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Early trauma hemorrhage-induced splenic and thymic apoptosis is gut-mediated and TLR4-dependent

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Abstract

Immune depression after trauma-hemorrhage has been implicated as an important factor in the pathogenesis of sepsis and septic-organ failure. Although recent studies have implicated immune cell apoptosis as an important factor in the evolution of this post-trauma immune suppressed state, neither the initial triggers that induce this response nor the cellular pathways through which these triggering pathways act have been fully defined. Thus, the current study tests the hypothesis that acute splenic and thymic immune cell apoptosis developing after trauma-hemorrhagic shock (T/HS) is due to gut-derived factors carried in intestinal lymph and that this T/HS lymph-induced immune depressed state is mediated through TLR4. The first set of experiments documented that T/HS caused both thymic and splenic immune cell apoptosis as measured by TUNEL and caspase-3 immunohistochemistry and that this increase in apoptosis was totally abrogated by mesenteric lymph duct ligation. In subsequent experiments, mesenteric lymph collected from animals subjected to T/HS or trauma-sham shock (T/SS) were injected into TLR4 deficient (TLR4_{mut}) mice or their wild-type (WT) littermates. T/HS, but not T/SS, lymph caused splenic apoptosis in the WT mice. However, the TLR4_{mut} mice were resistant to T/HS lymph-induced splenic apoptosis. Furthermore, the WT, but not the TLR4_{mut} mice developed splenic apoptosis after actual T/HS. In conclusion, gut-derived factors appear to initiate a sequence of events that leads to an acute increase in splenic and thymic immune cell apoptosis and this process is TLR4-dependent.

Keywords

Programmed Cell Death; Shock lymph

INTRODUCTION

It has become increasingly well recognized that both excessive inflammation and immunosuppression proceed concurrently in patients with severe trauma or sepsis and that both of these immuno-inflammatory responses can co-exist for prolonged periods of time (1). While extensive attention has focused on controlling the hyper-inflammatory response, by comparison until recently relatively little attention has been focused on the concurrent immune-depressive response. However, with the failure of a large number of clinical trials

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utilizing multiple different anti-inflammatory therapies and approaches (2), plus the work of Hotchkiss and others (3), more attention is now being focused on the depressed immune response component of the dysregulated post-shock immuno-inflammatory response. In this context, studies have shown that trauma-hemorrhage produces severe and prolonged immune depression, which increases host susceptibility to sepsis, a major cause of post-injury mortality (4). The loss of large numbers of lymphocytes and dendritic cells appears to contribute to this immune depressed state by impairing the host's ability to sustain an effective immune response, which in turn results in a failure to clear primary infections and an increased susceptibility to nosocomial infections (5). Studies showing that prevention of apoptosis in clinically relevant animal sepsis models increases survival provides support for the hypothesis that immune cell apoptosis is a relevant pathologic mechanism in sepsis and trauma (6-8).

In trauma-hemorrhagic shock (T/HS), not only has thymus, splenic, and peripheral blood lymphoid apoptosis been implicated in the development of post-T/HS immune dysfunction, but apoptosis has also been documented to occur in the intestine as well as other organs, such as the lungs (8,9). The factors initiating the sequence of events resulting in apoptosis has been thought to relate to a combination of the global (total body) ischemia-reperfusion response to shock in combination with the pro-inflammatory effects of tissue injury (8). However, an alternate hypothesis is that the primary initiator of the early post-T/HS systemic apoptotic response is due to factors released from the stressed gut rather than purely the global systemic response to hypotension and tissue injury. Support for this notion comes from work showing that intestinal lymph duct ligation (LDL) prevents/limits non-gut organ injury/dysfunction as well as neutrophil, endothelial cell and RBC dysfunction (10) plus studies showing that LDL limits pulmonary endothelial and parenchymal cell apoptosis (9). Thus, the current study was designed to test the hypothesis that gut-derived factors in mesenteric lymph are necessary and sufficient to induce an acute post-T/HS-induced immune cell apoptotic response. Our results supporting this hypothesis are the first to show a direct causal relationship between T/HS-induced gut injury and the development of immune organ apoptosis and thereby provide important insights into the physiologic mechanisms involved in inducing the systemic apoptotic response that occurs shortly after T/HS.

