Is Fas Ligand or Endotoxin Responsible for Mucosal Lymphocyte Apoptosis in Sepsis?

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**Background:** Apoptosis (Ao) is a normal constitutive process that seems to have pathological effects in diseases of immune deficiency and autoimmune disorders, as well as in certain lymphoid tissues during sepsis. Little is known about this process in mucosal lymphoid tissue, such as intestinal intraepithelial lymphocytes (IELs).

**Objectives:** To determine whether sepsis induces increased Ao in small intestinal IEL, whether this was associated with functional changes in cytokine gene expression in the IEL, and which mediators control this process and their impact on the survival of the mouse with sepsis.

**Design:** Male C3H/HeN (endotoxin-sensitive), C3H/HeJ (endotoxin-tolerant), and C3H/HeJ-FasLgld (endotoxin-tolerant/Fas ligand [FasL]–deficient) mice were subjected to sepsis (cecal ligation and puncture [CLP]) and IELs were harvested at 4 (early) or 24 hours (late sepsis). Alterations in the cell phenotype and Ao (TUNEL [terminal deoxynucleotidyl transferase–mediated deoxyuridine 5-triphosphate nick-end labeling] assay) were determined by 3-color flow cytometry. Cytokine gene expression was assessed by multiprobe RNase protection assay.

**Results:** At 4 hours after CLP, only the frequency of IEL which was CD8+ decreased markedly. By 24 hours after CLP, the number of CD8+ and CD4+ cells decreased while the proportion of double-negative cells showed a marked increase when compared with sham-controls. The percentage of Ao positive in CD8+ and CD4+ double-positive and double-negative cells increased markedly 24 hours after CLP concomitant with a significant ($P < .05$ vs sham-controls, Mann-Whitney $U$ test) increase in expression of the IL-2, IL-10, and IL-15 gene. These data collectively suggest that sepsis causes lymphocyte activation–induced Ao that may be mediated by FasL. Additional studies were done to determine if the increased Ao was due to either endotoxin or FasL. The results of studies with endotoxin-tolerant C3H/HeJ or FasL-deficient C3H/HeJ-FasLgld mice showed an increase in Ao in CD4+ and CD8+ cells from septic C3H/HeJ but not C3H/HeJ-FasLgld mice. With regard to septic mortality, our results indicated that there was a marked reduction in mortality in C3H/HeJ-FasLgld vs C3H/HeJ mice.

**Conclusions:** We conclude that the phenotypic changes associated with increased Ao may be a reflection of localized immune cell activation due to a FasL-mediated process and not endotoxin. Thus, FasL directly and/or indirectly contributes to higher septic mortality.

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**Despite the advent of a number of modern medical therapeutic approaches, sepsis and multiple organ failure remains one of the major causes of morbidity and mortality (as high as 60%) in the intensive care unit.** Many studies have suggested that development of multiple organ failure is strongly linked to the initial presentation of sepsis. However, the precise mechanism by which sepsis induces subsequent multiple organ failure is still poorly understood. In this respect, over the last few years, an increasing number of studies have focused on the contribution of the barrier failure of the gut in the initiation and development of these conditions. Intestinal intraepithelial lymphocytes (IELs) are a subpopulation of the mucosa-associated lymphoid tissue. In the small intestine, IELs lie within the epithelial lining, which serves as the only layer to separate the intestinal lumen from the internal environment. These cells are, therefore, constantly exposed to foreign antigens and, thus, are thought to play a key role in the modulation of the mucosal immune system. The important role of IELs in gut immunity has been demonstrated in the maintenance of epithelial integrity, cytolytic potential, and the secretion of multiple cytokines in vitro. Studies have shown that the cytotoxicity of small-intestinal IELs is enhanced and CD4+ helper T cells are lost in mice and hu-
MATERIALS AND METHODS

CECMICAL LIGATION AND PUNCTURE (CLP)

Polymicrobial sepsis was induced in mice with the methods described by Baker et al.19 and Ayala et al.20 Male inbred C3H/HeN.CrBcr (C3H/HeN endotoxin-sensitive) (Charles River Laboratories, Wilmington, Mass), C3H/HeJ (endotoxin-tolerant), and C3H/HeJ-FasL14 (endotoxin-tolerant/Fas ligand-deficient) (Jackson Laboratory, Bar Harbor, Me) mice, 7 to 10 weeks old, were lightly anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, Ill), 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 content through the punctured holes. The cecum was returned to the abdomen and the incision was closed in layers. Next, the mice were resuscitated with 0.8 mL of lactated Ringer solution by subcutaneous injection. For sham-controls, the cecum was neither ligated nor punctured. All procedures were carried out according to the National Institutes of Health Guidelines on Laboratory Animals and approved by the Animal Welfare Committee of Rhode Island Hospital, Providence.

