

## HEPARIN INHIBITS P388D1 CELLS ADHERENCE AND METASTASIS TO PERIPHERAL LYMPH NODES *IN VITRO* AND *IN VIVO*

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### ABSTRACT

*Heparin is known to attenuate tumor metastasis mainly by inhibiting the interaction between L-selectin and its ligand. However, the mechanism of heparin on lymphoma is unclear. This report demonstrates that both L-selectin/h IgG chimeric protein and heparin treatment can significantly inhibit the adhesion of P388D1 cells onto lymphatic sinusoids and marginal sinusoids in vitro, that heparin can attenuate P388D1 cell homing to lymph nodes in vivo at 12 hours, and that heparin significantly reduced P388D1 cells metastasis to lymph nodes 18 days after injection. These results indicate that heparin may act as a ligand for L-selectin on the P388D1 macrophage-like lymphoma cell line to attenuate tumor growth and metastasis.*

**Keywords:** heparin, P388D1 macrophage-like lymphoma cell line, metastasis, peripheral lymph nodes

Many reports show that carcinoma progression is associated with alterations of cell-surface glycosylation (1-4). Recently, it has been noted that carcinoma cell-surface mucins carrying sialyl Lewis<sup>x/a</sup> can be ligands for L-selectin, a cell adhesion molecule (5-7). Because L-selectin can mediate tumor cell interactions with leukocytes, an *in vivo* role for L-selectin in the metastatic spread of

tumors has been suggested (8,9), in particular L-selectin expression on some tumor cells (10,11). The ligands for L-selectin are also found on vascular endothelium, carcinoma cells and at various extravascular sites. L-selectin is thought to play a role in metastasis. Indeed, in our previous experiments, we found that the expression of L-selectin on the P388D1 cell line is a prerequisite for dissemination into peripheral lymph nodes (PLNs) (12).

Various studies have shown that heparin is not just an anticoagulant, but a complex set of multifunctional glycosaminoglycan molecules with many other potential biological effects (13-15). Several lines of experiments also have demonstrated that tumor metastasis in experimental animals can be inhibited by heparin (16-20), and a few clinical trials also suggest a beneficial effect of heparin in patients with cancer (21,22). Thus, some explanations for the heparin effect on cancer have been suggested including action as ligands for selectins. On the basis of our previous work, we proposed that heparin may have some biological effects on P388D1 cells. Here we test if heparin can attenuate P388D1 cell binding to peripheral lymph nodes *in vitro* and also if it can also reduce their metastatic potential to peripheral lymph nodes *in vivo*. These data may suggest that heparin does indeed act as a ligand for L-selectin in P388D1 macrophage-like

lymphoma cell line to attenuate tumor growth and metastasis.

## MATERIALS AND METHODS

### *Mice, Cell Line, and Reagents*

6-8-week-old DBA/2 mice were obtained from the animal facility of the Dalian Medical University (SPF). The P388D1 murine macrophage-like lymphoma cell line was purchased from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai). Trizol reagent, the RT-PCR kit and the DNA labeling and detection kit were obtained from TaKaRa Biotechnology Co. Ltd, China. Heparin was purchased from Shanghai Institute of Biological Products. Antibodies to murine L-selectin (MEL-14) anti-mouse IgG, PE-conjugated anti-mouse L-selectin (MEL-14) and PE-conjugated anti-mouse IgG used for flow cytometry were provided by BD Biosciences (Institute for Biomedical Research). The vital fluorescent dyes 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) was used for tagged P388D1 cells purchased from Fluka. Antibodies to murine CD147 was purchased from Santa Cruz Co. Other reagents produced in China and elsewhere were analytically pure.

### *Cell Culture Conditions*

The murine macrophage-like lymphoma cells P388D1 were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated newborn bovine serum (NBS), 100 units/ml of penicillin, and 100 g/ml of streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide.

### *Analysis of L-selectin Expression by Flow Cytometry*

To assess surface expression of L-selectin on P388D1 cells, cells were washed three

times with Hanks' balanced salt solution (HBSS) before blocking non-specific sites with 0.5% (v/v) bovine serum albumin (BSA) in HBSS. Then, cells were incubated with a PE-conjugated anti-mouse L-selectin and PE-conjugated anti-mouse IgG for 1h at room temperature (RT), washed with HBSS/BSA and HBSS, resuspended in HBSS/BSA for flow cytometry, and analyzed using a FACS Vantage SE™ flow cytometer (BD Biosciences).

