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THE DUAL FUNCTIONS OF RIP1 IN FAS-INDUCED HEPATOCYTE DEATH DURING SEPSIS

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Abstract

In examining the liver's response to sepsis our lab has found that septic hepatocytes exhibit a higher degree of necrosis when compared to septic thymocytes, which typically die through the canonical apoptotic pathway. Recently, an adaptor protein associated with the Fas/TNF death receptor pathway, Receptor Interacting Protein 1 (RIP1) has been shown to be critical for determining whether a cell's death is apoptotic or necrotic. We propose to test the central hypothesis that RIP1 activation by death receptor (Fas) during sepsis determines whether the hepatocytes fate is apoptotic vs. necrotic. We approached this problem by delivering RIP1 siRNA *in vivo* to C57BL/6 mice and observing changes in mortality after septic challenge. Contrary to our hypothesis, RIP1 silenced mice did not survive as long as scrambled sequence injected controls (22.2% vs. 50.0% 14 days post CLP, respectively). When we used a pharmacological/synthetic antagonist of RIP1 kinase, Necrostatin 1 (Nec-1) and examined the mortality of Nec1 treated mice there was no difference from the RIP1 siRNA treated mice (20.0% vs. 22.2% respectively). Further, we carried out a series of comparative histological studies which indicated that septic mice pre-treated with Nec-1 exhibited a preservation of liver glycogen stores (represented by Periodic Acid Schiff [PAS] stain) vs. siRNA treated mice which exhibit lower glycogen stores as well as altered morphology. Furthermore, the histological studies also revealed Nec1 treatment in septic mice increases caspase 3 activity. We speculate that these contradictory findings are due to the dual signaling responsibilities of RIP1, where the RIP1 kinase domain can induce death through Fas ligation while also initiating pro-survival signaling through NF κ B.

Keywords

Apoptotic vs necrotic cell death; RIP1 kinase; siRNA; kinase inhibition; mouse liver; CLP

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Introduction

Sepsis, the leading cause of mortality in surgical and medical intensive care units, is a condition in which the immune system no longer appears to respond to a pathogen adequately, oftentimes leading to multiple organ failure. While significant advances in supportive care have been made, very few therapeutic advances have been made based on mechanistic processes that underpin the development of septic morbidity/mortality. Our laboratory and others have shown that increased apoptosis of immune cells mediated by death receptor ligation contributes greatly to increased morbidity/mortality in the experimental setting. However the mechanism by which non-immune tissue cells are injured/die is less clear.

RIP1 is a multifunctional adaptor protein that has been implicated in many signaling functions associated with apoptosis as well as inflammation. RIP1 has three domains; a kinase domain, intermediate domain and a death domain [1]. The death domain of RIP1 has been shown to associate with many receptors, namely the TNF superfamily *i.e.*, TNFR1, Fas and TRAIL [2] [3]. RIP1 has been shown to be involved in switching apoptotic death to a necrotic cell death in L929 cells treated with the pan-caspase inhibitor z-VAD [3]. Recently, Cho *et.al.* reported that RIP1 and RIP3 associate to form the basis of a signalosome responsible for initiating programmed necrosis [4]. In studies done with RIP gene deficient (–/–) fibroblasts, RIP1 was shown to not be necessary to initiate Fas-induced apoptosis. However, RIP1 was necessary for survival of CD4/CD8 double positive lymphocytes derived from the thymus indicating RIP1 can act as a pro-survival signal in some cases [2]. Downstream pro-survival signals for RIP1 appear to be mediated through signaling via NFκB, p38 and/or JNK.

We previously reported in a mouse model of sepsis, immune cell populations in the spleen and thymus show an increase in Fas mediated apoptotic death. On the other hand, the liver (hepatocytes specifically) while also displaying aspects of an increase in apoptosis also showed that indices of necrosis were increased as well [5]. In light of the above information, we proposed to examine the hypothesis that RIP1 was involved in the alteration of the apoptotic death pathway to “programmed necrosis” in the liver. Below we describe those experiments designed to address this hypothesis and how our results have led us to consider an alternative role for RIP1 in the pathobiology of sepsis in liver, with a particular focus on its alternative role in cellular survival.

