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Linarin down-regulates phagocytosis, pro-inflammatory cytokine production, and activation marker expression in RAW264.7 macrophages

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Abstract Plant-extracted flavonoid glycosides have been reported to be bioactive compounds with pleiotropic functions, including antioxidant, anti-inflammatory, and anti-cancer effects. This study investigated the anti-inflammatory role of linarin (acacetin-7-rutinoside, which is found in *Chrysanthemum indicum* (Gam-Guk) and *Dendranthema zawadskii* (Gu-Jul-Cho)), on lipopolysaccharide-stimulated RAW264.7 macrophages. Linarin treatments exhibited no cytotoxicity up to a concentration of 30 μM, as assessed by MTT assay. The production of nitric oxide, an inflammatory mediator, was decreased by addition of linarin. The secretion of pro-inflammatory cytokines, interleukin-1β and interleukin-6, was significantly decreased in a dose-dependent manner. Linarin also decreased the phagocytic ability of macrophages following co-culture with fluorescent beads. In addition, expression levels of antigen-presenting surface markers, MHC II and CD80, were suppressed by linarin. Taken together, these results indicate that the flavonoid glycoside linarin has an anti-inflammatory effect, in part through the suppression of phagocytosis, cytokine production, and antigen presentation in macrophages.

Keywords: flavonoid, inflammation, cytokine, phagocytosis, antigen-presentation

Introduction

Inflammation plays a role in the biodefense system against harmful stimulation such as pathogens and injured/damaged tissue. This defense system consists of closely related innate and adaptive immune responses (1). Among the various immune cells involved in this complex response, macrophages are unique in that they are not only the most typical innate immune cells, but are also adaptive immune-triggering mediators. Furthermore, diet-induced activation of macrophages has been shown to be relevant to the onset of metabolic diseases, including obesity, diabetes, and coronary-artery disease (2).

Macrophages are sub-categorized into two types, those using classical activation (M1) and those requiring alternative activation (M2), and both types have different functional phenotypes. Classically activated macrophages are stimulated by interferon-γ and lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria, and have been reported to respond as follows (3). Phagocytosis acts as a primary defense through engulfment and decomposition of foreign molecules. Following phagocytosis, macrophages release inflammatory mediators including nitric oxide and canonical inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which further trigger recruitment and/or activation of other immune cells. Following classical stimulation, macrophages were reported to express antigen-presenting proteins, major histocompatibility complex (MHC) II and cluster of diffrentiation (CD) 80, on their cell surfaces, thereby further stimulating antigen-specific adaptive immune cells such as T- and B-lymphocytes (4,5).

Linarin, a major flavonoid glycoside compound (acacetin-7-rutionoside, Fig. 1) extracted from *Chrysanthemum indicum* (Gam-Guk) and *Dendranthema zawadskii* (Gu-Jul-Cho), has been shown to have pleiotropic bioactive benefits (6,7). It has been demonstrated to possess anti-oxidant, anti-cancer, sleep-enhancing, and neuroprotective effects (8-10). However, the studies on anti-inflammatory properties of linarin are relatively poor to date. Martinez-Vazquez *et al.* (10) reported anti-inflammatory effects of linarin in a mouse model of edema. Considering that *in vivo* inflammation mobilizes multiple

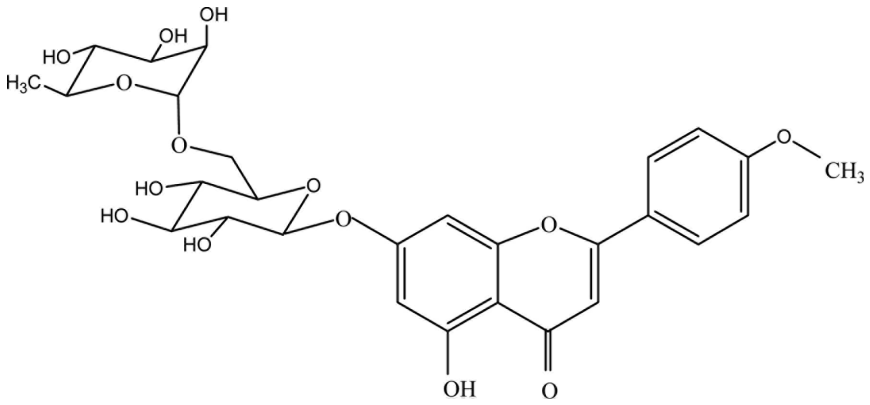

Fig. 1. The structure of linarin.

