

Ginseng and Salviae herbs play a role as immune activators and modulate immune responses during influenza virus infection

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Abstract

We have investigated the adjuvant roles of common herbal medicines (ginseng, Salviae) and their effects on early immune responses during influenza virus infection in a mouse model. Intranasal co-administration with inactivated influenza virus A (PR8) and ginseng or Salviae extract increased the levels of influenza virus specific antibodies and neutralizing activities compared to immunization with PR8 alone, and provided protective immunity. Salviae co-administration significantly enhanced IFN- γ and IL-2 cytokine producing splenocytes while ginseng induced high levels of IL-4 and IL-5 cytokine producing cells after challenge infection. Cells expressing an early activation marker CD69 and levels of a pro-inflammatory cytokine IL-6 were highly elevated in lungs from naïve mice during challenge virus infection, which might be a mechanism in lung inflammation leading to death. In contrast, immunized mice that were co-administered ginseng or Salviae modulated CD69 expressing immune cells, did not produce IL-6, and showed significant enhancement of influenza virus specific IgA antibody in lungs after challenge virus infection. Therefore, these results indicate that both ginseng and Salviae play a role as mucosal adjuvants against influenza virus as well as immuno-modulators during influenza virus infection.

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1. Introduction

Herbal medicines have been used in humans for a thousand years to treat medical illness or to improve physical performance. Among over 20,000 herbal medicines known to be in human use, very few have been proven to have efficacy by controlled experiments [1,2]. The herbal extracts contain a mixture of proteins and other organic chemicals and carbohydrates, which may include alkaloids, flavonoids, glycosides, saponins, and terpenes [3,4]. Since approximately one third of human drugs were originally derived from plant sources, research efforts have been encouraged towards identifying novel herbal products that have efficacy for treating disease [2].

Ginseng is one of the most well studied herbal medicines. A placebo-controlled study in normal human volunteers revealed that ginseng significantly increased functions of neutrophil, CD4 T, and natural killer cells [5]. In an *in vitro* study, ginseng was also found to increase the activities of peripheral blood mononuclear cell from AIDS patients and normal individuals [6]. Salviae species are used as traditional medicines around the world. Previous studies demonstrated that Salviae species improved renal disease [7], enhanced cardiovascular activities promoting blood flow [8], exhibited anti-inflammatory [9] and sedative effects [10], and ameliorated acute hepatotoxicity [11].

The potential adjuvant and/or immunomodulatory effects of these herbal medicines after mucosal immunization have not been previously studied. In this study, we investigated the possible roles of ginseng and Salviae extracts as mucosal adjuvants and their potential immunomodulatory functions.

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Immune responses were evaluated after intranasal immunization of mice with an inactivated influenza PR8 virus co-administered with the herbal extracts ginseng or *Salviae*. The adjuvant effects of ginseng and *Salviae* extracts were found to be comparable to those of the well-studied mucosal adjuvant, cholera toxin (CT) and a conventional adjuvant approved for human use, aluminium hydroxide. Also, the early activation status of immune cells in the lungs from naïve and immunized mice after challenge infection was determined.

2. Materials and methods

2.1. Virus

Influenza virus A/PR8 (PR8) was grown in 10-day old embryonated hen's eggs and purified from allantoic fluid by using a discontinuous sucrose gradient (15%, 30% and 60%) layers. Inactivation of the purified virus was performed by mixing the virus with formalin at a final concentration of 1:4000 (v/v) as described [12,13]. Inactivation of the virus was confirmed by plaque assay on confluent monolayer Madin-Darby canine kidney (MDCK) cells and inoculation of the virus into 10-day old embryonated hen's eggs. For challenge experiments, mouse adapted PR8 (provided by Dr. Huan Nguyen, University of Alabama at Birmingham, Birmingham, AL) was prepared as lung homogenates from intranasally infected mice.

2.2. Herbal extract preparation

Korean red ginseng (*Panax ginseng* C.A. Meyer) powder capsules (Korea Ginseng Co., South Korea) were previously used in clinical studies of individuals infected with human immunodeficiency virus type 1 [14]. An extract of the *Salviae radix* species has been previously used in animal models [7], in clinical studies, and is routinely prescribed by Oriental medicine-licensed doctors. Dried *Salviae radix* roots were kindly provided by Dr. In-S. Jung (Grace Acupuncture and Herbs Clinic, Duluth, GA). Dried *Salviae radix* herbs were ground into small pieces. Water extracts of both herbs were prepared as follows. Herbs were mixed with distilled water (ginseng: 80 mg/ml, *Salviae*: 36 mg/ml) and then heated to 80–90 °C for 2 h. The aqueous extracts were 0.45 µm membrane-filtered and kept at 4 °C until use. Approximately 20% of the weight of dried ginseng and *Salviae* herbs were extracted into the aqueous solution. Batch to batch preparations were consistent and showed similar effects in independent animal experiments. Herbal extract preparations were initially tested for their safety. Ginseng or *Salviae* extract (4 mg dose per mouse) was subcutaneously administered to mice daily for 7 days, and we did not observe any changes in body weight or sign of disease (data not shown).

2.3. Immunization and challenge

Female inbred BALB/c mice (Charles River) aged 6–8 weeks were used. For immunization we used 12 mice per group. Mice were intranasally immunized with 25 µg of inactivated PR8 alone or co-administered with 25 µg of inactivated PR8 and herbal extracts (in terms of dried ginseng or *Salviae* raw plant materials, 200 mg/kg body weight) at day 0 and 14. As control groups, mice were intranasally administered the same dose of ginseng or *Salviae* extract used for immunization at day 0 and 14. Also, for comparison, additional groups of mice were intranasally immunized with 25 µg of inactivated PR8 virus and 2 µg of cholera toxin (CT) or 3% of aluminium hydroxide at day 0 and 14. For virus challenges, isoflurane-anesthetized mice were infected with 750 PFU (5× the 50% lethal dose) in 50 µl of phosphate-buffered saline (PBS) per mouse at week 4 after the boost immunization. Mice were observed daily for 17 days to monitor changes in body weight and to record death.

