

An Effective Vaccine Strategy of Translating Apoptotic Tumor Cells into Cancer Vaccine

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Abstract

Cancer vaccine has been regarded as the most attractive treatment for the patients. Our previous work showed that cartilage polysaccharide of short-chain (CPS) could induce apoptosis of L1210 cell lines through mitochondria apoptosis pathway and mice treated with CPS didn't develop tumors after L1210 cell inoculation when compared to control mice without treatment. In present work, we developed a vaccine based on previous experiments and results showed that four in ten mice vaccinated didn't show proliferation of ascite tumors while the mice in the control group didn't show inhibition of tumor growth. We further identified the antibody with immunofluorescence and western blot from the serum of immunized mice which can recognize a protein with molecular weight of 35 kDa. Finally, we used mass spectrometry and identified the target recognized by the antiserum annexin A2, which is involved in cell proliferation, differentiation and apoptosis processes and is tightly associated with cancer progression and chemoresistance.

Keywords: cartilage polysaccharide, L1210 cells, apoptosis, annexin A2, anti-tumor immune

1. Introduction

Each year, more than 10 million people are diagnosed with cancer worldwide, while almost 8 million die from cancer or cancer-associated diseases and 12% of all deaths globally are due to tumors (Parkin, Bray, Ferlay, & Pisani, 2005). In spite of tremendous advances in the prevention, diagnosis, and therapy of cancer, there is still no cure. Currently, primary treatment of cancer involves surgery, radiotherapy, hormone therapy and cytotoxic drugs. Many forms of cancers are considered to be incurable, and treatment for many tumors results in only a minor increase in the time of survival (Vasso, 2011). So it is not surprising that researchers continuously work to develop new modalities for anti-cancer therapy in the worldwide (Yan, Chen, Liu, & Huang, 2010; Jain, 2010; Wang, Kaumaya & Cohn, 2010; Srivastava, Bosch, Wilson, Edelman, & Ostrand-Rosenberg, 2010; Akbulut, Tang, Akbulut, Maynard, & Deisseroth, 2010; Um *et al.*, 2010; Suzuki, Chang, Kitajima, & Takaku, 2010). Cancer vaccine or immunotherapy, with advances in molecular biology, is now giving new insights into the treatment of cancer, because of the identification of antigens and cytokines, the molecular events that trigger oncogenesis and the better understanding of immune system. So cancer vaccines represent the most attractive treatment modalities.

In April 2010, sipuleucel-T (Provenge[®], Dendreon Corporation) became the first therapeutic cancer vaccine to be approved by the U.S. Food and Drug Administration (Kantoff *et al.*, 2010; Higano *et al.*, 2009). As John Nemunaitis outlines in his article, the success of sipuleucel-T (Provenge[®], Dendreon Corporation) may only be the start of a wave of successes in the area of cancer vaccines (Nemunaitis, 2011).

Evidently, many cancer antigens are self-antigens and are also expressed by healthy cells of the body (Finn & Banachereau, 2010). But there is also evidence that the extent of post-translational modification may contribute to

the immunogenicity of cancer antigens (Farkas & Finn, 2010). Many researches (Akbulut, Tang, Akbulut, Maynard & Deisseroth, 1998; Gregory, 2000; Bellone et al., 2000) show that antigen presenting cell (APC) could effectively identify antigens that expressed by apoptotic tumor cells, then present to T cells and stimulate anti-tumor immune response. Other research findings (Obeid et al., 2007; Casares et al., 2005; Nowak et al., 2003; Locher et al., 2010) indicate that the mechanism of some anti-cancer drugs is to induce tumor cells apoptosis and enhance its immunogenic, so host' immune system could remove apoptotic tumor cells further. Induction of apoptotic cell death or post-translational modification of tumor cells may make cancerous cells recognized by immune system.

We reported cartilage polysaccharide of short-chain (CPS) that was extracted by our laboratory induce apoptosis of L1210 cells through mitochondria pathway in vivo previously (Liu, Yang, Song, Cao & Wang, 2009a). During the course of experiments we found mice that cured by CPS treatment no longer develop tumors when L1210 cells were inoculated again. We infer that apoptosis or post-translational modification of tumor cells may contribute to anti-tumor immunity. Based on previous results, we develop one such apoptotic tumor cell lysate-derived vaccine named CPS & cell vaccine. Now we extend our work by investigating whether it has anti-tumor effects on mouse ascites tumor.

2. Materials and Methods

2.1 Materials

TUNEL Cell Biol Toxicol assay Kits purchased from Promega (Madison, WI). Goat anti-rabbit IgG-FITC (sc-2012) purchased from Santa Cruz Biotechnology (Heidelberg, Germany). HISTOPAQUE-1083 purchased from Sigma-aldrich (USA).

2.2 Cartilage Polysaccharides of Short-chain

Porcine cartilage treated with 1% sodium carbonate, pH 10-11, for 3 h, and heated at 70°C for 30 min. Degradation by papain (1:500 w/v) was performed overnight and the substance was purified by DEAE-SephadexA-25 column to give a long-chain polysaccharide. The long-chain polysaccharide was degraded by hydroxyl radicals (3% H₂O₂) for 3 h to produce CPS and the CPS was precipitated by ethanol. After lyophilization, CPS was dissolved in cell culture medium or in physiological saline for in vitro or in vivo study. CPS derived from porcine cartilage were extracted and purified by our laboratory (Liu et al., 2009a; Liu, Song, Yang, Liu, & Zhang, 2007; Liu, Han, Zhang, Zhao, & Zhen, 2009b).

2.3 Cells and Animals

L1210 cells line (provided by the Immunological Laboratory of Tianjin Medical University). Healthy female DBA/2 mice of clean grade (mean body weight 20 g) were obtained from the Department of Experimental Animals, Academy of Military Medical Science and Beijing. The National Institutes of Health Guidelines for the care and use of laboratory animals were followed in all animal experimental procedures. The animals were allowed free for food and water at all times and were maintained on a 12 h light/dark cycle in a controlled temperature 25°C and humidity (50%±5%) environment for 1 week before use.

