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Polysaccharides of *Ganoderma lucidum* alter cell immunophenotypic expression and enhance CD56$^+$ NK-cell cytotoxicity in cord blood

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Abstract—In our previous study, a fucose-containing glycoprotein fraction (F3), isolated from the water-soluble extracts of *Ganoderma lucidum*, was shown to stimulate mice spleen cell proliferation and cytokine expression. We now further investigate the effect of F3 on the immunophenotypic expression in mononuclear cells (MNCs). When human umbilical cord blood (hUCB) MNCs were treated with F3 (10–100 μg/mL) for 7 days, the population of CD14$^+$CD26$^+$ monocyte/macrophage, CD83$^+$CD1a$^+$ dendritic cells, and CD16$^+$CD56$^+$ NK-cells were 2.9, 2.3, and 1.5 times higher than those of the untreated controls ($p < 0.05$). B-cell population has no significant change. T cell growth was, however, slightly inhibited and CD3 marker expression decreased ~20% in the presence of higher concentrations of F3 (100 μg/mL). We also found that F3 is not harmful to human cells in vitro, and after F3 treatment, NK-cell-mediated cytotoxicity was significantly enhanced by 31.7% ($p < 0.01$) at effector/target cell ratio (*E*/*T*) 20:1, but was not altered at *E*/*T* 5:1.

1. Introduction

Ganoderma lucidum, an oriental medical fungus, has been widely used to promote health and longevity in China and other Asian countries.[1] The fruiting bodies and cultured mycelia of Reishi are reported to be effective in the treatment of many diseases, such as chronic hepatopathy, hypertension, hyperglycemia, and neoplasia.[2] This medical fungus has also attracted great attention due to the fact that its polysaccharide fractions have anti-tumor activity.[3,4]

It has been demonstrated that administration of the crude or partially purified polysaccharides of Reishi could significantly inhibit the growth of locally implanted sarcoma.[5] Although the tumor inhibition activity may be related to the activation of host immune responses, the mechanism of the anti-tumor effects of Reishi is so far uncertain. Further investigation is thus required to demonstrate its effect and to understand the mechanism at the molecular level. Our previous study showed that an active glycoprotein component, isolated from the water soluble Reishi extract, designated fraction 3 (F3) is significantly active in stimulating mice spleen cell proliferation and cytokine expression.[4] The immunophenotypic effect of F3 on human immune cells has not yet been well documented, however. This study is intended to investigate this issue using the mononuclear cells isolated from human umbilical cord blood (UCB), which has been utilized as a source of hematopoitic stem cells, and to date, more than 2000 UCB transplantations have been performed worldwide. The mononuclear cells collected from cord blood comprise immature lymphocytes, which can be induced into different subsets of immune cells, due to their progenitor

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properties. Specific cell type differentiation and maturation can be observed by using flow cytometry via specific fluorescent monoclonal antibody staining.

Specific antibodies, used against cell surface markers, were chosen for this study, and are introduced as follows: CD3/TCR (T Cell Receptor) is a marker found on mature T cells during thymopoiesis;[6] CD19, a B cell specific antigen, is a critical signal transduction molecule that regulates B lymphocyte development, activation, and differentiation;[7] CD14 is a monocyte/macrophage differentiation marker;[8] CD26 is a cell surface protease, expressed on many cells of the immune system including some $CD4^+$ T cells and macrophage;[9] CD45 can be found on all nucleated hematopoietic cells;[10] anti-human CD56 and CD16 antibodies are both used for identification of natural killer (NK) cells and their subpopulations, according to the different expression of the surface antigens CD16 and CD56;[11] CD83 and CD1a are expressed on dendritic cells while anti-CD83 and anti-CD1a antibodies can serve as useful markers for human dendritic cells phenotypic characterization.[12]

