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Modulation of local and systemic immune responses by fermented garlic extract

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Abstract. Fermented garlic (FG), prepared by fermenting garlic with *Bacillus subtilis* for 3-15 days at 20-40˚C has been linked to immune-boosting properties. In the present study, FG was utilized to investigate its immunomodulatory effects using RAW264.7 cells, as well as BALB/c and C3H/HeN mice. Nitric oxide (NO) and pro-inflammatory cytokine levels were quantified in RAW264.7 cells treated with or without FG (0.016, 0.08, 0.4, 2 and 10 mg/ml), garlic extract (GE) (0.016, 0.08, 0.4, 2 and 10 mg/ml) and lipopolysaccharide (1 μg/ml). Peritoneal macrophages and splenocytes were obtained from BALB/c mice for *ex vivo* analyses. MTT and enzyme-linked immunosorbent assays were used to determine splenocyte proliferation, natural killer (NK) cell activity and cytokine production. The intestinal immunostimulatory *in vitro* effects of FG were also investigated. In a concentration-dependent manner, FG treatment markedly accelerated the proliferation of RAW264.7 cells, murine peritoneal macrophages and splenocytes. Moreover, FG initiated macrophage activation by significantly increasing the NO, tumor necrosis factor-α, interleukin (IL)-12 and IL-6 levels in RAW264.7 cells and peritoneal macrophages. In contrast to FG, GE promoted RAW264.7 cell proliferation at lower concentrations and toxicity at higher concentrations, while no detectable NO synthesis was observed at the concentrations tested. Likewise, the FG extract at larger concentrations fortified the intestinal immune system and improved bone marrow cell proliferation by significantly elevating IL-6 and granulocyte-macrophage colony-stimulating factor production in splenocytes and Peyer's patch cells of mice. In addition, the NK cell activities of splenocytes from FG-treated mice at lower doses against YAC-1 cells were significantly increased, which must have been fostered and activated by interferon-γ generated by splenocytes. On the whole, these findings provide relevant *in vitro* and *ex vivo* information that describes the mechanisms through which FG can strengthen a compromised immune system by influencing local and systemic gut immune responses directly or indirectly.

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Introduction

The human body has both an innate and adaptive immune system that operates in tandem, with phagocytes and natural killer (NK) cells driving innate immune responses and T and B lymphocytes driving adaptive immune responses (1,2). The overstimulation of the immune system is harmful, while a well-regulated immune system protects the body from infections and cancer growth (3). Innate immune cell activities, such as phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) are major strategies to suppress or eliminate microorganisms and tumor cells (4). In addition, activated macrophages release cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-12, and chemical mediators, such as nitric oxide (NO), prostaglandins and reactive oxygen species (ROS) that stimulate the proliferation of nearby innate and adaptive immune system cells (5). The RAW 264.7 murine macrophage cell line is a broadly applied model for the *in vitro* testing of new potential medicines on macrophage activity (6).

The main goal of Traditional medicine is to develop immunostimulators that are safer, entirely biodegradable and cost-effective in order to prevent diseases (7). Notably, several foods and dietary herbs have been consumed as formulations to improve the quality of life, and thus these can be employed as a valuable source of agents that can boost host immune responses by stimulating or suppressing both specific and non-specific immunity (8). In fact, the immunostimulatory qualities of natural herbs are due to their abundance in antioxidants and bioactive compounds, such as vitamins, phenolic acid and flavonoids (9). Therefore, the rigorous search for natural products or herbs and their immunoregulatory processes has markedly increased in recent years.

Garlic (*Allium sativum*) is a culinary and medicinal herb known for its antioxidant, anticancer, antibacterial, cholesterol-lowering, anti-inflammatory and other properties (10,11). The presence of sulfur-containing substances, such as allicin, alliin, ajoene and their metabolites, allyl mercaptan and allyl methyl sulfide, has been attributed to the diverse biological effects reported in garlic preparations and extracts (12,13).

As previously demonstrated, at low doses, garlic oil stimulates a Th1 immune response, whereas, at high levels, it promotes a type 2 T helper (Th2)-type response (14). Alliin, a garlic component, protects against lipopolysaccharide (LPS)-induced pro-inflammatory responses, as evidenced by the upregulation of anti-inflammatory genes and the downregulation of pro-inflammatory genes, such as IL-6 and monocyte chemoattractant protein-1 (15). In addition, allicin has been shown to improve the immunological response of peripheral blood cells, as well as macrophage phagocytic activity (16). Recently, fermented garlic extract (FGE) has been discovered to be a more effective immune booster and anti-tumor agent than fresh garlic extract (GE). This is due to the fact that the fermentation procedure and type of microbial strain change organosulfur compounds in raw garlic to S-allyl cysteine and S-allyl mercaptocysteine (17), and enhance antioxidant activity and immune-stimulating potency by increasing the levels of total polyphenols, tetrahydro-carboline derivatives, vitamins and flavonoids. In addition, proteolytic enzymes from microorganisms hydrolyze polyphenol complexes into free and soluble phenols, which are more active and effectively absorbed during fermentation (18-20).

Taking these tendencies into account, garlic and aged GEs have been widely examined for their immune-enhancing effects *in vitro* and *ex vivo* (15,21), although there are no available studies to date on the immunological modifying effects of FGE, at least to the best of our knowledge. As a result, in the present study, it was hypothesized that supplementation with FGE could exert immune-modulating effects. To determine this hypothesis, the present study focused on immunological responses in RAW264.7 cells, primary mouse splenocytes and NK cell cytotoxicity in BALB/c mice. In addition, the present study aimed to investigate whether FGE may contribute to the intestinal immune response of C3H/HeN mice.

Materials and methods

Preparation of samples. A total of 5 g of air-dried garlic (obtained from Dongsung farm of Dongsun Biopharmaceutical) was extracted with 100% water (100 ml) for 8 h at room temperature. The aqueous extract was filtered through filter paper (1 μm) and lyophilized to produce white GE powder. The GE was then stored at 4˚C.

Solid-state fermentation using *Bacillus subtilis (B. subtilis)* KCTC 1028 which was procured from KCTC (The Korean Collection for Type Cultures) for 3-15 days at 20-40˚C was performed to yield FG extract in the aqueous phase. The amounts of water, garlic and *B subtilis* were in the proportions of 85:5:1. The saccharides and *B. subtilis* were added to the water in the first fermentation step to activate the bacteria, and the garlic was then fermented for 3 days. The second fermentation step was preferably performed for 7 days at 20-40˚C. The fermented extract was then concentrated to 500 ml (4.2 Brix) in a concentrator before being dried in a freeze drier. Dried materials were suspended in sterile distilled water to make a stock solution of 10 mg/ml, which was then filtered through a 0.45-μm pore membrane and sterilized. Sub-stock solutions were then generated for tests by diluting (broth medium, Difco; BD Biosciences) to the required concentration, and the samples were further stored at 4˚C.

