Histones, DNA, and Citrullination Promote Neutrophil Extracellular Trap Inflammation by Regulating the Localization and Activation of TLR4

Highlights

- Neutrophil extracellular trap chromatin is proinflammatory at sub-lethal concentrations
- Synergy between histones and DNA is critical for sub-lethal signaling
- Citrullination is dispensable for NETosis but potentiates histone-mediated signaling
- Histones activate TLR4, while DNA recruits TLR4 to histone-containing endosomes

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In Brief

Tsourouktsoglou et al. discuss how the pro-inflammatory capacity of neutrophil extracellular traps (NETs) depends on chromatin fragmentation, citrullination, and synergistic interactions between citrullinated histones and DNA. Histones are the primary pro-inflammatory agents, and DNA potentiates sub-lethal signaling by recruiting TLR4 to histone-containing endosomes.
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SUMMARY

Neutrophil extracellular traps (NETs) promote atherosclerosis by inducing proinflammatory cytokines, but the underlying mechanism remains unknown. NET DNA is immunogenic, but given the cytotoxicity of NET histones, it is unclear how it activates cells without killing them. Here, we show that histones, DNA, citrullination, and fragmentation synergize to drive inflammation below the histone cytotoxicity threshold. At low concentrations, nucleosomes induce cytokines, but high concentrations kill cells before cytokines are produced. The synergy between histones and DNA is critical for sub-lethal signaling and relies on distinct roles for histones and DNA. Histones bind and activate TLR4, whereas DNA recruits TLR4 to histone-containing endosomes. Citrullination is dispensable for NETosis but potentiates histone-mediated signaling. Consistently, chromatin blockade or PAD4 deficiency reduces atherosclerosis. Inflammation is also reduced in infected mice expressing GFP-tagged histones that block TLR4 binding. Thus, chromatin promotes inflammation in sterile disease and infection via synergistic mechanisms that use signals with distinct functions.

INTRODUCTION

Inflammation is the underlying cause of many chronic diseases. The mechanisms that regulate sterile inflammation are still poorly understood. Endogenous molecules, known as damage-associated molecular patterns (DAMPs), play a central role in inducing the cytokines that regulate the process. DAMPs can leak out of damaged cells, or they can be released by immune cells in a regulated manner. For example, the release of neutrophil extracellular traps (NETs) via programmed cell death induces inflammation in conditions such as gout and atherosclerosis (Muñoz et al., 2016; Warnatsch et al., 2015). NETs act as endogenous DAMPs that prime monocytes and macrophages to produce interleukin (IL)-1β, a cytokine that promotes atherosclerosis along with other proinflammatory cytokines, such as IL-1α and IL-6 (Warnatsch et al., 2015). The mechanisms that link NETs to the production of proinflammatory cytokines remain unknown.

NETs are pleiotropic molecules composed of decondensed chromatin and proteins such as the cathelicidin LL-37, S100A8, and high-mobility group protein 1 (HMGB1) that have been linked to inflammation (Lande et al., 2007, 2011; Tian et al., 2007; Ullas et al., 2017; Urban et al., 2009; Urbonaviciute et al., 2008; Vogl et al., 2007). Extracellular DNA is also thought to induce IL-1β and type I interferons (IFNs) (Atianand and Fitzgerald, 2013) and is detected by Toll-like receptor (TLR)9, whereas cytosolic DNA induces the intracellular sensors cGas/STING and AIM2 (Fernandes-Alnemri et al., 2009; Gaidt et al., 2017; Hornung et al., 2009; Ishikawa and Barber, 2008; Sun et al., 2013). Extracellular DNA is also internalized and detected by the cytosolic DNA receptors (Chamilos et al., 2012; Gehrke et al., 2013; Lood et al., 2016; Paludan and Bowie, 2013). Consistently, oxidation of NET DNA during NET formation increases its IFN-inducing capacity via the activation of cGas/STING (Lood et al., 2016). However, immune responses to endogenous DNA in its native nucleosomal state have not been characterized. This is a critical issue, because histones are cytotoxic in vitro, and their release in the circulation causes lethality in mouse models of sepsis and liver injury (Abrams et al., 2013; Huang et al., 2011; Kumar et al., 2015; Xu et al., 2009, 2011). TLRs are implicated in these phenotypes, and injection of pure recombinant histones drives chemokine expression and leukocyte recruitment (Huang et al., 2011; Westman et al., 2015; Xu et al., 2011). A recent study also identified a cytotoxic role for NET histones in fibrous cap erosion in atherosclerotic lesions (Silvestre-Roig et al., 2019). However, these phenotypes have predominantly been attributed to the cytotoxic properties of histones and their ability to disrupt cell membranes rather than to direct signaling activity (Huang et al., 2011; Silvestre-Roig et al., 2019; Xu et al., 2009, 2011). It is, therefore, unclear how chromatin DNA or other NET components might activate immune cells to produce cytokines without killing them.
In addition, NET chromatin bears unique post-translational modifications. During NET formation, histones become citrullinated by protein arginine deiminase 4 (PAD4), and this process is thought to be essential for NET formation (Lesher et al., 2012; Papayannopoulos et al., 2010; Wang et al., 2009). This unique modification has facilitated the identification of NETs in vivo (Branz et al., 2014) but has also resulted in many studies interpreting the absence of citrullination as a lack in NET formation in PAD4-deficient animals without the use of additional NET markers. Moreover, the idea that citrullination is essential for NET formation has prohibited the investigation of a possible role for the modification in NET function.

In addition, little is known about the mechanisms that regulate cellular responses to NETs. Many TLR agonists act synergistically when presented together at low concentrations (Napolitani et al., 2005). Negative relationships between different classes of immune-sensing receptors have also been reported (Negishi et al., 2012). Together, these positive and negative relationships are thought to help refine immune responses (Tan et al., 2014). Synergy could be pivotal in the detection of NETs, as they contain an array of factors. Two NET proteins were shown to potentiate the detection of DNA by TLR9. LL-37 potentiates TLR9-mediated type-I IFN induction, whereas HMGBl enhances TLR9 activation via the receptor for advanced glycation products (RAGE) (Lande et al., 2007; Tian et al., 2007). DNA-bound proteins also increase the uptake of nucleic acids (Berthe loot et al., 2016; Chamilos et al., 2012; Sirois et al., 2013). However, the physiological relevance of these synergistic interactions is not well defined.

Here, we investigate the pro-inflammatory NET components and their corresponding receptors and dissect the mechanisms that regulate the responses to NETs that ultimately drive atherogenesis. We report, for the first time, a role for histone citrullination in NET function, which helped uncover the importance of histones as major pro-inflammatory components in NETs. We demonstrate that the pro-inflammatory capacity of NETs depends on chromatin fragmentation, citrullination, and synergistic interactions of citrullinated histones with DNA, where histones are the primary pro-inflammatory agents and DNA potentiates signaling below the histone cytotoxicity threshold via a novel mechanism.

