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ORIGINAL ARTICLE

# Identification of Functional Fragments in gMYL6 and the Mechanism of Improving NK Cell-Mediated Cytotoxicity

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## ABSTRACT

Background: In a previous study, unrecognized role of gMYL6 in the up-regulation of human NK cells' development and cytotoxicity was reported. Objective: To further elucidate the mechanism of action of small fragments recombinant of gMYL6 enhancing the NK cells activity. Methods: Mononuclear cells were isolated from umbilical cord blood (UCB) by density-gradient centrifugation and NK cells were propagated and cultured. The small peptides from the gMYL6, with ability to enhance the cytotoxicity of NK cells were screened by CCK-8 method and one of the most powerful peptides was identified for the next study. Flow cytometry were used to explore the proliferation and apoptosis of K562 cells, as well as the cell cycle arrest. The apoptosis of target cells was observed by AO/EB fluorescence staining, and the value of apoptosis was detected by flow cytometry. The method of protein imprinting was also used to explore the pathway of small peptides to enhance the NK cells activity. On the other hand, Real-time Quantitative PCR Detecting System was used to verify the mechanism of K562cell suppression. Results: Small D peptide significantly increased NK cells cytotoxicity and induced both cell cycle arrest at G2/M and apoptosis of K562 cell. Conclusion: Small D could be a novel promising peptide against cancers since it showed the capacity to promote the cord blood-derived NK cells' cytotoxicity.

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**Keywords: Cancer Immunotherapy, gMYL6, NK Cell, Small Molecule Peptide**

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## INTRODUCTION

Tumor immunotherapy is one of the most promising research directions in the field of anti-tumor. It has the potential to treat many types of tumors by enhancing the detection ability for tumor and using its own immune system to clear tumor cells, including immunomodulator and cellular immunotherapy, etc.(1-3). So far T cells have been the mainstay of cellular immunotherapy. But, there are some limitations for the T cell-based immunotherapy, such as the immune escape of tumor cells. NK cells are key components of innate immunity and can kill many cancer cells via direct killing. With the deepening of research on NK cells, it has received more and more attention in tumor immunotherapy. NK cells play a key role in the elimination of compromised host cells, such as tumor or virus-infected cells (4). Here, we reviewed the current applications of NK cells immunotherapy reported in works, such as, Kollipara PS (5) and others found that activation of NK cells with snake venom can inhibit the growth of lung cancer cells. Radice E et al (6) found that pretreatment of peripheral blood lymphocytes in patients with non-metastatic colorectal cancer with SKA-IFN-γ （IFN-γ prepared by sequential-kinetic-activation SKA） significantly increased the cytotoxicity of NK cells. Janneke S et al demonstrated that highly functional NK cells derived from human CD34+ haematopoietic stem and progenitor cells (HSPC) efficiently destructed ovarian carcinoma spheroids in vitro and killed intraperitoneal ovarian tumors in vivo (7).

The occurrence, development and metastasis of tumor are closely related to the inhibition of anti-tumor immune function of NK cells. The use of NK cells for immunotherapy relies on the availability of a great number of NK cells with optimal cytotoxic activity (8,9). However, there may be a loss in function or number of NK cells in cancer patients which is necessary to transfer to play a good anti-tumor effect. Therefore, to acquire a sufficiently large number of fully functional NK cells, many culture methods were developed to apply functional NK cells in the clinic (10).

Some studies have shown that monoclonal antibodies, small molecular compounds and polypeptide drugs can promote NK cell-mediated anti-tumor immune response. Among monoclonal antibodies, small molecular compounds, and polypeptide drugs, polypeptide drugs with moderate molecular weight between monoclonal antibodies and small molecular compounds have become the focus of anti-tumor drug research and development in recent years due to their low toxicity, low immunogenicity, strong tissues penetration force, and good specific binding capacity with tumor (11-12). In the development of peptide drugs, mining functional fragments based on the overall structure is an important research strategy. For example, thymopentin is an effective part of thymosin 2 secreted by the thymus. It has five amino acids but has the same physiological functions as thymosin 2. It has been used as a synthetic peptide drug in clinical practice (13-14).