NK-cells in UCB have been studied and are known to play an important role in immune surveillance against cancer and in the development of blood borne metastasis and local recurrence.[13] The levels of natural cytotoxicity decrease in the peripheral blood of patients with various types of cancer, compared to health controls.[14] NK-cell mediated cytotoxicity is modulated by various cytokines, including IL-1, IL-2, IL-12, and interferons.[15] A previous study has discovered that the polysaccharides isolated from *G. lucidum* can enhance the cytotoxic activity of NK-cells and stimulate tumor necrosis factor α and interferon-γ release,[16–18] respectively. We also found in our previous study[4] that F3 can enhance host immune response by stimulating production of cytokines. In order to investigate F3's influence on NK-activity, an experiment was designed to evaluate whether F3 might interfere with NK-cell lytic function. $CD56^+$ NK-cells were purified using magnetic beads, conjugated with anti-$CD56^+$ monoclonal antibodies, and cultured target cells (K562 cells) at different effector/target cell ratios for cytolytic comparison. Thereafter, the percentage of lyses against target cells was detected by Alamar Blue assay.[19]

The cytolytic activity of purified CB NK-cells was reported to be similar[20] to that of purified adult PB NK-cells. Its known that mature NK-cells express CD56 alone or in combination with CD16. The majority of adult peripheral blood (PB) NK-cells is $CD56^+16^+$, with a minor population of $CD56^+16^-$ cells. In this study, we also tested the effects of F3 on NK-cell surface marker expression in MNCs, isolated from the UCB of six volunteers.

2. Materials and methods

2.1. Isolation of UCB mononuclear cells

Human UCB from six healthy volunteers was drawn into EDTA-coated tubes. The blood was collected right after the full-term baby was delivered and before the placenta separated from the uterus. Using aseptic procedures, an 18-gauge needle was inserted into the umbilical vein and umbilical cord blood drawn for tests. Samples were stored at room temperature and processed within 24 h after collection. The umbilical cord blood (50–100 mL) was processed using density gradient centrifugation with Ficoll-Paque (density 1.077; Pharmacia Biotech; Uppsala, Sweden).

The buffy coat interface was retrieved and washed with *Dulbecco*'s phosphate buffered saline ([PBS] pH 7.4) and EDTA (0.2 mM). It was re-suspended in a complete culture medium, consisting of RPMI-1640, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin (Gibco BRL), and was then supplemented with 20% fetal bovine serum (FBS). Mononuclear cells isolated through these procedures were prepared at a final concentration of 10^6 cells/mL.

2.2. Flow cytometric analysis of UCB phenotypic changes

The mononuclear cells isolated from the six umbilical cord blood specimens were placed in six T75 culture flasks at 5×10^5 cells/mL density in preparation for the F3 treatment. After seeding of cells, the flasks were maintained in a 37 °C to 5% CO_2 incubator for 1 h to equilibrate before 100 μg/mL of *G. lucidum* F3 extracts were added to each culture. The F3 fraction was dissolved in PBS for all experiments. Control cultures were added with an equal volume of PBS without F3, while positive controls were treated with 100 μg/mL LPS (Sigma) from a Gram-negative cell wall.

Cells were cultured for 7 days after treatment. To proceed for flow cytometry, cells ($1–2 \times 10^6$) were pelleted and re-suspended in 2 mL of staining buffer (0.2 mM EDTA, 2% FBS in phosphate buffered saline [PBS]). Staining buffer (100 μL) containing 10 μL of fluorescence-conjugated antibody was added to the cell suspension for labeling. After incubation at 4 °C for 40 min, all samples were then centrifuged at 1500 rpm for 5 min, followed by washing of the pellets twice with washing buffer (0.2 mM EDTA, 2% FBS in phosphate buffered saline [PBS]). All monoclonal antibodies to surface antigens, including CD45, CD3, CD16, CD19, CD56, CD83, and CD1a, were obtained from Coulter Immunotech, USA.

2.3. Cell count and determination of proliferation

Cell numbers were determined using light microscopy, based on the ability of living cells to exclude trypan blue. Cell proliferation was assessed by their reducing activity on sodium (2,3)-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, inner salt (XTT).[21,22] Briefly, 100 μL of 2×10^5 cells/mL were incubated with different concentrations of F3 for 48 h. An XTT solution containing phenazine methosulfate was added to a final concentration of 0.2 mg/mL and 25 mM, respectively, for 4 h. Absorbance was measured with a spectrophotometer, using test and reference wavelengths of 450 and 650 nm, respectively. Each experiment was per-

formed in triplicate and repeated at least three times. Results were expressed as the mean ± SEM.