2.4. Sample collections

Blood samples were collected by retro-orbital plexus puncture before immunization and 2 weeks after priming and boost immunizations. Vaginal lavage fluids were collected by washing the vagina with 200 µl of PBS. Ten pieces of freshly voided feces were collected at 2 weeks after boost immunization, weighed, and resuspended in PBS with NaN₃ in a ratio of 1 mg of feces in 1 ml PBS solution. The feces in PBS were resuspended by vortexing vigorously until solutions were homogenous. Samples were then spun in a microcentrifuge for 10 min and supernatants were collected. For measurement of immune response parameters, six mice from each group were sacrificed at day 4 to 5 post-challenge, and six remaining mice were sacrificed at day 15 post-challenge. Lymphocytes from lung and spleen samples were collected from sacrificed mice, and used for ELISPOT and fluorescence activated cell sorting (FACS) analysis.

2.5. Antibody responses

IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA Ab titers to influenza virus were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [15]. Ninety-six-well microtiter plates (Nunc-Immuno Plate MaxiSorp™, Nunc Life Technologies, Basel, Switzerland) were coated with 100 µl of inactivated PR8 (4 µg/ml) in coating buffer (0.1 M Sodium Carbonate, pH 9.5) at 4 °C overnight. One hundred-fold diluted sera, 10-fold diluted saliva, and 10-fold diluted vaginal washes were added to wells to determine influenza specific binding antibody levels. The plates were then incubated with horseradish peroxidase-labeled goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnology) at 37 °C for 1.5 h and then the substrate *O*-Phenylenediamine (OPD) (Zymed, San Francisco, CA) in citrate-phosphate buffer (pH 5.0) containing

0.03% H₂O₂ (Sigma) was used to develop color. The optical density at 450 nm was read by an ELISA reader (model 680, Bio-Rad).

2.6. Virus neutralization assay

Neutralization assay was performed using MDCK cells following the procedure previously described [15]. Serum samples were serially diluted in Dulbecco's modified Eagle's medium (DMEM), and final volume of 190 μ l was mixed with 10 μ l of diluted virus stock containing approximately 100 infectious particles. The virus–serum mixtures were incubated at 37 °C for 1 h and then added to 6-well plates containing confluent MDCK cell monolayers. The plates were incubated at 37 °C for 1 h, and overlay medium was added containing DEAE dextran, non-essential amino acid, glutamine, and trypsin, and incubated for 2–3 days. The cells were then fixed with 20% ethanol and stained with 1% crystal violet. The neutralization activity was expressed as percentage reduction of plaque numbers.

2.7. Cytokine assays

All antibodies against mouse cytokines used in ELISPOT assays were purchased from PharMingen (San Diego, CA). Anti-mouse gamma interferon (IFN- γ), IL-2, IL-4 and IL-5 antibodies (3 μ g/ml in coating buffer) were used to coat Multiscreen 96-well filtration plates (Millipore). Freshly isolated splenocytes (1.5×10^6 cells) were added to each well and stimulated with inactivated influenza virus (2 μ g/ml). The plates were incubated for 36 h at 37 °C with 5% CO₂. Development and counting of ELISPOTs were performed as described [16]. Cytokine ELISA was performed as described previously [17]. Ready-Set-Go IL-6 and IFN- γ kits (eBioscience, San Diego, CA) were used for detecting cytokine levels in lung extracts following the manufacturer's procedures.

2.8. Lung viral titers, cytokines, and lymphocyte analysis

The whole lungs were collected at day 4 (or 5) and 15 post-challenge and minced between glass slides. Lung homogenates were centrifuged at 1000 RPM for 10 min to separate cells. The lung supernatants were frozen and kept at –70 °C until used for virus titer and cytokine assays. The lung cell pellets were suspended in red blood cell lysis buffer (Sigma) to remove red blood cells. Then 0.5×10^6 single cell suspensions were stained with cell phenotypic and activation marker antibodies, FITC conjugated-CD4, APC conjugated-CD8, APC conjugated-CD11b, and PE conjugated-CD69 for 30 min at 4 °C in 50 μ l 10% BCS buffer (PBS containing 10% bovine calf serum and 0.1% sodium azide). Cells were washed with 2% BCS, fixed with 1% paraformaldehyde and then analyzed by flow cytometry on a FACScan (Becton Dickinson). Fifty thousand events were counted for each sample and WINMDI 2.8 software (Scripps Research Institute Cytometry Software) was used for the analysis.

2.9. Statistics

All parameters were recorded for individuals within all groups. Immunization experiments were repeated to confirm the reproducibility of different batches of ginseng and Salviae extracts, and immune responses were found to be reproducible. Statistical comparisons of data were carried out using the ANOVA and Npar1-way Kruskal–Wallis test of the PC-SAS system. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Ginseng or Salviae extracts enhance humoral immune responses

To determine whether herbal extracts have mucosal adjuvant effects, groups of mice were intranasally immunized with inactivated influenza virus (PR8) plus ginseng or Salvia extract. Influenza virus specific binding antibodies were determined using an ELISA plate coated with inactivated influenza virus antigen (Fig. 1). Low levels of serum binding antibodies were induced similarly in all groups after priming. The levels of influenza virus specific IgG were significantly increased after a boost immunization ($P < 0.001$). Placebo groups that received ginseng or Salviae alone without inactivated PR8 virus did not show induction of influenza specific antibody indicating that antibody levels measured in immune

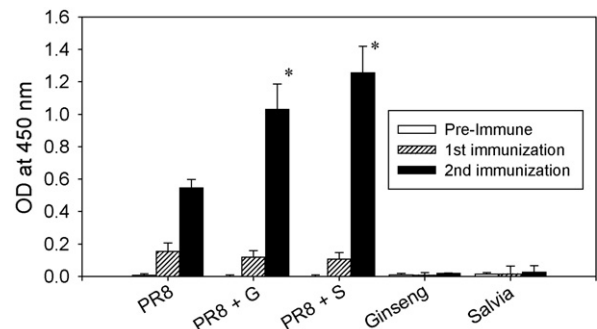


Fig. 1. Influenza virus specific total serum IgG antibody responses. Mice were intranasally immunized with inactivated influenza virus PR8/34 (PR8) alone (25 μ g) or with a mixture of PR8 and ginseng or Salviae (4 mg per mouse) on day 0 and 14. As placebo controls, mice were intranasally administered with ginseng or Salviae alone (4 mg per mouse). Blood samples were collected individually at 2 weeks after primary (first) and boost (second) immunizations, and 100-fold diluted sera used to determine total IgG by ELISA coated with inactivated influenza virus. Optical densities were read at 450 nm (OD₄₅₀), and results are expressed as the arithmetic mean (OD₄₅₀) and error bars indicate standard deviation (S.D.). Statistical significance is indicated between mice immunized with PR8 alone and mice with PR8/herbal extracts (* $P < 0.005$; ** $P < 0.0001$). PR8, immunization with inactivated influenza virus PR8 alone; PR8 + G, immunization with PR8 and ginseng extract (4 mg per mouse); PR8 + S, immunization with PR8 and Salviae extract (4 mg per mouse); Ginseng, administration with ginseng alone (4 mg per mouse); Salvia, administration with Salviae alone (4 mg per mouse).

