Regulation of Innate Immunity by DNA Damage Signaling

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von

Christopher J. Harbort
Bachelor of Science – Biology

Präsidentin der Humboldt-Universität zu Berlin

Prof. Dr. Ing. Dr. Sabine Kunst

Dekan der Lebenswissenschaftlichen Fakultät

der Humboldt-Universität zu Berlin

Prof. Dr. Bernhard Grimm

Gutachter/innen

1. Prof. Dr. Angela Kaindl
2. Prof. Dr. Klaus Osterrieder
3. Prof. Dr. Arturo Zychlinsky

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ZUSAMMENFASSUNG:

Eine Entzündung ist eine biologische Antwort auf einen schädlichen Reiz, etwa ein Pathogen oder eine beschädigte Zelle und dient dem Schutz gegen Infektionen, dem Entfernen beschädigten Gewebes und dem Einleiten von Reparaturvorgängen. Neutrophile sind phagozytische Zellen des angeborenen Immunsystems von Säugetieren. Ihre zerstörerische Kraft spielt einerseits eine essentielle Rolle bei der entzündlichen Bekämpfung von Mikroorganismen, birgt aber auch das Potential erheblicher Kollateralschäden. Um chronische Entzündungen und Erkrankungen zu vermeiden, müssen diese Zellen und ihre Funktionen deshalb streng reguliert werden. Die Neutrophilen selber nehmen an dieser Regulierung durch das Freisetzen von pro- und antiinflammatorischen Signalen teil, unter anderem produzieren sie proinflammatorische Zytokine oder initiieren rechtzeitig die Apoptose.

Ein Eckpfeiler der Regulierung von Zytokinproduktion und Zelltod ist der oxidative Burst, bei dem Neutrophile reaktive Sauerstoffspezies (ROS) bilden. Ein Ausbleiben des oxidativen Bursts, z. B. bedingt durch genetische Mutationen, führt zu einer als „septische Granulomatose“ (CGD) bezeichneten Krankheit, die sich durch eine dysregulierte angeborene Immunantwort und damit durch Immundeffizienz bei gleichzeitiger Autoinflammation auszeichnet. Die molekularen Ziele von ROS, welche diese Mechanismen regulieren, sind nicht zweifelsfrei identifiziert.

Wir haben „ataxia-telangiectasia mutated (ATM)“ Kinase, ein Hauptregulator der DNA-Schadensantwort (DDR), als einen ROS-abhängigen Modulator von Neutrophilen identifiziert. Mutationen in ATM führen zu der pleiotropischen Erkrankung „Ataxia Telangiectasia“. AT Patienten leiden nicht nur unter den Folgen der fehlerhaften DNA-Reparatur sondern zeigen auch Krankheitserscheinungen die stark an das inflammationsassoziierte CGD erinnern. Diese Beobachtung veranlasste uns, die Neutrophilen dieser Patienten genauer zu untersuchen.

Zunächst zeigen wir, dass Neutrophile von AT Patienten erhöhte Menge an Zytokinen produzieren und eine verlängerte Lebensspanne aufweisen. Diese Effekte entstehen durch eine erhöhte Aktivierung von p38 MAP Kinase. Wir zeigen außerdem, dass die Aktivierung der DDR die Zytokinproduktion unterdrückt und die Zellen in die Apoptose führt. Der oxidative Burst ist notwendig für die endogene Aktivierung von ATM und somit für die Regulierung dieser Prozesse in aktivierten Neutrophilen. Zu guter Letzt haben wir ein Mausmodell mit einer DDR Defizienz spezifisch in den Zellen der angeborenen Immunantwort entwickelt, das uns weitere in vivo Studien dieses Signalweges ermöglicht.

Diese Arbeit enthüllt einen neuartigen Mechanismus der Regulierung von Zytokinproduktion und Apoptose in Neutrophilen und etabliert die DDR als ein Ziel der ROS-gesteuerten Immunmodulation. In diesem Zusammenhang wird auch gezeigt, dass eine dysregulierte Neutrophilenaktivität dem hyperinflammatorischen Phänotyp von AT Patienten zugrundeliegen könnte. Wir glauben, dass inflammatorische Vorgänge die treibende Kraft hinter Teilen der Pathologie von AT Patienten sein könnten und somit ein potentielles Ziel für klinische Intervention darstellen. Dies ist besonders relevant, da AT bisher als unbehandelbar gilt.

SUMMARY:

Inflammation is a biological response to harmful stimuli, such as pathogens or damaged cells, aimed at protecting against infection, removing damaged tissues, and initiating repair. Neutrophils are phagocytic cells of the mammalian innate immune system whose destructive functions play an essential role in inflammation and microbial clearance, but also cause collateral damage to the host. Therefore, these processes must be tightly regulated to prevent chronic inflammation and disease. Neutrophils contribute to this regulation by exerting both pro- and anti-inflammatory signals, including the production of pro-inflammatory cytokines and the timely initiation of apoptosis.

A cornerstone of the regulation of neutrophil cytokine production and apoptosis is the oxidative burst, by which neutrophils generate reactive oxygen species (ROS). Deficiencies in the oxidative burst cause chronic granulomatous disease (CGD), a disorder characterized by dysregulated innate immune responses, leading to immunodeficiency and simultaneous autoinflammation. The downstream targets of ROS responsible for regulating these functions have not yet been fully identified.

We have identified ataxia telangiectasia mutated (ATM) kinase, a master regulator of the DNA damage response (DDR), as a ROS-dependent modulator of neutrophil responses. Mutations in ATM cause the pleiotropic disease Ataxia-telangiectasia (AT). In addition to disorders resulting from defective DNA repair, AT patients suffer from numerous manifestations that are reminiscent of the inflammation associated with CGD, leading us to examine their neutrophil responses.

We began by showing that neutrophils from AT patients overproduce pro-inflammatory cytokines and have a prolonged lifespan. This effect was mediated by increased activation of p38 MAP kinase. We additionally show that activation of the DDR in neutrophils suppresses cytokine production and can initiate apoptosis. Furthermore, the oxidative burst was found to be required for endogenous activation of ATM to regulate these processes in stimulated neutrophils. Finally, we have created a mouse model with innate immune cell-specific DDR deficiency which will allow further analysis and *in vivo* studies of this pathway.

This work reveals a novel mechanism for the regulation of neutrophil cytokine production and apoptosis, establishing the DDR as a downstream mediator of immune regulation by ROS. Furthermore, it indicates that dysregulated neutrophil responses may underlie a hyperinflammatory phenotype in AT patients. We propose that inflammation may be a driving force behind some of the pathology of AT, providing a potential target for clinical intervention for some symptoms of this currently untreatable disease.

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ABBREVIATIONS

53BP1	p53-binding protein 1
ANCA	anti-neutrophil cytoplasmic antibodies
AP-1	Activator protein-1
ARE	AU-rich elements
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
BRCA1/2	Breast Cancer gene 1/2
C/EBP	CCAAT-enhancer binding proteins
CC	Cysteine-cysteine
CGD	Chronic granulomatous disease
Chk1/2	Checkpoint kinase 1/2
CLR	C-type lectin receptor
CRISPR	Clustered regularly interspaced short palindromic repeats
CXC	Cysteine-X-Cysteine
CXCR1/2	CXC chemokine receptor 1/2
DAMP	Damage associated molecular pattern
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gy	Gray (unit of radiation)
(γ)-H2A.X	(gamma)-Histone variant H2A.X
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKLM	Heat-killed Listeria monocytogenes
IL-	Interleukin
IRF	Interferon regulatory factor
IκB	Inhibitor of kappa B
JNK	c-Jun N-terminal kinase
kD	Kilodalton
KU-55933	ATM inhibitor 2-(4-Morpholinyl)-6-(1-thianthrenyl)-4H-Pyran-4-one, 2-(Morpholin-4-yl)-6-(thianthren-1-yl)-4H-pyran-4-one

KU-60019	ATM inhibitor (2R,6S)-2,6-Dimethyl-N-[5-[6-(4-morpholinyl)-4-oxo-4H-pyran-2-yl]-9H-thioxanthen-2-yl]-4-morpholineacetamide
LPS	Lipopolysaccharide
LysM	Lysozyme M
MAMP	Microbial-associate molecular pattern
MAP(KKK)	Mitogen activated protein (kinase)
MCP	Monocyte chemotactic protein
MIP	Macrophage inflammatory protein
miRNA	MicroRNA
MPO	Myeloperoxidase
MOI	Multiplicity of infection
MRE11	Meiotic recombination 11
mRNA	Messenger ribonucleic acid
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Nbs1	Nijmegen breakage syndrome 1
NE	Neutrophil Elastase
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization domain-like receptor
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SLE	Systemic lupup erythematosus
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	Ultraviolet

INTRODUCTION

CHAPTER 1: INFLAMMATION

Inflammation is a complex response involving multiple tissues of the body aimed at removing microbial infections, potentially harmful foreign objects, and damaged tissues. A properly controlled inflammatory response is beneficial to the organism; it protects against infections, removes noxious irritants and damaged tissues, and initiates tissue growth, repair, and the restoration of homeostasis. However, this response can become pathological when dysregulated. Failure to mount a strong inflammatory response can leave an organism susceptible to infection, while an increased or prolonged response results in chronic inflammation, tissue damage and dysfunction, and disease. For this reason, inflammation must be tightly regulated by balancing pro- and anti-inflammatory functions on multiple molecular and cellular levels. Therefore, characterizing these regulatory mechanisms is important to understanding not only the healthy inflammatory response, but also diseases that arise when these regulatory mechanisms break down.

1.1 Structure of the inflammatory cascade

The players involved in an inflammatory response can be conceptually grouped into four categories forming a typical cascade: inducers, sensors, mediators, and effectors. Inflammation is induced by the local recognition of various stimuli, both exogenous and endogenous, and is then propagated by the production and release of inflammatory mediators. These mediators act as signals to different tissues, resulting in the establishment of an inflammatory state and the recruitment of dedicated immune cells to sites of inflammation. These recruited effector immune cells exert various proinflammatory and antimicrobial functions, amplifying the immune response and removing the foreign threat, but also causing collateral tissue damage. Once the foreign agent is successfully removed, the acute inflammatory response is followed by a resolution and repair phase, which ends the inflammatory response and restores homeostasis. If the acute inflammation fails to remove the foreign object or pathogen, or if the resolution and repair phase is not properly initiated, the inflammatory response continues and can become chronic.

1.1.1 Inducers of inflammation

Inflammation can be triggered by recognition of various foreign and endogenous stimuli that indicate the presence of an infection or tissue damage. Microbial associated molecular patterns (MAMPs) are a set of conserved molecular structures present on many species of

microorganisms, such as the lipopolysaccharide (LPS) of Gram-negative bacterial membranes, or the flagellin protein that makes up the flagella of motile bacteria. These MAMPs are highly conserved despite the evolution of immune receptors that recognize them, indicating that they are crucial for the viability of microbes[1].

In contrast to MAMPS, damage associated molecular patterns (DAMPs) are host-derived molecules whose presence indicates tissue damage has occurred, such as extracellular ATP[2]. The presence of these molecules in the extracellular environment indicates that non-programmed cell death or lysis has occurred, either in response to infection, stress, or injury.

1.1.2 Sensors of inflammatory stimuli

The innate immune systems of multicellular eukaryotes have evolved a diverse set of germline-encoded sensors that detect MAMPs and DAMPs in order to recognize the presence of infectious microorganisms and the occurrence of tissue damage[1]. These sensors are known as pattern recognition receptors (PRRs), and can be membrane-bound or cytoplasmically located. Binding of a PRR to its specific MAMP or DAMP leads to its activation and the induction of intracellular signaling cascades, which initiate production of inflammatory mediators and effector functions.

Four families of PRRs have been described to date: First, the membrane-bound Toll-like receptor family, which recognize diverse MAMPs and DAMPs[3]. Second, the c-type lectin receptors (CLRs), which recognize carbohydrates found on various microbes; zymosan for instance, a derivative of fungal cell walls, is sensed by the lectin receptor dectin-1[4]. Third, the nucleotide-binding oligomerization domain-like receptors (NLRs), which are cytosolic PRRs that sense various MAMPs from intracellular pathogens, as well as toxins and noxious crystals[5]. And fourth, cytosolic nucleic acid sensors, such as retinoic-acid inducible gene 1 (RIG-1)[6]. Upon ligand binding, these receptors trigger downstream signaling cascades via kinases, such as the mitogen activated protein (MAP) kinase families, and activate transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), and interferon regulatory factors (IRFs) to name a only few[7]. Activation of these pathways results in the transcription and translation of immune related genes, including inflammatory mediators, and the activation of immune cells to perform effector functions.

1.1.3 Mediators of inflammation

Inflammatory mediators are usually soluble factors produced locally at sites of infection and tissue damage. Generally, these mediators serve to activate and propagate an inflammatory state, attract immune cells, and activate effector mechanisms to facilitate removal of the foreign

invader. The list of inflammatory mediators and functions they elicit is extensive. They include cytokines, lipid mediators, complement factors, and vasoactive amines. These mediators also activate effector functions by priming and stimulating immune cells to release antimicrobial peptides, produce further inflammatory mediators, or differentiate and mature into active effector cells.

The production of inflammatory mediators begins locally at sites of infection or tissue damage. This local, initially small production of mediators sounds the alarm and recruits a wave of immune cells, with neutrophils being the first responders. These immune cells amplify the response by producing more proinflammatory mediators, which shape the ensuing immune response. This positive feedback system provides the inflammatory response the potential to magnify and expand to match the magnitude and nature of the insult until resolution begins and the process is actively shut down.

1.1.3.1 Cytokines

The most well studied and extensive class of inflammatory mediators is the cytokine family. Cytokines are produced by various cell types, exert pleiotropic effects on diverse target cells, and with some exceptions, often exhibit redundancy. These small, secreted signaling proteins can be classified into various different functional classes, including chemokines, proinflammatory cytokines, and anti-inflammatory cytokines, to name only a few[8].

1.1.3.1.1 Chemokines

The chemokine family of cytokines is named for their ability to induce chemotaxis in immune cells, making them responsible for the recruitment of effectors cells to sites of inflammation[9]. In addition to chemotaxis, chemokines also prime cells for other immune functions[10]. There are two main classes of chemokine based on the spacing of their amino-terminal cysteine residues: CXC chemokines, such as IL-8, are responsible for the recruitment of granulocytes, monocytes, and macrophages, though other cell types can also respond to these chemokines[11]. CC chemokines, such as MIP-1 α, recruit monocytes and drive them to differentiate into macrophages, as well as recruit other immune cells.

1.1.3.1.2 Proinflammatory cytokines

Proinflammatory cytokines are produced by many cell types; in the innate immune system, the principal sources are neutrophils and macrophages. Notable proinflammatory cytokines include interleukins IL-1α and IL-1β, IL-6, and tumor necrosis factor (TNF). These cytokines exert diverse, often redundant effects both locally and systemically to promote inflammation. These effects include the up-regulation of receptors on endothelial cell surfaces to

allow extravasation of immune cells to sites of inflammation, development of fever, induction of acute phase proteins from the liver, eliciting of effector functions, stimulation of further cytokine production by immune cells, and induction of cell death[12].

1.1.3.1.3 Anti-inflammatory cytokines

Cytokines can also exert anti-inflammatory and growth-promoting activities[13]. IL-1 receptor antagonist prevents proinflammatory IL-1 receptor signaling; IL-10 and transforming growth factor-β (TGF-β) are both able to inhibit the proinflammatory cytokine production and effector functions from neutrophils and macrophages. These signals are important for resolving the vicious circle of inflammation. Clearly the regulation of inflammation by cytokines is complex, complicated by concentration-dependence, cell type specificities, and cross talk among receptors and cytokines.

1.1.4 Effectors of inflammation

The mediators of inflammation induce a diverse range of molecular and cellular effector functions. In response to these inflammatory signals, various tissues of the body release antimicrobial peptides and opsonizing proteins, which facilitate pathogen recognition and killing, as well as trigger coagulation to limit dissemination. However, the most important effector functions elicited by inflammation in vertebrates are derived from the body's dedicated immune cells, which are recruited and activated by these mediators to perform diverse coordinated functions.

1.1.4.1 Cells and functions of the immune system

In vertebrates, the immune cells are divided into two separate arms: the innate immune system and the adaptive. The innate immune system responds quickly and powerfully by recognizing MAMPs conserved on almost all pathogens, and is in most cases robust enough to halt infections. The adaptive immune response, on the other hand, is much slower, requiring days or even weeks to develop responses that are specific to singular structures and may be specific to a single pathogen. However, once this adaptive response is successfully developed memory persists, allowing rapid and enhanced immune responses that can prevent re-infection with the same or similar pathogens.

1.1.4.1.1 Cells of the innate immune system

The **granulocytes** are the foot soldiers of the immune system - they are highly abundant, quick to respond, and well equipped with diverse antimicrobial mechanisms. These cells are distinguished by their cytoplasm full of granules packed with various antimicrobials and effectors

ready to be released. The most abundant granulocyte, and most important in infections, is the neutrophil[14]. Neutrophils circulate in the blood and once recruited to tissues contribute multiple effector functions, including phagocytosis, reactive oxygen species (ROS) production, and release of antimicrobials which will be reviewed in more detail below. The other granulocytes (eosinophils, mast cells, and basophils) are less abundant and are involved in allergies, asthma, and responses to multicellular parasites[15].

Monocytes, macrophages, and dendritic cells are also phagocytic cells of the innate immune system. Tissue resident macrophages are sentinel cells present throughout the body, ready to detect initial invasion by foreign objects and initiate immune responses by producing cytokines[16]. Monocytes are recruited from the circulation into sites of inflammation, where they differentiate into monocyte-derived tissue macrophages and dendritic cells. Resident and monocyte-derived macrophages contribute to immune responses by orchestrating cytokine production, phagocytosing pathogens, and clearing apoptotic cells. Though they are not as adept at microbial killing as neutrophils, macrophages and dendritic cells can instead act as antigen-presenting cells to initiate development of adaptive responses.

1.1.4.1.2 Cells of the adaptive immune system

The adaptive immune response consists of specific **B cells** and **T cells**. These cells produce novel antibodies and receptors through genomic recombination, giving them the ability to recognize a more diverse repertoire of foreign structures than PRRs alone. B cells secrete antibodies, which can opsonize pathogens to facilitate phagocytosis or neutralize virulence factors. T cells use their repertoire of receptors to exert cytotoxic effects on infected cells, facilitate activation of B cells, or shape immune responses by producing cytokines. These cells work in conjunction with the innate immune system to coordinate a successful inflammatory response.

CHAPTER 2: NEUTROPHIL FUNCTIONS IN INFLAMMATION

The neutrophil is the most abundant immune cell in humans, making up 50-70% of white blood cells[17]. They develop from precursor cells in the bone marrow, expressing the antimicrobials and signaling proteins they will use as mature neutrophils and storing them in membrane-bound organelles called granules[18]. Once mature, they are released into the bloodstream, where they patrol the body in search of signs of infection. Neutrophils express multiple cytokine receptors and PRRs, including the TLRs (all except TLR3[19]), CLRs, as well as antibody and complement receptors. This repertoire allows them to detect cytokines and MAMPs emanating from local sites of inflammation and respond by extravasating from the bloodstream into tissues. Once there, they travel along this chemotactic gradient towards the source of cytokine production where they are activated by host-derived inflammatory mediators and pathogen-derived products to perform their many inflammatory and antimicrobial functions.

Compared to other cells of the immune system, neutrophils are short-lived and quickly undergo apoptosis[20]. This serves to limit the collateral tissue damage from their destructive inflammatory functions, and also provides strong anti-inflammatory signals explained in detail in section 2.3. These neutrophil functions must be tightly controlled in order to balance both pro- and anti-inflammatory signals. Deciphering the mechanisms that regulate neutrophil function is thus important for understanding their role both in health and, when this regulation fails, in disease.

2.1 Neutrophil effector functions

Once the neutrophil reaches its final destination, the site of infection, it performs multiple proinflammatory and antimicrobial functions. Stimulated by the mixture of cytokines and MAMPs, neutrophils produce various proinflammatory cytokines, driving the inflammatory response. They also contribute to microbial clearance through degranulation, phagocytosis, ROS production, and formation of neutrophil extracellular traps (NETs). The powerful antimicrobials they deploy are indiscriminate in their toxicity, killing microbes while also causing substantial collateral damage to host tissues.

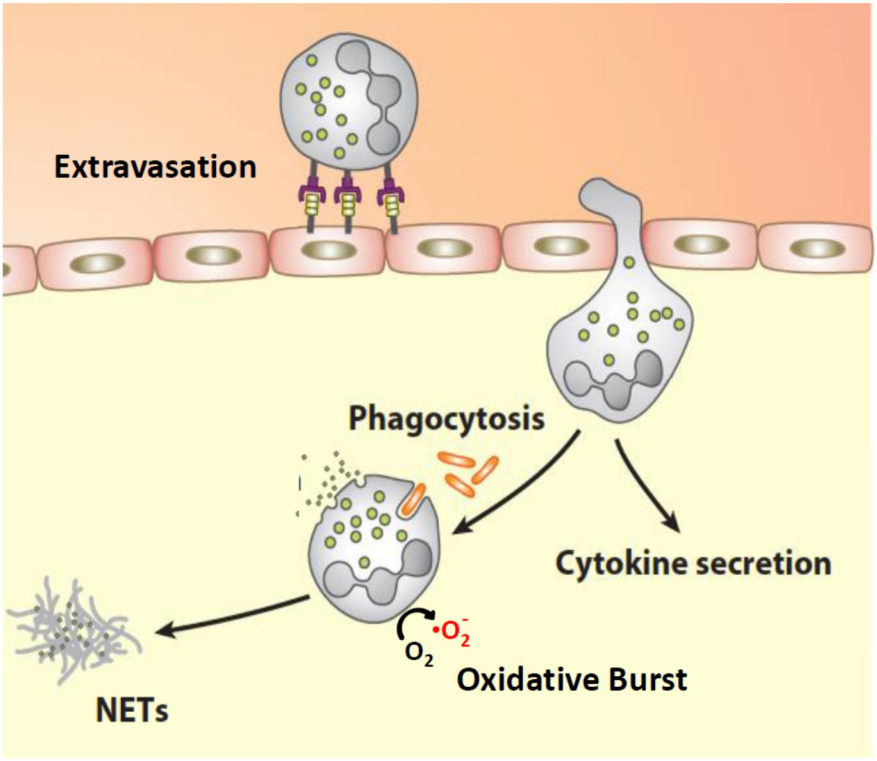


Figure 2.1: Neutrophil recruitment to sites of inflammation and effector functions, figure modified from[14]

2.1.1 Degranulation and phagocytosis

Neutrophils have evolved a specialized storage organelle, the granule, in which they pack their multitude of antimicrobials to limit their release until necessary[18]. These include *(a)* proteases which degrade virulence factors and extracellular matrix, *(b)* cationic peptides which bind to membranes and permeabilize cells, *(c)* nutrient-binding proteins which sequester essential nutrients, and *(d)* enzymes for production of ROS. Upon neutrophil activation, these granules are mobilized in various ways to utilize these weapons. Fusion of the granules with the plasma membrane results in degranulation, expelling the granule contents into the extracellular environment. The liberated antimicrobials are then free in the extracellular environment to target the invading microbes, and also cause damage to host bystander cells[21].

Alternatively, neutrophils that come into contact with microbes can engulf them by phagocytosis. The neutrophil then targets its granules to fuse with the phagosome, deploying its arsenal of antimicrobials into the lumen, maturing it into a harsh, destructive environment that kills the microbe.

2.1.2 The oxidative burst

Simultaneous to these processes, the neutrophil fires up its oxidative burst to produce another class of potent antimicrobial: reactive oxygen species (ROS). The oxidative burst generates a substantial amount of these highly volatile chemicals through the activity of a multimeric enzyme complex: the NADPH oxidase[22]. Components of this complex are anchored in the granule membranes, and others soluble in the cytoplasm; upon stimulation, these components assemble and ROS are produced both on the phagosomal and the plasma membranes (Figure 2.2). NADPH oxidase reduces molecular oxygen to the highly reactive molecule superoxide, which is then transformed into other species of ROS by further enzymes[23]. These various ROS species are able to modify and damage molecules through oxidation, including DNA, proteins, and lipids. This makes them potently microbicidal, but, a recurring theme with neutrophil antimicrobials, they are indiscriminate and equally damaging to host molecules[24]. The ability of ROS to modulate proteins by oxidation, a form of post-translational modification, also allows them to serve as important signaling molecules[23], a topic which will be discussed later.

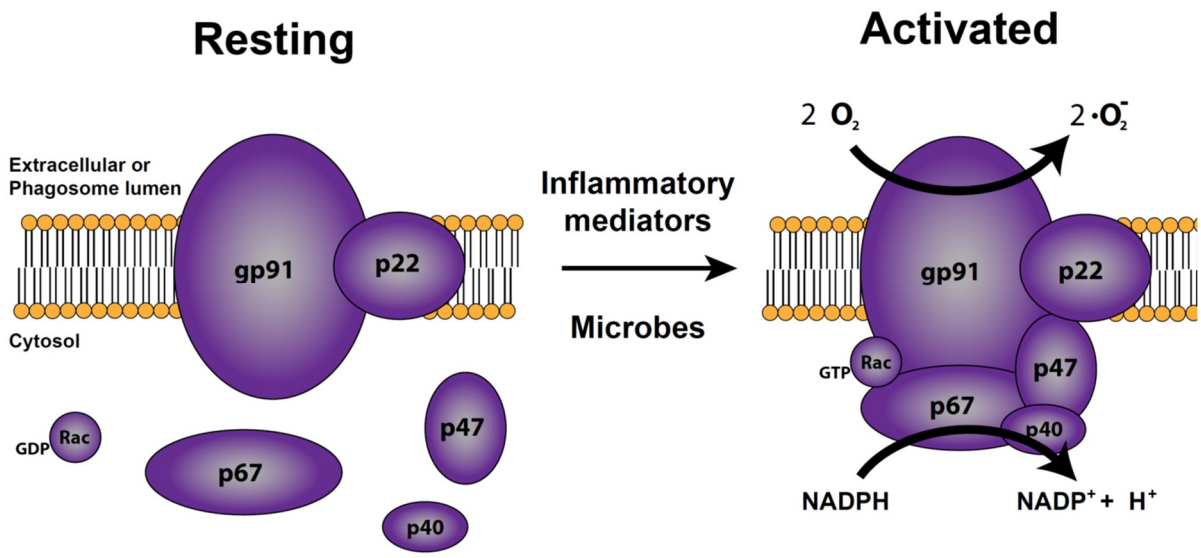


Figure 2.2: Schematic of NADPH oxidase complex components, their assembly, and activation upon stimulation

2.1.3 Neutrophil extracellular traps

In a last-ditch effort to attack foreign invaders, an overwhelmed neutrophil may commit to a specialized form of inflammatory cell death to produce a neutrophil extracellular trap (NET)[25]. During this active process, termed NETosis, the subcellular organelles break down, including the nuclear membrane[26]. The neutrophil's nucleus expands, mixing with the cytoplasm and granular contents. Eventually, the plasma membrane bursts and releases a web of chromatin

decorated with antimicrobials tethered to its stringy surface: a NET. A high local concentration of antimicrobials is maintained by their localization to the NET, increasing their efficiency in microbial killing while limiting their diffusion through the tissue[27]. NETs are not innocuous to the host, however; their release also contributes to organ damage and exposes normally-intracellular antigens to the extracellular world in the midst of an inflammatory environment, potentially driving development of autoimmunity[28].

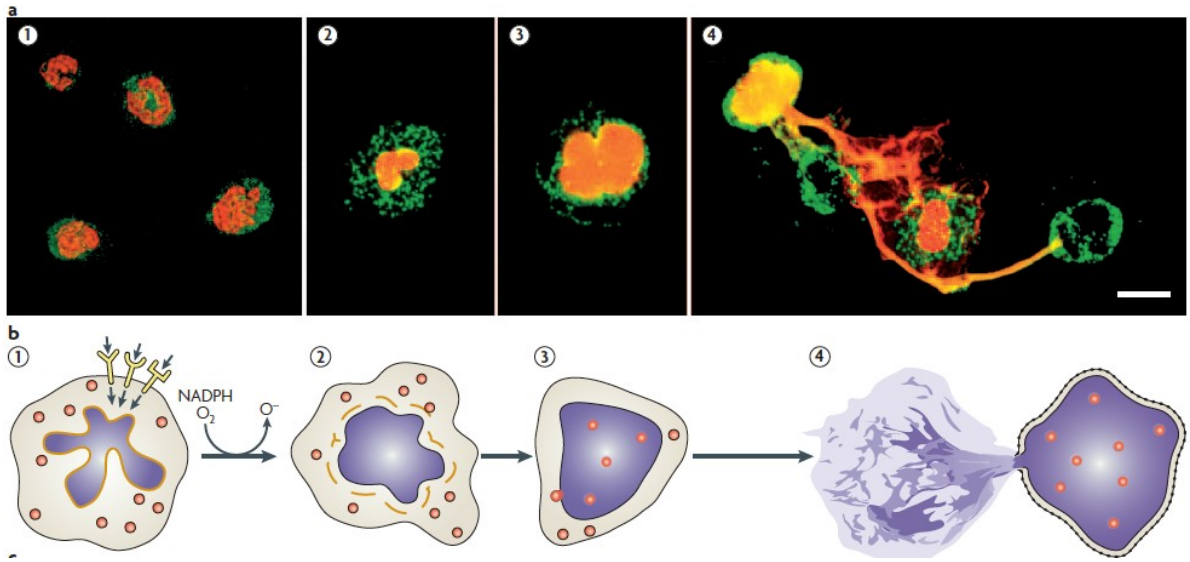


Figure 2.3: Stages of neutrophil extracellular trap (NET) formation, taken from[29]. **A)** Fluorescent micrograph of PMA-stimulated neutrophils forming NETs. Neutrophil elastase is labeled in green, and DNA in red. **B)** Schematic representation of NETosis

2.2 Neutrophil-derived cytokines

Throughout the engagement of its antimicrobial programs, the stimulated neutrophil is actively transcribing and secreting cytokines. In recent years it has become clear that the neutrophil is an important source of multiple cytokines[30]. They constitute the majority of immune cells present at the early stages of inflammation, outnumbering other immune cells by as much as two orders of magnitude[31], and thus set the stage for the inflammatory response.

2.2.1 Neutrophil cytokine repertoire

Neutrophils secrete chemokines, such as IL-8 and MIP-1α, and pro-inflammatory cytokines including IL-1β, IL-6 and TNF[30]. This diverse repertoire underlines their important roles not only in modulating innate immunity, but also in crosstalk with the adaptive immune system, antiviral response, and hematopoiesis. IL-8 is the most abundantly-produced cytokine by neutrophils and plays an important role in neutrophil biology and the inflammatory response.

2.2.2 Regulation of neutrophil IL-8 production

IL-8 was the first identified neutrophil chemokine that is induced by inflammatory mediators[32]. It is also known to be one of the most important neutrophil attractants, capable of inducing acute inflammation[33]. Many cell types, including endothelial and epithelial cells, macrophages, as well as neutrophils can produce IL-8 in response to inflammatory stimuli. Similarly, many cell types including neutrophils express the chemokine receptors CXCR1 and CXCR2, which allow them to detect and respond to IL-8 and other CXC chemokines.

2.2.2.1 *IL8* gene structure

The genes encoding IL-8 and several other related chemokines are clustered in a region on chromosome 4 in humans[34]. The *IL8* gene contains binding sites for multiple transcription factors and regulatory elements, including NF-κB, CCAAT/enhancer binding proteins (C/EBP), and activator protein-1 (AP-1)[35,36]. The downstream three-prime untranslated region (3'UTR) contains multiple AUUUA repeats, which contribute to the short half-life of its mRNA[37]. These various regulatory elements confer fine-tuned control of gene expression at the transcriptional and post-transcriptional level.

2.2.2.2 Transcriptional regulation of cytokine production

Stimulation of neutrophil extracellular receptors, PRRs and cytokine receptors, activate two main pathways to initiate and control transcription of cytokines: NF-κB and MAP kinase pathways[38] (see Figure 2.4). Both of these pathways are important for the up-regulation of immune response genes in neutrophils, but also play crucial roles during growth, development, and cell cycle regulation.

The transcription factor NF-κB exists as a homo- or heterodimer of various Rel family subunits p50 NF-κB1, p52 NF-κB2, p65 RelA, and/or c-Rel. In unstimulated cells it localizes to the cytoplasm bound to its inhibitor IκB (inhibitor of κB)[39]. In response to cell activation, IκB is hyperphosphorylated by IκB kinase (IKK), efficiently targeting it for proteolytic degradation. This dissociation from the NF-κB dimer frees the transcription factor to migrate to the nucleus and rapidly begin transcription of its target genes.

