and chemokines in this disease process has not been fully elucidated. iNKT cells are specialized lineage of T cells that recognize glycolipid antigens presented by the MHC class I-like molecule CD1d. The iNKT cells mediate various functions rapidly by producing a mixture of Th1 and Th2 cytokines and vast array of chemokines. Thus, iNKT cells can function as a bridge between the innate and adaptive immune systems, and orchestrate tissue inflammation. However, to our knowledge, there has been only one paper by Olson et al. reported the presence of iNKT cells in cardiac tissue obtained from acute Lyme carditis model. Therefore, the present study was the first that demonstrated the increased infiltration of iNKT cells in post-MI hearts (Figure 1).

Effects of the activation of iNKT cells by αGC in Post-MI heart.

The most important finding of this study was that the activation of iNKT cells by αGC improved survival and attenuated LV remodeling and failure after MI (Figures 2 and 3, Table 1). The beneficial effects of αGC were not attributable to its MI size-sparing effect, because the infarct size calculated as % LV circumference was comparable between MI+PBS and MI+αGC mice. Furthermore, its effects might not be attributable to those on hemodynamics, because blood pressure
and heart rate were not altered (Table 1). αGC, a glycosphingolipid, is a well-known iNKT cell receptor ligand that can specifically activate iNKT cells.\textsuperscript{13} It has been demonstrated that iNKT cells expand dramatically 2-3 days after \textit{in vivo} treatment with αGC, and return to the baseline level by approximately 9 days after treatment.\textsuperscript{21, 22} Moreover, the effects of iNKT cell stimulation may differ according to the timing of αGC administration. In the model of experimental autoimmune encephalomyelitis, early immunization with αGC protected against this disease, whereas later immunization potentiated it.\textsuperscript{23} In the present study, αGC injection significantly enhanced iNKT cell infiltration (Figure 1) and could effectively ameliorate post-MI LV remodeling and failure (Figures 2 and 3).

**Role of IL-10 in the inhibitory effects of iNKT cell activation by αGC.**

Another important finding of the present study was that the enhanced expression of IL-10 was involved in the inhibitory effects of iNKT cell activation against LV remodeling and failure (Table 2). These results are consistent with the previous findings that the therapeutic effects of αGC against TH1-like autoimmune diseases include two mechanisms such as a shift from T\textsubscript{H1} toward a T\textsubscript{H2} pattern\textsuperscript{9-11, 23} and the induction of immunosuppressive cytokine IL-10.\textsuperscript{9, 11, 12} The present study demonstrated that IL-10 was increased in non-infarcted LV from sham and MI animals in association with an increase in iNKT cells after the treatment with αGC at 7 days (Figure 6C and D). Interestingly, the enhanced expression of IL-10 gene by αGC persisted only in MI mice. These changes of IL-10 gene expression (Figure 6D) completely corresponded to those of iNKT cells (Figure 1B). Moreover, the inhibitory effects of αGC on LV remodeling and HF were reversed by anti-IL-10 receptor antibody and the treatment with only anti-IL-10 antibody of MI mice did not affect LV remodeling and HF (Table 2). Therefore, these data suggest that IL-10 is not associated with the development of LV remodeling and HF after MI without αGC, and IL-10 is involved in the beneficial effects of iNKT cell activation against post-MI remodeling and failure. These findings were consistent with a recent study by Krishnamurthy et al, in which LV dimension and function by echocardiography after MI did not differ between wild-type and IL-10-null mice.\textsuperscript{24}

