Factor VII-activating protease

Unraveling the release and regulation of dead cell nuclear damps

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CHAPTER 2

Histones, cell-free DNA, or nucleosomes: the immunity of extracellular chromatin unraveled

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ABSTRACT

In inflammation, extensive cell death may occur, which results in the release of chromatin components into the extracellular environment. Individually, the purified chromatin components double stranded (ds)DNA and histones have been demonstrated to display various pro-inflammatory effects, both *in vitro* and *in vivo*. However, DNA and histones are organized in nucleosomes in the nucleus, and released as such in inflammation. The extracellular effects of nucleosomes have not been studied as extensively as the separate effects of histones and dsDNA, but there appear to be some marked differences. Moreover, additional pathways seem to be involved to bring about their pro-inflammatory extracellular effects. Remarkably, little distinction between the different forms in which histones circulate has been made throughout literature. This is partly due to the limitations of existing techniques to differentiate between histones in their free or DNA-bound form. Here, we review the current understanding of immunostimulation by histones, dsDNA, and nucleosomes, and discuss the importance of techniques that in their detection differentiate between these different chromatin components.

INTRODUCTION

Upon infection, pathogen-associated molecular patterns (PAMPs) are able to initiate an inflammatory response in the host through the activation of pattern recognition receptors (PRRs), including the toll-like receptors (TLRs). PAMPs that are recognized by TLRs include lipopolysaccharides (LPS) derived from the cell wall of gram-negative bacteria, flagellin, double stranded RNA, unmethylated CpG sequences in DNA molecules, and others. The specific recognition of these evolutionarily distant, yet often conserved exogenous molecules by the immune system was proposed in 1989[1], alongside the postulation that the immune system distinguishes between self and non-self upon the initiation of an immune response. In 1994, this view was challenged by the danger-model, which suggested that the immune system does not distinguish between self and non-self, but instead primarily recognizes molecules released from damaged or dying cells, otherwise known as damage-associated molecular patterns (DAMPs), to detect danger[2]. These ideas were later combined in a model which proposed that the hydrophobic portions exposed on endogenous DAMPs as well as on exogenous PAMPs form the essential patterns that are shared and recognized by the immune system[3].

Indeed, various DAMPs that are released upon cellular damage or cell death are efficient inducers of inflammation. Well known nuclear DAMPs are histones and DNA, which are present in the nucleus in the form of a nucleosome complex. Cell-free histones and DNA have been found to independently of each other trigger either TLR-2 and -4 (histones)[4] or TLR-9 (DNA)[5]. Notably, various pro-inflammatory extracellular effects have been ascribed to these nuclear DAMPs, but several of these effects appear to be dictated by the form in which these molecules are present; histones may circulate freely or in complex with DNA in the form of a nucleosome. Remarkably, throughout the literature very little distinction between the different forms of histones and DNA in clinical samples is made. Moreover, in some articles the terms histones and nucleosomes are used interchangeably. In this review we introduce the currently known extracellular effects of cell-free histones and DNA, and compare the separate effects of each to the effects that are attributable to their complex in the form of extracellular nucleosomes. Furthermore, given that the extracellular effects of these molecules drastically differ, we provide an overview of the current techniques available to detect and quantify

cell-free histones, DNA, and nucleosomes in body fluids, and methods to distinguish between the presence of these molecules. This review highlights the importance of distinguishing between free histones and histones as part of a nucleosome complex and addresses a topic in nuclear DAMP research that deserves more attention.

Histone induced inflammation

Histones are highly basic proteins rich in arginine and lysine that form the building blocks of chromatin in eukaryotic cells and are highly conserved amongst species. In humans, an octamer consisting of two dimers of histone H2A and H2B and a tetramer of histone H3 and H4 forms a core around which 147 bp of DNA is wrapped ±1.67 times. The formed complex is referred to as a nucleosome[6]. The nucleosome structure plays an essential role in regulating gene transcription and facilitates efficient higher-order chromatin compaction. A fifth histone subtype, the linker histone H1, resides at the stretch of linker DNA that connects two nucleosomes and is essential in regulating chromatin compaction and transcriptional access to the nucleosome[7]. In addition to their vital intracellular functions, histones are widely recognized to bear important pro-inflammatory functions upon their release from the nucleus into the extracellular environment[8,9].

