Mechanisms of Neutrophil extracellular trap-mediated signalling in chronic inflammatory disease

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Declaration

I, Theodora Dorita Tsourouktsoglou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Neutrophil extracellular traps (NETs) serve as potent pro-inflammatory cues in chronic sterile inflammatory diseases such as atherosclerosis, by promoting the expression of IL-1β in monocytes and macrophages. However, the molecular basis for this important priming step is unknown. Here we show that histones, rather than DNA, are the major pro-inflammatory component of NETs, recognised by TLR4. Consistent with these findings, chromatin neutralisation blocks murine atherosclerosis and mice expressing EGFP-tagged histones, which interferes with TLR4 activation, have reduced inflammation in a pulmonary fungal infection. We find that monocytes and macrophages show different sensitivity towards histones, depending on the requirement for synergy with DNA. We propose that DNA affects histone-mediated signalling depending on the localization of TLR4. DNA enables histones to activate monocytes, where TLR4 is intracellular, at concentrations below the histones' cytotoxicity threshold, via the translocation of TLR4 to histone-containing endosomes. DNA is dispensable for histone-mediated signalling when TLR4 is expressed on the cell surface. We demonstrate that citrullination of histones and fragmentation of chromatin further potentiate activation. Moreover, we show that histone citrullination is dispensable for NET formation *in vitro* and *in vivo*, but it potentiates atherosclerosis in a sexual dimorphic manner. These synergistic mechanisms make chromatin a driver of chronic inflammation and control the detection of such ubiquitous endogenous DAMPs.

Impact Statement

Our work uncovers the molecular mechanisms that tune the response to omnipresent molecules and drive sterile inflammation. This work can aid the development of targeted therapies specific to chronic sterile inflammation, as well as provide useful insight for academic research on the field of innate immunity and DAMP recognition mechanisms.

We establish a novel role for extracellular chromatin as a major inflammatory signal that drives sterile inflammatory disease *in vivo*. We demonstrate that targeting chromatin can be effective against atherosclerosis and potentially other chronic inflammatory conditions. Current therapeutic approaches focus on neutralising the inflammatory mediator IL-1β, an approach that renders patients susceptible to infections since IL-1β is vital for host protection against pathogens. We identify a potential new therapeutic approach that targets the specific inducers of IL-1β in sterile inflammation, without interfering with the protective roles of IL-1β in infection.

Our work has also yielded important conceptual advances for innate immunity and inflammation research. We uncover a novel mechanism of synergy that regulates the response towards such ubiquitous molecules like histones and DNA. Our findings suggest that immune cells regulate their responses to DAMPs by changing TLR4 localization. This translocation is enabled by DNA co-stimulation, allowing monocytes to be activated by chromatin at concentrations far below its cytotoxic concentrations. These findings offer a significant contribution to the field, proving that DNA in reality plays a co-stimulatory role in inflammation, enhancing the sensitization to histones rather than DNA-bound proteins assisting DNA signalling. Finally, we uncover that PAD4 is not required for NET formation but potentiates the pro-inflammatory activity of NETs. Also, the role of PAD4 in atherogenesis is sexually dimorphic. Together these findings explain discrepancies in conclusions relating to the relevance of NETs and can lead to a better understanding of the high incidence of atherosclerosis in autoimmune disease patients, the majority of which are female.

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Abbreviations

AIM2	absent in melanoma 2
AP-1	activator protein 1
APC	antigen presenting cell
ApoE	apolipoprotein E
ASC	apoptosis-associated speck-like protein containing a CARD domain
BCR	B cell receptor
BMDM	bone marrow-derived macrophages
BSA	bovine serum albumin
CAPS	cryopyrin-associated periodic syndrome
CARD	caspase recruitment domain-containing protein
CCL2	C-C motif chemokine ligand 2
CCR2	C-C motif chemokine receptor type 2
CD	cluster of differentiation
CFU	colony-forming units
CG	cathepsin G
cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
CGD	chronic granulomatous disease
citH3	citrullinated Histone H3
Cl-am	chloride amidine
CLR	C-type lectin receptor
CxCR	chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	foetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor

GSDMD	gasdermin D
HBSS	hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high fat diet
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGB1	high mobility group box 1
ICAM1	intercellular adhesion molecule 1
IFN	interferon
IKK	inhibitor of nuclear factor kappa-B kinase
IL-	interleukin
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
IRI	ischaemia reperfusion injury
JNK	c-Jun N-terminal kinase
KO	knock-out
LDL	low density lipoprotein
LFA-1	lymphocyte function-associated antigen 1
LL-37	cathelicidin
LPS	lipopolysaccharide
LRR	leucine-rich repeat
M-CSF	macrophage colony-stimulating factor
MAPK	mitogen-activated protein kinase
MBP	maltose binding protein
MCP-1	monocyte chemoattractant protein
MD-2	lymphocyte antigen 96
MHC	major histocompatibility complex
miRNA	microRNA
MMP-9	metalloproteinase-9
MPL	monophosphoryl lipid A
MPO	myeloperoxidase
MSU	monosodium urate
MyD88	myeloid differentiation primary response gene 88
NADPH	nicotinamide adenine dinucleotide phosphate

NBD	nucleotide binding domain
NE	neutrophil elastase
NEMO	NF-kappa-B essential modulator
NET	neutrophil extracellular trap
NF-κB	nuclear factor-κB
NLR	nucleotide-binding oligomerisation domain (NOD)-like receptor
NLRP3	NOD-, LRR- and pyrin domain-containing 3
NOD	nucleotide-binding oligomerization domain
Nox2	NADPH oxidase 2
Nuc	nucleosomes
ODN	oligodeoxynucleotide
ORO	oil red o
PAD4	peptidylarginine deiminase 4
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PK	proteinase K
PKC	protein kinase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear cell
PMSF	phenylmethylsulfonyl fluoride
PPAR-α	peroxisome proliferator activated receptor-α
PR3	proteinase 3
PRR	pattern recognition receptor
PSGL	P-selectin glycoprotein ligand-1
RAGE	receptor for advanced glycation endproducts
RIG-1	retinoic acid-inducible gene 1
RLR	RIG-I-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species

S100A8	S100 Calcium Binding Protein A8
SDF-1/CXCL12	stromal derived factor 1
SEAP	secreted embryonic alkaline phosphatase
SLE	systemic lupus erythematosus
STING	stimulator of interferon genes
Syk	spleen tyrosine kinase
TAB	TAK1-binding protein
TAK1	TGFβ-activated kinase 1
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptors
TRAF-6	TNF receptor-associated factor 6
TRALI	transfusion-related acute lung injury
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VCAM	vascular cell adhesion protein
VLA4	very late antigen-4
WT	wild-type

Chapter 1. Introduction

Thesis outline

The immune system employs complex inflammatory responses in order to protect the host, heal damaged tissues and restore homeostasis. Immune cells have developed sophisticated responses towards pathogens, but also towards endogenous sterile stimuli originating from damaged host cells. These responses promote tissue repair but can also trigger chronic pathology, if they are not tightly regulated. Here, we investigate the molecular mechanisms that allow immune cells to communicate and orchestrate complex inflammatory programmes that lead to chronic sterile pathology. We identify chromatin as a major endogenous pro-inflammatory agent and dissect the contribution of its components. We uncover the synergistic mechanisms that regulate the response to chromatin and promote chronic inflammatory pathology during atherosclerosis.

Prologue

Inflammation is a complex response that recruits and programmes immune cells in response to invading pathogens and tissue injury. When properly regulated, inflammation is essential for the effective protection of the host, the healing of the damaged tissues and eventually the restoration of homeostasis.

One of the main triggers for an inflammatory response is an invading pathogen. This response is of critical importance for host defence. It constitutes the first line of defence towards the threat, aiding the containment and clearance of the infection. Control of infection is believed to be the main evolutionary force for the development of the inflammatory response.

Traditionally, immunologists believed that the immune system's function was strictly based around the discrimination of self or non-self, by identifying conserved pathogen-associated molecular patterns (PAMPs) or virulence factors. However, it is now becoming clear that the immune system also focuses on sensing and responding to danger, irrespective of the origin (Matzinger, 1994a). During tissue damage, endogenous molecules are released that trigger an inflammatory programme. The molecules that elicit these responses are vastly diverse, but converge to distinct inflammatory programmes.

Although inflammation is essential for protecting the host, it can also cause severe pathology. The effector mechanisms that the innate immune system utilises are potent and non-discriminating, causing damage and killing host cells. In an infection, the cost-benefit ratio is favourable to the host, quickly clearing the pathogen and the collateral damage is usually limited. When inflammation is triggered by a sterile stimulus which may not be causing damage to the host, the mechanisms that get initiated are of none or unknown benefit to the host. Inflammation-driven tissue damage becomes the dominant outcome and if the stimulus does not get cleared, this process can lead to genesis or exacerbation of disease. In all, the specific mechanisms that link stressful events and orchestrate the inflammatory programme towards non-microbial agents are generally unknown. The work presented here uncovers fundamental mechanisms responsible for shaping sterile inflammatory responses.

Sterile inflammation

Sterile inflammation is driven by various inorganic or organic components that are not of microbial origin. These components can induce injury to the host either directly or indirectly. Chronic inflammation arises when the responsible agents are not contained or cleared and the response persists. In this case, the prolonged production of inflammatory mediators by effector cells such as neutrophils and macrophages, drives tissue damage, fibrosis and excessive collagen accumulation, leading to detrimental chronic pathology. In many cases, whether the inflammatory response is serving a beneficial role to the host is not clear.

1.1 Triggers of sterile inflammation

Sterile inflammation can be triggered by a variety of different components. One of the main triggers of sterile inflammation are crystals. Chronic inhalation of naturally occurring minerals such as asbestos and silica, lead to interstitial pulmonary fibrosis by causing the continuous activation of alveolar macrophages (Mossman and Churg, 1998). Arthropathies are often caused by calcium crystal depositions in the joints and surrounding tissues that drive the destruction of the cartilage (Ea and Lioté, 2004). Sterile inflammation is also responsible for gout and pseudogout, were increased uric acid in the serum, causes the formation of monosodium urate (MSU) crystals that are deposited in all tissues and primarily around the joints. This causes neutrophil infiltration, cartilage destruction and fibrosis (Ragab et al., 2017). Finally, in atherosclerosis, the chronic deposition of cholesterol crystals in the arterial wall, leads to the development of atherosclerotic plaques due to the local recruitment of macrophages, neutrophils, T and B cells. The activation of these immune cells leads to inflammation and the expansion of plaques. Eventually, acute plaque rupture or thromboembolisms can block blood flow.

Inflammation also occurs in response to ischaemia reperfusion injury (IRI), that arises when blood supply to a tissue is stopped and reestablished. Hypoxia and the reinstatement of blood flow and oxygen causes inflammation (Eltzschig and Eckle, 2011). IRI occurs during acute myocardial and cerebral infarctions, acute kidney injury and organ transplantations.

In Alzheimer's disease, the characteristic neurotoxicity associates with the activation of microglial cells that surround b-amyloid containing plaques, driving the production of pro-inflammatory cytokines and reactive oxygen species (Weiner and Frenkel, 2006). Even in cancer, chronic inflammation can promote tumour development, dissemination and resistance to treatment (Shalapour and Karin, 2015).

1.2 Damage associated sterile inflammatory triggers

Another principal source of sterile inflammatory triggers are host-derived, non-infectious molecules that get released after cell death or tissue injury. These molecules are kept separate from their relevant receptor in intact healthy tissues, usually because they are intracellular components that will only be released upon cell membrane permeabilisation. In many cases, these signalling components can be kept separate by the compartmentalization that exists in healthy tissue, from cell membranes to the basement membrane and the surface epithelium and vascular endothelium. These non-microbial endogenous molecules are collectively termed damage-associated molecular patterns (DAMPs) and can activate pro-inflammatory pathways in the same way as PAMPs can. Such triggers include nuclear proteins like the high-mobility group box 1 (HMGB1), heat shock proteins and mitochondrial proteins and DNA (Iyer et al., 2009, Rabadi et al., 2012, Wu et al., 2010). In contrast, apoptosis which is an orchestrated cell death program that gets cleared, typically is immunologically silent.

1.3 Cell death as a source of DAMPs

The immune system has evolved mechanisms to limit its responses against unnecessary signals. The type of cell death is important in determining the quality and quantity of released DAMPs. These DAMPs are major determinants of the ensuing level of inflammation (Amarante-Mendes et al., 2018).

Apoptosis, the first programmed cell death type to be described, is characterised by chromatin condensation, fragmentation of the nucleus, shrinkage of the cell body and membrane blebbing. Eventually the cell disintegrates into fragments termed apoptotic

bodies (Amarante-Mendes and Green, 1999). Upon their death, these cells release location signals (like ATP and lysophosphatidylcholine) and cytokines that recruit phagocytes to clear the dead cell matter. However, the preservation of the intracellular structures and plasma membranes, post-translational modifications that dampen the inflammatory potential of DAMPs and alarmins, as well as the rapid cell clearance that follows the location signals, make apoptosis a death that does not drive sizable inflammation (Cullen et al., 2013, Hochreiter-Hufford and Ravichandran, 2013).

Another form of cell death is necrosis. A programmed type of necrosis is necroptosis that is considered more inflammatory than apoptosis. In necroptosis, cells undergo a plasma membrane permeabilisation which results in the release of intracellular content, which includes a variety of proteases, DNA and DAMPs such as HMGB1 (Grootjans et al., 2017). However, it is still very unclear whether the proinflammatory reaction caused is a direct effect of the DAMPs exposed via leakage of the intracellular content or if the material released can produce, activate or modify other DAMPs present in the extracellular space.

Pyroptosis is a regulated cell death form, mediated through the activity of caspase-1 (and caspase-11 in mice or 4 and 5 in humans). Caspases cleave Gasdermin-D (GSDMD) to create an N-terminal p30 fragment that causes the formation of pores in the plasma membrane, leading to the osmotic lysis of the cell (Aglietti et al., 2016, Ding et al., 2016, Liu et al., 2016). A key event in pyroptosis is the release of inflammatory cytokines such as IL-1β, along with other intracellular material that includes DAMPs and fragmented DNA (Fink and Cookson, 2006).

Finally, NET formation from neutrophils (described in detail on page 36) , is an important source of DAMPs in sterile and chronic inflammatory pathology (Papayannopoulos et al., 2011, Warnatsch et al., 2015). Many proteins on NETs have been linked to inflammation and cytotoxicity such as LL-37, HMGB-1, S100A8 and histones.

In summary, there are several known intracellular molecules that can arise from cell death and tissue injury and are pro-inflammatory such as nuclear proteins (HMGB-1) and DNA (nuclear and mitochondrial) as well as various peptides and different cellular chaperones (heat shock proteins).

1.4 Sensing of sterile inflammatory triggers – Pattern Recognition Receptors

The immune system is divided into innate and adaptive components, each with a specific role and responsibility. The adaptive immune system, mainly organised around T and B cells, employs a vast repertoire of antigen receptors. The diversity and size of this repertoire makes it probable for a lymphocyte to recognise an antigen, causing the activation and proliferation of that cell (Goldrath and Bevan, 1999). This is necessary for the generation of an efficient and highly specific immune response, but the selection and maturation process of these receptors can take up to five days, giving pathogens time to establish an infection. By contrast, the innate immune system is rapidly activated and defends the host without the time-demanding need for selection and maturation. The innate immune system employs an armament of effective but low-specificity mechanisms. These include a variety of antimicrobial peptides, reactive oxygen species and the alternative complement pathway. Their role is to initiate an inflammatory cascade to contain the infection and to activate the adaptive immune system. The primary distinction of the innate and adaptive system lies in immune recognition mechanisms.

To initiate an inflammatory response, triggering signals must be sensed by host cells. The innate immune system recognises an emerging infection or tissue stress. Antigen presenting cells (APCs) have their own system of discriminating between self-non self. Janeway, in 1989, suggested that APCs remain quiescent till they get activated via an assortment of germline-encoded pattern recognition receptors (PRRs) that recognize conserved characteristics/structures (Janeway, 1992). The cells that express PRRs are primarily monocytes, macrophages, dendritic cells and B cells, but also other lymphocytes, epithelial cells and fibroblasts.

Pattern recognition receptors are relatively non-specific as opposed to the receptors of the adaptive immune system. They recognise primarily PAMPs but it has become clear that they can also recognise “self” molecules by binding danger host signals produced after cell damage, as a result of infection or sterile cellular injury (Beg, 2002). Upon signal recognition, PRRs initiate multiple signalling pathways that orchestrate antimicrobial and proinflammatory responses (Akira et al., 2006).

There are five identified classes of PRRs. Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the cytoplasmic retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-Leucin Rich Repeats (LRR)-containing receptors (NLR), and absent in melanoma 2 (AIM2) receptors. Here we focus on TLRs as they are one of the primary pathways responsible for IL-1β transcription and therefore crucial in driving sterile pathology.

1.4.1 Toll-like receptors (TLRs)

One of the principal PRR families is the family of Toll-like receptors. They are well-conserved, representing an evolutionarily ancient host defense system. The receptor protein Toll was first identified as a gene controlling the correct dorso-ventral patterning embryo of Drosophila melanogaster. However, in adult flies (Hashimoto et al., 1988), Toll signalling induces the expression of several host defense mechanisms including antimicrobial peptides, and is critical for defense against gram-positive bacterial and fungal pathogens (Lemaitre et al., 1996). Homologs of Toll, named Toll-like receptors were found in other animals including mammals in which they are associated with resistance to viral, bacterial and fungal infections. In plants, proteins with domains resembling the ligand-binding regions of the TLRs are involved in the production of antimicrobial peptides, indicating the ancient association of these domains to the mechanisms of recognition and host defense.

There are 11 known TLRs in mammals and each is devoted to recognising a type of molecular elements. They can recognise molecular patterns of most microbes (PAMPs) and are expressed by many types of cells, including the phagocytic macrophages and dendritic cells, B cells and certain epithelial cells. They are either localized on the cell surface or intracellularly (in endosomes) in which case the ligands need to be internalized before they can be recognized. Their expression levels are modified rapidly in response to pathogens, cytokines and stress.

TLRs can be grouped into subfamilies based on their primary sequence and the type of ligands they recognise. For example, TLR1, TLR2 and TLR6 bind lipids, and TLR7, TLR8, TLR9 bind nucleic acids. However, some can identify various structurally dissimilar ligands. Particularly, TLR4 can recognise numerous different inducers ranging

from bacterial and viral proteins, to heat-shock proteins and other damage associated self molecules.

Structurally, TLRs are transmembrane proteins with an extracellular region of 19-25 copies of a leucine-rich-repeat (LRR) motif, that creates a horseshoe scaffold for ligand recognition (Bell et al., 2003). They have a cytoplasmic signalling region, called the Toll/IL-1R homology domain (TIR) as it is homologous to that of the interleukin-1 receptor (IL-1R) (Bowie and O'Neill, 2000). These conserved domains are crucial for signalling. Interestingly, even though the LRR domains are highly conserved, the different TLRs can be activated by ligands with various different structures. Upon binding of a ligand, TLRs form dimers or oligomers. The dimerisation allows the conformational change of the receptor which is required for the recruitment of downstream molecules via TIR-domain interactions (Dunne et al., 2003). Some TLRs need specific accessory proteins in order to recognise their ligands.

Signalling by TLRs in various cell types induces a diverse range of intracellular responses that together result in the production of inflammatory cytokines (such as IL-1β and interferon (IFN) IFN-α and IFN-β, the type I interferons) and chemotactic or antimicrobial molecules. Different ligands trigger the expression of different molecules based on the pathways involved. After activation, depending on which adaptor molecules will bind to TLRs, two distinct signalling pathways can be activated, leading to the production of proinflammatory cytokines and type 1 IFNs.

The classical pathway activates the NF-κB transcription factors. TLRs also activate several members of the interferon regulatory factor (IRF) transcription factor family through a second pathway and activate members of the activator protein 1 (AP-1) family, like c-Jun, through a different signaling pathway involving mitogen-activated protein kinases (MAPKs). NF-κB and AP-1 act primarily in the induction of expression of pro-inflammatory cytokines and chemotactic factors, whereas the IRF factors are particularly important for inducing antiviral type I interferons.

In more detail, in the MyD88-dependent pathway (after engagement of all TLRs except TLR3) upon binding of a ligand, MyD88 (via its TIR domain in the C-terminus) and Toll-interleukin 1 receptor adaptor protein (TIRAP) are allowed to interact with the TIR domain of the TLR’s cytoplasmic tail (Muzio et al., 1997). MyD88’s death domain (in the N-

terminus) recruits and activates IRAK4 and IRAK1 (via their respective death domains) (Suzuki et al., 2002, Wesche et al., 1997). The IRAK complex then recruits the TNF receptor associated factor-6 (TRAF-6, an E3 ubiquitin ligase) (Ye et al., 2002) and the complex disengages from the receptor and interacts with a complex consisting of the TNF activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2. This interaction causes the phosphorylation of TAK1 and TAB2, causing the complex to translocate to the cytoplasm. There, TAK1 has two important actions. It can activate JNK and p38 (MAPK14) which will activate the AP-1 family of transcription factors. It can also phosphorylate and activate IκB kinase complex which is composed of three proteins: IKKα, IKKβ and IKKγ (also known as NEMO). Activated IKK kinases phosphorylate IκB, which releases the p50 and p65 NF-κB proteins allowing them to translocate to the nucleus and initiate the transcription of genes encoding for pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Karin and Ben-Neriah, 2000).

TLR3 and TLR4 can stimulate the MyD88-independent or Toll/IL1 receptor domain-containing adapter inducing interferon-b (TRIF) - dependent pathway. The TRIF-related adapter molecule (TRAM) is important in the MyD88-independent signalling mediated by TLR4. Stimulation of TLR signalling via the MyD88-independent TRIF-dependent pathway also leads to activation of NF-κB and MAPK signalling pathways, mainly inducing the production of type I interferons, CD86 and IP-10.

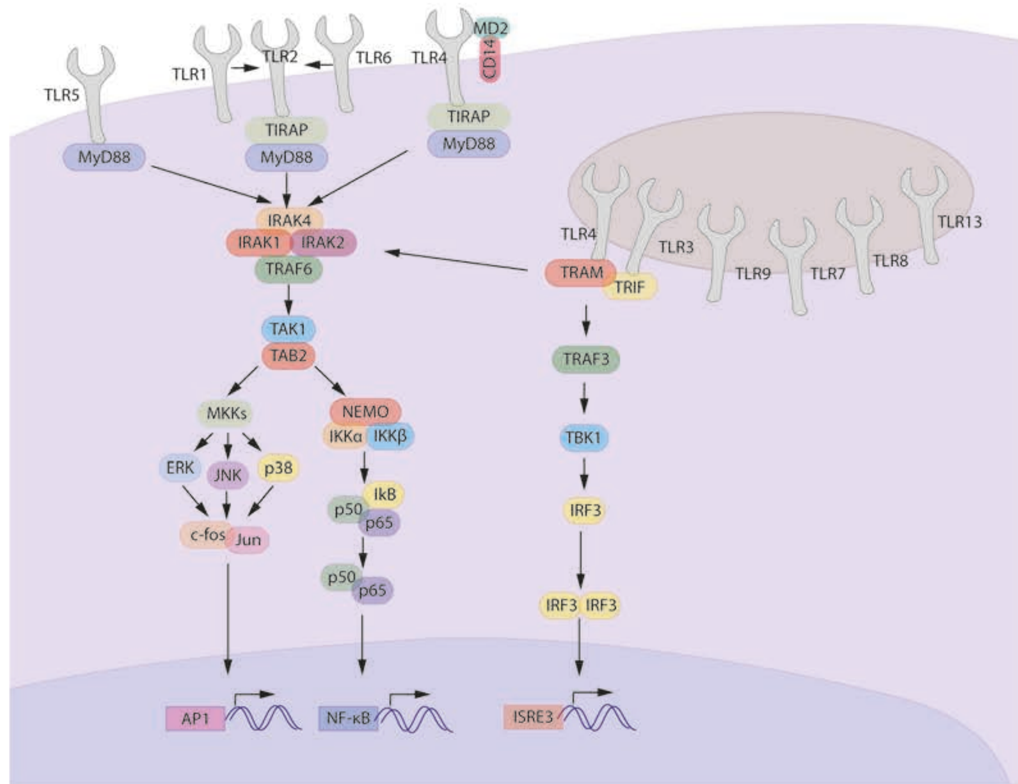

Figure 1. 1 The TLR signalling pathways and downstream effector molecules.

Overview of the key molecules involved in TLR signalling. TLR1, TLR2, TLR4, TLR5 and TLR6 are thought to be on the cell membrane. TLR1 and TLR6 heterodimerise with TLR2 to recognize their ligands. TLR3, TLR7 TLR8 and TLR9 are found intracellularly. The MyD88 pathway is triggered by all TLRs other than TLR3, leading to the transcription of genes under the control of AP1 and NF-κB transcription factors. TLR3 and TLR4 can drive the activation of the TRIF-dependent pathway, leading to the transcription of interferon genes.

1.4.2 TLR Synergy

In adaptive immunity, there is a requirement for two distinct signals for activation of B and T cells: the antigen must bind to the B or T cell receptor (BCR, TCR), and a second receptor-mediated signal is required to license the lymphocyte to respond. For example, in T cells, the TCR must bind an antigen-loaded MHC from an antigen presenting cell (APC), and then CD28 needs to also bind CD80 or CD86 on the APC to initiate T cell activation and proliferation. This molecular mechanism acts as a safeguard to prevent the accidental initiation of harmful autoimmune reactions. This two-signal model exists in innate immunity as well, specifically with TLR receptors (Napolitani et al., 2005). For

example, the activation of TLR receptors by microbial agonists (such as LPS), leads to inflammation, tissue damage and the activation of adaptive immunity. Yet, the same TLR, when activated by endogenous ligands like HMGB1, causes partial immune responses, inflammation is limited and adaptive immunity does not get activated. So, it is clear that the quality and magnitude of the response is regulated by additional factors. This is thought to be the mechanism by which the immune system can distinguish between commensal and pathogenic microbes. Secondary danger or virulence signals are required to be detected concomitantly, to achieve the relevant response (Matzinger, 1994b). Interestingly, antimicrobial responses are also shaped by cross-interference between different PRRs (Negishi et al., 2012).

Utilizing TLR synergy in immunotherapies has proven beneficial as vaccines against parasites that include a concomitant TLR agonist, enhance the parasite specific response. Specifically, the combination use of CpG and monophosphoryl lipid A (MPL) in *leishmania* vaccines to activate TLR9 and TLR4 respectively, has improved vaccine efficacy by driving synergic IL-12 production and inducing an effective T-cell response, leading to reduced parasite burden during disease (Raman et al., 2010).

However, the mechanisms of cooperation and crosstalk induced by different pattern recognition receptor signals remain unknown. The crosstalk between two different receptor-driven signals could be regulating the pathways at various levels, from the activation of downstream regulators to mRNA transcription levels, translation into protein or both. The mechanism that mediates synergy between different TLR pathways has been under intense investigation. TLR synergy is thought to involve the simultaneous activation of MyD88-dependent (TRIF-independent) and MyD88-independent (TRIF-dependent) pathways. For TLRs acting though the same pathway, the evidence for synergy is controversial.

