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Ginsenosides Rg1 and Re act as adjuvant via TLR4 signaling pathway

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ABSTRACT

Previous studies have demonstrated that ginsenosides Rg1 and Re extracted from the root of *Panax ginseng* C.A. Meyer have adjuvant properties. However, the molecular mechanisms behind their adjuvant activities remain unclear. In the present study, we first investigated the adjuvant effect of Rg1 and Re on the immune responses to a model antigen ovalbumin (OVA) in C3H/HeB mice as well as in C3H/HeJ mice carrying a defective toll-like receptor-4 (TLR4) gene, and then evaluated Rg1 and Re for their stimulation of phosphorylation of nuclear factor-kappa B (NF-κB) p65 in the macrophages from above two different strains of mice. In addition, Rg1 and Re were also evaluated for their induction of NF-κB in RAW-Blue™ cells. The results showed that Rg1 and Re had adjuvant activities in stimulating IgG, splenocyte proliferation, and mRNA expression of cytokines IL-4, IL-10, IL-12 and IFN-γ as well as transcription factors GATA-3 and T-bet by splenocytes in C3H/HeB mice but not in C3H/HeJ mice. Rg1 and Re induced phosphorylation of NF-κB p65 at Ser536 in macrophages from C3H/HeB mice but not from C3H/HeJ mice. Both Rg1 and Re induced expression of NF-κB in RAW-Blue™ cells. These results suggested that TLR4 signaling pathway is involved in the adjuvant activities of Rg1 and Re. Nevertheless, pretreatment with anti-TLR4 antibody suppressed the Re- but not Rg1-induced expression of NF-κB, indicating that Rg1 may trigger both extracellular and intracellular TLR4 by passing through the cell membrane while Re only activate extracellular TLR4 as it fails to enter inside of the cells to stimulate intracellular TLR4.

1. Introduction

The root of *Panax ginseng* C.A. Meyer (ginseng) is one of the most popular traditional Chinese medicines. Ginseng as a general tonic has been officially written in the Chinese Pharmacopoeia [1,2]. The global production of the root has been increased from 3500 tonnes in 2004 to 6000 tonnes in 2010, accounting for the second-highest selling herbs in the United States and Europe [3,4]. Ginseng saponins (GS), or ginsenosides, are believed to be one of the biologically ingredients in ginseng extracts. More than 40 ginsenosides have been identified in *P. ginseng* [5]. Recent investigations have shown that GS has adjuvant effects on the immune responses. Rivera et al. [6] have observed an increased antibody response to vaccination against porcine parvovirus (PPV) in guinea pigs when PPV antigen is mixed with a GS. Kong et al. [7] have found that GS has promoted both humoral and cellular immune responses to Newcastle disease (ND) vaccine in chickens. Qu et al. [8] reported significantly increased humoral and cellular immune responses against toxoplasmosis in ICR mice immunized with a recombinant *Toxoplasma gondii* SAG1 antigen and Rg1.

Adjuvant properties of GS may be related to its activation of the innate immunity. The antibody response can be enhanced by injection of GS at a separate site of the body from antigen [9], suggesting that GS might trigger the immune system in a non-specific manner. In another study for evaluation of ginsenoside Rb1 as an adjuvant in stimulation of the immune response to PPV vaccines [10], production of cytokines including IFN-γ, IL-2, IL-4, IL-10 and tumor necrosis factor α (TNF-α) has been effectively promoted, indicating that both Th1 and Th2 responses have been activated. Although much has been done regarding the adjuvant properties and immunomodulatory activities of ginseng, the molecular mechanisms remain unclear. Toll-like receptors (TLRs) are germline-encoded receptors expressed on innate immune cells, and play a critical role in the immune response to pathogens by sensing microorganisms. To date, 10 members of the TLR family in humans and 12 in mice have been identified [11–16]. In addition to the substances originated from microorganisms as ligands [17,18], TLR4 also recognizes plant-derived molecules such as taxol [19,20]. To investigate if GS are associated with TLR4 in their adjuvant activities, we evaluated the adjuvant effects of ginsenosides Rg1 and Re on the immune responses in C3H/HeB stain mice with a normal expression of *tlr4* gene as well as in C3H/HeJ stain mice with a point mutation at *tlr4* gene, and their effect on the phosphorylation

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of NF-κB p65 in the macrophages isolated from the two different mice.

2. Materials and methods

2.1. Chemicals and reagents

Ginsenosides Rg1 and Re extracted from the root of *P. ginseng* were purchased from Tauto Biotech Co. Ltd. (Shanghai, China). Ovalbumin (OVA) was the product of Sigma Chemical Co., USA. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Hyclone Laboratories, Inc. (Logan, UT).