### *Immunofluorescence Assay*

Expression of the adhesion molecule L-selectin was examined by the direct immunofluorescence method using MEL-14 against L-selectin. The cultured P388D1 cells were washed three times with PBS and then blocked with 5% (v/v) normal goat serum for 20 min. Thereafter, cells were incubated in PE-conjugated anti-mouse L-selectin and PE-conjugated anti-mouse IgG (control) at room temperature (1:100) for 60 min and washed thoroughly with PBS. Slides were sealed with 50% (v/v) glycerin in an alkaline buffer. Observation and photography were performed with an Olympus multifunction microscope (Olympus BX51, Japan).

### *Labeling of P388D1 cells with fluorescent dyes CFSE*

The P388D1 cells were labeled with the vital dye CFSE (23). In brief,  $1 \times 10^6$  P388D1 cells in 1ml of PBS were incubated with 5  $\mu$ M CFSE at 37°C for 10 min, then 1 ml of cold PBS was added to stop the reaction. Labeled cells were washed once and counted. After 1 day, the entire culture was harvested, cell viability and recovery were determined, and the labeling of P388D1 cells was analyzed by flow cytometry.

### *Immunohistochemistry*

For immunohistochemical staining with lymphoma marker CD147, slides were

dewaxed, rehydrated and boiled in 0.01 mol/l of citrate buffer (pH 6.0) for 30 min in a microwave oven. After endogenous peroxidase activity had been blocked, slides were incubated with CD147 (1:100) overnight at 4°C. Then the sections were incubated with HRP-conjugated anti-human antibody (1:1000) for 30 min at 37°C. The slides were finally stained with DAB and counterstained with haematoxylin.

#### *Cell Proliferation Assay*

The cytotoxic effect of heparin (50 and 100 and 150 units) and CFSE on P388D1 cells were examined. At designated time-points, numbers of cells were quantified using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The number of cells in each well was counted for a total of 3 days.

#### *In Vitro Adhesion Assay*

P388D1 cells (200  $\mu$ l,  $3 \times 10^5$ ) were incubated with frozen section of DBA/2 mouse lymph node. In this modification adhesion assay, the sections were allowed to stay in static conditions for 15 min at 37°C, followed by 5 min of rotation at 60 rpm, and static for 15 min at 7°C (24). Then adherent cells were fixed in 1% (v/v) glutaraldehyde, sections stained with hematoxylin and eosin, and number of cells adherent to the frozen section of DBA/2 mouse lymph node counted with the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

#### *Homing Assay*

One or 12 hours after CFSE-tagged P388D1 cells ( $1 \times 10^7$ ) with either saline or and heparin (100 units) were injected into the foot pads of recipient mice, mice were sacrificed and their peripheral lymph nodes collected and sectioned (n=6). Flow cytometry and hematoxylin and eosin (HE) staining were used to detect metastatic

P388D1 cells in peripheral lymph nodes to enhance sensitively (25).

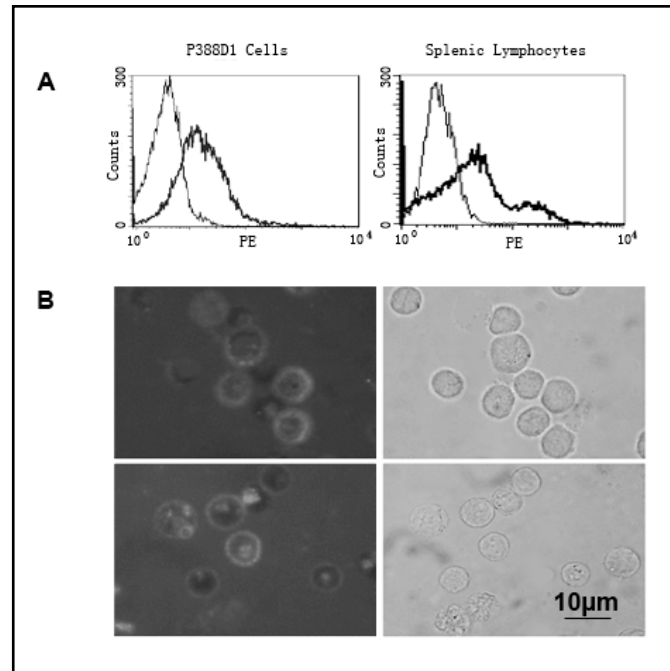
#### *RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)*

