Non-redundant role of the extracellular chaperone clusterin in host tolerance against extracellular histone-induced toxicity

One sentence summary: Protective role of clusterin during sepsis

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Abbreviations: CIRP, cold-inducible RNA binding protein; CLP, cecal ligation and puncture; CRP, C-reactive protein; CRT, calreticulin; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1 ; ICU, intensive care unit; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PRM, pattern recognition molecule; PTX, pentraxin; SPR, surface plasmon resonance.

Abstract

Increasing host tolerance to damage-associated molecular patterns may lower the intensity of tissue injury in diseases associated with massive cell death, such as sepsis. Extracellular histones, released by dying cells, have devastating effects and have emerged as pivotal mediators of cell death and tissue destruction in sepsis. We report that the extracellular chaperon clusterin binds to histones and inhibits in vitro their inflammatory, cytotoxic and platelet aggregation properties. Results from a prospective study reveal that the serum levels of clusterin collapse in septic shock patients at ICU admittance and normalized in surviving but not in non surviving patients. The in vivo inflammatory response in response to LPS or histones is increased in clusterin-deficient ($Clu^{-/-}$) mice compared to wild type mice. Moreover, $Clu^{-/-}$ mice are more susceptible to experimental endotoxemia and sepsis than wild type mice. Finally, the injection of clusterin lowers the mortality of mice with CLP. This study demonstrates that clusterin has a key role in maintaining tissue tolerance by neutralizing extracellular histones. Restoring clusterin thus appears as a therapeutic and promising option in sepsis and other severe diseases associated with histone-induced tissue injuries.

Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection[1,2]. An early antibiotic treatment associated with supportive cares is essential to control infection, but are often insufficient to prevent a lethal outcome[3]. Sepsis thus remains a leading cause of in-hospital mortality. Jamieson et al reported that host survival to severe sepsis or septic shock relies on a well balanced response including its resistance to infecton associated with an ability to decrease the magnitude of the inflammatory response and/or its negative impact on tissues, without directly targeting pathogens[4,5]. This pioneering study suggested that enhancing tissue tolerance to damage represents a promising complementary therapeutic option in sepsis[6].

Accumulating data underline the role of endogenous danger molecules released by dying cells in inducing severe inflammation and tissue damages. These harmful molecules, called damage-associated molecular patterns (DAMPs) or alarmins, are sensed by host cells via innate immunity receptors (pattern recognition molecules or PRM) and generate an inflammatory phenotype similar to the one induced by microbes. When released in excess, a condition that occurs during sepsis[7–9], DAMPs are detrimental to the host by overwhelming inflammation and inducing cell injury. Histones have emerged as endogenous DAMPs with potent inflammatory and devastating cytotoxic properties. Released by dying cells in the extracellular milieu, histones are actually recognized as major mediators of tissue injury in a variety of severe diseases, such as sepsis, trauma and ischemia[10–14]. In addition to promoting the secretion of proinflammatory cytokines, histones also induce platelet aggregation and cell death[15–17]. In septic patients, levels of plasma nucleosomes and histones correlate with disease activity and severity[18,19]. Injected in mice, histones induce a strong inflammatory syndrome associated with massive tissue injury[13]. Although the pro-inflammatory properties of histones appear dependent on TLR2/TLR4 or TLR9 signalling[14,15,17,20], their role in histone-induced cytotoxicity remains controversial[15,17,21].

Recent studies suggested that extracellular histones may represent therapeutic targets. Their neutralization with antibodies, heparin or activated protein C blocks their pro-inflammatory and pro-thrombotic activity, and reduces mortality in models of sterile inflammation and sepsis[13,22]. A similar protective role is also observed with some soluble PRM, such as the pentraxins CRP and PTX3[10,23]. However, the expression of these soluble PRM, such as PTX3

and CRP, is dramatically increased in sepsis patients, especially in non survivors[24,25], suggesting that elevated levels of these histone-neutralizing molecules are not systematically associated with protection.

Clusterin (also known as ApoJ), a 75-80 kDa heterodimeric disulphide-linked glycoprotein, is a multifaceted protein acting as an apoplipoprotein, a complement regulator and an extracellular chaperon[26]. As a chaperon, clusterin stabilizes a broad range of client proteins and keeps them soluble, inhibiting the formation of toxic aggregates[27]. The interaction of clusterin with client proteins induces their rapid clearance by hepatocytes[28]. Clusterin also confers cell protection by limiting apoptosis and necrosis in ischemia-reperfusion murine models[29]. Clusterin is found at relatively high concentrations in a variety of biological fluids[26,30]. In human, the concentrations of serum clusterin range from 50 to 400 µg/mL[31] and its expression is hugely increased in case of inflammation[32–34]. We had previously reported that clusterin opsonizes late apoptotic cells and favors their elimination by phagocytes[35], suggesting that clusterin may act as a soluble PRM. Interestingly, histones, which accumulate at the surface of late apoptotic cells[36], have been identified as privileged clusterin-binding elements[35]. The objective of this study was to evaluate the capacity of clusterin to interact with extracellular histones and to evaluate the status and role of clusterin during sepsis.

Results

Clusterin levels are decreased in septic shock patients

We first quantified serum clusterin at ICU admission in a retrospective cohort of septic shock patients. Compared to heathy patients, the levels of clusterin were significantly decreased and were lower in non-surviving than in surviving patients (Supplemental Fig. S1a). To confirm and detail these observations, we conducted a prospective study that included 110 patients with septic shock and severe sepsis (Table 1). Clusterin, PTX3 and CRP were quantified at ICU admittance (day 0), and then day 3 and day 7. Results confirmed that septic shock patients had lower levels of clusterin than healthy subjects (Fig. 1a), with a more pronounced decrease in non-surviving than in surviving patients (Fig. 1a). More precisely, the levels of clusterin were significantly reduced in non-survivors at ICU admittance and did not normalize at day 3 and day 7 while, in contrast, they tended to normalize at day 7 in survivors (Fig. 1a). In agreement with a previous study[25], the levels of PTX3 were strongly elevated, especially in non-surviving patients, and decreased in surviving and non-surviving patients at day 7 (Fig. 1b). The levels of CRP were elevated in both groups and progressively returned to basal levels between day 3 and day 7 (Supplemental Fig. S1b).

When septic shock patients were grouped according to the median concentration of clusterin at ICU admission, patients with levels <103 µg/mL had significantly higher SAPSII score, lower blood platelet count, higher blood lactic acid concentration and lower prothrombin time than patients with clusterin levels $\geq$ 103 µg/mL (Table 1).

As clusterin-client complexes may have led to underestimate clusterin quantification, we also analyzed clusterin by Western Blotting. Results confirmed a decrease of clusterin in the serums of septic shock patients, and showed that this decrease did not result from its degradation as no low molecular weight immunoreactive bands were detected (Supplemental Fig. S1c).

Interestingly, the expression of clusterin mRNA was elevated in freshly isolated PBMC from septic shock patients, compared to healthy subjects (Fig. 1c, left panel) and monocytes isolated from septic shock patients spontaneously produced clusterin in vitro and, contrary to IL-6, its production was not modulated upon stimulation (Fig. 1c, right panel). These data suggested that the decrease of circulating clusterin levels in septic shock patients did not result from an inhibition of production.

Clusterin interacts with histones

Clusterin chaperons a large variety of ligands, including damaged molecules[26]. As clusterin-client molecules are eliminated in vivo[28], we hypothesized that the decrease of clusterin levels in septic shock patients may result from its binding to danger molecules (i.e. histones, genomic DNA, HMGB1, CRT, HSP70, CIRP) present in the serums of sepsis patients[8]. Results showed that clusterin bound to immobilized histones, preferentially to histones H3 and H4 (Fig. 2a); low or no binding was observed on CRT, CIRP, genomic DNA, HMGB1 and HSP70 (Supplemental Fig. S2a). The interaction of clusterin with immobilized histones was higher than the one observed with CRP (Fig. 2a), used as a control[10]. The binding of clusterin to histones H3 and H4 was confirmed by SPR (data not shown). Interestingly, the addition of recombinant H4 in the serum of healthy subjects induced the formation of clusterin-H4 complexes (Fig. 2b), demonstrating that this interaction can occur in human serum.

Clusterin-histone complexes are detected in the serums of septic shock patients

We thus evaluated the presence of clusterin-H4 complexes in the serums of septic shock patients. Results showed that the levels of clusterin-H4 complexes were elevated in septic shock patients, especially in non-surviving compared to surviving patients (Supplemental Fig. S1d); clusterin-H4 complexes were low or undetectable in the serums of healthy subjects (Supplemental Fig. S1d). Immunoprecipitation confirmed the presence of circulating clusterin-histone complexes in the serums of septic shock patients (Supplemental Fig. S1e).

The levels of nucleosomes, released by dying cells[37], reflect severe tissue damages, especially in sepsis[18,38]. Elevated levels of circulating nucleosomes were detected in septic shock patients and, in agreement with previous studies[38], the highest levels were detected in non-survivors compared to survivors, especially at admittance and at day 3 (Fig. 1e and Supplemental Fig. 1f). Interestingly, the levels of circulating nucleosomes were inversely correlated with the levels of clusterin (Fig. 1f).

Clusterin dampens histone-induced inflammation

Histones induce the production of the inflammatory cytokines IL-6 and TNFα by monocytes (Supplemental Fig. S3a and[39,40]).We thus evaluated the capacity of clusterin to modulate histone-induced cytokine production. Results showed that clusterin reduced, in a dose-dependent manner, the production of TNFα and IL-6 by monocytes stimulated with non-toxic concentrations of histones (Fig. 3a). Importantly, clusterin did not modulate the production of

TNFα and IL-6 in response to LPS or IL-1β (Fig. 3a); clusterin alone did not induce IL-6 and TNFα secretion by monocytes (Fig. 3a). We next evaluated whether clusterin may neutralize the pro-inflammatory properties of histones contained in the serums of septic shock patients. Serums from selected patients induced the secretion of IL-6 and TNFα by monocytes, whereas serums from healthy subjects did not, unless supplemented with histones (Fig. 3b). The inflammatory potential of serums from septic shock patients was prevented by adding recombinant clusterin (Fig. 3b). In agreement with its capacity to prevent cell activation, clusterin inhibited the binding of histones to cell membranes (Supplemental Fig. S3b&c).

Clusterin protects against histone-induced cell death and platelet aggregation

In vitro, histones induce endothelial cell death and promote platelet aggregation[13,16]. We first investigated whether clusterin may protect cells from histone-induced toxicity. Results showed that clusterin protected monocytes from histone-induced death (Fig. 4a). Excessive NETosis is proposed as a mechanism of tissue injury during septic shock[41,42]. As expected, histones induced NETs at a similar level that PMA (Fig. 4b), used as a positive control[43]. Clusterin prevented histone-induced but not PMA-induced NETosis (Fig. 4b). Clusterin alone did not induce cell death (Fig. 4a&b). We and others have reported that circulating histones, present in the serums from septic shock patients, are cytotoxic[44]. Serums from septic shock patients, but not from healthy subjects, were toxic for monocytes and endothelial cells (Fig. 4c). Addition of recombinant clusterin to the serums of patients dramatically reduced their capacity to induce endothelial cell death (Fig. 4c).

Histones promote platelet aggregation and thrombosis when released into the circulation[45] and induce in vitro platelet aggregation[11]. We observed that histone-induced platelet aggregation was totally inhibited by clusterin (Fig. 4d).

Histones induce clusterin secretion

The expression of clusterin is increased in virtually all cell types in response to pro-inflammatory signals[31]. We thus evaluated the capacity of histones to induce clusterin production. Histones, LPS and IL-1β plus TNFα induced clusterin secretion by monocytes (Fig. 5a); the production of clusterin was associated to an increase of clusterin mRNA expression at 20 h (Fig. 5b). Western Blotting and microscopy revealed that human neutrophils have a preformed stock of clusterin (Supplemental Fig. S4) and, as reported[46], in platelets (data not shown). Histones, PMA and LPS induced a rapid release of clusterin by

platelets and neutrophils (Fig. 5c). These data show that histones and inflammatory cytokines trigger the secretion of clusterin by different cell types.