Table 1
Comparison of antibody titers to PR8 antigen after intranasal co-immunization with different adjuvants^a

Group ^b	IgG	IgG1	IgG2a	IgG2b	IgG3
PR8	$(1.3 \pm 0.19) \times 10^5$	$(1.2 \pm 0.29) \times 10^5$	$(3.0 \pm 1.1) \times 10^4$	$(1.6 \pm 0.47) \times 10^4$	$(1 \pm 0.23) \times 10^3$
PR8 + G	$(5 \pm 1.0)^* \times 10^5$	$(5 \pm 1.2)^* \times 10^5$	$(6.4 \pm 1.1)^* \times 10^4$	$(3.2 \pm 0.47)^* \times 10^4$	$(1 \pm 0.24) \times 10^3$
PR8 + S	$(1 \pm 0.23)^{**} \times 10^6$	$(1 \pm 0.27)^{**} \times 10^6$	$(6.4 \pm 0.64)^* \times 10^4$	$(3.2 \pm 0.45)^* \times 10^4$	$(1 \pm 0.27) \times 10^3$
PR8 + CT	$(1 \pm 0.25)^{**} \times 10^6$	$(2.6 \pm 0.24)^* \times 10^5$	$(2.6 \pm 0.2)^{**} \times 10^5$	$(1.3 \pm 0.24)^{**} \times 10^5$	$(1.6 \pm 0.25)^{**} \times 10^4$
PR8 + Al(OH) ₃	$(2.6 \pm 0.22)^* \times 10^5$	$(3.2 \pm 0.24) \times 10^4$	$(6.4 \pm 0.26)^* \times 10^4$	$(3.2 \pm 0.29)^* \times 10^4$	$(4 \pm 0.27)^* \times 10^3$

^a Antibodies specific to influenza virus PR8 were determined at 2 weeks after the boost (second) immunization. Titers are expressed as the highest dilution of serum having a mean optical density at 450 nm greater than the mean plus 2 standard deviations of similarly diluted naïve or placebo control serum samples. Results are expressed as the geometric mean titers \pm standard deviation of reciprocal serum dilutions.

^b PR8, Intranasal immunization with inactivated influenza PR8 virus alone; PR8 + G, inactivated influenza PR8 virus plus ginseng; PR8 + S, inactivated influenza PR8 virus plus Salviae; PR8 + CT, inactivated influenza PR8 virus plus CT; PR8 + Al(OH)₃, inactivated influenza PR8 virus plus Al(OH)₃.

* $P < 0.05$ between the PR8 group and a comparing group; ** $P < 0.005$ between the PR8 group and a comparing group.

sera are influenza specific (Fig. 1). Interestingly, after a boost immunization, groups of mice which received PR8 plus ginseng or Salvia extract showed 2–2.5-fold increases in antibody levels measured by OD at 450 nm, and 5–10-fold increases in antibody titers of total IgG antibody (Table 1) when compared with those in mice immunized with PR8 alone ($P < 0.005$). We found that one OD₄₅₀ unit of 100-fold diluted samples (Fig. 1) is equivalent to 1.6 mg/ml concentration based on the standard curve of purified mouse IgG antibody control (data not shown). These increases in total IgG antibody in groups of mice that received co-administration with ginseng or Salviae were comparable to those observed in the aluminium hydroxide or CT-adjuvanted group (Table 1).

To further characterize antibody production, serum IgG subtypes IgG1, IgG2a, IgG2b, IgG3 and IgA were determined after the boost immunization as shown in Table 1. All isotype antibodies binding to influenza viral antigen were observed in all groups of immune sera with IgG1 being the highest among the subtypes. Compared to that of the PR8 group the levels of subtype antibodies, particularly IgG1, were induced at 4–7-fold higher levels in groups of mice co-administered with PR8 and ginseng or Salviae extract ($P < 0.05$ and < 0.001 , respectively). It is interesting to note that the adjuvant effects of ginseng and Salviae on IgG1 subtype were 2–3-fold higher than that of the CT adjuvant group, whereas the highest levels of IgG2a, IgG2b, and IgG3 were found in the group with CT adjuvant group (Table 1). Also, ginseng or Salviae groups showed levels of IgG2a and IgG2b that were 2-fold higher than the PR8 control group, which were similar to that of the aluminium hydroxide adjuvanted group. This is consistent with the results of total IgG antibody in the PR8 plus ginseng or Salviae groups, which showed the higher level after the boost immunization (Fig. 1). These results indicate that both ginseng and Salviae extracts have an adjuvant effect in enhancing immune responses against the co-administered inactivated influenza virus PR8 antigen, and that their adjuvant effects are equivalent to those of CT or aluminium hydroxide.

Mucosal immune responses were evaluated to further determine the effects of herbal extracts. Mucosal secretions from vagina, and lung and fecal extracts were collected at 2 weeks after the boost immunization and ELISA was used to

detect mucosal IgA and IgG production. Co-administration of either ginseng or Salviae extract enhanced the levels of mucosal IgA and IgG antibodies against influenza virus in lung and fecal extract samples when compared with PR8 alone (Fig. 2A, B, E, and F). Salviae enhanced the levels of vaginal IgA responses while increased vaginal IgG antibody levels were observed with the ginseng group (Fig. 2C and D). These results indicate a positive effect of co-administration with ginseng or Salviae extract in enhancing mucosal antibody responses, particularly in lung IgA and IgG antibodies which are expected to play primary roles in preventing influenza virus infection.

3.2. Co-administration of ginseng or Salviae extract enhances neutralization activity

To determine the effects of herbal extracts on inducing neutralizing activities, serum samples collected at 2 weeks after the boost immunization were used in a standard plaque reduction assay on MDCK cells (Fig. 3). Mice immunized with PR8 plus Salviae or ginseng extract showed 3-fold higher levels of neutralizing activity (50% neutralization activity titer, 180) than mice with PR8 alone (50% neutralization activity titer, 60). The placebo groups of mice with administration of herbal extracts alone showed only a background level of neutralization activity similar to those of naïve controls (data not shown). Thus, these results indicated that co-immunization with Salviae or ginseng extract can enhance induction of neutralizing antibody against influenza virus.

