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p53-Dependent and -Independent Pathways of Apoptotic Cell Death in Sepsis¹

Richard S. Hotchkiss,^{2*} Kevin W. Tinsley,* Jia-Ji Hui,* Katherine C. Chang,* Paul E. Swanson,[†] Anne M. Drewry,[§] Timothy G. Buchman,[‡] and Irene E. Karl[§]

Sepsis induces extensive apoptosis of lymphocytes, which may be responsible for the profound immune suppression of the disorder. Two potential pathways of sepsis-induced lymphocyte apoptosis, Fas and p53, were investigated. Lymphocyte apoptosis was evaluated 20–22 h after sepsis by annexin V or DNA nick-end labeling. Fas receptor-deficient mice had no protection against sepsis-induced apoptosis in thymocytes or splenocytes. p53 knockout mice (p53^{-/-}) had complete protection against thymocyte apoptosis but, surprisingly, had no protection in splenocytes. p53^{-/-} mice had no improvement in sepsis survival compared with appropriately matched control mice with sepsis. We conclude that both p53-dependent and p53-independent pathways of cell death exist in sepsis. This differential apoptotic response of thymocytes vs splenocytes in p53^{-/-} mice suggests that either the cellular response or the death-inducing signal is cell-type specific in sepsis. The fact that p53^{-/-} lymphocytes of an identical subtype (CD8⁻CD4⁺) were protected in thymi but not in spleens indicates that cell susceptibility to apoptosis differs depending upon other unidentified factors. *The Journal of Immunology*, 2000, 164: 3675–3680.

Sepsis is currently the most common cause of patient death in many intensive care units (1). A hallmark of patients with sepsis is the development of anergy, which is typified by their loss of the delayed type hypersensitivity response (2). Septic patients do not respond to skin tests when challenged with Ags to which they were previously exposed and therefore to which they should have a positive response (e.g., *Candida*, mumps, etc.) (2). One possible factor that may be contributing to the immune suppression in sepsis is the profound loss of lymphocytes that occurs in sepsis. Animal studies have demonstrated that sepsis induces extensive lymphocyte apoptosis throughout the body (3–6). A recent postmortem study we conducted in patients who died of sepsis and multiple organ failure showed that lymphocyte apoptosis occurs in spleen, Peyer's patches, intestinal lamina propria, and lymphoid aggregates (7). A profound decrease in the circulating lymphocyte count (often decreased to <50% of normal) was associated with the lymphocyte apoptosis in 15 of 19 septic patients (7). Evidence supporting the importance of lymphocyte apoptosis on outcome in sepsis has recently been reported by Ayala and coworkers (8), who showed that septic mice deficient in FasL^{gld} (Fas ligand)³ had decreased mucosal B lymphocyte apoptosis and improved survival compared with controls. Recently our laboratory reported that transgenic mice that overexpressed the antiapoptotic protein Bcl-2 in T cells had complete protection against sepsis-induced T cell apoptosis as well as improved survival (9).

The purpose of this study was to examine potential molecular mechanisms of apoptosis. Fas, a member of the TNF receptor family, is a cell surface protein that is involved in both clonal deletion of autoreactive T cells and elimination of activated T cells (10, 11). Activation of Fas results in lymphocyte apoptosis via a caspase-8-mediated pathway. p53 is a stress-induced transcription factor that can be activated by a number of adverse stimuli including DNA damage, hypoxia, and reactive oxygen species. Increased expression of p53 protein under these conditions causes growth arrest or apoptosis (12, 13). These two potential apoptotic pathways may be interrelated because in some instances p53 activation may induce Fas expression (14, 15). The role of these two mechanisms was investigated in the mouse cecal ligation and perforation (CLP) model of sepsis, a clinically relevant model of the disorder, using genetic constructs.

Materials and Methods

Genetic mice models

Fas receptor-deficient. Fas receptor-deficient mice (MRL/MpJ-Fas^{lpr}) and normal age- and sex-matched controls (MRL/MpJ) were purchased from The Jackson Laboratory (Bar Harbor, ME; catalog nos. 00485 and 00486, respectively). These mice have a very low expression of the Fas receptor due to insertion of a retrotransposable element in an intron of the *fas* gene (10).

p53 knockout. Mice homozygous for p53 deficiency, (p53^{-/-}; see Ref. 12) and normal age- and sex-matched controls C57BL/6J (B6J) were also purchased from The Jackson Laboratory (catalog no. 002101). p53^{-/-} mice have a high spontaneous rate of lymphomas, and one mouse was excluded because of this complication.

