Increased apoptosis in lamina propria B cells during polymicrobial sepsis is FasL but not endotoxin mediated

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Am J Physiol Gastrointest Liver Physiol 280: G812–G818, 2001.—Recent studies from our laboratory demonstrated that mucosal lymphoid tissue such as Peyer’s patch cells and lamina propria (LP) B lymphocytes from mice shows evidence of increased apoptosis after sepsis that is associated with localized inflammation/activation. The mechanism for this is poorly understood. Endotoxin as well as Fas/Fas ligand (FasL) have been shown to augment lymphocyte apoptosis; however, their contribution to the increase of apoptosis in LP B cells during sepsis is not known. To study this, sepsis was induced by cecal ligation and puncture (CLP) in endotoxin-tolerant C3H/HeJ or FasL-deficient C3H/HeJ FasLgld (FasL–) mice and LP lymphocytes were isolated 24 h later. Phenotypic, apoptotic, and functional indexes were assessed. The number of LP B cells decreased markedly in C3H/HeJ mice but not in FasL-deficient animals at 24 h after CLP. This was associated with comparable alteration in apoptosis and Fas antigen expression in the B cells of these mice. Septic LP lymphocytes also showed increased IgA production, which was absent in the FasL-deficient CLP mice. Furthermore, Fas ligand deficiency appeared to improve survival of septic challenge. These data suggest that the increase in B cell apoptosis in septic animals is partially due to a Fas/FasL-mediated process but not endotoxin.

cecal ligation and puncture; programmed cell death; Fas ligand-deficient mouse

DESPITE THE SIGNIFICANT ADVANCES in clinical care, sepsis and multiple organ failure (MOF) are still common causes of morbidity and mortality in the surgical intensive care unit (26). Although the etiology of the development of MOF is poorly understood, it has been proposed that failure of the gut barrier may play a critical role in the initiation and development of this state. In this respect, the localized gut immune system is thought to be important in maintaining both gut function and integrity (9). However, the physiological and pathological mechanism by which the mucosal immune system contributes to the initiation and development of the syndrome of MOF is poorly understood.

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Apoptosis or programmed cell death is a tightly regulated cellular suicide program that plays a critical role in development and homeostasis in immune and central nervous systems of many organisms (29). However, the induction of apoptosis in certain immune cells is recognized as a pathological process that can be regulated by various agents, a number of which are also stress mediators that are released during trauma and sepsis (12). Previous studies from our laboratory (4) documented that lymphoid tissue such as thymus, bone marrow, and Peyer’s patch shows evidence of increased apoptosis after the onset of sepsis. One possible mechanism that might contribute to the onset of MOF, in which intestinal mucosal immune function might be affected after sepsis, could be related to pathological changes in the mechanisms controlling apoptosis in the intestinal immune system. However, little is known about the localized intestinal immune response to sepsis and/or the pathophysiological responses that control it. Intestinal lamina propria (LP) lymphocytes, located in close proximity to the intestinal lumen and mucosal barrier, serve a major role in immune surveillance and are functionally distinct from peripheral blood T lymphocytes (37). Although recent studies (4, 11, 17, 18, 20) demonstrated a correlation between increased apoptotic cell death in the intestinal mucosal immune cells (including LP lymphocytes) and septic challenge, little information is available concerning the mechanisms by which these apoptotic changes in the small intestinal LP B cell population are regulated.

In general, apoptosis of B lymphocytes is mediated through the B cell antigen receptor by antigens or through CD95/Fas receptor, a member of the tumor necrosis factor (TNF) receptor family (8, 24). Although B cell receptor ligation results in deletion by apoptosis, this is dependent on the developmental stage of the B cell as well as on the nature of the antigen and signals derived from coreceptors (21). Fas-mediated cell death is a component of activation-induced cell death and elimination of autoreactive T and B cells (8). Boirivant et al. (7) demonstrated that the increased apoptosis of stimulated human LP T lymphocytes is mediated by...
the Fas/Fas ligand (Fasl) pathway. Bacterial lipopoly-
saccharide (LPS) or endotoxin, a polyclonal B cell activ-
ator, has also been shown to induce increased apop-
tosis in B lymphocytes (36). In a previous report, we
(11) provided evidence of increased apoptosis in B lymph-
ocytes in LP of C3H/HeN endotoxin-sensitive mice
after the onset of polymicrobial sepsis. In addition, we
(4) also demonstrated that the increase of Peyer’s patch
B lymphocyte apoptosis is Fas mediated and associated
with activation of IgA release by these cells. To exam-
ine the potential mechanism(s) contributing to apopto-
sis, here we investigate whether endotoxin and/or Fas/Fasl pathway induces apoptosis in the LP
B cell population and the effect of this on B cell func-
tion such as IgA release during sepsis. To the extent
that these changes are linked to ability of the animal
to survive septic challenge, we also examined the impact
of deficiency of these mediators on the septic animal’s
mortality.