ISOLATION AND PURIFICATION OF IELs

The IELs were isolated using the method described by Davies and Parrott11 with minor modifications. Mice were killed 4 or 24 hours after CLP by methoxyflurane overdose and the small intestine was immediately removed and washed thoroughly with cold Hanks balanced salt solution (calcium free and magnesium free) (Gibco, Grand Island, NY). The Peyer’s patches, fat, and mesentery were dissected and removed and the gut was slit open and cut into segments (0.5-1 cm). The gut segments were incubated for 90 minutes at 37°C in 3 changes of 30 mL of Hanks balanced salt solution containing 1-mmol/L diithiothreitol and 1-mmol/L EDTA (Sigma Chemical Co, St Louis, Mo) with continuous shaking in a water bath. The cell suspension from 3 incubations were pooled, centrifuged, resuspended, and filtered by passing through a loosely packed nylon wool column at room temperature to remove mucus, tissue debris, and dead cells. The cells were washed twice with RPMI complete medium with 2% fetal calf serum. Isolated IELs were further purified on a colloid suspension (Percoll, Sigma) gradient.21 Eight milliliters of cells suspended in the 44% colloid suspension was overlaid onto 5 mL of the 67% colloid suspension in a 15-mL conical tube and centrifuged at 600 rpm for 20 minutes at 4°C. Cells collected from the interface of the 44% and 67% colloidal suspensions were washed twice and resuspended at 10^6 cells per milliliter of RPMI complete medium. The viability of the recovered cells was determined by trypan blue exclusion and was found to be greater than 90%.

CELL STAINING AND FLOW CYTOMETRIC ANALYSIS

To correlate the changes in the percentage of cells that were Aa positive with phenotypic expression, IELs were stained with a combination of phycoerythrine-conjugated anti-CD4 monoclonal antibody (mAb) (clone RM4-5, rat IgG2a), cyochrome-conjugated anti-CD8 mAb (clone 53-6.7, rat IgG2a) (Pharmingen, San Diego, Calif), and fluorescein isothiocyanate (FITC)-labeled TUNEL (terminal deoxynucleotidyl transferase [TdT]–mediated deoxyuridine 5-triphosphate [dUTP] nick-end labeling) (Boehringer, Indianapolis, Ind), an in situ cell death detection kit, for detection of Ao.

Three-color flow cytometric analysis was carried out according to methods previously described by our laboratory.16 The analysis was performed on a flow cytometer with an equal frequency of either γ δ or α β T-cell receptor) and divergent lineages (T cells develop with or without thymic influence).11,12 However, little is known about the mucosal, IEL immune response during sepsis or what mediators affect it.

Programmed cell death or apoptosis (Aa) is a cellular suicide program that is a regulated nonnecrotic form of cell death. For most cells, Aa usually is a constitutive response, but Aa can also be induced in certain immune cells such as T and B lymphocytes via immunologic and/or pathological processes.13 Apoptosis in activated T cells can be regulated by a variety of signals, of which the Fas (CD95):Fas ligand (FasL) pathway is best known.14 The induction of Fas/FasL appears to serve as an internal brake that restrains the antigen-activated T cell’s response. However, Aa is also regulated by many stress mediators that are reported to be released during trauma and sepsis.3 It has also been shown that intravenous administration of endotoxin to animals induces the release of a wide variety of mediators (eg, cytokines, hormones) that may account for circulatory and metabolic disturbances as well as immune dysfunction of the host. However, the contribution of these agents to the development of Aa and/or changes in mucosal immunity during sepsis are unclear.