types of leukocytes, the cellular and/or molecular mechanisms by which linarin suppressed the inflammations was not described. Han *et al*. (12) previously reported that linarin down-regulated TNF-α production in LPS-stimulated RAW264.7 cells. In that study, TNF-α was indirectly quantified by using a TNF-α sensitive cell line, WEHI-164. The expression of activation markers, i.e., CD80 and CD86 on RAW264.7 cells was also demonstrated without quantification and subsequent statistical analysis. Taken together, the previous studies indicate a potent role of linarin as an anti-inflammatory phytochemical.

Therefore, the aim of this study was to seek the anti-inflammatory effects of linarin on LPS-stimulated RAW264.7 macrophage cells and to investigate the multiple physiological responses.

Materials and Methods

Reagents Linarin (purity 98%) was purchased from ChemFaces (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic antimycotic solution were purchased from Hyclone (Logan, UT, USA). LPS, phosphate buffered saline (PBS), N-(1-naphthyl)-ethylenediamine, phosphoric acid, and sodium nitrite were purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT was purchased from Amresco (Solon, OH, USA). Enzyme-linked immuno-sorbent assay (ELISA) kits for TNF-α, IL-1β, and IL-6 and anti-mouse CD16/CD32 purified, anti-mouse CD80-FITC, and anti-mouse MHC Class II (I-A/I-E)-APC were purchased from eBioscience (San Diego, CA, USA). Fluoresbrite® plain yellow green (YG) 1.0 micron microspheres (2.6% solids-latex) were purchased from Polysciences (Warrington, PA, USA). Dimethyl sulfoxide (DMSO) and ethyl alcohol anhydrous (99.9%) were purchased from Daejung Chemical Co. (Siheung, Korea).

Cell culture and treatment The mouse macrophage RAW264.7 cell line was purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in DMEM supplemented with 10% fetal FBS and 1% antibiotic antimycotic solution (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin, 25 μg/mL amphotericin B) at 37°C in a 5% CO_2 incubator. Cells ($2x10^5$ cells/mL) were pretreated for 24 h with linarin dissolved in ethanol and then stimulated by incubating with 500 ng/mL of LPS for 12 or 24 h.

Cytotoxicity assay The cytotoxicity of linarin in RAW264.7 cells was assessed by MTT assay (13). Briefly, cells ($2x10^5$ cells/ mL) were seeded, exposed to linarin (0, 5, 10, 20, or 30 μM) for 24 h, and then incubated with LPS (500 ng/mL) for 12 h or 24 h. After treatment with linarin, MTT solution (5 mg/mL) was added at 10 μL/well, and cells were incubated for 2-4 h at 37°C before culture supernatant was removed. MTT formazan was prepared in 100 μL of DMSO, followed by assessment of absorbance at 540 nm using a micro plate reader (Bio-Rad, Hercules, CA, USA) to determine the cytotoxicity of linarin.

Nitric oxide quantification Nitric oxide in cell culture media was measured using Griess reagent (13,14). Cells ($2x10^5$ cells/ìL) were cultured with linarin (0, 5, 10, 20, 30 μM) in a 24-well plate for 24 h and stimulated with LPS for 12 or 24 h. Then, the culture supernatant was harvested, and 50 μL of Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) was mixed with an equal volume of the supernatant. After 10 min, the nitric oxide concentration was measured by absorbance using a micro plate reader (Bio-Rad) at 540 nm. Sodium nitrate served as a standard.

