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Protective effect of polysaccharides on simulated microgravity-induced functional inhibition of human NK cells

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ABSTRACT

Polysaccharides are believed to be strong immunostimulants that can promote the proliferation and activity of T cells, B cells, macrophages and natural killer (NK) cells. This study aimed to investigate the effects of five polysaccharides (*Grifola frondosa* polysaccharide (GFP), lentinan (LNT), *G. lucidum* polysaccharide (GLP), *Lycium barbarum* polysaccharide (LBP) and yeast glucan (YG)) on primary human NK cells under normal or simulated microgravity (SMG) conditions. Our results demonstrated that polysaccharides markedly promoted the cytotoxicity of NK cells by enhancing IFN-γ and perforin secretion and increasing the expression of the activating receptor NKp30 under normal conditions. Meanwhile polysaccharides can enhance NK cell function under SMG conditions by restoring the expression of the activating receptor NKG2D and reducing the early apoptosis and late apoptosis/necrosis. Moreover, the antibody neutralization test showed that CR3 may be the critical receptor involved in polysaccharides induced NK cells activation. These findings indicated that polysaccharides may be used as immune regulators to promote the health of the public and astronauts during space missions.

1. Introduction

Many polysaccharides extracted from plants or fungi have a variety of beneficial biological and physical properties (Borchers, Keen, & Gershwin, 2004; Borchers, Stern, Hackman, Keen, & Gershwin, 1999). Some of them can modulate the proliferation and activation of immunocompetent cells (Kim, Oh, & Park, 2003; Leung, Liu, Koon, & Fung, 2006) by enhancing the expression of cytokines (Hauer & Anderer, 1993) and surface receptors (Mueller, Hamprecht, & Anderer, 1989). Therefore, they are ideal candidates for immunostimulatory (Tzianabos, 2000) and anti-tumor (Wasser, 2002) without displaying clinical side effects.

During long space missions, astronauts and cosmonauts showed an overall decrease in immune function and experience an increased risk of infection (Sonnenfeld & Shearer, 2002). A number of studies have indicated that microgravity (MG) is the major factor causing immune dysfunction during space flight (Cogoli et al., 1993; Wang, An, Jiang, & Hang, 2011). MG can both decrease the number and suppress the activity of T and B lymphocytes, natural killer (NK) cells, dendritic cells, granulocytes, macrophages and hematopoietic stem cells (Crucian, Cubbage, & Sams, 2000; Maier, 2006; Meehan et al., 1992; Stowe et al., 1999). Studies on ground-simulated microgravity (SMG) also showed similar results (Martinelli et al., 2009). In recent years, the identification of bioactive compounds that can combat the adverse effects of microgravity on the immune system has received increased attention.

NK cells are an important link between innate and adaptive immunity and play a pivotal role in anti-inflammatory responses, tumor surveillance and autoimmune disorder regulation (Vivier, Nunes, & Vely, 2004). In our previous work, we found that the functions of primary human NK cells were significantly inhibited by SMG (Li et al., 2013). The identification of bioactive compounds that could restore the lost NK cell function under SMG conditions might provide a strategy for solving this problem.

NK cells express complement receptor type 3 (CR3) and Toll-like receptor 4 (TLR-4), which are well-known target receptors recognized by polysaccharides. Therefore NK cells are believed to be an important target of polysaccharide-mediated immunomodulation (Mian, Lauzon, Andrews, Lichty, & Ashkar, 2010; Ross & Vetvicka,

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1993). Initial studies have shown that polysaccharides (*e.g.*, *Ganoderma lucidum* (Chien et al., 2004), lentinan (Nanba & Kuroda, 1987) and MD-fraction, a polysaccharide derived from *Grifola frondosa* (Kodama, Asakawa, Inui, Masuda, & Nanba, 2005)) are able to modulate NK cells (human and mouse) activity by enhancing their cytotoxicity and cytokine expression level. Nevertheless, further studies have been rarely performed investigating the biological effects of these polysaccharides on human NK cells.

Five polysaccharides derived from plants or fungi, *G. frondosa* polysaccharide (GFP), lentinan (LNT), *G. lucidum* polysaccharide (GLP), *Lycium barbarum* polysaccharide (LBP) and yeast glucan (YG), are recognized as important immunostimulants and widely used as nutritional supplements and medicinal substances for health (Jin, Huang, Zhao, & Shang, 2013; Kodama, Komuta, Sakai, & Nanba, 2002; Matsumoto, Yamada, & Amagai, 1991). Therefore, this study systematically investigated the effects of the above polysaccharides on primary human NK cells under normal and SMG conditions. These polysaccharides were prepared at different concentration gradients, and their effect on NK cells proliferation was assessed *in vitro*. Then, CCK-8 method, real-time quantitative PCR, flow cytometry and ELISA were employed here to evaluate the cytotoxicity, receptor expression and cytokine/cytolytic protein secretion level of NK cell after polysaccharide administration. Furthermore, the TLR-4 and CR3 antibody neutralization assay was performed to explore the preliminary mechanism in polysaccharides induced NK cells activation.