Previous studies from our laboratory have indicated that a variety of immune cells from different compartments, such as thymocytes,15 bone marrow cells,16 Peyers patch B cells,16 macrophages,12 and small-intestinal lamina propria mononuclear cells,16 undergo apoptosis following the onset of polymicrobial sepsis. The data from Peyers patch and lamina propria lymphocytes suggest that increased Aa is associated with the activation of these cells during sepsis. However, there is little information concerning the response of IELs during sepsis on either a functional or a phenotypic level, as well as whether Aa is induced. The aim of this study, therefore, was to de-
CHANGE OF IEL POPULATIONS FROM C3H/HeN MICE FOLLOWING THE ONSET OF SEPSIS

Table 1 shows the percentage of apoptotic cells stained with CD4 and/or CD8 markers in sham-controls and CLP mice. A slight but significant decline of CD8 single-positive, CD4+CD8−, cells was observed at both 4 (Figure 1, top) and 24 (Figure 1, bottom) hours after CLP. However, the percentage of CD4 single-positive, CD4+CD8−, and CD4+CD8+, cells was not markedly decreased until 24 hours after CLP, while the percentage of CD4+CD8− cells was significantly increased at 24 hours.

SEPSIS INDUCES AN INCREASE IN THE FREQUENCY OF AN IN IELs FROM C3H/HeN MICE

To correlate the frequency of AN with phenotypic expression, IELs isolated from C3H/HeN mice were stained with a combination of anti-CD4 and anti-CD8 mAbs for phenotypic characterization and TUNEL assay for assessment of AN. Figure 2 shows the percentage of apoptotic cells stained with CD4 and/or CD8 markers in sham-controls and CLP mice at 4 (Figure 2, top) and 24 hours (Figure 2, bottom) following the onset of sepsis. At 24 but not at 4 hours, a significant increase in the frequency of AN in cells expressing CD4+CD8−, CD4+CD8+, and CD4+CD8− was observed in the septic mice. However, there were no important differences in the CD4+CD8− population between sham-controls and septic mice.
CYTOKINE GENE EXPRESSION IN IELs FROM C3H/HeN MICE

Expression of cytokine mRNA was analyzed by RPA. The results of cytokine gene expression from freshly isolated (unstimulated) IELs are shown in Figure 3. Using the commercial multiprobe set, IL-2, IL-10, IL-15, and IFN-γ genes were consistently detected in both sham-controls and CLP mice, while signals for IL-4, IL-5, IL-6, IFN-γ, and IL-13 genes (also included in the multiprobe set) were not detected in our system. Quantitative analysis of the expressed cytokine mRNA levels was estimated in the autoradiographic intensity normalizing to housekeeping gene (L32 or GAPDH) expression. Among the detected cytokine genes in IELs, there was a marked increase in IL-2, IL-10, and IL-10 mRNA evident 24 hours after CLP. However, expression of IFN-γ mRNA did not differ between sham-controls and CLP mice at either time point.

SEPSIS INDUCES AN INCREASE IN THE FREQUENCY OF A₀ IN IELs FROM C3H/HeJ (ENDOTOXIN-TOLERANT) BUT NOT C3H/HeJ-FasL<sup>−/−</sup> (ENDOTOXIN-TOLERANT/FasL-DEFICIENT) MICE

Because recent studies have indicated that Fas antigen and FasL may mediate A₀, seen to be associated with the activation of lymphocytes during an immune response, it was our hypothesis that the increase in lymphocyte A₀, seen here to be associated with increased cytokine expression might be reflective of the Fas/FasL-mediated process. To examine this, we conducted experiments to assess the frequency of A₀, with phenotypic expression in IELs at 24 hours after the onset of sepsis using a mutant mouse strain with a defective FasL gene (C3H/HeJ-FasL<sup>−/−</sup>)<sup>23</sup> and C3H/HeJ mice (as the endotoxin-tolerant background control). The percentages of both CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells were markedly decreased, while a marked increase in CD4<sup>+</sup>CD8<sup>+</sup> cells was seen in septic C3H/HeJ mice at 24 hours (Figure 4, top). However, these changes were not observed in C3H/HeJ-FasL<sup>−/−</sup> mice (Figure 4, bottom). A significant increase in the frequency of A₀ in cells expressing CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>+</sup>CD8<sup>−</sup> was observed in C3H/HeJ mice 24 hours after CLP (Figure 5, top). Interestingly, all of the IELs' phenotypic populations showed a slight trend toward lower apoptotic frequency in septic C3H/HeJ-FasL<sup>−/−</sup> mice although these were not statistically significant (Figure 5, bottom).