Cytokine quantification Pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, in cell-culture supernatants were quantified using ELISA kits, following the manufacturer's instructions. Briefly, 100 μL of diluted capture antibody (Ab) was added to each well and incubated overnight at 4°C. Supernatant was removed and washed 3-5 times, repeating the process between each step. Next, 200 μL of assay diluent was added to each well and incubated for 1 h. Then, 100 μL of purified standard included in the ELISA kits and culture supernatant were added to each well and incubated for 2 h. Next, 100 μL of mixed detection Ab and streptavidin conjugated with horse radish peroxidase (SAv-HRP) was added to each well. After washing, 100 μL of substrate solution was added and incubated for 30 min in

the dark. Next, 50 μL of stop solution was added to each well. Finally, the cytokine concentrations of the samples were calculated using a standard curve on the basis of absorbance measured by a micro plate reader (Bio-Rad).

Assessment of phagocytic activity Phagocytosis of macrophages was assessed by co-culture of cells with fluorescent beads following the manufacturer's instructions. Briefly, cells ($5x10^5$ cells/mL) were pretreated with linarin for 24 h and then incubated with 10 μL of $1x10^9$ particles/mL of yellow green latex-beads (bead size, 1.0 μm) for an additional 1 h at 37°C. Thereafter, the cultured cells were collected and centrifuged at 300× *g* for 5 min. Cells were washed three times with cold PBS, followed by flow cytometric analysis (Accuri C6 Cytometer; BD Biosciences, San Jose, CA, USA). The results of phagocytic activity were expressed as mean fluorescent intensity (MFI).

Analysis of cell surface markers Cell surface antigens of LPS-stimulated RAW264.7 cells were assessed with fluorescence-conjugated monoclonal antibodies. Cells ($2x10^5$ cells/mL) were treated at the designated conditions. Cells were harvested and incubated with purified anti-mouse CD16/CD32 mAbs at 4°C for 15 min for blocking of non-specific binding of Abs to fragment crystalizable region (Fc) receptors. Cells were further incubated with fluorescence-conjugated monoclonal antibodies, anti-mouse CD80 conjugated with fluorescein isothiocyanate (CD80-FITC), or anti-mouse MHC II (I-A/I-E) conjugated with allophycocyanin (MHC II-APC), for 15 min at 4°C. Following incubation with specific antibody, cells were washed three times in cold PBS and centrifuged at 300× *g* for 5 min. The MFI was determined for the 10,000 cells of each sample using a BD Accuri C6 flow cytometer (BD Biosciences). Histograms were generated and analyzed by Flowjo V10 software (Treestar, San Carlos, CA, USA).

Statistical analysis Data were expressed as the mean±SEM. Different letters in a set of data indicate significant differences by one-way analysis of variance (ANOVA) followed by Tukey's multiple test using PRISM 5.0 (GraphPad Software, La Jolla, CA, USA) (n=5, p<0.05). ns; no significant difference.

Results and Discussion

Cytotoxicity of linarin on RAW 264.7 The cytotoxicity of linarin at the concentrations of 0, 5, 10, 20, and 30 μM, was assessed by MTT assay. Specifically, this method measures the absorbance of purple MTT formazan that was reduced by dehydrogenase of mitochondria in living cells. As shown in Fig. 2, with the activation of cells by LPS for 12 h, there was not a significant difference in cell viability with treatment of linarin up to 20 μM. Interestingly, cell viability was significantly reduced to 80% by addition of 30 μM linarin followed by LPS treatment for 12 h. In contrast, LPS-stimulation of cells for 24 h

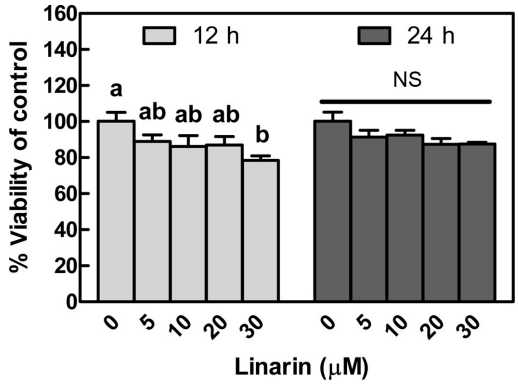

Fig. 2. Cytotoxicity assessment of linarin by MTT assay in RAW264.7 cells. RAW264.7 cells were incubated with linarin at various doses for 24 h, followed by LPS-stimulation for an additional 12 or 24 h.