Cells and cell culture. The RAW 264.7, a murine macrophage cell line, was obtained from the Korea Cell Line Bank (KCLB 40071) and was routinely cultured in DMEM (HyClone; Cytiva SH30243.01) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S) (GenDEPOT, Inc.) at 37˚C in a humidified chamber of 95% air and 5% CO_2 atmosphere.

Cell viability assay. RAW264.7 cells (2x10^6 cells/well) were seeded on 96-well plates and cultured for 24 h to evaluate the cell viability of the sample. Stock solutions of 10 mg/ml FGE were employed and were then diluted with medium to the necessary concentrations. The cells were then pre-treated for 24 h with 0.016-10 mg/ml of FGE, with untreated cells serving as a control. Following 24 h of incubation at 37˚C, the cells were examined using an EZ-cytox cell viability assay kit (Daeil Lab Service, Co., Ltd.) for 30 min before the absorbance was measured at 450 nm (Multiscan GO, Thermo Fisher Scientific, Inc.) to test cell viability. Viability was expressed as relative viability (%) with respect to the group treated with distilled water (control group).

Determination of NO levels. The Griess Reagent System (G2930; Promega Corporation) was used to measure nitric oxide levels in the cell culture supernatants. Briefly, the RAW264.7 cells were treated for 24 h with various concentrations of FGE (0.016, 0.08, 0.4, 2 and 10 mg/ml) and 1 μg/ml LPS (MilliporeSigma) as a control. The obtained supernatants were then incubated (1:1) with Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid]. The absorbance was then measured at 540 nm (Multiscan GO, Thermo Fisher Scientific, Inc.), and the NO concentration was calculated using a standard curve.

Determination of cytokine levels using enzyme-linked immunosorbent assay (ELISA). The levels of TNF-α, IL-6 and IL-12 in the cell culture supernatants were determined using a commercial sandwich ELISA kit: Mouse TNF-α ELISA set, cat. no. 555268, BD Biosciences; mouse IL-6 ELISA set, cat. no. 555240 BD Biosciences; and mouse IL-12 ELISA set, cat. no. 555165, R&D Systems, Inc.). For 24 h, the RAW264.7 cells and peritoneal macrophages were pre-treated with FGE (0.016, 0.08, 0.4, 2 and 10 mg/ml) and LPS (1 μg/ml) respectively. The culture medium was collected, and the TNF-α, IL-6 and IL-12 levels were measured in the supernatant using ELISA kits according to the manufacturer's instructions.

Supernatants were obtained from splenocytes that had been incubated in the presence or absence of various concentrations (0.016, 0.08, 0.4, 2 and 10 mg/ml) of FG and LPS (1 μg/ml) for 24 h to quantify the levels of IL-6, interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, the absorbance was determined using a spectrophotometer (Multiscan GO, Thermo Fisher Scientific, Inc.) at 450 nm, as per the manufacturer's instructions (mouse IL-6 ELISA set, cat. no. 555240, BD Biosciences; mouse INF-γ ELISA set, cat. no. DY485 R&D Systems, Inc.).

Mice. A total of 40 BALB/c mice 6 weeks old, weighing 18-20 g, female and 15 C3H/HeN mice (6 weeks old, weighing 16-18 g, female) were purchased from Core Tech Co., Ltd.

The mice were allowed to acclimatize to their environment for 1 week prior to the experiment on a 12-h light/dark cycle at 23˚C and 55% humidity. The mice were provided with access to water and a standard pellet diet *ad libitum*. The animal health and behavior was monitored daily and the animals were cared for according to the Guide for the Care and Use of Laboratory Animals of the Kyung Hee University. The experiment was carried out for 1.5 months, for which 40 BALB/c mice and 15 C3H/HeN mice were used. All animal health and behaviors were monitored daily. The 55 mice were euthanized by cervical dislocation at the end of the experiment. However, no mortality occurred during the experiment. The human endpoints used to determine when animals ought to be euthanized in the experiment included the inability to rest in a sternal position, being unable to eat or drink, respiratory issues, paralysis, uncontrollable bleeding and irreversible weight loss. The assessment criteria for the confirmation of death in the experiment included no heart rate, no signs of breathing, and dilated and unreactive pupils with light. All animal welfare considerations taken, including efforts to minimize suffering and distress, the use of analgesics or anesthetics (including the dose), or special housing conditions according to the Institutional Animal Care and Use Committee of Dongsung Cancer Center and Kyung Hee University guidelines. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUSBC-R-SPA 2018-0601) and the Institutional Animal Care and Use Committee of the Dongsung Cancer Center under protocol IACUC #ds002205112-EUTO3. The experiments were carried out in compliance with the ARRIVE guidelines.

Isolation of peritoneal macrophages from BALB/c mice. Peritoneal fluid was collected from the BALB/c mice on the 4th day following the administration of 1 ml 5% thioglycollate media (MilliporeSigma) intraperitoneally to extract murine peritoneal macrophages (MPMs). Subsequently, the cell suspension was dispensed and cultured for 4 h in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (cat. no. CM002-050, GenDEPOT) in an incubator (5% CO_2, 95% air). Furthermore, to remove non-adherent cells, the dishes were gently rinsed with the medium.

Peritoneal macrophage cell viability assay. The MPMs ($1x10^6$ cells/ml) were seeded in 96-well plates (SPL Life Sciences Co., Ltd.). To determine the effects of FGE and LPS on the viability of peritoneal macrophages, the cells were pre-treated with 0.016, 0.08, 0.4, 2 and 10 mg/ml FGE and LPS (1 μg/ml) for 24 h. A 100 μl solution of Ez-cytox/phosphate-buffered saline (PBS) was added, and the absorbance at 450 nm was measured using a microplate reader (Tecan Austria GmbH). Cell viability was expressed as the relative activity (%) with respect to the group treated with distilled water (control group).

Isolation of splenocytes. The splenocytes were harvested from the spleen following the cervical dislocation of BALB/c mice. A single-cell suspension was obtained by grinding the spleen under aseptic conditions and passing through a 100-μm cell strainer. Red blood cells were lysed with 0.2% NaCl lysis solution (cat. no. 00-4300, Thermo Fisher Scientific, Inc.) prior to being washed in PBS and kept in RPMI-1640 complete medium.

Determination of splenocyte proliferation. The effects of FGE or LPS on the cell proliferation of splenocytes were detected using the EZ-cytox cell viability assay kit (Daeil Lab Service, Co., Ltd.). Splenocytes were seeded into 96-well culture plates at a density of $2.5x10^6$ cells/ml and incubated at 37˚C for 24 h. The cells were then treated with various concentrations of FGE (0.016, 0.08, 0.4, 2 and 10 mg/ml) and LPS (1 μg/ml) at 37˚C in a 5% CO_2 incubator. A total of 20 μl EZ-cytox solution was added to each well and incubated for 3 h to assess splenocyte proliferation. The absorbance was measured at 450 nm using a microplate reader (Tecan Austria GmbH).

Isolation of Peyer's patch (PP) cells and bone marrow cells. PPs were removed aseptically from the exposed small intestine of the C3H/HeN mice. The tissue was sorted into single-cell suspensions by grinding and filtering through a 200-gauze metal mesh. Finally, harvested cells were washed twice with Hank's balanced saline solution (HBSS; cat. no. 14175095, Gibco; Thermo Fisher Scientific, Inc.), and suspended in a complete medium (RPMI-1640 with 10% FBS).

