Journal of Thrombosis and Haemostasis, **14**: 397–410 DOI: 10.1111/jth.13223

ORIGINAL ARTICLE

Extracellular histones reduce survival and angiogenic responses of late outgrowth progenitor and mature endothelial cells

H. A. MENA,* A. CARESTIA,* L. SCOTTI,† F. PARBORELL,† M. SCHATTNER* and S. NEGROTTO*
*Experimental Thrombosis Laboratory, Institute of Experimental Medicine (IMEX), National Academy of Medicine-CONICET; and
†Experimental Medicine and Biology Institute (IByME), CONICET, Buenos Aires, Argentina

To cite this article: Mena HA, Carestia A, Scotti L, Parborell F, Schattner M, Negrotto S. Extracellular histones reduce survival and angiogenic responses of late outgrowth progenitor and mature endothelial cells. *J Thromb Haemost* 2016; **14**: 397–410.

Essentials

- Extracellular histones are highly augmented in sites of neovessel formation, such as regeneration tissues.
- We studied histone effect on survival and angiogenic activity of mature and progenitor endothelial cells.
- Extracellular histones trigger apoptosis and pyroptosis and reduce angiogenesis *in vivo* and *in vitro*.
- Histone blockade can be useful as a therapeutic strategy to improve angiogenesis and tissue regeneration.

Summary. *Background:* Extracellular histones are highly augmented in sites of neovessel formation, like regeneration tissues. Their cytotoxic effect has been studied in endothelial cells, although the mechanism involved and their action on endothelial colony-forming cells (ECFCs) remain unknown. *Objective:* To study the effect of histones on ECFC survival and angiogenic functions and compare it with mature endothelial cells. *Methods and Results:* Nuclear morphology analysis showed that each human recombinant histone triggered both apoptotic-like and necrotic-like cell deaths in both mature and progenitor endothelial cells. While H1 and H2A exerted a weak toxicity, H2B, H3 and H4 were the most powerful. The percentage of apoptosis correlated with the percentage of ECFCs exhibiting caspase-3 activation and was zeroed by the pan-caspase inhibitor Z-VAD-FMK. Necrotic-like cell death was also suppressed by this compound and the caspase-1 inhibitor Ac-YVAD-CMK, indicating that histones triggered ECFC pyroptosis. All histones, at non-cytotoxic concentrations, reduced migration and H2B, H3 and H4 induced cell cycle arrest and impaired tubulogenesis via p38 activation. Neutrophil-derived histones exerted similar effects. *In vivo* blood vessel formation in the quail chorioallantoic membrane was also reduced by H2B, H3 and H4. Their cytotoxic and antiangiogenic effects were suppressed by unfractioned and low-molecular-weight heparins and the combination of TLR2 and TLR4 blocking antibodies. *Conclusions:* Histones trigger both apoptosis and pyroptosis of ECFCs and inhibit their angiogenic functions. Their cytotoxic and antiangiogenic effects are similar in mature endothelial cells and disappear after heparin addition or TLR2/TLR4 blockade, suggesting both as therapeutic strategies to improve tissue regeneration.

Keywords: antiangiogenesis effect; cell viability; endothelial cells; endothelial progenitor cells; histones.

Correspondence: Soledad Negrotto, Laboratorio de Trombosis Experimental, IMEX, CONICET-Academia Nacional de Medicina, Pacheco de Melo 3081, Buenos Aires (1425), Argentina.
Tel.: +5411 4805 5759; fax: +5411 4805 0712.
E-mail: solenegrotto@hematologia.anm.edu.ar

Received 17 July 2015
Manuscript handled by: W. Ruf
Final decision: P. H. Reitsma, 25 November 2015

Introduction

Histones are basic low-molecular-weight proteins that are responsible for DNA organization. In humans, there are five main types: the linker histone H1 and the core histones H2A, H2B, H3 and H4 [1]. Extracellular histones are derived from dying cells or neutrophil extracellular traps (NETs). Interestingly, NETs and histones were found to be highly increased not only in infectious diseases (sepsis) [2,3] but also in sterile inflammation [4], playing a role in the pathogenesis of several inflammatory disorders [5–10]. In addition to the vast cell death, an increase of NETs and histones during ischemia and wound repair has been reported, although their role in damage and development of tissue regeneration has not been clarified [11,12].

As to the mechanism of histone toxicity, it has been shown that they bind non-specifically to cell membrane phospholipids [13,14] and form pores [15] or use existing

channels to promote calcium influx and membrane depolarization [16], although activation of toll-like receptors (TLRs) 2 and 4 has been also reported [17–19]. Circulating histones have been shown to be mediators of trauma-associated lung injury in mice [20] and in several types of primary and transformed endothelial cells, which showed a decreased viability in culture after treatment with a mixture of calf thymus-derived histones, H3 or H4 [2,20–22]. Still, the mechanisms involved in histone-induced cytotoxicity on endothelial cells have not been completely elucidated.

Blood vessel endothelium is a key organ involved not only in the regulation of thrombus formation but also in tissue regeneration. Effective endothelial repair and development of new vessels require the contribution of both angiogenesis, which involves the migration and proliferation of mature endothelial cells in nearby tissues, and vasculogenesis, which is a process mediated by bone marrow (BM)-derived cells that have the potential to differentiate into mature endothelial cells and are collectively referred to as endothelial progenitor cells (EPCs) [23,24]. Different cell populations have been reported to play roles in vasculogenesis, but only one population contains cells, called endothelial colony-forming cells (ECFCs) (also known as late outgrowth EPCs), that are considered to be true endothelial progenitors and form neovessels *in vivo* [25]. Interestingly, NETs and histones were found to be vastly increased at sites were angiogenesis and vasculogenesis are active processes, including ischemic tissues [11] and wounds [12] and within the peritumoral area, where they are thought to contribute to cancer progression [9,26].

Based on this background, we studied the effect of histones on ECFC survival and angiogenic functions and compared it with mature endothelial cells from micro (human microvascular endothelial cells 1, HMEC1) and macrovascular (human umbilical vein endothelial cell, HUVEC) beds.

Methods

Cell culture

This study was performed according to institutional guidelines (National Academy of Medicine, Argentina) and received prior approval by the institutional ethics committee. All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Peripheral and umbilical cord blood and umbilical cords were collected from healthy donors. All individuals provided written informed consent for the collection of samples and subsequent analysis. Human umbilical vein endothelial cells (HUVECs) and ECFCs were obtained from the umbilical cord vein and blood, respectively, and characterized as previously described [27–29]. Characterization data are available upon request.

Human microvascular endothelial cells 1 (HMEC1) and the human alveolar basal epithelial cell line (A549) were obtained from the American Type Culture Collection. Primary human foreskin fibroblasts were kindly provided by Dr Marcelo De Campos-Nebel (Laboratory of Mutagenesis, IMEX, Buenos Aires, Argentina).

Endothelial cell treatment with histones

Endothelial cells in endothelial basal medium 2 (EBM2) with 2% fetal bovine serum (FBS) or endothelial growth medium 2 (EGM2) (Lonza, Walkersville, MD, USA) were treated with human recombinant H1, H2A, H2B, H3 or H4 (New England Biolabs, Ipswich, MA, USA). In some experiments, unfractioned heparin (BD Biosciences, San Jose, CA, USA), low-molecular-weight heparin (Northia, Buenos Aires, Argentina), Z-VAD-fmk (Biomol, Plymouth Meeting, PA, USA), Ac-YVAD-cmk, SB203580 (Enzo Life Sciences, San Diego, CA, USA), anti-histones (clone H11-4, Roche Diagnostics, Indianapolis, IN, USA), anti-human TLR4, anti-human TLR2 antibodies (eBioscience, San Diego, CA, USA) or the corresponding IgG were added 30 min before histones. Endotoxin contamination was discarded as similar results were observed in the presence of Polymyxin B (Sigma, St Louis, MO, USA) (data not shown).

Proliferation and viability assays

Endothelial cell proliferation was determined spectrophotometrically by measuring acid phosphatase activity (pNPP substrate, Sigma). Nuclear morphology was analyzed by fluorescence microscopy after acridine orange and ethidium bromide staining, and by flow cytometry after FITC-annexin-V binding and propidium iodide (PI) staining (BD Biosciences) [28,30].

Flow cytometry studies

Cells were fixed, permeabilized and then caspase-3 activation and cell cycle progression were determined by staining with FITC-conjugated antihuman caspase-3 or monoclonal antibody or Ki67 antigen and PI (BD Biosciences). Samples were acquired in a FACSCalibur flow cytometer (BD Biosciences) and analyzed by FCS Express V3 (De Novo Software, Glendale, CA, USA).

Measurement of angiogenic responses

Confluent cell monolayers were wounded and the extent of cell migration into the wounded area was measured after 18 h. Chemotaxis driven by EGM2 or stromal-derived factor 1 (SDF1) (Preprotech, Rocky Hill, NJ, USA) was

examined using transwells with 8.0-μm pore polycarbonate membrane inserts (Costar, Billerica, MA USA). The number of migrated cells was determined by counting under a high-power microscope. Tube formation on reduced growth factor basement membrane matrix (Geltrex™; Gibco, Grand Island, NY, USA) was examined by phase-contrast microscopy and the total number of branch points was quantified by analyzing images of the entire surface. Image analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) [28]. P38 phosphorylation levels were measured by Western blot using rabbit anti-phospho p38 (Tyr 182) followed by an HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) [28]. The quail chorioallantoic membrane (CAM) assay was performed as previously described [31]. In brief, after 2 days of *in ovo* and 5 days of *ex ovo* incubations, filter paper disks impregnated with EGM2 containing each histone (1 μM) or vehicle (control) were placed on CAMs from 7-day embryos for 48 h. After sacrificing the embryos by freezing, CAMs were fixed, dissected and placed on slides to register images under a stereomicroscope. The number of blood vessel branch points was counted within the area encompassing the entire paper disk.