2.4 Flow Cytometry Assay

Collected cells treated with CPS for 24 h, 48 h and 72 h, also 0 h cells (untreated with CPS) were used as control. Cells were washed in ice-cold PBS for three times. The cells were then fixed in 70% methanol, and DNA was stained with PI (50 mg/mL) containing 5 mg/mL RNase in 1:100 dilution. Cell cycle analysis was performed with a FACS can flow cytometer (Becton Dickinson, San Jose, CA). EXPO Software (Applied Cytometry Systems) was used to determine the cell cycle phase distribution after debris exclusion. The sub-G1/G0 cell fraction was considered as representative of apoptotic cells.

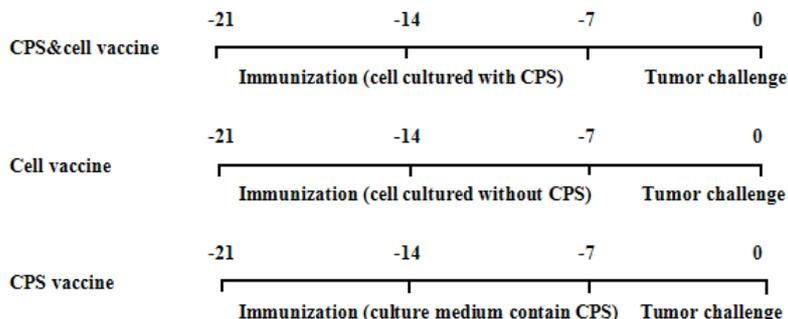
2.5 TUNEL Assay

The occurrence of apoptosis in L1210 cells was detected as described previously, by monitoring the presence of DNA fragmentation in situ. TUNEL staining was performed using death detection kit according to the manufacturer's instructions. Apoptotic cells were examined and photographed under a fluorescence microscope (Olympus, C5060-ADU and Japan).

2.6 Experimental Protocols

Forty female DBA/2 mice were randomly divided into four groups. One is CPS & cell vaccine group (n=10) received subcutaneous vaccination with immunogen I (L1210 cells cultured with 400µg/mL CPS for 48 h, and then put the cells and culture medium into liquid nitrogen for 5 times and 30s/time for freezing-thawing cells treatment.) three times at 1-week interval. Second is Cell vaccine group (n=10) received subcutaneous

vaccination with immunogen 2 (L1210 cells cultured without CPS for 48 h, and then put the cells and culture medium into liquid nitrogen for 5 times and 30s/time for freezing-thawing cells treatment.) three times at 1-week interval. Third is CPS vaccine group (n=10) received the same dose of culture medium (contain 400µg/mL CPS) that had been freezing-thawed, three times at 1-week interval. And then 1 week after the third time of vaccination in all above three groups, 2.0×10^5 L1210 cells were inoculated subcutaneously. Other 10 mice were used as blank group without any treatment. During the experiments, all mice were monitored for some general health indicators: body weight, general behavior, feeding and defecation, appearance of fur, etc.



2.7 Splenic Lymphocyte Proliferation Assay

Isolate lymphocytes from spleen of mice in different groups by HistoPaque-1083 density centrifugation, resuspend and add 100µl lymphocytes to 96-well plates, quadruplicate wells for each sample. Add 100µl ConA (5µg/mL) or LPS (10µg/mL) to each well. For control, 100µl of medium should be added to each control well. Incubate the plate at 37°C, 5% CO₂ and complete humidity atmosphere for 72 h. Pipet MTT solution into each well of the 96-well assay plate at 4 hours prior to the end of culture period, centrifuge and add 150µl DMSO each well. Record the absorbance at 570nm using a 96-well plate reader immediately after the incubation.

T cells stimulus index(TSI)= (ConA treatment-Background)/(Control-Background) (1)

B cells stimulus index(BSI)= (Lps treatment-Background)/(Control-Background) (2)

2.8 Cellular Immunofluorescence

L1210 cells washed with ice-cold PBS for three times, and then fixed with 3.7% Para formaldehyde for 25 min, permeabilized with 0.1% Triton X-100 for 5 min at room temperature. To block nonspecific antibody binding, samples were incubated with 0.1% BSA in PBS at room temperature for 30 min. Then samples were incubated with serum primary antibody (1:100 in 0.1% BSA) of different groups overnight at 4°C. Cells were incubated with FITC-conjugated goat anti-mouse secondary antibody (1:200 in 0.1% BSA) at 37°C in dark for 2 h on the next day. Finally cells were washed with PBS and examined using a fluorescence microscope (Olympus, C5060-ADU and Tokyo, Japan).

2.9 Western Blot

Cells were lysed by cell lysis buffer to obtain total proteins, adjust concentration of protein by the Pierce Protein Assay (Pierce, Rockford, IL) using BSA as the standard. SDS-PAGE or dimensional electrophoresis applied to separate cellular protein, and then transfer onto nitrocellulose membranes, incubated with mice serum. Tumor-specific antibodies in serum may reactive to specific protein of tumor cells in this experiment. All immunoblots were visualized by ECL.

2.10 Statistical Analysis

Experiments were performed at least three times with similar results. Values are expressed as means±SEM. For comparisons between groups, the SPSS One Way ANOVA analysis of variance was used. The P values less than 0.05 were considered to be statistically significant.

3. Results

3.1 Morphological Observation of Cells Apoptosis

Morphological changes of cells cultured with CPS were evaluated by hematoxylin and eosin (H&E) staining. First, cells were collected at 0 h and 48 h after treatment. They were then stained with H&E by conventional methods and dehydrated through a graded ethanol series to xylene, and dried in air. Some significant morphological changes could be found after CPS treated for 48 h in vitro (Figure 1C). Cell treatments were homogeneous on 0 h and 48 h which were untreated with CPS (Figure 1A and 1B). After 400µg/mL CPS treated

for 48 h, only one normal and integrity cell could be found under observation, other L1210 cells apoptosis with nuclear chromatin concentration, marginalized and cell shrinkage as well as apoptotic bodies observed under microscope (Figure 1C). The results demonstrate that apoptosis may happen in L1210 cells after culturing with 400 μ g/mL CPS for 48 h in vitro.

