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Original Article

Impacts of K562 cells towards activities of Toll-like receptor pathway of human mesenchymal stem cell-bone marrow

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Abstract: The study aim was to investigate the impacts of K562 cells towards the activities of Toll-like receptor pathway of human mesenchymal stem cell-bone marrow (HMSC-bm). The in vitro co-culture of HMSC-bm and K562 cells was set as the experiment group (HMSC-bm + K562), the HMSC-bm cultured alone was set as the control group (HMSC-bm), the expressions of six interested genes and their proteins, namely MyD88, P38, NF-κB, TAB1, TLR3 and TBK1, of the Toll -like receptor signaling pathway were detected and compared, as well as the secretions of such cytokines as IL-6, IL-8, TNF-α and IFN-α in the cell supernatant, which were regulated by the Toll-like receptor pathway. The expressions of MyD88, P38, TAB1 and TLR3 of the HMSC-bm + K562 group were higher than the HMSC-bm group, while that of TBK1 was lower, and the NF-κB expression showed no significant difference between the two groups ($P > 0.05$). Compared with the HMSC-bm group, the supernatant of HMSC-bm + K562 group exhibited the higher secretion levels of IL-6 and IL-8, while that of IFN-α was just contrary, and the differences were significant ($P < 0.05$). The secretion levels of TNF-α within the two groups were not significantly different ($P > 0.05$). The co-culture of K562 and HMSC-bm could induce the activity changes of Toll-like receptor pathway of HMSC-bm, which was beneficial towards the proliferation of K562 cells.

Keywords: Co-culture, HMSC-bm, K562, Toll-like receptor signaling pathway

Introduction

The Toll-like receptor signaling pathway was generally considered to be able to participate the immune defenses, which included the tumor immunity. Generally, the immunization was the self-protection methods of the host, while when the body suffered from a solid tumor, the two immune mechanisms, namely the anti-tumor mechanism and the pro-tumor mechanism, were simultaneously present in the tumor microenvironment [1]. And in the tumor microenvironment, the Toll-like receptor pathway was activated, thus playing a role of promoting the tumor growth through multiple mechanisms [2-7].

Different from solid tumors, the cell proliferation of hematologic neoplasm was affected by a sophisticated and complex regulatory system- the hematopoietic microenvironment of bone marrow. The stromal cells inside the hematopoietic microenvironment might produce the soluble cytokine, thus mediating the proliferation and differentiation of hematopoietic stem/progenitor cells, as well as playing a support role towards the hematopoiesis, meanwhile, these cytokines also had the regulatory effects towards tumor cells [8-11].

The bone marrow mesenchymal stem cells (BMMSCs) were the precursor cells of fibroblasts, osteoblasts, adipocytes, endothelial cells, muscle cells and others in the hematopoietic microenvironment, and could directly differentiate into the above cells under certain conditions [12], thus participating the hematopoietic regulation [13]. It was reported [14, 15] that the co-culture with normal BMMSCs could inhibit the growth of K562 and U937 cells. However, no matter the chronic myeloid leukemia or lymphoma, the growth of tumor cells would not be inhibited by the existence of BMMSCs inside the bone marrow microenvironment. So, it could

Table 1. List of primers used in RT-PCR

Gene	Primer sequences	Product size	Genbank registration No.
β-actin	5' atcatgtttgagaccttcaaca 3'	318 bp	BC002409
	5' catctcttgctcgaagtcca 3'		
NF-κB	5' CGGCTAACTTTTGTATTTT 3'	174 bp	NM_001001349.2
	5' GGGATGTAGTTTTCTGTTT 3'		
TAB1	5' GCTGGGGGACTTGTTTG 3'	345 bp	U49928.1
	5' GACTGGGAGATGGGGAG 3'		
TBK1	5' TGGCCAAAGGAAAAAAATGA 3'	187 bp	NM_013254.3
	5' GAGCAGAACCGCACCACT 3'		
TLR3	5' ACTGATGCTCCGAAGGG 3'	205 bp	NM_003265.2
	5' ACCAGGGTTTGCGTGTT 3'		
MYD88	5' ACACCGCCCATTCCACTTC 3'	331 bp	U70451.1
	5' GCTCCCTGCTCACATCATT 3'		
P38	5' CCGTTTGATGATTCCTT 3'	241 bp	AF015256.1
	5' CTTCTGTCCTCTGCTTG 3'		

be presumed that during the interactions of tumor cells and bone marrow microenvironment, certain changes that would benefit the tumor cell proliferation was induced.

