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Immunologic synergism with IL-2 and effects of cCHMIs on mRNA expression of IL-2 and IFN-γ in chicken peripheral T lymphocyte

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Abstract

Two compound Chinese herbal medicinal ingredients (cCHMIs) were prepared, respectively, with epimedium polysaccharide plus propolis flavone (cCHMIs 1) and astragalus polysaccharide plus ginsenoside (cCHMIs 2). In animal immune experiment, Newcastle disease vaccine was mixed, respectively, with two cCHMIs and IL-2 to vaccinate 15-day-old chicken in experimental groups. On days 7, 14, 21 and 28 after vaccination, the dynamic changes of serum antibody titers were tested by micro-method. In gene expression experiment, each cCHMIs, at three concentrations, was added into cultured chicken peripheral T lymphocyte. After cultivation of 7 h, the expression of IL-2 mRNA in the cell, 24 h, IFN-γ mRNA, were determined by semi-quantitative RT-PCR assay. The results showed that two cCHMIs, whether single or cooperative injection with IL-2, could significantly raise the antibody titers, while immunologic synergisms of two cCHMIs with IL-2 were unconspicuous. cCHMs 1 at three doses and cCHMIs 2 at high dose could remarkably promote the expression of IL-2 mRNA in chicken T lymphocyte, two cCHMIs at three doses, the expression of IFN-γ mRNA, which could explain why the immunologic synergism of cCHMIs with IL-2 was not obvious.

Keywords: Compound Chinese herbal medicinal ingredients; Antibody titer; IL-2; IFN-γ; mRNA expression

1. Introduction

Astragalus, *gensing* and *Epimedium* are health-promoting herbal medicine commonly used in China. Their use could be dated back more than 2000 years ago and be recorded in Shen Nong's Materia Medica written in the Han dynasty. Scientific investigation in the last two decades has revealed that their effective components, astragalus polysaccharide, ginsenoside and epimedium polysaccharide, possess immunoenhancement both *in vivo* and *in vitro* [1–7]. Propolis, a Chinese medicine found in last decades, its main components have anti-inflammation and immunoregulative activity [8–11].

Interleukin-2 (IL-2) is a kind of T cell growth factor. It can induce the differentiation and stimulate the reactivity of many kinds of killed cells, such as cytotoxic T lymphocyte, lymphokine activated killer, and promote B cells to proliferate, differentiate and secrete immunoglobulin (Ig). In addition, IL-2 can stimulate the expression of the MHC II type antigen, transferrin receptor and insulin receptor, and generate manifold cytokines, including interferon-γ (IFN-γ), interleukin-4, interleukin-5, interleukin-6, TNF-α, TNF-β, CSF, and so on. IFN-γ is another important cytokine. It was generated mainly by $CD4^+$ and $CD8^+$T cell stimulated by antigen and mitogen, secondly by NK cell. IFN-γ has antiviral effect and can regulate immune response.

In recent years, numerous studies had demonstrated that a lot of Chinese herbal medicinal ingredients not only promoted lymphocyte proliferation but also improved the

Abbreviations: APS, *Astragalus polysaccharide*; cCHMIs, compound Chinese herbal medicinal ingredients; CHMI, Chinese herbal medicinal ingredient; CMF, calcium and magnesium-free; ConA, concanavalin A; EPS, *Epimedium polysaccharide*; GS, *Ginsenosides*; HI, hemagglutination inhibition; IFN-γ, interferon-γ; IL-2, interleukin-2; ND, Newcastle disease; PF, *Propolis flavone*; RT-PCR, reverse transcription-polymerase chain reaction

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expression of cytokines so as to enhance the immunity. In previous researches, the authors had found that astragalus polysaccharide, ginsenoside, epimedium polysaccharide, propolis flavone and two cCHMIs made from them had better immunoenhancement. At the same time, it had been confirmed that the effect of cCHMIs was better than that of single CHMI, and CHMI with IL-2 had better cooperative effect [12,13].

In the study, two cCHMIs were prepared, respectively, with EPS plus PF (cCHMIs 1) and APS plus GS (cCHMIs 2). In animal immune experiment, Newcastle disease vaccine was mixed, respectively, with each of two cCHMIs and IL-2 to vaccinate 15-day-old chickens. The dynamic changes of serum antibody titers were tested by micro-method. In gene expression experiment, each cCHMIs, at three concentrations, was added into cultured chicken peripheral T lymphocyte. The mRNA expression of IL-2 and IFN-γ were determined by semi-quantitative RT-PCR assay. The purpose is to observe the immunologic synergism of cCHMIs with IL-2 and probe into their molecular mechanisms on immunoenhancement.

