J Microbiol Immunol Infect
2002;35:71-77



# Apoptosis contributes to the decrement in numbers of alveolar macrophages from rats with polymicrobial sepsis

**Min-Chi Lu[1,3], Tzu-An Liu[1], Miau-Rong Lee[2], Lina Lin[4], Weng-Cheng Chang[1]**

*Departments of [1]Physiology and [2]Biochemistry, School of Medicine, China Medical College; [3]Division of Infectious Diseases, Department of Medicine, China Medical College Hospital; and [4]National Taichung Institute of Technology, Taichung, Taiwan, ROC*

Received: August 30, 2001 Revised: January 20, 2002 Accepted: February 5, 2002

To investigate the effects of sepsis-related acute lung injury on the events of alveolar macrophages apoptosis and phagocytosis, cecal-ligated-and-punctured male Sprague-Dawley rats were employed as sepsis model. At the early (9 h) and late (20 h) stages of sepsis, cecal-ligated-and-punctured and sham-operated animals were sacrificed and their lungs were removed. Alveolar macrophages were isolated by bronchoalveolar lavage and counted. The results showed that the purity of alveolar macrophages from both groups was over 98% as stained by Giemsa. The number of alveolar macrophages in late-stage septic rats significantly decreased. Alveolar macrophages apoptosis was then evaluated by labeling with fluorescein-conjugated annexin-V and exclusion of propidium iodide. There were minimal levels of baseline apoptosis in sham-operated rats. Compared with that of sham-operated rats, cecal-ligated-and-puncture operation resulted in 2.5- and 3.2-fold time-dependent increases in the amount of apoptotic alveolar macrophages in early- and late-stage septic animals, respectively. Among cecal-ligated-and-punctured and sham-operated rats of 9 and 20 h, the ability of alveolar macrophages to phagocytize opsonized fluorescence particles did not change significantly. However, the total alveolar macrophages phagocytic capacity of septic animals reduced due to the decrease in the number of alveolar macrophages. We conclude that apoptosis contributes to the decrement in the number of alveolar macrophages in cecal-ligated-and-punctured rats. Considering that alveolar macrophages have important roles in the defense and immunoregulation of the lungs, these results suggest that the defensive ability of septic lungs may be reduced, and could explain, at least in part, the increased susceptibility of septic lungs to superimposed infections.

**Key words:** Acute lung injury, alveolar macrophage, apoptosis, sepsis

Sepsis has been one of the major causes of illness and death in the elderly and patients in the intensive care unit [1]. In spite of early intervention and aggressive treatment, sepsis still represents a major threat to hospitalized patients with a mortality approaching 50%. For patients who survive the initial episodes, complications may occur and result in a devastating condition [1]. The lung is one of the vital organs that are frequently injured during the process of sepsis [2]. The sepsis-induced impaired respiration often leads to the complication of adult respiratory distress syndrome, the most severe form of acute lung injury (ALI) [3,4]. Adult respiratory distress syndrome develops in 20% to 50% of patients with sepsis, and increases the mortality of sepsis to more than 50% to 80% [5].

Septic patients are especially susceptible to the development of respiratory tract infections, and they manifest more severe illness, which presumably is due to the insufficient defense and overwhelming lung injury. Alveolar macrophages (AM) are the first-line defenders of the lungs [6]. They are constantly exposed to both airborne and blood-borne pathogens and take essential roles against infections because of their capability to phagocytize and kill invading micro-organisms. During the initial phase of lung inflammation, recruitment of additional leukocytes is necessary when there are not enough local leukocytes for an adequate response. To induce and potentiate inflammatory and immune processes, AM release the required cellular mediators, such as tumor necrosis factor (TNF)-α and eicosanoids [7,8]. As a result, the amount and function of AM must be properly regulated. The critical role of AM in lung defense against infection has been shown by the high frequency of pneumonia in severely neutropenic patients and in cases of lung macrophage dysfunction [9].