MATERIALS AND METHODS

Animals. Male inbred C3H/HeJ (endotoxin tolerant) and
C3H/HeJ-FasL<sup>−/−</sup> mice (endotoxin tolerant/Fasl deficient; Jackson Laboratory, Bar Harbor, ME; Ref. 25), 6–8 wk old, were used in all experiments. The studies described here were carried out according to the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, Bethes-
da, MD 20892] and were approved by the Brown University
and Rhode Island Hospital Animal Welfare Committee.

Cecal ligation and puncture. Polymicrobial sepsis was in-
duced in mice according to the method described by Baker et
al. (5) and Ayala et al. (3). Briefly, mice were lightly anes-
ethetized with Metofane (methoxyflurane; Pitman-Moore,
Mundelein, IL), shaved at the abdomen, and scrubbed with
Betadine. A midline incision (1.5–2.0 cm) was made below
the diaphragm to expose the cecum. The cecum was ligated
and punctured twice with a 22-gauge needle and gently
compressed to extrude a small amount of cecal contents
through the punctured holes. The cecum was returned to the
abdomen, and the incision was closed in layers with 6-0
Ethilon suture (Ethicon, Somerville, NJ). The animals then
were resuscitated with 0.6 ml of lactated Ringer solution by
subcutaneous injection. For the sham-operated controls, the
animals were subjected to the same surgical procedure, i.e.,
laparotomy and cecal isolation, but the cecum was neither
ligated nor punctured. For the survival study, mice were
subjected to the cecal ligation and puncture (CLP) procedure
and monitored for mortality over a 28-day period.

Isolation and purification of LP mononuclear cells. LP
mononuclear cells (LPMC) were isolated using the method
described by Davies and Parrott (14) with some modifica-
tions. The animals were killed with Metofane (methoxyflurane; Pitman-Moore), the plates were developed with avidin-peroxidase-
H<sub>2</sub>O<sub>2</sub>-ACE (3-amino-9-ethylcarbazole in 0.1 mol/l sodium
acetate buffer, pH 5.0). The number of spots present in the
membrane was counted on a Mocha Image analysis system
(Jandel Scientific, Corte Madera, CA).

Light microscopic examination. A 2 cm-long section of il-
eum was excised and immediately fixed in 10% buffered
formaldehyde. Paraffin-embedded tissues were sectioned at

IgA ELISpot assay. An ELISpot assay was used to deter-
mine the number of LPMC secreting IgA (16). Purified LPMC
were cultured (10<sup>5</sup> cells/well) on monolonal anti-mouse IgA
(5 μg/ml; Pharmingen)-precoated (4°C, overnight) nitrocel-
losate filtration plates (Millipore, Bedford, MA). After washing
and incubation (4°C, overnight) with a secondary biotinyl-
ated mononclonal anti-mouse IgA antibody (2.5 μg/ml, Pharm-
ingen), the plates were developed with avidin-peroxidase-
H<sub>2</sub>O<sub>2</sub>-ACE (3-amino-9-ethylcarbazole in 0.1 mol/l sodium
acetate buffer, pH 5.0). The number of spots present in the
membrane was counted on a Mocha Image analysis system
(Jandel Scientific, Corte Madera, CA).
5 μm thick for staining with hematoxylin and eosin. In an attempt to roughly quantify the extent of apoptotic change present in these samples, 10 random fields at ×600 magnification (between villi and muscular layer in which the LP are found) were screened in a blinded fashion for each sample from a total of 3 animal samples in every group of animals. A total of B cells recovered from the small intestinal LP was significantly decreased in septic C3H/HeJ-FasLgld mice at 24 h. However, these changes were not observed in C3H/HeJ mice.