Clusterin is a non-redundant molecule that reduces histone-induced inflammation

Proteomic analyses have identified several plasma proteins that may interact with histones[47]. We therefore evaluated the role of serum clusterin in the protection against histones. We first evaluated the impact of clusterin depletion on the ability of serums from healthy subjects to prevent histone-induced cytokine production. The capacity of serums from healthy subjects to prevent histone-induced IL-6 and TNFα production was reduced after clusterin depletion (Fig. 6a) and the levels of IL-6 and TNF induced by histones were elevated in serum-free medium compared to medium supplemented with 10% of serum from healthy subjects (Fig. 6a). Similarly, the serum from wild type ($Clu^{+/+}$) mice was more efficient than that clusterin-deficient ($Clu^{-/-}$) mice in preventing histone-induced monocyte activation (Fig. 6a).

We next compared the inflammatory response of $Clu^{-/-}$ and $Clu^{+/+}$ mice to a sublethal (50 mg/kg) intravenous injection of histones. $Clu^{-/-}$ mice had higher levels of IL-6 and TNFα than $Clu^{+/+}$ mice (Fig. 6b); no difference was observed in response to PBS in both types of mice (Fig. 6b). As controls, the levels of IL-6 were similar in $Clu^{-/-}$ and $Clu^{+/+}$ mice after injection of IL-1β (Fig. 6c).

Protective role of clusterin against sepsis

A previous study reported that an intravenous injection of histones is lethal in mice[13]. We thus evaluated the capacity of endogenous clusterin to protect mice against a lethal challenge with histones. Upon intravenous injection of 100 mg/kg histones, all mice died within 15 min (Fig. 7a). Interestingly, co-injection of clusterin and histones delayed death and rescued 28.5 % of mice (Fig. 7a). Based on this result, we thus evaluated the capacity of clusterin to protect mice against an endotoxinic shock, reported to be dependent on histones[13], and to cecal ligation and puncture (CLP), a widely used murine sepsis model. The intraperitoneal injection of a non-lethal dose of LPS (50 mg/kg) in wild type mice induced a decrease of the levels of clusterin associated to an increase of circulating nucleosomes and clusterin-H4 complexes (Fig. 7b). Interestingly, the expression of clusterin was enhanced in the peritoneum (Supplemental Fig. S5a), spleen, liver and kidney of LPS-challenged mice (Supplemental Fig. S5b). These results showed that the decrease of clusterin levels in LPS-challenged mice reflected that in septic shock patients.

Clusterin deficient ($Clu^{-/-}$) mice were more sensitive than wild type ($Clu^{+/+}$) mice to an intraperitoneal injection of a lethal dose (75 mg/kg) of LPS (Fig. 7c). Importantly, splenocytes from $Clu^{-/-}$ and wild type mice produced similar levels of IL-6 upon in vitro stimulation with LPS, showing that both strains are similarly sensitive to LPS (Supplemental Fig. S5c). Finally, we observed that $Clu^{-/-}$ mice were more susceptible to CLP-induced death that $Clu^{+/+}$ mice (Fig. 7d) and that the injection of clusterin lowered the mortality of mice with CLP (Fig. 7e).

Discussion

Extracellular histones (released by dying cells) have emerged as critical mediators of tissue injury in severe diseases and their neutralization is protective against the tissue damages they induce. Soluble PRM can inhibit the inflammatory and cytotoxic properties of histones. In this study, we identified clusterin as a non-redundant histone-binding molecule that inhibits, in vitro and in vivo, their inflammatory and cytotoxic properties. Results also showed that the levels of clusterin collapsed in patients with severe sepsis and that the lack of normalization of clusterin, associated with elevated levels of extracellular histones, was predictive of death in septic patients. Finally, we show (i) that $Clu^{-/-}$ mice are more sensitive to endotoxemia and sepsis-like than $Clu^{+/+}$ mice and, (ii) that the injection of clusterin protects mice from sepsis-induced death. Collectively, these results identify that clusterin acts as an endogenous histone inhibitory molecule and suggest that normalization of its levels appears as a therapeutic alternative for the treatment of severe pathologies associated with a massive release of histones in the extracellular milieu, such as sepsis.

Clusterin is a pivotal regulator of extracellular proteostasis via its capacity to bind to and to participate to the elimination of misfolded proteins and protein aggregates[28,31]. We report that clusterin binds to histones, prototypic endogenous danger signals, and neutralizes their inflammatory and cytotoxic properties and their capacity to induce platelet aggregation. In agreement with a previous study[47], we observed that the interaction of histones with clusterin occurs in serums that contain several other histone-binding molecules, such as albumin, lipoprotein and complement associated proteins, suggesting that clusterin constitutes a privileged histone-binding element. This hypothesis is reinforced by the fact that supplementing serums from sepsis patients with clusterin reduced their inflammatory and cytotoxic properties.

The inflammatory versus cytotoxic properties of extracellular histones are dependent on their concentrations. At low concentrations, histones induce inflammatory cytokines via signalling PRR (TLR2, TLR4, TLR9 and NLRP3)[14,15,17,48]. At high concentrations, histones are cytotoxic, a property related to their ability to translocate within the cell membrane, forming channels that increase cell permeability that ultimately lead to cell death[49]. The protective role of clusterin may thus rely on its capacity to avoid the interaction of histones with cell membranes and signalling innate receptors. However, the mechanisms involved in clusterin-histones interaction remain unknown. Based on the literature, one can hypothesize that (i)

histones, which are amphipathic molecules[50], may interact with the hydrophobic globule-like domain of clusterin and, (ii) that positively charged residues of histones may interact with negatively charged carbohydrates of clusterin[51]. Interestingly, the binding capacity of clusterin to client proteins is enhanced in a mildly acidic milieu[52], a condition that is commonly found during sepsis[53,54].

The levels of serum clusterin are dramatically elevated during inflammation and its expression is increased in several cell types upon stimulation with inflammatory mediators and microbial moieties[31,55]. Our results identify histones as novel clusterin-inducing molecules. In agreement with the inflammatory status associated to sepsis, we observed that PBMC from sepsis patients constitutively produced clusterin and expressed, as reported[56], elevated levels of clusterin mRNA compared to healthy subjects. Nevertheless, the levels of clusterin collapsed while, in contrast, the levels of nucleosomes were elevated in patients, suggesting that the decrease of circulating clusterin would result from a massive and prolonged release of histones from damaged tissues, leading to an overflow of its buffering capacity. These results suggest that during severe sepsis, preformed and neosynthesized clusterin levels are not sufficient to maintain its concentration at levels sufficient to neutralize extracellular histones.

Other histone-binding proteins have been identified, such as albumin[57], thrombomodulin[58], heparins[59] and the pentraxins CRP and PTX3; these molecules, and especially pentraxins, have been reported protective against the deleterious properties of histones[10]. Previous studies have reported that PTX3, but not CRP[60], is protective in experimental models of endotoxemia and sepsis[23,61]. Nevertheless, and contrary to clusterin, the levels of PTX3 were strongly elevated in sepsis patients and correlated with a higher risk of death[62], suggesting that its protective role is limited in sepsis patients. One of the most intriguing observations of our study was that low levels of clusterin in sepsis patients were associated to a higher risk of death and negatively correlated with the levels of extracellular histones. A decrease of clusterin in sepsis patients has been previously reported[63,64] and elevated levels of extracellular histones are associated with disease severity in systemic inflammation (sepsis, cancer, autoimmune diseases)[65]. Moreover; the in vitro properties of histones were observed at concentrations similar to the ones found in the serums of patients[66]. Similar to our results, previous studies suspected that the protective roles of clusterin are dependent on its concentrations and clusterin to client ratio[27,67]. Collectively, these results suggest (i) that the

amounts of clusterin are no longer sufficient to limit the toxicity of histones, the levels of which are very high in patients with severe septicaemia and, (ii) that this clusterin deficiency is not compensated for by other histone binding elements, such as pentraxins, even though they are present at high levels in the patient serums. This hypothesis is supported by in vivo experiments showing that clusterin-deficient mice are more sensitive than wild type mice to experimental endotoxemia and sepsis.

Sepsis has been defined as a dysregulated response to infection, leading to tissue damages[68]. It is now accepted that sepsis-induced organ dysfunction results from the incapacity of the host to maintain or restore tolerance mechanisms[5,69]. Neutralizing endogenous danger signals generated during sepsis, and especially histones, is essential to prevent tissue destruction. Extracellular histones released by dying cells can then induce the killing of other cells, setting up a deleterious amplication loop of histone-induced cell death. A decrease in the levels of molecules protecting against the deleterious effects of histones would therefore have disastrous consequences. Our results demonstrate that clusterin has protective properties against histone-induced endothelial cell injury and thrombosis, two major features of septic shock[2,13] and that clusterin-deficient mice are susceptible to CLP-induced sepsis. We therefore hypothesized that in vivo injection of clusterin would reduce the death of mice induced by injection of histones and dampen the severity of experimental sepsis. Results showed that the administration of clusterin decreased histone-induced lethality in wild type mice and reduced the mortality of mice subjected to experimental sepsis. Our results are in agreement with previous studies reporting that neutralizing histones, by using antibodies or activated protein C, reduced the mortality of mice subjected to CLP-induced sepsis[13,70,71] or to ischemia/reperfusion[14,72]. Our results are in agreement with previous studies reporting that clusterin is protective in severely stressed tissues, especially in pathologies associated with massive cell death. As example, $Clu^{-/-}$ mice exhibit a decreased survival rate in ischemia-reperfusion injury model[73] and clusterin prevents grafted organs form damage under ischemic conditions[74,75] and limits in vivo the severity of autoimmune myocarditis[76].

Collectively, our study highlights a central role of clusterin in the host protection and tolerance to histone-mediated injury in sepsis. Maintaining elevated levels of clusterin appears as a promising therapeutic option in pathological conditions driven by histone toxicity and associated with clusterin consumption, such as sepsis. These results also identify histones as

clusterin-client molecules, reinforcing its role as a scavenging and clearing molecule involved in tissue protection against endogenous danger molecules.

Material and Methods

The protocols are detailed in the Supplementary Material and Methods.

Patients and healthy subjects

Blood from patients with sepsis admitted to the critical care departments of the university hospitals of Angers and Bordeaux were collected in accordance with the guidelines of the Ethics committee of the University Hospital of Angers (agreement 2013-26). Blood from healthy subjects were from the blood collection center of Angers (agreement ANG-2017-01).

Binding assays

The interaction of clusterin with partners was assessed by a solid phase assay. Purified histones, recombinant H2A, H2B, H3 and H4, HMGB1, HSP70, calreticulin (CRT), CIRP and microbial DNA were immobilized in 96-wells immunoassay plates (Nunc) before incubation with biotinylated clusterin, CRP or HSA). Plates were then incubated with streptavidin-HRP; bound proteins were detected using TMB. Results are expressed as OD values.

Analysis of clusterin, nucleosomes and clusterin-histone complexes

Human and mouse clusterin and circulating nucleosomes were quantified by commercial ELISA. Complexes formed by the interaction of clusterin with histone H4 were evaluated by a homemade assay using anti-clusterin and anti-H4 mAbs as capture and detection Abs, respectively. Bound Abs were detected using a HRP-conjugated anti-rabbit IgG Ab and the chromogenic substrate TMB. Results are expressed as OD values. The presence of clusterin-histones complexes in human serums was also evaluated by immunoprecipitation. Briefly, serums were incubated with an anti-Clu mAb immobilized to protein A/G column and the presence of histones in the eluted proteins was evidenced by Western Blotting using an anti-histone Ab; bound Abs were detected using a HRP-labeled anti-rabbit IgG Ab and chemilumiscence.

