

Original Article

Ginsenoside Rg1 activates bone marrow-derived dendritic cells in mice and acts as an effective anti-infection vaccine adjuvant

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Abstract: Background: Ginsenoside, the active ingredient extracted from *Panax ginseng*, is reported to enhance immune response. In the present study, we explored whether ginsenoside could activate the dendritic cells and work as an immune adjuvant in anti-infectious vaccines. Methods: The mouse dendritic cells were obtained from bone marrow cells being treated with GMSF and IL-4. The inflammatory cytokines were detected by ELISA assay. The transcript levels of chemokines were detected by real time PCR. Lymphoproliferative activity was detected by MTT assay. Pneumolysin antigen (PN) specific antibody titers were detected by ELISA assay. Results: The Rg1 promoted the secretion of inflammatory cytokines in a dose dependent manner, including tumor necrosis factor- α , interleukin-12p70 (IL-12 p70), IL-6 and IL-1 β . Moreover, it also increased the transcript levels of chemokines, such as IP-10, RANTES, MCP-1 and IL-8. In the animal model, BalB/c mice were immunized subcutaneously with 50 μ g of Rg1 plus 50 μ g of PN, 50 μ g of Rg1, or 50 μ g of PN alone. The mice were booster immunized after 1 week. After the final immunization, lymphoproliferative activity, PN-specific antibody titers and cytokines secretion were determined. The results showed that the splenocytes from mice immunized with Rg1 in combination with PN had a higher lymphoproliferative activity, developed a higher level of PN-specific antibody titer and produced higher levels of INF- γ and IL-4, compared with the other groups (** $P < 0.01$). Conclusion: Thus, our results demonstrated that ginsenoside Rg1 could effectively activate innate immune cells, especially dendritic cells and exert a potent adjuvant effects to elicit anti-infection immunity. It could have a potential to elicit immune effects to anti-tumor or infectious diseases.

Keywords: Ginsenoside, adjuvant, immune response, anti-infection

Introduction

Nowadays, vaccines are widely used to prevent various infectious diseases, including influenza, hepatitis B, hepatitis A, meningitis or even cervical cancer, etc [1-3]. However, there are still no effective vaccines to cure malaria, tuberculosis, AIDS and cancer [4-6]. Thus, new and effective vaccines are urgently to be found and explored, as well as the effective adjuvant with low toxicity. Traditional adjuvants are usually used to highlight the antigen-specific antibody titers and mostly polarize the immune system towards Th2 responses [7, 8]. Recently, new generation of adjuvants was studied, such as saponins [9, 10], cytokines [11, 12], heat shock family proteins [13] and Toll-like receptor agonists [14-16], and so on. The new generation

of adjuvants can activate innate immune response, which leads to the activation of professional antigen-presenting cells (APCs), or changes the type of immune response [17].

Ginseng is a kind of precious Chinese medicine, with high medicinal value, and has been used for a long history in China [18, 19]. It is used to treat diabetes, hypertension, hyperlipidemia, and heart failure when administered in the doses recommended in China [20-22]. One of the possible mechanisms was due to maintaining homeostasis, anti-inflammatory, anti-oxidant, anti-apoptotic, and immune-stimulatory activities [23]. It has been reported that ginseng has antiparasitic activity and the extracts, including ginsenosides and ginseng polysaccharides may contribute to treat the tropical

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disease malaria [24]. Ginsenosides are the active components which were extracted from the roots or leaves of the ginseng. More than 30 ginsenoside monomers have been isolated and identified. Ginsenoside Rg1 has been reported to have immune-modulating activities, including promoting the immune activity of T helper (Th) cells [25]. Su, F. thought ginsenosides Rg1 could activate both extracellular and intracellular Toll-like receptor 4 (TLR4) by passing through the cell membrane to regulate the immune response [26]. Moreover, Rg1 regulated the immune response in vivo with low haemolytic effect [27].

In the present study, we explored whether Rg1 could stimulate protective adaptive immune responses and identify its potential as a vaccine adjuvant. We used recombinant pneumolysin antigen (PN) as a candidate vaccine antigen, which had antigenicity without hemolytic activity, to evaluate the adjuvant effects of Rg1 in vitro and in vivo.

