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Modulation of cytokines production, granzyme B and perforin in murine CIK cells by *Ganoderma lucidum* polysaccharides

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Abstract

Ganoderma lucidum polysaccharides (*Gl*-PS) have shown a variety of immune modulating effects. Culture of immunologic effector cells termed cytokine induced killer (CIK) cells is dependent on exogenous cytokines. It is not known about effects of *Gl*-PS on cytokine production, perforin and granzyme B expression in CIK cells. We made use of CIK cells as a means to investigate interaction between *Gl*-PS and cytokines, and explore mechanism of *Gl*-PS acting on proliferation and anti-tumor activity of CIK cells. The results suggested that *Gl*-PS (400 or 100 μg/ml) promoting CIK cells proliferation and cytotoxicity were relevant to enhancing IL-2, TNF production, protein and mRNA expression of granzyme B and perforin in CIK cells through synergizing cytokines in decreasing doses of IL-2 and anti-CD3 by 75 and 50%, and maybe were irrelevant to nitric oxide (NO). These results confirmed that *Gl*-PS was a promising biological response modifier (BRM) and immune potentiator.
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Keywords: Polysaccharides; Cytokine; Perforin; Granzyme B; Antitumour activity; Immunomodulatory

1. Introduction

Ganoderma lucidum polysaccharides (*Gl*-PS) demonstrating an extensive biological activity are isolated from the fruit body of *Ganoderma lucidum* (Leyss, ex Fr.) Karst. (Lingzhi; Reishi), and have been reported to be effective in modulating immune functions, inhibiting tumor growth (Lin & Zhang, 2004), preventing oxidative damage (You & Lin, 2002), protecting liver, reducing serum glucose levels, along with no toxicity (Zhang, Wang, Ni, Teng, & Lin, 2002; Zhang, He, Yuan, & Lin, 2003). Of particular significance among these function is its immunomodulating activities, including promoting NK cells cytotoxicity, proliferation of lymphocytes induced by concanavalin A or lipopolysaccharide, the function of mononuclear phygocyte system, humoral immunity, maturation of dendrite cells derived from cultured murine bone marrow and immune response induced by dendrite cells, antagonizing the inhibitory effects of immunosuppressive agents and anti-tumor drugs on lymphocytes proliferation and immune activity (Lin & Zhang, 2004), potentiating interleukin (IL-1, IL-2, IL-3 and IL-6), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) production and their mRNA expression. Recent findings suggested that *Gl*-PS directly adding to the cultured medium neither induced tumor cells apoptosis nor restrained its proliferation in vitro, and the anti-tumor effect of *Gl*-PS was mediated by cytokines released from activated T lymphocytes and macrophages (Wang, Hsu et al., 1997; Wang, Khoo, Chen, Lin, Wong & Lin, 2002; Lin & Zhang, 2004).

Cytokine-induced killer (CIK) cells are highly efficient cytotoxic effector cells generated in vitro from lymphocytes by addition of IFN-γ, IL-2, IL-1 and an antibody against CD3. The advantage of using CIK cells instead of lymphokine activated killer (LAK) cells in immunotherapeutic protocol is based on CIK cells possessing a higher level of cytotoxicity and a higher proliferation rate than LAK cells. CIK cells are dependent on exogenous cytokines such as IL-2, IL-1 and IFN-γ to obtain activation, proliferation and cytotoxicity (Zoll, Lefterova, Finke,

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Trojaneck, Ebert and Micka, 1998). Without prior exposure to tumor cells, CIK cells display TCR-independent cytotoxicity against a number of tumor cell lines, using mainly a perforin-based mechanism (Verneris, Kornacker, Mailänder, & Negrin, 2000). Perforin and granzymes exist in CIK cells. Both of them play an important role in the lytic activity, which is main mechanism of CIK cells killing targets. Granzyme B clearly promotes DNA fragmentation and is directly involved in cell death (Marten, Renoth, von-Lilienfeld-Toal, Buttgereit, Schakowski and Glasmacher, 2001; Mehta, Schmidt-Wolf, Weissman, & Negrin, 1995). Several cytokines, including IL-2, IL-6, IL-7, IL-12 and IFN-γ can induce perforin and granzyme B mRNA expression, which correlates well with the cytotoxic activities of killer cells (Fitzpatrick, Makrigiannis, Kaiser, & Hoskin, 1996; Greene, Makrigiannis, Fitzpatrick, & Hoskin, 1997; Liu, Walsh, & Youn, 1995; Spitzer & Meadows, 1999). Little is known about effects of *Gl*-PS on cytokine production, perforin and granzyme B expression in CIK cells. In the present study, we made use of CIK cells as a means to investigate interaction between *Gl*-PS and cytokines that play a great role in the course of immune precursor cells proliferation and differentiating to CIK cells, and explored mechanism of *Gl*-PS acting on CIK cells proliferation and anti-tumor activity.

2. Materials and methods

2.1. Cells and animals

Mouse L929 fibrosarcoma cells obtained from Institute of Basic Medicine, Academy of Military Medical Sciences (Beijing, China) were maintained in RPMI-1640 medium with 10% inactivated fetal calf serum (FCS). Mouse P815 mastocytoma cells (NK-resistant) and mouse YAC-1 lymphoma cells (NK-sensitive) obtained from Department of Pathology, School of Basic Medical Science, Health Science Center, Peking University (Beijing, China) were maintained in RPMI-1640 medium with 10% inactivated FCS. Male (6–8 weeks old) C57BL/6 mice were purchased from Department of Experimental Animal, Health Science Center, Peking University (Beijing, China).