2.4. Flow cytometric acquisition

Flow cytometry was performed with a FACSclibur cytometer (Becton Dickinson). The instrument was set for two-color analysis using FACScomp software and was calibrated using Calibrite beads (Beckton Dickinson) with a threshold of 200 on FSC to exclude debris. Data were collected in list mode and analyses were performed using CellQuest software version 3.1f (Becton Dickinson) and Win MIDI version 2.8 software. At least 10,000 target cells were collected and analyzed. All the samples were tested in duplicate and the results presented as mean values.

$CD56^+$ natural-killer cells (NK-cells) isolated from UCB mononuclear cells were enriched by a positive magnetic-bead cell separation method (MACS, Miltenyi Biotec). In brief, we isolated MNC from the buffy coat of human umbilical cord blood by using Ficoll-Paque® as mentioned above, and passed cells through 30 μm nylon mesh (Milipore) to remove clumps. (The filter should be rinsed before use.) Filtered cells were washed twice with buffer (PBS containing 0.1% sodium azide, 1% human serum albumin, and 0.15% sodium citrate). The cell pellets were suspended in 500 μL of this buffer and 200 μL of FcR Blocking Reagent (Miltenyi Biotec) and incubated for 15 min on ice to block FcR. Then, 200 μL of $CD56^+$ microbeads per 10^8 total cells were added, followed by incubation for an additional 30 min on ice, and then washed twice with the buffer. The cells were re-suspended in 1 mL of the buffer. The magnetically labeled cells in 1 mL of the buffer were applied to two MACSy RS1 separation columns (Miltenyi Biotec) that had been equilibrated with the buffer in the magnetic field of the Vario MACSy separator (Miltenyi Biotec).[23] The negative cells were washed off the column with 2 mL of the buffer. Retained cells were eluted from the column outside the magnetic field by pipetting 2 mL of the buffer onto the column and using the plunger supplied with the column.

Aliquots of the sorted cells were stained by PC5-labeled anti-$CD56^+$ monoclonal antibody (Coulter Immunotech) to analyze the purity of $CD56^+$ NK-cells. The purity of the isolated NK-cells was determined by flow cytometry analysis and reached up to 95%.

2.5. Activation of effector cells

Highly enriched $CD56^+$ NK-cell suspensions were cultured in a medium supplemented with RPMI-1640 for 24 h (37 °C, 10% CO_2). Six different concentrations of F3 or of LPS (Fig. 2), ranging from 100 to 3.125 μg/mL by serial dilution, were added into cell suspensions for pre-incubation treatment prior to the subsequent cytotoxicity test. The control group was treated with PBS.

After 7 days of incubation, the cultures were washed twice with phosphate buffered saline ([PBS] pH 7.4) and then re-suspended in medium containing 20% FBS, ready for the cytotoxicity assay.

The results of the effect of F3 on $CD56^+$ NK-cell mediated cytotoxicity are expressed as ratios of survival of K562 cells of F3 treated groups versus the controls.

2.6. Preparation of target cells

K562 (CCL-243, ATCC), a human erythroleukemia cell line, was used as an NK-sensitive target for the cytotoxicity assays.

Cells were cultured in RPMI-1640 medium (Gibco Laboratories) containing 10% fetal bovine serum (FBS, Gibco) and 1% antibiotics (P/S, penicillin 100 IU/mL and streptomycin 100 μg/mL, Gibco) in 75 cm^2 culture flasks (Falcon) to a concentration of 2×10^5 cells/mL. On the day of testing, cells were washed once with PBS and re-suspended in a complete medium at a concentration of 2×10^5/mL.

2.7. Cytotoxicity assay

Different concentrations of activated effector cells, that is, F3-treated $CD56^+$ NK-cells, and target cells, that is, K562, were co-cultured in six-well plates (Falcon) in triplicate. The effector to target cell (*E*:*T*) ratios were 5:1, 20:1, and 80:1, respectively. The cytotoxic activity of NK-cells was measured using Alamar Blue (Alamar BioSciences, Sacramento, CA), which is a colorimetric indicator that changes color upon reduction when a membrane potential across a cell occurs.[18] Twelve hours after the co-culture of effector cells and target cells, Alamar Blue indicator was added to the culture wells at a ratio of 200 μL indicator to 2 mL of medium. The plates were then further incubated for 4 h at 37 °C. Absorbance of color was measured on an ELISA reader at wavelengths of 570 and 595 nm. Controls containing only medium and Alamar Blue reagent that had also been incubated for 4 h were also measured at the same wavelengths. Calculation was performed following suggestions in the manufacturer's manual.

