Immunopathology and Infectious Disease

CD8⁺ T Cells Promote Inflammation and Apoptosis in the Liver after Sepsis

Role of Fas-FasL

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Although studies blocking the Fas pathway indicate it can decrease organ damage while improving septic (cecal ligation and puncture, CLP) mouse survival, little is known about how Fas-Fas ligand (FasL) interactions mediate this protection at the tissue level. Here, we report that although Fas expression on splenocytes and hepatocytes is up-regulated by CLP and is inhibited by *in vivo* short interfering RNA, FasL as well as the frequency of CD8⁺ T cells are differentially altered by sepsis in the spleen (no change in FasL, decreased percentage of CD8⁺ and CD4⁺ T cells) versus the liver (increased FasL expression on CD8⁺ T cells and increase in percentage/number). Adoptive transfer of CLP FasL^{+/+} versus FasL^{-/-} mouse liver CD8⁺ T cells to severe combined immunodeficient or RAG1^{-/-} recipient mice indicated that these cells could induce inflammation. The FasL-mediated cytotoxic capacity of these septic mouse liver CD8⁺ T cells was shown by their ability to damage directly cultured hepatocytes. Finally, although CD8^{-/-} mice exhibited a reduction in both CLP-induced liver active caspase-3 staining and blood interleukin-6 levels, only FasL^{-/-} (but not CD8^{-/-}) protected the septic mouse spleen from increasing apoptosis. Thus, although truncating Fas-FasL signaling ameliorates many untoward effects of sepsis, the pathological mode of action is distinct at the tissue level. (*Am J Pathol* 2007, 171:87–96; DOI: 10.2353/ajpath.2007.061099)

Sepsis is a major cause of morbidity and mortality in patients in the intensive care unit, with approximately a third of the 750,000 annually reported cases resulting in death.¹ At the present time, treatment is supportive, and because most molecular-based treatments have failed clinically, there is an urgent need for a better understanding of the pathology of sepsis and its resultant organ failure.

With respect to organ damage and mortality associated with sepsis in mouse models, our laboratory and others have reported that it is at least in part attributable to the activation of the Fas-FasL signaling pathway and not TLR4.² Studies that have blocked Fas signaling, including Fas fusion protein (FasFP)^{3,4} and FasL^{-/-} mice,² have shown a reduction of liver damage and improved survival after sepsis. Most recently, we used Fas and caspase 8 short interfering RNA (siRNA) *in vivo* after sepsis. Both of these, given as a hydrodynamic, posttreatment bolus injection after cecal ligation and puncture (CLP), decreased apoptosis in the liver and the spleen, decreased indicators of liver damage, and reduced mortality after sepsis by 50%.⁵ Although in these studies we observed that the suppression of Fas and caspase 8 signal was maintained in the whole liver and spleen up until day 10 after injection, however, the mode of action of this siRNA treatment remains unknown at a cellular level of action. Clearly, any cell that takes up Fas siRNA or is affected by it, even after CLP, would be considered a potential target and therefore may be involved in septic morbidity. Sepsis induces extensive apoptosis of lymphocytes, and this has been suggested to contribute to immu-

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nosuppression and mortality.^{2,5-14} To elucidate further the mechanism by which the Fas pathway contributes to the pathology of sepsis, we sought to determine what immune and nonimmune cell types express increased Fas death receptor after CLP, therefore, serving as potential targets of Fas siRNA administration. By the same token, we also sought to establish cell types that express FasL and their contribution to Fas-FasL signaling in the liver and spleen after sepsis. Here, we report that in accordance with previous work, Fas is up-regulated in the liver and the spleen after sepsis, particularly in hepatocytes and lymphocytes, respectively. Last, we demonstrate that Fas ligand-expressing CD8⁺ T cells contribute to apoptosis and proinflammation in the liver but not the spleen after sepsis.

Materials and Methods

Animals

Male C57BL/6 mice, 6 to 8 weeks of age, were used as wild-type controls for all experiments. For adoptive transfer studies, male RAG1^{-/-} mice (B6.129S7-Rag1^{tm/Mom}/J) as well as male severe combined immunodeficient (SCID) mice (B6.CB17-Prkdc^{scid}/SzJ) were used as recipients. Donors for adoptive transfer experiments included female green fluorescent protein (GFP) transgenic C57BL/6-TgN (ACTbEGFP)/Osb and male FasL^{-/-} mice (B6.Smn.C3-FasL^{gld}/J). This strain was also used for apoptosis studies as well as CD8^{-/-} (B6.129S2-Cd8a^{tm/Mak}/J). All mice were purchased from Jackson Laboratories, Bar Harbor, ME. The studies described here were performed in accordance with the National Institutes of Health and The Guide for the Care and Use of Laboratory Animals, and were approved by the Brown University and Rhode Island Hospital institutional animal care and use committees.

