· 论著 ·

The effect of high mobility group box-1 protein on immune function of human T lymphocytes in vitro

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【Abstract】 Objective To investigate the effect of high mobility group box-1 protein (HMGB1) on immune function of human T lymphocytes in vitro and explore its potential role in cell-mediated immune dysfunction. **Methods** Fresh blood was obtained from healthy adult volunteers and peripheral blood mononuclear cells (PBMCs) were isolated, then rhHMGB1 was added to PBMCs. Cell viability was assessed by thiazolyl blue (MTT) assay. Four-color flow cytometric (FCM) analysis was used for the measurement of CD3, CD8 expression. Reverse transcription-polymerase chain reaction amplification was performed to detect respective gene expression of interleukin-2 (IL-2), IL-2 receptor (IL-2R) alpha. **Results** ①Proliferation of T lymphocytes was not affected by rhHMGB1 in low concentrations, while continued exposure of T cells to 500 - 1 000 μg/L rhHMGB1 for 48 hours resulted in a decrease in MTT assay. ② Different stimulating time and dosages of rhHMGB1 did not alter CD4 expression of $CD3^+$ T lymphocytes. rhHMGB1 stimulation provoked a dose-dependent and time-dependent increase in Th2 subset and lowering in ratio of Th1 to Th2. ③ Compared with the untreated cells, when the cells were co-incubated with rhHMGB1 (10 μg/L) for 12 hours, mRNA expressions of IL-2 and IL-2R were significantly up-regulated. At 48 hours, in contrast, gene expression was relatively lower in T cells after exposure to 100 - 1 000 μg/L rhHMGB1. **Conclusion** These data demonstrated that HMGB1 had a dual influence on immune functions of T lymphocytes.

【Key words】 high mobility group box-1 protein; immunity; T lymphocytes; proliferation; interleukin-2

高迁移率族蛋白 B1 对人 T 淋巴细胞免疫功能影响的体外研究 黄立锋，姚咏明，孟海东，赵晓东，董宁，于燕，盛志勇(100037 北京，解放军总医院第一附属医院全军烧伤研究所)

【摘要】 目的 观察高迁移率族蛋白 B1(HMGB1)对健康人 T 淋巴细胞增殖的影响，并对其机制进行初步探讨。**方法** 分离健康人外周血单个核细胞(PBMCs)，调整细胞浓度后接种于细胞培养板并加入重组人高迁移率族蛋白 B1(rhHMGB1)进行刺激。以四甲基偶氮唑盐微量Ⅱ酶反应比色法(MTT)检测细胞数量和细胞活性，观察 HMGB1 对 T 淋巴细胞增殖活性的影响。采用四色流式细胞术(FCM)分析 $CD3^+$淋巴细胞 CD4 表达。细胞中白细胞介素-2(IL-2)、IL-2α 受体(IL-2Rα)基因表达水平采用逆转录-聚合酶链反应(RT-PCR)分析。**结果** ①500～1000 μg/L rhHMGB1 作用 48 h 后 T 淋巴细胞增殖反应显著抑制，低于这一剂量对其增殖活性影响不显著。②不同 rhHMGB1 刺激时间和作用剂量对 $CD4^+$ T 淋巴细胞未造成明显改变，但 rhHMGB1能时间-剂量依赖性增加 Th2 亚群比例，并因此降低 Th1/Th2 比值，刺激后 T 淋巴细胞免疫功能出现 Th1 优势向 Th2 优势偏移。③经植物血凝素激活后 12 h T 淋巴细胞 IL-2 和 IL-2Rα 基因表达达到峰值；rhHMGB1与 T 淋巴细胞共同培养 12 h 后，10～100 μg/L 剂量可明显上调 IL-2 和 IL-2Rα 基因表达；而较高剂量 rhHMGB1(100～1 000 μg/L)刺激持续 48 h 上述效应衰竭，并表现出相反的变化趋势。**结论** HMGB1 对 T 淋巴细胞包括增殖、分化和细胞因子分泌等免疫功能具有直接调节效应。剂量蓄积和持续刺激可诱导 T 淋巴细胞功能亚群从促炎优势向抗炎优势转化。