2.2. Drugs

Ganoderma lucidum polysaccharides (*Gl*-PS), a kind of polysaccharide was isolated from boiling water extract of fruit bodies of *Ganoderma lucidum* (Leyss, ex Fr.) Karst followed by ethanol precipitation, dialysis and protein depletion using Sevag method. *Gl*-PS was glycopeptide with molecular weight of 584,900, and the ratio of polysaccharides to peptides is 93.61:6.49%. The polysaccharides were consisted of D-rhamnose, D-xylose, D-fructose, D-galactose, D-mannose and D-glucose with molar ratio of 0.793:0.964:2.944:0.167:0.389:7.94 and linked together by β-glycosidic linkages. The peptides contained 16 kinds of amino acid. *Gl*-PS was water-soluble powder and dissolved in serum-free RPMI 1640 medium (Gibco BRL), then filtered through a 0.22 μm filter and stored at 4 °C. It was further diluted, to indicated concentrations prior to each assay.

2.3. CIK cells preparation and supernatants collection

Nonadherent mononuclear cells from spleen of C57BL/6 mice were incubated at a concentration of 1×10^6/ml in completed RPMI 1640. Murine IFN-γ 1000 U/ml was added on d 0. After 24 h of culture, 50 ng/ml mAb against CD3, 300 U/ml IL-2 and 100 U/ml murine rIL-1 were added. Fresh medium containing IL-2 (150 U/ml) was added every 3 d to maintain a cell density of $0.2–0.5\times10^6$/ml at initial stages, and thereafter, $1.5–2.0\times10^6$/ml for a total of 14–21 d. Cytokines were purchased from PeproTech EC Ltd (London, UK). Experiment in vitro was divided into groups as follows: positive control group was prepared as above by routine protocol. Experimental group 1 or 2 was the same as routine protocol except additional stimulus *Gl*-PS (400 or 100 μg/ml) and decreasing dose of IL-2 and anti-CD3 by 75 and 50%. Addition soluble starch (400 or 100 μg/ml) or methylcellulose (400 or 100 μg/ml) instead of *Gl*-PS in the same protocol as experimental group served as negative control groups 1–4.

Supernatants were collected, respectively, from every group at initial CIK cells proliferative stages and in the course of CIK cells killing targets to detect IL-2, IFN-γ, NO, TNF production.

2.4. Measurements of nitric oxide (NO) production

NO production was quantified by the accumulation of nitrite in the supernatant by the standard Griess reaction.

2.5. TNF activity measurement

TNF levels were determined by the standard L929 cytotixic assay. The TNF level in the sample was determined using the standard curve of TNF on lognormal probability coordinates paper.

2.6. IL-2, IFN-γ production assay

Secretion of IL-2 and IFN-γ from supernatants was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Jingmei Biotech) according to the manufacturer's protocol. The minimum detection level of IL-2 and IFN-γ was 5 and 15 pg/ml.

2.7. Western blot analysis

Immunoblot analysis was used to determine protein production of perforin and granzyme B. The cell lysates

were electrophoresed on 12 and 8% SDS-PAGE for detection of granzyme B and perforin. The proteins were transferred to nitrocellulose membranes (Amersham, UK). The membranes were subsequently preblocked in T-TBS (TBS containing 0.1% Tween 20) containing 5% nonfat milk powder (blocking buffer) and then incubated with the primary antibody (goat polyclonal anti-perforin antibody and goat polyclonal anti-granzyme B antibody were purchased from Santa Cruz Biotechnology, Inc.) in blocking buffer at dilution of 1:100 overnight at 4 °C, followed by incubation with rabbit anti-goat IgG antibody conjugated to peroxidase. The antigen–antibody complex was visualized with Western blotting luminol reagent (ECL, Santa Cruz Biotechnology, Inc.). The bands were quantified with Gel Doc 2000 system and Quantity One software (BIO-RAD).

2.8. RNA extraction and semi quantitative RT-PCR

Cells were collected and washed once with diethyl pyrocarbonate (DEPC)-treated PBS, and the total RNA were extracted using TRIzol Reagent (Gibco BRL). RNA precipitates were dissolved in DEPC-treated water containing RNasin® Ribonuclease Inhibitor (Promega). The concentration of RNA was quantified spectrophotometrically. The mRNA expression of perforin and granzyme B in CIK cells was performed by reverse transcription polymerase chain reaction (RT-PCR) using Access RT-PCR system (Promega) according to the manufacture's procedures. PCR was performed for 28 cycles, which was chosen to ensure that amplification of all specific cDNA products were exponential with a 30 s denaturing step at 94 °C, 1 min annealing step at 60 °C, and a 2 min extension phase at 68 °C. Specific primer sequences of granzyme B (O'Connell, Pacheco-Silva, Nickerson, Muggia, Bastos and Kelley, 1993) perforin (Seko, Tsuchimochi, Nakamura, Okumura, Naito and Imataka, 1990; Youn, Liu, Kim, Young, Kwon and Kwon, 1991) or β-actin (Hoskin, Phu, & Makrigiannis, 1996) were as follows: for Granzyme B, sense 5′-CCC AGG CGC AAT GTC AAT-3′, anti-sense: 5′-CCA GGA TAA GAA ACT CGA-3′; for Perforin, sense 5′-AGG CAG CTG CTA ATA TCA AT-3′, anti-sense 5′-TGT GCT GTT TCT TCT TCT CC-3′; for β-actin, sense 5′-CTG GAG AAG AGC TAT GAG C-3′, anti-sense 5′-GTA ACG ACT GTC CTA CGT CTT-3′. The size of granzyme B, perforin or β-actin production were 330, 501, or 241 bp, respectively, DNA PCR products were eletrophoresed on a 2% agarose gel and stained with ethidium bromide and visualized by UV transillumination. The detected cDNA was compared to a molecular weight routine (marker) consisting of a 100, 200, 400, 500, 600, 800, 1000 bp ladder to confirm PCR product size. By using Gel Doc 2000 system and Quantity One software (BIO-RAD), PCR products were quantified by densitometric scanning. Perforin and granzyme B expression were normalized relative to the steady-state expression of β-actin used as internal control, respectively.