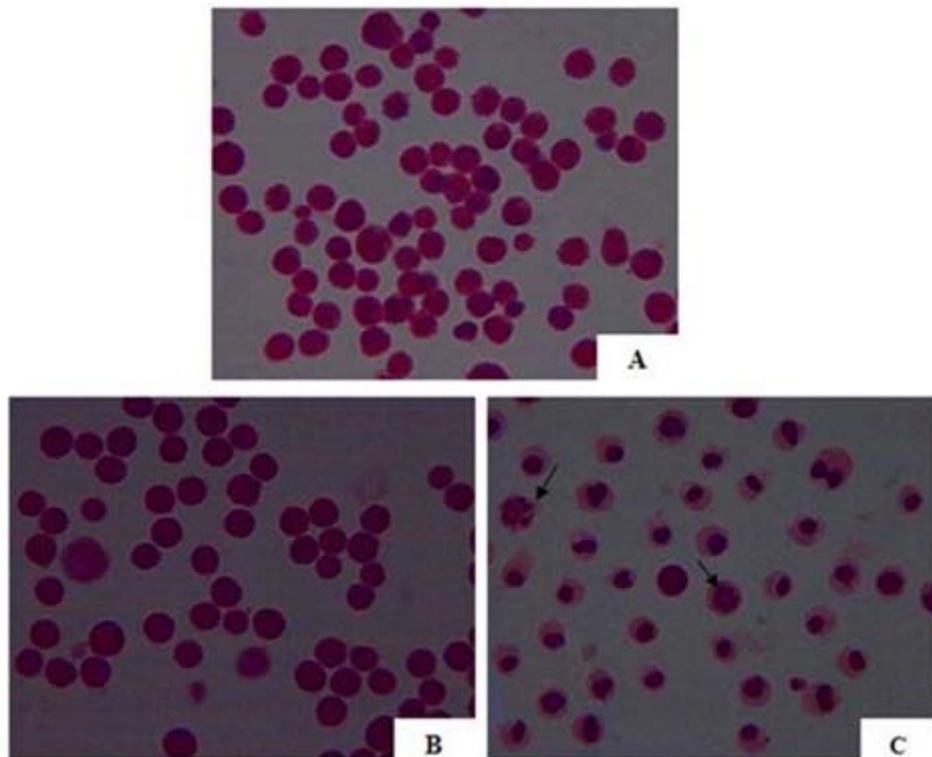


Figure 1. H & E staining of L1210 cells. Cells were treated with or without CPS for 48 h. Untreated cells were used as control. Cells morphology determined by microscopy. (A) Control, 0 h cells ($\times 40$ objective lens) (B) Cells treated without CPS for culturing 48 h ($\times 40$ objective lens) (C) Cells treated with 400 μ g/mL CPS for culturing 48 h ($\times 40$ objective lens)

3.2 Cell Cycle Analysis of Cells Apoptosis

To further investigate the biological effects of CPS on L1210 cells, cell cycle and apoptosis were analyzed. Cells cultured without CPS present a normal cell cycle profile (Figure 2B and Table 1) compared with 0 h cells (Figure 2A and Table 1). Co-cultured with CPS decreased the number of cells in G_0/G_1 phase, made cell cycle blocked to G_2/M phase, as well as significantly ($P < 0.01$) increased the number of cells in sub- G_0/G_1 phase, which were representative of apoptotic cells (Figure 2 and Table 1). These data suggested CPS could induce apoptotic cell death of L1210 cells in vitro.

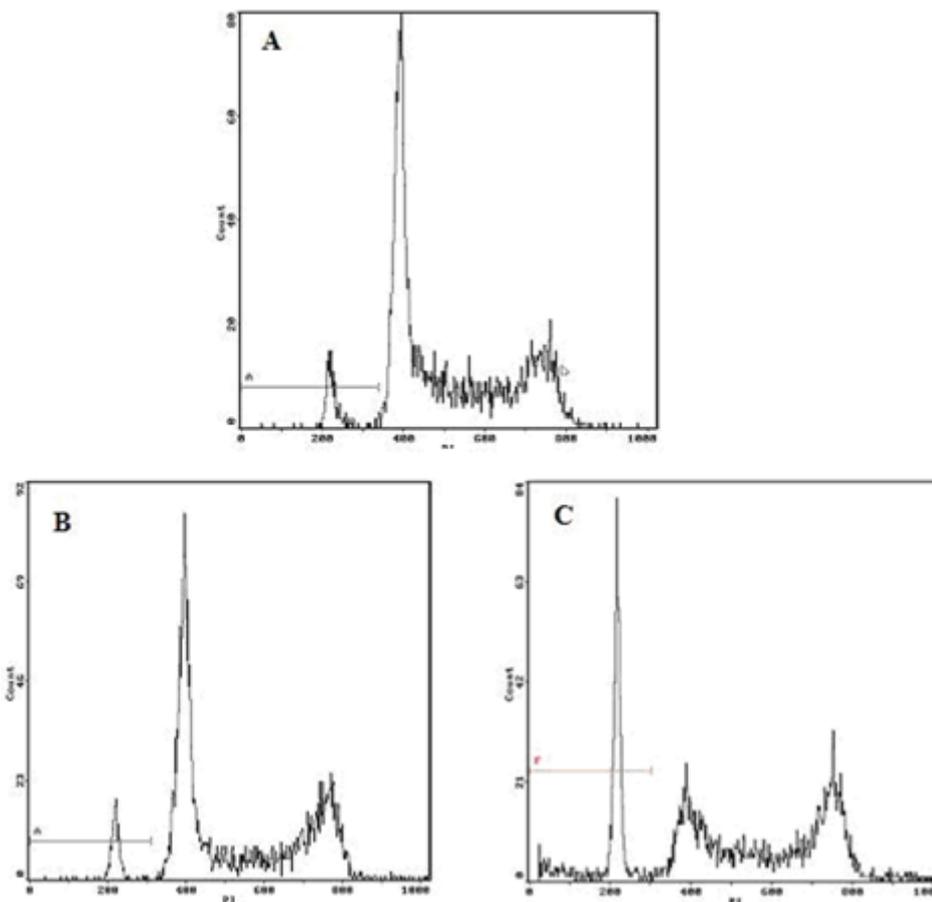


Figure 2. L1210 cells cycle analysis. Cells were collected on 48 h with or without 400 μ g/mL CPS treatment in vitro. Untreated 0 h cells were used as control. (A) 0 h cells (B) 48 h cells cultured without CPS (C) 48 h cells cultured with CPS

Table 1. Cell cycle analysis of L1210 cells with or without CPS.