Inspired by these facts, first of all, small peptides were designed and synthesized in this study. The α-helix is the most abundant secondary structural motif, representing a generic template for inhibitor design (15). Accordingly, four small peptides (A peptide, B peptide, C peptide and D peptide) were obtained by truncating the main α-helix fragment of goat myosin light chain 6 (gMYL6) which can up-regulate the toxicity of human NK cells in previous study (16), and their bioactivity of promoting the cytotoxicity of NK cells was evaluated. Ultimately, small D peptide can significantly increase cord blood-derived NK cell cytotoxicity and induce cell cycle arrest at G2/M and the cell apoptosis. Therefore, this research lays the foundation for further exploring the role of peptides on NK cells.

## MATERIALS AND METHODS

**Reagents and antibodies.** In this study, based on written informed consent, umbilical cord blood (UCB) units were obtained at birth after normal full-term delivery. The linear peptides were prepared by standard Fmoc solid-phase techniques on a CS336X automated peptide synthesiser (CSBio; U.S.A.) using repeated steps of amino acid coupling and Fmoc deprotection (17). Cell lysate and RNA extraction kits were purchased from Zhuang Meng Co., Ltd, China. Anti-human PRF1, GZMB, and LAMP1 antibodies were purchased from ABclonal, while FITC-CD3(clone UCHT1), APC-CD56 (clone HCD56), PerCP-Cy5.5-NKp30 (clone P30-15), and APC-NKG2D (clone 1D11) antibodies were purchased from BD Co., Ltd, USA. AO/EB. Fluorescent dyes were also purchased from Solarbio ,Co., Ltd, China. The Annexin V-FITC/PI kit as well as cell cycle stain were obtained from BD Co., Ltd, USA and stored at 4°C in the dark. Moreover, K562 cell line was obtained from the Chinese Academy of Medical Sciences and stored in liquid nitrogen containing 10% DMSO and 10% FBS. Cell culture medium RPMI and fetal bovine serum (FBS) were also purchased from CORNING, Co., Ltd, China. Besides, NK cell-related medium X-VIVO 15 was purchased from LONZA, Co., Ltd, China.

**Cell culture.** Umbilical cord blood samples were collected and the mononuclear cells (MNC) were isolated by ficoll-hypaque density-gradient centrifugation and cultured in the culture dishes using the basal media (18). Expanded MNC were differentiated and expanded using NK cell differentiation medium. Cell cultures were refreshed with new medium every 2~3 days. Cultures were maintained in a 5% $CO_2$ incubator with 95% humidity at 37°C.

**NK cells cytotoxicity to K562 cells.** After 20 days in culture, we mixed the NK and K562 cells at logarithmic growth phase in a 96-well plate (effector: target ratio=2:1). The effector cell wells, target cell wells, and blank wells were set at the same time. Then, 80 μl of the cell suspension was placed in 96-well plates and 20μl of different concentrations of the small peptide were added to the plates for the stimulation test. Afterwards, each well was added to 100 μL (40 μl effector cell, 40 μl target cell and 20 μl peptides) and cultivated for 8 h and 16 h with 5% $CO_2$ at 37°C. The 96-well plate was taken out of incubator, placed in a centrifuge, and centrifuged at 1000g for 5 min to aspirate the supernatant. After adding 100 μL of medium and 10 μL CCK-8 to each well, the cells were put into the incubator and cultured for further 4 h. Finally, the OD value was detected at 450nm wavelength. The specific killing of K562 cells by NK cells was calculated with the following formula: Cytotoxicity (%) = [1 - (target cell well OD value - effector cell well OD value) / target cell well OD value] x 100%.

**Cell-cycle assay.** K562 cells were seeded into 6-well plates at a density of $2 \times 10^6$. The concentration of NK cells was adjusted in $4 \times 10^6$ per well, and blank control and experimental groups with incorporation of small D peptide in culture were set for evaluation and measured with a volume of 2 ml. After incubation for 8 h and 16 h in an incubator at 37°C with 5% $CO_2$, the cells were collected and washed with PBS. Then, we proceeded with discarding the supernatant. The cells were washed with PBS once again after fixation with alcohol overnight at 4°C. After PI staining, the cell suspension was analyzed by flow cytometry.