Total RNA was isolated from P388D1 cells using the Trizol procedure. Equal amounts of RNA from each sample were added to the 20- $\mu$ l reaction mixture, and cDNA was synthesized by using the RT-PCR kit according to the manufacturer's protocol. PCR was carried out using a PCR thermo-cycler (ThermoHybaid UK & International). PCR primers were synthesized by TaKaRa Biotechnology Co. Ltd, China. CD147 PCR primers to amplify a 326 bp fragment were 5'GAGAGCTTGCGAAACTGGTC3' (forward), and 5' AACCAACACCAGGACCTCAG 3' (reverse). GAPDH primers (486 bp fragment) were 5' GGCCGTGAAGTCGTCAGAAC 3' (forward), 5' GCCACGATGCCCAGGAA 3' (reverse). The thermal cycles were: 94°C for 5 minutes, followed by denaturing at 94°C for 30 seconds, annealing at 64°C for 30 seconds extension at 72°C for 50 seconds and final extension at 72°C for 5 minutes. The PCR products were analyzed on a 1% (w/v) agarose gel. GAPDH was used to standardize the samples with equal cDNA.

#### *Experimental Metastasis in DBA/2 Mice*

P388D1 cells ( $3 \times 10^6$ ) were washed three times, and along with heparin (100 units) or saline injected into the foot pads of DBA/2 mice (n=10), and every 3 days thereafter. The mice were sacrificed and their peripheral lymph nodes were collected and sectioned after 18 days. We used hematoxylin and eosin to detect the metastatic tumor foci. To more precisely identify the metastatic P388D1 cells, we also detected lymphoma marker CD147 on P388D1 cells by RT-PCR and immunohistochemistry.

#### *Statistical Analysis*



*Fig. 1: The P388D1 murine macrophage-like lymphoma cell line exhibits expression of the homing receptor L-selectin. P388D1 cells were analyzed by flow cytometry and immunocytofluorescent assays. A) P388D1 cells and splenic lymphocytes (as control) were incubated with a PE-conjugated anti-mouse L-selectin (MEL-14) for flow cytometric analysis. B) In addition, P388D1 cells (top) and splenic lymphocytes (bottom) were incubated in PE-conjugated MEL-14 for immunocytofluorescent analysis under an Olympus multifunction micro-scope. Photo contrast of area is seen on right.*

All data are expressed as mean  $\pm$  SD. Student's t-test was used for statistical analysis. The chi-square test was used to compare the numbers of mice with metastatic tumors. A p value  $<0.05$  was considered statistically significant.

## RESULTS

### *Growth Rate of P388D1 In Vitro*

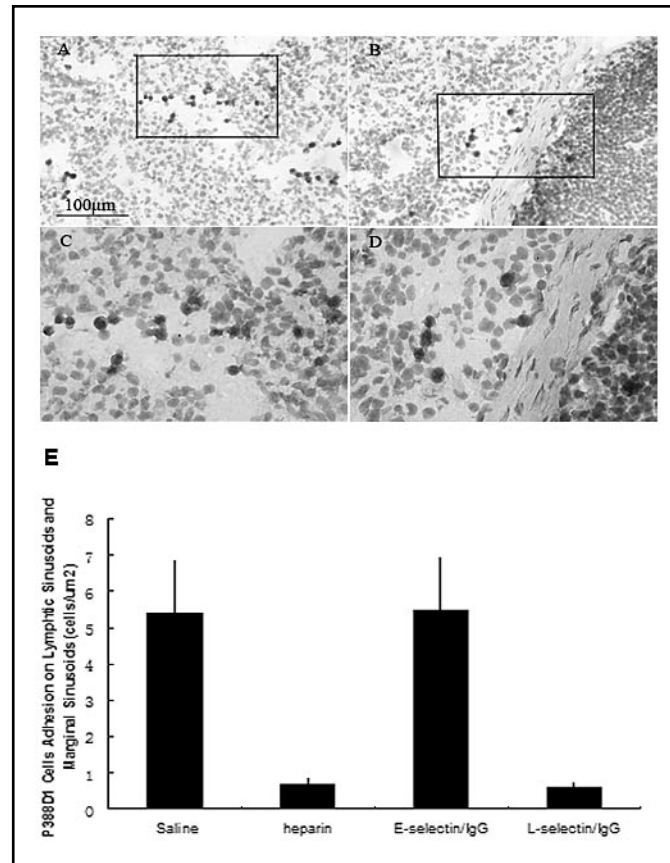
The cytotoxic effects of heparin (50 and 100 and 150 units) and CFSE-tagged P388D1 cells were examined. It was found that heparin and CFSE have no direct impact on the growth of P388D1 cells (12).