RESULTS

Citrullination Is Dispensable for NETosis in Atherosclerosis

Given the recent conflicting data surrounding the role of citrullination in NET formation, we sought to examine whether the phenotypes associated with PAD4 deficiency may be attributed to a role of citrullination in NET function (Claushuus et al., 2018; Kenny et al., 2017). First, we examined whether citrullination is implicated in NET formation induced by cholesterol crystals. Cl-amidine, a potent inhibitor of PADs, blocked histone citrullination effectively but failed to inhibit NET formation in human neutrophils induced by cholesterol crystals (Figure 1A). Measured in their native unfixed state, non-citrullinated NETs were equally decondensed. Un-citrullinated NETs contained typical NET markers such as neutrophil elastase (NE), myeloperoxidase (MPO), calprotectin (S100A8) and proteolytically processed histone H3 (Figures 1B and 1C). However, NETs formed in the presence of Cl-amidine lacked citrullinated histone H3 as assessed by immunofluorescence microscopy and western immunoblotting. We also probed for NET formation in the atherosclerotic lesions of ApoE and ApoE/PAD4-deficient mice fed on a high-fat diet for 6 and 16 weeks. We stained for citrullinated histone H3 and additional NET markers such as Ly6G, which we have previously shown to colocalize with NETs (Warnatsch et al., 2015) and MPO. Chromatin was detected by staining with PL2-3, an antibody isolated from mice with spontaneous autoimmunity that recognizes epitopes in histones H2A and H2B and in DNA (Lomsman et al., 1992).

We detected abundant NETs of similar size and distribution in both PAD4-deficient and PAD4-sufficient APO knockout mice (Figures 1D, 1E, and S1). The presence of non-citrullinated NETs in these lesions indicates that citrullination is not essential for NET formation in response to cholesterol crystals in vitro and in atherosclerotic lesions. Furthermore, it highlights the importance of additional chromatin markers for the detection of NETs in PAD4-deficient mice.

Nucleosomes and Citrullination Regulate Cytokine Production

Based on these findings, we hypothesized that citrullination could influence NET-mediated pathology by regulating the proinflammatory function of NETs. To test this hypothesis, we generated non-citrullinated NETs in the presence of Cl-amidine, solubilized them using restriction enzymes, and tested their capacity to induce IL-1β expression in human primary blood monocytes. Non-citrullinated NET fragments exhibited a lower capacity to upregulate IL-1β mRNA, IL-1x, and IL-6 (Figures 2A and S2). At these NET concentrations, mRNA levels yielded by non-citrullinated NETs were comparable to those yielded from treatment of citrullinated NETs with DNase I (Figure S2). Given that histones are the major citrullinated proteins in NETs, this finding suggested that histones could be important in NET-mediated cytokine induction.

To address this question, we first examined whether purified nucleosomes were sufficient to activate monocytes. We purified native nucleosomes from granulocytic human leukemia 60 (HL-60) cells and generated different-size fragments with micrococcal nuclease (Mnase), which digests the inter-nucleosomal linker DNA without digesting any DNA that is directly bound to histones. Nucleosomes were potent inducers of IL-1β, and their activating capacity increased as their fragment size decreased (Figure 2B). Activation was maximal with di-nucleosomes (0.4 kb) and mono-nucleosomes (0.2 kb) to levels comparable to those achieved with stimulation with lipopolysaccharide (LPS), suggesting that decondensation and partial nuclease processing are required for NET-mediated signaling. We also tested nucleosomes isolated from tissues of CAG::H2B-EGFP knocktransgenic mice that express a H2B-EGFP fusion protein. These nucleosome-EGFP (EGFP-Nuc) preparations failed to induce cytokine expression, indicating that the presence of a bulky EGFP domain on nucleosomes interfered with their pro-inflammatory activity when compared with control nucleosomes of human or mouse origin (Figures 2B and 2C). This finding suggested that cytokine induction involved histone recognition by specific receptors that...
was disrupted by EGFP rather than non-specific cytotoxicity. These results allowed us to investigate genetically whether histones promote inflammation in vivo. Given that H2B-EGFP-bearing nucleosomes did not exhibit proinflammatory activity in vitro, we used CAG::H2B-EGFP mice as a genetic tool to examine whether histones play a role in the induction of inflammation during pulmonary fungal infection, which promotes NET release (Branzk et al., 2014). We detected lower IL-1β concentrations in the bronchoalveolar lavage (BAL) of CAG::H2B-EGFP mice after 24 h of intratracheal infection with C. albicans (Figure 2D), in spite of the similar pulmonary fungal load (Figure 2E), suggesting that histones are important for the induction of this cytokine during acute fungal infection. Murine bone-marrow-derived macrophages (BMDMs) from CAG::H2B-EGFP mice responded to various proinflammatory agonists, including cit-H3.
the PL2-3 anti-chromatin antibody against different mono-nucleosome concentrations. The reduction in pro-inflammatory capacity of mono-nucleosomes was comparable upon neutralization of TLR2/4 or histone blockade with the PL2-3 Fab (Figure 3C). TLR2/4 neutralization also blocked the activation of human monocytes when recombinant cit-H3 precomplexed to NET DNA, LPS (0.5 ng/mL), or CpG (1 μM), indicating that histones engage primarily TLR4 (Figures 3E, 3F, and S3A–S3D). Notably, EGFP nucleosomes failed to activate the TLR4 reporter cells, indicating that TLR4 is sensitive to alterations in histone architecture, possibly involving steric hindrance by EGFP (Figure 3E).

To investigate whether histones physically interact with TLR4, we incubated HEK cells expressing either TLR2-hemagglutinin (HA) or TLR4-HA with recombinant citrullinated H3 or mono-nucleosomes. HA-tagged TLR4, but not TLR2, could be immunoprecipitated with a recombinant MBP-H3 fusion protein (Figure 3G) or antibodies against mono-nucleosomes (Figures 3H and 3I). Citrullination of nucleosomes using recombinant PAD4 increased binding to TLR4, suggesting a mechanism of action for the enhancement of NET-mediated signaling by this modification. Moreover, EGFP-Nuc preparations failed to bind TLR4, indicating that the inability of these molecules to induce IL-1β is attributed to the lack of physical association with TLR4. Interestingly, in situ citrullination of these EGFP-Nuc preparations reversed the binding inhibition exerted by EGFP. Therefore, changes in charge due to citrullination overcome the ability of EGFP to block TLR4, which indicates that citrullination can regulate histone–TLR4 interactions. Collectively our data demonstrate that native histones bind and activate TLR4. This
Figure 3. Histones Induce IL-1β by Binding and Activating TLR4

(A) Total IL-1β induced in BMDMs from WT or from TLR4-, TLR9-, or STING-deficient mice, incubated with increasing concentrations of mono-nucleosomes or LPS (0.5 ng/mL). Data are representative of three independent experiments and are represented as mean ± SD. Statistical comparison of TLR4−/−/− to WT via two-way ANOVA followed by Sidak’s multiple comparison test.

(B) Total IL-1β induced in untreated primary human monocytes or stimulated with cit-NETs alone or in the presence of neutralizing antibodies against TLR2 and TLR4. Statistical analysis by Mann-Whitney test. Data representative of three individual stimulations from two different donors and are represented as mean ± SD.

(legend continued on next page)
interaction is not affected by their association to DNA but is potentiated by citrullination.

**Histones Synergize with DNA in Cytokine Induction**

Since NET DNA has also been implicated in inflammation, we assessed the role of DNA by treating chromatin preparations with DNase I or benzonase, two nuclease that degrade both inter-nucleosomal and histone-bound DNA to completely remove it from nucleosomes. These DNA-free nucleosomes lost their capacity to potently activate monocytes (Figure 4A). These data suggested that, in addition to histones, DNA was also required for potent cytokine induction in human monocytes. The magnitude of the decrease in cytokine induction upon complete DNA digestion suggested a potential synergistic relationship between DNA and histones.

