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In vitro study of immunosuppressive effect of apoptotic cells*

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Abstract: Recent studies revealed that apoptotic cells are actively involved in immunosuppression and anti-inflammation. After being phagocytosed by macrophages, apoptotic cells can actively regulate cytokines secretion from lipopolysaccharide (LPS)-stimulated macrophages, in which the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) is increased while the pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β) and leukin-8 (IL-8) are suppressed. In this paper, we first present evidence that phagocytosed apoptotic cells regulate cytokine secretion of LPS-stimulated macrophages, but also inhibit the activation of T lymphocytes stimulated by ConA. These data suggest that apoptotic cells can alter the biological behavior of macrophages which gain immunosuppressive property.

Key words: Macrophages, Apoptosis, Cytokines, Immunosuppression
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INTRODUCTION

Apoptosis plays an important role in differentiation, development and pathophysiological processes such as inflammation, neoplasia and autoimmune diseases. For a long time, apoptotic cells per se and the clearance of apoptotic cells had been viewed as neutral in immune response. Recently many investigations suggested that apoptotic cells actively regulate the immune response (Voll *et al*., 1997; Fadok *et al*., 1998; 2000; Byrne and Reen, 2002). Apoptotic cells release latent and also active TGFβ1, a potent immunosuppresant, which contributes to the establishment of local immunosuppressive milieu (Chen *et al*., 2001). Reiter and Krammer (1999) found that necrotic tumor cells help macrophages kill tumor cells, but that, apoptotic tumor cells decrease the competence of macrophage in clearance of tumor cells, and enhance prompt the growth of tumor cells. In addition, phagocytosis of apoptotic cells was found to inhibit macrophage activity and finally stimulate the growth of Trypanosome cruzi (Frerie-de-Lima *et al*., 2000; Freitas Balanco *et al*., 2001). These foundings indicate that apoptotic cells can induce immune tolerance. Necrotic cells send a "danger signal" to the immune system and thus evoke strong immune response, while cells dying by apoptosis do not send a danger signal to the immune system, and finally induce T lymphocytes to become tolerant. However, if apoptotic cells are not engulfed effectively, they will undergo secondary lysis, which could send out danger signal to the immune system and finally result in immune response (Savill, 1998; Savill *et al*., 2002). This causes immunologists to give second thought to the significance and clearance of apoptotic cells. Savill *et al*.(2002) suggested that the phagocytosis of apopototic cells should not only be viewed as clearing aging cells to make room for functional cells, but also setting up the immunosuppressive milieu at the local site. Interestingly, some investigations revealed that after apoptotic cells were bounded or ingested by macrophages, the secretion of immunosuppressive cytokines such as IL-10 was increased markedly, whereas the secretion of

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pro-inflammatory cytokines such as TNFα, IL-12 and IL-1β were suppressed (Voll *et al*., 1997; Fadok *et al*., 2000; Byrne and Reen, 2002). These results imply that apoptotic cells or phagocytosis of apoptotic cells may actively regulate immune response. Here we also found that apoptotic cells, after being ingested by macrophages, can actively alter the biological behavior of macrophages, which acquire immunosuppressive property.

MATERIALS AND METHODS

Antibodies and reagents

RPMI-1640 and hank balanced salt solution (HBSS) were purchased from GIBCO (America). Lipopolysaccharide (LPS), canavalin A (ConA), FITC-Annexin-V and propidium iodide (PI) were purchased from BD Pharmingen (America). The monoclonal antibodies hamaster anti-mouse FITC-CD69 (Cat# HM4001), rat anti-mouse TRITC-CD3 (Cat# RM3406), rat anti-mouse PE-CD25 (Cat# RM6004) and Isotype control antibodies hamster IgG-FITC, rat IgG2a-TRI (Cat# R2a06), rat IgG1-PE (Cat# R104), rat Ig2a-PE (Cat# R2a04) were obtained from CALTAG (America). Monoclonal anti-TGFβ1 antibody (Cat# MAB2401) was from RD. Mouse TGFβ1, TNFα and MIP-2 ELISA sets were from American Biosource Company.