In 2009, Xu *et al.* demonstrated that intravenous injection of histones in mice was lethal within minutes, whilst anti-histone antibodies reduced mortality in LPS, TNF-α, and cecal ligation and puncture models of sepsis[10]. *In vitro*, it was shown that histones were cytotoxic when added to cultured endothelial cells. In a follow-up study, the authors demonstrated that, in addition to the cytotoxic effects, injection of sublethal doses of histones in mice resulted in high levels of TNF-α, IL-6, and IL-10, which was abrogated when using TLR-4 knock-out (KO) mice, but not in TLR-2 KO mice[4]. In addition, it was shown that histones signal via both TLR-4 and TLR-2 through the use of specific TLR-transfected HEK cells. Thereafter, these results were corroborated and extended by Allam *et al.*, who demonstrated that histones were cytotoxic to renal endothelial cells and tubular epithelial cells *in vitro*, stimulated bone marrow-derived dendritic cells (BMDCs) in a TLR2- and -4 dependent manner, and induced inflammation *in vivo* in a TLR-2 and -4 dependent manner[11]. In addition to studies on the effects of histones mediated via TLR-2 and TLR-4

signalling, Huang *et al.* demonstrated that TLR-9 KO mice were protected from histone-mediated ischemia/reperfusion (I/R) injury. It was found that exogenous histone infusion exacerbated I/R injury in wild-type (WT), but not in TLR-9 KO mice. The authors deduced that the exogenous histones likely served as a cofactor that amplified TLR-9 mediated signaling brought about by circulating DNA released from dying cells, although direct evidence for the role of endogenous DNA in the *in vivo* model was not presented. Another observation that further supports the induction of inflammation by histones was reported by Abrams *et al.*, who found that neutrophils that were incubated with purified histones released MPO and were activated to form neutrophil extracellular traps[12]. However, the direct involvement of TLRs in this process was not investigated. Important to note is that when investigating the role of TLR-9, the translation from mice to men is troublesome as TLR-9 can be found in macrophages, myeloid DCs, activated T-cells, plasmacytoid DCs, B-cells, and neutrophils in mice, whilst in humans TLR-9 expression is limited to plasmacytoid DCs, B-cells, and neutrophils. This results in a radically different inflammatory response towards TLR-9 agonists in mice compared to humans[13], which complicates nuclear DAMP research in animal models.

To understand the mechanisms involved in histone induced cytotoxicity, several observations reported in the literature provide insight. FITC-labeled histones were shown to bind to the surface of cultured EA.hy926 endothelial cells and subsequently induced an influx of Ca^{2+}, which resulted in cell lysis[12]. Likely, the affinity of histones for phosphodiester bonds does not only ensure their avid binding to DNA, but also to phosphodiester bonds in phospholipids, resulting in the integration of histones into the plasma membrane. The glycocalyx covering the cell surface appears to determine the sensitivity of different cell types to histone-induced cytotoxicity. Chaaban *et al.* demonstrated that CHO cells deficient in heparan-sulfate or with inhibited hyaluronan production, were markedly more sensitive to histone-induced cytotoxicity[14]. This suggests that the glycocalyx serves as a protective layer to prevent histone insertion into the plasma membrane. Notably, cell death was not inhibited by TLR-2 and TLR-4 neutralizing antibodies, indicating that these receptors are not involved in histone induced cytotoxicity in that experimental system. However, in a study by Ekaney *et al.*, a neutralizing anti-TLR-4 antibody did inhibit histone induced cytotoxicity of human microvascular endothelial

cells. Whether these differences result from differences in the cell lines used, a different inhibitory anti-TLR-4 antibody used, or distinct mechanisms of cell death is unclear.

An explanation for the discrepant sensitivity of histone cytotoxicity to neutralizing anti-TLR antibodies may be derived from two studies in which histones were found to activate the NLRP3 inflammasome, either in LPS-primed BMDCs[15], and in Kupffer cells upon liver ischemia/reperfusion injury[16]. Inflammasome activation may result in caspase-1 and caspase-11 dependent pyroptotic cell death in certain cell types (see review[17]), although so far the involvement of specific NLRP3 activation has not been linked directly to pyroptosis yet. Nevertheless, we hypothesize that TLR-mediated inflammasome activation by histones may result in pyroptotic cell death. Further studies are required to reveal whether this mechanism exists or not, and whether it proves to be another mechanism of histone-mediated cell death, in addition to cell death induced by plasma membrane integration of histones. Although inflammasome activation by histones was observed in TLR-4 deficient Kupffer cells, the involvement of other TLRs remains unexplored, as are the different cell types able to execute pyroptosis. For an overview of the immunostimulatory effects of histones, see **Figure 1**.