Engagement of many of the same receptors that activate the NF-κB pathway simultaneously trigger MAP kinase pathways[40]. Three families of MAP kinases exist, jun amino-terminal kinases (JNK), extracellular receptor kinases (ERK), and p38 MAP kinase. These kinases are activated by phosphorylation via a family of kinases known as MKKs (MAP kinase kinases), which are in turn activated by MKKKs (MKK kinases). This hierarchical cascade signals in parallel and with significant overlap, allowing for simultaneous induction and amplification of all three

kinase families. Active, phosphorylated JNK, ERK, and p38 then exert multiple functions, including the activation of several transcription factors, among them AP-1 and C/EBP. These various transcription factors converge on inflammatory genes, including IL-8 and other cytokines.

2.2.2.3 Post-transcriptional regulation of cytokine production

In addition to cytokine transcription, other parallel mechanisms are enacted which regulate their production post-transcriptionally. This post-transcriptional regulation has largely been attributed to the activity of p38 MAP kinase, which promotes stabilization of various mRNAs to increase their translation[35]. AU-rich elements (ARE) in the 3'UTR of the IL-8 mRNA, as well as other cytokine transcripts, contribute to their instability and short half-life, limiting their translation. ARE-binding proteins recognize and bind to these regions, and can promote either destabilization or stabilization of the mRNA. p38 has been shown to regulate various ARE-binding proteins, adding another level to the control of expression. Additionally, p38 activation can affect the phosphorylation state of ribosomal subunits, modulating translation[38]. Recently, it has become apparent that microRNAs also play a role in fine-tuning the production of cytokines[41]. These short RNA sequences have complementarity to a target mRNA, interacting with it to prevent its translation or induce its degradation.

It is clear that multiple pathways, transcription factors, and post-transcriptional mechanisms of regulation all contribute to fine-tuning the production of IL-8 and other cytokines. These processes act synergistically in response to stimulation to rapidly increase cytokine production.

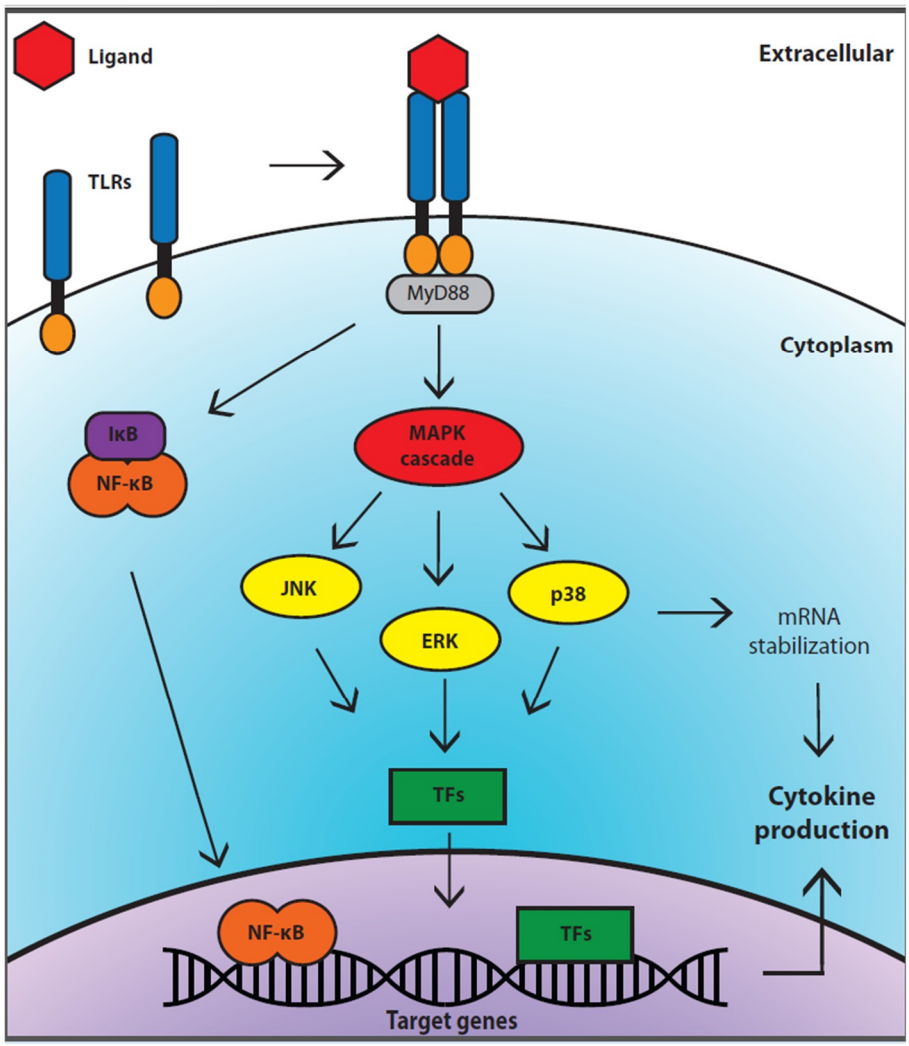


Figure 2.4: Generic PRR stimulation and intracellular signaling cascades converging on cytokine production

2.2.3 Roles of IL-8 in inflammation

The CXC chemokines are crucial inflammatory mediators that are responsible for many aspects of the inflammatory response. IL-8 is massively upregulated in response to the detection of microbes or tissue injury. The source of this initial IL-8 production is dependent on the tissue, but is likely to be resident macrophages[42]. This draws in an initial wave of neutrophils, which produce more IL-8, among other cytokines. These cytokines signal in an autocrine manner to activate neutrophils and in a paracrine manner to recruit and activate further cells.

IL-8 is recognized by CXCR1 and CXCR2, G-protein coupled receptors, which in turn activate protein kinase C[43]. This apical kinase is involved in the multiple pathways that activate the neutrophil's destructive functions. Stimulation of neutrophils with IL-8 induces chemotaxis and degranulation. It also primes them for their other functions; when the primed neutrophil

encounters microbes it responds quicker and with more force - producing more ROS and more efficiently phagocytosing[44,45].

These responses induced by IL-8 have been shown to be sufficient for the development of a strong acute inflammatory response *in vivo*. Injection of IL-8 into the skin produces a strong, sustained local inflammatory response that is characterized by the rapid infiltration of neutrophils, induction of multiple cytokines, and eventual recruitment of other immune cells[46,47]. Additionally, administration of neutralizing antibodies against IL-8, CXCR1/2, or antibody-mediated depletion of neutrophils prevents the development of inflammation in multiple animal models of inflammatory diseases[48]. Thus, neutrophils and IL-8 are both necessary and sufficient for the development of inflammation.

2.3 Neutrophils in the regulation of inflammation

2.3.1 Neutrophil apoptosis

The neutrophil comes preprogrammed with a biological clock that begins ticking towards its imminent death as soon as it is released from the bone marrow[49]. Reports of neutrophil lifespan *in vivo* are a point of contention, varying between 0.5 and 5 days[50], and likely depending on their activation status[51]. Indeed, the half-life of neutrophils *ex vivo* in culture is closer to 12 hours. Neutrophils undergo constitutive apoptosis even in the absence of any stimuli[20]. Their lifespan is increased when exposed to inflammatory stimuli, though still on the scale of mere days. This programmed cell death is central to regulating their functions and the resolution of inflammation.

Constitutive neutrophil apoptosis displays all the signs of classical apoptosis: the cell shrinks, the cytoplasm becomes vacuolated, mitochondria depolarize, the membrane lipid phosphatidylserine flips to the outer leaflet of the plasma membrane, and the genome becomes fragmented[52]. These apoptotic neutrophils no longer respond to stimuli, and become inert. As in classical apoptosis, this process is regulated by a balance of pro-survival and pro-apoptotic signals. The NF-κB and MAP kinase pathways are important regulators of these signals, along with transcription factor p53[53]. These contribute to production of antagonizing pro- and anti-survival signals; pro-survival signals reduce over time in both naive and stimulated neutrophils, eventually tipping the scales towards death.

2.3.2 Neutrophils in the resolution of inflammation

The nature of neutrophilic inflammation has the power to produce a devastating positive feedback loop: neutrophils damage tissues and produce proinflammatory cytokines, recruiting and activating more neutrophils. This destructive cycle has to be actively shut down in order to prevent chronic inflammation and organ failure. Not surprisingly, many mechanisms have evolved to tightly regulate inflammation at multiple levels, and involving different cell types.

Neutrophils themselves play a role in limiting their inflammatory functions; constitutive neutrophil apoptosis is a crucial player in the resolution of inflammation. Not only does it enforce a time limit on the neutrophil's destructive spree, it also provides a strong anti-inflammatory signal. As the neutrophil becomes apoptotic and unresponsive, the membrane lipid phosphatidylserine relocates to the extracellular face of the plasma membrane. This molecule serves as an "eat me" signal to macrophages, persuading them to phagocytose the neutrophil corpse[54]. This process, termed efferocytosis, effectively clears the tissue of apoptotic cells, preventing the release of their injurious contents should they become necrotic. It also serves a second purpose: ingestion of apoptotic neutrophils reprograms macrophages to adopt an anti-inflammatory phenotype[55]. In response, they secrete IL-10 and TGF-β, which inhibit the production of pro-inflammatory mediators, and promote tissue healing and growth[56,57]. Due to the extraordinary number of short-lived neutrophils present at sites of acute inflammation, efferocytosis must occur on an impressively large scale and provide strong anti-inflammatory instructions.

2.3.3 Neutrophil functions in disease

In addition to their importance during infections, neutrophils are implicated in diverse non-infectious diseases ranging from immunodeficiency, to autoinflammatory and autoimmune diseases, neurological diseases, and cancer. It's clear that neutrophil functions are a double-edged sword and must be tightly regulated, as imbalances producing either too little or too much neutrophilic inflammation are implicit in disease. Cytokine production, timely initiation of apoptosis, and clearance of apoptotic cells all contribute to this regulation. Alterations in these processes shift the balance of neutrophil functions and inflammation, and are implicit in multiple inflammatory diseases.

2.3.3.1 Neutrophil deficiencies

Neutropenia is a condition characterized by permanent or transient periods of low neutrophil blood counts. Neutropenia is highly associated with increased susceptibility to infection; the frequency, duration, and severity of infection inversely correlate with circulating

neutrophil counts[58,59]. Disruption of neutrophil antimicrobial mechanisms correspondingly leads to immunodeficiency. Various genetic disorders that disrupt degranulation[60], phagocytosis[61], NET formation[62,63], or the oxidative burst have been described[64]. These diverse diseases affecting neutrophils all share a common symptom - immunodeficiency and severe susceptibility to infection.

2.3.3.2 Neutrophils in inflammatory diseases

On the other side of the coin, increased or uncontrolled neutrophil activity is associated with the pathology of many diseases. Neutrophils contribute to excessive inflammation and are a major source of tissue damage in acute inflammation[65]. Increases in pro-inflammatory functions of neutrophils (cytokine production and NET formation) accompanied by decreases in anti-inflammatory functions (apoptosis and efferocytosis) are commonly documented in autoinflammatory and autoimmune diseases. This imbalance can lead to spontaneous inflammation without an exogenous stimulus, as well as exaggerated and prolonged inflammation in response to infection.

Neutrophilic dermatoses are a group of autoinflammatory conditions where hyperactive neutrophils drive the formation of sterile festering skin granulomas composed primarily of neutrophils[66]. Interestingly, IL-8 overexpression has been shown to be the smoking gun behind at least some forms of neutrophilic dermatosis[67]. Delayed initiation of apoptosis is also implicit in inflammatory diseases, such as arthritis[68]. Increased neutrophil recruitment and activation exposes neutrophil components through degranulation and NET formation, increasing the risk of developing anti-neutrophil autoantibodies, a phenomenon observed in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE)[28,68,69].

2.4 Chronic granulomatous disease and ROS-mediated regulation of neutrophil functions

A cornerstone of neutrophil function and regulation is the oxidative burst. The ROS it generates are important not only for direct microbial killing, but also in regulating multiple neutrophil functions, demonstrating the ability of ROS to act as important signaling molecules. Mutations in components of the NADPH oxidase machinery, and thus in ROS production, disrupt multiple neutrophil processes and result in chronic granulomatous disease (CGD)[70]. This disorder is paradoxically defined by immunodeficiency and simultaneous autoinflammation. The pathogenesis of CGD sheds light on the myriad ways the oxidative burst impacts the evolution of an inflammatory response and demonstrates the importance of neutrophils in microbial clearance and immune regulation.

2.4.1 Clinical manifestations of chronic granulomatous disease

CGD is caused by mutations in any subunit of the NADPH oxidase complex that compromise its ability to generate ROS. The most common form of CGD is caused by mutations in the gp91 subunit encoded on the X chromosome[71]. Patients suffer from recurrent and persistent infections, especially of A*spergillus*, *Staphylococcus*, *Serratia*, and *Salmonella*[72]. Concurrently, CGD patients suffer from exaggerated and spontaneous inflammatory responses. These are most apparent in the formation of persistent granulomas that can manifest in multiple organs and tissues, most commonly in the digestive tract[73]. These granulomas often form spontaneously, without any signs of a culpable infection. Highlighting the predisposition for inflammation in these patients, CGD is accompanied by increased incidence of colitis, arthritis, and various forms of lupus[73]. Heterozygous carriers of these mutations also develop these inflammatory conditions at increased frequencies[74]. Patients are treated with prophylactic antibiotics and antifungals to prevent infections, and when necessary, corticosteroids to treat inflammation[75].

2.4.2 Antimicrobial activity of ROS

The NOX2 NADPH oxidase is expressed exclusively in phagocytes of the innate immune system, and thus affects mainly neutrophils and macrophages. Immunodeficiency in CGD patients is caused by an inability of these cells, especially neutrophils, to efficiently kill microbes. The oxidative burst contributes to microbial killing by multiple mechanisms. ROS species themselves can be directly microbial at high concentrations, but they are also required for other neutrophil functions. ROS production stimulates the release of antimicrobial peptides from the granules and increases the activity of their stored proteases, such as neutrophil elastase, which are important for the destruction of microbes[76]. Additionally, the oxidative burst is required for NETosis; neutrophils from patients with CGD fail to form NETs in response to microbes[26]. Thus, multiple important antimicrobial mechanisms are neutralized in CGD neutrophils, rendering patients highly susceptible to infections.

2.4.3 ROS-mediated regulation of inflammation

The autoinflammation observed in CGD is similarly driven by the dysregulation of multiple pathways that depend on the oxidative burst. The result is an increase in proinflammatory cytokine production, compounded by a deficiency in pathways to resolve inflammation, a net imbalance that strongly favors inflammation.

2.4.3.1 Cytokine production

Neutrophils and macrophages from CGD patients have been documented to overproduce inflammatory cytokines, especially IL-8 from neutrophils and IL-1β from macrophages[77,78]. This phenomenon has been attributed to alterations in intracellular signaling pathways leading to increased transcription and translation of cytokines[79]. There is increasing evidence that oxidative modification of redox-active proteins can modulate their functions. Activation of MAP kinases, phosphatases, and transcription factors such as NF-κB have been shown to be modulated by oxidation, and are linked to the dysregulation of cytokine production observed in CGD[80,81]. In line with this, addition of hydrogen peroxide to stimulated neutrophils has been shown to reduce cytokine production, supporting an anti-inflammatory role for ROS[79]. In addition to producing more inflammatory cytokines, the decreased activation and release of proteases by ROS-deficient neutrophils results in less proteolytic degradation of cytokines in the extracellular environment. These compound effects increase the concentrations of cytokines present, and drive persistent activation of the immune response.

2.4.3.2 Apoptosis and efferocytosis

The production of ROS has been linked to apoptosis of stimulated neutrophils. CGD patients exhibit deficiencies in neutrophil apoptosis and the clearance of these apoptotic cells by macrophage efferocytosis. The altered intracellular signaling pathways mentioned above likely contribute to this, as the MAP kinase and NF-κB pathways can provide both pro-inflammatory and pro-survival signals[82]. The activation of stress responses to oxidative damage of cellular components is thought to also play a role in inducing apoptosis. The result of ROS deficiency is a prolonged neutrophil lifespan and decreased accumulation of phosphatidylserine (the “eat me” signal for macrophages) on the surface of apoptotic cells[83]. This delays the transition to an anti-inflammatory state triggered by macrophage efferocytosis. Delayed resolution is further magnified by ROS-deficient macrophages, which are unable to efficiently clear and digest apoptotic debris by efferocytosis[84]. This essentially precludes the production of pro-resolving cytokines IL-10 and TGF-β, but also results in persistence of debris from apoptotic neutrophils[85]. This is believed to be the force behind development of autoantibodies associated with autoimmunity in CGD patients.

2.5 Challenges of neutrophil research

Studies with neutrophils and macrophages from CGD patients have elucidated some of the mechanisms behind the antimicrobial and anti-inflammatory functions of the oxidative burst. However, the various downstream targets of ROS that mediate the changes observed in CGD cells

are still largely unknown. Elucidation of these pathways remains a challenge due to the intractability of neutrophils. Neutrophils do not replicate or survive long in culture, and generally already contain the proteins they need for their functions and no longer actively produce them. For these reasons, genetic manipulations that make screening possible - siRNA, viral transduction, or CRISPR-Cas - are not possible in neutrophils. Neutrophil studies must therefore often be undertaken with inhibitors and, for genetic evidence, analyses of primary neutrophils from patients.

CHAPTER 3: ATAXIA TELANGIECTASIA AND ATM KINASE

3.1 Ataxia Telangiectasia

Ataxia telangiectasia (AT) is an incurable, autosomal recessive genetic disorder occurring with a frequency of 1:40,000 - 100,000 live births[86]. AT is caused by mutations inactivating the kinase ataxia telangiectasia mutated (ATM), best known for its role as the orchestrator of the DNA damage response (DDR)[87]. AT is a pleiotropic disease, with patients presenting myriad symptoms, many of which are caused by deficient repair of DNA damage. Other symptoms, however, have not yet been explained, indicating pleiotropic functions of ATM.

3.1.1 Clinical features of ataxia telangiectasia

The hallmark symptoms of AT are progressive cerebellar neurodegeneration resulting in ataxia - a loss of coordination of voluntary muscle movement - and telangiectasia - a dilation of blood vessels. These are frequently accompanied by a wide range of other manifestations affecting multiple systems of the body including sterility, strong predisposition to cancer, developmental and growth defects, metabolic syndrome, and immunodeficiency[88]. Though often unaddressed, many reports in the literature describe hallmarks of autoinflammation in AT patients, including increased serum cytokine levels[89], markers of oxidative stress[90], cutaneous granulomatous lesions[91-94], development of autoantibodies[95,96], and pulmonary disease[97]. The most common causes of death in AT patients are cancer and chronic lung disease, each accounting for approximately one-third of mortalities[98,99].

The clinical symptoms of AT exist on a spectrum; the presence and severity of each can be variable. The relation between the wide spectrum of clinical aspects of AT and the genetic defect in ATM remains unclear[100,101]. It has become apparent in recent years that not all of these symptoms can be tied to a deficiency in the recognition and repair of DNA damage, and much research has focused on elucidating important roles of ATM in other cellular processes.

3.1.2 Diversity of *ATM* mutations

ATM is a large gene, containing 66 exons and spanning 150 kilobases, localized to chromosome 11q22-23 in humans. To date, over 400 unique disease-causing mutations in *ATM* have been identified in AT patients (Leiden Open Variation Database, November 2016). There are only few and isolated instances of founder effects in populations; most AT patients are compound heterozygotes carrying two differently mutated *ATM* genes[102]. Disease-causing mutations occur throughout the gene with no identified mutational hotspots. The most common types of mutations are substitutions and deletions, and are usually predicted to cause premature

translation termination and protein truncation, or to produce an unstable protein[103]. Some identified mutations cause aberrant transcript splicing expected to result in unstable or non-functional protein. AT can be caused by null mutations as well as hypomorphic mutations, which result in residual but significantly decreased levels and activity of ATM[104].

Because of the large number of mutations, the relative infrequency of the disease, and the confounding heterozygosity of patients, studies linking specific genotypes to cellular or clinical phenotypes are challenging. However, studies have shown that disease severity correlates with residual ATM activity; patients with detectable levels of residual ATM protein and activity have fewer and less severe symptoms, delayed onset of malignancy, and increased lifespan[104]. The heterogeneity may further be compounded by interactions with other genes. Four complementation groups within AT patients were originally identified based on cellular radio-resistant DNA synthesis[105], a result that remains unexplained now that AT has been linked to mutations in a single gene[106]. Because of this diversity and complexity, it still remains impossible to subdivide patients into clinical subtypes or predict symptoms or severity based on the genetic information.

3.2 ATM and the DNA damage response

ATM is a large (350 kDa) serine/threonine kinase best known for its role in the DNA damage response (DDR). It is a member of the phosphatidylinositol 3 kinase-like kinase (PIKK) family of kinases, which also includes ATR (ataxia-telangiectasia and RAD3-related) kinase and DNA-PK_{cs} (DNA-dependent protein kinase catalytic subunit), two other kinases involved in the DDR. These proteins contain a conserved kinase domain, a FRAP-ATM-TRRAP (FAT) domain, and a FAT c-terminal domain[107]. ATM exists as an inactive dimer which, in response to DNA damage, undergoes multiple post-translational modification events, including phosphorylations and acetylation. The marker of ATM activation is its autophosphorylation at serine 1981, which results in dissociation of the dimer to form an active monomer[108].

3.2.1 Mechanisms of DNA damage

Genomic DNA exists as two associated, complementary, antiparallel polynucleotide strands and is the medium for storage and transfer of hereditary information; its integrity and stability across generations are essential for life. DNA is not inert, however; it is under constant assault by chemicals, enzymes, radiation, and physical strains. These genotoxic compounds can induce different types of DNA damage by a multitude of mechanisms[109]. Breaking the bonds between nucleotides in the DNA backbone results in single- or double-strand breaks, which can lead to genomic instability and chromosomal rearrangements. Chemical modification or removal

of nucleotide bases can change complementary base-pairing between strands and lead to mutations during transcription and replication. Addition of adducts or cross-linkages between strands or nucleotides can change the three-dimensional shape of DNA. These alterations can preclude the recognition and function of DNA-binding proteins, transcription, and DNA replication.

One of the major sources of DNA damage is ROS. Various types of ROS can modify DNA by oxidation at multiple sites to create DNA lesions, including single and double-strand breaks, addition of adducts and cross-linkages, as well as base modification[110]. These ROS can be generated spontaneously or as byproducts of metabolism, and are estimated to induce on the order of 10^5 DNA insults per cell per day[111]. Exposure to ionizing ɣ-radiation is a potent inducer of both single- and double-strand breaks. UV radiation excites bases and leads to cross-linkages between nucleotides. Many enzymes also induce DNA damage: topoisomerases cleave DNA to relax and unwind it; enzymes involved in repairing mismatched or chemically-altered bases must induce strand breaks during the process of nucleotide removal and replacement. DNA damage can also be induced by genotoxic drugs. Etoposide is a drug which inhibits the re-ligation function of topoisomerase II, causing the accumulation of double-strand breaks[112]. Cisplatin, a genotoxic drug containing platinum complexes, catalyzes inter- and intra-strand crosslinks in DNA, causing transcriptional and replicational stress, and producing single- and double-strand breaks during its repair[113,114]. Treatment with irradiation, etoposide, or cisplatin can be used as tools to generate DNA damage in cells, and cancer treatment.

3.2.2 The DNA damage response

Upon DNA damage, various sensor proteins interact with ATM, ATR, and/or DNA-PK_{cs} to recruit them to sites of DNA damage. This leads to activation of these kinases and the initiation of a DDR, a complex signaling cascade that coordinates cell cycle arrest, DNA repair, or apoptosis[109]. The outcome of this response can be dependent on the cell type, cell cycle stage, and the severity of damage induced. ATM, ATR, and DNA-PK_{cs} are each activated most robustly in response to different types of DNA damage, and can initiate a DDR involving different substrates, although there is significant overlap in their downstream pathways[115]. ATM and DNA-PK_{cs} are both activated in response to double strand breaks, but initiate different repair pathways. ATM initiates repair by homologous recombination, a mechanism using the sister chromatid as a template to repair the damaged DNA[116]. In contrast, DNA-PK_{cs} promotes DNA repair by nonhomologous end-joining, which ligates strands of broken DNA without a template guide. ATR is activated by a wide spectrum of DNA damage, including single strand breaks, stalled replication forks, DNA adducts and cross-linking, but also by double strand breaks[115].

Although ATM and ATR are activated most efficiently by different forms of DNA damage, these pathways are not discrete, and repair often involves activation of both kinases[117-119]. Together these kinases orchestrate a complex network of responses involving many cellular pathways. In response to ionizing radiation, over 700 proteins are differentially phosphorylated in a manner dependent on ATM and ATR[120]; additionally, 75% of phosphorylation events in response to DNA damage do not occur at consensus ATM and ATR recognition sites[121]. This indicates that ATM and ATR act upstream of multiple complex kinase cascades, demonstrating the breadth of the DDR within the cell.

3.2.3 Activation and substrates of ATM

3.2.3.1 Activation by DNA damage

ATM is recruited to sites of DNA damage by a sensor complex composed of meiotic recombination 11 homolog (MRE11), radiation sensitive 50 (RAD50), and Nijmegen breakage syndrome 1 (NBS1), together known as the MRN complex[122]. This complex binds to naked DNA ends at the site of the break and recruits ATM by interaction specifically with NBS1. Once bound to this complex, ATM is activated by autophosphorylation and then performs many functions including amplification of DNA damage signaling, regulation of cell cycle checkpoints, chromatin remodeling, DNA repair, and initiation of apoptosis via phosphorylation of many downstream substrates (Figure 3.1). One of the initial events is local phosphorylation of histone variant H2A.X. Phosphorylated H2A.X (named γ-H2A.X) induces chromatin relaxation to facilitate repair and plays a role in recruiting numerous DDR proteins and amplifying DNA damage signaling[123]. H2A.X can be phosphorylated by ATM, ATR, and DNA-PK_{cs}, and serves as a robust marker for DNA damage. ATM also activates cell cycle checkpoints via phosphorylation of various substrates including p53 and checkpoint kinase 2 (CHK2), which prevent cell division until DNA is repaired[124].

Simultaneously, ATM initiates the DNA repair process by activating many factors that promote DNA end processing and ligation, including breast cancer susceptibility 1 and 2 (BRCA1 and BRCA2), p53 binding protein 1 (53BP1), and DNA ligases and cofactors[101]. ATM also dictates transcriptional and translational responses to fine-tune repair and determine cell fate. MAP kinase pathways, NF-κB, and transcription factor p53 can all be activated in response to DNA damage in an ATM-dependent manner[122]. In addition to their role in regulating immune response genes, these pathways upregulate repair enzymes, reinforce cell cycle checkpoints, activate stress responses, and modulate cell survival and apoptosis. Post-transcriptional control of expression is also achieved by modulation of microRNA biogenesis; a large number of microRNAs are upregulated upon DNA damage in an ATM-dependent manner[125]. Balancing the directions of

these various pathways is thought to determine whether the cell receives pro-survival or pro-apoptotic signals after DNA damage, and is likely decided by tissue-specific expression of various factors and the extent of damage, highlighting the exquisitely intricate nature of the DDR.

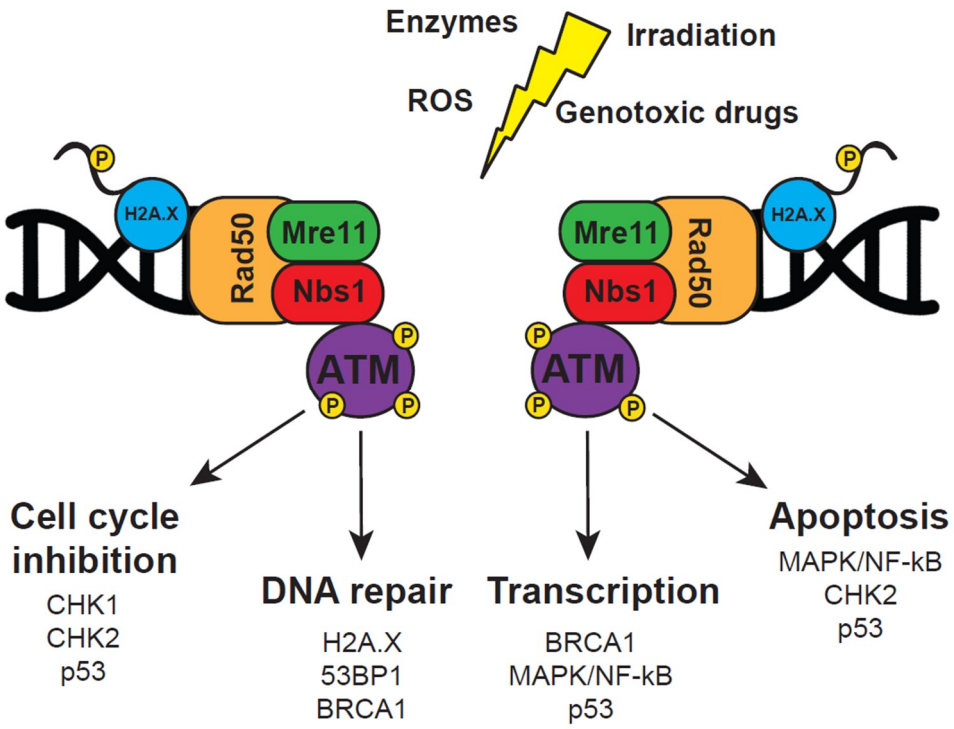


Figure 3.1: Schematic of ATM activation by DNA damage and its downstream substrates

3.2.3.2 ATM activation by oxidative stress

In addition to activation by DNA damage, a second mechanism of ATM activation directly by oxidation has recently been proposed. In this model ROS, such as H_2O_2, can oxidize ATM forming covalent intermolecular disulfide bonds between two ATM molecules via various cysteine residues[126]. Conformational changes in response to oxidation also result in ATM activation and autophosphorylation, but this activation is not dependent on DNA damage or the MRN complex. Oxidized ATM was shown *in vitro* to phosphorylate some of the classical substrates of ATM, including CHK2 and p53, but not others such as histone H2A.X. These results indicate a potential role for ATM outside the nucleus in response to stress factors other than DNA damage, particularly oxidative stress. Though *in vivo* demonstration of the importance of this second mechanism of ATM activation are challenging, because oxidative stress and DNA damage within a cell are impossible to isolate, some evidence suggests a role in AT pathogenesis. An *ATM* mutation has been identified (R3047X) which causes a truncation of the c-terminal region of ATM[127]. The protein expressed by this gene appears to respond normally to DNA damage via the

MRN complex, but is not activated by oxidation. Interestingly, patients harboring this mutation have been described as "AT variants;" they develop ataxia but not immunodeficiency, and their cells exhibit decreased radiosensitivity. This indicates that direct ROS-mediated activation of ATM may be physiologically relevant.

3.3 **Molecular pathogenesis of AT**

It is clear that ATM is a crucial member of the DDR; cells from AT patients have defective DNA damage signaling, decreased repair capacity, are sensitive to radiation and other genotoxic insults, and exhibit genomic instability. Many of the defining symptoms of AT can be ascribed to this impaired protection of the genome, including neurodegeneration, sterility, development of malignancy, and immunodeficiency. The molecular bases behind other pathologies have yet to be explained.

3.3.1 Neurodegeneration

The primary sign of AT, ataxia, is caused by progressive loss of Purkinje and granular neurons of the cerebellum. Neurons appear to be exquisitely sensitive to DNA damage and deficiencies in its repair. It is proposed that the cell loss leading to neurodegeneration derives from excessive unrepaired DNA lesions that eventually lead to the induction of apoptosis[128]. This is corroborated by the existence of overlapping neurodegenerative and neurodevelopmental manifestations being present in multiple syndromes caused by deficiencies in other DDR proteins, such as Nijmegen breakage syndrome[129], ataxia with oculomotor apraxia,[130], and AT-like disorder[131].

3.3.2 Cancer

The link between malignancy and DNA damage has been cemented in cancer biology for some time; genomic instability, recombination events between chromosomes, and mutation of proto-oncogenes drive tumorigenesis. Without a proper DDR, the frequency of these events is increased. Even being a heterozygous carrier of dysfunctional DDR genes, such as *ATM*, *BRCA1*, or *p53*, can greatly increase cancer predisposition[132,133]. In AT, the majority of malignancies are leukemias and lymphomas of T and B cell origin. This is often driven by defective repair of DNA breaks formed during V(D)J recombination[134]. This somatic recombination event occurs during T and B cell maturation to generate the diverse repertoire of T and B cell receptors. During this recombination event, double strand breaks (DSBs) are formed, which are repaired in an ATM-dependent manner. Failure to efficiently repair these breaks can result in aberrantly rearranged

receptor loci, sometimes fusing the locus with an oncogene and causing malignant transformation.

3.3.3 Immunodeficiency

Immunodeficiency is reported to affect over half of all AT patients[98]. The most common abnormalities are T and B cell lymphopenia, and absence or decrease in antibody isotypes IgA, IgE, and IgG subclasses. The type and degree of these immunodeficiencies can vary, and when present leave patients more susceptible to recurrent bacterial infections, especially of the sinopulmonary tract, though opportunistic infections are uncommon[135]. The origin of this immunodeficiency is also rooted in a defective DDR and V(D)J recombination. Persistent unrepaired DNA damage generated during recombination eventually leads to apoptosis of T and B cells, causing lymphopenia. Similarly, immunoglobulin class switching in mature B cells is again mediated by recombination involving DNA breaks. Without ATM and proper DNA damage signaling, this often results in apoptosis and prevents production of various immunoglobulin isotypes. The effects of ATM deficiency on cells of the innate immune system, however, have largely been neglected.