Cell culture and treatment

Human T lymphocyte Jurkat cells were purchased from Academy of Medical Science, China. They were cultured in RPMI-1640 containing 10% (*V*/*V*) heat-inactivated featl cattle serum (FCS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% (*V*/*V*) CO_2. Macrophages were prepared by peritoneal lavage from abdominal cavity ICR mice. Briefly, spleen cells were harvested and cultured in RPMI-1640 containing 10% heat-inactivated FCS for 20 h before stimulation by ConA. Jurkat T cells were used as the source of apoptotic cells. When the density of Jurkat T cells reached 5×10^6 ml^{-1}, the cells were irradiated by ultraviolet (254 nm) light for 10 min (Fadok *et al*., 1998) and then incubated for 2 h to obtain 60%~80% apoptosis cells, while necrosis cells were less than 5% (assessed by flow cytometry). To obtain necrotic (lysed) cells, Jurkat cells were irradiated by ultraviolet (254 nm) light for 20 min. The average percentage of lysis, assessed by trypan blue positive, was approximately 90%.

Female ICR mice (6~8 weeks old, 20~25 g) were obtained from Animal Experimental Center of Zhejiang University. The mice were stimulated with LPS (0.5 μg/g) intraperitoneally. After 3 d, the mice were sacrificed by CO_2 asphyxiation, and their peritoneal cavities were lavaged with HBSS. Cells were pelleted at 300 g (10 min) and resuspended in RPMI-1640. The cells were then plated at 4×10^6 cells per well in six-well tissue culture plates and allowed to adhere for 2 h. Nonadherent cells were washed off and the remaining macrophages were cultured in RPMI-1640 without serum. Apoptotic Jurkat cells were added at a ratio of five per macrophage, and then cultured for 24 h. Necrotic Jurkat cells or viable Jurkat cells were added to macrophages as control. Supernatants were harvested 24 h later.

For our experiment, the cells were divided into four group: Apoptotic group: apoptotic Jurkat cells were added to macrophages; Necrotic group: necrotic Jurkat cells were added to macrophages; Viable group: viable Jurkat cells were added to macrophages as a control group; Anti-TGFβ1 group: anti-TGFβ1 neutralizing antibody was added to macrophages together with apoptotic Jurkat cells.

Ratio of apoptotic cells induced by UV irradiation

After UV (254 nm) irradiation, the cells were resuspended in 100 μl HBSS, followed by the addition of 10 μl FITC-Annexin-V and 10 μl PI. The cells were then incubated for 15 min in the dark at room temperature, washed with HBSS, and then finally analyzed on an FACSC alibur flow cytometer. The ratio of apoptotic cells was calculated by the following equation:

$$\frac{\text{Number of FITC-Annexin-V positive and PI negative cells}}{\text{Total number of cells irradiated by ultraviolet light}}$$

Ratio of phagocytosis of apoptotic cells

In order to assess the percentage of phagocytosis of apoptotic cells, 5×10^6 previously labelled apoptotic Jurkat cells were added to each well of macrophages (the ratio of apoptotic Jurkat cells to

macrophages was 5:1), and incubated at 37 °C, with 5% CO_2 for 40 min. The plate was washed vigorously with RPMI-1640 to remove uningested apoptotic Jurkat cells. The macrophages were then collected and analyzed on FACSC alibur flow cytometer. The ratio of phagocytosis was calculated by the following equation:

$$\frac{\text{Number of FITC-Annexin-V positive and PI negative cells}}{\text{Total number of macrophages}}$$

Cytokine analysis

Supernatants were collected from culture plates 24 h after Jurkat cells addition and then stored at −70 °C for enzyme-linked immunoadsorbent assay (ELISA) analysis. Assays were performed according to the instructions provided with each ELISA set. Since ELISA assay measures only the active form of TGFβ1, total TGFβ1 was subjected to preactivation with acidification to pH 3 for 15 min and then returned to pH 7.0 prior to the ELISA assay. The plates were read in a BIO-Tek EL309 ELISA reader.