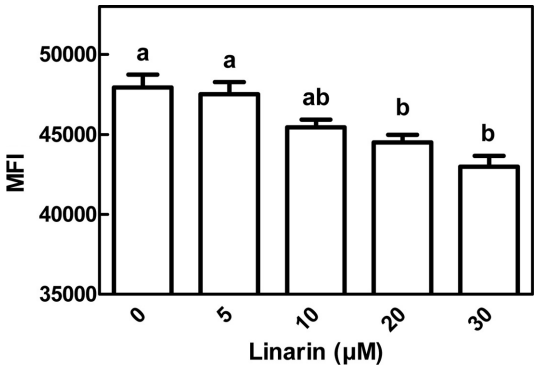

Fig. 3. Effect of linarin on phagocytosis of RAW264.7 cells as assessed by flow cytometric MFI. After pretreating cells with linarin (0, 5, 10, 20, or 30 μM) for 24 h, cells were co-cultured with fluorescent latex beads for an additional 1 h. MFI denotes mean fluorescent intensity.

exhibited no significant differences in cell viability. Indeed, cells treated with 30 μM linarin showed viability of ~90%. Considering that a rate of 80% viability of cells following treatments with natural compounds is generally considered non-cytotoxic, these results indicate that linarin had no strong influence on the viability of cells when used at concentrations less than 30 μM. This current observation is in accordance with a report by Han *et al*. (12), in which the toxicity of linarin in murine splenocytes was noted in the concentration range of 20-40 μg/mL.

Linarin inhibits the phagocytic activity of macrophages Phagocytosis, a "cellular chewing of foreign substances" by macrophages, is an initial step of innate immunity. Phagocytosis is initiated by interplay between macrophage surface receptors and ligands on the surfaces of particles (15). Phagocytic macrophages act via two immune responses. They mediate a death process through the digestion of pathogens via lysosomes, and they also make use of phagocytosis to direct antigens to MHC I and II (16). In order to assess the role of linarin in phagocytosis, FITC-conjugated latex beads were used as an antigen surrogate in this study. The phagocytic activities of the cells

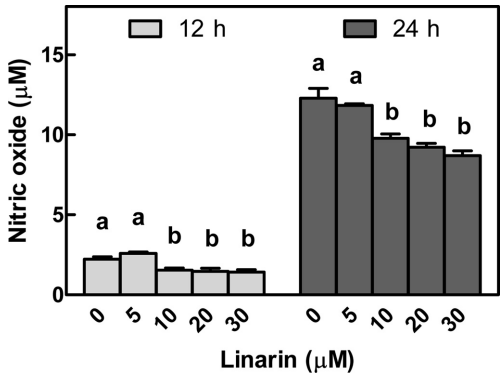

Fig. 4. Effect of linarin on nitric oxide production in LPS-induced RAW264.7 macrophages. RAW264.7 cells were incubated with linarin (0, 5, 10, 20, or 30 μM) for 24 h, followed by lipopolysaccharide stimulation for an additional 12 or 24 h. The amount of nitric oxide in culture media was quantified by Griess reagent.

following treatment with linarin at the indicated concentrations were measured using flow cytometric analysis and were expressed as MFI. As demonstrated in Fig. 3, linarin treatment resulted in a reduction in phagocytic ability of RAW264.7 cells in a dose-dependent manner, which insured the necessity of further functional studies in down-stream effector mediators, including production of nitric oxide and cytokines as well as antigen presentation. In this regard, the current observation is in accordance with a previous report that a flavonoid eriodictyol down-regulates the phagocytic properties of LPS-stimulated macrophages (17).