Isolation and activation of human monocytes

Human monocytes, isolated from healthy subjects as described[77], were cultured with histones, LPS or IL-1β, in the absence or presence of clusterin. In some experiments, monocytes were incubated with the serum from healthy subjects or septic shock patients diluted 1:10 (v:v); IL-6 and TNFα were quantified in the cell culture supernatants by ELISA.

Generation of neutrophil extracellular traps (NETs)

NETs were induced as described[78]. Briefly, neutrophils were seeded on poly-L-lysine-treated glass coverslips before stimulation with PMA or histones, without or with clusterin. Extracellular DNA was stained with the non-cell permeant DNA binding dye SYTOX Green. Fluorescence (λ_{ex}=485 nm; λ_{em}=538 nm) was recorded for 2 h at 10 min intervals; results are expressed as relative fluorescence between treated and non-treated cells. After fixation, slides were mounted in ProLongGold anti-fading reagent and images obtained using Nikon A1 R Si microscope.

Cytokine quantification

Human and mouse IL-6 and TNFα were quantified by ELISA in cell culture supernatants and serums, according to the manufacturer recommendations.

Platelet aggregation assay

Platelet-rich plasma (PRP) was isolated from healthy subjects. Platelets were resuspended in Tyrode's buffer containing or not histone H4, preincubated or not with 25µg/mL clusterin. Platelet aggregation was measured on a TH-V2 optical aggregometer.

Activation of murine splenocytes

Spleen cells isolated form wild type ($Clu^{+/+}$) and clusterin-deficient ($Clu^{-/-}$) mice were cultured in serum-free medium supplemented or not with 10% serum (v:v) from $Clu^{+/+}$ or $Clu^{-/-}$ mice and activated with histones or LPS. IL-6 was quantified in cell culture supernatants by ELISA.

Quantitative PCR

RNA isolation and retrotranscription were performed as described[79]. The expression of the mRNA encoding indicated proteins was analyzed by qPCR. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method using RPS18, EF1A, TBP, RPL13A and PPIA as references. Results are expressed as relative mRNA expression; primer sequences are available upon request.

Western Blotting analysis

Proteins were electophoretically separated on a 4-20% SDS-PAGE gel in non-reducing conditions and transferred to an Immobilon membrane. After saturation, membranes were incubated with goat polyclonal anti-human clusterin antibody and then with HRP-conjugated anti-goat IgG Ab; bounds antibodies were detected by chemiluminescence.

In vivo models

$Clu^{+/+}$ and $Clu^{-/-}$ mice injected intraperitoneally with LPS (experimental endotoxemia) or IL-1β or injected intravenously with histones, pre-incubated or not with clusterin; In some experiments, kidney, liver and spleen were harvested for further analysis. CLP-induced sepsis model was performed as described[13]. Mice were injected or not with clusterin at day 1, 2, 3 and 4 post-cecal ligation. Parameters analysed were survival, clusterin expression and levels of seric IL-6 and TNFα. Experiments were conducted according to institutional ethics committee of the Région Pays de la Loire (agreement 2012.193).

Statistical analysis

Data are shown as mean ± SEM and were analysed by the Mann Whitney test or the Kruskal Wallis test. Correlations were determined using Spearman's rank correlation test. Survival curves were done using Kaplan-Meir method and compared using the Log-rank test. $P<0.05$ was considered significant.

References

1. Adhikari NKJ, Fowler RA, Bhagwanjee S, Rubenfeld GD. Critical care and the global burden of critical illness in adults. *Lancet*. 2010;376(9749):1339-1346. doi:10.1016/S0140-6736(10)60446-1
2. Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med*. 2013;369(21):2063. doi:10.1056/NEJMc1312359
3. Rhodes A, Evans LE, Alhazzani W, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. *Crit Care Med*. 2017;45(3):486-552. doi:10.1097/CCM.0000000000002255
4. Jamieson AM, Pasman L, Yu S, et al. Role of tissue protection in lethal respiratory viral-bacterial coinfection. *Science*. 2013;340(6137):1230-1234. doi:10.1126/science.1233632
5. Medzhitov R. Septic shock: on the importance of being tolerant. *Immunity*. 2013;39(5):799-800. doi:10.1016/j.immuni.2013.10.012
6. Figueiredo N, Chora A, Raquel H, et al. Anthracyclines induce DNA damage response-mediated protection against severe sepsis. *Immunity*. 2013;39(5):874-884. doi:10.1016/j.immuni.2013.08.039
7. Denk S, Perl M, Huber-Lang M. Damage- and pathogen-associated molecular patterns and alarmins: keys to sepsis? *Eur Surg Res*. 2012;48(4):171-179. doi:10.1159/000338194
8. Timmermans K, Kox M, Scheffer GJ, Pickkers P. DANGER IN THE INTENSIVE CARE UNIT: DAMPS IN CRITICALLY ILL PATIENTS. *Shock*. 2016;45(2):108-116. doi:10.1097/SHK.0000000000000506
9. Wiersinga WJ, Leopold SJ, Cranendonk DR, van der Poll T. Host innate immune responses to sepsis. *Virulence*. 2014;5(1):36-44. doi:10.4161/viru.25436
10. Abrams ST, Zhang N, Dart C, et al. Human CRP defends against the toxicity of circulating histones. *J Immunol*. 2013;191(5):2495-2502. doi:10.4049/jimmunol.1203181
11. Abrams ST, Zhang N, Manson J, et al. Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med*. 2013;187(2):160-169. doi:10.1164/rccm.201206-1037OC
12. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999;285(5425):248-251. doi:10.1126/science.285.5425.248
13. Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. *Nat Med*. 2009;15(11):1318-1321. doi:10.1038/nm.2053
14. Huang H, Evankovich J, Yan W, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology*. 2011;54(3):999-1008. doi:10.1002/hep.24501
15. Allam R, Scherbaum CR, Darisipudi MN, et al. Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. *J Am Soc Nephrol*. 2012;23(8):1375-1388. doi:10.1681/ASN.2011111077
16. Saffarzadeh M, Juenemann C, Queisser MA, et al. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS ONE*. 2012;7(2):e32366. doi:10.1371/journal.pone.0032366
17. Xu J, Zhang X, Monestier M, Esmon NL, Esmon CT. Extracellular histones are mediators of death through TLR2 and TLR4 in mouse fatal liver injury. *J Immunol*. 2011;187(5):2626-2631. doi:10.4049/jimmunol.1003930
18. Zeerleder S, Stephan F, Emonts M, et al. Circulating nucleosomes and severity of illness in children suffering from meningococcal sepsis treated with protein C. *Crit Care Med*. 2012;40(12):3224-3229. doi:10.1097/CCM.0b013e318265695f

19. Zeerleder S, Zwart B, Wuillemin WA, et al. Elevated nucleosome levels in systemic inflammation and sepsis. *Crit Care Med*. 2003;31(7):1947-1951. doi:10.1097/01.CCM.0000074719.40109.95
20. Boulé MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med*. 2004;199(12):1631-1640. doi:10.1084/jem.20031942
21. Ekaney ML, Otto GP, Sossdorf M, et al. Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation. *Crit Care*. 2014;18(5):543. doi:10.1186/s13054-014-0543-8
22. Wildhagen KCAA, García de Frutos P, Reutelingsperger CP, et al. Nonanticoagulant heparin prevents histone-mediated cytotoxicity in vitro and improves survival in sepsis. *Blood*. 2014;123(7):1098-1101. doi:10.1182/blood-2013-07-514984
23. Daigo K, Nakakido M, Ohashi R, et al. Protective effect of the long pentraxin PTX3 against histone-mediated endothelial cell cytotoxicity in sepsis. *Sci Signal*. 2014;7(343):ra88. doi:10.1126/scisignal.2005522
24. Caironi P, Masson S, Mauri T, et al. Pentraxin 3 in patients with severe sepsis or shock: the ALBIOS trial. *Eur J Clin Invest*. 2017;47(1):73-83. doi:10.1111/eci.12704
25. Mauri T, Bellani G, Patroniti N, et al. Persisting high levels of plasma pentraxin 3 over the first days after severe sepsis and septic shock onset are associated with mortality. *Intensive Care Med*. 2010;36(4):621-629. doi:10.1007/s00134-010-1752-5
26. Wilson MR, Easterbrook-Smith SB. Clusterin is a secreted mammalian chaperone. *Trends Biochem Sci*. 2000;25(3):95-98. doi:10.1016/s0968-0004(99)01534-0
27. Yerbury JJ, Poon S, Meehan S, et al. The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. *FASEB J*. 2007;21(10):2312-2322. doi:10.1096/fj.06-7986com
28. Wyatt AR, Yerbury JJ, Berghofer P, et al. Clusterin facilitates in vivo clearance of extracellular misfolded proteins. *Cell Mol Life Sci*. 2011;68(23):3919-3931. doi:10.1007/s00018-011-0684-8
29. Wehrli P, Charnay Y, Vallet P, et al. Inhibition of post-ischemic brain injury by clusterin overexpression. *Nat Med*. 2001;7(9):977-979. doi:10.1038/nm0901-977
30. Rizzi F, Coletta M, Bettuzzi S. Chapter 2: Clusterin (CLU): From one gene and two transcripts to many proteins. *Adv Cancer Res*. 2009;104:9-23. doi:10.1016/S0065-230X(09)04002-0
31. Wyatt AR, Yerbury JJ, Ecroyd H, Wilson MR. Extracellular chaperones and proteostasis. *Annu Rev Biochem*. 2013;82:295-322. doi:10.1146/annurev-biochem-072711-163904
32. Charnay Y, Imhof A, Vallet PG, Kovari E, Bouras C, Giannakopoulos P. Clusterin in neurological disorders: molecular perspectives and clinical relevance. *Brain Res Bull*. 2012;88(5):434-443. doi:10.1016/j.brainresbull.2012.05.006
33. Swertfeger DK, Witte DP, Stuart WD, Rockman HA, Harmony JA. Apolipoprotein J/clusterin induction in myocarditis: A localized response gene to myocardial injury. *Am J Pathol*. 1996;148(6):1971-1983.
34. Zhou W, Guan Q, Kwan CCH, et al. Loss of clusterin expression worsens renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol*. 2010;298(3):F568-578. doi:10.1152/ajprenal.00399.2009
35. Cunin P, Beauvillain C, Miot C, et al. Clusterin facilitates apoptotic cell clearance and prevents apoptotic cell-induced autoimmune responses. *Cell Death Dis*. 2016;7:e2215. doi:10.1038/cddis.2016.113
36. Radic M, Marion T, Monestier M. Nucleosomes are exposed at the cell surface in apoptosis. *J Immunol*. 2004;172(11):6692-6700. doi:10.4049/jimmunol.172.11.6692