Materials & Methods

Mouse Model of Sepsis [6] [7]

C57BL/6 male mice, 8-12 week old, were anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL.). A midline incision (1.5 to 2cm) was made and the cecum was isolated, ligated with 4-0 silk and punctured twice with a 22-gauge needle. The cecum was compressed to extrude a small amount contents from the cecum. The cecum was returned to the peritoneal cavity and both layers were closed with 6-0 nylon sutures (Ethicon, Somerville, NJ). Two to three drops of lidocaine was applied to the peritoneum after closure for analgesia. The mice were resuscitated with 1.0 ml of lactated Ringer's solution subcutaneously. Sham controls were subjected to the same surgical procedure except the cecum was neither ligated nor punctured. All animal experiments, described herein, were done in accordance with National Institute of Health (Bethesda, MD) guidelines and as approved by the local animal use committee at Lifespan-Rhode Island Hospital.

Hepatocyte Isolation

Hepatocytes were isolated using the two-step perfusion method [8]. In brief, mice were anesthetized with isoflurane and subjected to ventral midline incision. The hepatic portal vein was isolated and cannulated with a 27 gauge needle. The liver was perfused for 4 minutes with bicarbonate buffer (HBSS, EDTA, HEPES and NaHCO_3) using a peristaltic pump (Masterflex; Cole-Palmer, Vernon Hills, IL). The liver was then perfused with a warm collagenase solution (Leibovitz's modified L-15 medium, glucose and collagenase IV) for 15 minutes, teased apart in the collagenase solution; percolated through a 25mL pipette and filtered through two layers of gauze. The resultant cells were suspended in RPMI 1640 medium + 5% fetal calf serum and centrifuged at $30 \times g$ for 4 minutes and the hepatocytes were then resuspended in CER I lysis buffer (NE-PER kit; Pierce Biotechnology, Rockford, IL.) to isolate cytosolic as well as the nuclear fractions.

Immunoprecipitation

Hepatocytes were lysed and incubated with Fas antibody ([M-20] SC-716, Lot D1304; Santa Cruz Biotechnology Inc., Santa Cruz, CA.) for an hour. The antibodies were pulled down with protein G coated agarose beads (SC-2003 Lot B1810; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Western Blot Analysis

Protein lysates of mouse hepatocytes were run on 10% Tris-glycine gels (Invitrogen, Carlsbad, CA). Blotting procedures, chemiluminescent detection, and densitometric analysis were performed as previously described by our laboratory [9]. Membranes were probed with either RIP1 polyclonal antibody (Cat. # 610458, Lot 04047 BD Transduction Laboratories™, San Diego, CA.) or NF κ B p65 polyclonal antibody (ab7970, Lot 870107 Abcam, Cambridge, MA.) and bands were detected at 76kD and 65 kD, respectively. GAPDH and Histone H2 antibodies were used as loading controls for the cytosolic and nuclear fractions, respectively.

Hydrodynamic siRNA Delivery

Mice were injected with 50 μ g RIP1 siRNA (5'-GAAUGAGGCUUACAACAG-3'; Dharmacon Inc., Rockford, IL.) in 2 mL saline via the lateral tail vein over an approximate 5 second period, as previously described [9] [10] [11]. Specificity of RIP1 knock down was determined by western analysis of hepatocyte lysates derived from mice that had received RIP1 siRNA or scrambled siRNA sequence treatment prior to the CLP procedure.

RIP1 Kinase Inhibition

Mice were injected intraperitoneally (*i.p.*) with 0.125mg Necrostatin 1 (Nec1) (Sigma Aldrich, St. Louis, MO.) in 0.2% DMSO daily for two days prior to CLP. Immediately following CLP the animals were given another 0.125mg dose of Nec1. Control animals were only injected with 0.2% DMSO. Subsequent Nec1 and control injections were given daily for the duration of the survival study. Twenty-four hours post-CLP the mouse livers were perfused and harvested for analysis.

Quantification of Serum Chemokines/Cytokines and Liver Enzymes

Blood was collected into a heparinized syringe via cardiac puncture of control or Nec1 treated mice. The red blood cells were separated by centrifugation and the serum was removed and stored at -80°C until analysis. IL-10, IL-6, MCP-1 and TNF- α levels were measured in the serum using a mouse cytometric bead array (BD cytometric bead array mouse inflammation kit, BD Biosciences, San Jose, CA) according to the manufacturers instructions. Alanine aminotransferase, (ALT) and Aspartate aminotransferase, (AST)

enzyme activities were measured using ALT and AST reagent sets (Biotron Diagnostics, Hemet, CA.).

