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Human CRP Defends against the Toxicity of Circulating Histones

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C-reactive protein (CRP) is an acute-phase protein that plays an important defensive role in innate immunity against bacterial infection, but it is also upregulated in many noninfectious diseases. The generic function of this highly conserved molecule in diseases that range from infection, inflammation, trauma, and malignancy is not well understood. In this article, we demonstrate that CRP defends the human body against the toxicity of histones released into the circulation after extensive cell death. In vitro, CRP significantly alleviates histone-induced endothelial cell damage, permeability increase, and platelet aggregation. In vivo, CRP rescues mice challenged with lethal doses of histones by inhibiting endothelial damage, vascular permeability, and coagulation activation, as reflected by significant reductions in lung edema, hemorrhage, and thrombosis. In patients, elevation of CRP significantly increases the capacity to neutralize extracellular histones in the circulation. We have also confirmed that CRP interacts with individual histones in vitro and forms CRP–histone complexes in serum from patients with both elevated CRP and histones. CRP is able to compete with phospholipid-containing liposomes for the binding to histones. This explains how CRP prevents histones from integrating into cell membranes, which would otherwise induce calcium influx as the major mechanism of cytotoxicity caused by extracellular histones. Because histone elevation occurs in the acute phase of numerous critical illnesses associated with extensive cell death, CRP detoxification of circulating histones would be a generic host defense mechanism in humans. The Journal of Immunology, 2013, 191: 2495–2502.

The pentraxin protein, C-reactive protein (CRP), has long been recognized as a major acute-phase protein involved in innate immunity and adaptive immunity (1, 2). Overall, CRP plays a crucial role in host defense against bacterial infection.

CRP is also significantly upregulated in many clinical scenarios without infection, such as trauma, pancreatitis, myocardial infarction, neoplasia, and inflammatory disorders. However, its use in clinical assessment is often as a nonspecific marker, and its role(s) in these diseases is largely unknown. In addition to opsonizing pathogens, CRP has also been found to opsonize damaged cells, including nuclear breakdown products, such as chromatin and histones to facilitate their clearance (7, 13–16). Because the presence of circulating nuclear breakdown products has been linked to the development of autoimmune diseases (17), CRP has been proposed as a therapeutic reagent in mouse models of these disorders (18–20).

Recently, histone toxicity has been demonstrated in vitro and in mice (21–27), which prompted us to investigate the potential of CRP in neutralizing these toxic effects. In this article, we demonstrate that CRP detoxifies histones both in vitro and in vivo with the major protective mechanism being through CRP–histone complex formation to block histone–cell membrane integration and the consequent increase in intracellular calcium.

Materials and Methods

Patients

Sera from patients on the Intensive Care Unit at the Royal Liverpool University Hospital, United Kingdom, with diagnoses of severe trauma, necrotizing pancreatitis, and severe sepsis were collected in accordance with protocol approved by the Local Research Ethics Committee and the Hospital Trust.

Animals

C57/BL6 male mice of average weight ~22 g from the Shanghai Laboratory Animal Center (Shanghai, China) were housed 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. These were also in compliance with British Home Office laws. Histones and CRP were injected through the tail veins with outcome monitored for up to 6 d in survival assays. To compare the

The online version of this article contains supplemental material.

Abbreviations used in this article: abcsFv, anthonite single-chain variable fragment; CRP, C-reactive protein; dCRP, denatured C-reactive protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, propidium iodide; PS, phosphatidylserine; sTM, soluble thrombomodulin; TAT, thrombin-antithrombin.

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pathological changes of different treatments, we euthanized mice at 1 or 4 h after injection. Blood was collected and serum isolated within 2 h and stored at −80°C. Organs were fixed in 4% (v/v) paraformaldehyde for 24 h and then stored in 70% (v/v) ethanol until embedded and sectioned.

**Tissue culture**

EAh926 (human endothelial) cell line was cultured in DMEM (Sigma) supplemented with 20% (v/v) FBS (Sigma). HUVECs were isolated as described previously (28) and cultured in DMEM supplemented with 20% (v/v) FBS and 5 ng/ml epidermal growth factor (Invitrogen) for use within three passages.

**Reagents**

Human CRP (Merck) was 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 −20°C. Antihistone single-chain variable fragment (ahscFv) was expressed in *E. coli* and purified, as previously described, with demonstration of anti-histone specificity (27). LPS contamination was monitored using E-Toxate reagents (Sigma). Recombinant histones (New England Biolabs) and calf thymus histones (Roche) were also monitored using E-Toxate.

