MEDICAL UNIVERSITY OF VIENNA

Paracrine factors released by γ-irradiated peripheral blood mononuclear cells inhibit neutrophil extracellular trap formation

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

Katharina Klas, BSc. MSc.

Supervisor:
Univ.-Prof. Univ.-Doz. Dr.med.univ. Hendrik Jan Ankersmit, MBA

Department of Thoracic Surgery
FFG Project APOSEC
Vienna Medical University of Vienna
Währinger Gürtel 18-20, 1090 Vienna, Austria

Vienna, 10/2022

Declaration

The experiments conducted to accomplish this thesis have been carried out at the Department of Thoracic Surgery and the Department of Dermatology, at the Medical University of Vienna under the supervision of Hendrik Jan Ankersmit and Michael Mildner. The project was financed by the Aposcience AG and peer reviewed third party funding as described in the manuscript section “Funding”. In vitro and ex vivo experiments were performed in cooperation with Michael Mildner, Anna Ondracek, Thomas Hofbauer and Karin Pfisterer. Peripheral blood mononuclear cell secretome was produced by the Austrian Red Cross Blood Transfusion Service for Upper Austria, Linz according to GMP-requirements. Interpretation of results, writing and experimental design leading to the publication underlying this thesis were accomplished under the supervision of Michael Mildner and Hendrik Jan Ankersmit.

Table of contents

List of figures

List of tables

Abstract English

Neutrophils, representing the largest population of circulating granulocytes, are equipped with a great variety of highly efficient effector functions. Historically, neutrophils have been considered as critical players of the immune system by phagocytosing invading pathogens or eliminating those by degranulation. However, in the past decades, a rather new effector function has received increasing interest. The discovery of the neutrophils capability to form and extrude net-like structures, so called neutrophil extracellular traps (NETs), via a process called NETosis, has shaped various research fields. NETs have been continuously linked to a plethora of pathologies. Research increasingly demonstrates the tremendous implication of NETs in disease progression and patient prognosis in autoimmune disorders, cardiovascular diseases, wound healing and tissue regeneration, cancer as well as acute respiratory distress syndrome and Covid-19. NETosis is a highly versatile process, following various signalling cascades depending on the inducing stimulus, and is yet not fully understood. However, pharmacological inhibition of NET formation, to alleviate disease burden and complications is of critically high interest. Previous studies have attributed potent immunomodulatory, regenerative and tissue-protective effects to the secretome of γ-irradiation-stressed peripheral blood mononuclear cells (PBMCsec). Nevertheless, the effect of PBMCsec on neutrophils has never been investigated. In this dissertation, it is demonstrated that PBMCsec prevents NET formation in *in vitro* experiments in calcium ionophore and phorbol 12 myristate 13 acetate (PMA) activated neutrophils. Furthermore, it was shown that the purified individual substance classes present in PBMCsec alone do not exert significantly reduced rates of NET formation. This indicates that an interplay of several substance classes or the whole secretome is required for this inhibitory effect. In depth analysis revealed that PBMCsec most likely inhibits NET formation via a dual mechanism. It was demonstrated that reactive oxygen species production is prevented upon PBMCsec-treatment, accompanied by the upregulation of anti-oxidative factors. Furthermore, one of the key enzymes required for NETosis, protein arginine deiminase 4 (PAD4), appears to be modulated by PBMCsec as a reduced enzymatic activity was observed. Together, these findings suggest a potent therapeutic potential of PBMCsec for a diverse set of NETs-associated diseases. These data lay the foundation for future disease-targeted studies investigating the *in vivo* effect of PBMCsec.

Abstract German

Neutrophile, die die größte Population zirkulierender Granulozyten darstellen, sind mit einer Vielzahl hocheffizienter Effektorfunktionen ausgestattet. In der Vergangenheit wurden Neutrophile als entscheidende Akteure des Immunsystems betrachtet, indem sie eindringende Pathogene phagozytieren oder diese durch Degranulation eliminierten. In den letzten Jahrzehnten hat jedoch eine neue Effektorfunktion zunehmend an Interesse gewonnen. Die Entdeckung der Fähigkeit, dass Neutrophile netzartige Strukturen, sogenannte extrazelluläre Traps (NETs), über einen als NETose bezeichneten Prozess zu bilden und auszuschütten, hat verschiedenste Forschungsgebiete zunehmend geprägt. NETs wurden fortlaufend mit einer großen Anzahl von Pathologien in Verbindung gebracht. Neue Forschungserkentnisse zeigen zunehmend die enorme Bedeutung von NETs für den Krankheitsverlauf und die Patientenprognose bei Autoimmunerkrankungen, Herz-Kreislauf-Erkrankungen, Wundheilung und Geweberegeneration, Krebs sowie akutem Atemnotsyndrom und Covid-19. NETose ist ein sehr vielseitiger Prozess, der je nach auslösendem Stimulus verschiedenen Signalkaskaden folgt und noch nicht vollständig verstanden ist. Die pharmakologische Inhibierung der NET-Bildung, zur Linderung von Krankheitslast und Komplikationen, ist jedoch von äußerst hohem Interesse. Frühere Studien haben dem Sekretom von durch γ-Strahlung gestressten mononukleären Zellen des peripheren Blutes (PBMCsec) starke immun-modulatorische, regenerative und gewebeschützende Wirkungen zugeschrieben. Dennoch wurde die Wirkung von PBMCsec auf Neutrophile bis dato noch nicht untersucht. In dieser Dissertation wird gezeigt, dass PBMCsec die NET-Bildung in *in vitro* Experimenten in Kalziumionophor- und Phorbol-12-Myristat-13-Acetat (PMA)-aktivierten Neutrophilen verhindert. Weiterhin konnte gezeigt werden, dass die in PBMCsec vorhandenen gereinigten Einzelsubstanzklassen allein keine signifikante Reduktion der NET-Bildung bewirken. Dies weist darauf hin, dass für diese Hemmwirkung ein Zusammenspiel mehrerer Substanzklassen bzw. des gesamten Sekretoms erforderlich ist. Eine detailiertere Analyse ergab, dass PBMCsec höchstwahrscheinlich die NET-Bildung über einen dualen Mechanismus hemmt. Es wurde gezeigt, dass die Produktion reaktiver Sauerstoffspezies bei PBMCsec-Behandlung verhindert wird, begleitet von der Hochregulierung antioxidativer Faktoren. Darüber hinaus scheint eines der für NETose erforderlichen Schlüsselenzyme, Protein-Arginin-Deiminase 4 (PAD4), durch PBMCsec moduliert zu werden, da eine verringerte enzymatische Aktivität beobachtet wurde. Zusammen deuten diese Ergebnisse auf ein starkes therapeutisches Potenzial von PBMCsec für eine Vielzahl von NET-assoziierten Erkrankungen hin. Diese Daten bilden die Grundlage für zukünftige krankheitsbezogene Studien zur Untersuchung der *in vivo* Wirkung von PBMCsec.

Publication arising from the thesis

Paracrine factors released by γ-irradiated peripheral blood mononuclear cells inhibit neutrophil extracellular trap formation

Katharina Klas, Anna S Ondracek, Thomas M Hofbauer, Andreas Mangold, Karin Pfisterer, Maria Laggner, Dragan Copic, Martin Direder, Daniel Bormann, Hendrik Jan Ankersmit, Michael Mildner

Antioxidants (Basel), 2022 Aug 11; 11(8):1559. doi:10.3390/antiox11081559. PMID: 36009277

Abbreviations

AAA	abdominal aortic aneurism
AAV	ANCA associated vasculitis
AMI	acute myocardial infarction
ANCA	anti-neutrophil cytoplasmic antibody
APCs	antigen presenting cells
ARDS	acute respiratory distress syndrome
BM	bone marrow
C/EBP-α	CCAAT/enhancer-binding protein alpha
CR..	complement receptor 1
CXCL12	chemokine stromal-derived factor 1
CXCR	CXC chemokine receptor
DAG	diacylglycerol
DAMPs	danger associated molecular patterns
DC	dendritic cell
DVT	deep vein thrombosis
ECM	extracellular matrix
ER	endoplasmic reticulum
ESC	embryonic stem cell
EVs	extracellular vesicles
G-CSF	granulocyte-colony stimulating factor
Gfi1	growth factor independent-1
GM-CSF	granulocyte-macrophage-colony stimulating factor
GMP	granulocyte-monocyte progenitor
GPCR	G protein-coupled receptor
HIF-1α	hypoxia inducible factor
HMGB1	high mobility group protein B1
HO-1	heme oxygenase 1
HSC	hematopoietic stem cell
HSP	heat shock protein
ICAM	intracellular adhesion molecule
IL..	interleukin
iNOS	inducible nitric oxide synthase
IP3	inositol-triphosphate
iPSC	induced pluripotent stem cell
JAK	janus kinase
MAC	macrophage
MAPK	mitogen activated protein kinase

MMP..	matrix metalloproteinase
MPO	myeloperoxidase
MS	multiple sclerosis
mTORC1	mammalian target of rapamycin
mtROS	mitochondiral reactive oxygen species
MVO	microvascular obstruction
NE	neutrophil elastase
NET	neutrophil extracellular traps
NETosis	neutrophil extracellular trap formation
NFκB	nuclear factor kappa B
NGAL	Lipocalin 2
NOD	nucleotide oligomerization domain
PAD4	protein arginine deiminase 4
PBMC	peripheral blood mononuclear cells
PBMCsec	secretome of γ-irradiated PBMCs
pDC	plasmacytoid dendritic cell
PG	prostaglandin
PKC	protein kinase C
PMA	phorbol 12 myristate 13 acetate
PR3	proteinase 3
RA	rheumatoid arthritis
ROS	reactive oxygen species
RvD	class D resolvin
RvT	class T resolvin
SERCA	sarco-endoplasmic reticulum calcium ATPase
SLE	systemic lupus erythematosus
SOCE	store operated calcium entry
T1D / T2D	type 1 / type 2 diabetes
TF	tissue factor
TLR	toll like receptor
TNF	tumour necrosis factor
VAMP	vesicle-associated membrane protein
VASP	vasodilator-stimulated phosphoprotein
VCAM	vascular adhesion molecule
VEGF	vascular endothelial growth factor
VLA4	integrin α4β1
VWF	von Willebrand factor

Acknowledgement

Conducting this thesis and all associated projects was only possible due to the support of Hendrik Jan Ankersmit who continuously contributed to my journey with new ideas and input.

I am deeply grateful to Michael Mildner for his invaluable advice on scientific as well as personal questions and for his immense knowledge and experience which he offered unceasingly throughout my time as PhD-student. He was the one of the main driving forces at times were I struggled.

My special thanks go to my colleagues in the Research Laboratory of the Department of Surgery and the Department for Dermatology. Most specifically, I would like to extend my sincere thanks to my PhD colleagues, Dragan, Daniel and Martin for a cherished time spent together in the lab as well as in social settings and for their unbroken support.

My deep gratitude goes to my family for paving my way and their continuous encouragement.

I would also like to express my deepest appreciation for my partner Thomas and thank for his tremendous understanding, unconditional support and aid on every step of the way.

INTRODUCTION

1 Neutrophil granulocytes

Human blood not only provides living cells and tissues with oxygen and nutrients, contains platelets contributing to clotting in case of damaged vessels, but also delivers immune cells, including granulocytes, monocytes, T and B cells, to sites of infection.[1] Neutrophil granulocytes, also referred as polymorphonuclear leukocytes, contribute critically to the innate immune system as first line defenders, act as modulators of adaptive immune responses and additionally aid maintaining homeostasis.[2-4] More than 10^{11} neutrophils are produced per day within the bone marrow (BM), making them the most common leukocyte found in the circulation.[5] The term polymorphonuclear leukocyte is derived from the uniquely characteristic segmented nucleus which precisely distinguishes neutrophils morphologically from other granulocytes as basophils or eosinophils.[6] Besides different nuclear morphology, the varying granulocyte populations exert diverse cell type-specific functions. Basophils are mainly considered to take part in chronic allergy and allergy-induced inflammation, function as antigen presenting cells (APCs), and regulate immune cell memory and Th2 cell function.[7-13] Eosinophils, the second least represented granulocyte population in the circulation, are critically involved in the control of parasitic infections but emerging evidence additionally suggests crucial contribution to bacterial and viral defence.[14] Neutrophils were initially considered as solely phagocytic cells which aid in the resolution of inflammation and clearance of infections by engulfing and destroying pathogens, foreign material as well as dead cells and damaged tissue.[4] However, increasing evidence accumulates indicating not only a pro-resolving involvement of neutrophils in various pathologies but also contribution to tissue damage in case of dysregulated effector functions.[4,15-21]

1.1 Neutrophil lineage committed granulopoiesis

Neutrophils are produced at extensively high rates in the BM on a daily basis from hematopoietic stem cells. Upon terminally differentiation, they leave the BM and progress to patrol the circulation for signs of disturbed homeostasis and infection.[22] In general, granulopoiesis describes the development of all cells belonging to the granulocyte-lineage with

the commonality of a high abundance of secretory granules. Specific neutrophil-lineage commitment is dependent on various factors including key transcription factors, inflammatory state, and environmental influence.[22] Granulocyte development is initiated with the differentiation of hematopoietic stem cells into several intermediate multipotent progenitors which differentiate into granulocyte-monocyte progenitors (GMP)(Figure 1).[5,22] Neutrophil lineage commitment is marked by further differentiation to myeloblasts followed by differentiation to promyelocytes, myelocytes, metamyelocytes and band cells. The final differentiation of band cells results in mature neutrophils.[23] To initiate commitment to myelopoiesis, the transcription factor PU.1 is of crucial importance. PU.1 expression increases throughout maturation from the promyelocyte stage.[24,25] While GMP development is dependent on high expression levels of PU.1, continuous high expression favours monocyte-macrophage development. The transcription factor CCAAT/enhancer-binding protein alpha (C/EBP-α) antagonizes PU.1 function, thereby driving granulocyte differentiation.[26] Additionally, granulocyte-colony stimulating factor (G-CSF), a target of C/EBP-α, further promotes neutrophil-lineage commitment in GMPs.[27] However, neutrophil complexity and heterogeneity is reflected by the fact, that other cytokines including interleukin-6 (IL6) and granulocyte-macrophage-colony stimulating factor (GM-CSF) can partially compensate in case of absence of G-CSF to ensure that at least some neutrophils are generated.[28,29] Another essential transcription factor is growth factor independent-1 (Gfi1), which is upregulated in stem cells at the point of granulocyte-lineage commitment (Figure 1). High expression of Gfi1 represses monocyte-lineage favouring transcription factors and restricts stem cell proliferation promoting progressive differentiation.[30-32] C/EBP-α further contributes to granulocytic lineage decision at early stages of neutrophil development by inhibiting the cell-cycle regulator E2F1.[33,34] Several transcription factors are required to ensure the further differentiation beyond promyelocytes. At the myelocyte stage, C/EBP-ε expression increases which is of vital importance for the transcription of granule proteins.[25,35,36] While C/EBP-α expression gradually decreases after the myeloblast stage, C/EBP-ε peak expression levels overlap at the myelocyte-metamyelocyte stage. Other C/EBP family members including C/EBP-β, C/EPB-γ, C/EBP-δ and C/EBP-ζ show continuously increasing expression levels from the metamyelocyte stage onwards.[25] During final maturation steps, neutrophil nuclei undergo several changes from a round shape to banded nuclei and eventually to a lobulated morphology. Progressive neutrophil differentiation is accompanied with changes in the expression of several factors involved in the retention or release of neutrophils from the BM. CXC chemokine receptor 4 (CXCR4) and integrin α4β1 (VLA4) are downregulated while CXCR2 and Toll-like receptor 4 (TLR4) are upregulated.[5] BM stromal cells express chemokine stromal-derived factor-1/SDF-1 (CXCL12) and vascular adhesion molecule 1 (VCAM1), ligands for CXCR4 and VLA4 respectively.[5] It was shown that CXCR4-CXCL12 interaction represents a key mechanism

retaining neutrophils within the BM.[37] Only 1-2% of neutrophils enter the circulation under homeostatic conditions, highlighting the tight regulation of neutrophil liberation from the BM.[38] G-CSF stimulates neutrophil release by interfering with CXCR4-CXCL12 interactions and upregulation of additional CXCR2 ligands by the endothelium outside of the BM.[38-40]

Neutrophils were initially believed to be short-lived cells with a restricted life-span of only 1.5 and 8 hours in mice and humans, respectively.[4,22] More recent studies revealed that under homeostatic conditions neutrophils appear to survive longer with an average circulatory lifespan of up to 12.5 hours in mice and 5.4 days in humans, however in this study all neutrophils, including those in the BM were assessed.[41] Several studies reported a several fold extended lifespan upon neutrophil activation due to infection or inflammation resulting in a persisting tissue infiltration. [38,42-44] Aged circulatory neutrophils eventually return to the BM or alternatively die in peripheral tissues, both fates resulting in phagocytosis by dendritic cells (DCs) or macrophages (MAC).[45,46] Phagocytosis of dying neutrophils was shown to function as feed-forward loop dampening neutrophil development in the bone marrow.[46] Both DCs and MAC produce IL23 upon neutrophil phagocytosis, thereby initiating IL17 production of T leukocytes, which in turn drives G-CSF dependent neutrophil precursor differentiation.[46-50]

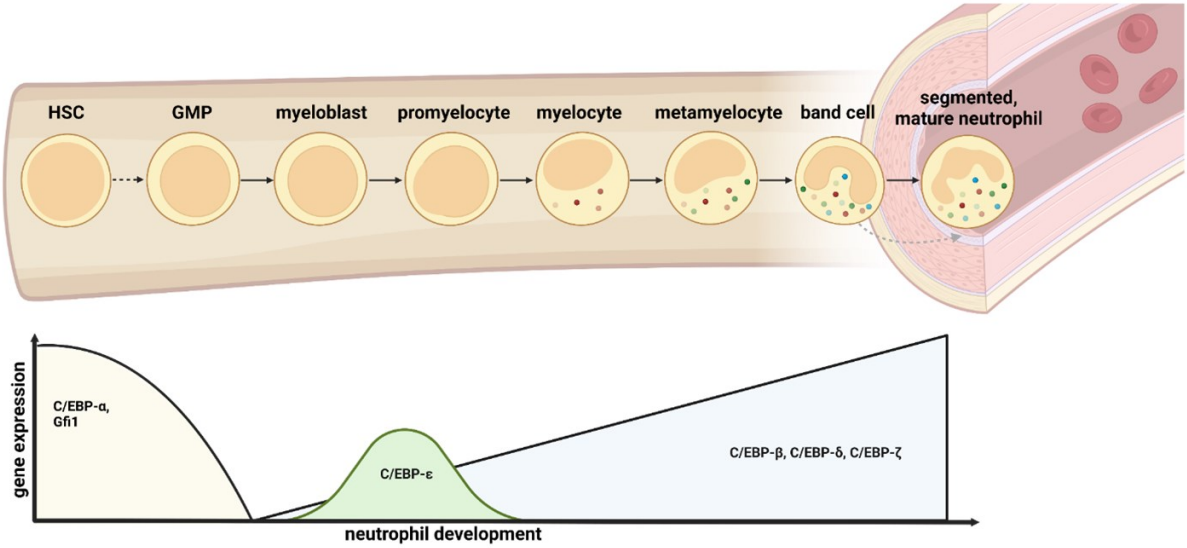


Figure 1 Gene expression during granulopoiesis.
Hematopoietic stem cells (HSC) differentiate upon G-CSF stimulus via several progenitor steps under the influence of C/EBP-α and Gfi1 into granulocyte-monocyte progenitors (GMP) within the bone marrow. C/EBP-ε expression peaks at myeloblast and promyeloblast stages. With progressing differentiation towards mature neutrophils C/EBP-β, C/EBP-δ and C/EBP-ζ expression increases and peaks when neutrophils reach a fully mature state and are released into the blood stream. This figure was created with BioRender.com.

1.2 Neutrophil effector functions

The decision of a neutrophil towards one of its effector functions, phagocytosis, degranulation or neutrophil extracellular trap (NET) formation, is a highly complex and yet poorly understood phenomenon.[51] Within the circulation, neutrophils function as counterpart to tissue-resident macrophages by phagocytosing cell remnants and apoptotic cells.[52,53] Certain cytokines, including the transcription factor GM-CSF, favour phagocytosis.[54-56] Additionally, caspase-mediated programmed cell death leads to the exposure of modified phosphatidylserine residues which facilitates the recognition of these cells for phagocytosis.[57,58] Recognition of phosphatidylserines, accompanied by firm adhesion by tethering receptors, including PSGL1 for activated platelets and Tim4 for apoptotic cells, triggers phagocytosis.[59,60] In contrast, the effect of neutrophil-platelet aggregates appears to be more complex and context dependent.[51] The formation of these aggregates initially depends on the interaction of platelet P-selectin and neutrophil PSGL1 receptor.[61-65] Upon binding, both neutrophils and platelets upregulate factors further promoting strong and stable adhesion including integrins on neutrophils and fibrinogen on platelets.[66,67] In dependence of overall environmental conditions, including metabolic, activation state and the interaction with the extracellular matrix, there are three possible outcomes of this interaction: 1) neutrophils dissociate from platelets thereby resolving the aggregate and may migrate towards sites of inflammation to exert effector functions [68-72], 2) neutrophils phagocytose platelets in response to recognition of features of activated platelets as for example anionic phospholipids [66,72-74], 3) the induction of NET formation.[75] In general, it appears as NET formation *in vivo* is triggered when inflammatory stimuli, regardless whether endogenous or microbial, exceeds a certain threshold of platelet activation.[61,76,77] Furthermore, certain stimuli, including monosodium urate crystals cannot be phagocytosed and alternatively trigger NET formation.[78] Taken together, although not yet fully understood, pH of the microenvironment, metabolic and activation state of neutrophils, overall inflammatory state of immune cells and within tissues, distinct signalling cues as well as the size of the triggering stimuli contribute to the delicate fate decision of neutrophil effector functions.[51] In contrast, degranulation is a tightly regulated receptor-coupled process requiring triggers distinctive of phagocytosis and NET formation.[79]

1.2.1 Degranulation

One key mechanism of neutrophil granulocytes is the rapid release of preformed granules, which are packed with toxic cargo including antimicrobial peptides and several proteases.[80,81] There are at least three different types of granules (primary/azurophilic, secondary/specific,

tertiary/gelatinase granules) and some further subtypes as well as secretory vesicles have been suggested.[82] It is believed that neutrophil granules contribute to the influence of both adaptive and innate immune responses.[80,83] Neutrophil granules are obtained sequentially during differentiation and are named according to their timely appearance and major contents (Table 1). Primary granules are already formed at the promyelocyte stage and contain a large set of antimicrobial proteins and factors with antimicrobial activity, including serine proteases, myeloperoxidase (MPO), defensins and lysozyme.[81,84] Granule proteases of primary granules were found to be activated prior to their incorporation into granules resulting in extensively potent and toxic contents that are readily available.[85] A high heterogeneity can be observed amongst primary granules as some are directed to cell surface trafficking, marked by expressing Slp1/JFC1 and Rab27, while others are prone to fuse with the phagosome lacking the before mentioned proteins.[86] Some granules were found to lack α-defensins while exhibiting all other characteristics of primary granules suggesting even greater granule heterogeneity.[87] Both, secondary and tertiary granules are formed from the myelocyte stage throughout band stage and share similar cargo and thus also functions.[81,87] Approximately two-third of the peroxidase-negative granules contain lipocalin, MMP9 and lactoferrin and are suggested to be a hybrid form of granule subtype.[87] Furthermore, additional categorization of tertiary granules containing ficolin 1 as a ficolin 1-rich subtype was suggested. However, little is known about this potential subtype and requires more detailed research.[88,89] During band and segmented stages of differentiation, secretory vesicles are formed by endocytosis.[90] In contrast to primary granules, which can also fuse with the phagosome, secondary as well as tertiary granules and secretory vesicles are restricted to releasing their contents by fusion with the plasma membrane and are additionally believed to aid in neutrophil adherence to previously activated endothelium.[87,90] Mobilization and release of granules and their content is a tightly regulated process allowing selective degranulation upon specific receptor-coupling leading to distinct signalling events specific for each granule type.[79] Neutrophil degranulation is triggered by the ligation of Fcγ receptors, Mac-1 or G protein-coupled receptors (GPCRs) by a diverse set of stimuli including chemokines, complement fragments, cytokines, bacterial products and endothelial adhesion molecules.[91] Priming stimuli such as tumour necrosis factor (TNF), platelet-activating factor or GM-CSF induce markedly enhanced degranulation responses.[92] Albeit different downstream signalling events are induced upon receptor ligation they all coincide to the induction of calcium flux and the activation of Rac2.[93] Selective granule extrusion is further mediated by their individual requirements of certain calcium concentrations.[87,93] Rac2, a member of the Rho GTPase subfamily, is required for actin skeleton remodelling thereby allowing granule mobilization.[94-97] Rac2 deficiency in mice results in impaired primary granule release while secondary and tertiary granule degranulation was not affected, indicating the presence of additional factors influencing actin reorganization at

least in mice.[96-99] GPCR or Fcγ receptor ligation results in the translocation of the Src-family kinase members Hck and Fgr to primary and secondary granules respectively.[100-102] Even though yet not well understood, the Src-family kinases appear to activate p38 mitogen activated protein kinase (MAPK) leading to actin rearrangement and facilitating the degranulation of all three granule types.[103] p38 MAPK is suggested to function in a similar manner to Rac2 as it is capable of inducing actin remodelling via heat shock protein 27 (HSP27).[104] An additional mode of action for selective degranulation is indicated by the ligation of toll like receptor 9 (TLR9) which activates NF-kB-mediated transcription of inducible nitric oxide synthase (iNOS).[105,106] It was observed in macrophages that iNOS is capable of activating Src-kinases and could therefore be the signalling pathway by which TLR9 ligation results in primary granule degranulation.[106,107] In addition to actin skeleton remodelling, an increased calcium concentration is an indispensable mechanism for functional degranulation. GPCR mediated calcium release is conducted via the activation of phospholipase C and subsequent inositol-triphosphate (IP_3) and diacylglycerol (DAG) production. IP_3 contributes to calcium liberation stored in the endoplasmic reticulum (ER) while DAG mediates protein kinase C (PKC) dependent store-operated channel activation.[108,109] Additionally, other stimuli such as hypoxia or FcγRIIA-mediated phagocytosis further enhance degranulation via AKT signalling or Src-family kinase mediated IP_3 and DAG production.[108,110] Activation of neutrophils via complement receptor 1 (CR1) and CR3 leads to the phosopholipase D dependent liberation of phosphatidic acid that can be converted into DAG, thus facilitating degranulation.[87] The final step of degranulation requires the fusion of the granule and target membrane. This mechanism is dependent on the binding of vesicle-associated membrane protein (VAMP also referred as SNARE) to cognate SNAREs (t-SNARE) found at the phagosome or plasma membrane.[111] The main SNAREs involved in neutrophil degranulation are syntaxin 6 and SNAP-23. Selective degranulation is further dependent on VAMP-SNARE interaction as VAMP-1 and VAMP-7 mediate the release of primary granules while VAMP-2 directs the release of tertiary granules.[112-114] The fusion of granules with phagosomes is exclusively observed for primary granules and seems to be mediated by Rab GTPases, specifically Rab5a.[101] Contrary, another Rab GTPase, Rab27a appears to be inflicted in degranulation of all three granule subtypes.[87] Released neutrophil granule contents such as MPO or elastase not only contribute to antimicrobial host defence but also function in an autocrine and paracrine manner modulating the mode of neutrophil cell death, life-span and function.[115-117] MPO is capable of regulating Mac-1 expression and activation of MAPK/ERK as well as PI3K/Akt pathways which contribute to the prolongation of neutrophil life-span by preserving anti-apoptotic proteins. This feed-forward loop may amplify neutrophil persistence and thus the inflammatory response.[115,118,119] Contrasting, a pro-resolving regulatory role was attributed to lactoferrin by its ability to stimulate

IL10 secretion from macrophages and selectively inhibiting activation and migration of neutrophils.[120,121]

Table 1 Nomenclature and key molecules found within neutrophil granules.

Nomenclature	Primary granules	Secondary granules	Tertiary granules	Secretory vesicles
Alternative nomenclature	Azurophilic granules	Specific granules	Gelatinase granules	
Identifying markers	Azurocidin, Myeloperoxidase (MPO), CD63	Lipocalin 2 (NGAL), CD66b	Gelatinase B (MMP9), CD11b	Albumin, CD45, Mac-1, CD13
Antimicrobial proteins	Defensins, Cap57, Lysozyme	Lactoferrin, Pentraxin 3, Lysozyme, Haptoglobin, $Gp91^{phox}$, $Gp22^{phox}$	Cathelicidin (CAP-18), Lysozyme, $Gp91^{phox}$, $Gp22^{phox}$	$Gp91^{phox}$, $Gp22^{phox}$
Proteases	Neutrophil elastase, Cathepsin G, Proteinase 3	uPA, MMP8	MMP25, MMP8	MMP25, Proteinase 3
Adhesion molecules		Mac-1, CD66, CD67	Mac-1, CD67	Mac-1, CD67
Receptors	CD63	uPAR, Laminin-receptor, thrombospondin receptor	Ficolin-1	Complement receptor 1, CD14, FCγR, C1q-receptor, formylpeptide receptor
Trafficking and docking	VAMP-1, VAMP-7, Rab5/Rab27a	VAMP-7	VAMP-2, VAMP-7	VAMP-7, Rab3d

The distinct distribution of individual molecules allows for the delicate differentiation between the three individual granule subtypes as well as the secretory vessels. In addition to the frequently used identifying markers, these granules and vesicles can further be distinguished by the presence or absence of certain antimicrobial proteins, functional proteases and receptors.