On the other hand, bone marrow cells were recovered from the femurs of the same mice. Single-cell suspensions were obtained by drawing femoral bone tissue into 1 ml of a syringe, and further, the collected tissue was treated several times with sterile 0.2% NaCl to remove all red blood cells. The obtained cells were then passed through a 100-μM cell strainer, centrifuged at 200 x g, 5 min at 4˚C and suspended in RPMI-1640 medium.

Bone marrow cell proliferation activity via PP. The cells from PPs were washed in 10% FBS containing RPMI-1640 and seeded at a density of $2x10^6$ cells/ml. For 5 days, aliquots of cell suspension (180 μl) derived from PPs were dispensed into a 96-well plate and cultured with 20 μl of diluted concentrations of FGE. The cultured supernatant (conditioned medium) was then tested for bone marrow cell proliferation and the presence of GM-CSF, an immunoreactive cytokine produced by PP cells.

Furthermore, the isolated bone marrow cells were adjusted to $2.5x10^5$ cells/ml and 100 μl were dispensed into each well of the PP cell culture. To examine the ability to grow on bone marrow cells, 50 μl of the resultant supernatant were mixed with 50 μl bone marrow cell suspension in DMEM full medium (DMEM; cat. no. SH30243.01, HyClone; Cytiva) and grown for 6 days. Bone marrow cell proliferation via PP cells stimulated with FGE was detected using the EZ-cytox cell viability assay kit (Daeil Lab Service, Co., Ltd.), and the absorbance (Multiscan GO, Thermo Fisher Scientific, Inc.) was measured at 450 nm to determine the relative activity (%) for myeloid cell proliferation.

Cytotoxic activity assay of NK cells from splenocytes against cancer cells. Female BALB/c mice (6-8 weeks old) were intravenously injected with 0.1, 1 and 10 mg/kg of FGE in the drug-treated groups and PBS in the vehicle-treated groups, for 4 days and 1 day before sacrifice. The mice were sacrificed by

cervical dislocation and the spleens were aseptically extracted. Splenocytes that had destroyed all red blood cells using 0.2% NaCl were recovered in serum-free medium (SFM) containing 1% penicillin/streptomycin (cat. no. CA005-010, GenDepot) and were adjusted to a density of 5×10^7 cells/ml.

Briefly, YAC-1 lymphoma cells (CVCL_2244; American Type Culture Collection) were used as target cells to detect NK cell cytotoxicity. The cytotoxic capacity of harvested mouse splenocytes as effector cells was investigated. Splenocytes and YAC-1 cells were co-cultured at 50:1, 25:1 and 12.5:1 ratios of effector:target cells (1×10^5 cells/ml) in 96-well plates. After 24 h of mixed culture of the target cell and the effector cell, the effector cell toxicity was determined. The amount of lactate dehydrogenase (LDH) generated in the target cell was measured using the LDH assay kit, and the tumor cell killing ability of NK cells was assessed using the following formula:

$$NK\ cell\ cytotoxic\ activity\ (\%) = \frac{\text{exerimental} - \text{effector spontaneous} - \text{target spontaneous}}{target\ maximum - target\ spontaneous} x100$$

Statistical analysis. The experimental results are expressed as the mean ± SD of the results of experiments performed in triplicate samples. Statistical analysis was performed using one-way ANOVA (with Graph Pad Prism 5.0 software, GraphPad Software, Inc.) followed by post hoc Tukey's multi comparison test. $P<0.05$ was considered to indicate a statistically significant difference. In addition, Duncan's multiple range tests were used to confirm the significance of each measurement value.

Results

Effect of FGE on the viability of RAW264.5 cells. The cytotoxic effects of FGE and GE on murine RAW264.7 macrophage cells were assessed before the immunomodulatory impact was established, as shown in Fig. 1A. The RAW264.7 cells treated with FGE at concentrations of 0.016, 0.08, 0.4, 2 and 10 mg/ml exhibited a viability of 161.7±14.46, 179.62±11.35, 169.79±11.30, 147.71±10.36 and 154±14.16%, respectively. On the other hand, the cells treated with GE at concentrations of 0.016, 0.08, 0.4, 2 and 10 mg/ml exhibited a viability of 123.83±8.08, 130.64±8.71, 143.27±11.34, 89.79±10.25 and 65.77±6.82%, respectively. Indeed, these findings revealed that FGE had no negative effect on macrophage viability at any of the concentrations examined. However, at the concentrations of 2 and 10 mg/ml, GE was cytotoxic to the RAW264.7 cells.

Effect of FGE on NO and cytokine production in RAW264.7 cells. To confirm the immunostimulatory activity of FGE, NO production was evaluated after the RAW264.7 macrophages were treated with FGE and GE. FGE treatment at 0.016, 0.08, 0.4, 2 and 10 mg/ml upregulated NO production in a concentration-dependent manner compared to the GE-treated and untreated cells (Fig. 1B). NO generation was markedly promoted by FGE at the concentration of up to 2 mg/ml, followed by a reduction at the concentration of 10 mg/ml. Nonetheless, GE equivalent to the concentrations of FGE did not increase NO levels in RAW264.7 cells. The production of cytokines in RAW264.7 macrophages was likewise assessed to assess the effects of FGE on immunological activation. The levels of all three immunostimulatory cytokines, TNF-α, IL-6 and IL-12, were enhanced by FGE treatment compared to the untreated cells, as shown in Fig. 1C-E. In comparison to the LPS-treated control, TNF-α production was observed to be greater at 2 and 10 mg/ml.

Immunostimulatory effects of FGE on peritoneal macrophage activity and cytokine production. The effects of FGE on peritoneal macrophage cell viability were examined by treating the cells with the respective concentrations of FGE (0.016, 0.08, 0.4, 2 and 10 mg/ml) or LPS (1 µg/ml) for 24 h. As regards the peritoneal macrophages, FGE had no effect on cell survival, while it significantly increased macrophage activity at 10 mg/ml (Fig. 2A). As shown in Fig. 2B and C, FGE at concentrations of 10 mg/ml also markedly increased the production of TNF-α and IL-6 by peritoneal macrophages in a concentration-dependent manner. In peritoneal macrophages, TNF-α production increased with 0.4 to 10 mg/ml of FGE, whereas IL-6 production increased with 0.016 to 10 mg/ml of FGE. In the evaluation of IL-12 production ability, however, a different aspect was observed compared with TNF-α or IL-6. IL-12 exhibited a 3-fold greater capacity for production at a low concentration level of 0.08 mg/ml of FGE than at the 10 mg/ml concentration. On the other hand, IL-12 production increased with FGE at the concentration of 0.08 mg/ml and then decreased with concentrations from 0.4 to 10 mg/ml. Nuclear factor κ-B (NF-κB) and interferon regulatory factor-1 (IRF-1) are involved in the production of IL-12, which is then activated by IFN-γ (22). T-cells and NK cells, which release a substantial amount of IFN-γ, are found in insignificant numbers in adherent peritoneal exudate cells (23). As a result, FGE treatment may have induced peritoneal macrophages to create more IL-12 in response to IFN-γ. Thus, FGE has the ability to stimulate peritoneal macrophages, prompting them to generate effector molecules, such as TNF-α, IL-6 and IL-12.

