Apoptotic Cells Protect Mice against Lipopolysaccharide-Induced Shock[1]

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LPS is a main causative agent of septic shock. There is a lack of effective therapies. In vitro studies have shown that uptake of apoptotic cells actively inhibits the secretion by activated macrophages (Mϕ) of proinflammatory mediators such as TNF-α and that such uptake increases the antiinflammatory and immunosuppressive cytokine TGF-β. We therefore investigated the protective effect of apoptotic cells against LPS-induced endotoxic shock in mice. The current report is the first study to demonstrate that administration of apoptotic cells can protect mice from LPS-induced death, even when apoptotic cells were administered 24 h after LPS challenge. The beneficial effects of administration of apoptotic cells included 1) reduced circulating proinflammatory cytokines, 2) suppression of polymorphonuclear neutrophil infiltration in target organs, and 3) decreased serum LPS levels. LPS can quickly bind to apoptotic cells and these LPS-coated apoptotic cells can be recognized and cleared by Mϕ in a CD14/thrombospondin/vitronectin receptor-dependent manner, accompanied with suppression of TNF-α and enhancement of IL-10 expression by LPS-activated Mϕ. Apoptotic cells may therefore have therapeutic potential for the treatment of septic shock. ***The Journal of Immunology,*** **2008, 180: 4978–4985.**

Bacterial LPS as a constituent of the cell wall of Gram-negative bacteria is a major causative agent of septic shock. In experimental animals, LPS challenge leads to pathophysiological changes similar to the human septic shock syndrome. During sepsis, LPS starts a complex cascade of events in responsive cells, particularly in monocytes, macrophages (Mϕ),[3] and polymorphonuclear neutrophils (PMNs) that leads to the production of endogenous mediators (1–4). LPS initiates rapid influx and activation of leukocytes, coupled with overproduction of proinflammatory mediators, such as TNF-α, IL-1, IL-6, IL-12, IFN-γ, eicosanoids, and NO free radicals, that are thought to underlie the tissue damage that precedes multiple organ dysfunction syndrome (i.e., loss of capillary integrity, multiple organ dysfunction, distributive shock, septic shock, and death) (5, 6). To date, it has been very difficult to identify effective therapies for the Gram-negative shock syndrome.

Among the cytokines induced by LPS, IL-1, TNF-α, IL-12, and IFN-γ are the key factors in the development of septic disease (7–9). However, therapies aimed at neutralizing these cytokines have proved largely ineffective in clinical trials (10, 11). Various newer approaches, including molecules that can bind LPS and neutralize its activities, could have potential clinical applications. The therapies at directed at neutralizing LPS by using anti-LPS Abs have been in clinical trials for a number of years but have so far demonstrated disappointing outcomes (12, 13). Therefore, there is still an urgent need for development of new therapeutic approaches to increase the survival of patients with septic shock.

Clearance of apoptotic cells by Mϕ promotes resolution of inflammation (14, 15). Fadok et al. observed that the phagocytosis of apoptotic cells by LPS-stimulated Mϕ decreased proinflammatory cytokine (IL-1β, IL-8, GM-CSF, TNF-α, and IL-10) secretion by driving TGF-β production (16). Similarly, macrophages secrete an increased amount of secretory leukocyte protease inhibitor when ingesting apoptotic cells, which could also contribute to the resolution of the inflammatory response (17). Voll et al. reported that the presence of apoptotic cells during monocyte activation with LPS increases their secretion of the antiinflammatory and immunoregulatory cytokine IL-10 and decreases secretion of the proinflammatory cytokines TNF-α, IL-1, and IL-12 (18). Furthermore, not only CD36 Ab but also exposure to apoptotic cells can modulate myeloid dendritic cell maturation in response to inflammatory signals, probably via IL-10 expression (19). These findings indicate that interaction with apoptotic cells not only prevents the release of toxic and immunogenic intracellular contents, but that it also stimulates myeloid cells (monocytes, macrophages, and dendritic cells) to express an antiinflammatory or suppressive phenotype. We therefore hypothesized that administration of apoptotic cells can be used for the treatment of LPS-induced sepsis.

To date, most of our knowledge about nonphlogistic phagocytosis of apoptotic cells has been focused on action in vitro, but the contributions of apoptotic cells in various inflammatory states in vivo and the significance in vivo of reducing inflammatory reaction remain to be fully clarified. In this study, mice challenged with high doses of LPS resulted in a syndrome resembling septic shock

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[3] Abbreviations used in this paper: Mϕ, macrophage; LAL, *Limulus* amebocyte lysate; PMN, polymorphonuclear neutrophil; TSP, thrombospondin; VnR, vitronectin receptor.

in humans (20, 21). We investigated the protective effect of apoptotic cells, demonstrating that i.v. administration of apoptotic cells up to 24 h after onset of endotoxemia protected mice from LPS-induced lethality, which was associated with significantly inhibited production of TNF-α, IL-12, and IFN-γ, decreased LPS concentration in serum, and reduction of PMN infiltration in the lungs, liver, heart, and kidneys. Apoptotic cells may therefore have therapeutic potential for the treatment of septic shock.

Materials and Methods

All chemicals were from Sigma-Aldrich unless otherwise indicated. Culture media and supplements were from Life Technologies, and sterile tissue culture plasticware was from Falcon Plastics.