Specifically, it has been reported that simultaneous stimulation of TLR2 and TLR4, causes the production of much greater levels of TNF than observed for each ligand alone (Sato et al., 2000). Also, TLR4 (which signals through MyD88 and/or TRIF), and TLR2 or TLR7 (both signal via MyD88), synergistically increased the production of IL-6 and IL-12p70 (Mitchell et al., 2010). In the same study, simultaneous stimulation of TLR7 with TLR2 or TLR9 (all MyD88-dependent) also caused synergistic production of pro-inflammatory cytokines in dendritic cells. In this study in particular, the double stimulation

of TLR4 and TLR7, or TLR2 and TLR7, drove synergistic increase in protein levels of the NF-κB p65 subunit versus single stimulations, explaining the synergic mechanism. Synergy in TNF and IL-6 production as well as NF-κB translocation to the nucleus has also been observed in bone marrow derived macrophages between agonists for TLR2, TLR5 or TLR9 (MyD88-dependent) together with TLR3 (MyD88-independent). However, with the exception of TLR9, this study could not identify synergy between simultaneous stimulation of any two MyD88-dependent TLRs. This suggests that synergy is more prevalent in combinations which induce both the MyD88 and TRIF pathways, which essentially mimic the dual activation of the two TLR4 downstream pathways (Bagchi et al., 2007).

Cooperativity might vary between different cell types. In mouse macrophages, stimulation of TLR4 and TLR9 causes a synergistic effect in the production of TNF, via enhanced MAPK mediated signalling (De Nardo et al., 2009b). However, in a different study, TLR4 and TLR9 costimulation in human monocyte derived dendritic cells caused the synergistic expression of IL-12p70 but not TNF. In these contradictory studies, the same TLR activators were used (LPS and CpG) suggesting that there are differences between synergic mechanisms between the different APCs.

TLRs can also synergize with other signalling pathways such as the Fcγ receptor and BCR pathways. For example, TLR2 and TLR4 signalling has been shown to augment FcR mediated phagocytosis (Sjoelund et al., 2014b). The analysis of the TLR2/4-induced phosphoproteome in macrophages, showed multiple targets within the FcγR-mediated phagocytosis pathway (Sjoelund et al., 2014a). The TLR-FcγR crosstalk may happen at the level of MAPK and components of the NF-κB pathway, but it can also occur closer to the Fcγ receptor at the level of Syk activation. It was shown that LPS driven activation of TLR4 activates Syk in neutrophils (Arndt et al., 2004). Also, Syk co-immunoprecipitated with TLR4 in neutrophils and their interaction was evident in monocytes as well (Zanoni et al., 2011). That suggests either a direct interaction between these molecules or a presence of a signalling complex. Finally, the nuclear protein HMGB-1, when associating with a CpG oligonucleotide, synergistically increased cytokine production by TLR9 and the receptor for advanced glycation end-products (RAGE) (Tian et al., 2007).

One of the main roles for receptor cooperativity, is to enable ligand presentation to the receptors. For example, it is now clear that nucleic acids which are ligands to TLR7 and TLR9, can be recognized and internalized by the BCR or Fc receptors for presentation and activation of the endosomal TLR7 and TLR9 (Chaturvedi et al., 2008, Rifkin et al., 2005). But the role of BCR in BCR/TLR9 cooperation is not simply the delivery and presentation of the ligand. After BCR activation, total and endosomal levels of TLR9 increase, suggesting that BCR directly regulates TLR9 (Busconi et al., 2007, Chaturvedi et al., 2008). Tight regulation of these pathways is crucial to host homeostasis as TLR7 and TLR9 exhibit opposing pathogenic and protective roles correspondingly in autoimmunity (Bossaller et al., 2016, Christensen et al., 2006, Santiago-Raber et al., 2010). Studies have revealed that BCR-proximal kinases Syk and Lyn govern the BCR-TLR7/TLR9 crosstalk. Lyn, which is associated with the BCR regulation was found to negatively regulate TLR7 and TLR9 activation in B cells *in vivo* (Hua et al., 2014, Lamagna et al., 2014). The mechanism is unknown but might be similar to DCs, where Lyn directly associates with IRF5 and thus inhibits the association of TRAF6 with NF-κB which promotes the activation of the transcription factor (Ban et al., 2016). On the other hand, the activation of Syk promotes the expression of TRAF6 in B cells from systemic lupus erythematosus (SLE) patients (Iwata et al., 2015). This might be the mechanism that allows Syk blockade to reduce TLR9 responses. Finally, another study attributes the BCR-TLR9 synergy mechanism on TAK1 since upon TAK1 inhibition or silencing, the synergistic effect was abrogated (Szili et al., 2014).

TLRs have also been shown to synergize via post transcriptional mechanisms by regulating microRNAs (miRNAs). MicroRNAs are short noncoding RNAs regulating protein production in various ways. TLR activation downregulates miRNAs that inhibit the translation of mRNA of the cytokines into protein (Naqvi et al., 2016). Whereas TLR signalling drives the transcription of cytokine mRNA molecules, other immune receptors such as FcγR could be downregulating the inhibitory miRNAs to allow mRNA translation to happen efficiently, thus potentiating the signal.

Finally, receptor synergy could also be driven simply by inducing close physical proximity. TLR4 (together with its co-receptor CD14) as well as the FcγRs exist on lipid rafts on the cell membrane and these rafts could promote receptor synergy by bringing the two types of receptors close.

In all, TLR synergy is thought to be important to refine immune responses. However, the hypersensitivity of these receptors to microbial endotoxins and other PAMPs suggest that TLRs evolved to be maximally activated by a single microbial ligand. Interestingly most studies of TLR synergy employed bacterial agonists that bear very high affinities for immune sensing receptors. In contrast, affinities towards endogenous DAMPs tend to be of a lower magnitude, suggesting that synergy might have evolved to better regulate the reactivity towards host injury. The molecular basis of these interactions as well as their biological significance still remain largely unknown.

1.5 Effector mechanisms of sterile inflammation

In response to the various inflammatory triggers, many mediators can be produced which in turn influence cell functions and tissues in a variety of ways. These mediators can be produced on demand by local tissue cells, leucocytes such as the tissue resident macrophages or mast cells, or they can be pre-formed and stored in granules and released upon the sensing of triggers. The mediators can be classified into different groups such as cytokines, chemokines and proteolytic enzymes, the vasoactive amines and peptides and fragments of complement components. Mediators also cause the production of other additional mediators, causing a snowball effect. Here, we focus on one of the main drivers of sterile inflammatory conditions, the proinflammatory cytokines of the interleukin-1 (IL-1) family.

1.5.1 Interleukin-1 cytokine family

Interleukins are cytokines secreted by a variety of cell types, allowing cells to communicate during an immune response. The IL-1 family of cytokines are central mediators in immune responses. They cover a wide range of biological functions and target a plethora of different cells and organs. More than any other cytokine group, the family of interleukin-1 cytokines are tightly associated with pathogenic inflammation, autoimmunity and infectious diseases. IL-1 can cause tissue malfunction, especially during chronic inflammation and autoimmune diseases (Dinarello et al., 2012, Medzhitov, 2008).

The family consists of 11 members. Most of the IL-1 family are pro-inflammatory (IL-1α, IL-1β, IL-18, IL-33, IL-36a, IL-36b, IL-36g), a few are anti-inflammatory either directly (IL-37 and IL-38) or by antagonizing the IL-1R or IL-36 receptor (IL-1RA and IL-36RA respectively) (Dinarello, 2018). IL-1, together with IL-33 and IL-18 have a central role in regulating innate immune cells and inflammation. Upon infection, tissue damage or other requirement for a rapid recruitment of hematopoietic cells, the immune system immediately responds with emergency generation of granulocytes in the bone marrow. IL-1 is responsible for the initiation of this critical process, but chronic IL-1 exposure causes the loss and damage of haematopoietic stem cells and their self-renewal capacity (Pietras et al., 2016). Additionally to driving the emergency granulopoiesis program, IL-1 drives the polarization of different myeloid and lymphoid cells.

IL-1 cytokines carry out their function upon binding to their receptors. The family of IL-1 receptors (IL-1R), encompass 10 transmembrane receptors with a similar architecture (Boraschi et al., 2018). The cell surface receptor IL-1 receptor type 1 (IL-1RI) recognizes both IL-1α and IL-1β. It is expressed on almost all cell types and allows IL-1 to drive the production of cytokines, chemokines and other inflammatory mediators. Upon binding of its ligand, the interleukin receptors dimerize via their intracellular Toll/IL-1R homologous region (TIR) domains, a signalling domain that they share with TLRs. This allows the recruitment of MyD88, which then via a series of IL-1R associated kinases (IRAKs) and the tumour necrosis factor receptor-associated factor 6 (TRAF6), leads to the activation of NF-κB, the c-Jun N-terminal kinase (JNK), p38, activator protein-1 (AP1), extracellular signal-regulated kinases (ERKs), the interferon-regulatory factor (IRF) and other mitogen-associated protein kinases (MAPKs) (Martin and Wesche, 2002). In the same way as TLRs, the repertoire of different IL-1 receptors, their differential expression levels as well as the different regulatory molecules offer the specificity of different responses.

IL-1 activity needs to be tightly regulated to prevent uncontrolled chronic inflammation that can lead to disease. This is accomplished by many check points that include the regulation of expression and maturation of the cytokine, intracellular signaling inhibitors, the generation of receptor antagonists (like the IL-1 family IL-1 receptor antagonist, IL-1ra) and decoy receptors like IL-1RII.

1.5.2 Generation of IL-1β

Within the IL-1 family, the best studied member is IL-1β. Undoubtedly, it plays a primary role in host defense against pathogens but it also holds a dominant role in many inflammatory diseases, including atherosclerosis. IL-1β is a potent pro-inflammatory cytokine that is generated by various different hematopoietic cells like monocytes and macrophages (in blood or tissue), dendritic cells and even brain microglia. It is expressed in response to signalling from TLRs and CLRs, other cytokines (including IL-1 cytokines themselves) and activated complement elements (Dinarello, 2011). It regulates crucial functions in sterile inflammation, promoting the upregulation of adhesion molecules responsible for the recruitment of neutrophils and monocytes on the surface of endothelial cells (Wang et al., 1995) and the production of other important pro-inflammatory mediators such as IL-17 by promoting the survival of Th17 cells (Sutton et al., 2006, Sutton et al., 2009). IL-1β has been shown to activate endothelial cells towards a pro-thrombotic and pro-inflammatory route (Nawroth et al., 1986). In atherosclerosis, Il-1β produced by activated macrophages leads to the production of monocyte-attracting chemokines like CCL2 and the platelet-derived growth factor, the proliferation of fibroblasts and smooth muscle cells, the arterial wall thickening and the eventual formation of plaques (Ross, 1999, Raines et al., 1989). In arthropathies such as gout, elevated production of IL-1β leads to joint inflammation and destruction.

IL-1β is released by inflammatory cells, after the cytokine is proteolytically processed by a large protein complex called inflammasome. The full length IL-1β must be cleaved by caspase-1 before the active mature cytokine is released. In order for caspase-1 to be active, its proenzyme form (procaspase-1), must be first recruited to the inflammasome, to undergo proximity induced autoactivation.

The inflammasomes are protein complexes that assemble in the cytosol of activated cells and activate caspase 1 (Martinon et al., 2002). Inflammasomes have a crucial role in host defence against pathogenic infection. They assemble in response to various infectious and harmful insults. There are now many characterised inflammasomes that get activated by distinct stimuli and lead to the activation of caspases. Each inflammasome is named after the specific PRR that gets activated to assemble the inflammasome. Most of the different sensor proteins have a nucleotide-binding domain

(NBD) followed by leucine-rich repeats (LRR). These scaffolding proteins are members of the NLR family. Out of the known inflammasomes, NLRP3 and AIM2 have been shown to be able to sense sterile molecules.

NLRP3 activation plays a central role in various sterile inflammatory diseases. Phagosomal uptake of a variety of structurally diverse stimuli such as cholesterol crystals (Duewell et al., 2010), MSU crystals (Martinon et al., 2006), asbestos and silica (Cassel et al., 2008, Dostert et al., 2008) as well as b-amyloid fibrils (Halle et al., 2008), is followed by phagosome-lysosome fusion and eventually the rupture of the phagolysosome. The release of the phagolysosomal contents into the cytoplasm, together with a rapid efflux of intracellular potassium lead to the assembly of the NLRP3 inflammasome (Di et al., 2018, Hornung et al., 2008). Subsequently, the adapter protein apoptosis associated speck-like protein containing a CARD domain (ASC), assembles into one large complex termed “ASC speck” (Cai et al., 2014, Lu et al., 2014). Many pro-caspase 1 molecules then associate with the ASC specks, leading to caspase-1 autoproteolytic processing and activation. The NLRP3 inflammasome can also be activated in response to PRR activation. For example, the activation of dectin-2, one of the primary C-type lectin receptors, by the fungus *Histoplasma capsulatum* can directly activate the NLRP3 inflammasome for caspase-mediated IL-1β release in DCs (Chang et al., 2017). CLRs are known to recognise DAMPs (Ahrens et al., 2012, Kiyotake et al., 2015, Yamasaki et al., 2008, Zhang et al., 2012), suggesting that similarly to *H. capsulatum*, DAMPs could potentially also activate the NLRP3 inflammasome directly, however this remains currently unknown.

Catalytically active caspase 1 is required for the release of IL-1β and IL-18 (Cerretti et al., 1992, Thornberry et al., 1992). Caspase 1 is activated via the canonical activation of the NLRP3 inflammasome whereas caspase 11 (for mice) and caspase 4 and 5 (in humans) are activated upstream of caspase 1 via the non-canonical NLRP3 pathway (Pellegrini et al., 2017). These are characterised as the inflammatory caspases. Other than IL-1 cytokine maturation, the activation of caspase 1 also leads to the cleavage of the pyroptotic mediator gasdermin D (GSDMD). Gasdermin D in turn, via its N-terminal fragment induces pyroptosis, a lytic form of cell death, further contributing to inflammation (Kayagaki et al., 2015, Shi et al., 2015).

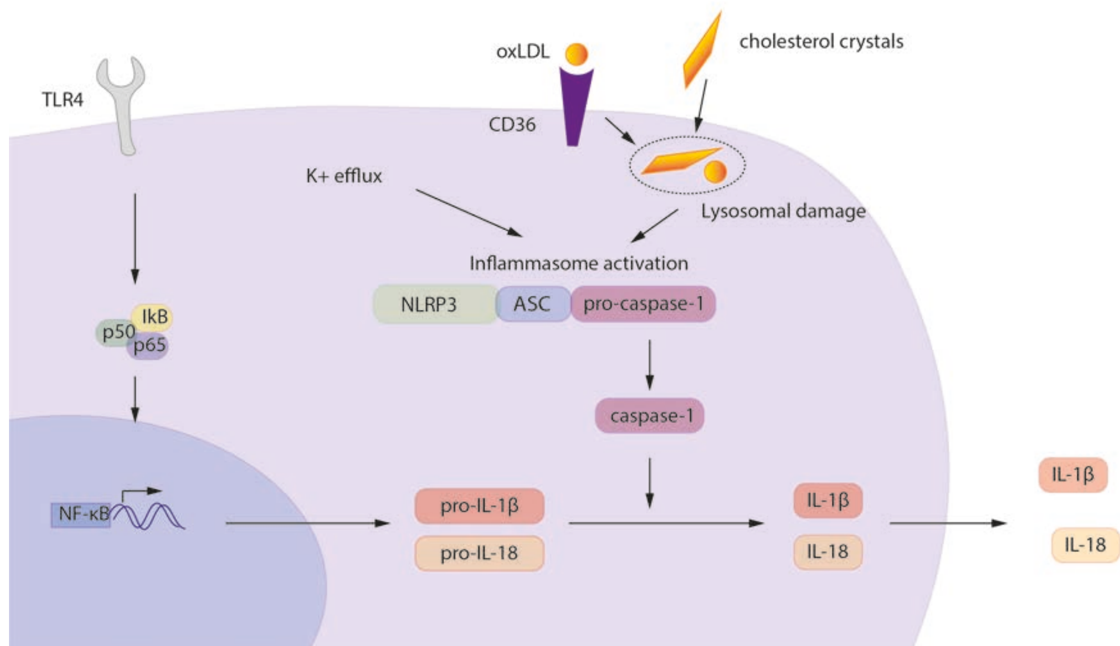

Figure 1. 2 Inflammasome activation and caspase mediated cytokine maturation.

The release of IL-1β requires two distinct signals: the first signal drives the transcription and synthesis of immature pro-IL-1β, and the second signal drives the inflammasome mediated maturation and release of IL-1β. Signals such as the uptake and degradation of lysosomes that contain oxLDL or cholesterol crystals, and the presence of extracellular ATP that drives K+ efflux cause the activation of the NLRP3 inflammasome. This drives the activation of caspase-1 which proteolytically processes pro-IL-1β to its mature form, allowing it to be secreted by the cell. The same process is required for the release of IL-18.

Innate immune cells in sterile inflammation

The cells of the innate immune system are key players in inflammation, both as effector cells that damage and repair tissues and as regulators of the inflammatory response. Here, we focus on the two main drivers of sterile inflammation, neutrophils and blood monocytes/macrophages.

1.6 Neutrophils

Neutrophils are the most abundant and potent effector cells of the immune system. They are rapidly recruited to sites of infection and play a major role in pathogen clearance. They are armed with a wide range of antimicrobials, most of which are made and stored in granules (Borregaard et al., 2007).

Neutrophils are equipped with four different types of granules: the azurophilic, specific, gelatinase and secretory granules (Borregaard and Cowland, 1997). The granules differ in exocytosis capacity and protein content. Azurophilic granules are created first and they contain a variety of proteases like neutrophil elastase (NE), cathepsin G (CG), and proteinase 3 (PR3) and antimicrobial proteins like myeloperoxidase (MPO), azurocidin, lysozyme and defensins (Borregaard et al., 2007). The next granules to be formed are the specific granules that contain collagenase, gelatinase, lysozyme and lactoferrin. Then, gelatinase granules form and contain arginase, gelatinase, lysozyme, CD11b and NADPH oxidase components. Lastly, the secretory vesicles contain mainly receptors for example cytokine receptors like the IFNαR, IFNγR, TNFR, receptors for chemokines like CXCR-1, CXCR-2, CXCR-4, MD-2, CD14, and TLRs (Borregaard et al., 2007). These antimicrobial weapons are crucial to defend the host against pathogens but can also cause great damage to the host tissues. Therefore, their employment is tightly regulated through the processes of phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs).

1.6.1 Neutrophil generation and recruitment

Neutrophils, the most abundant immune cells in the blood, are short lived, with a half-life of about 19 hours (Lahoz-Beneytez et al., 2016). Thus, neutrophils must be continuously replenished. In the bone marrow, the development of myeloid cells starts with a common myeloid progenitor (CMP) which gives rise to a multipotent granulocyte-monocyte progenitor (GMP) (Akashi et al., 2000). The GMP gives rise to a proliferative neutrophil precursor, which is committed to the neutrophil lineage. Recently it was shown that during neutrophil development in the bone marrow, neutrophils cluster in three subsets: pre-neutrophils (preNeus), immature neutrophils and mature neutrophils. The

proliferation-competent progenitors preNeu give rise to post-mitotic immature neutrophils which eventually differentiate into the segmented mature neutrophils. Throughout their development, these neutrophils subsets are characterised by distinct transcriptomic and functional signatures (Evrard et al., 2018).

Once in the blood, neutrophils patrol until they get recruited into tissues by sensing microbe presence or cytokines and chemokines. Neutrophils adhere to the vessel wall, roll and enter the endothelial wall by the process of extravasation. This migration process further primes and activates neutrophils. Initially, the endothelium near the site of infection or inflammation, becomes stimulated by pro-inflammatory molecules like histamine and the platelet activating factor (PAF) leading to the upregulation of selectins. Neutrophils bind selectins via their P-selectin glycoprotein ligand-1 (PSGL-1). These interactions, together with interactions between the neutrophil's integrins lymphocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4), endothelial intercellular adhesion molecule (ICAM-1) and the vascular cell adhesion protein 1 (VCAM-1), slow down the cells and eventually arrest them (Zarbock et al., 2011). After adhering, neutrophils crawl towards the chemoattractant cues, by cytoskeletal rearrangement and integrin recycling. These integrin contacts allow the cells to crawl vertically and even against the direction of the blood flow. Neutrophils finally transmigrate through the endothelium and into the vessel wall primarily between cells by breaking the cell-cell junctions (Filippi, 2019).

1.6.2 Neutrophil immune responses

Neutrophils, like all other innate immune cells, are equipped with a variety of different PRRs that allow them to sense microbes and tissue damage cues (Thomas and Schroder, 2013). The PRRs present on neutrophils include TLRs, CLRs, AIM2, NLRs and RIG-I. After recognition of PAMPs and DAMPs, neutrophils recruit their antimicrobial strategies to respond effectively.

Degranulation

Neutrophils release their granule contents in order to regulate local responses and to create a toxic, microbicidal environment. Granules release their content upon triggers

like the increase of intracellular Ca^{2+}. Degranulation plays a central role in modulating neutrophil biology as it exposes crucial components for neutrophil functions, such as superoxide anion production, cell adhesion and extravasation. Gelatinase granules have been shown to be used first, whereas the azurophilic granules are employed last (Kjeldsen et al., 1992, Sengeløv et al., 1995, Sengeløv et al., 1993). Some granules e.g. the specific granules fuse with the cell membrane, whereas others like the azurophilic preferentially fuse with the phagosomes, targeting the ingested microbes and exposing them to a variety of toxic molecules.

Phagocytosis

Phagocytosis is one of the primary neutrophil antimicrobial strategies and promotes the uptake of opsonised microbes and small particles into an intracellular phagosome. It is a three step process that begins with the binding of a particle that bears a ligand for the phagocytic receptors. The phagocytic receptors include the Fc receptor (FcR), a variety of PRRs (TLRs, NLRs and CLRs like dectin-1). The interaction of multiple receptors with the ligands cause the encirclement of the particle with the neutrophil's membrane (Griffin et al., 1975). The clustering of receptors causes the signal initiation via interaction of their ITAM motifs. This leads to a rearrangement of the cytoskeleton mediated by actin that leads to the uptake of the particle into the cytoplasm, enclosed in the phagosome. Neutrophil preformed granules then fuse with the phagosome, forming a phagolysosome. The secretion of granule contents into the phagosome initiates the digestion of the particle (Amulic et al., 2012, Kruger et al., 2015) .

Generation of reactive oxygen species (ROS)

PRR activation and other types of triggers like the chemical phorbol 12-myristate 13-acetate (PMA) cause the formation of reactive oxygen species (ROS) via a process called respiratory or oxidative burst. ROS include different molecules that vary in stability, activity and membrane permeability. In neutrophils, ROS is predominately produced by the multi-protein NADPH oxidase complex (Nox2). This complex either assembles on the plasma membrane and ROS are released extracellularly, or on the phagolysosomal membrane and ROS are pumped into the phagolysosome (Dupré-Crochet et al., 2013). After assembly, it transfers electrons on to molecular oxygen, producing superoxide anions inside the phagolysosome or extracellularly (El-Benna et al., 2008). Superoxide

anions dismutate to hydrogen peroxide which can be converted to hydroxyl radical •OH (Lambeth, 2004). Also, MPO can convert hydrogen peroxide to hypochlorous acid, which is a highly effective microbicidal (Levine and Segal, 2016, Rosen and Klebanoff, 1979). All oxygen derivatives are able to block microbial growth by causing damage to their proteins, DNA and lipids. Also, ROS are important for the formation of the antimicrobial NETs (described in detail in the next section). NET formation is blocked in mice that are deficient for Nox2 and renders them susceptible to fungal pulmonary infections (Röhm et al., 2014). However, there are reports of NET formation happening independently of Nox2, relying on ROS produced from mitochondria instead (Douda et al., 2015, Hosseinzadeh et al., 2016, Lood et al., 2016b). ROS are not only required to initiate chromatin decondensation that is essential to NET formation, they also contribute to the crosslinking of NET components, strengthening NET stability and contributing to microorganism capture (Csomós et al., 2016).

1.6.3 Neutrophil extracellular traps (NETs)

Another very important antimicrobial strategy of neutrophils, is the release NETs. NETs are extracellular web-like structures that consist of the cell’s cytosolic and granule proteins assembled on decondensed chromatin (Brinkmann et al., 2004b). NETs are able to trap, neutralize and kill fungi (Urban et al., 2006a), bacteria (Brinkmann et al., 2004b), viruses (Saitoh et al., 2012) and parasites (Abdallah et al., 2012). NET formation, or NETosis, is a form of programmed cell death **(Fig. 1.3)**.

The proteins initially identified on NETs were studied on neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA). This molecule causes the activation of protein kinase C (PKC) leading to the production of ROS and eventually the generation of NETs. These NETs contained 24 proteins, primarily histones, neutrophil elastase (NE, a serine protease also called ELANE), myeloperoxidase (MPO), cathelicidins, calprotectin, defensins, proteinase 3 and actin (Urban et al., 2009b). Further work has extended the list of NET associated proteins and it has become obvious that NET content varies with different stimuli. Whether and how the differences affect NET function is yet unclear.

1.6.4 NET release mechanism

NETosis is a slow, programmed, lytic cell death that starts with the arrest of the neutrophil's actin dynamics and depolarization (Metzler et al., 2014). The nuclear envelope disintegrates and the nucleus loses its lobular shape. Nuclear chromatin decondenses and expands into the cytoplasm of intact cells where it associates with cytoplasmic and granular contents (Fuchs et al., 2007). Several hours after stimulation, the cell membrane permeabilises and the NETs are released into the extracellular space where they continue to expand. An alternative mechanism that leads to NET release within minutes, termed non-lytic NETosis, has been reported to occur after stimulation with *Staphylococcus aureus* (Yipp et al., 2012) **(Fig. 1.3)**. This non-lytic response was identified *in vivo* via intravital microscopy and is performed predominately by a fraction of the first neutrophils to arrive at the site of infection.

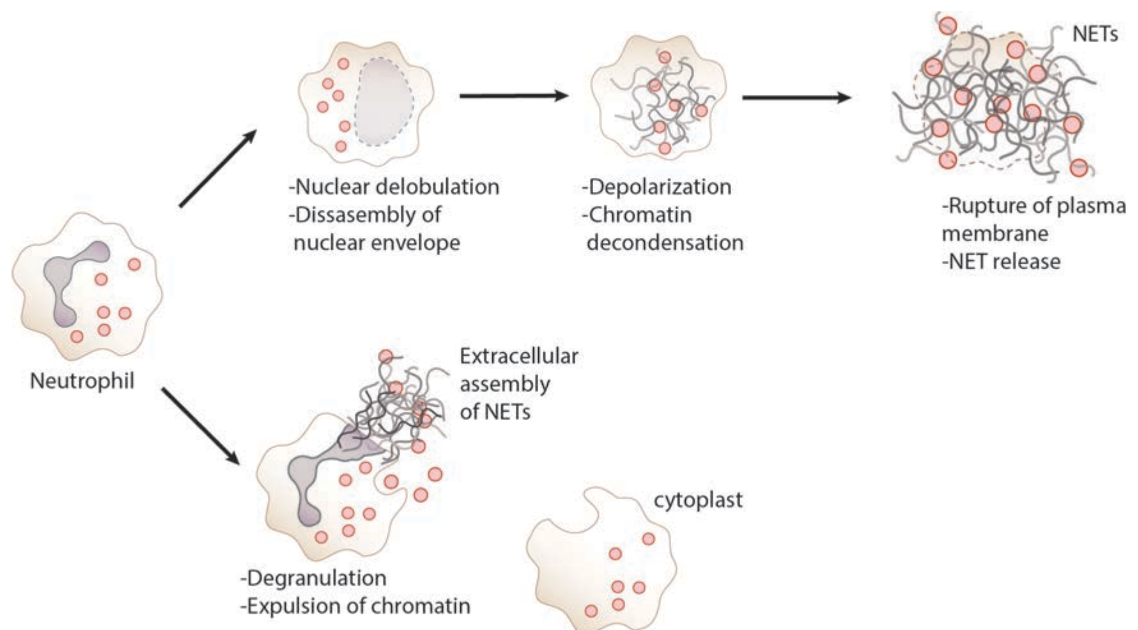

Figure 1. 3 Pathways of NET formation.