MATERIALS AND METHODS

Animals

Adult male specific pathogen-free Sprague-Dawley rats (Charles River, Boston, MA) weighing 325 g to 450 g or male TLR4 deficient, C3H/HeJ mice (TLR4_{mut}) and their wild-type, C3H/HeOuJ littermates (WT) weighing 20-30 g (Jackson Laboratories, Bar Harbor Maine) were used in the experiments. Adult male noncastrated Yorkshire pigs, weighing 25 to 35 kg were used (Animal Biotech Industries, Danboro, PA) for the lymph collection protocol. Animals underwent a 7-day acclimatization period with 12-hour light/dark cycles under barrier-sustained conditions, during which time they had free access to water and either Harlan Teklad laboratory chow (Teklad 22/5 Rodent diet [W] 8640, Harlan Teklad, Madison, WI) for the rodents or Purina pig chow (Purina Modified Mini-Pig Grower Diet 5081, Richmond, Ind). The animals were maintained in accordance with the guidelines of the National Institutes of Health guide for the Care and Use of Laboratory Animals, and the experiments were approved by the New Jersey Medical School Animal Care and Use Committee.

Experimental Design

In the first experiment, we tested the hypothesis that the pathogenesis of T/HS-induced splenic and thymic cellular apoptosis was causally related to T/HS-induced gut injury and the presence of biologically-active factors in the intestinal lymph of these animals. To accomplish this, we measured splenic and thymic cell apoptosis in the following 3 groups of rats; 1) rats subjected to trauma-sham shock (T/SS), 2) T/HS or 3) T/HS plus mesenteric lymph duct ligation (LDL). The rationale behind these groups was that if factors in the lymph from the T/HS rats was contributing to immune cell apoptosis, LDL would abrogate this effect by preventing intestinal lymph from reaching the systemic circulation. These measurements were made in animals sacrificed at 3 or 24 hrs after T/SS, T/HS or T/HS +LDL. To improve the accuracy of our results, apoptosis was measured by both TUNEL and caspase-3 immunohistochemistry (IHC) assays.

The above studies were carried out in male animals. However, preclinical shock and sepsis studies indicated that immune and inflammatory responses are better preserved in proestrus female than male animals. Thus, we tested whether T/HS-induced splenic cell apoptosis would be reduced in female rats.

To further test the hypothesis that gut-derived factors in mesenteric lymph were directly involved in triggering splenic cell apoptosis, T/SS or T/HS pig lymph was injected into WT mice. The rationale for using pig lymph instead mouse lymph is a practical one as the volume of lymph produced by a mouse is insufficient in quantity for use in our experiments. Additionally, we have previously used a pig lymph injection model for evaluating the systemic effects of toxic lymph. In this experiment, WT mice were challenged with T/HS or T/SS lymph (0.03 ml/gm over 3 hours) following which they were sacrificed, their spleens harvested and assessed for apoptosis via TUNEL and caspase-3 IHC. In addition, because of the increasingly documented role of danger signals and Toll-like receptors in mediating organ injury and immuno-inflammatory cell dysfunction after shock-trauma as well as in septic states (11-14), we expanded these lymph injection studies to include the TLR4_{mut} littermates of these wild-type mice.

Lastly, to further test the notion that TLR4 activation was involved in the pathway leading to T/HS-induced spleen cell apoptosis, we subjected TLR4_{mut} mice and their WT littermates to actual T/SS or T/HS. Then at 3 hrs post-T/HS, T/SS or lymph injection, the mice were sacrificed, their spleens harvested and splenic apoptosis was measured by IHC.

Immunohistochemistry Assays

Spleens and thymi were harvested atraumatically at sacrifice and were fixed in 10% (v/v) buffered formalin (pH 7.2), permeabilized following the manufacturer's instructions and then assessed for apoptosis by TUNEL and caspase-3 immunohistochemistry staining using commercially available kits (Trevigen, Gaithersburg, MD and Cell Signaling Technology, Inc., Beverly, MA). A minimum of forty randomly selected high power fields of each tissue section were evaluated by a trained pathologist in a blinded fashion. Data was then recorded as the number of positive cells per high power field.