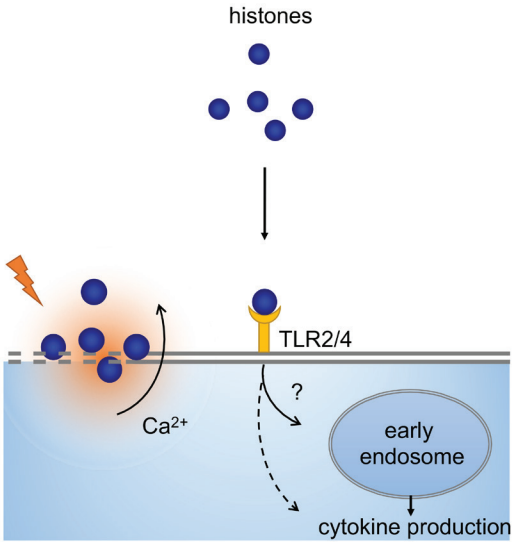

Figure 1. The immunostimulatory effects of histones
Purified histones disturb plasma membrane integrity, which induces a calcium flux, resulting in cellular lysis. In addition, histones have also been shown to signal via TLR-2 and -4. It is unclear whether TLR binding of histones induces their uptake and translocation into early endosomes.

Immunostimulatory effects of cell free DNA

DNA is a polymeric molecule that encodes the genetic information required for all life-forms on earth. However, upon release into the extracellular environment it may play a different role. Bacterial DNA is a potent immunostimulant as it contains unmethylated CpG motifs that provoke signalling via TLR-9[8]. In contrast, the CpG motifs in vertebrate DNA are mostly methylated[18]. Indeed, purified vertebrate DNA has repeatedly been found to inadequately activate TLR-9[19,20]. Furthermore, in a recent study by Bhagirath *et al.*, a comparison was made of the effects of purified protein-free, and therefore histone-free, nuclear DNA, mitochondrial DNA, and bacterial DNA on human neutrophil viability and IL-6 release. It was found that only mitochondrial and bacterial DNA, which contain unmethylated CpG motifs, increased neutrophil viability as a consequence of their activation[21]. Furthermore, only bacterial DNA induced IL-6 secretion from neutrophils.

Interestingly, in contrast to purified vertebrate DNA, complexed DNA, either as a nucleosome or in complex with anti-DNA antibodies, has been demonstrated to activate TLR-9 in cultured mouse BMDCs and spleen DCs[22,23], and also *in vivo* in mice[24]. Several explanations for the differences in TLR-9 stimulation by either purified or complexed DNA can be given. First, since TLR-9 in pDCs and B-cells is only located in the endosomal compartment, DNA needs to be endocytosed in order to activate TLR-9. Purified vertebrate DNA is not easily endocytosed[25], but several proteins that bind DNA may facilitate its uptake, including C1q[26], anti-DNA antibodies[27], and importantly, also histones[28]. Secondly, in addition to the recognition of unmethylated CpG motifs, the phosphodiester backbone of DNA has been demonstrated to efficiently dimerize TLR-9 in solution[29]. Thus, vertebrate DNA may activate TLR-9 in a sequence independent manner[30,31]. Strikingly, synthetically produced oligodinucleotides (ODNs) often contain a phosphorothioate-modified backbone in order to improve cellular uptake[32]. In an elegant study by Haas *et al.*, it was demonstrated that ODNs with this modified backbone have an antagonistic effect on TLR-9 signalling through their high affinity for TLR-9, and that only when a CpG motif was inserted into ODNs with this backbone these regained their immunostimulatory effects[31]. Unmodified DNA with a naturally occurring phosphodiester backbone was stimulatory regardless of the sequence. Results obtained with phosphorothioate-modified ODNs in

studies wherein the role of CpG motifs in TLR-9 stimulation were investigated may therefore require careful interpretation. Finally, in a more recent study it was shown that TLR-9 preferentially recognizes a curved DNA backbone[29]. Such bending of the DNA backbone presumably occurs in the DNA that wraps nucleosomes, and perhaps also in complexes of DNA with anti-DNA antibodies. In addition, it has become clear that cell free DNA may mediate TLR-9 independent immunostimulation via cytoplasmic DNA sensing mechanisms such as cyclic GMP-AMP synthase (cGAS), which results in activation of stimulator of interferon genes (STING). Initiation of this pathway by endogenous DNA, but also by dsDNA viruses that have invaded the cell, results in type I interferon secretion, thereby contributing to DNA-mediated immune activation (see review[33]). An important but so far unanswered question in this matter is whether nucleosomes that have been taken up by a cell are able to activate the cGAS-STING pathway.