3.3. Protection of mice from a lethal challenge

To investigate whether immunized mice are protected against lethal challenge, body weight changes and survival rates were monitored after challenge (Fig. 4A and B). The naïve and placebo (ginseng or Salviae alone) groups of mice with challenge infection showed a significant and progressive loss in body weight and shivering, indicating that these mice suffered severe illness due to PR8 viral challenge infection. By day 8 post-challenge, all mice in the naïve and placebo challenge group lost around 40% of body weight and died. In contrast, all immunized mice showed only 5–10% loss

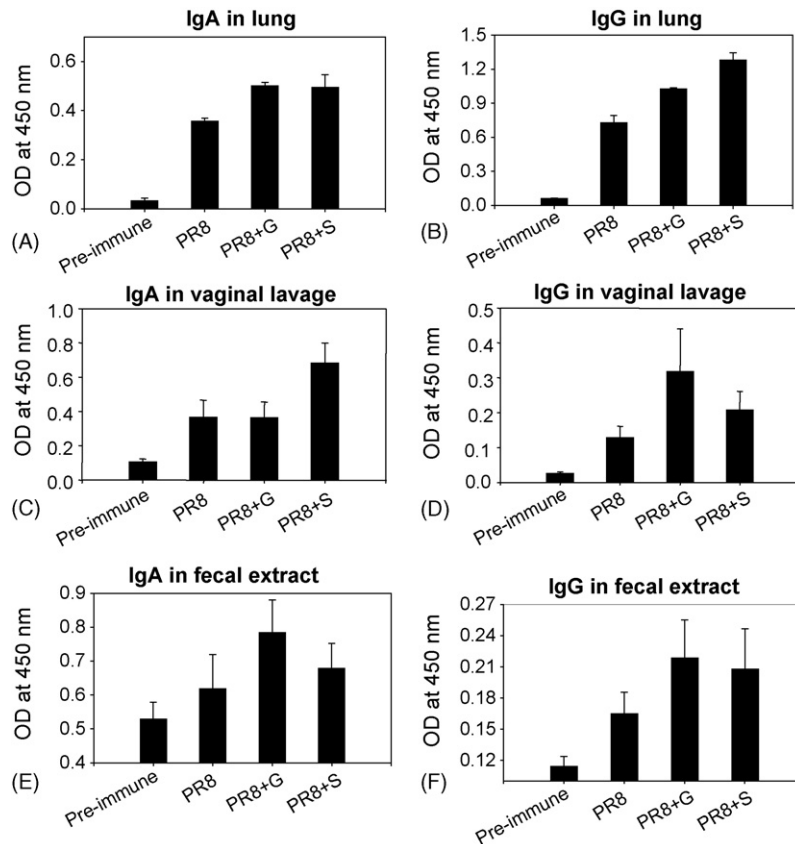


Fig. 2. Mucosal antibody responses. Vaginal lavage and fecal samples were collected at 2 weeks and lung samples at 4.5 weeks after the second immunization. ELISA was used to determine mucosal IgA and IgG antibody levels. Values of OD₄₅₀ were obtained from 2-fold diluted lung extracts (A, B), 10-fold diluted vaginal and fecal samples (C–F), and results represented as the arithmetic mean (OD₄₅₀) + S.D. Each data represents the arithmetic mean from 6 mice per group. Immunization groups are the same as described in Fig. 1.

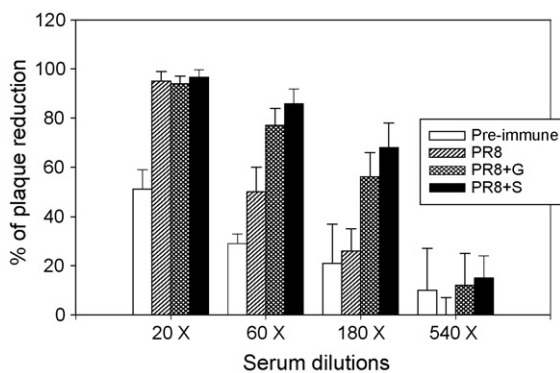


Fig. 3. Serum neutralizing activities. Virus-neutralizing antibody activities in sera collected at 2 weeks after the boost immunization were determined using the plaque assay. Diluted sera of individual mice (12 mice per group) were incubated with approximately 100 plaque forming unit of PR8 virus for 1 h at 37 °C. The mixtures were then applied to monolayers of confluent MDCK cells, and a standard plaque reduction assay was performed. Immunization groups are the same as described in Fig. 1. The placebo groups of mice immunized with ginseng or Salviae extract alone showed similar titers as pre-immune controls (data not shown).

in body weight between day 3–8 post-challenge, and then recovered to normal condition. There was no significance difference between groups immunized with ginseng or Salviae extract together or PR8 alone. Lung viral titers post-challenge were analyzed to determine the replication of challenge virus at day 4 and 5 post-challenge (Fig. 4C). The naïve and placebo groups that received PR8 challenge virus showed high viral titers reaching to 6 logs while no virus was detected in any immunized groups including the ginseng or Salviae herbal extract group. These results indicate that mice immunized with PR8 alone or PR8/ginseng or Salviae extract mixture can induce fully protective immune responses against lethal challenge.