Model of Sepsis

The surgical procedure to generate sepsis was performed as previously described.¹⁵ C57BL/6 male mice were lightly anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL). The abdomen was shaved and scrubbed with povidone-iodine (Betadine; Alcon Laboratories, Fort Worth, TX). A midline incision (1.5 to 2 cm) was made below the diaphragm. The cecum was isolated, ligated, punctured twice with a 22-gauge needle, and was gently compressed to extrude a small amount of cecal material. The cecum was returned to the abdomen, and the muscle and skin incisions were closed with 6-0 Ethilon suture material (Ethicon, Inc., Somerville, NJ). Before suturing the skin 2 to 3 drops of lidocaine (Abbott Laboratories) was administered to the wound for analgesia. The mice were subsequently resuscitated with 1.0 ml of lactated Ringer's solution subcutaneously. Sham controls were subjected to the same surgical laparotomy and cecal isolation, but the cecum was neither ligated nor punctured.

In Vivo Delivery of siRNA

Fas siRNA was obtained from Dharmacon RNA Technologies (Lafayette, CO). The target sequences used for Fas is as follows: 5'-AAGUGCAAGUGCAAACCAGAC-3'.¹⁶ For the adoptive transfer studies, a group of SCID and RAG1^{-/-} mice (four to five mice per group per strain) received 50 μ g of naked Fas siRNA/mouse 24 hours before adoptive transfer of CD8⁺ T cells. Typically, 50 μ g of siRNA was dissolved in 1.5 ml of phosphate-buffered saline (PBS) and was then injected rapidly (throughout a period of 5 seconds) into the tail vein. In apoptosis studies, mice received 50 μ g of Fas siRNA 30 minutes after CLP as described previously.⁵

Cell Isolation

Briefly, liver nonparenchymal cells (NPCs) were isolated via perfusion of the liver with 0.05% collagenase and 5% fetal calf serum in Hanks' balanced salt solution.¹⁷ The liver was then dissected and broken up, and hepatocytes were removed via slow centrifugation of 57 $\times g$. Cells in the supernatant were then centrifuged and separated on a 30% Histodenz gradient (weight by volume) (Sigma, St. Louis, MO). NPCs in the top layer were then washed and used for flow cytometry phenotyping or further cell isolations.

CD8⁺ T cells used for adoptive transfer experiments were isolated using MACS columns (Miltenyi Biotech, Auburn, CA). Typically, just under 10⁷ cells were resuspended in degassed running buffer consisting of 0.5% bovine serum albumin and 2 mmol/L ethylenediaminetetraacetic acid in PBS. Biotin-antibody cocktail, including 1 μ g of biotin-conjugated CD31 monoclonal antibody (BD Pharmingen, San Diego, CA)/10⁶ cells and anti-biotin microbeads (Miltenyi Biotech) were added to remove non-CD8⁺ T cells. The suspension was run through a MACS column (Miltenyi Biotech), and the purified CD8⁺ T-cell population was collected. This population was determined to be 95% pure by flow cytometry staining for CD8 on a BD FACSArray (BD Biosciences, San Jose, CA). This population was also counted after sham, CLP, and CLP/Fas siRNA procedures to determine the number of CD8⁺ T cells in the liver.

Viable hepatocyte populations were isolated using the two-step perfusion method as previously described.¹⁸ In brief, mice were anesthetized and subjected to laparotomy. The hepatic portal vein was cannulated, and the liver was perfused for 4 minutes with warm Hanks' balanced salt solution containing sodium bicarbonate, ethylenediaminetetraacetic acid, and HEPES using a peristaltic pump (Masterflex; Cole-Palmer, Vernon Hills, IL). The liver was then perfused with warm modified L-15 medium containing glucose and collagenase IV for 15 minutes. The liver was then gently broken up in the collagenase solution and filtered through a 100- μ m cell strainer with RPMI 1640 medium + 5% fetal calf serum and centrifuged at 30 $\times g$ for 4 minutes. The hepatocytes were then resuspended, viability assessed by trypan blue exclusion, and were subsequently cultured in HEPES-buffered RPMI 1640 medium containing insulin, sodium pyruvate, and 10% fetal calf serum.