Materials and methods

BM-derived DCs culture

Bone marrow-derived DCs were generated as described in the paper [28]. Briefly, bone marrow cells were obtained from C57BL/6 mice, and cultured in 6-well plates at 3.5×10^6 cells/well in 4 mL of complete RPMI 1640 supplemented with 10% FCS. Additionally, the complete RPMI 1640 was also including 2 mM L-glutamine, 100 µg/mL of streptomycin, 100 U/mL penicillin, 50 µM 2-ME (Life Technologies, Invitrogen, USA) in the presence of recombinant mouse GM-CSF (10 ng/mL; R&D Systems, Heidelberg, Germany) and recombinant mouse IL-4 (3 ng/mL; R&D Systems, Heidelberg, Germany). Roswell Park Memorial Institute (RPMI) 1640 medium (Cat. No. 11875093) was obtained from ThermoFisher Scientific Corporation. The cells were cultured at 37°C in a humidified incubator with 5% CO₂. Every twice day, half of the medium was removed and replaced with fresh medium. The BM-derived DCs were cultured for 7 days for the next experiments.

Identification of BM-derived DCs by FACS analysis

The DCs were treated with 10 ng/mL of recombinant mouse GM-CSF and 3 ng/mL recombinant mouse IL-4 for 7 days. The cells were col-

lected and washed. For phenotypic analysis, the DCs were fixed with 1% PFA and washed once by PBS. Then, the cells were incubated with FITC-labeled anti-mouse MHC class II (IA/IE) (clone M5/114.15.2 eBioscience, San Diego, CA, USA) and phycoerythrin-labeled anti-mouse CD11c (clone HL3). The cells were washed with PBS buffer and analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA).

DCs cytokine production

After 5 days of culture, BM-derived mouse DCs were adjusted to 5×10^5 cells/mL and treated with increasing concentrations of Rg1 (0.1 µg/mL, 0.5 µg/mL, 2.5 µg/mL and 12.5 µg/mL) for 48 h. Culture supernatants were harvested at 37°C and centrifuged for cytokines analysis by ELISA. The concentrations of TNF-α, IL-6, IL-1β and IL-12 p70 were determined according to the manufacturer's protocol (Neobioscience, Beijing, China). In another experiment, the splenocytes were restimulated with 1 µg/mL of PN for 24 h. The levels of IFN-γ and IL-4 in supernatants were measured by ELISA assay according to the manufacturer's protocol (Neobioscience, Beijing, China). All of the samples were analyzed in duplicate for cytokine levels.

RNA preparation and real-time RT-PCR

Immature BM-DCs were treated with 2.5 µg/mL of Rg1 for 24 hours for detection of chemokines, including interferon-γ-inducible protein 10 (IP-10), regulated upon activation normal T-cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and IL-8. Briefly, total RNA was isolated by an RNAPure kit (Biotek, China) according to the kit protocols. The RNA was retrotranscribed with MLV-reverse transcriptase (Invitrogen, USA). The reaction condition of real-time PCR (ABI Prism 7500, USA) was 40 cycles of 95°C for 12 s and 60°C for 1 min with SYBR Green. The comparative Ct method was used to quantify transcripts, normalizing for β-actin. All the primers used were listed as follows: IP-10, 5'-GGTCTGAGTGGGACTCAAGG-3' and 5'-TCTTTTCATCGTGGCAATG-3', RANTES, 5'-TCGTGCCACGTC AAGGAGTATTT-3' and 5'-ACTAGAGCAGCGATGACAGGGAA-3', monocyte chemotactic protein-1 (MCP-1), 5'-TGCATCTGCCCTAAGGCTTC-3' and 5'-AAGTGCTTGAGGTGTTTGTGG-3', IL-8, 5'-GTGCAGTTTTGCCAAGGAGT-3' and 5'-TT-