Neutrophil-derived histone isolation

Neutrophils were isolated from human peripheral blood by Ficoll Hypaque gradient centrifugation and dextran sedimentation, as described previously [32]. Histone release from neutrophils (1×10^6 mL^{-1}) was induced by stimulation with monosodium urate crystals (300 μg mL^{-1}, Sigma) at 37 °C for 4 h. Cell suspensions were first sonicated and then centrifuged at low speed ($180 \times g$, 5 min) and supernatants were kept at −80 °C until used. DNA was measured in the supernatants using SybrGreen in a fluorometer (Biotek, Winooski, VT, USA).

Statistical analysis

Results are expressed as means ± SEM. Significant differences ($P < 0.05$) were identified by one-way analysis of variance (ANOVA) followed by the Bonferroni test using the GraphPad software package (PRISM Version 5.0, San Diego, CA, USA).

Results

Differential cytotoxic effect of histones on ECFC and other cell types

We first analyzed the ability of individual human recombinant histones to promote cell death in late outgrowth endothelial progenitor cells (ECFCs). ECFC nuclear morphology analysis by fluorescence microscopy indicated that while H1 and H2A had a slight but significant cytotoxic effect only at 4 μM, H2B, H3 and H4 triggered both necrotic-like and apoptotic-like cell deaths after 24 h in a concentration-dependent manner (Fig. 1A and B). Similar results were observed by flow cytometry after annexin V and PI staining (Fig. 1C).

Kinetic studies revealed that necrotic-like cells were evident at 2 h after addition of histones (4 μM) and the percentage remains constant overtime (6 and 24 h later), whereas apoptosis was only observed at 24 h in a small proportion (Figure S1). The effect of histones on ECFC viability was compared with mature endothelial cells from macro and microvascular beds (HUVEC and HMEC1, respectively) and other cell types such as the human alveolar basal epithelial cell line (A549) and primary human foreskin fibroblasts. The sensitivity of each cell type to these proteins varied considerably. While ECFCs and HUVECs were equally sensitive to H2B, H3 and H4, only H2B and H3 induced HMEC1 death (Fig. 1D). Regarding the non-endothelial cell types, histone-mediated toxicity was drastically lower (Fig. 1D).

Activated caspase-1 is the main mediator of histone-induced cell death

Considering that there is enough evidence demonstrating that cells may undergo other forms of cell death that share nuclear morphology features with necrosis (e.g. necroptosis and pyroptosis) [33], our results led us to hypothesize that there are some cells that appear to be necrotic cells but in fact experienced some type of programmed cell death. To elucidate this hypothesis, ECFCs were preincubated with the pan-caspase inhibitor Z-VAD-fmk, which completely blocked apoptosis and, additionally, largely suppressed the necrotic-like process (Fig. 2A). To get a deeper insight into the mechanisms involved in histone-induced ECFC cell death, we next analyzed the involvement of caspases. We found that caspase-3 was activated in a small fraction of ECFCs after 24 h (Fig. 2B), which correlated with the percentage of apoptosis observed (Fig. 1A). Caspase-1 activation was also analyzed as this protease is often activated by pore-forming substances and is the main mediator of the inflammatory death process known as pyroptosis [34]. Interestingly, we found that histones triggered ECFC pyroptosis because pretreatment with the caspase-1 specific inhibitor Ac-YVAD-cmk markedly suppressed cell death at similar levels to Z-VAD-fmk (Fig. 2C). Both Z-VAD-fmk and Ac-YVAD-cmk exerted a similar inhibitory action of H3-induced cell death in HUVECs and HMEC1 (Fig. 2D), indicating that histones trigger pyroptosis not only in progenitor but also in mature endothelial cells.

H2B, H3 and H4 reduce ECFC proliferation through a cell cycle arrest induction

We next aimed to elucidate whether histones, at concentrations that were not toxic, were able to regulate other

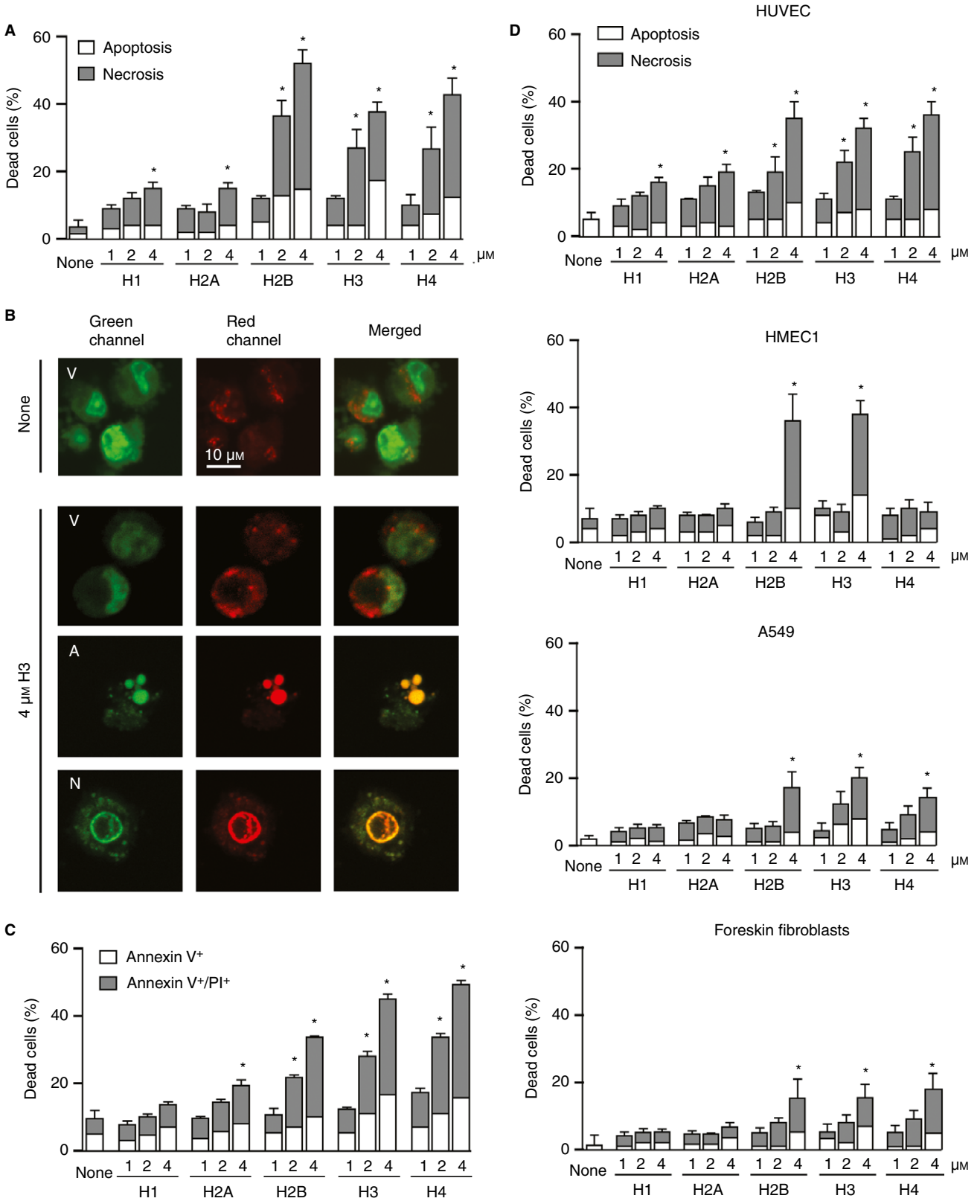


Fig. 1. Effect of histones on endothelial cell survival. (A and B) Endothelial colony-forming cells (ECFCs) were treated with histones at the indicated concentration and nuclear morphology was analyzed by acridine orange (green channel) and ethidium bromide (red channel) staining and fluorescence microscopy after 24 h. Images show viable (V), apoptotic-like (A) and necrotic-like cells (N) ($n = 5$). (C) Annexin-V and propidium iodide (PI) double staining was analyzed by flow cytometry after 24 h. (D) All cells were treated with histones at the indicated concentration and nuclear morphology was analyzed by fluorescence microscopy after 24 h ($n = 5$). *$P < 0.05$ vs. None, one-way ANOVA.

