Liver Injury After Invariant NKT Cell Activation by Free Alpha-galactosylceramide and Alpha-galactosylceramide-loaded Dendritic Cells

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Abstract. *Background/Aim: Both free alpha-galactosylceramide (αGalCer) and αGalCer-loaded dendritic cells (DCG) activate invariant natural killer T (iNKT) cells to varying degrees, with αGalCer inducing liver injury. We sought to evaluate liver injury by these two pathways. Materials and Methods: Mice were injected with αGalCer or DCG followed by analysis of serum alanine transaminase (ALT) activity levels, mortality and liver function. Results: While ALT levels were elevated after DCG in a tumor necrosis factor (TNF)-α-dependent manner, DCG did not cause lethal injury. More serious injury of liver CD31-positive endothelial cells ($CD31^+$ EC) was observed in mice treated with αGalCer than with DCG. Furthermore, liver $CD31^+$ EC of αGalCer-treated mice induced naïve liver lymphocytes to produce TNF-α. Conclusion: DCG treatment did not induce lethal liver injury. $CD31^+$ EC may play an antigen-presenting role to iNKT cells after αGalCer treatment and may be a cause of lethal injury.*

Invariant natural killer T (iNKT) cells are characterized by the expression of an invariant T cell receptor α chain encoded by *Vα14Jα18* in mice and *Vα24Jα18* in humans and recognize numerous endogenous and exogenous glycolipids presented by the major histocompatibility complex class I-like CD1d molecule (1, 2). Intravenous (*i.v.*) or intraperitoneal (*i.p.*) injection of free alpha-galactosylceramide (αGalCer), a well-studied exogenous glycolipid, activates iNKT cells and induces anti-tumor effects in various murine tumor models (3-5). Injection of free αGalCer also induces liver injury in mice. This particular liver damage is characterized by tumor necrosis factor (TNF)-α dependence, strain differences and serious damage in older mice (6-8).

There are two methods to activate iNKT cells by αGalCer *in vivo*: injection of free αGalCer and injection of CD1d-αGalCer complexes. CD1d-αGalCer complexes are generally produced by bone marrow-derived dendritic cells pulsed with αGalCer (DCG) *in vitro*. Although injection of both free αGalCer and DCG induced activation of iNKT cells to produce several cytokines (*e.g.* interferon (IFN)-γ, interleukin (IL)-4 and TNF) and gain cytolytic activity (9-10), each of these treatments causes iNKT cells to exhibit different properties: free αGalCer induces long-term iNKT cell anergy (defined as unresponsiveness to subsequent stimuli), while DCG induces a prolonged IFN-γ-producing iNKT cell response. Injection of DCG showed stronger tumor protection against a murine melanoma lung metastasis model because of this difference (10).

It is not clear whether DCG induces liver injury. Therefore, we analyzed liver injury after injection of free αGalCer and DCG in this study. Our results show that injection of DCG caused liver damage. Although serum alanine transaminase (ALT) activity levels were comparable after both treatments, only free αGalCer induced lethal liver damage in older mice. To further deepen the insights into mechanisms underlying liver injury induced by αGalCer and DCG, we investigated the function of liver cells after treatment. CD31-positive ($CD31^+$) hepatic endothelial cells (EC) from mice treated with free αGalCer caused naïve hepatic lymphocytes to produce TNF; these cells were shown to be more injured than EC from mice treated with DCG. These results deepen our understanding of liver injury after iNKT cell activation.