**Possible mechanisms of IL-10 for the attenuation of LV remodeling.**

IL-10 can inhibit the production of proinflammatory cytokines by macrophages and T\textsubscript{H1} cells,\textsuperscript{25, 26} and directly promote the death of inflammatory cells.\textsuperscript{27} Furthermore, beyond its suppressive effects on inflammatory gene synthesis, IL-10 also regulates extracellular matrix\textsuperscript{28} and angiogenesis.\textsuperscript{29} In the present study, the activation of iNKT cells by αGC decreased cardiac myocyte hypertrophy and apoptosis, and inhibited interstitial fibrosis possibly through inhibiting the zymographic MMP-2 level in non-infarcted LV (Figure 4). MMP-2 is ubiquitously distributed in cardiac myocytes and fibroblasts, and has been shown to play a crucial role in the development of cardiac remodeling after MI.\textsuperscript{30} Theoretically, an increase in MMP activity would result in a decrease in the MMP substrate, collagens,
whereas an inhibition of MMP would result in an increase in collagens. However, our previous study showed that the selective disruption of the MMP-2 gene attenuated interstitial fibrosis after MI.\textsuperscript{30} Therefore, the decrease in zymographic MMP-2 level by αGC might be involved in the attenuation of interstitial fibrosis in our model. On the other hand, MMP-9 is mainly expressed in infiltrating inflammatory cells such as neutrophils and T lymphocytes. Previous paper showed that subcutaneous injection of recombinant IL-10 suppressed inflammation and attenuated LV remodeling after MI in mice by inhibiting fibrosis via suppression of HuR/MMP-9 and by enhancing capillary density through the activation of STAT3.\textsuperscript{31} Moreover, the previous study by Burchfield et al showed that IL-10 from transplanted bone marrow mononuclear cells contributed to cardiac protection after MI in association with a decrease in T lymphocyte accumulation, reactive hypertrophy, and myocardial collagen deposition.\textsuperscript{32} However, in the present study, zymographic MMP-9 level was not affected by αGC, which was consistent with the infiltration of lymphocyte observed by immunohistochemical staining for CD3 (Figure 5). We also measured the protein levels of HuR/MMP-9 or STAT3 in the non-infarcted LV. However, these protein levels were not affected by αGC (data not shown).

**Role of other inflammatory cells and cytokines.**

In agreement with the increase in macrophage infiltration by αGC, MCP-1 gene expression was increased. αGC increased not only M1 macrophage but also M2 macrophage, which tune inflammatory responses and promote tissue repair.\textsuperscript{33} Therefore, the increase in M2 macrophage might neutralize the effect of the increased M1 macrophage and MCP-1. The present study also showed that TNF-α was increased in non-infarcted LV from MI+αGC (Figure 6). TNF-α is a proinflammatory cytokine considered to be cardiotoxic and induce LV dysfunction.\textsuperscript{34} However, in contrast, TNF-α has also protective effects during the maladaptive transition to HF.\textsuperscript{35} Indeed, the treatment of patients with HF with either soluble TNF receptor (RENEWAL) or an anti-TNF antibody (ATTACH) could not show clinical benefits.\textsuperscript{36, 37} Therefore, the increase in TNF-α by αGC would not necessarily lead to the aggravation of LV remodeling.

**Limitations.**

There are several limitations to be acknowledged in the present study. First, we could not directly demonstrate the location of iNKT cells by the immunohistochemical analysis using biotinylated CD1d dimer (BD Bioscience) with loading of αGC according to the previous report by Kamijyuku et al.\textsuperscript{38} We tried the double immunohistochemical staining using antibodies for anti-armenian hamster TCR-β-PE (BD Bioscience) and anti-mouse NK 1.1-APC (BD Bioscience) according to the newly published paper.\textsuperscript{39} Furthermore, we also performed in situ hybridization using Digoxigenin-labeled DNA probes for mouse Vα14Jα18. Unfortunately, however, we could not detect iNKT cells in situ in the heart. Even though we defined iNKT cells within the heart by using the gene expression as well as the flow cytometric analysis, further studies are needed to overcome some
technical difficulties of in situ detection and clarify this important issue. Second, the underlying mechanisms responsible for the activation of iNKT cells after MI remain to be established. To date, the endogenous ligand for iNKT cells has not been known. Based on our results using αGC, a glycosphingolipid, sphingolipid ceramide may be a crucial intermediate, since ceramide has been shown to be synthesized by long-chain fatty acids and actually increased in the heart after coronary microembolization.40 Third, the source of IL-10 production after the stimulation of αGC remains to be determined. IL-10 has been shown to be produced by iNKT cells themselves upon exogenous stimulation.41 In addition, IL-10 can be expressed and secreted from macrophages activated by iNKT cells.42, 43 In the present study, the activation of iNKT cells by αGC injection increased the infiltration of macrophage in sham and MI mice at 7 days, however, there was no difference in it between MI+PBS and MI+αGC at 28 days (Figure 6). Therefore, the main source of IL-10 production at later phase of αGC injection would be the cells other than macrophages.