SURVIVAL STUDY OF C3H/HeJ (ENDOTOXIN-TOLERANT) AND C3H/HeJ-FasL<sup>−/−</sup> (ENDOTOXIN-TOLERANT/FasL-DEFICIENT) MICE SUBJECTED TO CLP

To the extent the FasL deficiency might provide any protection against septic mortality, C3H/HeJ and C3H/HeJ-FasL<sup>−/−</sup> mice were subjected to the CLP procedure and monitored for their survival over a 7-day period. Figure 6
shows that although the survival rates of 2 mouse strains were not significantly different from each other until day 7, the latent and lower mortality rates were observed in C3H/HeJ-FasLgld mice consistently on days 2 through 7.

**COMMENT**

The murine small-intestinal IELs are a heterogeneous mononuclear cell population that contains approximately 80% to 90% CD3+ T cells.24 Most IEL T cells are CD4−CD8+ (approximately 50%-75%) and contain large intracytoplasmic granules of perforin and serine esterases, the concentration of which are increased on antigenic activation.12,25 These large granular lymphocytes are unusual for a T-cell population and are thought to mediate natural killer cell cytotoxic activity.26 Total viable IEL yield in our preparation from C3H/HeN mice was markedly decreased at 4 but not 24 hours after CLP. However, it should be noted that although at 24 hours total IEL yield did not differ between sham-control and septic animals, a marked increase in CD4+ CD8+ cells was observed in septic mice, which may be reflective of a route of entry for neutrophil, macrophage, and/or other non-T-cell populations. Thus, it appears that localized immune cell activation and/or the recruitment of inflammatory cells to the gut occurs during sepsis.

To assess the effect of polymicrobial sepsis on the T-cell subpopulations of IELs, the phenotypic expression was examined using 3-color flow cytometric analysis. Besides CD4+CD8+ T cells, IELs typically consist of about 7.5% CD4+CD8− and approximately 10% double-positive and 7.5% double-negative T cells.27 The percentage of CD4+CD8+ and CD4−CD8+ T-cell populations in the IEL preparations from sham-control C3H/HeN mice was consistent with those reported in the literature.27 Interestingly, only the percentage of CD4−CD8+ cells was markedly decreased in the septic mice at both 4 and 24 hours following the onset of sepsis. A similar finding was made in C3H/HeJ-FasLgld mice. Thus, we conclude that sepsis produces an early effect on the cytotoxic T-cell population that is not evident until late in the helper T cell phenotype.

The IELs, because of their anatomical location, are thought to play a critical role in the mucosal immune system through the surveillance of the local antigen environment. Although little is known about the potential function of IELs in vivo, many in vitro studies have been conducted to elucidate the biological function in this population. Consisting primarily of T cells, of which, approximately 70% are CD8+ cytotoxic T cells, IELs have been shown to possess both a competent tumor and microbial cytotoxic capacity.5 The IELs have also been shown to contribute to the maintenance of epithelial cell integrity,6 and they also appear to be involved in the maintenance of mucosal secretary IgA immune response.28 Fur-
thermore, IELs have been shown to secrete a variety of cytokines on stimulation and/or activation. In this regard, our study demonstrates that freshly isolated small-intestinal IELs from septic mice express higher levels of mRNA for IL-2, IL-10, and IL-15 genes but not for the IFN-γ gene. While these changes seem to be relatively small, we speculate that this is a part of the extended period of time required to isolate these ex vivo cells. These results, based on cytokine gene expression, also suggest that there seems to be no clear in vivo preference toward Th1 or Th2 phenotype in murine IELs. These findings are in agreement with those of Clevers et al, who showed that IELs from mice contain a mixture of Th1- and Th2-type cells. However, the increased release of these cytokines, i.e., interleukin (IL) 2, IL-15, suggests that these cells have most likely been activated in response to stimuli associated with the onset of sepsis. In this regard, the induction of T-cell activation sets in motion not only processes involved with the development of a cell-mediated immune response (such as lymphokine release, major histocompatibility complex antigen expression), but it also induces mechanisms to eventually contain such a response, i.e., via up-regulation of expression of Fas/FasL as well as other receptors-pathways that can induce apoptotic cell death (via autocrine, paracrine, and/or exocrine cytokine-mediator pathways).