2. Material and methods

2.1. Compound Chinese herbal medicinal ingredients

Astragalus polysaccharide (APS, a total polysaccharide containing 88.96% of glucose), *Epimedium polysaccharide* (EPS, a total polysaccharide containing 71.23% of glucose) and *Propolis flavone* (PF, a total flavone containing 70.99% of rutin) were prepared in our laboratory. *Ginsenosides* (GS, a total saponin containing 75% of Rbl) was bought from Yawei Pharmaceutical Co., Jilin, China [13]. Two cCHMIs were composed. cCHMIs 1 consisted of EPS and PF, cCHMIs 2, GS and APS, according to a certain proportion. Each cCHMIs was diluted with deionized water (Key laboratory of Nanjing Agricultural University) into certain concentration used in the animal immune experiment based on our previous results [4,13]. The total CHMIs contents of each dose in cCHMIs 1 and cCHMIs 2 were 0.75 and 1.875 mg, respectively. In gene expression experiment, each cCHMIs was diluted into three concentrations with RPMI-1640 without fetal bovine serum. cCHMIs 1 were 30, 15 and 7.5 μg/ml, respectively; cCHMIs 2 were 600, 300 and 150 μg/ml [12–15]. The diluted preparation were sterilized by pasteurization and detected for endotoxin by pyrogen tests [16]. When the endotoxin amount was accord with the standard of Chinese Veterinary Pharmacopoeia (less than 0.5 EU/ml), they were stored at 4 °C for the test.

2.2. Reagents

RPMI-1640 (GIBCO) supplemented with benzyipenicillin 100 IU/ml, streptomycin 100 IU/ml and 10% fetal bovine serum, was used for washing, re-suspending the cells, diluting the mitogen and culturing the cells. Concanavalin A (ConA, Sigma), as a mitogen, was dissolved into 0.5 mg/ml with RPMI-1640, filtered through a 0.22 μm syringe filter and stored at −20 °C.

Chicken recombinant interleukin-2 (IL-2, 10.0 mg/ml) was provided by Institute of Parasitology, College of Veterinary Medicine, Nanjing Agricultural University. Lymphocytes Separation Medium (Ficoll-Hypaque, ρ: 1.077 ± 0.002, No. 030728) was the product of Rong-sheng Biostix Shanghai Inc., trizol, Invitrogen company, diethyl pyrocarbnate (DEPC), Sai-jie Biology technique Inc., Oligod(t)$_{18}$, ribonuclease inhibitor, 2.5 mM dNTPs, 10 mM dNTPs, DL2000 Marker, reverse transcriptase (AMV), Taq DNA polymerase, Agarose, TaKaRa Biology technique Inc.

2.3. Vaccine

Inactivated ND vaccine virus (La Sota strain, No. 031228) was offered by Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Science. It was mixed, respectively, with two cCHMIs plus IL-2 (200 μg per dose), two cCHMIs, four CHMIs, IL-2 (200 μg per dose) and PBS to make into different adjuvant vaccine and non-adjuvant vaccine with the same antigen content.

2.4. Animal immune experiment

2.4.1. Animals

One-day-old White Roman chickens (male), purchased from Tangquan Poultry Farm, were housed in wire cages (60 cm × 100 cm) in air-conditioned rooms at 37 °C and lighted for 24 h at the beginning of pretrial period. The temperature was gradually declined to the room temperature and the light time to 12 h per day, which were kept constant in the following days. Chickens were fed with the commercial starter diet, provided by the feed factory of Jiangsu Academy of Agricultural Science.

2.4.2. Experimental design

Three hundred and thirty chickens were randomly divided into 11 groups. On 15 days old, their average titer of maternal antibody was 3.6 $\log_2$ and the average body weight was 112 g. Each chicken in Group 1–9 was intramuscularly injected, respectively, with 0.5 ml of vaccine containing different adjuvant, being cCHMIs 1-IL-2, cCHMIs 2-IL-2, cCHMIs 1, cCHMIs 2, PF, EPS, GS, APS and IL-2 in turn, while in Group 10, 0.5 ml non-adjuvant vaccine and in Group 11, 0.5 ml physiological saline, as controls. On days 7 (D_7), 14 (D_{14}), 21 (D_{21}), and 28 (D_{28}) after the vaccination, 10 chickens were sampled randomly from each group for determination of serum hemagglutination inhibition (HI) antibody titer by micro-method.