2.2. Animals

C3H/HeB ($TLR4^{+/+}$) and C3H/HeJ ($TLR4^{-/-}$) aged 5–8 weeks were obtained from the Model Animal Research Center of Nanjing University. Mice were housed in polypropylene cages with sawdust bedding in hygienically controlled environment. The temperature was controlled at 24 ± 1 °C and humidity at 50 ± 10%. Feed and water were supplied ad libitum. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.3. Immunization and sample collection

Either C3H/HeB ($TLR4^{+/+}$) or C3H/HeJ ($TLR4^{-/-}$) mice were randomly divided into 5 groups with each containing 6 animals of the same stain. The mice were subcutaneously immunized twice at 3-week intervals with saline (200 μl) or saline (200 μl) containing OVA (10 μg) alone or with Rg1 (50 μg), Re (50 μg) or LPS (25 μg). Two weeks after that, blood samples were collected from the orbital venous sinus for measurement of serum OVA-specific IgG; splenocytes were harvested for determination of lymphocyte proliferation, cytokines and transcription factors mRNA.

2.4. Determination of serum IgG

An indirect enzyme-linked immunosorbent assay (ELISA) was conducted to measure serum OVA-specific as described by Xiao et al. [21]. Briefly, polyvinyl 96-well microtiter plates were coated in 100 μl of OVA solution (5 μg/ml in 0.05 M carbonate buffer, pH 9.6). After washing, the wells were blocked with 5% FBS in PBS and incubated for 2 h. To measure IgG, 100 μl of diluted serum (1:1000) was added and the plates were incubated at 37 °C for 1 h. After another washing, 100 μl of goat anti-mouse IgG diluted in PBST (1:5000) was added and incubated at 37 °C for 1 h. Plates were washed again, and 100 μl of 3,3′,5,5′-tetramethyl benzidine (Exalpha Biologicals, Inc., USA) substrate solution (100 μg/ml of 0.1 M citrate-phosphate, pH 5.0) was added to each well and incubated for 15 min. The reaction was stopped using 50 μl of 2 M H_2SO_4. The optical density of the plates was read at 450 nm by an ELISA plate reader (Thermo Multiscan MK3, USA).

2.5. Lymphocyte proliferation

Spleen was collected 2 weeks after the booster immunization, and kept in Hank's balanced salt solution (HBSS, Sigma). The organ was minced and passed through a steel mesh to obtain a homogeneous cell suspension. To lyse contaminated erythrocytes, 0.83% NH_4Cl in 0.01 M Tris–HCl (pH 7.2) was added. After centrifugation ($380 \times g$ at 4 °C for 10 min), the pelleted cells were washed in HBSS and re-suspended in medium of RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FBS. Cell viability was estimated using the trypan blue exclusion [22] and the concentration of viable lymphocytes was more than 95%. Lymphocyte proliferation was assayed as previously described [23]. Briefly, splenocytes were seeded into a 96-well flat-bottom microtiter plate (Nunc) at 5.0×10^6 cells/ml, thereafter Con A (5 μg/ml), LPS (8 μg/ml), OVA (100 μg/ml) or medium was added giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 48 h. To each well, 50 μl of MTT solution (2 mg/ml) were added 4 h before the end of incubation. After centrifugation of the plates ($1400 \times g$, 5 min), untransformed MTT was removed carefully. To each well, 150 μl of a DMSO working solution (192 μl DMSO with 8 μl 1 M HCl). After 15 min, the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference. The stimulation index (SI) was calculated based on the formula: SI = the OD value for mitogen cultures/the OD value for non-stimulated cultures.

2.6. Quantification of target genes by real-time PCR

Splenocytes were prepared as above, and were seeded into a 24-well flat-bottom microtiter plate (Nunc) at 5×10^6 in 2 ml of RPMI medium. Thereafter 20 μl OVA (10 μg/μl) was added, and incubated at 37 °C. After 15 h treatment, cells were centrifuged 10 min ($380 \times g$ at 4 °C), and washed in ice-cold PBS, then subjected to RNA extraction. Splenocytes were lysed in 1 ml of RNAiso™ Plus (Takara, China) and RNA was isolated according to the manufacture's protocol. The concentration of RNA was determined at 260 nm. Reverse transcription was performed by mixing 1 μg of RNA with 5 μl iScript reagent (Bio-Rad) in a DEPC-treated tube, thereafter the final volume was adjusted to 20 μl with nuclease-free water. The reverse transcription was performed in a condition of 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, hold at 4 °C.

