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The Role of Hepatic Invariant NKT Cells in Systemic/Local Inflammation and Mortality during Polymicrobial Septic Shock

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NKT cells have been described as innate regulatory cells because of their rapid response to conserved glycolipids presented on CD1d via their invariant TCR. However, little is known about the contribution of the hepatic NKT cell to the development of a local and/or systemic immune response to acute septic challenge (cecal ligation and puncture (CLP)). We found not only that mice deficient in invariant NKT cells (Jα18−/−) had a marked attenuation in CLP-induced mortality, but also exhibited an oblation of the systemic inflammatory response (with little effect on splenic/peritoneal immune responsiveness). Flow cytometric data indicated that following CLP, there was a marked decline in the percentage of CD3+ α-galactosylceramide CD1d tetramer+ cells in the mouse C57BL/6J and BALB/c liver nonparenchymal cell population. This was associated with the marked activation of these cells (increased expression of CD69 and CD25) as well as a rise in the frequency of NKT cells positive for both Th1 and Th2 intracellular cytokines. In this respect, when mice were pretreated in vivo with anti-CD1d-blocking Ab, we observed not only that this inhibited the systemic rise of IL-6 and IL-10 levels in septic mice and improved overall septic survival, but that the CLP-induced changes in liver macrophage IL-6 and IL-10 expressions were inversely effected by this treatment. Together, these findings suggest that the activation of hepatic invariant NKT cells plays a critical role in regulating the innate immune/systemic inflammatory response and survival in a model of acute septic shock.

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4 Abbreviations used in this paper: CLP, cecal ligation and puncture; α-GalCer, α-galactosylceramide; iNKT, invariant NKT; MFI, mean fluorescence intensity; NPC, nonparenchymal cell.

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capacity, we found that Ab-treated septic mouse splenocytes produce less of the Th2/anti-inflammatory cytokine IL-10 and more of the Th1/proinflammatory cytokines IFN-γ and IL-2 than nonspecific Ab-treated septic mice (17). Intriguingly, from an innate regulatory perspective, we also observed that anti-CD1d Ab blockade markedly reduced the release of IL-6 and IL-10 in the blood of these same septic animals. However, whereas NKT cells represent ~1–3% of the normal splenic population, they comprise at least 10–20% more of the total T cell population in the liver (17–19). Because the liver has been reported to play a significant role in contributing to both the innate proinflammatory cytokine and acute-phase mediator response to infectious challenge or shock and is an organ that is often injured in the septic process (20–24), we set out to test the hypotheses that not only are hepatic iNKT affected by experimental polymicrobial septic shock, but they contribute to the pathology of this condition.

Materials and Methods

Animals

Male BALB/c, C57BL/6J (from The Jackson Laboratory), and C57BL/6-Jα18–/– (Jα18–/–; deficient in iNKT cells) (a gift from H. Taniguchi, Kanagawa, Japan) (25) mice, 8–10 wk of age, were used in all experiment procedures. In a subset of experiments, mice were treated with either 500 μg of rat anti-mouse CD1d (clone 1B1; BD Biosciences) or isotype control Ab, i.p., 18 h before subjecting them to CLP, as described previously (16). Research objectives and all animal protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and conducted in accordance with the Animal Welfare Act and National Institutes of Health guidelines.

Septic model

The CLP surgery established by Baker et al. (26) and modified by us (16) was used to induce polymicrobial septic shock in mice. In brief, the cecum was exposed by a midline incision and then tied off with a 3-0 silk ligature 1 cm from the distal end. The ligated portion was then subjected to two punctures with a 22-gauge needle: one at the distal end of the cecum, and one close to the site of ligation. CLP animals were in a state of septic shock by 24 h. To control for the effects of the anesthesia and laparotomy on the septic group, sham surgery mice were subject to all of the procedures above saving ligation and puncture of the cecum.

Isolation of liver nonparenchymal cells (NPC), splenocytes, bone marrow cells, and peritoneal leukocytes

Livers were first ground and suspended in PBS with 2% FBS. After three washes with the same medium, NPC were isolated from the remaining cell pellet using a density gradient of 40 and 70% Percoll, according to the methods of Pein and Biron (27).