Adoptive Transfer

To determine the effects of CD8⁺ T cells from septic and nonseptic donor mice, CD8⁺ T cells were isolated from the liver as described above and adoptively transferred into SCID or RAG1^{-/-} mouse recipients, livers of which were subsequently examined for pathological changes. However, before these studies, we initially attempted to establish the nature of the homing of these isolated cells to certain tissues. To do this, 1.0×10^6 CD8⁺ T cells from the liver of GFP transgenic mice were isolated and injected via tail vein into C57BL/6 recipients. Twenty-four hours after injection, the liver, thymus, spleen, heart, and lung were harvested from the recipients and flash-frozen in OCT. Frozen tissues were sectioned on a cryostat (Leica, Wetzlar, Germany), stained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and visualized using an Eclipse E400 fluorescence microscope (Nikon, Tokyo, Japan) to assess the presence of GFP cells. The presence of CD8⁺ T cells was measured as the index of the number of GFP cells versus the total number of 4,6-diamidino-2-phenylindole/nuclear-stained cells in a given field.

To assess the functional capacity of CD8⁺ T cells to injure/damage and/or inflame the liver, male C57BL/6 mice underwent sham or CLP, and a group of FasL^{-/-} mice underwent CLP. At this time, one group of SCID or RAG1^{-/-} recipients received a pretreatment of 50 μ g of Fas siRNA hydrodynamically. Twenty-four hours later, mice were sacrificed, livers harvested, and CD8⁺ T cells isolated as described above. CD8⁺ T cells (1.0×10^6) were then transferred to SCID and RAG1^{-/-} recipients via tail vein injection. Twenty-four hours later, SCID and RAG1^{-/-} recipients were sacrificed, and the liver and blood/plasma harvested for subsequent assays and histology. Histological changes in these sections were determined by a pathologist who was blinded to the treatment identity of the samples.

Electron Microscopy

For electron microscopic examination, the livers of the RAG1^{-/-} recipient mice were first perfused with 0.15 mol/L sodium cacodylate buffer for 2 minutes at a rate of 3 ml/minute using a peristaltic pump (Cole-Palmer Masterflex). The liver was subsequently perfusion-fixed with 2.5% glutaraldehyde in 0.15 mol/L sodium cacodylate buffer for 10 minutes, also at a rate of 3 ml/minute. The tissue was excised and immediately submersed in fixative at 4°C and prepared into 1-mm³ blocks. After overnight fixation, the tissue was washed with buffer and postfixed in 1% OsO₄ (osmium tetroxide) in 0.15 mol/L sodium cacodylate buffer for 1 hour at 4°C. After fixation, samples were rinsed in buffer and dehydrated in a graded acetone series and embedded in Spurr's epoxy resin. Semithin sections (1 μ m) were prepared using a Reichert Ultracut S microtome (Leica) then stained with methylene blue-azure II and evaluated for areas of interest. Ultrathin sections (50 to 60 nm) were prepared, retrieved onto 300-mesh copper grids, and contrasted with

uranyl acetate and lead citrate. Sections were examined using a Morgagni 268 transmission electron microscope, and images were collected with an AMT Advantage 542 charge-coupled device camera system (FEI Company, Hillsboro, OR).

Assessment of Interleukin (IL)-6, Mig, and IP-10

For adoptive transfer studies, the plasma of recipient mice was harvested 24 hours after injection to analyze circulating levels of IL-6 via a sandwich method enzyme-linked immunosorbent assay kit (BD Pharmingen), performed according to the manufacturer's instructions ($n = 4$ to 5 per group). Likewise, septic mouse liver Mig and IP-10 levels were also determined by Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN).

Western Blot

Protein lysates of mouse liver NPCs and spleen were run on 12% Tris-glycine gels (Invitrogen, Carlsbad, CA). Blotting procedures, chemiluminescent detection, and densitometric analysis were performed as previously described by our laboratory.¹⁹ Membranes were probed with Fas ligand rabbit anti-rat polyclonal antibody (Chemicon, Temecula, CA) and bands were detected at 32 and 40 kd.

Assessment of Apoptosis

For annexin V staining, 1.0×10^6 cells were washed twice with cold PBS and resuspended in 1 $\times$ annexin binding buffer (Abcam, Cambridge, MA). Cells (1.0×10^5) were then stained with annexin V-PE (BD Pharmingen) and 7-aminoactinomycin D (Serotec, Raleigh, NC) and incubated for 15 minutes at room temperature in the dark. Cells were then analyzed by flow cytometry.

For anti-active caspase 3 staining, paraffin slides underwent epitope retrieval using citrate buffer and serum-blocked using a 2% fetal calf serum solution. Sections were incubated with anti-active caspase 3 antibody (BD Pharmingen) for 1 hour and then blocked with a peroxidase-blocking solution. Sections were then incubated with anti-rabbit horseradish peroxidase secondary antibody. After washing slides were incubated with diaminobenzidine substrate solution and counterstained with Mayer's hematoxylin.

