Reduced Ceramides Are Associated with Acute Rejection in Liver Transplant Patients and Skin Graft and Hepatocyte Transplant Mice, Reducing Tolerogenic Dendritic Cells

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We set up this study to understand the underlying mechanisms of reduced ceramides on immune cells in acute rejection (AR). The concentrations of ceramides and sphingomyelins were measured in the sera from hepatic transplant patients, skin graft mice and hepatocyte transplant mice by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Serum concentrations of C24 ceramide, C24:1 ceramide, C16:0 sphingomyelin, and C18:1 sphingomyelin were lower in liver transplantation (LT) recipients with than without AR. Comparisons with the results of LT patients with infection and cardiac transplant patients with cardiac allograft vasculopathy in humans and in mouse skin graft and hepatocyte transplant models suggested that the reduced C24 and C24:1 ceramides were specifically involved in AR. A ceramide synthase inhibitor, fumonisin B$_1$ exacerbated allogeneic immune responses *in vitro* and *in vivo,* and reduced tolerogenic dendritic cells (tDCs), while increased P3-like plasmacytoid DCs (pDCs) in the draining lymph nodes from allogeneic skin graft mice. The results of mixed lymphocyte reactions with ceranib-2, an inhibitor of ceramidase, and C24 ceramide also support that increasing ceramide concentrations could benefit transplant recipients with AR. The results suggest increasing ceramides as novel therapeutic target for AR, where reduced ceramides were associated with the changes in DC subsets, in particular tDCs.

Keywords: acute rejection, ceramide, human, mice, tolerogenic dendritic cells, transplantation

INTRODUCTION

Improvements in surgical techniques and immunosuppressive regimens have increased the numbers of patients undergoing liver transplantation (LT) and enhanced their survival, although complications, such as acute and chronic rejection, can have a negative impact on patient health and survival (Choudhary et al., 2017; Song et al., 2014). Acute rejection (AR) occurs in 15%-25% of LT recipients (Rodriguez-Peralvarez et al., 2016), with the major cause of AR being allogeneic immune responses in the host, mediated primarily by T cells. Allo-reactive T cells become activated through interactions with major histocompatibility complex (MHC) molecules on donor cells, called the direct pathway, or with donor peptides presented by recipient MHC on antigen-presenting cells (APCs), called the indirect pathway (Siu et al., 2018). CD8 T cells have been shown to mediate murine cardiac graft rejection by interacting with dendritic cells (DCs) in secondary lymphoid tissues, such as the spleen and lymph nodes (drLNs) (Harper et al., 2015). Upon activation, naïve T cells differentiate into effector T cells, resulting in graft destruction (Marino et al., 2016). DCs play an important role in AR, acting as APCs to stimulate T cells. DCs can be divided into multiple subsets, such as conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (Merad et al., 2013). pDCs can secret type I interferon (IFN) and differentiate into cDCs (Reizis, 2019). Tolerogenic DCs (tDCs) are one of the key players in organ transplantation (Marin et al., 2018). They suppress T cell proliferation to prolong allograft survival in skin graft mice (Carretero-Iglesia et al., 2016).

The identification of non-invasive biomarkers specifically diagnostic of AR could improve long-term graft and patient survival. Many candidate biomarkers have been suggested based on immunological analyses, genomics, transcriptomics, and proteomics. The results of proton nuclear magnetic resonance analysis of hepatic bile from human LT recipients (Melendez et al., 2001) led to the development of metabolomics methods to analyze outcomes following transplantation (Baliga et al., 2003). Because the liver is a key organ involved in metabolism, metabolomics could provide particularly important information in liver dysfunction. Early studies mostly focused on relatively small molecules, such as lactate, glutamine, and citrate (Hrydziuszko et al., 2010; Serkova et al., 2007), but more recent studies have shown the lipidomic changes in transplant patients. The serum concentrations of phosphatidylcholines, lysophosphatidylcholines, polyunsaturated fatty acids, and sphingomyelins were found to be lower in renal transplant patients with than without AR (Zhao et al., 2014). By contrast, the serum concentrations of phospholipids, lysophopholipids, and sphingomyelins were found to be increased in biopsy samples from donor livers collected before surgery and later showing early allograft dysfunction (Cortes et al., 2014; Xu et al., 2015). Recent metabolomics studies reported that dihydro-ceramides and phospholipid molecules may be potential biomarkers of AR following LT (Liu et al., 2021; Mucke et al., 2018).

Ceramides are the neutral building block of sphingolipids, which are composed of sphingosine and fatty acid (Kita and Shimomura, 2022). Sphingolipids account for up to 5% of the human plasma lipidome (Zelnik et al., 2021). Ceramide is synthesized by three pathways: the *de novo*, sphingomyelin, and salvage/recycling pathways (Sokolowska and Blachnio-Zabielska, 2019). Sphinganine/sphingosine, sphingomyelin, and glucosylceramide can be hydrolyzed to produce ceramide by ceramide synthase, sphingomyelinase, and glucosylceramidase, respectively. Ceramides play important roles in apoptosis and autophagy (Morad and Cabot, 2013), and regulate ICAM-1 (intercellular adhesion molecule 1) function during T cell migration in multiple sclerosis (Lopes Pinheiro et al., 2016). Inflammation increases the ceramide contents in DCs (Ocana-Morgner et al., 2017), and a ceramide analogue is found to stimulate DCs to promote T cell responses to viral infection (Pritzl et al., 2015). Interestingly, CD8 T cells require ceramide to form ceramide-rich platforms in target cell membranes to initiate graft-versus-host disease (GVHD) (Rotolo et al., 2009), and ceramide synthase (CerS) 6 contributes to optimal allo-T cell activation, proliferation, and cytokine production in GVHD (Sofi et al., 2017). Ceramide synthase 6 generates C16 ceramide.

Despite these findings, the role of ceramide in AR remains relatively unclear. The present study therefore assessed the serum concentrations of sphingolipids during AR in LT patients and murine transplant models, compared with those of other complications, as well as the effects of reducing ceramide synthesis on immune cells in the draining drLNs from a murine skin graft model. This study is the first comprehensive study on the role of ceramide in AR, to our knowledge.

MATERIALS AND METHODS

Human serum collection and selection

The present study included adult patients who underwent LT surgery at the Asan Medical Center, Seoul, Korea. The study protocol was approved by the Institutional Review Board (IRB) of Asan Medical Center (approval No. 2011-0898), and all participants provided written informed consent. Standard care and treatment were provided to all the patients. The study was performed in accordance with the principles of the Declaration of Helsinki.