Programmed cell death, namely apoptosis, is the process of cell death that is genetically determined,

*Corresponding author: Dr. Weng-Cheng Chang, Department of Physiology, School of Medicine, China Medical College, 91, Shuei-Shieh Road, Taichung, 40421, Taiwan, ROC. E-mail: lumin chi@mail.cmc.edu.tw*

tightly controlled, and has distinctive morphological and biochemical features [10]. It has been demonstrated that apoptosis is important in the events of tissue injury, for example, the decrease in cellularity during the transition between granulation tissue and scarring during the development of fibrogenic lung disease [11]. Apoptosis occurs in sepsis [12]; however, its importance in the development of sepsis-related ALI is not completely understood. Among all the experimental models of sepsis and sepsis-like states, cecal ligation and puncture (CLP) in rats seems to mimic many events occurring in human sepsis. Therefore, in this study, we examined the total numbers of lavaged AM from early as well as late stages of CLP-induced sepsis rats as compared with those of sham-operated ones. Furthermore, we explored the degree of AM apoptosis to explain the changes of AM cellularity and the phagocytic ability of AM from animals of different septic stages.

## Materials and Methods

### Polymicrobial sepsis model

Respiratory disease-free 200- to 250-g male Sprague-Dawley rats were obtained from the Laboratory Animal Center of National Science Council (Taipei, Taiwan). Animals were housed under specific pathogen-free conditions at the Medical Sciences Building Animal Center of China Medical College (Taichung, Taiwan), and standard rat chow and water *ad libitum* were provided for at least 2 weeks before initiation of the experimental procedures. Before the experiments, rats were fasted overnight for about 10 h. Cecal ligation and puncture were performed as described in the literature [13] with some modification. Rats were anesthetized with ether, a 2-cm midline abdominal incision was made and the cecum was exposed. The cecum was then ligated using 4-0 silk just distal to the ileocecal valve without interfering the ileocecal content passage. Two holes were punctured with an 18-gauge needle at the approximately one third bottom of the cecum with leakage of inner contents out of cecum to ensure the patency of the punctured holes. The abdominal incision was closed in 2 layers using 6-0 monofilament Nylon, and normal saline (40 mL/kg body weight) was injected subcutaneously over nuchal area. The animals were allowed to recover in their cages. Sham-operated rats followed the same surgical procedures except that the cecum was neither ligated nor punctured. Early and late stages of sepsis refer to those animals sacrificed at 9 and 20 h, respectively, after CLP. For experiments on sham and sepsis in pair, cell number studies employed 7 and 4 animals and apoptosis and phagocytosis studies used 4 and 3 animals for 9 and 20 h time points, respectively.

### Bronchoalveolar lavage and isolation of alveolar macrophages

At 9 or 20 h post-operation, the rats were exsanguinated, and bronchoalveolar lavage of the lungs was performed using a polyethylene tube (PE-240, Clay Adams, Parsippany, NJ, US) wedging in the mid-portion of the trachea. About 105 mL of 4°C phosphate-buffered saline (PBS) solution were instilled in aliquots of 5 to 6 mL through the lumen of the tube. The lavage fluid was then gently aspirated with a syringe, and collected into a sterile bottle for cell isolation. A total of 18 to 20 times of lavage and mild message was applied to the lungs.

The lavage fluid from each animal (about 100 mL) was pooled and centrifuged at 1400 rpm for 10 min at 4°C. The cell pellet was washed with PBS, spun again at 1400 rpm for 10 min at 4 °C, and resuspended in 20 mL of PBS [14]. Total recovered cell number was determined by a hemacytometer and the yield was expressed as cells per rat. Immediately after lavage, cells were cytocentrifuged on glass slides at a concentration of $3 \times 10^4$ cells per slide with the use of Shandon cytospin 3 (Cheshire, England). After slides being airdried, the purity of lavaged cells was assessed by Giemsa stain and verified by nonspecific esterase stain. It was determined that AM comprised more than 98% of the cells in all experiments that follow.