Caspase 3 Activity Assay

As described previously by Chung and colleagues,²⁰ livers from C57BL/6 and CD8^{-/-} septic mice as well CD8^{-/-} mice posttreated with Fas siRNA were homogenized in the presence of lysis buffer containing dithiothreitol. Reaction buffer ($\times 2$) containing dithiothreitol and AFC-DEVD (caspase 3) (Biomol, Plymouth Meeting, PA) was added to 400 μ g of liver lysate. After a 1-hour incubation at 37°C, samples were read on a fluorescent plate

reader (FLx800; Bio-Tek Inc., Winooski, VT) at excitation 400 nm and emission 505 nm, and the extent of AFC release was reported in arbitrary fluorescent units.

Assessment of Cytotoxicity ex Vivo

Hepatocytes from naïve, sham, 4-hour CLP, as well as Fas siRNA 24-hour pretreated 4-hour CLP C57BL/6 mice were isolated as described. Hepatocytes were plated at a concentration of 2.0×10^4 /well in a 96-well plate. CD8⁺ T cells were added as effector cells at ratios of 1:1, 1:2, 1:5, and 1:10. Hepatocytes were also plated alone (as background control) and with 1% Triton X-100 (as a maximum release control). Cultures were left overnight and were subsequently analyzed to establish the percent cytotoxicity as measured by release of lactate dehydrogenase (LDH). LDH release was assessed as described in the manufacturer's instructions (BioVision, Mountain View, CA). Percent cytotoxicity was calculated as: (test sample – background)/(maximum release – background) $\times$ 100.

Flow Cytometry

Sham, CLP, and CLP/siRNA mice treated via hydrodynamic delivery, as well as naïve control mice were euthanized at 24 hours. The liver was perfused and NPCs isolated as described above. Kupffer cells were enriched by adherence on plastic. Nonadherent cells were obtained from the wash eluent, concentrated/washed by centrifugation, viability established by trypan blue exclusion, resuspended in PBS at 1.0×10^6 cells/ml, incubated with a nonspecific Fc blocker (BD Pharmingen), and stained for CD4, CD8, and Fas (BD Pharmingen) as previously described in our laboratory.²¹ The spleen was also harvested and the splenocytes processed in a comparable manner for staining for CD4 and CD8 expression. All cells were read (5000 events) on a BD FACSArya (BD Biosciences).

Plasma Liver Enzyme Levels

Blood from recipient mice treated with sham, wild-type CLP, or FasL^{-/-} CLP CD8⁺ T cells was collected 24 hours prior in a syringe containing 2 U of heparin, transferred to a microtube, and centrifuged at $10,000 \times g$ for 10 minutes at 4°C. Plasma samples were stored at –80°C until assayed. Plasma aspartate aminotransferase and alanine aminotransferase levels were determined using a kit (Biotron Diagnostics, Hemet, CA), according to the manufacturer's instructions.

Statistics

The data are presented as a mean and SE of the mean for each group. Differences in percentile (ie, apoptotic index percentage, Fas expression) data (following standard data transformation) and changes in IL-6 levels were considered to be significant at $P < 0.05$, as determined

Figure 1. A: Percentage of CD4⁺ or CD8⁺ T cells found in the liver and the spleen after CLP and CLP with Fas siRNA treatment. Black bars represent CD4⁺ T cells, and lined bars represent CD8⁺ T cells. B: Top: Extrapolated numbers of CD4⁺ and CD8⁺ cells from the spleens of CLP and CLP Fas siRNA-treated mice. Bottom: Numbers of CD8⁺ T cells isolated from the liver after CLP and CLP with Fas siRNA treatment. * $P < 0.05$ versus sham and CLP Fas siRNA, one-way analysis of variance followed by Student-Newman-Keuls multiple comparison, $n = 4$ per group.

using the one-way analysis of variance. Differences between multiple groups were established using Tukey's test or the Student-Newman-Keuls multiple comparison.

Results

T-Cell Populations Increase in the Liver after CLP

Although we have previously shown that *in vivo* Fas siRNA administration maintains suppression of Fas protein in the liver and spleen up to 10 days, while concomitantly improving survival after CLP, it was not clear which cell types were in fact the target of this treatment.⁵ This information is not only critical to understanding the nature of the siRNA's therapeutic effect but also should point to critical pathological events in the development of organ injury in this model of sepsis.

Previous studies have shown that T lymphocytes, particularly CD4⁺, CD8⁺, as well as B220⁺ B cells were lost in the spleen after CLP.^{2,5–14} In accordance with this, we have found these cell types were indeed lost in the spleen after CLP (Figure 1A). With Fas siRNA treatment, however, these populations were rescued after CLP. In the liver, on the other hand, there seemed to be an increase in the percentage of CD4⁺ and CD8⁺ cells after CLP that was diminished to sham levels after Fas siRNA treatment (Figure 1A). Numbers of these cells followed the same trend, indicating the change in percentage was not attributable to influx or loss of other cell types in the population. The number of CD4⁺ or CD8⁺ cells from the spleen extrapolated from the flow cytometry experiments is shown in Figure 1B, top graph. This was also supported by the observation that there was a twofold increase in the total number of CD8⁺ T cells isolated from the liver after CLP using Miltenyi columns. This was also decreased after Fas siRNA treatment (Figure 1B, bottom graph). Of note, the influx of liver T cells was not associated with a change in Mig or IP-10 levels because no

difference was seen in septic or sham mouse liver expression (data not shown).

