regulating IFN-γ production in CD4+ T cells: in the absence of polarizing signals, Ezh2 suppresses spontaneous generation of IFN-γ-producing cells via inhibition of Eomes expression. The findings presented herein provide a dramatic example of the importance of Ezh2 in establishing and maintaining phenotypic identity and indicate that without the ability to actively suppress alternative fates via chromatin modifications, dysregulated immune responses ensue.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were from CLEA Co. Mice with loxp sites (fl) flanking the SET domain of *Ezh2* (*Ezh2*$^{SET\text{-}fl/SET\text{-}fl}$) were generated as described (Hirabayashi et al., 2009) and backcrossed with C57BL/6 mice for ten generations. *Eomes*$^{Exon1\text{-}fl/Exon1\text{-}fl}$ mice were kindly provided by S. Reiner (Columbia University) (Intlekofer et al., 2008). *Eomes*$^{Exon1\text{-}fl/Exon1\text{-}fl}$ mice and *Tbx21*$^{-/-}$ mice (Szabo et al., 2002) were crossed with *Ezh2*$^{SET\text{-}fl/SET\text{-}fl}$ mice in our laboratory and bred with mice expressing transgenes for Cre recombinase. CD4-Cre mice were purchased from Taconic and OX40-Cre mice were kindly provided by N. Killeen (UCSF) (Klinger et al., 2009). For antigen-specific activation of CD4+ T cells, mice expressing the OTII-TCRαβ specific for residues 323–339 of the ovalbumin protein were used. All wild-type control mice used were sex-matched littermates. Controls were either Ezh2$^{SET\text{-}fl/SET\text{-}fl}$ Cre-negative or Ezh2$^{wt/wt}$ Cre-positive with similar experimental results obtained irrespective of Cre transgene expression. All mice were maintained under specific-pathogen-free conditions and animal care was conducted in accordance with the guidelines of Chiba University.

CD4+ T Cell Cultures

Naive (CD44^{lo}CD62L^{hi}) CD4+ T cell purification from spleens of mice and culture conditions for Th cell differentiation are described in the Supplemental Experimental Procedures.

Immunofluorescent Staining for Flow-Cytometric Analysis

The antibodies used for detection of surface molecules and intracellular staining are listed in the Supplemental Experimental Procedures. Flow cytometry data was acquired on a FACSCalibur or FACSCanto flow cytometer and results analyzed with FlowJo software (Tree Star).

Enzyme-Linked Immunosorbent Assay

A standard sandwich ELISA protocol was used to measure the concentrations of cytokines in cell-free culture supernatants and BAL fluid samples. Antibody pairs are listed in the Supplemental Experimental Procedures.

Knockdown Analysis

For knockdown of Gata3, the Mouse T cell Nucleofector Kit (Amaxa) was used according to the manufacturer's protocol. Th2 cells were transfected with 675 pmole of control random siRNA (AM4635) or siRNA for *Gata3* (s66482) from Applied Biosystems and cultured for 24 hr before analysis.

Quantitative Reverse Transcription-Polymerase Chain Reaction

RT-PCR was performed by standard protocols. A detailed description is included in the Supplemental Experimental Procedures. Probes and primers are listed in Table S3.

Microarray Data Collection and Analysis

Total cellular RNA was extracted with TRIzol reagent (Invitrogen). RNA was labeled with a 3′ IVT Express kit (Affymetrix) and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturer's protocols. Expression values were determined with GeneChip Operating Software (GCOS) software (Affymetrix).

Chromatin Immunoprecipitation

ChIP assays were performed as described in the Supplemental Experimental Procedures. Polyclonal anti-Ezh2 (pAB-039-50, Diagenode), anti-H3K27me3 (07-446, Millipore), anti-H3K9-Ac (06-599, Millipore), anti-H3K27ac (ab4729, Abcam), anti-p300 (N-15 sc-548, Santa Cruz), and anti-CBP (ab2832, Abcam) were used for immunoprecipitation. Probes and primers are listed in Table S3. Enrichment was calculated with the following formula: (specific antibody ChIP – control Ig ChIP)/input DNA. The highest enrichment for each antibody was set to 10 with all other positions calculated as a function of this value.

ChIP-Sequence

Data acquisition for ChIP-sequence was performed as described previously (Kanai et al., 2011). Read sequences were aligned to the mm9 mouse reference genome (University of California, Santa Cruz [UCSC], July 2007). Enrichment values were calculated for intervals from −5 kb to +3 kb relative to the transcriptional start site of each gene. A detailed description of the analysis protocol is included in the Supplemental Experimental Procedures.

Mouse Model of Allergic Asthma

Th2 cell polarized Ezh2 WT or OX40-Cre-induced *Ezh2*$^{\Delta SET/\Delta SET}$ OT-II CD4+ cells (2×10^6) were intravenously transferred to syngeneic C57BL/6 mice. These mice were then challenged 1 and 3 days later with aerosolized OVA (10 mg/ml) for 30 min. BAL was performed either 12 hr (for ELISA) or 48 hr (for analysis of airway inflammatory cells) after the last allergen challenge. mRNA and histological analysis was performed 48 hr after the last allergen challenge. A detailed description is included in the Supplemental Experimental Procedures.

Statistical Analysis

Unless otherwise indicated, p values were calculated with Student's t tests or ANOVA with Bonferroni's post-tests when multiple comparisons were performed.

ACCESSION NUMBERS

ChIP-seq and microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession numbers GSE51079 and GSE50729, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.09.012.

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REFERENCES

Aguado, E., Richelme, S., Nuñez-Cruz, S., Miazek, A., Mura, A.M., Richelme, M., Guo, X.J., Sainty, D., He, H.T., Malissen, B., and Malissen, M. (2002). Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science *296*, 2036–2040.

Allan, R.S., Zueva, E., Cammas, F., Schreiber, H.A., Masson, V., Belz, G.T., Roche, D., Maison, C., Quivy, J.P., Almouzni, G., and Amigorena, S. (2012). An epigenetic silencing pathway controlling T helper 2 cell lineage commitment. Nature *487*, 249–253.

Ansel, K.M., Djuretic, I., Tanasa, B., and Rao, A. (2006). Regulation of Th2 differentiation and Il4 locus accessibility. Annu. Rev. Immunol. *24*, 607–656.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349–353.

Di Meglio, T., Kratochwil, C.F., Vilain, N., Loche, A., Vitobello, A., Yonehara, K., Hrycaj, S.M., Roska, B., Peters, A.H., Eichmann, A., et al. (2013). Ezh2 orchestrates topographic migration and connectivity of mouse precerebellar neurons. Science *339*, 204–207.

Ezhkova, E., Pasolli, H.A., Parker, J.S., Stokes, N., Su, I.H., Hannon, G., Tarakhovsky, A., and Fuchs, E. (2009). Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell *136*, 1122–1135.

He, S., Wang, J., Kato, K., Xie, F., Varambally, S., Mineishi, S., Kuick, R., Mochizuki, K., Liu, Y., Nieves, E., et al. (2012). Inhibition of histone methylation arrests ongoing graft-versus-host disease in mice by selectively inducing apoptosis of alloreactive effector T cells. Blood *119*, 1274–1282.

Hirabayashi, Y., Suzki, N., Tsuboi, M., Endo, T.A., Toyoda, T., Shinga, J., Koseki, H., Vidal, M., and Gotoh, Y. (2009). Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. Neuron *63*, 600–613.

Hosokawa, H., Kimura, M.Y., Shinnakasu, R., Suzuki, A., Miki, T., Koseki, H., van Lohuizen, M., Yamashita, M., and Nakayama, T. (2006). Regulation of Th2 cell development by Polycomb group gene bmi-1 through the stabilization of GATA3. J. Immunol. *177*, 7656–7664.

Hosokawa, H., Tanaka, T., Suzuki, Y., Iwamura, C., Ohkubo, S., Endoh, K., Kato, M., Endo, Y., Onodera, A., Tumes, D.J., et al. (2013). Functionally distinct Gata3/Chd4 complexes coordinately establish T helper 2 (Th2) cell identity. Proc. Natl. Acad. Sci. USA *110*, 4691–4696.

Huang, W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57.

Hwang, E.S., Szabo, S.J., Schwartzberg, P.L., and Glimcher, L.H. (2005). T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science *307*, 430–433.

Intlekofer, A.M., Banerjee, A., Takemoto, N., Gordon, S.M., Dejong, C.S., Shin, H., Hunter, C.A., Wherry, E.J., Lindsten, T., and Reiner, S.L. (2008). Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science *321*, 408–411.

Jacob, E., Hod-Dvorai, R., Schif-Zuck, S., and Avni, O. (2008). Unconventional association of the polycomb group proteins with cytokine genes in differentiated T helper cells. J. Biol. Chem. *283*, 13471–13481.

Ji, H., Rintelen, F., Waltzinger, C., Bertschy Meier, D., Bilancio, A., Pearce, W., Hirsch, E., Wymann, M.P., Rückle, T., Camps, M., et al. (2007). Inactivation of PI3Kgamma and PI3Kdelta distorts T-cell development and causes multiple organ inflammation. Blood *110*, 2940–2947.

Kanai, A., Suzuki, K., Tanimoto, K., Mizushima-Sugano, J., Suzuki, Y., and Sugano, S. (2011). Characterization of STAT6 target genes in human B cells and lung epithelial cells. DNA Res. *18*, 379–392.

Kanno, Y., Vahedi, G., Hirahara, K., Singleton, K., and O'Shea, J.J. (2012). Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. Annu. Rev. Immunol. *30*, 707–731.

Kimura, M., Koseki, Y., Yamashita, M., Watanabe, N., Shimizu, C., Katsumoto, T., Kitamura, T., Taniguchi, M., Koseki, H., and Nakayama, T. (2001). Regulation of Th2 cell differentiation by mel-18, a mammalian polycomb group gene. Immunity *15*, 275–287.