### Assay for alveolar macrophage apoptosis

Evidence of apoptosis was determined by an apoptosis kit (Boehringer Mannheim, Mannheim, Germany) [15] evaluating the fluorescein-labeled annexin-V binding and the exclusion of propidium iodide (PI) by AM as described in the literature [16]. Alveolar macrophages obtained from CLP and sham-operated rats were resuspended in PBS at a concentration of $1 \times 10^6$ cells/mL and incubated in the presence of fluorescein-labeled annexin V, PI, or both at the same time. Flow cytometry analysis was performed on a Becton Dickson FACScalibur (Mountain View, CA, US) as described in the literature [17]. Excitation was at 488 nm (100 mW) with a Coherent 6W argon-ion laser (Becton Dickinson). For each particle, emission was measured with photomultipliers with $520 \pm 20$ and $620 \pm 35$-nm band-pass filters for fluorescein. Data were collected as 10 000 event list mode files and analyzed with CELLQuest software (Becton Dickinson Diagnostic Instrument System, Sparks, MD, US). Experiments were performed twice to guarantee reproducibility.

### Measurement of alveolar macrophage phagocytosis

Alveolar macrophages phagocytosis was determined as described in the literature [18]. The prewashed SPHERO carboxyl fluorescent (FL) particles (0.85 μm in diameter; PharMingen, San Diego, CA, US) were opsonized with fresh rat serum and diluted in RPMI 1640. Stored at 4°C, the prepared particles were sonicated for 1 min before use. The freshly isolated AM were incubated with FL particles at 37°C and shaken at 150 rpm. After 30 min of incubation, the phagocytosis by AM was stopped and the cells were fixed with paraformaldehyde solution. Cells were then washed in PBS to eliminate freely floating particles. A FACScalibur flow cytometer was then employed to measure the phagocytosis of FL particles by AM. Analyses were performed by counting 10 000 macrophages per sample. The percentage of phagocytic cells was defined as the percentage of macrophages that ingested one or more FL particles. Again, data were collected as 10 000 event list mode files and analyzed with CELLQuest software.

### Data and statistical analysis

Data from all experiments was expressed, where applicable, as means ± SEM. The data means of sham and sepsis animals at 9 or 20 h postoperation were compared by using the paired Student's t test and those of 9 and 20 h with the same surgical procedure were analyzed by using the unpaired Student's *t* test. In all comparisons, a *p* value of less than 0.05 was considered significant.

## Results

In the septic groups, most rats appeared only mildly ill, including fever, 9 h following CLP. Previous studies revealed that blood cultures at early septic stage [12]. Subsequently, the rats became lethargy and tachypneic, moved about less, and finally stopped drinking. A mortality of more than 90% was established 3 days after CLP operation in our preliminary experiments (data not shown) as well as in the literature [12].

### Cells recovered from bronchoalveolar lavage fluids

In all sham and septic rats, the recovered fluid volume was about 95% of the input volume. For each rat, about 100 mL of lavage fluid was collected and the isolated AM represented the majority of lung AM population, as described previously [19]. The purity of AM from lavage fluid was always over 98% by microscopic examination of cytocentrifuge preparation stained with a Giemsa stain and, for verification, with a non-specific esterase stain. The results from repeated experiments showed that the numbers of total AM obtained from 20-h septic lungs tended to decrease as compared with those from 9-h septic groups ($p$=0.058) (Fig. 1). Moreover, the AM number from 20-h septic rats was significantly less than that from 20-h sham ones ($p$<0.05) (Fig. 1).

### Analysis of alveolar macrophage apoptosis

The progressive decrease of AM cell number could be due to cell death during the process of sepsis. Under light microscopic examination, characteristic apoptotic features, including cell shrinkage and chromatin condensation, were evident in a lot of Giemsa-stained apoptotic AM from 9- and 20-h septic rats (Fig. 2). Cells from sham-operated animals showed only minimal apoptotic pictures. However, such traditional morphological study is not sensitive enough and is difficult for precise evaluation. For quantitative