To test whether synergy was involved, we performed titrations with purified NET DNA and recombinant histone H3. Histone H3 alone modestly activated monocytes at a concentration of 0.3 μM. The addition of DNA at sub-threshold concentrations dramatically enhanced monocyte responsiveness to histone H3 (Figure 3H). Furthermore, citrullination of recombinant histones using a purified recombinant PAD4 enzyme further potentiated monocyte stimulation. We also found that monocytes failed to produce IL-1β in response to concentrations of DNA up to 1.0 μg/mL, but that they were significantly sensitized to DNA stimulation in the presence of a non-activating concentration of histone H3 (Figure 4C). Therefore, synergy between histones and DNA confers the bulk of the pro-inflammatory activity of chromatin and NETs in human monocytes. Citrullination also influenced cytokine induction but played a less critical role as compared to DNA.

Given the ability of DNA to potentiate extracellular histone-mediated cytokine induction, we tested whether nucleosome DNA induced inflammatory signaling in our experimental conditions by examining the production of type I IFNs, which are known to be induced by DNA-mediated activation of TLR9 or STING. Purified citrullinated histone H3 alone or in the presence of NET DNA failed to induce type I IFNs (Figure 4D). Moreover, 10−7 M nucleosomes that were sufficient to potently induce IL-1β failed to up-regulate IFN-α or IFN-β in human monocytes and murine BMDMs (Figures 4E and 4F). The lack of TLR9 activation by purified histones and NET DNA or mono-nucleosomes was also confirmed using TLR9 reporter cell lines (Figures S3C and S3D). Therefore, these concentrations of extracellular NET DNA were unable to directly induce pro-inflammatory signaling in our experiments.

**DNA Regulates Intracellular TLR4 Localization**

Given that NET DNA did not influence histone binding to TLR4 in HEK cells, we hypothesized that the mechanism of synergy might involve changes in the cell biology of TLR4 in monocytes. Therefore, we examined the localization of TLR4 using flow cytometry by staining for intracellular, extracellular, and total TLR4. In human primary monocytes, TLR4 was completely absent from the cell surface and could only be detected intracellularly (Figure 5A). This led us to investigate whether DNA might promote the translocation of TLR4 to the cell surface to facilitate the recognition of extracellular chromatin. However, TLR4 localization remained intracellular after stimulation with NET DNA alone, histone H3, or a complex of the two (Figure 5B). Next, we investigated whether synergy might involve changes in intracellular TLR4 signaling. Consistent with this idea, inhibition of endosomal acidification with bafilomycin A reduced human monocyte responses to nucleosome stimulation (Figure 5C). To examine whether DNA induced changes in the intracellular TLR4 localization, we incubated human monocytes with MBP-H3 fusion protein alone or pre-complexed to NET DNA for 2 h. We stained for TLR4 and anti-MBP to visualize internalized extracellular histones and distinguish them from endogenous chromatin. In the presence of DNA, TLR4 was localized around 45% of the compartments containing internalized histone H3 (Figures 5D and 5E). Many of these compartments also sequestered Ras-related protein Rab-5 (Rab5), confirming that extracellular histones were being sequestered in early endosomes (Figure 5F). In the absence of DNA, TLR4 failed to translocate to histone-containing endosomes, suggesting that extracellular DNA exerted its synergistic effects by promoting the translocation of TLR4 to endosomes containing internalized histones.

**Synergy Enables Sub-lethal Signaling**

A synergistic relationship between different TLR agonists has been previously reported (Napolitani et al., 2005), but the physiological context of this phenomenon is not well defined. We reasoned that synergy may serve as a mechanism to enable cytokine

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(C) Total IL-1β induced in primary human monocytes upon titration of mono-nucleosomes alone or in the presence of antibodies against chromatin (PL2-3 Fab) and TLR2 and TLR4. Statistical analysis by two-tailed Student’s t test. Data are representative of three independent experiments and are represented as mean ± SD.

(D) Total IL-1β induced in primary human monocytes stimulated with recombinant cit-H3 (0.03 μM) and NET DNA (300 ng) in the presence or absence of anti-TLR2 and anti-TLR4 neutralizing antibodies. Statistical analysis by Mann-Whitney test. Data are representative of three independent experiments and are represented as mean ± SD.

(E and F) TLR4-mediated NF-κB activity assessed in a heterologous HEK-Blue cell system, by NF-κB-driven induction of secreted embryonic alkaline phosphatase (SEAP). (E) TLR4 HEK-Blue reporter cells were treated with different fragments of purified HL-60 chromatin or mouse EGFP-tagged nucleosomes or LPS (0.5 ng/mL). (F) TLR4 HEK-Blue reporter cells were stimulated with LPS (0.5 ng/mL) or CpG (1 μM) or recombinant citrullinated H3 alone or in complex with NET DNA. Data are representative of three (E) or five (F) independent experiments and are represented as mean ± SD.

(G) HEK cells expressing TLR4-HA or TR2-HA were incubated with recombinant H3 fused to maltose-binding protein (MBP-H3) alone or complexed to NET DNA. Cells were lysed, and interacting proteins were pulled down with amylase beads and immunoblotted with antibodies against HA. The total cell lysate (input) and the pull-down are depicted. Input was diluted 20× more than the pull-down.

(H) HEK cells expressing TLR4-HA were incubated alone (–) or with in vitro citrullinated or non-citrullinated human mono-nucleosomes or mouse EGFP-mono-nucleosomes. Lysates were immunoprecipitated using an antibody against histone H3 and immunoblotted with antibodies against HA. The total cell lysate (input) was diluted 20× more than the pull-down samples.

(I) Band intensities in (H) using ImageJ analysis.

*p < 0.05; **p < 0.01; ***p < 0.001; ns, p > 0.05.
loss in viability, human monocytes failed to produce any IL-1β upon exposure to nucleosomes at a concentration of 10⁻³/C₀.

We examined the relationship between mononucleosome uptake and cytokine induction below the histone cytotoxicity threshold. Thus, we found that monocytes were viable and could tolerate mononucleosomes for up to 16 h at concentrations of 10⁻⁷ M, but all histone-containing cells died upon exposure to nucleosome concentrations of 5 × 10⁻⁷ M or higher (Figures 6A and 6B). By comparison, nucleosomes activated human monocytes at concentrations as low as 10⁻⁹ M, with stimulation peaking at concentrations of 10⁻⁸ M to 10⁻⁷ M (Figure 6C). Consistent with a rapid loss in viability, human monocytes failed to produce any IL-1β upon exposure to nucleosomes at a concentration of 10⁻⁶ M, which indicates that, from a technical standpoint, optimal chromatin concentration is critical in the detection of cytokine induction. Therefore, histone cytotoxicity suppresses pro-inflammatory responses at high chromatin concentrations, while the synergy between histones and DNA enables human monocytes to respond to chromatin at concentrations that are well below the cytotoxicity threshold.

**Endogenous Histones Promote Atherosclerosis**

To examine whether extracellular chromatin was a major driver of NET-induced inflammation during atherosclerosis, we tested the effects of PAD4 deficiency and chromatin-blocking antibodies in a murine model of atherosclerosis. We treated female ApoE-deficient mice with either PL2-3 immunoglobulin G (IgG) or the Fab fragment. Treatment with the PL2-3 Fab antibody did not affect blood cholesterol and triglyceride concentrations (Figure 7A), indicating that extracellular chromatin is a major driver of local sterile inflammation that promotes atherosclerosis. To interrogate the basis of the differences in the in vivo efficacy against atherosclerosis between the full-length and the Fab version of the PL2-3, we compared their ability to block nucleosome-mediated induction of IL-1β in cultured murine BMDMs. The full-length antibody and the Fab fragment exhibited comparable capacity to block cytokine induction in vitro, indicating that the ineffectiveness of the full-length PL2-3 antibody is only relevant in vivo (Figure 7D). Interestingly, an antibody against histone H3 could not block the induction of IL-1β in vitro, indicating that...
chromatin-blocking efficiency may vary among different chromatin-targeting antibodies and may involve specific epitope recognition.

