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Rice-derived glucosylceramides up-regulate HLA-DR expression on myeloid dendritic cells to activate innate immune responses in healthy Japanese subjects: A randomized, placebo-controlled, double-blind trial

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ABSTRACT

Introduction: In a prior study, we found that rice-derived glucosylceramides (GlCer) improved overall health and alleviated common cold symptoms, including nasal congestion, sore throat, coughing, headache, muscle pain, and diarrhea. One of the underlying mechanisms may involve the enhanced activation of antigen-presenting cells (APC) through Mincle, a c-type lectin receptor, and toll-like receptors. However, the clinical impact of GlCer on cell-mediated innate immunity, especially concerning APC, is not yet fully understood.

Objective: Therefore, we performed a clinical study on the effects of commercially available rice-GlCer (Oryza Ceramide®) on blood dendritic cells (DC).

Methods: The study was planned as a randomized, placebo-controlled, double-blind trial. Oryza Ceramide®-PCD (OC-PCD, 20 mg) tablets, each containing 0.6 mg of GlCer, were used as the active tablet. Twenty-two healthy Japanese individuals were enrolled and randomly divided to an active group (n=11) or placebo group (n=11 For 8 weeks,

participants took three active tablets (60 mg of OC-PCD) or three placebo tablets daily. DC ratios and CD expression on DC during the intervention were set as the primary outcomes.

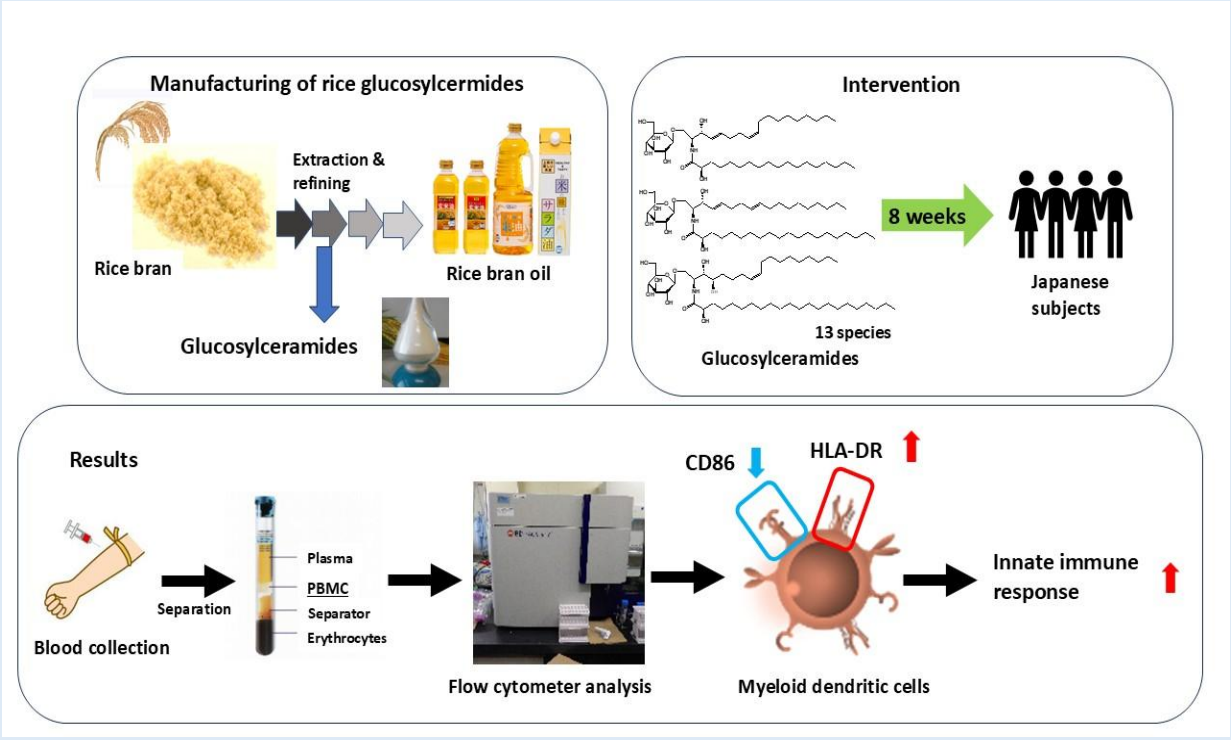
Results: All subjects finished the trial, and the per protocol set constituted 11 in each group. The myeloid DC (mDC) ratio and CD86 expression were significantly lower in the active group than in the placebo group. In contrast, HLA-DR expression on mDC was slightly higher in the active group. A stratified analysis of subjects with mean fluorescence intensity (MFI) for HLA-DR <60,000 revealed a significant high value in HLA-DR expression in the active group. A correlation was observed between changes in MFI values for HLA-DR and CD86 on mDC. However, plasmacytoid DC parameters did not change.

Novelty of the Study: Rice-derived GlCer was firstly clarified to enhance innate immune response specifically mDC to improve common cold symptoms through this clinical trial.

Conclusions: The oral administration of GlCer affected mDC by up-regulating the expression of HLA-DR and down-regulating CD86, which contribute to antigen presentation to T lymphocytes. The present results suggests that GlCer attenuate cold symptoms by enhancing the interaction between mDC and T lymphocytes.