Relative quantitation of GATA-3, T-bet and cytokines cDNA to β-actin was conducted on ABI 7500 (PE Applied Biosystems, USA) [24]. The primers and probes for target genes and β-actin were the same as described by Yuan et al. [23]. Amplification was carried out in a volume of 20 μl solution containing 2 μl of 10× PCR buffer, 2 μl of $MgCl_2$ (25 mmol/l), 2 μl of dNTPmix (2.5 mM), 0.4 μl of Tag DNA polymerase (Takara, China), 2 μl of cDNA template, 2 μl (5 μM) of each target gene and β-actin specific primers, 1 μl (5 μM) of target gene and β-actin specific probes. Reaction conditions were the standard conditions for the TagMan PCR (15 s denaturation at 95 °C, 30 s annealing at 60 °C) with 45 PCR cycles. Relative quantification between samples was achieved by the $2^{-\Delta\Delta CT}$ [25] method and calculated by software REST 2005 (gifted by Eppendorf company). The data was reported as the *n*-fold difference relative to target gene mRNA expression in the calibrator group (the mice immunized with saline) [25,26].

2.7. SEAP reporter assay

RAW-Blue™ cells (Invitrogen, San Diego, CA) are derived from RAW264.7 macrophages with chromosomal integration of pNiFty-SEAP, a plasmaid expressing secreted embryonic alkaline phosphatase (SEAP) gene under the control of an NF-κB-inducible ELAM-1 (E-selectin) promoter [27]. The macrophage cell lines were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and zeocineosin (200 μg/ml).

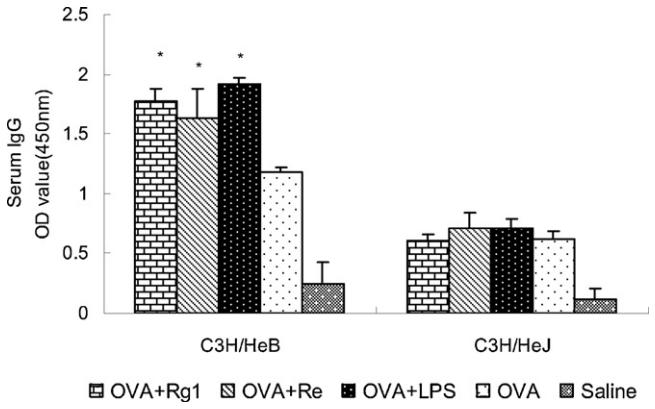

Fig. 1. Serum OVA-specific IgG responses in C3H/HeB (TLR4$^{+/+}$) and C3H/HeJ (TLR4$^{-/-}$) mice. Animals (n = 6/group) were subcutaneously injected with saline or OVA with or without Rg1, Re or LPS on days 1 and 21. Sera were collected 2 weeks after the second immunization for analysis of IgG levels using indirect ELISA. Data are expressed as mean ± SD. Bars designated as * are statistically different from the OVA group (P < 0.05).

The cell suspension (2×10^6 cells/ml) were pretreated in a 96-well plate with rabbit anti-mouse TLR4 antibody (20 μg/ml final, Novus Biologicals, Littleton, CO.) for 1 h at 37 °C. After washing, the cells were treated in saline or 30 μg/ml ginsenosides or 1 μg/ml LPS for 18 h. The supernatants were collected for SEAP secretion assay. QUANTI-Blue™ powder was dissolved in endotoxin-free water and sterile filtered (0.22 μm) to produce a QuantiQuanta-blue substrate. The cell supernatant (40 μl/well) were added to the substrate (160 μl/well) and incubated at 37 °C for 1 h. Absorbance was measured at 630 nm by an ELISA plate reader (Thermo Multi-scan MK3, USA).

2.8. Phosphorylation of NF-κB p65 in macrophages

Primary mouse macrophages were harvested from the peritoneal cavity of C3H/HeB or C3H/HeJ mice 4 days after intraperitoneal injection with 2 ml of 4% Brewer's thioglycollate broth (BBL, Sparks, MD) and cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 200 U/ml penicillin, and 200 μg/ml streptomycin in a 5% CO_2, 95% air atmosphere at 37 °C.

Peritoneal macrophages (1×10^6 cells/ml) were treated with 30 μg/ml ginsenosides or 1 μg/ml LPS in 6-well plates. After indicated periods, cells were harvested and lysed in a RIPA lysis buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM Na_3VO_4. The whole-cell lysates were prepared for p65 phosphorylation detection by Western blot.