1.2.2 Phagocytosis

Phagocytosis by neutrophils is less well described compared to other effector functions and the majority of the molecular understanding of phagocytosis is derived from studies based on macrophages.[122] However, together with macrophages neutrophils belong to professional phagocytes capable of internalizing opsonized bacteria, invading pathogens or dead cells.[51,122] So far, two general mechanisms of phagocytosis have been identified including the trigger mechanism and the zipper mechanism.[123,124] Distinct signalling by a limited number of pathogens including for example *Salmonella* or *Shigella*, initiates the trigger mechanism resulting in the formation of plasma membrane protrusions surrounding the engulfed target.[123,125] On the other hand, the zipper mechanism involves a large number of surface receptors that sequentially bind to target ligands thereby wrapping the target particles of a

great variety of pathogens.[125] The main receptor relevant for neutrophil phagocytosis is the complement receptor 3, a Fcγ class receptor recognizing IgG. Additionally, even though not considered as phagocytic receptors, NOD receptors and TLRs may increase phagocytosis upon activation.[123] Phagocytosis is initiated by lateral clustering of complement receptor 3 (CR3 or Mac-1) and regulated by a balance between complement C5a receptor (C5aR or CD88) and CR3.[110,126,127] Pharmacological inhibition or genetic deletion of C5aR as well as reduced expression of CR3 results in defective phagocytosis in both human and mouse neutrophils with subsequent defective intracellular killing of bacteria.[127,128] It appears as neutrophils are equipped with an intracellular self-regulating mechanism as certain granule components, including neutrophil elastase (NE), cathepsin G and proteinase 3, are capable of cleaving C5aR.[106,129] The influence of granule contents is further complexed by its context-dependent involvement. While C5a induces the release of NE and thereby the reduction of C5aR, TLR9 ligation mediates the additional release of proteinase 3, both leading to compromised phagocytosis.[106] Under normal conditions persuading phagocytosis, receptor activation induces lipid remodelling within the cell membrane accompanied by actin cytoskeleton rearrangement. This membrane reorganization is required for proper pathogen/particle internalization within the phagosome.[130] The initial composition of a fully formed phagosome is not automatically antimicrobial. Phagosome maturation describes the process by which the fully formed, yet not fully functional phagosome acquires the contents essential for intracellular killing.[122] To fully cover the events during neutrophil phagocytosis, it is noteworthy that this process shares several parallels with the endocytic pathway.[131] One major difference between macrophage and neutrophil phagocytosis is characterized by the fact that conventional endosomes as well as lysosomes are absent in neutrophils and not readily available.[122] The antimicrobial effect observed in neutrophil phagosomes is derived by their fusion with preformed granules and secretory vesicles.[132] Similar to other effector functions, phagocytosis impacts neutrophil life-span. Usually, neutrophil apoptosis is accelerated upon phagocytosis of opsonized bacteria in a reactive oxygen species (ROS)-dependent manner that results in caspase 8 activation.[43,133,134] Overall, it is still not fully understood how neutrophils decide whether to phagocytose or to form NETs.[135]

1.2.3 Neutrophil Extracellular Trap formation (NETosis)

Neutrophil extracellular trap (NET) formation was initially described as programmed cell death distinct from necrosis and apoptosis as potent host defence mechanism to trap and kill invading pathogens including a diverse set of bacteria, fungi and viruses.[136-141] Besides neutrophils, other leukocytes such as macrophages, basophils, eosinophils and mast cells were described

to release extracellular DNA resembling extracellular traps.[142-145] However, increasing evidence accumulates that NET formation, also called NETosis, does not inevitably result in cell death as vital NETosis may also occur without cell lysis.[139,146-149] Furthermore, NETosis is frequently considered as a double-edged sword of innate immunity as neutrophils are capable of forming NETs also in sterile inflammation thereby eliciting damage to host cells.[147,150,151] While the presence or increased occurrence of NETs has been associated to various pathologies, thereby demonstrating the pathophysiological relevance of NET formation, the exact molecular and cellular mechanisms underlying this process are not yet fully understood and are still continuously being discovered.[152] NETs mainly consist of condensed chromatin and DNA, forming web-like structures with a pore size of approximately 200 nm.[153] Nuclear proteins such as histones, a diverse set of granule proteins including NE and MPO, as well as cytosolic proteins and actin are attached to these structures.[154-156]

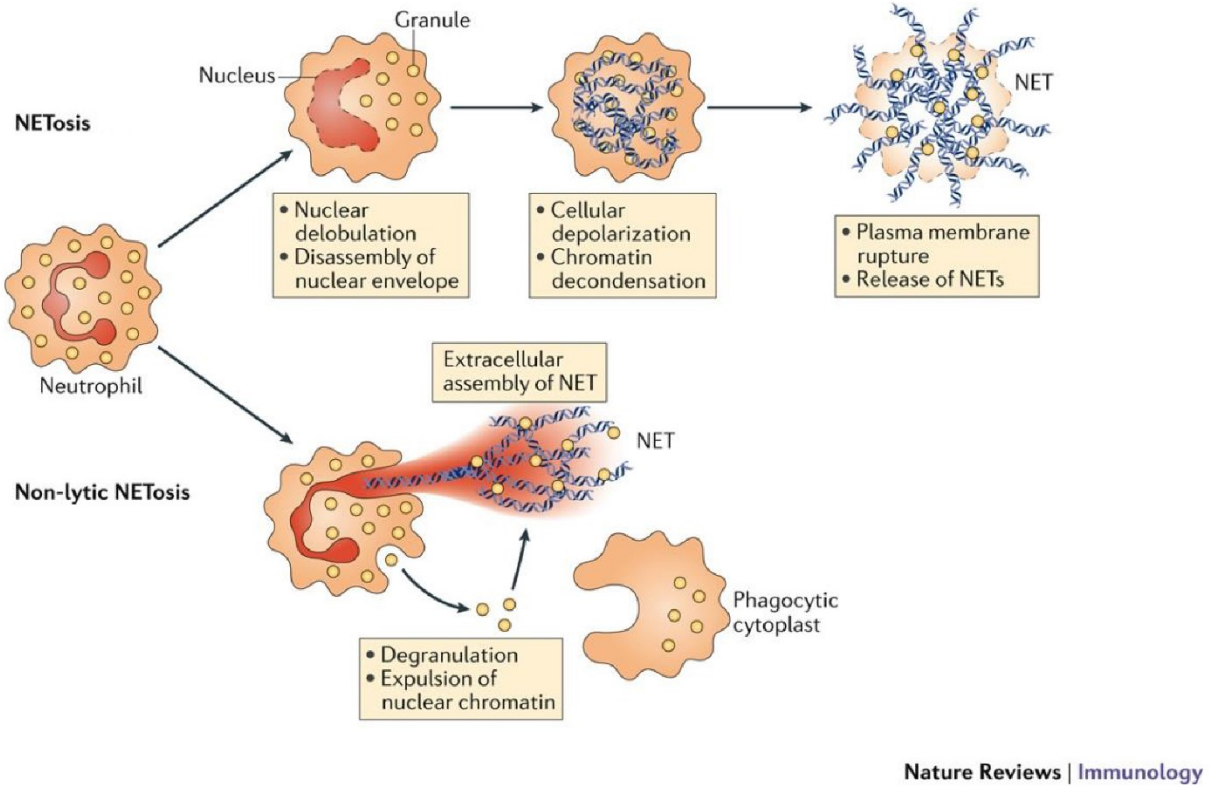


Figure 2 NET formation pathways (NETosis).
Two major NET formation pathways have been suggested. The classic and more common lytic form, usually termed as NETosis or NET formation is characterized by striking intracellular re- and disassembly of the nucleus and the cytoskeleton, followed by chromatin decondensation and membrane rupture eventually leading to the release of NETs and remnants of the cellular body. During non-lytic NET formation neutrophils only partially degranulate and extrude NET components for extracellular NET assembly. Neutrophils performing this form of NET formation remain capable of phagocytosis. Adapted from Papayannopoulos et al. 2018.[157]

1.2.3.1 Neutrophil activation and NETosis initiation

The ultimate prerequisite for NETosis is the preceding activation of neutrophils, as resting neutrophils do not form NETs in non-inflammatory conditions.[148] Neutrophil activation involves a broad spectrum of surface receptors.[148,158-160] Genetically modified mice representing with a deficiency in Toll-like receptor 2 (TLR2) or complement component 3, or with generally impaired interleukin-1 (IL1)-receptor/TLR signalling were unable to form NETs upon stimulation with *Staphylococcus aureus*, indicating an involvement in NETosis signalling in terms of specific bacterial stimuli.[148] Furthermore, ligands of tumour necrosis factor (TNF), Fc and G-protein-coupled receptors (GPCRs) were observed to initiate NETs formation.[158-160] In addition to this diverse set of surface receptors, neutrophils express surface proteins such as CD18, a β2 integrin, which enables them to form NETs upon encountering activated platelets, bacteria or viruses, which further highlights the miscellaneous activating possibilities of neutrophils.[76,160-162] Besides plasma membrane surface receptors, neutrophils additionally express nucleotide oligomerization domain (NOD)-like receptors which have been shown to induce NETosis upon activation.[152] Additional routes of neutrophil activation circumventing surface receptor ligation have been described for ROS or bacterial toxins, such as nigericin and ionomycin.[117,163,164] Activation of neutrophils leading to NETosis signalling initiation subsequently leads to increased intracellular calcium concentrations due to the liberation of calcium storages of the endoplasmic reticulum (ER).[165-168] Ligation of Fcγ receptors, GPCRs, complement receptors or TLR4 contribute to calcium release from the ER which is usually followed by additional calcium influx upon opening of plasma membrane channels.[165-168] Calcium chelation was observed to impair NET formation in stimuli-dependent manner as well as in dependence of extracellular or intracellular calcium chelation. Extracellular calcium chelation prevents phorbol myristate acetate (PMA)-, IL8-, calcium ionophore- (ionomycin and nicericin) and *Pseudomonas aeruginosa*-stimulated NET formation.[158,163,169] While intracellular calcium chelation also inhibits IL8, calcium ionophore- and PMA-induced NET release, it does not affect NET formation upon *Candida albicans* or group B *Streptococcus* stimulation.[163,170] Even though the exact cellular processes dependent on increased calcium concentrations during NETosis signalling are not yet entirely understood, increased calcium availability is widely accepted to be a key requirement for fully functional NET formation and release.[152]

1.2.3.2 Morphodynamics

A multitude of morphological changes of neutrophils have been well described in the context of migration to inflamed or infected tissue where high flexibility is inevitable for proper

transmigration and extravasation.[4] Several changes during NETosis initiation have been observed, however it is not yet clear whether the observed morphodynamics are required for functional NET formation.[152] It was observed that upon activation neutrophils spread prior to shedding of plasma membrane microvesicles followed by rounding up.[171,172] It is suggested that the increased cell spreading is accompanied by the activation or upregulation of extracellular matrix (ECM) surface receptors, which further promotes NETosis.[76,160,161,173,174] Nevertheless, it was shown that at least upon PMA stimulation, neutrophils enter NETosis despite being seeded on substrate surfaces lacking integrin ligands.[171] Plasma membrane microvesicles, shed by activated neutrophils, are annexin V positive and contain cytosolic granules.[172] It was suggested that these microvesicles may serve as messengers contributing to systemic effects, including for example the promotion of thrombosis.[175] Furthermore, it is hypothesized that the presence of annexin V subjects the microvesicles to phagocytosis by macrophages without inducing further inflammatory responses.[176] Despite the lack of complete understanding of the exact role and involvement of the neutrophil-derived microvesicles, it was shown that they contribute to the limitation of bacterial growth, activation of platelets, downregulation of macrophage activation and stimulate endothelial cells to secrete cytokines such as IL6 and IL8.[177-180]

1.2.3.3 Involvement of kinase signalling pathways

Elevated levels of calcium as well as cytokine engagement has been shown to be involved in the activation of several kinases and cell cycle regulators during NETosis.[158,181-183] The protein kinase C (PKC), specifically the isoforms PKCα, PKCβ1 and PKCζ, are activated by calcium and phorbol esters in a phospholipid-dependent manner. PKC serves as critical cell cycle regulator and mediates ionomycin-, PMA-, IL8-, *C. albicans*-, group B *Streptococcus*- and platelet-activating factor-induced NETosis.[158,163,181,184] Stimulation of neutrophils with PMA additionally revealed the involvement of cyclin-dependent kinase 6, regulating G_1/S phase transition during the cell cycle, as well as the Raf-MEK-ERK MAP kinase pathway.[182] Contrasting, monosodium urate crystal- as well as *S. aureus*-induced NET release is mediated by the SYK-PI3K-mTorc2 pathway.[170] Furthermore, activating mutations in the non-receptor tyrosine kinase Janus kinase 2 (JAK2) were observed to enhance neutrophils tendency to release NETs.[183] Taken together, this highlights the delicate stimulus-dependent heterogeneity of neutrophil downstream signalling.[152]

1.2.3.4 The role of reactive oxygen species

Reactive oxygen species (ROS) are dually implicated in NETosis, as they are a direct product of NET formation but also serve as activating stimuli for resting neutrophils.[117,163] In neutrophils ROS are either derived from mitochondria or NADPH oxidase.[117,163] An increase in ROS production is observed rapidly within 20 minutes after neutrophil stimulation with calcium ionophores, *C. albicans*, group B *Streptococcus*, PMA or *S. aureus*. Similar to calcium and kinase signalling, the production of ROS of different origin is highly stimulus dependent.[117,152,163] Furthermore, it was proposed that certain stimuli, including PMA or *S. aureus* seem to strictly depend on NADPH oxidase derived ROS, as neutrophils from patients suffering from chronic granulomatous disease lack functional NADPH oxidase and fail to form NETs upon stimulation.[185] The critical role of NADPH oxidase is further highlighted as inhibition of NADPH oxidase was shown to indirectly decrease histone citrullination by mediating protein arginine deiminase 4 (PAD4) activity, thereby compromising NET formation.[186,187] NADPH oxidase activation is mainly PKC-mediated but other kinase pathways, such as the c-Raf-MEK-ERK cascade as well as Akt, were shown to promote the assembly of functional NADPH oxidase.[181] The mode of action activating NADPH oxidase during NETosis appears to be highly stimulus-specific, since its induction in neutrophils stimulated with parasites was shown to be c-Raf-MEK-ERK signalling-dependent but PKC-independent.[188] It is noteworthy that both, PKC and the c-Raf-MEK-ERK cascade stimulate Mcl-1 expression, which is the main protein in neutrophils exerting an anti-apoptotic effect, which may explain the association with suppressed apoptosis in activated neutrophils dedicated to NETosis.[181,189]

Contrasting, *Leishmania donovani, Paracoccidioides brasiliensis, C. albicans*, calcium and potassium ionophores, soluble immune complexes, monosodium urate crystals as well as *S. aureus* do not depend on NADPH oxidase, thereby indicating an involvement of mitochondrial ROS (mtROS).[163,170,173,190-192] Stimulation of NADPH oxidase-deficient neutrophils lead to robust NETosis induction relying on mtROS, which is believed to be produced due to increased calcium concentrations. This mode of action is commonly referred as NADPH-independent NETosis.[189] However, it was shown that mtROS activates NADPH oxidase, thereby further promoting NETosis.[193] Based on these findings, it was suggested to omit the term “NADPH oxidase-dependent NETosis” and replace it with “mitochondria-dependent NETosis”.[189]

1.2.3.5 The involvement of protein arginine deiminase 4 in DNA decondensation

One of the main features distinguishing NETosis from other forms of cell death is marked by chromatin decondensation, which is not seen in necrosis, pyroptosis or apoptosis, where chromatin remains unchanged or becomes condensed.[194,195] During NETosis, chromatin heterogeneity is lost and decondensation is mediated by posttranslational histone modifications. In contrast to marginally described histone acetylation, citrullination of histones has been well described and is considered to drive NET formation.[164,196] Furthermore, histone modification by serine protease mediated cleavage is also considered as critical step during NET formation.[197] The PAD family comprises five isoforms, which are expressed in mammals and catalyse citrullination.[198,199] PAD4 is primarily expressed in granulocytes and is the only subtype that holds a nuclear localization signal.[200,201] However, precise localization has been observed inconsistently as cytosolic activity was reported, yet visualization of PAD4 showed a nuclear localization.[172,201,202] It was shown that PAD4 activation occurs in a calcium-dependent manner. Interestingly, *in vitro* PAD4 enzyme activity required higher calcium concentrations as were observed intracellularly in activated neutrophils, indicating that additional or alternative pathways are contributing to PAD4 activation.[203-205] Furthermore, it was suggested that ROS may also contribute to PAD4 activation, however, direct evidence has not been shown yet.[206] PAD4 specifically citrullinates histones H3, H4, H2A and the linker histone H1 at different arginines.[207-209] Neutrophils obtained from PAD4 knockout mice were shown to lack citrullinated histones and fail to undergo NETosis upon a plethora of stimuli.[187,210-212] In addition to neutrophil activation in an inflammatory milieu, PAD4 was also described to be required during sterile inflammation, including cancer and deep vein thrombosis.[212,213] Despite questionable specificity, pharmacological inhibition of PADs was described to reduce NET extrusion in primary human and mouse neutrophils. [214-219] However, contradictory reports describe NETosis lacking citrullinated histones upon PMA stimulation and some studies showed no inhibitory effect of pharmacological PAD inhibitors upon bacterial-, PMA- or calcium ionophore-induced NETosis.[163,184] Additionally, NETosis was observed in PAD4 knockout mice exposed to *C. albicans*.[220] It is suggested that other PAD family members, specifically PAD2 contribute to protein citrullination, thereby substituting potential PAD4 deficiency.[221-223] Even though delayed, chromatin decondensation was observed in PAD4-deficient neutrophil-like HL60 cells.[172] These findings propose that PAD4 enzymatic activity may be critical during a specific phase of decondensation, such as initiation, but not exclusively required for fully functional chromatin decondensation.[152] Nevertheless, it is widely accepted that PAD4 mediated histone citrullination is a prerequisite for end-stage NETosis.[163,224,225]

1.2.3.6 Cellular adaptations required for the release of NET content

Nuclear as well as cytoplasmic rearrangement during NETosis resembles morphological changes observed during migration and cell division. It involves lamin remodelling and pore formation in the nuclear envelope before it eventually ruptures.[117,171,226-228] Activation of neutrophils induces local discontinuities of the lamin networks which are believed, even though not yet fully proven, to be mediated by either PAD4 and/or PKC-induced phosphorylation.[171,227,228] The complete sequence of actions occurring during NETosis are not fully uncovered yet and several controversial observations have been described. These include that nuclear envelope vesiculation occurs during suicidal and vital NETosis.[117,139] During classical suicidal NETosis, this vesiculation is believed to mediate the disintegration of the nuclear envelope which subsequently facilitates the release of chromatin into the cytosol.[117,229] Furthermore, it is hypothesized that the nuclear envelope is vesiculated but not ruptured during vital NETosis. Electron microscopy of stimulated neutrophils suggests that these vesicles bud off of the outer nuclear membrane prior to exocytosis at the plasma membrane.[139] However, these findings have fuelled controversial discussions as it remains to elucidate how these vesicles are formed without rupturing the inner nuclear membrane yet forming with only a single-membrane.[152,172] Contrasting, high-resolution, live-cell imaging revealed that the nuclear membrane breaks at various sites instead of being disintegrated by vesiculation.[152,172]

To ensure proper release of nuclear contents into the extracellular space, a great variety of cytoskeletal rearrangements has to be performed including the disassembly of microtubules, actin filaments as well as vimentin intermediate filaments.[171,172,182,230] It was shown that the pharmacological stabilization of the actin cytoskeleton, by actin filament polymerization, resulted in compromised NET formation.[171,172] Contrary, NETosis was also observed to be impaired if drugs inducing actin filament depolymerisation were applied early after neutrophil stimulation, thus suggesting a specifically and temporally requirement of actin destabilization during later stages of NETosis.[152,171,172]

The molecular mechanisms underlying the extrusion of NET contents into the extracellular space remain mostly unknown. However, it has been suggested that plasma membrane rupture follows a multistep process with progressively increasing membrane permeability.[172] Despite contradictory findings, it was recently proposed that the eventual extracellular DNA release takes place as passive phenomenon due to chromatin-swelling mediated mechanical rupture, rather than being actively mediate as it appeared to be independent of MPO, glycolysis, metabolism and ATP.[171]

1.2.3.7 Suicidal versus non-lytic NETosis

The tremendous diversity of neutrophil effector functions is further expanded by their capability to undergo a non-lytic form of NETosis retaining other effector functions and not altering their viability. Initially, this phenomenon was described as vital NETosis observed in neutrophils in presence of LPS stimulated platelets.[147] Due to the discrepancy in describing a form of cell death using the term “vital”, the Cell Death Nomenclature Committee recommended omitting this specific terminology.[231] The extrusion of nuclear content from viable neutrophils was observed *in vivo* in skin infected with gram-positive bacteria. This process was induced by the activation of the complement system and interaction with TLR2. In this context, nuclear-free neutrophils retained functional chemotactic and phagocytic ability.[148] Furthermore, release of mitochondrial DNA was observed in GM-CSF primed neutrophils upon LPS stimulation in a NADPH oxidase-dependent mechanism.[145,232] Suicidal, or lytic NETosis and non-lytic NET extrusion can be distinguished by three fundamental differences. Firstly, certain stimuli, including microbial-specific molecular patterns, TLR2 and complement activation, seem to trigger non-lytic NET extrusion in a drastically more rapid fashion.[139,147,148] Secondly, non-lytic NET extrusion is associated with retaining other neutrophil effector functions such as degranulation and phagocytosis.[148,173] And lastly, molecular mechanisms required for non-lytic NET release differ as this phenomenon relies on vesicular trafficking of nuclear content to the plasma membrane where it is released eventually.[139]

1.3 Neutrophils and NETs in disease

Neutrophil effector functions, specifically NET formation has been linked to protection in various pathologies as means of host defence. NETs were shown to immobilize pathogens and prevent pathogen spreading as well as penetration into the bloodstream.[137,148] However, deficiency, mutation or impaired function of several factors required for functional NET extrusion, including for example MPO and NADPH oxidase, were associated with increased disease burden and poor prognosis in various pathologies.[185,233] Furthermore, patients suffering from defects of neutrophil functions, as seen in chronic granulomatous disease, Chédiak-Higashi disease, leukocyte adhesion deficiencies or the Papillon-Lefévre syndrome present with high incidences of recurrent infections, chronic non-resolving inflammation and hyper-inflammation, mainly due to the incompetency of proper pathogen clearance.[234-238] Accumulating evidence has linked dysregulated and excessive NET occurrence with heavily promoting host damage in infection as well as sterile inflammation and several pathologies including thrombosis, pulmonary diseases, rheumatoid arthritis, systemic lupus

erythematosus, sepsis, inflammatory skin diseases and chronic wounds, diabetes and associated complications, Covid-19 as well as heart failure to name only a few (Figure 3).[157,189,221,239-241]

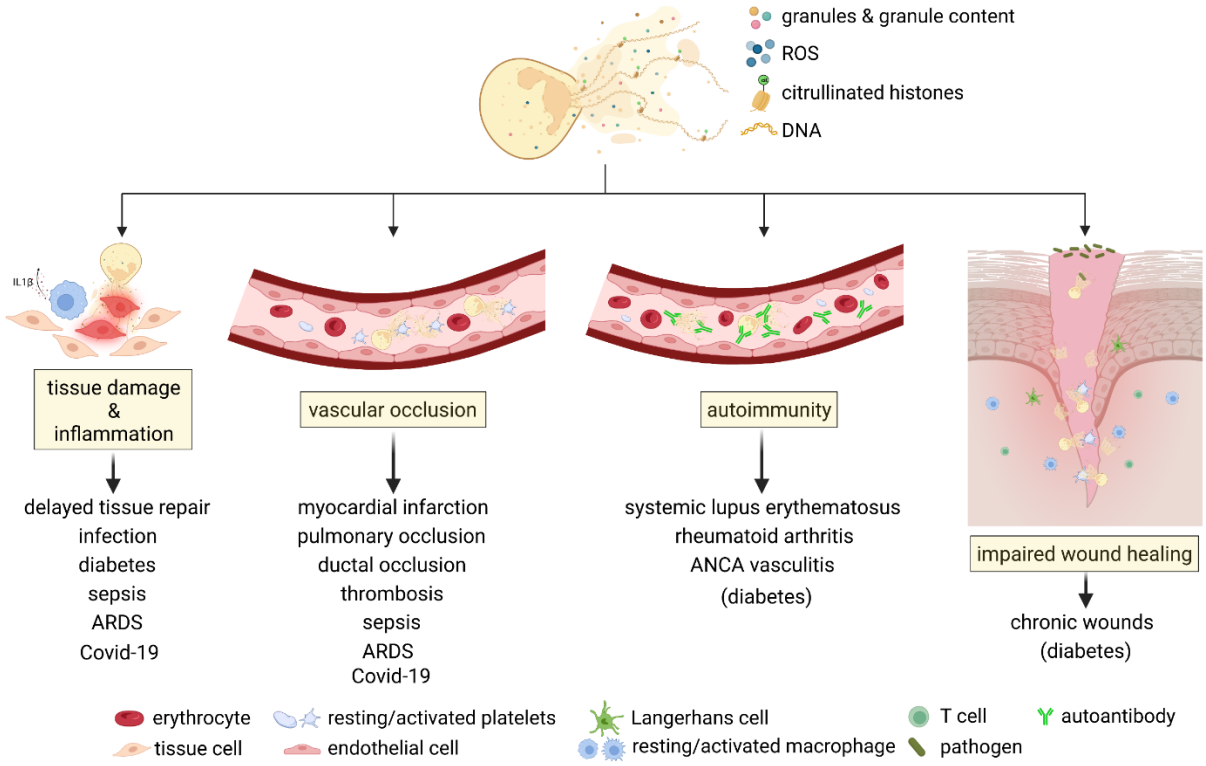


Figure 3 Implications of NETs in pathologies.
NETs and NET-contents are potent drivers of various diseases and pathologic conditions. Direct contact of NETs to tissue induces tissue damage and inflammation. Vascular occlusion is favoured by NETs and NET-platelet aggregates. Continuous exposure to NETs-contents may induce the production of autoantibodies resulting in autoimmunity. Prolonged and/or excessive infiltration and persistence of neutrophils and NETs within wounds is associated with delayed or impaired wound healing. Diseases named in this figure are not necessarily caused by NETs but strongly correlate with NET formation. This figure was created with BioRender.com

1.3.1 Cardiovascular diseases

1.3.1.1 Atherosclerosis

Atherosclerosis onset is marked by endothelial cell damage resulting in the deposition of lipids followed by plaque formation.[242] Endothelial cell damage may be neutrophil-induced or a result of hyperlipidemia, which was also shown to promote neutrophilia thereby indicating a feedforward loop with detrimental consequences.[243,244] NETs were detected in human plaques and in superficial erosions in close proximity of apoptotic endothelial cell clusters.[242,245] Murine models of atherosclerosis revealed a sterile inflammation-induced production of cytokines triggering neutrophils to form NETs.[246] The considerable influence of NETs in atherosclerosis was further proven as atherosclerotic plaques were approximately 3-fold smaller in ApoE/NE/proteinase 3 deficient mice compared to controls and DNase I treatment of control

animals showed a comparable plaque size reduction.[246] Inhibition of NET formation by using a PAD4 inhibitor, chlor-amidine, yielded a significant reduction in atherlosclerotic lesion size, delayed thrombosis in carotid artery, decreased neutrophil recruitment and NET formation.[247,248]

1.3.1.2 Vascular occlusion and thrombosis

NETs formed within the circulation provide a scaffold promoting deep vein thrombosis (DVT).[249] It was shown that DVT treatment with PAD4 inhibitors and DNase I prevented thrombosis in mouse models.[212,250] Release of von Willebrand factor (VWF) and P-selectin from the endothelium recruit neutrophils and initiate NETs formation.[249,251] P-selectin dependent accumulation of neutrophils further recruits platelets, which are then stimulated to produce thromboxane A2 that subsequently triggers endothelial cells to upregulate intercellular adhesion molecule 1 (ICAM1) surface expression, thereby ensuring firm adhesion of neutrophils.[252] Additionally, platelet-derived high mobility group protein B1 (HMGB1) as well as integrins and ROS promote NET formation.[251,253] Besides mechanical occlusion, Factor XIIa is recruited by NETs and contributes to coagulation and the mobilization of VWF, Factor XIIa and P-selectin containing Weibel-Palade bodies from endothelial cells.[253,254] Binding of NET derived histones to fibrin and VWF leads to additional recruitment of red blood cells and platelets.[249,250] Formation of neutrophil-platelet aggregates themselves also trigger a robust pro-coagulant response by enhancing the intravascular tissue factor activity.[250,255] Endogenous anticoagulants such as thrombomodulin may be degraded or modified by neutrophil proteases, specifically NE, or inactivated by oxidases.[256,257] Activated platelets are major contributors to thrombosis and are highly responsive to histone mediated activation via TLR2 and TLR4.[258,259]

1.3.1.3 Acute Myocardial infarction

Acute myocardial infarction (AMI) represents one of the most common cardiac emergencies and usually results of ischemic heart diseases such as coronary artery stenosis, thrombosis or the rupture of a coronary atherosclerotic plaque.[260,261] AMI is accompanied by cardiac wound healing, necrosis, inflammation and increased leukocyte influx into the infarcted zone.[261] High numbers of neutrophils infiltrate the infarcted area within hours post AMI where they interact with danger associated molecular patterns (DAMPs), released by necrotic and apoptotic myocytes, which in turn induces an inflammatory response.[262,263] Tissue damage, reduced resolution of inflammation and poor patient prognosis is associated with excessive neutrophil

infiltration or delayed regression due to accumulation of inflammatory mediators.[19-21,264] In addition to the preceding involvement of NETs in atherothrombosis generation and accumulation in coronary thrombi, NETs continue to interact with platelets during AMI and were shown to further promote platelet activation and express functional tissue factor.[265] Specifically histone H3 and H4 were found to induce thrombin generation in a platelet-dependent manner, thereby promoting thrombogenesis.[265,266] Both, DNA-histone complexes as well as double stranded DNA, which represent key components of NETs, were observed at increased levels at the culprit site and were found to be correlated with the coronary thrombus NET burden. Furthermore, the latter showed a positive correlation with the infarct size, which highlights the detrimental involvement of NETs in the infarcted myocardium. In terms of NET clearance, DNase activity was observed to negatively correlate with the area at risk, infarct size and NET burden.[264]

1.3.1.4 Anti-neutrophil cytoplasmic antibody associated vasculitis

Vasculitis describes a diverse group of conditions marked by inflammation of blood vessels leading to organ ischaemia and damage. Due to the enormous heterogeneity observed in vasculitis no consensus on exact nomenclature and characterization has been found yet. However, it can be classified broadly according to the size of the vessel involved (e.g. large: aorta; small: intraparenchymal arteries, arterioles, capillaries, venules) and the presence or absence of autoantibodies.[267] Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) describes an exceptional form of vasculitis characterized by tissue damage, endothelial injury and inflammation of blood vessels featuring the loss of tolerance towards the neutrophil granule proteins proteinase 3 (PR3) or MPO.[268] AAV is associated with severe organ damage or life-threatening complications and is typically characterized by microvascular endothelial inflammation leading to progressive tissue injury, fibrosis and ultimately to loss of function.[268] Several hypotheses have been established which factors could cause AAVs. On the one hand, it appears as genetic predispositions may be involved to a certain extent. However, it was also suggested that increased exposure of neutrophil antigens to DCs may induce loss of tolerance.[269-271] Immunological loss of tolerance of T cells and B cells to neutrophil granule proteins results in the development of ANCAs which not only target PR3 or MPO, but also contribute to neutrophil activation. Neutrophil activation by ANCAs leads to cytoskeletal alterations, generation of ROS and an upregulation of adhesion molecules, resulting in neutrophil accumulation at already stressed tissue sites, where they further promote tissue injury and additionally release autoantigens by degranulation of NET formation.[272-275] Released autoantigens are processed by antigen presenting cells with

subsequent antigen recognition by T cells, which eventually results in a vicious cycle of continuous inflammation, ANCA production and neutrophil activation.[276] Furthermore, AAVs are associated with an increased risk of cardiovascular events including myocardial infarction, stroke or cardiovascular death and additional dysregulation of either the immune or coagulation system reinforces the risk of venous thromboembolism.[277-279]

1.3.2 Autoimmunity

NETs, specifically certain components such as MPO, DNA, citrullinated proteins and proteinase 3, were proposed to promote autoimmune diseases by providing a source of self-antigens. This phenomenon was first discovered in kidney biopsies from patients presenting with ANCA-associated vasculitis and was further corroborated by studies evaluating NETs deposition in patients suffering from rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[157]

1.3.2.1 Rheumatoid arthritis

Antibodies targeting citrullinated proteins were detected in two thirds of RA patients.[280] RA is a chronic inflammatory disease primarily affecting the joints, however in severe manifestations it is associated with neutropenia, splenomegaly and excessive systemic inflammation.[280,281] It was shown that neutrophils derived from RA patients are highly prone to spontaneously form NETs as well as upon stimuli, including antibodies targeting citrullinated proteins, TNF-α, IL-17 and immune complexes.[282-284] This observation is due to a pre-activation of neutrophils by the rheumatoid factor which drives neutrophils to excessively produce ROS, form NETs and degranulate, thereby further extruding self-antigens.[285] Targeting PAD4 in animal models of RA using a pan-PAD inhibitor showed alleviation of disease burden, marked by reduced erosive changes and inflammation.[286] Furthermore, a therapeutic scheme using monoclonal antibodies against citrullinated histones showed similar results as PAD4 inhibition in a collagen-induced RA model.[287] However, a NETs-dependent mechanism driving RA severity has been challenged by the observation that the excessive citrullination of proteins, present in the synovial fluid of human arthritic joints, resembles citrullination seen in T cell-derived pore-forming toxin treated neutrophils.[288]

1.3.2.2 Systemic lupus erythematosus

SLE is an autoimmune disease characterized by large numbers of autoantibodies, which are mainly directed against nuclear antigens, the generation of immune complexes and abnormal myeloid as well as lymphoid cells.[222,289] Patients suffering from SLE show reduced NETs clearance, which is thought to result from impaired DNA degradation, due to DNase inhibitors and antibodies binding to NETs.[289] NETs are believed to worsen SLE severity by stimulating plasmacytoid dendritic cells (pDCs) to abundantly produce type I interferons via TLR9 and TLR7 signalling.[290-292] In addition to pDCs, NETs activate macrophages in a feedforward loop resulting in further neutrophil stimulation to produce NETs.[293] It is suggested that the prolonged persistence of NETs as well as individual NETs components lead to complement activation, which in turn further promotes disease activity.[294] Studies in a murine SLE model revealed that inhibition of NETs alleviated SLE pathogenesis by additionally reducing organ damage and reduction of vascular injury.[295,296] NETs targeted therapy may also suppress macrophage activation, thereby limiting immune complex formation, which contributes to the suppression of the adaptive immune response in SLE.[291]

1.3.2.3 Diabetes

Despite highly contrasting causative events, neutrophil effector functions and response to infection are exceedingly dysregulated in both, Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D).[297] While T1D represents an autoimmune disorder marked by T cell orchestrated pancreatic β cell loss accompanied by insulin deficiency, T2D is an acquired disease characterized by defective insulin production in combination with insulin resistance in the liver and skeletal muscle.[298,299] Patients with diabetes are reportedly at high risk of infection at the urinary tract, respiratory system and especially the skin.[300,301] Hyperglycaemia and advanced glycation end products are critical factors altering neutrophil function.[302,303] It was observed that neutrophil phagocytosis as well as chemotaxis were reduced in diabetic patients.[304] Other effector functions such as pro-inflammatory cytokine release, ROS production and NET formation, were found to be increased in diabetic patients.[305,306] Increased NET burden is associated with complications including retinopathy, attendant cardiovascular diseases and impaired resolution of infection, specifically in diabetic foot ulcera.[307] Elevated levels of glucose concentrations promote increased rates of NET formation as well as amplified ROS production.[305,308] This triggers a feedforward loop, since ROS serves as potent trigger of NET formation itself. Furthermore, it was found that NETs persist in patients with diabetes up to one year despite normalisation of their blood glucose levels.[309] Potent proteases such as NE

showed higher enzymatic activity in diabetic patients, further exacerbating inflammation.[310] Furthermore, both, human and murine neutrophils isolated from diabetic patients or experimentally induced diabetes were already primed to undergo NETosis.[305] Several therapeutic approaches targeting PAD4 or ROS showed promising results specifically in terms of improved wound healing.[305,311]

1.3.3 Wound healing

Wound healing is a tightly controlled process marked by four phases: haemostasis, inflammation, proliferation and subsequent remodelling. Dysregulation of either of these phases may result in delayed or impaired wound healing.[312] One of the first cell types recruited to the wound site are neutrophils.[15] NETs have increasingly gained attention in terms of delayed wound healing.[305,313,314] While neutrophil granule contents are partly required for functional wound healing, excessive granule and NETs components are linked to impaired wound healing.[315,316] Chronic wounds show elevated levels of proteases and particularly NE is capable of degrading proteins critical for structural maintenance including collagen, fibronectin and proteoglycans, leading to the disruption of cell connections.[316,317] MPO contributes to increased NET integrity and stability by crosslinking individual NETs, leading to inflammation and local tissue damage.[315,318] Other NET components such as histones are capable of integrating into phospholipid membrane bilayers, thereby compromising membrane permeability and integrity, resulting in increased calcium influx and ultimately cell death.[258,319] Other factors such as NET-derived MMP9 take part in ECM and intracellular matrix degradation, which potentially affects proper reepithelialisation.[4,320-322] In addition to direct effects, NET aggregates in the vasculature promote vascular occlusion via thrombus formation, leading to insufficient blood supply to the wounded area, which in turn is accompanied by delayed clearance of dead tissue as well as ischemia and ultimately impaired wound healing.[157,323,324] NETs may also dampen the formation of new capillaries via NE-mediated cleavage of wound healing associated growth factors, including platelet-derived growth factor and vascular endothelial growth factor.[325] The deleterious effect of NETs during wound healing is particularly noticeable in diabetic wounds. Hyperglycaemia itself sufficiently primes neutrophils to rapidly form NETs.[305] Counteracting the detrimental effect of NETs during wound healing by genetic PAD4 deletion was observed to improved wound healing in diabetic mice significantly.[326] However, hyperglycaemia is not a diabetes specific prerequisite as normoglycaemic, aseptic wound healing as similarly improved upon PAD4 knockdown.[305]

1.3.4 Cancer

The role of neutrophils and neutrophil effector functions has been described to be rather controversial and highly dependent on the type of cancer as well as disease progression stage and composition of the microenvironment. Neutrophils were reported to exert anti- as well as pro-cancer effects, as they are extremely versatile in adapting to various microenvironmental signals.[2,327] Several studies described the anti-cancer effect of neutrophils to be dependent on effector molecules, such as nitric oxide or NE, which either delay tumour growth and induce killing or are capable of directly killing cancer cells.[328,329] Furthermore, neutrophils were shown to indirectly limit tumour progression at early stages by stimulating macrophages, which in turn trigger the release of T cell derived IFNγ.[330] The high plasticity of neutrophils was highlighted by a study in early-stage human lung cancer, where immature neutrophils differentiated into a hybrid state, expressing an antigen-presenting-cell phenotype and cross-presented tumour antigens to T cells, thereby inducing an anti-tumour effect.[331] In contrast, accumulating evidence suggests that neutrophils may also contribute to tumour incidence, formation and growth, due to maintaining a chronic inflammatory state, genome instability, promoting angiogenesis and inducing DNA damage.[327,332-335] Neutrophils were also shown to amplify DNA damage and persuading cancer initiation in chemically induced tumorigenesis such as cigarette smoke.[336] Furthermore, neutrophil-derived ROS were shown to favour oxidative DNA damage mutations leading to intestinal cancer growth.[337] Neutrophils may exert proangiogenic effects, as the release of their vascular endothelial growth factor (VEGF)-reservoir stimulates neo-angiogenesis, thus promoting tumour growth.[338] Additionally, neutrophils may indirectly promote angiogenesis by releasing MMP9 which degrades the extracellular matrix, thereby forcing mesenchymal cells to secrete VEGF.[339] Interactions between circulating tumour cells and neutrophils were described to promote cycle progression as well as the metastatic capacity.[340] Studies showed that neutrophil depletion was accompanied by a reduction of complete inhibition of metastasis.[341]