Blood samples were collected weekly from LT recipients into STT BD Vacutainer tubes (BD Biosciences, USA) until 4 weeks after LT in February to December 2012. Serum samples were prepared within 2 h after blood collection and stored in a deep freezer for future use. Patients' medical records were reviewed, and those with and without AR were determined 1 year later. All the participants were ethnically Korean. The control group consisted of patients without rejection of similar ages and gender as the patients with AR. Rejection in some patients was confirmed by biopsy, whereas others were regarded as having experienced clinical rejection and were subsequently treated with standard rejection therapeutics without biopsy. A clinical diagnosis of AR was considered in patients with elevated alanine aminotransferase (ALT) and bilirubin levels without evidence of infection, thrombosis, and other complications. Of the over 300 patients who underwent LT during the study period, 59 patients with AR and 78 control patients were selected; their clinical and demographic characteristics are shown in Table 1. However, the numbers

Table 1. Demographic and clinical characteristics of LT recipients included in this study

	Control group (n = 78)	AR group (n = 59)	P value
Sex	M 60, F 18	M 46, F 13	>0.999
Age (y)	52 ± 7.446	51 ± 8.914	0.702
Type of operation	LD 68 (Dual 3), DD 10	LD 53 (Dual 3), DD 6	0.834
ABO compatibility	C 62, I 16	C 48, I 11	0.956
Original diseases	LC (HBV) 51	LC (HBV) 24	0.110
	LC (HBV, alcohol) 2	HBV-FHF 3	
	LC (HCV) 6	LC (HCV) 9	
	LC (NBNC) 2	LC (NBNC) 2	
	ALC LC 6	ALC LC 9	
	AIH 1	Toxic hepatitis-FHF 1	
	SBC 1	PSC 1	
	Budd-Chiari syndrome 1	Budd-Chiari syndrome 1	
	Retransplant 4	Retransplant 4	
	Liver hemangioma 1	HCC 1	
	FHF 1	Idiopathic PVT, LC 1	
	Idiopathic PVT 1	PBC 1	
	PBC 1	Fulminant hepatitis (r/o HAV AH) 1	
	-	Toxic hepatitis-Wilson's 1	
Days to rejection	-	14 ± 112.940	
Cold ischemic time (min)	88.5 ± 79.466	95 ± 79.756	0.807
Warm ischemic time (min)	40 ± 12.919	41 ± 16.906	0.269
Total ischemic time (min)	128 ± 84.534	136 ± 77.509	0.940
Pneumonia	Y 18, N 66	Y 10, N 49	0.372
Bacteremia	Y 2, N 69	Y 4, N 55	>0.999
Sepsis	Y 1, N 76	Y 1, N 58	>0.999
Any of pneumonia/bacteremia/sepsis	Y 21, N 57	Y 10, N 40	
MELD scores	12 ± 10.229	17 ± 9.759	0.260

Values are presented as mean ± SD.

LT, liver transplantation; AR, acute rejection; M, male; F, female; LD, living donor; Dual, dual transplantation; DD, deceased donor; ABO, ABO blood type; C, compatible; I, incompatible; LC, liver cirrhosis; HBV, hepatitis B virus; FHF, fulminant hepatic failure; HCV, hepatitis C virus; NBNC, non-HBV and non-HCV; ALC, alcoholic; AIH, autoimmune hepatitis; SBC, secondary biliary cirrhosis; PSC, primary sclerosing cholangitis; HCC, hepatocellular carcinoma; PVT, portal vein thrombosis; PBC, primary biliary cholangitis; r/o, rule out; HAV, hepatitis A virus; AH, acute hepatitis; Y, yes; N, no; MELD, model for endstage liver disease.

of samples at each time point varies, as not all samples could be collected in a timely manner.

Measurement of lipids using LC-MS/MS

Sphingolipids were measured as previously described (Koh et al., 2021). In brief, organic phases were collected after liquid-liquid extraction of the sera and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) equipped with 1290 HPLC (Agilent, Germany) and QTRAP 5500 (ABSciex, Canada).

Murine skin grafts

Murine skin grafts were transplanted from C57BL/6 donor mice (Orientbio, Korea) to allogeneic sex-and age-matched BALB/c recipient mice (Orientbio) using standard protocols with minor modifications (Rovira et al., 2013). Briefly, recipient mice were anesthetized with Zoletil 50 (Virbac, Korea) and Rompun (Bayer Korea, Korea). A graft bed on the left lateral thorax was prepared with scissors by removing an area of epidermis. Skin samples of donor mice measuring 8 mm × 8 mm were transferred to the prepared bed, sutured and then covered with Mepitel One (Molnlycke Health Care,

Australia), gauze, and surgical tape. The mice were sacrificed 7 days later, and blood and tissue samples were collected. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Asan Medical Center (approval No. 2015-12-144).

In some experiments, Fumonisin B_1 (Sigma-Aldrich, USA) at 2 mg/kg was subcutaneously injected into recipient mice 30 min before skin grafting. The mice were subcutaneously injected with DMSO (vehicle control) or Fumonisin B_1 at 2 mg/kg daily for 4 more days (Draper et al., 2011; He et al., 2006). All animal procedures using the inhibitor were approved by the IACUC of Asan Medical Center (approval No. 2021-12-255).

Murine hepatocyte transplantation

Mouse hepatocytes were isolated using a two-step EDTA/collagenase liver perfusion method (Weglarz et al., 2000) with minor modifications. All procedures were approved by the IACUC of Asan Medical Center (approval No. 2018-12-130). Perfusion medium I consisted of sterile phosphate-buffered saline (PBS) containing 1% (v/v) buffer A [1 M HEPES (pH 7.4), 5% (w/v) KCl], 0.5% 1 M glucose, and 0.1% 200 mM

EDTA, whereas perfusion medium II contained sterile PBS, 1% (v/v) buffer A, 2% 1 M HEPES, 0.5% 1 M glucose, and 2% 500 mM CaCl$_2$; the pH of both media was adjusted to 7.4 with NaOH. Type IV collagenase (Gibco, USA), at a concentration of 3 mg/ml, was added to perfusion medium II and filtered. Before liver perfusion, adult mice were anesthetized, and each mouse liver was perfused through the portal vein with perfusion medium I, followed by perfusion medium II. The largest lobe of each mouse liver was soaked in collagenase-containing perfusion medium II and chopped into small pieces, which were passed through a 100 μm nylon cell strainer (Falcon, USA). Hepatocytes were resuspended in PBS at a concentration of 10^6 cells per 100 μl. For hepatocyte transplantation, BALB/c mice were anesthetized and 50 μl of hepatocyte suspension, containing 0.5 × 10^6 cells, were injected into the mouse's largest liver lobe. After 7 days, whole blood was collected by heart puncture, and the serum was harvested and frozen at −80°C until assayed.