**DISCUSSION**

Our data indicate that endogenous externalized chromatin is a key driver of inflammation in sterile disease but can also amplify inflammation during infection. Chromatin components appear to have distinct modes of action that enable synergistic interactions. Histones are the main signaling mediator that activates the transcription of IL-1β in mononuclear cells by binding and activating TLR4. Instead, the contribution of chromatin DNA in direct immune signaling is limited by the histone cytotoxicity threshold. However, chromatin DNA serves a previously unknown function by regulating the cellular localization of intracellular TLR4 to promote TLR4 recruitment to endosomes containing internalized chromatin. We also excluded that DNA synergy could be due to avidity stemming from tethering multiple nucleosomes, since chromatin fragmentation increased its activation potential and DNA did not influence TLR4 reporter activity in cells that overexpress the receptor. This model differs from findings in plasmacytoid dendritic cells where proteins potenti-
DNA-mediated signaling upstream of type I IFN expression (Lande et al., 2007).

Our model also provides a new concept in our understanding of the mechanisms that drive synergy between different TLR agonists. Prior work suggested that synergy between microbial TLR agonists is mediated by the enhanced activation of the extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) pathways (De Nardo et al., 2009). Another mechanism involves direct physical interaction between different receptors. For instance, DNA-bound HMGB1 recruits RAGE into a complex with TLR9 to enhance myeloid differentiation primary response 88 (MyD88) recruitment (Tian et al., 2007). However, it is conceptually difficult to explain the synergistic action between two cues that converge on the same downstream pathways via separate receptors. Instead, synergy can be easily achieved if the two activators play distinct roles in the signal transduction cascade, with one signal promoting receptor recruitment and the second signal triggering receptor activation. This mode of action provides a new paradigm for synergy among endogenous TLR agonists.

Moreover, in this instance, our data provide an explanation for the physiological role of synergy between cytotoxic DAMPs. The requirement for synergy serves to enable sub-lethal signaling by weak agonists but also introduces opportunities for regulation. For example, circulating chromatin occurs in sepsis and other systemic inflammatory conditions (Xu et al., 2009). The requirement for DNA might serve to enable serum DNases to suppress the potent activation of blood monocytes by circulating chromatin via the degradation of DNA. Cells may also be able to tune their sensitivity to DAMPs by regulating the cellular localization of TLR4. Moreover, the model predicts that, at low concentrations, NETs are proinflammatory, while at high NET concentrations, histone cytotoxicity is likely to reduce inflammation. This mechanism may contribute to the anti-inflammatory effect of aggregated NETs in addition to the degradation of pro-inflammatory molecules by NET proteases (Leppkes et al., 2016; Schauer et al., 2014).

The requirement for chromatin fragmentation provides an additional layer for the regulation of inflammatory responses. NET decondensation and subsequent processing by endonucleases may allow immune cells to differentiate between chromatin originating from dead cells and chromatin from NETing neutrophils. The role of nucleases clearly depends on their ability to fragment chromatin partially or completely. Enzymes that fragment chromatin without degrading nucleosome-bound DNA enhance inflammation by generating pro-inflammatory mono-

Figure 6. Histone-DNA Synergy Enables Cytokine Induction below the Histone Cytotoxicity Threshold

(A) Human monocytes incubated with purified EGFP-tagged mono-nucleosomes at the indicated concentrations for 2 h stained with a live/dead dye and analyzed by flow cytometry. Data are representative of three independent experiments.

(B) Human monocytes incubated with purified EGFP-tagged mono-nucleosomes at the indicated concentrations for indicated time points as in (A). Time points depict percent total death or fraction of live GFP-positive cells over time for each concentration of nucleosomes. Data are representative of three independent experiments.

(C) Total IL-1β induced in human monocytes incubated with either LPS (55 pg/mL) or increasing concentrations of human mono-nucleosomes. Data are represented as mean ± SD.
nucleosomes, whereas enzymes that completely remove DNA, such as DNase I, suppress inflammation by disrupting the synergy between DNA and histones.

Histone modifications, such as citrullination, that are found extensively in NETs also regulate the pro-inflammatory capacity of histones. Prior work has implicated PAD4 in atherosclerosis, suggesting that the decrease in the phenotype was associated with a defect in NET formation (Knight et al., 2014; Liu et al., 2018). Another study reported no differences in plaque size between chimeric mice receiving WT control cells and mice receiving PAD4-deficient cells and concluded that NETs were not implicated in atherosclerotic lesion growth but affected fibrous cap thickness (Franck et al., 2018). Our experiments suggest that NETs still form in the plaques of PAD4-deficient animals but that citrullination enhances atherogenesis by potentiating the pro-inflammatory activity of histones. Compared to the more efficient plaque growth suppression by histone targeting or neutrophil protease deficiency (Warnatsch et al., 2015), the intermediate PAD4 deficient phenotype is consistent with a role for PAD4 in potentiating NET function rather than an absolute requirement for NET formation. Therefore, our observations support a role for PAD4 in atherogenesis but lead to a

Figure 7. Citrullinated NET Chromatin Promotes Atherosclerosis

(A) Aortic root cross-sections from female ApoE-deficient or ApoE/PAD4-deficient animals untreated or treated with vehicle (Veh, Fab elution buffer) or an anti-chromatin PL2-3 Fab for 6 weeks on a high-fat diet. Sections were stained with the lipid dye Oil Red O (red) and counterstained with hematoxylin (scale bars, 200 μm). Images are representative of at least 7 animals per group.

(B) Quantifications of average plaque size from several sections plotted as a fraction of lumen area from female ApoE-deficient or ApoE/PAD4-deficient animals untreated or treated with PBS or vehicle (Veh, Fab elution buffer) or an anti-chromatin PL2-3 full-length IgG or PL2-3 Fab for 6 weeks on a high-fat diet. Each point represents one animal. Statistical analysis by one-way ANOVA followed by Turkey’s multiple comparison test.

(C) Plasma concentrations of IL-1β from mice in (A). WT mice fed on regular chow until the same age were used as baseline controls. Each point represents one animal. Statistical analysis by Mann-Whitney test.

(D) Inhibition of total IL-1β induction in WT murine BMDMs stimulated with 10⁻⁸ M mono-nucleosomes in the presence of PL2-3 IgG or Fab, anti-histone H3 (α-H3 IgG), or a control IgG. Data are represented as mean ± SD.

*p < 0.05; **p < 0.01; ***p < 0.001; ns, p > 0.05.
reinterpretation of the mechanistic basis for PAD4-dependent phenotypes. Citrullination enhanced the binding of nucleosomes to TLR4, which provides a new mechanistic paradigm for citrullination and potentially other histone modifications in regulating the pro-inflammatory properties of NETs.

Our atherosclerosis and infection experiments now implicate chromatin histones not only as cytotoxic agents but also as signaling mediators that enhance inflammation during infection and sterile disease. Chromatin sensing might play beneficial or detrimental roles in potentiating inflammation during infection, depending on the context. The beneficial effects of DNase administration during infection argue toward a detrimental contribution of NET histones in hyperinflammatory scenarios (Pillai et al., 2016). Furthermore, uninfected mice tolerated the administration of the anti-chromatin PL2-3 Fab for several weeks. In contrast to animals deficient in neutrophil proteases that are known to affect inflammation in adipose tissues and elsewhere (Talukdar et al., 2012), we did not observe a statistically significant decrease in circulating cytokines as a result of PL2-3 Fab administration, whose effects were more localized, suggesting that systemic cytokines play a less important role in early atherogenesis. While the long-term effects of nucleosome targeting are unknown, targeting chromatin may provide a therapeutic avenue to treat chronic inflammatory diseases and acute inflammation by reducing pathogenic levels of cytokines without interfering with cytokine induction in response to microbial triggers.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107602.

