Erythropoietin prevents lymphoid apoptosis but has no effect on survival in experimental sepsis

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BACKGROUND: Lymphoid apoptosis in sepsis is associated with poor outcome, and prevention of apoptosis frequently improves survival in experimental models of sepsis. Recently, erythropoietin (EPO) was shown to protect against lipopolysaccharide (LPS)-induced mortality. As cecal ligation and puncture (CLP) is a clinically more relevant model of sepsis, we evaluated the effect of EPO on CLP-induced lymphoid tissue apoptosis and mortality.

METHODS: Young Wistar rats were subjected to polymicrobial sepsis by CLP. EPO (5,000 U/kg intraperitoneal) was administered 30 min before CLP and then 1 and 4 h after CLP. Spleen, thymus, and small intestine were harvested at 24 h and assessed for apoptosis by terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) and caspase-3 staining. A separate group of animals was followed up for mortality.

RESULTS: Splenic, thymic, and intestinal apoptosis was increased after CLP; administration of EPO significantly decreased apoptosis as determined by TUNEL and caspase-3 staining. Final survival in the CLP mortality study was 30% in both saline and EPO groups.

CONCLUSION: Our results provide the first evidence that EPO attenuates lymphoid apoptosis in the CLP model of sepsis. However, EPO is not associated with a survival benefit in the CLP model of sepsis.

Sepsis can be described as the systemic maladaptive response of the body to invasion by pathogenic microorganisms (1). It is an important cause of morbidity and mortality in children and adults worldwide. Patients with sepsis often present with evidence of infection, circulatory, and respiratory failure. This presentation is usually caused by an initial, severe proinflammatory response. If appropriate and timely treatment can be instituted, patients may survive this stage; however, an anti-inflammatory response frequently follows the proinflammatory phase (2).

Apoptosis has been implicated in the development of the anti-inflammatory phase during sepsis (3). Several studies have demonstrated increased lymphocyte apoptosis in animals and humans with sepsis and its relation to poor outcome (2,4–6). Although initial findings were in adults, lymphopenia

and apoptosis-associated depletion of lymphoid organs also have a role in sepsis-related death in critically ill children and neonates (7,8).

Uptake of apoptotic cells by phagocytic cells leads to production of anti-inflammatory cytokines or anergy, whereas uptake of necrotic cells causes secretion of proinflammatory cytokines. Therefore, in contrast to necrosis, apoptotic cell death does not produce inflammation but rather an immunosuppressive state (9). The importance of apoptosis in sepsis has been revealed by multiple experimental studies that demonstrated that prevention of lymphocyte apoptosis increases survival (10–13).

Among the candidates for a therapeutic approach is erythropoietin (EPO), a hematopoietic growth factor shown to have antiapoptotic and cytoprotective effects in both animal and human models of hypoxia–ischemia (14–17). We previously demonstrated that EPO decreases lipopolysaccharide (LPS)-induced thymic and splenic apoptosis (18). Recently, Aoshiba *et al.* (19) reported that administration of a large dose of EPO improves survival in endotoxemic shock, but not in a cecal ligation and puncture (CLP) model of sepsis. In their study, lymphoid apoptosis in the LPS group animals was reduced but CLP animals were not assessed for apoptosis, and it was not clear why EPO failed to improve survival in the CLP group (19).

Our aim in this study was to examine the effect of EPO on immune-cell apoptosis and survival specifically in the CLP model of sepsis.

RESULTS

EPO Attenuates Apoptosis Induced by CLP in the Ileum, Thymus, and Spleen

To investigate whether EPO administration is associated with a reduction of apoptosis in the ileum, terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining was performed. At 24 h, CLP was associated with an increased number of cells staining as TUNEL positive in the small intestine. EPO treatment significantly reduced the number of TUNEL-positive cells. The findings by TUNEL were confirmed with caspase-3 staining (**Figures 1** and **2**).