Mixed lymphocyte reaction (MLR)

Spleen tissue was homogenized with a syringe plunger in 5 mL RPMI 1640 (Welgene, Korea), filtered with a 100 μm nylon cell strainer (Falcon) and washed twice with 1× PBS. Red blood cells (RBCs) were lysed with 1× RBC lysis buffer (BioLegend, USA), and the cells were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS), 55 μM 2-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich), and 1% penicillin/streptomycin (Gibco), and the numbers of cells were counted using a hemocytometer. Target cells were isolated from C57BL/6 mouse spleens and effector cells were collected from the spleens of BALB/c mice that did and did not receive C57BL/6 skin grafts. Target cells were treated with 25 μg/ml mitomycin C (Sigma-Aldrich), incubated at 37°C for 30 min and washed 3 times with RPMI 1640 (Welgene). The cells were co-cultured in 96 well round bottom plates (Corning, USA) at an effector-to-target ratio of 1:1 in RPMI 1640 (WelGene) supplemented as above for 7 days. Indicated concentrations of fumonisin B$_1$ (Sigma-Aldrich), ceranib-2 (Cayman Chemical Company, USA) or C24 ceramide (Avanti Polar Lipids, USA) were added every 3 days as previously described (Draper et al., 2011). Cell proliferation rates were determined by CCK8 assay kits (Dojindo, Japan), following the manufacturer's protocols.

Flow cytometry

To isolate immune cells from draining drLNs, the tissue was homogenized with a syringe plunger in 5 ml RPMI 1640 (Welgene), filtered with a 100 μm nylon cell strainer (Falcon), and washed twice with 1× PBS. The cells were suspended in RPMI 1640 supplemented as above and the numbers of cells were counted using a hemocytometer. Cells from the drLN were incubated with fluorescence-conjugated antibodies and washed twice with 1× PBS containing 2% FBS (Welgene). Antibodies used for surface staining included anti-mouse CD3 (Clone 145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD44 (G44-26 or IM7), CD62L (MEL-14), CD11b (M1/70), CD45 (30-F11), CD11c (N418), MHC class II (M5/114.15.2), B220 (RA3-6B2), Ly6C (HK1.4), CD25 (PC61), CD86 (GL-1), and FoxP3 (FJK-16s). Antibodies and matched isotype controls

were purchased from BD Biosciences, BioLegend, or eBioscience (USA). Cells were pre-treated with rat anti-mouse CD16/32 monoclonal antibody (2.4G2; BD Biosciences) to block Fc receptor. Cell preparations were subjected to flow cytometry data on a CytoFlex flow cytometer (Beckman Coulter, USA) and the results were analyzed with FlowJo software (TreeStar, USA).

Differentiation of tolerogenic bone marrow-derived DCs (BMDCs) *in vitro*

Single cell suspension of the bone marrow from BALB/c mice was isolated and RBCs were lysed with 1× RBC lysis buffer (BioLegend). Cells were resuspended in RPMI 1640 supplemented as above, and the numbers of cells were counted. BMDCs were cultured in 6-well plates at 2 × 10^6 cells/well with 20 ng/ml recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF) (PeproTech, USA), 20 ng/ml recombinant murine IL-4 (PeproTech), 10 nM vitamin D3 and 1 μM fumonisin B$_1$ (Sigma-Aldrich) (Ferreira et al., 2011). DMSO was used as vehicle control. Medium containing GM-CSF, IL-4, vitamin D3, and Fumonisin B$_1$ was refreshed every 2 days. On day 7, DCs were stimulated with 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich). The DCs were harvested on day 8 and analyzed by flow cytometry.

Statistics

Lipid levels in patients were reported as least-square mean (lsmean) and SEM, and P values were obtained with SAS (ver. 9.4; SAS Institute, USA) using a generalized linear mixed model. All the data from animal experiments were reported as mean ± SEM and compared by Student's t-test or ANOVA using Prism ver. 6 (GraphPad Software, USA). A P value < 0.05 was regarded as statistically significant.

RESULTS

Ceramides were reduced by AR

Analysis of the serum concentrations of the ceramides C14, C16, C18, C18:1, C20, C24, and C24:1; the sphingomyelins C16:0, C18:0, and C18:1; sphinganine; and sphingosine in LT patients showed that the serum concentrations of the ceramides C24 and C24:1 and the sphingomyelins C16:0 and C18:1 were significantly lower in the AR than in the control group 1 week after LT (Fig. 1). In addition, the C18:1 sphingomyelin was lower in the AR group 2 and 3 weeks after LT, and the C24 ceramide was lower in the AR group 3 weeks after LT. Reductions in the ceramides C24 and C24:1 were first observed 1 week after LT, before the diagnosis of AR at an average 13.5 days (Table 1). ALT levels do not differ usually in a week (Kim et al., 2016).

