

# Activation of Triggering Receptor Expressed on Myeloid Cells-1 Protects Monocyte from Apoptosis through Regulation of Myeloid Cell Leukemia-1

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## Abstract

**Background:** Triggering receptor expressed on myeloid cells-1 (TREM-1) can amplify the proinflammatory response and may contribute to the pathogenesis of inflammatory disease such as sepsis. However, the role of TREM-1 in monocyte fate and the detailed molecular mechanisms evoked by TREM-1 are unknown.

**Methods:** Adenoviruses overexpressing TREM-1 were constructed and transfected into a monocytic cell line. After activation of TREM-1 by agonist antibody with or without lipopolysaccharide, apoptosis was induced and assayed

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## What We Already Know about This Topic

- In an inflammatory environment, monocytes escape natural apoptosis, and their accumulation (such in sepsis) may favor detrimental organ responses
- TREM-1 levels are thought to play a key role in this phenomenon

## What This Article Tells Us That Is New

- In human monocytes, overexpression of TREM-1 afforded protection from apoptosis, an effect involving two kinases (ERK and PI3/Akt) and increased Mcl-1 expression
- In septic patients, TREM-1 levels were inversely correlated to the magnitude of apoptosis in monocytes

using flow cytometry. The signaling pathways downstream of TREM-1 were illustrated by inhibitory experiments. Pro-apoptotic/antiapoptotic protein levels were measured using immunoblot. In addition, the relationship between the expression levels of TREM-1 in monocytes and the magnitude of monocyte apoptosis were analyzed in septic patients.

**Results:** Activation of TREM-1 protected monocytes from staurosporine-induced apoptosis. This characteristic was also obtained under lipopolysaccharide stimulation. The protection of TREM-1 against monocyte apoptosis was abrogated after inhibition of extracellular signal-regulated kinase or v-akt murine thymoma viral oncogene homologue signaling. Cross-linking of TREM-1 remarkably up-regulated myeloid cell leukemia-1 protein level, and inhibition of extracellular signal-regulated kinase or v-akt murine thymoma viral oncogene homologue resulted in the reduction of myeloid cell leukemia-1 expression. Inhibition of myeloid cell leukemia-1 abolished the antiapoptotic effect of TREM-1. Furthermore, in septic patients, TREM-1 levels were inversely correlated to the magnitude of apoptosis in monocyte.

**Conclusions:** TREM-1 played an important role in apoptosis in monocytes. Activation of TREM-1 protected monocytic cells from apoptosis through activation of both extracellular signal-regulated kinase and v-akt murine thymoma viral oncogene homologue pathways and increased

expression of myeloid cell leukemia-1 protein. These findings provide a novel additional mechanism for TREM-1-mediated hyperinflammatory response in monocytes.

**M**ONOCYTES are key guardians of the innate immune system. They play central roles in initiation and resolution of inflammation through diverse biologic activities.<sup>1,2</sup> Under physiologic circumstance, circulating monocytes undergo spontaneous apoptosis after a short-time existence, whereas in an inflammatory environment, the apoptotic programs are suppressed and monocytes survive with a longer lifespan.<sup>3</sup> These surviving monocytes accumulate in the inflamed sites and provoke a detrimental inflammatory response, which ultimately exacerbates the disorders.<sup>1-3</sup> Despite the fact that recent studies have largely progressed in the signaling pathways for apoptosis and antiapoptosis,<sup>4-6</sup> the mechanisms by which monocytes escape the apoptotic fate upon inflammatory stimuli are insufficiently understood.

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface receptor belonging to the immunoglobulin superfamily. TREM-1 is selectively expressed on neutrophils and a subset of monocytes.<sup>7</sup> TREM-1 acts synergistically with Toll-like receptors and Nod-like receptors to amplify proinflammatory responses.<sup>8,9</sup> In murine models with endotoxemia and septic peritonitis, neutralization of TREM-1 prevented hyperinflammatory responses and death.<sup>10</sup> In humans, increased levels of TREM-1 and/or a soluble form of TREM-1 have been observed in biologic fluids of patients suffering from a variety of inflammatory conditions.<sup>11,12</sup> Furthermore, persistently elevated monocytic TREM-1 expression indicated a fatal outcome of septic shock, whereas TREM-1 expression on neutrophils was not altered in patients with septic shock or under lipopolysaccharide stimulation *in vitro*.<sup>13,14</sup> These findings suggest that monocytic TREM-1, rather than neutrophil TREM-1, plays important roles in inflammatory disease such as sepsis. However, whether the levels of monocytic TREM-1 have an impact on cell fate during inflammation remains unknown.

The present study was therefore designed to determine the role of TREM-1 in apoptosis *via* overexpression of TREM-1 in human acute monocytic leukemia cell line (THP-1) cells, and the detailed signaling pathway participating in this activity evoked by TREM-1 was elaborated. Moreover, the relationship between TREM-1 levels and the magnitude of apoptosis in monocytes from septic patients was also investigated.

## Materials and Methods

### Subjects

From July 1, 2012, to September 4, 2012, patients newly hospitalized in the surgical intensive care unit at the University Hospital of Zhejiang University (Hangzhou, Zhejiang Province, People's Republic of China) were screened for eligibility.

The septic patients were enrolled in the study. The diagnosis of sepsis met the criteria recommended by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference.<sup>15</sup> The following items for each septic patient were recorded: age, sex, Acute Physiology and Chronic Health Evaluation II score, and Sequential Organ Failure Assessment score. Results of microbiologic culture were also recorded. All of the medical records of each patient were reviewed by two senior intensivists, and agreement on the diagnosis was achieved in all cases. Notable exclusion criteria included the following: age younger than 18 yr, life expectancy less than 24 h, human immunodeficiency virus-positive, treatment with long-term corticosteroids within 6 months or short-term corticosteroids within 4 weeks, chemotherapy or radiation therapy within 4 weeks, or a history of organ transplantation. The study was approved by the local institutional review boards (Hangzhou, Zhejiang Province, People's Republic of China), and informed consent was obtained from all patients or their relatives.