2. Materials and methods

2.1. Preparation of primary human NK cells and cell lines

Human myelogenous leukemia cells (K562, CCTCC Number: GDC037) were obtained from the China Center for Type Culture Collection (Wuhan, China). K562 cells and stimulating cells (irradiated, genetically modified K562 cells (Imai, Iwamoto, & Campana, 2005)) were maintained in RPMI-1640 media (Gibco, Rockville, MD, USA) supplemented with 10% fetal calf serum (FBS) (Sijiqing Hangzhou, China) and 100 μg/mL of penicillin and streptomycin (Genview, Carlsbad, CA, USA). All cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Primary human NK cells were obtained from peripheral blood mononuclear cells (PBMCs) using an *ex vivo* expansion method. Briefly, peripheral venous blood (~10 mL) was collected in heparinized tubes from healthy donors ($n = 8$) according to the informed consent guidelines of the ethics committee at the Northwestern Polytechnical University Hospital. Blood samples were diluted twofold using sterile phosphate-buffered saline (PBS) and overlaid onto lymphocyte separation medium (Haoyang, Tianjin, China). The samples were centrifuged at 400 × *g* for 30 min at room temperature. PBMCs were collected and washed twice with sterile PBS followed by re-suspension in complete RPMI-1640 media (Gibco, Rockville, MD, USA) supplemented with 10% FBS (Sijiqing, Hangzhou, China), 100 U/mL IL-2 (Peprotech, Rocky Hill, NJ, USA) and 100 μg/mL of penicillin and streptomycin (Genview, Carlsbad, CA, USA). The PBMCs were then counted and seeded onto six-well plates (Costar, Cambridge, MA, USA) with equal numbers of stimulating cells (irradiated genetically modified K562 cells, prepared as described by Imai et al., 2005). The PBMCs and stimulating cells were co-cultured at 37 °C in a 5% CO_2 atmosphere. Half of the culture medium was changed every two days after the second week. After a 21-day culture, 1×10^5 *ex vivo*-expanded NK cells were collected and stained with CD3/CD16$^+$CD56 (LeuTM-4/11c + 19, contains FITC-labeled CD3 (Leu-4), PE-labeled CD16 and PE-labeled CD56) monoclonal antibodies (mAbs) along with isotype-matched controls (IgG1-FITC/IgG2-PE) (BD Biosciences, San Jose, CA, USA). The cells were then analyzed using flow cytometry (BD FACSCalibur, San Jose, CA, USA).

2.2. Polysaccharide treatment, NK cell proliferation and cytotoxicity assay

GFP, GLP, LBP and LNT were purchased from Tianjin Cinorch Pharmaceutical Co. Ltd. (China). YG was purchased from Zhuhai Tiantian Biotechnology & Engineering Co. Ltd. (China). The carbohydrate content of the five polysaccharides (GFP, GLP, LBP, LNT and YG) was approximately 86.32%, 88.48%, 92.72%, 89.19% and 90.32%, respectively, as determined using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

For the proliferation assay, NK cells were cultured in 24-well plates at a density of 10^6 cells/mL in the presence of GFP, GLP, LBP, LNT or YG at concentrations of 10, 50, 100, 500 and 1000 mg/L, respectively. Equal volumes of PBS were added to control cells. All cells were incubated at 37 °C in a 5% CO_2 incubator. After 48 h, the NK cells were centrifuged at 1000 × *g* for 5 min, washed three times with sterile PBS, and re-suspended in 600 μL of RPMI-1640 media (IL-2 free). A 200-μL cell suspension was mixed with 20 μL of CCK-8 (Cell Counting Kit-8, Dojindo, Japan) per well in a 96-well plate in triplicate for each group. The cells were then incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. The optical density (OD) values were recorded at 450 nm.

For the cytotoxicity assay, K562 cells were used as the target cells for the cytotoxicity assay. NK cells (2×10^6) were removed from polysaccharide treatment (the polysaccharide concentration was 100 mg/mL) and washed three times with sterile PBS. The cells were then re-suspended in 400 μL of RPMI-1640 (IL-2 free). Using an effector:target ratio of 5:2, the NK cells (2×10^5) were plated into each well of a 96-well plate and mixed with K562 cells (8×10^4). The experiment was repeated in triplicate. The NK control well (effector cell control) contained 2×10^5 NK cells, and the K562 control well (target cell control) contained 4×10^4 K562 cells. The plate was incubated at 37 °C in a 5% CO_2 atmosphere. After incubation for 4 h, 20 μL of CCK-8 was added to each well, and the plate was incubated for an additional 2 h. The OD values were recorded at 450 nm, and the killing rate of the NK cells against the K562 cells was calculated according to the following formula as previously described:

$$\text{Killing rate}\,(\%) = \left[1 - \frac{\text{OD}_{e+t} - \text{OD}_e}{\text{OD}_t}\right] \times 100\%$$

where OD_e represents the average OD450 of triplicate wells for the NK cell control; OD_t represents the average OD450 of triplicate wells for the K562 cell control; and OD_{e+t} represents the average OD450 of triplicate wells for NK cells plus K562 cells.

2.3. TLR4 and CR3 neutralization

Anti-TLR4/MD2 complex antibody and anti-CR3 monoclonal antibodies (mAbs) were purchased from Abcam (Abcam Inc., Cambridge, MA, USA) and BD Biocience (BD Biocience Clontech, Franklin Lake, NJ, USA). Primary human NK cells were pre-incubated with the antibodies for 1 h before treated with polysaccharides. Cytotoxicity assay was performed using the methods described above.

2.4. Real-time quantitative PCR analysis

For real-time quantitative PCR (RT q-PCR) analysis, NK cells were treated with 100 mg/L GLP, LBP or LNT for 48 h, while equal volumes of phosphate buffered saline (PBS) for control. RT q-PCR and SYBR green random mixing methods (Ponchel et al., 2003) were used to estimate the mRNA levels of IFN-γ, perforin and granzyme-B, and four NK cell membrane receptors, NKG2A, NKG2D, NKp30,

Table 1
Primers used in real-time PCR analyses.

