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Prevention of Lymphocyte Apoptosis in Septic Mice with Cancer Increases Mortality

Amy C. Fox,* Elise R. Breed,†‡ Zhe Liang,†‡ Andrew T. Clark,* Brendan R. Zee-Cheng,* Katherine C. Chang,‡ Jessica A. Dominguez,§ Enjae Jung,* W. Michael Dunne,‖ Eileen M. Burd,§ Alton B. Farris,§ David C. Linehan,* and Craig M. Coopersmith †,‡

Lymphocyte apoptosis is thought to have a major role in the pathophysiology of sepsis. However, there is a disconnect between animal models of sepsis and patients with the disease, because the former use subjects that were healthy prior to the onset of infection while most patients have underlying comorbidities. The purpose of this study was to determine whether lymphocyte apoptosis prevention is effective in preventing mortality in septic mice with preexisting cancer. Mice with lymphocyte Bcl-2 overexpression (Bcl-2-Ig) and wild type (WT) mice were injected with a transplantable pancreatic adenocarcinoma cell line. Three weeks later, after development of palpable tumors, all animals received an intratracheal injection of Pseudomonas aeruginosa. Despite having decreased sepsis-induced T and B lymphocyte apoptosis, Bcl-2-Ig mice had markedly increased mortality compared with WT mice following P. aeruginosa pneumonia (85 versus 44% 7-d mortality; \( p = 0.004 \)). The worsened survival in Bcl-2-Ig mice was associated with increases in Th1 cytokines TNF-\( \alpha \) and IFN-\( \gamma \) in bronchoalveolar lavage fluid and decreased production of the Th2 cytokine IL-10 in stimulated splenocytes. There were no differences in tumor size or pulmonary pathology between Bcl-2-Ig and WT mice. To verify that the mortality difference was not specific to Bcl-2 overexpression, similar experiments were performed in Bim\( ^{−/−} \) mice. Septic Bim\( ^{−/−} \) mice with cancer also had increased mortality compared with septic WT mice with cancer. These data demonstrate that, despite overwhelming evidence that prevention of lymphocyte apoptosis is beneficial in septic hosts without comorbidities, the same strategy worsens survival in mice with cancer that are given pneumonia. The Journal of Immunology, 2011, 187: 000–000.
carcinoma cell line 3 wk prior to the onset of Pseudomonas aeruginosa pneumonia had a 24% increase in mortality compared with septic mice that were previously healthy (44).

In light of 1) the extensive literature demonstrating a survival benefit to preventing lymphocyte apoptosis in previously healthy mice and 2) the knowledge that mice with cancer (or other comorbidities) behave differently than previously healthy mice subjected to the identical septic insult, this study examined whether preventing lymphocyte apoptosis would improve survival in the clinically relevant model of sepsis in the setting of cancer.

Materials and Methods

Mice

Nine- to 16-wk-old mice were used for all experiments. Transgenic mice overexpressing human Bcl-2 in both T and B lymphocytes (Bcl-2-Fab mice) were generated as previously described (17, 47). Of note, Bcl-2 expression is significantly greater in the spleen than in the thymus in these animals. Bim−/− mice were initially generated at the Walter and Eliza Hall Institute of Medical Research (Melbourne, VIC, Australia) (48), but were subsequently bred for over 3 y at Washington University for experiments examining lymphocyte apoptosis and sepsis, where they were a gift from Dr. Richard Hotchkiss (Washington University School of Medicine) (21). Transgenic mice that overexpress Bcl-2 in the intestinal epithelium (Fobpl-Bcl-2 mice) were generated on an FVB/N background and were then backcrossed to C57BL/6 mice for >20 generations (49, 50). For all studies in this article, animals were compared with C57BL/6 littermates or controls bred in the same animal facility. Mice had free access to food and water and were maintained on a 12-h light-dark schedule in a barrier facility. All studies complied with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Animal Studies Committee of both Washington University and Emory University.