T/HS Rat Model

T/HS was induced as previously described (9). Briefly, the rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), following which the femoral artery and jugular veins were cannulated. After the lines were placed, a laparotomy was performed to create tissue injury. Subsequently, the abdomen was closed in two layers. In the animals that were subjected to hemorrhagic shock, the shed blood was withdrawn into a syringe containing heparin via the jugular vein catheter until the mean arterial pressure was reduced to 30-35

mmHg. Mean arterial pressure was maintained at this level for 90 minutes by reinfusing or withdrawing blood as needed. The shed blood was kept at 37°C. At the end of the shock period, the rats were resuscitated with their own shed blood. T/SS rats underwent the same procedure, but no blood was withdrawn or infused. In the subgroup of rats that underwent lymph duct ligation, the mesenteric lymph duct was identified, following which it was doubly ligated with 3-0 silk and divided to prevent the intestinal lymph from reaching the systemic circulation (9).

T/HS Mouse Model

Male mice were anesthetized with pentobarbital (60-80 mg/kg IP) and under strict asepsis, a 2.5 cm midline laparotomy was performed. Isoflurane was given if needed to maintain surgical level of anesthesia. Blood was withdrawn from the jugular vein until a mean arterial pressure (MAP) between 35-40 mmHg was obtained and maintained for 60 min. After 60 min, the mice were resuscitated with their shed blood. Sham-shock animals (T/SS) underwent cannulation of the femoral artery and jugular vein followed by a laparotomy; however, no blood was withdrawn and the MAP was kept within normal limits. At 3 hr, after the end of shock or sham shock period, the mice were sacrificed and the spleens were harvested for TUNEL and caspase-3 IHC.

Pig trauma-hemorrhagic shock and lymph collection model

Mesenteric lymph was collected from pigs for injection into WT or TLR4_{mut} mice. Pigs underwent mesenteric lymph duct cannulation, followed by T/HS or T/SS, as described previously (11). Briefly, male pigs (n=4-5/grp), that were fasted the evening before surgery, underwent anesthetic induction with ketamine (20 mg/kg IM) and xylazine (2 mg/kg IM) followed by endotracheal intubation and were then maintained on intravenous anesthesia using fentanyl and ketamine. Serial arterial blood gases were performed to maintain appropriate carbon dioxide and oxygen tension (35-40mm Hg and >75mm Hg, respectively). Pigs were subjected to T/HS which involved a laparotomy with mesenteric lymph duct cannulation and withdrawal of blood in a staged fashion to a MAP of 40mm Hg. The MAP was maintained until the base deficit reached -2 or the total shock period reached 3 hours. Animals were then resuscitated in a staged fashion with a combination of all shed blood and lactated ringers to a MAP of 80-100 mm Hg. T/SS animals underwent laparotomy with lymph duct cannulation but without blood withdrawal. All animals were euthanized 2 hours after completion of resuscitation using an intravenous bolus of KCl. Mesenteric lymph was collected in sterile tubes for 30 minutes before shock, during shock and on an hourly basis during the post shock period. The collected lymph specimens were centrifuged at 500 g for 20 minutes at 4°C to remove all cellular components, tested for sterility on MacConkey and blood agar plates, aliquoted and stored at -80°C. Lymph samples collected from the 1-3 hr post-T/SS and T/HS time period were used. Previously, we published that these banked lymph samples did not contain measurable levels of bacteria DNA and were devoid of endotoxin, using the limulus lysate assay (limit of detection, 0.06 endotoxin units) (11).

Lymph infusion protocol

Mice underwent laparotomy as well as internal jugular vein cannulation. Laparotomy was closed after 15 minutes using two layers of 3-0 silk suture. WT and TLR4_{mut} mice were infused with T/HS or T/SS lymph from randomly selected pigs from each group. The lymph used for each mouse was collected from an individual pig (pooled fractions collected during 1-3 hr post T/SS or T/HS). T/HS or T/SS pig lymph was infused via the jugular catheter at a rate of 10 µL/g body weight per hour for 3 hours as previously described (11,15). At the end of the 3 hour lymph infusion period, the mice were sacrificed and the spleens were harvested for TUNEL and caspase-3 IHC. The rationale for the lymph volume infused was based on the actual amount of lymph produced by the pigs (ml/kg/hr) subjected to T/HS or T/SS over

the shock and resuscitation period which was approximately 28-30 mL/kg (11). Dose-response pilot studies of pig T/HS lymph documented that lung injury occurred with doses of pig lymph as low as 10 μ L/g body weight. As this dose was determined to be the minimum dose sufficient to cause lung injury, we chose to use the same dose to evaluate if splenocyte apoptosis would occur after intravenous injection.