FGE enhances the splenocyte proliferative capacity (mitogen activity). Mitogen activity employing splenocytes revealed that the FGE-treated groups were able to proliferate splenocytes. FGE treatment led to a statistically significant increase in proliferative activity at the lowest concentration of 0.016 mg/ml in comparison to the distilled water-treated control. The proliferative activity induced by FGE at concentrations of 0.016-10 mg/ml was 1.30-2.32-fold higher than that of the distilled water-treated control. In particular, mitogen activity was similar to that of the LPS-treated positive control group at the maximum concentration of 10 mg/ml (Fig. 3A).

FGE modulates the IL-6, IFN-γ and GM-CSF cytokine levels. Splenocytes have a complex immune response mechanism where one cytokine is involved and other cytokines follow. Therefore, depending on the type of cytokine identified when the splenocytes are stimulated, this has been used as evidence of other immune responses (24). In the present study, the ability of FGE to affect hemopoietic and T helper 1 cell (Th1) cytokines was assessed by examining its immunomodulatory effects on the splenocyte secretion of IL-6, GM-CSF and IFN-γ. The secretion of IL-6, IFN-γ, and GM-CSF was measured in BALB/c splenocytes cultured in

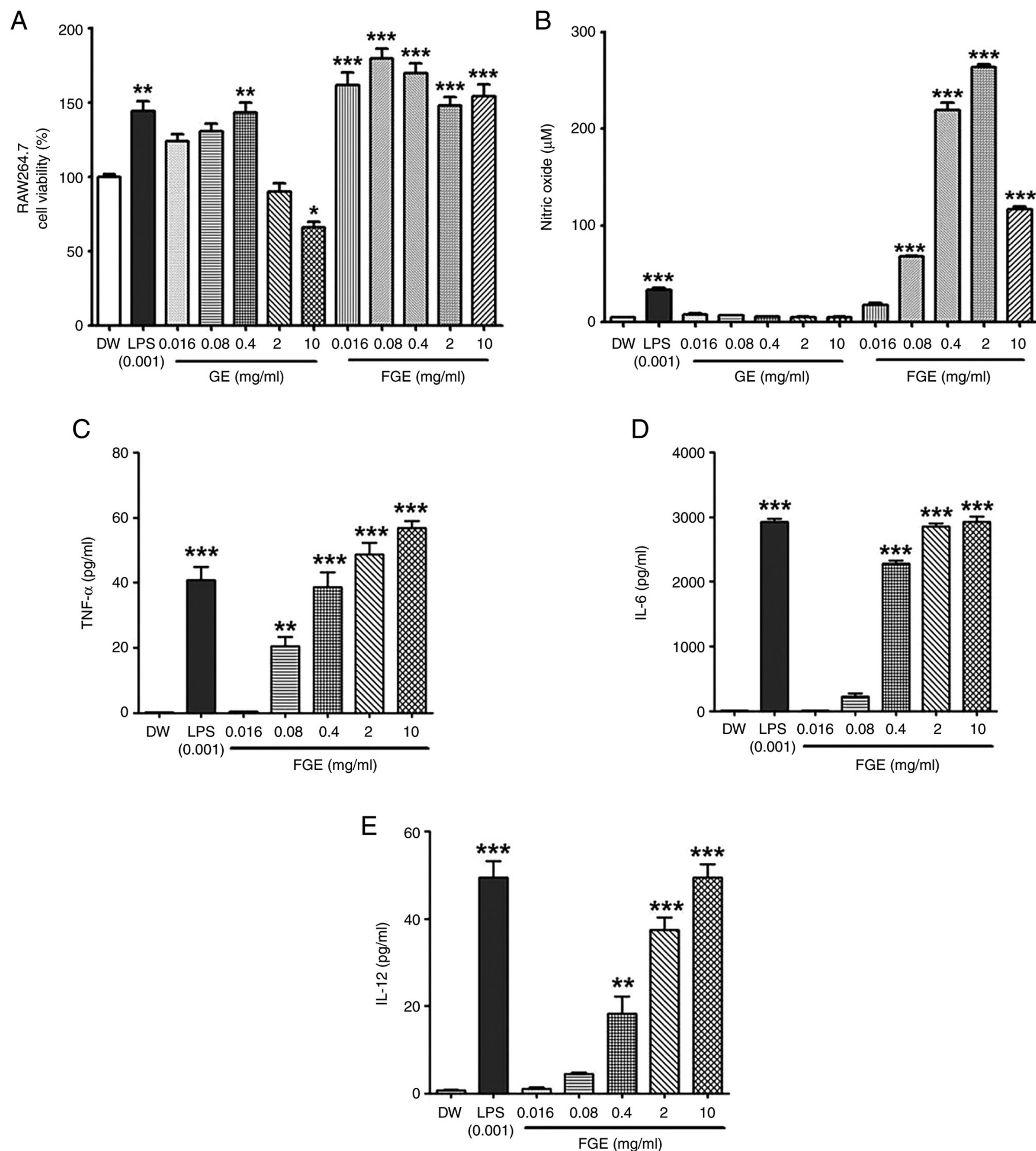

Figure 1. *In vitro* effect of FGE on cytotoxicity, and NO and cytokine production in RAW264.5 cells. (A) Effects of GE and FGE on cell viability during the first 24 h in RAW264.7 cells. FG exhibited no toxicity at any concentrations, while GE exhibited cytotoxicity at the concentrations of 2 and 10 mg/ml. MTT assays of RAW264.7 cells were used to assess the cytotoxicity of GE, FGE, LPS (0.001 mg/ml or 1 μg/ml) and DW treatments for 24 h. The effects of FGE and GE on (B) NO and only FGE on (C) TNF-α, (D) IL-6, and (E) IL-12 production were confirmed using ELISA. The data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, compared to the vehicle (DW)-treated control. DW, distilled water; FGE, fermented garlic extract; GE, garlic extract; LPS, lipopolysaccharide; NO, nitric oxide.

medium supplemented with the vehicle, LPS and FGE (0.016, 0.08, 0.4, 2, and 10 mg/ml). As demonstrated in Fig. 3B, at the lower concentration of 0.016 mg/ml, FGE had a minimal effect on IL-6 (Th17) cytokine production. However, the production of IL-6 was considerably increased following treatment with FGE from the concentration of 0.08 mg/ml in a concentration-dependent manner, as compared to the distilled water-treated negative control group. The secretion of GM-CSF was likewise increased and exhibited a similar trend as that of IL-6 (Fig. 3D). At the concentration of 0.08 mg/ml, FGE had a significant effect on IFN-γ (Th1) cytokine secretion, which then exhibited a concentration-dependent suppression at 0.4 mg/ml (Fig. 3C). These results demonstrated that FGE stimulated cytokine secretion (IL-6) at higher concentrations, while stimulating IFN-γ secretion at lower concentrations, suggesting that FGE can interfere with the Th1 and hematopoietic cytokine profiles.