Periodic Acid-Schiff (PAS) Stain of Glycogen Stores Quantification

Histological slides were stained with PAS and scanned with an Aperio Scan scope-CS (Aperio Technologies, Inc., Vista, CA) and 5-10 fields were selected at 200x magnification for each specimen. Area measurements were performed with iVision software (BioVision Technologies, Exton, PA). Positive staining was defined through intensity thresholding for total area stained in the field and percent region of interest (%ROI). A Pathologist specializing in gastroenterology, blinded to the groups, reviewed the slides for apoptosis and other morphological differences between samples.

Caspase 3 Staining

Mice were injected with Nec1, (as described above), or 0/2% DMSO, as a control. The two groups, Nec1 and DMSO were subjected to CLP or Sham surgery and the livers were perfused and fixed 24 hours post surgery. These livers were paraffin embedded and histological slides were cut. Tissue sections 5 microns in thickness were deparaffinized and rehydrated through graded alcohols to water. Tissue sections were subjected to heat induced antigen retrieval in 10 mM citrate buffer (pH 6.0) for 11 min, blocked using Peroxidase Block (Dako, Carpinteria, CA.) for 5 min. The slides were then blocked using Avidin Biotin Block for 10 minutes for each solution (Vector Labs, Burlingame, CA.). Sections were incubated with caspase 3 antibody overnight at 4°C. The slides were developed using the Envision Plus Dual Link and the Liquid DAB+ substrate kits (Dako, Carpinteria, CA.). The slides were counterstained in hematoxylin, dehydrated through graded alcohols to xylene and mounted with permount. The amount of Caspase 3 staining was quantified in the same manner described above.

Presentation of Data and Statistical Analysis

The results are presented as a mean $\pm$ SEM for each group. Mann-Whitney U-test was used to analyze the comparison between 2 groups. For data comparing more than 2 groups, a One- way ANOVA was executed followed by a Student-Newman-Keuls' test to determine the differences. The survival data were compared using the Log Rank test. $P = 0.05$ was considered statistical significance.

Results

Inhibition of Fas-FasL signal alters septic mouse liver RIP1 gene expression

We have previously reported that Fas-FasL signaling played a role in inducing the onset of apoptosis, most markedly in lymphoid tissues of the spleen [9] and small intestine [12]. Additionally, we reported that Fas-FasL signaling also seems to effect liver cell survival/damage [9]. While we demonstrated modest evidence of apoptosis, the majority of data for the liver point at necrotic and/or necro-apoptotic/non-classical apoptotic cell death [5]. One proposed role of RIP1 was in the regulation of the induction of classic apoptotic vs. programmed necrotic cell death [13]; we initially tested the hypothesis that RIP1 was involved in the alteration of the apoptotic death pathway leading to "programmed necrosis" in the liver. Therefore, in our initial experiment we simply attempted to determine the extent to which signaling via Fas-FasL led to the upregulation of RIP1 following the onset of sepsis. We found in doing anti-Fas antibody pull down of whole liver homogenates that when probed for the presence of RIP1 that there was a trend toward higher levels RIP1 association in the septic mice as compared to sham mice (Figure 1).

Knock-down of RIP1 gene expression decreased (not increased) septic mouse survival

Since RIP1 appeared to be associated with Fas and presumably death receptor-induced death in the liver during sepsis, we subsequently chose to determine if RIP1 expression played a role in septic mouse survival. To test this, we knocked down expression of RIP1 via *in vivo* hydrodynamic delivery of RIP1 siRNA (50 µg/ 2 mL saline/ mouse via the tail vein over 5 seconds) in a mouse model of sepsis and determined percent survival vs. control siRNA injected mice. Our rationale was that by knocking down RIP1 expression the death domain association with the Fas Associated Death Domain (a Fas-FasL downstream signaling intermediate) would be disrupted, thereby, reducing apoptosis (in the liver) and increasing survival. To demonstrate how effective hydrodynamic administration of RIP1 siRNA was in knocking down RIP1 mRNA, we measured protein levels, via western blot, of RIP1 in whole liver of both RIP1 siRNA and scrambled sequence siRNA (control) treated mice in response to CLP. We noted that there was a substantial reduction in the amount of RIP1 expression evident at 24 hours post-RIP1 siRNA administration as compared to control siRNA treatment (Figure 2). Surprisingly, when we assessed the effect of RIP1 siRNA pretreatment on septic mouse survival, as shown in Figure 3, we found that the RIP1 siRNA treated animals did poorer, which was the opposite of what our initial hypothesis would have predicted. Over the course of 14 days the animals that received RIP1 siRNA had a 22.2% survival rate (n=18) compared to 50.0% survival (n=18) in the control group. This indicates that RIP1 appears to be necessary for survival in septic injury of C57BL/6 mice.