Statistical analysis

Analysis of variance (ANOVA) with the post hoc Tukey-Kramer multiple comparison test was used for comparisons between multiple groups, whereas t tests were used for comparison between two groups. Results are expressed as mean $\pm$ SD. $p < 0.05$ was considered statistically significant.

RESULTS

As compared to rats subjected to T/SS, T/HS was associated with a significant 2-3 fold increase in both splenic and thymic immune cell apoptosis at 3 hours after the end of the T/HS period (Figure 1). Although the incidence of apoptosis in the spleens and thymi was several-fold higher when assayed by the TUNEL as compared to the caspase-3 assays, the relative ratio between the T/SS and T/HS groups were similar in both assays. As TUNEL is a more sensitive, but less specific measure of apoptosis, the greater number of positive cells versus caspase-3 is not unexpected. Furthermore, caspase-3, which measures a terminal effector protein in the caspase-dependant pathway of apoptosis, does not measure other mechanisms of apoptosis, such as caspase-independent apoptosis. The increase in T/HS-induced apoptosis was abrogated in the T/HS rats that had their mesenteric lymph ducts ligated to prevent their intestinal lymph from reaching the systemic circulation (Figure 1). This increase in T/HS-induced splenic and thymic immune cell apoptosis persisted for at least 24 hrs after T/HS as did the protective effect of LDL (Figure 2). In fact, the incidence of apoptosis was similar at both the 3 and 24 hr time points.

Because these studies were carried out in male rats and studies indicate that immune responses are better preserved in proestrus female than male rats after T/HS (16) and post-traumatic thymic lymphocyte apoptosis is abrogated in proestrus female rats (34), we measured splenic apoptosis by TUNEL and caspase-3 IHC in proestrus female rats subjected to T/SS or T/HS. The incidence of splenic apoptosis by TUNEL at 3 hrs after T/SS or T/HS was similar between the female rats subjected to T/SS or T/HS (9.16 ± 5.73 vs 7.21 ± 4.01 apoptotic cells/hpf). Caspase-3 staining also showed no difference in apoptosis between the T/SS or T/HS groups, (0.9 ± 0.7 vs 0.6 ± 0.2 apoptotic cells/hpf). Thus, T/HS-induced splenic apoptosis did not occur in proestrus female rats, in contrast to male rats.

Since T/HS-induced immune-cell apoptosis in the spleen and thymus was prevented by LDL, we next tested whether T/HS lymph was sufficient to recreate the splenic apoptotic response observed after actual T/HS by infusing T/HS or T/SS lymph samples into WT mice. The injection of T/HS lymph into WT mice was associated with a higher number of apoptotic splenic cells than the injection of T/SS lymph. As shown in Figure 3a, the WT mice injected with T/HS lymph had a 1.8-fold increase in splenic apoptosis by TUNEL, as compared to the mice injected with T/SS lymph. A similar 2-fold increase in splenic apoptosis was observed when apoptosis was identified by caspase-3 (Figure 3b). This increase in T/HS lymph-induced splenic apoptosis was more profound in the white pulp than the red pulp of the spleen indicating that lymphocyte apoptosis predominates. Thus, the *in vivo* injection of T/HS, but not T/SS, lymph was sufficient to induce a splenic apoptotic response. Recently it has been suggested that Toll-like and other receptors are involved in transducing the systemic immuno-inflammatory response after T/HS and other insults (14). Based on our work (11,12) and work of others (14) showing that TLR4 deficient mice are

relatively resistance to lung and other organ injuries as well as neutrophil activation after T/HS, we tested whether the T/HS-induced splenic apoptotic response would be abrogated in TLR4_{mut} as compared to their WT littermates. We found that, in contrast to the WT mice, T/HS lymph injection did not increase splenic apoptosis in the TLR4_{mut} mice whether measured by TUNEL (Figure 3c) or by caspase-3 (Figure 3d).

