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**Activation of Invariant Natural Killer T Cells by Alpha-Galactosylceramide
Ameliorates Doxorubicin-Induced Cardiotoxicity in Mice**

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Doxorubicin (DOX), one of the most important antineoplastic agents, is widely used to treat pediatric and adult cancers including breast cancer, leukemia, and lymphoma. Despite DOX-based therapies can provide beneficial effects on survival rate of cancer patients, DOX may induce cardiotoxicity in a dose-dependent manner, leading to limitation of cumulative dose of DOX in cancer treatment. The adverse effects of DOX range from subclinical left ventricular (LV) dysfunction to symptomatic heart failure. It has been reported that about 5% of cancer patients may suffer from heart failure when a cumulative dose of DOX reaches 400 mg/m², and 453 patients (approx. 0.9% of total heart transplantation) underwent heart transplantation due to DOX-induced cardiomyopathy from 1987 to 2010 in the U.S.¹ Until now, there is no specific biomarker that predicts cardiotoxicity and preventive therapy against cardiovascular complications after administrating DOX. In addition, acute cardiotoxicity of DOX often results in dose reductions and/or treatment delays which may hamper cancer patient's prognosis. Indeed, more than half of elderly patients in non-Hodgkin's lymphoma are reported to be treated with lower dose of DOX than expected.²

To avoid these adverse effects, precise mechanisms of DOX-induced cardiotoxicity are needed to be clarified and several studies have previously shown the involvement of oxidative stress and mitochondrial dysfunction, but prevention and treatment targeting these defects remain unestablished in the clinical settings.³ Recently, several molecular pathways such as immune responses and tissue injury have been proposed as another potential target for DOX-induced cardiotoxicity.⁴⁻⁶

Invariant natural killer T (iNKT) cells are a unique subset of T lymphocytes that recognize glycolipid antigens. They can rapidly and robustly produce a mixture of T-helper type 1 (Th1) and Th2 cytokines on activation in shaping subsequent immune responses. Indeed, iNKT cells can modulate tissue inflammation in various pathophysiological conditions.⁷ However, it remains unclear whether iNKT cells are involved in DOX-induced

cardiotoxicity. We previously found that a treatment with α -galactosylceramide (α GC), an specific activator of iNKT cells, prevent LV remodeling after myocardial infarction in mice by regulating immunologically disturbed conditions.⁸

We thus hypothesized that iNKT cells' activation could prevent DOX-induced cardiotoxicity. All experiments and animal care accorded with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, were approved by our institutional animal research committee, and conformed to the Hokkaido University Graduate School of Medicine Animal Care Guidelines for the Care and Use of Laboratory Animals.

Eight-week-old male C57BL/6J mice were intraperitoneally administered either DOX (20 mg/kg body weight; n=28) to induce DOX-induced cardiomyopathy or vehicle (distilled water; n=13) on Day 0, as described.⁹ DOX-administered mice were randomly divided into 2 groups: those treated with 0.1 μ g/g body weight of α GC (DOX+ α GC; n=14) and those treated with phosphate-buffered saline (PBS) (DOX+PBS; n=14) by intraperitoneal injections (twice; 4 days before and 3 days after DOX administration). Similarly, vehicle-administered mice were divided into 2 groups: those treated with 0.1 μ g/g body weight of α GC (Vehicle+ α GC; n=7) and those treated with PBS (Vehicle+PBS; n=6) by intraperitoneal injections (twice; 4 days before and 3 days after vehicle administration).

An echocardiography conducted on Day 14 revealed that the LV fractional shortening was significantly reduced in the DOX+PBS compared to the Vehicle+PBS, and the LV fractional shortening of the DOX+ α GC was similar to that of the Vehicle+PBS, indicating that administration of α GC improved the reduced LV systolic function caused by DOX (**Figure 1**). In contrast, the Vehicle+ α GC mice were comparable to the Vehicle+PBS mice in the LV fractional shortening (**Figure 1**).