3.3.4 Pathologies not explained by DNA repair

In addition to the clinical manifestations of ATM deficiency that can be linked to aberrant DNA damage repair and cell cycle control, AT patients present with multiple symptoms and syndromes that are not easily explained by DNA damage. These include insulin resistant diabetes, metabolic syndrome, increased oxidative stress and decreased mitochondrial function[87,122]. In recent years it has become clear that ATM plays a role in other pathways independent of DDR.

The increased risks of diabetes, metabolic syndromes, as well as growth impairment are indicative of a role for ATM in metabolic signaling. ATM has been shown to be activated by insulin signaling independently of DNA damage, and to then modulate pathways controlling glucose metabolism and growth[136]. ATM is also important in regulating the redox environment of the cell by stimulating production of NADPH and glutathione, a strong antioxidant[137]. AT patients and *ATM* knockout mice show increased levels of basal ROS[138] and signs of oxidative stress due to decreased production of antioxidants[100,139]. This increased oxidative stress further exacerbates the rates of DNA damage in these patients, as ROS are potent oxidizers of DNA and an important source of DNA damage. Antioxidant treatment in ATM-deficient mice has been shown to decrease cancer rates[140], and even rescue some of the metabolic defects and insulin resistance[141], and neuro-motor function[142].

3.3.5 Signs of inflammation

In addition to the multitude of symptoms already mentioned, many reports exist that indicate an inflammatory component to AT may exist. One study found significantly elevated levels of IL-8 in the serum of AT patients, despite having no signs of infection[89]. The incidence of autoimmune phenomena measured by presence of autoantibodies was reported at 45% in one cohort of patients[96]. Additionally, several reports exist in the literature of AT patients presenting with cutaneous granulomatous lesions[91-93]. In these cases, no infectious organism could be isolated from the lesions and they did not respond to antibiotics, although some resolved with anti-inflammatory treatments[143]. These symptoms, as well as some of the other classical symptoms of AT, are associated with inflammation, but their link to DNA damage and repair remains unclear.

3.4 **Aim of the study**

Prior to this study it was known that patients with AT suffer from a range of symptoms that are not clearly linked to deficiencies in DNA damage recognition or repair, but which have can be associated with inflammation. These patients also commonly have immunodeficiency. Research of this phenomenon has focused on the adaptive immune system, with so far no consideration of the function of the innate immune system in these patients. Many of the inflammatory symptoms described in AT patients have been shown in other diseases to involve dysregulation of neutrophil functions, which predispose patients to inflammation. We hypothesized that neutrophil functions may be altered in AT patients. This would indicate a new and interesting role for ATM and the DDR in immune regulation, and potentially shed light on the etiology of inflammation in AT. Furthermore, as ATM has recently been shown to be redox reactive and involved in regulation of oxidative stress, we hypothesized that ATM may be an important mediator of regulatory functions of the oxidative burst. To address these questions, neutrophil functions were assayed upon ATM inhibition, and more relevantly, with primary neutrophils isolated from AT patients. Cytokine production, NET formation, and apoptosis were altered in ATM-deficient neutrophils. Furthermore, activation of the DDR in stimulated neutrophils modulated these functions in a manner dependent on the oxidative burst, revealing a mechanistic link between ATM-deficient and NADPH oxidase-deficient neutrophil dysregulation. These abnormal neutrophil responses suggest that improper inflammation may be an underlying feature of some of the pathology of AT. Finally, a mouse model to specifically study the effects of DDR deficiency in neutrophils and macrophages in an *in vivo* system was developed.

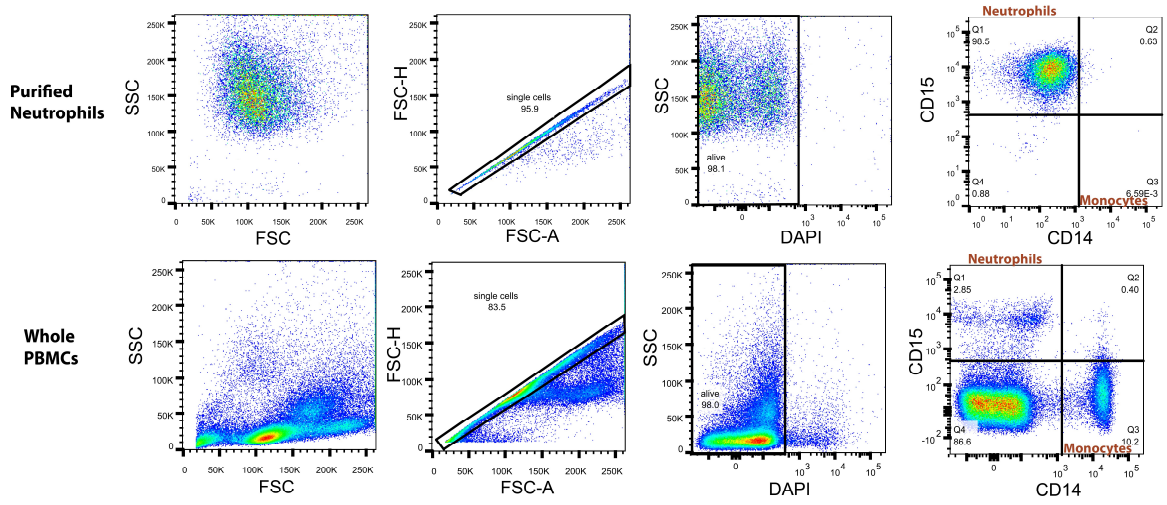
RESULTS

CHAPTER 4: ATM AND THE DDR REGULATE NEUTROPHIL FUNCTIONS

4.1 ATM inhibition increases neutrophil cytokine production and delays apoptosis

4.1.1 Neutrophils express functional ATM

Patients with AT were previously reported to have increased levels of the largely neutrophil-specific cytokine IL-8 in their serum[89], as well as increased markers of oxidative stress and tissue damage[138,144]. As these are indications of inflammation and neutrophil activation, we set out to determine the effects of ATM deficiency on neutrophil functions.



Isolation	% CD15+ Neutrophils	% CD14+ Monocytes
1	97.9	0.00403
2	95.8	0.00969
3	95	0.014
4	95.4	0
5	98.2	0
6	98.5	0.00659
7	98.8	0.01
8	97.2	0.00345
9	95	0.00832
Average:	96.86666667	0.006231111

Figure 4.1: Purity of routine neutrophil preparations. Human peripheral blood neutrophils were isolated by density separation and examined for purity by flow cytometry. **(a)** Example gating strategy for neutrophils (CD15+/CD14-) and monocytes (CD15-/CD14+) performed on purified neutrophils as well as whole peripheral blood mononuclear cells (PBMCs) as a control. **(b)** Purity of 9 routine neutrophil preparations (average purity = 96.87%).

As it was previously reported that neutrophils do not express key players of the DDR at the transcriptional level[145], the presence of functional ATM protein was assayed in human neutrophils. Neutrophils were isolated from peripheral blood of healthy donors by subsequent density separations, resulting in highly pure, unactivated neutrophils (purity > 95%; Figure 4.1). Using western blot, a protein of appropriate molecular weight (~350 kD) was detected with an antibody against whole ATM (Figure 4.2). In response to γ-irradiation-induced DNA damage, autophosphorylation of ATM was observed using a phospho-ATM specific antibody. This phosphorylation was abrogated by addition of KU-55933, a highly specific small molecule inhibitor of ATM[146], confirming the identity of the detected band as ATM.

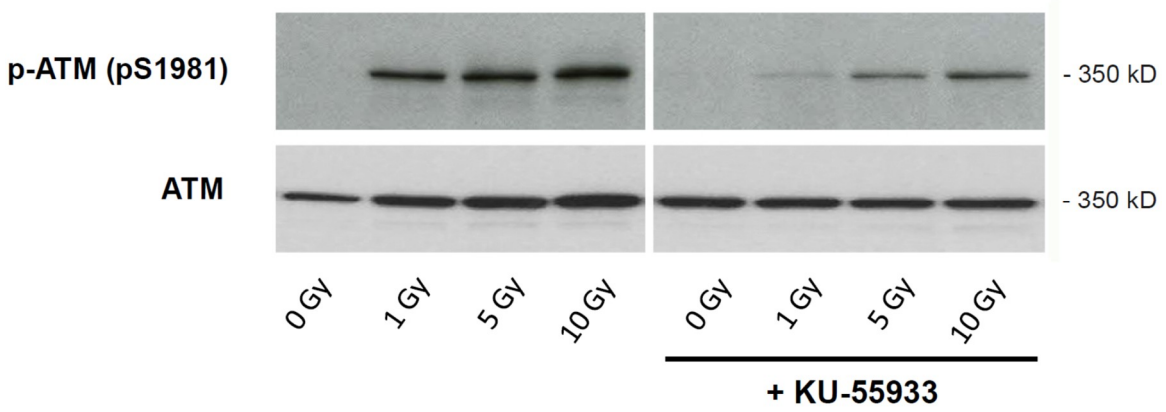


Figure 4.2: Neutrophils express functional ATM protein. Immunoblot analysis of whole and phosphorylated ATM in lysates of neutrophils exposed to ionizing γ-irradiation with and without ATM inhibitor KU-55933, 10μM. Whole cell lysates were made 1 hour after irradiation.

4.1.2 ATM and the DNA damage response are activated upon neutrophil stimulation

We next asked if ATM and the DDR are activated endogenously in neutrophils in response to physiologically relevant stimuli. Lysates from a time course of LPS stimulation were analyzed for phosphorylation of ATM and of histone variant γ-H2A.X, a marker of DNA damage. An accumulation of phosphorylated ATM and γ-H2A.X was observed over time in response to LPS stimulation (Figure 4.3a). Treatment with the ATM inhibitor abrogated phosphorylation of ATM, but not of H2A.X. To test if other neutrophil stimuli were capable of activating ATM, protein lysates were made from neutrophils stimulated with PMA, a diacylglycerol mimetic and potent activator of protein kinase C, and opsonized zymosan, a component of fungal cell walls. Upon treatment with these stimuli, phosphorylation of ATM and γ-H2A.X was also observed (Figure 4.3b).

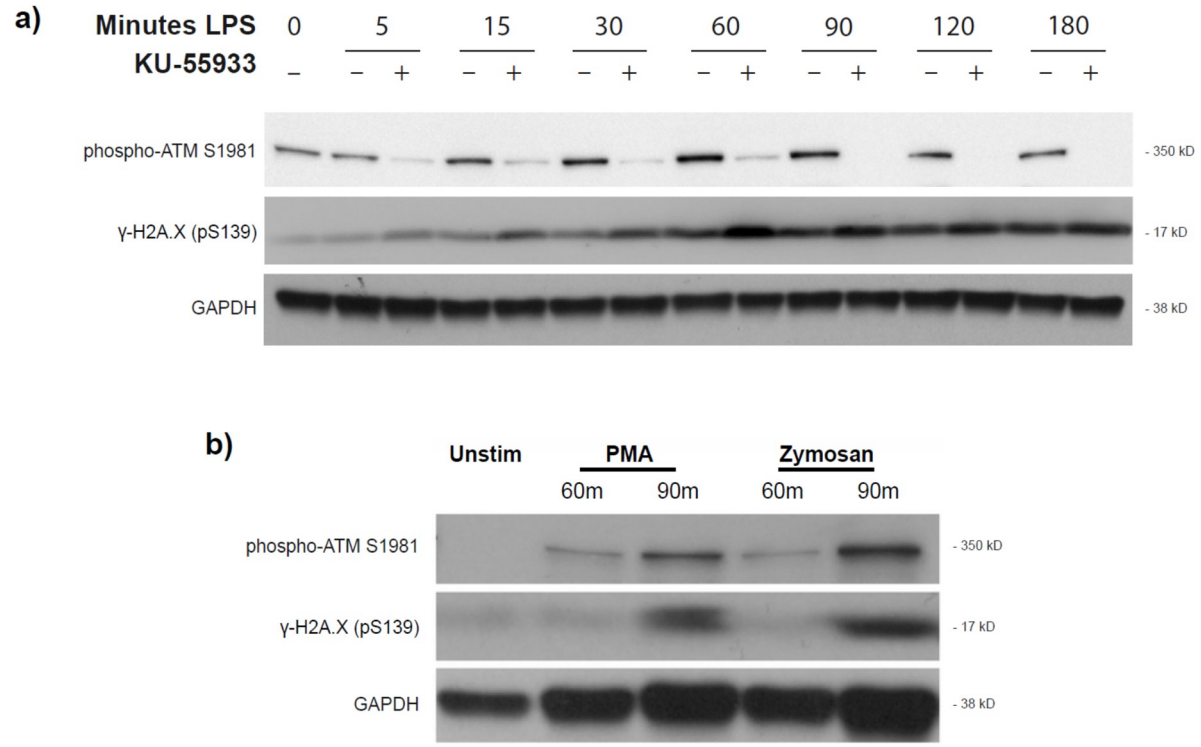


Figure 4.3: ATM and DNA damage marker γ-H2A.X are phosphorylated in stimulated neutrophils. (a) Immunoblot analysis of phospho-ATM and γ-H2A.X in lysates of LPS-stimulated neutrophils with and without KU-55933, 10μM. Neutrophil lysates were made at indicated time points after LPS (100ng/ml) stimulation. **(b)** Immunoblot analysis of phospho-ATM and γ-H2A.X in lysates of neutrophils stimulated for indicated times with PMA (50nM) or opsonized zymosan (10μg/ml).

Phosphorylation of ATM in response to PMA stimulation was also assayed by immunofluorescence. Neutrophils stimulated with PMA were stained with antibodies against phosphorylated ATM and analyzed by fluorescence microscopy. An accumulation of phospho-ATM in the nucleus of stimulated cells was observed over time (Figure 4.4). These results confirm that neutrophil express DNA damage proteins and have a functional DDR, which is induced by activating stimuli.

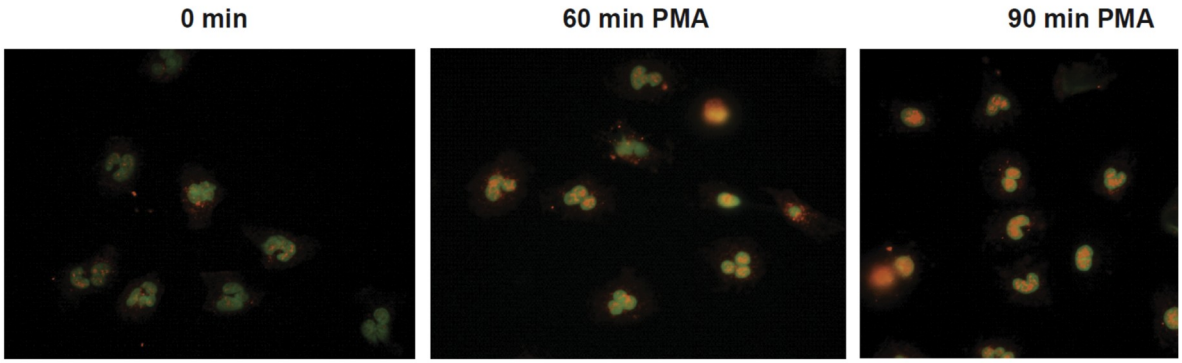


Figure 4.4: Phospho-ATM is observed in the nucleus of stimulated neutrophils. Immunofluorescence staining of pATM (red) and DNA (green) in PMA-treated neutrophils. At indicated time points, PMA-stimulated neutrophils were fixed with 2% PFA on glass coverslips, stained with sytox DNA dye (green) and anti-phospho-ATM antibody (red) and visualized by fluorescence microscopy.

4.1.3 ATM inhibition increases neutrophil and monocyte cytokine production

We next set out to examine the effects of ATM inhibition on neutrophil functions. Multiple neutrophil responses were measured, including the oxidative burst, phagocytosis, formation of neutrophil extracellular traps (NETs), cytokine production, and viability from peripheral blood-isolated human neutrophils cultured in the presence of the ATM inhibitor. Neutrophils efficiently mounted an oxidative burst, phagocytosed fixed bacteria, and formed NETs when ATM was inhibited, indicating that ATM activity is not required for these functions (Figure 4.5). ATM-inhibited neutrophils produced a greater initial oxidative burst than control cells.

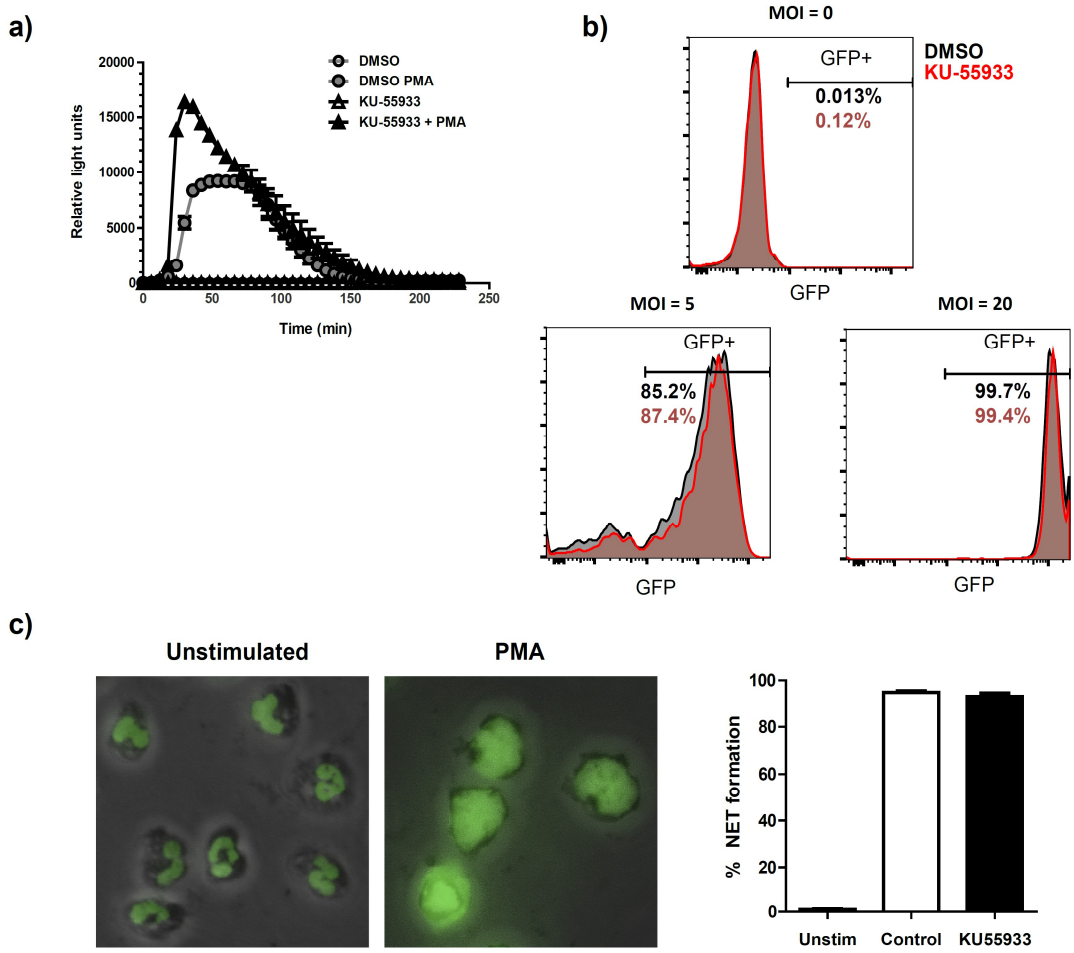


Figure 4.5: ATM activity is not required for ROS production, phagocytosis, or NET formation. (a) Oxidative burst in PMA-stimulated neutrophils with and without 10μM KU-55933. Neutrophils were incubated with luminol and horseradish peroxidase to measure ROS production by luminescence. Luminescence was measured kinetically in a luminometer and expressed as relative light units. **(b)** Phagocytosis of fixed GFP-expressing bacteria. Neutrophils were incubated for 30 minutes with fixed GFP-expressing *E. coli* at indicated multiplicity of infection (MOI), washed, and analyzed by flow cytometry to measure phagocytosis of bacteria. **(c)** Example images and quantification of NET formation. Neutrophils were stimulated with 50nM PMA for 3 hours to form NETs, fixed with 2% PFA, stained with sytox green DNA dye, and visualized by microscopy. During NETosis, nuclei lose their lobulated shape, expand, and then are released from the cell. The percentage of cells that formed NETs was analyzed by counting at least 500 cells per condition.

When measuring cytokine production from LPS-stimulated neutrophils, it was found that ATM inhibition caused significant increases in production of both IL-8 and MIP-1α. This increase occurred in a dose-dependent manner (Figure 4.6a), and could be reproduced with a second small molecule inhibitor of ATM, KU-60019[147] (Figure 4.6b).

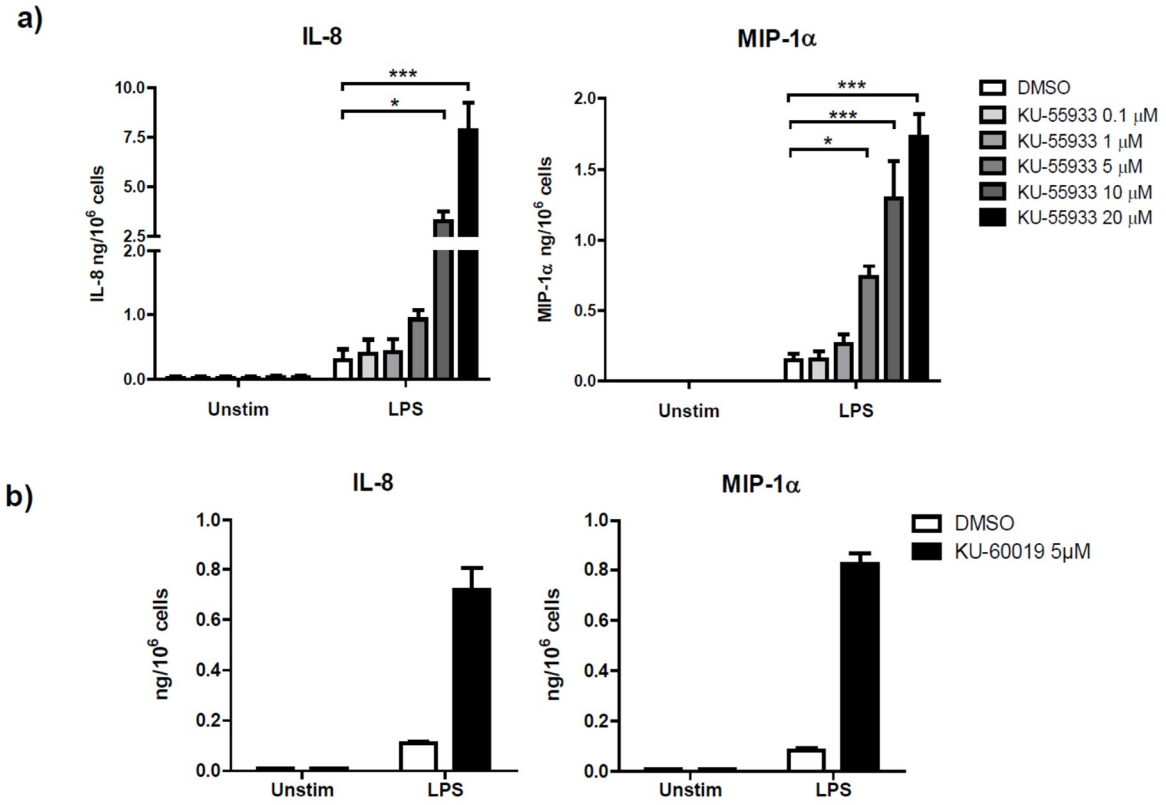


Figure 4.6: ATM-inhibition increases neutrophil cytokine production in response to LPS in a dose-dependent manner. (a) IL-8 and MIP-1α production from neutrophils stimulated with LPS in the presence of KU-55933. Neutrophils were incubated with KU-55933 at indicated concentrations and stimulated with 100ng/ml LPS. Cell-free supernatants were collected after 18 hours and cytokine concentrations determined by ELISA. Data are presented as the mean ± standard error of the mean (SEM) of 4 compiled experiments. Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 by repeated measures ANOVA followed by Dunnett's multiple comparison test. **(b)** Cytokine production from LPS-stimulated neutrophils treated with 5μM KU-60019. Data are presented as the mean ± standard deviation (SD) of technical replicates from a representative experiment.

To test if this effect was specific to stimulation with LPS, cytokine production was measured in response to a panel of extracellular receptor agonists known to activate neutrophils. Heat-killed *Listeria monocytogenes* (HKLM) stimulate cells mainly via activation of TLR2, LPS via TLR4, bacterial protein flagellin via TLR5, and opsonized zymosan from yeast cell walls via TLR2 and dectin-1. Inhibition of ATM increased levels of cytokine production in response to each of these stimuli (Figure 4.7).

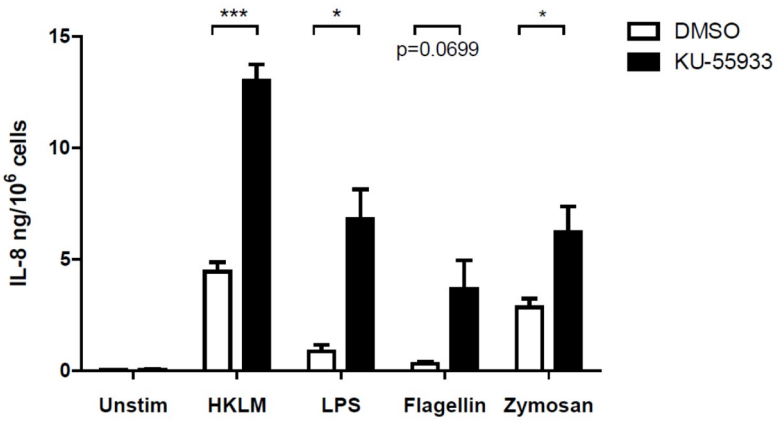


Figure 4.7: ATM-inhibition increases cytokine production in response to various pattern recognition receptor agonists. IL-8 production from neutrophils stimulated with a panel of PRR-agonists. Neutrophils were incubated with 10μM KU-55933 and stimulated for 18 hours with heat-killed *Listeria monocytogenes* (HKLM, MOI 100), LPS (100ng/ml), flagellin (1μg/ml), or opsonized zymosan (10μg/ml). IL-8 concentration in supernatants was measured by ELISA. Data are presented as the mean ± SEM of 3 compiled experiments. Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 by paired Student *t* test.

Cytokine production was also measured from human blood-isolated monocytes to test if the effect of ATM inhibition on cytokine production was specific to neutrophils. Monocyte production of several pro-inflammatory cytokines was also significantly increased by ATM inhibition (Figure 4.8).

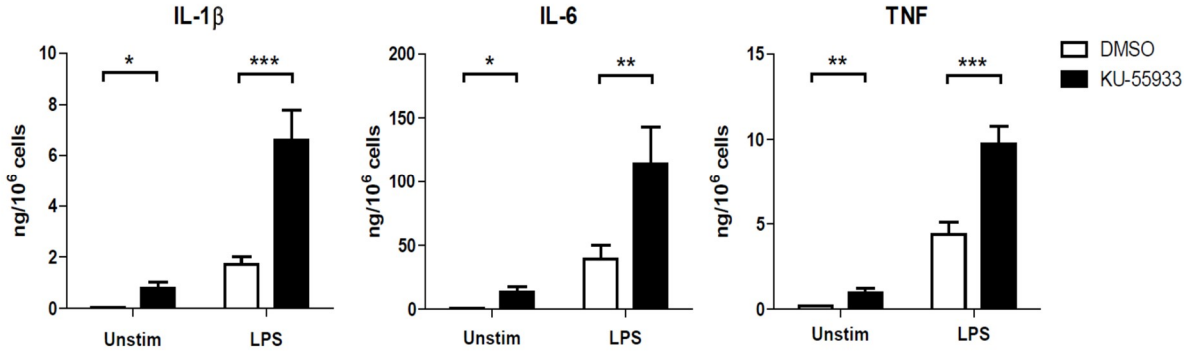


Figure 4.8: ATM inhibition increases monocyte cytokine production. LPS-stimulated production of IL-1β, IL-6 and TNF by isolated human monocytes. Monocytes were isolated from PBMCs by density separation followed by positive selection with CD14 microbeads, incubated with 10μM KU-55933, and stimulated with 100 ng/ml LPS. After 18 hours stimulation, supernatants were harvested and cytokines were measured by ELISA. Data are presented as the mean ± SEM from experiments with 10 blood donors. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by paired Student *t* test.

4.1.4 ATM inhibition mildly affects neutrophil lifespan

Neutrophil constitutive apoptosis is an important mechanism to limit their lifespan and inflammatory functions. Because ATM can activate p53 and induce apoptosis, we asked whether ATM deficiency may affect constitutive neutrophil apoptosis. Neutrophil viability was measured at 6 and 12 hours post-stimulation to determine if ATM inhibition affects the kinetics of apoptosis

induction. Neutrophil viability was measured via flow cytometry by staining with annexin V (which binds to phosphatidylserine exposed on the surface of apoptotic cells) and propidium iodide (PI, a non-cell-permeable vital DNA dye). Viable cells remain double negative, early apoptotic cells stain annexin V-positive/PI-negative, and late apoptotic and necrotic cells stain annexin V-/PI-double positive (Figure 4.9a). ATM inhibition did not affect viability of unstimulated neutrophils; however a mild increase in viability was observed upon ATM inhibition in LPS-stimulated neutrophils after 12 hours (Figure 4.9b)

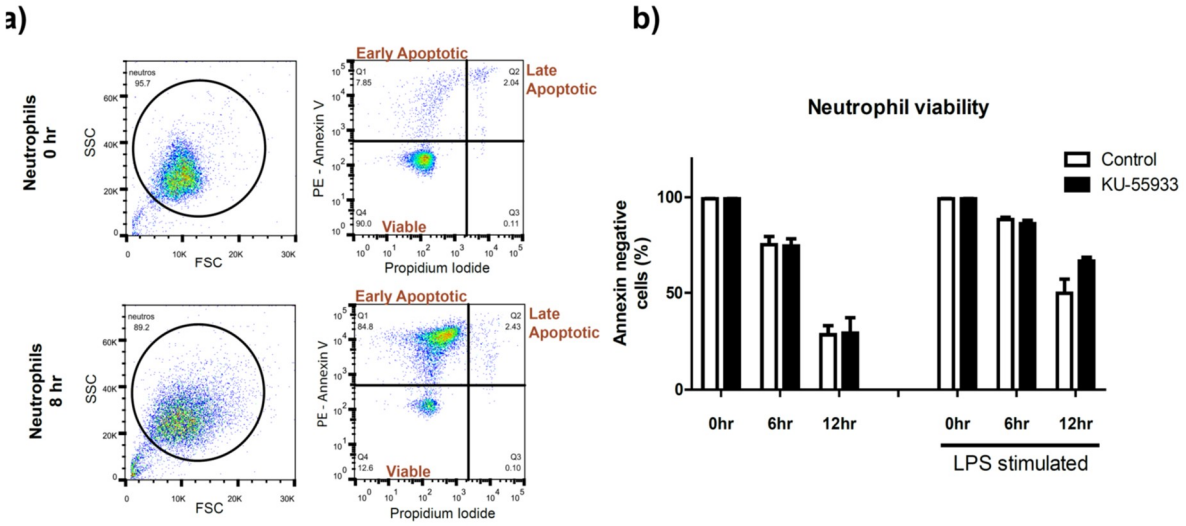


Figure 4.9: ATM inhibition mildly affects neutrophil lifespan. (a) Gating strategy for determining neutrophil viability by annexin V/PI staining in flow cytometry. Neutrophils were resuspended at indicated time points and stained with PE-annexin V and PI. Cells were analyzed by flow cytometry and gated to distinguish annexin V/PI double negative (viable cells), annexin V-positive/PI-negative (early apoptotic), and annexin V/PI double positive (late apoptotic/necrotic). At least 10,000 cells were analyzed per sample. **(b)** Viability curve of unstimulated and LPS-stimulated neutrophils. Neutrophils were incubated with KU-55933 (10μM) with and without LPS stimulation, collected, stained, and analyzed at indicated time points. Data are graphed as the mean ± SEM of data from three independent neutrophil donors.

4.2 Primary neutrophils from Ataxia Telangiectasia patients overproduce IL-8 and neutrophil extracellular traps, and have a prolonged lifespan

In order to verify the relevance of the phenotype with ATM inhibitors, we worked with a cohort of 14 AT patients, who have genetic deficiencies of ATM. One of these patients participated twice on separate occasions separated by more than six months. Data from three patients were not included in the results: one patient diagnosed with malignancy shortly after the date of blood donation, one patient who was being treated with prophylactic antifungal drugs known to affect neutrophil functions, and one patient diagnosed with cystic fibrosis and AT concurrently (final participating patients, n=12; mean age, 16 years). At the time of blood donation, participating

patients were not being treated with any medication, besides some receiving standard immunoglobulin transfer therapy. All patients were free of apparent infections and malignancy at the time of blood donation. AT diagnoses were confirmed by *ATM* gene sequencing (Table 4.1). All patients were unrelated and carried independent *ATM* mutations. Neutrophils isolated from sex-matched and approximately age-matched healthy donors were used as controls (n = 12; mean age, 28 years). Neutrophils were isolated and experiments performed with one AT patient and one control concurrently.