Analyses in mice showed that the serum concentrations of the ceramides C18:1, C24, and C24:1 were slightly lower in mice that had undergone allogeneic skin grafting and significantly lower in those of hepatocyte transplant at 1 week post transplantation. Sphingomyelin C16:0 was only reduced in mice following hepatocyte transplant, but not in skin graft mice (Fig. 2). The results suggest the reduction of ceramides be related to allogeneic immune responses, while those of sphingomyelins might be results of hepatic damages.
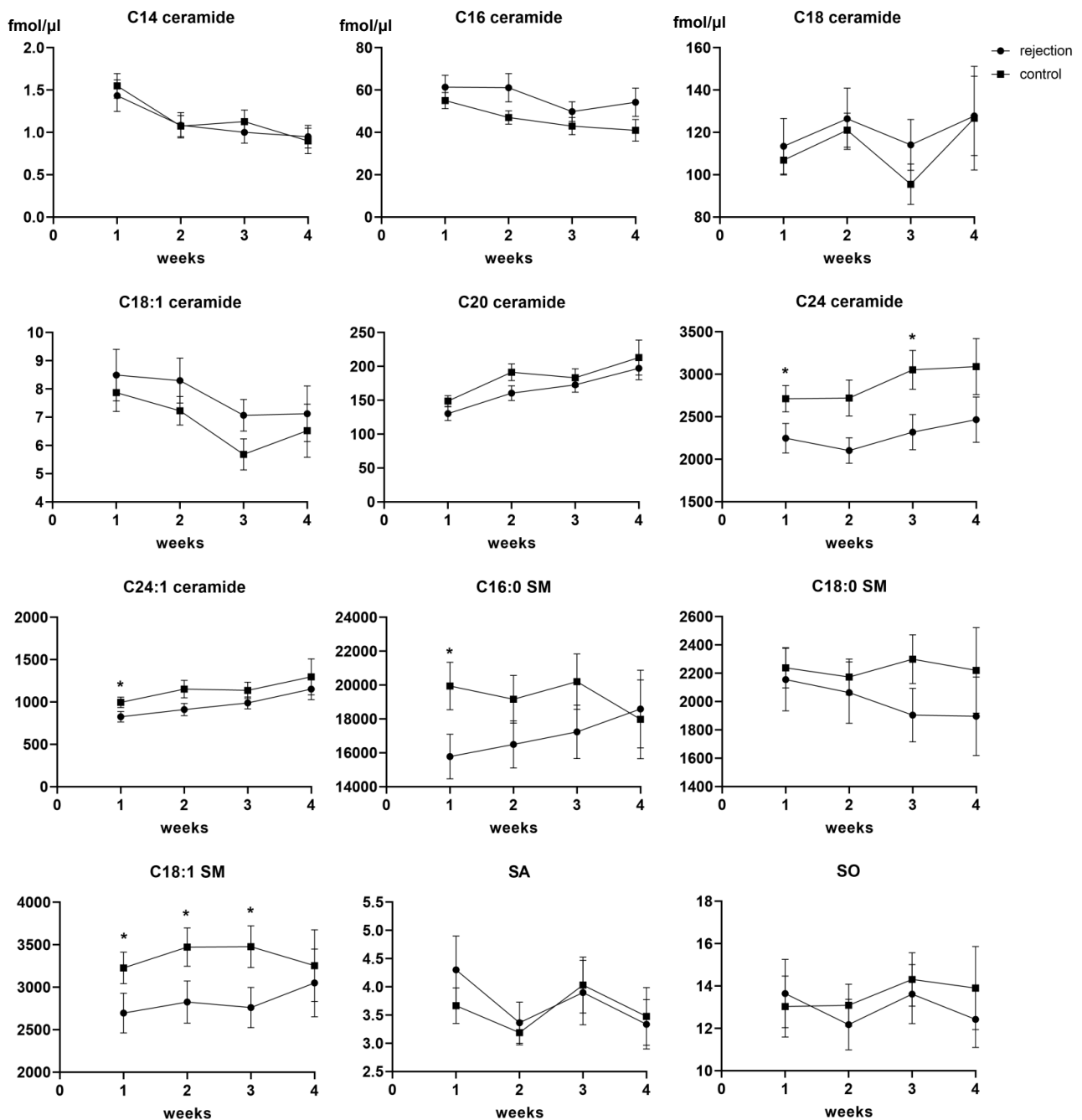
Fig. 1. Reduced ceramide and sphingomyelin concentrations in hepatic transplant patients with acute rejection. Serum concentrations (fmol/μl) of ceramides and sphingolipids from liver transplant patients were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), with results reported as least-square mean ± SEM and compared by Student's t-tests. Control; n = 58 for week 1, n = 67 for week 2, n = 52 for week 3, and n = 21 for week 4. Rejection; n = 38 for week 1, n = 39 for week 2, n = 40 for week 3, and n = 29 for week 4. *P < 0.05. SM, sphingomyelin; SA, sphinganine; SO, sphingosine.

To confirm that these results were specifically due to AR, the LT patients were re-categorized as who had pneumonia, sepsis, or infection, and who did not. Infection and acute inflammation were associated with reductions in C18:1 sphingomyelin and sphinganine, but not with significant reductions in ceramides (Supplementary Table S1, Supplementary Materials and Methods). Furthermore, the serum levels of sphingolipids were not reduced in cardiac graft patients with cardiac allograft vasculopathy (CAV) (Supplementary Tables S2 and S3). The sera were collected at least 1 year after sur-

A

B
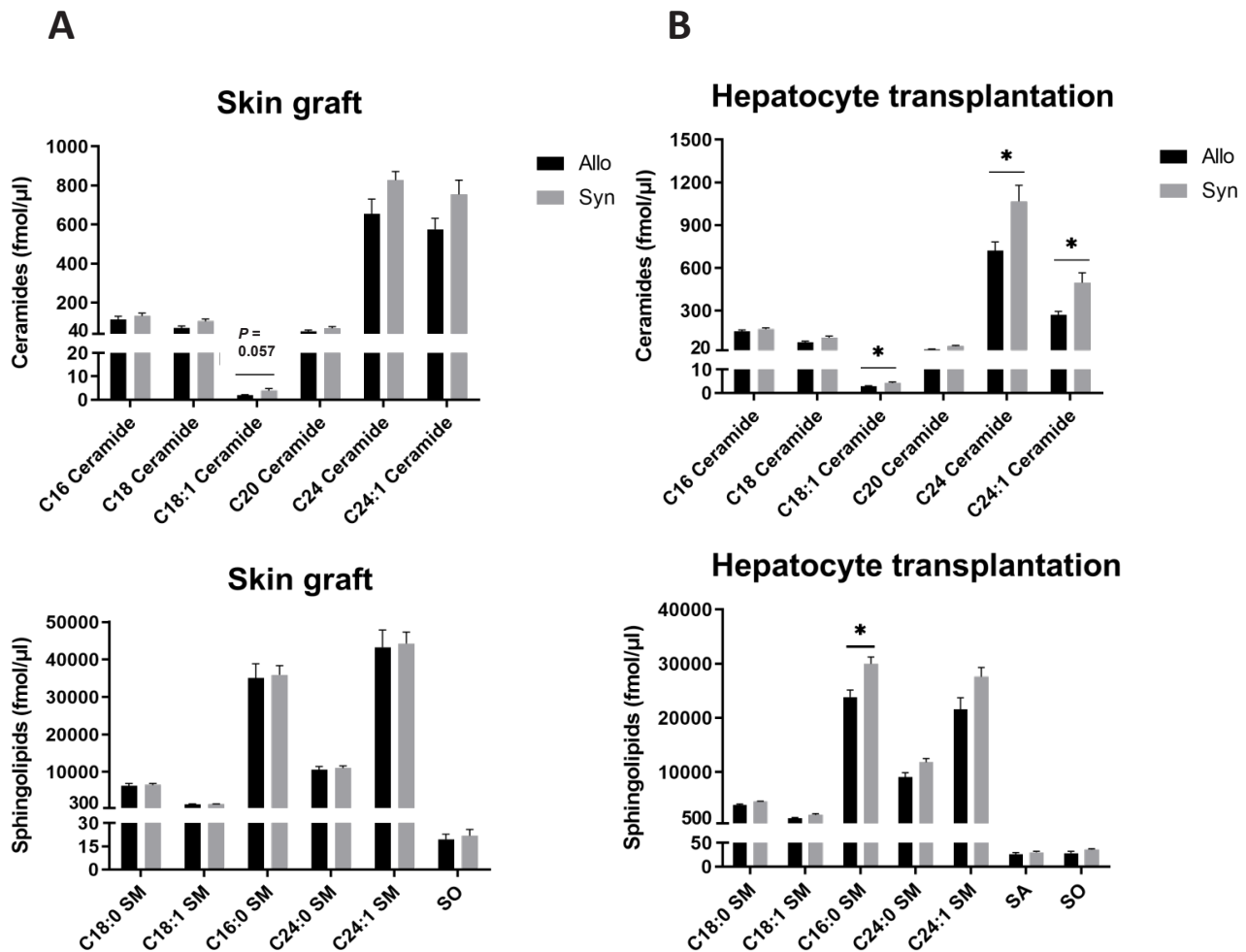
Fig. 2. Reduced ceramide and sphingomyelin concentrations in mice with allogeneic skin grafts and hepatocyte transplants. (A and B) Skin graft (A) and hepatocyte transplantation (B) were performed using female 8- to 12-week-old BALB/c mice as recipients and female 8- to 12-week-old C67BL/6 mice as allogeneic donors. Sera were collected 1 week later. Serum concentrations (fmol/μl) of ceramides and sphingolipids were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Sphinganine was not detectable in the sera from skin graft mice. Results are reported as mean ± SEM (n = 4 for upper panels and n = 5 for lower panels) and compared by Mann-Whitney U test. *$P < 0.05$. Allo, Allogeneic transplant; Syn, syngeneic skin transplant; SM, sphingomyelin; SO, sphingosine; SA, sphinganine.

gery. Taken together, these findings indicate that serum ceramide concentrations were reduced exclusively by AR, whereas serum sphingomyelins could be reduced by infection, acute inflammation, or liver damages.