37. Marsman G, Zeerleder S, Luken BM. Extracellular histones, cell-free DNA, or nucleosomes: differences in immunostimulation. *Cell Death Dis*. 2016;7(12):e2518. doi:10.1038/cddis.2016.410
38. Chen Q, Ye L, Jin Y, et al. Circulating nucleosomes as a predictor of sepsis and organ dysfunction in critically ill patients. *Int J Infect Dis*. 2012;16(7):e558-564. doi:10.1016/j.ijid.2012.03.007
39. Aziz M, Jacob A, Yang W-L, Matsuda A, Wang P. Current trends in inflammatory and immunomodulatory mediators in sepsis. *J Leukoc Biol*. 2013;93(3):329-342. doi:10.1189/jlb.0912437
40. Netea MG, van der Meer JWM, van Deuren M, Kullberg BJ. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol*. 2003;24(5):254-258. doi:10.1016/s1471-4906(03)00079-6
41. Gardiner EE, Andrews RK. Neutrophil extracellular traps (NETs) and infection-related vascular dysfunction. *Blood Rev*. 2012;26(6):255-259. doi:10.1016/j.blre.2012.09.001
42. Kovach MA, Standiford TJ. The function of neutrophils in sepsis. *Curr Opin Infect Dis*. 2012;25(3):321-327. doi:10.1097/QCO.0b013e3283528c9b
43. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ*. 2009;16(11):1438-1444. doi:10.1038/cdd.2009.96
44. Raffray L, Douchet I, Augusto J-F, et al. Septic shock sera containing circulating histones induce dendritic cell-regulated necrosis in fatal septic shock patients. *Crit Care Med*. 2015;43(4):e107-116. doi:10.1097/CCM.0000000000000879
45. Fuchs TA, Bhandari AA, Wagner DD. Histones induce rapid and profound thrombocytopenia in mice. *Blood*. 2011;118(13):3708-3714. doi:10.1182/blood-2011-01-332676
46. Tschopp J, Jenne DE, Hertig S, et al. Human megakaryocytes express clusterin and package it without apolipoprotein A-1 into alpha-granules. *Blood*. 1993;82(1):118-125.
47. Pemberton AD, Brown JK, Inglis NF. Proteomic identification of interactions between histones and plasma proteins: implications for cytoprotection. *Proteomics*. 2010;10(7):1484-1493. doi:10.1002/pmic.200900818
48. Huang H, Chen H-W, Evankovich J, et al. Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. *J Immunol*. 2013;191(5):2665-2679. doi:10.4049/jimmunol.1202733
49. Hariton-Gazal E, Rosenbluh J, Graessmann A, Gilon C, Loyter A. Direct translocation of histone molecules across cell membranes. *J Cell Sci*. 2003;116(Pt 22):4577-4586. doi:10.1242/jcs.00757
50. Kawasaki H, Koyama T, Conlon JM, Yamakura F, Iwamuro S. Antimicrobial action of histone H2B in Escherichia coli: evidence for membrane translocation and DNA-binding of a histone H2B fragment after proteolytic cleavage by outer membrane proteinase T. *Biochimie*. 2008;90(11-12):1693-1702. doi:10.1016/j.biochi.2008.07.003
51. Kapron JT, Hilliard GM, Lakins JN, et al. Identification and characterization of glycosylation sites in human serum clusterin. *Protein Sci*. 1997;6(10):2120-2133. doi:10.1002/pro.5560061007
52. Poon S, Rybchyn MS, Easterbrook-Smith SB, Carver JA, Pankhurst GJ, Wilson MR. Mildly acidic pH activates the extracellular molecular chaperone clusterin. *J Biol Chem*. 2002;277(42):39532-39540. doi:10.1074/jbc.M204855200
53. Fall PJ, Szerlip HM. Lactic acidosis: from sour milk to septic shock. *J Intensive Care Med*. 2005;20(5):255-271. doi:10.1177/0885066605278644

54. Lee S-W, Hong Y-S, Park D-W, et al. Lactic acidosis not hyperlactatemia as a predictor of in hospital mortality in septic emergency patients. *Emerg Med J*. 2008;25(10):659-665. doi:10.1136/emj.2007.055558
55. Shannan B, Seifert M, Leskov K, et al. Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. *Cell Death Differ*. 2006;13(1):12-19. doi:10.1038/sj.cdd.4401779
56. Bauer M, Giamarellos-Bourboulis EJ, Kortgen A, et al. A Transcriptomic Biomarker to Quantify Systemic Inflammation in Sepsis - A Prospective Multicenter Phase II Diagnostic Study. *EBioMedicine*. 2016;6:114-125. doi:10.1016/j.ebiom.2016.03.006
57. Lam FW, Cruz MA, Leung H-CE, Parikh KS, Smith CW, Rumbaut RE. Histone induced platelet aggregation is inhibited by normal albumin. *Thromb Res*. 2013;132(1):69-76. doi:10.1016/j.thromres.2013.04.018
58. Nakahara M, Ito T, Kawahara K, et al. Recombinant thrombomodulin protects mice against histone-induced lethal thromboembolism. *PLoS ONE*. 2013;8(9):e75961. doi:10.1371/journal.pone.0075961
59. Iba T, Hashiguchi N, Nagaoka I, Tabe Y, Kadota K, Sato K. Heparins attenuated histone-mediated cytotoxicity in vitro and improved the survival in a rat model of histone-induced organ dysfunction. *Intensive Care Med Exp*. 2015;3(1):36. doi:10.1186/s40635-015-0072-z
60. Hirschfield GM, Herbert J, Kahan MC, Pepys MB. Human C-reactive protein does not protect against acute lipopolysaccharide challenge in mice. *J Immunol*. 2003;171(11):6046-6051. doi:10.4049/jimmunol.171.11.6046
61. Dias AA, Goodman AR, Dos Santos JL, et al. TSG-14 transgenic mice have improved survival to endotoxemia and to CLP-induced sepsis. *J Leukoc Biol*. 2001;69(6):928-936.
62. Gibot S, Cravoisy A, Kolopp-Sarda M-N, et al. Time-course of sTREM (soluble triggering receptor expressed on myeloid cells)-1, procalcitonin, and C-reactive protein plasma concentrations during sepsis. *Crit Care Med*. 2005;33(4):792-796. doi:10.1097/01.ccm.0000159089.16462.4a
63. Høgåsen K, Mollnes TE, Brandtzaeg P. Low levels of vitronectin and clusterin in acute meningococcal disease are closely associated with formation of the terminal-complement complex and the vitronectin-thrombin-antithrombin complex. *Infect Immun*. 1994;62(11):4874-4880.
64. DeCoux A, Tian Y, DeLeon-Pennell KY, et al. Plasma Glycoproteomics Reveals Sepsis Outcomes Linked to Distinct Proteins in Common Pathways. *Crit Care Med*. 2015;43(10):2049-2058. doi:10.1097/CCM.0000000000001134
65. Holdenrieder S, Stieber P. Clinical use of circulating nucleosomes. *Crit Rev Clin Lab Sci*. 2009;46(1):1-24. doi:10.1080/10408360802485875
66. Semeraro F, Ammollo CT, Morrissey JH, et al. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood*. 2011;118(7):1952-1961. doi:10.1182/blood-2011-03-343061
67. Bettens K, Vermeulen S, Van Cauwenberghe C, et al. Reduced secreted clusterin as a mechanism for Alzheimer-associated CLU mutations. *Mol Neurodegener*. 2015;10:30. doi:10.1186/s13024-015-0024-9
68. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA*. 2016;315(8):801-810. doi:10.1001/jama.2016.0287
69. Medzhitov R, Schneider DS, Soares MP. Disease Tolerance as a Defense Strategy. *Science*. 2012;335(6071):936-941. doi:10.1126/science.1214935
70. Li Y, Liu Z, Liu B, et al. Citrullinated Histone H3 – A Novel Target for Treatment of Sepsis. *Surgery*. 2014;156(2):229-234. doi:10.1016/j.surg.2014.04.009

71. Kusano T, Chiang K-C, Inomata M, et al. A novel anti-histone H1 monoclonal antibody, SSV monoclonal antibody, improves lung injury and survival in a mouse model of lipopolysaccharide-induced sepsis-like syndrome. *Biomed Res Int*. 2015;2015:491649. doi:10.1155/2015/491649
72. Nakazawa D, Kumar SV, Marschner J, et al. Histones and Neutrophil Extracellular Traps Enhance Tubular Necrosis and Remote Organ Injury in Ischemic AKI. *J Am Soc Nephrol*. 2017;28(6):1753-1768. doi:10.1681/ASN.2016080925
73. Nguan CYC, Guan Q, Gleave ME, Du C. Promotion of cell proliferation by clusterin in the renal tissue repair phase after ischemia-reperfusion injury. *Am J Physiol Renal Physiol*. 2014;306(7):F724-733. doi:10.1152/ajprenal.00410.2013
74. Li S, Guan Q, Chen Z, Gleave ME, Nguan CYC, Du C. Reduction of cold ischemia-reperfusion injury by graft-expressing clusterin in heart transplantation. *J Heart Lung Transplant*. 2011;30(7):819-826. doi:10.1016/j.healun.2011.03.007
75. Guan Q, Li S, Yip G, Gleave ME, Nguan CYC, Du C. Decrease in donor heart injury by recombinant clusterin protein in cold preservation with University of Wisconsin solution. *Surgery*. 2012;151(3):364-371. doi:10.1016/j.surg.2011.09.032
76. McLaughlin L, Zhu G, Mistry M, et al. Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. *J Clin Invest*. 2000;106(9):1105-1113. doi:10.1172/JCI9037
77. Duluc D, Delneste Y, Tan F, et al. Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood*. 2007;110(13):4319-4330. doi:10.1182/blood-2007-02-072587
78. de Buhr N, von Köckritz-Blickwede M. How Neutrophil Extracellular Traps Become Visible. *J Immunol Res*. 2016;2016. doi:10.1155/2016/4604713
79. Preisser L, Miot C, Le Guillou-Guillemette H, et al. IL-34 and macrophage colony-stimulating factor are overexpressed in hepatitis C virus fibrosis and induce profibrotic macrophages that promote collagen synthesis by hepatic stellate cells. *Hepatology*. 2014;60(6):1879-1890. doi:10.1002/hep.27328

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Data and materials availability. All data necessary for the interpretation of results were included in the manuscript. Requests for information or materials should be addressed to the corresponding authors.

Figure legends

Figure 1. **Analysis of clusterin and histones in septic shock patients**. **a&b**. Levels of clusterin (Clu) (**a**) and PTX3 (**b**) were quantified by ELISA in the plasmas of surviving and non surviving septic shock patients at admittance at the ICU (D0), D3 and D7 (mean ± SEM). a, the dotted line corresponds to the mean clusterin levels in healthy subjects; PTX3 is undetectable in the plasma of healthy subjects. **c**. The expression of clusterin mRNA and capacity to produce clusterin upon in vitro stimulation with LPS or histones (24 h stimulation) was analyzed by qPCR and ELISA, respectively, in PBMC of 10 healthy subjects (HS) and 10 septic shock patients. Results are expressed in relative expression compared to GAPDH mRNA and in pg/mL, respectively (mean ± SEM). **d**. Levels of nucleosomes were evaluated by ELISA in the plasmas of surviving and non surviving septic shock patients at admittance at the ICU (D0), D3 and D7 (mean ± SEM). **e**. Correlation between the levels of circulating nucleosomes and clusterin levels in septic shock patients. **a-d**. *p<0.05; **p<0.01; ns, not significant (Mann Whitney U test).

Figure 2. **Clusterin binds to histones and form complexes ex vivo**. **a**. The binding of 1.5 nM biotinylated clusterin (Clu), CRP and HSA to immobilized recombinant H2A, H2B, H3, H4 and calf thymus histones was analyzed by ELISA. **b**. Serums from healthy subjects (n=5) were supplemented with the indicated concentrations of recombinant H4 and clusterin-H4 complexes were quantified by ELISA. **a-b**. Results are expressed in OD values (mean ± SEM, n=5).

Figure 3. **Clusterin prevents histone-induced IL-6 and TNFα production.** Monocytes (n=5) isolated from healthy subjects were stimulated or not with 50 µg/mL calf thymus histones, 50 ng/mL LPS or 50 ng/mL IL-1β, in the absence or presence of the indicated concentrations of clusterin (**a**) or incubated with 10% serum from septic shock patients supplemented or not with 25 µg/mL clusterin or 10% serum from healthy subject supplemented with 25 µg/mL clusterin or 50 µg/mL calf thymus histones (**b**). TNFα (**a**) and IL-6 (**b**) were quantified in the 24 h supernatants (mean ± SEM; Mann Whitney U test). **a-b**. *p<0.05; **p<0.01; ns, not significant (Mann Whitney U test).