RT-PCR analyses of MIP-2 and TNFα

Total RNA was extracted from macrophages 24 h after cells addition using Trizol according to manufacturer's instruction (Gibo BRL). cDNA was synthesized from 2 µg of RNA, with 1 µg of Oligo$(dT)_{15}$ in 20 µl reaction volume. PCR amplification was conducted in a total reaction volume of 25 µl, with 10^{-11} mol of each primer, 1.5 U Taq polymerase, 2 µl 25 mmol/L $MgCl_2$, 1 µl 10 mmol/L dNTP. PCR was performed at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 32 cycles. The following primers were used: MIP-2 sense 5′-CATCAGCATGGACCGCTACC-3′ and anti-sense 5′-CAGCCAGCAGAGCAGGAAGA-3′; TNFα sense 5′-CAGGCGGTGCCTATGTCTCA-3′ and anti-sense 5′-TCGGCTGGCACCACTAGTT G-3′.

The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed with UV light.

Spleen cells activation and analysis of CD69 and CD25

Twenty-four hours after Jurkat cells were added to macrophages, supernatants were collected from each group and acidify to pH 3 for 15 min and then corrected to pH 7.0. After this, 2×10^6~3×10^6 spleen cells were added to the supernatants of each group respectively. Each group was divided into two subgroups. One subgroup was stimulated by canavalin A (5 µg/ml) for 6 h, another subgroup for 20 h. Spleen cells were collected by centrifugation at 2000 r/min, then resuspended in 100 µl HBSS. Cells were either stained with anti-mouse monoclonal antibody FITC-CD69 (2 µl) and TRITC-CD3 (2 µl) for 20 min, or PE-CD25 (2 µl) and TRITC-CD3 (2 µl) for 20 min in the dark at room temperature. Isotype control antibodies were added as negative control. Spleen cells were washed once for flow cytometry analysis.

Statistical analysis

Data were expressed as mean±*SD*. Statistical analysis was performed using ANOVA for multiple comparisons with SPSS 10.0 software. P value <0.05 was considered significant.

RESULTS

Apoptosis ratio

UV induced apoptotic cells labelled by FITC-Annexin-V and PI were assessed by flow cytometry. The average percent apoptosis was 70%±8%, whereas necrosis (positive for PI) was <5% (Fig.1).

Ratio of phagocytosis of apoptotic cells

The macrophages which engulfed apoptotic cells labeled by FITC-Annexin-V were assessed by flow cytometry. The average percent phagocytosis was 50%±12% (Fig.2).

Macrophages cytokine production

To determine what effects the apoptotic cells have on the macrophages, the macrophages derived from mouse peritoneal lavage were exposed to apoptotic cells. Viable or necrotic cells were also added to macrophages as controls. Twenty-four hours after cells addition, the supernatants were collected and analyzed for cytokines. The results showed that macrophages ingesting apoptotic cells suppressed the production of MIP-2 and TNFα (P<0.01), but increased the secretion of TGFβ1 to the levels nearly two times compared to that of controls (P<0.01). In

addition, when anti-TGFβ1 antibody was added along with apoptotic Jurkat cells to macrophages, MIP-2 and TNFα production increased significantly. These results suggested that TGFβ1 played a key role in inhibiting the production of MIP-2 and TNFα (Fig.3).

Analyses of MIP-2 and TNFα by RT-PCR

To determine whether the suppression effects of apoptotic cells also happened at the level of transcription, semiquantitative RT-PCR was conducted to determine the mRNA levels of MIP-2 and TNFα in LPS-stimulated macrophages. As expected, phagocytosis of apoptotic cells decreased the mRNA levels of MIP-2 and TNFα in LPS-stimulated macrophages. On the other hand, TGFβ1 neutralizing antibody reversed the suppressive effects (Fig.4).