The splenic apoptotic response was increased in mice after actual T/HS as compared to mice subjected to T/SS (Figure 4a,b) indicating that mice and rats manifest a similar splenic apoptotic response after T/HS. Similar to what was observed after T/HS lymph injection, the splenic apoptotic response to actual shock did not occur in the TLR4_{mut} mice (Figure 4; 5) further supporting an important role for TLR4 in transducing the hemodynamic and tissue injury responses of T/HS into an immune cell apoptotic response. As in the T/HS lymph injection studies, the incidence of splenocyte apoptosis was increased in both the white pulp than red pulp of the WT mice (Figure 4c). Likewise, this effect was abated in the TLR4_{mut} mice (Figure 4d).

DISCUSSION

The major observation of the current study is that it shows for the first time a direct causal relationship between T/HS-induced gut injury and the development of immune organ apoptosis and suggests that the pathogenesis of this response occurs via a TLR4-dependent pathway induced by humoral factors carried to the systemic circulation by the intestinal lymphatics. The conclusion that humoral factors present in mesenteric lymph after T/HS are necessary and sufficient to induce an immune cell apoptotic response are based on the observations that lymph duct ligation prevented T/HS-induced splenic and thymic apoptosis while the injection of T/HS, but not, T/SS lymph into naïve mice resulted in a splenic apoptotic response similar to that observed after actual T/HS. The notion that T/HS lymph has cytotoxic properties has been previously reported for endothelial and pulmonary parenchymal cells utilizing both in vitro and in vivo model systems (9,11,17). However, T/HS lymph has also been shown to be causally responsible for neutrophil priming after T/HS and that this priming response was TLR4-dependent (12). Thus, it appears that the immuno-inflammatory-inducing properties of the humoral factors contained in T/HS lymph differ for neutrophils and lymphoid cells, but both are TLR4-dependent. Since the acute post-injury response of patients sustaining major trauma involves both increased neutrophil priming/activation and lymphocyte apoptosis/anergy (18,19), these results support the notion that these two discordant cellular responses to shock-trauma can both be traced back to gut injury adding support for the gut hypothesis of MODS (10).

The relative biologic and clinical importance of these observations are supported by preclinical and clinical studies indicating that both neutrophil activation (12,18) and immune cell apoptosis contribute to morbidity and mortality after major trauma or during sepsis (19,20). Consequently understanding the mechanisms that induce immune cell apoptosis and an immune depressed state may provide important potential therapeutic insights. In this regard, significant information has accumulated on the cellular signaling pathways involved in immune cell apoptosis after major trauma as well as sepsis. Human studies by Hotchkiss et al (20) documented that the primary lymphocyte populations at risk of developing apoptosis included CD4 and CD8 T-cells, B-cells and NK cells. Based on the observations that caspase-3, caspase-8 and caspase-9 molecules were activated, they concluded that both the extrinsic or death receptor apoptotic pathway (caspase-3 and caspase-8) as well as the mitochondrial apoptotic pathway (caspase-9) were involved in the lymphoid cell apoptotic process (20). Although lymphocyte caspase-3 activity was found to be increased, several preclinical trauma and sepsis studies did not find evidence to support a role for TNF or Fas ligand activation of the death receptor as the trigger which initiated the apoptotic response

(21,22). Nonetheless, blockade of the extrinsic caspase-3 or the mitochondrial caspase-9 apoptotic pathways was effective in preventing sepsis or trauma-induced lymphoid tissue apoptosis (22). Thus, although it appears likely that multiple pathways are involved in lymphocyte apoptotic cell death, less is known about the exogenous stressors involved in triggering this response. In the context of MODS, four groups of apoptosis-inducing stressors have received special attention including a) cytokines, b) reactive oxygen species associated with ischemia-reperfusion insults, c) heat shock proteins and d) glucocorticoids (23). However, no in vivo studies have documented a direct causal relationship between these or other stressors/factors and the apoptotic response, although many associations have been found. Consequently studies directed at defining the sequence of events, which results in T/HS-induced lymphoid tissue apoptosis are important. In this light, our studies indicating that the apoptotic cascade is initiated by factors released from the stressed gut are the first studies to definitively identify the exact source of the factors that initiate the induction of the acute systemic lymphoid tissue apoptotic response that occurs after T/HS and indicate that this effect occurs through a TLR4-dependent pathway.