Cancer model

The transplantable mouse pancreas adenocarcinoma cell line Pan02 was injected to induce cancer as described previously (51, 52). Pan02 cells were cultured in RPMI 1640 medium supplemented with 1% glutamine, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% FBS, and 1% penicillin–streptomycin (Cellgro, Herndon, VA). A total of 250,000 Pan02 cells were injected s.c. into each animal’s right inner thigh. Mice were then housed for 3 wk in a barrier facility to allow tumors to grow prior to the induction of sepsis. Animals given Pan02 cells developed well-defined, circumscribed tumors at the injection site, but the tumors were not metastatic and did not cause mortality in the period studied in this set of experiments. The longest axis of the tumor was used for measuring tumor size. In a recent study from our laboratory (44), animals given Pan02 cells 3 wk earlier were compared with healthy mice. Animals had similar body weights, liver function, kidney function, lung histology, wet-to-dry lung ratios, blood cytokine levels (TNF-α, IL-6, IL-10, IL-12, and MCP-1), bronchoalveolar lavage (BAL) cytokines (IL-6, IL-10, IL-12, and MCP-1), gut apoptosis, intestinal villus length, intestinal proliferation, hematocrit, WBC count, absolute neutrophil count, absolute lymphocyte count, and platelet count. In contrast, the presence of cancer caused lower levels of T lymphocyte and B lymphocyte apoptosis compared with healthy mice and also induced higher levels of TNF-α in BAL fluid.

Sepsis model

Three weeks after the injection of Pan02 cells, mice were made septic via direct intratracheal injection of P. aeruginosa (American Type Culture Collection 27853) (53). A midline cervical incision was made while using isoflurane anesthesia, and 40 μl of a solution of bacteria diluted in 0.9% NaCl (final concentration, 6 × 106 CFUs/ml) was injected directly into the trachea using a 29-gauge insulin syringe. Animals were then held vertically for 10 s to enhance delivery of bacteria into the lungs. Mice received a single 1-ml s.c. injection of 0.9% NaCl to replace insensible fluid losses following incision closure. Animals were either sacrificed 24 h after induction of pneumonia for tissue harvest or followed 7 d for survival. Of

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom) Effect of Bcl-2 overexpression on lymphocyte apoptosis in septic mice with cancer. T lymphocyte (A, B; n = 13–19 per group) and B lymphocyte apoptosis (C; n = 14–19 per group) was decreased in Bcl-2-Fab mice compared with WT mice whether assayed by active caspase 3 staining (A, C) or the TUNEL assay (B) by flow cytometry. Similar results were qualitatively seen on H&E-stained sections in which splenic apoptosis was elevated in septic WT mice with cancer (D), but lower in Bcl-2-Fab mice subjected to the same insult (E). Representative histomicrographs are shown at original magnification ×400. *p < 0.05.
FIGURE 2. Effect of Bcl-2 overexpression in lymphocytes on survival in septic mice with cancer. Mortality was nearly twice as high in Bcl-2-lg mice compared with WT mice 7 d after induction of P. aeruginosa pneumonia. *p < 0.05.

The addition of substrate buffer containing O-dianisidine and 0.005% hydrogen peroxide, myeloperoxidase (MPO) activity was measured at 470-nm wavelength over 6 min (Bio-Tek Instruments-µQuant Microplate Spectrophotometer, Winoski, VT). MPO activity was calculated as ΔOD/minute per microliter of BAL fluid.

**Bacterial cultures**

Blood was collected from the inferior vena cava, and BAL fluid was obtained as described above. BAL samples were serially diluted in sterile saline and plated on sheep blood agar plates. Plates were incubated overnight at 37°C and examined for colony counts 24 h later.