【关键词】 高迁移率族蛋白 B1； 免疫； T 淋巴细胞； 增殖； 白细胞介素-2

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High mobility group box-1 protein (HMGB1) is a non-histone, chromatin-binding protein, which was isolated from calf thymus over 30 years ago[1]. Recently, HMGB1 has been identified as a proin-flammatory cytokine that mediates endotoxin lethality, inflammation, and macrophage activation in sepsis[2-4]. It has been found in the serum of patients with acute (sepsis) and chronic (rheumatoid arthritis) inflammatory conditions[5-6], thus it may be involved in

maladaptive or autoimmune responses. Up to now, the biology of HMGB1 has been extensively studied as a proinflammatory cytokine of systemic inflammation, while little is known in regard to its potential contribution to the host cell-mediated immunity, particularly regarding human T lymphocytes.

Clinical data suggest that there is close relationship between the extent of impaired host defense and sepsis as well as multiple organ dysfunction syndrome. Studies have indicated that shift from Th1 to Th2 response was a contributory factor to marked suppression of cell-mediated immunity in sepsis, though the underlying mechanism has not been fully elucidated[7]. HMGB1 has recently been identified as a crucial cytokine that mediates the responses to infection, injury and inflammation. As HMGB1 appears to emerge late after a serious infection, a hope arises that it might be a potential therapeutic target in clinical practice. However, the biological activities of extracellular HMGB1 and its effect on the innate immune response have not been well illustrated. In this study, we investigated the effect of HMGB1 on immunity of human T lymphocytes in vitro and explored its potential role in cell-mediated immune dysfunction.

1 Materials and Methods

1.1 Experimental design: The study population comprised of 26 healthy volunteers. All of them with no detectable physical or clinical laboratory abnormality were demanded to refrain from taking any drug for one month. Eight volunteers [5 men and 3 women, mean age (24±9) years old, range 18-42 years old] participated in the experiment of polarization of the T-helper lymphocyte activity; Eight volunteers [4 men and 4 women, mean age (25±6) years old, range 22-37 years old] participated in the lymphocyte proliferation assay; Ten volunteers [7 men and 3 women, mean age (29±7) years old, range 25-42 years old] were enrolled for the experiment to determine the gene expressions of interleukin-2 (IL-2) and IL-2 receptor (IL-2R). Written informed consents were signed before the beginning of experiments. The experimental protocol was approved by the Ethical Review Committees of Postgraduate Medical College of PLA, Beijing.

1.2 Peripheral blood T lymphocyte isolation and counting: Thirty milliliters of heparinised blood was diluted in Hanks' balanced salt solution, and Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) was used for isolation and preparation of peripheral blood lymphocytes. The isolated lymphocytes were washed twice with phosphate buffered saline (PBS), centrifuged (500 g, 4 minutes), resuspended in basal medium (unstimulated culture) RPMI-1640 (Gibco-BRL, Gaithersburg, MD) containing 10% fetal calf serum (Seromed, Berlin, Germany), 1% penicillin/streptomycin (Sigma Chemical Co., St. Louis, MO), and 1% glutamine (ICN, Eschwege, Germany). Then cell populations were counted under a light microscope. Based on the different experimental demand, the peripheral blood mononuclear cells (PBMCs) suspension was diluted with RPMI-1640 to 2×10^9 cells/L.

1.3 Assessment of cell viability by methyl thiazolyl tetrazolium (MTT) assay: Cells (2×10^9 cells/L) were inoculated to 96-well plates with 0.2 ml per well and incubated for 4 hours at 37 ℃ in 5% CO_2. 1% phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO) was added to maintain cell proliferation and viability. At 24, 48, 60 hours, recombinant human HMGB1 (rhHMGB1, Sigma Chemical Co., St. Louis, MO) in different amount or PBS were added to the PBMCs suspension to make final concentration of HMGB1 of 0, 1, 10, 50, 100, 500 and 1 000 μg/L per well, respectively, with 4 wells for each concentration of HMGB1. Cells were incubated for 68 hours, and then 100 μl supernatant was procured. MTT (Sigma Chemical Co., St. Louis, MO) 20 μl was added to each well. After culturing for 4 hours, 100 μl Triton-ISOP solution was added. Optical density (OD) value per well at 560 nm wavelength was read in a plate reader 10 minutes later, and mean value was calculated.

