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Splenectomy Protects against Sepsis Lethality and Reduces Serum HMGB1 Levels

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High mobility group box 1 (HMGB1) is a critical mediator of lethal sepsis. Previously, we showed that apoptotic cells can activate macrophages to release HMGB1. During sepsis, apoptosis occurs primarily in lymphoid organs, including the spleen and thymus. Currently, it is unclear whether this accelerated lymphoid organ apoptosis contributes to systemic release of HMGB1 in sepsis.

In this study, we report that splenectomy significantly reduces systemic HMGB1 release and improves survival in mice with polymicrobial sepsis. Treatment with a broad-spectrum caspase inhibitor reduces systemic lymphocyte apoptosis, suppresses circulating HMGB1 concentrations, and improves survival during polymicrobial sepsis, but fails to protect septic mice following splenectomy. These findings indicate that apoptosis in the spleen is essential to the pathogenesis of HMGB1-mediated sepsis lethality. The Journal of Immunology, 2008, 181: 3535–3539.

S evere sepsis is the leading cause of death in intensive care units and accounts for 9.3% of deaths in the United States annually (1). High mobility group box 1 (HMGB1) is an intracellular DNA-binding protein that is a mediator in lethal sepsis (2–6). HMGB1 is released into the extracellular milieu during sepsis, and administration of recombinant HMGB1 to mice recapitulates many pathological signs of sepsis, including fever, intestinal barrier dysfunction, and tissue injury (2–3, 7–10). Neutralizing Abs directed against HMGB1 reverse the course of established sepsis (3–4, 6).

We recently showed that apoptotic cells can activate macrophages to release HMGB1 (11). Administration of a broad-spectrum caspase inhibitor to mice with polymicrobial sepsis significantly reduces serum HMGB1 concentrations and suppresses apoptosis in the thymus and spleen (11). Monoclonal neutralizing Abs against HMGB1 confer significant protection against organ damage, but do not prevent apoptosis, indicating that HMGB1 release is downstream of apoptosis on a common pathway to lethal organ damage in sepsis (11).

Little is known regarding the systemic regulation of HMGB1 release during lethal sepsis. However, it has been established that peripheral lymphoid organs, such as the thymus and spleen, are sites of extensive apoptosis in sepsis (12–15). Accordingly, we reasoned that regulation of apoptosis-induced systemic HMGB1 release might occur there. We therefore studied mice with lethal polymicrobial sepsis, and found that caspase-dependent apoptosis in the spleen is a specific and critical determinant of HMGB1 release, organ damage, and lethality.

Materials and Methods

Recombinant mouse TNF was obtained from R&D Systems. Tryptic soy agar was purchased from BD Biosciences. Neutral formalin solution (10%) was obtained from Sigma-Aldrich. Poly-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) and control peptide benzoxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA-FMK) were obtained from BD Biosciences. Anti-caspase 3 Abs (Cat. no. AF835) were from R&D Systems.

Animal experiments

Male 6–8 wk old BALB/c mice were purchased from Taconic Farms and allowed to acclimate for 7 days before experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Mice were housed in the animal facility of the Feinstein Institute for Medical Research under standard temperature, light, and dark cycles.

Cecal ligation and puncture (CLP)

Mice were subjected to CLP surgery as described previously (16). Following anesthesia with an i.m. injection of ketamine (75 mg/kg, Fort Dodge) and xylazine (20 mg/kg, Boehringer Ingelheim), a 15 mm midline abdominal incision was made to expose the cecum. After ligation at 5.0 mm from the cecal tip, the cecal stump was punctured once with a 22-gauge needle, and a small amount of stool (1 mm in length) was extruded. The cecum was returned to the abdominal cavity, and the incision was closed with running 6–0 prolene suture. All animals were administered normal saline resuscitation (20 ml/kg of body weight, injected subcutaneously), and a single dose of antibiotics (Primaxin, 0.5 mg/mouse in 200 μl sterile saline, injected subcutaneously, Merck) 15 min after surgery. Survival was monitored for 3 wk. To determine levels of HMGB1 and cytokines in the serum, parallel experiments were conducted and mice were euthanized 24 or 44 h after CLP to collect blood and tissues (livers, kidneys, hearts, small intestine, thymus, and spleens). In some experiments, septic mice were given caspase inhibitor (Z-VAD-FMK) or control peptide (Z-FA-FMK, 0.5 mg/mouse, i.p.) 90 min and 12 h after CLP. Animals were euthanized 24 h after CLP; spleen and thymus were collected for analyses of apoptosis. In survival experiments, caspase inhibitor or control peptide was administered 90
min after CLP and/or splenectomy surgeries, and then every 12 h for a total of 3 days. Survival was monitored for 3 wk.