2.9. Western blot

The protein of cell lysates was quantified by the BCA reagent (Sigma–Aldrich). The samples were diluted with 2× Laemmli sample buffer (1:1) and boiled for 5 min. After centrifugation at 12,000 × g in 4 °C for 5 min, 30 μl of diluted sample was subjected to 12% (w/v) separating and 5% stacking gel (SDS-polyacrylamide gel electrophoresis). The proteins were transferred onto the immobilon-p transfer membrane (Millipore Corporation, USA), which was washed and then blocked in 5% skimmed milk in TBS for 1 h at 37 °C in an incubating shaker. After three washes in TBST, the membrane was incubated overnight with phosphor-Ser536 p65 antibody (1:1000, Cell Signaling Techonology, Danvers MA) at 4 °C with gentle shaking. The membrane was washed three times in TBST, followed by incubating with 1:2000 goat anti-rabbit IgG (sc-2004) for 1 h at 4 °C. After the final wash, the immunoblot was examined by BeyoECL Plus (Beyotime Biotechnology, China) according to the manufacturer's instructions. The membrane was exposed to an X-ray film, and developed.

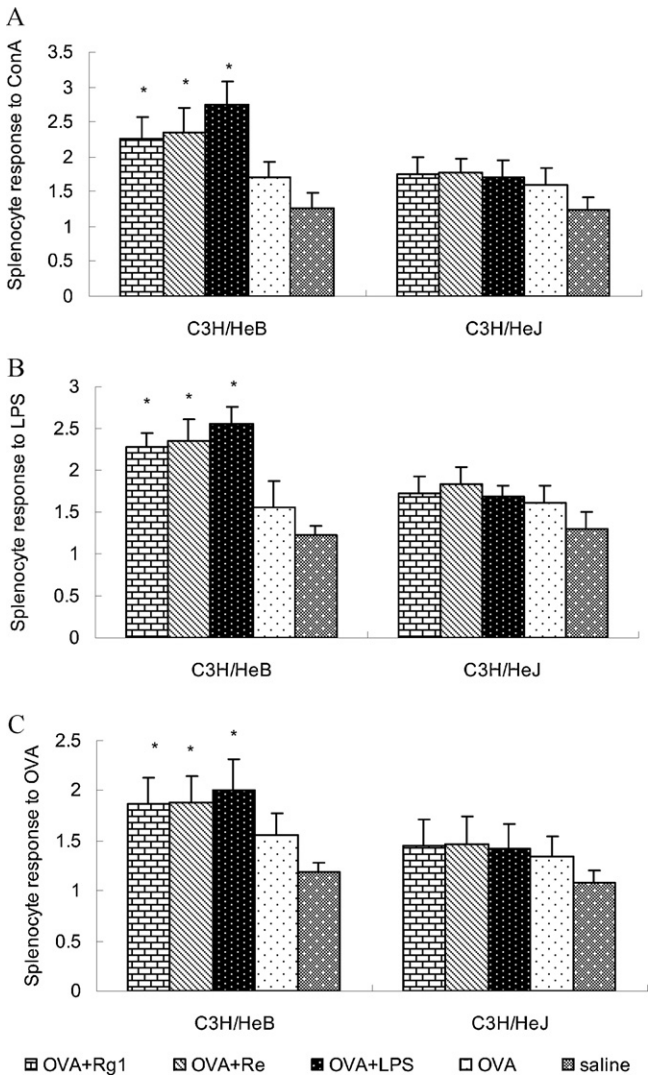

Fig. 2. Splenocyte proliferation in responses to ConA, LPS or OVA stimulation in C3H/HeB (TLR4$^{+/+}$) and C3H/HeJ (TLR4$^{-/-}$) mice. Animals (n = 6/group) were subcutaneously injected with saline or OVA with or without Rg1, Re or LPS on days 1 and 21. Splenocytes were prepared 2 weeks after the second immunization and cultured with ConA, LPS or OVA. Splenocyte proliferation was measured by the MTT method as described in the text, and shown as a stimulation index (SI). Data are expressed as mean ± SD. Bars designated as * are statistically different from the OVA group (P < 0.05).