Sepsis induced by LPS

BALB/c mice at 8 wk of age were used. A single dose of 2.8 mg/kg LPS (O11:B4) in 100 μl of sterile saline was administered i.v. Mice were then randomly divided into control and treatment groups. Mice in the treatment group were injected apoptotic PMNs i.v. with various doses of apoptotic cells at different time points after LPS injection. Mice in the control group were administrated with PBS (pH 7.4) after LPS injection. Serum samples and organs were collected. The general conditions and mortality were recorded for up to 3 wk after injection to ensure that no additional late deaths occurred.

Sepsis induced by cecal ligation and puncture (CLP)

For induction of sepsis by CLP, mice were anesthetized with pentobarbital (60 mg/kg) (Abbott Laboratories), a 1-cm midline incision was made on the anterior abdomen, and the cecum was exposed and ligated below the ileocecal junction without causing bowel obstruction. Four punctures were made in the cecum using a 21-gauge needle to induce lethal CLP sepsis, the cecum was returned to the abdomen, and the peritoneal wall and skin incision were closed. One hour later, 10 millions of apoptotic PMNs or PBS were injected into CLP mice i.v.

Measurement of PMN infiltration

Infiltrated PMNs in organs were detected by immunohistochemical staining. Paraffin sections were incubated with Ab against mouse PMN overnight at 4°C. Sections were then washed with PBS. Endogenous peroxidase was inactivated in 3% H_2O_2 in methanol, and then incubated with biotinylated link Ab and peroxidase-conjugated streptavidin (DakoCytomation). After being washed with PBS, sections were developed with 3,3′-diaminobenzidine to produce a brown color. Purified rabbit-nonspecific IgG was used as a negative control.

Isolation of peripheral blood PMNs and Mϕ

PMNs were isolated from the peripheral blood of healthy donors by dextran sedimentation and discontinuous plasma-Percoll (Pharmacia) density gradients using the methods described by Savill et al. (22). PMNs were aged in tissue culture for 24 h in Iscove's DMEM with 10% FBS and PMNs underwent apoptosis spontaneously in culture. Viability of >99% was confirmed by trypan blue exclusion, and apoptosis was verified by light microscopy of May-Giemsa-stained cytopreps. Human monocytes were isolated by counterflow centrifugation as described previously (23) and cultured in Iscove's DMEM with in 96-well plates (1×10^5 per well). After 1 h of incubation at 37°C and 5% CO_2, the wells were cultured for 7 days in Iscove's DMEM with 10% autologous serum to mature into Mϕ (22, 24). LPS-coated PMNs were prepared by incubating apoptotic PMNs with LPS (10 μg/ml) at 37°C for 30 min. The free LPS was washed away with PBS. Control apoptotic PMNs were treated identically except that LPS was absent.

Preparation of apoptotic PMNs

PMNs were aged in cultured for 24 h in Iscove's DMEM with 10% FBS and PMNs underwent apoptosis spontaneously in culture (22). The viability of >99% confirmed by trypan blue exclusion, and apoptosis verified by light microscopy of May-Giemsa-stained cytopreps, showed the typical apoptotic PMN characteristics, that is, condensed nuclei and vacuolated cytoplasm. The apoptotic and necrotic PMNs were also confirmed by annexin-V/propidium iodide staining and detection by FACS.

Phagocytosis assay

Apoptotic PMNs and LPS-coated PMNs were washed twice with PBS, suspended in Iscove's DMEM, and 5×10^5 cells were added to each washed well of Mϕ. After interaction for 30 min at 37°C in 5% CO_2 atmosphere, the wells were washed in saline at 4°C to remove non-ingested apoptotic cells. The wells were fixed with 2% glutaraldehyde and then stained for myeloperoxidase to reveal ingested cells. The proportion of Mϕ ingesting PMNs was counted using inverted light microscopy.

Cytokines in mice sera or supernatants of cell culture

Concentrations of cytokines such as IL-12, IL-6, IFN-γ, IL-10, and TNF-α in mouse sera were detected by BD Cytometric Bead Array (CBA) (BD Biosciences) according to the manufacturer's recommendations. In brief, 25 μl of the mixed capture beads was mixed with 25 μl of mouse serum and 25 μl of mouse inflammation PE detection reagent for 2 h at room temperature. The beads were washed and analyzed by a FACScan flow cytometer (BD Biosciences). Concentrations of TGF-β, IL-10, and TNF-α in mouse serum or in supernatants of cell culture were detected using ELISA kits (R&D Systems) according to the manufacturer's recommendations.

Measurement of LPS in mouse serum

The concentrations of LPS in mouse serum were detected by using *Limulus* amebocyte lysate (LAL) assay (Associates of Cape Cod) according to the instructions provided by the manufacturer. In brief, 50 μl of serum was added in triplicate to 50 μl of LAL in a pyrogen-free microplate well. After incubation at 37°C for 10 min, 100 μl of prewarmed substrate solution was added and then 100 μl of stop buffer was added at the 16-min time point. The absorbance was measured at 540–550 nm. One nanogram of endotoxin is equivalent to 5 endotoxin units.

LPS binding to apoptotic PMNs

Apoptotic PMNs were interacted with LPS (10 μg/ml) for 30 min at room temperature. LPS binding was detected by FACS using LPS Ab conjugated with FITC.