Klinger, M., Kim, J.K., Chmura, S.A., Barczak, A., Erle, D.J., and Killeen, N. (2009). Thymic OX40 expression discriminates cells undergoing strong responses to selection ligands. J. Immunol. *182*, 4581–4589.

Koyanagi, M., Baguet, A., Martens, J., Margueron, R., Jenuwein, T., and Bix, M. (2005). EZH2 and histone 3 trimethyl lysine 27 associated with Il4 and Il13 gene silencing in Th1 cells. J. Biol. Chem. *280*, 31470–31477.

Löhning, M., Richter, A., and Radbruch, A. (2002). Cytokine memory of T helper lymphocytes. Adv. Immunol. *80*, 115–181.

Makar, K.W., Pérez-Melgosa, M., Shnyreva, M., Weaver, W.M., Fitzpatrick, D.R., and Wilson, C.B. (2003). Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. Nat. Immunol. *4*, 1183–1190.

Nakayama, T., and Yamashita, M. (2008). Initiation and maintenance of Th2 cell identity. Curr. Opin. Immunol. *20*, 265–271.

O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. Mol. Cell. Biol. *21*, 4330–4336.

O'Shea, J.J., and Paul, W.E. (2010). Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science *327*, 1098–1102.

Onodera, A., Yamashita, M., Endo, Y., Kuwahara, M., Tofukuji, S., Hosokawa, H., Kanai, A., Suzuki, Y., and Nakayama, T. (2010). STAT6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of GATA3 expression in T helper type 2 cells. J. Exp. Med. *207*, 2493–2506.

Ranger, A.M., Oukka, M., Rengarajan, J., and Glimcher, L.H. (1998). Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. Immunity *9*, 627–635.

Reiner, S.L. (2007). Development in motion: helper T cells at work. Cell *129*, 33–36.

Schoenborn, J.R., Dorschner, M.O., Sekimata, M., Santer, D.M., Shnyreva, M., Fitzpatrick, D.R., Stamatoyannopoulos, J.A., and Wilson, C.B. (2007). Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. Nat. Immunol. *8*, 732–742.

Shen, X., Liu, Y., Hsu, Y.J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.C., and Orkin, S.H. (2008). EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol. Cell *32*, 491–502.

Su, I.H., Basavaraj, A., Krutchinsky, A.N., Hobert, O., Ullrich, A., Chait, B.T., and Tarakhovsky, A. (2003). Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. Nat. Immunol. *4*, 124–131.

Su, I.H., Dobenecker, M.W., Dickinson, E., Oser, M., Basavaraj, A., Marqueron, R., Viale, A., Reinberg, D., Wülfing, C., and Tarakhovsky, A. (2005). Polycomb group protein ezh2 controls actin polymerization and cell signaling. Cell *121*, 425–436.

Suto, A., Wurster, A.L., Reiner, S.L., and Grusby, M.J. (2006). IL-21 inhibits IFN-gamma production in developing Th1 cells through the repression of Eomesodermin expression. J. Immunol. *177*, 3721–3727.

Suzuki, A., Iwamura, C., Shinoda, K., Tumes, D.J., Kimura, M.Y., Hosokawa, H., Endo, Y., Horiuchi, S., Tokoyoda, K., Koseki, H., et al. (2010). Polycomb group gene product Ring1B regulates Th2-driven airway inflammation through the inhibition of Bim-mediated apoptosis of effector Th2 cells in the lung. J. Immunol. *184*, 4510–4520.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., and Glimcher, L.H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655–669.

Szabo, S.J., Sullivan, B.M., Stemmann, C., Satoskar, A.R., Sleckman, B.P., and Glimcher, L.H. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science *295*, 338–342.

Tan, J., Yang, X., Zhuang, L., Jiang, X., Chen, W., Lee, P.L., Karuturi, R.K., Tan, P.B., Liu, E.T., and Yu, Q. (2007). Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. *21*, 1050–1063.

Thomas, R.M., Gamper, C.J., Ladle, B.H., Powell, J.D., and Wells, A.D. (2012). De novo DNA methylation is required to restrict T helper lineage plasticity. J. Biol. Chem. *287*, 22900–22909.

Tofukuji, S., Kuwahara, M., Suzuki, J., Ohara, O., Nakayama, T., and Yamashita, M. (2012). Identification of a new pathway for Th1 cell development induced by cooperative stimulation with IL-4 and TGF-β. J. Immunol. *188*, 4846–4857.

Usui, T., Nishikomori, R., Kitani, A., and Strober, W. (2003). GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. Immunity *18*, 415–428.

Usui, T., Preiss, J.C., Kanno, Y., Yao, Z.J., Bream, J.H., O'Shea, J.J., and Strober, W. (2006). T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. J. Exp. Med. *203*, 755–766.

Wei, G., Wei, L., Zhu, J., Zang, C., Hu-Li, J., Yao, Z., Cui, K., Kanno, Y., Roh, T.Y., Watford, W.T., et al. (2009). Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity *30*, 155–167.

Yamashita, M., Ukai-Tadenuma, M., Miyamoto, T., Sugaya, K., Hosokawa, H., Hasegawa, A., Kimura, M., Taniguchi, M., DeGregori, J., and Nakayama, T. (2004). Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. J. Biol. Chem. *279*, 26983–26990.

Yamashita, M., Kuwahara, M., Suzuki, A., Hirahara, K., Shinnaksu, R., Hosokawa, H., Hasegawa, A., Motohashi, S., Iwama, A., and Nakayama, T. (2008). Bmi1 regulates memory CD4 T cell survival via repression of the Noxa gene. J. Exp. Med. *205*, 1109–1120.

Yang, Y., Xu, J., Niu, Y., Bromberg, J.S., and Ding, Y. (2008). T-bet and eomesodermin play critical roles in directing T cell differentiation to Th1 versus Th17. J. Immunol. *181*, 8700–8710.

Yu, Q., Zhou, B., Zhang, Y., Nguyen, E.T., Du, J., Glosson, N.L., and Kaplan, M.H. (2012). DNA methyltransferase 3a limits the expression of interleukin-13 in T helper 2 cells and allergic airway inflammation. Proc. Natl. Acad. Sci. USA *109*, 541–546.

Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587–596.

Zhu, J., Yamane, H., and Paul, W.E. (2010). Differentiation of effector CD4 T cell populations (*). Annu. Rev. Immunol. *28*, 445–489.

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Original article

Activation of invariant natural killer T cells by α-galactosylceramide ameliorates myocardial ischemia/reperfusion injury in mice

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ABSTRACT

Invariant natural killer T (iNKT) cells orchestrate tissue inflammation via regulating various cytokine productions. However the role of iNKT cells has not been determined in myocardial ischemia/reperfusion (I/R) injury. The purpose of this study was to examine whether the activation of iNKT cells by α-galactosylceramide (α-GC), which specifically activates iNKT cells, could affect myocardial I/R injury. I/R or sham operation was performed in male C57BL/6J mice. I/R mice received the injection of either αGC (I/R + αGC, n = 48) or vehicle (I/R + vehicle, n = 49) 30 min before reperfusion. After 24 h, infarct size/area at risk was smaller in I/R + αGC than in I/R + vehicle (37.8 ± 2.7% vs. 47.1 ± 2.5%, $P < 0.05$), with no significant changes in area at risk. The numbers of infiltrating myeloperoxidase- and CD3-positive cells were lower in I/R + αGC. Apoptosis evaluated by TUNEL staining and caspase-3 protein was also attenuated in I/R + αGC. Myocardial gene expression of tumor necrosis factor-α and interleukin (IL)-1β in I/R + αGC was lower to 46% and 80% of that in I/R + vehicle, respectively, whereas IL-10, IL-4, and interferon (IFN)-γ were higher in I/R + αGC than I/R + vehicle by 2.0, 4.1, and 9.6 folds, respectively. The administration of anti-IL-10 receptor antibody into I/R + αGC abolished the protective effects of αGC on I/R injury (infarct size/area at risk: 53.1 ± 5.2% vs. 37.4 ± 3.5%, $P < 0.05$). In contrast, anti-IL-4 and anti-IFN-γ antibodies did not exert such effects. In conclusion, activated iNKT cells by αGC play a protective role against myocardial I/R injury through the enhanced expression of IL-10. Therapies designed to activate iNKT cells might be beneficial to protect the heart from I/R injury.

1. Introduction

Early and successful myocardial reperfusion is the most effective strategy to reduce infarct size and preserve cardiac function after acute myocardial infarction (MI) [1]. Reperfusion after ischemia can salvage the ischemic myocardium, however, simultaneously it causes additional cell death and attenuates the beneficial effects of reperfusion itself, called myocardial ischemia/reperfusion (I/R) injury [2]. Inflammation has been shown to play a critical role in the pathophysiology of myocardial I/R injury [3], and various immune cells, such as neutrophils, T lymphocytes, monocytes/macrophages, and mast cells, are involved in myocardial I/R injury [4–7]. Recent study by Yang et al. demonstrated that $CD4^+$ T lymphocytes played an important role in the development of I/R injury and interferon (IFN)-γ was involved in their action by using Rag1 knockout mice lacking mature lymphocytes [5].

Invariant natural killer T (iNKT or type 1 NKT) cells are innate-like T lymphocyte population characterized by co-expressing NK lineage receptors and T cell receptors (TCR), and their TCR has invariant α-chain (Vα14-Jα18 in mice, and Vα24-Jα18 in humans) [8,9]. They are activated by recognizing glycolipid antigens presented by CD1d, a member of major histocompatibility complex (MHC) class I

Abbreviations: AAR, area at risk; αGC, α-galactosylceramide; IFN-γ, interferon-γ; IL, interleukin; iNKT, invariant natural killer T; I/R, ischemia/reperfusion; IS, infarct size; LV, left ventricle; MI, myocardial infarction; MNCs, mononuclear cells; MPO, myeloperoxidase; NK, natural killer; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; TCR, T cell receptor; T_H1, T-helper type 1; T_H2, T-helper type 2; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TTC, 2,3,5-triphenyltetrazolium chloride.