2.10. Statistical analysis

Data were expressed as means ± standard deviations (S.D). Duncan's test was used to compare the parameters between groups by using SPSS 13.0. P-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Ginsenosides Rg1 and Re enhance the serum IgG response to OVA in C3H/HeB (TLR4$^{+/+}$) but not in C3H/HeJ (TLR4$^{-/-}$) mice

Rg1, Re or LPS significantly increased the serum IgG response to OVA in C3H/HeB (TLR4$^{+/+}$) mice when compared with the mice

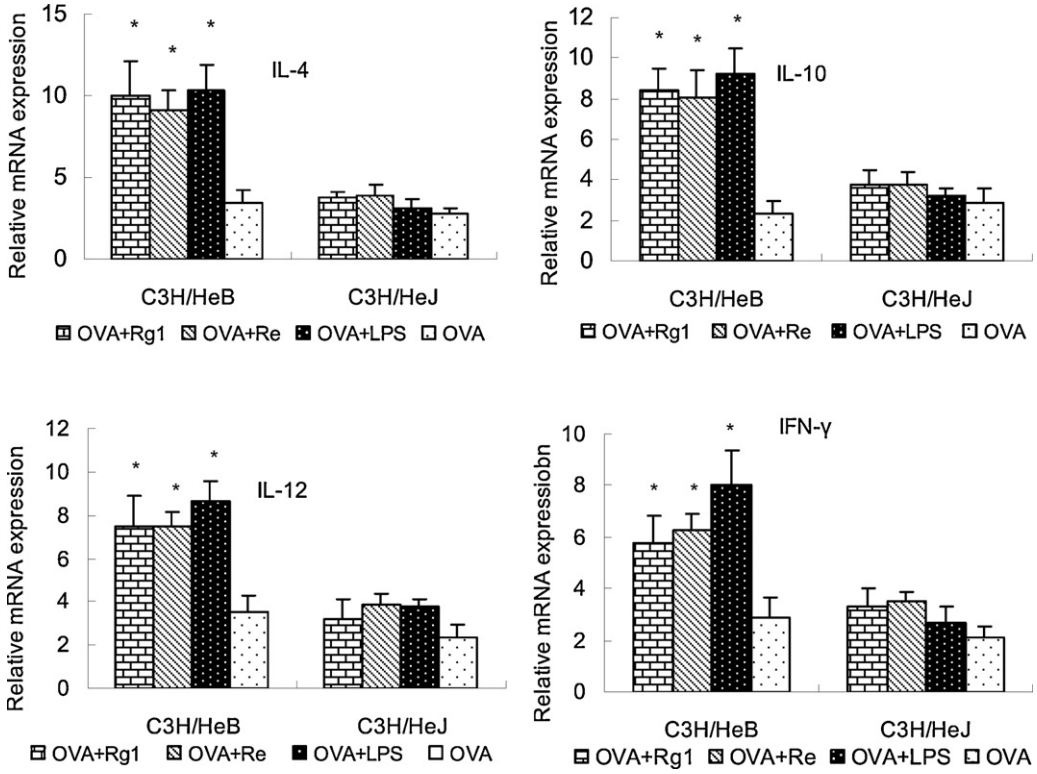

Fig. 3. mRNA expression of IL-4, IL-10, IL-12 and IFN-γ by splenocytes isolated from C3H/HeB (TLR4$^{+/+}$) and C3H/HeJ (TLR4$^{-/-}$) mice. Animals (n = 6/group) were subcutaneously injected with OVA with or without Rg1, Re or LPS on days 1 and 21. Splenocytes were prepared 2 weeks after the second immunization and cultured with OVA (100 μg/ml) for 15 h. Production of cytokine mRNA was analyzed by real-time PCR. Results are reported as n-fold difference relative to cytokine mRNA expression of calibrator samples from the mice injected with saline only (n = 6/group). Bars designated as * are statistically different from the OVA group (P < 0.05).

immunized with OVA alone, but no significantly increased IgG response was recorded in C3H/HeJ mice (TLR4$^{-/-}$) as shown in Fig. 1 (P < 0.05).

3.2. Ginsenosides Rg1 and Re enhance the splenocyte proliferative response to mitogen stimulation in C3H/HeB (TLR4$^{+/+}$) but not C3H/HeJ (TLR4$^{-/-}$) mice

Compared with the mice immunized with OVA alone, Rg1, Re or LPS significantly promoted splenocyte proliferative response to ConA, LPS and OVA in C3H/HeB mice (TLR4$^{+/+}$), but no significantly increased proliferation was recorded in C3H/HeJ (TLR4$^{-/-}$) mice as shown in Fig. 2 (P < 0.05).

3.3. Ginsenosides Rg1 and Re promote mRNA expression of cytokines and transcription factors by splenocytes in C3H/HeB (TLR4$^{+/+}$) but not C3H/HeJ (TLR4$^{-/-}$) mice

Compared with the mice immunized with OVA alone, Rg1, Re or LPS significantly increased the mRNA expression of cytokines IL-4, IL-10, IL-12 and IFN-γ as well as transcription factors GATA-3 and T-bet in C3H/HeB mice, but no significantly increased cytokines and transcription factors was recorded in C3H/HeJ mice as shown in Figs. 3 and 4 (P < 0.05).