Statistical analysis

Results are presented as means ± SEM. Differences between groups at any point in time were tested using ANOVA and were followed by the Bonferroni post hoc test. Two-sample comparisons were performed using the Student t test. Survival data were analyzed with Kaplan-Meier test ($p < 0.05$ was considered to be significant).

Results

Administration of apoptotic cells protected mice from endotoxic lethality

We used human apoptotic PMNs with >50% apoptotic cells as verified by May-Giemsa-stained cytopreps and >99% viability as assessed by trypan blue exclusion. The apoptotic and necrotic PMNs were also confirmed by annexin V/propidium iodide staining with detection by FACS (Fig. 1). The effect of administration of a single injection of apoptotic cells on survival of mice challenged with LPS (2.8 mg/kg) i.v. was investigated. After LPS challenge alone, mice showed signs of severe sepsis symptoms such as reduced mobility, conjunctivitis, diarrhea, and fur ruffling within 3 h. LPS-treated mice began to die at 8–12 h and all mice died within 3 days in the control group. In contrast, mice in the group injected i.v. with apoptotic cells (10×10^6/mouse) immediately after challenge with LPS exhibited fewer signs of sickness. Only 20% of treated mice died at day 7; that is, treatment with apoptotic cells resulted in 80% survival ($n = 12$, $p < 0.001$) (Fig. 2*A*). Late deaths in the treatment group were not observed during the 3 wk after LPS injection, indicating that apoptotic cell treatment conferred a complete and lasting protection against lethal endotoxemia. By contrast, human apoptotic cells (10×10^6/mouse) were injected into normal mice and no abnormalities were observed during 3 wk after injection.

To further examine whether the administration of apoptotic cells has a beneficial effect in another animal model, we induced sepsis in mouse by CLP. Without any treatment, 52% of mice (12 of 25)

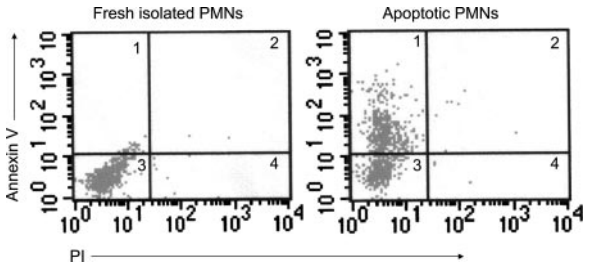

FIGURE 1. Annexin V vs propidium iodide (PI) density plots in logarithmic scale. PMNs were isolated from blood and aged in cultured for 24 h and PMNs underwent apoptosis spontaneously. Four populations are resolved. Fresh isolated PMNs are double negative and are seen in the lower left quadrant (3). Cells that are annexin V (+)/PI (−) (1) are apoptotic. The annexin V (+)/PI (+) cell population (2) has been described as secondary necrotic or advanced apoptotic. Cells in the last quadrant (4), which are annexin V (−)/PI (+), may be cells with stripped cytoplasmic membranes (leaving isolated nuclei), cells in late necrosis, or cellular debris.

died within 5 days. Mice with apoptotic cell treatment 1 h after CLP exhibited fewer signs of sickness. Less than 20% of treated mice (4 of 22) died in the first 3 days (Fig. 2*B*).

We next investigated whether delayed administration of apoptotic cells would also prevent mice from endotoxic lethality. Treatment with apoptotic cells (10×10^6/mouse) was initiated 1, 3, 6, and 24 h, respectively, after the onset of endotoxemia. Delayed

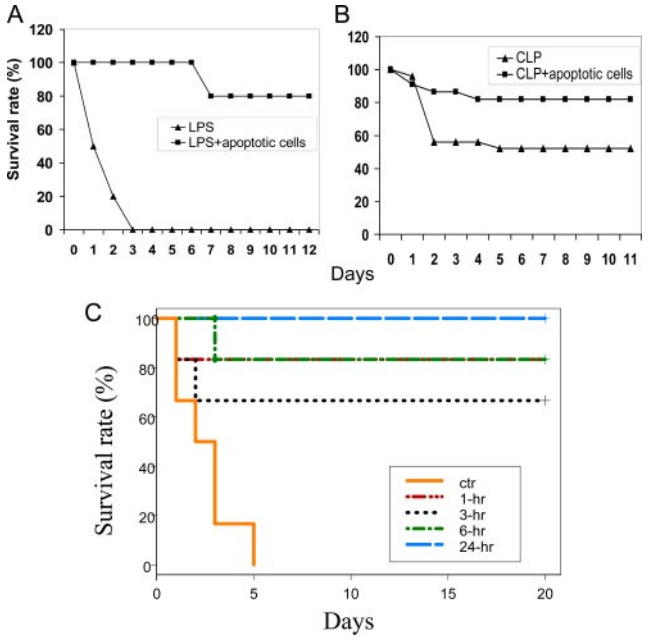

FIGURE 2. Effect of administration of apoptotic cells on survival in LPS- and CLP-induced sepsis. *A*, Mice were administered a single dose of apoptotic cells (10×10^6/mouse) i.v. at the time of LPS administration (2.8 mg/kg, i.v.). Apoptotic cells conferred significant protection against lethality ($n = 12$/group, $p < 0.05$). *B*, Mice had induced sepsis by CLP and then were administered a single dose of apoptotic cells (10×10^6/mouse) i.v. Apoptotic cells conferred significant protection against lethality ($n = 25$ for control group and $n = 22$ for apoptotic cell-treated group, $p < 0.05$). *C*, Effect of delayed administration of apoptotic cells on prevention of the endotoxin lethality. Mice in control group were injected with LPS (2.8 mg/mouse, i.v.). Treatment with apoptotic cells (10×10^6/mouse) was initiated 1, 3, 6, and 24 h after the onset of endotoxemia. Delayed treatment at all time points significantly protected mice from lethal shock ($n =$ 6/group, $p < 0.05$).