CLP also resulted in increased apoptosis in the thymus and spleen, as determined by TUNEL and caspase-3 staining.
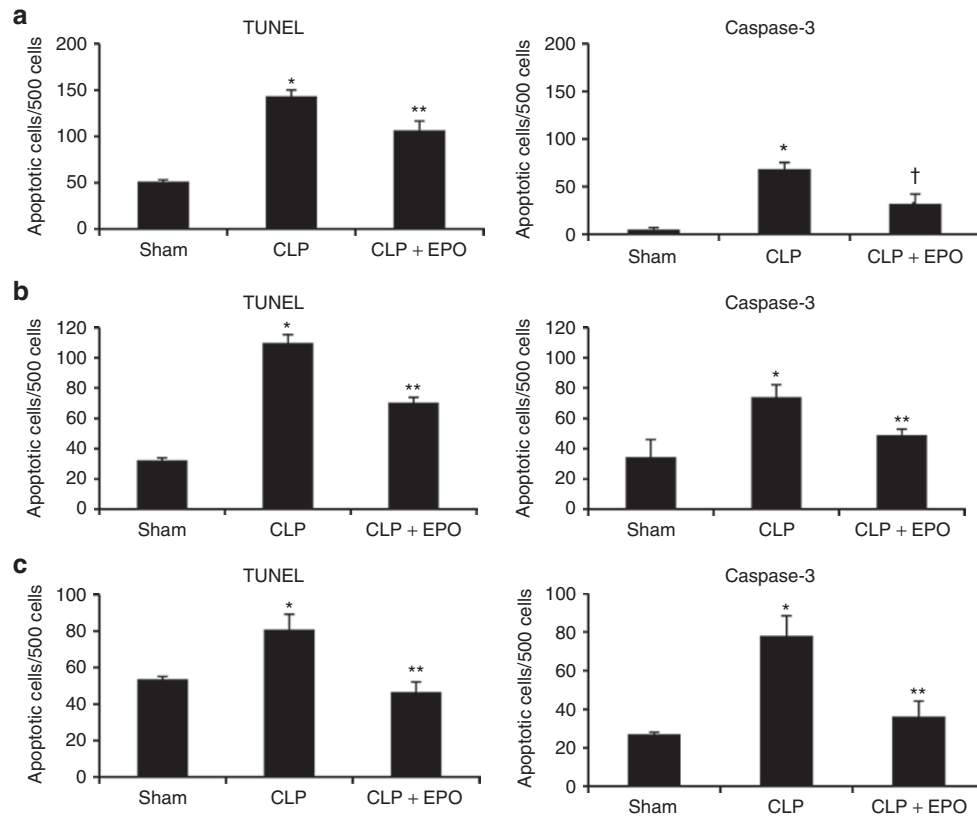
Figure 1. EPO inhibits apoptosis in the ileum, thymus, and spleen in the CLP model of sepsis. The numbers of terminal deoxynucleotidyl transferase nick-end labeling (TUNEL)-positive and caspase-3-positive cells per 500 cells counted in the (**a**) ileum, (**b**) thymus, and (**c**) spleen of septic animals. Animals in the sham group underwent laparotomy without cecal ligation and puncture. Animals in the control group (CLP group) underwent cecal ligation and puncture (CLP) and received saline. Animals in the CLP + erythropoietin (EPO) group underwent CLP and received EPO 30 min before and then 1 and 4 h after the procedure. Two animals in the CLP group expired before 24 h. *$P < 0.05$ vs. sham group. **$P < 0.05$ vs. CLP group. †$P = 0.01$ vs. CLP group.
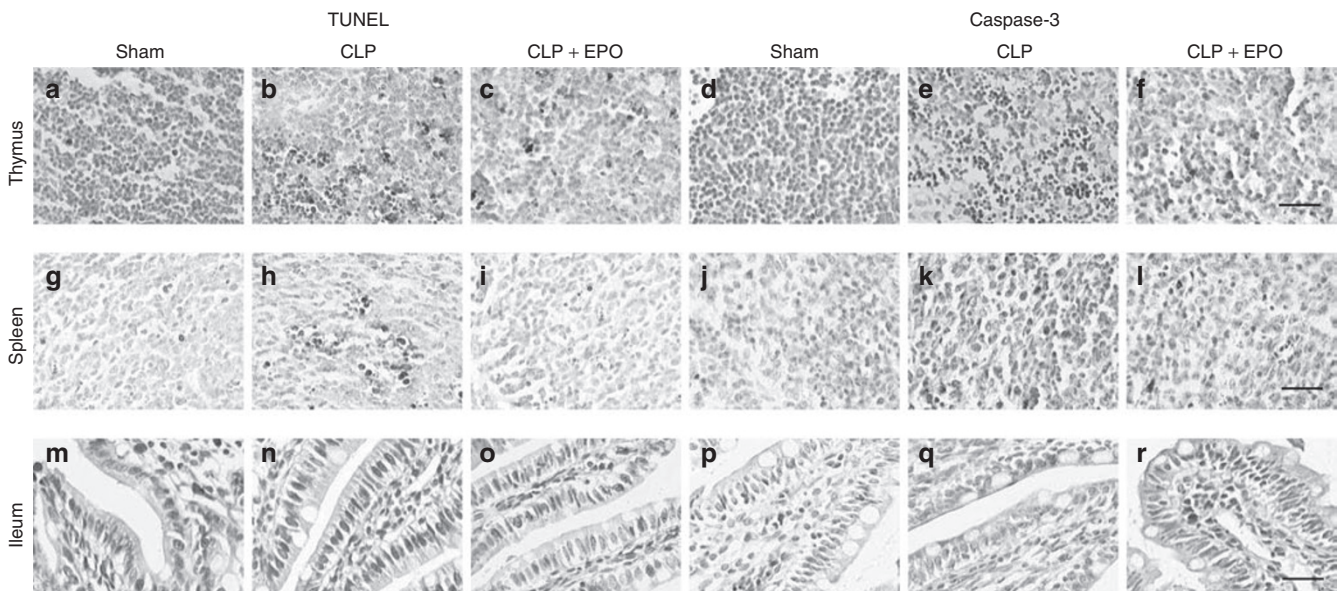