1.4 Determination of $CD4^+/CD3^+$ and CD69 expressions on T lymphocytes: Cell suspension was harvested, washed once with PBS, and centrifuged (500 g, 4 minutes), and the cells were resuspended in basal medium 200 μl RPMI-1640. 20 μl CD3-PerCP (Becton-Dickinson, San Jose, CA) and 5 μl CD8-APC (Becton-Dickinson, San Jose, CA) were added. Cells were stored at room temperature in darkness. Then They were washed once with 2 ml PBS, centrifuged (500 g, 4 minutes), resuspended in 300 μl RPMI-1640. Two samples of supernatant (100 μl) were added in two flow cytometry (FCM) test tubes. Two samples were labeled respectively as the following methods: ① Negative comparison staining: the method was the same with that of intracellular cytokine staining; ② Cell-stimulating marker CD69 staining: cells were incubated with 10 μl CD69-PE (Becton-Dickinson, San Jose, CA) for 20 minutes at 4 ℃ in the dark and then washed twice with FACS buffer PBS with 1% bovine specific albumin and 0.1% sodium azide before FACS acquisition.

Before the FCM analysis, PBMCs were cultured for 6 hours with 50 μg/L phorbol 12-myristate 13-acetate (PMA), 1 mg/L ionomycin, and 2 μmol/L GolgiStop. $CD8^+CD69^+$ T cells expressions were counted by gating on the $CD3^+$ cell population[8]. $(1-2)\times10^5$ cells were analyzed in each experiment using the Cell Quest program (Becton-Dickinson, San Jose, CA) after setting the quadrants using isotype controls.

1.5 IL-2, IL-2R gene expressions: Cells (2×10^9 cells/L) were inoculated in 24-well plates with 2 ml per well and incubated for 2 hours at 37 ℃ in 5% CO_2. Cultures were stimulated with 1% PHA. rhHMGB1/PBS was added in the PBMCs suspensions to make the final concentration of HMGB1 at 0, 10, 100, and 1 000 μg/L per well respectively. There were three parallel wells in each array. Incubated for 12, 48 hours respectively, supernatants and cells were collected.

Total cellular RNAs were isolated from cells using Qiagen RNase purification kits (Qiagen, Alameda, CA). The Multiprobe RNase protection assays (RPAs) were performed with 5 - 10 μg total RNA according to the manufacturer's directions (Pharmingen, San Diego, CA). Purity of nucleonic acid was determined as A_{260}/A_{280} ratio, the following formula calculation was performed to determine total RNA concentration: RNA concentration (g/L) = ($A_{260}\times40\times100$)/1 000. After DNase treatment for RNA specimens, the reverse transcription was performed using the reverse transcriptase and Oligo (dt) 15 Primer in ice bath for cDNA. Reverse transcription system included: 1.0 μg template (RNA), 0.5 μl rRNasin (4×10^7 U/L), 1.0 μl Oligo (dt) 15 Primer (0.5 g/L), 4.0 μl $MgCl_2$ (25 mmol/L), 2.0 μl 10 × reverse transcription buffer, 2.0 μl dNTP (10 mmol/L), 1.5 μl avian myeloblastosis virus (AMV) retroviridase (1×10^7 U/L), 20 μl non-RNase liquid. The suspension was mixed well, centrifuged briefly, heated in an aqueous bath for 1 hour at 42 ℃, AMV retroviridase was inactivated for 5 minutes at 95 ℃, immersed in ice bath for 5 minutes, centrifuged again, cDNA was kept at - 20 ℃ for later use.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the following primers: ① IL-2Rα, product size 298 bp[9], forward 5'- GAA TTT ATC ATT TCG TGG TGG GGC A -3', reverse 5'- TCT TCT ACT CTT CCT CTG TCT CCG -3'; ② IL-2, product size 457 bp[10], forward 5'- ATG TAC AGG ATG CAA CTC CTG TCT T-3', reverse 5'-GTC AGT GTT GAG ATG ATG ATG CTT TGA C-3'; ③ β-actin, product size 383 bp[10], forward 5'-GTG GGG CGC CCC AGG CAC CA -3', reverse 5'- GTC CTT AAT GTC ACG CAC GAT TTC -3'. Reverse transcription system for cDNA amplification included: 4 μl cDNA, 0.5 μl dNTP (10 mmol/L), 1.5 μl $MgCl_2$ (25 mmol/L), 1.0 μl upstream primer, 2.0 μl 10×reverse transcription buffer, 1.0 μl downstream primer, 2.0 μl Taq DNA pclymerase (0.5 g/L), 25 μl non-RNase liquid. Mixed well and centrifuged briefly, covered with 50 μl liquid paraffin, and amplification was performed in a thermal cycler (Perkin Elmer, USA).