Table I. *Effect of apoptotic cells on reduction of septic symptoms*

Observation Time[a]	Time of Administration for Apoptotic Cells after LPS Challenge[b]	Symptoms		
		Reduced Mobility	Conjunctivitis	Diarrhea
24 h	Control	++	++	++
	1 h	+	+	+
	3 h	++	++	+
	6 h	++	++	+
	24 h	+	+	−
48 h	Control	++	++	+
	1 h	+/−	+/−	−
	3 h	+	+	−
	6 h	+	+	−
	24 h	+/−	+	−
72 h	Control	++	++	+
	1 h	−	−	−
	3 h	+/−	+/−	−
	6 h	+/−	+/−	−
	24 h	−	−	−
96 h	Control	++	++	+
	1 h	−	−	−
	3 h	−	−	−
	6 h	−	−	−
	24 h	−	−	−

[a] Time after administration of apoptotic cells.
[b] Mice were injected with 10×10^6 apoptotic PMNs.
++ indicates that more than two-thirds of mice had symptoms; +, one-third to two-thirds of mice had symptoms; +/−, less than one-third of mice had symptoms; −, no mice had symptoms.

treatments at all time points not only significantly protected mice from lethal shock ($n = 6$/group, $p < 0.05$, Fig. 2*C*), but they also reduced the sepsis symptoms obviously (Table I). No late deaths occurred during the subsequent 3-wk period of observation. These results indicated that delayed administration of apoptotic cells in mice provided protection from LPS-induced lethal shock.

Administration of apoptotic cells attenuated LPS-induced organ damage

We further investigated whether injection of apoptotic cells would attenuate LPS-induced organ damage. The mice received apoptotic cells or PBS injection (as control) immediately after challenge

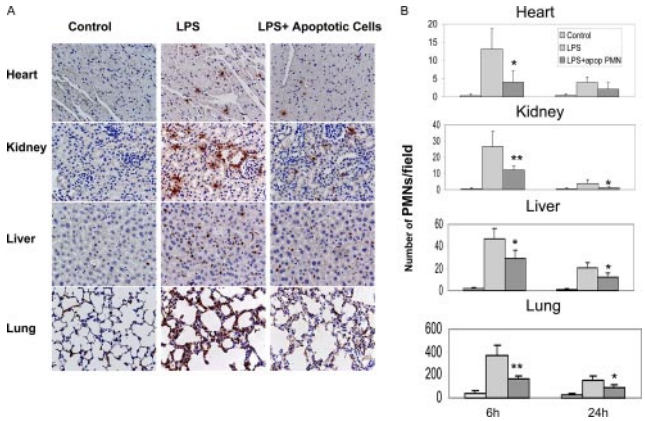

FIGURE 3. Effect of injection of apoptotic cells on PMN infiltration. The mice received apoptotic cells or PBS injection (as control) immediately after challenge with LPS (2.8 mg/kg, i.v.) and were sacrificed at 6 and 24 h after injection of apoptotic cells. *A*, The PMN staining (brown) in livers, lungs, kidneys, and hearts at 6 h after injection of apoptotic cells. *B*, Number of infiltrated PMNs by immunohistochemical staining ($n = 12$; *, $p < 0.05$; **, $p < 0.001$).