Taken together, these observations indicate that many questions regarding the immunostimulatory capacity of vertebrate DNA remain. However, it is clear that DNA mediates potent immunostimulatory effects,

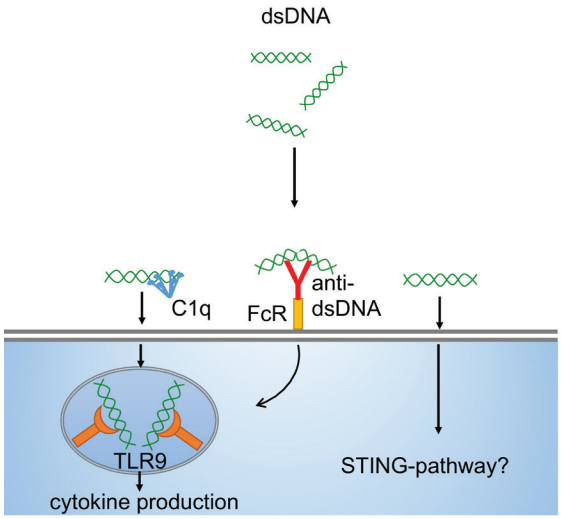

Figure 2. The immunosimulatory effects of dsDNA
Purified DNA is endocytosed and signals via TLR-9, or activates cytoplasmic DNA sensing mechanisms. Purified DNA is not easily endocytosed. Several proteins such as C1q, anti-dsDNA antibodies, and histones, appear to enhance dsDNA endocytosis. The constraints for TLR-9 signaling by dsDNA, including CpG content, the phosphodiester backbone, and DNA curvature, are discussed in the text.

both via TLR-9 stimulation as well as via cytoplasmic DNA sensing mechanisms (see **Figure 2**), and that the form in which DNA circulates, e.g. free or as a nucleosome or immune complex, modulates its immunostimulatory capacity. Furthermore, as discussed above, DNA may serve as a template to enhance TLR-2 and -4 signaling instigated by histones.

The distinct effects of histones and DNA when assembled in the form of nucleosomes

From the sections above it is clear that both extracellular histones and cell-free DNA have immunostimulatory effects on cells. However, a substantial body of evidence suggests that nucleosomes have markedly different extracellular effects when compared to free histones and DNA. Rönnefarth *et al.* demonstrated that upon incubation with nucleosomes purified from calf thymus, human neutrophils became activated with CD66b and CD11b upregulation, displayed increased phagocytosis of added microspheres, and secreted IL-8[34]. Interestingly, in this study, nucleosomes induced neutrophil activation and recruitment equally efficient in both WT and TLR-2/4 KO mice. In a continuation of the study, also TLR-9 was shown to be dispensable for nucleosome-induced neutrophil activation, although nucleosomes did induce TLR-9 upregulation and increased the response to alternative TLR-9 agonists[35]. In addition to neutrophils, nucleosomes have been demonstrated to also activate human and murine DCs , in a MyD88-independent pathway[36]. Given that MyD88 is a downstream signalling protein for all TLRs, with the exception of TLR-3, the involvement of these TLRs was excluded in this study. In contrast, nucleosomes derived from *Plasmodium falciparum* potently stimulated murine DCs in a TLR-9 dependent manner[23]. These results clearly suggest that immune activation by nucleosomes is in part determined by the species the nucleosomes derive from, and that activation may be initiated through distinct pathways in different cell types. It also suggests that immune activation by nucleosomes does not follow the same immune activation pathways that have been described for histones and/or DNA, or at least that additional pathways are also involved. To explain the immunostimulatory effects of nucleosomes, the presence of a specific cell surface receptor that binds nucleosomes has been postulated. Cell surface proteoglycans have been shown to be involved in nucleosome binding to cell surfaces, but the presence of a specific nucleosome

receptor has remained elusive[37–40].