Splenectomy

When mice were subjected to both CLP and splenectomy surgeries, they received splenectomy immediately before CLP. Mice were anesthetized with ketamine and xylazine as described above. The spleen was identified following a midline laparotomy incision, and removed after appropriate blood vessel ligation. Sham animals underwent laparotomy without removing the spleen.

Blood bacterial counts

Blood bacteria were recovered as previously described (3). In brief, 5 μl of whole blood was diluted with PBS and plated as 0.15 ml aliquots on tryptic soy agar plates. Colony forming units (CFU) were counted after overnight incubation at 37°C and expressed as CFU/ml blood.

Cytokine measurements

Serum HMGB1 concentrations were determined by Western immunoblotting analysis as previously described (2–3). HMGB1 concentrations were calculated with reference to standard curves generated with purified HMGB1 as described previously (3). Serum levels of TNF, IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-γ were measured using Cytometric Bead Array (BD Biosciences) according to the manufacturer’s instructions.

Analyses of tissue histology and apoptosis

Immediately after euthanasia, tissues were collected and fixed in 10% formalin, and sections were made and stained with H&E for morphologic evaluations.

For apoptosis analyses, TUNEL staining was performed by using ApoAlert DNA fragmentation assay kit (BD Biosciences) and caspase 3 expression was determined by using immunostaining kits from Vector Laboratories. Staining and analyses were performed by blinded observers. Quantifications were performed by counting a representative area of tissue section of each slide.

Statistical analysis

Data are presented as mean ± SEM. Differences between treatment groups were determined by a Student’s t test or one-way ANOVA followed by the least significant difference test or Fisher’s exact test (for survival experiments); p values <0.05 were considered statistically significant.
IL-12, and IFN-γ increases serum concentrations of Th-1 cytokines such as IL-2, seen in sepsis (12). We observed that splenectomy significantly decreased serum HMGB1 levels compared with sham-operated controls (Fig. 1C). Administration of Z-VAD-FMK significantly reduces serum HMGB1 levels as compared with septic mice treated with control peptide, Z-FA-FMK, to splenectomized or sham-operated mice during CLP sepsis. Consistent with our previous findings (11), administration of Z-VAD-FMK significantly reduces systemic HMGB1 release, we administered Z-VAD-FMK, or control peptide, Z-FA-FMK, to splenectomized or sham-operated mice during CLP sepsis. Consistent with previous studies (11, 13, 17), we found that treatment with Z-VAD-FMK significantly reduces splenic apoptosis as revealed by TUNEL staining (Fig. 2A). CLP also induces significant apoptosis in the cortex of the thymus (Fig. 2B). Administration of Z-VAD-FMK significantly reduces thymic apoptosis in splenectomized mice as shown by TUNEL (Fig. 2B). Active caspase 3 staining (data not shown). Previous studies showed that caspases are proteases that activate apoptotic cell death, and administration of agents that inhibit caspase activity improves organ function and increases survival in experimental sepsis (12–15, 17). Caspase inhibitors fail to protect mice lacking functional T and B lymphocytes, indicating that lymphocytes are critical to the caspase-dependent protective effects (17). To study the role of caspases and splenectomy in sepsis, we administered a broad-spectrum caspase inhibitor, Z-VAD-FMK, or negative control peptide, Z-FA-FMK, to splenectomized or sham-operated mice during CLP sepsis. Consistent with previous studies (11, 13, 17), we found that treatment with Z-VAD-FMK significantly reduces thymic apoptosis in splenectomized septic mice as shown by TUNEL (Fig. 2B). Active caspase 3 staining (data not shown). As expected, extensive apoptosis is present in the thymus of splenectomized mice in a magnitude comparable to sham-operated septic mice with intact spleen (Fig. 2B).