1.3.5 Acute respiratory distress syndrome and Covid-19

Acute respiratory distress syndrome (ARDS) represents a life-threatening respiratory pathology with increasing incidence and mortality rates.[342,343] Its severity was recalled upon the recent outbreak of Covid-19 as 29-42% of Covid-19 patients developed ARDS and 15-52% of these patients died.[344,345] ARDS patients typically developh extensive pulmonary oedema, diffuse bilateral pulmonary infiltrates and hypoxemia. Lungs of ARDS patients are marked by increased vascular permeability, leading to protein-rich fluid leaking into the alveolar space

and tremendous accumulation of activated immune cells, which compromises gas exchange and results in respiratory failure.[346,347] One hallmark of ARDS is the excessive infiltration of neutrophils in the inflamed lung.[348] Circulating neutrophils follow a chemokine gradient, released by damaged pulmonary tissue. Neutrophil recruitment and activation is not limited to either exogenous or endogenous inflammatory stimuli, nor to infectious or sterile inflammatory stimuli, thereby facilitating high levels of activation and promoting a potent neutrophil-driven immune response resulting in damaged tissue and lung dysfunction.[349-351] Accumulating NETs were observed to obstruct small airways and damage alveolar capillaries.[352] The role of neutrophils and specifically NETs during Covid-19 is highly complex and was shown to indirectly promote disease pathogenesis.[240] Patients with Covid-19 show endothelial inflammation, which in turn promotes coagulation as well as thrombus formation.[353,354] NETs associated biomarkers are elevated in the circulation during early infection response and are associated with disease severity and thrombosis.[355] NETs-mediated thrombosis during Covid-19 was proposed to be complement dependent. Covid-19 activates the complement cascade leading to thrombin mediated tissue factor (TF) expression in neutrophils, which results in TF carrying NETs with pro-coagulatory properties.[356] Furthermore, it was observed that live as well as heat-inactivated SARS-CoV-2 virus is capable of inducing NETs formation, most likely via binding to angiotensin converting enzyme 2 receptor on neutrophils.[357,358] Additionally, NETs were found to increase virus infectivity as NE can cleave S protein thereby facilitating virus entry into the cell.[359]

1.4 Pharmacological targeting of NETs

Indirect immunomodulatory inhibition of NET formation was observed in several studies using acetylsalicylic acid (Aspirin), which is an anti-thrombotic, non-steroidal drug with anti-inflammatory effects.[360,361] It inhibits thromboxane A2, thereby counteracting platelet aggregation and activation.[362] Upon activation, platelets show increased surface expression of P-selectin and secrete several mediators, such as platelet factor 4, CCL5 and HMBG-1, thus facilitating neutrophil binding, subsequent activation and NET formation.[147,251,363] Targeting platelets or blocking platelet-neutrophil interactions may serve as indirect anti-NET therapeutic approach. This was shown to be efficient in an endotoxin-triggered acute lung injury model, where pre-treatment with aspirin lead to reduced intravascular NET formation and decreased lung injury.[364,365] Another mechanism to indirectly prevent NET formation is the therapeutic administration of thrombomodulin, which is capable of limiting procoaculant responses and prevents NET formation in neutrophils co-cultured with activated platelets.[366]

Cyclosporine A, an immunosuppressive drug, directly inhibits NET formation by inhibiting the calcineurin pathway.[367] It was shown that a combinatory therapy of cyclosporine A together with ascomycin yielded potent inhibition of NET formation *in vitro.*[158] However, the benefit of strong immunosuppressant drugs targeting NETs is questionable as they are accompanied by potentially severe adverse effects and predisposing patients to recurring and more severe infections.[367]

Direct and irreversible inhibition of PAD4 activity, and thus reduced or inhibited NET formation, is mediated by chlor-amidine.[368] Systemic therapeutic administration in a murine model of SLE protected animals from NET-induced vascular damage, kidney injury as well as endothelial dysfunction.[296]

Prostaglandins (PGs) are endogenously synthesized enzymes that were attributed to exert both anti- as well as pro-inflammatory effects.[369] PGE2 was shown to counteract PMA-induced NET formation by modulating EP2 and EP4.[370] Defective intracellular bacterial killing was observed in a PGE2 overproducing mouse model, while EP2 and EP4 antagonist treatment could restore NET formation, thus suggesting that blocking PGE2-EP2 or PGE2-EP4 signalling axis restores NET formation.[371]

Certain complement components were shown to interact with neutrophils and trigger NET formation resulting in a vicious cycle as NET products can in turn activate the complement cascade.[372] A peptide inhibitor of complement C1 was observed to reduce MPO activity and therapeutic complement inhibition is already successfully employed in paroxysmal nocturnal hemoglobuinuria, a rare disease characterized by a variety of symptoms including haemolytic anemia, thrombosis, and renal insufficiency.[373,374]

The commonly used antidiabetic drug metformin induces AMP-activated protein kinase activity, which in turn prevents mitochondrial ROS production via mammalian target of rapamycin (mTORC1) inhibition.[375] *In vitro* studies revealed that metformin reduces overall NETs concentrations and particularly cell free DNA, proteinase 3, histones and elastase and additionally prevents the activation of NOX in neutrophils by preventing PKC-βII membrane translocation.[376,377]

DNase treatment is widely used in *in vitro* experiments and despite not preventing NET formation per se, it was observed to potently alleviate disease burden, by reducing NET-mediated tissue damage in different animal models.[305,378,379]

2 Cell-based therapy in regenerative medicine

The importance of regenerative medicine has increasingly gained attraction due to several studies providing promising results, meeting the aim of repairing or replacing damaged cells or organs with potentially regaining normal function.[380] Bone marrow transplants used to treat immune system or blood disorders are the most established forms of stem cell therapy.[381] Stem cell-based therapies comprise any therapeutic intervention for a disease that involves any type of human stem cells, including induced pluripotent stem cells (iPSCs), adult stem cells and embryonic stem cells (ESCs).[382] However, despite promising results, stem cell therapy is accompanied by several limitations. One of the biggest concerns is reflected by ethical conflicts using ESCs but also iPSCs, arising from the fact that processing of human embryos is required as well as the unlimited differentiation capacity potentially leading to tumour formation, respectively.[380] The efficacy of stem cell therapy was further denounced by initial clinical trials in the setting of AMI, revealing only minor clinical improvements upon autologous progenitor cell treatment.[383] Several studies yielded contradictory results, reporting on the one hand reduced risk of mortality as well as reduced re-hospitalization in patients suffering from heart failure upon autologous cell therapy, while other studies revealed no beneficial effect associated with stem cell therapy in AMI.[384,385] Mesenchymal stromal cells (MSCs), also referred as mesenchymal stem cells, have been of high interest in terms of therapeutic application due to their immune regulatory function and high safety profile.[386] It was shown that MSCs do not exert immunosuppressive functions at baseline, but require microenvironmental cues, including inflammation, to adopt an immunosuppressive phenotype.[386] Despite initially promising results, 28% of registered clinical trials have been completed and results were reported for only 2% of the completed clinical trials.[386] Immunoselection of mesenchymal precursor cells (MPCs) for STRO-1 expression, which is associated with high co-expression of STRO-3, was shown to be superior to unspecific adherence-isolated MSCs in terms of paracrine activity and thus potential cardiovascular therapeutic approaches.[387] Pluripotent, self-renewing MPCs with a high capacity for differentiation and proliferation were demonstrated to exert beneficial effects in animal models of MI by improving left ventricular (LV) function accompanied by reducing infarct expansion upon targeted delivery. Furthermore, already low concentrations of directly intra-myocardially injected MPCs showed therapeutic effects and resulted in alterations of MMP tissue inhibitor and MMP levels as well as collagen dynamics.[388] The beneficial effect of immunoselected STRO-3 positive MPCs was further demonstrated in a large animal model of monoarthritis, as MPC-treated animals showed reduced lymphocytic

and monocytic infiltration of synovial tissues, decreased cartilage erosions and overall decreased clinical signs of joint pain and swelling as well as decreased lameness.[389] Further investigation of the cardioprotective effects of STRO-3 positive MPCs in a rat model of MI revealed that not only the injection of MPCs but also the treatment with MPC-conditioned media decreased fibrosis and myocyte apoptosis, preserved LV dimensions and function and promoted neovascularization.[390]

The initial theory that transplanted stem cells would incorporate at injured sites and transdifferentiate into the required cell types thereby exerting tissue regenerative effects was further tremendously challenged by studies reporting that only a minor percentage, 1-5% of injected stem cells, engraft at the desired tissue site.[391-395] It was shown that the majority of injected cells was sequestered in the lymph nodes, bone marrow, lung, liver and spleen already a few hours post infusion.[393,396] These findings raised the question how the beneficial effects observed in stem cell therapy were obtained. Gnecchi et al. shifted the theory from direct stem cell induced immunomodulation towards a secretome-assisted effect, which does not solely rely on direct cell-cell interactions, but is supported by paracrine factors released from infused cells.[397,398] Conditioned media from mesenchymal stem cells, exposed to hypoxic stress, was shown to significantly decrease hypoxia-induced cell death in ventricular cardiomyocytes of rats.[397,399] Shortly thereafter, it was shown that the stem cell secretome deviates marginally from supernatants derived from peripheral blood mononuclear cells (PBMCs).[400] The controversy around stem cell therapy was further expanded when the immunomodulatory effect was proposed to be derived from the secretome of apoptotic stem cells and may even be similar to the secretome of any apoptotic cell type.[399,401] Apoptosis, representing a tightly regulated active process, is critically involved in the maintenance of homeostasis and constant self-renewal. It is known to drive strong immunomodulatory effects by directly secreting immunocompetent mediators or indirectly by activating dendritic cells and phagocytes.[399] A systemic decrease of inflammatory concentrations as well as a release of anti-inflammatory cytokines is observed upon infusion of apoptotic cells.[402,403] The broad spectrum of signals released by apoptotic cells serves to alarm their surrounding environment of dysregulated homeostasis or danger to promote immune responses, survival and stimulate cell proliferation.[399]

Using the secretome of stem cells instead of direct cell transfusion allows to circumvent stem cell therapy associated risk factors such as immune rejection and teratoma formation.[404]

2.1 Stressed PBMCs and their secretome

2.1.1 Cardiology

Ankersmit et al. demonstrated that an LPS-induced pro-inflammatory phenotype in PBMCs and monocytes was reduced by treatment with apoptotic γ-irradiated PBMCs.[405] The injection of irradiated PBMC suspensions in an *in vivo* rodent model of AMI resulted in reduced infarct size and improved functional parameters including end-systolic and end-diastolic diameters as well as ejection fraction.[405] Lichtenauer et al. sought to further elucidate these findings and investigating the pleiotropic effect of apoptotic PBMCs using the same rat model of AMI.[406] Irradiated PBMCs were injected either intravenously or intramyocardially upon onset of ischemia. Similar to previously reported results, treatment with irradiated PBMC cell suspensions yielded significantly reduced infarction sizes accompanied with improved remodelling post AMI. Furthermore, an increased homing of cells associated with a regenerative phenotype, including c-kit, IGF-1, FGF-2 and FLK-1 positive cells as well as macrophages, was observed. Ventricular remodelling appeared to be reduced due to a change in the ratio of collagenous and elastic fibres, favouring reduced scar formation.[406]

Interestingly, already in the first manuscript by Ankersmit et al, it was shown that the application of conditioned medium of irradiated PBMCs (PBMCsec) increased the production of VEGF and MMP9 in human primary keratinocytes and fibroblasts. This finding attributes anit-inflammatory and pro-angiogenic properties to PBMCsec for the first time and suggests a promising treatment option for AMI.[405]

Furthermore, this observation led to the development of a cell-derived yet cell-free therapeutic approach, in which the sole effects of paracrine factors, released by irradiated PBMCs (PBMCsec or APOSEC), were evaluated in an experimental AMI model in rats as well as in a porcine closed chest reperfused AMI model.[407] In both AMI models, a single intravenous dose of PBMCsec yielded a significant reduction of myocardial scar tissue. Furthermore, an improved cardiac output, higher ejection fraction and decreased infarct size was also observed in the porcine AMI model. *In vitro* experiments with primary human cardiomyocytes showed that treatment with PBMCsec prevented starvation-induced cell death. PBMCsec activated pro-survival signalling cascades, including c-Jun, CREB, Erk1/2 and AKT, while simultaneously increasing anti-apoptotic factors such as BAG1 and Bcl-2.[407] To further unravel the mode of action of PBMCsec during AMI, the effect of PBMCsec on platelets and microvascular obstruction (MVO) was assessed in a porcine closed chest reperfused AMI model.[408] The animals were treated with a single intravenous dose of PBMCsec 40 minutes post occlusion. Magnetic resonance imaging revealed significantly reduced areas of MVO in

PBMCsec treated animals compared to the control group. Furthermore, improved ventricle contraction capacity as well as ST segment resolution accompanied by reduced ventricular arrhythmias were observed in the PBMCsec treatment group. Platelet activation, representing a critical contributor to MVO, was observed to be significantly reduced, characterized by decreased levels of sCD40L, TSP-1, sP-selectin and PF-4. Additional *in vitro* experiments with isolated human and porcine platelets further corroborated the *in vivo* data, since despite strong activating stimuli, platelet aggregation was prevented by PBMCsec treatment. This mechanism was attributed to PBMCsec-induced upregulation of vasodilator-stimulated phosphoprotein (VASP).[408] Moreover, Pavo et al. showed an increased homing of $CD31^+$ cells at the border zone of infarcted area in a porcine closed chest AMI model, suggesting a PBMCsec-induced enhancement of angiogenesis, accompanied with increased accumulation of endogenous cardiac stem cells.[409] Gene expression patterns in infarcted, border zone and remote myocardium revealed strong downregulation of lipid metabolism-, inflammation- and apoptosis-associated genes such as caspase-1, stromal derived factor 2-like protein 1, TNF-α, arachidonate 15-lipoxygenase and claudin 3. In parallel, PBMCsec induced the upregulation of angiogenic growth factors such as insulin-like growth factor, as well as Kruppel-like factor which is known to function as a regulator of homeostasis, vascular tone and exert anti-atherogenic effects.[409] Attempting to unravel the underlying transcriptional changes and pharmacodynamics induced by PBMCsec treatment, transcriptomic analysis of the perfused heart, transition zone and non-perfused heart, as well as liver and spleen, revealed highly organ-specific alterations.[410] Overall, a uniform trend towards the downregulation of pro-inflammatory mediators was observed. PBMCsec induced strong expression of genes crucial for cardiomyocyte function in the infarcted area.[410] The immunomodulatory effects of PBMCsec were further addressed by Hötzenecker et al. in an experimental autoimmune myocarditis mouse model mimicking critical aspects of human inflammatory dilated cardiomyopathy.[411] Intraperitoneal injection of PBMCsec lead to almost complete inhibition of myocarditis characterized by sparse lymphocytic infiltration and absence of apoptotic or necrotic cardiomyocytes. Circulating levels of autoantibodies were partially reduced by PBMCsec treatment. Despite no statistical significance, a trend of reduced amounts of pro-inflammatory cytokines was observed in the PBMCsec group. One substantial finding of this study was that PBMCsec induces caspase-8-dependent apoptosis in $CD4^+$ T cells both *in vitro* and *in vivo* and lymphocytes derived from treated animals did not show a proliferative response upon stimulation.[411]

2.1.2 Neurology

Based on the early findings that PBMCsec ameliorates ischemia-induced tissue damage in AMI, Altmann et al. assessed the impact of rat- and human-PBMCsec treatment on ischemic lesion volumes in a rat middle cerebral artery occlusion model.[405-408,412] The right hemisphere was occluded and animals received either a single intraperitoneal dose of PBMCsec 40 minutes post-ischemia induction or an additional dose 24 hours post-surgery. Both treatment groups, allogenic PBMCsec and xenogenic PBMCsec, showed comparably reduced infarction volumes and improved neuroscores. Furthermore, cytoprotection mediating signalling cascades, such as Akt, c-Jun, Erk1/2 and CREB were activated in Schwann cells and astrocytes treated with PBMCsec *in vitro*. Administration of PBMCsec to cultured astrocytes and neurons lead to a dose dependent activation of CREB phosphorylation and increased length of newly formed neuronal sprouts.[412] In a second attempt, the effect of PBMCsec on spinal cord injury was investigated.[413] Neurological damage and deterioration is frequently induced upon spinal cord injury by an inflammatory response, ischemia and increased oxidative stress.[413] Administration of PBMCsec in a rat spinal cord injury model revealed substantially improved functional recovery and motor function. 28 days after trauma the PBMCsec-treated animals showed significantly smaller cavity formation marked by reduced areas of white matter. Furthermore, acute axonal injury was reduced and an increased vascular density was observed in the spinal cord. A reduction of oxidative stress was characterized by reduced expression levels of inducible nitric oxide synthase.[413]

2.1.3 Wound healing

Additionally, the beneficial effect of the secretome of non-stressed PBMCs was assessed in a full thickness wound mouse model.[414] Cell culture supernatants of freshly isolated PBMCs, cultured for 24 hours were applied to 6 mm punch biopsy wounds daily for three days and resulted in significantly elevated wound closure. Furthermore, treatment of primary human keratinocytes and fibroblasts induced migration but not proliferation *in vitro.* In line with previous findings, a tremendously enhanced infiltration of $CD31^+$ cells was observed, suggesting increased angiogenesis.[409,414] Application of the secretome of non-stressed PBMCs on endothelial cells further induced tube-formation and proliferation in an *in vitro* matrigel-based assay.[414] Hacker et al. further investigated the effect of the secretome of non-stressed PBMCs and PBMCsec in a full-thickness burn injury model in pigs.[415] Comparing the secretome of non-stressed and irradiated PBMCs during wound closure of burn wounds revealed a superiorly beneficial effect of PBMCsec in terms of wound healing. PBMCsec

treated wounds showed increased epidermal thickness, more advanced epidermal differentiation as well as increased numbers of rete ridges. Again, an enormously increased accumulation of $CD31^+$ cells was observed in PBMCsec treated wounds.[415] Furthermore, the beneficial effects of PBMCsec on wound healing were further assessed in a rodent epigastric flap model.[416] A single dose of PBMCsec in combination with fibrin sealant was administered intraoperatively immediately prior to wound closure and led to significantly reduced tissue necrosis rate compared to control or fibrin sealant only groups. While no difference was observed in the amount of lymphatic vessels, PBMCsec treatment resulted in a significant increase in von Willebrand Factor positive blood vessels 7 days postoperative. It was suggested that the beneficial effect of PBMCsec is exerted by reducing post-ischemic inflammation additionally to increased re-vascularization.[416] One critical factor during wound healing is a sufficient antimicrobial defence response. Robust antimicrobial activity against certain gram-negative and gram-positive bacteria, all known to be critically involved in the pathogenesis of diabetic foot ulcers, was observed.[417] The secretome of non-stressed PBMCs already displayed modest antimicrobial activity towards the gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* as well as gram-positive bacteria as for example *Staphylococcus aureus*, yet PBMCsec exhibited significantly stronger effects on these strains and inhibited their growth significantly.[417]

2.1.4 Components of the secretome

PBMCsec consists of a plethora of components including proteins, lipids, DNA, extracellular vesicles (EVs), which were attributed to exert regenerative effects.[418] Wagner et al. sought to dissect the molecular composition of EVs found in the secretome of non-stressed as well as irradiation-stressed PBMCs and their impact on wound healing.[418] Irradiation of PBMCs strikingly changed the composition, number and size of EVs and in contrast to non-irradiated PBMC secretome-derived EVs their molecular components exhibited strong association with regenerative processes. Irradiated PBMCs released higher rates of EVs containing a broad set of native as well as oxidized bioactive phospholipids. *In vitro* aortic ring sprouting assays as well as reporter gene assays revealed a reduced potency of individual subfractions of PBMCsec, including EVs, proteins and lipids, compared to the whole PBMCsec. Furthermore, PBMCsec was first applied in the context of diabetic wound healing in a full-thickness skin wound mouse model using $LepR^{db/db}$ diabetic mice. Topical administration of PBMCsec accelerated wound closure and a significantly reduced wound area was observed 25 days post wounding. Contrasting previous findings, enhanced angiogenesis marked by increased homing of $CD31^+$ cells was not observed.[418] Furthermore, it was observed that irradiation not

only changes the PBMC-derived EVs of the secretome but also induces changes in both miRNA and mRNA profiles. [419] A time-dependent alteration in miRNA and mRNA expression was reported. Comparing miRNA and mRNA expression data revealed a negative correlation between mRNA-miRNA and lead to the identification of a significantly downregulated transcription factor, the hepatic leukaemia factor, which together with a plethora of other regulated gene sets is critically involved in the modulation of irradiation-responsive pathways including endocytosis, MAPK signalling, cytokine-cytokine interactions and apoptosis.[419] More in depth analysis of the secretome further revealed that irradiation induces a tremendous alteration in gene expression patterns in PBMCs coding for secreted proteins.[420] Gene ontology assessment showed strong correlation of the altered genes with biological processes such as wound healing, leukocyte trafficking regulation and angiogenesis. Comparing PBMCsec with the secretome of non-stressed PBMCs further revealed drastically elevated levels of bioactive lipids, including triglycerides, cholesterol esters, free fatty acids, cholesterol, cholesterol sulfate and phospholipids. Particularly phospholipids appear to be highly susceptible to irradiation-induced changes, specifically those with intact but oxidized sn-2 chains.[420]

2.1.5 Irradiation induced apoptosis and necroptosis

PBMCsec represents a rather complex compound with several different cell types contributing to the resulting secretome. Simader et al. investigated whether certain subpopulations, including B cells, natural killer cells, $CD4^+$ and $CD8^+$ T cells as well as monocytes, account for the tissue-regenerative effect.[421] Individual isolated and purified cell populations were exposed to high-dose γ-irradiation, and subsequently their secretomes analysed using transcriptomics. It was demonstrated, that each cell type responds to γ-irradiation by a distinct cytokine production profile, death receptor signalling as well as different pro-angiogenic pathways.[421] Furthermore, it was observed that the beneficial effect by PBMCsec requires interactions of the individual subsets. Moreover, Simader et al. revealed that γ-irradiated PBMCs not only undergo apoptosis, but also necroptosis. In depth analysis showed that inhibition of apoptosis did not alter the pro-angiogenic effects of PBMCsec. However, inhibiting necroptosis abrogated the previously observed effect. TNF receptor superfamily member 1B was identified as the key molecule of necroptosis in PBMCs upon γ-irradiation.[421]

2.1.6 Immunomodulatory effects

In addition to improved wound healing, PBMCsec was found to reduce the inflammatory response and cellular infiltration in dendritic cell (DC)-mediated skin inflammation in mice.[422] PBMCsec prevented the differentiation of monocyte-derived DCs which was characterized by reduced expression of DC markers including MHC class II, CD11c and CD1a. DC maturation, lipopolysaccharide-induced cytokine secretion, DC-mediated immune cell proliferation as well as antigen uptake was significantly reduced upon treatment with PBMCsec. The presence of PBMCsec during monocyte-derived DC differentiation resulted in an impaired capability to prime naïve $CD4^+$ T cells into both T_{H1} and T_{H2} cells. *In situ* analysis of skin further revealed a modified DC phenotype. The key discovery was that these modifications appear to be based on PBMCsec-derived lipid-mediated immunomodulatory changes.[422] In another study, the immunomodulatory effect of PBMCsec on mast cell and basophil activation in the context of IgE-mediated hypersensitivity was investigated.[423] Experimentally induced mast cell degranulation in mouse ears was reduced by topical administration of PBMCsec. Several genes involved in Fc-receptor signalling as well as immune cell degranulation were significantly downregulated in murine mast cells. PBMCsec treatment of activated primary human dermal mast cells robustly inhibited α-IgE- and compound 48/80-induced mediator release *in vitro*. In addition to the suppression of mast cell degranulation, allergen driven activation of basophils derived from allergic donors was attenuated by PBMCsec treatment *in vitro*. Transcriptomic analysis of PBMCsec-treated basophils revealed a similar pattern including strong downregulation of gene sets relevant for Fc-receptor signalling and immune cell degranulation. In this study, lipids were found to be the major contributors of the observed immunomodulatory effects.[423]

Taken together, PBMCsec has been attributed a diverse set of mode of actions positively influencing various pathologies such as AMI, wound healing and inflammatory responses (Figure 4).

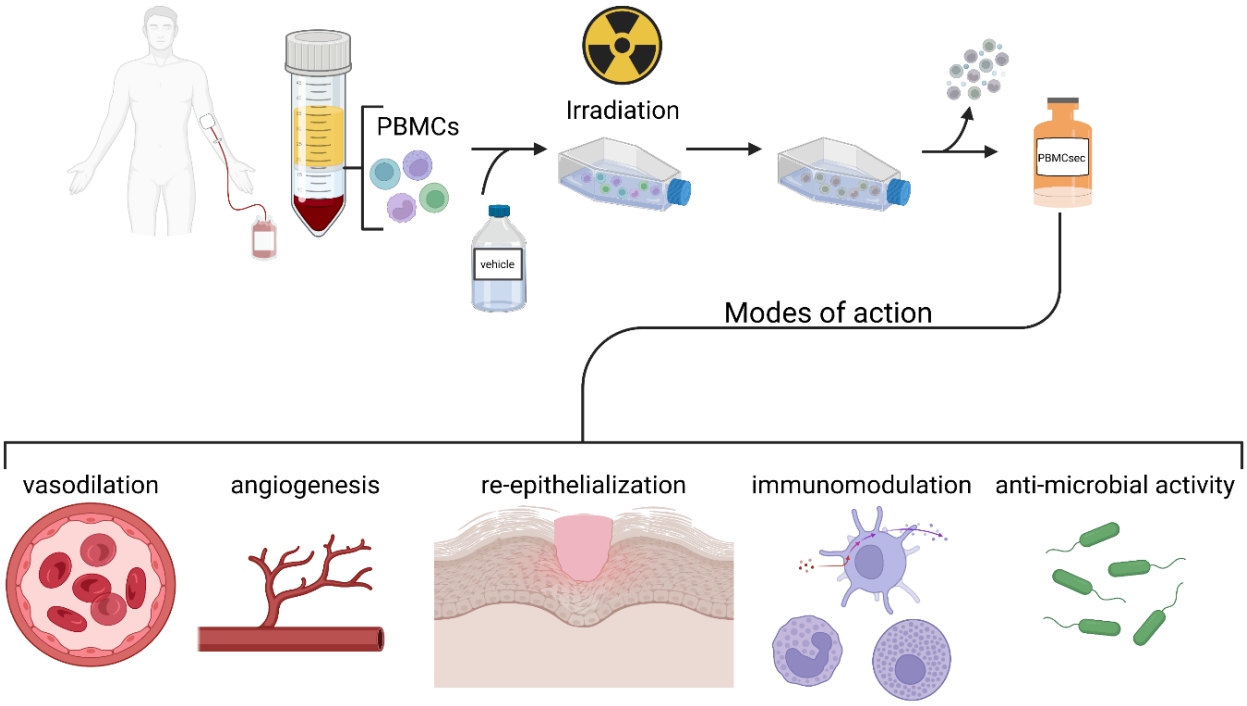


Figure 4 Modes of action of PBMCsec.
During the cultivation of irradiated PBMCs a plethora of paracrine factors is secreted. These factors have been attributed several modes of action with overall cytoprotective effects. The so far investigated modes of action include vasodilation, angiogenesis, re-epithelialization, immunomodulation of various cell types as well as anti-microbial activity. This figure was created with BioRender.com

2.1.7 Clinical trial

The first in-human application of PBMCsec was performed in the MARSYAS I trial (therein referred as APOSEC), a randomized, single-centre, placebo-controlled, double-blinded phase 1 trial. Healthy male volunteers received two 4 mm punch biopsy wounds on their upper arm and were treated with both placebo and autologous PBMCsec in NuGel for 7 consecutive days. The participants were randomly assigned into either a high dose or low dose group. The primary interest was focused on investigating the tolerability of PBMCsec followed by the impact on wound closure and re-epithelization. Recorded adverse events were all characterized as mild and unlikely to be related to the treatment. The main limitation of the study was the short intervention time which restricted the assessment of wound closure which revealed no significant differences.[424]

3 Aims of this thesis

Dysregulated NET formation is known to critically contribute to host damage in infectious conditions and (sterile) inflammation. The progression of several pathologies is closely associated with increased NET formation.[157]

Previous studies analysing the effect of PBMCsec in a diverse set of pathological conditions already provides deeper insights into potential mode of actions. Particularly the immunomodulatory effect is of high interest.[418,420,422,423]

However, the effect of PBMCsec on neutrophils in the context of NET formation has not been investigated so far.

The main aim of this thesis was to evaluate to what extent NET formation is influenced by PBMCsec treatment.

Furthermore, we aimed to unravel potential deviations in the potency of the PBMCsec subfractions to influence NET formation.

Finally, we sought to identify the mode of action by which PBMCsec influences NET formation.

RESULTS

1. Prologue

It was previously shown that PBMCsec exerts various anti-inflammatory, cytoprotective, immunomodulatory and pro-angiogenic effects in a diverse set of pathologies.[399] However, the influence of PBMCsec on neutrophils, particularly the formation of NETs, has not been elucidated yet, despite prominent involvement of neutrophils in the vast majority of investigated pathologies.[157]

The main objective of this thesis was to evaluate whether PBMCsec harbours any inhibitory capacity towards NET formation. Furthermore, we sought to investigate if certain substance classes present in PBMCsec are responsible for these effects and pursued to analyse whether molecular mechanisms critical for NET formation may be impaired.

1.1 Paper

antioxidants

MDPI

Article

The Effect of Paracrine Factors Released by Irradiated Peripheral Blood Mononuclear Cells on Neutrophil Extracellular Trap Formation

Katharina Klas [1,2], Anna S. Ondracek [3], Thomas M. Hofbauer [3], Andreas Mangold [3], Karin Pfisterer [4], Maria Laggner [1,2], Dragan Copic [1,2], Martin Direder [1,2], Daniel Bormann [1,2], Hendrik Jan Ankersmit [1,2,*,†] and Michael Mildner [4,*,†]

[1] Laboratory for Cardiac and Thoracic Diagnosis, Regeneration and Applied Immunology, Department of Thoracic Surgery, Medical University of Vienna, 1090 Vienna, Austria
[2] Aposcience AG, 1200 Vienna, Austria
[3] Division of Cardiology, Department of Internal Medicine II, Vienna General Hospital, Medical University of Vienna, 1090 Vienna, Austria
[4] Department of Dermatology, Medical University of Vienna, 1090 Vienna, Austria
* Correspondence: hendrik.ankersmit@meduniwien.ac.at (H.J.A.); michael.mildner@meduniwien.ac.at (M.M.)
† These authors contributed equally to this work.

Abstract: Neutrophil extracellular trap (NET)-formation represents an important defence mechanism for the rapid clearance of infections. However, exaggerated NET formation has been shown to negatively affect tissue-regeneration after injury. As our previous studies revealed the strong tissue-protective and regenerative properties of the secretome of stressed peripheral blood mononuclear cells (PBMCsec), we here investigated the influence of PBMCsec on the formation of NETs. The effect of PBMCsec on NET formation was assessed ex vivo in ionomycin stimulated neutrophils derived from healthy donors using flow cytometry, image stream analysis, and quantification of released extracellular DNA. The effect of PBMCsec on molecular mechanisms involved in NET formation, including Ca-flux, protein kinase C activity, reactive oxygen species production, and protein arginine deiminase 4 activity, were analysed. Our results showed that PBMCsec significantly inhibited NET formation. Investigation of the different biological substance classes found in PBMCsec revealed only a partial reduction in NET formation, suggesting a synergistic effect. Mechanistically, PBMCsec treatment did not interfere with calcium signalling and PKC-activation, but exerted anti-oxidant activity, as evidenced by reduced levels of reactive oxygen species and upregulation of heme oxygenase 1 and hypoxia inducible-factor 1 in PBMCsec-treated neutrophils. In addition, PBMCsec strongly inhibited the activation of protein arginine deiminase 4 (PAD4), ultimately leading to the inhibition of NET formation. As therapeutics antagonizing excessive NET formation are not currently available, our study provides a promising novel treatment option for a variety of conditions resulting from exaggerated NET formation.

Keywords: neutrophil; neutrophil extracellular traps (NETs); PAD4; ROS; secretome; peripheral blood mononuclear cell secretome

check for updates

Citation: Klas, K.; Ondracek, A.S.; Hofbauer, T.M.; Mangold, A.; Pfisterer, K.; Laggner, M.; Copic, D.; Direder, M.; Bormann, D.; Ankersmit, H.J.; et al. The Effect of Paracrine Factors Released by Irradiated Peripheral Blood Mononuclear Cells on Neutrophil Extracellular Trap Formation. *Antioxidants* **2022**, *11*, 1559. https://doi.org/10.3390/antiox11081559

Academic Editors: Mercedes Camacho Pérez de Madrid and Josep Julve

Received: 29 July 2022
Accepted: 10 August 2022
Published: 11 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Neutrophil granulocytes represent the main population of circulating leukocytes in the blood [1]. They exert a plethora of functions critical for maintaining immune homeostasis, and their contribution to immune regulatory mechanisms is of vital importance during infectious conditions [2]. Being amongst the first cell populations to be recruited to a site of infection, they use a broad machinery of defence mechanisms, including the production of reactive oxygen species (ROS), excretion of cytotoxic granules, phagocytosis of pathogens, and the formation of neutrophil extracellular traps (NETs), to fight invading

pathogens [3]. In addition to these functions, NET formation represents another potent defence mechanism for the elimination of pathogens [4]. Neutrophils are equipped with a vast array of surface receptors, including Toll-like and NOD-like receptors, G-protein coupled receptors, cytokine receptors, as well as Fc and complement receptors, rendering them highly responsive to a multitude of stimuli [5]. Some of these stimuli, such as ROS or bacterial toxins, are potent inducers of NETs and operate independently of the classical neutrophil activation pathways via surface receptors [6–8]. After induction of NET formation, intracellular Ca^{2+} levels increase due to influx and its release from the endoplasmic reticulum, promoting protein kinase C (PKC) activation and phosphorylation of $Gp91^{phox}$ [9]. Activation of PKC, in turn, leads to the assembly of functional NADPH oxidase, which generates reactive oxygen species (ROS) [10]. Furthermore, increased Ca^{2+} levels activate protein arginase deiminase 4 (PAD4) [11], which promotes chromatin decondensation by converting arginine residues of core histones H3 and H4 into citrulline [8]. In addition, ROS also lead to the gradual disassembly of the nuclear membrane, followed by the dispersion of chromatin throughout the cytoplasm, where it is decorated with granular and cytoplasmic contents [12]. Ultimately, chromatin, DNA, granular, and cytoplasmic contents are released into the extracellular space as NETs [6,13].