Denaturating cycle at 97 ℃ for 5 minutes was first done, and the condition for PCR reactions was shown in Table 1. The PCR production was electrophoresed in a 1.2% agarose gel containing 0.5 g/L ethidium bromide in Tris borate/ethylenediaminetetraacetic acid (EDTA) buffer. Bands were visualized by UV-transillumination. Pictures were analyzed with LEICA Q-500IW image analysis software (LEICA Q-500IW, Germany).

Table 1 Conditions for PCR reactions

cDNA	denaturation	renaturation	elongation	cycles
IL-2	95 ℃, 60 seconds	57 ℃, 60 seconds	72 ℃, 120 seconds	35
IL-2R	95 ℃, 45 seconds	55 ℃, 45 seconds	72 ℃, 90 seconds	35
β-actin	95 ℃, 30 seconds	55 ℃, 30 seconds	72 ℃, 60 seconds	30

1.6 Statistical analysis: Data were expressed as mean [standard deviation (SD)]. One-way ANOVA was used for the data as obtained at same stimulating time and different dosages, or same dosage and different stimulating time. The Mann-Whitney U test was performed for the data of heterogeneity of variance or abnormal distribution in groups. Statistical analysis for the data of two-sets comparisons was performed using the Student's t-test. The statistical packages Stata 8.0 was used for all statistical calculations. The significance of differences was set at $P<0.05$ and the highly significance of differences was set at $P<0.01$.

2 Results

2.1 The correlation between lymphocyte counts and MTT OD: Lymphocytes were inoculated at ($0.5-5)\times10^9$/L, as shown in Figure 1. Logarithm of lymphocyte counts and MTT OD were correlated with linear correlation, straight line equation was $Y = 0.85X - 5.33$ ($n = 26$, $r=0.990$, $P<0.01$). According to the principle that OD was more accurate in the range of 0.3 - 0.8, the lymphocyte count in MTT assay was elected as 2.0×10^9/L.

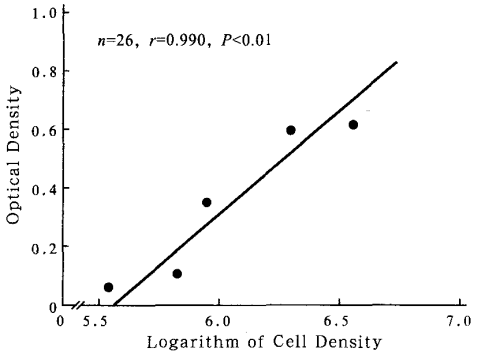


Figure 1 Correlation between lymphocyte counts and MTT OD

2.2 Changes in T lymphocyte proliferation stimulated with PHA: Changes in T lymphocyte proliferation stimulated with PHA for 5 days were shown in Figure 2. Viable count tapered off at 36 hours post-inoculation, entering the exponential growth phase at 36 - 72 hours, and cell

replication was exhausting after 72 hours. Based on these data, the incentive phase of HMGB1 was selected at the stage of 24 - 72 hours ($n=8$, $r=0.826$, $P<0.01$).

