

NK cell-mediated immunostimulatory effects of ethanol extract of *Morinda citrifolia* (noni) fruit

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Abstract

Background

Morinda citrifolia (Noni) is a plant that has long been used in various products such as foods and cosmetics. Although noni has been known to have immunostimulatory activity, detailed mechanism at the cellular level has not been fully elucidated yet. In this study, we focused on understanding as to how noni fruit can positively stimulate body's immune responses.

Methods

To do this, an ethanol extract of noni fruit (Mc-fEE) was prepared and administered for 30 days to male C57BL/6 mice for *in vivo* experiment. NK cell activity and cytokine production level were analyzed by flowcytometry, real-time PCR, and ELISA. Mc-fEE-triggered molecular events were detected from RAW264.7 cells and splenocytes using Western blotting and real-time PCR analyses.

Results

The mRNA expression levels of cytokines such as interleukin families, IFN- β , and TNF- α , were increased when cells were treated with Mc-fEE (0-200 μ g/ml) *in vitro*. Western blotting analysis showed that these increases occurred when immune response stimulating pathways were activated. In addition, flow cytometry was performed using spleens of mice orally treated with Mc-fEE (0-200 mg/kg) *in vivo*. Mc-fEE increased the NK cells population and the NK cells from the spleens were cytotoxic to YAC-1 cells, a T cell lymphoma. This was confirmed through LDH assay; several cytokines including IL-1 β , IFN- γ , and TNF- α were secreted *in vivo*.

Conclusion

In this paper, we confirmed that Mc-fEE has immunostimulatory effects by activation and increase of the NK cell population.

Background

The immune system protects against various diseases [1]. The immune system includes multiple proteins, cells, tissues, and organs and reacts to invading pathogens. Immune responses are either innate or adaptive [2]. The innate immune system is the first line of defense against external stimuli, acting in a non-specific manner. Innate immunity refers to a system that recognizes specific nonself-pathogen-associated molecular patterns (PAMPs) through indicated pattern-recognition receptors (PRRs). This leads to induction of downstream signaling pathways such as interferon regulatory factor (IRF), activator protein (AP)-1, or nuclear factor-kappa B (NF- κ B) pathways that ultimately regulate anti-pathogen

responses [3]. PAMPs are essential for the survival of microorganisms and are conserved highly for each species [4]. Examples of well-known PAMPs include lipopolysaccharide (LPS), a main constituent of the outer membrane of gram-negative bacteria, and Poly(I:C). LPS interacts with PRR toll-like-receptor 4 (TLR4), and Poly(I:C) interacts with TLR3 [4].

The innate immune system includes epithelial barriers; phagocytes, including macrophages and dendritic cells; and complement cascades [5]. Phagocytes mediate phagocytosis, plasma membrane-mediated engulfment of large particles; and professional phagocytes, mainly neutrophils and macrophages, remove pathogens or cell debris. The complement cascade proceeds through several steps [6]. Briefly, the sequence consists of opsonization, chemotaxis, cell lysis, and agglutination. Infected or damaged cells that require phagocytosis are tagged with opsonin-like antibodies (opsonization). Macrophages accumulate at the site of these cells through chemical signals (chemotaxis), and membrane attack complexes (MACs) are formed on the cell surface, resulting in cell lysis. Then, agglutination, the conglomeration of infected cells for easier antibody-mediated attack, occurs [7]. Macrophages may participate in antitumour activity due to the production of effector molecules such as NO, TNF- α and IL-1 β [8]. These macrophage-derived mediators have been recognized for their cytotoxic activities against cancer cells [9].

Natural killer (NK) cells are immune cells involved in innate immunity and possess various receptors that recognize abnormal cells, including infected cells or tumor cells [10]. Most immune cells must detect major histocompatibility complex (MHC) classes on the surfaces of infected cells before initiating an immune response, but NK cells have the unique ability to recognize and kill stressed cells in the absence of antibodies and MHC [11]. The interaction between macrophages and NK cells is a critical initial line of defense against pathogens including viruses, bacteria, parasites, and fungi. In order to fight infections, NK cells must be recruited and activated to release cytokines and exert cytotoxicity at the sites of inflammation. Activation via soluble mediators such as IL-12 and IL-18, and stimulation by direct cell-to-cell contact, are the most common ways for macrophages to prime NK cells [9, 12, 13].

When antigens are not eliminated completely by the innate immune response, a more specific process, the adaptive immune response, is induced [14]. The adaptive immune system, also known as the acquired immune system, kills pathogens highly specifically using specialized systemic cells that prevent pathogen growth. This is in contrast to innate immunity in which pathogens are killed extensively and non-specifically [15]. Adaptive immune system cells recognize antigens presented by antigen-presenting cells (APCs) such as B cells, dendritic cells, and macrophages. Signals are transmitted among immune cells, resulting in secretion of various cytokines, including tumor necrosis factor (TNF), interleukin (IL) family members, and interferon (IFN) family members [16]. The development of immunological memory, in which pathogens are remembered via memory B cells and memory T cells, is another key feature of adaptive immunity [17].

Adaptive immune responses also can be regulated by NK cells. Released cytokines such as TNF- α and IFN- γ from NK cells induce differentiation of CD4 + T cells to Th1 cells and maturation of APC cells.

Mature DCs induce the release of interleukins such as IL-12, which activates CD8 + T cells [18] [19].