Materials and Methods

Mice and reagents. Pathogen-free C57BL/6 female mice, 6- to 8-week-old and 42- to 55-week-old, were purchased from CLEA

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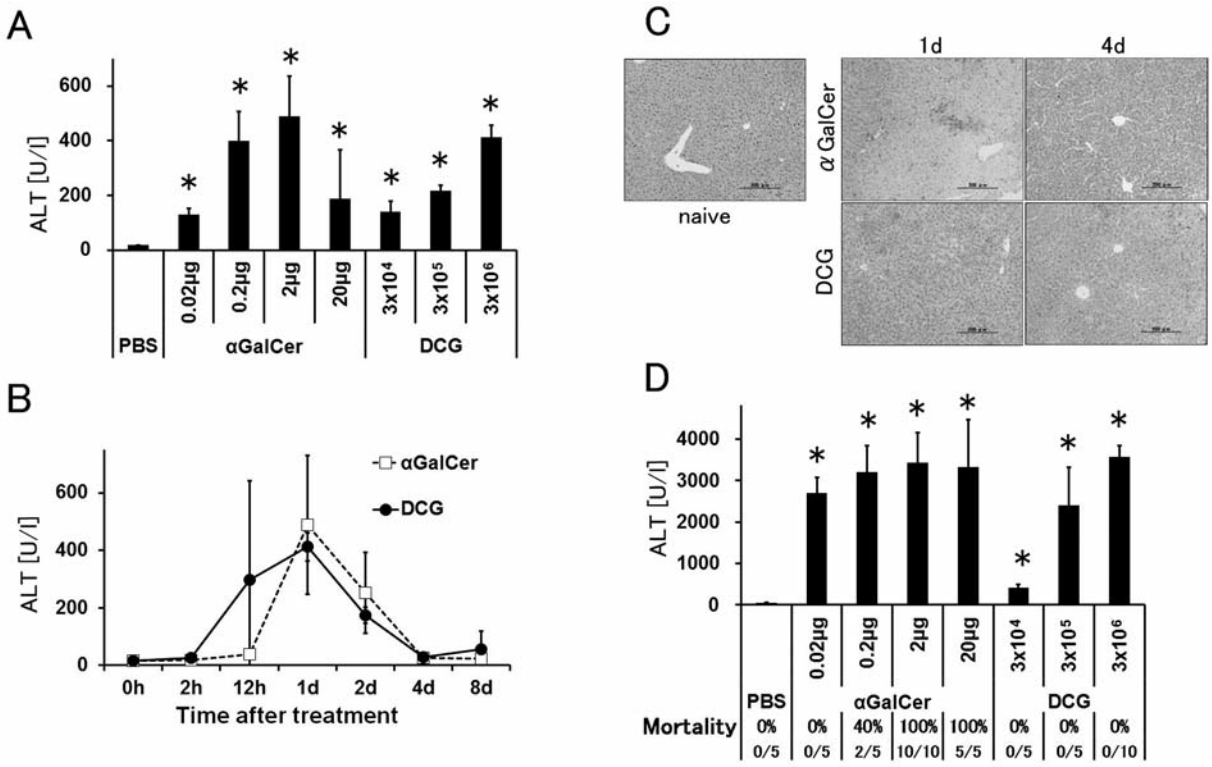

Figure 1. *Both free αGalCer and DCG i.v. injection induce liver injury in young and old mice. (A) Young, 6- to 8-week-old C57BL/6 mice were i.v. injected with PBS, 0.02 μg-20 μg of αGalCer or 3×10⁴-3×10⁶ DCG and ALT activity was measured 24 h later (n=4). Results are mean±SD; *p<0.05 vs. PBS control. (B) Young, 6- to 8-week-old C57BL/6 mice were i.v. injected with 2 μg of αGalCer or 3×10⁶ DCG and ALT activity was measured 2 h, 12 h, 1 d, 2 d, 4 d and 8 d later. Each data point is the mean±SD of the measurements from three mice. (C) Mouse livers were sectioned and stained with H&E. Liver damage induced by free αGalCer and DCG was assessed by light microscopy. (D) This experiment was carried out as in A, with 42- to 55-week-old C57BL/6 mice being injected with PBS, αGalCer or DCG (n=4). Results are mean±SD; *p<0.05 vs. PBS control. Mortality is also shown.*

Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions and studied in compliance with institutional guidelines of Kobe University (approval number: P090113). Mouse TNF-α antibody was purchased from R&D Systems (Minneapolis, MN, USA) and α-GalCer (KRN7000) was purchased from Funakoshi (Tokyo, Japan).

