PARIS XIII - SORBONNE PARIS NORD UNIVERSITY

Galilée Doctoral School Sciences, Technologies, Health

Pro-inflammatory activity and adjuvant effect of Neutrophil Extracellular Traps in physiological and pathological context

---

DOCTORAL THESIS

By

Ahmad HAIDAR AHMAD

INSERM UMR 1125

A thesis submitted in conformity with the requirements for the degree

DOCTOR IN BIOLOGY,

MAJOR MOLECULAR AND CELL BIOLOGY

Orally defended on March 1st, 2024

Jury:

Prof. DE CHAISEMARTIN Luc, Bichat - Claude-Bernard Hospital	Reporter
Dr. WITKO-SARSAT Veronique, Institut Cochin	Reporter
Dr. BITOUN Samuel, Bicêtre Hospital	Examiner
Dr. GARDANO Laura, Sorbonne Paris Nord University	Examiner
Prof. DECKER Patrice, Sorbonne Paris Nord University	Thesis director

ACKNOWLEDGMENTS

I am immensely grateful to my thesis committee members. I appreciate your time and expertise in evaluating my work.

I would like to express my deepest gratitude to Prof. DE-CHAISEMARTIN Luc for graciously accepting to review this work. It is certainly an honor to benefit from his profound knowledge of neutrophil extracellular traps.

I would like to extend my profound gratitude to Dr. WITKO-SARSAT Veronique for graciously accepting to review this work. Dr. Witko-Sarsat's profound expertise and understanding of neutrophils promise to add significant insights to our discussion.

I would like to express my sincere gratitude to Dr. BITOUN Samuel for accepting to be an examiner for this work. His specialized knowledge in rheumatology will add significant insights to our discussion.

I would like to express my deepest gratitude to Dr. GARDANO Laura for accepting to be an examiner for this work. Her depth of knowledge on the topic of B lymphocytes promises to enrich the conversation surrounding this work.

I would like to acknowledge the financial support received from GALILEE doctoral school that enabled me to carry out this research.

I would like to express my heartfelt gratitude to the individuals who have supported and guided me throughout the journey of researching and writing this thesis

First, I am deeply indebted to my thesis advisor, Prof. DECKER Patrice. The scientific guidance I obtained from you was vital in propelling my project to the fruitful heights it has attained.

I would particularly like to thank Li2P lab for providing me with the required equipment, laboratory facilities, and a collaborative research atmosphere. I owe a debt of gratitude to the countless hours spent in the laboratory. This work would not have been accomplished without their assistance.

Prof. BOISSIER Marie-Christophe, I would like to thank you for giving me the opportunity to really grow in your lab.

My heartfelt gratitude goes to the engineers Delphine, Roxane, and Magali, whose tremendous experience and assistance were critical to the successful completion of my thesis. You play a pivotal role in this laboratory. Your encouragement during the stressful moments has been a source of strength and motivation. Thank you so much for all of your assistance. I can’t thank you enough. I would not also forget to express my greatest gratitude to Mylene, who has been always welcoming to provide us with blood samples.

I would like to thank Dr. BITON Jerome for explaining correlation analysis using R software to me, as well as for your insightful remarks and constructive criticism during the team meeting. I see how you cultivate your creativity and open-mindedness, two essential characteristics of an excellent scientific researcher.

I would like to thank Dr. RIVIERE Elodie for correcting part of my manuscript and for her kindness.

I would not forget to thank former and current helpful lab members Prof. BESSIS Natacha, Dr. ASSIER Eric, Dr. SEMERANO Luca, Dr. SIGAUX Johanna, and Dr. PAOLINI Lea.

To my dear friends Dyhia, Lyna, Megane, Meryeme, Ghozlane and Tessnime, who have provided both moral support and occasional distractions, thank you for being there. I am glad for the numerous conversations and moments of levity that made my journey more enjoyable. Thank you for being there with me through thick and thin. Your friendship has been a source of motivation and encouragement. I wish you all the best!

I would also like to thank Antonin, who helped me with my experiments on mice models. Laure and Betty for your help in FACS sorting cells.

In addition, I am very appreciative to all of those who repeatedly rolled up their sleeves to donate their blood in the name of science.

Furthermore, I want to express my gratitude to Fadi, Darine, Leo and Enzo. Who have been like a second family. Your unwavering support and help, since I have arrived in France, have been invaluable to me.

I would like to express my deepest appreciation to my family, Mona, Hassouna, Ali and Yamen, whose unwavering support and love have been the cornerstone of my journey in completing this thesis. You have been my rock, my inspiration, and my motivation, and I am forever indebted to you.

My deepest gratitude also goes to my close friends, Firas, Hussein and Mounir. Thank you for providing encouragement and laughter when I needed it most.

Lastly, I want to thank my love, Rim, who is everything to me, you are the best thing that ever happened to me, you make absolutely everything better in my life, thank you for supporting me, I love you.

I dedicate this dissertation to my parents, Mona and Hassouna, who have encouraged and supported me every step of the way.

"Research is the process of going up alleys to see if they are blind."

Marston Bates

SOMMAIRE

LIST OF ABBREVIATIONS

2-DG 2-Deoxy-D-glucose

8-OG 8-oxoguanine

AAT Alpha-1 antitrypsin

ADCC Antibody-dependent cellular cytotoxicity

ACPA Anti-citrullinated protein antibodies

ACR American College of Rheumatology

Ag Antigen

AIM2 Absent in melanoma 2

ANAs Anti-nuclear antibodies

ANCA Anti-neutrophilic cytoplasmic antibody

APC Antigen presenting cell

ARDS Acute respiratory distress syndrome

ASC Antibody secreting cells

ATP Adenosine triphosphate

BCR B cell receptor

BM Bone marrow

BP Bullous pemphigoid

Breg Regulatory B cell

C1q Complement factor 1q

CarP Carbamylated protein

CCL chemokine ligand

CCR Chemokine receptor

CD Cluster of differentiation

CDK Cyclin-dependent kinase

CF Cystic fibrosis

cGAS Cyclic-GMP-AMP synthase

CGD Chronic granulomatous disease

CIA Collagen-induced arthritis

CitH3 Citrullinated histone 3

CMP Common myeloid progenitor

COVID Coronavirus disease 2019

CpG Oligodeoxynucleotides

CRs Complement receptors

CRP C-reactive protein

CTLA-4 Cytotoxic T-lymphocyte associated protein 4

CQ Chloroquine

CXCL Chemokine (C-X-C motif) ligand 1

CXCR CX chemokine receptors

DAMPs Damage-associated molecular patterns

DAS Disease activity score

DCs Dendritic cells

DCFDA Dichlorofluorescein diacetate

DCFH Dichlorofluorescein

DLBCL Diffuse Large B-cell lymphoma

DMARDs Disease-modifying antirheumatic drugs

DN Double negative

DNase Deoxyribonuclease

DOX Doxorubicin

DPI Diphenyleneiodonium

Ds Double strand

EC Endothelial cell

ELS Ectopic lymphoid structures

ERK Extracellular signal-regulated kinases

EULAR European League Against Rheumatism

ESR Erythrocyte sedimentation rate

exRNAs Extracellular RNA

FACS Fluorescence-activated cell sorting

FAO Fatty acid oxidation

Fc Fragment crystallizable

FcR Fragment crystallizable rceptor

FLS Fibroblast-like synoviocytes

GC Germinal center

G-CSF Granulocyte-colony stimulating factor

GLUT Glucose transporters

GM-CSF Granulocyte-macrophage colony-stimulating factor

GMP Granulocyte-monocyte progenitor

H2O2 Hydrogen peroxide

HDs Healthy donors

HLA Human leukocyte antigens

HMGB1 High mobility group box 1

HIV Human immunodeficiency virus

HS Hidradenitis suppurativa

HSP Heat shock protein

ICAM-1 Intercellular adhesion molecule-1

Ics Immune complexes

IFN Interferon

Ig Immunoglobulin

IL Interleukin

Kmal Lysine malonylation

LDNs Low-density neutrophils

LN Lymph node

LPS Lipopolysaccharide

LN Lupus Nephritis

MAPK Mitogen-activated protein kinases

MHC Major histocompatibility complex

MIP-2 Macrophage inflammatory protein 2

MLKL mixed-lineage kinase domain-like

MMP Matrix metalloproteinase

Mnase Micrococcal nuclease

MPO Myeloperoxidase

mtROS Mitochondrial ROS

MTX Methotrexate

NAC N-acetylcysteine

NDNs Normal density neutrophils

NADPH Nicotinamide adenine dinucleotide phosphate

NE Neutrophil elastase

NETs Neutrophil extracellular traps

NF-kB Nuclear factor kappa betta

NLRP3 NOD-like receptor protein 3

NO Nitric oxide

NOX NADPH oxidases

OA Osteoarthritis

OC Osteoclast

OLFM4 Olfactomedin 4

OxPhos Oxidative phosphorylation

PAI-1 Plasminogen activator inhibitor-1

PAD4 Peptidylarginine deiminase 4

PBMCs Peripheral blood mononuclear cells

pDCs plasmacytoid dendritic cells

PD-L1 Programmed death-ligand 1

PI3K phosphoinositide-3-kinase

PMA Phorbol myristate acetate

PMNs Polymorphonuclear leukocytes

PPP Pentose phosphate pathway

PRR Pattern recognition receptor

PSGL1 P-selectin glycoprotein ligand 1

RA Rheumatoid arthritis

RANKL Receptor activator of nuclear factor kappa beta (NFkB ligand)

RF Rheumatoid factor

ROS Reactive oxygen species

RTX Rituximab

S1P sphingosine-1-phosphate

SEM Scanning electron microscopy

SF Synovial fluid

SHM somatic hypermutation

SIRL Signal inhibitory receptor on leukocytes

SLE Systemic lupus erythematosus

SLPI secretory leukocyte protease

SLOs secondary lymphoid organs

SNP Single nucleotide polymorphism

SOD Superoxide dismutase

STING Stimulator of interferon genes

Stx Shiga toxin

T1D Type 1 diabetes

TCR T-cell receptor

TFH T follicular helper cells

TGF Transforming growth factor

TLR Toll-like receptors

TNFα Tumor necrosis factor-α

TNF-R1 Type 1 TNF receptor

Treg Regulatory T cells

VEGF vascular endothelial growth factor

WT Wild type

LIST OF FIGURES

LIST OF TABELES

INTRODUCTION

I. Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the most frequent form of white blood cell in the peripheral blood in human and are involved in a variety of physiological and pathological processes in the body (**1**). Neutrophils were identified more than 100 years ago. These cells were first observed by Paul Ehrlich, who developed a method for staining leukocytes in 1880 (**2**). Historically defined as the soldiers of our innate immune system, they are the first line of cells recruited at the site of infection against a wide range of pathogens, including bacteria, fungi, and protozoa. Neutrophils are produced in the bone marrow (BM) from a common myeloid progenitor cell during granulopoiesis. They first emerge in human clavicular bone marrow 10 to 11 weeks after fertilization (**3**). Neutrophil precursors can be found in the peripheral blood by the end of the first trimester, and mature cells develop between 14 and 16 weeks of pregnancy (**4**). It is generally thought that neutrophil formation begins with the common myeloid progenitor (CMP), from which the granulocyte-monocyte progenitor (GMP) develops (**5**). Neutrophil development is divided into two stages: the proliferative stage, during which GMP differentiate into myeloblasts, promyelocytes, and myelocytes, and the nonproliferative stage, in which proliferative precursors give rise to nonproliferative metamyelocytes, band cells, and mature neutrophils (**5, figure 1**).

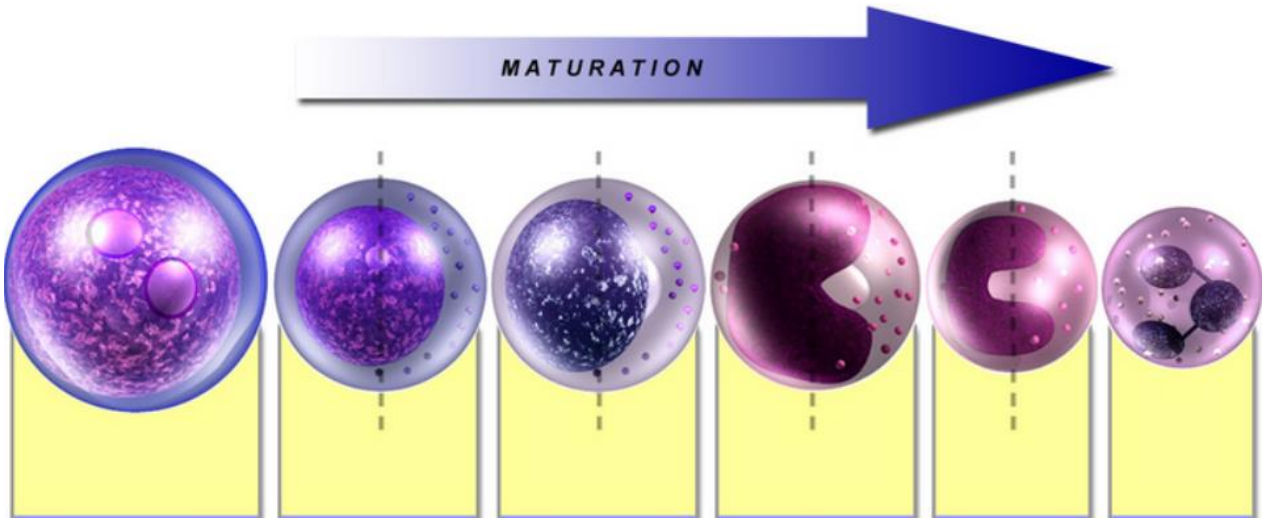


Figure 1 Neutrophils maturation diagram. Images demonstrating the steps of neutrophilic granulopoiesis. The myeloblast, the most immature stage, has a large nucleus with numerous nucleoli and a nongranular cytoplasm. During the promyelocyte stage, the cell expands and main granules develop in the cytoplasm. During the myelocyte stage, cell division stops and specialized granules develop in the cytoplasm. During the last phases of differentiation, the cell decreases and changes in nuclear morphology become more visible. The nucleus begins to indent during the metamyelocyte stage, acquiring a horseshoe shape as a band cell and eventually becoming multilobulated in the mature neutrophil.

Granules are formed at various phases of neutrophil growth. Notably, azurophil granules mark the transition from myeloblasts to promyelocytes; specific granules appear in the myelocyte/metamyelocyte stage; gelatinase granules are seen in band cells; and secretory vesicles are found in segmented cells (**6**).

Neutrophils are generated at a rate of 10^{11} per day, which can rise to 10^{12} per day during a bacterial infection (**5**). In adults, the approximate normal range for the number of white blood cells is 4,000 to 11,000 cells/microL, of which 60-70% are mature neutrophils circulating in the peripheral blood in human, and 10–25% in mice (**7**). Under homeostatic conditions, neutrophils enter the circulation, migrate to the tissues, where they perform their functions, and are finally eliminated by macrophages. In the circulation, mature neutrophils have a segmented nucleus and measure ~7-10 µm in diameter with a cytoplasm enriched with granules and secretory vesicles (**1**). Furthermore, the regulation of neutrophil circulation rates is an important feature of the local and systemic response to inflammatory stimuli. Neutrophil homeostasis in the peripheral circulation is tightly regulated and maintained by balancing neutrophil production in the bone marrow, neutrophil release from the bone marrow, and neutrophil elimination into the circulation. Mature neutrophils are retained in the bone marrow reserve and are continuously released into the circulation under physiological conditions (**8**). CXCR4/CXCL12 and VLA-4/VCAM-1 pathways are important regulators of neutrophil retention in the BM (**8**). In response to various infections, neutrophil release from BM increases rapidly and this process is mediated by CXCR2, whose ligands include the chemokines CXCL1, CXCL2, CXCL5, CXCL6, CXCL8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (**8**). In addition, stress and systemic inflammation are associated with circulating neutrophilia, and various inflammatory mediators, including leukotriene B4, the complement component C5a, Interleukin (IL)-8 and tumor necrosis factor-α (TNFα), have been shown to induce neutrophil migration when injected into mouse models (**8, 9**).

A. Neutrophil lifespan

In 1930, the lifetime of a rabbit neutrophil was initially predicted to be 3-4 days (**10**). However, neutrophils have always been considered short-lived cells, and traditional calculations based on ex-vivo survival in culture or half-life after adoptive transfer have shown that these cells survive only 8 to 12 hours in the circulation and up to 1 to 2 days in tissues, their renewal being slowed or

accelerated during an inflammatory response (**5, 11, 12**). But according to a study, the typical lifespan of neutrophils under basal conditions is 12.5 hours for mouse PMNs and 4 to 5 days for human PMNs, which is much longer than generally accepted (**13**). The methodological techniques used in this study to determine PMNs lifespan led to some criticism of the results and a potential overestimation of neutrophil lifespan in blood (**14**). However, during inflammation, factors including cytokines, chemokines, hormones, lipid mediators, and damage-associated molecular patterns (DAMPs), could increase neutrophil survival, ensuring the presence of neutrophils at the site of inflammation. As a result, these findings suggest that precise neutrophil lifespan remains challenging to estimate, raising the potential that neutrophil lifespan is very context-dependent (**15**).

B. Neutrophil migration

Neutrophils are the first line of cellular defense against invading pathogens in tissues, by crossing the endothelial cell (EC) barrier. Neutrophil tissue infiltration is essential for pathogen clearance and tissue repair, and it is tightly controlled because abnormal neutrophil accumulation in tissues causes tissue damage, and leads to severe pathological disorders as shown in multiple organ dysfunction syndrome, vascular inflammation and rheumatoid arthritis (RA) by abnormal production of pro-inflammatory cytokines, generation of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETs). The recruitment of neutrophils into tissue is a multi-step process known as the neutrophil extravasation cascade: tethering, rolling, adhesion, crawling and, finally, transmigration (16), involving various adhesion molecules and receptors. They normally migrate from capillaries to the site of inflammation, following the highest level of a chemotactic gradient (16).

1. Neutrophil transmigration mechanism

Neutrophil recruitment is initiated by changes on the surface of the endothelium caused by inflammatory mediators (including histamine, bacteria-derived peptides and different cytokines) generated by tissue-resident leukocytes when they come into contact with pathogens. These mediators stimulate the expression of adhesion molecules on the endothelium luminal surface (16). There are three types of selectins: L-selectin (CD62L), P-selectin (CD62P), and E-selectin (CD62E). They regulate neutrophil trafficking to sites of inflammation (16). Without selectins, inflammatory cell recruitment is significantly reduced (17). P-selectin is stored in Weibel-Palade

bodies within EC and can be rapidly translocated to the cell surface upon activation, E-selectin is inducible and is elevated in 90 minutes upon activation. L-selectin is constitutively expressed on the surface of circulating neutrophils (16). Circulated neutrophils are first captured onto the EC surface, due to the interaction between selectins and their glycosylated ligands, including P-selectin glycoprotein ligand 1 (PSGL1) expressed on the surface of neutrophils (16). Moreover, L-selectin expression by circulating neutrophils may facilitate subsequent catching to an already rolling neutrophil (18). This early adhesive interaction mediated by selectins is weak and temporary, and it promotes neutrophil tethering and rolling along the endothelium of blood vessels, which can be in the direction of or against the blood flow. To allow neutrophils to roll effectively, the dissociation of a selectin-ligand interaction at the cell's back should be balanced by the formation of another interaction at the cell's front. As neutrophils roll along the endothelial surface, they become activated in response to chemotactic factors released at the site of inflammation. This activation triggers a conformational change in the integrins expressed on the surface of neutrophils, such as β2 integrins (e.g., LFA-1). This leads to an enhanced affinity for cell adhesion ligands expressed on the endothelium. Activated integrins bind to their ligands, such as ICAM-1 (intercellular adhesion molecule-1), expressed on endothelial cells, allowing firm adherence and neutrophil arrest on the endothelial surface (16).

The adhesion step of the recruitment cascade prepares neutrophils for transmigration. This process allows the neutrophils to resist the shear forces exerted by the flowing blood. Once tightly tethered, neutrophils flatten and polarize in order to crawl on the endothelial lumen surface and identify a gap for transmigration across the endothelium barrier. This is known as diapedesis or transmigration, allowing neutrophils to exit the bloodstream and migrate to sites of infection or tissue damage. Interestingly, neutrophils may cross the EC barrier in two ways: between two EC (per acellular route) or straight through them (transcellular route) (**16**). Finally, the cells must pass through the pericyte layer and the venular basal membrane before reaching the inflamed interstitial tissues. Furthermore, when compared to circulating neutrophils, transendothelial cell-migrated and tissue-infiltrated neutrophils exhibit an extended survival, increased migration capacity, and increased cytotoxicity due to increased ROS production or NET formation or protease release (**19**).

However, in certain situations, neutrophils may reverse migration, leaving the tissue and returning to circulation. This process has been reported in a variety of circumstances, including during the inflammatory resolution phase (**20**).

2. Chemokines

Chemokines include CXCL8 (also known as IL-8) in humans, CXCL2 and CXCL5. Via the chemokine receptor CXCR2, they activate neutrophils and subsequently enhance their adhesion to the endothelium (**16**). Similarly, Toll-like receptors (TLR)-2 and TLR4 signals cause the expression of CXCR2 on the surface of neutrophils. CXCR2 levels correlate with disease severity during sepsis and neutrophils isolated from non-surviving patients express CXCR2 more than those derived from surviving patients (**21**).

Individual chemokines have distinct and overlapping functions in the recruitment of neutrophils throughout disease beginning and development. In inflammatory environement, such as in RA, neutrophils generate chemokines like CXCL2 and CCL3, and they also stimulate the production of chemokines like CXCL1, CXCL5, and CCL9 by fibroblast-like synoviocytes, endothelial cells, and macrophages (**22**). Although multiple pathophysiological changes occur during progression of disease, in ulcerative colitis, plasminogen activator inhibitor-1 (PAI-1) is upregulated by epithelial cells, and it can induce neutrophil-mediated chemokine expression of CXCL1 and CXCL5, and neutrophils recruitment to inflamed sites leads to local colon tissue injury in a mouse model (**23**). The transient-receptor-potential channel-kinase (TRPM7) Akt1/mTOR signaling regulate neutrophil transmigration in response to chemokine (**24**).

C. Neutrophil apoptosis

Neutrophils are usually released into the circulation within 10-24 h before migrating into tissues upon recruitment. Neutrophil cell death is an essential event to maintain neutrophil numbers during infection or inflammation and in homeostatic conditions as approximately 10^{11} neutrophils are generated every day in normal adult individuals (**5**). Indeed, after performing crucial functions, neutrophils trigger a spontaneous apoptotic program in response to various intracellular or extracellular factors. Apoptosis plays an important role in eliminating neutrophils from inflamed tissues and plays a crucial role in the resolution of inflammation. Thus, neutrophil apoptosis regulates both the duration and intensity of an inflammatory response, as well as the level of neutrophil-mediated tissue damage. Deficient neutrophil clearance by synovial macrophages may

be an essential factor contributing to the propagation of diseases such as RA (**25**). It is considered that neutrophils are mainly cleared from circulation in the liver, spleen, and bone marrow under physiological conditions.

Apoptosis is a non-inflammatory process and occurs with minimal damage to the surrounding tissue. Neutrophils are no longer functional at early stage of this death, accompanied by a decrease in the expression of surface receptors and adhesion molecules, and chemokine production, respiratory burst and degranulation are impaired (**26**). Thereafter apoptotic neutrophils are phagocytosed by other cells, mainly macrophages, which results in the release of anti-inflammatory compounds.

1. Delayed and accelerated apoptosis

However, during immune responses, neutrophil apoptosis can be accelerated or prolonged depending on specific cellular stimuli, and it has been demonstrated experimentally that a number of signaling pathways and inflammatory processes, including adhesion, migration, exposure to pro-inflammatory cytokines, and hypoxia, can delay or accelerate neutrophil apoptosis. Delayed neutrophil apoptosis has been linked to a variety of acute and chronic inflammatory disorders such as in RA (**27**). Signaling pathways including phosphoinositide 3-kinases (PI3K), extracellular signal-regulated kinases (ERK), and Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), triggered by different stimuli as granulocyte-colony stimulating factor (G-CSF), IL-8, C reactive protein, some bacteria, and lipopolysaccharides (LPS) can temporarily delay apoptosis and extend neutrophil life span in order to promote cell viability as they migrate from the bloodstream into infected and inflamed tissues (**28**). Likewise, GU-rich sequences in SARS-CoV-2 genome may delay spontaneous neutrophil apoptosis via TLR8-dependent pathways (**29**). A neutrophil subset with delayed apoptosis was identified in a recent study, which expresses the dual endothelin-1/signal peptide receptor (DEspR) and is associated with injury and mortality in patients with acute respiratory distress syndrome (ARDS). In neurologic rat model, reversing delayed apoptosis in $DEspR^+$ $CD11b^+$ neutrophils using anti-DEspR reduces neurologic symptoms (**30**). Interestingly, delayed apoptosis in ARDS neutrophils may enhance NET formation (**31**).

In contrast, multiple studies have revealed that ligation of the prototypic death receptor, such as pro-apoptotic B-cell lymphoma-2 (Bcl-2) family, could further accelerate apoptosis (**32**). However, the pro-apoptotic effects of death receptors can be suppressed in neutrophils by

inflammatory mediators present at sites of inflammation. Moreover, ROS could trigger neutrophil apoptosis in a P53-dependent manner (**33**). TNF-α exerts a biphasic effect on neutrophil apoptosis. Through accelerated degradation of the anti-apoptotic protein, myeloid cell leukemia 1 (Mcl-1), it may additionally accelerate neutrophil death at early stages (**34**), alternatively, TNF-α may induce a delay in PMN apoptosis via JNK/FoxO3a pathway (**35**). Moreover, human neutrophils express the cyclin-dependent kinase (CDK)-2, 7, and 9, and inhibiting these kinases may lead to accelerated apoptosis (**31**).

D. Neutrophil populations

Neutrophils have long been considered a homogeneous population with a conserved phenotype and function, with similar appearances microscopically once mature (**36**). Traditionally, neutrophils may be identified by their characteristic nuclear morphology, as well as histological hematoxylin and eosin staining. As long ago as 1920, it was reported that neutrophils are not the homogenous leucocyte subset commonly assumed and that circulating neutrophils might differ significantly in aspects such as phagocytosis, protein synthesis, and oxidative metabolism (**37**). Currently, neutrophils are classified into different subsets based on their characteristics, transcriptome, surface protein expression, and functions. Furthermore, several clusters of differentiation (CD) markers have been identified on human neutrophils isolated from different sites, activation levels, and contexts (**38**). Currently, CD10 (neutral endopeptidase), CD11b, CD14, CD15, CD16, CD66b and CD62L (L-selectin) are commonly used for identifying human neutrophil populations (**38**). They may be used also in combination with several receptors implicated in neutrophil functions including CD64 (FcγRI), CD32 (FcγRIIa), CD89 (FcαR, IgA receptor), complement receptors (CRs) CD35 (CR1), and CD11c/CD18 (CR4), and CD177 (a specific marker to neutrophils) (**39**). CD10 is used as a marker for distinguishing mature neutrophils from immature neutrophils (**40**). Beyond maturational changes, neutrophils shift phenotype with activation. Neutrophils are highly flexible, developing various phenotypes and subsets in response to a wide range of physiological (e.g., age-related effects) and pathological (e.g., inflammation and infection) conditions. As a result, many questions still exist, such as whether each subgroup of neutrophils exerts similar functions or not such as NET formation in response to stimuli, and if the produced NETs differ from one another.

Additionally, neutrophils express new surface markers important for their functions. Human neutrophil migration is associated with the emergence of surface ICAM-1, an increase in CD18, and a decrease in CD62L, CXCR1 and CXCR2. Activated neutrophils undergo a number of functional and phenotype modifications when compared to resting neutrophils. Some surface markers are lost upon activation, including CD62L, which is rapidly shed in activated neutrophils (**41**). However, a recent study showed that $CD62L^{low}$ neutrophils may not be considered activated mature neutrophils, but rather a different form of neutrophil that is recruited to the bloodstream in response to acute inflammation (**42**).

Neutrophil populations can be categorized based on certain characteristics, including specific surface markers, maturity, and density.

1. Based on maturity

Neutrophils undergo a series of maturation stages in the bone marrow before being released into circulation. These stages reflect the morphological changes that neutrophils undergo as they mature. Mature human neutrophils have a multilobular nucleus and exhibit an almost uniform signature of $CD11b^{+}CD16^{hi}CD62L^{hi}CD10^{hi}$ (**43**). Immature neutrophils present uncompleted segmentation of the nucleus. $CD16^{low}$ expressing neutrophils are considered as immature neutrophils because the nucleus of $CD16^{low}$ neutrophils is not completely segmented and is bigger in diameter than the nucleus of mature $CD16^{high}CD62L^{high}$ neutrophils with complete segmentation (**44**). Immature neutrophils express higher levels of CXCR4 than mature neutrophils, which may promote their retention in the bone marrow (**45**).

The degree of functional difference between immature and mature neutrophils remains to be determined because neutrophil differentiation is critical for their effector mechanisms. Traditionally, it was considered that immature neutrophils are less competent, having a lower capacity to fight infection, and lower migration capacity compared to mature neutrophils. For instance, the murine spleen is found to comprise mature $Ly6G^{high}$ neutrophils with high phagocytic capacity, as well as immature $Ly6G^{low}$ neutrophils with a conserved capacity for proliferation, restricted mobility, and low phagocytic capacity (**46**). In addition, the capacity of immature human neutrophils to produce NETs is reduced following interferon activation (**47**). However, it was recently discovered that immature neutrophil populations had a better phagocytic capacity and migration (**44**).

High numbers of immature neutrophils have been linked to a variety of inflammatory contexts. Immature $CD15^{pos}CD16^{neg}$ neutrophils are significantly elevated in RA bone marrow (**48**). In addition, immature neutrophils have been detected higher in patients with systemic lupus erythematosus (SLE) (**49**).

2. Normal and low-density neutrophils

Neutrophil granule density has also been used to identify neutrophil populations. It has been shown that during acute and chronic inflammation, a population of neutrophils co-sediments with peripheral blood mononuclear cells (PBMCs) following gradient centrifugation (Figure 2). As a result, this neutrophil population is known as low-density neutrophils (LDNs), LDNs have a comparable density to PBMCs and are isolated with them, as opposed to normal density neutrophils (NDNs), which segregate with other polymorphnuclear cells during density exclusion.

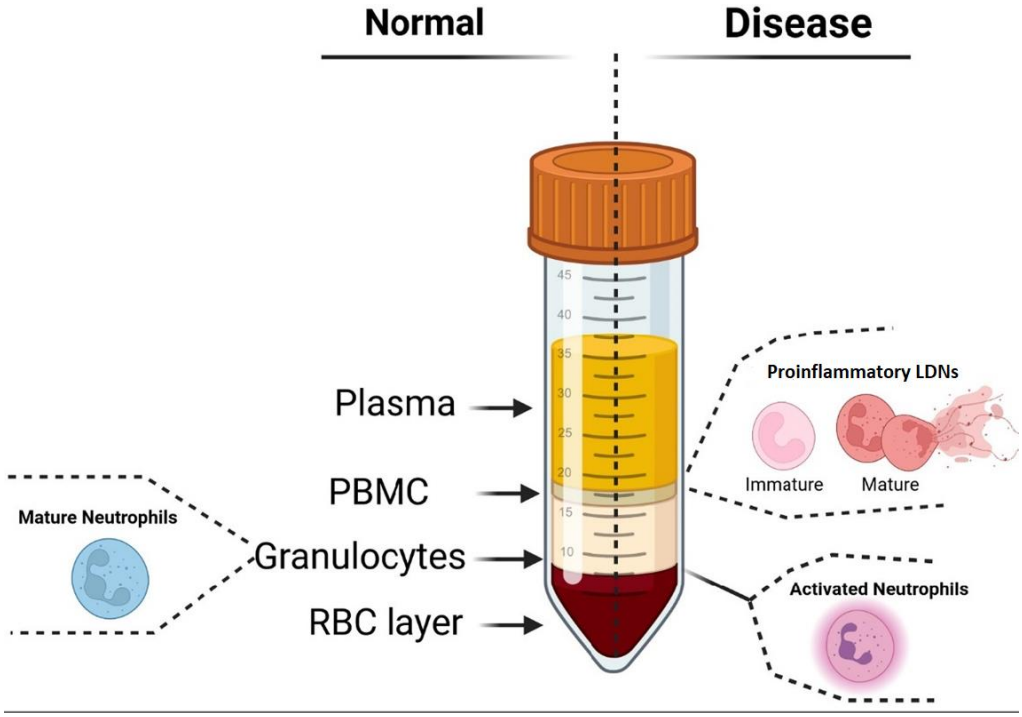


Figure 2 Separation of blood cells by Ficoll density gradient centrifugation. Low density neutrophils (LDNs) colocalize with mononuclear cells. After a Ficoll gradient centrifugation, heterogenous populations of neutrophils can be obtained from blood from normal (left) or inflammatory condition (right). Granulocytes (including normal dense neutrophils) sediment to the bottom of the tube on top of the red blood cell layer (RBC).

LDNs were originally identified in patients with SLE and RA. LDNs have been also identified in pregnancy, autoimmune diseases, cancer, infection, and inflammation, and are thought to have a role in pathogenesis (**50**).

The markers employed to identify LDNs varied among research and disease categories. In comparison to the traditional isolation approach, magnetic sorting followed by percoll density gradient is capable of separating high-purity LDNs and NDNs from blood (**51**). This methodology enabled the identification of the potential phenotype of LDNs and NDNs cells. Recently, CD98 has been used to identify LDNs relative to NDNs (**52**). In SLE, $CD98^{pos}$ LDNs have increased mitochondrial respiratory capacity compared to $CD98^{neg}$ NDNs (**52**). CD98 is a transmembrane protein that dimerises with L-type amino acid transporters. Furthermore, CD16 expression on LDNs is decreased in rheumatoid arthritis (**53**). Moreover, both immature and mature neutrophils are found in LDNs population. In severe Coronavirus disease (COVID-19) patients, both $CD10^{neg}$ (immature) and $CD10^{pos}$ (mature) LDN populations were significantly higher. In the context of COVID-19, $CD10^{low}$ LDNs have an immunosuppressive profile (**54**), but mature LDNs expressing higher levels of CD10 and CD16 are pro-inflammatory and are more likely to produce NETs (**54**).

01. Involvement in diseases

LDNs may be directly generated by inflammation. Increased LDN numbers are observed in many inflammatory environments. LDN counts were elevated in different diseases such as cancer, virus infection (human immunodeficiency virus (HIV), COVID-19), and autoimmune disorders compared with healthy donors (HDs), and correlate with disease activity (**50**). LDN levels are higher in advanced cancer patients compared to early-stage cancer patients or healthy controls (**55**), and there is an important association between high circulating LDN levels and resistance to anti-PD1/PDL1 immunotherapy in lung cancer (**56**). Furthermore, when activated by TNF or LPS, healthy NDNs can generate LDNs, implying that activation during various disease states might affect the production of LDNs from already circulating NDNs (**51**). Moreover, LDNs produce increased level of pro-inflammatory cytokines and chemokines in response to LPS compared to NDNs (**52**).

02. LDNs vs. NDNs functions

Role of LDNs and NDNs is still evolving, their role could vary in different disease states. SLE LDNs have a drastically different proteome and metabolic profile than SLE NDNs. LDNs exhibit increased pathways related to translational activity, intracellular trafficking, and type I IFN-induced protein pathways (**52, 57**). In vitro, SLE LDNs were able to activate T cells (**58**), while LDNs from HDs or cancer patients have been found to suppress T-cell proliferation (**51, 59**).

Additionally, high-purity LDNs are found to have similar ROS production and NET formation capacity in response to PMA and no difference in apoptosis compared to NDNs in healthy individuals (**51**). In contrast, RA LDNs exhibit a substantially altered transcriptome, expressing elevated levels of transcripts for granule proteins (including elastase, Matrix metalloproteases (MMPs) and Myeloperoxidase (MPO)) compared with RA NDNs (**53**).

3. Based on surface marker expression

Neutrophils can also be classified into different populations based on the expression of specific surface markers.

01. CD177 neutrophil subset

A marker that characterizes neutrophil subgroups is the surface glycoprotein CD177 which is only expressed in neutrophils and regulates transmigration across the endothelium. CD177 is a neutrophil-specific receptor that presents the proteinase 3 (PR3) antigen on the neutrophil surface. A study on the membrane expression of PR3 in neutrophils confirmed its bimodal distribution. The bimodal distribution of CD177 is therefore the same as that of membrane PR3 expression in a resting neutrophil (**60**).

CD177 is expressed by around 40% of neutrophils in healthy individuals (**61**). CD177 is localized in the specific granule, remaining in the granule membrane for fast mobilization to the surface upon cell activation. It reportedly plays a key role in the genesis of several inflammatory diseases. A population of $CD177^{pos}CD16^{pos}$ neutrophils is increased in patients with acute pancreatitis and it is associated with disease severity (**62**). In RA patients, the percentage of $CD177^{high}$ neutrophils is augmented and they show increased ROS production. Methotrexate (MTX) treatment was associated with a reduction in ROS production and CD177 expression (**39**). $CD177^{pos}$ neutrophils are also augmented in pregnancy, during bacterial infection, systemic lupus erythematosus, pathogen-induced colitis, cancer, and COVID-19 (**39, 61**).

The differential immunological functions of $CD177^{pos}$ and $CD177^{neg}$ neutrophils are not fully characterized. Studies of circulating $CD177^{pos}$ and $CD177^{neg}$ neutrophils demonstrate equivalent expression of integrins and Fc receptors, fibronectin adhesion, and in vivo migration to the inflamed joint (**63**). However, $CD177^{pos}$ neutrophils have been found to be more prone to NET formation and to produce more IL-22 and TGF-β than $CD177^{neg}$ neutrophils (**64**). Furthermore,

NET formation by $CD177^{pos}$ neutrophils was linked to tissue damage in diseases such as biliary atresia (BA) (**64**).

02. Olfactomedin 4 neutrophil

Olfactomedin 4 (OLFM4) is a neutrophil-specific granule protein, that belongs to the olfactomedin domain-containing glycoprotein family. It has been proposed that the expression of OLMF4 defines two such neutrophil subgroups, expressing or not OLFM4. OLFM4 is expressed in a subset of human and mice neutrophils (**65, 66**). Approximately 25% of neutrophils in healthy human donors express OLFM4 (**66**). OLFM4 expression appears in normal and low-density neutrophil populations (**67**). Similarly, OLFM4 expression increases with neutrophil maturation, and it is found to be not required for neutrophil development, phagocytosis, or migration (**66**).

The proteomic profiles of the fluorescence-activated cell sorting (FACS) -sorted OLFM4-defined neutrophil subsets from HDs are different (**67**). However, $OLFM4^{pos}$ and $OLFM4^{neg}$ neutrophils present similar phagocytosis, migration and ROS production (**65, 67**). Interestingly, the composition of NETs produced by these two subsets was different upon PMA stimulation, OLFM4 is only found in NETs produced by $OLFM4^{pos}$ neutrophils (**65**).

Studies have begun to elucidate the potential functions of OLFM4. OLFM4 expression in human neutrophils increases dramatically during sepsis and is associated with patient mortality (**68**). Moreover, OLFM4 has been identified as a new target for anti-neutrophilic cytoplasmic antibody (ANCA) -associated vasculitis (AAV) (**69**).

E. Neutrophil functions

Neutrophils play a crucial role in the immune system's defense against infections. Their primary function is to identify and destroy harmful microorganisms, such as bacteria and fungi, to protect the body from infections.

1. Phagocytosis

Phagocytosis is a biological process in which a cell detects, internalizes, and destroys a bacterium, fungus, or dead cell, which is essential for the maintenance of host health. Neutrophils and macrophages comprise the professional phagocytic cells. They are equipped with receptors that allow them to detect their targets and have a unique ability to engulf and thereby eliminate pathogens and cell debris. After recognizing the target particle, phagocytic receptors activate

signaling cascades that change lipids in the cell membrane and control the actin cytoskeleton in order to extend the cell membrane around the particle. Neutrophils, like macrophages, may phagocyte both opsonized and non-opsonized particles. The main opsonin receptors of neutrophils, Fc receptors, and β2 integrins, bind to immunoglobulin or complement-coated particles, respectively.

Receptor shedding is a method for the irreversible loss of transmembrane cell surface receptors by proteolysis of the receptor at the plasma membrane. TLR7/8 activation has been found to induce FcγRIIA shedding, thereby shifting neutrophil function from phagocytosis of immune complexes (ICs) to NET formation (**70**). In contrast, when priming neutrophils phagocytosis with a phagocytic signal, IC-mediated NET formation was totally impaired (**70**).

Neutrophil phagocytic function plays a beneficial and harmful role in different contexts. Bacterial phagocytosis by neutrophils is critical in the clearance of invading pathogens, particularly Staphylococcus aureus. Abnormal phagocytosis renders the host vulnerable to bacterial infections. In septic arthritis, an increased concentration of neutrophils in the joint as a result of locally photodynamic treatment promotes bacteria clearance and reduces tissue injury (**71**). In an inflammatory environment, phagocytosis may be beneficial, as impaired clearance of ICs by neutrophils induces increased complement C5a generation (**70**). Neutrophil phagocytic activity triggers the synthesis of several immunomodulatory factors, which recruit additional neutrophils, modulate subsequent neutrophil responses, and coordinate responses of other cell types like macrophages, dendritic cells and lymphocytes, thereby providing a link between innate and acquired immune responses.

The ability of neutrophils to phagocytose is known to be impacted by factors such as disease activity. Increased phagocytosis capacity was found in PMNs from SLE patients with high compared to low disease activity (**72**). Similarly, phagocytosis has been proven to be enhanced in neutrophils of RA patients compared to those of healthy individuals (**73**). Moreover, phagocytosis via opsonin was found to be increased in neutrophils from Anti-citrullinated protein antibodiies (ACPA) and rheumatoid factor (RF)-positive RA patients compared to seronegative patients (**73**).

2. Degranulation

Degranulation is the process by which neutrophils release the granules. Degranulation can occur at the plasma membrane to kill external pathogens. Intracellular degranulation involves the release

of granule contents within the cytoplasm of the neutrophil. Degranulation is necessary for pathogen killing, but it also influences the immune response during infectious and non-infectious disorders. Mature neutrophils have a multi-lobed nucleus, few mitochondria, little Golgi structure, and a highly granular cytoplasm containing vesicles. Neutrophils carry a membrane-bound organelles called the secretory vesicle and three types of cytoplasmic granules that influence cell function: primary or azurophilic granules, secondary or specific granules, and tertiary or gelatinase-containing granules (**74, figure 3**).

01. Granule subsets

Granule subsets are characterized by their protein composition and ability for mobilization. Primary granules are generated earliest during neutrophil development. They are called from their capacity to take up the basic dye azure A and store myeloperoxidase, lysozyme, defensins, most potent proteolytic (including neutrophil elastase (NE), proteinase 3, and cathepsin G), and bactericidal proteins, and seems to be the microbicidal compartment that is mobilized during phagocytosis (**6**). The specific granules class is made of granules that are smaller, do not have MPO, and are distinguished by the presence of the glycoprotein lactoferrin and antimicrobial substances such as neutrophil gelatinase-associated lipocalin, human cationic antimicrobial protein-18, and lysozyme (**6**). Gelatinase granules do not contain MPO, they are smaller than specific granules and contain few antimicrobial compounds, but they serve as a storage site for many metalloproteases, including matrix MMP-9, gelatinase, and leukolysin (**6**). Tertiary granules are important in the modulation of neutrophil biology because they contain critical components of primary functional responses of neutrophils during inflammation: superoxide anion production, cell adhesion (CD11b, CD11c), and extravasation (MMP-9, Rap1) (**6**). Recently, a fourth granule population enriched in the anti-microbial lectin ficolin-1 was identified. Ficolin-1 is present in tertiary granules; nevertheless, the authors discovered a second pool of ficolin-1-rich granules with a high ability for mobilization (**75**).

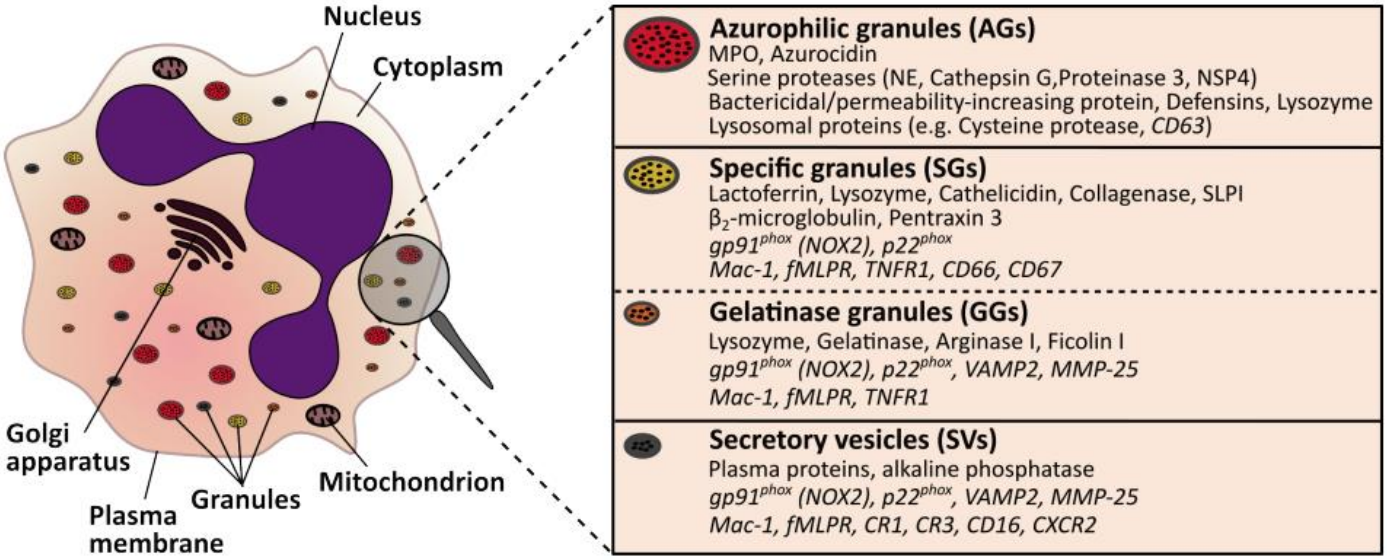


Figure 3. **Architecture of human neutrophil granulocytes.** Neutrophils exhibit two prominent morphological characteristics. lobulated nuclei and neutrophil granules. Neutrophil granules have a unique composition. Types of granules: azurophilic granules (AGs), secretory and gelatinase granules (SGs, GGs) and secretory vesicles (SVs). MPO: myeloperoxidase, NE: neutrophil elastase, NSP4: neutrophil serine protease 4, SLPI: secretory leukocyte protease inhibitor, CD: cluster of differentiation, Mac-1: macrophage-1 antigen, fMLPR: N-formyl-methionyl-leucyl-phenylalanine receptor, TNFR1: tumor necrosis factor receptor 1, VAMP2: vesicle-associated membrane protein 2, MMP-25: matrix metalloproteinase-25, CR1/3: complement receptor 1/3, CXCR2: CXC chemokine receptor 2.

02. Abnormal degranulation

Degranulation is triggered by neutrophil activation with microbial or inflammatory stimuli. Pro-inflammatory cytokines, such as TNF-alpha, GM-CSF or hypoxia promote neutrophil degranulation and increase the risk of tissue injury (**76**). Neutrophil degranulation is enhanced and promotes the pathogenesis of different disorders (**76**). Neutrophil degranulation can accelerate cancer development, by promoting the invasion and migration of cancer cells (**77**). Furthermore, NE is a serine protease found in azurophilic granules that plays a role in host defense against bacteria. NE is associated with several diseases. For instance, NE is abundant in the surface fluids of cystic fibrosis (CF) airways (**78**). However, patients with CF may be protected from SARS-CoV-2 viral infection by NE-mediated cleavage of the protein binding domain from the bronchial epithelia (**78**).

3. ROS production

A powerful weapon of neutrophils against bacterial and fungal infections is the generation of reactive oxygen species, also known as "oxidative burst." Small, reactive compounds generated from oxygen are referred to as ROS. These include non-radical molecules (with paired electrons),

such as singlet oxygen ($^1O2$), hydrogen peroxide (H2O2), organic peroxides (ROOH, hydroperoxides), and ozone (O3), as well as free radicals (molecules containing one or more free electrons), such as superoxide (O2•−), hydroxyl (OH•), peroxyl (ROO•), and alkoxyl (RO•) (**79**).

01. Mechanism of ROS production

001. NADPH oxidase

In an inflammatory environment, or in response to various microorganisms and soluble agonists, neutrophils generate ROS via different mechanisms. The major part is by inducing nicotinamide adenine dinucleotide phosphate (NADPH) pathway (oxidative burst of neutrophils is shown in figure 4), which is one of the main producers of ROS in PMNs (**80**), and which belongs to the NADPH oxidase (NOX) family (NOX1, NOX2/gp91 (phox), NOX3, NOX4, NOX5, DUOX1, and DUOX2). NOX is a protein complex composed of multiple membrane-associated subunits, which can assemble both at the phagosomal membrane as well as at the PMN surface. NADPH oxidase is expressed in phagocytic and non-phagocytic cells (**80**), and Nox2 is the well-known member of the NOX family.

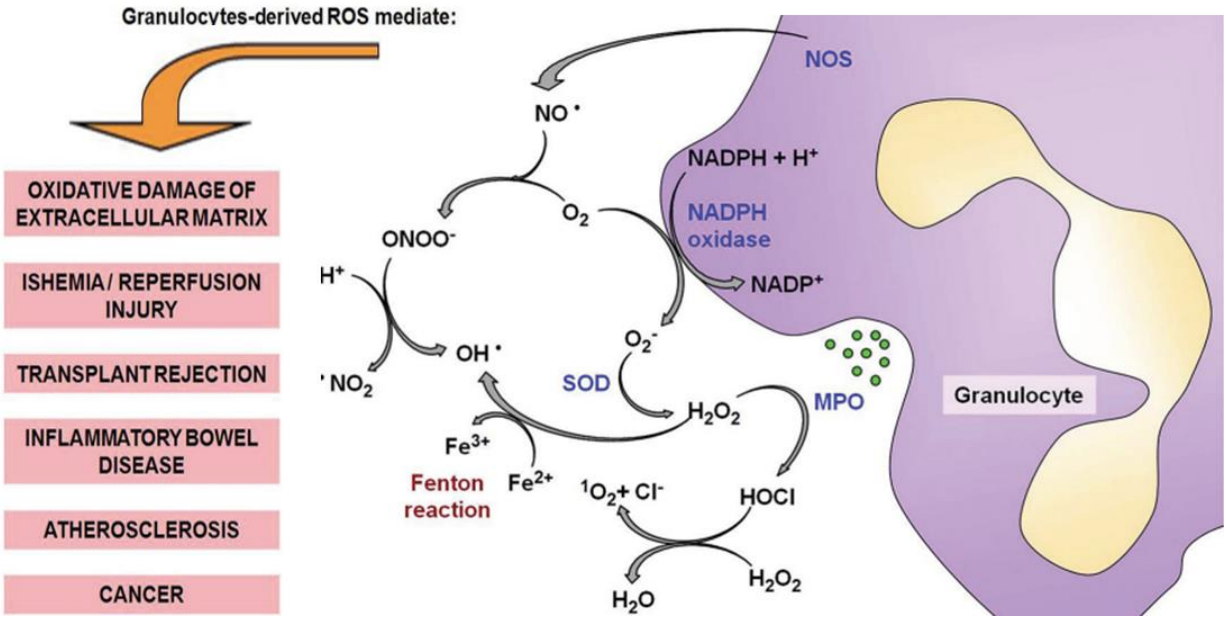


Figure 4. Chain of production of oxygenated free radicals in granulocytes, from oxygen (O2), under the effect of NADPH oxidase, transfer of H+ ions from NADPH and production of intermediate radicals superoxide ions (O2-) and hydrogen peroxides (H2O2).

002. Mitochondria

Mitochondria may play a role in regulating the motility and migration of neutrophils. It can also influence cell lifespan and the synthesis of inflammatory mediators. Moreover, mitochondria are another non-NADPH oxidase source of ROS within PMNs (**81**). Formation of mitochondrial ROS (mtROS) occurs primarily in the electron transport chain located in the inner mitochondrial membrane during oxidative phosphorylation. Neutrophils are primarily highly glycolytic cells, dependent on glucose, and produce little ATP from oxidative phosphorylation. Few mitochondria are present in the human neutrophils and have a very low and limited activity (**82**). It is therefore critical for neutrophil survival at sites of inflammation or infection where the local oxygen tension is generally very low. Stimuli such as intracellular $Ca2^{+}$ release which typically takes place downstream of pathogen recognition cause the production of mtROS in PMNs (**83**). Thus, mtROS are required for efficient NET formation from PMNs from chronic granulomatous disease (CGD) patients characterized by a genetic deficiency in the activity of the enzyme NOX (**83**), proving that the mitochondria are a significant alternative source of cellular ROS in the absence of NADPH oxidase. However, the interplay between specific ROS sources is not clear. Crosstalk between two major ROS sources, mitochondria and NADPH oxidases, is of particular interest (**84**).

02. ROS functions

After production, ROS can diffuse in cells and tissues before it reacts or becomes quenched. Each ROS has specific activities and diffusion distance (3-20 μm), which is related to their short lifetime (table 1), for example, the highly reactive $^{1}O2$ has a short diffusion range (10-50 nm), $O2•^{-}$ has diffusion distance of around 0.5 μm, OH• has a medium diffusion range (3 μm), while H2O2 has a relatively long diffusion range (>10 μm) (**85, 86**).

Table 1. Approximate half-lives of reactive oxygen species (ROS)

Molecule	Half-Life
$O2•^{-}$	30–40 μs
OH•	>1s
ROO•	7 s
RO•	1μs
$^{1}O2$	3.5μs
H_2O_2	chemically stable
ROOH	until 2 h

Neutrophils are capable of producing vast amounts of ROS; ROS can be released extracellularly into the environment at the site of infection or intracellularly in the phagolysosome following phagocytosis of bacteria. ROS are very effective in degrading ingested particles, including bacteria and fungi. Moreover, ROS can cross the membranes of bacterial pathogens and can oxidize DNA, proteins, lipids, and carbohydrates. Thereby ROS can regulate several redox-mediated pathological processes, including the release of primary and secondary granules (**83**), and NET generation in response to a few stimuli (**87**). Moreover, ROS play a role in the production of pro-inflammatory cytokines like TNF-α and macrophage inflammatory protein 2 (MIP-2) (**88**). Additionally, ROS production is correlated with neutrophil counts and infiltration in different pathology, such as COVID-19 (**89**), where they contribute to clinical features of acute disease in COVID-19 patients (**89**).

In the body, there is a balance between the production and elimination of free radicals. Defective ROS production allows bacteria to survive, repeatedly colonize different tissue sites, and cause sepsis (**90**).

03. ROS detection

ROS detection methods are essential in studying oxidative stress and its impact on various biological processes. There are several techniques available to detect and measure ROS levels in cells and tissues.

001. 2',7'-Dichlorofluorescein diacetate (DCFDA) Assay

DCFDA is a non-fluorescent probe that can passively diffuse into cells. Once inside the cell, DCFDA is deacetylated by cellular esterases to form 2',7'-dichlorofluorescin (DCFH). DCFH reacts with various ROS, including hydrogen peroxide and peroxynitrite, to form the fluorescent compound 2',7'-dichlorofluorescein (DCF). DCF fluorescence can be measured using fluorescence microscopy or flow cytometry to assess intracellular ROS levels (**91**).

002. Chemiluminescence Assay

This method measures the light emitted during the reaction of certain chemiluminescent probes with ROS. For example, luminol, when oxidized by ROS, emits light, and the intensity of the luminescence is proportional to the amount of ROS present (**92**).

4. Cytokine production

Cytokine production by neutrophils is a crucial aspect of the immune response. The most common type of invading cells in inflamed tissues are neutrophils, which frequently exceed mononuclear leukocytes. Thus, the fact that neutrophils clearly exceed other cell types implies that the contribution of neutrophil-derived cytokines might be crucial under certain conditions. According to multiple studies, in response to microenvironmental signals or constitutively, neutrophils express and release a variety of cytokines that modulate the immune response, including, the immunoregulatory cytokine IL-10 and pro-inflammatory cytokines IL-8, IL-6, TNF-α, IL-1β, IFN-α, G-CSF, and GM-CSF and chemokines, e.g. chemokine ligand (CCL)-2, CCL3 and chemokine receptor (CCR)-7 (**93, Figure 5**). CCR7 play an important role in neutrophil migration. Injection of complete Freund adjuvant (CFA) attracted neutrophils to draining lymph nodes in wild-type mice but not in CCR7-/- mice (**94**). Furthermore, PMNs can regulate T cell function. They can promote T cell response via secreting chemokines that recruit T cells to the site of inflammation, which has been proven in vivo models by production of CCL1, CCL2, and CCL5 (**95**). Also, neutrophils have been identified as effector cells in the regulation of B cell responses. PMNs produce BAFF after activation by G-CSF, which is important for B cell maturation and survival (**96**).

Additionally, neutrophils play a direct suppressive role via immuno-modulatory cytokine production. During mycobacterial infection, regulatory neutrophils release IL-10, which particularly targets IL-10R-expressing Th17 $CD4^+$ T cells, which may be crucial in regulating the otherwise uncontrolled Th17 response (**97**). However, production of IL-10 by neutrophils is a subject of active research. The potential for artifacts in the assessment of IL-10 synthesis by neutrophils due to the presence of monocytes in experimental preparations has been acknowledged in the scientific literature. Indeed, the findings of Marco Cassatella group suggest that neutrophils are unable to synthesize IL-10 due to a closed configuration of promoter, which prevents the transcription of the IL-10 gene (**98**).

While neutrophil-derived cytokines are essential for mounting an effective immune response and clearing infections, their dysregulation or excessive production can lead to chronic inflammation and tissue damage, contributing to various inflammatory diseases, such as rheumatoid arthritis.

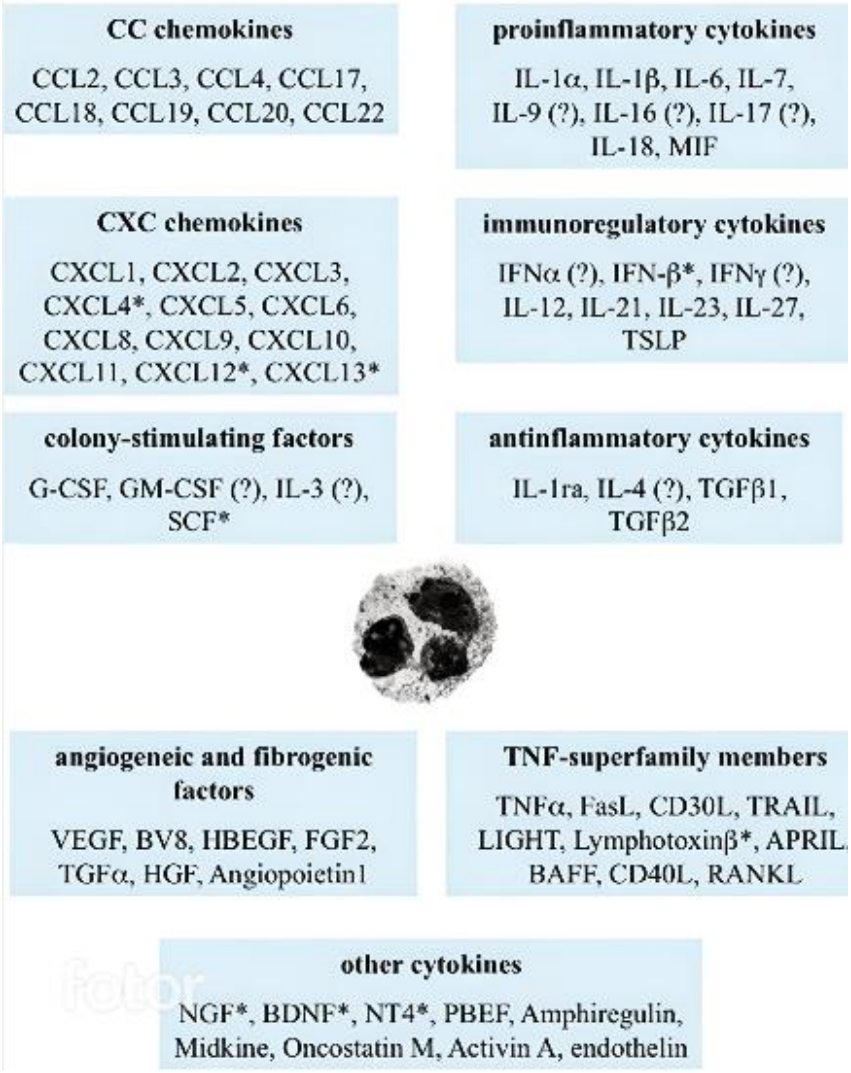


Figure 5. Cytokines expressed or produced by human neutrophils. Tecchio C et al, Front. Immunol.,2014.

F. Neutrophil extracellular traps (NETs)

Besides the functions mentioned above, another function in neutrophils known as NET formation (classically described NETosis) has been described. According to the majority of publications, NET release is a type of pathogen-induced active cell death that enables neutrophils to continue fighting germs after they have died. NET formation has been observed over the years and the classically described NETosis follows the activation of neutrophils by phorbol 12-myristate 13-acetate (PMA). In the 90s, a new rapid death of human neutrophils by the potent activator PMA,

with alterations distinct from typical apoptosis or necrosis has been reported (**99**). In 2004, Brinkmann et al. reported that neutrophils stimulated by PMA or IL-8, release a large web-like structure composed of decondensed chromatin and neutrophil antibacterial factors, coining the name neutrophil extracellular traps (**100**). PMA is a chemical compound that is often used in laboratory research to activate protein kinase C (PKC), a family of enzymes involved in various cellular processes. Moreover, PMA can activate the phosphoinositide-3-kinase (PI3K), a protein that also regulates the induction of autophagy, and may be one of the signaling pathways of NET formation (**101**). Importantly, auto-phagosome assembly is not necessary for NET formation (**101**).

Detailed scanning electron microscopy (SEM) examinations have revealed that NETs are formed of DNA segments interconnected with globular protein sections comprising 15-17 nm and 25 nm in diameter, respectively. These components combine to generate bigger threads with a diameter of 50 nm (**100**). It is also known that human and mouse neutrophils are not the only cells that form NETs. Release of extracellular traps (ET) has been reported in various immune cells (**102**).

1. Different forms of NET formation

In the years that followed Brinkmann's findings, research on NET formation exploded, revealing that this phenomenon can occur in multiple ways depending on the stimulus, and various signaling pathways were described. Different stimuli trigger different types of NET formation, and the same stimulus may trigger different mechanisms and types of NET formation. In our recent reviews, we comprehensively elucidated the diverse mechanisms governing NET formation (**103, 104**).

NET formation can occur both in physiological and pathological contexts. The molecular processes leading to the release of NETs are complex and varied. Two major mechanisms were described by which neutrophils can release NETs, suicidal and vital NETosis (**Figure 6**). In the first mechanism "suicidal NETosis", NETs are slowly released by neutrophil cells leading to cell lysis. The second strategy, termed vital NETosis, enables cells to continue functions such as phagocytosis after NET release. Suicidal NETosis occurs 2–4 hours after stimulation, whereas vital NETosis occurs rapidly about 5–60 minutes after stimulation. Moreover, recent investigations have shed light on a third mechanism for NET formation, described as "mitochondrial NETosis".

01. Suicidal NETosis

NETosis is the word that has historically been used to describe the release of NETs by dying cells. Suicidal NETosis, also known as lytic NETosis or classical NETosis, is often triggered by different ligands, complements, or cytokines. After this, the neutrophils undergo typical features of suicidal NETosis which requires raf-MEK-ERK activation of NADPH oxidase and ROS production (**105**). Raf-MEK-ERK is an early event upstream of Nox2 activation. This pathway also regulates the expression of Mcl-1, an anti-apoptotic protein, suggesting that Raf-MEK-ERK may inhibit apoptosis by promoting NETosis (**105**). It is clear that NOX-dependent NETosis agonists induce the formation of large amounts of ROS in neutrophils, such as PMA, viruses, bacteria, components of bacteria, and immunological stimulis such as antibodies which then cause the activation of different protein kinases (extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase, AKT kinase, PI3K) (**99, 100, 106**). Indeed, it remains unclear how ROS are involved in nuclear membrane disassembly and mixing of NET components. However, it is clear that ROS play a central role in starting the program. The hypothesis by which ROS induce NET release is that excess of ROS production causes DNA damage, which is then repaired by DNA repair machinery, leading to the complete opening of chromatin and following NETosis. DNA nucleotides could be oxidized by ROS (e.g., converting guanine to 8-oxoguanine(8-OG)). Excess of ROS generate genome-wide 8-OG, which can lead to a specific type of DNA mutation known as a transversion, specifically a G:C to T:A transversion, which represents a frequent somatic mutation in human cancers. The initial steps of DNA repair are needed to decondense chromatin in these cells (**107**). Inhibition of early steps of oxidative DNA damage repair (by APE1, PARP1 and DNA ligase inhibitors) suppresses NETosis (**107**). Furthermore, in the NOX-dependent pathway, in the absence of oxidants or in neutrophils from severely immunocompromised chronic granulomatosis patients (CGD) (these cells have deficiencies in NOX-mediated ROS production), PMNs have been shown to be defective in NET formation in response to PMA (**106, 108**). This deficiency in NET generation in CGD PMNs is caused by mutations in the gene encoding NOX and disrupts the ability of the complex to generate ROS (**108**). Additionally, pharmacological inhibition, for example by using hydroxymethylglutaryl coenzyme A reductase inhibitor, which blocks the oxidative burst of PMNs, leads to decreased NETosis against staphylococci (**109**). Similarly, diphenyleneiodonium (DPI), a NADPH oxidase inhibitor, and N-acetylcysteine (NAC), the general ROS scavenger, decrease NET formation in response to PMA (**107**). There is now growing

evidence to suggest that some stimuli induce NETs independently of NADPH oxidase (**110**). NETosis induced by certain strains of S. aureus has been shown to be both NOX-dependent and independent (**111**).

One of the critical steps in NET formation is the decondensation of chromatin. In addition to ROS, proteases such as (MPO and NE) translocate to the nucleus, and contribute to chromatin decondensation by cleaving histones. Additionnaly, NE is in charge of cytoskeleton and nuclear membrane decomposition, enabling nuclear content and cytoplasm to mix together (**112**). NE activity is also essential for MPO translocation to the nucleus (**112**). At the same time, peptidylarginine deiminase 4 (PAD4) translocate to the nucleus. It replaces the positively charged histone arginine residue with a neutral citrulline residue., by a mechanism called citrullination. Citrullination of histones H3 and H4 decreases the electrostatic interaction between histones and DNA, thereby promoting chromatin decondensation (**113**).

As the chromatin decondenses, the nuclear envelope becomes disrupted, allowing the mixing of nuclear contents with cytoplasmic components. This leads to the integration of antimicrobial proteins and enzymes stored in neutrophil granules with the decondensed chromatin. The activated neutrophil undergoes cell lysis, resulting in the rupture of the plasma membrane. This release allows the extrusion of the chromatin-protein complexes as NETs into the extracellular space (**114**). This procedure took place 3-4 hours after the initiating of stimulation.

Suicidal NETosis is a tightly regulated process involving multiple molecular events, including the activation of NADPH oxidase, chromatin decondensation, granule mixing, and plasma membrane rupture. Dysregulation of this process has been implicated in various inflammatory and autoimmune diseases, highlighting the importance of understanding the mechanisms underlying suicidal NETosis for therapeutic interventions.

02. Vital NETosis

Vital NETosis, also known as non-lytic NETosis or alternative NETosis, is an alternative pathway of NET formation that does not involve cell lysis or the sacrifice of neutrophils. Unlike suicidal NETosis, which results in the death of neutrophils, vital NETosis allows neutrophils to remain viable and continue their functions after releasing NETs (**115**). This form of NET formation has gained increasing attention in recent years. Similar to suicidal NETosis, neutrophils are activated by various stimuli, such as, calcium ionophore microbial pathogens, immune complexes, or

inflammatory mediators. Activation can occur through the recognition of specific receptors, such as Toll-like receptors or Fc receptors, on the neutrophil surface (**111, 115, 116**). Lipopolysaccharide, additionally triggers rapid NET release in vivo via vital NETosis, which does not involve cell lysis and is mediated by TLR4 on platelets (**116**). This pathway is a rapid process and takes about 5–60 min after stimulation.

As the neutrophils become activated, the chromatin within the nucleus undergoes decondensation. However, unlike in suicidal NETosis, the nuclear envelope remains intact during vital NETosis. The decondensed chromatin is extruded into the extracellular space via transport vesicles without breach of the plasma membrane, forming NETs (**111, 115**). Nuclear chromatin traffics from the nucleus to the extracellular environment via vesicles in an oxidant-independent manner (**115**). Importantly, vital NETosis allows neutrophils to retain their viability and functional capabilities. After releasing NETs, neutrophils can continue their immune functions, including phagocytosis and the production of ROS (**115**). This is in contrast to suicidal NETosis, where the neutrophils undergo cell lysis and are ultimately eliminated.

Both vital NETosis and suicidal NETosis represent distinct strategies of NET formation with different implications in immune responses and disease pathogenesis. For example, vital and non-vital NETosis are present in normal and preeclamptic pregnancies (**117**). Vital NETosis is thought to play a role in maintaining immune functions while minimizing tissue damage. It is considered a more controlled and regulated process, allowing neutrophils to contribute to host defense without sacrificing themselves. In contrast, Suicidal NETosis is typically associated with acute or intense immune responses to pathogens, contributing to pathogen clearance but also leading to tissue damage in certain contexts (**115**).

03. Mitochondrial NET formation

A third form of NET formation has been described. There is growing evidence that viable neutrophils release mitochondrial DNA (mtDNA) and granule proteins without causing cell damage. This process requires an active NADPH oxidase, cytoskeleton rearrangements, and glycolytic ATP generation, all of which are functionally dependent on mitochondria (**118**). Recently, it has been shown that viable eosinophils generate extracellular traps within 5 minutes after activation (**119**). Moreover, the priming of neutrophils with GM-CSF followed by a brief activation of TLR4 or the complement C5a receptor, results in another type of ROS-dependent

vital NETosis associated with the release of mtDNA but not nuclear DNA (**120**). This mechanism is described as mitochondrial NETosis. Mitochondrial NETosis is a type of regulated cell death in which neutrophils release NETs that are enriched in mtDNA.

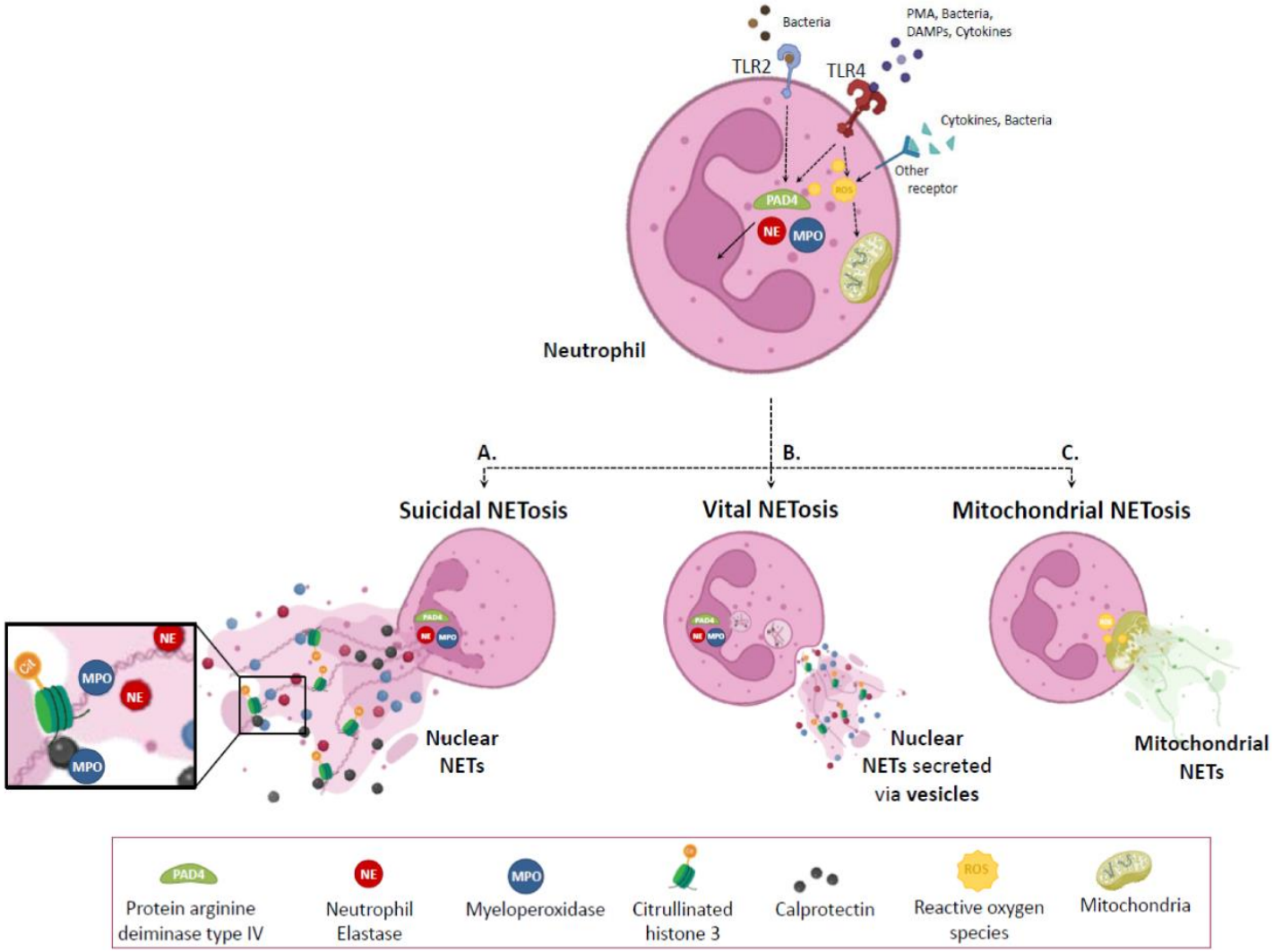


Figure 6. Several types of NET formation. M Castaño et al, International Journal of Molecular Sciences 2023. The production and release of NETs can occur via three distinct processes: (A) suicidal, (B) vital, and (C) mitochondrial NETosis. The recognition of stimuli by neutrophil membrane receptors initiates a signaling cascade. The protein arginine deiminase type IV (PAD4), boosts neutrophil elastase (NE) and myeloperoxidase (MPO) translocation to the nucleus, and may raise reactive oxygen species (ROS) levels. PAD4 catalyzes histone 3 citrullination (citH3) in nuclear NETs releases, whereas NE and MPO decondensed chromatin. TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; PMA, phorbol myristate acetate; DAMPs, damage-associated molecular patterns.

2. NET formation is a distinct form of cell death

Otherwise, this form of cell death is characterized by disruption of the nuclear and granule membranes and release of uncondensed chromatin into the cytoplasm. However, this observation received little attention until the appearance of research done by Brinkmann et al. that showed in 2004 that this mysterious cell death indeed represents the third defense strategy of neutrophils, in addition to phagocytosis and degranulation (**100**). These findings were supported by different studies, using detailed in vitro cellular imaging, which showed that NETotic cells do not exhibit apoptotic phenotype and signs such as eat-me signaling, caspase activity, nuclear chromatin condensation, and internucleosomal DNA cleavage. Moreover, in contrast to apoptotic cells, both the nucleus and the granule membrane are degraded during NETosis, while plasma integrity is preserved (**106**). Interestingly, different inhibitor has been described to switch NETosis to apoptosis, such as tonicity of saline (NaCl) (**121**), or histone deacetylase inhibitors (**122**), which are important in understanding the regulation of NETosis and apoptosis in neutrophils. For instance, hesperetin, which has anti-inflammatory and antioxidant activities, may block NET formation and shift neutrophil death from NETosis to apoptosis, reducing NET-related intestinal barrier damage and suggesting a unique protective role in intestinal barrier dysfunction during sepsis (**123**).

Necrosis is among the various modes of cell death. NETs can be difficult to distinguish from necrosis using traditional methods such as immunofluorescence microscopy or ELISA. Unlike traditional approaches, Raman microscopy, a laser-based microscopic method that can provide detailed information about chemical structure, phase and polymorph, crystallinity, and molecular interactions, could clearly distinguish between NETs and necrotic cells. Using this approach, it has been shown that necrotic cells appear to have more cellular material than NETs, particularly larger quantities of lipids (**124**). However, NETosis and necroptosis can have particularly intricate relationships with one another (**125, 126**). The well-studied type of controlled necrosis, necroptosis, depends on the activation of RIPK1/3 and then phosphorylation of the pseudokinase mixed-lineage kinase domain-like (MLKL). However, it has been shown that the RIPK1 inhibitor and MLKL inhibitor reduce NET generation by neutrophils, which indicates that RIPK3-dependent necrosis functions as an upstream activator of NET generation (**125**).

Additionally, pyroptosis is a non-apoptotic form of cell death characterized by membrane rupture and release of proinflammatory cytokines including cytokines like IL-1β and IL-18 with cell lysis through gasdermin D (GSDMD) pore formation (**127**). The key to pyroptosis is the activation of inflammatory caspases and GSDMD. There is conflicting information about the crosstalk of NET formation pathways and pyroptosis, and it has become obvious that both mechanisms overlap and interact at various stages. A study showed that GSDMD triggers granule permeabilization during NET formation and also localizes to the plasma membrane of PMA-stimulated neutrophils (**128**). It is also interesting to note that certain studies have indicated that the rupture of the plasma membrane during ROS-dependent NET formation is mediated by gasdermin D, thereby associating NETs with pyroptosis (**129**). However, some studies showed that NET formation upon activation with C5a or LPS is gasdermin D-independent (**130**). Interestingly, murine neutrophils are resistant to canonical pyroptosis, but can undergo NET formation when they detect cytoplasmic LPS. Suggesting that NET formation can use an alternative pathway than pyroptosis (**131**).

3. Metabolic requirements for NET formation

Tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OxPhos), pentose phosphate pathway (PPP), and fatty acid oxidation (FAO) are among the metabolic processes necessary to meet the energetic, biosynthetic, and functional needs of active neutrophils. Under pathological situations, neutrophils can adapt to the tissue environment by modifying their metabolic activity via multiple metabolic pathways. In recent years, researchers have focused on the metabolic requirements for NET formation. Glycolysis and ATP generation were discovered as a critical metabolic step in the formation of NETs (**132**). It has been observed that glycolytic ATP generation contributes to the formation of NETs by promoting microtubule network assembly (**132**). Additionally, Opa1 is essential for glycolysic ATP synthesis, and mice lacking Opa1 in neutrophil populations exhibit less antibacterial defensive capabilities, providing clear evidence for the role of glycolysis in NET formation (**132**). Similarly, hyperglycemia has been shown to increase NET formation by type 2 diabetes (T2D) patient's neutrophils (**133**).

Moreover, glucose is required for NET-induced PMA in human neutrophils (**134**), and glycolysis inhibition reduces NET formation in response to PMA (**134, 135**). It has also been observed that the reduction in NET formation triggered by GM-CSF and C5a is due to 2-Deoxy-D-glucose (2-DG) blocking glycolysis in human and murine neutrophils (**132**).

The pentose phosphate pathway is an alternative glucose-dependent metabolic track in neutrophils that has been seen in both resting and active neutrophils as well as during NET formation (**135**). PPP is engaged in the NOX-dependent ROS generation and hence contributes to NET formation (**135**).

Moreover, neutrophils are frequently detected in hypoxic inflammatory tissues with enhanced lactate. It was recently shown that human neutrophils exogenously treated with lactate generate NETs (**136**).

4. NET composition and induction

NET formation can be induced by various stimuli, including infectious agents, inflammatory mediators, and immune complexes. NET formation occurs through several signaling mechanisms depending on the stimulus (**137**). Furthermore, NETs are web-like structures composed of DNA, and is highly enriched in core histones but also includes high levels of granule proteins, such as cathepsin G, and proteinase, MPO, NE, or cytosolic proteins such as S100 proteins. NETs comprise around 800 proteins in total, the majority of which are associated with autoimmunity, inflammation, and lupus. Many of the proteins discovered in NETs exhibit one or more post-translational modifications such as methionine sulfoxide, thiol oxidation, deamination, and phosphorylation. Moreover, its composition differs depending on the stimulus and can be related to the pathological context (**138, 139**). NET proteomic analysis of Type 1 diabetes (T1D) patients was distinct from that of HDs (**140**). NETs from T1D patients were enriched in metabolic proteins. This might be an adaptive mechanism used by activated T1D neutrophils to prevent impaired glycolysis and, as a result, dysregulated NET formation (**140**). Similarly, proteomic analysis of NETs from RA and SLE PMNs induced by PMA or ionomycin revealed that there are some proteins that are significantly different between RA and SLE NETs, such as histone H2B, which is higher in SLE. There is also an extensive range of post-translationally modified proteins in RA and SLE compared to HDs (**141**). Moreover, acetylated, methylated residues and mtDNA in NETs were higher in SLE-derived NETs compared to HD-derived NETs (**142, 143**).

There are a variety of stimuli that can induce NET formation, as detailed below:

01. Microbial pathogens

Many bacteria, fungi, and parasites can stimulate neutrophils to release NETs as a defense mechanism against microbial invasion. Bacteria that escape phagocytosis by PMNs by forming

large aggregates or interfering with phagosome maturation can induce NET release. Several gram-positive and gram-negative bacteria, including *Streptococcus pneumonia*, *Escherichia coli* and *Helicobacter pylori* (**144**), as well as components of the bacterial cell wall such as LPS can induce NET formation (**145**). Similarly, toxins produced by bacteria such as Shiga toxin (Stx)-producing *Escherichia coli* have shown a capacity to induce NET formation (**146**).

Furthermore, several studies have described NETs in infections with fungi, *Candida albicans, Candida auris* and parasites including *Leishmania amazonensis* or *Entamoeba histolytica* have a capacity to trigger NET formation by neutrophils (**144**). Moreover, *Toxoplasma gondii* causes the production of NETs, which reduces the viability of T. gondii (**147**).

Furthermore, neutrophils could sense human immunodeficiency virus (HIV)-1 by TLR7 and TLR8 to produce NETs (**148**). However, bacteriophages like the *P. aeruginosa* phage didn't trigger NET release (**149**).

02. Inflammatory mediators

Various inflammatory mediators, including cytokines and chemokines, can trigger NET release. For instance, IL-6, IFNγ, TNF-α, IL-17 and chemokines have been reported to stimulate NET formation (**144, 150**). Moreover, IL-8 is a chemokine that plays a crucial role in neutrophil recruitment and activation. It has been shown to induce NET formation by promoting neutrophil activation and ROS production (**151**). IL-1β appears to increase NET formation by promoting neutrophil NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome activation in neutrophils (**152**). Indeed, NET accumulation in atherosclerotic lesions is reduced by IL-1β antagonists (**152**).

NET formation can also be induced by reactive oxygen species such as hydrogen peroxide (**106**). ROS can be produced by neutrophils themselves or by other cells in the inflammatory microenvironment.

Moreover, antibodies such as IgG and IgA can directly stimulate neutrophils to release NETs (**125**). IgA is a much more potent inducer of NET formation than IgG (**153**). Moreover, Immune complexes formed by the binding of antibodies to their target antigens can activate neutrophils and induce NET release (**153**). These complexes can arise in autoimmune diseases, such as systemic

lupus erythematosus, rheumatoid arthritis, or vasculitis. IgA immune complexes in RA patients' plasma and synovial fluid can induce NET release through FcαRI on neutrophils (**154**).

03. In vitro most well studied stimulis

001. PMA

The protein kinase C agonist, phorbol 12-myristate 13-acetate, is by far the most effective and frequently used inducer of NET formation in experimental systems. PMA is a phorbol chemical and is obtained from the plant Croton tiglium. It was originally shown to be a tumor promoter in mouse skin, and subsequent research revealed that the carcinogenic impact was linked to PKC activation. PMA is now often employed in trials to induce tumor growth and leukocyte activation. Despite being an artificial stimulus, PMA is well-known and widely used in research to study neutrophils. Furthermore, nanomolar concentrations of PMA are known to induce neutrophil degranulation, metabolic changes and ROS production (**155**), and it is the stimulus widely used in vitro to induce NET formation (**124**). Protein kinase C isoforms may mediate the formation of neutrophil extracellular traps (**124**). PMA has been criticized for a lack of biological relevance. Indeed, PMA uses pathway related to physiological simuli (**137**). Recently, it has been shown that Raman spectral signatures of NETs elicited by PMA and LPS were strikingly comparable (**122**). However, proteomic analysis of NETs has shown differences between PMA and LPS-induced NETs (**139**).

The PMA-induced NET formation has been instrumental in studying the biology and functional significance of NETs in various research contexts. However, it is important to note that PMA-induced NET formation may not entirely replicate the physiological conditions found in vivo. Nonetheless, the use of PMA has provided valuable insights into the mechanisms and consequences of NET formation, furthering our understanding of the role of NETs in immune responses and disease pathogenesis.

002. Calcium ionophore

These molecules act by altering the intracellular ion balance, particularly calcium ($Ca2^{+}$) levels, which play a crucial role in the signaling pathways involved in NET formation. One specific ionophore known for its ability to induce NETs is ionomycin. A23187 is a calcium ionophore that induces a huge calcium influx, while nigericin increases potassium effluxes in cells, which also

results in a calcium influx. A23187, unlike LPS and PMA, did not cause morphological changes in the nuclei (**138**).

The ionophore-induced NET formation provides a useful experimental tool for studying the molecular mechanisms and functional consequences of NETs. The disruption of intracellular calcium homeostasis by ionophores represents an artificial but effective means to trigger NET release. However, it is important to note that the precise signaling events and molecular pathways triggered by ionophores in NET formation may still require further investigation to fully understand their specific contributions in different contexts and disease conditions.

5. NET formation by various PMN population, LDNs vs. NDNs

NET formation can vary among different neutrophil populations and under different conditions.

Both LDNs and NDNs are capable of forming NETs, but there can be differences in the characteristics and regulation of NET formation between these two populations. LDNs isolated from healthy individuals appear to generate fewer NETs than NDNs in response to PMA stimulation, in contrast to LDNs isolated from chronic inflammatory diseases which produced an increased number of NETs (**Table 2**). In RA, LDNs have lower chemotaxis and phagocytosis activities, delayed apoptosis, and reduced NETs formation in response to PMA (**53**). Overall, there is strong evidence for neutrophils harmful effects in RA, but no clear evidence that LDNs play specialized functions in this disease. In addition, in SLE, LDNs have been found to spontaneously release mtDNA (**156**). Extracellular mtDNA stimulates plasmacytoid dendritic cells and promotes $CD4^+$ T cell activation, which are important in the development of SLE.

Table 2. NET formation by NDNs vs LDLs neutrophils

	LDNs NET formation	LDNs phenotype
Healthy individuals	Fewer PMA-induced NETs compared to NDNs in healthy individuals (**51**).	$CD15^{+}CD10^{+}$ $CD16^{+}CD66b^{+}$
Systemic Lupus Erythematosus	Enhanced spontaneous NET formation vs. SLE NDNs and healthy NDNs (**157**).	$CD14^{-}CD15^{+}$
Rheumatoid arthritis	No difference vs. RA NDNs or healthy NDNs (**53**).	$CD14^{-}$ $CD15^{+}$ $CD10^{+}$ $CD16^{+}$
Spontaneous abortion	Enhanced PMA-induced NETs vs. LDNs from un-pregnant and normally pregnant women (**158**).	$CD45^{+}CD15^{+}CD14^{-}$
Psoriasis	Enhanced PMA-induced NETs vs. psoriatic NDNs (**159**).	$CD14^{-}$ $CD15^{+}$ $CD10^{+}$
Cystic fibrosis (CF)	Less PMA-induced NETs compared to CF NDNs (**51**).	$CD15^{+}CD10^{+}$ $CD16^{+}CD66b^{+}$

6. Antimicrobial function of NETs

Neutrophil extracellular traps are a type of antimicrobial defense mechanism used by neutrophils. NETs have been shown to be effective against a variety of bacteria, including Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Streptococcus pneumonia (**144**). NETs are thought to play an important role in the innate immune response to bacterial infection. PMNs undergo NET production in response to the protozoan pathogen T. gondii and prevent Toxoplasma infection (**147**). They are particularly effective against bacteria that are resistant to antibiotics. Immunofluorescence analyses revealed the presence of proteins such as gelatinase (from tertiary granules), lactoferrin (from specific granules), neutrophil elastase, cathepsin G, myeloperoxidase (from azurophilic granules), and other cytoplasmic proteins with antimicrobial function in NETs (**160**).

Similarly, histones are the most abundant NET-bound proteins. Histones have the most potent antimicrobial properties in NET, and they can help kill bacteria that are trapped in NETs (**161**). Cathepsin G, a granular serine protease, is necessary for Neisseria clearance by NETs (**162**). Antibodies against histones or cathepsin G were demonstrated to impair the bactericidal capacity of NETs, demonstrating the antibacterial importance of the NET-bound proteins (**100, 162**). DNA

of NET exhibit antibacterial action that could be hampered by enzymatic and cationic treatments that degrade or eliminate DNA's ability to chelate cations (**163**). NET antimicrobial proteins work together with the DNA scaffold of the NETs to create a highly effective defense mechanism against microbial infections. Note that DNA-protein complexes also function as immunological triggers, recruiting and activating immune cells for bacterial clearance (**164**).

The combined action of trapping pathogens and releasing antimicrobial molecules contributes to the rapid elimination of invading microorganisms, bolstering the host's immune response to infections.

7. NETs components act as DAMPs

NET formation promotes the release of nuclear contents into the extracellular environment, which is analogous to the process of DAMPs. DAMPs are naturally occurring compounds that trigger and intensify the non-infectious inflammatory response. DAMPs are released into the extracellular environment following sterile or viral tissue injury and interact with pattern-recognition receptors (PRRs) such as toll-like receptors and the NLRP3 inflammasome. DAMPs initiate the innate immune response and protect against external danger signals by activating innate immune cells such as neutrophils, tissue macrophages, and dendritic cells. NLRP3 activation by DAMPs such as extracellular ATP, hyaluronan, and uric acid can activate caspase-1 and promote the release of IL-1 and IL-18 via inflammasome formation. The number of DAMPs has continuously expanded with the advancement of relevant research, primarily including high-mobility group protein B1 (HMGB1), cell-free DNA (cfDNA), circulating DNA, histones, and mitochondrial DNA (**165**). However, chromatin-associated molecules, which include nuclear DNA and histones, extracellular RNA, mitochondrial DNA, DNA or RNA-binding proteins, and extracellular traps, may be classed as chromatin-associated molecular patterns (CAMPs) (**166**). Furthermore, NETs may contain several DAMPs, such as DNA, RNA, and protein components such as histones and HMGB-1, antimicrobial peptides such as LL37, and enzymes such as myeloperoxidase which are released and contribute to their antimicrobial action. In this section, I will focus primarily on the NETs DAMPs found in RA.

01. DAMPs closely related to NETs in RA

The major symptoms of RA include joint swelling, pain, and stiffness caused by inflammation of the synovial membrane. It is clear that DAMPs are associated with RA development.