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**AUTHOR CONTRIBUTIONS**

T.-D.T. designed and performed the majority of the experiments. M.I. designed plasmids, generated recombinant protein, and performed flow cytometry. A.W., D.H., and Q.W. contributed to experiments. V.P. conceived and supervised the study. V.P. and T.-D.T. wrote the manuscript.

**DECLARATIONS OF INTEREST**

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-HA tag antibody | Abcam  | Ab9110; RRID: AB_307019 |
| Histone H3 (citrulline R2+R8+R17) (anti-cit-H3) | Abcam  | Ab5103; RRID: AB_304752 |
| Rabbit IgG, polyclonal - Isotype Control | Abcam  | Ab37415; RRID: AB_2631996 |
| TLR4 antibody       | Abcam  | Ab22048; RRID: AB_446735 |
| Mouse IgG1 kappa isotype control antibody | Antibodies online | ABIN2704378; RRID: AB_2833099 |
| S100A8 antibody     | Antibodies online | ABIN111892; RRID: AB_2833100 |
| PE Mouse Anti-Human TLR4 (CD284), Clone TF901 | BD biosciences | 564215; RRID: AB_2738674 |
| Alexa Fluor 647-anti-mouse-Ly6G-antibody | Biolegend | 127610; RRID: AB_1134159 |
| Maltose Binding Protein (MBP) antibody | Biolegend | 906901; RRID: AB_2565070 |
| Rab5 antibody       | Cell Signaling Technology | C8B1 - 3547; RRID: AB_2300649 |
| Neutrophil elastase antibody | GeneTex  | GTX72042; RRID: AB_383332 |
| TLR2 neutralizing antibody | Invivogen | pab-hstlr2; RRID: AB_11124921 |
| TLR4 neutralizing antibody | Invivogen | pab-hstlr4; RRID: AB_11125132 |
| PL2-3 anti-chromatin mouse antibody | Losman et al., 1992 | N/A |
| Histone H3 antibody | Millipore | 07-690; RRID: AB_417398 |
| Myeloperoxidase antibody | R&D | AF3667; RRID: AB_2250866 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| AnykD Criterion TGX Precast Midi Protein Gel | Bio-Rad Laboratories | 5671124 |
| Trans-Blot Turbo Midi PVDF membrane | Bio-Rad Laboratories | 1704157 |
| Histone H3 (human recombinant) | Cayman Chemical | 10263 |
| SYTOX green         | Invitrogen | S7020 |
| LPS-SM (LPS from S. minnesota R595) | Invitrogen | tlr1-smlps |
| FSL-1               | Invitrogen | tlr1-fsl |
| CD14 microbeads     | MACS Miltenyi | 130-050-201 |
| Chloride-amidine    | Merk Millipore | 506282 |
| BseRI               | New England biolabs | R0581S |
| PacI                | New England biolabs | R0547S |
| Ndel                | New England biolabs | R0111S |
| AflII               | New England biolabs | R0541S |
| Amylose Resin high flow | New England Biolabs | E8022S |
| Micrococcal Nuclease (MNase) | New England Biolabs | M0247S |
| Cholesterol         | Sigma | C8667 |
| cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail | Sigma-Aldrich | 4693159001 |
| Bafilomycin A1 from Streptomyces griseus | Sigma-Aldrich | B1793 |
| Benzonase® Nuclease | Sigma-Aldrich | E1014 |
| ProLong Gold Antifade Mountant | ThermoFisher scientific | P36930 |
| DAPI(4',6-Diamidino-2-Phenylindole, Dihydrochloride) | ThermoFisher scientific | D1306 |
| LIVE/DEAD Fixable Blue Dead Cell Stain Kit | ThermoFisher scientific | L34961 |
| **Critical Commercial Assays** |        |            |
| Human IFN-beta ELISA Kit | R&D systems | 41410-1 |
| Limulus ameocyte lysate assay | ThermoFisher scientific | 88282 |
| Pierce Fab Preparation Kit | ThermoFisher scientific | 44985 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| IL-1 beta Human Uncoated ELISA Kit | ThermoFisher scientific | 88-7261-77 |
| IL-1 beta Mouse Uncoated ELISA Kit | ThermoFisher scientific | 88-7013-88 |
| Interferon alpha (Cell culture) Human ELISA Kit | ThermoFisher scientific | 411001 |
| IFN-alpha/IFN-beta 2-Plex Mouse ProcartaPlex Panel | ThermoFisher scientific | EPX02A-22187-901 |

**Experimental Models: Cell Lines**

| Human HL-60 HL-60 (ATCC® CCL-240) | ATCC | CCL-240 |
| HEK-Blue hTLR2 | Invivogen | hkb-htr2 |
| HEK-Blue hTLR4 | Invivogen | hkb-htr4 |
| HEK-Blue hTLR9 | Invivogen | hkb-htr9 |
| HEK-Blue Null1 Cells | Invivogen | hkb-null1 |
| HEK-Blue Null2 Cells | Invivogen | hkb-null2 |
| 293/hTLR2-HA | Invivogen | 293-htr2ha |
| 293/hTLR4-HA | Invivogen | 293-htr4ha |
| 293/Null | Invivogen | 293-null |

**Experimental Models: Organisms/Strains**

| WT C. albicans SC5314 | Arturo Zychlinsky | N/A |
| TLR9 / C57BL/6-J-Tlr9<sup>flbM<sub>Mmjax</sub></sup> | Hemmi et al., 2000 | N/A |
| TLR4 / B6(Cg)-Tlr4<sup>tm1.2Karp<sub>J</sub></sup> | Hoshino et al., 1999 | N/A |
| STING / B6(Cg)- Sting1<sup>tm1.2Cam<sub>B</sub></sup> | Ishikawa and Barber, 2008 | N/A |
| ApoE / B6.129P2-Apoetm1Unc<sub>J</sub> | Piedrahita et al., 1992 | N/A |
| ApoE/PAD4 / B6.Cg-Pad4<sup>[tm1.1kow]</sup> | This paper | N/A |
| CAG::H2-EGFP / B6.Cg-Tg[HIST1H2BB/EGFP]<sup>1Pa/J</sup> | The Jackson Laboratory | 006069; RRID: IMSR_JAX:006069 |

**Oligonucleotides**

| human ODN 2006 (ODN 7909) | Invivogen | trl-2006 |
| BarnHi-H3.1-Forward primer: 5'-CGCGGATCCATGGCTCGTACTAAGCAG-3' | Sigma-Aldrich | N/A |
| BarnHi-hPAD4-forward: 5'-CACGGATCCATGGCCAGGGGACATTG-3' | Sigma-Aldrich | N/A |
| SalI-hPAD4-Reverse: 5'-GCGGTCGACTCAGGGCACCATGTTCC-3' | Sigma-Aldrich | N/A |
| Xhol-H3.1-Reverse primer: 5'-GAGCTCGAGTTACGCCCCTCTCOCGG-3' | Sigma-Aldrich | N/A |
| TaqMan® Gene Expression Assay, Assay ID: Hs00174092_m1, human IL1A | ThermoFisher scientific | 4331182 |
| TaqMan® Gene Expression Assay, Assay ID: Hs00174131_m1, human IL6 | ThermoFisher scientific | 4331182 |
| TaqMan® Gene Expression Assay, Human HPRT1 Hs02800695_m1 | ThermoFisher scientific | 4331182 |
| TaqMan® Gene Expression Assay, human IL1B Hs01555410_m1 | ThermoFisher scientific | 4331182 |