Linarin suppresses the production of nitric oxide in RAW264.7 cells

Nitric oxide is synthesized by nitric oxide synthase (NOS), which has three isoforms; neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (17). In macrophages, nitric oxide produced by inducible NOS (iNOS) of cells activated by LPS and/or IFN-γ plays a key role in the onset of the inflammation that is part of antimicrobial and cytotoxic effects (18). As presented in Fig. 4, the production of nitric oxide was decreased by linarin treatment at the concentrations of 10-30 μM, as determined by the addition of Griess reagent followed by colorimetric measurement. Excess linarin over 10 μM exhibited no additional effects, indicating that nitric oxide production was not dose-dependent. Similarly to the current observation, acacetin, an aglycon of linarin, was reported to down-regulate the expression of iNOS (19).

Effect of linarin on the secretion of pro-inflammatory cytokines

Cytokines have a variety of functions in the immune system including recruitment and activation of immune cells. Pro-inflammatory cytokines secreted by LPS-stimulated macrophages are reported to include IL-1β, IL-6, and TNF-α (20). IL-1β, IL-6, and TNF-α are typical inflammatory response regulation cytokines that have similar functions but also individual characteristics. IL-6 plays a role as a differentiation factor of T/B-lymphocytes for antibody production, while IL-1β has a high potential for inflammation by inducing PGE_2 production. TNF-α is known as an endotoxin-induced factor (21). Using commercially available ELISA kits, these three cytokines were quantified using the standards provided. As shown in Fig. 5, IL-6 (panel A) production by