Age (years)	**Mutation**	**Effect**
4	[c.6679C>T (p.Arg2227Cys)] [Deletion of exon 65] [unclear variant c.8671T>G]	2227Arginine>Cysteine substitution Loss of C-terminus, nonsense mediated mRNA decay unclear, possibly benign polymorphism
9	[c.610G>T (p.Gly204*)] [Deletion of exons 32-34]	Termination Deletion of at least 2kb not described before
10	[c.7172C>T (p.Ala2391Val)] homozygous	Unknown
11	[c.7875_7876delTGinsGC (p.Asp2625_Ala2626delinsGluPro)] [IVS53-2A>C (codon 2544del159nt) (Ex54)]	missense variant Deletion of exon 54
12	*same as above*	*same as above*
11	[c.7517-7520delGAGA (p.Arg2506*fs*)] homozygous	Deletion leading to frameshift
14	[c.4303A>T, p.K1435*] [c.8620C>T, p.Q2874*]	Termination Termination
20	[c.5979_5983delTAAAG (p.S1993R*fs*)] homozygous	Deletion leading to frameshift and premature stop
22	[c.4303A>T (p.Lys1435*)] [c.8620C>T (p.Glu2874*)]	Termination Termination
25	[c.1110C>G (p.Tyr370*)] [IVS54-3T>G]	Termination Aberrant splicing, skipping exon 55
25	[IVS14+2 T>G] [IVS20-581del4]	In-frame deletion of codons 601-633 Pseudeo-exon inclusion
26	[IVS63del17kb] homozygous	Exons 64-65 deleted

Table 4.1: Ataxia Telangiectasia cohort patient data. Data of participating AT patients, including age at time of blood donation, sequenced *ATM* mutation and predicted mutation effect according to Leiden Open Variation Database.

Neutrophils from patients with AT produced significantly more IL-8 in response to LPS than healthy controls (Figure 4.10a). NET formation was simultaneously measured from patient neutrophils. In response to stimulation with PMA, neutrophils robustly release NETs (~95% after 3 hours, Figure 4.5). NET formation was measured at an early time point, 2 hours post-stimulation, in order to detect potential increases in NETosis. NET formation was assessed by measuring increases in the nuclear area, as the nucleus expands and is released during NET

formation[148]. PMA-stimulated neutrophils from AT patients exhibited larger average nuclear area, and thus increased NET formation, compared to healthy controls (Figure 4.10b).

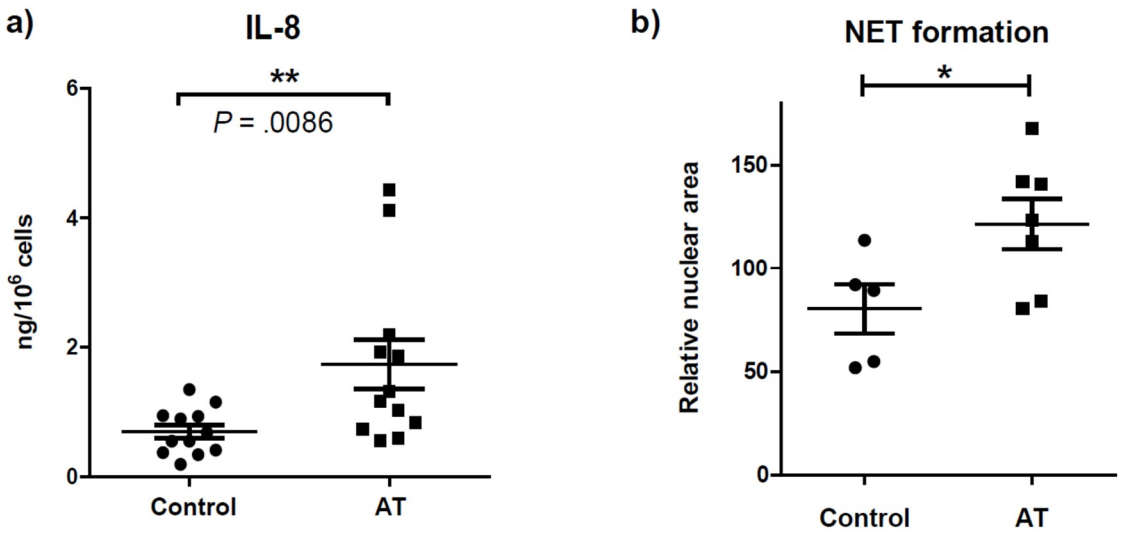


Figure 4.10: Primary neutrophils isolated from AT patients produce more IL-8 and NETs. (a) IL-8 production from neutrophils isolated from AT patients and healthy controls after 18 hours stimulation with LPS (100ng/ml) (P = 0.0086, Mann-Whitney *U* test). IL-8 was measured in supernatants by ELISA. **(b)** NET formation in response to PMA-stimulation by AT patients (n=7) and controls (n=5). Cells were fixed with 2% PFA after 2 hours PMA stimulation and stained with DNA dye sytox green. Images were taken on a fluorescence microscope, and relative nuclear area was automatically quantified in Image J. Horizontal lines represent mean ± SEM. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by Mann-Whitney U test.

Neutrophil apoptosis was concurrently measured and showed that neutrophils from AT patients remained viable longer than those from healthy controls (Figure 4.11). This result was obtained using three independent readouts of apoptosis: annexin V surface staining (Figure 4.11a), terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Figure 4.11b), and the SubG1 assay (Figure 4.11c-d). The TUNEL assay measures DNA fragmentation in apoptotic cells by incorporating a tail of fluorescent nucleotides onto broken DNA ends, which is then visualized microscopically. TUNEL-positive cells are apoptotic. The SubG1 assay measures DNA content in cells by flow cytometry. Viable cells contain a full diploid genome content (equivalent to a cell in G1 phase of the cell cycle); however, the genome of apoptotic cells is fragmented, some of which diffuse out of the nucleus of fixed cells during washes, resulting in an apparent DNA content below that of a cell in G1 phase (example plot in Figure 4.11c). This decrease is detectable by flow cytometry and can be used to separate viable and apoptotic cells. Using these independent methods, a delay in apoptosis initiation in neutrophils from AT patients was confirmed.

Taken together, these data with ATM inhibitors complemented by experiments with primary cells from AT patients show that deficient ATM activity results in increased production of cytokines and NETs, and a delayed initiation of apoptosis. This phenotype suggests an

important role for ATM in regulating neutrophil functions and inflammation, and indicates that AT patients may have dysregulated innate immune responses.

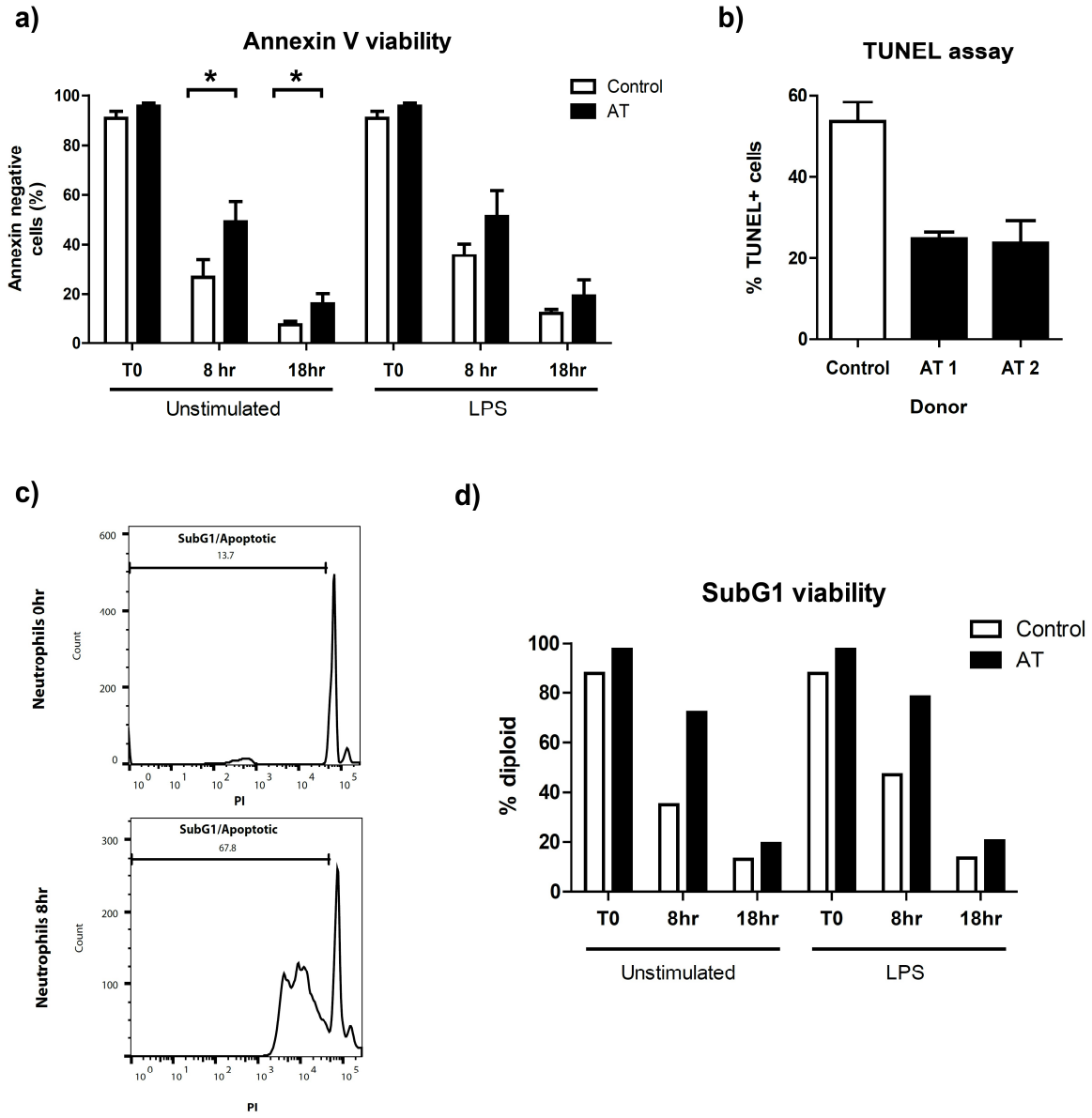


Figure 4.11: Primary neutrophils from AT patients have an increased lifespan compared to healthy controls. **(a)** 18-hour viability curve of neutrophils isolated concurrently from AT patients (n = 6-8) and healthy controls (n = 7-9) measured by annexin V/PI staining. At indicated time points, neutrophils were collected, stained with annexin V/PI and analyzed by flow cytometry. Data are plotted as Mean ± SEM. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by Mann-Whitney *U* test at each time point. **(b)** Assessment of spontaneous neutrophil apoptosis by TUNEL assay. Neutrophils seeded on coverslips were fixed after 12 hours in culture and labeled by TUNEL. Labeled DNA was visualized by microscopy and percent TUNEL-positive cells was determined by counting at least 200 cells from three independent images. **(c)** Example histogram and gating strategy of flow cytometry data for determination of neutrophil viability by SubG1 assay. At indicated time points, neutrophils were collected, fixed in ethanol, stained with PI, and analyzed by flow cytometry. Apoptotic cells stain less positively for PI. **(d)** 18-hour viability curve of neutrophils isolated concurrently from a single AT patient and healthy control as measured by SubG1 DNA content. Results from a single experiment.

4.3 Increased MAP kinase activation in ATM-deficient neutrophils results in increased expression of cytokines and NET formation

We next set out to determine the mechanism by which ATM inhibition affects neutrophil functions. Specifically, we asked whether the prolonged viability of ATM-inhibited neutrophils was sufficient to increase cytokine production, or if these are two discrete phenomena. In the previous experiments, cytokines measurements represented the cumulative production of cytokines over the lifespan of the neutrophils (~18 hours). Thus, the increased cytokine production and decreased cell death could not be resolved. We set out to determine if these phenomena could be resolved, and on which level the production of IL-8 is increased.

4.3.1 Increased IL-8 production is independent of prolonged lifespan.

Neutrophil cytokine production and cell death were measured at 12 hours post stimulation, when neutrophils were still partially viable and differential cell death would be less pronounced. By this earlier time point, only a slight increase in cell viability was observed with the ATM inhibitor, but IL-8 production was largely increased (Figure 4.12). This difference was quantified by calculating the amount of IL-8 produced per viable cell at the 12-hour time point; [ng of IL-8 in supernatant]÷[Number of viable cells]. Expressing the data in this way shows that the amount of IL-8 produced by a viable neutrophil in a given time period is increased when ATM is inhibited.

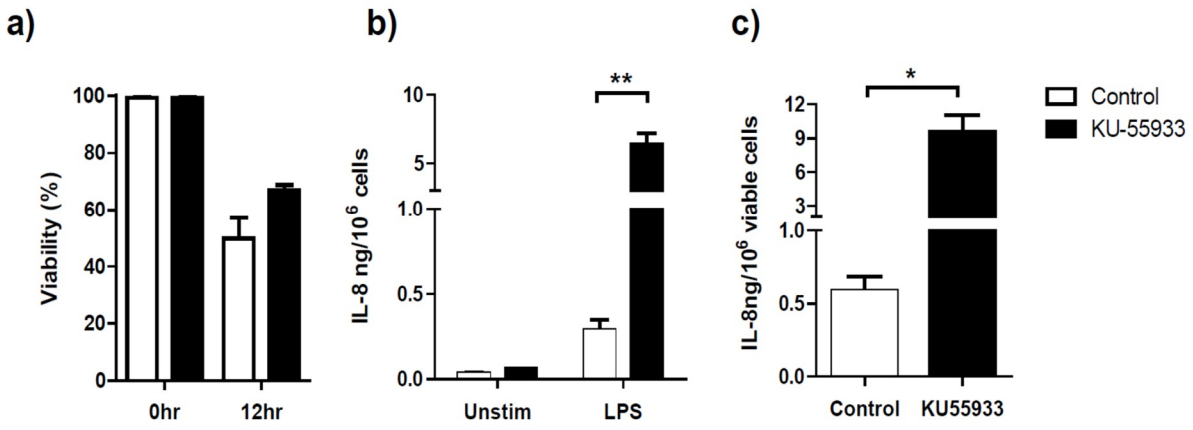


Figure 4.12: ATM-inhibited neutrophils produce IL-8 at an increased rate. (a) Viability of neutrophils 12 hours after stimulation with LPS (100ng/ml). Viability was measured by annexinV/PI staining as before. **(b)** IL-8 production from LPS-stimulated neutrophil after 12-hours normalized to total cells seeded per well. **(c)** IL-8 production from (b) normalized to cell viability at 12 hours post-LPS stimulation, calculated as [total IL-8 production at 12 hours]/[fraction of cells viable after 12 hours]. Data are presented as the mean ± SEM of 3 compiled experiments. Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 by paired Student *t* test.

To further assess the contribution of apoptosis to limiting neutrophil cytokine production, experiments were performed with Pifithrin-μ, a small molecule inhibitor of p53

activity. Inhibition of p53 completely abrogated cell death over a 20-hour period (Figure 4.13a), but did not increase the production of IL-8 in response to LPS during this period (Figure 4.13b). This indicates that neutrophil cytokine production is controlled by a mechanism independent of the initiation of cell death and that an increased lifespan alone is not sufficient for increased IL-8 production.

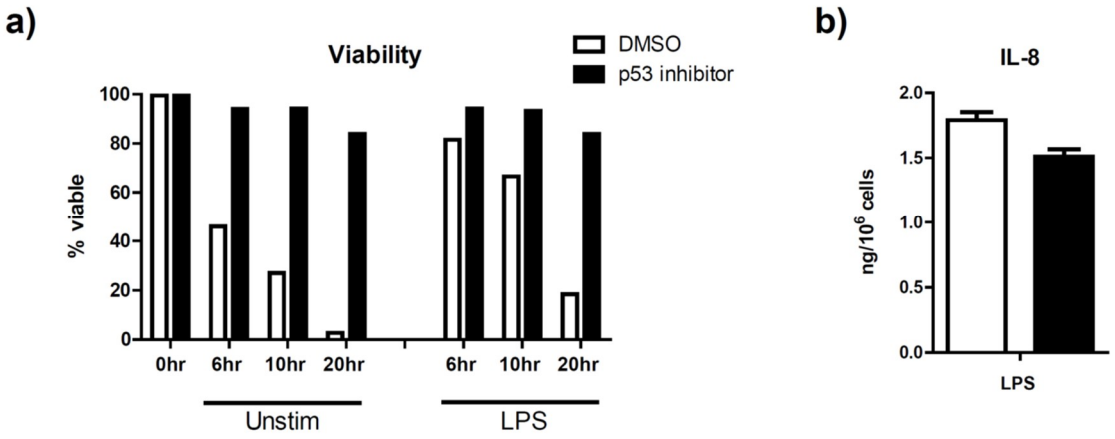


Figure 4.13: Increased neutrophil lifespan is not sufficient for increased cytokine production. (a) Viability time course of isolated human neutrophils incubated with p53 inhibitor pifithrin-μ (1 μM) and stimulated with LPS (100ng/ml). Neutrophil viability was measured at indicated time points by annexin V/PI staining. **(b)** IL-8 production from LPS-stimulated cells after 20 hours. IL-8 was measured in cell-free supernatants by ELISA.

4.3.2 ATM-inhibited neutrophils transcribe and translate cytokines at an increased rate

To confirm that ATM-inhibited neutrophils produce cytokines at an increased rate, their production was measured at the level of both mRNA transcription and protein translation. mRNA was isolated from LPS-stimulated neutrophils, reverse transcribed into cDNA, and used as a template for qPCR reactions. qPCRs were performed to amplify cytokine targets and the housekeeping gene β-microglobulin. mRNA levels of both IL-8 and MIP-1α were increased when ATM was inhibited over a 12-hour time course, showing that transcription of these genes is enhanced in the absence of ATM activity (Figure 4.14a). Additionally, increased levels of multiple other pro-inflammatory cytokine transcripts were detected upon stimulation in the presence of ATM inhibitor (Figure 4.14b).

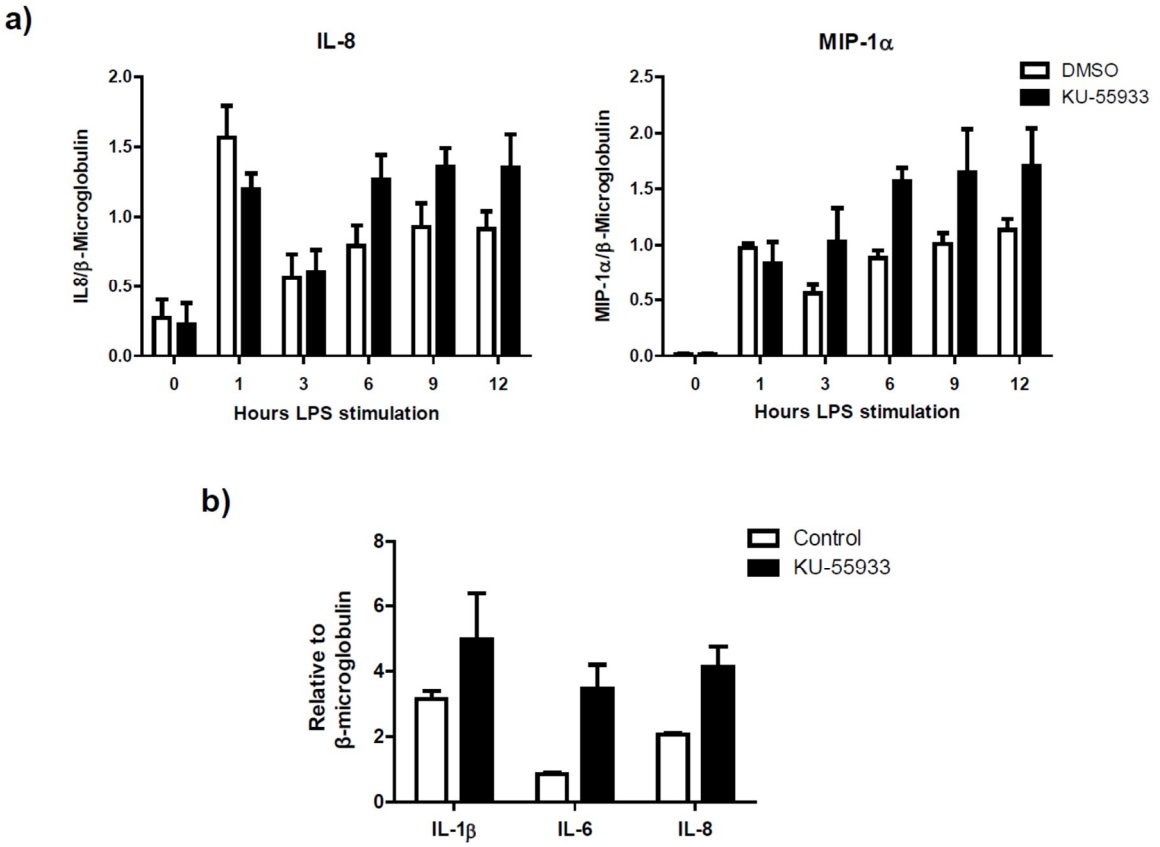


Figure 4.14: Cytokine transcripts are increased in ATM-inhibited neutrophils. (a) Quantitative PCR (qPCR) analysis of relative IL-8 and MIP-1α mRNA transcript in cDNA from a time course of neutrophils stimulated with LPS (100ng/ml) with and without KU-55933 (10μM). **(b)** qPCR analysis of multiple cytokine transcripts in cDNA from neutrophils stimulated for 1 hour with LPS (100ng/ml) in the presence of KU-55933 (10μM). At indicated time points, neutrophils were lysed, mRNA was extracted and reverse transcribed into cDNA and used for qPCR in triplicate. Data are expressed as the relative amount of cytokine transcript divided by the relative amount of housekeeping gene β2-microglobulin. Relative transcript levels were calculated by extrapolation from a standard curve made from serial dilutions of a pooled sample. Data were analyzed by StepOnePlus software. Data represent the mean ± SEM of three independent experiments.

IL-8 production was then measured by intracellular cytokine staining to determine if protein production was increased, and was not an effect on secretion. Secretion was inhibited with Brefeldin A and neutrophils were stimulated, resulting in the intracellular accumulation of cytokines. IL-8 levels were then measured by intracellular immunofluorescence staining. The fluorescence intensity, and thus the intracellular level of IL-8, was increased in ATM-inhibited cells as early as 3 hours post-stimulation (Figure 4.15). The difference between control and ATM-inhibited cells continues to grow until 20 hours post-stimulation. These data, along with the p53 inhibition experiments, support the conclusion that ATM inhibition increases the rate of cytokine production at the level of transcription and translation, and is not an effect of a prolonged lifespan.

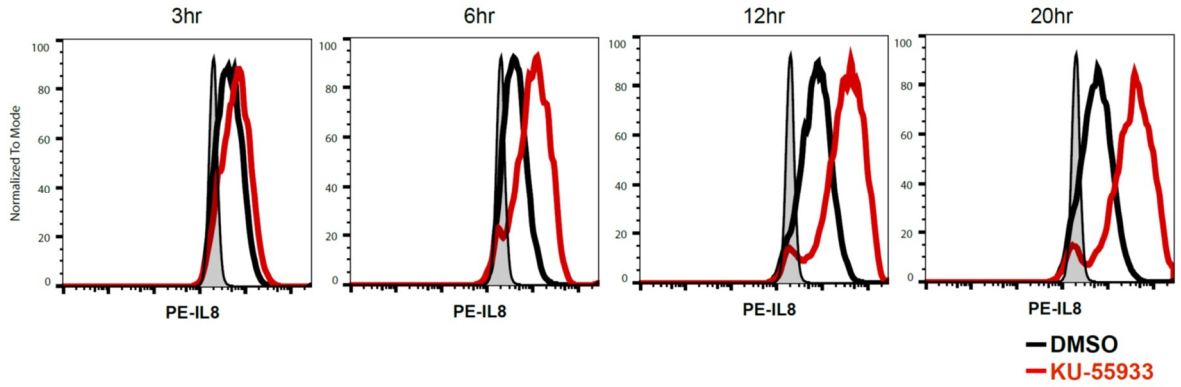


Figure 4.15: ATM inhibition increases neutrophil IL-8 production on the cellular level. Intracellular IL-8 immunofluorescence staining of LPS-stimulated neutrophils. Cells were incubated with Brefeldin A to block secretion and stimulated with LPS with or without KU-55933 (10μM). At indicated time points, neutrophils were fixed with 2% PFA, permeabilized, and stained with fluorescence-conjugated anti-IL-8 antibody and analyzed by flow cytometry. At least 10,000 cells were analyzed per sample. Gray fill represents unstimulated neutrophils.

4.3.3 ATM inhibition increases MAP kinase activation

To understand how ATM inhibition leads to increased transcription and translation of cytokines, the pathways upstream of transcription in response to inflammatory stimuli were examined. Neutrophil stimulation via TLR signaling leads to phosphorylative activation of MAP kinases, including p38 and ERK, as well as activation of the transcription factor NF-κB. Activation of these pathways culminates in the transcription and translation of immune-related genes, including pro-inflammatory cytokines.

Activation of MAP kinases and NF-κB were measured to determine if ATM-inhibition affects these pathways. Whole cell protein lysates of neutrophils stimulated with LPS in the presence and absence of ATM inhibitor were made over a three-hour time course and analyzed by western blot. Upon LPS stimulation, phosphorylation of MAP kinases p38 and ERK were observed, as well as transient degradation of IκBα, an inhibitor of NF-κB and proxy readout for its activation. Interestingly, when ATM was inhibited, the phosphorylation of p38 was increased and prolonged compared to controls (Figure 4.16a). Levels of IκBα and phosphorylated p38 and ERK were quantified by densitometry (Figure 4.16b). Levels of p38 phosphorylation were consistently increased across this time course, while differences in IκBα and phosphorylated ERK were mild and only observed at later time points.

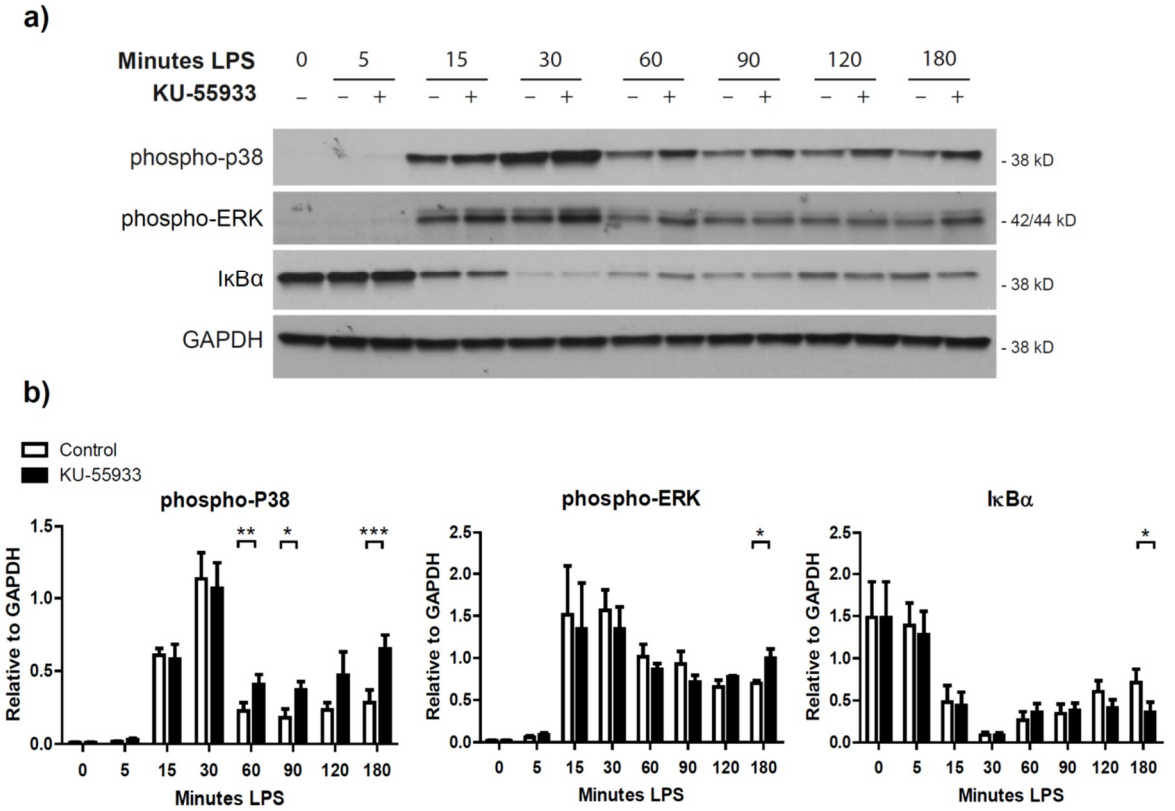


Figure 4.16: Phosphorylation of MAP kinase p38 is increased in the absence of ATM activity in LPS-stimulated neutrophils. (a) Western blot analysis of phosphorylation of MAP kinases p38 and ERK, and IκBα degradation in response to LPS. Neutrophil whole cell lysates were made at indicated time points after LPS stimulation with and without KU-55933 (10μM) and analyzed by western blot with antibodies specific to phosphorylated p38, pERK, IκBα, and loading control GAPDH. Blot is representative of 4 experiments. **(b)** Quantification of relative levels of phospho-p38, phospho-ERK, and IκBα from western blots in (a). Quantification was performed using ImageJ software densitometry analysis and expressed as signal relative to GAPDH loading control. Data represent the mean ± SEM of quantification of 4 experiments. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by paired Student *t* test at each time point.

As ATM inhibition increased p38 phosphorylation in response to LPS, we next examined whether ATM activity directly affects p38 activation. Phosphorylation of ATM and p38 was assayed by immunoblot in lysates of unstimulated and LPS-stimulated neutrophils which were treated with ATM inhibitor and/or γ -irradiation. Lysates were made 1 hour post-stimulation, when differences in p38 phosphorylation were first observed in Figure 4.16. As before, ATM inhibition increased p38 phosphorylation in LPS-stimulated neutrophils. However, ATM activation by irradiation did not affect p38 activation (Figure 4.17).

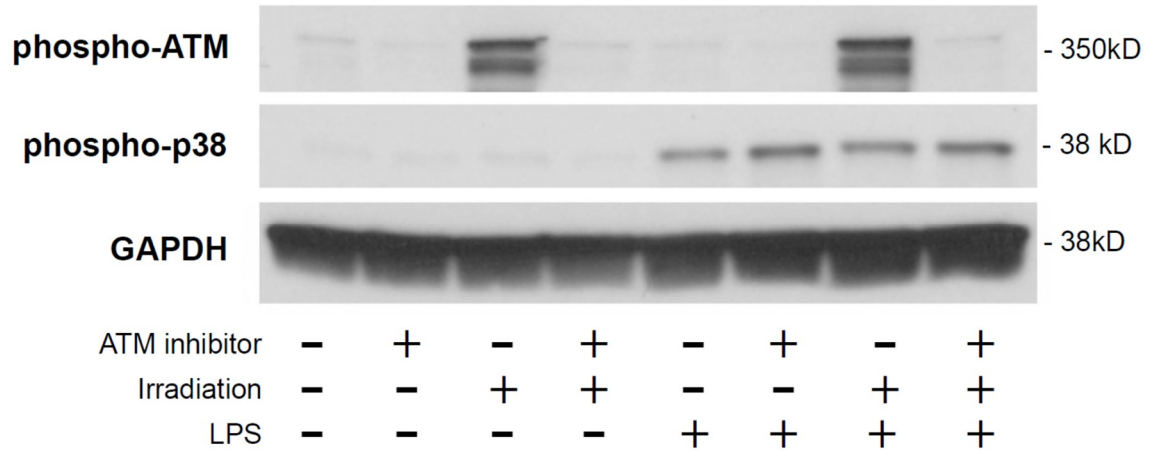


Figure 4.17: ATM activation by DNA damage does not induce p38 phosphorylation. (a) Immunoblot analysis of p38 and ATM phosphorylation in response to irradiation-induced DNA damage and LPS stimulation. Neutrophils were incubated with KU-60019, exposed to 10 Gy irradiation and then stimulated with LPS (100ng/ml). Whole cell lysates were made 60 minutes after LPS stimulation and analyzed by western blot with antibodies specific to phosphorylated pATM, p38, and loading control GAPDH.