### *The P388D1 Murine Lymphoid Neoplasm Cell Line Exhibits Expression of the Homing Receptor L-selectin*

To explore the role of L-selectin in the macrophage-like lymphoma P388D1 cell line, we detected the expression of L-selectin on P388D1 cells. Flow cytometry analysis demonstrated that P388D1 cells expressed L-selectin ( $41 \pm 2\%$  (mean  $\pm$  SD) positive,  $n = 4$ ) (Fig. 1A). The immunofluorescence assay showed that L-selectin was present on the surface of P388D1 cells ( $34 \pm 2\%$  (mean  $\pm$  SD) positive,  $n = 4$ ) (Fig. 1B), showing that the P388D1 cell line exhibited expression of the homing receptor L-selectin. We used expression of L-selectin by C57BL/6 mouse splenic lymphocytes as a control.

### *Heparin and L-selectin/h IgG Chimeric Protein Blocks Adhesion of P388d1 Cells to Lymph Nodes In Vitro*

The adhesion assay with static and rotary conditions demonstrated that P388D1



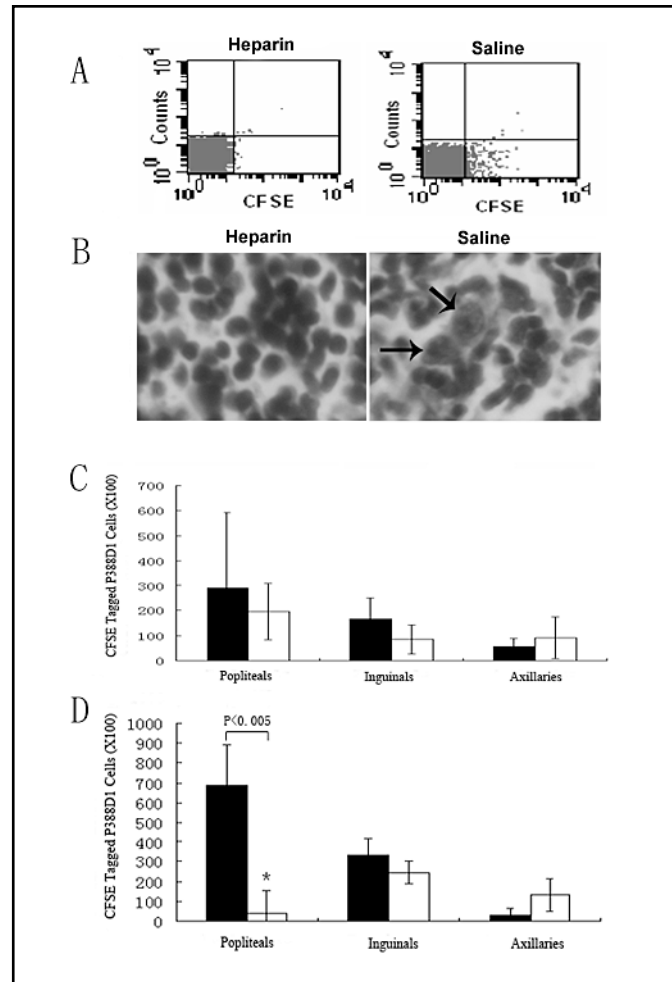
**Fig. 2.** Adhesion of P388D1 cells to lymph nodes is blocked by heparin and L-selectin/h IgG chimeric protein in a modification of the Stamper-Woodruff assay. P388D1 cells were shown to bind specifically to endothelium of lymphatic sinusoids (A,C) or to marginal sinusoid (B,D). Boxed areas in A and B are enlarged in C and D, respectively. The results of four independent assays show heparin and L-selectin/h IgG chimeric protein significantly inhibits binding of P388D1 to lymphatic sinusoids and marginal sinusoid (E). (Values represent mean $\pm$ SD,  $p < 0.001$ ,  $p < 0.001$ ).