Apoptosis is a constitutive process for most of the cells that carry out their own death through activation of an internally encoded suicide program and initiate Ao. However, Ao can also be induced in some immune cells through the antigenic engagement of the T-cell or B-cell receptor complex (activation-induced cell death) or by various factors (e.g., glucocorticoids, inflammatory cytokines, nitric oxide) released during pathological insult. Studies from our laboratory and others indicated that increased Ao is evident in a variety of immune cell types (T and B cells, macrophages, and granulocytes) from different organs and tissues including the thymus, bone marrow, spleen, Peyer’s patches, and lamina propria mononuclear cells following the onset of sepsis. Our results show that the apoptotic frequency of T lymphocytes in intestinal IEL preparation from endotoxin-sensitive C3H/HeN mice was increased in septic mice. A marked increase in the frequency of Ao in CD4+CD8−, CD4+CD8+, and CD4+CD8+ T cells was evident 24 hours after CLP. This indicates that induction of Ao is an event that is not evident in this cell population until the late hypodynamic-hypometabolic stage of polymicrobial sepsis, a period in which organ perfusion and oxygenation are expected to be compromised in the gut. These findings are in keeping with our previous findings in B cells from the Peyer’s patch and bone marrow, in which a marked increase in apoptotic frequency was not detected until the late time point (24 hours after CLP). However, the results differ from the thymus, peritoneal macrophage, and lamina propria mononuclear cells in which the onset of Ao was detected as early as 4 hours after CLP.

It has been suggested that activated T cells up-regulate the expression of the Fas/FasL gene, and undergo activation-induced Ao. Fas ligand (FasL or CD95L, members of the tumor necrosis factor superfamily) interacts with their receptors, Fas (APO-1 or CD95, members of the tumor necrosis factor receptor superfamily), to regulate important functions in immune cell activation, differentiation, proliferation, and survival. Endotoxin (a key mediator of gram-negative bacteria) has been shown to induce thymic Ao, however, the dosage of lipopolysaccharide required to induce marked Ao was 50 µg or higher per mouse (intraperitoneally; equivalent to 2.5 mg of lipopolysaccharide per kilogram of body weight). This concentration of lipopolysaccharide typically produces blood levels of endotoxin.
Over the last few years, it has become apparent that the intestine as an immunological organ may play not only an important role in the maintenance of gut barrier function but in the evolution of multiple organ failure associated with shock and sepsis. However, little is known about the pathobiologic characteristics of these changes and what regulates them. We, therefore, have attempted to examine the effect of sepsis on the phenotype as well as functional response of one compartment of the mucosal immune system, the IEL. Our findings indicate that sepsis induces an increase in Aα, in different populations of IELs that include CD4+CD8−, CD4+CD8+, CD4+CD8−, and/or CD4+CD8− T lymphocytes. A marked increase in IL-2, IL-10, and IL-15 mRNA gene expression in IELs was observed 24 hours after the onset of sepsis. These results suggest that the increase in Aα in intestinal IELs is associated with changes in cytokine gene expression following the onset of sepsis; there is localized immune cell activation leading to activation-induced apoptotic cell death during sepsis; and this IEL apoptotic response mediated by Fasl and FasL deficiency seems to provide a survival advantage during sepsis. Thus, the use of agents that inhibit FasL during sepsis might not only be useful in preventing the potential pathological effects of sepsis-induced gut lymphoid Aα but the associated septic mortality.