Overview of the two pathways of NET formation. NETosis is a cell death pathway that is starts with the delobulation of the nucleus and the nuclear envelope disassembly. This leads to cellular depolarisation and chromatin decondensation, followed by the rupture of the cell membrane and the release of NETs. Non-lytic NETosis can occur separately of cell death and is characterised by the expulsion of chromatin and degranulation. The NET components then assemble extracellularly. During this process, active anucleated cytoplasts are left behind that continue to phagocytose microorganisms.

The receptors that promote NET release are not well defined, however, there are reports implicating specific receptors with NET formation. For example, in contrast to dectin-1 which has been shown to suppress NET formation, dectin-2 which recognises mannose-containing structures on fungi like *C. albicans*, promotes the association of neutrophils with the hyphal forms and the release of NETs (Loures et al., 2015). LPS mediated activation of TLR4 on platelets in sepsis, was shown to drive NET formation via the association of activated platelets with neutrophils (Clark et al., 2007). Finally, LPS was also shown to induce the upregulation of complement C3a receptor on neutrophils, leading to NET formation (Guglietta et al., 2016).

At a molecular level, the mechanism of NET formation involves several granule proteins such as NE and MPO. In resting neutrophils, NE resides in the azurophilic granules and together with other proteins like CG, MPO and azurocidin, they form the protein complex named azurosome. After ROS production by the NADPH oxidase, the H_2O_2 causes NE to disassociate from the azurosome in an MPO-dependent manner. MPO is also required to activate the proteolytic activity of NE and the protease gets released into the cytoplasm (Metzler et al., 2014) where it degrades filamentous F-actin. The degradation of actin causes neutrophil immobilization. Subsequently, NE translocates into the nucleus where it partially digests the histones, causing the DNA to relax and expand. MPO follows NE into the nucleus, causing further decondesation. *In vitro*, this process has been reported to take 4-6 hours (Fuchs et al., 2007, Papayannopoulos et al., 2010). Inhibition of MPO's enzymatic activity does not block NETosis but delays it (Metzler et al., 2011). However its role is crucial as shown by studies on neutrophils of chronic granulomatous disease (CGD) patients (Fuchs et al., 2007) or with total MPO deficiency (Metzler et al., 2011). The same is true for NE, as shown by studies that used NE-deficient mice or NE inhibitors in studying pulmonary infections, sepsis, cancer (Albrengues et al., 2018, Branzk et al., 2014, Cools-Lartigue et al., 2013, Kolaczkowska et al., 2015, Papayannopoulos et al., 2010).

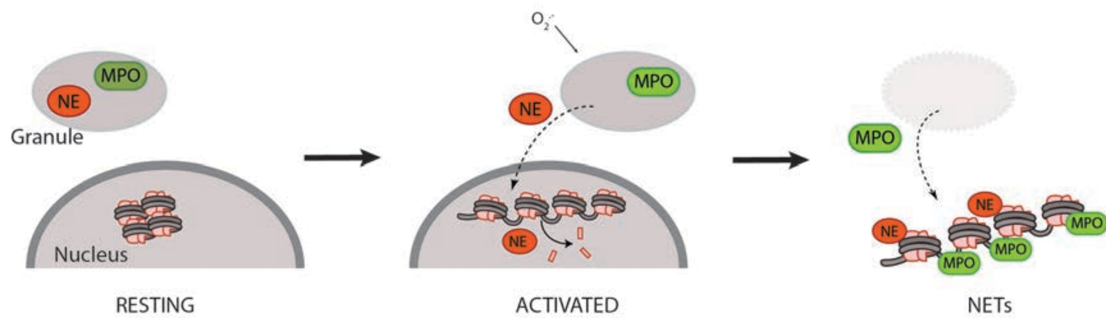

Figure 1. 4 Model of molecular mechanism of NET release. In resting neutrophils, myeloperoxidase (MPO) and neutrophil elastase (NE) are stored in the azurophilic granules. Upon activation, reactive oxygen species are produced, inducing the translocation of NE to the nucleus. There, NE cleaves histones, leading to chromatin decondensation. Finally, MPO binds to chromatin in the later stages, further promoting decondensation, leading to NET release. Modified image from (Papayannopoulos et al., 2010)

1.6.5 Regulation of NET formation

Aberrant NET release has been linked to several inflammatory and autoimmune diseases. In order to avoid NET mediated pathology, NET formation must be tightly controlled. One factor that can regulate NET formation is the size of the particle (microbe or sterile stimulants like crystals). When small particles are phagocytosed, the fusion of azurophilic granules with the phagosome results in the sequestration of NE away from the nucleus. However, in the case of particles too large to be phagocytosed, NE will be allowed to translocate to the nucleus to cause chromatin decondensation. Moreover, NE is released into the cytoplasm, degrading the actin cytoskeleton, and blocking phagocytosis in the process (Branzk et al., 2014, Pieterse et al., 2016). This mechanism allows neutrophils to form NETs selectively in response to microbes that can evade phagocytosis, minimizing NET-mediated pathology. This regulation has been shown to be disrupted in mice with deficiencies in phagocytosis. For example, mice lacking dectin-1 that mediates fungal phagocytosis, have extensive NET-mediated pathology after being challenged with small fungi (Branzk et al., 2014). Smaller microorganisms can also evade phagocytosis and drive NETosis by aggregating as observed with *mycobacterium bovis* (Branzk et al., 2014). Other microorganisms have developed mechanisms to evade NET formation by inhibiting the mechanisms of NET release. Specifically, *Pseudomonas aeruginosa* binds Siglec-9 on neutrophils and inhibits ROS generation, as well as drives the production of IL-10 (Khatua et al., 2012). Also, there are pathogens that have

developed the ability to degrade NETs that form, via the secretion of endonucleases (Beiter et al., 2006, Buchanan et al., 2006, Sumby et al., 2005, Thammavongsa et al., 2013).

1.6.6 NET citrullination

During NET formation, histones are post-translationally modified in a process called citrullination, facilitating chromatin decondensation (Wang et al., 2009a). Citrullination is the post-translational modification mediated by peptidylarginine deiminases (PAD). PAD4, the enzyme responsible for histone citrullination in neutrophils, replaces ketimine groups of arginine residues with ketone groups, changing them into citrulline residues, in a calcium dependent manner. This causes a change to a less positive charge on histones. Citrullination of histone 3 has been used as a marker for NET formation reporting NET release reliably (Branzk et al., 2014). However, other stimuli such as T-cell derived perforin/granzyme B pore formins that are implicated in rheumatoid arthritis can also induce hypercitrullination of a large number of proteins without causing NET release (Romero et al., 2013).

Inhibiting PAD4 causes decreased histone citrullination and many studies have reported a block in NET formation, suggesting citrullination is a process required for chromatin decondensation during NET formation. However, it seems that the role of citrullination might be dependent on the stimulant. For example, some reports argue that NETs induced by phorbol-12-myristate-13-acetate (PMA) are not citrullinated (Kenny et al., 2017, Radic and Neeli, 2013) but physiological NET stimulants such as cholesterol crystals and fungi induce citrullination (Branzk and Papayannopoulos, 2013, Warnatsch et al., 2015). More recent studies have suggested that depending on the stimulus, citrullination occurs during NET formation but is not required for NETs to form (Kenny et al., 2017, Warnatsch et al., 2015). Adding to the confusion around this issue, many studies examining the role of PAD4 in NET formation, employ citrullinated histone 3 or 4 as the only marker for demonstrating the lack of NET formation (Leshner et al., 2012a, Li et al., 2010b, Wang et al., 2009b). This has caused difficulties in understanding whether citrullination is required for NET formation and has yet to allow any studies to explore whether it plays a role in NET function.

It is unclear whether histone citrullination is sufficient to cause chromatin decondensation without the activity of NE. Inhibition of NE activity abrogates chromatin decondensation but histone H3 still gets citrullinated (Branzk et al., 2014). This suggests that histone citrullination is a characteristic of the NET formation process that happens independent of NE activity. Moreover, recent findings support that citrullination plays a role at a late stage of chromatin decondensation by mediating the disassembly of mononucleosomes to free DNA and histones (Chen et al., 2016). In all, the role of citrullination on NETs is not completely understood and while it may not play a crucial role in NET formation, it could be relevant to NET function.

1.6.7 NETs in immune defence

NETs contain an array of proteins that are important in phagocytosis, degranulation and regulation of cytokine secretions, making it challenging to identify their contribution in immune defence separate of their other processes. From the initial NET description, it was suggested that NETs are responsible for trapping microbes, targeting them with a high local concentration of antimicrobials (Brinkmann et al., 2004b). The role of NETs in supporting host defence was clear after gene therapy of a CGD patient suffering from aspergillosis restored NET formation and allowed the elimination of the pathogen (Bianchi et al., 2009). It is not clear whether NETs actively kill microbes but they can trap and prevent dissemination as well as facilitate the work of antimicrobial proteins and phagocytes. For example, in the case of the helminth Strongyloides stercoralis, NETs are induced but unsuccessful in directly killing the larvae. However, NETs trap the larvae and facilitate their killing by macrophages and neutrophils (Bonne-Annee et al., 2014). Also, bacterial strains that express DNases and are able to degrade NETs are more virulent (Buchanan et al., 2006). The microbicidal capacity of NETs depends on the microbe. For example, NETs form in response to *Neisseria gonorrhoeae* but do not kill it. In contrast, NETs can control the growth of *C. albicans* and *A. fumigatus* and are able to kill Lactobacillus crispatus (Bonne-Annee et al., 2014, McCormick et al., 2010, Urban et al., 2006b) .

The ability of NETs to kill fungi is attributed to antifungal NET proteins such as calprotectin, an ion chelator that scavenges manganese and zinc, starving the fungi of essential nutrients (Bianchi et al., 2011, Urban et al., 2009a). Moreover, histones

(Kawasaki and Iwamuro, 2008), DNA (Halverson et al., 2015), cathelicidins and defensins (Ganz, 2003, Tecle et al., 2010) are potent antimicrobial components of the NETs but whether they contribute to NET-mediated killing is not well-defined.

1.6.8 NETs in immune pathology

Although NETs are protective against infection, they have also been associated with pathology in different conditions. The pathogenic roles of PAD4 have been attributed to aberrant NET formation. Blocking or genetically abrogating PAD4 protects animals against acute kidney injury, rhabdomyolysis, haemorrhage and sepsis, and even cancer. NETs have been also implicated in the development of thrombosis (Fuchs et al., 2010, von Brühl et al., 2012), vessel occlusion with thrombi (Jimenez-Alcazar et al., 2017) and transfusion-related acute lung injury (TRALI) (Thomas et al., 2012). NETs are also a source of self-antigens increasing the risk for autoimmune disease. However, NETs can also drive the resolution of inflammation by degrading pro-inflammatory cytokines and chemokines via serine proteases (Schauer et al., 2014).

During sepsis, neutrophils and NETs are critical in controlling the pathogens and improve survival in the early stages of the septic shock (Luo et al., 2014, McDonald et al., 2012, Meng et al., 2012). At the same time, NETs have been shown to contribute to organ damage and sepsis mortality (Biron et al., 2018, Czaikoski et al., 2016). Interestingly, in a recent study of a mouse model of sepsis, the reduction of NETs with DNase or a partial PAD4 deficiency (PAD4 +/-) decreases tissue injury and improves survival. Total PAD4 deficiency had even less tissue injury but the bacterial load and inflammation increased due to reduced immune protection. Also, even though digestion of NETs by DNases is detrimental to pathogen control, the digestion of NETs in a controlled manner by two blood DNases is essential in preventing NET-mediated vascular occlusion(Jiménez-Alcázar et al., 2017). These studies are great examples demonstrating the dual role of NETs in both immune protection and pathology.

NETs are directly cytotoxic to endothelial cells (Saffarzadeh et al., 2012, Villanueva et al., 2011) and damage the endothelium in transfusion-related acute lung injury (Thomas et al., 2012). Excessive formation of NETs during pulmonary fungal infections also damages the lung epithelium (Branzk et al., 2014). Blocking NET formation by NE or

PAD4 deficiency prevents hepatic damage in a *S. aureus* sepsis model (Kolaczkowska et al., 2015). Histones are cytotoxic through compromising the integrity of the plasma membrane. However, even though blocking NET histones with neutralizing antibodies reduced toxicity against endothelial and epithelial cell lines (Saffarzadeh et al., 2012), NETs and neutrophils seem to not be the primary source of circulating histones in sepsis (Hamaguchi et al., 2015). In opposition, NETs have been

In addition, NETs drive chronic sterile inflammatory pathology by priming macrophages to produce IL-1β. This mechanism has been linked to the development of atherosclerosis and gout (Mitroulis et al., 2011, Warnatsch et al., 2015). However, the molecular mechanism that allows NET-mediated IL-1β expression from macrophages remains unclear. Some of the NET proteins have been connected to inflammation, like LL-37, HMGB-1 and S100A8 (Lande et al., 2011, Lande et al., 2007b, Papayannopoulos, 2018, Tian et al., 2007, Ulas et al., 2017, Urban et al., 2009b, Urbonaviciute et al., 2008, Vogl et al., 2007). Moreover, NETs drive autoimmunity in SLE by promoting type-I IFNs production in plasmacytoid dendritic cells (Garcia-Romo et al., 2011, Lande et al., 2011, Villanueva et al., 2011). NET DNA becomes oxidised during NET formation causing an increase in its interferonic capacity via the cGas/STING pathway activation, but it remains unclear how NETs gain access to the cytoplasm in order to activate cGas (Lood et al., 2016b).

Overall, NETs are a rich source of DAMPs that can drive chronic sterile inflammation via numerous mechanisms that need to be better characterized.

1.7 Monocytes and Macrophages

Macrophages are cells of our innate immune system that are present in all tissues and necessary for the maintenance of homeostasis. Macrophages are either tissue resident or derive from infiltrating blood monocytes. Tissue resident macrophages are generated from precursor cells in the yolk sac or the foetal liver (Ginhoux et al., 2010, Yona et al., 2013). These macrophages are self-renewing, maintaining the population separately to the circulating monocytes (Hashimoto et al., 2013).

Blood monocytes develop in the bone marrow and then enter the circulation or populate the spleen. Monocytes in the blood play a critical role in the inflammatory response to

different stimuli. Like neutrophils, they express several different PRRs, and can phagocytose microorganisms and dying cells. They are divided into two subsets, mainly identified by their Ly6C expression levels: the short-lived inflammatory monocytes that migrate to sites of infection and inflammation where they trigger inflammatory responses (classical or inflammatory monocytes) which are $Ly6C^{high}$, and a “resident subset” that have a longer half-life and migrate into non inflamed tissues (nonclassical or resident monocytes) which are $Ly6C^{low}$ (Geissmann et al., 2003).

Monocyte recruitment to sites of inflammation is essential for host defence. The monocyte chemoattractant protein-1 (chemokine C-C ligand 2, CCL2) and its receptor CCR2 on monocytes are important in the migration of monocytes from the bone marrow into the blood stream (Serbina and Pamer, 2006, Tsou et al., 2007). Monocytes in the blood move via chemotaxis into tissues, following chemoattractants produced by pathogens or by host cells at sites of invasion or injury such as monocyte chemoattractant protein 1 (MCP-1), the matrix metalloproteinase-9 (MMP9) and stromal cell-derived factor-1 (SDF-1)/ CXCL12.

Early adhesion of monocytes to the endothelium of the blood vessels is mediated by the vascular cell adhesion molecule-1 (VCAM-1). Cells adhere to the endothelial wall with the help of cell membrane integrins (CD11a, CD11b, CD11c and CD18) and the molecules ICAM-1 and ICAM-2 on the endothelial cells. Finally, movement is arrested and cells migrate through the endothelium into the sub-endothelial site of inflammation. There, they give rise to tissue-resident macrophages and dendritic cells (Gerszten et al., 1999, Zhang et al., 2011).

As soon as the leave the blood stream, these monocytes that express high levels of lymphocyte antigen 6C ($Ly6C^{hi}$), rapidly differentiate into macrophages and dendritic cells in a process mediated by the macrophage colony stimulating factor (M-CSF). They produce numerous proinflammatory cytokines such as IL-1β, TNF-α, as well as matrix metalloproteinases (MMPs) to digest dead cells and necrotic tissue. Monocytes are detrimental to the outcome of different pathologies such as cerebral ischemia and atherosclerosis as has been proven by deficiencies or blocking of the CCR2-CCL2 axis, VCAM-1 and M-CSF (Boring et al., 1998a, Cybulsky et al., 2001, Dimitrijevic et al., 2007, Gu et al., 1998, Smith et al., 1995).

Finally, during the second phase of an inflammatory response, a different set of monocytes that are Ly6C^{low} get recruited to the sites of inflammation and express anti-inflammatory factors such as IL-10, TGF-β and the vascular endothelial growth factor (VEGF). They are recruited during the reparative phase and promote wound healing.

1.8 Atherosclerosis

Atherosclerosis is a disease of the medium and large arteries and the principal killer in the western and developing world (Mozaffarian et al., 2015). In this chronic condition, hyperlipidaemia causes the formation of plaques in the arterial walls. As a result of plaque formation, the arterial wall progressively thickens and constricts the blood flow through the vascular lumen. Eventually, plaque rupture and the development of thrombi is the main cause of coronary disease and death.

1.8.1 Plaque initiation

High cholesterol in the blood is considered one of the main causes of atherosclerosis. The presence of elevated levels of low density lipoprotein (LDL) cholesterol in the plasma (even levels characterised as “average” for the western world), causes changes to the endothelial cell permeability of the arteries. This triggers the accumulation of lipids and specifically LDL into the arterial wall, where they adhere and aggregate into the matrix. After the LDL particles collect in the sub-endothelial space, they undergo oxidation and other chemical modifications. *In vitro*, LDL oxidation is promoted by monocytes, endothelial cells and smooth muscle cells.

In a normal artery, the walls have a trilaminar structure. In contact with the blood flow is a layer of endothelial cells resting on a basement membrane. The intimal layer (*intima*) contains a few scattered smooth muscle cells within the extracellular matrix. That extracellular matrix is the barrier between the intima and the *media*. The *media* consists of a layer of tightly packed smooth muscle cells. Dendritic cells and macrophages reside within the aortic *intima* and *adventitia* (the outermost layer) even before atherogenesis.

1.8.2 Leucocyte adhesion, chemoattraction and activation

The first step after the initiation of an atherogenic diet (high in cholesterol) and the consequent deposition of lipids to the arterial walls, is the attachment of leukocytes to the endothelial cells lining the *intima* (Poole and Florey, 1958). This early adhesion step of monocytes and T-cells from the blood flow to the areas of atheroma initiation, is mediated by the vascular cell adhesion molecule-1 (VCAM-1) expressed by the endothelial cells in response to cholesterol, specifically at regions prone to formation of lesions, and mediates leucocyte adhesion (Cybulsky and Gimbrone, 1991, Li et al., 1993). The expression of VCAM-1 begins early after the introduction of atherogenic diet. Modified lipoprotein particles such as oxidized phospholipids and short-chain aldehydes, together with products of lipoprotein oxidation mediated by the transcription factor NF-κB (Collins and Cybulsky, 2001) in the blood flow, are thought to be aiding the maintenance of chronic inflammation in the *intima*. VCAM-1 expression is induced by the pro-inflammatory cytokines (mainly IL-1β, TNF-α) present in the lesions.

VCAM-1 plays a central role in atherogenesis. A strong connection was made after a hypomorphic mutant of VCAM-1 was sufficient to decrease lesion development in atherosclerotic mice (Cybulsky et al., 2001). P- and E-selectin assist the recruitment of leukocytes to the atherosclerotic lesions. Their supportive role was uncovered after the deletion of P- and E-selectin resulted in smaller fatty lesions and plaques in animals susceptible to atherogenesis (Dong et al., 1998, Johnson et al., 1997).

However, VCAM-1 expression is not ubiquitous on the arterial wall but rather, it is focused to specific areas. The branching sites of the arteries and areas that withstand a disturbed and non-laminar flow of blood are predisposed to the formation of lesions. These sites express molecules like superoxide dismutase that combat oxidative stress, or nitric oxide synthase that inhibits the activation of NF-κB (De Caterina et al., 1995, Topper and Gimbrone Jr, 1999). These result in the inhibition of VCAM-1 expression in these areas and therefore its expression is limited to the sites with disturbed flow making these much more susceptible to atherogenesis.

After adhering to the endothelial cells of the walls, the leukocytes transmigrate into the subendothelial space using the junctions between the endothelial cells, following a

chemoattractant gradient. Oxidised LDL, the major cholesterol carrier, induces the secretion of the monocyte chemoattractant protein-1 (MCP-1) and other chemoattractants from the endothelial and smooth muscle cells (Cushing et al., 1990). The gradient of MCP-1 is responsible for the recruitment of monocytes into the intima. In fact, atherosclerotic animals lacking MCP-1 or its receptor CCR2, show a striking reduction in monocyte accumulation and lipid levels on the lesion (Boring et al., 1998b, Gu et al., 1998). Similar roles have been attributed to IL-8, the interferon-γ (IFN- γ)-inducible CXC chemokines (for T cell recruitment) and eotaxin (for mast cell recruitment) (Boisvert et al., 1998, Haley et al., 2000, Mach et al., 1999).

Once monocytes inhabit the arterial intima, they quickly acquire macrophage characteristics. Macrophages take up modified lipoproteins present in the tissue and store them in droplets in their cytoplasm. These lipid-loaded macrophages, known as foam cells, are a characteristic of the fatty streak, the early atherosclerotic lesion, and proliferate inside the intima (Robbins et al., 2013). Fatty streaks primarily comprise macrophage foam cells, a few smooth muscle cells and an *intima* rich in proteoglycans. M-CSF is found to be overexpressed in these areas (Clinton et al., 1992, Rosenfeld et al., 1992), driving the monocyte to foam macrophage switch. M-CSF has been shown to be responsible for atherosclerotic lesion formation (Rajavashisth et al., 1998, Smith et al., 1995). Additionally, granulocyte-macrophage colony stimulating factor (GM-CSF) also contributes to inflammation in the atheroma, by supporting the survival of monocytes that contain MPO that produces hypochlorous acid in plaques, driving oxidative stress and inflammation (Sugiyama et al., 2001).

1.8.3 Plaque formation

Hyperlipidaemia increases the numbers of circulating monocytes and neutrophils in the blood. Excess cholesterol that gets deposited in the subintimal area, leads to the precipitation of cholesterol crystals. Cholesterol crystals and immune cell numbers inside the lesions increase gradually causing the development of pathology. Neutrophils have been shown to accumulate in large arteries upon initiation of high-fat diet (Jongstra-Bilen et al., 2006, Van Leeuwen et al., 2008). Neutrophils then attach and roll onto the arterial lumen in a process mediated by P- and E-selectins, where they get activated and secrete the contents of their granules. Myeloperoxidase, azurocidin and proteinase-3 are

neutrophil granule contents that have been shown to play a role in the adhesion of monocytes and permeability of the endothelium (Lee et al., 2003, Sugiyama et al., 2004, Taekema-Roelvink et al., 2001). These neutrophil proteins also play an important role in macrophage maturation and activation towards an inflammatory phenotype.

Cholesterol crystals are taken up in macrophage phagosomes. This process is triggered by the CD36 receptor (Sheedy et al., 2013) and leads to the activation of the NLRP3 inflammasome, via the damage of the phagolysosomes (Duewell et al., 2010). NLRP3 deficient atherosclerotic mice show a significant reduction in lesion size and cytokine production (Duewell et al., 2010). The secretion of IL-1β and other pro-inflammatory cytokines drives pathology by causing increased permeability of the vasculature and upregulation of chemokines that lead to increased myeloid cell recruitment, establishing fatty streaks and furthering plaque lesion growth. Leucocyte infiltration can also lead to capillary leakage, vasodilation and hypotension.

After these fatty streaks are formed, the budding atheroma slowly develops into a more complicated pathologic lesion. In this stage, the intima thickens, smooth muscle cells proliferate and produce extracellular matrix making the lesion grow in size. Also, the lipid pool (core) of the lesion retains a calcium deposit, a process termed microcalcification. After the establishment of an early atheroma, it’s progression might stabilise and the lesion will have a small lipid core and a thick fibrous cap, or the plaque will become increasingly vulnerable with a larger lipid pool and necrotic core. If inflammation persists further, the lipid core grows and macrophages in the fibrous cap get activated via pro-inflammatory cytokines such as IL-1β and secrete metalloproteinases that degrade the extracellular matrix (Galis et al., 1994, Saren et al., 1996, Sukhova et al., 1999). Also, pro-inflammatory cytokines affect the generation of new collagen. These phenomena, together with local microcalcification (Vengrenyuk et al., 2006) and macrophage cell death (Kolodgie et al., 2000), cause the thinning of the fibrous cap, rendering it fragile and susceptible to rupture. The thin fibrous cap mainly consists of collagen and very few smooth muscle cells. During plaque rupturing, the blood meets with the necrotic core components. Coagulation is activated after the blood comes in contact with tissue factor, a macrophage and foam cell membrane protein which drives coagulation, and a thrombus is created.

Cholesterol crystals drive neutrophils to form neutrophil extracellular traps (NETs) in the atherosclerotic lesions (Warnatsch et al., 2015). NETs can prime macrophages directly to induce the expression of inactive pro-IL-1β. In addition, NET components such as CG and cathelicidins, have been shown to attract monocytes, further driving pathology. In all, NETs play a critical role in driving atherogenesis but the specific NET components and mechanism responsible remain unknown.

