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A novel synthetic, host-defence peptide protects against organ injury/dysfunction in a rat-model of severe hemorrhagic shock

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Running head: Pep19-4LF in hemorrhagic shock

MINI-ABSTRACT (50 words)

This study evaluated the role of host-defence/antimicrobial peptides in trauma-associated hemorrhagic shock (HS). Trauma-associated HS resulted in the release of the host defence peptide LL-37. The synthetic host defence peptide Pep19-4LF attenuated the HS-associated organ injury/dysfunction in the rat by activating pro-survival pathways and by inhibiting local and systemic inflammation.

ABSTRACT

Objective: To evaluate (i) levels of the host-defence/antimicrobial peptide LL-37 in patients with trauma and hemorrhagic shock (HS) and (ii) the effects of a synthetic host-defence peptide on multiple organ failure (MOF) associated with HS in rats.

Background: There are no specific interventions, which reduce MOF in HS. Injury and infection triggers the release of host-defence/antimicrobial peptides, the function of which in HS is unknown.

Methods: LL-37 was measured in plasma from 48 trauma/HS patients admitted to an urban major trauma center. Rats were submitted to HS followed by resuscitation and treated during resuscitation with the synthetic host-defence/antimicrobial peptide Pep19-4LF (66 or 333 µg/kg x h) or vehicle.

Results:

Plasma levels of LL-37 were elevated in patients with trauma/HS. In anesthetized rats subjected to HS and resuscitation, Pep19-4LF attenuated the HS-induced kidney dysfunction, liver injury and lung inflammation. Pep19-4LF enhanced (kidney/liver) the phosphorylation of (i) protein kinase B (Akt) and (ii) endothelial nitric oxide synthase (eNOS) and, hence, activated the Akt/eNOS survival pathway. Pep19-4LF also attenuated the HS-induced (i) translocation of p65 from cytosol to nucleus, (ii) phosphorylation of IKK on Ser^{176/180} and (iii) phosphorylation of IκBα on Ser^{32/36} resulting in inhibition of NF-κB and formation of NF-κB-dependent pro-inflammatory cytokines. Pep19-4LF prevented the release of TNFα caused by heparan sulfate in human mononuclear cells by binding to this DAMP.

Conclusions: Trauma-associated HS results in release of the host-defence/antimicrobial peptide LL-37. The synthetic host-defence/antimicrobial peptide Pep19-4LF attenuates the organ injury/dysfunction associated with HS by activating pro-survival and anti-inflammatory pathways.

Keywords

Antimicrobial peptides, hemorrhagic shock, multiple organ failure, NF- κ B pathway, LL-37

INTRODUCTION

Severe injuries account for 9 % of the deaths worldwide.¹ Although guidelines for the early management of hemorrhagic shock (HS; including resuscitation and organ support strategies) have decreased the rates of immediate (on scene/within 60min) and early (emergency department and operating room/within 1-4h) deaths,² post-injury multiple organ failure (MOF) is still associated with significant morbidity and mortality². Therapeutic agents that reduce the incidence and severity of MOF following HS could, therefore, have a major global impact on patient outcomes and resource utilization. The MOF after HS is associated with excessive systemic inflammation, secondary to the release of damage-associated molecular patterns (DAMPs) from extensive tissue damage and ischemia reperfusion injury.³ To date, there are no specific pharmacological interventions used clinically to prevent MOF following/associated with HS.

Host-defence/antimicrobial peptides are known for over 100 years and form part of the innate immune system of insects, plants, and vertebrates by defending the host against invading microorganisms.^{4,5,6} Although these peptides differ in sequence and structure, they are predominantly short (10 – 50 amino acids) amphipathic molecules.⁶ The most extensively studied host-defence/antimicrobial peptide in humans is the cathelicidin-derived peptide LL-37.⁶ LL-37 exhibits strong bactericidal properties, but at the same time neutralizes pathogenic factors released during injury/infection including lipopolysaccharide (LPS) or lipoprotein (LP).⁷ In addition to the interaction with PAMPs (pathogen associated molecular patterns), LL-37 modulates the inflammatory response induced by DAMPs and, hence, modulates many physiological host functions including inflammation, angiogenesis and wound healing.^{6,8} Thus, host-defence/antimicrobial peptides are attractive candidates for the development of novel therapeutic interventions in infectious and inflammatory diseases.⁶ The systemic application of LL-37 as a potential drug in man, however, is limited by its toxicity.^{9,10} The challenge is to develop synthetic

host-defence/antimicrobial peptides (mimetics) that have little or no adverse effects. Peptide 19-4LF (Pep19-4LF) is one of several new synthetic host-defence/antimicrobial peptides, which belongs to the class of synthetic anti-lipopolysaccharide peptides (SALP = synthetic anti-LPS peptides).^{11,12} However, in addition to binding LPS, these peptides exhibit potent anti-inflammatory effects in experimental sepsis by interacting with a variety of PAMPs and DAMPs.^{13,12,14,15}

The role of host-defence/antimicrobial peptides in HS is unknown. Therefore, the aims of the present study were to (i) investigate the plasma levels of LL-37 in patients with trauma with or without HS and (ii) to explore the effects of Pep19-4LF on the organ injury/dysfunction associated with HS. We report here for the first time, that (i) the plasma levels of LL-37 are elevated in patients with trauma/HS (when compared with trauma without HS) and that (ii) Pep19-4LF attenuates the HS-associated organ injury/dysfunction. Mechanistically, Pep19-4LF has pro-survival and anti-inflammatory properties, as it activates the Akt/eNOS cell survival pathways and attenuates the activation of the nuclear factor kappa B (NF- κ B) pathway in the rat *in vivo*. Moreover, Pep19-4LF exhibits its anti-inflammatory activity, at least in part, by directly interacting/binding to the DAMP heparan sulfate in human mononuclear cells (MNCs) *in vitro*. These data suggest that Pep19-4LF may prevent the MOF in patients caused by trauma-associated HS, which, in turn, may improve outcome in these patients.

METHODS

Study population and human outcome measurements

Details relating to the study population and human outcome measurements are provided in the *supplemental*.

Use of human subjects-ethic statement

All patients or their legal representative gave written informed consent. Before inclusion of the first individual, the local National Health Service Research Ethics Committee (REC: 07/Q0603/29) approved this study, which was performed in accordance with the Declaration of Helsinki in its latest form. The use of plasma from healthy volunteers was approved by the ethics committee of the University Hospital Aachen (EC Nr. 206_09, 5 January 2010).

Use of experimental animals-ethic statement

The experimental protocols used in this study have been approved by the Animal Welfare Ethics Review Board (AWERB) of Queen Mary University of London and the study was performed under license issued by home office (Procedure Project License; PPL: 70/7348). Animal care was in accordance with the Home Office guidance on Operation of Animals (Scientific Procedures Act 1986) published by Her Majesty's Stationery Office and the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Hemorrhagic shock and quantification of organ injury and dysfunction

This study was carried out on 46 male Wistar rats (Charles River Ltd, Margate, UK) weighing 230-350 g receiving a standard diet and water *ad libitum*. Hemorrhagic shock and quantification of organ injury and dysfunction were performed as described previously in this journal (*supplemental Fig. 1*).¹⁶

Experimental design

Rats were randomly allocated to the following groups: Sham + vehicle (n = 11), sham + Pep4LF (n = 6), HS + vehicle (n = 12), HS + Pep4LF-LD (n = 4), HS + Pep4LF-HD (n = 8). Rats were administered vehicle (saline 1.5 ml/kg/h) or Pep19-4LF (low dose (LD) = 66 µg/kg x h; high dose (HD) = 333 µg/kg x h) continuously for 4 hours after resuscitation using infusion pump for rodents

(PHD2000, 70-2000; Harvard apparatus Massachusetts, U.S). The doses of Pep19-4LF used in this study were based on efficacy seen in previous *in vitro* and *in vivo* studies.^{13,12,14,15}

Immunoblot analysis

Western blot was performed as previously described.¹⁷

Cytokine analysis

Concentrations of serum cytokines were determined using a commercially available cytometric bead array (BD Biosciences, San Jose, CA or BioLegend, San Diego, CA) following the manufacturer/product specific protocol.

Immunohistochemistry

Lung samples were obtained at the end of the experiment and fixed in formalin for 48 h and immunohistochemistry was performed as described previously.¹⁷

Determination of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined as an indicator of neutrophil accumulation into the lungs and performed as described previously.¹⁶

Human mononuclear cells study

Mononuclear cells (MNC) were isolated from heparinized blood samples obtained from healthy donors as described previously.¹¹

Isothermal titration calorimetry

Microcalorimetric experiments of peptide binding to heparan sulfate were performed using isothermal titration calorimeter as described before.¹⁵

Statistics

Unless otherwise stated, the data is expressed as median and standard error or described in box and whisker format showing medians, interquartile ranges and full ranges of n observations, where n represents the number of animals/experiments studied. Statistical analysis was carried out using Prism 6 for Mac OS X (GrapPad, San Diego, CA, USA). The distribution of the data was assessed using D'Agostino's K-squared test or Kolmogorov–Smirnov test. Unless otherwise stated, normal distributed data were assessed by 1 or 2-way analysis of variance followed by Bonferroni post hoc test. Unless otherwise stated, not normally distributed data were analyzed with a non-parametric test (Kruskal-Wallis followed by Dunn test). A $p < 0.05$ was considered to be significant.