We in vitro co-cultured HMSC-bm and K562 cells. As well as setting the K562 cells cultured alone as the control group 1, and the 10000 u/ml recombinant human interferon α2b -treated K562 cells as the control group 2. It was found that after cultured for 72 h, the K562 cells of the 3 groups grew slowly from 0 to 24 h, while the continued culture exhibited that the proliferation rate of K562 cells was gradually accelerated. This suggested that HMSC-bm impacted the growth of K562 cells. We set the in vitro co-culture of HMSC-bm and K562 cells as the experiment group (HMSC-bm + K562), and HMSC-bm cultured alone as the control group (HMSC-bm), and detected the expressions of six interested genes and their proteins, namely MyD88, P38, NF-κB, TAB1, TLR3 and TBK1 of the Toll-like receptor signaling pathway, as well as the secretion levels of IL-6, IL-8, TNF-α and IFN-α in the supernatants of the 2 groups, aiming to preliminarily study the impacts of K562 cells towards the Toll-like receptor pathway of HMSC-bm.

Materials and methods

Cell and cell culture

The K562 cells were provided by the key Laboratory of Hematology, Hebei Province. HMSC-bm was purchased from Sciencell Co., USA.

The recovered K562 cells were added into the RPMI1640 medium, which contained 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin, and cultured at 37°C and 5% CO_2. The culture medium was replaced once 24 hr after the cells were recovered, after that, the medium was changed once every three days, when the K562 cells covered 80 to 90% area, the passage could be performed.

Culture of HMSC-bm: The disposable plastic flask was used, and the high glucose DMEM medium, which contained 100 U/ml penicillin, 100 ug/ml streptomycin and 10% FBS, was used as the complete medium, the recovered HMSC-bm was cultured at 37°C and 5% CO_2, with half medium changed 24 hr later, after that, the medium was totally changed once every 3 days, the inverted microscope was used to observe the situations of cell growth. 7 to 8 days later, when HMSC-bm appeared 80~90% wall-adherence and fusion, the passage could be performed.

Co-culture of K562 cells and HMSC-bm: 1 ml HMSC-bm was seeded in 96-well plates, with the density as 1 × 105/ml, and incubated at 37°C and 5% CO_2 until its wall-adherent degree reached 80% to 90%. 1 ml K562 cells in the logarithmic growth phase were then added onto the HMSC-bm cell layer, with the same density (1 × 105/ml).

RT-PCR analysis

RT-PCR was used to determine the expression of MyD88, P38, NF-κB, TAB1, TLR3 and TBK1. Total RNA was extracted using Trigol reagent (Invitrogen, USA) according to the manufacturer's instructions. The primers used are listed in **Table 1**. The conditions for PCR were as follows: denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec (β-actin), or 55°C for 30 sec (MyD88 and TAB1), or 50°C for 30 sec (P38 and TBK1), or 53°C for 30 sec (NF-κB), or 51°C for 30 sec (TLR3) and extension at 72°C for 30 sec, 35 cycles, and a final 10 min at 72°C. PCR products were visual-