Figure 4. **Clusterin prevents histone-mediated cell death and platelet aggregation**. **a**. PBMC from healthy subjects (n=5) were incubated with 50 µg/mL calf thymus histones

without or with 25 µg/mL clusterin. Cell death was monitored by flow cytometry at 24 h and results were expressed as the percentage of Annexin V^- living cells. **b**. Neutrophils from healthy subjects (n=6) were stimulated or not with 50 µg/mL histones or 30 nM PMA, in the absence or presence of 25 µg/mL clusterin. Extracellular DNA was labeled using SYTOX green and fluorescence was quantified after 1 h incubation (left panel); right panel, fluorescence microscopy images of SYTOX staining (one of three experiments). **c**. Endothelial cells were cultured in medium supplemented with 10% serum from healthy subjects or from septic shock patients, supplemented or not with 25 µg/mL clusterin. Cell death was monitored by flow cytometry at 24 h and the results expressed as the percentage of Annexin V^+ dead cells (n=6). **d**. Human platelets isolated from healthy subjects (n=4) were incubated with 50 µg/mL histones, without or with 25 µg/mL clusterin. Results are expressed as an index of aggregation. **a-d**. *p<0.05, **p<0.01; ns, not significant (Mann Whitney U test).

Figure 5. **Histones induce clusterin production**. **a**. Monocytes from healthy subjects were stimulated with 25 µg/mL calf thymus histones, 50 ng/mL IL-1β + 50 ng/mL TNFα or with 100 pg/mL LPS. The production of clusterin was quantified by ELISA in the 24 h supernatants. **b**. PBMC from healthy subjects were stimulated with 25 µg/mL calf thymus histones and clusterin mRNA was analyzed at the indicated time-points by qPCR; results are reperesentative of one of 4 experiments. **c**. Human platelets (left panel) and neutrophils (right panel) were stimulated or not with 25 µg/mL calf thymus histones, 50 nM PMA or 200 ng/mL LPS and clusterin was quantified by ELISA in the 2 h supernatants. Results are expressed in pg/mL (neutrophils) or in pg/mL/10^6 elements (platelets) (n=5). **A,c**. *p<0.05 (Kruskal-Wallis test).

Figure 6. **Non redundant role of clusterin in histone-induced inflammation**. **a**. Left panel, murine splenocytes were incubated or not with 50 µg/mL calf thymus histones in serum free medium supplemented with 10% serum from wild type ($Clu^{+/+}$) or clusterin-deficient ($Clu^{-/-}$) mice. Right panel, human monocytes were incubated or not with 50 µg/mL calf thymus histones in serum free medium supplemented with 10% non depleted or clusterin-depleted serums. IL-6 and TNFα were quantified in 24 h supernatants (n=5). **b**. $Clu^{+/+}$ and $Clu^{-/-}$ mice were injected with 50 mg/kg histones or PBS. Circulating IL-6 and TNFα were quantified at 2 h. **c**. $Clu^{+/+}$ and $Clu^{-/-}$ mice were injected with 10 ng IL-1β or PBS. IL-6 was quantified in the

serums and peritoneal fluids collected at 2 h. **a-c**. *p<0.05, **p<0.01; ns, not significant (Mann Whitney U test).

Figure 7. **Clusterin protects mice from experimental sepsis**. **a**. Wild type mice (n=7) were injected intravenously with PBS, 100 mg/kg calf thymus histones or 100 mg/kg histones plus 30 mg/kg clusterin. **b**. Wild type mice (n=7) were injected intraperitoneally with 50 mg/kg LPS. The levels of clusterin, nucleosomes and clusterin-H4 complexes were determined at the indicated time points (mean ± SEM). **c-d**. $Clu^{+/+}$ and $Clu^{-/-}$ mice were injected intraperitoneally with 75 mg/kg LPS (**c**) or subjected to experimental sepsis (CLP) (**d**). **e.** Wild type mice were subjected to CLP and were injected with 50 µg clusterin at day 1, day 3 and day 5. **a,c-e**. Survival curves were done using Kaplan Meir method and compared using the Log-rank test. * p<0.05; **p<0.01. **b**. * p<0.05; **p<0.01 (Kruskal-Wallis test).

Supplementary Figure S1. **a.** The levels of clusterin were evaluated by ELISA in the serums of healthy subjects and of septic shock patients (n=50) (mean ± SEM). **b**. Levels of CRP were quantified by ELISA in the plasmas of surviving and non surviving septic shock patients at admittance at the ICU (D0), D3 and D7 (mean ± SEM). **c**. Western blotting analysis of clusterin in the plasmas of patients (left panel) and healthy subjects (right panel). 75 µg of proteins were loaded in each lane. **d**. The levels of clusterin-H4 complexes were evaluated by ELISA in the serums of septic shock patients and healthy subjects (mean ± SEM). **e**. Serums from two septic shock patients were immunoprecipitated with an anti-clusterin mAb and the presence of Clu-histone complexes was revealed by Western-Blotting with an anti-histone Ab. Results are representative of one out of 2 experiments. **f**. The levels of nucleosomes were evaluated by ELISA in the serums of septic shock patients and healthy subjects. **a,b,d,f**. *p<0.05, **p<0.01, ***p<10^{-3}; ns, not significant (Mann Whitney U test).

Supplementary Figure S2. The binding of 1.5 nM biotinylated clusterin to immobilized BSA, genomic DNA, high mobility group box 1 (HMGB1), calreticulin (CRT), Hsp70, cold inducible RNA-protein (CIRP), and calf thymus histones, was evaluated by ELISA. Results are representative in OD values (mean ± SD, n=5).

Supplementary Figure S3. **a.** Monocytes from healthy subjects were incubated in serum-free medium with increasing concentrations of calf thymus histones. IL-6 and TNFα were quantified by ELISA in the 24 h supernatants (n=5, mean ± SEM). ***p<10^{-3} (Kruskal-Wallis

test). **b-c**. MonoMac 6 cells were incubated for 10 min with 4 µg/mL AF488-labeled histones and increasing concentrations of clusterin. The binding of histones was evaluated by flow cytometry (**b**) or confocal fluorescence microscopy (**c**). **b**. Results, expressed as a percentage of inhibition of AF488-labeled histones, are representative of one of 3 independent experiments. **c**. Confocal microscopy images of MM6 cells incubated with 8 µg/mL AF488-labeled histones in the absence (left panel) or presence (right panel) of 150 µg/mL clusterin. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Scale bar, 30 µm.

Supplementary Figure S4. The expression of clusterin by neutrophils isolated from healthy subjects was analyzed by Western Blotting (each lane corresponds to cell extracts obtained from independent subjects) and by confocal microscopy (representative results from 1 of 4 independent experiments).

Supplementary Figure S5. **a**. The levels of clusterin were determined by ELISA in the serums of wild type mice injected intraperitoneally with PBS or 50 mg/kg LPS and collected at the indicated times. **b**. Western blotting analysis of clusterin expression in the spleen, liver and kidney of wild type mice injected with 50 mg/kg of LPS or PBS (n=2). 75 µg of proteins were loaded in each lane. Results are representative of one of two experiments. **c**. Splenocytes from $Clu^{+/+}$ or $Clu^{-/-}$ mice (n=5) were stimulated or not with 50 µg/mL histones or 50 ng/mL LPS. IL-6 was quantified by ELISA in the 24 h supernatants. **a,c**. *$p<0.05$; **$p<0.01$; ns, not significant (Mann Whitney U test).

Table 1. Baseline characteristics of patients.

	All patients	Serum Clu < 103 µg/mL	Serum Clu ≥ 103 µg/mL	
	n=110	**n=46**	**n=64**	**P**
Baseline characteristics				
Sex (M/F)	76/34	23/11	41/23	0.178
Age (years)	66.2 ± 13.8 [20-88]	67.3 ± 12.0	65.3 ± 15.0	0.456
Weight (Kg)	78.0 ± 18.9 [44-140]	81.4 ± 17.0	75.5 ± 19.9	0.114
SOFA	9.0 ± 3.7 [1-19]	10.6 ± 3.7	7.8 ± 3.3	**<0.001**
SAPSII	49.8 ± 18.3 [14-123]	54.6 ± 17.2	46.4 ± 18.5	**0.021**
Organ Support at ICU admission, n (%)				
Mechanical ventilation	64 (58.2)	29 (63.0)	35 (54.7)	0.381
PaO_2/FiO_2	214.2 ± 137 [28-757]	212.4 ± 128	215.7 ± 146	0.925
Invasive/non invasive ventilation	54/10	26/3	28/7	0.428
Renal replacement therapy	16 (14.5)	5 (10.9)	11 (17.2)	0.353
Use of vasopressors	84 (76.4)	37 (80.4)	47 (73.4)	0.394
Laboratory values at admission				
Serum creatinine (µmol/L)	180.3 ± 171 [26-1311]	184.7 ± 132	178.8 ± 185	0.859
Hemoglobin (g/dL)	11.0 ± 2.1 [5.3-16.4]	11.3 ± 2.6	10.8 ± 1.7	0.219
White blood cells (G/L)	14.7 ± 9.4 [0.3-41.4]	13.8 ± 10.3	15.3 ± 8.7	0.394
Blood neutrophils (G/L)	13.2 ± 9.4 [0.0-37.7]	12.3 ± 9.6	13.8 ± 9.3	0.506
Platelets (G/L)	200.0 ± 158 [14.0-999.0]	130.0 ± 87	249.3 ± 178	**<0.001**
Blood lactate (mmol/L)	2.65 ± 3.1 [0.3-25.0]	3.37 ± 2.7	2.1 ± 3.3	**0.037**
Prothrombin time (%)	57.4 ± 20 [10-96]	51.6 ± 21	61.5 ± 19.1	**0.015**
Serum fibrinogen (g/L)	5.32 ± 2.1 [1.2-10.3]	5.4 ± 2.3	5.3 ± 2.0	0.720
Microbiological analysis; n (%)				
Positive blood cultures	38 (34.5)	19 (41.3)	19 (29.7)	0.208
Beta-hemolytic streptococcus	13 (11.8)	7 (15.2)	6 (9.4)	0.382
Staphylococcus	11 (10.0)	4 (8.7)	7 (10.9)	0.758
Gram negative rods	29 (26.4)	16 (34.8)	13 (20.3)	0.124
Polymicrobial	7 (6.4)	2 (4.3)	5 (7.8)	0.696
Other	1 (0.9)	0 (0)	1 (1.6)	1.000
Organ support at any time of ICU stay; n (%)				
Mechanical ventilation	65 (59.1)	30 (65.2)	35 (54.7)	0.327
Renal replacement therapy	22 (20.0)	11 (23.9)	11 (17.2)	0.384
Use of vasopressors	86 (78.2)	37 (80.4)	49 (76.6)	0.628
ICU length of stay, days	11.2 ± 14.7 [1-90]	11.0 ± 15.5	11.3 ± 14.3	0.916
ICU mortality; n (%)	19 (17.3)	14 (30.4)	5 (7.8)	**0.002**