cells easily adhered to the lymphatic sinusoids (Fig. 2A,2C) and marginal sinusoid (Fig. 2B,2D). To determine whether heparin is involved in recognition of the position of lymphatic sinusoids and marginal sinusoid, we tested the blocking effects of heparin and L-selectin/h IgG chimeric protein on sections of lymph nodes. The cells adhering to lymphatic sinusoids and marginal sinusoid were determined with the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). The data indicate that the adhesion to lymphatic sinusoids and marginal sinusoid can be blocked by heparin and

L-selectin/h IgG chimeric protein (Fig. 2E) ( $p < 0.001$  for both).

#### Homing Assay Results

We found a significant reduction in CFSE-tagged P388D1 cells in lymph nodes by flow cytometry and hematoxylin and eosin (H&E) staining after 12 hours in heparin-treated mice (compared to saline controls) (Fig. 3D) ( $p < 0.005$ ). Although no significant difference was seen at 1 hr (Fig. 3C), cells formed significant metastatic colonies in the lymph nodes after 12 hours (Fig. 3D).



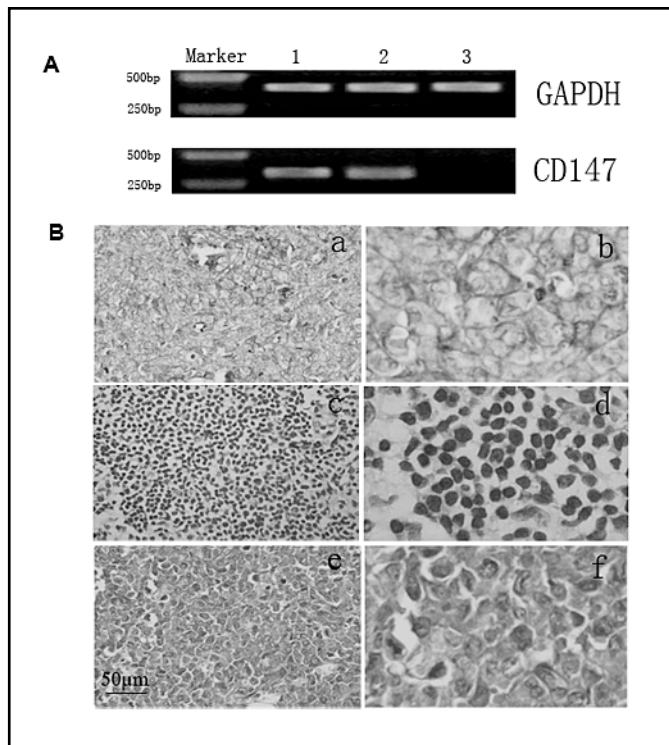
**Fig. 3. Homing assays.** Typical representation of CFSE+ P388D1 cells dissemination to lymph node by flow cytometry analysis (A) and H&E (B) staining (arrow indicates the metastasis P388D1 cells) ( $\times 400$ ). C and D represent the results of heparin inhibited CFSE+ P388D1 cells dissemination to lymph node at 1 hour and 12 hours, respectively. The metastasis of heparin-treatment group to popliteal nodes was significantly lower than control group after 12 hours (■ saline treatment group; □ heparin treatment group).