S100A8/9/11/12 are important members of the calcium-binding protein S100 family, and were shown to be overexpressed in RA patients' synovial tissue, synovial fluid, and blood (**167**). Indeed, it has been shown by immunocytochemistry that S100A8/A9 is simultaneously released with DNA during NET formation (**168**). In addition, joint inflammation can cause cellular stress and an increase in heat shock protein 70 (Hsp70) level in synovial tissue and HSP70 is thought to enhance inflammation by activating macrophages via TLR2 signaling (**169**). However, HSP70 is found among NET components and released as DAMPs during NET formation (**170**), suggesting NETs as a possible source of HSP70 molecule in RA. Furthermore, HMGB1 (the first known DAMPs) concentration was enhanced in the serum and synovial fluid of RA patients (**171**). HMGB1 was found in PMA-induced NETs (**139**). On the other hand, in experimental animal models, neutralization of HMGB1 can preserve cartilage from degradation and prevent bone deterioration caused by RA (**172**).

02. Histones

NETs are mostly composed of extracellular histones. Histones concentrations were shown to be higher in RA serum (**173**). Also they have been found in high concentration in synovial fluid arthritic mice (**174**). Neutrophil histones in RA synovial fluid would interact with other cells in the joint, such as macrophages and synoviocytes (**174**). By causing lytic cell death in synoviocytes and macrophages, histones may contribute to synovial inflammation (**174**). In addition to the direct effect of histones in the activation of inflammatory cells, PAD4 enzyme released during NET formation, citrullinates arginine residues in the core histones H2A, H3, and H4, leading indirectly to the production of autoantibodies directed against citrullinated histones (**175**), which supports a role for NETs in the generation of antigens targeted by autoantibodies.

03. DNA

DNA is an important component of NETs and a powerful immune stimulatory component. The clinical significance of NET DNA in inflammation is demonstrated by auto-inflammatory and autoimmune symptoms found in individuals and mouse models with deoxyribonuclease (DNase) deficiency (discussed in part 11).

There are several pathways by which DNA can be recognized and contribute to inflammation. The major DNA sensors responsible for DNA-driven immune responses include TLR-9, absent in

melanoma 2 (AIM2) and cyclic-GMP-AMP synthase (cGAS). These DNA sensors are found in neutrophils and cell populations in contact with NETs.

001. TLR9

TLR9 is a type of receptor protein that is found in various immune cells. TLR9 is located in the endosomes of immune cells. TLR9 may also be present on the cell surface; it can be found on the surface of splenic dendritic cells (DCs), rat peritoneal mast cells, and human PBMCs after activation. It is also found on the surface of human and murine neutrophils (**176**). Once TLR9 detects the presence of DNA patterns, it triggers a signaling cascade that leads to the production of pro-inflammatory cytokines and the activation of the immune response. In B cells, TLR9, has been shown to mediate cell responses to nucleic acid. Especially TLR9 stimulation via oligonucleotides CpG induce B cell activation and increased CD40 expression (**177**).

Aside from its involvement in direct neutrophil activation and its role in NET formation, TLR9 is also involved in the recognition of NETs and hence in NET-mediated inflammation. TLR9 expression increases in several diseases. TLR9 may be activated in RA by tissue injury, cell death, NETs, or necrosis, resulting in increased synovial swelling. Indeed, increased levels of cfDNA, DNA-histone, or DNA-MPO complexes (NETs markers) have been found in the serum of RA patients (**178**). TLR9 antagonist and cationic nanoparticle scavenger of cell-free DNA reduce RA symptoms such as ankle and tissue swelling, as well as bone and cartilage degradation (**179**). Many studies indicate that NET DNA activates TLR9 in immune and non-immune cells. In atherosclerosis, DNA from NETs promotes neutrophil recruitment by activating TLR9 in macrophages and producing IL-8 (**151**). Moreover, chronic and excessive NET production may contribute to pulmonary fibrosis in a lung inflammation model via activating TLR9 in fibroblasts (**180**).

Besides, several studies have suggested that protein-binding NETs may aid in TLR9 DNA recognition. The DNA-LL37 complex is taken up by plasmacytoid dendritic cells (pDCs) and causes IFN-I production through TLR9 (**181**). Remarkably, the ability of LL37 peptides to activate TLR signaling is not limited to TLR9.

002. cGAS-STING

cGAS, first discovered in 2013, is a cytosolic DNA sensor, that may recognize chromatin in the nucleus as well as double stranded DNA (dsDNA) in the cytosol, nucleus, and mitochondria. It

catalyzes the generation of cyclic GMP-AMP (cGAMP) from ATP and GTP. 2′3-cGAMP binds to the adaptor stimulator of interferon genes (STING) in the endoplasmic reticulum. STING subsequently activates the transcription factors TANK-binding kinase 1 and IB kinase, which phosphorylate the transcription factor, interferon regulatory factor 3 (IRF3), driving an interferons type I response and production of inflammatory cytokines such as type I interferons, TNF, IL-1β, and IL-6, which are associated with initiation and maintenance of autoimmune disorders. Unlike TLR9, cGAS involvement is limited to NET detection rather than NET formation by neutrophils. However, a study showed that cGAS may play a role in NET formation; cGAS−/− neutrophils exhibit less LPS-induced NET formation than in wild-type (WT) cells (**182**).

Extracellular release of DNA during NET formation has been linked to the activation of cGAS. NETs are powerful inducers of IFN-I in STING-dependent immune sensing, especially when the DNA is oxidized, making it more resistant to degradation and hence more interferonogenic (**183**). Exogenous NETs induce epithelial cell damage via cGAS-STING leading to acute lung injury in a mouse model (**184**). Similarly, NETs could contribute to brain injury and neuronal death by promoting NLRP1 inflammasome activation in a STING-dependent manner (**185**). Recent research found important mechanical insights on how cGAS detects DNA of NETs. Researchers discovered that upon phagocytosis by immune cells, DNA from NETs translocate to the cytosol and activates cGAS (**186**). NETs treated with DNase-I revealed a lower potential to activate cGAS than undigested NETs (**184**). Furthermore, DNA binding proteins, such as the mitochondrial transcription factor A (TFAM) or HMGB1 (present in NETs), have been demonstrated to activate cGAS (**187**).

003. AIM2

AIM2 (that refers to Absent in Melanoma 2) is a component of inflammasomes in innate immune cells. The AIM2 inflammasome helps the body defend against a variety of bacterial and viral infections. AIM2 binds dsDNA from viruses, bacteria, endogen DNA from apoptotic bodies, NETs, and mtDNA and allows for complex oligomerization; its interaction with the adaptor protein, apoptosis-associated speck-like protein containing CARD (ASC), leads to the production of the pro-inflammatory cytokines IL-1β and IL-18 (**188**). AIM2 is found in a variety of organs and cells, including the spleen, small intestine, peripheral blood, and leukocytes. It is constitutively expressed in macrophages and dendritic cells. Furthermore, T-cell receptor (TCR) stimulation

reduced AIM2 expression in T cells. BCR activation had a similar effect on B cells (**188**). However, AIM2 is abundantly expressed in lupus patients' B cells and triggers B-cell differentiation (**189**).

AIM2 has also been linked to the development of psoriasis, arthritis, and other autoimmune illnesses by recognizing cytosolic self-DNA. Indeed, AIM2 expression was shown to be increased in RA synovium but not in OA synovium (**190**), and seems to play a role in RA occurrence and development. Besides, a recent study found a positive correlation between AIM2, IL-1β, CRP level, and RA clinical features (**190**).

Additionally, AIM2 was found to have a role in NET detection in an LPS-induced acute respiratory distress syndrome (ARDS) model, where intracellular NET-DNA binds the AIM2 receptor, activating the inflammasome and causing alveolar macrophage pyroptosis (**191**). Another study discovered AIM2 within NETs. It was found that AIM2 binding to NETs protected them from DNase-I degradation, implying that extracellular AIM2-NET interactions may enhance prolonged IFN-I signaling (**192**). NETs in psoriasis activate the AIM2 inflammasome via the p38 mitogen-activated protein kinases (MAPKs) signaling pathway, resulting in the generation of IL-1β. However, DNase-I treatment of NETs only marginally lowers IL-1β release, indicating that this was independent of DNA (**193**).

04. Extracellular RNA

Extracellular RNA (exRNAs) are a diverse category of ribonucleic acids that include messenger, ribosomal, micro (miRNAs), long non-coding (lncRNAs), and circular RNAs. These RNAs can be released by cells in free form, attached to proteins or phospholipids. Numerous types of exRNAs, such as miRNAs, ribosomal RNA, and lncRNAs, play a role as key DAMPs and have been implicated in regulating inflammatory processes at various levels and leukocyte recruitment. Despite the focus on DNA and protein components, another key cellular biomolecule, RNA, has recently been discovered in NETs. Several studies have reported the presence of RNA in NETs (**194, 195, 196**). Confocal microscopy revealed the presence of RNA in PMA-induced NETs (**195**). RNA NETs may be internalized by endothelial cells in TLR and actin cytoskeleton-dependent manner and induce inflammation via type I IFN-stimulated genes (**194**). Moreover, different NET-binding peptides could promote RNA internalization by cells. NETs contain the alarmins LL-37

which have RNA binding activity (**197**) and ECs or pDC exposed to NETs take up LL-37 that colocalizes with neutrophil-derived RNA (**194**).

The impact of NET-derived RNA in RA pathogenesis is not fully understood. However, RNAs are known to play a role in the development of RA. For instance, miR-let7b has been shown to promote arthritis via interferogenic activity on pDCs due to its ability to act as a TLR7 ligand, and it may also induce M1 pro-inflammatory macrophage polarization (**198**). MiR-let7b has been detected in NETs (**199**). This may suggest that NET is the source of exRNA in RA and is capable of initiating an immune response that leads to RA pathogenesis.

8. NET immunomodulatory function

Besides their antimicrobial function in trapping and neutralizing pathogens, recent research has shed light on NETs immunomodulatory role, influencing various aspects of the immune response (**103**). NETs immunomodulatory function involves interacting with immune cells, influencing cytokine production, and contributing to the resolution of inflammation. For instance, NETs downregulate cytokine production by monocyte-derived dendritic cells in response to LPS (**200**).

This suggests a potential regulatory role for NETs in modulating the immune response. A different aspect of NET immunomodulatory activity is that LL-37 binding NETs may directly bind the S1 domain of SARS-CoV-2, mask angiotensin-converting enzyme 2 receptors, and limit SARS-CoV-2 infection (**201**). Additionally, one of the most critical anti-inflammatory roles of NETs is their ability to neutralize pro-inflammatory molecules, such as cytokines and chemokines. During NET formation, neutrophils release a range of proteases and granule contents into the extracellular space. Some of these components can directly degrade or sequester inflammatory mediators, thereby preventing their sustained action and dampening the inflammatory cascade (**202**). Furthermore, NETs contain antimicrobial proteins, such as lactoferrin which may exert anti-inflammatory function (**203**).

While NETs can promote the release of pro-inflammatory cytokines, they also participate in the generation of anti-inflammatory cytokines like IL-10 and TGF-ß by macrophages (**204**). Additionnaly, NETs was shown to support regulatory macrophages M2b polarization (**204**). Moreover, NETs can help to control the immune response and prevent it from becoming too excessive. This can be helpful in preventing the development of autoimmune diseases. For

instance, PAD4 deficiency exacerbates acute inflammation and enhances tissue damage following myocardial infarction in mice, which can be attributed in part to a lack of NETs (**204**).

9. NET analysis and quantification

NET quantification refers to the measurement or assessment of the levels or presence of NETs in biological samples. Quantifying NETs can provide valuable information about the extent of neutrophil activation, the intensity of inflammatory processes, and their potential contribution to various diseases.

Several techniques are used to quantify NETs in research and clinical settings (**figure 7**).

01. Fluorescence microscopy

The most popular method for detecting NETs is to use conventional fluorescence microscopy to identify decondensed chromatin with a DNA-binding dye and its co-localization with granular or histone proteins. Currently, immunofluorescence imaging is one of the best approaches for demonstrating NET presence in tissue (**205**). Recently developed semi-automated NET measurement methods based on microscopy are labor-intensive, and several metrics frequently fail to conform uniformly over a broad range of images (**206**). The results obtained in different laboratories to estimate the quantity of released NETs are difficult to compare since diverse approaches were utilized and the readout was prone to individual bias in many publications. As a result, a method for standarized quantification of NET formation is required. A novel approach for identifying and quantifying NET formation was described, combining high-throughput live in situ cell imaging with a computer algorithm for data analysis (**207**). In recent years, there has been an increase in interest in developing software tools to measure NETs in fluorescence microscopy images. Trapalyzer, a computer tool that quantifies NETs automatically, was recently developed (**208**). Using these techniques, a new NET marker has been developed, such as the lipid peroxidation marker 4-hydroxynonenal (4-HNE). It showed strong colocalization with NETs and was associated with neutrophil infiltration of lung tissues, indicating that it may be used as an effective marker for NET identification (**209**).

02. Electron microscopy

Another method for NET visualization is electron microscopy. Electron microscopy (EM) has been a valuable technique in visualizing NETs due to its high resolution, allowing researchers to examine the ultrastructure of cellular components at a detailed level. Scanning electron microscopy

(SEM) appears to be the central choice for visualizing NET formation, it can provide three-dimensional images of the external structure of NETs (**100**). In this study, they employed SEM to investigate NET formation in mouse neutrophils. Moreover, this approach has been used to identify the formation of extracellular traps by cells other than neutrophils, such as macrophages (**210**).

While electron microscopy is effective for visualising the structure of NETs, it cannot provide information on the dynamic mechanisms involved in NET formation. For real-time observations, other imaging techniques such as fluorescence microscopy or live-cell imaging may be more suitable. Moreover, the tremendous energy of the electron beam can occasionally affect the structure of a material, resulting in a misleading image.

03. Flow cytometry

Flow cytometry can be employed to quantify NETs in vivo and in vitro by measuring the fluorescence intensity of specific markers associated with NET formation. Neutrophils can be identified based on their characteristic surface markers (e.g., CD66b) and NETs can be detected using fluorescently labeled antibodies against NET-associated proteins. For example, flow cytometry has been used to quantify NET in the bronchoalveolar lavage fluid (BALF) of mice upon pulmonary infection with S. aureus (**211**). In this study, anti-citrullinated histone H3 and anti-Ly6G were used to stain NET and neutrophils respectively (**211**). Cytometry-based techniques consistently fail to detect externalized NETs because sample processing eliminates NETs and fragile cells while detecting early-stage NET formation with nuclear swelling. Moreover, flow cytometry does not provide information on the morphology and structural details of NETs. Electron microscopy or immunofluorescence microscopy remains necessary for a detailed understanding of NETs.

04. ELISA

Enzyme-linked immunosorbent assay (ELISA) assay can be used to measure specific markers or components of NETs in biological samples. Cells undergoing NET formation release protein-DNA complexes; apoptotic and necrotic neutrophils produce a minial and no significant amount of these complexes (**212**). NETs may be detected in undiluted plasma samples using sandwich ELISA with single anti-MPO, anti-NE, or anti-citrullinated H3 capture antibodies and anti-DNA detection antibodies (**213**). Elisa is a well-established method for measuring NETs in blood or different

samples such as bronchoalveolar lavage fluid (**214**). After adding the peroxidase substrate tetramethylbenzidine (TMB) and ending the reaction, the observed optical density (OD 450 nm) is proportional to the quantity of NETs released in the sample. The data is often reported as absorbance values, or quantified DNA content using an in-house created standard.

05. Picogreen

Since NETs are predominantly composed of DNA, DNA quantification methods can be employed to estimate NET levels. A typical method is multi-well plate-based quantification of externalized cell-free DNA in supernatant using the Picogreen kit, or in situ detection of DNA using membrane-impermeable dyes such as sytoxgreen (**106, 206**). Furthermore, the PicoGreen test lacks the sensitivity to detect a very modest amount of NETs. However, these approaches do not allow precise identification of NET-associated DNA since detected DNA might be the product of neutrophil necrosis or late apoptosis, and morphological alterations remain equivocal.

It is important to note that each quantification method has its strengths and limitations, and the choice of method depends on the research objectives, sample type, and available resources. Using fluorescence microscopy in conjunction with complementary techniques may provide the most detailed understanding of NETs. Additionally, the interpretation of NET quantification data should be done in conjunction with other clinical and experimental parameters to gain a comprehensive understanding of the underlying biological processes.

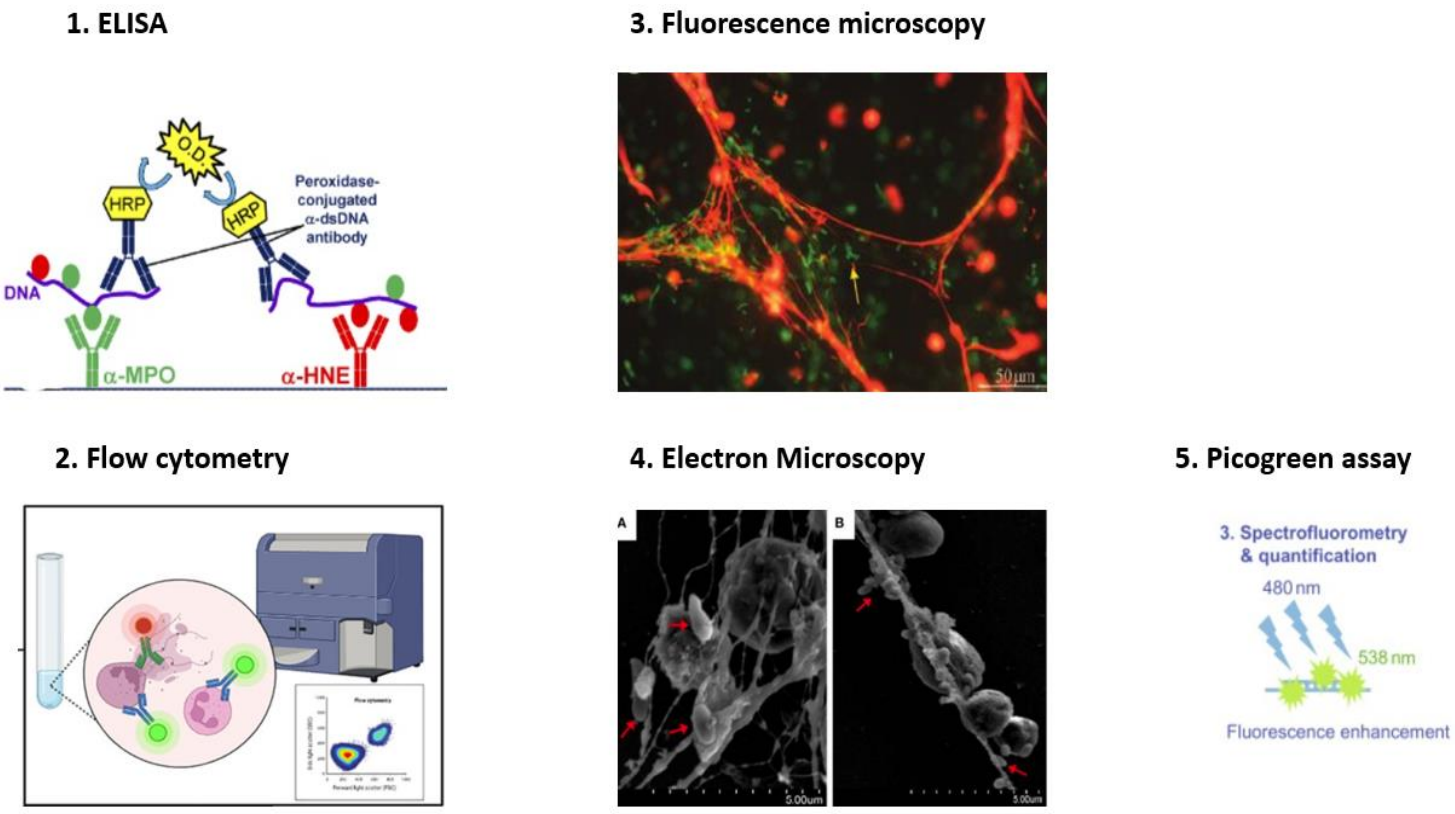


Figure 7. Common methods used to quantify NETs in research.

10. The mechanisms of NET degradation

NET clearance is required to maintain the proper balance of NET formation and degradation. Given the central role of NETs in several diseases, the removal of NETs from the circulation and tissues is critical for preventing inflammation and auto-immune responses in the host. NETs' physiological persistence in circulation or tissues is uncertain, with studies ranging from hours (**215**), to weeks (**216**).

01. DNase elimination of NET

DNase, also known as deoxyribonuclease, is an enzyme that degrades DNA molecules by cleaving the phosphodiester bonds between nucleotides. DNase enzymes play an essential role in various biological processes, including DNA replication, DNA repair, and gene expression regulation.

Due to the potentially harmful actions of NETs, NET turnover is strictly regulated by circulating extracellular DNases that eliminate NETs. DNases are expressed across multiple tissues. DNase I is the principal nuclease found in blood and other body fluids that cleaves extracellular dsDNA. It

is primarily generated by the pancreas and kidneys. DNase I and MNase (Micrococcal nuclease) are two of the most often reported nucleases in NET degradation studies (**217**). Indeed, the administration of neutralizing antibodies against nucleases impairs NET degradation (**218**). The importance of DNases in NET neutralization is highlighted by their presence in pathogenic bacteria, which release NET-cleaving DNases to aid in their escape from the host defense system. The ability of Leptospira to cause persistent infection and achieve effective colonization in a variety of hosts is dependent on NET escape (**219**).

Cleavage with DNases is one of the most important physiological processes in maintaining a low concentration of circulating free DNA and maintaining tolerance to self-DNA. However, DNase activity is decreased in some diseases such as cancer, leading to NET accumulation (**217**). Serum DNase I activity was shown to be considerably lower in RA patients than in healthy control individuals, and this may be responsible for the increased NET formation as well as playing a role in the pathogenesis of RA (**220**). Furthermore, it has been shown that DNase-deficient mice present accumulated splenic antibody-secreting cells reactive to dsDNA (**221**).

02. NET elimination by macrophages

DNase I in physiological quantities is insufficient to completely destroy NETs (**222**), indicating that this structure requires further assistance in decomposition. DNase degradation of NETs is followed by intracellular degradation by macrophages (**222**), highlighting a prominent role of macrophages in NET degradation and resolution of inflammation. Recent studies have shown that inhibition of macropinocytosis in mice with thrombus resulted in a protracted clearance of the clot; also, the quantity of NETs inside the thrombus was increased (**223**). Moreover, by eliminating NETs, synovial fluid macrophages may play a significant role in the resolution of NET-induced gouty inflammation (**224**). There was a significant negative correlation identified between the percentage of macrophages and the quantity of NETs in the synovial fluid of gouty arthritis patients (**224**).

Local macrophage density in human aortic aneurysms was found to be inversely related to surrounding NETs in intraluminal thrombi as well as the vessel wall (**223**). Pro-inflammatory activation of macrophages and dendritic cells primes them for increased NET intake and degradation. MSU crystals have been shown to improve synovial fluid macrophage NET engulfment capacity (**224**). Macrophages are recognized as essential regulators of extracellular

DNA degradation because they phagocyte NET elements without eliciting an inflammatory response (**222**). It has been shown that PMA–NET degradation by macrophages takes place in lysosomes (**222**). Moreover, NET clearance by macrophages is aided by the extracellular digestion of large fragments of NETs by DNase I produced by macrophages, as well as opsonization of NETs with complement factor 1q (C1q) (**222**).

03. NET degradation defects

NET degradation defects refer to the impairment or dysfunction in the clearance of NETs from the body. Proper degradation and clearance of NETs are essential to prevent excessive inflammation and tissue damage. However, defects in the mechanisms responsible for NET degradation can lead to the persistence of NETs, contributing to the pathogenesis of various diseases especially SLE and vasculitis (**104**). Inadequate NET disintegration can serve as a source of immunogens derived from these structures, such as DNA, histones, enzymes, and other NET components. Compromised NET clearance causes an accumulation of NETs in inflammatory sites, resulting in greater inflammation and the presence of NET autoantigens for a longer period (**104**). This might potentially break self-tolerance and hence exacerbate the underlying autoimmune reaction.

All of the processes involved in the degradation of NETs, as explained above, can be hampered. Low DNase activity and functional impairment can be induced by the production of anti-DNase inhibitors (and/or anti-DNase neutralizing autoantibodies) or by DNase gene mutations. A mutation in the deoxyribonuclease 1 like 3 (DNase1L3) gene has also been linked to SLE (**225**). Moreover, in some autoimmune diseases, autoantibodies targeting NET components can interfere with the degradation of NETs. These autoantibodies can prevent the binding of DNase enzymes to NETs, impairing their clearance. For example, in SLE, autoantibodies against NET components have been shown to inhibit DNase activity and impede NET degradation (**226**). Similarly, RA patients have impaired capacity to degrade NETs, and RA sera had reduced ability to degrade NETs compared to HD sera (**227**).

Furthermore, impaired phagocytic capacity of cells, particularly macrophages, and neutrophils, can contribute to defects in NET degradation. Conditions such as acute respiratory distress syndrome, which is characterized by low effectiveness of efferocytosis, can result in compromised NET clearance (**228**).

11. NET inhibition

The regulation of NET formation is a complex process involving multiple mechanisms and molecules. Natural or synthetic anti-NET treatment may reduce exacerbated immune response, hyper-inflammation, immuno-thrombosis, and other problems. Understanding the interplay between NET inducers and inhibitors is crucial for maintaining a balanced immune response and preventing NET-associated pathologies. Ongoing research continues to uncover new endogenous and exogenous inhibitors and their mechanisms of action, providing potential targets for therapeutic interventions aimed at modulating NET formation.

01. Endogenous NET inhibitors

Endogenous NET inhibitors are naturally occurring molecules produced by the body that regulate or inhibit the formation or activity of NETs. Endogenous NET inhibitors, such as neonatal NET-inhibitory factor, suppress key NET formation factors such as histone citrullination, nuclear decondensation, and PAD4 activity (**229**).

DNase I was one of the first endogenous inhibitors of NET formation that was described (discussed above). Recently, several endogenous NET inhibitors were described. For example, A1ATm358 in the placental matrix belongs to the neonatal regulatory element that regulates NET formation in the perinatal environment (**229**). In addition, Serpins (serine protease inhibitors) are a family of proteins that regulate the activity of proteases involved in various physiological processes. Some serpins, such as alpha-1 antitrypsin (AAT) and proteinase inhibitor 9 (PI-9), have been shown to inhibit NET formation. These serpins bind to and inhibit the activity of neutrophil elastase, a protease involved in NET release, thereby limiting NET formation (**230**). AAT are produced by hepatocytes and released in the blood. AAT concentrations in the blood increase fourfold during infection and inflammation in RA (**231**). Therefore, AAT plays an anti-inflammatory role and ameliorates joint inflammation and pain in models of arthritis (**231**). Similarly, secretory leukocyte protease inhibitor (SLPI) is an endogenous protein produced by various cells, including neutrophils and epithelial cells. SLPI has antimicrobial properties and can also inhibit the formation of NETs. SLPI binds to histones in the nucleus of neutrophils, which are a major component of NETs, and prevents their interaction with DNA, thereby inhibiting NET formation (**232**). Moreover, heparin is a naturally occurring polysaccharide that acts as an anticoagulant. It has been found to possess inhibitory effects on NET formation. Heparin can directly interact with histones, which are

essential for NET structure and stability, and disrupt their binding to DNA, leading to the inhibition of NET formation. Heparin treatment has the capability for managing patients with severe COVID-19 by acting as an anti-inflammatory, anti-NET and improving lung oxygenation (**233**).

02. Exogenous NET inhibitors

Exogenous NET inhibitors are substances or compounds that are derived from external sources, such as drugs or therapeutic agents, which have been developed to specifically target and inhibit the formation or activity of NETs (**Table 3**). These inhibitors are designed to intervene in the excessive or dysregulated NET formation seen in various pathological conditions. Further research into the suppression of NET formation pathway, as well as drugs that degrade NETs, might give novel therapeutic methods for autoimmune disorders.

DNase-based therapeutics refer to the use of recombinant DNase I or DNase I-like enzymes as therapeutic agents to treat various conditions characterized by excessive or dysregulated extracellular DNA. Recombinant DNase I or DNase I-like enzymes have been developed as exogenous NET inhibitors. These enzymes function by directly degrading the DNA backbone of NETs, promoting their breakdown and clearance (discussed above). For instance, drugs such as dornase alfa (the recombinant form of the human DNase I enzyme) showed successful NET degradation (**234**). It has been approved for clinical use in certain conditions characterized by excessive mucus production, such as cystic fibrosis, and COVID-19 (**234**). DNase-based therapeutics show promise in limiting NET-associated damage and inflammation.

Among exogenous NET inhibitors strategies is the nanomedicine. Active NET-targeting strategies aimed at improving drug homing while minimizing systemic toxicity are being researched in the field of nanomedicine. Recently, genetic engineering approaches have been employed to create cell membranes loaded with active targeting ligands, allowing the generation of nanoformulations with improved functionality that may be customized to specific applications. A similar platform was shown to target NET by genetically engineered cell membrane hybrid liposomes to specifically target the ablation of NETs. A cell membrane engineered to express a NET-binding protein was hybridized with liposomes loaded with DNase demonstrating successful NET removal (**235**). Likewise, stroke homing peptides (SHP) are short amino acid sequences or peptides that have been designed or discovered to possess an affinity for specific targets and have the ability to selectively bind to and accumulate at the site of injury, allowing for targeted drug delivery,

imaging, or therapeutic interventions. Recently, SHP-guided deoxyribonuclease 1 used to degrade NETs has been described (**236**).

NET inhibitors target specific steps in this process to regulate or suppress NET formation. Various molecular and pharmacological agents have been investigated for their ability to inhibit NETs, such as inhibitors of protein kinases, ROS scavengers, anti-inflammatory agents, and immunomodulatory molecules. Inhibitors of PAD enzymes, such as Cl-amidine and BB-Cl-amidine, have been developed to block the citrullination process and prevent excessive NET release (**237**). These inhibitors show potential in reducing NET-mediated tissue damage and inflammation. In addition, inhibitors targeting ROS production, such as NAC or DPI, have been investigated as potential exogenous NET inhibitors (**238**). These compounds aim to limit the oxidative burst and subsequent NET release by neutrophils.

Moreover, medicines such as metformin and dexamethasone, and antibiotics may have immunomodulatory effects by inhibiting NET formation as well as the production of pro-inflammatory cytokines. Azithromycin and other macrolides have considerable anti-inflammatory benefits through inhibiting the formation of NETs (**239**).

Identifying and eliminating invading NETs is critical for treating NET-associated illnesses, although effective treatments remain difficult. However, to yet, no therapeutic treatment has been established to stop the progression of multi-organ dysfunction caused by neutrophils/NETs.

Table 3. **Potential anti-NETs therapeutics**

Pharmacological compounds	**Target**	**Mode of action**
Dexamethasone (240**)**	TLR2, TLR4	further research is required to understand the precise mechanism.
Dornase alfa (234**)**	DNA	Degrade NET DNA.
Azithromycin (239**)**	Cytokines including IL-6 and IL-8	Immuno-modulating effects by influencing the activation and migration of neutrophils.
Metformin (241**)**	mTORC1, AMPK	Antidiabetic, inhibits mitochondrial ROS, inactivates the PKC-NOX pathway blunting NET formation.
Cl-amidin (237**)**	PAD4	Inhibition of PAD4 required for NET formation.
N-acetylcysteine + DPI (238**)**	ROS	Inhibits ROS production dependent NET formation.

12. Role of NETs in the pathogenesis of various diseases

NET involvement in inflammatory illness has been comprehensively detailed in our two recent reviews (**103, 104**). This part will primarily focus on the role of NETs and probable interplay with B cells in the pathogenicity of various inflammatory diseases.

01. NETs in systemic lupus erythematosus

Systemic Lupus Erythematosus is an autoimmune disease characterized by abnormalities in adaptive immunity induced by genetic predisposing factors and various environmental exposures, resulting in the loss of self-tolerance. This results in extensive tissue damage and inflammation in organs such as the joints, skin, brain, lungs, kidneys, and blood vessels. SLE is defined biologically by the presence of antibodies directed against self-DNA in the blood. Furthermore, neutrophils play an important role in autoimmune reactivity and disease progression. There have been reports of qualitative abnormalities in various neutrophil functions in SLE, and PMNs demonstrate pathogenic features such as enhanced NET formation in lupus neutrophils (**153**). Indeed, NETs are found massively in the skin and kidneys of those patients (**157**).

Moreover, SLE is associated with polyclonal B cell abnormalities, increased B cell hyper-reactivity, and the production of self-reactive autoantibodies. Pathogenic autoantibodies

are characteristic of this illness and have been proven to play a vital role in many indications of lupus, and B cells are undoubtedly critical participants in SLE pathogenesis. Indeed, in lupus, NETs constitute a source of self-antigens for B lymphocytes. Antigens such as double-stranded DNA (dsDNA) and antimicrobial proteins are exposed by NETs, and it has been demonstrated that serum from active SLE patients binds more strongly to the DNA of NETs, indicating the existence of anti-NETs antibodies (**226**). Similarly, SLE autoantibodies can bind antigens present on NETs, e.g. anti-LL37 (**153**). Indeed, by activating both the BCR and TLR9 on lupus B cells, NETting neutrophils carrying LL37-DNA complexes promote lupus B cells to produce IgG and NET-specific autoantibodies in an antigen-dependent manner (**242**). Additionally, IgG2 isotype class switch recombination, which is the major autoantibody in serum and renal glomeruli of lupus Nephritis (LN) patients, is induced by NETs isolated from SLE patients, and this leads to the development of renal lesions typical of LN (**243**). Likewise, H2A is present in NETs, and anti-H2A IgG2 levels have been demonstrated to correlate with SLE activity score and to distinguish between SLE patients with renal complications and other SLE patients (**244**). Additionnaly, NET level has been correlated with an increase in anti-dsDNA production in lupus patients' sera (**157**), demonstrating that there is an interaction between NET and B cell activation to produce anti-NET antibodies, and that the existence of these NETs and anti-NETs plays a significant role in illness progression and aggravation. Otherwise, B cell-produced anti-NET shield the NETs from nucleases and protects them from degradation (**245**).

02. NETs in hidradenitis suppurativa

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disorder that develops cutaneous inflamed nodules and abscesses. HS lesions include dermal tunnels with elevated molecular inflammatory expression, including loricrin, filaggrin, lipocalin-2, CXCL-1 and CXCL-8 synthesis, leading to significant neutrophil infiltration into the skin. Furthermore, NETs were abundant within the epithelial border of the dermal tunnels (**246**), as well as the increased spontaneous NET formation by PMNs from the peripheral circulation of HS patients (**246**). Furthermore, B cells form follicle-like structures within the dermis (**247**). This enhances the possibility that NETs, which are abundant in the skin, to interact with cutaneous B cells, activating them. Indeed, HS patients develop antibodies against NETs derived in vitro from HS patients; HS sera also show significantly elevated antibodies to citrullinated proteins found in NETs, such as tenascin, ApoA1, and ApoE, fibrinogen A, citrullinated filaggrin, indicating that NETs increase

autoantigen generation in HS lesional skin and peripherally (**246**). It has also been shown that NETs are associated with disease severity and progression, and that they can generate a type I IFN response in the skin by priming pDC, which leads to indirect B cell activation and plasma cell differenciation, resulting in an aberrant adaptive immune response (**246**).

03. NETs in bullous pemphigoid

Bullous pemphigoid (BP) is an incapacitating autoimmune sub-epidermal blistering disease that predominantly affects the elderly. Tissue-bound and circulating autoantibodies to hemidesmosomal antigens, such as BP180, characterize this disease. Neutrophils are the most abundant cells in the inflammatory infiltrate of BP patients' skin (**Figure 8**). NETs were found in lesional skin biopsies and serum of BP patients and correlate with disease activity (**248**). Neutrophils from BP patients produced more NETs than the neutrophils of HD patients (**248**). BP sera cause NET formation by BP PMNs but not by control PMNs (**249**). This indicates that BP neutrophils are in a pre-activated state and were primed to undergo NET fromation. Additionally, in remission patients, NETs appear at the extremity of the blister where the dermis splits from the epidermis and have decreased with time following treatment (**248**).

NETs influence B-cell activation and antibody production in BP; NETs promote BP B cells to differentiate into placmocytes and NET-activated B cells produce more total IgG and anti-BP180 (**248**). Likewise, ICs purified from the sera of BP patients could induce increased NET formation in BP neutrophils via FcR and PAD4 activation (**248**).

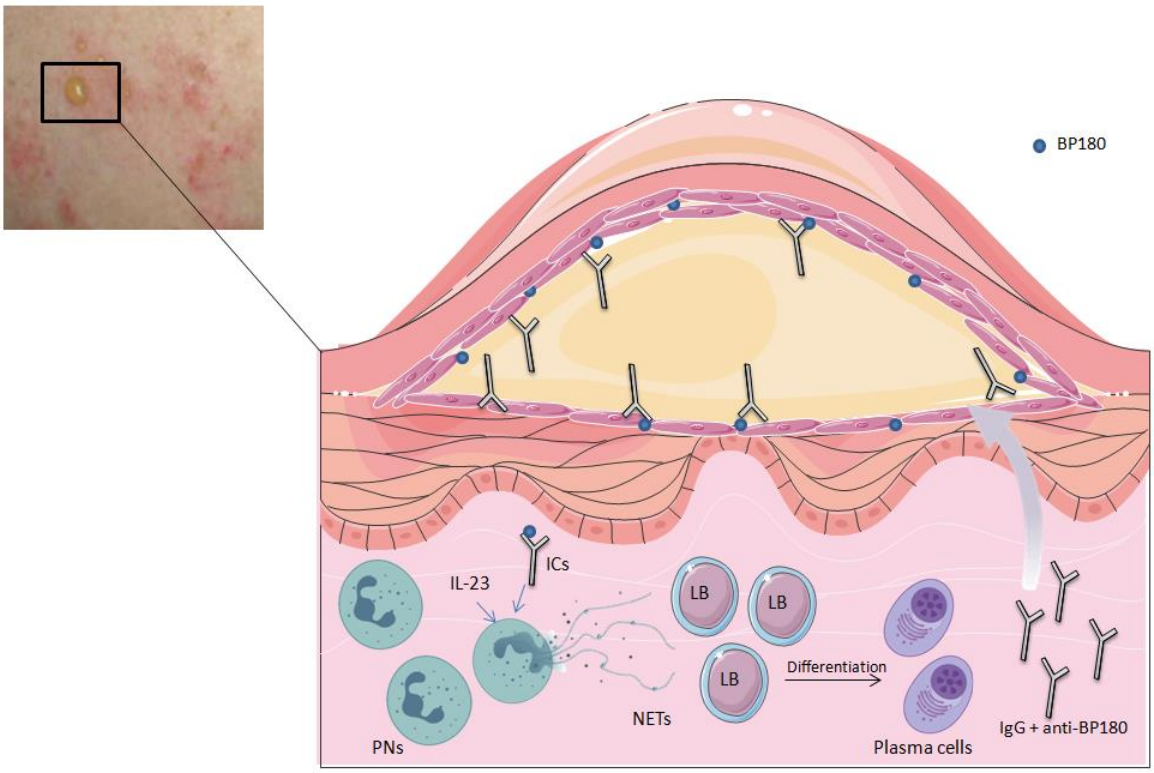


Figure 8. Typical cutaneous manifestations in a patient with bullous pemphigoid, tense blisters surrounded by erythematousoedematous lesions. LB: B lymphocyte, NETs: neutrophil extracellular traps.

04. NETs in cancer

The role of NETs in tumor progression is still being studied; evidence suggests a link between intra-tumoral NET deposition and tumor progression in both experimental models and human cancer patients. NETs have a role in the growth, development, and metastasis of several cancer types. Cancer cell metastasis is aided by NETs in pre-metastatic livers. NET-DNA binding to the DNA sensor CCDC25 on tumor cells, causes cytoskeleton remodeling, migration, adhesion, and proliferation (**251**). Likewise, in breast cancer, when spreading cancer cells arrived in the lungs (a common location of metastatic colonization in breast cancer) they interacte with neutrophils and stimulated neutrophils to create NETs by secreting G-CSF (**252**). Additionally, there is evidence to suggest that cancer can predispose neutrophils to release NETs. A significant increase in NET formation was observed in isolated neutrophils from 28-day tumor-bearing mice without any additional stimulus, implying that NET formation occurs in these mice (**253**).

Furthermore, diffuse large B-cell lymphoma (DLBCL) is a kind of lymphoma that affects B cells, which is the most prevalent type of non-Hodgkin lymphoma in adults. IL-8 highly secreted by DLBCL cells binds CXCR2 and mediates NET formation in murine neutrophils (**253**). NETs can independently promote DLBCL proliferation and migration through the activation of TLR9, indeed TLR9 knockdown inhibits growth and lymph node metastasis of DLBCL (**253**). Moreover, $CD5^+$ B cells have been identified as an aggressive subtype of DLBCL; NETs enhance $CD5^+$ B–cell proliferation by activating NFk-B, and neither apoptotic nor necrotic neutrophils stimulate $CD5^+$ B–cell proliferation in vitro. This could aid the transition from autoimmunity to lymphoma and DLBCL progression (**254**).

Additionally, NETs have emerged as potential biomarkers for various cancers due to their involvement in disease progression and metastasis. For instance, five NETs-related prognostic signature (CD93, CRISPLD2, KCNJ15, IRAK4, and MAPK3) have a good performance in predicting the prognosis of bladder cancer (**255**).

II. B cells

B cells or B lymphocytes are a key player of the adaptive immune response and are responsible for humoral immunity.

A. B cell development

B cell development is a tightly controlled process, which takes place according to a precise chronology in different organs and tissues of the body. It is characterized by progression through a series of checkpoints defined by rearrangement and expression of immunoglobulin genes to ultimately generate mature naïve B cells in the peripheral blood. Human B-lineage cells are found in a variety of tissues during early fetal development. However, the BM is the only site of B-lymphogenesis after birth.

Early B cell populations in the bone marrow pass sequentially through a series of phenotypically distinct stages including pro-, pre- and immature B cell pools. From B progenitor to immature B stage, they undergo a series of genetic rearrangements aimed at producing a functional B cell receptor (BCR) (**256, figure 9**). Differentiation of pro-B into pre-B b allows the formation of the so-called pre–B-cell antigen receptor (pre-BCR) complex. Upon expression of pre-BCR, pre-B

cells proliferate and undergo rearrangement of their Igκ, then λ light chain (L) genes allowing the differentiation into immature B cells carrying a BCR (a functional IgM isotype) on their surface. Once a functional BCR is on the membrane, it must be examined for its capacity to bind self-antigens to ensure that only a small number of auto-reactive B cells are released (central tolerance) (**256**).

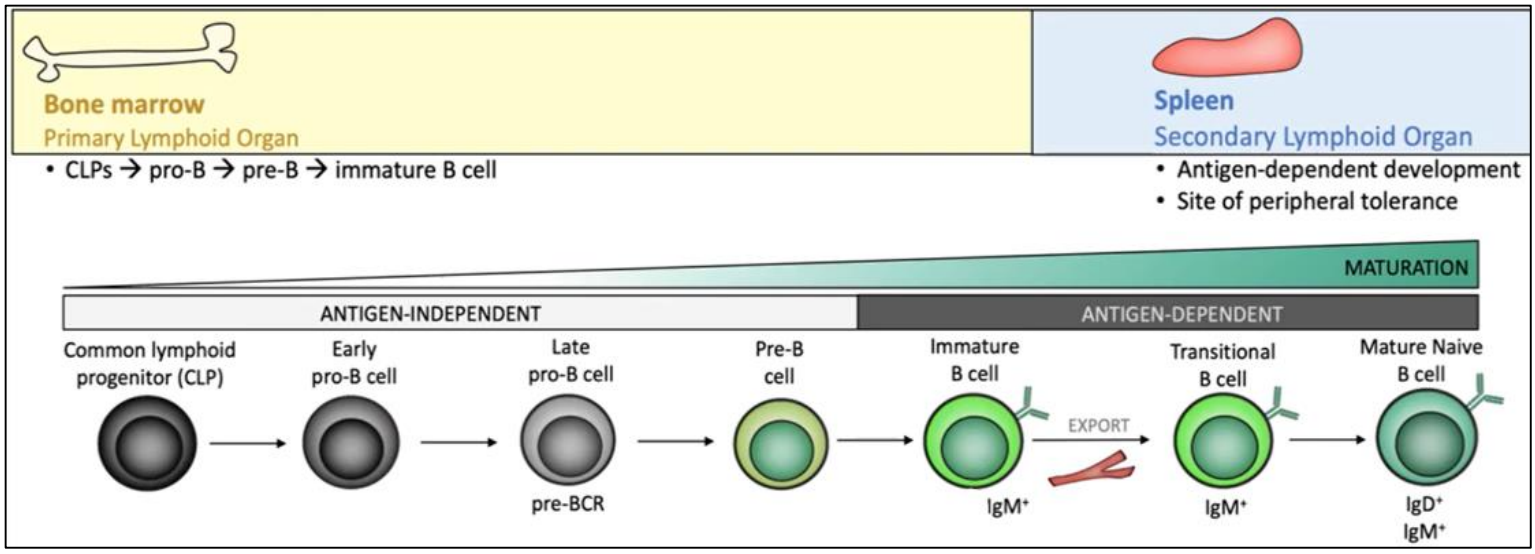


Figure 9. B cell development in bone marrow and spleen.

B. Identification of B cells

The rise of phenotypic markers along the B cell development starts with CD34, followed by CD38, CD10 (the earliest canonical Pro/Pre-B cell marker), CD19, CD20, and ending with immunoglobulin heavy chain IgH expression, indicative of immature B cells ready to leave the bone marrow. CD19 and CD20 are both expressed on B-cell subsets in blood but CD20 expression is lost during terminal B-cell differentiation into antibody-secreting cells (**257**). A relatively limited number of surface phenotypic markers, including CD19, CD20, IgD, CD27, CD38, CD138 and CD24, can be used to identify all parental populations of human peripheral B lymphocytes (**258**).

Human B lymphocytes are classified into four major subgroups based on the expression of IgD and CD27 (**257, figure 10**). Precursor B cells develop from hematopoietic stem cells to immature B cells in the bone marrow and then mature into naïve B cells ($CD19^{+}IgD^{+}CD27^{-}$) in the periphery. IgD is an immunoglobulin expressed on the surface of naive B cells, whereas IgD down-regulation and increased CD27 expression on B cells indicate the expression of immunoglobulin genes that

have undergone somatic hypermutation (SHM) in the germinal center (GC) within lymphoid organ B-cell follicles, giving rise to memory B cells (**257**). IgD and IgM are first expressed on naïve B cells and are thereafter downregulated following isotype switching (**259**). Naive B cells develop into memory B cells after recognizing antigens in the secondary lymphoid organs (**257**). In GC, high-affinity B cells are chosen through interactions with follicular dendritic cells and antigen-specific follicular helper T cells (**260**). This GC reaction produces high-affinity switching memory (SM) B cells ($CD19^+IgD^-CD27^+$) and long-lived plasma cells (**260**). In the extrafollicular T cell-independent or GC-independent pathway, innate signals such as Toll-like receptor signals promote the production of non-switched memory B cells ($CD19^+IgD^+CD27^+$) (**260**). Double negative (DN) B cells ($CD19^+IgD^-CD27^-$) are a small, poorly known B cell subgroup that was originally identified in individuals with systemic lupus erythematosus (**261**). This fraction has attracted the interest of researchers in recent years due to its high prevalence in individuals with autoimmune and infectious disorders, as well as its association with pro-inflammatory and autoimmune features (**262**). DN B cells encountered less SHM than CD27-expressing counterparts, which is consistent with their absence of CD27 expression (**263**). DN B-cell population can be divided into four subtypes with different origins and functional capacities: DN1 B cells (Switched-memory B-cell precursor), DN2 B cells, DN3 B cells (DN2 precursor), and DN4 B cells (IgE^+ Switched-memory B-cell precursor) (**262**). In autoimmune fibrotic disorders, as well as in COVID-19, DN3 B cells may be involved in tissue inflammation and fibrosis (**264**).

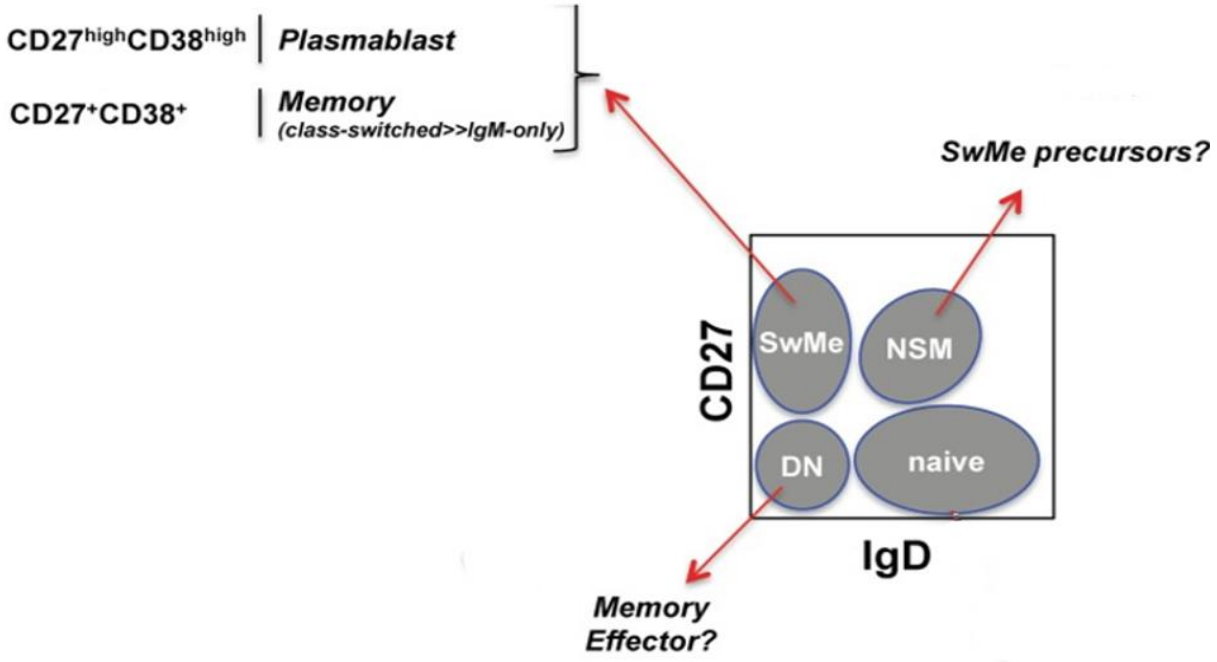


Figure 10 B cell subsets based on IgD and CD27 expression. Schematized flow cytometry plot indicates four core B cell subsets defined by CD27 and IgD expression. SwMe, switched memory; DN, double-negative; NSM, non-switched memory and naïve B cells. Adapted from *Kaminski DA, Front Immunol, 2012.*

C. Anatomical distribution of B cells

B cells are distributed throughout the body in specific anatomical locations to carry out their functions. B cell development begins in the bone marrow, where hematopoietic stem cells differentiate into precursor B cells. Mature B cells migrate then to lymph nodes (LN) through circulation. During their transit in lymph nodes, B cells migrate through various microenvironments where they meet antigens, get activated, and develop into effector cells. B cell migration to LN is tightly controlled (**265**). CXCL13 has been identified as a key regulator of B-cell migration (**265**). Human LN B cells express surface CCR7 and CXCR5. $CXCR5^{low}$ B cells are found to no longer migrate efficiently in response to CXCL13 (**265**). After several hours of surveillance, B cells may leave lymphoid tissues mediated by sphingosine-1-phosphate (S1P) via their S1PR1 receptor (**266**). The precise mechanisms regulating the time that B cells spend in lymphoid tissue have not been fully defined. Recent research has revealed that lymphocyte entrance and exit from lymphoid tissues has a circadian aspect, with more lymphocytes accumulating in lymphoid tissues during periods of increased physical activity (**267**).

An increased amount of chemokines occurs during inflammation. For instance, expression of numerous homeostatic and inflammatory chemokines with the ability to impact lymphocyte trafficking into tissue are increased in the synovium and synovial fluid of RA patients. B cells are abounding in the synovium and are organized into follicular structures (**268**). The chemokine expression pattern of B cell migration, CXCL13, is expressed in ectopic germinal centers in the synovium of RA patients (**269**). However, it is unclear whether auto-reactive B cells develop inside ectopic follicles and germinal centers in the synovium or are recruited from draining LNs.

D. Activation of B Cells

B cells are capable of recognizing antigens that attach to their surface immunoglobulin receptors, resulting in the production of soluble antibodies, which mediates the humoral immune response via pathogen neutralization, opsonization, and complement fixation.

An antigen-specific signal via the TCR and a co-stimulatory signal from an antigen-presenting cell (APC) are required for naive T cell activation. Like T cells, naive B cell activation requires antigen recognition by the Ig receptor and additional signals from a $CD4^+$ T cell (thymus-dependent) or, in certain situations, directly from microbial components or DAMPs (thymus-independent). Early cell-surface modifications linked with B cell activation include CD40, CD80, CD86, and CD69 upregulation (**270**).

1. T cell-dependent B cell activation

Thymus-dependent B cell responses, also known as T cell-dependent B cell activation, need antigen detection by both B cells and T cells. This type of activation is essential for the generation of high-affinity antibodies and the development of immunological memory. APCs ingest antigens in the tissues and circulate to lymph node T cell-rich zones through the lymphatics, where they present antigenic peptides linked to major histocompatibility complex (MHC) class II molecules to naive T $CD4^+$ lymphocytes. TFH cells subsequently relocate to the T-B cell interface, where they engage with antigen-specific B cells. The same antigen binds to a specific BCR in lymph node follicles, resulting in internalization by receptor-mediated endocytosis, processing into smaller peptide fragments through a process called antigen processing, and cell-surface presentation on MHCII molecules. When the T cell recognizes the peptide-MHCII complex through the interaction of the TCR, triggers the expression of CD40 ligand (CD154), which binds to CD40 on the B cell, causing B cell proliferation and differentiation (**271**). CD40 is a key co-

receptor that is involved in T cell-dependent B cell activation, immunoglobulin class switching, and the establishment of humoral memory. T cell-dependent B cell activation contributes to clinical symptoms in different illnesses, and blocking CD40L-CD40 communication between T and B cells may prevent disease severity (**272**). Other molecules like, IFN-γ, IL-4, IL-10, and IL-21 are also secreted by T cells and are necessary for Ig isotype switching (**273**). These signals are important for the activation and differentiation of both B and T cells. T-B cell interactions cause B cell proliferation in germinal centers, as well as somatic hypermutation and affinity maturation of their Igs, which results in the development of long-lived antibody-secreting plasma cells and memory B cells (**274**).

2. T cell independent B cell activation

T cell-independent (TI) responses are further classified into two types: type I (TI-I) can be induced by antigens containing ligands of Toll-like receptors, while type II (TI-II) is elicited by antigenic substances with repetitive multiple epitopes, which include bacterial capsular polysaccharides (capPS) of certain bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitides*, which are used as vaccines against these bacteria (**275**). Pneumovax is a human vaccination made up of capPS from 23 pneumococcal serotypes that are considered as model TI antigens. In humans, Pneumovax vaccination produces serum reactions that persist for up to 5 years (**276**).

In the absence of TLR signaling, TI-II antigens robustly induce BCR cross-linking, responsible for rapid B cell activation, proliferation, plasmablastic differentiation and antibody production (**277**). TI responses, in contrast to t-cell dependent responses, are incapable of generating fully established GC or a memory response characterized by affinity maturation and a greater, quicker response of B cells to antigens rechallenge. However, certain evidence suggests that specific types of B cell memory in mice arise in response to distinct model TI antigens, as well as the creation of rapidly collapsing GC (**278**).

III. Rheumatoid arthritis (RA)

Rheumatoid arthritis is a chronic autoimmune disorder that affects millions of people worldwide with women affected two to three times more commonly than men. Numerous studies have examined RA prevalence, with estimates of a global prevalence of 0.24% (**279**). The French doctor

Augustin Jacob Landré-Beauvais was the first to identify and categorize this disease in 1880 (**280**). It primarily affects the joints and causes inflammation, leading to joint swelling and destruction. The main symptoms include muscle weakness, tight or painful joints in the lower extremities that limit physical activity, as well as changes to gait and balance that may increase the risk of falls in RA patients. The first signs of systemic arthritis are usually seen and felt in the joints of the hands or feet but can affect all joints, including the spine. RA is also considered a systemic autoimmune disease that can also affect other organs of the body, such as the lungs, heart, and eyes (**281**). Moreover, elevated incident cardiovascular disease risk has been found in RA patients compared to patients without RA (**282**).

RA evolution happens by inflammatory eruption causing joint destruction and irreversible disability in the absence of an effective treatment, making it essential to diagnose and treat RA early. Furthermore, RA is a chronic, long-term joint disorder that may persist for decades and even for life. RA pathogenesis remains unclear, it involves a complex interplay between genetic, epigenetic and environmental factors resulting in a cascade of immune reactions. RA is a heterogeneous disease, with variable clinical appearance among patients, and pathogenic mechanisms involved between individuals with the same diagnosis as well as between various disease stages. However, recent data from the literature incriminate innate immunity cells in the development of RA lesions. In this part, I will describe the etiology and pathogenesis of RA before focusing on the preponderant role of B cells and NETs in this pathology.

A. Disease course

The disease course of RA can vary among individuals, but it generally follows several phases (**figure 11**). The first phase refers to overall susceptibility to RA when there are no symptoms or observable persistent immunological abnormalities. Environmental factors interact with a genetic predisposition promoting susceptibility to RA. These interactions may lead to the second phase of preclinical RA. Multiple studies show a phase of RA development marked by autoantibody and other biomarker abnormalities in the absence of and prior to the manifestation of clinically identifiable inflammatory arthritis that characterizes RA. This period is called preclinical rheumatoid arthritis (pre-RA) (**figure 11**) (**283**). Anti-citrullinated protein antibodies are a specific type of autoantibody that plays a critical role in the diagnosis and pathogenesis of RA. There is emerging evidence that the generation of ACPAs in genetically susceptible individuals begins long

before clinically visible joint inflammation and that the triggering of this autoimmunity might begin in mucosal epithelial surfaces, such as the lungs (**284**). High-resolution computer tomography (HRCT) examinations have demonstrated that lung abnormalities (interstitial lung disease) can be present in ACPA-positive patients prior to the beginning of RA joint symptoms, as well as in newly diagnosed ACPA-positive RA (**284**). Furthermore, increased citrullination and lymphocyte infiltration have been seen in the lungs of patients with early untreated RA (**285**). Recently it has been shown that T cell-driven B cell differentiation, which results in local class switching and somatic hypermutation, is seen in the lungs before and during the early stages of ACPA-positive RA (**286**). Some studies have found increases in specific cytokines and chemokines, such as IL1α and interferonγ-induced protein 10, before RF, as well as ACPA-positive in the serum of pre-RA patients (**287**). Furthermore, numerous studies have demonstrated that C-reactive protein (CRP), a routinely used inflammatory marker in clinical practice, increases with time in pre-RA patients (**288**).

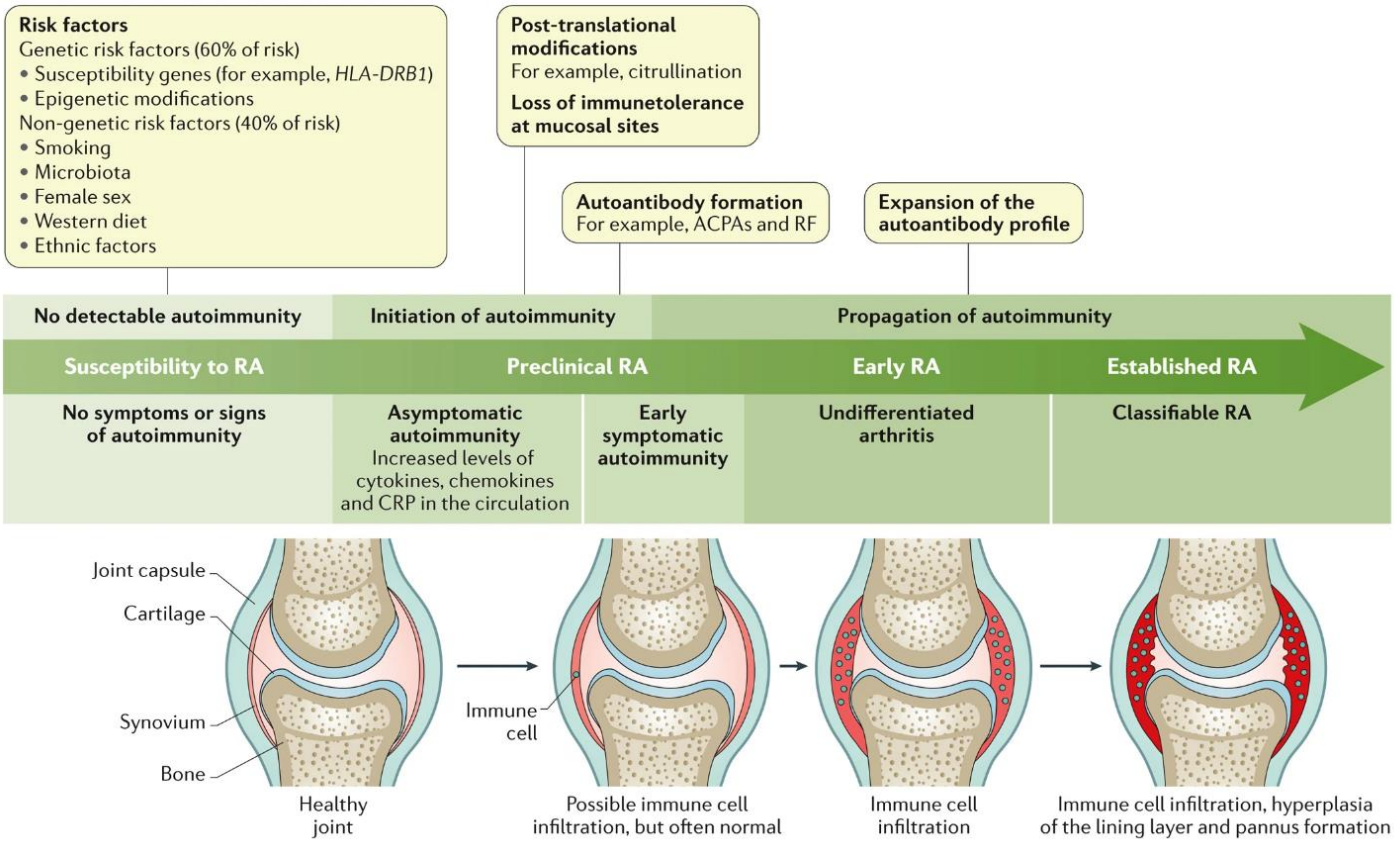


Figure 11. Nature Reviews Disease Primers. Rheumatoid arthritis (RA) is caused by both hereditary and non-genetic risk factors, and many risk factors are probably necessary before RA is initiated. Disease progression involves initiation and propagation of autoimmunity against modified self-proteins, which can occur years before the onset of subclinical synovitis (inflammation of the synovium) and clinical symptoms.

Pre-RA is followed by the early inflammatory phase which is marked by the onset of symptoms such as joint pain, stiffness, and swelling. This phase typically occurs over weeks to months and is characterized by the initiation of synovitis, which is caused by an inflammation of the synovial tissue (**283**). During this phase, there is an increase in the production of inflammatory cytokines and the infiltration of immune cells into the synovial tissues, leading to joint inflammation. Several studies have suggested that mucosal mechanisms may play a role in the progression from pre-RA to clinically evident RA. Evidence suggests that IgG ACPAs were enhanced the earliest in Pre-RA, whereas IgA ACPAs emerged around the time of clinically apparent RA (**289**).

As the disease progresses, RA enters the established phase, which is characterized by persistent and chronic inflammation in multiple joints (**283**). This phase can lead to joint damage, erosion of cartilage and bone, and structural deformities. The inflammation can extend to other tissues and organs, leading to systemic symptoms, such as fatigue, fever, and weight loss. At this stage, the diagnosis of RA is confirmed based on clinical, laboratory, and imaging findings.

B. Diagnosis of RA

Before 2010, the diagnosis of RA was based on a set of criteria established by the American College of Rheumatology (ACR) dating from 1987 (**290**). But these criteria have been criticized for their lack of specificity, mainly for early RA. Therefore, in 2010, new classification criteria were proposed by the ACR and the European League Against Rheumatism (EULAR) (**291**), in order to diagnose RA earlier and thus increase the chances of therapeutic efficacy. The diagnosis of RA requires patients to have swelling in at least one joint on clinical examination. A sensitive examination of the affected joint is then used to confirm the diagnosis, ultrasound, or MRI, as well as serological biomarkers (RF and ACPAs), and acute phase reactants (ESR and CRP). Lastly, a scoring system has been used to categorize RA patients (**Table 4).**

Type of joint damage	*(0-5)*
1 large joint	0
10 medium or large joints	1
1-3 small joints	2
4.10 small joints	3
>10 joints (at least one small joint)	5
SEROLOGIE	***(0-3)***
FR^- and $ACPA^-$	0
At least one weakly positive test	2
At least one strongly positive test	3
SYNOVITIS DURATION	***(0-1)***
< 6 weeks	0
> 6 weeks	1
ACUTE PHASE REACTIONS	***(0-1)***
Neither CRP nor high ESR	0
High CRP and ESR	1

Table 4 ACR and EULAR classification criteria

A total score of ≥6 is needed to classify a patient as having definite RA. The large joints correspond to the shoulders, elbows, hips, knees and ankles. The small joints encompass joints metacarpophalangeal, interphalangeal proximal, metatarsophalangeal II to V, interphalangeal joints of the thumb.

Diagnosing rheumatoid arthritis can be a complex process, as the symptoms can vary from person to another and can mimic other types of arthritis. The diagnosis of RA is mainly based on clinical manifestations (joint swelling, redness, and warmth) and serum biomarkers such as RF and anti-CCP antibodies, also known as ACPAs, with the latter being more disease-specific. Indeed, although anti-CCP autoantibodies are an important characteristic of RA, two main types of RA patients have been described, seropositive and negative RA. Seropositive RA refers to the presence of RF and/or anti-CCP antibodies in a person diagnosed with RA (60%-80% of people with RA are seropositive) (**292**), on the contrary some individuals are negative for these autoantibodies (seronegative RA). A negative anti-CCP result does not however rule out the disease as these antibodies are not found in all patients. In addition, RF testing looks for a group of non-specific antibodies. RF can be present in up to 70% of individuals with RA, although they can also be present in healthy individuals who never develop rheumatoid arthritis. Several illnesses, including hepatitis, persistent infections, or other inflammatory conditions or autoimmune diseases, might cause RF to be elevated (**293**). Moreover, RA patients often have an elevated erythrocyte sedimentation rate (ESR) or CRP level, which may indicate the presence of an inflammatory process in the body, CRP has a strong relationship with disease activity (**294**). However, up to 30% of patients have completely normal blood tests.

1. Disease activity score

Disease activity of RA is usually assessed by using the DAS28 index proposed by EULAR and developed in 1995 (**291**). DAS stands for 'disease activity score', and the number 28 refers to the 28 joints that are examined in the assessment (shoulder, elbow, wrist, metacarpophalangeal, proximal interphalangeal, knee). The first DAS was based on an examination of 44 joints (DAS44) (**295**), and this was later followed by a reduced and simplified version based on 28 joints, DAS28. DAS28 scores were firstly referred to DAS28 (ESR) and are composed of four components, including the number of swollen joints, the number of tender joints, the patient's global health score and was originally using erythrocyte sedimentation rate (ESR) as the inflammation marker, ranging from 0 to 9.4 (**295**). DAS28 (ESR) values are calculated as follows = 0.56*(TJC28) + 0.28*(SJC28) + 0.014*GH + 0.33*log(ESR), where TJC = tender joint count and SJC = swollen joint count and GH= patient assessment of disease. The validated threshold values of DAS28 using ESR are 2.6 for remission, 3.2 for low disease activity, and 5.1 for high disease activity (**295**). Indeed, in 2007, Fransen et al. proposed DAS28-CRP, which would use the same thresholds as DAS28-ESR but instead measure C-reactive protein (**296**), since CRP is a preferential measure of inflammation compared to ESR, with ESR being confounded by age, sex, anemia, time of day, plasma viscosity, and aberrant red blood cell size and shape. DAS28 CRP values is calculated as follows: DAS28 (CRP) = 0.56*(TJC28) +0.28*(SJC28) + 0.014*GH + 0.36*log(CRP+1) + 0.96 (**297**).

C. Risk factors

RA is a multifactorial disease and the exact origin of RA is still not completely elucidated. However, it is known that in RA, immunological tolerance to autologous proteins (including collagen, and fibrinogen) is impaired for various reasons, leading to the development of autoantibodies against autoantigens. Several factors may intervene to disrupt immune system functions and promote RA development: hormonal, environmental, psychological, genetic, infectious factors and others. The degree of involvement of these different factors in the development of RA is not known.

1. Genetic factors

RA has a strong genetic component, the genetic risk for RA that has been estimated by scientific studies is about 50% (**298**). Several genes have been associated with an increased risk for disease

development. Genetic susceptibility factors for RA have been known for many years, and over time new genes were identified as shown in figure 12.

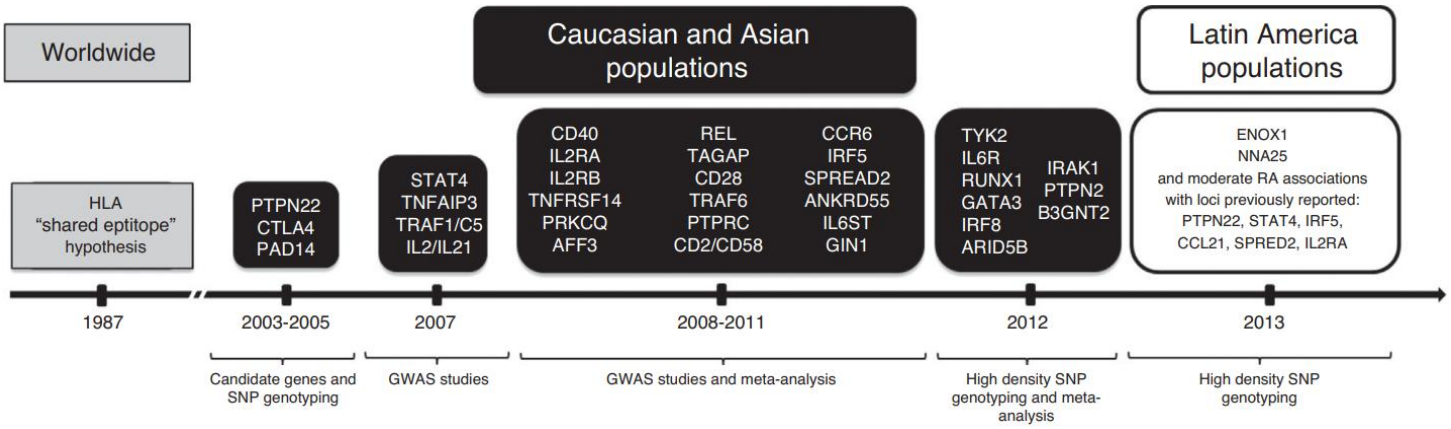


Figure 12. Timeline of discovery of several genes associated with RA. Castro-Santos P, Rev Bras Reumatol Engl Ed (2016).

The major histocompatibility complex locus contains the primary genes responsible for RA susceptibility. Over 250 genes encoding the antigen proteins for T-cell recognition are found in this locus, including the HLA-DRB1 gene, which is the most widely studied gene and has the strongest genetic relationship with RA, with a 3-fold increased risk of RA (**299**). Certain HLA-DR alleles may influence the development and progression of RA, although these alleles differ by ethnicity and geographical location. For instance, HLA-DRB1*0401 alleles encode unfavorable variants of the shared epitope (SE), with a conserved sequence of five amino acids linked to the disease. Predisposing HLA-DRB1 SE alleles are found in 64–70% of RA patients and 55% of their healthy relatives (**300**). The precise immunological implications of their expression are not clear but according to the shared epitope theory, certain alleles with this conserved sequence are associated with the pathogenesis of RA because they enable antigen-presenting cells to mispresent of arthritogenic self-peptides or molecular mimicry with foreign antigens to T cells, leading to autoimmune reactions that are directly involved in the pathogenesis of RA (**301**).

Prior to 2007, other genes were described, such as protein tyrosine phosphatase non-receptor 22 (PTPN22), peptidyl arginine deiminase type IV (PADI4), cytotoxic T-lymphocyte antigen 4 (CTLA4), the B-cell cell surface receptor gene (CD40), and the chemokine receptor genes (e.g., CCR6), signal transducer and activator of transcription 4 protein (STAT4), and tumors necrosis factor-receptor associated factor 1 and complement component 5 (TRAF1/C5) related genes are

the main genetic factors associated with an ACPA-positive subtype, although interferon regulatory factor 5 (IRF-5) is only found in the ACPA-negative subtype (**302**).

For instance, single nucleotide polymorphism (SNP) is frequently found in RA patients, increasing the individual susceptibility to RA development. Ming Li et al. found a SNP (rs6427528) at the 1q23 region (near enough to the CD84 locus) in a GWAS study of 2,706 RA patients that was associated with changes in the disease activity scores of treated patients with the anti-tumor necrosis factor (TNF) etanercept (**303**).

It is quite hard to understand how genetic diversity contributes to the emergence of RA. Some studies have hypothesized that genetic predisposition might lead to RA only when encountering certain environmental conditions. Such genetic-environment interaction has been demonstrated between the HLA alleles and inhaled pollutants (**304**).

2. Bacterial and virus infections

Much emphasis has been placed on the infectious etiology of RA. Intercurrent infections are widely known to be associated with RA recurrence. Many bacteria, including Porphyromonas gingivalis (P. gingivalis), Proteus mirabilis (P. mirabilis), Epstein-Barr virus (EBV), and mycoplasma, have been implicated in the etiology of RA in clinical and animal model research (**305**). The presence of microbial components in RA tissues adds to the evidence of a link between infection and RA. DNA of P. gingivalis, mycoplasma, parvovirus, EBV, and cytomegalovirus (CMV) have all been found in RA patients' synovial fluid, synovial membranes, or serum (**305**).

Periodontitis is a serious gum infection and the most commonly associated RA disease, a recent meta-analysis found that individuals with periodontitis had a 69% higher incidence of RA than healthy groups (**306**). Another study found that moderate to severe periodontitis was more common in RA patients (51%) than in osteoarthritis patients (26%) of the same age and gender (**307**).

The relationship between infections and RA is complex. P. gingivalis is a major periodontal pathogen and expresses the citrullinating enzyme peptidyl arginine deiminase. As a result, P. gingivalis infection contributes to protein citrullination which might result in the production of ACPA and the development of RA (**308**). Additionally, P. gingivalis could induce NET formation, and NETs are immunogenic in RA and are a source of antigens for ACPA production (**309**).

Furthermore, infections can cause immune dysregulation and alter the balance of immune cells and cytokines. P. endodontics is found in high numbers in the saliva microbiota profiles of individuals with early-onset RA compared with healthy controls; this bacterium generates collagenases and protease enzymes, which can also play a role in tissue degradation (**310**). P. intermedia and Tannerella forsythia have also been found in the synovial fluid of RA patients, they express BspA, a surface adhesion protein, which stimulates the release of bone-resorbing pro-inflammatory cytokines IL-6, IL-8, and TNF-α (**311**).

3. Sex factor

Sex differences in RA have been studied from a variety of perspectives, including incidence, phenotype, comorbidities, response to treatment, and prognosis, although the underlying causes are uncertain. The prevalence of RA is more common in women than in men with a 4 women:1 man ratio at younger ages (<50 years old) and a 2:1 ratio at older ages (>60 years). However, this ratio decreases with age at onset (**312**). Women have a poorer outcome result than men in terms of disease activity and disability (**313**), but joint damage is the same for men and women. In an arthritis mouse model, males had an earlier onset of arthritis and more severe implications on joints, bones, and kidneys (**314**). Furthermore, gender variations in disease activity indices, particularly DAS28-ESR, have been the most studied. Women had higher DAS, a poorer prognosis (**315**), and were less likely to achieve remission than men (**316**). The relationship between sex difference and CRP level, as well as the difference between DAS28-ESR and DAS28-CRP, has been investigated, but only a few studies have assessed the influence of sex differences on DAS28-CRP (**317**).

Indeed, hormones are believed to contribute to RA pathogenesis, when compared to premenopausal women, perimenopausal women with RA were less likely to achieve remission (**318**). Moreover, the dopaminergic receptor D1DR on RA B cells appears to be sex-specific, it is overexpressed on B cells from female RA patients compared to male RA patients and healthy donors. D1DR expression is found to correlate with disease duration and functional disability in RA females (**319**).

4. Environmental factors

Environmental factors, such as smoking, nutritional consumption, and physical activity, impact the development and progression of RA. Many environmental risk variables interact with genetic risk factors.

01. Smoking

Many environmental factors, dietary and lifestyle factors have been associated with an increased risk for RA, and the strongest of these is exposure to tobacco, particularly among individuals carrying the HLA-DRB1 genotypes linked to RA, indicating a possible connection between genetic and environmental factors in the pathogenesis of RA. Multiple studies have described odds ratios of the link between smoking and RA higher than 2, and it is estimated that smoking exposure accounts for 20-30% of the environmental risk for RA (**320**). Importantly, various aspects of the smoking-RA interaction may moderate the elevated risk of RA development. Primarily, smoking may contribute to the activation of PAD enzymes in the serum of smokers, which leads to the citrullination of proteins (**321**), resulting in the presentation of citrullinated antigens and, eventually, the production of ACPAs. Smoking is most significantly related to ACPA-positive RA (**320**). Although increasing oxidative stress is one of the major mechanisms for the development of RA, nicotine from tobacco smoke can increase oxidative stress in the body. An association between serum cotinine (toxic ingredients of tobacco smoke) and rheumatoid arthritis has been found in a recent epidemiological study in the US general population (**322**).

02. Nutrition

In recent years, there has been an increase in interest in the role of nutrition in the development of RA. In this context, nutrition has both direct and indirect roles in disease development by providing anti-inflammatory dietary components and by influencing body mass index (BMI) and visceral fat storage. A recent study reported that high fiber intake was related to a lower incidence of RA (**323**). Several observational studies also show that a high intake of vegetables, seafood, and adherence to the Mediterranean diet is related to a lower risk of RA (**324**), although the findings are not entirely consistent. A highly explored issue in the context of nutrition and RA disease treatment is Omega-3, whether consumed through fish or taken using nutritional supplements. A recent systematic review and meta-analysis comparing omega-3 to placebo found that omega-3 had an

effect on pain relief and swollen joint count (**325**). Furthermore, high magnesium (Mg) diet may suppress arthritis, by expanding $Foxp3^+$ Treg cells and the production of IL-10 (**326**).

03. Other environmental factors

Physical exercise may be another modifiable risk factor for RA that could apply to a broader subset of patients. Encouragingly, increasing leisure physical activity was found to be related to a lower incidence of RA (**327**).

Other environmental and lifestyle parameters, as well as their function in the development of RA, have been studied. Low socioeconomic status, as indicated by a low degree of formal education, has been associated with an increased risk of RA in studies (**328**). Moreover, exposure to minerals, such as silica, during daily activities may be associated with an increased risk of RA (**329**).

The climatic environment may influence immune cell proportion and function and may be related to the pathogenic process of RA. By comparing two RA cohorts from different locales (Tsukuba and Karuizawa), which range in height by 1000 m and, as a result, have significant variations in average air temperature and atmospheric pressure. RA patients in Tsukuba, compared to Karuizawa had a significant increase in Th1, Tph cells, and a significant decrease in Th17 and $CD8^+$ Treg in T cell subpopulations, as well as a significant increase in class-switched memory B cells and a significant decrease in unswitched memory B, naive B cells in B cell subpopulations (**330**).

D. Role of neutrophils in the pathogenesis of RA

Neutrophils play a complex role in the pathogenesis of RA. Their dysfunction and excessive activation can contribute to the inflammation and tissue damage observed in RA.

1. Neutrophil migration in RA

RA is recognized by joint inflammation. The influx of immune cells, including macrophages, neutrophils, B cells, and T cells, into the synovial compartment defines the early phases of RA. Neutrophils are the most common joint-infiltrating cells in RA (**331**), and most RA animal models depend on neutrophils to induce joint inflammation. Chronic inflammation in the RA synovium is caused by the production of a number of mediators, including chemokines. Local stimulation of neutrophils in the synovial vasculature allows them to migrate transendothelially into inflammatory tissues. Neutrophil migration in RA may be induced by cytokines such as TNF-α,

IL-1, IL-6, IL-8, IL-15, IL-17a, IL-22, IL-23, IFN-γ, GM-CSF and G-CSF (**332**). Moreover, plasma and synovial fluid (SF) CCL2, CCL3, CCL4, CXCL5, and CXCL10 concentrations are higher in RA patients compared to other types of arthritis (**333**). Chemokine production has been shown to change throughout RA stages. CCL4, CXCL4, CXCL7, and CXCL13 are expressed early on, while CCL3 and CCL9 were produced later (**333**). In addition, chemokine receptor CCR1, CXCR2, and CXCR4 expression are higher on purified SF PMNs than on bone morrow PMNs from arthritic joints (**22**). Similarly, neutrophils from RA patients are found to express high levels of CCR2 compared to PMN from healthy donors, and pharmacologic inhibition of CCR2 is found to decrease neutrophil infiltration into the joints in an arthritic model in mice (**334**). In vitro, cell-free synovial fluid induces increased L-selectin expression; one of the first neutrophil adhesion molecules to come in contact with the endothelium, and induces neutrophil chemotaxis (**335**).

Neutrophils additionally deliver chemokines on themselves into the joint and participate in their own recruitment by producing IL-1β thereby inducing the production of neutrophil-active chemokines from structural cells of the joint (**22**). Another cell type participates in neutrophil migration, in collagen-induced arthritis (CIA) model, synovial macrophages and resident synovial fibroblasts exacerbate neutrophil-driven joint injury by increasing neutrophil recruitment into joints through the production of the chemokine CXCL2 (**336**). Furthermore, synovial fibroblasts exposed to IL-18, CCL19, and CCL21 secrete more vascular endothelial growth factor (VEGF), which may induce angiogenesis in the synovium and exacerbate immune infiltration (**336**).

2. PMN apoptosis in RA

Neutrophil apoptosis is dysregulated in RA. Delayed apoptosis within synovial joints contributes to chronic inflammation, immune cell recruitment, and prolonged release of proteolytic enzymes. Apoptosis is delayed in both blood and synovial fluid neutrophils from RA patients. these cells have an altered phenotype, increased expression of protein Mcl-1 and decreased levels of activated caspase-9. Decreased apoptosis is accompanied by increased NET production in RA SF neutrophils (**337**). Emodin, a traditional medicine for RA, promotes apoptosis and inhibites NETosis and autophagy in neutrophils (**337**). Similarly, the delay of neutrophil apoptosis in RA is returned to normal levels after methotrexate therapy (**27**). the microenvironment of RA synovial fluid promotes neutrophil survival; it has been demonstrated that synovial fluids from RA patients

are capable to delay the apoptosis of neutrophils (**338**). Because of their extended lifespan, these cells have an increased capacity to mediate host tissue damage.

Many cytokines generated during inflammation including GM-CSF, TNFα, IL1β, IL-9, IL-15, and interferons, which are increased in both blood and synovial fluid, are most likely to delay neutrophil apoptosis (**338**). Meanwhile, IL-6 can inhibit the apoptosis of neutrophils from RA patients in vitro (**338**). Other pro-inflammatory mediators in RA, such as leukotriene B4 which activates NF-kB may increase the anti-apoptotic Mcl-1epression and induce delayed RA neutrophil apoptosis (**339**). Likewise, CRP is routinely assessed as a marker of systemic inflammation in RA and CRP levels are often persistently elevated in patients with RA. It has been shown that CRP delays neutrophil apoptosis and may therefore contribute to the amplification of the inflammatory response (**340**).

3. Enhanced neutrophil activities in RA

In the context of RA, neutrophils exhibit heightened activities and altered phenotype, characterized by increased expression of activation markers such a s CD11b, reduced leukosialin and CD43 expressions, and the upregulation of an azurophil granule protein (CD63) (**342**). Moreover, RA neutrophils exhibit distinct alterations in their energy metabolism, reflecting a shift in the cellular metabolic profile. Notably, metabolites such as ATP (adenosine triphosphate), ADP (adenosine diphosphate), GTP (guanosine triphosphate), and glutathione demonstrate significant increases in RA neutrophils. In contrast, glucose level is decreased in RA neutrophils compared to HD neutrophils (**342**).

RA PMNs exhibit an aberrant immune response characterized by the production of elevated levels of pro-inflammatory cytokines. RA neutrophils exhibit increased TNF mRNA expression and elevated NF-κB activity compared with controls (**343**). Moreover, SF neutrophils from patients with RA have found to produce significantly more IL-8 and IL-1β compared to peripheral blood neutrophils (**344**).

4. ROS production by PMNs in RA

ROS and nitric oxide (NO) are generated at sites of inflammation in various joint disorders. ROS are found to play a significant role in the pathogenesis of rheumatoid arthritis (**345**). RA patients showed a marked increase in ROS formation in the blood (**345**). RA PMNs exhibit heightened oxidative stress and increased spontaneous superoxide (O2-) (**346**). Different stimuli in RA can

induce ROS production by neutrophils, such as synovial fluid from RA (**347**). Moreover, hypoxia in the joint cavity can also induce ROS production as demonstrated by in Vivo PET/MRI Imaging (**348**). Elevated ROS blood amount is accompanied by increased lipid peroxidation, protein oxidation, and DNA damage (**345**). In RA patients, ROS levels show a high correlation with disease activity. They have a strong correlation with C-reactive protein and clinical parameters and can be used as an indirect predictor of the degree of synovial inflammation in RA patients (**349**).

5. NET formation in RA

The involvement of NETs in the pathophysiology of RA has been effectively reported in our recent review (**103**). In RA, neutrophils infiltrate the synovial joints and can produce NETs, which are implicated in RA pathogenesis. RA SF neutrophils lose their migratory characteristics and get retained inside the joint, generating signals that increase joint injury and inflammation through the recruitment and activation of both innate and adaptive immune cells (**347**). Consequently, inhibition of neutrophil differentiation and maturation or inhibition of NET formation obstructs the induction of arthritis mouse models (**350**).

01. Enhanced NET formation in RA

The aberrant formation of NETs from neutrophils has been demonstrated in the pathogenesis of RA. When compared to healthy controls, peripheral blood neutrophils from RA patients exhibit higher NET formation (**351, 352, 353**). During RA, NETs participate in joint tissue lesions by activating various immune cells, NETs also serve as a source of neo-antigens leading to auto-antibody production. It has been shown that pain severity is positively correlated to higher NET production in mice (**354**). The activation of the SHIP1/MAPK/TNF pathway is required for increased NET formation in RA (**353**). Signal inhibitory receptor on leukocytes-1 (SIRL-1) is decreased in RA neutrophils compared to HDs. It has been demonstrated that ligation of SIRL-1 to neutrophils suppressed ROS production and NET formation (**355**). Also, a SIRL-1 expression was negatively correlated to NET formation in RA (**355**), which suggests a potential implication of SIRL-1 in the regulation of NET formation in RA.

Several stimuli have been implicated in inducing NET release during RA. ACPAs have shown a potential to drive NET formation in vitro (**351**). The RA joint or serum contain stimuli that might cause NET formation. For example, IL-40 is a new B-cell-associated cytokine. Elevated serum IL-40 was detected in RA patients in the early stages and is related to enhanced NET formation (**356**).

IL-8, TNF and IL-17A collaborate to trigger NET formation in RA neutrophils, with TNF priming considerably increasing NET formation caused by IL-17A (**351**).

02. NETs as source of autoantigens in RA

NETs have emerged as a significant source of autoantigens in RA which potentially trigger the production of autoantibodies. In the context of RA, the release of NETs by activated neutrophils provides a source of self-antigens that contribute to the immune dysregulation and perpetuation of the disease. Citrullinated proteins are known to be ACPA targets and may play a role in RA development. NETs are a source of both citrullinated and carbamylated autoantigens (**351, 356**). ACPA and anti-carbamylated proteins (anti-carP) are associated with poor RA prognosis (**351, 356**). Levels of citrullinated histone H3 (CitH3)-expressing NETs was greater in neutrophils from at-risk RA patients compared to controls (**357**). Moreover, higher quantities of extracellular citrullination were only identified in anti-CCP-positive RA patients' blood (**358**). ACPAs, especially IgA ACPAs, are generated in the lungs of RA patients due to enhanced citrullinated protein-expressing NETs (**357**).

The dysregulation of these post-translational modifications in RA can lead to the generation of neo-autoantigens. Internalization of NETs containing modified autoantigens by fibroblast-like synoviocytes (FLS) endows them with antigen-presenting cell capacities and induces anti-citrulline/carbamyl harmful adaptive immunity (**359**). Synovial B cells within ectopic lymphoid structures in RA joints were found to produce high-affinity ACPAs targeting NETs (**360**). Increased synovial PAD activity is responsible for the loss of tolerance to citrullinated proteins, and systemic citrullination might indicate an increased risk of developing citrulline-specific autoimmunity (**357**).

Similar to citrullination, carbamylation is another post-transitional modification seen in NETs and act as autoantigens in RA. Carbamylated protein-DNA complexes appear in high concentrations in RA plasma and was associated with RA severity (**356**). RA NETs contain carbamylated LL37 (carLL37). CarLL37 levels were observed to be higher in RA patients' plasma and synovial fluid as compared to healthy controls (**361**). Patients with RA generate autoantibodies against carNET proteins (**356**). Anti-carLL37 antibodies have been detected in RA sera and synovial fluid, and they are correlated with bone erosion scores in RA patients (**361**).

03. NETs induce several immune cell activation

In RA, NETs have been implicated in the activation of several immune cells. We have already shown that RA-derived NETs can activate macrophages and neutrophils (**352**). Moreover, citrullinated H2B histones are found in NET and can activate macrophages to generate TNF while also propagating neutrophil activation (**362**). These findings suggest that citrullinated proteins found in NETs cause an immunological response in vivo. Additionally, interactions between synovial NET and FLSs promote an inflammatory environment susceptible to a pathogenic immune response. NETs have been shown to enhance inflammatory responses and promote a pro-inflammatory gene signature in RA synovial fibroblasts, including the production of IL-6, IL-8, chemokines and adhesion molecules (**351, 363**). Furthermore, NET uptake by FLS promotes an inflammatory phenotype and an enhanced expression of HLA class II (**359**). Neutrophil elastase exists in NETs, increases peptidyl arginine deiminase-2 release by FLSs, and citrullination of extracellular matrix proteins (**363**). In particular, humanized HLA-DRB*04:01 transgenic mice can develop ACPAs after being immunized with NETs-loaded FLS. These ACPAs could recognize α-enolase, citrullinated fibrinogen, and vimentin, all of which are RA-associated (**359**).

Impact of NETs extend on other immune cells, such as osteoclast. It has been shown that NETs promote osteoclast formation which contributes to the destructive bone changes characteristic of RA (**364**). Similarly, through induction of the maturation of DCs (upregulation of co-stimulatory molecules, such as CD80, CD86, and MHC class II), NETs also play a crucial role in the induction and proliferation of Th1 pathogenic cells in CIA model (**365**).