Hepatic macrophages, peritoneal macrophages, splenocytes, and/or bone marrow cells were isolated, as previously described by our laboratory (28–30). The capacity of splenic T cells or adherent cell cultures to produce cytokines in response to the T cell stimulus anti-CD3 Ab or LPS (as a well-defined phagocyte stimulant), respectively, was also assessed, as described previously (28, 29).

Cytokine levels

Blood was collected by cardiac puncture, plasma separated, and frozen stored until needed for assay. BD-Cytometric Bead Assay using a BD-FACSArray (BD Biosciences) (31) was applied to determine the proinflammatory or Th1/Th2 cytokine levels in blood or cell supernatants.

Flow cytometry

NPC were stained with PE-conjugated anti-CD3, anti-CD4, anti-CD69, anti-CD25, and anti-CD1d Abs (BD Biosciences); allophycocyanin-conjugated anti-F4/80, anti-CD11c, and anti-NK1.1 Abs (BD Biosciences); α-galactosylceramide (α-GalCer)-loaded, as well as unloaded CD1d tetramer (provided by the tetrramer facility at the National Institutes of Health) and the appropriate Ab isotypic-chromaphore-conjugated controls (BD Biosciences). Incubation was performed at room temperature for 20 min in 100 μl of staining buffer (~1% BSA and 0.01% sodium azide in PBS). Samples were then read and analyzed (to delineate specific from nonspecific staining) using a BD FACSArray and software, as previously described (32).
Intracellular cytokine staining

The detection of intracellular cytokine expression was conducted, as previously described in our laboratory (33). For these studies, freshly purified NPC harvested 24 h post-CLP were first incubated 2 h with brefeldin A (3 μg/ml; eBioscience) at 37°C in complete RPMI 1640 medium. Cell surface staining was then performed by incubation with allophycocyanin-Cy7-labeled anti-CD3 Abs and allophycocyanin-labeled anti-NK1.1 Abs (for C57BL/6J mice) or anti-DX5 Abs (for BALB/c mice) for 45 min at 4°C in the dark. Note that we used anti-NK1.1 or anti-DX5 Ab as opposed to α-GalCer-loaded CD1d tetramer, on C57BL/6J or BALB/c mice, respectively, because the tetramer staining was lost during the latter steps of the intracellular cytokine staining protocol. Cells were then permeabilized using freshly prepared Fix’Perm reagent (eBioscience) and incubated with PE-labeled anti-IL-2, anti-IL-6, anti-IL-10, anti-IL-4, anti-TNF-α, anti-MCP-1, anti-IFN-γ Abs, or the appropriate isotype controls (eBioscience). After washing twice, cells were analyzed by flow cytometry (32).

Survival study

Age-matched groups of C57BL/6J and iNKT-deficient (Jα18−/−) mice or anti-mouse IgG- and anti-mouse CD1d-treated BALB/c mice were subject to CLP, and their survival was monitored and recorded for 14 days.

Statistical analysis

The data are presented as a mean ± SEM for each group. Differences in the experimental means for flow cytometric and cytokine values were considered significant if p < 0.05 as determined using the Kruskal-Wallis One Way Analysis of Variance on Ranks with SigmaStat Version 3.0 (SPSS). Differences in survival curves were considered significant by the log-rank survival analysis at p < 0.05 (SigmaStat Version 3.0; SPSS).

Results

Septic shock-induced mortality and the systemic inflammatory response, but not splenic or peritoneal cytokine release capacity, are substantially reduced by the genetic deficiency of iNKT cells

When we conducted a survival study on age-matched male C57BL/6J and iNKT (Jα18−/−)-deficient mice, we found that a significant survival advantage could be detected as early as 48 h following CLP in the Jα18−/− mice and remained evident even at 14 days post-CLP (Fig. 1A). In line with this, we observed that in Jα18−/− mouse, CLP-induced blood systemic cytokine response was essentially ablated (Fig. 1B).