2.8. Statistical analysis

Data were analyzed using a paired or unpaired Student's *t*-test, with 95% confidence.

3. Results

3.1. Flow cytometric assay for UCB phenotypic changes

We evaluated the immuno-phenotypic changes of mononuclear cells treated with F3, and the results are shown in Figure 1. Our experiments also indicate that F3 is not harmful to human cells in vitro (Fig. 2). The concentration of F3 was selected based on our preliminary study. The $CD14^+CD26^+$ monocyte/macrophage expression was increased by a factor of 2.9-fold after F3 treatment, when compared to the controls (Fig. 1a). $CD83^+CD1a^+$

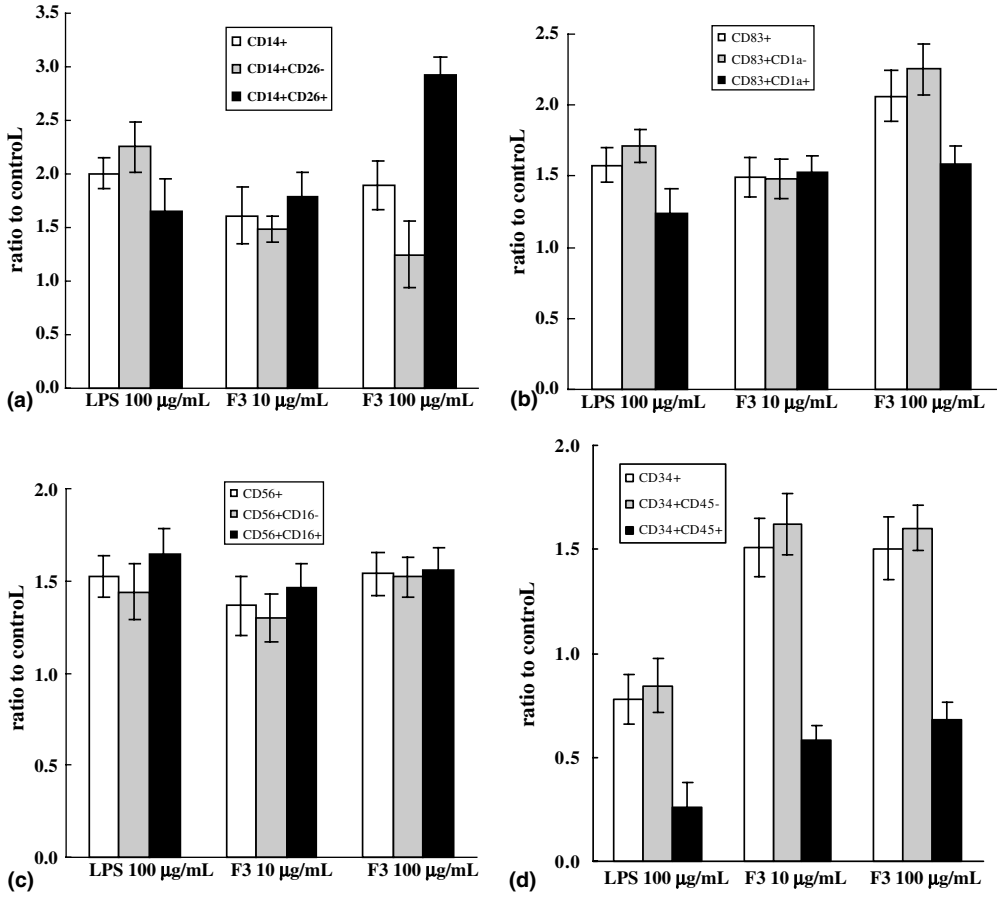

Figure 1. Flow cytometric analysis of three different lymphocytes cell subtype. Flow cytometric analysis of (a) $CD14^+$ monocyte/macrophage; (b) $CD83^+$ dendritic cells; (c) $CD56^+$ NK-cells; (d) $CD34^+$ hematopoietic stem cell.