RESULTS

Plasma concentrations of the host defence AMP cathelicidin LL-37

Figure 1 shows plasma concentrations of LL-37 in healthy volunteers and trauma patients recruited from an urban major trauma center. The median age of the healthy volunteers were 47 (32-53) years with 80% male. Further demographic and clinical parameters of trauma patients are described in table 1. Admission blood samples of trauma patients were obtained within 2 h of injury (Table 1). When compared to healthy volunteers, trauma patients (n = 24) and trauma hemorrhage patients (n = 23) (defined as patients who received greater than or equal to two units of packed red blood cells on admission) showed significantly higher plasma levels of LL-37. When compared to trauma patients, the plasma levels of LL-37 were significantly higher in trauma hemorrhage patients (Fig. 1).

Pep19-4LF attenuates the decline in blood pressure during resuscitation after HS

When compared to sham-operated rats, HS-rats treated with vehicle showed a significant decline in mean arterial pressure (MAP) after resuscitation (Fig.2). Intravenous administration of high-dose Pep19-4LF (333 µg/kg x h, in 0.9% saline) significantly attenuated the decline in MAP observed during the resuscitation period in HS-rats, while the low dose of Pep19-4LF (66 µg/kg x h, in 0.9% saline) had no significant effect. In contrast, the high dose of Pep19-4LF had no effect of MAP in sham-operated rats (Fig. 2).

Pep19-4LF attenuates the organ injury and dysfunction caused by HS

When compared to sham-operated rats, rats subjected to HS treated with vehicle exhibited a renal dysfunction, as indicated by significant increases in serum urea (Fig. 3A) and creatinine (Fig. 3B), and a significant decline in creatinine clearance (Fig. 3C). HS-rats also exhibited significant

increases in alanine aminotransferase, aspartate aminotransferase, amylase and lipase, indicating the development of liver and pancreatic injury, respectively (Fig. 3E-G). In addition, HS-rats exhibited a significant increase in serum lactate, indicating global ischemia (Fig. 3D). When compared to vehicle-treated rats, intravenous administration of high-dose Pep19-4LF significantly attenuated the organ injury and dysfunction as well as the rise in lactate caused by HS (Fig. 3A-H). Although the lower dose of Pep19-4LF reduced pancreatic injury, it had no effect on any of the other parameters measured (Fig. 3A-H).

Pep19-4LF attenuates lung inflammation caused by HS

Having shown that Pep-4LF-treatment attenuates kidney dysfunction and liver injury, we next investigated the effects of Pep-4LF on lung inflammation measured as recruitment of macrophages (CD68-positive cells) and neutrophil activation (MPO activity) into the lung. When compared to sham-operated rats, we found a significant increase in CD68-positive cells and MPO-activity in lungs of HS-rats treated with vehicle (Fig. 4A-C). Treatment of HS-rats with Pep19-4LF significantly attenuated the recruitment of macrophages and neutrophil activation caused by HS (Fig. 4A-C).

Pep19-4LF attenuates the increase in interleukin (IL)-6 and monocyte chemotactic protein-1 (MCP-1) caused by HS

Having shown that Pep-4LF attenuates the activation of NF- κ B in kidney and liver, we next investigated the effects of Pep-4LF on the formation of pro- and anti-inflammatory cytokines caused by HS. When compared to sham-operated rats, HS-rats treated with vehicle developed a significant increase in serum IL-6 and MCP-1. In HS-rats, we also observed increases in serum IL-10 and C-X-C motif ligand 1 (CXCL1), but these effects were not significant (Fig. 5A,B).

Treatment of HS-rats with Pep19-4LF abolished the increases in IL-6, MCP-1, IL-10, and CXCL1 caused by HS (Fig. 5A-D).

Pep19-4LF attenuates the activation of NF- κ B (liver and kidney) caused by HS

Having shown that Pep19-4LF significantly attenuates kidney dysfunction and liver injury caused by HS, we next explored the potential mechanism(s) underlying the observed beneficial effects of high dose of Pep19-4LF. When compared to sham-operated rats, HS-rats treated with vehicle exhibited a significant increase in the nuclear translocation of the p65 subunit of NF- κ B in kidney and liver (Fig. 6A,E) as well as a significantly increased degree of phosphorylation of I κ B kinase α and β (IKK α/β) on Ser^{176/180} and of I κ B γ on Ser^{32/36} in both kidney and liver (Fig. 6B,C,F,G). The intravenous administration of high-dose Pep19-4LF attenuated the nuclear translocation of the NF- κ B subunit p65, the phosphorylation of IKK α/β on Ser^{176/180}, and of I κ B α on Ser^{32/36} in both liver and kidney (Fig. 6B,C,F,G).

Pep19-4LF increases activation of Akt and eNOS in kidney and liver after HS

As activation of the Akt-survival pathway is known to reduce HS-induced organ dysfunction^{16,17} we next investigated whether the high dose of Pep19-4LF activates Akt in kidney and liver of HS-rats (Fig. 6D,I). Moreover, we investigated the phosphorylation of eNOS in kidney and liver (Fig. 6E,J). When compared to sham-operated rats, HS-rats treated with vehicle showed a significant reduction in the phosphorylation of Akt on Ser⁴⁷³ and eNOS on Ser¹¹³ in both kidney and liver (Fig. 6D,E,I,J). In contrast, treatment of HS-rats with Pep19-4LF attenuated the decline in Akt phosphorylation on Ser⁴⁷³ and of eNOS phosphorylation on Ser¹¹³ in kidney and liver, when compared to HS rats treated with vehicle (Fig. 6D,E,I,J).

Pep19-4LF inhibits the heparan sulfate-induced TNF α secretion in human peripheral blood mononuclear cells

As discussed above, traumatic injury and trauma-associated HS result in release a variety of endogenous TLR ligands, including heparan sulfate ¹⁸. We report here that heparan sulfate stimulates the release of TNF α from human MNCs, and that this effect is reduced/abolished in a concentration-dependent manner by Pep19-4LF (Fig. 7A).

Pep19-4LF exhibits a strong binding to heparan sulfate

To gain a better understanding of the mechanism(s) by which Pep19-4LF reduces the formation of TNF α in human MNC challenged with heparan sulfate, we investigated the potential binding of Pep19-4LF to heparan sulfate by isothermal titration calorimetry. There was a strong exothermic reaction between the two reactants, running into a saturation of binding at higher mass ratios (Fig. 7B). This high affinity binding was characteristic for a chemical complex reaction, and may explain that the binding epitopes of heparan sulfate to TLR4 are hidden by the peptide.

Pep19-4LF does not show cytotoxic activity

Finally, possible cytotoxic effects of Pep19-4LF were studied in the hemolysis assay with RBC as sensitive target cells for cytotoxicity. Pep19-4LF caused only a very small degree of hemolysis in concentrations of up to 100 μ g/ml (Fig. 7C).

DISCUSSION

The main findings of this study are that (i) plasma levels of the host-defence/antimicrobial peptide LL-37 are elevated in patients with trauma-associated HS, and that (ii) the synthetic host-defence/antimicrobial peptide Pep19-4LF attenuates the renal dysfunction and liver injury caused by HS in the rat. Pep19-4LF also reduced both the local (lung) and the systemic (rise in plasma IL-6 and MCP1) inflammation caused by HS. Pep19-4LF activated the Akt/eNOS cell survival pathway and attenuated the activation of NF- κ B in kidney/liver of HS-rats. Moreover, Pep4LF attenuated the release of TNF α caused by the DAMP heparan sulfate in human peripheral blood MNCs.

Trauma-hemorrhage is associated with the release of DAMPs (from the host) and PAMPs (from bacteria after bacterial translocation from the gut), which drive inflammation and contribute to tissue/organ-damage. It is unclear whether trauma-hemorrhage also triggers the release of host-defence/antimicrobial peptides, but the serum of trauma patients does have an enhanced antimicrobial capacity, which limits the growths and ultimately kills Gram-negative and Gram-positive bacteria.¹⁹ We report here for the first time that trauma leads (within 2 h) to a significant increase in the plasma levels of the host-defence/antimicrobial peptide LL-37. Most notably, the highest levels of LL-37 were found in patients with trauma complicated by severe hemorrhage (Fig.1). LL-37 is primarily released by T cells and natural killer cells²⁰. Notably, natural killer cells are elevated and activated within 2 h of injury in trauma patients who later develop MOF, suggesting that these cells contribute to the rapid rise in plasma LL-37.²¹

Having found that trauma-HS increases plasma LL-37, we next performed a reverse translational approach and investigated whether pharmacological intervention with a synthetic host-defence/antimicrobial peptides attenuates the MOF associated with HS in rats. As the therapeutic

use of LL37 in man is limited by its systemic toxicity in therapeutic doses,^{9,10} we synthesized Pep19-4LF, which does not cause any significant adverse effects (hemolysis) in the doses used (Fig. 7C).