3.4. Anti-TLR4 antibody blocks Re- but not Rg1-increased activity of SEAP in murine macrophages

Fig. 5 indicates that SEAP activity was enhanced in the culture of RAW-Blue™ cells when incubated with Rg1, Re or LPS. Pretreatment of RAW-Blue™ cells with anti-TLR4 antibody blocked Re- and LPS- but not Rg1-increased activity of SEAP.

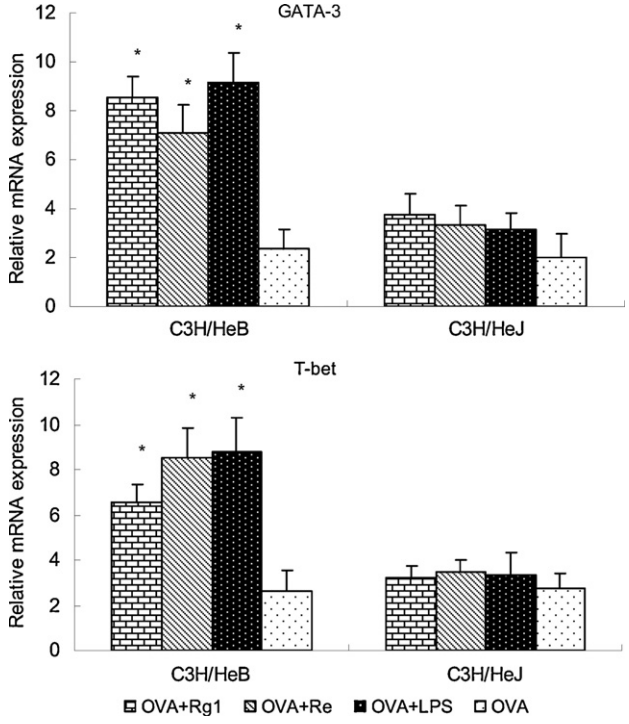

Fig. 4. mRNA expression of GATA-3 and T-bet by splenocytes isolated from C3H/HeB (TLR4$^{+/+}$) and C3H/HeJ (TLR4$^{-/-}$) mice. Animals (n = 6/group) were subcutaneously injected with OVA with or without Rg1, Re or LPS on days 1 and 21. Splenocytes were prepared 2 weeks after the second immunization and cultured with OVA (100 μg/ml) for 15 h. Production of cytokine mRNA was analyzed by real-time PCR. Results are reported as n-fold difference relative to cytokine mRNA expression of calibrator samples from the mice injected with saline only (n = 6/group). Bars designated as * are statistically different from the OVA group (P < 0.05).

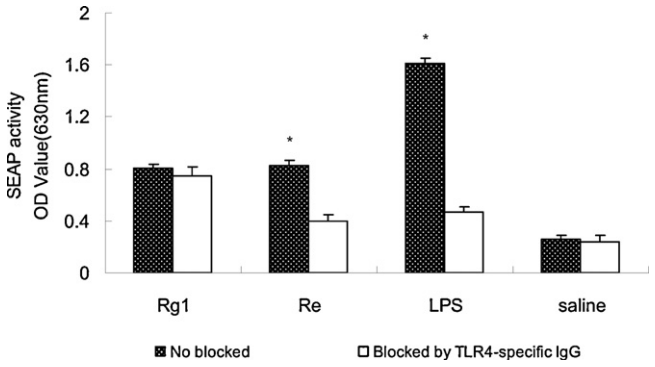

Fig. 5. Activity of the SAEP released from RAW-Blue™ cells. RAW-Blue™ cells were treated with anti-TLR4 antibody (5 μg/ml), and then cultured with Rg1 (30 μg/ml), Re (30 μg/ml) or LPS (1 μg/ml). After that, QUANTI-Blue™ was added to measure the activity of SEAP. Data are expressed as mean ± SD ($n = 3$). Bars designated as * are statistically different from the group blocked by TLR4-specific IgG ($P < 0.05$).

3.5. Ginsenoside Rg1 induces phosphorylation of NF-κB p65at Ser-536 in the macrophages isolated from C3H/HeB (TLR4$^{+/+}$) but not C3H/HeJ (TLR4$^{-/-}$) mice

Both Rg1 and LPS treatments led to p65 phosphorylation at serine 536 in a time-dependent manner. The phosphorylation of NF-κB p65 started to be detected at 30 min and reached to the peak at 50 min after C3H/HeB macrophages were cultured with Rg1 (Fig. 6A). While, the earliest phosphorylation was found at 10 min and peaked at 30 min when C3H/HeB macrophages were cultured with LPS (Fig. 6B). In contrast, no detectable phosphorylation of NF-κB p65 was found in C3H/HeJ macrophages cultured with either Rg1 or LPS.