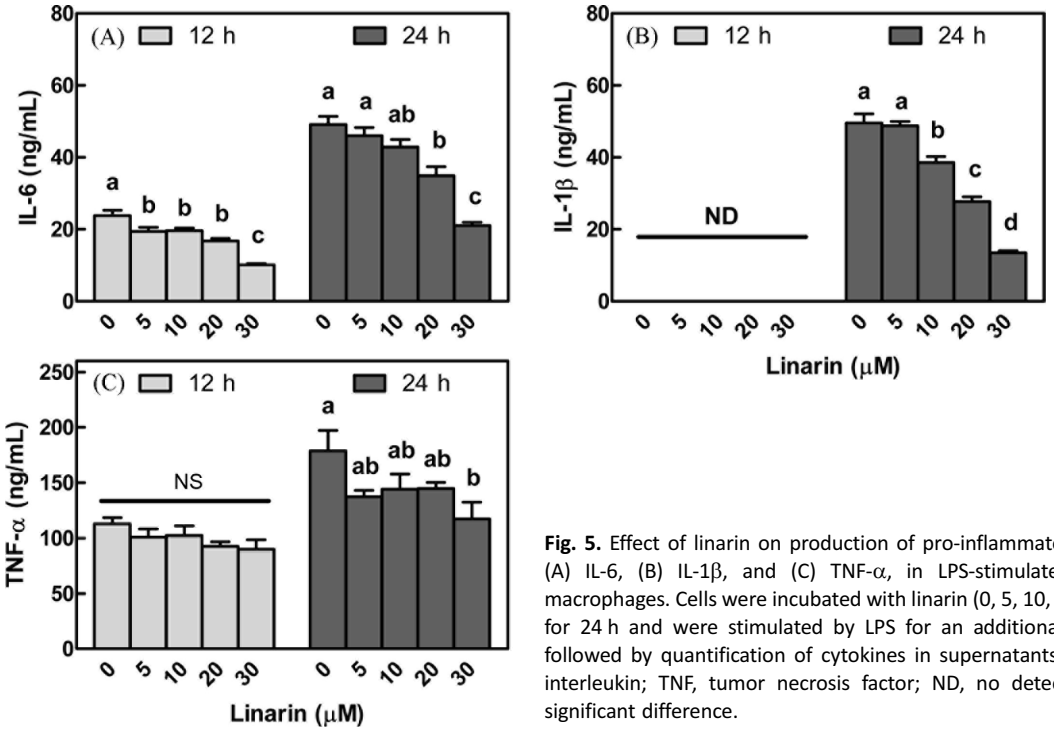

Fig. 5. Effect of linarin on production of pro-inflammatory cytokines, (A) IL-6, (B) IL-1β, and (C) TNF-α, in LPS-stimulated RAW264.7 macrophages. Cells were incubated with linarin (0, 5, 10, 20, or 30 μM) for 24 h and were stimulated by LPS for an additional 12 or 24 h, followed by quantification of cytokines in supernatants by ELISA. IL, interleukin; TNF, tumor necrosis factor; ND, no detection; NS, no significant difference.

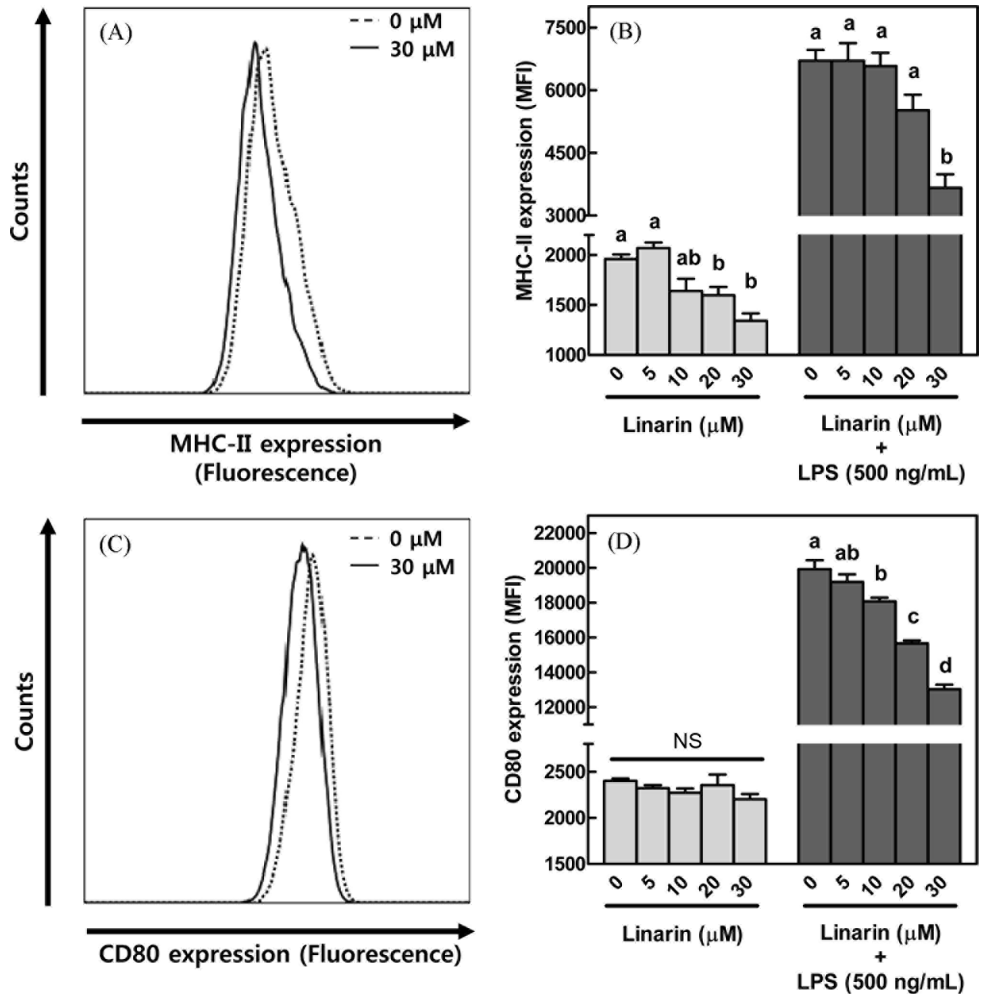

Fig. 6. Effect of linarin on the expression of activation markers in LPS-stimulated RAW264.7 macrophages. Cells stained with specific antibodies to major histocompatibility complex (MHC) II or cluster of diffrentiation (CD) 80 were analyzed by flow cytometry. (A and C) The representative histograms of MHC II (A) and CD80 (C) expression in linarin-treated (30 μM, black) cells vs. control (0 μM , dotted). (B and D) The statistical analyses of MHC II (B) and CD80 (D) expression. The expression levels of specific surface molecules are presented in terms mean fluorescent intensity (MFI).