Fumonisin B₁ treatment promoted the proliferation of alloreactive cells *in vitro* and *in vivo*

Because these results suggested that the reduction of ceramides could exacerbate AR, the effect of the inhibition of ceramide synthesis on allogeneic immune responses was assessed in MLR using mouse splenocytes. Fumonisin B₁, a ceramide synthase inhibitor, was found to promote the proliferation of effector cells in a dose-dependent manner (Fig. 3A). To confirm the results *in vivo*, fumonisin B₁ or vehicle (DMSO) was injected into recipient mice, followed by skin grafting. One week later, splenocytes were isolated and proliferation

assays were performed. These results showed that fumonisin B₁-treated cells from allogeneic skin grafted mice proliferated more than vehicle control (Fig. 3B).

To investigate the effect of reduced ceramide synthesis on immune cells, mice received skin grafts, followed by injection of fumonisin B₁ for 5 consecutive days. T cell and DC subsets in the drLN obtained 7 days after skin grafting were analyzed to determine the immune cell populations affected by reduced ceramide. Flow cytometry was performed to assess the numbers of CD44^{lo}CD62L^{lo} effector, CD44^{hi}CD62L^{lo} memory, and CD44^{lo} CD62L^{hi} naïve T cells (Supplementary Fig. S1A) and CD4^{+}CD25^{+}FoxP3^{+} regulatory T cells (Treg) (Supplementary Fig. S1B). T cell populations were not significantly altered by fumonisin B₁ in the drLN following skin grafting (Fig. 3C). DCs have been classified as CD11c^{+}B220^{−}Ly6C^{−}MHCII^{+} cDCs and CD11c^{+}B220^{+} pDCs (Merad et al., 2013). Majority of the
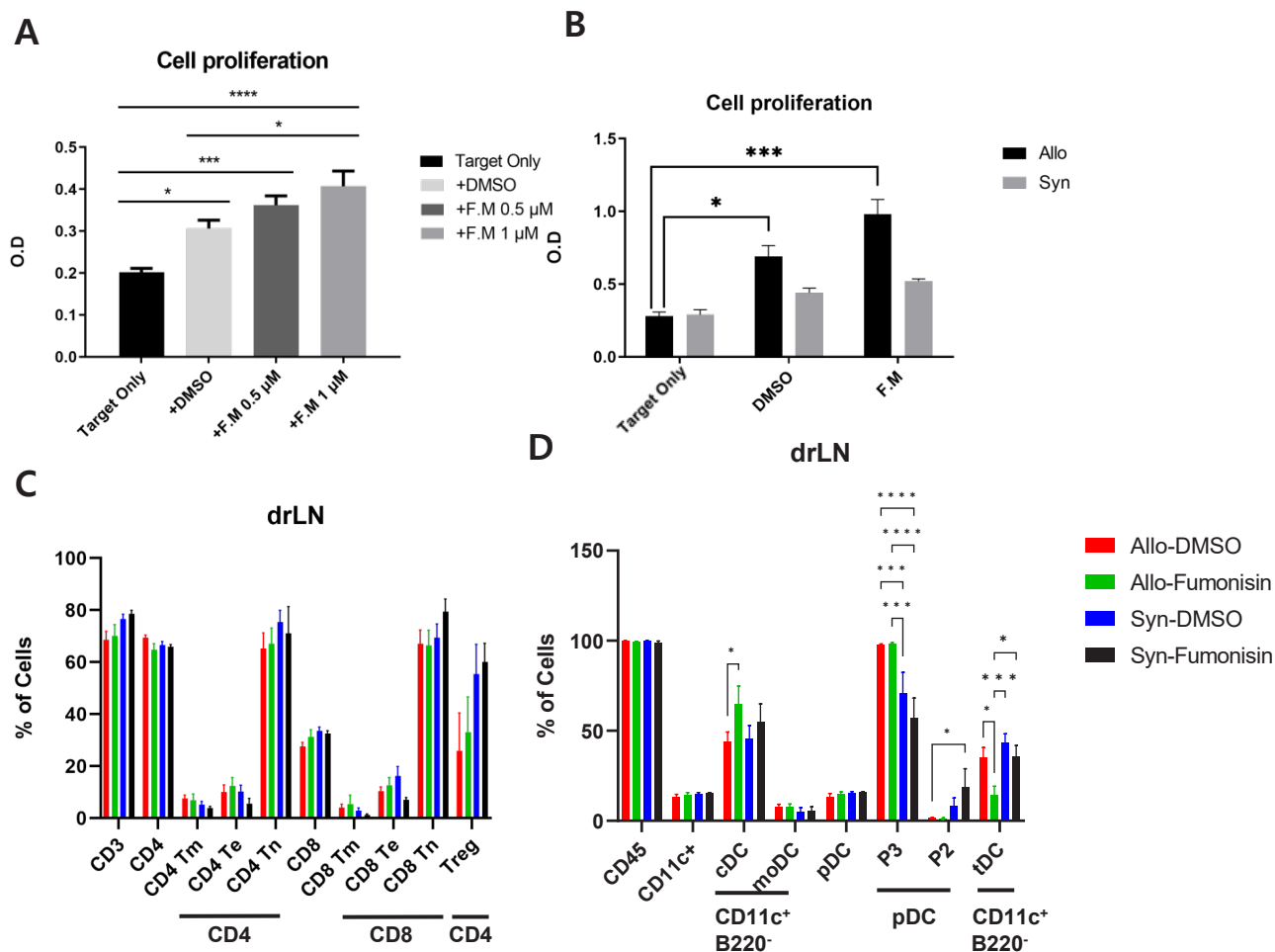
**Fig. 3. Fumonisin B₁, a ceramide synthase inhibitor, increases the proliferation of allo-reactive cells.** Mixed lymphocyte reactions were performed with splenocytes from female 8- to 12-week-old BALB/c mice as effectors and from female 8- to 12-week-old C57BL/6 mice as allogeneic target or stimulant, cocultured for 1 week at a ratio of 1:1 in the presence or absence of the indicated concentrations of fumonisin B₁. Target cells were pre-treated with mitomycin C. (A) Cell proliferation was assessed by CCK8 assays. The results are the means of six independent experiments. The error bars represent SEM. Results were compared by ANOVA with Tukey's multiple comparison tests. O.D, optical density. Target only, C57BL/6 splenocytes pre-treated with mitomycin C; DMSO, coculture of effector and stimulant cells in the presence of vehicle control; F.M, coculture of effector and stimulant cells in the presence of fumonisin B₁. (B) Splenocytes were prepared from mice that had been administered fumonisin B₁ 1 week after skin grafting. Recipients were BALB/c mice and allogeneic donors were C67BL/6 mice. Proliferation was assessed by CCK8 assays after another 1 week in culture. Target only, splenocytes treated with mitomycin C as background control; DMSO, vehicle control; F.M, fumonisin B₁-administered; Allo, allogeneic; Syn, syngeneic. The results shown are the means for 5-8 mice. The error bars represent SEM. Results were compared by ANOVA with Tukey's multiple comparison. (C) T cell subtypes in the draining lymph nodes (drLN) of mice 1 week after skin grafting. The results shown are the mean ± SEM (n = 6-9 mice, for Treg, n = 3-4 mice). Results were compared by ANOVA. Tm, memory T cells; Te, effector T cells; Tn, naïve T cells; Treg, regulatory T cells. (D) DCs in the drLN 1 week after skin grafting. The results shown are the means for five mice. The error bars represent SEM (n = 5-6). Groups were compared by ANOVA with Tukey's multiple comparison. DC, dendritic cells; cDC, conventional DCs; moDC, monocyte-derived DCs; pDC, plasmacytoid DCs; P3, P3-like pDC; P2, P2-like pDC; tDC, tolerogenic DCs. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$.