Figure 2. Representative images of thymus, spleen, and ileum. Terminal deoxynucleotidyl transferase nick-end labeling and caspase-3 immunostaining in the (**a–f**) thymus, (**g–l**) spleen, and (**m–r**) ileum is shown 24 h after cecal ligation and puncture (CLP) (scale bar = 50 μm). EPO, erythropoietin.

Similar to the ileum, administration of EPO reduced the number of apoptotic cells significantly (**Figures 1** and **2**). This finding was consistent with our previous research in which we demonstrated that EPO attenuates lymphocyte apoptosis in spleen and thymus in an LPS model of sepsis (18).
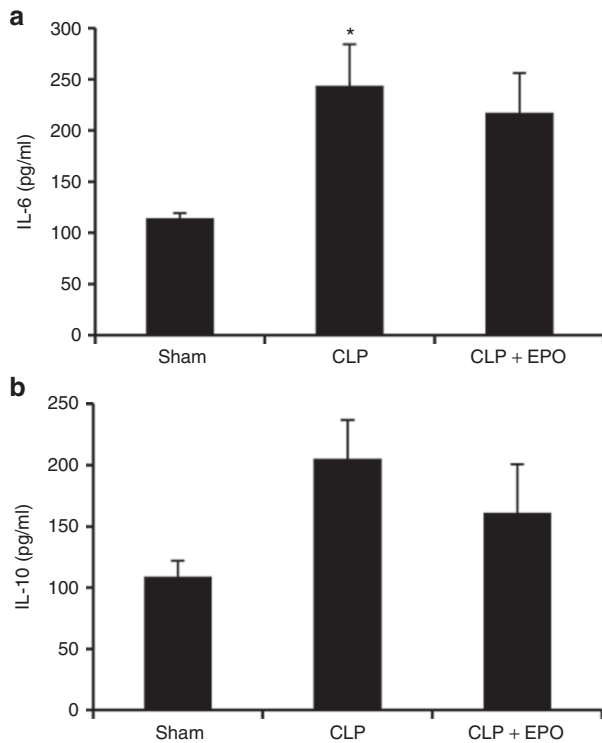
a