Presentation of data and statistical analysis. Data are presented as means ± SE for each group. Differences in percentile data (i.e., percentage of apoptosis positive and Fas positive) were considered to be significant if \( P < 0.05 \), as determined by the Mann-Whitney \( U \)-test. Differences in the extent of the apoptotic scale of the histology data were considered to be significant if \( P < 0.05 \), as analyzed by Student’s \( t \)-test. The survival data were compared using Fisher’s exact test and considered to be significant at \( P < 0.05 \).

RESULTS

Polymicrobial sepsis induces changes in LP B cell population from C3H/HeJ (endotoxin tolerant) but not C3H/HeJ-FasLgld mice (endotoxin tolerant/FasL deficient). Phenotypes of isolated LPMC were characterized by staining the surface markers with specific monoclonal antibodies. Figure 1 demonstrates that the percentage of cells positively stained with anti-B220 (B cells) in sham-operated animals was ~35–40% of the total LPMC from both mouse strains. The percentage of B cells recovered from the small intestinal LP was significantly decreased in septic C3H/HeJ mice at 24 h. However, these changes were not observed in C3H/HeJ-FasLgld mice.

![Fig. 1. Percentage of lamina propria mononuclear cells (LPMC) positively stained anti-B220 in C3H/HeJ and C3H/HeJ-FasLgld mice. B220-positive population was markedly decreased in septic C3H/HeJ but not in C3H/HeJ-FasLgld mice 24 h after cecal ligation and puncture (CLP). Values are means ± SE; \( n = 6 \) mice/group. * \( P < 0.05 \) vs. sham operated (Mann-Whitney \( U \)-test).](storage/082/0185/18554012.png)

Polymicrobial sepsis increases apoptotic frequency in LP B cells from C3H/HeJ but not C3H/HeJ-FasLgld mice as determined by TUNEL staining. It should be noted that the overall baseline percentage of apoptosis was high, including that for cells from the sham-operated animals (~36%). The reason for this may be due in part to the nature of the purification procedure performed here, which is relatively time consuming and involves treatment with collagenase to dissociate the connective tissue. However, that having been said, the yield and apoptotic rate of the LP B cells obtained here were consistent with the literature (14) and our previous experience using this method of isolation in C3H/HeN mice (11). Nonetheless, when comparing the effects of FasL deficiency on LP B cell apoptosis, we observed that although a significant increase in the frequency of apoptosis in cells expressing B220 was seen in C3H/HeJ mice 24 h after CLP, there was no significant change in the B cell population apoptosis between sham-operated and CLP C3H/HeJ-FasLgld mice (Fig. 3). Although the percentage of apoptosis in LP B cells from FasLgld CLP mice was lower than that in HeJ CLP mice, it did not reach statistical significance.

**Light microscopy.** To confirm that the changes seen in the ex vivo isolated cells represented in vivo changes in the tissue, paraffin-embedded tissue sections of ileum were stained with hematoxylin and eosin and evaluated by light microscopy. Figure 4, A and C, which exhibits typical ileal morphology, represents...
samples from sham-operated C3H/HeJ and C3H/HeJ-FasL<sup>gld</sup> mice. Samples from septic C3H/HeJ mice consistently show marked changes in tissue cellularity consistent with the onset of apoptosis and intestinal injury. In this regard, an increased number of shrunken pyknotic cells with condensed nuclei are seen within the LP (Fig. 4B). These changes are characteristic of apoptosis. However, these types of morphological changes were significantly less evident in ileum taken from septic C3H/HeJ-FasL<sup>gld</sup> mice (Fig. 4D). Using the morphological apoptotic scoring system of Hotchkiss et al. (18), we found that the extent of apoptosis confined to regions of LP was significantly higher in C3H/HeJ mice 24 h after CLP (1.2 ± 0.058 vs. 3.27 ± 0.088 for C3H/HeJ sham operated vs. CLP, respectively; *P < 0.05 vs. sham operated, Student’s t-test; n = 3/group), whereas there were no significant changes in apoptosis between sham-operated and CLP C3H/HeJ-FasL<sup>gld</sup> mice. However, the apoptotic score in LP B cells from FasL<sup>gld</sup> CLP mice was markedly lower than that in HeJ CLP mice (1.17 ± 0.033 vs. 1.6 ± 0.15, FasL<sup>gld</sup> sham operated vs. CLP, respectively; *P < 0.05 vs. C3H/HeJ CLP, Student’s t-test; n = 3/group).