Figure 1

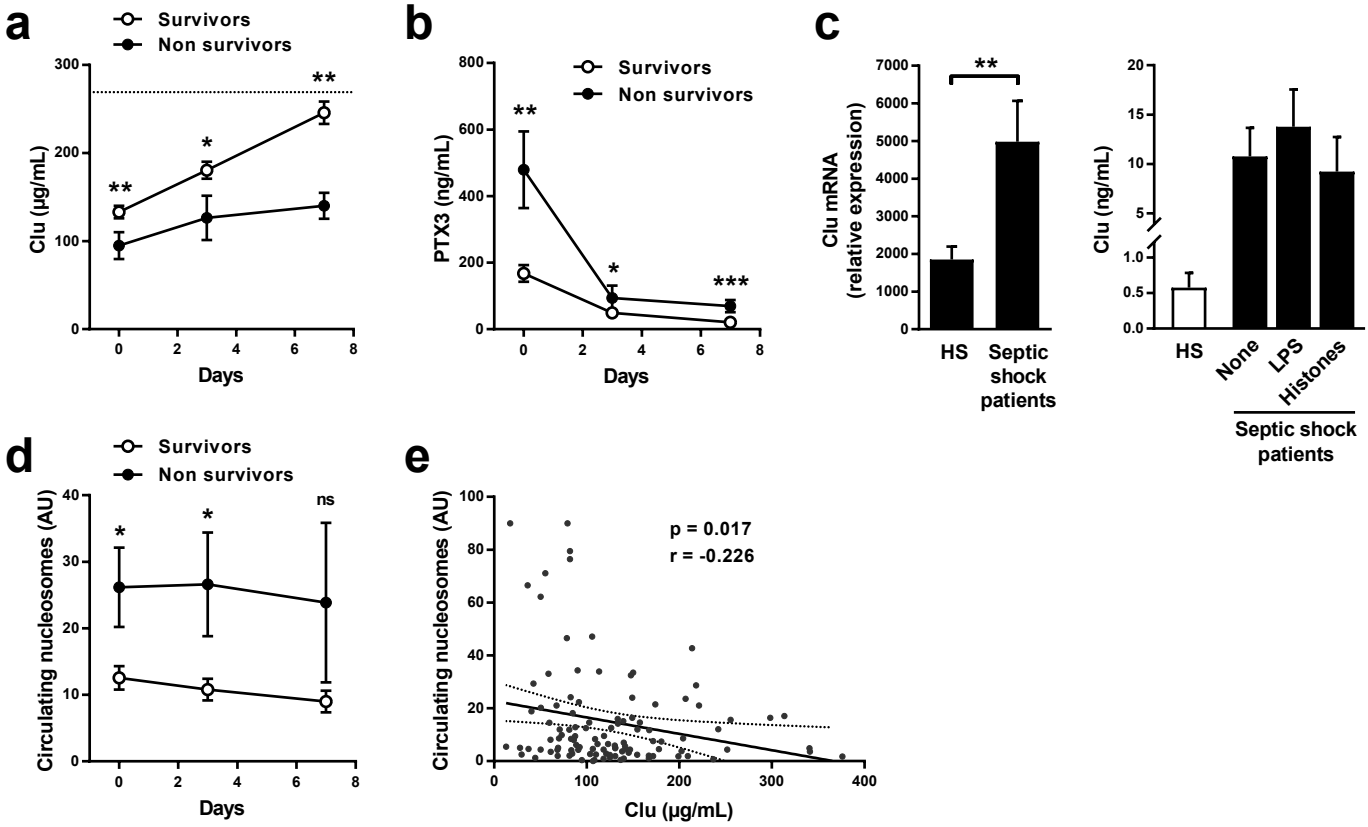

a
Survivors
Non survivors
Clu (µg/mL)
Days
b
Survivors
Non survivors
PTX3 (ng/mL)
Days
c
Clu mRNA (relative expression)
HS
Septic shock patients
Clu (ng/mL)
HS
None
LPS
Histones
Septic shock patients
d
Survivors
Non survivors
Circulating nucleosomes (AU)
Days
ns
e
Circulating nucleosomes (AU)
p = 0.017
r = -0.226
Clu (µg/mL)

Figure 2

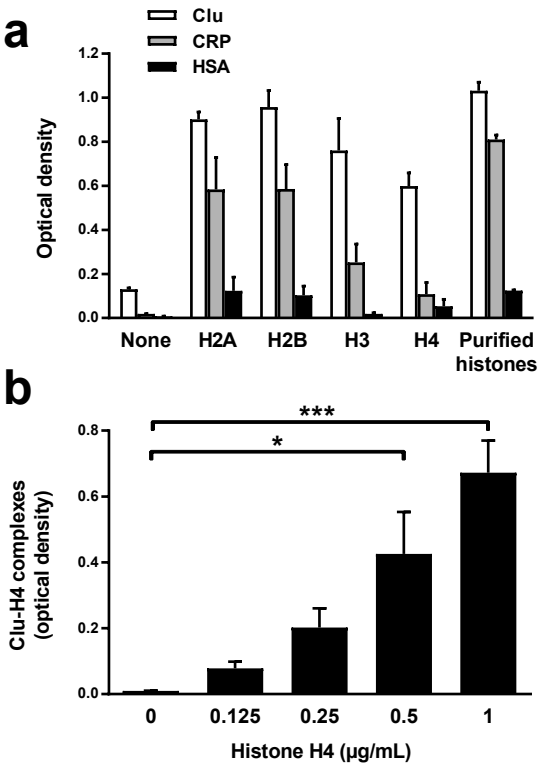

a
Clu
CRP
HSA
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0.0
None
H2A
H2B
H3
H4
Purified histones
b
***
*
Clu-H4 complexes (optical density)
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
Histone H4 (µg/mL)

Figure 3

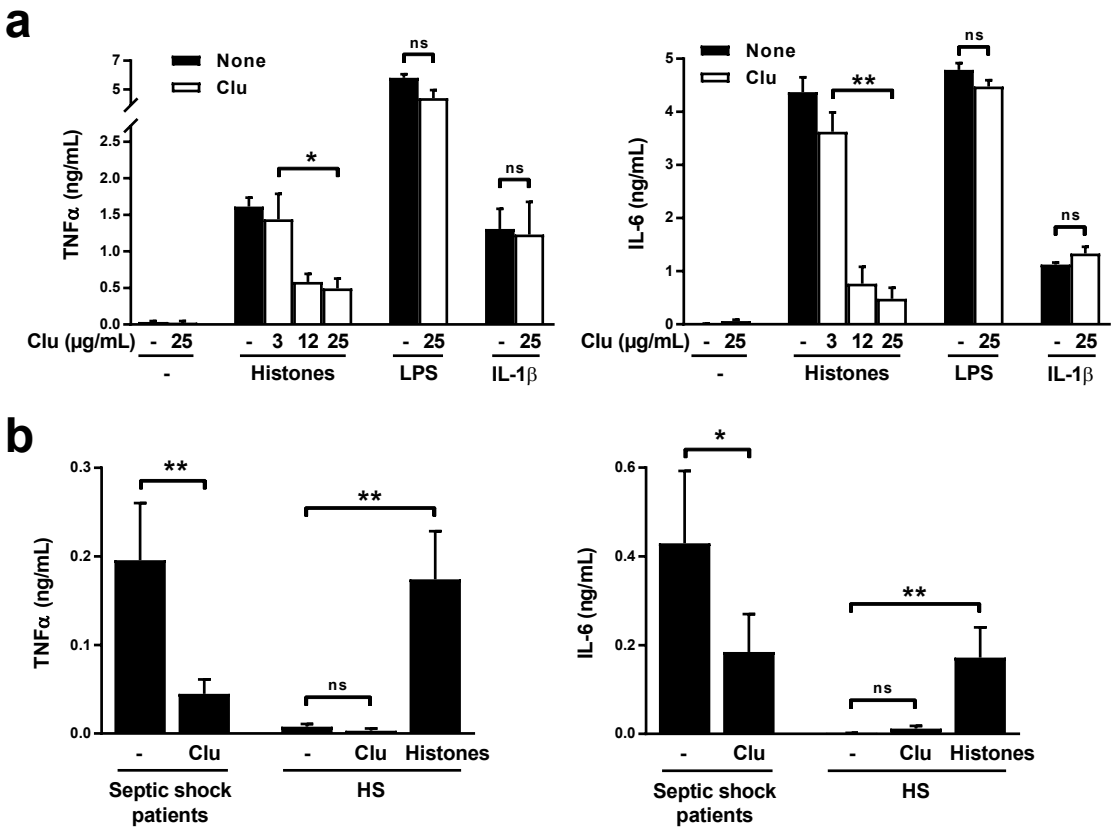

a
None
Clu
TNFα (ng/mL)
IL-6 (ng/mL)
Clu (µg/mL)
- 25
- 3 12 25
- 25
- 25
-
Histones
LPS
IL-1β
ns
*
**
b
Septic shock patients
HS
Clu
Histones

Figure 4

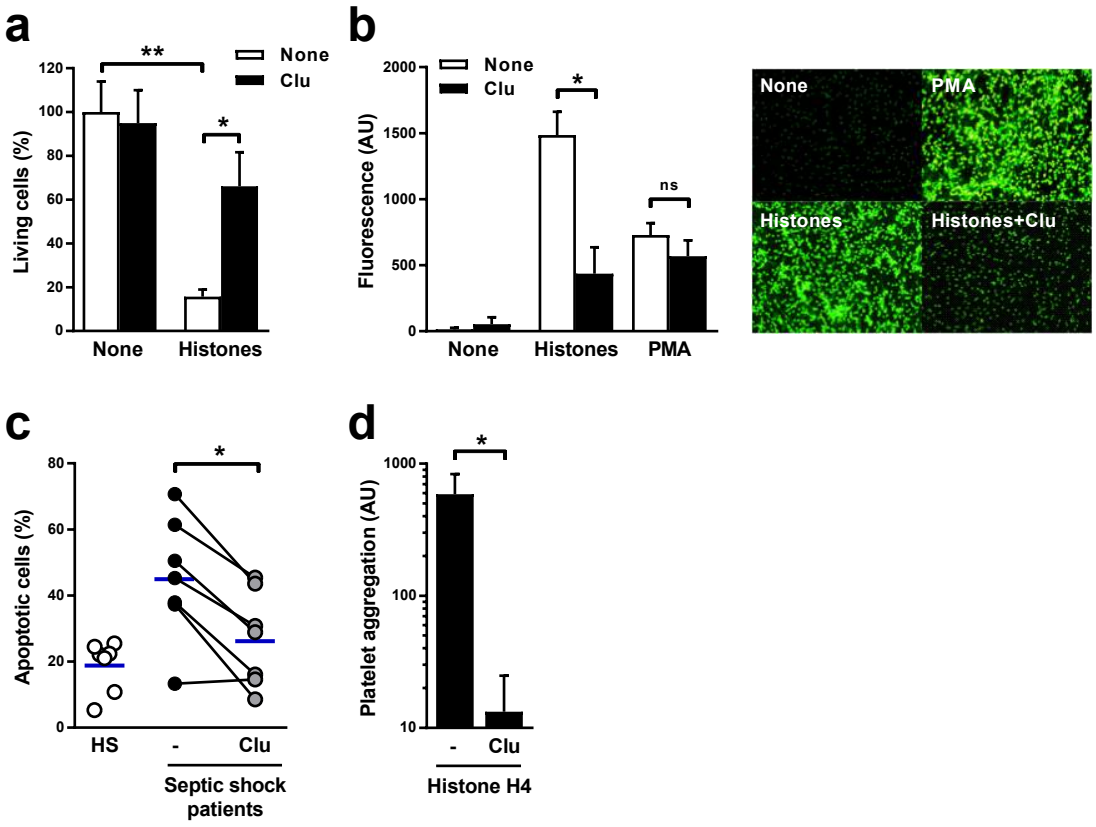

a
**
*
None
Clu
Living cells (%)
120
100
80
60
40
20
0
None
Histones
b
None
Clu
*
ns
Fluorescence (AU)
2000
1500
1000
500
0
None
Histones
PMA
None
PMA
Histones
Histones+Clu
c
*
Apoptotic cells (%)
80
60
40
20
0
HS
-
Clu
Septic shock patients
d
*
Platelet aggregation (AU)
1000
100
10
-
Clu
Histone H4