**Apoptosis assays by AO/EB staining and flow cytometry.** Different treatment cells were washed with ice-cold phosphate buffer saline (PBS) and fixed with formalin (4%, w/v). Cell nuclei were counterstained with acridine orange (AO) and ethidium bromide (EB) (AO: 100 μg mL−1, EB: 100 μg mL−1) for 2 min. The cells were observed with a fluorescence microscope (Nikon, Yokohama, Japan) with excitation at 350 nm and emission at 460 nm. On the other side, different treatment cells were washed three times with PBS and collected for

FITC-AnnexinV/PI staining assay. Based on the protocol of Cell Apoptosis Detection Kit, the cells were resuspended in 1x buffer at a concentration of $1 \times 10^6$ / ml (10x Annexin V binding buffer was diluted to 1x with distilled water). Then, 100 µl of solution ($1 \times 10^5$) was transferred to a 5 ml centrifuge tube. Cells were incubated upon addition of 5 µl of both of Annexin V- FITC and PI in the dark for 15 min. The stained cells were immediately analyzed by flow cytometry (ACEA NovoCyte).

**Western blot.** Cells were collected and lysed in 1 mL RIPA lysis buffer containing 2 µL protease inhibitor. Thereafter, the lysates were subject to sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis and transferred to PVDF membrane. The membrane was blocked with milk powder solution (5% skim milk in Tris saline with Tween [TBST]) for 2 h. After blocking, the membrane was incubated overnight with primary antibodies at 4°C, washed with 1 × TBST buffer four times, probed with secondary antibody for 2 h, washed again with 1 × TBST buffer four times, and finally visualized with ECL substrate.

**Quantitative real-time polymerase chain reaction analysis.** Total RNA was extracted from K562 cell using the FOREGENE kit and the concentration of RNA was measured. 500 ng RNA was reverse-transcribed with a Zhuang Meng Reverse Transcription Kit. The transcriptional reaction conditions were 45°C for 15 min and 85°C for 5 min. Then, the obtained cDNA products were used to quantify mRNA expression level according to the SYBR enzyme description. The reaction (after joining SYBR) conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5s and 60°C for 30s according to manufacturer's instructions. All primers are listed in Table 1.

**Table 1. Quantitative Amplification Primers.**

| Primer Names | Primer Sequences |
|---|---|
| **PRF1 primers (forward)** | 5′-TGATGCCACCATTCCAGGAG-3′ |
| **PRF1 primers (reverse)** | 5′-CAGAGACAGGGGGCACTTG-3′ |
| **GZMB primers (forward)** | 5′-CCTTCCTGAGAAGATGCAACCAA-3′ |
| **GZMB primers (reverse)** | 5′-AAGGAAAAGTCTGCATCTGCCCT-3′ |
| **LAMP1 primers (forward)** | 5′-CACGTAGGACGCATGAAGGT-3′ |
| **LAMP1 primers (reverse)** | 5′-CAGGATCACCCCGAATGTCA-3′ |
| **GAPDH primers (forward)** | 5′-GAAAGCCTGCCGGTGACTCC-3′ |
| **GAPDH primers (reverse)** | 5′-AGGAAAAGCATCACCCGGAG-3′ |

**Statistical Analysis.** All experiments were carried out at least three times. The statistical significance of results was analyzed by homogeneity of variance test, and then tested with Student's t test using SPSS 13.0. $P < 0.05$ was considered statistically significant.

## RESULTS

**The development and differentiation of NK cells derived from human umbilical cord blood.** First, mononuclear cells were isolated from UCB samples and cultured in a certain concentration of inducer and amplification medium. During this period, the status of the cells was regularly observed and the expanded cells were tested by flow cytometry. After 2-3 weeks, a large population (85.67% of the cells are mature NK cells) was represented by $CD56^{+}CD3^{-}$ cells with NK cells' features (Figure 1B). Further analysis of the expression of NK receptors on $CD56^{+}CD3^{-}$ cells revealed a high percentage of expression of their activating receptors NKp30 and NKG2D (NK activating receptors )(Figure 1C, D).