#### *Heparin Inhibits P388D1 Dissemination into Lymph Node In Vivo*

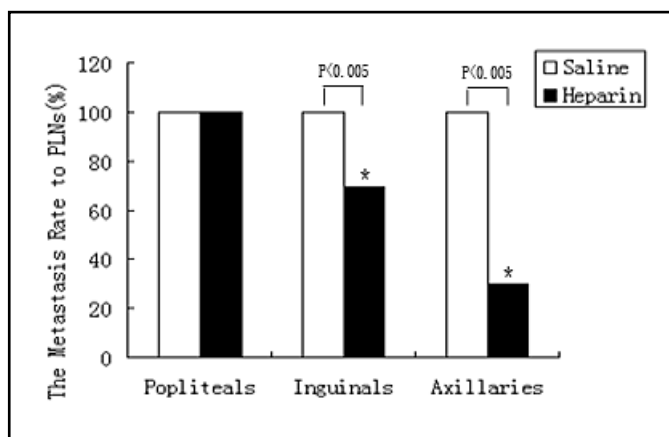
RT-PCR demonstrated mRNA of lymphoma marker CD147 in P388D1 cells and lymph nodes metastasized by P388D1 cells, but not in normal lymph nodes (Fig. 4A). Immunohistochemistry displayed expression of CD147 in lymph nodes with metastasized P388D1 cells (Figs. 4Ba,4Bb), but not in normal lymph nodes (Figs.

4Bc,4Bd) or in saline-treated controls (Figs. 4Be,4Bf).

Analysis of metastasis rates (Fig. 5) showed that the heparin-treated groups have significantly altered growth rate of P388D1 lymphoma with reduced frequency of tumor development (inguinal: 7/10,  $p < 0.005$ ; axillary: 3/10,  $p < 0.005$ ), compared to saline treated group (inguinal: 10/10; axillary: 10/10).



**Fig. 4.** Heparin inhibits dissemination of P388D1 cells in vivo. (A) RT-PCR detection showed that P388D1 cells expressed CD147 (lane 1, P388D1 cells; lane 2, lymph node metastasized with P388D1 cells; lane 3, normal lymph node). GAPDH was used as a loading control. (B) Results of immunohistochemical staining with lymphoma marker CD147. Positive staining was demonstrated for lymph nodes metastasized with P388D1 cells at low (a) and high (b) power magnification. Both normal mouse lymph nodes at low (c) and high (d) power magnification and control (saline injections) lymph nodes at low (e) and high (f) power magnification lack positive staining.



**Fig. 5.** Lymph node dissemination of P388D1 cells is blocked by heparin in vivo. Analysis of the metastasis rate of dissemination of P388D1 cells to lymph nodes demonstrate significantly lower metastasis in the heparin-treatment group to inguinal and axillary nodes than in the control groups ( $p < 0.005$ ;  $p < 0.005$ ).

## DISCUSSION

P388D1 cells represent a macrophage-like lymphoid neoplastic cell line. In our present work, we have explored heparin inhibition of P388D1 cell adhesion and dissemination to peripheral lymph node *in vitro* and *in vivo*. We demonstrated that heparin can inhibit P388D1 cell binding to peripheral lymph nodes *in vitro* and reduce metastasis potential to peripheral lymph nodes of P388D1 cells *in vivo* as shown by homing assay and experiment metastasis assays.

In experimental studies, heparin's affect on progression and metastasis of cancer is complex. It has been shown to inhibit thrombin and fibrin formation induced by cancer cells due to their anticoagulant function (26). In addition, heparins bind to growth factors and ECM proteins and also affect proliferation and migration as well as angiogenesis in tumors (27,28). Furthermore, heparins have been found to inhibit expression of oncogenes, such as *c-fos* and *c-myc* (29,30). Heparins also can interfere with the binding of selectins to their ligands, and heparin is a potent inhibitor of selectin-mediated interaction (31,32). In this study, we have found that heparin inhibits P388D1 cells adherence and metastasis to peripheral lymph node *in vitro* and *in vivo* and propose that this effect may be due to inhibition of the binding of L-selectin to their ligands.

## ACKNOWLEDGMENTS

Wei Wei and Yunfei Zuo contributed equally to this work. We thank Pin Yan and Yuejian Liu for their help with the immunofluorescence assay and analysis by flow cytometry, respectively. This work was supported by a grant from National Natural Science Foundation of China (30470400 and 30870550).

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