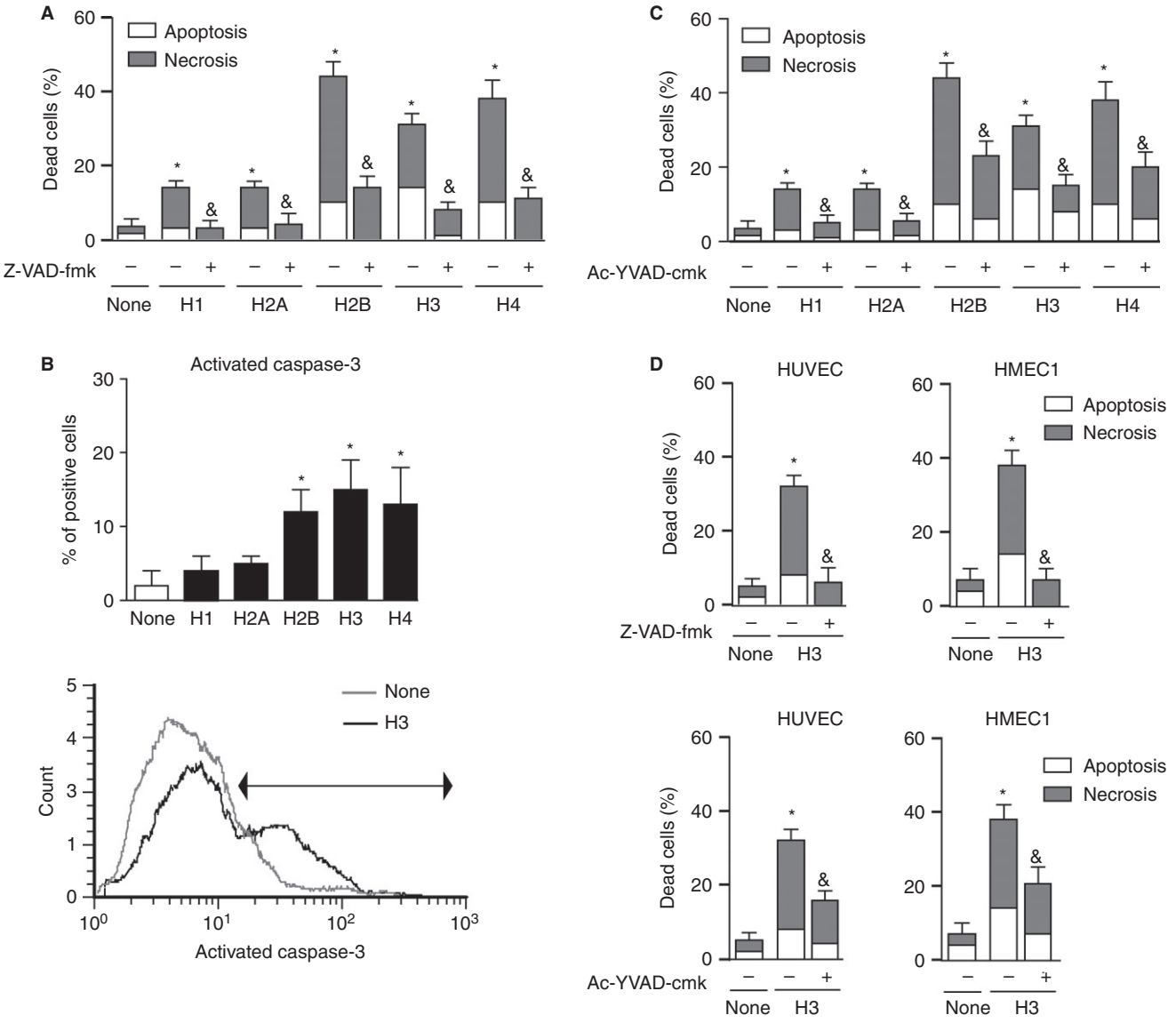
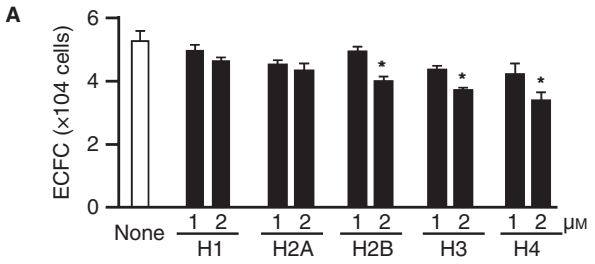


Fig. 2. Histones induce a caspase-dependent endothelial cell death. (A) Cells were preincubated with Z-VAD-fmk (30 μM) for 30 min and then treated with histones (4 μM). Nuclear morphology was analyzed by fluorescence microscopy after 24 h ($n = 5$). (B) Cells were treated with histones (4 μM) and caspase-3 activation was analyzed by flow cytometry after 24 h. The histogram represents one of six independent experiments. (C) Cells were preincubated with Ac-YVAD-cmk (50 μM) for 30 min and then treated with histones (4 μM). Nuclear morphology was analyzed by fluorescence microscopy after 24 h ($n = 4$). (D) Cells were preincubated with Z-VAD-fmk (30 μM) and Ac-YVAD-cmk (50 μM) for 30 min and then treated with H3 (4 μM). Nuclear morphology was analyzed by fluorescence microscopy after 24 h ($n = 3$). $^{*}P < 0.05$ vs. None; $^{\&}P < 0.05$ vs. histone without Z-VAD-fmk or Ac-YVAD-cmk, one-way ANOVA.

endothelial cell functions. Whereas in histone toxicity studies cells were maintained in EBM2 containing 2% FBS, in order to induce ECFC functional responses, cells were cultured in EGM2 (EBM2 supplemented with 2% FBS and growth factors). As expected, under these conditions, the effectiveness of histone in inducing cell death decreased significantly, whereas H1 and H2A were innocuous at any concentration tested, and H2B, H3 and H4 induced cell death only when used at 4 μM (21 ± 3*, 18 ± 4* and 17 ± 2*% of cell death by nuclear morphology, $n = 4$, $^{*}P < 0.05$ vs. none). Based on these data, angiogenic responses were examined upon cell treatment with 1–2 μM histones, concentrations that failed to affect cell survival.

When proliferation was examined by measuring cell phosphatase activity (pNPP assay), treatment with H2B, H3 and H4 significantly decreased cell number (Fig. 3A). Cell counts in a Neubauer chamber yielded similar results (data not shown). Flow cytometric analysis of the proliferation marker Ki67 antigen and PI showed that while H1 and H2A failed to modify cell cycle progression at any concentration tested, H2B, H3 and H4 induced cycle arrest in G0 at the expense of cells in G1 or S/G2/M phases when used at a concentration of 2 μM (Fig. 3B),



B

% of + cells	ECFC None	H1	H2A	H2B	H3	H4
G0	7 ± 3	10 ± 4	5 ± 2	17 ± 3*	21 ± 4*	22 ± 3*
G1	72 ± 6	79 ± 5	73 ± 4	64 ± 7*	54 ± 2*	70 ± 6
S/G2/M	17 ± 5	12 ± 3	17 ± 4	18 ± 3	20 ± 2	8 ± 3*

C

% of + cells	HUVEC None	H3
G0	19 ± 2	29 ± 5*
G1	70 ± 5	68 ± 7
S/G2/M	11 ± 3	3 ± 2*

% of + cells	HMEC1 None	H3
G0	8 ± 3	31 ± 2*
G1	85 ± 6	59 ± 5*
S/G2/M	6 ± 4	10 ± 3

Fig. 3. Effect of histones on proliferation of endothelial cells. (A) Cells were treated with histones at the indicated concentration and proliferation was analyzed by pNPP assay after 48 h ($n = 5$). (B and C) Cells were treated with histones (2 μM) for 48 h and cell cycle progression was analyzed by Ki-67 antigen and propidium iodide double staining and flow cytometry ($n = 4$) ($n = 3$). *$P < 0.05$ vs. None, one-way ANOVA.

but not 1 μM (data not shown). Similar results were obtained in mature endothelial cells treated with H3 (Fig. 3C). These data indicate that these three histones inhibit endothelial cell proliferation through induction of cell cycle arrest.

Histones reduce endothelial cell migration

The extent of ECFC proliferation and migration into the cell monolayer wounded area was significantly impaired by H2B, H3 and H4 (1 μM), but not H1 and H2A (Fig. 4A). Nevertheless, a higher but still innocuous concentration of H1 and H2A (2 μM) exerted an effective inhibition (Fig. 4A). When this effect was compared with mature endothelial cells, notably, the percentage of wound closure was similar in untreated ECFCs, HUVECs and HMEC1 (55 ± 09%, 62 ± 05% and 60 ± 05%, respectively) and H3 pretreatment similarly impaired this process in all cells (Fig. 4B). In the presence of cycloheximide, H2B, H3 and H4 exerted a similar inhibitory action in ECFCs (Fig. 4C), demonstrating that, despite their antiproliferative effect, these three histones also inhibited cell migration. These results were confirmed by using transwell cell culture inserts, where ECFC chemotaxis in response to EGM2 or SDF1 was significantly reduced by 1 μM H2B, H3 and H4 (Fig. 4D).