3.4. Herbal extracts enhance cytokine responses

Production of cytokines is an indicator of cellular immune responses. To determine the effects of ginseng and Salviae extract co-administration on inducing cytokine production after challenge, spleens were collected at day 15 post-challenge and cytokine-producing splenocytes were determined by ELISPOT in response to stimulation with inactivated influenza virus (Fig. 5). Immunization with PR8 showed increased levels of splenocytes producing cytokines

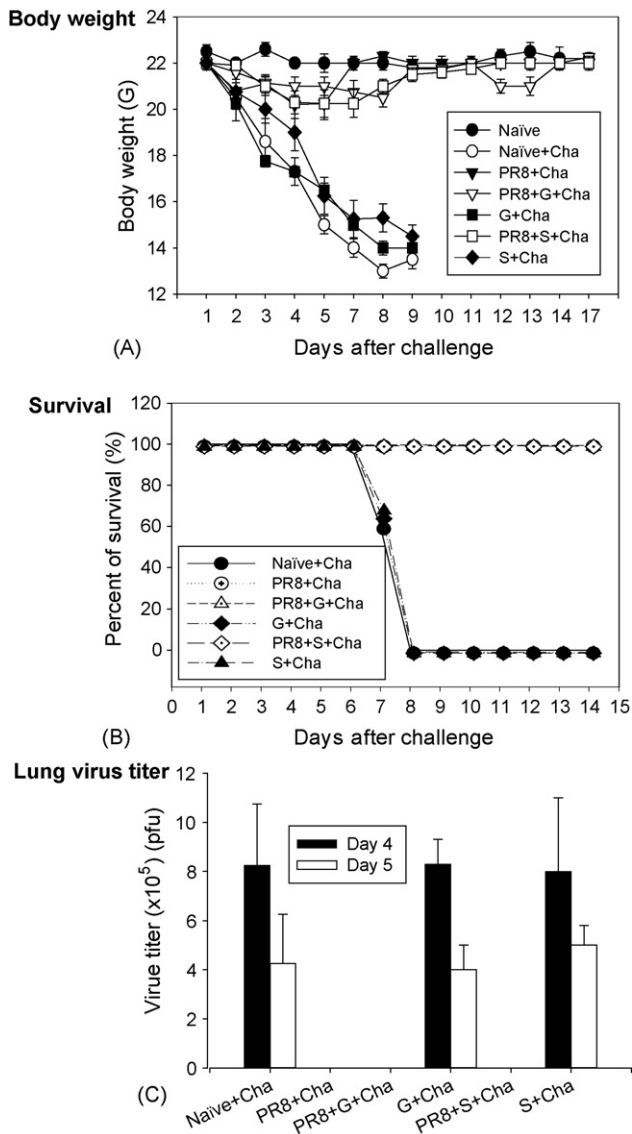


Fig. 4. Protection from the lethal challenge infection. (A) Changes in body weight after challenge infection. At week 4 after the second immunization, naïve or immunized mice were intranasally infected with 750 PFU ($5 \times$ the 50% lethal dose) of mouse-adapted PR8 virus. Mice were monitored for changes in body weight daily for 17 days. (B) Survival rate. All mice in the naïve and placebo groups died at day 7 and 8 post-challenge. (C) Lung influenza virus titer was detected at day 4 and 5 post-challenge. Naïve + Cha, naïve mice infected with challenge virus; PR8 + Cha, mice immunized with PR8 alone then challenge infection; PR8 + G + Cha, mice co-administered with PR8 and ginseng then challenge infection; PR8 + S + Cha, mice co-administered with PR8 and Salviae then challenge infection; G + Cha, mice administered with ginseng alone then challenge infection; S + Cha, mice administered with Salviae alone then challenge infection.

IFN- γ , IL-2, IL-4, and IL-5 compared to those from naïve mice. Co-immunization with ginseng or Salviae extract showed significantly enhanced levels of the cytokine-producing cells compared to the immunization with PR8 alone. Interestingly, inclusion of Salviae increased levels of IFN- γ and IL-2 producing cells by 5–7-fold when compared to the PR8 immunized group (Fig. 5A and B). In contrast,

ginseng co-immunization showed 4–5-fold enhanced levels of IL-4 and IL-5 producing splenocytes compared to those in PR8 immunized mice (Fig. 5C and D). Salviae was more likely to induce Th1 type cytokines (IFN- γ , IL-2) whereas ginseng augmented Th2 type cytokine responses (IL-4, IL-5). The placebo groups of mice that received ginseng or Salviae extract alone did not show the production of cytokines specific to influenza virus stimulation (data not shown). These results indicate that co-administration with herbal extract significantly enhanced influenza specific cytokine producing lymphocytes.

3.5. Immunization reduces early lymphocyte activation after challenge infection

The status of early lymphocyte activation in the lung during respiratory viral infection is not well understood. To evaluate the early events in immune cell activation and the effects of immunization with inactivated PR8 and ginseng or Salviae extract during influenza virus infection, we analyzed lymphocytes in the lungs after intranasal challenge with PR8. Lungs were collected from individual mice at day 4 post-challenge. To examine the lung lymphocyte populations in detail, lung cells were prepared and stained with cell surface marker antibodies, CD4, CD8, CD11b, and an early activation marker, CD69 (Fig. 6). From the flow cytometry profiles of each phenotypic population out of total lymphocytes gated (Fig. 6), results on CD4, CD8, and CD11b cells and CD69⁺ populations of their corresponding phenotypic lymphocytes were summarized in Fig. 7.

CD69 is expressed on activated lymphocytes but not on resting cells [18]. Analysis of naïve mice indicated that CD69⁺ populations were present in small percentages in CD4⁺ T, CD8⁺ T cells, and CD11b⁺ monocyte/macrophage populations (Figs. 6 and 7). Also, all groups of immunized mice showed similar levels of cells in CD69⁺ populations as naïve mice prior to challenge infection (data not shown). However, percentages of cells in gated CD69⁺ populations were increased by 7–60-fold during early influenza virus infection in naïve mice depending on cell phenotypes (7-fold in CD69⁺CD4⁺, 9-fold in CD69⁺CD8⁺ T cells, and 60-fold in CD69⁺CD11b⁺ phenotypic cells) (Fig. 6A and B). In contrast, the PR8 immunized group did not show such significant increases in CD69⁺ populations after challenge. The group receiving ginseng showed a moderate increase in levels of CD69⁺CD4⁺ cells compared to the PR8 group after challenge. The Salviae group showed a 2-fold increase in CD69⁺CD4⁺ and CD69⁺CD11b⁺ phenotypic cell populations compared to the PR8 immunized group.

In summary, these results indicate that CD69⁺ cell populations were significantly increased in naïve mice after challenge, which may be involved in development of lung inflammation. Immunization with inactivated influenza virus significantly reduced CD69⁺ cell populations in lung after challenge infection, and co-administration of ginseng or Salviae extract modulated the levels of CD69⁺ populations.