CD11c⁺B220⁺ cells were Ly6C⁻, thus we further divided pDCs to Ly6C⁺/⁻MHC II⁺/⁻ P2-like and Ly6C⁻MHC II⁺ P3-like pDCs (Majdoubi et al., 2019) (Supplementary Figs. S1C and S2). Monocyte-derived DCs (moDCs) were defined as CD11c⁺B220⁻Ly6⁺ (Merad et al., 2013; Sharma et al., 2018). Fumonisin B₁ significantly increased P3-like pDCs and slightly decreased P2-like pDCs in the drLN from allogeneic skin graft mice. Notably, CD11c⁺B220⁻MHCII⁻Ly6C⁻ tDCs (Marin et al., 2018) were sig-

nificantly reduced in the drLN from allogeneic skin graft mice by fumonisin B₁ (Fig. 3D). The results suggest that fumonisin B₁ changed DC subsets which plays a role in AR.

The effect of fumonisin B₁ on tDC population was confirmed with BMDC *in vitro*. Tolerogenic DC was differentiated from mouse BM cells in the presence of fumonisin B₁ (Fig. 4A, Supplementary Fig. S3). As seen in Figs. 4B and 4C, the treatment of fumonisin B₁ reduced tDC population among

A

Tolerogenic BMDC differentiation
In vitro

B

C
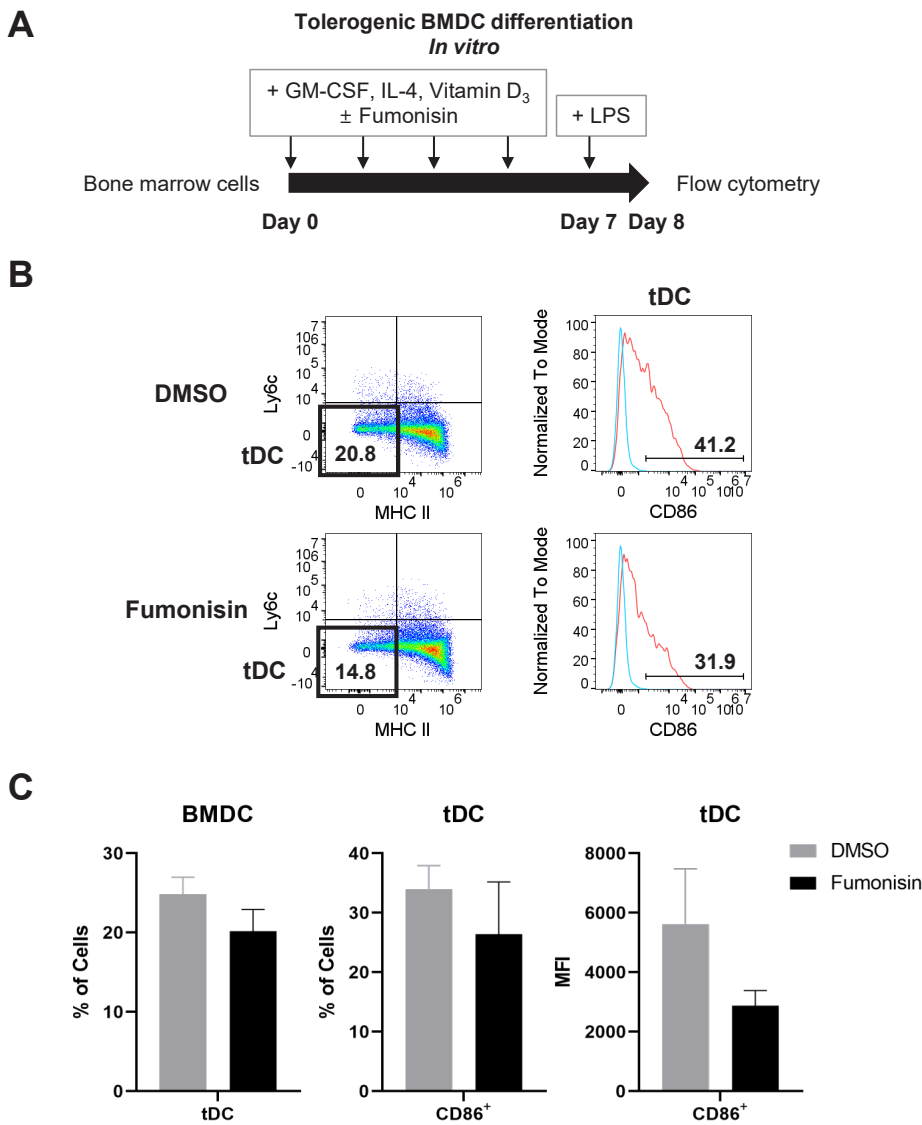
Fig. 4. Fumonisin B1 reduced tolerogenic DCs *in vitro*. (A) Experimental scheme of the differentiation of tDCs from bone marrow. (B) Representative flow cytometric plots of tDCs and CD86 expression from three independent experiments. Gating strategies are displayed in Supplementary Fig. S3. (C) Summary of tDC population (%) of BMDC (left), CD86$^+$ tDC population (%) (middle), and mean fluorescence indices (MFI) of CD86 (right). The results are the means of three independent experiments. The error bars represent SEM (n = 3). $P > 0.05$ (not significant). BMDC, bone marrow-derived dendritic cell; GM-CSF, granulocyte-macrophage colony stimulating factor; LPS, lipopolysaccharide; tDC, tolerogenic DC.