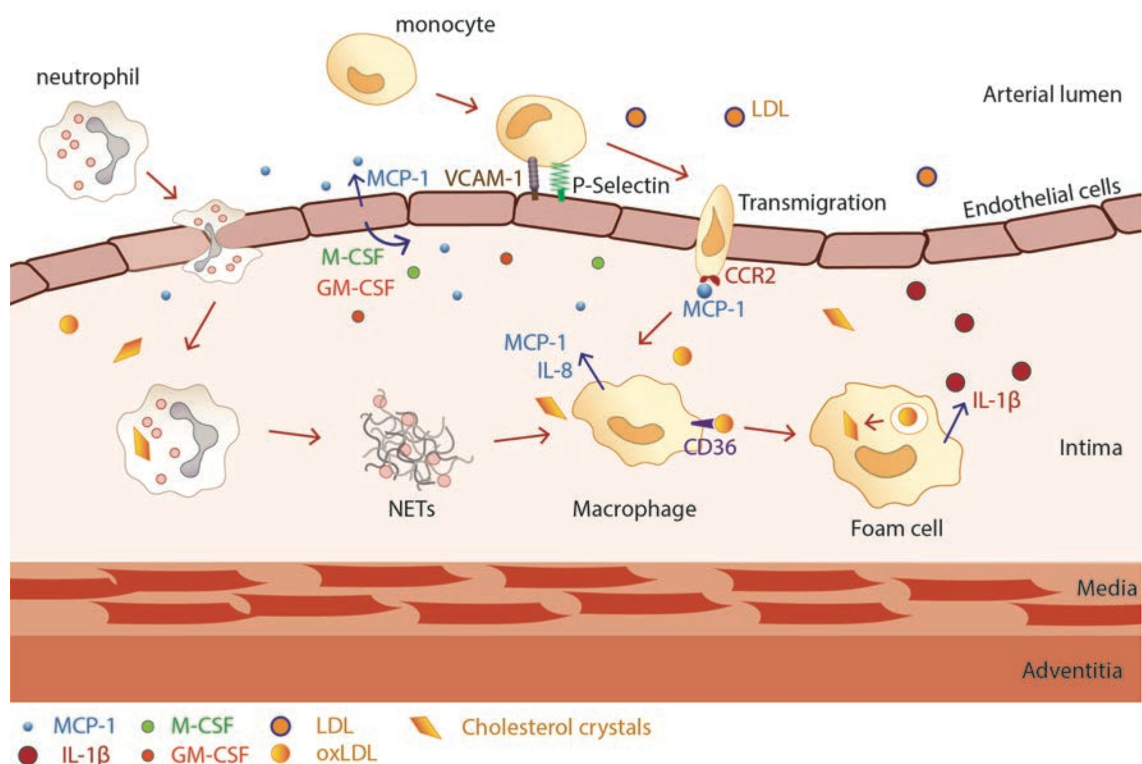

Figure 1. 5 Molecular mechanism of plaque formation in the arteries. Low density lipoprotein (LDL) enters the endothelium where it gets oxidised by macrophages and smooth muscle cells. This leads to the upregulation of adhesion molecules on the endothelial surface and the recruitment of monocytes to the forming lesion. Monocytes transmigrate into the intima, where they differentiate into macrophages due to M-CSF and GM-CSF produced by endothelial cells. Macrophages take up lipoproteins via their scavenger receptors (CD36) and form cholesterol crystals. Neutrophils form NETs in response to cholesterol crystals and NETs prime macrophages to produce IL-1β. At the same time, cholesterol crystals activate the NLRP3 inflammasome in macrophages, causing finally the release of IL-1β.

1.8.4 Targeting IL-1β to prevent inflammation

Preventing inflammation directly or indirectly is the main focus of existing therapies towards atherosclerosis. The main drugs known to be effective against atherosclerosis target lipid biosynthesis like statins that directly inhibit hydrocymethylglutaryl coenzyme A reductase (HMG-CoA reductase). This prevents cholesterol synthesis, reducing the adhesion of leukocytes, macrophage proliferation and therefore immunopathology (Aikawa et al., 2001). Additionally, fibric acid derivatives are used that activate the PPAR-α pathway, a major pathway regulating lipid metabolism. This causes VCAM-1 expression inhibition and tissue factor by the cells in the atherosclerotic lesion (Marx et al., 2001, Marx et al., 1999).

The main role of IL-1β in immune defence is in the acute response to pathogens. IL-1β is protective against bacterial, fungal and viral infections. It drives the rapid recruitment and activation of neutrophils and macrophages in order to phagocytose pathogens and to release toxic nitrogen and oxygen radicals (Sahoo et al., 2011). However, IL-1β plays a critical role in driving immune pathology. IL-1 has been tightly associated with heart disease as it regulates endothelial and smooth muscle cell mitogenesis, leukocyte adherence, lipoprotein metabolism, the thrombogenic response of endothelial cells and vascular permeability. In atherosclerotic plaque formation, IL-1β stimulates the vascular smooth muscle cells via the upregulation of TGF-β. It is also responsible for the endothelial cell expression of adhesion molecules (Bochner et al., 1991). Also, IL-1β upregulates IL-17, a T cell-derived cytokine, which promotes neutrophil recruitment via the chemokines CXCL1 and CXCL2 (Miller et al., 2006, Park et al., 2005).

Studies of neutralisation of IL-1β have provided a lot of insight into the role of the cytokine in inflammatory conditions. There has been a large amount of preclinical studies that support specifically targeting IL-1β. The beginning of IL-1 targeting came with the introduction of a recombinant form of the IL-1 Receptor antagonist (IL-1Ra), Anakinra (Amgen) (Furst, 2004). This molecule blocks IL-1α and IL-1β and has been approved to treat the symptoms of rheumatoid arthritis, cryopyrin-associated periodic syndrome (CAPS) and Still's disease. Anakinra is also beneficial in gout attacks that are unresponsive to standard treatment.

The proinflammatory programme that IL-1β drives in the development of atherosclerosis has led to a clinical trial of an anti- IL-1β antibody, named Canakinumab. Under the clinical trial CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study),

10061 patients were treated with increasing doses of the antibody and the investigated end-points were non-fatal myocardial infarction, non-fatal stroke or cardiovascular death (Ridker et al., 2017a). The trial was completed, proving that IL-1β neutralization is effective at preventing recurrence of cardiovascular disease, specifically reducing the risk of stroke and cardiovascular disease related deaths in high risk patients by 15-17%. An unexpected finding of this trial was that IL-1β neutralisation also protected the patients against arthritis, lung cancer and gout (Ridker et al., 2017b).

The development of IL-1 blocking approaches have aided in understanding the role of IL-1 in inflammation. Interestingly, blocking IL-1 has revealed an underlying inflammatory basis for diseases that were not thought to be inflammatory such as type 2 diabetes (Larsen et al., 2007), hearing loss (Klein and Horneff, 2010, Rynne et al., 2006) and heart failure (Abbate et al., 2010, Van Tassell et al., 2012).

These findings suggest that by further exploring the regulatory mechanisms and the role of the different members of the IL-1 family, current therapeutic tools could be used towards a multitude of diseases with previously unappreciated inflammatory component. Even though these approaches have proven effective towards the control of sterile and auto-inflammation, targeting IL-1 increases the risk of life-threatening infections or even adverse responses to vaccines. Therefore, alternative and more specific anti-inflammatory strategies need to be explored.

1.9 Recognition of chromatin

As mentioned previously, NETs have been shown to drive atherosclerosis by directly activating macrophages to produce IL-1β. However, the specific NET components and mechanism responsible remain unknown. NETs are composed of DNA, histones and various antimicrobial proteins. Here, we describe the mechanisms of recognition of DNA and histones by the innate immune system.

1.9.1 Endogenous DNA recognition

Nucleic acids are sensed by a variety of receptors. TLRs present in the endosome are a predominant group of receptors that recognise invading nucleic acids. TLR3 recognises double stranded RNA (Alexopoulou et al., 2001), TLR7 and TLR8 recognise single stranded RNA (Diebold et al., 2004, Heil et al., 2004, Lund et al., 2004), and TLR9 recognises DNA. Bacterial genomic DNA is a stimulant for TLR9 (Hemmi et al., 2000, Krieg, 2002). It includes unmethylated CpG dinucleotides that get recognised by TLR9 (Ahmad‐Nejad et al., 2002, Latz et al., 2004). DNA viruses also contain genomes rich in CpG motifs and activate TLR9 to drive the production of pro-inflammatory cytokines and type I interferons (Hochrein et al., 2004, Lund et al., 2003). The motifs can only be recognized by TLR9 after degradation of DNA to single strands and the acidification/maturation of endosomes. CpG motifs are very rare and highly methylated in mammalian genomes, which makes them non-immunostimulatory.

DNA in the cytosol activates intracellular sensors like the cGas/STING and AIM2 pathways. STING is an adaptor of the endoplasmic reticulum that is critical for responding to bacterial and viral pathogens via IFN-β induction (Ishikawa and Barber, 2008). STING plays a role in driving chronic inflammation triggered by self-DNA (Ahn et al., 2014) and contributes to autoimmune diseases and in DNA adjuvancy (Ishikawa et al., 2009). Upon stimulation with DNA, cyclic-GMP-AMP (cGAMP) is generated after the cGAS synthase binds to DNA in the cytosol. cGAMP then binds and activates STING driving interferon production (Sun et al., 2013).

Cytosolic DNA can also drive inflammasome activation and lead to the maturation of cytokines such as IL-1β and IL-18. Absent in melanoma 2 (AIM2) is a protein complex that binds DNA and detects bacterial and viral pathogens. In response to cytoplasmic DNA, AIM2 activates NF-kB and caspase-1, driving IL-1β and IL-18 production and maturation (Hornung et al., 2009).

Extracellular self-DNA from dying cells can still signal via the cytosolic DNA receptors. Host DNA is not presented naked and usually associates with proteins. For example, by forming complexes with endogenous proteins such as LL37 or other NET components self-DNA can activate TLR9 (Chamilos et al., 2012, Lande et al., 2007a). Also, DNA associating with HMGB1, the nuclear DNA-binding protein that is released from cells undergoing necrosis, synergistically activate TLR9 cooperating with RAGE, a multiligand PRR (Tian et al., 2007). RAGE and LL37 also increase the nucleic acid uptake further

facilitating signalling (Bertheloot et al., 2016, Chamilos et al., 2012, Sirois et al., 2013). Finally, like bacterial DNA, self-DNA gets oxidised during NETosis, increasing its ability to be recognised and drive inflammatory responses (Gehrke et al., 2013, Lood et al., 2016a).

In conclusion, several DNA sensors have been described in the last few years, providing new insight into the mechanisms of innate immune response to DNA. Interestingly, structural analysis of some of the DNA sensors (e.g. AIM2 and cGAS), have confirmed that DNA recognition is sequence-independent as the receptors form electrostatic interactions with the DNA sugar-phosphate backbone. This suggests that there are additional unknown checkpoints to ensure receptor activation in cases of infection or tissue damage, only as required.

1.9.2 Histone recognition and cytotoxicity

Self-DNA released from dying cells or via NET formation, associates with histones. These evolutionarily conserved basic proteins are present in every eukaryotic cell. They are essential to the tight packaging of DNA. By binding to the negatively charged DNA, they form the nucleosome, the basic unit of structure in chromatin. A nucleosome core contains an octamer of histones, consisting of two of each core histone H2A, H2B, H3 and H4 and superhelical DNA wrapped around it (Luger et al., 1997). Histone H1 is a linker histone that binds on to the complete nucleosome core to form a higher order structure (Allan et al., 1980). All histones share a similar structure. Histones H1, H2A and H2B are lysine-rich and H3 and H4 are arginine rich.

Other than their DNA organisation role, histones are important in regulating gene transcription, via post-translational modifications like the acetylation, methylation and phosphorylation of their N-terminal tails (Bannister and Kouzarides, 2011).

Histones also act as antimicrobial factors. They have been shown to penetrate and destroy bacterial cell membranes. Interestingly, in some cases the antibacterial activity of histones partially depends on their cleavage by a bacterial protease (Tagai et al., 2011). Histones have been shown to exhibit antimicrobial activity against viruses (Hoeksema et al., 2015, Tamura et al., 2003), fungi (Kashima, 1991) and parasites

(Guimaraes-Costa et al., 2009, Wang et al., 2011). They comprise 70% of the protein composition of NETs (Urban et al., 2009a). Antibody neutralisation of histones has been shown to prevent bacterial killing, proving an important role for histones on the antimicrobial capacity of NETs (Brinkmann et al., 2004a).

Histones are known to be cytotoxic and free circulating histones have been directly associated with increased acute inflammation and lethality in sepsis or liver injury in mice (Abrams et al., 2013b, Huang et al., 2011, Kumar et al., 2015, Xu et al., 2011, Xu et al., 2009). The injection of excess amounts of recombinant histones drives TLR4 and TLR2 dependent inflammation and lethality in septic models and anti-histone antibodies are protective. TLR2 and TLR4 deficient mice are protected towards histone-cytotoxicity driven pathology. Histones have also been shown to be cytotoxic towards endothelial and lung epithelial cells (Saffarzadeh et al., 2012) and to induce thrombosis (Ammollo et al., 2011, Semeraro et al., 2011). Also, histones induce the expression of tissue factor (TF), the initiator of blood coagulation by vascular endothelial cells and macrophages, in a TLR2 and TLR4 dependent manner (Yang et al., 2016). C-reactive protein, an acute-phase molecule that gets upregulated in many sterile inflammatory diseases, was shown to modulate histone damaging effects by directly binding to plasma histones and decreasing injury to endothelial cells (Abrams et al., 2013a). Finally, recent work has shown that histone H4 binds and lyses smooth muscle cells, causing tissue damage in arteries and aggravating atherosclerotic inflammation (Silvestre-Roig et al., 2019).

Many of the studies that explored histone cytotoxicity in inflammatory conditions employ excess recombinant histone administration *in vivo* or acute infections that cause massive cell death, making it difficult to uncover whether inflammation is caused by direct histone signalling or by the DAMPs that are indirectly released via histone cytotoxicity. Most importantly, the physiological contribution of endogenous histones in the pathogenesis of sterile inflammatory conditions has not been investigated. Given the cytotoxicity elicited by histones, it is unclear how chromatin might act as a potent signal to immune cells, activating them but without killing them.

1.10 Aims of thesis

Here, we investigate the molecular mechanisms that allow immune cells to orchestrate complex inflammatory programmes that lead to chronic sterile pathology. NETs are potent drivers of sterile inflammation. Previous work from our group demonstrated that NETs are proinflammatory by activating macrophages to produce IL-1β (Warnatsch et al., 2015). With this work, we are aiming to uncover what are the specific NET components responsible for NET-mediated inflammation. Moreover, we are looking to identify the responsible monocyte and macrophage receptors as well as the key mechanisms that regulate cellular responses to these NET components.

Chapter 2. Materials & Methods

Cholesterol crystals preparation

Cholesterol (Sigma-Aldrich, C8667) 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 and thawing cycles. The crystals were spun down and resuspended in phosphate-buffered saline (PBS, Thermo fisher, 10010056), at a concentration of 5mg/ml. All reagents were sterile and endotoxin-free, as confirmed by the Limulus amoebocyte lysate assay (Thermo fisher scientific, 88282).

Human peripheral blood neutrophil and monocyte isolation

Peripheral blood was isolated from de-identified consenting healthy adult volunteers, according to approved protocols of the ethics board of the Francis Crick Institute and the Tissue Act. Briefly, 6ml of whole heparinised blood was overlaid over 6ml of histopaque 1119 (Sigma, 1119) in falcon tubes and centrifuged with a gentle break at 800 x g for 20 minutes. The plasma was kept aside, and the PBMC and PMN layers were separated to be used for isolating monocytes and neutrophils respectively.

Neutrophils were isolated as described before (Aga et al., 2002). The neutrophil layer was washed with 10ml Hank's Balanced Salt Solution (HBSS) without Mg^{2+}, Ca^{2+} or phenol red (Thermo Scientific, SH3058801) containing 0.1% plasma (HBSS - -). After centrifugation at 300 x g for 10 minutes, cells were resuspended in 2ml of HBSS - -. A percoll (GE healthcare, 17-5445-02) gradient was then prepared in 15ml falcons from 2ml layers of 85%, 80%, 75%, 70% and 65% percoll diluted in PBS, in that order. The cells were overlaid on the top of the percoll gradient and centrifuged with gentle break at 800 x g for 20 minutes. The neutrophil layer was removed and washed with 10ml HBSS- - and then resuspended in 2ml HBSS- -. Neutrophils were counted using a Neubauer haemocytometer. For plating, neutrophils were resuspended in HBSS with Mg^{2+}, Ca^{2+} without phenol red (HBSS++, Thermo scientific, SH3026801).

For the isolation of CD14 positive monocytes, the PBMC layer was counted using a Neubauer haemocytometer and washed in 25ml PBS supplemented with 0.5% heat inactivated foetal bovine serum (FBS), centrifuged at 300 x g for 10 minutes. Cells were resuspended in 80ul of PBS + 0.5% FBS and 20ul of CD14 microbeads (MACS Miltenyi, 130-050-201) for every 1 x 10^7 cells and incubated on ice for 15 minutes. The cells were then washed in 25ml PBS + 0.5% FBS, centrifuged at 300 x g for 10 minutes and resuspended in 15 ml PBS + 0.5% FBS, passed through 3 LS columns (MACS Miltenyi, 130-042-401) on a magnetic surface. After washing the columns using PBS + 0.5% FBS, columns were removed from the magnetic surface and flushed with 5ml PBS + 0.5% FBS per column. Cells were counted using the Neubauer haemocytometer before being used. For plating, cells were resuspended in HBSS ++ supplemented with 10% FBS.

Human monocyte derived macrophage generation

After isolation from peripheral blood, human CD14 positive monocytes were cultured for 6 days at 5x10^6 cells per petri dish in 16ml RPMI-1640 supplemented with GlutaMAX™ (Gibco) and 25mM HEPES (Gibco), 10% FBS, 100 U/ml penicillin and 100ug/ml streptomycin (Sigma, P4333) and 10nM recombinant human GM-CSF (MACS Miltenyi Biotec) or M-CSF (. Macrophages were then lifted with PBS (without Ca^{2+} or Mg^{2+}) supplemented with 2mM EDTA and cultured in RPMI 1640 supplemented with GlutaMAX™ and 25mM HEPES, 10% FBS, 100 U/ml penicillin and 100ug/ml streptomycin on coverslips in 24 well plates or in 96 well plates for nucleosome stimulations or were stained for flow cytometry analysis.

Bone marrow-derived macrophage generation

Bone marrow was isolated from the femur and tibia from WT or CAG::H2-EGFP mice or mice deficient in TLR4 or TLR9 or STING (Ishikawa and Barber, 2008, Hemmi et al., 2000, Hoshino et al., 1999). Red blood cells were lysed in 2ml of ACK lysis buffer (ThermoFisher Scientific, A1049201) for 1 minute. After washing and counting, 5x10^6 cells per petri dish were cultured in 10ml DMEM supplemented with GlutaMAX (ThermoFisher scientific, 31331028), 20% L929 cell culture supernatant (in-house preparation), 10mM HEPES (Lonza, BE17-737E), 1% L-glutamine (Thermo Fisher

Scientific, 25030024), 10% FBS (Invitrogen), 1% sodium pyruvate (Sigma, S8636), 100U/ml penicillin and 100ug/ml streptomycin (ThermoFisher Scientific, 15140122) and 0.05mM 2-mercaptoethanol (ThermoFisher Scientific, 50mM, 31350010) for 7 days. 10ml of fresh medium were added on day 4 of culture. Dishes were washed in PBS and cells were incubated with 5ml 2.5mM EDTA (Invitrogen) in PBS with 5% FCS for 10 minutes at 4°C. Cells were washed, counted and then cultured in DMEM with 1% FBS, 1% L-glutamine, 100U/ml penicillin and 100ug/ml streptomycin, 10µM HEPES and 0.05mM 2-mercaptoethanol.

NET induction, imaging and preparation

Isolated neutrophils were plated at $5x10^4$ cells per well in the presence of 200µM PAD inhibitor Cloride-amidine (Cl-amidine) (Merk Millipore, 506282) or DMSO (Sigma, D2650) in 24 well plates in HBSS++. After settling for 30-60 minutes, neutrophils were stimulated with 0.5mg/ml cholesterol crystals.

For NET quantitation, after 4 hours incubation at 37°C with cholesterol crystals, 0.1µM Sytox green (Invitrogen, S7020) was added, NET formation was assessed by fluorescent microscopy. Images were captured using a LEICA DMIRB microscope (20x objective) and the Micro-Manager software (Edelstein, 2014). NET release was quantitated using ImageJ software as previously described (Papayannopoulos et al., 2010). Briefly, the Sytox fluorescence images were transformed into binary images and the area of each Sytox positive event was quantitated. Area values were then distributed into bins of increasing area sizes. Finally, the results were plotted as the area of each Sytox positive event over the total number of cells as assessed by phase-contrast microscopy in the same field, in the respective bins.

For immunofluorescence analysis of NETs, after four hours of cholesterol crystal stimulation of $5x10^4$ cells per well on glass coverslips, cells were fixed for 20 minutes at room temperature with 2% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100 (Sigma) in PBS. Slides were washed with PBS and blocked with 2% bovine serum albumin (BSA) and 2% donkey serum in PBS for 1 hour at room temperature. Samples were then stained with primary antibodies in blocking buffer for 2 hours, washed with PBS, incubated with secondary antibodies in blocking buffer for 1 hour and washed with

PBS again. Samples were counterstained with DAPI (Life Technologies) before being mounted onto glass microscopy slides in ProLong Gold mounting medium (Thermo scientific, P36934) and examined by confocal microscopy. Images were analysed with ImageJ v2.0 software. Primary antibodies: anti-histone 3 citrulline R2+R8+R17 (Abcam, ab5103), anti-human neutrophil elastase (GeneTex, GTX72042) and anti-human myeloperoxidase (R&D, AF3667). Secondary antibodies: Alexa Fluor 488–conjugated donkey anti-goat, Alexa Fluor 568–conjugated donkey anti-rabbit and Alexa Fluor 647–conjugated donkey anti-mouse (Invitrogen).

For NET preparations (NET preps), isolated human blood neutrophils were plated at $1.2x10^6$ cells per well in the presence of 200µM PAD inhibitor Cloride-amidine (Cl-amidine) or DMSO in 6 well plates in HBSS++. After 1 hour, cells were stimulated overnight with cholesterol crystals at 0.5mg/ml. The culture medium was then removed and a restriction enzyme mix containing BseRI, PacI, NdeI and AflII (5U/ml in Cut Smart NEB buffer, all New England biolabs, R0581S, R0547S, R0111S, R0541S) 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, P7581) after a Proteinase K (New England Biolabs) treatment (100ug/ml, 2hours at 56°C). Equal amounts of NETs containing 120ng of DNA were analysed by Western-blot using anti-histone H3 citrulline R2+R8+R17 (Abcam, ab5103), anti-human histone H3 (Millipore, 07-690) anti-human myeloperoxidase (R&D, AF3667) and anti-human S100A8 (antibodies online, ABIN111892).

Nucleosome isolation

3-$6x10^8$ HL-60 cells or cells from homogenated lung, liver and spleen of CAG::H2-EGFP transgenic mice (Hadjantonakis and Papaioannou, 2004), were lysed on ice in 10ml of lysis buffer containing 20mM HEPES pH7.5, 0.25M Sucrose, 3mM $MgCl_2$, 20mM KCl, 0.1% NP-40, 1mM DTT, 0.4mM PMSF and 1x cOmplete (protease inhibitor tablet, Sigma-Aldrich, 11836170001). The lysate was washed 3 times in lysis buffer and then layered over equal volume of lysis buffer containing 2M sucrose and the layers were spun at 800 x g for 15 minutes at 4°C with gentle deceleration. Pelleted nuclei were resuspended in 4ml of 20mM HEPES pH7.5, 3mM $MgCl_2$, 0.2mM EGTA, 1mM DTT, 0.4mM PMSF and 1x cOmplete. Then, 4ml of the same buffer supplemented with 0.6M

KCl and 10% glycerol was added in a dropwise fashion using a Pasteur pipette over gentle vortex. After a 10 minute incubation on ice, the preparation was pelleted at 17500 x g for 30 minutes. The pellet was resuspended using a tight dounce homogeniser in 10ml of a buffer containing 20mM HEPES pH7.5, 0.4M NaCl, 1mM EDTA, 5% glycerol, 1mM DTT, 0.5mM PMSF and 1x cOmplete. Sample was centrifuged at 10000 x g for 10 minutes, pellets were resuspended in 3-5ml of 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 Potter-Elvehjem homogeniser for 40-50 strokes. After a final centrifugation at 10000 x 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, 87718). To digest the nucleosome preparations, a final concentration of 3mM $CaCl_2$ was added for digestion with 10U/ml micrococcal nuclease (MNase, New England Biolabs) or 2mM $MgCl_2$ for the digestion with 50U/ml Benzonase or 20U/ml DNase I (both Sigma, E1014 and D5025). Presence and size of DNA was assessed by electrophoresis after Proteinase K treatment on a 1% agarose gel. When nucleosomal DNA was digested enough, the enzymatic activity was chelated with 50mM EGTA, and the salt concentration was increased to 0.65M NaCl for storage. 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 (Sigma, ISB1L). 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-CGCGGATCCATGGCTCGTACTAAGCAG-3 and an XhoI-H3.1-Reverse primer: 5-GAGCTCGAGTTACGCCCTCTCCCCGC-3. It was then inserted into the pMAL-c2x plasmid cleaved with BamHI and SalI (New England Biolabs). Finally, a TEV protease cleavage sequence was introduced preceding the H3.1 using BclI (New England biolabs) and BamHI. MBP-FXa-TEV-H3.1 (described as MBP-H3) or MBP-FXa (described as MBP) were then expressed in BL21(DE3) Escherichia coli cells. Cultures were grown in Lysogeny broth (LB) medium at 37°C. Expression was induced

at OD_{600}= 0.4 with 200 μM IPTG and cells were incubated at 37°C for 3h. MBP-H3 and MBP were purified in 20mM Tris pH7.4, 200mM NaCl and 1mM DTT using Amylose Resin high flow (New England Biolabs). Protein elution was achieved by adding 10mM Maltose (Sigma-Aldrich).

hPAD4 was amplified from a cDNA library of human neutrophils using the primers BamHI-hPAD4-forward: 5'-CACGGATCCATGGCCCAGGGGACATTG-3' and SalI-hPAD4-Reverse: 5'-GCGGTCGACTCAGGGCACCATGTTCC-3' and subsequently cleaved with BamHI/SalI (New England biolabs). hPAD4 was then ligated to a pBH4 plasmid backbone, containing a 6His-tag on the N-terminal site, previously linearized with BamHI and SalI. 6His-hPAD4 was then recombinantly expressed in BL21(DE3) E.coli cells. Cultures were grown in Lysogeny broth (LB) medium at 37°C. Protein expression was induced at OD_{600}= 0.4 with 200 μM IPTG and cells were incubated at 25°C for 5h. 6His-hPAD4 was purified in 20 mM Tris pH8, 300 mM NaCl and 10 mM Imidazole using Ni-NTA beads. Protein elution was achieved increasing the imidazole concentration to 250mM. The eluate was then dialyzed against 20mM Tris pH8, 200mM NaCl. 1mM DTT and 2mM $CaCl_2$. Ion exchange chromatography was performed using a Q-Sepharose column (Resource™ Q, GE lifesciences, 1ml) and the sample was eluted with a 200-1M NaCl gradient. The fractions containing the enzyme were identified by tandem mass spectrometry and enzymatic activity was confirmed on recombinant histones (Cayman).

Analysis of cytokine expression

All different types of cells used here were isolated from consenting healthy adult volunteers or mice as described earlier. Human monocytes were plated in 96 well flat bottom plates, pre-coated with poly-D-lysine (PDL, Millipore) at $5x10^4$ cells/well in HBSS with Ca^{2+} and Mg^{2+} supplemented with 10% heat inactivated foetal calf serum (Gibco). Human macrophages were plated in 24 well plates after differentiation, at $2x10^5$/well in RPMI with GlutaMax, 10% FBS and 100 U/ml penicillin and 100ug/ml streptomycin. BMDMs were plated in 24 well plates after differentiation, at $1x10^5$/well DMEM with 1% FBS, 1% L-glutamine, 100U/ml penicillin and 100ug/ml streptomycin, 10µM HEPES and 0.05mM 2-mercaptoethanol. Macrophages were incubated on plated overnight before stimulating. Where stated, cells were preincubated with 5ug/ml anti-TLR2 and anti-TLR4

neutralizing antibodies (Invivogen, pab-hstlr2 and pab-hstlr4) or 10μM DNA signalling inhibitory ODN TTAGGG (A151) (Invivogen), for 1 hour before being stimulated with the indicated stimulants.