E. Role of B cells in the pathogenesis of RA

B cells have an important role in RA beyond the production of autoantibodies, as they additionally produce soluble factors (cytokines and chemokines), present antigens to T cells, and form B cell aggregates in the synovium (**366**). The RA synovial tissue structure resembles (in some places) secondary lymphoid tissue, with T cell and B cell differentiation sites. In RA patients, ectopic lymphoid structures (ELS) in the synovium are present at both early and late stages of the disease and have been associated with autoantibody titers, inflammatory cytokine levels, and disease severity (**366**). Importantly, B cells provide a critical function in T cell activation. T cell activation in RA patients is associated with an expansion of B lymphocytes in ectopic lymphoid structures (**367**). In arthritis mouse model, B cell depletion resulted in the dissociation of B cell follicular

structures and a significant decrease in IL-1ß and IFN-γ levels, demonstrating that B cells influence the capacity of T cells and macrophages to produce these pro-inflammatory cytokines (**367**). In addition to ectopic structure formation, B-cell activation markers are higher in patients with early RA. The increase of B-cell activation markers in very early RA indicates that B-cell activation is an early pathogenic event in RA (**368**). Moreover, a dysregulation of cytokines production by B cells was observed in RA. B cells from seropositive RA patients secrete less IL-10 after in-vitro stimulation compared to HDs (**369**).

B cell recruitment into the joints plays a significant role in the pathogenesis of the disease. When compared to B cells from peripheral blood, SF B cells significantly upregulated the surface expression of CCR1, CCR2, CCR4, CCR5, and CXCR4 (**370**). The increase of B lymphocytes in the joints is primarily mediated by synovial microenvironment-derived chemokines. B cell recruitment in the inflamed synovium is significantly influenced by CXCL13 and CCL20 (**370**). CXCL13 and CCL20, specific ligands of CXCR5 and CCR6, respectively, have been found in the synovium of individuals with chronic RA (**269**). CXCL13 is a particularly effective chemoattractant for B cells (**371**).

1. Altered B cell phenotype and repertoire in RA

RA B cells show altered peripheral B cell homeostasis and functions, showing a shift in the B-cell phenotype and repertoire. Nevertheless, patients with RA show peripheral B-cell frequencies similar to those of healthy controls (**372**). A recent study suggests that IL-27 which is increased in RA serum notably enhances immune alterations of B cells from RA patients via activating the mTOR signaling pathway (**373**). For instance, mTOR phosphorylation in B cells was further enhanced in RA patients (**373**).

01. RA B cell repertoire

In RA, B cell populations undergo significant changes. Several studies showed decreased switched memory ($IgD^{-}CD27^{+}$) and increased naïve ($IgD^{+}CD27^{-}$) B cells in blood of RA patient compared to healthy individuals (**371**). This is consistent with the concept that RA patients have deficiencies in tolerance checkpoints, resulting in an abundance of polyreactive cells in the mature naive B-cell compartment (**374**). Aside from the distortion in naive B cells, RA patients have an increase in the double negative ($IgD^{-}CD27^{-}$) B-cell fraction, which is particularly noticeable when comparing $ACPA^{+}$ RA patients to controls (**375**). Recently, single-cell RNA sequencing studies of RA

synovial tissue revealed four B cell states: naïve, memory, age-associated B cells, and plasma cells (**376**). Additionally, characterization of B cell subpopulations in RA patient synovial fluid showed an accumulation of switched memory ($IgD^{-}CD27^{+}$) and double-negative memory ($IgD^{-}CD27^{-}$) B cell (**377**). The pathogenic functions of B cells, particularly plasmablasts and switched memory B cells, which trigger the development of RA by generating ACPA autoantibodies, presenting autoantigen, and secreting cytokines, have been widely investigated (**378**).

02. RA B cell phenotype

B cells have an altered phenotype in RA. Analyzing circulating B cells by mass cytometry showed increased expression of HLA-DR on RA B cells compared to healthy donors (**379**), which is consistent with more activated B cells. Moreover, a B cell subset that expresses ICOSL has been found expanded in RA SF and peripheral blood of RA patients compared to HDs and OA and has an intense capacity to produce inflammatory cytokines and the capability to activate autoreactive T cells (**380**). Similarly, single-cell transcriptomics of RA synovium revealed an abundant population of synovial B cells with elevated expression of activation markers including CD69 and CD83 (**268**). This subset has increased expression of chemokine receptors and chemotactic factors involved in ELS formation, including lymphotoxin (LT)-α, LT-β, and IL-6 (**268**). Furthermore, B cells in RA may exhibit altered expression of surface markers. CD79a is a B cell-specific antigen that is expressed throughout the maturation of B cells, making it one of the most diagnostically sensitive and specific Pan-B cell markers for routine immunophenotypic research. A study found that the density of synovial CD79a-positive cells was greater in RA patients compared to OA patients and was substantially associated with joint erosion (**381**).

2. B cell involvement in the pathogenesis of RA

01. Antibody production

Autoantibodies are mostly released and generated by autoreactive B cells once they differentiate into plasma cells. The antibody repertoire in RA recognizes a wide range of antigens, including collagen type II, citrullinated proteins and carbamylated proteins. RFs and ACPA are the two most well-investigated autoantibodies. According to current research, the two main types of autoreactive B cells in RA are ACPA-positive B cells and RF-positive B cells (**382**). However, the development of ACPA-positive B cells and RF-specific B cells differs; ACPA-positive B cells undergo more

germinal center responses than RF-positive B cells (**383**). ACPA-positive B cells had a higher rate of somatic hypermutation and class switching than RF-specific B cells (**383**).

Activated T cells, particularly $CD4^+$ T cells, play a critical role in providing help to B cells during antibody production. T cells interact with B cells in the germinal centers of secondary lymphoid organs. Antibody generation by B cells is extremely dependent on T helper cells, notably C-X-C chemokine receptor type 5 $(CXCR5)^+$ follicular T helper cells (**384**). There is substantial evidence that the percentage of $CXCR5^+$ follicular T helper cells in circulation is increased in RA patients (**385**).

001. ACPA

ACPAs are autoantibodies that specifically target proteins containing citrullinated residues, which are post-translational modifications of the amino acid arginine. The process of ACPA production starts with the citrullination of proteins. Peptidyl arginine deiminases (PADs), a family of enzymes, catalyze the conversion of arginine to citrulline. PAD4 is expressed in several cell types including neutrophils (also present in NETs), and macrophages, and recently it has been found in platelets (**386**). More than 20 ACPA-recognized molecules have been studied (**387**). ACPA targets include synovium and synovial fluid, cartilage (**388**), the lungs (**389**), and inflammatory cells such as neutrophils (**390**). Indeed, only a few ACPA targets, e.g. citrullinated fibrinogen, citrullinated vimentin, citrullinated α-enolase peptide 1, and citrullinated Tenascin-C have been determined to be present in the joint (**391**).

The list of RA-associated autoantibodies continues to expand. Platelet aggregates are frequently found in the blood and joints of individuals with RA, and microparticles released as a result of platelet activation accumulate in their joints. Recently, it has been found that ACPAs may identify a large amount of citrullinated proteins found in platelets (**392**).

002. Pathogenicity of ACPAs and RFs

Autoantibodies such as RFs and ACPAs play several roles in the pathogenesis of RA. ACPAs can form immune complexes by binding to citrullinated proteins in the joints. These immune complexes contribute to the chronic inflammation and tissue damage observed in RA. Immune complexes containing RFs or ACPAs stimulate the complement system, resulting in the formation of C5a which can induce joint injury (**393**). In RA patients, autoantibodies against citrullinated vimentin can also promote osteoclast differentiation to mediate bone degradation (**394**). ACPAs

can contribute to the pathogenesis of RA by directly activating immune cells (**395**). It has been shown that ACPAs induce NETs formation by neutrophils. Additionally, ACPAs have been demonstrated to activate macrophages and stimulate the generation of pro-inflammatory cytokines via an immune-complex-mediated mechanism involving Fc receptors (**396**). The incorporation of RFs in the ACPA-IC boosted this impact (**393**). Furthermore, ACPAs can interact with various immune cells, further amplifying the inflammatory response. ACPAs might bind to fibroblasts in RA patients' inflamed synovia but not in healthy, non-inflamed joints (**397**). The binding of ACPAs influenced cell adhesion and promoted cell migration (**397**).

02. B cells act as APC in RA

The role of B cells in RA has historically focused on the functions of autoantibody production. In rheumatoid arthritis, B cells play a crucial role not only as antibody-producing cells but also as antigen-presenting cells. B cells may become increasingly important in activating autoreactive T cells. Furthermore, RF^+ B cells are thought to play a crucial role in antigen presentation (**398**). They may take up antigen-Ig immune complexes via their RF-specific membrane Ig receptors (**398**). B cells then digest and deliver antigen peptides, inducing T-cell activation (**398**).

The initial activation of naïve $CD4^+$ cells is dependent on sustained signaling via TCR engagement of peptide/MHC complexes (e.g. HLA-DR) as well as APC costimulatory molecule interaction. CD40L, CD28 on T cells, and CD40, CD80/CD86 on APCs are the most well-studied costimulatory pathways. B cell costimulatory molecule expression may be important for T cell activation. CD86 and CD40 on B cells are primarily providing costimulatory signals to T cells. Reciprocally, the CD40-CD40L pathway is an important co-stimulatory pathway that drives T cell-dependent B cell activation. Additionally, BCR signaling has been shown to be important in the development of autoreactive B cells in RA (**399**). According to studies on mouse models, antigen-specific B lymphocytes are required as APCs for autoantibodies production, autoreactive T cell activation, and the development of autoimmune arthritis due to their expression of MHC class II and co-stimulatory molecules CD80 and CD86 (**400**). More evidence that T-cell responses in RA synovitis can be dependent on B cells is that in severe combined immunodeficiency (SCID) mice xenotransplanted with RA synovial tissue enriched in B cells, treatment with a monoclonal anti-CD20 antibody resulted in disruption of GCs, and impairment of T cell activation, as well as a decrease in the production of T cell-derived cytokines (**367**).

Analyzing circulating B cells by mass cytometry showed increased expression of HLA-DR on RA B cells compared to healthy donors (**382**). HLA-DR molecules present peptides to follicular helper T cells that promote the generation of IgG antibodies, implying that RA-associated HLA-DR molecules may present peptides to TFH cells to aid in the generation of ACPAs. Furthermore, citrullinated proteins are important autoantigens that influence the development of RA disease. Research suggested that HLA-DRB1 alleles might bind citrullinated peptides and deliver them to T helper cells that recognize citrullinated proteins (**401**). Another study discovered that HLA-DR alleles might bind to PAD4 and employ it as a carrier to internalize and process the PAD4-citrullinated protein complex, as well as present the PAD4 peptides to T helper cells, resulting in the production of IgG antibodies to multiple citrullinated proteins (**402**). Similarly, RA patients show both antibodies and T cell responses to PAD4, which suggests that the target for helper T cells implicated in the formation of ACPAs may be PADs rather than citrullinated antigens (**402**).

Furthermore, RA patients had a higher proportion of CD86-expressing naive and memory B cells than healthy controls, suggesting an expanded active state among these subpopulations, which is expected to promote more effective interaction with pathogenic T cells. CD86 expression is higher on plasmablasts, being important for antibody production, and on memory B cells, as compared to naive B cells, where CD86 expression has been described as low or undetectable (**403**). Existing study has shown that CD80/86-CD28 costimulatory signals are necessary for arthritis induction and the establishment of specific T-cell activation in CIA model (**404**).

CD40-CD40L interactions promote T and B cell differentiation and activatiom. The presence of CD40L on the membrane of an activated T cell promotes the development of memory B cells and long-lived plasma cells. It has been established that early CD40L expression results in B-cell activation, Ig secretion, isotype switching, and memory formation. However, CD40L is considerably up-regulated in T cells in RA, and the amount of soluble CD40L in RA patients is associated with autoantibody levels and disease activity (**405**). The interaction of CD40 expressed on the surface of activated B cells with CD40L expressed on activated $CD4^+$ T cells is required for the immune response progression (**406**). VIB4920 (a CD40L inhibitor) has been proven in clinical studies to decrease B cell activation and differentiation while also reducing disease activity in RA patients (**407**).

Aside from antigen presentation, B cells can activate pathogenic T-cell responses via cytokine release.

3. B cell derived cytokines and their role in RA

B cells have emerged as significant contributors to the pathogenesis of rheumatoid arthritis through their ability to produce various cytokines. In RA, B cells display an aberrant cytokine profile that further amplifies the inflammatory response within the joints and promotes disease progression. TNF-α IFN-γ, IL-6 and IL-1β are among the cytokines secreted by B cells and that participate in inflammation (**408**).

01. RANKL

The TNF cytokine superfamily member, receptor activator of NF-kappaB ligand (RANKL) is a major osteoclastogenic molecule. Binding of RANKL and its receptor (RANK) triggers tumor necrosis factor receptor-associated factor 6 (TRAF6), nuclear factor-kappa B (NF-kB), and mitogen-activated protein kinases (MAPKs), which promote osteoclast (OC) differentiation by regulating the expression of osteoclast-related genes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and MMP9.

RANKL was expressed in RA animal models within 4 days after the onset of arthritis (**409**). RANKL/ osteoprotegerin play a crucial role in bone destruction by osteoclasts in animal models of arthritis, as evidenced by the fact that RANKL knockout (KO) mice did not have localized bone loss despite continuous joint inflammation (**410**). Patients with RA showed a positive correlation between the SF cell count and RANKL level, which may suggest a relationship with active inflammation and more destructive joint disease (**411**). Furthermore, the baseline RANKL level in blood was demonstrated to predict bone deterioration after 5 and 11 years of follow-up in patients with early, untreated RA (**412**).

Stimulated human memory B cells, notably from RA patients, are shown to produce RANKL in quantities exceeding that produced by T cells (**413**). Both synovial fluid and synovial tissue B cells from patients with RA spontaneously express RANKL (**413**). Evidences suggest that B lymphocytes may influence bone via several ways. Thus, the production of RANKL and other cytokines by synovial B cells, such as IL-1, IL-6, and TNF, indicates they may directly or indirectly activate OCs via antibody-independent processes. The factors that govern RANKL upregulation in B cells are beginning to emerge. IFN-γ significantly increased RANKL expression in B cells,

shifting them toward a more osteoclastogenic phenotype (**414**). The anti-RANKL monoclonal antibody can improve the rat CIA model, showing that the anti-RANKL monoclonal antibody has some potential and may be useful in further research into the mechanism of RA therapy (**415**).

02. TNF-α

TNF-α is an inflammatory cytokine that is encoded in the major histocompatibility complex region as a trimeric protein. TNF-α and its receptors TNFR1/TNFR2 are the most important members of a gene superfamily of ligands and receptors that regulate vital biological activities. The main molecular mechanism responsible for TNF pro-inflammatory effects is TNFR1 signaling. TNF expression is higher in RA patients, and transgenic mice with TNF overexpression develop autoimmune arthritis (**416**). Recent studies combining single-cell transcriptomics and mass cytometry have shown that B and T cells are also the primary source of TNF-α in RA synovium (**388**). TNF-α is a potent pro-inflammatory cytokine involved in the recruitment and activation of immune cells, such as macrophages and neutrophils, leading to the production of additional pro-inflammatory mediators.

TNF-α is a key cytokine in RA. TNF-α may stimulate synovial fibroblasts, hyperplasia, and recruit inflammatory cells (**417**). TNF-α upregulates the expression of B-cell activating factor (BAFF) and hVCAM1 by FLSs, and increased T-FLS cell interaction which may play a role in B cell recruitment (**418**). Similarly, TNFα may trigger ICAM1 expression on FLS and promote FLS-T cell interaction, which increases T cell activation, differentiation, and proliferation (**419**). Moreover, TNF-activated osteoclasts are also critical for the progression of RA disease, and activated osteoclasts in RA result in synovial hyperplasia and angiogenesis (**420**), suggesting that B cell-derived TNF-α may contribute to bone resorption in RA. Additionally, TNF-α stimulates the production of matrix metalloproteinases, enzymes that degrade extracellular matrix components, leading to tissue damage and joint destruction (**421**).

03. IL-6

IL-6, another important RA regulator, was discovered in 1986 as a secreted factor that stimulated immunoglobulin production. B cells are a prominent source of IL-6 in the chronic phase of autoimmune disorders such as RA (**422**). RA patients with higher levels of IL-6 reported worse health-related quality of life than those with medium or low levels (**421**). IL-6 is released by synovial fibroblasts and B cells in the RA synovium (**388**). IL-6 plays a role in bone resorption by

osteoclasts in joints because it enhances RANKL expression on osteoblasts and synovial cells, resulting in osteoclast differentiation and pannus development. For instance, IL-6 directly induced RANKL expression in RA-FLS (**423**). Furthermore, it has been shown that IL-6 in synergy with TNF could induce osteoclast differentiation, thereby contributing to the pathology of inflammatory arthritis associated with joint destruction (**424**).

IL-6 functions through the IL-6 receptor (IL-6R). There are two types of this receptor: membrane-bound (IL-6R) and soluble (sIL-6R). T cells, B cells, monocytes, osteocytes, and osteoblasts primarily express the membrane-bound receptor, whereas the soluble form is generated by differential splicing or cleavage of the IL-6R extracellular domain. Sarilumab is a monoclonal antibody that suppresses IL-6-mediated synovial damage by targeting both the membrane-bound and soluble forms of the IL-6 receptor (**425**).

04. IL-1β

In rheumatoid arthritis, interleukin-1 beta (IL-1β) is a key pro-inflammatory cytokine that plays a significant role in the pathogenesis and progression of the disease. IL-1β is produced by various cells, including immune cells, including B cells (410) and resident joint cells, and it exerts multiple effects on various cell types involved in RA. IL-1β, is elevated in the serum of patients with RA and is positively correlated with disease activity (**426**). Within the joint, IL-1β contributes to joint damage by increasing the release of degradative enzymes such as matrix metalloproteinases, cathepsins, and mast cell proteases (**427**). IL-1β also increases the development of osteoclasts, which contributes to bone erosions that impair joint function (**428**). In the presence of IL-1β, TNF-α can enhance the production of RANKL by B cells, increasing the development of osteoclasts. Additionally, IL-1β promotes angiogenesis, within the synovium. It stimulates the production of angiogenic factors, such as vascular VEGF and basic fibroblast growth factor (bFGF), leading to increased blood vessel formation in the synovial tissue. The newly formed blood vessels contribute to the influx of immune cells into the joints and provide nutrients to the expanding synovial pannus (**429**).

05. IFN-γ

B cell-derived IFN-γ is a critical checkpoint for B cell autoimmune responses. B cells aggravated proteoglycan-induced arthritis by producing IFN-γ (**430**). Mice with IFN-γ deficiency that was restricted to B cells were totally resistant to the disease (**430**). B cell IFN-γ production may thereby

cause autoimmunity by acting on T cells. Notably, increased IFN-γ signaling in T cells might result in an increase in TFH cell accumulation and, as a result, uncontrolled autoimmunity (**431**).

4. Bregs in RA

Bregs are immunosuppressive cells that induce immunological tolerance by producing IL-10, IL-35, and TGF-β, preventing the development of pathogenic T cells and other pro-inflammatory lymphocytes (**432**). The impairment of Bregs in RA contributes to the dysregulated immune response observed in this autoimmune disease. Bregs in healthy individuals are characterized by the expression of specific cell surface markers and the secretion of immunosuppressive cytokines. The phenotypic characterization of Bregs in RA is an active area of research, and while some markers have been identified, there is still ongoing investigation to fully understand their specific phenotype in this disease (**432**). It has been reported that the proportions IL-10-producing Breg $CD19^+IL10^+$, granzyme B-producing Breg $CD19^+GrB^+$, $CD19^+Foxp3^+$, and $CD19^+TGF\beta^+$ Bregs were significantly decreased in RA patients (**433**). GrB-producing Bregs were substantially decreased in RA patients and might be recovered following efficient therapy with disease remission in RA (**433**). Moreover, B cells expressing the lymphocyte activation gene-3 (LAG3) have been discovered as a new regulatory B cell subpopulation. $LAG3^+$ B lymphocytes were shown to be significantly lower in RA patients as compared to healthy individuals, and the frequency of $LAG3^+$ B cells was inversely associated with tender joint count (**434**).

5. Anti B-cell therapy

Targeting B cells has emerged as an effective therapeutic strategy in the management of rheumatoid arthritis.

01. Targeting Bruton's Tyrosine Kinase BTK

Bruton's Tyrosine Kinase BTK is a non-receptor cytoplasmic tyrosine kinase that is part of the TEC family of kinases. It is widely expressed in monocytes/macrophages, dendritic cells, mast cells, osteoclasts, and B cells. Antigen binding by BCR initiates intracellular signaling cascades that result in BTK phosphorylation, thereby promoting the survival, proliferation, and differentiation of B cells. In order to control of B-cell proliferation and function, recent research shows that targeting BTK might be an effective therapeutic strategy for RA (**436**). Recently, a novel BTK inhibitor, TAS5315, was shown to suppress the progression of inflammation and joint

destruction in CIA, and to suppress the expression of CD69, CD86, and MHC class II in mouse B lymphocytes (**437**).

02. Rituximab

Rituximab (RTX), a monoclonal antibody target CD2 which is found on white blood cells called B cells. RTX may decrease disease activity in RA patients. However, several clinical trials have shown that Rituximab has a lower effect on B cell numbers in the synovium than in the circulation. All patients with RA in a serial synovial biopsy trial of RTX treatment reported essentially total depletion of circulating B cells following therapy, but only a slight decrease in synovial B cells (**438**).

F. Fibroblast-like synoviocytes in RA

Fibroblast-like synoviocytes, also known as synovial fibroblasts, are the most common cell type in the synovial intima. FLSs control the composition of the extracellular matrix and synovial fluid, keeping cartilage surfaces lubricated and nourished. They are composed of two to three layers of cells and constitute 75-80% of all synoviocytes in normal human synovium. In the inflamed rheumatoid synovium, the healthy two- to three-layer structure is transformed into a pannus-like structure, a hyperplasic synovium including an increased number of activated FLSs and macrophages that extends into the joint, and invading and degrading the cartilage matrix, promoting joint destruction (**439**). RA-FLSs are the primary inner lining cells and targets in RA. It has been reported that SARS-CoV-2 affects the severity of RA, probably by inducing inflammation in FLS (**440**). The migration and invasion of RA FLSs is the major cause of joint cartilage degradation (**441**). FLSs from RA patients can exhibit tumor cell features, including invasive characteristics (**442**). The hypoxia microenvironment is a key element in RA synovial tissues, and plays a role in promoting RA-FLS migration and invasion. HIF-1 is a member of the hypoxia-inducible factors family that has been shown to be strongly expressed in RA-FLS in order to adapt to a hypoxic environment after sensing a change in oxygen levels in the joint microenvironment. HIF-1 expression in RA-FLS has been linked to the severity of arthritis, synovial hyperplasia, and angiogenesis (**443**). Moreover, FLSs can protect themselves from apoptosis by stimulating autophagy via increasing ROS levels (**444**). LKB1 plays an essential role

in oxidative stress; it may regulate the AMPK-mediated SLC7A11-NOX4-ROS pathway to control FLS migration (**445**).

In chronic inflammatory conditions such as RA, FLS become hyperactive and contribute to joint damage. Activated FLS expression of MHC class II molecules is related to synovial inflammation. NETs trigger upregulation of HLA-DR on FLSs (**359**). HLA-DR$^+$ FLS produce soluble mediators such as the pro-inflammatory cytokines IL-6 and IL-15, as well as the chemokines CCL2, CXCL9, and CXCL12, as well as adhesion molecules ICAM1 and VCAM1, suggesting interactions with leukocytes (**356**). ICAM1 expression on FLSs, promotes FLS-T cell interaction, which increases T cell activation and proliferation (**419**). FLS cytokine signatures were discovered to be linked with the number of infiltrating CD4$^+$ T cells in RA synovial tissue (**419**). Additionally, environmental factors are clearly involved in the occurrence and development of RA. It has been shown recently that FLS can internalize microplastics and exacerbate cartilage damage (**446**).

Studies have shown that NETs can directly interact with FLSs and trigger a variety of responses. NETs can induce FLS activation and promote a pro-inflammatory gene signature in FLSs (**362, 369**). In RA, B cells can produce autoantibodies, including ACPAs. ACPAs are shown to induce NET formation and these NETs may activate FLS to generate IL-6, IL-8 and IL1β (**447**). Also, ACPA can promote FLS migration (**397**). Additionally, Gasdermin D (GSDMD) in RA serum could induce NET formation and GSDMD-dependent NET production aided in the activation and proliferation of FLS, accelerating cartilage and bone degradation and increasing RA disease activity (**448**). Meanwhile, a recent study identified that FLS from RA patients might internalize NETs and deliver arthritogenic peptides to T cells, resulting in autoimmunity and cartilage destruction (**359**).

G. T cells in RA

Autoreactive T cells, particularly CD4$^+$ T cells, are responsible for inflammation in RA joints. In most animal models of RA, CD4$^+$ T cells are essential for the development of the disease. Typical histology of RA synovium demonstrates extensive infiltration of CD4 T cells not only in the joint but also in the tendon sheath (**449**). Also, higher CD4/CD8 T cell ratios were found in RA patients, including early-RA, compared to healthy controls (**450**). Similarly, CD20$^+$ T cell frequency has

been found higher in the blood of RA patients compared to healthy individuals (**451**). $CD20^+$ T cells are increased in mice with CIA and produce high levels of pro-inflammatory cytokines (GM-CSF, TNF-α, IL-17, and IFN-γ) (**452**).

In RA, the T cell repertoire is influenced by genetic factors and microbial triggers. $CD8^+$ T lymphocytes specific for Epstein-Barr virus, cytomegalovirus, and influenza virus were shown to be more abundant in synovial fluid than in peripheral blood (**453**).

RA-associated T cell pathogenicity can be assessed by evaluating lymphocyte proliferation, flow cytometric measurement of activation markers (e.g., CD69, CD40L/CD154) and the cytokine-secreting profile. IFN-γ is the most commonly used cytokine marker for T cell activation, but other cytokines can also be tested to investigate Th1 cell types (IFN-γ, TNF-α, IL-2, IL-6, IL-12, and IL-21), Th2 cell types (IL-4, IL-5, IL-10, and IL-13) and Th17 cell types (IL-17). The majority of the $CD4^+$ T cells that infiltrate the synovium express activation markers, including HLA-DR and CD69 (**454, 455**). In early RA patients, $CD4^+PD\text{-}1^+$ cells within the inflamed synovial tissue had increased expression of CXCL13, which are associated with B cell help (for example recruitment of B cells) (**456**). Moreover, RA patients' circulating T cells produce more ROS as the disease progresses (**457**).

H. RA treatment

The treatment of rheumatoid arthritis aims to control inflammation, relieve symptoms, prevent joint damage, and improve overall quality of life. The main goal of treatment is to achieve remission, which means that the symptoms are under control and there is no evidence of active disease. The management of RA usually involves a combination of medications, lifestyle changes, and sometimes surgery. It's important to note that the treatment approach may vary depending on the severity of the disease and individual patient factors.

1. Disease-modifying antirheumatic drug (DMARDs)

DMARDs are a family of drugs indicated for the treatment of numerous inflammatory arthritis, including RA. DMARDs are classified in many types, conventional synthetic DMARDs (csDMARDs), targeted synthetic DMARDs (tsDMARDs) and biologic DMARDs (bDMARDs).

01. Conventional synthetic DMARDs

csDMARDs are the traditional drugs. cDMARDs are immunosuppressive drugs that do not affect particular immune system components. Example methotrexate, leflunomide, hydroxychloroquine… The most commonly used cDMARDs for RA is methotrexate.

Methotrexate: Methotrexate (MTX) is an agent of the antimetabolite class, used in the treatment of certain cancers and autoimmune diseases. It inhibits dihydrofolate reductase, a vital enzyme in folic acid metabolism (**458**). Gubner and collegues proved the anti-cancer effects of MTX in 1951, resulting in remission in breast cancer (**459**).

It was scientifically proven to be an effective and successful therapy for RA by the year 1985 (**460**). It has been a mainstay in RA therapy for several decades and is considered one of the most effective and well-tolerated DMARDs. Methotrexate is typically the first-line treatment for RA due to its proven efficacy, safety profile, and relatively low cost compared to other DMARDs (**461**).

The addition of other conventional synthetic DMARDs, such as sulfasalazine and hydroxychloroquine, biological DMARDs or alternative treatments may be substituted for or added to MTX (**462**).

02. Targeted synthetic DMARDs

These are inhibitors of the Janus Kinase (JAK) enzyme, involved in the signaling of numerous cytokines. Examples include tofacitinib and baricitinib (**463**).

03. Biologic DMARDs

Biologic DMARDs are highly specific and target a specific pathway of the immune system. Biotherapy is increasingly used in the treatment of RA. Its main aim is to reduce the frequency, duration and intensity of relapses, and to reduce overall rheumatic activity until clinical remission is achieved. But with some of these drugs, there is an increased risk of inducing haemopathies and infections, which often evolve rapidly and severely (**464**).

Etanercept: Etanercept is the first tumor necrosis factor inhibitor to be approved for the treatment of rheumatoid arthritis. It is an immunoadhesin-like fusion protein combining the P75 fraction of the soluble TNF-alpha receptor with a human IgG1 Fc fragment (**465**).

Infliximab: Infliximab (Remicade) is a chimeric monoclonal antibody designed to bind to TNF and prevent it from causing inflammation. Other TNF-targeting antibodies have since been developed, as well as antibodies targeting other cytokines such as IL-1β (**466**).

Antibodies targeting, for example, the IL-6 receptor (Tocilizumab) or the CD20 (RTX) surface molecule expressed by B lymphocytes are also available (**467**).

OBJECTIVES OF THE THESIS

Background: Neutrophil extracellular traps are web-like structures composed of DNA and various proteins including granule proteins and antimicrobial proteins released by neutrophils to trap and neutralize invading pathogens, such as bacteria, fungi, and parasites. They were initially identified as a mechanism to combat infections, but their implications extend beyond this primary function. We have already shown that NETs may activate immune cells such as macrophages and neutrophils (**352**). Other researches have shown that NETs can directly activate fibroblasts (**359**), T cells (**468**), and autoreactive memory B cells (**242**). However, all studies on B cells and NETs have focused on NETs as a source of antigen that can activate B cells, resulting in antibody production. The direct interaction of NETs with B cells has not been investigated, neither in physiological nor pathological contexts.

Aim 1: to demonstrate that NETs have pro-inflammatory properties, acting as DAMPs, and directly activate B cells, particularly naïve and non-autoimmune B cells in an antigen-independent manner.

Background: The most abundant NET component is DNA (**87, 100**). Toll-like receptors 9 are the principal receptors that detect DNA (**469**). Several studies report the involvement of TLR9 in various cell activation by NETs. NET production contributes to pulmonary fibrosis in the lung inflammation model via activating TLR9 in fibroblasts (**180**). Additionally, DNA from NETs was found to promote neutrophil recruitment by activating TLR9 in macrophages and production of IL-8 (**151**). NETs also can trigger NET formation by neutrophils by activating TLR9 (**470**). Furthermore, Gestermann et al. demonstrated that the DNA-LL37 complex, which mimics the NET structure, may activate memory B cells in a TLR9-dependent manner (**242**). However, the signaling pathway and the involvement of TLR9 in naive B cell activation by natural NETs have not been investigated. Moreover, several proteins, including C1q and LL-37, have been discovered in NETs or have been shown to deposit in NETs (**471, 472**). LL-37 is found to facilitate DNA uptake by cells (**473**). B cells express various receptors for C1q and LL-37. We have already demonstrated that C1q and LL-37 can modulate macrophage activation by NETs (**352**). These proteins can act synergistically with NETs to activate cells. However, the implication of these proteins has not been investigated in B cell activation by NETs.

Aim 2: to comprehend the processes by which NETs activate B cells, to investigate the involvement of TLR9 in B cell activation by NETs, and to identify the signaling pathway implicated in this mechanism.

Background: In rheumatoid arthritis, accumulated NETs were found in RA due to increased NET formation and impaired NET degradation (**178**). The impaired clearance of NETs allows them to accumulate and persist in the synovial fluid and tissue of the inflamed joints (**178**). The role of NETs in the pathophysiology of RA has been well-studied. Furthermore, RA NET composition differs from HDs NETs (**141**). Moreover, as mentioned earlier, NETs contain various components, including DNA, histones, and citrullinated proteins. In RA, citrullinated proteins are considered autoantigens. NETs have been identified as a potential source of antigens in RA and NETs can indirectly activate B cells leading to auto-antibodies production (**474**). However, the direct activation of RA B cells by NETs and the innate function of NET-activated B cells in RA have not been studied. Additionally, several lines of evidence indicate that B cells are more activated in RA patients (**372, 408**).

Aim 3: to characterize RA B-cell response to NETs and to determine whether this activation is exacerbated in RA as a result of RA NET composition or increased B cell responsiveness.

Background: B cells play a crucial role in the adaptive immune response by serving as both antibody-producing cells and antigen-presenting cells. As APCs, B cells are involved in the activation of T cells (**475**). Additionally, neutrophils are the primary producers of ROS. ROS can also act as signaling molecules to activate various immune cells, including neutrophils themselves, macrophages and dendritic cells (**476**). This leads to the release of pro-inflammatory cytokines and chemokines, which attract more immune cells to the site of infection or tissue damage. The involvement of NET-activated B cells in neutrophil and T cell activation has not been explored.

Aim 4: to investigate the consequences of B cell activation by NETs on other immune cells such as T cells and neutrophils.

RESULTS

I. Article 1

Article in preparation

Neutrophil Extracellular Traps trigger polyclonal B lymphocyte activation independently of antigen specificity toward a pro-inflammatory response

*Ahmad Haidar Ahmad[1,2], Maxime Batignes[1,2], Delphine Lemeiter[1,2], Dyhia Melbouci[1,2], Elodie Segura[3], Luca Semerano[1,2,4], and Patrice Decker[1,2]**

[1]University Sorbonne Paris Nord, [2]Inserm UMR 1125, Li2P, Bobigny, France, [3]PSL University, Curie Institute, U932 Inserm, Paris, France and [4]Avicenne Hospital, Rheumatology Department, AP-HP, Bobigny, France.

*Corresponding author: Prof. Patrice Decker, University Sorbonne Paris Nord, Inserm UMR 1125, Li2P, 1 rue de Chablis, 93017 Bobigny, France. Phone: + 33 1 48 38 77 91; Fax: +33 1 48 38 88 93; e-mail: patrice.decker@univ-paris13.fr

Keywords: Neutrophils, Neutrophil extracellular traps, DAMPs, B cells, polyclonal activation, pro-inflammatory responses, antigen presenting cell, rheumatoid arthritis.

Abstract. Activated polymorphonuclear neutrophils (PMNs) expel neutrophil extracellular traps (NETs), comprised of DNA and proteins. Described as anti-microbial, emerging evidence suggests that NETs can become immunogenic. Increased NET formation has been reported in rheumatoid arthritis (RA). Our previous findings shown that NETs are pro-inflammatory on resting macrophages. We propose a novel mechanism wherein NETs act as damage-associated molecular patterns (DAMPs) on B lymphocytes, to induce their polyclonal activation, independently of antigen specificity. This mechanism may be amplified in RA patients. Thus, we determined whether NETs directly activate B lymphocytes, even non-autoimmune and non-memory B cells, and we investigated the consequences of this activation on other immune cells.

Methods. Blood PMNs and peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from healthy donors (HDs)/RA patients. Total or naïve B lymphocytes were subsequently purified from PBMC by negative magnetic sorting. NETs were induced *in vitro* by phorbol myristate acetate (PMA) stimulation on adherent PMNs, followed by enrichment and characterization. B lymphocytes were cultured in the presence/absence of HDs/RA NETs, as well as lipopolysaccharide (LPS) and CpG-oligonucleotide. Similar experiments were conducted with cells from both wild-type and Toll-like receptor 9 (TLR9)-deficient mice. Cell purity/phenotype/activation were estimated by flow cytometry. Cytokine/IgG secretion/production were quantified by ELISA/flow cytometry. Additionally, gene expression profiling in NET-activated B cells was assessed by RNA sequencing. Functional consequences of NET-activated B cells were evaluated on PMNs by measuring migration and reactive oxygen species (ROS) production. The impact on T cells was analyzed through monitoring proliferation and cytokine production in T-B cell co-culture assays.

Results. NETs induced a robust up-regulation of HLA-DR and the co-stimulatory molecules CD40, and CD86 by total B lymphocytes both in HDs and RA patients. Notably, similar results were obtained when analyzing naïve B cells. We revealed that both HDs and RA NETs effectively activate B cells. Remarkably, RA B cells exhibited a more pronounced response to NETs, indicative of an enhanced sensitivity in the inflammatory setting. Moreover, naïve/total B lymphocytes secreted interleukin (IL)-8, IL-6, Tumor necrosis factor-α (TNF-α), and total IgG but not IL-10 in response to NETs. Cytokine production (CD19-gated) was confirmed by intracellular cytokine staining. Interestingly, NET responsiveness was observed in both normal and TLR9-

deficient B cells, highlighting the independence of TLR9 signaling in this process. Moreover, NET-activated B cells trigger PMN ROS production and recruitment, T cell proliferation and cytokine production. Finally, NETs upregulate pro-inflammatory genes in B cells, while down-regulating regulatory genes.

Conclusions. Our study demonstrates that NETs directly trigger polyclonal B cell activation, even in naïve B lymphocytes, in a TLR9-independent manner, leading to both activated antigen-presenting cell (APC) phenotype, and pro-inflammatory cytokine profile. The consequences of NET-induced B cell activation encompass T cell activation and PMN recruitment/activation. This mechanism may be pathogenic in inflammatory autoimmune diseases characterized by increased NET formation/pro-inflammatory activity. Thus, we identified a possible mechanism that contributes to B cell activation, providing a novel way by which PMNs can modulate adaptive immunity.

Introduction

In recent years, the immune system's innate and adaptive components have been recognized as interconnected players in the orchestration of immune responses. Neutrophil extracellular traps (NETs) are web-like structures composed of DNA charged with hundreds of proteins, including histones and antimicrobial proteins that neutrophils release to capture and eliminate pathogens (**1**). However, recent insights challenge the classic notion of NETs as primarily antimicrobial defenders, demonstrating their ability to elicit immunogenic responses. We have previously shown that NETs can directly activate resting macrophages (**2**). Moreover, NETs contain a wide range of damage-associated molecular patterns (DAMPs), including nuclear and cytoplasmic components such as DNA, RNA, histones, and highly inflammatory compounds such as myeloperoxidase (MPO), antimicrobial peptide (e.g. LL-37), calprotectin and others. These DAMPs can be recognized by immune cells and promote inflammation. However, excessive or dysregulated NET formation and subsequent DAMP release can contribute to chronic inflammation and autoimmune diseases, such as rheumatoid arthritis (RA) (**3, 4**).

B cells are a critical component of the immune system and play a significant role in inflammation. One of the primary functions of B cells is the production of antibodies. B cells can also participate in inflammation through antigen presentation and cytokine production. Previous research has mostly focused on NETs as sources of autoantigens, the triggering of B cell activation and subsequent antibody production (**3**). We have already found that NETs may be recognized by anti-citrullinated protein antibodies (ACPAs) from RA patients, and activate macrophages (**2**). However, NETs contain various components capable of stimulating B cells directly. It has been demonstrated that LL37-DNA complexes derived from NETs, trigger antibody production by human memory B cells, by inducing toll-like receptor 9 (TLR9) and B-cell receptor (BCR) activation (**5**). NETs may have a potential impact on B cells. Nevertheless, the comprehensive impact of NETs on B cells, particularly concerning their innate activity, remains unexplored in both inflammatory and physiological contexts. Here, we explored the connection between NETs and B cell activation, in both HDs and RA patients, aiming to elucidate the differential responses and potential implications for inflammation mechanisms and consequences of this interaction.

Here we report that NETs are pro-inflammatory and can directly activate total and even naïve B cells in an antigen-independent manner, leading to increased pro-inflammatory cytokines

production and up-regulation of activation markers essential for antigen-presenting cell function. Strikingly, this activation is amplified in RA patients, is modulated by the presence of LL-37 and C1q, and does not require DNA receptor TLR9 to activate B cells. Consequently, we also showed that NET-activated B cells could activate T cells leading to T cell proliferation and production of cytokines and trigger neutrophil recruitment and reactive oxygen species (ROS) production to amplify the inflammation.

Materials and methods

Donors and patients

Peripheral blood was drawn into heparinized vacutainers from RA patients from the Avicenne Hospital-rheumatology Department (CCP Ile de France, NI-2016-11-01) and from healthy donors (HDs) from the Etablissement Français du Sang (EFS) (agreement-13/A/107), Bobigny, France. The diagnosis of RA was determined according to the 2010 criteria of the European League Against Rheumatism. RA patients included in this study were ACPA-positive and were not under biotherapy; they were either untreated or on methotrexate, with or without oral corticosteroids. Informed consent were obtained from patients.

Synovial fluid was collected from the inflamed joint of certain RA patients (n=5; 1 patient was receiving biotherapies, and 4 were not receiving biotherapies) and used to isolate synovial neutrophils and other mononuclear cells (MNCs).

Mice

C57BL/6 were obtained from Janvier Labs (Le Genest-Saint-Isle). Mouse experiments have been approved by the local ethics committee (Darwin Committee of the University Sorbonne Paris Nord).

Human neutrophil isolation

Neutrophils were isolated from healthy donors or RA patients by density gradient centrifugation using Polymorphprep ™, at 500g for 40 minutes and without brake at 20°C. Both PMN and peripheral blood mononuclear cells (PBMCs) layers were collected.

The remaining red blood cells were lysed using hypotonic ACK-buffer (NH4Cl, KHCO3, and EDTA). Neutrophil count and viability were measured by acridine orange/propidium iodide staining and analyzed by Luna-FL™ Automated Fluorescence Cell Counter. PMN purity was determined by flow cytometry.

Bone marrow derived neutrophil isolation (BMDN)

Femurs and tibias of naive C57BL/6 mice were extracted and immediately flushed in sterile conditions with PBS/10% FBS and processed through a 100 μm filter to remove aggregates. The bone marrow suspension was collected in a sterile tube and centrifuged at 300g for 8 minutes.

Neutrophils were magnetically separated (negative selection) using the mouse neutrophil isolation Kit (MiltenyiBiotec) according to the manufacturer's instructions. Cells were counted and resuspended in RPMI medium containing FBS. BMDN purity was determined by flow cytometry.

Neutrophil Extracellular Trap production

Human or mouse neutrophils were seeded (at $1.5x10^6$ cells/ml) in RPMI 1640 (Gibco) medium onto a 1-well chamber slide (Labteck II) containing 0.001% Poly-L-lysine (sigma). Cells were allowed to attach for 1 hour before being stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma) at a final concentration of 50 nM for human PMNs and 100 nM for mouse BMDNs. Treated human or mouse neutrophils were incubated for a further 4 or 16 h, respectively, at 37°C to allow NET formation. After incubation, the culture medium was carefully removed without disturbing the adherent neutrophils, and cells were washed gently twice with phosphate-buffered saline (PBS). Finally, enzymatic digestion with DNase I (10 U/ml, Sigma) was used to detach NETs from the chamber slide surface. The DNase reaction was stopped using EDTA (3mM). NETs were obtained following two 10 minutes of centrifugations of supernatants at 300 and 16000 g. Alternatively, another chamber slide was incubated in the absence of neutrophils following the same steps as previously described. These preparations, described as NET-Buffer (BF), served as a control for NETs.

Before use in culture, DNA and proteins contents of NETs were characterized and measured for each preparation. To quantify protein, the absorbance of prepared samples containing NETs or controls was measured at 280 nm using Nanodrop spectrophotometry. DNA of NETs was measured using the Picogreen DNA quantification kit (Invitrogen) according to the manufacturer's instructions. DNA and proteins were also analysed by agarose electrophoresis (1.5%) and SDS-PAGE (12.5 %), respectively.

Human total and naïve B cell separation

Miltenyi Biotec MACS Kit was used to purify human $CD19^+$ total B cells and $CD19^+IgD^+CD27^-$ naive B cells from healthy donors or RA patients. Briefly, PBMCs isolated by Polymorphprep or Ficoll gradient extraction were counted and resuspended in 40 µl/10^7 cells of MACS buffer [PBS containing 0.5% bovine serum albumin (Sigma Aldrich)]. Then a precise volume of antibody cocktail according to cell number was added, followed by the addition of MicroBeads, and incubated for 10 min at 4°C. Cells were magnetically separated using magnetic columns (Miltenyi

Biotec) after one or more washes with buffer. Purified cells were washed twice with culture medium after isolation and analyzed by flow cytometry.

Mouse total B cell separation

Total B cells were isolated from mouse spleens. C57BL/6 mice, aged 8-12 weeks, were euthanized by approved ethical procedures, and spleens were harvested. Cell suspension was prepared by gently crushing the spleen on a 70 µm cell strainer. Red blood cells were lysed with ACK buffer, and the remaining cells were washed with PBS. B cells were then isolated using a positive selection (Miltenyi Biotec MACS Kit) according to the manufacturer's instructions. The purity of isolated B cells was assessed by flow cytometry.

Culture

Total and naive B cells ($0.5*10^6$/ml) isolated from mice or HD or RA blood by magnetic sorting were cultured for 3 days in IMDM medium with 10% FBS (eurobio) in a 96-well culture plate, with or without soluble NETs diluted to 25% (initial concentration between 10-20 µg/ml), or with NET buffer with the same dilutions as those used for NETs. As a positive control, cells were stimulated with Typhimurium lipopolysaccharides (LPS) and oligodeoxynucleotide (CpG) (2395, Invivogen) at final concentrations of 1µg/ml and 2µM respectively. Culture supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA).

In certain experimental conditions, isolated B cells were cultured in the presence of GolgiPlug™ (BD Biosciences) and GolgiStop™ (BD Biosciences) for a duration of 12 hours to assess intracellular cytokine production.

For RNA sequencing analysis, total and naïve B cells were stimulated with NET or NET-buffer for a duration of 6 hours. Following the stimulation period, RNA extraction was performed using a standardized protocol.

RNA extraction

RNA was isolated from NET and BF-stimulated naïve and total B cells using the RNeasy kit (Qiagen) according to manufacturer protocol. RNA was snap-frozen and stored at -80°C. mRNA concentration and quality were assessed using the Agilent Bioanalyzer Nano chip. RNA integrity (RIN) was > 7.0.

RNA sequencing

Libraries were prepared according to Illumina's instructions accompanying the TruSeq Stranded mRNA Library Prep Kit (Illumina). 500 ng of RNA was used for each sample. Library length profiles were controlled with the LabChip GXTouchHT system (Perkin Elmer). Sequencing was performed in the same sequencing unit of NovaSeq (Illumina) (100-nucleotide-length reads, paired ends) with an average depth of 15 million reads per sample.

RNA-seq analysis

Genome assembly was based on the Genome Reference Consortium (hg38). Quality of RNA-seq data was assessed using FastQC. Reads were aligned to the transcriptome using 127 STAR. Differential gene expression analysis was performed using DESeq2. Genes with low number of counts (<10) were filtered out.

T-B cell co-culture

$CD4^+$ T cell isolation

Total $CD4^+$ T cells were magnetically separated (negative selection) using the $CD4^+$ T Cell Isolation Kit, human (MiltenyiBiotec) according to the manufacturer's instructions. Isolated T cell purity was analyzed by flow cytometry.

Autologous co-culture

Autologous total $CD4^+$ T cells and $CD19^+$ B cells were alternatively isolated as described above. Then T cells were cryopreserved using dimethyl sulfoxide (DMSO) and autologous $CD19^+$ B cells were cultured for 3 days with NETs as described above. After 3 days, frozen T cells were thawed. Then, T cells were labeled with cell trace violet tracker. T cells were then resuspended in IMDM medium containing FBS and activated with anti-CD3 (Clone OKT3, invitrogen) and anti-CD28 (Clone CD28.2, BD Biosciences) antibodies. At the same time, B cells were washed twice with IMDM medium. Activated B cells were added to T cell culture at a 1:2 ratio, and cultured for 4 days at 37°C. Supernatants were collected at 4 days and assessed for IFN-γ, TNF, and IL-17 by ELISA. T cell proliferation was monitored by flow cytometry.

Mixed lymphocyte reaction

$CD19^+$ B cells were isolated and cultured as previously described. After 3 days of culture, $CD4^+$ T cells were isolated from a different non-HLA-matched healthy donor blood, labeled with cell

trace violet tracker, and cultured in IMDM medium containing FBS. At the same time, B cell culture was stopped by washing cells. B cells were next added to the T cell culture at a 1:2 ratio and incubated for 4 days at 37°C. Supernatants were collected at 4 days and assessed for IFN-γ, TNF, and IL-17 by ELISA. T cell proliferation was monitored by flow cytometry.

ELISA

ELISA was used to quantify the concentration of cytokines in B cell culture and B-T cells co-culture supernatants: human IL-6 (BD Bioscience), human IL-8 (BD Bioscience), human IL-10 (BD Bioscience), human TNFα (BD Bioscience), human IFN-γ (R&D Systems), human IL-17 (R&D Systems), mouse MIP-2 (R&D Systems), mouse IL-10 (R&D Systems) and human total IgG (MABTECH).

Flow cytometry

Flow cytometry was employed to analyze the expression of surface and intracellular markers in order to assess cell phenotype, purity, or activation.

Neutrophils purities were determined by labeling cells with FITC-conjugated anti-CD66b (a specific neutrophil marker), monoclonal antibody (IgM,k) [isotypic control, a FITC-conjugated mouse IgM,k monoclonal antibody (BD, Bioscience)], and PE-CY7-conjugated anti-CD11b (IgG1,k) [isotypic control a PE-CY7-conjugated mouse IgG1,K monoclonal antibody (BD, Bioscience)].

BMDN purity was determined by labeling cells with FITC-conjugated anti-Ly6G monoclonal antibody (IgM,k) [isotypic control, a FITC-conjugated mouse IgM,k monoclonal antibody (BD, Bioscience)], and PE-CY7-conjugated anti-CD11b (IgG1,k) [isotypic control a PE-CY7-conjugated rat IgG1,K monoclonal antibody (BD, Bioscience)].

We characterized freshly isolated B cells using the following monoclonal anti-human antibodies: APC-conjugated anti-CD19, BB515-conjugated anti-CD27, BV510-conjugated anti-IgD, PE-CY7-conjugated anti-HLA-DR, PE-CY5-conjugated anti-CD86, BV421-conjugated anti-CD40 (BD, Bioscience). B cells were defined as CD19-expressing cells, IgD and CD27 were used to discriminate memory from naïve subsets. HLA-DR, CD40, and CD86 were used to assess the activation status of freshly purified B cells.

Purity of isolated $CD4^+$ T cell purities were determined by labeling cells with PE-Cy7-conjugated anti-CD3 and BV421-conjugated anti-CD4. T cells were identified by double expression of CD4 and CD3.

Cells were acquired on a FACS Canto II (BD, Bioscience) flow cytometry and analyzed using FACS Diva software (BD, Bioscience).

Chemotaxis assay

PMN migration assay was performed using the μ-Slide Chemotaxis (ibidi GmbH) according to the manufacturer's instructions. Cells in RPMI medium ($7.5*10^6$/ml) were suspended in a gel mixture composed of Collagen I, bovine (Gibco), and NaOH (1M), at a final cell concentration of $2x10^6$ cells/ml and then loaded into the central chamber of the migration slide. After incubation and cell attachment, the two reservoirs were filled either with supernatants of cultures from NET-stimulated B cells or NET-Buffer-stimulated B cells. Interleukin-8 (100 ng/ml, Immunotools) was used as a control chemoattractant. The medium was used as a negative control with no chemoattractant. Live cell imaging was performed using a microscope taking a picture every 1 minute over a period of 60 minutes. 60–100 single cells were tracked manually in each experiment. The assay was independently repeated at least four times.

Luminol ROS detection assay

Neutrophils ($1*10^6$/ml) were incubated with fresh supernatants from 3 days culture of B cells (NER-stimulated or not), in the presence of 100μM luminol (Sigma Aldrich). As a control, stimuli were cultured without B cells. Luminol can measure extracellular and intracellular ROS. The free radical combines with luminol to produce a light signal. Luminol-enhanced chemiluminescence was measured continuously for 4 hours on a TriStar2 plate reader.

Statistical analysis

In all figures, data are shown as the mean value and the standard error of the mean (SEM) of pooled experiments. In pooled data, each donor was tested in triplicates and then the mean was used for subsequent analysis. To determine the stimulatory activity of NET, we compared target cells cultured with NET to target cells cultured with the NET purification buffer. According to data distribution, groups were compared using two tailed Mann-Whitney test or Wilcoxon matched-pairs signed rank test. In correlation analysis, Spearman rank correlation test was employed to

assess the relationships between variables. For $p \leq 0.05$, difference was considered statistically significant.

Results

Total and naïve B cells from healthy donors are directly activated by NETs

We have previously shown that NETs can activate macrophages in healthy donors (**2**). In order to better investigate the pro-inflammatory impact of NETs, we aimed to investigate their effect on purified B cells. Firstly, because memory and naive B cells respond to stimulatory signals differently (**6**), we tested the effect of HD-derived NETs on B cell sub-populations among PBMCs from HDs. We have found that both naïve and memory B cells are activated in PBMCs stimulated with NETs, as shown by CD86 upregulation (**Supplemental Fig. 1**). Therefore, to confirm the direct B cell activation by NETs, especially on naïve B cells, we conducted experiments using sorted $CD19^+$ total B cells, and sorted $CD19^+IgD^+CD27^-$ naïve B cells from HDs. Sorted cells were cultured for 72 hours with NETs or with its negative control, NET Buffer (BF). Agonists of TLR4 (LPS) and TLR9 (CpG-ODN 2395) were used as a positive control. B cell activation was estimated by measuring cytokine production and the expression of activation markers HLA-DR and the costimulatory molecules CD40 and CD86. The results demonstrated that NETs effectively activate purified total and naïve B cells, inducing increased expression of the mean fluorescence intensity (MFI) of HLA-DR, CD40 and CD86 compared to NET buffer (**Fig. 1A**). Furthermore, to determine whether NETs induce B cells to produce cytokines, supernatant from stimulated B cells was collected at 72 hours and analyzed for the presence of IL-6, IL-8, IL-10 and TNF. Both naïve and total B cells produced pro-inflammatory cytokines IL-6, IL-8 and TNF in response to NETs (**Fig. 1B**). The activation of naïve B cells, that has not been exposed to any antigen, among HD PBMCs by NETs suggested a polyclonal effect of NETs on B cells. Interestingly, NET-activated B cells didn't produce the immuno-modulatory cytokine IL-10 (**Supplemental Fig. 2**). Additionally, cytokine production specifically by NET-activated B cells was confirmed by intracellular staining (**Supplemental Fig. 3**). Moreover, despite a short culture duration of total B cells with NETs, we observed increased total IgG levels in NET- and LPS+CpG-stimulated total B cells supernatants compared to BF-stimulated B cells supernatants (**Fig. 1B**).

B cell activation by NETs is aided by C1q and LL-37

The regulation of B cell activation is complex and influenced by various factors. We aimed to investigate the involvement of two immune modulators, C1q and LL-37, in the mechanism of B

cell activation by NETs. C1q, a crucial component of the complement system, exhibits binding affinity for NETs and can interact with various B cell receptors, thereby modulating their activation (**7, 8**). Moreover, LL-37, a human antimicrobial peptide, possesses immunomodulatory properties and can influence B cell activation. LL-37 can facilitate DNA delivery to TLR9 and promote the production of pro-inflammatory cytokines by B cells (**5, 9**). Given these properties, we investigated the effects of C1q or LL-37 on NET-stimulated B cells. Firstly, to dissect the direct impact of these proteins, total B cells were stimulated with the NET negative control (BF) in the presence of either LL-37 or C1q. C1q display suppressive properties by significantly reducing the expression of HLA-DR and CD40 on B cells (**Fig. 2A**). Moreover, C1q exhibits a suppressive influence on the production of IL-6 (**Fig. 2B**), emphasizing its multifaceted role in dampening B cell activation. Conversely, LL-37 demonstrates a more modest effect on B cell CD40 expression only (**Fig. 2A**). However, in the presence of NETs, C1q and LL-37 both showed an amplifying impact on B cell activation. TNF production and HLA-DR, CD40, and CD86 expression on B cells were considerably higher when stimulated with NETs in the presence of C1q than when treated with NETs alone (**Fig. 2A, B**). Similarly, LL-37 in the presence of NETs increases CD40 expression and TNF production by B cells (**Fig. 2A, B**). In conclusion, we demonstrated that C1q and LL-37 operate simultaneously with NETs to further activate B cells.

RNA-sequencing reveals that NETs trigger a complex pro-inflammatory profile in total and naïve B cells

To further understand the impact of NETs on B cells, we performed a transcriptome study to analyze the differential gene expression between total and naïve B cells stimulated by NETs compared to NET buffer. Our transcriptomic analysis reveals a complex pro-inflammatory profile triggered by NETs in B cells. Over 6000 genes were differentially expressed in both total and naïve B cells following NET stimulation. RNA sequencing (RNAseq) data confirmed the pro-inflammatory profile of NET-activated total and naïve B cells. Principal component analysis (PCA) identified two distinct clusters, where NET-activated B cells were clearly distinct from BF-stimulated B cells (**Fig. 3A**). Total and naïve B cells, when exposed to NETs, exhibit a dynamic modulation of gene expression, contributing to the orchestration of a multifaceted pro-inflammatory response. NET-activated total and naïve B cells were enriched in RNA coding for pro-inflammatory compounds, including cytokines e.g. IL-8, RANKL, IL-1b, fibroblast growth

factor (FGF2), lymphotoxin a, metalloproteinases (e.g. MMP2, MMP7) and chemokines (such as CCL7 and CCL13). In contrast, immunomodulatory genes such as IL10R was downregulated following activation with NETs in both total and naïve B cells (**Fig. 3B, C**).

RA B cells are more activated than HD B cells

Several studies have reported a high expression of activation markers on B cells among total PBMCs from RA patients (**10, 11**). To dissect alterations of the B cell activation status in patients with RA, we analyzed the expression of activation markers using multicolor flow cytometry on blood B cells freshly isolated from RA patients (9 RA patients) and compared them to healthy donors (n=12). As expected, we also detected significantly elevated expression of CD40, CD86 and HLA-DR (frequencies and/or MFI) on freshly purified B cells from RA patients compared to HD B cells (**Supplemental Fig. 4**), indicating a status of high activation. None of the expression of the above markers on RA B cells was found correlated with the activity of the disease represented by DAS28-CRP. Furthermore, to investigate the B cell activation status at the site of inflammation in RA patients, we compared the expression of the above markers on RA B cells among mononuclear cells (MNCs) freshly isolated using Ficoll gradient density, from blood or from synovial fluid (SF) from inflamed joint of the same patient (n=5). Therefore, we revealed an even more activated B cell phenotype in SF, characterized by higher expression of CD86 and HLA-DR but not CD40 compared to blood from the same RA patient (**Fig. 4**), indicating that B cells in synovial fluid may become more activated. The data underscored the potential role of B cells in the local inflammatory milieu.

Both HD and RA NET activate B cells but RA B cells are more sensitive to NETs than HD B cells

The aberrant formation of NETs from neutrophils has been demonstrated in the pathogenesis of RA. We have already demonstrated that RA PMNs produce more NETs than HD PMNs, and that RA-derived NETs are more efficient in activating neutrophils and macrophages compared to HD-derived NETs (**2**). Additionally, it is well known that NET composition from PMNs from patients with RA have a specific composition (**12**). We aimed to test whether the difference in NET composition may modulate B cell activation. To approve that, we stimulated total HD B cells with NET derived from PMNs from HDs or RA patients. Both NETs were used at the same DNA concentration, as measured by picogreen. In the current study and contrary to expectations, our

results revealed that both RA-derived NETs and HD-derived NETs exhibited similar capacities to activate B cells. Both NETs induced similar levels of cytokine production and expression of activation markers by B cells (**Fig. 5A**).

Furthermore, since we found that RA B cells are highly activated, we aimed to determine whether B cells from RA patients differ in their ability to respond to NETs. To this purpose, we measured HD and RA total B cell responses to HD-derived NETs. Interestingly, our findings revealed that RA B cells exhibit a heightened response to NETs compared to HD B cells. Following stimulation with HD-derived NETs, RA B cells displayed significantly elevated expression levels of HLA-DR, CD40, and CD86 compared to HD B cells. The fold-increase (NET/BF) in CD40 expression was particularly pronounced in RA B cells, ranging from over 2-fold to more than 3-fold compared to HD B cells (**Fig. 5B**). Although not shown, no difference was found when comparing cytokines production by both HD and RA B cells in response to NETs. Importantly, in RA patients, CD40 expression by NET-activated B cell demonstrated a significant and positive correlation to disease activity (DAS28-CRP; R= 0.6818; p= 0.0251; **Fig. 5C**). This correlation sheds light on the intricate interplay between CD40-exprssing B cells and the pathogenicity of NETs in the chronic inflammation characteristic of RA.

Taken together, CD40, CD86, and HLA-DR on B cells are elevated on blood and SF B cells from RA patients, and are amplified after NET stimulation, suggesting NETs as a potential inducer of B cell activation in RA.

TLR9 in not required to induce B cell activation in response to NETs

Having established that NETs can directly activate B cells, this study aimed to dissect the underlying receptors and signaling pathways involved in this intricate mechanism. Given the predominant DNA composition of NETs, we investigated the role of the DNA receptor TLR9 in B cell activation using B cells purified from wild-type (WT) and TLR9-deficient (TLR9-/-) mice. As a control, the TLR9 agonist oligodeoxynucleotide 2006 (ODN 2006) was employed to assess the TLR9-dependent response. Surprisingly, both WT and TLR9 -/- B cells demonstrated comparable activation in response to NET stimulation, with no significant difference observed in CD86 expression (**Fig. 6A**). NETs induced a 2-fold increase in CD86 expression in both WT and TLR9 -/- B cells. Moreover, the production of MIP-2 by B cells in response to NETs was similar between WT and TLR9 -/- B cells (**Fig. 6B**). Conversely, TLR9 -/- B cells did not exhibit activation

in response to the TLR9 agonist ODN 2006. This finding strongly indicates that B cell activation by NETs is independent of TLR9 signaling.

NET-activated B cells act as antigen-presenting cells leading to T-cell activation

We next aimed to elucidate the impact of B cell activation by NETs on the activation of other immune cells, particularly T cells. Because we found that NET-activated B cells exhibit an activated antigen-presenting cell (APC) phenotype, via the expression of high levels of HLA-DR, CD40 and CD86 and production of pro-inflammatory cytokines, we aimed to investigate how NET-activated B cells may influence T cell activation and cytokine production.

Firstly, we intended to investigate whether the presence of autologous NET-activated B cells influences T cell activation by anti-CD3 and anti-CD28 antibodies, which mimics stimulation by antigen-presenting cells. $CD4^+$ T cells were co-cultured with autologous B cells that had been stimulated or not for 72 hours (**Fig. 7A**). To assess B-cell stimulatory activity, T cell proliferation and cytokine production were measured after 4 days of co-culture. We showed that in the presence of autologous B cells, T cells are more activated. They release more TNF, and proliferate more than T cells alone in response to anti-CD3 and anti-CD28 (e.g. 22% vs. 46%, $p < 0.05$) (**Fig. 7B**). Interestingly, this impact was increased when B cells had already been stimulated with NETs compared to NET-buffer (e.g. 69% vs. 47%, $p < 0.05$). In the absence of B cells, NETs had no effect on T cells. In summary, our results indicate that upon TCR activation, NET-activated B cells provide additional co-stimulatory signals to T cells, triggering their activation.

Next, using a different approach, we sought to confirm the potential of NET-activated B cells to act as APCs and stimulate $CD4^+$ T cell responses. In this experiment, a mixed lymphocyte reaction assay (MLR) was conducted in which B cells stimulated for 72 hours with either NETs or NET-buffer were co-cultured with allogeneic $CD4^+$ T cells (**Fig. 7C**). Interestingly, our findings revealed that NET-activated B cells induced a significantly higher proliferation of $CD4^+$ T cells in the MLR assay compared to B cells stimulated with control buffer (**Fig. 7D**). This result indicates that NET-activated B cells possess an enhanced capacity to function as APCs, leading to increased $CD4^+$ T cell activation and expansion. However, while $CD4^+$ T cell proliferation was heightened in the presence of NET-activated B cells, no significant increase in cytokine production was

observed in the co-culture, suggesting that the impact of NET-activated B cells may be more prominent at the level of T cell expansion rather than cytokine secretion (data not shown).

NET-activated B cells trigger neutrophil recruitment and ROS production

Our RNA sequencing data revealed upregulated mRNA levels of various chemokines in NET-activated B cells, and subsequent ELISA analysis confirmed the secretion of significant amounts of IL-8 in the culture supernatants of NET-activated B cells. Given that these cytokines, particularly IL-8, are known to induce neutrophil migration and function in inflammatory settings (**13**), we sought to investigate the consequences of B cell activation by NETs on neutrophil recruitment and activation (**Fig. 8A**). In order to assess the consequence of B cell activation by NETs on neutrophil recruitment, we performed a chemotaxis assay. We used chemotaxis slide which includes a chamber consisting of two large reservoirs that are connected by a narrow observation area. Neutrophils were seeded in the narrow in a matrix gel, and supernatants from culture of B cells stimulated with NET or NET-Buffer were added into the two reservoirs (**Fig. 8B, top**). Migration towards reservoir containing NETs- or NET-Buffer-B cell supernatants was monitored and recorded using a microscope. In order to analyze the directionality of cell migration, the chemotaxis tool was used to manually track neutrophil trajectories, and the maximum number of cells was tracked. Averaged forward migration indices (FMIs) were calculated indicating the direction of cell migration. Our results demonstrated that neutrophils preferentially migrated towards NET-activated B cell supernatants compared to B cell supernatants stimulated with control buffer (**Fig. 8B, bottom**). The chemotactical behavior suggests a potential role for NET-activated B cells in facilitating neutrophil recruitment and migration in the inflammatory microenvironment.

Secondly, neutrophils are renowned as the primary producers of reactive oxygen species (ROS) among immune cells. ROS can initiate signaling pathways involved in the immune response. We subsequently proceeded to examine the effects of B cell activation by NETs on ROS production by neutrophils, shedding light on the potential impact of NET-activated B cells on neutrophil function. Supernatants from B cells were collected after 3 days of culture. Neutrophils were then cultured with these supernatants in the presence of luminol, a compound that emits light when oxidized by ROS. The intensity of the light emitted in the luminol ROS assay is proportional to

the amount of ROS present in the neutrophil culture. The kinetics of ROS production by PMNs in response to B cells supernatants over 2 hours are reported in (**Fig. 8C**). We found that compared to BF-stimulated B cells, supernatants of NET-activated B cells induce a significant ROS production by neutrophils. LPS+CpG-stimulated B cells were also able to trigger ROS production (**Fig. 8C**), indicating a broader capacity of activated B cells to influence neutrophil function. Additionally, to exclude the possibility that the stimulus (NET or LPS+CpG) are still present in the supernatants and may directly activate neutrophils to produce ROS, we duplicated the culture with the same stimulus and culture conditions, but without the presence of B cells. We show that among these supernatants, only LPS+CpG supernatants without B cells were able to induce ROS production (**Fig. 8C**). This indicates that such stimuli may still be present in the supernatants after 3 days of culture. Importantly, in the absence of B cells, supernatants containing NETs did not induce ROS generation. emphasizing that factors produced by B cells in response to NETs are responsible for triggering neutrophil activation and ROS production.

Discussion

Complex interactions between various immune cells and their signaling molecules play an important role in the maintenance of immune homeostasis. NETs, originally described as an important defense mechanism against microbial invaders, have emerged as significant contributors of both antimicrobial defense and immune dysregulation. In this article, we examined in detail the role of NETs, particularly their interaction with B lymphocytes, and the potential implications for autoimmune conditions, with a focus on rheumatoid arthritis.

To date, few studies have addressed B cell activation by NETs and there are no data on naïve B cells in a non-autoimmune context. B cells can act as potent antigen presenting cell and also influence immune response by producing cytokines (**14**). However, most of these studies have focused on indirect B cell activation by NETs, e.g. via synovial fibroblasts, or dendritic cells, or via immune complexes, and all of these studies were interested in NETs as source of auto-antigens which led to B cell activation and subsequent antibody production (**15, 16**). In addition, NETs are composed of DNA, histones and proteins (including neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, leukocyte proteinase 3, lactoferrin and others). NET-components can provide co-stimulatory signals to B cells through the engagement of different receptors, e.g. Toll-like

receptor. Therefore, we wondered whether NETs activate B cells through their enriched composition.

In the present study, we provided evidence that NETs are pro-inflammatory, act as DAMPs and could directly activate total and naïve B cells from healthy donors and from patients with RA. We demonstrated that NET-activated B cells produce high level of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α, but not IL-10 and express high level of HLA-DR, and the co-stimulatory molecules CD40 and CD86, essential molecules for APC function. Moreover, The RNA-sequencing data reveals the upregulation of major pro-inflammatory genes, shedding light on the molecular pathways triggered by NETs. The intricate network of gene expression includes mediators of inflammation, signaling molecules, and immune response effectors. This detailed analysis enables us to uncover the specific genes that govern the pro-inflammatory response of B cells to NETs. Furthermore, the current study has also revealed that B cell activation by NETs is amplified by proteins such as C1q and LL-37 and doesn't require TLR9 receptor.

Additionally, in rheumatoid arthritis, accumulated NETs were found in RA due to increased NET formation and impaired NET degradation. NETs have also been detected in the synovial fluid and synovial tissues of affected joints. They are believed to contribute to the chronic inflammation and tissue damage seen in RA (**17**). We have already shown that NETs can activate various immune cells, including macrophages and neutrophils, through several mechanisms (**2**). Several lines of evidence indicate that B cells are more activated in RA patients. B cells in the synovial tissue and fluid of affected joints are often organized into lymphoid structures known as ectopic lymphoid structures, suggesting ongoing B cell activation and local immune responses within the joints. Moreover, our findings highlight a distinct alteration in the activation status of B cells in RA patients vs. HDs, with increased activation markers both in peripheral blood and, notably, at the site of inflammation in the synovial fluid. This heightened expression of activation markers suggests that B cells in RA patients are primed and more responsive to stimuli. Several factors may contribute to the increased activation of B cells in RA. We demonstrated that in RA, NETs belong to the factors involved in the activation of B cells, and we showed that RA B cells are more sensitive to NETs than HD B cells. This finding sheds light on the pathophysiology of RA and the potential implications of NETs and B cells for disease progression. However, no difference was observed regarding the activity of RA vs. HD-derived NETs.

Moreover, our findings show that RA B cells are more prone to activation by NETs compared to HD B cells, as evidenced by increased expression of activation markers, notably CD40. The observed correlation between CD40 expression on NET-activated RA B cells and disease activity suggests that CD40-expressing B cells may be involved in the chronic inflammation that characterizes RA. This new perspective on the interaction between NETs and B cells in RA improves our understanding of the disease's pathogenic mechanisms.

The ability of B cells to produce pro-inflammatory cytokines and act as APCs in response to NET stimulation has several implications for autoimmune diseases. In the current study, we demonstrated that NET-activated B cells can act as APC and induce T cell activation and proliferation. Suggesting that in auto-immune diseases, this phenomenon plays a role in exacerbating autoreactive T cells activation and consequently auto-antibody production, emphasizing the potent immunomodulatory effects of NETs in the context of B-T cell interactions

Besides, we showed that B cell activation by NETs can have downstream effects on neutrophil recruitment and activation. NET-activated B cells can release pro-inflammatory cytokines, such as IL-8, which is potent chemoattractants for neutrophils. These phenomena can recruit neutrophils to the site of infection or inflammation, where they can further exacerbate inflammation and the release of additional NETs. Moreover, the findings show that NET-activated B cells trigger ROS production by PMNs and recruit PMNs, indicating a coordinated immune response involving communication between innate and adaptive immunities.

In summary, this study provides compelling evidence that NETs have a profound impact on B lymphocytes, driving their activation, cytokine production, and interactions with other immune cells. Notably, B cells from RA patients exhibit an enhanced response to NETs, which may contribute to the autoimmune and inflammatory features of the disease. Understanding the mechanisms underlying NET-induced B cell activation is crucial for unraveling the pathogenesis of autoimmune conditions like RA and may open new avenues for the development of targeted therapeutic strategies aimed at modulating the immune response in these disorders.

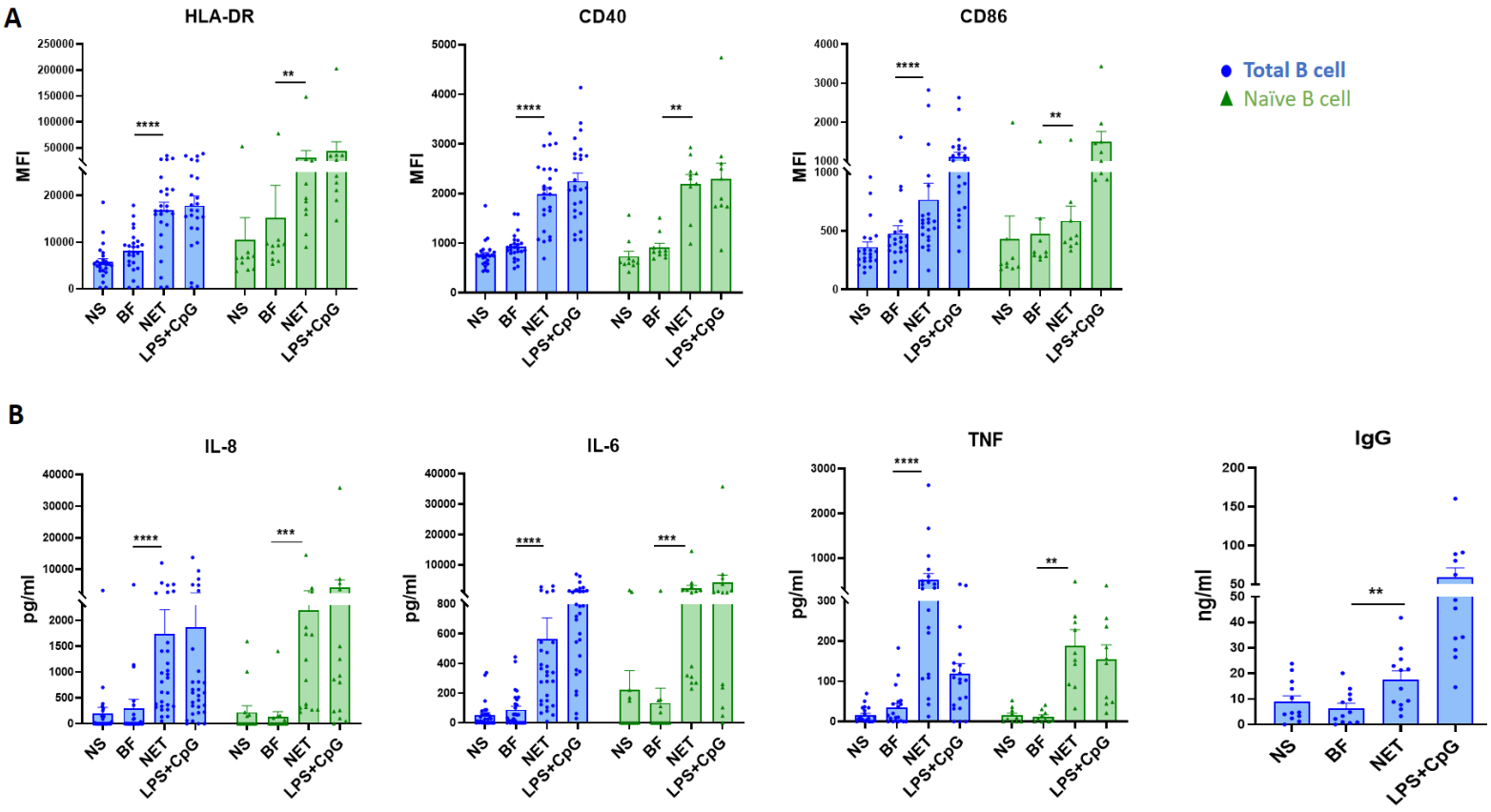


Figure 1. **Total and naïve B cells from healthy donors are directly activated by NETs.** Total B cells (in blue) and naïve B cells (in green) were purified from healthy donors using magnetic sorting (negative selection) and cultured for 3 days with HD-derived NETs, its purification buffer (BF), or TLR4 and 9 agonists (LPS and CpG, respectively). **(A).** Flow cytometry was used to examine the expression of the activation molecules CD86, HLA-DR, and CD40. **(B).** ELISA was used to measure cytokine and IgG secretion. Graphs represent MFI or concentration (pg/ml, ng/ml) (mean values ± SEM of pooled data) from independent experiments (n= 25 for total B cells, and n=10 for naïve B cells). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (Wilcoxon matched-pairs signed rank test). MFI**,** Mean fluorescence intensity; LPS, lipopolisacharide; CpG, oligodeoxynucleotide; NETs, neutrophil extracellular traps; BF**,** NET-Buffer.

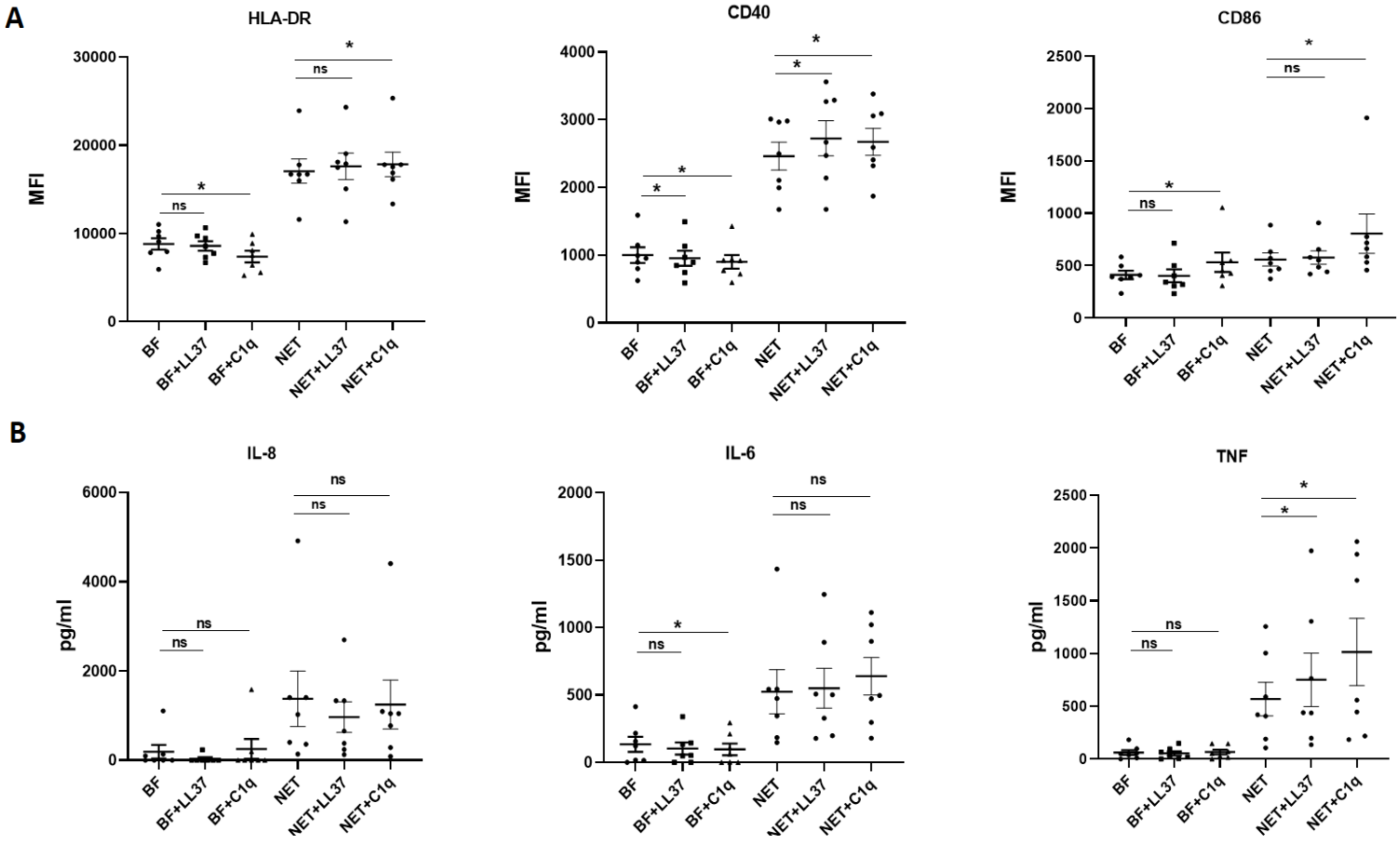


Figure 2. **B cell activation by NETs is aided by C1q and LL-37.** Total B cells were cultured for 3 days with HD-derived NETs, its purification buffer (BF), with or without C1q and LL-37 at a final concentration of 50 µg/ml and 10 µg/ml respectively. **(A).** Flow cytometry was used to examine the expression of the activation molecules CD86, HLA-DR, and CD40. **(B).** ELISA was used to measure cytokines secretion. Graphs represent MFI or concentration (mean values ± SEM of pooled data) from independent experiments (n=7). *$p < 0.05$ (Wilcoxon matched-pairs signed rank test). MFI**,** Mean fluorescence intensity; NETs, neutrophil extracellular traps; BF, NET-Buffer.

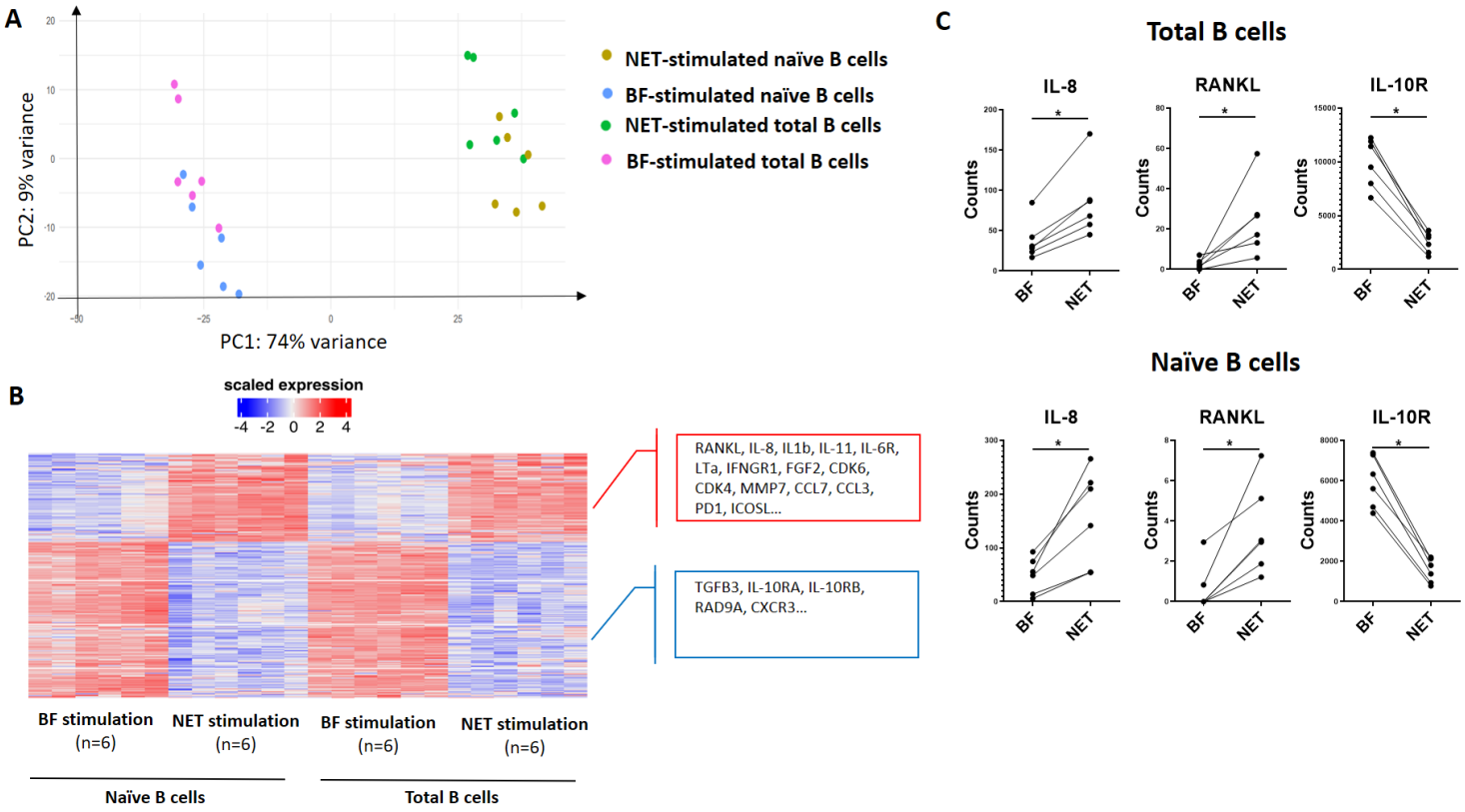


Figure 3. RNA-sequencing reveals that NETs trigger a complex pro-inflammatory profile in total and naïve B cells. Total and naïve B cells from HDs were cultured for 6 hours with HD-derived NETs or its purification buffer (BF). RNA-sequencing (RNA-Seq) was performed for total and naïve B cells stimulated with NETs compared to NET-buffer. **(A)** Principal component analysis (PCA) of gene expression profiles from total/naïve B cells stimulated with NET or NET-buffer. **(B)** Heat map showing the RNAs upregulated (red) and downregulated (blue) in NET-activated total/naïve B cells compared to NET-buffer. **(C)** Differential expression of key genes in total B cells (top) and naïve B cells (bottom) when stimulated by NETs compared to NET buffer. Graphs represent 6 independent RNA-sequencing for total or naïve B cells isolated from the same donor, activated by a pool of six independent NET preparations.

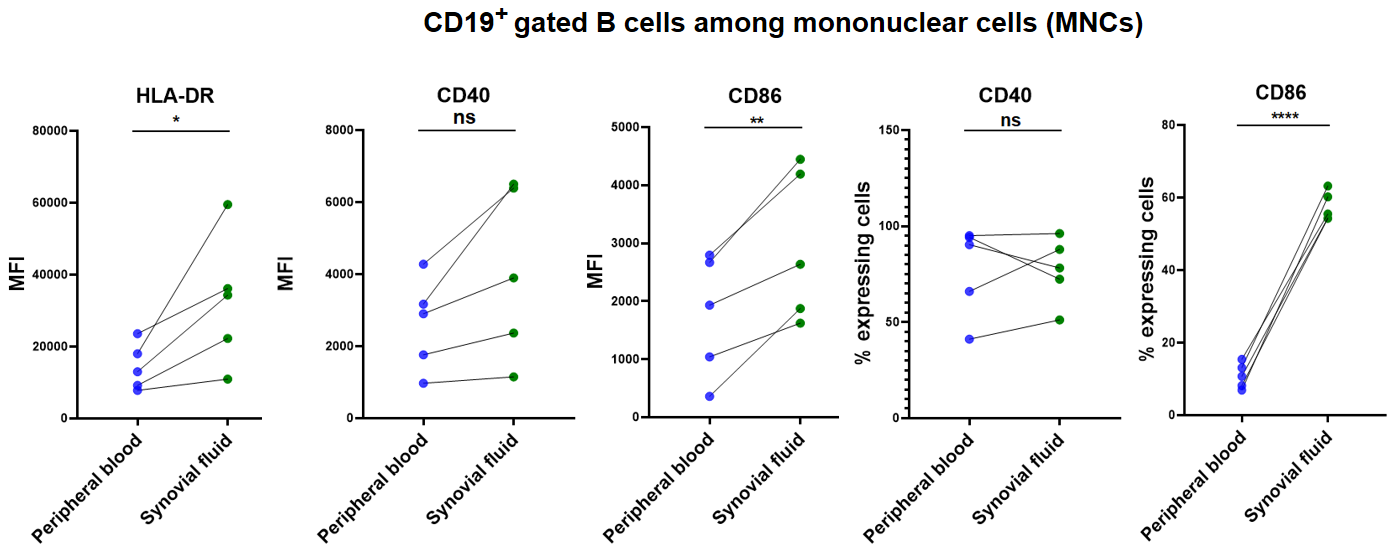


Figure 4. Synovial fluid of RA patients exhibit enhanced B cell activities compared to peripheral blood. Mononuclear cells (MNCs) were purified from RA patients blood or synovial fluid using density separation by Ficoll, and directly analyzed by flow cytometry to examine the expression of activation markers CD40, CD86 and HLA-DR on B cells. Graphs represent MFI and % of activation marker on $CD19^+$-gated B cells among total mononuclear cells of 5 independent experiments. $*p < 0.05$, $**p < 0.01$ (Paired t test). %, Frequency; MFI, Mean fluorescence intensity.