**Recombinant DNA**

| pMAL-c2X plasmid | Addgene | 75286 |

**Software and Algorithms**

| Fiji/ImageJ version 2.0.0 | https://imagej.net/Contributors | https://imagej.net/Fiji |
| FlowJo version 10 | Becton Dickinson | https://www.flowjo.com |
| GraphPad Prism Version 7 | GraphPad Inc. | https://www.graphpad.com/scientific-software/prism/ |

**Other**

| L929 cell culture supernatant | The Francis Crick Institute – Cell services core facility | N/A |
LEAD CONTACT AND MATERIALS AVAILABILITY

Lead contact
This study did not generate new unique reagents. Further information should be requested by the Lead Contact, Venizelos Papayannopoulos (veni.p@crick.ac.uk).

Materials availability
Requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Venizelos Papayannopoulos (veni.p@crick.ac.uk).

Data and Code Availability
This study did not generate or analyze datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
All mice were bred and maintained on a C57BL/6J background in a pathogen free, 12-hour light-dark cycle environment. All experiments were performed under an approved project license and following the UK Home Office regulations under the Animals Scientific Procedures Act 1986 (ASPA). ApoE/PAD4 deficient mice were generated by crossing ApoE deficient (Piedrahita et al., 1992) on a C57BL/6J background with PAD4 deficient mice (Hemmers et al., 2011) on a C57BL/6J background for at least 10 generations. 8-10 week old female mice were used in all experiments unless otherwise specified.

Human blood samples
Peripheral blood was isolated from consenting healthy adult volunteers, according to approved protocols of the ethics board of the Francis Crick Institute and the Tissue act.

Human cell lines
HL-60 is an acute promyelocytic leukemia cell line isolated from a 36-year-old Caucasian female. The cells were expanded in suspension in IMDM supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100U/ml penicillin and 100µg/ml streptomycin. HEK-Blue TLR cells (Invivogen) are human embryonic kidney 293 (HEK293) engineered cell lines that stably co-express the relevant TLR gene and an inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. Cells along with their parental lines were maintained in DMEM supplemented with 4.5g/L glucose and 2mM L-glutamine, 10% heat-inactivated fetal calf serum (GIBCO), 100U/ml penicillin, 100µg/ml streptomycin, 100µg/ml Normocin (Invivogen) and the relevant selection antibiotic as per manufacturer instructions (Invivogen). 293/TLR-HA cells are generated by stable transfection of the HEK293 cell line with the TLR gene fused to the influenza hemaglutinin (HA) tag. Cells along with their parental lines were maintained in DMEM supplemented with 4.5g/L glucose and 2mM L-glutamine, 10% heat-inactivated fetal calf serum (GIBCO), 50U/ml penicillin, 50µg/ml streptomycin, 100µg/ml Normocin (Invivogen) and 10µg/ml blasticidin as per manufacturer instructions (Invivogen). All cell lines were maintained in a 5% CO₂ humidified incubator at 37°C.

METHOD DETAILS

Cholesterol crystal preparation
Cholesterol (Sigma-Aldrich) was solubilized in 95% Ethanol by incubating at 65°C, at a concentration of 12.5mg/ml. Cholesterol crystals were formed by 5 consecutive freezing/thawing cycles, spun down at 13000rpm and resuspended in phosphate-buffered saline (PBS, Gibco), at a concentration of 5mg/ml. Reagents were sterile and endotoxin-free, as confirmed by Limulus amebocyte lysate assay (Thermo fisher scientific).

Neutrophil isolation, NET imaging and preparation
Neutrophils were isolated with a two-step purification protocol using histopaque separation followed by a discontinuous Percoll gradient (Aga et al., 2002). Isolated neutrophils were plated in the presence or absence of 200 µM PAD inhibitor Cloride-amidine (Clamide) (Merk Millipore). After 30 min settling, the neutrophils were stimulated with 0.5mg/ml cholesterol crystals. After 4 hours of incubation at 37°C, NET formation was confirmed by visualization using 0.1 μM SYTOX green (Invitrogen) and images were captured using a LEICA DMIRB microscope (20x objective) and analyzed using Fiji/ImageJ software. NET release was quantitated using ImageJ software as previously described (Papayannopoulos et al., 2010) and results were plotted using the frequency function in Microsoft Excel as the area of distribution of SYTOX+ events relative to total cells counted by phase-contrast microscopy. For immunofluorescence analysis of NETs, 4 hours after cholesterol crystal stimulation, cells were fixed for 20 min at RT with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Subsequently, samples were blocked with 2% bovine serum albumin (BSA) and 2% donkey serum in phosphate-buffered saline and incubated with anti-histone 3 citrulline R2+R8+R17 (Abcam),
anti-human neutrophil elastase (GeneTex) and anti-human myeloperoxidase (R&D) antibodies, followed by Alexa Fluor 488-conjugated donkey anti-goat, Alexa Fluor 568-conjugated donkey anti-rabbit and Alexa Fluor 647-conjugated donkey anti-mouse (all by Invitrogen). Samples were counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride; ThermoFisher Scientific) before being mounted in ProLong Gold (ThermoFisher Scientific) and examined by confocal microscopy. Images were analyzed with ImageJ v2.0 software. For NET preparations, after overnight incubation with cholesterol crystal stimulation, the culture medium was removed and a restriction enzyme mix containing BseRI, Pacl, Ndel and AlIII (SU/ml in Cut Smart NEB buffer, New England Biolabs) was added and incubated at 37°C for 30-60 min to achieve partial NET digestion. NET DNA concentration was determined using Quant-iT PicoGreen dsDNA Reagent (Thermo Fisher Scientific) after a Proteinase K (New England Biolabs) treatment (100ug/ml for 2hours at 56°C). Equal amounts of NETs containing 120ng of DNA were analyzed by Western-blot using anti-histone H3 citrulline R2+R8+R17 (Abcam), anti-human histone H3 (Millipore) anti-human myeloperoxidase (R&D) and anti-human S100A8 (antibodies online).