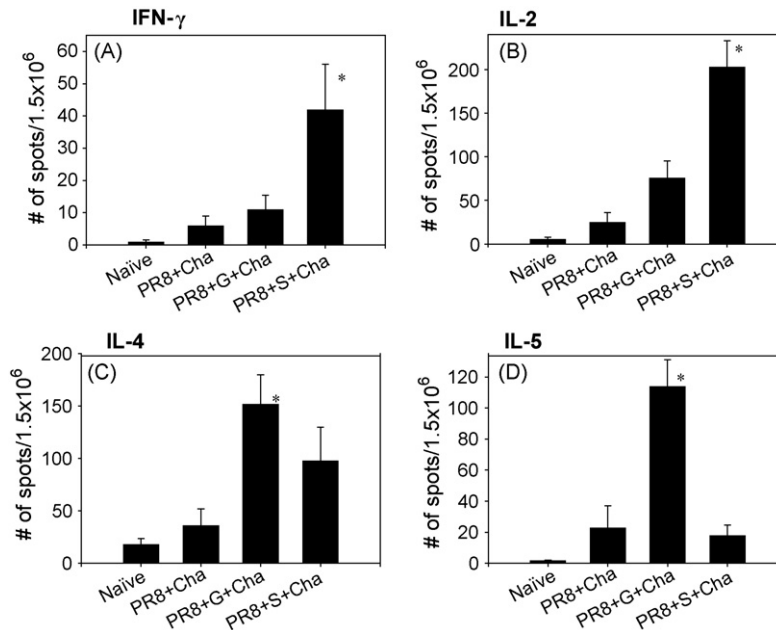


Fig. 5. Quantitative analysis of cytokine-producing splenocytes. Cytokine (IFN- γ , IL-2, IL-4, IL-5) producing splenocytes isolated at day 15 post-challenge were determined by ELISPOT after stimulation with inactivated influenza virus. The spots for cytokine-producing cells from the spleen were counted and expressed based on 1.5×10^6 cells. Bars indicate standard deviations from six mice per group. * $P < 0.05$ (between PR8 and PR8 + herbal extracts). Immunization groups are the same as described in Fig. 4.

3.6. Cytokine and virus specific IgA antibody levels in the lung post-challenge

Like activated CD69⁺ lymphocytes, cytokines are involved in clearing virus as well as in inflammation causing tissue damage. After challenge infection, cytokines in lung extracts were determined using cytokine ELISA (Fig. 8A). A pro-inflammatory cytokine IL-6 was detected at a significantly high level in naïve mice infected with challenge virus whereas little or no IL-6 was observed in the groups of immunized mice both before and after challenge infection. IFN- γ was also increased by 10-fold in naïve mice after challenge infection, and was approximately 2.5-fold higher than those in the groups of immunized mice at day 4 post-challenge (Fig. 8B). In addition, there were no significant changes in IFN- γ levels in immunized mice before and after challenge (at day 4). Interestingly, at day 15 post-challenge, IFN- γ in lung extracts was observed in the PR8 group but little or no IFN- γ was detected in groups receiving ginseng or Salviae and virus.

To determine the induction of lung IgA levels which might have played a role in providing protection from challenge infection, PR8 virus specific IgA antibody was determined (Fig. 9). There was no IgA specific to PR8 detected in naïve mice with challenge infection. At day 4 post-challenge, all groups of mice immunized showed enhanced levels of PR8 specific lung IgA antibodies. At day 15 post-challenge, groups of mice co-immunized with ginseng or Salviae showed significant enhancements in IgA levels compared to the PR8 immunized mice (Fig. 9).

These results indicate that highly increased levels of IL-6 and IFN- γ cytokines induced in naïve mice after viral infection may be involved in lung inflammation. Immunization with inactivated virus plus ginseng or Salviae induced high levels of virus specific IgA antibodies in lung, which may play a beneficial role in long-term protection.

4. Discussion

In this study, we have investigated whether herbal extracts (ginseng, Salviae) would have an adjuvant role in enhancing immune responses against influenza virus antigens, and their effects on early events in lymphocyte activation during influenza virus infection using a mouse model. We observed that ginseng or Salviae extract increased the levels of influenza virus specific antibodies and neutralizing activities when co-administered with inactivated virus. Salviae resulted in significant enhancement of IFN- γ and IL-2 cytokine responses after challenge infection while ginseng induced high levels of IL-4 and IL-5 cytokine producing lymphocytes. Finally, we observed that herbal extracts modulated leukocytes expressing CD69, an early activation marker, and induced high levels of lung IgA antibody after challenge infection. These results indicate that both ginseng and Salviae extracts have an adjuvant role as well as immunomodulatory functions.

Salviae species have been used widely to treat certain human diseases. Studies with Salviae extracts performed in chronic hepatitis B virus infected patients in combina-

