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Akt Decreases Lymphocyte Apoptosis and Improves Survival in Sepsis

Ursula Bomhhardt,* Katherine C. Chang,† Paul E. Swanson,§ Tracey H. Wagner,† Kevin W. Tinsley,† Irene E. Karl,‡ and Richard S. Hotchkiss‡†

Sepsis induces extensive death of lymphocytes that may contribute to the immunosuppression and mortality of the disorder. The serine/threonine kinase Akt is a key regulator of cell proliferation and death. The purpose of this study was to determine whether overexpression of Akt would prevent lymphocyte apoptosis and improve survival in sepsis. In addition, given the important role of Akt in cell signaling, T cell Th1 and Th2 cytokine production was determined at 24 h after surgery. Mice with overexpression of Akt had a marked improvement in survival compared with wild-type littermates, i.e., 94 and 47% survival, respectively, p < 0.01. In wild-type littermates, sepsis caused a marked decrease in IFN-γ production, while increasing IL-4 production 2-fold. In contrast, T cells from Akt transgenic mice had an elevated production of IFN-γ at baseline that was maintained during sepsis, while IL-4 had little change. Akt overexpression also decreased sepsis-induced lymphocyte apoptosis via a non-Bcl-2 mechanism. In conclusion, Akt overexpression in lymphocytes prevents sepsis-induced apoptosis, causes a Th1 cytokine propensity, and improves survival. Findings from this study strengthen the concept that a major defect in sepsis is impairment of the adaptive immune system, and suggest that strategies to prevent lymphocyte apoptosis represent a potential important new therapy. The Journal of Immunology, 2004, 172: 7583–7591.

Sepsis is defined as the severe inflammatory response that accompanies overwhelming infection (1). This highly lethal condition is characterized by extensive apoptosis of cells of the immune system (2–6). Recent evidence suggests that the profound lymphocyte apoptosis which occurs during sepsis results in impaired host immune defenses and that prevention of lymphocyte cell death improves survival (2, 4, 5, 7). The serine/threonine kinase Akt (protein kinase B) is a potent regulator of cell proliferation and cell survival and prevents cell death in a variety of settings (8–15). Akt has been demonstrated to decrease apoptosis due to dexamethasone, activated Ras pathway, UV irradiation, and other conditions (8, 11, 14–16). Akt also plays an important role in lymphocyte signaling, for instance in thymocytes leading to increased and sustained activation of the Src kinase Lck and the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway (15). T cells that overexpress Akt have increased expression of activation markers and promote inflammation (14).

Although Akt has been shown to prevent apoptotic cell death, the precise mechanism by which Akt exerts this protective effect is not known. Potential downstream targets of Akt include forkhead proteins (10), Bcl-xL (11), NF-κB (17), Bad (18), caspase 8 (19), and glycogen synthase kinase (20). Importantly, lymphocytes that overexpress Bcl-2, a close relative of Bcl-xL, are resistant to sepsis-induced apoptosis and confer a survival advantage in transgenic mice (7).

Previously, we have shown that transgenic expression of a constitutively active form of Akt1 (myr Akt) influences thymocyte selection and enhances peripheral T cell survival (15). In addition, myr Akt was found to increase T cell reactivity, cell cycle progression, and production of Th1 as well as Th2 cytokines after in vitro stimulation (21). The focus of the present study was to determine whether lymphocytes from mice that express a constitutively active Akt would be protected against sepsis-induced lymphocyte apoptosis and have improved survival in the disorder. T cells that overexpress Akt have been shown to have increased expression of activation markers and promote inflammation (14, 15). We also investigated whether Akt could modulate the impaired T cell activation and cytokine production that are hallmarks of sepsis.