4. Discussion

Recent investigations showed that Rg1 and Re had adjuvant properties to enhance the immune responses in animals. Sun et al. [28] reported that OVA, when injected with Rg1 or Re, induced significantly higher IgG, IgG1 and IgG2a responses than OVA was injected alone in mice. Song and co-workers [29] observed that influenza vaccine (H_3N_2), when injected with Re, elicited significant higher specific IgG, IgG1, IgG2a and IgG2b, HI titers, lymphocyte proliferation responses as well as IFN-γ and IL-5 secretions. Sun et al. [30] also reported a synergistic activity of Rg1 and alum in promotion of Th1/Th2 immune responses. Similarly, the present study demonstrated that Rg1 and Re significantly promoted IgG responses in C3H/HeB mice but not in C3H/HeJ (TLR4$^{-/-}$) mice (Fig. 1). The enhanced IgG was associated with simultaneously up-regulated transcription factors T-box (T-bet) and GATA-binding protein-3 (GATA-3) expressed in T-cells in C3H/HeB mice as shown in Fig. 4, leading to the increased expression of Th1 (IL-12 and IFN-γ) and Th2 (IL-4 and IL-10) as shown in Fig. 3.

The lymphocyte proliferation depends on the mitogen used. ConA stimulates T cell whereas LPS stimulates B cell [31]. In Rg1- or Re-adjuvanted group, significantly enhanced lymphocyte proliferation was induced by Con A (Fig. 2A) and LPS (Fig. 2B) in C3H/HeB mice but not in C3H/HeJ mice, indicating that both T and B cells were activated. In order to induce antibody production, triggered