CLP model of sepsis

The CLP model of sepsis is a widely utilized, clinically relevant model of sepsis (peritonitis) that has been validated in many laboratories (5, 16). Multiple Gram-negative and Gram-positive organisms are obtained on blood culture from CLP mice (5). In anesthetized mice, the cecum is isolated, ligated with 4-0 silk, and punctured once with a 26-gauge needle. Sham-operated mice had cecal manipulation only. At 20–22 h postsurgery, CLP and sham mice were killed, and thymi and spleens were removed for study.

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³ Abbreviations used in this paper: FasL, Fas ligand; CLP, cecal ligation and perforation; PI, propidium iodide.

Evaluation of apoptosis

Fluorescent TUNEL. Thymi and spleens from Fas-deficient and matched control mice were excised and placed in 10% paraformaldehyde. Paraffin-embedded tissue slices were dewaxed, rehydrated, and evaluated using an apoptosis detection kit (Boehringer Mannheim, Indianapolis, IN) as described previously (5, 9). Tissue sections were examined at $\times 200$ magnification by fluorescence microscopy, and a minimum of three random fields were evaluated. The percentage of area of the field that was positively labeled for apoptosis was calculated using an image analysis program (Metamorph; Universal Imaging, West Chester, PA) as described previously (9).

Flow cytometry: cell phenotyping and quantification of apoptosis.

Thymi and spleens from the various groups of mice discussed previously were gently glass ground to dissociate the cells, which were then washed twice in PBS with 1% BSA and 0.01% sodium azide. The degree of cell apoptosis was quantified using a commercially available annexin V/propidium iodide product (apoptosis detection kit; R&D Systems, Minneapolis, MN) as described previously (9). Mouse T and B subsets were determined using a variety of cychrome- or PE-labeled anti-CD Abs (PharMingen, San Diego, CA) as described previously (9). Flow cytometric analysis (50,000 events/sample) was performed on FACSCaliber (Becton Dickinson, San Jose, CA).

Survival studies in sepsis. Additional groups of p53^{-/-} and B6J mice underwent CLP and survival was recorded. The methods for the survival studies in the mouse CLP model have been described previously (9). Briefly, an investigator blinded to the identity of the mice performed CLP in eight p53^{-/-} and eight B6J mice. Approximately 1 h after CLP, the mice received metronidazole (35 mg/kg) and ceftriaxone (50 mg/kg). The mice were allowed free access to food and water, and survival was recorded for 6 days.

Statistical analysis

Data are reported as the mean \pm SEM. Data were analyzed using the statistical software program Prism (GraphPad Software, San Diego, CA). Data for the percentage of apoptosis determined by flow cytometry were analyzed using one-way ANOVA, except where stated. Differences in group survival were determined using Fischer's exact *p* test. The *p* values ≤ 0.05 were accepted as significant.

Results

Flow cytometry: annexin V labeling and cell phenotyping in Fas-deficient and MRL/MpJ mice

Thymocytes and splenocytes from septic Fas-deficient mice ($n = 8$) had a marked increase in annexin V-positive and propidium iodide (PI)-negative stained cells (indicative of apoptosis) compared with sham-operated Fas-deficient mice ($n = 7$; 11.2 ± 1.0 vs 3.5 ± 0.4 for septic and sham thymocytes, respectively, and 10.0 ± 0.7 vs 4.0 ± 0.4 for septic and sham splenocytes, respectively; $p < 0.001$ (Fig. 1)).

MRL/MpJ mice (the control background strain of mice for the Fas receptor-deficient mice) also had a marked increase in annexin V-positive and PI-negative labeled thymocytes and splenocytes from septic ($n = 9$) vs sham ($n = 6$) mice (12.0 ± 0.4 vs 3.6 ± 0.3 for septic and sham thymocytes, respectively, and 10.7 ± 0.5 vs 4.3 ± 0.5 for septic and sham splenocytes, respectively; $p < 0.001$ (Fig. 1)).