Trial Registration: UMIN-CTR: UMIN000055299

Keywords: rice; glucosylceramide; HLA-DR; dendritic cells; CD86



Graphical Abstract: Rice-derived glucosylceramides up-regulate HLA-DR expression on myeloid dendritic cells to activate innate immune responses.

INTRODUCTION:

Food-derived glucosylceramides (GlCer) have been approved to claim benefits for epidermis hydration and barrier functions as functional foods in Japan under the Food with Functional Claims System [1,2]. Clinical findings on GlCer revealed the suppression of transepidermal water loss (TEWL), which refers to the epidermal barrier and moisturizing ability [3]. We previously confirmed that the intake of rice-derived GlCer inhibited TEWL [4]. The inhibition of TEWL by specific GlCer molecules has been linked to increased levels of filaggrin and corneodesmosin, which are proteins that promote water retention in the stratum corneum [5, 6]. Another health benefit of GlCer is immunomodulation, as β-GlCer binds to macrophage-inducible c-type lectin (Mincle) receptors, which recognize pathogens on the surface of antigen-presenting cells (APCs) and trigger immune responses similar to those against invading mycobacteria and fungi without inducing toxicity. After the recognition of pathogens, sensing signals trigger APCs to induce innate immunological responses, such as phagocytosis and cytokine production [7]. Mincle also detects sterols and β-GlCer provided from dead cell membranes [8], and mediates the formation of neutrophil extracellular traps, which capture pathogens in innate immune responses [9]. In addition to Mincle binding, β-GlCer was shown to modulate toll-like receptor (TLR)-4/lipopolysaccharide (LPS) responses without directly binding to TLR-4 on macrophages [10]. Thus, TLRs and c-type lectin receptors are crucial for pathogen-sensing. The lipid A portion of LPS has been suggested to share structural similarities with the cellular lipid ceramide [11].

However, a previous report demonstrated that ceramide does not attach to TLR-4; instead, lipopolysaccharide (LPS) appears to heighten immune reactions by increasing ceramide levels [12].

Regarding the immunological effects of rice-derived GlCer, we previously reported that the ingestion of rice-derived GlCer attenuated cold symptoms and reduced T lymphocyte counts in Japanese subjects [13]. In the Foods with Function Claims system in Japan, *Lactococcus* bacteria are approved to claim immunomodulatory effects that enhance innate immune responses through the activation of plasmacytoid dendritic cells (pDC) [14, 15]. Therefore, examining the effects on plasmacytoid and myeloid dendritic cells (pDC and mDC) is crucial to clarify the innate immunomodulatory activity of rice-derived GlCer. Hence, we investigated the influences of rice GlCer on blood DC responses.

MATERIALS AND METHODS

Subjects and grouping: All subjects were selected from employees of our company between August 23 and 30, 2024. Inclusion criteria were Japanese adults without any diseases (20 years or older). Exclusion criteria were described in our previous report [13].

Additionally, subjects who had received a COVID-19 vaccination within the previous 3 months were excluded. The 22 members selected were asked to keep the notice described in the previous report [13]. The subjects were also asked to refrain from brushing teeth after 8:00 am on the day of blood collection.

Test samples and allocation*:* As test preparations, white tablets including Oryza Ceramide® (OC)-PCD were prepared in our facility. An active tablet included 20 mg of OC-PCD (0.6 mg of GlCer), 45 mg of crystalline cellulose, 80.5 mg of hydrogenated maltose starch syrup, 2.25 mg of silicon dioxide, and 2.25 mg of calcium stearate. OC-PCD composed of 40% concentrated rice bran extract and 60% γ-cyclodextrin. Placebo tablets contained 20 mg of γ-cyclodextrin instead of 20 mg of OC-PCD. We packed active or placebo tablets in a container bag with a white or yellow mark on the package. Information linking the samples to the assigned groups

was rigorously protected until the study was completed. Test tablets were allocated by class randomization to equalize the allocation ratio (1:1). Allocation was performed according to the previously reported procedure [13].

Study protocol and outcomes: This randomized, placebo-controlled, double-blind, parallel-group study was performed at Oryza Oil & Fat Chemical Co., Ltd. The protocol was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN000055614). Subjects were randomly assigned to either a placebo group (11 adults) or OC group (11 adults), with the allocation being stratified. Subjects were instructed to take 3 tablets (OC or placebo) daily after breakfast for a period of eight weeks. The primary outcome was the activation markers (HLA-DR and CD86) of peripheral blood DC, including mDC and pDC. These parameters were examined using blood collected at baseline and after 1, 2, 4, and 8 weeks of the intervention.