Primer	Sequence (5′→3′)	Amplicon size (bp)
IFNγ	F: TGC-AGG-TCA-TTC-AGA-TGT-AGC R: GGA-CAT-TCA-AGT-CAG-TTA-CCG	248
Perforin	F: GGG-ACA-ATA-ACA-ACC-CCA-TCT R: GGA-ATT-TTA-GGT-GGC-CAT-GAT	174
Granzyme-B	F: AGA-TCG-AAA-GTG-CGA-ATC-TGA R: TTC-GTC-CAT-AGG-AGA-CAA-TGC	138
NKG2A	F: TCA-TTG-TGG-CCA-TTG-TCC-TGA-GG R: AGC-ACT-GCA-CAG-TTA-AGT-TCA-GC	291
NKG2D	F: ATC-GCT-GTA-GCC-ATG-GGA-ATC-CG R: AGA-CAT-ACA-AGA-GAC-CTG-GCT-CTC	213
NKp30	F: TGA-GAT-TCG-TAC-CCT-GGA-AGG R: CAC-TCT-GCA-CAC-GTA-GAT-GCT	235
NKp44	F: TCC-AAG-GCT-CAG-GTA-CTT-CAA R: GAT-TGT-GAA-TCG-AGA-GGT-CCA	163
β-Actin	F: CAG-ATC-ATG-TTT-GAG-ACC-TTC R: TGT-GGT-GGT-GAA-GCT-GTA-GCC	250

and NKp44. β-actin was used as the reference gene (Table 1). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative changes in gene expression (Livak & Schmittgen, 2001).

All of the primers had a similar efficacy in the PCR amplification as β-actin primers (data not shown). Total RNA was extracted from 1×10^7 NK cells in each group using TRIzol (TRIzol reagent, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA concentrations were determined by spectrophotometry, and RNA quality was evaluated through electrophoresis on RNA-denatured gels. cDNA was obtained using the TransScriptTM One-Step gDNA Removal and cDNA Synthesis SuperMix kits (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. RT q-PCR was performed using the TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The reaction mixtures were incubated for 30 min at 48 °C, followed by 30 s at 94 °C and 40 amplification cycles of 5 s at 94 °C, 15 s at 55 °C, and 10 s at 72 °C (fluorescence measurement step). At the end of 40 cycles, a melting curve analysis was performed to confirm the presence of only a single amplified product of the expected size.

2.5. Quantification of NK cell cytokine secretion

For cytokine quantification, NK cells (8×10^5) from each polysaccharide-treated group were centrifuged and washed three times with sterile PBS and re-suspended in 400 μL of RPMI-1640 (IL-2 free). K562 cells (1.6×10^5) were added to stimulate the NK cells (Denman et al., 2012). The co-culture cells were incubated for 4 h at 37 °C in a 5% CO_2 atmosphere. The supernatants were collected, and IFN-γ, perforin and granzyme-B were quantitated using ELISA kits (BD Bioscience, CA, USA). The ELISA assay was performed twice for each sample. OD values were recorded at 450 nm. A standard curve was generated according to the OD values of standards using Curve Expert 13.0 software. The concentration of cytokines in the supernatants was calculated from these standard curves.

2.6. Determination of NK receptor expression using flow cytometry

NK cells (5×10^5) from each polysaccharide-treated group and control group were centrifuged, washed 3 times with PBS, divided into 4 groups (1×10^5 cells each) and immunostained with PE-conjugated mouse anti-human NKG2A (R&D Systems, MN, USA), NKG2D, NKp30 and NKp44 (BD Biosciences, CA, USA) mAbs following analyzed by flow cytometry.

2.7. NK cell exposure to SMG and polysaccharide treatment

NK cells were randomly divided into two groups: NK cells in the SMG group were rotated around a horizontal axis in a 2D-RWV (a time-averaged gravity vector of 10^{-2} g with a revolution of 30 r/min, developed by the China Astronaut Research and Training Center), and NK cells in the 1GC (1 g control) group were cultured under normal gravity (gravity level = 1 g) conditions. The two groups of NK cells were cultured in IL-2-free RPMI-1640 media (supplemented with 10% FBS and 100 μg/mL of penicillin and streptomycin). The 2D-RWV cultures were maintained at 37 °C in a 5% CO_2 atmosphere for 48 h.

The NK cells were prepared as described above, and polysaccharides were added to the SMG culture system. The groups were divided as follows: SMG + GLP group, SMG + LBP group, SMG group and 1G control group. The cytotoxicity and NK receptor expression assays were performed according to previous methods. For the apoptosis assay, NK cells (1×10^5) of each group were centrifuged, washed 3 times with PBS, and labeled with Annexin V-FITC and PI (Annexin V-FITC Apoptosis Detection kit, Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's instructions to detect apoptotic cells.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software (IBM, New York, USA). Data were presented as the mean ± SD. The results were analyzed using an analysis of variance (ANOVA). Multiple comparisons used the LSD and SNK tests to evaluate the significance of differences between groups. Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Expanded primary human NK cells and proliferation assay of NK cells after polysaccharide treatment

After a 21-day *ex vivo* expansion, human NK cells were harvested and stained with CD56 + 16-PE and CD3-FITC mAbs. The percentage of NK cells (CD56 + 16^+ $CD3^-$) was determined using flow cytometric analysis (Fig. 1A and B). The mean percentage of NK cells in the post-expansion PBMCs was 92.37% ± 1.22% ($n = 4$).

The result of NK cells proliferation assay showed that when the concentration of the polysaccharides was less than 50 mg/L, the OD_{450} value of each polysaccharide treatment group was not markedly different from that of the control (Fig. 1C). However, at the concentration of 100 mg/L, the positive effect of the polysaccharides on NK cells proliferation was apparent. Subsequently, when the polysaccharides concentration was further increased, the toxic effects of them were observed. The OD_{450} of each group was significantly decreased compared with the control at the 1000 mg/L polysaccharides treatment.

3.2. Polysaccharides promoted the activities of NK cells under normal conditions

The effect of polysaccharides administration on NK cells cytotoxicity was tested. Three polysaccharides, GLP, LBP and LNT, had positive effect on the cytotoxicity of NK cells after 48 h of treatment. The NK cell cytotoxicities of the GLP, LBP and LNT groups were 96.43 ± 2.14%, 97.0 ± 2.38% and 96.20 ± 2.05% respectively, and were significantly increased from those of the control (87.46 ± 2.62%, $p < 0.05$) (Fig. 2A). These results indicated that GLP, LBP and LNT had a better effect on NK cells cytotoxicity compared with GFP and YG. Thus, GLP, LBP and LNT were appropriate candidates for use in subsequent experiments.