The degree of apoptosis in the various cell phenotypes in thymi and spleens of septic and sham-operated Fas-deficient and MRL/MpJ mice is demonstrated in Fig. 2. Sepsis caused a statistically significant increase in apoptosis in all T cell phenotypes in thymi ($p < 0.01$) and in T and B splenocytes ($p < 0.001$) of both strains of mice (Fig. 2). There were no statistical differences in the degree of sepsis-induced apoptosis in cells from Fas-deficient mice vs those from MRL/MpJ mice.

Flow cytometry: annexin V labeling and cell phenotyping in p53^{-/-} and B6J mice

Flow cytometry using dual labeling with annexin V and PI was performed in splenocytes and thymocytes from a limited number

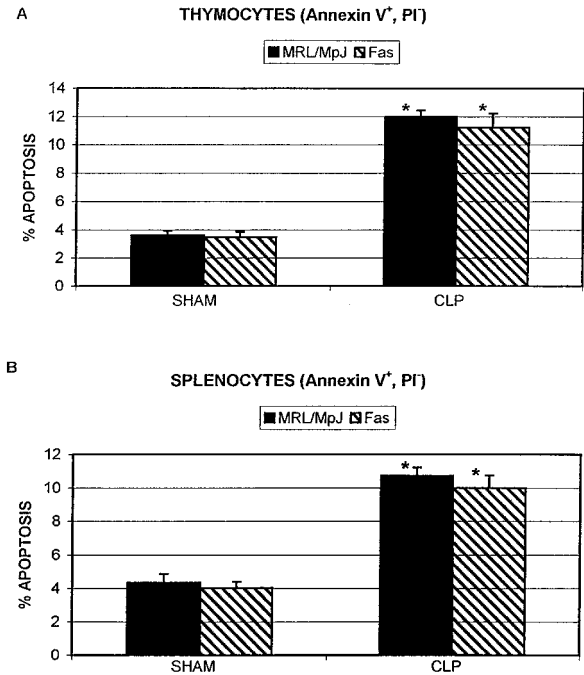


FIGURE 1. Percentage of apoptotic cells in Fas-deficient mice determined by annexin V and propidium iodide staining. Thymocytes (A) and splenocytes (B) from mice that underwent CLP to induce sepsis or sham surgery were dissociated and labeled with annexin V and PI using a commercially available apoptosis detection kit (see *Materials and Methods*). Cells were examined by flow cytometry, and those cells that were annexin V-positive but PI-negative were graded as apoptotic. Note the increase in apoptosis in thymocytes and splenocytes from septic Fas-deficient and MRL/MpJ mice (the control mice for Fas-deficient mice) vs sham-operated mice. *, $p < 0.001$.

of septic and sham B6J mice (three sham and three septic) and p53^{-/-} mice (two sham and three septic) (Fig. 3). Because of the limited number of mice in this part of the study (due to decreased availability of p53^{-/-} mice), data were compared by student's *t* test. Similar to results in the Fas-deficient and MRL/MpJ mice, sepsis caused a marked increase in annexin V-positive and PI-negative labeled thymocytes ($p < 0.02$) and splenocytes ($p < 0.05$) in B6J mice (Fig. 3). Surprisingly, thymocytes but not splenocytes from septic p53^{-/-} mice had no increase in apoptosis (Fig. 3).

To examine effects of sepsis on apoptosis in the various cell phenotypes, a larger number of p53^{-/-} and B6J mice were examined (eight septic and five sham p53^{-/-} mice; 11 septic and six sham B6J mice). Data were analyzed by ANOVA. The increase in apoptosis in septic vs sham B6J mice occurred in all cell types of thymi and spleens, i.e., CD8⁺CD4⁻, CD8⁻CD4⁺, CD8⁺CD4⁺, CD3⁺19⁻, and CD3⁻CD19⁺. In some cases, over 20% of cells were apoptotic (Fig. 4).

Similar to findings in septic B6J cells, splenocytes of all subtypes from septic p53^{-/-} mice had a marked increase in annexin V-positive cells compared with cells from sham-operated p53^{-/-} mice. There was no difference in the percentage of apoptotic splenocytes in septic B6J vs septic p53^{-/-} mice.