Materials and Methods

Transgenic mice

Construction of human CD2-myr Akt1 (myr Akt) transgenic mice and expression of myr Akt in thymocytes and peripheral T cells by Western blot analysis have been described previously (15). Myr Akt expression in T cells was also verified via flow cytometry using an anti-phospho-Akt Ab from Cell Signaling (Beverly, MA), catalog 9277. Mice transgenic for Akt1 were identified using PCR of tail snips, as previously described (15). Mice used in the experimental protocols were backcrossed more than five generations to C57BL/6 mice (B6) and were 6–12 wk old. Preliminary studies demonstrated no difference in the effect of heterozygous or homozygous Akt overexpression on apoptosis or cytokine production (data not shown). Therefore, mice that were either heterozygous or homozygous for Akt overexpression and their negative wild-type littermates were used for studies.
Cecal ligation and puncture (CLP) model of sepsis

The CLP model of sepsis is a widely used, clinically relevant model of sepsis (peritonitis) that has been validated in many laboratories (2–4). Multiple Gram-negative and Gram-positive organisms are obtained on blood culture from CLP mice (4). Mice weighing 17–25 g were housed for at least 3 days before manipulations. 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. The abdomen was closed in two layers, and the mice were injected with 1.0 ml of 0.9% saline s.c. At 20–22 h postsurgery, CLP and sham mice were killed, and thymus and spleens were removed for study.

Survival studies in sepsis

Additional groups of mice underwent CLP and were included in survival studies. The methods for the survival studies in the mouse CLP model have been described previously (7). Briefly, an investigator blinded to the identity of the mice performed CLP in the various groups of mice. Approximately 1 h after CLP, the mice received imipenem (1 mg/mouse). The antibiotics were repeated every 12 h for 48 h and then discontinued. The mice were allowed free access to food and water, and survival was recorded for 7 days.

Cytokine analysis

ELISPOT (Cellular Technology, Cleveland, OH) was performed, as per the manufacturer’s instructions, to determine the effects of overexpression of Akt on stimulated T cell Th1 and Th2 cytokine production in splenocytes. Approximately 24 h after CLP or sham surgery, splenocytes were isolated by gently grinding the moistened organ between the ends of two frosted microscope slides, followed by filtering through sterile mesh wire, as described previously (4). Because the Akt transgenic mice have a larger percentage of CD4 T cells in the spleen compared with wild-type mice, magnetic cell sorting (positive selection) of CD4 T cells (Miltenyi Biotec, Auburn, CA) was used to isolate these cells (~90% purity via flow cytometry). Equal numbers of CD4 T cells (0.5 × 10⁶) from the transgenic and wild-type mice were used for ELISPOT determinations. Cells were loaded on plates precoated with capture Ab (BD PharMingen, San Diego, CA) for IFN-γ or IL-4 and stimulated with anti-CD3 and anti-CD28 mAbs, as described previously (22). Cell culture medium was RPMI 1640 with 50 μM 2-ME, 2 mM glutamine, supplemented with 10% FCS. Cell incubation was maintained for 24 or 48 h for IFN-γ and IL-4, respectively. A secondary HRP-labeled detecting Ab was added and detected with 3-aminophenylphosphate as a substrate. Images of the individual wells were obtained, and comparison between the intensities of the individual wells was performed using a software program from the manufacturer (Cellular Technology) that counted the number of spots on the ELISPOT plate. The spots are proportional to the number of cytokine-producing cells.

Flow cytometry detection of apoptosis

Briefly, splenocytes were obtained from the different treatment groups ~24 h after sham or CLP surgery, and apoptosis was quantified using a commercially available fluorescein-labeled annexin V (7-aminoactinomycin D, 7-AAD) kit (apoptosis detection kit; R&D Systems, Minneapolis, MN), as described previously (4, 7). The lymphocyte phenotypes were identified using fluorescently labeled mAbs directed against lymphocyte surface markers (BD PharMingen); B cells, CD4 T cells, CD8 T cells, and CD3 T cells. Flow cytometric analysis (10,000–50,000 events/sample) was performed on FACS Calibur (BD Biosciences, San Jose, CA).