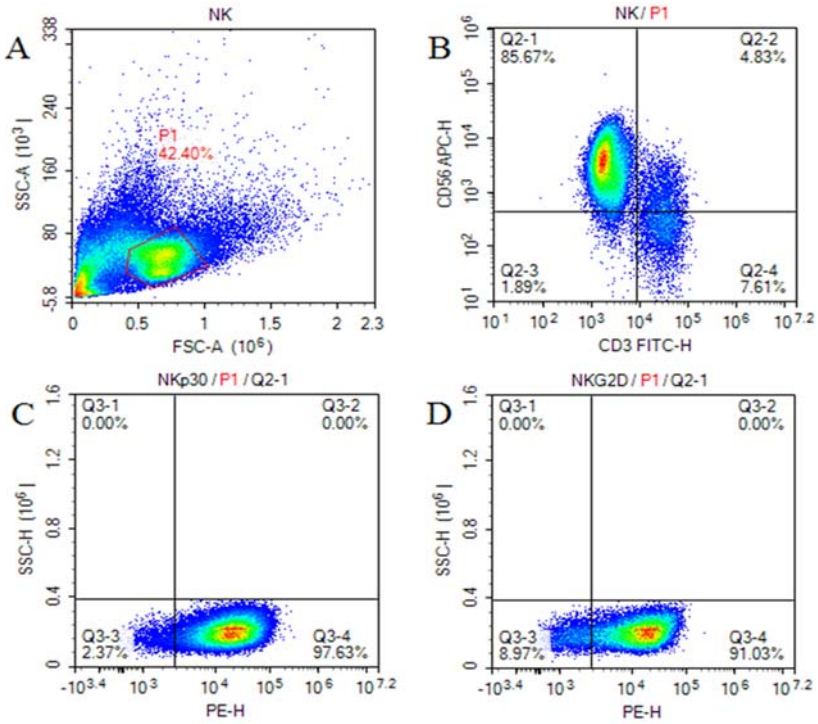


**Figure 1. Data of flow cytometry analysis of mature NK cells induced by human umbilical cord blood.** (A) Scatter plot of the collected cells. (B) The expressions of CD3 and CD56 in mature NK cells. We also identified the activation of NK cells by the expression of activating receptors NKp30 and NKG2D. (C) The percentage of NKp30 expressed in NK cells is 97.6%. (D) The percentage of NKG2D is 91%.

**Peptides from gMYL6 Enhance NK Cell Cytotoxicity.** To investigate the potential role of gMYL6 peptides from in human NK cell cytotoxicity, cultured NK cells were maintained in different concentration (25 μM, 50 μM, and 100 μM) of small peptides for 8 h and 16 h. Subsequently, we used the CCK-8 reagent to detect the cytotoxicity of NK cells. The activity of cytolytic treated with small D peptide increased up to a maximum of 10.63% at 25 μM. Other small peptides also had variable degrees of improving effect on the cytotoxicity of NK cells (Table 2 and Figure 2).

**Table 2. Increasing rate of cytotoxicity of NK cells under the action of active peptides.**

| Group | Amino Acid Sequence | rate of increase (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 25 μM | | 50 μM | | 100 μM | |
| | | 8h | 16h | 8h | 16h | 8h | 16h |
| **A peptide** | EFKEAFQLFDRTGD GKI | 1.25 ± 0.89 | 1.32 ± 0.26 | 1.78 ± 0.66 | — | 0.64 ± 0.11 | — |
| **B peptide** | LYSQCGDVMRALG QNP | — | — | 0.72 ± 0.21 | 3.04 ± 1.47 | — | — |
| **C peptide** | NAEVLKVLGNPKS DEM NVKV | 8.22 ± 1.02 | — | 5.75 ± 0.54 | 3.22 ± 1.8 | — | — |
| **D peptide** | LDFEHFLPMLQTV AK | 10.6 ± 1.73 | 5.52 ± 0.95 | 5.98 ± 0.32 | 4.20 ± 0.55 | 3.06 ± 0.42 | — |

The results also indicated that small peptides retained and improved the cytotoxicity activity of NK cells against tumor cells and small D peptide could be the main functional fragment of gMYL6 exerting cytotoxicity.