Nucleosome isolation
HL-60 cells or cells from homogenized lung, liver and spleen of WT or CAG::H2B-EGFP transgenic mice (Hadjantonakis and Papaioannou, 2004), were lysed on ice in a buffer containing 20mM HEPES pH7.5, 0.25M Sucrose, 3mM MgCl2, 20mM KCl, 0.1% NP-40, 1mM DTT, 0.4mM PMSF and 1x Complete protease inhibitor cocktail tablet (Sigma-Aldrich). The lysate was then layered over equal volume of lysis buffer containing 2M sucrose and spun at 800 g for 20 min. Pelleted nuclei were resuspended in 20mM HEPES pH7.5, 3mM MgCl2, 0.2mM EGTA, 1mM DTT, 0.4mM PMSF and 1x Complete protease inhibitor cocktail tablet. An equal volume of the same buffer supplemented with 0.6M KCl and 10% glycerol was added over gentle vortex. After a short incubation on ice, the preparation was pelleted at 17500 g. The pellet was resuspended using a douncer in 20mM HEPES pH7.5, 0.4M NaCl, 1mM EDTA, 5% glycerol, 1mM DTT, 0.5mM PMSF and 1x Complete. After centrifugation at 10000 g, pellets were resuspended in a high salt buffer containing 20mM HEPES pH7.5, 0.65M NaCl, 1mM EDTA, 0.34M sucrose, 1mM DTT, 0.5mM PMSF and 1x Complete, and were homogenized using a tight dounce for 40-50 strokes. After a final centrifugation at 10000 g to remove any unopened nuclei and nuclear debris, the supernatant was dialyzed overnight into a low salt buffer of 20mM HEPES pH7.5, 0.1M NaCl, 1mM EDTA, 1mM DTT and 0.5mM PMSF, using a 2kDa dialysis cassette (Thermo Fisher Scientific). To digest the nucleosome preparations, a final concentration of 3mM CaCl2 was added for digestion with 10U/ml micrococcal nuclease (MNase, New England Biolabs) or 2mM MgCl2 for the digestion with 50U/ml Benzonase or 20U/ml DNase I (both Sigma-Aldrich). DNA amounts and fragment size were assessed by electrophoresis after Proteinase K treatment on a small sample at a 1% agarose gel. Nucleosomal DNA digestions were terminated by Ca2+ chelation with 50mM EGTA and the salt concentration was increased to 0.65M NaCl. To prepare Proteinase K-digested nucleosomes, after MNase digestion to a 0.2kb DNA length, preps were incubated with proteinase K and then heat-inactivated at 99°C. Lack of intact proteins was confirmed by electrophoresis and InstantBlue Coomassie Stain (Expeidon Protein Solutions). EGTA was dialyzed away using a 2kDa dialysis cassette and any aggregates were removed with high speed centrifugation before use.

Generation of recombinant MBP-Histone H3 and hPAD4 enzyme
Histone H3.1 was amplified from a cDNA library derived from mouse splenocytes using a BamHI-H3.1-Forward primer: 5-CGGGATCTAGGTCGTACATAGGAG-3 and an Xhol-H3.1-Reverse primer: 5-GAGCTCGAGTTACGCCCTCTCCCCGC-3. A TEV protease cleavage sequence was introduced preceding the H3.1 using BclI (New England BioLabs). Constructs were expressed in BL21(DE3) (Biolabs). A TEV protease cleavage sequence was introduced preceding the H3.1 using BclI (New England BioLabs) and BamHI. The PCR fragment was digested ad gel purified, then ligated to a pMAL-c2x plasmid cleaved with BamHI and SalI (New England BioLabs) was added and incubated at 37°C for 30-60 min to achieve partial NET digestion. NET DNA concentration was determined using Quant-iT PicoGreen dsDNA Reagent (Thermo Fisher Scientific) after a Proteinase K (New England Biolabs) treatment (100ug/ml for 2hours at 56°C). Equal amounts of NETs containing 120ng of DNA were analyzed by Western-blot using anti-histone H3 citrulline R2+R8+R17 (Abcam), anti-human histone H3 (Millipore) anti-human myeloperoxidase (R&D) and anti-human S100A8 (antibodies online).

Analysis of cytokine expression in human monocytes
Peripheral blood mononuclear cells (PBMCs) were isolated from consenting healthy adult volunteers by centrifugation over a Histopaque-1119 gradient (Sigma-Aldrich). CD14-positive monocytes were isolated using MACS CD14 microbeads (MACS Miltenyi Biotec) according to manufacturer instructions. Cells were plated in HBSS with Ca2+ and Mg2+ supplemented with 10% heat-inactivated
fetal calf serum (GIBCO). In some cases, cells were preincubated with 5ug/ml anti-TLR2 and anti-TLR4 neutralizing antibodies (Invivogen, pab-hstlr2 and pab-hstlr4) or 50nM Bafilomycin A1 (Sigma-Aldrich) before being stimulated with the indicated concentrations of NET fragments preparations (500ng/ml as per DNA content, with or without pretreatment with 20U/ml DNase I for 2 hours). Nucleosome preparations (prepared from either HL-60 or H2B-EGFP cells), recombinant human Histone H3 (Cayman), recombinant human Histone H3 citrullinated overnight at 37°C with PAD4 (100µM Histone-3 with 25mM recombinant human PAD4) preincubated with NET DNA (purified from NET fragment preparations cleaned of protein content, using the QiAquick Gel extraction kit by Qiagen following manufacturer’s instructions), CpG ODN 2006 (Invivogen) or LPS from Salmonella Minnesota R595 (Invivogen). All stimulants except for LPS, were pretreated with 50ug/ml of Polymyxin B (Invivogen) to neutralise any potential endotoxin contamination. After overnight incubation, 0.1% NP-40 and 1x complete protease inhibitor tablet were added to the medium and the lysates were analyzed for human IL-1β, IFN-α and IFN-β via ELISA according to the manufacturer’s instructions. For cytokine mRNA level assessment, monocytes were incubated with the indicated stimulants for 2 hours at 37°C. Total cellular RNA was isolated using the Trizol reagent/Chloroform/Isopropanol (Sigma-Aldrich) method. 2 µg of RNA were then used to perform reverse transcription and generate cDNA using the Transcriptor high fidelity cDNA synthesis kit from Roche according to the manufacturer’s instructions. 

**Analysis of cytokine expression in BMDMs**

Bone marrow was isolated from the femur and tibia from WT C57BL/6J mice or animals deficient in TLR4 or TLR9 or STING or CAG::H2B-EGFP transgenic animals (Hadjantonakis and Papaioannou, 2004; Hemmi et al., 2000; Hoshino et al., 1999; Ishikawa and Barber, 2008). Red blood cells were lysed using ACK lysis buffer (GIBCO) and the remaining cells were cultured in DMEM supplemented with 20% L929 cell culture supernatant (The Francis Crick Institute – cell services core facility), 10mM HEPES (Lonza), 1% L-glutamine (GIBCO), 10% FCS (Invitrogen), 100 U/ml penicillin and 100µg/ml streptomycin (GIBCO) and 0.05mM 2-mercaptoethanol (GIBCO) for 7 days. Cells were washed in PBS and collected using 2.5mM EDTA (Invitrogen) in PBS with 5% FCS. Cells were then cultured overnight in DMEM with 1% FCS, 1% L-glutamine, 100µl penicillin and 100µg/ml streptomycin, 10 µM HEPES and 0.05mM 2-mercaptoethanol. Cells were stimulated with the indicated concentrations of HL-60 nucleosome preparations, recombinant citrullinated histone H3 (Cayman) with DNA, CpG ODN2006 (Invivogen) or LPS from Salmonella Minnesota R595 (Invivogen). All stimulants except for LPS, were pretreated with 50ug/ml of Polymyxin B (Invivogen) to neutralise any potential endotoxin contamination. 1% NP-40 and 1x complete was added to the medium and the lysates were analyzed for mouse IL-1β, IFN-α and IFN-β via ELISA (ThermoFisher Scientific) according to the manufacturer’s instructions.

**Immunoprecipitation**

293 cell lines stably expressing the relevant TLR gene fused at the 3’ end to the influenza hemaglutinin (HA) tag were obtained by Invivogen and cultured according to the manufacturer’s instructions. 293TLR2-HA and 293TLR4-HA cells were stimulated for 2 hours with 1 µM MBP-H3 or 1 µM of MBP (preincubated with or without 80ng of NET DNA), 1 µM Nucleosomes from HL-60 or from H2B-EGFP cells (preincubated with or without recombinant human PAD4). Samples were then lysed in a buffer containing 50mM Tris pH-8, 100mM NaCl, 1mM EDTA, 0.05M NP-40, 0.1% sodium deoxycholate, 0.1% SDS and 1x Complete protease inhibitor cocktail (Sigma-Aldrich). MBP-H3 and MBP stimulated samples were incubated with Amylose Resin high flow (New England Biolabs) and Nucleosome stimulated lysates were incubated with a rabbit Histone H3 antibody (Millipore) and subsequently incubated with Sepharose Fast flow beads (Sigma-Aldrich). Samples were eluted in Laemmli buffer before being analyzed on a western blot using an anti-HA tag antibody.