Pharmacological inhibition of RIP1 kinase does not improve survival but alters NFκB translocation in response to sepsis

The observation that inhibition of RIP1 expression actually suppressed survival in septic animals, did not support our original hypothesis that; RIP1 expression was involved in septic morbidity/mortality (Figure 3). Thus, this implies that RIP1's capacity to contribute to the onset of programmed cell death, apoptotically and/or necrotically, may not be its central role in the septic animal. Given the reported ability of RIP1 to increase survival as well as inflammatory cell signaling, via, NFκB activation etc, in other model systems [1] [13], we alternatively hypothesized that the presence of the RIP1 protein was necessary to propagate signaling involved in supporting the animal's overall survival. To assess this, we attempted to specifically inhibit RIP1 kinase activity (i.e., death induction) while preserving the other RIP1 domains (death domain and the RIP homotypic interaction motif) for use in functions like NFκB signaling, etc. [14], via daily *in vivo* treatment with the RIP1 kinase inhibitor, Nec1 [15] [16]. Similar to the study we performed with RIP1 siRNA animals, we assessed the survival of Nec1 treated mice to polymicrobial sepsis (Figure 4). What we observed was 25% (n=16) of the Nec1 treated mice survived vs. 50% (n=16) of the control mice. These results were similar to the RIP1 siRNA study suggesting the RIP1 kinase domain is critical in the animal's survival following murine polymicrobial sepsis. To the extent that kinase inhibition via Nec1 treatment altered NFκB nuclear translocation/activation (something that would not be anticipated [14]), we noted a significant change between the degree of NFκB nuclear translocation seen in the septic DMSO vehicle treated mice and the Nec1 treated animals (Figure 5) indicating the kinase domain is necessary for NFκB translocation.

Necrostatin1 treatment increases markers of liver injury and pro-inflammatory cytokine levels

To determine if Nec1 treatment had any effect on the inflammatory response, we measured serum cytokines by cytometric bead array (Figure 6). Nec1 treatment appears to significantly increase TNF-α, MCP-1, IL-10 and IL-6 compared to DMSO CLP group. Furthermore, Nec1 seems to have no apparent effect on liver damage during sepsis as ALT and AST activities remain the same in both Nec1 and control groups.

Necrostatin 1 treatment alters liver glycogen stores

We assessed the effect of *in vivo* Nec1 pre-treatment on liver metabolism following the onset of sepsis. To do this, we examined the morphological changes in the liver after 2 days of pre-treatment (0.125mg Nec1 in 0.2% DMSO *i.p. once per day*) followed by another injection immediately post-CLP. The livers were taken 24 hours post-CLP, fixed and stained with Periodic Acid-Schiff stain (PAS) as a measurement of glycogen stores/production in the liver. Here we noted obvious changes in glycogen stores, in Nec1 injected CLP mice vs. sham and vehicle treated CLP mice. The sham liver sections are replete with glycogen stores while vehicle treated CLP sections lost their glycogen stores. Nec1 pre-treatment appeared to preserve glycogen stores in the liver (Figure 7). These results were confirmed by a pathologist blinded to the treatments of the samples. Both PAS and hematoxylin and eosin (H&E) stained slides were analyzed by the pathologist. The presence of glycogen in the PAS stained slides did not correspond to any pathology with respect to the H&E slides. In fact, there were not a lot of substantial morphological changes noted in the H&E slides except for mildly reactive hepatocytes, which were seen in roughly equal distribution between all of the experimental groups.

Necrostatin 1 treatment increases activity of caspase 3 in the liver

We then interrogated the level of activated caspase 3 in the same samples we had previously stained for glycogen. Although the pathologist observed minimal signs of apoptosis, caspase 3 activity is significantly higher in Nec1 treated livers than DMSO treated or sham livers (Figure 8).