Analysis of liver transaminase. Blood samples were obtained from the tail artery at the indicated times and kept at 4˚C overnight to allow the serum to separate from the clot. Serum preparations were collected following centrifugation at 1,000 × *g* for 15 min. Serum ALT activity levels were determined by using the Alanine Aminotransferase Kit (Wako, Tokyo).

Cytokine assays. The serum concentration of TNF was measured using a CBA assay kit (BD Biosciences, San Diego, CA, USA).

Antibodies and flow cytometry. The following monoclonal antibodies (mAbs) for flow cytometry were purchased from BD Biosciences: Anti-mouse CD1d, CD31, CD95, F4/80, TNFR1 and TNFR2. Flow cytometry data were acquired with a FACSCalibur (Becton Dickinson, San Jose, CA, USA) or MofloXDP (Beckman Coulter, Fullerton, CA, USA) instrument and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Cell preparation. For preparation of liver cells and hepatic lymphocytes, blood was removed by injecting Hank's balanced salt solution (HBSS) through the portal vein. The liver was surgically removed and incubated with 1 mg/ml collagenase type II solution for 30 min at 37˚C. Isolated hepatocytes and endothelial cells were washed and purified in HBSS using low-speed centrifugation at 40 × *g* (11-13). For cell sorting, liver cells were stained with anti-CD31-phycoerythrin (PE) mAb for 30 min at 4˚C and sorted by a Moflo XDP at 4˚C. Hepatic lymphocytes were obtained by centrifugation at 700 × *g*, re-suspended in 33% percoll and centrifuged at 700 × *g* for 15 min (11). Bone marrow-derived DCG were generated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS) and αGalCer, as described previously (9, 14, 15).

Cytokine production. Hepatocytes (2×10⁴ cells) and CD31⁺ EC (2×10⁴ cells) from PBS-, αGalCer- and DCG-immunized mice were cultured with or without naïve hepatic lymphocytes (4×10⁵ cells) in 200 μl RPMI-1640 medium containing 10% FCS in 96-round-

bottom-well plates for 20 h. The supernatants were collected and TNF and IFN-γ production was measured using a CBA assay kit.
Statistical analysis. Differences were analyzed using the Student's t-test and log-rank test. A $p<0.05$ was considered statistically significant.

Results

While i.v. injection of αGalCer or DCG induced liver injury, DCG did not induce lethal liver injury in older C57BL/6 mice. To assess whether DCG caused liver injury, αGalCer and DCG were *i.v.* transferred into young (6- to 8-week-old) C57BL/6 mice. ALT activity was used as a measure for liver injury. As previously described, the maximum DCG dose that could be studied was 3×10^6 DCG, because 1×10^7 DCG induced sudden death due to lung thrombosis (9). A significant increase in serum ALT was detected 24 h after injection with both 0.02-20 μg of αGalCer and 3×10^4-10^6 DCG (Figure 1A, B). The liver damage induced by αGalCer and DCG was confirmed histologically by the presence of hepatic necrosis one day after injection and widened intercellular space on day 4 (Figure 1C). The numbers and proportion of iNKT cells increase with aging; injection of free αGalCer into older C57BL/6 mice induced lethal liver injury (6, 8). Therefore, we examined how older mice respond to DCG. All 42- to 55-week-old C57BL/6 mice treated with 2 μg and 20 μg of αGalCer died within 48 h with high ALT activity levels. Although older mice treated with DCG showed severe hepatic damage (ALT>3,000 U/l in the 3×10^6 DCG group), they ultimately survived (Figure 1D).

Neutralization of TNF-α counteracted liver injury caused by free αGalCer and DCG. Liver injury induced by free αGalCer has been proven to be dependent on TNF-α (6). We compared the release of TNF into the serum of mice injected with free αGalCer and DCG. Both free αGalCer and DCG increased serum TNF concentrations; however, responses to free αGalCer were higher and more rapid (Figure 2A). Next, we analyzed the effect of a neutralizing antibody against TNF-α in this model. Use of polyclonal anti-TNF-α antibody significantly reduced liver injury caused by both free αGalCer and DCG (Figure 2B).