b

Figure 3. Erythropoietin (EPO) does not significantly change serum interleukin (IL)-6 and IL-10 levels. (**a**) IL-6 levels in sham-operated animals, animals that underwent cecal ligation and puncture (CLP) and received saline only (CLP group), and animals that underwent CLP and received EPO (CLP + EPO group). (**b**) IL-10 levels in sham-operated animals, animals that underwent CLP and received saline only (CLP), and animals that underwent CLP and received EPO (CLP + EPO). *$P < 0.05$ vs. sham group.
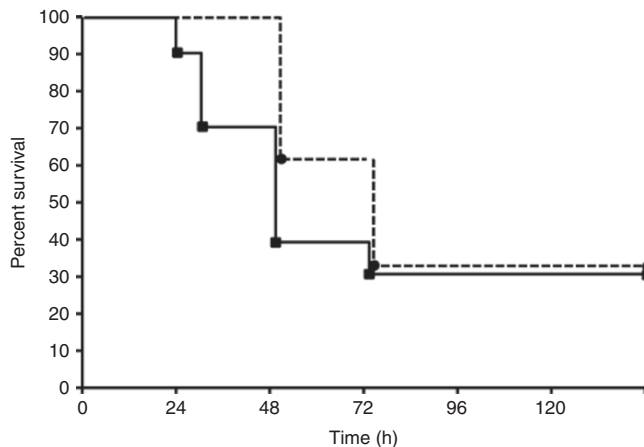

Figure 4. Erythropoietin (EPO) does not improve survival in cecal ligation and puncture (CLP) sepsis. Rats undergoing CLP received 5,000 U/kg EPO (squares) or saline (circles), 30 min before and 1 and 4 h after procedure. Survival was monitored for 7 d.

EPO Does Not Affect IL-6 or IL-10 Cytokine Levels in CLP

To better understand some of the mechanisms whereby EPO exerts its effects during the CLP model of sepsis, we analyzed serum interleukin-6 (IL-6) and IL-10 levels at 24 h after the CLP procedure. These data showed that as compared with sham animals, serum levels of IL-6 ($P < 0.05$) and IL-10 ($P >$ 0.05) increased in animals undergoing CLP. EPO reduced levels of IL-6 and IL-10, but not significantly (**Figure 3**).

EPO Does Not Reduce Mortality Induced by CLP

To determine whether EPO reduced mortality owing to sepsis, CLP was performed. Animals received 5,000 U/kg EPO (*n* = 10) or equivalent volume of normal saline (*n* = 10) intraperitoneally at 30 min before and then 1 and 4 h after the procedure. The administration of EPO conferred no survival benefit over normal saline, and survival in both groups was 30% at 7 d (*P* = 0.82; **Figure 4**). No deaths occurred in sham-operated animals (*n* = 4; data not shown).

DISCUSSION

In this study, we show for the first time that administration of EPO in a CLP model of sepsis attenuates apoptosis in the spleen, thymus, and small intestine of young rats. Yet, despite a reduction in apoptosis, we were not able to demonstrate a survival benefit with EPO in the CLP model of sepsis.