Morinda citrifolia is a plant species in the genus *Moringa*, native to Southeast Asia including China and Australasia [20, 21]. This plant has been used in foods, cosmetics, and traditional medicines for its anti-fungal, anti-oxidative, or stamina-boosting effects [22, 23]. In particular, immunomodulatory activities of noni-derived components such as polysaccharide-rich substance from the fruit juice have been published by demonstrating their anti-cancer activities [24, 25], modulating the immune system via activating cannabinoid 2 receptors, and promoting the production of IFN- γ [26]. In addition, most of the studies have described the immunomodulatory activities under immunosuppression conditions [13, 27–29]. Different studies have demonstrated in the metabolic and excretory organs [30], as well as the effect on the adaptive immune system, managed by B and T lymphocytes [31, 32]. However, detailed mechanism as to how this fruit can stimulate immune system under normal healthy conditions is not yet fully addressed. Therefore, in this paper, we focused on exploring immunoregulatory role of an ethanol extract of fruit of noni (*Morinda citrifolia*) (Mc-fEE) in the innate immune system under normal and healthy conditions by testing the activation levels of macrophage and NK cells, and understanding the molecular mechanisms of these effects using healthy mice, primary splenocytes, RAW264.7 cells, a murine macrophage-like cells, and Yac-1 cells, a mouse T cell lymphoma and isolated primary NK cells.

Methods

Materials and antibodies

Mc-fEE (also called Nonitri[®]) and β -glucan were provided by COSMAXNBT Inc. (Korea). RAW264.7 cells (ATCC number TIB-71) were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and YAC-1 (KCLB number 40160) cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Roswell Park Memorial Institute (RPMI) 1640 media, antibiotics (penicillin-streptomycin solution), and phosphate buffered saline (PBS) were purchased from Hyclone (Logan, UT, USA). Stain buffer (FBS) and specific antibodies [FITC CD3, PE CD4, PE-Cy[™]7 CD8, FITC CD11c, PE CD335 (NKp46), PE-Cy[™]7 F4/80] were purchased from BD Biosciences (Sparks, MD, USA). NK Cell Isolation Kit (mouse), buffer (0.5% BSA, 2mM EDTA in PBS, pH 7.2), and autoMACS[®] Rinsing Solution (2 mM EDTA in PBS, pH 7.2) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Antibodies for p65, p50, c-Jun, c-Fos and their phosphor-specific antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for β -actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). TNF- α ELISA kit (Quantikine[™] ELISA, Cat No. : MTA00B) was purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of Mc-fEE and High-performance liquid chromatography (HPLC) analysis

Mc-fEE was provided from COSMAX NBT Inc. (Korea). Briefly, dried noni fruits were extracted with 10% ethanol at 80°C for 4 hours. Noni filtrates were evaporated, and the final yield of Mc-fEE was 20% (w/w).

During the *in vitro* studies, the Mc-fEE stock solution was made by dissolving Mc-fEE stock with DMSO at a concentration of 100 mg/ml. When each experiment was performed, the stock solution was diluted to the desired final concentration of 0, 50 and 200 µg/ml using the suitable culture medium. The Mc-fEE dissolved well in DMSO and was sterilized by heating at 85°C for 20 minutes.

For standardization of this extract, high-performance liquid chromatography (HPLC) analysis was utilized to determine phytochemical profile of Mc-fEE with a standard compound, deacetylasperulosidic acid.

Cell culture

RAW264.7 cells and YAC-1 cells were cultured in RPMI 1640 media with 10% inactivated FBS, glutamine, and 1% antibiotics at 37°C under 5% CO₂.

Animals

Male C57BL/6 mice (6–8 weeks old, 18–20 g) were purchased from OrientBio (Sungnam, Korea). Mice were caged in groups of 5 under a 12-hour light and dark cycle and fed a pelleted diet and tap water *ad libitum*. For *in vivo* experiments, mice were administered vehicle or Mc-fEE (50 mg/kg and 200 mg/kg) orally once a day for 30 days. All animal studies were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Sungkyunkwan University. (Suwon, Korea; Approval ID: SKKUIACUC2021-01-62-1)

Cell viability test

The cell viability test was analyzed by a MTT assay as previously described [33]. RAW264.7 cells were seeded at a concentration of 1×10^6 cells/ml per well in a 96-well-plate and incubated overnight. After incubation, Mc-fEE (0-200 µg/ml) was applied at a concentration 4 times higher than the final concentration, and cells were incubated for 24 hours. Next, 100 µl of supernatant of each well was removed, and 10 µl of MTT solution (10 mg/ml in PBS, pH 7.4) was added for 3 hours at 37°C under 5% CO₂. After incubation, 100 µl of MTT stop solution (15% sodium dodecyl sulphate) was added, and the plate was incubated overnight. The absorbance was measured at 570 nm using a multi-reader Spectramax 250 (BioTex, Bad Friedrichshall, Germany), and the group values were normalized.

Nitric oxide assay

RAW264.7 cells were seeded in a 96-well-plate at a concentration of 1×10^6 cells/ml; after 24 hours, cells were treated with or without polymyxin B (40 µg/ml). After incubation for 30 minutes, Mc-fEE (0-200 µg/ml) or LPS (1 µg/ml) applied to each group. Cells were incubated for 24 hours, and 100 µl of supernatant was isolated to new 96-well-plates. Obtained supernatants were mixed with 100 µl of Griess reagent, and the absorbance was measured at 540 nm using a multi-reader Spectramax 250 (BioTex, Bad Friedrichshall, Germany).

Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells were plated in a 96-well-plate at a concentration of 1×10^6 cells/ml; after 24 hours, cells were treated with Mc-fEE (0-200 $\mu\text{g/ml}$) for an additional 24 hours. Next, 100 μl of supernatant was used to measure the amount of TNF- α using ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

mRNA analysis using quantitative real-time Polymerase Chain Reaction (PCR)

RAW264.7 cells were treated with Mc-fEE (0-200 $\mu\text{g/ml}$) and harvested after 24 hours. Mice were dosed orally with Mc-fEE (0-200 mg/kg), and spleens were isolated from the mice. Total RNA from cell lysates and splenocytes was acquired using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from total RNA (1 μg) as previously reported [34]. The mRNA expression levels of IFN- β , TNF- α , IFN- γ , and interleukins (6, 10, 1 β , 12b) were obtained using quantitative real-time PCR with SYBR Premix Ex Taq (Takara, Japan) on a thermal cycler (Bio-Rad, USA). The gene expression results were normalized as the ratio of optimal density relative to GAPDH. The primers used in this experiment are listed in Table 1.

Table 1
Primer sequences used in a quantitative real-time PCR.

Gene	Direction	Sequences (5' to 3')
IL-6	Forward	GAC AAA GCC AGA GTC CTT CAG AGA
	Reverse	CTA GGT TTG CCG AGT AGA TCT C
IFN- β	Forward	AAG AGT TAC ACT GCC TTT GCC ATC
	Reverse	CAC TGT CTG CTG GTG GAG TTC ATC
TNF- α	Forward	TGC CTA TGT CTC AGC CTC TT
	Reverse	GAG GCC ATT TGG GAA CTT CT
IL-1 β	Forward	GTG AAA TGC CAC CTT TTG ACA GTG
	Reverse	CCT GCC TGA AGC TCT TGT TG
IL-12b	Forward	TGG AGC ACT CCC CAT TCC TA
	Reverse	GAG CTT GCA CGC AGA CAT TC
IFN- γ	Forward	TGG CTG TTT CTG GCT GTT ACT
	Reverse	GTT GCT GAT GGC CTG ATT GTC
GAPDH	Forward	CAC TCA CGG CAA ATT CAA CGG CAC
	Reverse	GAC TCC ACG ACA TAC TCA GCA C

Western blotting analysis

RAW264.7 cells (2.5×10^6 cells/ml) were seeded in 3-cm plates and incubated overnight at 37°C under 5% CO₂. After incubation, cells were treated with either Mc-fEE (200 µg/ml) or β-glucan (50 µg/ml) for the indicated time (0–60 min). Cells were isolated with cold PBS and prepared for western blotting analysis as previously described [35]. Proteins were analyzed using specific antibodies diluted in 3% BSA buffer (1:2500) and detected using an ECL reagent.

Flow cytometry analysis

Male C57BL/6 mice (15 mice per group) were injected with Mc-fEE (0–200 mg/kg) once a day for 30 days. Spleens were isolated and ground with RPMI 1640 media. Ground splenocytes were suspended in stain buffer (1% BSA, 0.1% sodium azide). The cell suspension was stained with the specific antibodies for 40 minutes at 4°C, and the fluorescence was detected using a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

NK cell isolation and LDH cytotoxicity assay

Male C57BL/6 mice (15 mice per group) were injected with Mc-fEE (0–200 mg/kg) once a day for 30 days. Total spleens were obtained and ground with RPMI 1640 media. Ground splenocytes, 3×10^7 cells, were aliquoted into e-tubes and centrifuged at 300 g for 10 minutes. The supernatants were removed, and the cells were suspended in 120 µl of buffer (0.5% BSA, 2mM EDTA in PBS, pH 7.2). Then, 30 µl of NK Cell Biotin-Antibody Cocktail was added to each e-tube, and cells were pipetted to be resuspended. After incubation using a rotator at 4°C for 5 minutes, 1 ml of buffer was added to each e-tube, the tube was centrifuged at 300 g for 10 minutes, and the supernatants were removed. After adding 240 µl of buffer, 60 µl of Anti-Biotin MicroBeads was added and incubated for 10 minutes at 4°C using a rotator. LS columns were inserted into the MACS Separator and washed with 2 ml of autoMACS® rinsing solution (2mM EDTA in PBS, pH 7.2). Buffer was added so that the volume of the cell suspension was at least 500 µl, and NK cells were obtained through column flow. After centrifuging at 3000 rpm for 5 minutes, the obtained NK cells were suspended in RPMI 1640 media and plated at 10^5 cells/100 µl and 5×10^4 cells/100 µl in round bottom 96-well-plates. YAC-1 cells were co-cultured with NK cells at a concentration of 10^4 cells/100 µl. The cells were incubated at 37°C under 5% CO₂ for 6 hours. The round bottom 96-well-plates were centrifuged at 250 g at room temperature for 10 minutes, and the supernatants were transferred to a new 96-well-plate. Next, 100 µl of reaction solution (catalyst : dye solution = 1 : 45) was added to each well and incubated at room temperature for 5–15 minutes while blocking light. After that, 50 µl of 1 N HCl was added to stop the reaction, and absorbance was measured at 490 nm.

$$\text{Cytotoxicity (\%)} = \frac{A - \text{lowcontrol}}{\text{highcontrol} - \text{lowcontrol}} \times 100$$

A: [effector – target cell mix] – [effector cell control]

high control: target cells + Triton X-100

low control: only target cells

Statistical analysis

For statistical comparisons, a Student's *t*-test was used to determine the statistical significances of the difference between values for the multiple experimental and control groups. Data were expressed as the mean $\pm$ standard error, and the results were obtained from at least three independent experiments of 5 samples (enzyme assay), 5 samples (*in vitro* experiments), and 15 mice per group (*in vivo* experiments). A *p*-value < 0.05 was considered statistically significant.