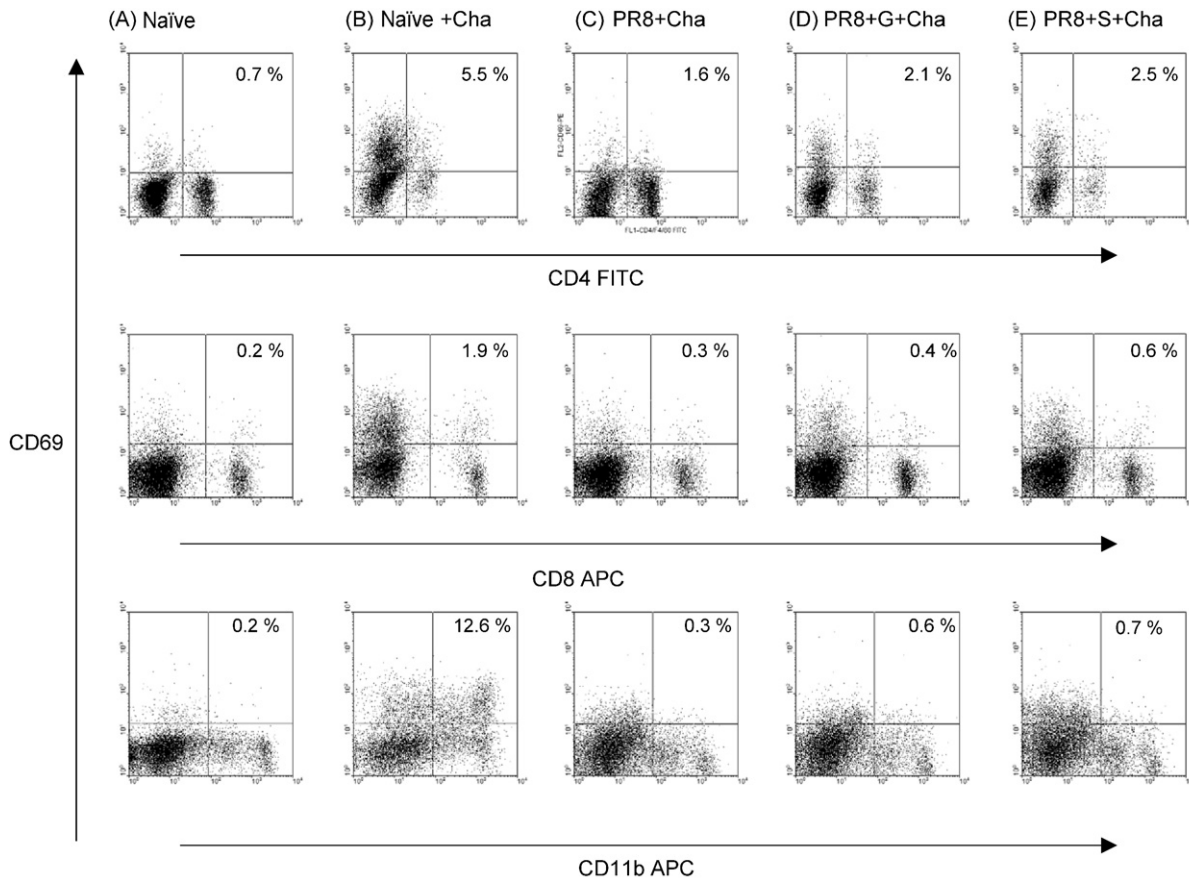


Fig. 6. Representative flow cytometry profiles indicating increased populations expressing the CD69 early activation marker. Single cell suspensions were prepared from lungs isolated at day 4 post-challenge and stained with cell surface and CD69 marker antibodies. Fifty thousand events of stained splenocytes were collected by FACS and gated lymphocyte populations were analyzed by WINMDI software. Panel A, naïve mice; Panel B, naïve mice with challenge infection; Panel C, PR8 alone immunized mice with challenge infection; Panel D, PR8 + G + Cha, mice co-immunized with PR8 and ginseng then challenge infection; Panel E, PR8 + S + Cha, mice co-immunized with PR8 and Salviae then challenge infection. Numbers indicate percentages based on the gated total populations. CD69⁺CD4⁺, CD69⁺CD8⁺, CD69⁺CD11b⁺ populations were significantly elevated in the naïve mice with challenge infection.

tions with several different herbs showed beneficial effects, resulting in a higher rate of recovery from further disease progression than control groups [19–22]. However, the potential adjuvant and/or immunomodulatory effects of Salviae have

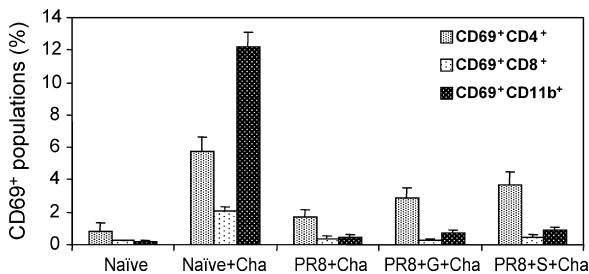


Fig. 7. Lung immune cell populations with CD69⁺ phenotypes before and after challenge. Each phenotype of cells was gated by FACS analysis and results are expressed as percentile of each population based on the total gated populations (four individual mouse in each group). CD69⁺CD4⁺, CD69⁺CD8⁺, and CD69⁺CD11b⁺ phenotypic cells are expressed as percentiles based on total gated populations (Fig. 6). Groups denoted as Naïve + Cha, PR8 + Cha, PR8 + G + Cha, and PR8 + S + Cha are described in Fig. 4.

not been previously reported. Our study clearly demonstrates an adjuvant role for Salviae extract. Co-administration of Salviae extract significantly enhanced both mucosal IgA and IgG antibodies as well as binding and neutralizing antibody levels in blood. Also, mice immunized with PR8 plus Salviae showed significant enhancement of IFN- γ and IL-2 cytokines as well as a moderate increase in IL-4 cytokine production by splenocytes. These results suggest that Salviae extract can be developed as a promising adjuvant for enhancing humoral and cellular immunity.

Ginseng is one of the most well studied herbal medicines and appears to have multiple effects including an immunomodulatory function. Recent studies demonstrated that ginseng extract alone or in combination with aluminium hydroxide played an adjuvant role against inactivated porcine parvovirus and bacterial antigens [23–25] and that ginseng treatment reduced lung pathology caused by chronic *Pseudomonas aeruginosa* [26]. All these previous studies were performed by subcutaneous immunization. We investigated the effect of ginseng as a potential mucosal adjuvant against influenza virus antigens. We found that intranasal

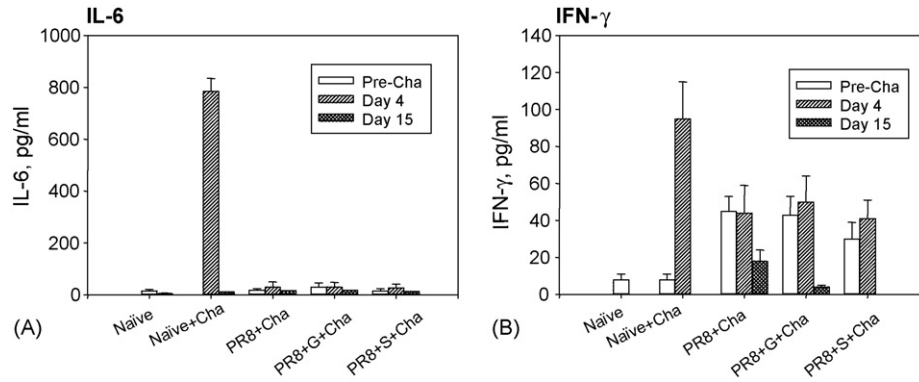


Fig. 8. Cytokines in lung post-challenge. Lung extracts were prepared at three time points: at 4 weeks after the boost immunization (pre-cha), at day four post-challenge (day 4), and at day 15 post-challenge. Naïve mouse samples were used as a control. Cytokines in lung extracts were determined using ELISA, and results were geometric mean values obtained from 4 mice at each time point. (A) IL-6; (B) IFN- γ . Immunization groups are the same as described in Fig. 4.

co-administration of ginseng resulted in not only significant increases in serum IgG antibody but also in lung IgA antibody levels. Although addition of ginseng increased the levels of all cytokines (IFN- γ , IL-2, IL-4, IL-5) analyzed, increases in IL-4 and IL-5 were more prominent than other cytokines indicating a Th2 type response. Regardless of the route of immunization, our study and others demonstrate that ginseng can play a role as an adjuvant for co-administered antigens.

