Reduction of circulating histone toxicity is a major function of C-reactive protein after extensive tissue damage

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Abbreviations: APC, activated protein C; CRP, C-reactive protein; ahscFv: anti-histone single-chain variable fragments; IL-6: interleukin-6; IL-1β: interleukin-1β; FITC: fluorescein isothiocyanate;

Running title: C-reactive protein reduces the toxic effects of histones *in vitro* and *in vivo*

ABSTRACT(150 words)

C-reactive protein (CRP) is a major acute phase protein. Although known to interact with chromatin, nucleosomes and histones, its functional roles are not clearly understood. Using both i*n vitro* and *in vivo* models and samples from patients, this study demonstrates for the first time that CRP plays important roles in reducing the toxic effects of histones released into the circulation after extensive cell death. CRP protects endothelial cells by preventing histone integration into the cell membrane and thus reducing Ca^{2+} influx. *In vivo*, circulating histones cause endothelial damage, increased microvascular permeability, coagulation activation and IL-6 secretion. The latter induces CRP production in hepatocytes to form a negative feedback loop, a possible evolutionally conserved mechanism to limit secondary damage after extensive tissue injury. However, CRP responses lagged behind the histone surge following severe trauma. This indicates a time window for histone toxicity and also for potential clinical interventions using anti-histone therapy.

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C-reactive protein (CRP) has long been recognized as a major acute phase protein. Earlier studies have demonstrated that CRP activates complements, opsonizes pathogens such as *Streptococcus pneumoniae* and facilitates their clearance[1,2]. However, CRP is also upregulated in many non-infectious diseases, such as trauma, pancreatitis, myocardial infarction, autoimmune diseases and cancers. Further studies have found that CRP binds to circulating nucleosomes and histones[3,4]. This has assigned a functional role for CRP in preventing the development of autoimmune diseases through clearance of nuclear breakdown products [5-7].

During cell death, chromatin is cleaved into multiples of oligo- and mono-nucleosomes, which are released extracellularly[8] and further degraded into individual histones, heterodimers[9] as well as free DNA[10]. However, these are rarely detected in blood unless there is extensive cell death[11] as they are rapidly cleared by the liver[12]. A few reports have indicated the toxic potential of histones. Exogenous application of the histone H1 induced cytotoxicity in MCF-7 cells by increasing intracellular calcium levels[13]. Extracellular histones induce membrane permeability changes in thymus cells[14]. More recently, the toxicity of histones to endothelial cells has been demonstrated both *in vitro* and *in vivo*[15].

Here, we demonstrate for the first time that CRP plays an important role in the acute phase response to injury by reducing circulating histone toxicity. CRP protects endothelial cells by forming CRP-histone complexes to block histone integration into the cell membrane, which is an essential step for histone toxicity. Histone integration enhances membrane permeability to allow Ca^{2+} influx, which increases intracellular Ca^{2+} levels to cause cell damage. Resultant pathological changes include increased microvascular permeability and coagulation activation in both the animal model and patients with trauma, with direct correlation to histone concentrations. Histones also stimulate IL-6 release to induce CRP synthesis by human hepatocytes. This feedback loop would serve to limit the secondary damage by histones after extensive cell death. However, sufficient CRP elevation takes at least 4-6h whilst histones are released immediately after injury. A time window therefore exists for potential clinical intervention to minimize the toxicity of acutely released histones.

RESULTS

Both modified and non-modified histones are toxic to endothelial cells

The toxicity of histones was analyzed using flow cytometry based on the principle of chromatin loss by damaged cells[16] (Fig. 1a). Toxicity of calf thymus histones to EAhy926 cells was dose-dependent (Fig. 1b). To rule out contamination, histone-containing solutions were extensively dialyzed against saline or filtered (5KD cutoff). Dialyzed histones remained toxic but not the buffer that passed through the filter. To exclude species variation, histones from U937, a human monocyte-like cell line, were isolated[17] and found to have similar toxicity (Fig. 1b). Recombinant (non-modified) or isolated (modified) individual histones also showed toxicity as did H2A/H2B and H3/H4 dimers (Fig. 1c), demonstrating that histone toxicity is not due to contamination by small chemicals or other types of nuclear proteins.

CRP reduces histone toxicity to endothelial cells through complex formation

CRP, over 50 μg/ml, significantly protected EAhy926 cells treated with 20 μg/ml histones (Fig. 1d) and the protection reached a plateau from 100 μg/ml CRP. To achieve a maximal protective effect, 250 μg/ml CRP (0-500 μg/ml in patients) was used in further experiments unless specified (Fig. 1e). Similar results were obtained on primary human umbilical vein endothelial cells (Fig. 1e). CRP, unlike APC, did not cleave or degrade histones and pre-treating EAhy926 cells with CRP for 1-4 h did not reduce histone toxicity if CRP was washed away prior to histone treatment (data not shown), suggesting that CRP protection relies on complex formation. Previous reports mainly used I^{125}-labeled CRP and demonstrated that CRP interacted with H1, H2B and H3 but not H4[3,4,18]. Our gel overlay assay showed a similar pattern (Fig. 1f). However, when CRP or individual histones were immobilized on an optical biosensor, both H4 and H3 showed far stronger binding to CRP than H1, H2A and H2B (Fig. 1g&h). It was reported that CRP cross-links only to H4 in solution[19]. These discrepancies may be due, in part, to limitations

of the detection methods used. In fact, CRP bound to all tested histones and accordingly protected EAhy926 cells treated with H1, H2A, H2B, H3 or H4 ($p<0.05$) (Fig.1i).

CRP blocks histone integration onto the cell membrane, which is an essential step in inducing toxicity

As to the mechanism(s) involved in histone toxicity, the available literature points to a histone-induced increase in membrane permeability[20]. FITC labeled-histones in culture medium started to enrich in endothelial cell membranes after 10 min incubation (Fig 2a, A-C). Pre-incubated with CRP, FITC-histones aggregated in the culture medium or inside cells but did not concentrate on the cell membrane (Fig. 2a, D-F). Since anti-histone antibodies can reduce histone toxicity[15], we produced an anti-histone recombinant antibody (ahscFv) which strongly binds to H1, H3 and H4 but weakly to, H2A and H2B (Supplemental Fig. 1) for confirming the specificity of CRP effects on histones. Here, ahscFv showed similar effects to CRP (G-I), confirming CRP directly affects histones but not the cell membrane. Truncated histones lost their toxicity once their membrane binding properties[14] were disrupted (Supplemental Table 1). However, deletion of H1 N-terminal 110 amino acids (aa), H2A C-terminus 68aa or H3 C-terminal 62aa did not block their membrane binding and toxicity. APC-cleaved FITC-histones increased their internalization but reduced membrane binding and toxicity (Supplemental Fig. 2). These data strongly suggest that histones inside cells are not toxic whilst membrane binding is essential to their toxicity, at least to endothelial cells.