In addition to differences in immune activation by histones and nucleosomes, the cytotoxic effects described for histones do not appear to apply to nucleosomes. Studies wherein purified nucleosomes were injected in mice to study their clearance lack any mention of cytotoxic effects, even at doses of up to 1 mg nucleosomes[41]. Of note, injection of 1.25 mg of purified histones is lethal within 1 h[10]. The half-life of injected nucleosomes (2-85 μg) was estimated to be around 4 minutes, although at higher doses, going up to 1 mg, the clearance of nucleosomes was greatly impaired, suggesting that saturation of the clearance mechanism had been reached. That nucleosomes do not provoke cytotoxic effects was confirmed *in vitro* by Abrams *et al.*, whom demonstrated that isolated nucleosomes did not induce cell death of cultured endothelial cells, unless nucleosomes were degraded by brief sonication or upon their incubation with serum[42]. Nonetheless, nucleosomes have been described to induce necrotic cell death specifically in cultured lymphocytes, whilst DNA and histones did not induce necrosis determined by counting propidium iodide positive, annexin V negative cells[43]. Given that nucleosomes contain DNA, the binding of nucleosomes to lymphocytes, which was also described in this study, may however have affected the quantification of necrotic cells by propidium iodide staining. Nevertheless, in the same study it was found that upon injection of nucleosomes in mice, the number of spleen cells, presumably lymphocytes, significantly decreased, whilst there were no signs that lymphocytes had migrated to other organs. Notably, also in this study, the mice did not display signs of inflammation upon injection with nucleosomes.

The potent nuclear derived DAMP high-mobility group box 1 (HMGB1) may interact with nucleosomes. HMGB1 stimulates cells via TLR-4 and the receptor for advanced glycation endproducts (RAGE) signaling[44]. Upon binding of HMGB1 to nucleosomes, the resulting nucleosome-HMGB1 complex may induce different cellular effects than either HMGB1 or nucleosomes alone. Nucleosome-HMGB1 complexes have been found in the circulation of SLE patients and were shown to induce the secretion of IL-1β, IL-6, IL-10, and TNF-α from human macrophages, and induce the expression of costimulatory molecules in human DCs[45]. Interestingly, nucleosomes without HMGB1 were not immunostimulatory in this study. These results suggest that

HMGB1 may form a key component of nucleosomes that directly determines their immunostimulatory capacity. Indeed, HMGB1 was shown to bind more avidly to RAGE in the presence of CpG DNA and augmented IFN-γ production by CpG-stimulated human plasmacytoid dendritic cells (pDCs), possibly by enhancing CpG DNA uptake[46]. Worth noting, in the previously mentioned neutrophil stimulation studies by Ronnefarth *et al.* the presence of HMGB1 in their nucleosome preparations was excluded, and neutrophil activation occurred readily[34]. Taken together, the described observations suggest that the prerequisites for immunostimulation by nucleosomes may well be cell-type dependent, and require further investigation. We have summarized the different routes by which nucleosomes mediate their extracellular effects in a schematic illustration in **Figure 3**.

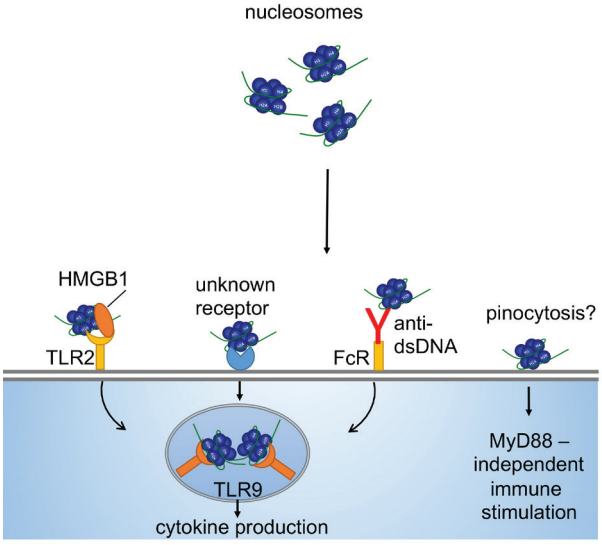

Figure 3. The immunostimulatory effects of nucleosomes
In contrast to purified histones, dsDNA, or mixed preparations, nucleosomes appear to follow additional and different routes of immunostimulation, also depending on the cell type it encounters. Similar to dsDNA, anti-dsDNA antibodies increase the uptake of nucleosomes by phagocytic cells. Moreover, purified nucleosomes with bound HMGB1 mediate immunostimulation of human macrophages via TLR-2. In contrast, purified nucleosomes lacking HMGB1 are stimulatory to neutrophils and dendritic cells in a MyD88-independent manner, indicating that stimulation by nucleosomes is also cell-type specific. In contrast to histones, nucleosomes do not appear cytotoxic. Given that nucleosomes were repeatedly found to bind to the plasma membrane, the existence of a nucleosome-specific receptor has been proposed, but this receptor has thus far not been identified. Finally, it is unclear whether nucleosomes that have been taken up by cells are able to stimulate intracellular DNA sensing mechanisms.