### Isolation of Monocytes

Ten milliliters of EDTA-anticoagulated peripheral whole blood was drawn from each patient within 24 h after diagnosis of sepsis. Monocytes were isolated as described previously.<sup>16,17</sup> In brief, the blood was diluted 1:1 with phosphate-buffered saline (PBS). The diluted sample was added on the top of Hypaque-Ficoll solution (Sigma-Aldrich, St. Louis, MO) and centrifuged. The layer with mononuclear cells was removed and washed twice with PBS. Then, the cells were resuspended in Roswell Park Memorial Institute 1640 medium supplied with 2% fetal calf serum, and further separated from contaminating lymphocytes by adherence to plastic wells for 1 h at 37°C. Adherent monocytes were washed extensively with PBS to remove residual non-adherent cells. Meanwhile, neutrophils were prepared by aspiration of the neutrophil-rich pellet and hypotonic lysis of the contaminating erythrocytes.<sup>16,17</sup> Trypan blue staining revealed the isolated cells with greater than 95% viability.

### Real-Time Quantitative Polymerase Chain Reaction

Total RNA of the isolated monocytes and neutrophils was extracted using TRIzol<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA). Complementary DNA was generated by reverse transcription of 1 µg of total RNA. The expression levels of TREM-1 were quantified by real-time polymerase chain reaction using the housekeeping gene  $\beta$ -actin as an internal control. Quantification was performed by the  $2^{-\Delta\Delta C_T}$  method, as described previously.<sup>17</sup>

### Cells

THP-1 cells were maintained in Roswell Park Memorial Institute 1640 medium with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The 293A cells were grown in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells in

the exponential growth phase were used in the experiments. The culture medium and supplements were from GIBCO (Life Technologies, Grand Island, NY).

### Construction of Adenoviral Vector

Construction of adenoviral vector containing the gene coding TREM-1 was performed as described previously.<sup>18</sup> The recombinant adenoviral vector, Ad.TREM-1, was packed and amplified using the 293A cell line. A control vector, which did not contain any transgene, was constructed in the same way and named Ad.V. Viral titers were determined using Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA). A stock titer ranging from  $10^{11}$ – $10^{12}$  plaque-forming units/ml was applied in the following experiments.

### Expression of TREM-1 in THP-1 Cells

THP-1 cells ( $2 \times 10^6$  cells) were infected with Ad.TREM-1 or Ad.V. at high multiplicity of infection (*i.e.*, 250) in serum-free Roswell Park Memorial Institute 1640 medium. Two hours later, fetal bovine serum was added to a final concentration of 10% and the cells were cultured for another 72 h. Expression of TREM-1 in THP-1 cells was determined by Western blot and flow cytometric analysis.

### Stimulation of Transfected THP-1 Cells

To study TREM-1-mediated activation, a 24-well flat-bottom plate (Corning, Lowell, MA) was precoated with 10  $\mu$ g/ml agonist anti-TREM-1 antibody (MAB1278; R&D Systems, Minneapolis, MN) or an isotype control antibody (mouse IgG1; R&D Systems) or PBS at 4°C overnight. After washing twice with sterile PBS, the transfected cells were added to the wells, and the plate was centrifuged for 10 min at 1,200 rpm. Then, the cells were maintained for 24 h at 37°C with 5% carbon dioxide in air. Staurosporine (0.6  $\mu$ g/ml; Enzo Life Sciences, Farmingdale, NY) or dimethyl sulfoxide (Sigma-Aldrich) was added 4 h before the cells were collected for analysis.

For lipopolysaccharide (Sigma-Aldrich) stimulation, 100 ng/ml lipopolysaccharide was added simultaneously when the transfected cells were plated into the antibody-coated wells. To investigate the role of extracellular signal-regulated kinase (Erk) and/or phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homologue (Akt) in the TREM-1 signaling pathway, 50 mM mitogen-activated protein kinase inhibitor PD98059 (Sigma-Aldrich) and/or 100 nM PI3K inhibitor wortmannin (Sigma-Aldrich) were added to the transfected cells 1 h before the antibody stimulation.

To illustrate the importance of myeloid cell leukemia-1 (Mcl-1) in TREM-1-mediated protection against apoptosis, a cyclin-dependent kinase inhibitor, Roscovitine (Sigma-Aldrich), which could specifically down-regulate Mcl-1,<sup>19–21</sup> was added into the transfected cells simultaneously with staurosporine in a final concentration of 20  $\mu$ M.

### Flow Cytometry and Detection of Apoptosis

For measuring the magnitude of apoptosis in monocytes and neutrophils in the septic patients, 100  $\mu$ l of whole blood was incubated with 2 ml of lysing solution in each polypropylene tube for 15 min at room temperature. After being washed with PBS two times, the cells were resuspended in annexin V binding buffer, and incubated with 5  $\mu$ l of CD14 or 20  $\mu$ l of CD66 (BD Biosciences, San Jose, CA) in the appropriate tubes for 15 min at room temperature in the dark. After incubation with annexin V–fluorescein isothiocyanate (Biouniquer, Hong Kong, People's Republic of China) for another 10 min, 400  $\mu$ l of PBS was added to each tube for flow cytometric analysis.

Apoptosis in THP-1 cells was analyzed using an annexin V–fluorescein isothiocyanate apoptosis detection kit (Biouniquer) according to the manufacturer's instructions. Briefly, after washed with cold PBS, the cells were resuspended in 500  $\mu$ l of annexin V–fluorescein isothiocyanate binding buffer, and then 5  $\mu$ l of annexin V–fluorescein isothiocyanate and 5  $\mu$ l of propidium iodide were added. The cells were centrifuged gently and incubated for 10 min at room temperature in the dark. Each test contained appropriate controls. The data were acquired and analyzed using flow cytometry.