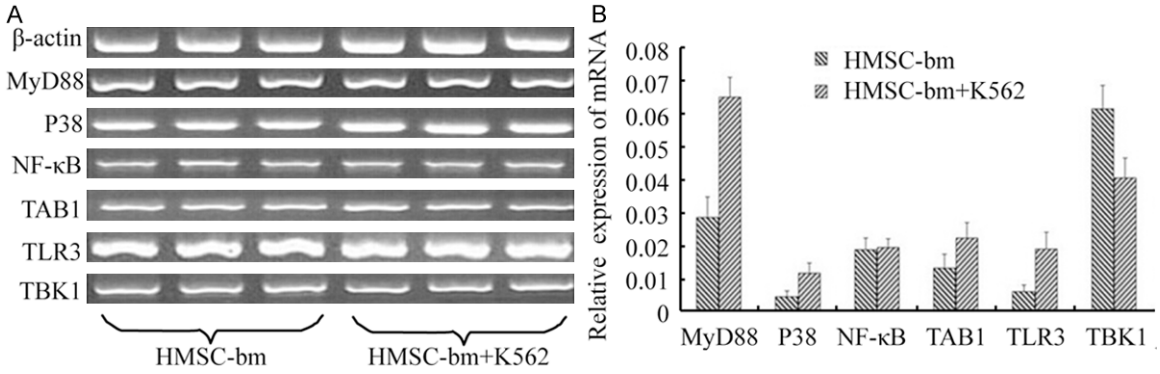

Figure 1. Results of RT-PCR assay of the 2 groups. A. The mRNA expressions of MyD88, p38, NF-κB, TAB1, TLR3 and TBK1 in the experimental group and the control group were determined by RT-PCR. B. Relative mRNA expression among MyD88, p38, NF-κB, TAB1, TLR3 and TBK1 in the experimental group and the control group.

ized by gel electrophoresis on 1.5% agarose (w/v) gel, and then viewed using an ultraviolet photometry (UVP) bioimaging system.

Western blot analysis

Total protein was extracted and quantified according to the manufacturer's protocol. Each equal amount of protein was loaded on sodium dodecyl sulfate-polyacrylamide gel at 100 V for 2 h, and then the protein was transferred to polyvinylidene fluoride (PVDF)-membranes. The membranes were blocked in 5% fat-free milk at room temperature for 2 h, and the blots were stained with specific primary antibodies, including Anti-MyD88 (abcam, Cambridge, UK), anti-p38a(BD, USA), anti-NF-κB (abcam, Cambridge, UK), anti-TAB1 (abcam, Cambridge, UK), Anti-TLR3 (abcam, Cambridge, UK), Anti-TBK1 (abcam, Cambridge, UK), and anti-GAPDH antibodies (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were then washed and incubated with goat anti-rabbit HRP-IgG (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, and observed with a chemiluminescent substrate. Bound immunoglobulins were removed from the membranes by washing twice with Restore™ Western Blot Stripping Buffer, and the signal was visualized by enhanced chemiluminescence and detected using the ChemiDoc XRS + system (Bio-Rad, Hercules, CA, USA); the signal was analyzed by the Image Lab (ECL). GAPDH was used as an internal control.

Enzyme linked immunosorbent assay (ELISA)

The cell supernatant was collected, in which the contents of IL-6, IL-8, TNF-α and IFN-α were detected with the human tumor necrosis factor α (TNF-α) ELISA KIT (Wuhan cusabio, Wuhan, China), human hypersensitive interleukin-6 (hs-IL6) ELISA KIT (Wuhan cusabio, Wuhan, China), human interleukin 8 (IL-8/CXCL8) ELISA KIT (Wuhan cusabio, Wuhan, China) and human α-interferon (IFN-α) ELISA KIT (Wuhan cusabio, Wuhan, China).

Statistical analysis

Data are presented as means ± standard deviation (SD). All the statistical analyses were performed using SPSS 19.0 software. The statistical analysis of results was carried out using Student's t-test and one-way ANOVA analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RT-PCR

The primers' information was shown in **Table 1**. As shown in **Figure 1**, the gene mRNA expression levels of MyD88, P38, TAB1 and TLR3 of the experiment group were higher than the control group, and the differences were significantly ($P < 0.05$), while that of TBK1 gene mRNA of the experiment group was significantly lower than the control group ($P < 0.05$); the expressions of gene mRNA NF-κB between the two groups showed no significant difference ($P > 0.05$).

Western blot

As shown in **Figure 2**, the protein expression levels of MyD88, P38, TAB1 and TLR3 of the