In conclusion, iNKT cells have a protective effect on LV remodeling and failure after MI via enhanced IL-10 expression. Therefore, therapies designed to activate iNKT cells may be beneficial against the development of post-MI heart failure.

ACKNOWLEDGEMENTS
We thank Kaoruko Kawai, Akiko Aita, and Miwako Fujii for excellent technical assistance.

SOURCES OF FUNDINGS
This study was supported by grants from the Ministry of Education, Science, and Culture (17390223, 20590854, 20117004, 21390236), and Hokkaido Heart Association Grant for Research.

DISCLOSURES
None

REFERENCES
Cytotoxic T lymphocytes are activated following myocardial infarction and can recognize and kill healthy myocytes in vitro. J Mol Cell Cardiol. 2000;32:2141-2149.


26. Frangogiannis NG, Mendoza LH, Lindsey ML, Ballantyne CM, Michael LH, Smith CW, Entman ML. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. *J Immunol.* 2000;165:2798-2808.


Table 1. Echocardiography, Hemodynamics, and Organ Weights in Experiment 2

<table>
<thead>
<tr>
<th></th>
<th>Sham+PBS</th>
<th>Sham+αGC</th>
<th>MI+PBS</th>
<th>MI+αGC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>522±10</td>
<td>522±12</td>
<td>531±16</td>
<td>520±13</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.4±0.1</td>
<td>3.4±0.04</td>
<td>5.4±0.1*</td>
<td>5.0±0.1*†</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.1±0.03</td>
<td>2.1±0.04</td>
<td>4.5±0.1*</td>
<td>4.1±0.1*†</td>
</tr>
<tr>
<td>FS, %</td>
<td>38.2±0.7</td>
<td>38.3±0.6</td>
<td>16.5±0.6*</td>
<td>18.8±0.6*†</td>
</tr>
<tr>
<td>AWT, mm</td>
<td>0.63±0.01</td>
<td>0.62±0.01</td>
<td>0.31±0.01*</td>
<td>0.30±0.01*</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>0.68±0.02</td>
<td>0.68±0.01</td>
<td>0.97±0.01*</td>
<td>0.96±0.02*</td>
</tr>
<tr>
<td><strong>Hemodynamics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, /min</td>
<td>507±9</td>
<td>499±9</td>
<td>485±23</td>
<td>495±11</td>
</tr>
<tr>
<td>Mean AoP, mmHg</td>
<td>78.1±2</td>
<td>77.7±2</td>
<td>75.0±3</td>
<td>79.3±1</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>1.7±0.3</td>
<td>2.3±0.1</td>
<td>10.7±1.1*</td>
<td>6.6±0.6*†</td>
</tr>
<tr>
<td>LV +dP/dt, mmHg/s</td>
<td>15625±623</td>
<td>14972±398</td>
<td>7352±697*</td>
<td>9386±476*†</td>
</tr>
<tr>
<td>LV -dP/dt, mmHg/s</td>
<td>9983±697</td>
<td>9130±691</td>
<td>5045±482*</td>
<td>5861±286*</td>
</tr>
<tr>
<td><strong>Organ Weights</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body wt, g</td>
<td>25.1±0.3</td>
<td>24.9±0.2</td>
<td>24.5±0.4</td>
<td>24.8±0.3</td>
</tr>
<tr>
<td>Heart wt/Body wt, mg/g</td>
<td>4.6±0.1</td>
<td>4.5±0.1</td>
<td>6.8±0.2*</td>
<td>6.1±0.1*†</td>
</tr>
<tr>
<td>Lung wt/Body wt, mg/g</td>
<td>5.2±0.03</td>
<td>5.2±0.1</td>
<td>7.2±0.7*</td>
<td>5.9±0.2†</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>-</td>
<td>-</td>
<td>56±2</td>
<td>55±1</td>
</tr>
</tbody>
</table>

LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness; AoP, aortic pressure; LVEDP, left ventricular end-diastolic pressure; wt, weight. Data are mean ± SEM. *P<0.05 vs. Sham+PBS, †P<0.05 vs. MI+PBS.
Table 2. Echocardiography, Hemodynamics, and Organ Weights in Experiment 3

<table>
<thead>
<tr>
<th></th>
<th>MI+αGC</th>
<th>MI+ anti-IL-10 receptor antibody</th>
<th>MI+αGC+ anti-IL-10 receptor antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>516±18</td>
<td>519±16</td>
<td>510±18</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>4.8±0.1</td>
<td>5.4±0.1*</td>
<td>5.4±0.1*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.9±0.1</td>
<td>4.6±0.1*</td>
<td>4.6±0.1*</td>
</tr>
<tr>
<td>FS, %</td>
<td>19±0.8</td>
<td>14.5±0.7*</td>
<td>15.4±0.7*</td>
</tr>
<tr>
<td>AWT, mm</td>
<td>0.30±0.01</td>
<td>0.37±0.06</td>
<td>0.35±0.06</td>
</tr>
<tr>
<td>PWT, mm</td>
<td>0.98±0.02</td>
<td>1.02±0.02</td>
<td>0.99±0.04</td>
</tr>
<tr>
<td><strong>Hemodynamics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, /min</td>
<td>518±16</td>
<td>487±17</td>
<td>515±22</td>
</tr>
<tr>
<td>Mean AoP, mmHg</td>
<td>86±3</td>
<td>81±4</td>
<td>82±2</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5.4±0.8</td>
<td>10.8±0.7*</td>
<td>11.4±3.3*</td>
</tr>
<tr>
<td>LV +dP/dt, mmHg/s</td>
<td>10441±661</td>
<td>6555±1031</td>
<td>7719±1284</td>
</tr>
<tr>
<td>LV -dP/dt, mmHg/s</td>
<td>6847±569</td>
<td>4119±364</td>
<td>5774±1236</td>
</tr>
<tr>
<td><strong>Organ Weights</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body wt, g</td>
<td>25.2±0.5</td>
<td>24.7±1.3</td>
<td>25.8±0.6</td>
</tr>
<tr>
<td>Heart wt/Body wt, mg/g</td>
<td>6.3±0.3</td>
<td>8.8±0.9</td>
<td>6.9±0.5</td>
</tr>
<tr>
<td>Lung wt/Body wt, mg/g</td>
<td>5.5±0.1</td>
<td>10.9±2.1*</td>
<td>7.9±1.0*</td>
</tr>
<tr>
<td>Infarct size, %</td>
<td>56±2</td>
<td>54±2</td>
<td>56±4</td>
</tr>
</tbody>
</table>

LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness; AoP, aortic pressure; LVEDP, left ventricular end-diastolic pressure; wt, weight. Data are mean ± SEM. *P<0.05 vs. MI+αGC.
**FIGURE LEGENDS**