Annexin V is an accepted marker for acute detection of apoptosis in cells that have not undergone fixation, but it cannot be used in cells that have been fixed for intracellular protein detection. To relate intracellular expression of Akt (vide infra) and apoptosis, i.e., to determine whether cells that expressed Akt were resistant to apoptosis, the CaspACE FITC-VAD-FMK in situ marker kit (Promega, Madison, WI; catalog G7461) was used, as per the manufacturer’s instructions.

Flow cytometry detection of Akt and active caspase 3

Intracellular staining of splenocytes for active Akt was performed using the protocol recommended by Cell Signaling, and by Tazzari et al. (23). Briefly, dissociated splenocytes were fixed in 1% paraformaldehyde, permeabilized in ice-cold methanol, and incubated with an anti-phospho-Akt Ab (Ser473) (Cell Signaling; catalog 9271). The secondary Ab used to identify the primary Ab was a donkey anti-rabbit Ab (Jackson Immunoresearch Laboratories). For detection of active caspase 3, an Ab specific to the cleaved short fragment of caspase 3 was used, per the manufacturer’s instructions (Cell Signaling; catalog 9664). The cells were fixed in 1% paraformaldehyde, followed by permeabilization with methanol. The secondary Ab was a donkey anti-rabbit Ab (Jackson Immunoresearch Laboratories).

Western blot analysis of Bcl-2

Spleens from Akt transgenic mice or wild-type littermates were removed, and the cells were dissociated by gentle grinding between the frosted ends of two microscope slides. To have equal numbers of T cells for analysis of Bcl-2, CD3 T cells were isolated using the Miltenyi Biotec magnetic bead protocol, as per the manufacturer’s instructions. Approximately 5 × 10⁶ CD3-positive T cells were obtained from each animal, and protein extractions were prepared. A total of 20 μg of protein/well was loaded onto a 12% SDS-PAGE gel, and electrophoresis was performed. The protein was transferred to a nitrocellulose membrane and blocked for 2 h at room temperature with skim milk. Next, the primary Ab was incubated overnight at 4°C with skim milk. The primary Ab against mouse Bcl-2 (BD PharMingen) was detected by HRP-conjugated Ab (BD PharMingen) and the SuperSignal West Fento (Pierce, Rockford, IL).

Western blot analysis of B cells for myr Akt

For Western blot analysis of B cells, splenic cells from wild-type and Akt transgenic mice were treated with anti-CD4 (RL174.2) and anti-CD8 (31.68.1) hybridoma supernatant and low toxin baby rabbit complement (Cedarlane, Hornby, Ontario, Canada). Purity of B cells was verified by search Laboratories, West Grove, PA). For detection of active caspase 3, an Ab specific to the cleaved short fragment of caspase 3 was used, per the manufacturer’s instructions (Cell Signaling; catalog 9664). The cells were fixed in 1% paraformaldehyde, followed by permeabilization with methanol. The secondary Ab was a donkey anti-rabbit Ab (Jackson Immunoresearch Laboratories).

FIGURE 1. Overexpression of Akt improves survival. Transgenic mice with a constitutively active Akt on a CD2 promoter or their wild-type littermates underwent CLP to induce sepsis (see Materials and Methods). Survival was recorded for 7 days. Three separate studies were performed, and the results of the three studies were combined. Mice with overexpression of Akt had a statistically significant improvement in survival compared with wild-type mice, p = 0.0021.

FIGURE 2. Gray scale photomicrographs of ELISPOT for IFN-γ. Twenty-four hours after surgery, splenocytes were isolated from sham- or CLP-operated Akt transgenic mice or wild-type littermates. CD4 T cells (5 × 10⁶) were isolated by Miltenyi Biotec magnetic microbeads and plated on individual wells for 24 h. Production of IFN-γ was determined by a colorimetric reaction involving avidin-HRP-mediated oxidation of a substrate to produce the color reaction (depicted in gray scale). The four photomicrographs are from four mice in the same experiment. Note the dramatic decrease in IFN-γ in CD4 T cells from wild-type (B6) mice that had undergone CLP to induce sepsis. Transgenic Akt mice maintained a robust production of IFN-γ.