To the extent that iNKT cells might be playing a role in acute septic immune cell dysfunction, we assessed the degree to which local tissue immune responsiveness was altered (as an index of immune morbidity) following CLP in iNKT cell-deficient Jα18−/− mice (25) vs background control animals. Surprisingly, whereas we documented/reaffirmed the functional decline in the capacity of splenocytes to produce Th1 cytokines as well as peritoneal macrophages’ abilities to produce proinflammatory mediators after sepsis (Fig. 1, C and D) that has been previously reported (28, 32, 34–37), genetic deficiency of iNKT cells only partially restored Th1 cytokine (IFN-γ and TNF-α) release in splenocytes (Fig. 1C) and had no effect on peritoneal macrophage cytokine release (Fig. 1D).
FIGURE 3. Liver iNKT cells present with increased expression of activation makers after CLP. Flow cytometric analysis indicates that on αGalCer-loaded CD1d tetramer+ cells derived from the livers of C57BL/6J mice subjected to CLP (as compared with sham), there is an increase in the expression of the activation markers CD69 and CD25. A. Representative dot plots are presented for 24 h post-sham and CLP liver NPC preparations. The regions delineating positive staining CD1d tetramer alone, CD69 alone, dual-positive, as well as dual-negative cells were set using the appropriate unloaded tetramer and Ab isotype controls. B. From these dot plots a histogram was derived for CD1d tetramer+ cells that illustrate the typical increase in the MFI seen for these cells obtained from the CLP sample. C. Histograms of the cumulative changes in MFI for CD69 CD1d tetramer+ indicate that at 12 and 24 h post-CLP there was a marked increase in the fluorescent intensity of CD69 on this cell subpopulation in the liver. D. Histograms of the cumulative changes in MFI of no less than nine independent animals sampled per group for CD25 CD1d tetramer+ indicate that at 24 h post-CLP there was also a significant increase in the fluorescent intensity of CD25 on this same cell subpopulation. Results of no less than six to nine independent animals sampled per group are given as mean ± SEM. *, Indicates significance at p < 0.05 vs the sham group.

The frequency of liver iNKT cells declines over the first 24 h following septic insult

Because the liver is one of the largest sites of resident NKT cells in the mouse (18, 19) and because we and others have previously shown that substantial aspects/amounts of the cytokines seen during the systemic proinflammatory response to experimental sepsis and/or shock (20, 38) are initially derived from the liver, we attempted to determine what effect CLP had on this resident iNKT cell subpopulation. Looking initially at a typical flow dot plot (Fig. 2A) and associated histogram (Fig. 2B) of NPC derived 24 h following either sham protocol or CLP, it can be seen that there is a ~70% decline in the number of liver CD3+ tetramer+ cells/iNKT cells after sepsis (18). When these data are collated for several repeat experiments, we observed that whereas the percentage of liver iNKT cells for septic mouse samples remained the same as the sham animals’ cells at 4 h, it was significantly decreased at 12 and 24 h post-CLP by ~45% (Fig. 2C). Furthermore, we observed that the frequency of CD3+NK1.1+ closely correlated with the percentages seen on the CD3+ tetramer+ cells detected in this study in the liver (Fig. 2D). To the degree that the loss in liver iNKT cells was not an actual decline, we observed not only that there was no decline in the mean fluorescence intensity (MFI) of the CD3+ tetramer+ staining within the iNKT cell population (Fig. 2, A and B; e.g., the sum of 8–9 animals’ samples/group for the MFI for CD1d tetramer+ expressions was 2554 ± 97 for sham and 2383 ± 80 for CLP (24 h post)) and no significant differences in the total NPC yield from sham or CLP livers (Fig. 2E), but also that these cells did not appear to have migrated elsewhere. This latter conclusion was based on the observation that when the same staining was performed in the spleen and bone marrow cells, no increase in tetramer-positive iNKT cells was found (data not shown). To the extent that the decline in NKT cells was associated with a marked change in the expression of CD1d APCs present in the liver, we noted no significant change in the extent of CD1d+ expression in either the whole NPC population (sham, 60.3 ± 5.7% vs CLP, 62.5 ± 1.5% CD1d+, n = 6–8 mice per group) or the F4/80+CD1d+ or CD11c+CD1d+ subpopulations following CLP on these cells by flow cytometric assessment (data not shown). That said, from our data, because we do not know whether there is an actual loss in Vα14-Jα18 TCR expression at a message or protein level on these cells, we cannot absolutely preclude the possibility that the apparent decline of (or at least some aspect of it) NKT cells observed in this study might not be accounted for by loss of the iNKT cell TCR expression during activation process itself.