Neutrophil functions, specifically the extrusion of NETs, are considered beneficial during infection [14]. However, dysregulated or extensive NET formation may result in undesirable tissue damage [15,16] and is linked to many inflammatory disorders, including sepsis, asthma, lupus, rheumatologic diseases, as well as diabetes [17]. Additionally, neutrophils receive increasing interest in cancer research as potential drivers of metastasis [18]. Furthermore, NETs are discussed as potential inducers of endothelial tissue damage, leading to various forms of vasculitis [19]. The accumulation of neutrophils, as well as the entailing activation and NET formation, at the culprit site lesion during acute coronary syndrome or acute myocardial infarction, is associated with poor disease prognosis and an increased long-term mortality rate [20–22]. In addition to systemic disorders, excessive NET formation is also associated with locally impaired or prolonged tissue regeneration, due to increased neutrophil-derived ROS in the microenvironment of the injury [16,23–26].

Recent advances in cell-derived, yet cell-free medicinal products have increasingly gained attention in regenerative medicine [27,28]. Although initial research on cell-free therapeutic agents focused on secretomes derived from stem cells, we could demonstrate that the secretome of peripheral blood mononuclear cells (PBMC) exhibits comparable regenerative effects [29–35]. The potency of the PBMC-derived secretome (PBMCsec) was further increased by exposing PBMCs to 60 Gy γ-irradiation, which induces apoptosis and necroptosis, resulting in the release of a plethora of pro-regenerative paracrine factors [29]. Lichtenauer et al. showed strong regenerative potential of PBMCsec in rodent and porcine models of acute myocardial infarction [35]. These pioneering findings laid the foundation for further studies, which identified a broad spectrum of therapeutic implications for PBMCsec in a vast variety of pathologic conditions, including chronic heart failure after myocardial infarction [36], cerebral ischemia [35], burn injury [35], diabetic wound healing [30], and acute spinal cord injury [35]. Furthermore, strong anti-inflammatory properties of PBMCsec have been demonstrated in the context of myocarditis [35], as well as inflammatory skin conditions [34]. The observed tissue-regenerative effect of PBMCsec is based on a complex interplay of various biologically active agents produced and released by stressed PBMCs [30,32,35]. The broad action spectrum of PBMCsec has been intensively investigated and revealed promising treatment opportunities, where anti-inflammatory [37,38], anti-microbial [33], tissue-regenerative [32], pro-angiogenic [29,30], and vasodilatory [35] properties are important.

Although PBMCsec possesses compelling immunomodulatory effects [35], potential anti-inflammatory and stabilizing effects on (activated) neutrophils have not been investigated so far. Hence, we sought to investigate the effect of PBMCsec on NET formation.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki and applicable local regulations. Use of human neutrophils was approved by the institutional ethical review board of the Medical University of Vienna (Vienna, Austria) (protocol code 1539/2017). Written informed consent was obtained from all donors.

2.2. Generation of PBMCsec

PBMCsec was produced in compliance with good manufacturing practice by the Austrian Red Cross, Blood Transfusion Service for Upper Austria (Linz, Austria) as previously described [30,34]. Briefly, the PBMCs were enriched using Ficoll-Paque PLUS (GE Healthcare, Chicago IL, USA) density gradient centrifugation. Cell suspensions were adjusted to 2.5×10^7 cells/mL and exposed to 60 Gy γ-irradiation (IBL 437, Isotopen Diagnostik CIS GmbH, Dreieich, Germany). Subsequently, cells were cultured in phenol red-free CellGenix GMP DC medium (CellGenix, Freiburg, Germany) for 24 h. Cells, as well as cellular debris, were removed by centrifugation. The conditioned supernatants containing the secretome were filtered through 0.22 µm filters followed by viral clearance using Theraflex methylene blue technology (MacoPharma, Mouvaux, France). The secretomes were lyophilized and sterilized by high-dose γ-irradiation (25,000 Gy, Gammatron 1500, Mediscan, Seibersdorf, Austria). CellGenix GMP DC medium without cells was used as vehicle control. The GMP batches A000918399131, A00918399136, and A000918399132 were used in this study. The stock concentration of one vial lyophilized secretome equals to 25 units/mL.

2.3. Fractionating PBMCsec

The lipid fraction was purified according to Folch et al. (PMID: 13428781) with minor modifications. Briefly, one part reconstituted PBMCsec was mixed with 9 parts 2:1 (*vol*/*vol*) chloroform–methanol and, subsequently, excessively vortexed. Then, 0.7 M of formic acid was used to acidify the emulsion (a one-fourth volume of the chloroform–methanol mix) and homogenized by thorough shaking. Phase separation was obtained by leaving the samples on ice for 30 min. The lower, organic phase was further applied to rotary vacuum evaporation (475 mbar, 100 rpm, 60 °C water bath temperature) in order to eliminate solvents. The protein fraction was isolated by combining four times the volume of ice-cold acetone (VWR Chemicals, PA, USA) to one volume of reconstituted PBMCsec, followed by thorough vortexing and incubation at −20 °C for 60 min, to obtain a protein precipitate. After centrifuging the sample at 18,000× *g* for 10 min, ice-cold acetone was added to the protein pellet, briefly vortexed and centrifuged at 18,000× *g* for 10 min. Acetone was discarded and remaining acetone was allowed to evaporate at room temperature. Finally, the protein pellet was resuspended in 0.9% NaCl in the initially used volume. DNA was isolated by adding equal amounts of isopropanol (Merck Millipore, MA, USA) as PBMCsec and 1/10 volume of 7.5 M sodium acetate (Merck Millipore) and incubated at −20 °C for 1 h. After centrifugation for 5 min at 18 000× *g* the DNA pellet was washed twice with 1 mL 70% Ethanol (Merck Millipore) followed centrifugation at 18,000× *g* for 5 min. The DNA pellet was allowed to dry for 10 min at room temperature prior to resuspension in double distilled, nuclease-free H_2O. Extracellular vesicles were obtained by ultracentrifugation at 110,000× *g* for 2 h at 4 °C, as previously described [30]. To ensure comparability, all fractions were used in the same concentrations as are present in PBMCsec. All fractions were tested separately and in a combined form. To reconstitute the fractions, equal volumes of each fraction were combined and further diluted to the equivalent concentration of PBMCsec.

2.4. Neutrophil Isolation

Neutrophils were isolated using the MACSxpress Whole Blood Neutrophil Isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to manufacturer's instructions. In brief, magnetic beads were resuspended in 2 mL of buffer A. One-fourth of the total amount of processed blood of magnetic beads and buffer B were added to the blood

and incubated at room temperature for 5 min under constant gentle rotation. Blood and isolation cocktail mix were placed in the MACSxpress Separator (Miltenyi Biotec) and allowed to separate for 15 min. Clear, neutrophil-containing top phase was transferred into a fresh tube and washed with basal RPMI 1640 without phenol red (Thermo Fisher Scientific, Waltham, USA). If required, a red blood cell lysis was performed using a Red Blood Cell Lysis Buffer (Abcam, Cambridge, UK) for 10 to 15 min at room temperature. Neutrophils were resuspended in basal RPMI 1640 in an assay dependent concentration without phenol red for further use.

2.5. Induction of NET Formation

Either isolated neutrophils or whole blood samples after red blood cell lysis were pre-treated with 2 units/mL PBMCsec or equivalent vehicle medium for 20 min at 37 °C. Cells were then stimulated with 5 µM ionomycin (Sigma Aldrich, St. Louis, MO, USA) or 100 nm phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich), or 10 µM Thapsigargin (Abcam) for 2 h at 37 °C unless indicated otherwise.

2.6. Flow Cytometry

After stimulation with indicated compounds, cells were centrifuged and stained with anti-citrullinated Histone H3 antibody (ab5103, Abcam) to detect NETs, anti-CD66b antibody (pacific-blue conjugated mouse anti-human, clone G10F5, BioLegend, San Diego, CA, USA) and anti-CD15 antibody (4hycoerythrin-cyanine 7 conjugated mouse anti-human, clone W6D3, BioLegend) to identify neutrophils. Flow cytometric analysis was performed using BD FACSCanto II and BD FACSDiva software (version 6.1.3) (BD Pharmingen, San Jose, CA, USA).

2.7. Cell Viability Assay

Incucyte Cytotox Dye for Counting Dead Cells (Sartorius, Goettingen, Germany) was used according to the manufacturer's instructions. In brief, cells were treated as indicated, followed by the addition of 250 nm cytotox green dye for staining 100 µL cell suspension in a concentration of 4×10^6 cells/mL condition in a 96-well plate. Cell death was assessed over the indicated time periods in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Microplate reader (BMG Labtech, FLUOstar OPTIMA) and the BMG Labtech Optima software (software version 2.20Rs, BMG Labtech, Ortenberg, Germany).

2.8. EZ4U Cell Proliferation and Cytotoxicity Assay

EZ4U (Biomedica, Vienna, Austria) assay was performed according to manufacturer's instructions. Briefly, the substrate was dissolved in 2.5 mL activator solution and pre-warmed to 37 °C. Then, a 20 µL substrate was added to 200 µL cell suspension at a concentration of 4×10^6 cells per condition in a 96-well plate and incubated for 2 h. Continuous absorbance measurements at 450 nm were performed using a microplate reader (BMG Labtech, FLUOstar OPTIMA) and the BMG Labtech Optima software (software version 2.20Rs).

2.9. ROS Production Measurement

ROS production was measured using the DCFD/H2DCFDA cellular ROS assay kit (Abcam). Cells were treated as indicated and the assay was performed as recommended by the manufacturer.

2.10. Ca^{2+} Flux Measurement

Ratiometric calcium flux measurements with Fura Red were performed as described by Wendt et al., with minor modifications [39]. In brief, a cell suspension of 4×10^6 cells per condition, either isolated neutrophils or whole blood, pre-treated with PBMCsec or vehicle for 20 min as indicated, were washed, resuspended in 400 µL full medium containing 1 µM

Fura Red (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and incubated for 30 min at 37 °C. Cells were washed once with medium, resuspended in 4 mL medium and incubated for another 30 min at 37 °C. Subsequently, cells were rested on ice for up to 30 min. Data were acquired on a FACSAria III flow cytometer (BD Bioscience, San Jose, CA, USA). Before intracellular calcium flux measurement, 1 mL of Fura Red-loaded cells was transferred to a FACS tube and pre-warmed for 5 min at 37 °C in a water bath. The cells were kept at 37 °C during the whole measurement. The baseline response was recorded for 30 s prior to stimulation with 5 µM ionomycin. Changes in calcium mobilization were recorded for a total of 120 s. Fura Red was excited using a 405 nm violet laser and a 561 nm green laser and changes in emission were detected with a 635 LP, 660/20 BP, and a 655 LP, 795/40 BP filter set, respectively. The 'Fura Red Ratio' over time was calculated using the Kinetics tool in FlowJo software (version 9.3.3, Tree Star Inc., Ashland, OR, USA) as follows:

$$Fura\ Red\ Ratio = \frac{increase\ of\ 405\ \text{nm}\ induced\ emission}{decrease\ of\ 561\ \text{nm}\ induced\ emission}$$

2.11. DNase Activity Measurement

DNase activity was measured by incubating 0.25 µg/µL Lambda DNA with 0.5 M acetate/NaOH at pH 4.8 (Merck Millipore), 50 mM $CaCl_2$(Merck Millipore), 50 mM $MgCl_2$ (Sigma-Aldrich, St. Louis, MO, USA), 40 mM 2-mercaptoethanol (Merck Millipore) and either 2 units/mL PBMCsec or equivalent vehicle control or DNase I (Thermo Fisher Scientific) as positive control for 1 h at 37 °C. 10 µL of each sample were loaded into a 1% agarose gel with gel red (Biotium, Fremont, CA, USA) together with 2 µL 6x loading dye (Thermo Fisher Scientific). Electrophoresis was performed at 200 V for 35 min. DNase activity was determined by absence of λ-DNA.

2.12. Proteome Profiler

The Human Apoptosis Array kit (R&D Systems, Minneapolis, MN, USA) was used in accordance with the manufacturer's instructions with no modifications. Isolated neutrophils were treated as indicated and cell lysates of 4×10^6 cells per condition of 4 individual donors were pooled.

2.13. PAD4 Inhibitor Assay

The inhibitory capacity of PBMCsec was measured using the PAD4 inhibitor Screening Assay kit (ammonia, Cayman Chemical, Ann Arbor, MI, USA) and performed according to manufacturer's instructions.

2.14. Western Blot Analysis

For Western blot analysis, cells were lysed in 1x Laemmli sample Buffer (Bio-Rad Laboratories, CA, USA) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich), After sonication SDS-PAGE electrophorese was performed on 4–20% gradient gels (Criterion TGX Precast Gels, Bio-Rad Laboratories). Proteins were electrotransferred onto 0.2 µM nitrocellulose membranes (Trans-Blot Turbo, Bio-Rad Laboratories) and immunodetected using primary antibodies against pan phospho-PKC (βII Ser660) antibody (#9371, Cell Signaling Technology, Danvers, MA, USA). This antibody detects endogenous PKC α, β I, β II, δ, ε, η and θ isoforms phosphorylated at carboxy-terminal residue homologous to serine 660 of PKC βII. Peroxidase-conjugated secondary antibody were detected with the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, MA, USA), according to manufacturer's instructions. A Ponceau S solution (Sigma-Aldrich) staining served as equal loading control.

2.15. Statistics

Statistical analyses were performed using Prism 8.0.1 (Graph Pad Prism). Data were shown ± standard deviation (SD). One-way ANOVA and Sidak's multiple comparisons test were performed and * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$.

3. Results

3.1. PBMCsec Inhibits NET Formation

Although immunomodulatory properties of PBMCsec have been well described in a variety of different cell types [30,34,40], a potential effect of PBMCsec on neutrophils has not yet been explored. To investigate whether PBMCsec interferes with experimentally induced NET formation, we pre-incubated human whole blood with 2 units/mL PBMCsec or an equivalent dose of vehicle prior to stimulation with 5 µM ionomycin. Unstimulated or PBMCsec-treated samples showed few citH3-positive neutrophils, identified by $CD15^+CD66b^+citH3^+$ staining (Figure 1A, upper panel and Figure 1B, 2.4 ± 0.97% and 1.6 ± 1.22% positive cells, respectively, Figure S1A). The addition of ionomycin strongly induced NET formation, as demonstrated by a significant increase in citH3-positive cells (54.52 ± 14.41%) after two hours of incubation (Figure 1A, bottom panel and Figure 1B). This effect was almost completely abolished by pre-incubation with PBMCsec before ionomycin treatment (3.9 ± 1.28% citH3-positive neutrophils). A dose titration of PBMCsec revealed that 2 U/mL was the lowest dose with NET inhibiting activity (Figure S1C). By contrast, vehicle treatment showed only a weak reduction in NET-formation (27.94 ± 17.71% citH3-positive neutrophils) (Figure 1A, bottom panel, and Figure 1B). As DNA is one of the major constituents of NETs [4], we analysed the amount of extracellular DNA in ionomycin-stimulated samples using cytotox green staining (Figure 1C). After two hours, only a weak cytotox green signal was detected in untreated, PBMCsec, or vehicle treated samples. Although stimulation with ionomycin resulted in a drastic increase in extracellular DNA (Figure 1C), the addition of PBMCsec almost completely inhibited the release of DNA after ionomycin stimulation. To exclude the possibility that the observed effect was due to a direct inhibitory effect of PBMCsec on ionomycin, we also used PMA (100 nM), another well-described inducer of NET formation. As shown in Figure 1D, PMA treatment of PBMCsec pre-incubated neutrophils led to a comparable inhibition of DNA release. Additionally, we evaluated the metabolic activity of ionomycin-activated neutrophils [41]. Ionomycin-activation resulted in a prominent decrease in metabolic activity which, in contrast to vehicle treatment, was completely abolished by the addition of PBMCsec (Figure S1B). Immunostaining of neutrophils for citH3 showed classical NET-structures after ionomycin treatment, which were completely absent in the presence of PBMCsec (Figure 1E). Taken together, these findings indicate that treatment of experimentally activated neutrophils with PBMCsec significantly reduces the formation of NETs.

3.2. A Synergistic Effect of Different Substance Classes Inhibits NET Formation

PBMCsec is composed of different substance classes, including free DNA, lipids, proteins, and extracellular vesicles [34,35,38,42] (Figure 2A). Thus, we further aimed to investigate to what extent the individual fractions contribute to the inhibitory effects on NET formation (Figure 2B,C). Therefore, PBMCsec and its fractions were added to whole blood prior to ionomycin stimulation and citH3 levels were analysed (Figures 2B,C and S2). Ionomycin treatment showed a significant increase in $citH3^+$ neutrophils, which was almost completely abolished by the addition of PBMCsec. In contrast, purified fractions showed only partial inhibition of ionomycin-induced histone citrullination, indicating that the inhibitory effect of PBMCsec requires the complex interplay of all fractions of the secretome. Stimulation with the reconstituted fractions of PBMCsec fully restores the inhibitory activity of NET formation (Figure 2B,C).

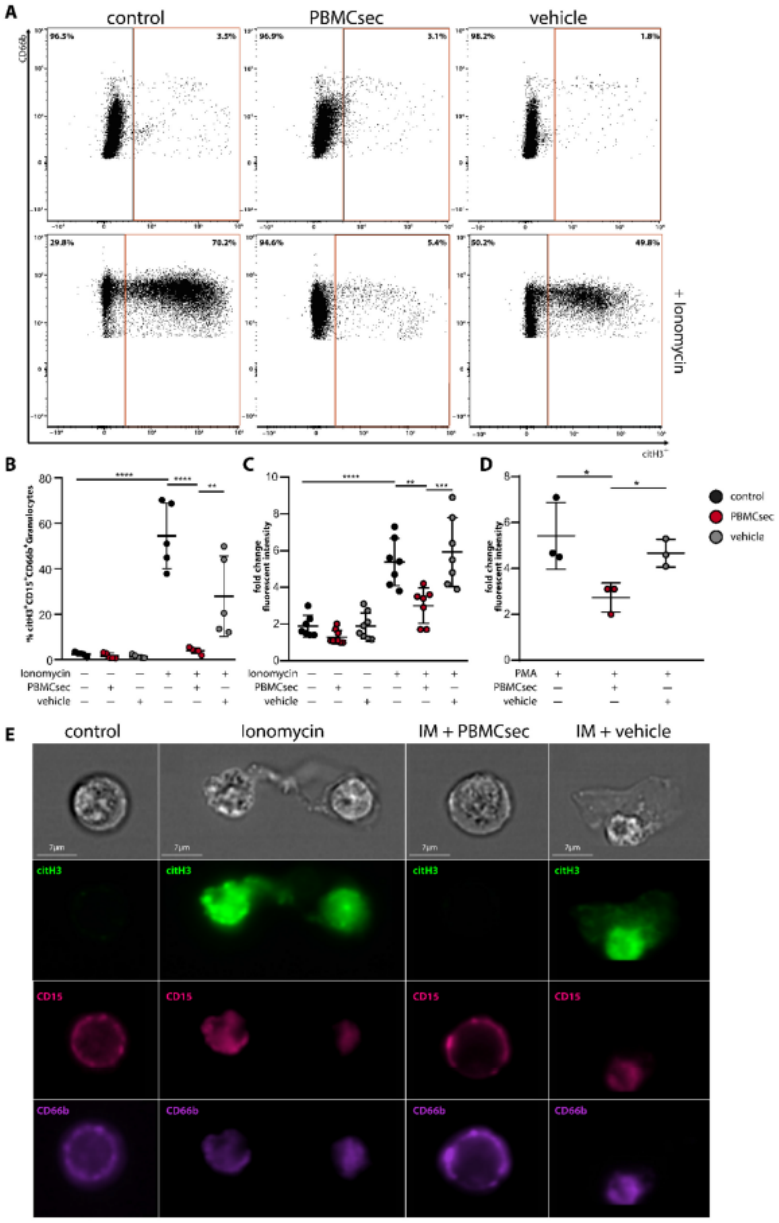


Figure 1. PBMCsec inhibits NET formation. Erythrocyte-lysed blood was treated with PBMCsec or vehicle for 20 min and subsequently stimulated with ionomycin (IM) for 2 h and analysed with flow cytometry, cytotox staining, and image stream analysis. (**A**) Neutrophils were identified in flow cytometry as $CD66b^{+}CD15^{+}$ cells and NET-forming neutrophils were characterized by citH3. Control, PBMCsec, or vehicle treated samples are shown in the top panel and ionomycin-activated neutrophils are shown in the bottom panel. $n = 5$. One representative sample is shown out of five

replicates summarized in (**B**). (**C**) Extracellular DNA content was measured using cytotox staining of neutrophils after pre-treatment with PBMCsec or vehicle and subsequent activation for 2 h with (**C**) IM or (**D**) PMA. Fold change increase in relative fluorescent intensity is shown after two hours of stimulation relative to time point zero/start of stimulation/minute one after induction of NETs. (**E**) Visualization of IM-activated neutrophils was performed using image stream analyses. Untreated (control) and IM-PBMCsec treated neutrophils did not show citH3$^+$ staining. IM and IM-vehicle treated neutrophils showed robust citH3$^+$ staining of cells and additional extracellular structures (indicative for NETs). Green, citH3; magenta, CD15; purple, CD66b; n = 2. One representative sample is shown. Data are represented as individual values with mean and error bars indicate SD, one-way ANOVA and Sidak's multiple comparisons test. * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$.

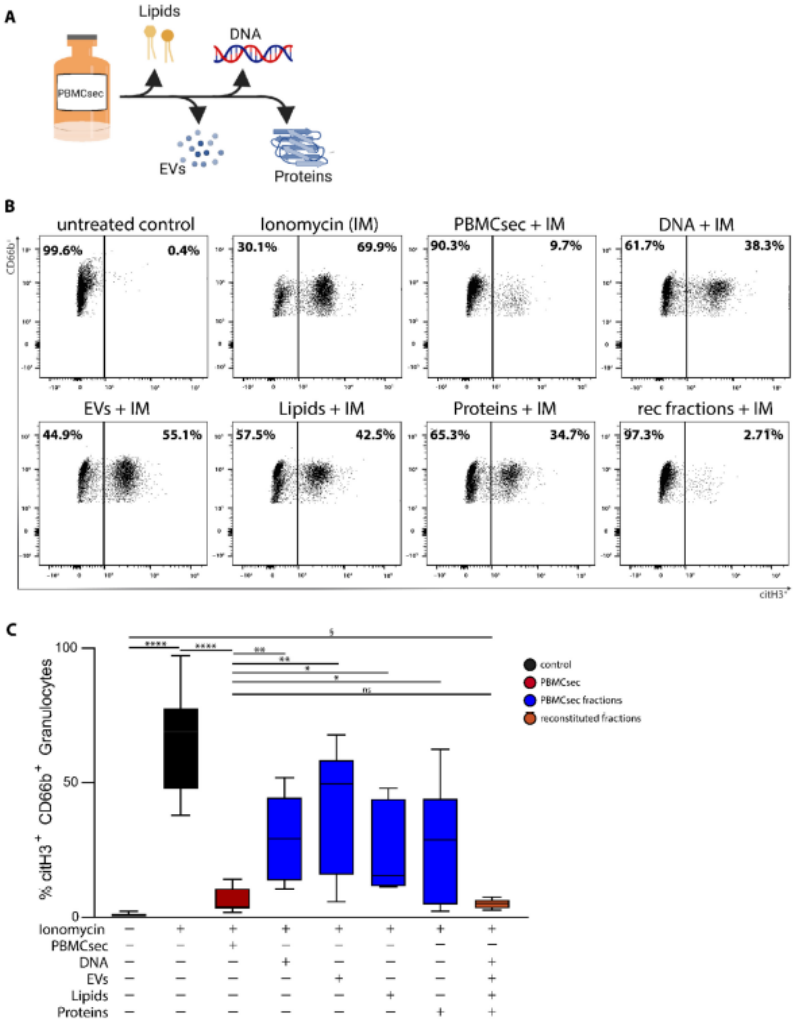


Figure 2. Isolated substance classes of PBMCsec show a synergistic effect on NET-inhibition. (**A**) Schematic depiction of the isolated and tested substance classes of PBMCsec. This scheme was

created with BioRender.com, accessed on 2 June 2022 (**B**) Neutrophils were identified in flow cytometry as $CD66b^{+}CD15^{+}$ cells and NET formation was characterized by citH3+ signal. rec fractions + IM = reconstituted fractions + ionomycin. n = 6. One representative sample out of six is shown, summarized in (**C**) one-way ANOVA and Sidak's multiple comparisons test. Data are represented as mean and error bars indicate SD. § = ANOVA without multiple comparison tests, $p =< 0.0001$; * $p < 0.0332$, ** $p < 0.0021$, **** $p < 0.0001$.

3.3. PBMCsec Does Not Show DNase-Activity

As digestion of NETs by DNAses is the main NETs-clearing mechanism [43], we next investigated whether PBMCsec displays DNAse activity. Therefore, we incubated λDNA with PBMCsec and analysed DNA degradation (Figure 3A). Compared to recombinant DNAse I, which completely digested λDNA, PBMCsec showed no DNA degrading activity (Figure 3A). Since this in vitro assay was optimized for DNAse I only, we further tested potential NETs-degrading properties of PBMCsec in whole blood ex vivo. For this purpose, we stimulated whole blood with ionomycin and applied PBMCsec either prior to or two hours after ionomycin treatment (Figure 3B). In contrast to neutrophils treated with PBMCsec prior to their activation, treatment two hours after induction of NET formation did not reduce the amount of citH3 positive neutrophils (Figure 3C,D and Figure S3). These data demonstrate that PBMCsec does not degrade pre-formed NETs by DNases, suggesting an active intervention in the NET-forming signalling cascade.

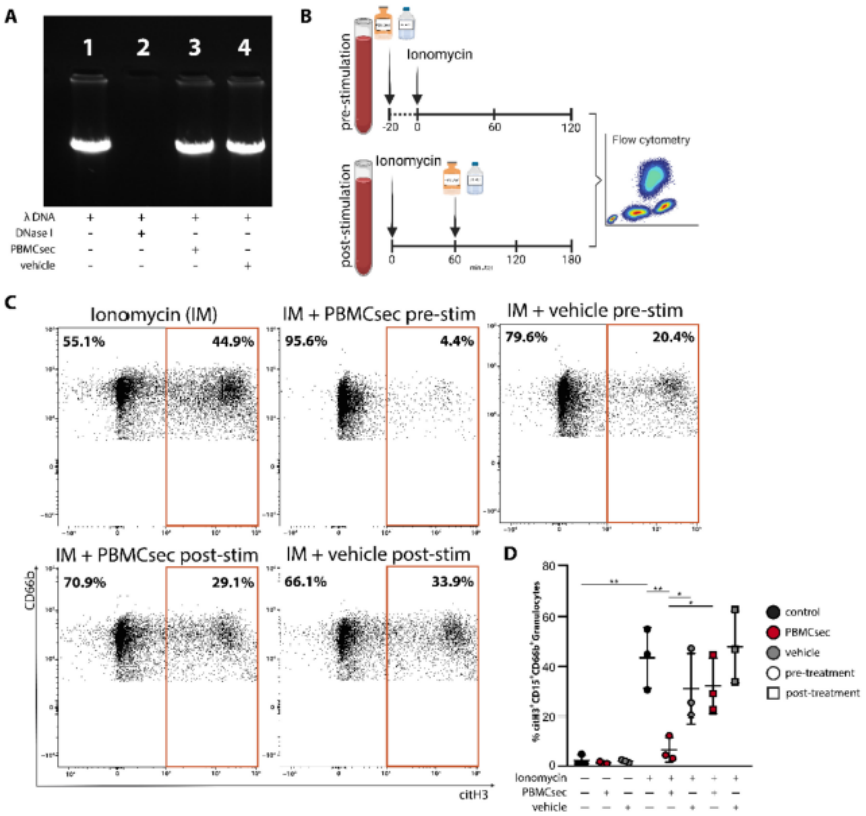


Figure 3. PBMCsec inhibits NET formation by a DNase-independent mode of action. (**A**) DNase

activity was measured in a cell-free assay by co-incubation of PBMCsec or vehicle with λ-DNA. DNase I was used as positive control. $n = 3$, one representative sample is shown. (**B**) Schematic depiction of the adapted neutrophil stimulation protocol for the measurement of potential DNase activity in a cell-based assay. This scheme was created with BioRender.com, accessed on 2 June 2022 (**C**) Neutrophils were identified as $CD66b^+CD15^+$ cells and citH3+ signal was used to characterize NET formation. $n = 3$, one representative experiment is shown. (**D**) Statistical summary of all biological donors is shown. Data are represented as individual values with mean and error bars indicate SD. One-way ANOVA and Sidak's multiple comparisons test. * $p < 0.0332$, ** $p < 0.0021$.

3.4. PBMCsec Inhibits NET Formation by Preventing ROS Production and PAD4 Activity

Induction of NETs requires an increase in intracellular calcium levels [8,9,11]. We, therefore, first investigated whether PBMCsec interferes with ionomycin-induced calcium influx. Analysis of intracellular calcium signalling, using a Fura Red based flow cytometry approach, revealed that pre-treatment of whole blood with PBMCsec only marginally reduced calcium influx after addition of ionomycin (Figure 4A,B). The decline in calcium flux was only transient and returned to control values rapidly. No significant difference was observed between PBMCsec and vehicle treatment, suggesting that the observed decrease in the calcium influx is not sufficient to affect NET formation. In addition, we also tested Thapsigargin, an irreversible inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, which prevents the storage of excess intracellular calcium in the endoplasmic reticulum [44]. The addition of 10 µM Thapsigargin induced NET formation comparably to ionomycin (Figure S4). Furthermore, PBMCsec was able to counteract Thapsigargin-induced NET formation (Figure S4) very efficiently. Together, these findings indicate that PBMCsec does not interfere with calcium flux in neutrophils and suggest that its NET-inhibiting action is mediated downstream of calcium flux. As increased intracellular calcium concentrations promote the activity of PKC [9], we next investigated PKC phosphorylation by Western blot analysis, using a pan phosphor-PKC antibody. As this antibody detects several phosphorylated isoforms of PKC, an assignment to a specific isoform was not possible. However, we were not able to detect differences in the amount of phosphorylated PKC after pre-treating neutrophils with PBMCsec or vehicle (data not shown), suggesting that the inhibitory action of PBMCsec is also downstream of PKC.

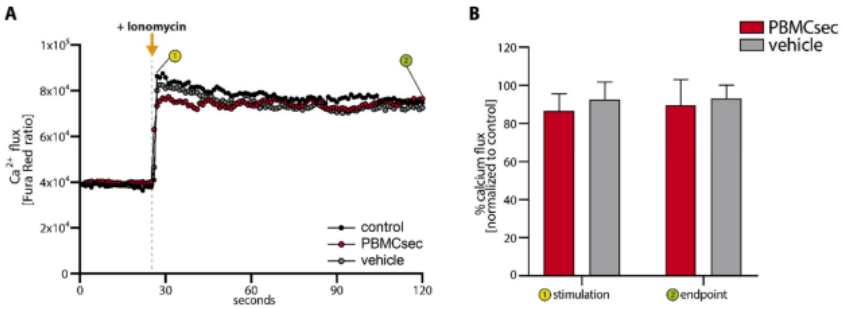


Figure 4. PBMCsec inhibits NET formation without interfering with calcium flux. (**A**) Ratiometric calcium flux was measured with Fura Red in neutrophils. Neutrophils were observed for approximately 30 s to record a baseline Fura Red ratio indicating homeostatic calcium flux prior to the addition of IM and subsequent analysis for a total of 120 s. (**B**) Statistical analysis of the percent reduction in calcium flux in PBMCsec or vehicle treated neutrophils compared to control samples is shown for the time points of stimulation (①, addition of IM) and the endpoint (②,120 s) $n = 4$. Error bars indicate SD. No statistically significant reduction was observed for PBMCsec or vehicle treatment.

Since activation of the NET signalling pathway down-stream of NADPH leads to the production of ROS and activation of PAD4 [8,10,11], we next investigated whether PBMCsec

exerts its inhibitory activity by modulating these processes. We, therefore, investigated ionomycin-induced production of ROS using the cell permeant reagent 2′,7′-dichlorofluorescin diacetate (DCFDA). Ionomycin treatment of PBMCsec-stimulated neutrophils resulted in a significant decrease in ROS production, as compared to ionomycin treatment alone (Figure 5A). By contrast, pre-treatment with vehicle showed no inhibitory effect (Figure 5A). Analysis of protein expression revealed that PBMCsec inhibited ionomycin-induced down-regulation of known anti-oxidative factors, including hemoxygenase-1 (HO-1) [45] and hypoxia inducible factor 1 alpha (HIF-1α) [46] in purified human neutrophils (Figures 5B and S5B), which was not observed in vehicle-treated cells.