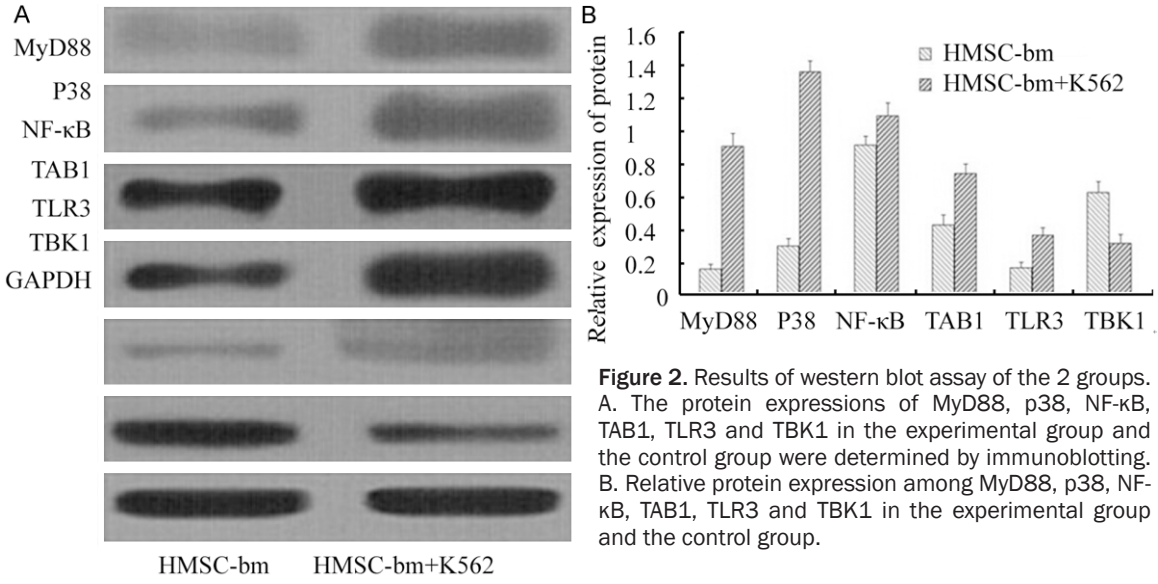

Figure 2. Results of western blot assay of the 2 groups. A. The protein expressions of MyD88, p38, NF-κB, TAB1, TLR3 and TBK1 in the experimental group and the control group were determined by immunoblotting. B. Relative protein expression among MyD88, p38, NF-κB, TAB1, TLR3 and TBK1 in the experimental group and the control group.

experiment group were higher than the control group, while that of TBK1 protein was lower than the control group, and that of NF-κB protein was not significantly different, these trends were consistent with the results of gene assay.

ELISA

As shown in **Figure 3** and **Table 2**, compared with the control group, the supernatant of the experiment group secreted higher levels of IL-6 and IL-8, while that of IFN-α was contrary, and the differences were significant ($P < 0.05$). The secretion levels of TNF-α between the two groups were not significantly different ($P > 0.05$).

Discussion

According to whether the Toll-like receptors (TLRs) could interact with the adapter protein MyD88, the Toll-like signaling pathways could be divided into two categories: classic MyD88-dependent pathway and non-MyD88-dependent pathway [16]. In the classic MyD88-dependent pathway, the activated MyD88 interacted with IRAK4, formed the TLR- MyD88-IRAK receptor complex, thereby promoting the IRAK4-mediated IRAK1 phosphorylation. After the phosphorylation, IRAK1 would bind the TNF-receptor-associated factor 6 (TRAF6), activated the latter and mediated two signaling pathways: one was to induce the activation of MAPK, thereby activating the transcription factor of protein-1, so it was called as the TLR-MyD88/IRAK- mitogen-activated protein kinase (MAPK) pathway; the other one was the TLR-MyD88/IRAK-NF-κB inductive kinase/NF-κB pathway, these two pathways both might ultimately mediate the production and releasing of a large number of pro proinflammatory cytokines and chemokines such as IL-6, IL-8, IL-12 and TNF-α, etc. besides TLR3, other TLRs could mediate the activation of Toll-like receptor pathway through this pathway [17, 18]. While TLR3 could combine with TRIF, thus activating the non-MyD88-dependent pathway, and its ultimate biological effect was to promote the expression of interferon -α/β [2]. In addition, TLR4 could also activate the non-MyD88-independent pathway with the collaboration of TRAM [19].