Purification of peripheral blood mononuclear cells (PBMC): Blood was independently collected using 8-mL BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes - Sodium Heparin (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) on weeks 0, 1, 2, 4, and 8 of the intervention for analyses. The tubes were centrifuged at 1,800×*g* at room temperature within 2 hr of sampling. After centrifugation, the layer containing PBMC was collected. One mL of BD PharmLyse™ Lysing buffer (Becton, Dickinson and Company) was added, and hemolysis was induced by an incubation for 15 min. Additionally, the tubes were washed twice with 10 mL of phosphate-buffered saline [PBS(-)] (FUJIFILM Wako Pure Chemicals Co., Osaka, Japan). After centrifugation for 5 minutes at 300×*g*, the upper layer of the solution was discarded, and cell pellets were suspended in 0.5 mL of CELLBANKER 1 Plus (ZENOAQ, Fukushima, Japan). Cell pellets were then stored in liquid nitrogen until the flow cytometric analysis.

Flow cytometric analysis*:* PBMC were stained with a fluorescent dye conjugated to the following antibodies: human HLA-DR-Alexa488, CD86-PE, CD123-BB700, CD11c-RB780, neutropilin-1 (CD304, BDCA4)-Alexa647, CD3-APC-H7, CD14-APC-H7, and CD19-APC-H7 (Becton, Dickinson and Company). The blocking was conducted using Human BD Fc Block™ and BD MonoBlock Leukocyte Stain Buffer (Becton, Dickinson and Company) to avoid non-specific reactions between mononuclear cells and APC fluorescence. Cell viability was measured by 7-AAD staining (Becton, Dickinson and Company) (Figure 1).

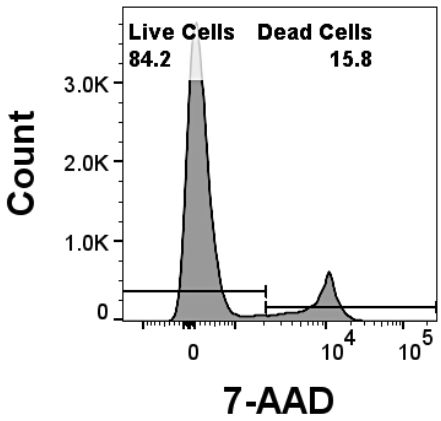


Figure 1. Counts of live or dead PBMC BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes were used to prepare PBMC. Dead cells were stained by 7-AAD. The survival ratio of PBMC was >80% and cells were used for DC gating.

The following reagents were utilized for the isotype control: mouse IgG1 κ-RB780, mouse IgG1 κ-APC-H7, mouse IgG2b κ-APC-H7, mouse IgG2b κ-Alexa647, and mouse IgG2b κ-RB780 (Becton, Dickinson and Company). Brilliant Stained Buffer (Becton, Dickinson and Company) was used as the staining buffer. After staining, cells underwent two washes with FACS buffer [5% fetal bovine serum and 2 mM EDTA in PBS(-)] prior to being suspended. The analysis was performed using a BD FACS-Lyric™ flow cytometer (Becton, Dickinson and Company). The resulting data were then subjected to a thorough analysis using FlowJo v10.10 software. The gating strategy of mDC and pDC is described in Figure 2.