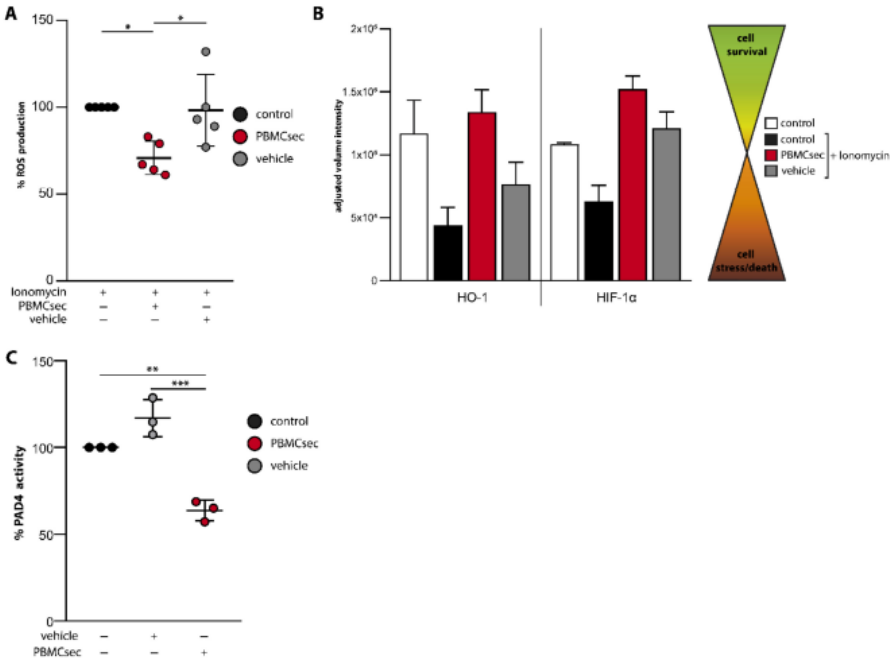


Figure 5. PBMCsec prevents ROS production and PAD4 activity. (**A**) ROS production in IM stimulated and PBMCsec or vehicle treated neutrophils normalized to IM-induced ROS production. $n = 5$ (**B**) Analysis of protein levels of HO-1 and HIF-1α of isolated neutrophils stimulated with ionomycin and treated with PBMCsec or vehicle, using a proteome profiler is shown. Cell lysates of four individual donors and experiments were pooled. Error bars indicate SD of two technical replicates. (**C**) Enzymatic activity of PAD4 was measured in a cell-free assay upon co-incubation of PBMCsec or vehicle with the PAD4-substrate and normalized to untreated control. $n = 3$, data are represented as individual values with mean and error bars indicate SD, unless indicated otherwise. One-way ANOVA and Sidak's multiple comparisons test was performed where significances are indicated. * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$.

The activation of PAD4 with subsequent histone-citrullination represents one of the final steps in the NETs signalling pathway [11]. Thus, we next investigated PAD4 activity in a cell-free assay. Although vehicle treatment did not show PAD4 inhibiting properties, PBMCsec inhibited PAD4 activity by approximately 40% (Figure 5C). Together, these data demonstrate that PBMCsec exerts its inhibitory activity on NET formation by reducing intracellular ROS production and preventing PAD4 activation.

4. Discussion

The formation of NETs is a highly effective first line defence mechanism against invading pathogens [17]. However, there is growing evidence that excessive NETs formation contributes to tissue damage and the induction of auto-immune diseases [16,47–49]. Although several substances, including acetylsalicylic acid, cyclosporine A [50], metformin [51] and chloroquine [52], have been shown to influence NET formation, therapeutic drugs targeting NET formation are so far not available. In the current study, we provide evidence that PBMCsec effectively inhibited NET formation by reducing ROS production and PAD4 activation, thereby providing a novel potential cell-derived but cell-free therapeutic intervention for NET-associated diseases.

The tissue regenerative and anti-inflammatory action spectrum of PBMCsec is multifaceted [29,32,34,37,38,53,54], and most of its beneficial effects have been shown to require the interplay of several components of the secretome [30,55]. Indeed, we also found that NET formation was only fully inhibited when neutrophils were treated with the whole secretome or reconstituted purified fractions. Since we observed NETs inhibition at different steps of the NETs signalling pathway, we hypothesize that individual secretome fractions act on different signalling molecules. Recently, Laggner et al. showed that lipids present in PBMCsec attenuate skin inflammation and allergic reactions by targeting dendritic cell function [34], as well as mast cell and basophil activation, respectively [56], suggesting that lipids are mainly responsible for the anti-inflammatory activities of PBMCsec. Several lipid species have been detected in PBMCsec, including phosphatidylserines, lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatdiylethanolamines, and resolvins [34]. Interestingly, several studies described a NET-inhibitory or NET-resolving action of resolvins [57–59]. Spinosa et al. demonstrated a decreased NET burden accompanied by reduced abdominal aortic aneurysm in reslovin-treated mice [57]. In addition, neutrophils derived from resolvin-treated mice showed less susceptibility to ionomycin-induced NET formation [58]. Although both of these studies identified less NET formation in the presence of resolvins, Chiang et al. showed that NETs formed after *Staphylococcus aureus* infection were more efficiently cleared by macrophages after treatment with reslovins [59]. These data indicate an important function of resolvins in the prevention of NET formation and/or resolution of NETs. Therefore, it is conceivable that the partial inhibition of NET formation observed by PBMCsec-lipids may be explained by the variety of resolvins found in PBMCsec [34]. However, whether PBMCsec-derived resolvins or other lipid classes are indeed involved in PBMCsec-induced NETs inhibition needs to be determined in future studies.

In addition to the lipid fraction, PBMCsec-derived proteins also showed a strong inhibitory action on NET formation. Previous studies demonstrated that addition of either bovine or human serum albumin to ionomycin-treated cells almost completely blocked NET formation by chelating calcium [60]. However, as we only detected a slight decrease in calcium influx after treatment with PBMCsec and vehicle, a sole albumin-dependent effect is unlikely. Additionally, PBMCsec treatment also abrogated Thapsigargin-induced NETosis, which indicates that PBMCsec-mediated NETosis inhibition occurs without interfering with calcium flux or the cells' capability to store excess calcium into intracellular storage units, such as the endoplasmic reticulum. Furthermore, we also showed comparable effects when NET formation was induced with PMA, which induces NETs in a calcium-independent manner. Together, our data suggest a calcium- and albumin-independent mode of action of the protein fraction of PBMCsec. Further in-depth proteomics analyses of PBMCsec-derived proteins are required to elucidate whether a single protein or a combination of proteins is responsible for the inhibition of NET formation.

Our data suggest the inhibition of ROS production and PAD4 activation as the two major modes of action for the reduction in NET formation by PBMCsec. Oxidative stress, especially the generation of ROS, is a hallmark of NET formation [10,61] and HSPs are known to effectively block excessive ROS production [62]. Our study revealed that PBMCsec inhibited hemeoxygenase 1 (HO-1 or HSP32) and HIF-1α downregulation during

ionomycin-induced NET formation. Both HO-1 and HIF-1α have been shown to promote neutrophil survival by reducing ROS levels [63] and via Akt and NFκB signalling under stress, respectively [46,64]. These data suggest that PBMCsec contributes to the stabilization of the delicate balance of pro- and anti-oxidative processes by regulating the expression of HSPs, thereby preventing neutrophil-induced tissue damage. Since HO-1 is also known to downregulate adhesion molecules and chemokines required for neutrophil infiltration [45], PBMCsec may alleviate inflammatory responses by reducing neutrophil infiltration in damaged and inflamed tissue. However, further studies are required to unravel the exact mechanism by which PBMCsec counteracts ROS production as it is not yet clear whether it functions as ROS-scavenger, inhibits the liberation of ROS from mitochondria or if it interferes with the functional assembly of NADPH oxidase subunits.

PAD4 is one of the most prominently investigated factors critical for NET formation, and PAD inhibitors have been extensively studied in the context of a broad variety of diseases, including multiple sclerosis [65], myocardial infarction [66], and rheumatoid arthritis [67]. However, the exact mechanism of PAD4 inhibition is not yet fully understood [67]. Our data indicate that stressed PBMCs secrete factors that serve as PAD4 inhibitors. Interestingly, Yost et al. identified a group of peptides in umbilical cord blood with strong PAD4-inhibiting effects, leading to inhibition of NETs [68]. Sequence analyses identified α1-antitrypsin, a serine protease inhibitor, known to possess immunomodulatory and anti-inflammatory properties [69], as the main PAD4-inhibiting factor. Interestingly, α1-antitrypsin is synthesized by circulating monocytes and, therefore, a component of PBMCsec (Figure S6) [70]. According to our quantification analysis, 25–30 ng/mL SERPINA1 are present in two units of PBMCsec. This enzyme inhibitor has been considered as an acute phase protein, which contributes to the inhibition of NET formation by targeting a vast array of factors contributing to NET formation [71]. Further studies are needed to identify the PAD4-inhibiting factor(s) in PBMCsec.

5. Conclusions

In summary, we have demonstrated a strong NETs-inhibitory activity of PBMCsec via a dual mechanism. Specifically the identification of a PAD4 inhibitor, produced naturally in the human body, as well as the prevention of ROS production might strongly improve the treatment of diseases associated with excessive NET formation, such as rheumatoid arthritis [67], multiple sclerosis [65], sepsis [17], heart failure, and myocardial infarction [66]. Pre-clinical toxicological evaluation of PBMCsec has already been performed without the occurrence of major adverse events after topical and intravenous application (LPT, study number 35015). Therefore, our study has paved the way for a clinical study in humans, assessing the potency of PBMCsec in NETs-associated diseases in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11081559/s1, Graphical abstract: PBMCsec inhibits NET formation; Figure S1: Gating strategy, PBMCsec improves neutrophil metabolic activity; Figure S2: Flow cytometric analysis of spontaneous NET formation; Figure S3: Flow cytometric analysis of unstimulated neutrophils; Figure S4: PBMCsec inhibits Thapsigargin-induced NETosis; Figure S5: Gating strategy and purity of isolated neutrophils; Figure S6: SERPINA1 abundance in PBMCsec.

Author Contributions: Conceptualization, K.K., M.M., and H.J.A.; methodology, K.K., M.M., A.S.O., and T.M.H.; validation K.K., M.M., A.S.O., T.M.H.; formal analysis, K.K.; investigation, K.K., A.S.O., T.M.H., and K.P.; resources, H.J.A.; data curation, K.K., D.C., M.D., and D.B.; writing—original draft preparation, K.K. and M.M.; writing—review and editing, K.K., M.M., M.L., and A.M.; visualization, K.K.; supervision, M.M.; project administration, K.K. and M.M.; funding acquisition, H.J.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research project was financed in part by the Austrian Research Promotion Agency grant "APOSEC" (852748 and 862068, 2015–2019), by the Vienna Business Agency "APOSEC to clinic," (2343727, 2018–2020), and by the Aposcience AG under group leader H.J.A., M.M. was funded by Aposcience AG.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethical Review Board of the Medical University of Vienna (protocol code 1539/2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article or Supplementary Materials.

Acknowledgments: We thank H.P. Haselsteiner and the CRISCAR Familienstiftung for their belief in this private–public partnership to augment basic and translational clinical research.

Conflicts of Interest: The Medical University of Vienna has claimed financial interest. H.J.A. holds patents related to this work (WO 2010/079086 A1; WO 2010/070105 A1).

References

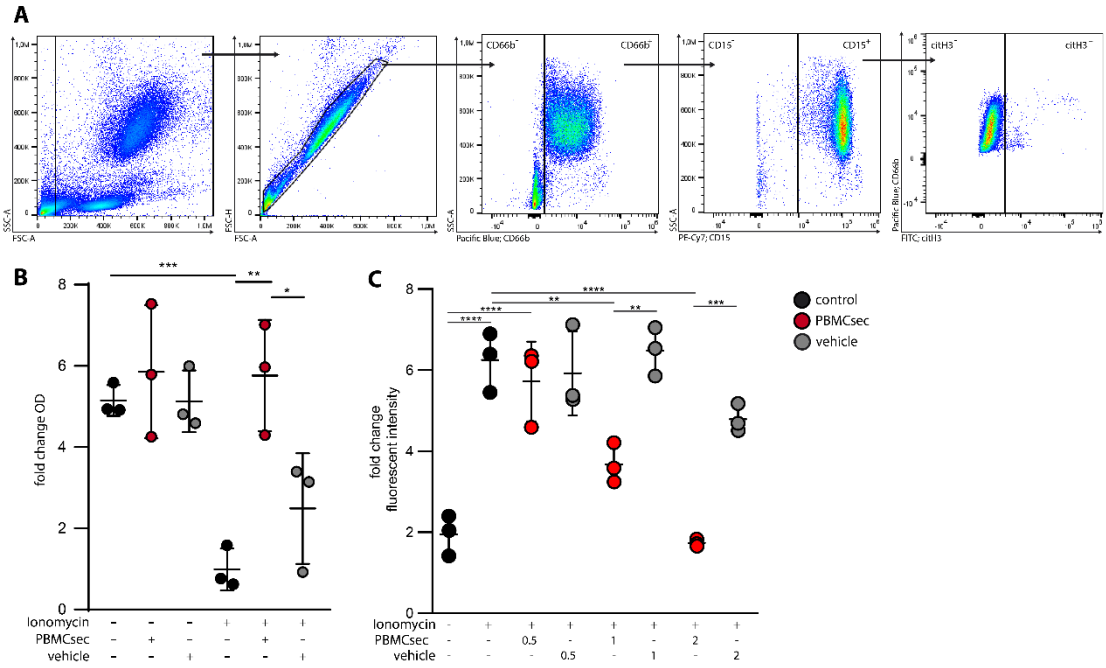
1. Rosales, C. Neutrophil: A cell with many roles in inflammation or several cell types? *Front. Physiol.* **2018**, *9*, 113.
2. Nicolás-Ávila, J.Á.; Adrover, J.M.; Hidalgo, A. Neutrophils in Homeostasis, Immunity, and Cancer. *Immunity* **2017**, *46*, 15–28. [CrossRef]
3. Brinkmann, V.; Zychlinsky, A. Beneficial suicide: Why neutrophils die to make NETs. *Nat. Rev. Microbiol.* **2007**, *5*, 577–582. [CrossRef] [PubMed]
4. 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] [PubMed]
5. Futosi, K.; Fodor, S.; Mócsai, A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int. Immunopharmacol.* **2013**, *17*, 638–650. [CrossRef] [PubMed]
6. 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]
7. Kenny, E.F.; Herzig, A.; Krüger, R.; Muth, A.; Mondal, S.; Thompson, P.R.; Brinkmann, V.; von Bernuth, H.; Zychlinsky, A. Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife* **2017**, *6*, e24437. [CrossRef]
8. Wang, Y.; Li, M.; Stadler, S.; Correll, S.; Li, P.; Wang, D.; Hayama, R.; Leonelli, L.; Han, H.; Grigoryev, S.A.; et al. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J. Cell Biol.* **2009**, *184*, 205–213. [CrossRef]
9. Kaplan, M.J.; Radic, M. Neutrophil Extracellular Traps: Double-Edged Swords of Innate Immunity. *J. Immunol.* **2012**, *189*, 2689–2695. [CrossRef]
10. Sollberger, G.; Tilley, D.O.; Zychlinsky, A. *Neutrophil Extracellular Traps: The Biology of Chromatin Externalization*; Cell Press: Cambridge, MA, USA, 2018; Volume 44, pp. 542–553.
11. Rohrbach, A.S.; Slade, D.J.; Thompson, P.R.; Mowen, K.A. Activation of PAD4 in NET formation. *Front. Immunol.* **2012**, *3*, 360. [CrossRef]
12. Papayannopoulos, V.; Metzler, K.D.; Hakkim, A.; Zychlinsky, A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* **2010**, *191*, 677–691. [CrossRef]
13. Neeli, I.; Dwivedi, N.; Khan, S.; Radic, M. Regulation of extracellular chromatin release from neutrophils. *J. Innate Immun.* **2009**, *1*, 194–201. [CrossRef] [PubMed]
14. Kolaczkowska, E.; Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **2013**, *13*, 159–175. [CrossRef]
15. Thålin, C.; Hisada, Y.; Lundström, S.; Mackman, N.; Wallén, H. Neutrophil Extracellular Traps. *Arterioscler. Thromb. Vasc. Biol.* **2019**, *39*, 1724–1738. [CrossRef]
16. Wang, J. Neutrophils in tissue injury and repair. *Cell Tissue Res.* **2018**, *371*, 531–539. [CrossRef]
17. Fine, N.; Tasevski, N.; McCulloch, C.A.; Tenenbaum, H.C.; Glogauer, M. The Neutrophil: Constant Defender and First Responder. *Front. Immunol.* **2020**, *11*, 571085. [CrossRef]
18. Wculek, S.K.; Malanchi, I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature* **2015**, *528*, 413–417. [CrossRef]
19. Gupta, A.K.; Joshi, M.B.; Philippova, M.; Erne, P.; Hasler, P.; Hahn, S.; Resink, T.J. Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. *FEBS Lett.* **2010**, *584*, 3193–3197. [CrossRef]
20. Mangold, A.; Ondracek, A.S.; Hofbauer, T.M.; Scherz, T.; Artner, T.; Panagiotides, N.; Beitzke, D.; Ruzicka, G.; Nistler, S.; Wohlschläger-Krenn, E.; et al. Culprit site extracellular DNA and microvascular obstruction in ST-elevation myocardial infarction. *Cardiovasc. Res.* **2021**, *118*, 2006–2017. [CrossRef]
21. Mangold, A.; Hofbauer, T.M.; Ondracek, A.S.; Artner, T.; Scherz, T.; Speidl, W.S.; Krychtiuk, K.A.; Sadushi-Kolici, R.; Jakowitsch, J.; Lang, I.M. Neutrophil extracellular traps and monocyte subsets at the culprit lesion site of myocardial infarction patients. *Sci. Rep.* **2019**, *9*, 16304. [CrossRef]
22. Distelmaier, K.; Winter, M.P.; Dragschitz, F.; Redwan, B.; Mangold, A.; Gleiss, A.; Perkmann, T.; Maurer, G.; Adlbrecht, C.; Lang, I.M. Prognostic value of culprit site neutrophils in acute coronary syndrome. *Eur. J. Clin. Investig.* **2014**, *44*, 257–265. [CrossRef] [PubMed]

23. Fadini, G.P.; Menegazzo, L.; Rigato, M.; Scattolini, V.; Poncina, N.; Bruttocao, A.; Ciciliot, S.; Mammano, F.; Ciubotaru, C.D.; Brocco, E.; et al. NETosis delays diabetic wound healing in mice and humans. *Diabetes* **2016**, *65*, 1061–1071. [CrossRef] [PubMed]
24. Kitching, A.R.; Anders, H.J.; Basu, N.; Brouwer, E.; Gordon, J.; Jayne, D.R.; Kullman, J.; Lyons, P.A.; Merkel, P.A.; Savage, C.O.S.; et al. ANCA-associated vasculitis. *Nat Rev Dis Prim.* **2020**, *6*, 71. [CrossRef] [PubMed]
25. Meegan, J.E.; Yang, X.; Beard, R.S.; Jannaway, M.; Chatterjee, V.; Taylor-Clark, T.E.; Yuan, S.Y. Citrullinated histone 3 causes endothelial barrier dysfunction. *Biochem. Biophys. Res. Commun.* **2018**, *503*, 1498–1502. [CrossRef] [PubMed]
26. Klopf, J.; Brostjan, C.; Eilenberg, W.; Neumayer, C. Neutrophil extracellular traps and their implications in cardiovascular and inflammatory disease. *Int. J. Mol. Sci.* **2021**, *22*, 559. [CrossRef]
27. Natallya, F.; Herwanto, N.; Prakoeswa, C.; Indramaya, D.; Rantam, F. Effective healing of leprosy chronic plantar ulcers by application of human amniotic membrane stem cell secretome gel. *Indian J. Dermatol.* **2019**, *64*, 250. [CrossRef] [PubMed]
28. Karpov, A.A.; Puzanov, M.V.; Ivkin, D.Y.; Krasnova, M.V.; Anikin, N.A.; Docshin, P.M.; Moiseeva, O.M.; Galagudza, M.M. Non-inferiority of microencapsulated mesenchymal stem cells to free cells in cardiac repair after myocardial infarction: A rationale for using paracrine factor(s) instead of cells. *Int. J. Exp. Pathol.* **2019**, *100*, 102–113. [CrossRef] [PubMed]
29. Ankersmit, H.J.; Hoetzenecker, K.; Dietl, W.; Soleiman, A.; Horvat, R.; Wolfsberger, M.; Gerner, C.; Hacker, S.; Mildner, M.; Moser, B.; et al. Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium. *Eur. J. Clin. Investig.* **2009**, *39*, 445–456. [CrossRef] [PubMed]
30. Wagner, T.; Traxler, D.; Simader, E.; Beer, L.; Narzt, M.S.; Gruber, F.; Madlener, S.; Laggner, M.; Erb, M.; Vorstandlechner, V.; et al. Different pro-angiogenic potential of γ-irradiated PBMC-derived secretome and its subfractions. *Sci. Rep.* **2018**, *8*, 18016. [CrossRef]
31. Mildner, C.S.; Copic, D.; Zimmermann, M.; Lichtenauer, M.; Direder, M.; Klas, K.; Bormann, D.; Gugerell, A.; Moser, B.; Hoetzenecker, K.; et al. Secretome of Stressed Peripheral Blood Mononuclear Cells Alters Transcriptome Signature in Heart, Liver, and Spleen after an Experimental Acute Myocardial Infarction: An In Silico Analysis. *Biology* **2022**, *11*, 116. [CrossRef] [PubMed]
32. Beer, L.; Zimmermann, M.; Mitterbauer, A.; Ellinger, A.; Gruber, F.; Narzt, M.S.; Zellner, M.; Gyöngyösi, M.; Madlener, S.; Simader, E.; et al. Analysis of the secretome of apoptotic peripheral blood mononuclear cells: Impact of released proteins and exosomes for tissue regeneration. *Sci. Rep.* **2015**, *5*, 16662. [CrossRef]
33. Kasiri, M.M.; Beer, L.; Nemec, L.; Gruber, F.; Pietkiewicz, S.; Haider, T.; Simader, E.M.; Traxler, D.; Schweiger, T.; Janik, S.; et al. Dying blood mononuclear cell secretome exerts antimicrobial activity. *Eur. J. Clin. Investig.* **2016**, *46*, 853–863. [CrossRef] [PubMed]
34. Laggner, M.; Copic, D.; Nemec, L.; Vorstandlechner, V.; Gugerell, A.; Gruber, F.; Peterbauer, A.; Ankersmit, H.J.; Mildner, M. Therapeutic potential of lipids obtained from γ-irradiated PBMCs in dendritic cell-mediated skin inflammation: PBMC lipid secretome and DC functions. *eBioMedicine* **2020**, *55*, 102774. [CrossRef] [PubMed]
35. Beer, L.; Mildner, M.; Gyöngyösi, M.; Ankersmit, H.J. Peripheral blood mononuclear cell secretome for tissue repair. *Apoptosis* **2016**, *21*, 1336–1353. [CrossRef] [PubMed]
36. Pavo, N.; Zimmermann, M.; Pils, D.; Mildner, M.; Petrási, Z.; Petneházy, Ö.; Fuzik, J.; Jakab, A.; Gabriel, C.; Sipos, W.; et al. Long-acting beneficial effect of percutaneously intramyocardially delivered secretome of apoptotic peripheral blood cells on porcine chronic ischemic left ventricular dysfunction. *Biomaterials* **2014**, *35*, 3541–3550. [CrossRef]
37. Panahipour, L.; Kargarpour, Z.; Laggner, M.; Mildner, M.; Ankersmit, H.J.; Gruber, R. TGF-β in the Secretome of Irradiated Peripheral Blood Mononuclear Cells Supports In Vitro Osteoclastogenesis. *Int. J. Mol. Sci.* **2020**, *21*, 8569. [CrossRef]
38. Panahipour, L.; Kochergina, E.; Laggner, M.; Zimmermann, M.; Mildner, M.; Ankersmit, H.J.; Gruber, R. Role for lipids secreted by irradiated peripheral blood mononuclear cells in inflammatory resolution in vitro. *Int. J. Mol. Sci.* **2020**, *21*, 4694. [CrossRef]
39. Wendt, E.R.; Ferry, H.; Greaves, D.R.; Keshav, S. Ratiometric Analysis of Fura Red by Flow Cytometry: A Technique for Monitoring Intracellular Calcium Flux in Primary Cell Subsets. *PLoS ONE* **2015**, *10*, e0119532. [CrossRef]
40. Hacker, S.; Mittermayr, R.; Nickl, S.; Haider, T.; Lebherz-Eichinger, D.; Beer, L.; Mitterbauer, A.; Leiss, H.; Zimmermann, M.; Schweiger, T.; et al. Paracrine Factors from Irradiated Peripheral Blood Mononuclear Cells Improve Skin Regeneration and Angiogenesis in a Porcine Burn Model. *Sci. Rep.* **2016**, *6*, 25168. [CrossRef]
41. Schimek, V.; Strasser, K.; Beer, A.; Göber, S.; Walterskirchen, N.; Brostjan, C.; Müller, C.; Bachleitner-Hofmann, T.; Bergmann, M.; Dolznig, H.; et al. Tumour cell apoptosis modulates the colorectal cancer immune microenvironment via interleukin-8-dependent neutrophil recruitment. *Cell Death Dis.* **2022**, *13*, 113. [CrossRef]
42. Simader, E.; Traxler, D.; Kasiri, M.M.; Hofbauer, H.; Wolzt, M.; Glogner, C.; Storka, A.; Mildner, M.; Gouya, G.; Geusau, A.; et al. Safety and tolerability of topically administered autologous, apoptotic PBMC secretome (APOSEC) in dermal wounds: A randomized Phase 1 trial (MARSYAS I). *Sci. Rep.* **2017**, *7*, 6216. [CrossRef] [PubMed]
43. Jiménez-Alcázar, M.; Rangaswamy, C.; Panda, R.; Bitterling, J.; Simsek, Y.J.; Long, A.T.; Bilyy, R.; Krenn, V.; Renné, C.; Renné, T.; et al. Host DNases prevent vascular occlusion by neutrophil extracellular traps. *Science* **2017**, *358*, 1202–1206. [CrossRef] [PubMed]
44. Ribeiro, D.; Freitas, M.; Rocha, S.; Lima, J.L.F.C.; Carvalho, F.; Fernandes, E. Calcium Pathways in Human Neutrophils—The Extended Effects of Thapsigargin and ML-9. *Cells* **2018**, *7*, 204. [CrossRef] [PubMed]
45. Seldon, M.P.; Silva, G.; Pejanovic, N.; Larsen, R.; Gregoire, I.P.; Filipe, J.; Anrather, J.; Soares, M.P. Heme oxygenase-1 inhibits the expression of adhesion molecules associated with endothelial cell activation via inhibition of NF-kappaB RelA phosphorylation at serine 276. *J. Immunol.* **2007**, *179*, 7840–7851. [CrossRef] [PubMed]

46. Zhang, Z.; Yao, L.; Yang, J.; Wang, Z.; Du, G. PI3K/Akt and HIF-1 signaling pathway in hypoxia-ischemia (Review). *Mol. Med. Rep.* **2018**, *18*, 3547–3554. [CrossRef] [PubMed]
47. 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] [PubMed]
48. Boufenzer, A.; Carrasco, K.; Jolly, L.; Brustolin, B.; Di-Pillo, E.; Derive, M.; Gibot, S. Potentiation of NETs release is novel characteristic of TREM-1 activation and the pharmacological inhibition of TREM-1 could prevent from the deleterious consequences of NETs release in sepsis. *Cell. Mol. Immunol.* **2021**, *18*, 452–460. [CrossRef] [PubMed]
49. Barnado, A.; Crofford, L.J.; Oates, J.C. At the Bedside: Neutrophil extracellular traps (NETs) as targets for biomarkers and therapies in autoimmune diseases. *J. Leukoc. Biol.* **2016**, *99*, 265–278. [CrossRef]
50. Gupta, A.K.; Giaglis, S.; Hasler, P.; Hahn, S. Efficient Neutrophil Extracellular Trap Induction Requires Mobilization of Both Intracellular and Extracellular Calcium Pools and Is Modulated by Cyclosporine A. *PLoS ONE* **2014**, *9*, e97088.
51. Menegazzo, L.; Scattolini, V.; Cappellari, R.; Bonora, B.M.; Albiero, M.; Bortolozzi, M.; Romanato, F.; Ceolotto, G.; Vigili de Kreutzeberg, S.; Avogaro, A.; et al. The antidiabetic drug metformin blunts NETosis in vitro and reduces circulating NETosis biomarkers in vivo. *Acta Diabetol.* **2018**, *55*, 593–601. [CrossRef]
52. Boone, B.A.; Murthy, P.; Miller-Ocuin, J.; Doerfler, W.R.; Ellis, J.T.; Liang, X.; Ross, M.A.; Wallace, C.T.; Sperry, J.L.; Lotze, M.T.; et al. Chloroquine reduces hypercoagulability in pancreatic cancer through inhibition of neutrophil extracellular traps. *BMC Cancer* **2018**, *18*, 678. [CrossRef]
53. Hoetzenecker, K.; Zimmermann, M.; Hoetzenecker, W.; Schweiger, T.; Kollmann, D.; Mildner, M.; Hegedus, B.; Mitterbauer, A.; Hacker, S.; Birner, P.; et al. Mononuclear cell secretome protects from experimental autoimmune myocarditis. *Eur. Heart J.* **2015**, *36*, 676–685a. [CrossRef] [PubMed]
54. Lichtenauer, M.; Mildner, M.; Baumgartner, A.; Hasun, M.; Werba, G.; Beer, L.; Altmann, P.; Roth, G.; Gyöngyösi, M.; Podesser, B.K.; et al. Intravenous and intramyocardial injection of apoptotic white blood cell suspensions prevents ventricular remodelling by increasing elastin expression in cardiac scar tissue after myocardial infarction. *Basic Res. Cardiol.* **2011**, *106*, 645–655. [CrossRef] [PubMed]
55. Simader, E.; Beer, L.; Laggner, M.; Vorstandlechner, V.; Gugerell, A.; Erb, M.; Kalinina, P.; Copic, D.; Moser, D.; Spittler, A.; et al. Tissue-regenerative potential of the secretome of γ-irradiated peripheral blood mononuclear cells is mediated via TNFRSF1B-induced necroptosis. *Cell Death Dis.* **2019**, *10*, 729. [CrossRef] [PubMed]
56. Laggner, M.; Acosta, G.S.; Kitzmüller, C.; Copic, D.; Gruber, F.; Altenburger, L.M.; Vorstandlechner, V.; Gugerell, A.; Direder, M.; Klas, K.; et al. The secretome of irradiated peripheral blood mononuclear cells attenuates activation of mast cells and basophils. *eBioMedicine* **2022**, *81*, 104093. [CrossRef] [PubMed]
57. Spinosa, M.; Su, G.; Salmon, M.D.; Lu, G.; Cullen, J.M.; Fashandi, A.Z.; Hawkins, R.B.; Montgomery, W.; Meher, A.K.; Conte, M.S.; et al. Resolvin D1 Decreases Abdominal Aortic Aneurysm Formation by Inhibiting NETosis in a Mouse Model. *J. Vasc. Surg.* **2018**, *68*, 93S. [CrossRef]
58. Cherpokova, D.; Jouvene, C.C.; Libreros, S.; DeRoo, E.P.; Chu, L.; de La Rosa, X.; Norris, P.C.; Wagner, D.D.; Serhan, C.N. Resolvin D4 attenuates the severity of pathological thrombosis in mice. *Blood* **2019**, *134*, 1458–1468. [CrossRef]
59. Chiang, N.; Sakuma, M.; Rodriguez, A.R.; Spur, B.W.; Irimia, D.; Serhan, C.N. Resolvin T-series Reduce Neutrophil Extracellular Traps. *Blood* **2021**, *139*, 1222–1233. [CrossRef] [PubMed]
60. Neubert, E.; Senger-Sander, S.N.; Manzke, V.S.; Busse, J.; Polo, E.; Scheidmann, S.E.F.; Schön, M.P.; Kruss, S.; Erpenbeck, L. Serum and serum albumin inhibit in vitro formation of Neutrophil Extracellular Traps (NETs). *Front. Immunol.* **2019**, *10*, 12. [CrossRef] [PubMed]
61. Hakkim, A.; Fuchs, T.A.; Martinez, N.E.; Hess, S.; Prinz, H.; Zychlinsky, A.; Waldmann, H. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat. Chem. Biol.* **2011**, *7*, 75–77. [CrossRef] [PubMed]
62. Ikwegbue, P.C.; Masamba, P.; Oyinloye, B.E.; Kappo, A.P. Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. *Pharmaceuticals* **2018**, *11*, 2. [CrossRef]
63. Li, X.; Schwacha, M.G.; Chaudry, I.H.; Choudhry, M.A. Heme Oxygenase-1 Protects against Neutrophil-Mediated Intestinal Damage by Down-Regulation of Neutrophil p47 phox and p67 phox Activity and O_2^- Production in a Two-Hit Model of Alcohol Intoxication and Burn Injury. *J. Immunol.* **2008**, *180*, 6933–6940. [CrossRef] [PubMed]
64. Walmsley, S.R.; Print, C.; Farahi, N.; Peyssonnaux, C.; Johnson, R.S.; Cramer, T.; Sobolewski, A.; Condliffe, A.M.; Cowburn, A.S.; Johnson, N.; et al. Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. *J. Exp. Med.* **2005**, *201*, 105–115. [CrossRef]
65. Sarswat, A.; Wasilewski, E.; Chakka, S.K.; Bello, A.M.; Caprariello, A.V.; Muthuramu, C.M.; Stys, P.K.; Dunn, S.E.; Kotra, L.P. Inhibitors of protein arginine deiminases and their efficacy in animal models of multiple sclerosis. *Bioorgan. Med. Chem.* **2017**, *25*, 2643–2656. [CrossRef] [PubMed]
66. Du, M.; Yang, W.; Schmull, S.; Gu, J.; Xue, S. Inhibition of peptidyl arginine deiminase-4 protects against myocardial infarction induced cardiac dysfunction. *Int. Immunopharmacol.* **2020**, *78*, 106055. [CrossRef]
67. Jones, J.E.; Causey, C.P.; Knuckley, B.; Slack-Noyes, J.L.; Thompson, P.R. Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. *Curr. Opin. Drug Discov. Dev.* **2009**, *12*, 616–627.