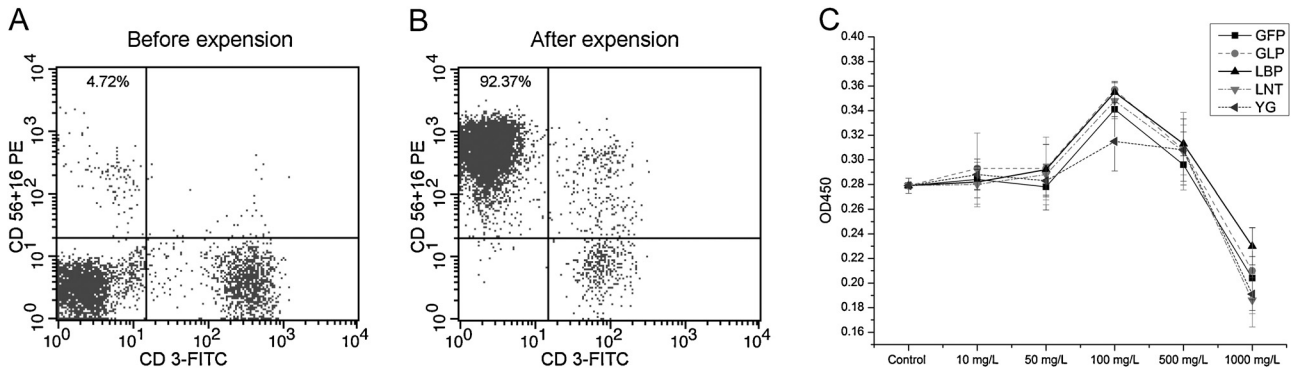

Fig. 1. Pre- and post-expanded PBMCs and NK cells proliferation after polysaccharides treatment. (A) Pre-expansion PBMCs were analyzed by flow cytometry. The percentage of NK cells (CD56+16+ CD3−) in the PBMCs was 4.72%. (B) Post-expansion PBMCs were analyzed by flow cytometry. The percentage of NK cells in the PBMCs was 92.37%. (C) OD450 value variations of NK cells after 48 h of polysaccharides treatment.

IFN-γ was significantly increased in the GLP and LBP groups ($p < 0.05$) on the mRNA level (Table 2), which were up-regulated 3.4-fold and 2.5-fold, respectively. Similarly, the mRNA levels of perforin and granzyme-B were also up-regulated by GLP administration; they were 2.1-fold and 1.8-fold enhanced, respectively ($p < 0.05$). On the protein level, IFN-γ was 586.33 ± 37.65 pg/mL in the supernatant of the GLP group and 646 ± 84.26 pg/mL in the LBP group compared with 400.33 ± 28.57 pg/mL in the control group ($p < 0.05$). Higher levels of perforin were detected in the supernatants of each group. The perforin level was significantly enhanced only in the GLP-treated group (797.67 ± 142.78 ng/mL) compared with the control group (466.33 ± 90.07 ng/mL) ($p < 0.05$) (Fig. 2B). The results showed that IFN-γ and perforin were markedly enhanced at both the protein and mRNA level which indicated they were key molecules modulated by polysaccharides in the process of NK cell activation. The expression of granzyme-B was also slightly enhanced by polysaccharide administration at the mRNA level, but the protein was undetectable in the supernatant upon ELISA analysis. This finding may be explained by the fact that NK cells lysis of target cells through direct granzyme-B releasing into the target cells rather than releasing it into the culture supernatant.

The expression of the surface activating receptor NKp30 was significantly up-regulated 2.6-fold, 3.5-fold and 2.5-fold after GLP, LBP and LNT treatment, respectively ($p < 0.05$) (Table 2). Moreover cytofluorimetric analysis revealed that only the NKp30 activating receptor showed an obvious change in expression after polysaccharides administration (Fig. 2C and D). The expression rate of NKp30 in each polysaccharide-treated group was 93.22 ± 3.98% (GLP), 94.02 ± 4.47% (LBP) and 91.10 ± 4.78% (LNT) compared with 81.18 ± 3.85% in the control group. The expression of the other receptors NKG2A, NKG2D and NKp44 were not significantly changed after polysaccharides administration. NKp30 is an important activating receptor expressed on the surface of NK cells and a member of the natural cytotoxicity receptor (NCR) family (Ferlazzo, Tsang, Moretta, Melioli, Steinman, & Munz, 2002). When stimulated by its ligand, NKp30L, NKp30 can promote NK cell secretion of TNF-α and IFN-γ (Vitale et al., 2005). The present results showed that polysaccharide administration could enhance NKp30 expression, and this may partly explain the increased cytotoxicity and IFN-γ expression observed in NK cells.

3.3. Polysaccharides can restore the function of NK cells under SMG conditions

Rotating wall vessel (RWV) bioreactors can suspend cell cultures by rotating them around a horizontal axis at a constant velocity to achieve a time-averaged gravity vector of 10^{-2} g. The present results showed that once NK cells were cultured under SMG conditions, the cytotoxicity of NK cells was significantly decreased compared with the 1G control group. Meanwhile polysaccharides were added to the NK cells cultures, GLP and LBP restored the cytotoxicity of the NK cells under SMG conditions, (Fig. 3A). The cytotoxicity of the NK cells was 74.79 ± 2.53% in the SMG + GLP group and 78.51 ± 3.17% in the SMG + LBP group compared with 65.54 ± 5.21% in the SMG group (without any polysaccharides treatment) and 89.5 ± 3.5% in the 1G control group. Generally it is suggested that the polysaccharides have the positive effects on the cytotoxicity of NK cells under SMG conditions.