Time	Sub-G ₀ /G ₁ (%)	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
0 h	6.27 \pm 1.36	38.00 \pm 2.52	43.40 \pm 3.34	18.60 \pm 2.05
48 h (treated without CPS)	6.25 \pm 0.75	45.20 \pm 5.03	32.30 \pm 2.17	22.50 \pm 3.39
48 h (treated with CPS)	23.40 \pm 2.23**	20.30 \pm 1.69**	48.00 \pm 5.17	31.70 \pm 2.46**

** $P < 0.01$ vs. 0 h

L1210 cells were treated with or without 400 μ g CPS/mL for 48 h. Cell cycle analysis was performed by flow cytometry. 0 h cells were used as control

3.3 TUNEL Assay

Many apoptotic cells could be found after 400 μ g/mL CPS co-cultured for 48 h as shown in Figure 3, and the apoptosis rate was (47.39% \pm 1.23%), while the one of 0 h was just (4.25% \pm 0.73%). Cells that cultured without CPS for 48 h had no difference with 0 h cells ($P > 0.05$, Figure 3B and 3C). The results demonstrated CPS could prevent cells survival and induce apoptosis in vitro.

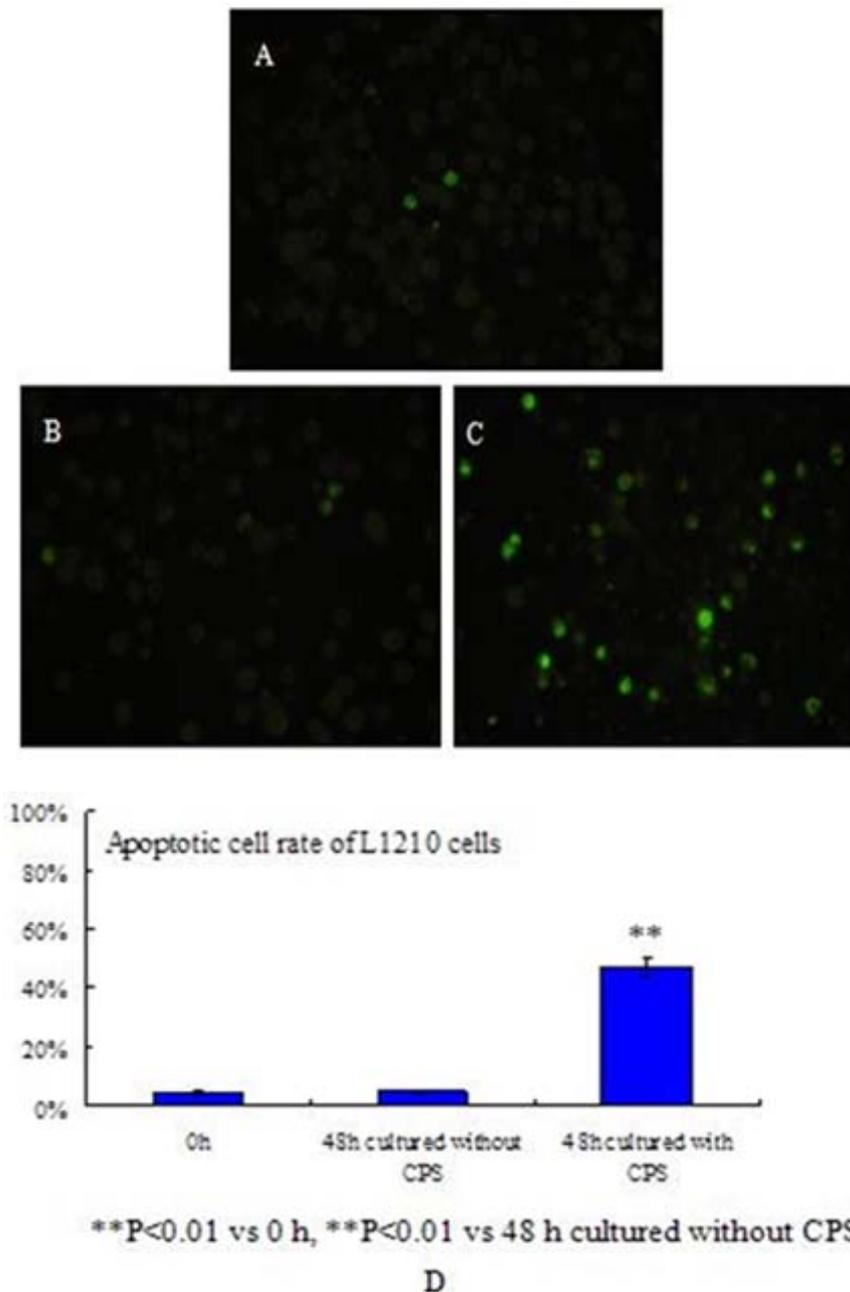
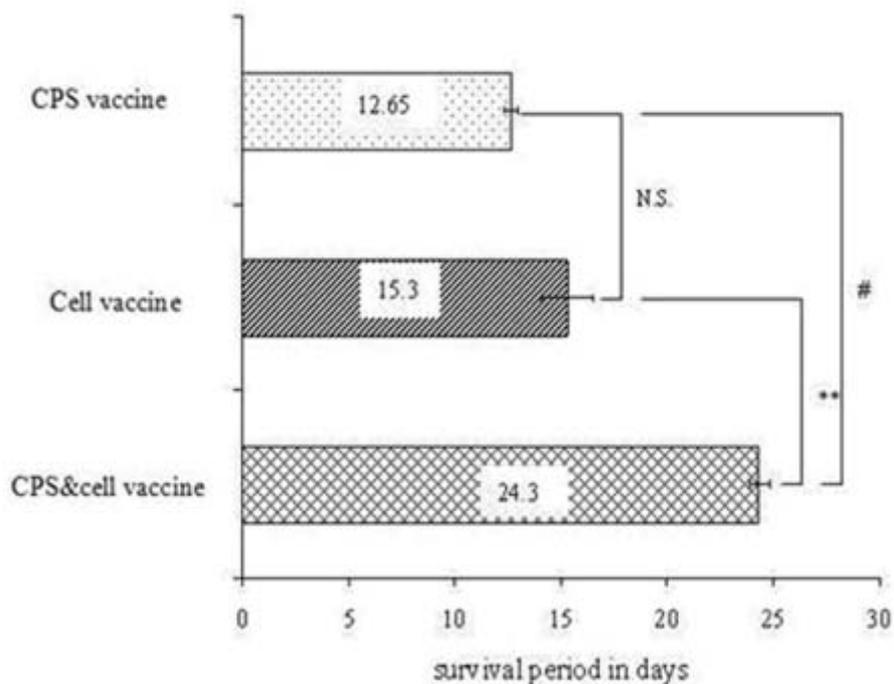