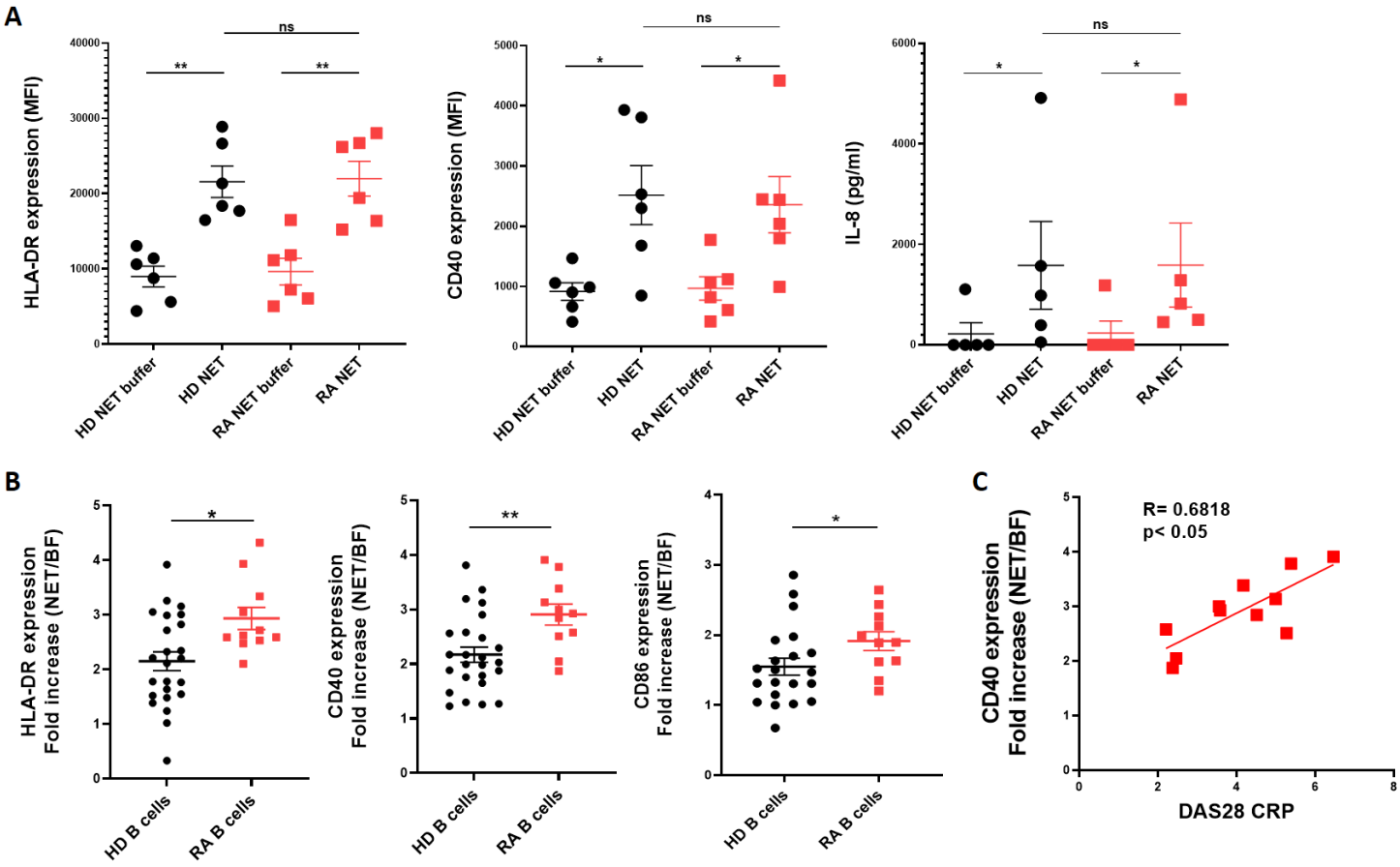


Figure 5. Both HD and RA NET activate B cells but RA B cells are more sensitive to NETs than HD B cells. (A) Total B cells were purified from healthy donors using magnetic sorting (negative selection) and cultured for 3 days with HD-derived NETs or RA-derived NETs, or their purification buffer (BF) and analyzed by flow cytometry. Graphs represent MFI or concentration (pg/ml) (mean values ± SEM of pooled data) from independent experiments (n=6). **(B)** Total B cells from RA patients (n=11) and from HDs (n=21) were cultured with HD-derived NET for 3 days and analyzed by flow cytometry. Graphs represent the fold increase expression of activation markers in response to NETs vs BF. $*p < 0.05$, $**p < 0.01$ (Mann Whitney test). **(C)** Correlation between NET-induced CD40 expression on RA B cells and RA severity calculated by DAS28-CRP (n=11). MFI**,** Mean fluorescence intensity. R, Spearman correlation coefficient.

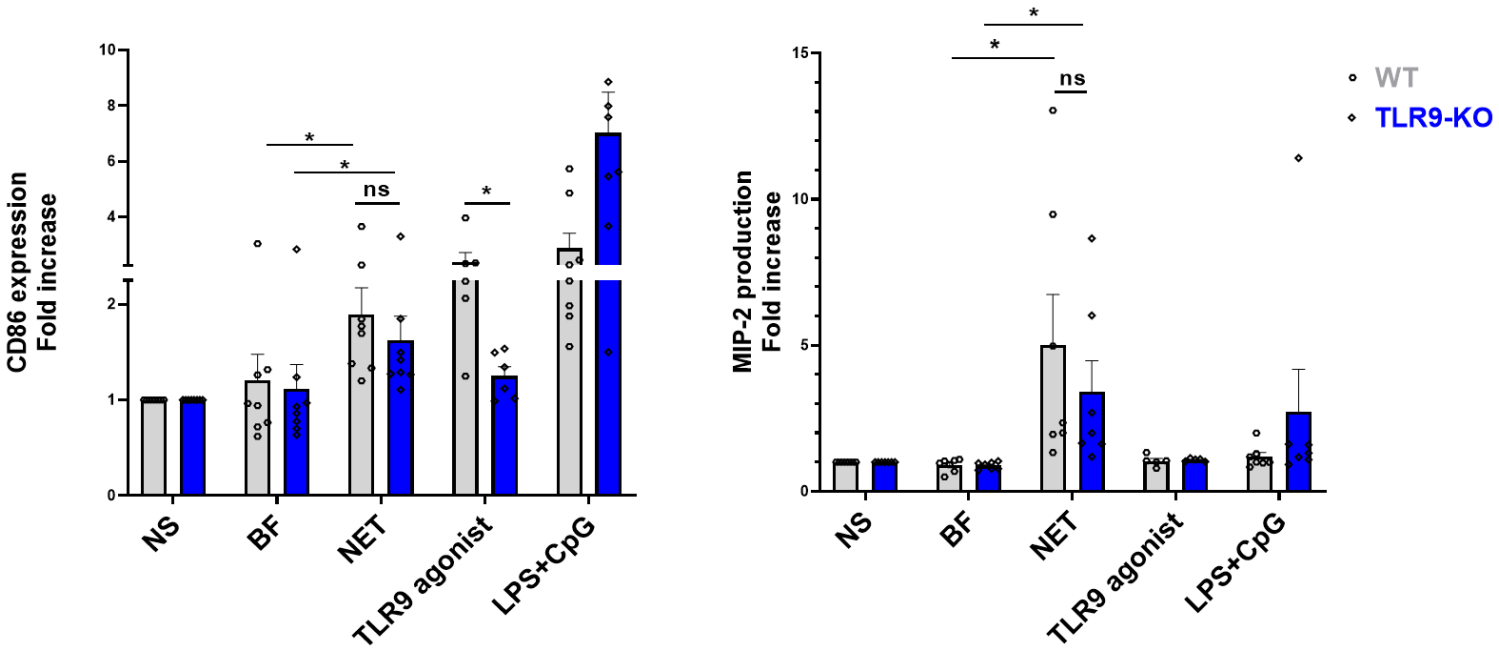


Figure 6. TLR9 is not required to induce B activation in response to NETs. Total B cells were purified from spleens of WT and TLR9-/- mice using magnetic sorting and cultured for 3 days with purified mouse NETs, its purification buffer (NET buffer), or TLR4 and 9 agonists (LPS and CpG, respectively). **(A)** Flow cytometry was used to examine the expression of the activation molecule CD86. **(B)** ELISA was used to measure cytokine production. Graphs represent the fold increase of MFI or concentration from 8 independent experiments. *$p < 0.05$ (Wilcoxon matched-pairs signed rank test).

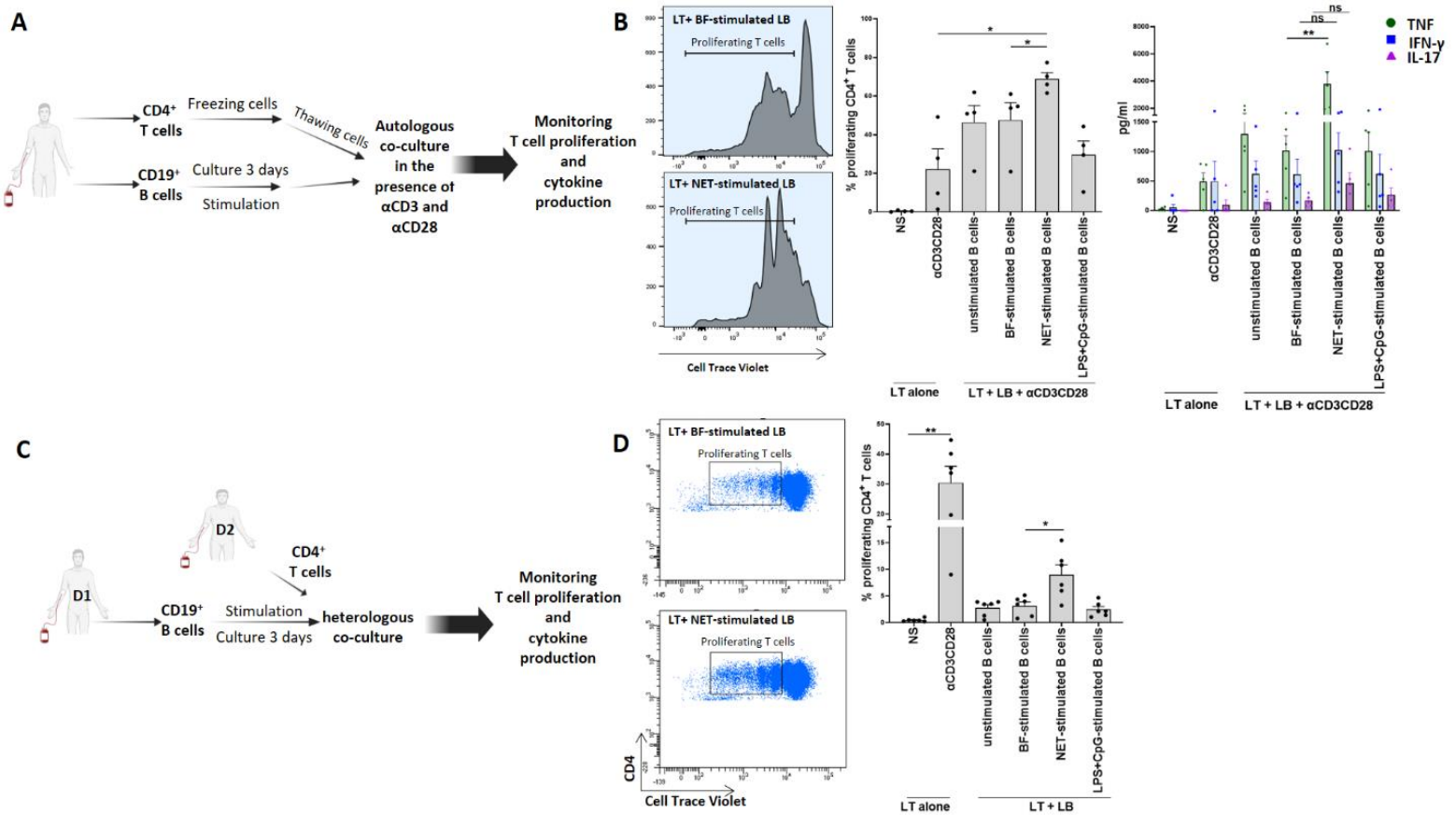


Figure 7. NET-activated B cells act as antigen-presenting cells leading to T-cell activation. Activated total B cells were co-cultured with T cells at 1:2 B/T ratio. Flow cytometry was used to measure proliferation after 4 days of the co-culture. ELISA was used to measure cytokine production. **(A)** Schematic representation of the autologous study design. **(B)** Autologous $CD19^+$ B cells and $CD4^+$ T cells were co-cultured for 4 days in the presence of anti-CD3 and anti-CD28 antibodies. Graphs represent the percentage of T-cell proliferation, and cytokine concentrations (mean values ± SEM of pooled data) from 5 independent experiments. **(C)** Schematic representation of the allogenic study design. **(D)** Heterologous $CD19^+$ B cells and $CD4^+$ T cells were co-cultured for 4 days. Graphs represent the percentage of T-cell proliferation (mean values ± SEM) from 6 independent experiments. Representative flow cytometry histograms and dot-plots are shown. $*p < 0.05$, $**p < 0.01$ (Mann Whitney test). D, donor; NS, non-stimulated, α, anti-; NETs, neutrophil extracellular traps; BF, NET-buffer.

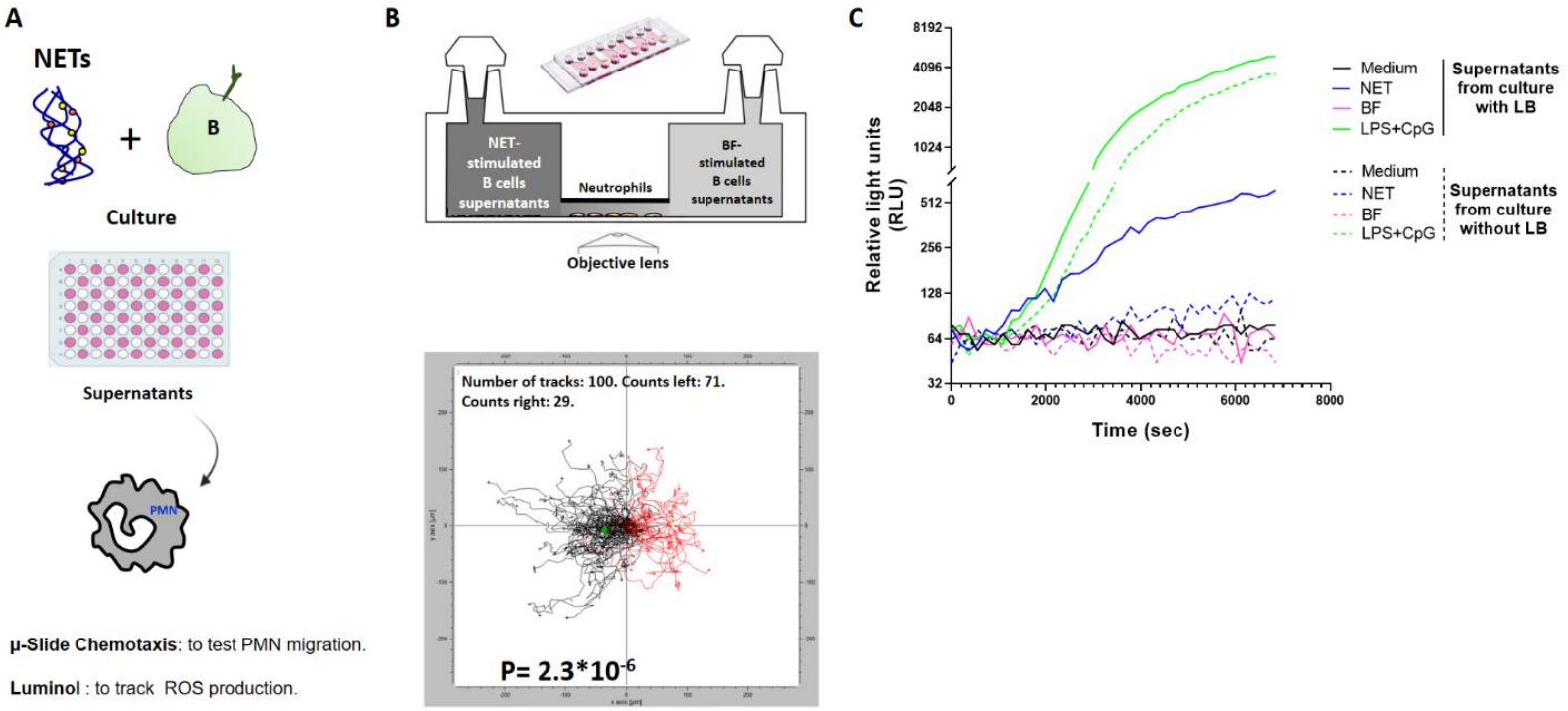
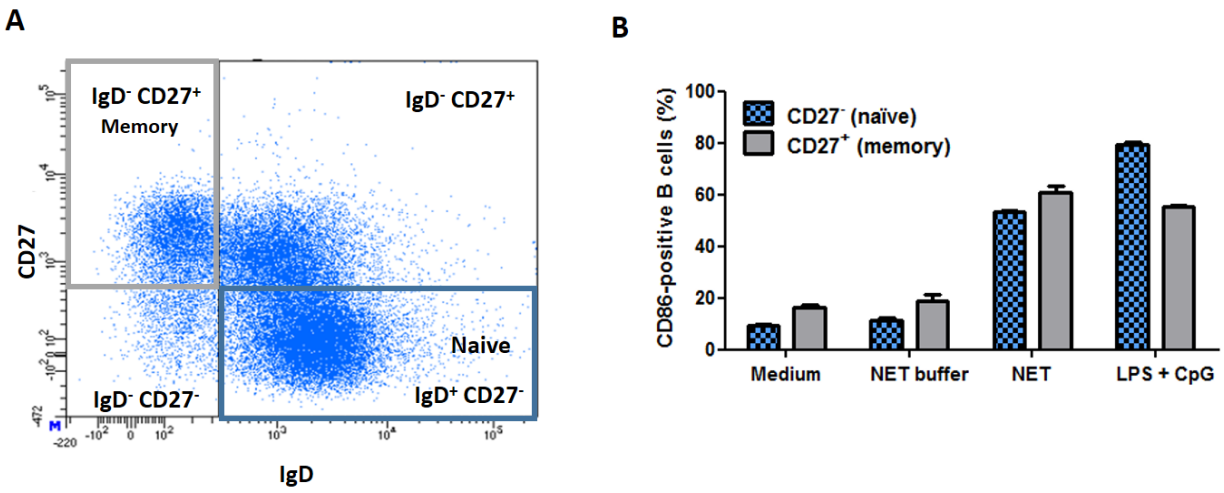
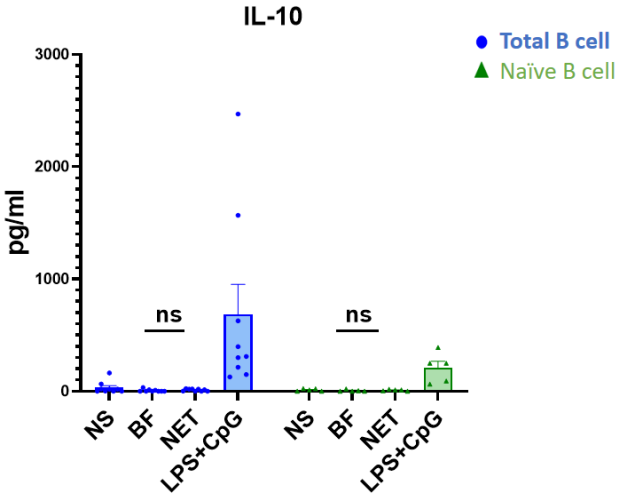


Figure 8. NET-activated B cells trigger neutrophil recruitment and ROS production. (A) Schematic representation of the study design. Total B cells were cultured with HD-derived NETs, its purification buffer, or TLR4 and 9 agonists (LPS and CpG, respectively). After 3 days supernatants were collected and used to stimulate freshly isolated neutrophils. **(B, top)** µ-Slide Chemotaxis was used to track neutrophils migration toward NET or BF-stimulated B cell supernatants as described in the materials and methods. **(B, bottom)** Trajectory paths of 100 randomly picked single cells are shown. Black paths depict cells with left migration, toward NET-activated B cell supernatants, and red paths depict cells with right migration toward BF-stimulated B cell supernatants. Data are from one experiment representative of 4 independent experiments. **(C).** Neutrophil ROS generation was measured in a luminol-dependent chemiluminescence assay as described in materials and methods. Neutrophils ($2 * 10^5$ cells) were cultured with B cell supernatants in the presence of luminol (100 µM) for 2hours. Data were representative of 5 experiments with similar results. **PMNs:** Neutrophils; **BF**: NET-buffer; **ROS**: Reactive oxygen species.

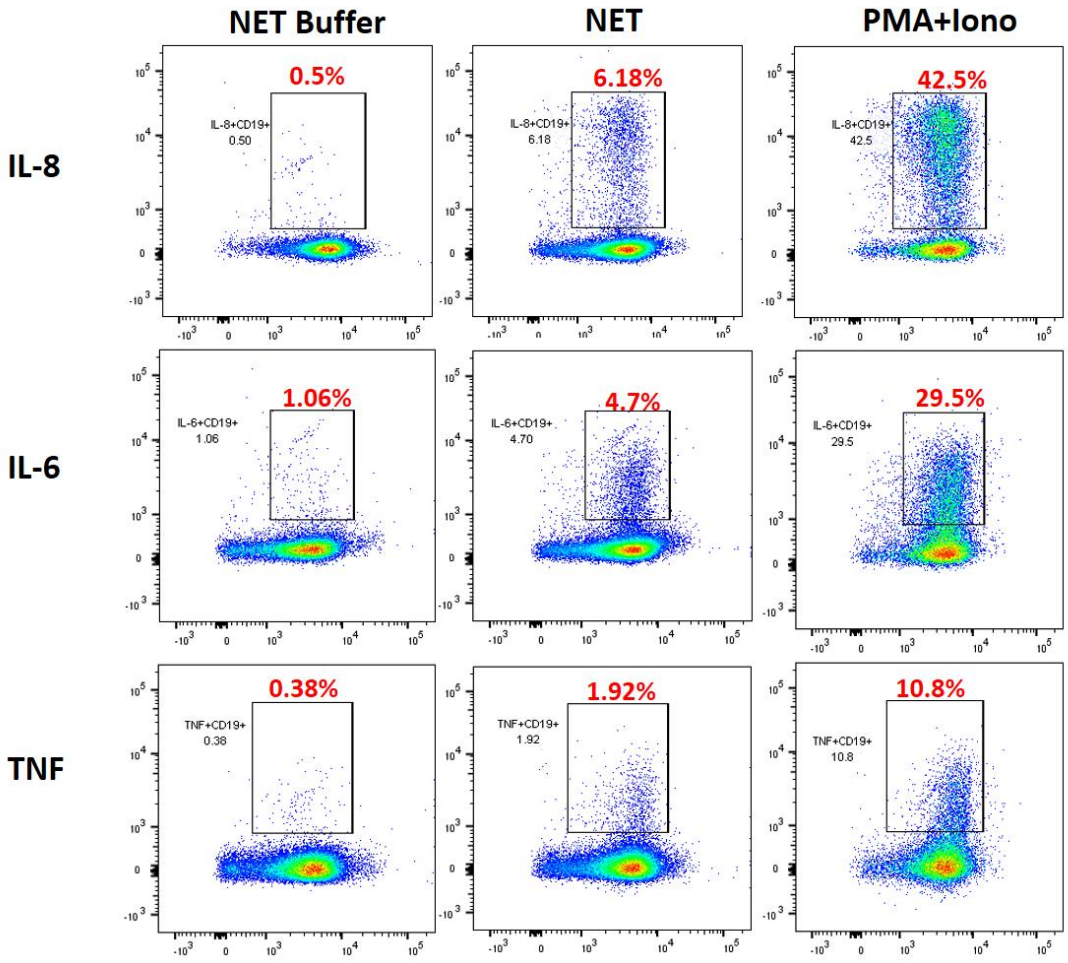


Supplementary Figure 1. Both naïve and memory B cells are activated by NETs. Healthy donor PBMCs were cultured for 3 days with purified NETs, its purification buffer (NET buffer), or TLR agonists and then analyzed by flow cytometry gating on B lymphocytes ($CD19^+$ cells). **(A)** In addition, IgD and CD27 staining was used to distinguish naïve ($CD27^-IgD^+$) and memory ($CD27^+IgD^-$) B cells. **(B)** Expression of activation molecules on each subset were then measured. One representative donor (of 2) is shown (with means and SEM of triplicates). PBMCs: peripheral blood mononuclear cells, CD: Cluster of differentiation.

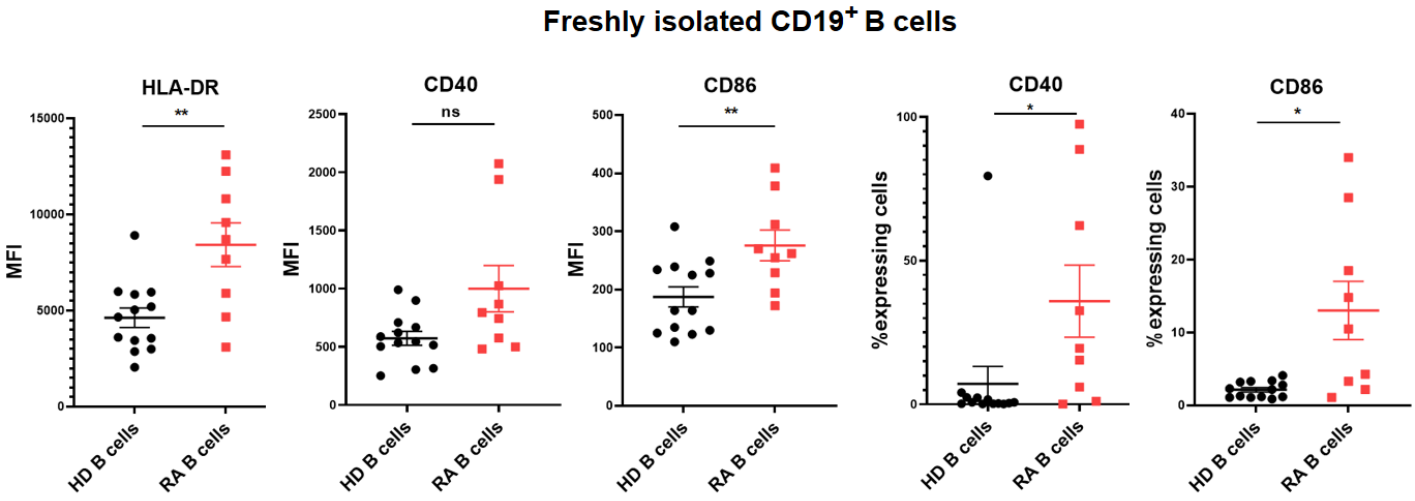


Supplementary Figure 2. IL-10 is not produced by total and naïve B cells in response to NETs. Total B cells (in blue) and naïve B cells (in green) were purified from healthy donors using

magnetic sorting (negative selection) and cultured for 3 days with HD-derived NETs, its purification buffer (BF), or TLR4 and 9 agonists (LPS and CpG, respectively). Production of the immuno-modulatory cytokine IL-10 has been measured by ELISA. Graph represent concentration (pg/ml) (mean values ± SEM) from independent experiments (n= 9 for total B cells, and n=5 for naïve B cells). ns > 0.05 (Wilcoxon matched-pairs signed rank test). MFI**,** Mean fluorescence intensity; LPS, lipopolisacharide; CpG, oligodeoxynucleotide; NETs, neutrophil extracellular traps; BF**,** NET-Buffer.



Supplementary Figure 3. Cytokine production by human B cells has been confirmed using flow cytometry gating on CD19^{+} cells. Total B cells were cultured for 12 hours. with NET, its purification buffer (NET buffer), and PMA+Iono as positive control in the presence of protein transport inhibitor. Cells were fixed/permeabilized and stained for cytokines. One representative donor (of 2) are shown. PMA**,** phorbol-12-myristate-13-acetate; Iono, Ionomycin.



Supplementary Figure 4. Enhanced B cell activity in RA patients compared to healthy donors. Total B cells were purified from RA patients or healthy donors PBMCs using magnetic sorting (negative selection), and directly analyzed by flow cytometry to examine the expression of activation markers CD40, CD86 and HLA-DR. Graphs represent MFI and frequencies (%) of activation markers on $CD19^+$ B cells from HDs (n=12) and RA patients (n=9). *$p < 0.05$, **$p < 0.01$ (Mann Whitney test). DAS**,** Disease activity score; **%:** Frequency, MFI**:** Mean fluorescence intensity.

References

1. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532-5. doi: 10.1126/science.1092385. PMID: 15001782.

2. Ribon M, et al. Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37. J Autoimmun. 2019 Mar;98:122-131. doi: 10.1016/j.jaut.2019.01.003. Epub 2019 Jan 28. PMID: 30704942.

3. Melbouci D, et al. Neutrophil extracellular traps (NET): not only antimicrobial but also modulators of innate and adaptive immunities in inflammatory autoimmune diseases. RMD Open. 2023 Aug;9(3):e003104. doi: 10.1136/rmdopen-2023-003104. PMID: 37562857.

4. Haidar Ahmad, A, et al. Polymorphonuclear Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus: More Complicated Than Anticipated. Immuno 2022, 2, 85-103. https://doi.org/10.3390/immuno2010007.

5. Gestermann N, et al. Netting Neutrophils Activate Autoreactive B Cells in Lupus. J Immunol. 2018 May 15;200(10):3364-3371. doi: 10.4049/jimmunol.1700778. Epub 2018 Apr 9. PMID: 29632142.

6. Carpenter EL, et al. Collapse of the CD27+ B-cell compartment associated with systemic plasmacytosis in patients with advanced melanoma and other cancers. Clin Cancer Res. 2009 Jul 1;15(13):4277-87. doi: 10.1158/1078-0432.CCR-09-0537. Epub 2009 Jun 23. PMID: 19549767; PMCID: PMC2896505.

7. Leffler Jet al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol. 2012 Apr 1;188(7):3522-31. doi: 10.4049/jimmunol.1102404. Epub 2012 Feb 17. PMID: 22345666.

8. Kouser L, et al. Emerging and Novel Functions of Complement Protein C1q. Front Immunol. 2015 Jun 29;6:317. doi: 10.3389/fimmu.2015.00317. PMID: 26175731; PMCID: PMC4484229.

9. Hurtado P, et al. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. J Immunol. 2010 Feb 1;184(3):1425-35. doi: 10.4049/jimmunol.0902305. Epub 2009 Dec 30. PMID: 20042575.

10. Gottenberg JE, et al. Markers of B-lymphocyte activation are elevated in patients with early rheumatoid arthritis and correlated with disease activity in the ESPOIR cohort. Arthritis Res Ther. 2009;11(4):R114. doi: 10.1186/ar2773. Epub 2009 Jul 23. PMID: 19627580; PMCID: PMC2745796.

11. Wang Y, et al. Rheumatoid arthritis patients display B-cell dysregulation already in the naïve repertoire consistent with defects in B-cell tolerance. Sci Rep. 2019 Dec 27;9(1):19995. doi: 10.1038/s41598-019-56279-0. PMID: 31882654; PMCID: PMC6934703.

12. Chapman EA, et al. Caught in a Trap? Proteomic Analysis of Neutrophil Extracellular Traps in Rheumatoid Arthritis and Systemic Lupus Erythematosus. Front Immunol. 2019 Mar 11;10:423. doi: 10.3389/fimmu.2019.00423. PMID: 30915077; PMCID: PMC6421309.

13. Casale TB, et al. Combination of IL-8 plus TNF alpha induces additive neutrophil migration. Allergy Asthma Proc. 1999 Nov-Dec;20(6):361-3. doi: 10.2500/108854199778251852. PMID: 10624491.

14. Lund FE, et al. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. Curr Dir Autoimmun. 2005;8:25-54. doi: 10.1159/000082086. PMID: 15564716.

15. Carmona-Rivera C, et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. Sci Immunol. 2017 Apr;2(10):eaag3358. doi: 10.1126/sciimmunol.aag3358. Epub 2017 Apr 14. Erratum in: Sci Immunol. 2020 Jan 31;5(43): PMID: 28649674; PMCID: PMC5479641.

16. Lande R, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011 Mar 9;3(73):73ra19. doi: 10.1126/scitranslmed.3001180. PMID: 21389263; PMCID: PMC3399524.

17. Schneider AH, et al. Neutrophil extracellular traps mediate joint hyperalgesia induced by immune inflammation. Rheumatology (Oxford). 2021 Jul 1;60(7):3461-3473. doi: 10.1093/rheumatology/keaa794. PMID: 33367912.

II. Scientific review 1

Immuno 2022, 2(1), 85-103; https://doi.org/10.3390/immuno2010007

In this review, we explore the multifaceted properties of human PMN and discuss recent evidence linking PMNs and NETs to the pathogenesis of both SLE and RA.

immuno

MDPI

Review

Polymorphonuclear Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus: More Complicated Than Anticipated

Ahmad Haidar Ahmad [1,2], Dyhia Melbouci [1,2] and Patrice Decker [1,2,*]

[1] Li2P, University Sorbonne Paris Nord, 1 rue de Chablis, 93017 Bobigny, France; ahmad.haidarahmad@edu.univ-paris13.fr (A.H.A.); dyhia.melbouci@univ-paris13.fr (D.M.)
[2] Inserm UMR 1125, 1 rue de Chablis, 93017 Bobigny, France
* Correspondence: patrice.decker@univ-paris13.fr; Tel.: +33-1-48-38-77-91

Abstract: Polymorphonuclear neutrophils (PMN) are the most abundant leucocytes in the circulation in humans. They represent a heterogeneous population exerting diverse functions through several activities. Usually described as typical pro-inflammatory cells, immunomodulatory properties of PMNs have been reported. Among others, once activated and depending on the stimulus, PMNs expel neutrophil extracellular traps (NET) in the extracellular space. NETs are complexes made of DNA and granule proteins representing an innate immune mechanism fighting infections. Nevertheless, an excess of NET formation might be involved in the development of inflammatory or autoimmune responses. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are two chronic, inflammatory, autoimmune diseases of unknown etiology and affecting mostly women. Several abnormal or non-classical functions of PMNs or PMN sub-populations have been described in SLE and RA. Particularly, NETs have been suggested to trigger pro-inflammatory responses by exposing pro-inflammatory mediators. Likewise, NETs may be the targets of autoantibodies or even might trigger the development of autoantibodies by exposing autoantigens. In the present review, we will summarize heterogeneous properties of human PMNs and we will discuss recent evidence linking PMNs and NETs to the pathogenesis of both SLE and RA.

Keywords: neutrophils; neutrophil extracellular traps; rheumatoid arthritis; systemic lupus erythematosus; inflammation; autoimmunity; immunomodulation; extracellular chromatin

Citation: Haidar Ahmad, A.; Melbouci, D.; Decker, P. Polymorphonuclear Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus: More Complicated Than Anticipated. *Immuno* **2022**, *2*, 85–103. https://doi.org/10.3390/immuno2010007

Academic Editors: Jan Willem Cohen Tervaert and Carlo Perricone

Received: 8 November 2021
Accepted: 27 December 2021
Published: 7 January 2022

1. Introduction

Polymorphonuclear neutrophils (PMN) represent more than 50% of blood leukocytes in humans. Circulating PMNs were thought classically to have a relatively short half-life. However, more recently, their in vivo lifespan has been estimated to 5.4 days in human blood [1] and to 120 h for tissue PMNs in vivo in zebrafish [2]. Moreover, PMN survival is increased under inflammatory conditions.

PMNs are among the first cells recruited to sites of inflammation. They are classically described as pro-inflammatory cells. Their classical functions comprise: (1) phagocytosis (via direct recognition of pathogens through receptors or via receptors for opsonins); (2) degranulation (different types of granules being present in PMNs with primary granules (containing, for example, proteinase 3, myeloperoxidase (MPO), cathepsin G, neutrophil elastase or defensins (belonging to antimicrobial peptides)), secondary granules (containing, for example, lactoferrin, cathelicidin (LL-37 in humans, an antimicrobial peptide)), tertiary granules (containing, for example, gelatinases, such as matrix metalloproteinase (MMP) 2 and MMP9) and secretory vesicles (containing, for example, receptor for the C1q complement protein)); (3) production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) which display bactericidal activities or are involved in cell–cell communication; (4) secretion of cytokines (e.g., tumor necrosis factor (TNF)) and chemokines

(e.g., interleukin (IL)-8). More recently, activated PMNs have been shown to produce and release neutrophil extracellular traps (NET), complexes of DNA and proteins from granules [3]. Nevertheless, immunomodulatory and regulatory or even immunosuppressive functions have also been attributed to PMNs.

The cell surface molecule CD66b is exclusively expressed by granulocytes in humans [4], especially PMNs and eosinophils. However, as in blood 95% of them are PMNs, CD66b is classically used as a PMN marker. Eosinophils can be further excluded by CD16 staining to discriminate neutrophils ($CD16^{+}$) from eosinophils ($CD16^{-}$). It should be noted that in mice Ly-6G should be used as a PMN marker, and not Gr-1, which is also detected, for example, on monocytes [5]. Nevertheless, PMNs are not a homogenous population. Naïve circulating PMNs are $CD54^{low}$ $CXCR1^{high}$ whereas naïve PMNs activated in vitro and tissue-resident PMNs are $CD54^{low}$ $CXCR1^{low}$. Interestingly, a subset of human PMNs able of retrograde migration from tissues to peripheral blood (reverse transmigration) is characterized by the $CD54^{high}$ $CXCR1^{low}$ phenotype [6]. During systemic inflammation or after severe injury, three circulating PMN populations with different morphologies and phenotypes have been described: band cells ($CD16^{dim}$/$CD62L^{bright}$), PMNs with hypersegmented nucleus ($CD16^{bright}$/$CD62L^{dim}$) and PMNs with a conventional segmented nucleus ($CD16^{bright}$/$CD62L^{bright}$), similar to PMNs found under normal conditions [7]. Likewise, a PMN subset defined as $CD49d^{+}VEGFR1^{high}CXCR4^{high}$ has been observed during hypoxia [8]. PMNs from the peripheral blood can also be classified according to their density after isolation by density-gradient centrifugation. High-density neutrophils correspond to conventional PMNs (also named normal-density neutrophils (NDN)), whereas low-density neutrophils (LDN) are detected at higher concentrations in patients with pathologic conditions (essentially autoimmune and autoinflammatory diseases, cancer, infections) or some non-pathologic situations (e.g., pregnancy). LDNs were first described as cells contaminating peripheral blood mononuclear cells (PBMC) prepared by Ficoll-Hypaque density centrifugation from patients with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) [9] and were later purified, characterized and defined as low-density granulocytes (LDG) in SLE patients [10]. PMN heterogeneity is also observed in tissues. Several phenotypes and functions of tissue PMNs have been reported, especially in tumors. PMNs able to infiltrate tumor tissue are termed tumor-associated neutrophils (TAN). Their heterogeneity has been observed during tumor progression as their phenotype and functions vary greatly, as reviewed in [11], with the description of anti- and pro-tumor TANs, initially described as N1 and N2 neutrophils [12]. Origin of TAN subsets may result from the activation status of PMNs or their differentiation from progenitors depending on the environment. However, the latter TAN subsets will not be further developed in the present review.

Upon activation, PMNs may produce NETs. Firstly described as a defense mechanism against bacteria [3], NETs can be induced by a variety of pathogens and during sterile inflammation as well, in response to several damage-associated molecular patterns (DAMP). According to the stimulus triggering NET formation, NET composition differs and NET formation is associated or not with PMN death. Likewise, NET formation has been associated with a range of activities, from antibacterial to immunomodulatory or even pro-inflammatory activities. Therefore, by exposing pro-inflammatory mediators, NETs may behave as a DAMP or be a source of DAMPs. Moreover, NETs may be a source of autoantigens by exposing or releasing in the extracellular space self-proteins (either in excess, or normally not exposed, or modified (e.g., citrullinated or after proteolytic cleavage)).

As a consequence of the different properties mentioned above, PMNs participate not only in the host defense against pathogens, but also in sterile inflammation, tumor progression or control, wound healing and tissue repair. Regarding sterile inflammation, PMNs have been suggested to play a key role in several pro-inflammatory autoimmune diseases, particularly in RA and SLE.

SLE is a systemic chronic inflammatory disease affecting 0.05% of the population, especially women (9♀/1♂) between 15 to 50 years old. It is characterized mainly by skin,

kidney, joint and central nervous system manifestations. SLE is triggered by a combination of genetic and environmental factors. It is defined as an autoimmune disease and is associated with the production of more than 100 different autoantibodies [13]. Among the targets recognized, nucleosome (the basic DNA packing unit in chromatin) is a major lupus autoantigen. It is found at higher concentrations in the circulations of SLE patients [14–16] and both autoreactive nucleosome-specific B [17] and T-helper [18] cells have been reported. Actually, SLE patients develop autoantibodies recognizing the whole nucleosomal complex (nucleosome-restricted antibodies) or its constituents, namely either histones or DNA. Of note, anti-double-stranded (ds) DNA autoantibodies represent a lupus marker.

RA is also a chronic, autoimmune, inflammatory disease. It affects 0.5% of the population with a 3♀/1♂ratio. RA leads to joint destruction and is associated with systemic manifestations. Particularly, RA is a major risk factor for cardiovascular diseases. As many autoimmune diseases, RA is triggered by a combination of genetic (essentially genes coding for HLA-DR molecules) and environmental (e.g., smoking) factors. RA is associated with the production of the disease-specific anti-citrullinated protein antibodies (ACPA) [19,20], which are present in about 70% of patients and are useful for both RA diagnosis and eventually prognosis [21]. ACPA-positive RA patients develop a more severe and erosive disease with extra-articular manifestations. ACPAs recognize different target proteins and some ACPAs are pathogenic (e.g., by inducing osteoclastogenesis [22]).

The aim of the present review is to summarize evidence linking human PMNs to the pathogenesis of SLE and RA, with a special focus on NETs. We will describe how human PMNs are activated, the consequences on immune cell activation, their potential involvement in inducing autoantibody production and the downstream pathogenic mechanisms. To better understand the potential pathogenicity of PMNs, we will first briefly introduce the complexity of human PMNs and NET formation.

2. Interaction of PMNs with Other Immune Cells and Non-Classical Functions of PMNs

Insight into PMNs has evolved during recent years and non-classical functions of PMNs have been identified. First of all, PMN plasticity has been demonstrated. Indeed, PMNs may acquire phenotypic and functional properties of antigen-presenting cells, like cell surface expression of MHC II and costimulatory molecules as well as antigen presentation [23,24]. The latter results suggest that PMNs are also directly involved in adaptive immune responses. Even cross-presentation was reported [25,26]. PMN plasticity in terms of phenotype and function may depend on the microenvironment.

PMNs or PMN subsets with unexpected activities have been reported. Steady-state PMNs from healthy individuals express a functional cell surface Toll-like receptor (TLR) 9 [27] and part of them are able to secrete interferon (IFN)-α upon activation [28]. Even a sub-population expressing a functional T-cell receptor leading to IL-8 secretion upon specific engagement has been reported [29].

PMNs communicate with diverse immune cells and link innate and adaptive immunities. Thus, PMNs interact and activate dendritic cells (DC) [30]. Likewise, PMNs and the pro-inflammatory Th17 lymphocytes chemoattract each other [31]. In addition, PMNs help B lymphocytes to produce immunoglobulins (Ig) [32]. PMNs also interact and/or affect macrophages/monocytes, for example, by promoting the secretion of mature IL-1β by lipopolysaccharide (LPS)-primed macrophages [33] or by favoring adhesion of classical monocytes through the release of cathelicidins [34]. Particularly, PMNs display immunomodulatory activities. We have reported that PMNs cooperate with regulatory T lymphocytes (Treg) to sustain their activity [35]. Likewise, PMNs may produce the regulatory cytokine IL-10 under particular conditions [36]. Activated PMNs also secrete soluble CD66b (soluble CEACAM8) [37], which has a dual activity as it is known to costimulate B lymphocytes or to inhibit TLR2 response. Interestingly, even immunosuppressive functions have been reported for PMNs. In healthy individuals, $CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}$ PMNs inhibiting T-cell proliferation have been observed following endotoxin challenge [7]. These PMNs exhibit a decreased adhe-

sion to activated endothelium [38]. Likewise, immunosuppressive $CD10^+$ PMNs inhibiting IFN-γ production by T lymphocytes have been discovered in healthy individuals treated with granulocyte colony-stimulating factor for stem cell mobilization [39]. This subset belongs to LDNs. Actually, immunosuppressive NDNs have also been reported, but only in pathological situations such as cancer patients and HIV-1 infection. PMNs have also been shown to decrease IFN-γ secretion by invariant natural killer T cells, a mechanism requiring cell–cell contact and live PMNs [40]. Likewise, PMNs impair pro-inflammatory cytokine secretion by PBMCs in response to *C. Albicans* or a TLR4 agonist (LPS); however, here probably via PMN-derived proteases [41].

Similarly, PMNs may also be immunomodulatory via the production of NETs. We have shown that NETs inhibit the secretion of the pro-inflammatory cytokine IL-6 by LPS-stimulated macrophages [42]. Similarly, NETs inhibit myeloid DC activation in response to LPS [43]. NETs have also been shown to control inflammation by degrading cytokines, in situations where PMN concentrations are huge, as in gout [44]. In this context, NETs aggregate and act via serine proteases. One may thus ask whether the mechanism described by Gresnigt et al. does not partly rely on NETs or NET-derived proteases. On the contrary, NETs can prime T lymphocytes [45], suggesting that an excess of NETs might become pathogenic. Similarly, NETs induce polyclonal activation of memory B lymphocytes from healthy individuals [46]. One can hypothesize that PMNs might also communicate with other cell types through NET formation.

Nevertheless, excessive PMN activation and NET release can be deleterious, and are for example associated with SARS-CoV-2 pathogenesis as recently reviewed [47].

3. Neutrophil Extracellular Trap Formation, Function and Clearance

NETs were first described in 2004 [3]. In response to phorbol myristate acetate (PMA, a protein kinase C agonist), IL-8 or LPS, PMNs were originally shown to release in the extracellular environment structures composed of nuclear DNA filaments decorated with histones (namely chromatin filaments) and associated with granule proteins, but devoid of membrane. Importantly, these fibers are capable of trapping and killing gram-positive and gram-negative bacteria, highlighting a new innate immune mechanism to neutralize and remove pathogenic bacteria by neutrophils. Since this original description, the definition of NETs has evolved, both in terms of composition and function [48]. Thus, in addition to bacteria, NETs are involved in host defense against parasitic [49] and fungal [50,51] infections. NETs also display antiviral properties [52]. It has been proposed that NETs are one of the means used by PMNs to degrade microbes that have a too large diameter to be phagocytosed [53].

NETs bind to pathogens by electrostatic interactions between histones or DNA constituents of NETs and the membrane of microbes, thus preventing their dissemination. NETs inactivate virulent factors of the captured pathogens and eliminate them. To do this, numerous proteins have been shown associated with NETs. Twenty-four proteins have been identified by Urban et al. in PMA-induced NETs, including some cytoplasmic proteins [54]. Since then, proteomic analyses revealed that the composition of NETs prepared from PMNs purified from healthy individuals is more complex and may vary according to the stimulus [55,56]. Interestingly, the antimicrobial effect mediated by NETs has been shown to pass especially through histones [3], LL-37 [57] and calprotectin (S100A8/A9) [54]. Some of these proteins kill microbes by forming membrane pores. Likewise, an antimicrobial activity has been reported for DNA [58]. Conversely, as a defense mechanism, bacteria have developed evading strategies against NETs, for example, via the secretion of nucleases degrading NETs [59] or virulence factors inhibiting the activity of antimicrobial peptides [57]. Although, reciprocally, LL-37 confers resistance of NETs against bacterial nucleases [60]. However, NETs also act as a physical barrier to limit bacterial biofilm dissemination [61].

The original description by Brinkmann et al. was associated with the death of PMNs and was named NETosis [3], a process different from apoptosis or necrosis [62]. NETosis,

sometimes named suicidal NETosis, occurs in 2–4 h and is the canonical mechanism. Since then, different mechanisms and pathways of NET formation have been described [63,64], leading to PMN death or not, depending on the stimulus. In the latter case, this process is named vital (or live) NETosis. Therefore, it is recommended to use "NETosis" only when NET extrusion is accompanied with PMN death, or at least "suicidal" or "vital" should be mentioned. Otherwise, the correct term is "NET formation" [48]. Accordingly, NET formation is triggered by several stimuli: parasites [65], bacteria like *Staphylococcus aureus* and their products such as LPS [62], *Candida albicans* [66,67] or HIV-1 [52] but also by immobilized immune complexes (IC) [68], activated platelets [69] or cytokines like IL-8 [3].

Vital NETosis is accomplished by rapidly (less than one hour) ejecting mitochondrial DNA, instead of nuclear DNA, bound to granule proteins [70]. Mitochondrial NETs are generally produced after priming PMNs with granulocyte-macrophage colony-stimulating factor, followed by stimulation with LPS or complement factor 5a (C5a) and preserve membrane integrity. These NETs were subsequently shown to kill bacteria [71,72].

Another non-suicidal pathway of NET formation has been described, leading to NETs made of nuclear DNA. Activation of PMNs by *Staphylococcus aureus* in vivo and in vitro results in nuclear condensation which is followed by the separation of inner and outer membranes of the nucleus. Subsequently, transport vesicles containing nuclear DNA are formed and burgeon through the plasma membrane into the extracellular space, without breach of the plasma membrane in rapid kinetics (5–60 min). Once in the external environment the vesicles rupture and NETs are released [73,74]. This mechanism is also bactericidal, although those NETs display a limited proteolytic activity.

Recently, NET formation was compared in response to several stimuli [64]. All the five stimuli tested induced NETs composed essentially of chromosomal DNA, displaying bactericidal activity and leading to PMN death (NETosis). Nevertheless, different pathways were triggered during NET formation. However, stimulation by *Staphylococcus aureus* or granulocyte-macrophage colony-stimulating factor followed by stimulation with LPS or C5a was not tested in that study. Therefore, one cannot firmly conclude that all stimuli inducing NET formation lead to NETs made of nuclear DNA and in a suicidal way.

Thus, several mechanisms of NET formation exist, triggered by different pathways and leading or not to PMN death. Depending on the stimulus, formed NETs differ in their composition (nuclear versus mitochondrial DNA, containing histones or not, eventually enriched in some post-translational modifications, . . .). A minimal common definition for NETs may be complexes made of DNA and proteins from granules (e.g., neutrophil elastase, MPO), eventually with other associated proteins.

Once released, NETs are cleared by different mechanisms. NETs are normally degraded by deoxyribonuclease 1 (DNase1) present in sera from healthy individuals [75] and can be degraded in vitro by DNase1-like 3 secreted by myeloid DCs [76]. Moreover, NETs are engulfed by macrophages, a process facilitated when NETs are opsonized by C1q [77] or LL-37 [76], and are then degraded intracellularly.

4. Involvement of PMNs in RA and SLE

Although PMNs are beneficial in a physiological situation, they may behave differently in a pathological context through increased release of ROSs and increased secretion of pro-inflammatory cytokines. For instance, activated PMNs release granules containing proteases able to process pro-inflammatory cytokines into mature active forms [78]. Particularly, NETs are protective in response to several types of infections, but NET formation is also triggered during sterile inflammation and may cause tissue damages or immune cell activation. Therefore, uncontrolled activation of PMNs or deficient resolution of inflammation, e.g., due to prolonged PMN survival, may be deleterious. Similarly, an excess of NET formation or impairment of the NET clearance mechanisms described above may lead to enhanced release of DAMPs and autoantigens, potentially modified by post-translational modifications or PMN proteases, generating neo-epitopes.

Former studies have shown that PMNs are activated in SLE [79] and RA [80,81]. As mentioned above, PMN activation may lead to NET formation. Moreover, PMNs are present in affected tissues both in SLE [82] and RA [83,84] patients. Because anti-dsDNA autoantibodies are a SLE marker and because circulating chromatin is detected at higher concentrations in SLE patients [15] and correlates with disease activity [16], NETs have been considered as a putative lupus autoantigen. Similarly, because ACPAs are a RA marker and because NET formation is associated with protein citrullination in some cases, it has been hypothesized that NETs might induce or be recognized by ACPAs. In both cases, NETs may form ICs with those autoantibodies and trigger downstream pathogenic mechanisms. More recently, anti-NET antibodies have been reported in RA and SLE [85].

For all these reasons, PMNs and NETs have been intensively studied in the last years in both diseases. We will here discuss the potential pathogenic roles of PMNs and particularly of NETs in both RA and SLE. Key observations supporting these hypotheses are presented in Table 1 (PMNs) and Table 2 (NETs). Potential pathogenic mechanisms involving PMNs and NETs are depicted in Figure 1A (SLE) and Figure 1B (RA).

4.1. Involvement in SLE Pathogenesis

Some alterations were reported for PMNs in SLE. PMNs express CD11b (a subunit of Mac-1) and single-nucleotide polymorphisms in the ITGAM locus (coding for CD11b) are associated with SLE. PMNs from individuals with nonsynonymous variant alleles of ITGAM show a significant impaired Mac-1-mediated and IFN-γ-mediated phagocytosis as well as impaired adhesion [86]. Similarly, activated PMNs from SLE patients shed FcγRIIA, potentially leading to an impaired clearance of ICs [87]. Moreover, an increase in PD-L1-expressing PMNs has been reported in SLE, especially in patients with high disease activity [88]. Likewise, the frequency of IL-17$^+$ PMNs is increased in SLE patients in comparison to healthy individuals [89]. In addition, lupus PMNs extrude oxidized mitochondrial DNA-protein complexes and these complexes are interferogenic, as they stimulate plasmacytoid DC (pDC) to secrete IFN-α [90], a key lupus cytokine. Indeed, IFN-α promotes e.g., the differentiation of SLE monocytes into DCs. A subset of LDNs, named LDGs, has been observed in SLE [10]. In contrast to immunosuppressive LDNs described earlier, LDGs are pro-inflammatory cells. They display an activated phenotype and secrete higher levels of pro-inflammatory cytokines, have a normal bactericidal activity and capacity to synthesize H_2O_2, but show an impaired phagocytic activity. Importantly, LDGs express increased levels of IFN-α mRNA. Particularly, LDGs are non-suppressive in SLE and activate T lymphocytes to produce pro-inflammatory cytokines [91].

In SLE, extracellular chromatin (especially nucleosomes) is found in the circulation and deposits in kidneys [92]. We have shown that nucleosomes are not only a major lupus autoantigen but also behave like a DAMP. Particularly, extracellular nucleosomes trigger PMN activation, leading to the secretion of the pro-inflammatory cytokine IL-8 [93]. The latter is also a chemokine and is particularly efficient in recruiting PMNs, leading to an amplification loop. Interestingly, recognition of nucleosomes by PMNs occurs independently of TLR9 [94], a classical DNA receptor. Importantly, in response to nucleosomes PMNs secrete IFN-α [28], a cytokine involved in SLE pathogenesis, and produce NETs [28], fueling the microenvironment in autoantigens and pro-inflammatory mediators. PMNs primed to produce IFN-α were actually observed in the bone marrow of SLE patients [95]. These PMNs also produce B-cell factors like BAFF and APRIL, whereas alterations in B-cell development were observed in the bone marrow. Cell-free chromatin activates also natural killer cells, at least partly by up-regulating plasma membrane MICA expression on monocytes or PMNs [96]. In addition to free nucleosomes, lupus ICs containing DNA and anti-DNA antibodies also stimulate PMNs to secrete IL-8 [97].

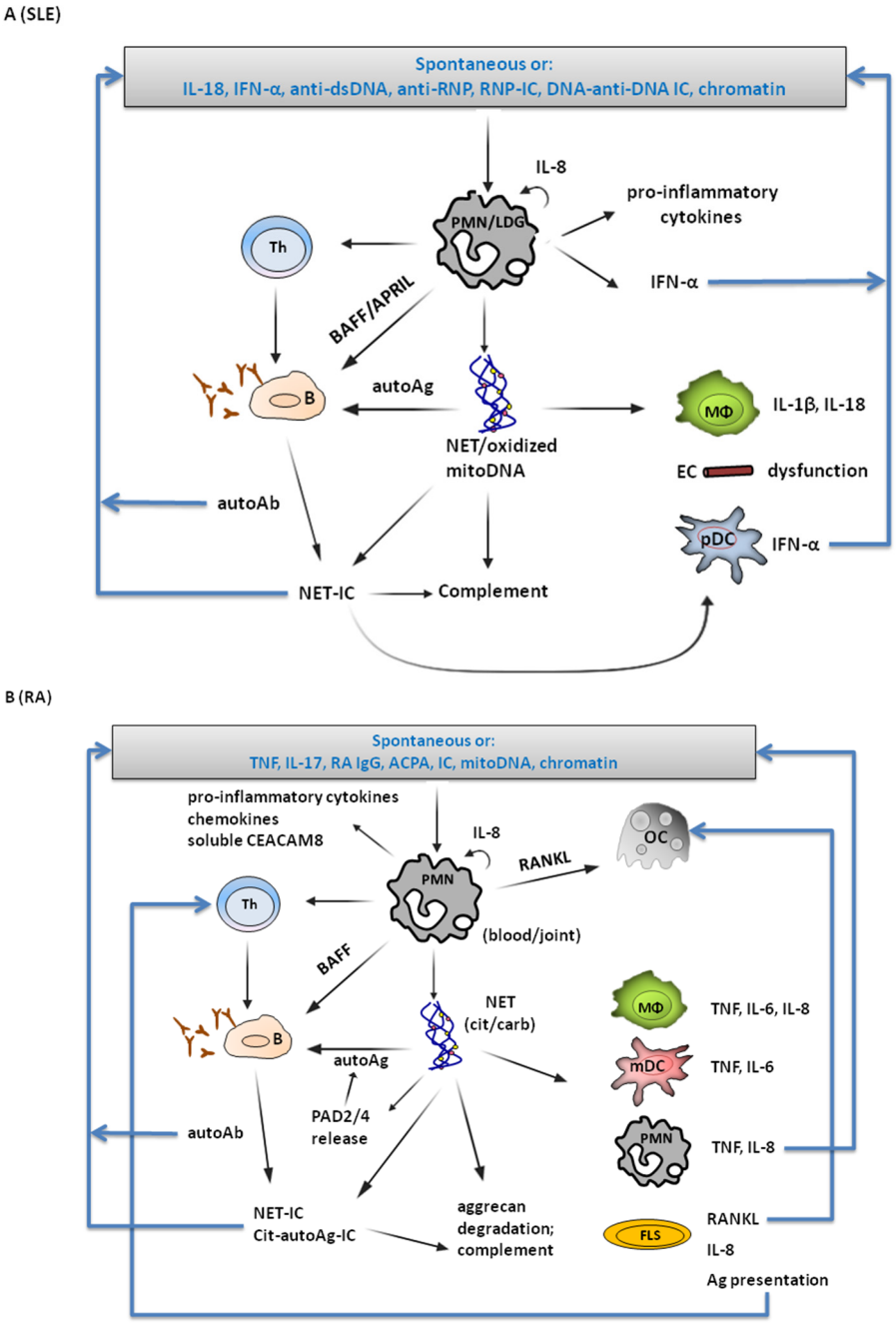


Figure 1. Potential pathogenic mechanisms involving PMNs and NETs in SLE (**A**) and RA (**B**). PMNs are activated either spontaneously or in response to disease-associated stimuli, potentially leading to NET formation. The consequences of PMN activation (e.g., antigen presentation, cytokine secretion) and NET formation (e.g., release of immunomodulatory molecules and autoantigens, potentially modified, such as by released PADs) on different cell types and activation of the complement system are depicted. NETs (modified or not) may act directly or via NET-containing immune complexes. Amplification loops are shown. The figure focuses on PMNs and NETs; other pathogenic mechanisms are involved. The downstream consequences of cytokines, immune complexes or activated target cells are not presented. ACPA, anti-citrullinated protein antibody; Ag, antigen; autoAb, autoantibody; autoAg, autoantigen; B, B lymphocyte; carb, carbamylated; cit, citrullinated; ds, double-stranded; EC, endothelial cell; FLS, fibroblast-like synoviocyte; IC, immune complex; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; LDG, low-density granulocyte; mDC, myeloid dendritic cell; mitoDNA, mitochondrial DNA; MΦ, macrophage; OC, osteoclast; PAD, peptidyl-arginine deiminase; pDC, plasmacytoid dendritic cell; RNP, ribonucleoprotein; Th, T-helper lymphocyte; TNF, tumor necrosis factor.

Many studies focused on the role of NETs in SLE. LDGs have a higher capacity to form NETs and NETs are observed in skin and kidney lesions of patients [82]. Importantly, anti-dsDNA autoantibodies purified from SLE patients trigger NET formation in PMNs from healthy individuals [98]. Moreover, several NET clearance mechanisms are impaired in SLE. Serum DNase1 activity is decreased in SLE patients [99] and loss-of-function variants of DNase1-like 3 have been reported [100]. Likewise, in a subset of SLE patients, NET degradation by serum DNase1 is impaired, due to either the presence of DNase1 inhibitors or anti-DNase1 antibodies, which is associated with kidney manifestations [75]. Moreover, serum C1q concentrations are decreased in SLE patients [101] and therefore the NET clearance mechanism described for macrophages [77] is probably impaired. This may result in activation of the complement cascade upon deposition of residual C1q on NETs [102].

Several studies analyzed the capacity of NETs to activate immune cells, supporting their role as a source of DAMPs, inducing important SLE cytokines. Lupus LDGs spontaneously produce NETs which stimulate IL-1β and IL-18 secretion by LPS-primed macrophages from healthy individuals [103]. Once secreted, IL-18 induces NET formation. Both secretion of mature (cleaved) IL-1β and activation of caspase-1 were noted, indicating involvement of inflammasome. Similarly, NETs from healthy donors and SLE patients increase calcium flux in macrophages from healthy donors and SLE patients [104]. Moreover, NETs from lupus LDGs are enriched in MMPs and damage endothelial cells in vitro [105]. NETting PMNs are able to stimulate IFN-α secretion by pDCs [106], whereas PMNs stimulated by ribonucleoprotein-containing ICs release oxidized mitochondrial DNA inducing IFN-α secretion in PBMCs [107]. Similarly, PMNs stimulated with anti-ribonucleoprotein autoantibodies produce NETs able to stimulate IFN-α secretion by pDCs [108]. Those NETs contain LL-37, a DNA-binding protein facilitating uptake by pDCs and recognition by TLR9. The NET activity in SLE may depend on a specific NET composition. A particular protein composition of NETs has been associated with lupus nephritis [109], whereas in patients with active SLE, NETs are enriched in IL-17A [110]. Likewise, IL-33 is complexed with NETs and amounts of such complexes are correlated with disease activity. These NETs induce IFN-α production by pDCs in an IL-33 receptor-dependent manner [111].

In agreement with the hypothesis that NETs may be a source of autoantigens, patient antibodies binding NETs have been reported [75,85,102]. Those antibodies recognize the NET structure, without additional information on their fine specificity, and can, therefore, be named anti-NET autoantibodies. Compared to NETs from healthy individuals, SLE NETs contain increased amounts of acetylated and methylated histones [112] and some of these post-translational modifications are targeted by lupus IgG autoantibodies (e.g., acetylation of histone H4 at lysine 16) [113]. Recently, human monoclonal anti-DNA antibodies generated from SLE B cells have been demonstrated to recognize NETs [114]. Moreover, autoantibodies against NET proteins are present in SLE patients, as anti-neutrophil elastase, anti-LL-37, anti-MPO, anti-cathepsin G or anti-lactoferrin antibodies (as reviewed in [115,116]) and, inversely, some antibody reactivities associated with SLE target NET proteins or NET-binding proteins (e.g., anti-C1q antibodies). These data are reminiscent of anti-neutrophil cytoplasmic antibodies (ANCA) associated with the pathogenesis of autoimmune small vessel vasculitis (SVV). Most SVV patients have detectable ANCAs specific for proteinase 3 or MPO. Interestingly, NETs have been shown to be a source of proteinase 3 and MPO, whereas ANCAs induce NET formation in normal PMNs [117]. Former studies have shown that mice immunized with human PMNs develop anti-MPO and anti-lactoferrin antibodies [118]. Although the role of ANCAs is less evident in SLE, lupus patients have circulating ANCAs, including anti-LL-37 antibodies [106]. Interestingly, memory B lymphocytes purified from SLE patients produce ANCAs, including anti-LL37 antibodies, when stimulated with NETs [46].

As a consequence of all those NET-recognizing autoantibodies, NETs may also be pathogenic in ICs. For instance, induction of IFN-α secretion by pDCs was observed with NETs in the presence of anti-LL-37, anti-DNA or anti-human neutrophil peptide antibodies

and was stronger than with NETs alone [106]. Interestingly, IFN-α secretion was abolished in the presence of a TLR9 antagonist, suggesting the recognition of the DNA moiety of NETs. Therefore, in addition to classical downstream pathogenic mechanisms induced by ICs in SLE, NET-containing ICs trigger mechanisms mediated by their particular composition.

4.2. Involvement in RA Pathogenesis

In RA, PMNs are present in the synovial fluid of patients [83]. In addition, PMNs are found in large numbers at the pannus-cartilage interface in early RA [84] and heavily infiltrate the synovial tissue in the first weeks of RA onset [119].

As described in SLE, RA PMNs also display particular characteristics and alterations. Thus, RA synovial fluid PMNs express class II MHC molecules and induce T-cell proliferation in a class II MHC-dependent manner [120]. Moreover, synovial fluid PMNs trans-differentiate into DC-like cells [121], evidencing plasticity. Actually, RA synovial fluid is anti-apoptotic on PMNs cultured under hypoxia [122], which mimics conditions existing in vivo within joints. RA synovial fluid PMNs express a high amount of BAFF at the mRNA level [123], suggesting a role of PMNs in supporting B-cell activation. In fact, RA synovial fluid PMNs express a lower amount of plasma membrane BAFF than blood PMNs, whereas TNF induces BAFF release, suggesting that joint infiltrating PMNs shed BAFF upon local stimulation by synovial fluid TNF [124]. PMNs also produce TNF, a key cytokine in RA, as well as IL-8 and IL-6, cytokines also associated with RA and particularly RA synovial fluid PMNs spontaneously express those three cytokines at the mRNA level [123]. RA synovial fluid PMNs display a gene signature of oxidative stress [125]. Interestingly, RA synovial fluid contains high amounts of mitochondrial DNA and the latter induces RANKL expression in normal PMNs. Regarding circulating cells, RA blood PMNs express RANKL [126], a key molecule in osteoclast differentiation, linking PMNs to bone erosions in patients. Membrane TNF expression is increased on RA PMNs before TNF inhibitor therapy in comparison to PMNs from healthy individuals, but returns to baseline expression after successful therapy with TNF inhibitor [80]. In addition, apoptosis is delayed in blood PMNs from RA patients before TNF inhibitor therapy [80]. We have recently reported that PMNs cooperate with regulatory T lymphocytes to sustain the activity of the latter in healthy individuals, whereas this mechanism is deficient in RA patients [35]. By comparing paired blood and synovial fluid PMNs from RA patients with active disease, synovial fluid PMNs had increased expression of chemokines [127]. Particularly, hypoxia-inducible factor-1α signaling is up-regulated in synovial fluid PMNs. Importantly, a combination of PMN biomarkers may predict response to TNF inhibitor therapy in RA [128]. Finally, LDGs have also been observed in RA patients. By comparing RA PMNs and RA LDGs, both populations differ by their transcriptome and RA LDGs may represent an immature population with lower constitutive rates of apoptosis [129]. Although the pro-inflammatory activity of LDGs might be less evident in RA than in SLE, only RA LDGs were able to secrete IL-1β after stimulation [129].

Table 1. Involvement of PMNs in SLE and RA.

	SLE	**RA**
PMNs are activated	[79]	[80]
PMNs detected in affected tissues	[82]	[83,84]
Pro-inflammatory LDGs [1]	[10]	[129]
Altered PMN phenotype	[87,88]	[80,120,121,123,126]
PMNs express/produce key disease cytokines	IFN-α [28]	TNF [123]

[1] IFN, interferon; LDGs, low-density granulocytes; PMNs, polymorphonuclear neutrophils; TNF, tumor necrosis factor.

Numerous studies suggest that NETs are involved in RA pathogenesis. RA PMNs have a higher capacity to produce NETs in vitro, either spontaneously [130,131] or after stimulation [42,130]. Particularly, pro-inflammatory cytokines induce NET formation,

especially TNF and IL-17A [130]. Moreover, NETs are present not only in the synovial fluid of RA patients [132], but also in rheumatoid nodules [130]. NET formation is associated with the release of active peptidyl-arginine deiminase (PAD) 2 and 4, enzymes involved in protein citrullination, either as free extracellular PAD2/PAD4 or bound to NETs [132]. Importantly, active PADs are present in RA synovial fluids [132]. The latter results link NET formation to local citrullination in vivo and the potential induction of ACPA production in RA patients. Interestingly, we and others have shown that ACPA-rich IgGs purified from RA patients bind to NETs [42,130] or even induce NET formation [130]. NET binding and NET induction were confirmed with purified ACPAs [133]. Similarly, sera from RA patients recognize activated PMNs and NETs (and especially citrullinated histone H4) [134] and monoclonal antibodies generated from RA synovial B lymphocytes have a strong reactivity against citrullinated histones and PMA-induced NETs [135]. It should be noted that citrullination can occur during NET formation (at different levels and in response to some stimuli), and thus NETs may be a source of RA autoantigens and be either the targets of ACPAs or even the true autoantigen triggering ACPA production. Likewise, RA patients develop also antibodies against carbamylated proteins (anti-CarP) which are predictive for a more severe disease [136]. Yet, NETs externalize carbamylated proteins and carbamylated NETs from RA patients are particularly efficient in activating fibroblast-like synoviocytes (FLS) and macrophages [137]. Especially, NETs up-regulate RANKL in RA FLSs, linking NETs to osteoclastogenesis. In addition, some RA patients were shown to develop ANCAs [138]. ANCAs may recognize different NET proteins and NETs may become pathogenic in the form of ICs. For instance, anti-lactoferrin antibodies are found in the serum of RA patients and lactoferrin-containing ICs stimulate TNF secretion by macrophages [139]. Moreover, classical effector functions of ICs might be triggered, as mentioned for SLE patients. In turn, PMNs are activated by ICs [140], especially by ICs from the synovial fluid of RA patients [141], leading to an amplification loop.

Potential roles of NETs in RA physiopathology have been investigated. NETs activate RA FLSs, which are critical cells involved in joint damage, leading to pro-inflammatory cytokine secretion by FLSs [130]. NETs are also internalized by FLSs, triggering class II MHC molecules up-regulation, and this is associated with presentation of citrullinated antigens to T-cells; RA FLSs loaded with NETs activate citrullinated-vimentin-specific $CD4^+$ T lymphocytes from RA patients [133]. Importantly, NETs are directly involved in articular cartilage damage by degrading aggrecan in a neutrophil elastase-dependent manner [142]. We have shown that NETs are pro-inflammatory on resting macrophages and PMNs. NETs activate both cell types to secrete pro-inflammatory cytokines, but not IL-10. Moreover, RA NETs were more active than NETs from healthy individuals in terms of their capacity to induce IL-8 secretion, while inducing minimal IL-10 secretion [42]. Similarly, activation of myeloid DCs from healthy individuals by RA NETs is stronger than with NETs from healthy individuals [131].

Interestingly, although the source of chromatin is unknown, extracellular cell-free chromatin is also observed in the synovial fluid in RA [83] and deposits on the cartilage surface, and in addition chromatin-containing ICs deposit in the joint tissue of RA patients [143]. Thus, some of the DAMP activities of chromatin on PMNs described above in SLE may also occur in RA, like PMN activation [93], as observed with RA PMNs [28], leading to the secretion of the key RA cytokine TNF in addition to IL-8 and IL-6 [94], as well as induction of NET formation [28]. Moreover, we have reported that chromatin-stimulated PMNs secrete a soluble form of CEACAM8 (also named CD66b), a granulocyte-specific protein, and that concentrations of soluble CEACAM8 are elevated in the synovial fluid of RA patients [37]. Interestingly, RA LDGs express higher levels of CEACAM8 mRNA than RA blood PMNs [129]. As in SLE, the origin of extracellular chromatin in RA is unknown and part of it may come from NETs. Therefore, cell-free chromatin may behave as a DAMP in a disease non-specific manner. Reciprocally, if part of this chromatin has a different composition in RA in comparison to SLE, or if the response of RA PMNs to chromatin differs from SLE PMNs, chromatin may act in a more SLE-specific way.

Table 2. Involvement of NETs in SLE and RA.

	SLE	RA
Higher NET [1] formation	[82]	[42,130,131]
Impaired NET clearance	[75,102]	[144]
Anti-NET antibodies	[75,85,102]	[85]
NETs detected in affected tissues	Skin, kidney [82]	Rheumatoid nodules [130] Synovial fluid [132]
Disease-specific antibodies bind NETs	Anti-dsDNA [114]	ACPA-rich IgGs [42,130] Purified ACPAs [133]
Disease-specific antibodies induce NETs	Anti-dsDNA [98]	ACPA-rich IgGs [130] Purified ACPAs [133]
NETs directly activate immune cells or key cells involved in disease pathogenicity	Immune cells [103,104,106–108] Endothelial cells [105]	Immune cells [42,131,137] FLSs [130,137]
NETs in immune complexes activate immune cells	[106]	[139]

[1] ACPAs, anti-citrullinated protein antibodies; ds, double-stranded; FLSs, fibroblast-like synoviocytes; NETs, neutrophil extracellular traps.

Defects in NET/chromatin clearance mechanisms might participate in RA pathogenesis, although this area of investigation is less developed than in SLE. Actually, sera from RA patients display a lower capacity to degrade NETs in vitro than sera from healthy individuals [144]. Likewise, the capacity of RA sera to degrade DNA in vitro is impaired in comparison to healthy individuals, especially in RA patients with high disease activity [145]. According to the literature, serum DNase1 may at least partly contribute to NET degradation. Although DNase1-like 3 was shown to be unaffected in RA patients [146], the missense variant rs35677470 at the DNase1-like 3 locus is potentially associated with the development of RA [147]. Other clearance mechanisms might be involved and defective in RA.

According to the above data, accumulation of NETs and/or cell-free chromatin may be deleterious in RA as a source of DAMPs and autoantigens. As mentioned, NET formation is increased in RA PMNs. More recently, several studies have reported that cell-free chromatin or NET-derived products are found at higher concentrations in the circulation of RA patients. Indeed, ELISAs have been developed to measure extracellular MPO-DNA complexes or neutrophil elastase-DNA complexes as well as cell-free nucleosomes. Although they might be indicative in some approaches, those ELISAs are only surrogate markers of NET formation.

5. Conclusions

Many data support a key role for PMNs in RA and SLE pathogenesis. Many of them suggest that PMNs are involved through the formation of NETs while others highlight PMN dysfunctions. Several pathogenic mechanisms are potentially common to RA and SLE, but they are probably finely regulated in response to stimuli which are more disease specific. The next step will be to determine the nature of the disease-specific stimuli triggering PMN activation and NET formation in vivo. Another key step will consist in determining the precise PMN response in each disease, the mechanisms and pathways governing NET activities, and to which extent NET composition varies in different pathologies.

Indeed, NET composition likely affects their activities. Using PMNs prepared from healthy individuals and activated in vitro, it has been shown that the composition of NETs depends on the stimulus [55,56]. Most importantly, NET composition also varies depending on the disease [55]. One study reported a NET composition specific to PMNs isolated from SLE patients with nephritis [109]. The origin of NETs and/or chromatin (PMN sub-populations, tissue or circulating PMNs) might also contribute to different activities.

Other cell types (eosinophils [148], basophils [149], mast cells [150], monocytes [151] and lymphocytes [152]) have been reported to release extracellular traps or DNA and, therefore, their contribution to the mechanisms described above should be analyzed in RA and SLE.

In conclusion, PMNs (and NETs) can be both pro- and anti-inflammatory and this probably partly depends on the stimuli triggering PMN activation/NET formation and

the microenvironment. Uncontrolled PMN activation, excessive NET formation, and/or impaired NET clearance may become pathogenic. As a consequence, NETs may behave both as an adjuvant and as a source of DAMPs and autoantigens, influencing both innate and adaptive immunity. The complex NET composition and the repertoire of antibodies recognizing NETs suggest a broad family of anti-NET antibodies may exist, as described for the anti-nucleosome antibody family in SLE patients.

Author Contributions: Writing—original draft preparation, A.H.A., D.M. and P.D.; writing—review and editing, P.D.; visualization, P.D.; supervision, P.D.; project administration, P.D.; funding acquisition, P.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by INSERM and the University Sorbonne Paris Nord.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge Natacha Bessis (University Sorbonne Paris Nord, Inserm UMR 1125, Bobigny, France) for critical reading of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the writing of the manuscript.

References

1. Pillay, J.; Den Braber, I.; Vrisekoop, N.; Kwast, L.M.; De Boer, R.J.; Borghans, J.A.; Tesselaar, K.; Koenderman, L. In vivo labeling with $2H_2O$ reveals a human neutrophil lifespan of 5.4 days. *Blood* **2010**, *116*, 625–627. [CrossRef]
2. Dixon, G.; Elks, P.M.; Loynes, C.A.; Whyte, M.K.; Renshaw, S.A. A method for the in vivo measurement of zebrafish tissue neutrophil lifespan. *ISRN Hematol.* **2012**, *2012*, 915868. [CrossRef]
3. Brinkmann, V.; Reichard, U.; Goosmann, C.; Fauler, B.; Uhlemann, Y.; Weiss, D.S.; Weinrauch, Y.; Zychlinsky, A. Neutrophil extracellular traps kill bacteria. *Science* **2004**, *303*, 1532–1535. [CrossRef]
4. Skubitz, K.; Micklem, K.; van der Schoot, E. CD66 and CD67 cluster workshop report. In *Leukocyte Typing V*; Schlossmann, S.F., Boumsell, L., Gilks, W., Harlan, J., Kishimoto, T., Morimoto, C., Ritz, J., Springer, T.A., Tedder, T.F., Todd, R.F., Eds.; Oxford University Press: Oxford, UK, 1995; Volume 1, pp. 889–899.
5. Bao, Y.; Cao, X. Revisiting the protective and pathogenic roles of neutrophils: Ly-6G is key! *Eur. J. Immunol.* **2011**, *41*, 2535–2538. [CrossRef]
6. Buckley, C.D.; Ross, E.A.; McGettrick, H.M.; Osborne, C.E.; Haworth, O.; Schmutz, C.; Stone, P.C.; Salmon, M.; Matharu, N.M.; Vohra, R.K.; et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J. Leukoc. Biol.* **2006**, *79*, 303–311. [CrossRef]
7. Pillay, J.; Kamp, V.M.; van Hoffen, E.; Visser, T.; Tak, T.; Lammers, J.W.; Ulfman, L.H.; Leenen, L.P.; Pickkers, P.; Koenderman, L. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J. Clin. Investig.* **2012**, *122*, 327–336. [CrossRef]
8. Massena, S.; Christoffersson, G.; Vagesjo, E.; Seignez, C.; Gustafsson, K.; Binet, F.; Herrera, H.C.; Giraud, A.; Lomei, J.; Westrom, S.; et al. Identification and characterization of VEGF-A-responsive neutrophils expressing CD49d, VEGFR1, and CXCR4 in mice and humans. *Blood* **2015**, *126*, 2016–2026. [CrossRef]
9. Hacbarth, E.; Kajdacsy-Balla, A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. *Arthritis Rheum.* **1986**, *29*, 1334–1342. [CrossRef]
10. Denny, M.F.; Yalavarthi, S.; Zhao, W.; Thacker, S.G.; Anderson, M.; Sandy, A.R.; McCune, W.J.; Kaplan, M.J. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J. Immunol.* **2010**, *184*, 3284–3297. [CrossRef]
11. Lecot, P.; Sarabi, M.; Pereira, A.M.; Mussard, J.; Koenderman, L.; Caux, C.; Bendriss-Vermare, N.; Michallet, M.C. Neutrophil Heterogeneity in Cancer: From Biology to Therapies. *Front. Immunol.* **2019**, *10*, 2155. [CrossRef]
12. Fridlender, Z.G.; Sun, J.; Kim, S.; Kapoor, V.; Cheng, G.; Ling, L.; Worthen, G.S.; Albelda, S.M. Polarization of tumor-associated neutrophil phenotype by TGF-β: "N1" versus "N2" TAN. *Cancer Cell* **2009**, *16*, 183–194. [CrossRef]
13. Sherer, Y.; Gorstein, A.; Fritzler, M.J.; Shoenfeld, Y. Autoantibody explosion in systemic lupus erythematosus: More than 100 different antibodies found in SLE patients. *Semin. Arthritis Rheum.* **2004**, *34*, 501–537. [CrossRef]
14. Rumore, P.M.; Steinman, C.R. Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. *J. Clin. Investig.* **1990**, *86*, 69–74. [CrossRef]

15. Amoura, Z.; Piette, J.C.; Chabre, H.; Cacoub, P.; Papo, T.; Wechsler, B.; Bach, J.F.; Koutouzov, S. Circulating plasma levels of nucleosomes in patients with systemic lupus erythematosus: Correlation with serum antinucleosome antibody titers and absence of clear association with disease activity. *Arthritis Rheum.* **1997**, *40*, 2217–2225. [CrossRef]
16. Williams, R.C., Jr.; Malone, C.C.; Meyers, C.; Decker, P.; Muller, S. Detection of nucleosome particles in serum and plasma from patients with systemic lupus erythematosus using monoclonal antibody 4H7. *J. Rheumatol.* **2001**, *28*, 81–94.
17. Amoura, Z.; Koutouzov, S.; Chabre, H.; Cacoub, P.; Amoura, I.; Musset, L.; Bach, J.F.; Piette, J.C. Presence of antinucleosome autoantibodies in a restricted set of connective tissue diseases: Antinucleosome antibodies of the IgG3 subclass are markers of renal pathogenicity in systemic lupus erythematosus. *Arthritis Rheum.* **2000**, *43*, 76–84. [CrossRef]
18. Lu, L.; Kaliyaperumal, A.; Boumpas, D.T.; Datta, S.K. Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *J. Clin. Investig.* **1999**, *104*, 345–355. [CrossRef]
19. Schellekens, G.A.; de Jong, B.A.; van den Hoogen, F.H.; van de Putte, L.B.; van Venrooij, W.J. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Investig.* **1998**, *101*, 273–281. [CrossRef]
20. Girbal-Neuhauser, E.; Durieux, J.J.; Arnaud, M.; Dalbon, P.; Sebbag, M.; Vincent, C.; Simon, M.; Senshu, T.; Masson-Bessiere, C.; Jolivet-Reynaud, C.; et al. The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues. *J. Immunol.* **1999**, *162*, 585–594.
21. Schellekens, G.A.; Visser, H.; de Jong, B.A.; van den Hoogen, F.H.; Hazes, J.M.; Breedveld, F.C.; van Venrooij, W.J. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.* **2000**, *43*, 155–163. [CrossRef]
22. Harre, U.; Georgess, D.; Bang, H.; Bozec, A.; Axmann, R.; Ossipova, E.; Jakobsson, P.J.; Baum, W.; Nimmerjahn, F.; Szarka, E.; et al. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. *J. Clin. Investig.* **2012**, *122*, 1791–1802. [CrossRef]
23. Sharma, S.; Davis, R.E.; Srivastva, S.; Nylen, S.; Sundar, S.; Wilson, M.E. A Subset of Neutrophils Expressing Markers of Antigen-Presenting Cells in Human Visceral Leishmaniasis. *J. Infect. Dis.* **2016**, *214*, 1531–1538. [CrossRef]
24. Vono, M.; Lin, A.; Norrby-Teglund, A.; Koup, R.A.; Liang, F.; Lore, K. Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. *Blood* **2017**, *129*, 1991–2001. [CrossRef]
25. Beauvillain, C.; Delneste, Y.; Scotet, M.; Peres, A.; Gascan, H.; Guermonprez, P.; Barnaba, V.; Jeannin, P. Neutrophils efficiently cross-prime naive T cells in vivo. *Blood* **2007**, *110*, 2965–2973. [CrossRef]
26. Davey, M.S.; Morgan, M.P.; Liuzzi, A.R.; Tyler, C.J.; Khan, M.W.A.; Szakmany, T.; Hall, J.E.; Moser, B.; Eberl, M. Microbe-specific unconventional T cells induce human neutrophil differentiation into antigen cross-presenting cells. *J. Immunol.* **2014**, *193*, 3704–3716. [CrossRef]
27. Lindau, D.; Mussard, J.; Wagner, B.J.; Ribon, M.; Ronnefarth, V.M.; Quettier, M.; Jelcic, I.; Boissier, M.C.; Rammensee, H.G.; Decker, P. Primary blood neutrophils express a functional cell surface Toll-like receptor 9. *Eur. J. Immunol.* **2013**, *43*, 2101–2113. [CrossRef]
28. Lindau, D.; Mussard, J.; Rabsteyn, A.; Ribon, M.; Kotter, I.; Igney, A.; Adema, G.J.; Boissier, M.C.; Rammensee, H.G.; Decker, P. TLR9 independent interferon alpha production by neutrophils on NETosis in response to circulating chromatin, a key lupus autoantigen. *Ann. Rheum. Dis.* **2014**, *73*, 2199–2207. [CrossRef]
29. Puellmann, K.; Kaminski, W.E.; Vogel, M.; Nebe, C.T.; Schroeder, J.; Wolf, H.; Beham, A.W. A variable immunoreceptor in a subpopulation of human neutrophils. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 14441–14446. [CrossRef]
30. Costantini, C.; Calzetti, F.; Perbellini, O.; Micheletti, A.; Scarponi, C.; Lonardi, S.; Pelletier, M.; Schakel, K.; Pizzolo, G.; Facchetti, F.; et al. Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NK-derived IFNγ: Role of CD18, ICAM-1, and ICAM-3. *Blood* **2011**, *117*, 1677–1686. [CrossRef]
31. Pelletier, M.; Maggi, L.; Micheletti, A.; Lazzeri, E.; Tamassia, N.; Costantini, C.; Cosmi, L.; Lunardi, C.; Annunziato, F.; Romagnani, S.; et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood* **2010**, *115*, 335–343. [CrossRef]
32. Puga, I.; Cols, M.; Barra, C.M.; He, B.; Cassis, L.; Gentile, M.; Comerma, L.; Chorny, A.; Shan, M.; Xu, W.; et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat. Immunol.* **2012**, *13*, 170–180. [CrossRef]
33. Sadatomo, A.; Inoue, Y.; Ito, H.; Karasawa, T.; Kimura, H.; Watanabe, S.; Mizushina, Y.; Nakamura, J.; Kamata, R.; Kasahara, T.; et al. Interaction of Neutrophils with Macrophages Promotes IL-1β Maturation and Contributes to Hepatic Ischemia-Reperfusion Injury. *J. Immunol.* **2017**, *199*, 3306–3315. [CrossRef]
34. Wantha, S.; Alard, J.E.; Megens, R.T.; van der Does, A.M.; Doring, Y.; Drechsler, M.; Pham, C.T.; Wang, M.W.; Wang, J.M.; Gallo, R.L.; et al. Neutrophil-derived cathelicidin promotes adhesion of classical monocytes. *Circ. Res.* **2013**, *112*, 792–801. [CrossRef]
35. Batignes, M.; Santinon, F.; Boissier, M.C.; Decker, P.; Bessis, N. Neutrophils and Regulatory T Lymphocytes (Treg) Cooperate to Sustain Treg Activity but This Interaction Is Altered in Rheumatoid Arthritis Patients [abstract]. *Arthritis Rheumatol.* **2019**, *71*, 163–164.
36. Piskin, G.; Bos, J.D.; Teunissen, M.B. Neutrophils infiltrating ultraviolet B-irradiated normal human skin display high IL-10 expression. *Arch. Dermatol. Res.* **2005**, *296*, 339–342. [CrossRef] [PubMed]
37. Ribon, M.; Mussard, J.; Semerano, L.; Singer, B.B.; Decker, P. Extracellular Chromatin Triggers Release of Soluble CEACAM8 Upon Activation of Neutrophils. *Front. Immunol.* **2019**, *10*, 1346. [CrossRef] [PubMed]

38. Kamp, V.M.; Pillay, J.; Lammers, J.W.; Pickkers, P.; Ulfman, L.H.; Koenderman, L. Human suppressive neutrophils CD16bright/CD62Ldim exhibit decreased adhesion. *J. Leukoc. Biol.* **2012**, *92*, 1011–1020. [CrossRef] [PubMed]
39. Marini, O.; Costa, S.; Bevilacqua, D.; Calzetti, F.; Tamassia, N.; Spina, C.; De Sabata, D.; Tinazzi, E.; Lunardi, C.; Scupoli, M.T.; et al. Mature $CD10^{+}$ and immature $CD10^{-}$ neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood* **2017**, *129*, 1343–1356. [CrossRef]
40. Wingender, G.; Hiss, M.; Engel, I.; Peukert, K.; Ley, K.; Haller, H.; Kronenberg, M.; von Vietinghoff, S. Neutrophilic granulocytes modulate invariant NKT cell function in mice and humans. *J. Immunol.* **2012**, *188*, 3000–3008. [CrossRef]
41. Gresnigt, M.S.; Joosten, L.A.; Verschueren, I.; van der Meer, J.W.; Netea, M.G.; Dinarello, C.A.; van de Veerdonk, F.L. Neutrophil-mediated inhibition of proinflammatory cytokine responses. *J. Immunol.* **2012**, *189*, 4806–4815. [CrossRef]
42. Ribon, M.; Seninet, S.; Mussard, J.; Sebbag, M.; Clavel, C.; Serre, G.; Boissier, M.C.; Semerano, L.; Decker, P. Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37. *J. Autoimmun.* **2019**, *98*, 122–131. [CrossRef]
43. Barrientos, L.; Bignon, A.; Gueguen, C.; de Chaisemartin, L.; Gorges, R.; Sandre, C.; Mascarell, L.; Balabanian, K.; Kerdine-Romer, S.; Pallardy, M.; et al. Neutrophil extracellular traps downregulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells. *J. Immunol.* **2014**, *193*, 5689–5698. [CrossRef]
44. Schauer, C.; Janko, C.; Munoz, L.E.; Zhao, Y.; Kienhofer, D.; Frey, B.; Lell, M.; Manger, B.; Rech, J.; Naschberger, E.; et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat. Med.* **2014**, *20*, 511–517. [CrossRef] [PubMed]
45. Tillack, K.; Breiden, P.; Martin, R.; Sospedra, M. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J. Immunol.* **2012**, *188*, 3150–3159. [CrossRef]
46. Gestermann, N.; Di Domizio, J.; Lande, R.; Demaria, O.; Frasca, L.; Feldmeyer, L.; Di Lucca, J.; Gilliet, M. Netting Neutrophils Activate Autoreactive B Cells in Lupus. *J. Immunol.* **2018**, *200*, 3364–3371. [CrossRef] [PubMed]
47. Dotan, A.; Muller, S.; Kanduc, D.; David, P.; Halpert, G.; Shoenfeld, Y. The SARS-CoV-2 as an instrumental trigger of autoimmunity. *Autoimmun. Rev.* **2021**, *20*, 102792. [CrossRef]
48. Boeltz, S.; Amini, P.; Anders, H.J.; Andrade, F.; Bilyy, R.; Chatfield, S.; Cichon, I.; Clancy, D.M.; Desai, J.; Dumych, T.; et al. To NET or not to NET: Current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ.* **2019**, *26*, 395–408. [CrossRef]
49. Abi Abdallah, D.S.; Lin, C.; Ball, C.J.; King, M.R.; Duhamel, G.E.; Denkers, E.Y. Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. *Infect. Immun.* **2012**, *80*, 768–777. [CrossRef]
50. Urban, C.F.; Reichard, U.; Brinkmann, V.; Zychlinsky, A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol.* **2006**, *8*, 668–676. [CrossRef]
51. Bianchi, M.; Hakkim, A.; Brinkmann, V.; Siler, U.; Seger, R.A.; Zychlinsky, A.; Reichenbach, J. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* **2009**, *114*, 2619–2622. [CrossRef]
52. Saitoh, T.; Komano, J.; Saitoh, Y.; Misawa, T.; Takahama, M.; Kozaki, T.; Uehata, T.; Iwasaki, H.; Omori, H.; Yamaoka, S.; et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* **2012**, *12*, 109–116. [CrossRef]
53. Branzk, N.; Lubojemska, A.; Hardison, S.E.; Wang, Q.; Gutierrez, M.G.; Brown, G.D.; Papayannopoulos, V. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* **2014**, *15*, 1017–1025. [CrossRef]
54. Urban, C.F.; Ermert, D.; Schmid, M.; Abu-Abed, U.; Goosmann, C.; Nacken, W.; Brinkmann, V.; Jungblut, P.R.; Zychlinsky, A. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog.* **2009**, *5*, e1000639. [CrossRef]
55. Chapman, E.A.; Lyon, M.; Simpson, D.; Mason, D.; Beynon, R.J.; Moots, R.J.; Wright, H.L. Caught in a Trap? Proteomic Analysis of Neutrophil Extracellular Traps in Rheumatoid Arthritis and Systemic Lupus Erythematosus. *Front. Immunol.* **2019**, *10*, 423. [CrossRef]
56. Petretto, A.; Bruschi, M.; Pratesi, F.; Croia, C.; Candiano, G.; Ghiggeri, G.; Migliorini, P. Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. *PLoS ONE* **2019**, *14*, e0218946.
57. Lauth, X.; Kockritz-Blickwede, M.; McNamara, C.W.; Myskowski, S.; Zinkernagel, A.S.; Beall, B.; Ghosh, P.; Gallo, R.L.; Nizet, V. M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. *J. Innate Immun.* **2009**, *1*, 202–214. [CrossRef] [PubMed]
58. Halverson, T.W.; Wilton, M.; Poon, K.K.; Petri, B.; Lewenza, S. DNA is an antimicrobial component of neutrophil extracellular traps. *PLoS Pathog.* **2015**, *11*, e1004593. [CrossRef]
59. Derré-Bobillot, A.; Cortes-Perez, N.G.; Yamamoto, Y.; Kharrat, P.; Couvé, E.; Da Cunha, V.; Decker, P.; Boissier, M.C.; Escartin, F.; Cesselin, B.; et al. Nuclease A (Gbs0661), an extracellular nuclease of Streptococcus agalactiae, attacks the neutrophil extracellular traps and is needed for full virulence. *Mol. Microbiol.* **2013**, *89*, 518–531. [CrossRef]
60. Neumann, A.; Vollger, L.; Berends, E.T.; Molhoek, E.M.; Stapels, D.A.; Midon, M.; Friaes, A.; Pingoud, A.; Rooijakkers, S.H.; Gallo, R.L.; et al. Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial nucleases. *J. Innate Immun.* **2014**, *6*, 860–868. [CrossRef] [PubMed]