**Histone cytotoxicity assay**

A propidium iodide (PI) method was used, as previously described (29). In brief, EAh926 cells were grown to 70–90% confluence and treated with histones or histones preincubated with CRP or activated protein C for 30 min in DMEM supplemented with 2% (v/v) FBS. After 1 h of incubation, the medium was removed to a fresh tube, and the remaining cells were detached using Versene and transferred to the same tube. The cells were pelleted, washed, and fixed in 70% (v/v) ethanol for 30 min at −20°C before staining with 20 μg/ml PI. Flow cytometric analysis of PI-stained damaged nuclei results in a broad peak of hypodiploid particles, clearly separated from the distinct diploid DNA peak of viable cells.

**Quantification of circulating histones, histone–CRP complexes, CRP, soluble thrombomodulin, and thrombin-antithrombin**

A Cell Death Detection ELISA PLUS kit (Roche Diagnostics) was modified for measuring histone–CRP complexes in serum by replacing anti-DNA

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**FIGURE 1.** CRP reduces histone-induced endothelial cell damage and vascular hyperpermeability. (A) The toxic effects of different histone concentrations on EAh926 cells in the presence of 2% (v/v) FBS. Means ± SD are shown, *p < 0.05 compared with untreated (ANOVA). (B) Dose response of CRP in protecting EAh926 cells treated with 40 μg/ml histones in 2% (v/v) FBS medium. Means ± SD are shown, and ANOVA test showed CRP from 50 μg/ml significantly increased cell survival rates (p < 0.05). (C) Comparison of histone (40 μg/ml) toxicity with both primary (HUVEC) and immortalized (EAh926) human endothelial cells in 2% (v/v) FBS medium with activated protein C as positive control. *p < 0.05 compared with other groups (ANOVA). (D) sTM in the blood of mice 4 h after i.v. injection of saline (control), 10 mg/kg CRP, 50 mg/kg histones and combined histones and CRP (Hist+CRP). Means ± SD of circulating sTM in mice (n = 10 per group) are shown; *p < 0.05 compared with other groups (ANOVA). (E) Inhibitory effects of CRP (250 μg/ml) on 50 μg/ml histone-induced endothelial permeability (*Materials and Methods*). Mean ± SD of fold changes to untreated (UT) controls are shown. *p < 0.05 compared with other groups (ANOVA). (F) Wet/dry weight ratios of lungs from mice (n = 10 per group) euthanized 4 h after i.v. injection of saline (control), 50 mg/kg histones, or histones + 10 mg/kg CRP (Hist+CRP). *p < 0.05 compared with control (ANOVA).
Ab with HRP-conjugated anti-CRP mAb (Abcam). ELISA kits were also used to determine CRP (Diamed), soluble thrombomodulin (STM), and thrombin-antithrombin (TAT; Cusabio Biotech) levels in serum. Each sample was performed in duplicate. Histone H3 in plasma were detected by Western blotting using anti-histone H3 Ab (Abcam) and calculated using human recombinant histone H3 protein as standard.

**Permeability assay**

The in vitro permeability was analyzed in a dual-chamber system using Evans blue–labeled BSA, as described previously (30). In brief, EAhy926 cells were grown on the upper chamber of Transwell polycarbonate membranes (Corning) to a confluent monolayer and treated with histones in DMEM with 2% (v/v) FBS for 1 h. Permeability was then assessed by replacing the media in the upper chamber with 100 μl Evans blue–BSA and in the lower chamber with 500 μl DMEM with 1% (v/v) BSA. After 10 min, a 100 μl aliquot was taken from the lower chamber, and absorbance was measured at 650 nm using a spectrometer. In vivo permeability assay was carried out as follows: wild type C57BL/6 male mice of 22 g were injected i.v. with saline (control), 60 mg/kg histones (LD 50; nonlethal dose) preincubated without or with 10 mg/kg CRP (Hist+CRP); n = 10 per group) 4 h after injection of 50 mg/kg histones (nonlethal dose) preincubated with or without 10 mg/kg CRP (Hist+CRP); *p < 0.05 compared with histones alone (Student’s t test). Student’s t test). Mice (10 per group) were injected with saline (100% lethal dose) without (control) or with preincubation with CRP at 1.6 mg/ml, 75 mg/kg histones (Hist alone), or 10 mg/kg CRP (Hist+CRP) for 4 h. Pulmonary edema was quantified by measuring the wet/dry weight ratio of the right lung. Wet weight was obtained immediately after euthanasia and dry weight after 4 d of drying at 60˚C.