2.9. Statistical analysis

Data were analysed using one-way ANOVA followed least-significant difference (LSD). P values below 0.05 were considered statistically significant.

3. Results

3.1. Regulation of Gl-PS on cytokines production at stage of CIK cells induction and proliferation

Our previous studies demonstrated that *Gl*-PS (400 or 100 μg/ml) could decrease the doses of IL-2 and anti-CD3 by 75 and 50%, respectively, in inducing CIK cells by synergizing cytokines in lower doses, and had insignificant change on proliferation, cytotoxicity and phenotype of CIK cells induced by cytokines in higher doses alone. But addition soluble starch (SS) or methylcellulose (MC) (400 or 100 μg/ml) instead of *Gl*-PS, proliferation and cytotoxicity of CIK cells were significantly lower than that of CIK cells induced by cytokines in higher doses alone, and influences of addition equal volume of serum-free RPMI 1640 medium instead of SS or MC on proliferation and cytotoxicity of CIK cells were almost equal to that of addition SS or MC and cytokines in decreasing dose of IL-2 and anti-CD3 by 75 and 50%. Therefore, SS and MC were chosen as negative control agents (data not showed) (Zhu & Lin, 2004).

Nonadherent murine splenocytes cultured in complete medium without any cytokine showed small in size and nucleus were dense. Size and nucleus of CIK cells became large, and large granules emerged in cytoplasm that increased and nucleus was loose. There was no significant difference in CIK cells morphology between positive control induced by cytokines in higher dosed alone and experimental groups induced by *Gl*-PS (400 or 100 μg/ml) synergizing cytokines in lower doses (Fig. 1).

NO levels in CIK cells supernatants from different groups described in materials and methods were undetectable on d 1–3, 5, 10 in the culture, NO production was very low ($<0.93\pm0.12$ μM) on d 15 and no significant difference existed in different groups.

Compared with positive control group, TNF level in CIK cells supernatants harvested on d 1 from experimental groups changed insignificantly, but was higher than that in negative control groups. TNF level in supernatants of every groups decreased markedly and was undetectable on d 4 of CIK cells culture (Fig. 2).

IL-2 level in CIK cells supernatants harvested from every group peaked on d 2. IL-2 levels in CIK cells supernatants harvested on d 1–3 from experimental groups were lower than that in positive control group but higher than that in negative control groups significantly (Fig. 3).

The changes of IFN-γ level in CIK cells supernatants harvested on d 1–3 from every group were not significant.

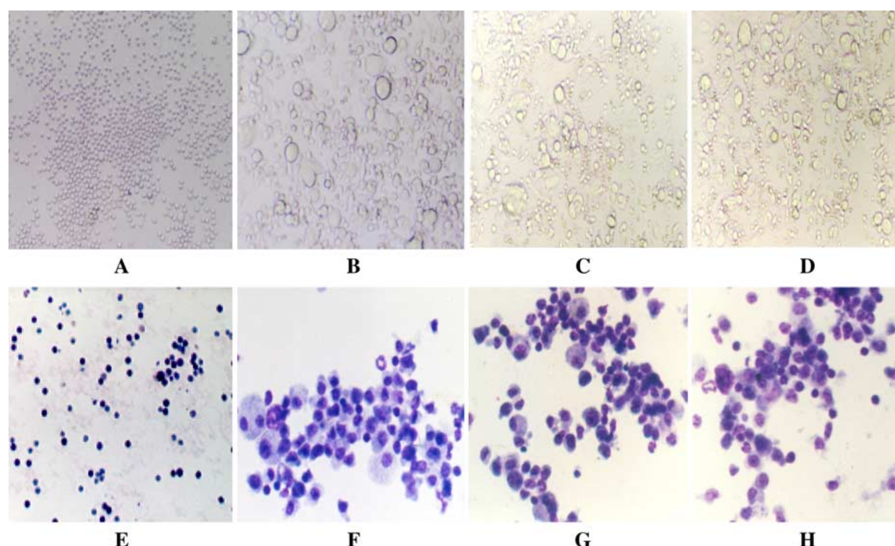

Fig. 1.

IFN-γ level in CIK cells supernatants from every group peaked on day 1 and then reduced markedly to a lower amount after 48 h, and was only in the minimum detectable bounds on d 3 (Fig. 4).

3.2. Regulation of Gl-PS on cytokines production in the course of CIK cells and tumor cells coculture

Supernatants in 24 h cocultures of P815 or YAC-1 cells and CIK cells induced by different protocol on d 15 described in materials and methods at an effector: target ratio of 20:1 were harvested, respectively. NO level in supernatants of effectors and targets cocuture in every group was minimum detectable and insignificant from each other and from that in CIK cells supernatants on d 15 in every group. NO production in YAC-1 or P815 cells culture supernatants was not detectable.

TNF levels in supernatants from 24 h cocultures of P815 or YAC-1 cells and CIK cells in experimental groups were insignificant from that in positive control group, but higher than that in negative control groups. TNF levels in supernatants of P815 or YAC-1 cells or CIK cells induced by different protocol on d 15 were low and insignificant from each other (Fig. 5).