NET preparations were generated as described earlier. Nucleosomes were isolated from the HL-60 cell line or the CAG::H2-EGFP transgenic mice, and were treated with MNase, DNase, benzonase or proteinase K and heat inactivated as described earlier. Recombinant human histone H3 (Cayman) was either kept untreated or was citrullinated overnight at 37°C with recombinant PAD4 (100μM Histone-3 with 25nM recombinant human PAD4) and was 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). Stimulants were preincubated with the anti-chromatin 10^{-8}M PL2-3 antibody IgG or Fab or with the anti-histone H3 antibody (Merk Millipore, 07-690) or a control IgG isotype (abcam). In all cases other than LPS stimulations, stimulants were preincubated for a minimum of 15 minutes with polymyxin B at a final well concentration of 50ug/ml (Invivogen, tlrl-pmb) to block any endotoxin contamination.

Cells were stimulated with the indicated concentrations of stimulants described above or with 1μM of human CpG ODN2006 or mouse CpG ODN1668 (Invivogen), or LPS from Salmonella Minnesota R595 (Invivogen) at indicated concentrations. After overnight incubation, 0.1% NP-40 and 1x cOmplete was added to the medium for 2 minutes before the lysates were spun down and analysed for human or mouse IL-1β via ELISA (eBioscience) according to the manufacturer’s instructions. Human monocyte lysates were diluted 1:4 and incubated for 2 hours at room temperature on the ELISA plate, human macrophages were diluted 1:2 and incubated overnight at 4°C, and mouse BMDM lysates were incubated undiluted overnight at 4°C.

For cytokine mRNA level assessment, $1x10^6$/well human peripheral blood CD14 positive monocytes were plated in a 6 well plate in HBSS++ +10%FBS and stimulated with 300ng/ml citrullinated or non-citrullinated NET preps for 2 hours at 37°C. Total cellular RNA was isolated using the TriReagent/Chloroform/Isopropanol (Sigma-Aldrich) method. 2ug of isolated RNA were then used to perform reverse transcription and generate cDNA using the Transcriptor high fidelity cDNA synthesis kit (Roche, 5091284001) using anchored-oligo(dT)18 primer. Gene expression was measured using TaqMan Universal

PCR Master Mix (Life technologies, 4304437) on a microamp fast optical 96 well reaction plate (Life technologies, 4346906) with IL-1β, IL-1α, IL-6 and HPRT-1 specific primers (Life technologies, Hs01555410_m1, Hs02800695_m1)on a 7900HT Fast Real Time PCR System (Applied biosystems). The cycling threshold for each cytokine was analysed and then normalized to that of HPRT1. Then, the relative gene expression was normalised over the naïve expression levels.

Immunoprecipitation

293 cell lines stably expressing the relevant TLR gene fused at the 3' end to the influenza hemagglutinin (HA) tag (Invivogen, 293-htlr2ha and 293-htlr4-ha and parental line 293-null) were cultured according to the manufacturer's instructions, in DMEM (ThermoFisher Scientific) supplemented with 4.5g/L glucose, 10% FBS, 50U/ml penicillin, 50ug/ml streptomycin, 100ug/ml Normocin and 2mM L-glutamine. $1x10^6$ 293/TLR2-HA and 293/TLR4-HA cells were plated in 6 well plates and stimulated for 2 hours with 1µM MBP or 1µM of MBP-H3 (preincubated with or without 80ng of purified NET DNA), 1µM Nucleosomes from HL-60 or from H2B-EGFP cells (preincubated with or without recombinant human PAD4). Samples were then lysed in 1ml of lysis buffer containing 50mM Tris pH8, 100mM NaCl, 1mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS and 1x cOmplete protease inhibitor (Sigma-Aldrich) and 20ul were kept aside to be ran on the western blot as the input sample. MBP-H3 (+/- NET DNA) and MBP sample lysates were incubated with 40ul of Amylose Resin high flow (New England Biolabs) overnight at 4°C. After 3 washes with PBS, the beads were resuspended in 1x SDS Laemmli buffer, boiled at 95°C for 10 minutes and ran on a Criterion TGX precast gel (Any-KD, Biorad) for western immunoblotting. For the Nucleosome stimulated lysates, the lysates were incubated with a rabbit Histone-H3 antibody (Millipore, 07-690) for 1 hour at 4°C and subsequently incubated with 30ul of Sepharose Fast flow beads (Sigma-Aldrich) per sample for 1 hour at 4°C. Samples were eluted by incubating at 97°C for 10 minutes in 1x SDS Laemmli buffer before being resolved on a Criterion TGX precast gel.

Western blot analysis

Samples were resolved on a Criterion TGX precast gel (Any-KD, Biorad) and transferred on a Trans-Blot Turbo Midi PVDF membrane (Biorad) using the Trans-Blot Turbo Transfer System (Biorad, 1704155). The membranes were then blocked in 5% BSA in tris-buffered saline and tween-20 (TBS-T) and incubated with anti-human myeloperoxidase (R&D, AF3667), anti-human histone H3 (Millipore, 07-690), anti-histone H3 citrulline R2+R8+R17 (Abcam, ab5103), anti-human S100A8 (antibodies online, ABIN111892), and anti-HA tag antibody (Abcam, ab9110) in 5% BSA for 2 hours at room temperature, except for the anti-histone H3 citrulline antibody that was incubated overnight at 4°C. After washing the membranes in TBS-T, they were incubated with the relevant species HRP-conjugated secondary antibodies (Thermo scientific) for 1 hour at room temperature and washed again in TBS-T. Finally, the membranes were incubated with enhanced chemiluminescent substrate (ECL, Thermo Fisher, 32106) and developed onto a photo film.

TLR reporter line assay

HEK-Blue reporter cell lines overexpressing human TLR2 or TLR4 (as well as the respective parental lines) were obtained by Invivogen (hkb-htlr2, hkb-htlr4) and cultured according to the manufacturer’s instructions. These transfected HEK293 cell lines detect stimulants of the respective overexpressed receptors by induction of secreted embryonic alkaline phosphatase (SEAP) under the control of TLR specific genes. Cells were stimulated with recombinant histone H3 (untreated or citrullinated with recombinant PAD4), preincubated with or without 100ng/ml of purified NET DNA, or nucleosomes purified from HL-60, EGFP-Nucleosomes purified from CAG::H2-EGFP expressing mice, LPS (from Salmonella Minnesota R595, Enzo) or CpG ODN2006 (Invivogen) or FSL-1 (Invivogen). The levels of SEAP induction were determined with HEK-Blue Detection (Invivogen), according to the manufacturer’s instructions. In brief, cells were washed and counted and resuspended at $4.5x10^{5}$/ml in HEK-Blue Detection medium before being added onto the stimulants. After overnight stimulation, the absorbance was measured using a microplate reader (Fluostar Omega, BMG labtech) at 655nm.

Atherosclerosis model

All mice were housed in a pathogen free, 12-hour light-dark cycle environment. All experiments were performed under an approved project license and following the Home Office guidelines.

ApoE/PAD4 deficient mice were generated by crossing ApoE deficient with PAD4 deficient mice (Zhang et al., 1992, Hemmers et al., 2011) on a C57BL/6J background. Male or female mice of 8-10 weeks old were fed for 6 or 16 weeks on a high fat diet (60% energy from fat, Testdiet). For the chromatin neutralisation experiments, ApoE-/- were injected once a week for 6 weeks with 0.8mg/kg of the Fab fragment of the mouse anti-chromatin PL2-3 antibody (Losman et al., 1992), full length IgG or Fab fragment after digestion (using ThermoFisher Scientific, Pierce^{TM} Fab preparation Kit, 44985) or equivalent volume of PBS or vehicle (PBS passed through the Pierce^{TM} Fab preparation Kit). To assess the therapeutic capacity of the chromatin neutralisation, after 6 weeks of high fat diet, ApoE deficient mice were switched to the standard diet and were injected once a week for 6 weeks with the PL2-3 Fab or vehicle.

Mice were sacrificed by terminal anaesthesia (pentobarbital intraperitoneal injection) and exsanguinated from the jugular vein for a terminal blood plasma sample (collected in heparin containing tubes). Blood was centrifuged at 2000 x g for 15 minutes to isolate the plasma layer. A PBS perfusion of the arterial system was performed via the left ventricle of the heart. The heart was fixed with 4% PFA overnight at 4°C and then dehydrated overnight with 20% sucrose. Hearts were embedded in Optimum Cutting Temperature compound (OCT, VWR, 361603E) 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 10µm, collected on glass slides and stored at -80°C. Sections were then stained with Oil Red O (Sigma, O0625) in 60% isopropyl alcohol for 10minutes, washed in water and counterstained with Mayer's haematoxylin (Sigma, MHS1). Images were acquired using the Olympus Slidescanner VS-120 and analysed using OlyVia software (Olympus). The relative plaque lesion area was calculated by using the averages from several sections measured using Adobe Photoshop and ImageJ software.

For each section, the plaque area was analysed proportionally to the total volume of the aortic root lumen area.

For immunofluorescence staining of the atherosclerotic lesions, aortic root sections were first dried for 30 min at room temperature followed by a wash in water to remove OCT. Sections were permeabilised for 2 minutes with 0.2% Triton-X in PBS. Slides were washed in PBS and non-specific binding was blocked with 2% donkey serum in PBS for 1 hour in room temperature. Sections were incubated with primary antibodies in 2% donkey serum in PBS for 2 hours at room temperature. After three washes, sections were incubated with secondary antibodies in 2% donkey serum in PBS for 1 hour at room temperature. Samples were washed, counterstained with DAPI (Life Technologies) and mounted in ProLong Gold (Molecular Probes) before being examined by confocal microscopy. Images were analysed with ImageJ v2.0 software. Primary antibodies: anti-histone 3 citrulline R2+R8+R17 (Abcam ab5103) and anti-mouse myeloperoxidase (R&D, AF3667). Secondary antibodies: Atto 550-conjugated anti-chromatin PL2-3 ((Losman et al., 1992)), Alexa 647-conjugated anti-mouse Ly6G (Biolegend, 127610, Clone 1A8) anti-goat Alexa 488, anti-rabbit Alexa 647 or anti-rabbit Alexa 488, anti-mouse Alexa 488.

Plasma samples from total blood of C57BL/6J wild type (not on high fat diet) or APOE -/- after 6 weeks on high fat diet were analysed for cytokine expression after a 1:2 dilution in PBS, according to manufacturer's protocol for mouse Il-1β ELISA (ThermoFisher Scientific, 88-7013-88). For cholesterol and triglyceride levels, plasma samples were diluted 1:4 in PBS and ran at the cobas c111 machine (Roche) using the reagents for cholesterol (Chol2, Roche, 04718917) and triglycerides (Trig, Roche, 04657594), following manufacturer's instructions.

Candida albicans pulmonary infection

Wild type *Candida albicans* (SC5314, clinical isolate) was cultured overnight in yeast extract-peptone-dextrose (YEPD) medium at 37ºC before being subcultured in YEPD medium for 4 hours. Female wild type and CAG::H2-EGFP transgenic mice of 8-12 weeks were infected intratracheally with $1x10^6$ *C. albicans* in 50ul PBS. To assess microbe load, animals were sacrificed 24 hours post infection via terminal anaesthesia (pentobarbital intraperitoneal injection), lungs were homogenized in 1ml sterile PBS and

serial dilutions of the homogenates were plated onto sabourad dextrose agar plates. Colonies were counted after incubating the plates at 37°C overnight. To analyse cytokines in bronchoalveolar lavage (BAL), animals were sacrificed 24 hours post infection, lungs were rinsed with 500µl sterile PBS and mouse IL-1β was measured by ELISA in undiluted samples (ThermoFisher Scientific, according to manufacturer's protocol).

Flow cytometry

CD14+ human monocytes, were stimulated with the indicated concentrations of H2B-EGFP Nucleosomes. At the indicated timepoints, samples were stained with anti-mouse CD11b antibody (clone M1/70, Biolegend) and LIVE/DEAD™ Fixable blue dead cell stain kit (Thermo Fisher) and then fixed with 4% PFA (Sigma), before acquisition on the cell analyser LSR Fortessa (BD).

For intracellular and extracellular TLR4 localization in different mouse cells:
Human monocytes, human macrophages and murine BMDMs, isolated as described above, were stained for extracellular and intracellular TLR4 with anti-human TLR4 (clone TF901, BD Biosciences) or anti-mouse TLR4 (clone SA15-21, BioLegend) by using the Foxp3 staining buffer set (eBioscience). Also, terminal blood was collected from terminally anaesthetised WT mice via cardiac puncture, overlaid on Histopaque 1119 and spun at 800 x g for 20 minutes under gentle break. Cells were washed with PBS + 2% FBS, overlaid over 80% percoll, and spun at 800 x g for 20 minutes under gentle break. For the alveolar macrophages, lungs of terminally anaesthetised WT mice were washed with 1.5ml of PBS and cells were spun down at 300 x g for 10 minutes. Cells were plated in V-bottom 96 well plates in PBS + 5% FBS, and left to rest at 37°C for 3 hours. After that, cells were washed in PBS without FBS, and resuspended in an antibody mix that included the Live/Dead dye (1:1000, molecular probes, L34957), CD3/CD19-PercPcy5.5 (1:200), Ly6G-FITC (1:200), Ly6C-BV7111 (1:500), CD11b-PE Cy7 (1:500), CD11c-APC Cy7 (1:200), Siglec F-BV421 (1:200, BD biosciences E50-2440) and TLR4-PE or TLR4-APC (BioLegend, SA15-21) or Isotype-controls (1:100), for extracellular staining. Cells were then incubated for 20 minutes at 4°C, washed in PBS and resuspended in 1x Fix/Perm (eBioscience, 5523) for 30 minutes at 4°C. Cells were then washed and resuspended in 1x Permeabilisation solution twice. For total TLR4

staining, cells underwent a second round of incubation with TLR4-PE or TLR4-APC or isotypes (1:100) for 20 minutes at 4°C. After 2 washes in 1x Permeabilisation solution, all cells were resuspended in PBS + 2% FBS, before acquisition on the cell analyser LSR Fortessa (BD). Also, human monocytes after stimulation with 1µM of MBP-H3 and 300ng/ml NET DNA were stained for extracellular and intracellular TLR4 with anti-human TLR4 (clone TF901, BD Biosciences) or anti-mouse TLR4 (clone SA15-21, BioLegend) by using the Foxp3 staining buffer set (eBioscience). All data were analysed using FlowJo software v10.

Immunofluorescence microscopy studies of fixed cells

$5x10^4$ human CD14 positive monocytes and human monocyte derived macrophages on glass coverslips were stimulated with 0.5µM of MBP-Histone H3 preincubated with or without 300ng/ml NET DNA. After 2 hours, cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature. Samples were permeabilized with 0.5% Triton X-100 in PBS for 2 minutes. Samples were washed in PBS and blocked with 2% BSA and 2% Donkey serum in PBS for 1 hour at room temperature. Then, samples were stained with antibodies against TLR4 (abcam, ab22048), Rab5 (C8B1, Cell Signalling Technology, 3547) MBP (BioLegend, 906901) overnight at 4°C. Samples were washed and stained with donkey anti-mouse Alexa 488, donkey anti-rat Alexa 568 and donkey anti-rabbit Alexa 647 (all Invitrogen). Samples were washed and counterstained with DAPI before being 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 analysed with ImageJ v2.0 software.

Statistical analysis

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: $p>0.05$, *: $p\leq0.05$, **: $p\leq0.01$, ***: $p\leq0.001$.

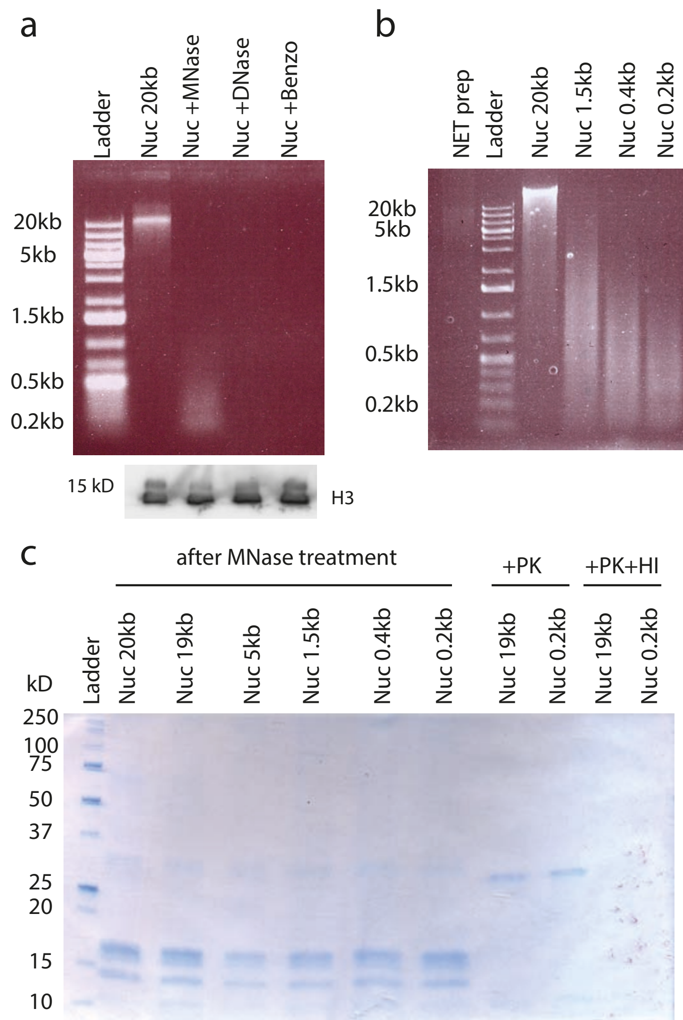

Figure 2. 1 DNA and protein content of different material preparations.

a. DNA and histone content of HL-60 nucleosome preparations untreated or treated with MNase, DNase or Benzonase, resolved by 1% agarose electrophoresis or SDS-page electrophoresis respectively. **b.** DNA content of NET preparation or HL-60 nucleosomes untreated (Nuc 20kd) or treated with MNase for different lengths of time to obtain fragments of decreasing size. **c.** Analysis of protein content by SDS-page electrophoresis followed by coomassie blue staining of HL-60 nucleosomes untreated (Nuc 20kd) or treated with MNase for different lengths of time, followed by proteinase K treatment (+ PK) and then heat inactivation (+PK +HI).

Results

Chapter 3. NET histones are major pro-inflammatory agents in a TLR4 dependent manner

Introduction

NETs drive inflammation in various conditions such as gout and atherosclerosis. In atherosclerosis, chronic stimulation of the immune system by sterile triggers leads to the formation of plaques on the arterial walls, constricting the blood flow and eventually leading to plaque rupture and death (Ross, 1999). Previous work from our group demonstrated that NETs are potent drivers of local inflammation by activating macrophages to produce IL-1β (Warnatsch et al., 2015). Specifically, mice lacking the main proteases neutrophil elastase and proteinase 3 that drive chromatin decondensation and NET formation, were protected from atherosclerosis as were mice that were treated with DNase I that digests NETs. While this work identified NETs as key drivers of inflammation, the specific NET components that drive NET-mediated inflammatory responses remain unknown. The aim of the work presented here is to uncover the mechanisms of NET-mediated pathology by first identifying the key inflammatory moieties in NETs and subsequently understanding the mechanisms that regulate cellular responses to these NET components.

3.1 Citrullination is not required for NET formation in atherosclerosis

One key feature in NETs is the posttranslational modification known as citrullination, that is mediated by the enzyme protein arginine deiminase (PAD) PAD4. Citrullination is found extensively on NET histones and is thought to promote NET formation by mediating chromatin decondensation (Leshner et al., 2012b, Papayannopoulos et al., 2010, Wang et al., 2009a). However, recent work has hinted that the requirement of citrullination for NET formation may depend on the different stimuli. Therefore, it is important to understand whether the phenotypes that are associated with PAD deficiency could be due to an unknown role for citrullination in NET function.

To test this hypothesis, we first examined whether citrullination is required for NET formation *in vitro*. We incubated neutrophils with cholesterol crystals in the presence or absence of the potent PAD4 inhibitor Chloride amidine (Cl-am), and assessed NET formation. NETs forming after PAD4 inhibition were equally decondensed, as measured by DNA decondensation in their native, unfixed state **(Fig. 3.1a)**. NETs that formed after Cl-amidine inhibition, lack histone H3 citrullination as assessed by immunofluorescence as well as western immunoblot but contain the typical NET markers, such as myeloperoxidase (MPO), neutrophil elastase (NE), calprotectin (S100A8), partially cleaved histones (H3) **(Fig. 3.1b and c)**. These data confirm that PAD4 inhibition using Chloride amidine, effectively blocks histone citrullination but does not inhibit chromatin decondensation nor NETosis in cholesterol crystal-stimulated human neutrophils.

In order to assess the role of NET citrullination in NET formation *in vivo*, we employed a model of murine atherosclerosis (Zhang et al., 1992). In this model, mice deficient in apolipoprotein E (ApoE), a protein that is essential for the normal metabolism of cholesterol, spontaneously develop arterial plaques when fed on a high fat diet (HFD). NETs form inside the atherosclerotic lesions of the aortic root (Warnatsch et al., 2015). ApoE deficient animals were crossed with a PAD4 deficient mouse line (Hemmers et al., 2011) to create atherosclerotic mice deficient in citrullination.

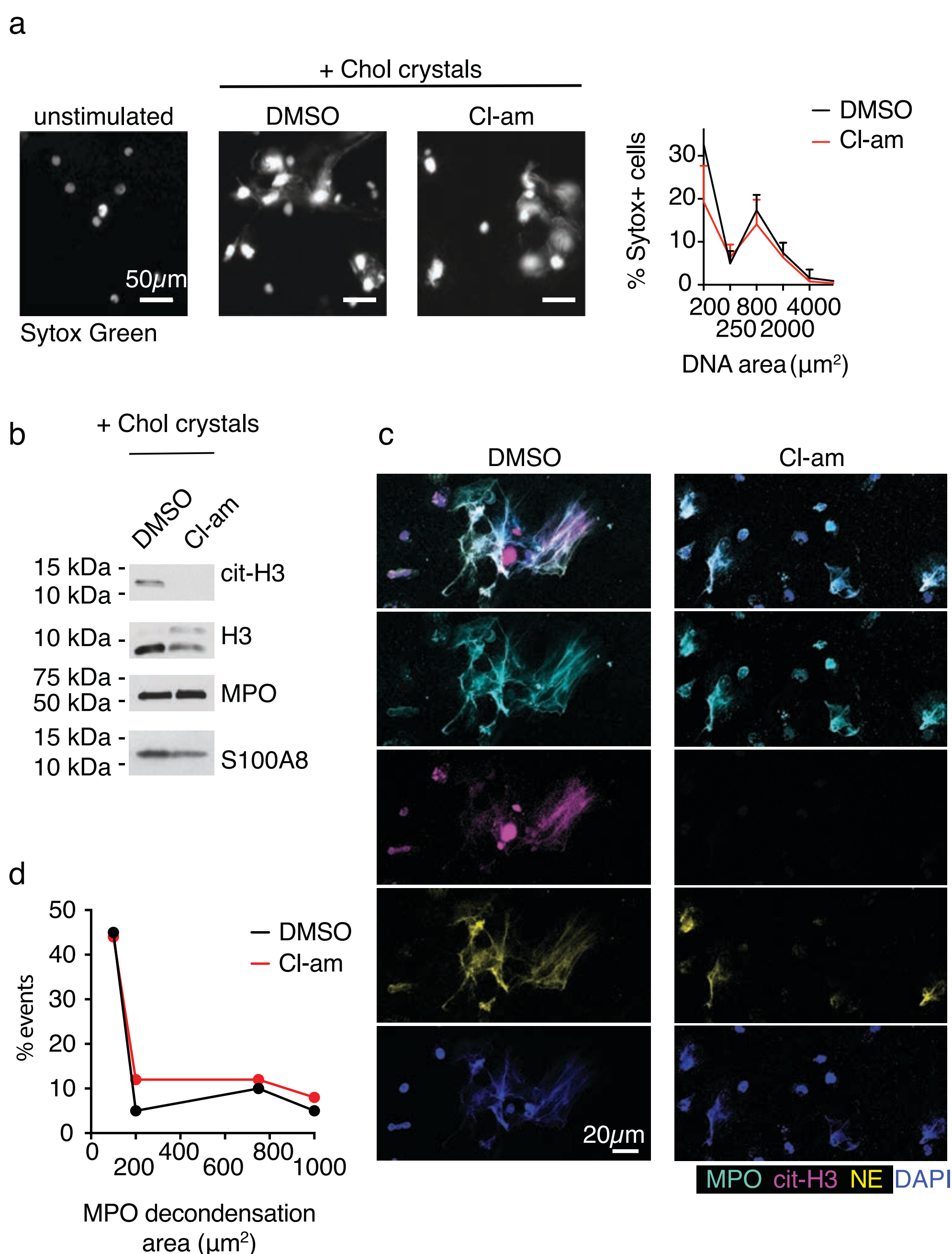

Figure 3. 1 Histone citrullination is not required for NET formation *in vitro.* left: NET release detected by Sytox Green in undisturbed primary human neutrophils, either treated with Cl-amidine (Cl-am) or with DMSO, incubated for 4 hours in the absence or presence of cholesterol crystals. Scale bar: 50µm. right: Quantification of NET formation measured as an increase in extracellular DNA decondensation. The area of each Sytox stained nucleus is measured by microscopy and plotted as a frequency of Sytox positive dead cells falling within a range of nuclear areas per 100 total cells. **b.** Western immunoblotting of NET preparations as in (a) stained for the NET markers MPO, cit-H3, S100A8 and histone H3. **c.** Cells from (a) were fixed and immunostained for citrullinated histone H3 (cit-H3, magenta), myeloperoxidase (MPO, cyan), neutrophil elastase (NE, yellow) and DNA (DAPI, blue). Scale bar: 20µm. Data (a, b and c) are representative of at least three independent experiments. **d.** Quantitation of NET release from human

neutrophils as in (c), measured by extracellular MPO area. Area of each event measured by microscopy and plotted as a frequency of MPO positive areas falling within a range of decondensation sizes per 100 total cells.

We find NETs inside the atherosclerotic lesions of both the ApoE and ApoE/PAD4 deficient mice after 6 and 16 weeks of feeding them on the high fat diet **(Fig. 3.2a and b)**. Citrullination of histone H3 has been traditionally used as a marker for NET formation, reporting NET release reliably (Branzk et al., 2014). To identify non citrullinated NETs *in vivo* we employ a variety of non-citrullinated markers, such as Ly6G (neutrophil marker previously shown to be found on NETs), MPO, an anti-chromatin antibody (chro) that binds the H2A/H2B/DNA complex (Losman et al., 1992) and DAPI. We found that NETs had a similar distribution and area size in the tissues of the two strains **(Fig. 3.2b)** but histone citrullination was absent on the NETs in ApoE/PAD4 deficient aortic lesions.

These findings suggest that citrullination is not required for NET formation against cholesterol crystals *in vitro* and during atherosclerosis *in vivo*. It also emphasizes the requirement for development of additional NET-specific markers to be able to detect the presence of non-citrullinated NETs and assess their function.