The frequency of activated liver iNKT cells and liver macrophages that express pro/anti-inflammatory cytokines is elevated at 24 h following septic insult

Because studies have suggested that iNKT cells can be readily activated (18, 39), we subsequently attempted to determine the extent to which the tetramer+ cells were expressing the activation markers CD69 and CD25. What we found was whereas there was a marked overall decline in the frequency of septic mouse CD1d tetramer+ cells (Fig. 2, C and D), these residual cells exhibited a marked increase in CD69 and CD25 expressions, as determined by the rise in MFI (Fig. 3).
Moreover, because iNKT cells have been reported to rapidly up-regulate the release of IL-4, IFN-γ (9), as well as several other Th1/Th2 cytokines (12–14), we attempted to determine whether the frequency of NKT cells expressing the cytokines IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, and MCP-1, by intracellular cytokine staining/flow cytometric assessment, was altered. What we observed in liver NKT cells derived from septic C57BL/6J mice was that there was a marked increase in the frequency of TNF-α⁺, IL-6⁺, and IL-10⁺ cells with a trend, although nonsignificant rise in IL-4⁺ and IFN-γ⁺ cells (Fig. 4, A–E). No change was seen in IL-2⁺ or MCP-1⁺ cell percentages (data not shown).

Because previous studies have indicated not only that liver NKT cells may interact with local tissue macrophages to activate them (11), but that liver macrophages contribute to aspects of the developing systemic proinflammatory cytokine response encountered in the septic animal (20), we concomitantly examined the extent of change in frequency of liver macrophages (F4/80⁺ cells) that were positive for the inflammatory cytokines, TNF-α, IL-6, and IL-10. Not surprisingly, what we observed was a marked rise in the septic mouse percentage of liver macrophages that was positive for all three of these cytokines (Fig. 4, F–H).

Inhibition of Ag presentation/activation via CD1d blocks proinflammatory cytokine release, septic mortality, and the CLP-induced potentiation of hepatic macrophage/Kupffer cell cytokine production

Because we had observed that the residual liver iNKT cell population, although in decline, was apparently activated and producing cytokines, we attempted to ascertain whether inability to activate iNKT cells (by blocking their ability to see Ag presented in the context of CD1d) altered the capacity to not only induce a systemic proinflammatory cytokine response, CLP mortality, but also potentiate septic hepatic macrophage cytokine expression. What we found was that, as seen in Jx18⁻/⁻ mice, BALB/c mice pretreated with the CD1d-blocking Ab 18 h before being subjected to CLP not only exhibited lower levels of circulating TNF-α, IL-6, MCP-1 (commonly assessed markers of the proinflammatory response (40, 41)), and IL-10 (a typical index of the anti-inflammatory response (42, 43)) (Fig. 5A), but showed a decrease in NKT cell’s capacity to produce TNF-α/IL-6/IL-10 and IL-4 (Fig. 6, A–D). Most importantly, this was associated with a marked improvement in septic survival when compared with rat IgG-treated mice (Fig. 5B). Interestingly, when we assessed the extent to which the frequency of septic liver macrophage intracellular inflammatory cytokine expression was altered by anti-CD1d-blocking Ab pretreatment, we found that whereas the percentage of IL-6⁺ macrophages markedly declined (when compared with IgG-treated mouse samples), the rise in TNF⁺ cells was not attenuated (Fig. 6, F and G). Furthermore, the frequency of IL-10⁺ septic mouse F4/80⁺ cells was significantly increased by anti-CD1d-blocking Ab pretreatment (Fig. 6H).

Of note, whereas we initially chose to use C57BL/6J mice, because they served as the genetic background mouse for the Jx18⁻/⁻-deficient mouse, we decided to use the BALB/c mouse for the last part of this study because of the following: 1) we wanted to determine the extent to which the contribution of the NKT cells to septic morbidity was mouse strain specific (44, 45); 2) we had previously looked at changes in septic mouse splenic responses using this strain (16); and 3) our initial observations that relative to the C57BL/6J that the CLP-induced changes in frequency (a decline in the percentage of NKT cells) and activation status (concomitant increases in both CD69 and CD25) of the BALB/c’s hepatic NKT cell subpopulation were comparable (Fig. 7). That said, whereas the aspects of NKT cell percentage decline in the face of
Discussion