Histone binding to the cell membrane causes hyper-permeability to ions, which is inhibited by CRP

Whole-cell currents were recorded using the perforated patch configuration from single EAh926 cells, as previously described[21]. Histones induced large dose-dependent inward currents that were reversible for short exposure times (Fig 2b). The development of inward current indicates a net influx of positively charged ions, such as Ca^{2+} and Na^{+}, or the efflux of negatively charged ions, such as Cl^{-}. We found that intracellular Ca^{2+} was elevated to 50, 200 and 1000 nM after exposure to 10, 20 and 50 μg/ml histones, respectively (Fig. 2c). The elevation was abolished when extracellular Ca^{2+} was removed (Fig. 2d), indicating that Ca^{2+} influx is the major cause. It is well known that a sustained increase in cytosolic Ca^{2+} can lead to cell death in many cell types[22-25]. By controlling the extracellular Ca^{2+} concentration, we found that the extent of histone-induced cell damage positively correlated to extracellular Ca^{2+} concentrations (2e). Pre-incubating histones with CRP or ahscFv dramatically suppressed both inward whole cell currents (2f) and intracellular Ca^{2+} elevations (2g) (Supplemental Fig. 3) and suggests Ca^{2+} influx as the downstream cause of histone toxicity.

CRP reduces histone-induced endothelial permeability both *in vitro* and *in vivo*

Elevation of intracellular Ca^{2+} is involved in the pathway used by inflammatory factors, e.g. thrombin, to induce permeability changes[26]. Histones increased permeability of an endothelial monolayer in a dose-dependent manner (Fig. 3a). A significant change occurred after 40 min exposure to 20 μg/ml histones (Fig. 3b), which is later than that induced by thrombin (within 10 min) and suggests that their mechanisms may differ. CRP, APC and ahscFv abolished the increased endothelial permeability induced by 20-50 μg/ml histones (Fig. 3c). All individual histones significantly increased permeability (Fig. 3d). To confirm that circulating histones are able to increase vascular permeability *in vivo*, the ratio of wet/dry weights of lung were monitored[27]. A sublethal dose, 50 mg/kg histones significantly increased the ratio ($p<0.05$), which was also inhibited by co-injection of 10 mg/kg CRP or ahscFv ($p<0.05$) (3e).

CRP protects histone-treated mice

Intravenous injection of 50 mg/kg histones or CRP alone did not kill any mice. Injection of 75 mg/kg histones killed all mice within an hour. When low-dose CRP (1.6 mg/kg) was co-infused with histones (75 mg/kg), survival times were prolonged. When CRP was increased to 5 mg/kg, 2/6 mice survived for over 6 days and 4/6 mice died between 7-11 h after injection. With 10 mg/kg CRP, 7/7 mice survived for over 6 days (Fig. 3f). Log-rank test showed significant differences in the survival times between the 4 groups ($p<0.001$). Ten mg/kg ahscFv had the same protective effect. Histological examination showed multifocal alveolar hemorrhage and edema with capillary microthrombi in lungs and kidneys of the histone alone group (Fig. 3g,

A&B) to indicate increased microvascular permeability and coagulation activation. Similarly, mice euthanized 4 h after injection of 75 mg/kg histones+10 mg/kg CRP (panel C) or 10 mg/kg ahscFv (panel D) showed lung congestion and edema but much less lung hemorrhage. No capillary microthrombi were observed in these animals. Sections from mice euthanized at 24 h (panel E) and 6 days (panel F) after sublethal dose of histones (50 mg/kg) showed numerous neutrophils within lung capillaries (E, arrows) and perivascular rim of macrophages with fewer lymphocytes (F, arrow).

Both exogenous and endogenous histones damage endothelial cells and activate coagulation *in vivo*, which is inhibited by CRP

Immunohistological staining showed that injected histones accumulated in the membrane of endothelial cells. Faint staining was also found on the membrane of blood cells (Fig. 4a, panel A) but no such staining was found in control mice (panel B). In histones+CRP groups, histone accumulation in the endothelial membrane became less obvious (panel C). To demonstrate histone-induced endothelial damage and coagulation activation in mice, levels of soluble thrombomodulin (sTM), an endothelial cell membrane protein which can be shredded and released upon cell damage[28], and TAT, a recognized marker of coagulation activation[29] were measured. sTM and TAT increased about 2 fold in 50 mg/kg histones+saline group compared to saline alone. When histones were pre-incubated with 10 mg/kg CRP and then injected into mice, sTM and TAT levels became significantly lower than in the histones+saline group (Fig.4b, c). To explore the effect of endogenous histones, we established a modified mice model of severe trauma based on previous reports[30] by dropping a heavy object onto each leg of anesthetized mouse. H3 was detectable after trauma (Fig. 4d). The levels in trauma+saline (CRP-) group and trauma+CRP (CRP+) group were not significantly different (4e) but sTM and TAT levels were significantly higher in trauma+saline group than that in control and trauma+CRP groups ($P<0.05$)(Fig. 4f&g), indicating that endogenous histones released from tissue injury was similar to exogenous histones in causing endothelial cell damage and coagulation activation, which can be inhibited by CRP.

Extensive cell death in trauma patients elevates circulating histones to levels that exceed neutralization in blood

Levels of circulating nucleosomes of 250 trauma patients (Supplemental Table 2) on admission were associated with severity estimated by injury severity scores (ISS) (Fig. 5a). Western blotting, determined the levels of non-degraded histone H3 (Fig. 5b) from which the total circulating histones could be estimated. In patients with major trauma, the calculated total circulating histones ranged from 10 to 230 μg/ml within 4h after injury with Median: 28.6, Quartile: 13.7, 58.9 μg/ml in a group of 52 major trauma patients. When applied in DMEM medium, 10 μg/ml histones showed significant toxic effects on cultured endothelial cells. When applied in serum or plasma (using PPACK, a non-calcium chelating anticoagulant[31]) these effects were not evident until histones reached 20 and 40 μg/ml, respectively, suggesting normal blood could neutralize at least 10-30 μg/ml histones. Components in serum and plasma that neutralize histones are not clear. Patients had significantly higher sTM (Median: 4600.3 Quartile: 3648.6, 5497.1 pg/ml) and TAT (Median 81.2, Quartile 33.7, 110.3 ng/ml) than healthy donors (sTM Median: 2389.3 Quartile: 1123.1, 3011.9 pg/ml, TAT Median: 6.0 Quartile: 5.6, 6.4 ng/ml, n=20) (Median test, $P<0.01$). Although the correlation between H3 and ISS was poor (Fig. 5c, $r=0.36$, $p<0.05$), the levels of sTM and TAT in the 52 trauma patients were well correlated to circulating histones (Fig. 5d&e, $r=0.55$ and $r=0.56$, $P<0.001$). These results are consistent with the finding in animal experiments and suggest that in trauma patients, high levels of circulating histones are toxic.

Complexes between CRP and circulating histones limit damage to endothelial cells in patients

Further dynamic analysis of a group of 7 patients with simple blunt injury showed that circulating nucleosomes and histones sharply increased on admission and were significantly reduced to 20-30% of original levels by 72 h after injury (Fig. 5f). CRP levels started to increase at 4-6 h after injury to peak at around 24 h. At this time, CRP-histone complexes became detectable (Fig. 5f). This data indicates that CRP elevation lagged at least 4 h behind the histone surge. Serum containing about 50 μg/ml histones collected within 4 h after injury was toxic to cultured endothelial cells (Fig. 5h), which could be

neutralized by adding CRP and ahscFv, or 100 nM APC (Fig 5g). This suggests that the toxicity comes from histones within these sera. In contrast, sera collected after 24 h, which contained both high levels of histones (rebound after surgery) and CRP showed no significant toxic effects. Similar results were obtained with sera from patients with severe sepsis and necrotizing pancreatitis (Data not shown). These observations support the idea that endogenous CRP elevation neutralizes the toxic effects of circulating histones in many clinical scenarios.