4.3.4 Increased cytokine production upon ATM inhibition is dependent on MAP kinases

The contributions of MAP kinases p38 and ERK to the cytokine overproduction phenotype of ATM-inhibited cells were tested using well characterized small molecule inhibitors. Inhibition of ERK reduced the production of IL-8, while p38 inhibition almost completely abrogated IL-8 production (Figure 4.18a). Notably, the increase in IL-8 production upon ATM inhibition was reduced in the presence of ERK inhibitor, and completely reversed by the p38 inhibitor, suggesting that these MAP kinases are involved in the ATM-dependent regulation of IL-8 production. Similarly, inhibition of p38 or ERK decreased NET formation (Figure 4.18b). As PMA is such a strong NET-inducer, an increase in NET formation *in vitro* upon ATM inhibition could not be observed. However, these data indicate that p38 and ERK positively regulate NET formation, and support the hypothesis that increased p38 activation in ATM-deficient cells increases NET production.

Taken together, these data show that in the absence of ATM activity, stimulated neutrophils transcribe and produce increased levels of inflammatory cytokines independently of their prolonged lifespan. Additionally, they display increased and prolonged activation of MAP kinases, especially p38. Activity of this MAP kinase contributes to cytokine production and NET formation, and is involved in the overproduction of cytokines when ATM is inhibited.

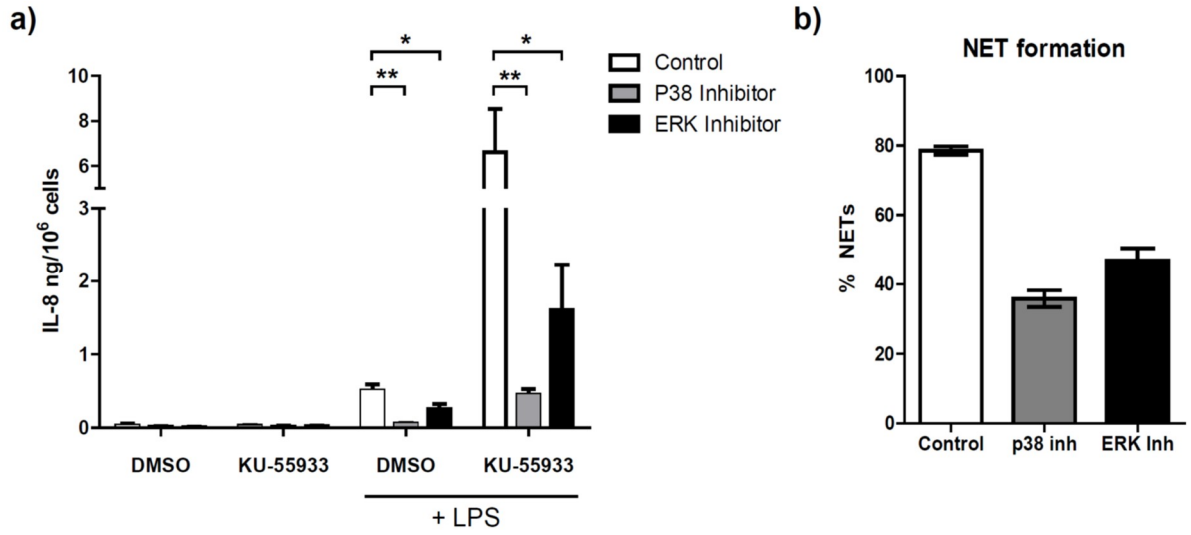


Figure 4.18: Overproduction of IL-8 in ATM-inhibited neutrophils is dependent on MAP kinases. (a) IL-8 production from LPS-stimulated neutrophils incubated with ATM inhibitor KU-55933 in combination with inhibitors of MAP kinases p38 (SB203580, 5μM) and ERK (PD98059, 5μM). IL-8 was measured in supernatants by ELISA. Data represent the mean ± SEM of 5 experiments. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by repeated measures ANOVA followed by Dunnett's multiple comparison test. **(b)** NET formation in PMA-stimulated neutrophils is decreased by inhibition of p38 and ERK. Neutrophils were fixed after 2 hours of PMA stimulation and NETs quantified by microscopy with sytox green DNA dye. At least 500 cells per condition were counted.

4.4 The DNA damage response negatively regulates neutrophil cytokine production

4.4.1 Inhibition of DNA damage signaling increases cytokine production.

The DDR is a complex, hierarchical signaling network involving multiple damage sensors, signal transducers, and effector proteins that initiate appropriate cellular responses (see Chapter 3 introduction). ATM and ATR are apical kinases in this response, which are activated by distinct forms of DNA damage. ATM efficiently phosphorylates and activates CHK2, while ATR activates CHK1, though some crosstalk and redundancy exists. The responses downstream of these kinases are distinct, but have some overlap and convergence on common effectors, including p53 (Figure 4.19a). We asked whether the modulation of neutrophil cytokine production and lifespan are directly mediated by ATM, or occur via other DNA damage sensors and their downstream effectors.

To address this question, a panel of well-characterized inhibitors of various key players in DNA damage signaling cascade was used. Neutrophil viability was measured after 7 hours of stimulation, and cytokine production after 18 hours while inhibiting ATM, ATR, CHK1, CHK2, or p53. Cytokine production was significantly increased when ATM, ATR, or CHK2 were inhibited (Figure 4.19b, red bars). Neutrophil viability was increased by inhibition of p53 and CHK2 (Figure 4.19b, black bars). These results indicate that members of the DDR acting downstream of ATM

and ATR may be the important effectors in regulating cytokine production and apoptosis, and suggest a role for CHK2 and p53.

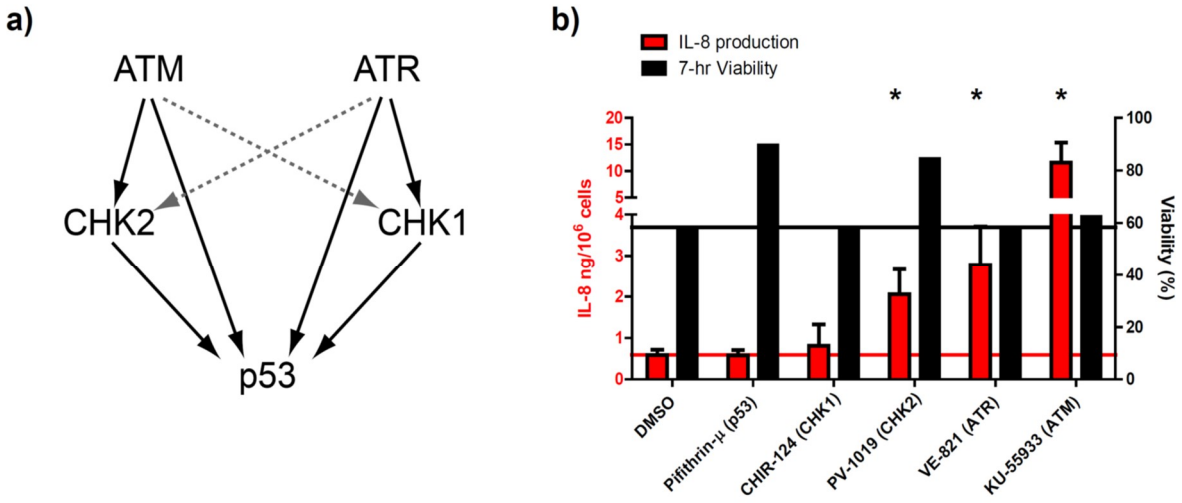


Figure 4.19: Inhibition of multiple members of the DDR increases neutrophil cytokine production and life span. (a) Schematic of DNA damage signaling cascade members. ATM activates CHK2, while ATR activates CHK1. Crosstalk between ATM-CHK1 and ATR-CHK2 can also occur (gray dashed lines). Each of these four kinases can phosphorylate and modulate p53. **(b)** IL-8 production (red bars, left y-axis) and viability (black bars, right axis) of LPS-stimulated neutrophils incubated with inhibitors of members of the DDR. IL-8 was measured in the supernatants of stimulated cells after 18 hours. Viability was measured at 7 hours post-stimulation by flow cytometry. Red and black lines show IL-8 production and viability values, respectively, of control neutrophils for reference. Neutrophils were incubated with inhibitors of p53 (pifithrin-μ, 1 μM), CHK1 (CHIR-124, 1 μM), Chk2 (PV1019, 10 μM), ATR (VE-821, 1 μM) or ATM (KU-59933, 10μM) and then stimulated with LPS (100ng/ml). Cytokine data are presented as the mean ± SEM of 5 experiments. Viability data were obtained from counting at least 10,000 cells in a representative experiment.

That inhibition of ATM or ATR, as well as their substrates CHK2 and p53, recapitulated phenotypes of cytokine production and apoptosis suggests that some redundancy and compensation in these pathways may exist. Inhibitors of ATM and ATR were used in combination to determine if simultaneous inhibition of both arms of the DDR can synergize their effects on cytokine production. Indeed, simultaneous inhibition of both kinases resulted in a further increase in IL-8 production (Figure 4.20). These data together indicate a role for CHK2, or a substrate of CHK2, in regulating cytokine production, as well as p53-mediated apoptosis. Additionally, ATM and ATR exhibit some redundancy in activation of these pathways.

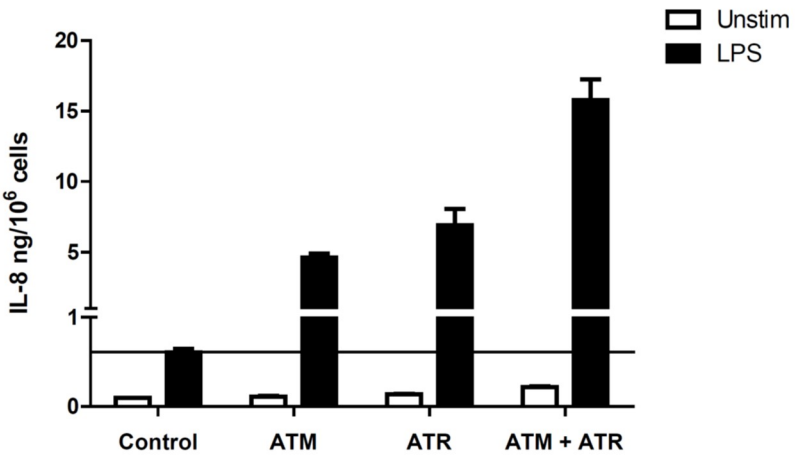


Figure 4.20: Inhibition of ATM and ATR synergistically increases cytokine production. Neutrophil IL-8 production in the presence of inhibitors of ATM (KU55933, 10 μM) and ATR (VE-821, 5 μM) alone and in combination. Cell-free supernatants were harvested after 18 hours and cytokines were measured by ELISA. Data are presented as the mean ± SD of triplicates from a representative experiment.

4.4.2 Exogenous induction of DNA damage dampens cytokine production

Our findings that inhibition of DNA damage signaling results in increased cytokine production and NET formation, as well as delayed apoptosis suggest that the DDR negatively regulates neutrophil activation and lifespan. We hypothesized that exogenous induction of DNA damage and activation of ATM would suppress cytokine production, promote apoptosis and potentially decrease NET formation. To address this, several exogenous DNA-damaging agents were used as tools to activate the DDR. Ionizing γ-irradiation activates ATM by directly inducing DNA double strand breaks. Etoposide is a topoisomerase-II poison, inhibiting its ability to re-ligate DNA after inducing DNA double strand nicks. Cisplatin activates the DDR by catalyzing the formation of DNA-crosslinks and adducts, leading to transcriptional and replicational stress.

Treatment of neutrophils with these compounds activated DNA damage signaling, as evidenced by the phosphorylation of ATM and histone H2A.X (Figure 4.21a). Neutrophils were treated with various concentrations of DNA damaging agents, then stimulated with LPS to induce cytokine production. DNA damage-induction reduced the amount of cytokines produced by neutrophils in a dose-dependent manner (Figure 4.21b). Interestingly, etoposide and ionizing radiation exerted an effect on cytokine production without affecting neutrophil viability (Figure 4.21c). Treatment with cisplatin, however, also increased rates of apoptosis. We additionally confirmed that the decrease in cytokine production in response to ionizing radiation was dependent on ATM. Irradiation caused a dose-dependent decrease in cytokine production from control cells, but had no effect on ATM-inhibited neutrophils (Figure 4.21d).

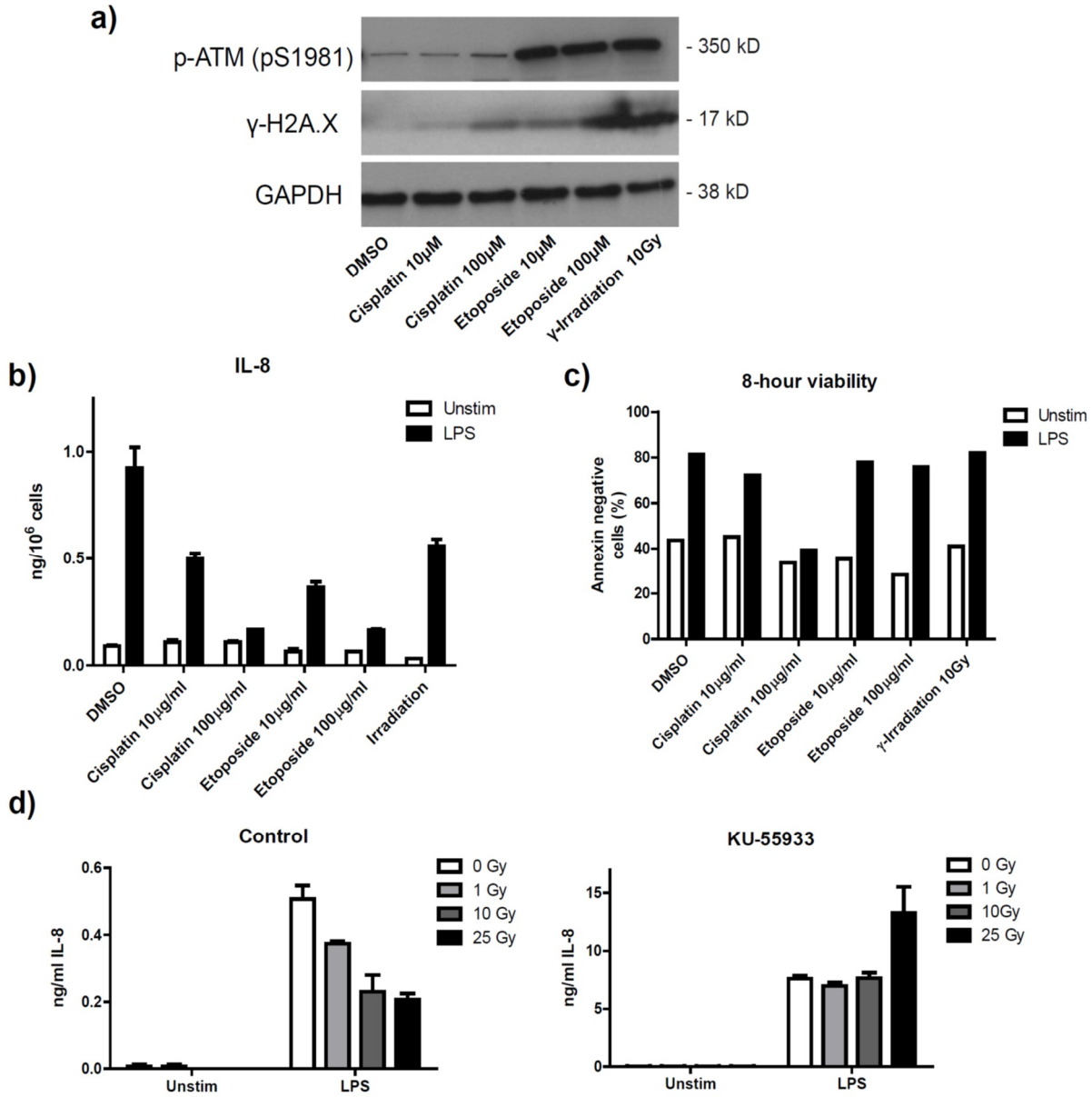


Figure 4.21: Activation of the DDR suppresses neutrophil cytokine production. (a) Immunoblot of phospho-ATM and γ-H2A.X in response to treatment with cisplatin, etoposide, and ionizing radiation. Neutrophils were treated with 10 or 100μM cisplatin and etoposide, or exposed to 10 Gy ionizing radiation. Whole cell lysates were made after 2 hours and analyzed by western blot. **(b)** 18 hour IL-8 production of neutrophils after DNA damage induction. Neutrophils were treated with indicated doses of cisplatin, etoposide or ionizing radiation for one hour, and then stimulated with LPS (100ng/ml). Data are presented as the mean ± SD of technical replicates from a representative of 3 experiments. **(c)** Neutrophil viability 8 hours post-stimulation measured by annex V/PI staining and flow cytometry. 10000 cells per condition were analyzed. **(d)** IL-8 production from neutrophils exposed to increasing doses of ionizing radiation with and without ATM inhibitor (KU-55933, 10μM). Neutrophils were treated with ATM inhibitor or vehicle control, then exposed to indicated doses of irradiation before stimulation with LPS (100 ng/ml). IL-8 was measured in cell-free supernatants by ELISA. Data represent the mean ± SD of technical replicates of a single experiment.

Induction of NET formation, however, was largely unaffected by DNA damage treatments. High doses of irradiation or etoposide treatment did not significantly decrease NET formation in response to PMA (Figure 4.22).

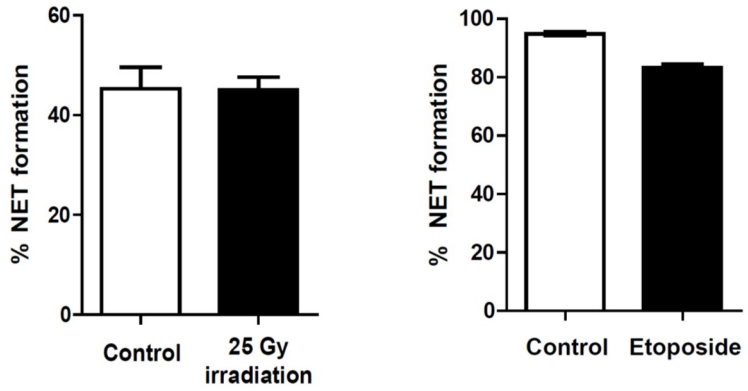


Figure 4.22: DNA damage induction does not suppress PMA NET formation. PMA-stimulated NET formation in neutrophils treated with etoposide or ionizing radiation. Neutrophils were exposed to 25 Gy irradiation or one hour of treatment with 100μM etoposide before stimulation with PMA to form NETs. Percent NET formation was quantified by sytox green staining and counting at least 500 cells in fluorescent micrographs.

These results show that activation of DNA damage signaling can negatively regulate neutrophil production of cytokines. Additionally, this regulation can occur independently of apoptosis induction, as in the case of etoposide and exposure to ionizing radiation.

CHAPTER 5: NEUTROPHIL OXIDATIVE BURST ACTIVATES ATM TO REGULATE NEUTROPHIL FUNCTIONS

5.1 Activation of the DDR is dependent on the oxidative burst

We have shown that DNA damage signaling is activated in stimulated neutrophils, and that this response regulates MAP kinase signaling, cytokine production, and apoptosis. The relevant mechanism responsible for inducing DNA damage and ATM activation, however, remained to be identified. One prime candidate was the oxidative burst. This is one of the hallmarks of neutrophil activation, generating large amounts of ROS via the NADPH oxidase complex. These ROS function simultaneously as antimicrobial chemicals and as signaling molecules known to play a role in regulating neutrophil functions. These volatile compounds indiscriminately oxidize molecules with which they come in contact, and have been shown in other contexts to be able to damage DNA as well as directly activate ATM by oxidation[126].

5.1.1 ROS scavenger pyrocatechol neutralizes the oxidative burst

Each of the stimuli used to activate neutrophils in our previous experiments induced an oxidative burst (Figure 5.1). To test the dependence of ATM activation on ROS production, a general ROS scavenger, pyrocatechol, was used. A titration was performed to determine the lowest concentration at which the oxidative burst was completely neutralized. Incubation with pyrocatechol decreased ROS production in a dose dependent manner (Figure 5.2a); 30μM was found to be the lowest dose at which ROS production was neutralized to background levels, and was then used in following experiments.

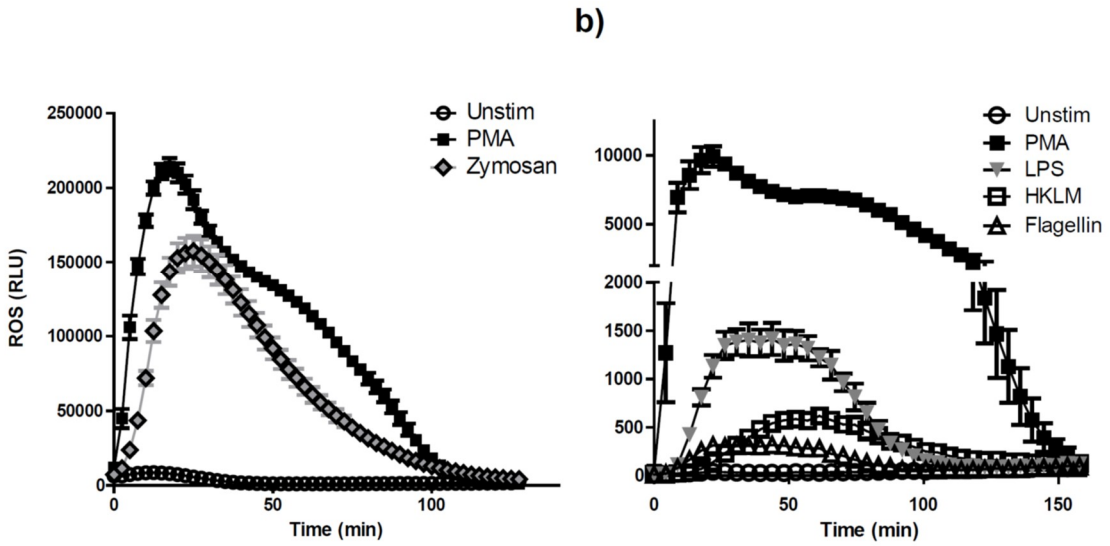


Figure 5.1: Neutrophil ROS production in response to various stimuli. (a) Neutrophils were incubated with luminol and horseradish peroxidase and stimulated with 50 nM PMA, 10 µg/ml opsonized zymosan, or **(b)** 100 ng/ml LPS, MOI 100 heat killed *Listeria monocytogenes*, and 1 µg/ml flagellin to stimulate the oxidative burst. ROS production was measured kinetically by luminescence in relative light units (RLU) in a luminometer.

5.1.2 ROS scavenging abrogates DDR activation in stimulated neutrophils

We then asked whether neutralizing the oxidative burst affects ATM activation. Neutrophils were incubated with pyrocatechol to scavenge ROS and stimulated with PMA for immunofluorescence analysis of ATM phosphorylation. After stimulation with PMA, phospho-ATM strongly accumulated in the nucleus of control cells, but was decreased in ROS-scavenged cells (Figure 5.2b). Similarly, phosphorylation of ATM, as well as some of its downstream substrates, γ-H2A.X and BRCA-1, were assayed by immunoblot. In neutrophils stimulated with PMA and opsonized zymosan, robust phosphorylation of these DNA damage proteins was observed, but was completely abrogated by the addition of pyrocatechol (Figure 5.2c). Importantly, irradiation, which damages DNA independently of the oxidative burst, still successfully induced phosphorylation of these proteins in both control and ROS-scavenged cells, showing that the DDR was not inhibited by pyrocatechol.

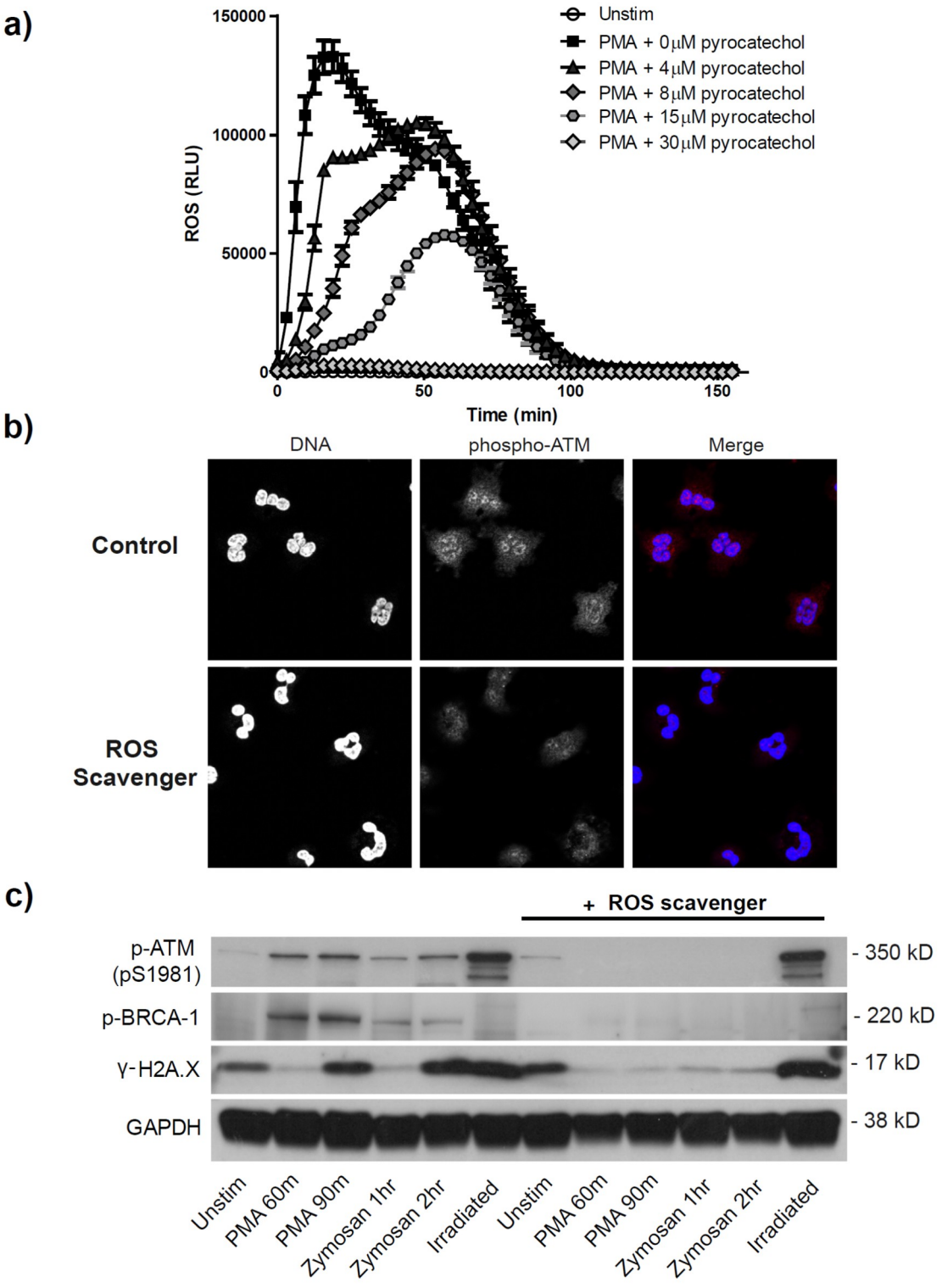


Figure 5.2: ROS scavenging abrogates activation of the DDR in stimulated neutrophils. (a) Determination of pyrocatechol dose for complete neutralization of the oxidative burst. Neutrophils were incubated with luminol, horseradish peroxidase, and pyrocatechol, and then stimulated with 50 nM PMA to induce an oxidative burst. ROS production was measured kinetically in a luminometer. **(b)** Immunofluorescence staining of pATM (red) and DNA (blue) in control and ROS-scavenged neutrophils stimulated with PMA. Neutrophils were treated with 30μM pyrocatechol or vehicle control, stimulated for 60 minutes with PMA, then fixed and stained with anti-pATM antibody and Hoechst DNA dye for immunofluorescence microscopy. **(c)** Immunoblot analysis of phosphorylation of ATM, BRCA-1, and γ-H2A.X and GAPDH. Neutrophil whole cell lysates were made at indicated time points after stimulation with PMA (50 nM), opsonized zymosan (10 μg/ml) or 5 Gy ionizing radiation in the presence of 30μM pyrocatechol, and then analyzed by western blot with indicated antibodies.

5.1.3 Neutrophil oxidative burst is required for ATM activation

Pyrocatechol is a general scavenger of ROS, and is not specific for ROS production by NADPH oxidases. In addition to the oxidative burst, ROS are produced at basal levels as byproducts of metabolic reactions and mitochondrial respiration. In order to conclusively pin down the source of ROS activating ATM, the ability of neutrophils from patients with chronic granulomatous disease (CGD) to activate ATM was tested. CGD is a genetic disease caused by mutations in components of the NADPH oxidases, resulting in neutrophils unable to mount an oxidative burst.

ROS production was measured in stimulated neutrophils isolated from a healthy control and a CGD patient to confirm that the CGD patient was completely deficient in the oxidative burst (Figure 5.3a). The phosphorylation of ATM was then assessed in stimulated neutrophils by western blot. Phospho-ATM was detected in activated neutrophils from healthy controls, but was markedly decreased in CGD neutrophils, indicating that NADPH oxidase-derived ROS are the salient activators of DNA damage signaling in stimulated neutrophils (Figure 5.3b).

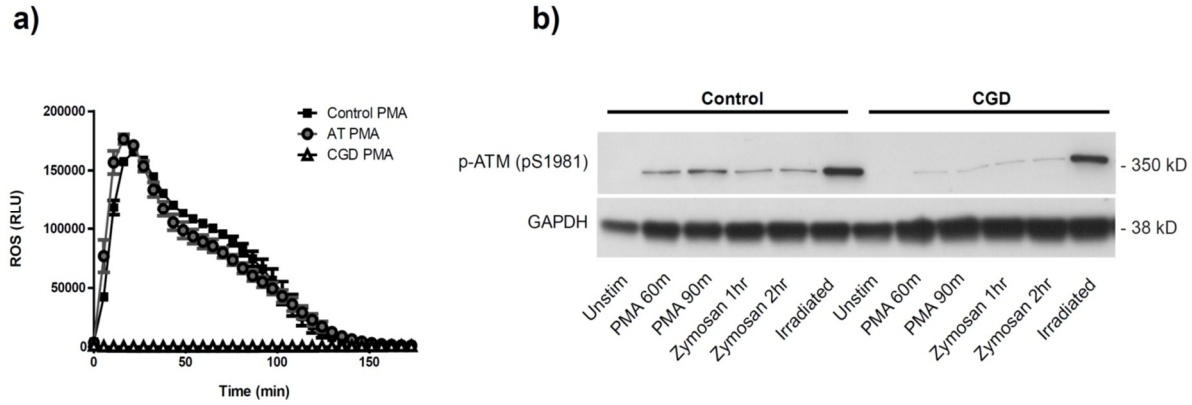


Figure 5.3: Neutrophils from Chronic Granulomatous Disease patients fail to mount an oxidative burst and activate ATM. (a) ROS production by neutrophils from a CGD patient, an AT patient, and a healthy control stimulated with PMA. Neutrophils were isolated concurrently from patients and a healthy control, incubated with luminol and horseradish peroxidase, and stimulated with PMA (50 nM). ROS production was measured kinetically in a luminometer. **(b)** Western blot analysis of pATM in whole cell lysates of stimulated neutrophils from CGD patient and healthy control. Isolated neutrophils were stimulated for indicated time points with PMA (50 nM) or opsonized zymosan (10 µg/ml), or exposed to 5 Gy ionizing radiation and allowed to recover for one hour. Whole cell lysates were made and analyzed by western blot with antibodies against pATM and GAPDH.

5.2 DNA damage operates downstream of the oxidative burst to modulate cytokine production

The discovery that the oxidative burst activates ATM suggests a potential link between CGD and AT. Interestingly, the phenotype observed in ATM-deficient neutrophils, increased cytokine production and delayed apoptosis, has previously been described for CGD neutrophils.

Indeed, the hyperinflammatory pathology of CGD is ascribed to the dysregulation of these neutrophil functions caused by the deficient oxidative burst[149]. Based on the similar reported neutrophil phenotype observed in ROS or ATM deficiency, we hypothesized that they are involved in the same pathway regulating these functions.

To support this model, cytokine production was measured from neutrophils isolated concurrently from a CGD patient and an AT patient. AT neutrophils produced normal levels of ROS when stimulated, while CGD neutrophils did not (Figure 5.3a), however, both produced more IL-8 than a healthy control (Figure 5.4a). Corroborating these results, neutrophils from a healthy donor treated with pyrocatechol to scavenge ROS, or with ATM inhibitor both resulted in increased cytokine production compared to control cells (Figure 5.4b).