It was reported that [20], the serum IL-6 level in the CML patients were higher than the healthy persons, Corrado et al [21] studied and found that the CML cells released and stimulated the bone marrow stromal cells to produce IL-8, thus regulating the malignant phenotypes of leukemia cells in vivo and in vitro. The study described that IL-6 and IL-8 might have much more important significance in the pathogenesis of CML. Our experimental results showed that after co-cultured with the K562 cells, the gene expression levels of MyD88, P38 and TAB1 in the classic MyD88-dependent pathway of HMSC-bm were higher than the control group, the activation of these genes would inevitably mediate

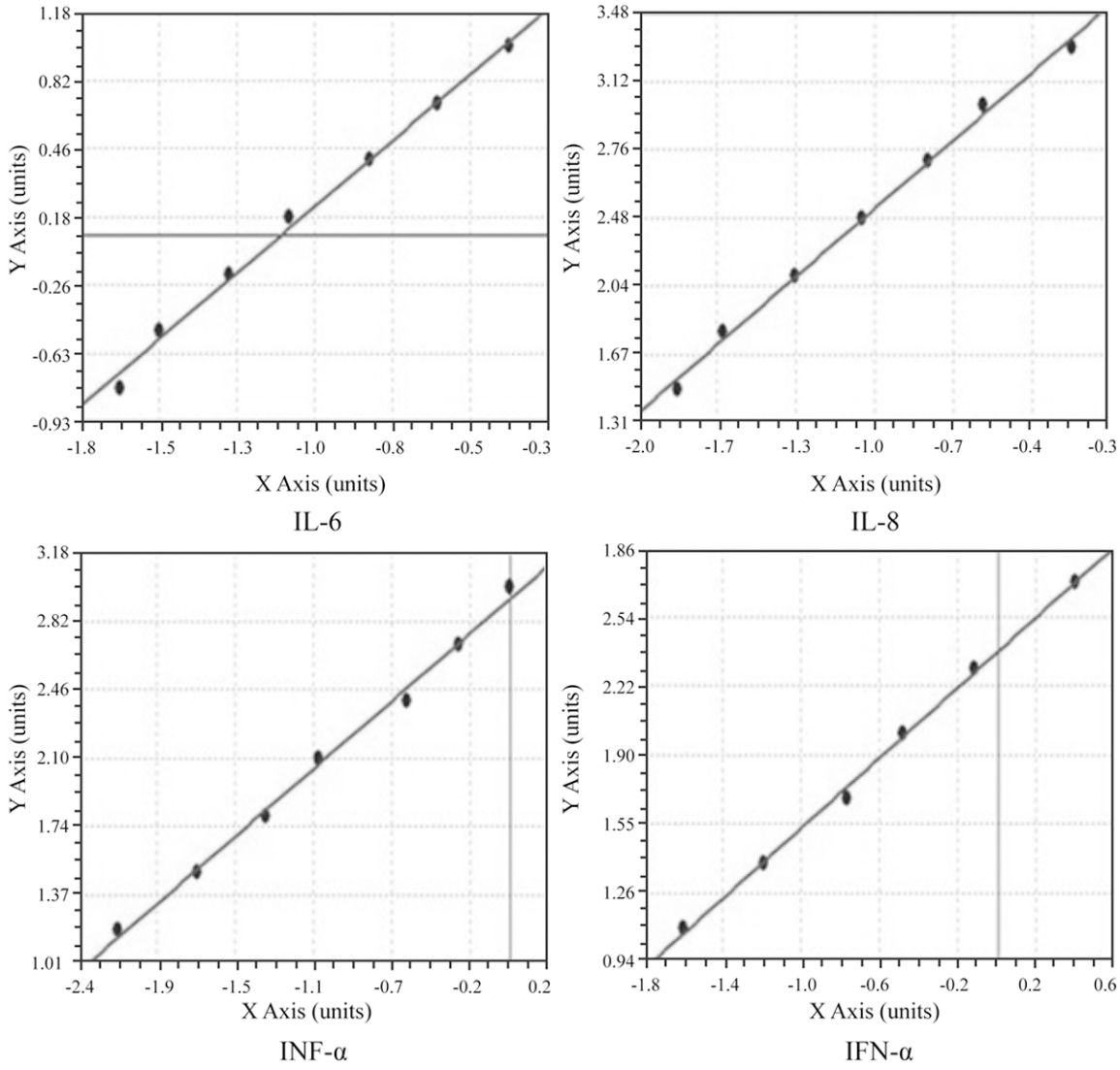

Figure 3. Standard curve of ELISA (IL-6, IL-8, TNF-α and IFN-α).