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like molecules, and rapidly secrete a mixture of large amount of T-helper type 1 (T_H1) and T_H2 cytokines, such as IFN-γ, interleukin (IL)-10 and IL-4 in shaping subsequent adaptive immune responses [10]. Thus, iNKT cells can function as a bridge between the innate and adaptive immune systems, and orchestrate tissue inflammation.

iNKT cells have been demonstrated to play a protective role in various autoimmune and inflammatory diseases such as type 1 diabetes, experimental allergic encephalomyelitis, rheumatoid arthritis, and enteritis [11–15]. We have also reported that the activation of iNKT cells by α-galactosylceramide (αGC), a specific activator for iNKT cells [16], can attenuate the development of left ventricular (LV) remodeling and failure after MI created by chronic ligation of coronary artery in mice [17]. The activation of iNKT cells by αGC has also been reported to protect the liver against I/R injury in mice via IL-13 production [18]. However, no previous studies have examined the effects of iNKT cell activation by αGC on myocardial I/R injury.

Therefore, the purpose of the present study was to determine whether the activation of iNKT cells by αGC could attenuate myocardial I/R injury. We also determined whether the protective effects on attenuated myocardial I/R injury might involve the activation of anti-inflammatory cytokines including IL-10.

2. Materials and methods

Detailed methods are available in the Online Supplementary Material.

2.1. Animals

C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animals were used for experiments at 10 to 12 weeks of age (weight 23–27 g). Mice were bred in a pathogen-free environment and kept under a constant 12-h light–dark cycle at a temperature of 23 °C to 25 °C. Standard chaw and water were provided.

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.

2.2. Experimental design

2.2.1. Experiment 1: effects of αGC on iNKT cell and cytokine expression in the normal mice

To confirm that αGC could activate iNKT cells in the heart similar to the spleen, C57BL/6J mice were sacrificed 0, 24, and 72 h after αGC (Funakoshi Co., Ltd., Tokyo, Japan) injection (0.1 μg/g body weight i.p., n = 9 for each group) and the proportion of iNKT cells in the heart and spleen were measured by flow cytometric analysis [17].

To determine that αGC could induce the changes of cytokines in the blood and the heart within 24 h, another group of C57BL/6J mice were sacrificed 0, 0.5, 1, 3, 6, 12, and 24 h after single injection of αGC (n = 6 for each group). Serum levels of IL-10, IL-4, and IFN-γ were measured by ELISA and their gene expressions in the heart were measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). TCR in iNKT cells has invariant α-chain encoded by Vα14-Jα18 gene segment in mice, and $J\alpha18^{-/-}$ mice lack iNKT cells. To confirm whether the changes of cytokines by the injection of αGC were due to the activation of iNKT cells, iNKT cell-deficient ($J\alpha18^{-/-}$) mice were sacrificed after single injection of αGC and same measurements were performed. They were provided from Dr. M. Taniguchi (RIKEN, Yokohama, Japan) and backcrossed 10 times to C57BL/6J.

2.2.2. Experiment 2: effects of αGC on myocardial I/R injury

Myocardial I/R surgery or sham operation was performed in C57BL/6J mice according to the methods described previously [5]. After anesthesia, the left coronary artery was ligated for 45 min. Ischemia was confirmed by bleaching of the myocardium. Reperfusion was initiated by releasing the ligature. Sham-operated mice underwent a similar procedure without ligation. αGC (0.1 μg/g body weight i.p.) was administered 30 min before reperfusion to specifically activate iNKT cells. As control, the same volume of vehicle was administered into sham and I/R mice.

Mice were sacrificed 24 h after reperfusion (sham + vehicle, n = 22; sham + αGC, n = 22; I/R + vehicle, n = 49, I/R + αGC, n = 48). These mice were divided into groups for some measurements. Another groups of mice were sacrificed 72 h after reperfusion for flow cytometric analysis (n = 9 for each group), because iNKT cells have been reported to be invisible by flow-cytometric detection 24 h after αGC administration [19]. Additional mice were sacrificed 72 h after reperfusion for RT-PCR analysis (n = 7–8 for each group). To confirm early protective effect of αGC, C57BL/6J mice received I/R surgery with vehicle or αGC, and sacrificed 2 h after reperfusion to measure infarct size (I/R + vehicle, n = 7; I/R + αGC, n = 8). Furthermore, to confirm the effect of αGC-induced reduction of infarct size on long-term LV function and remodeling, echocardiography and hemodynamic measurement were performed at 28 days after reperfusion (I/R + vehicle, n = 8; I/R + αGC, n = 8).

To confirm whether the effect of αGC on infarct size in I/R was due to the activation of iNKT cells, $J\alpha18^{-/-}$ mice received I/R surgery with vehicle or αGC and sacrificed 24 h after reperfusion to measure infarct size (n = 5 for each group).

Furthermore, to examine the role of various cytokines in the effects of αGC on myocardial I/R injury, rat anti-IL-10 receptor monoclonal antibody (200 μg/mouse, i.p., BD Pharmingen, San Diego, CA), rat anti-IL-4 monoclonal antibody (250 μg/mouse, i.p., R&D System, Inc.), or rat anti-IFN-γ monoclonal antibody (150 μg/mouse, i.p., R&D System, Inc.) was administered 90 min before I/R surgery and infarct size was measured 24 h after reperfusion. The doses of these antibodies were chosen based on the previous study of their efficacy [18,19,20]. We also confirmed that the changes of serum IL-4 or IFN-γ levels were completely inhibited by identical antibodies. Rat IgG1κ was used as control. αGC was administered 30 min before reperfusion (I/R + αGC + rat IgG1κ, n = 8; I/R + αGC + anti-IL-10R, n = 8; I/R + αGC + anti-IL-4, n = 7; I/R + αGC + anti-IFN-γ, n = 9).

Finally, to examine the role of IFN-γ on myocardial I/R injury, rat anti-IFN-γ monoclonal antibody (150 μg/mouse, i.p., R&D System, Inc.) or IgG1κ was administered 90 min before I/R surgery and infarct size was measured 24 h after reperfusion (n = 6 for each).

2.3. Statistical analysis

Data are expressed as means ± SE. The Student *t* test was performed for comparison between 2 independent groups. For multiple-group comparisons, one-way ANOVA followed by the Dunnett's test or the Tukey's test was performed. A value of $P < 0.05$ 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 agree to the manuscript as written.

3. Results

3.1. Experiment 1: effects of αGC on iNKT cell and cytokine expression in the normal mice

3.1.1. Proportion of iNKT cells after αGC administration

After αGC administration, splenic iNKT cells disappeared at 24 h and were increased at 72 h (Supplemental Fig. 1, upper panel) in consistency with the previous report [19]. The number of cardiac iNKT cells itself was lower than that of splenic iNKT cells. However, they were increased 72 h after αGC administration in parallel to splenic

iNKT cells (Supplemental Fig. 1, lower panel). Similar results were observed in 3 independent experiments.

3.2. Serum levels and myocardial gene expression of cytokines after αGC administration

After the administration of αGC, serum IL-10 and IL-4 levels were rapidly increased and peaked at 1 h and 3 h respectively, and serum IFN-γ levels were increased later and peaked at 12 h (Supplemental Fig. 2A), which was consistent with the previous reports [21,22]. Gene expression of IL-10, IL-4, and IFN-γ in the LV was increased within 24 h and peaked at later phase than serum levels (Supplemental Fig. 2B).

3.3. Specificity of αGC for iNKT cells

αGC did not increase serum levels and myocardial gene expression of IL-10, IL-4, and IFN-γ in Jα18$^{-/-}$ mice (Fig. 1).

3.4. Experiment 2: effects of αGC on myocardial I/R injury

3.4.1. Body weight and hemodynamics

There was no difference in body weight among all groups. Systolic blood pressure was significantly lower in I/R mice compared to sham mice, however, which was not affected by αGC. Diastolic blood pressure and heart rate did not differ among 4 groups.

3.5. iNKT cells

Representative flow cytometric analyses from 4 groups of mice are shown in Fig. 2A. The proportion of iNKT cells 72 h after reperfusion was increased up to 2.7-fold in I/R + vehicle compared to sham + vehicle. αGC significantly increased these proportion of iNKT cells both in sham up to 17.5-fold ($P = 0.042$) and I/R mice up to 10.3-fold ($P = 0.004$) (Fig. 2B). Similar results were observed in 3 independent experiments.

3.6. I/R injury and LV function

The administration of αGC into I/R mice decreased infarct size. IS/AAR was significantly smaller in I/R + αGC than in I/R + vehicle (37.8 ± 2.7% vs. 47.1 ± 2.5%, $P = 0.018$) without significant changes in AAR/LV (58.5 ± 2.3% vs. 58.9 ± 2.7%, P = NS) at 24 h after reperfusion (Fig. 3A). In consistent with these results, serum level of troponin-I was also lower in I/R + αGC than in I/R + vehicle (5.8 ± 0.8 ng/mL vs. 8.7 ± 0.7 ng/mL, $P = 0.016$, Fig. 3B). At 2 h after reperfusion, IS/AAR was also smaller in I/R + αGC than in I/R + vehicle (20.7 ± 1.8% vs. 29.9 ± 2.8%, $P = 0.014$) without significant changes in AAR/LV (Supplemental Fig. 3). To examine whether the reduction in infarct size by αGC was due to the activation of iNKT cells, Jα18$^{-/-}$ mice were used. There were no differences in IS/AAR between Jα18$^{-/-}$ + I/R + αGC and Jα18$^{-/-}$ + I/R + vehicle (34.9 ± 4.7% vs. 35.6 ± 4.1%, P = NS) and in AAR/LV between groups (51.3 ± 6.4% vs. 59.0 ± 2.4%, P = NS) at 24 h after reperfusion (Supplemental Fig. 4).