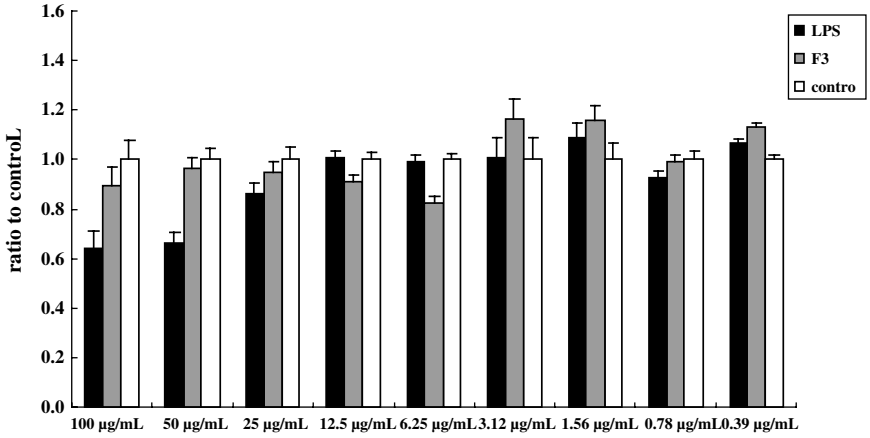

Figure 2. XTT assay of F3 on human umbilical cord blood MNC proliferation.

dendritic cells and $CD16^+CD56^+$ NK-cells treated with F3 (10–100 μg/mL) also reached 2.3 times and 1.5 times higher than those of the untreated controls ($p < 0.05$), respectively, as shown in Figure 1b and c. Interestingly, primary observation revealed that the expression of CD3 decreased by 3% and 20% in cultures with F3 concentration of 10 and 100 μg/mL, respectively. However, as this is not statistically significant, further tests with various concentrations of treatment will be performed. There were no significant changes in $CD19^+$ MNCs with the current F3 concentrations (Table 1).

3.2. Lysis of K562 cells after enrichment of $CD56^+$ NK-cells

After enrichment of $CD56^+$ NK-cells by magnetic separation, NK-cells with F3 at seven different concentrations ranging from 0 to 100 μg/mL were pre-incubated

Table 1. Summary of cell subtypic changes after treatments

		LPS 100 μg/mL	F3 10 μg/mL	F3 100 μg/mL
$CD56^+$ NK-cell		152.4 ± 11%	136.5 ± 16%	153.7 ± 12%
Subtype	$CD56^+CD16^-$	143.9 ± 15%	129.5 ± 13%	152.0 ± 11%
	$CD56^+CD16^+$	164.6 ± 14%	146.6 ± 13%	156.2 ± 12%
$CD14^+$ monocyte/macrophage		200.6 ± 31%	161.3 ± 22%	189.8 ± 17%
Subtype	$CD14^+CD26^-$	225.0 ± 23%	148.8 ± 12%	124.8 ± 31%
	$CD14^+CD26^+$	164.9 ± 15%	179.4 ± 26%	292.1 ± 23%
$CD83^+$ dendritic cells		157.7 ± 12%	149.4 ± 14%	206.4 ± 18%
Subtype	$CD83^+CD1a^-$	171.3 ± 17%	148.0 ± 11%	225.6 ± 12%
	$CD83^+CD1a^+$	124.3 ± 21%	153.0 ± 13%	159.1 ± 19%
$CD34^+$ hematopoietic stem cell		78.0 ± 12%	151.0 ± 14%	151.0 ± 15%
Subtype	$CD34^+CD45^-$	84.3 ± 13%	162.0 ± 15%	160.1 ± 11%
	$CD34^+CD45^+$	25.8 ± 12%	58.0 ± 7%	67.7 ± 9%
$CD3^+$ T cell		132.4 ± 19%	97.3 ± 11%	79.3 ± 10%
$CD19^+$ B cell		107.8 ± 21%	105.2 ± 27%	107.1 ± 14%

for 7 days. For the cytotoxicity analysis, the tests were done at the effector/target cells ratios of 5:1, 20:1, and 80:1, respectively.

Alamar Blue, a colorimetric indicator that changes from oxidized (nonfluorescent, blue) form to a reduced (fluorescent, red) form after uptake by living cells, is used for detecting target cell survival.