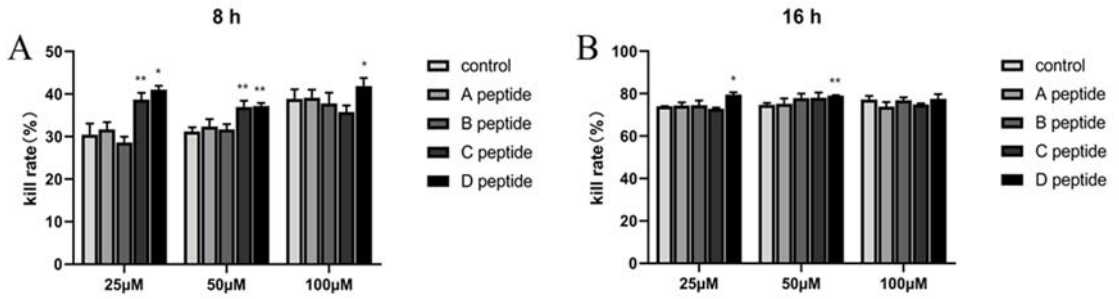


**Figure 2. The cytotoxicity of NK cells to K562 cells.** (A) The cytotoxicity of NK cells treated by each group of small peptides for 8 h against K562 cells at 25 μM, 50 μM and 100 μM. (B) The cytotoxicity of NK cells treated by each group of small peptides for 16 h against K562 cells at 25 μM, 50 μM and 100 μM (*p<0.05, **p<0.01).

**Effects of NK Cells Treated by D peptide on Cell Cycle of K562 Cells.** Flow cytometry was used to analyze the K562 cell-cycle phase distribution. The results showed that when treated with D peptide, the number of K562 cells at G1 and S phase decreased, while they increased at G2 phases (Figure 3). The changed number of cells at different cell-cycle phases suggested that small D peptide could help NK cells inhibit the growth of K562 cells.