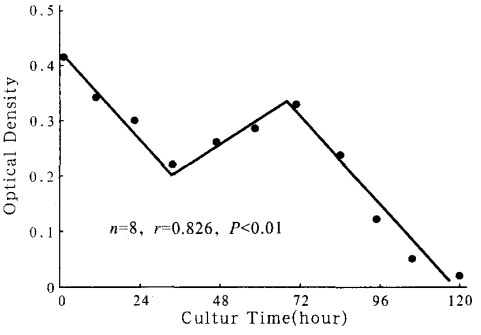
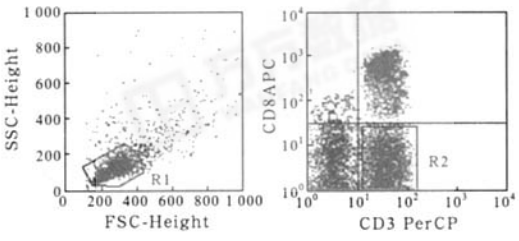


Figure 2 Changes in T lymphocyte proliferation stimulated with PHA

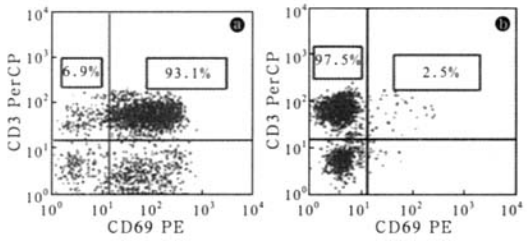
2.3 Changes in T lymphocyte proliferation: With same dosage (1 - 1 000 μg/L) but different time of action, no significant difference in T lymphocyte proliferation was noted. However, continued exposure of T cells to 500 - 1 000 μg/L rhHMGB1 resulted in inhibition of their proliferation. No significant difference in T lymphocyte proliferation was observed after being stimulated with different dosage of rhHMGB1 at 12 or 24 hours. After 48 hours, the effect of rhHMGB1 in different dosage on T lymphocyte proliferation showed significant difference ($P=0.045\ 3$). T lymphocyte reproductive activities with 0, 1 μg/L of rhHMGB1 were stronger than with 500 or 1 000 μg/L (both $P<0.05$), and a highly significant difference was found among 10, 500, and 1 000 μg/L (both $P<0.01$) (Figure 3). Data showed that continued exposure of T cells to high dosages of rhHMGB1 for 48 hours resulted in marked decrease in MTT density.

2.4 CD69 expression: Application of CD3/CD8 gating to examination of Th1/Th2 cells in FCM is a new approach for the more accurate and sensitive detection of Th1/Th2 cells (Figure 4). CD69 expression was an important cell-stimulating marker. Higher than 85% in CD69/CD3 ratio was demanded for good intracellular cytokine staining. PBMCs were cultured for 6 hours with intracellular cytokine excitomotor (PMA, ionomycin, GolgiStop). $CD69^+$ T cells expression value was consistent with the standard demand (Figure 5).



R1: $CD4^+$; R2: $CD3^+CD8^-$

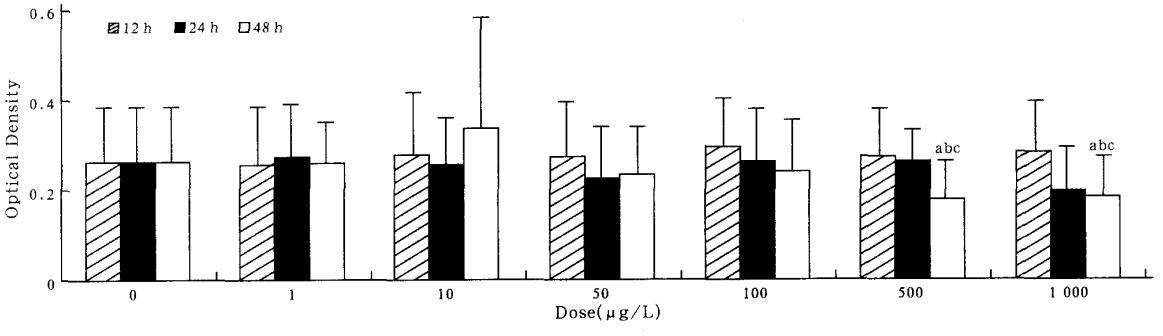
Figure 4 Gates for FCM analysis of CD4 expression and Th1/Th2 subsets