61. Thanabalasuriar, A.; Scott, B.N.V.; Peiseler, M.; Willson, M.E.; Zeng, Z.; Warrener, P.; Keller, A.E.; Surewaard, B.G.J.; Dozier, E.A.; Korhonen, J.T.; et al. Neutrophil Extracellular Traps Confine Pseudomonas aeruginosa Ocular Biofilms and Restrict Brain Invasion. *Cell Host Microbe* **2019**, *25*, 526–536. [CrossRef] [PubMed]
62. Fuchs, T.A.; Abed, U.; Goosmann, C.; Hurwitz, R.; Schulze, I.; Wahn, V.; Weinrauch, Y.; Brinkmann, V.; Zychlinsky, A. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **2007**, *176*, 231–241. [CrossRef]
63. Parker, H.; Dragunow, M.; Hampton, M.B.; Kettle, A.J.; Winterbourn, C.C. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J. Leukoc. Biol.* **2012**, *92*, 841–849. [CrossRef] [PubMed]
64. Kenny, E.F.; Herzig, A.; Kruger, R.; Muth, A.; Mondal, S.; Thompson, P.R.; Brinkmann, V.; Bernuth, H.V.; Zychlinsky, A. Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife* **2017**, *6*, e24437. [CrossRef]
65. DeSouza-Vieira, T.; Guimaraes-Costa, A.; Rochael, N.C.; Lira, M.N.; Nascimento, M.T.; Lima-Gomez, P.S.; Mariante, R.M.; Persechini, P.M.; Saraiva, E.M. Neutrophil extracellular traps release induced by Leishmania: Role of PI3Kgamma, ERK, PI3Ksigma, PKC, and [Ca^{2+}]. *J. Leukoc. Biol.* **2016**, *100*, 801–810. [CrossRef]
66. Kenno, S.; Perito, S.; Mosci, P.; Vecchiarelli, A.; Monari, C. Autophagy and Reactive Oxygen Species Are Involved in Neutrophil Extracellular Traps Release Induced by C. albicans Morphotypes. *Front. Microbiol.* **2016**, *7*, 879. [CrossRef] [PubMed]
67. Metzler, K.D.; Fuchs, T.A.; Nauseef, W.M.; Reumaux, D.; Roesler, J.; Schulze, I.; Wahn, V.; Papayannopoulos, V.; Zychlinsky, A. Myeloperoxidase is required for neutrophil extracellular trap formation: Implications for innate immunity. *Blood* **2011**, *117*, 953–959. [CrossRef]
68. Behnen, M.; Leschczyk, C.; Moller, S.; Batel, T.; Klinger, M.; Solbach, W.; Laskay, T. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. *J. Immunol.* **2014**, *193*, 1954–1965. [CrossRef]
69. Caudrillier, A.; Kessenbrock, K.; Gilliss, B.M.; Nguyen, J.X.; Marques, M.B.; Monestier, M.; Toy, P.; Werb, Z.; Looney, M.R. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J. Clin. Investig.* **2012**, *122*, 2661–2671. [CrossRef]
70. Yousefi, S.; Mihalache, C.; Kozlowski, E.; Schmid, I.; Simon, H.U. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* **2009**, *16*, 1438–1444. [CrossRef]
71. Amini, P.; Stojkov, D.; Wang, X.; Wicki, S.; Kaufmann, T.; Wong, W.W.; Simon, H.U.; Yousefi, S. NET formation can occur independently of RIPK3 and MLKL signaling. *Eur. J. Immunol.* **2016**, *46*, 178–184. [CrossRef]
72. Amini, P.; Stojkov, D.; Felser, A.; Jackson, C.B.; Courage, C.; Schaller, A.; Gelman, L.; Soriano, M.E.; Nuoffer, J.M.; Scorrano, L.; et al. Neutrophil extracellular trap formation requires OPA1-dependent glycolytic ATP production. *Nat. Commun.* **2018**, *9*, 2958. [CrossRef]
73. Pilsczek, F.H.; Salina, D.; Poon, K.K.; Fahey, C.; Yipp, B.G.; Sibley, C.D.; Robbins, S.M.; Green, F.H.; Surette, M.G.; Sugai, M.; et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J. Immunol.* **2010**, *185*, 7413–7425. [CrossRef] [PubMed]
74. Yipp, B.G.; Petri, B.; Salina, D.; Jenne, C.N.; Scott, B.N.; Zbytnuik, L.D.; Pittman, K.; Asaduzzaman, M.; Wu, K.; Meijndert, H.C.; et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat. Med.* **2012**, *18*, 1386–1393. [CrossRef]
75. Hakkim, A.; Furnrohr, B.G.; Amann, K.; Laube, B.; Abed, U.A.; Brinkmann, V.; Herrmann, M.; Voll, R.E.; Zychlinsky, A. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 9813–9818. [CrossRef]
76. Lazzaretto, B.; Fadeel, B. Intra- and Extracellular Degradation of Neutrophil Extracellular Traps by Macrophages and Dendritic Cells. *J. Immunol.* **2019**, *203*, 2276–2290. [CrossRef]
77. Farrera, C.; Fadeel, B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J. Immunol.* **2013**, *191*, 2647–2656. [CrossRef]
78. Padrines, M.; Wolf, M.; Walz, A.; Baggiolini, M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett.* **1994**, *352*, 231–235. [CrossRef]
79. Molad, Y.; Buyon, J.; Anderson, D.C.; Abramson, S.B.; Cronstein, B.N. Intravascular neutrophil activation in systemic lupus erythematosus (SLE): Dissociation between increased expression of CD11b/CD18 and diminished expression of L-selectin on neutrophils from patients with active SLE. *Clin. Immunol. Immunopathol.* **1994**, *71*, 281–286. [CrossRef]
80. Wright, H.L.; Chikura, B.; Bucknall, R.C.; Moots, R.J.; Edwards, S.W. Changes in expression of membrane TNF, NF-{kappa}B activation and neutrophil apoptosis during active and resolved inflammation. *Ann. Rheum. Dis.* **2011**, *70*, 537–543. [CrossRef]
81. Wright, H.L.; Moots, R.J.; Edwards, S.W. The multifactorial role of neutrophils in rheumatoid arthritis. *Nat. Rev. Rheumatol.* **2014**, *10*, 593–601. [CrossRef]
82. Villanueva, E.; Yalavarthi, S.; Berthier, C.C.; Hodgin, J.B.; Khandpur, R.; Lin, A.M.; Rubin, C.J.; Zhao, W.; Olsen, S.H.; Klinker, M.; et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J. Immunol.* **2011**, *187*, 538–552. [CrossRef]
83. Yu, D.; Rumore, P.M.; Liu, Q.; Steinman, C.R. Soluble oligonucleosomal complexes in synovial fluid from inflamed joints. *Arthritis Rheum.* **1997**, *40*, 648–654. [CrossRef]
84. Mohr, W.; Pelster, B.; Wessinghage, D. Polymorphonuclear granulocytes in rheumatic tissue destruction. VI. The occurrence of PMNs in menisci of patients with rheumatoid arthritis. *Rheumatol. Int.* **1984**, *5*, 39–44. [CrossRef]

85. De Bont, C.M.; Stokman, M.E.M.; Faas, P.; Thurlings, R.M.; Boelens, W.C.; Wright, H.L.; Pruijn, G.J.M. Autoantibodies to neutrophil extracellular traps represent a potential serological biomarker in rheumatoid arthritis. *J. Autoimmun.* **2020**, *113*, 102484. [CrossRef] [PubMed]
86. Zhou, Y.; Wu, J.; Kucik, D.F.; White, N.B.; Redden, D.T.; Szalai, A.J.; Bullard, D.C.; Edberg, J.C. Multiple lupus-associated ITGAM variants alter Mac-1 functions on neutrophils. *Arthritis Rheum.* **2013**, *65*, 2907–2916. [CrossRef]
87. Lood, C.; Arve, S.; Ledbetter, J.; Elkon, K.B. TLR7/8 activation in neutrophils impairs immune complex phagocytosis through shedding of FcgRIIA. *J. Exp. Med.* **2017**, *214*, 2103–2119. [CrossRef] [PubMed]
88. Luo, Q.; Huang, Z.; Ye, J.; Deng, Y.; Fang, L.; Li, X.; Guo, Y.; Jiang, H.; Ju, B.; Huang, Q.; et al. PD-L1-expressing neutrophils as a novel indicator to assess disease activity and severity of systemic lupus erythematosus. *Arthritis Res. Ther.* **2016**, *18*, 47. [CrossRef] [PubMed]
89. Lopez, P.; Rodriguez-Carrio, J.; Caminal-Montero, L.; Mozo, L.; Suarez, A. A pathogenic IFNalpha, BLyS and IL-17 axis in Systemic Lupus Erythematosus patients. *Sci. Rep.* **2016**, *6*, 20651. [CrossRef]
90. Caielli, S.; Athale, S.; Domic, B.; Murat, E.; Chandra, M.; Banchereau, R.; Baisch, J.; Phelps, K.; Clayton, S.; Gong, M.; et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J. Exp. Med.* **2016**, *213*, 697–713. [CrossRef]
91. Rahman, S.; Sagar, D.; Hanna, R.N.; Lightfoot, Y.L.; Mistry, P.; Smith, C.K.; Manna, Z.; Hasni, S.; Siegel, R.M.; Sanjuan, M.A.; et al. Low-density granulocytes activate T cells and demonstrate a non-suppressive role in systemic lupus erythematosus. *Ann. Rheum. Dis.* **2019**, *78*, 957–966. [CrossRef]
92. Van Bruggen, M.C.; Kramers, C.; Walgreen, B.; Elema, J.D.; Kallenberg, C.G.; van den Born, J.; Smeenk, R.J.; Assmann, K.J.; Muller, S.; Monestier, M.; et al. Nucleosomes and histones are present in glomerular deposits in human lupus nephritis. *Nephrol. Dial. Transplant.* **1997**, *12*, 57–66. [CrossRef]
93. Rönnefarth, V.M.; Erbacher, A.I.; Lamkemeyer, T.; Madlung, J.; Nordheim, A.; Rammensee, H.G.; Decker, P. TLR2/TLR4-independent neutrophil activation and recruitment upon endocytosis of nucleosomes reveals a new pathway of innate immunity in systemic lupus erythematosus. *J. Immunol.* **2006**, *177*, 7740–7749. [CrossRef]
94. Lindau, D.; Rönnefarth, V.; Erbacher, A.; Rammensee, H.G.; Decker, P. Nucleosome-induced neutrophil activation occurs independently of TLR9 and endosomal acidification: Implications for systemic lupus erythematosus. *Eur. J. Immunol.* **2011**, *41*, 669–681. [CrossRef]
95. Palanichamy, A.; Bauer, J.W.; Yalavarthi, S.; Meednu, N.; Barnard, J.; Owen, T.; Cistrone, C.; Bird, A.; Rabinovich, A.; Nevarez, S.; et al. Neutrophil-mediated IFN activation in the bone marrow alters B cell development in human and murine systemic lupus erythematosus. *J. Immunol.* **2014**, *192*, 906–918. [CrossRef] [PubMed]
96. Hervier, B.; Ribon, M.; Tarantino, N.; Mussard, J.; Breckler, M.; Vieillard, V.; Amoura, Z.; Steinle, A.; Klein, R.; Kotter, I.; et al. Increased Concentrations of Circulating Soluble MHC Class I-Related Chain A (sMICA) and sMICB and Modulation of Plasma Membrane MICA Expression: Potential Mechanisms and Correlation With Natural Killer Cell Activity in Systemic Lupus Erythematosus. *Front. Immunol.* **2021**, *12*, 633658. [CrossRef] [PubMed]
97. Means, T.K.; Latz, E.; Hayashi, F.; Murali, M.R.; Golenbock, D.T.; Luster, A.D. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J. Clin. Investig.* **2005**, *115*, 407–417. [CrossRef]
98. Patino-Trives, A.M.; Perez-Sanchez, C.; Perez-Sanchez, L.; Luque-Tevar, M.; Abalos-Aguilera, M.C.; Alcaide-Ruggiero, L.; Arias-de la Rosa, I.; Roman-Rodriguez, C.; Segui, P.; Espinosa, M.; et al. Anti-dsDNA Antibodies Increase the Cardiovascular Risk in Systemic Lupus Erythematosus Promoting a Distinctive Immune and Vascular Activation. *Arterioscler. Thromb. Vasc. Biol.* **2021**, *41*, 2417–2430. [CrossRef]
99. Chitrabamrung, S.; Rubin, R.L.; Tan, E.M. Serum deoxyribonuclease I and clinical activity in systemic lupus erythematosus. *Rheumatol. Int.* **1981**, *1*, 55–60. [CrossRef]
100. Al Mayouf, S.M.; Sunker, A.; Abdwani, R.; Abrawi, S.A.; Almurshedi, F.; Alhashmi, N.; Al Sonbul, A.; Sewairi, W.; Qari, A.; Abdallah, E.; et al. Loss-of-function variant in DNASE1L3 causes a familial form of systemic lupus erythematosus. *Nat. Genet.* **2011**, *43*, 1186–1188. [CrossRef]
101. Bijl, M.; Reefman, E.; Horst, G.; Limburg, P.C.; Kallenberg, C.G. Reduced uptake of apoptotic cells by macrophages in Systemic Lupus Erythematosus (SLE): Correlates with decreased serum levels of complement. *Ann. Rheum. Dis.* **2006**, *65*, 57–63. [CrossRef]
102. Leffler, J.; Martin, M.; Gullstrand, B.; Tyden, H.; Lood, C.; Truedsson, L.; Bengtsson, A.A.; Blom, A.M. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J. Immunol.* **2012**, *188*, 3522–3531. [CrossRef]
103. Kahlenberg, J.M.; Carmona-Rivera, C.; Smith, C.K.; Kaplan, M.J. Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages. *J. Immunol.* **2013**, *190*, 1217–1226. [CrossRef]
104. Barrera-Vargas, A.; Gomez-Martin, D.; Carmona-Rivera, C.; Merayo-Chalico, J.; Torres-Ruiz, J.; Manna, Z.; Hasni, S.; Alcocer-Varela, J.; Kaplan, M.J. Differential ubiquitination in NETs regulates macrophage responses in systemic lupus erythematosus. *Ann. Rheum. Dis.* **2018**, *77*, 944–950. [CrossRef]
105. Carmona-Rivera, C.; Zhao, W.; Yalavarthi, S.; Kaplan, M.J. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. *Ann. Rheum. Dis.* **2015**, *74*, 1417–1424. [CrossRef]

106. Lande, R.; Ganguly, D.; Facchinetti, V.; Frasca, L.; Conrad, C.; Gregorio, J.; Meller, S.; Chamilos, G.; Sebasigari, R.; Riccieri, V.; et al. Neutrophils Activate Plasmacytoid Dendritic Cells by Releasing Self-DNA-Peptide Complexes in Systemic Lupus Erythematosus. *Sci. Transl. Med.* **2011**, *3*, 73ra19. [CrossRef]
107. Lood, C.; Blanco, L.P.; Purmalek, M.M.; Carmona-Rivera, C.; De Ravin, S.S.; Smith, C.K.; Malech, H.L.; Ledbetter, J.A.; Elkon, K.B.; Kaplan, M.J. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat. Med.* **2016**, *22*, 146–153. [CrossRef] [PubMed]
108. Garcia-Romo, G.S.; Caielli, S.; Vega, B.; Connolly, J.; Allantaz, F.; Xu, Z.; Punaro, M.; Baisch, J.; Guiducci, C.; Coffman, R.L.; et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci. Transl. Med.* **2011**, *3*, 73ra20. [CrossRef]
109. Bruschi, M.; Petretto, A.; Santucci, L.; Vaglio, A.; Pratesi, F.; Migliorini, P.; Bertelli, R.; Lavarello, C.; Bartolucci, M.; Candiano, G.; et al. Neutrophil Extracellular Traps protein composition is specific for patients with Lupus nephritis and includes methyl-oxidized alphaenolase (methionine sulfoxide 93). *Sci. Rep.* **2019**, *9*, 7934. [CrossRef]
110. Frangou, E.; Chrysanthopoulou, A.; Mitsios, A.; Kambas, K.; Arelaki, S.; Angelidou, I.; Arampatzioglou, A.; Gakiopoulou, H.; Bertsias, G.K.; Verginis, P.; et al. REDD1/autophagy pathway promotes thromboinflammation and fibrosis in human systemic lupus erythematosus (SLE) through NETs decorated with tissue factor (TF) and interleukin-17A (IL-17A). *Ann. Rheum. Dis.* **2019**, *78*, 238–248. [CrossRef]
111. Georgakis, S.; Gkirtzimanaki, K.; Papadaki, G.; Gakiopoulou, H.; Drakos, E.; Eloranta, M.L.; Makridakis, M.; Kontostathi, G.; Zoidakis, J.; Baira, E.; et al. NETs decorated with bioactive IL-33 infiltrate inflamed tissues and induce IFNalpha production in SLE patients. *JCI Insight* **2021**, *6*, e147671.
112. Pieterse, E.; Hofstra, J.; Berden, J.; Herrmann, M.; Dieker, J.; Van Der Vlag, J. Acetylated histones contribute to the immunostimulatory potential of neutrophil extracellular traps in systemic lupus erythematosus. *Clin. Exp. Immunol.* **2015**, *179*, 68–74. [CrossRef] [PubMed]
113. Liu, C.L.; Tangsombatvisit, S.; Rosenberg, J.M.; Mandelbaum, G.; Gillespie, E.C.; Gozani, O.P.; Alizadeh, A.A.; Utz, P.J. Specific post-translational histone modifications of neutrophil extracellular traps as immunogens and potential targets of lupus autoantibodies. *Arthritis Res. Ther.* **2012**, *14*, R25. [CrossRef]
114. Lou, H.; Wojciak-Stothard, B.; Ruseva, M.M.; Cook, H.T.; Kelleher, P.; Pickering, M.C.; Mongkolsapaya, J.; Screaton, G.R.; Xu, X.N. Autoantibody-dependent amplification of inflammation in SLE. *Cell Death Dis.* **2020**, *11*, 729. [CrossRef] [PubMed]
115. Knight, J.S.; Kaplan, M.J. Lupus neutrophils: 'NET' gain in understanding lupus pathogenesis. *Curr. Opin. Rheumatol.* **2012**, *24*, 441–450. [CrossRef]
116. Knight, J.S.; Carmona-Rivera, C.; Kaplan, M.J. Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. *Front. Immunol.* **2012**, *3*, 380. [CrossRef]
117. Kessenbrock, K.; Krumbholz, M.; Schonermarck, U.; Back, W.; Gross, W.L.; Werb, Z.; Grone, H.J.; Brinkmann, V.; Jenne, D.E. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat. Med.* **2009**, *15*, 623–625. [CrossRef]
118. Rauova, L.; Gilburd, B.; Zurgil, N.; Blank, M.; Guegas, L.L.; Brickman, C.M.; Cebecauer, L.; Deutsch, M.; Wiik, A.; Shoenfeld, Y. Induction of biologically active antineutrophil cytoplasmic antibodies by immunization with human apoptotic polymorphonuclear leukocytes. *Clin. Immunol.* **2002**, *103*, 69–78. [CrossRef]
119. Mourao, A.F.; Canhao, H.; Sousa, E.; Cascao, R.; da Costa, J.B.; de Almeida, L.S.; Oliveira, M.E.; Gomes, M.M.; Queiroz, M.V.; Fonseca, J.E. From a neutrophilic synovial tissue infiltrate to a challenging case of rheumatoid arthritis. *Acta Reumatol. Port.* **2010**, *35*, 228–231. [PubMed]
120. Cross, A.; Bucknall, R.C.; Cassatella, M.A.; Edwards, S.W.; Moots, R.J. Synovial fluid neutrophils transcribe and express class II major histocompatibility complex molecules in rheumatoid arthritis. *Arthritis Rheum.* **2003**, *48*, 2796–2806. [CrossRef]
121. Iking-Konert, C.; Ostendorf, B.; Sander, O.; Jost, M.; Wagner, C.; Joosten, L.; Schneider, M.; Hansch, G.M. Transdifferentiation of polymorphonuclear neutrophils to dendritic-like cells at the site of inflammation in rheumatoid arthritis: Evidence for activation by T cells. *Ann. Rheum. Dis.* **2005**, *64*, 1436–1442. [CrossRef]
122. Cross, A.; Barnes, T.; Bucknall, R.C.; Edwards, S.W.; Moots, R.J. Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. *J. Leukoc. Biol.* **2006**, *80*, 521–528. [CrossRef]
123. Yeo, L.; Toellner, K.M.; Salmon, M.; Filer, A.; Buckley, C.D.; Raza, K.; Scheel-Toellner, D. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis. *Ann. Rheum. Dis.* **2011**, *70*, 2022–2028. [CrossRef]
124. Assi, L.K.; Wong, S.H.; Ludwig, A.; Raza, K.; Gordon, C.; Salmon, M.; Lord, J.M.; Scheel-Toellner, D. Tumor necrosis factor alpha activates release of B lymphocyte stimulator by neutrophils infiltrating the rheumatoid joint. *Arthritis Rheum.* **2007**, *56*, 1776–1786. [CrossRef]
125. Contis, A.; Mitrovic, S.; Lavie, J.; Douchet, I.; Lazaro, E.; Truchetet, M.E.; Goizet, C.; Contin-Bordes, C.; Schaeverbeke, T.; Blanco, P.; et al. Neutrophil-derived mitochondrial DNA promotes receptor activator of nuclear factor kappaB and its ligand signalling in rheumatoid arthritis. *Rheumatology* **2017**, *56*, 1200–1205. [CrossRef]
126. Poubelle, P.E.; Chakravarti, A.; Fernandes, M.J.; Doiron, K.; Marceau, A.A. Differential expression of RANK, RANK-L, and osteoprotegerin by synovial fluid neutrophils from patients with rheumatoid arthritis and by healthy human blood neutrophils. *Arthritis Res. Ther.* **2007**, *9*, R25. [CrossRef]

127. Wright, H.L.; Lyon, M.; Chapman, E.A.; Moots, R.J.; Edwards, S.W. Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps. *Front. Immunol.* **2020**, *11*, 584116. [CrossRef] [PubMed]
128. Wright, H.L.; Cox, T.; Moots, R.J.; Edwards, S.W. Neutrophil biomarkers predict response to therapy with tumor necrosis factor inhibitors in rheumatoid arthritis. *J. Leukoc. Biol.* **2017**, *101*, 785–795. [CrossRef]
129. Wright, H.L.; Makki, F.A.; Moots, R.J.; Edwards, S.W. Low-density granulocytes: Functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. *J. Leukoc. Biol.* **2017**, *101*, 599–611. [CrossRef]
130. Khandpur, R.; Carmona-Rivera, C.; Vivekanandan-Giri, A.; Gizinski, A.; Yalavarthi, S.; Knight, J.S.; Friday, S.; Li, S.; Patel, R.M.; Subramanian, V.; et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci. Transl. Med.* **2013**, *5*, 178ra40. [CrossRef]
131. Papadaki, G.; Kambas, K.; Choulaki, C.; Vlachou, K.; Drakos, E.; Bertsias, G.; Ritis, K.; Boumpas, D.T.; Thompson, P.R.; Verginis, P.; et al. Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation. *Eur. J. Immunol.* **2016**, *46*, 2542–2554. [CrossRef] [PubMed]
132. Spengler, J.; Lugonja, B.; Ytterberg, A.J.; Zubarev, R.A.; Creese, A.J.; Pearson, M.J.; Grant, M.M.; Milward, M.; Lundberg, K.; Buckley, C.D.; et al. Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid. *Arthritis Rheumatol.* **2015**, *67*, 3135–3145.
133. Carmona-Rivera, C.; Carlucci, P.M.; Moore, E.; Lingampalli, N.; Uchtenhagen, H.; James, E.; Liu, Y.; Bicker, K.; Wahamaa, H.; Hoffmann, V.; et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci. Immunol.* **2017**, *2*, eaag3358.
134. Pratesi, F.; Dioni, I.; Tommasi, C.; Alcaro, M.C.; Paolini, I.; Barbetti, F.; Boscaro, F.; Panza, F.; Puxeddu, I.; Rovero, P.; et al. Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann. Rheum. Dis.* **2014**, *73*, 1414–1422.
135. Corsiero, E.; Bombardieri, M.; Carlotti, E.; Pratesi, F.; Robinson, W.; Migliorini, P.; Pitzalis, C. Single cell cloning and recombinant monoclonal antibodies generation from RA synovial B cells reveal frequent targeting of citrullinated histones of NETs. *Ann. Rheum. Dis.* **2016**, *75*, 1866–1875.
136. Shi, J.; Knevel, R.; Suwannalai, P.; van der Linden, M.P.; Janssen, G.M.; van Veelen, P.A.; Levarht, N.E.; van der Helm-van Mil, A.H.; Cerami, A.; Huizinga, T.W.; et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17372–17377.
137. O'Neil, L.J.; Barrera-Vargas, A.; Sandoval-Heglund, D.; Merayo-Chalico, J.; Aguirre-Aguilar, E.; Aponte, A.M.; Ruiz-Perdomo, Y.; Gucek, M.; El Gabalawy, H.; Fox, D.A.; et al. Neutrophil-mediated carbamylation promotes articular damage in rheumatoid arthritis. *Sci. Adv.* **2020**, *6*, eabd2688. [CrossRef]
138. Mustila, A.; Paimela, L.; Leirisalo-Repo, M.; Huhtala, H.; Miettinen, A. Antineutrophil cytoplasmic antibodies in patients with early rheumatoid arthritis: An early marker of progressive erosive disease. *Arthritis Rheum.* **2000**, *43*, 1371–1377.
139. Hu, L.; Hu, X.; Long, K.; Gao, C.; Dong, H.L.; Zhong, Q.; Gao, X.M.; Gong, F.Y. Extraordinarily potent proinflammatory properties of lactoferrin-containing immunocomplexes against human monocytes and macrophages. *Sci. Rep.* **2017**, *7*, 4230.
140. Fossati, G.; Bucknall, R.C.; Edwards, S.W. Insoluble and soluble immune complexes activate neutrophils by distinct activation mechanisms: Changes in functional responses induced by priming with cytokines. *Ann. Rheum. Dis.* **2002**, *61*, 13–19.
141. Robinson, J.J.; Watson, F.; Bucknall, R.C.; Edwards, S.W. Role of Fc gamma receptors in the activation of neutrophils by soluble and insoluble immunoglobulin aggregates isolated from the synovial fluid of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **1994**, *53*, 515–520.
142. Carmona-Rivera, C.; Carlucci, P.M.; Goel, R.R.; James, E.; Brooks, S.R.; Rims, C.; Hoffmann, V.; Fox, D.A.; Buckner, J.H.; Kaplan, M.J. Neutrophil extracellular traps mediate articular cartilage damage and enhance cartilage component immunogenicity in rheumatoid arthritis. *JCI Insight* **2020**, *5*, e139388. [CrossRef] [PubMed]
143. Monach, P.A.; Hueber, W.; Kessler, B.; Tomooka, B.H.; BenBarak, M.; Simmons, B.P.; Wright, J.; Thornhill, T.S.; Monestier, M.; Ploegh, H.; et al. A broad screen for targets of immune complexes decorating arthritic joints highlights deposition of nucleosomes in rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15867–15872. [CrossRef] [PubMed]
144. Bach, M.; Moon, J.; Moore, R.; Pan, T.; Nelson, J.L.; Lood, C. A Neutrophil Activation Biomarker Panel in Prognosis and Monitoring of Patients with Rheumatoid Arthritis. *Arthritis Rheumatol.* **2020**, *72*, 47–56. [CrossRef]
145. Perez-Sanchez, C.; Ruiz-Limon, P.; Aguirre, M.A.; Jimenez-Gomez, Y.; Arias-de la Rosa, I.; Abalos-Aguilera, M.C.; Rodriguez-Ariza, A.; Castro-Villegas, M.C.; Ortega-Castro, R.; Segui, P.; et al. Diagnostic potential of NETosis-derived products for disease activity, atherosclerosis and therapeutic effectiveness in Rheumatoid Arthritis patients. *J. Autoimmun.* **2017**, *82*, 31–40. [CrossRef] [PubMed]
146. Zhao, Q.; Yang, C.; Wang, J.; Li, Y.; Yang, P. Serum level of DNase1l3 in patients with dermatomyositis/polymyositis, systemic lupus erythematosus and rheumatoid arthritis, and its association with disease activity. *Clin. Exp. Med.* **2017**, *17*, 459–465. [CrossRef] [PubMed]
147. Westra, H.J.; Martinez-Bonet, M.; Onengut-Gumuscu, S.; Lee, A.; Luo, Y.; Teslovich, N.; Worthington, J.; Martin, J.; Huizinga, T.; Klareskog, L.; et al. Fine-mapping and functional studies highlight potential causal variants for rheumatoid arthritis and type 1 diabetes. *Nat. Genet.* **2018**, *50*, 1366–1374. [CrossRef] [PubMed]

148. Yousefi, S.; Gold, J.A.; Andina, N.; Lee, J.J.; Kelly, A.M.; Kozlowski, E.; Schmid, I.; Straumann, A.; Reichenbach, J.; Gleich, G.J.; et al. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat. Med.* **2008**, *14*, 949–953. [CrossRef]
149. Morshed, M.; Hlushchuk, R.; Simon, D.; Walls, A.F.; Obata-Ninomiya, K.; Karasuyama, H.; Djonov, V.; Eggel, A.; Kaufmann, T.; Simon, H.U.; et al. NADPH oxidase-independent formation of extracellular DNA traps by basophils. *J. Immunol.* **2014**, *192*, 5314–5323. [CrossRef]
150. von Kockritz-Blickwede, M.; Goldmann, O.; Thulin, P.; Heinemann, K.; Norrby-Teglund, A.; Rohde, M.; Medina, E. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* **2008**, *111*, 3070–3080. [CrossRef] [PubMed]
151. Granger, V.; Faille, D.; Marani, V.; Noel, B.; Gallais, Y.; Szely, N.; Flament, H.; Pallardy, M.; Chollet-Martin, S.; de Chaisemartin, L. Human blood monocytes are able to form extracellular traps. *J. Leukoc. Biol.* **2017**, *102*, 775–781. [CrossRef] [PubMed]
152. Ingelsson, B.; Soderberg, D.; Strid, T.; Soderberg, A.; Bergh, A.C.; Loitto, V.; Lotfi, K.; Segelmark, M.; Spyrou, G.; Rosen, A. Lymphocytes eject interferogenic mitochondrial DNA webs in response to CpG and non-CpG oligodeoxynucleotides of class C. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E478–E487. [CrossRef]

III. Scientific review 2

RMD Open 2023;9:e003104. doi: 10.1136/rmdopen-2023-003104

In this review, we provide an examination of the multifaceted roles of PMNs and NETs in immune responses, shedding light on their potential contributions to various diseases and offering insights into the intricate balance between pro-inflammatory and anti-inflammatory activities.

RMD Open
Rheumatic & Musculoskeletal Diseases

REVIEW

Neutrophil extracellular traps (NET): not only antimicrobial but also modulators of innate and adaptive immunities in inflammatory autoimmune diseases

Dyhia Melbouci, Ahmad Haidar Ahmad, Patrice Decker

To cite: Melbouci D, Haidar Ahmad A, Decker P. Neutrophil extracellular traps (NET): not only antimicrobial but also modulators of innate and adaptive immunities in inflammatory autoimmune diseases. *RMD Open* 2023;**9**:e003104. doi:10.1136/rmdopen-2023-003104

Received 24 February 2023
Accepted 14 May 2023

Inserm UMR 1125, Li2P, Université Sorbonne Paris Nord—Campus de Bobigny, Bobigny, Île-de-France, France

Correspondence to
Prof. Patrice Decker;
patrice.decker@univ-paris13.fr

ABSTRACT

Polymorphonuclear neutrophils (PMN) represent one of the first lines of defence against invading pathogens and are the most abundant leucocytes in the circulation. Generally described as pro-inflammatory cells, recent data suggest that PMN also have immunomodulatory capacities. In response to certain stimuli, activated PMN expel neutrophil extracellular traps (NET), structures made of DNA and associated proteins. Although originally described as an innate immune mechanism fighting bacterial infection, NET formation (or probably rather an excess of NET together with impaired clearance of NET) may be deleterious. Indeed, NET have been implicated in the development of several inflammatory and autoimmune diseases as rheumatoid arthritis or systemic lupus erythematosus, as well as fibrosis or cancer. They have been suggested as a source of (neo)autoantigens or regulatory proteins like proteases or to act as a physical barrier. Different mechanisms of NET formation have been described, leading to PMN death or not, depending on the stimulus. Interestingly, NET may be both pro-inflammatory and anti-inflammatory and this probably partly depends on the mechanism, and thus the stimuli, triggering NET formation. Within this review, we will describe the pro-inflammatory and anti-inflammatory activities of NET and especially how NET may modulate immune responses.

KEY MESSAGES

⇒ Neutrophil extracellular traps (NET) display both pro-inflammatory/anti-inflammatory activities and this dual activity is regulated by the environment, which influences NET composition.
⇒ NET may either be immunomodulatory, antigenic, immunogenic or even pathogenic.
⇒ NET are a source of both damage-associated molecular patterns and autoantigens, including in sterile inflammation.
⇒ NET constituents can be modified during NET formation and targeted by autoantibodies.

INTRODUCTION

Polymorphonuclear neutrophils (PMN) are classically defined as terminally differentiated, non-dividing and short-lived cells dying after a few hours. They represent more than 50% of blood leucocytes in humans and are described as typical pro-inflammatory cells. They are among the first cells recruited at inflammatory sites. Traditionally, PMN are thought to carry out their functions through elementary mechanisms, namely phagocytosis, production of reactive oxygen and nitrogen species or release of granules containing proteases and antimicrobial peptides. They secrete chemokines and pro-inflammatory cytokines as well, like IL-8 and TNF. Although they represent key innate immune cells involved in response to infections, PMN are also activated during sterile inflammation, for example, in response to endogenous ligands and especially damage-associated molecular patterns (DAMP). Therefore, they can exert beneficial or detrimental and even pathogenic roles. Surprisingly, although PMN are described as pro-inflammatory cells, they were until recently relatively sparsely studied in inflammatory diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), on which we will focus in this review. Actually, PMN have gained more interest since the discovery of neutrophil extracellular traps (NET). Indeed, PMN and especially NET may represent a source of both autoantigens and DAMP. Here we will discuss the immunomodulatory activities as well as the pathogenicity of NET, with a special emphasis on these two diseases. Although

the aim of the present review is not to detail the different signalling pathways leading to NET formation, we will discuss critical points to avoid confusion with other biological processes.

PMN: MORE SOPHISTICATED THAN BELIEVED

Because distinct PMN sub-populations may differ in their capacity to produce NET or may form NET of variable composition (see below), we will shortly emphasise PMN heterogeneity and plasticity. Vision on PMN has evolved during recent years. It was thought classically that circulating PMN have a relatively short half-life, but an in vivo lifespan of 5.4 days has been reported for human blood PMN,[1] and PMN survival is believed to be increased under inflammatory conditions.

Moreover, non-classical functions of PMN have been identified. Indeed, and as recently reviewed,[2] PMN may behave as antigen-presenting cells, produce type I interferon (IFN-I), communicate with several immune cell types (such as natural killer cells, dendritic cells (DC), pro-inflammatory Th17 lymphocytes, macrophages/monocytes but also regulatory T lymphocytes) and display also immunomodulatory functions, like secretion of soluble CEACAM8, IL-10 production or even B cell-helper PMN.[3] The latter was especially reported in the mouse.[4] All those data suggest that PMN link innate and adaptive immunities.

In addition to non-classical functions, neutrophil heterogeneity has become apparent with the description of PMN sub-populations in blood and tissues.[5] Heterogeneity of human blood PMN was very recently confirmed by single-cell RNA-sequencing analysis.[6] Particularly interesting in the context of RA and SLE, pro-angiogenic PMN (recruited by vascular endothelial growth factor (VEGF)-A) have been reported in hypoxic tissues[7] whereas low-density granulocytes (LDG) are enriched in the circulation of patients with diverse inflammatory diseases (detailed below).

Interestingly, particular functions or subsets have been identified in inflammatory autoimmune diseases. Thus, RA PMN differentiate into DC-like cells[8] and express RANKL[9] which plays a key role in osteoclastogenesis. Moreover, PMN from patients with RA express B-cell activating factor and IFN-α.[10] Synovial fluid PMN from patients with RA have a higher capacity to express cell surface MHC II and to induce T-cell proliferation[11] and are more sensitive to non-apoptotic cell death triggered by Siglec-9.[12] Particularly, RA synovial fluid is anti-apoptotic for PMN cultured under hypoxia,[13] mimicking the in vivo situation within joints, whereas RA synovial fluid PMN express higher levels of chemokines than autologous RA peripheral blood PMN.[14] Regarding subsets, LDG with pro-inflammatory properties were first observed in SLE[15] and shown to activate T lymphocytes.[16] LDG are also present in patients with RA and present different characteristics compared with autologous classical RA PMN, for example, altered transcriptome and lower NET formation in response to phorbol myristate acetate (PMA).[17]

Among the new neutrophil functions described during the last two decades, the formation of NET is particularly intriguing and exciting. It became a topic with intense research and is of particular interest in the field of inflammatory autoimmune diseases. It has also generated debates and controversies, especially potential NET triggers as well as mechanisms leading to NET formation and pathways involved, or methods to identify, characterise and analyse NET. The implication of NET in pathological conditions has shed new light on PMN, especially in SLE and RA.

UNDERSTANDING PROPERLY NET FORMATION IN THE CONTEXT OF RA AND SLE

Because NET composition may vary depending on the stimulus or the mechanism/pathway triggered during NET formation (see below) and may influence NET immunological properties, a brief overview of NET formation mechanisms is presented. NET were first discovered in 2004 by Brinkmann and colleagues.[18] They have shown that stimulation of neutrophils with PMA, lipopolysaccharides (LPS) or IL-8 induces the release in the extracellular environment of structures composed of nuclear DNA filaments decorated with histones (namely chromatin) and granule proteins but devoid of membrane. Numerous proteins have been shown associated with NET, including some cytoplasmic proteins.[19] Importantly, these fibres are capable of trapping and killing gram-positive and gram-negative bacteria, defining a new approach to neutralise and remove pathogenic bacteria by PMN.

Since Brinkmann's work, the composition and role of NET have received a lot of interest and have been better characterised, without reaching a consensus.[20] In addition to bacteria like *Staphylococcus aureus* and their products such as LPS,[21] NET are induced by or even involved in host defence against fungal[22–25] and parasitic[26 27] infections. They also display antiviral properties.[28] Additional NET-inducing stimuli include activated platelets[29] and, especially interesting in RA and SLE, immobilised immune complexes[30] or cytokines.[18] Particularly, NET may form in response to a variety of DAMP or alarmins during sterile inflammation (eg, HMGB1[31] or LL-37[32]), a situation where NET are potentially pathogenic.

After NET extrusion in Brinkmann's original model,[18] neutrophils were shown to die in 2–4 hours by a process which differs from apoptosis or necrosis[21] and named NETosis.[33] This is the canonical mechanism and is sometimes named suicidal NETosis. However, it is known now that several mechanisms and pathways are involved in NET formation,[34 35] depending on the stimulus, and not all of them lead to cell death. The latter alternative mechanisms are named vital (or live) NETosis. Thus, the term 'NETosis' should only be used when NET extrusion is accompanied with neutrophil death, or at least 'suicidal'

or 'vital' should be included. Otherwise, the correct term is 'NET formation'.[20]

However, the most used in vitro stimulus is PMA, which strongly induces classical NETosis through activation of the multimeric NADPH oxidase 2 (NOX2) complex.[36] NOX2 produces reactive oxygen species (ROS) which are required for NETosis.[21] ROS primarily trigger activation of neutrophil elastase (NE). Myeloperoxidase (MPO) acts synergistically with NE to enhance chromatin decondensation.[37 38] Some NET inducers activate peptidyl-arginine deiminase 4 (PAD4), which citrullinates, for example, histones, favouring chromatin decondensation.[39] In response to PMA, histone citrullination is very low, but detectable in primary human PMN. All these molecular events lead to disintegration of the nuclear envelope. As a result, the cytoplasm and karyoplasm become intertwined, the plasma membrane ruptures and NET are released into the extracellular space.[21]

Regarding non-suicidal pathways, formation of NET containing genomic DNA has also been described. Indeed, activation of PMN by *S. aureus* in vivo and in vitro results in early nuclear condensation which is followed by the separation of inner and outer membranes of nucleus. Subsequently, transport vesicles containing nuclear DNA are formed and burgeon through the plasma membrane into the extracellular space, without breach of the plasma membrane in rapid kinetics (5–60 min). Once in the external environment, the vesicles rupture and NET are released.[40 41] This mechanism is NOX-independent and bactericidal. Intact anuclear PMN or cytoplasts have been observed in vivo and may retain some functions, such as chemotaxis and phagocytosis. This mechanism was later on named vital NETosis.[42]

In addition, NET formation by viable PMN can be accomplished by rapidly (1 hour) ejecting mitochondrial (instead of nuclear) DNA bound to granule proteins in a ROS-dependent manner.[43] These NET do not contain histones but do contain granule proteins. Production of NET containing mitochondrial DNA is generally observed after priming PMN with GM-CSF, followed by stimulation with LPS or complement factor 5a (C5a), and preserves membrane integrity. This process does not require ATG5-dependent autophagy.[44] These NET were subsequently shown to kill bacteria.[45 46]

However, citrullination levels were not evaluated in the latter two models. The function of PAD4 in NET production is the subject of much discussion, especially in the field of RA because citrullinated proteins are targeted by autoantibodies. Several studies suggested the need for PAD4 to induce NET formation in response to specific stimuli.[47–49] However, other reports highlight that NET can be formed in the absence of functional PAD4.[35] The latter study suggests that citrullination occurs during NET formation but that PAD activity is not necessary. Furthermore, the presence of citrullinated histones on NET may be the result of extracellular citrullination by PAD enzymes, which are released when neutrophils are activated.[50]

Thus, several mechanisms of NET formation, triggered by different pathways, exist and lead to PMN death or not. Depending on the stimulus, the composition of NET differs (nuclear vs mitochondrial DNA, containing histones or not, possibly enriched in some post-translational modifications). A minimal common definition for NET may be complexes made of DNA and proteins from granules (eg, NE, MPO), possibly with other associated proteins.

One point has to be discussed in the context of RA and SLE. It has been proposed that NET-like structures might result from other processes confused with NETosis, namely leucotoxic hypercitrullination or defective mitophagy.[51] Leucotoxic hypercitrullination would be triggered by pore-forming proteins like immune proteins, bacterial toxins, or calcium ionophores. However, Parker *et al*[34] have shown that ionomycin induces the release of DNA associated with MPO. This is the definition of NET. More recently, Kenny *et al*[35] reported that the calcium ionophore A23187 triggers the release of structure made of genomic DNA and NE, with bactericidal activity and leading to PMN death, namely NETosis. Thus, both studies reported NET formation and one confirmed the involvement of NETosis, although additional pathways might be involved. Importantly, NET induced by calcium ionophores are highly citrullinated, suggesting that NET induced by pore-forming stimuli might be a source of citrullinated autoantigens in RA. Regarding defective mitophagy, it is a normal PMN process. Most cells eliminate damaged mitochondria via mitophagy.[52] In contrast, mitophagy is defective in PMN.[53] Instead, they release mitochondrial content into the extracellular space, like mitochondrial DNA–proteins complexes. If mitochondrial DNA is oxidised, it is redirected to lysosomes for degradation.[53] However, SLE PMN or IFN-α-primed healthy PMN exposed to lupus anti-ribonucleoprotein (RNP) autoantibodies extrude oxidised mitochondrial DNA–proteins complexes rather than routing mitochondrial DNA to lysosomes.[53] Similarly, SLE PMN or healthy PMN exposed to RNP-containing immune complexes release extracellular oxidised mitochondrial DNA which is interferogenic.[54] It is however unclear whether oxidised mitochondrial DNA released in response to the latter stimuli is physically associated with granule proteins and whether these mitochondrial DNA–proteins complexes are antibacterial, and thus whether these structures strictly comply with the definition of NET. In contrast, and as described above, GM-CSF+LPS/C5a stimulation induces NET made of mitochondrial DNA and granule proteins with antimicrobial activity. Nevertheless, NET induced by calcium ionophores or anti-RNP autoantibodies/immune complexes are highly relevant in the context of SLE and RA as (1) oxidised mitochondrial DNA is pro-inflammatory, (2) anti-oxidised mitochondrial DNA autoantibodies are present in a fraction of patients with SLE and (3) citrullinated proteins are the targets of the

RA-specific anti-citrullinated protein antibodies (ACPA), autoantibodies present in about 70% of patients.

PHYSIOLOGICAL FUNCTIONS AND ANTIMICROBIAL ACTIVITY OF NET

NET react with and/or are induced by a variety of pathogens. Particularly, NET are protective in response to a range of bacterial infections.

To support a physiological role of NET, the existence of NET in vivo was first demonstrated both in experimental shigellosis in rabbits and in spontaneous human appendicitis.[18] Moreover, NET were shown to enhance bacteria trapping in vivo in mice on cooperation with platelets activated through Toll-like receptor (TLR) 4.[55] Then, vital vesicular NET formation was confirmed in vivo during Gram-positive skin infections in mice and humans.[41] Interestingly, NET have been observed in saliva and blood PMN exposed to saliva undergo NET formation.[56] Those NET display high resistance to deoxyribonucleases (DNase) and high capacity to kill bacteria, suggesting their involvement in the antimicrobial defence and/or tissue homeostasis at the oral mucosa.

NET have been shown to bind to bacteria, either Gram-positive or Gram-negative ones.[18] They degrade bacterial virulence factors, for example, IpaB from *Shigella flexneri* or α-hemolysin from *S. aureus*, via serine proteases. Particularly, NET display an extracellular bactericidal activity, as demonstrated by the extracellular bacterial killing, which is reduced after digestion of NET with nucleases. NET-associated factors contribute to their antimicrobial activity and the presence of antimicrobial molecules on NET may be a way to locally increase their concentrations. Actually, NET-bound cathepsin G and NE process and activate the pro-inflammatory cytokines IL-1α and IL-36,[57] amplifying the inflammatory response to fight pathogens. Interestingly, the antimicrobial effect mediated by NET passed especially through histones,[18] cathelicidin (LL-37 in humans)[58] as well as calprotectin (S100A8/A9).[19] It should be noted that histones have been shown in the past to be bactericidal.[59] Some of these proteins kill microbes by forming membrane pores. Likewise, an antimicrobial activity has been reported for DNA.[60] Moreover, it has been proposed that PMN release NET only in response to pathogens too large to be phagocytosed in order to selectively neutralise them, whereas phagocytosis inhibits NET release.[61]

NET also act as a physical barrier to limit bacterial biofilm dissemination.[62] During keratitis induced by *Pseudomonas aeruginosa*, PMN are recruited to the bacterial biofilm formed on the cornea and form NET at the base of the biofilm. In mice, NET create a 'dead zone' barrier which impedes bacterial dissemination into the brain. However, NET formation in turn amplifies biofilm formation, promoting ocular pathology. Thus, a tight regulation of NET formation and its intensity is required.

As a defence mechanism, bacteria have developed evading strategies against NET, for example, via the secretion of nucleases degrading NET[63] or virulence factors inhibiting the activity of antimicrobial peptides.[58] Some bacteria express a surface nuclease degrading NET to escape killing by NET.[64] To evade NET, *Neisseria meningitides* is able to modify the lipid A moiety of its LPS or to upregulate its zinc uptake receptors,[65] whereas *Streptococcus pneumoniae* modifies its surface charge or produces a capsule.[66] Reciprocally, the antimicrobial peptide LL-37 present in NET confers resistance of NET against bacterial nucleases.[67]

NET were also observed in the cerebrospinal fluid of patients with pneumococcal meningitis.[68] However, NET formation appears deleterious in this context, as in a rat model of meningitis NET hinder bacterial clearance in the central nervous system. This result exemplifies the dual activity of NET and the required balance between beneficial versus pathological effects of NET.

Although NET are usually beneficial in response to infections, NET may become pathogenic in some particular cases, when NET formation is (locally) too intense or when NET are not efficiently cleared or do not form aggregated NET (aggregates formed at high PMN density; see 'Beneficial activities of NET in diseases' section). Pathogenic NET may also form during sterile inflammation in response to injury or disease-associated triggers, some of them being endogenous self-molecules such as DAMP or cytokines.

IMPACT OF NET ON THE REGULATION OF IMMUNE RESPONSES

For the reasons mentioned above, we focused here on studies either using classical NET inducers (essentially PMA, LPS, crystals, autoantibodies/immune complexes, cytokines but also calcium ionophores) or depicting well characterised NET containing DNA (genomic or mitochondrial) and proteins and not on studies dealing only with extracellular DNA release. Due to their potential pathogenic activity in SLE, complexes made of oxidised mitochondrial DNA in response to anti-RNP autoantibodies/RNP-containing immune complexes will also be mentioned. Moreover, we focused on data generated with primary cells and not cell lines, either for generating NET or for cells targeted by NET. The data described below refer to the immunomodulatory activities of NET rather than their role as autoantigens.

Besides the physiological and protective effects of NET in response to invading pathogens, NET have been described in several diseases, especially in inflammatory and/or autoimmune diseases (table 1) and fibrosis. NET can be beneficial and protective, for example, through anti-inflammatory activity, but in most cases they have a detrimental and pathogenic role in these diseases where they stimulate immune responses through their pro-inflammatory, antigenic (which is particularly true in RA and SLE) and immunogenic activities. NET work as a source of autoantigens and DAMP or may even be considered as DAMP themselves. As such, they may be key endogenous ligands involved in sterile inflammation.

Table 1 Involvement of NET formation in inflammatory autoimmune diseases

Diseases	Potential NET inducers	Effects proposed	Mechanims reported
Systemic lupus erythematosus (SLE)	Autoantibodies±IFN-α[53 100 116 153]	Pathogenic	Source of autoantigens[92 111 127 154]
	Immune complexes[54]		Plasmacytoid DC activation: key lupus cytokine IFN-α induced[53 54 99 100 116]
	Nucleosomes[94]		Macrophage activation[91 96]
			Impaired endothelium-dependent vasorelaxation[97] and activation of endothelial cells by NET-associated RNA[98]
Rheumatoid arthritis (RA)	Autoantibodies[101 105]	Pathogenic	Source of autoantigens[72 101 105–107 109 111]
	Cytokines[101]		Macrophage activation: key RA cytokine TNF induced[72]
			Neutrophil activation: key RA cytokine TNF induced[72]
			Myeloid DC activation[102]
			Fibroblast-like synoviocyte activation and antigen presentation[101 105 109 115]
			Cartilage degradation[115]
			Monocyte differentiation into osteoclasts[117]
Autoimmune small-vessel vasculitis	Autoantibodies[84]	Pathogenic	Induction of respiratory burst-activating ANCA[85]
Diabetes	Glucose[128]	Pathogenic	Impaired wound healing[128]
			Myeloid DC activation and induction of IFN-γ-producing T lymphocytes[129]
Psoriasis	?	Pathogenic	IL-17 exposure[82]

ANCA, anti-neutrophil cytoplasmic antibodies; DC, dendritic cells; IFN, interferon; NET, neutrophil extracellular traps.

Physiological modulatory activities of NET

Using a short differentiation protocol (3 days with M-CSF), NET have been shown to be taken up by human monocyte-derived macrophages.[69] Healthy donor NET did not induce (either pro-inflammatory or anti-inflammatory) cytokine secretion by resting macrophages from healthy donors, although a slight but significant induction of IFN-α was observed (table 2), and this was thus described as a silent process (figure 1). Nevertheless, concentrations of NET used to stimulate macrophages were not indicated. Interestingly, NET-mediated induction of IFN-α was also reported for healthy donor plasmacytoid DC (pDC).[70] Likewise, stimulation of healthy donor monocyte-derived DC with NET from healthy donors was not associated with DC activation as estimated by HLA-DR and CD80/83/86 expression[71] (figure 1). Cytokine secretion was reported to be unaffected.

On the contrary, using a 6-day differentiation protocol, we have shown that NET from healthy donors induce the secretion of pro-inflammatory cytokines, but not immunomodulatory IL-10, by resting M-CSF-differentiated monocyte-derived macrophages.[72] Activation was associated with HLA class I and class II as well as CD86 upregulation. A similar pro-inflammatory response was triggered by healthy donor NET on unprimed healthy donor PMN (figure 1). One plausible hypothesis to explain the difference with above results is that highly mature or differentiated macrophages are more responsive to NET. Similarly, macrophages have been shown to phagocytose NET and to express IFN-I after cGAS activation.[73] In agreement with our results and a pro-inflammatory response induced by NET, although NET do not trigger cytokine secretion by healthy macrophages or DC, they potentiate IL-1β, TNF and IL-6 secretion by LPS-stimulated macrophages (3 day differentiation protocol)[69] as well as IL-1β and IFN-γ secretion by LPS-stimulated DC.[74] Interestingly, NET also influence cells from adaptive immunity (figure 2A). In healthy individuals, NET can directly prime resting $CD4^+$ T lymphocytes.[75] NET-induced activation is TCR-dependent but does not trigger T-cell proliferation. In coculture, NET induce cluster formation, upregulation of the activation markers CD25 and CD69, and phosphorylation of the TCR-associated signalling kinase ZAP70 in $CD4^+$ T cells. Moreover, NET increase T-cell responses to specific antigens, making T cells capable of being activated by sub-optimal stimuli. Although NET-primed $CD4^+$ T lymphocytes do not proliferate, they do proliferate and secrete IFN-γ in the presence of resting DC and without specific antigens (a suboptimal stimulus). Importantly, this study also confirms indirectly the stimulatory

Table 2 Involvement of NET in the regulation of human immune responses

Context	Activation by NET	Inhibition by NET	Mechanims reported
In healthy individuals	Activation of plasmacytoid DC		IFN-α induction[70]
	Activation of resting macrophages		Pro-inflammatory cytokines secreted[72]
			Induction of type I IFN[69 73]
			CD86 and HLA up-regualted[72]
	Activation of unprimed PMN		Pro-inflammatory cytokines secreted[72]
	Enhanced activation of LPS-primed macrophages		IL-1β, TNF and IL-6 secretion[69]
	Enhanced activation of LPS-primed DC		IL-1β and IFN-γ secretion[74]
	$CD4^+$ T cell priming		CD25 and CD69 up-regulated[75]
	Memory B cell activation		Total IgG secretion[76]
	Activation of the complement system		e.g., C1q binding[127]
	Activation of cytokines		Processing by NET proteases[57]
		Inhibition of LPS-stimulated macrophages	Reduced IL-6 secretion[72]
		Inhibition of LPS-stimulated myeloid DC	Reduced TNF, IL-8, IL-12 and IL-10 secretion[71 74]
			Reduced HLA-DR, CD40, CD80 and CD86 expression[71]
Pathological			
In RA	Activation of resting macrophages		IL-8, IL-6 and TNF secretion[72]
	Activation of unprimed PMN		IL-8 and TNF secretion[72]
	Activation of myeloid DC		IL-6 and TNF secretion[102]
			HLA-DR and CD86 up-regulated[102]
	Activation of fibroblast-like synoviocytes		IL-6 and IL-8 secretion[101]
			MHC class II up-regulated[115]
In SLE	Activation of resting macrophages		Calcium flux increased[96]
			IL-6 and TNF secretion[96]
	Activation of LPS-primed macrophages		IL-1β and IL-18 secretion[91]
	Activation of plasmacytoid DC		IFN-α induction[54 70 99 100 116]
	Memory B cell activation		ANCA secretion[76]
	Activation of the complement system		e.g., C1q binding[127]
In type 1 diabetes	Activation of myeloid DC		Pro-inflammatory cytokines secreted[129]
			Maturation markers up-regulated[129]
In gout		Degradation of cytokines[78]	

Direct effects of NET on immune cells or molecules are shown. NET-containing immune-complexes are not presented here. Consequences on B and T cells are shown in figure 2.
ANCA, anti-neutrophil cytoplasmic antibodies; DC, dendritic cells; IFN, interferon; LPS, lipopolysaccharides; NET, neutrophil extracellular traps; PMN, polymorphonuclear neutrophils; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

potential of NET on DC. Indeed, when cocultured with DC and only in the presence of NET, both unprimed purified $CD4^+$ and $CD8^+$ T cells were activated. Regarding $CD4^+$ lymphocytes, NET trigger activation of both naïve and memory cells in the presence of DC.[75] Similarly, NET are able to trigger polyclonal activation of memory B lymphocytes from healthy individuals and to induce total IgG secretion.[76] Using complexes made of purified DNA and granule proteins (especially LL-37) to mimic NET, authors have shown that these structures are internalised and activate B lymphocytes partly in a TLR9-dependent manner.

Beneficial activities of NET in diseases

In gout, monosodium urate crystals induce NET formation[77] which limits inflammation by degrading cytokines (especially pro-inflammatory cytokines, but also IL-10) and chemokines.[78] This anti-inflammatory property of NET may happen only at sites where PMN are highly concentrated, generating aggregated NET, as observed in gout tophi.[78] In addition, inflammation may lead to the release of extracellular histones and the latter contribute to endothelial dysfunction.[79] Interestingly, at high concentrations, NET have been shown to proteolytically degrade extracellular free histones (not NET-bound histones), at least in vitro, resulting in attenuated histone-mediated cytotoxicity in cell cultures.[80] In patients suffering from chronic granulomatous disease, characterised by NOX2 mutations, ROS-dependent NET formation is impaired and results in recurrent bacterial and fungal infections; in these immunodeficient patients,

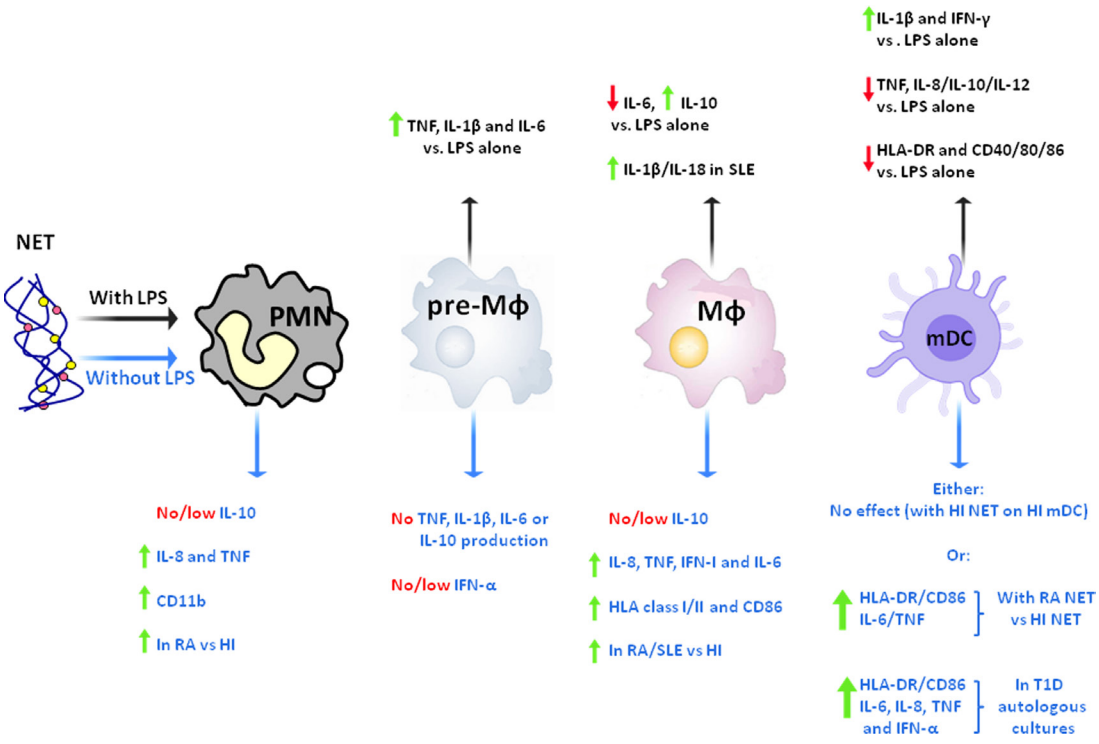


Figure 1 Direct effects of NET on myeloid cells. Direct interaction of NET with different cell types and its consequences in the absence (blue arrows, lower part) or presence (black arrows, upper part) of LPS. Effects of NET-containing immune complexes are not depicted. Data are pooled from human studies, both in healthy individuals and patients with inflammatory autoimmune diseases. Mechanisms highlighted as stronger in patients as compared with healthy individuals refer to data with either NET from patients or target cells from patients. HI, healthy individuals; IFN-I, type I interferon; LPS, lipopolysaccharides; mDC, myeloid dendritic cells; MΦ, macrophages; NET, neutrophil extracellular traps; PMN, polymorphonuclear neutrophils; pre-MΦ, macrophages obtained after only 3 day differentiation; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.

NET formation and protection against *Aspergillus* infection can be restored by gene therapy targeting NOX.[23] Also, NET have been suggested to form a physical barrier during acute necrotising pancreatitis and peritonitis in order to isolate necrotic areas from surrounding healthy tissues, limiting spreading of necrosis-associated pro-inflammatory mediators.[81]

Pathological NET-mediated mechanisms in inflammatory autoimmune diseases

On the contrary, a pathogenic role of NET has been reported in several inflammatory conditions, like acute pancreatitis, sterile lung inflammation, vascular occlusion during severe bacterial infection, chorioamnionitis, atherosclerosis, fibrosis, thrombosis and cancer. Regarding inflammatory autoimmune diseases (table 1), NET are present in skin biopsies from psoriatic patients and contain the pro-inflammatory cytokine IL-17A, which is involved in psoriasis pathogenesis.[82] Similarly, NET were observed in situ in lesional skin biopsy of patients with bullous pemphigoid and blister fluids from patients induce NET formation ex vivo.[83] Interestingly, in autoimmune small-vessel vasculitis, anti-neutrophil cytoplasmic antibodies (ANCA) trigger in vitro formation of NET which are found deposited in inflamed kidneys of patients.[84] Moreover, injection of NET-loaded DC induces ANCA production in mice.[85] In addition, NET may participate in the severity of COVID-19 and reciprocally SARS-CoV-2 infection has been suggested as a trigger of autoimmunity.[86] Actually, SARS-CoV-2 triggers the release of NET[87] which are detected in lung microthrombi of patients[88] and COVID-19 adult patients display a reduced capacity to degrade NET.[89]

RA and SLE

Particularly, it is now believed that NET may also play a pathogenic role in SLE or RA, as a result of increased NET formation, decreased NET clearance and/or altered composition.[2 14]

In SLE, a PMN sub-population (LDG) has a higher capacity to spontaneously form NET in vitro and NET are detected in skin and kidney lesions from patients.[90] The same group suggested that IL-18 induced NET release by LDG.[91] Moreover, sera from a subgroup of lupus patients have an impaired NET-degrading activity, which correlates with high titers of anti-double stranded DNA autoantibodies (precisely a lupus marker).[92] These patients develop NET-binding antibodies. It was recently shown that AIM2 and IFI16 are autoantigens binding NET, protecting them from DNase1 degradation.[93] Circulating nucleosomes, a DAMP and classical lupus autoantigen, triggers NET formation,[94] leading to an

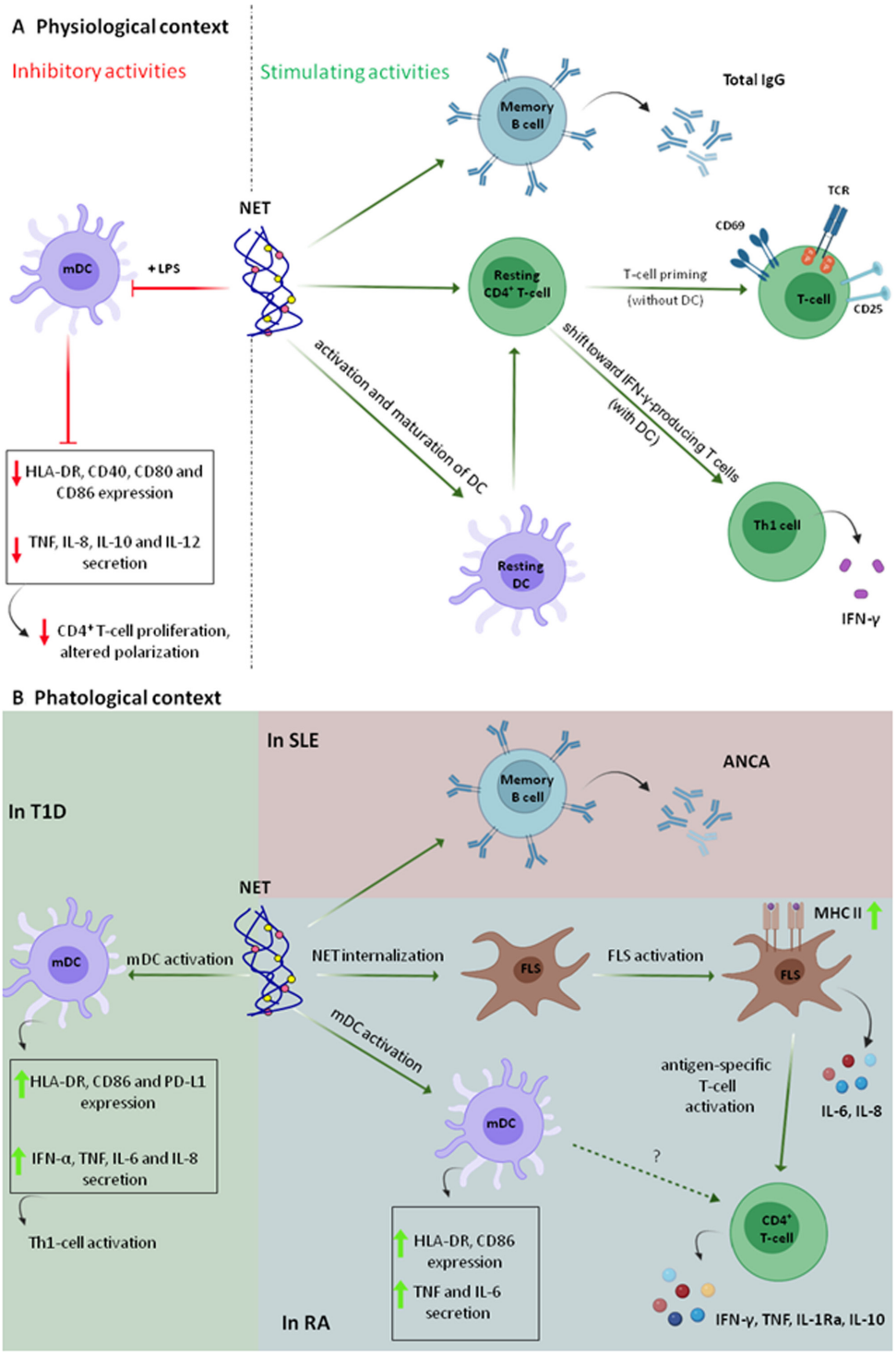


Figure 2 Direct and indirect effects of NET on B and T lymphocytes. (A) NET effects in a physiological context. On the left part (red arrows), NET inhibit myeloid DC activation in response to LPS, leading to impaired T-cell response. On the right panel (green arrows), NET directly activate memory B lymphocytes to secrete total IgG. NET can also prime resting CD4$^+$ T cells, leading to CD25 and CD69 upregulation, as well as ZAP70 phosphorylation, without requiring dendritic cells. Likewise, NET stimulate resting DC to activate resting CD4$^+$ T cells, favouring a Th1-like response. Data are pooled from studies with NET and target cells from healthy individuals. (B) NET effects in a pathological context. In type 1 diabetes (green background), NET stimulate myeloid dendritic cells to activate Th1 lymphocytes. In SLE (pink background), NET directly activate memory B lymphocytes to secrete ANCA. Finally, in RA (blue background), NET activate fibroblast-like synoviocytes on internalisation and NET peptides are presented to antigen-specific CD4$^+$ T cells, leading to their activation. NET also activate myeloid dendritic cells, which potentially (dotted line) stimulate T lymphocytes. Effects of NET-containing immune complexes are not depicted in this figure. ANCA, anti-neutrophil cytoplasmic antibodies; DC, dendritic cells; FLS, fibroblast-like synoviocytes; LPS, lipopolysaccharides; mDC, myeloid DC; NET, neutrophil extracellular traps; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.

amplification loop of the inflammatory process. Likewise, plasmas from patients with SLE induce a stronger release of NET by normal PMN than plasma from healthy volunteers and high levels of NET formation are associated with increased plasma levels of antinuclear antibodies, anti-double stranded DNA antibodies and a high IFN signature in patients with SLE.[95] Regarding the pathogenic mechanisms triggered (table 2), lupus NET spontaneously produced by LDG stimulate IL-1β and IL-18 secretion by LPS-primed macrophages from healthy individuals.[91] Mature (cleaved) IL-1β was secreted and associated with caspase-1 activation, indicating inflammasome involvement. In addition, NET induced in PMN from healthy donors and patients with SLE increase calcium flux in macrophages from healthy donors and patients with SLE, whereas NET spontaneously formed by lupus LDG were less efficient[96] (figure 1 and table 2). This increased calcium flux may be partly triggered by activation of CXCR4 on macrophages by ubiquitinated proteins present in NET. NET induced in classical SLE PMN also trigger TNF and IL-6 secretion by lupus macrophages; especially TNF secretion, but also IL-10, was higher in lupus macrophages versus normal macrophages in response to NET. In support of a pathogenic role of NET in SLE, LDG-derived NET impair endothelium-dependent vasorelaxation in vitro.[97] Actually, these NET extrude RNA and induce IFN-I-stimulated genes once internalised by endothelial cells.[98] In SLE, IL-33 is complexed with NET and the amount of NET-IL-33 complexes is correlated with disease activity. These complexes trigger IFN-α (a key lupus cytokine) production by pDC in an IL-33 receptor-dependent manner.[99] In addition, lupus anti-RNP autoantibodies induce formation of NET containing LL-37, a DNA-binding protein facilitating DNA uptake by pDC, in SLE but not healthy PMN.[100] Those anti-RNP-induced lupus NET trigger the activation of healthy pDC and the secretion of IFN-α in a TLR9-dependent manner.[100] Likewise, PMN stimulated with RNP-containing immune complexes release oxidised mitochondrial DNA inducing IFN-α production in PBMC in a Stimulator of Interferon Genes-dependent manner.[54] Such complexes are also spontaneously released by lupus LDG. Lupus patients produce also circulating ANCA, for example, anti-LL-37 antibodies. Importantly, in patients with ANCA-positive SLE but not in healthy donors, anti-LL-37-induced NET (which also contain LL-37) trigger the production of ANCA, and especially anti-LL-37 antibodies, by memory SLE B cells in an antigen-dependent manner[76] (figure 2B).

As observed in SLE, RA PMN (both blood and synovial fluid PMN) produce more NET in vitro than PMN from healthy individuals, either spontaneously or in response to LPS or PMA,[72 101 102] and NET are present in both rheumatoid nodules[101] and the synovial fluid of patients with RA.[50] Actually, RA serum and RA synovial fluid increase NET formation by healthy PMN.[14 101 102] Similar results were obtained in the mouse collagen-induced arthritis model in which bone marrow PMN from diseased mice produce more NET in vitro than PMN from normal mice.[102] In another RA mouse model, NET have been suggested to mediate joint hyperalgesia.[103] Interestingly, PMA-stimulated PMN release NET containing alarmin S100A11 (calgizzarin), which behaves as a DAMP in RA.[104] In addition, we and others have shown that ACPA-rich IgG purified from patients with RA bind to NET[72 101] or even induce NET formation.[101] NET binding and NET induction were confirmed with purified ACPA.[105] Likewise, RA sera recognise activated PMN and NET[106] and monoclonal antibodies generated from RA synovial B lymphocytes have a strong reactivity against citrullinated histones and PMA-induced NET.[107] As NET formation can be associated with citrullination (at different levels and in response to some stimuli), NET may be a source of RA autoantigens and either the targets of ACPA or even the true autoantigen triggering ACPA production. Moreover, NET formation is associated with the release of active PAD2 and PAD4 in the extracellular milieu, either free or bound to NET,[50] which is potentially associated with the citrullination of extracellular autoantigens locally in affected tissues. NET formation in RA joints may thus be linked to citrullination and potentially to recognition by ACPA or even induction of ACPA production, triggering pathogenic mechanisms such as immune complex deposition in tissues and complement activation. Interestingly, in an RA model consisting of HLA-DR4-transgenic mice in which fibroblast-like synoviocytes loaded with RA NET were injected in the synovial space, animals develop ACPA and show impaired cartilage integrity, without however overt arthritis.[105] In addition to ACPA, patients with RA develop antibodies against carbamylated proteins, which predict a more severe disease,[108] and NET externalise carbamylated proteins[109] such as carbamylated LL-37[110]; these NET are thus also the targets of RA autoantibodies, including anti-carbamylated LL-37 antibodies. Recently, anti-NET antibodies have been reported in RA, SLE, Sjögren's syndrome and scleroderma.[111] Regarding the mechanism involved, we have recently shown that RA NET are more efficient in activating macrophages and PMN than healthy donor NET, leading to the secretion of the pro-inflammatory cytokines IL-8, IL-6 and the key RA cytokine TNF, with minimal induction of IL-10[72] (figure 1). These data indicate that, in RA, both NET formation and the pro-inflammatory activity of NET are increased in comparison to healthy individuals (when activity is defined by the capacity of NET to trigger in target cells a cytokine secretion profile in favour of pro-inflammatory vs anti-inflammatory cytokines). Actually, we have recently demonstrated that NET from patients with RA activate several myeloid cell sub-populations towards a pro-inflammatory profile (Seninet *et al*, submitted). The fact that RA NET are more efficient in inducing pro-inflammatory cytokines might be explained by a slightly different NET composition. Proteomic analyses revealed that the nature of the stimulus or origin of the PMN (healthy individuals vs autoimmune patients) may influence the composition of NET.[112–114] Similarly, NET from

patients with RA have been shown to activate (although moderately) myeloid DC from healthy volunteers, and with a higher activity than healthy donor NET, as shown by HLA-DR/CD86 upregulation and IL-6/TNF secretion.[102] NET are also directly involved in articular cartilage damage in RA by disrupting the cartilage matrix in a NE-dependent manner.[115] In RA, autoantibodies induce NET formation in vitro and RA NET stimulate fibroblast-like synoviocytes (the cells invading cartilage in RA) to secrete IL-6 and IL-8[101] and to upregulate MCH class II on internalisation of NET[105]; NET indirectly lead to T-cell activation via presentation of NET peptides (figure 2B). In turn, RA synovial fluid $CD8^+$ T cells induce NET formation.[116] Similarly, in an in vitro mouse cell culture model, ovalbumin-loaded DC have a higher capacity to stimulate ovalbumin-specific $CD4^+$ T cells from OT-II mice when DC are treated with NET from collagen-induced arthritis mice as compared with NET from control mice, as shown by the increased frequency of IFN-γ-producing Th1 cells,[102] demonstrating increased antigen-presenting cell functions after NET treatment. Recently, carbamylated NET (as those observed in patients with RA) have been shown to directly trigger monocyte differentiation into osteoclasts, supporting a role of NET in bone erosion.[117]

However, in different experimental lupus models, using NOX2-deficient or PAD4-deficient mice, NET have been suggested to be protective or without influence[118–120]; nevertheless NOX2 and PAD4 are also involved in cellular processes other than NET formation and might play different roles at different stages of the disease. As mentioned above, NOX-independent NET formation has been reported, whereas PAD activity may be involved but not required for NET formation. Interestingly, recent data suggest that NET formation partly occurs in a NOX-independent manner in vivo in patients with SLE.[121] One should also take into account that the PMN blood frequency is much lower in mice than in humans, probably affecting NET impact. On the opposite, PAD inhibition protects from lupus manifestations in the MRL/*lpr* mouse model,[122] whereas disease severity is reduced in PAD4-deficient animals in the TNF-α-transgenic mouse model of RA.[123] In a recently described new RA mouse model (collagen-induced arthritis with G-CSF administration), PAD4-deficient animals present lower levels of citrullinated histones in the blood and the synovial tissue and develop a less severe disease[124]; in this model, oral administration of a PAD4 inhibitor able to block NET formation in vitro reduces arthritis development.[125] Interestingly, NET have been suggested to contribute to lupus nephritis in MPO-deficient mice; these mice developed more severe nephritis with increased deposition of NET in glomeruli than wild-type mice.[126] The latter result indicates that effects of MPO and NET are not necessarily linked, which makes sense as MPO is also present in monocytes. Moreover, some stimuli do not require ROS to induce NET formation and even PMN from patients with inactive NOX may produce NET; similar observations were reported regarding MPO.[34 35]

Finally, the stimuli triggering NET formation in vivo are unknown and therefore it is tricky to speculate on the NET mechanism/pathway involved in these autoimmune patients or mice.

It should be noted that physiological NET clearance mechanisms are impaired in patients with SLE and RA (as recently reviewed[2]), amplifying NET-mediated pathological mechanisms in patients. Thus, both in patients with RA and SLE, defective NET clearance may result in activation of the complement system on deposition of residual complement factor 1q (C1q) on NET,[127] as well as enhanced release of autoantigens and DAMP.

Diabetes

NET also impair wound healing, especially in diabetes[128] where PMN from patients (with either type 1 or type 2 diabetes) extruded more DNA spontaneously or after ionomycin stimulation than PMN from healthy controls. Likewise, PMN from healthy individuals extruded more DNA in response to PMA or ionomycin when exposed to high glucose concentration. In addition to priming, it should be noted that glucose alone also stimulated DNA release.[128] Thus, altered metabolic regulation, for example, via the glycolytic pathway, may predispose PMN to form NET. Furthermore, energy metabolism and mitochondria involvement in NET release is supported by the fact that glycolytic ATP production is required for NET formation after GM-CSF priming and subsequent C5a stimulation.[46] Indeed, PMN lacking the mitochondrial protein OPA1 have a decreased ATP production through glycolysis and fail to release NET. Potential pathogenic consequences of NET release have been described in type 1 diabetes. In monocyte-derived DC cultures with autologous NET, patient NET contribute to a significant increase in DC maturation markers and inflammatory cytokine production as compared with normal NET-DC cultures. DC capacity to induce IFN-γ-producing T lymphocytes (both Th1 and $CD8^+$ cells) was enhanced in DC-T cell cocultures with patient NET and this does not result from the direct effect of NET on T cells (figure 2B). Using RNA-seq analysis, patient NET were shown to downregulate TGF-β while upregulating IFN-α in healthy donor DC.[129]

All the results described in that section suggest that NET behave as a source of DAMP as well as autoantigens and we suggest they may also behave as an adjuvant, influencing both innate and adaptive immunities. They can trigger directly or indirectly pro-inflammatory and antigen-specific responses. NET may also contribute to sustained inflammation by inhibiting efferocytocis. Impaired efferocytosis is a hallmark of several autoimmune diseases and is involved in delayed resolution of inflammation. Indeed, extracellular cold-inducible RNA-binding protein (a DAMP present at high concentration in the blood and especially in the synovial fluid of patients with RA[130]) induces NET that inhibit efferocytosis.[131]

In addition to direct effects on cells, NET can also act indirectly via immune complexes. NET are targets of autoantibodies in several diseases (eg, ACPA in RA, NET-binding antibodies in SLE, ANCA in patients with vasculitis) although the fine specificity of those antibodies is not always characterised. For example, what is recognised in NET by lupus autoantibodies is not clear (DNA, maybe only partly? Individual NET components? Or are they true 'anti-NET' antibodies recognising tertiary NET structure, that is, NET-restricted antibodies?). In RA, there are probably also non-ACPA antibodies binding to NET, for example, anti-carbamylated protein antibodies. Among classical ANCA, anti-proteinase 3 and anti-MPO are the most frequent autoantibodies. The resulting immune complexes may become pathogenic on recognition through Fc receptors. NET can also become more active in immune complexes. In SLE, anti-LL-37 antibodies induce formation of NET containing LL-37.[70] Although NET alone stimulate pDC to secrete IFN-α, this secretion is strongly enhanced in the presence of anti-LL-37 antibody, which is abrogated by a TLR9 inhibitor.[70] As other immune complexes, NET-containing immune complexes may bind and activate the complement system and be even more strongly recognised by cells expressing complement receptors. NET-containing immune complexes may be more efficient stimuli after internalisation and activation of intracellular receptors (eg, endosomal TLR9 recognising DNA in NET). Indeed, although mammalian self DNA normally poorly stimulates TLR9, it may activate TLR9 when it reaches this receptor or on enforced translocation to endosomes.[132–134]

NET and the regulation of inflammation

On the contrary, NET may be anti-inflammatory or may at least limit inflammation in some circumstances (figure 1 and table 2). Indeed, we have shown that NET inhibit the response of LPS-stimulated macrophages, leading to reduced IL-6 secretion, whereas IL-10 secretion was enhanced.[72] This was observed with both NET from healthy individuals or patients with RA and on both macrophages from healthy individuals or patients with RA. Interestingly, although NET activate also resting PMN, LPS-stimulated PMN are not responsive to inhibition by NET.[72] Similarly, NET from healthy individuals partly inhibit LPS-induced maturation of normal myeloid DC, as shown by reduced HLA-DR, CD40, CD80 and CD86 upregulation as well as reduced secretion of both pro-inflammatory (eg, TNF or IL-8) and immunomodulatory cytokines (IL-10).[71] This was associated with a lower ability of DC to stimulate $CD4^+$ T lymphocyte proliferation and altered $CD4^+$ T lymphocyte polarisation (reduced IL-10 and Th1/Th17 cytokine secretion, increased Th2 cytokines; figure 2A). NET-mediated inhibition of DC maturation in the presence of LPS was confirmed in healthy subjects, showing in addition inhibition of IL-12 secretion.[74] Such inhibitory activities of NET on macrophages were not observed by Farrera *et al*, especially not the impaired IL-6 secretion by LPS-stimulated macrophages.[69] This might be explained by the different protocol used to differentiate macrophages from monocytes in that study, harvesting macrophages after 3 days instead of 6 days, which probably generates less differentiated cells.

Finally, NET are immunomodulatory by targeting directly key immune proteins (table 2). Free NET (not only NET-containing immune complexes) bind C1q and activate the complement cascade, leading to the production of the C5a anaphylatoxin.[127] Thus, non-degraded NET may participate in complement consumption and inflammation in SLE. These NET-complement complexes may also be recognised by cells expressing complement receptors. On the opposite, NET may directly degrade some PMN-derived cytokines, through NET-associated serine proteases, to resolve inflammation. Interestingly, patients affected by Papillon-Lefèvre syndrome are characterised by nonfunctional PMN serine proteases and intense periodontal inflammation; although PMN from these patients have been reported to display impaired canonical NET formation, they aberrantly release some NET-like DNA structures nearly devoid of NE and unable to degrade inflammatory mediators.[135] However, NET-bound proteases can also process and activate pro-inflammatory cytokines.[57] Therefore, excessive NET formation and activation of such cytokines may convert a protective response in a detrimental pro-inflammatory and tissue-damaging reaction, both in the context of infection and sterile injury.

WHAT HAVE WE LEARNED FROM IN VITRO STUDIES?

The present review clearly indicates that NET are extracellular structures composed of DNA and a set of proteins that may vary depending on the stimuli, the physiological or pathological context and, accordingly, to the pathways and/or the mechanisms triggered during NET formation, although many of these proteins are overlapping. Some of them may be modified or altered, either by post-translational modifications (eg, citrullination), partial degradation or cleavage as observed with histones.[19] Therefore, we believe that the simple measure of cell-free DNA, without at least testing whether DNA forms complexes with proteins and testing known NET proteins (or even better characterising associated proteins) should not be used as a surrogate marker to estimate the presence of NET in biological fluids or cell culture supernatants. Likewise, citrullinated histone H3 is found during NET formation, but not in response to all stimuli and at different levels, and therefore is not a clear NET marker. Moreover, citrullination of histones is not only and specifically induced during NET formation and thus additional tools have to be used simultaneously to refer to NET. As an alternative, NET-detecting sandwich ELISA have been developed that measure extracellular complexes made of DNA and MPO or NE. This approach is more NET-associated but not specific; it might also only partly reflect the extent of NET formation. Indeed, these

ELISA may only detect NET induced by some stimuli and they assume that such complexes are only released during NET formation and not during other cellular processes or by monocytes which also express MPO and also release extracellular traps.[136] DNA–MPO complexes are also released during monocyte extracellular trap formation[136] and are thus not specific to PMN. Measuring DNA–NE complexes might appear an alternative (NE being theoretically more PMN-specific than MPO) but NE is also detected in extracellular traps produced by monocytes.[136] The best NET characterisation remains thus the visualisation of DNA–proteins complexes using a combination of stainings for both DNA and multiple proteins, for example, by fluorescence microscopy.

Regarding in vitro cell activation assays with NET prepared from isolated neutrophils, we recommend to use NET obtained from adherent activated PMN (detached by vigorous pipetting or mild nuclease digestion) rather than NET-containing cell culture supernatants to avoid transferring on target cells the stimulus or, for example, cytokines induced by that stimulus. Likewise, adding target cells directly on adherent NET is not recommended as some NET-inducing stimuli or the triggered cytokines may adhere to plastic.[72] These approaches can however be used for NET staining, screening of NET-recognising antibodies and possibly for memory B-cell restimulation. Again, NET used for cell stimulation must be characterised, as cell activation mechanisms differ from those triggered by cell-free DNA.

In all cases, we suggest using primary PMN instead of cell lines and, when working with mouse PMN, Ly-6G$^+$ and not Gr-1$^+$ cells should be used.[137] For cell activation assays, NET prepared from highly purified PMN should be used.

Although PMA is the best characterised inducer of NET, using more physiological stimuli to analyse the impact of NET in different mechanisms and pathologies would be important for a better understanding. However, PMA is also one of the most often used inducers because it triggers high levels of NET formation; anyway, PMA allows measuring the capacity of PMN to release NET. Actually, there are not so many natural and disease-specific or even disease-associated NET stimuli described. In SLE, stimulation of PMN with anti-RNP antibodies or RNP-containing immune complexes leads to the release of NET made of oxidised mitochondrial DNA which are interferogenic. However, those NET have to be better characterised. In RA, some cytokines or autoantibodies have been reported to induce NET, however often at low levels.

PERSPECTIVES

The data described here raise several questions. Other cell types (eosinophils,[138] basophils,[139] mast cells,[140] and more recently monocytes[136] and even lymphocytes[141]) have been reported to release extracellular traps or DNA. In the latter case, these structures do not contain antibacterial proteins. As their composition may differ from NET, their capacity to modulate immune cell responses and their role in inflammatory and/or autoimmune diseases should be tested. Likewise, as PMN subpopulations have been described in blood and tissues, it would be interesting to compare activities of NET prepared from these different PMN subtypes, first in healthy individuals, and then in patients suffering from different inflammatory and/or autoimmune diseases. Activities of NET are probably influenced by their composition. NET content and NET-associated proteins were originally characterised by immunofluorescence in NET induced in vitro with normal PMN.[18] NET composition was confirmed on PMA-stimulated healthy PMN by mass spectrometry analysis and proteins were quantified by immunoblotting.[19] Then, proteomic analyses on total blood PMN revealed that NET composition differs in healthy individuals according to the stimulus used (PMA vs calcium ionophore).[112] Similar results were obtained with normal PMN stimulated with PMA, calcium ionophore or LPS but showing in addition that post-translational modifications of NET proteins are influenced by the stimulus.[113] Moreover, NET composition also slightly varies according to diseases, as shown with NET induced in vitro by PMA or calcium ionophore on RA versus SLE PMN.[112] Likewise, in vitro, RA-associated stimuli as TNF, rheumatoid factor and RA IgG induce NET with different compositions in PMN from healthy individuals.[101] In addition, IgM rheumatoid factor induce NET containing citrullinated proteins in control PMN.[105] In SLE, NET induced in vitro by LPS stimulation contain lower amounts of ubiquitinated proteins.[96] Similarly, using PMA-stimulated PMN, it has been shown that NET composition is modified in patients with SLE as compared with healthy individuals and even characterises different SLE subsets, especially those with severe disease i.e. with nephritis.[114] Particularly, NET from patients with active SLE are enriched in IL-17A and tissue factor, as evidenced by immunofluorescence and immunoblotting.[142] Replicating proteomic analyses with NET induced by physiological and disease-related stimuli would support hypotheses on NET functions. These data suggest also that epigenetic modifications might control NET activities. Although additional studies will be required to support this hypothesis, NET have been suggested to trigger macrophage activation through the binding of NET ubiquitinated proteins (potentially histones) to CXCR4.[96] In addition, carbamylated NET from patients with RA are particularly efficient in activating fibroblast-like synoviocytes.[109] Likewise, NET induced by particular lupus immune complexes are enriched in oxidised mitochondrial DNA.[54] Additional studies support disease-associated differences in NET composition. Compared with NET from healthy individuals, SLE NET contain increased amounts of acetylated and methylated histones[143]; some of these post-translational modifications are targets of lupus IgG autoantibodies, as acetylated histone H4.[144] Finally, proteomic analyses

revealed the presence of several post-translational modifications in NET, which vary according to the stimulus, and are potentially different between healthy individuals and autoimmune patients.[112 113]

CONCLUSIONS

NET are not only antimicrobial but also antigenic, immunogenic and pro-inflammatory/anti-inflammatory by exposing immunomodulatory molecules, and may behave as a DAMP depending on the microenvironment. This dual pro-inflammatory/anti-inflammatory activity was also observed with aggregated NET. Indeed, aggregated NET may also become pro-inflammatory; they are produced in pancreatic ducts in response to pancreatic juice and promote pancreatic inflammation by occluding pancreatic ducts.[145] Although NET directly act on different immune cell types, resulting in enhanced or impaired cell activity, those effects can be modulated by cofactors able to bind NET, such as C1q and LL-37 which recognise DNA, provided the target cells express cell surface receptors for C1q or LL-37.[72] Further studies will be required to determine to which extent inhibiting NET formation and/or accumulation could be a therapeutic option in autoimmune patients. For example, stimulating signalling via the inhibitory receptor SIRL-1 in PMN using specific antibodies inhibits NET formation in vitro.[146] Finally, as different antibodies recognise NET (eg, ACPA, ANCA, anti-carbamylated protein antibodies), we wonder whether some of them belong to the anti-chromatin antibodies family first described in SLE or whether it is time to define a new anti-NET antibodies family.