**Intestinal epithelial apoptosis**

Apoptotic cells were quantified in 100 contiguous, well-oriented crypt-villus units using H&E staining and active caspase-3 staining (53). Apoptotic cells were identified on H&E-stained sections by morphologic criteria. Cells with characteristic nuclear condensation and fragmentation were considered to be apoptotic. For active caspase-3 staining, intestinal sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 10 min. Ag retrieval was performed by placing slides in Ag Decloaker (Biocare Medical, Concord, CA) and heating them in a pressure cooker for 45 min. Slides were then blocked with 20% goat serum (Vector Laboratories, Burlingame, CA) and incubated with rabbit polyclonal anti-active caspase-3 (1:100; Cell Signaling Technology, Beverly, MA) overnight at 4°C. The following day, slides were incubated with goat anti-rabbit biotinylated secondary Ab (1:200; Vector Laboratories) for 45 min at room temperature followed by Vectorstain Elite ABC reagent (Vector Laboratories) for 60 min. Slides were then washed, incubated with 0.05% diaminobenzidine in PBS for 10 min, counterstained with hematoxylin, and then mounted.

**Statistics**

All data were tested for Gaussian distribution using the Shapiro-Wilk normality test. If data were found to have a Gaussian distribution, comparisons were performed using the Student's t test, and results were presented as mean ± SEM. If data did not have a Gaussian distribution, comparisons were performed using the Mann–Whitney U test and were presented as median ± range. Survival studies were analyzed using the log-rank test. Data were analyzed using the statistical software program Prism 4.0 (GraphPad, San Diego, CA).

**Results**

In all experiments, mice were injected with the transplantable pancreatic adenocarcinoma cell line Pan02. Three weeks later, animals were given P. aeruginosa pneumonia. As a result, all animals developed sepsis in the setting of preexisting cancer.

**Bcl-2 overexpression in lymphocytes increases mortality despite preventing sepsis-induced lymphocyte apoptosis**

Transgenic mice overexpressing human Bcl-2 in both T and B lymphocytes (Bcl-2-lg mice) had decreased levels of T lymphocyte apoptosis compared with wild type (WT) littermates by both

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ns, not significant.
WT mice (p2-) that Bcl-2- followed for survival after the induction of sepsis. Despite the fact (Fig. 1) apoptosis was also observed on H&E-stained splenic sections.

To determine whether the local host response played a role in the plasma inducers an upregulation of Th1 cytokines in BAL fluid but not plasma.

Bcl-2 overexpression in lymphocytes in septic mice with cancer induces an upregulation of Th1 cytokines in BAL fluid but not plasma.

To determine whether the local host response played a role in the elevated mortality in septic Bcl-2-Ig mice, BAL cytokines were measured in transgenic and WT mice (Table I). Concentrations of the Th1 cytokines TNF-α (p = 0.008) and IFN-γ (p = 0.03) were significantly higher in Bcl-2-Ig than WT mice. In contrast, when plasma cytokines were assayed, no significant differences were detected between septic Bcl-2-Ig and WT mice with cancer (Fig. 2).

Bcl-2 overexpression in lymphocytes in septic mice with cancer decreases production of IL-10 in stimulated lymphocytes.

Lymphocytes isolated from the spleens of septic Bcl-2-Ig mice that were incubated ex vivo with anti-CD3/28 had lower production of the Th2 cytokine IL-10 compared with WT littermates (p = 0.004; Table III). Other stimulated cytokines were similar in septic mice with cancer, regardless of whether they overexpressed Bcl-2 in their lymphocytes.

Bcl-2 overexpression in lymphocytes does not alter tumor size.

To determine whether overexpression of an antiapoptotic protein in lymphocytes caused a secondary effect on cancer growth, tumor size was measured in Bcl-2-Ig and WT mice. No difference in tumor size was identified, regardless of whether an animal overexpressed Bcl-2 in its lymphocytes (Fig. 3).

Bcl-2 overexpression in lymphocytes does not alter pneumonia severity.