The administration of Pep19-4LF significantly attenuated the fall in blood pressure (Fig. 2) as well as the rise in serum lactate caused by HS (Fig. 3D). Thus, Pep19-4LF reduces the delayed vascular decompensation and organ/tissue ischemia probably due to increased microvascular perfusion secondary to increased perfusion pressure. Moreover, Pep19-4LF significantly attenuated the liver injury, renal dysfunction, pancreatic injury and lung inflammation caused by HS (Fig. 3 A, B, E, F, G, H). As neutrophils and macrophages play an important role in HS-associated lung inflammation, we evaluated the degree of macrophage infiltration (measured as number of CD68-positive cells) and the degree of neutrophil activation (measured as MPO-activity) in the lung. HS resulted in a significant increase in the number of macrophages in the lung as well as a significant increase in MPO-activity, both of which was attenuated by the treatment of HS-rats with Pep19-4LF (Fig. 4). Neutrophils and macrophages release (via degranulation) pro-inflammatory cytokines, such as IL-6 or MCP-1 which importantly contribute to acute lung injury/inflammation.²² The pro-inflammatory cytokines IL-6 and MCP-1 are important mediators of alterations associated with organ dysfunction and even lethality following HS and resuscitation.^{23,24,25,26} For instance, a monoclonal antibody against IL-6 reduces the organ dysfunction and inflammation caused by HS.⁴² Indeed, we report here that Pep19-4LF also attenuates the rise in serum IL-6 and MCP-1 caused by HS in the rat (Fig. 5).

What, then, are the mechanisms by which Pep19-4LF attenuates HS-associated organ injury/dysfunction? There is good evidence that PAMPs and DAMPs released during trauma-HS interact with Toll-like receptors (i.e. TLR2 and TLR4) resulting in activation of NF- κ B.^{27,28,29} Indeed,

we observed a significant increase in (a) the nuclear translocation of NF- κ B subunit p65 (Fig. 6 A, E) and (b) the degree of phosphorylation of IKK α/β on Ser^{176/180} (Fig. 6B,F) and of I κ B α on Ser^{32/36} (Fig. 6C,G) in liver/kidneys of rats with HS. This activation of NF- κ B in key target organs was attenuated in HS-rats treated with Pep19-4LF during resuscitation. I κ B α masks the nuclear localization signals of NF- κ B proteins and sequesters NF- κ B as an inactive complex in the cytoplasm, thereby inhibiting NF- κ B.^{30,31} Signal-induced proteolytic degradation of I κ B α , which has been phosphorylated by I κ B kinases (IKK α/β) liberates NF- κ B to translocate to the nucleus.³¹ Subsequently, NF- κ B activates the transcription of a number of genes involved in producing pro-inflammatory cytokines and chemokines known to result in the transcription of a multitude of pro-inflammatory cytokines, chemokines and proteins that are widely implicated in the pathophysiology of MOF.^{17,16} Thus, the organ protective effects of Pep19-4LF in HS are associated with a significant reduction in the activation of the NF- κ B pathway, which in turn accounts for the reduced formation of IL-6 and MCP-1 (see above).

We also investigated the effects of HS with or without Pep19-4LF on the degree of activation of the Akt-survival pathway (Fig. 6). When compared to sham rats, HS rats treated with vehicle showed a significantly decreased phosphorylation of Akt on Ser⁴⁷³ (indicating reduction in activity of this kinase) in kidney and liver, which makes these organs less resistant to organ injury (see below). In contrast, Pep19-4LF attenuated the decline in Akt-activation caused by HS (Fig. 6D, H). Akt is a member of the phosphoinositide-3-kinase (PI3K) signal transduction enzyme family. When activated (phosphorylated on Ser⁴⁷³) by its upstream regulator PI3K, Akt controls inflammatory response, chemotaxis and apoptosis.³² Most notably, activation of the Akt-survival pathway reduces organ injury in many conditions associated with ischemia/inflammation including ventilation-induced lung injury, sepsis-induced organ dysfunction, myocardial infarction, and HS-

induced organ dysfunction.^{33,34,35,36,16} Moreover, activation of Akt results in phosphorylation and activation of eNOS at Ser¹¹⁷⁷ that enhances the formation of small amounts of NO, which is pivotal for the preservation of microvascular perfusion and, hence, reducing organ injury.^{37,34,38} We report here that the degree of eNOS phosphorylation on Ser¹¹⁷⁷ is significantly higher in HS-rats treated with Pep19-4LF indicating activation of eNOS and enhanced formation of NO in the microcirculation at least of kidney and liver. We propose that the enhanced formation of NO by eNOS in HS-rats treated with Pep19-4LF contributes to improved microcirculatory perfusion resulting in better tissue oxygenation and lower lactate levels (Fig 3D). Thus, the organ protective effects of Pep19-4LF in HS are associated with a significant activation of the Akt/eNOS survival pathway.

Traumatic injury and trauma-associated HS result in the release of a variety of endogenous TLR ligands, including heparan sulfates.^{27,28,29,39} Moreover, the degradation of the glycocalyx (and subsequent liberation of heparan sulfates) induces remote organ injury after trauma/hemorrhagic shock,^{40, 41,42,43} suggesting heparan sulfate as a potential therapeutic target for Pep19-4LF. Indeed, using isothermal titration calorimetry, we found a strong Coulomb interaction between Pep19-4LF and heparan sulfate, as indicated by strong exothermic reaction running into a saturation characteristic (Fig. 7B). These results are in line with our recent findings, reporting a strong Coulomb interaction between the related peptide Pep2.5 and heparan sulfate.¹⁵ To investigate whether Pep19-4LF inhibits the release of TNF α caused by heparan sulfate in human cells, we exposed human MNCs to heparan sulfate in the presence or absence of Pep19-4LF. Most notably, Pep19-4LF attenuated the release of TNF α caused by heparin sulfate in these cells in a dose-related fashion (Fig. 7A) indicating that Pep19-4LF is able to prevent the activation of human cells by the DAMP heparin sulfate. Thus, these findings indicate, that the organ protective and

anti-inflammatory effects of Pep19-4LF in HS are, at least partly, associated with its interaction with relevant DAMPs, such as heparan sulfate.

In conclusion, we report here for the first time that trauma and trauma-haemorrhage result in a significant release of the host-defence/antimicrobial peptide LL-37. As the systemic administration of higher doses of LL-37 leads to adverse effects, we have synthesized a small host-defence/antimicrobial peptide, Pep19-4L. Like LL-37, Pep19-4LF neutralizes the effects of LPS and lipoproteins.¹² LL-37 also interacts with and attenuates the effects of several DAMPs.^{6,8} We report here that Pep19-4LF abolishes the release of TNF α caused by heparan sulfate in human mononuclear cells. In addition, Pep19-4LF attenuates the organ injury/dysfunction caused by severe hemorrhage and resuscitation in the anesthetized rat. This protective effect of Pep19-4LF was associated with activation of the Akt/eNOS-survival pathway (kidney and liver), which increases the resistance of these organs to injury. In addition, Pep19-4LF also attenuates the activation of NF- κ B in these organs, resulting in the reduced formation of the pro-inflammatory cytokines IL-6 and MCP-1. Thus, we propose that Pep19-4LF may be useful to reduce the organ injury/dysfunction and inflammation caused by severe hemorrhage and resuscitations in patients with trauma.

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AUTHOR CONTRIBUTIONS

Conception and design: NY, LM, and CT; Animal experiments: NY; Cell culture experiments: LH, TG, WC, and KB; Human sample analysis: NY, LM, EZ, GM, TS; Animal sample analyses: NY, LM, GP, FC, BV, MC, and TS; Clinical study and patient data analyses: LM, JS, KB, GM, and TS; Peptide development and synthesis: KB, LH, TG, WC. Statistical analyses: NY, LM, and CT; Drafting the manuscript for important intellectual content: NY, LM, EZ, GP, FC, BV, MC, JS, LH, TG, WC, KB, GM, TS, KB, and CT; All authors reviewed and approved the manuscript.

ADDITIONAL INFORMATION

Supplementary information accompanies this paper.

Competing financial interests: KB has a patent for the structure of the synthetic antimicrobial peptide 19-4LF (Brandenburg Antiinfektiva, Borstel, Germany): Patent-No: PCT/EP2009/002565.

KB chief scientific officer and TS is chief medical officer of Brandenburg Antiinfektiva GmbH. All the other authors declare no conflicts of interest.

LEGENDS TO FIGURES

FIGURE 1. LL-37 plasma levels in human healthy volunteers and trauma patients

Plasma concentrations of the cathelicidin LL-37 were assessed in control healthy volunteers (n = 10) and in trauma patients (n = 24) as well as in trauma hemorrhagic patients (n = 23). Data are expressed as box and whisker min to max for n number of observations. + = mean value. *P < 0.05 vs. healthy; §P < 0.05 vs. trauma (Kruskall-Wallis test with Dunn's multiple comparisons test).

FIGURE 2. Pep19-4LF attenuates the decline in MAP during resuscitation after HS.

HS-rats received continuous administration of low-dose (LD; 66 µg/kg x h) or high-dose (HD; 333 µg/kg x h) of Pep-4LF or saline (vehicle) throughout 4 h after resuscitation. Sham animal were used as control and received saline or high-dose of Pep19-4LF. The MAP was recorded during the whole experiment. The following groups were studied: sham + vehicle (n = 11); sham + Pep4LF-HD (n = 6); HS + vehicle (n = 12), HS + Pep4LF-LD (n = 4); HS + Pep19-4LF-HD (n = 8). Data are expressed as mean ± SEM for n number of observations. Statistical analysis was performed using 2-way ANOVA followed by Bonferroni post hoc test. *P < 0.05 vs HS + vehicle. ANOVA indicated analysis of variance; SEM, standard error of the mean.