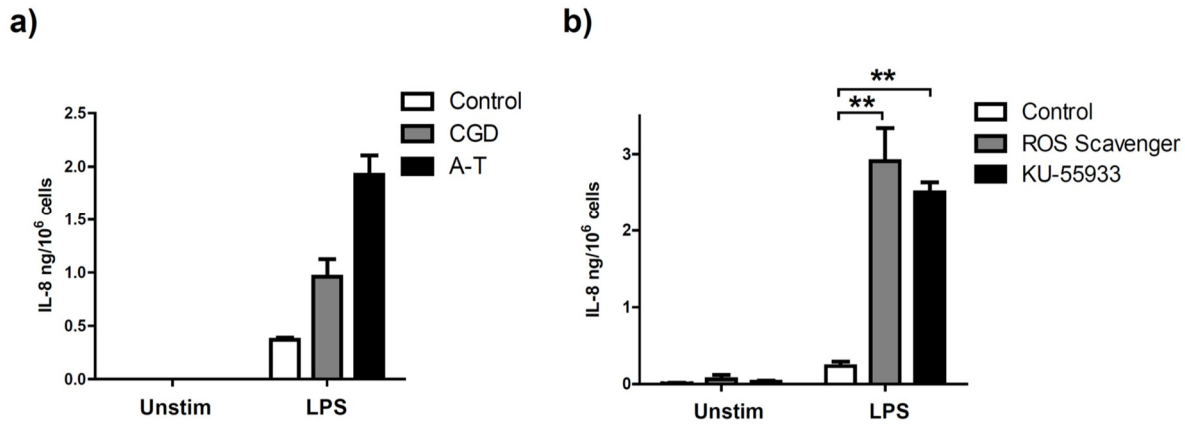


Figure 5.4: Deficient ROS production or ATM activity similarly result in overproduction of IL-8. (a) IL-8 production from neutrophils isolated from a healthy control, a patient with CGD, and a patient with AT. Neutrophils were isolated concurrently from patients and a control and stimulated for 18 hours with LPS (100 ng/ml). IL-8 was measured in supernatants by ELISA. Data are presented as mean ± SD of technical replicates of a single experiment. **(b)** IL-8 production from neutrophils isolated from a healthy donor and incubated with ROS scavenger or ATM inhibitor. Isolated neutrophils were incubated with pyrocatechol (30 μM), KU-55933 (10 μM), or vehicle control, and stimulated 18 hours with LPS (100 ng/ml). Data are presented as mean ± SEM of results from 3 experiments. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by Repeated Measures ANOVA with Bonferroni multiple comparison test.

As we have described a ROS-dependent activation of ATM in stimulated neutrophils, and an identical phenotype in ROS- or ATM-deficient cells, we hypothesized that ATM is one of the downstream substrates of the oxidative burst that are important for regulating neutrophil cytokine production. This model was tested by asking if the cytokine overproduction phenotype of ROS-scavenged neutrophils could be countered by exogenously inducing DNA damage, thus restoring activation of ATM and the DDR. As before, neutrophils incubated with pyrocatechol overproduced IL-8 when stimulated; however, when DNA damage was induced by adding etoposide, the cytokine production from ROS-scavenged cells was markedly reduced (Figure 5.5). These results show that the activation of ATM and DNA damage signaling occurs via ROS

produced by the oxidative burst, and is involved in the mechanism of ROS-dependent regulation of cytokine production and apoptosis.

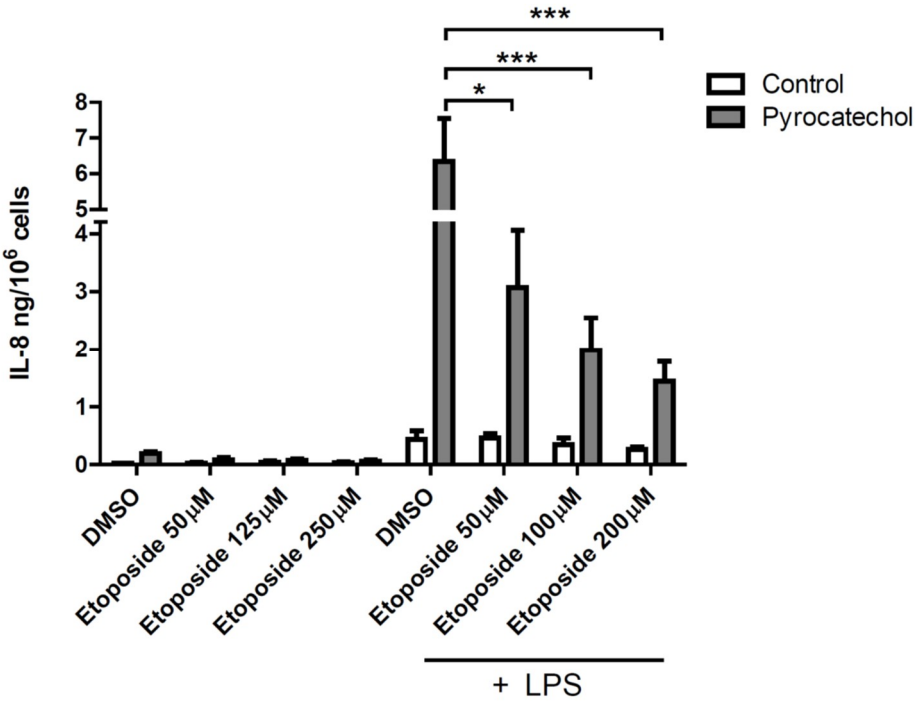


Figure 5.5: Exogenous induction of DNA damage complements overproduction of IL-8 in ROS-scavenged neutrophils. Isolated neutrophils were incubated with pyrocatechol (30 μM) and indicated concentrations of etoposide, and then stimulated with LPS (100 ng/ml). IL-8 production was measured in supernatants after 18 hours by ELISA. Data are presented as mean ± SEM of results from 3 experiments. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by Repeated Measures ANOVA with Bonferroni multiple comparison test.

CHAPTER 6: A MOUSE MODEL OF PHAGOCYTE-SPECIFIC DDR DEFICIENCY

Our data indicate strong similarities between the hyperinflammatory phenotype of CGD (ROS-deficient) neutrophils and AT (ATM-deficient) neutrophil responses to stimulation *in vitro*. This phenotype in neutrophils and macrophages is thought to be the driving force behind the chronic inflammation observed in CGD patients. We, therefore, hypothesized that hyperinflammatory responses may also occur in AT patients. We set out to determine if these results could be translated into a mouse model of DDR deficiency in order to determine the relevance of these results *in vivo*.

6.1 Residual Atm protein and activity is present in neutrophils from mice carrying the *Atm*^{tm1Awb} mutant allele

A mouse line harboring the *Atm*^{tm1Awb} mutant allele of *Atm* (the mouse homologue of ATM) was obtained for experiments. This mutation was made by insertion of a neomycin cassette into exon 38 of *Atm*, introducing a premature stop codon[150]. The truncated protein that should result from this mutation corresponds to a described mutation which creates an unstable protein that has been described in multiple AT patients. To determine if Atm and the DDR similarly play an important role in regulating myeloid cell responses in murine cells as shown in human cells, experiments were performed with neutrophils and macrophages isolated from these mice.

It was first tested whether *Atm*$^{-/-}$ mouse neutrophils and macrophages recapitulate the phenotype observed in human neutrophils. Peritoneal-elicited neutrophils isolated from *Atm* mutant mice and littermate wildtype controls were stimulated overnight with LPS, PMA, opsonized zymosan, or fungal pathogen *Candida albicans*. Bone marrow-derived macrophages were differentiated *in vitro*, then stimulated overnight with LPS. The production of multiple pro-inflammatory cytokines was measured, including KC, the mouse homologue of IL-8. No significant differences in cytokine production were observed in with *Atm*$^{-/-}$ neutrophils (Figure 6.1) or macrophages (Figure 6.2) in response to stimulation.

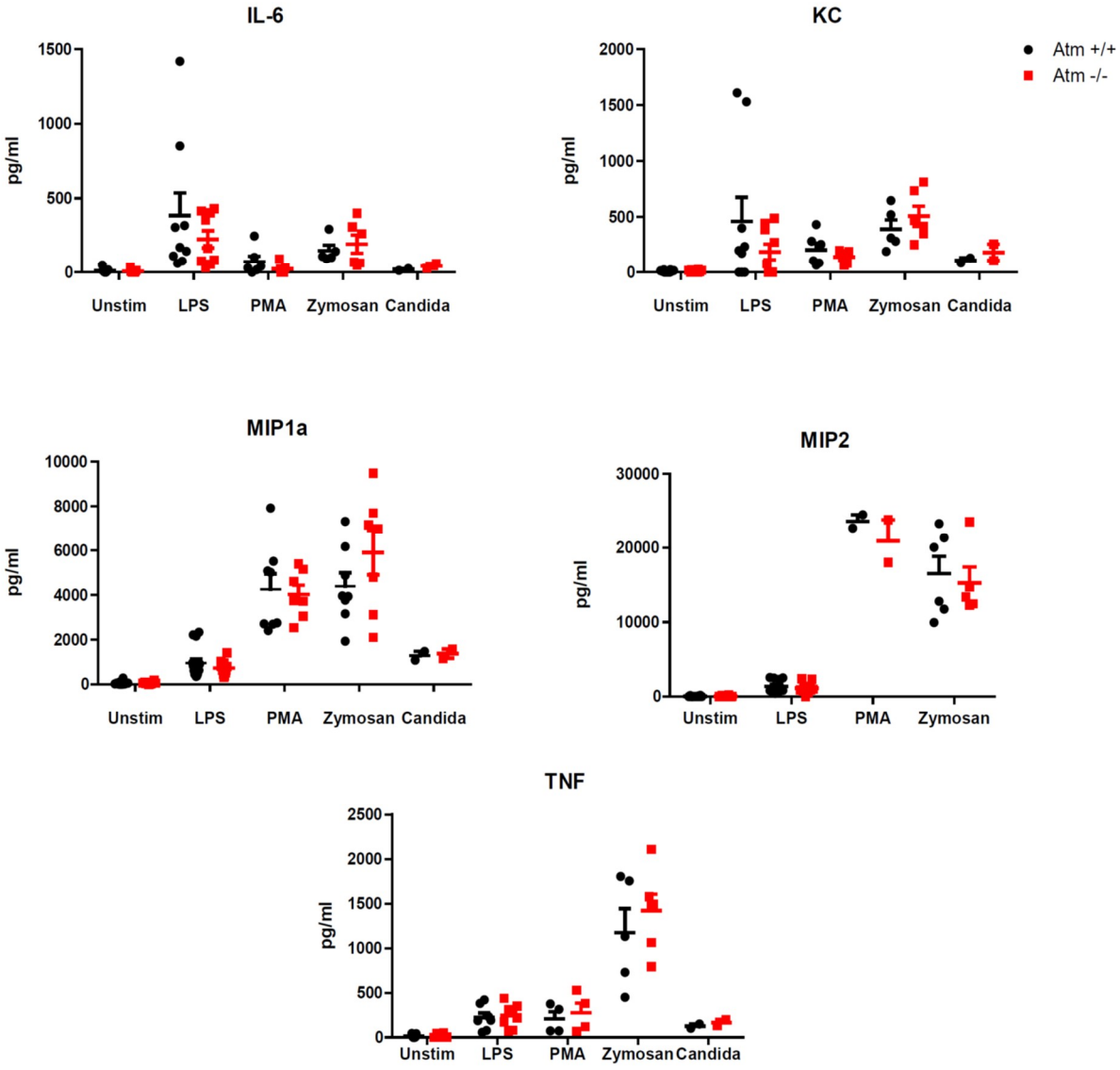


Figure 6.1: Cytokine production from peritoneal elicited neutrophils from *Atm*^{tm1Awb} mice. Neutrophils were elicited into the peritoneal cavity by sterile casein injection, then isolated by density centrifugation and stimulated overnight with LPS (200ng/ml), PMA (50nM), opsonized zymosan (10 µg/ml) or *C. albicans* (MOI 1). Supernatants were collected after 18 hours and used for cytokine measurements by ELISA or Bioplex analysis. Data shown are pooled from 2-4 experiments with 1-3 mice per genotype per experiment. Each dot represents mean cytokine production from a single mouse measured by 3 technical replicates. Lines indicate the average ± SEM of all mice per condition. No significant differences by Mann-Whitney U test.

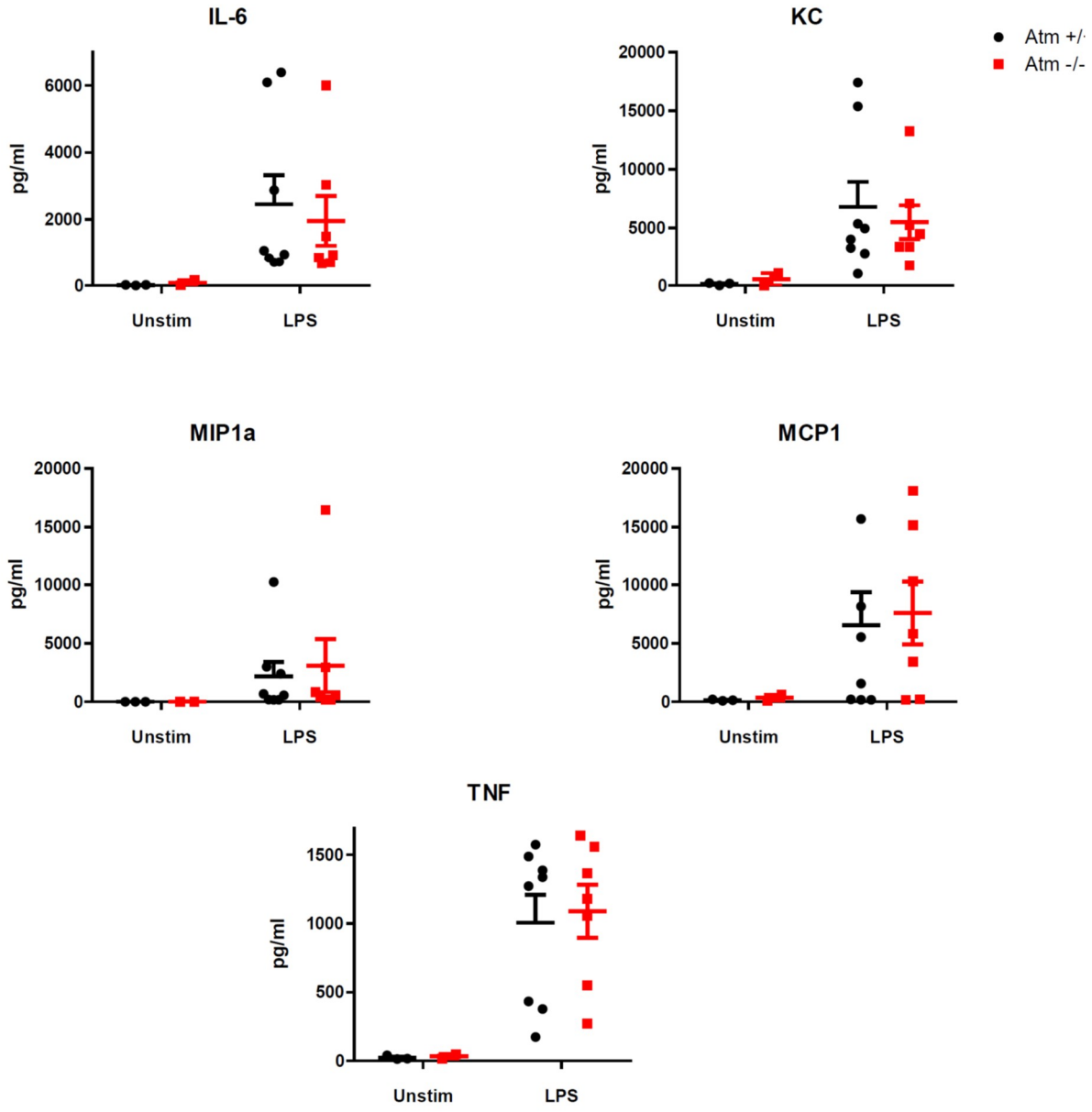


Figure 6.2: Cytokine production from bone marrow-derived macrophages from *Atm*^{tm1Awb} mice. Total bone marrow cells were differentiated into macrophages *in vitro* and then stimulated 18 hours with LPS (200ng/ml). Cytokines were measured in supernatants by ELISA or by Bioplex analysis. Data shown are pooled from 3 experiments with macrophages from 1-3 mice per genotype per experiment. Each dot represents mean cytokine production from a single mouse measured by 3 technical replicates. Lines indicate the average ± SEM of all mice per condition. No significant differences by Mann-Whitney U test.

To explain this discrepancy with the human neutrophil data, Atm levels were assayed in purified *Atm*$^{-/-}$ mouse neutrophils. Although no Atm mRNA or protein is detectable in peripheral tissues of *Atm*^{tm1Awb} mice, in some tissues a novel mRNA has been found which has successfully spliced out the inserted cassette, and is translated into a stable, functional ATM protein[151]. This may explain why this mouse model of AT does not recapitulate many aspects of the human disease. Neutrophils from mice carrying this mutation were found to have residual levels of ATM, which were detectable by western blot. This residual protein level also retained activity, as it was

successfully phosphorylated in response to γ-irradiation-induced DNA damage (Figure 6.3). This residual level of Atm in neutrophils may be sufficient for cytokine regulation and explain why no phenotype was observed.

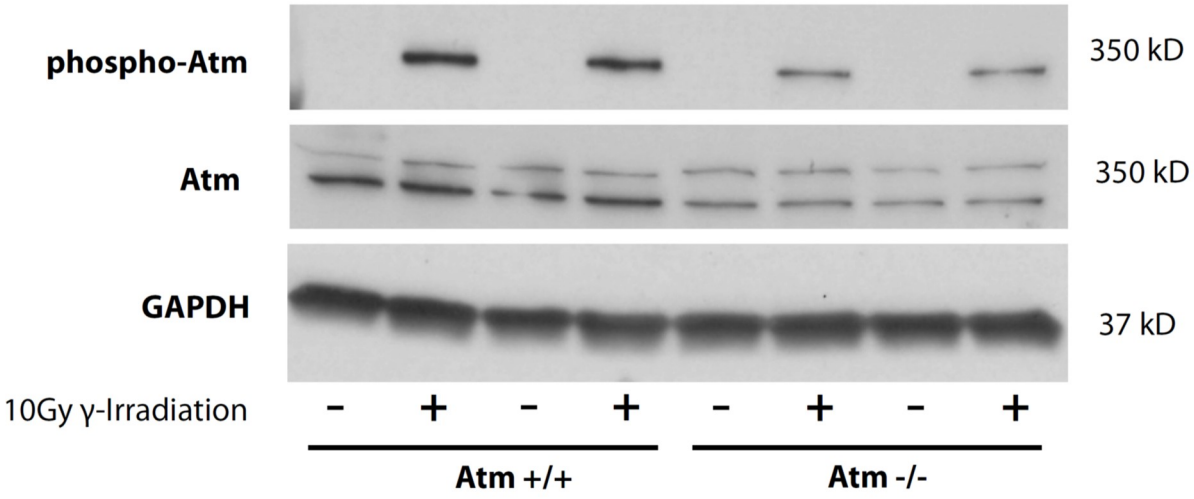


Figure 6.3: *Atm*^{tm1Awb} mouse neutrophils maintain residual levels of functional Atm. Immunoblot analysis of whole- and phospho-Atm levels in mouse peritoneal elicited neutrophils in response to ionizing radiation. Isolated peritoneal neutrophils were exposed to 10 Gy ionizing irradiation, allowed to recover for one hour, and then used for whole cell lysates. Lysates were analyzed by western blot with antibodies specific to indicated proteins.

6.2 Creation of a phagocyte-specific Nbs1-deficient mouse model

Multiple characteristics of mouse models of Atm deficiency make them problematic for studying the role of the DNA damage signaling in myeloid cells in vivo. Residual Atm protein and activity in neutrophils from *Atm*^{tm1Awb} mice make them unsuitable for studying the effects of Atm deficiency in the innate immune system. Additionally, somatic mutations of *Atm* cause many different pleiotropic phenotypes in the whole organism, which confounds interpretation of *in vivo* models of infection and inflammation. Simultaneous inhibition of ATM and ATR in human neutrophils resulted in increased cytokine production (Figure 4.19), indicating that deficiency in one of these kinases may be at least partially compensated for by the other. To resolve these two issues, the Cre-Lox recombinase system was used to create a deletion of *Nbs1* (the mouse homologue of NBS1) specifically in neutrophils and macrophages. Nbs1 is an important member of the MRN DNA damage-sensing complex, which recruits Atm as well as Atr to sites of DNA damage, resulting in their activation by phosphorylation (see Figure 3.1). Thus, Nbs1-deficient cells would have impaired activation of both Atm- and Atr-mediated DNA damage signaling. This model also provides a tool to discern Atm activation by DNA damage from direct activation by oxidation. Nbs1-deficient cells have normal levels of Atm that are expected to be activated normally by ROS, but will not be activated by ROS-induced DNA damage, which requires Nbs1.

To generate a mouse with a myeloid-specific deletion of *Nbs1*, three separate mouse lines were interbred: ***Nbs1*⁻:** an *Nbs1* null allele with deletion of exon 6, leading to an unstable protein. Mice with this allele breed heterozygous, as the homozygous mutant is embryonic lethal[152]. ***Nbs1*^{flox}:** an *Nbs1* allele containing *loxP* sites flanking exon 6. This allele results in fully functionally Nbs1 protein, but when Cre recombinase is expressed, exon 6 is excised, resulting in a null mutation similar to *Nbs1*⁻ [152]. ***LysM-Cre*:** a targeted insertion of *Cre* recombinase cDNA into the endogenous *Lysozyme M* locus, resulting in Cre expression exclusively in neutrophils and macrophages[153].

Mice were bred in such a way that experimental animals and various littermate controls would be generated (Figure 6.4a). The experimental myeloid-specific *Nbs1* knockout mice created by this system have the genotype ***Nbs1*$^{-/flox}$ *LysM-Cre*$^{+}$**, harboring one *Nbs1* null allele, one *Nbs1* floxed allele, and one copy of *Cre* under the LysM promoter (Figure 6.4b). Because the effects of DNA damage are being studied, and Cre expression can induce background DNA damage even in the absence of floxed alleles[154], the proper control animals should also express Cre. The relevant control in this case are animals of the genotype ***Nbs1*$^{+/flox}$ *LysM-Cre*$^{+}$**, which contain one wildtype and one floxed *Nbs1* allele, and one copy of *Cre* under the *LysM* promoter. Neutrophils and macrophages of these animals experience the same levels of DNA damage as the experimental genotype, as they will also express Cre and have one floxed gene excised, but will still express functional Nbs1 protein from the wildtype allele.

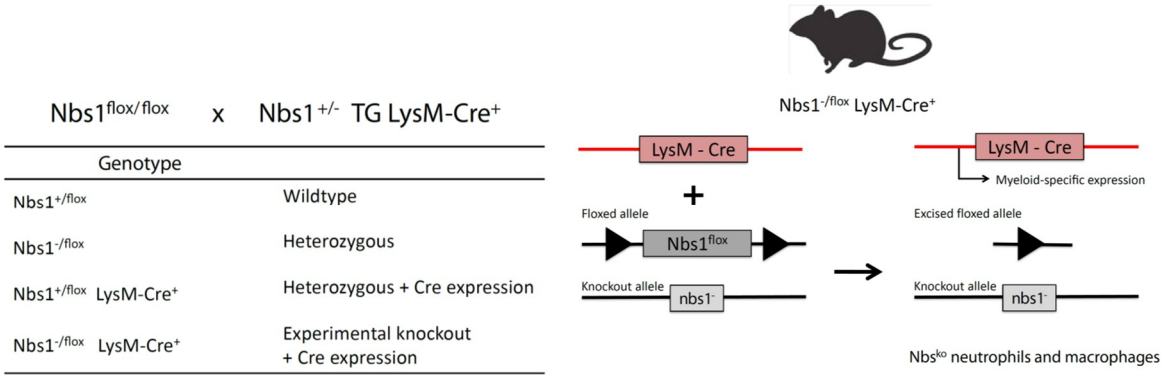


Figure 6.4: Breeding scheme for myeloid-specific Nbs1 knockout mice and resulting genotypes. (a) Breeding *Nbs1*$^{flox/flox}$ with *Nbs*$^{+/-}$ *LysM-Cre* mice results in four genotypes, including the experimental *Nbs1*$^{-/flox}$ *LysM-Cre* genotype, which results in Nbs1-deficient myeloid cells. **(b)** Genotype of experimental animals. Expression of Cre recombinase in myeloid cells excises the *Nbs1*^{flox} allele, resulting in Nbs1-deficient cells.

6.3 Nbs1-deficient macrophages overproduce cytokines

Bone marrow cells from each of these genotypes successfully differentiated into viable macrophages with normal morphology *in vitro*, indicating that innate immune cells can successfully develop in the absence of Nbs1. To examine the efficiency of removal of the *Nbs1*^{flox} allele and the ability of the different genotypes to activate Atm in response to DNA damage, lysates were made from bone marrow-derived macrophages exposed to ionizing radiation and analyzed by immunoblot. An efficient reduction of Nbs1 protein as well as Atm phosphorylation in response to DNA damage was observed in macrophages from experimental knockout animals (Figure 6.5). Interestingly, Nbs1-deficient macrophages also produced significantly increased levels of multiple inflammatory cytokines when stimulated with LPS (Figure 6.6). These results confirm that DNA damage signaling modulates cytokine production in murine innate immune cells, similar to what we have reported in human neutrophils. Additionally, it suggests that the activation of Atm and its substrates by DNA damage, as opposed to direct oxidation by ROS, is a relevant mechanism for this regulation.

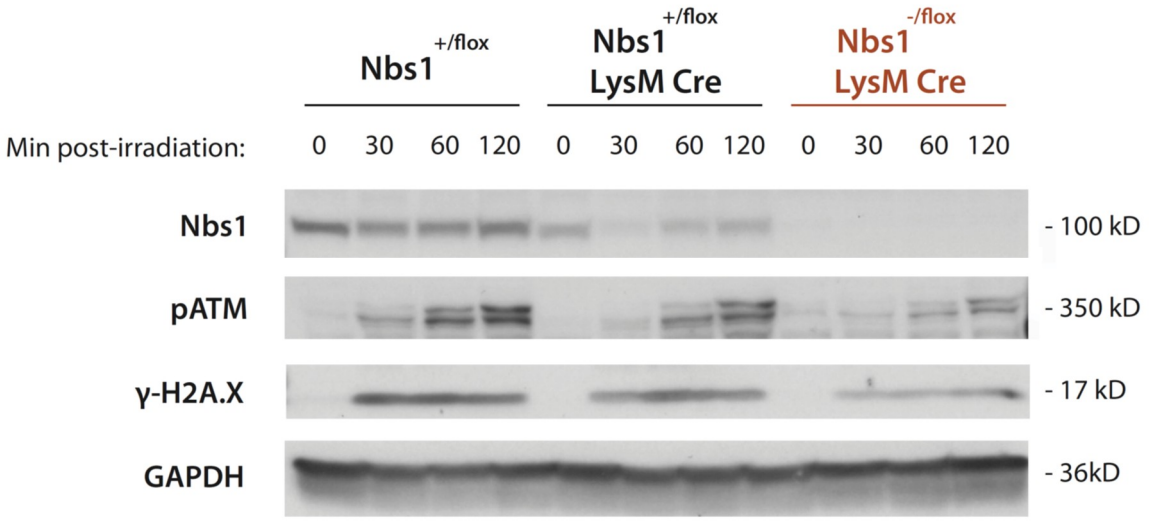


Figure 6.5: Immunoblot analysis of Nbs1, phospho-Atm, and γ-H2A.X in Nbs1-deficient macrophages. Bone marrow-derived macrophages were differentiated from mice of indicated genotypes. Mature macrophages were exposed to 5 Gy γ-irradiation, and whole cell lysates were made at indicated time points after irradiation. Lysates were analyzed by western blot for Nbs1, phospho-Atm, γ-H2A.X, and loading control GAPDH.

These data show that a myeloid-specific knockout of Nbs1 is more suitable than the Atm^{tm1Awb} mouse model for studies of the effects of the DDR on myeloid cell-derived inflammation. These mice have no observed developmental or growth defects, in contrast to the somatic *Atm* or *Nbs1* mutant mice. As such, this conditionally Nbs1-deficient mouse model is well suited for *in vivo* studies of immune responses during infection. The specificity of the Nbs1-deletion, limited

to cells of the innate immune system, will allow any discovered phenotype to be attributed to the contribution of DNA damage signaling in neutrophils and macrophages.

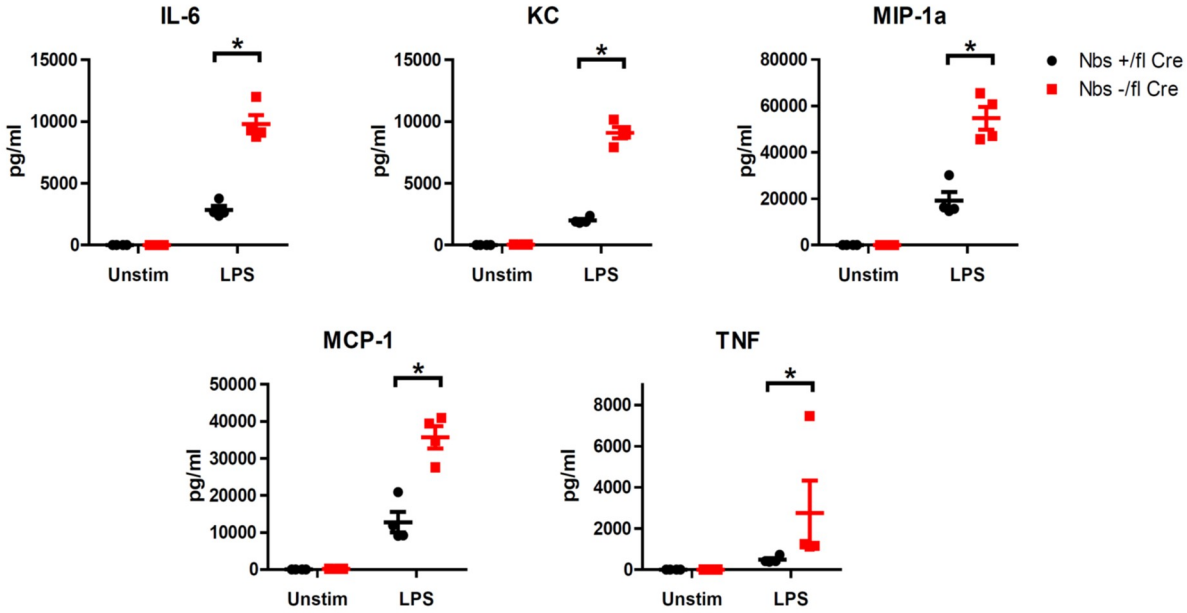


Figure 6.6: Nbs1-deficient macrophages overproduce inflammatory cytokines. Total bone marrow cells were differentiated into macrophages *in vitro* and then stimulated 18 hours with LPS (200ng/ml). Cytokines were measured in supernatants by ELISA or Bioplex analysis. Data shown are pooled from experiments with macrophages from 4 mice per genotype. Dots represent the mean of triplicate technical replicates from single mice. Lines indicate the average ± SEM of all mice per condition. Asterisks indicate significant increases: *P<.05, **P<.01, ***P<.001 by Mann-Whitney *U* test.

DISCUSSION

CHAPTER 7: DISCUSSION

Prior to this study it was known that ROS are important regulators of neutrophil functions and that deficiency in the oxidative burst results in immunodeficiency as well as hyperinflammatory neutrophil responses, which drive disease. The mechanism and downstream substrates of ROS-mediated regulation of inflammation remain incompletely understood. We identified ATM and the DDR as downstream mediators regulating cytokine production, NET formation, and apoptosis in a ROS-dependent manner. Though the exact mechanisms remain elusive, this work shows that alterations in MAP kinase activation are involved. Deficiency in DNA damage signaling increases production of pro-inflammatory cytokines and NETs, and delays apoptosis in neutrophils. A similar cellular phenotype is observed in CGD, where it drives a pathological inflammatory phenotype, and suggests that a similar inflammatory predisposition may exist in AT. Underlying chronic inflammation may exacerbate symptoms of the disease, as well as provide a potential explanation for the etiology of multiple symptoms that are prevalent in AT but which are not completely understood in the context of DDR deficiency. These results suggest that inflammation may be a potential treatment target that could improve some of the symptoms of this so far untreatable disease.

7.1 ATM and the DDR regulate neutrophil functions

7.1.1 Functional ATM is expressed in neutrophils and activated upon stimulation

We examined whether neutrophils express functional ATM and other DDR proteins. This is an interesting biological question, as neutrophils are terminally differentiated, non-dividing cells, and they likely do not have a long enough life span to accumulate mutations that may hamper their functions. We found that neutrophils express full length ATM, which becomes phosphorylated in response to DNA damage, and that addition of a specific small molecule ATM inhibitor prevents this phosphorylation. Phosphorylation of histone H2A.X and BRCA1 were also observed, indicating that neutrophils maintain a functional DDR. Furthermore, these DDR proteins were phosphorylated in neutrophils upon activation with various stimuli, including LPS, zymosan, and PMA.