Interestingly, similar properties have been reported for NET and extracellular chromatin in patients with SLE and RA, especially their DAMP activity, for example, PMN activation.[94] Nucleosomes represent a key lupus autoantigen. They are present at higher concentrations in the circulation of patients as compared with healthy individuals[147 148] and deposit in kidneys and skin. Similarly, chromatin is present in the synovial fluid of inflamed joints in patients with RA[149] and deposits in affected joints.[150] Part of circulating nucleosomes might derive from NET due to their auto-catabolic activity,[151] especially from the smooth stretches which are probably only composed of histones and DNA and have dimensions similar to nucleosomes.[19] Because it might influence their activity, it would be interesting to determine the cell and tissue origin of circulating nucleosomes and/or NET by analysing nucleosome positioning.[152]

Acknowledgements The authors acknowledge Professor Natacha Bessis (University Sorbonne Paris Nord, Inserm UMR 1125, Bobigny, France) for critical reading of the manuscript.

Contributors PD designed, supervised, wrote and edited the manuscript. DM and AHA participated in writing the original draft.

Funding This work was supported by University Sorbonne Paris Nord and Inserm.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

ORCID iD

Patrice Decker http://orcid.org/0000-0002-8155-2792

REFERENCES

1 Pillay J, den Braber I, Vrisekoop N, *et al*. In vivo labeling with 2H2O reveals a human neutrophil LifeSpan of 5.4 days. *Blood* 2010;116:625–7.
2 Haidar Ahmad A, Melbouci D, Decker P. Polymorphonuclear neutrophils in rheumatoid arthritis and systemic lupus erythematosus: more complicated than anticipated. *Immuno* 2022;2:85–103.
3 Puga I, Cols M, Barra CM, *et al*. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol* 2012;13:170–80.
4 Zhang X, Majlessi L, Deriaud E, *et al*. Coactivation of Syk kinase and Myd88 Adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* 2009;31:761–71.
5 Buckley CD, Ross EA, McGettrick HM, *et al*. Identification of a Phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukoc Biol* 2005;79:303–11.
6 Wigerblad G, Cao Q, Brooks S, *et al*. Single-cell analysis reveals the range of transcriptional States of circulating human neutrophils. *J Immunol* 2022;209:772–82.
7 Massena S, Christoffersson G, Vågesjö E, *et al*. Identification and characterization of VEGF-A-responsive neutrophils expressing Cd49D, Vegfr1, and Cxcr4 in mice and humans. *Blood* 2015;126:2016–26.
8 Iking-Konert C, Ostendorf B, Sander O, *et al*. Transdifferentiation of Polymorphonuclear neutrophils to Dendritic-like cells at the site of inflammation in rheumatoid arthritis: evidence for activation by T cells, Ann. *Annals of the Rheumatic Diseases* 2005;64:1436–42.
9 Poubelle PE, Chakravarti A, Fernandes MJ, *et al*. Differential expression of RANK, RANK-L, and Osteoprotegerin by Synovial fluid neutrophils from patients with rheumatoid arthritis and by healthy human blood neutrophils, arthritis Res. *Arthritis Res Ther* 2007;9:R25.
10 Yeo L, Toellner K-M, Salmon M, *et al*. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis, Ann. *Annals of the Rheumatic Diseases* 2011;70:2022–8.
11 Cross A, Bucknall RC, Cassatella MA, *et al*. Synovial fluid neutrophils transcribe and Express class II major Histocompatibility complex molecules in rheumatoid arthritis. *Arthritis Rheum* 2003;48:2796–806.
12 von Gunten S, Yousefi S, Seitz M, *et al*. Siglec-9 Transduces apoptotic and Nonapoptotic death signals into neutrophils depending on the proinflammatory cytokine environment. *Blood* 2005;106:1423–31.
13 Cross A, Barnes T, Bucknall RC, *et al*. Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. *J Leukoc Biol* 2006;80:521–8.
14 Wright HL, Lyon M, Chapman EA, *et al*. Rheumatoid arthritis Synovial fluid neutrophils drive inflammation through production of Chemokines. *Front Immunol* 2021;11:584116.
15 Denny MF, Yalavarthi S, Zhao W, *et al*. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I Ifns. *J Immunol* 2010;184:3284–97.
16 Rahman S, Sagar D, Hanna RN, *et al*. Low-density Granulocytes activate T cells and demonstrate a non-suppressive role in systemic lupus erythematosus, Ann. *Ann Rheum Dis* 2019;78:957–66.
17 Wright HL, Makki FA, Moots RJ, *et al*. Low-density Granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. *J Leukoc Biol* 2017;101:599–611.
18 Brinkmann V, Reichard U, Goosmann C, *et al*. Neutrophil extracellular traps kill bacteria. *Science* 2004;303:1532–5.

19 Urban CF, Ermert D, Schmid M, *et al*. Neutrophil extracellular traps contain Calprotectin, a cytosolic protein complex involved in host defense against Candida albicans, Plos. *PLoS Pathog* 2009;5:e1000639.
20 Boeltz S, Amini P, Anders H-J, *et al*. To NET or not to NET:Current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ* 2019;26:395–408.
21 Fuchs TA, Abed U, Goosmann C, *et al*. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;176:231–41.
22 Urban CF, Reichard U, Brinkmann V, *et al*. Neutrophil extracellular traps capture and kill Candida albicans yeast and Hyphal forms. *Cell Microbiol* 2006;8:668–76.
23 Bianchi M, Hakkim A, Brinkmann V, *et al*. Restoration of NET formation by Gene therapy in CGD controls Aspergillosis. *Blood* 2009;114:2619–22.
24 Metzler KD, Fuchs TA, Nauseef WM, *et al*. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 2011;117:953–9.
25 Kenno S, Perito S, Mosci P, *et al*. Autophagy and reactive oxygen species are involved in neutrophil extracellular traps release induced by C. albicans Morphotypes. *Front Microbiol* 2016;7:879.
26 Abi Abdallah DS, Lin C, Ball CJ, *et al*. Toxoplasma Gondii triggers release of human and mouse neutrophil extracellular traps. *Infect Immun* 2012;80:768–77.
27 DeSouza-Vieira T, Guimarães-Costa A, Rochael NC, *et al*. Neutrophil extracellular traps release induced by Leishmania: role of Pi3Kgamma, ERK, Pi3Ksigma, PKC, and [Ca2+. *J Leukoc Biol* 2016;100:801–10.
28 Saitoh T, Komano J, Saitoh Y, *et al*. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency Virus-1, cell host. *Cell Host & Microbe* 2012;12:109–16.
29 Caudrillier A, Kessenbrock K, Gilliss BM, *et al*. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest* 2012;122:2661–71.
30 Behnen M, Leschczyk C, Möller S, *et al*. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil Granulocytes via Fcgammariiib and Mac-1. *J Immunol* 2014;193:1954–65.
31 Tadie J-M, Bae H-B, Jiang S, *et al*. Hmgb1 promotes neutrophil extracellular trap formation through interactions with toll-like receptor 4, am. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2013;304:L342–9.
32 Neumann A, Berends ETM, Nerlich A, *et al*. The antimicrobial peptide LL-37 facilitates the formation of neutrophil extracellular traps. *Biochem J* 2014;464:3–11.
33 Steinberg BE, Grinstein S. Unconventional roles of the NADPH oxidase: signaling, ion homeostasis, and cell death. *Sci STKE* 2007;2007:e11.
34 Parker H, Dragunow M, Hampton MB, *et al*. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J Leukoc Biol* 2012;92:841–9.
35 Kenny EF, Herzig A, Krüger R, *et al*. Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife* 2017;6:e24437.
36 Hakkim A, Fuchs TA, Martinez NE, *et al*. Activation of the RAF-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol* 2011;7:75–7.
37 Papayannopoulos V, Metzler KD, Hakkim A, *et al*. Neutrophil Elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol* 2010;191:677–91.
38 Metzler KD, Goosmann C, Lubojemska A, *et al*. A myeloperoxidase-containing complex regulates neutrophil Elastase release and actin Dynamics during Netosis. *Cell Rep* 2014;8:883–96.
39 Leshner M, Wang S, Lewis C, *et al*. Pad4 mediated Histone Hypercitrullination induces Heterochromatin Decondensation and Chromatin unfolding to form neutrophil extracellular trap-like structures. *Front Immunol* 2012;3:307.
40 Pilsczek FH, Salina D, Poon KKH, *et al*. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J Immunol* 2010;185:7413–25.
41 Yipp BG, Petri B, Salina D, *et al*. Infection-induced Netosis is a dynamic process involving neutrophil Multitasking in vivo. *Nat Med* 2012;18:1386–93.
42 Yipp BG, Kubes P. Netosis: how vital is it *Blood* 2013;122:2784–94.
43 Yousefi S, Mihalache C, Kozlowski E, *et al*. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* 2009;16:1438–44.
44 Germic N, Stojkov D, Oberson K, *et al*. Neither Eosinophils nor neutrophils require Atg5-dependent Autophagy for extracellular DNA trap formation. *Immunology* 2017;152:517–25.
45 Amini P, Stojkov D, Wang X, *et al*. NET formation can occur independently of Ripk3 and MLKL signaling. *Eur J Immunol* 2016;46:178–84.
46 Amini P, Stojkov D, Felser A, *et al*. Neutrophil extracellular trap formation requires Opa1-dependent Glycolytic ATP production. *Nat Commun* 2018;9:2958.
47 Hosseinzadeh A, Thompson PR, Segal BH, *et al*. Nicotine induces neutrophil extracellular traps. *J Leukoc Biol* 2016;100:1105–12.
48 Lewis HD, Liddle J, Coote JE, *et al*. Inhibition of Pad4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol* 2015;11:189–91.
49 Tatsiy O, McDonald PP. Physiological stimuli induce Pad4-dependent, ROS-independent Netosis, with early and late events controlled by discrete signaling pathways. *Front Immunol* 2018;9:2036.
50 Spengler J, Lugonja B, Ytterberg AJ, *et al*. Release of active Peptidyl arginine Deiminases by neutrophils can explain production of extracellular Citrullinated Autoantigens in rheumatoid arthritis Synovial fluid. *Arthritis Rheumatol* 2015;67:3135–45.
51 Konig MF, Andrade F. A critical reappraisal of neutrophil extracellular traps and Netosis mimics based on differential requirements for protein Citrullination. *Front Immunol* 2016;7:461.
52 Ashrafi G, Schwarz TL. The pathways of Mitophagy for quality control and clearance of mitochondria. *Cell Death Differ* 2013;20:31–42.
53 Caielli S, Athale S, Domic B, *et al*. Oxidized mitochondrial Nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med* 2016;213:697–713.
54 Lood C, Blanco LP, Purmalek MM, *et al*. Neutrophil extracellular traps enriched in Oxidized mitochondrial DNA are Interferogenic and contribute to lupus-like disease. *Nat Med* 2016;22:146–53.
55 Clark SR, Ma AC, Tavener SA, *et al*. Platelet Tlr4 activates neutrophil extracellular traps to Ensnare bacteria in septic blood. *Nat Med* 2007;13:463–9.
56 Mohanty T, Sjögren J, Kahn F, *et al*. A novel mechanism for Netosis provides antimicrobial defense at the oral mucosa. *Blood* 2015;126:2128–37.
57 Clancy DM, Henry CM, Sullivan GP, *et al*. Neutrophil extracellular traps can serve as platforms for processing and activation of IL-1 family Cytokines. *FEBS J* 2017;284:1712–25.
58 Lauth X, von Köckritz-Blickwede M, McNamara CW, *et al*. M1 protein allows group A Streptococcal survival in phagocyte extracellular traps through Cathelicidin inhibition. *J Innate Immun* 2009;1:202–14.
59 Hirsch JG. Bactericidal action of Histone. *J Exp Med* 1958;108:925–44.
60 Halverson TWR, Wilton M, Poon KKH, *et al*. DNA is an antimicrobial component of neutrophil extracellular traps, Plos. *PLoS Pathog* 2015;11:e1004593.
61 Branzk N, Lubojemska A, Hardison SE, *et al*. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol* 2014;15:1017–25.
62 Thanabalasuriar A, Scott BNV, Peiseler M, *et al*. Neutrophil extracellular traps confine Pseudomonas Aeruginosa ocular Biofilms and restrict brain invasion. *Cell Host Microbe* 2019;25:526–36.
63 Derré-Bobillot A, Cortes-Perez NG, Yamamoto Y, *et al*. Nuclease A (Gbs0661), an extracellular Nuclease of Streptococcus Agalactiae, attacks the neutrophil extracellular traps and is needed for full virulence . *Molecular Microbiology* 2013;89:518–31. 10.1111/mmi.12295 Available: http://doi.wiley.com/10.1111/mmi.2013.89.issue-3
64 Beiter K, Wartha F, Albiger B, *et al*. An Endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. *Curr Biol* 2006;16:401–7.
65 Lappann M, Danhof S, Guenther F, *et al*. In vitro resistance mechanisms of Neisseria Meningitidis against neutrophil extracellular traps. *Mol Microbiol* 2013;89:433–49.
66 Wartha F, Beiter K, Albiger B, *et al*. Capsule and D-Alanylated Lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. *Cell Microbiol* 2007;9:1162–71.
67 Neumann A, Völlger L, Berends ETM, *et al*. Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial Nucleases. *J Innate Immun* 2014;6:860–8.
68 Mohanty T, Fisher J, Bakochi A, *et al*. Neutrophil extracellular traps in the central nervous system hinder bacterial clearance during Pneumococcal meningitis. *Nat Commun* 2019;10:1667.
69 Farrera C, Fadeel B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol* 2013;191:2647–56.

70 Lande R, Ganguly D, Facchinetti V, *et al*. Neutrophils activate Plasmacytoid Dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* 2011;3:73ra19.
71 Barrientos L, Bignon A, Gueguen C, *et al*. Neutrophil extracellular traps Downregulate Lipopolysaccharide-induced activation of monocyte-derived Dendritic cells. *J Immunol* 2014;193:5689–98.
72 Ribon M, Seninet S, Mussard J, *et al*. Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37. *Journal of Autoimmunity* 2019;98:122–31.
73 Apel F, Andreeva L, Knackstedt LS, *et al*. The cytosolic DNA sensor cGAS recognizes neutrophil extracellular traps. *Sci Signal* 2021;14:eaax7942.
74 Lazzaretto B, Fadeel B. Intra- and extracellular degradation of neutrophil extracellular traps by Macrophages and Dendritic cells. *J Immunol* 2019;203:2276–90.
75 Tillack K, Breiden P, Martin R, *et al*. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol* 2012;188:3150–9.
76 Gestermann N, Di Domizio J, Lande R, *et al*. Netting neutrophils activate Autoreactive B cells in lupus. *J Immunol* 2018;200:3364–71.
77 Mitroulis I, Kambas K, Chrysanthopoulou A, *et al*. Neutrophil extracellular trap formation is associated with IL-1Beta and Autophagy-related signaling in gout, Plos. *PLoS ONE* 2011;6:e29318.
78 Schauer C, Janko C, Munoz LE, *et al*. Aggregated neutrophil extracellular traps limit inflammation by degrading Cytokines and Chemokines. *Nat Med* 2014;20:511–7.
79 Xu J, Zhang X, Pelayo R, *et al*. Extracellular Histones are major mediators of death in sepsis. *Nat Med* 2009;15:1318–21.
80 Knopf J, Leppkes M, Schett G, *et al*. Aggregated nets Sequester and Detoxify extracellular Histones. *Front Immunol* 2019;10:2176.
81 Bilyy R, Fedorov V, Vovk V, *et al*. Neutrophil extracellular traps form a barrier between necrotic and viable areas in acute abdominal inflammation. *Front Immunol* 2016;7:424.
82 Lin AM, Rubin CJ, Khandpur R, *et al*. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. *J Immunol* 2011;187:490–500.
83 Giusti D, Bini E, Terryn C, *et al*. NET formation in Bullous Pemphigoid patients with relapse is modulated by IL-17 and IL-23 interplay. *Front Immunol* 2019;10:701.
84 Kessenbrock K, Krumbholz M, Schönermarck U, *et al*. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009;15:623–5.
85 Sangaletti S, Tripodo C, Chiodoni C, *et al*. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid Dendritic cells toward ANCA induction and associated Autoimmunity. *Blood* 2012;120:3007–18.
86 Dotan A, Muller S, Kanduc D, *et al*. The SARS-Cov-2 as an instrumental trigger of Autoimmunity. *Autoimmun Rev* 2021;20:102792.
87 Veras FP, Pontelli MC, Silva CM, *et al*. SARS-Cov-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *J Exp Med* 2020;217:e20201129.
88 Middleton EA, He X-Y, Denorme F, *et al*. Neutrophil extracellular traps contribute to Immunothrombosis in COVID-19 acute respiratory distress syndrome. *Blood* 2020;136:1169–79.
89 Carmona-Rivera C, Zhang Y, Dobbs K, *et al*. Multicenter analysis of neutrophil extracellular trap dysregulation in adult and pediatric COVID-19, JCI. *JCI Insight* 2022;7:e160332.
90 Villanueva E, Yalavarthi S, Berthier CC, *et al*. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose Immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* 2011;187:538–52.
91 Kahlenberg JM, Carmona-Rivera C, Smith CK, *et al*. Neutrophil extracellular trap-associated protein activation of the Nlrp3 Inflammasome is enhanced in lupus Macrophages. *J Immunol* 2013;190:1217–26.
92 Hakkim A, Fürnrohr BG, Amann K, *et al*. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis, Proc. *Proc Natl Acad Sci USA* 2010;107:9813–8.
93 Antiochos B, Trejo-Zambrano D, Fenaroli P, *et al*. The DNA sensors Aim2 and Ifi16 are SLE Autoantigens that bind neutrophil extracellular traps. *Elife* 2022;11:e72103.
94 Lindau D, Mussard J, Rabsteyn A, *et al*. Tlr9 independent interferon alpha production by neutrophils on Netosis in response to circulating Chromatin, a key lupus Autoantigen. *Ann Rheum Dis* 2014;73:2199–207.
95 van der Linden M, van den Hoogen LL, Westerlaken GHA, *et al*. Neutrophil extracellular trap release is associated with Antinuclear antibodies in systemic lupus erythematosus and anti-phospholipid syndrome, rheumatology. *Rheumatology* 2018;57:1228–34.
96 Barrera-Vargas A, Gómez-Martín D, Carmona-Rivera C, *et al*. Differential Ubiquitination in nets regulates macrophage responses in systemic lupus erythematosus, Ann. *Ann Rheum Dis* 2018;77:annrheumdis–2017.
97 Carmona-Rivera C, Zhao W, Yalavarthi S, *et al*. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix Metalloproteinase-2, Ann. *Ann Rheum Dis* 2015;74:1417–24.
98 Blanco LP, Wang X, Carlucci PM, *et al*. RNA Externalized by neutrophil extracellular traps promotes inflammatory pathways in endothelial cells. *Arthritis Rheumatol* 2021;73:2282–92.
99 Georgakis S, Gkirtzimanaki K, Papadaki G, *et al*. Nets decorated with bioactive IL-33 infiltrate inflamed tissues and induce Ifnalpha production in SLE patients, JCI. *Insight* 2021;6:e147671.
100 Garcia-Romo GS, Caielli S, Vega B, *et al*. Netting neutrophils are major Inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* 2011;3:73ra20.
101 Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, *et al*. Nets are a source of Citrullinated Autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013;5:178ra40.
102 Papadaki G, Kambas K, Choulaki C, *et al*. Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation. *Eur J Immunol* 2016;46:2542–54.
103 Schneider AH, Machado CC, Veras FP, *et al*. Neutrophil extracellular traps mediate joint hyperalgesia induced by immune inflammation, rheumatology. *Rheumatology* 2021;60:3461–73.
104 Navrátilová A, Bečvář V, Baloun J, *et al*. S100A11 (Calgizzarin) is released via Netosis in rheumatoid arthritis (RA) and stimulates IL-6 and TNF secretion by neutrophils. *Sci Rep* 2021;11:6063.
105 Carmona-Rivera C, Carlucci PM, Moore E, *et al*. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci Immunol* 2017;2:eaag3358.
106 Pratesi F, Dioni I, Tommasi C, *et al*. Antibodies from patients with rheumatoid arthritis target Citrullinated Histone 4 contained in neutrophils extracellular traps, Ann. *Ann Rheum Dis* 2014;73:1414–22.
107 Corsiero E, Bombardieri M, Carlotti E, *et al*. Single cell cloning and Recombinant Monoclonal antibodies generation from RA Synovial B cells reveal frequent targeting of Citrullinated Histones of nets, Ann. *Ann Rheum Dis* 2016;75:1866–75.
108 Shi J, Knevel R, Suwannalai P, *et al*. Autoantibodies recognizing Carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage, Proc. *Proc Natl Acad Sci USA* 2011;108:17372–7.
109 O'Neil LJ, Barrera-Vargas A, Sandoval-Heglund D, *et al*. Neutrophil-mediated Carbamylation promotes Articular damage in rheumatoid arthritis. *Sci Adv* 2020;6:eabd2688.
110 O'Neil LJ, Oliveira CB, Sandoval-Heglund D, *et al*. Anti-Carbamylated LI37 antibodies promote pathogenic bone Resorption in rheumatoid arthritis. *Front Immunol* 2021;12:715997.
111 de Bont CM, Stokman MEM, Faas P, *et al*. Autoantibodies to neutrophil extracellular traps represent a potential serological biomarker in rheumatoid arthritis. *J Autoimmun* 2020;113:S0896-8411(20)30102-5:102484.:.
112 Chapman EA, Lyon M, Simpson D, *et al*. Caught in a trap? Proteomic analysis of neutrophil extracellular traps in rheumatoid arthritis and systemic lupus erythematosus. *Front Immunol* 2019;10:423.
113 Petretto A, Bruschi M, Pratesi F, *et al*. Neutrophil extracellular traps (NET) induced by different stimuli: A comparative Proteomic analysis. *PLoS One* 2019;14:e0218946.
114 Bruschi M, Petretto A, Santucci L, *et al*. Neutrophil extracellular traps protein composition is specific for patients with lupus nephritis and includes methyl-Oxidized Alphaenolase (Methionine Sulfoxide 93). *Sci Rep* 2019;9:7934.
115 Carmona-Rivera C, Carlucci PM, Goel RR, *et al*. Neutrophil extracellular traps mediate Articular cartilage damage and enhance cartilage component Immunogenicity in rheumatoid arthritis, JCI. *JCI Insight* 2020;5:e139388.
116 Jung J, Lee JS, Kim YG, *et al*. Synovial fluid Cd69(+)Cd8(+) T cells with tissue-resident phenotype mediate Perforin-dependent Citrullination in rheumatoid arthritis. *Clin Transl Immunology* 2020;9:e1140.
117 O'Neil LJ, Oliveira CB, Wang X, *et al*. Neutrophil extracellular trap-associated Carbamylation and Histones trigger Osteoclast

formation in rheumatoid arthritis, Ann. *Ann Rheum Dis* 2023;82:630–8.
118 Campbell AM, Kashgarian M, Shlomchik MJ. NADPH oxidase inhibits the pathogenesis of systemic lupus erythematosus. *Sci Transl Med* 2012;4:157ra141.
119 Kienhöfer D, Hahn J, Stoof J, *et al*. Experimental lupus is aggravated in mouse strains with impaired induction of neutrophil extracellular traps, JCI. *Insight* 2017;2:e92920.
120 Gordon RA, Herter JM, Rosetti F, *et al*. Lupus and proliferative nephritis are Pad4 independent in murine models, JCI. *Insight* 2017;2:e92926.
121 Pieterse E, Rother N, Yanginlar C, *et al*. Cleaved N-terminal Histone tails distinguish between NADPH oxidase (NOX)-Dependent and NOX-independent pathways of neutrophil extracellular trap formation, Ann. *Ann Rheum Dis* 2018;77:1790–8.
122 Knight JS, Subramanian V, O'Dell AA, *et al*. Peptidylarginine Deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/Lpr mice, Ann . *Ann Rheum Dis* 2015;74:2199–206.
123 Shelef MA, Sokolove J, Lahey LJ, *et al*. Peptidylarginine Deiminase 4 contributes to tumor necrosis factor alpha-induced inflammatory arthritis. *Arthritis & Rheumatology* 2014;66:1482–91. 10.1002/art.38393 Available: http://doi.wiley.com/10.1002/art.v66.6
124 Fukui S, Gutch S, Fukui S, *et al*. The prominent role of hematopoietic Peptidyl arginine Deiminase 4 in arthritis: Collagen- and granulocyte colony-stimulating factor-induced arthritis model in C57Bl/6 mice. *Arthritis Rheumatol* 2022;74:1139–46.
125 Gajendran C, Fukui S, Sadhu NM, *et al*. Alleviation of arthritis through prevention of neutrophil extracellular traps by an orally available inhibitor of protein arginine Deiminase 4, SCI. *Sci Rep* 2023;13:3189.
126 Odobasic D, Muljadi RCM, O'Sullivan KM, *et al*. Suppression of Autoimmunity and renal disease in Pristane-induced lupus by myeloperoxidase. *Arthritis Rheumatol* 2015;67:1868–80.
127 Leffler J, Martin M, Gullstrand B, *et al*. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol* 2012;188:3522–31.
128 Wong SL, Demers M, Martinod K, *et al*. Diabetes primes neutrophils to undergo Netosis, which impairs wound healing. *Nat Med* 2015;21:815–9.
129 Parackova Z, Zentsova I, Vrabcova P, *et al*. Neutrophil extracellular trap induced Dendritic cell activation leads to Th1 polarization in type 1 diabetes. *Front Immunol* 2020;11:661.
130 Yoo IS, Lee SY, Park CK, *et al*. Serum and Synovial fluid concentrations of cold-inducible RNA-binding protein in patients with rheumatoid arthritis. *Int J Rheum Dis* 2018;21:148–54. 10.1111/1756-185X.12892 Available: http://doi.wiley.com/10.1111/apl.2018.21.issue-1
131 Chen K, Murao A, Arif A, *et al*. Inhibition of Efferocytosis by extracellular CIRP-induced neutrophil extracellular traps. *J Immunol* 2021;206:797–806.
132 Yasuda K, Yu P, Kirschning CJ, *et al*. Endosomal translocation of vertebrate DNA activates Dendritic cells via Tlr9-dependent and -Independent pathways. *J Immunol* 2005;174:6129–36.
133 Barton GM, Kagan JC, Medzhitov R. Intracellular localization of toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat Immunol* 2006;7:49–56.
134 Lindau D, Mussard J, Wagner BJ, *et al*. Primary blood neutrophils express a functional cell surface toll-like receptor 9. *Eur J Immunol* 2013;43:2101–13.
135 Hahn J, Schauer C, Czegley C, *et al*. Aggregated neutrophil extracellular traps resolve inflammation by proteolysis of Cytokines and Chemokines and protection from Antiproteases. *FASEB J* 2019;33:1401–14.
136 Granger V, Faille D, Marani V, *et al*. Human blood monocytes are able to form extracellular traps. *J Leukoc Biol* 2017;102:775–81.
137 Bao Y, Cao X. Revisiting the protective and pathogenic roles of neutrophils: Ly-6G is key *Eur J Immunol* 2011;41:2535–8.
138 Yousefi S, Gold JA, Andina N, *et al*. Catapult-like release of mitochondrial DNA by Eosinophils contributes to Antibacterial defense. *Nat Med* 2008;14:949–53.
139 Morshed M, Hlushchuk R, Simon D, *et al*. NADPH oxidase-independent formation of extracellular DNA traps by Basophils. *J Immunol* 2014;192:5314–23.
140 von Köckritz-Blickwede M, Goldmann O, Thulin P, *et al*. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* 2008;111:3070–80.
141 Ingelsson B, Söderberg D, Strid T, *et al*. Lymphocytes eject Interferogenic mitochondrial DNA Webs in response to Cpg and non-Cpg Oligodeoxynucleotides of class C, Proc. *Proc Natl Acad Sci USA* 2018;115:E478–87.
142 Frangou E, Chrysanthopoulou A, Mitsios A, *et al*. Redd1/Autophagy pathway promotes Thromboinflammation and fibrosis in human systemic lupus erythematosus (SLE) through nets decorated with tissue factor (TF) and Interleukin-17A (IL-17A). *Ann Rheum Dis* 2019;78:238–48.
143 Pieterse E, Hofstra J, Berden J, *et al*. Acetylated Histones contribute to the Immunostimulatory potential of neutrophil extracellular traps in systemic lupus erythematosus. *Clin Exp Immunol* 2014;179:68–74.
144 Liu C, Tangsombatvisit S, Rosenberg JM, *et al*. Specific post-Translational Histone modifications of neutrophil extracellular traps as Immunogens and potential targets of lupus Autoantibodies, arthritis Res. *Arthritis Res Ther* 2012;14:R25.
145 Leppkes M, Maueröder C, Hirth S, *et al*. Externalized Decondensed neutrophil Chromatin Occludes Pancreatic ducts and drives Pancreatitis. *Nat Commun* 2016;7:10973.
146 Van Avondt K, Fritsch-Stork R, Derksen RHWM, *et al*. Ligation of signal inhibitory receptor on Leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus, Plos. *PLoS ONE* 2013;8:e78459.
147 Amoura Z, Piette JC, Chabre H, *et al*. Circulating plasma levels of Nucleosomes in patients with systemic lupus erythematosus: correlation with serum Antinucleosome antibody titers and absence of clear association with disease activity. *Arthritis Rheum* 1997;40:2217–25.
148 Rumore PM, Steinman CR. Endogenous circulating DNA in systemic lupus erythematosus. occurrence as Multimeric complexes bound to Histone. *J Clin Invest* 1990;86:69–74.
149 Yu D, Rumore PM, Liu Q, *et al*. Soluble Oligonucleosomal complexes in Synovial fluid from inflamed joints. *Arthritis & Rheumatism* 1997;40:648–54. 10.1002/art.1780400409 Available: http://doi.wiley.com/10.1002/art.v40:4
150 Monach PA, Hueber W, Kessler B, *et al*. A broad screen for targets of immune complexes decorating Arthritic joints highlights deposition of Nucleosomes in rheumatoid arthritis, Proc. *Proc Natl Acad Sci USA* 2009;106:15867–72.
151 Pisareva E, Mihalovičová L, Pastor B, *et al*. Neutrophil extracellular traps have auto-Catabolic activity and produce Mononucleosome-associated circulating DNA. *Genome Med* 2022;14:135.
152 Snyder MW, Kircher M, Hill AJ, *et al*. Cell-free DNA comprises an in vivo Nucleosome Footprint that informs its tissues-of-origin. *Cell* 2016;164:S0092-8674(15)01569-X:57–68.:.
153 Patiño-Trives AM, Pérez-Sánchez C, Pérez-Sánchez L, *et al*. Anti-dsDNA antibodies increase the cardiovascular risk in systemic lupus erythematosus promoting a distinctive immune and vascular activation, Arterioscler. *ATVB* 2021;41:2417–30.
154 Lou H, Wojciak-Stothard B, Ruseva MM, *et al*. Autoantibody-dependent amplification of inflammation in SLE. *Cell Death Dis* 2020;11:729.

DISCUSSION AND PERSPECTIVES

Complex interactions between various immune cells and their signaling molecules play an important role in the maintenance of immune homeostasis. NETs, originally described as an important defense mechanism against microbial invaders, have emerged as significant contributors of both antimicrobial defense and immune dysregulation. In this work, we examined in detail the role of NETs, particularly their interaction with B lymphocytes, and the potential implications for autoimmune conditions, with a focus on RA.

The pro-inflammatory effect of NETs on B cells is one of the interesting results addressed here. This finding illustrates the considerable interplay between neutrophils and other immune cells, indicating that NETs might be powerful mediators of inflammation. Furthermore, the current work offers a new mean in which NETs act as DAMPs to activate polyclonaly B cells, independently of antigens specificity. The ability of NETs to influence the behavior of B cells highlights the complexity and adaptability of immune responses. This finding sheds new light on the etiology of autoimmune disorders, notably RA, which is characterized by B cell hyperactivity.

I. NET induction

In this study we used a non-physiological stimulus, PMA, to induce NET formation. PMA has been criticized for a lack of biological relevance. However, PMA is a well-characterized and reproducible stimulus for NET formation, which allows researchers to study the effects of NETs on cell function. PMA provides a reliable and standardized method to trigger NET formation across different experimental setups. This consistency is valuable for comparative studies and reproducibility. Its standardized induction and potency make it suitable for various experimental settings. Moreover, it mimics strong NET formation. Finally, PMA induces NET formation by activating pathways related to physiological stimuli (**137**).

NETs employed in this study were extensively characterized by measuring DNA and proteins contained in NETs, and immunofluorescence was used to observe NET structure and to evaluate the presence and colocalization of primary proteins associated to NETs with the DNA, such as MPO, NE, histones, and others.

Additionally, PMA may inadvertently stimulate other cell types besides neutrophils, leading to confounding effects in the experimental results. However, our NET induction procedure includes

repeated washings of NET to remove any interfering molecules. Moreover, our negative control (NET-buffer), which was also prepared with PMA but in the absence of PMNs, had no or just a slight effect on cells, suggesting that PMA was not transferred with NETs.

It is believed that PMA-induced NET formation may not faithfully replicate the complexity and specificity of NETosis in response to different pathogens. However, it has been shown recently that Raman spectral signatures of NETs elicited by PMA and LPS (as physiological stimulus) were comparable, with very minor changes (**124**). Raman spectroscopy is a non-destructive chemical analysis technique that provides detailed information about chemical structure and molecular interactions. However, proteomic analysis of these NETs has shown some differences (**139**).

Therefore, NET induction with more physiological triggers, e.g. immune complexes, cytokines, or RA serum, will lead to a more comprehensive understanding of NET impact in RA context.

Nonetheless, the use of PMA has provided valuable insights into the mechanisms and consequences of NET formation, furthering our understanding of the role of NETs in immune responses and disease pathogenesis.

II. Activation of B cells by NETs

After B cell activation, several activation markers are upregulated on the surface of B cells, including HLA-DR, CD40, CD69, CD86 and others (**257**). B cells may also undergo changes in their intracellular signaling and gene expression profiles, leading for example to cytokine production. In the present study, we used flow cytometry to examine the expression of the aforementioned markers and intracellular cytokine production, ELISA to assess cytokine and antibody secretion, and RNA sequencing to identify gene expression in B cells following NET activation.

To date, few studies have addressed B cell activation by NETs. B cells can act as APC, and also influence immune response by producing cytokines (**477**). However, most of these studies have focused on indirect B cell activation by NETs, via synovial fibroblasts, or dendritic cells, or via immune complexes (**153, 359**). All of these studies were interested in NETs as a source of auto-antigens which led to B cell activation and subsequent antibody production. Nevertheless, NETs can provide co-stimulatory signals to B cells through the engagement of Toll-like receptors, which might promote B cell activation.

In the present study, we provided evidence that NETs are pro-inflammatory, act as DAMPs and could directly activate total and naïve B cells from healthy donors and from patients with RA. We demonstrate that both in healthy individuals and RA patients, NETs induce the upregulation of HLA-DR and co-stimulatory molecules CD40 and CD86 on B lymphocytes. This suggests that NETs can activate B cells and potentially enhance their antigen-presenting capabilities. The upregulation of these molecules is crucial for effective interaction with other immune cells, such as T cells, and is indicative of B cell activation. Moreover, purified naïve/total B lymphocytes respond to NETs by secreting various cytokines, including IL-8, IL-6, TNFα, and total IgG. This indicates that NETs can elicit an inflammatory response from B cells. Notably, the absence of IL-10 production suggests that the B cell response to NETs is primarily pro-inflammatory and may be involved in promoting inflammation or autoimmune responses. We may test a broader panel of cytokines, both pro or anti-inflammatory, by multiplex assays. Elevated levels of pro-inflammatory cytokines, particularly TNF and IL-6, have been associated with various autoimmune diseases, including RA and SLE (**422, 478**). This finding suggests that the interaction between NETs and B cells might be a contributing factor in the pathogenesis of these conditions. The study might have relevance in understanding the immune dysregulation seen in these autoimmune diseases.

Furthermore, NET-activated total and naïve B cells were enriched in mRNA coding for pro-inflammatory compounds, including cytokines e.g. IL-8, RANKL, IL-1β, IL-11, Fibroblast Growth Factor (FGF2), lymphotoxin α, metalloproteinases e.g. MMP2, MMP7, chemokines such as CCL7 and CCL13, and others. In contrast, immunomodulatory genes such as IL10R, and TGFbR2 were downregulated following activation with NETs in both total and naïve B cells. The upregulation of pro-inflammatory genes in NET-activated B cells underscores their role in amplifying the overall inflammatory response. This finding sheds light on the multifaceted roles of NETs in immune responses and has implications for immune dysregulation.

A. NETs induce total IgG production by B cells

Total B cells were cultured for 3 days with NETs or LPS+CpG as positive control. Despite the short period of culture, total IgG were secreted by NET-activated B cells as measured by ELISA. LPS and CpG were also able to induce IgG production by B cells. In this experiment, total B cells were used, and plasma cells may be present among these cells. Plasma cells are the primary

producers of antibodies. The frequency of these cells was not investigated in our culture following stimulation with NETs. However, before culture, the frequencies of memory ($CD19^+CD27^+IgD^-$) and naive ($CD19^+CD27^-IgD^+$) B cells were analyzed. Both naive and memory B cells are known to progress to a plasma-cell following stimulation by cytokines (IL-21) (**479**). Plasma cells derived from $CD27^+$ B cells are primarily IgG^+, while those from $CD27^-$ B cells are IgM^+. Plasmablast ($CD27^+CD38^+$) and plasma cell ($CD27^+CD38^+CD138^+$) formation starts at day 6 after T-Cell-Independent (TI) stimulation with CpG and IL-2 (**480**). Other studies showed that memory and naïve B-cell stimulation via TLR7/8 induces greater differentiation than stimulation via TLR9 and occurs after 4 days of culture (**481**).

Furthermore, we found in some cultures of naïve b cells stimulated by NETs, an increased IgG secretion. Five percent of sorted naive B cells were $CD19^+CD27^-IgD^-$. The ability of these cells to generate IgG has not been studied. However, IgG production by naive B cells has already been seen in supernatants following IL-21 stimulation but after 10 days of culture (**479**).

Additionally, B cells in RA patients can produce autoantibodies such as RF and ACPAs. NETs and ACPA production in RA is an area of active research. NETs act as source of auto-antigens and induce ACPA production by B cells. The direct influence of NETs on ACPA secretion has not yet been explored. Following NET stimulation, we noticed that RA B cells generate IgG. However, ACPA-containing IgG antibodies were not measured. The finding raises the possibility that NETs may contribute to the autoimmune response in RA, either by directly stimulating autoantibody production or by exacerbating the underlying immune dysregulation. Monitoring IgG levels in response to NETs might provide insights into the progression of RA. Moreover, targeting the NET-mediated pathways that drive IgG production might provide a means to mitigate RA and reduce inflammation.

B. RA B cells are more activated than HD B cells

The high expression of HLA-DR, CD40 and CD86 on B cells from RA patients is a significant observation and highlights the heightened activation and stimulatory capacity of these B cells in the context of the disease. Several lines of evidence indicate that B cells are more activated in RA patients. Moreover, we and other studies have shown that B cells in RA patients exhibit an activated phenotype. They express higher levels of activation markers, such as HLA-DR, CD86, and CD40, compared to B cells from healthy individuals (**372, 379**). Using flow cytometry, we

confirmed the upregulation of these markers on freshly purified B cells from patients with RA compared to those from HDs. This heightened expression of activation markers suggests that B cells in RA patients are primed and more responsive to stimuli. Additionally, it's possible that the overactive B cells are producing more autoantibodies or engaging in interactions with other immune cells, further perpetuating the inflammatory cascade.

Furthermore, the synovial fluid and tissue is the battleground in RA, where the autoimmune attack is concentrated. The synovial microenvironment is distinct from the systemic circulation. It contains factors and signals that may promote the activation and recruitment of immune cells. Moreover, B cells in the synovial tissue and fluid of affected joints are often organized into lymphoid structures known as ectopic lymphoid follicles, suggesting ongoing B cell activation and local immune responses within the joints. Expression of activation markers on B cells from RA synovial fluid vs. peripheral blood has not been explored. In our study, we compared the expression of the above markers on SF B cells among mononuclear cells compared to B cells from the peripheral blood. Our findings demonstrate that B cells from the synovial fluid of RA patients indeed exhibit heightened active phenotypes compared to those in the peripheral blood. The heightened B cell activity within the synovial fluid suggests that these cells may play a more direct role in the pathogenesis of the joint-specific autoimmunity in RA. CD86 expression was most prevalent in SF B cells, confirming a potential involvement of this marker in RA pathogenesis.

CD28 and CTLA-4 are the two primary receptors on the surface of T cells that bind CD86. Binding to CD28 stimulates lymphocytes, which boosts the immune response, whereas binding to CTLA-4 suppresses lymphocyte activation, reducing immunity (**482**). CD86 expression on B cells can impact the balance between different types of immune responses. The interaction between CD86 and T cells can influence the differentiation of T cells into various subsets, including pro-inflammatory Th1 and Th17 cells (**483**). In certain autoimmune conditions, B cells can have both pathogenic and regulatory roles. CD86 expression on B cells can influence their ability to interact with T cells and regulate immune responses. In some cases, CD86-expressing B cells might promote regulatory T cell responses that help control autoimmunity (**484**). Here I suggest that CD86 expression on B cells is higher in RA, in order to provide signals to regulate the immune responses. However, we didn't analyze the correlation between CD86 expression on B cells and frequencies of T regulatory cells in RA.

C. RA B cells are more prone to activation by NETs and NETs act as immune amplifiers

On the flip side, the pro-inflammatory response induced by NETs in B cells raises questions about the potential role of NETs in autoimmune diseases. Dysregulated immune responses, characterized by excessive inflammation, are central to many autoimmune disorders. If NETs contribute also to the activation of self-reactive B cells, they may be implicated in the pathogenesis of autoimmune diseases.

Accumulated NETs were found in RA due to increased NET formation and impaired NET degradation. NETs have also been detected in the synovial fluid and synovial tissues of affected joints (**354**). They are believed to contribute to the chronic inflammation and tissue damage seen in RA. We have already shown that RA-derived NETs can activate various immune cells, including monocytes and neutrophils, through several mechanisms (**485**).

In another study, we have shown that RA neutrophils produce more NETs compared to HD neutrophils (**342**). Also, RA NET composition differs from HD NETs (**141**). So we intended to explore whether the RA NET composition had any effect on B cell activation. Indeed, we showed no difference between response of B cells to NETs from both HDs and RA patients, excluding a protein composition difference of RA NETs. However, in this experiment, we used NETs from HDs or RA patients at the same DNA concentration. In various contexts, the DNA composition of NETs might vary. For instance, NETs from SLE patient neutrophils have enhanced quantity of mitochondrial DNA compared to HDs NETs (**142**), and presence of mitochondrial DNA could enhance the inflammatory response. Thus, presence of mitochondrial DNA in RA NETs has not been studied in our study.

Furthermore, the heightened expression of activation markers suggests that B cells in RA patients are primed and more responsive to stimuli. We found that RA B cells are more prone to activation by NETs compared to HD B cells. HLA-DR, CD40 and CD86 expression was significantly higher on RA B cells following stimulation with NETs compared to HD B cells. NETs as DAMPs may be detected by innate receptors such as TLR. Various TLRs are overexpressed in RA patients (**486**). This might explain why RA B cells are so reactive to NET stimulation compared to HD B cells. However, several factors may contribute to the increased activation of B cells in RA. We demonstrated that in RA, NETs are belonging the factors involved in this activation.

Additionally, higher expression of HLA-DR, CD40, and CD86 on B cells suggests an increased potential for antigen presentation and co-stimulation. This may lead to a more robust immune response, including the production of autoantibodies and inflammatory cytokines.

D. CD40 expression by NET-activated RA B cell is significantly correlated to disease activity

CD40 is a co-stimulatory molecule expressed on B cells, among other immune cells. Its primary role is to facilitate interactions between B cells and T cells and to promote antibody production. Activation via CD40 plays a crucial role in immune responses, and its dysregulation can contribute to the development of autoimmune diseases (**271**). CD40 on B cells interacts with CD40 ligand (CD40L) on activated T cells. Expression of CD40L on RA $CD4^+$ T cells is associated with active disease (**487**).

Disease activity in RA is associated with the extent of inflammation, pain, and number of affected joint. Monitoring disease activity is essential for disease management and treatment decisions. In this study, we found an elevated CD40 expression on RA B cells activated by NETs. CD40 expression was positively and significantly correlated to disease activity estimated by DAS28.CRP. When disease is more active, it suggests that B cells are more sensitive to NETs, which, in turn, can reflect increased immune responses and inflammation associated with more active disease.

Further research is needed to elucidate the precise mechanisms by which NET stimulation leads to increased CD40 expression on B cells and how this relates to disease activity.

In summary, the correlation between CD40 expression by NET-activated RA B cells and disease activity indicates that CD40 expression may be a valuable indicator of disease severity and a potential target for therapeutic interventions.

III. Understanding the mechanism by which NETs activate B cells

We investigated the precise molecular mechanisms underlying NET-induced B cell activation, as well as the signaling pathways involved and the downstream effects on other immune cells, which are all critical stages in completely comprehending the complexities of this phenomenon.

A. B cell activation by NETs is modulated by C1q and LL-37

Several proteins in the inflammatory environment can affect B cell activation. In this study, we tested the impact of C1q and LL-37 on B cell activation by NETs.

The complement system is a part of the immune system that consists of a group of proteins (such as C1q). These proteins play a crucial role in enhancing the immune response. We have already shown that C1q complement protein may enhance the NET-induced IL-8 secretion by macrophages (**352**). Moreover, some complement components can directly interact with B cells to promote their activation and differentiation into antibody-producing cells. In healthy individuals, C1q is involved in the activation of primed B lymphocytes to produce IgM or IgG isotypes (**488**). Our RNAseq data shows that both naïve and total B cells upregulate the expression of one of C1q receptor, complement component 1q subcomponent binding protein (C1qbp), following stimulation with NETs. C1qbp was assumed to function as a receptor for the globular part of C1q, playing an important role in the inflammatory response (**489**).

Additionally, the cathelicidin peptide LL-37 is known to have the ability to bind nucleic acids and enhance their detection by endosomal TLRs (**197**). likewise, LL37-DNA complexes in NETs have been shown to directly activate human memory B (**242**).

Firstly, in the absence of NETs, we found that LL-37 had no direct influence on B cell activation marker expression or cytokine production. In contrast, C1q has an inhibitory effect on B cells through decreasing IL-6 production and CD40 and HLA-DR expression. Conversely, LL-37 demonstrates a more modest effect on B cell CD40 expression only. In the presence of NETs, we have found that B cell activation by NETs is modulated in the presence of C1q and LL-37. LL-37 and C1q didn’t act similarly. TNF production and HLA-DR, CD40, and CD86 expression on B cells were considerably higher when stimulated with NETs in the presence of C1q than when treated with NETs alone. In summary, we found that inflammatory mediators like C1q and LL-37, which are generated during inflammation, can function synergistically with NETs to further activate B cells. The modulation of B cell activation by C1q and LL-37 suggests that the immune response is finely regulated at multiple levels. Additional investigation is required to understand how these proteins interact with NETs. These proteins or peptides could be targeted or manipulated in a therapeutic approach to either enhance or dampen the immune response,

depending on the clinical context. For example, in autoimmune diseases, targeting C1q and LL-37 might be explored to reduce excessive immune activation.

On the other hand, it's important to investigate how the roles of C1q and LL-37 in B cell activation by NETs change in various disease states and at different disease kinetics/phases. For example, the dynamics might differ in infections, autoimmune diseases, and cancer. A comprehensive understanding of these interactions can lead to better targeted therapies.

B. NETs activate B cells in a TLR9 independent manner

NETs contain various immune-stimulatory molecules, and they are mostly composed of DNA. Indeed, DNA is recognized by TLR9, a toll-like receptor that preferentially recognizes DNA. We found that NETs activate B cells in a TLR9-independent manner. Gestermann et al. demonstrated that LL37-DNA complexes, an artificial complex that mimics NET structure, trigger polyclonal B cell activation via TLR9. With NET stimulation, the response of memory B cell was only partially TLR9-independent (**242**). Despite significant research, unlike natural NETs, which can vary in composition depending on the triggering stimuli and neutrophil state, mimicking NET could limit the natural function of NETs due to their complex structure and properties. Different protein combinations in the NETs operate synergistically or antagonistically. For instance, DNA-MPO and DNA-NE complexes fail to achieve NETs natural function, they fail to puncture the bacterial cell wall or affect their viability (**490**).

The finding underscores the complexity of the immune response to NETs. It implies the involvement of alternative pattern recognition receptors or mechanisms in sensing NET components and triggering B cell activation. Further research is needed to identify these receptors and understand their role.

Other receptors on B cells could recognize NETs DNA, such as absent in melanoma 2 (AIM2), cyclic-GMP-AMP synthase (cGAS) and NLRP3. For instance, THP1 cells in which cGAS or STING was deleted did not respond to NETs (**186**). Implication of these receptors in B cell activation by NET should be evaluated. However, we do not see IFN-I in contrast to Apel. Indeed, these receptors are not totally DNA specific, which may make it difficult to determine whether the primary cell activators are DNA or proteins in NETs. For instance, DNA binding proteins, such as

the mitochondrial transcription factor A (TFAM) or HMGB1 (present in NET), have been demonstrated to activate cGAS (**177**). Finally, NET recognition may occur via proteins.

IV. Consequences of B cell activation by NETs on immune cells

The consequences of NET-activated B cells extend beyond their direct activation. These hyper-activated B cells may impact other immune cells, such as neutrophils and T cells, orchestrating a complicated network of interactions. The suggested amplification of this mechanism in RA patients emphasizes the clinical significance and consequences of our results. Unraveling the complexities of how NETs influence B cell activity holds potential for the development of therapeutics aimed at reducing the abnormal immune responses observed in autoimmune illnesses.

A. NET-activated B cells act as antigen presenting cells and stimulate T cells

We found that following NET activation, B cells upregulate HLA-DR, CD40, and CD86, all of which are required for antigen-presenting cell activity. It is known that B cells can act as effective APC and become increasingly important in activating T cells. Therefore, our study further explored the consequences of B cell activation by NETs on T cells. In this experiment, we performed a mixed lymphocyte reaction (MLR) assay. Similar results were observed in the autologous setup. NET-activated B cells were co-cultured with T cells from different individuals with different human HLA types. The capacity of B cells to serve as stimulator cells for a primary MLR assay has already been determined (**491**). Our results showed that NET-activated B cells play the role of APC and induce T-cell proliferation, whereas LPS+CpG-activated B cells didn't induce T cell proliferation. We found by ELISA that in contrast to NETs, LPS+CpG induced IL-10 production by purified B cells (Supplemental figure 2). Thus, B cells activated by LPS and CpG may continue producing IL-10 in B-T cell co-culture, an immuno-modulatory cytokine that may inhibit T cell activation. Also, LPS-activated B cells were found to suppress T cell proliferation in a dendritic and T cells co-culture, probably due to IL-10 and PD-L1 expression (**492**).

However, one limitation of these experiments is that B cells were washed before co-culture with heterologous T cells. Therefore, pro-inflammatory cytokines generated by NET-activated B cells are removed as well. These cytokines would work synergistically with activated B cells to further stimulate T cells. B cells were washed to eliminate NETs from supernatants because NETs are

known to activate T cells (**468**), and we primarily looked at the effect of B cells on T cells in co-culture. However, that study showed that T cell proliferation hadn't been induced by NETs (**468**), indicating that NET-activated B cells are the main cause of the inducible effect in our experiment. Moreover, as a control, we performed the culture with but without B cells.

Moreover, the main cytokines secreted by B cells in response to NETs were, IL-6, IL-8, and TNF. For instance, IL-6 and TNF, are already known to promote activation and proliferation of naïve and memory T cells (**493, 494**). IL-8 is not typically involved in the direct activation of T cells; it is primarily considered as a potent chemoattractant of T cells (**495**).

Another crucial aspect of B cell function is APC. In the context of NET stimulation, B cells can engulf extracellular DNA from NETs that may contain antigens derived from pathogens or self-antigens in the case of autoimmune diseases. Once internalized, B cells process and present these antigens to T cells, initiating adaptive immune responses. This antigen presentation can lead to the activation of T cells and subsequent immune reactions.

Moreover, in an inflammatory environment, such as in RA inflamed joint, activated B cells can interact with other immune cells and contribute to the formation of ectopic lymphoid structures. B cells within these lymphoid structures can present antigens to T cells, further fueling the immune response and sustaining the chronic inflammation seen in RA. Furthermore, NETs contain citrullinated peptides and PAD4. It has been show that HLA-DRB1 alleles might bind citrullinated peptides and deliver them to T helper cells that recognize citrullinated proteins, or might bind to PAD4 and employ it as a carrier to internalize and process the PAD4-citrullinated protein complex to present the PAD4 peptides to T helper cells, resulting in the production of IgG antibodies to multiple citrullinated proteins. Our study suggests that in the inflamed joint, NET may enhance APC activity of B cells to present auto-antigens from NETs or other auto-antigens to T cells, exacerbating autoreactive T cells development and auto-antibodies production.

B. NET-activated B cells trigger ROS production and neutrophil recruitment

We have demonstrated that neutrophils may activate B cells through NET formation. Further, we aimed to determine whether B cells and neutrophils undergo an amplification cycle.

Firstly, using a highly sensitive chemiluminescence assay, we showed that supernatants of NET-activated B cells induce ROS production by neutrophils. Luminol can detect the following types of reactive oxygen species; Hydrogen Peroxide (H2O2), Superoxide Anion (O2·-), and Hydroxyl Radical (·OH). RA patients show a marked increase in ROS formation in the blood (**345**). In addition, synovial fluid from RA patients can trigger ROS production by neutrophils (**347**). This group suggested that the major cytokines involved in ROS induction by SF were IFNγ, TNFα, IL-1β, IL-6, and G-CSF. Additionally, IL-8 is known to induce ROS production by neutrophils (**496**). Importantly, the majority of the above cytokines were found induced in B cells following NET activation. In this study, we present a potential mechanism involved in this elevated ROS level in RA patients.

Secondly, the results of this study show that B cells activated by NETs produce chemo-attractant which triggers neutrophil recruitment. The precise chemo-attractant involved in this mechanism was not determined. Several studies have shown that IL-8 and TNFα induce neutrophil migration when injected into mouse models (**9**). These two cytokines were induced in B cells by NETs. Other chemokines, e.g. CCL2 and CCL3, which are similarly involved in neutrophil recruitment were shown to be increased in NET-activated B cells (RNAseq data). Antibodies could be added with supernatants of B cells into the reservoir of ibidi slide to block cytokines (**497**), in order to determine which cytokines in the NET-activated supernatants are the main one responsible for neutrophil recruitment.

Neutrophil tissue infiltration is essential for pathogen clearance and tissue repair, and it is tightly controlled because abnormal neutrophil accumulation in tissues causes tissue damage. We suggest that, in RA, NET-activated B cells induce neutrophil migration into RA inflamed joint. RA SF neutrophils lose their migratory abilities and get trapped inside the joint (**347**). This causes signals to be produced that attract and activate both innate and adaptive immune cells to enhance joint damage and inflammation.

V. RNAseq data suggest that NET-activated B cells may activate immune cells other than neutrophils and T cells

Our RNAseq results revealed a substantial amount of pro-inflammatory genes that were up-regulated after B cells were activated by NETs, suggesting that the consequences of B cell

activation by NETs may have an important impact on a large number of immune cells. In this study, we have only focused on the consequenses on neutrophils and T lymphocytes. For instance, NET-activated B cells showed high expression of RANKL, and it is known that B cells are a primary source of RANKL in rheumatoid arthritis, according to cytokine mRNA profiling (**422**), and RANKL can trigger osteoclast activation and maturation. Additionally, in vitro studies have revealed that RANKL released by B cells can promote monocyte maturation into osteoclasts, resulting in bone destruction in RA (**415**). Our data suggest NETs as a potential mediator of RANKL production by B cells and as a player in bone erosion in RA. In the same context, NET-activated B cells also showed increased expression of matrix metalloproteinase (MMP) enzymes, MMPs promote inflammatory processes and thereby contribute to the development of rheumatoid arthritis (**498**).

RNAseq additionally revealed that after NET stimulation, B cells expressed a significant level of Lymphotoxin α. Importantly, T and B cells in RA create ectopic lymphoid structures in the joint, where they interact and activate each other (**367, 377**). Indeed, it is known that the aggregated T cell and B cell infiltration in ectopic lymphoid structures is maintained by Lymphotoxin α and β released by B cells (**499**).

Furthermore, our results demonstrated that B cell activation by NETs promoted neutrophil recruitment. RNAseq data also revealed a wide number of increased chemokine expressions, including CCL3, CCL2, CCL3L1, CCL7 and others. These chemokines, especially CCL3 may attract more inflammatory cells to migrate and accumulate in the joint, and these inflammatory cells produce more inflammatory factors in the synovial environment, exacerbating the local inflammatory response in RA (**500**).

VI. B cell activation by NETs may be beneficial! Role in infection

NETs are primarily associated with the defense against microbial pathogens, where they trap and kill bacteria and fungi (**103, 104**). Indeed, the finding that NETs can stimulate pro-inflammatory cytokine production by B cells, and indirect ROS production by neutrophils suggests that this interaction may be part of the immune system's strategy to combat infections. Cytokines produced by NET-activated B cells may play essential roles in recruiting and activating various immune cells, as we have shown with neutrophils, to the site of infection. This can help in the elimination of pathogen.

Moreover, NET-activated B cells can act as APCs. In the context of infections, B cells can efficiently present pathogen-derived antigens to T cells, facilitating a targeted immune response.

Additionally, the observed T cell proliferation suggests that NET-activated B cells can also contribute to the activation and expansion of cytotoxic T cells. This is crucial for the clearance of intracellular pathogens.

VII. Suggested therapy

Our findings illustrated an aggressive mechanism in which NETs activate B cells, and these cells then exert a pro-inflammatory response by generating pro-inflammatory cytokines and acting as APC. The mechanism which may aggravate inflammation in various circumstances.

This mechanism might be targeted at several points. NETs are the main players in this activation mechanism. Numerous molecules have been developed to specifically target and inhibit the formation or activity of NETs. Drugs such as dornase alfa (the recombinant form of the human DNase I enzyme) showed successful NET degradation (**234**) and have been approved for clinical use in certain conditions characterized by excessive mucus production, such as cystic fibrosis, and COVID-19 (**234**). However, this medication has never been tested in an arthritic model. It would be interesting to examine this medication in arthritis animal models, such as CIA, with an emphasis on B cell activation and phenotype.

Furthermore, we found that NET-activated B cells expressed high level of CD40, CD86 and HLADR. Targeting these molecules might help to reduce the pro-inflammatory effects of B cell activation by NETs shown in this study. Abatacept (CTLA-4Ig) has been used effectively to treat autoimmune illnesses. Abatacept suppresses T cell co-stimulation and activation by binding to CD80 and CD86 on the surface of B cells, resulting in the down-regulation of inflammatory mediators (**501**).

In conclusion, we have used RNA-seq and different experimental analysis to describe a pro-inflammatory properties of NETs, which trigger polyclonal B cells activation including production of pro-inflammatory cytokines, total IgG, and up-regulation of activation markers. We propose this altered activation may be involved in the severity of RA and explains the function of neutrophils and NETs in the pathogenesis of the disease.

REFERENCES

1. Hellebrekers P, et al. Neutrophil phenotypes in health and disease. Eur J Clin Invest. 2018 Nov;48 Suppl 2(Suppl Suppl 2):e12943. doi: 10.1111/eci.12943. Epub 2018 May 25. PMID: 29682724; PMCID: PMC6282827.

2. Ehrlich P. 1879. Über die specifischen Granulationen des Blutes. Arch Anat Physiol (Leipzig) 571–579.

3. Slayton WB, et al. The first-appearance of neutrophils in the human fetal bone marrow cavity. Early Hum Dev. 1998 Dec;53(2):129-44. doi: 10.1016/s0378-3782(98)00049-8. PMID: 10195706.

4. Lawrence SM, et al. The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis. Microbiol Mol Biol Rev. 2018 Feb 7;82(1):e00057-17. doi: 10.1128/MMBR.00057-17. PMID: 29436479; PMCID: PMC5813886.

5. Dancey JT, et al. Neutrophil kinetics in man. J Clin Invest. 1976 Sep;58(3):705-15. doi: 10.1172/JCI108517. PMID: 956397; PMCID: PMC333229.

6. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. Immunol Rev. 2016 Sep;273(1):11-28. doi: 10.1111/imr.12440. PMID: 27558325.

7. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol. 2004 Mar 1;172(5):2731-8. doi: 10.4049/jimmunol.172.5.2731. PMID: 14978070.

8. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. Trends Immunol. 2011 Oct;32(10):452-60. doi: 10.1016/j.it.2011.06.008. Epub 2011 Aug 11. PMID: 21839682; PMCID: PMC3470857.

9. Terashima T, et al. Release of polymorphonuclear leukocytes from the bone marrow by interleukin-8. Blood. 1998 Aug 1;92(3):1062-9. PMID: 9680376.

10. Weiskotten HG. The Normal Life Span of the Neutrophile (Amphophile) Leucocyte (Rabbit): The Action of Benzol IX. Am J Pathol. 1930 Mar;6(2):183-190.3. PMID: 19969897; PMCID: PMC2007285.

11. Mayadas TN, et al. Neutrophil beta2 integrins: moderators of life or death decisions. Trends Immunol. 2005 Jul;26(7):388-95. doi: 10.1016/j.it.2005.05.002. PMID: 15922663.

12. Basu S, et al. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. Blood. 2002 Aug 1;100(3):854-61. doi: 10.1182/blood.v100.3.854. PMID: 12130495.

13. Pillay J, et al. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood. 2010 Jul 29;116(4):625-7. doi: 10.1182/blood-2010-01-259028. Epub 2010 Apr 21. PMID: 20410504.

14. Tofts PS, et al. Doubts concerning the recently reported human neutrophil lifespan of 5.4 days. Blood. 2011 Jun 2;117(22):6050-2; author reply 6053-4. doi: 10.1182/blood-2010-10-310532. PMID: 21636720.

15. Geering B, et al. Living and dying for inflammation: neutrophils, eosinophils, basophils. Trends Immunol. 2013 Aug;34(8):398-409. doi: 10.1016/j.it.2013.04.002. Epub 2013 May 9. PMID: 23665135.

16. Voisin MB, et al. Neutrophil transmigration: emergence of an adhesive cascade within venular walls. J Innate Immun. 2013;5(4):336-47. doi: 10.1159/000346659. Epub 2013 Mar 2. PMID: 23466407; PMCID: PMC6741604.

17. Asaduzzaman M, et al. P-selectin glycoprotein-ligand-1 regulates pulmonary recruitment of neutrophils in a platelet-independent manner in abdominal sepsis. Br J Pharmacol. 2009 Jan;156(2):307-15. doi: 10.1111/j.1476-5381.2008.00021.x. Epub 2009 Jan 16. PMID: 19154425; PMCID: PMC2697831.

18. Bargatze RF, et al. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. J Exp Med. 1994 Nov 1;180(5):1785-92. doi: 10.1084/jem.180.5.1785. PMID: 7525838; PMCID: PMC2191733.

19. Allen C, et al. Neutrophil cerebrovascular transmigration triggers rapid neurotoxicity through release of proteases associated with decondensed DNA. J Immunol. 2012 Jul 1;189(1):381-92. doi: 10.4049/jimmunol.1200409. Epub 2012 Jun 1. PMID: 22661091; PMCID: PMC3381844.

20. de Oliveira Set al. Neutrophil migration in infection and wound repair: going forward in reverse. Nat Rev Immunol. 2016 May 27;16(6):378-91. doi: 10.1038/nri.2016.49. PMID: 27231052; PMCID: PMC5367630.

21. Souto FO, et al. Essential role of CCR2 in neutrophil tissue infiltration and multiple organ dysfunction in sepsis. Am J Respir Crit Care Med. 2011 Jan 15;183(2):234-42. doi: 10.1164/rccm.201003-0416OC. Epub 2010 Aug 23. PMID: 20732989.

22. Chou RC, et al. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. Immunity. 2010 Aug 27;33(2):266-78. doi: 10.1016/j.immuni.2010.07.018. PMID: 20727790; PMCID: PMC3155777.

23. Wang X, et al. Plasminogen Activator Inhibitor-1 Potentiates Neutrophil Infiltration and Tissue Injury in Colitis. Int J Biol Sci. 2023 Apr 9;19(7):2132-2149. doi: 10.7150/ijbs.75890. PMID: 37151884; PMCID: PMC10158018.

24. Nadolni W, et al. TRPM7 Kinase Is Essential for Neutrophil Recruitment and Function via Regulation of Akt/mTOR Signaling. Front Immunol. 2021 Feb 15;11:606893. doi: 10.3389/fimmu.2020.606893. PMID: 33658993; PMCID: PMC7917126.

25. Cuda CM, et al. The inflammatory role of phagocyte apoptotic pathways in rheumatic diseases. Nat Rev Rheumatol. 2016 Aug 23;12(9):543-58. doi: 10.1038/nrrheum.2016.132. PMID: 27549026; PMCID: PMC5297631.

26. Jones J et al. Apoptosis is associated with reduced expression of complement regulatory molecules, adhesion molecules and other receptors on polymorphonuclear leucocytes: functional relevance and role in inflammation. Immunology. (1995) 86:651–60.

27. Weinmann P, et al. Delayed neutrophil apoptosis in very early rheumatoid arthritis patients is abrogated by methotrexate therapy. Clin Exp Rheumatol. 2007 Nov-Dec;25(6):885-7. PMID: 18173925.

28. Colotta F, et al. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood. 1992 Oct 15;80(8):2012-20. PMID: 1382715.

29. Gardiman Eet al. SARS-CoV-2-Associated ssRNAs Activate Human Neutrophils in a TLR8-Dependent Fashion. Cells. 2022 Nov 26;11(23):3785. doi: 10.3390/cells11233785. PMID: 36497044; PMCID: PMC9738506.

30. Herrera VLM, et al. Anti-DEspR antibody treatment improves survival and reduces neurologic deficits in a hypertensive, spontaneous intracerebral hemorrhage (hsICH) rat model. Sci Rep. 2023 Feb 15;13(1):2703. doi: 10.1038/s41598-023-28149-3. PMID: 36792616; PMCID: PMC9932093.

31. Song C, et al. Delayed neutrophil apoptosis may enhance NET formation in ARDS. Respir Res. 2022 Jun 13;23(1):155. doi: 10.1186/s12931-022-02065-y. PMID: 35698192; PMCID: PMC9190136.

32. Croker BA, et al. Fas-mediated neutrophil apoptosis is accelerated by Bid, Bak, and Bax and inhibited by Bcl-2 and Mcl-1. Proc Natl Acad Sci U S A. 2011 Aug 9;108(32):13135-40. doi: 10.1073/pnas.1110358108. Epub 2011 Jul 18. PMID: 21768356; PMCID: PMC3156212.

33. Xiang Y, et al. ROS-activated CXCR2+ neutrophils recruited by CXCL1 delay denervated skeletal muscle atrophy and undergo P53-mediated apoptosis. Exp Mol Med. 2022 Jul;54(7):1011-1023. doi: 10.1038/s12276-022-00805-0. Epub 2022 Jul 21. PMID: 35864308; PMCID: PMC9356135.

34. Cross A, et al. The dual effects of TNFalpha on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. Blood. 2008 Jan 15;111(2):878-84. doi: 10.1182/blood-2007-05-087833. Epub 2007 Oct 17. PMID: 17942758.

35. Chen D, et al. TNF-α Induces Neutrophil Apoptosis Delay and Promotes Intestinal Ischemia-Reperfusion-Induced Lung Injury through Activating JNK/FoxO3a Pathway. Oxid Med Cell Longev. 2021 Dec 29;2021:8302831. doi: 10.1155/2021/8302831. PMID: 35003520; PMCID: PMC8731283.

36. Wigerblad G, et al. Single-Cell Analysis Reveals the Range of Transcriptional States of Circulating Human Neutrophils. J Immunol. 2022 Aug 15;209(4):772-782. doi: 10.4049/jimmunol.2200154. PMID: 35858733; PMCID: PMC9712146.

37. Gallin JI. Human neutrophil heterogeneity exists, but is it meaningful? Blood. 1984 May;63(5):977-83. PMID: 6324930.

38. Lakschevitz FS, et al. Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry. Exp Cell Res. 2016 Mar 15;342(2):200-9. doi: 10.1016/j.yexcr.2016.03.007. Epub 2016 Mar 9. PMID: 26970376.

39. Göhring Ket al. Neutrophil CD177 (NB1 gp, HNA-2a) expression is increased in severe bacterial infections and polycythaemia vera. Br J Haematol. 2004 Jul;126(2):252-4. doi: 10.1111/j.1365-2141.2004.05027.x. PMID: 15238147.

40. Marini O, et al. Mature CD10+ and immature CD10- neutrophils present in G-CSF-treated donors display opposite effects on T cells. Blood. 2017 Mar 9;129(10):1343-1356. doi: 10.1182/blood-2016-04-713206. Epub 2017 Jan 4. Erratum in: Blood. 2017 Jun 15;129(24):3271. PMID: 28053192.

41. Monteseirín J, et al. L-selectin expression on neutrophils from allergic patients. Clin Exp Allergy. 2005 Sep;35(9):1204-13. doi: 10.1111/j.1365-2222.2005.02320.x. PMID: 16164449.

42. Tak T, et al. Human CD62Ldim neutrophils identified as a separate subset by proteome profiling and in vivo pulse-chase labeling. Blood. 2017 Jun 29;129(26):3476-3485. doi: 10.1182/blood-2016-07-727669. Epub 2017 May 17. PMID: 28515092.

43. Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. Blood Cells Mol Dis. 2002 Mar-Apr;28(2):260-74. doi: 10.1006/bcmd.2002.0513. PMID: 12064921.

44. van Grinsven E, et al. Immature Neutrophils Released in Acute Inflammation Exhibit Efficient Migration despite Incomplete Segmentation of the Nucleus. J Immunol. 2019 Jan 1;202(1):207-217. doi: 10.4049/jimmunol.1801255. Epub 2018 Nov 30. PMID: 30504419.

45. Evrard M, et al. Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. Immunity. 2018 Feb 20;48(2):364-379.e8. doi: 10.1016/j.immuni.2018.02.002. PMID: 29466759.

46. Deniset JF, et al. Splenic Ly6Ghigh mature and Ly6Gint immature neutrophils contribute to eradication of S. pneumoniae. J Exp Med. 2017 May 1;214(5):1333-1350. doi: 10.1084/jem.20161621. Epub 2017 Apr 19. PMID: 28424248; PMCID: PMC5413339.

47. Martinelli S, et al. Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem. 2004 Oct 15;279(42):44123-32. doi: 10.1074/jbc.M405883200. Epub 2004 Aug 9. PMID: 15302890.

48. Ohtsu S, et al. Enhanced neutrophilic granulopoiesis in rheumatoid arthritis. Involvement of neutrophils in disease progression. J Rheumatol. 2000 Jun;27(6):1341-51. PMID: 10852252.

49. Bennett L, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med. 2003 Mar 17;197(6):711-23. doi: 10.1084/jem.20021553. PMID: 12642603; PMCID: PMC2193846.

50. Ning X, et al. Low-Density Granulocytes in Immune-Mediated Inflammatory Diseases. J Immunol Res. 2022 Jan 31;2022:1622160. doi: 10.1155/2022/1622160. PMID: 35141336; PMCID: PMC8820945.

51. Hardisty GR, et al. High Purity Isolation of Low Density Neutrophils Casts Doubt on Their Exceptionality in Health and Disease. Front Immunol. 2021 Jun 8;12:625922. doi: 10.3389/fimmu.2021.625922. PMID: 34168640; PMCID: PMC8217868.

52. Martin KR, et al. CD98 defines a metabolically flexible, proinflammatory subset of low-density neutrophils in systemic lupus erythematosus. Clin Transl Med. 2023 Jan;13(1):e1150. doi: 10.1002/ctm2.1150. PMID: 36653319; PMCID: PMC9849148.

53. Wright HL, et al. Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. J Leukoc Biol. 2017 Feb;101(2):599-611. doi: 10.1189/jlb.5A0116-022R. Epub 2016 Sep 6. PMID: 27601627.

54. Silvin A, et al. Elevated Calprotectin and Abnormal Myeloid Cell Subsets Discriminate Severe from Mild COVID-19. Cell. 2020 Sep 17;182(6):1401-1418.e18. doi: 10.1016/j.cell.2020.08.002. Epub 2020 Aug 5. PMID: 32810439; PMCID: PMC7405878.

55. Meyer C, et al. Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. Cancer Immunol Immunother. 2014 Mar;63(3):247-57. doi: 10.1007/s00262-013-1508-5. Epub 2013 Dec 20. PMID: 24357148.

56. Arasanz H, et al. Circulating Low Density Neutrophils Are Associated with Resistance to First Line Anti-PD1/PDL1 Immunotherapy in Non-Small Cell Lung Cancer. Cancers (Basel). 2022 Aug 9;14(16):3846. doi: 10.3390/cancers14163846. PMID: 36010840; PMCID: PMC9406164.

57. Bashant KR, et al. Proteomic, biomechanical and functional analyses define neutrophil heterogeneity in systemic lupus erythematosus. Ann Rheum Dis. 2021 Feb;80(2):209-218. doi: 10.1136/annrheumdis-2020-218338. Epub 2020 Sep 28. PMID: 32988843; PMCID: PMC7855438.

58. Rahman S, et al. Low-density granulocytes activate T cells and demonstrate a non-suppressive role in systemic lupus erythematosus. Ann Rheum Dis. 2019 Jul;78(7):957-966. doi: 10.1136/annrheumdis-2018-214620. Epub 2019 Apr 30. PMID: 31040119; PMCID: PMC6585283.

59. Kumagai Y, et al. Surgical Stress Increases Circulating Low-Density Neutrophils Which May Promote Tumor Recurrence. J Surg Res. 2020 Feb;246:52-61. doi: 10.1016/j.jss.2019.08.022. Epub 2019 Sep 24. PMID: 31561178.

60. Halbwachs-Mecarelli L, et al. Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. FEBS Lett. 1995 Oct 23;374(1):29-33. doi: 10.1016/0014-5793(95)01073-n. PMID: 7589506.

61. Silvestre-Roig C, et al. Neutrophil Diversity in Health and Disease. Trends Immunol. 2019 Jul;40(7):565-583. doi: 10.1016/j.it.2019.04.012. Epub 2019 May 31. PMID: 31160207; PMCID: PMC7185435.

62. Zhang J, et al. CD177 Inhibits Neutrophil Extracellular Trap Formation and Protects against Acute Pancreatitis in Mice. J Clin Med. 2023 Mar 27;12(7):2533. doi: 10.3390/jcm12072533. PMID: 37048616; PMCID: PMC10094768.

63. Bai M, et al. CD177 modulates human neutrophil migration through activation-mediated integrin and chemoreceptor regulation. Blood. 2017 Nov 9;130(19):2092-2100. doi: 10.1182/blood-2017-03-768507. Epub 2017 Aug 14. PMID: 28807980; PMCID: PMC5680608.

64. Zhang R, et al. CD177+ cells produce neutrophil extracellular traps that promote biliary atresia. J Hepatol. 2022 Nov;77(5):1299-1310. doi: 10.1016/j.jhep.2022.06.015. Epub 2022 Jul 5. PMID: 35803543.

65. Welin A, et al. The human neutrophil subsets defined by the presence or absence of OLFM4 both transmigrate into tissue in vivo and give rise to distinct NETs in vitro. PLoS One. 2013 Jul 29;8(7):e69575. doi: 10.1371/journal.pone.0069575. PMID: 23922742; PMCID: PMC3726694.

66. Alder MN, et al. Olfactomedin 4 marks a subset of neutrophils in mice. Innate Immun. 2019 Jan;25(1):22-33. doi: 10.1177/1753425918817611. Epub 2018 Dec 11. PMID: 30537894; PMCID: PMC6661892.

67. Lundquist H, et al. The Olfactomedin-4-Defined Human Neutrophil Subsets Differ in Proteomic Profile in Healthy Individuals and Patients with Septic Shock. J Innate Immun. 2022 Nov 30:1-14. doi: 10.1159/000527649. Epub ahead of print. PMID: 36450268.

68. Kangelaris KN, et al. A neutrophil subset defined by intracellular olfactomedin 4 is associated with mortality in sepsis. Am J Physiol Lung Cell Mol Physiol. 2021 May 1;320(5):L892-L902. doi: 10.1152/ajplung.00090.2020. Epub 2020 Dec 23. PMID: 33355521; PMCID: PMC8174834.

69. Arnold S, et al. Spectrum of ANCA-specificities in eosinophilic granulomatosis with polyangiitis. A retrospective multicentre study. Clin Exp Rheumatol. 2023 Apr;41(4):936-942. doi: 10.55563/clinexprheumatol/299yme. Epub 2023 Apr 5. PMID: 37073637.

70. Lood C, et al. TLR7/8 activation in neutrophils impairs immune complex phagocytosis through shedding of FcgRIIA. J Exp Med. 2017 Jul 3;214(7):2103-2119. doi: 10.1084/jem.20161512. Epub 2017 Jun 12. PMID: 28606989; PMCID: PMC5502427.

71. Tanaka M, et al. Photodynamic therapy can induce a protective innate immune response against murine bacterial arthritis via neutrophil accumulation. PLoS One. 2012;7(6):e39823. doi: 10.1371/journal.pone.0039823. Epub 2012 Jun 26. PMID: 22761911; PMCID: PMC3383702.

72. Gullstrand B, et al. Combination of autoantibodies against different histone proteins influences complement-dependent phagocytosis of necrotic cell material by polymorphonuclear leukocytes in systemic lupus erythematosus. J Rheumatol. 2012 Aug;39(8):1619-27. doi: 10.3899/jrheum.111511. Epub 2012 Jul 1. PMID: 22753651.

73. de Siqueira MB, et al. Enhanced neutrophil phagocytic capacity in rheumatoid arthritis related to the autoantibodies rheumatoid factor and anti-cyclic citrullinated peptides. BMC Musculoskelet Disord. 2015 Jun 30;16:159. doi: 10.1186/s12891-015-0616-0. PMID: 26123215; PMCID: PMC4486699.

74. Borregaard N, et al. Neutrophil granules: a library of innate immunity proteins. Trends Immunol. 2007 Aug;28(8):340-5. doi: 10.1016/j.it.2007.06.002. Epub 2007 Jul 12. PMID: 17627888.

75. Rørvig S, et al. Ficolin-1 is present in a highly mobilizable subset of human neutrophil granules and associates with the cell surface after stimulation with fMLP. J Leukoc Biol. 2009 Dec;86(6):1439-49. doi: 10.1189/jlb.1008606. Epub 2009 Sep 9. PMID: 19741154.

76. Gierlikowska B, et al. Phagocytosis, Degranulation and Extracellular Traps Release by Neutrophils-The Current Knowledge, Pharmacological Modulation and Future Prospects. Front Pharmacol. 2021 May 4;12:666732. doi: 10.3389/fphar.2021.666732. PMID: 34017259; PMCID: PMC8129565.

77. Chen ZX, et al. LPCAT1 enhances the invasion and migration in gastric cancer: Based on computational biology methods and in vitro experiments. Cancer Med. 2023 May 15. doi: 10.1002/cam4.5991. Epub ahead of print. PMID: 37184260.

78. Kummarapurugu AB, et al. Neutrophil Elastase decreases SARS-CoV-2 Spike protein binding to human bronchial epithelia by clipping ACE-2 ectodomian from the epithelial surface. J Biol Chem. 2023 May 13:104820. doi: 10.1016/j.jbc.2023.104820. Epub ahead of print. PMID: 37187291; PMCID: PMC10181948.

79. Sies H, Berndt C, Jones DP. Oxidative Stress. Annu Rev Biochem. 2017 Jun 20;86:715-748. doi: 10.1146/annurev-biochem-061516-045037. Epub 2017 Apr 24. PMID: 28441057.

80. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007 Jan;87(1):245-313. doi: 10.1152/physrev.00044.2005. PMID: 17237347.

81. Peng S, et al. Established and emerging roles for mitochondria in neutrophils. Immunol Rev. 2023 Mar;314(1):413-426. doi: 10.1111/imr.13158. Epub 2022 Nov 4. PMID: 36331270.

82. Maianski NA, et al. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. Cell Death Differ. 2004 Feb;11(2):143-53. doi: 10.1038/sj.cdd.4401320. PMID: 14576767.

83. Vorobjeva N, et al. Mitochondrial permeability transition pore is involved in oxidative burst and NETosis of human neutrophils. Biochim Biophys Acta Mol Basis Dis. 2020 May 1;1866(5):165664. doi: 10.1016/j.bbadis.2020.165664. Epub 2020 Jan 8. PMID: 31926265.

84. Dikalov S. Cross talk between mitochondria and NADPH oxidases. Free Radic Biol Med. 2011 Oct 1;51(7):1289-301. doi: 10.1016/j.freeradbiomed.2011.06.033. Epub 2011 Jul 6. PMID: 21777669; PMCID: PMC3163726.

85. Kuimova MK, et al. Singlet oxygen in a cell: spatially dependent lifetimes and quenching rate constants. J Am Chem Soc. 2009 Jan 14;131(1):332-40. doi: 10.1021/ja807484b. PMID: 19128181.

86. Mikkelsen RB, et al. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003 Sep 1;22(37):5734-54. doi: 10.1038/sj.onc.1206663. PMID: 12947383.

87. Brinkmann V, Laube B, Abu Abed U, Goosmann C, Zychlinsky A. Neutrophil extracellular traps: how to generate and visualize them. J Vis Exp. 2010 Feb 24;(36):1724. doi: 10.3791/1724. PMID: 20182410; PMCID: PMC3125121.

88. Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. J Exp Med. 2011 Mar 14;208(3):417-20. doi: 10.1084/jem.20110367. Epub 2011 Feb 28. PMID: 21357740; PMCID: PMC3058577.

89. Veenith T, Martin H, Le Breuilly M, Whitehouse T, Gao-Smith F, Duggal N, Lord JM, Mian R, Sarphie D, Moss P. High generation of reactive oxygen species from neutrophils in patients with severe COVID-19. Sci Rep. 2022 Jun 21;12(1):10484. doi: 10.1038/s41598-022-13825-7. PMID: 35729319; PMCID: PMC9212205.

90. Lu J, Liu J, Li A. Roles of neutrophil reactive oxygen species (ROS) generation in organ function impairment in sepsis. J Zhejiang Univ Sci B. 2022 Jun 15;23(6):437-450. doi: 10.1631/jzus.B2101075. PMID: 35686524; PMCID: PMC9198233.

91. Dixit R, et al. Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy. Plant J. 2003 Oct;36(2):280-90. doi: 10.1046/j.1365-313x.2003.01868.x. PMID: 14535891.

92. Jantean L, et al. Measurement of reactive oxygen species production by luminol-based assay in Nicotiana benthamiana, Arabidopsis thaliana and Brassica rapa ssp. rapa. Plant Biotechnol (Tokyo). 2022 Dec 25;39(4):415-420. doi: 10.5511/plantbiotechnology.22.0823a. PMID: 37283611; PMCID: PMC10240919.

93. Tecchio C, et al. Neutrophil-derived cytokines: facts beyond expression. Front Immunol. 2014 Oct 21;5:508. doi: 10.3389/fimmu.2014.00508. PMID: 25374568; PMCID: PMC4204637.

94. Beauvillain C, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. Blood. 2011 Jan 27;117(4):1196-204. doi: 10.1182/blood-2009-11-254490. Epub 2010 Nov 4. PMID: 21051556.

95. Kish DD, et al. Neutrophil expression of Fas ligand and perforin directs effector CD8 T cell infiltration into antigen-challenged skin. J Immunol. 2012 Sep 1;189(5):2191-202. doi: 10.4049/jimmunol.1102729. Epub 2012 Jul 18. PMID: 22815291; PMCID: PMC3424302.

96. Scapini P, et al. G-CSF-stimulated neutrophils are a prominent source of functional BLyS. J Exp Med. 2003 Feb 3;197(3):297-302. doi: 10.1084/jem.20021343. PMID: 12566413; PMCID: PMC2193843.

97. Doz E, et al. Mycobacteria-infected dendritic cells attract neutrophils that produce IL-10 and specifically shut down Th17 CD4 T cells through their IL-10 receptor. J Immunol. 2013 Oct 1;191(7):3818-26. doi: 10.4049/jimmunol.1300527. Epub 2013 Aug 30. PMID: 23997221.

98. Tamassia N, et al. Cutting edge: An inactive chromatin configuration at the IL-10 locus in human neutrophils. J Immunol. 2013 Mar 1;190(5):1921-5. doi: 10.4049/jimmunol.1203022. Epub 2013 Jan 25. PMID: 23355741.

99. Takei H, et al. Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. J Leukoc Biol. 1996 Feb;59(2):229-40. doi: 10.1002/jlb.59.2.229. PMID: 8603995.

100. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532-5. doi: 10.1126/science.1092385. PMID: 15001782.

101. Germic N, et al. Neither eosinophils nor neutrophils require ATG5-dependent autophagy for extracellular DNA trap formation. Immunology. 2017 Nov;152(3):517-525. doi: 10.1111/imm.12790. Epub 2017 Aug 7. PMID: 28703297; PMCID: PMC5629432.

102. Vorobjeva NV, Chernyak BV. NETosis: Molecular Mechanisms, Role in Physiology and Pathology. Biochemistry (Mosc). 2020 Oct;85(10):1178-1190. doi: 10.1134/S0006297920100065. PMID: 33202203; PMCID: PMC7590568.

103. Melbouci D, et al. Neutrophil extracellular traps (NET): not only antimicrobial but also modulators of innate and adaptive immunities in inflammatory autoimmune diseases. RMD Open. 2023 Aug;9(3):e003104. doi: 10.1136/rmdopen-2023-003104. PMID: 37562857.

104. Haidar Ahmad, A, et al. Polymorphonuclear Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus: More Complicated Than Anticipated. Immuno 2022, 2, 85-103. https://doi.org/10.3390/immuno2010007.

105. Hakkim A, et al. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat Chem Biol. 2011 Feb;7(2):75-7. doi: 10.1038/nchembio.496. Epub 2010 Dec 19. PMID: 21170021.

106. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007 Jan 15;176(2):231-41. doi: 10.1083/jcb.200606027. Epub 2007 Jan 8. PMID: 17210947; PMCID: PMC2063942.

107. Azzouz D, Khan MA, Palaniyar N. ROS induces NETosis by oxidizing DNA and initiating DNA repair. Cell Death Discov. 2021 May 18;7(1):113. doi: 10.1038/s41420-021-00491-3. PMID: 34001856; PMCID: PMC8128883.

108. Bianchi M, Hakkim A, Brinkmann V, Siler U, Seger RA, Zychlinsky A, Reichenbach J. Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood. 2009 Sep 24;114(13):2619-22. doi: 10.1182/blood-2009-05-221606. Epub 2009 Jun 18. PMID: 19541821; PMCID: PMC2756123.

109. Chow OA, von Köckritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL, Gallo RL, Monestier M, Wang Y, Glass CK, Nizet V. Statins enhance formation of phagocyte extracellular traps. Cell Host Microbe. 2010 Nov 18;8(5):445-54. doi: 10.1016/j.chom.2010.10.005. PMID: 21075355; PMCID: PMC3008410.

110. Parker H, et al. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. J Leukoc Biol. 2012 Oct;92(4):841-9. doi: 10.1189/jlb.1211601. Epub 2012 Jul 16. PMID: 22802447.

111. Pilsczek FH, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol. 2010 Dec 15;185(12):7413-25. doi: 10.4049/jimmunol.1000675. Epub 2010 Nov 22. PMID: 21098229.

112. Papayannopoulos V, et al. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 2010 Nov 1;191(3):677-91. doi: 10.1083/jcb.201006052. Epub 2010 Oct 25. PMID: 20974816; PMCID: PMC3003309.

113. Konig MF, Andrade F. A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination. Front Immunol. 2016 Nov 4;7:461. doi: 10.3389/fimmu.2016.00461. PMID: 27867381; PMCID: PMC5095114.

114. Burgener SS, Schroder K. Neutrophil Extracellular Traps in Host Defense. Cold Spring Harb Perspect Biol. 2020 Jul 1;12(7):a037028. doi: 10.1101/cshperspect.a037028. PMID: 31767647; PMCID: PMC7328462.

115. Yipp BG, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med. 2012 Sep;18(9):1386-93. doi: 10.1038/nm.2847. PMID: 22922410; PMCID: PMC4529131.

116. Clark SR, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med. 2007 Apr;13(4):463-9. doi: 10.1038/nm1565. Epub 2007 Mar 25. PMID: 17384648.

117. Guillotin F, et al. Vital NETosis vs. suicidal NETosis during normal pregnancy and preeclampsia. Front Cell Dev Biol. 2023 Jan 5;10:1099038. doi: 10.3389/fcell.2022.1099038. PMID: 36684420; PMCID: PMC9849884.

118. Yousefi S, et al. Untangling "NETosis" from NETs. Eur J Immunol. 2019 Feb;49(2):221-227. doi: 10.1002/eji.201747053. Epub 2019 Jan 15. PMID: 30629284.

119. Radonjic-Hoesli S, et al. Adhesion-induced eosinophil cytolysis requires the receptor-interacting protein kinase 3 (RIPK3)-mixed lineage kinase-like (MLKL) signaling pathway, which is counterregulated by autophagy. J Allergy Clin Immunol. 2017 Dec;140(6):1632-1642. doi: 10.1016/j.jaci.2017.01.044. Epub 2017 Apr 12. PMID: 28412393.

120. Yousefi S, et al. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ. 2009 Nov;16(11):1438-44. doi: 10.1038/cdd.2009.96. Epub 2009 Jul 17. PMID: 19609275.

121. Nadesalingam A, et al. Hypertonic Saline Suppresses NADPH Oxidase-Dependent Neutrophil Extracellular Trap Formation and Promotes Apoptosis. Front Immunol. 2018 Mar 8;9:359. doi: 10.3389/fimmu.2018.00359. PMID: 29593709; PMCID: PMC5859219.

122. Hamam HJ, Palaniyar N. Histone Deacetylase Inhibitors Dose-Dependently Switch Neutrophil Death from NETosis to Apoptosis. Biomolecules. 2019 May 11;9(5):184. doi: 10.3390/biom9050184. PMID: 31083537; PMCID: PMC6571947.

123. Chen F, et al. Hesperetin attenuates sepsis-induced intestinal barrier injury by regulating neutrophil extracellular trap formation via the ROS/autophagy signaling pathway. Food Funct. 2023 May 11;14(9):4213-4227. doi: 10.1039/d2fo02707k. PMID: 37067254.