FIGURE 3. Pep19-4LF attenuates the organ injury and dysfunction caused by HS.

(A) Serum urea, (B) serum creatinine, (C) creatinine clearance (CCr), (D) serum lactate, (E) serum alanine transaminase (ALT), (F) serum aspartate transaminase (AST), (G) serum amylase, and (H) serum lipase of HS or sham-operated rats. All parameters were assessed 4 h subsequent to HS. HS-rats received continuous administration of low-dose (LD; 66 µg/kg x h) or high-dose (HD; 333 µg/kg x h) of Pep-4LF or saline (vehicle) throughout 4 hours after resuscitation. Sham animal were used as control and received saline or high-dose of Pep19-4LF. Data are shown as box and

whiskers, showing medians, interquartile range, and full range. The following groups were studied: sham + vehicle (n = 11); sham + Pep4LF-HD (n = 6); HS + vehicle (n = 12), HS + Pep4LF-LD (n = 4); HS + Pep19-4LF-HD (n = 8). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. §*P* < 0.05 vs sham + vehicle and **P* < 0.05 vs HS + vehicle. ANOVA indicated analysis of variance.

FIGURE 4. Pep19-4LF attenuates lung inflammation caused by HS.

Representative images for CD68 as a macrophage marker (200 fold), (B) quantitative analysis of the number of CD68-positive cells/mm², (C) MPO activity in lungs of HS or sham-operated rats. The scale bar represents 100µm. All parameters were assessed 4 h subsequent to HS. HS-rats received continuous administration of vehicle (saline) or Pep19-4LF of (333 µg/kg x h) throughout 4 hours after resuscitation. Sham animal were used as control and received saline. Data are shown as box and whiskers, showing medians, interquartile range, and full range. The following groups were studied: sham + vehicle (n =4); HS + vehicle (n = 6), HS + Pep19-4LF HD (n = 6). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. §*P* < 0.05 vs sham + vehicle and **P* < 0.05 vs HS + vehicle. ANOVA indicated analysis of variance.

FIGURE 5. Pep19-4LF attenuates the increase in IL-6 and MCP-1 caused by HS.

The serum concentrations of (A) interleukin (IL)-6, (B) monocyte chemotactic protein-1 (MCP-1), (C) IL-10, and (D) C-X-C motif ligand 1 (CXCL1) were determined using a cytometric bead array in sham and HS rats treated with vehicle or Pep19-4LF (333 µg/kg x h) throughout 4 h after resuscitation. All parameters were assessed 4 h subsequent to HS. Data are presented as box and whiskers format, showing medians, interquartile range, and full range. (E) Heatmap of measured cytokines. The following groups were studied: sham + vehicle (n = 11); HS + vehicle (n = 12), HS + Pep19-4LF-HD (n = 8). Statistical analysis was performed using 1-way ANOVA

followed by Bonferroni post hoc test. $\S P < 0.05$ vs sham + vehicle and $*P < 0.05$ vs HS + vehicle. ANOVA indicated analysis of variance.

FIGURE 6. Pep19-4LF attenuates the activation of NF- κ B and increases the activation of Akt and eNOS in kidney and liver after HS.

The nuclear translocation of p65 subunit of NF- κ B (kidney (A), liver (F)), and the phosphorylation of Ser^{176/180} on IKK α/β (kidney (B), liver (G)), Ser^{32/36} on I κ B α (kidney (C), liver (H)), Ser⁴⁷³ on Akt (kidney (D), liver (I)), and Ser113 on eNOS (kidney (E), liver (J)) of sham and HS rats treated with vehicle or high-dose of Pep19-4LF (333 μ g/kg x h) upon resuscitation were determined by Western blotting. Protein expression was measured as relative OD. Data are shown as box and whiskers, showing medians, interquartile range, and full range. The following groups were studied: sham + vehicle (n =4); HS + vehicle (n = 4), HS + Pep19-4LF-HD (n = 4). Statistical analysis was performed using 1-way ANOVA followed by Bonferroni post hoc test. $\S P < 0.05$ vs sham + vehicle and $*P < 0.05$ vs HS + vehicle. ANOVA indicated analysis of variance; OD, optical density.

FIGURE 7. Pep19-4LF interacts with heparan sulfate

Inhibitory effect of Pep10-4LF on heparan sulfate-induced TNF α release in human peripheral blood mononuclear cells from healthy donors. Pep19-4LF was added at the indicated weight ratios of the concentrations of heparan sulfate to Pep19-4LF. (B) Enthalpy of the Pep19-4LF-heparan sulfate binding. Isothermal calorimetric titration of a 1 – 4 mM Pep19-4LF solution into a 200 μ g/ml heparan sulfate dispersion. The enthalpy changes at each injection were measured and the area underneath each injection peak was integrated and plotted against the weight ratio of the concentrations of Pep19-4LF to heparan sulfate. A downward peak corresponds to an exothermic reaction, and an upward peak corresponds to an endothermic reaction. (C) Red blood cells, obtained from citrated human blood were suspended at a concentration equivalent to 5% of the

normal hematocrit. Pep19-4LF was added at different concentrations and the supernatants were analyzed for haemoglobin. Results are expressed as the percentage released with respect to sonicated controls (100% release) or controls processed without peptide (0% release).

Table 1. Admission Characteristics & Outcomes

	Trauma No Haemorrhage	Trauma Haemorrhage	P-value*
Admission Characteristics			
N	24	23	-
Age	43 (35-49)	32 (24-51)	0.190
Male (%)	24 (100)	16 (69.6)	0.003‡
Blunt Mechanism (%)	23 (95.8)	15 (65.2)	0.008‡
Time Injury-Sample (mins)	94 (76-108)	100 (59-116)	0.686
Admission Base Deficit	0 (-2-1)	11 (8-22)	<0.001
Admission Lactate	2 (1-3)	8 (6-11)	<0.001
Pre-baseline PRBC (units)	0 (0-0)	1 (0-3)	<0.001
24hr PRBC (units)	0 (0-0)	10 (5-12)	<0.001
ISS	23 (20-29)	29 (25-41)	0.004
AIS Head	0 (0-0)	0 (0-0)	0.371
AIS Chest	4 (3-5)	3 (2-5)	0.904
AIS Abdomen	2 (0-3)	2 (0-3)	0.444
AIS Extremity	2 (0-4)	3 (0-5)	0.097
Outcomes			
28-day mortality (%)	0 (0)	6 (26.1)	0.007‡
Ventilator Days	0 (0-2)	3 (3-7)	<0.001
Critical Care LOS	0 (0-6)	9 (4-20)	<0.001
Hospital LOS	15 (8-38)	18 (4-51)	<0.773
PRBC: Packed Red Blood Cells; ISS: Injury Severity Score; AIS: Abbreviated Injury Severity Score; LOS: Length of Stay. Median (interquartile range) reported unless specified. *Trauma hemorrhage vs. No hemorrhage Mann-Whitney U-test unless ‡Chi-Squared test.			

SUPPLEMENTARY INFORMATION

A novel synthetic antimicrobial peptide protects against organ injury/dysfunction in a rat-model of severe hemorrhagic shock

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Heinbockel, Thomas Gutschmann, Wilmar-Alexander Correa-Vargas, Klaus Brandenburg,
Gernot Marx, Tobias Schuerholz, Karim Brohi, Christoph Thiemermann

METHODS

Study population and human outcome measurements

Study setting and participants

Trauma patients who presented to an urban major trauma centre were recruited to an ongoing, prospective observational cohort study called the 'Activation of Coagulation and Inflammation in Trauma Study II' (ACIT-II). This study was originally established in 2008 to investigate the biological mechanisms underlying acute traumatic coagulopathy and the inflammatory response to trauma. Adult trauma patients who require trauma team activation on admission were eligible for inclusion. Exclusion criteria included age under 16 years, transfer from another hospital, arrival-time greater than 120 min from injury, pre-hospital administration of greater than 2000 ml crystalloid, greater than 5 % burns, severe liver disease, known bleeding abnormality (including anticoagulant medication), refused consent and vulnerable patients.

Patient selection

Trauma patients for this study (n = 47) were identified from the available ACIT-II cohort based on their Injury Severity Score (ISS) and blood product requirements during resuscitation. Patients were included if they had an ISS score greater than or equal to 16. Trauma Haemorrhage patients were defined as patients who received greater than or equal to two units of packed red blood cells (PRBC) on admission. Control patients (No Haemorrhage) received no PRBCs during the first 24 hours of their admission. We subsequently excluded patients with a head Abbreviated Injury Severity (AIS) score greater than three in order to ensure the groups were not skewed on the basis of severe traumatic brain injury. Furthermore, we included healthy volunteers (n = 10) as a control group.

Sample and data collection

Admission data were collected on patient demographics, mechanism of injury, blood product use and baseline physiology. Arterial blood gas analysis for base deficit (BD) and lactate was performed during the trauma team resuscitation as part of normal care processes. Admission bloods were drawn within 2 hours of injury. Whole blood was collected in 4.5 ml citrated vacutainer tubes and centrifuged at 3400 rpm for 10 minutes. The plasma supernatant was centrifuged again at the same settings, and the double-spun plasma was subsequently stored in aliquots at -80 °C. Patient outcomes including 28-day mortality, ventilator days, critical care and hospital length of stay were recorded.