Table 2. ELISA results of IL-6, IL-8, TNF-α and IFN-α in the cell supernate

	HMSC-bm + K562 co-culture	HMSC-bm single culture
IL-6	25.21±0.30*	14.62±0.92
IL-8	6022.64±134.71**	127.26±4.49
TNF-α	36.33±7.03#	44.17±3.09
IFN-α	40.11±0.61##	53.66±4.29

Note: *compared with HMSC-bm single culture group $P < 0.05$, **compared with HMSC-bm single culture group $P < 0.05$, #compared with HMSC-bm single culture group $P < 0.05$, ##compared with HMSC-bm single culture group $P > 0.05$.

the production and release of such downstream cytokines as IL-6 and IL-8, and the ELISA results confirmed that the levels of IL-6 and IL-8 within HMSC-bm when co-cultured with the K562 cells were higher than those when cultured alone, and the differences were significant ($P < 0.05$).

The activation of NF-κB could promote the secretion of TNF-α, and TNF-α could also promote the expression of NF-κB [22] by a positive feedback mechanism. In this study, the expressions of NF-κB within the two groups had no significant difference ($P > 0.05$), and the levels of TNF-α secreted in the supernatants of the 2 groups had no significant difference ($P > 0.05$), prompted us that the TLR- MyD88/IRAK-NF-κB pathway might not participate the interactions between the K562 cells and HMSC-bm.

Under the normal circumstances, the activated TLR3 could mediate the apoptosis of cells through the non-MyD88-dependent pathway [23-26]. In our study found that the TLR3 expression levels of the experiment group was higher than the control group, while the expression of its downstream TBK1 was lower than the control group, thus the IFN-α secretion level detected in the cell supernatants of the experiment group was lower than the control group. We hypothesized that the co-culture with the K562 cells could stimulate HMSC-bm to express the higher levels of TLR3, but certain mechanism reduced the activities of TLR3 downstream pathway, therefore, the release of IFN-α was downregulated.

In summary, whether co-cultured with the K562 cells was an important factor to affect the activities of Toll-like receptor signaling pathway of HMSC-bm. The classical MyD88-dependent pathway activated and upregulated the release of IL-6 and IL-8, which might have a role of promoting the occurrence and development of CML; the inhibition of non-MyD88-dependent pathway would downregulate the expression of IFN-α, which would inevitably weaken the inhibition of HMSC-bm towards theβ-catenin expression by the K562 cells. It was shown that [23], after the sequence-specific siRNA downregulated the expression of β-catenin inside the K562 cells, the subcutaeous implantation of these K562 cells into the BALB/c nude mice revealed that the tumor survival rate of this group was significantly lower than the control group (implanted the K562 cells that were transfected with unrelated sequence plasmid DNA) and the non-treatment group (implanted the K562 cells that were transfected with non-plasmid DNA), indicating that the downregulation of β-catenin could inhibit the tumorigenicity of CML, and weakening the β-catenin inhibition meant to promote the progress of CML.

Thus, it could be seen that the co-culture process of K562 and HMSC-bm could induce the activity changes of Toll-like receptor pathway of HMSC-bm, and these changes were beneficial to the proliferation of K562 cells, it could be inferred that the CML cells had the same induction in vivo towards BMMSCs, thus resulting in the tumor microenvironment that was beneficial to the occurrence and development of CML.

Disclosure of conflict of interst

None.

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References

[1] Bhardwaj N. Harnessing the immune system to treat cancer. J Clin Invest 2007; 117: 1130-1136.

[2] Akira S, Uematsu S and Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124: 783-801.

[3] Alvero AB, O'Malley D, Brown D, Kelly G, Garg M, Chen W, Rutherford T and Mor G. Molecular mechanism of phenoxodiol-induced apoptosis in ovarian carcinoma cells. Cancer 2006; 106: 599-608.

[4] Kelly MG, Alvero AB, Chen R, Silasi DA, Abrahams VM, Chan S, Visintin I, Rutherford T and Mor G. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res 2006; 66: 3859-3868.

[5] Chen CJ, Kono H, Golenbock D, Reed G, Akira S and Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med 2007; 13: 851-856.

[6] Chen R, Alvero AB, Silasi DA and Mor G. Inflammation, cancer and chemoresistance: taking advantage of the Toll-Like receptor signaling pathway. Am J Reprod Immunol 2007; 57: 93-107.