FGE influences the activity of the gut immune-system through PP stimulation. First, the effect of garlic fermentation broth on bone marrow cells was evaluated. As shown in Fig. 4A, the garlic fermented broth exhibited a concentration-dependent

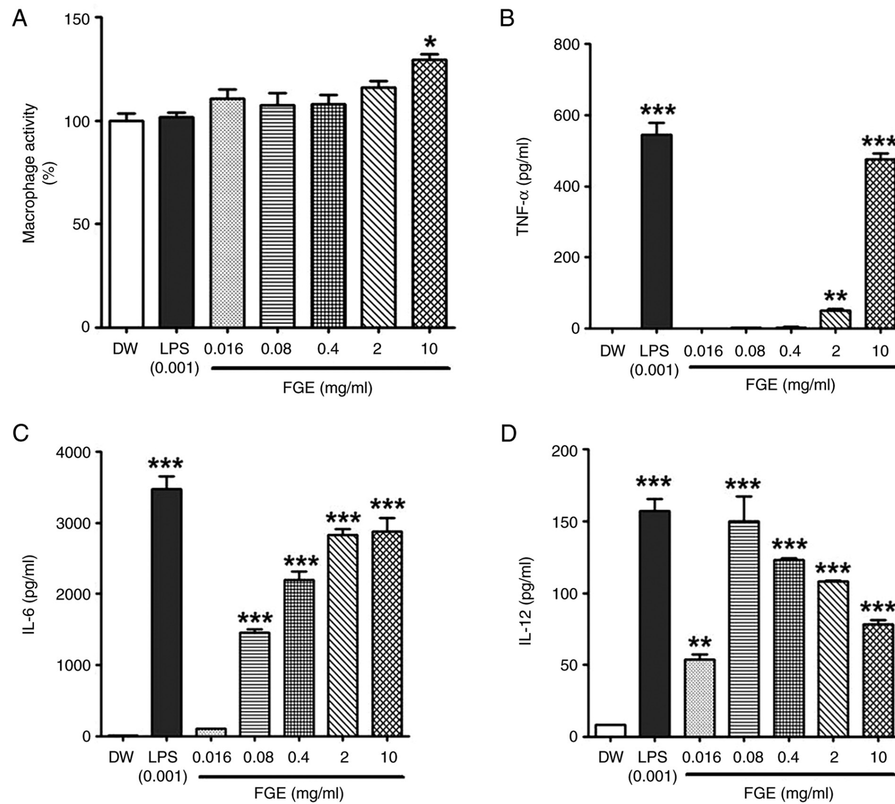

Figure 2. *Ex vivo* effect of FGE on cell viability and cytokine production in peritoneal macrophages. Effects of FGE at concentrations of 0.016-10 mg/ml on the (A) viability of peritoneal macrophages, and (B) TNF-α, (C) IL-6, and (D) IL-12 production were measured using ELISA. Values are shown as the mean ± standard deviation (n=3). LPS (0.001 mg/ml or 1 μg/ml) was used as a positive control and DW as a negative control. *P<0.05, **P<0.01 and ***P<0.001, compared to the vehicle (DW)-treated control. DW, distilled water; FGE, fermented garlic extract; GE, garlic extract; LPS, lipopolysaccharide.

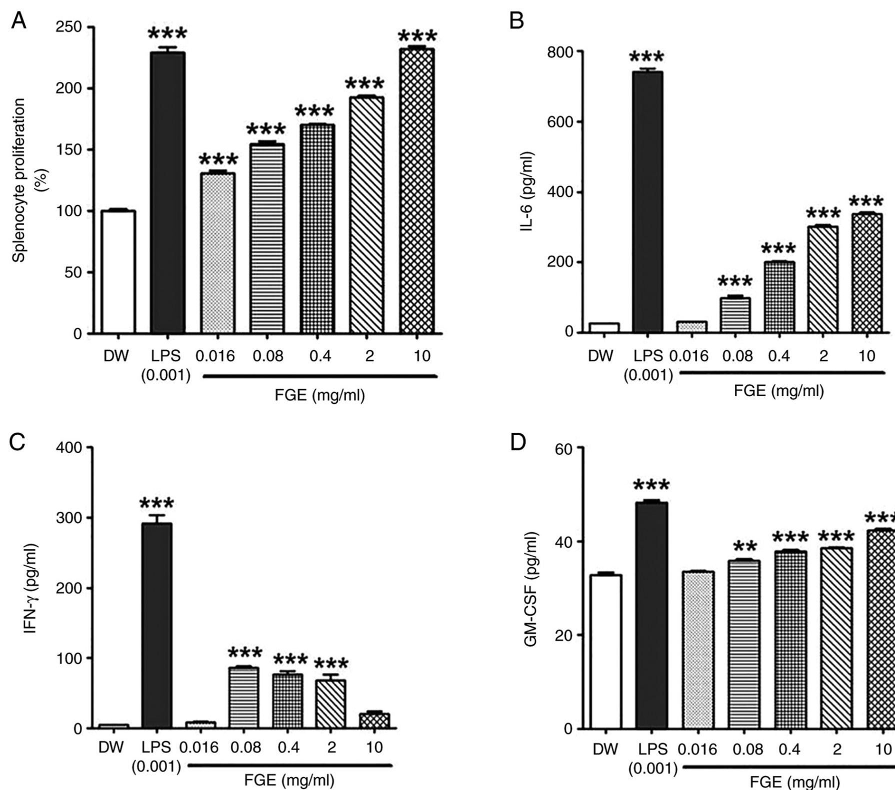

Figure 3. FGE enhances spleen cell proliferation and cytokine production. The effects of FGE at concentrations ranging from 0.016-10 mg/ml on (A) splenocyte cell proliferation and the secretion of (B) IL-6, (C) INF-γ and (D) GM-CSF cytokines secreted by BALB/c splenocytes. LPS (0.001 mg/ml or 1 μg/ml) was used as a positive control and DW as a negative control. Values are shown as the mean ± standard deviation (n=3). **P<0.01 and ***P<0.001, compared to the vehicle (DW)-treated control. DW, distilled water; FGE, fermented garlic extract; GE, garlic extract; LPS, lipopolysaccharide.