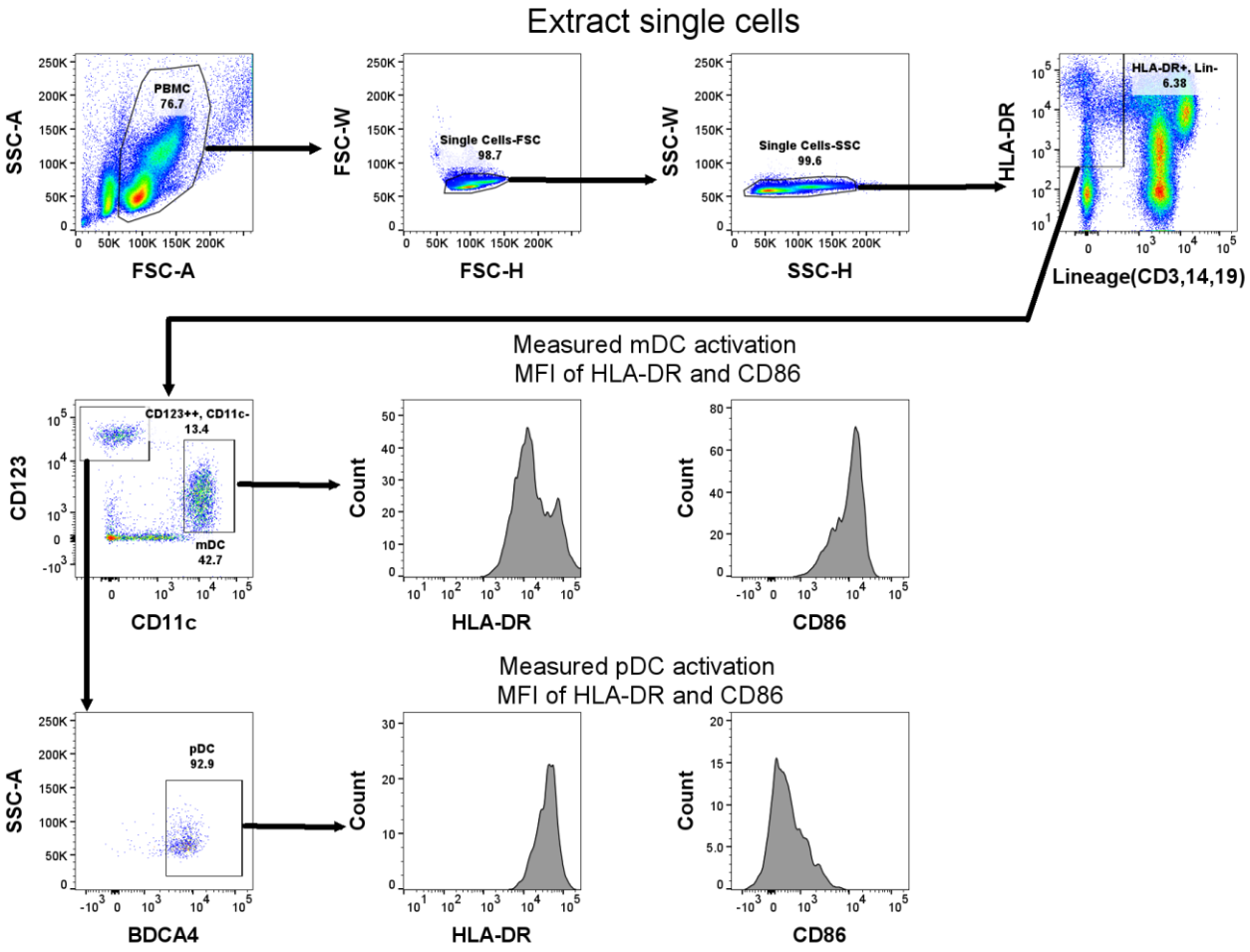


Figure 2. Gating strategies for mDC and pDC from PBMC.

mDCs were defined as cells with HLA-DR$^+$Lineage (CD3, 14, 19)$^-$CD11c$^+$ and mean fluorescence intensity values (MFI) for HLA-DR and CD86 were used as markers of activated mDC. pDCs were defined as being HLA-DR$^+$Lineage (CD3, 14, 19)$^-$CD123^{++}BDCA4$^+$ and the mean MFI of HLA-DR and CD86 were used as markers of activated pDC. In isotype control staining, a non-specific reaction was not observed between the fluorescent dye and cells (Figure 3). Therefore, the observed outcomes of this experiment indicated an antigen-antibody reaction.

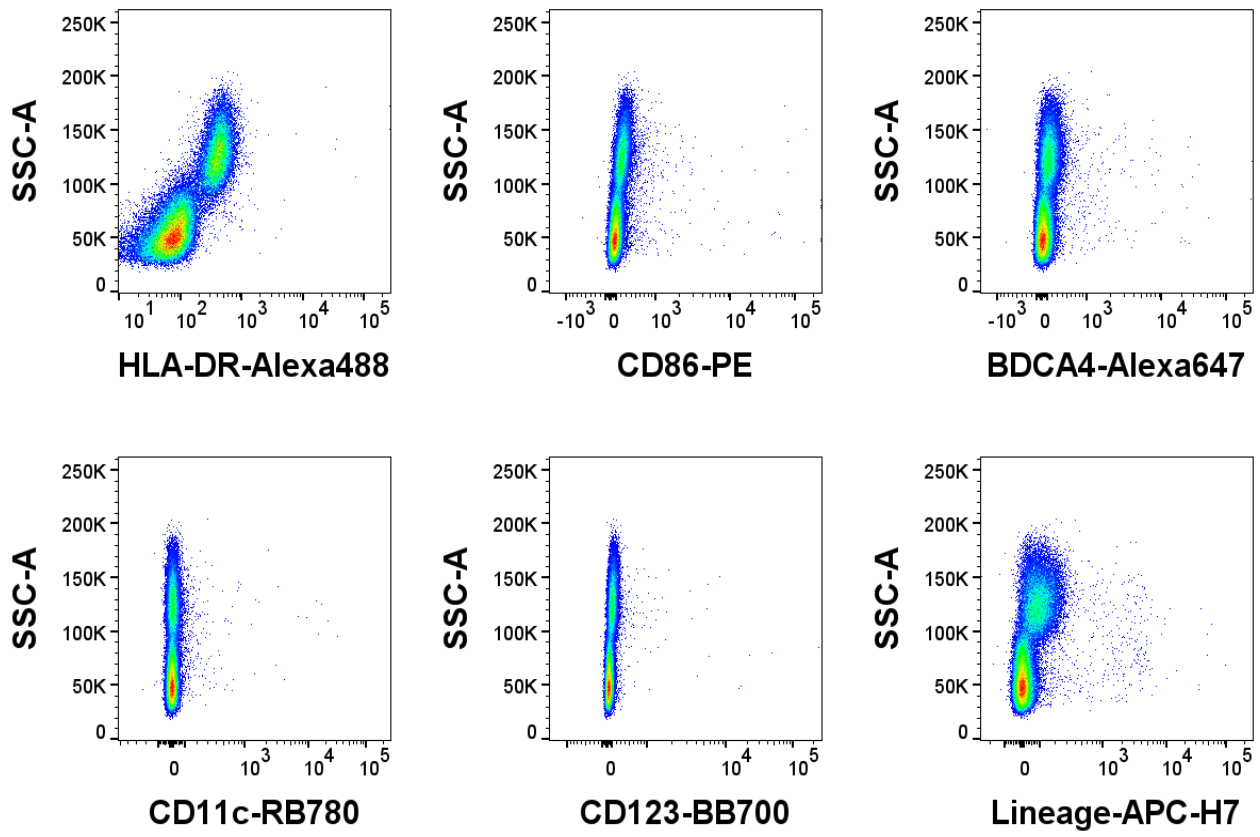


Figure 3. Reactivities of the isotype control of fluorescent dyes on PBMC. To assess the reactivities of fluorescent dyes for antibodies and PBMC, isotype control staining was performed. Since MFI was <1,000, no fluorescent dyes reacted to non-specific reactions. Therefore, cells with MFI >1,000 were regarded positive cells in subsequent experiments.