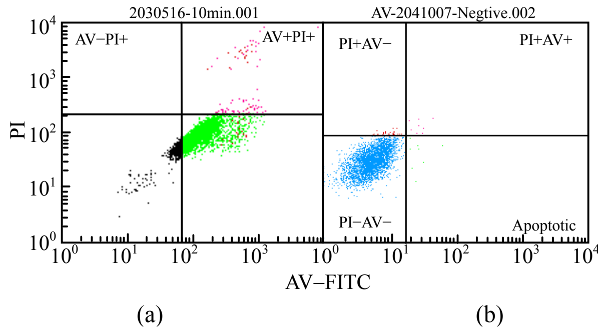
Expression of CD69, CD25, on ConA-activated T lymphocytes

TGFβ1 is one of the most potent immunoregulatory molecules. Macrophages that engulfed apoptotic cells increased TGFβ1 production and decreased MIL-2 and TNFα secretion. This phenomenon suggested that apoptotic Jurkat cells may induce macrophages to possess anti-inflammatatory or immunosuppressive property. In order to test this hypothesis, 2×10^6~3×10^6 spleen cells were added to the supernatants of each group with ConA stimulation for 6 h. After this we collected spleen cells and analyzed the expression of CD69 and CD25 on ConA-activated T lymphocytes. CD69 and CD25 are routinely used as markers for the activation of T lymphocytes (Crabtree, 1989). The percentages of CD69- and CD25-positive T lymphocytes in apoptotic group were 19.698%±8.25% and 15.18%±7.04% respectively, which were significantly lower than those corresponding percentages in necrotic and viable group. To investigate whether the increased production of TGFβ1 induced by phagocytosis of apoptotic cells was the main reason for the inhibition of the expression of CD69 and CD25, TGFβ1 neutralizing antibody was used to counteract the effects of TGFβ1. As expected, after adding TGFβ1 neutralizing antibody, the percentage of CD69- and CD25-positive T lymphocytes stimulated by ConA were increased even higher than those in necrotic and viable group. Therefore, these data suggested that the suppression of the expression of CD69 and CD25 depended on the increased secretion of TGFβ1 induced by phagocytosis of apoptotic cells. Furthermore, when the stimulation of T lymphocytes by ConA lasted for 20 h, the percentages of CD69- and CD25-positive lymphocytes showed no significant differences among groups (data not shown). So it showed that the suppression effect was transient (Fig.5).

DISCUSSION

Efficient phagocytosis of apoptotic cells is of great importance in vivo, because the clearance of apoptotic cells prior to lysis is critical to prevent inflammation (Savill, 1998; Savill *et al*., 2002; Rovere *et al*., 1999). Engulfment of apoptotic cells prior to lysis prevents the release of immunogenic materials of apoptotic cells and thus ensures the protection of neighboring cells from being damaged by leaked cellular contents of apoptotic cells. Our studies showed that with the clearance of apoptotic cells, production of proinflammatory cytokines such as TNFα and MIP-2 were inhibited through increased TGF-β1 secretion. Furthermore, the inhibition effects occurred at the level of transcription, as the mRNA levels for TNFα and MIP-2 in apoptotic cells treated macrophages were decreased. Anti-TGF-β1 antibody restored the expression of TNFα and MIP-2 at both mRNA and protein levels. This suggested that the increased production of TGF-β1 plays a key role in inhibiting the generation of TNFα and MIP-2. On the contrary, necrotic cells did not have the same effect. Taken together, our results demonstrated that apoptotic cells, after being phagocytosed, actively suppress inflammatory/immune response through increased TGF-β1 secretion.