In considering the role of TLR4 in the induction of lymphoid tissue apoptosis, one must consider the danger model first proposed by Matzinger et al over decade ago (24). This model has helped explain the pathogenesis of organ injury and systemic inflammation in certain sterile, non-bacterial inflammatory states, such as thermal injury or trauma-shock (11,13,14). In essence, the danger model proposes that endogenous host-derived molecules from damaged cells and tissues activate the immune system to cause a systemic inflammatory response. These endogenous molecules are called alarmins or danger-associated molecular pattern molecules (DAMPs) while the receptors they bind to are called danger-associated molecular pattern receptors, which act as sensors of tissue injury. The Toll-like receptors are one such receptor family that have been identified as playing major roles in the pathogenesis of microbial as well as sterile inflammatory states. Within that family of receptors, TLR4 appears prominent. Although the function of the TLR4 receptor was originally thought to identify gram-negative bacteria, it is now also recognized as being one of the receptors sensing endogenous alarmins generated after tissue injury and/or shock (25). Consequently, we and others have investigated the role TLR4 activation plays in the development of sterile-inflammatory states, such as T/HS and found that T/HS-induced organ injury involves TLR4 activation (11-14,25). The observation that TLR4 deficient mice, in contrast to their wild-type littermates, do not develop lymphoid tissue apoptosis after actual T/HS or an in vivo challenge with T/HS lymph indicates that TLR4 activation mediates acute post-T/HS immune depression as well as systemic inflammation. If further studies validate this role of TLR4 activation as an important step in the rapidly acquired immune depressed state as well as the increased systemic inflammatory response after trauma-shock, the TLR4 receptor would be a potential early therapeutic target whose modulation might abrogate both the immune depression and hyper-inflammation observed after T/HS. However, due to the complexity of the in vivo response to injury, shock or sepsis as well as the potential benefits of TLR4 activation, much more must be understood before this potential therapeutic strategy could be applied clinically.

Although the in vivo administration of intestinal T/HS lymph is sufficient to induce splenic apoptosis and it does so through a TLR4-dependent pathway, identification of the exact factors in the lymph which are responsible for this lymphoid apoptotic effect remain elusive. We have previously reported aliquots of the stored porcine T/HS lymph samples used in this study are sterile and do not contain measurable levels of endotoxin or bacterial DNA (11). Thus, it appears that non-microbial factors within the T/HS lymph are responsible for its apoptotic-inducing activity. Although protein factors in T/HS lymph appear to contribute to its biologic activity (26), studies investigating protein factors in lymph, including extensive proteomic studies, have not been fully rewarding, although they suggest that the biologic

activity of T/HS lymph is not due to cytokines, heat shock proteins nor currently recognized TLR4 protein alarmins (27-29). Less is known about the lipid factors in T/HS lymph, although lipid factors appear to contribute to lymph's biologic activity (30,31). Although work continues, this inability to isolate and characterize the putative mediators in T/HS lymph has been a major impediment to clarifying the mechanisms by which T/HS lymph induces its various biologic effects.