Polymicrobial sepsis increases number of LP IgA-secreting B cells from C3H/HeJ mice but not C3H/HeJ-FasL<sup>gld</sup> mice. IgA secretion in LP B cells was used as the potentially functional index for the increased apoptosis during sepsis. An ELISpot analysis was performed to determine the ability of LP B cells to secrete IgA. The result in Fig. 5 shows that C3H/HeJ mice had significantly more LP B cells that secreted IgA after the onset of sepsis. Again, this was not observed in FasL-deficient mice.

Survival study of C3H/HeJ and C3H/HeJ-FasL<sup>gld</sup> mice subjected to CLP. To the extent the FasL deficiency might provide protection against septic mortality, C3H/HeJ and C3H/HeJ-FasL<sup>gld</sup> mice were subjected to the CLP procedure and monitored for survival over a 28-day period. Table 1 shows that although the survival rates of two mouse strains were not significantly different from each other until day 7, a latent and lower mortality rate was observed in C3H/HeJ-FasL<sup>gld</sup> mice consistently on days 2–28.

Fig. 3. Percentage of apoptotic LPMC (as determined by terminal dUTP nick-end labeling) that are B220-positive phenotype in C3H/HeJ and C3H/HeJ-FasL<sup>gld</sup> mice. At 24 h after CLP, a marked increase of apoptosis in B220-positive population was seen in septic C3H/HeJ but not in C3H/HeJ-FasL<sup>gld</sup> mice. Values are means ± SE; n = 6 mice/group. *P < 0.05 vs. sham operated (Mann-Whitney U-test).

Fig. 4. Hematoxylin and eosin staining of representative ileum sections 24 h after sham operation or CLP (magnification, ×600). A: C3H/HeJ sham operated. B: C3H/HeJ CLP. C: C3H/HeJ-FasL<sup>gld</sup> sham operated. D: C3H/HeJ-FasL<sup>gld</sup> CLP. Note that ileum from C3H/HeJ CLP mice (B) exhibits disruptions of the cellular architecture within the tissue. Cells (arrows) show feature characteristics of apoptosis, i.e., nuclear condensation, pyknosis, and shrinking cytoplasm. These morphological changes were not observed in the ileum taken from either sham-operated animals (A and C) or FasL-deficient CLP mice (D).
DISCUSSION

Our (11) previous study using C3H/HeN, which is an endotoxin-sensitive mouse strain, indicated that sepsis induces an increased apoptosis in both T and B lymphocytes from small intestinal LP. In this regard, little information is available concerning the nature of the mediator(s) responsible for the induction of this increase in apoptosis seen in mucosal B cells during sepsis. Endotoxin or LPS, a component of Gram-negative bacterial cell wall, is a potent B lymphocyte mitogen and macrophage/monocyte activator that acts to induce apoptosis in T and B cells in vivo and in vitro (10, 36, 38). Moreover, endotoxin is an important inducer of the septic response. Therefore, endotoxin appeared to be a good candidate mediator that might be responsible for LP B cell death during polymicrobial sepsis. It has also been indicated that Fas/FasL interactions are directly involved in induction of apoptosis in T and B lymphocytes. Recently, studies from our laboratory (4) indicated that intestinal Peyer’s patch B cell apoptosis is markedly increased during sepsis, and this appears to involve Fas/FasL. Moreover, Boirivant et al. (7) reported that the increased apoptosis in in vitro stimulated LP T cells is mediated by the Fas/FasL pathway. These findings suggest that Fas/FasL evidently is another candidate for the study of sepsis-induced B cell apoptosis. Thus genetic mutations in Fas (lpr) or FasL (gld) with endotoxin-tolerant background (C3H/HeJ) may provide a powerful tool for analyzing the possible role of these mediators in mucosal B cell apoptosis during sepsis.