Echocardiography and hemodynamic data at 28 days after reperfusion were shown in Table 1. LV end-diastolic dimension did not differ between the 2 groups, whereas LV end-systolic dimension was significantly decreased in I/R + αGC compared to I/R + vehicle, which resulted in greater fractional shortening in I/R + αGC. Furthermore, anterior wall thickness including infarct region was preserved in I/R + αGC. There were no differences in HR, BP, and LV ± dP/dt between groups. LV end-diastolic pressure was decreased in I/R + αGC.

3.7. Inflammatory cell infiltration

Immunohistochemical analysis revealed that the number of MPO (as a marker of neutrophil)- and CD3 (as a marker of T lymphocyte)-positive

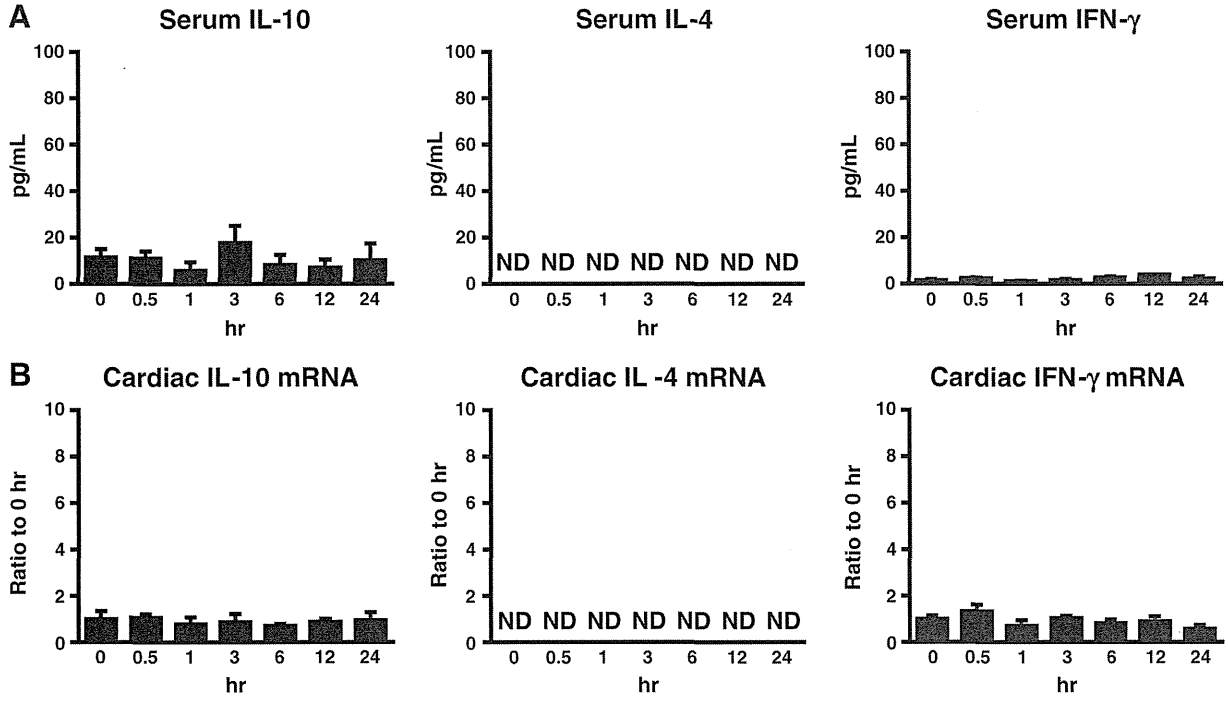

Fig. 1. Specificity of αGC for iNKT cells. (A) Serum levels of IL-10, IL-4 and IFN-γ at 0, 0.5, 1, 3, 6, 12 and 24 h after αGC intraperitoneal injection into Jα18$^{-/-}$ mice (0.1 μg/g body weight). n = 3 for each group. (B) Quantitative analysis of IL-10, IL-4 and IFN-γ mRNA expression in the myocardium after αGC injection into Jα18$^{-/-}$ mice. n = 3 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. 0 h. ND, not detected.

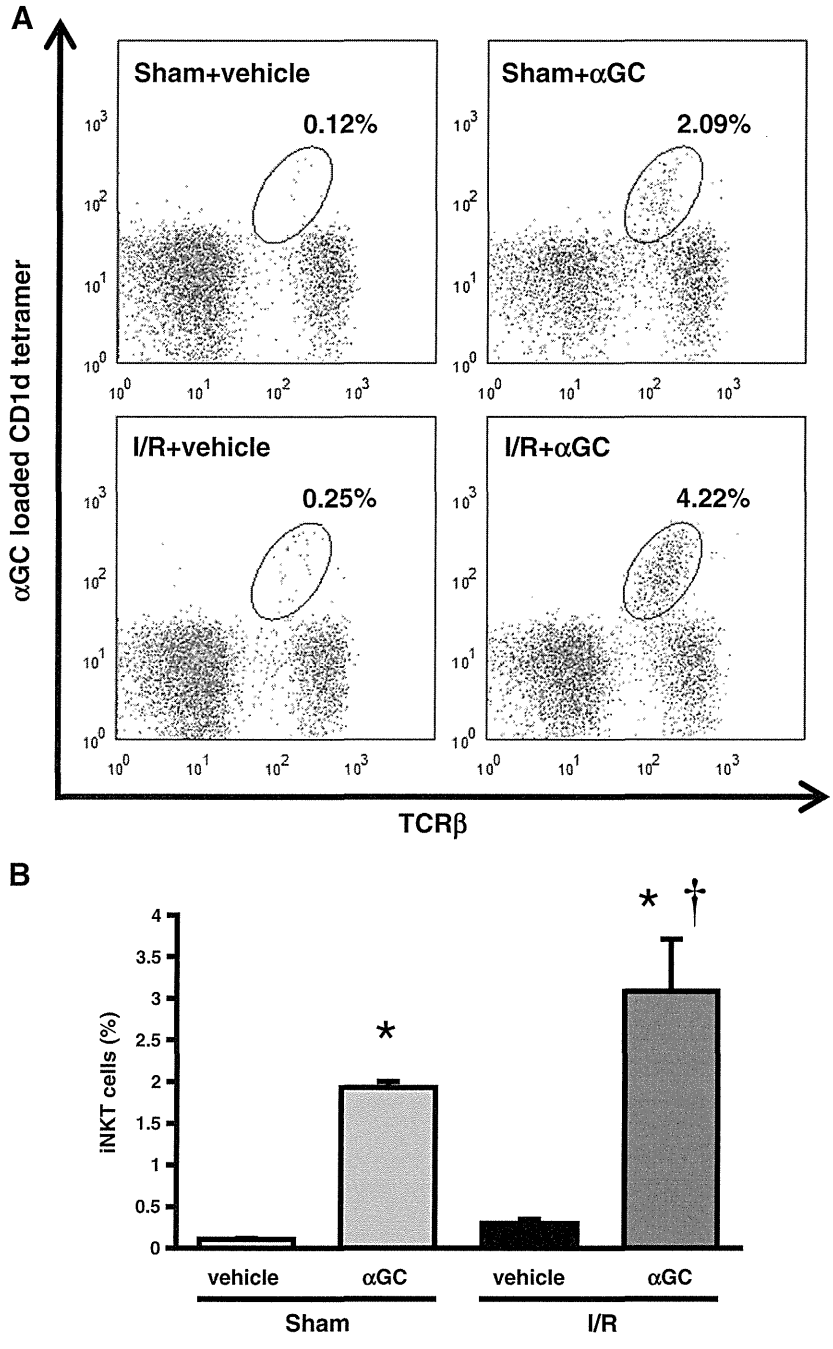

Fig. 2. The proportion of iNKT cells in the heart from 4 groups of mice. (A) Representative flow cytometric analyses of cardiac mononuclear cell (MNC) suspensions 72 h after reperfusion from sham + vehicle, sham + αGC, I/R + vehicle and I/R + αGC. Cardiac MNCs from 3 different mice for each group were pooled and analyzed. Circles indicate the population of iNKT cells. (B) Summary data for the proportion of iNKT cells. n = 3 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle.

cells and the ratio of MAC3 (as a marker of macrophage)-positive area in the ischemic myocardium were increased in I/R + vehicle compared to sham + vehicle. Administration of αGC into I/R mice significantly ameliorated the infiltration of MPO- and CD3-positive cells in I/R mice. In contrast, there were no significant differences in MAC3-positive area between I/R + vehicle and I/R + αGC (Figs. 4A, B).

Flow cytometric analysis also showed that $CD45^+$ cells (leukocytes), $CD45^+Ly6G^+$ cells (neutrophils), and Ly6Chigh monocytes in the ischemic heart were decreased in I/R + αGC compared to I/R + vehicle (Supplemental Fig. 5). In contrast, $CD45^+CD68^+$ cells excluding monocytes (macrophages) were comparable between groups (Supplemental Fig. 5).

3.8. TUNEL staining and caspase-3 protein

There were rare TUNEL-positive nuclei both in sham + vehicle and sham + αGC mice. There were some cardiomyocytes with TUNEL-positive nuclei in I/R + vehicle and I/R + αGC (Fig. 5A). The number of TUNEL-positive cardiomyocytes in the ischemic LV was increased in I/R + vehicle compared to sham + vehicle. It was significantly decreased in I/R + αGC compared to I/R + vehicle (Fig. 5B). Full length caspase-3 protein levels were significantly increased in the ischemic myocardium from I/R + vehicle compared to sham + vehicle, which was consistent with previous papers [23,24], and this increase was also inhibited in I/R + αGC (Fig. 5C).