ⓐ: stimulated; ⓑ: unstimulated

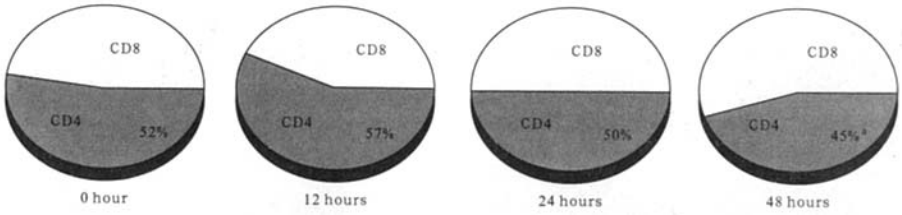
Figure 5 CD69 expression in T cells stimulated with PMA and ionomycin

2.5 Changes in $CD4^+/CD3^+$ ratio: The Th1 expression level was markedly lowered when cells were cultured for 72 hours ($P<0.01$), the changes in CD4, Th2 expression levels and ratio of Th1/Th2 were not statistically significant. It was shown that incubation with rhHMGB1 in different dosages ($F=2.527\ 5$, $P=0.010\ 9$) and different



Note: [a] $P<0.05$ as 500 μg/L, 1 000 μg/L groups vs. 0 μg/L group; [b] $P<0.05$ as 500 μg/L, 1 000 μg/L groups vs. 1 μg/L group; [c] $P<0.01$ as 500 μg/L, 1 000 μg/L groups vs. 10 μg/L group

Figure 3 Changes in T lymphocytes proliferation stimulated with rhHMGB1



Note: statistical significance, $^aP<0.05$ as 48 hours group vs. 12 hours group

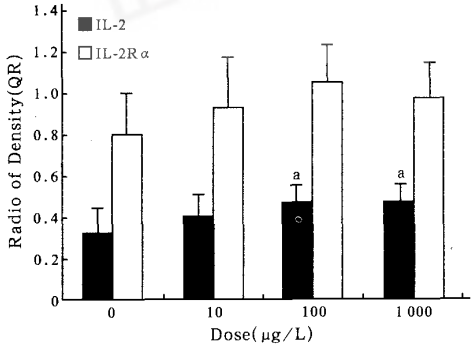
Figure 6 Changes in $CD4^+/CD8^+$ ratio of T lymphocyte stimulated with 1 000 μg/L rhHMGB1

time ($F=4.915\ 9$, $P=0.010\ 5$) could significantly influence T lymphocytes surface antigen CD4 expression. With the same time (12, 48 hours) but different dosages, no significant difference in CD4 expression was found, but significant differences in CD4 expression at 24 hours were found ($F=3.214\ 4$, $P=0.023\ 9$). At same dosage (1, 10, 100, 1 000 μg/L) but different time it was shown that there were significant differences in CD4 expression with dosages of 10 μg/L ($F=3.355\ 0$, $P=0.032\ 9$) and 1 000 μg/L ($F=3.826\ 7$, $P=0.020\ 5$). It was showed that $CD4^+/CD3^+$ ratio at 48 hours was significantly lower than that at 12 hours ($P<0.05$) when stimulated with 1 000 μg/L rhHMGB1. The results suggested that continued exposure of T cells to 500 - 1 000 μg/L rhHMGB1 would result in down-regulation of CD4 expression, thus, reducing the lymphocyte activities to receive antigen presentation (Figure 6).

2.6 Changes in mRNA expressions of IL-2 and IL-2Rα: T cell multiplication was activated with PHA stimulation and the gene expressions reached the peak value at 8 - 12 hours. Then, gene expression levels of IL-2 descended quickly, but high levels of IL-2Rα gene expressions persisted for 48 hours.

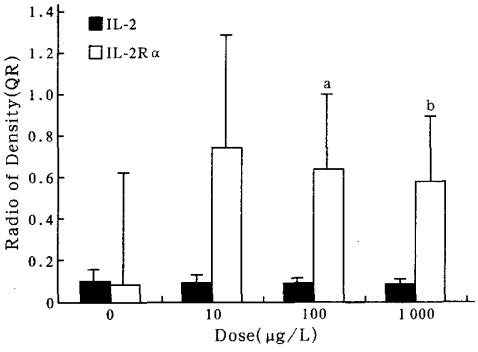
At 12 hours, it showed that there was a significant difference in IL-2 mRNA expressions with different dosages of rhHMGB1 ($F=4.687\ 2$, $P=0.007\ 3$). IL-2 mRNA expressions increased with stimulation of rhHMGB1 in 100 μg/L ($P<0.01$), and this effect was more obvious at 1 000 μg/L ($P<0.01$). There was no marked difference in IL-2Rα mRNA expressions at different dosages (Figure 7).