FasL Is Up-Regulated on Liver NPCs but Not Splenocytes

Previously, we have shown that Fas receptor is up-regulated in the liver and the spleen after CLP.⁵ With respect to FasL, we analyzed expression in the liver NPC fraction as well as in the spleen via Western blot. Although FasL increased on liver NPCs after CLP, it did not seem to change in the spleen (Figure 2A). To elucidate further what cell type in the liver NPC fraction was expressing FasL, CD8⁺ T cells were isolated, and as shown in Figure 2B, CD8⁺ T cells were found to express more FasL after CLP when compared with sham. Densitometric representation of these changes in FasL is shown in Figure 2C.

CD8⁺ T Cells Adoptively Transferred from Septic Mice to Naïve Immunodeficient Mice Induced Liver Damage and Inflammation

Because we found that CD8⁺ T cells appeared to increase by almost twofold in the liver after CLP and were expressing increased FasL, we speculated, based on the work of Sherwood and colleagues,^{22,23} that these cells might have the capacity to promote localized tissue injury/inflammation and/or damage to the liver. In an attempt to test this hypothesis, we established an adoptive transfer system that would allow us to determine the effect of CD8⁺ T cells from septic and nonseptic mice in immunodeficient recipients (SCID and RAG1^{-/-}; the absence of functional recipient lymphoid cells should allow assessment of donor lymphocyte effects).

However, before transferring cells from sham and/or CLP mice of differing backgrounds we first felt it was important to establish the extent to which isolated liver CD8⁺ T cells actually homed back to the liver. We observed that fluorescent CD8⁺ T cells isolated from the liver of transgenic GFP-overexpressing donor mice primarily seemed to home back to the liver of the recipient and, to a lesser extent, the thymus (Figure 3, A and B). Alternatively, as expected, no evidence of these transferred cells was seen in the lung, heart, or spleen (taken as negative controls).

In the functional adoptive transfer study, donor CD8⁺ T cells from wild-type mice that had undergone CLP seemed to cause extensive vacuolization and morphological changes in the SCID or RAG1^{-/-} recipient liver (Figure 3C). Looking at these sections via electron microscopy (Figure 3D), there seems to be phagocytic or endocytic vacuoles on the periphery of the hepatocytes in the recipient mice that received only wild-type septic CD8⁺ T cells. This type of change was not seen to this extent in either the sham or FasL^{-/-} CLP groups. To the extent that this was not a result of a tissue damage/necrotic injury, we observed no marked changes in aspartate aminotransferase and/or

Figure 2. A: Expression of Fas ligand in liver NPCs and splenocytes after CLP. B: Expression of Fas ligand on CD8⁺ T cells isolated from the liver after CLP. Western blots representative of three separate trials. In both blots, read across the top from CLP, - indicates sham, and + indicates CLP. C: Densitometry of FasL Western blots A (top graph) and B (bottom graph).

alanine aminotransferase in the serum of SCID and RAG1^{-/-} recipients. This suggests that the adoptively transferred cells were not initiating substantial or significant evidence of necrosis in this system (Figure 4A). There was, however, a significant increase in serum IL-6 that was not seen in the animals that received a pretreatment of Fas siRNA, or the animals that received FasL^{-/-} CD8⁺ T cells (Figure 4B). To determine whether the adoptively transferred CD8⁺ T cells were having an apoptotic effect in the livers of the recipients, liver sections were stained for active caspase 3. The

Figure 3. **A:** Index of GFP CD8⁺ donor T cells in tissues of wild-type C57BL/6 recipients per 100 cells per high-powered field (HPF). ND, not detectable. **B:** Representative fluorescent micrograph of GFP CD8⁺ T cells in the liver of wild-type C57BL/6 recipient. **C:** Liver sections of SCID and RAG1^{-/-} recipient mice after adoptive transfer of CD8⁺ T cells from septic wild-type C57BL/6 or C57BL/6-gld (FasL^{-/-}) mice. **D:** Electron microscopy of RAG1^{-/-} recipient mice as correlated in **C**.