Ethics, adherence, and compliance: The present study complied with the Declaration of Helsinki (2013 revision) and conducted in conformity with ethical considerations as described in previous work [13]. The protocol was admitted by the Ethics Committee of Takara Clinic (Medical Corporation Seishinkai, Tokyo, Japan) on August 7, 2024 (Approved ID:2408-oryza-240725-TC). All participants accepted a full explanation about the protocol and purpose of the study before consenting of participation. All subjects were employees of Oryza Oil & Fat Chemical Co., Ltd.

Statistical analysis: Results are exhibited as the mean and SD. The unpaired *t*-test was used to compare the placebo and active groups. A probability value <5% was considered to indicate a significant difference (*: p <0.05, **: p <0.01).

RESULTS

Study performance: The present trial was conducted between September 24, 2024 and November 19, 2024. During the study period, no subjects in either group withdrew before completing the 8-week intervention (Figure 4). Therefore, all subjects were included in analyses. The average ages of subjects in the OC-PCD and placebo groups were 44.0±13.8 and 38.9±12.7 years, respectively.

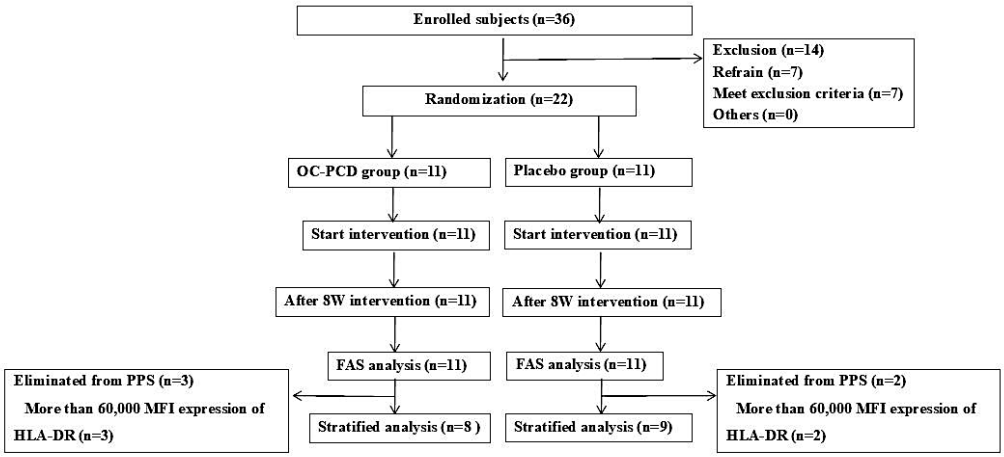


Figure 4. Flowchart of the clinical trial.

Blood DC parameters: Baseline mDC ratios in PMBC in the placebo and OC-PCD groups were 1.51±0.55% and 1.97±0.89%, respectively, while pDC ratios were 0.64±0.34% and 0.75±0.51%, respectively. After 1, 2, 4, and 8 weeks of the intervention, mDC ratios were significantly lower in the OC-PCD group than in the placebo group (Figure 5). Baseline MFI of HLA-DR on mDC in the placebo and OC-PCD groups were 53,785±12,800 and 50,607±14,324, respectively. Furthermore, the expression of HLA-DR was slightly higher in the OC-PDC group without significant difference than in the placebo group. On the other hand, after 1, 4, and 8 weeks of the intervention, CD86 expression on mDC was significantly reduced by OC-PDC. Baseline MFI of CD86 on mDC in the placebo and OC-PCD groups were 10,754±2,373 and 11,320±2,013, respectively. No significant differences were obserbed in pDC parameters between the groups.