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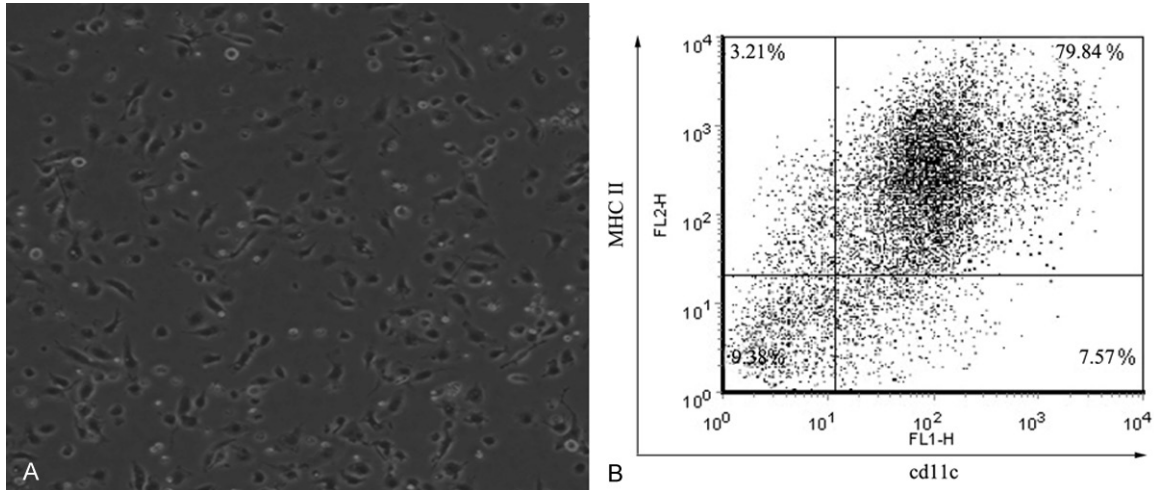


Figure 1. Identification of bone marrow-derived DCs in C57BL/6 mice. A. The bone marrow-derived dendritic cells were cultured as described in Material and Methods. The bone marrow cells were treated with GM-CSF plus IL-4 for 7 days to obtain the BM-derived DCs. The BM-derived DCs were observed by microscopy (original magnification, $\times 100$). B. The BM-DCs were stained with MHC II⁺ and CD11c⁺ antibodies and the identification of BM-DCs were analyzed by FACS on day 7.

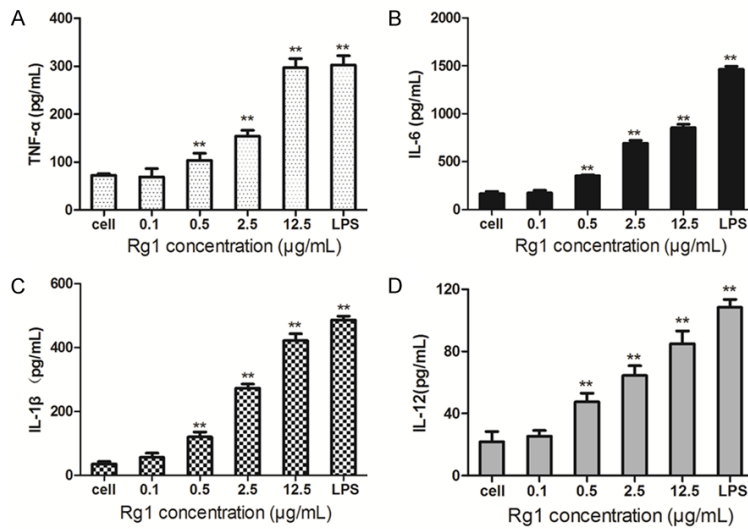


Figure 2. Ginsenoside Rg1 promotes the secretion of cytokines in bone marrow-derived DCs. Cultured BM-DCs were treated with increasing concentrations of Rg1 (0.1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 2.5 $\mu\text{g/mL}$ and 12.5 $\mu\text{g/mL}$) for 24 h. The levels of TNF- α (A), IL-6 (B), IL-1 β (C) and IL-12 p70 (D) in supernatants were measured by ELISA assay. Data are shown as mean concentrations (pg/mL) \pm SD. ** $P < 0.01$, compared with untreated cells.