Sepsis is a systemic reaction induced by microorganisms when they enter the body. For many years, an uncontrolled hyperinflammatory state, triggered by the entering microorganisms, was held responsible for much of the morbidity and mortality in sepsis. However, more recent studies have demonstrated that in many patients who survive the initial proinflammatory surge, an anti-inflammatory state ensues in which apoptosis of immune cells has a central role (1,6,20). It is known that in contrast to necrotic cell death, apoptotic cell death can have significant anti-inflammatory consequences because the uptake of apoptotic cells by phagocytic cells leads to the release of anti-inflammatory cytokines. The hypoimmune state and the apoptosis-associated depletion of immune cells may in turn cause inability to clear the primary infection or development of nosocomial infections, ultimately leading to demise of the host (8). Attenuation of apoptosis, therefore, appears to be a logical treatment target in sepsis (21,22). Indeed, numerous experimental studies have demonstrated that prevention of lymphocyte apoptosis in sepsis improves survival (10,11,23).

EPO, a hematopoietic growth factor with antiapoptotic properties, has been shown to have cytoprotective effects in various organs and tissues (14,15,17). We previously demonstrated that EPO attenuates splenic and thymic apoptosis in an endotoxic sepsis model (18). Recently, with mounting knowledge about its cytoprotective effects, a role for EPO in the treatment of sepsis has been suggested (16).

Antiapoptotic and tissue protective effects of EPO have been demonstrated most vigorously in models of ischemia–reperfusion injury affecting the brain, intestine, heart, and kidney, among other organs and tissues (14,24–26). More recently, studies have indicated that EPO has cytoprotective effects in experimental models of inflammation and sepsis. In a murine model of zymosan-induced inflammation and organ failure, treatment with EPO attenuated lung, liver, and pancreatic injury; renal dysfunction; and mortality (15). Pretreatment with EPO before LPS administration in a rat model of lung injury attenuated histological lung injury and edema, and

LPS-mediated myeloperoxidase activity (27). EPO also significantly attenuated renal dysfunction in mice induced by LPS, possibly reversing adverse effects on renal superoxide dismutase. Furthermore, continuous EPO receptor activator was shown to preserve creatinine clearance and tubular function in a CLP model of sepsis in rats (28). In a model of LPS-induced sepsis, Aoshiba *et al.* demonstrated that EPO reduces apoptosis in the lung, liver, small intestine, spleen, and thymus, findings consistent with our previous work (18,19). Our current results, which show attenuation of apoptosis by EPO in the thymus, spleen, and small intestine of rats undergoing CLP, appear therefore to be consistent with the literature suggesting an antiapoptotic role of EPO in various models of inflammation and sepsis.

In models of ischemia–reperfusion injury, protective effects of EPO may be mediated by the inhibition of several proinflammatory cytokines, including IL-6 and tumor necrosis factor-α (TNF-α) (24,29). Although in our study animals in the EPO group had slightly lower IL-6 and IL-10 cytokine levels than control animals, we were not able to show a statistically significant decrease. In this respect, our results appear to be similar to other studies of endotoxic shock in mice and pigs showing unchanged cytokine levels with EPO (19,30). However, existing literature on the effects of EPO on cytokine levels in sepsis is not consistent. In conscious rats, EPO was reported to increase the release of IL-6, IL-1b, and TNF-α along with markers of organ injury in LPS-treated animals (31). In humans, a single low dose of EPO given before a bolus injection of endotoxin leads to increased levels of TNF-α and IL-6 with no effect on IL-10. However, EPO was shown to attenuate platelet and leukocyte adhesion as well as blood–brain barrier dysfunction and TNF levels in CLP mice (32). Therefore, additional research is needed to better define the role, if any, of cytokines in the actions of EPO in models of inflammation and sepsis.

Despite multiple experimental studies clearly demonstrating improved survival with reduction of lymphocyte apoptosis in sepsis, we were not able to achieve a similar effect with EPO. Aoshiba *et al.*, in their study of endotoxemic shock, reported that survival benefit was achieved through the use of EPO in mice only if the agent was administered within 2 h of LPS administration, and there was no protection if EPO was given either before LPS or was started later than 2 h. Moreover, they were not able to establish a survival benefit in the CLP model of sepsis, similar to our current results (19). Mortality in endotoxemic sepsis is frequently related to a proinflammatory cytokine storm. If EPO does not change or even increases proinflammatory cytokine levels in reaction to LPS, as suggested by some studies in humans and animals, it is unlikely that EPO will have a beneficial effect in the LPS-induced cytokine surge or on mortality that results from it (31,33).