Discussion

We have shown that mice in which we had knocked down RIP1 gene/protein expression with siRNA treatment exhibit a diminished capability to withstand a septic challenge. Furthermore, use of a pharmacological inhibitor of the RIP 1 kinase domain yields similar results. These two results indicate that the kinase domain of RIP1 is necessary for survival in a murine model of polymicrobial sepsis. We have also demonstrated by western blot that blocking the RIP1 kinase domain by Nec1 treatment decreases NF κ B p65 nuclear translocation in septic conditions. This result supports reports that RIP1 is integral to inducing NF κ B translocation but, suggests the kinase domain may be involved contrary to the reports that implicate the intermediate domain [19] [20]. We observed these effects in the results of the serum cytokine measurements, namely, IL-6, IL-10, MCP-1 and TNF- α , were all significantly elevated in Nec1 treated mice. Increased cytokines, together with the increased levels of hepatic enzymes ALT and AST, suggests that Nec1 treatment is damaging to hepatocytes.

Through our histological studies of the livers of RIP1 gene knock down mice post-CLP, we observed an altered distribution of glycogen in the hepatocytes. Similarly, inhibition of RIP1 kinase by Nec1 also displays alteration in glycogen distribution. When contrasted with the levels of glycogen in scrambled sequence and vehicle controls these results suggest the kinase domain of RIP1 may contribute to cell signaling that alters cellular energy consumption during sepsis. These data are in keeping with Zhang *et al* [17] who showed that RIP1, (through its homotypic interaction with RIP3), was necessary for regulation of glycogen phosphorylase activity. They further showed that RIP1 and RIP3 were necessary for activation of the metabolic enzyme, glycogen phosphorylase, *in vivo*. Furthermore, another group recently published that the RIP1 kinase domain is necessary for the stable formation of Complex IIb [4]. Based on the aforementioned published results we suspect what might be happening in our system is that Nec1 has inhibited the kinase domain of RIP1; therefore, RIP1 and RIP3 can no longer autophosphorylate each other to regulate

glycogen phosphorylase [17]. As a result, more glycogen remains in the liver during the initial systemic inflammatory response, which, we presume, provides energy to activate caspases (see Figure 8). It is important to note that, RIP1, in addition to its versatile signaling capability, after initiator caspases are activated is cleaved by caspase 8 thus eliminating the necroptotic pathway. Once RIP1 is cleaved it becomes ubiquitinated and subsequently degraded by the proteasome [19]. As we have demonstrated here that RIP1 is essential for necroptotic death through Fas and others have reported that RIP1 is a critical glycogen metabolism regulator by its interaction with RIP3 [17][18]. Perhaps the continuous inhibition of RIP1 kinase by Nec1 during sepsis leads to a condition where once the glycogen is consumed in the liver apoptosis will cease. As such, as long as there is energy present, *i.e.* glycogen, the effect of Nec1 on the ability of RIP1 and RIP3 to associate and form a functional complex IIB might then be hidden. Once the energy is consumed we begin to see differences in the survival of Nec1 treated animals vs. control. Nec1 treated mice displayed significantly lower NF κ B translocation 24 hours after CLP (see Figure 5). We would expect NF κ B translocation to be an early event peaking 2-4 hours after CLP [23]. Coupled with the significant increase in systemic levels of IL-10, IL-6, MCP-1 and TNF- α at 24 hours post-CLP in the Nec1 treated group (see Figure 6) may suggest that the cytokines are from other sources and not exclusively from the hepatocytes.

Nevertheless, the data reported here suggest several possible roles for RIP1 in the liver during septic injury. More questions remain to be answered, like what is the effect of long term Nec1 administration in these animals. As shown in the Nec1 survival study these animals have a modest early survival benefit but go on to die in a sustained manner over time, while the control group survival curve plateaus. It would be interesting to examine the livers at multiple time points, *i.e.* 6 hours and seven days, to delineate any other changes in death markers, serum cytokines, and architecture in the liver.

Finally, recent publications have linked IKK (a regulator of NF κ B translocation), to autophagy, whereby IKK is sufficient to induce autophagy in nutritionally deprived cells [21]. Given the role of RIP1 in energy metabolism, as depicted by the effect of RIP1 silencing/Nec1 treatment on liver glycogen levels, and as a member of the IKK complex it may be worth examining markers of autophagy after Nec1 treatment in the liver.