Results

Mc-fEE shows immunostimulatory effects *in vitro*

To check the immunostimulatory effects *in vitro*, we first conducted multiple experiments with RAW264.7 cells. First, we treated RAW264.7 cells with Mc-fEE for 24 hours at concentrations of 50 μ g/ml and 200 μ g/ml to test cytotoxicity of this extract using a MTT assay (Fig. 1a). The result shows that there was no interference of cell viability up to 5% upon treatment with Mc-fEE in RAW264.7 cells. Next, nitric oxide (NO) level was not increased by Mc-fEE at concentrations of 50 and 200 μ g/ml. This was determined by comparing Mc-fEE-treated cell responses to those of LPS-treated ones. In contrary, LPS significantly upregulated the NO level. In addition, after treatment with Mc-fEE, the response to polymyxin B treatment was assessed. Upon treatment with Mc-fEE at concentrations of 50 and 200 μ g/ml and polymyxin B, the findings showed that polymyxin B did not significantly effect NO production of Mc-fEE, on the other hand, significantly inhibited the positive LPS control. The results of this NO assay suggest the absence of endotoxin (Fig. 1b). Additionally, we performed an ELISA to determine the protein expression level of TNF- α , and the results showed that TNF- α production level was increased significantly with Mc-fEE treatment at concentrations of 50 μ g/ml and 200 μ g/ml (Fig. 1c). Also, the mRNA expression levels of various cytokines were detected by quantitative real-time PCR. Mc-fEE at concentrations of 200 μ g/ml significantly upregulated the expression levels of several cytokines, including proinflammatory cytokines of IL-6, IFN- β , TNF- α , and IL-1 β , and IL-12b, which could induce NK cell activation (Fig. 1d). This confirmed that Mc-fEE has immunostimulatory effects. Finally, we obtained the phytochemical profiles of Mc-fEE by using high-performance liquid chromatography (HPLC) analysis. As shown in Fig. 1e, by comparing with the standard content, we confirmed that Mc-fEE contains deacetylasperulosidic acid (DAA), and it is an iridoid compound mainly found in plants [36]. Also, it is well known to have various bioactivities, including anti-inflammatory, anti-cancer, and antioxidant effects [37] [38]. It was confirmed that Mc-fEE has immunostimulatory effects, through the increase of these pro-inflammatory cytokines, especially cytokines known as immune stimulant.

Mc-fEE mediates the NF- κ B and AP-1 pathway to have immunostimulatory effects

To determine the mechanism of the increase in those cytokines, we compared Mc-fEE treatment at concentrations of 200 μ g/ml responses to those of a family member of β -D-glucose polysaccharides, β -

glucan. This molecule is present in the cell wall of fungi or bacteria and is known to induce an immune response by interacting with receptors of macrophages [39]. The levels of phosphorylation of p65 and p50, NF- κ B subunits, as well as c-Jun and c-Fos, AP-1 subunits were increased for both β -glucan- and Mc-fEE-treated cells (Fig. 2a, 2b). The data indicate that the immunostimulatory efficacy of Mc-fEE *in vitro* is a result of enhanced activity of NF- κ B and AP-1 pathways, involved in the regulation of immune response.

Mc-fEE increases the expression levels of NK cells population in the spleen

We next tested the immune-stimulating efficacy of Mc-fEE *in vivo*. For 4 weeks, mice were administered orally 50 mg/kg or 200 mg/kg concentration of Mc-fEE, and at both concentrations, the body weights of the mice increased similarly to that of the untreated group (Fig. 3a). This suggests that Mc-fEE is not toxic *in vivo*. There were no significant differences in spleen and thymus weights as a result of Mc-fEE administration (Fig. 3b, 3c). The *in vivo* immunostimulatory effects of Mc-fEE were evaluated by measuring the populations of immune cells, especially NK cells. The results are depicted in Fig. 3. We performed flow cytometry analysis and confirmed the population of several immune cells in the mice spleen, including Tc, Th, NK, and dendritic cells, as well as macrophages as shown in Supplementary Fig. 1 and Fig. 3d. Among these various types of immune cells, Mc-fEE significantly increased the population of NK cells (Fig. 3e). Therefore, supporting the *in vitro* findings and evaluating the immunostimulatory efficacy *in vivo*, Mc-fEE has immune-enhancing ability through induction of increases in the population of NK cells, which contribute to a vital role in early defense against infection.

Mc-fEE shows immunostimulatory effects ex vivo

Last, we conducted an additional experiment to determine whether the activation levels of increased NK cells were increased by Mc-fEE. Also, whether the increase population of NK cells could provide the NK cytotoxic activity of Mc-fEE since NK cells are known to target cancer cells and are cytotoxic to these cells. Therefore, as depicted in Fig. 4a, in order to evaluate potential NK cells activity of Mc-fEE, the cytotoxicity of NK cells was measured by co-culture of NK cells isolated from mice spleens and YAC-1, a mouse T cell lymphoma which is sensitive to the cytotoxic activity of NK cell. Experiments were carried out a ratio NK cells to YAC-1 cells of 5:1 or 10:1. NK cells from Mc-fEE-treated mice had greater cytotoxicity to YAC-1 cells compared to untreated mice (Fig. 4a), with Mc-fEE 200 mg/kg showed more effective for cytotoxicity activity against YAC-1 cells in lower ratio of NK cells to YAC-1 cells (E/T = 5). Whereas in lower doses of Mc-fEE, 50 mg/kg, had significantly effect of cytotoxicity in E/T = 10. Furthermore, the upregulated levels of NK cell activation were assessed at the mRNA level; and the expression levels of IL-1 β , IFN- γ , and TNF- α , well-known NK cell activation markers, in the splenocytes, from total spleen cells, were increased significantly at doses of 200 mg/kg (Fig. 4b). Mc-fEE showed immunostimulatory efficacy by enhancing the activities of NK cells, which participates an important role in innate immune response against infection, including cancer cells without antigen stimulation.