For TREM-1 expression analysis, Ad.TREM-1- or Ad.V-transfected cells ( $1 \times 10^6$ ) were blocked with 10% fetal bovine serum in PBS at room temperature for 30 min and then incubated with anti-TREM-1 antibody or mouse IgG1 isotype antibody in PBS with 3% (weight/volume) bovine serum albumin at room temperature for 1 h. After washing with PBS, the cells were incubated with Alex Fluor 555-conjugated goat anti-mouse secondary antibody (Life Technologies) at 4°C for 30 min. Finally, the cells were analyzed using an LSR II flow cytometer (BD Biosciences) with FlowJo software (Tree Stars, Ashland, OR).

### Western Blot Analysis

Cells were collected and lysed in lysis buffer (50 mM Tris [pH 7.4], 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin supplemented with 1 mM phenylmethanesulfonyl fluoride) at 4°C for 40 min. The lysate was centrifuged every 10 min, followed by centrifugation at 14,000 rpm for 15 min at 4°C. Cytoplasmic proteins were extracted using a cytoplasmic protein extraction kit (Beyotime, Shanghai, People's Republic of China) according to the manufacturer's instructions.

The protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). Fifty micrograms of cytoplasmic protein or 25–30  $\mu$ g of total protein was applied for detection of either cytoplasmic cytochrome *c* and proapoptotic/antiapoptotic proteins or TREM-1 protein, respectively. The proteins were denatured by heating at 70°C for 10 min in 4 $\times$  NuPAGE LDS sample buffer (Life Technologies), and separated by NuPAGE Bis-Tris gel

electrophoresis (Life Technologies). The proteins were blotted onto polyvinylidene fluoride membrane (Millipore, Billerica, MA). Then, the membrane was blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 and incubated overnight with the following antibodies: rabbit anti-Bcl-2, rabbit anti-Bcl-xL, rabbit anti-Bak, rabbit anti-Bax, rabbit anti-Mcl-1, and rabbit anti-cytochrome *c* (all from Epitomics, Inc., Burlingame, CA); goat anti-TREM-1 (D-20; Santa Cruz Biotechnologies, Santa Cruz, CA); and mouse anti- $\beta$ -actin (Sigma-Aldrich). The membrane was washed three times for 5–10 min each with Tris-buffered saline with 0.05% Tween-20. Antibody binding was visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce) after incubation with corresponding horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA).  $\beta$ -Actin was tested as a protein control.

### Statistical Analysis

Data are expressed as median with range or mean  $\pm$  SD where applicable. The relationship between the expression levels of TREM-1 and the magnitude of apoptosis was assessed by using the Spearman correlation test. Differences between the differently treated groups *in vitro* were analyzed by one-way analysis of variance followed by the Bonferroni multiple comparison test. Statistical analysis was performed using GraphPad Prism 5.00 for Windows (GraphPad Software,

Inc., La Jolla, CA). A two-tailed value of  $P < 0.05$  was considered statistically significant.

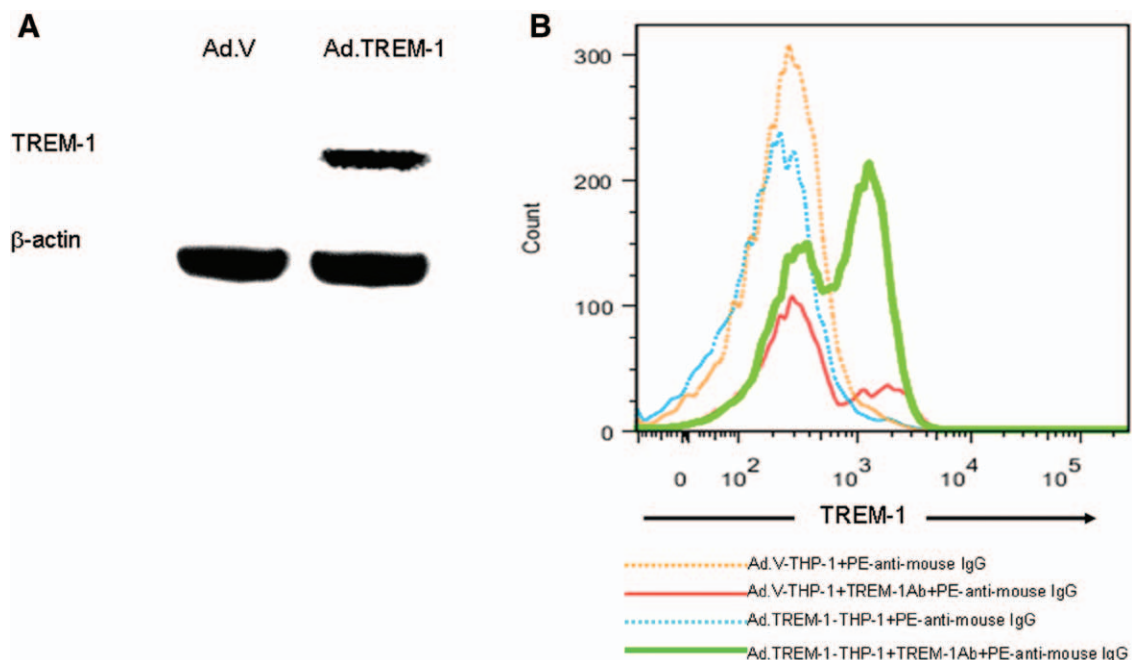
## Results

### Expression of TREM-1 Protein on THP-1 Cells

After infection with Ad.TREM-1 or Ad.V. in THP-1 cells for 72 h, both Western blot and flow cytometric analysis showed an obviously elevated level of TREM-1 in Ad.TREM-1-transfected THP-1 cells (fig. 1). Because of the transfection efficiency, two peaks were observed in the flow cytometric analysis, in which the left one represented the untransfected cells not expressing TREM-1 and the right one represented the transfected cells that did express TREM-1 (fig. 1B).