IL-6 secretion triggered by circulating histones induces CRP production in a feedback mechanism

We noticed that levels of circulating histones were positively correlated to IL-6 (Fig. 6a) and IL-1β (Supplemental Fig 4) in trauma patients but the latter only increased significantly in a few patients. Since IL-6 is the essential regulator of CRP expression in hepatocytes[32,33], this observation suggests that an IL-6-mediated link between histones and CRP exists. Previous reports show that T lymphocytes, endothelial cells, macrophages and neutrophils secrete IL-6[34]. In trauma, IL-6 reached a peak value around 2h after injury[35] which suggests that pre-synthesized IL-6 was released from storage. We found that isolated leukocytes from human peripheral blood released high levels of IL-6 from 2h after stimulation with 50 μg/ml histones (Fig. 6b) and the release was inhibited by 100 μg/ml CRP (Fig. 6c). Medium containing the released IL-6 stimulated CRP production in hepatocytes (Fig. 6d). Intravenous injection of histones into mice stimulated IL-6 release, which peaked at 4h (Fig. 6e). Similarly, IL-6 was significantly increased in the trauma model (Fig. 6f). Flow cytometry showed that IL-6 existed in lymphocytes, neutrophils and monocytes from healthy donors (6g). Immunohistochemical staining showed that IL-6 was positive in normal mouse blood cells and bronchi epithelial cells (Fig. 6h). These data demonstrate that circulating histones are major factors in stimulating IL-6 release in trauma to trigger CRP production and also explain why IL-6 increase is in proportion to the magnitude of the surgical stress[36]. The data also suggest that a physiologically relevant negative feedback loop exists (Fig. 6i), which could have an important impact on the survival from trauma or other diseases accompanied by extensive cell death or tissue injury.

DISCUSSION

Elevation in CRP levels has long been recognized as a non-specific response to many pathological situations, including infection, inflammation, neoplasm and injury. Here we demonstrate for the first time that the generic role of CRP is to combat the toxicity of histones released after extensive cell death. CRP elevation in trauma is a direct response to circulating histone-stimulated IL-6 secretion and is able to reduce secondary damage by forming CRP-histone complexes.

It is reported that histones bind to different phospholipids[37-40], which are major components of the cell membrane. Here, we confirm that histone integration into the cell membrane and the resultant Ca^{2+} influx are the major mechanism of histone toxicity to endothelial cells. CRP, APC and ahscFv protect these cells by reducing histone-membrane interaction.

Evidence of endothelial damage *in vivo* by both exogenous and endogenous histones is provided by the elevation of sTM. Increased endothelial permeability is another indicator. In histone-treated mice, lung oedema, haemorrhage, and increased wet/dry lung weight ratio reflect the vascular permeability changes. Thrombosis and elevated TAT levels may be partly due to endothelial damage. However, other factors, such as impairing protein C activation[41], histone-induced neutrophil extracellular traps (NET)[42] and platelet activation[41,43,44] (Supplemental Fig. 5, 6) are also involved. AhscFv but not CRP is able to inhibit H3 or H4 induced-platelet aggregation and suggests that the mechanism for histone-induced platelet activation is different from that of histone-induced endothelial damage. This differential rescue effect of CRP might be helpful in not compromising the histone-induced haemostatic response to injury whilst dampening histone damage to endothelial cells, in particular.

CRP and APC are the currently known natural molecules with anti-histone properties. Their circulating levels are low in healthy adults but CRP can increase 1000 fold to reach 500 μg/ml in the acute phase of many diseases. Protein C levels can decrease in conditions, such as severe sepsis. We also noticed that CRP elevation is mediated by IL-6, which directly responds to the increase in circulating histones. Therefore, CRP elevation in acute phase responses represents a powerful mechanism in humans to combat the toxicity of circulating histones.

Although circulating histone levels in patients with trauma did not reach the concentrations used in mice to induce lethality, their levels far exceeded the normal blood capacity to neutralize histone toxicity. As observed, histone-induced secondary injury occurred and may promote organ failure by damaging endothelial cells, increasing micro-vascular permeability and activating blood coagulation. We noticed that in the acute phase of severe trauma, nucleosomes and histones are released immediately after tissue injury while it takes up to 6 h or more for CRP to rise to active levels[45]. This indicates a time window for histones to exert their toxic effects before they are neutralized by CRP. Therefore, a potential critical time period for clinical intervention exists for anti-histone therapy. From a therapeutic standpoint, APC is an anti-coagulant with bleeding as a major side effect and this would prevent its use in many acute injury states. CRP activates the complement system, which might cause further tissue damage. The potential is there for specific anti-histone therapy and in this study, we have developed an ahscFv (Supplemental Fig. 1) which protects cultured endothelial cells and mice exposed to histones. Further optimization to improve efficacy and reduce possible side effects could hold promise and provide hope for much needed solutions in the acute management of severe trauma and life-threatening injuries.

ONLINE METHODS

Tissue culture. EAhy926 (human endothelial), Hep3B (human hepatocytes from ATCC) and U937 (human monocytic) cell lines was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 20% Fetal Bovine Serum (FBS, Sigma). Human umbilical vein endothelial cells (HUVECS) were isolated as described previously[46]. The detached cells were cultured in DMEM medium supplemented with 20% FBS and 5 ng/mL epidermal growth factor (Invitrogen) and used within 3 passages.

Reagents. Human CRP (Merck) proteins were extensively dialyzed and concentrated. After confirmation of the pentamer form of each protein by HPLC gel filtration, CRP at final concentrations of 1, 2 or 4 mg/ml was stored at -80°C. Anti-histone scFv was expressed in *E. coli* and purified using his-binding resin. LPS contamination was also monitored using E-Toxate™ reagents (Sigma). Similarly, recombinant histones (New England Biolab) and calf thymus histones (Roche) were also monitored using E-Toxate™. Any reagents with LPS over 0.1 EU/mg were passed through a Detoxi-Gel™ column (Pierce) to remove LPS and reconfirmed as LPS-free prior to use.

Isolation of human histones. Histones were isolated from the human monocytic U937 cell line using the acid extraction method [17]. Isolated histones were concentrated using a Vivaspin protein ultrafiltration device (5,000 MWCO) (Sartorius Stedim biotech) and their purity ascertained by 15% SDS/PAGE stained with Coomassie Brilliant Blue. H1, H2A, H2B, H3 and H4 migrated with correct molecular weights and the overall purity was over 95%. The preparation did not contain detectable LPS and other nuclear proteins.

Gel overlay assay. Equal amount of proteins (3 μg) were subjected to SDS-PAGE. One gel was stained with Coomassie Brilliant Blue to check equal loading and the other gel were electro-blotted onto PVDF membranes, which were then incubated at 4°C overnight with biotinylated CRP. The CRP bound to histones was detected using streptavidin –HRP (Sigma).

Binding assays using a biosensor. Binding reactions were carried out in an IAsys two-channel resonant mirror biosensor (Affinity Sensors), as described previously[47]; CRP purified from human ascites (Merck) was immobilized on aminosilane surface using BS^3 (Perbio, Chester, UK). One μM of each class of human recombinant histones was applied to the surface and the binding curves recorded. The recombinant histones were also immobilized on aminosilane surfaces separately and 5.5 μg/ml of CRP in solution was applied. This is to ensure its pentameric form during the binding assays and to rule out the possibility that pentameric CRP may be interrupted during immobilization and surface regeneration.