**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 (31). Recorded membrane currents were filtered at 5 kHz, digitized using a Digidata 1320A interface (Axon Instruments), and analyzed using pCLAMP software. Histones (20 μg/ml), CRP (250 μg/ml), or histones preincubated with CRP for 30 min were added to the extracellular solution and applied to the cell by bath superfusion. All experiments were performed at room temperature (18–22˚C).

**Measurement of intracellular calcium**

Intracellular Ca2+ concentration was determined by measuring fluorescence emission at 510 nm during excitation at 340 and 380 nm according to published protocols (32) with fura 2-AM as the fluorescent probe. Fluorescence was monitored continuously using a Hitachi F-7000 fluorescence spectrometer, and intracellular Ca2+ concentration was calculated using the software provided. Ca2+ influx was stimulated by adding calf thymus histones to the fura 2–loaded EAhy926 cells.

**Fluorescent staining**

ahscFv and histones were labeled using a FluoroTag FITC conjugation kit (Sigma), and CRP was conjugated with Cy5 (GE Healthcare) separately. Free FITC or Cy5 was removed using a Sephadex G25 column. EAhy926 cells were fixed with 4% (w/v) paraformaldehyde, permeabilized with 1% (v/v) Triton X-100, and blocked with 5% (w/v) defatted milk before the co-staining with 10 μg/ml FITC-ahscFv and Cy5-CRP for 1 h. Images were taken using LSM 710 confocal microscope. For histone–plasma membrane interactions, EAhy926 cells were seeded in glass-bottom dishes for 24 h and incubated with FITC-histones (10 μg/ml) alone or with CRP, denatured CRP (dCRP) (250 μg/ml), and liposomes (100 mM) made of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidyserine (PS). The images were taken after 10-min incubation using LSM 710 confocal microscope.

**Binding assays**

Gel overlay was used to determine which histones bind CRP. In brief, equal amounts of proteins were subjected to SDS-PAGE. One gel was stained with Coomassie brilliant blue to ensure equal loading and the other electroblotted onto polyvinylidene difluoride membranes, which were incubated at 4˚C overnight with 3 μg/ml CRP. Bound CRP was detected using anti-CRP-HRP (Sigma). In addition, the competitive binding of histones to CRP or

![Figure 2](http://www.jimmunol.org/)  
**FIGURE 2.** CRP inhibits histone-induced platelet aggregation and coagulation activation. (A) Effect of human recombinant histones on washed platelets. Platelet aggregation was measured after 40 μg/ml individual histone H3 or H4 was added to freshly isolated platelets from healthy donors in the absence or presence of 250 μg/ml CRP. A typical curve of light transmission is shown. (B) H3–induced platelet aggregation was set up as 100%. Means ± SD of relative percentages in platelet aggregation from four independent measurements using platelets isolated from different donors are shown. *p < 0.05 compared with control (ANOVA); ‡p < 0.05 compared with histone alone (Student t test). (C) Mice (10 per group) were injected i.v. with saline (control), 60 mg/kg histones (LD50) preincubated without or with 10 mg/kg CRP. Blood was taken 15 min after injection, and platelet count normalized against hematocrit (HCT) is shown. #p < 0.05 compared with control (ANOVA); *p = 0.012 compared with histones alone (Student t test). (D) Mean ± SD of circulating TAT levels in mice (n = 10 per group) 4 h after injection of 50 mg/kg histones (nonlethal dose) preincubated without or with 10 mg/kg CRP (Hist+CRP); *p < 0.05 compared with other groups (ANOVA).