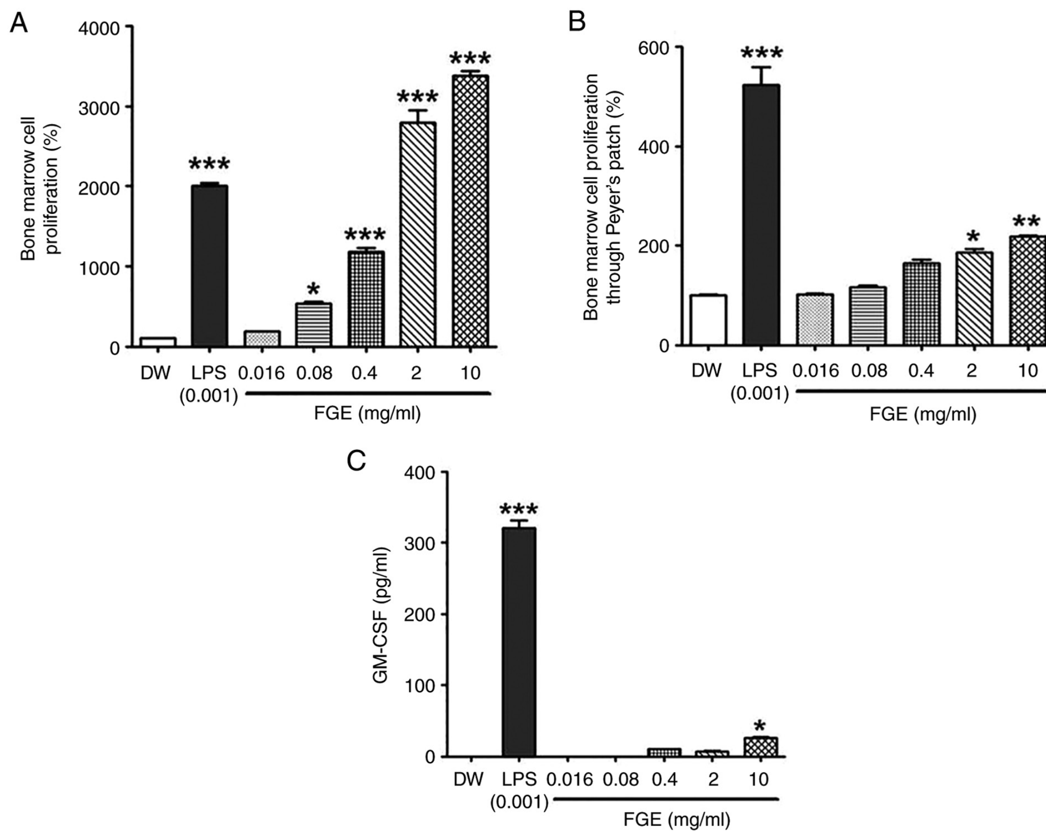

Figure 4. FGE enhances intestinal immune activity in C3H/HeN mice. Effects of FGE at concentrations of 0.016-10 mg/ml on (A) bone-marrow-cell proliferation, (B) bone-marrow-cell proliferation through Peyer's patch cells, and (C) GM-CSF production by Peyer's patch cells. LPS (0.001 mg/ml or 1 μg/ml) was used as a positive control and DW as a negative control. Values are shown as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001, compared to the vehicle (DW)-treated control. DW, distilled water; FGE, fermented garlic extract; GE, garlic extract; LPS, lipopolysaccharide.

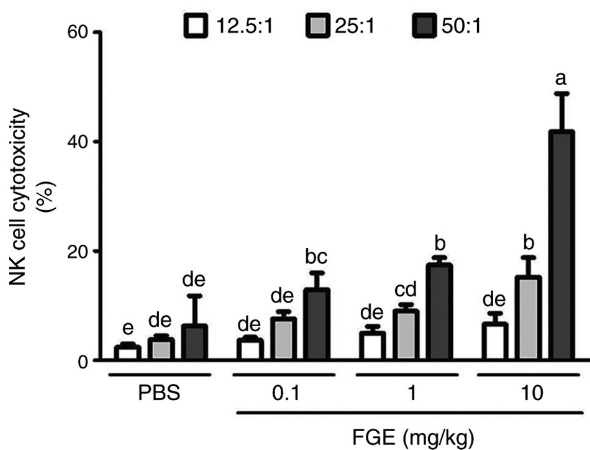

Figure 5. FGE enhances natural killer cell activity in splenocytes from BALB/c mice. Splenocytes from mice injected intravenously with 0.1, 1 and 10 mg/kg of FGE were incubated with target YAC-1 cells at varied effector-target ratios (50:1, 25:1 and 12.5:1). After 24 h, cell death was determined by the release of lactate dehydrogenase. Phosphate-buffered saline (physiologic saline)-treated groups were used as a negative control. Data are expressed as NK cell cytotoxic activity (%) and as the mean ± standard deviation, n=3. Duncan's multiple range tests were used to confirm the significance of each measurement value. Bars labeled by different letters (a-e) indicate significant differences (P<0.05). NK, natural killer; FGE, fermented garlic extract.

trend to enhance the proliferation of bone marrow cells. FGE at all concentration ranges (0.016-10 mg/ml) significantly enhanced the proliferation of bone marrow cells compared to the distilled water-treated control group. It exhibited cell proliferative activity (2.8-3.4-fold) compared to the LPS-treated positive control group.

On the other hand, by measuring the bone marrow cell proliferative activity mediated by PP cells, the effects of various concentrations of FGE on the intestinal immune system were also determined. In the present study, PP cells were stimulated directly with FGE at 0.016-10 mg/ml, and the supernatant was subsequently administered to a bone marrow cell culture to mimic hematopoietic growth factors (HGFs). As shown in Fig. 4B, FGE at the highest concentration of 10 mg/ml exerted the most prominent promoting effect on bone marrow cell proliferative activity through PPs. T-cells and B-cells comprise PP cells, where T-cells are the primary source of CSF and cytokines (25,26). In the present study, the level of the hematopoietic cytokine, GM-CSF, increased following treatment with 0.4 to 10 mg/ml FGE, with the maximum production observed at 10 mg/ml (Fig. 4C). As a consequence, the 10 mg/ml concentration of FG exceedingly stimulated HGFs from specialized immune cells, such as T-cells and intestinal PP cells, culminating in bone marrow cell differentiation. FGE, however, did not promote bone marrow cell proliferation and cytokine production as much as when compared to the LPS-treated positive control. Therefore, these findings indicate that FGE is helpful in increasing intestinal immunostimulatory activity.