that are considerably higher than the circulating levels of this agent detected in patients37 or mice20 with sepsis. Furthermore, previous studies from our laboratory have demonstrated that genetically related strains of mice, C3H/HeJ (endotoxin-sensitive strain) and C3H/HeN (endotoxin-tolerant strain) showed no difference in survival. HeN (endotoxin-sensitive strain) and C3H/HeJ (endotoxin-tolerant strain) showed no difference in survival rate when they were subjected to CLP.30 In this respect, we attempted to determine whether Fas/FasL and/or endotoxin contribute to the increased Aα in IELs during sepsis. C3H/HeJ (endotoxin-tolerant) and C3H/HeJ-FasLgld (a mouse mutant strain of endotoxin-tolerant/FasL-deficient) mice were subjected to CLP and the frequency of Aα in IELs was determined. A marked increase in Aα in CD4+CD8− and CD4+CD8+ cells was observed in endotoxin-tolerant C3H/HeJ mice. Alternatively, IEL T cells from C3H/HeJ-FasLgld showed a slight (though not significant) decrease in Aα after CLP. These findings, therefore, indicate that FasL but not endotoxin mediates the induction of Aα in IELs, since sepsis causes IEL T-cell Aα in both endotoxin-sensitive and -tolerant mice. This is in keeping with the studies reported by Hiramatsu et al.34 who showed by in situ TUNEL that CLP induced Aα in different organs including the gut by an endotoxin-independent pathway.

In light of this, we speculated that Fas/FasL-mediated Aα may play an important role in morbidity and/or mortality during sepsis. We, therefore, attempted to determine the relationship between FasL deficiency to the mouse’s survival by subjecting C3H/HeJ and C3H/HeJ-FasLgld mice to CLP and comparing their mortality. A significantly lower mortality rate was observed in FasL-deficient mice after CLP. On the surface, this would seem to be in keeping with the hypothesis that an overzealous gut inflammatory response associated with increased Fas/FasL-mediated Aα, may be contributing to not only the dysfunction of the septic gut barrier but also to the associated morbidity/mortality seen in sepsis. However, although it is tempting to speculate that the protective effects on survival seen here might be related to the decreased Aα in the gut IELs, we recognize that these protective effects of FasL deficiency may be directed at other immune tissues or cells (eg, phagocytes that can act as a source of FasL) and/or at nonimmune cells or tissues that also respond to the FasL gene product.30

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DISCUSSION

John C. Marshall, MD, Toronto, Ontario This work is intriguing and important in 2 separate domains. One is the inference that the gut immune system potentially has a role in survival in a model of intra-abdominal infection. The second is that you have shown us an endotoxin-independent and Fas-dependent pathway to mortality in a classic model of intra-abdominal sepsis. There are wonderful possibilities for that sort of work in the future. I have 3 questions that I wanted to ask you.

The first one is that in your manuscript you raise the possibility that when you see an increased number of double-negative cells in the gut wall that you may be looking at infiltration by nonlymphocytes, and I wonder if you have done any studies to see if there are increased numbers of neutrophils in the gut; in other words, to see whether or not the intraepithelial lymphocyte population is suppressing inflammation and you are losing a suppressive influence?

Second, I am wondering if you have looked at all at what might be responsible for the changes in phenotype and susceptibility to apoptosis? Is it a flow-dependent phenomenon? If you don’t resuscitate the animals, do you see a worsening of it? Or is it secondary to changes in the bacterial flora, since we know that the bacterial flora of the small intestine will change during cecal ligation and puncture.

And then finally, I wanted to ask you with regard to the survival studies you have done, whether you have done histology. This to my knowledge is the first model that I have seen that really reflects what happens in human multiple organ failure, which is that mortality is not early but it is late, and the fact that your survival curves diverge at about 7 days is most intriguing. I am wondering if you have looked to see if there is increased evidence of inflammation in the liver or the lungs of the HeJ but not GLD mice?

Dr Chung: Thank you, Dr Marshall. With regard to your first question about double-negative cell population increase, it’s possible that neutrophil and other cells are from other compartments. We haven’t looked at the neutrophil phenotype due to some limitation of the cell yield. The cell yield sometimes is pretty low. It’s more important in 2 separate domains. One is the inference that the gut immune system potentially has a role in survival in a model of intra-abdominal infection. The second is that you have shown us an endotoxin-independent and Fas-dependent pathway to mortality in a classic model of intra-abdominal sepsis. There are wonderful possibilities for that sort of work in the future.