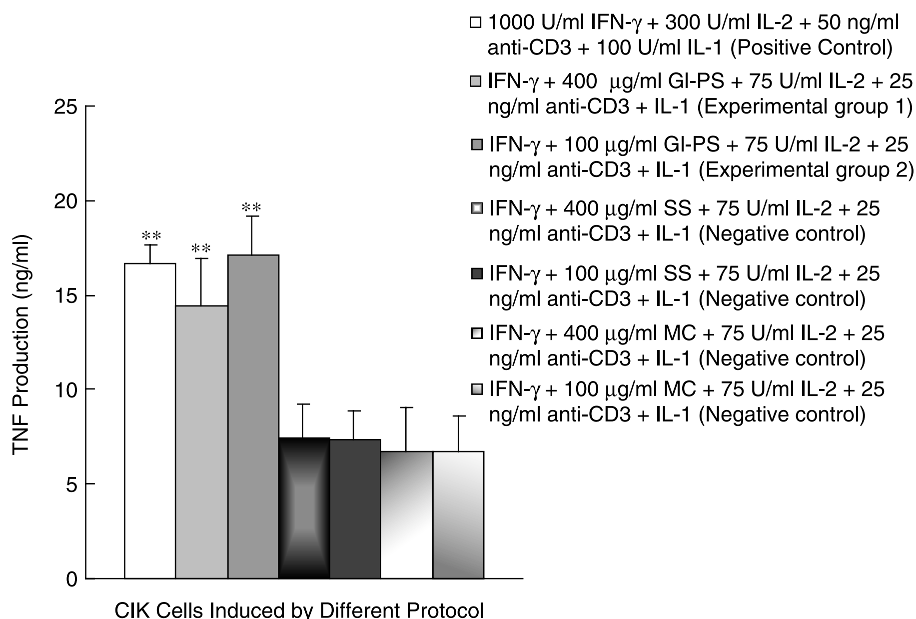

Fig. 2. Effects of *Gl*-PS on TNF production by CIK cells. CIK cells culture supernatants from different protocol described in legend above were harvested on d 1, respectively. $^{**}P<0.01$ *vs* negative controls. The dose of IFN-γ (1000 U/ml) and IL-1 (100 U/ml) was equal in every group. SS, soluble starch; MC, methylcellulose.

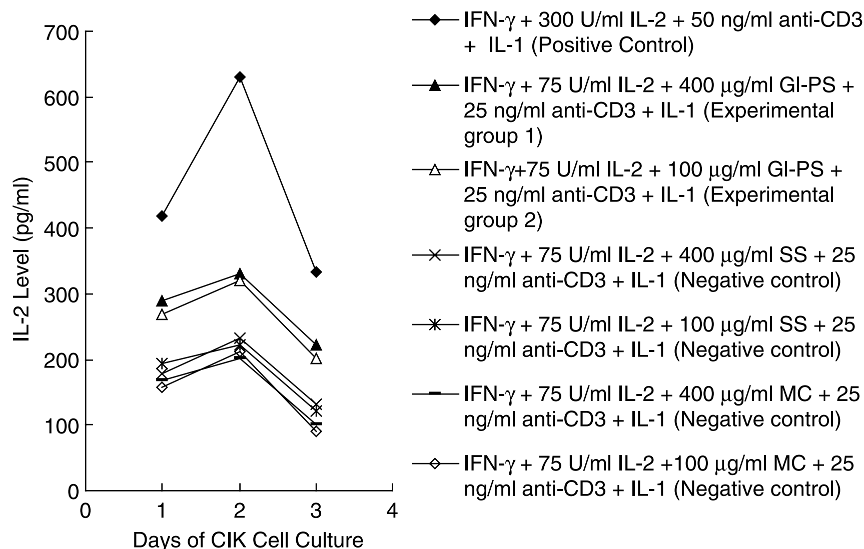

Fig. 3. Effects of *Gl*-PS on IL-2 level in CIK cells culture supernatants. CIK cells culture supernatants from different protocol described in legend above were harvested on d 1–3, respectively. The dose of IFN-γ (1000 U/ml) and IL-1 (100 U/ml) was equal in every group. SS, soluble starch; MC, methylcellulose.

IFN-γ production in supernatants of P815, YAC-1 cells or CIK cells induced by different protocol on d 15, or in 24 h coculture supernatants of P815 or YAC-1 cells and CIK cells from different groups was undetectable by ELISA.

3.3. Regulation of Gl-PS on protein production of granzyme B in CIK cells

Granzyme B within cytoplasmic granules has molecular mass of 32 kDa (Isaaz et al., 1995). Fig. 6 demonstrated that protein of granzyme B was expressed in CIK cells. Granzyme B expression of CIK cells in negative control group added methylcellulose (100 μg/ml) was regarded as 100%. It showed that protein of granzyme B expression in CIK cells in experimental groups induced by *Gl*-PS (400 or 100 μg/ml) and cytokines in lower doses were higher than that in negative control groups, but insignificant from positive control groups induced by cytokines in higher doses alone.