Surprisingly, CD3⁺CD19⁻, CD8⁻CD4⁺, CD8⁺CD4⁻, and CD8⁺CD4⁺ thymocytes from septic p53^{-/-} mice did not have increased annexin V labeling compared with sham-operated p53^{-/-} mice (Fig. 4B). Also, the percentage of apoptotic thymocytes from septic p53^{-/-} mice was less than that in thymocytes from septic B6J mice ($p < 0.01$).

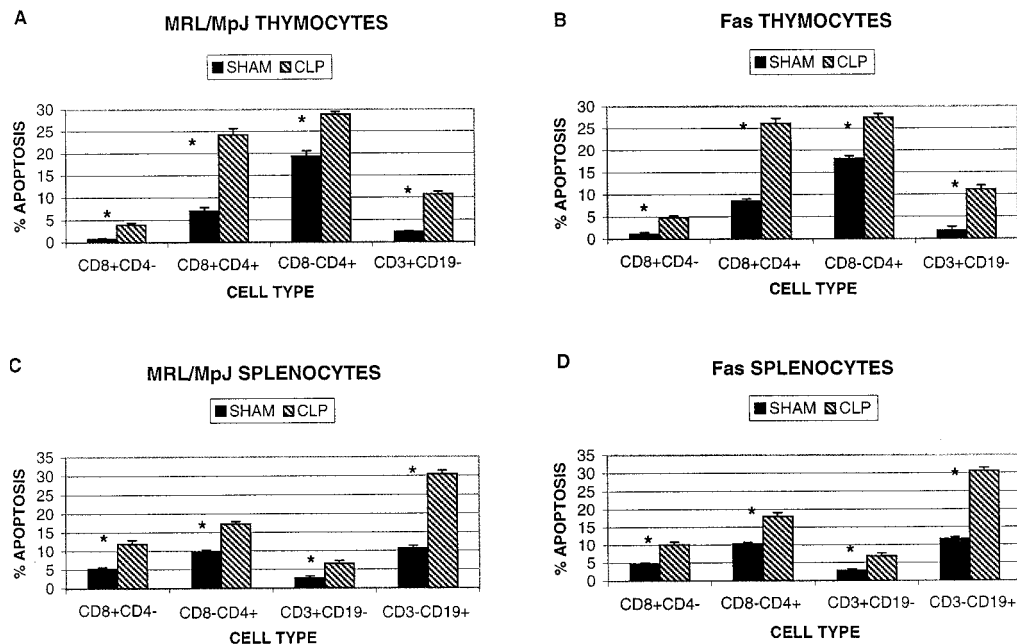


FIGURE 2. Percentage of apoptotic cells (phenotypes) in Fas-deficient and MRL/MpJ mice determined by annexin V staining. Thymocytes and splenocytes were dissociated, and apoptotic cells were detected using fluorescein-labeled annexin V and flow cytometry. T and B cells were detected using fluorescently labeled Abs to CD markers. Note that the increase in the percentage of apoptosis in thymocytes (A and B) and splenocytes (C and D) from Fas-deficient and MRL/MpJ mice that underwent CLP vs thymocytes and splenocytes from sham-operated mice.

The absence of apoptosis in thymi of septic $p53^{-/-}$ mice was confirmed by light microscopy. An observer blinded to sample identity scored thymi from septic $p53^{-/-}$ mice as having no increase in apoptosis compared with sham-operated mice (data not shown). Representative examples of hematoxylin and eosin-stained thymi from septic $p53^{-/-}$ and B6J mice are demonstrated in Fig. 6. Thymocytes from the septic $p53^{-/-}$ mice are normal in appearance, whereas large numbers of thymocytes from the septic B6J mice showed classic nuclear features of pyknosis and karyorrhexis, which are diagnostic of apoptosis.

Sepsis survival study

There was no difference in sepsis survival in the two groups of mice ($p53^{-/-}$ ($n = 8$) vs B6J ($n = 8$) mice) (Fig. 5). At the end of 6 days, there was a 37.5% survival in the $p53^{-/-}$ mice and a 25% survival in the B6J mice.