To further explore the specific role of spleen apoptosis and systemic HMGB1 release, we administered Z-VAD-FMK, or control peptide, Z-FA-FMK, to splenectomized or sham-operated mice during CLP sepsis. Consistent with our previous findings (11), administration of Z-VAD-FMK significantly reduces serum HMGB1 levels as compared with septic mice treated with control
peptide (Fig. 2C). In contrast, treatment of splenectomized septic mice with Z-VAD-FMK increases serum HMGB1 levels (Fig. 2C) and fails to protect against sepsis lethality (survival = 40% in control group vs 25% in Z-VAD-FMK treated group, n = 11 mice per group, p = NS). Administration of Z-VAD-FMK to splenectomized, nonseptic mice does not influence serum HMGB1 levels (Fig. 2C). There is no statistical difference in blood bacterial counts between caspase inhibitor or control peptide-treated splenectomized mice or sham-splenectomized controls, suggesting that these effects are not due to changes in bactericidal clearance mechanism (Fig. 2D). Together, these findings indicate that caspase-dependent apoptosis in the spleen, rather than the thymus, is specifically responsible for HMGB1 release in this model of polymicrobial sepsis, and specific prevention of spleen apoptosis protects animals against HMGB1-mediated sepsis lethality.

Discussion

Apoptosis of lymphocytes during sepsis can result in maladaptive cytokine responses, including increased release of the lethal proinflammatory cytokine HMGB1 (11). Numerous studies demonstrate that prevention of excessive lymphocyte apoptosis significantly improves survival in experimental models of sepsis (12–15, 17–18). Adoptive transfer of T lymphocytes over-expressing the anti-apoptotic protein Bcl-2 into mice, and over-expression of Bcl-2 in T lymphocytes of transgenic mice, are both protective against sepsis lethality (17–18). Administration of compounds that block pro- tease involved in activating programmed cell death (e.g., caspase inhibitors) can also reduce organ damage and mortality in experimental sepsis (13–15, 17).

Previous studies from our laboratory show that administration of the broad-spectrum caspase inhibitor Z-VAD-FMK inhibits lymphocyte apoptosis in the thymus and spleen, reduces systemic HMGB1 release, and improves survival in sepsis (11). Other studies indicate that HMGB1 is released during apoptotic cell death in a time-dependent manner, and this release can be reduced by Z-VAD-FMK (19–20). In this study, we examined the relationship between lymphoid tissue apoptosis, HMGB1 release, and organ injury during murine polymicrobial sepsis. Our findings indicate that prevention of apoptosis in the spleen via splenectomy significantly inhibits systemic HMGB1 release, reduces liver injury, and protects against sepsis lethality (Fig. 3). In addition, splenectomy does not decrease apoptosis in the thymus, which suggests that HMGB1 release is dependent upon organ-specific lymphoid apoptosis. Administration of Z-VAD-FMK to splenectomized, septic mice significantly inhibits thymic apoptosis, but fails to reduce systemic HMGB1 release or improve survival, further suggesting that apoptosis in the spleen, rather than in the thymus, contributes to HMGB1-mediated sepsis lethality. HMGB1 levels are elevated in splenectomized mice treated with Z-VAD-FMK, but these results are not statistically significant as compared with animals with intact spleens plus CLP (Fig. 2C). It is likely that in splenectomized animals, the progression of tissue injury in other organs contributes to elevated HMGB1.

Excessive lymphocyte apoptosis during sepsis may result in a shift from a Th1-dependent to a Th2-dependent cytokine response, compromising the host’s ability to defend against invasive pathogens (12). In this study, we observed that elimination of splenic apoptosis via splenectomy significantly increases levels of the Th1 cytokines IL-2, IL-12, and IFN-γ 44 h after the onset of sepsis; Th2 cytokines such as IL-4 and IL-10 remain unchanged (Fig. 1D). This suggests that local splenocyte apoptosis is critical to the modulation of systemic cytokine production. Despite this potentially beneficial Th1-mediated response, we found no difference in circulating bacterial counts between splenectomized and sham-splenectomized mice 24 h after the onset of sepsis.

Our results raise important questions regarding the precise immunological role of the spleen in sepsis (21–23). Splenectomized patients are reported to be at higher risk for developing rapidly progressive, lethal septic shock due to overwhelming bacterial infection (24–26). In this study, we show, however, that splenectomy does not impair bacterial clearance or worsen survival during polymicrobial abdominal sepsis treated with antibiotics. On the contrary, prevention of apoptosis in the spleen via splenectomy significantly reduces liver damage, reduces HMGB1 release, and decreases lethality. It may be advantageous to develop pharmacological compounds that specifically protect the critical pool of lymphocytes in the spleen that regulate HMGB1 release and sepsis lethality (Fig. 3). Additional studies are needed to further explore the relationship between caspase-dependent lymphocyte apoptosis in the spleen and systemic release of HMGB1 during lethal sepsis.

Disclosures

K.J.T. is a consultant to MedImmune, Inc., Gaithersburg, MD.

References