To quantify iNKT cells' infiltration into the heart tissue, mononuclear cells were isolated from the heart tissue of following mice on Day 14; Vehicle+PBS (n=4), DOX+PBS (n=7),

and DOX+ α GC (n=6). A flow cytometric analysis revealed that the ratio of iNKT cells to mononuclear cell infiltrated into the heart tissue was significantly higher in the DOX+ α GC than the Vehicle+PBS and the DOX+PBS mice (**Figure 2**).

We also conducted histomorphometric analysis of the heart tissue from following mice; Vehicle+PBS (n=5), DOX+PBS (n=10), and DOX+ α GC (n=14). Picro-sirius red staining revealed that the ratio of fibrosis area to the heart tissue was markedly higher in the DOX+PBS than in the Vehicle+PBS, which was completely attenuated in the DOX+ α GC (**Figure 3**). The sections of heart tissue were also stained with antibody (Ab) against mouse Iba1 (rabbit anti-mouse Iba1 polyclonal Ab), which revealed that the number of Iba1⁺ macrophages in the heart tissue was significantly elevated in the DOX+ α GC compared to the Vehicle+PBS and the DOX+PBS (55.4 \pm 3.2 cells/mm² vs. 21.7 \pm 2.0 cells/mm² and 37.5 \pm 5.9 cells/mm², P <0.05) in consistent with the changes in infiltration ratio of iNKT cells to the heart tissue.

Finally, to quantify cardiac gene expression, total RNA was extracted from the heart tissue. A TaqMan quantitative PCR analysis showed that mRNA expressions of Arginase 1 and Retnla (M2 macrophage markers) were markedly enhanced in the DOX+ α GC compared to the DOX+PBS (Arginase 1: 2.5 \pm 0.4 vs. 1.6 \pm 0.3 [relative ratio to the Vehicle+PBS], P =0.08; Retnla: 2.4 \pm 0.5 vs. 1.1 \pm 0.2 [relative ratio to Vehicle+PBS], P <0.05), while those of iNOS and MCP-1 (M1 macrophage markers) were comparable between groups (data not shown). Because M2 macrophages play a pivotal role in anti-inflammatory and fibrotic responses during the tissue injury and repair processes,¹⁰ iNKT cells' activation may reduce cardiac fibrosis caused by DOX-induced cardiotoxicity via enhanced M2 macrophage polarization.

We thus provide the first report that the iNKT cells' activation by α GC-administration ameliorates DOX-induced LV dysfunction and cardiac fibrosis in mice. Facing aging

population, the development of adjuvant remedy that reduces the risk of DOX-induced cardiotoxicity may contribute to lower frequency of dose reductions and/or treatment delays in cancer treatment. Therapy targeting iNKT cells' activation could be a promising candidate of novel preventive and therapeutic strategy against DOX-induced cardiotoxicity.

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Author contribution

NI, AS, SK, TY and ST contributed to the conception or design of the work. YO, NI and SK drafted the manuscript. YO, NI, AS, IN, NK, KY and AN contributed to the acquisition, analysis, or interpretation of data for the work. All the authors critically revised the manuscript. All gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Figure legends

Figure 1. Activation of iNKT cells by α GC ameliorates DOX-induced left ventricular dysfunction in mice.

Summarized data of left ventricular FS evaluated by echocardiography on Day 14 in 4 groups of mice; Vehicle+PBS (n=6), Vehicle+ α GC (n=7), DOX+PBS (n=10), and DOX+ α GC (n=14). Data are mean \pm SEM. * P <0.05 vs. Vehicle+PBS, † P <0.05 vs. DOX+PBS by one-way ANOVA followed by the Tukey test. α GC, α -galactosylceramide; DOX, doxorubicin; FS, fractional shortening; iNKT, invariant natural killer T.

Figure 2. α GC administration increases iNKT cells' infiltration into heart tissue in mice.