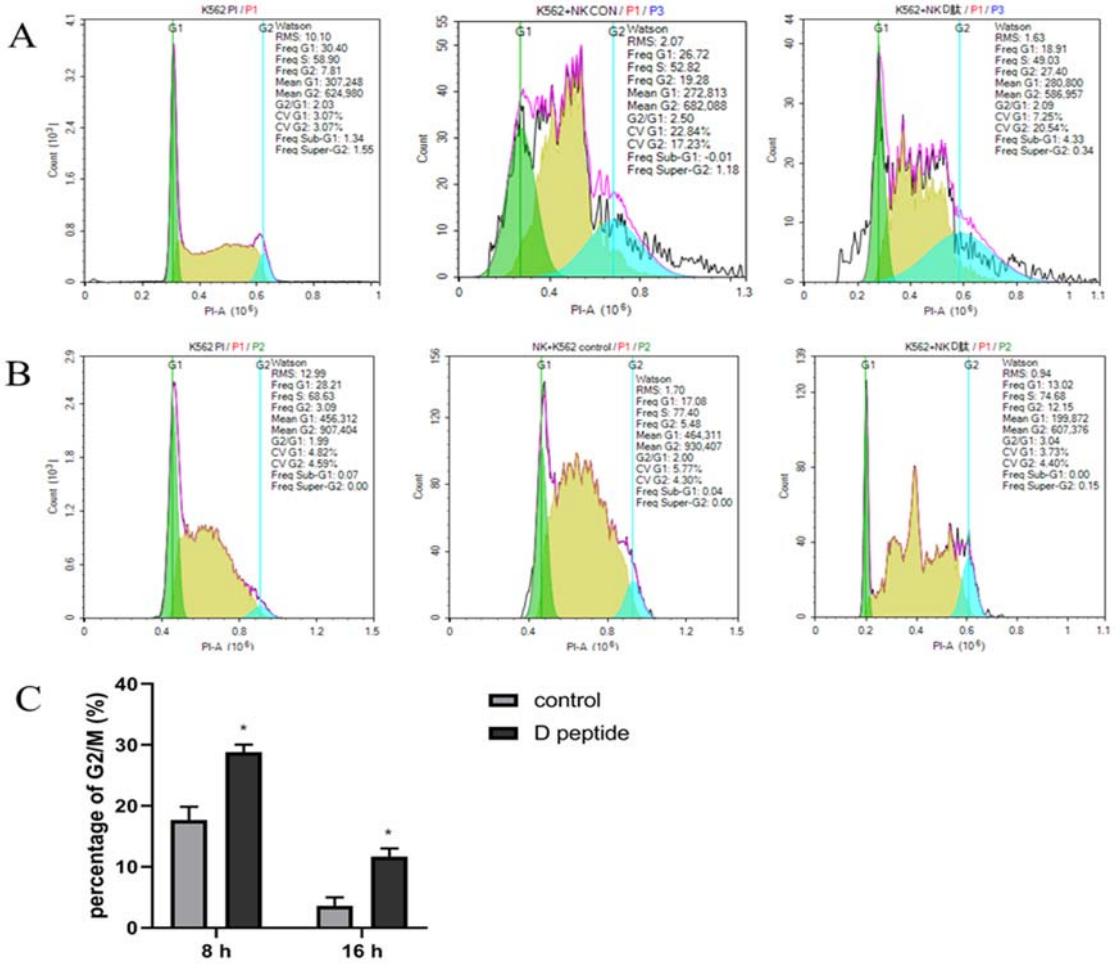


**Figure 3. The effects of NK cells treated by peptide D on the cell cycle of K562 cells.** Treated NK cells by D peptide for (A) 8 hours, (B) 16 hours were associated with a decreased percentage of cells in the G1 and S phases and an increased percentage of cells in the G2 phase compared with control cells. (C) The percentage of cells in the G2/M phase treated by D peptide compared with control cells（*p<0.05).

**Effects of NK Cells Treated by D Peptide on Apoptosis of K562 Cells.** Apoptosis sometimes occurs as a defense mechanism such as in immune reactions. In this study, the apoptosis of K562 cells induced by NK cells was detected by flow cytometry. To investigate the effect of NK cells treated by D peptide on apoptosis, the apoptosis was assayed by AO/EB staining and flow cytometry. As shown in Figure 4, in the control (a) at 8 h and (c) at 16 h, the percentage of apoptotic cells is 28.67% and 45.33%, respectively. After treatment of NK cells by D peptide of 25 μM, the percentages of apoptotic cells were 33.33% at 8 h and 52.67% at 16 h, respectively. This showed that D peptide could help NK cells induce apoptosis in K562 cells. Further investigation by flow cytometry revealed the similar results. As shown in Figure 5, the improvement rate of apoptotic cells in experimental group compared with the control group is 3.8% at 8 h and 4.0% at 16 h, respectively. These results showed that D peptide could enhance the cytotoxicity of NK cells to K562 cells and induce the apoptosis of K562 cells through apoptosis pathway.

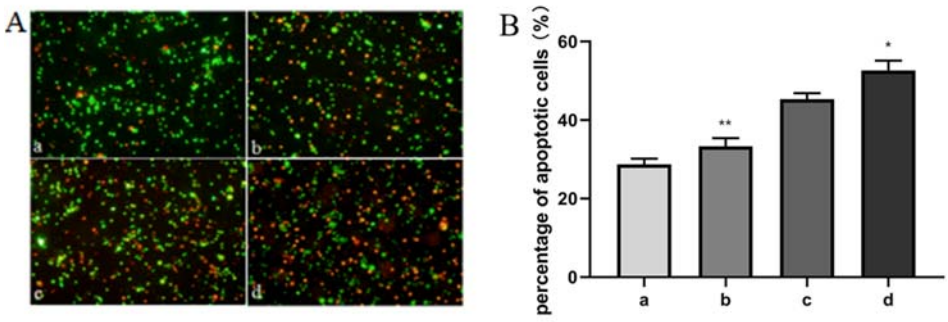


**Figure 4. The apoptosis of K562 cells was analyzed by both fluorescent (AO/EB) staining Cells emitting green fluorescence indicative of healthy growth cells and cells emitting red fluorescence indicating that the cells have died or apoptosed.** (A) Representative images of fluorescent microscopy data: a) represents the morphology of cells in the control group, b) represents the morphology of cells in the experimental group (NK cells treated by D peptide). Both groups of cells were stained for 8 hours c) indicates the morphology of cells in the control group, and d) indicates the morphology of cells in the experimental group. Both groups of cells were stained for 16 hours. (B) Quantitative analysis of the apoptotic percentage of cells in each group (*p<0.05, **p<0.01).