In light of the differences identified in BAL cytokines, pneumonia severity was assessed to determine whether alterations in pulmonary pathology were responsible for the mortality difference between Bcl-2-Ig and WT mice. Both pneumonia severity and percentage of lung tissue with inflammation were similar between Bcl-2-Ig and WT mice (Fig. 4A, 4B). No differences were detected between the groups in atelectasis (ranging from none to moderate in Bcl-2-Ig mice, ranging from focal to moderate in WT mice) or vascular congestion (focal to moderate in all animals, without differences between groups). In addition, there were no differences in neutrophil infiltration and degranulation in BAL fluid as measured by MPO activity between Bcl-2-Ig and WT mice (Fig. 4C). Bacteria were not detectable in BAL fluid 24 h after tracheal instillation of P. aeruginosa (n = 5–6 mice per group).

Septic Bim−/− mice with cancer have increased mortality compared with WT mice.

To determine whether the alteration in mortality was specific to Bcl-2-Ig mice or potentially more generalizable, survival studies were repeated in Bim−/− mice that were injected with Pan02 cells as described above and made septic via P. aeruginosa pneumonia 3 wk later. Septic Bim−/− mice with cancer had an 18% increase in mortality compared with WT mice with cancer (p = 0.046; Fig. 5).

Bcl-2 overexpression in the intestinal epithelium has no effect on mortality.

To determine whether the effect of altering sepsis-induced apoptosis was lymphocyte specific, a survival study was performed on transgenic mice that overexpressed Bcl-2 in the intestinal epithelium (Fabpl-Bcl-2 mice). There was no difference in survival between septic Fabpl-Bcl-2 mice with cancer and septic WT mice with cancer (Fig. 6A). However, unlike either septic Bcl-2-Ig mice with cancer or previously healthy septic Fabpl-Bcl-2 mice, no protection against sepsis-induced apoptosis was identified in the cell type where the transgene was expressed in this set of experiments (Fig. 6B, 6C).

Discussion

This study demonstrates that prevention of sepsis-induced lymphocyte apoptosis in mice with cancer increases mortality. These results contrast significantly from extensive literature demonstrating that prevention of lymphocyte apoptosis in a peritonitis model of sepsis improves survival in mice that were healthy prior to their septic insult. In addition, animals with the identical genetic alteration in apoptosis signaling respond to sepsis in contradictory manners depending on whether an animal has cancer prior to the onset of sepsis and the model of sepsis used. Both Bcl-2-Ig mice and Bim−/−.
mice have increased survival if pneumonia is initiated in previously healthy mice (17, 21, 54), but have decreased survival if pneumonia is initiated in mice with preexisting cancer.

A differential host response appears to play a role in explaining this discrepancy. Bcl-2-Ig mice with cancer have increases in their Th1 cytokines TNF-α and IFN-γ in BAL fluid 24 h after the onset of pneumonia compared with WT septic mice with cancer. In addition, when their splenocytes are stimulated ex vivo, they have decreased production of the Th2 cytokine IL-10. These findings suggest that there is an exaggerated inflammatory response in septic mice with cancer when lymphocyte apoptosis is prevented. This increased proinflammatory response is both local and systemic in nature given the findings in BAL fluid and stimulated splenocytes.

The inflammatory response following sepsis is complex. Either too much or too little inflammation can damage the host by causing unintended organ failure or leaving the host susceptible to secondary infections, respectively. Some degree of inflammation is beneficial, because immunoparalyzed hosts are at risk for fatal infectious complications. However, the appropriate amount of inflammation varies with both the clinical scenario and the length of time elapsed after the initial insult. Too much inflammation can be as harmful as too little inflammation, and it is likely that the increased inflammatory state in the lungs and the decreased ability of splenocytes to secrete compensatory anti-inflammatory cytokines played a role in the excessive mortality seen in this study.