LL37-ELISA

Plasma-levels of the cathelicidin-derived human AMP LL-37 in patients were quantified using a commercially available ELISA kit (Cambridge Bioscience Ltd, Cambridge, UK) by following the manufacturer/product specific protocol.

Immunoblot analysis

Semi-quantitative immunoblot analyses of nuclear translocation of p65 and the phosphorylation of I κ B α , IKK α/β , Akt, and eNOS were carried out in tissue samples as described before.¹ Briefly, lung, kidney and liver samples were homogenized in buffer and centrifuged at 4000 rpm for 5 min at 4°C. To obtain the cytosolic fraction, supernatants were centrifuged at 14000 rpm at 4°C for 40 min. The pelleted nuclei were re-suspended in extraction buffer and centrifuged at 14,000 rpm for 20 min at 4°C. Protein content was determined on both nuclear and cytosolic extracts using bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, Rockford, IL). Proteins were separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, which was incubated with a primary antibody [mouse anti-total Akt (1:1000); mouse anti-I κ B α pSer^{32/36} (1:1000); rabbit anti-NF- κ B p65

(1:1000); rabbit anti-eNOS pSer¹¹³ (1:1000)]. Membranes were incubated with a secondary antibody conjugated with horseradish peroxidase (1:2000) for 30 min at room temperature and developed with ECL detection system. The immunoreactive bands were visualized by autoradiography and the densitometric analysis was performed using Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and incubated with β -actin monoclonal antibody (1:5000) and subsequently with an anti-mouse antibody (1:10000) to assess gel-loading homogeneity. Densitometric analysis of the related bands is expressed as relative optical density, and normalized using the related sham-operated band.

Peptide-synthesis

The synthesis and purification of Pep19-4LF was performed at the Research Center Borstel, Germany as described previously.² The amino acid sequence of this 19´mer is GKKYRRFRWKFKGKLFLFG. Pep19-4LF was amidated at the C-terminal end and had a purity of > 95% as measured by HPLC and MALDI-TOF mass spectrometry.²

Immunohistochemistry

Lung samples were obtained at the end of the experiment and fixed in formalin for 48 h and immunohistochemistry was performed as described previously¹. Briefly, lung tissue was embedding in paraffin and processed to obtain 4- μ m sections. After deparaffinization, the slides were then incubated with rabbit anti-CD68 antibody ED1 (1:400; catalog no. MCA341R; AbD Serotec) for 1 h at 37°C and afterwards incubated for 30 min with labelled polymer-HRP antibody. Counterstaining was performed with Harris hematoxylin. Images were acquired using a NanoZoomer Digital Pathology Scanner (Hamamatsu Photonics K.K. Japan) and analyzed using the NDP Viewer software. The numbers of CD68 positive cells were counted in 10 randomly selected fields (200 \times) in a double-blinded manner by three independent investigators.

Hemorrhagic shock and quantification of organ injury and dysfunction

Hemorrhagic shock and quantification of organ injury and dysfunction were performed as described previously³. Rats were anesthetized by sodium thiopentone (120 mg/kg *i.p.* for induction, followed by 10 mg/kg *i.v.* for maintenance). We performed cannulation of the trachea, femoral artery (for measuring blood pressure), and carotid arteries (for blood withdrawal), jugular vein (for drug administration), and bladder (for collecting urine). We withdrew blood (up to 1 mL/min) via the cannula inserted in the carotid artery in order to achieve a fall in mean arterial pressure (MAP) to 27 to 32 mmHg. Thereafter, MAP was maintained at this level for a period of 90 min either by further withdrawal of blood during the compensation phase or administration shed blood during the decompensation phase. At 90 min after initiation of hemorrhage (or when 25% of the shed blood had to be re-injected to sustain MAP at 27 to 32 mmHg), animals were resuscitated with the remaining shed blood (mixed with 100 IU/mL heparinized saline) (over a period of 5 min) plus a volume of Ringer's lactate identical to the volume of blood spent during decompensation. During the last 3 h of resuscitation, urine was obtained for the estimation of creatinine clearance. Then, blood samples were collected via the carotid artery for measurement of lactate (Accutrend Plus Meter, Roche Diagnostics, West Sussex, UK) and organ injury/dysfunction parameters. Under deep anesthesia, the heart was removed to terminate the experiment. Blood samples were centrifuged to separate serum, which was used for the determination of urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase, amylase and creatine kinase (CK) by an external contract research facility (IDEXX Laboratories Ltd, West Yorkshire, UK) in a blinded fashion. In addition, lung, kidney and liver samples were taken and stored at -80 °C for further analysis. Sham-operated rats were used as control and underwent identical surgical procedures, but without hemorrhage or resuscitation. Blood pressures during the

experiment were measured by powerlab®, and recorded and analyzed by Labchart® (AD instruments Ltd, Dunedin, New Zealand).

Human mononuclear cells study

Mononuclear cells (MNC) were isolated from heparinized blood samples obtained from healthy donors as described previously.⁴ The cells were re-suspended in medium (RPMI 1640), and their number was equilibrated at 5×10^6 cells/ml. For stimulation, 200 μ l MNC (1×10^6 cells) was transferred into each well of a 96-well culture plate. Heparan sulfate and the mixtures of heparan sulfate and peptide were pre-incubated for 30 min at 37°C and added to the cultures at 20 μ l per well. The cells were then incubated for 4 h at 37°C with 5% CO₂. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 x g and stored at - 20°C until immunological determination of tumor necrosis factor alpha (TNF α) was carried out with a sandwich enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody against TNF α (clone 6b; Intex AG, Switzerland) and described previously in detail.⁴

Isothermal titration calorimetry (ITC)

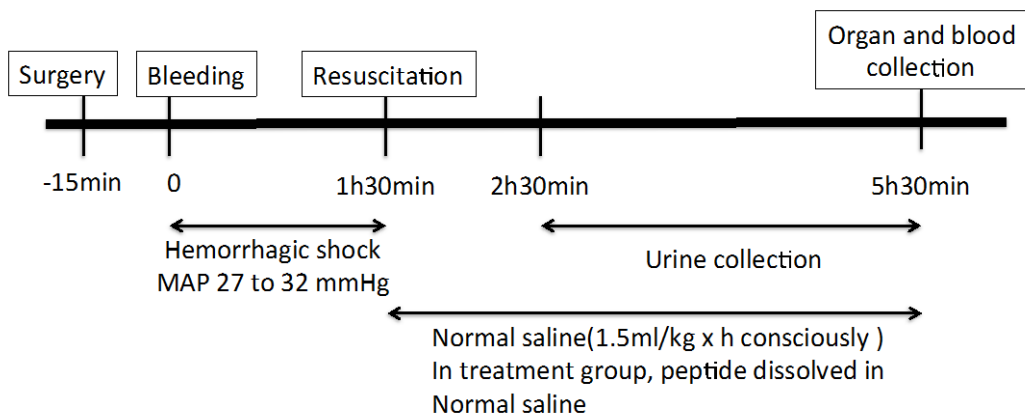
Microcalorimetric experiments of peptide binding to heparan sulfate were performed on a MSC isothermal titration calorimeter (MicroCal Inc., Northampton, MA) at 37 °C as described before.⁵ Briefly, after thorough degassing of the samples, Pep19-4LF (1 to 4 mM in 20 mM HEPES, pH 7.0) was titrated to a heparan sulfate suspension (200 μ g/ml in 20 mM HEPES, pH 7.0). The enthalpy change during each injection was measured by the instrument, and the area underneath each injection peak was integrated (Origin; MicroCal) and plotted against the weight ratio of the concentrations of peptide to heparan sulfate. Titration of the pure peptide into HEPES buffer resulted in a negligible endothermic reaction due to dilution. All experiments were carried out in duplicate.

Hemolysis assay

Red blood cells (RBC) were obtained from citrated human blood by centrifugation (1,500 x g; 10 min), washed three times with isotonic 20 mM phosphate- NaCl buffer (pH 7.4), and suspended in the same buffer at a concentration equivalent to 5% of the normal hematocrit. Forty-microliter aliquots of this RBC suspension were added to 0.96 ml of Pep19-4LF dilutions prepared in the same isotonic phosphate solution, incubated at 37°C for 30 min, and centrifuged (1,500 x g, 10 min). The supernatants were analyzed spectrophotometrically (with absorbance at 543 nm) for hemoglobin, and results were expressed as the percentage released with respect to sonicated controls (100% release) or controls processed without peptide (0% release).