Discussion

We investigated the immunostimulatory role of Mc-fEE *in vitro* using RAW264.7 cells and *in vivo* using mice. Since it is relatively common for natural extracts, including Mc-fEE, to be contaminated with environmental LPS due to the ubiquitous nature of LPS, first we evaluated the effect of Mc-fEE on the production of NO treated with polymyxin B (40 µg/ml), an inhibitor of gram-negative bacteria [40] to exclude the further possibility that the effect of Mc-fEE in the next examinations may in part of LPS contamination. The results of NO assay suggest the absence of endotoxin. Next, when Mc-fEE was used to treat murine macrophage RAW264.7 cells, the expression of various cytokines involved in the immune response increased. In particular, IL-12, consisting of a heterodimeric structure of IL-12a and b, stimulates the functions of T cells. IL-12 increases the expression levels of TNF-α and IFN-γ, and the cytotoxic activity of NK cells [41]. In addition, IL-1β and TNF-α have been shown to be a mediator of tumor cytostasis. The macrophage-derived these products are considered important mediators of tumour cytotoxicity [9]. Despite there is different discussion regarding IL-6 as anti- or pro- inflammatory activity, it also has been confirmed that IL-6 had a proinflammatory cytokine, IL-6 becomes a protagonist since it predominately induces pro-inflammatory signaling and regulates massive cellular processes [42, 43]. We confirmed that the mRNA expression level of these cytokines were upregulated when murine macrophage RAW264.7 cells were treated with Mc-fEE *in vitro*, and in line with the mRNA expression, the production level of TNF-α was also increased. The extracellular release of TNF-α, used as a marker of the immune-enhancing effect, also significantly increased (Fig. 1). Consistent with previous works [9, 27, 44–46], these findings indicate that Mc-fEE had an immunostimulatory activity in macrophages *in vitro* by inducing the mRNA expression of proinflammatory cytokines. The mechanism for this increase in cytokine expression was tested, and one finding was the increased activation levels of subunits of transcription factors of the NF-κB and AP-1 pathways. This suggests that immune response-induced signaling was increased [47]. Activated NF-κB pathways induce the survival, activation, and differentiation of multiple innate immune cells and T cells [48]. The activation of transcription factors of the AP-1 pathway induces the differentiation of T regulatory cells that suppress autoimmune diseases by suppressing self-reactivity [49]. We discovered that Mc-fEE has immune-enhancing efficacy by activating the NF-κB and AP-1 pathways at the cellular level by phosphorylating of p50 and p65, NF-κB subunits, as well as c-Jun and c-Fos, AP-1 subunits (Fig. 2). Regarding *in vivo* study, in mice spleens injected with Mc-fEE, the immune cell populations were measured using flow cytometry assay. Also, in accordance with our previous studies using *M. citrifolia* fruit water extract [45], The NK cell population significantly increased with Mc-fEE oral treatment (Fig. 3). NK cells and cytotoxic T lymphocytes (CTLs) are generally involved with its anti-cancer properties [28]. Last, the level of LDH released from damaged cells after co-culture with YAC-1 cells, a T cell lymphoma, was assessed. Several papers have described that immunomodulation contributes to the anticancer activity of noni [13, 28, 50]. It is prompted us to evaluate the potential activity of cytotoxicity of the increased population of NK cells by the Mc-fEE. The increased cytotoxicity of NK cells by Mc-fEE was established through the increase in LDH level in the group administered Mc-fEE compared to the untreated group (Fig. 4). However, Mc-fEE 200 mg/kg showed more effective in E/T = 5, whereas Mc-fEE 50 mg/kg had a greater effect of cytotoxicity in E/T = 10.

Conceivably, indicating that with higher doses of Mc-fEE, 200 mg/kg, it only needed a lower ratio of NK cells to target YAC-1 cells. Whereas a higher ratio of NK cells to YAC-1 cell of Mc-fEE 200 mg/kg serves as a decreased activity of its cytotoxicity. It may also be expected that there is a certain upper limit, as a large number of cells were co-cultured in each 96-well-plate. Moreover, in agreement with *in vitro study*, the effect of Mc-fEE in splenocytes was examined by measuring the expression of cytokines, IL-1 β , TNF- α , and IFN- γ . Mc-fEE also enhanced the expression of IL-1 β , TNF- α , and IFN- γ , a potent macrophage activator [51] in splenocytes. Taken together, these findings, the *in vivo* and *ex vivo*, suggest that Mc-fEE has immunostimulatory effects under normal immune response conditions by elevating the activity of NK cells in spleen of mice, thereby participate to target such a one of cancer cells, YAC-1 cells. This results could support several previous studies regarding anti-tumor activity and immunomodulatory effect from the fruit of noni [13, 27–29].