Histones impair cord-like structure formation through p38 activation

The formation of cord-like structures by ECFCs was also markedly inhibited by H2B, H3 and H4, but not by H1 and H2A (Fig. 5A). The comparison between different endothelial cell types showed that untreated ECFCs, HUVECs and HMEC1 displayed 117 ± 15, 72 ± 12 and 73 ± 09 branch points after 18 h, respectively, and the three of them were equally sensitive to H3 (Fig. 5B). A higher concentration (2 μM) of H1 and H2A failed to modify the number of branch points displayed by ECFCs (data not shown). Of note, histone-induced impairment of tubulogenesis was not affected by Z-VAD-fmk (data not shown), suggesting that this effect is independent of caspase activation and the toxic action of histone. As others [35] and we [28] have previously demonstrated that p38 is an inhibitory pathway of ECFC-mediated tubulogenesis, in the next experiments we explored whether the activation of this kinase is responsible for the histone-induced antiangiogenic effect. Our results showed that the extent of tubulogenesis inhibition mediated by each histone correlated with their individual capacity for activating or phosphorylating p38 (Fig. 5C). Moreover, tubulogenesis impairment mediated by histones was reduced significantly in the presence of the p38 inhibitor SB203580 (Fig. 5D).

Histones reduce blood vessel formation in vivo

To examine the *in vivo* relevance of our findings, the antiangiogenic activity of histone was evaluated using the quail chorioallantoic membrane (CAM) assay, a specific angiogenesis model [31]. Filter paper disks impregnated with EGM2 containing H2B, H3, H4 (1 μM) or vehicle (control) were placed on CAMs for 48 h. Our results showed that H2B, H3 and H4 drastically decreased the number of vascular branch points compared with control values (Fig. 6), demonstrating that histone-mediated antiangiogenic effects are relevant not only *in vitro* but also *in vivo*.

Molecules involved in histone-endothelial cell interaction

Due to their basic nature and positive charge, histones can be neutralized by highly negative charged molecules such as heparin [16]. As expected, we observed that cell death was fully suppressed when ECFCs were exposed to 4 μM of H2B, H3 or H4 in the presence of 100 μg mL^{-1} unfractionated heparin or low-molecular-weight heparin (Fig. 7A). In addition, tubulogenesis impairment mediated by 1 μM H2B, H3 or H4 was completely prevented by all heparins, each at 25 μg mL^{-1} (Fig. 7B). Heparin

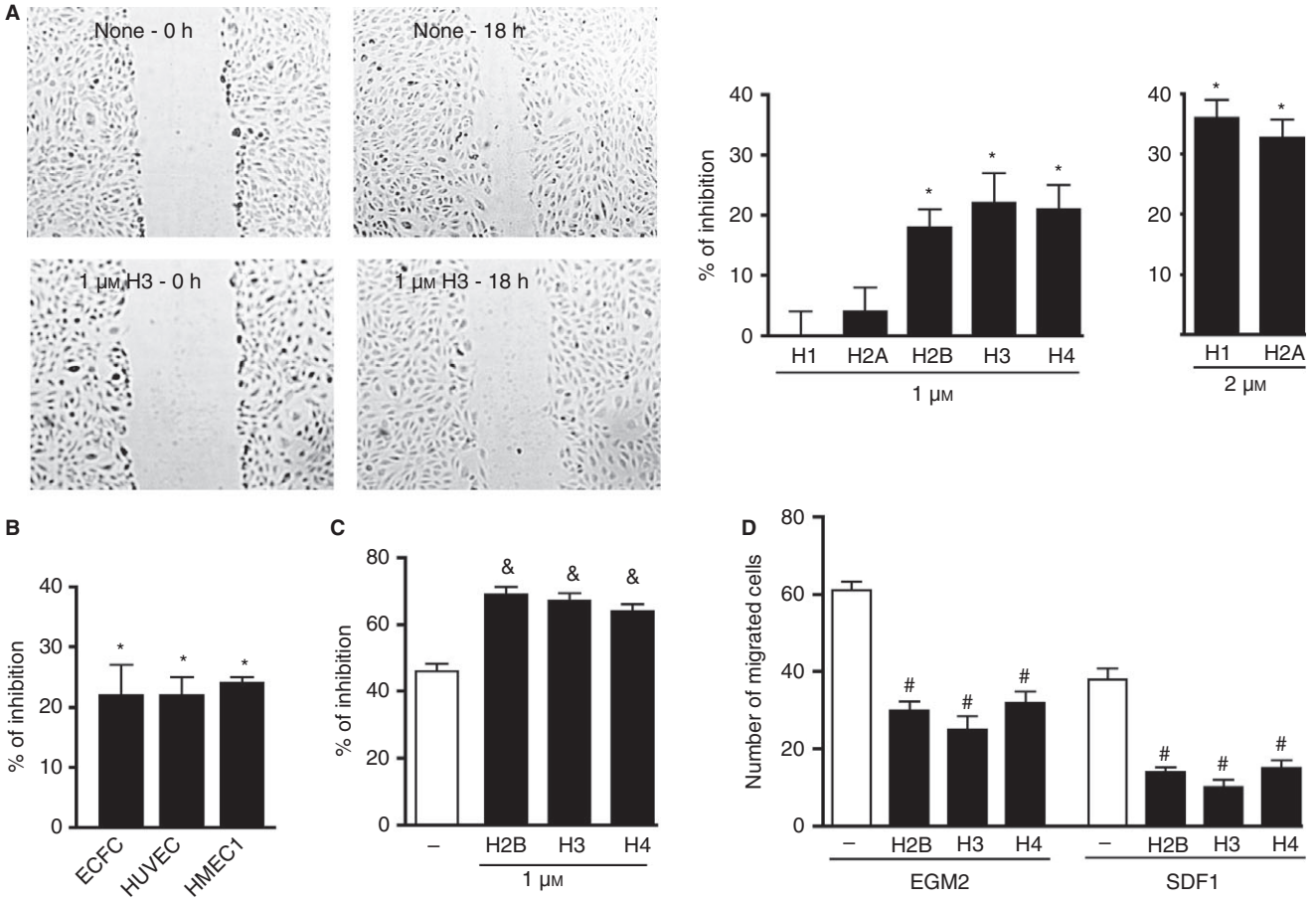


Fig. 4. Effect of histones on cell migration. (A) Confluent endothelial colony-forming cell (ECFC) monolayers were wounded and then treated with histones. The extent of cell migration and proliferation into the wounded area was calculated by analyzing images at 0 and 18 h with ImageJ software. Images represent five independent experiments. Results are expressed as percentage of inhibition relative to untreated controls (None). (B) The extent of cell migration and proliferation was compared between ECFCs, human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells 1 (HMEC1) treated with H3 (1 μM). (C) ECFC migration was also evaluated in the presence of 1 μg mL^{-1} cycloheximide ($n = 4$). (D) ECFCs were treated with H2B, H3 and H4 (1 μM) and migration in response to endothelial growth medium 2 (EGM2) or stromal cell-derived factor 1 (SDF1) (20 ng mL^{-1}) was determined by using transwell inserts. The number of migrated cells was counted under the light microscope ($n = 3$). $^{*}P < 0.05$ vs. None; $^{\&}P < 0.05$ vs. cycloheximide alone; $^{\#}P < 0.05$ vs. EGM2 or SDF1 alone, one-way ANOVA.

alone showed no effects (data not shown). Furthermore, histone-mediated toxic and antiangiogenic effects were significantly reduced by a neutralizing antibody against histones (Figure S2).

As we have previously demonstrated that the effect of some histones on platelet function is mediated, in part, by TLR2 and TLR4 [36,37] and HMEC1 toxicity mediated by calf thymus histones is mediated by TLR4 [18], we next evaluated whether these receptors were involved in the effects of histone on ECFCs. ECFC pretreatment with equal amounts of blocking antibodies against TLR2 or TLR4 (20 μg mL^{-1}), but not control IgG, significantly reduced the impairment of survival and tubulogenesis mediated by histones (Fig. 7C and D, respectively). Interestingly, a higher concentration of each blocking antibody (40 μg mL^{-1}) showed a similar inhibitory effect; however, when both TLR2 and TLR4 blocking antibodies were used in combination (20 μg mL^{-1} each), a complete prevention of H3-mediated cytotoxicity and tubulogenesis disruption was observed (Fig. 7C and D, respectively). Heparins and TLR2/4 blockade also reduced H3-mediated toxicity and antiangiogenic effects in HUVECs and HMEC1 (Figure S3).