Materials

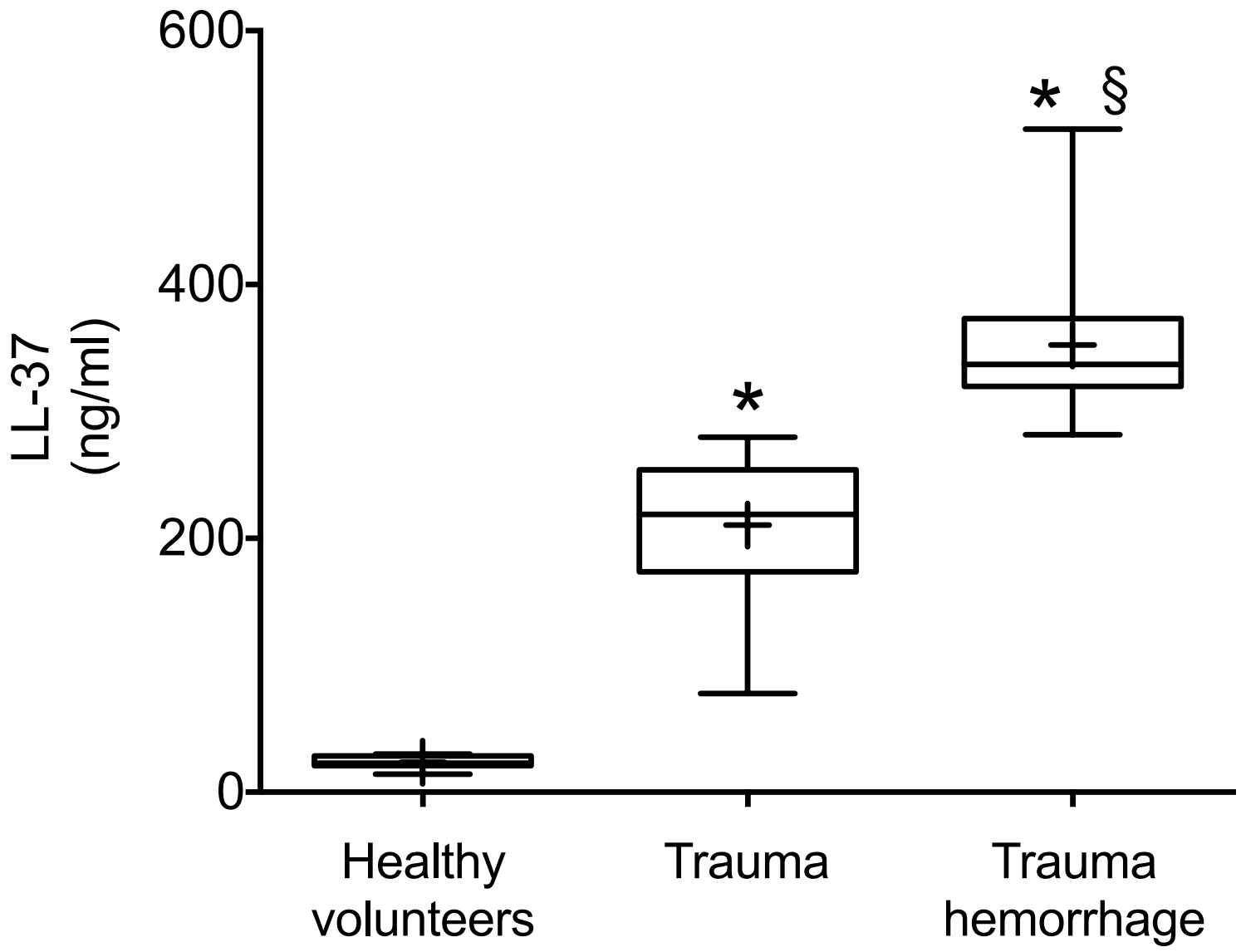
Unless otherwise stated, all compounds were from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Ringer's Lactate was from Baxter Healthcare Ltd (Deerfield, IL); Thiopental sodium from Archimedes Pharma Limited (Reading, UK).