The origin of circulating nucleosomes

So far, the origin and mechanisms of nucleosome release have not been studied in large detail. Although the obvious source of extracellular nucleosomes are dying or damaged cells, the mechanisms by which nucleosomes are released into the extracellular environment appear multifold. An important factor in facilitating nucleosome release may be the type of cell death that occurs, e.g. apoptosis, necrosis, pyroptosis, necroptosis, and others. For example, upon apoptosis, caspase-activated DNase (CAD) induces the fragmentation of DNA into oligonucleosomes, a process that does not take place in necrosis. Indeed, nucleosomes have been found on the surface of apoptotic cells[47]. Furthermore, apoptotic cells passively leak nucleosomes, whilst several plasma proteins such as Factor VII-activating protease (FSAP), which is activated upon contact with late apoptotic or necrotic cells, facilitate efficient release of chromatin from late apoptotic cells[48]. In addition to FSAP, Factor H has recently been found to bind nucleosomes and purified Factor H mediated their release from apoptotic cells[49]. However, this mechanism remains to be validated in full serum or plasma. In addition to these plasma proteins and intracellular nucleases, circulating nucleases also play an important part. For example, for FSAP mediated nucleosome release from necrotic cells, serum DNase activity was required to fragment chromatin before its release into the extracellular environment[50]. Interestingly, it is known that C1q may in its turn increase serum DNase I activity, resulting in enhanced necrotic chromatin clearance[26], whilst C1q also enhanced efferocytosis of late apoptotic cells[51]. The synergistic effects of these plasma factors in facilitating the release and clearance of dead cell chromatin remain to be elucidated. However, the mechanisms of chromatin release are beginning to be unraveled. For instance, FSAP efficiently cleaved linker histone H1 in necrotic cells. Since histone H1 mediates the higher order compaction of chromatin (see review[52]), H1 cleavage by FSAP may form a crucial step in the release of chromatin from dying cells. It is clear that chromatin release proceeds in a highly regulated manner and that multiple nucleases, both intracellular as well as extracellular, in combination with various plasma proteins are involved in this regulation. Impaired functionality of these factors, for example of DNase I, has been linked to systemic lupus erythematosus (SLE), whilst the release of chromatin from late apoptotic cells by FSAP has been shown to be inhibited in patients with SLE[53,54].

In addition to the release of chromatin from dying non-myeloid cells, activated neutrophils may undergo a form of cell death whereby their chromatin is excreted into the extracellular environment to form so-called neutrophil extracellular traps (NETs)[55]. Notably, this type of cell death has been described to occur in other cell types, e.g. mast cells, basophils, and macrophages, as well (see review[56]). NETs are decorated with neutrophil proteases and have been demonstrated to efficiently trap and kill pathogens such as bacteria, fungi and parasites[55]. Interestingly, NETs have been demonstrated to be cytotoxic to lung epithelial cells and mouse glomerular endothelial cells *in vitro*[57,58]. This effect was, in part, mediated by the histones present in NETs as NETs remained toxic upon DNA digestion, whilst anti-histone antibodies partially protected cells against NET induced cytotoxicity. As mentioned above, several studies have demonstrated that in contrast to NETs, purified nucleosomes are not toxic to cultured endothelial cells. Several explanations may be provided for the apparent differences in the cytotoxicity of NETs and nucleosomes. First, during NETosis, histones are processed by elastase[59] while peptidylarginine deiminase-4 (PAD4) converts the highly charged arginine residues in histones to more neutral citrulline, which results in a more open chromatin structure[60]. These modifications may possibly result in an increased exposure of cytotoxic histones when compared to purified unmodified nucleosomes. Secondly, the anti-microbial proteases present in NETs may confer cytotoxicity as well. Indeed, an MPO inhibitor decreased the cytotoxicity of NETs, while an elastase inhibitor had no effect[57]. Finally, the length of extracellular chromatin, which is much longer in NETs compared to purified nucleosomes, might also mediate cytotoxicity. In conclusion, extracellular chromatin may derive from different origins ranging from dying non-myeloid cells to NETting neutrophils, and the release of chromatin from these cells appears tightly controled (**Figure 4**). However, it is currently unclear to what extent the origin of the chromatin determines its cytotoxicity.