Histone cytotoxicity assay. A propidium iodide method was used, as previously described[16]. Briefly, EAhy926 cells were grown to 70-90% confluence in 24-well plates. Cells were washed with PBS and then incubated with histones with or without human CRP in DMEM (Sigma) medium supplemented with 2% FBS, for 1 h under 5% CO_2 at 37°C. When histones and CRP or APC was used, treatments were prepared 30 minutes prior to incubation with the cells. After 1 h incubation, cell supernatant was removed and cells were detached with Versene (GIBCO). Cells and supernatant were then combined and washed twice in PBS (Fisher Scientific International Inc.) and fixed in 70% ethanol for 30 min at -20°C. Cells were then centrifuged at 500g for 5 min and stained with propidium iodide (final concentration of 20 μg/ml). Flow cytometric analysis of propidium iodide-stained damaged nuclei results in a broad peak of hypodiploid particles, clearly separated from the distinct diploid DNA peak of viable cells.

Permeability assay. The permeability of a confluent monolayer of endothelial cells was analyzed in a dual chamber system using Evans blue-labeled BSA, as described previously[48]. In brief, 100 μl ($5x10^5$) EAhy926 cells were seeded on the upper chamber of Transwell polycarbonate membranes (24 well, Polycarbonate filters, 0.4 μm pore size, Corning) and the lower chambers filled with 500 μl of DMEM (supplemented with 20% FBS), cells were then grown for 2 days at 37°C to obtain a monolayer. Permeability was assessed by replacing the media in the upper chamber with 100 μl Evans Blue-BSA (0.67 mg/ml Evans blue in DMEM containing 4% BSA) and in the lower

chamber with 500 µl media (DMEM supplemented with 4% BSA). After 10 min, a 100 µl aliquot was taken from the lower chamber and absorbance measured at 650nm using a spectrometer.

FITC labeling and confocal microscopy. The histones were labeled using FluoroTag^{TM} FITC conjugation kit (Sigma) according to the manufacturer's instructions and free FITC was separated from FITC-proteins using sephadex G25 column. The FITC- labeled protein was added into cultured EAhy926 cells to a final concentration of 10 µg/ml on a 35 mm glass-bottomed dish. The images were taken using LSM 710 confocal microscopy.

Electrophysiology. Whole-cell currents were recorded using the perforated patch configuration from single EAhy926 cells using an Axopatch 200B amplifier (Axon Instruments) as previously described[49,50] Recorded membrane currents were filtered at 5 kHz, digitized using a Digidata 1320A interface (Axon Instruments) and analyzed using pCLAMP software. Patch pipettes were pulled from borosilicate glass capillaries (outer diameter 1.5 mm, inner diameter 1.17 mm; Harvard Apparatus) and fire-polished to give a final resistance of ~ 5 MΩ when filled with pipette-filling solution containing 210 µg/ml Amphotericin B (Sigma-Aldrich). Histones (20 µg/ml), CRP (250 µg/ml) and/or anti-histone scFv (250 µg/ml) were added to the extracellular solution and applied to the cell by bath superfusion. Histones were incubated with CRP or antibody for at least 30 minutes prior to application to the cells. All experiments were performed at room temperature (18–22°C), and the results are expressed as the mean ± S.E.M.

Measurement of intracellular calcium. Intracellular calcium concentration $[Ca^{2+}]_i$ was determined by measuring fluorescence emission at 510 nm during excitation at 340 nm and 380 nm according to a published protocol[51] and Fura-2AM as a fluorescent probe. EAhy926 cells were loaded with 3 µM Fura-2AM for 20 min and then washed with Calcium Assay Buffer (pH7.4) (120 mM NaCl, 4.7 mM KH_2PO_4, 1.2 mM MgCl, 1.25 mM $CaCl_2$, 10 mM Glucose, 20 mM Hepes). Fluorescence was monitored continuously using a Hitachi F-7000 fluorescence spectrometer, and $[Ca^{2+}]_i$ was calculated using the software provided with the instrument. Ca^{2+} influx was stimulated in EAhy926 cells with the addition of calf thymus histones (Roche) or recombinant human histones to the Fura-2 loaded cells.

Animals. C57/BL6 male mice of average weight ~22g from the SLAC Experimental Animal Centre (Shanghai, China) were kept and used at the Research Center of Gene Modified Mice, State Education Ministry Laboratory of Developmental Genes & Human Diseases, Southeast University, China. All procedures were performed according to State laws and monitored by local inspectors. This is also in compliance with British Home Office laws.

Immunohistochemical staining. Paraffin-embedded samples on glass slides were de-waxed and rehydrated followed by antigen retrieval using DAKO PT-Pre-Treatment link system at 97°C. After blocking any endogenous peroxidase activity, the sections were incubated with anti-histone H3 antibody (Abcam) diluted 1:100, and anti-IL-6 (R&D) 1:50 for 2 hours. To confirm their specificity, these antibodies were preincubated with histone H3 or IL-6 protein (molar ration 1:2) and secondary antibody alone as controls to validate each lot of staining. After washing, anti-rabbit secondary antibody-HRP labeled polymer (DAKO EnVision+ System-HRP (DAB) kit), was applied to the slides and incubated for 30 min. The bound antibody was visualized using DAB+chromagen for 1-5 min, followed by counterstaining with Mayer's haemalum and soaking in lithium carbonate (Sigma-Aldrich), After dehydration, the slides were mounted with coverslips using DPX (BDH) mountant and left to dry overnight. Images of the stained samples were taken using Olympus Microscopy and Nikon ACT-1 software.

***In vivo* permeability assay.** Wild type C57BL/6 male mice of 22 gram body weight were challenged with 50 mg/kg histones (i.v.), histones+10 mg/kg CRP, and histones+10 mg/kg anti-histone scFv for 4 h. Pulmonary edema was quantified by measuring the wet to dry weight ratio of the right lung. Wet weight is obtained immediately after extirpation and dry weight after 4 days of drying at 60°C. For histological analysis the cardiac lung lobe was formaldehyde fixed, paraffin embedded and 10 µm microsections were analyzed by HE staining.

Mouse trauma model. The mouse trauma model was created by the fall of heavy object to 4 limbs of anesthetized mice according to

previous reports[30]. Blood was taken with citrate as anti-coagulant from tail veins before and 60 min, 4 h after injury. The plasma was separated by centrifugation and stored at -80°C before analysis. The mice were euthanized at 4 h and organs were taken and fixed by 4% paraformaldehyde for 24 h followed by 70% ethanol.

Quantification of nucleosomes, histones, histone-CRP complexes, CRP, sTM, TAT, IL-6, and IL-1β in patient plasma. To quantify the amount of circulating nucleosomes in patients, a Cell Death Detection ELISA^{PLUS} assay (Roche Diagnostics) was used according to manufacturer's instructions. The assay for nucleosomes (histone-DNA complexes) is based on a quantitative sandwich-ELISA whereby two monoclonal antibodies generated against DNA (single and double stranded) and histones (H1, H2A, H2B, H3 and H4) capture mono- and oligo-nucleosomes. For histone-CRP complexes, the same kit was used by replacing anti-DNA antibody with HRP-conjugated anti-CRP monoclonal antibody. The CRP ELISA kit was from DiaMed. ELISA kits for sTM and TAT were purchased from Cusabio Biotech. ELISA kit for IL-6 and IL-1β were from Invitrogen. Each sample was performed in duplicate. Histone H3 in plasma were detected by Western blotting using anti-histone H3 antibody (Abcam) and calculated using human recombinant histone H3 protein (New England Biolabs) as standards.