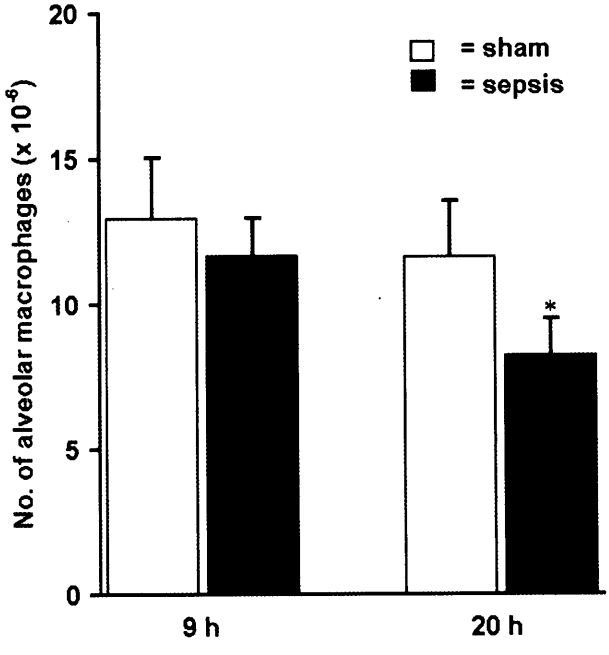


**Fig. 1.** The numbers of alveolar macrophages from sham and CLP-sepsis rats. At 9 h (early stage) or 20 h (late stage) post-operation, bronchoalveolar lavage was performed and alveolar macrophages was recovered. Total recovered cell number was determined by counting cells with the use of a hemacytometer and the yield was expressed as total isolated cells per rat. Results are represented as mean ± SEM (n = 7 in 9 h groups; n = 4 in 20 h groups). The data means of sham and sepsis animals were compared by using the paired Student's t test; means of different treatments at the same hour post-operation were compared by using the unpaired Student's *t* test.
*Significantly different from 20 h sham group ($p$<0.05).

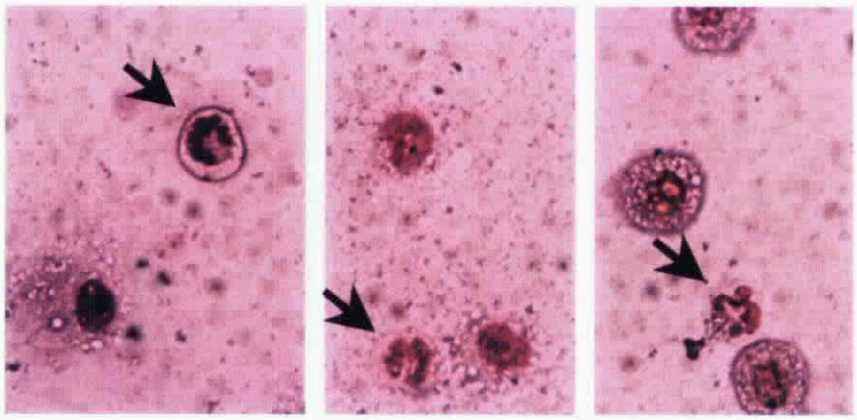

**Fig. 2.** Representative apoptotic picture of Giemsa-stained alveolar macrophages in 20-h septic rats. A lot of apoptotic cells (arrow) were observed under light microscopic examination at 400x magnification. Characteristic apoptotic features, including cell shrinkage and chromatin condensation, were evident. Cells from 20-h sham-operated animals showed only minimal levels of apoptosis (not shown).

investigation, AM retrieved by lung lavage from sepsis and sham animals at 9 h and 20 h post-CLP and sham operation, respectively, were assessed for their binding of fluorescein-labeled annexin V and exclusion of PI. Representative profiles of light-scattering properties (forward side scattering and side scattering) from AM of 20 h sham-operated and septic rats are demonstrated in Figure 3A. Figure 3B shows the uptakes of annexin V and PI by these AM. Cells that were stained positive for annexin V and negative for PI were undergoing apoptosis. As shown in Figure 4, AM retrieved from 9- and 20-h sham animals underwent minimal and similar levels of baseline apoptosis, with mean ± SEM of 9.7% ± 2% and 9.7% ± 2.5%, respectively. Compared with 9- and 20-h sham rats, CLP operation resulted in significant (2.5- and 3.2-fold) increases in the number of apoptotic AM from their respective septic animals. Moreover, there is a time-dependent increase in the amount of apoptotic AM during the progression of sepsis from early (9 h; 24.4% ± 2.2%) to late stages (20 h; 30.7% ± 0.9%) ($p<0.05$).