In our prior description of why septic mice with cancer were more likely to die than septic mice that were previously healthy (both WT C57BL/6 mice), we found that sepsis induced an increase in T and B lymphocyte apoptosis in all animals (44). However, septic mice with cancer had decreased T and B lymphocyte apoptosis compared with previously healthy septic mice. These findings imply that some degree of lymphocyte apoptosis may be beneficial in a subset of septic hosts in light of the fact that 1) mice with cancer have lower levels of sepsis-induced apoptosis than previously healthy mice despite higher mortalities, and 2) preventing this apoptosis leads to a further increase in mortality. Thus, an important implication of this study is that prevention of lymphocyte apoptosis may not always be a beneficial therapeutic strategy, and it may be a harmful strategy in select patient groups with a specific disease process. This issue is directly relevant because of the large amount of interest in translating anti-apoptotic therapies to the bedside of patients with sepsis based on overwhelming evidence in animal models and observational human studies suggesting that lymphocyte apoptosis is harmful in sepsis.

One possible explanation for the discrepancy between our results and those published in the existing literature is the difference in the model used. The vast majority of mouse studies demonstrating the benefit of preventing lymphocyte apoptosis use the cecal ligation and puncture model, and there is overwhelming evidence supporting apoptosis prevention in previously healthy mice with peritonitis. In contrast, this study uses a pneumonia model. To our knowledge, there is only one published study examining lymphocyte apoptosis prevention in pneumonia, in which overexpression of Bcl-2 in lymphocytes resulted in a trend toward improved survival in P. aeruginosa pneumonia (54). A similar experiment performed for this study also demonstrates a nonsignificant 16% improvement in survival in Bcl-2-Ig mice subjected to P. aeruginosa pneumonia compared with WT mice (data not shown). Based on this finding, it is possible that Bcl-2 overexpression confers either a small survival benefit or has no meaningful benefit in previously healthy mice subjected to pneumonia. Each of these results would be at least somewhat different from previously healthy mice subjected to peritonitis, where apoptosis prevention confers a marked survival benefit. Importantly, the results also differ significantly from mice with cancer that subsequently are given pneumonia, demonstrating that the addition of cancer as a comorbidity alters the host response by changing an intervention (i.e., lymphocyte apoptosis prevention) that may ordinarily be either beneficial or neutral into one that is detrimental to host survival. Although both peritonitis and pneumonia models induce sepsis, they do not induce an identical inflammatory response. Both Bcl-2-Ig mice and Bim−/− mice have increased basal numbers of lymphocytes compared with WT animals. In a pneumonia model, this baseline increase in effector cells could lead to profound changes in a stressed host. As such, this increase in effector cells might be more physiologically significant than prevention of lymphocyte apoptosis and functionally overwhelm it, resulting in the observed proinflammatory response. This explanation could help to explain the differential result.

**FIGURE 5.** Effect of knocking out the proapoptotic factor Bim on survival in septic mice with cancer. Mortality was higher in Bim−/− mice than in WT mice (n = 11–14 per group) 7 d after induction of P. aeruginosa pneumonia. *p < 0.05.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 6.** Effect of Bcl-2 overexpression on intestinal epithelial apoptosis and survival in septic mice with cancer. No difference in mortality was observed between Fabpl-Bcl-2 mice and WT mice 7 d after induction of P. aeruginosa pneumonia (A, n = 27–29 per group). Despite the fact that Bcl-2 was overexpressed in the intestinal epithelium in transgenic mice, intestinal epithelial apoptosis was similar by both H&E staining (B) and active caspase-3 staining (C) in Fabpl-Bcl-2 mice and WT mice. n = 12–13 per group.

![Figure 6](http://www.jimmunol.org/)
between mice subjected to peritonitis versus those subjected to pneumonia, although it would not explain the difference in survival between Bcl-2 overexpression in mice that were healthy prior to the onset of pneumonia versus those that had cancer prior to the onset of pneumonia.

Although the vast majority of preclinical studies use healthy, 6–12-wk-old mice, these animals actually model a patient population that almost never gets septic and is unlikely to die of the disease in the rare instance that it develops. Sepsis is a disease that most commonly affects the elderly or patients with comorbidities, or both; however, the typical study using young, previously healthy mice is the equivalent of studying the disease in a 13 y old (55, 56) without any medical history. Interventions that may be beneficial in this age group may lose their efficacy or actually be harmful in either aged patients or those with chronic comorbidities, who have markedly different inflammatory milieus at baseline.