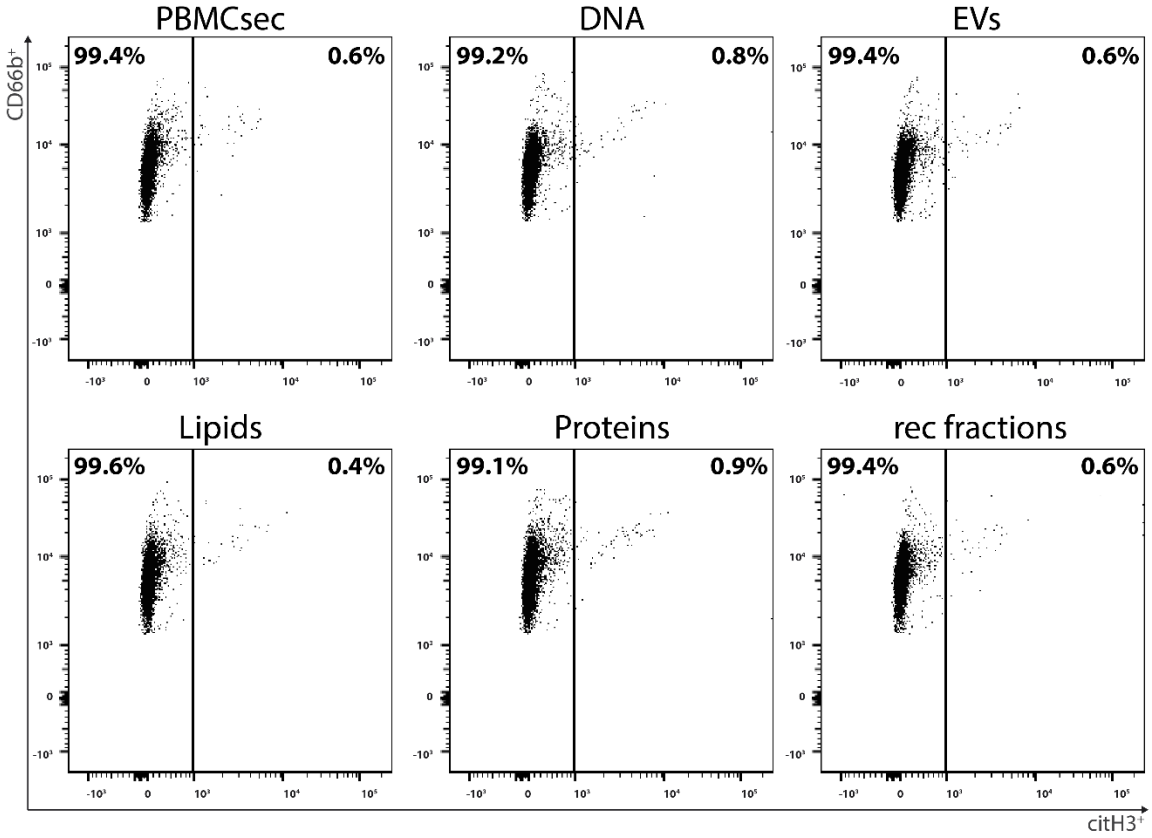
68. Yost, C.C.; Schwertz, H.; Cody, M.J.; Wallace, J.A.; Campbell, R.A.; Vieira-De-Abreu, A.; Araujo, C.V.; Schubert, S.; Harris, E.S.; Rowley, J.W.; et al. Neonatal NET-inhibitory factor and related peptides inhibit neutrophil extracellular trap formation. *J. Clin. Investig.* **2016**, *126*, 3783–3798. [CrossRef]
69. Janciauskiene, S.M.; Bals, R.; Koczulla, R.; Vogelmeier, C.; Köhnlein, T.; Welte, T. The discovery of α1-antitrypsin and its role in health and disease. *Respir. Med.* **2011**, *105*, 1129–1139. [CrossRef]
70. Van Furth, R.; Kramps, J.A.; Diesselhof Den Dulk, M.M.C. Synthesis of α1-anti-trypsin by human monocytes. *Clin. Exp. Immunol.* **1983**, *51*, 551–557.
71. Janciauskiene, S.; Wrenger, S.; Immenschuh, S.; Olejnicka, B.; Greulich, T.; Welte, T.; Chorostowska-Wynimko, J. The multifaceted effects of Alpha1-Antitrypsin on neutrophil functions. *Front. Pharmacol.* **2018**, *9*, 341. [CrossRef] [PubMed]

1.2 Supplementary Figures

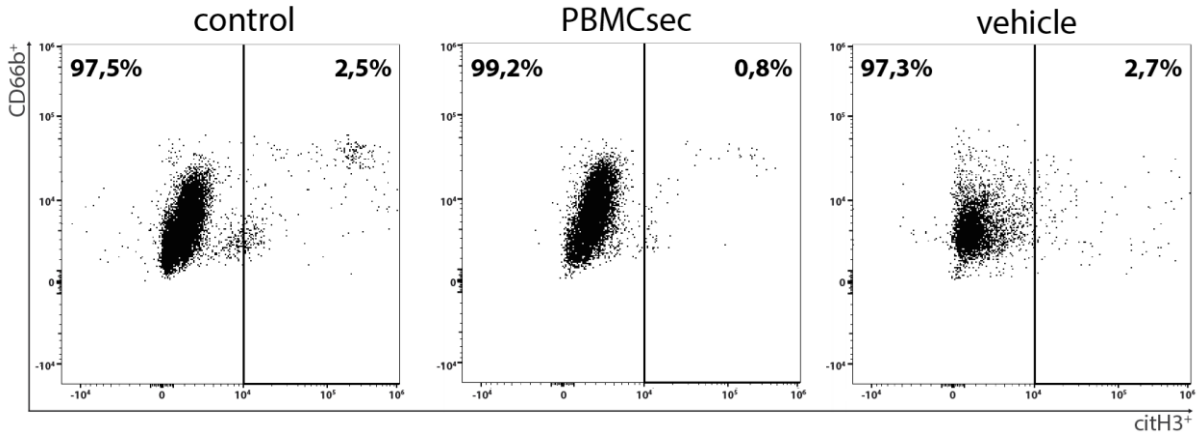


Supplementary Figure 1 PBMCsec improves neutrophil metabolic activity and inhibits NETosis in a dose-dependent manner

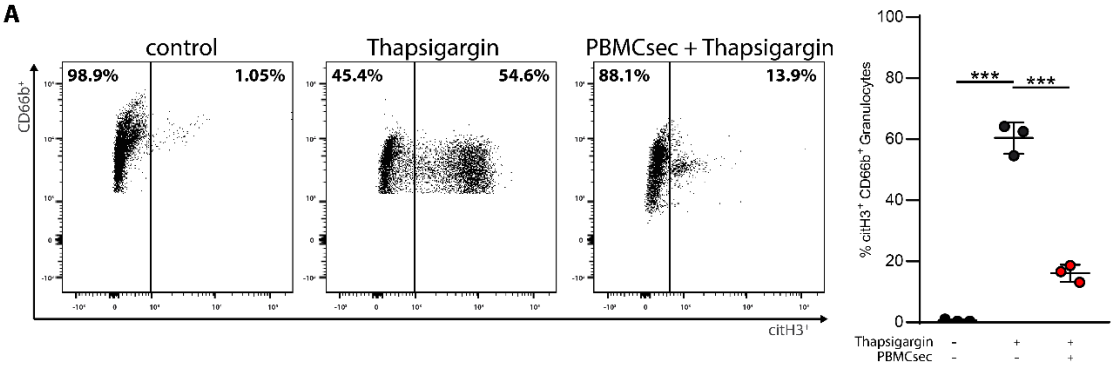
A) Flow cytometry gating strategy for erythrocyte lysed blood samples is shown. (B) Metabolic activity of neutrophils was measured using an absorbance based assay (EZ4U). Vehicle treated neutrophils did not show altered metabolic activity compared to untreated control samples. PBMCsec treatment appeared to partially promote metabolic activity of non-activated neutrophils. IM treatment resulted in a significant reduction of metabolic activity of neutrophils which was abolished by PBMCsec treatment. Vehicle treatment could not restore homeostatic metabolic activity in IM-activated neutrophils. C) Extracellular DNA content was measured using cytotox staining of neutrophils after pre-treatment with PBMCsec or vehicle in a dose dependent manner. Data are represented as mean ± SD, one-way ANOVA and Sidak's multiple comparisons test. *p<0.0332, **p<0.0021, ***p<0.0002



Supplementary Figure 2 Flow cytometric analysis of spontaneous NET formation
Flow cytometric analysis of untreated control neutrophils and neutrophils treated with PBMCsec derived substance classes in absence of an activating stimulus is shown. Neutrophils were identified as CD66b+CD15+ cells and NET formation was characterized by additional citH3+ signal. n = 3, one representative experiment is shown.

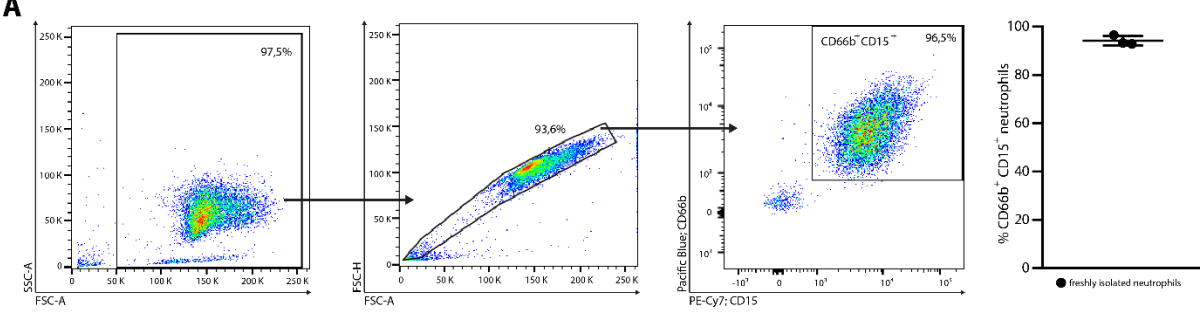


Supplementary Figure 3 Flow cytometric analysis of unstimulated neutrophils
Flow cytometric analysis of untreated control neutrophils and neutrophils treated with PBMCsec or vehicle in absence of an activating stimulus after two hours is shown. Neutrophils were identified as CD66b+CD15+ cells and NET formation was characterized by additional citH3+ signal. n = 3, one representative experiment is shown.

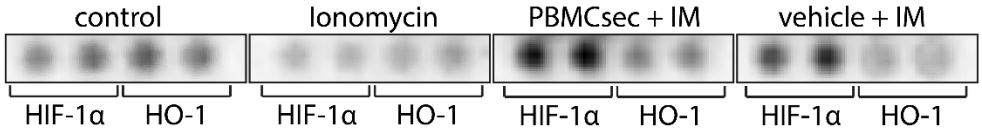


Supplementary Figure 4 PBMCsec inhibits Thapsigargin-induced NETosis
Neutrophils were analysed using flow cytometry and identified by $CD66b^+CD15^+$ signal. NETosis was identified by $citH3^+$ positive signal. Untreated control samples were compared to Thapsigargin-activated cells as well as Thapsigargin-activated and PBMCsec-treated cells. Data are represented as mean ± SD, one-way ANOVA and Sidak's multiple comparisons test. ***$p<0.0002$

A



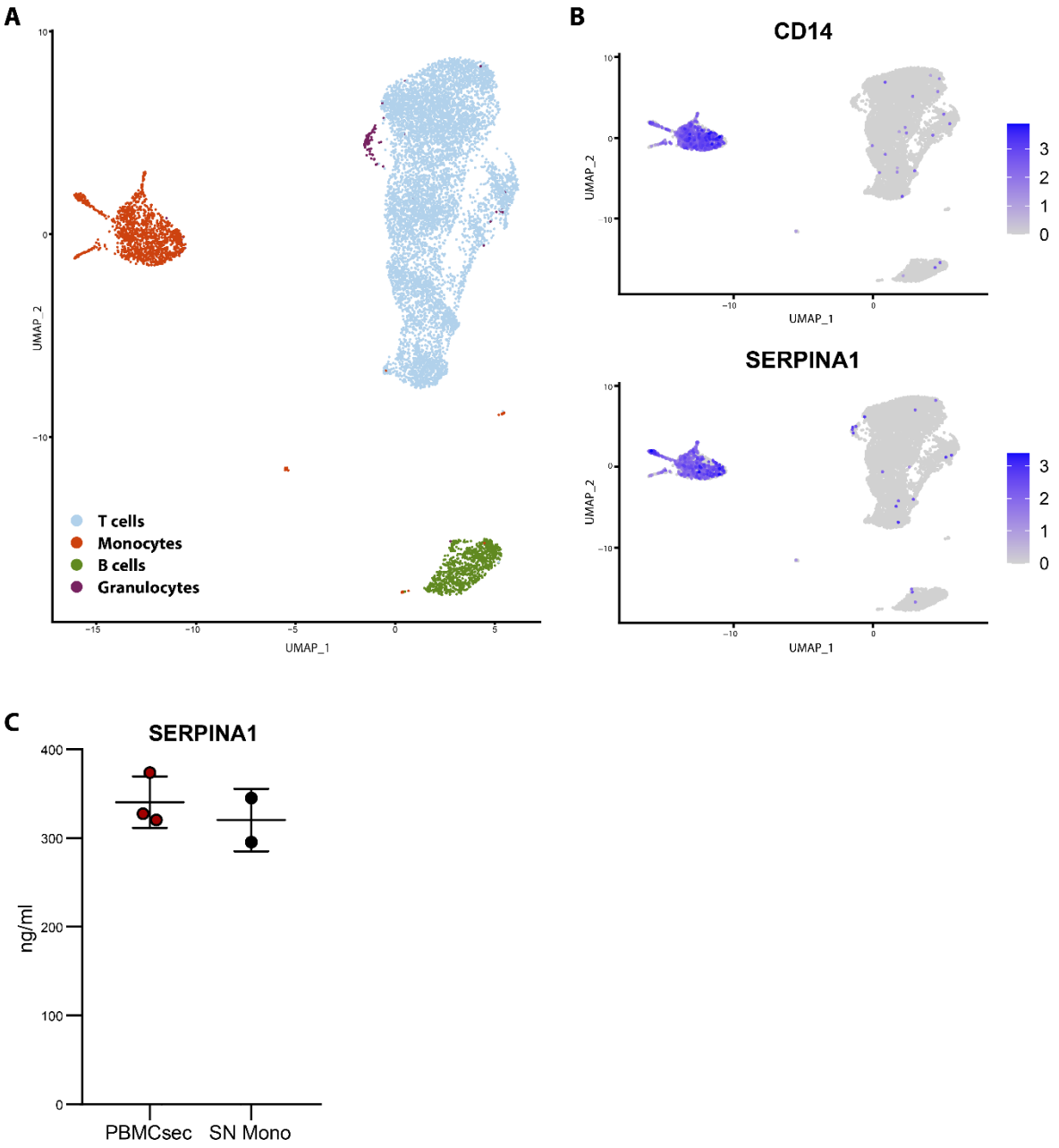
B



Label	Type	Adjusted volume intensity	# of analysed pixels
control	HIF-1α	109 825	13
control	HIF-1α	108 123	13
Ionomycin	HIF-1α	72 267	13
Ionomycin	HIF-1α	54 072	13
PBMCsec + IM	HIF-1α	160 245	13
PBMCsec + IM	HIF-1α	145 165	13
vehicle + IM	HIF-1α	112 189	13
vehicle + IM	HIF-1α	130 932	13
control	HO-1	136 363	13
control	HO-1	98 343	13
Ionomycin	HO-1	34 494	13
Ionomycin	HO-1	54 476	13
PBMCsec + IM	HO-1	121 447	13
PBMCsec + IM	HO-1	147 245	13
vehicle + IM	HO-1	64 068	13
vehicle + IM	HO-1	89 974	13

Supplementary Figure 5 Gating stragety and purity of isolated neutrophils and protein analysis of HIF-1α and HO-1

A) Flow cytometry gating strategy for the analysis of neutrophil purity after magnetic bead isolation is shown. Neutrophil purity was assessed by the percentage of $CD66b^+CD15^+$ cells and ranged from 92.9% to 96.5%. B) Analysed images of the proteome profiler of the protein levels of HO-1 and HIF-1α. Isolated neutrophils of four individual donors were stimulated with ionomycin and treated with PBMCsec or vehicle. Cell lysates of four individual donors and experiments were pooled. Raw analysis values are shown in the table below.



Supplementary Figure 6 SERPINA1 abundance in PBMCsec
Single cell RNA sequencing analysis of erythrocyte lysed whole blood shows (A) UMAP cluster depiction of captured cell populations. (B) Monocyte cluster were identified by the expression of CD14. SERPINA1 expression was almost exclusively found in the $CD14^{+}$ monocyte cluster. (C) SERPINA1 concentration in PBMCsec was analysed using ELISA. Supernatants of monocytes (SN Mono), cultured for 24 hours, were used as positive controls.

DISCUSSION

1 General discussion

Neutrophils belong to the most powerful immune cells mainly due to their capacity to form NETs as line defence mechanism of the innate immune system.[16] Despite their important function in host protection and pathogen elimination, accumulating evidence proves a more complex involvement of neutrophil effector functions in health and disease.[15,157] Particularly NET formation has been associated to detrimental host tissue damage and was shown to contribute to the progression of several diseases and pathologic conditions.[15,157] A broad range of studies was performed aiming to pharmacologically target NET formation or NET products, however, therapeutic drugs designed to specifically target NET formation are not yet available for in-patient use.[158,377,425] In this thesis, the effect of PBMCsec on NET formation in primary human neutrophils was investigated in an *ex vivo* setting. Our results provide evidence that PBMCsec effectively inhibits NET formation.

1.1 NET-derived extracellular DNA and histones

The overall reported inhibition of NET formation due to the application of PBMCsec treatment of activated neutrophils is indispensably associated with reduced extrusion of extracellular DNA and (modified) histones. Approximately 70% of the NETs components account for core histones and DNA.[156] Both, exhibit a dual role in host defence and possess potent anti-microbial as well as pro-inflammatory properties.[426] However, extracellular DNA serves as sign for tissue damage or programmed cell death and histones promote pro-inflammatory responses as they serve as danger associated molecular patterns if present in the extracellular space.[319,427] Modified histones, specifically citH3, were shown to majorly contribute to the pathogenesis of several pathologic conditions including acute lung injury, the disruption of the microvascular endothelial barrier and trigger positive feedback mechanisms which further potentiates NET formation.[428] Considering these detrimental effects of NET-derived DNA and histones, our findings suggest PBMCsec as potent treatment option for pathologic conditions strikingly influenced by increased availability of citH3 and extracellular DNA within tissues and circulation.

1.2 Synergy of different PBMCsec-substance classes

Previous studies already reported that several beneficial effects of PBMCsec treatment depend on the interplay of multiple subfractions of the secretome.[418,421] In concordance with these observations, we also observed that full inhibition of NET formation was only achieved in activated neutrophils upon treatment with the whole PBMCsec. As several PBMCsec derived fractions exerted partial inhibitory capacity we suggest that either individual factors within the protein and lipid fractions or a combination of factors found in these fractions act on different signalling molecules at different steps of the NETs signalling pathway.

It was previously shown that particularly the PBMCsec-derived lipid fraction possesses highly immunomodulatory functions as treatment of skin inflammation and allergic reactions with PBMCsec-lipids dampened dendritic cell function and reduced mast cell and basophil activation.[422,423] Furthermore, more in depth analysis of PBMCsec components revealed that it contains several lipid species such as phosphatidylserines, phosphatidylcholines, lysophosphatidylcholines, lyso-phosphatidylethanolamines, phosphatidylethanolamines and resolvins.[422] Analysis of the resolvins in particular revealed that several resolvins including resolvin D1 (RvD1), RvD2, RvD3, RvD4 and RvE1, were present at increased levels in PBMCsec compared to the secretome of non-irradiated PBMCs (unpublished data).

Resolvins have been previously associated with decreasing NETosis and alleviating abdominal aortic aneurism (AAA) disease burden.[429] Particularly RvD1 treatment was identified as potent NETosis inhibitor and was accompanied by decreased levels of the pro-inflammatory cytokines IL-1beta and IL-6. In parallel, the anti-inflammatory cytokine IL-10 was found to be increased upon RvD1 treatment. The specific NETs marker, citH3, was further found to be drastically decreased in RvD1 treated mice.[429] RvD4 was therapeutically administered in an experimental animal model of deep vein thrombosis and resulted in significantly enhanced resolution of thrombosis.[430] It was shown that RvD4 treatment reduces the number of neutrophils within the thrombus while promoting the recruitment of monocytes. RvD4 was attributed to exert anti-inflammatory effects by various mechanisms including the downregulation of adhesion molecules on immune cells, reducing the production of pro-inflammatory cytokines and diminishing leukocyte-endothelial cell interactions.[430] Several resolvins of the resolving T series (RvTs) were shown to act on neutrophils in a dual mechanism. On the on hand, they actively target yet unidentified mediators of the NETosis signalling pathway thereby reducing NET formation independently of the NET-triggering

stimuli. On the other hand, RvTs enhance NET-clearance by macrophages *in vitro* and *in vivo*.[431]

Taken together, accumulating evidence indicates that certain resolvins specifically target NET-formation, while others target neutrophil function and infiltration and some resolvins promote NET clearance by inducing increased phagocytosis in macrophages.[429-432] These studies led us to hypothesize that the NETosis inhibiting effect is in part due to the several resolinvs present in PBMCsec, and they might favour a shift in neutrophil effector functions as resolvins were observed to promote phagocytosis in macrophages.[432]

Furthermore, besides the lipid fraction, we also observed an inhibitory effect on NET formation by the proteins derived from PBMCsec. Previous studies revealed that calcium chelation by bovine or human serum albumin was sufficient to block NET formation in ionomycin-treated cells.[433] However, other stimuli, such as PMA, were shown to robustly induce NETosis despite the addition of bovine or human serum albumin.[433] Nevertheless, PMA-induced NET formation was similarly inhibited by PBMCsec treatment as calcium ionophore-induced NET formation. To further unravel which PBMCsec-derived protein or group of proteins is responsible for the observed partial inhibition of NET formation, proteomics analyses are required.

Since we observed only a partial inhibition of NET formation with the individual fractions but a strong inhibitory effect with reconstituted fractions, our data clearly indicate that this effect of PBMCsec is mediated by an interplay of the subfractions yielding a synergistic effect.

1.3 Inhibition of NETosis by a DNase-independent mechanism

Attempting to unravel the mode of action by which PBMCsec inhibits NET formation, we first had to investigate whether the observed NETs reduction was due to an active mechanism or a bystander result of DNA-degradation by DNases present in PBMCsec. Endogenous DNases function as potent host defence mechanism to maintain tissue integrity and degrade the DNA backbone of NETs.[378] Therefore, we tested whether PBMCsec possesses active DNases capable of degrading DNA and NETs. We neither observed DNA degradation in a cell-free assay nor could detect a reduction of citH3, indicating that PBMCsec does not degrade preformed NETs. It was previously shown that the variability of endogenous DNase activity critically affects for example vascular occlusion and autoimmune diseases as reduced DNase activity is associated with severely worsened patient prognosis.[378,434] Presence of DNase

inhibitors, genetic mutations, or the loss of endogenous DNases was observed in SLE resulting in decreased NETs clearance and aggravated disease progression.[434] Pharmacological targeting of DNase activity has been previously suggested as potential means to alleviate NETs-induced disease burden in SLE patients. However, this approach is at a preliminary state and specific circulating DNase inhibitors in SLE patients are not yet identified.[434] In the context of other diseases such as respiratory diseases, cancer, sepsis, and neurological disorders, DNase treatment resulted in a thoroughly positive effect on disease prognosis.[435] However, several studies fail to prove, that the observed beneficial effects of DNase treatment are due to degradation of NETs or the prevention of coagulation. Furthermore, neither timing, route of administration or dose are coherent in the different studies.[435] While beneficial effects of therapeutically administered DNases were partially reported, PBMCsec offers therapeutic means to inhibit NET formation rather than degrading NETs, thereby preventing host damage by other factors released during NET formation.

1.4 Calcium flux in neutrophils

Calcium represents a second messenger which is of tremendous importance for a great assortment of intracellular signalling cascades.[436] Previous studies have highlighted its importance in immune cells and particularly in neutrophils, as it was shown that calcium is critically involved in oxidative stress, neutrophil activation, inflammatory processes and cell death.[437-439] Independent of the mode of induction, NETosis relies on calcium as either initial inducer or downstream second messenger.[158,166] Considering the important role of this molecule we investigated the calcium flux in ionomycin-activated and PBMCsec-treated neutrophils. Our data indicate that the observed inhibitory effect of PBMCsec is not due to calcium scavenging as there was only a marginal decrease in calcium flux observed, which was quickly restored to similar levels as detected in control samples. While store operated calcium entry (SOCE) has been comprehensively investigated, another yet poorly understood receptor-dependent mechanism has been suggested.[436] SOCE, a two-step process, is accompanied by the depletion of intracellular calcium stores from the endoplasmic reticulum via sarco-endoplasmic reticulum calcium ATPase (SERCA) pumps. Subsequently, stromal interacting molecules, together with Orai proteins and transient receptor potential channels, forward this information to plasma membrane channels, which results in opening of calcium channels in order to replenish the depleted stores. [436] Thapsigargin, an irreversible inhibitor of SERCA pumps, has previously been used to force intracellular calcium store depletion.[436,440] We used Thapsigargin as stimulus to exclude that PBMCsec-treated neutrophils circumvent

NETosis by sequestration of excess calcium into intracellular calcium stores. Our data revealed that Thapsigargin not only serves as potent NETosis inducer itself, but also that the addition of PBMCsec prevented NET formation. Together, these data indicate that the mode of action of PBMCsec is mediated by influencing other factors than calcium that are involved further downstream in the NETosis signalling cascade.

1.5 Akt and NFκB signalling

Both Akt and NFκB signalling have been associated with NETosis. It was shown that activation of Akt is critical for both, PMA and calcium ionophore-induced NETosis.[441-444] Interestingly, our group has previously demonstrated that PBMCsec itself functions as potent inducer of Akt activation in a diverse set of cell types, such as primary human keratinocytes, fibroblasts, endothelial cells, Schwann cells as well as astrocytes.[412,414] Akt is known to suppress caspase signalling thereby promoting cell survival by blocking apoptosis.[443] The previously reported cytoprotective effects mediated by PBMCsec have partially been attributed to Akt activation. However, in terms of neutrophils and particularly NETosis, our data indicate that, despite mediating Akt activation in a celltype independent manner, the NETosis inhibiting activity by PBMCsec is most likely due to the modulation of other factors further downstream of the Akt signalling cascade or generally independent of this pathway.

However, it was previously demonstrated that activation of the Akt signalling pathway inhibits Raf-MEK-ERK signalling.[445,446] Interestingly, it was shown that pharmacological inhibition of Raf, as well as other members of the Raf-MEK-ERK signalling pathway, prevented NETosis.[181] Furthermore, Hakkim et al. demonstrated that Raf-MEK-ERK inhibitors also block the production of ROS due to the inability of ERK to phosphorylate $p47^{phox}$, thus impairing functional NADPH oxidase assembly.[181]

Furthermore, immunofluorescence staining of NFκB in ionomycin-activated neutrophils was marked by strong activation of NFκB. Interestingly, treatment of activated neutrophils with PBMCsec did not prevent or interfere with NFκB activation, however, also the immunofluorescence images clearly demonstrated that PBMCsec-treatment results in the inhibition of NET formation (data not shown). Together, these findings suggest that PBMCsec modulates other players involved in NETosis, that either overrule, function independently, or further downstream of NFκB.

1.6 Prevention of ROS production

One hallmark feature of NETosis is oxidative stress, and particularly the production of ROS.[117] Our data indicate, that one of the critical modes of action of PBMCsec in the inhibition of NET formation is the prevention of ROS production. Furthermore, our study revealed that HSPs were strongly down-regulated upon ionomycin-induced activation of NETosis. However, PBMCsec treatment of activated neutrophils counteracted the downregulation of heme oxygenase 1 (HO-1 or HSP32) as well as hypoxia inducible factor 1 alpha (HIF-1α).

1.6.1 Heme oxygenase 1

Previous studies have revealed that HO-1 upregulation occurs upon pathophysiological stimuli including endotoxemia, oxidative stress, ischemia, trauma-haemorrhage and inflammation.[447-450] Furthermore, it has been reported, that this enzyme critically contributes to the protection against oxidative tissue injury.[451] HO-1 is known to reduce factors such as $p47^{phox}$ and $p67^{phox}$, which are two important cytosolic proteins required for the assembly of functional NADPH oxidase.[451,452] Particularly the release of superoxide anion from neutrophils requires activation of functional NADPH oxidase.[451] During oxidative burst, activated neutrophils are capable of producing approximately 10nmol per minute of superoxide anion per one million neutrophils.[453] Neutrophil-derived ROS are known to directly act on endothelial cells reducing cell integrity and barrier function. During the development of lesions in atherosclerosis, ROS were reported to contribute to endothelial cell apoptosis. Additionally, ROS produced by neutrophils, particularly if excessively produced, have been linked to directly damage tissue in inflammatory bowel disease and potentially inducing gastrointestinal cancer.[91] Furthermore, HO-1 knockout in a murine model of renal ischemia-reperfusion injury was reported to exacerbate disease burden by inducing an upregulation of vascular cell adhesion molecule-1, thereby favouring increased neutrophil adhesion and the activation of inflammatory responses.[454]

Thus, our data suggest that PBMCsec mediated maintenance of high levels of HO-1, similar to those observed in neutrophils in a non-activated state, indirectly contributes to impaired ROS production by preventing the translocation of the proteins $p47^{phox}$ and $p67^{phox}$ required for the assembly of the NADPH oxidase complex. Moreover, PBMCsec treatment may further mitigate inflammatory responses by impeding neutrophil adhesion.

1.6.2 Hypoxia inducible factor

The transcriptional regulator HIF-1α is known to induce metabolic switches required for cell survival in response to hypoxic, stressed, infected or inflamed tissues. HIF-1α stabilization is required for the regulation of cellular metabolism and the mediation of gene expression.[455,456] We observed a robust decrease of HIF-1α protein content upon neutrophil activation with ionomycin, which was counteracted by PBMCsec treatment. Interestingly, several studies have reported contradictory results on the influence of ionomycin and other calcium ionophores in regard to the increased or decreased protein levels of HIF-1α.[457-460] It was previously suggested, that HIF-1α protein increase is a result of decreased intracellular calcium concentrations and attenuated proline hydroxylase activity.[459,460] Conversely, other studies reported that HIF-1α protein abundance was not affected by ionomycin. However, the same group reported an increase in transcriptional activity of HIF-1α.[458] Furthermore, it was reported that ionomycin-induced elevation of calcium concentration lead to the activation of a degradation pathway of HIF-1α.[457] Nevertheless, it is noteworthy, that these inconsistent and in part contradictory findings have to be interpreted carefully as these results are based on different cell types and calcium inducing agents and are not consistent with respect to normoxia or hypoxia.

It was previously shown that the loss of HIF-1α lead to tremendously impaired glycolysis and energy generation in macrophages and neutrophils, thereby resulting in heavily impaired effector functions including phagocytosis, intracellular killing of phagocytosed pathogens and migration.[461] Furthermore, evidence was provided, that HIF-1α critically contributes to neutrophil survival by suppressing apoptosis via NFB signalling.[462] Together, these observations lead us to hypothesize that the observed PBMCsec-induced upregulation of HIF-1α in activated neutrophils is a NETosis independent mode of action. We suggest, that while PBMCsec treatment impairs NET formation, it contributes to the maintenance of a functional immune response in neutrophils via HIF-1α promoted phagocytosis and intracellular killing pathogens, accompanied by reduced neutrophil apoptosis. Together, despite not sufficiently proven yet, this mode of action would provide a potent anti-NETs specific therapeutic approach with beneficial effects in terms of wound healing where proper pathogen clearance is essential while NET formation may impair wound healing.[305,314] Furthermore, inhibited NET formation in combination with other effector functions remaining functional would be beneficial particularly in pathologies marked by the presence of autoantibodies targeting neutrophils or NETs-specific components.[463]

1.7 PAD4 inhibition

PAD4 mediated histone citrullination represents one of the main features during NETosis, distinguishing it precisely from other forms of cell death.[195] PAD4 has been of high interest in research as a potential therapeutic target to inhibit NETosis due to its critical involvement in various pathologies including RA, multiple sclerosis (MS), cancer, sepsis, ischemia-reperfusion injury, heart failure and myocardial infarction.[464-466] Nevertheless, despite intense research, the particular mechanism how PAD4 inhibition is achieved, is not yet fully understood.[464] Besides the prevention of ROS production, our data indicate another mode of action by acting on PAD4 activity. We observed a PAD4 inhibition by PBMCsec in a cell-free assay. This finding suggests that as a response of stress, PBMCs secrete paracrine factors that function as PAD4 inhibitors. Interestingly, umbilical cord blood derived neutrophils from preterm and term infants fail to undergo NETosis upon stimulation.[467] This lacking immune-competency is quickly gained at day three post-delivery. Sequence analysis of proteins present in day 0 umbilical cord blood revealed several protein and peptide clusters to be different from venous blood plasma at day 28 post-delivery.[467] The sequence of one particular peptide was identified to be identical to the carboxy terminus of α-1-antitrypsin. This peptide showed strong NETosis inhibitory capacity even if applied to LPS-stimulated adult neutrophils.[467] The mode of action of α-1-antitrypsin was reported to be via PAD4 inhibition.[467] Furthermore, α-1-antitrypsin, also referred as SERPINA1, is known to inhibit neutrophil elastase and proteinase 3 as well as other intracellular and cell surface proteases.[468] It was previously reported, that circulating monocytes synthesize α-1-antitrypsin.[469] In line with these findings, using single cell RNA sequencing analysis, we found significantly high SERPINA1 expression levels in monocytes from healthy donors. Furthermore, we observed similar amounts of SERPINA1 in PBMCsec as in the supernatant of cultured monocytes. Together, these findings indicate, that PBMCsec derived SERPINA1 contributes to the inhibition of PAD4 and ultimately the prevention of NET formation.

2 Conclusion

In summary, the observations of this study attribute a strong NETs-inhibitory activity to PBMCsec. The identification of a dual mechanism by impairing two of the major key events during NETosis, ROS production and PAD4 activity (Figure 5), provides future therapeutic opportunities for a diverse set of diseases associated with NETosis, including for example myocardial infarction, heart failure, sepsis and rheumatoid arthritis.[16] PBMCsec has already been subjected to a pre-clinical toxicological assessment including intravenous and topical application, without any reported major adverse events (LPT, study number 35015). Hence, this study has paved the way for the therapeutic administration of PBMCsec in the context of NETs-associated diseases.