3.4. Regulation of Gl-PS on protein production of perforin in CIK cells

Molecular mass of mouse perforin protein is 70 kDa determined by SDS-PAGE under reducing conditions

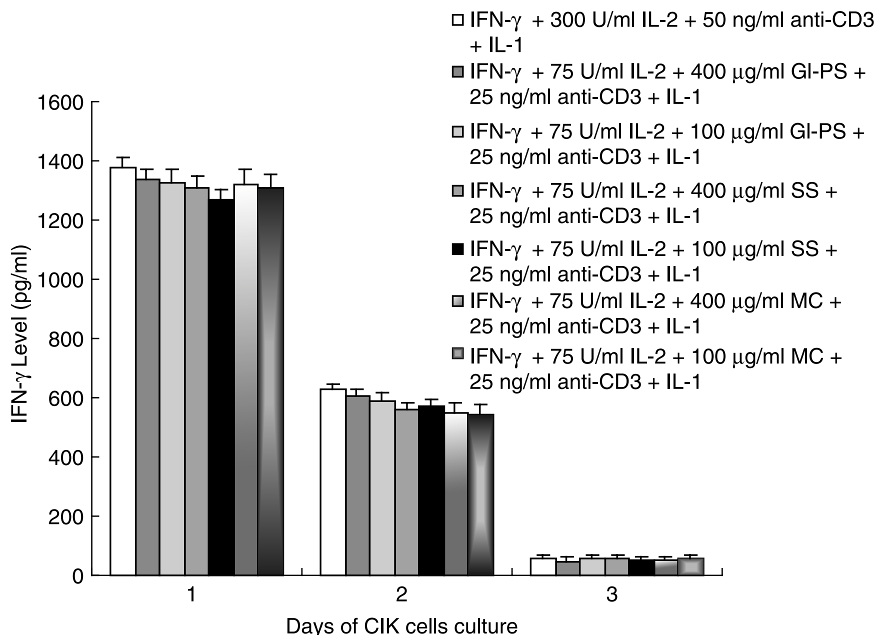

Fig. 4. Effects of *Gl*-PS on IFN-γ level in CIK cells culture supernatants. CIK cells culture supernatants from different protocol described in legend above were harvested on d 1–3, respectively. The dose of IFN-γ (1000 U/ml) and IL-1 (100 U/ml) was equal in every group. SS, soluble starch; MC, methylcellulose.

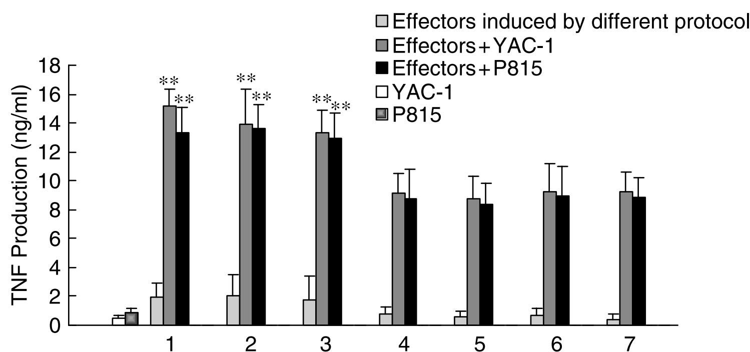

Fig. 5. Effects of *Gl*-PS on TNF production in cocultures of CIK and target cells. Number at *x*-axis meant effectors induced by different protocol described in materials and methods. (1) CIK cells were induced by routine protocol as positive control group. (2) or (3) CIK cells were induced by additional stimuli *Gl*-PS (400 or 100 μg/ml) and decrease dose of IL-2 and anti-CD3 by 75 and 50% served as experimental groups. (4)–(7) SS (400 or 100 μg/ml, 4 and 5) or MC (400 μg/ml or 100 μg/ml, 6 and 7) was used as negative control groups. Supernatants in culture of effectors, targets or in cocultures of CIK cells and P815 or YAC-1 cells at an effector: target ratio of 20:1 were harvested, respectively, for measurement TNF production. $^{**}P<0.01$ vs. negative controls.

(Young, Hengartner, Podack, & Cohn, 1986). Protein of perforin expression of CIK cells in negative control group added methylcellulose (400 μg/ml) was regarded as 100%. Fig. 7 showed that protein of perforin expression in CIK cells in experimental groups added *Gl*-PS (400 or 100 μg/ml) and cytokines in lower doses was higher than that in negative control groups, but insignificant from positive control group added cytokines in higher doses alone.

3.5. *Gl-PS modulated mRNA expression of granzyme B and perforin by CIK cells*

CIK cells cultured between d 10 and 28 have the greatest cytotoxic activity (Schmidt-wolf, Negrin, Kiem, Blume, & Weissman, 1991). Semiquantitative RT-PCR was used to determine the effect of *Gl*-PS on granzyme B and perforin mRNA expression by CIK cells on d 14. A β-actin PCR was performed to control for equal loading of PCR products onto the agarose gel. Figs. 8 and 9 demonstrated that mRNA of granzyme B and perforin was expressed in CIK cells. It also showed that granzyme B and perforin mRNA expression by CIK cells in experimental groups added *Gl*-PS (400 or 100 μg/ml) and cytokines in lower doses were higher than that in negative control groups, but insignificant from positive control group added cytokines in higher doses alone. Both of granzyme B and perforin mRNA expression had insignificant differences in negative control groups.