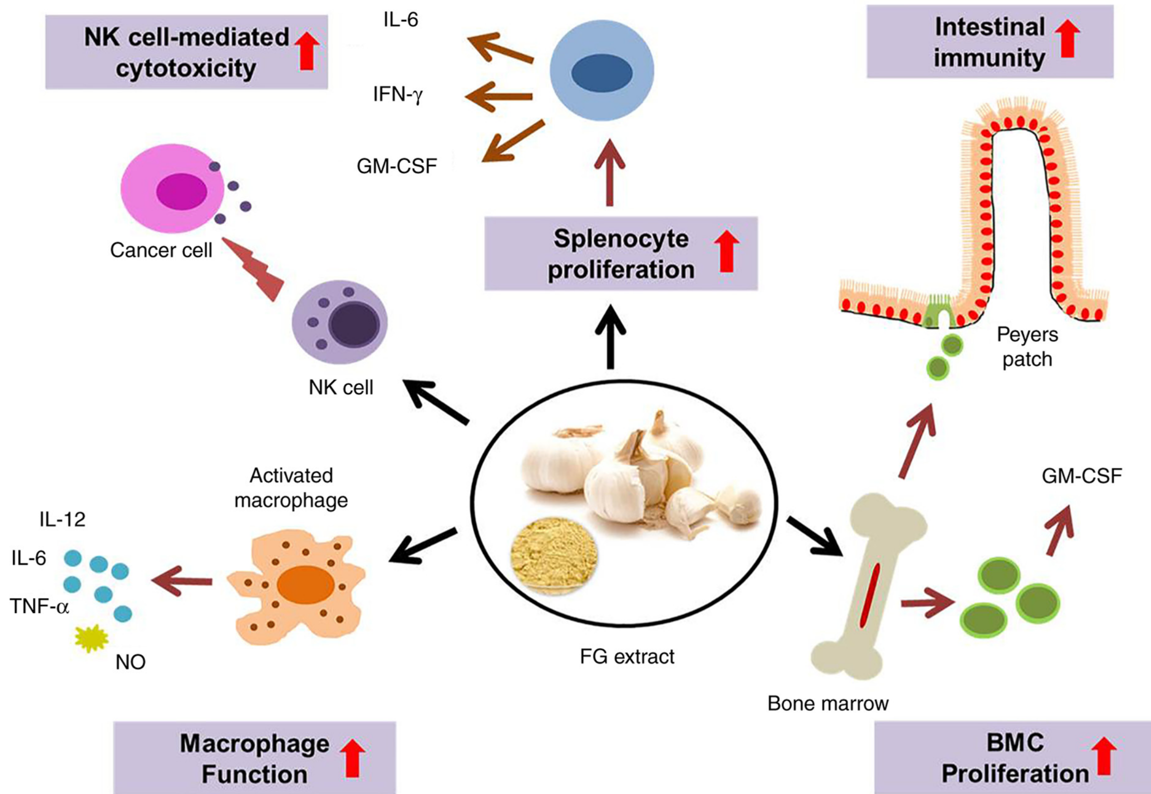

Figure 6. Conceptual diagram of FG and its proposed targets for immunomodulation. FG, fermented garlic; NK, natural killer; GM-CSF, granulocyte-macrophage colony-stimulating factor; BMC, bone marrow cell.

Effects of FGE on the cytotoxic activity of NK cells from BALB/c mice. In the present study, 6-week-old BALB/c mice were treated with PBS and, following the intravenous administration of FGE (0.1, 1 and 10 mg/kg), mouse splenocytes were employed to assess NK cell activity using YAC-1 cells as target cells. As a result, the effector (NK cells) and target (cancer cells) were compared to the saline-treated control in a ratio-dependent manner. At the ratio of 12.5:1, NK cells isolated from mice administered intravenously with physiological saline killed 2.4% of YAC-1 cells, whereas splenocytes from 0.1, 1 and 10 mg/kg FGE-treated mice killed 3.7 to 6.6% of YAC-1 cells. PBS-NK cells eliminated YAC-1 cells at a rate of 3.8% when the ratio was 25:1; however, FGE-NK cells killed cancer cells at a rate of 7.6-15.2%. When the ratio of NK cells to cancer cells was set to 50:1, PBS-NK cells cleared 6.3% of cancer cells, whereas FGE-NK cells eliminated 41.8% of YAC-1 cells. Treatment with FGE increased cytotoxic NK cell activity in the spleen and demonstrated that they can enhance the innate immune response to tumor cells (Fig. 5).

Discussion

In the present study, FG was prepared by fermentation with *B. subtilis*. It has been previously demonstrated that extracts from garlic fermented with *B. subtilis* are promising for the treatment of cardiovascular disease and blood cholesterol levels; the elevated level of stable nitrite present in the garlic contributes to these positive effects (27). However, for the first time, to the best of our knowledge, the present study established that FG prepared by using *B. subtilis* exerted immune-stimulating and non-toxic effects *in vitro* and *ex vivo*. The present study demonstrated the immunomodulatory properties of FG *in vitro* in a model of RAW264.7 cells. Macrophages, which are phagocytizing immune cells, are known to aid the body's innate and adaptive immunological responses (28). Following treatment with FGE, the present study determined that FGE activated macrophages by releasing immunostimulatory cytokines, such as TNF-α, IL-6, and IL-12 from RAW264.7 cells. In macrophages, FG promoted immunostimulatory cytokine production in a concentration-dependent manner. It is known that the excessive secretion of immunomodulatory factors by macrophages is known to serve as a chronic inflammatory factor or to be lethal to macrophages (29). As a result, the present study evaluated whether FGE cytotoxicity was observed in RAW264.7 cells. It was confirmed that FGE at concentrations ranging from 0.016 to 10 mg/ml was not cytotoxic to RAW264.7 macrophages, although FGE considerably promoted cell multiplication by 50 to 80% in comparison to the control. At the concentration of 10 mg/ml, FGE also increased peritoneal macrophage proliferation to the greatest extent. GE in comparison to FGE exerted a less prominent promoting effect on cell proliferation at low concentrations, while cytotoxicity was observed at higher concentrations. These findings suggest that FGE is not toxic to cells, and the immunomodulators generated in macrophages by FGE strengthen the human immune system in comparison to GE.

NO is an inflammatory mediator generated by macrophages and neutrophils after L-arginine activates nitric oxide synthase (30). Previous research has demonstrated that arginine in aged garlic and nitrites contained in fermented garlic

contribute to the promotion of NO generation (18,31-33). The present study also found that FGE markedly increased NO generation in RAW264.7 cells in a concentration-dependent manner in comparison to the distilled water control and GE-treated groups. Although GE promoted RAW264.7 cell proliferation at lower concentrations, it was unable to increase NO production at all concentrations. On the contrary, FGE increased cell proliferation and NO production (which reached maximum levels at the concentration of 2 mg/ml FGE). However, a concentration of FG (0.08 mg/ml) that stimulated cell proliferation, but did not produce NO suggests that the increased NO production by FGE may not result in increased macrophage proliferation. Such NO generated through garlic extracts has been proven to induce expansion and circulation by relaxing the muscles of blood vessels (34-36). Other research also suggests that FGEs, due to their greater NO generation capabilities, may have a vasorelaxing effect (33). Therefore, further research is required to dissect the role of FG in vascular endothelial function. Increased NO production, on the other hand, contributes to macrophage phagocytic activity as macrophages are innate immune cells that proliferate host defense against infections (37). In addition, FGE increased the production of pro-inflammatory cytokines, such as IL-6 and TNF-α in mouse peritoneal macrophages, which was consistent with the results obtained *in vitro*. However, a higher level and different pattern of IL-12 production were detected at a lower concentration of FGE in comparison to that in RAW264.7 cells. Kim *et al* (23) discovered that the activation of NF-κB and IRF-1 proteins by IFN-γ plays a role in the production of IL-12. Furthermore, T-cells, NK cells that release a substantial amount of IFN-γ, are found in insignificant numbers in adherent fractions of peritoneal exudate cells (23). Thus, FG therapy may have caused peritoneal macrophages to create more IL-12 in response to released IFN-γ; however, RAW264.7 cells were unable to develop such a response. They exhibited a higher INF-γ level at lower FGE concentrations and a lower INF-γ level with increasing FGE concentrations, which was consistent with the findings on splenocytes. These results are in accordance with those of previous research, in that in comparison to unfermented garlic, heat-dried garlic fermented with *Lactobacillus plantarum* boosted PBMC proliferation and NO production, and suggested that these biological activities were linked to greater phenolic and flavonoid content released by microorganisms during the fermentation process (19). Moreover, the present study found that fermenting garlic with *B. subtilis* increased macrophage proliferation, cytokine production and NO generation.