The findings in septic Fabspl-Bcl-2 mice reinforce the importance of cancer as a comorbidity. Previously healthy Fabspl-Bcl-2 mice do not have the increase in sepsis-induced gut epithelial apoptosis seen in WT animals subjected to the same insult and have improved survival following sepsis (24, 57). In contrast, when these animals have cancer prior to the induction of sepsis, they neither prevent sepsis-induced apoptosis nor improve survival. Although the finding that Bcl-2 was ineffective in preventing sepsis-induced gut apoptosis was surprising, we have previously described that these animals fail to prevent sepsis-induced intestinal apoptosis in the absence of lymphocytes (58), which suggests that the strength of apoptotic signaling (in the intestine at least) may be modifiable by host factors outside of the local environment. Furthermore, although the loss of the survival benefit conferred by gut-specific Bcl-2 was not as striking as the worsening of survival conferred by lymphocyte-specific Bcl-2 in septic mice with cancer, the results are consistent in that both the host response to sepsis and attempts to alter this response may be determined, at least in part, prior to the onset of infection.

This study has a number of limitations. Because all nonsurvival studies were performed at a single time point (24 h), the experimental design did not allow for a dynamic assessment of the temporal changes in the host response. Next, although WT C57BL/6 mice were subjected to the same model of sepsis, mortality was significantly higher in experiments examining the effect of Bim<sup>−/−</sup> mice than in those examining the effect of Bcl-2-<i>tg</i> mice. There is no clear explanation for this finding, because the experiments were performed by the same person (A.C.F.). Next, the decrease in sepsis-induced lymphocyte apoptosis in Bcl-2-<i>tg</i> mice was small. We believe this decrease is small because sepsis-induced lymphocyte apoptosis is significantly lower in mice with cancer than in previously healthy mice (44). Previous work from our laboratory has shown that T lymphocyte apoptosis in Pan02-bearing septic mice is 10.4% and that T lymphocyte apoptosis in sham mice with cancer is 3.5% (44). In a comparison of these prior results with data shown in Fig. 1B, Bcl-2 decreased sepsis-induced T lymphocyte apoptosis to levels seen in sham mice. The fact that mortality was decreased in Bcl-2-<i>tg</i> mice and Bim<sup>−/−</sup> mice, both of which have improved survival following cecal ligation and puncture in previously healthy mice (17, 21, 54), strengthens our findings. However, all transgenic and knockout mice used in this study have lifelong genetic alterations that could lead to chronic changes in other immune effector cells, which could affect the outcome when mice are subjected to pneumonia. As such, an alternate strategy initiating either Abs or drugs that inhibit apoptosis after the onset of pneumonia might have more clinical relevance and in theory a different effect on mortality.

Despite these limitations, these results lead to a new paradigm in understanding lymphocyte apoptosis in sepsis. There continues to be overwhelming evidence that lymphocyte apoptosis is harmful in hosts that were healthy prior to the onset of septic peritonitis. However, prevention of lymphocyte apoptosis by chronic genetic alterations in the mitochondrial pathway is harmful in mice with cancer that subsequently develop sepsis from pneumonia. Thus, the results do not support the view that sepsis-induced apoptosis is a maladaptive response independent of the host or insult; instead, they suggest a response that is modifiable by pre-existing host comorbidities and a model of sepsis used in which a basal level of lymphocyte cell death may be beneficial under the correct circumstances. These results should be repeated using other types of tumors, other comorbidities, other models of sepsis, and other apoptosis inhibitor strategies to determine their generalizability. These results should also be considered in designing entry criteria for future clinical trials aimed at preventing lymphocyte apoptosis in sepsis.

Disclosures

The authors have no financial conflicts of interest.

References