4. Discussion

Gl-PS, a high-molecular-weight heteroglycans which principal component is β-D-glucan showed immunological activity, such as promoting cellular immunity and humoral immunity (Lin & Zhang, 2004). Biological and immunopharmacological activities depend on polysaccharides conformation, molecular mass, solubility in water and triple-helical structure. Soluble starch is an α-(1,4)-bonding glucose polymer, and methylcellulose links by uniform β-(1,4) glycosidic linkages. Both of them do not show

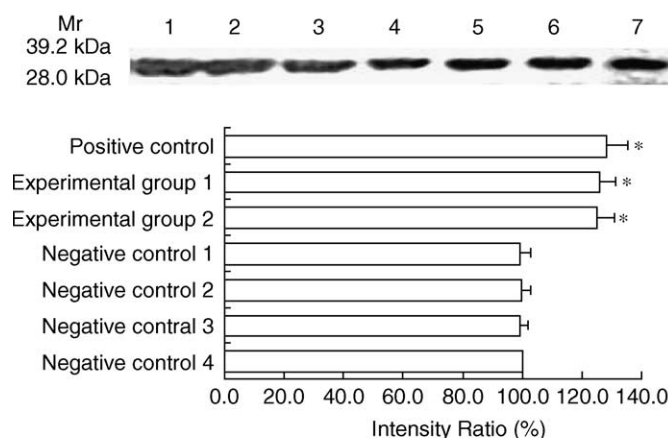

Fig. 6. Regulation of *Gl*-PS on protein express of granzyme B in CIK cells. CIK cells in different groups were collected, and total protein was extracted using lysis buffer solution. The expression of granzyme B protein was performed by western blot. (1) Positive control group. (2) and (3) Experimental group 1 and 2 added *Gl*-PS (400 or 100 μg/ml). (4) and (5) Negative control group 1 and 2 added SS (400 or 100 μg/ml). (6) and (7) Negative control group 3 and 4 added MC (400 or 100 μg/ml). $^{*}P<0.05$ vs. negative control 1 to 4.

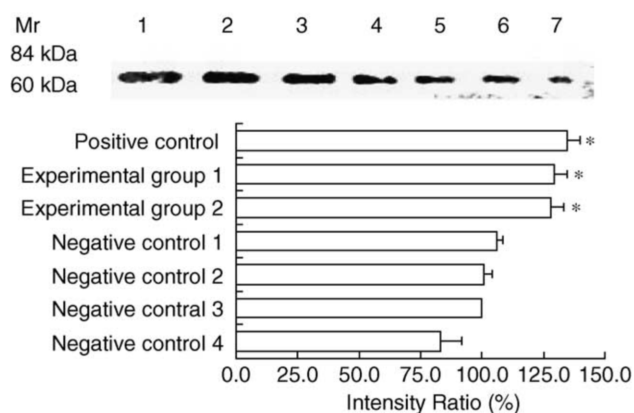

Fig. 7. Regulation of *Gl*-PS on protein express of perforin in CIK cells. CIK cells in different groups were collected, and the expression of perforin protein was performed by western blot. (1) Positive control group. (2) and (3) Experimental group 1 and 2 added *Gl*-PS (400 or 100 μg/ml). (4) and (5) Negative control group 1 and 2 added SS (400 or 100 μg/ml). (6) and (7) Negative control group 3 and 4 added MC (400 or 100 μg/ml). $^{*}P<0.05$ vs. negative control 1 to 4.

activities for lacking helical structure (Chen, 1997) and served as negative control in our studies. IL-2 induces T lymphocytes proliferation, differentiation and selectly maintains long-term growth of T lymphocytes, and plays an important role in CIK cells surviving, activation and proliferation in vitro. IL-1 and IFN-γ has no effect on CIK cells proliferation (Schmidt-wolf et al., 1991; Zoll et al., 1998). Previous studies suggested that *Gl*-PS (300 μg/ml) could enhance cytotoxicity of NK cells and LAK cells and cytokines production including IL-2 by activated murine splenocytes in votro (Lin & Zhang, 2004). We have reported previously that *Gl*-PS (400 or 100 μg/ml) could decrease the doses of IL-2 and anti-CD3 by 75 and 50%, respectively, in inducing CIK cells by synergizing cytokines in lower doses, and had insignificant change on proliferation, cytotoxicity and phenotype of CIK cells induced by cytokines in higher doses alone (Zhu & Lin, 2004). In the present study, we demonstrated that addition *Gl*-PS (400 or 100 μg/ml) synergizing cytokines in lower doses in experimental groups promoted IL-2 production at first 24 h of CIK cells culture, and then reached maximal level at 48 h. Although this IL-2 production was lower