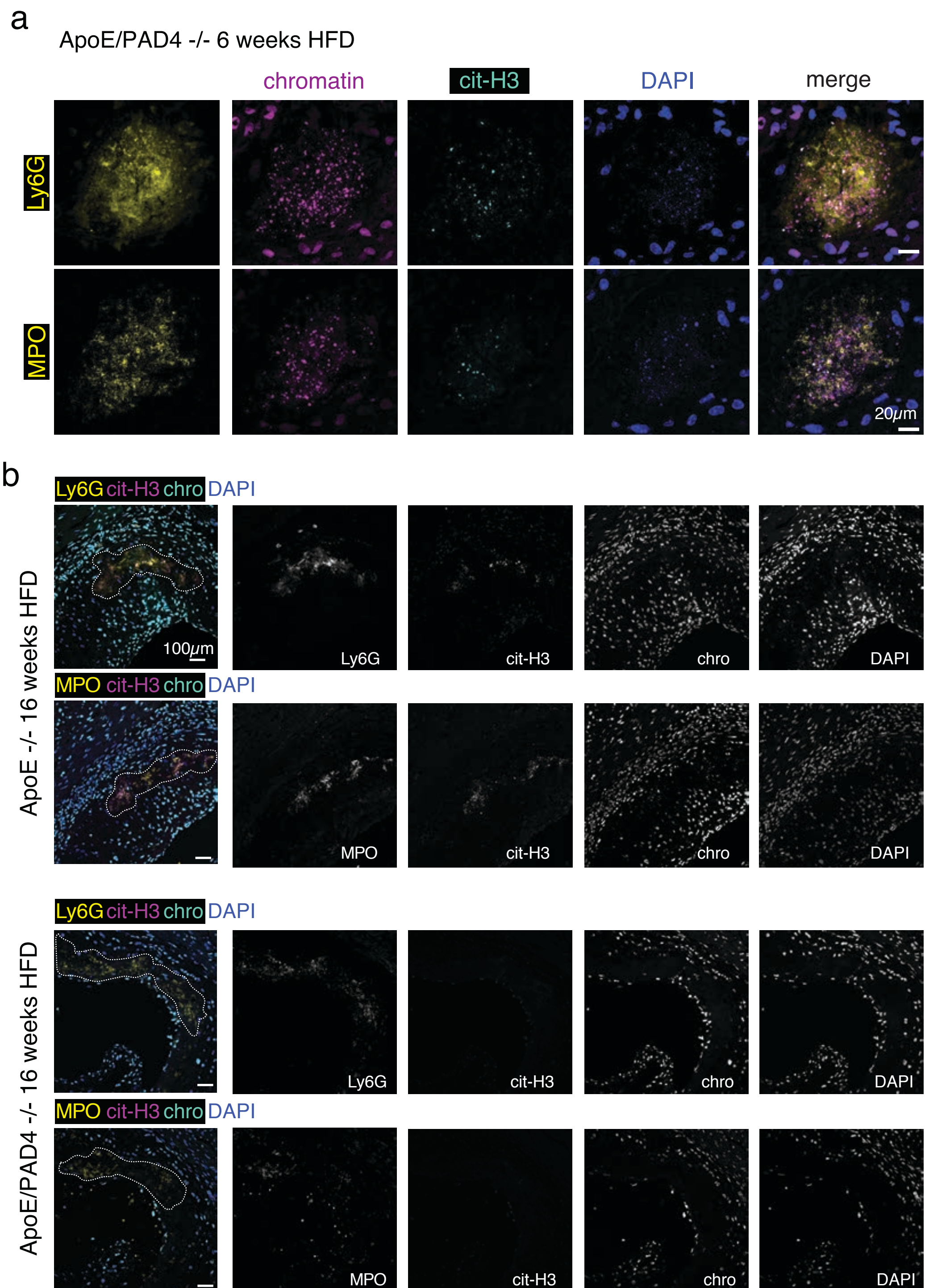

Figure 3. 2 Histone citrullination is not required for NET formation in vivo. a. Representative micrographs of consecutive aortic sections (top and bottom row) from ApoE/PAD4 deficient animals after 6 weeks on high fat diet, fluorescently immunostained for the neutrophil marker Ly6G (yellow, top panel) which is also found in NETs, the anti-chromatin PL2-3 antibody (chromatin, magenta), antibodies against citrullinated histone H3 (cit-H3, cyan), myeloperoxidase (MPO, yellow, bottom panel), and DAPI (blue). Scale

bar: 20µm. Data representative of three animals. **b.** Representative micrographs of consecutive aortic sections (upper and lower panel) from ApoE (upper rows) and ApoE/PAD4 (lower rows) deficient animals after 16 weeks on high fat diet, fluorescently immunostained for the neutrophil marker Ly6G (yellow, top column) the anti-chromatin PL2-3 antibody (magenta), antibodies against citrullinated histone H3 (cit-H3, cyan), MPO (yellow, bottom row), and DAPI (blue). Scale bar: 100µm. Data representative of three animals.

3.2 Nucleosomes are pro-inflammatory via TLR4

Since citrullination is not required for NET formation in atherosclerosis, we questioned whether it plays a role in NET function and specifically NET-mediated pro-inflammatory signalling. For that reason, preparations of NETs from neutrophils stimulated with cholesterol crystals were generated in PAD4 sufficient conditions or under PAD4 inhibition and solubilised with restriction enzymes. These NET preparations had citrullinated (cit-NET) or non-citrullinated histones respectively (non-cit NET) (see also **Fig. 3.1b**). The ability of these preparations to drive pro-inflammatory cytokines was then tested using primary human blood monocytes.

It must be noted here that in all our IL-1β protein analysis we focused on total levels of IL-1β, rather than the mature secreted cytokine. It has been shown that cholesterol crystals are sufficient to drive NET formation in neutrophils at concentrations that also drive inflammasome activation in monocytes (Duewell et al., 2010, Warnatsch et al., 2015).

We found that non citrullinated NETs had a lower capacity to induce IL-1β, IL-1α and IL-6 production in monocytes **(Fig. 3.3a)**. Since citrullination was an important factor for NET-mediated signalling and histones carry the majority of citrullination, we hypothesised that histones might be important for NET-mediated cytokine induction.

To address the role of histones and chromatin in NET-mediated signalling, we first investigated whether nucleosomes are sufficient to activate monocytes. We used the human leukaemia (HL-60) granulocytic cell line to isolate native granulocytic nucleosomes. Nucleosomes were treated with micrococcal nuclease (MNase) at variable

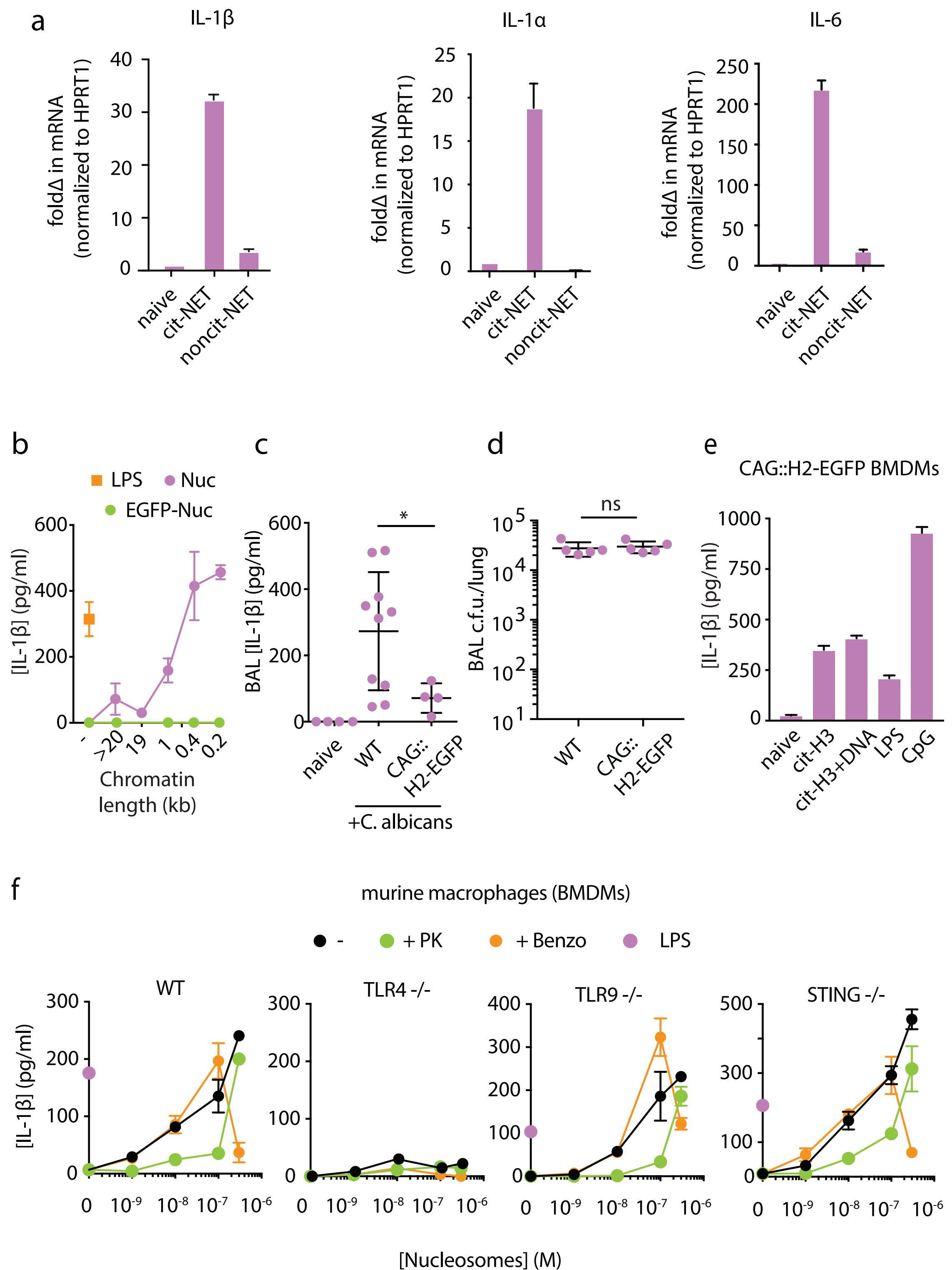

Figure 3. 3 NET-mediated activation of human primary monocytes depends on citrullinated histones and DNA. a. IL-1β, IL-1α and IL-6 mRNA expression in human monocytes in response to NET fragments generated by restriction enzyme digest of cholesterol crystal-stimulated neutrophils that were treated either with DMSO or Cl-amidine to generate citrullinated-NETs (cit-NET) and non-citrullinated NETs (noncit-

NET) respectively, analysed by RT-PCR, normalized to HPRT1 expression. Statistical analysis by the Mann-Whitney test. Data are representative of two individual stimulations per two donors. **b.** Total IL-1β induced in primary human monocytes untreated or stimulated with a single concentration of nucleosomes (10^{-7}M) purified from neutrophil-like human leukemia-60 (HL-60) cells (Nuc) or from transgenic mice that overexpress a H2B-EGFP fusion protein (EGFP-Nuc) and digested for different times with micrococcal nuclease (MNase) to generate different size nucleosome fragments. For larger ranges, depicted chromatin sizes are the mean size of samples that contain a gradient of fragments. Bacterial lipopolysaccharide (LPS) (0.5ng/ml) was used as a positive control. Data are representative of three independent experiments. **c.** IL-1β in bronchoalveolar lavage (BAL) of WT and transgenic mice expressing a H2B-EGFP tagged fusion protein infected intratracheally with *C. albicans* for 24 hours. Statistical analysis by Mann-Whitney test. **d.** the fungal load of the lungs of animals from (c). Statistical analysis by Mann-Whitney test. **e.** Total IL-1β induced in BMDMs from H2B-EGFP mice stimulated with citrullinated H3 (1µM) with or without purified NET DNA (300ng/ml), LPS (0.5ng/ml) or human optimised stimulatory CpG (1µM). **f.** Total IL-1β induced in BMDMs from WT, TLR4, TLR9 and STING deficient mice, stimulated with increasing concentrations of DNA-bearing mononucleosomes, DNA-free naked nucleosomes treated with Benzonase (Benzo) or proteinase K treated and heat inactivated mononucleosomes to degrade histones (PK) or LPS (0.5ng/ml). Data representative of three independent experiments.

times to generate fragments of different size. MNase digests the intra-nucleosomal DNA but does not digest DNA bound to histones. We found that HL-60 nucleosomal preparations strongly induce IL-1β and MNase digestion, to create smaller fragments, increases their activating potential **(Fig. 3.3b)**. Specifically, monocyte activation peaked with di-nucleosomes and mono-nucleosomes of 0.4 and 0.2kb DNA size. The level of activation with the small nucleosome fragments directly compares to lipopolysaccharide stimulation, a potent monocyte activator. This suggests that decondensation and nuclease activity promote NET-mediated signalling.

Interestingly, nucleosome fragments that were prepared from tissues of animals expressing an EGFP fusion histone protein (CAG::H2B-EGFP, (Hadjantonakis and Papaioannou, 2004)) did not induce cytokine expression. This result suggests that the presence of EGFP fused with histones, interferes with their signalling capacity, suggesting that histones directly interact with monocyte receptors. Therefore, cytokine induction might involve specific interactions with monocyte receptors that require histone recognition.

Since the EGFP-bearing nucleosomes did not elicit an inflammatory response in monocytes *in vitro*, we used the transgenic mice as a genetic tool to study the role of histone signalling in inflammation during a pulmonary fungal infection. Pulmonary *C.*

albicans infection promotes NET release (Branzk et al., 2014). Interestingly, after 24 hours of *C. albicans* lung infection, the bronchoalveolar lavage (BAL) of the H2B-EGFP mice contained significantly lower concentrations of IL-1β compared to WT control mice **(Fig. 3.3c)** despite bearing comparable fungal load in the lungs of both groups **(Fig. 3.3d)**, suggesting that histone-mediated signalling plays an important role in inducing this cytokine during an acute fungal infection. Moreover, bone marrow derived macrophages (BMDMs) from the CAG::H2B-EGFP animals, where able to be activated by stimuli like recombinant histone H3 and NET DNA as well as with LPS and an activatory CpG ODN (CpG) **(Fig. 3.3e)**, indicating that the defects in IL-1β production were not due to lack of immune cell responsiveness but possibly caused by the inefficient activation potential of the EGFP-bearing chromatin.

Taken together, these data suggest that extracellular histones play a critical role in NET mediated signalling and PAD4 may regulate inflammation by potentiating their proinflammatory capacity.

3.3 Nucleosome histones bind and activate TLR4

As discussed in the introduction, the receptors responsible for the proinflammatory capacity of endogenous histones in sterile inflammation are not known. TLR2 and TLR4 have been previously implicated in sensing NETs (Warnatsch et al., 2015). Moreover, studies have previously shown that histone cytotoxicity-mediated pathology is inhibited in TLR2 and TLR4 deficient animals (Xu et al., 2011). Finally, TLR4 deficient atherosclerosis-prone mice show significant protection against plaque development (Ding et al., 2012).

In order to identify the relevant PRRs required for sensing chromatin, we employed bone marrow derived macrophages deficient in TLR4, TLR9 or STING. WT murine macrophages responded potently to mononucleosomes. Proteinase K treatment inhibited nucleosome-mediated activation of macrophages, indicating that histones are the main inflammatory component of nucleosomes for macrophages **(Fig. 3.3f)**. The pre-treatment of nucleosomes with benzonase to remove DNA, did not reduce IL-1β production suggesting that DNA was not required for nucleosome-mediated macrophage stimulation. Interestingly, naked DNA-free nucleosomes appeared to be cytotoxic at

lower concentrations than nucleosomes carrying DNA (discussed further in next chapter). Importantly, TLR4 deficient macrophages failed to respond to nucleosomes, indicating that histone detection was mediated by TLR4. In contrast, TLR9 and STING deficiency did not affect nucleosome detection. These data indicated that TLR4 is the main and only receptor promoting chromatin-mediated IL-1β induction in murine macrophages.

We also probed the potential implication of TLR4, TLR2 and any DNA receptors in chromatin sensing in human monocytes. The pre-treatment of cells with neutralising antibodies against TLR2 and TLR4 (TLR2/4i) blocked IL-1β induction by citrullinated NET fragments **(Fig. 3.4a)**, suggesting that NETs drive IL-1β induction by engaging TLR2/4. In contrast to BMDMs, treating monocytes with an inhibitory ODN that blocks TLR9 and other DNA receptors, reduced IL-1β induction, uncovering a role for DNA in human monocyte responses to NETs.

Furthermore, we probed the role of these human monocyte receptors in sensing nucleosomes and recombinant histone H3. Consistently with the monocyte NET stimulation, TLR2 and TLR4 neutralising antibodies reduced the ability of monocytes to detect mononucleosomes **(Fig. 3.4b)**. The inhibitory ODN blocked monocyte activation less efficiently than TLR2/4 neutralisation, confirming the prime role of histones in driving nucleosome-mediated signalling. When comparing the efficiency of TLR neutralising with the anti-chromatin Fab (PL2-3), we found that the anti-chromatin Fab and TLR2/4 neutralisation blocked IL-1β induction comparably at the lower nucleosome concentrations of 10^{-8}M. However, at higher nucleosome concentrations, the efficiency of the anti-chromatin Fab dropped as the ligand concentration increased, consistent with the ligand concentration surpassing the blocking capacity of the antibody. The most efficient inhibition of monocyte stimulation *in vitro* was achieved by TLR2/4 neutralisation together with the inhibitory ODNi. The critical role of TLR4 was also confirmed by activating monocytes with recombinant cit-H3 and NET DNA as agonists **(Fig. 3.4c)**. Therefore, TLR4 is required for chromatin signalling in both human monocytes and BMDMs. Moreover, DNA plays an important role for chromatin-mediated signalling in human monocytes but it is dispensable for chromatin detection in murine BMDMs.

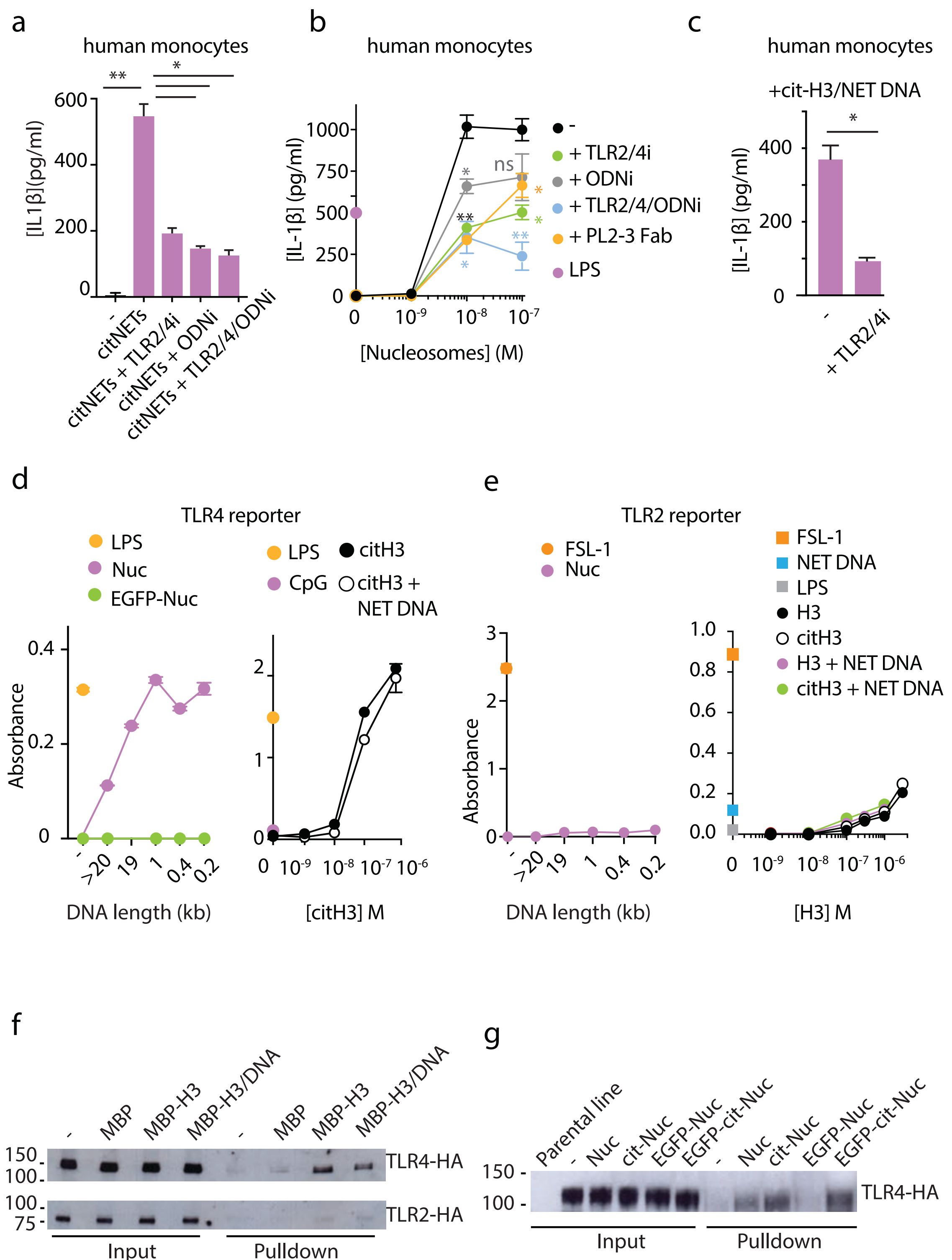

Figure 3. 4 Histones drive IL-1β induction via TLR4. a. Total IL-1β induced in primary human monocytes stimulated with cit-NETs alone, or after pre-treatment with TLR2 and TLR4 neutralising antibodies and/or inhibitory ODN. Statistical analysis by Mann-Whitney test. Data representative of three individual stimulations from two different donors. **b.** Total IL-1β induced in primary human monocytes after stimulation with

increasing concentrations of mononucleosomes alone or after pre-treatment with antibodies against chromatin (PL2-3 Fab), TLR2, TLR4 or inhibitory ODNi or combinations. Statistical analysis by two-tailed unpaired student t test, comparing each treatment to the Nucleosomes stimulation. Data representative of three independent experiments. **c.** Total IL-1β induced in primary human monocytes stimulated with recombinant cit-H3 (0.03μM) and NET DNA (100ng/ml) in the presence or absence of TLR2 and TLR4 neutralising antibodies. Statistical analysis by Mann-Whitney test. Data representative of at least three independent experiments. **d** and **e.** TLR4 (d) and TLR2 (e) mediated NF-kB activity assessed by induction of secreted embryonic alkaline phosphatase (SEAP) in the heterologous HEK-Blue cell system. Cells were stimulated with different size of purified HL-60 nucleosomes or EGFP-tagged nucleosomes or LPS (0.5ng/ml), CpG (1μM) or FSL-1 (50ng/ml). Also, cells were stimulated with different concentrations of recombinant cit-H3 alone or together with NET DNA (100ng/ml). Data representative of at least three independent experiments. **f.** HEK cells expressing TLR4-HA or TLR2-HA were incubated with recombinant H3 fused to maltose-binding protein (MBP-H3) alone or together with NET DNA. Cells were lysed and interacting proteins were pulled down with amylose beads and immunoblotted with anti-HA antibodies. The total cell lysate (input) was diluted 20x more than the pulldown samples. **g.** HEK cells expressing TLR4-HA were incubated alone or with HL-60 mononucleosomes or EGFP-tagged nucleosomes, either in the native state or after citrullination. Cells were lysed and interacting proteins were immunoprecipitated with an anti-H3 antibody and immunoblotted with anti-HA antibodies. The total cell lysate (input) was diluted 20x more than the pulldown samples.

In order to examine whether histones are sufficient to activate TLR receptors we used a heterologous HEK-Blue TLR2 or TLR4 reporter cell system. In these cell lines, the TLR mediated NF-kB activation is detected by the concomitant production of a secreted embryonic alkaline phosphatase (SEAP). We found that TLR4 was sufficient to activate the NF-kB pathway in response to nucleosome and histone stimulations **(Fig. 3.4d)**. Nucleosomes bearing an EGFP-histone fusion protein do not activate the TLR4 reporter cells, suggesting that histone architecture might be important for TLR4 activation. HEK-Blue cells that express TLR2 showed very low or no sensitivity to histones or nucleosomes **(Fig. 3.4e)**, further proving that chromatin histones primarily engage TLR4.

Finally, to identify whether histones physically interact with TLR4, we utilised cell lines that overexpress HA-tagged TLR4 or TLR2. We stimulated these cells with recombinant H3 or mononucleosomes. We found that HA-tagged TLR4 could be immunoprecipitated with recombinant MBP-H3 **(Fig. 3.4f)** or with antibodies against mononucleosomes **(Fig. 3.4g)**. Furthermore, we found that EGFP-nucleosomes can not bind TLR4, suggesting that these nucleosomes can not induce IL-1β due to lack of physical association with TLR4. Interestingly, citrullinating these EGFP-nucleosomes *in vitro* overcame the binding inhibition TLR4 binding suggesting that that charge changes by citrullination could

overcome EGFP-mediated TLR4 blocking. We conclude that native histones bind and activate TLR4 and these interactions are not affected by the association of histones with DNA.

To conclude, our data demonstrate that histones are the major pro-inflammatory moieties of chromatin and NETs in human monocytes and BMDMs, due to their ability to activate TLR4. Interestingly, we find that DNA plays a role in NET and nucleosome-mediated activation of human monocytes, but it is dispensable in the activation of BMDMs. This suggests a difference between monocytes and BMDMs in sensing extracellular chromatin. DNA appeared to play a secondary regulatory role in human monocytes via an unknown mechanism.

Also, we showed that non-citrullinated NETs form *in vivo* in atherosclerotic plaques. Our results suggest that NETs still form in the plaques of PAD4 deficient mice and citrullination potentiates the pro-inflammatory activity of histones and NETs. Citrullination of NETs and fragmentation of DNA are important in driving chromatin-mediated signalling but the mechanisms remain unknown.

Chapter 4. Histones synergise with DNA to promote inflammation below the histone cytotoxicity threshold

Introduction

NETs are an important source of histone bearing DNA and other inflammatory molecules. It has been shown that free extracellular histones are cytotoxic and have been linked to acute inflammation and lethality *in vivo*. So far, it has not been clear how histone-bearing DNA might be an inflammatory signal, activating immune cells without killing them. This is a paradox, because to date, the pro-inflammatory capacity of histones has been attributed to their cytotoxic properties (Xu et al., 2011, Xu et al., 2009). Our experiments in chapter 3 indicated that DNA together with histones mediate IL-1β induction in human monocytes. Moreover, we observed heterogenicity in immune cell specificity towards chromatin, since DNA-mediated signalling appeared to be dispensable in BMDMs. In this chapter, we examined the specific contribution of these two factors towards cytokine induction and the mechanisms that regulate their capacity to activate different immune cells. Finally, we explored how these mechanisms contribute to the ability of chromatin to promote inflammation.

4.1 Histones and DNA act synergistically to promote inflammation

Our experiments in chapter 3 suggested that a DNA receptor plays a critical role in NET sensing. To probe the contribution of NET DNA, we treated nucleosomes with DNase I and Benzonase, which are endonucleases that digest the histone-bound DNA as well as the intra-nucleosomal DNA. Intriguingly, DNA-free nucleosomes were not able to

potently activate monocytes at the concentrations tested **(Fig. 4.1a)**. This suggested that potent chromatin stimulation of monocytes involves the synergistic interactions of DNA with histones.