$CD31^+$ hepatic EC from free αGalCer-treated mice induced naive hepatic lymphocytes to produce TNF. Using propidium iodide (PI) uptake as a measure for apoptosis, we compared CD31-negative ($CD31^-$) hepatocytes (HC) and $CD31^+$ EC after PBS, αGalCer or DCG treatment. The percentage of PI-positive EC from αGalCer-treated mice was significantly higher than those from DCG-treated mice (Figure 3B). Since both HC and EC express CD1d on the cell surface (Figure 3A), both cell sets are able to present antigen to iNKT cells. We then examined how HC and EC induce hepatic lymphocytes to produce TNF. EC from mice treated with free αGalCer caused naïve hepatic lymphocytes to produce statistically higher TNF and IFN-γ levels (Figure 3C and D).

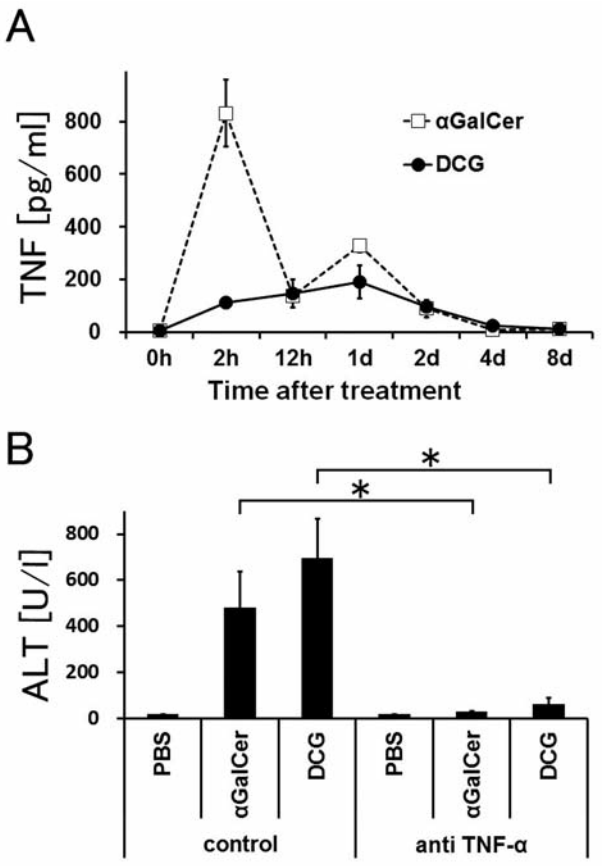

Figure 2. *Alleviation of free αGalCer- and DCG-induced liver injury by TNF-α neutralization. (A) Six- to 8-week-old C57BL/6 mice were i.v. injected with 2 μg of αGalCer or 3×10^6 DCG and TNF activity was measured 2 h, 12 h, 1 d, 2 d, 4 d and 8 d later. Each data point is the mean±SD of the measurements from three mice. (B) Six- to 8-week-old C57BL/6 mice were i.p. injected with polyclonal anti-TNF-α IgG, i.v. injected with PBS, 2 μg of αGalCer or 3×10^6 DCG 30min later and ALT activity was measured 24 h later (n=3). Results are mean±SD; *p<0.05.*

Discussion

Although injection of both free αGalCer and DCG activate iNKT cells *in vivo*, the pathways are different in certain aspects. DCG may be able to activate iNKT cells directly *in vivo*. On the other hand, free αGalCer is taken up by CD1d-positive cells, with these cells presenting CD1d-αGalCer complex to iNKT cells *in vivo*. Endosomes play a pivotal role in the uptake and presentation of αGalCer (16). Specialized antigen-presenting cells, such as dendritic cells, may play a role in antigen presentation to iNKT cells; however, how