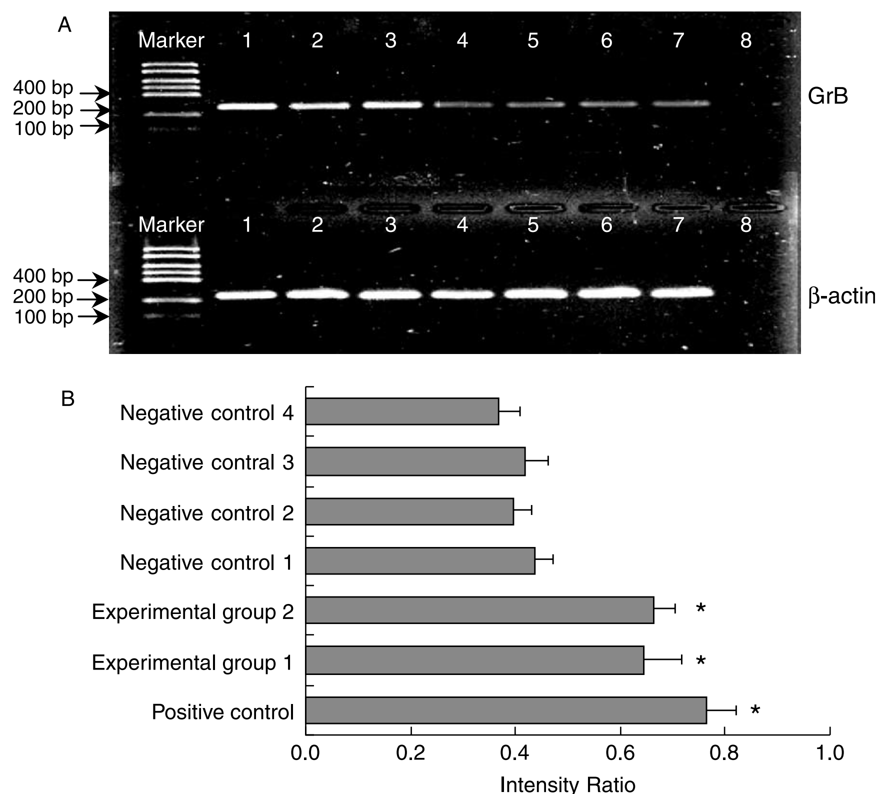

Fig. 8. Influences of *Gl*-PS on mRNA expression of granzyme B (Gr B) by CIK cells. (A) (1) Positive control group. (2) and (3) Experimental group 1 and 2 added *Gl*-PS (400 or 100 μg/ml). (4) and (5) Negative control group 1 and 2 added SS (400 or 100 μg/ml). (6) and (7) Negative control group 3 and 4 added MC (400 or 100 μg/ml). (8) Substitute nuclease-free water for the RNA template in the reaction for a negative control reaction. (B) Following 14 d of culture, mRNA was isolated and reverse transcribed. A semiquantitative RT-PCR with primers of specific for granzyme B in CIK cells induced by different protocol was performed. PCR products were quantified by densitometric scanning and granzyme B expression was normalized relative to the steady-state expression of β-actin used as internal control (intensity ratio: granzyme B to β-actin). $^{*}P<0.05$ vs. negative control group 1 to 4.

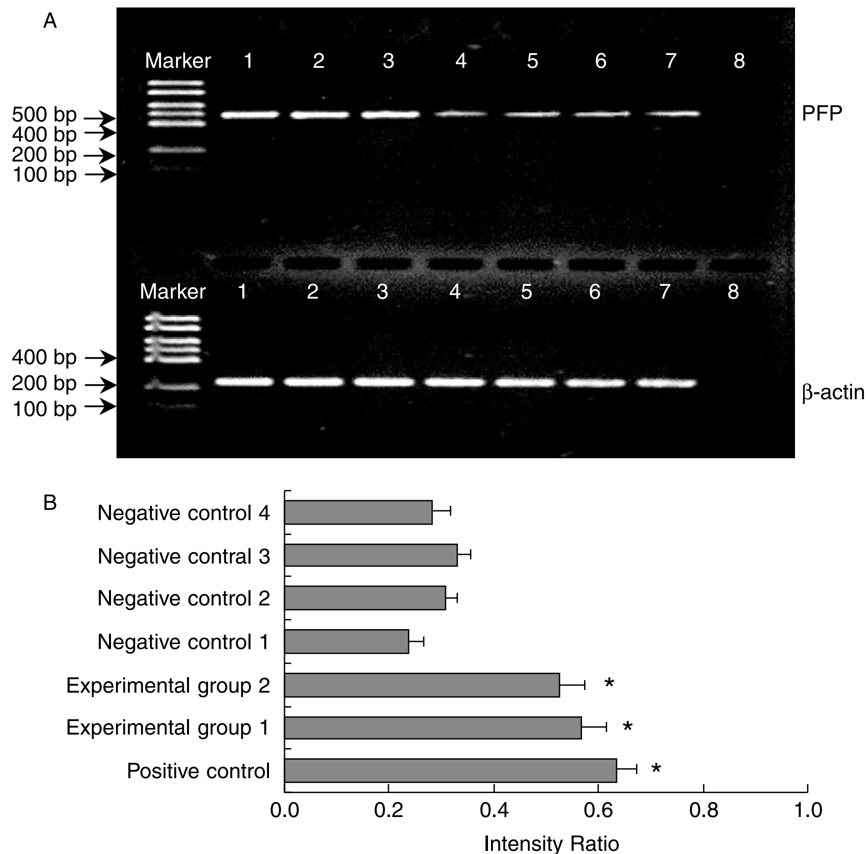

Fig. 9. Influences of *Gl*-PS on mRNA expression of perforin by CIK cells. (A) (1) Positive control group. (2) and (3) Experimental group 1 and 2 added *Gl*-PS (400 or 100 μg/ml). (4) and (5) Negative control group 1 and 2 added SS (400 or 100 μg/ml). (6) and (7) Negative control group 3 and 4 added MC (400 or 100 μg/ml). (8) Substitute nuclease-free water for the RNA template in the reaction for a negative control reaction. (B) Following 14 d of culture, mRNA was isolated and reverse transcribed. A semiquantitative RT-PCR with primers of specific for perforin in CIK cells induced by different protocol was performed and PCR products were quantified by the same methods as granzyme B. $^{*}P<0.05$ vs. negative control group 1 to 4.

significantly than that in CIK cells induced by cytokines in higher doses alone in positive control group, positive control group and experimental groups showed no change in CIK proliferation. We speculated that IL-2 level in experimental groups added *Gl*-PS (400 or 100 μg/ml) reached the range of optimal cooperation doses. Because stimulating CIK cells proliferation need IL-2 plus anti-CD3 within an optimal concentration range. The optimal dilution of anti-CD3 activating T lymphocytes alone was $1{:}10^5$ to $1{:}10^3$. A very low concentration of anti-CD3 (1/100,000 dilution or 10 ng/ml) was sufficient to induce CIK cells expansion synergizing IL-2 with plateau concentrations between 67 and 500 U/ml. That meant effect of IL-2 dose at 67–500 U/ml on CIK cells proliferation was equal. The anti-CD3 was added only during the initial culture. Once the cells were activated, no anti-CD3 needed (Ochoa, Gromo, Alter, Sondel, & Bach, 1987; Ting, Hargrove, & Yun, 1988; Zoll et al., 1998).