To determine how the polysaccharides restored the cytotoxicity of NK cells under SMG conditions, the four surface receptors were

Table 2
Gene expression profiles of IFN-γ, perforin, granzyme-B, NKG2D, NKG2A, NKp30 and NKp44 in NK cells after 48 h of polysaccharide treatment.

Gene	GLP group ΔΔCt	LBP group ΔΔCt	LNT group ΔΔCt
IFN-γ	−1.77 ± 0.23 $2^{-\Delta\Delta Ct} = 2^{2.02} = 3.4$ (fold)*	−1.31 ± 0.32 $2^{-\Delta\Delta Ct} = 2^{2.31} = 2.5$ (fold)*	−0.22 ± 0.16 $2^{-\Delta\Delta Ct} = 2^{0.22} = 1.16$ (fold)
Perforin	−1.07 ± 0.41 $2^{-\Delta\Delta Ct} = 2^{1.07} = 2.1$ (fold)*	−0.28 ± 0.43 $2^{-\Delta\Delta Ct} = 2^{0.28} = 1.21$ (fold)	−0.46 ± 0.37 $2^{-\Delta\Delta Ct} = 2^{0.46} = 1.38$ (fold)
Granzyme-B	−0.88 ± 0.22 $2^{-\Delta\Delta Ct} = 2^{0.88} = 1.8$ (fold)*	−0.13 ± 0.31 $2^{-\Delta\Delta Ct} = 2^{0.13} = 1.09$ (fold)	−0.46 ± 0.29 $2^{-\Delta\Delta Ct} = 2^{-1.96} = 1.38$ (fold)
NKG2A	−0.32 ± 0.31 $2^{-\Delta\Delta Ct} = 2^{0.32} = 1.3$ (fold)	−0.05 ± 0.16 $2^{-\Delta\Delta Ct} = 2^{0.\circ 5} = 1.0$ (fold)	−0.17 ± 0.21 $2^{-\Delta\Delta Ct} = 2^{0.17} = 1.13$ (fold)
NKG2D	−0.31 ± 0.33 $2^{-\Delta\Delta Ct} = 2^{0.31} = 1.2$ (fold)	−0.37 ± 0.24 $2^{-\Delta\Delta Ct} = 2^{0.37} = 1.3$ (fold)	−0.44 ± 0.32 $2^{-\Delta\Delta Ct} = 2^{0.44} = 1.36$ (fold)
NKp30	−1.37 ± 0.37 $2^{-\Delta\Delta Ct} = 2^{1.37} = 2.6$ (fold)*	−1.81 ± 0.24 $2^{-\Delta\Delta Ct} = 2^{1.81} = 3.5$ (fold)*	−1.33 ± 0.21 $2^{-\Delta\Delta Ct} = 2^{1.33} = 2.5$ (fold)*
NKp44	−0.41 ± 0.29 $2^{-\Delta\Delta Ct} = 2^{0.41} = 1.32$ (fold)	−0.72 ± 0.34 $2^{-\Delta\Delta Ct} = 2^{0.72} = 1.64$ (fold)	0.29 ± 0.27 $2^{-\Delta\Delta Ct} = 2^{-0.29} = 0.82$ (fold)

RT q-PCR using the SYBR green random mixing method was employed to test mRNA levels. β-Actin served as the internal standard control.
* $p < 0.05$ compared with control group. The data represent the mean ± SD of six independent experiments. One-Way ANOVA and LSD test.