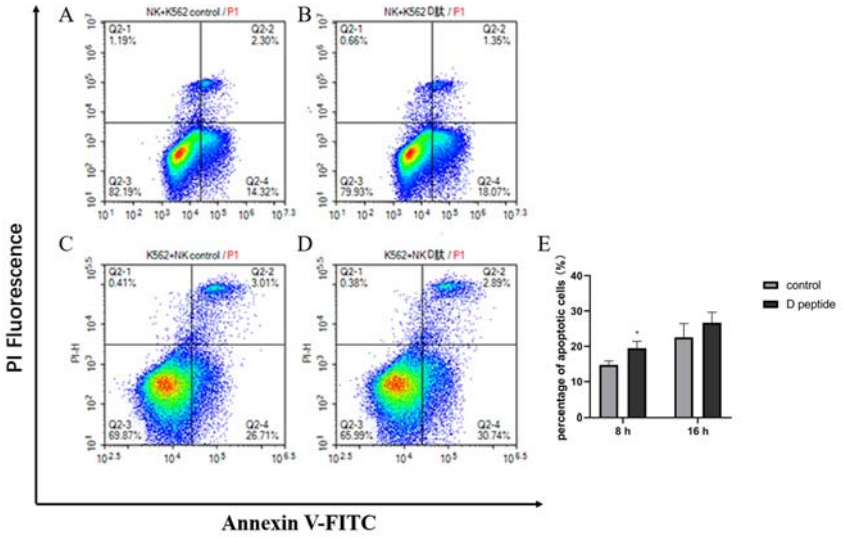


**Figure 5. Flow cytometry analysis of apoptosis in K562 cells.** (A) The percentage of apoptotic cells was 14.32% in the control group for 8 h, (B) The percentage of apoptotic cells was 18.07% in the experimental group (NK cells treated by 25 μM D peptide) for 8 h , (C) The percentage of apoptotic cells was 26.71% in the control group for 16 h, (D) The percentage of apoptotic cells was 30.74% in the experimental group (NK cells treated by 25μM D peptide) for 16 h, and (E) Quantitative analysis of the percentage of apoptotic cells treated by D peptide for 8 h and 16 h compared with control cells (*p<0.05).

**Phenotypic profile of NK cells treated by D peptide.** To elucidate the effects of D peptide on NK cells' cytotoxicity, the expression of perforin and CD107a was analyzed (Figure. 6A). As shown in Figure 6B and 6C, at 25 μM, D peptide significantly increased the expression of perforin and CD107a in NK cells compared to blank control after both 8h and 16 h incubation. Furthermore, The protein expression of PRF1, GZMB, and LAMP1 was determined by Western blot analysis. The results showed that compared with the control group, the PRF1 gene was highly expressed after 8h incubation with small D peptide. The experimental results were also statistically significant (p<0.05).