## Phagocytosis assessment

To further examine the phagocytic capability of septic and sham AM, opsonized carboxyl FL particles were employed. In a preliminary experiment, the lavaged cells were incubated with FL particles and then cytocentrifuged onto glass slides with the use of a cytospin. It was demonstrated that most of the particles were phagocytized into AM cytoplasm and few adhered to the surface of the cells as observed by a fluorescence microscopy (data not shown). Representative flow cytometric profiles of phagocytosis by AM are shown in Figure 5. Histograms of cell number versus FL intensity from AM of 9-h sham-operated (Fig. 5A) and 9-h CLP-sepsis (Fig. 5B) animals were plotted. No FL particle treatment cells (thick lines) are specified as the negative control. With FL particle incubation (thin line),

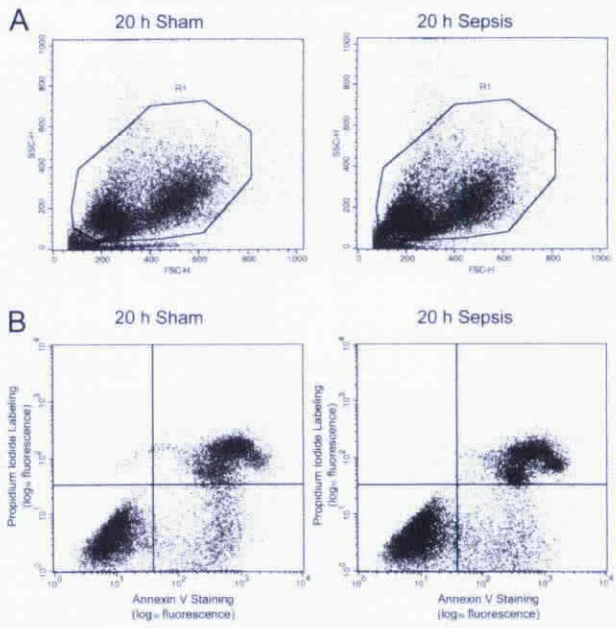


**Fig. 3.** Flow cytometric analysis of alveolar macrophage apoptosis. **(A)** Representative profiles of light-scattering properties (forward side scattering [FSC] and side scattering [SSC]) from alveolar macrophages of 20-h sham-operated and septic rats are demonstrated. **(B)** The uptakes of annexin V and propidium iodide by alveolar macrophages are shown. Cells that were stained positive for annexin V and negative for propidium iodide (in the right lower quadrant) were undergoing apoptosis.

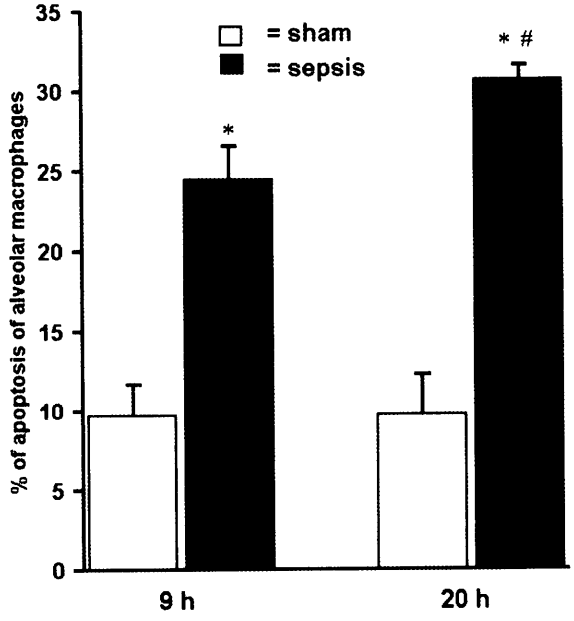


**Fig. 4.** The apoptosis of alveolar macrophages from 9 and 20 h rats of both sham and sepsis rats. The apoptotic cells were determined by their binding with fluorescein-labeled annexin V and absence of propidium iodide staining, which were analyzed by a flow cytometer. Data are expressed as an apoptotic percentage of the counted alveolar macrophages, and the results represent the mean ± SEM (n = 4 in 9 h groups and 3 in 20 h groups).
*$p<0.05$ versus corresponding 9- or 20-h sham
#$p<0.05$ versus 9-h sepsis.

a non-ingesting population on the left and another particle-ingesting population on the right can be clearly distinguished. Among sepsis and sham-operated rats of 9 and 20 h, the particle-ingesting AM population did not change significantly (Fig. 6).