BMDC and CD86 expression on tDC by percentages and MFI, although the differences were not statistically significant. In summary, fumonisin B$_1$, a ceramide synthase inhibitor, reduced tDC and their activation, thus the reduced ceramides could aggravate AR process by reducing tDC activity.

Increasing ceramide reduces allogeneic immune responses

Ceranib-2 is an inhibitor of acid ceramidase, potentially resulting in the accumulation of ceramide. Treatment of ceranib-2 during MLR inhibited the proliferation of alloreactive effector cells in a dose-dependent manner (Fig. 5A). In line with the results, C24 ceramide also reduced the proliferation of alloreactive cells (Fig. 5B). C24 ceramide was most prominently expressed at quantity and significantly reduced in the sera from human LT patients as well as those of mice following skin grafting and hepatocyte transplantation (Figs. 1 and 2). These findings indicate that the effect of reduced ceramides may be reversed by increasing ceramide concentration.

DISCUSSION

The present study showed that the concentrations of the ceramides C24 and C24:1 were lower in the sera of LT recipients with than without AR, and in the sera of mice that had undergone allogeneic skin grafting and hepatocyte transplantation, but not in the sera of LT recipients with bacterial infection, pneumonia, or sepsis, or cardiac transplant patients with CAV. The results show that the reduced ceramides were not results of infection or inflammation. CAV, a vascular disease unique to cardiac transplant patients, starts to occur approximately 1 year after surgery and affects patient long-term survival (Taylor et al., 2006). CAV is a form of Th1-mediated delayed-type hypersensitivity, but distinguished from AR (Merola et al., 2017). Nonetheless, the lipid profiles of CAV are not similar to those of LT with AR, implying that the reduced ceramides provide disease-specific information for AR. The present study also found that the serum concentrations
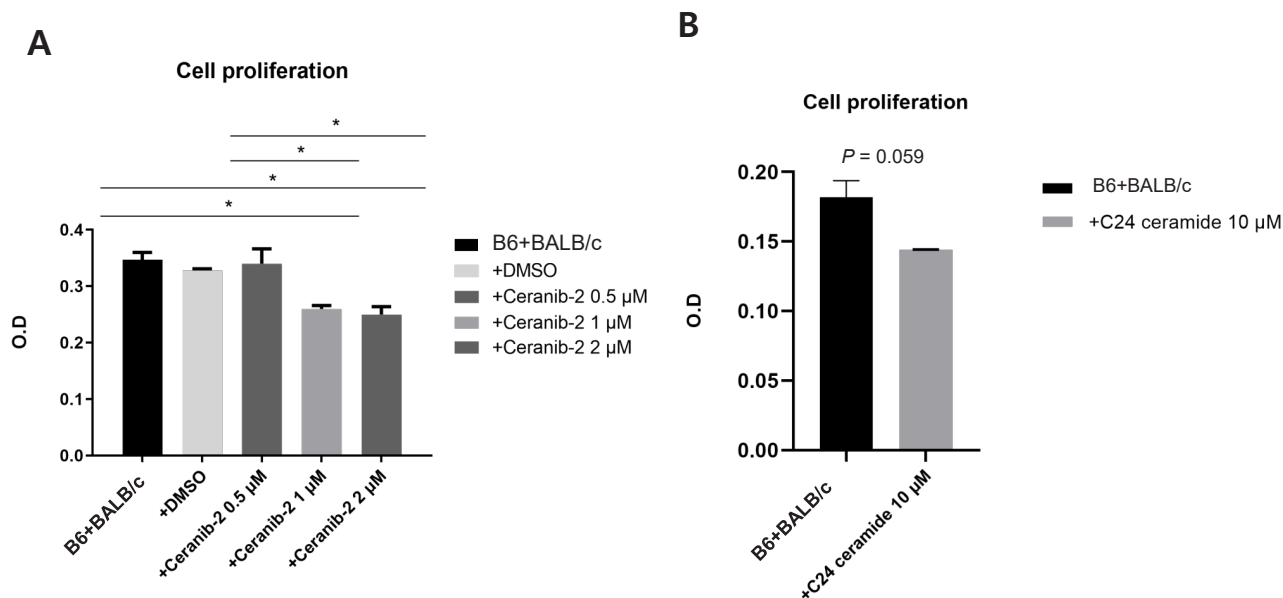
Fig. 5. Ceranib-2, an acid ceramidase inhibitor, and C24 ceramide reduce the proliferation of allo-reactive cells. (A) Following cell treatment with mitomycin C, mixed lymphocyte reactions (MLR) were performed with splenocytes from female 8- to 12-week-old BALB/c mice as effectors and from female 8- to 12-week-old C57BL/6 as allogeneic stimulants, cocultured for 1 week at a ratio of 1:1 in the presence or absence of the indicated concentrations of ceranib-2. Cell proliferation was assessed by CCK8 assays. The results are the means of three independent experiments. The error bars represent SEM (n = 3). Results were compared by ANOVA with Tukey's multiple comparison. $*P < 0.05$. B6+BALB/c, coculture of effector and stimulant cells; +DMSO, coculture of effector and stimulant cells in the presence of vehicle control; +Ceranib-2, coculture of effector and stimulant cells in the presence of ceranib-2 at the indicated concentrations. O.D, optical density. (B) MLR were performed in the presence or absence of the indicated concentrations of C24 ceramide as described above. The results are the means of three independent experiments. The error bars represent SEM (n = 3). B6+BALB/c, coculture of effector and stimulant cells; +C24 ceramide, coculture of effector and stimulant cells in the presence of C24 ceramide at 10 μM.

of C16:0 sphingomyelin were reduced in LT patients with AR and in mice following hepatocyte transplantation, but not in skin graft mice. These findings suggest that the levels of specific sphingolipids were altered by different causes, rather than AR. The gene expression involved in ceramide synthesis was significantly reduced in the spleens of mice that had undergone skin grafting, but not in the livers (Supplementary Fig. S4). In particular, the expression of *Cers3, 4,* and *5* was significantly reduced in the spleens of the allogeneic skin graft mice, suggesting that the reduced ceramides in the sera could be results of the reduced expression of the ceramide synthase genes in the circulating immune cells, not of the liver damages in hepatic transplant patients with AR. The mechanisms controlling specific lipid levels and gene expression await further investigation.