The highest level of cytotoxicity was noted at an *E*/*T* ratio of 20:1 when the effector cells were pre-incubated with 100 μg/mL F3 concentration. In the experiments done in triplicate, NK-cytotoxicity increased by 31.7% ($P < 0.01$) and 20.1% ($P < 0.05$) after pre-treatment with 100 and 50 μg/mL of F3, respectively, when compared to the untreated controls (Fig. 3a and c). The cytotoxicity at an *E*/*T* ratio of 5:1 was not significant, compared to the controls. On the other hand, when *E*/*T* ratios were as high as 80:1, no high cytotoxicity effect was observed, likely due to the over-saturation of cell numbers (data not shown). Effector cells pre-treated with the same concentrations of LPS were used as

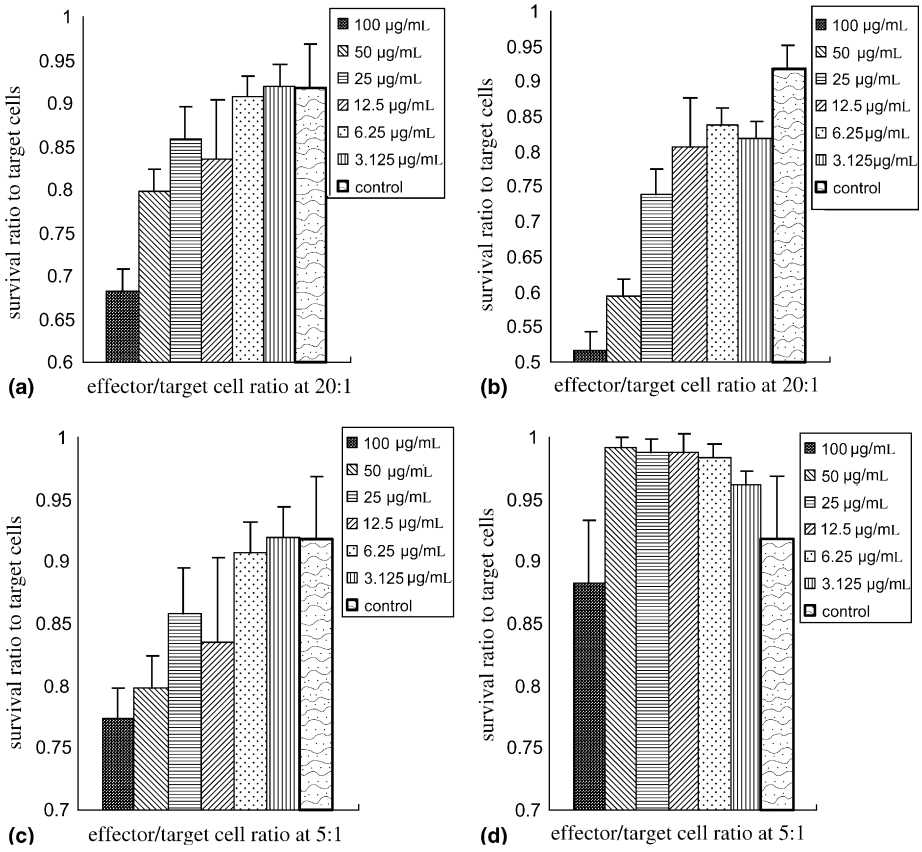

Figure 3. Effect of F3 on $CD56^+$ NK-cell mediated cytotoxicity. (a) NK-cells were pre-incubated with the indicated concentrations of F3 for 7 days. K562 cell lysis was assessed by Alamar Blue assay at an effector/target ratio of 20:1. (b) $CD56^+$ NK-cells were pre-incubated with LPS. (c) and (d) Effector cells/target cells (*E*/*T*) ratio at 5:1, F3-treated NK-activity compared with LPS-treated NK-activity at various concentrations. Results are expressed as a percentage of survival to the control. Data are the mean ± SD of three independent experiments (*, $p < 0.05$; **, $p < 0.01$ as compared to the control group without F3 treatment).