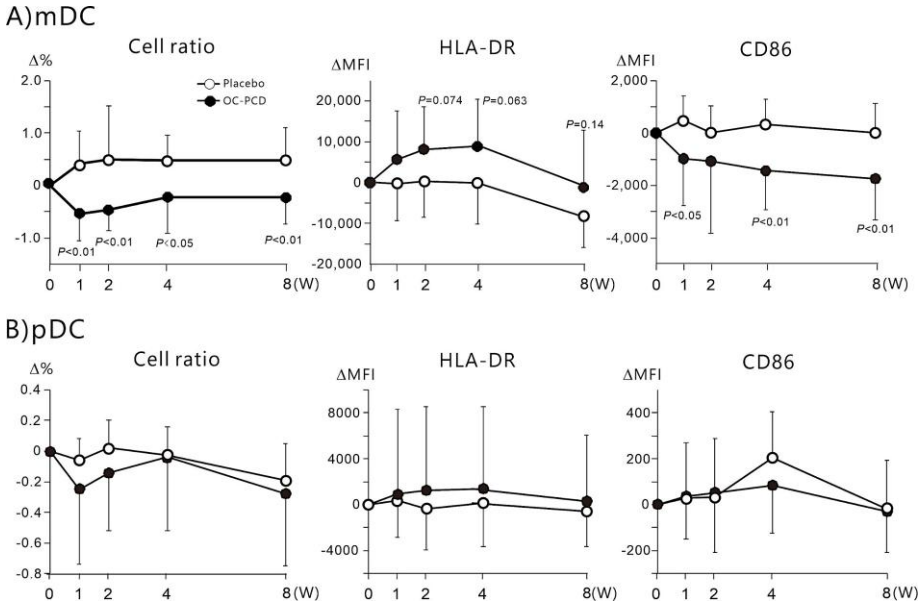


Figure 5. Effects of OC-PCD on mDC and pDC parameters in PMBC. A) The mDC ratio and expression of HLA-DR and CD86 in PBMC. B) The pDC ratio and expression of HLA-DR and CD86 in PBMC. Each point represents the mean and SD (n=11).

The mDC/pDC ratio was also calculated. The baseline numbers of mDC in the placebo and OC-PCD groups were 1687±578 and 2084±922 cells/1×10^5 PBMC, respectively. The baseline numbers of pDC in the placebo and OC-PCD groups were 639±330 and 750±506 cells/1×10^5 PBMC, respectively. As shown in Figure 6, the mDC/pDC ratio gradually increased in the placebo group, but was significantly lower in the OC-PCD group after 2 and 4 weeks of the intervention.

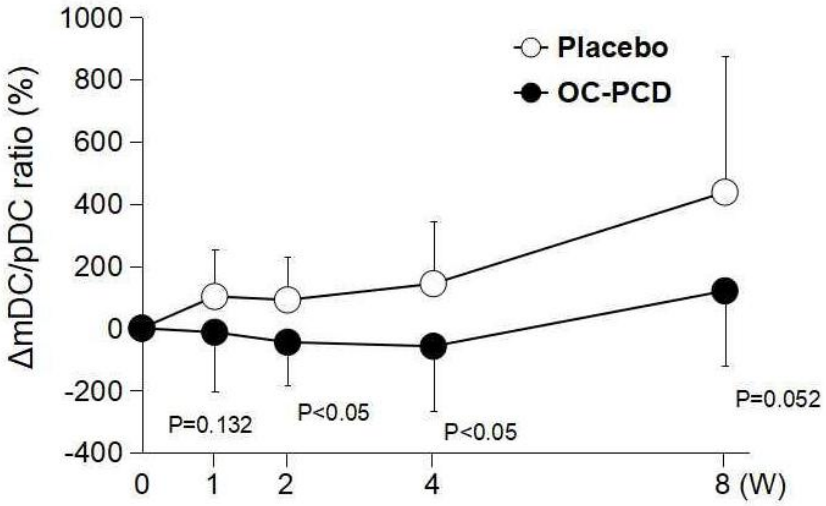


Figure 6. Changes in the mDC/pCD ratio during the intervention. Each point represents the mean with SD (n=11).

Since the expression of HLA-DR on mDC was slightly increased by OC-PCD, a stratified analysis was conducted of subjects with lower MFI of HLA-DR. Figure 7 shows the expression of HLA-DR on the mDC of subjects with MFI <60,000 as the baseline. HLA-DR expression was significantly higher in the OC-PCD group than in the placebo group after 2, 4, and 8 weeks of the intervention.

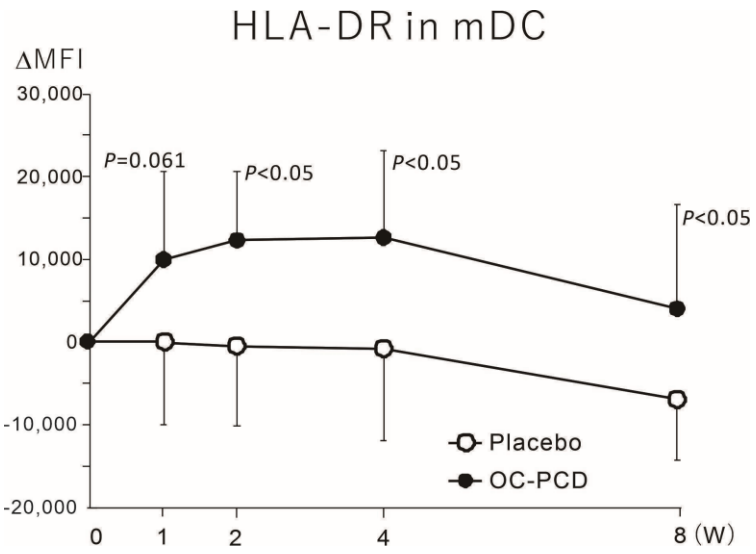


Figure 7. Stratified analysis of HLA-DR expression on mDC in subjects with the low expression of HLA-DR. Subjects with MFI of HLA-DR on mDC >60,000 as the baseline (n=5) were excluded from the analysis. Each point represents the mean and SD (n=8-9).