In the study by Aoshiba *et al.*, it is uncertain why EPO failed in the CLP group because apoptosis was not assessed in animals undergoing CLP. We demonstrated that EPO reduces apoptosis in lymphoid tissue in the CLP model of sepsis; however, this did not result in a survival advantage. These findings

suggest that EPO, even if it prevents lymphoid tissue apoptosis, does not improve survival in the CLP model of sepsis.

In this study, we chose to administer early high-dose EPO based on the experimental data showing that lymphoid apoptosis in the CLP model of sepsis starts very early and is most crucial during the early phase of illness (34–36). Even 24 h after a single 5,000 U/kg intraperitoneal dose of EPO, plasma concentrations remain at a high level of >1,000 mU/ml, and levels do not return to baseline before 48 h (37). We therefore believe that a significant amount of apoptosis expected to occur following CLP was prevented with the doses we administered (as seen in the reduced lymphoid apoptosis at 24 h). However, we cannot completely rule out that later or further doses of EPO might have had some positive effect on survival. Of note, Aoshiba *et al.* did administer EPO for a longer period (several days) without any additional survival benefit.

Other rare circumstances have been reported in which, despite prevention of lymphoid apoptosis, no survival benefit could be achieved. In an experimental study of LPS-induced acute lung injury, reduction of intestinal apoptosis via overexpression of the antiapoptotic protein Bcl-2 failed to result in a survival advantage in LPS-treated animals (38). Moreover, sepsis-induced T and B lymphocyte apoptosis is prevented in myeloid differentiation primary response protein 88 (MyD88)-knockout mice; nevertheless, there is a significant increase in mortality. Considering the role MyD88 has in the recognition of invading pathogens, it is possible that prevention of lymphocyte apoptosis is only protective if the host inflammatory response to microorganisms is not disturbed (39,40). Recently, Rodrigues *et al.* demonstrated that administration of continuous EPO receptor activator, an EPO with a long half-life, decreases expression of Toll-like receptor 4 and nuclear factor-κB in the kidney of septic rats (28). It is possible that EPO in the CLP model of sepsis is interfering with the host response to pathogens by decreasing the expression of Toll-like receptor 4 or through other mechanisms. The results of such interference could include, among others, decreased plasma levels of pro- and anti-inflammatory cytokines, as reported in the study of Rodrigues *et al.* (28). Indeed, serum levels of IL-6 and IL-10 in our CLP + EPO study group were lower than in the control group, although these changes did not reach a significant degree.

Unfortunately, we have no data on other cytokines, such as TNF-α or interferon-γ, that could have provided better information regarding the inflammatory state of the host. Moreover, it would have been interesting to have data on Toll-like receptor 4 and MyD88 expression to understand whether EPO interferes with host response signaling in this way.

Conclusion

With clearly established knowledge on the role of lymphoid apoptosis in the pathobiology of sepsis, antiapoptotic treatment approaches are gaining interest. We found that EPO, although effective in reducing lymphoid tissue apoptosis in the CLP model of sepsis, is not associated with a survival benefit in the CLP model of experimental sepsis. Despite promising

earlier reports on its benefits in the LPS model, further research is needed about the possible actions of EPO before it can be considered as an antiapoptotic agent for clinical use in sepsis.

METHODS

The experiments were performed in adherence to US National Institutes of Health guidelines on the use of experimental animals after approval by the Dokuz Eylul University Medical Faculty Animal Experiments Ethics Committee. During the study, animals were handled by or under the supervision of a veterinarian and all sedation was provided by an anesthesiologist.