Whole blood flow cytometry analysis. To identify the population of IL-6 containing white blood cells, 100 µl of whole blood was incubated at room temperature for 15 minutes with 200 µl of Fixation Medium (Fix&Perm®Reagent A, AN GRUB, Austria) then washed in 10 mls PBS by centrifugation for 5 min at 300g. The cells were then resuspended in 200 µl Permeabilization Medium (Fix&Perm®Reagent B, AN GRUB, Austria) and incubated at room temperature for a further 15 min. Cells were then washed in 10 ml PBS and centrifuged for 5 min at 300g. The white blood cell pellet was then resuspended in 100 µl PBS containing 5% BSA and either 0.5 µg Rat Anti-Human IL-6-PE (BD Pharmingen™, UK) or Normal Rat IgG-PE antibody control. After 30 min incubation the cells were washed twice in 100 µl PBS then resuspended in 100 µl PBS for immediate flow cytometric analysis. To identify the neutophil, monocyte and lymphocyte populations, cells were stained with Anti-Human CD15-FITC (BD Pharmingen™, UK), Anti-Human CD14-FITC (BD Pharmingen™, UK) and Anti-Human CD3-FITC (BD Pharmingen™, UK), respectively.

Statistical analysis. Intergroup differences were analyzed using ANOVA followed by the Student-Newman-Keuls test. Two group comparison before and after treatment used Student t test. Animal survival time was analyzed using log-rank test. Association analysis used simple linear correlation.

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AUTHOR CONTRIBUTIONS

STA conducted and analyzed *in vitro* assays of histone toxicity (including protein preparations), membrane binding, endothelial permeability assay and intracellular $[Ca^{2+}]_i$ measurement. NZ and WY did the animal experiments. TTL produced scFv anti-histone antibody and truncated histones. CD conducted and analyzed the electrophysiology experiments. FB did the ELISA assays for IL-β and IL-6 and immunohistological staining. SSW conducted *in vitro* endothelial permeability assay. AK did histological examinations. YA conducted apoptosis analysis and JT did the platelet aggregation assay. JM and KB collected human plasma and did nucleosome ELISA. GW designed, organized the project and wrote the manuscript as well as carried out the binding assays. CHT supervised the whole project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

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Figure legends

Figure 1. The toxicity of histones to cultured endothelial cells can be inhibited by CRP through CRP-histone complex formation. (a) Typical FACS of untreated and histone-treated EAhy926 cells after PI staining, 2N represents intact surviving cells after treatment and <2N represents cells that have lost chromatin and designated as non-surviving cells (Materials and Methods). (b) Survival rate of EAhy926 cells after various treatments. Mean±SD from at least 3 independent experiments are shown. ANOVA tests show that calf thymus histones, dialyzed calf thymus histones and human histones cause significant damage of EAhy926 cells ≤(p 0.03) while BSA or acid-treated BSA had no harmful effect on these cells ($p \geq 0.2$). (c) Toxic effects of individual human recombinant (hr)/calf thymus (cth) histones and calf thymus H2A/H2B and H3/H4 complexes on cultured EAhy926 cells. Cells were treated with 20 μg/ml mixed histones from calf thymus as a control and 20 μg/ml of individual histones or complexes (10 μg/ml of each component). Mean ±SD of cell survival rates from at least 3 independent experiments are shown. *indicates significant reduction of cell survival rates after treatment ($p<0.05$). (d) Dose response of CRP in protecting EAhy926 cells treated with 20 μg/ml calf thymus histones. Means±SD of cell survival rates after treatment from 3 experiments are shown and ANOVA test showed that CRP significantly increased cell survival rates ($p<0.05$). (e) Protective effects of CRP and APC on both HUVECS and EAhy926 cells treated with histones. Both types of cells were cultured to 70-80% confluence and treated with calf thymus histones 20 μg/ml alone or in combination with APC 10 nM or CRP 250 μg/ml as described in Materials and Methods. Means±SD from 3 independent experiments are showed. *indicates a significant reduction of cell survival rates compared with untreated cells (UT) ($p<0.05$). (f) Gel overlay assay to study CRP interaction with immobilized histones (Materials and Methods). Upper panel: gel overlay shows that CRP binds to H1, H2B and H3. Lower panel: Coomassie blue stained gel (n=S100P multimer, m=S100P monomer). (g) and (h) Biosensor assay to study interaction between histones and CRP. CRP (g) or individual histones (h) were immobilized on biosensor surfaces as described in Material and Methods. Binding curves of each human recombinant histone presenting their relative binding strengths to immobilized CRP or vice versa are shown in (g) and (h), respectively. (i) CRP protection of EAhy926 cells treated with 20 μg/ml individual human recombinant histones. Percentages of cells (means±SD) rescued by 250 μg/ml CRP from 4 independent experiments are shown, i.e. cell survival rate differences between cells treated with histones alone and histones +CRP. *indicates significant increases of cell survival rate by CRP addition ($p \leq 0.035$, Student t test).

Figure 2. Membrane binding and calcium influx determine histone toxicity to endothelial cells and could be inhibited by CRP. (a) Confocal images of EAhy926 cells incubated with FITC-histones (10 μg/ml) (panels A-C), FITC-histones+CRP (250 μg/ml) (panels D-F) and FITC-histones+anti-histones scFv (250 μg/ml) (panels G-I). Panels A, D, and G are F show FITC staining (green), panels B,E and H are phase contrast and panels C,F and I are the superimposed images of FITC and phase contrast. Arrows indicate membrane binding of FITC-histones. (b) Representative whole-cell currents recorded from EAhy926 cells when histones (20 μg/ml) were applied to the extracellular bathing solution as indicated. The currents generated by the application of histones were reversible on short exposure (30–60 seconds). (c) An example of elevation of intracellular Ca^{2+} recorded by Hitachi F-7000 fluorescence spectrometer when EAhy926 cells were exposed to different concentrations of histones. (d) An example of intracellular Ca^{2+} elevation triggered by histones (20 μg/ml) that was nearly abolished by removal of Ca^{2+} from extracellular medium. (e) The mean±SD of survival rates of cells incubated with medium containing 0-3 mM Ca^{2+} in the presence or absence of 20 μg/ml histones from 3 independent experiments. *indicate significant reduction of cell survival rate compared to that without calcium (ANOVA test $P<0.05$) (f) and (g) show the inhibitory effects of CRP (250 μg/ml) and anti-histone scFv (250 μg/ml) on histone-induced whole cell current and intracellular Ca^{2+}. Mean±SD from 3 independent experiments are shown. *indicate significant increase compared to all other groups. ǂ indicates significant lower compared to histone alone ($P=0.021$) but significantly higher than the rest of groups ($P<0.05$).