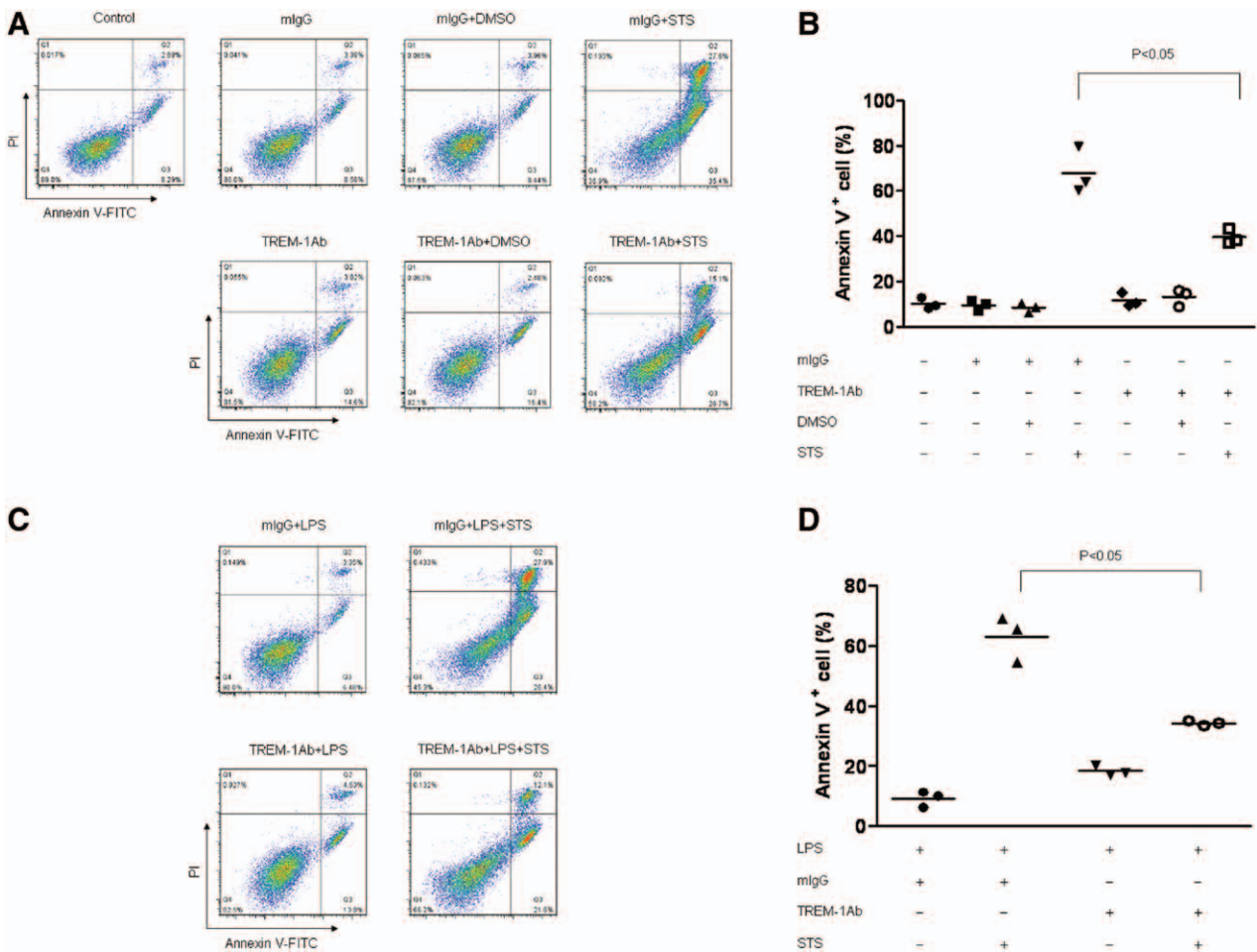
### Activation of TREM-1 Protected Monocytes from Apoptosis

To determine the effect of TREM-1 on apoptosis, Ad.TREM-1-transfected THP-1 cells were treated with agonist anti-TREM-1 antibody for 24 h and stimulated with 0.6  $\mu$ g/ml staurosporine for 4 h. Apoptosis analysis was performed by annexin V/propidium iodide staining and flow cytometry. Activation of TREM-1 significantly rescued the cells from apoptosis induced by staurosporine (fig. 2, A and B). However, TREM-1 activation did not change the fate of Ad.V-transfected THP-1 cells from staurosporine-triggered apoptosis (data not shown).



**Fig. 1.** Adenovirus expressing TREM-1 infection of THP-1 cells up-regulates TREM-1 expression. THP-1 monocytic cells were collected after 72 h of infection. (A) Ad.TREM-1- and Ad.V-infected cell lysates (30  $\mu$ g in each lane) were analyzed by immunoblot.  $\beta$ -Actin was used as a loading control. Results are representative of two independent experiments. (B) Ad.TREM-1- and Ad.V-infected THP-1 cells were performed by flow cytometric analysis. Results are representative of three independent experiments. Ad.TREM-1 = adenovirus expressing TREM-1; Ad.V = control adenovirus; PE = phycoerythrin; THP-1 = human acute monocytic leukemia cell line; TREM-1 = triggering receptor expressed on myeloid cells-1.





**Fig. 2.** Activation of TREM-1 protects monocytes from staurosporine-induced apoptosis. Adenovirus expressing TREM-1-infected THP-1 monocytic cells were cultured on TREM-1Ab or mouse IgG or phosphate-buffered saline-coated 24-well plate for 24 h, in combination with 0.6  $\mu$ M staurosporine or dimethyl sulfoxide for the last 4 h. Cells were stained with annexin V-fluorescein isothiocyanate/propidium iodide for flow cytometric analysis. Results are representative of three independent experiments (A). Data are presented as scatter plots with the mean imposed on the scatter (B). In some cases, 100 ng/ml lipopolysaccharide was added to mimic an inflammatory milieu and apoptosis was analyzed as described above. Results are representative of three independent experiments (C) and data are presented as scatter plots with the mean imposed on the scatter (D). DMSO = dimethyl sulfoxide; FITC = fluorescein isothiocyanate; LPS = lipopolysaccharide; mlgG = mouse IgG; STS = staurosporine; THP-1 = human acute monocytic leukemia cell line; TREM-1Ab = triggering receptor expressed on myeloid cells-1 antibody.