This study does have its limitations. First, only early time points after T/HS were studied with measurements being made at 3 and 24 hrs after T/HS. Thus, although gut-derived factors contained in lymph appear critical in the pathogenesis of the early, acute lymphoid organ apoptotic response after T/HS, non-gut-mediated mechanisms may be involved at later time points or in microbial-based septic states. Second, the gut may not be the only source of factors inducing an immune cell apoptotic response, since injured tissues can be a source of systemic alarmins and other immune-modulating molecules (32). Additionally, although both TUNEL and Caspase-3 immunohistochemistry were used to identify apoptotic cells and the results of these two assays correlated with each other, a higher percentage of the cells were TUNEL than Caspase-3 positive. This may be based on the fact that the TUNEL assay is less specific for apoptosis than caspase-3, since the TUNEL assay can not always distinguish apoptosis from necrosis (33). Although identifying and understanding the mechanism of cell death (apoptosis versus necrosis) is very important for a number of reasons, this distinction does not negate the key fact that T/HS promotes lymphoid cell death. Of note, a difference in the level of TLR4 expression on mice versus human lymphocytes may call into question the translatability of our findings to human subjects. However, other investigators have shown that human T-lymphocytes contain TLR4 proteins intracellularly despite a paucity of extracellular expression and an increased level of extracellular expression after T-lymphocyte activation (35). Thus, whether this difference between species ultimately reduces the effect of TLR4 activation on lymphocyte apoptosis remains to be seen. Lastly, the possibility of cross species reactivity contributing to an increase in apoptosis can not be entirely ruled out as we did not specifically test the immunogenicity of the pig lymph. However, there are a few considerations that lead us to believe that this effect, if it in fact occurs, is minimal and/or controlled for in our experiments. First, the statistical difference between the levels of lymphocyte apoptosis in the naïve WT mice subjected to actual T/SS or T/HS were similar to that of the mice injected with T/HS or T/SS pig lymph. If an immunologic cross species event was occurring, the amount of apoptosis should theoretically be equivalent in the T/SS and the T/HS injection groups and the level of apoptosis should be significantly higher in the T/SS lymph injected than the actual T/SS group. Since this was not the case, the likelihood of a significant cross species immunologic event in the pig lymph injected mice is less likely. Additionally, we have previously shown that injection of pig and rat T/HS lymph but not T/SS lymph into mice leads to *in vivo* increases in lung injury, RBC rigidity and PMN activation (12, 30), supporting the differential effects of T/HS versus T/SS lymph further indicating that our current observations are not a result of an immunologic interaction with the lymph itself.

In summary, the results of this study show that factors originating from the stressed gut and carried in the lymphatics are sufficient to induce an early lymphoid tissue apoptotic response and that this response is TLR4-dependent. The fact that the same stored porcine T/HS lymph samples that caused *in vivo* lymphoid tissue apoptosis have the ability to prime neutrophils through a TLR4-dependent pathway (12) indicates that gut-derived factors are capable of simultaneously leading to an immune-depressed state as well as inducing systemic inflammation. This divergent property of gut-derived factors, provides a potentially important insight to help explain the emerging paradox of how major trauma-hemorrhage

can lead to immune depression at the same time that it causes a systemic hyper-inflammatory response and inflammatory-mediated organ injury.

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Figure 1. Splenic and thymus immune cell apoptosis at 3 hrs after T/SS, T/HS or T/HS+LDL as measured by TUNEL (A, C) or caspase-3 (B, D) immunohistochemistry. Data expressed as Mean $\pm$ SD number of positive cells per hpf. N=5 animals per group. * $p < 0.01$ vs other groups.

Figure 2.

Splenic and thymus immune cell apoptosis at 24 hrs after T/SS, T/HS or T/HS+LDL as measured by TUNEL (A, D) or caspase-3 (B, D) immunohistochemistry. Data expressed as Mean $\pm$ SD number of positive cells per hpf. N=5 animals per group. * $p < 0.01$ vs other groups.

Figure 3.

A) Injection of T/HS but not T/SS lymph into WT mice increased splenic immune cell apoptosis as measured by the TUNEL assay or B) by caspase-3 IHC. C) TLR4_{mut} mice did not manifest an increase in splenic immune cell apoptosis after T/HS lymph injection when assayed by TUNEL or D) by caspase-3 IHC. Data expressed as Mean ± SD number of positive cells per hpf. N=8 animals per group. * p < 0.01 and ** p < 0.05 vs T/SS group.

Figure 4.

A) T/HS increases splenic immune cell apoptosis in WT but not TLR4_{mut} mice as measured by the TUNEL assay and B) a similar effect was observed with caspase-3 IHC. C) The increase in T/HS-induced splenic apoptosis by TUNEL involved both white and red pulp. D) In the TLR4_{mut} mice, apoptosis was not increased in white or red pulp after T/HS. Data expressed as Mean $\pm$ SD number of positive cells per hpf. N=5-6 mice per group. * $p < 0.01$ vs all other groups. ** $p < 0.05$ vs all other groups.

Figure 5.

A) Spleen of a WT mouse subjected to T/HS vs. B) Spleen of a TLR4_{mut} mouse subjected to T/HS at 200× magnification demonstrating decreased apoptosis in the TLR4 deficient mice on TUNEL staining (dark staining cells indicated by arrow).