China). Twenty-four C57BL/6 male mice (6 to 8 weeks old) were kept in specific pathogen-free conditions. They were randomly divided into four groups of 6 mice each. The groups were as following: Rg1 in combination with PN antigen immunized group, PN immunized group, Rg1 immunized group and PBS immunized group. Animals were injected subcutaneously twice on days 1 and 14, with 20 μg PN antigen alone, 20 μg of Rg1 alone, 20 μg of PN antigen plus 20 μg of Rg1, or with PBS. The experiment was approved by the Institutional Animal Care and Use Committee of first affiliated Hospital of Harbin medical university.

ATGAATTCTCAGCCCTCTTCAAAAATTCTC-3',
 β -actin, 5'-AGAGGGAAATCGTGCCTGAC-3' and
 5'-CAATAGTGATGACCTGGCCGT-3'.

Antigen, adjuvant and mice immunization

Ginsenosides Rg1 was purchased from Hongjiu Biotechnology (Jilin, China). The mutant pneumolysin antigen (PN) used was purchased from HaiGui Biosciences Corporation (Shanghai,

Splenocyte proliferation

One week after the final immunization, splenocytes were prepared as described in the paper [29]. The cells were adjusted to 2×10^5 cells/mL in RPMI 1640 supplemented with 10% FCS and restimulated with 1 $\mu\text{g/mL}$ PN for 48 h at 37°C in a 5% CO₂ humid incubator. Cell proliferation was then determined with the MTT assay [30].

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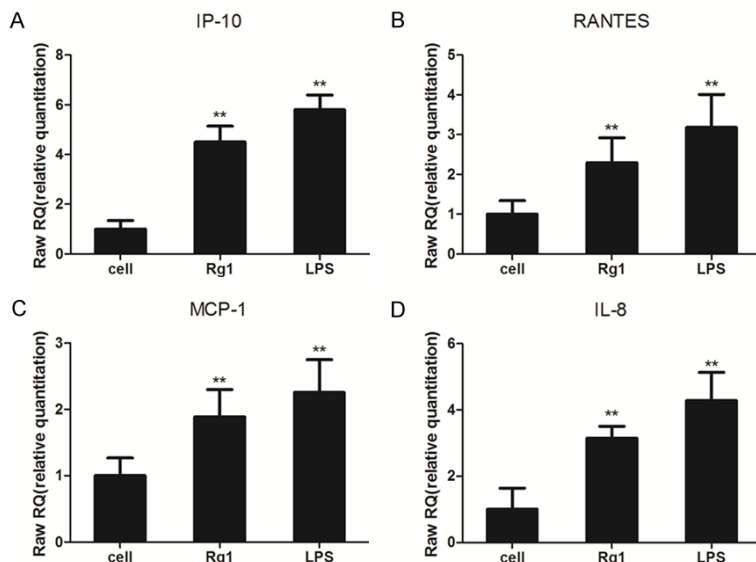


Figure 3. Ginsenoside Rg1 increases the transcription levels of chemokines in bone marrow-derived DCs. Cultured BM-DCs were treated with 2.5 $\mu\text{g}/\text{mL}$ of Rg1 for 24 hours. The transcription levels of IP-10 (A), RANTES (B), MCP-1 (C) and IL-8 (D) were detected by real time PCR assay. Untreated cells were used as negative controls and LPS-treated cells were used as positive controls. ** $P < 0.01$, compared with untreated cells.