To mimic an inflammatory environment *in vitro*, 100 ng/ml lipopolysaccharide was added into the transfected THP-1 cells and the cells were treated as described above. Similarly, activation of TREM-1 dramatically reduced staurosporine-induced apoptosis upon lipopolysaccharide stimulation (fig. 2, C and D).

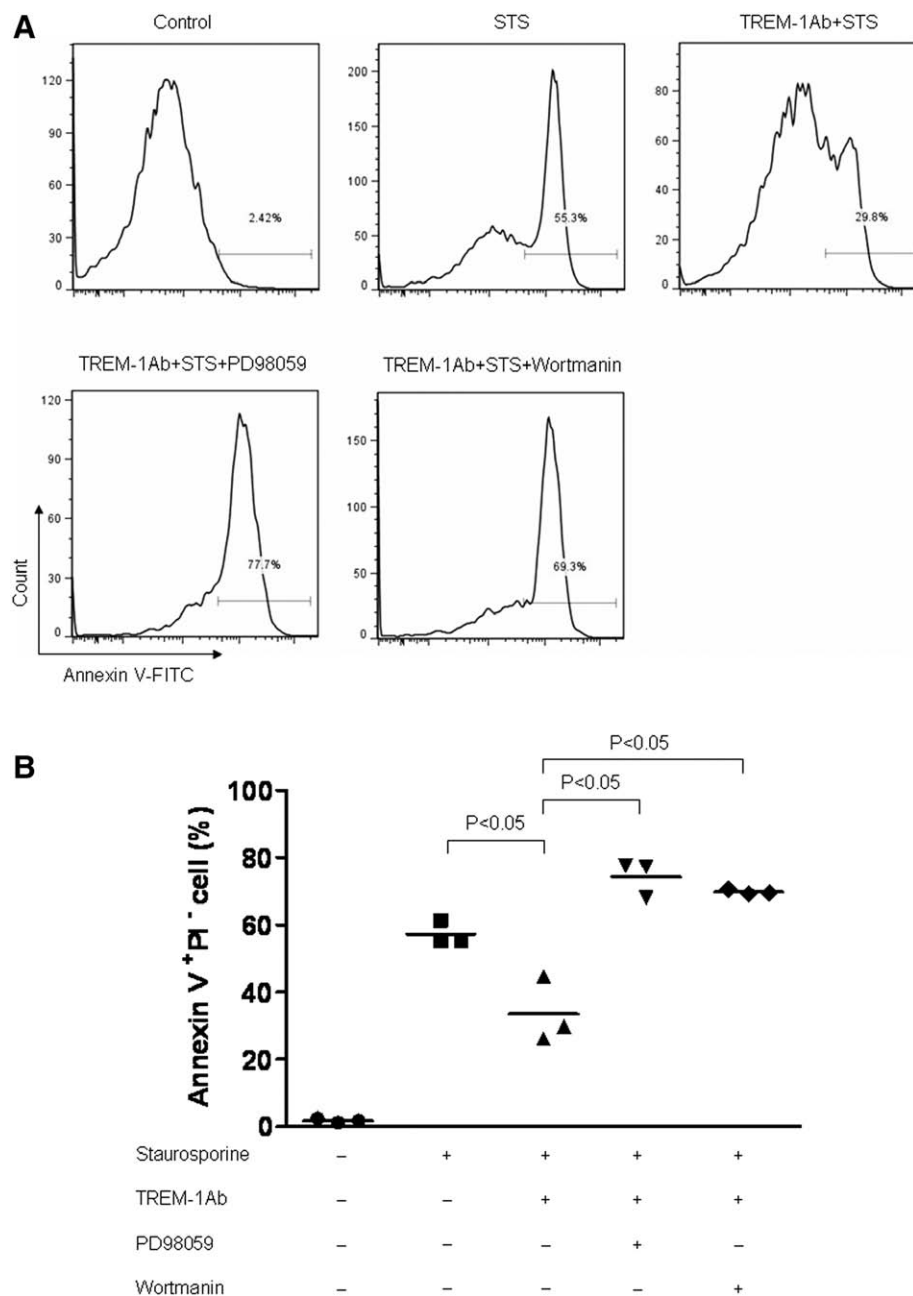
#### TREM-1-Promoted Survival of Monocytes Was Associated with Erk and PI3K/Akt Pathways

Activation of TREM-1 leads to tyrosine phosphorylation of several proteins, including Erk and PI3K/Akt.<sup>22,23</sup> To make certain which pathway might be involved in TREM-1-mediated protection against apoptosis, Erk and PI3K/Akt pathways were specifically inhibited by the mitogen-activated protein kinase inhibitor PD98059 and the PI3K inhibitor wortmannin before activation of TREM-1. Either inhibition of Erk or

inhibition of the PI3K/Akt pathway reversed the protective effect of TREM-1, causing the monocytes to enter into early apoptosis (fig. 3).

#### Activation of TREM-1 Up-Regulated Mcl-1 Expression in Monocytes

To find out which molecule might be critically responsible for TREM-1-mediated protection against monocytic apoptosis, the expression levels of proapoptotic/antiapoptotic proteins and cytoplasmic levels of cytochrome *c* were analyzed using Western blot analysis. As shown in figure 4A, activation of TREM-1 reduced staurosporine-induced cytochrome *c* release to the cytosol but did not influence the expression levels of the proapoptotic proteins Bak and Bax or the antiapoptotic proteins Bcl-2 and Bcl-xL. In contrast, activation of TREM-1 not only increased the basal expression