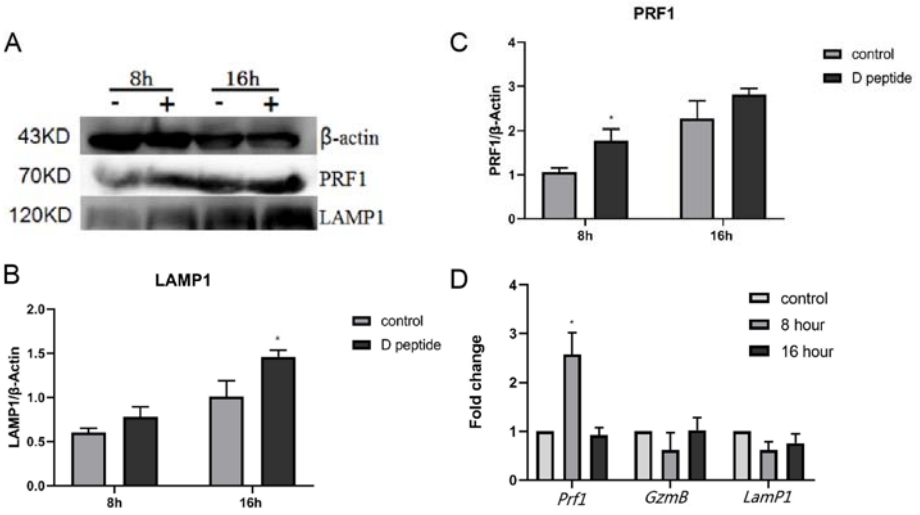


**Figure 6. The NK cells treated by D peptide are highly functional.** A: The expression of perforin and CD107a protein of NK cells after incubation with D peptide for 8 h and 16 h, B: Quantitative analysis of the expression of perforin protein in NK cells treated by D peptide for 8 h and 16 h compared with the control cells, C: Quantitative analysis of the expression of CD107a protein in NK cells treated by D peptide for 8 h and 16 h compared with the control cells, and D: The NK cells were incubated in the absence or presence of D peptide for 8 hours or 16 hours and then underwent qPCR analysis (*p<0.05).

## DISCUSSION

Due to the advantages of low molecular weight and toxicity, few side effects, easy synthesis, and low cost, many kinds of anti-tumor peptides have received extensive attention and entered clinical research. However, the key point to the development of peptide drugs lies in the discovery of lead peptide molecules. Previous studies have determined that goat myosin light chain 6 (gMYL6) could enhance the activity of NK cells, a component of goat anti-cancer active peptides obtained from immunized goat spleens (19). However, being a recombinant protein, gMYL6 also has some problems, including the high production cost and bad tissue penetration.

In this study, to identify the smallest functional fragment maintaining the activity of gMYL6, we designed four peptides according to the secondary structure of gMYL6. Although many hot spots were presented on the protein surface, these residues were generally not contiguous within the protein sequence as a result of protein folding.

The α-helix is the main secondary structural composition in protein which has captured the interest of many researchers. Alpha helix has shown to be able to mediate a number of critical treatment-related PPI interfaces (20-21). All the four isolated peptide sequences we designed in the present study came from the α-helix structure of gMYL6. We ultimately identify one peptide with the same activity as gMYL6. To examine the action mechanism NK cells treated by D peptide in K562 cells, we further determined the effect of NK cells on K562 cell cycle distribution by flow cytometry. G2/M arrest and apoptosis are common phenomena after DNA damage. After 8 h and 16h treatment, the K562 cells treated with NK cells (treated by D peptide of 25 μM) exhibited an increase in the proportion of K562 cells in the G2/M phase compared with the untreated K562 cells. The combined treatment also induced remarkable G2/M arrest, which in

turn induced cell-killing effects. After the combined treatment, the growth cycle of K562 cells stopped at the G2/M phase indicating that the growth and proliferation of tumor cells were inhibited in cell cycle. From both AO/EB staining and FITC-AnnexinV/PI staining assays, it could be seen that NK cells treated with D peptide significantly increased apoptosis in K562 cancer cells and the apoptosis rate of K562 cells increases along time. It is therefore suggested that D peptide could play a role in apoptosis induction. There are multiple ways for NK cells to kill target cells. Release of perforin and granzyme is the primary way and is closely related to activation of NK cells (22). In the present study, we first detected the cytotoxicity to K562 cells of NK cells treated by D peptide, we found that the cytotoxicity of NK cells enhanced. We also considered that the enhanced cytotoxicity of NK cells may be a result of releasing perforin. To verify this, we tested perforin and CD107a. The role of perforin is to perforate holes on the surface of the target cells and lyse the target cells (23). CD107a is an important membrane protein of NK cells related to the killing activity of NK cells (24). As shown in Figure 6, in the D peptide of 25 μM group, the expression of perforin and CD107a in NK cells has been significantly increased. Further study at the genetic level, the D peptide of 25 μM group also resulted in high expression of the cytotoxic effect gene PRF1. According to the findings of the present work, the small identified peptide can increase the cytotoxic activity of NK cells by enhancing the secretion of perforin and CD107a proteins and eventually inhibit the growth of tumor cells via inducing apoptosis of tumor cells. This peptide has also the potential of developing either small peptides as additives for NK cells culture in vitro or drug candidates for cancer immunotherapy.

## ACKNOWLEDGEMENTS

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