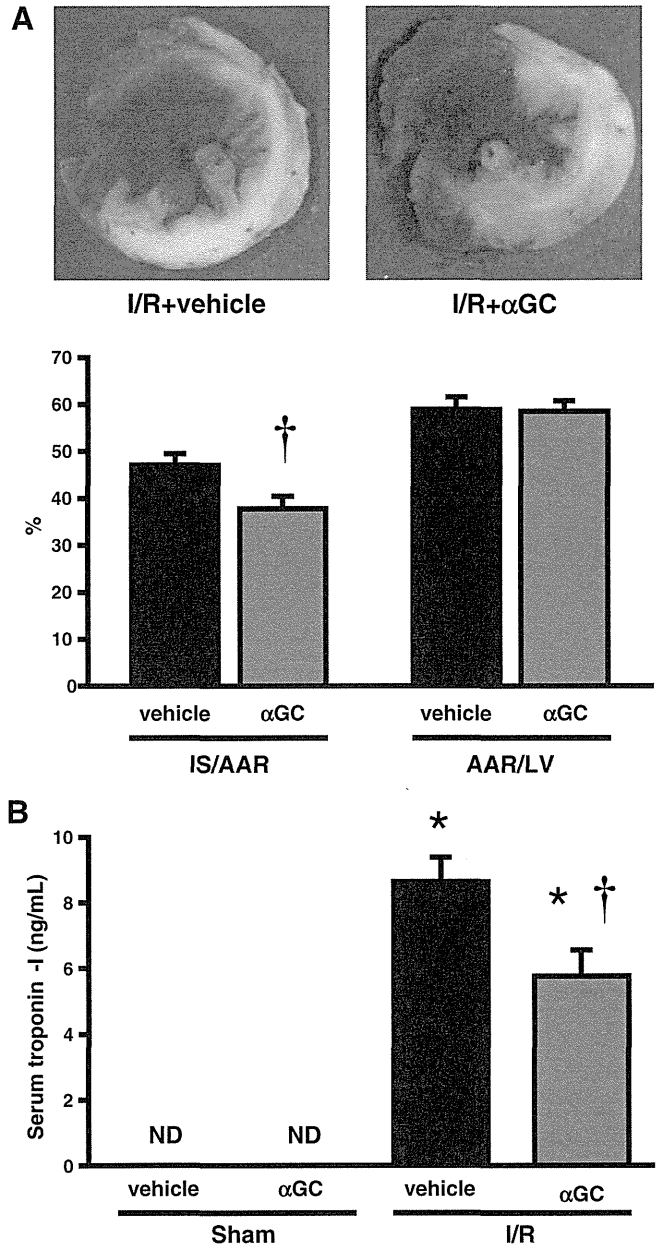

Fig. 3. Effects of αGC on myocardial I/R injury at 24 h. (A) Representative pictures of Evans Blue and TTC-stained LV sections from I/R + vehicle and I/R + αGC (upper panels). IS/AAR and AAR/LV 24 h after reperfusion in I/R + vehicle (n = 16) and I/R + αGC (n = 14) mice (lower panel). (B) Serum levels of troponin-I 24 h after reperfusion in sham + vehicle (n = 8), sham + αGC (n = 8), I/R + vehicle (n = 16) and I/R + αGC (n = 14) mice. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle. ND, not detected.

3.9. Serum and myocardial cytokines, and chemokines

Very small amounts of serum IL-10 and IFN-γ levels were detected and IL-4 was not detected in either sham + vehicle or I/R + vehicle mice. In contrast, αGC extremely increased these cytokine levels in both sham and I/R mice (Fig. 6A). At 24 h after reperfusion, gene expression of IL-10, IL-4, and IFN-γ in the ischemic myocardium tended to be increased in I/R + vehicle compared to sham + vehicle, and they were significantly increased in I/R + αGC compared to I/R + vehicle (Fig. 6B). These gene expressions were also measured at 72 h after reperfusion in another set of mice. Gene expression of IL-10 was significantly increased in both I/R + vehicle and I/R + αGC, and tended to be increased by αGC in sham and I/R (Supplemental Fig. 6A). IL-4 and IFN-γ were increased in I/R + αGC compared to I/R + vehicle (Supplemental Figs. 6B, C).

Gene expression of TNF-α, IL-1β, and TGF-β1 was significantly increased in I/R + vehicle compared to sham + vehicle. In contrast to IL-10, IL-4, and IFN-γ, TNF-α, and IL-1β were lower in I/R + αGC than I/R + vehicle (Fig. 6B). MCP-1, ICAM-1, and VCAM-1 were increased in I/R + vehicle (Supplemental Fig. 7). MCP-1 was decreased in I/R + αGC compared to I/R + vehicle (Supplemental Fig. 7), which could inhibit infiltration of inflammatory cells (Fig. 4 and Supplemental Fig. 5). In contrast, there was no difference in ICAM-1, and VCAM-1 was rather increased in I/R + αGC compared to I/R + vehicle (Supplemental Fig. 7).

3.10. Effects of neutralization of IL-10, IL-4, and IFN-γ on αGC-treated I/R mice

Representative pictures showed that the administration of anti-IL-10 receptor monoclonal antibody into I/R + αGC mice increased infarct size compared to I/R + αGC + rat IgG1κ. In contrast, the administration of anti-IL-4 and anti-IFN-γ did not affect it (Fig. 7, upper panels). IS/AAR was significantly greater in I/R + αGC + anti-IL-10R than I/R + αGC + rat IgG1κ (53.1 ± 5.2% vs. 37.4 ± 3.5%, $P = 0.046$) with no significant changes in AAR/LV (54.3 ± 2.0% vs. 54.7 ± 2.9%, P = NS) (Fig. 7, lower panel).

3.11. Effects of neutralization of IFN-γ on I/R mice

Representative pictures showed that the administration of anti-IFN-γ monoclonal antibody into I/R mice decreased infarct size compared to I/R + rat IgG1κ (Supplemental Fig. 8, upper panels). IS/AAR was significantly smaller in I/R + anti-IFN-γ than I/R + rat IgG1κ (38.0 ± 3.7% vs. 49.7 ± 1.9%, $P = 0.020$) with no significant changes in AAR/LV (56.4 ± 4.1% vs. 57.8 ± 1.5%, P = NS) (Supplemental Fig. 8, lower panel).

4. Discussion

The present study demonstrated that the activation of iNKT cells by αGC ameliorated myocardial I/R injury, accompanied by the decreases in inflammatory cell infiltration, apoptosis, and pro-inflammatory cytokines. Furthermore, the neutralization of αGC-induced increase in expression of IL-10 by receptor antibody abolished the protective effects of αGC on I/R injury. This is the first report to provide direct evidence for the protective effects of iNKT cell activation by αGC on myocardial I/R injury.

4.1. Activation of iNKT cells by αGC in the heart

αGC has been well known to activate iNKT cells, and they rapidly produce various cytokines such as IL-10, IL-4, and IFN-γ [25]. In parallel to these changes, iNKT cell-surface receptors, including TCR and NK1.1, become downregulated, which render iNKT cells invisible by flow-cytometric detection [19,26]. The downregulation of TCR remains until at least 24 h. Then, iNKT cells rapidly proliferate and increase to the peak level 72 h after αGC administration. The activation of iNKT cells by αGC has been observed in various organs, such as spleen, liver, lung, and kidney [18,19,26–28]. We previously demonstrated that the proportion of iNKT cells was increased within the heart 7 days after αGC administration [17]. In the present study, we confirmed that αGC increased cardiac iNKT cells in parallel to splenic iNKT cells (Supplemental Fig. 1) and rapidly enhanced the expression of cytokine genes within the heart (Supplemental Fig. 2B), however, αGC had no effect in iNKT cell-deficient ($J\alpha18^{-/-}$) mice (Fig. 1). Therefore, these findings indicate that αGC can specifically activate iNKT cells, which results in the production of cytokines in the heart.