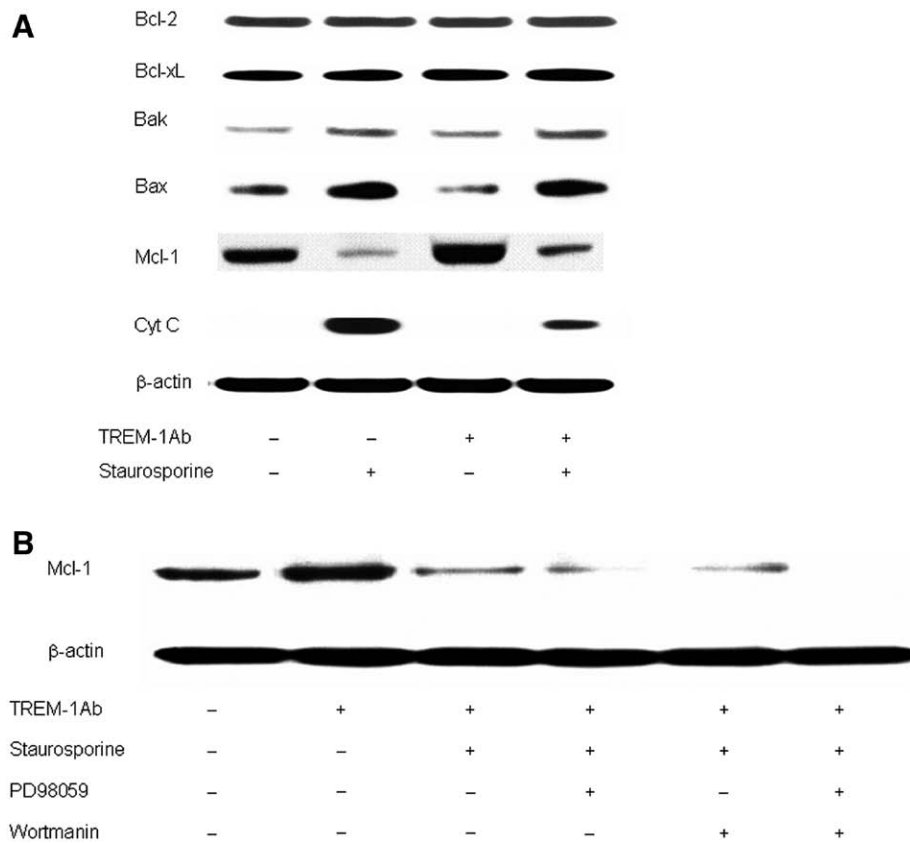


**Fig. 3.** The protective effect of TREM-1 on monocyte survival is mediated by Erk and Akt signaling. Adenovirus expressing TREM-1–infected THP-1 monocytic cells were cultured with 50 mM PD98059 (Erk inhibitor) or 100 nM wortmannin (Akt inhibitor) for 1 h. Then, the cells were added to TREM-1Ab– or phosphate-buffered saline–coated 24-well plate for 24 h, in combination with or without 0.6  $\mu$ g/ml staurosporine for the last 4 h. Cells were stained with annexin V–fluorescein isothiocyanate/propidium iodide and analyzed by flow cytometry. Results are representative of three independent experiments (A) and data are presented as scatter plots with the mean imposed on the scatter (B). Akt = v-akt murine thymoma viral oncogene homologue; Erk = extra-cellular signal–regulated kinase; FITC = fluorescein isothiocyanate; PI = propidium iodide; STS = staurosporine; THP-1 = human acute monocytic leukemia cell line; TREM-1Ab = triggering receptor expressed on myeloid cells-1 antibody.

level of Mcl-1 but also remarkably reversed staurosporine-triggered down-regulation of Mcl-1 in Ad.TREM-1–transfected cells.

Because inhibition of the Erk and PI3K/Akt pathways abolished the protective role of TREM-1 against apoptosis, we supposed that regulation of Mcl-1 expression

might be the downstream event of the Erk and PI3K/Akt pathways responsible for TREM-1–mediated protection of monocytic apoptosis. Western blot analysis showed that inhibition of the Erk and PI3K/Akt pathways either alone or jointly suppressed the expression level of Mcl-1 (fig. 4B).



**Fig. 4.** TREM-1-mediated pathways regulate Mcl-1 protein levels. (A) Adenovirus expressing TREM-1-infected THP-1 monocytic cells were cultured on TREM-1Ab- or phosphate-buffered saline-coated 24-well plate for 24 h, in combination with or without 0.6  $\mu$ g/ml staurosporine for the last 4 h. Cells were collected and lysed for Western blot analysis with related antibodies against proapoptotic/antiapoptotic proteins and cytochrome c. The result is representative of three independent experiments. (B) Adenovirus expressing TREM-1-infected THP-1 monocytic cells were cultured with 50 mM PD98059 and/or 100 nM wortmannin for 1 h. Then, the cells were added to TREM-1Ab- or phosphate-buffered saline-coated 24-well plate for 24 h, in combination with or without 0.6  $\mu$ g/ml staurosporine for the last 4 h. The Mcl-1 protein level in the cell lysate was detected using Western blot analysis. The result is representative of three independent experiments. Cyt C = cytochrome c; Mcl-1 = myeloid cell leukemia-1; THP-1 = human acute monocytic leukemia cell line; TREM-1Ab = triggering receptor expressed on myeloid cells-1 antibody.