2.4.3. Serum HI antibody assay [17,18]

Blood samples (1.0 ml per chick) were drawn into Eppendorf tubes from the main brachial vein of chicken and allowed

to clot at 37 °C for 2 h prior to collect serum. Serum was separated by centrifugation and stored at −20 °C for use. Briefly, two-fold serial dilution of serum, after inactivated at 56 °C for 30 min, were made in a 96-well, V-shaped bottom microtiter plate containing 50 μl of CMF–PBS in all wells and then 50 μL of NDV antigen (4 HA units) was added into all the wells except for the last row served as the controls. Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 10 min at 37 °C. Then, 50 μl of a 1% rooster erythrocytes suspension was added to each well and re-incubated for 30 min. A positive serum, a negative serum, erythrocytes, and antigens were also included as controls. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI.

2.5. The experiment of gene expression [19–21]

2.5.1. Preparation of lymphocyte

Blood samples were collected from the main brachial vein of 1-month-age chicken and transferred immediately into aseptic capped tubes with sodium citrate, then diluted with an equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After 20 min centrifugation at 800 × *g*, a white cloud-like lymphocytes' band was collected and washed twice with RPMI-1640 media without fetal bovine serum. Cell viability was assessed by trypan blue exclusion. The resulting pellet was re-suspended to 5×10^6/ml with RPMI-1640 media and incubated in 24-well culture plates with 2 ml/well, then different concentrations cCHMIs were added into and each sample seeded four wells. ConA with a working concentration 20 μg/ml was used for the purification of lymphocytes. The lymphocyte was collected to isolate the RNA to amplify IL-2 and IFN-γ after the plates were incubated at 39.5 °C for 7 h and 24 h in a humid atmosphere of 5% CO_2.

2.5.2. Total RNA isolation

Total RNA was isolated from peripheral T lymphocyte by using Trizol reagent following the illustration provided by the manufacturer. The quantity of isolated RNA was determined by ultraviolet spectrophotometry, the optical density was 1.8–2.0.

2.5.3. Reverse transcription (RT)

RT was performed according to literature [22]. Briefly, the system of reverse transcription was 20 μl, containing 13 μl of total RNA, 1 μl Oligo$(dT)_{18}$, 1 μl 10 mM dNTP, 4 μl 5× Buffer, 0.5 μl Rnasin (40 U/μl), 0.5 μl AMV (5 U/μL) at 40 °C for 1 h. A tube with no reverse transcriptase was included in the RT reaction as a negative control. First strand-cDNAs were stared at −80 °C until used.

2.5.4. Amplification of cDNA and quantitation of cytokine gene expression

The semi-quantitative RT-PCR technique to detect cytokine mRNA was adapted from reports [23,24]. The primer sequences used were as follows:

chicken IL-2: 5′-GGGCAGATTCTTATTTTTTGCAGATA-3′ (sense), 5′-AATACCCATATGTGCAAAGTACTGA-3′ (antisense);
chicken IFN-γ: 5′-GAGCCATCACCAAGA-3′ (sense), 5′-GAGCACAGGAGGTCATAA-3′(antisense);
chicken β-actin: 5′-ACGTCGCACTGGATTTCGAG-3′ (sense), 5′-TGTCAGCAATGCCAGGGTAC-3′(antisense).

Specific cytokine gene segments were amplified in reaction system containing 1.5 μl $MgCl_2$ (25 mM), 2.0 μl dNTP (2.5 mM), 2.5 μl 10× buffer, 2.0 μl cDNA, 15.5 μl ddH_2O, 0.5 μl TaqE, 20 pM primers. The PCR condition of IL-2 mRNA: at 94 °C for 4 min (denaturation 94 °C for 50 s; annealing 52 °C for 50 s; polymerization 72 °C for 50 s), 30 cycles, the reaction was extended at 72 °C for 10 min; the PCR condition of IFN-γ mRNA: at 94 °C for 4 min (denaturation 94 °C for 50 s; annealing 50 °C for 50 s; polymerization 72 °C for 50 s), 29 cycles, the reaction was extended at 72 °C for 10 min.