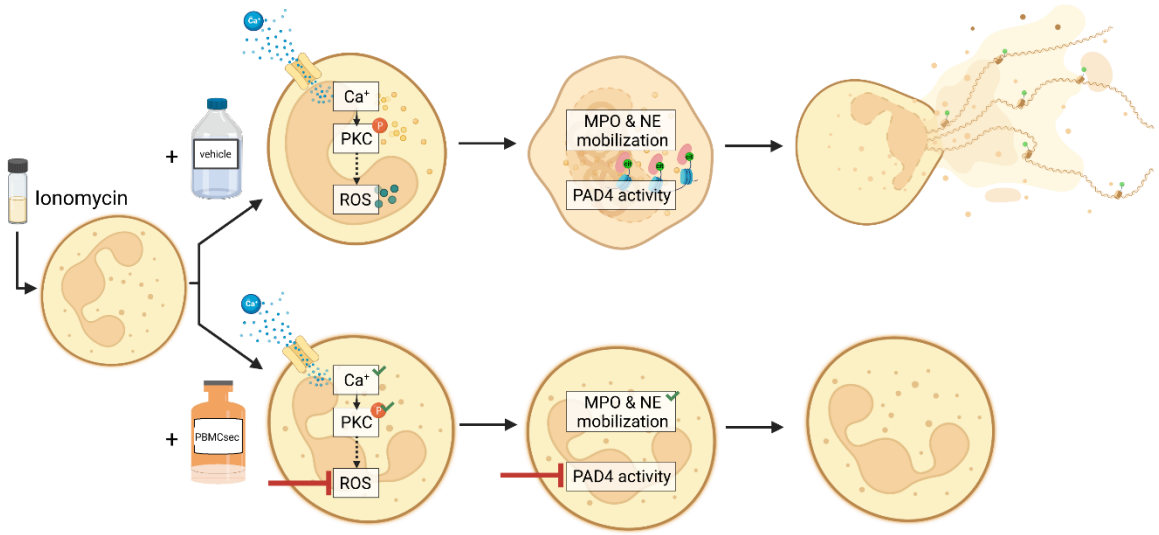


Figure 5 Graphical overview on the suggested inhibition of NETosis by PBMCsec.
Treatment of ionomycin activated neutrophils results in increased calcium influx, thereby initiating the NETosis cascade. While PBMCsec does not influence the activation and phosphorylation of Protein Kinase C (PKC), it prevents reactive oxygen species (ROS) production by a yet undefined mechanism. Myeloperoxidase (MPO) as well as neutrophil elastase (NE) activity remain unaltered by PBMCsec treatment. One of the hallmarks of NETosis, histone hypercitrullination by protein arginine deiminase 4 (PAD4), is most likely impaired by SERPINA1, present in PBMCsec.

3 Future prospective

This study has demonstrated the potent therapeutic potential of PBMCsec in terms of NETosis. Nevertheless, future studies are required to fully delineate the precise mode of action of PBMCsec. These studies should involve a comprehensive investigation of the potential cross-regulation of the Akt and Raf-MEK-ERK pathway. This will help to understand whether the observed prevention of ROS production may result from impaired ERK signalling. Furthermore, analysis of the translocation and activation of NADPH oxidase subunits such as $p67^{phox}$ and $p47^{phox}$, the latter being a target of ERK, would offer further valuable insight into the modulated NETosis signalling pathway. Lastly, as the lipid and protein fraction were observed to exert the most promising effects as single substance classes, meticulous identification of individual factors present in the secretome should be performed. Lipidomics and proteomics analyses of the secretome will most likely contribute to the understanding how and why PBMCsec exerts its cytoprotective and anti-inflammatory actions in such a broad spectrum of different cell populations.

MATERIALS & METHODS

The materials and methods used for this study are described in the aforementioned manuscript.

REFERENCES

1 Dean, L. *Blood Groups and Red Cell Antigens [Internet]*. (National Center for Biotechnology Information 2005).

2 Nicolas-Avila, J. A., Adrover, J. M. & Hidalgo, A. Neutrophils in Homeostasis, Immunity, and Cancer. *Immunity* **46**, 15-28, doi:10.1016/j.immuni.2016.12.012 (2017).

3 McKenna, E. *et al.* Neutrophils: Need for Standardized Nomenclature. *Front Immunol* **12**, 602963, doi:10.3389/fimmu.2021.602963 (2021).

4 Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* **13**, 159-175, doi:10.1038/nri3399 (2013).

5 Rosales, C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Front Physiol* **9**, 113, doi:10.3389/fphys.2018.00113 (2018).

6 Elaine M. Keohane, Catherine N. Otto & Walenga, J. M. *Rodak's Hematology: Clinical Principles and Applications*. Sixth Edition edn, (Elsevier, 2020).

7 Karasuyama, H., Mukai, K., Tsujimura, Y. & Obata, K. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat Rev Immunol* **9**, 9-13, doi:10.1038/nri2458 (2009).

8 Mukai, K., Obata, K., Tsujimura, Y. & Karasuyama, H. New insights into the roles for basophils in acute and chronic allergy. *Allergol Int* **58**, 11-19, doi:10.2332/allergolint.08-RAI-0059 (2009).

9 Maddur, M. S., Kaveri, S. V. & Bayry, J. Basophils as antigen presenting cells. *Trends Immunol* **31**, 45-48, doi:10.1016/j.it.2009.12.004 (2010).

10 Denzel, A. *et al.* Basophils enhance immunological memory responses. *Nat Immunol* **9**, 733-742, doi:10.1038/ni.1621 (2008).

11 Kawakami, T. Basophils now enhance memory. *Nat Immunol* **9**, 720-721, doi:10.1038/ni0708-720 (2008).

12 Sokol, C. L. & Medzhitov, R. Role of basophils in the initiation of Th2 responses. *Curr Opin Immunol* **22**, 73-77, doi:10.1016/j.coi.2010.01.012 (2010).

13 Gibbs, B. F., Streatfield, C. & Falcone, F. H. Basophils as critical orchestrators of Th2-type immune responses. *Expert Rev Clin Immunol* **5**, 725-734, doi:10.1586/eci.09.47 (2009).

14 Ramirez, G. A. *et al.* Eosinophils from Physiology to Disease: A Comprehensive Review. *Biomed Res Int* **2018**, 9095275, doi:10.1155/2018/9095275 (2018).

15 Wang, J. Neutrophils in tissue injury and repair. *Cell Tissue Res* **371**, 531-539, doi:10.1007/s00441-017-2785-7 (2018).

16 Fine, N., Tasevski, N., McCulloch, C. A., Tenenbaum, H. C. & Glogauer, M. The Neutrophil: Constant Defender and First Responder. *Front Immunol* **11**, 571085, doi:10.3389/fimmu.2020.571085 (2020).

17 Gupta, A. K. *et al.* Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. *FEBS Lett* **584**, 3193-3197, doi:10.1016/j.febslet.2010.06.006 (2010).

18 Thalin, C., Hisada, Y., Lundstrom, S., Mackman, N. & Wallen, H. Neutrophil Extracellular Traps: Villains and Targets in Arterial, Venous, and Cancer-Associated Thrombosis. *Arterioscler Thromb Vasc Biol* **39**, 1724-1738, doi:10.1161/ATVBAHA.119.312463 (2019).

19 Distelmaier, K. *et al.* Prognostic value of culprit site neutrophils in acute coronary syndrome. *Eur J Clin Invest* **44**, 257-265, doi:10.1111/eci.12228 (2014).

20 Mangold, A. *et al.* Neutrophil extracellular traps and monocyte subsets at the culprit lesion site of myocardial infarction patients. *Sci Rep* **9**, 16304, doi:10.1038/s41598-019-52671-y (2019).
21 Mangold, A. *et al.* Culprit site extracellular DNA and microvascular obstruction in ST-elevation myocardial infarction. *Cardiovasc Res*, doi:10.1093/cvr/cvab217 (2021).
22 Sollberger, G., Tilley, D. O. & Zychlinsky, A. Neutrophil Extracellular Traps: The Biology of Chromatin Externalization. *Dev Cell* **44**, 542-553, doi:10.1016/j.devcel.2018.01.019 (2018).
23 von Vietinghoff, S. & Ley, K. Homeostatic regulation of blood neutrophil counts. *J Immunol* **181**, 5183-5188, doi:10.4049/jimmunol.181.8.5183 (2008).
24 Iwasaki, H. *et al.* Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* **106**, 1590-1600, doi:10.1182/blood-2005-03-0860 (2005).
25 Bjerregaard, M. D., Jurlander, J., Klausen, P., Borregaard, N. & Cowland, J. B. The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood* **101**, 4322-4332, doi:10.1182/blood-2002-03-0835 (2003).
26 Ostuni, R., Natoli, G., Cassatella, M. A. & Tamassia, N. Epigenetic regulation of neutrophil development and function. *Semin Immunol* **28**, 83-93, doi:10.1016/j.smim.2016.04.002 (2016).
27 Rieger, M. A., Hoppe, P. S., Smejkal, B. M., Eitelhuber, A. C. & Schroeder, T. Hematopoietic cytokines can instruct lineage choice. *Science* **325**, 217-218, doi:10.1126/science.1171461 (2009).
28 Seymour, J. F. *et al.* Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity, perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival. *Blood* **90**, 3037-3049 (1997).
29 Liu, F., Poursine-Laurent, J., Wu, H. Y. & Link, D. C. Interleukin-6 and the granulocyte colony-stimulating factor receptor are major independent regulators of granulopoiesis in vivo but are not required for lineage commitment or terminal differentiation. *Blood* **90**, 2583-2590 (1997).
30 Laslo, P. *et al.* Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* **126**, 755-766, doi:10.1016/j.cell.2006.06.052 (2006).
31 Zarebski, A. *et al.* Mutations in growth factor independent-1 associated with human neutropenia block murine granulopoiesis through colony stimulating factor-1. *Immunity* **28**, 370-380, doi:10.1016/j.immuni.2007.12.020 (2008).
32 Velu, C. S., Baktula, A. M. & Grimes, H. L. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood* **113**, 4720-4728, doi:10.1182/blood-2008-11-190215 (2009).
33 Pulikkan, J. A. *et al.* Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood* **115**, 1768-1778, doi:10.1182/blood-2009-08-240101 (2010).
34 D'Alo, F. *et al.* The amino terminal and E2F interaction domains are critical for C/EBP alpha-mediated induction of granulopoietic development of hematopoietic cells. *Blood* **102**, 3163-3171, doi:10.1182/blood-2003-02-0479 (2003).
35 Yamanaka, R. *et al.* Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proc Natl Acad Sci U S A* **94**, 13187-13192, doi:10.1073/pnas.94.24.13187 (1997).
36 Lekstrom-Himes, J. & Xanthopoulos, K. G. CCAAT/enhancer binding protein epsilon is critical for effective neutrophil-mediated response to inflammatory challenge. *Blood* **93**, 3096-3105 (1999).

37 Eash, K. J., Means, J. M., White, D. W. & Link, D. C. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood* **113**, 4711-4719, doi:10.1182/blood-2008-09-177287 (2009).
38 Summers, C. *et al.* Neutrophil kinetics in health and disease. *Trends Immunol* **31**, 318-324, doi:10.1016/j.it.2010.05.006 (2010).
39 Kohler, A. *et al.* G-CSF-mediated thrombopoietin release triggers neutrophil motility and mobilization from bone marrow via induction of Cxcr2 ligands. *Blood* **117**, 4349-4357, doi:10.1182/blood-2010-09-308387 (2011).
40 Eash, K. J., Greenbaum, A. M., Gopalan, P. K. & Link, D. C. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest* **120**, 2423-2431, doi:10.1172/JCI41649 (2010).
41 Pillay, J. *et al.* In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* **116**, 625-627, doi:10.1182/blood-2010-01-259028 (2010).
42 Colotta, F., Re, F., Polentarutti, N., Sozzani, S. & Mantovani, A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80**, 2012-2020 (1992).
43 Filep, J. G. & Ariel, A. Neutrophil heterogeneity and fate in inflamed tissues: implications for the resolution of inflammation. *Am J Physiol Cell Physiol* **319**, C510-C532, doi:10.1152/ajpcell.00181.2020 (2020).
44 Mora-Jensen, H. *et al.* Technical advance: immunophenotypical characterization of human neutrophil differentiation. *J Leukoc Biol* **90**, 629-634, doi:10.1189/jlb.0311123 (2011).
45 Casanova-Acebes, M. *et al.* Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* **153**, 1025-1035, doi:10.1016/j.cell.2013.04.040 (2013).
46 Stark, M. A. *et al.* Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* **22**, 285-294, doi:10.1016/j.immuni.2005.01.011 (2005).
47 Aggarwal, S., Ghilardi, N., Xie, M. H., de Sauvage, F. J. & Gurney, A. L. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* **278**, 1910-1914, doi:10.1074/jbc.M207577200 (2003).
48 Forlow, S. B. *et al.* Increased granulopoiesis through interleukin-17 and granulocyte colony-stimulating factor in leukocyte adhesion molecule-deficient mice. *Blood* **98**, 3309-3314, doi:10.1182/blood.v98.12.3309 (2001).
49 Schwarzenberger, P. *et al.* Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *J Immunol* **164**, 4783-4789, doi:10.4049/jimmunol.164.9.4783 (2000).
50 Schwarzenberger, P. *et al.* IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J Immunol* **161**, 6383-6389 (1998).
51 Manfredi, A. A., Ramirez, G. A., Rovere-Querini, P. & Maugeri, N. The Neutrophil's Choice: Phagocytose vs Make Neutrophil Extracellular Traps. *Front Immunol* **9**, 288, doi:10.3389/fimmu.2018.00288 (2018).
52 Hepburn, A. L. The LE cell. *Rheumatology (Oxford)* **40**, 826-827, doi:10.1093/rheumatology/40.7.826 (2001).
53 Pisetsky, D. S. The LE cell: crime scene or crime stopper? *Arthritis Res Ther* **14**, 120, doi:10.1186/ar3878 (2012).
54 Hellberg, L. *et al.* Proinflammatory stimuli enhance phagocytosis of apoptotic cells by neutrophil granulocytes. *ScientificWorldJournal* **11**, 2230-2236, doi:10.1100/2011/413271 (2011).

55 Esmann, L. *et al.* Phagocytosis of apoptotic cells by neutrophil granulocytes: diminished proinflammatory neutrophil functions in the presence of apoptotic cells. *J Immunol* **184**, 391-400, doi:10.4049/jimmunol.0900564 (2010).
56 Galati, G. *et al.* In vivo administration of GM-CSF promotes the clearance of apoptotic cells: effects on monocytes and polymorphonuclear leukocytes. *J Leukoc Biol* **67**, 174-182, doi:10.1002/jlb.67.2.174 (2000).
57 Tyurin, V. A. *et al.* Oxidatively modified phosphatidylserines on the surface of apoptotic cells are essential phagocytic 'eat-me' signals: cleavage and inhibition of phagocytosis by Lp-PLA2. *Cell Death Differ* **21**, 825-835, doi:10.1038/cdd.2014.1 (2014).
58 Kagan, V. E. *et al.* A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J Immunol* **169**, 487-499, doi:10.4049/jimmunol.169.1.487 (2002).
59 Manfredi, A. A., Rovere-Querini, P. & Maugeri, N. Dangerous connections: neutrophils and the phagocytic clearance of activated platelets. *Curr Opin Hematol* **17**, 3-8, doi:10.1097/MOH.0b013e3283324f97 (2010).
60 Elliott, M. R. & Ravichandran, K. S. The Dynamics of Apoptotic Cell Clearance. *Dev Cell* **38**, 147-160, doi:10.1016/j.devcel.2016.06.029 (2016).
61 Kim, S. J. & Jenne, C. N. Role of platelets in neutrophil extracellular trap (NET) production and tissue injury. *Semin Immunol* **28**, 546-554, doi:10.1016/j.smim.2016.10.013 (2016).
62 Sreeramkumar, V. *et al.* Neutrophils scan for activated platelets to initiate inflammation. *Science* **346**, 1234-1238, doi:10.1126/science.1256478 (2014).
63 Theoret, J. F., Bienvenu, J. G., Kumar, A. & Merhi, Y. P-selectin antagonism with recombinant p-selectin glycoprotein ligand-1 (rPSGL-Ig) inhibits circulating activated platelet binding to neutrophils induced by damaged arterial surfaces. *J Pharmacol Exp Ther* **298**, 658-664 (2001).
64 Singbartl, K., Forlow, S. B. & Ley, K. Platelet, but not endothelial, P-selectin is critical for neutrophil-mediated acute postischemic renal failure. *FASEB J* **15**, 2337-2344, doi:10.1096/fj.01-0199com (2001).
65 Lefer, A. M., Campbell, B., Scalia, R. & Lefer, D. J. Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion: role of selectins. *Circulation* **98**, 1322-1328, doi:10.1161/01.cir.98.13.1322 (1998).
66 Maugeri, N. *et al.* Neutrophils phagocytose activated platelets in vivo: a phosphatidylserine, P-selectin, and {beta}2 integrin-dependent cell clearance program. *Blood* **113**, 5254-5265, doi:10.1182/blood-2008-09-180794 (2009).
67 Evangelista, V., Manarini, S., Coller, B. S. & Smyth, S. S. Role of P-selectin, beta2-integrins, and Src tyrosine kinases in mouse neutrophil-platelet adhesion. *J Thromb Haemost* **1**, 1048-1054, doi:10.1046/j.1538-7836.2003.00214.x (2003).
68 Gardiner, E. E. *et al.* Regulation of P-selectin binding to the neutrophil P-selectin counter-receptor P-selectin glycoprotein ligand-1 by neutrophil elastase and cathepsin G. *Blood* **98**, 1440-1447, doi:10.1182/blood.v98.5.1440 (2001).
69 Kornerup, K. N., Salmon, G. P., Pitchford, S. C., Liu, W. L. & Page, C. P. Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *J Appl Physiol (1985)* **109**, 758-767, doi:10.1152/japplphysiol.01086.2009 (2010).
70 Huo, Y. *et al.* Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* **9**, 61-67, doi:10.1038/nm810 (2003).

71 Peters, M. J. *et al.* Circulating platelet-neutrophil complexes represent a subpopulation of activated neutrophils primed for adhesion, phagocytosis and intracellular killing. *Br J Haematol* **106**, 391-399, doi:10.1046/j.1365-2141.1999.01553.x (1999).
72 Maugeri, N. *et al.* An intense and short-lasting burst of neutrophil activation differentiates early acute myocardial infarction from systemic inflammatory syndromes. *PLoS One* **7**, e39484, doi:10.1371/journal.pone.0039484 (2012).
73 Maugeri, N. *et al.* Clearance of circulating activated platelets in polycythemia vera and essential thrombocythemia. *Blood* **118**, 3359-3366, doi:10.1182/blood-2011-02-337337 (2011).
74 Ma, R. *et al.* Phosphatidylserine-mediated platelet clearance by endothelium decreases platelet aggregates and procoagulant activity in sepsis. *Sci Rep* **7**, 4978, doi:10.1038/s41598-017-04773-8 (2017).
75 Bilyy, R. O. *et al.* Macrophages discriminate glycosylation patterns of apoptotic cell-derived microparticles. *J Biol Chem* **287**, 496-503, doi:10.1074/jbc.M111.273144 (2012).
76 McDonald, B., Urrutia, R., Yipp, B. G., Jenne, C. N. & Kubes, P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe* **12**, 324-333, doi:10.1016/j.chom.2012.06.011 (2012).
77 Deniset, J. F. & Kubes, P. Recent advances in understanding neutrophils. *F1000Res* **5**, 2912, doi:10.12688/f1000research.9691.1 (2016).
78 Schauer, C. *et al.* Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med* **20**, 511-517, doi:10.1038/nm.3547 (2014).
79 Lacy, P. & Eitzen, G. Control of granule exocytosis in neutrophils. *Front Biosci* **13**, 5559-5570, doi:10.2741/3099 (2008).
80 Cassatella, M. A., Ostberg, N. K., Tamassia, N. & Soehnlein, O. Biological Roles of Neutrophil-Derived Granule Proteins and Cytokines. *Trends Immunol* **40**, 648-664, doi:10.1016/j.it.2019.05.003 (2019).
81 Rorvig, S., Ostergaard, O., Heegaard, N. H. & Borregaard, N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. *J Leukoc Biol* **94**, 711-721, doi:10.1189/jlb.1212619 (2013).
82 Mollinedo, F. Neutrophil Degranulation, Plasticity, and Cancer Metastasis. *Trends Immunol* **40**, 228-242, doi:10.1016/j.it.2019.01.006 (2019).
83 Mantovani, A., Cassatella, M. A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11**, 519-531, doi:10.1038/nri3024 (2011).
84 Lominadze, G. *et al.* Proteomic analysis of human neutrophil granules. *Mol Cell Proteomics* **4**, 1503-1521, doi:10.1074/mcp.M500143-MCP200 (2005).
85 Kettritz, R. Neutral serine proteases of neutrophils. *Immunol Rev* **273**, 232-248, doi:10.1111/imr.12441 (2016).
86 Munafo, D. B. *et al.* Rab27a is a key component of the secretory machinery of azurophilic granules in granulocytes. *Biochem J* **402**, 229-239, doi:10.1042/BJ20060950 (2007).
87 Yin, C. & Heit, B. Armed for destruction: formation, function and trafficking of neutrophil granules. *Cell Tissue Res* **371**, 455-471, doi:10.1007/s00441-017-2731-8 (2018).
88 Clemmensen, S. N., Udby, L. & Borregaard, N. Subcellular fractionation of human neutrophils and analysis of subcellular markers. *Methods Mol Biol* **1124**, 53-76, doi:10.1007/978-1-62703-845-4_5 (2014).

89 Rorvig, 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* **86**, 1439-1449, doi:10.1189/jlb.1008606 (2009).
90 Uriarte, S. M. *et al.* Comparison of proteins expressed on secretory vesicle membranes and plasma membranes of human neutrophils. *J Immunol* **180**, 5575-5581, doi:10.4049/jimmunol.180.8.5575 (2008).
91 Mayadas, T. N., Cullere, X. & Lowell, C. A. The multifaceted functions of neutrophils. *Annu Rev Pathol* **9**, 181-218, doi:10.1146/annurev-pathol-020712-164023 (2014).
92 Miralda, I., Uriarte, S. M. & McLeish, K. R. Multiple Phenotypic Changes Define Neutrophil Priming. *Front Cell Infect Microbiol* **7**, 217, doi:10.3389/fcimb.2017.00217 (2017).
93 Freeman, S. A. & Grinstein, S. Phagocytosis: receptors, signal integration, and the cytoskeleton. *Immunol Rev* **262**, 193-215, doi:10.1111/imr.12212 (2014).
94 Mitchell, T., Lo, A., Logan, M. R., Lacy, P. & Eitzen, G. Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling. *Am J Physiol Cell Physiol* **295**, C1354-1365, doi:10.1152/ajpcell.00239.2008 (2008).
95 Sun, C. X., Magalhaes, M. A. & Glogauer, M. Rac1 and Rac2 differentially regulate actin free barbed end formation downstream of the fMLP receptor. *J Cell Biol* **179**, 239-245, doi:10.1083/jcb.200705122 (2007).
96 Abdel-Latif, D., Steward, M. & Lacy, P. Neutrophil primary granule release and maximal superoxide generation depend on Rac2 in a common signalling pathway. *Can J Physiol Pharmacol* **83**, 69-75, doi:10.1139/y04-123 (2005).
97 Abdel-Latif, D. *et al.* Rac2 is critical for neutrophil primary granule exocytosis. *Blood* **104**, 832-839, doi:10.1182/blood-2003-07-2624 (2004).
98 Eitzen, G. *et al.* Proteomic analysis of secretagogue-stimulated neutrophils implicates a role for actin and actin-interacting proteins in Rac2-mediated granule exocytosis. *Proteome Sci* **9**, 70, doi:10.1186/1477-5956-9-70 (2011).
99 Bengtsson, T., Dahlgren, C., Stendahl, O. & Andersson, T. Actin assembly and regulation of neutrophil function: effects of cytochalasin B and tetracaine on chemotactic peptide-induced O2- production and degranulation. *J Leukoc Biol* **49**, 236-244, doi:10.1002/jlb.49.3.236 (1991).
100 Mocsai, A., Ligeti, E., Lowell, C. A. & Berton, G. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J Immunol* **162**, 1120-1126 (1999).
101 Perskvist N, R. K., Kulyté A, Stendahl O. Rab5a GTPase regulates fusion between pathogen-containing phagosomes and cytoplasmic organelles in human neutrophils. . *Journal of Cell Science* **115**, 1321-1330, doi: 10.1242/jcs.115.6.1321 (2002).
102 Kovacs, M. *et al.* The Src family kinases Hck, Fgr, and Lyn are critical for the generation of the in vivo inflammatory environment without a direct role in leukocyte recruitment. *J Exp Med* **211**, 1993-2011, doi:10.1084/jem.20132496 (2014).
103 Mocsai, A. *et al.* Kinase pathways in chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein kinase activated by Src family kinases. *J Immunol* **164**, 4321-4331, doi:10.4049/jimmunol.164.8.4321 (2000).
104 Jog, N. R. *et al.* Heat shock protein 27 regulates neutrophil chemotaxis and exocytosis through two independent mechanisms. *J Immunol* **178**, 2421-2428, doi:10.4049/jimmunol.178.4.2421 (2007).
105 Jozsef, L., Khreiss, T., El Kebir, D. & Filep, J. G. Activation of TLR-9 induces IL-8 secretion through peroxynitrite signaling in human neutrophils. *J Immunol* **176**, 1195-1202, doi:10.4049/jimmunol.176.2.1195 (2006).

106 Sekheri, M., El Kebir, D., Edner, N. & Filep, J. G. 15-Epi-LXA4 and 17-epi-RvD1 restore TLR9-mediated impaired neutrophil phagocytosis and accelerate resolution of lung inflammation. *Proc Natl Acad Sci U S A* **117**, 7971-7980, doi:10.1073/pnas.1920193117 (2020).
107 Maa, M. C. *et al.* The iNOS/Src/FAK axis is critical in Toll-like receptor-mediated cell motility in macrophages. *Biochim Biophys Acta* **1813**, 136-147, doi:10.1016/j.bbamcr.2010.09.004 (2011).
108 Lodge, K. M., Cowburn, A. S., Li, W. & Condliffe, A. M. The Impact of Hypoxia on Neutrophil Degranulation and Consequences for the Host. *Int J Mol Sci* **21**, doi:10.3390/ijms21041183 (2020).
109 Hamada, K., Miyatake, H., Terauchi, A. & Mikoshiba, K. IP3-mediated gating mechanism of the IP3 receptor revealed by mutagenesis and X-ray crystallography. *Proc Natl Acad Sci U S A* **114**, 4661-4666, doi:10.1073/pnas.1701420114 (2017).
110 Freeman, S. A. *et al.* Integrins Form an Expanding Diffusional Barrier that Coordinates Phagocytosis. *Cell* **164**, 128-140, doi:10.1016/j.cell.2015.11.048 (2016).
111 Hu, C. *et al.* Fusion of cells by flipped SNAREs. *Science* **300**, 1745-1749, doi:10.1126/science.1084909 (2003).
112 Catz, S. D. & McLeish, K. R. Therapeutic targeting of neutrophil exocytosis. *J Leukoc Biol* **107**, 393-408, doi:10.1002/JLB.3RI0120-645R (2020).
113 Mollinedo, F. *et al.* Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. *J Immunol* **177**, 2831-2841, doi:10.4049/jimmunol.177.5.2831 (2006).
114 Mollinedo, F., Martin-Martin, B., Calafat, J., Nabokina, S. M. & Lazo, P. A. Role of vesicle-associated membrane protein-2, through Q-soluble N-ethylmaleimide-sensitive factor attachment protein receptor/R-soluble N-ethylmaleimide-sensitive factor attachment protein receptor interaction, in the exocytosis of specific and tertiary granules of human neutrophils. *J Immunol* **170**, 1034-1042, doi:10.4049/jimmunol.170.2.1034 (2003).
115 Lau, D. *et al.* Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc Natl Acad Sci U S A* **102**, 431-436, doi:10.1073/pnas.0405193102 (2005).
116 Pasparakis, M. & Vandenabeele, P. Necroptosis and its role in inflammation. *Nature* **517**, 311-320, doi:10.1038/nature14191 (2015).
117 Fuchs, T. A. *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231-241, doi:10.1083/jcb.200606027 (2007).
118 Dzhagalov, I., St John, A. & He, Y. W. The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood* **109**, 1620-1626, doi:10.1182/blood-2006-03-013771 (2007).
119 El Kebir, D., Jozsef, L., Pan, W. & Filep, J. G. Myeloperoxidase delays neutrophil apoptosis through CD11b/CD18 integrins and prolongs inflammation. *Circ Res* **103**, 352-359, doi:10.1161/01.RES.0000326772.76822.7a (2008).
120 Bournazou, I. *et al.* Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *J Clin Invest* **119**, 20-32, doi:10.1172/JCI36226 (2009).
121 Lutaty, A. *et al.* A 17-kDa Fragment of Lactoferrin Associates With the Termination of Inflammation and Peptides Within Promote Resolution. *Front Immunol* **9**, 644, doi:10.3389/fimmu.2018.00644 (2018).
122 Lee, W. L., Harrison, R. E. & Grinstein, S. Phagocytosis by neutrophils. *Microbes Infect* **5**, 1299-1306, doi:10.1016/j.micinf.2003.09.014 (2003).
123 Nordenfelt, P. & Tapper, H. Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* **90**, 271-284, doi:10.1189/jlb.0810457 (2011).

124 Gierlikowska, B., Stachura, A., Gierlikowski, W. & Demkow, U. Phagocytosis, Degranulation and Extracellular Traps Release by Neutrophils-The Current Knowledge, Pharmacological Modulation and Future Prospects. *Front Pharmacol* **12**, 666732, doi:10.3389/fphar.2021.666732 (2021).
125 Jaumouille, V. & Waterman, C. M. Physical Constraints and Forces Involved in Phagocytosis. *Front Immunol* **11**, 1097, doi:10.3389/fimmu.2020.01097 (2020).
126 Guo, R. F. & Ward, P. A. Role of C5a in inflammatory responses. *Annu Rev Immunol* **23**, 821-852, doi:10.1146/annurev.immunol.23.021704.115835 (2005).
127 Mollnes, T. E. *et al.* Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* **100**, 1869-1877 (2002).
128 Berger, M., Sorensen, R. U., Tosi, M. F., Dearborn, D. G. & Doring, G. Complement receptor expression on neutrophils at an inflammatory site, the Pseudomonas-infected lung in cystic fibrosis. *J Clin Invest* **84**, 1302-1313, doi:10.1172/JCI114298 (1989).
129 van den Berg, C. W. *et al.* Mechanism of neutrophil dysfunction: neutrophil serine proteases cleave and inactivate the C5a receptor. *J Immunol* **192**, 1787-1795, doi:10.4049/jimmunol.1301920 (2014).
130 Fairn, G. D. & Grinstein, S. How nascent phagosomes mature to become phagolysosomes. *Trends Immunol* **33**, 397-405, doi:10.1016/j.it.2012.03.003 (2012).
131 Mukherjee, S., Ghosh, R. N. & Maxfield, F. R. Endocytosis. *Physiol Rev* **77**, 759-803, doi:10.1152/physrev.1997.77.3.759 (1997).
132 Faurschou, M. & Borregaard, N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* **5**, 1317-1327, doi:10.1016/j.micinf.2003.09.008 (2003).
133 Mayadas, T. N. & Cullere, X. Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol* **26**, 388-395, doi:10.1016/j.it.2005.05.002 (2005).
134 Filep, J. G. & El Kebir, D. Neutrophil apoptosis: a target for enhancing the resolution of inflammation. *J Cell Biochem* **108**, 1039-1046, doi:10.1002/jcb.22351 (2009).
135 Ley, K. *et al.* Neutrophils: New insights and open questions. *Sci Immunol* **3**, doi:10.1126/sciimmunol.aat4579 (2018).
136 Berends, E. T. *et al.* Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. *J Innate Immun* **2**, 576-586, doi:10.1159/000319909 (2010).
137 Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535, doi:10.1126/science.1092385 (2004).
138 Lappann, M. *et al.* In vitro resistance mechanisms of Neisseria meningitidis against neutrophil extracellular traps. *Mol Microbiol* **89**, 433-449, doi:10.1111/mmi.12288 (2013).
139 Pilsczek, F. H. *et al.* A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J Immunol* **185**, 7413-7425, doi:10.4049/jimmunol.1000675 (2010).
140 Saitoh, T. *et al.* Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* **12**, 109-116, doi:10.1016/j.chom.2012.05.015 (2012).
141 Urban, C. F., Reichard, U., Brinkmann, V. & Zychlinsky, A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol* **8**, 668-676, doi:10.1111/j.1462-5822.2005.00659.x (2006).
142 Chow, O. A. *et al.* Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe* **8**, 445-454, doi:10.1016/j.chom.2010.10.005 (2010).
143 Morshed, M. *et al.* NADPH oxidase-independent formation of extracellular DNA traps by basophils. *J Immunol* **192**, 5314-5323, doi:10.4049/jimmunol.1303418 (2014).

144 von Kockritz-Blickwede, M. *et al.* Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* **111**, 3070-3080, doi:10.1182/blood-2007-07-104018 (2008).
145 Yousefi, S. *et al.* Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat Med* **14**, 949-953, doi:10.1038/nm.1855 (2008).
146 Adrover, J. M. *et al.* Programmed 'disarming' of the neutrophil proteome reduces the magnitude of inflammation. *Nat Immunol* **21**, 135-144, doi:10.1038/s41590-019-0571-2 (2020).
147 Clark, S. R. *et al.* Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* **13**, 463-469, doi:10.1038/nm1565 (2007).
148 Yipp, B. G. *et al.* Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med* **18**, 1386-1393, doi:10.1038/nm.2847 (2012).
149 Yipp, B. G. & Kubes, P. NETosis: how vital is it? *Blood* **122**, 2784-2794, doi:10.1182/blood-2013-04-457671 (2013).
150 Sorvillo, N., Cherpokova, D., Martinod, K. & Wagner, D. D. Extracellular DNA NET-Works With Dire Consequences for Health. *Circ Res* **125**, 470-488, doi:10.1161/CIRCRESAHA.119.314581 (2019).
151 Wong, S. L. & Wagner, D. D. Peptidylarginine deiminase 4: a nuclear button triggering neutrophil extracellular traps in inflammatory diseases and aging. *FASEB J*, fj201800691R, doi:10.1096/fj.201800691R (2018).
152 Thiam, H. R., Wong, S. L., Wagner, D. D. & Waterman, C. M. Cellular Mechanisms of NETosis. *Annu Rev Cell Dev Biol* **36**, 191-218, doi:10.1146/annurev-cellbio-020520-111016 (2020).
153 Pires, R. H., Felix, S. B. & Delcea, M. The architecture of neutrophil extracellular traps investigated by atomic force microscopy. *Nanoscale* **8**, 14193-14202, doi:10.1039/c6nr03416k (2016).
154 Chapman, E. A. *et al.* Caught in a Trap? Proteomic Analysis of Neutrophil Extracellular Traps in Rheumatoid Arthritis and Systemic Lupus Erythematosus. *Front Immunol* **10**, 423, doi:10.3389/fimmu.2019.00423 (2019).
155 Petretto, A. *et al.* Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. *PLoS One* **14**, e0218946, doi:10.1371/journal.pone.0218946 (2019).
156 Urban, C. F. *et al.* Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog* **5**, e1000639, doi:10.1371/journal.ppat.1000639 (2009).
157 Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* **18**, 134-147, doi:10.1038/nri.2017.105 (2018).
158 Gupta, A. K., Giaglis, S., Hasler, P. & Hahn, S. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. *PLoS One* **9**, e97088, doi:10.1371/journal.pone.0097088 (2014).
159 Keshari, R. S. *et al.* Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. *PLoS One* **7**, e48111, doi:10.1371/journal.pone.0048111 (2012).
160 Rossaint, J. *et al.* Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. *Blood* **123**, 2573-2584, doi:10.1182/blood-2013-07-516484 (2014).
161 Mohanty, T. *et al.* A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa. *Blood* **126**, 2128-2137, doi:10.1182/blood-2015-04-641142 (2015).