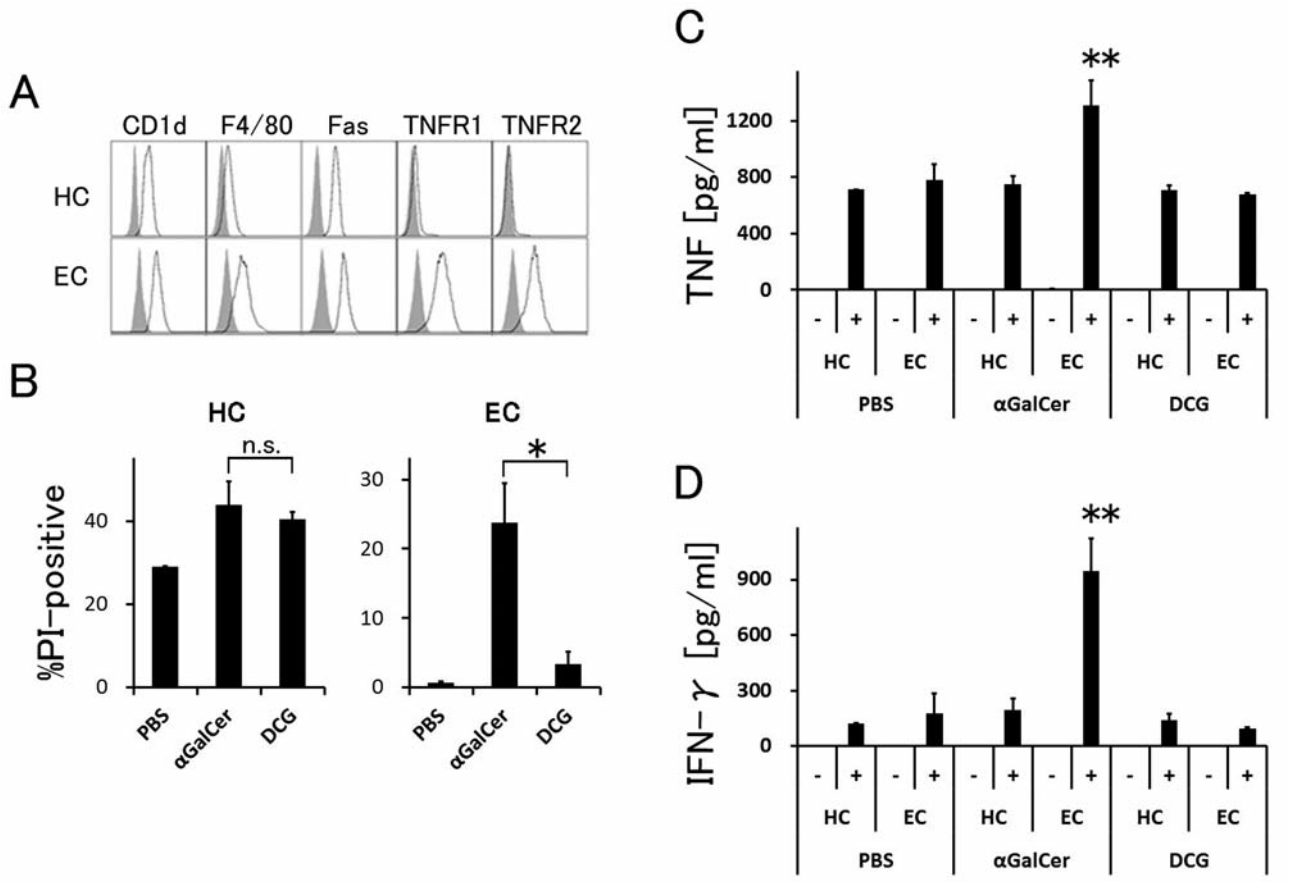

Figure 3. *Liver CD31+ EC from free αGalCer-treated mice caused naïve liver lymphocytes to produce TNF-α and IFN-γ. (A) Representative summary of flow cytometry analysis of CD31− HC and CD31+ EC. (B) Six- to 8-week-old C57BL/6 mice were i.v. injected with PBS, 2 μg of αGalCer or 3×10^6 DCG and the percentage of PI-positive HC and EC was measured 12 h later. (n=3). Results are mean±SD; *p<0.05. (C) Six- to 8-week-old C57BL/6 mice were i.v. injected with PBS, 2 μg of αGalCer or 3×10^6 DCG. HC and EC were obtained from immunized mice 2 h later and were co-cultured with or without naïve hepatic lymphocytes for 20 hours. The TNF concentration was then measured. (n=3). Results are mean±SD; *p<0.05 for EC immunized with αGalCer and co-cultured with naïve hepatic lymphocyte compared to others. (D) This experiment was carried out as in B but, instead, the concentration of IFN-γ was measured. (n=3). Results are mean±SD; *p<0.05 for EC immunized with αGalCer and co-cultured with naïve hepatic lymphocyte compared to others. PI, Propidium iodide; n.s, non significant; HC, hepatocytes; EC, endothelial cells.*

other CD1d-positive cells participate in this reaction is not clear. Interestingly, although Kupffer cells are major producers of TNF-α and play a pivotal role in a murine model of T cell and TNF-α-dependent liver injury (17), liver damage by free αGalCer is not reduced in Kupffer cell-depleted mice (6). Thus, the player responsible for antigen presentation to iNKT cells in the liver remains unknown.

HC are dominant liver cells and non-parenchymal liver cells include liver sinusoidal EC, Kupffer cells and hepatic stellate cells. We focused on HC and EC as injury of both cell sets may directly relate to liver damage, Kupffer cells are not critical during free αGalCer liver damage (6) and hepatic stellate cells are rare. In ovalbumin (OVA) transgenic mice, EC and Kupffer cells activate $CD8^+$ T cells from OVA-specific H-$2K^b$-restricted TCR transgenic mice at very low antigen concentrations, while HC and hepatic stellate cells do not (13). In concanavalin A-induced acute hepatitis, which is also induced by iNKT cell activation, EC are damaged by autophagic induction before HC (18). Using CD31 