Discussion

An important implication of the present work regards the nature of the complex regulation of apoptosis. The finding that T lymphocyte apoptosis in sepsis is $p53$ -dependent in thymus but not in spleen highlights the point that the cell death program can be independently regulated even within the same cell type and illustrates how specifically the decision of life vs death is controlled by the cell. Some of the organ-specific differences in T cell death in sepsis may be due to differences in immature vs mature cells. Immature thymocytes have a high rate of spontaneous apoptosis as the body seeks to eliminate potentially autoreactive lymphocytes (17). However, the fact that there was also a site-specific effect of sepsis in the more differentiated $CD8^+CD4^-$ T cells (i.e., apoptosis was not increased in sepsis in $CD8^+CD4^-$ T cells in thymi, but it was in spleens) indicates that the differences in apoptosis may not be due solely to changes in cell maturation. The ultimate decision to undergo apoptosis may be influenced by local environmental factors such as the paracrine mediators that are present in

sepsis. The importance of local factors on apoptosis may also explain the contradictory results of various studies examining specific mechanisms of apoptosis and underscores the necessity to employ *in vivo* models whenever possible.

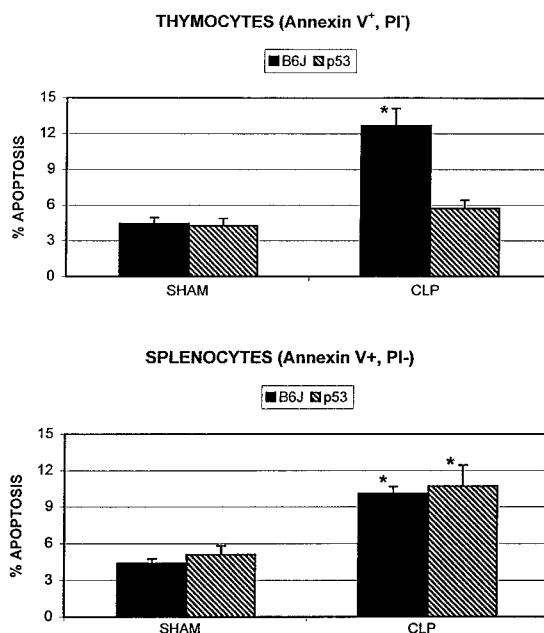


FIGURE 3. Percent apoptotic cells in $p53^{-/-}$ and B6J mice determined by annexin V and propidium iodide staining. Thymocytes (upper panel) and splenocytes (lower panel) from septic (CLP) or sham-operated mice were dissociated and labeled with annexin V and PI and were examined by flow cytometry as described previously. There was a marked increase in the percentage of apoptosis in thymocytes and splenocytes from septic vs sham B6J mice. In $p53^{-/-}$ mice, there was an increase in splenocyte apoptosis but not in thymocyte apoptosis in sepsis. *, $p < 0.05$.