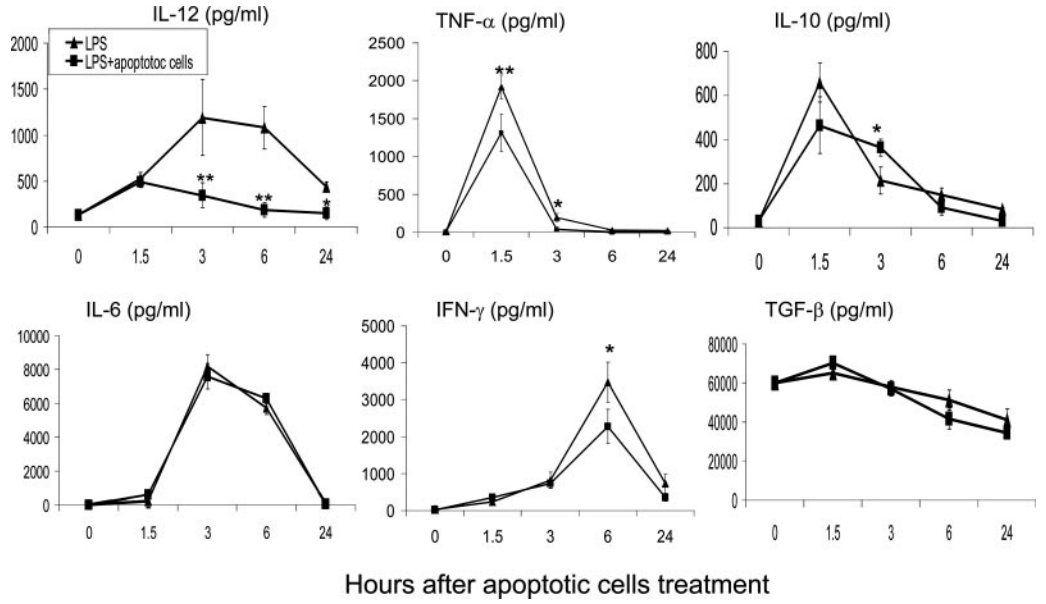

FIGURE 4. Effect of administration of apoptotic cells on cytokine production after challenge with LPS in vivo. The mice received apoptotic cells or PBS injection (as control) immediately after challenge with LPS (2.8 mg/kg, i.v.). Heart blood was collected at various time points after injection of apoptotic cells, and cytokines were assessed by BD Cytometric Bead Array and ELISA ($n = 6$; *, $p < 0.05$; **, $p < 0.001$).

with LPS (2.8 mg/kg, i.v.) and were sacrificed at 6 and 24 h after injection of apoptotic cells. The livers, lungs, kidneys, and hearts were collected for PMN staining. In mice injected with LPS alone, light microscopic examination showed PMN infiltration and hemorrhage in the lung, liver, heart, and kidney (Fig. 3*A*). However, these changes were attenuated in mice treated with apoptotic cells. Because PMN infiltration significantly correlated with the severity of inflammation, we assayed the number of infiltrated PMNs by immunohistochemical staining. Six hours after LPS challenge, the number of infiltrated PMNs in liver, lung, heart, and kidney were 46.9 ± 9.8, 372.3 ± 89.5, 13.1 ± 5.6, and 26.5 ± 9.3, respectively. However, in the apoptotic cell-treated mice, the infiltrated PMNs were 29.5 ± 7.5, 166.8 ± 24.8, 4.1 ± 3.1 and 12.1 ± 2.3, respectively (Fig. 2*B*, $n = 12$, $p < 0.05$). Similar patterns were observed at 24 h (Fig. 3*B*).

Apoptotic cell treatment decreased cytokine levels in vivo

We next assayed the levels of cytokines in the serum from apoptotic cell-treated and untreated mice at the indicated time points. Mice received apoptotic cells (or PBS as control) 1.5 h after being injected i.v. with 2.8 mg/kg LPS. Heart blood was collected at various time points after injection of apoptotic cells, and cytokines were assessed by BD Cytometric Bead Array and ELISA. In the control group receiving LPS above, the level of IL-12 peaked at 3 h and declined after 6 h. In comparison, the level of TNF-α reached a peak at 1.5 h and markedly declined thereafter. Apoptotic cell treatment resulted in significantly lower circulating levels of IL-12, TNF-α, and IFN-γ compared with the control group at certain time points, while IL-6 and TGF-β were not affected (Fig. 4). The level of IL-10 in apoptotic cell-treated mice at 3 h was significantly higher than that of the control group (Fig. 4). These results demonstrated that apoptotic cell treatment could reduce LPS-induced proinflammatory responses in vivo.

Administration of apoptotic cells reduced LPS concentrations in mice

We further measured the plasma concentrations of LPS in apoptotic cell-treated and control mice. Mice injected with LPS (2.8 mg/kg,

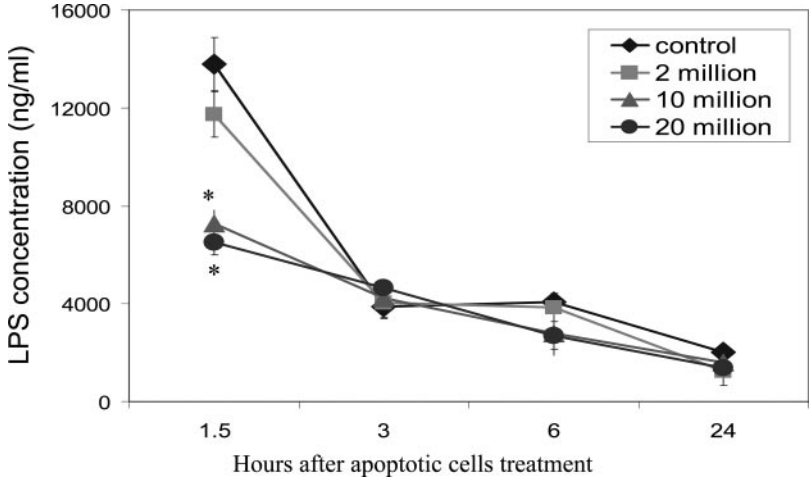

FIGURE 5. Effect of administration of apoptotic cells on plasma LPS levels after challenge with LPS in vivo. Mice received various doses of apoptotic cells (2×10^6, 10×10^6, and 20×10^6/mouse) immediately after injection with LPS (2.8 mg/kg, i.v.). Heart blood was collected at various time points after injection of apoptotic cells, and LPS was detected by LAL assay ($n = 6$; *, $p < 0.05$).