RAG1^{-/-} recipient mice that received CD8⁺ T cells from wild-type sham mice and CD8⁺ T cells from septic FasL^{-/-} mice exhibited no caspase 3 staining. The RAG1^{-/-} groups (no pretreatment and Fas siRNA pretreated) that received CD8⁺ T cells from wild-type septic mice also did not have a significant change in active caspase 3 in the liver.

Figure 4. **A:** Serum levels of liver enzymes alanine aminotransferase and aspartate aminotransferase in SCID (black bars) and RAG1^{-/-} (open bars) recipient mice after adoptive transfer of CD8⁺ T cells from septic wild-type C57BL/6 (CLP) or C57BL/6-gld (FasL^{-/-} CLP) mice. One group of recipient mice received a pretreatment of Fas siRNA before adoptive transfer of CD8⁺ T cells from septic wild-type C57BL/6 (F/CLP). **B:** Serum levels of IL-6 in SCID (black bars) and RAG1^{-/-} (open bars) after adoptive transfer as described in **A**. **P* < 0.05, Tukey test, *n* = 4 to 5 per group.

CD8⁺ T Cells Contribute to Apoptosis and Inflammation after CLP in the Liver

Because the donor environment of the adoptive transfer system may not fully represent/recapitulate a septic environment, apoptosis and inflammation were also compared in wild-type septic mice and septic mice that lack CD8⁺ T cells. Unlike the adoptive transfer study, active caspase 3 staining was much more evident in the wild-type septic animals versus the sham (Figure 5, B and A, respectively). This was present to a much lesser extent in septic CD8^{-/-} mice (Figure 5C). These results were repeated measuring active caspase 3 fluorometrically (Figure 5D). Although the septic CD8^{-/-} group showed a decrease in active caspase 3 compared with the septic wild-type animals, the septic CD8^{-/-} group of animals posttreated with Fas siRNA did not show a further reduction in active caspase 3, suggesting that another cell type may be expressing FasL in this process. Similar to what was seen in the adoptive transfer experiments, IL-6 levels were significantly decreased in septic CD8^{-/-} mice as compared with septic C57BL/6 mice (Figure 5E), whereas liver enzyme levels did not change (data not shown).

Although these data suggest that CD8⁺ T cells have an inflammatory or cytotoxic effect, it does not document whether this is a direct or indirect effect. To assess this, we established an *ex vivo/in vitro* co-culture assay in which we could examine the capacity of our CD8⁺ T cells to produce a cytotoxic effect/LDH release from primary

Figure 5. **A:** Active caspase 3 staining in liver of C57BL/6 mouse 24 hours after sham procedure. **B:** Active caspase 3 staining in liver of C57BL/6 mouse 24 hours after CLP. **C:** Active caspase 3 staining in liver of CD8^{-/-} (B6.129S2-Cd8a^{tm/Mak/J}) mouse 24 hours after CLP. **D:** Caspase 3 activity in liver of septic wild-type and septic CD8^{-/-} mice. **P* < 0.05 versus sham and both CLP CD8^{-/-} groups, Tukey test, *n* = 4 per group. **E:** Serum levels of IL-6 in wild-type sham and CLP mice as well as CD8^{-/-} CLP mice. **P* < 0.05 versus sham and CLP CD8^{-/-} groups, Tukey test, *n* = 3 to 4 per group.

hepatocyte culture isolates. In this *ex vivo* setting, we found that CD8⁺ T cells from wild-type septic mice promoted more cytotoxicity in 4-hour CLP-primed hepatocytes than septic CD8⁺ T cells lacking FasL (Figure 6). Four-hour CLP-primed hepatocytes from animals that received a 24-hour pretreatment of Fas siRNA were even less likely to be vulnerable to cytotoxicity, especially when co-cultured with FasL^{-/-} septic CD8⁺ T cells. This suggests that target cells must be primed to express an antigen (eg, Fas) recognized by wild-type (FasL-expressing) CD8⁺ T cells for optimal cytolytic activity.

CD8⁺ T Cells Do Not Contribute to Splenocyte Apoptosis after CLP

Finally, in contrast to the liver, it seems that CD8⁺ T cells are not necessary for splenocyte apoptosis because there was little change in the amount of splenocyte apoptosis seen in CD8^{-/-} mice versus wild-type mice after

CLP (Figure 7). FasL^{-/-} mice, however, exhibited significantly less splenocyte apoptosis, reaffirming the notion that Fas-FasL signaling contributes to apoptosis in the spleen after sepsis.