Representative flow cytometric assessment of cardiac mononuclear cells and summarized data of population of iNKT cells in the heart tissue from 3 groups of mice; Vehicle+PBS (n=4), DOX+PBS (n=7), and DOX+ α GC (n=6) on Day 14. Cardiac mononuclear cells from 3 different types of mice in each group were stored and analyzed. iNKT cells were gated as the α GC-loaded CD1d tetramer⁺ and TCR β ⁺ population. The inset numbers are a percentage of the gated region of the samples. Data are mean \pm SEM. * P <0.05 vs. Vehicle+PBS, † P <0.05 vs. DOX+PBS by one-way ANOVA followed by the Tukey test.

Figure 3. Activation of iNKT cells by α GC ameliorates DOX-induced cardiac fibrosis in mice.

Representative high-power photomicrographs of heart tissue sections stained with Picro-sirius red staining and summarized data of ratio of fibrosis area to the heart tissue from three groups of mice; Vehicle+PBS (n=5), DOX+PBS (n=10), and DOX+ α GC (n=14). Three fields were randomly selected from one coronal section in each mouse. Within each field, Picro-sirius red positive fibrosis area was quantified and fibrosis area ratio was then calculated as the sum of

three fibrosis areas divided by the sum of three heart tissue areas excluding intramyocardial coronary artery areas. Scale bar=200 μm . Data are mean \pm SEM. * P <0.05 vs. Vehicle+PBS, † P <0.05 vs. DOX+PBS by one-way ANOVA followed by the Tukey test.

Figure 1

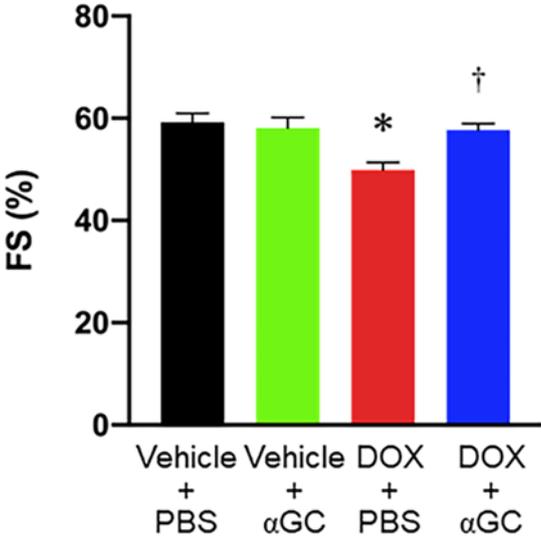


Figure 2

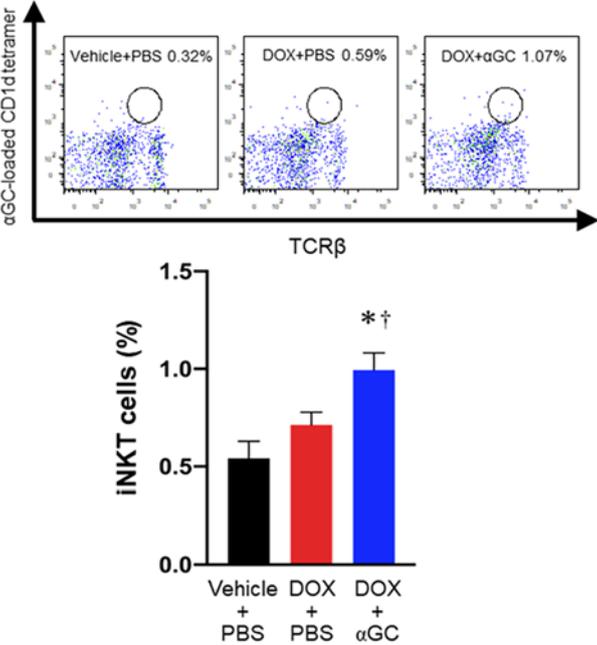


Figure 3