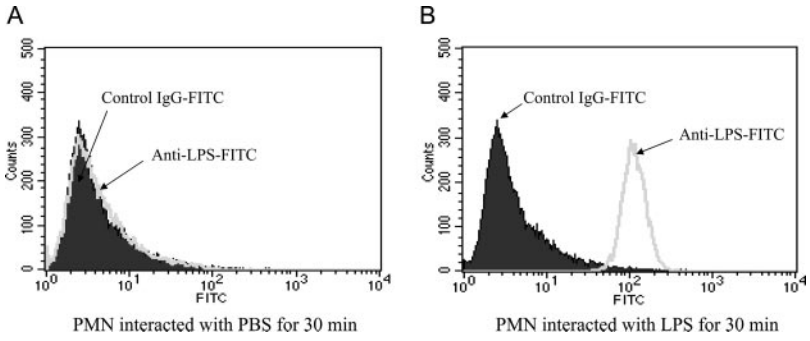

FIGURE 6. LPS binding to apoptotic PMNs. Apoptotic PMNs were interacted with PBS (*A*) or 10 μg/ml LPS (*B*) for 30 min at room temperature. LPS binding was detected by FACS using LPS Ab conjugated with FITC. Results shown are representative of three experiments.

i.v.) were sacrificed immediately and exhibited a LPS concentration in serum of 25,000 ± 1,638 ng/ml as detected by LAL assay. Mice received PBS (as control) or various doses of apoptotic cells (2×10^6, 10×10^6, and 20×10^6/mouse) at 1.5 h after being injected i.v. with 2.8 mg/kg LPS. Heart blood was collected at various time points after injection of apoptotic cells, and LPS was detected by LAL assay. In the control group, the circulating LPS concentration was 13,780 ± 1,110 ng/ml at 1.5 h and 2018 ± 221 ng/ml at 24 h after injection of PBS. Injection of apoptotic cells at 10×10^6 and 20×10^6/mouse significantly attenuated the concentrations of LPS in the sera of mice at 1.5 h, while injections of 2×10^6/mouse had no effects (n = 6/group, Fig. 5).

Binding of apoptotic PMNs to LPS

Our data showed that circulating LPS can be quickly reduced by administration of apoptotic cells at 1.5 h after LPS injection. Thus, we hypothesized that decreased LPS levels in serum may be due to LPS binding to apoptotic cells. Apoptotic PMNs were interacted with 10 μg/ml LPS for 30 min at room temperature and then labeled with LPS Ab conjugated with FITC. LPS binding was detected by flow cytometry. Fig. 6 demonstrates that most apoptotic PMNs can bind LPS.

LPS accelerated the clearance of apoptotic PMNs by human monocyte-derived Mϕ

Our data showed that LPS can bind to apoptotic cells. To determine whether LPS-coated apoptotic cells can be taken up by Mϕ quickly and safely, assays for phagocytosis of LPS-coated apoptotic PMNs were performed. Apoptotic PMNs were incubated with LPS at 10 μg/ml for 30 min and then LPS-coated apoptotic PMNs were interacted with Mϕ for 30 min. When compared with Mϕ taking up untreated apoptotic PMNs, a greater proportion of Mϕ was able to ingest LPS-coated PMNs (22.6 ± 2.8% for apoptotic PMNs and 43.9 ± 5.6% for LPS-coated apoptotic PMNs, $n = 12$, $p < 0.05$; Fig. 7*A*). This increase was also apparent when phagocytosis was expressed as the number of apoptotic cells ingested per 100 Mϕ (23.5 ± 10.7 for apoptotic PMNs and 57.3 ± 9.5 for LPS-coated PMNs, $n = 12$, $p < 0.05$; Fig. 7*B*). Furthermore, the number of apoptotic cells within 100 phagocytically competent Mϕ (i.e., those Mϕ taking up PMNs) was also increased by LPS coating from 130.0 ± 8.4 to 180.0 ± 5.3 ($n = 12$, $p < 0.05$; Fig. 7*B*).

LPS-increased phagocytosis was CD14- and thrombospondin (TSP)-dependent

Previous studies have shown that human monocyte-derived Mϕ recognition of apoptotic PMN used TSP/$\alpha_v\beta_3$ vitronectin receptor (VnR) integrin (22, 24). To determine what recognition mechanism was used by Mϕ recognizing and ingesting LPS-coated apoptotic PMNs, we investigated whether TSP/$\alpha_v\beta_3$ and CD14, a receptor for LPS, are involved. Uptake of apoptotic PMNs was specifically reduced by TSP mAb (A6.1) and RGDS (Arg-Gly-Asp-Ser) peptide (which is an inhibitor of VnR), but not by RGES (Arg-Gly-Glu-Ser) peptide, CD14 mAbs (61D3), and control mAb (Fig. 8). When applied to LPS-coated apoptotic PMNs, 61D3, A6.1, and RGDS had proportional inhibitory effects (Fig. 8). These results suggested that LPS-increased phagocytosis involved recruitment of CD14-dependent recognition to the TSP/VnR system.

LPS-coated apoptotic PMNs inhibit TNF-α and IL-10 release by LPS-stimulated Mϕ

Previous studies have shown that Mϕ uptake of apoptotic cells via CD14-dependent recognition does not release proinflammatory