Annexin V Detection of Apoptosis

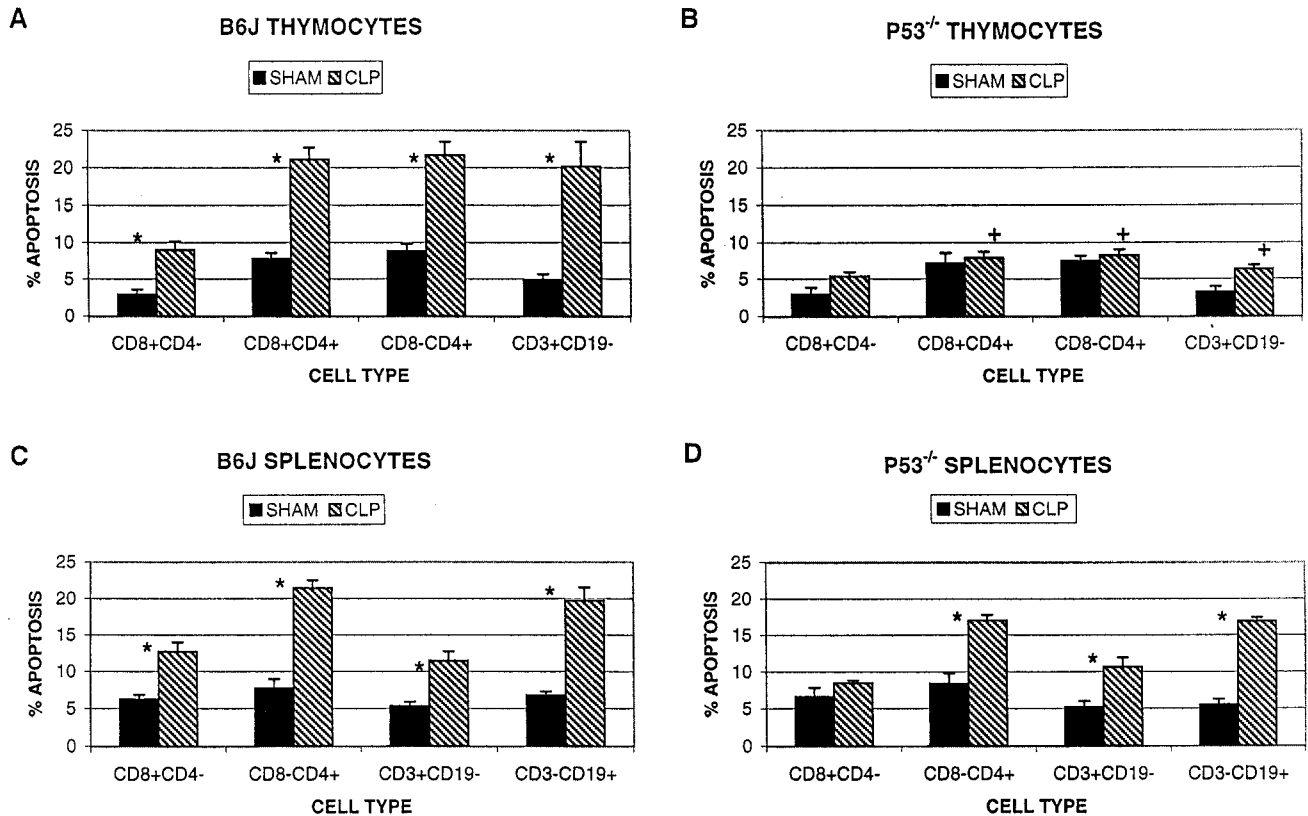


FIGURE 4. Percentage of apoptotic cells (phenotypes) in $p53^{-/-}$ and B6J mice determined by annexin V staining. Thymocytes (A and B) and splenocytes (C and D) were dissociated and apoptotic cells were detected using fluorescein-labeled annexin V and flow cytometry. T and B cells were detected using fluorescently labeled Abs to CD markers. Sepsis caused an increase in apoptosis in all phenotypes in the B6J mice (A and C) (i.e., all T cell subsets and in B cells; *, $p < 0.01$ septic vs sham B6J). Similar to the results in Fig. 3, $p53^{-/-}$ had no increase in apoptosis in any T cell subset in thymocytes (B). However, splenocytes from septic $p53^{-/-}$ did have an increase in apoptosis compared with splenocytes from sham $p53^{-/-}$ mice (*, $p < 0.01$) (D). Similar to results in Fig. 2, the degree of apoptosis in thymocytes from septic $p53^{-/-}$ mice was no different from that in sham-operated $p53^{-/-}$ mice, but it was less than the apoptosis in thymocytes of B6J mice. +, $p < 0.01$.

The results showing that thymocytes from $p53^{-/-}$ mice did not undergo apoptosis in sepsis may shed light on the nature of the apoptotic stimulus in sepsis. p53 is a stress-induced transcription factor, and recent work has implicated the cellular redox system as

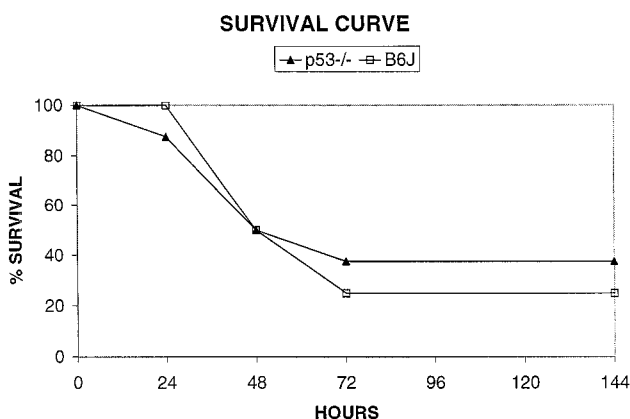


FIGURE 5. Sepsis survival study in $p53^{-/-}$ and B6J mice. Eight $p53^{-/-}$ mice and eight B6J mice underwent CLP to induce sepsis. Approximately 1 h after CLP, mice received antibiotics (35 mg/kg metronidazole and 50 mg/kg ceftriaxone). Survival was recorded for 6 days, and there was no difference in outcome in the two groups of mice.