Since the mechanisms that may account for the levels of extracellular chromatin in the circulation are manifold, this raises the question from which cell types the circulating nucleosomes originate. Circulating nucleosome levels are increasingly being used as a marker for NETosis[61–64], but may in fact be derived from various cell tissues and cell types. Although in several human diseases and murine models nucleosome levels indeed appear to

correlate with neutrophil activation as determined by circulating elastase levels[65], no assays currently exist that distinguish between NET-derived or non-myeloid cell-derived chromatin. Importantly, PAD4-deficient mice, which are reportedly impaired in NET formation, displayed similarly increased levels of circulating nucleosomes upon LPS-challenge as wild-type mice, indicating that nucleosomes not only derived from NETting neutrophils[66]. In a different study, Sun *et al.* studied the origin of circulating DNA through plasma DNA tissue mapping. They found that the largest part of circulating DNA in cancer patients and in patients that had undergone a bone marrow or liver transplantation was lymphocyte derived[67]. Notably, neutrophils contributed significantly to circulating cell-free DNA. Future studies employing this technique may help to elucidate the origin of circulating DNA in inflammatory disease.

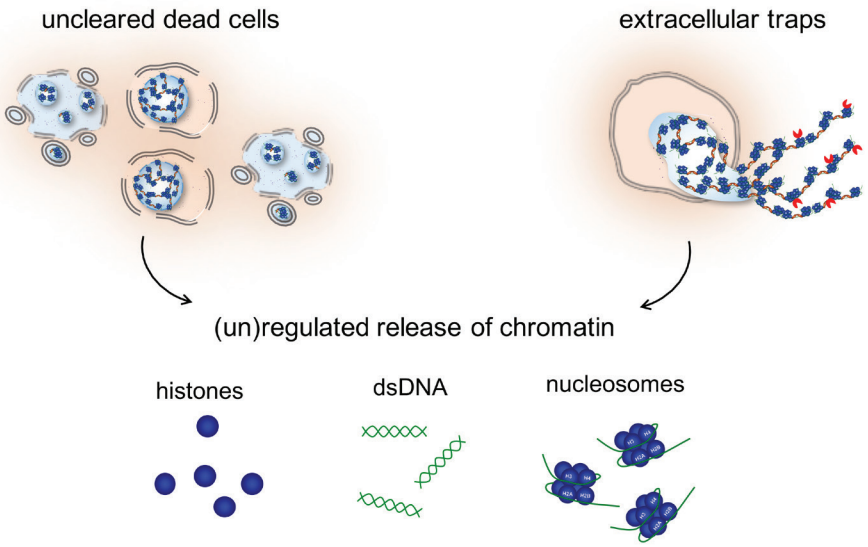

Figure 4. The origin of histones, dsDNA and nucleosomes
Upon insufficient clearance of dead cells, or the induction of (neutrophil) extracellular traps, chromatin components are released into the extracellular environment. This release may occur passively, but several plasma proteins are known to regulate the release of chromatin (components) from dead cells.

Detection of circulating histones and nucleosomes in disease

Circulating histones and nucleosomes have been frequently found in patients suffering from a wide range of inflammatory conditions, including sepsis[68], traumatic injury and surgery[12,69–71], cerebral stroke[72], chronic obstructive pulmonary disease (COPD)[73], systemic lupus erythematosus[74], multiple organ failure[71], disseminated intravascular coagulation (DIC)[75], thrombotic microangiopathies[76], sickle cell disease[61], paroxysmal nocturnal hemoglobinuria (PNH)[77], and cancer[78,79]. More importantly, circulating levels of nucleosomes correlate with the length of hospital stay in sickle cell disease[61], the severity of stroke[72], an increased risk of deep vein thrombosis (DVT)[80], are associated with mortality in children suffering from meningococcal sepsis[81], and may serve as a predictive marker for chemotherapy response in cancer patients[79], and mortality in trauma injury[69].