At 48 hours, no significant differences were observed in IL-2 mRNA expressions with stimulation of rhHMGB1 in different dosages, but a diminishing tendency of IL-2 mRNA expression was found with increasing concentration of rhHMGB1 ($P=0.059$). There was a significant difference in IL-2Rα mRNA expressions with different dosages of rhHMGB1 ($H=1.220$, $P=0.045$). The IL-2Rα mRNA expressions were down-regulated with 100 μg/L rhHMGB1 stimulation ($P<0.05$), and the down-regulation was very obvious with 1 000 μg/L rhHMGB1 stimulation ($P<0.01$) (Figures 8 and 9).



Note: $^aP<0.01$ as 10 μg/L, 100 μg/L, 1 000 μg/L groups vs. 0 μg/L group

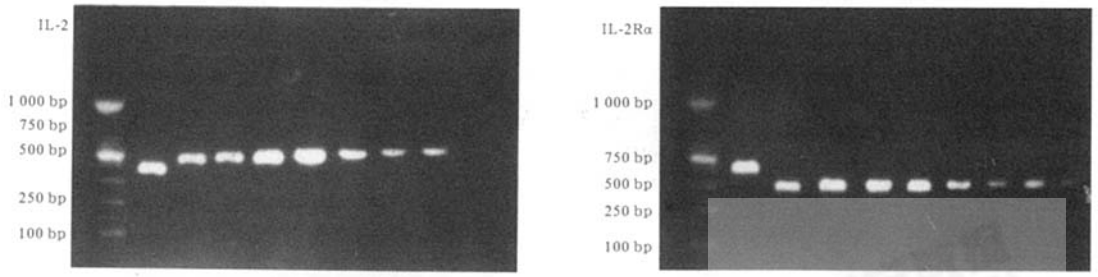
Figure 7 Effect of rhHMGB1 on mRNA expressions of IL-2 and IL-2Rα in T lymphocyte at 12 hours after incubation



Note: $^aP<0.05$, $^bP<0.01$ as 10 μg/L, 100 μg/L, 1 000 μg/L groups vs. 0 μg/L group

Figure 8 Effect of rhHMGB1 on mRNA expressions of IL-2 and IL-2Rα in T lymphocyte at 48 hours after incubation

Experimental results showed that the IL-2 mRNA expressions were up-regulated with increasing rhHMGB1 stimulation at 12 hours in vitro. However, the IL-2, IL-2Rα mRNA expressions were inhibited after 48 hours in response to stimulation with a high dose of rhHMGB1. The experimental results suggested that HMGB1 might have a dual



The sequences (from the left to the right) were Marker, primer β-actin, 0 μg/L(12 hours), 10 μg/L(12 hours), 100 μg/L(12 hours), 1 000 μg/L(12 hours), 0 μg/L(48 hours), 10 μg/L(48 hours), 100 μg/L(48 hours), and 1 000 μg/L(48 hours), respectively

Figure 9 Changes in mRNA expressions of IL-2 and IL-2Rα in T lymphocytes after stimulation with rhHMGB1

regulatory effect on T cells immune activity.

3 Discussion

Sepsis is an indocile challenge to human health and economical development world wide. Mortality rate of severe sepsis and septic shock may reach 30%-80%[11], and it has become the leading cause of death of non-cardiac diseases in different countries, mainly because its underlying pathogenetic mechanisms have not been fully elucidated[12]. The key point in treatment of sepsis is to control inflammatory response in a reasonable degree. The discovery of HMGB1 as a contributory pathogenetic factor in sepsis is of great interest as some investigators found that the inhibition of HMGB1 release is important in controlling inflammatory response in sepsis.