124. Lelliott PM, et al. Single-cell Raman microscopy with machine learning highlights distinct biochemical features of neutrophil extracellular traps and necrosis. Sci Rep. 2023 Jun 21;13(1):10093. doi: 10.1038/s41598-023-36667-3. PMID: 37344494; PMCID: PMC10284916.

125. Kazimirskii AN, et al. IgG Stimulates the Formation of Neutrophil Extracellular Traps and Modifies Their Structure. Bull Exp Biol Med. 2023 Apr;174(6):806-809. doi: 10.1007/s10517-023-05794-2. Epub 2023 May 9. PMID: 37160604.

126. Desai J, et al. PMA and crystal-induced neutrophil extracellular trap formation involves RIPK1-RIPK3-MLKL signaling. Eur J Immunol. 2016 Jan;46(1):223-9. doi: 10.1002/eji.201545605. Epub 2015 Nov 30. PMID: 26531064.

127. Liu X, Zhang Z, et al. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature. 2016 Jul 7;535(7610):153-8. doi: 10.1038/nature18629. PMID: 27383986; PMCID: PMC5539988.

128. Sollberger G, et al. Gasdermin D plays a vital role in the generation of neutrophil extracellular traps. Sci Immunol. 2018 Aug 24;3(26):eaar6689. doi: 10.1126/sciimmunol.aar6689. PMID: 30143555.

129. Chen KW, et al. Noncanonical inflammasome signaling elicits gasdermin D-dependent neutrophil extracellular traps. Sci Immunol. 2018 Aug 24;3(26):eaar6676. doi: 10.1126/sciimmunol.aar6676. PMID: 30143554.

130. Stojkov D, et al. NET formation is independent of gasdermin D and pyroptotic cell death. Sci Signal. 2023 Jan 24;16(769):eabm0517. doi: 10.1126/scisignal.abm0517. Epub 2023 Jan 24. PMID: 36693132.

131. Dubyak GR, et al. Pyroptosis in neutrophils: Multimodal integration of inflammasome and regulated cell death signaling pathways. Immunol Rev. 2023 Mar;314(1):229-249. doi: 10.1111/imr.13186. Epub 2023 Jan 19. PMID: 36656082; PMCID: PMC10407921.

132. Amini P, et al. Neutrophil extracellular trap formation requires OPA1-dependent glycolytic ATP production. Nat Commun. 2018 Jul 27;9(1):2958. doi: 10.1038/s41467-018-05387-y. PMID: 30054480; PMCID: PMC6063938.

133. Wang L, et al. Hyperglycemia Induces Neutrophil Extracellular Traps Formation Through an NADPH Oxidase-Dependent Pathway in Diabetic Retinopathy. Front Immunol. 2019 Jan 8;9:3076. doi: 10.3389/fimmu.2018.03076. PMID: 30671057; PMCID: PMC6331470.

134. Rodríguez-Espinosa O, et al. Metabolic requirements for neutrophil extracellular traps formation. Immunology. 2015 Jun;145(2):213-24. doi: 10.1111/imm.12437. PMID: 25545227; PMCID: PMC4427386.

135. Li Y, et al. Neutrophil metabolomics in severe COVID-19 reveal GAPDH as a suppressor of neutrophil extracellular trap formation. Nat Commun. 2023 May 5;14(1):2610. doi: 10.1038/s41467-023-37567-w. PMID: 37147288; PMCID: PMC10162006.

136. Awasthi D, et al. Glycolysis dependent lactate formation in neutrophils: A metabolic link between NOX-dependent and independent NETosis. Biochim Biophys Acta Mol Basis Dis. 2019 Dec 1;1865(12):165542. doi: 10.1016/j.bbadis.2019.165542. Epub 2019 Aug 29. PMID: 31473341.

137. Kenny EF, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. Elife. 2017 Jun 2;6:e24437. doi: 10.7554/eLife.24437. PMID: 28574339; PMCID: PMC5496738.

138. de Bont CM, et al. Stimulus-dependent chromatin dynamics, citrullination, calcium signalling and ROS production during NET formation. Biochim Biophys Acta Mol Cell Res. 2018 Nov;1865(11 Pt A):1621-1629. doi: 10.1016/j.bbamcr.2018.08.014. Epub 2018 Aug 25. PMID: 30327203.

139. Petretto A, et al. Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. PLoS One. 2019 Jul 8;14(7):e0218946. doi: 10.1371/journal.pone.0218946. PMID: 31283757; PMCID: PMC6613696.

140. Bissenova S, et al. NET Proteome in Established Type 1 Diabetes Is Enriched in Metabolic Proteins. Cells. 2023 May 5;12(9):1319. doi: 10.3390/cells12091319. PMID: 37174719; PMCID: PMC10177393.

141. Chapman EA, et al. Caught in a Trap? Proteomic Analysis of Neutrophil Extracellular Traps in Rheumatoid Arthritis and Systemic Lupus Erythematosus. Front Immunol. 2019 Mar 11;10:423. doi: 10.3389/fimmu.2019.00423. PMID: 30915077; PMCID: PMC6421309.

142. Wang H, et al. Neutrophil Extracellular Trap Mitochondrial DNA and Its Autoantibody in Systemic Lupus Erythematosus and a Proof-of-Concept Trial of Metformin. Arthritis Rheumatol. 2015 Dec;67(12):3190-200. doi: 10.1002/art.39296. PMID: 26245802.

143. Pieterse E, et al. Acetylated histones contribute to the immunostimulatory potential of neutrophil extracellular traps in systemic lupus erythematosus. Clin Exp Immunol. 2015 Jan;179(1):68-74. doi: 10.1111/cei.12359. PMID: 24758196; PMCID: PMC4260898.

144. Kaplan MJ, et al. Neutrophil extracellular traps: double-edged swords of innate immunity. J Immunol. 2012 Sep 15;189(6):2689-95. doi: 10.4049/jimmunol.1201719. PMID: 22956760; PMCID: PMC3439169.

145. Liu Y, et al. LPS-TLR4 pathway exaggerates alcoholic hepatitis via provoking NETs formation. Gastroenterol Hepatol. 2023 May 5:S0210-5705(23)00332-1. English, Spanish. doi: 10.1016/j.gastrohep.2023.05.002. Epub ahead of print. PMID: 37150251.

146. Feitz WJC, et al. Shiga Toxin 2a Induces NETosis via NOX-Dependent Pathway. Biomedicines. 2021 Dec 1;9(12):1807. doi: 10.3390/biomedicines9121807. PMID: 34944623; PMCID: PMC8698832.

147. Abi Abdallah DS, et al. Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. Infect Immun. 2012 Feb;80(2):768-77. doi: 10.1128/IAI.05730-11. Epub 2011 Nov 21. PMID: 22104111; PMCID: PMC3264325.

148. Saitoh T, et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe. 2012 Jul 19;12(1):109-16. doi: 10.1016/j.chom.2012.05.015. PMID: 22817992.

149. Roach DR, et al. Human Neutrophil Response to Pseudomonas Bacteriophage PAK_P1, a Therapeutic Candidate. Viruses. 2023 Aug 12;15(8):1726. doi: 10.3390/v15081726. PMID: 37632068; PMCID: PMC10458410.

150. Keshari RS, et al. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. PLoS One. 2012;7(10):e48111. doi: 10.1371/journal.pone.0048111. Epub 2012 Oct 26. PMID: 23110185; PMCID: PMC3482178.

151. An Z, et al. Neutrophil extracellular traps induced by IL-8 aggravate atherosclerosis via activation NF-κB signaling in macrophages. Cell Cycle. 2019 Nov;18(21):2928-2938. doi: 10.1080/15384101.2019.1662678. Epub 2019 Sep 8. PMID: 31496351; PMCID: PMC6791689.

152. Yalcinkaya M, et al. Cholesterol accumulation in macrophages drives NETosis in atherosclerotic plaques via IL-1β secretion. Cardiovasc Res. 2023 May 2;119(4):969-981. doi: 10.1093/cvr/cvac189. PMID: 36537208; PMCID: PMC10153645.

153. Lande R, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011 Mar 9;3(73):73ra19. doi: 10.1126/scitranslmed.3001180. PMID: 21389263; PMCID: PMC3399524.

154. Aleyd E, et al. IgA Complexes in Plasma and Synovial Fluid of Patients with Rheumatoid Arthritis Induce Neutrophil Extracellular Traps via FcαRI. J Immunol. 2016 Dec 15;197(12):4552-4559. doi: 10.4049/jimmunol.1502353. Epub 2016 Nov 9. PMID: 27913645.

155. Brown GE, Stewart MQ, Liu H, Ha VL, Yaffe MB. A novel assay system implicates PtdIns(3,4)P(2), PtdIns(3)P, and PKC delta in intracellular production of reactive oxygen species by the NADPH oxidase. Mol Cell. 2003 Jan;11(1):35-47. doi: 10.1016/s1097-2765(03)00005-4. PMID: 12535519.

156. Miao N, et al. Oxidized mitochondrial DNA induces gasdermin D oligomerization in systemic lupus erythematosus. Nat Commun. 2023 Feb 16;14(1):872. doi: 10.1038/s41467-023-36522-z. PMID: 36797275; PMCID: PMC9935630.

157. Villanueva E, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol. 2011 Jul 1;187(1):538-52. doi: 10.4049/jimmunol.1100450. Epub 2011 May 25. PMID: 21613614; PMCID: PMC3119769.

158. Ye H, et al. Dysregulated low-density granulocyte contributes to early spontaneous abortion. Front Immunol. 2023 Feb 23;14:1119756. doi: 10.3389/fimmu.2023.1119756. PMID: 36911722; PMCID: PMC9995479.

159. Teague HL, et al. Neutrophil Subsets, Platelets, and Vascular Disease in Psoriasis. JACC Basic Transl Sci. 2019 Feb 25;4(1):1-14. doi: 10.1016/j.jacbts.2018.10.008. PMID: 30847414; PMCID: PMC6390681.

160. Biswas S, et al. Understanding the Role of Antimicrobial Peptides in Neutrophil Extracellular Traps Promoting Autoimmune Disorders. Life (Basel). 2023 Jun 1;13(6):1307. doi: 10.3390/life13061307. PMID: 37374090; PMCID: PMC10301823.

161. Tanaka Y, et al. Dual Roles of Extracellular Histone H3 in Host Defense: Its Differential Regions Responsible for Antimicrobial and Cytotoxic Properties and Their Modes of Action. Antibiotics (Basel). 2022 Sep 13;11(9):1240. doi: 10.3390/antibiotics11091240. PMID: 36140018; PMCID: PMC9495139.

162. Lappann M, et al. In vitro resistance mechanisms of Neisseria meningitidis against neutrophil extracellular traps. Mol Microbiol. 2013 Aug;89(3):433-49. doi: 10.1111/mmi.12288. Epub 2013 Jun 25. PMID: 23750848.

163. Halverson TW, et al. DNA is an antimicrobial component of neutrophil extracellular traps. PLoS Pathog. 2015 Jan 15;11(1):e1004593. doi: 10.1371/journal.ppat.1004593. PMID: 25590621; PMCID: PMC4295883.

164. Tsourouktsoglou TD, et al. Histones, DNA, and Citrullination Promote Neutrophil Extracellular Trap Inflammation by Regulating the Localization and Activation of TLR4. Cell Rep. 2020 May 5;31(5):107602. doi: 10.1016/j.celrep.2020.107602. PMID: 32375035.

165. Decker P, et al. Nucleosome, the main autoantigen in systemic lupus erythematosus, induces direct dendritic cell activation via a MyD88-independent pathway: consequences on inflammation. J Immunol. 2005 Mar 15;174(6):3326-34. doi: 10.4049/jimmunol.174.6.3326. PMID: 15749864.

166. Nofi CP, et al. Chromatin-Associated Molecular Patterns (CAMPs) in sepsis. Cell Death Dis. 2022 Aug 12;13(8):700. doi: 10.1038/s41419-022-05155-3. PMID: 35961978; PMCID: PMC9372964.

167. Baillet A, et al. Synovial fluid proteomic fingerprint: S100A8, S100A9 and S100A12 proteins discriminate rheumatoid arthritis from other inflammatory joint diseases. Rheumatology (Oxford). 2010 Apr;49(4):671-82. doi: 10.1093/rheumatology/kep452. Epub 2010 Jan 25. PMID: 20100792.

168. Sprenkeler EGG, et al. S100A8/A9 Is a Marker for the Release of Neutrophil Extracellular Traps and Induces Neutrophil Activation. Cells. 2022 Jan 11;11(2):236. doi: 10.3390/cells11020236. PMID: 35053354; PMCID: PMC8773660.

169. Martin CA, et al. Aberrant extracellular and dendritic cell (DC) surface expression of heat shock protein (hsp)70 in the rheumatoid joint: possible mechanisms of hsp/DC-mediated cross-priming. J Immunol. 2003 Dec 1;171(11):5736-42. doi: 10.4049/jimmunol.171.11.5736. PMID: 14634081.

170. Shao S, et al. Neutrophil Extracellular Traps Promote Inflammatory Responses in Psoriasis via Activating Epidermal TLR4/IL-36R Crosstalk. Front Immunol. 2019 Apr 5;10:746. doi: 10.3389/fimmu.2019.00746. PMID: 31024570; PMCID: PMC6460719.

171. Goldstein RS, et al. Cholinergic anti-inflammatory pathway activity and High Mobility Group Box-1 (HMGB1) serum levels in patients with rheumatoid arthritis. Mol Med. 2007 Mar-Apr;13(3-4):210-5. doi: 10.2119/2006–00108.Goldstein. PMID: 17597834; PMCID: PMC1899837.

172. Schierbeck H, et al. Monoclonal anti-HMGB1 (high mobility group box chromosomal protein 1) antibody protection in two experimental arthritis models. Mol Med. 2011 Sep-Oct;17(9-10):1039-44. doi: 10.2119/molmed.2010.00264. Epub 2011 Jun 7. PMID: 21666956; PMCID: PMC3188862.

173. Peng W, et al. Increased levels of serum histone H4 and activated protein C in patients with active rheumatoid arthritis. Clin Exp Rheumatol. 2018 May-Jun;36(3):455-461. Epub 2017 Dec 27. PMID: 29303701.

174. Jung J, et al. Extracellular histones aggravate autoimmune arthritis by lytic cell death. Front Immunol. 2022 Aug 11;13:961197. doi: 10.3389/fimmu.2022.961197. PMID: 36032105; PMCID: PMC9410568.

175. Janssen KMJ, et al. Autoantibodies against citrullinated histone H3 in rheumatoid arthritis and periodontitis patients. J Clin Periodontol. 2017 Jun;44(6):577-584. doi: 10.1111/jcpe.12727. Epub 2017 May 23. PMID: 28370244.

176. Lindau D, et al. Primary blood neutrophils express a functional cell surface Toll-like receptor 9. Eur J Immunol. 2013 Aug;43(8):2101-13. doi: 10.1002/eji.201142143. PMID: 23686399.

177. Kielbassa K, et al. Ibrutinib sensitizes CLL cells to venetoclax by interrupting TLR9-induced CD40 upregulation and protein translation. Leukemia. 2023 Jun;37(6):1268-1276. doi: 10.1038/s41375-023-01898-w. Epub 2023 Apr 26. PMID: 37100883; PMCID: PMC10244160.

178. Song W, et al. Neutrophil Extracellular Traps Tied to Rheumatoid Arthritis: Points to Ponder. Front Immunol. 2021 Jan 29;11:578129. doi: 10.3389/fimmu.2020.578129. PMID: 33584645; PMCID: PMC7878527.

179. Fischer A, et al. The involvement of Toll-like receptor 9 in the pathogenesis of erosive autoimmune arthritis. J Cell Mol Med. 2018 Sep;22(9):4399-4409. doi: 10.1111/jcmm.13735. Epub 2018 Jul 11. PMID: 29992753; PMCID: PMC6111819.

180. Zhang S, et al. Neutrophil extracellular traps activate lung fibroblast to induce polymyositis-related interstitial lung diseases via TLR9-miR-7-Smad2 pathway. J Cell Mol Med. 2020 Jan;24(2):1658-1669. doi: 10.1111/jcmm.14858. Epub 2019 Dec 10. PMID: 31821687; PMCID: PMC6991674.

181. Garcia-Romo GS, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med. 2011 Mar 9;3(73):73ra20. doi: 10.1126/scitranslmed.3001201. PMID: 21389264; PMCID: PMC3143837.

182. Suksawad N, et al. Cyclic GMP-AMP Synthase (cGAS) Deletion Reduces Severity in Bilateral Nephrectomy Mice through Changes in Neutrophil Extracellular Traps and Mitochondrial Respiration. Biomedicines. 2023 Apr 18;11(4):1208. doi: 10.3390/biomedicines11041208. PMID: 37189826; PMCID: PMC10136362.

183. Gehrke N, et al. Oxidative damage of DNA confers resistance to cytosolic nuclease TREX1 degradation and potentiates STING-dependent immune sensing. Immunity. 2013 Sep 19;39(3):482-95. doi: 10.1016/j.immuni.2013.08.004. Epub 2013 Aug 29. PMID: 23993650.

184. Zhao J, et al. NETs Promote Inflammatory Injury by Activating cGAS-STING Pathway in Acute Lung Injury. Int J Mol Sci. 2023 Mar 7;24(6):5125. doi: 10.3390/ijms24065125. PMID: 36982193; PMCID: PMC10049640.

185. Cao Y, et al. Inhibition of neutrophil extracellular trap formation attenuates NLRP1-dependent neuronal pyroptosis via STING/IRE1α pathway after traumatic brain injury in mice. Front Immunol. 2023 Apr 14;14:1125759. doi: 10.3389/fimmu.2023.1125759. PMID: 37143681; PMCID: PMC10152368.

186. Apel F, et al. The cytosolic DNA sensor cGAS recognizes neutrophil extracellular traps. Sci Signal. 2021 Mar 9;14(673):eaax7942. doi: 10.1126/scisignal.aax7942. PMID: 33688080.

187. Andreeva L, et al. cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein-DNA ladders. Nature. 2017 Sep 21;549(7672):394-398. doi: 10.1038/nature23890. Epub 2017 Sep 13. PMID: 28902841.

188. Uresti-Rivera EE, et al. AIM2-inflammasome role in systemic lupus erythematous and rheumatoid arthritis. Autoimmunity. 2022 Nov;55(7):443-454. doi: 10.1080/08916934.2022.2103802. Epub 2022 Jul 26. PMID: 35880661.

189. Yang M, et al. AIM2 deficiency in B cells ameliorates systemic lupus erythematosus by regulating Blimp-1-Bcl-6 axis-mediated B-cell differentiation. Signal Transduct Target Ther. 2021 Sep 14;6(1):341. doi: 10.1038/s41392-021-00725-x. PMID: 34521812; PMCID: PMC8440614.

190. Chen Y, et al. Expression of AIM2 in Rheumatoid Arthritis and Its Role on Fibroblast-Like Synoviocytes. Mediators Inflamm. 2020 Oct 26;2020:1693730. doi: 10.1155/2020/1693730. PMID: 33162829; PMCID: PMC7605934.

191. Li H, et al. Neutrophil Extracellular Traps Augmented Alveolar Macrophage Pyroptosis via AIM2 Inflammasome Activation in LPS-Induced ALI/ARDS. J Inflamm Res. 2021 Sep 21;14:4839-4858. doi: 10.2147/JIR.S321513. PMID: 34588792; PMCID: PMC8473117.

192. Antiochos B, et al. The DNA sensors AIM2 and IFI16 are SLE autoantigens that bind neutrophil extracellular traps. Elife. 2022 May 24;11:e72103. doi: 10.7554/eLife.72103. PMID: 35608258; PMCID: PMC9129876.

193. Cao T, et al. Neutrophil extracellular traps promote keratinocyte inflammation via AIM2 inflammasome and AIM2-XIAP in psoriasis. Exp Dermatol. 2023 Apr;32(4):368-378. doi: 10.1111/exd.14711. Epub 2022 Nov 23. PMID: 36401800.

194. Blanco LP, et al. RNA Externalized by Neutrophil Extracellular Traps Promotes Inflammatory Pathways in Endothelial Cells. Arthritis Rheumatol. 2021 Dec;73(12):2282-2292. doi: 10.1002/art.41796. Epub 2021 Oct 22. PMID: 33983685; PMCID: PMC8589882.

195. Francesca Bork, et al. naRNA is a canonical neutrophil extracellular trap (NET) component and novel inflammation-amplifying composite DAMP. bioRxiv 2022.07.26.499571; doi: https://doi.org/10.1101/2022.07.26.499571.

196. Ding W, et al. A neutrophil extracellular traps-associated lncRNA signature predicts the clinical outcomes in patients with lung adenocarcinoma. Front Genet. 2022 Nov 7;13:1047231. doi: 10.3389/fgene.2022.1047231. PMID: 36419832; PMCID: PMC9676361.

197. Herster F, et al. Neutrophil extracellular trap-associated RNA and LL37 enable self-amplifying inflammation in psoriasis. Nat Commun. 2020 Jan 8;11(1):105. doi: 10.1038/s41467-019-13756-4. PMID: 31913271; PMCID: PMC6949246.

198. Kim SJ, et al. Identification of a Novel Toll-like Receptor 7 Endogenous Ligand in Rheumatoid Arthritis Synovial Fluid That Can Provoke Arthritic Joint Inflammation. Arthritis Rheumatol. 2016 May;68(5):1099-110. doi: 10.1002/art.39544. PMID: 26662519; PMCID: PMC5221504.

199. Linhares-Lacerda L, et al. Neutrophil extracellular trap-enriched supernatants carry microRNAs able to modulate TNF-α production by macrophages. Sci Rep. 2020 Feb 17;10(1):2715. doi: 10.1038/s41598-020-59486-2. PMID: 32066757; PMCID: PMC7026108.

200. Barrientos L, et al. Neutrophil extracellular traps downregulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells. J Immunol. 2014 Dec 1;193(11):5689-98. doi: 10.4049/jimmunol.1400586. Epub 2014 Oct 22. PMID: 25339673.

201. Aloul KM, et al. Upregulating Human Cathelicidin Antimicrobial Peptide LL-37 Expression May Prevent Severe COVID-19 Inflammatory Responses and Reduce Microthrombosis. Front Immunol. 2022 May 12;13:880961. doi: 10.3389/fimmu.2022.880961. PMID: 35634307; PMCID: PMC9134243.

202. Schauer C, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nat Med. 2014 May;20(5):511-7. doi: 10.1038/nm.3547. Epub 2014 Apr 28. PMID: 24784231.

203. Pan Y, et al. Evaluation of the Anti-Inflammatory and Anti-Oxidative Effects of Therapeutic Human Lactoferrin Fragments. Front Bioeng Biotechnol. 2021 Nov 30;9:779018. doi: 10.3389/fbioe.2021.779018. PMID: 34917601; PMCID: PMC8671034.

204. Eghbalzadeh K, et al. Compromised Anti-inflammatory Action of Neutrophil Extracellular Traps in PAD4-Deficient Mice Contributes to Aggravated Acute Inflammation After Myocardial Infarction. Front Immunol. 2019 Oct 1;10:2313. doi: 10.3389/fimmu.2019.02313. PMID: 31632398; PMCID: PMC6779806.

205. Schöenfeld L, et al. Immunofluorescence Imaging of Neutrophil Extracellular Traps in Human and Mouse Tissues. J Vis Exp. 2023 Aug 18;(198). doi: 10.3791/65272. PMID: 37677039.

206. Kraaij T, et al. A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes. Autoimmun Rev. 2016 Jun;15(6):577-84. doi: 10.1016/j.autrev.2016.02.018. Epub 2016 Feb 27. PMID: 26925759.

207. Singhal A, et al. An Imaging and Computational Algorithm for Efficient Identification and Quantification of Neutrophil Extracellular Traps. Cells. 2022 Jan 6;11(2):191. doi: 10.3390/cells11020191. PMID: 35053307; PMCID: PMC8773682.

208. Ciach MA, et al. Trapalyzer: a computer program for quantitative analyses in fluorescent live-imaging studies of neutrophil extracellular trap formation. Front Immunol. 2023 Jun 8;14:1021638. doi: 10.3389/fimmu.2023.1021638. PMID: 37359539; PMCID: PMC10285529.

209. Hosseini A, et al. Transcriptional Insights of Oxidative Stress and Extracellular Traps in Lung Tissues of Fatal COVID-19 Cases. Int J Mol Sci. 2023 Jan 31;24(3):2646. doi: 10.3390/ijms24032646. PMID: 36768969; PMCID: PMC9917045.

210. Lu C, et al. Sperm induce macrophage extracellular trap formation via phagocytosis-dependent mechanism. Biol Reprod. 2023 Sep 12;109(3):319-329. doi: 10.1093/biolre/ioad068. PMID: 37402702.

211. Poli V, et al. Quantitative cytofluorimetric analysis of mouse neutrophil extracellular traps. STAR Protoc. 2023 Jun 22;4(3):102372. doi: 10.1016/j.xpro.2023.102372. Epub ahead of print. PMID: 37352106.

212. Caudrillier A, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. J Clin Invest. 2012 Jul;122(7):2661-71. doi: 10.1172/JCI61303. Epub 2012 Jun 11. PMID: 22684106; PMCID: PMC3386815.

213. Thålin C, et al. Validation of an enzyme-linked immunosorbent assay for the quantification of citrullinated histone H3 as a marker for neutrophil extracellular traps in human plasma. Immunol Res. 2017 Jun;65(3):706-712. doi: 10.1007/s12026-017-8905-3. PMID: 28161762; PMCID: PMC5440486.

214. Bendib I, et al. Neutrophil Extracellular Traps Are Elevated in Patients with Pneumonia-related Acute Respiratory Distress Syndrome. Anesthesiology. 2019 Apr;130(4):581-591. doi: 10.1097/ALN.0000000000002619. PMID: 30676417.

215. Kolaczkowska E, et alP. Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. Nat Commun. 2015 Mar 26;6:6673. doi: 10.1038/ncomms7673. PMID: 25809117; PMCID: PMC4389265.

216. Jhunjhunwala S, et al. Neutrophil Responses to Sterile Implant Materials. PLoS One. 2015 Sep 10;10(9):e0137550. doi: 10.1371/journal.pone.0137550. PMID: 26355958; PMCID: PMC4565661.

217. Santocki M, et al. On Neutrophil Extracellular Trap (NET) Removal: What We Know Thus Far and Why So Little. Cells. 2020 Sep 11;9(9):2079. doi: 10.3390/cells9092079. PMID: 32932841; PMCID: PMC7565917.

218. Oliveira CB, et al. Neutralizing Anti–DNase 1 and –DNase 1L3 Antibodies Impair Neutrophil Extracellular Traps Degradation in Hidradenitis Suppurativa. J Invest Dermatol. 2023 Jan;143(1):57-66. doi: 10.1016/j.jid.2022.06.024. Epub 2022 Aug 4. PMID: 35934056; PMCID: PMC9771923.

219. Kumar A, et al. Deciphering the Role of Leptospira Surface Protein LigA in Modulating the Host Innate Immune Response. Front Immunol. 2021 Dec 16;12:807775. doi: 10.3389/fimmu.2021.807775. PMID: 34975922; PMCID: PMC8716722.

220. Xu XY, et al. Correlation of DNase I in serum and synovial fluid with inflammatory activity in patients with rheumatoid arthritis. Nan Fang Yi Ke Da Xue Xue Bao. 2016 Aug 20;36(9):1204-1208. Chinese. PMID: 27687651.

221. Wang CL, et al. DNase I and Sivelestat Ameliorate Experimental Hindlimb Ischemia-Reperfusion Injury by Eliminating Neutrophil Extracellular Traps. J Inflamm Res. 2023 Feb 21;16:707-721. doi: 10.2147/JIR.S396049. PMID: 36852300; PMCID: PMC9961174.

222. Farrera C, et al. Macrophage clearance of neutrophil extracellular traps is a silent process. J Immunol. 2013 Sep 1;191(5):2647-56. doi: 10.4049/jimmunol.1300436. Epub 2013 Jul 31. PMID: 23904163.

223. Haider P, et al. Neutrophil Extracellular Trap Degradation by Differently Polarized Macrophage Subsets. Arterioscler Thromb Vasc Biol. 2020 Sep;40(9):2265-2278. doi: 10.1161/ATVBAHA.120.314883. Epub 2020 Jul 16. PMID: 32673525; PMCID: PMC7447175.

224. Jeong JH, et al. Neutrophil extracellular trap clearance by synovial macrophages in gout. Arthritis Res Ther. 2021 Mar 19;23(1):88. doi: 10.1186/s13075-021-02472-4. PMID: 33741037; PMCID: PMC7977263.

225. Al-Mayouf SM, et al. Loss-of-function variant in DNASE1L3 causes a familial form of systemic lupus erythematosus. Nat Genet. 2011 Oct 23;43(12):1186-8. doi: 10.1038/ng.975. PMID: 22019780.

226. Lou H, et al. Autoantibody-dependent amplification of inflammation in SLE. Cell Death Dis. 2020 Sep 9;11(9):729. doi: 10.1038/s41419-020-02928-6. PMID: 32908129; PMCID: PMC7481301.

227. Bach M, et al. A Neutrophil Activation Biomarker Panel in Prognosis and Monitoring of Patients With Rheumatoid Arthritis. Arthritis Rheumatol. 2020 Jan;72(1):47-56. doi: 10.1002/art.41062. Epub 2019 Dec 3. PMID: 31353807; PMCID: PMC6935396.

228. Grégoire M, et al. Impaired efferocytosis and neutrophil extracellular trap clearance by macrophages in ARDS. Eur Respir J. 2018 Aug 2;52(2):1702590. doi: 10.1183/13993003.02590-2017. PMID: 29946009.

229. Yost CC, et al. Neonatal NET-inhibitory factor and related peptides inhibit neutrophil extracellular trap formation. J Clin Invest. 2016 Oct 3;126(10):3783-3798. doi: 10.1172/JCI83873. Epub 2016 Sep 6. PMID: 27599294; PMCID: PMC5096809.

230. Hudock KM, et al. Alpha-1 antitrypsin limits neutrophil extracellular trap disruption of airway epithelial barrier function. Front Immunol. 2023 Jan 10;13:1023553. doi: 10.3389/fimmu.2022.1023553. PMID: 36703990; PMCID: PMC9872031.

231. Biswas S, et al. Identification of novel autoantigen in the synovial fluid of rheumatoid arthritis patients using an immunoproteomics approach. PLoS One. 2013;8(2):e56246. doi: 10.1371/journal.pone.0056246. Epub 2013 Feb 13. PMID: 23418544; PMCID: PMC3572018.

232. Zabieglo K, et al. The inhibitory effect of secretory leukocyte protease inhibitor (SLPI) on formation of neutrophil extracellular traps. J Leukoc Biol. 2015 Jul;98(1):99-106. doi: 10.1189/jlb.4AB1114-543R. Epub 2015 Apr 27. PMID: 25917460; PMCID: PMC4467168.

233. White D, et al. Heparin resistance in COVID-19 patients in the intensive care unit. J Thromb Thrombolysis. 2020 Aug;50(2):287-291. doi: 10.1007/s11239-020-02145-0. Erratum in: J Thromb Thrombolysis. 2020 Jun 22;: PMID: 32445064; PMCID: PMC7242778.

234. Okur HK, et al. Preliminary report of in vitro and in vivo effectiveness of dornase alfa on SARS-CoV-2 infection. New Microbes New Infect. 2020 Sep;37:100756. doi: 10.1016/j.nmni.2020.100756. Epub 2020 Sep 7. PMID: 32922804; PMCID: PMC7476504.

235. Wang Z, et al. Cell membrane derived liposomes loaded with DNase I target neutrophil extracellular traps which inhibits colorectal cancer liver metastases. J Control Release. 2023 May;357:620-629. doi: 10.1016/j.jconrel.2023.04.013. Epub 2023 Apr 24. PMID: 37061194.

236. Wang CY, et al. Neutrophil extracellular traps as a unique target in the treatment of chemotherapy-induced peripheral neuropathy. EBioMedicine. 2023 Apr;90:104499. doi: 10.1016/j.ebiom.2023.104499. Epub 2023 Mar 2. PMID: 36870200; PMCID: PMC10009451.

237. Shen W, et al. Inhibition of Neutrophil Extracellular Traps Formation by Cl-Amidine Alleviates Lipopolysaccharide-Induced Endometritis and Uterine Tissue Damage. Animals (Basel). 2022 Apr 29;12(9):1151. doi: 10.3390/ani12091151. PMID: 35565576; PMCID: PMC9100562.

238. Mutua V, et al. A Review of Neutrophil Extracellular Traps (NETs) in Disease: Potential Anti-NETs Therapeutics. Clin Rev Allergy Immunol. 2021 Oct;61(2):194-211. doi: 10.1007/s12016-020-08804-7. PMID: 32740860; PMCID: PMC7395212.

239. Bystrzycka W, et al. Azithromycin and Chloramphenicol Diminish Neutrophil Extracellular Traps (NETs) Release. Int J Mol Sci. 2017 Dec 8;18(12):2666. doi: 10.3390/ijms18122666. PMID: 29292737; PMCID: PMC5751268.

240. Wan T, et al. Dexamethasone Inhibits S. aureus-Induced Neutrophil Extracellular Pathogen-Killing Mechanism, Possibly through Toll-Like Receptor Regulation. Front Immunol. 2017 Feb 9;8:60. doi: 10.3389/fimmu.2017.00060. PMID: 28232829; PMCID: PMC5299007.

241. Wang G, et al. Metformin inhibits neutrophil extracellular traps-promoted pancreatic carcinogenesis in obese mice. Cancer Lett. 2023 May 28;562:216155. doi: 10.1016/j.canlet.2023.216155. Epub 2023 Apr 6. PMID: 37030634.

242. Gestermann N, et al. Netting Neutrophils Activate Autoreactive B Cells in Lupus. J Immunol. 2018 May 15;200(10):3364-3371. doi: 10.4049/jimmunol.1700778. Epub 2018 Apr 9. PMID: 29632142.

243. Bertelli R, et al. Neutrophil Extracellular Traps in Systemic Lupus Erythematosus Stimulate IgG2 Production From B Lymphocytes. Front Med (Lausanne). 2021 Apr 12;8:635436. doi: 10.3389/fmed.2021.635436. PMID: 33912575; PMCID: PMC8072216.

244. Bruschi M, et al Serum IgG2 antibody multicomposition in systemic lupus erythematosus and lupus nephritis (Part 1): cross-sectional analysis. Rheumatology (Oxford). 2021 Jul 1;60(7):3176-3188. doi: 10.1093/rheumatology/keaa767. PMID: 33374003; PMCID: PMC8487649.

245. Hakkim A, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A. 2010 May 25;107(21):9813-8. doi: 10.1073/pnas.0909927107. Epub 2010 May 3. PMID: 20439745; PMCID: PMC2906830.

246. Byrd AS, et al. Neutrophil extracellular traps, B cells, and type I interferons contribute to immune dysregulation in hidradenitis suppurativa. Sci Transl Med. 2019 Sep 4;11(508):eaav5908. doi: 10.1126/scitranslmed.aav5908. PMID: 31484788.

247. Hoffman LK, et al. Integrating the skin and blood transcriptomes and serum proteome in hidradenitis suppurativa reveals complement dysregulation and a plasma cell signature. PLoS One. 2018 Sep 28;13(9):e0203672. doi: 10.1371/journal.pone.0203672. PMID: 30265680; PMCID: PMC6162087.

248. Fang H, et al. Neutrophil extracellular traps contribute to immune dysregulation in bullous pemphigoid via inducing B-cell differentiation and antibody production. FASEB J. 2021 Jul;35(7):e21746. doi: 10.1096/fj.202100145R. PMID: 34151465.

249. Giusti D, et al. NET Formation in Bullous Pemphigoid Patients With Relapse Is Modulated by IL-17 and IL-23 Interplay. Front Immunol. 2019 Apr 4;10:701. doi: 10.3389/fimmu.2019.00701. PMID: 31019514; PMCID: PMC6458298.

250. Yang L, et al. DNA of neutrophil extracellular traps promotes cancer metastasis via CCDC25. Nature. 2020 Jul;583(7814):133-138. doi: 10.1038/s41586-020-2394-6. Epub 2020 Jun 11. PMID: 32528174.

251. Park J, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med. 2016 Oct 19;8(361):361ra138. doi: 10.1126/scitranslmed.aag1711. PMID: 27798263; PMCID: PMC5550900.

252. Demers M, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. Proc Natl Acad Sci U S A. 2012 Aug 7;109(32):13076-81. doi: 10.1073/pnas.1200419109. Epub 2012 Jul 23. PMID: 22826226; PMCID: PMC3420209.

253. Nie M, et al. Neutrophil Extracellular Traps Induced by IL8 Promote Diffuse Large B-cell Lymphoma Progression via the TLR9 Signaling. Clin Cancer Res. 2019 Mar 15;25(6):1867-1879. doi: 10.1158/1078-0432.CCR-18-1226. Epub 2018 Nov 16. PMID: 30446590.

254. Sangaletti S, et al. Defective stromal remodeling and neutrophil extracellular traps in lymphoid tissues favor the transition from autoimmunity to lymphoma. Cancer Discov. 2014 Jan;4(1):110-29. doi: 10.1158/2159-8290.CD-13-0276. Epub 2013 Nov 4. PMID: 24189145.

255. Tu Y, Mao Z. Identification and Validation of Molecular Subtype and Prognostic Signature for Bladder Cancer Based on Neutrophil Extracellular Traps. Cancer Invest. 2023 Apr;41(4):354-368. doi: 10.1080/07357907.2023.2179063. Epub 2023 Feb 16. PMID: 36762827.

256. Winkler TH, Mårtensson IL. The Role of the Pre-B Cell Receptor in B Cell Development, Repertoire Selection, and Tolerance. Front Immunol. 2018 Nov 15;9:2423. doi: 10.3389/fimmu.2018.02423. PMID: 30498490; PMCID: PMC6249383.

257. Sanz I, et al. Challenges and Opportunities for Consistent Classification of Human B Cell and Plasma Cell Populations. Front Immunol. 2019 Oct 18;10:2458. doi: 10.3389/fimmu.2019.02458. PMID: 31681331; PMCID: PMC6813733.

258. Kaminski DA, et al. Advances in human B cell phenotypic profiling. Front Immunol. 2012 Oct 10;3:302. doi: 10.3389/fimmu.2012.00302. PMID: 23087687; PMCID: PMC3467643.

259. Geisberger R, Lamers M, Achatz G. The riddle of the dual expression of IgM and IgD. Immunology. 2006 Aug;118(4):429-37. doi: 10.1111/j.1365-2567.2006.02386.x. PMID: 16895553; PMCID: PMC1782314.

260. Bortnick A, Allman D. What is and what should always have been: long-lived plasma cells induced by T cell-independent antigens. J Immunol. 2013 Jun 15;190(12):5913-8. doi: 10.4049/jimmunol.1300161. PMID: 23749966; PMCID: PMC4234153.

261. Huang W, et al. The effect of anti-CD40 ligand antibody on B cells in human systemic lupus erythematosus. Arthritis Rheum. 2002 Jun;46(6):1554-62. doi: 10.1002/art.10273. PMID: 12115186.

262. Chung MKY, et al. Functions of double-negative B cells in autoimmune diseases, infections, and cancers. EMBO Mol Med. 2023 Jun 5:e17341. doi: 10.15252/emmm.202217341. Epub ahead of print. PMID: 37272217.

263. Fecteau JF, Côté G, Néron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. J Immunol. 2006 Sep 15;177(6):3728-36. doi: 10.4049/jimmunol.177.6.3728. PMID: 16951333.

264. Allard-Chamard H, et al. Extrafollicular IgD-CD27-CXCR5-CD11c- DN3 B cells infiltrate inflamed tissues in autoimmune fibrosis and in severe COVID-19. Cell Rep. 2023 May 30;42(6):112630. doi: 10.1016/j.celrep.2023.112630. Epub ahead of print. PMID: 37300833; PMCID: PMC10227203.

265. Park SM, et al. Migratory cues controlling B-lymphocyte trafficking in human lymph nodes. Immunol Cell Biol. 2021 Jan;99(1):49-64. doi: 10.1111/imcb.12386. Epub 2020 Sep 15. PMID: 32740978.

266. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Annu Rev Immunol. 2012;30:69-94. doi: 10.1146/annurev-immunol-020711-075011. Epub 2011 Dec 5. PMID: 22149932.

267. Druzd D, et al. Lymphocyte Circadian Clocks Control Lymph Node Trafficking and Adaptive Immune Responses. Immunity. 2017 Jan 17;46(1):120-132. doi: 10.1016/j.immuni.2016.12.011. Epub 2017 Jan 10. PMID: 28087238; PMCID: PMC5263259.

268. Meednu N, et al. Dynamic spectrum of ectopic lymphoid B cell activation and hypermutation in the RA synovium characterized by NR4A nuclear receptor expression. Cell Rep. 2022 May 3;39(5):110766. doi: 10.1016/j.celrep.2022.110766. PMID: 35508128; PMCID: PMC9234997.

269. Shi K, et al. Lymphoid chemokine B cell-attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. J Immunol. 2001 Jan 1;166(1):650-5. doi: 10.4049/jimmunol.166.1.650. PMID: 11123349.

270. Kaminski DA, et al. Advances in human B cell phenotypic profiling. Front Immunol. 2012 Oct 10;3:302. doi: 10.3389/fimmu.2012.00302. PMID: 23087687; PMCID: PMC3467643.

261. Pelton BK, et al. Cytokine-independent progression of immunoglobulin production in vitro by B lymphocytes from patients with systemic lupus erythematosus. Clin Exp Immunol. 1991 Feb;83(2):274-9. doi: 10.1111/j.1365-2249.1991.tb05627.x. PMID: 1825187; PMCID: PMC1535253.

272. Herrock O, et al. Inhibiting B cell activating factor attenuates preeclamptic symptoms in placental ischemic rats. Am J Reprod Immunol. 2023 Apr;89(4):e13693. doi: 10.1111/aji.13693. Epub 2023 Feb 27. PMID: 36794639; PMCID: PMC10009902.

273. Ozaki K, et al. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002 Nov 22;298(5598):1630-4. doi: 10.1126/science.1077002. PMID: 12446913.

274. Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol. 2008 Jan;8(1):22-33. doi: 10.1038/nri2217. PMID: 18097447.

275. Weller S, et al. T-independent responses to polysaccharides in humans mobilize marginal zone B cells prediversified against gut bacterial antigens. Sci Immunol. 2023 Jan 27;8(79):eade1413. doi: 10.1126/sciimmunol.ade1413. Epub 2023 Jan 27. PMID: 36706172; PMCID: PMC7614366.

276. Musher DM, et al. Safety and antibody response, including antibody persistence for 5 years, after primary vaccination or revaccination with pneumococcal polysaccharide vaccine in middle-aged and older adults. J Infect Dis. 2010 Feb 15;201(4):516-24. doi: 10.1086/649839. PMID: 20092407.

277. Vos Q, et al. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. Immunol Rev. 2000 Aug;176:154-70. doi: 10.1034/j.1600-065x.2000.00607.x. PMID: 11043775.

278. Obukhanych TV, et al. T-independent type II immune responses generate memory B cells. J Exp Med. 2006 Feb 20;203(2):305-10. doi: 10.1084/jem.20052036. Epub 2006 Feb 13. PMID: 16476769; PMCID: PMC2118207.

279. Safiri S, et al. Global, regional and national burden of rheumatoid arthritis 1990-2017: a systematic analysis of the Global Burden of Disease study 2017. Ann Rheum Dis. 2019 Nov;78(11):1463-1471. doi: 10.1136/annrheumdis-2019-215920. Epub 2019 Sep 11. PMID: 31511227.

280. Landré-Beauvais AJ. The first description of rheumatoid arthritis. Unabridged text of the doctoral dissertation presented in 1800. Joint Bone Spine. 2001 Mar;68(2):130-43. English, French. doi: 10.1016/s1297-319x(00)00247-5. PMID: 11324929.

281. Kim JW, et al. Systemic Manifestations and Complications in Patients with Rheumatoid Arthritis. J Clin Med. 2020 Jun 26;9(6):2008. doi: 10.3390/jcm9062008. PMID: 32604884; PMCID: PMC7356332.

282. Barkhane Z, et al. Comparison of Cardiovascular Outcomes in Patients With and Without Rheumatoid Arthritis: A Meta-Analysis of Observational Studies. Cureus. 2023 Jun 13;15(6):e40348. doi: 10.7759/cureus.40348. PMID: 37456442; PMCID: PMC10339149.

283. Gerlag DM, et al. EULAR recommendations for terminology and research in individuals at risk of rheumatoid arthritis: report from the Study Group for Risk Factors for Rheumatoid Arthritis. Ann Rheum Dis. 2012 May;71(5):638-41. doi: 10.1136/annrheumdis-2011-200990. Epub 2012 Mar 2. PMID: 22387728; PMCID: PMC3329228.

284. Reynisdottir G, et al. Structural changes and antibody enrichment in the lungs are early features of anti-citrullinated protein antibody-positive rheumatoid arthritis. Arthritis Rheumatol. 2014 Jan;66(1):31-9. doi: 10.1002/art.38201. PMID: 24449573.

285. Reynisdottir G, et al. Signs of immune activation and local inflammation are present in the bronchial tissue of patients with untreated early rheumatoid arthritis. Ann Rheum Dis. 2016 Sep;75(9):1722-7. doi: 10.1136/annrheumdis-2015-208216. Epub 2015 Nov 3. PMID: 26530319.

286. Joshua V, et al. Rheumatoid arthritis specific autoimmunity in the lung before and at the onset of disease. Arthritis Rheumatol. 2023 May 16. doi: 10.1002/art.42549. Epub ahead of print. PMID: 37192126.

287. Deane KD, et al. The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner. Arthritis Rheum. 2010 Nov;62(11):3161-72. doi: 10.1002/art.27638. PMID: 20597112; PMCID: PMC2980824.

288. Nielen MM, et al. Simultaneous development of acute phase response and autoantibodies in preclinical rheumatoid arthritis. Ann Rheum Dis. 2006 Apr;65(4):535-7. doi: 10.1136/ard.2005.040659. Epub 2005 Aug 3. PMID: 16079166; PMCID: PMC1798080.

289. Kelmenson LB, et al. Timing of Elevations of Autoantibody Isotypes Prior to Diagnosis of Rheumatoid Arthritis. Arthritis Rheumatol. 2020 Feb;72(2):251-261. doi: 10.1002/art.41091. Epub 2019 Dec 26. PMID: 31464042; PMCID: PMC6994340.

290. Arnett FC, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988 Mar;31(3):315-24. doi: 10.1002/art.1780310302. PMID: 3358796.

291. Aletaha D, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010 Sep;69(9):1580-8. doi: 10.1136/ard.2010.138461. Erratum in: Ann Rheum Dis. 2010 Oct;69(10):1892. PMID: 20699241.

292. Choi S, Lee KH. Clinical management of seronegative and seropositive rheumatoid arthritis: A comparative study. PLoS One. 2018 Apr 6;13(4):e0195550. doi: 10.1371/journal.pone.0195550. PMID: 29624625; PMCID: PMC5889180.

293. Ingegnoli F, et al. Rheumatoid factors: clinical applications. Dis Markers. 2013;35(6):727-34. doi: 10.1155/2013/726598. Epub 2013 Nov 13. PMID: 24324289; PMCID: PMC3845430.

294. Orr CK, et al. The Utility and Limitations of CRP, ESR and DAS28-CRP in Appraising Disease Activity in Rheumatoid Arthritis. Front Med (Lausanne). 2018 Aug 3;5:185. doi: 10.3389/fmed.2018.00185. PMID: 30123796; PMCID: PMC6085449.

295. van Riel PL, Renskers L. The Disease Activity Score (DAS) and the Disease Activity Score using 28 joint counts (DAS28) in the management of rheumatoid arthritis. Clin Exp Rheumatol. 2016 Sep-Oct;34(5 Suppl 101):S40-S44. Epub 2016 Oct 18. PMID: 27762189.

296. Fransen J, Welsing PM, De Keijzer RM, Van Riel PL. Disease activity scores using C-reactive protein: CRP may replace ESR in the assessment of RA disease activity. Ann Rheum Dis. 2004;62(Suppl 1):151.

297. Wells G, et al. Validation of the 28-joint Disease Activity Score (DAS28) and European League Against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate. Ann Rheum Dis. 2009 Jun;68(6):954-60. doi: 10.1136/ard.2007.084459. Epub 2008 May 19. PMID: 18490431; PMCID: PMC2674547.

298. Terao C, et al. Recent Advances in Defining the Genetic Basis of Rheumatoid Arthritis. Annu Rev Genomics Hum Genet. 2016 Aug 31;17:273-301. doi: 10.1146/annurev-genom-090314-045919. Epub 2016 May 23. PMID: 27216775; PMCID: PMC6727201.

299. Kerlan-Candon S, et al. HLA-DRB1 gene transcripts in rheumatoid arthritis. Clin Exp Immunol. 2001 Apr;124(1):142-9. doi: 10.1046/j.1365-2249.2001.01498.x. PMID: 11359453; PMCID: PMC1906025.

300. Ärlestig L, et al. Antibodies against cyclic citrullinated peptides of IgG, IgA and IgM isotype and rheumatoid factor of IgM and IgA isotype are increased in unaffected members of multicase rheumatoid arthritis families from northern Sweden. Ann Rheum Dis. 2012 Jun;71(6):825-9. doi: 10.1136/annrheumdis-2011-200668. Epub 2011 Nov 29. PMID: 22128080; PMCID: PMC3371219.

301. Carlé C, et al. Characteristics of the (Auto)Reactive T Cells in Rheumatoid Arthritis According to the Immune Epitope Database. Int J Mol Sci. 2023 Feb 21;24(5):4296. doi: 10.3390/ijms24054296. PMID: 36901730; PMCID: PMC10001542.

302. Korczowska I. Rheumatoid arthritis susceptibility genes: An overview. World J Orthop. 2014 Sep 18;5(4):544-9. doi: 10.5312/wjo.v5.i4.544. PMID: 25232530; PMCID: PMC4133460.

303. Cui J, et al. Genome-wide association study and gene expression analysis identifies CD84 as a predictor of response to etanercept therapy in rheumatoid arthritis. PLoS Genet. 2013 Mar;9(3):e1003394. doi: 10.1371/journal.pgen.1003394. Epub 2013 Mar 28. PMID: 23555300; PMCID: PMC3610685.

304. Criswell LA, et al. Smoking interacts with genetic risk factors in the development of rheumatoid arthritis among older Caucasian women. Ann Rheum Dis. 2006 Sep;65(9):1163-7. doi: 10.1136/ard.2005.049676. Epub 2006 Aug 3. PMID: 16887863; PMCID: PMC1798304.

305. Li S, et al. Microbial Infection and Rheumatoid Arthritis. J Clin Cell Immunol. 2013 Dec;4(6):174. doi: 10.4172/2155-9899.1000174. PMID: 25133066; PMCID: PMC4131749.

306. Qiao Y, et al. Rheumatoid arthritis risk in periodontitis patients: A systematic review and meta-analysis. Joint Bone Spine. 2020 Dec;87(6):556-564. doi: 10.1016/j.jbspin.2020.04.024. Epub 2020 Jun 25. PMID: 32593704

307. Dissick A, et al. Association of periodontitis with rheumatoid arthritis: a pilot study. J Periodontol. 2010 Feb;81(2):223-30. doi: 10.1902/jop.2009.090309. PMID: 20151800.

308. Ceccarelli F, et al. Porphyromonas gingivalis in the tongue biofilm is associated with clinical outcome in rheumatoid arthritis patients. Clin Exp Immunol. 2018 Nov;194(2):244-252. doi: 10.1111/cei.13184. Epub 2018 Sep 19. PMID: 30009382; PMCID: PMC6194338.

309. Bryzek D, et al. Triggering NETosis via protease-activated receptor (PAR)-2 signaling as a mechanism of hijacking neutrophils function for pathogen benefits. PLoS Pathog. 2019 May 20;15(5):e1007773. doi: 10.1371/journal.ppat.1007773. PMID: 31107907; PMCID: PMC6544335.

310. Lombardo Bedran TB, et al. Porphyromonas endodontalis in chronic periodontitis: a clinical and microbiological cross-sectional study. J Oral Microbiol. 2012;4. doi: 10.3402/jom.v4i0.10123. Epub 2012 Jan 5. PMID: 22232719; PMCID: PMC3253302.

311. Hasebe A, et al. Biological activities of Bacteroides forsythus lipoproteins and their possible pathological roles in periodontal disease. Infect Immun. 2004 Mar;72(3):1318-25. doi: 10.1128/IAI.72.3.1318-1325.2004. PMID: 14977934; PMCID: PMC356049.

312. Kvien TKet al. Epidemiological aspects of rheumatoid arthritis: the sex ratio. Ann N Y Acad Sci. 2006 Jun;1069:212-22. doi: 10.1196/annals.1351.019. PMID: 16855148.

313. Tengstrand B, et al. The influence of sex on rheumatoid arthritis: a prospective study of onset and outcome after 2 years. J Rheumatol. 2004 Feb;31(2):214-22. PMID: 14760787.

314. Schuh BM, et al. Sex differences in long-term effects of collagen-induced arthritis in middle-aged mice. Front Physiol. 2023 Jun 28;14:1195604. doi: 10.3389/fphys.2023.1195604. PMID: 37449011; PMCID: PMC10337783.

315. Sokka T, et al. Women, men, and rheumatoid arthritis: analyses of disease activity, disease characteristics, and treatments in the QUEST-RA study. Arthritis Res Ther. 2009;11(1):R7. doi: 10.1186/ar2591. Epub 2009 Jan 14. PMID: 19144159; PMCID: PMC2688237.

316. Dörner Tet al. Early and late responses in patients with rheumatoid arthritis who were conventional synthetic disease-modifying anti-rheumatic drug inadequate responders and were treated with tocilizumab or switched to rituximab: an open-label phase 3 trial (MIRAI). Clin Exp Rheumatol. 2019 Nov-Dec;37(6):937-945. Epub 2019 Apr 16. PMID: 31025930.

317. Iikuni N, et al. The influence of sex on patients with rheumatoid arthritis in a large observational cohort. J Rheumatol. 2009 Mar;36(3):508-11. doi: 10.3899/jrheum.080724. Epub 2009 Feb 4. PMID: 19208610.

318. Daraghmeh DN, et al. Female reproductive status and exogenous sex hormone use in rheumatoid arthritis patients treated with tocilizumab and csDMARDs. Rheumatology (Oxford). 2023 Feb 1;62(2):583-595. doi: 10.1093/rheumatology/keac357. PMID: 35731132; PMCID: PMC9891436.

319. Wieber K, et al. Dopamine receptor 1 expressing B cells exert a proinflammatory role in female patients with rheumatoid arthritis. Sci Rep. 2022 Apr 8;12(1):5985. doi: 10.1038/s41598-022-09891-6. PMID: 35396380; PMCID: PMC8993840.

320. Klareskog L, et al. Prevention of autoimmune rheumatic disease: state of the art and future perspectives. Ann Rheum Dis. 2010 Dec;69(12):2062-6. doi: 10.1136/ard.2010.142109. PMID: 21097657.

321. Vejlstrup A, et al. Release of active peptidylarginine deiminase into the circulation during acute inflammation induced by coronary artery bypass surgery. J Inflamm Res. 2019 May 29;12:137-144. doi: 10.2147/JIR.S198611. PMID: 31213874; PMCID: PMC6549760.

322. Lei T, et al. Comprehensive evaluation of serum cotinine on human health: Novel evidence for the systemic toxicity of tobacco smoke in the US general population. Sci Total Environ. 2023 May 31:164443. doi: 10.1016/j.scitotenv.2023.164443. Epub ahead of print. PMID: 37268133.

323. Bäcklund R, et al. Adherence to dietary guidelines, and the risk of developing rheumatoid arthritis-results from a nested case-control study. Rheumatology (Oxford). 2023 May 15:kead216. doi: 10.1093/rheumatology/kead216. Epub ahead of print. PMID: 37184858.

324. Zhan Y, et al. Interaction between rheumatoid arthritis and mediterranean diet on the risk of cardiovascular disease for the middle aged and elderly from National Health and Nutrition Examination Survey (NHANES). BMC Public Health. 2023 Mar 31;23(1):620. doi: 10.1186/s12889-023-15478-1. PMID: 37003993; PMCID: PMC10067192.

325. Sigaux J, et al. Impact of type and dose of oral polyunsaturated fatty acid supplementation on disease activity in inflammatory rheumatic diseases: a systematic literature review and meta-analysis. Arthritis Res Ther. 2022 May 7;24(1):100. doi: 10.1186/s13075-022-02781-2. PMID: 35526074; PMCID: PMC9077862.

326. Laragione T, et al. Magnesium increases numbers of Foxp3+ Treg cells and reduces arthritis severity and joint damage in an IL-10-dependent manner mediated by the intestinal microbiome. EBioMedicine. 2023 May 16;92:104603. doi: 10.1016/j.ebiom.2023.104603. Epub ahead of print. PMID: 37201335; PMCID: PMC10203746.

327. Liu X, et al. Long-Term Physical Activity and Subsequent Risk for Rheumatoid Arthritis Among Women: A Prospective Cohort Study. Arthritis Rheumatol. 2019 Sep;71(9):1460-1471. doi: 10.1002/art.40899. Epub 2019 Jul 19. PMID: 30920773; PMCID: PMC6717001.

328. Bergström U, et al. Smoking, low formal level of education, alcohol consumption, and the risk of rheumatoid arthritis. Scand J Rheumatol. 2013;42(2):123-30. doi: 10.3109/03009742.2012.723744. Epub 2012 Nov 6. PMID: 23126587.

329. Cavalin C, et al. Crystalline silica exposure in patients with rheumatoid arthritis and systemic sclerosis: a nationwide cross-sectional survey. Rheumatology (Oxford). 2023 Aug 1;62(8):2707-2715. doi: 10.1093/rheumatology/keac675.

330. Kondo Y, et al. Effect of climatic environment on immunological features of rheumatoid arthritis. Sci Rep. 2023 Jan 24;13(1):1304. doi: 10.1038/s41598-022-27153-3. PMID: 36693893; PMCID: PMC9873807.

331. Asif Amin M, et al. Synovial cellular and molecular markers in rheumatoid arthritis. Semin Immunopathol. 2017 Jun;39(4):385-393. doi: 10.1007/s00281-017-0631-3. Epub 2017 May 11. PMID: 28497350; PMCID: PMC5521018.

332. Chen W, et al. Neutrophil Function in an Inflammatory Milieu of Rheumatoid Arthritis. J Immunol Res. 2018 Dec 3;2018:8549329. doi: 10.1155/2018/8549329. PMID: 30622982; PMCID: PMC6304923.

333. Elemam NM, et al. Role of Chemokines and Chemokine Receptors in Rheumatoid Arthritis. Immunotargets Ther. 2020 Mar 9;9:43-56. doi: 10.2147/ITT.S243636. PMID: 32211348; PMCID: PMC7074856.

334. Talbot J, et al. CCR2 Expression in Neutrophils Plays a Critical Role in Their Migration Into the Joints in Rheumatoid Arthritis. Arthritis Rheumatol. 2015 Jul;67(7):1751-9. doi: 10.1002/art.39117. PMID: 25779331.

335. Björkman L, et al. Neutrophil recruitment to inflamed joints can occur without cellular priming. J Leukoc Biol. 2019 Jun;105(6):1123-1130. doi: 10.1002/JLB.3AB0918-369R. Epub 2018 Dec 20. PMID: 30570778.

336. Amin MA, et al. Interleukin-18 induces angiogenic factors in rheumatoid arthritis synovial tissue fibroblasts via distinct signaling pathways. Arthritis Rheum. 2007 Jun;56(6):1787-97. doi: 10.1002/art.22705. PMID: 17530707.

337. Zhu M, et al. Emodin ameliorates rheumatoid arthritis by promoting neutrophil apoptosis and inhibiting neutrophil extracellular trap formation. Mol Immunol. 2019 Aug;112:188-197. doi: 10.1016/j.molimm.2019.05.010. Epub 2019 Jun 5. PMID: 31176198.

338. Ottonello L, et al. Synovial fluid from patients with rheumatoid arthritis inhibits neutrophil apoptosis: role of adenosine and proinflammatory cytokines. Rheumatology (Oxford). 2002 Nov;41(11):1249-60. doi: 10.1093/rheumatology/41.11.1249. PMID: 12421997.

339. Li KX, et al. LTB4-induced anti-apoptosis and infiltration of neutrophils in rheumatoid arthritis. Clin Exp Rheumatol. 2020 May-Jun;38(3):543-551. Epub 2020 Feb 14. PMID: 32105594.

340. Khreiss T, et al. Loss of pentameric symmetry of C-reactive protein is associated with delayed apoptosis of human neutrophils. J Biol Chem. 2002 Oct 25;277(43):40775-81. doi: 10.1074/jbc.M205378200. Epub 2002 Aug 26. PMID: 12198121.

341. Lopez S, et al. Neutrophil expression of tumour necrosis factor receptors (TNF-R) and of activation markers (CD11b, CD43, CD63) in rheumatoid arthritis. Clin Exp Immunol. 1995 Jul;101(1):25-32. doi: 10.1111/j.1365-2249.1995.tb02272.x. PMID: 7621589; PMCID: PMC1553289.

342. Chokesuwattanaskul S, et al. Metabolic Profiling of Rheumatoid Arthritis Neutrophils Reveals Altered Energy Metabolism That Is Not Affected by JAK Inhibition. Metabolites. 2022 Jul 15;12(7):650. doi: 10.3390/metabo12070650. PMID: 35888774; PMCID: PMC9321732.

343.Wright HL, et al. Changes in expression of membrane TNF, NF-{kappa}B activation and neutrophil apoptosis during active and resolved inflammation. Ann Rheum Dis. 2011 Mar;70(3):537-43. doi: 10.1136/ard.2010.138065. Epub 2010 Nov 24. PMID: 21109521.

344. Beaulieu AD, et al. Differential expression of two major cytokines produced by neutrophils, interleukin-8 and the interleukin-1 receptor antagonist, in neutrophils isolated from the synovial fluid and peripheral blood of patients with rheumatoid arthritis. Arthritis Rheum. 1994 Jun;37(6):855-9. doi: 10.1002/art.1780370613. PMID: 8003057.

345. Mateen S, et al. Increased Reactive Oxygen Species Formation and Oxidative Stress in Rheumatoid Arthritis. PLoS One. 2016 Apr 4;11(4):e0152925. doi: 10.1371/journal.pone.0152925. PMID: 27043143; PMCID: PMC4820274.

346. Inaba M, et al. Increased basal phosphorylation of mitogen-activated protein kinases and reduced responsiveness to inflammatory cytokines in neutrophils from patients with rheumatoid arthritis. Clin Exp Rheumatol. 2008 Jan-Feb;26(1):52-60. PMID: 18328147.

347. Wright HL, et al. Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps. Front Immunol. 2021 Jan 5;11:584116. doi: 10.3389/fimmu.2020.584116. PMID: 33469455; PMCID: PMC7813679.

348. Fuchs K, et al. In Vivo Hypoxia PET Imaging Quantifies the Severity of Arthritic Joint Inflammation in Line with Overexpression of Hypoxia-Inducible Factor and Enhanced Reactive Oxygen Species Generation. J Nucl Med. 2017 May;58(5):853-860. doi: 10.2967/jnumed.116.185934. Epub 2017 Feb 9. PMID: 28183987.

349. Datta S, Kundu S, et al. Correlation of oxidant status with oxidative tissue damage in patients with rheumatoid arthritis. Clin Rheumatol. 2014 Nov;33(11):1557-64. doi: 10.1007/s10067-014-2597-z. Epub 2014 Apr 10. PMID: 24718487.

350. Yang F, et al. Inhibition of NET formation by polydatin protects against collagen-induced arthritis. Int Immunopharmacol. 2019 Dec;77:105919. doi: 10.1016/j.intimp.2019.105919. Epub 2019 Oct 23. PMID: 31655341.

351. Khandpur R, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013 Mar 27;5(178):178ra40. doi: 10.1126/scitranslmed.3005580. PMID: 23536012; PMCID: PMC3727661.

352. Ribon M, et al. Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37. J Autoimmun. 2019 Mar;98:122-131. doi: 10.1016/j.jaut.2019.01.003. Epub 2019 Jan 28. PMID: 30704942.

353. Cheng Z, et al. SHIP1 is required for the formation of neutrophil extracellular traps in rheumatoid arthritis. Int Immunopharmacol. 2023 Feb;115:109625. doi: 10.1016/j.intimp.2022.109625. Epub 2022 Dec 29. PMID: 36586275.

354. Schneider AH, et al. Neutrophil extracellular traps mediate joint hyperalgesia induced by immune inflammation. Rheumatology (Oxford). 2021 Jul 1;60(7):3461-3473. doi: 10.1093/rheumatology/keaa794. PMID: 33367912.

355. Wang L, et al. Signal inhibitory receptor on leukocytes-1 regulates the formation of the neutrophil extracellular trap in rheumatoid arthritis. Mol Immunol. 2022 Nov;151:242-251. doi: 10.1016/j.molimm.2022.09.008. Epub 2022 Sep 28. PMID: 36182788.

356. Navrátilová A, et al. IL-40: A New B Cell-Associated Cytokine Up-Regulated in Rheumatoid Arthritis Decreases Following the Rituximab Therapy and Correlates With Disease Activity, Autoantibodies, and NETosis. Front Immunol. 2021 Oct 21;12:745523. doi: 10.3389/fimmu.2021.745523. PMID: 34745117; PMCID: PMC8566875.

357. Okamoto Y, et al. Association of Sputum Neutrophil Extracellular Trap Subsets With IgA Anti-Citrullinated Protein Antibodies in Subjects at Risk for Rheumatoid Arthritis. Arthritis Rheumatol. 2022 Jan;74(1):38-48. doi: 10.1002/art.41948. Epub 2021 Nov 25. PMID: 34369110; PMCID: PMC8712364.

358. Yu K, et al. Novel method to quantify peptidylarginine deiminase activity shows distinct citrullination patterns in rheumatoid and juvenile idiopathic arthritis. Front Immunol. 2023 Jan 30;14:1111465. doi: 10.3389/fimmu.2023.1111465. PMID: 36793709; PMCID: PMC9923157.

359. Carmona-Rivera C, et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. Sci Immunol. 2017 Apr;2(10):eaag3358. doi: 10.1126/sciimmunol.aag3358. Epub 2017 Apr 14. Erratum in: Sci Immunol. 2020 Jan 31;5(43): PMID: 28649674; PMCID: PMC5479641.

360. Corsiero E, et al. Single cell cloning and recombinant monoclonal antibodies generation from RA synovial B cells reveal frequent targeting of citrullinated histones of NETs. Ann Rheum Dis. 2016 Oct;75(10):1866-75. doi: 10.1136/annrheumdis-2015-208356. Epub 2015 Dec 9. PMID: 26659717; PMCID: PMC5036240.

361. O'Neil LJ, et al. Anti-Carbamylated LL37 Antibodies Promote Pathogenic Bone Resorption in Rheumatoid Arthritis. Front Immunol. 2021 Sep 14;12:715997. doi: 10.3389/fimmu.2021.715997. PMID: 34594331; PMCID: PMC8477029.

362. Sohn DH, et al. Local Joint inflammation and histone citrullination in a murine model of the transition from preclinical autoimmunity to inflammatory arthritis. Arthritis Rheumatol. 2015 Nov;67(11):2877-87. doi: 10.1002/art.39283. PMID: 26227989; PMCID: PMC4626401.

363. Carmona-Rivera C, et al. Neutrophil extracellular traps mediate articular cartilage damage and enhance cartilage component immunogenicity in rheumatoid arthritis. JCI Insight. 2020 Jul 9;5(13):e139388. doi: 10.1172/jci.insight.139388. PMID: 32484790; PMCID: PMC7406272.

364. O'Neil LJ, et al. Neutrophil extracellular trap-associated carbamylation and histones trigger osteoclast formation in rheumatoid arthritis. Ann Rheum Dis. 2023 May;82(5):630-638. doi: 10.1136/ard-2022-223568. Epub 2023 Feb 3. PMID: 36737106.

365. Papadaki G, et al. Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation. Eur J Immunol. 2016 Nov;46(11):2542-2554. doi: 10.1002/eji.201646542. Epub 2016 Oct 5. PMID: 27585946; PMCID: PMC5476297.

366. Bombardieri M, et al. Ectopic lymphoid neogenesis in rheumatic autoimmune diseases. Nat Rev Rheumatol. 2017 Mar;13(3):141-154. doi: 10.1038/nrrheum.2016.217. Epub 2017 Feb 9. PMID: 28202919.

367. Takemura S, et al. T cell activation in rheumatoid synovium is B cell dependent. J Immunol. 2001 Oct 15;167(8):4710-8. doi: 10.4049/jimmunol.167.8.4710. PMID: 11591802.

368. Gottenberg JE, et al. Markers of B-lymphocyte activation are elevated in patients with early rheumatoid arthritis and correlated with disease activity in the ESPOIR cohort. Arthritis Res Ther. 2009;11(4):R114. doi: 10.1186/ar2773. Epub 2009 Jul 23. PMID: 19627580; PMCID: PMC2745796.

369. Rincón-Arévalo H, et al. Atypical phenotype and response of B cells in patients with seropositive rheumatoid arthritis. Clin Exp Immunol. 2021 May;204(2):221-238. doi: 10.1111/cei.13576. Epub 2021 Mar 2. PMID: 33459349; PMCID: PMC8062998.

370. Armas-González Eet al. Role of CXCL13 and CCL20 in the recruitment of B cells to inflammatory foci in chronic arthritis. Arthritis Res Ther. 2018 Jun 7;20(1):114. doi: 10.1186/s13075-018-1611-2. PMID: 29880013; PMCID: PMC5992813.

371. Legler DF, et al. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. J Exp Med. 1998 Feb 16;187(4):655-60. doi: 10.1084/jem.187.4.655. PMID: 9463416; PMCID: PMC2212150.

372. Catalán D, et al. B cells from rheumatoid arthritis patients show important alterations in the expression of CD86 and FcgammaRIIb, which are modulated by anti-tumor necrosis factor therapy. Arthritis Res Ther. 2010;12(2):R68. doi: 10.1186/ar2985. Epub 2010 Apr 15. PMID: 20398308; PMCID: PMC2888223.

373. Tang Y, et al. Altered peripheral B lymphocyte homeostasis and functions mediated by IL-27 via activating the mammalian target of rapamycin signaling pathway in patients with rheumatoid arthritis. Clin Exp Immunol. 2021 Dec;206(3):354-365. doi: 10.1111/cei.13663. Epub 2021 Oct 5. PMID: 34558072; PMCID: PMC8561691.

374. Samuels J, et al. Impaired early B cell tolerance in patients with rheumatoid arthritis. J Exp Med. 2005 May 16;201(10):1659-67. doi: 10.1084/jem.20042321. PMID: 15897279; PMCID: PMC2212916.

375. Moura RA, et al. B-cell phenotype and IgD-CD27- memory B cells are affected by TNF-inhibitors and tocilizumab treatment in rheumatoid arthritis. PLoS One. 2017 Sep 8;12(9):e0182927. doi: 10.1371/journal.pone.0182927. PMID: 28886017; PMCID: PMC5590747.

376. Zhang F, et al. Defining inflammatory cell states in rheumatoid arthritis joint synovial tissues by integrating single-cell transcriptomics and mass cytometry. Nat Immunol. 2019 Jul;20(7):928-942. doi: 10.1038/s41590-019-0378-1. Epub 2019 May 6. PMID: 31061532; PMCID: PMC6602051.

377. Floudas A, et al. Pathogenic, glycolytic PD-1+ B cells accumulate in the hypoxic RA joint. JCI Insight. 2020 Nov 5;5(21):e139032. doi: 10.1172/jci.insight.139032. PMID: 33148884; PMCID: PMC7710281.

378. Nakken B, et al. B-cells and their targeting in rheumatoid arthritis--current concepts and future perspectives. Autoimmun Rev. 2011 Nov;11(1):28-34. doi: 10.1016/j.autrev.2011.06.010. Epub 2011 Jul 14. PMID: 21777703.

379. Wang Y, et al. Rheumatoid arthritis patients display B-cell dysregulation already in the naïve repertoire consistent with defects in B-cell tolerance. Sci Rep. 2019 Dec 27;9(1):19995. doi: 10.1038/s41598-019-56279-0. PMID: 31882654; PMCID: PMC6934703.

380. Ding S, et al. Inducible costimulator ligand (ICOSL) on CD19+ B cells is involved in immunopathological damage of rheumatoid arthritis (RA). Front Immunol. 2022 Nov 2;13:1015831. doi: 10.3389/fimmu.2022.1015831. PMID: 36405702; PMCID: PMC9666393.

381. Mo YQ, et al. Synovial infiltration with CD79a-positive B cells, but not other B cell lineage markers, correlates with joint destruction in rheumatoid arthritis. J Rheumatol. 2011 Nov;38(11):2301-8. doi: 10.3899/jrheum.110615. Epub 2011 Oct 15. PMID: 22002013.

382. Malmström V, Grönwall C. The parallel worlds of ACPA-positive and RF-positive B cells. Nat Rev Rheumatol. 2018 Nov;14(11):626-628. doi: 10.1038/s41584-018-0094-5. PMID: 30266975.

383. Lu DR, et al. T Cell-Dependent Affinity Maturation and Innate Immune Pathways Differentially Drive Autoreactive B Cell Responses in Rheumatoid Arthritis. Arthritis Rheumatol. 2018 Nov;70(11):1732-1744. doi: 10.1002/art.40578. Epub 2018 Sep 24. PMID: 29855173; PMCID: PMC6203609.

384. Fortea-Gordo P, et al. Two populations of circulating PD-1hiCD4 T cells with distinct B cell helping capacity are elevated in early rheumatoid arthritis. Rheumatology (Oxford). 2019 Sep 1;58(9):1662-1673. doi: 10.1093/rheumatology/kez169. PMID: 31056653.

385. Arroyo-Villa I, et al. Constitutively altered frequencies of circulating follicullar helper T cell counterparts and their subsets in rheumatoid arthritis. Arthritis Res Ther. 2014 Dec 5;16(6):500. doi: 10.1186/s13075-014-0500-6. PMID: 25475240; PMCID: PMC4275955.

386. Koushik S, et al. PAD4: pathophysiology, current therapeutics and future perspective in rheumatoid arthritis. Expert Opin Ther Targets. 2017 Apr;21(4):433-447. doi: 10.1080/14728222.2017.1294160. Epub 2017 Feb 22. PMID: 28281906.

387. Wegner N, et al. Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis. Immunol Rev. 2010 Jan;233(1):34-54. doi: 10.1111/j.0105-2896.2009.00850.x. PMID: 20192991.

388. Haag S, et al. Identification of new citrulline-specific autoantibodies, which bind to human arthritic cartilage, by mass spectrometric analysis of citrullinated type II collagen. Arthritis Rheumatol. 2014 Jun;66(6):1440-9. doi: 10.1002/art.38383. PMID: 24470447.

389. Ytterberg AJ, et al. Shared immunological targets in the lungs and joints of patients with rheumatoid arthritis: identification and validation. Ann Rheum Dis. 2015 Sep;74(9):1772-7. doi: 10.1136/annrheumdis-2013-204912. Epub 2014 May 9. PMID: 24817415.

390. Spengler J, et al. Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid. Arthritis Rheumatol. 2015 Dec;67(12):3135-45. doi: 10.1002/art.39313. PMID: 26245941; PMCID: PMC4832324.

391. Bonifacio AF, et al. Novel autoantibodies in rheumatoid arthritis. Reumatismo. 2019 Apr 1;71(1):1-12. doi: 10.4081/reumatismo.2019.1102. PMID: 30932437.

392. Xu M, et al. Platelets derived citrullinated proteins and microparticles are potential autoantibodies ACPA targets in RA patients. Front Immunol. 2023 Jan 24;14:1084283. doi: 10.3389/fimmu.2023.1084283. PMID: 36761728; PMCID: PMC9902922.

393. Laurent L, et al. IgM rheumatoid factor amplifies the inflammatory response of macrophages induced by the rheumatoid arthritis-specific immune complexes containing anticitrullinated protein antibodies. Ann Rheum Dis. 2015 Jul;74(7):1425-31. doi: 10.1136/annrheumdis-2013-204543. Epub 2014 Mar 11. PMID: 24618262.

394. Harre U, et al.. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. J Clin Invest. 2012 May;122(5):1791-802. doi: 10.1172/JCI60975. Epub 2012 Apr 16. PMID: 22505457; PMCID: PMC3336988.

395. Wu S, et al. Anti-citrullinated protein antibodies are associated with neutrophil extracellular trap formation in rheumatoid arthritis. J Clin Lab Anal. 2021 Mar;35(3):e23662. doi: 10.1002/jcla.23662. Epub 2020 Nov 28. PMID: 33249645; PMCID: PMC7957993.

396. Clavel C, et al. Among human macrophages polarised to different phenotypes, the M-CSF-oriented cells present the highest pro-inflammatory response to the rheumatoid arthritis-specific immune complexes containing ACPA. Ann Rheum Dis. 2016 Dec;75(12):2184-2191. doi: 10.1136/annrheumdis-2015-208887. Epub 2016 Mar 23. PMID: 27009917.

397. Sun M, et al. Anticitrullinated protein antibodies facilitate migration of synovial tissue-derived fibroblasts. Ann Rheum Dis. 2019 Dec;78(12):1621-1631. doi: 10.1136/annrheumdis-2018-214967. Epub 2019 Sep 3. PMID: 31481351; PMCID: PMC6900251.

398. Leadbetter EA, et al. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature. 2002 Apr 11;416(6881):603-7. doi: 10.1038/416603a. PMID: 11948342.

399. Wardemann H, et al. B-cell self-tolerance in humans. Adv Immunol. 2007;95:83-110. doi: 10.1016/S0065-2776(07)95003-8. PMID: 17869611.

400. O'Neill SK, et al. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. J Immunol. 2005 Mar 15;174(6):3781-8. doi: 10.4049/jimmunol.174.6.3781. PMID: 15749919.

401. Hill JA, et al. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol. 2003 Jul 15;171(2):538-41. doi: 10.4049/jimmunol.171.2.538. PMID: 12847215.

402. Auger I, et al. Peptidylarginine Deiminase Autoimmunity and the Development of Anti-Citrullinated Protein Antibody in Rheumatoid Arthritis: The Hapten-Carrier Model. Arthritis Rheumatol. 2020 Jun;72(6):903-911. doi: 10.1002/art.41189. Epub 2020 Apr 26. PMID: 31820586; PMCID: PMC7317357.

403. Axelsson S, et al. A combination of the activation marker CD86 and the immune checkpoint marker B and T lymphocyte attenuator (BTLA) indicates a putative permissive activation state of B cell subtypes in healthy blood donors independent of age and sex. BMC Immunol. 2020 Mar 20;21(1):14. doi: 10.1186/s12865-020-00343-2. PMID: 32197584; PMCID: PMC7082969.

404. Tellander AC, et al. Interference with CD28, CD80, CD86 or CD152 in collagen-induced arthritis. Limited role of IFN-gamma in anti-B7-mediated suppression of disease. J Autoimmun. 2001 Aug;17(1):39-50. doi: 10.1006/jaut.2001.0527. PMID: 11488636.

405. Peters AL, et al. CD40 and autoimmunity: the dark side of a great activator. Semin Immunol. 2009 Oct;21(5):293-300. doi: 10.1016/j.smim.2009.05.012. PMID: 19595612; PMCID: PMC2753170.

406. Karnell JL, et al. Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond. Adv Drug Deliv Rev. 2019 Feb 15;141:92-103. doi: 10.1016/j.addr.2018.12.005. Epub 2018 Dec 13. PMID: 30552917.

407. Karnell JL, et al J. A CD40L-targeting protein reduces autoantibodies and improves disease activity in patients with autoimmunity. Sci Transl Med. 2019 Apr 24;11(489):eaar6584. doi: 10.1126/scitranslmed.aar6584. PMID: 31019027.

408. Wu F, et al. B Cells in Rheumatoid Arthritis：Pathogenic Mechanisms and Treatment Prospects. Front Immunol. 2021 Sep 28;12:750753. doi: 10.3389/fimmu.2021.750753. PMID: 34650569; PMCID: PMC8505880.

409. Stolina M, et al. RANKL is a marker and mediator of local and systemic bone loss in two rat models of inflammatory arthritis. J Bone Miner Res. 2005 Oct;20(10):1756-65. doi: 10.1359/JBMR.050601. Epub 2005 Jun 6. PMID: 16160733.

410. Pettit AR, et al. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol. 2001 Nov;159(5):1689-99. doi: 10.1016/S0002-9440(10)63016-7. PMID: 11696430; PMCID: PMC1867076.

411. Quaresma TO, et al. Comparative study of the synovial levels of RANKL and OPG in rheumatoid arthritis, spondyloarthritis and osteoarthritis. Adv Rheumatol. 2023 Mar 15;63(1):13. doi: 10.1186/s42358-023-00294-3. PMID: 36922894.

412. Geusens PP, et al. The ratio of circulating osteoprotegerin to RANKL in early rheumatoid arthritis predicts later joint destruction. Arthritis Rheum. 2006 Jun;54(6):1772-7. doi: 10.1002/art.21896. PMID: 16736519.

413. Meednu N, et al. Production of RANKL by Memory B Cells: A Link Between B Cells and Bone Erosion in Rheumatoid Arthritis. Arthritis Rheumatol. 2016 Apr;68(4):805-16. doi: 10.1002/art.39489. PMID: 26554541; PMCID: PMC4956406.

414. Ota Y, et al. Generation mechanism of RANKL(+) effector memory B cells: relevance to the pathogenesis of rheumatoid arthritis. Arthritis Res Ther. 2016 Mar 16;18:67. doi: 10.1186/s13075-016-0957-6. PMID: 26980135; PMCID: PMC4793760.

415. Lv D, et al. Evaluation of the anti-RANKL monoclonal antibody in rheumatoid arthritis rats. Arch Rheumatol. 2022 Nov 4;38(1):22-31. doi: 10.46497/ArchRheumatol.2023.9240. PMID: 37235123; PMCID: PMC10208611.

416. Keffer J, et al. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO J. 1991 Dec;10(13):4025-31. doi: 10.1002/j.1460-2075.1991.tb04978.x. PMID: 1721867; PMCID: PMC453150.

417. McInnes IB, et al. Pathogenetic insights from the treatment of rheumatoid arthritis. Lancet. 2017 Jun 10;389(10086):2328-2337. doi: 10.1016/S0140-6736(17)31472-1. PMID: 28612747.

418. Yoon SS, et al. B Cell Adhesion to Fibroblast-Like Synoviocytes Is Up-Regulated by Tumor Necrosis Factor-Alpha via Expression of Human Vascular Cell Adhesion Molecule-1 Mediated by B Cell-Activating Factor. Int J Mol Sci. 2021 Jul 2;22(13):7166. doi: 10.3390/ijms22137166. PMID: 34281218; PMCID: PMC8267633.

419. Kugler M, et al. Cytokine-directed cellular cross-talk imprints synovial pathotypes in rheumatoid arthritis. Ann Rheum Dis. 2023 Jun 21:ard-2022-223396. doi: 10.1136/ard-2022-223396. Epub ahead of print. PMID: 37344156.

420. Yokota K. Osteoclast differentiation in rheumatoid arthritis. Immunol Med. 2023 Jun 13:1-6. doi: 10.1080/25785826.2023.2220931. Epub ahead of print. PMID: 37309864.

421. Strand V, et al. High levels of interleukin-6 in patients with rheumatoid arthritis are associated with greater improvements in health-related quality of life for sarilumab compared with adalimumab. Arthritis Res Ther. 2020 Oct 20;22(1):250. doi: 10.1186/s13075-020-02344-3. PMID: 33081825; PMCID: PMC7574446.

422. Yeo L, et al. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis. Ann Rheum Dis. 2011 Nov;70(11):2022-8. doi: 10.1136/ard.2011.153312. Epub 2011 Jul 8. PMID: 21742639; PMCID: PMC3184241.

423. Hashizume M, et al. IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF-alpha and IL-17. Rheumatology (Oxford). 2008 Nov;47(11):1635-40. doi: 10.1093/rheumatology/ken363. Epub 2008 Sep 11. PMID: 18786965.

424. Yokota K, et al. Characterization and Function of Tumor Necrosis Factor and Interleukin-6-Induced Osteoclasts in Rheumatoid Arthritis. Arthritis Rheumatol. 2021 Jul;73(7):1145-1154. doi: 10.1002/art.41666. Epub 2021 May 27. PMID: 33512089; PMCID: PMC8361923.

425. Boyapati A, et al. Sarilumab plus methotrexate suppresses circulating biomarkers of bone resorption and synovial damage in patients with rheumatoid arthritis and inadequate response to methotrexate: a biomarker study of MOBILITY. Arthritis Res Ther. 2016 Oct 6;18(1):225. doi: 10.1186/s13075-016-1132-9. PMID: 27716324; PMCID: PMC5052933.

426. Ren C, et al. IL-37 alleviates TNF-α-induced pyroptosis of rheumatoid arthritis fibroblast-like synoviocytes by inhibiting the NF-κB/GSDMD signaling pathway. Immunobiology. 2023 May;228(3):152382. doi: 10.1016/j.imbio.2023.152382. Epub 2023 Apr 4. PMID: 37075579.

427. Ruscitti P, et al. The role of IL-1β in the bone loss during rheumatic diseases. Mediators Inflamm. 2015;2015:782382. doi: 10.1155/2015/782382. Epub 2015 Apr 12. PMID: 25954061; PMCID: PMC4410538.

428. Nakamura I, Jimi E. Regulation of osteoclast differentiation and function by interleukin-1. Vitam Horm. 2006;74:357-70. doi: 10.1016/S0083-6729(06)74015-8. PMID: 17027523.

429. Elshabrawy HA, et al. The pathogenic role of angiogenesis in rheumatoid arthritis. Angiogenesis. 2015 Oct;18(4):433-48. doi: 10.1007/s10456-015-9477-2. Epub 2015 Jul 22. PMID: 26198292; PMCID: PMC4879881.

430. Olalekan SA, et al. B cells expressing IFN-γ suppress Treg-cell differentiation and promote autoimmune experimental arthritis. Eur J Immunol. 2015 Apr;45(4):988-98. doi: 10.1002/eji.201445036. Epub 2015 Feb 11. PMID: 25645456; PMCID: PMC4438566.

431. Lee SK, et al. Interferon-γ excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. Immunity. 2012 Nov 16;37(5):880-92. doi: 10.1016/j.immuni.2012.10.010. PMID: 23159227.

432. Chekol Abebe E, et al. The Role of Regulatory B Cells in Health and Diseases: A Systemic Review. J Inflamm Res. 2021 Jan 12;14:75-84. doi: 10.2147/JIR.S286426. PMID: 33469337; PMCID: PMC7811483.

433. Xu L, et al. Impairment of Granzyme B-Producing Regulatory B Cells Correlates with Exacerbated Rheumatoid Arthritis. Front Immunol. 2017 Jun 30;8:768. doi: 10.3389/fimmu.2017.00768. PMID: 28713386; PMCID: PMC5491972.

434. Guo Y, et al. Changes in peripheral CD19(+)Foxp3(+) and CD19(+)TGFβ(+) regulatory B cell populations in rheumatoid arthritis patients with interstitial lung disease. J Thorac Dis. 2015 Mar;7(3):471-7. doi: 10.3978/j.issn.2072-1439.2015.02.11. PMID: 25922727; PMCID: PMC4387448.

435. Hu S, et al. Diminished LAG3+ B cells correlate with exacerbated rheumatoid arthritis. Ann Med. 2023 Dec;55(1):2208373. doi: 10.1080/07853890.2023.2208373. PMID: 37143367; PMCID: PMC10165927.

436. Arneson LC, et al. Bruton's Tyrosine Kinase Inhibition for the Treatment of Rheumatoid Arthritis. Immunotargets Ther. 2021 Aug 28;10:333-342. doi: 10.2147/ITT.S288550. PMID: 34485183; PMCID: PMC8409514.

437. Akasaka D, et al. Novel Bruton's tyrosine kinase inhibitor TAS5315 suppresses the progression of inflammation and joint destruction in rodent collagen-induced arthritis. PLoS One. 2023 Feb 23;18(2):e0282117. doi: 10.1371/journal.pone.0282117. PMID: 36821545; PMCID: PMC9949657.

438. Kavanaugh A, et al. Assessment of rituximab's immunomodulatory synovial effects (ARISE trial). 1: clinical and synovial biomarker results. Ann Rheum Dis. 2008 Mar;67(3):402-8. doi: 10.1136/ard.2007.074229. Epub 2007 Jul 20. PMID: 17644541; PMCID: PMC2754142.

439. Bustamante MF, et al. Fibroblast-like synoviocyte metabolism in the pathogenesis of rheumatoid arthritis. Arthritis Res Ther. 2017 May 31;19(1):110. doi: 10.1186/s13075-017-1303-3. PMID: 28569176; PMCID: PMC5452638.

440. Lee AR, et al. SARS-CoV-2 spike protein promotes inflammatory cytokine activation and aggravates rheumatoid arthritis. Cell Commun Signal. 2023 Mar 2;21(1):44. doi: 10.1186/s12964-023-01044-0. PMID: 36864432; PMCID: PMC9978284.

441. Lefèvre S, et al. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. Nat Med. 2009 Dec;15(12):1414-20. doi: 10.1038/nm.2050. Epub 2009 Nov 8. PMID: 19898488; PMCID: PMC3678354.

442. Faramarzi F, et al. Inhibitory Effects of Cold Atmospheric Plasma on Inflammation and Tumor-Like Feature of Fibroblast-Like Synoviocytes from Patients with Rheumatoid Arthritis. Inflammation. 2022 Dec;45(6):2433-2448. doi: 10.1007/s10753-022-01703-3. Epub 2022 Jun 17. PMID: 35713788.

443. Chen J, et al. HIF-1α dependent RhoA as a novel therapeutic target to regulate rheumatoid arthritis fibroblast-like synoviocytes migration in vitro and in vivo. J Orthop Translat. 2023 Jun 6;40:49-57. doi: 10.1016/j.jot.2023.05.004. PMID: 37346290; PMCID: PMC10279694.

444. Redza-Dutordoir M, et al. Interactions between reactive oxygen species and autophagy: Special issue: Death mechanisms in cellular homeostasis. Biochim Biophys Acta Mol Cell Res. 2021 Jul;1868(8):119041. doi: 10.1016/j.bbamcr.2021.119041. Epub 2021 Apr 17. PMID: 33872672.

445. Lee HR, et al. LKB1 Regulates Inflammation of Fibroblast-like Synoviocytes from Patients with Rheumatoid Arthritis via AMPK-Dependent SLC7A11-NOX4-ROS Signaling. Cells. 2023 Apr 26;12(9):1263. doi: 10.3390/cells12091263. PMID: 37174663; PMCID: PMC10177119.

446. Lihua C, et al. Microplastics aggravates rheumatoid arthritis by affecting the proliferation/migration/inflammation of fibroblast-like synovial cells by regulating mitochondrial homeostasis. Int Immunopharmacol. 2023 Jul;120:110268. doi: 10.1016/j.intimp.2023.110268. Epub 2023 May 16. PMID: 37201404.

447. Wu S, et al. Anti-citrullinated protein antibodies are associated with neutrophil extracellular trap formation in rheumatoid arthritis. J Clin Lab Anal. 2021 Mar;35(3):e23662. doi: 10.1002/jcla.23662. Epub 2020 Nov 28. PMID: 33249645; PMCID: PMC7957993.