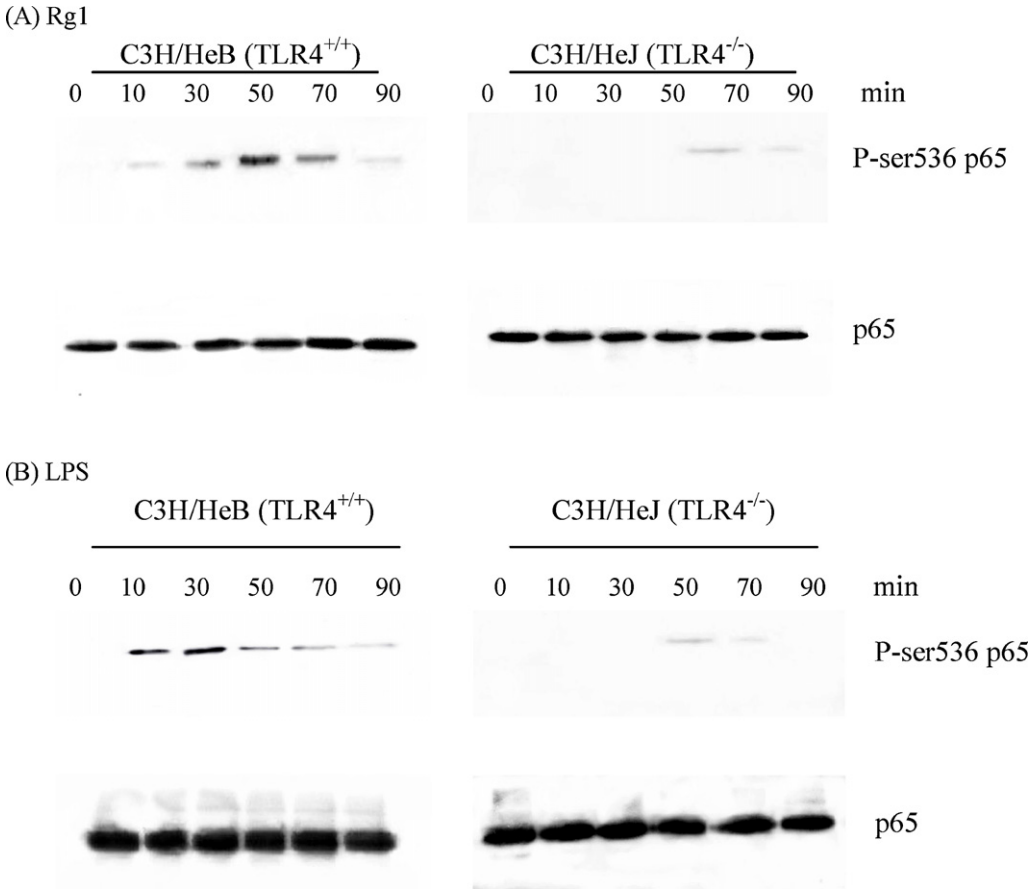

Fig. 6. Ser-536 phosphorylation of NF-κB p65 in the macrophages isolated from C3H/HeB (TLR4$^{+/+}$) and C3H/HeJ (TLR4$^{-/-}$) mice. Macrophages harvested from the peritoneal cavity of C3H/HeB and C3H/HeJ mice were cultured with Rg1 or LPS for the indicated period. The cell lysates were immunoprecipitated with the anti-phospho-Ser536-p65 and anti-p65 antibodies. Results are representative of three independent experiments.

B lymphocytes are required for clonal expansion. The enhanced lymphocyte responses to OVA (Fig. 2C) paralleled the increased OVA-specific IgG in C3H/HeB mice injected with OVA plus Rg1 or Re (Fig. 1). As Rg1 and Re showed adjuvant activities in C3H/HeB mice with a normal *tlr4* gene but not in C3H/HeJ stain mice having a point mutation at *tlr4* gene, we hypothesized that the adjuvant effects of Rg1 and Re may be related to TLR4 signaling pathways.

Nuclear factor-kappaB (NF-κB) is an important transcription factor responsible for regulation of various proinflammatory cytokines expression and plays a crucial role in the innate immunity [32]. In this study, the activity of Rg1 and Re as NF-κB inducer was evaluated by the SEAP reporter assay in RAW-Blue™ cells. The cells stably express a SEAP gene induced by NF-κB. However, basal expression of the SEAP gene is too low, and usually induced by stimuli, such as LPS. The SEAP secreted to media was measured by a SEAP assay kit. Activation of TLR4 is closely related to the expression of NF-κB [33,34]. Increased activity of SEAP as shown in Fig. 5 indicates that both Rg1 and Re activated TLR4 and induced expression of NF-κB in the cells.

Although Rg1 and Re share a similar chemical structure with a dammarane skeleton, Rg1 had an additional rhamnose at C-6 position [35], and the two ginsenosides presented contradictory behaviors in a TLR4-blocking assay. After pretreated with anti-TLR4 antibody, SEAP activity was completely damped when RAW-Blue™ cells were cultured with Re, but remained stable when the cells were stimulated with Rg1 (Fig. 5). The different effects of Rg1 and Re on the activity of SEAP released from RAW-blue™ cells pretreated by anti-TLR4 antibody might be explained by the hypothesis that Rg1 may penetrate into the cells and bind to the intracellular TLR4, which initiated a signaling cascade including NF-κB activation while Re may not be able to pass the cell membrane, and failed to activate the TLR4 when the extracellular portion of TLR4 was blocked by anti-TLR4 antibody. However, further study is needed to elucidate the exact reasons.

Phosphorylation of NF-κB p65 subunit at the site of serine-536 (phospho-Ser536-p65) is important for regulation of transcriptional activity and nuclear localization [36]. In order to clarify the relation between Rg1 and TLR4, we determined the phosphorylation by measuring phospho-Ser536-p65 in Rg1-treated macrophages isolated from C3H/HeB and C3H/HeJ mice. Fig. 6 showed that Rg1 as well as LPS induced NF-κB p65 subunit phosphorylation in the macrophages of C3H/HeB mice but not of C3H/HeJ mice (Fig. 6A and B). This result could be due to the reason that Rg1- and LPS-induced phosphorylations were TLR4-mediated, and the phosphorylation did not take place in the macrophages of C3H/HeJ stain mice because the cells had a point mutation at *tlr4* gene with a damaged TLR4 signaling pathway.

In summary, the present study demonstrated that the adjuvant activities of Rg1 and Re were TLR4-dependent. A model antigen OVA, when injected with Rg1 or Re, induced significantly higher IgG response, splenocyte proliferation, and mRNA expression of cytokines IL-4, IL-10, IL-12 and IFN-γ and transcription factors GATA-3 and T-bet by splenocytes in C3H/HeB mice but not C3H/HeJ mice. The Rg1- or Re-enhanced immune responses were associated with activated phosphorylation of NF-κB p65. Rg1 triggered both extracellular and intracellular TLR4 by passing through the cell membrane while Re only triggered extracellular TLR4 as it failed to pass through the cell membrane to activate intracellular TLR4.

Acknowledgement

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