We examined the relationship between HLA-DR and CD86 expression on mDC in all subjects (Figure 8). A strong correlation (R^2=0.746) was detected between HLA-DR and CD86 expression after 2 weeks of the intervention (Figure 8). Correlations were also observed after 1 (R^2=0.541) and 4 weeks of the intervention (R^2=0.629). A weak correlation (R^2=0.323) was noted after 8 weeks of the intervention.

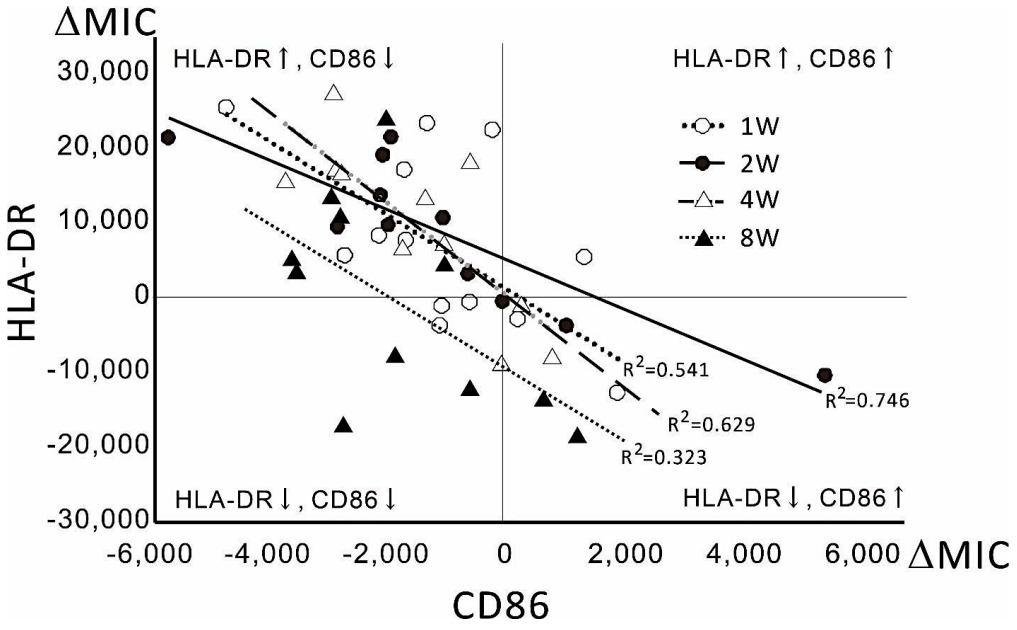


Figure 8. Correlations of changes in MFI of HLA-DR and CD86 expression on mDC in all subjects.

DISCUSSION

The present results revealed that rice-derived GlCer (OC-PCD) increased the HLA-DR expression and reduced that of CD86 on mDC. This is the first study to show that plant-derived GlCer enhanced the APC parameters that contribute to innate immune responses. HLA-DR plays a pivotal role in transmitting antigen information to T lymphocytes as a primary cell surface receptor. HLA-DR is also an MHC class II receptor encoded by the human leukocyte antigen complex and is mainly expressed on the surface of APC, including mDC [16]. In the transmission process of antigen information, HLA-DR binds to T-cell receptors (TCR) [17] and the T lymphocytes that accept antigen information contribute to acquired immune responses, such as cytokine production and cytotoxic responses [18]. In contrast to the increase observed in HLA-DR expression, CD86 expression on mDC was significantly inhibited by rice-derived GlCer. Regarding the negative and positive expression of CD, which is used to identify mDC, such as $CD1c^+$, $CD11c^+$, $CD14^-$, and $HLA\text{-}DR^+$ cells [19], the expression of CD86, CD83, CD80 and HLA-DR was previously shown to be up-regulated in the maturation process of mDC [20]. In addition, CD80 and CD86 were found to co-stimulate T lymphocytes though CD28 as a supportive transmitter of APC activation [21]. In the present study, we were unable to examine CD80 expression because of detection limitations by the flow cytometer's lasers. However, rice-derived GlCer suppressed the expression of CD86 significantly more than that of HLA-DR. Therefore, as one hypothesis, GlCer may promote the transmission of antigen signals mainly through the interaction between HLA-DR and TCR, which is a major route of T lymphocyte activation. Antigen-derived signals are transmitted by HLA-DR to TCR, while the secondary non-specific signals of APC are transmitted by co-stimulators, such as CD80, CD86, and CD83 [22]. A co-stimulatory signal of CD80 and CD86 [23] is regarded as a supportive signal to T lymphocytes. Therefore, rice-derived GlCer may stimulate a major signal transmission pathway in order to

transport antigen-specific information to T lymphocytes, which, in turn, trigger a stronger adaptive immune response. As a result of the up-regulated expression of HLA-DR, the co-stimulatory response by CD80 and CD86 appeared to be suppressed by GlCer. A previous study reported that some species of lactic acid bacteria activated both HLA-DR and CD80/86 [15], which means major and co-stimulative signals to activate T lymphocytes. Therefore, the mechanisms underlying DC activation by rice-derived GlCer may differ from those by lactic acid bacteria. However, reduction of co-stimulative signals is possible to induce immune tolerance [24]. Thus, further analysis of DC surface antigen referring cell status and phase is needed to evaluate actual DC condition such as fully mature and semi-matured [25]. Moreover, evaluation of monocyte activation by GlCer is also required as recently paramylon was newly admitted to indicate immunomodulative function in Japan by CD80 activation on monocytes [26].