Figure 3. CRP reduces histone-enhanced endothelial permeability and protects mice injected with lethal dose of histones. (a) Endothelial permeability changes measured by a dual chamber system using Evans blue-labeled BSA when confluent EAhy926 cells were treated with 0-50 µg/ml histones. Mean±SD of absorbance from 5 independent experiments are shown. * indicate significant increase of permeability compared to untreated cells ($P<0.05$). (b) the time course of 20 µg/ml histone-induced permeability changes. Mean±SD of absorbance from 5 independent experiments are shown. * indicate significant increase of permeability ($P<0.05$). (c) The inhibitory effects of APC, CRP (250 µg/ml) and anti-histone scFv (250 µg/ml) on 20 µg/ml histone-induced permeability increase. Mean±SD of fold changes to untreated (UT) from 3 independent experiments are shown.*indicates significant increase compared to all other groups. (d) the fold changes of endothelial permeability induced by different histones (2 µM). Mean±SD of fold changes to untreated from 3 independent experiments are shown. *indicate significant increase compared to untreated (ANOVA test, $P<0.05$). (e) the dry/wet weight ratios of lungs from the mice (10 mice in each group) euthanized 4 h after intravenous injection of with saline (control), 50 mg/kg histones, histones+10 mg/kg CRP or anti-histone scFv. * indicate significant increase in ratio, caused by fluid leakage into lungs (ANOVA test, $P<0.05$). (f) Survival curves represent the survival fractions of mice injected intravenously with 75 mg/kg of histones (group 1, n=5), 75 mg/kg histones + 1.6 mg/kg CRP (group 2, n=4), 75 mg/kg histones + 5 mg/kg CRP (group 3, n=6) and 75 mg/kg histones + 10 mg/kg CRP (group 4, n=7). (g) histological changes in mice after treatment with histones and CRP. Haematoxylin staining of sections from mouse that died after injection with 75 mg/kg histones: lung (panel A) and kidney (panel B) showed microthrombi in lung capillaries and glomerular tufts, respectively (arrows). Mice euthanized 4 h after injection of histones (75 mg/kg) + CRP (10 mg/kg) (Panel C) or anti-histone scFv (Panel D) showed lung congestion and edema (arrows) but no microvescular thrombi. Sections from mice euthanized at 24 h (panel E) and 6 days (panel F) after non-lethal dose of histones (50 mg/kg) showed numerous neutrophils within lung capillaries (arrows) (panel E) and perivascular rim of macrophages with fewer lymphocytes (arrow) (panel F). Bar =20 µm.

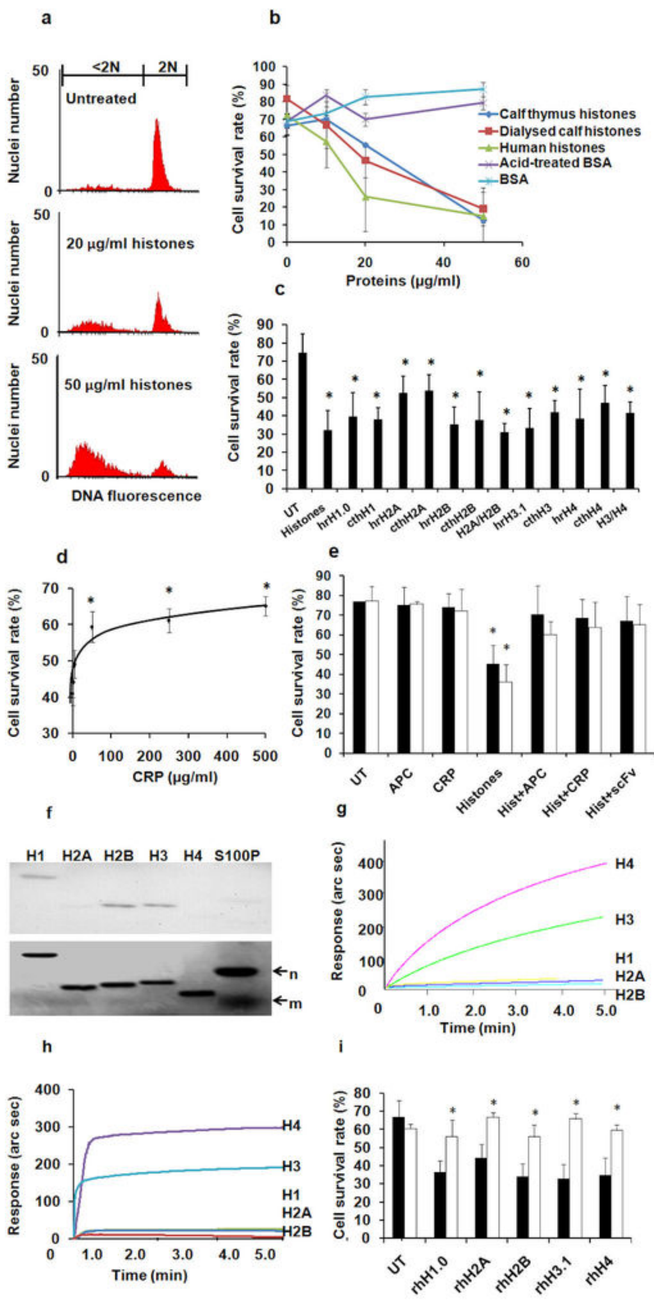
Figure 4. High levels of exogenous and endogenous circulating histones cause endothelial damage and coagulation activation. (a) Immunohistochemical staining of histone H3 in tissues from mice injected intravenously with saline (left), 50 mg/kg histones (middle) and histones+CRP (10 mg/kg) (right). Red arrows indicate the nuclei of endothelial cells and blue arrows indicate the association of histones with the plasma membranes of endothelial cells. (b) and (c) show the levels of circulating sTM and TAT in mice (10 mice per group) injected with saline (control), CRP (10 mg/kg), histones (50 mg/kg) and CRP+histones. Mean±SD are shown. *indicates significant increase when compared to other groups ($P<0.05$, n=6/group). (d-g) Mice trauma model (10 mice per group). (d) An example of western blotting of histone H3 in the plasma taken from mice before (1) and 1 h (2), 4 h (3) after trauma or anesthetics only (Control). In CRP+trauma group, 10 mg/kg CRP was intravenously injected 10 min before trauma. (e) Averages of histone H3 determined by Western blotting. No significant difference between CRP- and CRP+ groups but significant increase in both groups after trauma. (f) and (g) show the changes of sTM and TAT in circulation. Mean±SD are shown. *indicate a significant increase ($P<0.05$) compared to other groups.

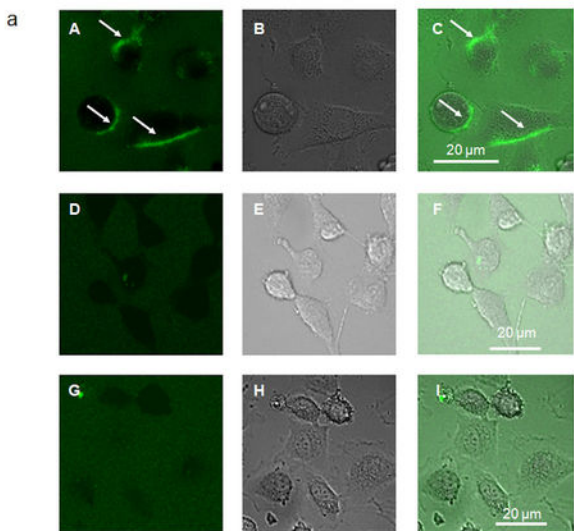
Figure 5. The levels and dynamic changes of circulating histones and CRP in patients with severe trauma. (a) The levels of circulating nucleosomes at admission measured using ELISA kit were significantly increased with the severity of trauma estimated by ISS in a group of 250 patients (Supplemental Table 2). *ANOVA test, $P<0.05$). (b) an example of a Western blot measuring the levels of full length histone H3 in the plasma of trauma patients. The standard H3 recombinant protein was from New England Biolabs. (c) correlation between H3 and ISS in a group of 52 patients ($r=0.36$, $P<0.01$). (d) and (e) correlation of H3 with sTM ($r=0.55$, $P<0.001$) and TAT（$r=0.56$, $P<0.001$）in the same group of patients. (f) dynamic changes of Histone H3, CRP and CRP-histone complexes in a group of 7 trauma patients. The highest values of each parameter were designated as 100%. The absolute values were presented in Supplemental Fig. 7. (g) The toxic effect of sera (50% in cell