Four- to 8-wk-old young male Wistar rats, weighing 90–130 g, were housed in constant temperature at 14:10 h periods of light and dark exposure with food and water freely available. The CLP model of sepsis that reproduces many of the clinical features of sepsis was used (41). Briefly, a midline laparotomy was performed on a temperature-regulated table under halothane anesthesia delivered through a face mask. During surgery, concentrations of end-tidal CO_2 and halothane were monitored with the use of a capnograph (Anesthesia Gas Monitor 1304; Bruel & Kjaer, Naerum, Denmark). After externalization, the cecum was ligated below the ileocecal valve with 4-0 silk. Then two perforations were made with a 20-gauge needle. The abdomen was closed in two layers, followed by a 30 ml/kg subcutaneous injection of normal saline. In sham-operated animals, the abdomen was opened but the cecum was neither ligated nor punctured. After surgery, animals were housed in individual cages.

For the experiments, rats were randomly divided into three groups. The first two groups ($n = 8$ each) received either saline (1 ml/kg) or human EPO (Roche, Basel, Switzerland) (5,000 U/kg) through intraperitoneal injection 30 min before and then 1 and 4 h after CLP. A third group served as sham ($n = 4$). Following halothane anesthesia, the thymus, spleen, and ileum were removed 24 h after CLP and fixed in formalin (Sigma Aldrich, St Louis, MO). For analysis of survival after CLP, the condition of animals (EPO group $n = 10$ and saline group $n = 10$) was evaluated daily for up to 7 d after the procedure.

Quantification of Apoptosis

Tissues were fixed in 10% buffered formalin overnight and embedded in paraffin after tissue processing. Serial sections (5 mm) were cut for histologic staining. Apoptotic cells were quantified by TUNEL and caspase-3 immunostaining. Apoptotic cells were quantified in a minimum of five random ×400 magnification fields. Quantitation was performed in both organs by an investigator blinded to sample identity.

Caspase-3 Immunostaining

Sections were mounted on poly-L-lysin-coated slides. The avidin-biotin-peroxidase method was performed using the primary monoclonal antibody against caspase-3 (1:100 dilution; Neomarkers, Fremont, CA). Briefly, the sections were deparaffinized and endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxidase in phosphate-buffered saline at room temperature for 10 min. After a 10-min microwave treatment, primary antibody was applied for 30 min at room temperature and washed in phosphate-buffered saline. Linking antibody and streptavidin-peroxidase complex (Neomarkers) were added consecutively for 10 min at room temperature and washed in phosphate-buffered saline. The peroxidase activity was visualized with diaminobenzidine (Sigma, St. Louis, MO) applied for 5 min. Appropriate positive and negative controls were also labeled with the primary antibody.

In Situ Cell Death Detection: TUNEL

To detect DNA fragmentation in cell nuclei, TUNEL assay was applied to the paraffin sections by using a commercial kit (In Situ Cell Death Detection Kit-POD, Roche). Paraffin-embedded tissues were dewaxed and then treated with 20 mg/ml proteinase K (Roche, Germany) for 10 min. After treatment with 0.3% H_2O_2 in methanol for 10 min and 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, the sections were incubated with TUNEL reaction mixture for 60 min at 37 °C. Further incubation with peroxidase-conjugated antibody

was performed for 30 min at 37 °C. The sections were stained with diaminobenzidine solution for 10 min at room temperature and then counterstained with hematoxylin.

Cytokine Analysis

At the time of killing, blood was obtained by cardiac puncture for determination of cytokine levels. After centrifugation (14,000 rpm, 4 °C, 5 min), serum was stored at −80 °C for later analysis. Standard enzyme-linked immunosorbent assay procedures described by the manufacturer (Invitrogen, Camarillo, CA) were used for determination of serum levels of IL-6 and IL-10.

Statistical Analyses

Analyses were performed with SPSS 11.0 (SPSS, Chicago, IL). For comparison between groups, one-way ANOVA and *post hoc* Tukey test were used. Survival analyses were performed by Kaplan–Meier analysis. Measurements considered not to be normally distributed were analyzed by Kruskal–Wallis one-way ANOVA, followed by Mann–Whitney's *U*-test, when appropriate. A *P* value < 0.05 was considered significant.

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