In conclusion, the data presented here suggests that the kinase domain of RIP1 plays a role in the alterations seen in cell survival and death in the liver during sepsis. We speculate these confounding results are due to the dual signaling responsibilities of RIP1. RIP1 through its kinase domain can complex with RIP3 to form complex IIB, inducing necrotic death through Fas ligation. However, RIP1 can also initiate pro-survival signaling and the maintenance of a competent immune response through NF κ B activation, which may account for its effect on septic animal survival.

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Fig. 1.

Fas signaling upregulates RIP1 expression. Whole-liver homogenates from septic or sham mice were immunoprecipitated with Fas antibody and immunoblotted for the presence of RIP1. The image is representative of a typical Western blot, sham on the left and CLP on the right. The graph is representative of the densitometry quantitation ($n = 4$) expressed as image density value over GAPDH. Not statistically significant (n.s.), $P > 0.05$, Mann-Whitney U test.

Fig. 2.

Receptor interacting protein 1 siRNA efficiency. Whole-liver homogenates from RIP1 or scrambled siRNA-treated septic mice were immunoprecipitated with Fas antibody and immunoblotted for RIP1. Western blot image represents scrambled sequence mice on the left and RIP1 siRNA mice on the right. The graph represents the densitometry measurements of the Western blot and illustrates the fold change in integrated density value of RIP1 siRNA compared with scrambled sequence over GAPDH. * $P < 0.05$, Mann-Whitney U test, $n = 4$ /group.

Fig. 3. Receptor interacting protein 1 siRNA survival study. Receptor interacting protein 1 siRNA (n = 18) and scrambled sequence (n = 18) mice were subjected to CLP and observed over 14 days for survival. Fifty percent of the mice given scrambled siRNA survived vs. 22.2% of RIP1 siRNA mice. * $P < 0.05$, log-rank test.

Fig. 4. Necrostatin 1 survival study. Necrostatin 1-treated ($n = 16$) and DMSO injection control ($n = 16$) mice were subjected to CLP and observed over 14 days for survival. Fifty percent of the mice given 0.2% DMSO survived vs. 25% of Nec1-treated mice. $*P < 0.05$, log-rank test.

Fig. 5.

Necrostatin 1 treatment does not significantly alter NF- κ B nuclear translocation. Nuclear proteins from hepatocytes treated with either Nec1 or DMSO control, from sham and CLP mice, were separated by polyacrylamide gel electrophoresis and immunoblotted for NF- κ B p65. Image is representative of a typical blot. Intensity of the signal was measured by densitometry and expressed as a ratio of p65 over histone H2. Sham (n = 3), CLP (n = 4-7).

* $P < 0.05$ vs. DMSO control, Mann-Whitney U test.

Fig. 6.

Inflammatory cytokines and liver enzymes in the serum increase in Nec1-treated septic mice. Serum cytokines were measured by cytometric bead array ($n = 3-4$) per group. Serum ALT and AST levels ($n = 3-4$) per group. * $P < 0.05$ vs. sham; # $P < 0.05$ vs. DMSO; n.s. $P > 0.05$, one-way ANOVA.

Fig. 7.

Comparison of liver glycogen in RIP1-silenced and Nec1-injected mice. Periodic acid Schiff-stained liver sections of Nec1, control (0.2% DMSO), and RIP1 siRNA- treated CLP mice and Nec1-treated sham mice. Periodic acid Schiff shows up as red staining in these samples. The graph indicates a quantification of PAS staining in the slides. Sham column is representative of a combination of sham animals injected with 0.2% DMSO, Nec1, and no treatment. Control column is representative of three animals injected with 0.2% DMSO and subjected to CLP. Necrostatin 1 column is representative of eight animals injected with Nec1 and subjected to CLP. siRNA column represents three animals injected with RIP1 siRNA. * $P < 0.05$ vs. DMSO control, one-way ANOVA, $n = 3-8$ /group.

Fig. 8.

Necrostatin 1 treatment increases caspase 3 activation at 24 h after CLP. Necrostatin 1-treated and DMSO injection control CLP livers were compared with Nec1- treated sham livers to determine levels of active caspase 3. Graph represents the percent of active caspase 3 stain detected in the region of interest (ROI). * $P < 0.05$ vs. DMSO control, one-way ANOVA ($n = 4/\text{group}$).