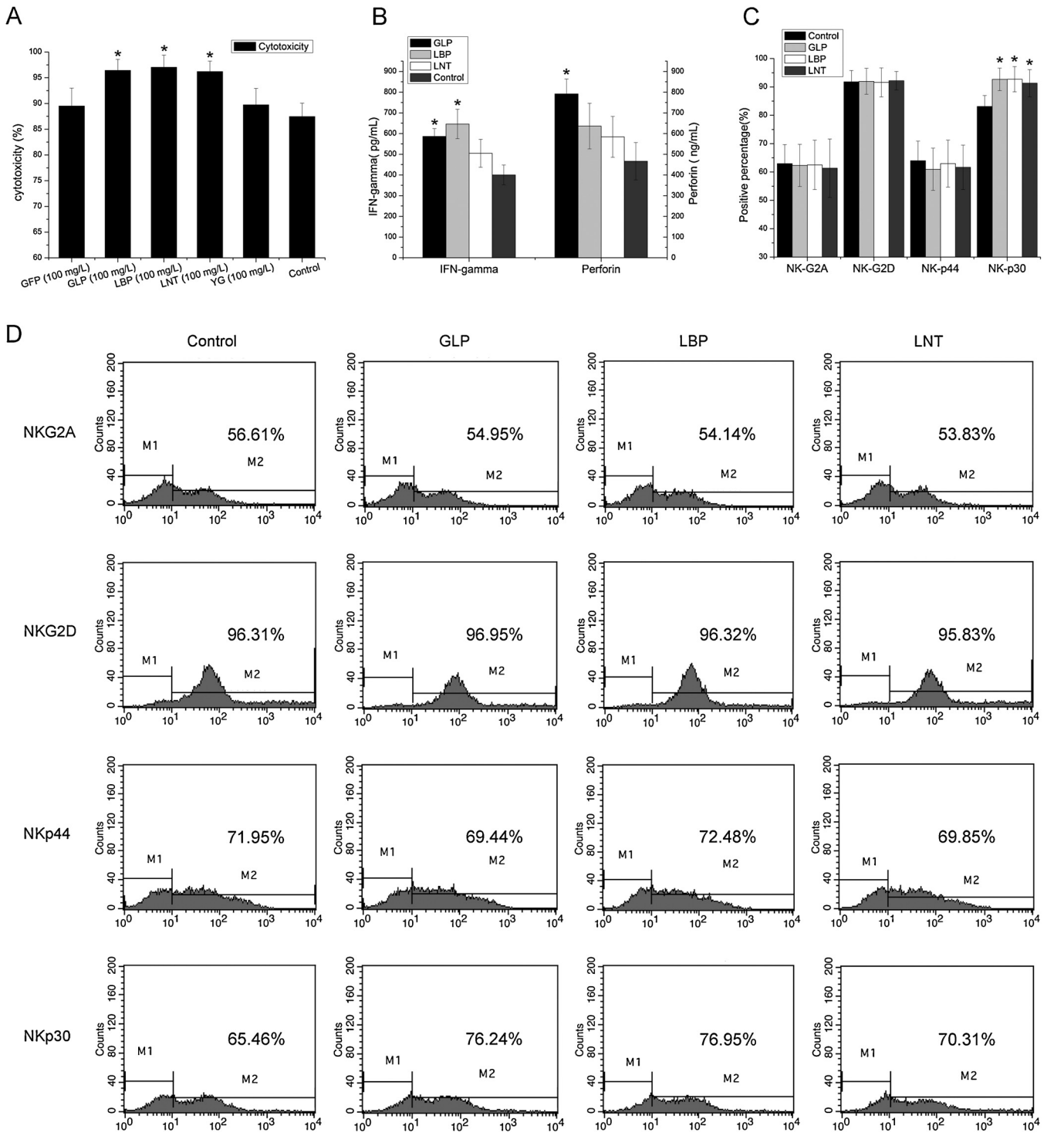

Fig. 2. Cytotoxicity, cytokine/cytolytic protein and receptors expression in NK cells after polysaccharides treatment under normal conditions. (A) NK cells cytotoxicity after polysaccharides treatment. (B) IFN-γ and perforin levels secreted by NK cells after polysaccharides treatment. (C) The receptor expression of NK cells after polysaccharides treatment. (D) Representative flow cytometry analysis of NK cells from one donor. Each column represents the mean ± SD from four independent experiments. One-way ANOVA and LSD tests were performed; $^{*}p < 0.05$ compared with the control.

re-tested. The results showed that the expression of NKG2A and NKG2D on NK cells was markedly decreased compared with the control group, and polysaccharides treatment restored the expression of NKG2D and up-regulated NKp30 (Fig. 3B and C). The expression rates of NKG2D in the SMG + GLP and SMG + LBP groups were 68.24 ± 2.64% and 71.86 ± 1.96%, respectively, in comparison with 61.31 ± 2.74% expression in the SMG group ($p < 0.05$). Polysaccharides treatment also increased NKp30 expression under SMG conditions. The NKp30 expression level in the two polysaccharide-treated groups were 93.35 ± 2.22% (SMG + GLP group) and 89.25 ± 4.31% (SMG + LBP group), which was a significant increase compared with 79.68 ± 3.20% expression in the SMG group. These results showed that the expression of the activating receptor NKp30 was up-regulated under SMG and normal conditions by polysaccharide administration. However, NKG2D was only up-regulated under the SMG condition, which