Apoptotic cells have been viewed as a source of autoantigen (Casciola-Roen *et al*., 1994; Mevorach *et al*., 1998). It was intriguingly noted that apoptotic cells do not evoke immune response in vivo, even in the liver, in which is the main site of phagocytosis of apoptotic cells (Crispe *et al*., 2000). As explained in Matzinger's "Danger Model" theory, antigens from apoptotic cells are processed and presented to T lymphocyte by macrophages after being phagocytosed. Since apoptotic cells do not send danger signals to the immune system, the macrophages, acting as



(a) (b)

Fig.1 The ratio of apoptosis induced by UV

(a) 5×10^6 Jurkat T-cells irradiated by UV (254 nm) for ten minutes, labelled by FITC-Annexin-V and PI, and subjected to flow cytometry analysis. The apoptosis ratio was 70%±10%. The necrosis ratio was less than 5%. This figure was representative of five independent experiments; (b) Jurkat T-cells without irradiation were included as negative control

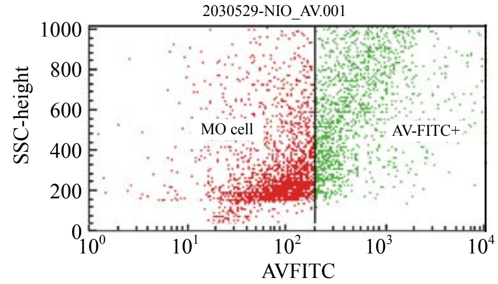
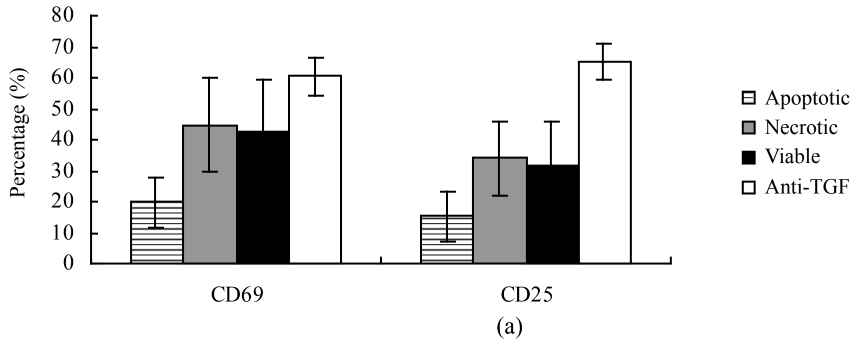
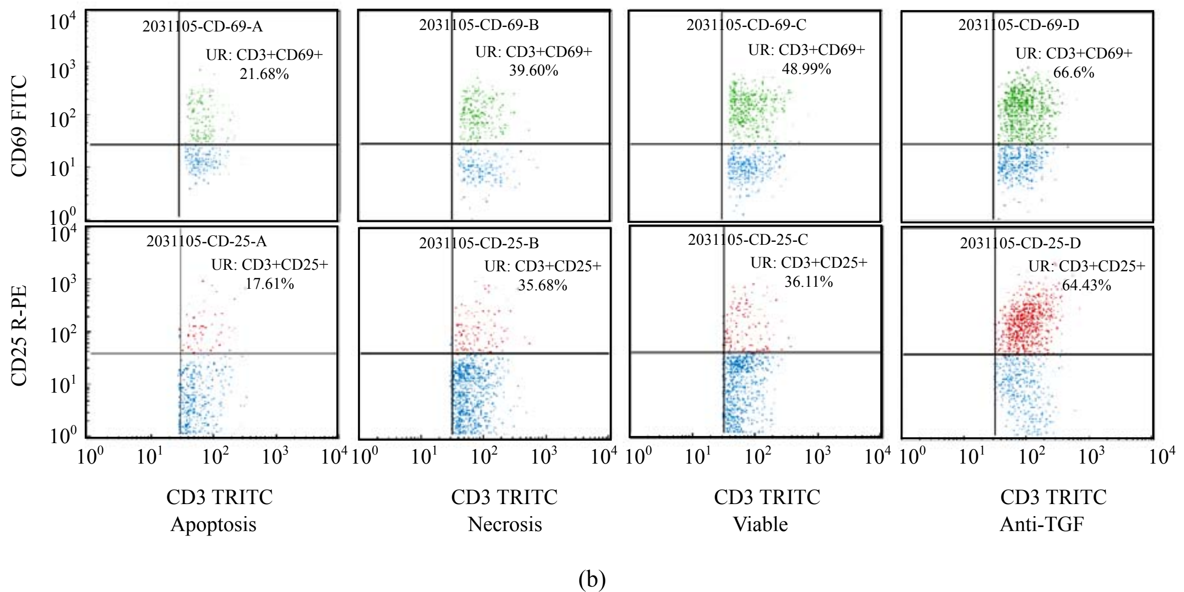