## Discussion

Although quite a few studies have demonstrated that apoptosis occurs in cells of various organs in septic animals [15,20], this is the first link of AM cell loss, as a possible contributing factor causing deteriorated lung defense in sepsis, to apoptosis. In this study, AM from septic rat lungs exhibited an increase in the percentage of apoptosis. With the progression from early to late stages, the change in AM apoptotic proportion became more prominent and was accompanied by a decrease in cell number of lavaged AM. It is likely that the change in AM apoptosis justified most of the change in AM cellularity.

Several lines of evidence in this investigation supported this conclusion. The ALI-associated increase in the apoptosis of septic AM developed as early as 3 h after CLP (data not shown), and the statistically significant decrease in AM number was present at 20 h in this study. This result is compatible with the observation that the annexin-V recognizes the early process of apoptosis and the completion of apoptotic process needs more than 4 to 6 h [10]. Apart from the increased amount of cell death, the decrement in AM number could be due to inadequate supply of their precursor monocytes from peripheral circulation. Seeing that the half life of AM in the lung is in the range of months, there would only be few, or negligible,

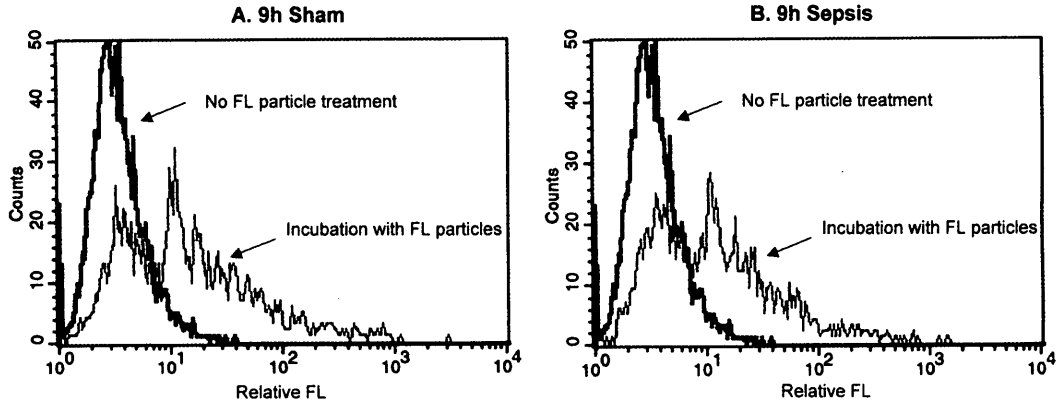


**Fig. 5.** Representative flow cytometric profiles of phagocytosis by alveolar macrophages. Lavage cells were incubated with opsonized fluorescence particles and analyzed for the particle-ingested cell populations. Histograms of cell number versus fluorescence intensity from alveolar macrophages of **(A)** 9-h sham-operated; and **(B)** 9-h CLP-sepsis animals were plotted. No fluorescence particle treatment cells (thick lines) are designated as the negative control. With fluorescence particle incubation (thin lines), a non-ingesting population on the left and another particle-ingesting population on the right can be clearly distinguished.