Splenocyte proliferation, together with an increase in cytokine production, leads to early humoral and cell-mediated immune enhancement (38). In the present study, for the first time, to the best of our knowledge, the immunomodulatory effects of FGE were assessed by determining mitogen activity in splenocytes. It was discovered that FGE promoted splenocyte proliferation in a concentration-dependent manner and led to a higher mitogen activity with a higher concentration than the LPS-treated group. As a major macrophage-activating cytokine, INF-γ plays a critical role in cell-mediated adaptive immunity against intracellular parasitic bacteria (39). In response to antigen recognition, a cluster of differentiation 4^+ ($CD4^+$) Th1 cells produce INF-γ, which is amplified by the production of IL-12 and IL-18 (40). As a result, in the present study, INF-γ followed a nearly identical trend to macrophage-derived IL-12 production. Splenocyte-produced IL-6 stimulates B-cell development and functions as a co-stimulator for T-cells and hepatocytes. It also promotes T-cell proliferation and stimulates the production of antibodies that trigger B-cell differentiation (41). In the present study, FGE at a low concentration level of 0.08 mg/ml led to a notable increase in IL-6 production (21.3-fold that of the distilled water control group). Following that, as the concentration increased, the production exhibited a tendency to gradually decrease. On the other hand, FGE also exhibited a concentration-dependent tendency to increase the production of splenocyte-derived GM-CSF, a powerful hematopoietic cell stimulator. By acting on the bone marrow precursor, GM-CSF facilitates the development of diverse circulating leukocytes of the innate immune system (42). As a result, FGE increased splenocyte secretion of the hemopoietic cytokines IL-6 and GM-CSF.

In the present study, the activity of *ex vivo* splenic cytotoxic NK cells was assessed to determine whether there were any links between FGE and tumor-related immunity. The effect of FGE on NK cell activation and cytotoxicity on YAC-1 cells, which lack class I major histocompatibility complex (MHC) molecules, was investigated in BALB/c mice by co-cultivation of isolated splenocytes with the NK cell sensitive YAC-1 target cell. The results revealed a concentration-related significant effect on NK cytotoxic activity in relation to the control in all ratios of effector cell to the target cell. These associations indicate that FG exerts antitumorigenic activities through the promotion of NK cell responses to YAC-1 tumor cell lysis. *In vitro* cytotoxic studies have revealed that aged garlic extract significantly improves the cytotoxic capability of human peripheral blood NK cells against K562 tumor cells (43,44), which is consistent with the findings of the present study. Thus, the ability of FGE to stimulate immune cell IL-12 release must have aided the generation of INF-γ-producing NK cells. As demonstrated herein and in previous research, the inhibition of tumor cell lines, such as YAC-1 may be further aided by extract-induced NK cells (43).

The small intestine's lumen is exposed to a range of bacteria and antigens, and gut-associated lymphoreticular tissues, such as PPs play a role in immune monitoring and intramucosal immune response (45). M-cells, which are specialized cells, transport external antigens directly from the lumen to antigen-presenting cells. After lymphocytes triggered by antigens travel to the mesenteric lymph nodes, activated T- and B-cells flow into the circulation via the thoracic duct, augmenting the immune response. Therefore, if the food item stimulates the PP in the colon, it could be produced as a functional food that helps the body's systemic immune response (46). To the best of our knowledge, there are no available studies to date on the impact of fermented garlic on the gut immune system via PP cells. Hence, the present study experiment is the first to demonstrate that FG increased bone marrow cell proliferation via PP cells. As PP cells are comprised of T- and B-cells, and T-cells produce GM-CSF, it can be hypothesized that FGE increased T-cell activation and the production of the hematopoietic growth factor, GM-CSF. As a result, it was found that FGE strongly promoted splenocytes to create the hematopoietic cytokines,

IL-6 and GM-CSF, which were sufficient to cause mouse bone marrow cells to proliferate and differentiate into various hematopoietic cells. Therefore, FG has the potential to significantly proliferate both murine peritoneal macrophages and splenocytes in a concentration-dependent manner. Hence, to confirm the link with systemic immunity, GM-CSF generation in the culture supernatant of PP cells with FGE and bone marrow cell proliferation by treatment with PPs were assessed in the present study. As a result, FGE may act as an inducer for strengthening the weakened intestinal immune system and promoting intestinal immunity through a functional diet.

Taken together, the present study strongly suggests that FG stimulates NK cell activity, the proliferation of macrophages, and splenic T- and B-cells, suggesting that it can improve cell-mediated immunity. FGE can enhance the cellular immune response by upregulating Th1 cytokines. It has intestinal immunostimulatory activity and positive effects on hematopoiesis and the production of immune cells. A conceptual diagram of FG and its proposed targets for immunomodulation is presented in Fig. 6.

In conclusion, the present study demonstrates that FG has immunomodulatory properties in both *ex vivo* and *in vitro* settings. Its action is mediated by the stimulation of macrophage functions, the production of NO and the lytic activity of NK cells-induced by INF-γ. The production of IL-6 and GM-CSF by FG plays a crucial role in bone marrow cell proliferation, which involves hematopoietic regulation. Furthermore, FG also has a favorable effect on intestinal health by increasing gut immunity through immune cell stimulation and cytokine production. Several hypotheses can be advanced to explain the mechanism underlying the reported modulation of local and systemic immune responses through intestinal immune responses by FG, which may be related to the substantial number of transformable organosulfur components formed by *B. subtilis*. However, further studies are warranted to concentrate on isolating the active components found in FGE and assess the impact of these compounds on a variety of immune system parameters. On the whole, FG may have an advantage in the development of nutraceutical products and functional agents that can help strengthen and sustain a strong immune system.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

All the experiments were devised by YWK and PG. Experiments were carried out by PG at the Kyung Hee University Skin Biotechnology Center and Dong-Sung Cancer Center. PG wrote the manuscript, prepared all the figures and revised the manuscript. YWK supervised the study and provided advice on the design, as well as manuscript preparation. JL assisted with the experiments and in manuscript preparation. TBTM and RS assisted with the study design and manuscript preparation, and revised the manuscript. YWK and PG confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUSBC-R-SPA 2018-0601) and the Institutional Animal Care and Use Committee of the Dongsung Cancer Center under protocol IACUC #ds002205112-EUTO3. The experiments were carried out in compliance with the ARRIVE guidelines.

Patient consent for publication

Not applicable.

Competing interests

Note that the authors are affiliated with the company (Dongsung Biopharmaceutical) that provided the fermented garlic whose effects have been investigated in this study.

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