**Figure 1.** (A) Representative flow cytometric assessment of cardiac mononuclear cells obtained from Sham+PBS, Sham+αGC, MI+PBS, and MI+αGC at day 7 and 28. Cardiac mononuclear cells from 5 different mice for each group were pooled and analyzed. The experiments were performed 3 times. iNKT cells were gated as the αGC-loaded dimer X tetramer^TCR-β^ population. The inset numbers are a percentage of the gated region of the samples. (B) Gene expression of Vα14/Jα18 in non-infarcted LV from sham+PBS, sham+αGC, MI+PBS and MI+αGC 7 days (n=6) and 28 days (n=4) after surgery. They were normalized to GAPDH gene expression and expressed as ratio to sham+PBS values. Data are expressed as means ± SE. *P<0.05 vs. Sham+PBS, †P<0.05 vs. MI+PBS.

**Figure 2.** (A) Percent survival of Sham+PBS (n=10), Sham+αGC (n=10), MI+PBS (n=31) and MI+αGC (n=27) mice shown by Kaplan-Meier method. (B) Representative M-mode echocardiographic images obtained from sham+PBS, sham+αGC, MI+PBS and MI+αGC. AW indicates anterior wall; PW, posterior wall; EDD, end-diastolic diameter.

**Figure 3.** (A) Representative high-power photomicrographs of LV cross-sections stained with Masson’s trichrome from sham+PBS (a), sham+αGC (b), MI+PBS (c) and MI+αGC (d) and summary data of myocyte cross-sectional area and collagen volume fraction in 4 groups of mice (n=6). Scale bar, 20 μm. (B) Representative photomicrographs TUNEL staining of LV sections from MI+PBS (a) and MI+αGC (b) and summary data for the number of TUNEL-positive cells in the non-infarcted LV (n=6). Scale bar, 20 μm. Data are expressed as means ± SE. *P<0.05 vs. Sham+PBS, †P<0.05 vs. MI+PBS.

**Figure 4.** Representative LV zymographic MMP-2 and MMP-9 activities in non-infarcted LV at 7 days after surgery (A) and their densitometric analysis (B and C; n=5 for each). P, positive control. Data are expressed as means ± SE. *P<0.05 vs. Sham+PBS, †P<0.05 vs. MI+PBS.

**Figure 5.** Representative photomicrographs of LV cross-sections stained with (A, upper panel) anti MAC3 and (B, upper panel) anti CD3 in Sham+PBS, Sham+αGC, MI+PBS and MI+αGC. Summary data of the numbers of (A, lower panel) MAC3 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, †P<0.05 vs. MI+PBS.

**Figure 6.** Quantitative analysis of gene expression of CD11c (A), Arginase (B), MCP-1 (C), RANTES (D), IFN-γ (E), TNF-α (F), IL-6 (G), and IL-10 (H) in the non-infarcted LV at day 7 (n=6) and 28 (n=4) after surgery. Gene expression was normalized to GAPDH and depicted as the ratio to Sham+PBS. Data are expressed as means ± SE. *P<0.05 vs. Sham+PBS, †P<0.05 vs. MI+PBS.
Novelty and Significance

What Is Known?

- Chronic tissue inflammation plays an important role in the development of left ventricular (LV) dysfunction and LV remodeling.

- Invariant natural killer T (iNKT) cells are a specialized lineage of T cells with NK marker. These cells produce a mixture of Th1 and Th2 cytokines and a vast array of chemokines to orchestrate tissue inflammation.

- iNKT cells play a protective role in experimental autoimmune and inflammatory diseases.

What New Information Does This Article Contribute?

- iNKT cells could be detected in normal heart and their infiltration was increased in non-infarcted LV after myocardial infarction (MI).

- The activation of iNKT cells by α-galactosylceramide (αGC) improved survival and ameliorated LV remodeling and failure after MI in mice, accompanied by decreases in interstitial fibrosis, cardiomyocyte hypertrophy, and apoptosis.