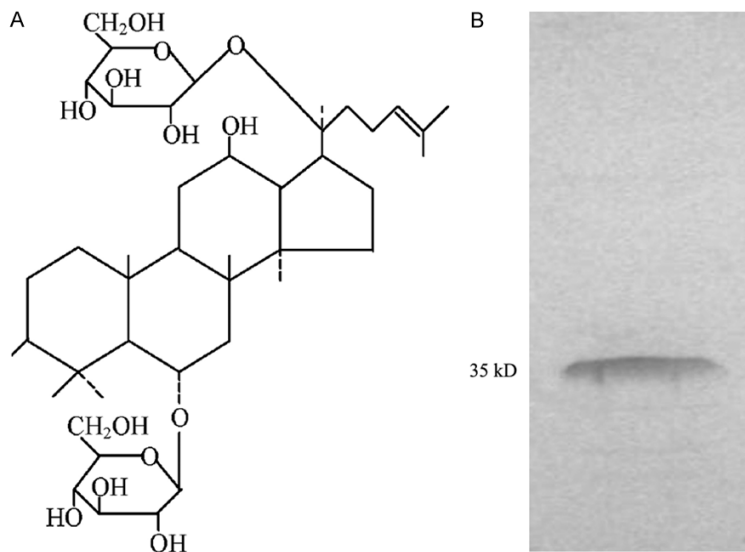


Figure 4. The adjuvant and antigen. A. Chemical structure of ginsenoside Rg1. The molecular formula is $\text{C}_{42}\text{H}_{72}\text{O}_{14}$ and the molecular weight is 801.02. B. The PN antigen was identified by SDS-PAGE. The molecular weight of PN is 35 kD.

Results

Identification of bone marrow-derived DCs in C57BL/6 mice

The dendritic cells were obtained and cultured from bone marrow cells of C57BL/6 mice as

described in Material and Methods. DC cells were cultured in complete medium of RPMI 1640 supplemented with 10 ng/mL of recombinant mouse GM-CSF and 10 ng/mL of recombinant mouse IL-4 for 7 days. Cell morphology was photographed after being cultured for 7 days (Figure 1A). FACS assay was performed to determine the rate of bone marrow-derived dendritic cells. As shown in Figure 1B, 79.84% of the dendritic cells exhibited the characteristic DC-specific marker CD11c^+ .

Ginsenoside Rg1 promotes the secretion of cytokines in bone marrow-derived DCs

In order to test whether ginsenoside Rg1 could promote the secretion of cytokines, such as $\text{TNF-}\alpha$, IL-6, IL-1 β and IL-12, DCs were treated with increasing concentrations of Rg1 for 24h and ELISA assay was performed according to the kit protocols. As shown in Figure 2, Rg1 induced a dose-dependent increase in cytokines production in BM-derived DCs. All the results demonstrated that ginsenoside Rg1 could promote the production of cytokines in dendritic cells and induce inflammatory response to activate innate immune responses.

Ginsenoside Rg1 increases the transcription levels of chemokines in bone marrow-derived DCs

To test whether Rg1 promotes the secretion of chemokines in BM-derived DCs, real time PCR assay was performed to test the mRNA levels of chemokines. The dendritic cells were treated with 2.5 $\mu\text{g}/\text{mL}$ of Rg1 for 24 hours and the mRNA levels of interferon- γ -inducible protein 10 (IP-10), regulated upon activation normal T-cell expressed and secreted (RANTES), monocyte che-

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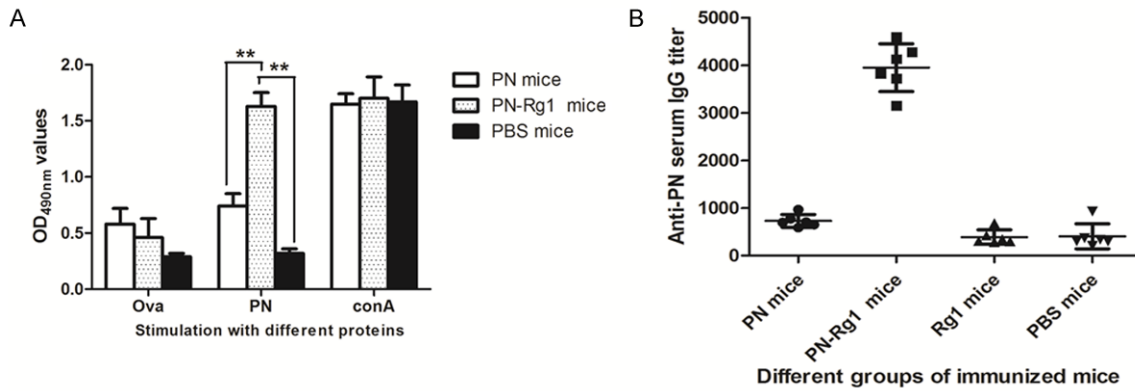


Figure 5. Anti-PN serum IgG titer increases in PN-Rg1 immunized mice. Mice were cultured and immunized as described in Materials and Methods. Seven days after the final inoculation, peripheral blood samples were collected from the tails in each mouse. The serum antibody titers were measured by ELISA assay. Each group contained 6 mice.