Initial triggers of the sepsis may be Gram-positive and -negative bacteria, tissue injury (when extensive) or ischemia, and, less often, fungi or viruses (2, 46). The polymicrobial septic shock model we used in this study introduced both necrotic and infectious material into circulation, which in turn is thought to serve as the stimulus for the biphasic immune response seen clinically (47). Although the search for endogenous/exogenous NKT ligands continues, our model shows that septic injury/insult is capable of activating these cells in the liver, and this occurs both in mice from a C57BL/6J strain background. Furthermore, at least in a model of acute onset mortality, we show that NKT cells not only contribute to the development of proinflammation in this setting, but that this is at least in part through a CD1d Ag-mediated event that appears to involve the activation of endogenous liver adherent cell populations. This is an important point because several recent studies have illustrated that iNKT cells not only express pattern recognition receptors, i.e., TLR-4 (48), TLR-2 (49), and TLR-9 (50), which should allow them to sense these microbial components present in the septic animal, but also respond to defined Gram-negative infectious challenge by potentiating the proinflammatory response.

These data differ with reports by Faunce et al. (13, 51, 52), which indicate that following an injury, such as burn, the iNKT cell, via its release of IL-4, plays a role in suppressing the developing cell-mediated adaptive immune response. This is not to say that we have not previously seen some aspects of an immune-suppressive phenotype developing in these mice (3). However, in this study, we have reported that both the lack of iNKT cells and functional blockade of iNKT cell signaling with anti-CD1d-blocking Ab treatment led to the decline of proinflammatory (TNF-α, MCP-1, and IL-6) as well as anti-inflammatory IL-10 release into circulation. Four possible reasons for differences in our findings may relate to the following: 1) the background of the animals used in the studies by Faunce et al. was solely BALB/c, which we (Fig. 6D) and others (44, 45) have shown exhibits a more Th2-shifted character/higher IL-4 than seen in C57BL/6J; 2) the nature of the insult, our model being infectious as well as tissue injury vs nonlethal burn alone; 3) that all our approaches in this study are pretreatment by the nature (in the case of the Jα18−/− mouse) and in the timing of anti-CD1d blockade treatment; and 4) the high/rapid (first 48–72 h) mortality (vs nonlethal burn injury (13)). Thus, we cannot preclude the possibility that in a model of chronic-low mortality sepsis, we might not be able to see the subsequent development of an immune-suppressive iNKT cell phenotype. However, this does not mitigate the primary observations that in the face of an acute septic shock/challenge, which activates the liver iNKT cells, these cells appear to play a critical stimulatory role in the developing proinflammatory status of the animal, which is associated with its mortality.

Our findings also diverge from data recently reported by Etogo et al. (53) that indicated that neither a change in cytokine production nor overall mortality was observed in response to acute septic shock in CD1d-deficient mice. Several possible explanations come to mind, as follows: first, genetic deficiency of CD1d on the APC, although producing an animal that is low in iNKT cells, may not be equivalent in all fashions to the iNKT cell-deficient, Jα18−/− mouse; second, the application of the anti-CD1d-blocking Ab may have a more restricted and/or transient effect than what the lack of the CD1d gene produces.

Our study also supports recent observations that iNKT cells have the capacity to mediate the development of a proinflammatory response by their interaction with other local cell type(s), such as tissue macrophages and/or local dendritic cells in the liver (11). Along with our previous observation that Kupffer cell depletion substantially reduces the systemic inflammatory response encountered following septic shock (21, 54), we found in this study that pretreatment with Abs, which block iNKT cell activation (55), significantly inhibited CLP priming for liver macrophage IL-6, but not TNF-α release. This is in keeping with findings we have made.
looking with ex vivo liver macrophage preparations derived from hypotensive shock (56) or CLP (57) mice. At present, we know little concerning the level of action of the iNKT cell’s effect on macrophages. Studies by Wesley et al. (11), in a divergent model of viral infection, suggest that soluble mediators such as IFNs and other cytokines may be important in this study. That said, our observation that liver NKT cell subpopulation differs in the nature of the intracellular cytokine pattern across strains (C57BL/6J vs BALB/c) implies that the production of soluble mediators, such as these cytokines, may not be as critical as cell-cell interactions that the NKT cell has with local liver leukocytes. However, until we can screen the septic liver NKT cells to determine what possible cell surface candidates might be involved here (studies presently outside the scope of this work), we can only speculate as to what those interactions might be.