Neutrophil-derived histones also decrease ECFC survival and tubulogenesis

In order to analyze the effect of histones derived from a physiological source, histone release from neutrophils was induced by stimulation with monosodium urate crystals and supernatants containing neutrophil-derived histones were assessed. ECFCs were incubated with supernatants from unstimulated neutrophils (0.11 ± 0.03 μg mL^{-1}) or undiluted supernatants derived from activated neutrophils (0.88 ± 0.03 μg mL^{-1}) at 1 : 2 or 1 : 4 dilutions. While supernatants from unstimulated neutrophils failed to

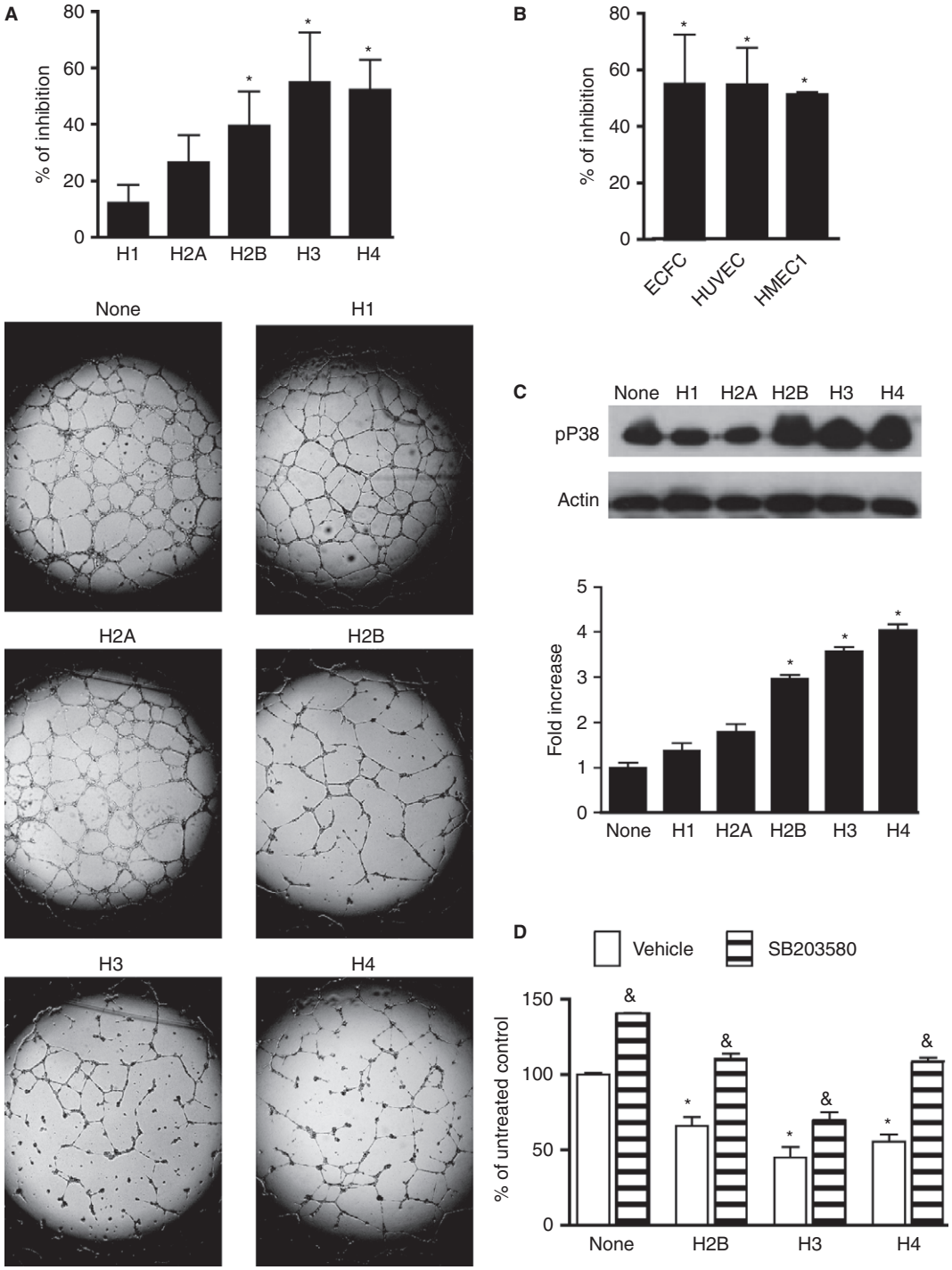


Fig. 5. Effect of histones on the formation of cord-like structures. (A) Endothelial colony-forming cells ECFCs were treated with histones at 1 μM. The number of branch points was calculated by analyzing images at 18 h with ImageJ software. Images represent six independent experiments. (B) Tubule formation was compared between ECFCs, human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells 1 (HMEC1) treated with H3 (1 μM). Results are expressed as percentage of inhibition relative to untreated controls (None). (C) ECFCs were treated with histones (1 μM) and the p38 phosphorylation levels were detected after 15 min by immunoblotting. Each membrane was reprobed with αActin antibody to calculate the relative integrated optic density (IOD) using Gel-Pro software ($n = 4$). (D) ECFCs were treated with histones (1 μM) in the presence or absence of p38 inhibitor (SB203580, 25 μM) and the number of branch points was determined after 18 h ($n = 3$). $^{*}P < 0.05$ vs. None; $^{\&}P < 0.05$ vs. histone without SB203580, one-way ANOVA.

modify ECFC viability, those from activated neutrophils promoted cell death in a concentration-dependent manner (Fig. 8A). Tubulogenesis was also significantly impaired by a non-cytotoxic dilution of activated neutrophil supernatants (1 : 4 dilution) (Fig. 8B). The involvement of histones in both processes was confirmed by the

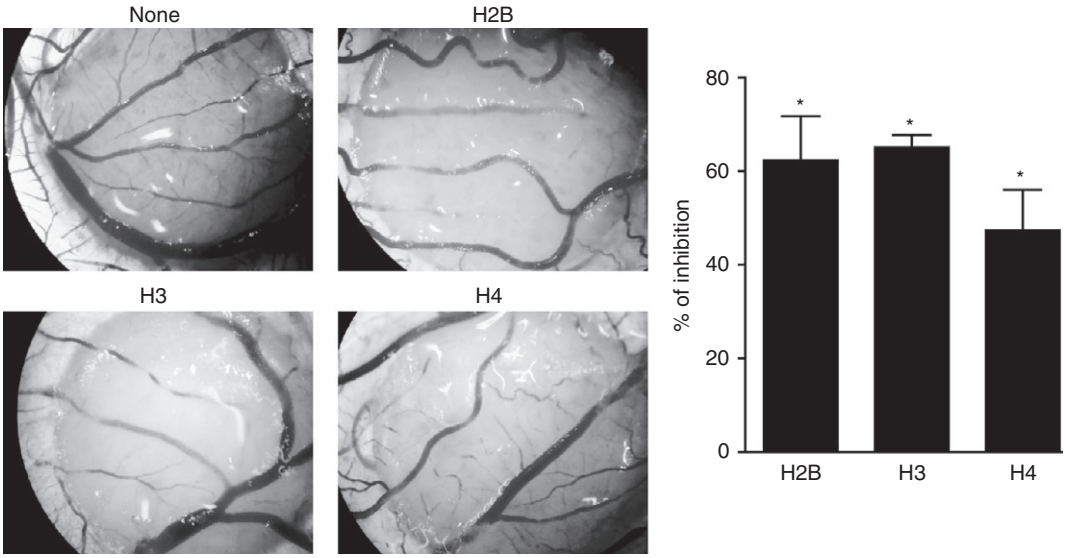


Fig. 6. Effect of histones on *in vivo* blood vessel formation. Filter paper disks impregnated with endothelial growth medium 2 (EGM2) containing each histone (1 μM) or vehicle (None) were placed on quail chorioallantoic membranes (CAMs) for 48 h. The number of blood vessel branch points was counted within the area encompassing the entire paper disk. Images were taken under a stereomicroscope and represent five independent experiments. Original magnification 30×. *$P < 0.05$ vs. None.

addition of heparin or the neutralizing antibody against histones, which partially reversed the deleterious effect on ECFC survival and tubule formation (Fig. 8A and B).

Discussion

We have demonstrated that human recombinant histones triggered both apoptosis and pyroptosis in late outgrowth endothelial progenitor cells and significantly inhibited their angiogenic responses, such as proliferation, migration/chemotaxis and cord-like structure formation. Moreover, histone-mediated antiangiogenic effects were also observed in an *in vivo* model of blood vessel formation on the quail chorioallantoic membrane. Their cytotoxic and antiangiogenic effects were similar in ECFCs and mature endothelial cells, but not in other cell types, and disappeared after addition of heparin and TLR2/TLR4 blockade. Moreover, our results showing that heparin and a histone blocking antibody partially reduced cell death and impairment of tubulogenesis mediated by neutrophil-derived histones indicate that our results were mimicked by histones from a physiological source.

The cytotoxic properties of histones have been extensively reported *in vivo* and *in vitro* in different cell types, including mature endothelial cells from different sources [2,20,21,38,39]. Most of these studies showed that endothelial cell viability, as measured by PI staining, is decreased by a mixture of histones derived from calf thymus, which show many differences to human histones, as discussed throughout this section. In the present work, using human recombinant individual histones, we confirmed and further extended these observations in mature endothelial cells from macro- and microvascular beds (HUVECs and HMEC1, respectively) through both the analysis of nuclear morphology by fluorescence microscopy and phosphatidylserine exposure by flow cytometry, and we described for the first time that histone-mediated cytotoxicity also affects ECFCs. In addition, we observed that toxicity was higher in endothelial cells than in non-endothelial cells, suggesting that the endothelium is particularly sensitive to extracellular histones.