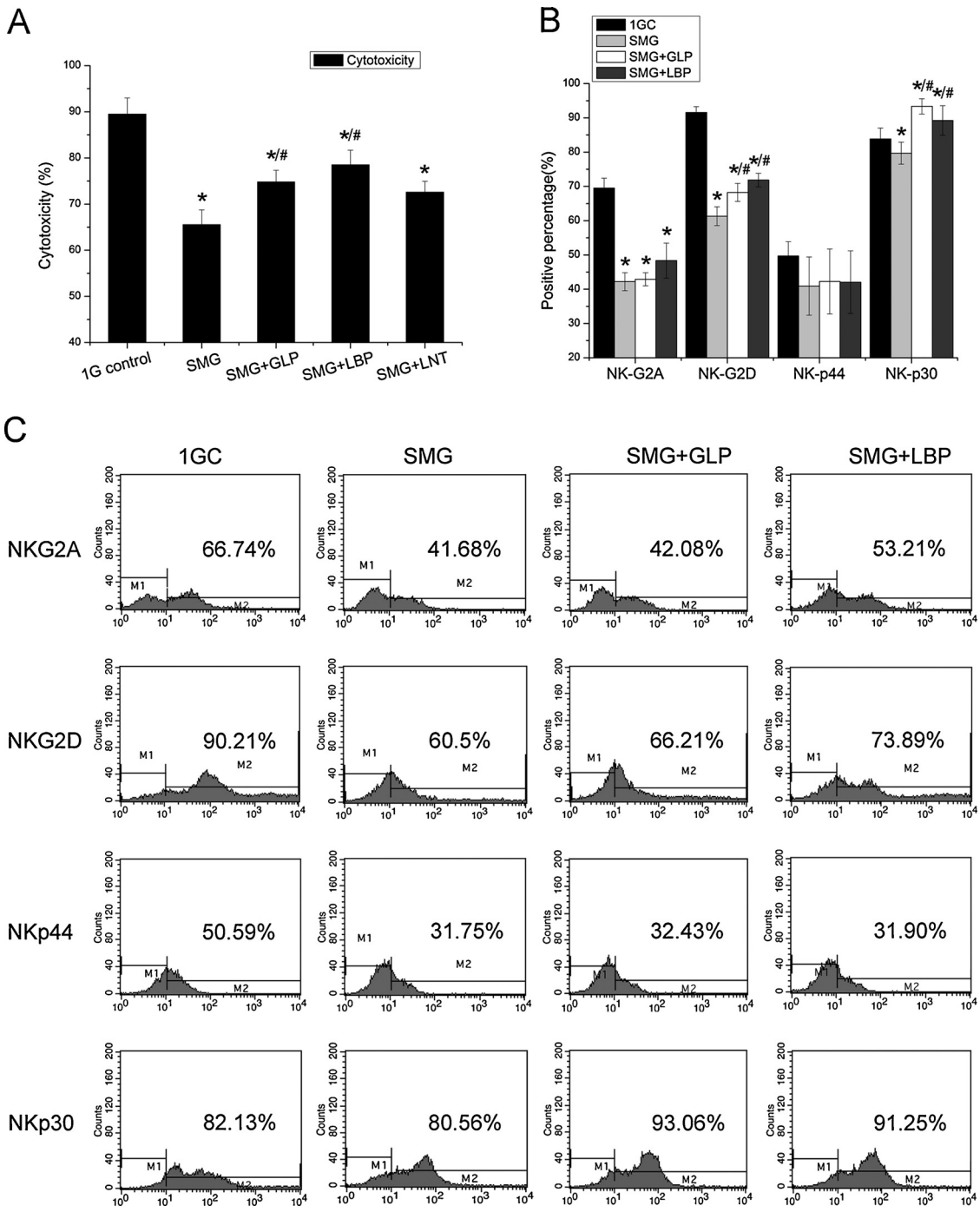

Fig. 3. Cytotoxicity and receptors expression in NK cells after polysaccharides treatment under SMG conditions. (A) The effect of polysaccharides on NK cell cytotoxicity under SMG conditions. (B) The effect of polysaccharides on NK cell receptor expression under SMG conditions. (C) Representative flow cytometry analysis of NK cells from one donor. Each column represents the mean ± SD from four independent experiments. One-way ANOVA and LSD tests were performed; $^{*}p < 0.05$ compared with the 1G control. $^{\#}p < 0.05$ compared with the SMG group.

may be because under normal conditions, NKG2D expression was too high to show a difference between groups. Meanwhile, under SMG conditions, NK cells NKG2D expression was significantly down-regulated, and polysaccharide treatment restored its expression to some extent.

Furthermore, it is noteworthy that the polysaccharides prevented the SMG-induced apoptosis and necrosis of NK cells. Fig. 4 depicts an Annexin V/PI double staining analysis of NK cells after 48 h of polysaccharides administration under SMG conditions. Obvious NK cell early apoptosis (Annexin V^+/PI^-) and late apoptosis/necrosis (Annexin V^+/PI^+) were observed when the cells were cultured under SMG conditions. Treatment with the two polysaccharides decreased the SMG-induced apoptosis and necrosis of NK cells and increased the percentage of viable cells. The early apoptosis rates were 15.84 ± 3.14% (SMG group), 12.7 ± 1.28% (SMG + GLP group), 10.54 ± 1.96% (SMG + LBP group) and 7.61 ± 1.21% (1G control group). Thereinto, the early apoptosis rates in the LBP-treated group decreased significantly compared with the SMG group ($p < 0.05$). The late apoptosis/necrosis rates in the GLP and LBP groups decreased to 8.26 ± 2.47% and 8.89 ± 2.26%, respectively, compared with 20.78 ± 2.31% in the SMG group ($p < 0.05$). Correspondingly, the viability rates of NK cells

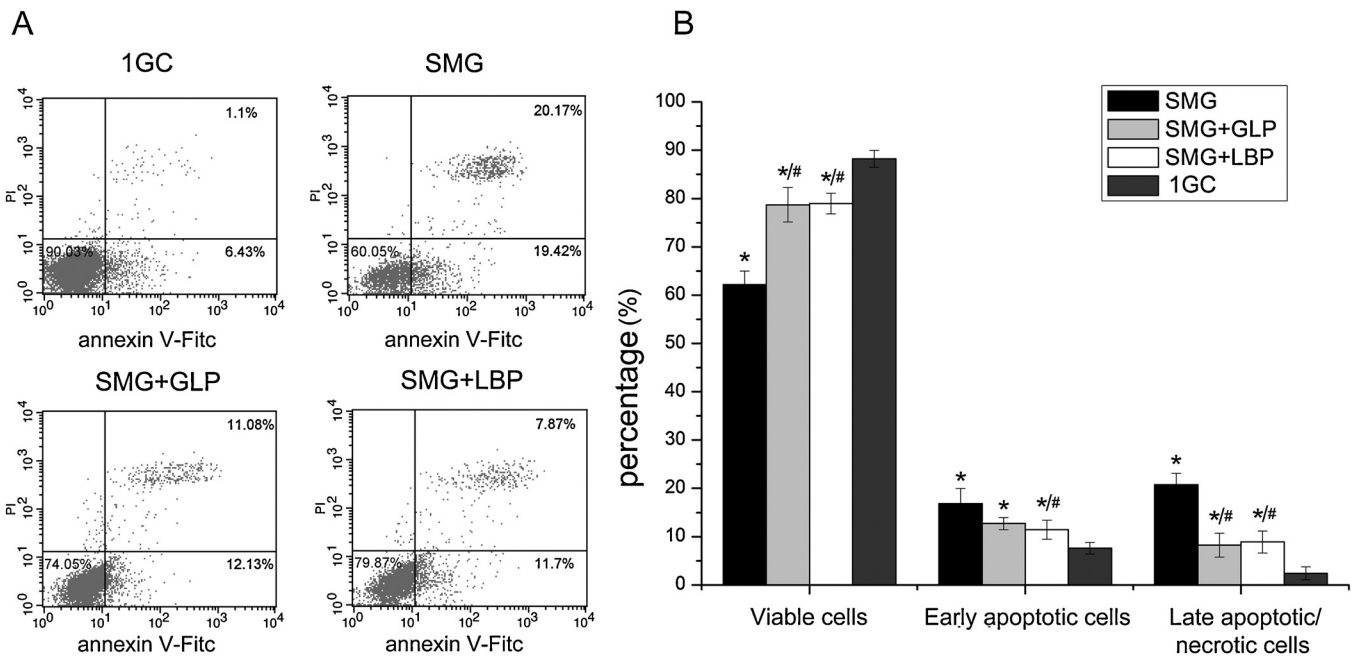

Fig. 4. Apoptosis and necrosis of NK cells after polysaccharides treatment under SMG conditions. (A) A representative flow cytometry plot of NK cells from one donor. The percentages of viable (Annexin V−/PI−), early apoptotic (Annexin V+/PI−), and late apoptotic/necrotic (Annexin V+/PI+) NK cells are shown. (B) The percentage of apoptosis in these cells is summarized in the bar graph; each column represents the mean ± SD from four independent experiments. Multivariate ANOVA and LSD tests were performed; $^{*}p < 0.05$ compared with the 1G control. $\#p < 0.05$ compared with the SMG group.