The PCR products were electrophoresed with 1.2% agarose gel and stained with ethidium bromide. The quantity of PCR products was analyzed by densitometer. The relative value of IL-2 or IFN-γ mRNA = (the value of IL-2 or IFN-γ mRNA)/(the value of β-actin), which can mean the high or low of expression of IL-2 or IFN-γ mRNA. All experiments were carried out in triplicate.

2.6. Statistical analysis

Data are expressed as the mean ± S.D. Duncan's multiple range test was used to determine the difference among cCHMIs and control groups. Differences between means were considered significant at $p < 0.05$.

3. Results

3.1. The dynamic changes of serum antibody titer

The dynamic changes of antibody titer were listed in Table 1. The antibody titers in two cCHMIs-IL-2 groups and two cCHMIs groups were significantly higher than those of non-adjuvant group on each time-point ($p < 0.05$) and significantly higher than those of IL-2 adjuvant group on days 7 and 14 after vaccination ($p < 0.05$). Between two cCHMIs-IL-2 groups and two cCHMIs groups, there were no differences at each time-point. The antibody titers in PF group were significantly higher than those of non-adjuvant group and IL-2 group on day 7 after vaccination and mostly lower than those of cCHMIs 1-IL-2 group and cCHMs 1 group at other time-points ($p < 0.05$). The antibody titers of EPS group were

Table 1
The dynamic changes of serum antibody titer (log_2)

Group	D_7	D_{14}	D_{21}	D_{28}
cCHMIs 1-IL-2	5.5 ± 0.52^{bcd}	7.8 ± 0.41^{a}	6.7 ± 0.55^{a}	5.0 ± 0.63^{ab}
cCHMIs 2-IL-2	6.7 ± 0.52^{a}	7.3 ± 0.52^{ab}	6.5 ± 0.55^{a}	5.3 ± 0.52^{a}
cCHMIs 1	6.0 ± 0.00^{b}	7.7 ± 0.52^{a}	6.5 ± 0.55^{a}	5.5 ± 0.55^{a}
cCHMIs 2	6.7 ± 0.52^{a}	7.3 ± 0.52^{ab}	6.2 ± 0.75^{ab}	5.5 ± 0.55^{a}
PF	5.7 ± 0.52^{bc}	6.7 ± 0.89^{bc}	5.7 ± 0.41^{bc}	4.7 ± 0.52^{bc}
EPS	4.5 ± 0.55^{e}	6.8 ± 0.41^{bc}	6.3 ± 0.52^{ab}	4.5 ± 0.55^{bc}
GS	5.3 ± 0.52^{cd}	6.3 ± 0.52^{c}	6.3 ± 0.82^{ab}	5.2 ± 0.75^{ab}
APS	5.5 ± 0.55^{cd}	6.3 ± 0.52^{c}	5.8 ± 0.75^{abc}	5.0 ± 0.00^{ab}
IL-2	5.0 ± 0.00^{de}	6.5 ± 0.55^{c}	6.5 ± 0.55^{a}	4.8 ± 0.75^{abc}
Non-adjuvant	4.5 ± 0.55^{e}	6.3 ± 0.52^{c}	5.5 ± 0.55^{c}	4.2 ± 0.41^{c}
control	3.3 ± 0.55^{f}	3.0 ± 0.52^{d}	2.8 ± 0.55^{d}	2.5 ± 0.55^{d}

Column data marked without the same superscripts (a–f) differ significantly ($p < 0.05$).

significantly higher than those of non-adjuvant group on day 21 after vaccination and lower than those of cCHMIs 1-IL-2 group and cCHMIs 1 group on days 7 and 14 after vaccination ($p < 0.05$). The antibody titers of GS group were significantly higher than those of non-adjuvant group on days 7, 21 and 28 after vaccination and lower than those of cCHMI 2-IL-2 group and cCHMI 2 group on days 7 and 14 after vaccination ($p < 0.05$). The antibody titers of APS group were significantly higher than those of non-adjuvant group on days 7 and 28 after vaccination and lower than those of cCHMIs 2-IL-2 group and cCHMIs 2 group on days 7 and 14 after vaccination ($p < 0.05$).