The time course of IFN-γ addition was found to be crucial in enhancing cytotoxicity. Only if added 24 h before the addition of IL-2, could IFN-γ increase cytotoxicity. After exposure to IFN-γ, the expression of receptor sites of IL-2 on lymphocytes increased (Schmidt-wolf et al., 1991). More importantly, addition IFN-γ prior to IL-2 and anti-CD3 might select for a population of cells to resist Fas-mediate apoptosis since CIK cells up-regulated both the Fas receptor and FasL over time in culture (Verneris et al., 2000). The combination of IFN-γ and anti-CD3 and IL-2 combined the proliferative effect of the anti-CD3 plus IL-2 treatment with the increase in cytotoxicity by IFN-γ. The addition of IL-1 improved the cytotoxicity slightly, but significantly induced the expression of IL-2 receptor (Schmidt-wolf et al., 1991). So we used equal dose of IFN-γ and the same addition order as positive control group to ensure effector of experimental groups against Fas-mediate apoptosis. IFN-γ level in CIK cells supernatants harvested on d 1–3 from every group were not significant different. This indicated that cytotoxicity of CIK cells in experimental groups would not reduce due to IFN-γ. Our study showed that *Gl*-PS (400 or 100 μg/ml) combined with cytokines in decreasing doses of IL-2 and anti-CD3 by 75 and 50% promoted IL-2 production within an optimal synergizing concentration and thus made CIK cells attain the same effect of proliferation and cytotoxicity as positive

control group added cytokines in higher doses alone. We were unable to detect any IFN-γ production in coculture supernatants of CIK cells and target tumor cells. The results suggested that CIK cells probably did not secret IFN-γ to kill tumor cells directly. Previous reports pointed out that IFN-γ, IL-1 had no effect on cell proliferation but there was an increase in cytotoxic activity when combined with anti-CD3. However, addition of TNF had no effect on CIK activity (Schmidt-wolf et al., 1991). We cannot demonstrate higher TNF level in positive control group and experimental groups than that in negative control group was related to CIK cells proliferation, but whether it maybe exerts other influence on CIK cells need further investigation. The equal noticeable TNF production was detected in coculture of target cells and CIK cells in positive control group and experimental groups, and TNF was secreted markedly in the course of CIK cells directly contacting with targets. So it was probable that TNF participate in anti-tumor activity of CIK cells.

NO attracts people attention as a tumoricidal and signal molecule. Recent findings indicated that protective and toxic effects of NO were frequently seen in parallel. Function of NO in inhibiting T and B cell proliferation was observed (Bogdan, 2001). We used nonadherent mononuclear cells as immune precursors of CIK cells in initial culture. NO production was undetectable in every group supernatants in the course of CIK proliferation. So action of NO in inhibiting T cell proliferation did not existed. Our results corresponded to al-Ramadi's findings that NO production was not observed in cultures consisting of nonadherent splenocytes (al-Ramadi, Meissler, Huang, & Eisenstein, 1992). We were unable to detect any noticeable NO production in course of CIK cells killing targets. The results suggested that NO might not be tumoricidal molecule produced by CIK cells.

Without prior exposure to tumor cells, CIK cells exert TCR-independent cytotoxicity employing mainly perforin/granzyme B mechanism. CIK cells secrete benzyloxycarbony-1-lysine thiobenzyl ester (BLT) esterase, which is a marker for granule content including granzyme B, upon stimulation with anti-CD3. The CIK effector co-expression NKT markers show maximum release of BLT esterase. Granzyme B is the major protease that induces DNA fragmentation in tumor target cells by CIK cells (Verneris et al., 2000). Previous studies demonstrated that perforin and granzyme B expression was highly relevant to cytotoxic activity (Mehta et al., 1995). Cytokines including IL-2, IFN-γ, TNF-α induce perforin and granzyme B expression (Fitzpatrick et al., 1996; Liu, Joag, Kwon, & Young, 1990; Liu et al., 1995; Robinet, Branellec, Termijtelen, Blay, Gay and Chouaib, 1990). Some polysaccharides with bioactivity such as carrageenan, heparan sulfate and *Gl*-PS promote NK, CTL cytotoxic activity, which is relevant to perforin and granzyme B (Abe, Kawamura, Kawabe, Watanabe, Gejyo and Abo, 2002; Wrenshall, Carlson, Cerra, & Platt, 1994; Lin & Zhang, 2004). We demonstrated that *Gl*-PS (400 or 100 μg/ml) improved protein and mRNA expression of perforin and granzyme B in CIK cells by synergizing cytokines in decreasing dose of IL-2 and anti-CD3 by 75 and 50%. This influences on protein and mRNA expression of perforin and granzyme B in CIK cells were similar to that of cytokines in higher doses alone. The expression of perforin and granzyme B correlated well with cytotoxicity of CIK cells. The results obtained from the present study suggested that the mechanisms of *Gl*-PS promoting CIK cells proliferation and cytotoxicity were relevant to enhancing IL-2, TNF production, protein and mRNA expression of granzyme B and perforin in CIK cells, and maybe were irrelevant to NO.

Our results confirmed that *Gl*-PS was a promising biological response modifier (BRM) and immune potentiator, and suggested the usefulness of combining with other immunotherapy.

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