Fig.2 The ratio of phagocytosis of apoptotic cells

Jurkat T-cells were labelled by FITC-Annexin-V 10 μl and added into macrophages. The phagocytosis ratio was calculated by the following equation: (Number of FITC-Annexin-V positive macrophages)/(Total number of macrophages). Data were determined by flow cytometer. The ratio of phagocytosis of apoptotic cells was 46.88%. Dot plots are gate on forward light scatter (FS), thus excluding dying Jurkat cells. This figure was representative of five experiments



(a)



(b)

Fig.5 Phagocytosis of apoptotic cells negatively influence ConA driven T cell activation (a) Phagocytosis of apoptotic Jurkat cells suppressed the activation of T lymphocytes driven by ConA. Anti-TGFβ1 neutralizing antibody reversed the suppression effects. Data are displayed as $\overline{\chi}\pm SD$ for 5 experiments (n=5). P<0.01 apoptosis vs necrosis, viable Jurkat cells and anti-TGFβ1 respectively; (b) The representative picture of five experiments

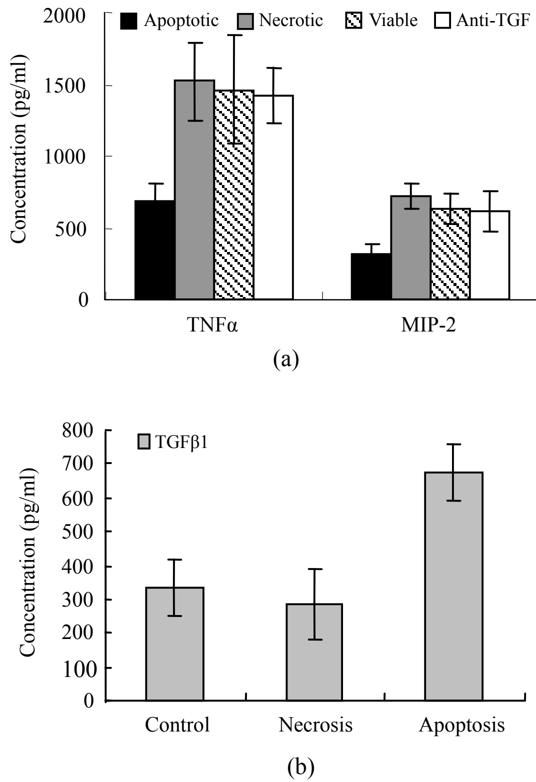


Fig.3 The effects of phagocytosis of apoptotic Jurkat cells on macrophages cytokine production. Phagocytosis of apoptotic Jurkat cells suppressed the production of TNFα and MIP-2 (a), but increased the production of TGFβ1 (b). Anti-TGFβ1 neutralizing antibody reversed the inhibitory effects derived from macrophages which had ingested apoptotic cells. Data are displayed as $\bar{\chi}\pm SD$ for 13 experiments (n=13). P<0.01 apoptosis vs necrosis, viable Jurkat cells and anti-TGFβ1 respectively