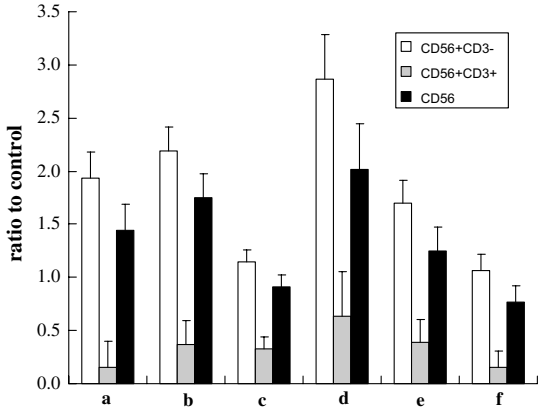

Figure 4. Difference in NK subtypes when cells were pre-treated with 100 μg/mL F3 versus equal volume of PBS. A relatively high ratio of $CD56^+CD3^-$ NK-cells (114–286%) was detected after cells were cultured in F3 (100 μg/mL) for a week compared to control ones (legend of a, b, c, d, e, and f were six individual volunteers).

positive controls and NK-cytotoxicity enhanced by F3 was comparable to these positive controls. NK-cytotoxicity increased significantly by 48.2% and 40.7% ($P < 0.01$), respectively, in pre-incubation with 100 and 50 μg/mL LPS (Fig. 3b and c). Treatment with F3 produced comparable cytotoxicity effects to treatment with LPS.

3.3. Individual variance

In order to investigate whether minor subtypes of NK-cells were affected by F3, UCB MNCs were collected from six volunteers. These MNCs were cultured for 7 days after treatment with 100 μg/mL of F3. NK-cells were then harvested and tested for cell surface markers (CD56 and CD3) using two-color flow cytometric analysis. Control groups were treated with PBS only. The number of $CD3^-CD56^+$ NK-cells increased significantly (114–286%) in certain individuals. All the six samples showed a big increase in $CD3^-CD56^+$ NK-cells, but a decrease (by 36.5–84.7%) in $CD3^+CD56^+$ NKT cells (Fig. 4).

These data imply that the phenotypic change of NK-cells might be the cause of enhancement of the NK-cell cytotoxicity effect, and treatment with high concentrations of F3 may decrease the T cell growth. The cytotoxicities of different subtypic NK-cells need further investigation.

4. Discussion

The immunomodulating effects of *Ganoderma lucidum* polysaccharides have been studied using adult peripheral blood mononuclear cells (PBMC) as the source of effector cells, and LAK-cells were generated by incubating with cytokines, such as IL-2, etc. In this study, we took advantage of human umbilical cord blood (UCB) mononuclear cells (MNC) as cord blood is known to possess more progenitor cells and is an excellent candidate for studying the effect of F3 on mononuclear cell subsets. In light of the increasing commercial storage of private umbilical cord blood, it is quite possible that in the future, these cells will be used more often for different studies. In this study, the MNCs from hUCB contain cells other than NK-cells that could cause lysis in a non-MHC manner. The $CD56^+$ NK-cells were thus highly purified in order to reduce the effects of F3 on cytotoxicity mediated by other cells.

The UCB samples from different individuals after F3 treatments exhibited a significant immune response, predominantly involving $CD3^+CD56^+$ NKT cells and $CD3^-CD56^+$ NK-cells, suggesting that both NK-cells and NKT cells may play important roles in protecting the newborn against infection. NKT cells comprise less than 0.1% of lymphocytes in adult peripheral blood and umbilical cord blood.[23] Our observations indicate that the proportion of NKT cells of UCB decreases, while the proportion of NK-cells increases, after F3 treatment. This may provide some information to understand, which NK-cells provide the newborn with a phenotypically distinct cytotoxic function.

Our results demonstrate that incubation with F3 alone not only increases the cytotoxicity of $CD56^+$ NK-cells against the NK-sensitive tumor-cell line K562 but also alters the expression of cell surface markers. In addition, incubation with LPS led to a significant increase in cytotoxicity in all the cases.

In summary, we have demonstrated that the polysaccharide fraction F3 from Reishi is able to stimulate the $CD56^+$ NK-cytotoxicity against the tumor cell line used in our experiments. Further study of the effect of F3 on other cytokines is in order ongoing to develop new immunotherapeutic strategies that will enhance the anti-tumor activity with human NK-cells.

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