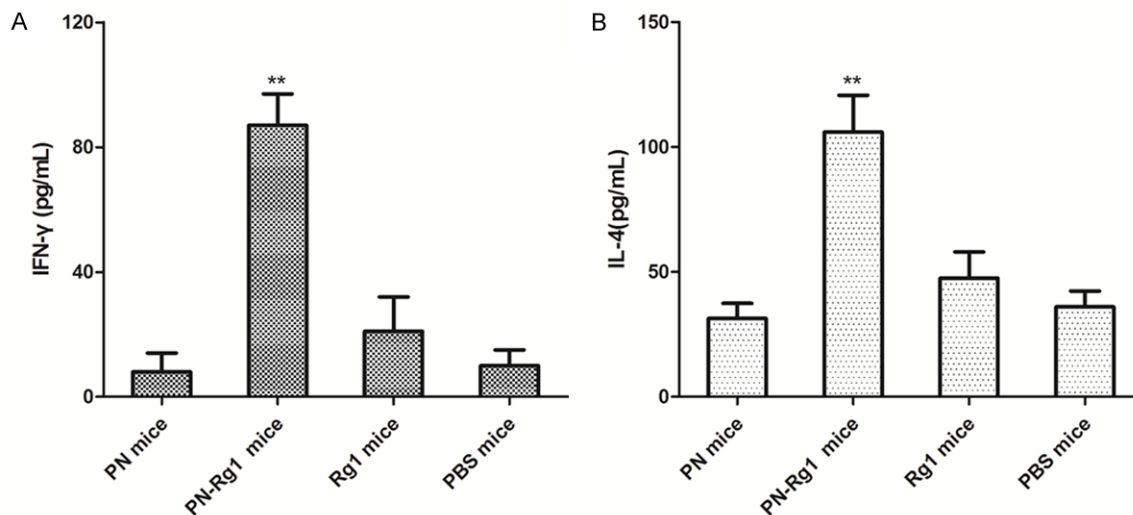


Figure 6. Serum IFN- γ and IL-4 levels are significantly up-regulated in PN-Rg1-immunized mice. The mice were cultured and immunized as described in Material and Methods. Seven days after final immunization, splenocytes were isolated and harvested from immunized mice following in vitro restimulation with 1 μ g/mL of PN for 24 h. The levels of IFN- γ and IL-4 in supernatants were measured by ELISA assay. Data are shown as the means \pm SD. ** $P < 0.01$, compared with PN-immunized mice.

moattractant protein-1 (MCP-1) and IL-8 were detected. As shown in **Figure 3**, treatment with Rg1 significantly increased the transcription levels of IP-10, RANTES, MCP-1 and IL-8 in BM-derived DCs. The results demonstrated that DCs could induce and regulate the activation of T-lymphocytes by secreting chemokines to recruit them.

PN-specific splenocyte significantly proliferates in PN-Rg1 immunized mice

In order to test whether Rg1 could be used as the vaccine adjuvant, we selected a special antigen in combination with Rg1 to immunize

the C57BL/6 mice. Specifically, the C57BL/6 mice were randomly divided into four groups including PN in combination with Rg1 (PN-Rg1 group), PN alone (PN group), Rg1 alone (Rg1 group) or PBS control (PBS group). The mice were immunized twice on days 0, and 14. The chemical structure of Rg1 was shown in **Figure 4A**. The purity of antigen was more than 99% (**Figure 4B**). One week after final immunization, splenocytes from each group were in vitro-stimulated with special antigen PN, irrelevant antigen OVA or positive control antigen ConA for 24 h. As shown in **Figure 5A**, in vitro stimulated with PN antigen, the splenocytes significantly

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proliferated in PN-Rg1 immunized mice than that in PN-immunized mice or PBS-immunized mice. While there was no statistical difference among the three groups stimulated with irrelevant antigen OVA or positive control protein ConA.