Fas (also known as CD95 or Apo-1) is a member of the TNF/nerve growth factor receptor superfamily, which regulates immune cell differentiation, proliferation, activation, and survival on ligation with FasL (belonging to TNF superfamily) or anti-Fas antibody (25). Fas expression is induced on activated lymphocytes and in nonhemopoietic tissue such as liver, heart, lung, and kidney (32). Fas expression on B cells has been demonstrated in many laboratories (8, 31) to be critical for activation-induced cell death and elimination of autoreactive B cells. Daniel and Krammer (13) reported that Fas expression is upregulated in B cells that undergo activation-induced apoptosis. In this regard, determination of Fas expression may be useful to provide insight into the role of the Fas/FasL pathway in LP B cell apoptosis during sepsis. Our results using endotoxin-tolerant (C3H/HeJ) and FasL-deficient mice (C3H/HeJ-FasLgld) showed that LP B cells exhibited increased apoptosis in line with enhanced Fas expression only in septic endotoxin-tolerant mice, which are not deficient in FasL. However, Fas expression in both sham-operated and CLP FasL-deficient mice was higher than in endotoxin-tolerant sham-operated mice. This is in agreement with the finding by Weintraub et al. (33) that Fas expression is increased on B cells in the B6/gld mouse spleen compared with normal B6 control mice. This is also in keeping with the finding that these B cells from B6/gld mice were more susceptible to apoptosis mediated by added soluble anti-Fas antibody (33). However, the mechanism of the upregulated Fas expression in the LP B cells during sepsis remains unknown.

That said, it is also clear that a number of different apoptotic mediators appear to be contributing to the induction of lymphoid apoptosis during sepsis (4, 20). In this respect, the apoptotic response to Fas does not appear to be a universal feature in all lymphoid tissues examined in septic mice. Recent studies (6, 23) have also indicated that stimulation of thymocyte, but not splenocyte, apoptosis is a p53-mediated but Fas-independent pathway (using p53 knockout and Fas receptor-deficient (FasLgld) mice). This lack of a Fas-mediated effect has also been documented in previous studies from our laboratory (1), in which it was shown that increased thymocyte apoptosis was not reduced in FasL-deficient (FasLgld) mice in sepsis. In addition, recent studies (4) have also shown that Peyer’s patch B cell, but not T cell, apoptosis is Fas mediated after septic challenge. Thus, although the current results indicate that LP B cell apoptosis appears to be mediated by Fas, we do not suggest that this is a mechanism that can account for all lymphoid apoptosis encountered in septic mice. Moreover, studies (15, 22, 27) have also indicated that stimulation of B cells with addition of endotoxin, CD40 ligand, or interleukin-4 (IL-4) has been demonstrated to upregulate Fas expression. However, endotoxin appears not to

Table 1. Survival of C3H/HeJ vs. C3H/HeJ-FasLgld mice after cecal ligation and puncture

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<th>Day</th>
<th>1</th>
<th>2</th>
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Values are given as no. of surviving mice/no. of total mice; n = 25 mice/group. FasL, Fas ligand; FasLgld, C3H/HeJ-FasLgld. *P < 0.05 vs. C3H/HeJ mice (Fisher’s exact test).
be a key player in this process according to our data above. With respect to CD40 ligand, the interaction of CD40 ligand and its receptor CD40 is thought to result in activation and induction of Fas expression in B cells, which then are highly susceptible to Fas-mediated killing by T cells (27). However, CD40 ligand staining with antibody did not show changes in LP lymphocytes in our hands (data not shown). Recent studies from our laboratory (11) show that sepsis can induce increased apoptosis in LPMC, including T and B cells, that is associated with upregulating cytokine (IL-2, IL-10, and IL-15) mRNA expression. These cytokines might also play a role in the induction of Fas expression in LP B cells. However, inhibition of these cytokines or the genetic deficiency of these agents and the effect on LP B cell apoptosis have not been assessed. Finally, although we have shown here that LP B cell apoptosis is mediated by Fas but not endotoxin, this does not rule out the possible effects of the intact microorganism and/or a combination of other enterotoxins and/or exotoxins that might be released on the induction of Fas-mediated apoptosis seen here. However, further study is needed to better delineate the nature of the pathogens or toxins present, besides endotoxin, before such experiments can be conducted.