448. Kaifeng Zhou, et al. GSDMD-dependent neutrophil extracellular traps formation contributes to fibroblast-like synoviocyte activation in rheumatoid arthritis, 13 April 2021, Research Square doi.org/10.21203/rs.3.rs-401936/v1.

449. Kaibara N, et al. Comparative histopathological analysis between tenosynovitis and joint synovitis in rheumatoid arthritis. Histopathology. 2008 Jun;52(7):856-64. doi: 10.1111/j.1365-2559.2008.03050.x. Epub 2008 May 6. PMID: 18462359.

450. Su R, et al. Stratified distribution of Th17 and Treg cells in patients with multi-stage rheumatoid arthritis. Arthritis Res Ther. 2023 Apr 4;25(1):55. doi: 10.1186/s13075-023-03041-7. PMID: 37016395; PMCID: PMC10071616.

451. Eggleton P, et al. Frequency of Th17 CD20+ cells in the peripheral blood of rheumatoid arthritis patients is higher compared to healthy subjects. Arthritis Res Ther. 2011;13(6):R208. doi: 10.1186/ar3541. Epub 2011 Dec 15. PMID: 22171710; PMCID: PMC3334661.

452. Pan P, et al. Aberrant pro-inflammatory responses of CD20+ T cells in experimental arthritis. Cell Immunol. 2023 May;387:104717. doi: 10.1016/j.cellimm.2023.104717. Epub 2023 Apr 6. PMID: 37075620.

453. Fazou C, et al. Epitope specificity of clonally expanded populations of CD8+ T cells found within the joints of patients with inflammatory arthritis. Arthritis Rheum. 2001 Sep;44(9):2038-45. doi: 10.1002/1529-0131(200109)44:9<2038::AID-ART353>3.0.CO;2-1. PMID: 11592365.

454. Afeltra A, et al. Expression of CD69 antigen on synovial fluid T cells in patients with rheumatoid arthritis and other chronic synovitis. Ann Rheum Dis. 1993 Jun;52(6):457-60. doi: 10.1136/ard.52.6.457. PMID: 8323399; PMCID: PMC1005072.

455. Yamada H, et al. Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints. J Rheumatol. 2011 Aug;38(8):1569-75. doi: 10.3899/jrheum.101355. Epub 2011 May 1. PMID: 21532055.

456. Lowe K, et al. Transcriptomic profiling of programmed cell death 1 (PD-1) expressing T cells in early rheumatoid arthritis identifies a decreased CD4 + PD-1 + signature post-treatment. Sci Rep. 2023 Feb 17;13(1):2847. doi: 10.1038/s41598-023-29971-5. PMID: 36801909; PMCID: PMC9938264.

457. Lee HR, et al. Reduction of Oxidative Stress in Peripheral Blood Mononuclear Cells Attenuates the Inflammatory Response of Fibroblast-like Synoviocytes in Rheumatoid Arthritis. Int J Mol Sci. 2021 Nov 17;22(22):12411. doi: 10.3390/ijms222212411. PMID: 34830290; PMCID: PMC8624216.

458. Rajagopalan PT, et al. Interaction of dihydrofolate reductase with methotrexate: ensemble and single-molecule kinetics. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13481-6. doi: 10.1073/pnas.172501499. Epub 2002 Oct 1. PMID: 12359872; PMCID: PMC129699.

459. GUBNER R, et al. Therapeutic suppression of tissue reactivity. II. Effect of aminopterin in rheumatoid arthritis and psoriasis. Am J Med Sci. 1951 Feb;221(2):176-82. PMID: 14799481.

460. Tian H, Cronstein BN. Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. Bull NYU Hosp Jt Dis. 2007;65(3):168-73. PMID: 17922664.

461. Singh JA, et al. 2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. Arthritis Rheumatol. 2016 Jan;68(1):1-26. doi: 10.1002/art.39480. Epub 2015 Nov 6. PMID: 26545940.

462. Shams S, et al. The Therapeutic Landscape of Rheumatoid Arthritis: Current State and Future Directions. Front Pharmacol. 2021 May 28;12:680043. doi: 10.3389/fphar.2021.680043. PMID: 34122106; PMCID: PMC8194305.

463. Pang M, Sun Z, Zhang H. Biologic DMARDs and targeted synthetic DMARDs and the risk of all-cause mortality in rheumatoid arthritis: A systematic review and meta-analysis. Medicine (Baltimore). 2022 Aug 12;101(32):e29838. doi: 10.1097/MD.0000000000029838. PMID: 35960132; PMCID: PMC9371573.

464. de Castro CT, et al. Real-world effectiveness of biological therapy in patients with rheumatoid arthritis: Systematic review and meta-analysis. Front Pharmacol. 2022 Aug 11;13:927179. doi: 10.3389/fphar.2022.927179. PMID: 36034836; PMCID: PMC9402894.

465. Haraoui B, Bykerk V. Etanercept in the treatment of rheumatoid arthritis. Ther Clin Risk Manag. 2007 Mar;3(1):99-105. doi: 10.2147/tcrm.2007.3.1.99. PMID: 18360618; PMCID: PMC1936291.

466. Perdriger A. Infliximab in the treatment of rheumatoid arthritis. Biologics. 2009;3:183-91. doi: 10.2147/btt.2009.3099. Epub 2009 Jul 13. PMID: 19707407; PMCID: PMC2726073.

467. Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol. 2006 May;6(5):394-403. doi: 10.1038/nri1838. PMID: 16622478.

468. Tillack K, et al. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. J Immunol. 2012 Apr 1;188(7):3150-9. doi: 10.4049/jimmunol.1103414. Epub 2012 Feb 20. PMID: 22351936.

469. Hemmi H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000 Dec 7;408(6813):740-5. doi: 10.1038/35047123. Erratum in: Nature 2001 Feb 1;409(6820):646. PMID: 11130078.

470. Agarwal S, et al. Disruption of Neutrophil Extracellular Traps (NETs) Links Mechanical Strain to Post-traumatic Inflammation. Front Immunol. 2019 Oct 24;10:2148. doi: 10.3389/fimmu.2019.02148. PMID: 31708911; PMCID: PMC6821718.

471. Leffler Jet al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol. 2012 Apr 1;188(7):3522-31. doi: 10.4049/jimmunol.1102404. Epub 2012 Feb 17. PMID: 22345666.

472. Stephan A, et al. LL37:DNA complexes provide antimicrobial activity against intracellular bacteria in human macrophages. Immunology. 2016 Aug;148(4):420-32. doi: 10.1111/imm.12620. PMID: 27177697; PMCID: PMC4948035.

473. Nakagawa Y, Gallo RL. Endogenous intracellular cathelicidin enhances TLR9 activation in dendritic cells and macrophages. J Immunol. 2015 Feb 1;194(3):1274-84. doi: 10.4049/jimmunol.1402388. Epub 2014 Dec 29. PMID: 25548223; PMCID: PMC4297737.

474. Karmakar U, et al. Crosstalk between B cells and neutrophils in rheumatoid arthritis. Immunology. 2021 Dec;164(4):689-700. doi: 10.1111/imm.13412. Epub 2021 Sep 8. PMID: 34478165; PMCID: PMC8561113.

475. Rastogi I, et al. Role of B cells as antigen presenting cells. Front Immunol. 2022 Sep 8;13:954936. doi: 10.3389/fimmu.2022.954936. PMID: 36159874; PMCID: PMC9493130.

476. Wang X, et al. The Role of Reactive Oxygen Species in the Rheumatoid Arthritis-Associated Synovial Microenvironment. Antioxidants (Basel). 2022 Jun 13;11(6):1153. doi: 10.3390/antiox11061153. PMID: 35740050; PMCID: PMC9220354.

477. Lund FE, et al. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. Curr Dir Autoimmun. 2005;8:25-54. doi: 10.1159/000082086. PMID: 15564716.

478. Biton J, et al. Interplay between TNF and regulatory T cells in a TNF-driven murine model of arthritis. J Immunol. 2011 Apr 1;186(7):3899-910. doi: 10.4049/jimmunol.1003372. Epub 2011 Feb 23. PMID: 21346237.

479. Deenick EK, et al. Naive and memory human B cells have distinct requirements for STAT3 activation to differentiate into antibody-secreting plasma cells. J Exp Med. 2013 Nov 18;210(12):2739-53. doi: 10.1084/jem.20130323. Epub 2013 Nov 11. PMID: 24218138; PMCID: PMC3832925.

480. Marsman C, et al. Optimized Protocols for In-Vitro T-Cell-Dependent and T-Cell-Independent Activation for B-Cell Differentiation Studies Using Limited Cells. Front Immunol. 2022 Jun 29;13:815449. doi: 10.3389/fimmu.2022.815449. PMID: 35844625; PMCID: PMC9278277.

481. Auladell M, et al. Distinguishing naive- from memory-derived human B cells during acute responses. Clin Transl Immunology. 2019 Nov 13;8(11):e01090. doi: 10.1002/cti2.1090. PMID: 31844520; PMCID: PMC6851823.

482. 1091. Linsley PS, et al. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunity. 1994 Dec;1(9):793-801. doi: 10.1016/s1074-7613(94)80021-9. Erratum in: Immunity 1995 Feb;2(2):following 203. PMID: 7534620.

483. Fraussen J, et al. B cells of multiple sclerosis patients induce autoreactive proinflammatory T cell responses. Clin Immunol. 2016 Dec;173:124-132. doi: 10.1016/j.clim.2016.10.001. Epub 2016 Oct 4. PMID: 27717695.

484. Catalán D, et al Immunosuppressive Mechanisms of Regulatory B Cells. Front Immunol. 2021 Apr 29;12:611795. doi: 10.3389/fimmu.2021.611795. PMID: 33995344; PMCID: PMC8118522.

485. Seninet S, et al. Neutrophil extracellular traps from rheumatoid arthritis patients differentially activate myeloid cell sub-populations towards a pro-inflammatory profile. JLB (in revesion).

486. Huang QQ, Pope RM. The role of toll-like receptors in rheumatoid arthritis. Curr Rheumatol Rep. 2009 Oct;11(5):357-64. doi: 10.1007/s11926-009-0051-z. PMID: 19772831; PMCID: PMC2913446.

487. Berner B, et al. Increased expression of CD40 ligand (CD154) on CD4+ T cells as a marker of disease activity in rheumatoid arthritis. Ann Rheum Dis. 2000 Mar;59(3):190-5. doi: 10.1136/ard.59.3.190. PMID: 10700427; PMCID: PMC1753086.

488. Young KR Jr, et al. Complement subcomponent C1q stimulates Ig production by human B lymphocytes. J Immunol. 1991 May 15;146(10):3356-64. PMID: 1902854.

489. Ghebrehiwet B, et al. gC1q-R/p33, a member of a new class of multifunctional and multicompartmental cellular proteins, is involved in inflammation and infection. Immunol Rev. 2001 Apr;180:65-77. doi: 10.1034/j.1600-065x.2001.1800106.x. PMID: 11414365.

490. Yang T, et al. Synthetic neutrophil extracellular traps dissect bactericidal contribution of NETs under regulation of α-1-antitrypsin. Sci Adv. 2023 Apr 28;9(17):eadf2445. doi: 10.1126/sciadv.adf2445. Epub 2023 Apr 28. PMID: 37115934; PMCID: PMC10146876.

491. Krieger JI, Chesnut RW, Grey HM. Capacity of B cells to function as stimulators of a primary mixed leukocyte reaction. J Immunol. 1986 Nov 15;137(10):3117-23. PMID: 2945857.

492. Boldison J, et al. Dendritic cells license regulatory B cells to produce IL-10 and mediate suppression of antigen-specific CD8 T cells. Cell Mol Immunol. 2020 Aug;17(8):843-855. doi: 10.1038/s41423-019-0324-z. Epub 2019 Nov 14. PMID: 31728048; PMCID: PMC7395736.

493. Li B, et al. IL-6 Promotes T Cell Proliferation and Expansion under Inflammatory Conditions in Association with Low-Level RORγt Expression. J Immunol. 2018 Nov 15;201(10):2934-2946. doi: 10.4049/jimmunol.1800016. Epub 2018 Oct 12. PMID: 30315140; PMCID: PMC6324200.

494. Mehta AK, Gracias DT, Croft M. TNF activity and T cells. Cytokine. 2018 Jan;101:14-18. doi: 10.1016/j.cyto.2016.08.003. Epub 2016 Aug 13. PMID: 27531077; PMCID: PMC5305780.

495. Taub DD, et al. T lymphocyte recruitment by interleukin-8 (IL-8). IL-8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both in vitro and in vivo. J Clin Invest. 1996 Apr 15;97(8):1931-41. doi: 10.1172/JCI118625. PMID: 8621778; PMCID: PMC507263.

496. Keshavarzian A, et al. Increased interleukin-8 (IL-8) in rectal dialysate from patients with ulcerative colitis: evidence for a biological role for IL-8 in inflammation of the colon. Am J Gastroenterol. 1999 Mar;94(3):704-12. doi: 10.1111/j.1572-0241.1999.00940.x. PMID: 10086655.

497. Zengel P, et al. μ-Slide Chemotaxis: a new chamber for long-term chemotaxis studies. BMC Cell Biol. 2011 May 18;12:21. doi: 10.1186/1471-2121-12-21. PMID: 21592329; PMCID: PMC3118187.

498. Grillet B, et al. Matrix metalloproteinases in arthritis: towards precision medicine. Nat Rev Rheumatol. 2023 Jun;19(6):363-377. doi: 10.1038/s41584-023-00966-w. Epub 2023 May 9. PMID: 37161083.

499. Myers RC, et al. Lymphotoxin α1β2 expression on B cells is required for follicular dendritic cell activation during the germinal center response. Eur J Immunol. 2013 Feb;43(2):348-59. doi: 10.1002/eji.201242471. Epub 2012 Dec 5. PMID: 23112125; PMCID: PMC3753018.

500. Croia C, Bursi R, Sutera D, Petrelli F, Alunno A, Puxeddu I. One year in review 2019: pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol. 2019 May-Jun;37(3):347-357. Epub 2019 May 10. PMID: 31111823.

501. Lorenzetti R, et al. Abatacept modulates CD80 and CD86 expression and memory formation in human B-cells. J Autoimmun. 2019 Jul;101:145-152. doi: 10.1016/j.jaut.2019.04.016. Epub 2019 May 1. PMID: 31054942.

ANNEXE

Article 2: Neutrophil extracellular traps from rheumatoid arthritis patients differentially activate myeloid cell sub-populations towards a pro inflammatory profile. **Journal of Leukocyte Biology (Revision in preparation).**

Article 2

In this article, we delved into the role of NETs in the activation of various cell sub-populations within the myeloid lineage, with a specific focus on rheumatoid arthritis. My contribution consisted in investigating the mechanism involved in NET-mediated activation, focusing in glycolysis and ROS.

Background 1:

In the context of RA, we observed increased NET formation, indicating a potential contribution to the disease pathology. Notably, NETs from RA patients exhibited heightened pro-inflammatory activity compared to those from healthy individuals (1). RA-derived NETs were more efficient in activating neutrophils and macrophages compared to NETs from healthy individuals, suggesting a disease-specific influence on the composition and functional characteristics of these extracellular traps.

Aim 1:

We previously demonstrated the pro-inflammatory impact of NETs on resting non-polarized macrophages, and our current investigation extends this understanding to various cell sub-populations within the myeloid lineage in response to RA-derived NETs.

Neutrophils, dendritic cells, monocytes, non-polarized M0, pro-inflammatory M1-like or immuno-regulatory M2c-like macrophages were cultured overnight in the presence of RA-derived NETs. Culture supernatants were collected and cytokines were measured by ELISA.

Background 2:

IL-1β is a potent pro-inflammatory cytokine that is involved in a wide range of diseases, including autoimmune diseases, infectious diseases, and cancer. It is produced via the inflammasome in a variety of cell types, including macrophages, PMNs, and dendritic cells. When inflammasome is activated, it cleaves the pro-inflammatory cytokines pro-IL-1β into its active form.

In PMNs, IL-1β production can be triggered by a single signal (**2**). This is because PMNs contain pre-formed pro-IL-1β, which may be rapidly cleaved into active IL-1β upon stimulation by NETs.

In macrophages, IL-1β production is typically triggered by two signals: a priming signal and an activating signal. The priming signal can be provided by a variety of stimuli, including LPS, TNF-α, and IFN-γ. The activating signal is typically provided by a PAMP or a DAMP (**2**). We hypothesized that RA-derived NETs can act as DAMPs and can induce inflammasome activation which has not been described yet in RA context.

Aim 2:

We were interested on studying inflammasome activation by RA-derived NETs in myeloid cells.

Neutrophils and monocytes were cultured overnight in the presence of RA-derived NETs. To stimulate IL-1β secretion, cells were stimulated with ATP for the last four hours. Culture supernatants were collected and cytokines were measured by ELISA.

Background 3:

Neutrophils and monocytes generate ROS during their activities (**3, 4**). ROS are small, highly reactive molecules that can damage cells and tissues. However, ROS also play important roles in a variety of cellular processes, including cell signaling, immunity and metabolism. ROS can activate the MAPK signaling pathway or JAK/STAT signaling pathway, which leads to increased production of pro-inflammatory cytokines (**5**). DPI, a NOX inhibitor, is a compound that has been used for its potential impact on modulating the oxidative burst of neutrophils and monocytes.

Moreover, neutrophils and monocytes rely heavily on glycolysis for energy production, a process essential for their functions. 2-Deoxy-D-glucose (2-DG) is a glucose analog that has been studied for its potential roles in various physiological and pathological conditions. Inhibiting glycolysis with 2-DG may impact the energy balance of these cells.

The involvement of glycolysis and ROS in neutrophils and monocytes activation by NETs has not yet been explored.

Aim 3:

To identify the mechanism by which NETs promote myeloid cell activation, by focusing on the role of glycolosis and ROS.

Neutrophils and monocytes were cultured overnight in the presence of RA-derived NETs. A glucose analog, 2-DG was used to block glycolosis and the NADPH oxidase inhibitory DPI was used to block ROS production in cells. Culture supernatants were collected and cytokines were measured by ELISA.

1. Ribon M, et al. Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37. J Autoimmun. 2019 Mar;98:122-131. doi: 10.1016/j.jaut.2019.01.003. Epub 2019 Jan 28. PMID: 30704942.

2. Netea MG, et al. IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog. 2010 Feb 26;6(2):e1000661. doi: 10.1371/journal.ppat.1000661. PMID: 20195505; PMCID: PMC2829053.

3. Winterbourn CC, et al. Reactive Oxygen Species and Neutrophil Function. Annu Rev Biochem. 2016 Jun 2;85:765-92. doi: 10.1146/annurev-biochem-060815-014442. Epub 2016 Apr 6. PMID: 27050287.

4. Canton M, et al. Reactive Oxygen Species in Macrophages: Sources and Targets. Front Immunol. 2021 Sep 30;12:734229. doi: 10.3389/fimmu.2021.734229. PMID: 34659222; PMCID: PMC8515906.

5. Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. J Exp Med. 2011 Mar 14;208(3):417-20. doi: 10.1084/jem.20110367. Epub 2011 Feb 28. PMID: 21357740; PMCID: PMC3058577.

Neutrophil extracellular traps from rheumatoid arthritis patients differentially activate myeloid cell sub-populations towards a pro-inflammatory profile

Sarra Seninet[1,2], Dyhia Melbouci[1,2], Ahmad Haidar Ahmad[1,2], Mylène Petit[1,2,3], Marie-Christophe Boissier[1,2,3], Elodie Segura[4], Luca Semerano[1,2,3] and Patrice Decker[1,2]*

[1]University Sorbonne Paris Nord, Li2P, [2]Inserm UMR 1125 and [3]Avicenne Hospital, Rheumatology Department, Assistance Publique-Hôpitaux de Paris (AP-HP), 93000 Bobigny, France, [4]Institut Curie, PSL University, Inserm U932, Immunity and Cancer, 75005 Paris, France.

*Corresponding author: Prof. Patrice Decker, University Sorbonne Paris Nord, Li2P, Inserm UMR 1125, UFR SMBH, 1 rue de Chablis, 93017 Bobigny Cedex, France. Email: patrice.decker@univ-paris13.fr

Abbreviations: ACPA, anti-citrullinated protein antibodies; FCS, fetal calf serum; GM-CSF, granulocyte/macrophage colony-stimulating factor ; IFN, interferon; IL, interleukin; LPS, lipopolysaccharides; M-CSF, macrophage colony-stimulating factor; NET, neutrophil extracellular traps; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear neutrophils; RA, rheumatoid arthritis; TNF, tumor necrosis factor

Keywords: neutrophils; neutrophil extracellular traps; macrophages; monocytes; myeloid cells; inflammation; rheumatoid arthritis; pathophysiology.

Abstract

Activated polymorphonuclear neutrophils (PMN) release neutrophil extracellular traps (NET). Increased NET formation and/or impaired NET clearance have been reported in chronic inflammatory diseases like rheumatoid arthritis (RA). We have previously shown that NET are pro-inflammatory on resting non-polarized macrophages. Particularly, this response was enhanced with NET from RA patients as compared with NET from healthy individuals. We now compared the pro-inflammatory activity of NET on cell sub-populations of the myeloid lineage, focusing on the effects of RA NET on target cells from healthy individuals. Results were then confirmed with RA target cells. We show that RA NET activate monocytes, PMN, macrophages as well as dendritic cells, leading to the secretion of pro-inflammatory cytokines. Particularly, NET trigger the secretion of RA-associated pro-inflammatory cytokines, but not the immuno-modulatory cytokine IL-10. Both pro-inflammatory macrophage and dendritic cell sub-populations strongly respond to NET. Importantly, even immuno-modulatory macrophage respond to NET. In macrophages and PMN, NET induce the secretion of IL-1β; whereas it occurs upon activation of the inflammasome in macrophages and with NET working as the priming stimulus, PMN do not need priming and directly produce IL-1β in response to NET. In conclusion, abnormal accumulation of NET in the extracellular space may be a major trigger capable to activate several myeloid cell sub-populations within a pathogenic pro-inflammatory response.

Introduction

Neutrophil extracellular traps (NET) are expelled by activated polymorphonuclear neutrophils (PMN). Originally described as an immune response against bacteria,[1] NET may become pathogenic, as observed in rheumatoid arthritis (RA).[2] NET are composed of DNA and proteins from granules, as well as additional proteins and their composition may vary according to the stimulus inducing NET (nuclear vs. mitochondrial DNA, containing histones or not, potentially enriched in post-translational modifications). Moreover, NET may be a source of autoantigens or danger-associated molecular patterns. Therefore, NET may be antigenic or immuno-stimulatory and their activity varies depending on their composition and thus depending on the NET-inducing stimulus or their origin (physiological vs. pathogenic NET, with possibly different activities in different diseases).

RA is a chronic inflammatory and autoimmune disease of unknown etiology. It affects 0.5% of the adult population and leads to joint destruction. Macrophages and PMN are key players in RA pathophysiology. PMN are activated in RA[3] and are recruited to inflamed joints.[4,5] Moreover, RA PMN have an increased capacity to produce NET[6,7,8] and NET are detected in affected tissues.[6,9] Likewise, macrophages are activated in RA[10] and an increase in the number of macrophages in the synovial tissue is an early event correlating with disease activity[11] and the level of joint erosion.[12] Particularly, resident macrophages in the affected synovial tissue release tumor necrosis factor (TNF) and interleukin (IL)-1β, the master regulators of chronic joint inflammation in RA. Macrophages are classified in three homeostatic populations, i.e. namely macrophages involved in host defense (M1 macrophages, with pro-inflammatory activity), or tissue repair (M2a, wound-healing) or immuno-regulation (M2c, with anti-inflammatory activity). The three sub-populations can be generated in vitro using interferon (IFN)-γ, IL-4 or IL-10, respectively.[13] In RA, it is

believed that macrophage homeostasis is disturbed in favor of an enhanced response of pro-inflammatory macrophage subsets as compared to immuno-modulatory macrophages. Once activated, both PMN and macrophages secrete the RA-associated cytokines TNF, IL-8 and IL-1β.

NET are believed to be pathogenic in RA. They activate several cell types involved in RA pathogenesis, such as PMN, macrophages and fibroblast-like synoviocytes, as recently reviewed.[2] They are also recognized by the RA-specific anti-citrullinated protein antibodies (ACPA) autoantibodies. We have previously shown that NET activate resting M0 macrophages.[8] Interestingly, RA NET demonstrated a higher capacity to trigger the secretion of pro-inflammatory cytokines than NET from healthy individuals. However, the effect of NET (and the underlying mechanisms) may vary depending on the subset or the level of polarization of the target cell. In the present study, we analyzed the response of different myeloid cell subsets to RA NET. We analyzed several myeloid cell populations, including monocytes, monocyte-derived macrophages as well as PMN, and all experiments were performed with primary cells. Especially, in macrophages we compared non-polarized, pro-inflammatory and immuno-modulatory sub-populations. Moreover, we analyzed additional pro-inflammatory populations of both macrophages and dendritic cells never tested with NET so far. Finally, we tested if NET can prime IL-1β secretion. We show that NET have the capacity to trigger the secretion of pro-inflammatory cytokines, but not IL-10, in all the myeloid cell sub-populations tested.

Methods

Human samples. EDTA-blood from unselected healthy individuals (Etablissement Français du sang, Bobigny, agreement 13/A/107) and from RA patients (Rheumatology Department, Avicenne Hospital, APHP, Bobigny) was used. RA patients fulfilled the American College of Rheumatology-European League Against Rheumatism 2010 criteria. All RA patients were positive for ACPA and were not treated by biologic therapies. Informed consents were collected and experiments were approved by the local ethics committee CPP Paris Ile de France (NI-2016-11-01).

Mice. C57BL/6 mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Mouse experiments have been approved by the local ethics committee (Darwin Committee of the University Sorbonne Paris Nord).

Cell isolation and culture

All experiments were performed with fresh primary cells. PMN and peripheral blood mononuclear cells (PBMC) were isolated from whole blood by dextran sedimentation using Polymorphprep (Axis Shield) as previously described.[14] Contaminating red blood cells were lysed using ACK buffer (NH_4Cl, $KHCO_3$, EDTA). Alternatively, PBMC were isolated from cytapheresis samples by density gradient on Ficoll Hypaque (Eurobio). Monocytes were purified from PBMC by CD14-positive selection using magnetic beads (Miltenyi Biotec). Purity of monocytes and PMN was verified by flow cytometry.

Monocyte-derived macrophages were prepared according to Ambarus *et al*.[15] Macrophages were differentiated from human monocytes in perfluoroalkoxy polymer culture inserts (Savillex) using 100 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF (ImmunoTools), in X-VIVO 15 medium (Lonza)) for seven days, or with 50 ng/ml for 4 days in IMDN medium (Thermo Fischer) containing 10% fetal calf serum (FCS) and then polarized for three days with 50 ng/ml IFN-γ or IL-10 (ImmunoTools), leading to non-polarized (without additional cytokines), pro-inflammatory (with IFN-γ) or immuno-regulatory (with IL-10) macrophages. The corresponding populations will be referred to M0, M1-like and M2c-like macrophages, respectively, according to Mantovani et al.[16] Alternatively, pro-inflammatory macrophages, as well as pro-inflammatory monocyte-derived dendritic cells, were prepared in the presence of an aryl hydrocarbon receptor antagonist (SR1) or agonist (FICZ), respectively, as previously reported.[17] Briefly, blood CD14+ monocytes were isolated from healthy donors' PBMC as described above. Monocytes ($2x10^6$ cells/mL) were cultured for 5 days in RPMI-Glutamax medium (GIBCO) supplemented with antibiotics (penicillin and streptomicin) and 10% FCS in the presence of 100 ng/mL M-CSF (Miltenyi), 5 ng/mL IL-4 (Miltenyi) and 5 ng/mL TNF-a (Miltenyi), in the presence of 8 µM SR1 (Cayman chemicals) or 60nM FICZ (Enzo Life Sciences). The phenotype of macrophage sub-populations and dendritic cells was verified by flow cytometry.

To prepare mouse bone marrow-derived macrophages, bone marrow was flushed from femurs and tibiae and red blood cells were lysed with ACK buffer. Bone marrow cells were resuspended in IMDM medium supplemented with 10% FCS and containing either recombinant mouse M-CSF (30 ng/ml) or granulocyte/macrophage colony-stimulating factor (GM-CSF, 50 ng/ml,

ImmunoTools) for seven days according to Fleetwood *et al*.[18] Only adherent cells were considered as macrophages.

NET were prepared as previously reported.[8] Isolated PMN were seeded on poly-L-lysine-coated (0.001%, Sigma-Aldrich) borosilicate chamber slides (NUNC), settled for 30 minutes, and activated with 50nM of phorbol myristate acetate (Sigma-Aldrich) in RPMI 1640 medium (Thermo Fisher). After four hours, the chambers were washed twice with PBS (Thermo Fisher) and NET were detached from the glass by mild deoxyribonuclease 1 (DNase 1, Sigma-Aldrich) digestion (10U/ml, 30 min). The reaction was stopped by 3 mM EDTA and the supernatants containing soluble NET were harvested and centrifuged at 300 g for 10 min to remove any intact cells. The upper phase was collected, and NET were enriched by a second centrifugation step (16,000 g, 10 min, to remove cell debris) and again the upper phase was collected and frozen. As a control, the same procedure was followed but without PMN to prepare the corresponding NET purification buffer. NET were quantified by fluorescence using PicoGreen, a dye for quantification of soluble double-stranded DNA (Life Technologies) and spectrophotometry (NanoDrop technology). NET were characterized by 16% SDS-PAGE and 1.5% agarose gel. Preparations of 10-20 μg/ml (of DNA, as determined spectrophotometrically by measuring optical density at 260 nm) were used in cell cultures.

Cells (0.75-1x10^6 cells /ml) were cultured in X-VIVO 15 (monocytes, M0 macrophages), IMDM-10% FCS (human M1, M2 and mouse macrophages) or RPMI-10% FCS medium (PMN, pro-inflammatory macrophages and dendritic cells). Cells were then cultured with the NET purification buffer (the true negative control) or stimulated with NET (50% v/v) or lipopolysaccharides (LPS) from S. typhimurium (Sigma-Aldrich). To stimulate IL-1β secretion, cells were in some cases

additionally stimulated with 1 mM ATP (Sigma-Aldrich) for the last four hours. Cell culture supernatants were collected at 18 hours (PMN) or 24 hours and frozen.

ELISA

Cytokine secretion was estimated by sandwich ELISA in cell culture supernatants, according to the manufacturer's recommendations, using kits from BD Biosciences (human IL-6, IL-8, IL-10 and TNF) and R&D Systems (human IL-1β, mouse MIP-2 and mouse IL-10). Concentrations are depicted as mean ± SEM of pooled data or mean ± SD of triplicates in representative experiments.

Flow cytometry

For PMN and monocytes, the phenotype and purity was determined by staining with monoclonal antibodies specific for CD66b (FITC-conjugated, clone G10F5) or CD14 (PE-conjugated, clone M5E2), respectively, or the corresponding isotype controls, at 4° C in staining buffer (PBS containing 5 % heat-inactivated FCS, 100 μg/ml human γ-globulin (Calbiochem), 0.02 % sodium azide) and according to classical protocols. For M0/M1/M2 macrophages, cells were first incubated with human FcR Blocking Reagent (Miltenyi) according to the associated protocol and then stained with anti-human CD200R receptor-BV421 (clone OX-108), anti-human CD14-BV510 (clone MφP9), anti-human CD206-BB515 (clone 19.2), anti-human CD64-PE-Cy™7 (clone 10.1), anti-human CD32-APC (clone FLI8.26) and anti-human CD16-APC-H7 (clone 3G8). All antibodies were purchased from BD Biosciences. Cell viability was estimated by propidium iodide staining. Cells were acquired on a FACS Canto II cell analyzer (BD Biosciences) and data were analyzed using FACS Diva (BD Biosciences). For pro-inflammatory macrophages

and dendritic cells, cells were stained for 30 minutes at 4°C with TruStain blocking solution (Biolegend), anti-CD16 FITC (BioLegend, clone 3G8) and anti-CD1a APC (BioLegend, clone HI149). DAPI (Fischer Scientific, 100ng/mL) was added immediately prior to acquisition. Cells were acquired on a FacsVerse instrument (BD Biosciences).

Statistical analyses

In all figures, legends indicate whether pooled data (with mean ± SEM) or representative experiments (mean ± SD of triplicates) are shown, the number of donors, the number of independent experiments and the number of independent NET preparations. To determine the stimulatory activity of NET, we compared target cells cultured with NET to target cells cultured with the NET purification buffer. To estimate the capacity of ATP to amplify IL-1β secretion by stimulated cells, we compared the target cells of interest cultured with the studied stimulus without ATP to cells cultured with the stimulus and ATP. Groups were compared using Wilcoxon matched-paired signed rank tests or paired *t*-test after having checked that both groups follow a Gaussian distribution and have similar variances. Data were analyzed using GraphPad Prism software ($p \leq 0.05$ was considered significant).

Results

RA NET activate M0 macrophages from both healthy individuals and RA patients

We have previously shown that NET activate resting non-polarized M0 macrophages from healthy individuals.[8] In the present work, we assessed the pro-inflammatory potential of NET from RA patients by analyzing their effect on different target cells from healthy individuals. We first characterized in more details the impact of RA NET on M0 macrophages and then extended our study to different cell populations of the myeloid lineage. We show that RA NET activate M0 macrophages (representative macrophage phenotypes are presented in supplemental Figure 1) from healthy individuals, leading to the secretion of the pro-inflammatory cytokine IL-8 (Figure 1A) and TNF (Figure 1B). LPS (a Toll-like receptor 4 agonist) was used as a positive control. Secretion of the immuno-modulatory cytokine IL-10 was not observed (data not shown). To confirm those results in a pathological context, RA M0 macrophages were cultured with RA NET. As observed above, RA NET trigger the secretion of IL-8 (Figure 1C) and TNF (Figure 1D). Interestingly, although modest, RA NET significantly induce IL-1β secretion by RA macrophages, but only in the presence of ATP Figure 1E), suggesting NET may prime the inflammasome. Similarly, LPS-induced IL-1β secretion is enhanced by ATP, indicating inflammasome involvement (Figure 1F). Indeed, ATP after LPS priming is a known NLRP3 activator.

NET activate both pro-inflammatory and immuno-modulatory macrophages

Because macrophage homeostasis is impaired in RA, we next compared the response of macrophage sub-populations to NET by culturing pro-inflammatory M1-like macrophages (differentiated with IFN-γ) and immuno-modulatory M2c-like macrophages (differentiated with

IL-10) with RA NET. Representative macrophage phenotypes are presented in supplemental Figure 1. RA NET trigger IL-8 secretion by M1 macrophages from healthy individuals (Figure 2A). Interestingly, even immuno-modulatory M2c macrophages secrete that pro-inflammatory cytokine in response to NET (Figure 2B), whereas NET do not induce IL-10 secretion (data not shown). Moreover, both M1 (Figure 2C) and M2c (Figure 2D) macrophages from RA patients are activated in the presence of RA NET, indicating that macrophages from RA patients and healthy individuals respond in a similar manner to NET. To confirm the capacity of NET to activate different macrophage subsets, mouse macrophages differentiated in the presence of GM-CSF or M-CSF (defined as M1-like and M2-like, respectively[19]) were stimulated with mouse NET. Both macrophage sub-populations respond to NET by secreting the pro-inflammatory cytokine MIP-2, a mouse IL-8 homologue (Figure 3A and 3B), whereas IL-10 was not induced in the two populations (Figure 3C and 3D), although they both produced IL-10 in response to LPS.

Pro-inflammatory macrophages and dendritic cells produce pro-inflammatory cytokines in response to NET

To confirm the ability of RA NET to stimulate pro-inflammatory macrophages from healthy individuals, we focused on macrophages differentiated in the presence of an aryl hydrocarbon receptor antagonist as recently reported.[17] In parallel, we analyzed pro-inflammatory dendritic cells differentiated in the presence of an aryl hydrocarbon receptor agonist. Representative phenotypes are presented in supplemental Figure 2. In agreement with above results, NET trigger the secretion of IL-8 (Figure 4A), as well as IL-6 (Figure 4B), by macrophages. Interestingly, a similar induction of cytokines was observed with dendritic cells (Figure 4C and 4D). Importantly,

TNF and IL-10 were not secreted by NET-activated macrophages or dendritic cells (data not shown).

NET trigger activation of monocytes

Monocytes can differentiate into either macrophages or dendritic cells. We therefore also analyzed the response of monocytes to NET. Monocytes from healthy individuals are activated by RA NET and secrete IL-8 (Figure 5A). A low secretion of TNF was also observed, without induction of IL-6 or IL-10 by NET (data not shown). Particularly, NET were unable to trigger IL-1β secretion, even after ATP stimulation, although a strong secretion was observed after stimulation by LPS and ATP (data not shown). Similarly, NET-stimulated RA monocytes secrete IL-8, as well as TNF (Figure 5B).

NET-activated PMN secrete IL-8 as well as IL-1β

Finally, we analyzed the capacity of RA NET to activate PMN. First, stimulation of PMN from healthy individuals with RA NET shows not only a significant induction of IL-8 secretion (Figure 6A), but also of IL-1β secretion (Figure 6B). IL-1β secretion did not require ATP and was not clearly enhanced by ATP (data not shown). We next analyzed RA PMN in response to RA NET and, similarly, both IL-8 and IL-1β secretion was triggered (Figures 6C and 6D, respectively) and secretion was also not increased with ATP. Neither RA PMN nor PMN from healthy individuals secrete IL-10 upon stimulation with NET (not shown).

Discussion

Beside their beneficial role during infections, NET are believed to be pro-inflammatory and pathogenic in certain circumstances, due to increased formation and/or impaired clearance, as observed in some chronic inflammatory autoimmune diseases such as RA. In RA, NET may be involved both in the initiation and in the perpetuation/amplification of the inflammatory process. Thus, it is essential to dissect the effect of NET on different cellular targets and at different level of polarization. Here, in the first study comparing the response of several myeloid cell populations to RA NET (performed with primary cells), we demonstrate that all NET-stimulated myeloid cells tested are activated to secrete several pro-inflammatory cytokines involved in RA pathogenesis (IL-8, IL-6, IL-1β and TNF) but not the immuno-modulatory cytokine IL-10. This is also the first demonstration of NET-induced cytokine secretion by blood monocytes. Likewise, we have analyzed myeloid cell populations never tested with RA NET so far, i.e. pro-inflammatory macrophages/dendritic cells differentiated with an aryl hydrocarbon receptor antagonist/agonist, and we report that they are both activated by NET. Especially, among macrophages we have compared the response of pro-inflammatory and immuno-modulatory sub-populations and data were confirmed with mouse macrophages.

We have previously shown that NET can stimulate PMN and M0 macrophages to produce IL-8.[8] In the latter study, we mainly reported the capacity of NET to activate these cells, without focusing on RA NET, and using pooled data from normal and RA PMN/macrophages. In the present study, we performed a more detailed analysis and on a larger sample size. Here we show that, in addition to IL-8, NET from RA patients induce TNF secretion by M0 macrophages, and both macrophages from healthy individuals and RA patients responded to RA NET. Similarly, we show here that NET trigger IL-8 secretion by PMN from both healthy individuals and RA patients. Particularly,

we show now that RA NET induce IL-1β secretion in both M0 macrophages and PMN. In M0 macrophages, NET-induced IL-1β secretion occurred only in the presence of ATP, as observed with LPS and ATP, suggesting activation of the NLRP3 inflammasome. Indeed, IL-1β secretion after activation of the NLRP3 inflammasome requires two signals: priming (e.g. with LPS) and a secondary stimulus (e.g. ATP). Therefore, we have shown that NET can work as a primer for IL-1β secretion in macrophages. NET have already been shown to induce IL-1β release, but in LPS-primed macrophages.[19,20] In those studies, even NET from healthy individuals induced IL-1β release by macrophages from healthy individuals. The latter report used M0 macrophages obtained by a slightly different protocol, namely macrophages obtained after 3 days, generating probably less mature cells. Here, we have tested whether NET may prime the NLRP3 inflammasome. Moreover, we mainly observed IL-1β secretion in RA macrophages. Interestingly, TNF (the key cytokine in RA) has been shown to sensitize macrophages to ATP and to trigger IL-1β secretion in a NLRP3-dependent manner,[21] and activation of the NLRP3 inflammasome has been suggested in RA patients.[22] Moreover, M0 macrophages have been shown to represent the most pro-inflammatory sub-population in response to typical RA stimuli, namely ACPA-containing immune complexes.[23] In PMN, induction of IL-1β secretion did not require priming of PMN, suggesting that either RA NET directly trigger IL-1β secretion independently of the inflammasome or that NET are able to trigger inflammasome activation in the absence of priming or a second signal. NET-induced activation of PMN may represent an amplification loop, leading to a chronic inflammation vicious circle. Indeed, once activated PMN release NET and NET in turn activate PMN which notably secrete IL-8, a known PMN chemoattractant, also able to trigger NET formation.[24,25] The reason why NET directly induce IL-1β secretion in PMN is unclear. As observed in the present study, canonical inflammasome activation (priming with LPS and then

stimulation by ATP or DNA) trigger IL-1β secretion by human PMN.[26] Moreover, components of the NLRP3 inflammasome are also expressed in PMN and this pathway is operational,[27] as robust production of IL-1β by LPS-primed PMN stimulated with ATP was observed in a NLRP3 inflammasome-dependent manner. The NET component potentially involved in NLRP3 activation is unknown and need further investigation. However, histones have been shown to trigger IL-1β secretion through activation of the NLRP3 inflammasome in LPS-primed dendritic cells.[28]

The stimulatory potential of NET was next confirmed on macrophage sub-populations. Human pro-inflammatory M1 macrophages and even immuno-modulatory M2c were activated by RA NET and secreted IL-8 but not IL-10. This capacity of NET to trigger activation of different macrophage subsets, either pro-inflammatory or immuno-modulatory, was conserved in two different mammal species, as it was also observed with mouse macrophages. Results were also confirmed with another pro-inflammatory subset, namely human macrophages differentiated in the presence of an aryl hydrocarbon receptor antagonist. Thus, the pro-inflammatory activity of RA NET was observed in three different types of human pro-inflammatory macrophages (M0, M1, aryl hydrocarbon receptor antagonist-derived). Interestingly, we obtained similar results in a pro-inflammatory subset of dendritic cells recently described. Actually, both inflammatory macrophages and dendritic cells have been described in the synovial fluid of RA patients.[29] Whereas, no effect of NET from healthy donors were observed on DC from healthy donors in another study,[30] RA NET were shown to activate dendritic cells,[7] supporting our observations. This was however performed with a different dendritic cell subset, and not with pro-inflammatory dendritic cells. Likewise, IL-8 induction by NET-stimulated dendritic cells has been reported,[31] but this was not confirmed with RA NET and was not tested with pro-inflammatory dendritic cells.

Finally, we analyzed precursors of some macrophage and dendritic cell populations, and we demonstrate that monocytes are also responsive to RA NET and secrete IL-8 and TNF, both in healthy individuals and RA patients, without secreting IL-10. Early studies have documented monocyte activation in RA, in blood and in the synovial fluid, both at the onset of the disease and during its chronic phase.[32,33] We show here that a potential RA-associated stimulus, namely NET, may participate in this process in patients. More recently, it was shown that monocytes from RA patients have a defective differentiation into M2-like macrophages and preferentially mature toward M1-like macrophages.[34] It may be interesting to determine the impact of NET on different monocytes subsets, namely classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes,[35] both at the level of monocyte activation and monocyte differentiation.

In conclusion, RA NET trigger a pro-inflammatory response in multiple cellular players involved in rheumatoid synovitis: monocytes, macrophages, dendritic cells and PMN. The cytokine secretion profile induced reflects the cytokines known to be pivotal in the pathogenesis of RA. The consequences of the NET-induced activation of myeloid cells on other immune cells, especially T and B lymphocytes, still need to be determined. NET, or increased NET levels, may become pathogenic, partly as a result of a defective NET clearance. Indeed, sera from RA patients have an impaired capacity to degrade NET in vitro in comparison to healthy individuals.[36]

References

[1] V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss et al., Neutrophil extracellular traps kill bacteria, Science 303 (2004) 1532-1535.

[2] A. Haidar Ahmad, D. Melbouci, P. Decker, Polymorphonuclear Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus: More Complicated Than Anticipated, Immuno 2 (2022) 85-103.

[3] H.L. Wright, B. Chikura, R.C. Bucknall, R.J. Moots, S.W. Edwards, Changes in expression of membrane TNF, NF-{kappa}B activation and neutrophil apoptosis during active and resolved inflammation, Ann. Rheum. Dis. 70 (2011) 537-543.

[4] W. Mohr, B. Pelster, D. Wessinghage, Polymorphonuclear granulocytes in rheumatic tissue destruction. VI. The occurrence of PMNs in menisci of patients with rheumatoid arthritis, Rheumatol. Int. 5 (1984) 39-44.

[5] D. Yu, P.M. Rumore, Q. Liu, C.R. Steinman, Soluble oligonucleosomal complexes in synovial fluid from inflamed joints, Arthritis Rheum. 40 (1997) 648-654.

[6] R. Khandpur, C. Carmona-Rivera, A. Vivekanandan-Giri, A. Gizinski, S. Yalavarthi, J.S. Knight et al., NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis, Sci. Transl. Med. 5 (2013) 178ra40.

[7] G. Papadaki, K. Kambas, C. Choulaki, K. Vlachou, E. Drakos, G. Bertsias et al., Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation, Eur. J. Immunol. 46 (2016) 2542-2554.

[8] M. Ribon, S. Seninet, J. Mussard, M. Sebbag, C. Clavel, G. Serre et al., Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37, J. Autoimmun. 98 (2019) 122-131.

[9] J. Spengler, B. Lugonja, A.J. Ytterberg, R.A. Zubarev, A.J. Creese, M.J. Pearson et al., Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid, Arthritis Rheumatol. 67 (2015) 3135-3145.

[10] R.W. Kinne, B. Stuhlmuller, G.R. Burmester, Cells of the synovium in rheumatoid arthritis. Macrophages, Arthritis Res. Ther. 9 (2007) 224.

[11] P.P. Tak, T.J. Smeets, M.R. Daha, P.M. Kluin, K.A. Meijers, R. Brand et al., Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity, Arthritis Rheum. 40 (1997) 217-225.

[12] D. Mulherin, O. Fitzgerald, B. Bresnihan, Synovial tissue macrophage populations and articular damage in rheumatoid arthritis, Arthritis Rheum. 39 (1996) 115-124.

[13] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (2008) 958-969.

[14] V.M. Rönnefarth, A.I. Erbacher, T. Lamkemeyer, J. Madlung, A. Nordheim, H.G. Rammensee et al., TLR2/TLR4-independent neutrophil activation and recruitment upon endocytosis of nucleosomes reveals a new pathway of innate immunity in systemic lupus erythematosus, J. Immunol. 177 (2006) 7740-7749.

[15] C.A. Ambarus, S. Krausz, M. van Eijk, J. Hamann, T.R. Radstake, K.A. Reedquist et al., Systematic validation of specific phenotypic markers for in vitro polarized human macrophages, J. Immunol. Methods 375 (2012) 196-206.

[16] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, Trends Immunol. 25 (2004) 677-686.

[17] C. Goudot, A. Coillard, A.C. Villani, P. Gueguen, A. Cros, S. Sarkizova et al., Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages, Immunity. 47 (2017) 582-596.

[18] A.J. Fleetwood, T. Lawrence, J.A. Hamilton, A.D. Cook, Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation, J. Immunol. 178 (2007) 5245-5252.

[19] J.M. Kahlenberg, C. Carmona-Rivera, C.K. Smith, M.J. Kaplan, Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages, J. Immunol. 190 (2013) 1217-1226.

[20] C. Farrera, B. Fadeel, Macrophage clearance of neutrophil extracellular traps is a silent process, J. Immunol. 191 (2013) 2647-2656.

[21] L. Franchi, T. Eigenbrod, G. Nunez, Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation, J. Immunol. 183 (2009) 792-796.

[22] R.J. Mathews, J.I. Robinson, M. Battellino, C. Wong, J.C. Taylor, S. Eyre et al., Evidence of NLRP3-inflammasome activation in rheumatoid arthritis (RA); genetic variants within the NLRP3-inflammasome complex in relation to susceptibility to RA and response to anti-TNF treatment, Ann. Rheum. Dis. 73 (2014) 1202-1210.

[23] C. Clavel, L. Ceccato, F. Anquetil, G. Serre, M. Sebbag, Among human macrophages polarised to different phenotypes, the M-CSF-oriented cells present the highest

pro-inflammatory response to the rheumatoid arthritis-specific immune complexes containing ACPA, Ann. Rheum. Dis. 75 (2016) 2184-2191.

[24] T.A. Fuchs, U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn et al., Novel cell death program leads to neutrophil extracellular traps, J. Cell Biol. 176 (2007) 231-241.

[25] A. Teijeira, S. Garasa, M.D.C. Ochoa, A. Cirella, I. Olivera, J. Glez-Vaz et al., Differential Interleukin-8 thresholds for chemotaxis and netosis in human neutrophils, Eur. J. Immunol. 51 (2021) 2274-2280.

[26] M. Bakele, M. Joos, S. Burdi, N. Allgaier, S. Poschel, B. Fehrenbacher et al., Localization and functionality of the inflammasome in neutrophils, J. Biol. Chem. 289 (2014) 5320-5329.

[27] A.K. Mankan, T. Dau, D. Jenne, V. Hornung, The NLRP3/ASC/Caspase-1 axis regulates IL-1beta processing in neutrophils, Eur. J. Immunol. 42 (2012) 710-715.

[28] R. Allam, M.N. Darisipudi, J. Tschopp, H.J. Anders, Histones trigger sterile inflammation by activating the NLRP3 inflammasome, Eur. J. Immunol. 43 (2013) 3336-3342.

[29] E. Segura, M. Touzot, A. Bohineust, A. Cappuccio, G. Chiocchia, A. Hosmalin et al., Human inflammatory dendritic cells induce Th17 cell differentiation, Immunity. 38 (2013) 336-348.

[30] L. Barrientos, A. Bignon, C. Gueguen, L. de Chaisemartin, R. Gorges, C. Sandre et al., Neutrophil extracellular traps downregulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells, J. Immunol. 193 (2014) 5689-5698.

[31] B. Lazzaretto, B. Fadeel, Intra- and Extracellular Degradation of Neutrophil Extracellular Traps by Macrophages and Dendritic Cells, J. Immunol. 203 (2019) 2276-2290.

[32] I. Fujii, M. Shingu, M. Nobunaga, Monocyte activation in early onset rheumatoid arthritis, Ann. Rheum. Dis. 49 (1990) 497-503.

[33] M.G. Ridley, G. Kingsley, C. Pitzalis, G.S. Panayi, Monocyte activation in rheumatoid arthritis: evidence for in situ activation and differentiation in joints, Br. J. Rheumatol. 29 (1990) 84-88.

[34] A. Paoletti, J. Rohmer, B. Ly, J. Pascaud, E. Riviere, R. Seror et al., Monocyte/Macrophage Abnormalities Specific to Rheumatoid Arthritis Are Linked to miR-155 and Are Differentially Modulated by Different TNF Inhibitors, J. Immunol. 203 (2019) 1766-1775.

[35] L. Ziegler-Heitbrock, P. Ancuta, S. Crowe, M. Dalod, V. Grau, D.N. Hart et al., Nomenclature of monocytes and dendritic cells in blood, Blood 116 (2010) e74-e80.

[36] M. Bach, J. Moon, R. Moore, T. Pan, J.L. Nelson, C. Lood, A Neutrophil Activation Biomarker Panel in Prognosis and Monitoring of Patients With Rheumatoid Arthritis, Arthritis Rheumatol. 72 (2020) 47-56.

Acknowledgements and Funding

The authors would like to thank Dr. Magali Breckler (University Sorbonne Paris Nord, Li2P, Inserm UMR 1125, Bobigny, France) for technical help in cell culture.

This work was supported by INSERM and University Sorbonne Paris Nord.

Author contributions

S.S., D.M., A.H.A. and E.S. performed experiments, analyzed and interpreted data. M.P., M.C.B. and L.S. selected patients, collected and analyzed clinical data, analyzed and interpreted data. P.D. performed some experiments, analyzed and interpreted data, designed experiments, supervised the study and wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors have no conflict to disclose.

Figure titles and legends

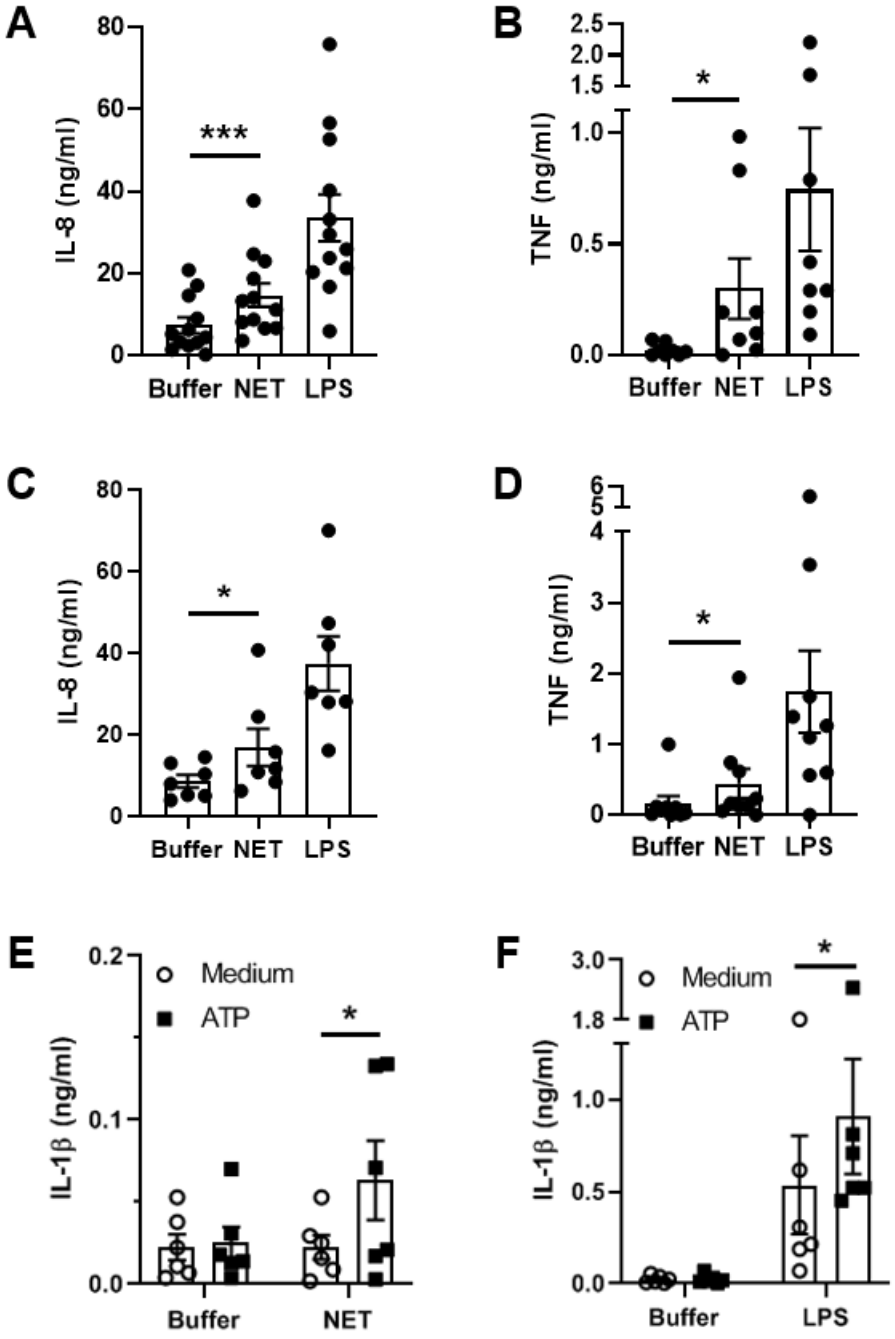


Figure 1. RA NET activate resting M0 macrophages from healthy individuals and RA patients. Non-polarized M0 macrophages from healthy individuals (A and B) or RA patients (C-F) were cultured with RA NET, the NET purification buffer (Buffer) or LPS in the

presence/absence of ATP. IL-8 (A, C), TNF (B, D) or IL-1β (E, F) secretion was estimated. Each dot represents an independent individual. Data are from 12 (A), 8 (B), 7 (C), 9 (D) and 6 (E, F) independent experiments using independent NET preparations. *, $p < 0.05$; ***, $p < 0.0005$ (Wilcoxon matched-paired signed rank test). Means and SEM are depicted.

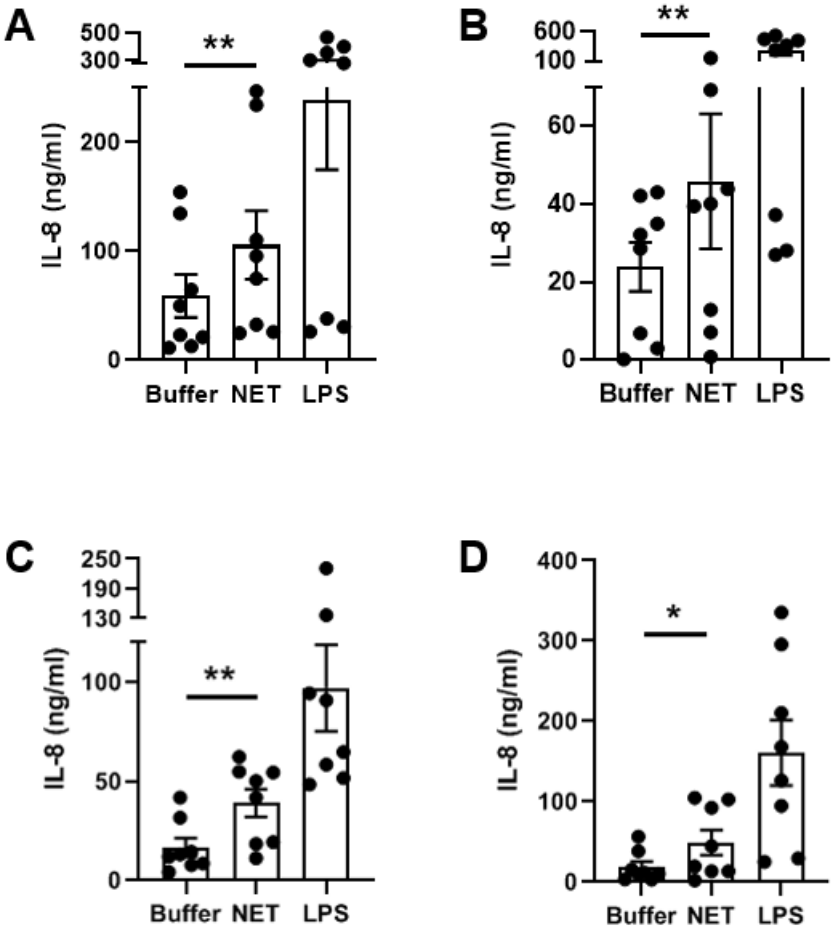


Figure 2. RA NET activate both M1 and M2c macrophages from healthy individuals and RA patients. IFN-γ-polarized M1 macrophages (A and C) and IL-10-polarized M2c macrophages (B and D) from healthy individuals (A and B) or RA patients (C and D) were cultured with RA NET, the NET purification buffer (Buffer) or LPS and IL-8 secretion was estimated. Each dot represents an independent individual. Data are from 8 independent experiments using independent NET

preparations. *, $p < 0.05$, **, $p < 0.01$ (Wilcoxon matched-paired signed rank test). Means and SEM are depicted.

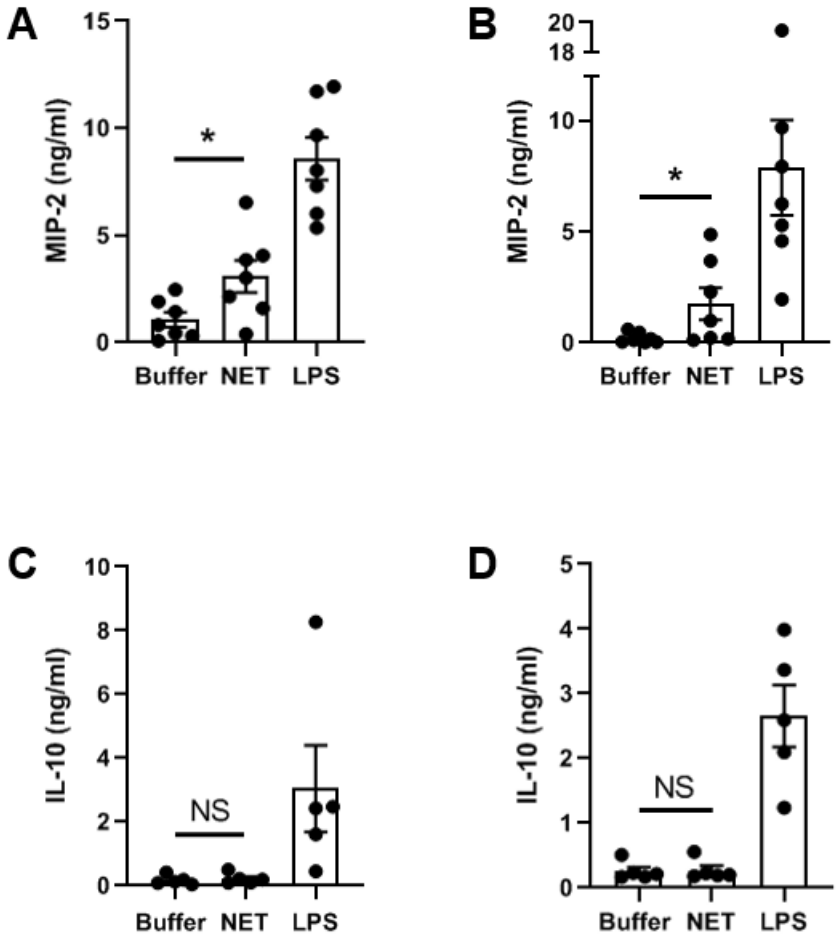


Figure 3. Different mouse macrophage subsets secrete the pro-inflammatory cytokine MIP-2 after stimulation by NET. GM-CSF-polarized (M1-like) macrophages (A and C) and M-CSF-polarized (M2-like) mouse macrophages (B and D) were cultured with mouse NET, the NET purification buffer (Buffer) or LPS and MIP-2 (A, B) or IL-10 (C, D) secretion was estimated. Each dot represents an independent macrophage preparation. Data are from 7 (A, B) or 5 (C, D) independent experiments using independent mouse NET preparations. *, $p < 0.05$ (Wilcoxon matched-paired signed rank test). Means and SEM are depicted. NS, not significant.

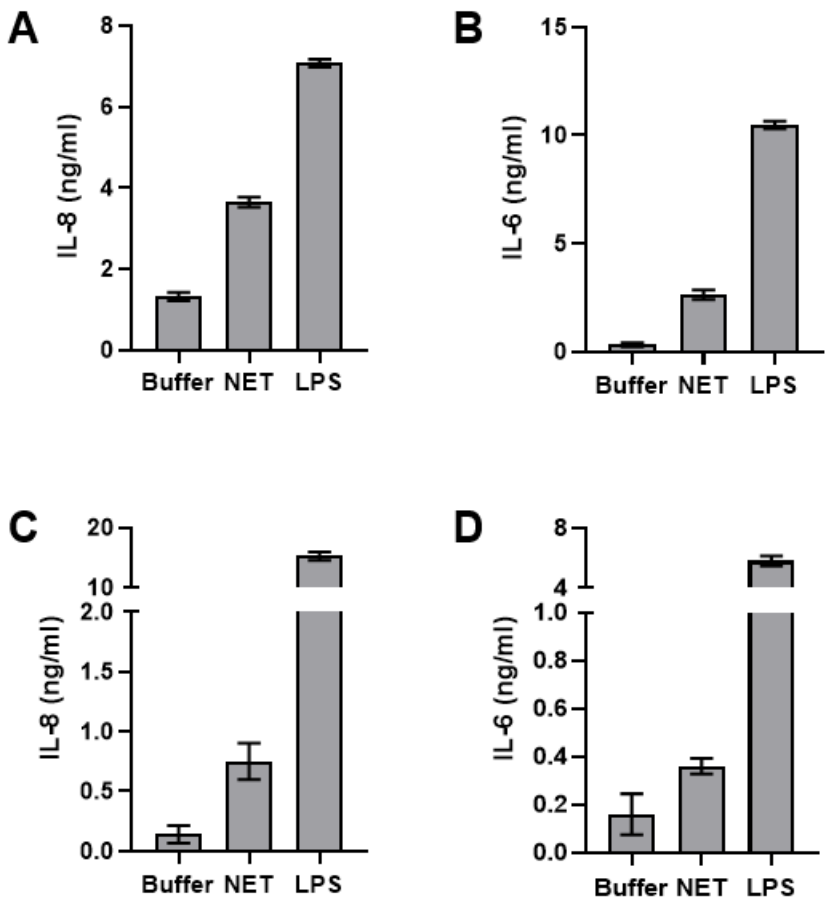


Figure 4. Pro-inflammatory macrophages and dendritic cells produce pro-inflammatory cytokines in response to RA NET. Healthy donor macrophages differentiated in the presence of an aryl hydrocarbon receptor antagonist (A and B) and dendritic cells differentiated in the presence of an aryl hydrocarbon receptor agonist (C and D) were cultured with RA NET, the NET purification buffer (Buffer) or LPS. IL-8 (A, C) and IL-6 (B, D) secretion was estimated. Shown is one representative experiment out of four independent experiments performed with four independent healthy individuals and two independent RA NET preparations (macrophages) or out of six independent experiments performed with four independent healthy individuals and three independent RA NET preparations (dendritic cells). Means and SD of triplicates are depicted.

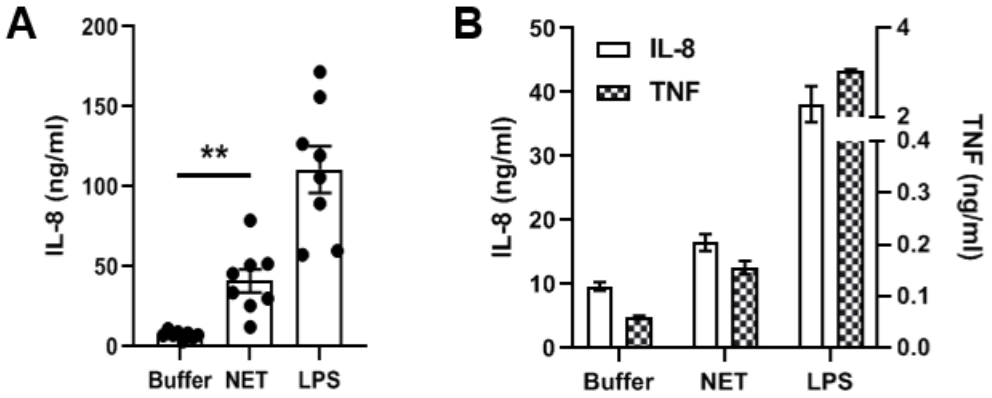


Figure 5. NET trigger activation of monocytes. Monocytes purified from healthy individuals (A) or RA patients (B) were cultured with NET, the NET purification buffer (Buffer) or LPS and IL-8 and TNF secretion was estimated. (A) Each dot represents an independent individual. Data are from 8 independent experiments using independent NET preparations. **, $p < 0.01$ (paired *t*-test). Means and SEM are depicted. (B) Shown is one representative experiment. Means and SD of triplicates are depicted.

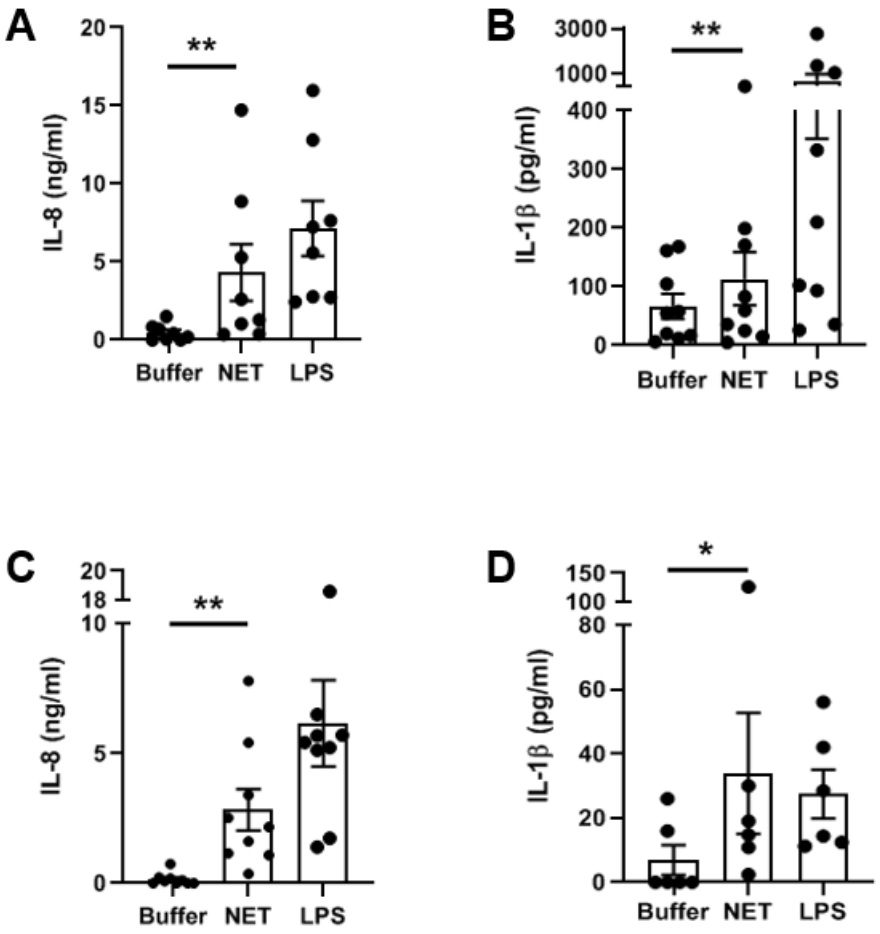
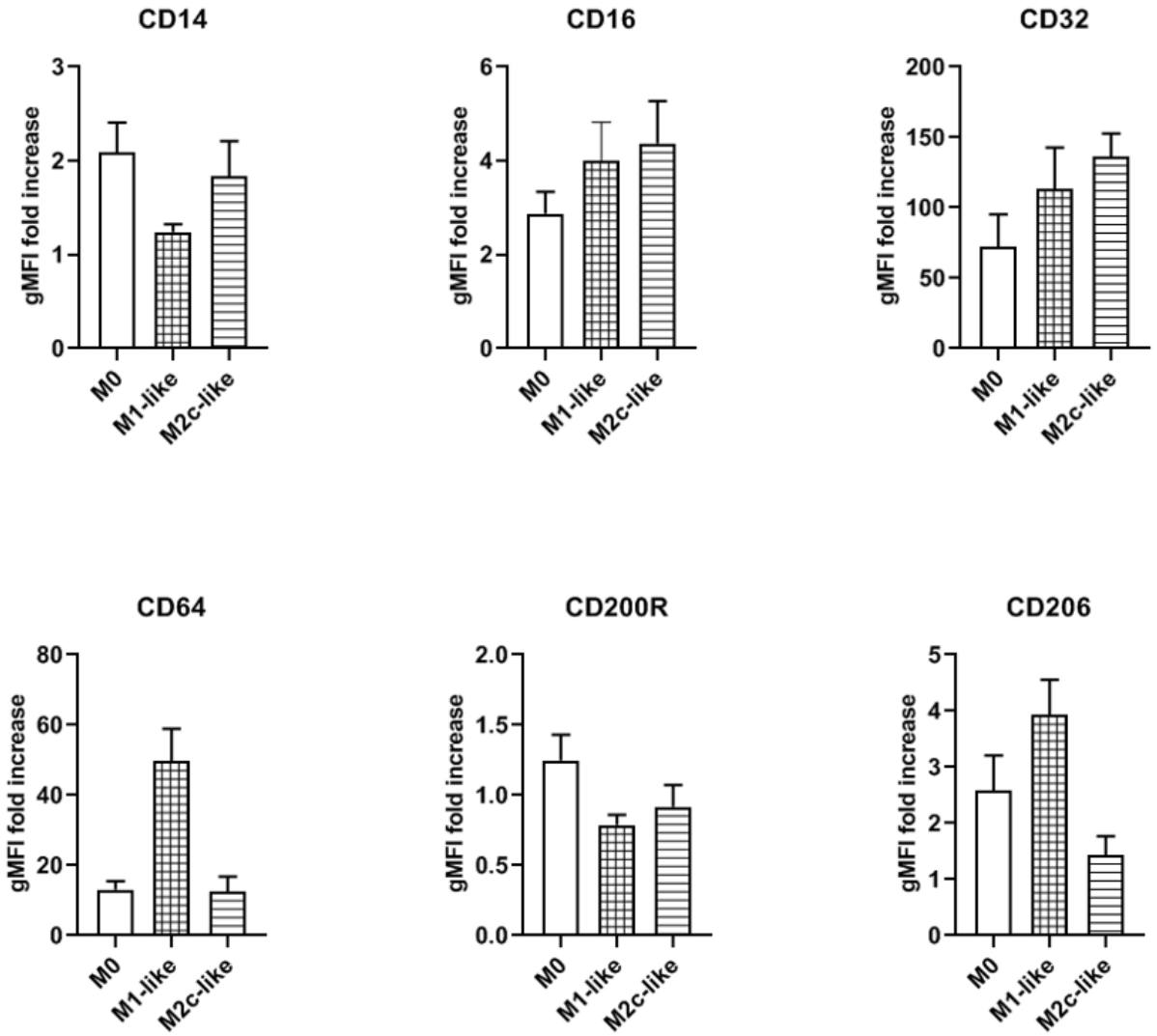


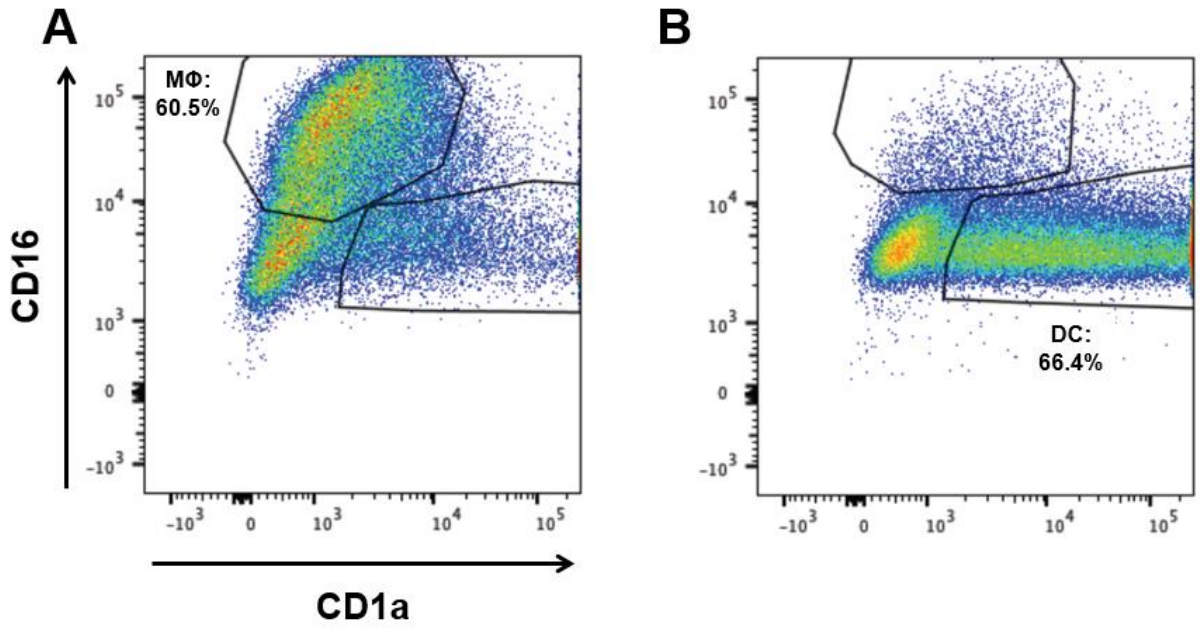
Figure 6. PMN secrete IL-8 and IL-1β in response to RA NET. PMN isolated from healthy individuals (A and B) or RA patients (C and D) were cultured with RA NET, the NET purification buffer (Buffer) or LPS and IL-8 (A, C) and IL-1β (B, D) secretion was estimated. Each dot represents an independent individual. Data are from 8 (A), 9 (B, C), or 6 (D) independent experiments using independent NET preparations. *, $p < 0.05$, **, $p < 0.01$ (Wilcoxon matched-paired signed rank test). Means and SEM are depicted.

Supplemental figure titles and legends



Supplemental Figure 1. Phenotypic characterization of M0, M1-like and M2c-like macrophage preparations. Purified monocytes were differentiated as described in the Methods section for seven days. Macrophages were then stained for specific cell surface makers with monoclonal antibodies or respective isotype controls and analyzed by flow cytometry. For each marker, the fold increase between the geometric mean fluorescence intensity (gMFI) measured after antibody and isotype staining is shown. Note that this is the combination of all markers, and

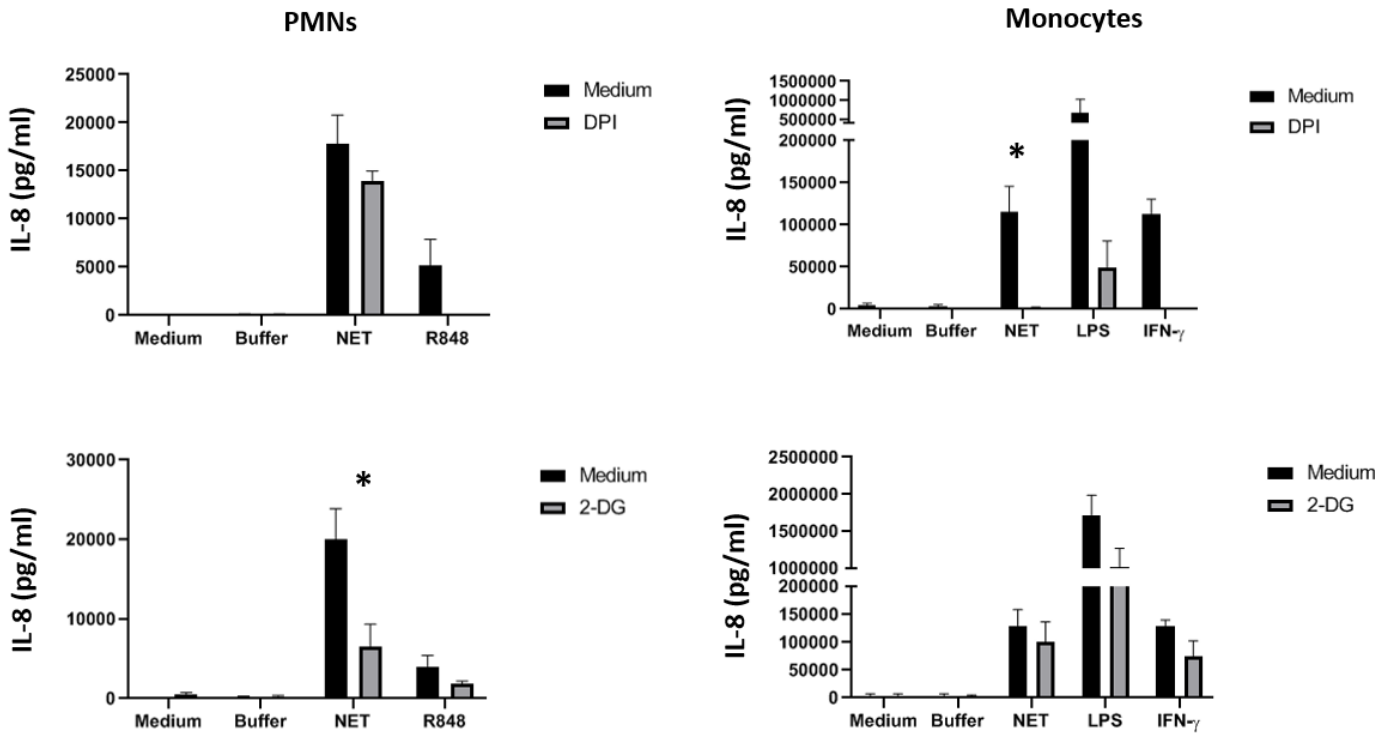
not any individual marker, that confirms the differentiation into the macrophage population of interest. Data are pooled from five independent experiments. Means and SEM are shown.



Supplemental Figure 2. Phenotypic characterization of pro-inflammatory macrophage and dendritic cell preparations. Purified monocytes were differentiated as described in the Methods section for five days. Macrophages (A) and dendritic cells (B) were then stained for CD1a and CD16 with monoclonal antibodies or respective isotype controls and analyzed by flow cytometry. The percentage of cells of interest is indicated. Shown is one representative experiment of four independent experiments using cells from independent healthy individuals. DC, dendritic cells; MΦ, macrophages.

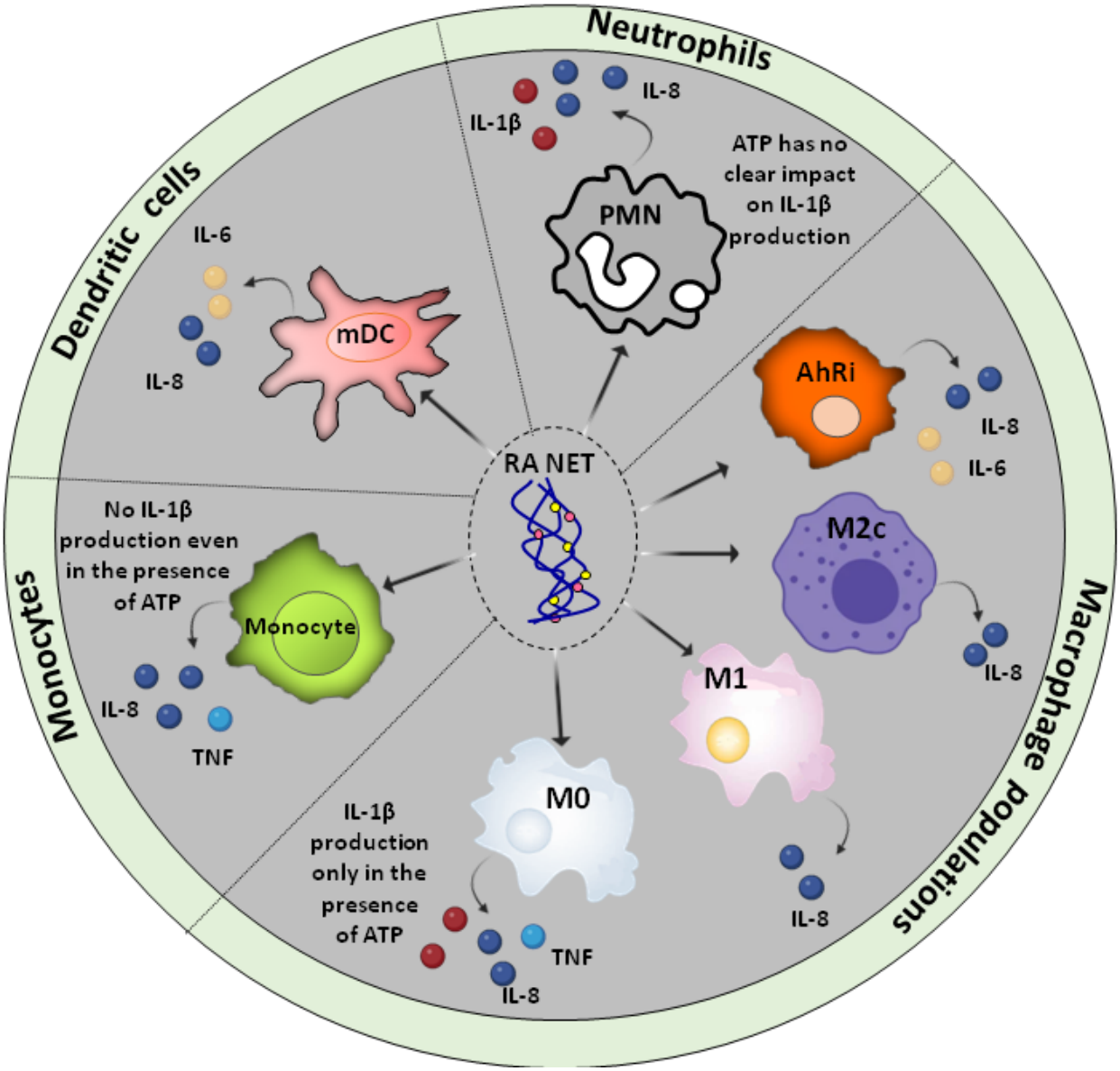
These results were recently obtained; they are presented here as a draft but will be integrated in the new version of the manuscript that is in preparation to be submitted soon (**additional figure)**.

Firstly, we reported that in the presence of an inhibitor of ROS production, monocytes but not neutrophils significantly reduced their production of IL-8 in response to NETs (**additional figure)**. Secondly, when we investigated the involvement of glycolysis in the response to NETs, we discovered that inhibiting glycolysis with 2-DG reduced IL-8 production by neutrophils but not by monocytes.



Additional figure. Involvement of glycolysis and ROS in neutrophil and monocyte activation by NETs. PMN and monocytes isolated from healthy individuals were cultured with RA NET, the NET purification buffer (Buffer) or LPS, IFN-γ and R848 in the presence of a ROS inhibitor (DPI), or a glycolysis inhibitor (2-DG). IL-8 secretion was measured by ELISA.

Featured image



Summary sentence: NET from RA patients trigger a pro-inflammatory response in multiple cellular players involved in rheumatoid synovitis and induce cytokines pivotal in the pathogenesis of RA.

CONFERENCES

1. Neutrophil Extracellular Traps trigger polyclonal B lymphocyte activation independently of antigen specificity toward a pro-inflammatory response.

Ahmad Haidar Ahmad, Maxime Batignes, Delphine Lemeiter, Dyhia Melbouci, Luca Semerano, Elodie Segura and Patrice Decker.

Poster presentation, GREMI meeting, 2023, Paris, France.

2. Neutrophil Extracellular Traps trigger polyclonal B lymphocyte activation independently of antigen specificity toward a pro-inflammatory response.

Ahmad Haidar Ahmad, Maxime Batignes, Delphine Lemeiter, Dyhia Melbouci, Luca Semerano, Elodie Segura and Patrice Decker.

Oral communication, International Congress of Immunology, 2023, Cape Town, South Africa.

3. Neutrophil Extracellular Traps trigger polyclonal B lymphocyte activation independently of antigen specificity toward a pro-inflammatory response.

Ahmad Haidar Ahmad, Maxime Batignes, Delphine Lemeiter, Dyhia Melbouci, Luca Semerano, Elodie Segura and Patrice Decker.

Poster presentation, 42th European Workshop for Rheumatology Research (EWRR), 2023, Dublin, Ireland.

4. Neutrophil Extracellular Traps trigger polyclonal B lymphocyte activation independently of antigen specificity toward a pro-inflammatory response.

Ahmad Haidar Ahmad, Maxime Batignes, Delphine Lemeiter, Dyhia Melbouci, Luca Semerano, Elodie Segura and Patrice Decker.

Oral communication, Club REM, 2023, Saint-Etienne, France.

5. Neutrophil Extracellular Traps trigger polyclonal B lymphocyte activation independently of antigen specificity toward a pro-inflammatory response.

Ahmad Haidar Ahmad, Maxime Batignes, Delphine Lemeiter, Dyhia Melbouci, Luca Semerano, Elodie Segura and Patrice Decker.

Poster presentation, French Society of Immunology (SFI), 2021, Paris, France.

Summary. Activated neutrophils (PMNs) expel neutrophil extracellular traps (NETs). NETs serve as a defense mechanism against pathogens. However, the role of NETs extends beyond their antimicrobial function, with implications in various physiological and pathological processes, including autoimmune disorders. Increased NET formation has been reported in rheumatoid arthritis (RA); a chronic inflammatory disease affecting joints. We have previously shown that NETs are pro-inflammatory on resting macrophages. Indeed, NETs contain several molecules with immunostumulatory properties. We suggest that NETs act as damage-associated molecular patterns (DAMPs) on B lymphocytes inducing their polyclonal activation, independently of antigen specificity; a new mechanism by which NETs might contribute to immune dysregulation, particularly in the context of RA.

We demonstrate by flow cytometry, ELISA and RNA sequencing (RNAseq) that NETs could directly activate total and naïve B cells toward a robust pro-inflammatory profile, characterized by high production of pro-inflammatory cytokines and the upregulation of HLA-DR and the co-stimulatory molecules CD40 and CD86 which are important for antigen presenting cell (APC) function. This activation is amplified in RA patients. We show also that this mechanism is modulated by the presence of C1q and LL-37 proteins, and didn't required the Toll-like receptor 9 (TLR9). Beyond B cell activation, our findings shed light on the domino effect initiated by NETs. NET-activated B cells subsequently act as APCs and trigger T cell activation, bolstering the adaptive immune response. NET-activated B cells also induce the recruitment and activation of neutrophils. This potential crosstalk highlights the versatile nature of NETs beyond their conventional role in antimicrobial defense.

Résumé. Les neutrophiles activés (PNNs) expulsent des 'Neutrophil extracellular traps' (NETs). Les NETs constituent un mécanisme de défense contre les agents pathogènes. Cependant, le rôle des NETs va au-delà de leur fonction antimicrobienne, avec des implications dans divers processus physiologiques et pathologiques, y compris les troubles auto-immuns. Une augmentation de la formation des NETs a été signalée dans la polyarthrite rhumatoïde (PR); une maladie inflammatoire chronique touchant les articulations. Nous avons précédemment montré que les NETs sont pro-inflammatoires sur les macrophages au repos. En effet, les NETs contiennent plusieurs molécules ayant des propriétés immunostumulatrices. Nous suggérons que les NETs agissent comme des motifs moléculaires associés aux dommages (DAMPs) sur les lymphocytes B en induisant leur activation polyclonale, indépendamment de la spécificité de l'antigène ; un nouveau mécanisme par lequel les NETs pourraient contribuer à la dérégulation immunitaire, en particulier dans le contexte de la PR.

Nous avons démontré par cytométrie de flux, ELISA et séquençage des ARN (RNAseq) que les NETs pouvaient directement activer les cellules B totaux et naïves vers un profil pro-inflammatoire robuste, caractérisé par une production élevée de cytokines pro-inflammatoires et une surexpression de HLA-DR et des molécules de co-stimulation CD40 et CD86 qui sont importantes pour la fonction de cellules présentatrices d'antigènes (CPA). Cette activation est amplifiée chez les patients atteints de PR. Nous avons également montré que ce mécanisme est modulé par la présence des protéines C1q et de LL-37 mais qu'il ne nécessite pas le 'Toll-like receptor 9' (TLR9). Au-delà de l'activation des cellules B, nos résultats mettent en lumière l'effet domino initié par les NETs. Les cellules B activées par les NETs agissent ensuite comme CPA et déclenchent l'activation des cellules T, renforçant ainsi la réponse immunitaire adaptative. Les cellules B activées par les NETs induisant également le recrutement et l'activation des neutrophiles. Cette interaction potentielle met en évidence la nature polyvalente des NETs au-delà de leur rôle conventionnel dans la défense antimicrobienne.