culture medium) taken at different time points from a group of 7 trauma patients on cultured endothelial cells, EAhy926. The mean±SD are shown. * indicate a significant reduction compared to normal (ANOVA test, P<0.05). (h) Serum containing about 50 µg/ml histones (estimated by H3) taken at admission was incubated with EAhy926 cells (50% serum in medium alone, or supplemented with 100 nM APC, 250 µg/ml CRP, and 200 µg/ml anti-histone scFv). Cell survival rates are presented. *indicate significant reduction when compared with other groups (ANOVA test， P<0.05).

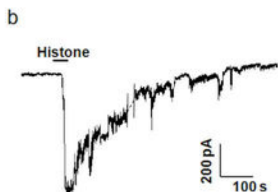
Figure 6. Circulating histones trigger the release of IL-6 and subsequently induce CRP production. (a) Circulating IL-6 significantly increased in severe trauma and correlated with circulating histone H3 in a group of 52 patients (r=0.61, P<0.001). (b) and (c) histones stimulated IL-6 release from isolated leukocytes. Blood was taken from 3 healthy donors and the leukocytes were isolated using gradient centrifugation and cultured in DMEM medium containing 20% FBS in the absence (UT) and presence of 50 µg/ml histones, 250 µg/ml CRP, 5 nM LPS or histones+CRP. Mean levels of IL-6 in culture medium after 4 h incubation as determined by an ELISA kit (Invitrogen) are shown in (b) and a time course is shown in (c). * indicate significant increase of IL-6 released to culture medium compared to untreated. ǂ indicates significant increase compared to other group (ANOVA test, P<0.05). The medium with histones collected from above cultures at 16 h was passed through an anti-histone scFv sepharose column to eliminate most of histones and the pass through was added into Hep 2B (ATCC) cell culture to induce CRP production. The CRP in the medium was detected using ELISA kit and is shown in (d). * indicate that CRP was significantly produced and released from 8 h. (e) the IL-6 in the plasma of mice (n=10) before and after injection of 50 mg/kg histones. Mean±SD are shown. * indicate significant increases from 1 h after injection. (f) the IL-6 in the plasma of mice (n=10) before and 4 h after trauma. (g) an example of flow cytometry analysis of peripheral blood from healthy donors using rat IgG-PE (black, class control), rat anti-human IL-6 PE (blue), and anti-CDs FITC (purple, R1, anti-CD15; R2, anti-CD14; R3, anti-CD3 corresponding to the gates R1, R2 and R3 to represent neutrophils, monocytes and lymphocytes, respectively). (h) An example of immunohistochemical staining of a lung section from a normal mouse with anti-IL-6 antibody (Abcam). This staining could be blocked by pre-incubation of the antibody with IL-6 protein. Red arrow indicates the positive staining of bronchi epithelial cells and black arrow indicates positive staining of blood cells. (i) the diagram of the proposed feedback loop.

Figure 1

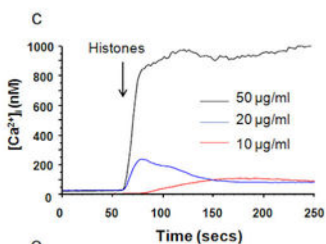




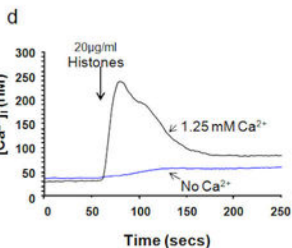
a
A
B
C
20 µm
D
E
F
20 µm
G
H
I
20 µm



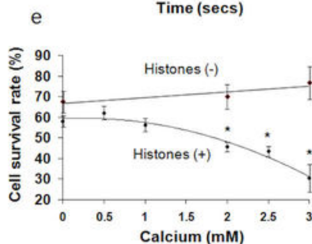
b
Histone
200 pA
100 s



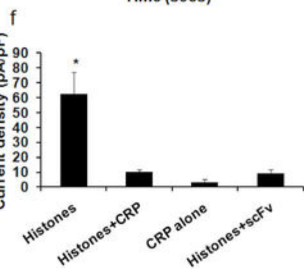
c
Histones
50 µg/ml
20 µg/ml
10 µg/ml
$[Ca^{2+}]_i$ (nM)
Time (secs)



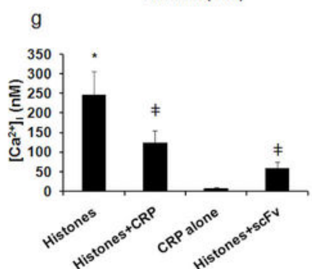
d
20µg/ml
Histones
1.25 mM Ca^{2+}
No Ca^{2+}
$[Ca^{2+}]_i$ (nM)
Time (secs)



e
Histones (-)
Histones (+)
Cell survival rate (%)
Calcium (mM)



f
Current density (pA/pF)
Histones
Histones+CRP
CRP alone
Histones+scFv



g
$[Ca^{2+}]_i$ (nM)
Histones
Histones+CRP
CRP alone
Histones+scFv

a

Absorbance (650nm)

0.00 0.05 0.10 0.15 0.20 0.25

0 10 20 30 40 50

Histones (µg/ml)

c

Fold change

0.0 0.5 1.0 1.5 2.0 2.5 3.0

UT
Histones
Hist+CRP
Hist+APC 10 nM
Hist+APC 100 nM
Hist+scFv

b

Absorbance (650nm)

0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35

0 20 40 60 80 100 120

Time (min)

d

Fold change

0 1 2 3 4 5 6 7 8 9

UT
ctHistones
hrH1.0
ctH1.0
hrH2A
hrH2B
hrH3.1
hrH3.2
hrH3.3
ctH3
hrH4
Thrombin

e

Lung wet /dry weight ratio

0 1 2 3 4 5 6 7 8

Control
Histones
Hist+CRP
Hist+scFv

f

Survival fraction

0 0.2 0.4 0.6 0.8 1

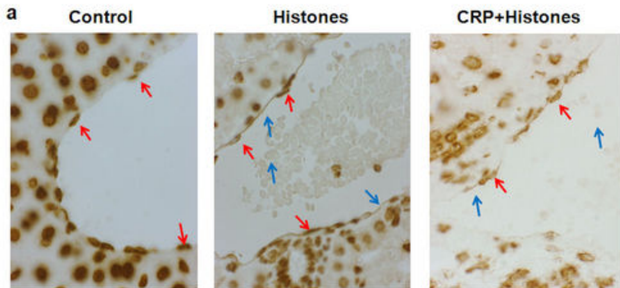
0.00 10.00 20.00 30.00

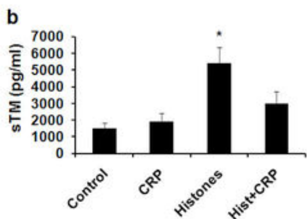
Time (h)