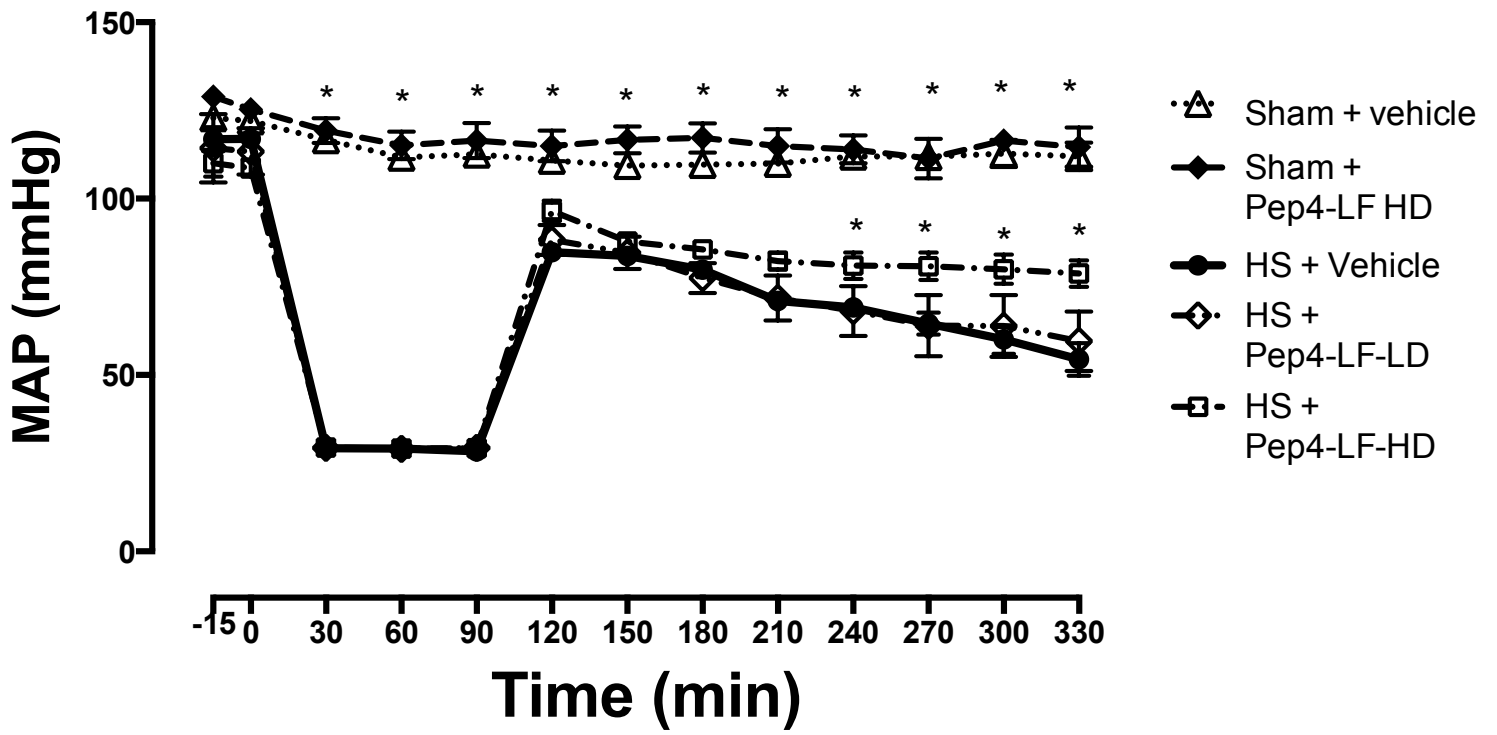


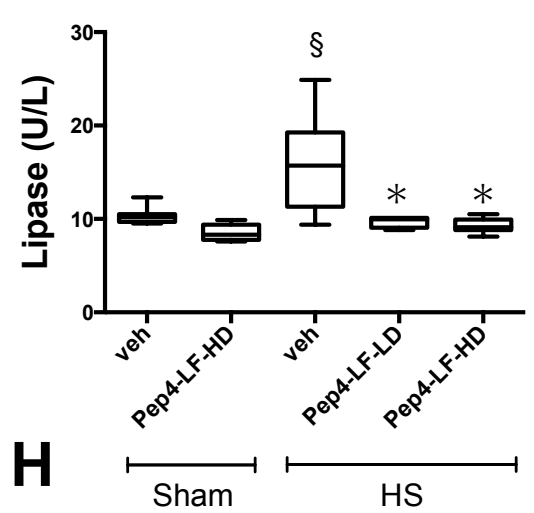
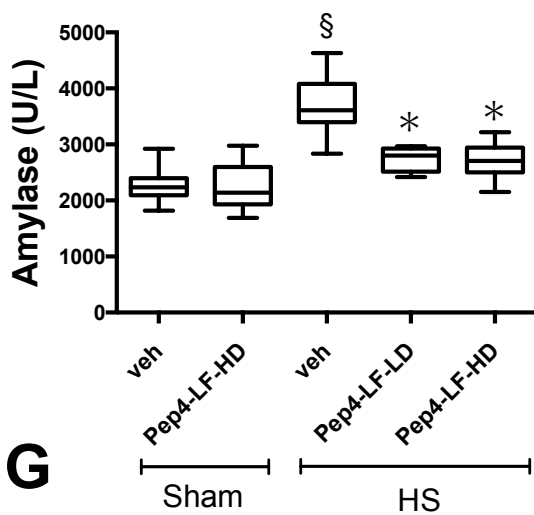
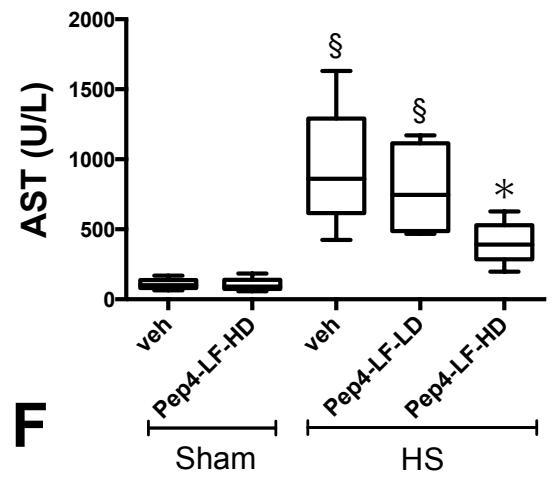
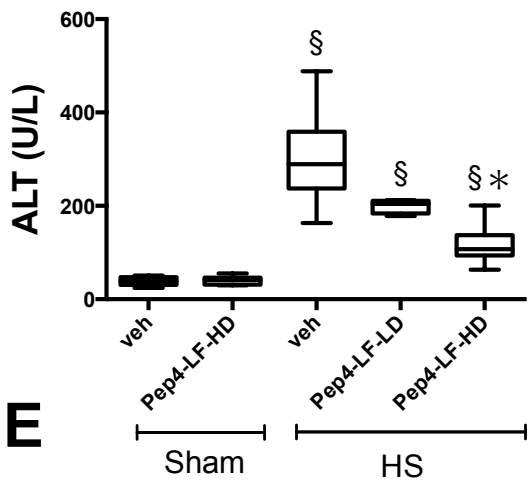
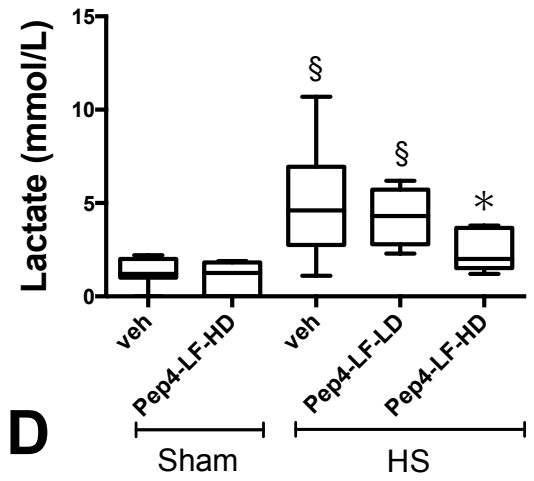
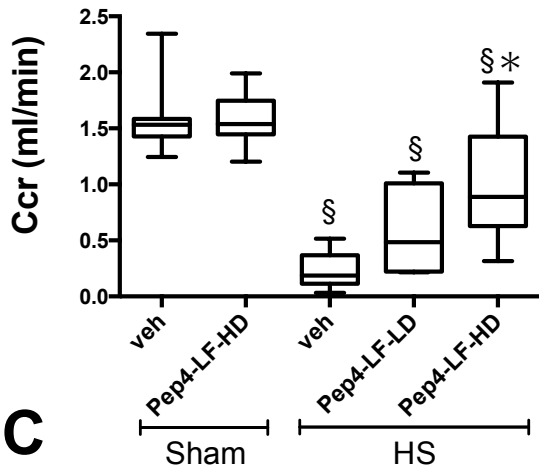
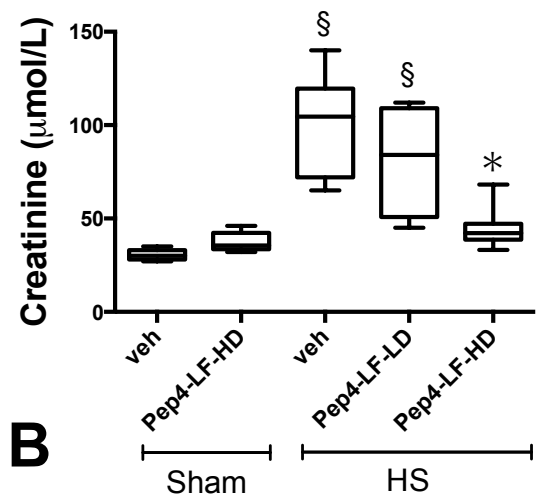
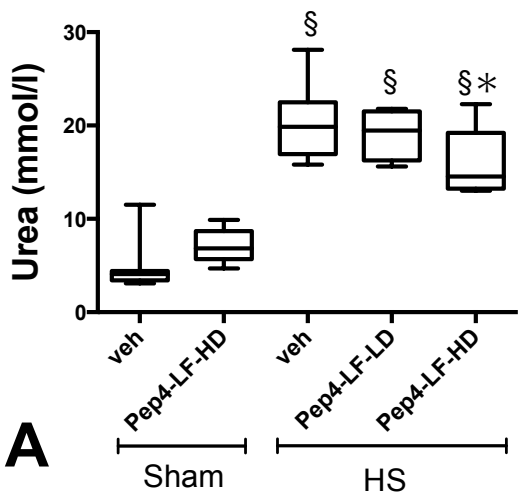
Supplemental Figure 1. Entire study course of hemorrhagic shock in rats.

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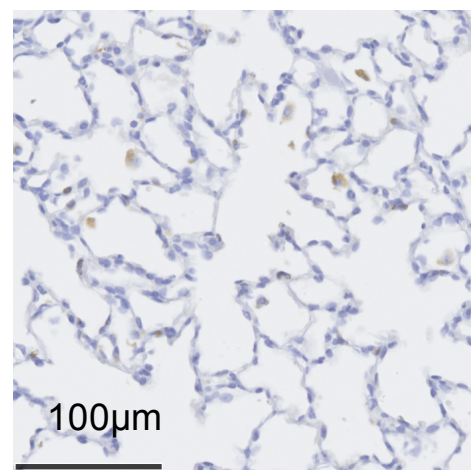
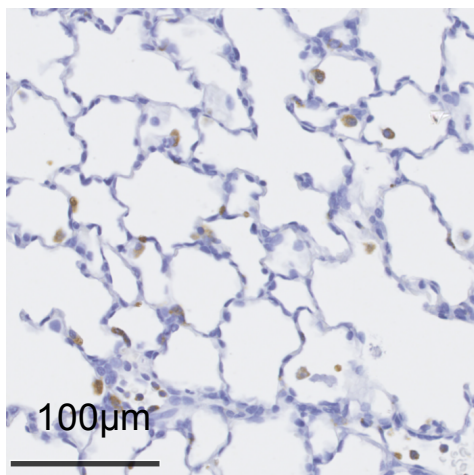
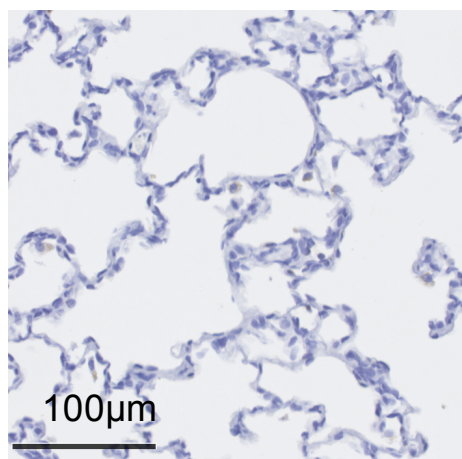




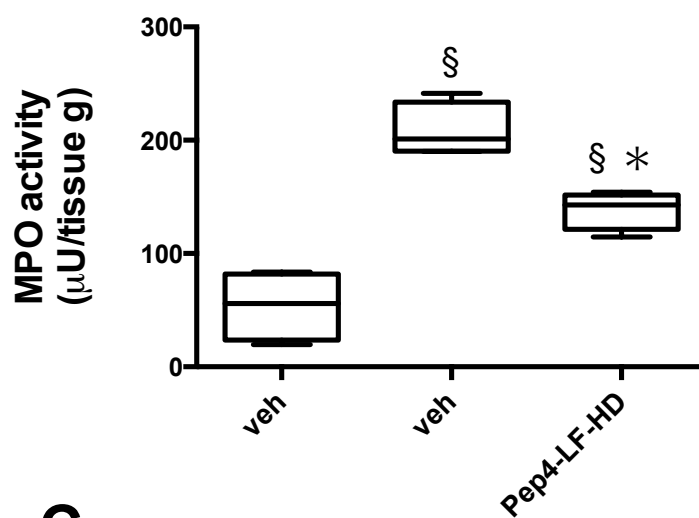
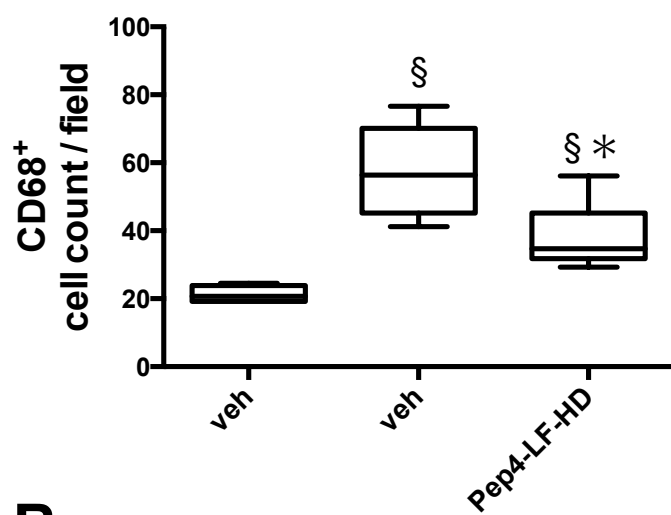
Sham+vehicle