Figure 5

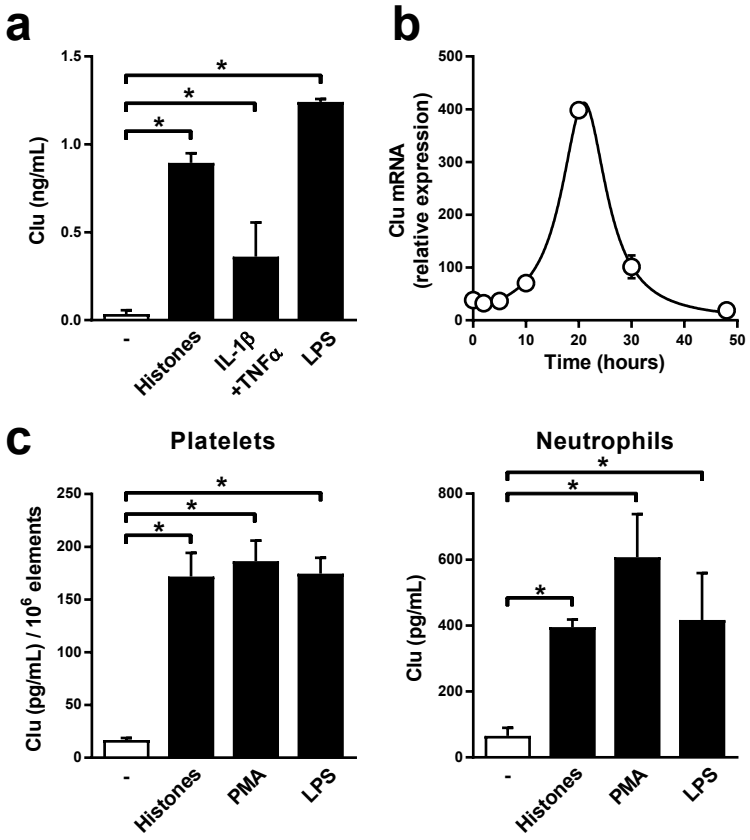

a
Clu (ng/mL)
1.5
1.0
0.5
0.0
*
*
*
-
Histones
IL-1β
+TNFα
LPS
b
Clu mRNA
(relative expression)
500
400
300
200
100
0
0
10
20
30
40
50
Time (hours)
c
Platelets
Clu (pg/mL) / 10^6 elements
250
200
150
100
50
0
*
*
*
-
Histones
PMA
LPS
Neutrophils
Clu (pg/mL)
800
600
400
200
0
*
*
*
-
Histones
PMA
LPS

Figure 6

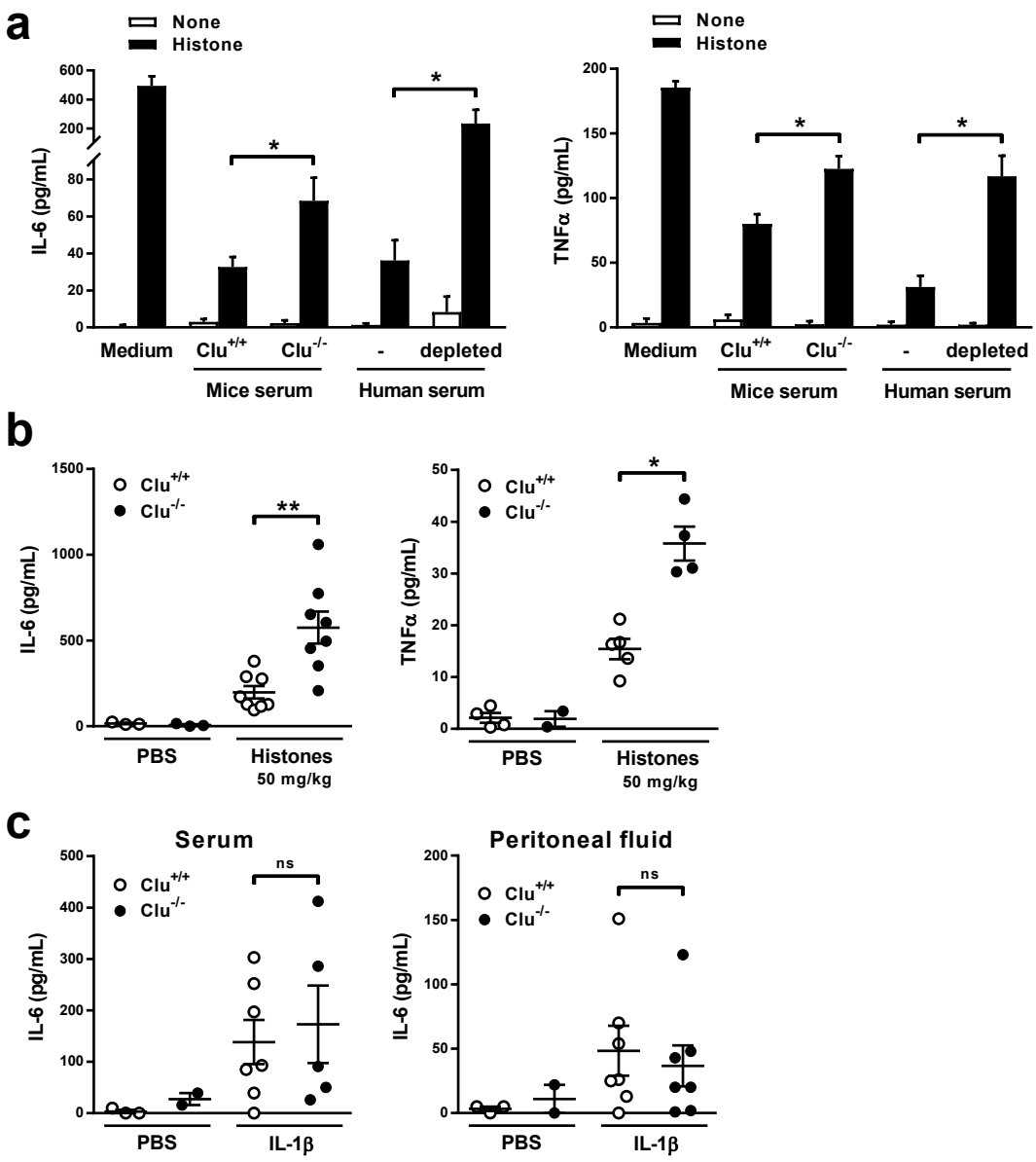

a
None
Histone
IL-6 (pg/mL)
TNFα (pg/mL)
Medium
Clu+/+
Clu-/-
-
depleted
Mice serum
Human serum
b
Clu+/+
Clu-/-
IL-6 (pg/mL)
TNFα (pg/mL)
PBS
Histones
50 mg/kg
c
Serum
Peritoneal fluid
ns
IL-6 (pg/mL)
PBS
IL-1β

Figure 7

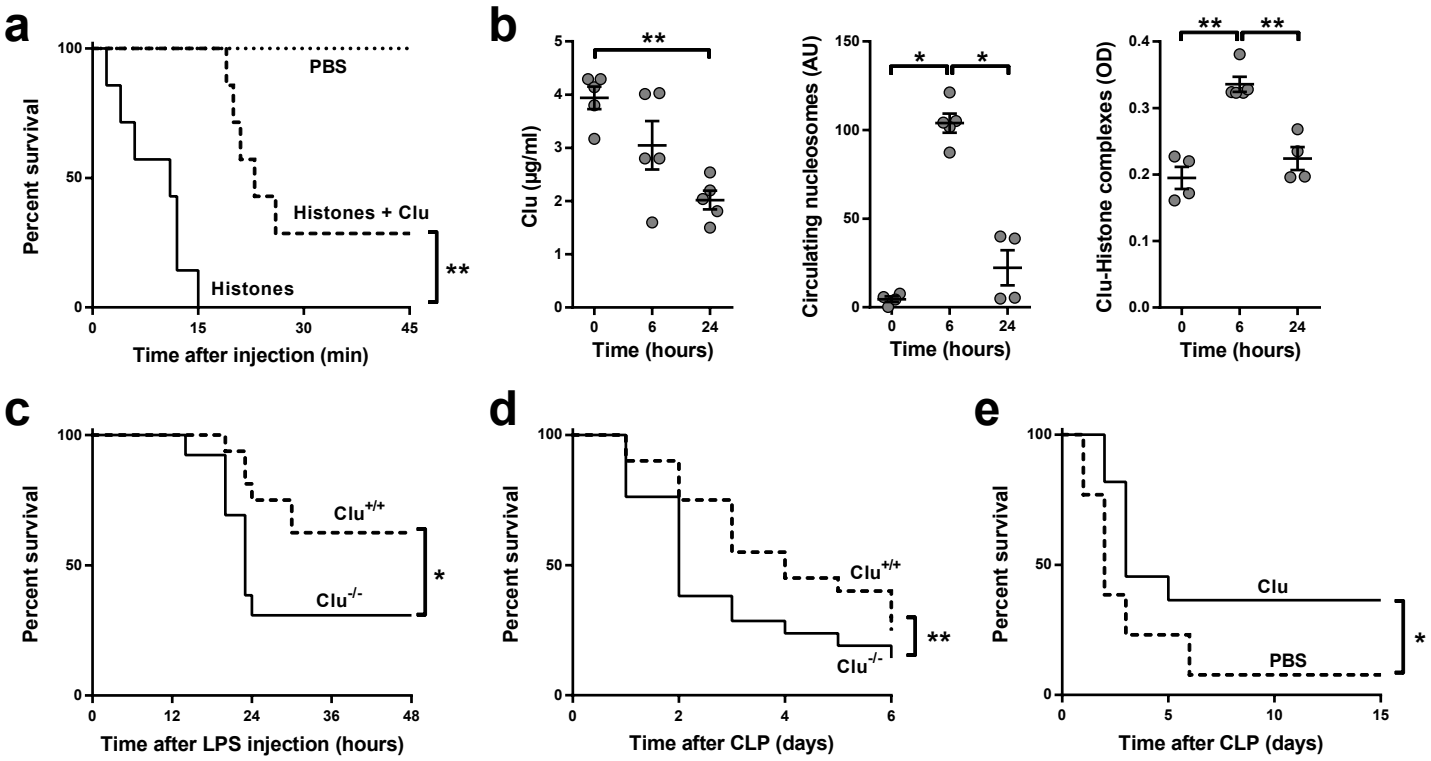

a
Percent survival
Time after injection (min)
PBS
Histones + Clu
Histones
**
b
Clu (µg/ml)
Time (hours)
Circulating nucleosomes (AU)
Clu-Histone complexes (OD)
c
Clu+/+
Clu-/-
Time after LPS injection (hours)
d
Time after CLP (days)
e
Clu
PBS