To detect the presence of circulating histones or nucleosomes, several assays are currently in use. The presence of histones in patient samples is easily visualized by means of immunoblot and this assay has been used extensively[4,10,12,73,82]. However, using immunoblot it is not possible to make a distinction between freely circulating histones, histones bound to DNA, or histones that are part of a nucleosome complex. Similarly, several ELISAs have been developed that quantify specific histone subtypes[24,71,73,75,83]. These assays often make use of polyclonal antibodies raised against histones and it is therefore unlikely that the antibodies used in these assays will solely detect free histones. Alternatively, ELISAs have been developed that specifically detect nucleosomes. Our own in-house developed nucleosome assay makes use of a monoclonal anti-histone H3 catching antibody and a monoclonal detection antibody that recognizes a structural epitope formed by histone H2A, H2B and DNA[84]. This ensures specificity for nucleosomes and we have not observed any cross-reactivity with purified free histones in this assay. A similar ELISA was developed by Roche (Cell Death Detection ELISA^{PLUS}) wherein a monoclonal anti-histone antibody is used as a catching antibody in combination with a monoclonal anti-DNA antibody for detection and which has been widely used[12,69,70,72,79,82,85–88].

In addition to the ELISA-based measurement of circulating nucleosomes, PCR-based approaches to quantify circulating cell-free DNA are also readily established. Since all cell-free DNA seems to be circulating in

complex as nucleosomes[89,90], the PCR-based approach appears to deliver very comparable results to a nucleosome ELISA[91], although PCR-based approaches may be more laborious and require more patient material. Important to note when developing such PCR method to quantify cell free DNA however, are the different chromatin fragment lengths that may be found in the circulation. Since most DNA seems to circulate in mono- or di-nucleosome fragments[89,90], caution should be taken when choosing primer sets for PCR that amplify DNA fragments shorter than 147 bp in length. With respect to the available nucleosome ELISAs, it is not clear how these assays respond to longer stretches of chromatin.

The nucleosome specific ELISAs in particular allow for a reliable measurement of circulating chromatin fragments. Regrettably, however, the absence of assays that specifically detect free histones has sometimes led investigators to assign certain effects to free histones whereas it is unclear whether these effects are in fact attributable to nucleosomes instead. In a study by Abrams *et al.*, immunoblot was used to detect histones and combined with a nucleosome ELISA to simultaneously detect nucleosomes in samples obtained from severe trauma patients[12]. Initially both histone and nucleosome levels were high in the first hours after trauma, but nucleosome levels dropped after 24 hr whilst histone levels remained elevated for up to 72 hr after hospitalization. The blot used to quantify histones was not shown but the density of the bands was assessed using densitometry. It is unclear whether the histones measured at 24 hr and 72 hr were free or whether they remained (partly) complexed with DNA but became undetectable in ELISA.

In the seminal paper of Xu *et al.* on the importance of TLR-2 and TLR-4 in ''histone-mediated'' immune signaling in ConA challenged mice, nucleosomes were immunoprecipitated from a mouse sample with an antibody against DNA-H2A-H2B. Strikingly, no residual histone H3 was detected upon analysis of the supernatant on immunoblot, which indicates that all histones were present as part of a nucleosome complex and were not circulating freely. This was correctly pointed out by the authors of the study, and it thus appears that not free histones but nucleosomes are responsible for the TLR-2 and TLR-4 mediated induction of inflammation observed in their study. Since in most *in vitro* studies nucleosomes induced TLR-independent immunostimulation, it is unclear how to interpret the *in vivo* data. It is possible however, that the

nucleosome preparations used for most *in vitro* studies, which often consist of mono- and di-nucleosomal fragments, are unable to efficiently cross-link TLR-2 and TLR-4, in contrast to the larger (NET) fragments that may be present locally at inflammatory sites *in vivo*. This is supported by the observation of Xu *et al.* that histone signaling via TLR-2 and TLR-4 in TLR-transfected HEK293 cells was enhanced in the presence of exogenously added DNA[4]. However, it is worth noting that the length of circulating cell-free DNA was determined to predominantly represent the mononucleosome size. Since the extracellular effects of histones and nucleosomes appear to be different and they may be subject to different clearance/degradation kinetics, this stipulates the importance of specific assays for chromatin components and a clear use of the terms histones and nucleosomes.

CONCLUSIONS

The important nuclear DAMPs histones and DNA induce immune activation independently of each other in various cell types, whilst histones are in addition also cytotoxic. However, when present in the form of a nucleosome complex, the cytotoxicity of histones seems to be missing, whereas the signaling pathways triggered by nucleosomes are not limited to TLRs and require further investigation. Furthermore, the release of chromatin from dying non-myeloid cells as well as from neutrophils in the form of NETs appears to be highly regulated. To measure circulating chromatin components, various techniques are in use that detect their targets with different degrees of specificity, so care should be taken when selecting a technique and attributing effects to either individual chromatin components, or nucleosomes.

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