Conclusion

We found that Mc-fEE had an immunostimulatory effect by enhancing the population and activation of NK cells *in vitro* and *in vivo*. Mc-fEE (0-200 μ g/ml) can induce the mRNA expression levels of cytokines such as IL-6, IFN- β , TNF- α , IL-1 β , and IL-12b in RAW264.7 cells via the activation of NF- κ B and AP-1 pathways. Moreover, Mc-fEE (0-200 mg/kg) increases the population of NK cells and upregulates their cytolytic activity. This was demonstrated when isolated NK cells from Mc-fEE-treated mice were co-cultured with YAC-1 cells, a T cell lymphoma, and confirmed through LDH assay. These results strongly suggest that Mc-fEE has immunostimulatory effects by elevating the activity of NK cells (Fig. 5).

Declarations

Acknowledgements

Not applicable.

Author Contributions

Conceptualization, H.K., L.R., S.-Y.C., and J.Y.C.; formal analysis, H.K., L.R., and J.Y.C.; investigation, H.K., L.R., Y.H.H., and S.-Y.C.; writing—original draft preparation, H.K., L.R., and J.Y.C.; writing—review and editing, J.Y.C.; supervision, J.Y.C.; funding acquisition, S.-Y.C. and J.Y.C. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The in vivo experiment was conducted according to the guidelines of the Declaration of Helsinki and all procedures were carried out in compliance with the ARRIVE guidelines which was approved by the Institutional Review Board (or Ethics Committee) of Sungkyunkwan University (Protocol code: SKKUIACUC2021-01-62-1, and Approval date: 2020.06.04).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

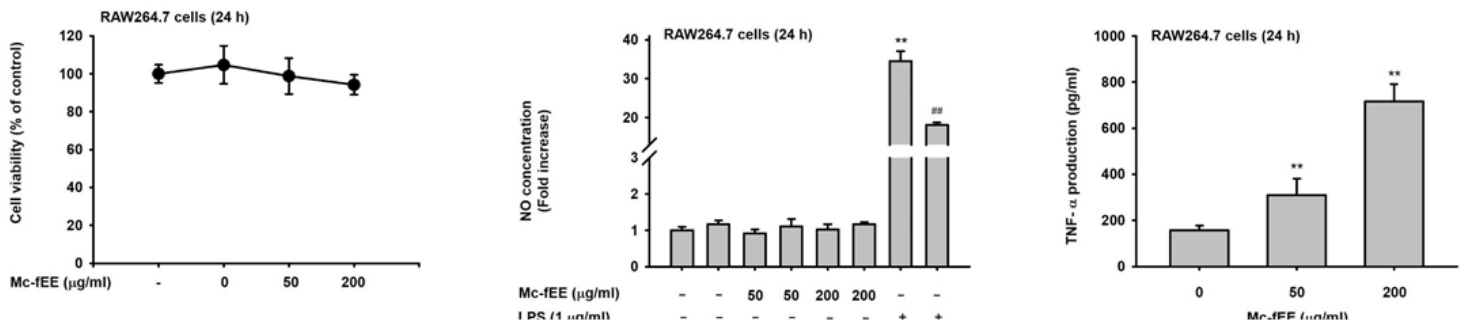


Figure 1

Immunostimulatory effects of Mc-fEE *in vitro*. (a) Mc-fEE (0-200 mg/ml) was used to treat RAW264.7 cells for 24 hours, and cell viability was measured by MTT assay. (b) Polymyxin B (40 mg/ml) was used to treat RAW264.7 cells for 30 minutes, Mc-fEE (0-200 mg/ml) or LPS (1 mg/ml) was used to treat these cells for 24 hours, and NO production was measured by NO assay. (c) TNF-α production level was detected by ELISA in supernatants of Mc-fEE (0-200 mg/ml)-treated RAW264.7 cells. (d) RAW264.7 cells treated with Mc-fEE (0-200 mg/ml) were used to detect various immune response-related pro-inflammatory cytokines, including IL-6, IFN-β, TNF-α, IL-1β, and IL-12β. (e) The phytochemical profiles were detected by using HPLC analysis with comparing the standard component, deacetylasperulosidic acid. Results (b-d) are expressed as mean ± SD (n = 4-5) and similar experimental data were obtained from an additional independent experiment performed under same conditions. *: $p < 0.05$, **: $p < 0.01$ compared to the normal group. ##: $p < 0.01$ compared to LPS alone.