Figure 3. TUNEL staining of L1210 cells. Cells were collected on 0 h, 48 h with or without CPS treatment. Untreated 0 h cells were used as control. (A) 0 h cells ($\times 40$ objective lens) (B) 48 h cells cultured without CPS ($\times 40$ objective lens) (C) 48 h cells cultured with $400\mu\text{g}/\text{mL}$ CPS ($\times 40$ objective lens) (D) Apoptotic cells rate

3.4 Immune Effects of Different Vaccine Groups

The survival time of different immune groups after L1210 cells inoculation in the duration of 30 days were observed and showed in Figure 4. Mice in CPS & cell immune group live longer than other vaccine groups ($P < 0.01$), and the exciting results found that four mice in ten with no proliferation of ascites tumor. It indicated that the anti-tumor effects of DBA/2 mice may induced by apoptotic cells or different post-translational modification in L1210 cells. No mice acquired anti-tumor immunity in other two groups.



#P<0.01 vs CPS vaccine; **P<0.01 vs Cell vaccine; N.S.:no difference.

Figure 4. Survival periods of mice with L1210 ascites tumors of different immune groups. Thirty female DBA/2 mice were randomly divided into three groups, the CPS & cell vaccine group (n=10) received subcutaneous vaccination with freezing-thawing L1210 cells that cultured with 400µg/mL CPS for 48 h. 2.0×10⁵ L1210 cells were inoculated after immunization. The Cell vaccine group (n=10) received subcutaneous vaccination with freezing-thawing L1210 cells that cultured without CPS for 48 h. 2.0×10⁵ L1210 cells were inoculated after immunization. The CPS vaccine group (n=10) received the same dose of culture medium (contain 400µg/mL CPS) that had been freezing-thawed, 2.0×10⁵ L1210 cells were inoculated subcutaneously.

Average survival periods in days of each group = $\bar{a} = \frac{\sum_{i=1}^n \text{every mouse survival days}}{n}$

3.5 Spleen Lymphocyte Proliferation

The cellular protein and DNA synthesis were significantly increased when lymphocyte stimulated by specific antigen or non-specific mitosis original. The ability of spleen lymphocyte proliferation in blank group, Cell vaccine, CPS vaccine and CPS & cell vaccine group were tested in this assay. It verified the stimulated rate of B cells and T cells in CPS&cell vaccine group were better than Cell or CPS alone vaccine group, but worse than blank group (Table 2). It suggested the mixed-culture system of CPS and apoptotic cells as total-tumor cells vaccine could significantly (P<0.01) increased the levels of humoral and cellular immunity.

Table 2. Lymphocyte proliferation of mice in different groups ($\bar{x} \pm s$) (n=10)

group	ConA(SI)	LPS(SI)
Blank	12.36±0.25	9.07±0.48
CPS vaccine	1.79±0.26	2.03±0.17
Cell vaccine	1.84±0.10	1.64±0.03
CPS&cell vaccine	4.89±0.21**	4.32±0.34**

** P<0.01 vs. Cell vaccine group or CPS vaccine group

3.6 Alcine Blue Staining

The alcine blue dye could combine with polysaccharides of cells. Control cells that untreated with CPS with little coloring, only glycoprotein or proteoglycan on cellular membrane were stained (Figure 5A). More and more alcine blue combined with cells when 400 μ g/mL CPS co-cultured for 24 h, 48 h and 72 h (Figure 5B-D). The experiment results indicated CPS could enhance the permeability of membrane, and may bind to substance that have polysaccharide receptors on cellular membrane or in cytoplasm by entering into cells, therefore may assemble to a new specific tumor antigen.