HS+vehicle

HS+Pep-4LF



A

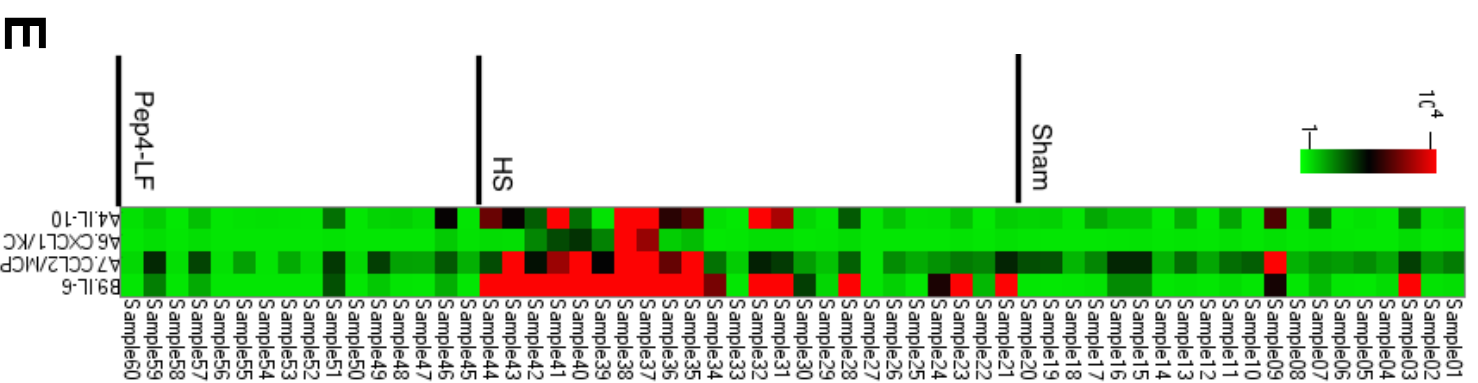
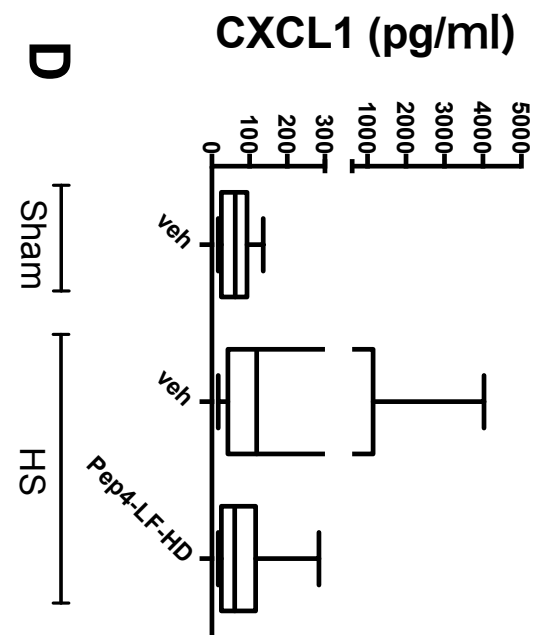
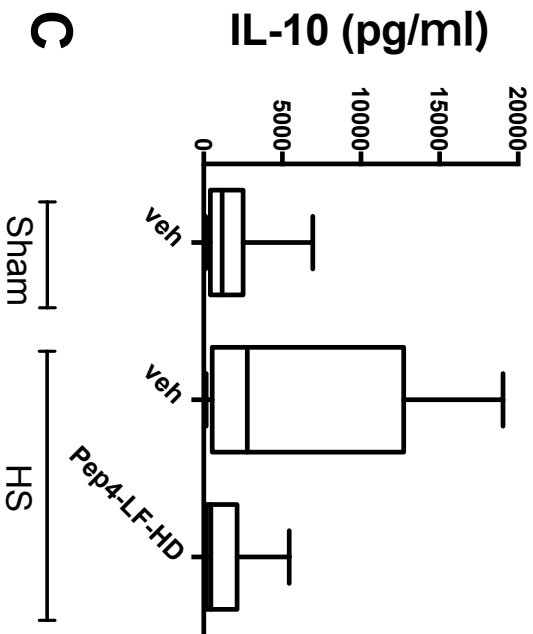
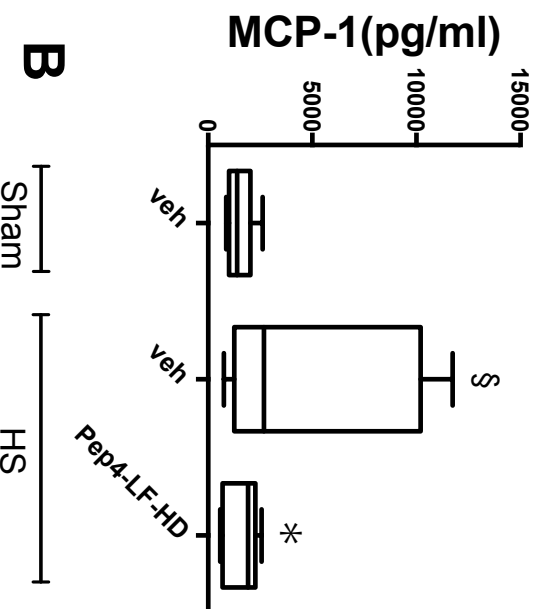
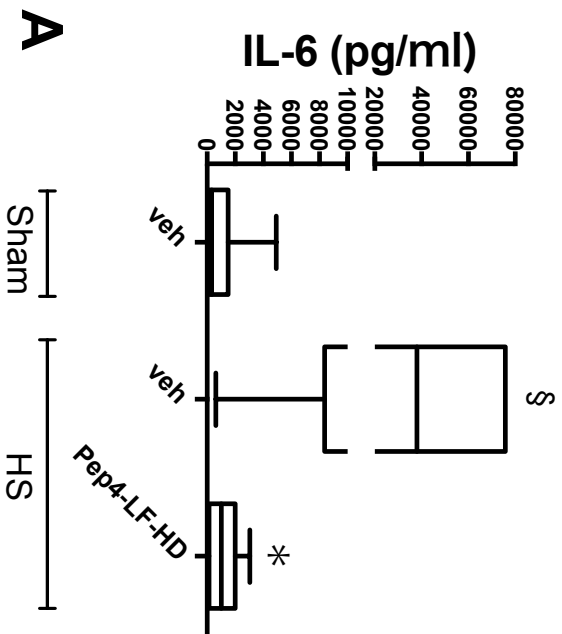


B

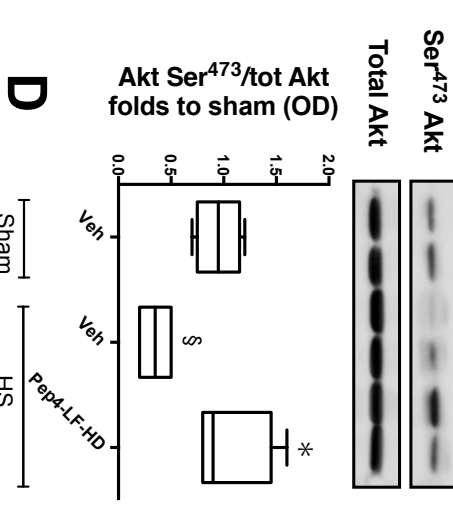
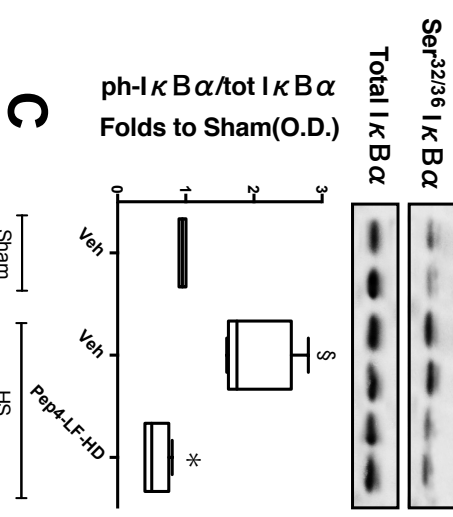
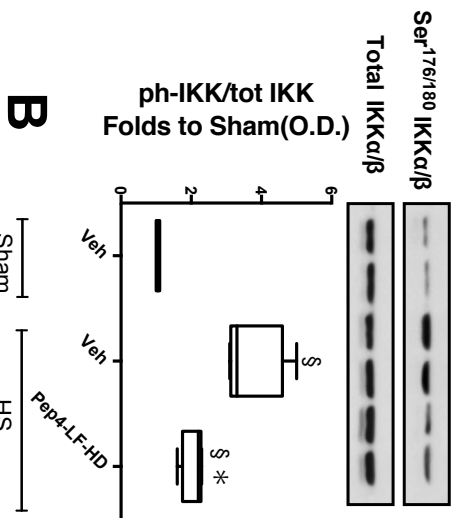
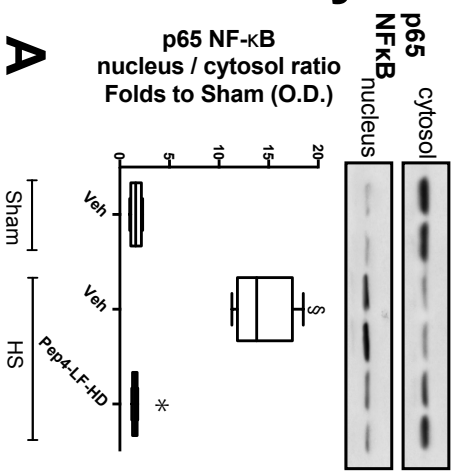
Sham HS

C

Sham HS



Kidney



Liver