Two methods, TUNEL staining and light microscopy of hematoxylin and eosin-stained ileal sections, were used to detect apoptosis in our study. TUNEL staining is highly sensitive but low in specificity; however, conventional light microscopy is low in sensitivity but highly specific when characteristic apoptotic morphology is present (18, 34). Overall, there was a high level of baseline apoptosis in isolated LP B cells from all mice as detected by TUNEL. However, sepsis did induce a marked increase in apoptosis in these cells from C3H/HeJ but not from FasL-deficient mice. Although the percentage of apoptosis in cells from FasL-deficient CLP mice is lower than that from C3H/HeJ CLP mice, they are not statistically significant. These results are consistent with light microscopy examination of samples in which an increase in apoptosis is observed in the ileum of C3H/HeJ but not FasL-deficient septic mice. Furthermore, these morphological differences in the extent of apoptosis between FasL-deficient CLP and C3H/HeJ CLP mice are statistically significant.

With respect to IgA secretory response in septic C3H/HeJ mice, the increase in apoptosis appears to be associated with enhanced endogenous IgA secretion, which is in accord with our previous studies of Peyer’s patch B cells (4). We further identify that this observation was also endotoxin independent and FasL mediated. Fas-mediated induction of apoptosis significantly contributes to elimination of clonal proliferation and regulation of lymphocyte number and turnover in various lymphoid tissues, particularly those continuously exposed to antigen challenge (30) such as the mucosal lymphocytes. Therefore, the increase of apoptosis in intestinal LP B cells along with augmented IgA secretion seen after sepsis would appear to be the result of activation-induced apoptosis. Initially, it may have been speculated that this increase in apoptosis in the activated lymphocytes may be a mechanism to maintain homeostasis that may protect the host from an excessive immune response. However, during overwhelming inflammation, as is thought to be encountered after CLP, aggravated activation-induced apoptosis of lymphocytes may result in humoral immune cell dysfunction. Interestingly, Teodorczyk-Injeyan et al. (28) indicated that nonspecific polyclonal Ig synthesis initially increases and then declines over time after burn injury. The eventual failure of the late Ig release corresponded to higher risk for fatal infectious complications (28). However, this aberration in IgA release in critical illness appears to be due to impairment of antibody synthesis to specific antigens (35) as opposed nonspecific IgA release.

The present findings show that polymicrobial sepsis induces increased LP B cell apoptosis, which is mediated by Fas/FasL pathway. In addition to identifying the mediator(s) responsible for increased apoptosis, we also performed a survival study using endotoxin-tolerant (C3H/HeJ) and endotoxin-tolerant/FasL-deficient mice (C3H/HeJ-FasL-def) in an attempt to assess the role of Fas/FasL in morbidity and/or mortality associated with sepsis. It should be noted that the survival rate was not different between endotoxin-sensitive (C3H/HeN) and -tolerant mice (C3H/HeJ) subjected to CLP operation in our previous study (2). Thus it appears that the significantly lower rate of late mortality observed in FasL-deficient mice after the onset of polymicrobial sepsis is due to FasL and is not a result of the concomitant inability to respond to endotoxin. Although it is tempting to speculate that the protective effects on survival seen here might be related to the decreased apoptosis in the gut LP lymphocytes, we recognize that these protective effects of FasL deficiency may be directed at other immune tissues (phagocytes) and/or at nonimmune cells that also express or perceive FasL. Nonetheless, the significance of these findings and the relative importance of these potentially pathological apoptotic changes, seen here in septic animal lymphoid tissue, have been recently confirmed by Hotchkiss et al. (19). In that study, Hotchkiss et al. illustrated not only that mice overexpressing the human Bcl-2 gene product (which antagonizes the apoptotic response) showed decreased lymphoid apoptosis after CLP but also that their survival was markedly improved (19). The degree that this survival advantage was related to the onset of lymphoid apoptosis was based on the finding (19) that RAG-2 mice, deficient in both mature T and B cells, survive septic challenge quite poorly compared with the background controls.

In summary, the increased apoptosis in intestinal LP B lymphocytes after the onset of sepsis appears to be mediated through Fas/FasL pathway. Furthermore, FasL deficiency is also involved in reduction of morbidity and/or mortality seen with septic insult. In light of this, we feel that further studies examining the role of FasL are warranted to clarify the site(s) of action of this agent and potential application of FasL antago-
nists as a posttreatment (therapeutic) agent during sepsis.

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