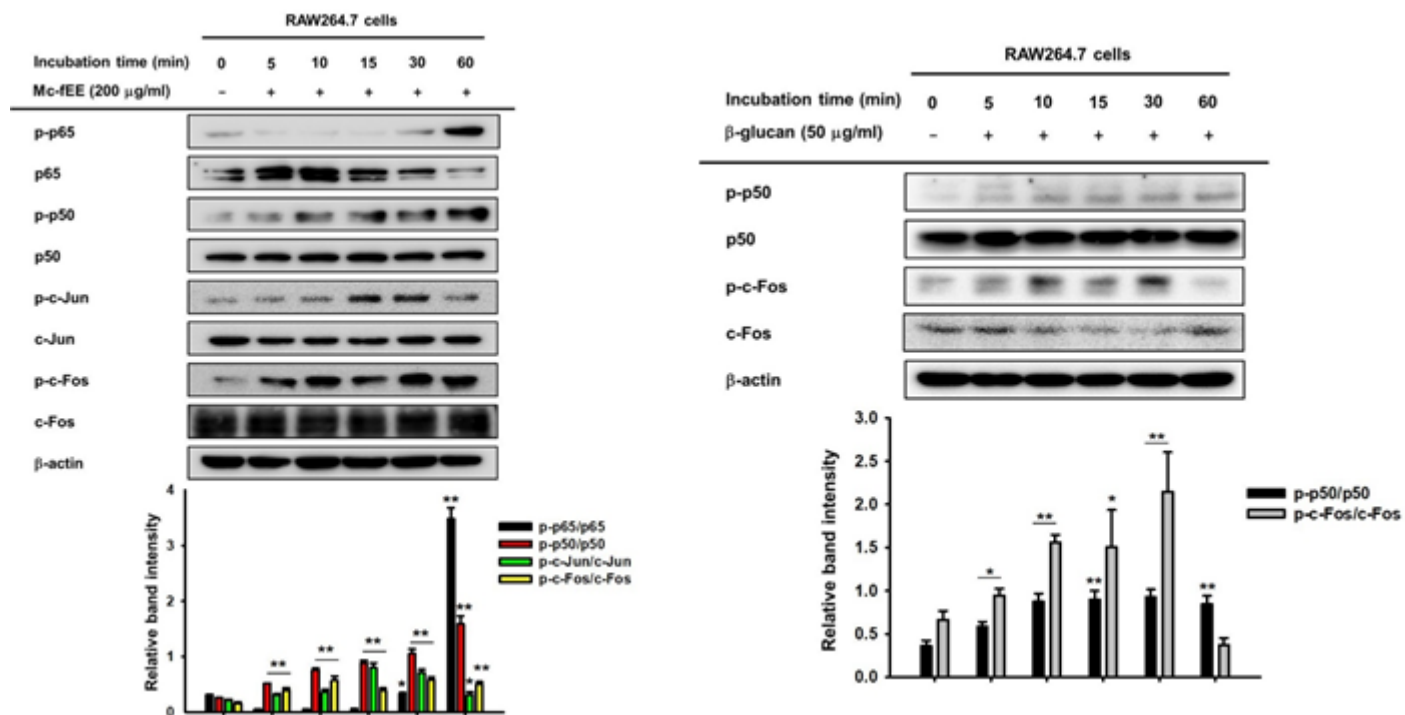


Figure 2

NF-κB and AP-1 pathway-mediated immunostimulatory effects of Mc-fEE. (a) Mc-fEE was used at a concentration of 200 mg/ml to treat RAW264.7 cells for indicated times. Western blotting analysis was conducted with total and phosphorylated antibodies of NF-κB subunits (p65, p50) and AP-1 subunits (c-Jun, c-Fos). (b) Total and phosphorylated p50 and c-Fos were determined in β-glucan-treated RAW264.7 cells in a time-dependent manner. The data are expressed as means + SD of three independent experiments. *: $p < 0.05$, **: $p < 0.01$ compared to the normal group.

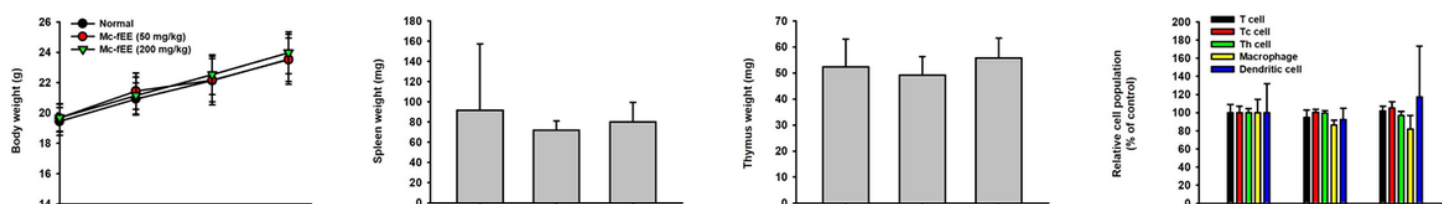


Figure 3

***In vivo* effects of Mc-fEE on populations of NK cells in mice spleens.** (a) Body weights of Mc-fEE (0-200 mg/kg)-treated mice were measured once a week for 4 weeks. (b, c) Spleen or thymus weights were obtained from Mc-fEE (0-200 mg/kg)-treated mice. (d) T cells CD3+, Tc cells CD3+/CD4-/CD8+, Th cells CD3+/CD4+/CD8-, macrophages F4/80+, and dendritic cells CD11c+ were isolated from the spleens of orally treated Mc-fEE (0-200 mg/kg) mice. The populations of cells were detected by flow cytometry analysis. (e) Populations of NK cells NKp46+ from orally treated Mc-fEE mice spleens were measured by flow cytometry analysis and plotted. The data are expressed as mean $\pm$ SD (n = 15). *: $p < 0.05$ compared to the normal group.