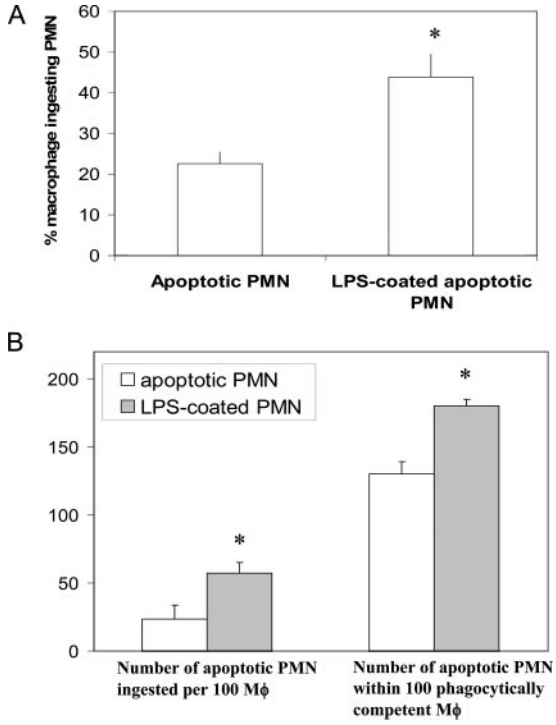

FIGURE 7. Effect of LPS on clearance of apoptotic PMNs by human monocyte-derived Mϕ. Apoptotic PMNs were incubated with LPS at 10 μg/ml for 30 min and then LPS-coated apoptotic PMNs were interacted with Mϕ for 30 min. *A*, Percentage of Mϕ taking up PMNs. *B*, Phagocytic capacity of Mϕ taking up cells. Phagocytosis was expressed as the number of cells ingested per 100 Mϕ and the number of cells within 100 phagocytically competent Mϕ (i.e., those Mϕ taking up PMNs). The data were expressed as means ± SEM, and similar results were obtained from three independent experiments (*, $p < 0.05$).

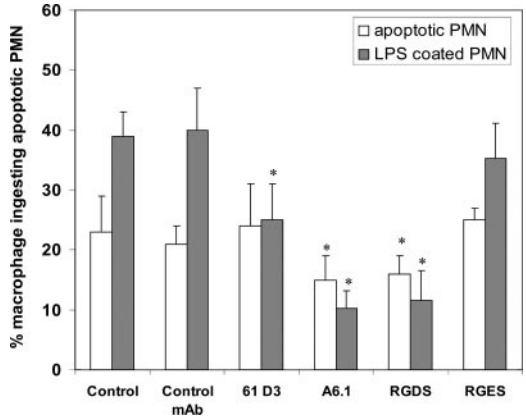

FIGURE 8. Specific inhibition of Mϕ recognition of LPS-coated PMNs by inhibitors of LPS/TSP/$\alpha_v\beta_3$. Uptake of apoptotic PMNs was specifically reduced by TSP mAb (A6.1) and RGDS peptide, but not by control peptide (RGES), CD14 mAbs (61D3), and control mAb. Recognition of LPS-coated apoptotic PMN was inhibited by 61D3, A6.1, and RGDS. The data were expressed as means ± SEM, and similar results were obtained from three independent experiments (*, $p < 0.05$).

mediators (25), while LPS clearance through CD14 activates Mϕ to release proinflammatory cytokines (26). We therefore investigated whether proinflammatory cytokine release was triggered in Mϕ that ingested LPS-coated PMNs. Mϕ interacted with 10 μg/ml LPS or culture medium as control for 12 h and then washed were incubated with LPS-coated PMNs for 30 min. Noningested cells were washed away and Mϕ were incubated with culture medium for 24 h. TNF-α and IL-10 in supernatants of cell culture were detected by ELISA. Exposure of Mϕ to LPS resulted in significant TNF-α and IL-10 expression. By contrast, Mϕ uptake of LPS-coated PMNs down-regulated LPS-stimulated Mϕ production of TNF-α and increased secretion of IL-10 (Fig. 9). Furthermore, LPS-coated PMNs alone did not stimulate Mϕ expression of TNF-α (Fig. 9). These data demonstrated that LPS promotes proinflammatory cytokine release, whereas Mϕ clearance of LPS-coated PMNs, like apoptotic PMNs, can inhibit LPS-enhanced TNF-α expression.

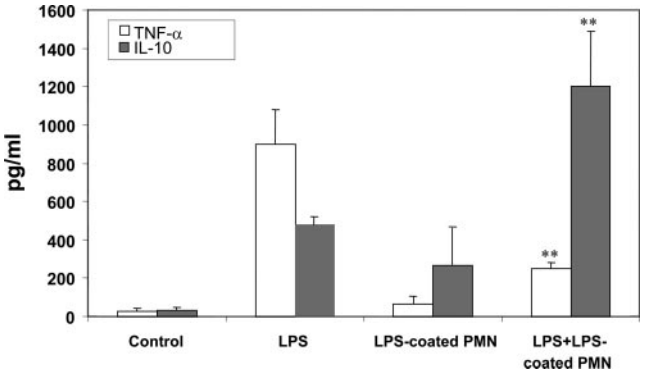

FIGURE 9. TNF-α and IL-10 release by Mϕ phagocytosing LPS-coated PMNs. Mϕ were interacted with 10 μg/ml LPS or culture medium as control for 12 h, and washed Mϕ were then incubated with LPS-coated PMNs or untreated PMNs for 30 min. Noningested cells were washed away and Mϕ were incubated with culture medium for 24 h. TNF-α and IL-10 in supernatants of cell culture were detected by ELISA. The data are expressed as means ± SEM, and similar results were obtained from three independent experiments (**, $p < 0.001$).