Regarding the effectiveness of individual histones in inducing cell death, the study by Abrams *et al.* [14] compared human histones and found that they exerted a similar potency in reducing the viability of the human endothelial cell line EA.hy926. On the other hand, Xu *et al.* [2] showed that calf thymus-derived H1, H2A and H2B barely induced toxicity of EA.hy926 and HUVECs, while H3 and H4 were the most potent. In agreement with this study, we observed a slight damage after H1 and H2A treatments as well as a powerful deleterious effect of H3 and H4 but, in contrast, we found that H2B induced a strong cytotoxic action on ECFCs, HMEC1 and HUVECs. The variances in cytotoxicity between individual histones might be associated with the different amino-acid sequences of the histones, and thus their different tertiary structures, which could cause differences in their interactions with endothelial cell receptors. In this sense, although the four core histones (H2A, H2B, H3 and H4) have similar three-dimensional structures, the histone fold regions of H2B, H3 and H4 exhibit the greatest similarity and H2A differs considerably from the other three histones [40]. Furthermore, the linker histone H1 does not contain the histone fold motif [41]. Alternatively, the difference in potency among these proteins observed

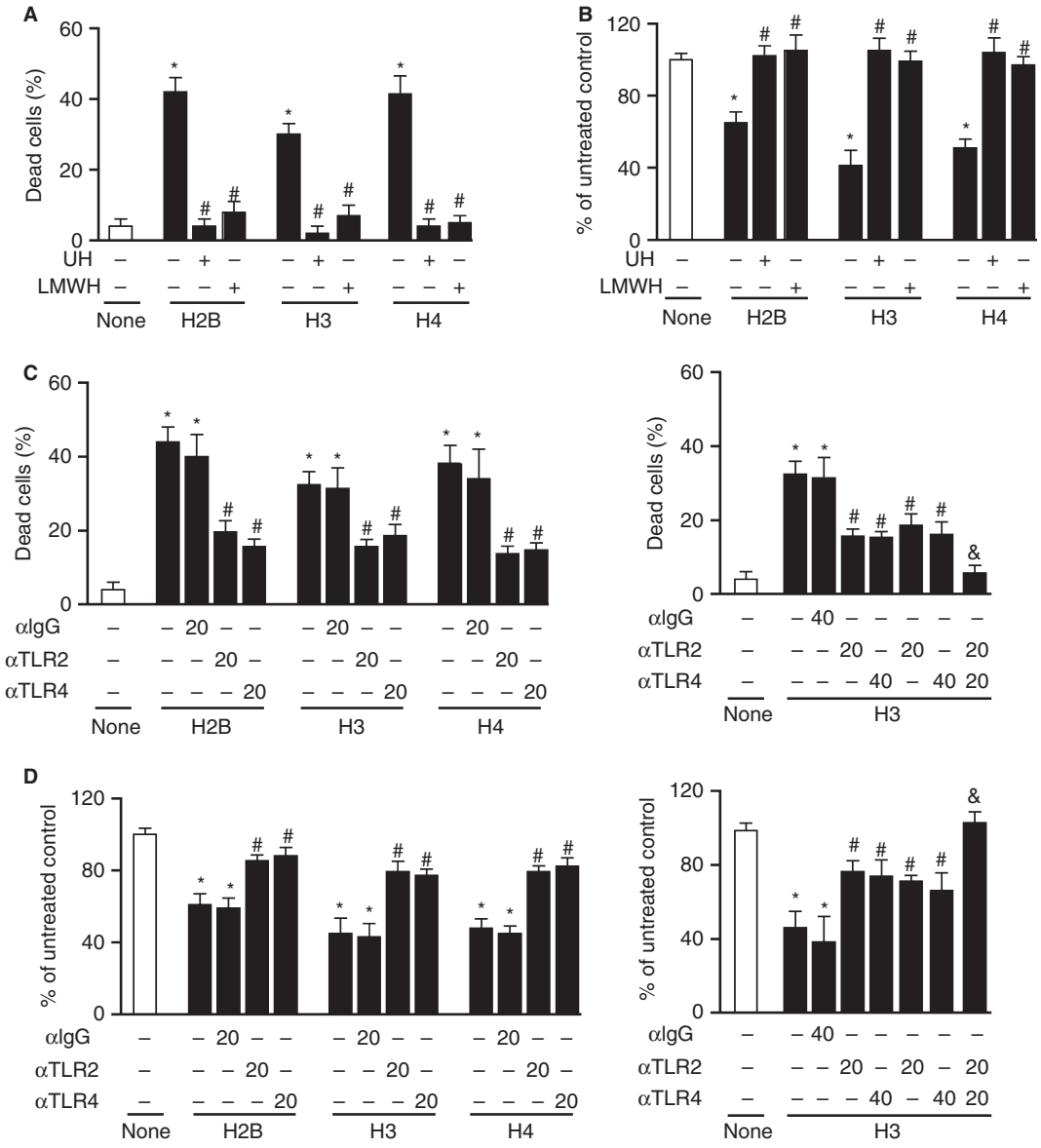


Fig. 7. Molecules involved in histone-endothelial colony-forming cell (ECFC) interaction. (A) ECFCs were treated with histones (4 μM) in the presence or absence of unfractioned heparin (UH) or low-molecular-weight heparin (LMWH) (100 μg mL^{-1}) and the percentage of cell death was determined by fluorescence microscopy after 24 h ($n = 3$). (B) ECFCs were treated with histones (1 μM) in the presence or absence of the different heparins (20 μg mL^{-1}) and tubulogenesis was assessed after 18 h ($n = 4$). (C) ECFCs were treated with histones (4 μM) in the presence or absence of αTLR2, αTLR4 or αIgG (20 or 40 μg mL^{-1}) and the percentage of cell death was determined by fluorescence microscopy after 24 h ($n = 3$). (D) ECFCs were treated with histones (1 μM) in the presence or absence of αTLR2, αTLR4 or αIgG (20 or 40 μg mL^{-1}) and tubulogenesis was assessed after 18 h ($n = 4$). *$P < 0.05$ vs. None; $^{\#}P < 0.05$ vs. histones alone or plus αIgG; $^{\&}P < 0.05$ vs. histone plus αTLR2 or αTLR4 alone, one-way ANOVA.

in different disease models might be due to a selective histone release in each situation; however, this interesting issue remains to be investigated.

The analysis of nuclear morphology showed that histones trigger both necrotic-like cells, which were rapidly evidenced at 2 h post-treatment and remained constant after 24 h, and apoptotic-like cells, which were only observed after 24 h. In line with our findings, it has been previously observed mixed populations of dead cells have been previously observed after treatment with pore-forming substances or membrane depolarization [42,43]. Regarding the mechanisms of histone-mediated cell death, caspase-3 was activated in a small percentage of ECFCs, which correlated with the percentage of apoptosis observed by nuclear morphology examination. In this sense, apoptosis induction by histones has been described before in neurons, which undergo mitochondrial apoptosis after H1 treatment [44]. In addition, TUNEL-positive renal cells were significantly reduced in anti-histone-treated mice in a model of aggravated kidney injury [17]. We also showed that the

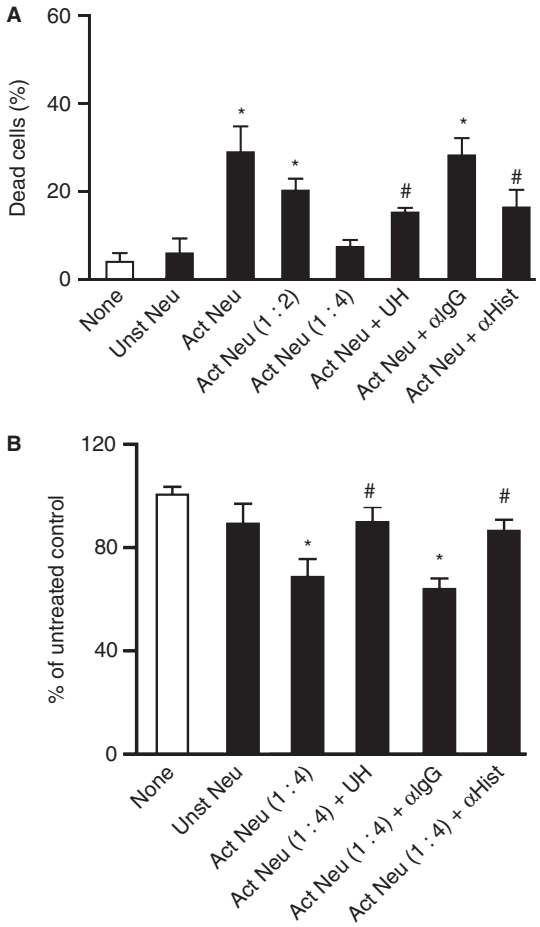


Fig. 8. Effect of neutrophil-derived histones on endothelial colony-forming cell (ECFC) survival and tubulogenesis. ECFCs were incubated with unstimulated neutrophil supernatants (Unst Neu), undiluted activated neutrophil supernatants (Act Neu), 1 : 2 dilution or 1 : 4 dilution. Supernatants were incubated in the presence or absence of unfractioned heparin (UH, 100 μg mL^{-1}), a blocking antibody against histones (αHist) or αIgG (50 μg mL^{-1}). (A) Cell viability and (B) tubulogenesis were assessed after 24 and 18 h, respectively ($n = 3$). $^{*}P < 0.05$ vs. None or Unst Neu; $^{\#}P < 0.05$ vs. Act Neu alone or plus αIgG, one-way ANOVA.

pan-caspase inhibitor Z-VAD-fmk suppressed not only apoptosis but also much of the necrotic-like process. Considering that this process was in addition prevented by the caspase-1 inhibitor Ac-YVAD-cmk at similar levels to Z-VAD-fmk, it seems that the cell population that shares nuclear morphology features with necrosis in fact undergoes pyroptosis upon histone treatment. It has been demonstrated in neutrophils that pore-forming substances promoted a programmed necrosis that involved NLRP3 inflammasome [43] and depended on caspase activation as Z-VAD-fmk fully blocked this process. By contrast, Z-VAD-fmk failed to inhibit dermal and lung endothelial cytotoxicity triggered by *Plasmodium falciparum* histones [22]. Thus, histone-mediated cell death appears to be a complex process that might depend on the nature of histone source, the cell type or model used.