#### **Inhibition of Mcl-1 Abrogated the Protection of TREM-1 against Monocytic Apoptosis**

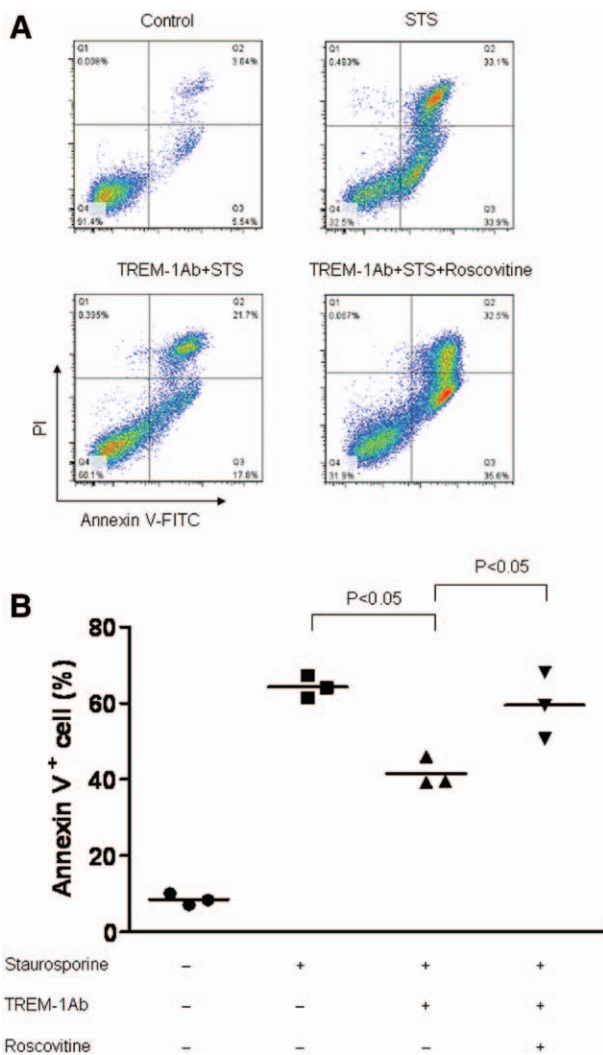
The role of Mcl-1 on TREM-1-mediated protection against monocytic apoptosis was further confirmed by an inhibitory experiment. Roscovitine, a cyclin-dependent kinase inhibitor, has been found to down-regulate the survival protein Mcl-1 at both the message RNA level and the protein level.<sup>19–21</sup> In the current study, treatment of the agonist-activated Ad.TREM-1-transfected cells with 20  $\mu$ M Roscovitine for 4 h did suppress the protein levels of Mcl-1 (data not shown). This suppression further abrogated the protective effect of TREM-1 against apoptosis (fig. 5), confirming that Mcl-1 was a key molecule accounting for TREM-1-mediated signaling pathway against apoptosis.

#### **Correlation of the TREM-1 Levels and the Magnitude of Apoptosis in Monocytes in Septic Patients**

To further confirm the role of TREM-1 in apoptosis observed above, the relationship between the expression

levels of TREM-1 and the magnitude of monocyte apoptosis was assessed in septic patients. During the study period, 34 patients were initially approached for screening of sepsis. After review by two senior intensivists, 26 patients agreed to be diagnosed as septic and were enrolled in the study. The basic characteristics of the septic patients are listed in table 1. Peritonitis (26.9%) and pneumonia (26.9%) were the most frequent initial diagnoses for the patients. Other diagnoses included obstructive cholecystitis, severe acute pancreatitis, and others. Microbiologic inspection evidenced that 16 patients experienced a documented infection. The other 10 patients showed clinically suspected infections as stated by the senior intensivists. The major sources of infection were the respiratory tract (42.3%) and the abdomen (23.1%).

The expression levels of TREM-1 and the magnitude of apoptosis in monocytes were successfully detected in all of the patients. Correlation analysis showed that the expression levels of TREM-1 were inversely correlated to



**Fig. 5.** Specific inhibition of Mcl-1 abrogates the protective effect of TREM-1 against monocyte apoptosis. Adenovirus expressing TREM-1-infected THP-1 monocytic cells were cultured on TREM-1Ab- or phosphate-buffered saline-coated 24-well plate for 24 h, in combination with or without 0.6  $\mu$ g/ml staurosporine and 20  $\mu$ M Roscovitine for the last 4 h. Cells were stained with annexin V–fluorescein isothiocyanate/propidium iodide and analyzed by flow cytometry. The result is representative of three independent experiments (A) and data are presented as scatter plots with the mean imposed on the scatter (B). FITC = fluorescein isothiocyanate; Mcl-1 = myeloid cell leukemia-1; STS = staurosporine; TREM-1Ab = triggering receptor expressed on myeloid cells-1 antibody.

the magnitude of apoptosis in monocytes ( $r = -0.49$ ,  $P = 0.01$ ) (fig. 6A).

In addition, the expression levels of TREM-1 and the magnitude of apoptosis in neutrophils were measured in 22 of 26 septic patients. Although the expression levels of TREM-1 were not significantly correlated with the magnitude of apoptosis ( $r = -0.28$ ,  $P = 0.21$ ) (fig. 6B), a trend that the neutrophils with higher expression levels of TREM-1 have less magnitude of apoptosis was observed.

**Table 1.** Basic Characteristics of the Septic Patients

Characteristics	Value
No. of patients	26
Age, yr	
Median	71.5
Range	28–87
Male sex, No. (%)	15 (57.7)
SOFA score	
Median	6
Range	1–12
APACHE II score	
Median	16
Range	5–27
Length of ICU stay, days	
Median	4
Range	1–16
ICU survival, No. (%)	22 (84.6)

APACHE II = Acute Physiology and Chronic Health Evaluation II; ICU = intensive care unit; SOFA = Sequential Organ Failure Assessment.