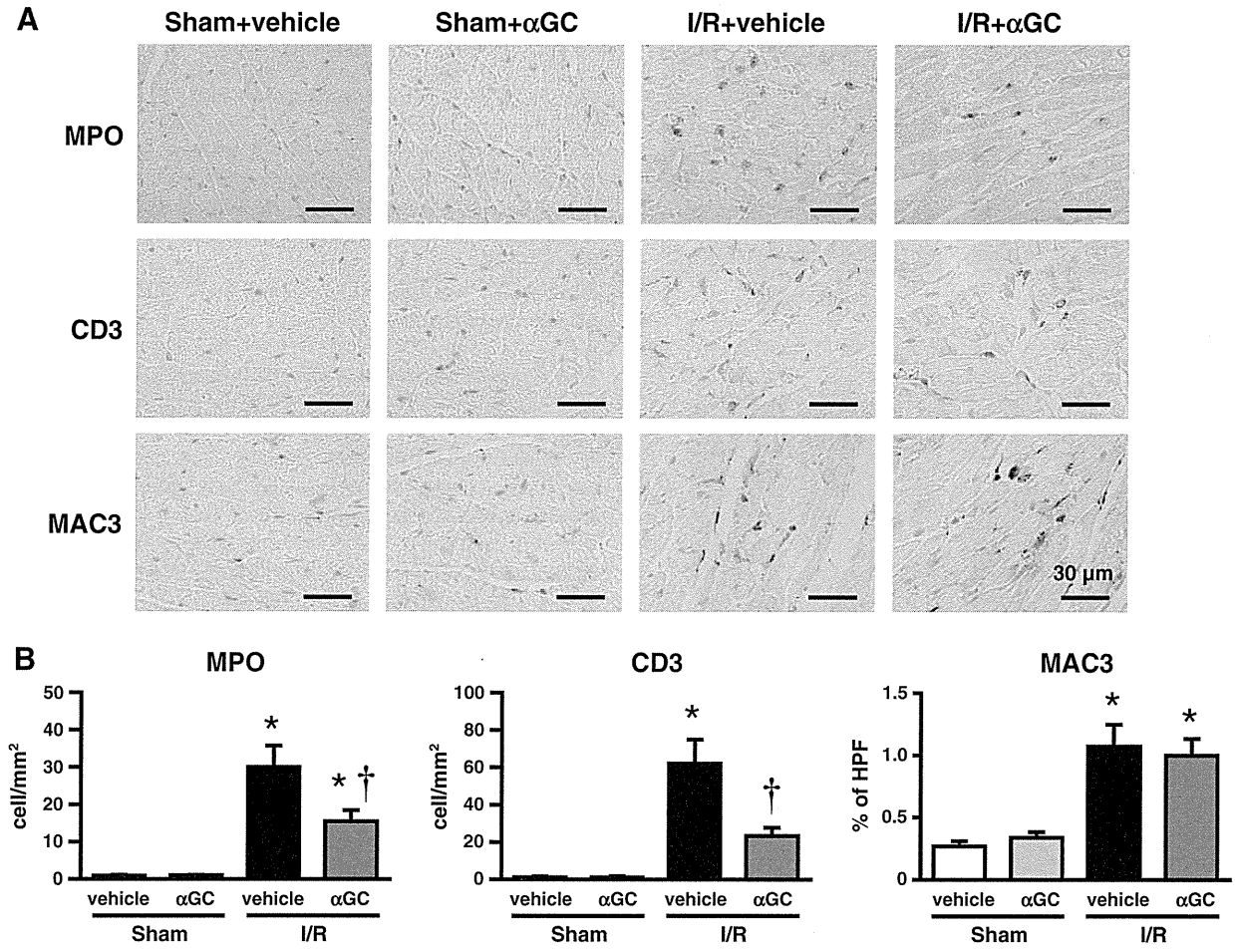

Fig. 4. Effects of αGC on the number of infiltrating inflammatory cells in ischemic myocardium. (A) Representative photomicrographs of LV sections stained with anti-myeloperoxidase (MPO), anti-CD3 and anti-MAC3 24 h after reperfusion. (B) Summary data for the number of MPO-positive cells and CD3-positive cells and the ratio of MAC3-positive area. n = 7–8 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle. HPF, high power field.

4.2. Myocardial I/R injury and cytokines

It is well known that various cytokines are involved in myocardial I/R injury [29,30]. These cytokines are produced by several types of cells, such as neutrophils, lymphocytes, macrophages, and endothelial cells, and play an important role in the pathogenesis of myocardial I/R injury. Endogenous TNF-α and IL-1 play as a mediator of inflammatory reactions, whereas, IL-10 and TGF-β have cardioprotective effects on myocardial I/R injury. Previous studies demonstrated that the blocking of pro-inflammatory cytokines or the administration of cardioprotective cytokines reduced infarct size [31–36]. On the other hand, the increases in IL-4 and IFN-γ are characteristic of the activation of iNKT cells [9]. IFN-γ has been reported to promote myocardial I/R injury [5], and the effect of IL-4 on myocardial I/R injury has not been elucidated. In the present study, αGC administration decreased infarct size (Fig. 3) and infiltrating inflammatory cells (Fig. 4 and Supplemental Fig. 5) in association with the decrease in the expressions of pro-inflammatory cytokines, TNF-α and IL-1β (Fig. 6B). Simultaneously, serum levels and gene expression of IL-10, IL-4, and IFN-γ were increased after αGC administration (Figs. 6A, B).

To determine the role of these cytokines in αGC-induced amelioration of myocardial I/R injury, we neutralized IL-10, IL-4, and IFN-γ on αGC-treated I/R mice. Anti-IL-10R monoclonal antibody canceled the protective effects of αGC in I/R mice, but not anti-IL-4 and IFN-γ monoclonal antibody (Fig. 7), indicating that IL-10 was involved in the protective effects of αGC in myocardial I/R injury. Yang et al. reported that INF-γ had deleterious effects on myocardial I/R injury [5]. We also showed that a single treatment with anti-IFN-γ monoclonal antibody reduced infarct size after I/R injury without αGC (Supplemental Fig. 8). In our results, the reduction in infarct size by αGC was the same as that by anti-IFN-γ monoclonal antibody, and

Table 1
Echocardiogram and hemodynamics 28 days after reperfusion.

	I/R + vehicle	I/R + αGC
Echocardiography	n = 8	n = 8
LVEDD, mm	3.7 ± 0.1	3.5 ± 0.1
LVESD, mm	3.0 ± 0.1	2.7 ± 0.1†
FS, %	18.9 ± 0.9	24.0 ± 1.1†
AWT, mm	0.64 ± 0.02	0.71 ± 0.01†
PWT, mm	0.80 ± 0.02	0.82 ± 0.02
Hemodynamics	n = 6	n = 7
Heart rate, bpm	456 ± 25	449 ± 20
Systolic BP, mm Hg	108.2 ± 6.2	108.1 ± 3.7
Diastolic BP, mm Hg	78.8 ± 4.7	76.5 ± 1.9
LVEDP, mm Hg	5.2 ± 0.4	1.9 ± 0.3†
LV +dP/dt, mm Hg/s	10,633 ± 1824	11,841 ± 1284
LV −dP/dt, mm Hg/s	7365 ± 1600	7286 ± 1105

LVEDD indicates left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness; BP, blood pressure; LVEDP, left ventricular end-diastolic pressure. Data are expressed as means ± SE.
† $P < 0.05$ vs. I/R + vehicle.

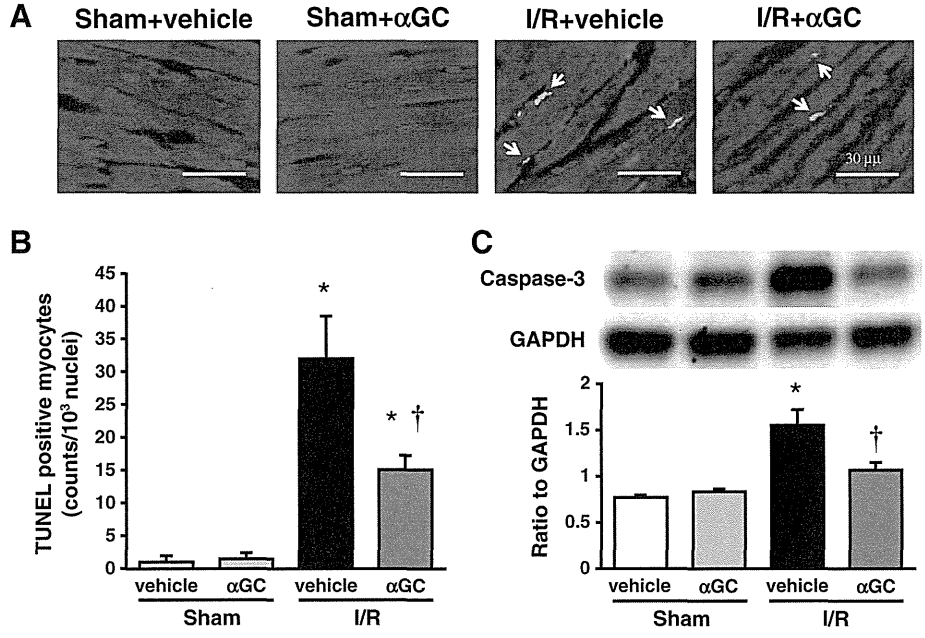

Fig. 5. Effects of αGC on apoptosis. (A) Representative photomicrographs of TUNEL-stained LV sections. TUNEL-positive nuclei (green), myoglobin (red), and DAPI (blue). Arrows indicate TUNEL-positive cells. (B) Summary data for the number of TUNEL-positive cells. n = 7–8 for each group. (C) Representative immunoblotting analysis and the summary data for caspase-3/GAPDH. n = 6 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle.