Anti-PN serum IgG titer increases in PN-Rg1 immunized mice

Next, we detected the anti-PN IgG titers in PN-Rg1 group, PN group, Rg1 group or PBS group by ELISA assay. As shown in **Figure 5B**, the serum levels of anti-PN IgG titer were significantly higher in PN-Rg1 group than that in PN group or PBS group (** $P < 0.01$). There was no significant difference between PN-immunized group and Rg1-immunized group or PBS-immunized group ($P > 0.05$). All the results obviously demonstrated that Rg1 worked as an efficient vaccine adjuvant to prime antigen-specific immune response.

Serum IFN- γ and IL-4 levels are significantly up-regulated in PN-Rg1-immunized mice

Furthermore, we detected the cytokines secretion levels in serum of PN-Rg1 group, PN group, Rg1 group and PBS group. Briefly, splenocytes from the immunized mice were in vitro-stimulated with 1 $\mu\text{g}/\text{mL}$ of PN for 24 h, the serum IFN- γ and IL-4 levels were determined by ELISA assay. As shown in **Figure 6**, the mean value of IFN- γ and IL-4 in the PN-Rg1 group was significantly higher than in the PN group, Rg1 group or PBS group ($P < 0.01$). All the results obviously revealed that Rg1, as a vaccine adjuvant, could induce the PN-specific cellular and humoral immune responses.

Discussion

Streptococcus pneumoniae (Spn) is a key pathogen for pneumonia, meningitis and otitis media [31-33]. It is also the main pathogen to induce diseases in immunocompromised patients, or infants, young children and the elderly diseases in the world [34-36]. With the discovery of resistant strains of the *Streptococcus pneumoniae*, the effects of the vaccine in prevention of infection are increasingly important [37, 38]. In the present study, we used pneumococcal hemolysin antigen (PN) as the candidate antigen, which was encoded by pneumococcal

hemolysin gene without the carboxy-terminal 33 nucleotides, expressed and purified in *E. coli*. Thus, PN antigen had shown no hemolytic activity but retained the immunogenicity to prime immune responses and to keep the safety of the vaccines. Additionally, we used C57BL/6 mouse as the animal model, and explored the adjuvant effects of Rg1.

Dendritic cells are major antigen-presenting cells (APCs) that play the key role in the adaptive immune system. Firstly, we used increasing concentrations of Rg1 to treat BM-derived DCs in vitro. The results obviously showed that Rg1 could enhance the cytokines production, including TNF- α , IL-6, IL-1 β and IL-12. The Rg1-induced inflammatory cytokines secretion could effectively activate innate immune responses. Moreover, Rg1 also increased the transcription of chemokines, including IP-10, RANTES, MCP-1 and IL-8. IP-10 is a C-X-C chemokine that activated T cells and macrophages and attracted T cells into the inflammatory site. RANTES activates human basophil granulocytes and plays a regulatory role in inflammatory processes. MCP-1 (CCL2) is one of the important chemokines that promote migration and infiltration of monocytes or macrophages. Thus, Rg1 acted as a new adjuvant, which played an important role to activate the innate immune responses by promoting the cytokines and chemokines.

Furthermore, we tested the adjuvant activity in a mouse model. As expected, the mice immunized with PN in combination with Rg1 showed a higher level of anti-PN IgG titers, as well as the more antigen-specific splenocyte proliferation activity. Additionally, the splenocytes from mice immunized with Rg1 in combination with PN produced higher levels of IFN- γ and IL-4 when in vitro stimulated with PN protein. All the results demonstrated that Rg1, as an adjuvant of PN antigen, promoted the mixed Th1/Th2 immune response in vivo. This was consistent with that ginsenoside Rg1 and aluminum hydroxide synergistically promoted immune responses to ovalbumin in BALB/c mice [39]. In conclusion, Rg1 may be used as an effective new vaccine adjuvant to enhance the innate and adaptive immune responses.

Disclosure of conflict of interest

None.

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