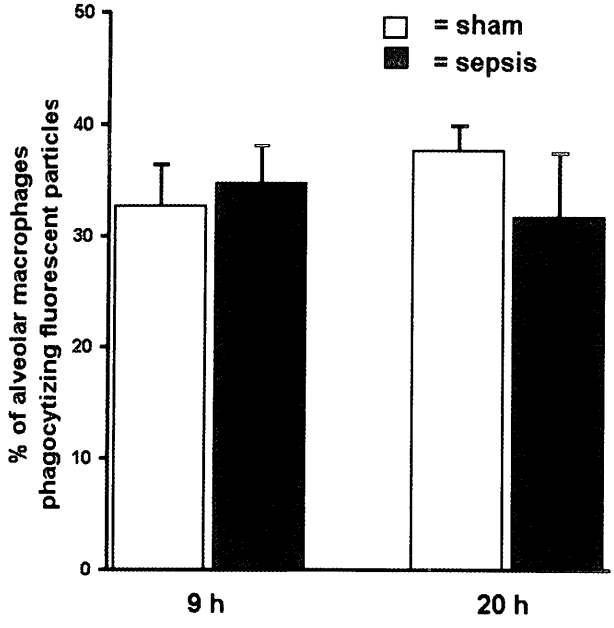


**Fig. 6.** The phagocytosis of alveolar macrophages from 9- and 20-h rats of both sham and sepsis groups. The apoptotic cells were determined by their ability to phagocytize opsonized fluorescent particles, which were than analyzed by a flow cytometer. Data are expressed as a phagocytosis percentage of the counted alveolar macrophages, and the results represent the mean ± SEM (n = 4 in 9 h groups and 3 in 20 h groups). No significant differences between groups were observed.

decrease in AM number if the transformation of monocytes into AM was stopped during the initial 20 h of sepsis. Necrosis is another possible explanation for the sepsis-related cell loss; however, we did not observe obvious changes in AM necrosis in 9- and 20-h septic rats as compared with that in sham ones (data not shown). It would be plausible to infer from these data that an increase in apoptosis was responsible for the sepsis-related decrease of AM cell number.

A few studies have investigated the functional changes of AM from animals with systemic sepsis or sepsis-like state. For AM, endotoxin resulted in decreases of phagocytosis activity in rats [21] and dogs [22], bactericidal activity in rats [23], and TNF production in human and mice [24]. On the other hand, Ayala *et al* [25] demonstrated that CLP operation caused little activation of mice AM in terms of interleukin (IL)-1, IL-6, and TNF production. In this study, the ability of AM to phagocytize opsonized FL particles was not significantly different among rats of sham and sepsis at 9 and 20 h. In porcine model of sepsis induced by *Pseudomonas* infection, on the contrary, Shennib *et al* [26] demonstrated a decrease in phagocytic ability of AM. It is possible that the processes of repeated wash and centrifugation, attachment of AM by the non-biological FL particles, or different sepsis model made the difference. Identification of this mechanism, however, is beyond the scope of this study. In this investigation, we demonstrated that there was a decrease in AM cell number, for which the total phagocytic capacity of AM from septic lung would certainly deteriorate.

Increases in apoptosis of end organs, including the lungs, were demonstrated in several models of sepsis, such as endotoxic shock [15] and CLP [20], and the degree of apoptosis correlated with serum TNF-α levels [15]. Acute lung injury, as demonstrated by the increased AM apoptosis of septic rats in this study, developed very early in hyperdynamic phase (9 h) and progressively worsened through late hypodynamic phase (20 h). Likewise, peritoneal and liver macrophages from CLP septic mice were shown to exhibit increased apoptosis over cells from sham animals [27]. In these septic peritoneal and liver macrophages, lipopolysaccharide *in vitro* resulted in a marked increase in apoptosis [28]. For human AM, Bingisser *et al* [8] discovered that lipopolysaccharide was the only inducer of apoptosis among the different bacterial cell wall components they tested, and TNF-α and IL-1β were not capable of enhancing the spontaneous rate of apoptosis. It is possible that lipopolysaccharide released by gram-negative bacteria was responsible for the sepsis-induced AM apoptosis in CLP model.

In summary, the sepsis-related decrease in cell number of AM was likely due to the increased AM apoptosis. Since the rat CLP model relates closely to the pathogenesis of events occurring in humans during sepsis, data in this study may have clinical relevance. Acute lung injury is one of the most common septic complications [2] and the subsequent respiratory therapy likely introduces more infection into the already damaged lungs. Because AM are important for inflammation and immune responses [9], the enhanced apoptosis in septic AM may compromise the antimicrobial defense of the lung and explain, at least in part, the prevalence of pulmonary infections in septic patients.

## Acknowledgments

This work was supported by a grant from National Science Council (NSC-89-2314-B-039-025) and partly from the China Medical College and Hospital.

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