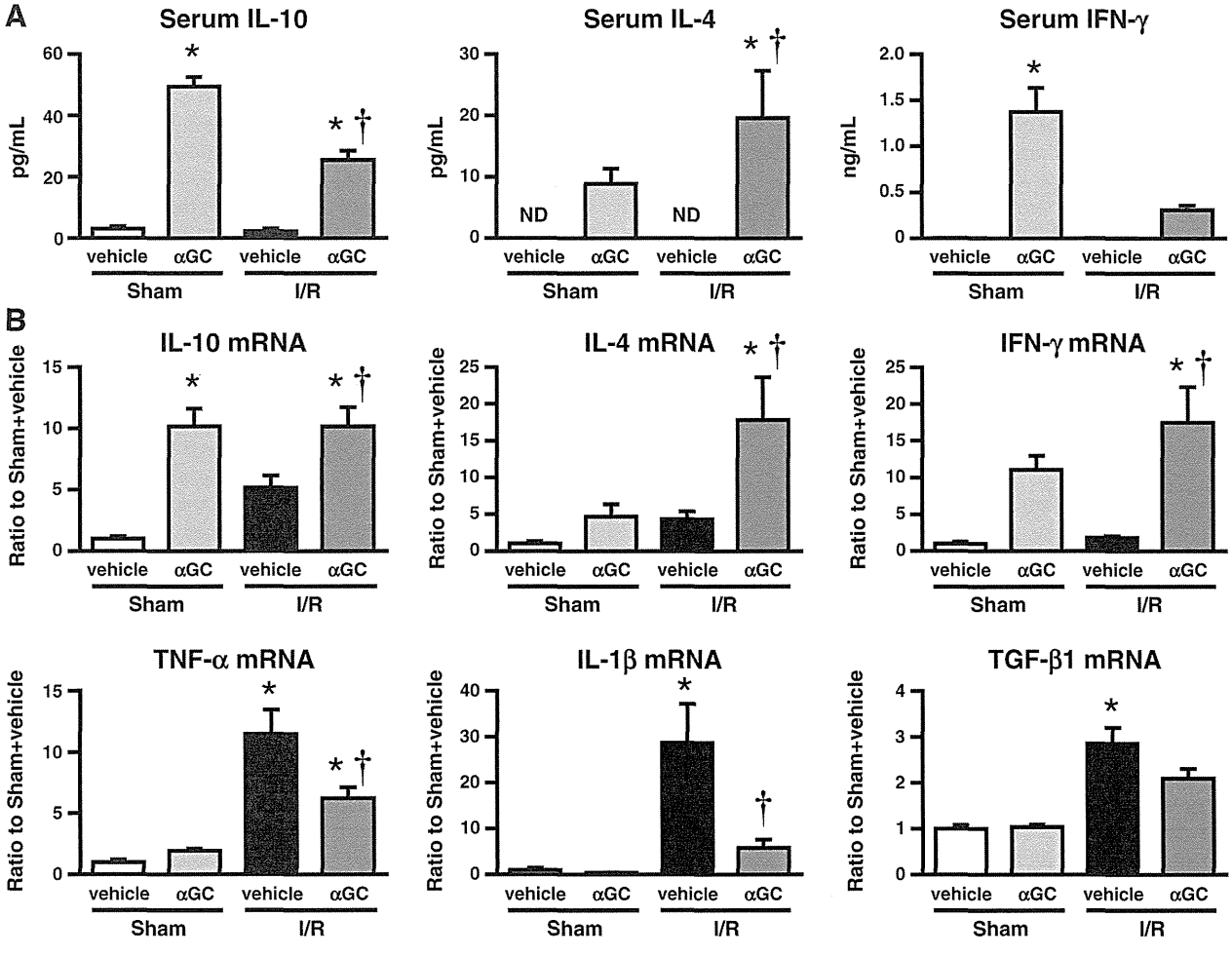

Fig. 6. Effects of αGC on serum levels and myocardial gene expression of cytokines. (A) Serum levels of IL-10, IL-4 and IFN-γ 24 h after reperfusion. (B) Quantitative analysis of mRNA expression of IL-10, IL-4, IFN-γ, TNF-α, IL-1β, and TGF-β1 in ischemic myocardium 24 h after reperfusion. n = 8 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. sham + vehicle. †$P < 0.05$ vs. I/R + vehicle. ND, not detected.

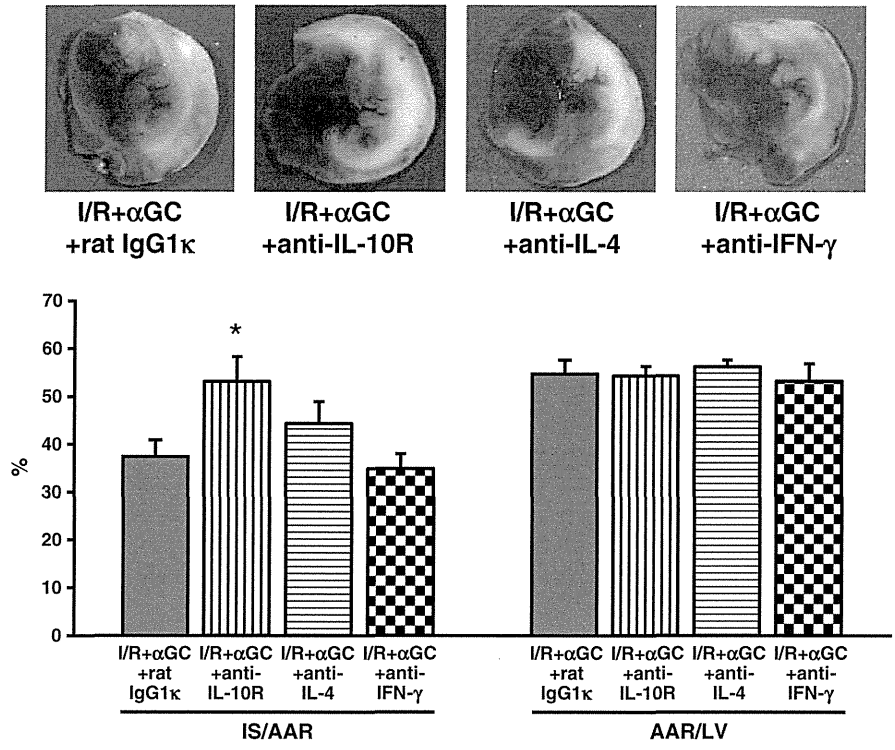

Fig. 7. Effects of neutralizing antibodies on αGC-induced amelioration of I/R injury. Representative pictures of Evans Blue and TTC-stained LV sections from I/R + αGC + rat IgG1κ, I/R + αGC + anti-IL-10 receptor mAb (IL-10R), I/R + αGC + anti-IL-4 mAb and I/R + αGC + anti-IFN-γ mAb (upper panels). Summary data for IS/AAR and AAR/LV 24 h of reperfusion (lower panel). n = 7–9 for each group. Data are expressed as means ± SE. *$P < 0.05$ vs. I/R + αGC + rat IgG1κ.

there was no additional reduction in infarct size by αGC and anti-IFN-γ monoclonal antibody. These results suggested that IL-10-dependent beneficial effects of αGC may be due to the inhibition of INF-γ-dependent deleterious effects on myocardial I/R injury. Indeed, it has been reported that IL-10 inhibits the expression of IFN-γ-induced genes [37].

We showed that infarct size was smaller in I/R + αGC than I/R + vehicle also at 2 h after reperfusion (Supplemental Fig. 3). Serum IL-10 was rapidly increased and peaked at 1 h after αGC injection, in contrast, gene expression of IL-10 in the LV was not increased at early phase after αGC injection (Supplemental Fig. 2). Therefore, the improvement in infarct size by αGC may be due to the increased serum IL-10, i.e. systemic activation of iNKT cells.

4.3. Protective effects of IL-10 on myocardial I/R injury

IL-10 is well known as a potent anti-inflammatory cytokine [38], and has been shown to play an important role in myocardial I/R injury [33]. It has been reported that endogenous IL-10 inhibits the production of TNF-α and serves to protect the reperfused myocardium through the suppression of neutrophil recruitment [33]. IL-10 has been reported to suppress the expression of CC chemokine gene including MCP-1 [39]. Exogenous IL-10 administration ameliorates myocardial I/R injury by inhibiting adherence of leukocytes to vascular endothelium [34], and by decreasing the production of pro-inflammatory cytokines through Signal Transducers and Activator of Transcription (STAT)-3 pathway [35]. It has also been shown that remote ischemic preconditioning has protective effects against myocardial I/R injury by the upregulation of IL-10 in the remote muscle and the release into circulation [40]. Moreover, IL-10 induces protection against myocardial injury by preventing apoptosis through the reduced phosphorylation of p38MAPK and the enhanced phosphorylation of STAT3 [41]. TNF-α and IL-1β promotes apoptosis in cardiac myocytes [42,43], which is also inhibited by IL-10. In the present study, we demonstrated that αGC administration ameliorated myocardial I/R injury (Fig. 3) with upregulating serum and myocardial IL-10 (Fig. 6). This was accompanied with the decreases in the infiltration of inflammatory cells into myocardium (Fig. 4 and Supplemental Fig. 5) and the gene expression of pro-inflammatory cytokines (Fig. 6B), and the reduction in apoptosis after I/R (Fig. 5). Our results suggest that the activated iNKT cells by αGC inhibit inflammatory response and cardiomyocyte apoptosis via the production of IL-10.

We previously demonstrated that administration of αGC into mice 1 day and 4 days after MI surgery ameliorated LV remodeling without affecting infarct size and these beneficial effects were also mediated by the enhanced expression of IL-10 in the heart [17]. On the other hand, in the present study, we demonstrated that αGC administration decreased infarct size. The discrepancy in the effects of αGC on infarct size was possibly due to the differences in experimental model assessing different pathophysiological processes (more angiogenesis, more fibrosis vs. accentuated inflammation and apoptosis in the acute setting) and the timing of αGC treatment (1 day after MI surgery vs. 30 min before reperfusion). The administration of αGC into MI mice was performed too late to salvage ischemic myocardium.

4.4. Clinical implication

The present study demonstrated that αGC administration during the ischemic period before reperfusion reduced infarct size. These findings suggest that αGC can be a novel agent in patients with acute MI to reduce I/R injury. In addition, based on our previous study of postinfarct heart failure [17], αGC administration may attenuate also LV remodeling and reduce mortality after MI. To date, several clinical trials (Phase I/II) using activated iNKT cells by αGC have been conducted in patients with cancer [44–48]. No severe adverse events were observed in these trials.

4.5. Limitations

There are several limitations to be acknowledged in the present study. First, we could not directly demonstrate the activation of iNKT cells within the heart 24 h after αGC administration because cell-surface receptors were downregulated. We tried double immunohistochemical staining of anti-TCRβ and anti-NK1.1 according to the newly published paper [49]. Furthermore, we also performed in situ hybridization using digoxigenin-labeled DNA probes for mouse Vα14Jα18. Unfortunately, however, we could not detect iNKT cells by these methods in the heart. Further studies are needed to overcome some technical difficulties of iNKT cell-detection and clarify this important issue. Alternatively, we demonstrated its activation by showing the increase of cytokine gene expressions and the similar time course of iNKT cell proportion within the spleen. Second, the source of IL-10 production after the stimulation of αGC remains to be determined. We tried to isolate iNKT cells using cell sorter and perform in vitro experiments. Unfortunately, however, we could not isolate sufficient amount of iNKT cells to perform in vitro experiments. We consider that there still might be some technical difficulties in in vitro experiments using isolated iNKT cells. Therefore, we could not directly demonstrate the source of IL-10 production. IL-10 has been shown to be produced by iNKT cells themselves on exogenous stimulation [50]. However, αGC-activated iNKT cells may stimulate other immune cells to produce IL-10. IL-10 can be also expressed and secreted from macrophages activated by iNKT cells. Moreover, in myocardial I/R injury, it has reported that CD5 positive T lymphocytes are the predominant source of IL-10 in the ischemic and reperfused heart. However, immunohistochemical analysis and flow cytometric analysis revealed that there was no difference in the infiltration of macrophage in the ischemic myocardium between I/R + αGC and I/R + vehicle, and other inflammatory cells were rather decreased in I/R + αGC compared to I/R + vehicle (Fig. 4 and Supplemental Fig. 5). Further investigations are required to elucidate the mechanism of IL-10 production after αGC administration in I/R mice. Third, we observed that iNKT cells were increased and endogenously activated in ischemic myocardium after I/R, however, the clear evidence on the role of iNKT cells in I/R injury has not been shown in the present study. We performed I/R injury experiment using iNKT cell deficient $J\alpha18^{-/-}$ mice. Unexpectedly, preliminary results showed that infarct size after I/R injury tended to be decreased in $J\alpha18^{-/-}$ mice compared to C57BL/6J control mice (IS/AAR in I/R + vehicle group from Fig. 3 vs. IS/AAR in $J\alpha18^{-/-}$ + I/R + vehicle group from Supplemental Fig. 4). These results suggest that endogenously activated iNKT cells may be involved in the development of I/R injury, even though endogenous ligand for the activation of iNKT cells has never been elucidated. Therefore, the discrepancy in these results may be due to the difference in the methods to activate iNKT cells; endogenous ligand vs. exogenously administrated αGC. Finally, protein levels of several cytokines could not be detected in the heart by ELISA in the present study. This may be due to short half-life, and smaller amount of cytokines protein in the heart than in the serum.