a preeminent target for p53's death-inducing capability (13, 18). Polyak et al. (13) examined gene transcripts induced by p53 expression before the onset of apoptosis. Of 7202 transcripts identified, only 14 were found to be markedly increased in p53-expressing cells compared with control cells. Many of these 14 newly identified gene targets of p53 encode proteins that generate or respond to oxidative stress. Thus, the concept has developed from this and similar studies that p53-induced death-specific transcripts may generate a burst of reactive oxygen species that triggers the mitochondrial-mediated apoptosis pathway (13, 18, 19). Recent work from our laboratory supports a role for reactive oxygen species in sepsis-induced apoptosis. Knockout mice totally deficient in the key antioxidant enzyme Cu/Zn superoxide dismutase had a marked increase in thymocyte apoptosis during sepsis (20).

In addition to induction of genes involved in the redox pathway, two other proapoptotic genes known to be induced by p53 are *fas* (15) and *bax* (21). The present findings demonstrating that Fas receptor-deficient mice did not have decreased thymocyte apoptosis in sepsis indicate that p53-induced Fas expression is not the likely pathway of cell death in T cells during sepsis. It is also unlikely that p53 is triggering apoptosis by induction of *bax* because of our recent study reporting that *bax*-deficient mice did not have decreased thymocyte or splenocyte apoptosis in sepsis compared with matched controls (9).