Discussion

These studies have demonstrated that CD8⁺ T cells, which express increased FasL and appear increased in number by twofold in the liver after the induction of polymicrobial sepsis, play a role in liver injury and inflammation. Further, although in the spleen there is a loss of lymphocytes because of apoptosis,^{6,9,10,15,24} the liver seems to have an influx of T cells, particularly CD4⁺ and CD8⁺. There are two main hypotheses at this point in time for accumulation of CD8⁺ T cells in the liver. In a study by Dong and colleagues,²⁵ it is suggested that there is a role for B7-H1 in the deletion (apoptosis) of antigen-activated CD8⁺ T cells in the liver coinciding with the "graveyard hypothesis." This graveyard hypothesis attempts to explain how activated T cells that are already undergoing apoptosis in the circulation (ie, already moribund) accumulate in the liver. This may or may not fit this scenario

Figure 6. Percent cytotoxicity as measured by LDH release from C57BL/6 naïve, sham, CLP, or Fas siRNA-pretreated CLP hepatocytes co-cultured with C57BL/6 sham, CLP, or CLP C57BL/6-gld (FasL^{-/-}) CD8⁺ T cells at a target/effector ratio of 1:5. **P* < 0.05 versus sham and CLP BL6-gld; #*P* = 0.001 versus sham, one-way analysis of variance followed by Student-Newman-Keuls multiple comparison, *n* = 4 to 5 per group.

Figure 7. Apoptosis of splenocytes after CLP in wild-type, CD8^{-/-}, and FasL^{-/-} mice. **P* < 0.05 versus CLP-FasL^{-/-} groups, Tukey test, *n* = 3 to 4 per group.

because earlier phenotyping experiments we have done using flow cytometry and annexin V staining have suggested that these cells are not going to the liver to die because at our 24-hour time point, they do not seem to be apoptotic. On the other hand, suppression of a death receptor (such as Fas siRNA) resulting in a decreased accumulation of T cells in the liver (as we show in Figure 1) does seem to be reminiscent of this graveyard hypothesis. However, liver chemokine expression studies that we have done for Mig and IP-10 do not show an increase, or any change for that matter, in these chemokines that would necessarily call these CD8⁺ T cells to the liver, either. One hypothesis by van Griensven and colleagues²⁶ is that these cells may actually be trapped, or sequestered, in the liver because of increased expression of ICAM-1 after CLP. ICAM-1^{-/-} mice exhibit decreased organ damage and a decrease in the invasion of lymphocyte subpopulations including CD4⁺ and CD8⁺. This study fits in with the "killing field hypothesis" suggested by Crispe and colleagues,²⁷ which proposes that the liver is a trap for activated T cells in the liver for subsequent killing. It is uncertain at this time why or how CD8⁺ T cells are accumulating in the septic liver because more studies need to be done to answer this question.

Irrespective of how the CD4⁺ and CD8⁺ cell numbers were increased, the increase in number was associated with liver damage and apoptosis.⁵ To determine whether the increase in CD8⁺ T cells had a detrimental effect on the liver, we used an adoptive transfer system to measure the effect of septic CD8⁺ T cells on the liver. To do this, immunodeficient SCID and RAG1^{-/-} mice were used, both of which lack functional B and T cells. Two different strains were used because SCID mice are common recipients for adoptive transfer but are sometimes considered leaky in that they can spontaneously develop partial immune reactivity, especially on the C57BL/6 background as is used here.²⁸ Therefore, RAG1^{-/-} were also used because they are considered a nonleaky SCID. In an effort to address the local tissue/cell Fas expression, we also pretreated a group of the immunodeficient mice with 50 μ g of Fas siRNA injected hydrodynamically 24 hours before adoptive transfer. This time frame was chosen because we have previously shown that Fas siRNA given hydrodynamically is taken up in the liver and can maintain suppression of Fas expression from 1 day and up to 10 days after injection.⁵ Recipients then either received liver CD8⁺ T cells from C57BL/6 donor mice that had undergone sham or CLP, or from FasL^{-/-} donor mice that had undergone CLP. General histological examination of the recipient livers 24 hours after adoptive transfer showed normal liver structure and sinusoids in the recipients receiving sham donor CD8⁺ T cells. Those animals receiving wild-type septic donor CD8⁺ T cells seemed to have more liver injury, including vacuolization. Electron microscopy revealed these vacuoles to be of phagocytic or endocytic nature.^{29,30} The mechanism of this vacuolization is not known at this time, except that it does seem to be Fas-related because the livers of the mice that received donor FasL^{-/-} CD8⁺ T cells exhibited larger areas of essentially normal liver structure but had