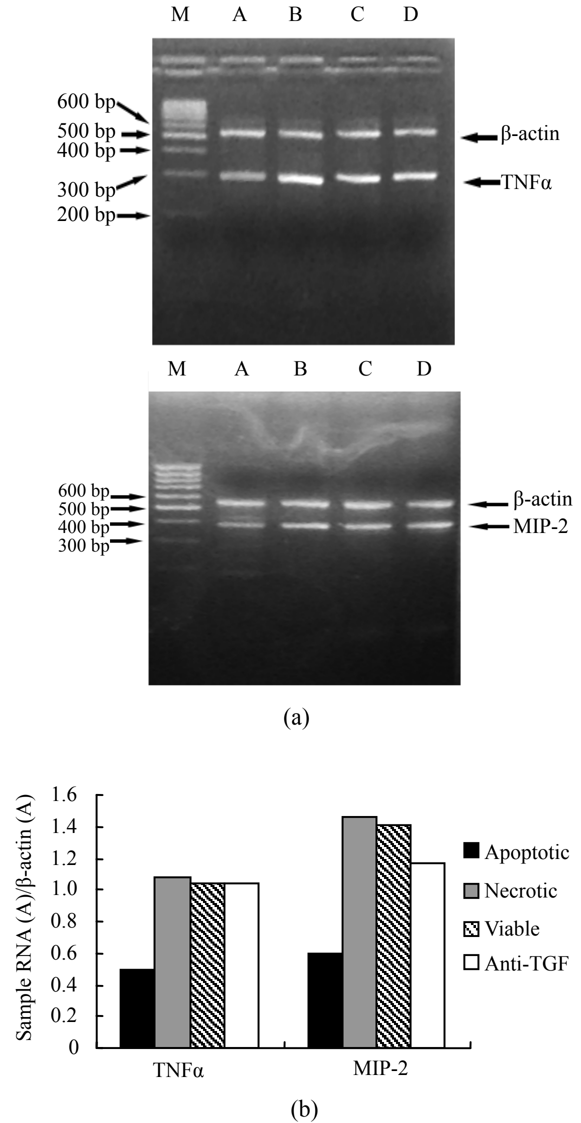


Fig.4 Analysis of MIP-2 and TNFα by RT-PCR (a) RNA isolated and assessed for MIP-2 and TNFα by RT-PCR. The suppression effects of MIP-2 and TNFα mRNA were observed in macrophages ingesting apoptotic Jurkat cells. TGFβ1 neutralizing antibody reversed the suppression effects. M, marker; A, phagocytosis of apoptotic Jurkat cells; B, phagocytosis of necrotic Jurkat cells; C, viable Jurkat cells; D, TGFβ1 neutralizing antibody; (b) Band intensity was expressed as relative absorbance units. For semiquantitative analysis, the ratio between the sample RNA and β-actin was calculated

phagocytic antigen-presenting cells, do not send co-stimulation signal to T cells, thus T cells become tolerant to antigens from apoptotic cells (Matzinger, 1994). We found that after being phagocytosed by macrophages, apoptotic cells actively regulate the biologic behavior of macrophages through increased TGFβ1 secretion and decreased proinflammatory cytokines production. It is well known that TGFβ1 is a potent immunosuppressant. The increased secretion of TGFβ1 may contribute to establish an immunosuppressive milieu that suppresses the activation of T lymphocyte. In our study, we found that the expression of CD69 and CD25 in T lymphocytes stimulated by ConA was inhibited through the increased TGF-β1 secretion, and that anti-TGF-β1 antibody can reverse the effects.

Based on our observation, we concluded that apoptotic cells actively alter the biological behavior of macrophages through increasing TGF-β1 secretion and thus make macrophages possess immunosuppressive property. These findings may provide us with new insights into the clearance of apoptotic cells.

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