3 Abbreviations used in this paper: CLP, cecal ligation and puncture; 7-AAD, 7-aminoactinomycin D.
Germany) for the indicated time points. A total of 2/H11003 B cells; H11001 T cell populations in the sham-operated Akt and wild-type mice showed that the Akt transgenics had a greater percentage of CD4 T cells, but a smaller 1 mM DTT, 1 mM orthovanadate, 2 erophosphate, 1% Triton X-100, 10% glycerol, 50 mM NaF, 0.04% azide, tional to the number of cytokine-producing cells; see Fig. 2. Technology) that counted the number of spots in each sample. The spots are proportion- microwells was performed using a software program from the manufacturer (Cellular Technology) that counted the number of spots in each sample. The spots are proportional to the number of cytokine-producing cells; see Fig. 2. staining for CD3 expression (145.2C11; BD PharMingen) and routinely was greater than 90%. Isolated B cells were left untreated or were stimu- lated with anti-IgM F(ab')2, Ab (10 µg/ml; Sigma-Aldrich, TaulKirchen, Germany) for the indicated time points. A total of 2 × 10^6 B cells was lysed in buffer containing 20 mM HEPES, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 50 mM NaF, 0.04% azide, 1 mM DTT, 1 mM orthovanadate, 2 µM leupeptin, and 0.4 mM PMSF for 30 min on ice. Protein extracts were separated on 8% SDS-PAGE, electroblotted to nitrocellulose membrane, and analyzed for expression of ac- tive Akt using anti-phospho-Akt (Ser473 ) Ab (Cell Signaling). The blot was reprobed with anti-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA) to control protein loading. Primary Abs were detected by goat anti-rabbit or rabbit anti-goat Ab coupled with HRP (both Jackson ImmunoResearch Laboratories) and SuperSignal West Pico (Pierce).

Immunohistochemical staining for phospho-Akt and phospho-forkhead

For immunohistochemical staining of phospho-Akt, a rabbit anti-phospho- Akt Ab from Cell Signaling (catalog 9277) was used, as per the manufac-
Akt overexpression caused increased CD4 T cells

As noted previously (15), flow cytometry revealed an increase in the percentage of CD4 T cells in splenocytes from the Akt transgenics vs wild-type littermate mice (Figs. 3A and 4). In spleens of sham-operated wild-type littermate mice, the percentage of CD4 T cells was 24.2 ± 1.3%, whereas the percentage of CD4 T cells in Akt transgenic mice was 46.6 ± 1.8%, \( p < 0.05 \) (Fig. 3A). In contrast, the percentage of B cells (CD19+) was decreased in the sham-operated Akt transgenics (30.1 ± 2.5%) compared with sham-operated wild-type littermate controls (48.0 ± 3.7%), and this most likely represents a compensatory effect of the increased percentage of CD4 T cells (Fig. 3B). The percentage of CD8 T cells in sham-operated Akt transgenic vs wild-type littermate mice was not different (Fig. 3C).

In splenocytes from wild-type littermate mice, sepsis caused a marked increase in apoptosis of CD3 and CD4 T and B cells (CD19+) (Fig. 5, A–C). In contrast, apoptosis was decreased in CD3 T and B cells from Akt mice with sepsis compared with

(Fig. 2; Table I). Compared with septic wild-type littermates, Akt transgenic mice have a \(-3\)-fold increase in IFN-\( \gamma \) (Fig. 2; Table I). IL-4 production in sham-operated Akt transgenics was slightly increased compared with wild-type littermate sham mice. Interestingly, the response to the septic insult was markedly different in the wild-type littermates vs Akt transgenic mice. IL-4 production increased \( >2\)-fold in the wild-type littermate septic mice, but no increase occurred in Akt transgenic mice. This indicates that Akt transgenic CD4 T cells react to the induced sepsis with a Th1-biased cytokine profile, whereas wild-type littermate CD4 T cells show a shift toward a Th2 cytokine immune response.