Figure S1

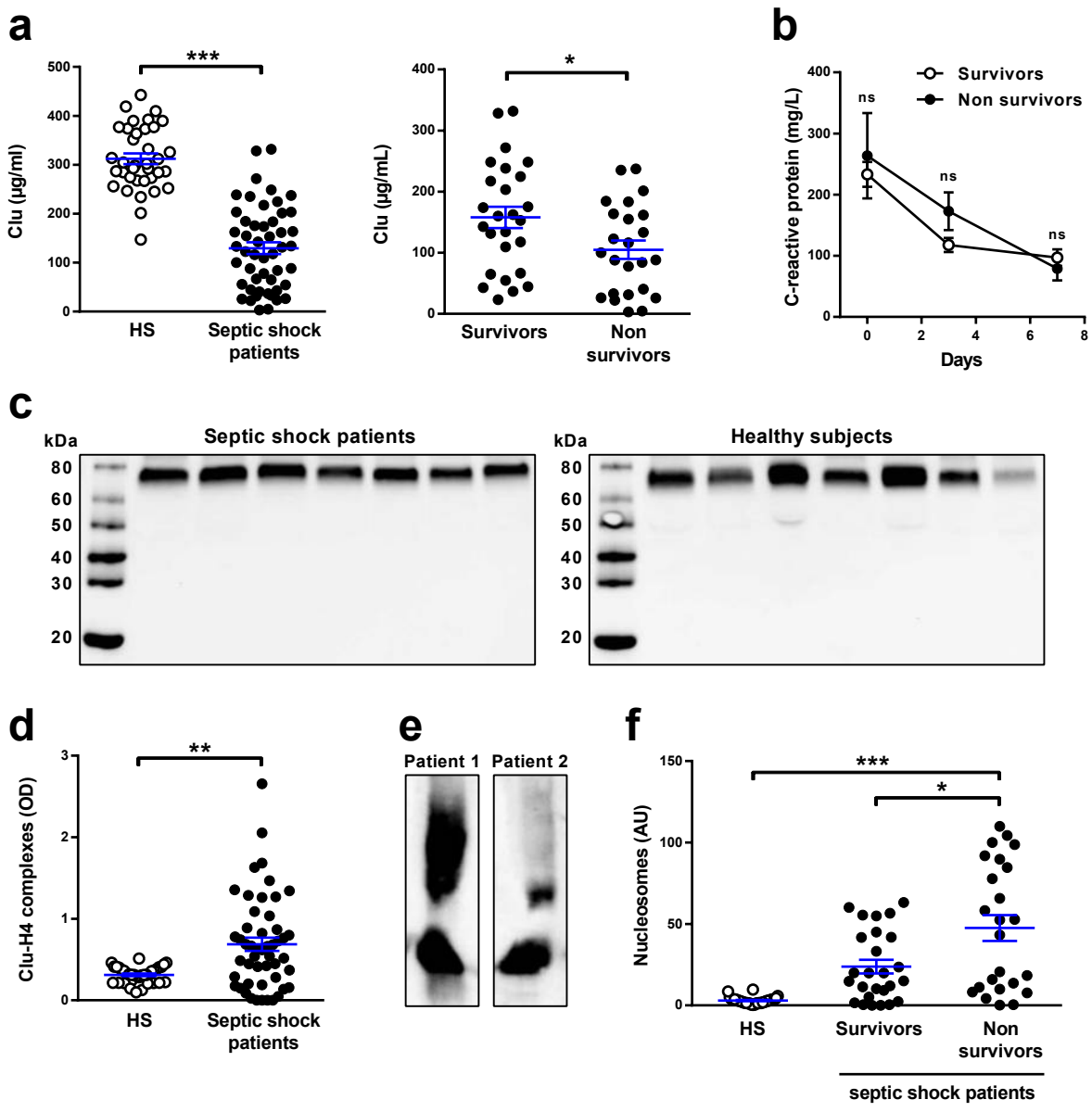

a
***
Clu (µg/ml)
500
400
300
200
100
0
HS
Septic shock patients
*
Clu (µg/mL)
400
300
200
100
0
Survivors
Non survivors
b
Survivors
Non survivors
C-reactive protein (mg/L)
ns
ns
ns
400
300
200
100
0
0
2
4
6
8
Days
c
kDa
Septic shock patients
80
60
50
40
30
20
kDa
Healthy subjects
80
60
50
40
30
20
d
**
Clu-H4 complexes (OD)
3
2
1
0
HS
Septic shock patients
e
Patient 1
Patient 2
f
***
*
Nucleosomes (AU)
150
100
50
0
HS
Survivors
Non survivors
septic shock patients

Figure S2

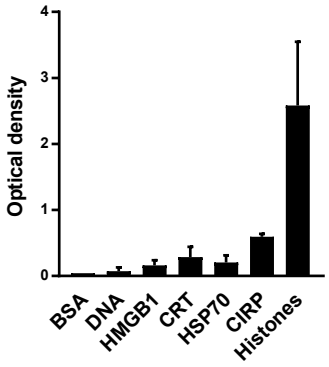

Optical density
4
3
2
1
0
BSA
DNA
HMGB1
CRT
HSP70
CIRP
Histones

Figure S3

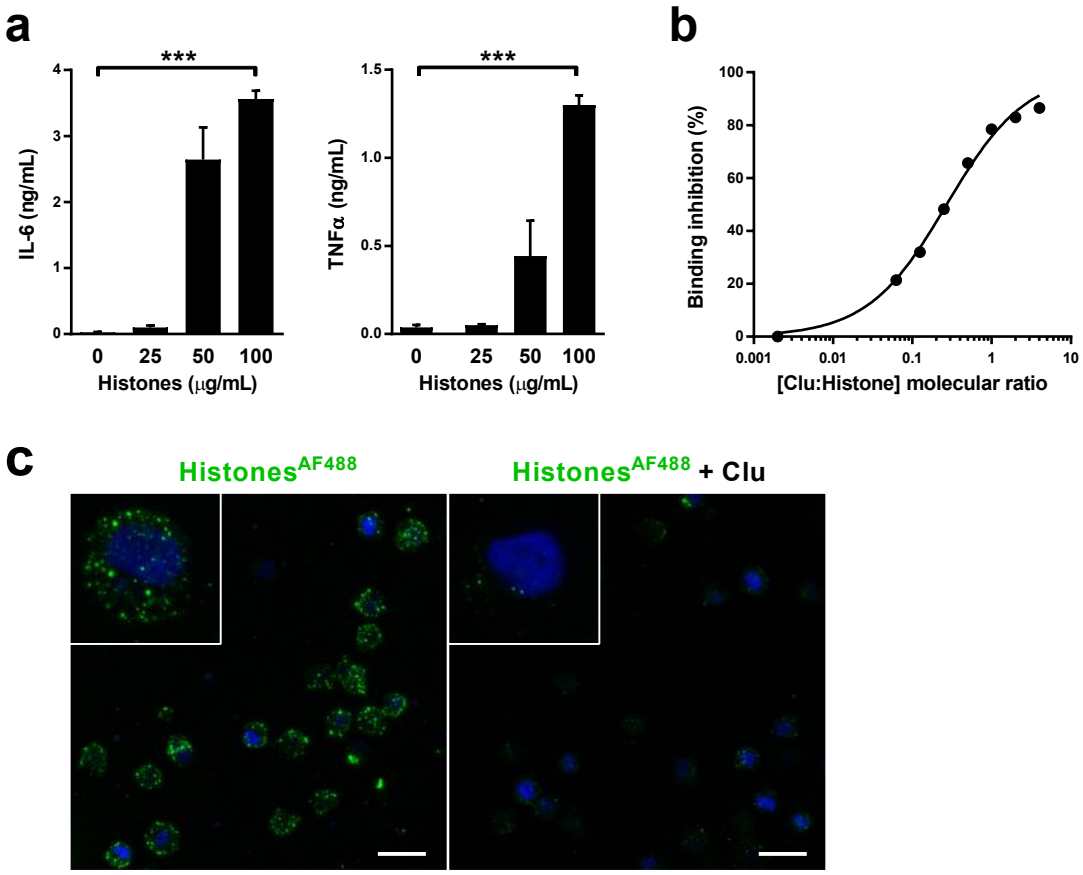
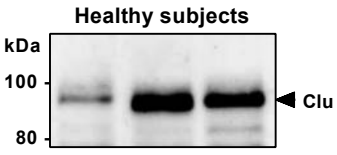

a
***
IL-6 (ng/mL)
0
1
2
3
4
0
25
50
100
Histones (μg/mL)
***
TNFα (ng/mL)
0.0
0.5
1.0
1.5
0
25
50
100
Histones (μg/mL)
b
Binding inhibition (%)
0
20
40
60
80
100
0.001
0.01
0.1
1
10
[Clu:Histone] molecular ratio
c
Histones^AF488
Histones^AF488 + Clu

Figure S4



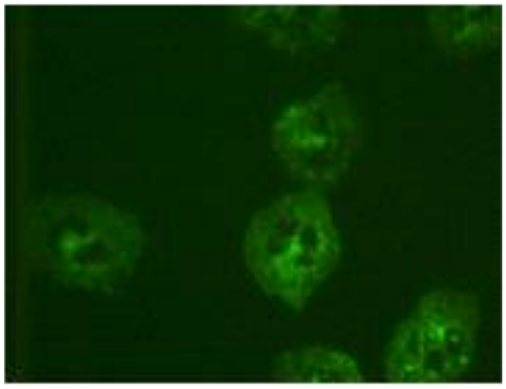
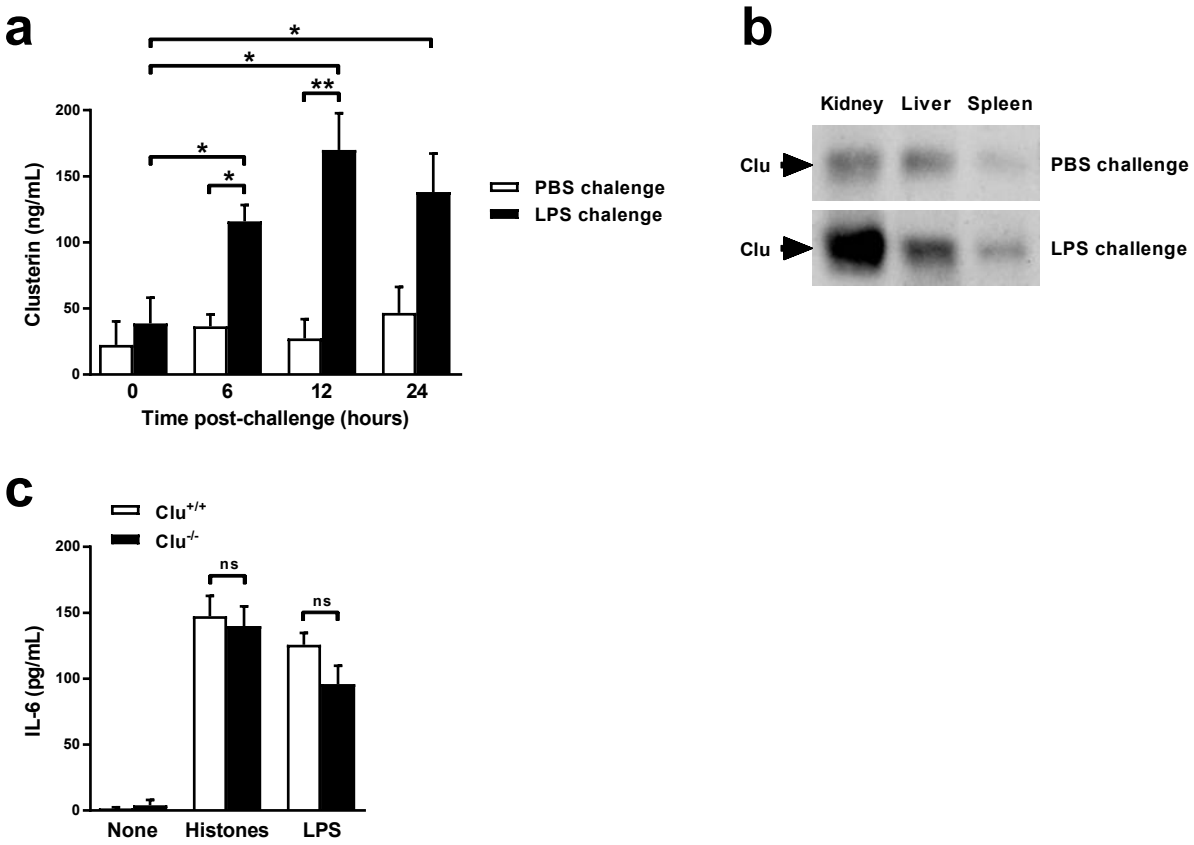


Figure S5


a
*
*
**
*
*
200
150
100
50
0
Clusterin (ng/mL)
0
6
12
24
Time post-challenge (hours)
PBS chalenge
LPS chalenge
b
Kidney
Liver
Spleen
Clu
PBS challenge
Clu
LPS challenge
c
Clu+/+
Clu-/-
ns
ns
200
150
100
50
0
IL-6 (pg/mL)
None
Histones
LPS