![Figure 3](http://www.jimmunol.org/)  
**FIGURE 3.** CRP protects mice challenged with lethal doses of histones by reducing lung edema, hemorrhage, and thrombosis. (A) Survival curves representing survival fractions of mice injected i.v. with 75 mg/kg histones (100% lethal dose) without (n = 10) or with preincubation with CRP at 1.6 mg/ml, 2.5 mg/ml, and 10 mg/kg CRP for 4 h after injection (n = 7). Mean ± SD of relative percentages in platelet aggregation from four independent measurements using platelets isolated from different donors are shown. *p < 0.05 compared with control (ANOVA); ‡p < 0.05 compared with histone alone (Student t test). (B) Pathological changes of lungs from mice. H&E stained (a–c) and immunohistochemically stained (d–f) with anti-fibrin Ab to demonstrate thrombosis. Lung sections from mice were injected with saline (a, d), 75 mg/kg histones (b, e), or histones preincubated with 10 mg/kg CRP (c, f). Black and blue arrows in (b) indicate lung edema and hemorrhage, respectively. Black arrow in (c) indicates the increased thickness of the alveolar wall. Red arrows indicate thrombosis (e) and fibrin deposition (f). Scale bar, 50 μm.
phospholipids was examined by a competitive ELISA. In brief, histones were coated on ELISA plates and incubated with different doses of liposomess mixed with 50 μg/ml CRP. The bound CRP was detected with anti–CRP-HRP, and the absorbance at 450 nM was used to represent the relative amount of CRP that bound to coated histones. The liposomes were prepared as described previously (33) using PS and PE at a ratio 1:2 to reflect the ratio in plasma membrane. PC, another major phospholipid in cell membranes, was omitted to avoid the possibility of its interaction with CRP (34).

**Immunohistochemical staining**

Paraffin-embedded lung sections were dewaxed and rehydrated followed by Ag retrieval using DAKO PT-Pre-Treatment link system. Anti-fibrin Ab (Abcam) and EnVision+ kit (DAKO) was used to probe fibrin. Images were taken using Olympus Microscopy and Nikon ACT-1 software.

**Platelet aggregation assay**

Blood was withdrawn from healthy donors, and freshly isolated washed platelets (35) were treated with recombiant human histone H3 and H4 (New England Biolabs), preincubated with or without CRP. Platelet aggregation was conducted using a Born Aggregometer (pap 8, Bio-DATA).

**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 with animal survival time analyzed using a log-rank test. Association analysis used simple linear correlation. Mean ± SD are from at least three independent experiments.

**Results**

**CRP reduces histone-induced endothelial cell damage and permeability increase**

Extracellular histones have been demonstrated to be toxic to endothelial cells (24). Fig. 1A showed that calf thymus histones in culture medium with 2% (v/v) FBS are significantly toxic to cultured EAhy926 cells, a human endothelial cell line. The toxicity of 40 μg/ml histones to EAhy926 cells can be significantly reduced when CRP was >50 μg/ml (Fig. 1B). BSA, as control, was unable to block histone toxicity to these cells (data not shown). Primary HUVECs isolated from cords were also used and showed similar responses to indicate that histone toxicity and CRP protection are not cell-line–specific effects (Fig. 1C). Activated protein C, which can cleave histones (24), was also used as positive control and could reduce histone toxicity in this assay.

In vivo translation of these data are shown in Fig. 1D–F. sTM, a circulating marker of endothelial damage, increased significantly in mice injected with a nonlethal infusion of histones at 50 mg/kg. When this was coinjected with 10 mg/kg CRP, sTM levels decreased significantly (Fig. 1D). Because the immediate consequence of endothelial damage is an increase in vascular permeability, preincubation with CRP was able to reduce significantly histone-induced permeability in vitro (Fig. 1E) and in vivo (Fig. 1F). Collectively, these data indicate that CRP can inhibit extracellular histone-induced endothelial damage and its consequences.

**CRP inhibits histone-induced platelet aggregation and coagulation activation**

CRP significantly inhibited histone-induced platelet aggregation by up to 50% but did not block it completely even at a very high concentration of 250 μg/ml (Fig. 2A, 2B). In mice, coinfusion of CRP (10 mg/kg) partially alleviated thrombocytopenia induced by injection of 60 mg/kg histones (Fig. 2C). Activation of coagulation, as measured by TAT complexes, was also significantly reduced in mice injected with 50 mg/kg histones + 10 mg/kg CRP compared with histone treatment alone (Fig. 2D). These data strongly indicate that CRP is able to attenuate histone-induced platelet and thrombin activation. The significant but incomplete blockage of these effects by CRP might serve to retain vital hemorrhagic processes whereas limiting excessive histone-induced damage at sites of vascular injury.