As to what stimulates/activates the liver iNKT cells during the early response to septic shock, we believe, based on our prior studies (16) and the data presented in this study using pretreatment with blocking Abs against CD1d-mediated Ag presentation, that CD1d and its associated Ag are involved. This is important because the Ag presented by as well as the structure of CD1d is highly conserved. In fact, whereas it was known that the Ags presented by CD1d were glycosylceramides, which could be replaced by the marine sponge product α-Gal-Ser (18, 58), it was not until the last few years that glycosylceramides in the bacterial pathogen pattern (in mammals) as well as a mammalian intracellular source of glycosylceramides, the glycosphingolipids/Gb3, are identified (10). This means that the Ag for presentation via CD1d could be derived from either a microbial agent and/or the host tissue via tissue injury. Another aspect of indirect support for this concept comes from a recent study by Kattan et al. (59), which indicated that supplement/treatment of CLP rats with a component of plasma lipoproteins, apolipoprotein E, an agent that is thought to bind and traffic Ag through CD1d to NKT cells, markedly increased septic morbidity/mortality. As mentioned before, recent studies also indicate that in response to septic shock, iNKT cells express receptors for the bacterial products, such as endotoxin (48), bacterial lipoproteins (49), and CpG DNA (50). However, there is controversy concerning whether or not iNKT cells can directly respond to these stimulants through these classic TLRs. In fact, a recent study by Nagarajan and Kronenberg (60) indicates that at least the response to in vivo endotoxin is mediated not through iNKT cell TLRs or Ov141i TCR/CD1d interaction, but via the stimulation by IL-12 and IL-18 produced by LPS-stimulated APCs. Nonetheless, the host response to CLP is often more complicated than the response to endotoxin or mono-specific Gram-negative bacterial challenge. In this respect, it has been shown that mice, which lack the capacity to respond to endotoxin due to a defect in TLR4, although being resistant to lethality and marked proinflammation response to endotoxin or mono-specific Gram-negative bacterial challenge. In this respect, it has been shown that mice, which lack the capacity to respond to endotoxin due to a defect in TLR4, although being resistant to lethality and marked proinflammation observed in TLR4-competent animals in response to endotoxemia (61, 62), are at least as susceptible, if not more so, to infectious bacterial challenge or polymicrobial septic in the form of CLP (63–69). Thus, whereas our initial data suggest that CD1d may be important to the early innate response to CLP, as used in this study, further studies are needed to determine the exact nature of the stimuli that contribute to iNKT cell response seen in CLP.

As to what happens to these iNKT cells following activation in the septic animal, it is difficult to say, because, as others have reported with diverse iNKT cell TCR stimuli (18, 19, 70), the frequency of CD3 Tetramer+ cells markedly declined in the septic mice despite expressing increased levels of the activation marker CD69 and CD25, thus suggesting that at least a portion of the apparent loss of these cells is actually related to their differentiation during the activation process. Nonetheless, although not an exhaustive assessment of various other lymphoid compartments,
which liver NKT cells were affected by septic shock and their possible contribution to its development from a pathological perspective. Thus, we did not initially use a therapeutic (posttreatment) approach in this study. Nonetheless, we believe these findings suggest that hepatic iNKT cells play a critical role in regulating the innate immune/systemic inflammatory response and survival in a model of acute septic shock.

**Disclosures**

The authors have no financial conflict of interest.

**References**


**FIGURE 7.** BALB/c mice present with a decreased frequency and an increased activation of liver iNKT after CLP. A. Using flow cytometric markers CD3+CD1d tetramerT for mouse NKT cells, we observed that the frequency of these cells was also declining in the liver of CLP BALB/c mice (a similar observation was also made with CD3+DX5+; data not shown). Cumulative alterations in MFI for both CD69 (B) and CD25 (C) for CD1d tetramerT indicate that at 24 h post-CLP, they were also elevated on BALB/c mouse liver NPC. No less than five independent BALB/c mice sampled per group. Results are given as mean ± SEM. *, Indicates significance at p < 0.05 vs the sham group.