SEPTIC THYMI

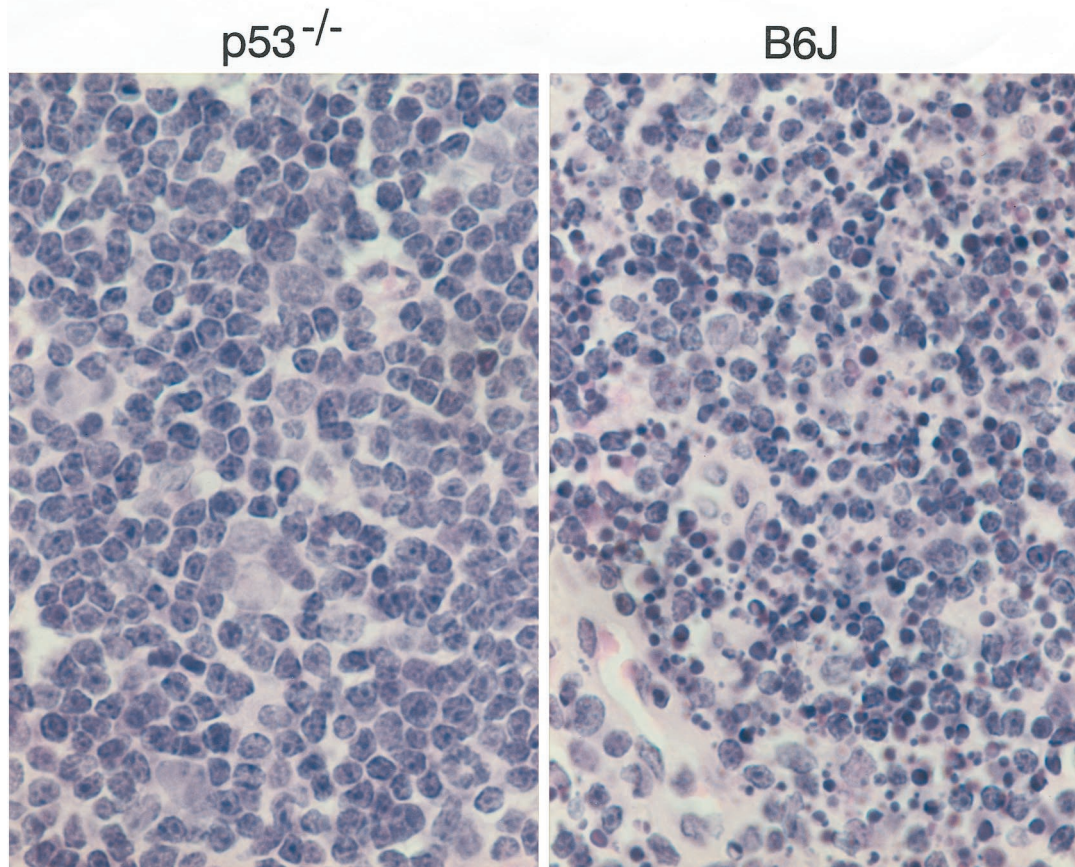


FIGURE 6. Light microscopy of hematoxylin and eosin-stained thymi from septic $p53^{-/-}$ and septic B6J mice. Note the normal-appearing morphology of thymocytes from the septic $p53^{-/-}$ knockout mice. Thymocytes from the septic B6J mice demonstrate small compacted and fragmented nuclei, which are characteristic findings in apoptosis. Magnification is $\times 1000$.

The current results documenting that Fas receptor-deficient mice (Fas^{lpr}) did not confer protection from apoptosis in sepsis agree with work by Ayala et al. (22), who reported that mice deficient in $FasL^{gld}$ did not have protection against sepsis-induced apoptosis in thymi. Of note, the $FasL^{gld}$ mice used in the study of Ayala et al. were on a C3H/HeJ background, an endotoxin-resistant strain. However, it is unlikely that the endotoxin-resistant strain employed in Ayala's study (22) affected lymphocyte apoptosis, given studies that have demonstrated that endotoxin-resistant mice are not protected against sepsis-induced apoptosis (23, 24). Interestingly, work from Ayala's laboratory (24) also demonstrated that Peyer's patch B lymphocyte apoptosis was significantly reduced in $FasL^{gld}$ mice with sepsis. Thus, Ayala's findings of a differential apoptotic response in various lymphoid tissues during sepsis supports the concept that regulation of lymphocyte cell death is influenced by local mediators or stimuli.

Previous studies from our laboratory have shown that prevention of lymphocyte apoptosis in sepsis improves survival in the disorder (9). In this regard, we demonstrated that mice that overexpress the antiapoptotic protein Bcl-2 selectively in T cells have improved survival compared with non-Bcl-2-overexpressing matched controls with sepsis. However, the failure of the $p53^{-/-}$ mice to have improved survival in sepsis compared with the controls (B6J) is not surprising because the $p53$ knockout conferred only selected T cell protection. The only population of T cells that were protected by $p53$ knockout were the T cells in the thymus. Thus, the majority of T cells still underwent apoptosis in sepsis.

Currently, two pathways of lymphocyte apoptosis are postulated to exist (25). The first pathway is a receptor-mediated pathway that involves members of the TNF-R family. The TNF p55 receptor and Fas receptor are two members of this family. Engagement of either of these receptors triggers apoptosis via a caspase-8-mediated mechanism. The current study, which demonstrates that Fas receptor deficiency does not block apoptosis in sepsis (as well as additional unpublished studies in our laboratory that show that TNF p55 knockout mice are not protected from sepsis-induced lymphocyte apoptosis), suggests that this pathway is not operative in sepsis.

The second presumed pathway of cell apoptosis is thought to occur by a mitochondrial-mediated pathway (25). Cytochrome C, which is released from the mitochondria, binds to apoptosis activating factor and forms a complex that results in activation of caspase-9. $p53$ is known to induce apoptosis by this mitochondrial pathway, although the exact target of activated $p53$ is unknown (26). The present study demonstrates that thymocyte but not splenocyte apoptosis occurs via a $p53$ -mediated mitochondrial pathway in sepsis. Other findings from our laboratory support this conclusion. Although not all investigators agree, a prevailing view is that the antiapoptotic protein Bcl-2 prevents mitochondrial-mediated apoptosis but is ineffective in receptor-operated (Fas- or TNF-mediated) apoptosis (27). Previously we have shown that transgenic mice that overexpress Bcl-2 in either T cells or B cells have complete protection against sepsis-induced lymphocyte apoptosis (9, 28). Recently we also demonstrated that caspase-9 is

activated in sepsis-induced thymocyte apoptosis (29). Taken together, these studies strongly support the concept that lymphocyte apoptosis in sepsis is mediated by the mitochondrial pathway.

In summary, sepsis induces thymocyte but not splenocyte apoptosis by a p53-dependent pathway. The Fas-mediated apoptotic pathway is not responsible for in vivo splenic or thymic apoptosis in sepsis. The cell decision to commit apoptosis is likely influenced by many factors including cell maturation and local environmental factors.

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