- An increase in the expression of interleukin (IL)-10 by αGC was involved in the favorable effects for LV remodeling after MI.

iNKT cells regulate tissue inflammation by producing a mixture of Th1 and Th2 cytokines. Although chronic tissue inflammation is involved in the development of LV remodeling and failure, the pathophysiological role of iNKT cells in these processes have not been elucidated. Our study shows that infiltration of iNKT cells was increased in non-infarcted LV and their activation by αGC improved survival and ameliorated LV remodeling and failure after MI via enhanced expression of IL-10. These findings indicate a previously undescribed protective effect of iNKT cells on LV remodeling and failure after MI. Given that iNKT cells can bridge innate and adaptive immune systems, they could act as an upstream regulator of cytokine networks in the heart. Therapies designed to regulate iNKT cells and to modulate cytokine network may be beneficial in ameliorating LV remodeling and failure.
Figure 1

(A) Flow cytometry analysis of αGC-loaded dimer X tetramer expression on Day 7 and Day 28.

(B) Graph showing the ratio of Vol.14Jα18 (Ratio to Sham+PBS) for different groups on Day 7 and Day 28.
The Activation of Natural Killer T Cells Ameliorates Post-Infarct Cardiac Remodeling and Failure in Mice
Mochamad Ali Sobirin, Shintaro Kinugawa, Masashige Takahashi, Arata Fukushima, Tsuneaki Homma, Taisuke Ono, Kagami Hirabayashi, Tadashi Suga, Putri Azalia, Shingo Takada, Masaru Taniguchi, Toshinori Nakayama, Naoki Ishimori, Kazuya Iwabuchi and Hiroyuki Tsutsui

Circ Res. published online August 10, 2012;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/08/10/CIRCRESAHA.112.270132

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/08/10/CIRCRESAHA.112.270132.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Detailed Methods

An expanded Methods section is available in the online Data Supplement at http://cireres.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.1 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 Vα14Jα18 (a specific marker of iNKT cells).2 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 α-galactosylceramide (αGC; 0.1μg/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 αGC was chosen based on the previous study of its efficacy.2 Thus, the experiment was performed in the following 4 groups of mice; sham+PBS (n=10), sham+αGC (n=10), MI+PBS (n=31), and MI+αGC (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+αGC, n=10 for MI+PBS, and n=16 for MI+αGC). 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/amylene hydrate (avertin; 2.5% wt/vol, 8 µL/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 caudal 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-µm 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.

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.

**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 10^5 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.1

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 ºC for 45 min. Tissue was then triturated through nylon mesh and centrifuged (1400 rpm, 5min, 4 ºC). Red blood cells were lysed with Tris-NH₄Cl solution. Cardiac mononuclear cells were isolated by density-gradient centrifugation with 33% Percoll™, as previously described.4 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 αGC, followed by detection with phycoerythrin (PE)-conjugated anti-mouse IgG1 mAb (BD) according to the manufacturer’s protocol.5 After washing, cells were stained with a combination of fluorescein isothiocyanate (FITC)-anti-TCRβ 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 \( \alpha \)14Jα18, 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 αGC-Treated Post-MI Hearts**

MI mice were divided into the following 3 groups of mice; MI+αGC (n=18), MI+anti-IL-10 receptor antibody (n=12), and MI+αGC+anti-IL-10 receptor antibody (n=19). αGC was administered identically as in Experiment 2. Anti-IL-10 receptor antibody (500µg/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.6

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+αGC group.

**Experiment 4: Specificity of αGC for NKT Cells**

Vα14+ NKT cell-deficient Jα18−/− (Jα18 KO) mice were provided from Dr. M. Taniguchi (RIKEN, Yokohama, Japan) and backcrossed 10 times to C57BL/6J.7 Sham and MI mice were created in male Jα18 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+αGC, KO+MI+PBS, and KO+MI+αGC. 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 ± 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 Figures and Figure Legends

Online Figure I. 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.
Online Figure II. Quantitative analysis of gene expression of MCP-1 (A), RANTES (B), TNF-α (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 ± SE. *P<0.05 vs. Sham+PBS.
Supplemental References