Fumonisin B$_1$ inhibits sphingosine-sphinganin-transferases and ceramide synthases and is therefore competitive inhibitor of sphingolipid biosynthesis and metabolism. The inhibition results in increased concentrations of sphinganine, sphingosine and their 1-phosphate metabolites and in decreased concentrations of complex sphingolipids, such as ceramides (He et al., 2006; Zitomer et al., 2009). We do not exclude the possibility that the effect of fumonisin B$_1$ on allogeneic immune responses might be caused not only by reduced ceramides but also by changes of multiple sphingolipids. Fumonisin B$_1$ elevates sphinganine and sphingosine, and their

1-phosphates (Riley and Merrill, 2019). Sphingosine-1-phosphate (S1P) is well known to play roles in immune cell signaling. Inhibition of sphingosine kinase-2 suppresses inflammation and attenuates graft injury after liver transplnataion in rats (Liu et al., 2012), and S1P receptor 3 (S1PR3)-deficient DCs protect mice from kidney ischemia-reperfusion injury (Bajwa et al., 2016). Taken together, the changes of multiple sphingolipids by fumonisin B$_1$ may affect DC populations and AR. Nonetheless, allogeneic immune responses were increased by inhibiting ceramide synthase and reduced by inhibiting acid ceramidase, suggesting that accumulation of ceramides have a therapeutic effect on AR. Although the acid ceramidase inhibitor, ceranib-2, likely results in the accumulation of ceramides, ceramides are synthesized by multiple pathways, indicating that no single inhibitor is sufficient to deplete or accumulate ceramides. Ceramides may be metabolized by other pathways, ceramide levels may not be altered drastically by blocking a single pathway. The sphingomyelin synthase inhibitor, tricyclodecan-9-yl-xanthogenate (D609), has been shown to increase ceramide levels and inhibit the proliferation of macrophages, microglia, and astrocytes (Gusain et al., 2012). In addition, the glucose analogue N-butyl-deoxynojirimycin (NB-DNJ) was found to inhibit the enzymes glucosylceramide synthase and glucosylceramidase, and is currently approved for treatment of type 1 Gaucher's disease. Additional studies using combinations of inhibitors,

such as ceranib-2, D609 and NB-DNJ, are likely needed to verify the therapeutic effects of accumulating ceramides. Importantly, C24 ceramide reduced allogeneic immune responses, supporting the therapeutic role of increasing ceramides in AR.

Ceramides are involved in cell death processes, including apoptosis and autophagy (Morad and Cabot, 2013). The ceramides C24 and C24:1 have been reported to mediate apoptosis in various cell types, including DCs and neutrophils (Kanto et al., 2001; Miguet et al., 2001; Seumois et al., 2007). However, fumonisin B_1 did not increase total CD11c$^+$ DCs or DC subsets in the drLN. These findings suggest that ceramides act as signaling molecules in T cells and DCs, rather than as an inducer of cell death. Furthermore, ceramide can be hydrolyzed by ceramidase to produce sphingosine which is further phosphorylated by sphingosine kinases to produce S1P. S1P exert a role as a signaling molecule through five different cell surface receptors. S1PR1 plays roles in T cell egression and the regulation of S1P gradients between lymphoid organs and circulatory fluids, while S1PR2 antagonizes migration towards chemokines (Baeyens et al., 2015). Recently, the role of S1PR2/3 axis in LT is revealed; hepatic ischemia-reperfusion injury triggers S1P-mediated NETosis which is a form of neutrophil cell death leading to the release of extracellular chromatin by promoting neutrophil extracellular traps (Hirao et al., 2023). Fingolimod (FTY720), an agonist at S1PR1, 3, 4 and 5 has also been suggested to prevent transplant rejection (Japtok and Kleuser, 2009).

CD8 T cells mediate murine cardiac graft rejection during interactions with DCs in secondary lymphoid tissues, such as the spleen and drLNs (Harper et al., 2015). We found that CD11c$^+$B220$^+$MHC II$^+$Ly6C$^-$ P3-like pDC subset was increased in drLN by fumonisin B_1 treatment *in vivo*, although the changes were not specifically related with AR. In moDC, Ly6C$^{-/+}$MHC II$^{-/+}$ P2 cells are considered immature and Ly6C$^-$ MHC II$^+$ P3 cells are fully differentiated (Majdoubi et al., 2019). In this study, P2-like pDCs were slightly reduced by fumonisin B_1, while P3-like pDCs increased. pDCs have been shown to contribute to inflammatory responses and to the proinflammatory activation of cytotoxic T cells and cDCs (Chistiakov et al., 2014). Treg could be induced by activated pDCs, that is correlated with low peptide-MHC II densities on APCs (Guery and Hugues, 2013). In our study, MHC II^{low} P2-like pDCs were reduced in allogeneic skin graft mice, while MHC II$^+$ P3-like pDCs were increased, implying less Treg differentiation might be induced by pDCs. pDC-driven induction of Treg correlates with spontaneous acceptance of kidney allograft (Oh et al., 2020). The functions of the subsets of pDCs await further investigation. Importantly, tDCs were significantly reduced by fumonisin B_1, supporting the role of tDCs in AR. Not only tDC population but CD86 expression was also downregulated on tDC by fumonisin B1 *in vitro*. CD86 is an activation marker of DC (Yoo and Ha, 2016). The roles of tDCs have been well described in organ transplantation (Marin et al., 2018). Donor-derived tDCs are shown to be safe and effective to suppress rejection in corneal and liver transplant patients (Hattori et al., 2012; Macedo et al., 2021), and autologous tDCs were evaluated in kidney transplant patients (Moreau et al., 2023). Thus, the changes in

DC subsets by reduced ceramide may boost antigen-specific immune responses in AR. It is interesting to know that ceramides are more abundant in the Treg than conventional T cells (Apostolidis et al., 2016). Although the cell population of Treg was not changed by fumonisin B_1, the functionality of Treg might be affected by reduced ceramide.

In conclusion, the present study showed that ceramides could be potential biomarkers or therapeutic targets for AR, but further studies awaits basic research on the underlying mechanisms as well as clinical research with larger cohort including more deceased donor-LT and non-Korean patients, as this study was conducted with ethnically Korean patients with a majority of living donor LT.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

AUTHOR CONTRIBUTIONS
H.J.Y., J.-J.K., S.H., and N.K. designed the research. H.J.Y., Y.Y., Y.K., S.J.K., P.H.T., T.K., M.K.K., J.H., and E.T. performed the experiments. Y.-I.Y., C.-S.A., G.-W.S., G.-C.P., S.-G.L., J.-J. K., D.-H.J., and S.H. collected samples. H.J.Y., Y.Y., Y.K., S.J.K., Y.-I.Y., P.H.T., T.K., M.K.K., J.H., and N.K. analyzed results. H.J.Y., J.-J.K., D.-H.J., S.H., and N.K. secured fundings. H.J.Y., Y.Y., Y.K., D.-H.J., S.H., and N.K. wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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