To evaluate whether DNA and histones synergize to induce IL-1β, we performed titrations of one stimulus and assessed the effect of the other on the potential to drive cytokine expression. We found that histone H3 could moderately activate monocytes alone, but adding NET DNA at concentrations that were not sufficient to drive IL-1β expression alone, dramatically enhanced the monocyte response towards histones **(Fig. 4.1b)**. Moreover, citrullinating histones with a purified recombinant PAD4, further enhanced their stimulation potential. In addition, when we titrated DNA, sub-threshold histone concentrations significantly sensitised monocytes to DNA **(Fig. 4.1c)**. Together, these experiments proved that histones and DNA synergise to drive the pro-inflammatory capacity of chromatin. Also, the synergy of histones with DNA is far more important than histone citrullination in mediating cytokine induction.

4.2 Synergy between histones and DNA enables activation below the histone cytotoxicity threshold

An important mechanism for inflammatory regulation is the synergic activity amongst various agonists of Toll-like receptors (TLR). This allows a higher pro-inflammatory response to be elicited when more than one agonists are present together at low concentrations than when they're present individually at higher concentrations. As opposed to pathogen-derived agents, host-derived DAMPs tend to have a lower affinity to PRRs and synergy might be critical to regulate the reaction towards host injury.

We hypothesized that the synergy between histones and DNA might serve to enable the activation of immune cells below the histone cytotoxicity threshold. To test this hypothesis we designed an experiment that measured EGFP nucleosome uptake (isolated from CAG::H2B-EGFP mice) and cell death by flow cytometry **(Fig. 4.1d)**. These experiments indicated that monocytes were viable and could take mononucleosomes for 16 hours at 10^{-7}M concentrations of histones. However, at higher concentrations nucleosomes were cytotoxic **(Fig. 4.1d** and **e)**. By comparison, nucleosomes primed monocytes at concentrations as low as 10^{-9}M, with the activation

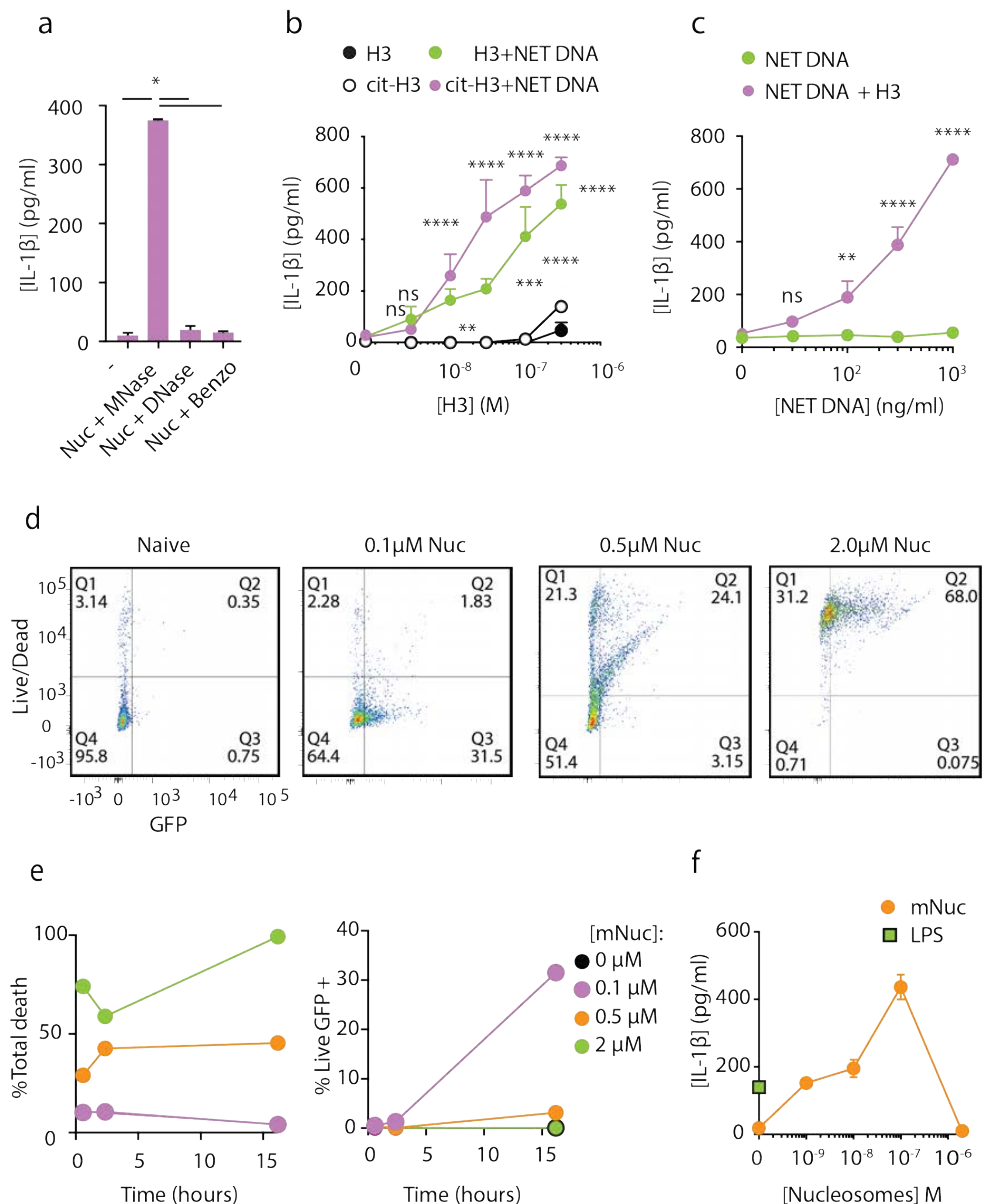

Figure 4. 1 Synergy of histones with DNA ensures activation below the histone cytotoxicity threshold. a. Total IL-1β induced in primary human monocytes stimulated with 10^{-7}M HL-60 mononucleosomes containing 0.2kb DNA fragments after MNase digestion (+MNase), naked DNA-free nucleosomes generated with DNase I (+DNase) or benzonase (+Benzo). Statistical analysis by Mann-Whitney test. Data are representative of two independent stimulations per two donors. **b** and **c.** Total IL-1β induced in primary human monocytes stimulated with increasing concentrations of (b) recombinant histone H3 or *in vitro* citrullinated recombinant H3 (cit-H3) alone or preincubated with low concentrations of DNA purified from human NETs (NET DNA, 100ng/ml) or (c) NET DNA preincubated with 10^{-7}M recombinant histone H3. Statistical

analysis by 2-way ANOVA followed by (b) Turkey's multiple comparisons test or (c) Sidak's multiple comparisons, comparing each double stimulation to its relevant single stimulant. Data are representative of at least (b) five independent experiments and (c) two independent stimulations in two independent experiments. **d.** Primary human monocytes incubated with purified EGFP-tagged mononucleosomes at the indicated concentrations for 2 hours, stained with a live/dead stain and analysed by flow cytometry with the help of Marianna Ioannou. Data representative of two independent experiments. **e.** Death and fraction of live GFP-positive cells from (d) graphed over time for each concentration of nucleosomes. **f.** Total IL-1β induced in primary human monocytes stimulated with either LPS (55pg/ml) or increasing concentrations of HL-60 mononucleosomes. Data representative of three independent experiments.

peaking between 10^{-8} and 10^{-7}M **(Fig. 4.1f)**. These data suggested that due to the synergistic interactions between histones and DNA, human monocytes were sensitive to nucleosomes at concentrations that were much lower than the nucleosomes cytotoxicity threshold. We reasoned that at cytotoxic nucleosome concentrations, monocytes would die before they could be activated. Consistent with this hypothesis, monocytes stimulated with 10^{-6}M nucleosomes did not produce any IL-1β, consistent with the nucleosome cytotoxic capacities. These findings together indicate that the synergy of histones with DNA enables monocytes to respond to chromatin at concentrations that are much lower that its cytotoxic threshold. However, EGFP nucleosomes were shown to be unable to bind TLR4 directly **(Fig. 3.4f)**. Therefore, it is important to repeat these experiments with nucleosomes that are able to bind TLR4 as the nucleosome concentrations which monocytes can tolerate might change upon active TLR4 signalling.

4.3 Nucleosome sensing is DNA-independent in murine macrophages

As we uncovered in chapter 3, DNA synergy is dispensable for activation of BMDMs by chromatin. In order to investigate whether other TLR agonists were still able to act synergistically in BMDMs as opposed to NET DNA, we titrated recombinant citrullinated histone H3 with sub-threshold concentrations of either purified NET DNA or of a human optimised stimulatory CpG. The human-optimised CpG analogue was preferred for these experiments in order to have better control of DNA receptor activation. When compared to the extremely potent mouse-optimised CpG, the human-optimised molecule had a lower activation potential. Consistent with the previous experiments on BMDMs activated by nucleosomes, citrullinated histone H3 could drive IL-1β induction, and that was dependent on TLR4 **(Fig. 4.2a)**. The activation of WT BMDMs by citrullinated histone H3 was critically enhanced by sub-threshold concentrations of human-optimised CpG. This

potentiation was abolished in TLR9 deficient BMDMs suggesting that CpG acts synergistically with histones via TLR9 in BMDMs. In contrast, purified NET DNA failed to synergise with citrullinated histone H3 in driving IL-1β production, indicating that even though BMDMs are capable of synergy between histones and DNA, this mechanism is not engaged by chromatin DNA. Therefore, the mechanisms employed by monocytes and macrophages in response to inflammatory chromatin must be different. Finally, these experiments also suggest that in human monocytes, CpG and NET DNA enhance histone-mediated TLR4 signalling via different mechanisms.

To investigate whether extracellular nucleosomal DNA could instead promote an alternative signalling in BMDMs, we also measured the production of interferons. Even though nucleosomes at 10^{-7}M concentration were sufficient to induce IL-1β to levels comparable to LPS activation in BMDMs, these same lysates contained low IFN-β protein concentrations **(Fig. 4.2b**). In other words, at concentrations that were sufficient to drive IL-1β induction, nucleosomes were unable to drive cytokines primarily induced by DNA receptors. This suggests that when presented extracellularly, chromatin DNA fails to activate TLR9 signalling at concentrations tested.

Since we uncovered a differential role of DNA in human monocytes and murine BMDMs, we investigated whether these differences were due to cell type differences or the species origins. We isolated murine monocytes (m-monocytes) and stimulated them with histone H3 alone or pre-complexed with NET DNA. Similar to the human monocytes, DNA potently enhanced the sensitivity of murine monocytes to histone H3 (**Fig. 4.2c**). These data indicate that cellular sensitivity to histones is dependent on the cell type and can be tuned by cellular differentiation.

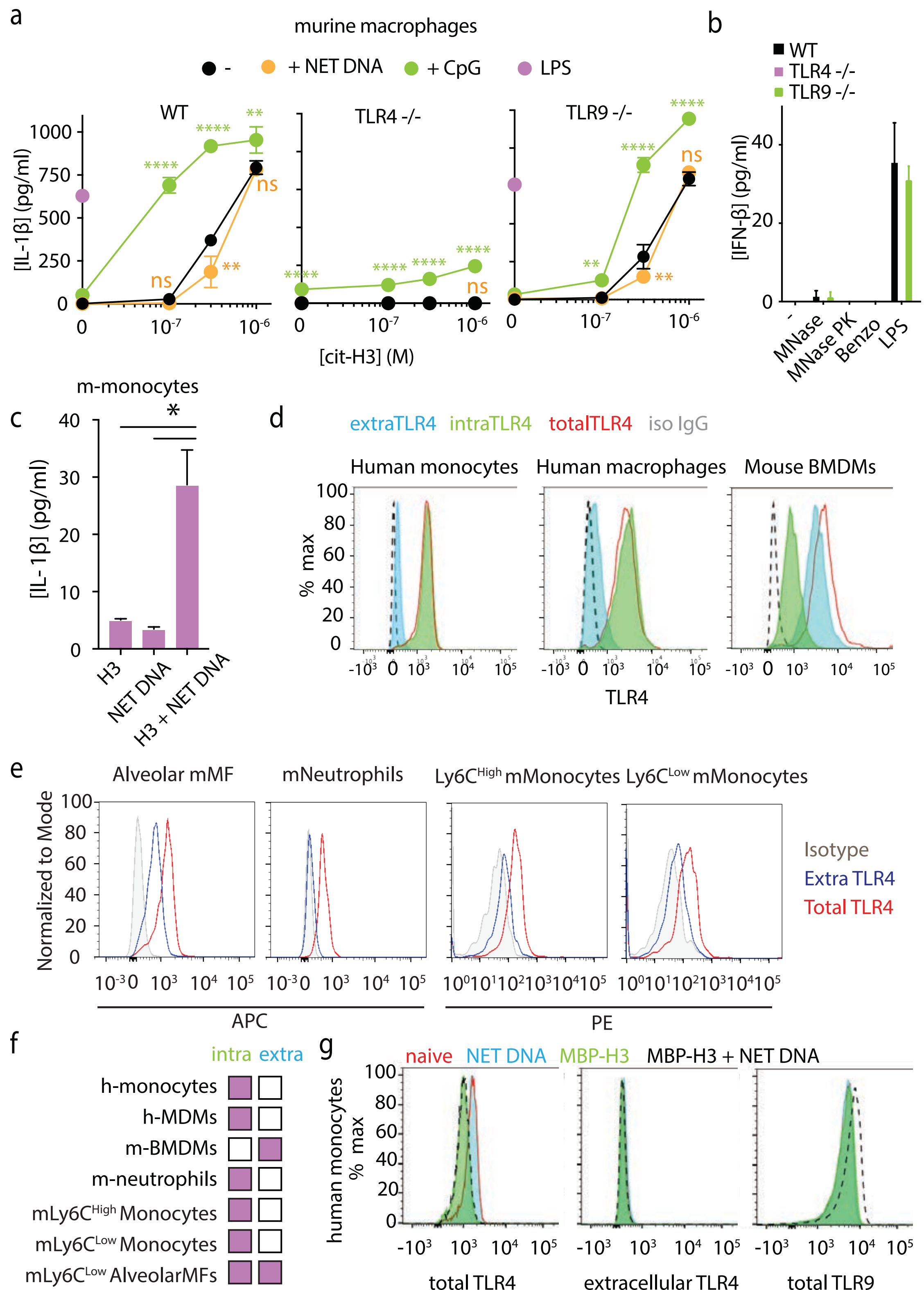

Figure 4. 2 DNA is redundant in BMDMs and TLR4 localisation in immune cells. a. BMDMs from WT, TLR4 or TLR9 deficient mice incubated with increasing concentrations of *in vitro* citrullinated recombinant H3 (cit-H3) alone, or in the presence of purified NET DNA (300ng/ml) or human preferred CpG (CpG) or LPS (0.5ng/ml). Statistical analysis by 2-way ANOVA followed by Dunnett's multiple comparison. Data representative of two

independent stimulations from two independent experiments. **b.** IFN-β production in the supernatant of BMDMs stimulated with 0.5μM nucleosomes treated with MNase, MNase followed by proteinase K and heat inactivation or Benzonase or LPS 90.5ng/ml). Data representative of two independent stimulations from two independent experiments. **c.** Total IL-1β induced in murine WT monocytes after stimulation with 10^{-7}M histone H3 alone or preincubated with low concentrations of DNA purified from human NETs (NET DNA, 1000ng/ml). Statistical analysis by Mann-Whitney test. **d.** TLR4 localization analysis by flow cytometry in human monocytes, human monocyte derived macrophages or mouse BMDM showing cell surface expression of TLR4 (extraTLR4, without cell permeabilisation), total TLR4 (totalTLR4) and therefore extrapolating the intracellular TLR4 (intraTLR4). Data acquired with the help of Marianna Ioannou. **d.** TLR4 localization analysis by flow cytometry in alveolar macrophages (mMF), murine blood neutrophils and Ly6C^{hi} and Ly6C^{low} monocytes, showing cell surface expression of TLR4 (extraTLR4) and total TLR4 (totalTLR4). Data acquired with the help of Nathalia de Vasconcelos. **e.** Summary of TLR4 localisation in immune cells analysed in c and d. **f.** Total and extracellular TLR4 and total TLR9 levels in human monocytes analyzed by flow cytometry after 2 hour stimulations with NET DNA (blue, 300ng/ml) or histone H3 fused to MBP (MBP-H3, green, 0.5μM) or MBP-H3 precomplexed with NET DNA (black).

4.4 DNA induces synergy via the regulation of TLR4 localization

To uncover the mechanism of synergy between histones and DNA, we took advantage of the differential effect of DNA on histone signalling in human monocytes and BMDMs. We hypothesised that the differential requirement for DNA synergy might be linked to differences in the cellular localization of TLR4. Therefore, we examined the cellular localization of TLR4 in these cells by flow cytometry and immunostaining. We found that in human monocytes and human monocyte-derived macrophages, TLR4 was completely absent from the cell surface and could only be detected intracellularly **(Fig. 4.2d** and **f)**. In contrast, in mouse BMDMs, TLR4 predominantly localized extracellularly, on the cell surface. This suggested that DNA-mediated synergy might depend on the localization of the receptor. We looked at a variety of murine immune cells, extracted from the lungs via bronchoalveolar lavage and the blood. TLR4 was exclusively intracellular in murine neutrophils, Ly6C^{hi} and Ly6C^{low} monocytes. In contrast, alveolar macrophages had both intra and extracellular amounts of TLR4 **(Fig. 4.2e** and **f)**.

To understand the mechanism of DNA-mediated synergy, we first examined whether DNA might be driving the translocation of TLR4 to the cell surface in monocytes by assessing the localisation of TLR4 after stimulation with histone, NET DNA or a complex of the two. Monocytes are known to increase TLR4 mRNA levels after activation with LPS (Muzio et al., 2000), however, we found total TLR4 protein levels to be slightly decreased after stimulation with recombinant histone and TLR9 increased subtly after

histone stimulation together with NET DNA **(Fig. 4.2g)**. These experiments also demonstrated that none of these stimulations drove the translocation of TLR4 to the cell surface. Based on these results, we reasoned that DNA-mediated synergy may involve changes in the signalling of intracellular TLR4 rather than via its translocation to the plasma membrane.

To investigate this possibility, we studied the effects of DNA stimulation on the localization of intracellular TLR4 in human monocytes. We stimulated human monocytes with MBP-H3 fusion protein alone, or in complex with purified NET DNA and immunostained for TLR4 and anti-MBP. Staining the MBP tag allowed us to distinguish the uptaken extracellular histone from the endogenous histones of the cells. Interestingly, in the presence of DNA, TLR4 localized around histones in contained cellular compartments **(Fig. 4.3a)**. In many cases, these compartments were also Rab5 positive, suggesting that extracellular histones were being sequestered in endosomal compartments **(Fig. 4.3b)**. In the absence of DNA, TLR4 failed to translocate to histone-containing endosomes and remained generally cytosolic. These data suggested that extracellular DNA might be achieving its synergistic effects by promoting the translocation of TLR4 to histone containing compartments.

We then investigated whether the fact that DNA drives TLR4 translocation to histone-containing endosomes in monocytes correlates with the induction of IL-1β. For that purpose we differentiated human monocytes to macrophages which also have intracellular TLR4 as assessed by flow cytometry **(Fig. 4.2d)**. Interestingly, we did not observe any TLR4 translocation to histone-containing compartments upon stimulation with histone and DNA **(Fig. 4.3c)**. Consistently, even though human macrophages can respond to LPS, both GM-CSF and M-CSF derived macrophages are not able to respond to the same nucleosome concentrations that drive potent activation in monocytes, even in the presence of DNA **(Fig. 4.3d)**. Therefore, macrophages are desensitized towards chromatin via unknown mechanisms that seem to block TLR4 translocation to the histone-containing compartments. These data suggest that TLR4 recruitment to histone containing endosomes is essential for inflammatory signalling.

In conclusion, we show that monocytes can sense histones and that could depend on the localisation of the receptor. In cells were TLR4 is expressed on the cell surface, histones are sufficient to drive the inflammatory cytokine response. However, in cells

were TLR4 is intracellular, DNA is required to promote the translocation of TLR4 to endosomal compartments that contain the internalised histone. By translocating there, the histone concentration required for activation is lowered below the histone cytotoxicity threshold. This suggests that cells may be regulating their responses to DAMPs by a mechanism that involves the change of TLR4 localization. DNA may therefore in reality play a co-stimulatory role in inflammation, enhancing the sensitization to histones rather than DNA-bound proteins assisting DNA-mediated signalling. Here, we uncover a synergistic mechanisms of the innate immune system tuning the response to such omnipresent molecules as histones and DNA. The regulation of intracellular localization of TLR4 by DNA, introduces an additional checkpoint for activation by extracellular histones.

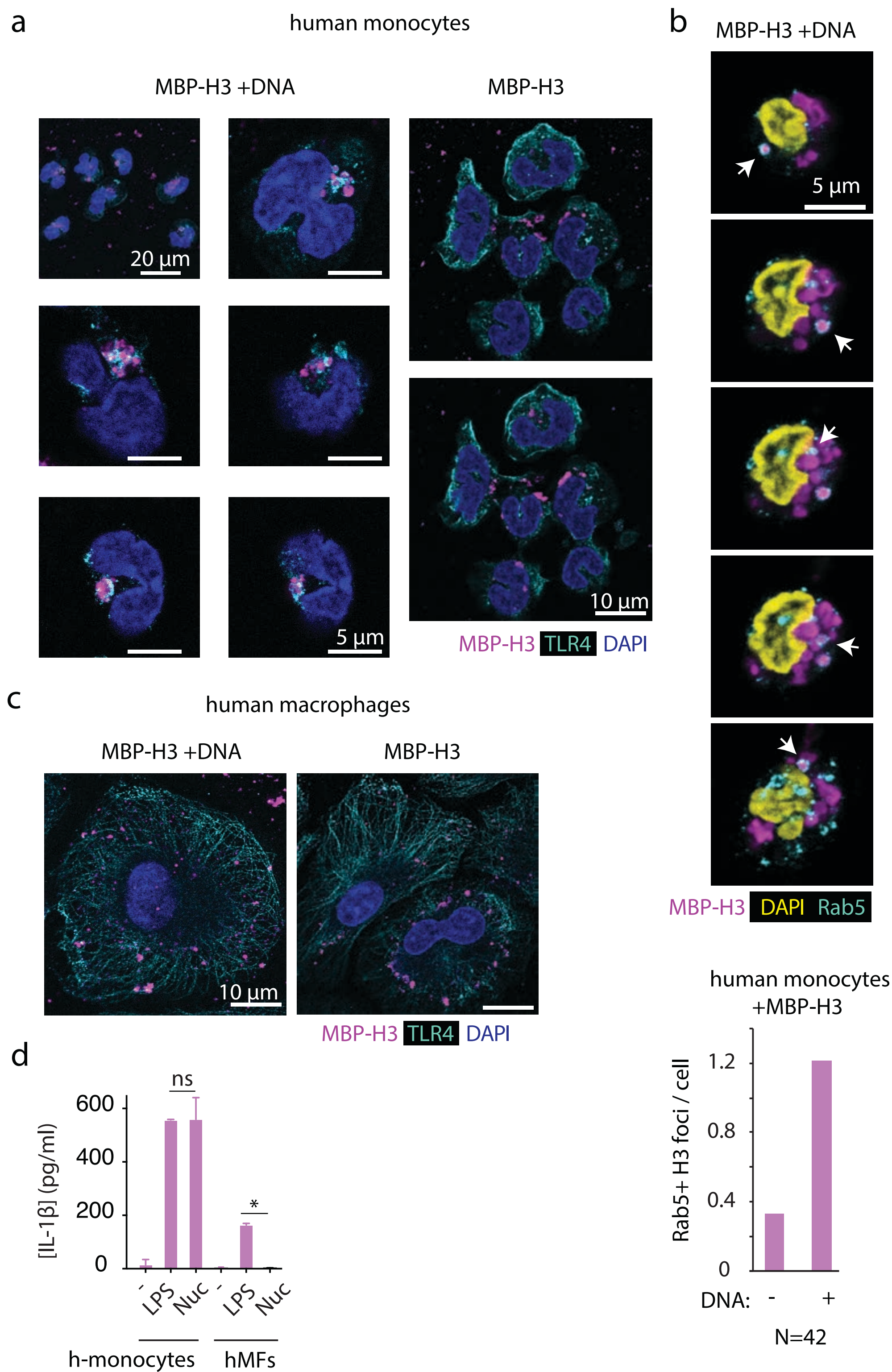

Figure 4. 3 DNA drives synergy by inducing intracellular TLR4 translocation to histone-containing compartments. a. Immunofluorescence confocal micrographs of primary human monocytes incubated with recombinant histone H3 fused with maltose binding protein (MBP-H3) alone, or preincubated with NET DNA, stained for DAPI (nuclear DNA, blue), MBP to detect exogenous histone H3 (magenta) and TLR4 (cyan).

Upper left panel in MBP-H3+DNA stimulations depicts a max projection of 5 sections from a single image (scale bar 20µm). The other 5 panels depict higher magnifications of single confocal sections of cells from the upper left panel (scale bars 5µm). For MBP-H3 stimulation, both micrographs are different z-stack confocal sections for same cells (scale bar 10µm) **b.** Rab5 positive endosomes (arrows) depicted in a z-stack immunofluorescence confocal micrograph sequence of human monocytes incubated with MBP-H3 preincubated with NET DNA, stained for DAPI (yellow), MBP (magenta) and Rab5 (cyan). **c.** Immunofluorescence confocal micrographs of primary human monocyte derived macrophages incubated with recombinant histone H3 fused with maltose binding protein (MBP) alone, or preincubated with NET DNA, stained for DAPI (nuclear DNA, blue), MBP to detect exogenous histone H3 (magenta) and TLR4 (cyan). **d.** Total IL-1β produced by primary human monocytes or human monocyte derived macrophages (hMFs) stimulated with LPS (0.5ng/ml) or 10^{-7}M mononucleosomes (Nuc). Statistical analysis by Mann-Whitney test. Data representative of at least three independent experiments.

Chapter 5. Histones promote atherosclerosis

Introduction

In atherosclerosis, the immune system's inflammatory response to high cholesterol in the blood leads to the formation of plaques on the arterial walls, constricting the blood flow and eventually leading to plaque rupture and death. NETs directly drive local inflammation, by activating macrophages to produce IL-1β. We have shown that histones and DNA are the main pro-inflammatory component of NETs *in vitro*. Here we explore the role of chromatin in driving sterile inflammation *in vivo*.

5.1 Endogenous histones promote atherosclerosis

In order to understand whether histones and chromatin are drivers of chronic sterile inflammation *in vivo*, we employed the antibody against the H2A-H2B-DNA chromatin complex termed PL2-3 on our atherosclerosis mouse model. We compared the full length IgG and the Fab fraction of the antibody.

We previously showed that the Fab fragment of the chromatin antibody significantly reduced mononucleosome activation of human monocytes (**Fig. 3.4b** on page 81). We further confirmed the neutralising effect of this chromatin Fab antibody (PL2-3 Fab) on murine bone marrow derived macrophages stimulated with mononucleosomes **(Fig. 5.1a)**. The full length chromatin antibody was less potent in inhibiting IL-1β induction, and interestingly, the treatment of mononucleosomes with two histone antibodies, did not have any effect on inhibiting IL-1β production, similar to a control IgG. These results suggested that an antibody needs to neutralise both histones and DNA in order to effectively block the inflammatory activity of chromatin on murine monocytes.