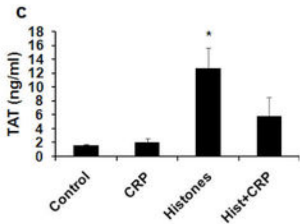
g

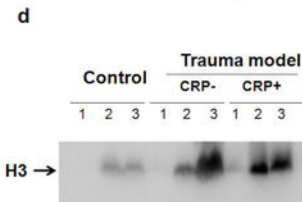
A B C D E F

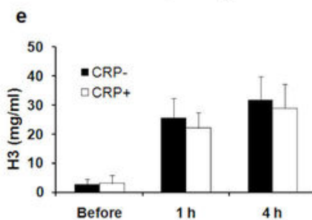
V

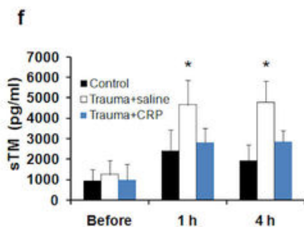

a
Control
Histones
CRP+Histones

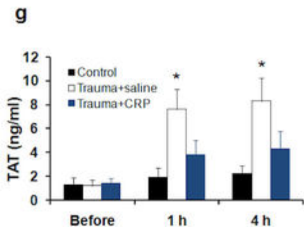

b
sTM (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
*
Control
CRP
Histones
Hist+CRP

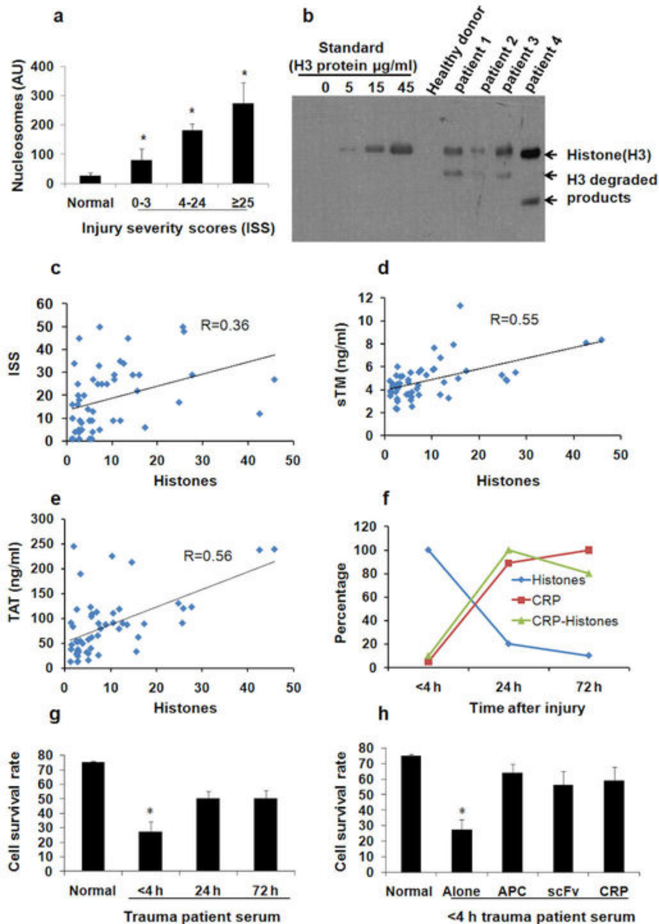

c
TAT (ng/ml)
18
16
14
12
10
8
6
4
2
0
*
Control
CRP
Histones
Hist+CRP


d
Trauma model
Control
CRP-
CRP+
1 2 3 1 2 3 1 2 3
H3

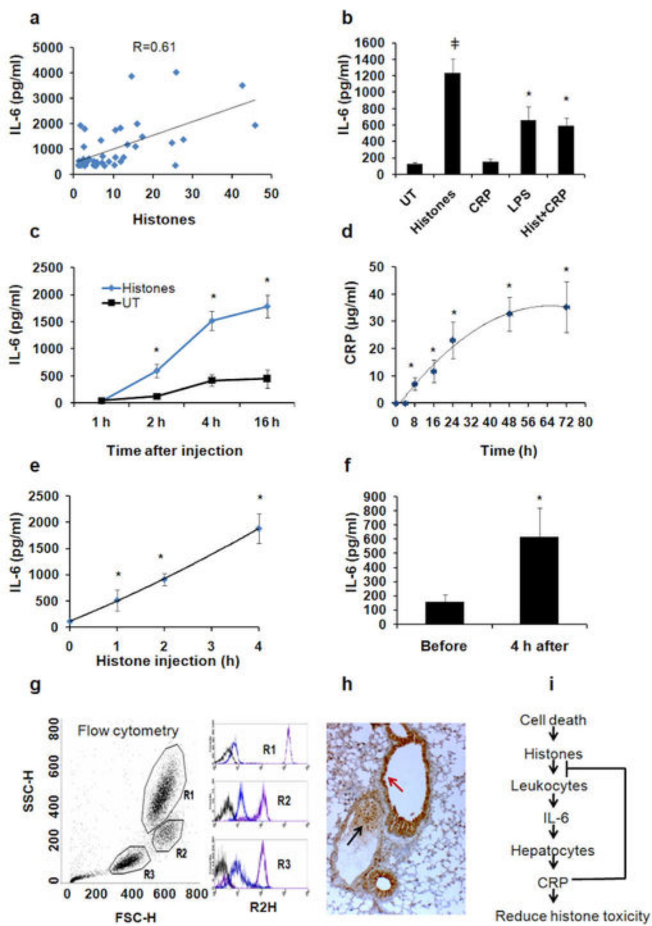

e
H3 (mg/ml)
50
40
30
20
10
0
CRP-
CRP+
Before
1 h
4 h


f
sTM (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
Control
Trauma+saline
Trauma+CRP
*
*
Before
1 h
4 h


g
TAT (ng/ml)
12
10
8
6
4
2
0
Control
Trauma+saline
Trauma+CRP
*
*
Before
1 h
4 h



a
Nucleosomes (AU)
400
300
200
100
0
*
Normal
0-3
4-24
≥25
Injury severity scores (ISS)
b
Standard
(H3 protein μg/ml)
0 5 15 45
Healthy donor
patient 1
patient 2
patient 3
patient 4
Histone(H3)
H3 degraded
products
c
ISS
R=0.36
Histones
d
sTM (ng/ml)
R=0.55
Histones
e
TAT (ng/ml)
R=0.56
Histones
f
Percentage
Histones
CRP
CRP-Histones
<4 h
24 h
72 h
Time after injury
g
Cell survival rate
Normal
<4 h
24 h
72 h
Trauma patient serum
h
Cell survival rate
Normal
Alone
APC
scFv
CRP
<4 h trauma patient serum


a
R=0.61
IL-6 (pg/ml)
Histones
b
IL-6 (pg/ml)
UT
Histones
CRP
LPS
Hist+CRP
c
Histones
UT
IL-6 (pg/ml)
1 h
2 h
4 h
16 h
Time after injection
d
CRP (µg/ml)
Time (h)
e
IL-6 (pg/ml)
Histone injection (h)
f
IL-6 (pg/ml)
Before
4 h after
g
Flow cytometry
SSC-H
FSC-H
R1
R2
R3
R2H
h
i
Cell death
Histones
Leukocytes
IL-6
Hepatocytes
CRP
Reduce histone toxicity