In a clinical study, an 8-week intervention with rice-derived GlCer suppressed common cold symptom scores, such as nose congestion, sour throat, cough, cephalalgia, muscle ache, and diarrhea [13]. In addition, changes in blood T lymphocytes negatively correlated with cumulative days with cold symptoms. Therefore, rice-derived GlCer may prevent and suppress common cold symptoms through the activation of T lymphocytes, which indicates the enhancement of acquired immune responses by lymphocytes. On the other hand, this study showed that rice-derived GlCer potentiated the expression of HLA-DR on mDC, which plays a crucial role in the mDC-T-lymphocyte interaction for T lymphocytes-related acquired immune responses. Collectively, previous findings and the present results on rice-derived GlCer suggest that they promote the activation of T lymphocytes, which attenuate cold symptoms by interacting with mDC.

Dietary sphingolipids, including ceramides, have been theorized to interact with the host immune system [27]. Both *in vitro* and *in vivo* experimental results indicated that sphingolipids exerted antibacterial activities against Gram-positive and -negative morbific bacteria [28,29]. A previous study confirmed the preventive effects of sphingolipids against severe infectious diseases involving innate and immediate defense events in several epithelial tissues [30]. In the lipid-absorbing process of GlCer, ceramides are transported through lymph ducts. Orally ingested GlCer and Cer are metabolized to a sphingoid and incorporate into intestinal membranes, where they are then recomposed into ceramides and go into the lymph ducts [31]. This a reasonable and effective route that may directly affect immune cells, such as lymphocytes and APC.

As one of the mechanisms responsible for the activation of DC, GlCer were shown to bind to Mincle, a c-type lectin receptor [32]. Mincle senses dead cells, fungi, and mycobacteria and its activation not only triggers cytokine production and neutrophil filtration, but also induces acquired immunity, such as T-lymphocyte responses and antibody production. The hydrophobic site of a lipid structure, such as ceramide, and hydrophilic sites on the surface of mycobacteria bind to Mincle receptors [33]. The development of vaccine adjuvants and cancer treatments has been attempting based on this phenomenon [34 -35].

The reason for the reduction in mDC and mDC/pDC ratios following the GlCer intervention is not yet understood. The pDC ratio, but not the mDC ratio, in PMBC generally decreases with aging [36, 37]. On the other hand, mDC have been shown to increase in cases of infectious or inflammatory diseases, such as multiple sclerosis [38], malaria infection [39], and chronic obstructive pulmonary disease [40]. In the present study, the GlCer intervention significantly reduced the mDC ratio. As a naturally occurring compound exerting similar effects, silibinin derived from *Plasmodium malariae*, which exhibits immunomodulatory activity, decreased blood mDC in liver transplant patients [41]. Therefore, silibinin appears to reduce mDC with recovery from

inflammation and surgery. Similar to silibinin, GlCer may suppress inflammatory cold symptoms by decreasing mDC through the curing process. Based on these findings, decreases in mDC by rice-derived GlCer through their immunomodulatory activities appear to be beneficial for the ingredient of functional foods like GRAS certified processed foods [42].

Scientific Innovation and Practical Implications: The result suggests that GlCer ingestion provides novel immunomodulative way to prevent infectious disease by enhancing innate immune response instead of polysaccharides and lactic acid bacteria in the functional food sector.

CONCLUSION

In conclusion, rice-derived GlCer promoted the expression of HLA-DR on mDC, which transmits antigen information to T lymphocytes. In contrast, GlCer down-regulated the expression of CD86 and reduced the mDC ratio, which suggests supportive effects for immunomodulatory activity. These effects of rice-derived GlCer may contribute to the attenuation of cold symptoms and T lymphocye responses we previously reported.

List of abbreviations: GlCer, glucosylceramides; TEWL, transepidermal water loss; Mincle, macrophage-inducible c-type lectin; APC, antigen-presenting cell; TLR, toll-like receptor; LPS, lipopolysaccharide; pDC, plasmacytoid dendritic cell; mDC, myeloid DC; OC, Oryza Ceramide®, SD; standard deviation; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; MFI, mean fluorescence intensity; TCR, T-cell receptor;

Competing Interests: The authors declare no conflicts of interest associated with this manuscript.

Authors' contributions: The authors' contributions to this study were as follows: Miyasaka K. was responsible for Conceptualization and Methodology; Miyasaka K., Yoneda A., and Kubo M. contributed to Data curation and Formal analysis; Takeda S. and Shimoda H. provided Resources; Shimoda H. contributed to Investigation, Methodology, Project administration, Resources and Visualization; Shimoda H. was responsible for Investigation and Supervision; Shimoda H. handled Writing - Original draft preparation; Shimoda H. and Miyasaka K. contributed to Writing - Review and editing.

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