3.2. The changes of gene expression

3.2.1. The variation of IL-2 mRNA expression

The IL-2 mRNA level of cCHMIs 1 at high, middle and low dose groups and cCHMIs 2 at high dose group were significantly higher than that of control group ($p < 0.05$), in which the high dose group of cCHMIs 1 was the best. The IL-2 mRNA level of EPS, PF, APS and GS were significantly higher than that of control ($p < 0.05$), in which the ratio of PF was the best. The IL-2 mRNA level of cCHMIs 1 at high and middle dose were significantly higher than those of PF and EPS ($p < 0.05$) those of control groups and most CHMI groups (Fig. 1).

3.2.2. The variation of lFN-γ mRNA expression

The IFN-γ mRNAlevel of cCHMIs 1 and cCHMIs 2 at high, middle and low dose groups were significantly higher than that of control group ($p < 0.05$), in which the ratio of cCHMIs 1 at middle dose group was the best. The IFN-γ mRNA level of PF and APS groups were significantly higher than that of control group ($p < 0.05$). The IFN-γ mRNA level of cCHMIs 1 at three dose groups were significantly higher than those of PF and EPS groups ($p < 0.05$). The IFN-γ

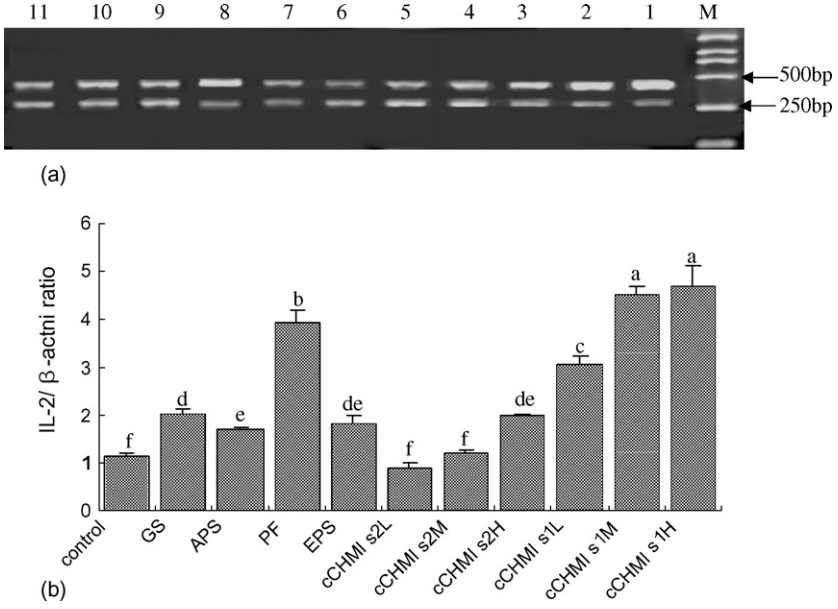

Fig. 1. The RT-PCR electrophoiesis photo (a) and analysis results (b) of IL-2 mRNA expression. M, DNA marker DL 2000; line 1–11: $cCHMI_{1H}$, $cCHMI_{1M}$, $cCHMI_{1L}$, $cCHMI_{2H}$, $cCHMI_{2M}$, $cCHMI_{2L}$, EPS, PF, APS, GS, control group in turn.