**CRP protects mice challenged with lethal doses of histones by reducing lung edema, hemorrhage, and thrombosis**

A total of 75 mg/kg histones killed all mice within an hour. Survival times were prolonged with one in seven mice surviving for >6 d when low-dose CRP (1.6 mg/kg) was coinfused. When CRP level was increased to 5 mg/kg, 3 of 7 mice survived for >6 d and 4 of 7 mice died between 7 and 11 h. At 10 mg/kg CRP, all seven mice survived >6 d (Fig. 3A). A log-rank test showed significant differ-

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**FIGURE 4.** Acute CRP elevation in patients reduces histone-induced toxicity to endothelial cells. (A) Sera of healthy individuals (normal) and of patients at different time points after severe trauma (1, 4, 24, and 72 h) were incubated with EAhy926 cells and expressed in relation to circulating histone and endogenous (Endo) CRP levels. Survival rate of untreated cells was set as 100%. Means ± SD of normalized cell survival rates from three independent experiments were presented; *p < 0.05 compared with normal (ANOVA). (B) CRP–histone complex levels in sera from seven patients at different time points (1, 4, 24, and 72 h) after severe trauma were detected using modified Cell Death ELISA kit (Roche) by replacing anti–DNA-HRP with anti–CRP-HRP. Means ± SD are presented; *p < 0.05 compared with first time point (ANOVA). (C and D) The toxic effect of sera from patients with necrotizing pancreatitis (C) or severe sepsis (D) on the survival rate of EAhy926 cells in relation to CRP levels (endogenous [Endo] concentration and/or exogenous [Exo] CRP addition). Means ± SD of normalized cell survival rates from three repeated experiments using the same serum are presented; *p < 0.05 compared with other groups (ANOVA).
ences in survival times among the four groups (p < 0.001). Histological examination showed that mice that died at 1 h after injection of 75 mg/kg histones alone had obvious lung edema and hemorrhage (Fig. 3Bb), but mice euthanized 1 h after injection of 75 mg/kg histones + 10 mg/kg CRP had less edema and hemorrhage, although congestion and increased alveolar wall thickness were observed (Fig. 3Bc). Immunohistochemical staining for fibrin showed obvious thrombosis in lungs from mice injected with 75 mg/kg histones alone (Fig. 3Be), but not in mice injected with histones preincubated with 10 mg/kg CRP. However, fibrin deposition was clearly observed in lungs (Fig. 2Bf). These pathological changes support the finding that CRP reduces histone-induced endothelial damage and coagulation activation.

Acute CRP elevation in patients reduces histone toxicity to endothelial cells

When serum samples from patients with severe trauma were incubated with endothelial cells, those collected within 4 h of injury could induce significant cell death (Fig. 4A). However, this effect was not observed at later time points of 24 and 72 h despite histone levels remaining elevated. The functional difference could be because of endogenous CRP levels, which were not raised within 4 h but were >150 μg/ml at 24 and 72 h after trauma. Indeed, this corresponded to significant elevation of CRP–histone complex levels, but only at 24 and 72 h (Fig. 4B). These data suggest that CRP is a major factor in combating histone-induced toxicity in patients.

To confirm this, we collected sera from patients with necrotizing pancreatitis (Fig. 4C) and severe sepsis (Fig. 4D), which are conditions also associated with extensive cell damage. Sera that contained high histone levels but low CRP concentrations were toxic to endothelial cells, whereas sera that were high in both CRP and histones were much less toxic. If exogenous CRP was added to sera with high histone levels, their toxic effects could be attenuated. These data indicate that elevated CRP is able to reduce clinically toxicity of circulating histones.

The mechanism of CRP detoxification is through preventing histone–cell membrane interaction by complex formation

We previously showed that histone-induced toxicity relied on membrane interaction and calcium influx (27). Preincubation of histones with CRP significantly reduced cell membrane association of FITC-labeled histones (Fig. 5A), as well as histone-induced inward currents (Fig. 5B, 5C) and calcium influx (Fig. 5D, 5E). These data strongly indicate that masking the membrane binding sites of histones is the mechanism of CRP detoxification.

The proposed mechanism of histone–cell membrane interaction is their affinity to phosphate groups of major phospholipids in the cell membrane, such as PC, PS, and PE (27, 36, 37), because phosphate groups exist in both DNA and bilayer lipid membranes. Preincubation of FITC-labeled histones with liposomes made of PC, PS, and PE significantly reduced the binding of histones to the cell membrane (Fig. 6A). Functionally, this significantly reduced the toxic effects of histones on endothelial cells (Fig. 6B). These data support the hypothesis that phospholipids on cell membranes are the major targets of histones.