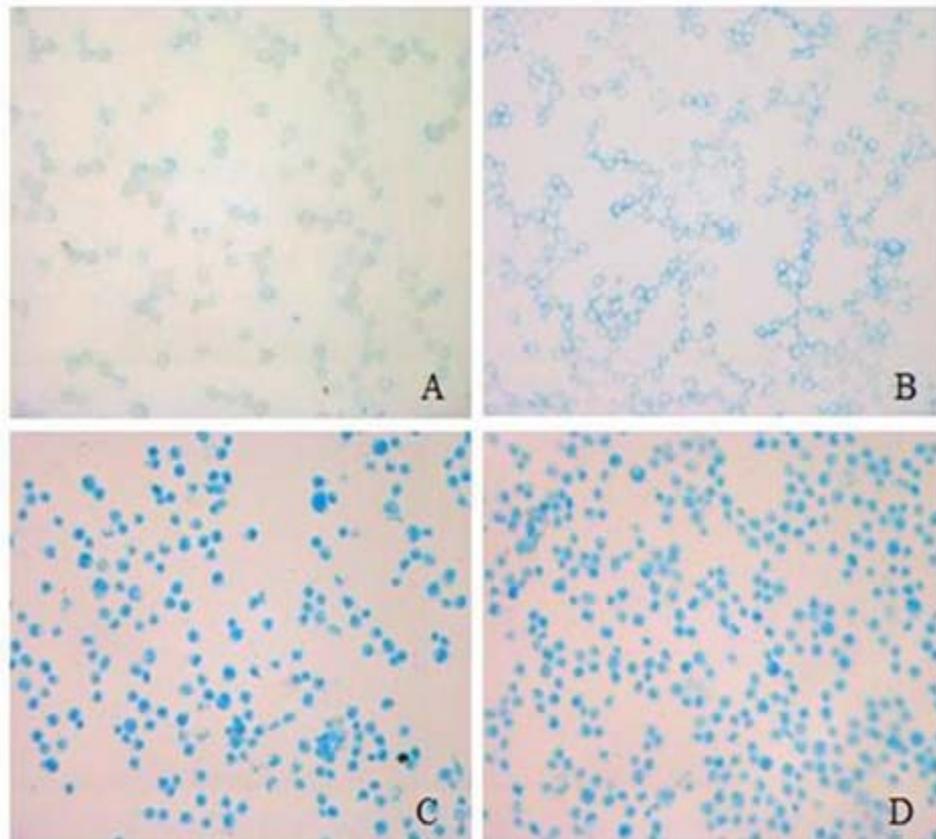


Figure 5. Alcian blue staining of L1210 cells. Cells were treated with or without CPS. Untreated cells were used as control. Cells morphology was determined by microscopy. (A) Control, 0 h cells ($\times 20$ objective lens) (B) Cells treated with 400 μ g/mL CPS for culturing 24 h ($\times 20$ objective lens) (C) Cells treated with 400 μ g/mL CPS for culturing 48 h ($\times 20$ objective lens) (D) Cells treated with 400 μ g/mL CPS for culturing 72 h ($\times 20$ objective lens)

3.7 Antibody Identification by Cellular Immunofluorescence

The specific bonds between antibody and L1210 cells were detected by cellular immunofluorescence. The serum of mice in CPS vaccine group, Cell vaccine group and CPS & cell vaccine group were collected respectively as antibody I. The specific antibody present in serum of successfully immunized mice, it can specifically bind to antigen that exists on tumor cell membrane or in cytoplasm (Figure 6).

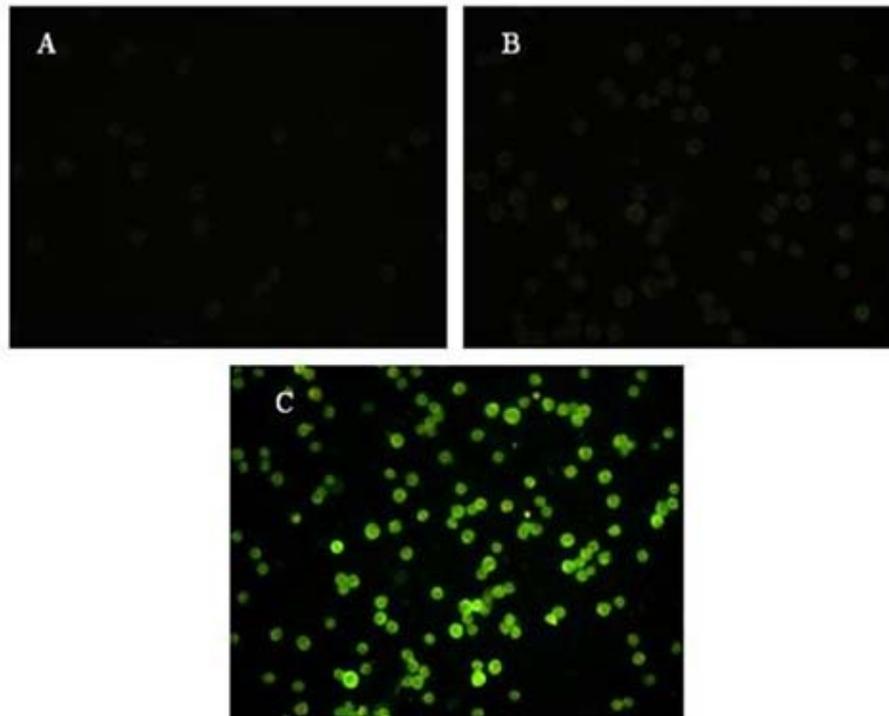


Figure 6. Immunofluorescent staining for antibody test. Normal L1210 cells were made to cell smears, serum of different groups were used as antibody I (A) Serum of mice in CPS vaccine group (B) Serum of mice in Cell vaccine group (C) Serum of successfully immunized mice in CPS & Cell vaccine group

3.8 Antibody Identification by Western Blot

To further investigate the mechanism of anti-tumor immunity, the experiments of SDS-PAGE and Western Blot were applied. Total proteins of L1210 cells were obtained by protein electrophoresis and CBB (Coomassie Brilliant Blue) staining (Figure 7A). The antibody could combine with proteins of about 35kDa in L1210 cells cultured with or without CPS specifically, while higher expressed with cells that treated by CPS (Figure 7C). It indicated the cellular protein may turn into tumor-specific antigen by CPS modified. The same results were obtained by two-dimensional electrophoresis and Western Blot (Figure 8). The protein (PI was 7-8) of about 35kDa in L1210 cells modified by CPS became antigen could stimulate mice anti-tumor immune response.

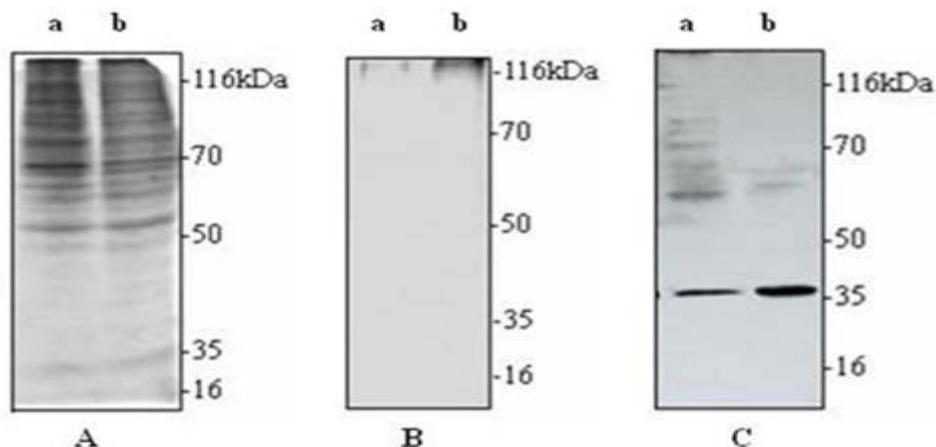


Figure 7. Results of proteins CBB staining and Western blot. (a) L1210 cells that cultured without CPS (b) L1210 cells that cultured with 400 μ g/mL CPS for 48 h.