wild-type littermate mice with sepsis (Fig. 5, A and B). Surprisingly, apoptosis was actually increased in CD4 T cells from sham-and CLP Akt-overexpressing mice compared with lymphocytes from wild-type littermate mice (Fig. 5C). Importantly, however, there was no further increase in apoptosis of CD4 lymphocytes from Akt transgenic mice with development of sepsis (Fig. 5C). There was no difference in apoptosis in CD8 T cells in wild type vs Akt (Fig. 5D).

To specifically compare apoptosis in cells that overexpressed Akt, double staining for Akt and apoptosis was performed using an anti-phospho-Akt Ab and the CaspACE FITC-VAD-FMK in situ marker, which detects caspase-cleaved substrates, respectively. Using this method, ~5–12% of CD3 T cells were positive for phospho-Akt, whereas the percentage of CD3 cells in wild-type littermate mice that were positive for phospho-Akt was negligible (Fig. 6). To determine whether overexpression of Akt protected against sepsis-induced apoptosis, the percentage of apoptotic CD3-positive T cells that expressed Akt was compared with the percentage of apoptotic CD3-positive T cells that did not express Akt from the same mouse. This value was determined by comparing the ratio of the percentage of cells in the upper right quadrant (apoptotic cells that express Akt) with the percentage of cells in the upper left quadrant (nonapoptotic cells that express Akt) vs the ratio of the percentage of cells in the lower right quadrant (apoptotic non-Akt-expressing cells) to the lower left quadrant (non-apoptotic non-Akt-expressing cells). In sepsis, the percentage of Akt-expressing CD3 T cells that were apoptotic was 4.7 ± 3.7%, whereas the percentage of apoptotic non-Akt-expressing CD3 T cells was 7.3 ± 4.1%, and this difference was statistically significant (n = 10), p < 0.05. This represented a ~36% reduction in sepsis-induced lymphocyte apoptosis in cells expressing Akt.

As an additional method to verify the protective effect of Akt against sepsis-induced apoptosis, double staining for Akt and active caspase 3 was performed on CD3-positive splenocytes. In wild-type littermates, sepsis caused an increase in CD3 T cells that were positive for active caspase 3, i.e., 3.9 ± 0.6 (n = 4) and 8.6 ± 0.5 (n = 2) for sham and septic animals, respectively. This difference was statistically significant (p < 0.01). In the Akt transgenic mice, the percentage of apoptotic CD3 splenocytes in sham animals was increased above baseline at 6.2 ± 0.2 (n = 4). (Cells that overexpress Akt have been noted to have an increase in baseline apoptosis, but to be resistant to apoptotic stimuli; see Discussion.) However, the important point is that there was no increase in T cell apoptosis with sepsis in the Akt transgenic mice, i.e., the percentage of CD3 splenocytes positive for active caspase 3 was 7.4 ± 0.7 (n = 3).

The percentage of T cells determined to be positive for phospho-Akt by flow cytometry was compared with conventional immunohistochemical staining for phospho-Akt of paraffin-embedded tissues. Although not quantified, immunohistochemical staining for phospho-Akt in formalin-fixed spleen slices showed an obviously larger percentage of T cells positive for phospho-Akt as compared with the percentage of splenocytes positive for phospho-Akt, as determined by flow cytometry (Fig. 7). In the T cell regions of the spleen, i.e., periarteriolar lymphoid sheaths, the majority of cells were positive for phospho-Akt in the Akt-overexpressing mice (Fig. 7). Thus, flow cytometry seems to underestimate the percentage of cells that overexpressed Akt. Immunohistochemical staining for phospho-Akt was negligible in wild-type littermate mice (data not shown).