**Western blot analysis**

Samples were resolved on a Criterion TGX precast gel (Any-KD, Bio-Rad Laboratories) and transferred on a Trans-Blot Turbo Midi PVDF membrane (Bio-Rad Laboratories) via semi-dry transfer. The membranes were then blocked in 5% BSA and incubated with anti-human myeloperoxidase (R&D), anti-human histone H3 (Millipore), anti-histone H3 citrulline R2+R8+R17 (Abcam), anti-human S100A8 (antibodies online), or anti-HA tag antibody (Abcam) and then incubated with HRP-conjugated secondary antibodies (Thermo scientific). Finally, the membranes were incubated with enhanced chemiluminescent substrate (ECL, ThermoFisher Scientific) and developed onto a photo film.

**TLR reporter line assay**

HEK-Blue reporter cell lines overexpressing human TLR2, TLR4 or TLR9 (as well as the respective parental lines) were obtained by Invivogen. These cell lines detect stimulants of the respective overexpressed receptors by induction of secreted embryonic alkaline phosphatase (SEAP). Cells were stimulated with recombinant Histone H3 (citrullinated with recombinant PAD4), preincubated with or without 100ng/ml of purified NET DNA, or 0.1 µM Nucleosomes purified from HL-60, EGFP-Nucleosomes purified from Cag::H2B-EGFP mice, 0.5ng/ml LPS (from Salmonella Minnesota R595, Enzo) or 1 µM CpG ODN2006 (Invivogen) or 100ng/ml FSL-1 (Invivogen). The levels of SEAP induction are determined with HEK-Blue Detection (Invivogen), according to the manufacturer’s instructions and absorbance was monitored using a microplate reader (Fluostar Omega, BMG labtech).
Mice and atherosclerosis model

Female mice of 8-10 weeks old were fed for 6 or 16 weeks on a high fat diet (60% energy from fat, Testdiet). ApoE deficient mice were injected once a week for 6 weeks under high fat diet with 0.8mg/kg of the full IgG or the Fab fragment of the mouse anti-chromatin PL2-3 antibody (Losman et al., 1992) (prepared using the Pierce™ Fab preparation Kit by Thermo Fisher Scientific) or equivalent volume of PBS or vehicle (PBS passed through the Pierce™ Fab preparation Kit). Mice were sacrificed by terminal anesthesia and exsanguinated from the jugular vein for a terminal blood plasma sample that was collected in heparin-containing tubes. A PBS perfusion of the arterial system was then performed via the left ventricle of the heart. The heart was fixed with 4% PFA and then dehydrated overnight with 20% sucrose. Hearts were embedded in Optimum Cutting Temperature compound (OCT, VWR) and frozen in a dry ice cooled slurry of absolute ethanol. The sections that comprised the aortic root as determined by the presence of the aortic valve leaflets, were serially sectioned on a Leica CM3050 S Cryostat at a thickness of 10 µm, collected and stored at −80°C. Sections were then stained with Oil Red O (Sigma-Aldrich) in 60% isopropyl alcohol and hematoxylin (RAL Diagnostics). Images were acquired using the Olympus Slidescaner VS-120 and analyzed using OlyVia software (Olympus). The relative plaque lesion area was calculated by using the averages from several consecutive sections measured using Adobe Photoshop and ImageJ software. For each section, the plaque area was analyzed proportionally to the total volume of the aortic root. For immunofluorescence staining of the atherosclerotic lesions, aortic root sections were first dried for 30 min at RT followed by incubation with 2% donkey serum, before being incubated with anti-histone 3 citrulline R2+R8+R17 (Abcam) and anti-mouse myeloperoxidase (R&D) as well as Alexa 647-conjugated anti-mouse Ly6G (Biolegend). Samples were counterstained with DAPI (ThermoFisher Scientific) before being mounted in Pro-Long Gold (ThermoFisher Scientific) and examined by confocal microscopy. Images were analyzed with ImageJ v2.0 software. Plasma samples from total blood of C57BL/6J wild-type (not on high fat diet) or APOE deficient mice after 6 weeks on high fat diet were analyzed for cytokine expression according to manufacturer’s protocol for mouse II-1β (ThermoFisher Scientific). For cholesterol and triglyceride levels, plasma samples were analyzed using the cobas c111 machine (Roche), following manufacturer’s instructions.

For the C. albicans pulmonary infection, wild-type C. albicans (SC5314 clinical isolate) was cultured overnight in yeast extract peptone dextrose (YPED) medium at 37°C before being subcultured in YEPD medium for 4 hours. Male and female wild-type and CAG::H2B-EGFP mice of 7-12 weeks were infected intratracheally with 1x10^6 C. albicans in PBS. To assess microbe load, animals were sacrificed 24 hours post infection, lungs were homogenized in PBS and were plated onto sabouraud dextrose agar plates. To analyze cytokines in bronchoalveolar lavage (BAL), animals were sacrificed 24 hours post infection, lungs were rinsed with PBS and mouse IL-1β was measured by ELISA (ThermoFisher scientific).

Flow cytometry

CD14+ human monocytes, were stimulated with the indicated concentrations of H2B-EGFP nucleosomes for the indicated times or 0.5µM MBP-H3 and 300ng/ml NET DNA for 2hours. For cytotoxicity assay, samples were stained with LIVE/DEAD™ Fixable blue dead cell stain kit (Thermo Fisher) and then fixed with 4% PFA (Sigma). For TLR4 localization, CD14+ human monocytes were stained extracellularly, intracellularly or both with anti-human TLR4 (clone TF901, BD Biosciences) or Isotype control (mouse IgG1,k, clone MOPC-21, BD Biosciences) by using the Foxp3 staining buffer set (eBioscience). All data were acquired on the cell analyzer LSR Fortessa (BD) and analyzed using FlowJo software v10.

Immunofluorescence microscopy studies of TLR4 localization

5x10^4 human CD14 positive monocytes were stimulated with 0.5 µM of MBP-Histone H3 preincubated with or without 300ng/ml NET DNA. After 2 hours, cells were fixed in 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Samples were blocked with 2% BSA and 2% Donkey serum in PBS and stained with antibodies against TLR4 (abcam,), Rab5 (C8B1, Cell Signaling Technology) MBP (BioLegend). Samples were washed and stained with donkey anti-mouse Alexa -488, donkey anti-rat Alexa 568 and donkey anti-rabbit Alexa 647 (all from Invitrogen) before being counterstained with DAPI and mounted in ProLong Gold mounting medium on glass slides and examined by confocal microscopy using the Leica TCS SP5 confocal laser scanning microscope. Images were analyzed with ImageJ v2.0 software.

QUANTIFICATION AND STATISTICAL ANALYSIS

All quantitative data that has been collected from experiments is expressed as mean ± SD as indicated in the figure legends. All statistical analyses were performed on GraphPad Prism Version 7. The significance level for all comparisons was set at 0.05. The type of test employed for each comparison is described on the relevant figure legend. All tests employed were two-tailed. Annotations ns: p > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.