To the best of our knowledge, this is the first report showing that histones inhibit angiogenic responses of mature endothelial cells and ECFCs. Our findings are relevant because recent evidence demonstrated that NET and histone levels are highly augmented in damaged tissues where vascular repair and formation of new vessels are key events, such as ischemic areas during reperfusion [11] and wounds of diabetic mice, where healing was delayed [12]. We found that H2B, H3 and H4 induced cell cycle arrest and all histones reduced EGM2- and SDF1-driven migration of ECFCs as well as mature endothelial cells. Moreover, H2B, H3 and H4 exerted an inhibitory action on ECFC cord-like structure formation, which correlated with p38 phosphorylation levels triggered by each histone and was significantly reduced by the p38 inhibitor SB203580, indicating that histones impaired tubulogenesis through the activation of p38, a recognized inhibitory pathway of tubulogenesis [35,45–47]. These results are in agreement with Gillrie *et al*. [22], who demonstrated that *P. falciparum*-derived histones activate endothelial p38, and in line with our previous findings showing that up-regulation of microvessel formation by acidic preconditioning in ECFCs is mediated in part by p38 suppression [28]. Interestingly, impairment of all angiogenic responses was observed at a concentration of histones that failed to induce cell death, suggesting that histones might be considered for treatments of diseases that aim to achieve endothelial quiescence and antiangiogenic effects with minimal cytotoxicity (e.g. cancer and glaucoma). Moreover, our data showing that ECFCs were targeted by the toxicity and antiangiogenic action of histones are of great relevance because ischemia, or other stressful conditions, damages tissues so severely that the regeneration of these areas largely depends on the migration of endogenous endothelial progenitor cells from bone marrow or cell therapy. In both cases, avoiding the harmful effect of histones could greatly help successful regeneration.

Using the quail chorioallantoic membrane, we demonstrated that histone-mediated antiangiogenic effects are relevant not only *in vitro* but also *in vivo*. Of note, both toxic and antiangiogenic effects of these proteins were observed at concentrations that can be attained *in vivo* in various species and diseases [2,20,48]. In humans, it has been reported that in patients with severe blunt trauma, total non-degraded circulating histone levels ranged from 10 to 230 μg mL^{-1} within 4 h of injury, which was significantly higher than in healthy donors (~2.3 μg mL^{-1}). Indeed, serum from these patients was toxic to cultured endothelial cells once histone levels exceeded 50 μg mL^{-1} [20]. In agreement with these findings, we here show that concentrations starting from 15 μg mL^{-1} histones (~1 μM) are enough to inhibit endothelial cell angiogenic responses both *in vitro* and *in vivo*, and above 45 μg mL^{-1} histones (~3 μM) the effect is cytotoxic. Moreover, histone levels at the site of injury are expected

to be higher than in plasma although to date there are no studies reporting the local concentration of histones, possibly because it is difficult to measure due to their association with cells [49], DNA and alterations in blood flow [50].

The molecules interacting with or mediating the effects of histones on the different cell types have not yet been fully identified. Due to their basic nature and positive charge, histones can be neutralized by highly negative charged molecules such as heparin, resulting in a complete inhibition of their cytotoxic properties, as shown in an *in vivo* sepsis model [51,52]. Similar to these studies, we observed a full suppression of histone-induced cell death by both unfractioned and low-molecular-weight heparins, which exerted a comparable inhibitory effect. In addition, all heparins completely abrogated histone-mediated impairment of tubulogenesis.

As to the mechanism involved in histone-mediated endothelial cell toxicity, it has been proposed to be a result of increased cell membrane permeability [14,17,22] as well as the activation of TLR2 and TLR4 [17–19, 53]. In agreement with these findings, we here show that both TLR2 and TLR4 are mediators of histone-induced endothelial cell death. In contrast, Abrams *et al.* [14] observed that TLR2 and TLR4 blocking failed to prevent histone cytotoxicity, although they used a mixture of calf-thymus histones and the EA.hy926 cell line, which could account for the discrepancies between our results. We also demonstrated that TLR2 and TLR4 mediate histone antiangiogenic activity as blockade of these receptors significantly reduced the impairment of cord-like structure formation by these proteins. These data, together with those showing that heparin prevented tubulogenesis suppression mediated by histones, strongly support the use of heparin or TLR2/TLR4 blockade as potential therapeutic strategies to improve tissue regeneration, as they prevent cell damage and yet preserve tubule formation and, in the case of heparin, exert a well-known up-regulation of endothelial cell proliferation.

Altogether, the present findings lead to the question: are the different observations of heparin, p38 inhibition and TLR2/TLR4 neutralization mechanistically integrated? It has been demonstrated that heparin binds and forms complexes with histones in a charge-dependent manner, preventing them interacting with cell surfaces [16,51,52]. On the other hand, TLR signaling classically leads to MAPK p38 activation in many cell types, including progenitor and mature endothelial cells [54–56]. Therefore, it is conceivable that either TLR neutralization or heparin addition impair TLR histone interactions, resulting in a significant reduction of histone-mediated effects on endothelial cells. Nonetheless, more research is needed to clarify this issue.

In summary, our present findings show that histones triggered apoptosis and pyroptosis of ECFCs and reduced their angiogenic activity to a similar extent to that seen in mature endothelial cells. Histone-mediated antiangiogenic effects were also observed in an *in vivo* model of blood vessel formation. The fact that the cytotoxic and antiangiogenic effects of histones were fully suppressed by unfractioned and low-molecular-weight heparins and reduced by TLR2 and TLR4 blocking antibodies demonstrates that heparin administration and TLR2/TLR4 blockade are possible strategies for improving tissue regeneration.

Addendum

H. A. Mena conducted most of the experiments, interpreted the data and wrote the paper A. Carestia performed neutrophil experiments L. Scotti and F. Parborell performed the *in vivo* experiments and critically revised the study M. Schattner performed data analysis and critical revision of the intellectual content and S. Negrotto designed and directed the study, and carried out data analysis, critical writing and revision of the intellectual content.

Acknowledgements

This work was financially supported by ANPCyT grants (PICT 1393/10, 0612/13 and 0733/11). We thank the obstetrics services of Hospital Rivadavia (Buenos Aires) for providing umbilical cord and cord blood samples.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Kinetic study of histone-induced ECFC cell death.
Fig. S2. Histone-mediated effects were suppressed by a blocking antibody against histones.
Fig. S3. Molecules involved in the interaction between histones and HUVECs or HMEC1.

References

1 Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 1997; **389**: 251–60.
2 Xu J, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F, Esmon CT. Extracellular histones are major mediators of death in sepsis. *Nat Med* 2009; **15**: 1318–21.
3 Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Devinney R, Doig CJ, Green FH, Kubes P. Platelet TLR4 activates neutrophil extracellular