Western blotting for Bcl-2

Western blots for Bcl-2 in splenic CD3 T cells from Akt transgenic and wild-type littermates showed abundant Bcl-2 in all spleens, but there was no difference in the amount of Bcl-2 in CD3 splenic T cells between the two groups (Fig. 8).

Western blotting of B cells for myr Akt

The CD2 minigene cassette was used in construction of the Akt transgene. Although the use of this construct would induce expression of Akt primarily in T cells, additional expression has been reported to occur in B cells as well (24). Myr Akt expression in T cells, as previously shown by Western blot (15), correlates with the reduced apoptosis of Akt transgenic T cells after sepsis induction compared with wild-type T cells. To determine whether the protection against sepsis-induced B cell apoptosis observed in the Akt transgenic mice (Fig. 5) might be due to myr Akt expression in B cells, Western blotting of B cells was performed (see Materials and Methods). B cells were stimulated with anti-IgM F(ab′)2 Abs or LPS. As demonstrated in Fig. 9, myr Akt was present only in the

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**FIGURE 6.** Akt protects against sepsis-induced lymphocyte apoptosis. Twenty-four hours after surgery, isolated splenocytes were incubated with CaspACE FITC-VAD-FMK to detect apoptosis. After a 60-min incubation, the cells were fixed and labeled with an anti-phospho-Akt Ab. Transgenic mice are positive for phospho-Akt (upper left quadrant in each panel), while wild-type mice have essentially no cells positive for phospho-Akt. The numbers in parentheses represent the percentage of cells in that quadrant. Overexpression of Akt protects against apoptosis, as evidenced by the fact that only a small percentage of cells that overexpress Akt are apoptotic. This is apparent by examining the flow diagrams and noting that the percentage of cells in the upper right quadrant compared with the percentage of cells in the lower right quadrant is smaller compared with the percentage of cells in the lower left quadrant.
B cells from the transgenic mice, while endogenous active Akt was present in B cells from wild-type littermates and transgenic mice. Immunohistochemical staining for phospho-forkhead showed a marked increase in cells positive for phospho-forkhead (Fox 03) in Akt transgenics vs wild-type littermate mice (Fig. 10). Only rare positive cells were present in wild-type littermate mice, whereas Akt transgenic mice had foci of positive cells that were located in both T cell regions (periarteriolar sheaths) and to a lesser degree in splenic follicles. The phospho-forkhead staining was confined to the cytoplasm of the cell with little to absent nuclear staining. Clusters of cells demonstrating the typical features of apoptosis, i.e., compacted and fragmented nuclei, were present in both Akt transgenic and wild-type littermate mice. Although not systematically quantitated, few cells that were positive for phospho-forkhead were observed to be undergoing apoptosis.

**Discussion**

The ability of Akt to cause a doubling in survival in a clinically relevant model of sepsis was the most impressive finding of the present study and provides insight into the pathophysiology of the disorder. There are several potential mechanisms responsible for the survival benefit in transgenic mice whose lymphocytes overexpress Akt. First, Akt decreased sepsis-induced lymphocyte apoptosis in both T and B cells. Previous studies have shown that sepsis induces a profound apoptosis-mediated depletion of T and B lymphocytes. Furthermore, investigations demonstrate that prevention of lymphocyte apoptosis in sepsis improves survival, most likely by augmenting the adaptive immune system (2–4, 20). For example, transgenic mice whose lymphocytes overexpress the antiapoptotic protein Bcl-2 are resistant to sepsis-induced lymphocyte apoptosis and have markedly improved survival (7, 20). Another potential mechanism for the protective effect of Akt is the increased activation state of the T cells that overexpress Akt. As typified by the septic wild-type mice (Table I), sepsis induces a shift in lymphocyte cytokine production from a proinflammatory Th1 to an anti-inflammatory Th2 profile (25–29). This cytokine shift to a Th2 profile in sepsis may compromise the ability of the host to combat the invading microorganisms. Of particular potential relevance, CD4 T cells from the Akt transgenic mice with sepsis produced a large amount of the Th1 cytokine IFN-γ compared with wild-type septic mice (Table I). IFN-γ has been shown to improve survival in a variety of infectious models, including sepsis (22). A small clinical study in patients with sepsis also demonstrated improved survival in patients treated with IFN-γ (30).