It is also known that CRP binds to the phosphocholine group of lysoPC (34). However, lysoPC is exposed only after the lipid bilayer of cell membranes has been disturbed, such as during necrosis and apoptosis (38). Therefore, CRP does not bind to normal cell membranes. In this study, we found that incubating CRP with EAhy926 cells for 1–4 h did not reduce histone-induced toxicity if CRP were washed away before histone treatment (data not shown). This experiment also indicates that CRP prevention of histone–membrane interaction is not due to CRP association with cell membranes.

In line with our findings of the existence of circulating CRP–histone complexes, the interaction between CRP and histone has been reported previously but with some discrepant findings (13, 14, 39–41). To clarify these discrepancies, we immunofluorescently stained EAhy926 cells with Cy5-CRP and FITC-ahsFcV to avoid any nonspecific staining of IgGs. Their colocalization observed in cell nuclei strongly indicates that CRP binds to histones (Fig. 6C).

Furthermore, a gel overlay assay showed that all histones could bind CRP (Fig. 6D) and that CRP could protect endothelial cells treated with the individual histones (Fig. 6E). Using competitive ELISA, we found that CRP competed with liposomes in binding histones (Fig. 6F, 6G) to explain how CRP blocks histone–plasma membrane integration (Fig. 5A).

Discussion

The acute-phase response is a complex series of reactions initiated in response to infection, inflammation, injury, or malignancy. Elevation in CRP levels has long been recognized in this context, but despite many reports into its properties, clear understanding of its generic function has been lacking (42). In part, this is because no mechanism of this phylogenetically ancient and well-conserved molecule has been described, although a few
nucleotide polymorphisms that do not alter the protein sequence have been identified (43). This would suggest a critical functional role for CRP. Although the concept of CRP acting to detoxify and clear damaging products of cell destruction was hypothesized back in 1981 (44), this study demonstrates for the first time, to our knowledge, that high levels of CRP in the acute clinical phase plays a major role in combating the toxicity of circulating histones.

**FIGURE 6.** CRP–histone complex formation reduces histone-induced cell damage. (A) Confocal images of living EAhy926 cells incubated with FITC-histones (10 μg/ml, left panel) or FITC-histones + liposomes made of PC, PS, and PE (right panel). Scale bar, 50 μm. Arrow in left panel indicates membrane binding of FITC-histones. (B) The protective effects of different concentrations of liposomes on histone-induced toxicity to EAhy926 cells in the presence of 2% (v/v) FBS. Means ± SD are shown. *p < 0.05 compared with histones alone (ANOVA). (C) Dual staining of EAhy926 cells with FITC-ahscFv (a), Cy5-CRP (b), phase contrast (c), and superimposed images (d) to demonstrate the colocalization of CRP with histones. All images were taken using a Zeiss LSM710 confocal microscope. (D) Gel overlay assay to study CRP interaction with immobilized histones. A total of 1 μg of each type of human recombinant histones (H1, H2A, H2B, H3.1, H4) and 6 μg S100P protein (negative control) were subjected to a gel overlay assay with 3 μg/ml CRP. Coomassie blue stained gel (upper panel) shows the gel loading (n = S100P multimer, m = S100P monomer). Overlaid with CRP and detected with anti-CRP Ab (lower panel). (E) CRP protection of EAhy926 cells treated with 40 μg/ml individual human recombinant histones in the presence (white) or absence (black) of 250 μg/ml CRP. Means ± SD from four independent experiments are shown; *p < 0.05 in cell survival compared with untreated (UT), *p ≤ 0.035 in relation to CRP addition (Student t test). (F) Competitive ELISA assay to demonstrate that CRP and phospholipids (PE+PS) competitively interact with histones. ELISA plates were coated with histones or buffer alone and incubated with 2 mM liposomes made of PS+PE (1:2) mixed with 50 μg/ml CRP. After washing, the bound CRP was detected using anti–CRP-HRP. Means ± SD of the absorbance from three independent experiments are shown. *p < 0.001 compared with other groups (ANOVA), *p = 0.007 compared with group 1 (Student t test). (G) A typical dose–response curve of the competitive ELISA with 0–200 mM liposomes. The bound CRP was detected using anti–CRP-HRP, and the means of absorbance from three independent experiments are presented.
In this study, we demonstrate that CRP could significantly alleviate histone-induced endothelial damage and coagulation activation, which are the major toxic effects of extracellular histones. CRP protective effects are due to its interaction with histones, and this has been further confirmed by coimmunofluorescent staining and gel overlay assay. How CRP binds histones is not clear, but positively charged histones are more likely to bind the predominantly negatively charged central pore of the CRP pentamer in a similar way to that of complement C1q. Because complement factor H has been reported to bind dCRP (nonnative pentameric CRP), we also denatured CRP by heating at 70°C for 1 h as described in a previous report (4). The dCRP strongly aggregated with FITC-labeled histones, and greatly reduced the binding of histones to the cell membrane and reduced histone toxicity to endothelial cells (Supplemental Fig. 1). These data indicate that both native and nonnative pentameric CRP are able to bind histones and reduce their toxic effects.