In our *in vivo* model, the treatment of atherosclerotic mice with the PL2-3 Fab fragment did not change the levels of cholesterol or triglycerides accumulating in the blood over 6 weeks on high fat diet **(Fig. 5.1b)**, but the size of atherosclerotic plaques was significantly reduced in treated mice of either sex **(Fig. 5.1c and d)**. IL-1β concentrations in the plasma were also decreased in mice receiving the PL2-3 Fab, but this difference was not statistically significant **(Fig. 5.1f)**. Interestingly, the full length PL2-3 IgG antibody was not able to block plaque formation **(Fig. 5.1d)**. This suggested that NET binding antibodies might have to lack the effector region in order to block inflammation effectively *in vivo*. This would be consistent with previous reports proposing a pathogenic role for NET-containing immune complexes, in autoimmune pathology. Alternatively, the smaller Fab fragment could be more efficient in penetrating the arterial wall and blocking chromatin mediated signalling.

To investigate whether chromatin blocking could also act therapeutically as well as preventatively for sterile inflammation, we allowed animals to first develop plaques for 6 weeks and then administered the antibody for the following 6 weeks while the animals were switched back to the standard diet. In this case, the administration of the PL2-3 Fab antibody following initial plaque formation could not rescue pathology, suggesting that atherosclerotic pathology could not be reversed after the establishment of initial inflammation **(Fig. 5.1g)**. Interestingly, even though the weight of these animals plateaued after switching back to standard diet at 6 weeks (data not shown), the plaques continued to grow to almost double the size in the next 6 weeks **(Fig. 5.1g** compared to **Fig. 5.1d)**. This could be because cholesterol persists in circulation after switch to standard diet, or because plaques continue to grow once established for 6 weeks. In the latter situation, the growth of already established plaques is independent of direct chromatin signalling.

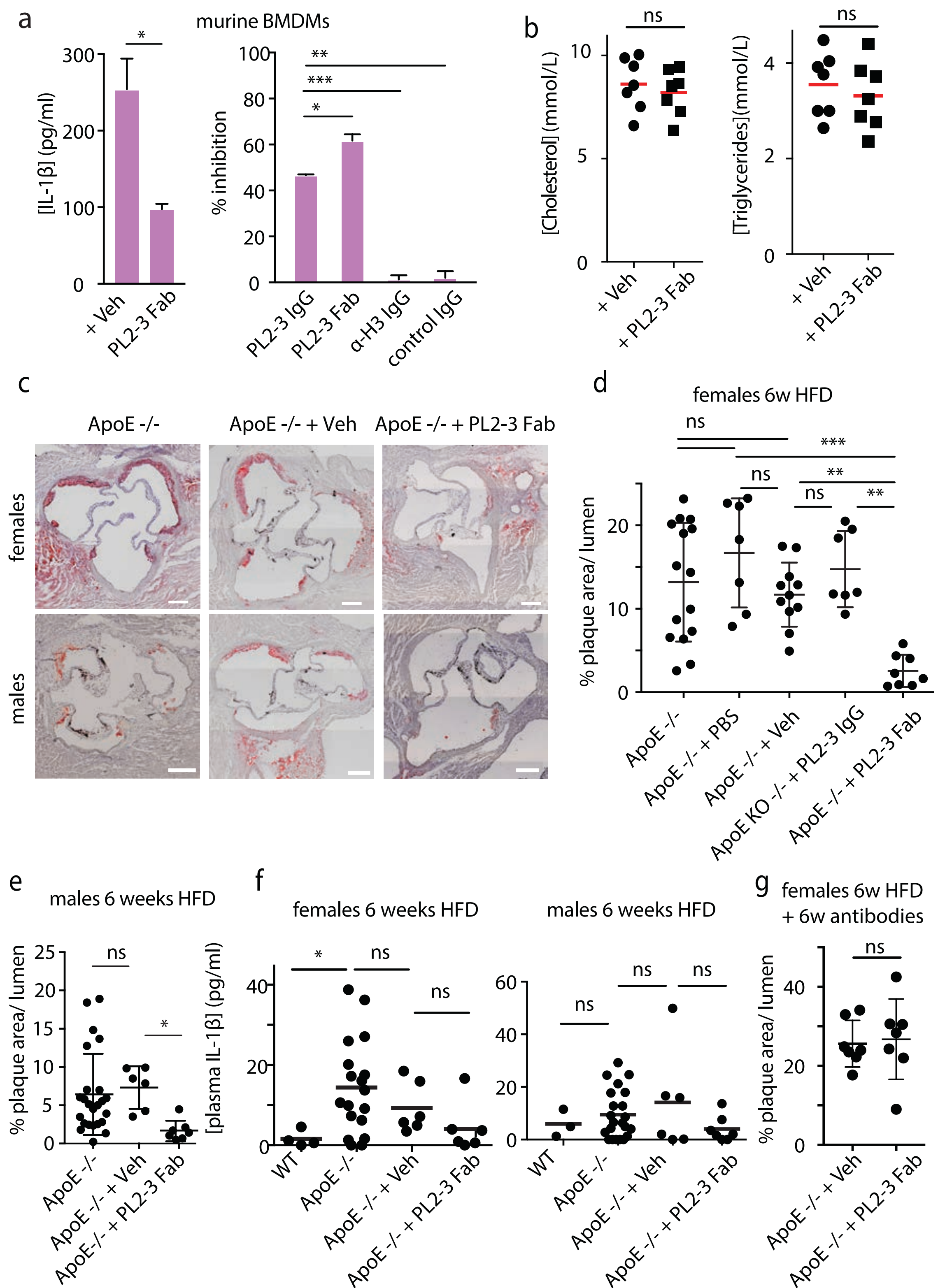

Figure 5. 1 Chromatin neutralisation prevents atherosclerosis. a. (left) Total IL-1β induced in murine BMDMs from WT mice treated with 10^{-8}M mononucleosomes in the presence of Fab elution buffer (Veh) or the PL2-3 anti-chromatin Fab (PL2-3). (right) Fraction of inhibition of total IL-1β induction in WT BMDMs stimulated with 10^{-8}M mononucleosomes in the presence of PL2-3 full length IgG or Fab, or a combination of two anti-histone H3 antibodies or a control IgG. Statistical analysis by Mann-Whitney

test. Data are representative of two independent stimulations from two experiments. **b.** Cholesterol and triglyceride levels in the plasma of female ApoE deficient mice after 6 weeks on high fat diet while treated with PL2-3 anti-chromatin Fab (PL2-3 Fab) or Fab elution buffer (Veh). Each point represents one animal. Statistical analysis by Mann-Whitney test. **c.** Aortic root cross-sections from female or male ApoE deficient animals either untreated or treated with Fab elution buffer (Veh) or the PL2-3 anti-chromatin Fab (PL2-3 Fab) fed on high fat diet (HFD) for 6 weeks. Sections were stained with the Oil Red O lipid dye (red) and counterstained with haematoxylin. Scale bar: 200μm Images are representative of >7 animals per group. **d** and **e.** Quantitation of average plaque size from multiple sections per aortic root, plotted as the fraction of lumen area in female (d) or male (e) ApoE deficient animals either untreated or treated with the PL2-3 anti-chromatin full length IgG or Fab or PBS or Fab elution buffer (Veh) fed on high fat diet for 6 weeks. Each point represents one animal. Statistical analysis by one-way ANOVA followed by Turkey's multiple comparison test. **f.** IL-1β concentration in plasma from female or male mice treated as in (d) and (e) or WT mice fed on standard chow until same age. Each point represents one animal. Statistical analysis by one-way ANOVA followed by Turkey's multiple comparison test. **g.** Quantitation of average plaque size from multiple sections per aortic root, plotted as a fraction of lumen area in female ApoE deficient animals after 12 weeks. First fed for 6 weeks on high fat diet for and then treated with Fab elution buffer (Veh) or the PL2-3 anti-chromatin full length IgG or Fab while switched on standard diet for following 6 weeks. Each point represents one animal. Statistical analysis by Mann-Whitney test.

5.2 Citrullination contributes to atherosclerotic pathology in a sexual dimorphic fashion

During NET formation, histones are citrullinated by protein arginine deiminase 4 (PAD4). In chapter 3, we demonstrated that citrullination is not required for NETosis in atherosclerosis and PAD4 deficient mice contain NETs in their atherosclerotic plaques similarly to PAD4 sufficient animals **(Fig. 3.2 a** and **b,** page 75**)**. We also revealed that citrullinated NETs and recombinant histones exhibit a higher capacity to upregulate IL-1β, IL-1α and IL-6 than non-citrullinated equivalents. In order to investigate the contribution of citrullination in chromatin-mediated inflammation *in vivo*, we studied ApoE/PAD4 deficient mice.

Citrullination on NETs enhances plaque formation moderately *in vivo*, reflecting the modest role that citrullination played *in vitro*. Interestingly, this was detected only in female ApoE/PAD4 mice **(Fig. 5.2a and b)**, but not in males. The role of PAD4 only in female mice suggests that while NET chromatin drives disease in both genders, PAD4 activity is responsible for the increased susceptibility of females to atherosclerosis.

However, more animals need to be analysed in order to confirm the sexual dimorphism findings. Our results also correlate with a recent report that showed that PAD4 did not influence atherogenesis in male mice (Franck et al., 2018). In this study, the authors did not investigate female mice and interpreted these results as evidence that NETs are not implicated in atherosclerosis. Yet, they also report that PAD4 influenced fibrous cap thickness in male mice, suggesting that not all PAD4-related phenotypes are sexually dimorphic. Overall, much work is still needed in order to understand the basis for this sexual dimorphism.

To conclude, our results indicate that extracellular chromatin is a major driver of sterile inflammatory disease and plays a key role in the early inflammatory events that promote the formation of atherosclerotic lesions. Using a chromatin neutralising Fab antibody fragments, we reveal that inflammation can be prevented in the initial stages of atherosclerosis, but not after establishment of initial pathology. Moreover, our data indicate that the presence of the Fc-binding motif might be negating the atheroprotective effect of chromatin neutralisation. Finally, we show that histone modifications also contribute in enhancing the pro-inflammatory capacity of chromatin *in vivo*, however this is only true for female mice.

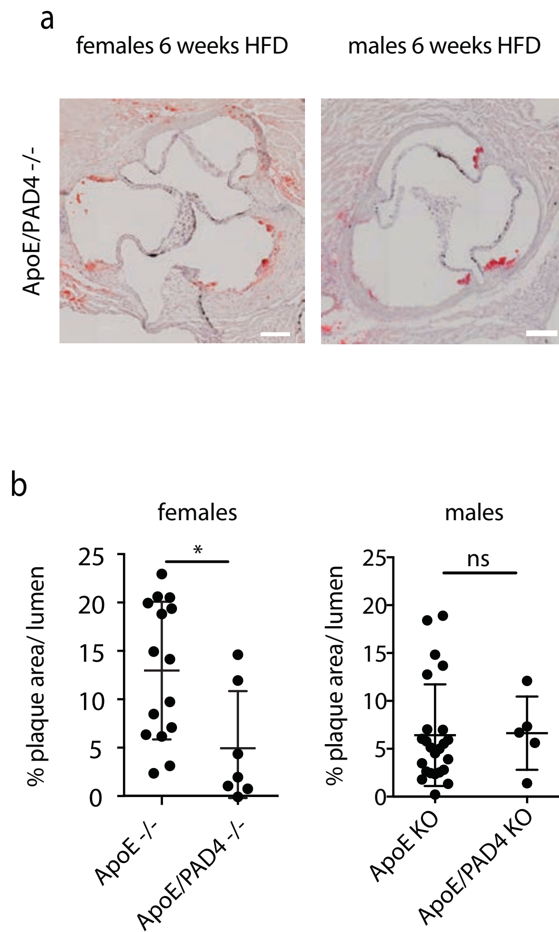

Figure 5. 2 PAD4 deficiency reduces atherosclerosis in female mice but not male. **a.** Aortic root cross-sections from female (left) or male (right) ApoE/PAD4 deficient animals fed on high fat diet (HFD) for 6 weeks. Sections were stained with the Oil Red O lipid dye (red) and counterstained with haematoxylin. Scale bar: 200μm Images are representative of >5 animals per group. **b.** Quantitation of average plaque size from multiple sections per aortic root, plotted as a fraction of lumen area in female (left) or male (right) ApoE and ApoE/PAD4 deficient fed on high fat diet for 6 weeks. Each point represents one animal. Statistical analysis by Mann-Whitney test.

Chapter 6. Discussion

The work presented here described previously unknown mechanisms by which extracellular chromatin originating from sources such as NETs, promotes inflammation and directly contributes to sterile inflammatory conditions such as atherosclerosis. The main effector cells in atherosclerosis are monocyte-derived macrophages (Landsman et al., 2009). We uncovered that the proinflammatory capacity of chromatin is mediated by histones activating TLR4 on monocytes and macrophages. In its native state extracellular chromatin signals via histones and DNA plays an optional regulatory role that our data suggest it may depend on the cellular localization of TLR4 **(Fig. 6.1)**.

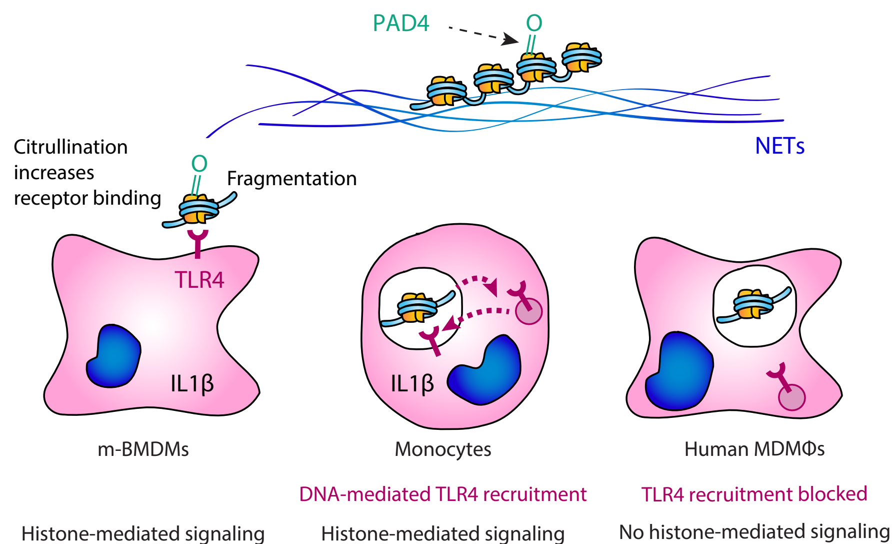

Figure 6. 1 Model depicting mechanism regulating NET-driven inflammation.

PAD4-mediated citrullination and fragmentation enhance the pro-inflammatory capacity on NET chromatin. The differential localization of TLR4 determines the requirement for DNA in recruiting. Cells with extracellular TLR4 respond to chromatin histones without any requirement for DNA. In contrast, monocytes with intracellular TLR4 required DNA to recruit TLR4 to histone-containing compartments. Finally, differentiation of human monocytes to macrophages switches off their sensitivity to chromatin by blocking the TLR4 recruitment to endosomes.

Synergy between different TLR agonists seems to rely on various mechanisms. Previous studies using potent TLR agonists suggested that microbial agonists mediate synergy by enhancing the activation of the MAPK and ERK pathways (De Nardo et al., 2009a). Also, co-receptors such as RAGE, after recognising HMGB1, increase the recruitment of MyD88 to TLR9 during DNA activation (Tian et al., 2007). We found that both monocytes and macrophages are equipped to respond to low sub-lethal concentrations of nucleosomes but differ in the underlying mechanisms that potentiate sensitivity. Due to histone cytotoxicity on higher concentrations of chromatin, DNA has minimal contribution in chromatin-mediated immune signalling. Our work proposed that DNA is required to potentiate histone signalling in monocytes.

In the case of our work with recombinant histone and NET DNA titrations, the sigmoidal switch-like responses to histones reveal the multivalent cooperative interactions. The addition of DNA to histones in the TLR4 reporter system, does not alter the shape of the response to the latter stimulant, which is an indication that DNA does not act by influencing the intrinsic cooperative mechanism or the direct recognition of histones by TLR4 on the plasma membrane. We have also eliminated the possibility that DNA-mediated synergy could affect avidity by tethering multiple nucleosomes together, since chromatin fragmentation with MNase increases its activation potential.

The cooperativity between DNA and histones plays a critical role in human monocyte responses where TLR4 is intracellular. In these cells, DNA correlates with the translocation of TLR4 to endosomes that contain uptaken chromatin. In contrast, when TLR4 is expressed on the cell surface, DNA is dispensable for histone mediated signalling. Mouse macrophages were capable of synergistic mechanisms between histones or LPS (LPS synergy data not shown) and a CpG stimulatory oligonucleotide, mediated by TLR4 and TLR9. However, we could not identify any cooperativity driven by chromatin-derived extracellular DNA and DNA also did not mediate TLR4 translocation to histone containing compartments in these cells. This suggested that chromatin DNA and CpG act via different mechanisms in macrophages.

These differences in the two cell types we examined might allow us to understand how cells respond to chromatin *in vivo*. Atherosclerotic pathology could be inhibited by DNase I treatments, demonstrating that chromatin activation of monocyte-derived macrophages *in vivo* depends on DNA (Chao et al., 2016, Liu et al., 2018, Warnatsch et al., 2015). Our

data suggested that TLR4 localisation may provide an additional checkpoint for monocytes to respond to extracellular histones, requiring DNA co-stimulation. Therefore, the differential responses to chromatin indicate that responses to DAMPs might be regulated by altering TLR4 localisation. That would consist a novel model for synergy between DAMPs.

The sensitivity towards chromatin fragmentation and histone citrullination adds layers of regulation. The decondensation of chromatin and subsequent processing by nucleases during NET formation may be important to potently activate monocytes and macrophages *in vivo*. These checkpoints may allow cells to differentiate between chromatin deriving from dead cells or from extracellular traps. Through these mechanisms, cells are able to respond to chromatin below the cytotoxic threshold of histones when appropriate, allowing for specificity in the response towards such ubiquitous molecules. Our work indicated that processive nucleases like DNase I and benzonase could disrupt chromatin-driven inflammatory signalling, through eliminating the synergy of DNA with histones. On the other hand, nucleases that fragment chromatin such as MNase, increase NET-driven inflammation due to the generation of potent proinflammatory mononucleosomes.

Citrullination, a post-translational modification found excessively on NETs, further contributed to the proinflammatory potential of histones. PAD4-mediated citrullination has been thought to be required for NET formation, however, many studies have not used sufficient markers for claiming absence of NETs in immunostainings of PAD4 deficient conditions (Li et al., 2010a, Wang et al., 2009a). A recent study investigated the role of citrullination in NET formation by examining the accessibility of DNA upon PMA activation (Chen et al., 2016). This study suggests that citrullination mediates the disassembly of DNA and histones from mononucleosomes in the later stages of NET formation. We find that mononucleosomes lose their signalling capacity upon DNase or Proteinase K treatments. Based on their hypothesis, mononucleosomes would lose their signalling capacity upon citrullination, contradicting our findings. However, one should consider that their findings are based on DNA accessibility. Changes in DNA accessibility for example via partial MNase digestion, might allow for DNA to present as accessible via the ATAC-see method but it would likely be very difficult to determine the lack of histones. Also, the removal of histones from DNA by post translational modifications is likely to be very challenging, due to the very high isoelectric point of non-citrullinated

histones (9.80-10.25). Therefore, even though these findings should be taken into consideration, further studies would need to be performed to determine the true molecular changes that occur to chromatin due to citrullination of histones.

Previous studies have linked PAD4 in atherosclerosis, by suggesting that PAD4 deficiency protected mice due to a defect in NET formation (Knight et al., 2014, Liu et al., 2018). A different report described no differences in atherosclerotic plaque formation between male chimeric mice that received a WT control or a PAD4 deficient bone marrow transplant, suggesting that NETs were not implicated in lesion development, but PAD4 did affect the thickness of fibrous caps (Franck et al., 2018). Also, PAD4 deficient animals were protected against inflammation that caused kidney and liver injury in a renal ischemia reperfusion model, however the sex of the animals was not indicated (Rabadi et al., 2016). Our work identifies NETs developing in the plaques of PAD4 deficient mice, but citrullination potentiates the proinflammatory capacity of histones, resulting in enhanced pathology. In comparison to NE/PR3 deficiency (Warnatsch et al., 2015) and chromatin antibody targeting, PAD4 deficiency leads to an intermediate phenotype. This is consistent with PAD4 playing a role in potentiating NET function, instead of PAD4 being required for NET formation in atherosclerosis.

Moreover, the contradictory PAD4 data in the literature can be explained by the sexually dimorphic dependence of atherogenesis on PAD4. These differences indicate that the elevated propensity for atherosclerosis in female mice might be related to PAD4 activity. PAD4 and NETs have been associated with autoimmune diseases like SLE (Smith and Kaplan, 2015). Although in the general population the risk for atherosclerosis is higher in males, atherosclerosis is prevalent in SLE patients, the majority of which are women (Feldman et al., 2013, Schoenfeld et al., 2013). Therefore, the sexual dimorphism associated with the PAD4 phenotype in mice suggests that the enzyme may be implicated in mechanisms underlying the prevalence of atherosclerosis in SLE.

Our findings propose a regulatory role for citrullination that affects the function of NETs and raise the issue of potential sex differences in these mechanisms. Our data also lead to a requirement for reinterpretation of previous studies where PAD4 deficiency was equated to a NET deficiency. However, the mechanism by which citrullination potentiates histone activity remains unclear. We do not detect any significant differences in the activation of the TLR4 reporter line from citrullinated or non-citrullinated histones. Yet,

citrullination increased the binding capacity of nucleosomes to TLR4, indicating a possible mechanistic basis in the regulation of the inflammatory capacity of extracellular histones.

In sepsis, histones have been observed in circulation and anti-histone antibodies are protective (Xu et al., 2009). Also, injecting an excess of recombinant histones in mice drives TLR-dependent inflammation and lethality (Xu et al., 2011). Clodronate liposomes which do not trigger systemic inflammation induce the release of circulating histones at levels that are much higher than any microbial sepsis challenge model (unpublished data from Marianna Ioannou). The histone amounts injected in these papers are 50-fold higher than the endogenous concentrations released by clodronate liposome treatment, making these findings extremely artificial. Therefore, it remains unclear whether the inflammation caused in these acute models is triggered by histones directly or by the different DAMPs that get released because of excessive histone-mediated cytotoxicity. In our atherosclerotic model, we identify endogenous chromatin as a driver of chronic sterile inflammatory disease under physiological conditions. Nevertheless, our data clearly show that histone-mediated inflammation is associated with signalling in viable cells at low concentrations, rather than inflammation caused by their cytotoxic roles at higher concentrations.

The physiological role for sensing extracellular chromatin is still not clear. Our fungal infection experiments in transgenic mice that express EGFP-bearing histones suggest that sensing extracellular chromatin might be beneficial in enhancing inflammation to protect against harmful pathogens. In our chromatin neutralising experiments, uninfected animals could tolerate the administration of the antibody for several weeks without presenting any immune defence compromise. In contrast to the NET-deficient ApoE animals that lack NE/PR3, we only observe a decrease in plaque formation after chromatin neutralisation and not in circulating cytokines. This suggests that the effects of chromatin neutralisation were more confined than a deficiency in neutrophil proteases that have been shown to contribute to inflammation elsewhere in these models, such as the adipose tissue and liver (Talukdar et al., 2012).

Moreover, our data suggest that the presence of the Fc-binding motif may play a decisive role *in vivo*. The full-length antibody against chromatin did not block atherosclerotic plaque formation but the removal of its Fc-binding domain is protective. These results

align with prior reports that demonstrate that immune complexes cause NET formation and drive autoimmune pathology (Behnen et al., 2014). On the other hand, full-length anti-histone antibodies can rescue sepsis mortality in mice likely due to the neutralization of histone cytotoxicity rather than pro-inflammatory activity. How these antibodies operate during sepsis is not well understood since the physical state of free circulating histones and the mechanisms of inflammation are unclear.

Even though the long-term effects of chromatin targeting are currently not understood, we unveil a new potential therapeutic avenue to treat chronic inflammatory pathology, that does not interfere with the anti-microbial responses, an unavoidable side effect of targeting inflammatory cytokines and other downstream signalling mediators.

6.1 Future plans

This work reveals endogenous chromatin as a potent driver of inflammation. We uncover that histones, rather than DNA, are the major pro-inflammatory component of chromatin and NETs. Chromatin is recognised by TLR4 and the sensing mechanisms differ in cells, based on the localisation of TLR4. We find that DNA enables histones to activate monocytes, where TLR4 is intracellular, at concentrations below the histones' cytotoxicity threshold, via the translocation of TLR4 to histone-containing endosomes. DNA is dispensable for histone-mediated signalling when TLR4 is expressed on the cell surface. In the future, we plan to identify the mechanism by which DNA drives the translocation of TLR4 to histone-containing compartments. Initially, we want to describe the precise intracellular localization of TLR4 in unstimulated blood monocytes before recruitment to histone compartments. Moreover, it will be interesting to uncover whether DNA directly interacts with TLR4, or via a different unknown receptor. Since we have optimised the experimental set up in order to investigate DNA synergic mechanisms in mouse monocytes, we will use genetic knock-outs to examine the involvement of different DNA receptors such as TLR9, cGAS, STING and AIM2.

Furthermore, we show that histone citrullination is dispensable for NET formation *in vitro* and *in vivo*, but it potentiates atherosclerosis in a sexual dimorphic manner. The mechanism that underlies this sexual dimorphism remains unknown. Therefore, we

would like to explore potential differences of monocytes and macrophages from male or female animals in response to citrullinated histones as well as differences in NET formation. Additionally, we would like to assess the effect of sexual hormones on the activation of these cells.

Finally, we find that the administration of chromatin blocking antibodies *in vivo* can significantly reduce sterile inflammation. Interestingly, this protective effect was only achieved when administering the Fab fragments of these antibodies. In opposition, the full length IgG was unable to protect animals against atherosclerosis. It would be of great interest to identify whether the Fab fragment achieves its protective effect due to the lack of immune complex formation of the Fc domain, or due to a more efficient tissue penetrance because of its smaller size. To test this, we have now developed a PL-2-3 antibody that has a mutated Fc domain, making it unable to bind to its receptor. Also, we would like to explore whether chromatin and NET-mediated pro-inflammatory signalling can contribute to other sterile inflammatory pathologies on mouse models of rheumatoid arthritis and tumour metastasis. *In vitro*, it would be interesting to uncover the exact step at which the antibody interferes with chromatin-mediated signalling. Of note, we would like to investigate whether the antibody interferes with the uptake of histones in monocytes or with the translocation of TLR4 to the histone containing compartments and its ability to signal.

Appendix

List of antibodies used

Antibody name	Catalogue number	Company
Histone H3 (citrulline R2+R8+R17) (anti-cit-H3)	Ab5103	Abcam
HA tag antibody	Ab9110	Abcam
TLR4 antibody	Ab22048	Abcam
S100A8 antibody	ABIN111892	Antibodies online
Mouse IgG1 kappa isotype control antibody	ABIN2704378	Antibodies online
Alexa Fluor 647-anti-mouse Ly-6G antibody	127610	Biolegend
Maltose Binding Protein (MBP) antibody	906901	Biolegend
Rab5 antibody	C8B1 - 3547	Cell Signalling Technology
Neutrophil elastase antibody	GTX72042	GeneTex
TLR2 neutralizing antibody	pab-hstlr2	Invivogen
TLR4 neutralizing antibody	pab-hstlr4	Invivogen
Histone H3 antibody	07-690	Millipore
Myeloperoxidase antibody	AF3667	R&D

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