Discussion

In vitro studies showed that uptake of apoptotic cells actively inhibits the secretion by activated Mϕ of proinflammatory mediators such as TNF-α and that it increases the antiinflammatory and immunosuppressive cytokine TGF-β (16, 18, 27). The current study is the first to demonstrate that administration of apoptotic cells can protect mice from LPS-induced death, even when apoptotic cells were administered 24 h after LPS challenge. The beneficial effects of administration of apoptotic cells included 1) reduced circulating proinflammatory cytokines, 2) suppression of PMN infiltration in target organs, and 3) decreased serum LPS levels.

The underlying mechanisms of the beneficial effect of apoptotic cells, however, require further clarification in the future. Activated Mϕ are the major cells of the immune system to produce proinflammatory cytokines such as TNF-α, IL-12, and IL-1. TNF-α may play a role in the initiation or progression of multiple organ failure in septic shock (28), and there is an association between TNF-α and severity of shock and mortality (29–31). TNF-α can induce vascular endothelial cells to express adhesion molecules (E-selectin and integrins such as CD11a/CD18b), stimulating migration of PMNs (32, 33), and therefore mediate the inflammatory changes in the multiple organs, resulting in PMN-dependent organ damage (34). IL-12 is another product of Mϕ that is important in sepsis pathogenesis. It is rapidly produced by phagocytic cells after stimulation with LPS (35). IL-12 participates in the amplification of the inflammatory and immune responses, which is essential in the development of septic shock and multiple organ failure (36).

Therefore, inhibiting secretion of these cytokines from activated Mϕ is a key issue for sepsis treatment. Our study showed that administration of apoptotic cells can significantly reduce the production of IL-12, TNF-α, and IFN-γ. One likely mechanism for this effect was demonstrated by our in vitro studies demonstrating that apoptotic cells, even if coated with LPS, increased the secretion by LPS-stimulated macrophages of the antiinflammatory and immunoregulatory cytokine IL-10 and decreased secretion of the proinflammatory cytokine TNF-α. These in vivo data were also corroborated by a recent study demonstrating that phagocytosis of apoptotic cells by activated macrophages results in strong inhibition of IL-12 gene expression (37).

Infiltration of PMNs into the major organs such as the lung and liver is associated with multiple organ damage and is one of the hallmarks of a systemic inflammatory response induced by endotoxin (38–40). In our present study, LPS injection caused increased PMN infiltrations in the lung, liver, heart, and kidney, which were partially reversed by apoptotic cell administration. The mechanisms by which apoptotic cell administration decreased PMN infiltration in multiple organs will require clarification in the future. However, ICAM-1 mediates PMN migration across the vascular membrane and into tissues, leading to organ injury. TNF-α is involved in the regulation of ICAM-1 expression. Apoptotic cell administration can decrease TNF-α level, and may therefore lead to down-regulation of ICAM-1 and reduced PMN infiltration.

In numerous clinical trials, attempts to target molecular events occurring in sepsis "downstream" of LPS have not been associated with improved survival. Some have suggested that agents able to remove endotoxin from the blood of septic patients would be of considerable clinical value. The data presented here suggest the possibility that, in addition to protection via suppression of proinflammatory cytokine expression, apoptotic cell treatment can also protect from endotoxin shock via direct binding and removal of LPS. We demonstrated that

apoptotic cells can directly bind LPS with high affinity and that this binding may lead to more rapid reduction of serum LPS concentrations after a bolus dose.

As apoptotic cells are quickly coated by LPS, we investigated how Mϕ recognize these LPS-coated apoptotic cells and whether phagocytosis of these cells is antiinflammatory. There are number of receptors for LPS such as CD14 (41, 42), macrophage scavenger receptor class A (43), and TLR4 (44, 45). The TLR4 family plays a central role in activation of innate immune system. LPS is thought to bind directly to TLR4, and it initiates the transmembrane signaling via an adaptor protein MyD88, leads to activation of NF-κB, and induces a number of inflammatory mediators (46). In our study, we showed that CD14, but not TL4, is one of the receptors for LPS-coated apoptotic cell uptake (data not shown). Additionally, mAbs to TSP and $\alpha_v\beta_3$ inhibit LPS-coated PMN uptake. Our study also showed that phagocytosis of LPS-coated PMNs is safe and nonphlogistic, as engulfment of LPS-coated PMN suppresses TNF-α and enhances IL-10 expression by LPS-activated Mϕ. These results further confirm the observation of Devitt et al., who reported that CD14 mediates Mϕ recognition and phagocytosis of apoptotic cells without release of proinflammatory cytokines (25). Our data suggested that CD14 may cooperate with TSP and VnR to take up LPS-coated apoptotic PMN (i.e., CD14 might be involved in recognizing or tethering apoptotic cells to Mϕ (26)) and that additional receptors such as $\alpha_v\beta_3$ are needed for LPS-coated apoptotic cell uptake.

Taken together, these results reveal that apoptotic cells have therapeutic potential for experimental septic shock by reducing release of the proinflammatory cytokines and circulating LPS concentration. More importantly, apoptotic cells can be administered significantly later after the acute phase response (24 h). Thus, treatment with apoptotic cells may offer a new strategy for the management of patients with sepsis and septic shock. However, note that the amount of PMN infused is rather high. Although we showed that 10×10^6 PMNs is efficient to protect mice against septic shock, further investigation is needed to understand how many cells would be efficient for humans. Furthermore, it would be possible to trigger PMN apoptosis combined with lesser amounts of apoptotic PMN injection.

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Disclosures

The authors have no financial conflicts of interest.

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