(A) SDS-PAGE and CBB staining (B) Results of Western Blot that serum of mice in blank group as antibody I (C) Results of Western Blot that serum of mice successfully immunized as antibody I .

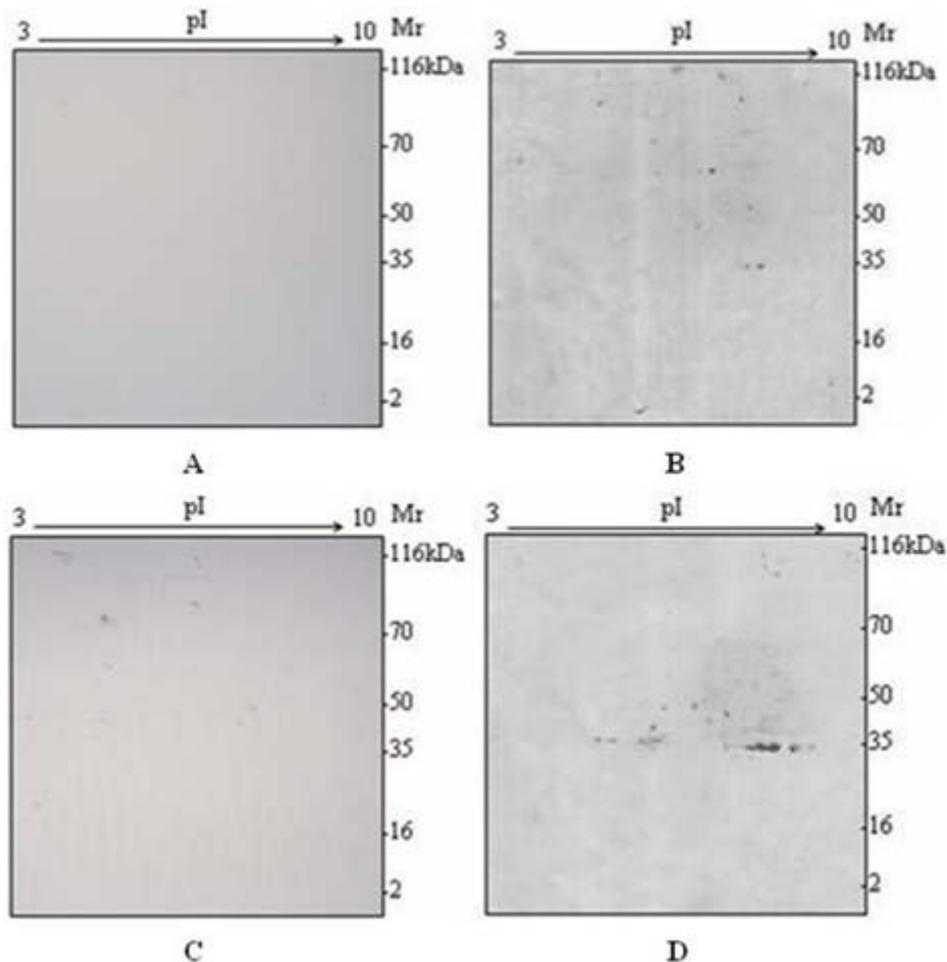


Figure 8. Two-dimensional electrophoresis and Western Blot. (A) L1210 cells that un-treated with CPS, serum of mice in blank group as antibody I (B) L1210 cells that un-treated with CPS, serum of mice successfully immune as antibody I (C) L1210 cells that co-cultured with 400 μ g/mL CPS for 48 h, serum of mice in blank group as antibody I (D) L1210 cells that co-cultured with 400 μ g/mL CPS for 48 h, serum of mice successfully immunized as antibody I .

3.9 Protein Identification

According to the results of Western Blot (Figure 7C and 8D), the specific protein point was cut from two-dimensional electrophoresis gel (Figure 9B) and measured mass spectrometry. Searched the results in NCBI (gi|168983831) and matched with relevant peptide sequence. The result suggested it was a known protein named Annexin A2. Its isoelectric point was 7.55 and molecular weight was 38.65 kDa. Analysis by Image Master Software, the de-regulated expression of Annexin A2 in L1210 cells that cultured with CPS (Figure 9B vs. Figure 9A) and the position of protein point was upward. That indicated Annexin A2 may have different modification after translation and CPS modified Annexin A2 had the potential to be tumor-specific antigen.