Because histones released after acute cellular and nuclear damage may also be present in the form of histone–DNA complexes, we have additionally checked their toxicity profile and any blocking effect by CRP. Using isolated nucleosomes (Supplemental Fig. 2A–C) and histones from swine liver, prepared as described previously (45), we demonstrate that swine histones have similar toxicity to cultured endothelial cells as with recombinant or calf thymus histones (data not shown). However, intact nucleosomes are not toxic (Supplemental Fig. 2D), but degraded nucleosomes, either from brief sonication or incubation with serum, showed toxicity to endothelial cells. Significantly, this could be reduced by CRP and dCRP (Supplemental Fig. 2D). To confirm this finding, genomic DNA fragments were mixed with isolated histones to create artificial histone–DNA complexes, as described previously (25). Again, we found that the complexes showed similar toxicity to histones alone, but that DNA alone was not toxic (Supplemental Fig. 3). These data suggest that the toxicity of histone–DNA complexes to endothelial cells is predominantly caused by histones, although histone–DNA complexes are stronger activators of TLR2 and TLR4 receptors and subsequent release of cytokines than histones alone (25). Similarly, CRP or dCRP can significantly reduce this toxicity (Supplemental Fig. 3). As to the clinical implications, our previous study has shown that circulating histone–DNA complexes were detectable only within 24 h after severe trauma, and that free histones remain detectable after 72 h (27). This suggests that histone–DNA complexes have been quickly degraded into histones. Collectively, these data would indicate that in vivo toxicity is caused by both histone–DNA complexes and histones alone in the circulation, and that CRP can block both forms, as confirmed by adding CRP to serum from patients in the immediate phases after severe trauma (Fig. 4A).

As to the mechanism of histone toxicity, a previous report showed that histones increased cell membrane permeability (46), but a recent report showed that anti-TLR4 or anti-TLR2 Ab could rescue mice from fatal liver injury to suggest that TLR4 and TLR2 may be involved in histone toxicity (25). We demonstrate that histones integrate onto the endothelial cell membrane both in vitro and in vivo to induce calcium influx, which is essential for histone toxicity, at least to endothelial cells (27) and platelets (22). CRP protection relies on the formation of CRP–histone complexes, which prevents the integration of histones into the cell membrane and consequently reduced histone-induced calcium influx and cell damage. We also found the aggregation of FITC-labeled histones after incubation with CRP both in tissue culture medium and inside cells. Whether CRP enhances the clearing of circulating histones still requires further investigation.

Histones can bind different phospholipids (36, 37, 47, 48), and as these are major components of the cell membrane, we speculate that histones bind to the phospholipid bilayer in the plasma membrane in a manner similar to the interaction between the DNA phosphodiester backbone and the histone cores (49). In this study, we demonstrate that artificial liposomes made of phospholipids compete with the cell membranes for histone binding and significantly reduce histone-induced toxicity. CRP is also able to compete with phospholipids for binding to histones, and this indicates that the major mechanism underlying the protective effects of CRP in vitro and in vivo is in its interference with the histone association to cell membranes. Therefore, CRP binds histones, as in its role in opsonizing pathogens, to block their toxic effect on cells and to possibly facilitate their clearance. This action defends the human body from the toxicity of circulating histones, especially in the acute phase of many critical illnesses.

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Disclosures
The authors have no financial conflicts of interest.

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