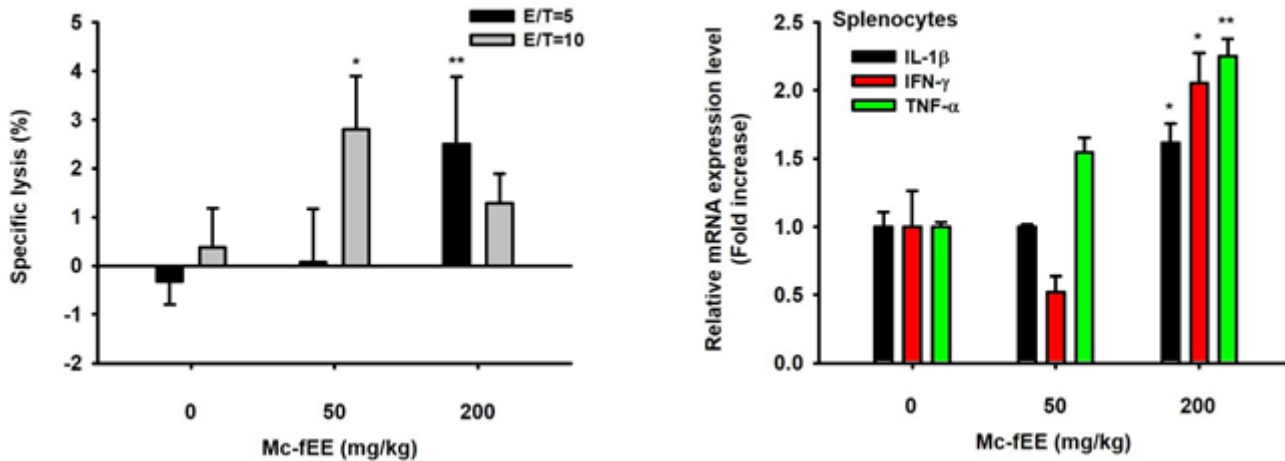


Figure 4

Immunostimulatory effects of Mc-fEE *in vivo* by promoting NK cell activities. (a) Specific lysis levels of YAC-1 cells by activated NK cells from Mc-fEE-treated mice spleens were measured by cell-mediated LDH assay. (b) Relative mRNA expression levels of IL-1b, IFN-g, and TNF-a were detected from Mc-fEE-treated mice splenocytes using quantitative real-time PCR. The data are expressed as mean $\pm$ SD (n = 3) with an additional experiment under same conditions. *: $p < 0.05$, **: $p < 0.01$ compared to the normal group.

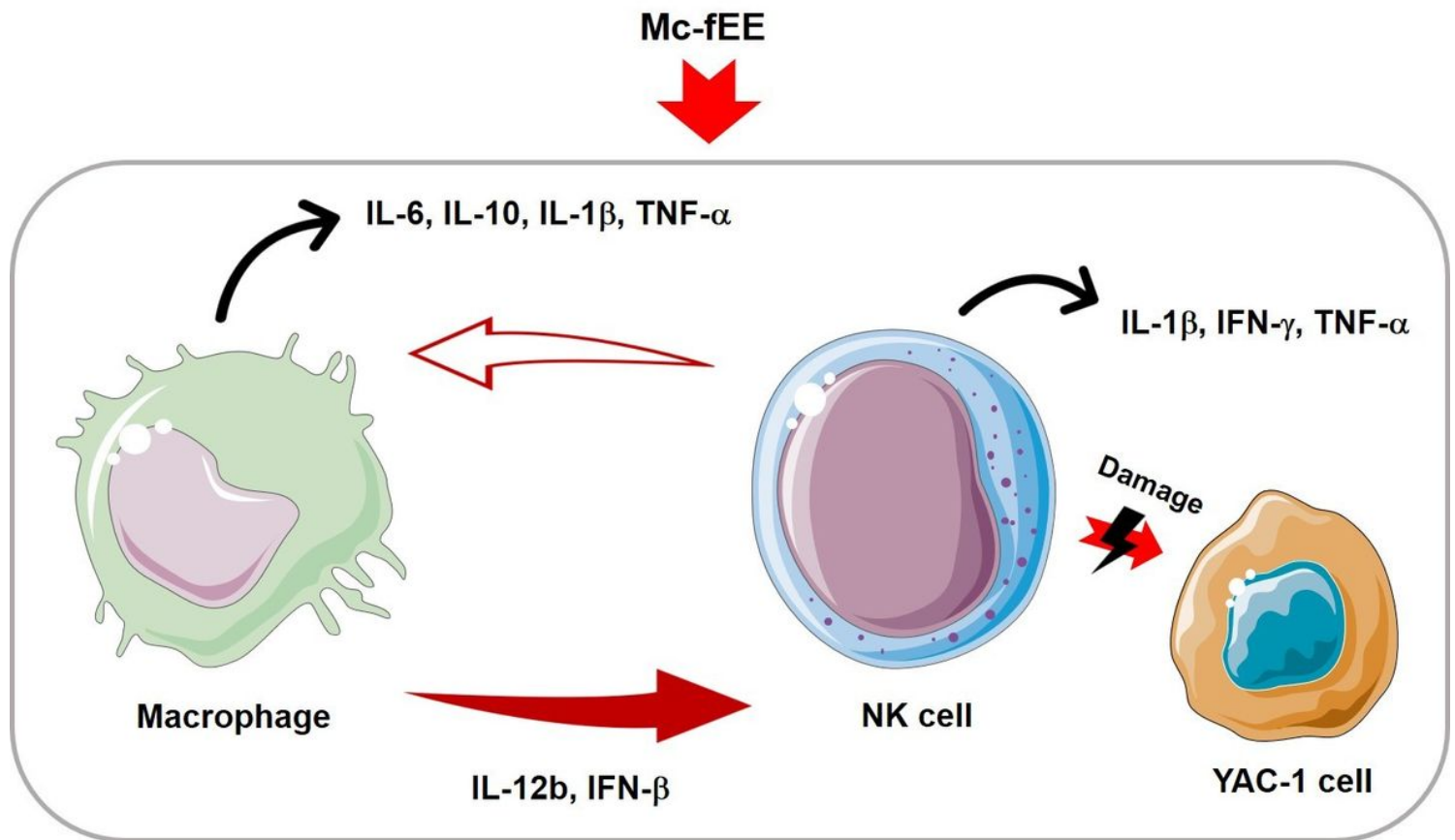


Figure 5

Scheme for Mc-fEE-mediated immunostimulatory effects

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