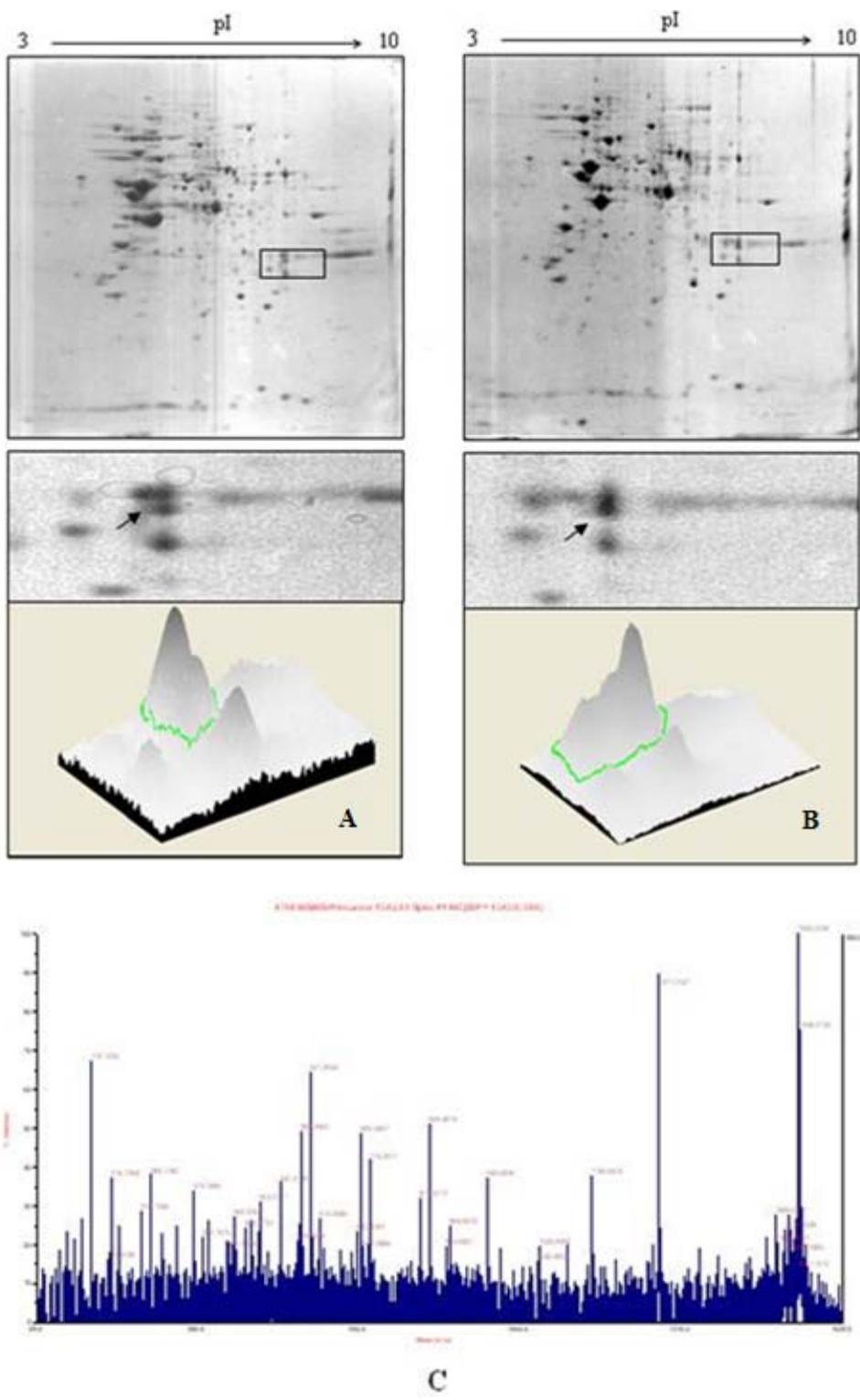


Figure 9. Changes of proteins in L1210 cells testified by two-dimensional electrophoresis. (A) L1210 cells treated without CPS (B) L1210 cells treated with 400µg/mL CPS for 48 h (C) PMF map

4. Discussion

In previous research we found that CPS intraperitoneal injection could inhibit the proliferation of L1210 cells in vivo. The mechanism of its inhibited effects is to induce L1210 cells apoptosis. Subsequently, we also found that cells could not proliferate when cured mice inoculated with L1210 cells again. According to documents (Fadok,

Warner, Bratton & Henson, 1998; Gregory, 2000; Obeid *et al.*, 2007; Casares *et al.*, 2005; Nowak *et al.*, 2003) and our experiment results, we inferred that the anti-tumor immunity activated during the course of apoptosis, so L1210 cells could not proliferate again in ascites of cured mice. The same experimental phenomena also were observed in mice S180 and H22 ascites tumor.

In this paper, we induced L1210 cells apoptosis by CPS co-cultured *in vitro*, and then prepared apoptotic cells lysate as immunogen to vaccine healthy DBA/2 mice. We found four mice in ten with no proliferation of ascites tumor in CPS & cell vaccine group. It indicated that apoptotic cells could induce anti-tumor immune effects of DBA/2 mice. In control-treated groups, cultured cells or CPS alone vaccine, L1210 cells progressively grow and overwhelm DBA/2 mice.

The mechanism of pre-apoptosis and anti-tumor immune was further discussed. L1210 cells of alcine blue staining show that the permeability of cellular membrane enhanced and CPS may bind to substance that had polysaccharide receptors on cellular membrane or in cytoplasm by entering into cells therefore assemble to a new specific tumor antigen (Figure 5). Specific bond of antigen and antibody were observed in the experiments of cellular immunofluorescence and Western Blot (Figure 6-8). Then two-dimensional electrophoresis gel and mass spectrometry were applied to identify that was what protein? The result showed it was a known protein named Annexin A2 (Figure 9), the de-regulated expression of Annexins A2 has been shown by CPS treated.

Annexin A2 exists in cellular membrane, cytoplasm and extra-cellular, could combine with phospholipids, cytoskeleton protein and dependent on Ca^{2+} , so it is classified to A subfamily in Annexins super protein family (Ursula Rescher & Volker Gerke, 2004; Patrick & Harvey, 1994). Annexin A2 participate in a series of cellular activities, such as exocytosis, phagocytosis, cellular proliferation, differentiation and apoptosis (Mussunoor & Murray, 2008). Annexin A2 in particular has been positively associated with malignant progression (Mai, Waisman & Sloane, 2000) and resistance to chemotherapy (Chuthapisith, *et al.*, 2009). Several reports (Mai, Waisman & Sloane, 2000; Chuthapisith *et al.*, 2009; Takano *et al.*, 2008) have shown that up-regulation of Annexin A2 levels is positively associated with cancer progression and chemoresistance, nevertheless the function of Annexin A2 during these processes remained unknown. Accumulating evidence suggest that interactions between Annexin A2 and its binding proteins play an important role in the tumor microenvironment and act together to enhance cancer metastasis. We identified Annexin A2 in L1210 cells was de-regulated by CPS co-cultured. It could be inferred that the CPS could decrease the malignant of L1210 cells, restrain its functions in tumor microenvironment and as immunoadjuvant to modify Annexin A2 furthermore activate mice immune system.

We will extend our work by investigating whether apoptotic tumor cells-derived vaccine could works in an immunotherapy model. Annexin A2 plays an important role in signal pathway transmission in cytoplasm. It has the potential value to be developed to an effective therapy target.

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