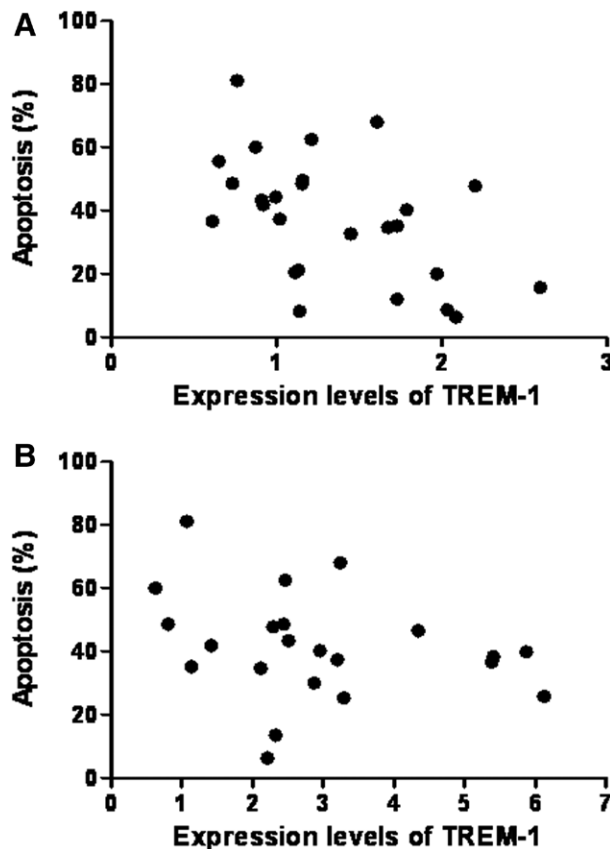
## Discussion

The current study found that, in septic patients, TREM-1 levels were inversely correlated to the magnitude of apoptosis in monocytes. Overexpression of TREM-1 in monocytes protected the cells from apoptosis. This protective effect of TREM-1 was attributed to elevated expression of Mcl-1 through activation of both Erk and PI3K/Akt pathways.

In chronic inflammatory diseases and in the tumor microenvironment, extended survival of monocytes leads to a persistent inflammatory milieu, which contributes to the pathogenesis of diseases.<sup>1–3</sup> An early increase in the apoptosis of blood monocytes was associated with improved survival in patients with varying degrees of sepsis.<sup>24</sup> Accumulated studies suggest a critical role of monocytic TREM-1 in inflammation; however, direct evidence concerning TREM-1 on the fate of monocytes upon inflammation was absent. Using adenovirus-mediated overexpression of TREM-1 in monocytes, this study demonstrated that activation of TREM-1 protected monocytes from apoptosis. This protection was also effective under lipopolysaccharide stimulation, further certifying a direct role of TREM-1 on monocyte survival in inflammation. Furthermore, the present study first observed that monocytes with higher expression levels of TREM-1 had less magnitude of apoptosis in septic patients. In light of the role of monocytes and apoptosis in the context of the pathophysiology of sepsis, this result suggests that TREM-1 may participate in the pathogenesis of sepsis *via* regulating the apoptotic process of monocytes. Considered together, these findings provide insight into an additional mechanism for TREM-1 amplifying the inflammatory response *via* promoting inflammatory cell survival.

To date, most studies regarding the TREM-1 pathway have focused on its regulation of inflammation or





**Fig. 6.** Correlation of the expression levels of triggering receptor expressed on myeloid cells-1 (TREM-1) and the magnitude of apoptosis in monocytes ( $r = -0.49$ ,  $P = 0.01$ ) (A) and neutrophils ( $r = -0.28$ ,  $P = 0.21$ ) (B) in septic patients.

its synergistic role with Toll-like receptors and Nod-like receptors.<sup>7,11,22</sup> TREM-1-induced effects in monocytes are mediated by tyrosine phosphorylation of Erk, phospholipase C- $\gamma$ , Akt, and the release of intracellular calcium, in which the Erk and Akt pathways are two important signaling cascades regulating cell survival.<sup>7,22</sup> Studies have demonstrated that Erk and PI3K/Akt signaling orchestrates antiapoptotic mechanisms in multiple steps of apoptotic processes.<sup>25–28</sup> In this study, inhibition of either the Erk pathway or the PI3K/Akt pathway impaired the protective effect of TREM-1 against apoptosis of monocytes, suggesting that both Erk and Akt signaling activated *via* cross-linking TREM-1 contributes to the protection of TREM-1 on monocyte survival.

Cytochrome *c* release to cytosol plays a central role in the mitochondrial apoptotic pathway, which is controlled by Bcl-2 family members.<sup>29</sup> Our results demonstrated that activation of TREM-1 inhibited staurosporine-induced cytochrome *c* release. This allowed us to propose that TREM-1 and its downstream Erk and Akt signaling most likely interfere with the Bcl-2 family members to promote monocyte survival. However, under staurosporine stimulation, neither the proapoptotic proteins Bax and

Bak nor the antiapoptotic molecules Bcl-2 and Bcl-xL were affected after TREM-1 activation. Interestingly, cross-linking of TREM-1 not only increased the basal level of Mcl-1 protein but also remarkably elevated staurosporine-down-regulated Mcl-1 protein level. Mcl-1 is an antiapoptotic member of the Bcl-2 family with a short half-life and is a highly regulated protein. Regulation of Mcl-1 expression can occur at multiple levels.<sup>30,31</sup> Previous studies have proved that the PI3K/Akt-dependent pathway could modulate Mcl-1 expression at the transcriptional level, and Erk signaling could regulate Mcl-1 stability and prolong its half-life.<sup>32–34</sup> Consistent with these reports, the importance of TREM-1-activated Erk and Akt signaling pathways in regulating Mcl-1 was supported by the observation that inhibition of Erk and/or Akt resulted in the reduction of Mcl-1 expression and loss of cell viability. Furthermore, inhibition of Mcl-1 abrogated the protective effect of TREM-1 on cellular lifespan. Overall, these observations proved an essential role of Mcl-1, regulated by both Erk and PI3K/Akt pathways *via* cross-linking of TREM-1, in promoting monocyte survival.

TREM-1 is also expressed on neutrophils. However, the expression levels of TREM-1 on neutrophils in septic patients were comparable to those in nonseptic patients and healthy volunteers.<sup>13</sup> In the current study, a correlation between the expression levels of TREM-1 and the magnitude of apoptosis in neutrophils in septic patients was also not observed. Further studies to determine this relationship in a large cohort and to demonstrate the role of TREM-1 in neutrophils using an appropriate cell line are warranted.

In summary, the present study found that TREM-1 levels were inversely correlated to the magnitude of apoptosis in monocytes in septic patients. Activation of TREM-1 exerted a protective effect against apoptosis of monocytic cells through activation of both Erk and PI3K/Akt pathways and an increase of the Mcl-1 protein level. TREM-1 protecting monocytes from apoptosis not only illustrates a novel additional role of TREM-1 in the pathophysiology of sepsis, but also provides potential therapeutic implications for certain inflammatory diseases in which TREM-1 plays important roles.

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