[7] Huang B, Zhao J, Shen S, Li H, He KL, Shen GX, Mayer L, Unkeless J, Li D, Yuan Y, Zhang GM, Xiong H and Feng ZH. Listeria monocytogenes promotes tumor growth via tumor cell Toll-like receptor 2 signaling. Cancer Res 2007; 67: 4346-4352.

[8] Asosingh K, Gunthert U, Bakkus MH, De Raeve H, Goes E, Van Riet I, Van Camp B and Vanderkerken K. In vivo induction of insulin-like growth factor-I receptor and CD44v6 confers homing and adhesion to murine multiple myeloma cells. Cancer Res 2000; 60: 3096-3104.

[9] Gibson LF. Survival of B lineage leukemic cells: signals from the bone marrow microenvironment. Leuk Lymphoma 2002; 43: 19-27.

[10] Granziero L, Circosta P, Scielzo C, Frisaldi E, Stella S, Geuna M, Giordano S, Ghia P and Caligaris-Cappio F. CD100/Plexin-B1 interac-

tions sustain proliferation and survival of normal and leukemic CD5+ B lymphocytes. Blood 2003; 101: 1962-1969.
[11] Lévesque JP, Helwani FM and Winkler IG. The endosteal 'osteoblastic' niche and its role in hematopoietic stem cell homing and mobilization. Leukemia 2010; 24: 1979-1992.
[12] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
[13] Barry FP and Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 2004; 36: 568-584.
[14] Lin YM, Pu LM, Yang SJ, Gao S and Zhang GZ. [Influence of human mesenchumal stem cells on cell proliferation and chemo-sens-itivity of K562 cells]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2006; 14: 308-312.
[15] Lin YM, Zhang GZ, Lu ZX, Leng ZX, Bu LS and Gao S. Study of apoptosis gene expression in U937 cells induced by adhesion culture with mesenchymal stem cell. Chin J Hematol 2006; 27: 249-253.
[16] Beg AA. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol 2002; 23: 509-512.
[17] Akira S and Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4: 499-511.
[18] O'Neill LA and Bowie AG. The family of five: TIR-domaincontaining adaptors in Toll-like receptor signaling. Nat Rev Immunol 2007; 7: 353-364.
[19] Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M and Seya T. TIR-containing adaptor molecule (TICAM)-2, a bridging adaptor recruiting to Toll-like receptor TICAM- 1 that induces interferon-beta. J Biol Chem 2003; 278: 49751-49762.
[20] Panteli KE, Hatzimichael EC, Bouranta PK, Katsaraki A, Seferiadis K, Stebbing J and Bourantas KL. Serum interleukin (IL)-1, IL-2, sIL-2Ra, IL-6 and thrombopoietin levels in patients with chronic myeloproliferative diseases. Br J Haematol 2005; 130: 709-715.
[21] Corrado C, Raimondo S, Saieva L, Flugy AM, De Leo G and Alessandro R. Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an interleukin 8-dependent survival of leukemia cells. Cancer Lett 2014; 348: 71-76.
[22] Shires GT, Fisher O, Murphy P, Williams S, Barber A, Johnson G, Davis B and Pahulu S. Recombinan activated protein C induces dose-dependent changes in inflammatory mediators, tissue damage, and apoptosis in vivo rat model of sepsis. Surg Infect 2007; 8: 377.
[23] Wang GR, Li ZJ, Li CH, Li Q and Qiu LG. Influence of siRNA interfering β-caten- in on tumorigenicity in vivo of K562 cell line. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2011; 19: 861-864.
[24] Seya T, Shime H, Takaki H, Azuma M, Oshiumi H and Matsumoto M. TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis. Oncoimmunology 2012; 1: 917-923.
[25] Thapa RJ, Nogusa S, Chen P, Maki JL, Lerro A, Andrake M, Rall GF, Degterev A and Balachandran S. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. Proc Natl Acad Sci U S A 2013; 110: E3109-E3118.
[26] McAllister CS, Lakhdari O, Pineton de Chambrun G, Gareau MG, Broquet A, Lee GH, Shenouda S, Eckmann L and Kagnoff MF. TLR3, TRIF, and caspase-8 determine double-stranded RNA-induced epithelial cell death and survival in vivo. J Immunol 2013; 190: 418-427.