5. Conclusions

Activated iNKT cells by αGC play a protective role against myocardial I/R injury through the enhanced expression of IL-10. Therapies designed to activate iNKT cells might be beneficial to protect the heart from I/R injury.

Disclosures

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2013.06.004.

References

[1] Cannon CP, Gibson CM, Lambrew CT, Shoultz DA, Levy D, French WJ, et al. Relationship of symptom-onset-to-balloon time and door-to-balloon time with mortality in patients undergoing angioplasty for acute myocardial infarction. JAMA 2000;283:2941–7.
[2] Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med 2007;357:1121–35.
[3] Zuidema MY, Zhang C. Ischemia/reperfusion injury: the role of immune cells. World J Cardiol 2010;2:325–32.
[4] Hawkins HK, Entman ML, Zhu JY, Youker KA, Berens K, Dore M, et al. Acute inflammatory reaction after myocardial ischemic injury and reperfusion. Development and use of a neutrophil-specific antibody. Am J Pathol 1996;148:1957–69.
[5] Yang Z, Day YJ, Toufektsian MC, Xu Y, Ramos SI, Marshall MA, et al. Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on CD4+ T lymphocytes. Circulation 2006;114:2056–64.
[6] Birdsall HH, Green DM, Trial J, Youker KA, Burns AR, MacKay CR, et al. Complement C5a, TGF-beta 1, and MCP-1, in sequence, induce migration of monocytes into ischemic canine myocardium within the first one to five hours after reperfusion. Circulation 1997;95:684–92.
[7] Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, et al. Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation 1998;98:699–710.
[8] Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? Nat Rev Immunol 2004;4:231–7.
[9] Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol 2007;25:297–336.
[10] Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. Curr Opin Immunol 2008;20:358–68.
[11] Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, et al. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat Med 2001;7:1052–6.
[12] Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, et al. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat Med 2001;7:1057–62.
[13] Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, et al. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. J Exp Med 2001;194:1801–11.
[14] Miellot A, Zhu R, Diem S, Boissier MC, Herbelin A, Bessis N. Activation of invariant NK T cells protects against experimental rheumatoid arthritis by an IL-10-dependent pathway. Eur J Immunol 2005;35:3704–13.
[15] Ronet C, Darche S, Leite de Moraes M, Miyake S, Yamamura T, Louis JA, et al. NKT cells are critical for the initiation of an inflammatory bowel response against *Toxoplasma gondii*. J Immunol 2005;175:899–908.
[16] Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 1997;278:1626–9.
[17] Sobirin MA, Kinugawa S, Takahashi M, Fukushima A, Homma T, Ono T, et al. Activation of natural killer T cells ameliorates postinfarct cardiac remodeling and failure in mice. Circ Res 2012;111:1037–47.
[18] Cao Z, Yuan Y, Jeyabalan G, Du Q, Tsung A, Geller DA, et al. Preactivation of NKT cells with alpha-GalCer protects against hepatic ischemia–reperfusion injury in mouse by a mechanism involving IL-13 and adenosine A2A receptor. Am J Physiol Gastrointest Liver Physiol 2009;297:G249–58.
[19] Crowe NY, Uldrich AP, Kyparissoudis K, Hammond KJ, Hayakawa Y, Sidobre S, et al. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. J Immunol 2003;171:4020–7.
[20] Mabalirajan U, Dinda AK, Kumar S, Roshan R, Gupta P, Sharma SK, et al. Mitochondrial structural changes and dysfunction are associated with experimental allergic asthma. J Immunol 2008;181:3540–8.
[21] Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 2001;413:531–4.

[22] Parekh VV, Singh AK, Wilson MT, Olivares-Villagomez D, Bezbradica JS, Inazawa H, et al. Quantitative and qualitative differences in the in vivo response of NKT cells to distinct alpha- and beta-anomeric glycolipids. J Immunol 2004;173:3693–706.
[23] Duan W, Yang Y, Yan J, Yu S, Liu J, Zhou J, et al. The effects of curcumin post-treatment against myocardial ischemia and reperfusion by activation of the JAK2/STAT3 signaling pathway Basic Res Cardiol;107:263.
[24] Bharti S, Golechha M, Kumari S, Siddiqui KM, Arya DS. Akt/GSK-3beta/eNOS phosphorylation arbitrates safranal-induced myocardial protection against ischemia–reperfusion injury in rats. Eur J Nutr;51:719–727.
[25] Van Kaer L. α-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. Nat Rev Immunol 2005;5:31–42.
[26] Wilson MT, Johansson C, Olivares-Villagomez D, Singh AK, Stanic AK, Wang CR, et al. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. Proc Natl Acad Sci U S A 2003;100:10913–8.
[27] Aoyagi T, Yamamoto N, Hatta M, Tanno D, Miyazato A, Ishii K, et al. Activation of pulmonary invariant NKT cells leads to exacerbation of acute lung injury caused by LPS through local production of IFN-gamma and TNF-alpha by Gr-1 + monocytes. Int Immunol 2011;23:97–108.
[28] Li L, Huang L, Vergis AL, Ye H, Bajwa A, Narayan V, et al. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia–reperfusion injury. J Clin Invest 2010;120:331–42.
[29] Frangogiannis NG, Youker KA, Rossen RD, Gwechenberger M, Lindsey MH, Mendoza LH, et al. Cytokines and the microcirculation in ischemia and reperfusion. J Mol Cell Cardiol 1998;30:2567–76.
[30] Zhang M, Chen L. Status of cytokines in ischemia reperfusion induced heart injury. Cardiovasc Hematol Disord Drug Targets 2008;8:161–72.
[31] Gurevitch J, Frolkis I, Yuhas Y, Lifschitz-Mercer B, Berger E, Paz Y, et al. Anti-tumor necrosis factor-alpha improves myocardial recovery after ischemia and reperfusion. J Am Coll Cardiol 1997;30:1554–61.
[32] Venkatachalam K, Prabhu SD, Reddy VS, Boylston WH, Valente AJ, Chandrasekar B. Neutralization of interleukin-18 ameliorates ischemia/reperfusion-induced myocardial injury. J Biol Chem 2009;284:7853–65.
[33] Yang Z, Zingarelli B, Szabo C. Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. Circulation 2000;101:1019–26.
[34] Hayward R, Nossuli TO, Scalia R, Lefer AM. Cardioprotective effect of interleukin-10 in murine myocardial ischemia–reperfusion. Eur J Pharmacol 1997;334:157–63.
[35] Manukyan MC, Alvernaz CH, Poynter JA, Wang Y, Brewster BD, Weil BR, et al. Interleukin-10 protects the ischemic heart from reperfusion injury via the STAT3 pathway. Surgery 2011;150:231–9.
[36] Lefer AM, Ma XL, Weyrich AS, Scalia R. Mechanism of the cardioprotective effect of transforming growth factor beta 1 in feline myocardial ischemia and reperfusion. Proc Natl Acad Sci U S A 1993;90:1018–22.
[37] Ito S, Ansari P, Sakatsume M, Dickensheets H, Vazquez N, Donnelly RP, et al. Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma-induced genes by suppressing tyrosine phosphorylation of STAT1. Blood 1999;93:1456–63.
[38] Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683–765.
[39] Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA, et al. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. J Immunol 1999;163:1537–44.
[40] Cai ZP, Parajuli N, Zheng X, Becker L. Remote ischemic preconditioning confers late protection against myocardial ischemia–reperfusion injury in mice by upregulating interleukin-10. Basic Res Cardiol 2012:107:277–107:.
[41] Krishnamurthy P, Rajasingh J, Lambers E, Qin G, Losordo DW, Kishore R. IL-10 inhibits inflammation and attenuates left ventricular remodeling after myocardial infarction via activation of STAT3 and suppression of HuR. Circ Res 2009;104:e9-18.
[42] Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock KL, et al. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. J Clin Invest 1996;98:2854–65.
[43] McTiernan CF, Lemster BH, Frye C, Brooks S, Combes A, Feldman AM. Interleukin-1 beta inhibits phospholamban gene expression in cultured cardiomyocytes. Circ Res 1997;81:493–503.
[44] Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res 2002;8:3702–9.
[45] Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, et al. Therapeutic activation of Valpha24 + Vbeta11 + NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. Blood 2004;103:383–9.
[46] Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, et al. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. Clin Cancer Res 2005;11:1910–7.
[47] Uchida T, Horiguchi S, Tanaka Y, Yamamoto H, Kunii N, Motohashi S, et al. Phase I study of alpha-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. Cancer Immunol Immunother 2008;57:337–45.
[48] Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I–II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. J Immunol 2009;182:2492–501.
[49] Barral P, Sanchez-Nino MD, van Rooijen N, Cerundolo V, Batista FD. The location of splenic NKT cells favours their rapid activation by blood-borne antigen. EMBO J 2012;31:2378–90.
[50] Sonoda KH, Faunce DE, Taniguchi M, Exley M, Balk S, Stein-Streilein J. NK T cell-derived IL-10 is essential for the differentiation of antigen-specific T regulatory cells in systemic tolerance. J Immunol 2001;166:42–50.