Previous studies from many groups, including our own, have shown that Akt has complex effects on cell survival and prevention of apoptosis (10–15). Pancreatic β cells expressing myr Akt1 had
an increase in basal apoptosis, but were resistant to streptozotocin-
induced apoptosis (13). The increase in baseline apoptosis in Akt-
overexpressing cells is unknown, but may be related to the effect
of Akt on cell cycle and cell size (31, 32). Further evidence that
the antiapoptotic effect of Akt is cell type and context speci
fic has been
provided by previous studies in which we documented that myr
Akt did not prevent thymocyte apoptosis secondary to a variety
of apoptogenic stimuli, but did prevent splenocyte apoptosis under
these same conditions (15). This failure of myr Akt to protect
thymocytes may be related to the fact that Akt provides stronger
survival signals for mature peripheral T cells than for thymocytes
that might be related to the subcellular localization of transgenic
Akt (15, 33). Similarly, myr Akt did not confer protection against
sepsis-induced apoptosis in thymocytes, even though these cells
expressed myr Akt (our unpublished data), but myr Akt did protect
splenocytes.

In the present study, three independent methods of evaluation of
apoptosis were used, i.e., annexin V labeling, fluorescent labeling
of caspase-cleaved substrates, and active caspase 3 staining. There
was an increase in lymphocyte apoptosis at baseline in Akt-over-
expressing mice, as noted by both annexin V and active caspase 3
staining. However, both methods also showed that sepsis did not
increase lymphocyte apoptosis in Akt-overexpressing mice (Fig. 5,
A–C, and results for active caspase 3). The increase in lymphocyte
apoptosis in the sham-operated Akt transgenic mice was somewhat
unexpected, but, as noted previously, a similar increase in baseline
apoptotic rate was noted in Akt-expressing pancreatic β cells (13).
The increased rate of apoptosis in lymphocytes from the Akt trans-
genic mice may be related to their activation state; activated lymph-
cytes are more susceptible to apoptosis in certain situations
(34). T cells from the Akt transgenic mice were more reactive
compared with T cells from wild-type mice, as evidenced by the
increased production of IFN-γ in the former compared with the
latter. Previous studies from our laboratory confirmed that Akt
increased T cell responsiveness by enhancing Lck and Erk activ-
ties (15). Of note, blinded evaluation of spleens from septic and
sham-operated Akt transgenics and wild-type littermate mice, in-
cluding immunohistochemical staining for Akt and TUNEL, con-
formed the protective effect of Akt against sepsis-induced apoptosis
in the spleen (data not presented).

Despite the increase in baseline apoptosis in the Akt transgenics
vs wild-type littermate mice, there was no increase in apoptosis in
CD4 T cells during sepsis, and therefore, the Akt transgenics most
likely had an effective increase in the numbers of lymphocytes that
were resistant to sepsis-induced apoptosis. Importantly, studies
from our laboratory have shown that adoptive transfer of normal
lymphocytes (lymphocytes that are not resistant to sepsis-induced
apoptosis) does not confer resistance to mortality in sepsis (our
unpublished findings). Therefore, one possible factor in the im-
proved sepsis survival in the Akt transgenic mice may have been
the increase in the number of lymphocytes that were resistant to
cell death during sepsis. Most likely, the improved survival in the

FIGURE 10. Phospho-forkhead is expressed in Akt transgenic mice. Immunohistochemical staining for phospho-forkhead (Fox 03) in spleens from Akt
transgenic mice (top two panels) and wild-type mice (bottom two panels). The cells that are positive for phospho-forkhead are identified by the brown
cytoplasmic stain. Note that few cells are positive for phospho-forkhead in the wild-type mouse, but many cells are positive in the Akt transgenic; ×100
magnification.
Akt transgenic mice was multifactorial and included increased numbers of activated apoptosis-resistant lymphocytes with a Th1 cytokine type propensity.