A previous report[145] found that neutrophils do not express multiple DDR proteins; however, expression was measured only at the mRNA level. The source of this discrepancy is likely that naïve neutrophils are transcriptionally rather inactive until they are stimulated; their arsenal of proteins are transcribed and translated during their development and are generally

sufficient for their short lifespan without continued transcription in mature cells[14]. For instance, myeloperoxidase would also be overlooked if one only measured mRNA in mature neutrophils, although it is the most abundant protein in these cells.[155]

7.1.2 Neutrophil preparation purity

ATM and other DDR proteins detected derive from neutrophils, as our isolation method by two density separation steps results in a highly pure neutrophil population, >95% purity, with essentially no contamination of monocytes. This is imperative for cytokine assays, where small contaminating populations have led to misinterpretations[156]. Monocytes can secrete several fold higher levels of cytokines than neutrophils on a per-cell basis and can therefore skew cytokine production assays[31]. Additionally, increased cytokine production upon addition of ATM inhibitors excludes confounding effects of purity, as the cells in control and inhibited samples are from the same preparation, and thus have the same purity. Furthermore, these results were corroborated by intracellular cytokine staining, which conclusively shows cytokine production in neutrophils.

7.1.3 ATM deficiency increases neutrophil cytokine production.

Using multiple lines of evidence, this work shows that innate immune cells deficient in ATM activity overproduce inflammatory cytokines in response to activating stimuli. This result was obtained with primary human neutrophils in experiments with two ATM inhibitors, and more importantly, confirmed with neutrophils from AT patients who have genetic deficiencies of ATM. Cytokine production from primary human monocytes was likewise increased by ATM inhibition, indicating that the effect is not neutrophil-specific. Finally, macrophages from mice deficient in Nbs1 showed decreased activation of ATM and correspondingly overproduced cytokines. Interestingly, the effect was observed in production of multiple chemokines and cytokines, including IL-1β, IL-6, IL-8, MIP-1α, and TNF. These results show that DDR deficiency in innate phagocytes in both humans and mice increases their production of proinflammatory cytokines, suggesting a role for this pathway in regulating inflammatory responses.

The increased production of cytokines in the absence of ATM activity was observed by measuring protein concentration in cell supernatants, intracellular protein levels, as well as mRNA. These data support the conclusion that the production of cytokines is increased by ATM deficiency and is not an artifact of altered secretion, cytokine breakdown, or other changes in ATM-inhibited cells.

7.1.4 Primary neutrophils from AT patients overproduce cytokines

To confirm the results with ATM inhibitors, we worked with a cohort of patients with AT, who have genetic mutations in ATM. These patients harbor diverse mutations in ATM. Participating patients were free of signs of infection, inflammation, or malignancy at the time of blood donation. Neutrophils from AT patients produced, on average, nearly 3-fold more IL-8 than controls. The range of cytokine production from AT patients is larger than that observed in controls, which may correlate with ATM activity. Patients do not all harbor null mutations, but are often hypomorphic, still retaining some ATM activity[107]. It is known that disease severity and symptoms correlate with residual ATM protein and activity[104]. A dose-dependent effect of the ATM inhibitor was also observed, indicating that residual ATM activity can attenuate the cytokine overproduction phenotype. We unfortunately were not able to perform experiments to determine levels of residual ATM activity in patients to determine if it correlated with the cytokine production, and this is not discernible from the genetic data alone.

7.1.5 Cytokine overproduction is independent of increased lifespan

We began our investigation of the mechanisms behind the increased cytokine production in ATM-deficient cells by demonstrating that it is not merely a byproduct of an increased lifespan. Inhibition of p53 completely blocked apoptosis over an 18-hour period, but did not lead to increased IL-8 production. This shows that cytokine production is regulated independently of cell death, and that an increased lifespan is not sufficient to increase cytokine production. Furthermore, we measured cytokine production and viability at an early time point before significant differences in apoptosis occur, and calculated the average rate of production per viable cell at this time point. This revealed an increased output of cytokines. This is further corroborated by intracellular IL-8 stain, where increased staining was observed as early as 3 hours after stimulation; the difference between the two populations continues to grow up to 20 hours post stimulation. Furthermore, in qPCR assays, the relative quantity of cytokine mRNA was normalized to that of a house-keeping gene, showing that transcript levels are increased on the cellular and not the population level.

7.1.6 The DNA damage response negatively regulates cytokine production

To gain further insights into the mechanism by which ATM influences cytokine production, the effects of ATM activation on cytokine production were examined by exogenously inducing DNA damage. Treatment with γ-radiation, etoposide, or cisplatin, all commonly used to damage DNA and activate the DDR, activated ATM and decreased cytokine production in a dose-dependent manner. The decrease in cytokine production in response to irradiation was

dependent on ATM. These results indicate that the DDR is an important negative regulator of cytokine production, and its absence leads to cytokine overproduction.

Consistent with these findings, multiple lines of evidence have recently emerged that point to a role of ATM in suppressing cytokine production and inflammation. In human dendritic cells, ATM negatively regulates IL-23 production[157]. Inhibition of ATM increased IL-23 production, while ATM activation attenuated it. Furthermore, pharmacological induction of the DNA damage response by anthracyclines, a class of commonly used chemotherapeutics, reduces levels of pro-inflammatory cytokines and increases survival in septic mice[158]. This protective effect was partially mediated by ATM, although the researchers conclude that this occurs via a lung-specific mechanism not mediated by immune cells.

Härtlova et al.[159] report that ATM deficiency in macrophages increases production of type I interferon. However, they do not suggest a direct role of ATM in reducing interferon production. Rather, they propose that unrepaired DNA lesions in AT cells activate the stimulator of IFN genes (STING) pathway via cytosolic DNA sensors, which primes the type I interferon system. According to their mechanism, treatment of macrophages with DNA damaging agents increases cytokine production by activating STING; however, we observe a decrease in cytokine production in response to DNA damage in neutrophils. This result may be explained by the lack of functional activation of STING in neutrophils[160]. Thus, ATM may exert its anti-inflammatory role via multiple mechanisms, both direct and indirect in various cell types. Alternatively, ATM may play a different role in the interferon pathway, which is distinct from the pathway leading to transcription of the proinflammatory cytokines examined in this study.[161]

It is unclear whether the exact mechanism we described is neutrophil-specific or is also relevant in other cell types. We have not examined the mechanism of cytokine overproduction observed in ATM-deficient monocytes or macrophages. Because AT patients are able to donate only small volumes of blood, we were unfortunately not able to sufficiently isolate primary monocytes for experiments. Given these reports, it will be interesting to examine which of these mechanisms, or if both, play a role in cytokine regulation in these cells.

7.1.7 Involvement of other DDR proteins

Our data show that ATM is not the sole member of the DDR that regulates neutrophil functions. Inhibition of fellow DNA damage signaling kinase ATR or downstream kinase Chk2 also increases cytokine production. Additionally, inhibition of Chk2 and p53 increase neutrophil lifespan. The DDR is highly redundant, with multiple kinases converging on common substrates and signaling loops that enable signal amplification and fine-tuned modulation of responses[162]. Activation of Chk2 and p53 can be modulated by both ATM and ATR (see Figure 4.19a), highlighting the interplay and redundancy in these DNA damage sensors. Simultaneous inhibition

of ATM and ATR synergistically increases cytokine production, suggesting that these pathways may converge on Chk2 to regulate cytokine production.

Chk2 is a kinase with multiple described downstream substrates involved in cell cycle regulation and DNA repair[163]. The important substrate(s) for regulating cytokine production have not yet been identified. Chk2 physically interacts with and negatively regulates MAP kinase ERK, though the exact mechanism remains unclear[164]. This result is in agreement with our data, suggesting that Chk2 acts downstream of ATM and ATR to negatively regulate MAP kinase signaling.

7.1.8 Increased MAP kinase signaling drives cytokine overproduction in ATM-inhibited cells

As cytokine production is increased at both the protein and mRNA levels, and occurs independently of cell death, the signaling events upstream of cytokine transcription and translation were investigated. Activation of p38 was increased in ATM-inhibited cells from one hour post-stimulation on. No significant differences in ERK or NF-κB were observed at early time points, but both were increased by 3 hours post-stimulation. In accordance with other reports[36], we were unable to detect activation of JNK in LPS-stimulated neutrophils (data not shown).

We showed that cytokine overproduction in ATM-inhibited cells is dependent on p38 and ERK activity. Inhibitors of p38 and ERK reduced cytokine production in control neutrophils, with p38 inhibition almost completely abrogating IL-8 production. Additionally, simultaneous inhibition of ATM and p38 completely negated the cytokine overproduction phenotype and restored IL-8 production to control levels. Co-inhibition of ATM and ERK similarly reduced IL-8 overproduction, but less efficiently.

Our data underscores the role of MAP kinases as positive regulators of inflammation. Activation of MAP kinases and NF-κB are known to synergistically regulate IL-8 production via multiple mechanisms, both transcriptionally and post-transcriptionally[35]. NF-κB itself is a transcription factor for immune related genes. MAP kinases contribute to activating further transcription factors, such as AP-1 and C/EBP, which coordinately regulate cytokine production. Increased activation of these pathways observed upon ATM-inhibition may be responsible for the increased transcription of cytokines. Furthermore, p38 also contributes to post-transcriptional regulation of IL-8 by stabilizing its mRNA[165]. Therefore, increased p38 activation may be responsible for the observed increases of both transcription and translation of IL-8. Increases in p38 activity are implicated in the pathology of inflammatory diseases, such as chronic obstructive pulmonary disease[166] and cystic fibrosis[167].

An effect similar to our findings in neutrophils has been observed in hematopoietic stem cells[168] as well as endothelial cells[169]. In these reports, elevated ROS levels led to increased p38

activation in ATM-deficient cells, but not in control cells. These findings, together with ours, strongly suggest an important role for ATM in modulating the activity of p38 in response to oxidative stress.

7.1.9 DNA damage activation of MAP kinases and NF-κB

Modulation of the MAP kinase and NF-κB pathways by DNA damage signaling is well established. These pathways are involved in stress signaling and regulation of the cell cycle and apoptosis. The interactions between these pathways are complex and reports are often contradictory. This is likely due to context-specific factors, such as the nature and extent of DNA damage, stage of the cell cycle, and the type of tissue in which the DNA damage occurs.

7.1.9.1 MAPKs

DNA damage signaling can activate p38 and ERK in an ATM-dependent manner[170,171]. Interestingly, we observed the contrary in neutrophils; DNA damage activated ATM but did not lead to MAP kinase activation or cytokine production. ATM inhibition, on the other hand, increased p38 activation and cytokine production. Though the mechanism linking ATM and MAP kinases in neutrophils is not yet elucidated, there are potential explanations from the literature that could explain these contrasting results. ATM activates MAPK phosphatase 1, a negative regulator of both p38 and ERK[172], showing that the DDR can provide positive and negative MAP kinase regulation. The specific signals involved in determining in which direction to shift MAP kinase signaling remain unclear. Furthermore, there appears to be specificity for certain isoforms of MAP kinases activated by DNA damage. Ionizing irradiation activates isoform p38γ, which contributes to cell cycle checkpoint maintenance[173]. We did not observe p38 activation by ATM in primary neutrophils by irradiation, which may derive from cell-type specificity. The predominant isoform of p38 in neutrophils responsible for inflammatory responses is p38α, whereas p38γ is not expressed[174]. The discrepancies in these various mechanisms involved in the DDR via cell type-specific manners underscore the importance of working with primary cells and not cell lines when at all possible.

7.1.9.2 NF-κB

There are multiple reports showing that ATM triggers the NF-κB pathway and activates cell survival genes to regulate apoptosis. Furthermore, NF-κB activation by ATM has been shown to induce IL-8 expression in metastatic cancer cells[175] and immortal cell lines[176]. Our work suggest this mechanism may not be relevant in neutrophils, as ATM activation did not induce or increase IL-8 production, but rather decreased it; likewise, ATM inhibition did not decrease NF-κB activation or IL-8 production, but rather increased it. This suggests that ATM-dependent NF-κB

activation may occur in only specific cells, perhaps dependent on tissue-specific expression of various cofactors and interactors involved in this pathway. For instance, activation of NF-κB by ATM has been shown to simultaneously inhibit cAMP responsive element-binding (CREB) protein-mediated gene regulation[177], a factor known to cooperate with NF-κB to regulate IL-8 production in neutrophils[178]. This could potentially explain how NF-κB activation by ATM may not be sufficient for IL-8 expression.

Another important point is that many reports of ATM-dependent NF-κB activation are based on results from cancer cells or immortalized cell lines[179-183]. In contrast, multiple studies with primary cells from both humans and mice were unable to reproduce this NF-κB activation in response to DNA damage, further supporting a cell-type dependent mechanism[184-187]. This may reflect a fundamental difference between cancerous or immortalized cell lines and normal primary cells. Cancer cells often have increased or constitutive NF-κB signaling, providing pro-survival and proliferative signals even in situations that would normally halt the cell cycle, such as the presence of DNA damage[188].

7.1.10 The DDR and NET formation

NET formation in response to PMA stimulation was measured in neutrophils isolated from AT patients. As PMA is a potent NET inducer that results in close to 100% NET formation, increases in NET formation are not easily detected. Therefore, NET formation was quantified at an early time point when many cells are still in the process of making NETs. NET formation was measured by quantifying the nuclear area, which increases as the nucleus expands and then is released from the cell during NETosis. By measuring nuclear area as a proxy for NET formation at a time point before all cells have ruptured and released NETs, a quantitative readout is obtained of not only how many cells are making NETs, but the dynamics with which this occurs[189].

A significant increase in nuclear area was observed at this early time point in neutrophils from AT patients, indicating they were making NETs more rapidly. This increased propensity to undergo NETosis may be relevant in an *in vivo* context, where other signals likely inform the neutrophil's decision of whether or not to make a NET. MAP kinase inhibition decreased NET formation, as has previously been reported[190], indicating that their activity positively drives NETosis. The increased activation of p38 in the absence of ATM activity may then be responsible for the increased NET formation in AT patients.

In contrast, however, neutrophils from healthy donors incubated with the ATM inhibitor did not produce increased NETs. This discrepancy between primary AT neutrophils and ATM-inhibited neutrophils could be explained by neutrophil priming. Neutrophil priming by exposure to cytokines is a well-described phenomenon that contributes to their activation[45]. AT patients are reported to have increased IL-8 in the serum, and we have shown that their neutrophils

produce more IL-8 when stimulated. AT cells may thus be pre-activated by exposure to cytokines *in vivo* before and during isolation. This priming effect could increase their activation and propensity to make NETs. This may not occur in neutrophils from healthy donors, which are incubated with ATM inhibitor for only 15-30 minutes before activation.

DNA damage induction by neither irradiation nor etoposide had a significant effect on NET formation, even at the high doses used. This may again be a result of using such a strong NET stimulus as PMA. Neutrophil activation pharmacologically by PMA may be too robust to see any negative regulatory effects from DNA damage.

Further experiments must be done in order to understand the role of ATM in NET formation. One important factor is finding a relevant *in vitro* stimulus that induces NET formation less efficiently than PMA. This would better allow detection of regulation of NETosis. More importantly, an *in vivo* model of NET formation during infection would provide more physiologically relevant information. The neutrophil exerts multiple effector functions simultaneously during *in vivo* infections, and integrates various signals to regulate these functions. Thus, testing the effect of ATM deficiency in an *in vivo* NET assay will be preferable to *in vitro* assays.

7.1.11 The DDR and apoptosis

7.1.11.1 ATM deficiency increases neutrophil lifespan

Neutrophil viability was measured in the presence of the ATM inhibitor and only a mild increase in viability over 12 hours was observed. In primary neutrophils from AT patients, however, a consistent increase in viability was observed by measuring apoptosis via different readouts. Staining with annexin V and vital DNA dyes showed a consistent increase in viability of AT neutrophils at 8 and 18 hours post-stimulation. Similarly, in single experiments viability of AT neutrophils was increased as measured by TUNEL and SubG1 assays for DNA fragmentation. We showed that neutrophil activation induces a DDR and deficiency in this response increases lifespan, suggesting that this pathway is one of the signals contributing to neutrophil apoptosis.

Neutrophil apoptosis is executed via p53[20]. P53-dependent apoptosis is one possible outcome of the activation of the DDR, mediated by phosphorylation of p53 by various DNA damage proteins. In addition to ATM and ATR, Chk2 can modulate p53 activity by phosphorylation. Inhibition of Chk2 prolonged neutrophil lifespan, indicating that DDR regulation of neutrophil apoptosis acts via Chk2. Interestingly, Chk2 also increased cytokine production, though p53 did not. Therefore, the pathway leading from ATM activation to regulation of cytokine production and apoptosis may branch from Chk2 and be mediated by different targets.

Increased neutrophil lifespan may be influenced by concurrently increased cytokine production. Autocrine stimulation of neutrophils with IL-8 and other cytokines provides an anti-apoptotic signal that increases their lifespan[191]. Further experiments would be required to conclusively separate these phenomena. Simultaneous treatment with anti-cytokine neutralizing antibodies could shed more light on this question. Another experiment that could address this is differential labeling of control and AT neutrophils in a co-culture experiment. Here both populations would be exposed to the same cytokine concentrations in culture, and differential staining would allow measuring of apoptosis in both populations at once.

7.1.11.2 Induction of neutrophil apoptosis by DNA damage

The effects of ATM and DNA damage on neutrophil apoptosis remain elusive and somewhat contradictory. Genetic deficiency or inhibition of ATM delay the initiation of apoptosis and prolong lifespan. However, activation of ATM via DNA damage did not consistently affect neutrophil lifespan. Treatment with cisplatin decreased the lifespan of stimulated neutrophils, while treatment with etoposide or γ-radiation had no effect. These three treatments damage DNA by different mechanisms, with etoposide and irradiation more efficiently producing double strand breaks and activating ATM. Cisplatin produces adducts that result in single and double strand breaks, and the activation of ATR and ATM. Disentangling these arms of the DDR is a difficult task due to significant overlap of substrates and mechanisms. Therefore, we can currently only speculate on the different outcomes of DDR activation.

One explanation for this discrepancy could lie in the two distinct mechanisms of ATM activation. ATM can be activated directly by ROS, or indirectly via DNA damage. It is speculated that ROS-activated and DNA damage-activated ATM may have different effects, as they have different substrate specificities[126] and cellular localization. The majority of ATM is found in the nucleus, but a significant fraction is present in the cytosol[124]; it is currently thought that the cytoplasmic reservoir responds to oxidative stress, and the nuclear fraction to DNA damage. Their specificities may also be influenced by the cofactors and substrates present in these different compartments[100]. The methods used in our experiments to exogenously activate ATM do so via DNA damage. ATM activation in stimulated neutrophils is dependent on ROS, and potentially occurs via either or both mechanisms. Thus, our experimental methods of inducing DNA damage may not represent the full spectrum of ATM activation in activated neutrophils. ATM may thus affect cytokine production and apoptosis via two distinct downstream mediators, one that is downstream of DNA-damage activated ATM that regulates cytokine production, and one that is exclusively downstream of ROS-activated ATM that affects apoptosis, and thus is not affected by cisplatin, etoposide or irradiation.

Teasing apart these two mechanisms is a challenge, as ROS and DNA damage will occur simultaneously, but may be possible with the right tools. Ideally, experiments would be performed with an ATM mutant that responds to DNA damage but not oxidative stress. AT patients harboring such a mutation exist[126]; primary neutrophils from these patients would be an excellent tool to examine these pathways. Due to the genetically intractable nature of neutrophils, these mutations cannot be introduced into primary cells, and it is imperative to perform these experiments with neutrophils, given the cell-specific effects discussed above. Neutrophil-like cell lines that partially reproduce neutrophil functions are available[192] and can be genetically manipulated; these may prove a valuable tool, provided they reproduce the mechanisms we have found in primary neutrophils, as they are immortalized cancer cells. Finally, NBS1-deficient cells, from patients with Nijmegen breakage syndrome or our mouse line, represent a unique tool in which DNA-damage activation of ATM is blocked, but ROS activation remains. These tools will prove valuable in pinpointing mechanisms of ROS-mediated ATM activation in the future.

7.2 The oxidative burst activates ATM to regulate neutrophil functions

7.2.1 ROS activation of ATM and the DDR

We showed efficient activation of ATM and other members of the DDR in neutrophils upon stimulation. Abrogation of this activation in ROS-scavenged and primary CGD cells conclusively shows that DDR activation in neutrophils is dependent on NADPH oxidase-dependent ROS production. It is well accepted that ROS can cause oxidative damage to DNA and induce a DDR; furthermore, neutrophil-derived ROS induce DNA damage in neighboring cells[193]. However, this is to our knowledge the first report to investigate the activation and significance of the DDR in neutrophils.

ATM is activated in response to DNA damage, but also reportedly by direct oxidation of cysteine residues in a manner independent of DNA[126]. We have not yet resolved these two potential mechanisms in neutrophils to completely determine their significance. However, several lines of evidence support a role for DNA damage-induced ATM activation, without ruling out additional contribution of ROS-activated ATM. First, neutrophil stimulation leads to H2A.X phosphorylation in a ROS-dependent manner and occurs when ATM is inhibited. H2A.X is phosphorylated by multiple kinases, including ATM, ATR, and DNA-PK, in response to DNA damage, explaining its phosphorylation even when ATM is inhibited. Furthermore, ROS-activated ATM does not phosphorylate H2A.X in the absence of DNA damage. Finally, mouse macrophages deficient in Nbs1, required for DNA damage-activation but not direct ROS-activation, overproduce cytokines similar to ATM-deficient neutrophils. These results strongly indicate that DNA damage is sufficient to induce a DDR and regulate cytokine production.

These results, however, to do not rule out a role for ROS-activated ATM. Our results indicate that ROS-activated, rather than DNA damage-activated, ATM may be important in regulating neutrophil apoptosis. As discussed in section 7.1.11.2, there are tools that may allow resolution of these mechanisms of ATM activation, including primary neutrophils from AT patients deficient in only one of these pathways and our Nbs1-deficient mouse model. Furthermore, a similar study of neutrophil functions in patients with Nijmegen breakage syndrome, caused by deficiency in NBS1, could shed light on these mechanisms. Comparing the phenotype of their neutrophils to those of AT patients and healthy controls could clarify the impact of ROS-activated and DNA damage-activated ATM in regulating neutrophil functions.

7.2.2 The DDR acts downstream of ROS to regulate cytokine production

We showed that deficiencies in the oxidative burst and the DDR both result in overproduction of cytokines by stimulated neutrophils, suggesting that ROS and the DDR act in the same pathway. Primary neutrophils from an AT patient and a CGD patient isolated concurrently both produced more IL-8 than the healthy control processed in parallel. This data is from a single experiment and must be taken with a grain of salt; a larger study with sufficient patients would be necessary to properly compare these populations. Congruently, there is much literature showing that human CGD phagocytes, as well as those from a mouse model, overproduce cytokines. Further supporting the similarity between these deficiencies, scavenging ROS or inhibiting ATM both increase cytokine production from healthy neutrophils.

ROS-scavenged neutrophils fail to activate ATM upon stimulation, and overproduce cytokines similarly to ATM-deficient neutrophils. These data suggest that the DDR acts downstream of ROS to regulate cytokine production. This was confirmed by inducing DNA damage in ROS-scavenged neutrophils, which abrogated the cytokine overproduction phenotype.

7.2.3 A model of cytokine regulation by ROS and DNA damage

We described a cellular phenotype in neutrophils from AT patients that shares some characteristics with those from CGD neutrophils; both overproduce inflammatory cytokines and delay apoptosis. In doing so, we identified ATM and the DDR as one of the targets downstream of the oxidative burst that is important for the ROS-regulation of neutrophil functions. Based on our results, we propose a model (**Figure 7.1**) in which stimulation of neutrophils activates MAP kinase signaling cascades and cytokine production, and simultaneously triggers the oxidative burst. The ROS produced activate ATM and DNA damage signaling, either by damaging DNA or by direct oxidation of ATM. The DDR then plays a role in regulating MAP kinase signaling, especially p38, and apoptosis via p53 to regulate neutrophil inflammatory responses. A fundamental

difference in the phenotypes of these cells, however, is their ability to make NETs: CGD neutrophils fail to undergo NETosis, while AT neutrophils may make even more NETs than healthy controls.

Deficiency in either the oxidative burst (CGD) or the DDR (AT) results in increased p38 activation and overproduction of inflammatory cytokines, and in AT patients, NETs. At the same time, decreased activation of p53 results in delayed apoptosis. These results not only further our mechanistic understanding of neutrophil function, but also expose an interesting link between these two disparate diseases that provides a new perspective on the pathology of AT.

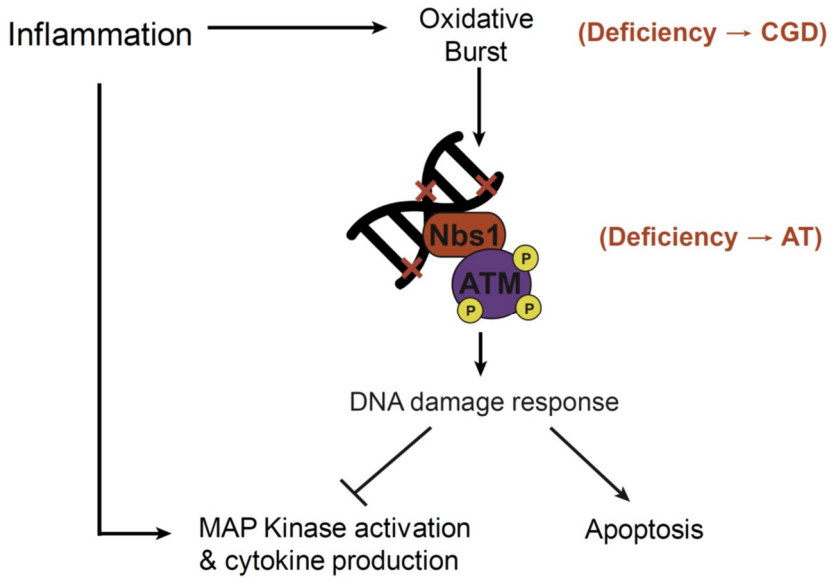


Figure 7.1: Model of oxidative burst and DNA damage response regulation of neutrophil functions.

7.3: Clinical significance

7.3.1 AT and CGD: similar diseases?

ROS are ubiquitous and promiscuous molecules which can modify various cellular macromolecules. In addition to their proposed antimicrobial importance, they contribute to the regulation of signaling pathways and transcription by oxidizing protein residues[194]. These reactions are post-translational modifications that can alter activity and substrate specificity of kinases, phosphatases, and transcription factors. In neutrophils, the oxidative burst regulates antimicrobial responses[76], apoptosis[83], autophagy[195], hypoxia and oxidative stress responses[196], chemotaxis[197,198], and NETosis[26]. Our data suggest that ATM and the DDR represent one of the

pathways affected by ROS in neutrophils. It is clear that ROS impact many cellular functions; the DDR represents only one of these. Similarly, the DDR is implicated in many more processes than ROS-regulation of neutrophil functions, most notably of course is repair of DNA damage in all cells. We propose that although CGD and AT are very different diseases, their phenotypes may intersect in the innate immune system due to involvement of both ROS and ATM in regulating innate immune cell functions.

7.3.2 AT: an inflammatory disease?

The similarities we have described between AT and CGD neutrophils may have important implications for our understanding of some of the pathologies associated with AT. The chronic inflammatory pathology of CGD is attributed to multiple effects of ROS-deficiency in phagocytes of the innate immune system. Overproduction of inflammatory cytokines and delayed apoptosis create a compound inflammatory predisposition that leads to hyperinflammatory responses and delayed resolution of inflammation. We have shown the same cellular phenotype in ATM-deficient phagocytes. Therefore, the hyperinflammatory responses of CGD patients may similarly be present in AT.

Examining the spectrum of pathologies associated with AT and CGD, it becomes clear that not only are there striking clinical similarities between these two diseases, but many features of AT can be linked to or exacerbated by inflammation. Non-resolving sterile granulomas[91,199], pulmonary inflammation[97,200], and increased incidence of autoimmune phenomena[96,201] are common to both AT and CGD. Furthermore, experimental models of colitis[202,203] and pulmonary inflammation[204,205] are exacerbated in mouse models of AT and CGD. In CGD this pathology is attributed to hyperactive phagocytes, but the cause of these symptoms in AT was not understood. Our model suggests that an autoinflammatory reaction caused by dysregulated neutrophil functions may be the culprit.

AT patients are extremely susceptible to cancer due to their increased accumulation of mutations and genomic rearrangements. Neutrophils exert multiple cancer-promoting activities; therefore, the hyperactive neutrophil responses we have described may be an important driver of malignancy as well. Neutrophil-derived ROS are potent inducers of DNA damage in bystander cells at sites of inflammation, thought to be the major driving force behind inflammation-associated tumorigenesis[193]. The oncogenic potential of ROS is likely even higher in AT patients, as they would not only experience more oxidative damage from prolonged inflammation, but the lack of a proper DDR results in decreased DNA repair and accumulation of mutations. Furthermore, cytokines such as IL-8 have been shown to provide proliferative signals that promote tumor growth[206]. Finally, proteases released by activated infiltrating neutrophils degrade tissue matrix, providing a paved road for tumor metastasis[207,208].

Even the hallmark symptom of AT, neurodegeneration, may be affected by inflammation. The reason neurons are so sensitive to ATM deficiency is still not understood. Some propose that neurons are simply more susceptible to DNA damage[128]. Others propose that as post-mitotic cells, AT neurons are not sensitive to DNA damage, but rather have dysregulated control of the cell cycle[209]. Even others propose that as highly metabolically active cells, neurons experience increased oxidative stress, for which ATM is an important sensor and regulator[210]. Inflammation has been implicated in multiple neurodegenerative diseases, such as Alzheimer's and Parkinson's disease[211]. Though signs of inflammation have not been observed in brains of AT patients or mice, systemic inflammation has been shown to induce cell cycle abnormalities and cell death in cerebral Purkinje cells in $ATM^{-/-}$ mice[212]. This suggests that underlying inflammation even outside of the brain may be a contributing factor to cell death in ATM-deficient neurons. Mutating ATM in *Drosophila* led to induction of immune response genes in glial cells, and neurodegeneration[213]. Surprisingly, selective knockdown of ATM in glial cells, but not neurons, led to inflammation and neuron cell death. Therefore, in this model, neuron cell death was caused not by ATM deficiency in the neurons, but by local inflammation from ATM-deficient glial cells.

There are many signs of inflammation in the clinical spectrum of AT. Our work on the innate immune cells of these patients suggests that hyperactive phagocytes may be one component underlying this inflammation. We do not, however, suggest that it is the single source of pathology in the disease. AT patients often have decreased numbers or altered classes of mature lymphocytes. These cells, especially certain classes of T cells, also play a role in regulating immune responses; their deficiencies can also cause inflammatory phenotypes[214]. Inflammation in AT may represent additive effects of multiple factors.

7.3.3 Treating inflammation in AT

There is currently no treatment available for AT, other than immunoglobulin therapy for immunodeficient patients and palliative care. Remarkably, recent clinical trials found that treatment with anti-inflammatory steroids improved neurological symptoms for AT patients[215,216]. It is unclear if this effect was due to the immunosuppressive properties of the drug, its antioxidant properties, or both. It will be interesting to see if other symptoms associated with AT will be alleviated by this treatment as well. Our work further strengthens the argument that targeting inflammation may be a promising clinical intervention for some of the symptoms of this devastating disease, including reducing risks of cancer, autoimmunity, chronic inflammation and even neurodegeneration.

7.3.4 DNA damage as an anti-inflammatory treatment

The identification of the ATM as a regulator of neutrophil functions and inflammation suggests that induction of a DDR can have immunosuppressive effects. Indeed, Figuero et al.[158] showed that pharmacological induction of the DDR using anthracyclines reduces levels of proinflammatory cytokines and increases survival in septic mice. This effect is dependent on ATM; however, they identify autophagy responses in the lung as being important for this protection. Our data suggests that this effect may also derive from reduced cytokine production in innate immune cells. Transient treatment with drugs that activate ATM or the DDR could provide immunomodulatory effects useful for treating acute inflammation as in sepsis, or in other inflammatory diseases.

7.4: A mouse model for phagocyte-specific DDR deficiency

7.4.1 ATM mice have residual ATM activity

We found that *ATM*^{tm1Awb} mouse neutrophils have residual ATM protein that is phosphorylated in response to DNA damage. This phenomenon has been observed in other tissues of this mouse line by Li et al[151]. The *Atm*^{tm1Awb} allele was created by insertion of a neomycin cassette, introducing a premature stop codon. However, it was found that in some tissues a splicing event is able to remove the cassette, resulting in an almost full-length, catalytically active form of Atm. This residual Atm activity may explain why the mouse model of AT only weakly recapitulates aspects of the human disease. Neutrophils and macrophages from these mice did not reproduce the cytokine overproduction phenotype observed in human AT cells, indicating that this residual Atm activity may be sufficient for regulation of cytokine production.