Our preliminary results had proved that HMGB1 was essential in the process of T lymphocyte adaptive immune responses. The studies in murine models showed that low doses of HMGB1 amplified the cytokine cascade during systemic inflammation. HMGB1, as a cytokine, significantly increased the release of TNF-α in a dose-dependent manner. However, with prolonged (24 - 72 hours) and high dosage (1 000 μg/L) of stimulation with HMGB1, the functions of macrophages and T lymphocyte proliferation were inhibited, and apoptosis of T cells intervened (data was not shown). When T cells were treated with HMGB1 in different dosages for different duration, they would transform into different subsets of Th1 and Th2. The fact that high dose of HMGB1 can transform Th1 to Th2 subset implies that there is an immune suppression in this event[13-14]. Based on this finding, in order to offer the theoretical evidences to interpret its clinical significance of immunologic competence, we designed to investigate the effects of HMGB1 on human T lymphocyte immune functions.

In humans approximately 80% of lymphocytes in peripheral blood are T-lymphocytes which mature in the thymus[15]. Many cellular factors and bioactive substances play a regulatory role in the proliferation of lymphocytes. Cellular DNA synthesis and mitosis are important symbols of cellular proliferation. A costimlatory signal is required for proliferation and differentiation of lymphocytes delivered through T-cell and antigen presenting cells (APCs) interaction. IL-2 is also thought to be able to stimulate T-lymphocytes proliferation to secrete immunoglobulins, and to enhance cellular immune responses[16]. Stimulation of T cells by antigen in absence of co-stimulatory signal can not cause T-cell response. Our experimental results indicated that T lymphocyte cells gradually proliferated after being stimulated by PHA, and it peaked at 24 hours. PHA nearly had no effects on the function of mononuclear cells which were capable to secrete cytokines, and it could not activate the mononuclear cells proliferation. HMGB1 in a low dose appeared to be a stimulatory signal for T lymphocyte proliferation, but continued exposure of T cells to high concentration of rhHMGB1 could result in a marked decrease in MTT density. The results showed that lymphocyte proliferation could be induced by HMGB1 at relative low dose. On the other hand, it was restrained by HMGB1 in high concentration in a time-dependent manner, and this was likely to be the direct cause of reduced growth index and decreased number of lymphocytes of the lymphoid organs in vivo[17].

Excessive release of anti-inflammatory cytokines is an important mechanism of immunosuppression in sepsis[18]. Recently, studies have been shown that there is an abnormal polarization of Th1 and Th2 cells in peripheral blood in patients with severe sepsis[19-20]. Because cytokines might play an important role in inducing $CD4^+$ T ancillary cells differentiation, we speculate that HMGB1 as a late cytokine might influence immune function shift of T lymphocytes from Th1 subset to Th2 subset. Our results showed that Th1/Th2 ratios were significantly decreased after 24 hours of 100 μg/L HMGB1 stimulation, or with 1 000 μg/L HMGB1 stimulation for 12 hours. The results indicate that a prolonged exposure to high dose of HMGB1 can lead to a shift of Th1 to Th2. The abnormal polarization of T cells brings about changes in immune state from continual autoimmune responses to immunological depression[19].

Some clinical observations reported that the levels of IL-2 and other proinflammatory cytokines began to decrease after 24 hours in septic patients, however, the level of soluble IL-2R (sIL-2R) increased at the same time in serious trauma, infection and septic patient[21]. Our data showed that levels of IL-2 mRNA expression were up-regulated after 12 hours of 10 - 1 000 μg/L HMGB1 stimulation in a dose-dependent manner. There was a close relation between decreased IL-2R expressions and increased HMGB1 after 48 hours. As the mediation of APCs such as monocytes, macrophages, and dendritic cells is excluded in vitro, it is our speculation that the mechanism of mediation of IL-2/IL-2R mRNA expressions of lymphocytes might be related to the receptors on cell surface. Up to now, receptors which can crosslink with HMGB1 and transduct the immunoregulation signals have not been identified on surfaces of lymphocyte cells. Whether there are other molecules on the lymphocyte surfaces which posses characteristics of the receptors of cytokines to activate the transductions of signals and genetic transcriptions is an unknown subject calling for further investigation.

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