only minor evidence of vacuolization. This suggests that septic donor CD8⁺ T cells expressing FasL seem to contribute to aspects of the injury seen in the immunodeficient recipient livers. However, the absence of active caspase 3 staining suggests that this is not attributable to the induction of apoptosis per se. The changes in IL-6 levels, however, suggest that these FasL-expressing cells also initiate aspects of inflammation because this proinflammatory response was not seen in the animals receiving donor FasL^{-/-} CD8⁺ T cells. Ligation of Fas, as well as activation of FADD or caspase 8, has been shown to lead to downstream activation of NF- κ B and transcription of proinflammatory cytokines.^{31,32} The liver sections of our recipient mice also showed clusters of proinflammatory cells, most likely neutrophils, in the groups that received wild-type septic donor CD8⁺ T cells. Normally, acute inflammation is characterized by vasodilatation, fluid exudation, and neutrophil infiltration. Although, from a response-to-infection standpoint, acute inflammation is beneficial, severe inflammation can lead to the systemic inflammatory response syndrome. This syndrome is characterized by hyperinflammation and can result in multiorgan injury, shock, and even death.³³ These processes of inflammatory IL-6 release and neutrophil influx have also both been observed as components of the liver's response to polymicrobial sepsis seen in rodents subjected to CLP.^{34–36}

Interestingly, the events observed in the liver were not consistent with those occurring in the spleen. In accordance with previous studies,^{2,5–14} we also show here a loss of T cells, particularly CD4⁺ and CD8⁺ lineage, after CLP is most likely via apoptosis. Even though Fas receptor is increased in the spleen after CLP,⁵ we and others have shown there is little overt change in FasL,^{37,38} suggesting that increased apoptosis in the spleen seen after sepsis is attributable to increased Fas only. In some cases, particularly in the liver, it has been reported that the Fas receptor can self-aggregate to autoinduce apoptosis.^{39,40} However, we believe that this is probably not the case in the spleen because FasL^{-/-} mice exhibited a significant reduction in apoptosis after CLP, implying that this is a death ligand-driven process. This, as well as the observation that Fas siRNA treatment after CLP decreases splenocyte apoptosis and improves survival,⁵ reaffirms the role of Fas-FasL signaling in onset of splenocyte apoptosis after sepsis. In this respect it has been reported that in septic patients, in which leukocytes have been shown to exhibit an increase in Fas receptor expression, this state of higher Fas expression has been correlated with an increased rate of mortality.⁴¹ A similar phenomenon for FasL has also been recently observed in the bacteremic baboon's spleen.⁴² This evidence coincides with the notion that during the immune hyporesponsive stage of sepsis the direct loss of functional immune cells via apoptosis contributes to the animal's inability to defend itself against the lethal polymicrobial challenge.⁹ Because we were not able to see a change in apoptosis in the septic CD8^{-/-} mouse spleens versus the septic wild-type spleens, but a significant decrease in the septic FasL^{-/-} spleens, this suggests that another cell type is expressing FasL and may be responsible for inducing

some of the apoptosis normally seen. As has been mentioned previously, survival studies done by Sherwood and colleagues^{22,23} show that CD8-deficient mice have a potentiation of survival when further depleted of natural killer (NK) cells using anti-asialo GM1 antibody. Many studies have looked at the TRAIL-mediated⁴³ and FasL-mediated cytotoxicity of NK cells in the liver.^{44,45} In the spleen, however, it seems invariant NKT cells are capable of expressing FasL and eliciting apoptosis of Fas-expressing lymphocytes,^{46,47} acting either as sentinels ready to kill or assisting in tolerance induction by promoting apoptosis of lymphocytes via Fas/FasL.

In summary, the studies presented here support the possibility of a detrimental role for FasL-expressing CD8⁺ T cells in liver injury, particularly via activation of local tissue inflammation, possible endocytic processes by hepatocytes, as well as apoptotic signaling after CLP. However, the studies with donor FasL^{-/-} mouse cells adoptively transferred to SCID/RAG1^{-/-} mice suggest that there may be another FasL-expressing cell, potentially NK, that also contributes to apoptosis in the liver. Alternatively, our observation in the spleen indicates that unlike the T lymphocytes of the liver, the Fas/FasL-mediated signaling that occurs here seems to be a result of increased death receptor expression and is not a consequence of overt induction of FasL expression on effector T cells. Together, these findings indicate that although many of the deleterious events seen in this septic model can be ameliorated by truncating Fas-FasL signaling,³⁻⁵ the mode of sepsis-induced death receptor-driven immune/organ dysfunction/injury is as diverse as the target organs being examined. Future studies should include determining whether CD8⁺ T cells are antigen activated, which thus may point to a better understanding of CD8⁺ T cells accumulation in the liver. In addition, studies should be conducted to determine whether CD8⁺ T cells and NK or NKT cells are acting in synergy to promote injury in the liver and apoptosis in the spleen. It is, thus, our hope that future studies will not only help elucidate the mechanism by which CD8⁺ T-cell activation and/or immigration to the liver, as well as the resultant target cell changes in Fas signaling, contribute to liver as well as splenic injury but allows to develop better therapeutic approaches to migrating the development of multiple organ failure resultant from sepsis.

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