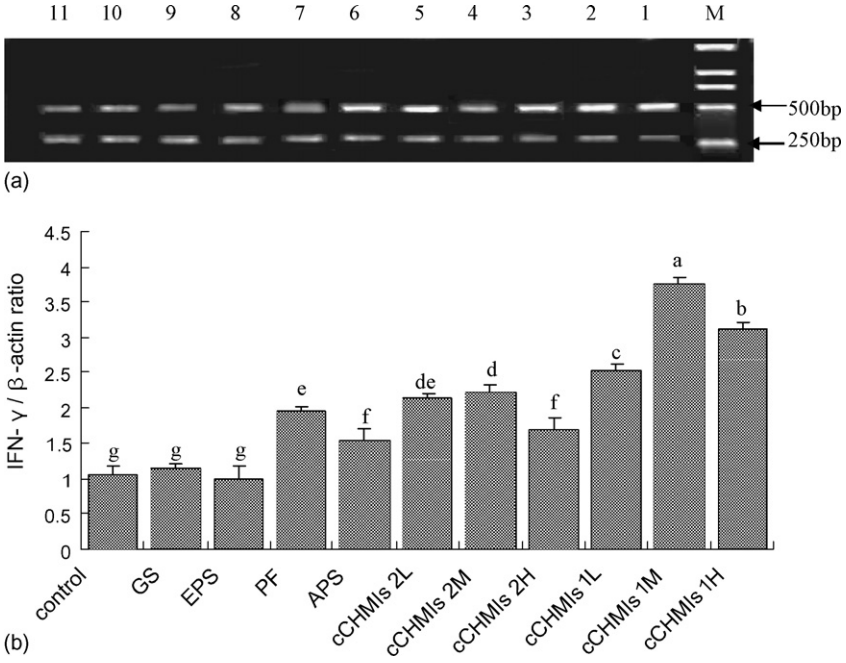

Fig. 2. The RT-PCR electrophoresis photo (a) and analysis results (b) of IFN-γ mRNA expression. M, DNA marker DL 2000; line 1–11: $cCHMI_{1H}$, $cCHMI_{1M}$, $cCHMI_{1L}$, $cCHMI_{2H}$, $cCHMI_{2M}$, $cCHMI_{2L}$, APS, PF, EPS, GS, control group in turn.

mRNA level of cCHMIs 2 at middle and low dose groups were significantly higher than those of GS and APS groups ($p < 0.05$) (Fig. 2).

4. Discussion

The results of animal immune experiments showed that the antibody titers of whether cCHMIs-IL-2 group or cCHMIs group were significantly higher than those of non-adjuvant group at each time-point, and the antibody titers of single CHMI group were significantly higher than those of non-adjuvant group at some time-points ($p < 0.05$). Those confirm that the cCHMIs-IL-2, cCHMIs and single CHMI have obvious immunologic enhancement.

Moreover, the antibody titers of cCHMIs-IL-2 groups and cCHMIs groups were significantly higher than those of single CHMI group and IL-2 group at some time-points ($p < 0.05$), but there were not significant differences between cCHMIs-IL-2 and cCHMIs groups. Those manifest that cCHMIs-IL-2 and cCHMIs possess stronger immunoenhancement as compared with CHMI, while there is not obviously cooperative effect between cCHMIs and IL-2. Thus, cCHMIs should be firstly selected as new-type immunopotentiator of Chinese herbal medicine.

Up to now there are few reports about effects of cCHMIs on animal cytokine expression in veterinary field. This study determined the effect of cCHMIs on mRNA expression of IL-2 and IFN-γ in chicken peripheral T lymphocyte. The results showed that cCHMIs 1 at three doses and cCHMIs 2 at high dose could markedly promote the expression of IL-2 gene, especially in cCHMIs 1 at high and middle doses the relative expression level of IL-2 were three-fold higher than control group, which indicated that cCHMIs 1 could strongly induce the generation of IL-2. In all of three doses of two cCHMIs groups, the expression levels of IFN-γ mRNA were significantly higher than that in control, which manifested that they could significantly improved the secretion of IFN-γ.

In immune system, immune cell and immune molecule can mutually regulate and form many immune regulation networks. IL-2-IFN-NKC system is one of immune regulation network. In this network, IL-2 plays important role in regulating generation of IFN and the activity of NKC. On the surface of NKC there are IL-2 receptors (IL-2R). Under the inducement of mitogen and antigen, Th cell secrete IL-2. After IL-2 combine with IL-2R of NKC, NKC will secrete IFN. IFN can act on precells of NKC and make them synthesize and express IL-2R. The cells can continue to proliferate after they accept the action of IL-2 [25]. IFN also participates in immune response mediated by T cell, B cell and other immune cells. IFN-γ is one of IFN, thus it is an important member of IL-2-IFN-NKC immune regulation network [26]. Our study showed that cCHMIs 1 and cCHMIs 2 not only promoted the expression of IL-2 mRNA but also improved the expression of IFN-γ mRNA, thus strengthen immunity. This may be one of their molecular mechanisms.

Many researches proved that four CHMIs selected by us could remarkably improve expression of IL-2 mRNA. APS could obviously increase expression of IL-2 mRNA and IL-2R mRNA in splenic lymphocyte of wound rats *in vitro* [27]. EPS could make the level of IL-2 and IL-2 mRNA recover normal in rats with chronic renal failure [28]. GS could enhance the genetation and release of IL-2 [29]. In this

research, we found that cCHMIs possessed stronger effects as compared with single CHMI, which could explain why there was not obvious immunologic synergism between cCHMIs and IL-2. Because cCHMIs themselves could induce the body to generate plenty of IL-2, it was difficult to display significant strengthening effect for cCHMIs when exogenous IL-2 was administrated again.

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