antibody and cell sorting techniques, we separated $CD31^-$ HC and $CD31^+$ EC. We showed that $CD31^+$ EC were more severely injured after free αGalCer treatment than after DCG treatment. Moreover, EC from mice treated with free αGalCer caused naïve hepatic lymphocytes to produce TNF; EC from mice treated with DCG did not. These results indicate that EC may play a role in antigen presentation to iNKT cells after free αGalCer treatment and that injury of EC may be a cause of lethal liver injury.

Recently, free αGalCer and CD1d-αGalCer complexes have been reported to induce innate and acquired anti-tumor immunity in a murine model (19), while human clinical trials have been conducted as well (20-24). There was no critical hepatic toxicity observed in these trials and only 1 of 25 patient showed Grade 1 ALT elevation in one clinical trial (24). The reason why hepatic injury was rare in these trials requires consideration: (i) The timing of blood collection might not have been optimal to detect liver damage because our results showed ALT levels peaked on day one and declined to a lower level four days after treatment. (ii) The dose of treatment might have been too small to induce liver damage, as we showed dose-dependent liver injury after both free αGalCer and DCG treatment. (iii) The hepatic toxicity induced by iNKT cells could be low in humans or could vary individually because of strain differences that have been reported in a murine αGalCer liver injury model. Nevertheless, potential hepatic toxicity in human clinical trials should always be taken into account.

In summary, the present work describes hepatic toxicity after iNKT cell activation and demonstrates that $CD31^+$ EC may play a role in antigen presentation to iNKT cells after free αGalCer treatment. DCG treatment induced dose- and TNF-α-dependent liver damage without lethal damage. Taken together, these results provide an explanation on how free αGalCer induces liver damage, thus allowing for a better mechanistic understanding of liver injury following iNKT cell activation.

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Disclosure Statement

The Authors declare no financial or commercial conflict of interest.

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