The underlying mechanisms by which ginseng or *Salviae* extract play a role as an adjuvant and potentially as an immunomodulator is not understood yet. Herbal extracts prepared in hot aqueous solution contain a mixture of protein and organic chemical as well as carbohydrate components. Although we detected the presence of proteins (data not shown), proteins may not be the active components since extracts were prepared in hot water. Recent studies demonstrated that human immunodeficiency virus type-1 (HIV-1) infected individuals who had taken Korean red ginseng for over 10 years showed significantly improved clinical parameters including slower decreases in CD4 T cells and lower

copy number of viral RNA [14,27]. Also, purified ginsenosides from the *Panax ginseng*, a kind of saponin, were shown to have adjuvant effects, which were equivalent to those of aluminium hydroxide [25]. A water-soluble polysaccharide isolated from *Salviae officinalis* species was shown to have a mitogenic activity as indicated by induction of the proliferation of thymocytes [28]. An extract of *Salviae* root was demonstrated to inhibit the mast cell-derived IgE allergic reaction [29] and tanshinone components of *Salviae* moderately inhibited the production of IFN- γ [30]. This result is consistent with our finding of reduction of proinflammatory cytokines after challenge infection in a group of mice co-administered with PR8 and *Salviae*. However, more research is needed to better understand the immunomodulatory roles of herbal extracts and to identify the active components.

Over-reaction of the immune response induces infiltration of lymphocytes, neutrophils and macrophages in lung tissue, and is known to be involved in the pathogenicity of the influenza virus [31,32]. CD69, an activation marker, is transiently expressed on activated lymphocytes at the sites of active immune responses *in vivo* and selectively in chronic inflammatory infiltrates, but is not detected in resting lymphocytes [33,34]. However, the activation status of these immune cells during influenza virus infection is not well understood. Here, we demonstrated extremely high levels of CD69⁺ cells in the lungs of unimmunized mice infected with influenza virus. All phenotypes of leukocytes analyzed including CD4⁺, CD8⁺, and CD11b⁺ cells expressed higher levels of CD69 in infected naïve mice than those in immunized mice with challenge infection. In addition, the over-production of inflammatory cytokines in response to microbial infection also seems to be involved in immunopathology. The level of IL-6 was demonstrated to have a positive correlation with lung inflammation [35,36]. Although IFN- γ deficiency was found to be associated with a more intense inflammatory response [37], high levels of IFN- γ might be also involved in inflammation [38]. High levels of IFN- γ and pro-inflammatory cytokine IL-6 were detected in lungs of infected naïve mice. However, immunized mice did not

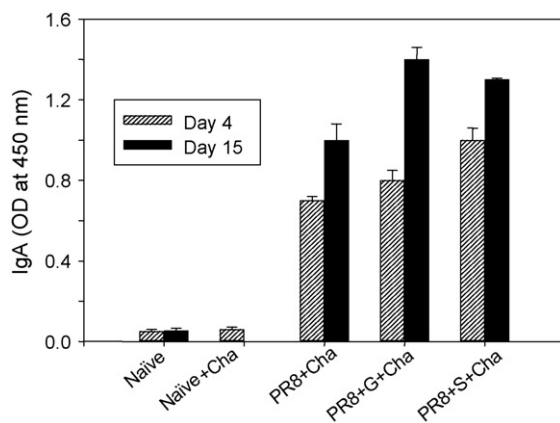


Fig. 9. Influenza virus specific IgA in lung before and after challenge. Influenza virus specific IgA antibody was determined in 10-fold diluted lung extract samples collected at day 4 and 15 post-challenge. Immunization groups are the same as described in Fig. 4.

show significant changes in levels of lung IFN- γ and did not induce the production of lung IL-6 before and after challenge, and cleared virus by day 4 post-challenge. Our results therefore provide the first evidence that high levels of CD69⁺ leukocytes in combination with over-production of pro-inflammatory cytokines may be involved in lethal damage to the lung leading to death.

CD69 is also known to be involved in activating lymphocyte functions in addition to its modulating inflammatory responses [34]. The presence of CD4⁺ populations expressing CD69 was shown to have a correlation with enhanced clearance of influenza virus in an IL-18 knockout mouse model [39]. After challenge, we found that co-administration of *Salviae* to immunized mice resulted in moderate increases in levels of CD69⁺CD4⁺ and CD69⁺CD11b⁺ populations in lung compared to PR8 immunization, and these levels of CD69⁺ cells were maintained even 2 weeks after challenge (data not shown). Coincidentally, influenza virus specific lung IgA antibody levels were moderately increased and maintained in groups of immunized mice receiving ginseng or *Salviae* compared to the PR8 immunized mice. It is of interest to further investigate the significance of maintaining the influenza virus specific lung IgA antibody levels in groups of mice co-immunized with ginseng or *Salviae* herbal extract.

Herbs are believed to be safe and effective without clear scientific evidence. Our preliminary results using a mouse model suggest that use of traditional herbal medicines regarding human health needs some caution. In mice infected with influenza virus without vaccination, administration of ginseng or *Salviae* did not show any beneficial effects (data not shown). Daily administration of ginseng or *Salviae* by either oral or systemic route for a week prior to influenza virus infection resulted in a partial protective effect of 20–30%, which is far below the level of protection from the inactivated influenza virus vaccination (data not shown).

In summary, the herbal medicines ginseng and *Salviae* have been found to have an adjuvant role against co-administered inactivated influenza viral antigen in a mouse model. CD69 expressing leukocytes may have dual functions, a pro-inflammatory function possibly exerting damage to the tissue as well as functional activation of leukocytes leading to host defense for the clearance of infectious agents. Thus, maintaining certain thresholds of CD69⁺ cells may be critically important to avoid immunopathogenesis from viral infection and to induce protective immunity. Importantly, ginseng and *Salviae* seem to have also an immunomodulatory function affecting the levels of CD69⁺ leukocytes and modulating cytokine production as well as serving immune activators in enhancing lung IgA antibody.

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