The mechanism for the protective effect of Akt in preventing sepsis-induced lymphocyte apoptosis is unknown. Akt has a number of downstream targets, e.g., forkhead proteins, pro- and antiapoptotic Bcl-2 family members, NF-κB, etc., that potentially could be involved (16–19). Previous studies have shown that mice that overexpress the antiapoptotic protein Bcl-2 in T or B cells are resistant to sepsis-induced lymphocyte apoptosis and have improved survival (7, 20). In the present study, no increase in expression of Bcl-2 was found in lymphocytes from Akt transgenic vs wild-type mice. This finding does not rule out the possibility that Akt may modulate other Bcl-2 family members. In this regard, Jones et al. (11) showed that lymphocytes that overexpress gag Akt had increased expression of the antiapoptotic protein Bcl-xL.

In a previous report, we showed that myr Akt transgenic T cells stimulated in vitro show a reduced nuclear translocation of NF-AT and NF-κB p65/RelA and RelB proteins (21). Because both of these transcription factors have been shown to be involved in regulating apoptosis (35, 36), it is possible that the reduced apoptosis of Akt transgenic T cells in sepsis is connected to reduced expression of apoptosis-mediating ligands such as Fas ligand or inflammatory proteins that are regulated by NF-AT and NF-κB proteins, respectively. In gag Akt transgenic T cells, Fas-mediated cell death was strongly reduced due to a failure of recruitment of caspase 8 to the fas-associated death domain signaling complex (19). Because myr Akt can replace CD28 costimulatory signals in T cell activation and proliferation (21), it is also possible that the apoptotic signals induced in sepsis are counteracted by the positive signals provided by myr Akt on T cell activation and cell cycle progression. As demonstrated in the present study, another potential mechanism for the antiapoptotic effect of Akt is increased expression of the phosphorylated forkhead family member FOXO3 (37, 38). Immunohistochemical staining showed a large increase in the percentage of lymphocytes expressing cytosolic phospho-forkhead in Akt transgenic mice vs wild-type mice (Fig. 7). FOXO3 can act as a proapoptotic transcription factor, and Akt-mediated phosphorylation of FOXO3 is thought to prevent its translocation to the nucleus, thereby preventing transcription of proapoptotic genes, including Bin and Fas (37, 38).

The appropriate response of the host to invading pathogens is clonal expansion of T and B cells to combat the microorganisms (2–5). Numerous studies have shown that in sepsis there is a marked apoptosis-induced depletion of lymphocytes rather than the expected lymphocyte proliferation. Additional studies demonstrate that prevention of lymphocyte apoptosis by overexpression of Bcl-2 improves survival, and thus have provided support for the concept that loss of lymphocytes in sepsis is a key pathogenic mechanism in the lethal disorder. Recent work by Iwata et al. (39) using transgenic mice that overexpress Bcl-2 in a number of cell lines other than lymphocytes has also shown improved survival in sepsis. Therefore, these investigators postulated that the protective effect of Bcl-2 may be independent of its ability to prevent lymphocyte death and instead may be related to a secretion product that is released by cells that overexpress Bcl-2. The current findings demonstrating that overexpression of a second antiapoptotic protein, i.e., Akt, also prevents sepsis-induced lymphocyte apoptosis, does not increase Bcl-2 and improves sepsis survival strengthen the hypothesis that the loss of lymphocytes is critical in sepsis and that prevention of lymphocyte apoptosis may represent an effective new therapy in sepsis.

In summary, overexpression of Akt decreased sepsis-induced lymphocyte apoptosis, increased production of the Th1 cytokine IFN-γ, increased cytosolic localization of phospho-forkhead, and improved survival in sepsis. The results bolster the important role of the lymphocyte in sepsis and open new possible targets for therapy of sepsis.

References