RAW264.7 cells was significantly suppressed by the addition of linarin in a dose-dependent manner. IL-1β (panel B) production was not observed following 12 h of stimulation. However, IL-1β secretion into the supernatant following 24 h of activation was remarkably decreased as the linarin concentration was increased. As for the effect of linarin on TNF-α production (panel C), there were no significant effects on LPS-stimulated cells over 12 h. However, the production of TNF-α was shown to be reduced by addition of linarin. In this regard, Han *et al*. (12) previously reported that supernatant from RAW264.7 cells cultured with linarin suppressed the growth of TNF-α sensitive cell line, indirectly indicating that linarin might affect the down-regulation of TNF-α production. The current study confirmed the hypothesis by direct measurement of TNF-α using ELISA. Taken together, these data indicate that linarin has potent effects on pro-inflammatory cytokine production machinery in macrophages. Molecular studies will be required to further investigate these effects.

Linarin modulates the expression of MHC II and CD80 in LPS-induced RAW264.7 Macrophages are unique in that they are categorized as innate immune cells by their lineage but also serve as professional antigen-presenting cells that further trigger adaptive immune cells, specifically T/B-lymphocytes (22,23). In this antigen-presentation, activated macrophages up-regulate the expression of surface proteins, MHC II and CD80. MHC II is a surface receptor that captures peptides of foreign and self proteins and presents them to the receptors of T lymphocytes (24). CD80 is a member of the B7 family, which also includes CD86 (25), which presents antigen by interacting with CD28 on the surfaces of T lymphocytes, resulting in T lymphocyte activation and differentiation (26). Figure 6A is a representative histogram of linarin-treated (dotted) and control (black) macrophages. By the measurement of MFIs of cells under various linarin treatments, the expression level of MHC II was determined to decrease in a dose-dependent manner following the addition of linarin (Fig. 6B). Likewise, expression of CD80 was

assessed as presented in Fig. 6C. Linarin also exhibited a reduction in CD80 expression in a dose-dependent manner, as depicted in Fig. 6D. Han *et al.* (12) previously attempted to assess the suppressive effects of linarin on costimulatory molecules on RAW264.7 cells, though the quantitative analysis of the surface expression was not clearly demonstrated. The current study took the advantage of well-developed fluorescence quantification methods to statistically analyze the suppressive effects of linarin. These data imply that linarin suppresses the expression of specific proteins on the surfaces of macrophages, as well as the dependent antigen-presentation, though more detailed studies are required for verification of this finding.

This study investigated the anti-inflammatory effects of linarin on LPS-stimulated RAW264.7 murine macrophages. Linarin had a dramatic inhibitory effect on inflammatory responses without cytotoxicity when used at concentrations up to 30 μM. Specifically, phagocytosis, expression of activation markers, and production of nitric oxide, IL-6, IL-1β, and TNF-α were dramatically regulated by linarin in a dose-dependent manner. To date, mechanistic studies of the anti-inflammatory role played by linarin are lacking, though many *in vivo* studies have tentatively indicated a use for linarin in treatment/prevention of inflammation, such as in mouse ear edema (27). Recently, acacetin, which contains a methoxy group on the B ring, was reported to be a stronger inhibitor of inflammation in LPS-induced macrophages (19). In accordance with this finding, the current study reports pleiotropic effects of linarin on macrophages though dissection of the mechanisms of these effects are still required.

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Disclosure The authors declare no conflict of interest.

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