in the polysaccharide-treated groups were markedly increased (78.69 ± 3.57%, SMG + GLP group; 78.96 ± 5.14%, SMG + LBP group) compared with the SMG group (62.17 ± 2.79%).

3.4. *Polysaccharides may promote the activity of NK cells via CR3-mediated signaling pathways*

The basic mechanism underlying polysaccharide-mediated immunostimulation is related to macrophage stimulation and modulation of the complement system (Schepetkin & Quinn, 2006). Specific receptors on cells are pattern recognition molecules, which can recognize polysaccharide polymers. These pattern recognition molecules include Toll-like receptors-4 (TLR-4), CD14, Complement receptor 3 (CR3, also known as CD11b/CD18, or Mac-1), scavenger receptor, dectin-1 and mannose receptor, they can activate macrophages by binding to polysaccharides (Rice et al., 2002; Taylor et al., 2002).

It has been proved TLR-4 and CR3 are also expressed on human NK cells (Mian, Lauzon, Andrews, Lichty, & Ashkar, 2010; Ross & Vetvicka, 1993). TLR4, one of the identified TLRs, has been reported to act as a receptor for lipopolysaccharide (LPS), component of the outer membrane of Gram-negative bacteria (Hoshino et al., 1999; Poltorak et al., 1998; Poltorak, Ricciardi-Castagnoli, Citterio, & Beutler, 2000; Takeuchi et al., 1999) and many of polysaccharides isolated from plants or fungi (Ando et al., 2002; Han et al., 2003; Rock, Hardiman, Timans, Kastelein, & Bazan, 1998). CR3 is considered as an iC3b receptor and adhesion molecule (Hogg & Berlin, 1995), and it is one of the most important phagocytic receptors for recognizing microbial pathogens (Springer, 1994). In the present study, the anti TLR-4 and anti CR3 antibodies (Abs) were used here to explore the possible way polysaccharide activate NK cells. NK cells were pre-incubation by anti TLR-4 and anti CR3 Abs respectively following polysaccharides treatments. The results showed that after pre-incubation of anti CR3 antibody, the polysaccharides increased cytotoxicity of NK cells was no longer representing (Fig. 5). The cytotoxicities of NK cells in GLP, LBP and LNT groups were 86.52%, 85.92% and 86.73% respectively when compared with the 86.26% of control. However, the increased cytotoxicity of NK cells in each polysacchraides treated group with absence of anti CR3 antibody was observed, which were 95.24% (GLP group), 96.81% (LBP group) and 95.72% (LNT group). The anti TLR-4 antibody did not show the similar negative effect on polysaccharides increased cytotoxicity of NK cells. These results suggested that polysaccharides may promote the activity of NK cells *via* CR3-mediated signaling pathway. Further studies are needed to determine whether other receptors are involved in the polysaccharide-mediated enhancement of NK cell activity and whether a cooperative effect exists between the polysaccharides.

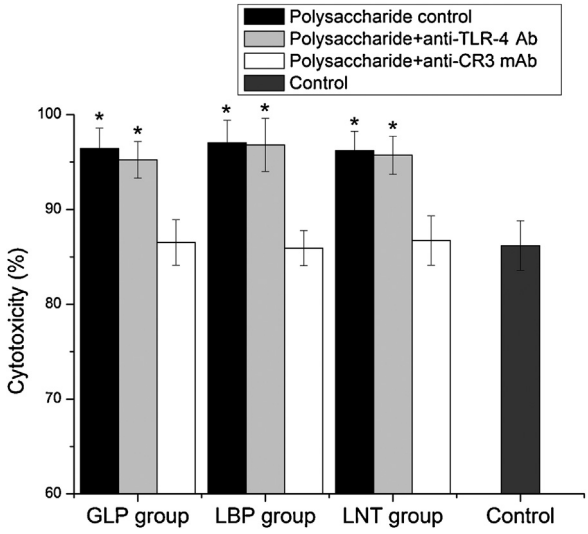

Fig. 5. Cytotoxicity of NK cells in antibodies neutralization assay. Each column represents the mean ± SD from four independent experiments. One-way ANOVA and LSD tests were performed; $^{*}p < 0.05$ compared with the control.

4. Conclusion

In conclusion, we investigated the effects of five polysaccharides derived from plants or fungi on primary human NK cells under normal or SMG conditions. It is suggested that polysaccharides can promote the cytotoxicity of NK cells by increasing NKp30 expression and the secretion of cytokine and cytolytic proteins. Among the five polysaccharides, GLP, LBP and LNT were more efficient immunostimulants at stimulating NK cell activities, and GLP was the best at promoting the secretion of cytokines. Furthermore, GLP and LBP were able to restore the function of NK cells under SMG conditions. These positive influences, including increasing the cytotoxicity of NK cells, prevented SMG-induced apoptosis and necrosis and up-regulated the expression of the activating receptors NKG2D and NKp30 in NK cells. The preliminary mechanism in polysaccharides promoting NK cells activation may be involved in the CR3 signaling pathway. The immunoregulatory effect of polysaccharides on NK cells may provide a strategy for overcoming the effects of microgravity on human NK cells during long space missions.

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