traps to ensnare bacteria in septic blood. *Nat Med* 2007; **13**: 463–9.
4 Cui BB, Tan CY, Schorn C, Tang HH, Liu Y, Zhao Y. Neutrophil extracellular traps in sterile inflammation: the story after dying? *Autoimmunity* 2012; **45**: 593–6.
5 Doring Y, Soehnlein O, Weber C. Neutrophils cast NETs in atherosclerosis: employing peptidylarginine deiminase as a therapeutic target. *Circ Res* 2014; **114**: 931–4.
6 Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, Punaro M, Baisch J, Guiducci C, Coffman RL, Barrat FJ, Banchereau J, Pascual V. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* 2011; **3**: 73ra20.
7 Knight JS, Kaplan MJ. Lupus neutrophils: 'NET' gain in understanding lupus pathogenesis. *Curr Opin Rheumatol* 2012; **24**: 441–50.
8 Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, Grone HJ, Brinkmann V, Jenne DE. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009; **15**: 623–5.
9 Demers M, Krause DS, Schatzberg D, Martinod K, Voorhees JR, Fuchs TA, Scadden DT, Wagner DD. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. *Proc Natl Acad Sci USA* 2012; **109**: 13076–81.
10 Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum Immunol* 2005; **66**: 1146–54.
11 Oklu R, Albadawi H, Jones JE, Yoo HJ, Watkins MT. Reduced hind limb ischemia-reperfusion injury in Toll-like receptor-4 mutant mice is associated with decreased neutrophil extracellular traps. *J Vasc Surg* 2013; **58**: 1627–36.
12 Wong SL, Demers M, Martinod K, Gallant M, Wang Y, Goldfine AB, Kahn CR, Wagner DD. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med* 2015; **21**: 815–9.
13 Zhao H, Bose S, Tuominen EK, Kinnunen PK. Interactions of histone H1 with phospholipids and comparison of its binding to giant liposomes and human leukemic T cells. *Biochemistry* 2004; **43**: 10192–202.
14 Abrams ST, Zhang N, Dart C, Wang SS, Thachil J, Guan Y, Wang G, Toh CH. Human CRP defends against the toxicity of circulating histones. *J Immunol* 2013; **191**: 2495–502.
15 Kleine TJ, Lewis PN, Lewis SA. Histone-induced damage of a mammalian epithelium: the role of protein and membrane structure. *Am J Physiol* 1997; **273**: C1925–36.
16 Gamberucci A, Fulceri R, Marcolongo P, Pralong WF, Benedetti A. Histones and basic polypeptides activate Ca2+/cation influx in various cell types. *Biochem J* 1998; **331**(Pt 2): 623–30.
17 Allam R, Scherbaum CR, Darisipudi MN, Mulay SR, Hagele H, Lichtnekert J, Hagemann JH, Rupanagudi KV, Ryu M, Schwarzenberger C, Hohenstein B, Hugo C, Uhl B, Reichel CA, Krombach F, Monestier M, Liapis H, Moreth K, Schaefer L, Anders HJ. Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. *J Am Soc Nephrol* 2012; **23**: 1375–88.
18 Ekaney M, Otto G, Sossdorf M, Sponholz C, Boehringer M, Loesche W, Rittirsch D, Wilharm A, Kurzai O, Bauer M, Claus R. Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation. *Crit Care* 2014; **18**: 543.
19 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**: 2626–31.
20 Abrams ST, Zhang N, Manson J, Liu T, Dart C, Baluwa F, Wang SS, Brohi K, Kipar A, Yu W, Wang G, Toh CH. Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med* 2013; **187**: 160–9.
21 Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, Lohmeyer J, Preissner KT. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS ONE* 2012; **7**: e32366.
22 Gillrie MR, Lee K, Gowda DC, Davis SP, Monestier M, Cui L, Hien TT, Day NP, Ho M. Plasmodium falciparum histones induce endothelial proinflammatory response and barrier dysfunction. *Am J Pathol* 2012; **180**: 1028–39.
23 Liman TG, Endres M. New vessels after stroke: postischemic neovascularization and regeneration. *Cerebrovasc Dis* 2012; **33**: 492–9.
24 Jujo K, Ii M, Losordo DW. Endothelial progenitor cells in neovascularization of infarcted myocardium. *J Mol Cell Cardiol* 2008; **45**: 530–44.
25 Critser PJ, Yoder MC. Endothelial colony-forming cell role in neoangiogenesis and tissue repair. *Curr Opin Organ Transplant* 2010; **15**: 68–72.
26 Demers M, Wagner DD. Neutrophil extracellular traps: a new link to cancer-associated thrombosis and potential implications for tumor progression. *Oncoimmunology* 2013; **2**: e22946.
27 Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; **52**: 2745–56.
28 Mena HA, Lokajczyk A, Dizier B, Strier SE, Voto LS, Boisson-Vidal C, Schattner M, Negrotto S. Acidic preconditioning improves the proangiogenic responses of endothelial colony forming cells. *Angiogenesis* 2014; **17**: 867–79.
29 Smadja DM, Bieche I, Uzan G, Bompais H, Muller L, Boisson-Vidal C, Vidaud M, Aiach M, Gaussem P. PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system. *Arterioscler Thromb Vasc Biol* 2005; **25**: 2321–7.
30 Negrotto S, Pacienza N, D'Atri LP, Pozner RG, Malaver E, Torres O, Lazzari MA, Gomez RM, Schattner M. Activation of cyclic AMP pathway prevents CD34(+) cell apoptosis. *Exp Hematol* 2006; **34**: 1420–8.
31 Scotti L, Abramovich D, Pascuali N, Durand LH, Irusta G, de Zuniga I, Tesone M, Parborell F. Inhibition of angiopoietin-1 (ANGPT1) affects vascular integrity in ovarian hyperstimulation syndrome (OHSS). *Reprod Fertil Dev* 2014. doi:10.1071/RD13356.
32 Lapponi MJ, Carestia A, Landoni VI, Rivadeneyra L, Etulain J, Negrotto S, Pozner RG, Schattner M. Regulation of neutrophil extracellular trap formation by anti-inflammatory drugs. *J Pharmacol Exp Ther* 2013; **345**: 430–7.
33 Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* 2014; **15**: 135–47.
34 Casson CN, Shin S. Inflammasome-mediated cell death in response to bacterial pathogens that access the host cell cytosol: lessons from legionella pneumophila. *Front Cell Infect Microbiol* 2013; **3**: 111.
35 Seeger FH, Haendeler J, Walter DH, Rochwalsky U, Reinhold J, Urbich C, Rossig L, Corbaz A, Chvatchko Y, Zeiher AM, Dimmeler S. p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. *Circulation* 2005; **111**: 1184–91.
36 Carestia A, Rivadeneyra L, Romaniuk MA, Fondevila C, Negrotto S, Schattner M. Functional responses and molecular mechanisms involved in histone-mediated platelet activation. *Thromb Haemost* 2013; **110**: 1035–45.
37 Semeraro F, Ammollo CT, Morrissey JH, Dale GL, Friese P, Esmon NL, Esmon CT. Extracellular histones promote thrombin

generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* 2011; **118**: 1952–61.
38 Gillrie MR, Krishnegowda G, Lee K, Buret AG, Robbins SM, Looareesuwan S, Gowda DC, Ho M. Src-family kinase dependent disruption of endothelial barrier function by Plasmodium falciparum merozoite proteins. *Blood* 2007; **110**: 3426–35.
39 Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, Liao X, Billiar T, Xu J, Esmon CT, Tsung A. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology* 2011; **54**: 999–1008.
40 Arents G, Moudrianakis EN. The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. *Proc Natl Acad Sci USA* 1995; **92**: 11170–4.
41 Marino-Ramirez L, Hsu B, Baxevanis AD, Landsman D. The Histone Database: a comprehensive resource for histones and histone fold-containing proteins. *Proteins* 2006; **62**: 838–42.
42 Xiao AY, Wei L, Xia S, Rothman S, Yu SP. Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. *J Neurosci* 2002; **22**: 1350–62.
43 Satoh T, Kambe N, Matsue H. NLRP3 activation induces ASC-dependent programmed necrotic cell death, which leads to neutrophilic inflammation. *Cell Death Dis* 2013; **4**: e644.
44 Gilthorpe JD, Oozeer F, Nash J, Calvo M, Bennett DL, Lumsden A, Pini A. Extracellular histone H1 is neurotoxic and drives a pro-inflammatory response in microglia. *F1000Res* 2013; **2**: 148.
45 Wu Y, Wang Q, Cheng L, Wang J, Lu G. Effect of oxidized low-density lipoprotein on survival and function of endothelial progenitor cell mediated by p38 signal pathway. *J Cardiovasc Pharmacol* 2009; **53**: 151–6.
46 Kuki S, Imanishi T, Kobayashi K, Matsuo Y, Obana M, Akasaka T. Hyperglycemia accelerated endothelial progenitor cell senescence via the activation of p38 mitogen-activated protein kinase. *Circ J* 2006; **70**: 1076–81.
47 Huh JE, Nam DW, Baek YH, Kang JW, Park DS, Choi DY, Lee JD. Formononetin accelerates wound repair by the regulation of early growth response factor-1 transcription factor through the phosphorylation of the ERK and p38 MAPK pathways. *Int Immunopharmacol* 2011; **11**: 46–54.
48 Bosmann M, Grailer JJ, Ruemmler R, Russkamp NF, Zetoune FS, Sarma JV, Standiford TJ, Ward PA. Extracellular histones are essential effectors of C5aR- and C5L2-mediated tissue damage and inflammation in acute lung injury. *FASEB J* 2013; **27**: 5010–21.
49 Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, Aarden LA. Nucleosome-releasing factor: a new role for factor VII-activating protease (FSAP). *FASEB J* 2008; **22**: 4077–84.
50 Ammollo CT, Semeraro F, Xu J, Esmon NL, Esmon CT. Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. *J Thromb Haemost* 2011; **9**: 1795–803.
51 Wildhagen KC, de Garcia Frutos P, Reutelingsperger CP, Schrijver R, Areste C, Ortega-Gomez A, Deckers NM, Hemker HC, Soehnlein O, Nicolaes GA. Nonanticoagulant heparin prevents histone-mediated cytotoxicity in vitro and improves survival in sepsis. *Blood* 2014; **123**: 1098–101.
52 Fuchs TA, Bhandari AA, Wagner DD. Histones induce rapid and profound thrombocytopenia in mice. *Blood* 2011; **118**: 3708–14.
53 Kumar SV, Kulkarni OP, Mulay SR, Darisipudi MN, Romoli S, Thomasova D, Scherbaum CR, Hohenstein B, Hugo C, Muller S, Liapis H, Anders HJ. Neutrophil extracellular trap-related extracellular histones cause vascular necrosis in severe GN. *J Am Soc Nephrol* 2015; **26**: 2399–413.
54 Banerjee A, Gerondakis S. Coordinating TLR-activated signaling pathways in cells of the immune system. *Immunol Cell Biol* 2007; **85**: 420–4.
55 He J, Xiao Z, Chen X, Chen M, Fang L, Yang M, Lv Q, Li Y, Li G, Hu J, Xie X. The expression of functional Toll-like receptor 4 is associated with proliferation and maintenance of stem cell phenotype in endothelial progenitor cells (EPCs). *J Cell Biochem* 2010; **111**: 179–86.
56 Wilhelmsen K, Mesa KR, Lucero J, Xu F, Hellman J. ERK5 protein promotes, whereas MEK1 protein differentially regulates, the Toll-like receptor 2 protein-dependent activation of human endothelial cells and monocytes. *J Biol Chem* 2012; **287**: 26478–94.