Although we have not observed an *in vitro* phenotype in cells from *Atm*^{tm1Awb} mice, some reports of hyperinflammatory *in vivo* responses can be found in the literature. In an experimental model of induced colitis, *Atm*$^{-/-}$ mice experience increased inflammatory response, morbidity and mortality[202]. Similarly, in an experimental model of acute lung injury, *Atm*$^{-/-}$ mice had increased immune cell recruitment, cytokine levels, and tissue damage in the lungs[204]. The mechanism behind the increased inflammation in these models is not known. The results, however, further suggest that inflammatory responses are exacerbated by ATM deficiency, supporting an anti-inflammatory role of the DDR in other cell types as well.

7.4.2 Phagocyte-specific mouse model of DDR deficiency

The *Atm*^{tm1Awb} mice present two principle problems for potential *in vivo* studies of the contribution of the DDR in innate phagocytes: they are an incomplete knockout, and every tissue

is affected by Atm deficiency. To bypass these caveats, a mouse line was bred with a specific deletion of Nbs1 in the innate phagocyte compartment. This was achieved by combining one Nbs1-knockout allele, one floxed allele, and Cre recombinase expressed under the lysozyme M promoter, expressed exclusively in phagocytes. The resulting experimental mice have Nbs1-deficient neutrophils, monocytes, and macrophages, but the other tissues are unaffected. This will be an advantage for *in vivo* studies, as any discovered phenotypes can be attributed to changes exclusively in the innate immune system.

Nbs1 was specifically targeted for two reasons. First, Nbs1 is required for activation of ATM in response to DNA damage[217], and also contributes to the activation of ATR[218]. Thus, Nbs1 deficiency may more efficiently preclude a DDR and prevent compensation via redundant pathways. Second, Nbs1 deficiency can be used as a tool to tease apart the effects of DNA damage-activated and ROS-activated ATM. Cells deficient in Nbs1 fail to activate ATM in response to DNA damage; however, normal expression of functional ATM is maintained and can respond to oxidative stress. Our *in vitro* data with Nbs1 deficient macrophages shows that they overproduce cytokines, suggesting a DNA damage-dependent role for ATM in regulating cytokine production.

This thesis began with a definition of inflammation as a complex response involving multiple tissues of the body aimed at removing microbial infections, potentially harmful foreign objects, and damaged tissues. As such, inflammation cannot be reproduced and studied *in vitro*; it is too complex. We have used multiple effector functions of cells of the innate immune system as proxy readouts of inflammation *in vitro*. These effects must be studied in an *in vivo* system to conclusively determine if they are sufficient to alter the inflammatory process and result in pathological responses. We have created a tool that will allow these questions to be answered in a more conclusive way.

7.5 Conclusion

This work furthers our understanding of neutrophil biology and the regulation of their functions by shedding light on a new mechanism of the anti-inflammatory effects of the oxidative burst. We demonstrated that NADPH oxidase-derived ROS initiates a DDR in neutrophils, and that various members of this cascade, including ATM, Chk2, and p53, are important factors regulating cytokine production, NET formation, and apoptosis. Furthermore, we have shown that they exert control over these functions by modulating MAP kinase signaling. This mechanistic connection between ROS, DNA damage, and inflammation uncovers an unexpected link between AT and CGD, and provides a new lens through which to examine the pleiotropic symptoms of AT: inflammation. Our findings suggest that the DDR could be a novel therapeutic target for inflammatory diseases. Furthermore, it suggests that inflammation may be a driving force behind the pathology of AT,

providing a possible target for clinical intervention for some symptoms of this yet-untreatable, devastating disease.

MATERIALS AND METHODS

CHAPTER 8: MATERIALS AND METHODS

8.1 Materials

8.1.1 Buffers and solutions

Phosphate-buffered saline (PBS)	
137 mM	NaCl
2.7 mM	KCl
4.3 mM	Na_2PO_4
1.47 mM	KH_2PO_4

Laemmli SDS loading buffer 6x	
480 mM	Tris HCL, pH 6.8
12% (v/v)	SDS
60% (v/v)	Glycerol
600 mM	DTT

Tris-glycine SDS running buffer	
25 mM	Tris Base
192 mM	glycine
3.5 mM	SDS

10 x TBS-T	
20 mM	Tris, pH 7.6
150 mM	NaCl
1 % (v/v)	Tween 20

Microscopy blocking buffer	
Base	PBS
5% (v/v)	Normal donkey serum
5% (v/v)	Normal mouse serum
15% (v/v)	Cold water fish gelatin
1% (w/v)	Bovine serum albumin
0.05% (v/v)	Tween 20

Neutrophil cytokine and apoptosis media	
Base	RPMI 1640
100 U/ml	Pen/Strep antibiotics
2 mM	L-glutamine
10% (v/v)	FCS

NET assay media	
Base	RPMI 1640
100 U/ml	Pen/Strep antibiotics
2 mM	L-glutamine
10 mM	HEPEs buffer
1% (w/v)	Human serum albumin

Western transfer buffer	
25 mM	Tris Base
192 mM	glycine
20% (v/v)	methanol

Flow cytometry staining buffer	
Base	PBS
1% (w/v)	Bovine serum albumin
5% (v/v)	Normal mouse serum

DNA isolation buffer	
Base	H_2O
10 mM	Tris pH 8.0
100 mM	NaCl
10 mM	EDTA
0.5 %	SDS

8.1.2 Cell culture reagents

Reagent	Product Number	Manufacturer
Heparin natrium	H-NA-25000	Ratiopharm
Histopaque	11191	Sigma-Aldrich
Percoll	17-0891-01	GE Healthcare
CD14 microbeads	130-050-201	Miltenyi
RPMI	12633020	Gibco
Fetal calf serum	12003C	Sigma-Aldrich
L-Glutamine	G7513	Sigma-Aldrich
Penicillin/Streptomycin	P4333	Sigma-Aldrich
HEPES	H0887	Sigma-Aldrich
PMA	P 8139	Sigma-Aldrich
LPS from *S. typhimurium*	ALX-581-011-L002	Enzo Scientific
HKLM	11-C02-MM	Invivogen
Flagellin	15-D07-MM	Invivogen
Zymosan	Z4250	Sigma-Aldrich

8.1.3 Pharmacological inhibitors

Inhibitor	Target	Chemical name	Concentration	Manufacturer
KU-55933	ATM	2-(4-Morpholinyl)-6-(1-thianthrenyl)-4H-pyran-4-one	10μM	Selleck, Cayman Chemical
KU-60019	ATM	2-((2S,6R)-2,6-dimethylmorpholino)-N-(5-(6-morpholino-4-oxo-4H-pyran-2-yl)-9H-thioxanthen-2-yl)acetamide	5μM	Selleck, Cayman Chemical
Pifithrin-μ	p53	2-phenyl-ethynesulfomide	1μM	Selleck
SB203580	p38	4-(4-(4-fluorophenyl)-2-(4-(methyl-sulfinyl)phenyl)-1H-imidazol-5-yl)pyridine	5μM	Selleck
PD98059	MEK/ERK	2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one	5μM	Selleck
CHIR-124	Chk1	(S)-3-(1H-benzo[d]imidazol-2-yl)-6-chloro-4-(quinuclidin-3-ylamino)quinolin-2(1H)-one	1μM	Selleck
PV-1019	Chk2	2-[4-(4-Chlorophenoxy)phenyl]-3H-benzimidazole-5-carboxamide	10μM	Sigma-Aldrich
VE-821	ATR	3-amino-6-[4-(methylsulfonyl)phenyl]-N-phenyl-2-pyrazinecarboxamide	1μM	Selleck
Protease Inhibitor cocktail		Mix P8340 for mammalian cell and tissue extracts	1:100	Sigma
GW311616A	Elastase	(3S,3aS,6aR)-3-Isopropyl-1-(methane-sulfonyl)-4-[4-(1-piperidinyl)-2(E)-butenoyl] perhydropyrrolo[3,2b]pyrrol-2(1H)-one hydrochloride	10 μg/ml	Sigma

8.1.4 Antibodies and staining reagents

8.1.4.1 Flow cytometry staining and analysis reagents

Antigen/fluorophore	Species	Concentration	Number	Manufacturer
CD15 - FITC	Mouse	1:20	555401	BD Biosciences
CD14 - PE	Mouse	1:20	557154	BD Biosciences
Annexin V - PE	-	5 µl/sample	51-65875X	BD Biosciences
Propidium Iodide	-	5 µg/ml	556463	BD Biosciences
7-AAD	-	2.5 µg/ml	51-68981E	BD Biosciences
FxCycle PI/RNase solution	-	-	F10797	Thermo Fischer
IL-8 - PE	-	5 µg/ml	G265-8	BD Biosciences

8.1.4.2 Antibodies used for Immunoblot assays

Antigen	Species	Concentration	Number	Manufacturer
ATM (human)	Rabbit	1:1000	#2873	Cell Signaling Technology
phospho-ATM (pS1981, human)	Rabbit	1:1000	#13050	Cell Signaling Technology
ATM (mouse)	Mouse	1 µg/ml	ab78	Abcam
phospho-ATM (pS1981, mouse)	Rabbit	1:1000	#5883	Cell Signaling Technology
GAPDH	Rabbit	1:2000	#5174	Cell Signaling Technology
γ-H2A.X (pS139)	Mouse	1:1000	05-636	Merck Millipore
phospho-BRCA1 (pS1524)	Rabbit	1:1000	#9009	Cell Signaling Technology
phospho-p38 (pT180/pY182)	Mouse	1:2000	#9216	Cell Signaling Technology
phospho-ERK (pT202/pY204)	Rabbit	1:1000	#3192	Cell Signaling Technology
IκBα	Rabbit	1:1000	#9242	Cell Signaling Technology
NBS1	Rabbit	1 µg/ml	-	Pineda Antikörper, kindly provided by Prof. Dr. M. Digweed
Rabbit IgG-HRP conjugate	Secondary	1:3000	#7074	Cell Signaling Technology
Mouse IgG-HRP conjugate	Secondary	1:3000	#7076	Cell Signaling Technology
Restore Plus™ stripping buffer			46428	Thermo Fischer
Mouse IgG-HRP conjugate	Secondary	1:3000	#7076	Cell Signaling Technology

8.1.4.3 Antibodies and stains for immunofluorescence and microscopy assays

Antigen/fluorophore	Species	Concentration	Number	Manufacturer
Anti-phospho-ATM (pS1981)	Mouse	10 µg/ml	MA1-2020	Thermo Fischer
Hoechst	-	5 µg/ml	H6024	Sigma
Sytox Green	-	500 nM	S7020	Thermo Fischer
Mouse IgG Alexa fluor 594	Goat	10 µg/ml	A11005	Thermo Fischer
ProLong antifade mountant	-	-	P36931	Thermo Fischer
DeadEnd Fluorimetric TUNEL assay	-	-	G3250	Promega

8.1.4.4 ELISA and Bioplex kits used for cytokine quantification

Reagent	Product number	Manufacturer
DuoSet® hIL-8 ELISA kit	DY208	R&D systems
DuoSet® hMIP-1α ELISA kit	DY270	R&D systems
DuoSet® hIL-1β ELISA kit	DY201	R&D systems
DuoSet® hTNF ELISA kit	DY210	R&D systems
DuoSet® hIL-6 ELISA kit	DY206	R&D systems
DuoSet® mIL-6 ELISA kit	DY406	R&D systems
DuoSet® mKC ELISA kit	DY453	R&D systems
DuoSet® mTNF ELISA kit	DY410	R&D systems
DuoSet® mIL-1 ELISA kit	DY401	R&D systems
DuoSet® mMIP-1α ELISA kit	DY450	R&D systems
DuoSet® mMIP-2 ELISA kit	DY452	R&D systems
DuoSet® mMCP-1 ELISA kit	DY479	R&D systems
Bio-Plex Pro™ Human Cytokine 17-plex	M5000031YV	Bio-Rad
Bio-Plex Pro™ Mouse Cytokine 23-plex	M60009RDPD	Bio-Rad
Bio-Plex Calibration Kit	171203060	Bio-Rad

8.2: Methods

8.2.1 Donor consent

All donors gave consent to blood drawing in accordance with the Helsinki Declaration. Blood samples were collected with approval from the ethical committees of each institution. Informed consent was provided by all patients, or by their parents, in the case of children. At the time of blood donation participants displayed no signs of infection or malignancy. Patients were receiving no treatments or medications apart from regular intravenous immunoglobulin therapy. AT patients whose mutations were to be sequenced gave consent to genetic analysis.

ATM mutation sequencing and genetic analysis was performed by the Center for Genomics and Transcription (CeGaT, Tübingen, Germany) using multiplex ligation-dependent probe amplification and quantitative PCR.

8.2.2 Cell isolation

8.2.2.1 Human neutrophil isolation

Human neutrophils were isolated by two subsequent density separations as previously described[26]. Heparinized (5 I.U./ml) venous blood was layered over an equal volume of Histopaque 1119 and centrifuged at 800 x g for 20 minutes with acceleration and brakes set at 7 and 3, respectively. The upper PBMC layer was discarded (or used for monocyte isolation, below), and the neutrophil-rich histopaque layer was collected, washed with PBS and pelleted by centrifugation at 300 x g for 10 minutes. The pellet was resuspended in 2 ml PBS and layered over a discontinuous discontinuous PBS-buffered Percoll gradient (85%, 80%, 75%, 70%, 65% in 2ml layers), and centrifuged at 800 x g for 20 minutes. The lower neutrophil band was collected, washed, and resuspended in PBS. Cell density was determined using a Neubauer chamber or CASY cell counter. Neutrophils were used as soon as possible after isolation.

8.2.2.2 Human monocyte isolation

Human monocytes were isolated by positive selection using CD-14 microbeads and magnetic selection. PBMCs were obtained from the same preparation as neutrophils after Histopaque separation (8.7.2.1 Human neutrophil isolation). The PBMC fraction was washed and resuspended in cold PBS containing 1% BSA and 2 mM EDTA. Monocytes were isolated according to Miltenyi isolation protocol. Platelets were removed by two sequential low-speed centrifugations at 200 x g for 10 minutes, discarding the supernatant. PMBCs were resuspended in the same buffer, counted by CASY, and incubated 15 minutes with magnetic CD-14 microbeads at 4 °C. The cell suspension was then allowed to pass through a magnetic MACS LD column by

gravity flow. The column was washed three times with 5 ml cold MACS buffer and the flowthrough was discarded. Monocytes were eluted from the column, washed and resuspended in PBS, counted by CASY cell counter and used as soon as possible after isolation.

8.2.2.3 Mouse neutrophil and bone marrow macrophage isolation

See section 8.2.12 Animal experiments

8.2.2.4 Cell preparation purity assessment by flow cytometry

Neutrophil and monocyte purity was assessed via flow cytometry by CD-14 and CD-15 staining. Approximately 10^5 cells were used for staining. Cells were washed in flow cytometry staining buffer and resuspended in 100µl. Cells were incubated 15 minutes on ice with Fc-block (BD Biosciences), followed by addition of CD-14 and CD-15 staining antibodies for 30 minutes. Cells were washed twice by centrifugation for 5 minutes at 300 x g and resuspended in 1 ml flow cytometry staining buffer. Data was recorded using a MACSQuant cytometer (Miltenyi), and analyzed using FlowJo software. At least 10,000 cells were measured per sample. Neutrophils were defined as CD15-high/CD14-low; monocytes as CD-15-negative/CD14-high.

8.2.3 Protein detection by immunoblot

8.2.3.1 Lysate preparation

Neutrophils were resuspended in cytokine and apoptosis media and incubated in 1.5-ml eppendorf tubes, with $2x10^6$ cells in 1 ml per tube. Inhibitors and/or stimuli were added and cells were incubated for indicated time points at 37°C with 750 RPM shaking. At time points, cells were centrifuged 5 minutes at 500 x g and resuspended in 100 µl cold PBS supplemented with protease inhibitors, elastase inhibitor and phosphatase inhibitor. Lysates were made by addition of 100 µl of hot 2x Laemmli SDS loading buffer. Lysates were briefly sonicated and kept at -20°C until use.

8.2.3.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein lysates were resolved by SDS-PAGE on Criterion™ TGX gels (BioRad). For ATM detection, samples were run on 4-15% gradient Tris-glycine gels in Tris-glycine SDS running buffer. For all other proteins, samples were run on 10% Tris-glycine gels. Prior to loading on to gels, samples were boiled for 5 minutes and centrifuged at 10,000 x g for 5 minutes. Electrophoresis was performed in Tris-glycine SDS running buffer at 100 V for 90-120 minutes until samples were sufficiently separated by size as estimated by pre-stained size markers.

8.2.3.3 Protein Transfer

Proteins that were separated by SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF, Amersham Hybond 0.2, GE Healthcare) membranes for immunodetection. PVDF membranes were activated by soaking in methanol. SDS gel and activated membrane were assembled with presoaked filter paper in western transfer buffer and transferred in a wet blotting chamber at 4°C onto PVDF membranes. For ATM detect, transfer was performed overnight at 50mA; for all other proteins transfer was performed for 2 hours at 200 mA.

8.2.3.4 Immunodetection on membranes

Membranes were washed in 1x TBS-T, blocked for 1 hour in either 5% skim milk powder or BSA in TBS-T, according to antibody manufacturer protocol, to saturate unspecific binding. Membranes were incubated overnight at 4°C in blocking buffer with protein-specific primary antibody (see section 8.1.4.2 for antibody specifics). Membranes were washed 3x for 10 minutes each in excess TBS-T, and incubated 30 minutes with HRP-conjugated secondary antibody in 5% skim milk in TBS-T with shaking. Membranes were washed 3x for 10 minutes each. To visualize proteins by enhanced chemiluminescence, membranes were incubated for 1 minute with HRP substrate solution (BD Bioscience). The reaction of HRP with its substrate leads to chemiluminescent radiation, which is detected by film and developed to visualize proteins. Membranes were stripped with Restore plus western blot stripping buffer (Thermo Fischer) and used for re-probing.

8.2.4 Phospho-ATM staining and immunofluorescence microscopy

8.2.4.1 Cell culture and stimulation

For immunofluorescence assays, neutrophils were resuspended at $2x10^5$ cells per ml in NET assay media and seeded on glass coverslips at a density of 10^5/well of a 24-well tissue culture-treated plate in 500 µl. Cells were incubated with appropriate inhibitors for 30 minutes at 37 °C, then stimulated by addition of PMA to final 50 nM. At indicated time points cells were fixed by addition of PFA to 2% final.

8.2.4.2 Staining and image acquisition

Coverslips were washed twice by inversion onto 500 µl droplets of PBS for 5 minutes. Neutrophils were then permeabilized by subversion in ice-cold acetone for 5 minutes, and then blocked at 37°C for 1 hour in microscopy blocking buffer. Samples were stained in blocking buffer with mouse anti-human phospho-ATM (Ser 1981) (Thermo scientific), followed by secondary antibody goat-anti mouse IgG conjugated to Alexa Fluor 568 (Invitrogen). Coverslips were mounted on ProLong Gold antifade mountant with DNA dye DAPI (Thermo Fisher Scientific).

Images were taken with a Leica SP8 confocal microscope (Leica) with Leica 63x/1.30 objective at 25°C. Micrograph merges were made in ImageJ software and cropped in Adobe Photoshop.

8.2.5 Oxidative burst chemiluminescence assay

ROS production was measured by luminol chemiluminescence. HRP catalyzes the oxidation of luminol by hydrogen peroxide generated by the oxidative burst; luminol chemiluminesces upon oxidation, which is measured quantitatively in a luminometer. Neutrophils were seeded in 96-well plates at a concentration of 1 x 10^5 cells per well in 100 µl of NET assay media supplemented with 50µM luminol and 1.2 units/ml HRP (both from Sigma). After 30 minutes incubation at 37°C, cells were stimulated by addition of 100 µl of 2x solutions of indicated stimuli in media. Luminescence was measured kinetically in a VICTOR Light luminescence counter (Perkin Elmer).

8.2.6 Phagocytosis of fluorescent bacteria

Neutrophil phagocytosis was assessed by incubation with fixed fluorescent bacteria and analyzed by flow cytometry. *E. coli* strain UTI89 expressing GFP, described in[219], were grown overnight at 37°C with agitation, measured by optical density and brought to a concentration of 10^8/ml using the conversion $OD_{600}1$ = 5.7 x 10^8 bacteria/ml . Bacteria were fixed for 30 minutes with 2% PFA, then washed 3x in PBS by centrifugation 5 minutes at 10,000 x g. Neutrophils, 10^5/ml in neutrophil cytokine and apoptosis media, were incubated in 1.5-ml eppendorf tubes at 37°C with shaking for 30 minutes with DMSO or ATM inhibitor. Bacteria were added to neutrophils at the indicated MOI for 30 minutes, then fixed with 2% PFA. Cells were washed by centrifugation 5 minutes at 300 x g and resuspended in PBS and measured by flow cytometry on a MACSQuant (Miltenyi) and analyzed by FlowJo software.

8.2.7 NET formation assays

8.2.7.1 Culture and stimulation

For NETosis assays, neutrophils were resuspended at 2x10^5 cells per ml in NET assay media and seeded at a density of 10^5/well of a 24-well tissue culture-treated plate in 500 µl. Cells were incubated with appropriate inhibitors for 30 minutes at 37 °C, then stimulated by addition of PMA to final 50 nM. At indicated time points cells were fixed by addition of PFA to 2% final.

8.2.7.2 Quantification of NET formation

NET formation was assessed by counting and measuring nuclear area. After at least 30 minutes of fixation, saponin was added to 0.1% to permeabilize intact cells and 500nM sytox

green DNA dye was added to visualize nuclei and NETs. Cells and NETs were visualized on a Leica Leitz DM IRB inverted epifluorescence microscope with Leica 20x/0.40 objective, at 25°C. For percent NET formation analysis, images were counted by eye to differentiate cells from NETs (see figure 4.5c) in 4-5 images taken in different locations of the same well; 200-500 cells were counted per condition. For nuclear area quantification, sytox images were analyzed using ImageJ image-processing software as previously described[148]. For experiments with a single donor +/- inhibitor, data were plotted as the mean of the average nuclear areas per image of a condition. For experiments comparing healthy controls and patients, data are plotted as the mean of the average nuclear areas calculated for each donor from multiple images.

8.2.8 Cytokine production assays

8.2.8.1 Cell culture and stimulation conditions for cytokine production

Neutrophils or monocytes were resuspended at 10^6/ml in neutrophil cytokine and apoptosis media. Cells were seeded in triplicate wells per condition at a density of 10^5 cells/well of a 96-well tissue culture-treated plate in 100 µl with relevant inhibitors, and incubated for 30 minutes at 37 °C. Cells were then stimulated by addition of 100 µl of a 2x stock of stimulus. Unless otherwise noted, stimulation was carried out overnight, around 18 hours. Cell-free supernatants were then collected by briefly centrifuging the plate and careful aspiration of 50-100 µl with a multichannel pipette. Supernatants were stored in 96-well plates at -80°C until analysis. Stimuli were used at the following final concentrations: 100ng/ml lipopolysaccharide, 10µg/ml opsonized Zymosan, MOI 100 heat killed Listeria monocytogenes, or 1µg/ml flagellin. Zymosan was opsonized with pooled human plasma from multiple donors.

8.2.8.2 Cytokine measurement by Enzyme-linked immunosorbent assay (ELISA)

ELISAs allow the quantification of proteins based on antibody binding specificity. Sandwich ELISAs rely on protein capture by a capture antibody immobilized on the well and subsequent detection of bound proteins with a detection antibody that recognizes a different epitope of the protein. The secondary antibody is labeled with biotin, allowing detection with streptavidin-HRP and conversion of a quantitative colorimetric substrate. Cytokines were measured by DuoSet ELISA assays per manufacturers instructions. Briefly, plates were coated with capture antibody overnight at room temperature, washed, and 100 µl of diluted samples were added. For neutrophils, samples were generally diluted 1:2 for unstimulated samples and 1:5 – 1:8 for stimulated samples to bring within the range of the standard solutions. Samples were incubated for 2 hours, the plate was washed and detection antibody was added for 2 hours. The plate was then washed, incubated with streptavadin-HRP, washed again, followed by addition of

substrate solution. Substrate development was stopped with acid stop and measured in a Spectramax M5 microplate reader (Molecular Devices) and data was analyzed by SoftMax® Pro software. Data were expressed as cytokine produced per 10^6 cells and plotted as the mean of triplicates from a single experiment, or the means of multiple pools experiments each calculated from triplates.

8.2.8.3 Cytokine measurement by Bio-plex assay

To simultaneously detect multiple cytokines in single supernatant samples multiplex assays were performed according to manufacturer's instructions (BioRad). Multiplex assays are capture sandwich immunoassays, in which the first antibody is coupled to a dye-conjugated polystyrene bead. A biotinylated detection antibody is followed by streptavidin-PE binding for quantification. The combination of differentially-dyed beads specific for individual cytokines allows quantification of mixtures of cytokines in a fluorescence analyzer. Briefly, the filter plate was washed, polystyrene beads were added, and the plate was washed again. Then 50 µl of cell culture supernatants were added per well. For neutrophils, samples were diluted 1:4, for monocytes, 1:10. After 30-minute incubation on a plate shaker, the plate was washed, detection antibodies were added, and incubated for 30 minutes with shaking. The plate was washed, and streptavadin-PE was added. After a 15-minute incubation, the plate was washed and beads were resuspended in assay buffer and measured on a Bio-Plex 200 system (BioRad). Data were expressed as cytokine produced per 10^6 cells and plotted as the mean of triplicates from a single experiment, or the means of multiple pools experiments each calculated from triplates.

8.2.9 Apoptosis assays

8.2.9.1 Cell culture

Neutrophils from cytokine production assays were used at indicated time points for apoptosis assays. After removing supernatants, cells were resuspended by pipetting, collected, washed, and used for apoptosis analyses.

8.2.9.2 Annexin V viability assay

Neutrophil viability was determined using the PE-Annexin V apoptosis kit per manufacturers instructions(BD Biosciences). Briefly, neutrophils were resuspended at the indicated time points, washed in 1x Annexin V binding buffer, stained with PE-Annexin V and vital dye propidium iodide (PI) or 7-AAD, and then measured by flow cytometry on a MACSQuant (Miltenyi) or FACSCalibur (BD). Analysis was performed using FlowJo software. At least 10,000 cells were measured per sample. Annexin V/vital dye double negative neutrophils were defined as viable.

8.2.9.3 SubG1 viability assay

Neutrophil viability was measured by SubG1 DNA content as previously described[220]. Neutrophils were washed and fixed on ice by slowly adding ethanol to final 70% (v/v) and incubated on ice for at least one hour or stored at -20°C. Fixed cells were washed twice by centrifugation at 1800 RPM at 4°C, resuspended in 0.5 ml FxCycle PI/RNase solution (Thermo Fischer) and incubated 30 minutes at room temperature. Cells were washed by centrifugation at 300 x g, resuspended in PBS, and measured by flow cytometry in a MACSQuant (Miltenyi). At least 10,000 cells per sample were acquired. Data was analyzed in FlowJo software.

8.2.9.4 Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) assay

Neutrophil apoptosis was measured by TUNEL assay, which incorporates tails of fluorescent nucleotides onto broken DNA ends generated by genome fragmentation during apoptosis, and visualized microscopically. Neutrophils were seeded at a density of $2x10^5$ cells/well on glass coverslips in a 24-well plate in 500 µl of neutrophil cytokine and apoptosis media. After 12 hours culture, cells were fixed with 2% PFA, then labeled with DeadEnd fluorometric TUNEL system (Promega). Coverslips were washed twice in PBS, then cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. Coverslips were washed twice in PBS, then equilibrated in reaction buffer for 10 minutes. TdT reaction mixture was added and slides were incubated for 60 minutes at 37°C. Incorporation of nucleotides was stopped by incubation in 2x SSC buffer for 15 minutes. Coverslips were washed three times in excess PBS for 5 minutes each and mounted on Prolong Antifade mounting media with DAPI (Thermo Fisher) to counterstain nuclei. Fluorescence images were obtained on a Leica DM R upright epifluorescence microscope with Leica 20x/0.5 objective at 25°C. Percent TUNEL-positive (apoptotic) cells were determined by counting at least 200 cells across three images per coverslip. Data were plotted as the mean of the percent TUNEL-positive cells from three images per donor.

8.2.10 Quantitative Real Time PCR

8.2.10.1 Stimulation, RNA extraction, and cDNA conversion

RNA extracted from stimulated neutrophils was reverse transcribed into cDNA for qPCR analysis. Neutrophils, 10^7 per sample, were cultured in 10 ml of neutrophil cytokine and apoptosis media in 15 ml Falcon tubes at 37°C with rotation. Indicated inhibitors were added and cells were incubated for 30 minutes before addition of 100ng/ml LPS. At indicated time points post-stimulation, neutrophils were collected by centrifugation at 500 x g for 10 minutes. Cell pellets were resuspended in lysis buffer from RNeasy mini kit (Qiagen) and frozen at -80°C until

RNA isolation. RNA was isolated according to RNeasy mini kit using QIAShredder (Qiagen) homogenization. cDNA was made using High-capacity RNA-to-cDNA kit (Thermo Fischer) according to manufacturer's protocol.

8.2.10.2 qPCR and analysis

Quantative Real-time PCR was performed on StepOnePlus Real-Time PCR System with 2x Fast SYBR Green master mix (Thermo Fischer) according to protocol with cDNA made from 20 ng of RNA per reaction. Serial 1:5 dilutions of pooled samples served as a reference standard. Data were analyzed using StepOnePlus real-time PCR system and software (Thermo Fischer) and expressed as the relative amount of cytokine product as determined from pooled standard curve divided by relative amount of β2- microglobulin product at each time point.

Target	**Forward**	**Reverse**
IL-1β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACTGAC
IL-6	GACCCAACCACAAATGCCA	GTCATGTCCTGCAGCCACTG
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT
MIP-1α	AGCTGACTACTTTGAGACGAGCA	CGGCTTCGCTTGGTTAGGA
β-Microglobulin	CTCCGTGGCCTTAGCTGTG	TTTGGAGTACGCTGGATAGCCT

Table 8.1: qPCR primers, previously verified[19].

qPCR reaction conditions (in 20μl)	
10μl	SYBR green master mix (2x)
500 nM	Forward primer
500 nM	Reverse primer
20 ng	Template cDNA
To final 20μl	RNase-free water

Table 8.2: qPCR reaction conditions

Step	**Temperature** (°C)	**Duration**	**Cycles**
Amplitaq polymerase activation	95	20 sec	HOLD
Denature	95	3 sec	40
Anneal/extend	60	30 sec	HOLD
Melt curve analysis			

Table 8.3: qPCR thermocycling conditions

8.2.11 Intracellular IL-8 staining

Neutrophils were stimulated in the presence of a secretion inhibitor, resulting in intracellular accumulation of cytokines, which was measured my immunostaining by flow cytometry. Neutrophils were cultured in cytokine and apoptosis media supplemented with 3 µg/ml Brefeldin A to inhibit cytokine secretion, and stimulated with LPS as for cytokine production assays. At indicated time points, neutrophils were resuspended and fixed with 2% PFA, washed and resuspended in PBS with 1% BSA and stored at 4°C. Cells were permeabilized with 0.1% saponin, washed and resuspended in flow cytometry buffer, then stained with PE-mouse anti-human IL-8 or isotype control antibody from BD Pharminogen, and measured by flow cytometry on a MACSQuant cytometer (Miltenyi). At least 10,000 cells were measured per sample. Data were analyzed using FlowJo software.

8.2.12 Animal experiments

8.2.12.1 Approval of animal experiments

Animal experiments are in compliance with the German animal protection law and have been officially approved by the Landesamt für Gesundheit und Soziales, Berlin.

8.2.12.2 Isolation of peritoneal-elicited neutrophils

Mouse peritoneal cells were collected 5 hours after infection of 1 ml thioglycolate into the peritoneal cavity of 10-12 week old mice. The peritoneum was lavaged with 10 ml of PBS to collect the elicited cells. Cells were washed, resuspended in 1 ml PBS, mixed with 9 ml PBS-buffered Percoll solution, and centrifuged at 6,000 x g for 30 minutes to separate cells. The neutrophil-rich layer was isolated, washed, then cultured for cytokine secretion experiments, as described in the procedures for human neutrophils.

8.2.12.3 Differentiation of bone marrow-derived macrophages

Whole bone marrow cells were isolated by flushing femurs from 8-12 week old mice. After washing, cells were seeded in 10-cm non-tissue culture-treated plastic petri dishes at a density of $2x10^7$ per dish in BMM media. On day 3, 5 ml of fresh BMM media was added to each dish. On day 7, macrophages were harvested by washing twice with PBS to remove non-adherent cells, then incubating 15 minutes in cold PBS on ice to de-attach macrophages. Cells were washed in PBS, counted by CASY cell counter, then cultured for cytokine secretion experiments, as described in procedures for human neutrophils and monocytes, except in BMM media instead of neutrophil media.

8.3 Statistical analysis

Where indicated, statistics were performed on results of multiple compiled experiments with an n of 3-5. Raw measurements were analyzed in Graph Pad prism 5 software using the indicated statistical tests. In all figures statistical significance is indicated as follows: * P<.05, ** P<.01, *** P<.001

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 06.07.2009 ist mir bekannt.

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Prof. Dr. Med. Joachim Roesler	Universitätsklinikum Dresden
Prof. Dr. Med. Angela Kaindl	Charité - Universitätsmedizin Berlin

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