162 Raftery, M. J. *et al.* beta2 integrin mediates hantavirus-induced release of neutrophil extracellular traps. *J Exp Med* **211**, 1485-1497, doi:10.1084/jem.20131092 (2014).
163 Kenny, E. F. *et al.* Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife* **6**, doi:10.7554/eLife.24437 (2017).
164 Wang, Y. *et al.* Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol* **184**, 205-213, doi:10.1083/jcb.200806072 (2009).
165 Dixit, N. & Simon, S. I. Chemokines, selectins and intracellular calcium flux: temporal and spatial cues for leukocyte arrest. *Front Immunol* **3**, 188, doi:10.3389/fimmu.2012.00188 (2012).
166 Immler, R., Simon, S. I. & Sperandio, M. Calcium signalling and related ion channels in neutrophil recruitment and function. *Eur J Clin Invest* **48 Suppl 2**, e12964, doi:10.1111/eci.12964 (2018).
167 Kandasamy, K., Bezavada, L., Escue, R. B. & Parthasarathi, K. Lipopolysaccharide induces endoplasmic store Ca2+-dependent inflammatory responses in lung microvessels. *PLoS One* **8**, e63465, doi:10.1371/journal.pone.0063465 (2013).
168 Schappe, M. S. *et al.* Chanzyme TRPM7 Mediates the Ca(2+) Influx Essential for Lipopolysaccharide-Induced Toll-Like Receptor 4 Endocytosis and Macrophage Activation. *Immunity* **48**, 59-74 e55, doi:10.1016/j.immuni.2017.11.026 (2018).
169 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* **92**, 841-849, doi:10.1189/jlb.1211601 (2012).
170 van der Linden, M., Westerlaken, G. H. A., van der Vlist, M., van Montfrans, J. & Meyaard, L. Differential Signalling and Kinetics of Neutrophil Extracellular Trap Release Revealed by Quantitative Live Imaging. *Sci Rep* **7**, 6529, doi:10.1038/s41598-017-06901-w (2017).
171 Neubert, E. *et al.* Chromatin swelling drives neutrophil extracellular trap release. *Nat Commun* **9**, 3767, doi:10.1038/s41467-018-06263-5 (2018).
172 Thiam, H. R. *et al.* NETosis proceeds by cytoskeleton and endomembrane disassembly and PAD4-mediated chromatin decondensation and nuclear envelope rupture. *Proc Natl Acad Sci U S A* **117**, 7326-7337, doi:10.1073/pnas.1909546117 (2020).
173 Byrd, A. S., O'Brien, X. M., Johnson, C. M., Lavigne, L. M. & Reichner, J. S. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans. *J Immunol* **190**, 4136-4148, doi:10.4049/jimmunol.1202671 (2013).
174 Byrd, A. S. *et al.* NETosis in Neonates: Evidence of a Reactive Oxygen Species-Independent Pathway in Response to Fungal Challenge. *J Infect Dis* **213**, 634-639, doi:10.1093/infdis/jiv435 (2016).
175 Hrachovinova, I. *et al.* Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. *Nat Med* **9**, 1020-1025, doi:10.1038/nm899 (2003).
176 Gordon, S. & Pluddemann, A. Macrophage Clearance of Apoptotic Cells: A Critical Assessment. *Front Immunol* **9**, 127, doi:10.3389/fimmu.2018.00127 (2018).
177 Timar, C. I. *et al.* Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood* **121**, 510-518, doi:10.1182/blood-2012-05-431114 (2013).
178 Pluskota, E. *et al.* Expression, activation, and function of integrin alphaMbeta2 (Mac-1) on neutrophil-derived microparticles. *Blood* **112**, 2327-2335, doi:10.1182/blood-2007-12-127183 (2008).

179 Gasser, O. *et al.* Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res* **285**, 243-257, doi:10.1016/s0014-4827(03)00055-7 (2003).
180 Mesri, M. & Altieri, D. C. Endothelial cell activation by leukocyte microparticles. *J Immunol* **161**, 4382-4387 (1998).
181 Hakkim, A. *et al.* Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol* **7**, 75-77, doi:10.1038/nchembio.496 (2011).
182 Amulic, B. *et al.* Cell-Cycle Proteins Control Production of Neutrophil Extracellular Traps. *Dev Cell* **43**, 449-462 e445, doi:10.1016/j.devcel.2017.10.013 (2017).
183 Wolach, O. *et al.* Increased neutrophil extracellular trap formation promotes thrombosis in myeloproliferative neoplasms. *Sci Transl Med* **10**, doi:10.1126/scitranslmed.aan8292 (2018).
184 Radic, M. & Neeli, I. Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. *Front Immunol* **4**, 38, doi:10.3389/fimmu.2013.00038 (2013).
185 Bianchi, M. *et al.* Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* **114**, 2619-2622, doi:10.1182/blood-2009-05-221606 (2009).
186 Neeli, I., Dwivedi, N., Khan, S. & Radic, M. Regulation of extracellular chromatin release from neutrophils. *J Innate Immun* **1**, 194-201, doi:10.1159/000206974 (2009).
187 Li, P. *et al.* PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* **207**, 1853-1862, doi:10.1084/jem.20100239 (2010).
188 Fonseca, Z. *et al.* Entamoeba histolytica Induce Signaling via Raf/MEK/ERK for Neutrophil Extracellular Trap (NET) Formation. *Front Cell Infect Microbiol* **8**, 226, doi:10.3389/fcimb.2018.00226 (2018).
189 Vorobjeva, N. V. & Chernyak, B. V. NETosis: Molecular Mechanisms, Role in Physiology and Pathology. *Biochemistry (Mosc)* **85**, 1178-1190, doi:10.1134/S0006297920100065 (2020).
190 Gabriel, C., McMaster, W. R., Girard, D. & Descoteaux, A. Leishmania donovani promastigotes evade the antimicrobial activity of neutrophil extracellular traps. *J Immunol* **185**, 4319-4327, doi:10.4049/jimmunol.1000893 (2010).
191 Mejia, S. P., Cano, L. E., Lopez, J. A., Hernandez, O. & Gonzalez, A. Human neutrophils produce extracellular traps against Paracoccidioides brasiliensis. *Microbiology (Reading)* **161**, 1008-1017, doi:10.1099/mic.0.000059 (2015).
192 Chen, K. *et al.* Endocytosis of soluble immune complexes leads to their clearance by FcgammaRIIIB but induces neutrophil extracellular traps via FcgammaRIIA in vivo. *Blood* **120**, 4421-4431, doi:10.1182/blood-2011-12-401133 (2012).
193 Vorobjeva, N. *et al.* Mitochondrial permeability transition pore is involved in oxidative burst and NETosis of human neutrophils. *Biochim Biophys Acta Mol Basis Dis* **1866**, 165664, doi:10.1016/j.bbadis.2020.165664 (2020).
194 de Vasconcelos, N. M., Van Opdenbosch, N., Van Gorp, H., Parthoens, E. & Lamkanfi, M. Single-cell analysis of pyroptosis dynamics reveals conserved GSDMD-mediated subcellular events that precede plasma membrane rupture. *Cell Death Differ* **26**, 146-161, doi:10.1038/s41418-018-0106-7 (2019).
195 Goldmann, O. & Medina, E. The expanding world of extracellular traps: not only neutrophils but much more. *Front Immunol* **3**, 420, doi:10.3389/fimmu.2012.00420 (2013).
196 Hamam, H. J., Khan, M. A. & Palaniyar, N. Histone Acetylation Promotes Neutrophil Extracellular Trap Formation. *Biomolecules* **9**, doi:10.3390/biom9010032 (2019).

197 Papayannopoulos, V., Metzler, K. D., Hakkim, A. & Zychlinsky, A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol* **191**, 677-691, doi:10.1083/jcb.201006052 (2010).
198 van Beers, J. J., Zendman, A. J., Raijmakers, R., Stammen-Vogelzangs, J. & Pruijn, G. J. Peptidylarginine deiminase expression and activity in PAD2 knock-out and PAD4-low mice. *Biochimie* **95**, 299-308, doi:10.1016/j.biochi.2012.09.029 (2013).
199 Thompson, P. R. & Fast, W. Histone citrullination by protein arginine deiminase: is arginine methylation a green light or a roadblock? *ACS Chem Biol* **1**, 433-441, doi:10.1021/cb6002306 (2006).
200 Nakashima, K. *et al.* Molecular characterization of peptidylarginine deiminase in HL-60 cells induced by retinoic acid and 1alpha,25-dihydroxyvitamin D(3). *J Biol Chem* **274**, 27786-27792, doi:10.1074/jbc.274.39.27786 (1999).
201 Nakashima, K., Hagiwara, T. & Yamada, M. Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes. *J Biol Chem* **277**, 49562-49568, doi:10.1074/jbc.M208795200 (2002).
202 Sun, B. *et al.* Citrullination of NF-kappaB p65 promotes its nuclear localization and TLR-induced expression of IL-1beta and TNFalpha. *Sci Immunol* **2**, doi:10.1126/sciimmunol.aal3062 (2017).
203 Nakayama-Hamada, M. *et al.* Comparison of enzymatic properties between hPADI2 and hPADI4. *Biochem Biophys Res Commun* **327**, 192-200, doi:10.1016/j.bbrc.2004.11.152 (2005).
204 Kearney, P. L. *et al.* Kinetic characterization of protein arginine deiminase 4: a transcriptional corepressor implicated in the onset and progression of rheumatoid arthritis. *Biochemistry* **44**, 10570-10582, doi:10.1021/bi050292m (2005).
205 Krause, K. H., Campbell, K. P., Welsh, M. J. & Lew, D. P. The calcium signal and neutrophil activation. *Clin Biochem* **23**, 159-166, doi:10.1016/0009-9120(90)80030-m (1990).
206 Neeli, I., Khan, S. N. & Radic, M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol* **180**, 1895-1902, doi:10.4049/jimmunol.180.3.1895 (2008).
207 Cuthbert, G. L. *et al.* Histone deimination antagonizes arginine methylation. *Cell* **118**, 545-553, doi:10.1016/j.cell.2004.08.020 (2004).
208 Hagiwara, T., Hidaka, Y. & Yamada, M. Deimination of histone H2A and H4 at arginine 3 in HL-60 granulocytes. *Biochemistry* **44**, 5827-5834, doi:10.1021/bi047505c (2005).
209 Christophorou, M. A. *et al.* Citrullination regulates pluripotency and histone H1 binding to chromatin. *Nature* **507**, 104-108, doi:10.1038/nature12942 (2014).
210 Thanabalasuriar, A. *et al.* Neutrophil Extracellular Traps Confine Pseudomonas aeruginosa Ocular Biofilms and Restrict Brain Invasion. *Cell Host Microbe* **25**, 526-536 e524, doi:10.1016/j.chom.2019.02.007 (2019).
211 Kolaczkowska, E. *et al.* Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nat Commun* **6**, 6673, doi:10.1038/ncomms7673 (2015).
212 Martinod, K. *et al.* Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. *Proc Natl Acad Sci U S A* **110**, 8674-8679, doi:10.1073/pnas.1301059110 (2013).
213 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* **109**, 13076-13081, doi:10.1073/pnas.1200419109 (2012).

214 Jones, J. E. *et al.* Synthesis and screening of a haloacetamidine containing library to identify PAD4 selective inhibitors. *ACS Chem Biol* **7**, 160-165, doi:10.1021/cb200258q (2012).
215 Muth, A. *et al.* Development of a Selective Inhibitor of Protein Arginine Deiminase 2. *J Med Chem* **60**, 3198-3211, doi:10.1021/acs.jmedchem.7b00274 (2017).
216 Lewis, H. D. *et al.* Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol* **11**, 189-191, doi:10.1038/nchembio.1735 (2015).
217 Wu, S. Y. *et al.* Candida albicans triggers NADPH oxidase-independent neutrophil extracellular traps through dectin-2. *PLoS Pathog* **15**, e1008096, doi:10.1371/journal.ppat.1008096 (2019).
218 Hosseinzadeh, A., Thompson, P. R., Segal, B. H. & Urban, C. F. Nicotine induces neutrophil extracellular traps. *J Leukoc Biol* **100**, 1105-1112, doi:10.1189/jlb.3AB0815-379RR (2016).
219 Tatsiy, O. & McDonald, P. P. Physiological Stimuli Induce PAD4-Dependent, ROS-Independent NETosis, With Early and Late Events Controlled by Discrete Signaling Pathways. *Front Immunol* **9**, 2036, doi:10.3389/fimmu.2018.02036 (2018).
220 Guiducci, E. *et al.* Candida albicans-Induced NETosis Is Independent of Peptidylarginine Deiminase 4. *Front Immunol* **9**, 1573, doi:10.3389/fimmu.2018.01573 (2018).
221 Hasler, P., Giaglis, S. & Hahn, S. Neutrophil extracellular traps in health and disease. *Swiss Med Wkly* **146**, w14352, doi:10.4414/smw.2016.14352 (2016).
222 Liu, Y. *et al.* Peptidylarginine deiminases 2 and 4 modulate innate and adaptive immune responses in TLR-7-dependent lupus. *JCI Insight* **3**, doi:10.1172/jci.insight.124729 (2018).
223 Wang, Y., Chen, R., Gan, Y. & Ying, S. The roles of PAD2- and PAD4-mediated protein citrullination catalysis in cancers. *Int J Cancer* **148**, 267-276, doi:10.1002/ijc.33205 (2021).
224 Konig, M. F. & Andrade, F. A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination. *Front Immunol* **7**, 461, doi:10.3389/fimmu.2016.00461 (2016).
225 Sofoluwe, A., Bacchetta, M., Badaoui, M., Kwak, B. R. & Chanson, M. ATP amplifies NADPH-dependent and -independent neutrophil extracellular trap formation. *Sci Rep* **9**, 16556, doi:10.1038/s41598-019-53058-9 (2019).
226 Chen, K. W. *et al.* Noncanonical inflammasome signaling elicits gasdermin D-dependent neutrophil extracellular traps. *Sci Immunol* **3**, doi:10.1126/sciimmunol.aar6676 (2018).
227 Gosswein, S. *et al.* Citrullination Licenses Calpain to Decondense Nuclei in Neutrophil Extracellular Trap Formation. *Front Immunol* **10**, 2481, doi:10.3389/fimmu.2019.02481 (2019).
228 Li, Y. *et al.* Nuclear envelope rupture and NET formation is driven by PKCalpha-mediated lamin B disassembly. *EMBO Rep* **21**, e48779, doi:10.15252/embr.201948779 (2020).
229 Ellenberg, J. *et al.* Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J Cell Biol* **138**, 1193-1206, doi:10.1083/jcb.138.6.1193 (1997).
230 Metzler, K. D., Goosmann, C., Lubojemska, A., Zychlinsky, A. & Papayannopoulos, V. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. *Cell Rep* **8**, 883-896, doi:10.1016/j.celrep.2014.06.044 (2014).

231 Galluzzi, L. *et al.* Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* **25**, 486-541, doi:10.1038/s41418-017-0012-4 (2018).
232 Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I. & Simon, H. U. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* **16**, 1438-1444, doi:10.1038/cdd.2009.96 (2009).
233 Milligan, K. L. *et al.* Complete Myeloperoxidase Deficiency: Beware the "False-Positive" Dihydrorhodamine Oxidation. *J Pediatr* **176**, 204-206, doi:10.1016/j.jpeds.2016.05.047 (2016).
234 Anderson, D. C. & Springer, T. A. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu Rev Med* **38**, 175-194, doi:10.1146/annurev.me.38.020187.001135 (1987).
235 Bunting, M., Harris, E. S., McIntyre, T. M., Prescott, S. M. & Zimmerman, G. A. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. *Curr Opin Hematol* **9**, 30-35, doi:10.1097/00062752-200201000-00006 (2002).
236 Roberts, H. *et al.* Characterization of neutrophil function in Papillon-Lefevre syndrome. *J Leukoc Biol* **100**, 433-444, doi:10.1189/jlb.5A1015-489R (2016).
237 West, B. C. Chediak-Higashi syndrome neutrophils are characterized by the absence of both normal azurophilic granules. *Am J Pathol* **122**, 177-189 (1986).
238 Holland, S. M. Chronic granulomatous disease. *Clin Rev Allergy Immunol* **38**, 3-10, doi:10.1007/s12016-009-8136-z (2010).
239 Yang, S. C., Tsai, Y. F., Pan, Y. L. & Hwang, T. L. Understanding the role of neutrophils in acute respiratory distress syndrome. *Biomed J* **44**, 439-446, doi:10.1016/j.bj.2020.09.001 (2021).
240 Pastorek, M., Dubrava, M. & Celec, P. On the Origin of Neutrophil Extracellular Traps in COVID-19. *Front Immunol* **13**, 821007, doi:10.3389/fimmu.2022.821007 (2022).
241 Ling, S. & Xu, J. W. NETosis as a Pathogenic Factor for Heart Failure. *Oxid Med Cell Longev* **2021**, 6687096, doi:10.1155/2021/6687096 (2021).
242 Megens, R. T. *et al.* Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thromb Haemost* **107**, 597-598, doi:10.1160/TH11-09-0650 (2012).
243 Giugliano, G. *et al.* Leukocyte count in peripheral arterial disease: A simple, reliable, inexpensive approach to cardiovascular risk prediction. *Atherosclerosis* **210**, 288-293, doi:10.1016/j.atherosclerosis.2009.11.009 (2010).
244 Friedman, G. D., Klatsky, A. L. & Siegelaub, A. B. The leukocyte count as a predictor of myocardial infarction. *N Engl J Med* **290**, 1275-1278, doi:10.1056/NEJM197406062902302 (1974).
245 Quillard, T. *et al.* TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J* **36**, 1394-1404, doi:10.1093/eurheartj/ehv044 (2015).
246 Warnatsch, A., Ioannou, M., Wang, Q. & Papayannopoulos, V. Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* **349**, 316-320, doi:10.1126/science.aaa8064 (2015).
247 Liu, Y. *et al.* Myeloid-Specific Deletion of Peptidylarginine Deiminase 4 Mitigates Atherosclerosis. *Front Immunol* **9**, 1680, doi:10.3389/fimmu.2018.01680 (2018).
248 Knight, J. S. *et al.* Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res* **114**, 947-956, doi:10.1161/CIRCRESAHA.114.303312 (2014).

249 Brill, A. *et al.* von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood* **117**, 1400-1407, doi:10.1182/blood-2010-05-287623 (2011).
250 Fuchs, T. A. *et al.* Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A* **107**, 15880-15885, doi:10.1073/pnas.1005743107 (2010).
251 Etulain, J. *et al.* P-selectin promotes neutrophil extracellular trap formation in mice. *Blood* **126**, 242-246, doi:10.1182/blood-2015-01-624023 (2015).
252 Rossaint, J. *et al.* Directed transport of neutrophil-derived extracellular vesicles enables platelet-mediated innate immune response. *Nat Commun* **7**, 13464, doi:10.1038/ncomms13464 (2016).
253 von Bruhl, M. L. *et al.* Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med* **209**, 819-835, doi:10.1084/jem.20112322 (2012).
254 Sporn, L. A., Marder, V. J. & Wagner, D. D. Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell* **46**, 185-190, doi:10.1016/0092-8674(86)90735-x (1986).
255 Engelmann, B., Luther, T. & Muller, I. Intravascular tissue factor pathway--a model for rapid initiation of coagulation within the blood vessel. *Thromb Haemost* **89**, 3-8 (2003).
256 Takano, S., Kimura, S., Ohdama, S. & Aoki, N. Plasma thrombomodulin in health and diseases. *Blood* **76**, 2024-2029 (1990).
257 Glaser, C. B. *et al.* Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation. *J Clin Invest* **90**, 2565-2573, doi:10.1172/JCI116151 (1992).
258 Xu, J. *et al.* Extracellular histones are major mediators of death in sepsis. *Nat Med* **15**, 1318-1321, doi:10.1038/nm.2053 (2009).
259 Semeraro, F. *et al.* Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* **118**, 1952-1961, doi:10.1182/blood-2011-03-343061 (2011).
260 Crea, F. & Libby, P. Acute Coronary Syndromes: The Way Forward From Mechanisms to Precision Treatment. *Circulation* **136**, 1155-1166, doi:10.1161/CIRCULATIONAHA.117.029870 (2017).
261 Chapman, A. R. *et al.* High-Sensitivity Cardiac Troponin and the Universal Definition of Myocardial Infarction. *Circulation* **141**, 161-171, doi:10.1161/CIRCULATIONAHA.119.042960 (2020).
262 Ma, Y., Yabluchanskiy, A. & Lindsey, M. L. Neutrophil roles in left ventricular remodeling following myocardial infarction. *Fibrogenesis Tissue Repair* **6**, 11, doi:10.1186/1755-1536-6-11 (2013).
263 Daseke, M. J., 2nd *et al.* Neutrophil signaling during myocardial infarction wound repair. *Cell Signal* **77**, 109816, doi:10.1016/j.cellsig.2020.109816 (2021).
264 Mangold, A. *et al.* Coronary neutrophil extracellular trap burden and deoxyribonuclease activity in ST-elevation acute coronary syndrome are predictors of ST-segment resolution and infarct size. *Circ Res* **116**, 1182-1192, doi:10.1161/CIRCRESAHA.116.304944 (2015).
265 Stakos, D. A. *et al.* Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction. *Eur Heart J* **36**, 1405-1414, doi:10.1093/eurheartj/ehv007 (2015).
266 Noubouossie, D. F. *et al.* In vitro activation of coagulation by human neutrophil DNA and histone proteins but not neutrophil extracellular traps. *Blood* **129**, 1021-1029, doi:10.1182/blood-2016-06-722298 (2017).

267 Watts, R. A., Hatemi, G., Burns, J. C. & Mohammad, A. J. Global epidemiology of vasculitis. *Nat Rev Rheumatol* **18**, 22-34, doi:10.1038/s41584-021-00718-8 (2022).
268 Kitching, A. R. *et al.* ANCA-associated vasculitis. *Nat Rev Dis Primers* **6**, 71, doi:10.1038/s41572-020-0204-y (2020).
269 Nogueira, E. *et al.* Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. *Nephrol Dial Transplant* **25**, 2209-2217, doi:10.1093/ndt/gfp783 (2010).
270 Pendergraft, W. F., 3rd *et al.* Autoimmunity is triggered by cPR-3(105-201), a protein complementary to human autoantigen proteinase-3. *Nat Med* **10**, 72-79, doi:10.1038/nm968 (2004).
271 Sangaletti, S. *et al.* Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood* **120**, 3007-3018, doi:10.1182/blood-2012-03-416156 (2012).
272 Jennette, J. C. & Nachman, P. H. ANCA Glomerulonephritis and Vasculitis. *Clin J Am Soc Nephrol* **12**, 1680-1691, doi:10.2215/CJN.02500317 (2017).
273 Tse, W. Y., Nash, G. B., Hewins, P., Savage, C. O. & Adu, D. ANCA-induced neutrophil F-actin polymerization: implications for microvascular inflammation. *Kidney Int* **67**, 130-139, doi:10.1111/j.1523-1755.2005.00063.x (2005).
274 Kuligowski, M. P. *et al.* Antimyeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. *Blood* **113**, 6485-6494, doi:10.1182/blood-2008-12-192617 (2009).
275 Johnson, P. A., Alexander, H. D., McMillan, S. A. & Maxwell, A. P. Up-regulation of the granulocyte adhesion molecule Mac-1 by autoantibodies in autoimmune vasculitis. *Clin Exp Immunol* **107**, 513-519, doi:10.1046/j.1365-2249.1997.d01-956.x (1997).
276 Hutton, H. L., Holdsworth, S. R. & Kitching, A. R. ANCA-Associated Vasculitis: Pathogenesis, Models, and Preclinical Testing. *Semin Nephrol* **37**, 418-435, doi:10.1016/j.semnephrol.2017.05.016 (2017).
277 Emmi, G. *et al.* Thrombosis in vasculitis: from pathogenesis to treatment. *Thromb J* **13**, 15, doi:10.1186/s12959-015-0047-z (2015).
278 Robson, J. *et al.* Damage in the anca-associated vasculitides: long-term data from the European vasculitis study group (EUVAS) therapeutic trials. *Ann Rheum Dis* **74**, 177-184, doi:10.1136/annrheumdis-2013-203927 (2015).
279 Morgan, M. D. *et al.* Increased incidence of cardiovascular events in patients with antineutrophil cytoplasmic antibody-associated vasculitides: a matched-pair cohort study. *Arthritis Rheum* **60**, 3493-3500, doi:10.1002/art.24957 (2009).
280 McInnes, I. B. & Schett, G. The pathogenesis of rheumatoid arthritis. *N Engl J Med* **365**, 2205-2219, doi:10.1056/NEJMra1004965 (2011).
281 Dwivedi, N. *et al.* Felty's syndrome autoantibodies bind to deiminated histones and neutrophil extracellular chromatin traps. *Arthritis Rheum* **64**, 982-992, doi:10.1002/art.33432 (2012).
282 Sur Chowdhury, C. *et al.* Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther* **16**, R122, doi:10.1186/ar4579 (2014).
283 Behnen, M. *et al.* Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcγRIIIB and Mac-1. *J Immunol* **193**, 1954-1965, doi:10.4049/jimmunol.1400478 (2014).
284 Khandpur, R. *et al.* NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* **5**, 178ra140, doi:10.1126/scitranslmed.3005580 (2013).

285 Apel, F., Zychlinsky, A. & Kenny, E. F. The role of neutrophil extracellular traps in rheumatic diseases. *Nat Rev Rheumatol* **14**, 467-475, doi:10.1038/s41584-018-0039-z (2018).
286 Willis, V. C. *et al.* N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. *J Immunol* **186**, 4396-4404, doi:10.4049/jimmunol.1001620 (2011).
287 Chirivi, R. G. S., Jenniskens, G. J. & Raats, J. M. H. Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis. *Journal of Clinical & Cellular Immunology*, doi:10.4172/2155-9899.S6-006 (2013).
288 Romero, V. *et al.* Immune-mediated pore-forming pathways induce cellular hypercitrullination and generate citrullinated autoantigens in rheumatoid arthritis. *Sci Transl Med* **5**, 209ra150, doi:10.1126/scitranslmed.3006869 (2013).
289 Hakkim, A. *et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* **107**, 9813-9818, doi:10.1073/pnas.0909927107 (2010).
290 Guiducci, C. *et al.* Autoimmune skin inflammation is dependent on plasmacytoid dendritic cell activation by nucleic acids via TLR7 and TLR9. *J Exp Med* **207**, 2931-2942, doi:10.1084/jem.20101048 (2010).
291 Lande, R. *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* **3**, 73ra19, doi:10.1126/scitranslmed.3001180 (2011).
292 Garcia-Romo, G. S. *et al.* Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med* **3**, 73ra20, doi:10.1126/scitranslmed.3001201 (2011).
293 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* **190**, 1217-1226, doi:10.4049/jimmunol.1202388 (2013).
294 Leffler, J. *et al.* Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol* **188**, 3522-3531, doi:10.4049/jimmunol.1102404 (2012).
295 Knight, J. S. *et al.* Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Invest* **123**, 2981-2993, doi:10.1172/jci67390 (2013).
296 Knight, J. S. *et al.* Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. *Ann Rheum Dis* **74**, 2199-2206, doi:10.1136/annrheumdis-2014-205365 (2015).
297 Dowey, R., Iqbal, A., Heller, S. R., Sabroe, I. & Prince, L. R. A Bittersweet Response to Infection in Diabetes; Targeting Neutrophils to Modify Inflammation and Improve Host Immunity. *Front Immunol* **12**, 678771, doi:10.3389/fimmu.2021.678771 (2021).
298 DeFronzo, R. A. Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am* **88**, 787-835, ix, doi:10.1016/j.mcna.2004.04.013 (2004).
299 Atkinson, M. A. *et al.* How does type 1 diabetes develop?: the notion of homicide or β-cell suicide revisited. *Diabetes* **60**, 1370-1379, doi:10.2337/db10-1797 (2011).
300 Ndosi, M. *et al.* Prognosis of the infected diabetic foot ulcer: a 12-month prospective observational study. *Diabet Med* **35**, 78-88, doi:10.1111/dme.13537 (2018).
301 Carey, I. M. *et al.* Risk of Infection in Type 1 and Type 2 Diabetes Compared With the General Population: A Matched Cohort Study. *Diabetes Care* **41**, 513-521, doi:10.2337/dc17-2131 (2018).

302 Kang, Y. *et al.* Effects of advanced glycation end products on neutrophil migration and aggregation in diabetic wounds. *Aging (Albany NY)* **13**, 12143-12159, doi:10.18632/aging.202924 (2021).
303 Wang, L. *et al.* Hyperglycemia Induces Neutrophil Extracellular Traps Formation Through an NADPH Oxidase-Dependent Pathway in Diabetic Retinopathy. *Front Immunol* **9**, 3076, doi:10.3389/fimmu.2018.03076 (2018).
304 Shetty, N., Thomas, B. & Ramesh, A. Comparison of neutrophil functions in diabetic and healthy subjects with chronic generalized periodontitis. *J Indian Soc Periodontol* **12**, 41-44, doi:10.4103/0972-124x.44089 (2008).
305 Wong, S. L. *et al.* Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med* **21**, 815-819, doi:10.1038/nm.3887 (2015).
306 Karima, M. *et al.* Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis. *J Leukoc Biol* **78**, 862-870, doi:10.1189/jlb.1004583 (2005).
307 Njeim, R. *et al.* NETosis contributes to the pathogenesis of diabetes and its complications. *J Mol Endocrinol* **65**, R65-r76, doi:10.1530/jme-20-0128 (2020).
308 Johnson, J. *et al.* Oxidative Stress in Neutrophils: Implications for Diabetic Cardiovascular Complications. *Antioxid Redox Signal* **36**, 652-666, doi:10.1089/ars.2021.0116 (2022).
309 Carestia, A. *et al.* NETosis before and after Hyperglycemic Control in Type 2 Diabetes Mellitus Patients. *PLoS One* **11**, e0168647, doi:10.1371/journal.pone.0168647 (2016).
310 Zurawska-Płaksej, E., Piwowar, A., Knapik-Kordecka, M. & Warwas, M. Activities of neutrophil membrane-bound proteases in type 2 diabetic patients. *Arch Med Res* **45**, 36-43, doi:10.1016/j.arcmed.2013.10.003 (2014).
311 Das, S. K., Yuan, Y. F. & Li, M. Q. Specific PKC βII inhibitor: one stone two birds in the treatment of diabetic foot ulcers. *Biosci Rep* **38**, doi:10.1042/bsr20171459 (2018).
312 Guo, S. & Dipietro, L. A. Factors affecting wound healing. *J Dent Res* **89**, 219-229, doi:10.1177/0022034509359125 (2010).
313 Leppkes, M. *et al.* Updates on NET formation in health and disease. *Semin Arthritis Rheum* **49**, S43-S48, doi:10.1016/j.semarthrit.2019.09.011 (2019).
314 Sabbatini, M., Magnelli, V. & Reno, F. NETosis in Wound Healing: When Enough Is Enough. *Cells* **10**, doi:10.3390/cells10030494 (2021).
315 Csomos, K. *et al.* Protein cross-linking by chlorinated polyamines and transglutamylation stabilizes neutrophil extracellular traps. *Cell Death Dis* **7**, e2332, doi:10.1038/cddis.2016.200 (2016).
316 Kawabata, K., Hagio, T. & Matsuoka, S. The role of neutrophil elastase in acute lung injury. *Eur J Pharmacol* **451**, 1-10, doi:10.1016/s0014-2999(02)02182-9 (2002).
317 Phillips, T. *et al.* Aberrant recruitment of leukocytes defines poor wound healing in patients with recessive dystrophic epidermolysis bullosa. *J Dermatol Sci* **100**, 209-216, doi:10.1016/j.jdermsci.2020.10.009 (2020).
318 Aratani, Y. Myeloperoxidase: Its role for host defense, inflammation, and neutrophil function. *Arch Biochem Biophys* **640**, 47-52, doi:10.1016/j.abb.2018.01.004 (2018).
319 Silk, E., Zhao, H., Weng, H. & Ma, D. The role of extracellular histone in organ injury. *Cell Death Dis* **8**, e2812, doi:10.1038/cddis.2017.52 (2017).
320 Zhao, R., Liang, H., Clarke, E., Jackson, C. & Xue, M. Inflammation in Chronic Wounds. *Int J Mol Sci* **17**, doi:10.3390/ijms17122085 (2016).
321 Menke, N. B., Ward, K. R., Witten, T. M., Bonchev, D. G. & Diegelmann, R. F. Impaired wound healing. *Clin Dermatol* **25**, 19-25, doi:10.1016/j.clindermatol.2006.12.005 (2007).

322 Reinke, J. M. & Sorg, H. Wound repair and regeneration. *Eur Surg Res* **49**, 35-43, doi:10.1159/000339613 (2012).
323 Korkmaz, H. I. *et al.* Neutrophil extracellular traps coincide with a pro-coagulant status of microcirculatory endothelium in burn wounds. *Wound Repair Regen* **25**, 609-617, doi:10.1111/wrr.12560 (2017).
324 Fuchs, T. A., Bhandari, A. A. & Wagner, D. D. Histones induce rapid and profound thrombocytopenia in mice. *Blood* **118**, 3708-3714, doi:10.1182/blood-2011-01-332676 (2011).
325 Eustache, J. H. *et al.* Casting A Wide Net On Surgery: The Central Role of Neutrophil Extracellular Traps. *Ann Surg* **272**, 277-283, doi:10.1097/SLA.0000000000003586 (2020).
326 Martins, V. F. *et al.* Surgical site peptidylarginine deaminase 4 (PAD4), a biomarker of NETosis, correlates with insulin resistance in total joint arthroplasty patients: A preliminary report. *PLoS One* **16**, e0245594, doi:10.1371/journal.pone.0245594 (2021).
327 Jaillon, S. *et al.* Neutrophil diversity and plasticity in tumour progression and therapy. *Nat Rev Cancer* **20**, 485-503, doi:10.1038/s41568-020-0281-y (2020).
328 Cui, C. *et al.* Neutrophil elastase selectively kills cancer cells and attenuates tumorigenesis. *Cell* **184**, 3163-3177 e3121, doi:10.1016/j.cell.2021.04.016 (2021).
329 Finisguerra, V. *et al.* MET is required for the recruitment of anti-tumoural neutrophils. *Nature* **522**, 349-353, doi:10.1038/nature14407 (2015).
330 Ponzetta, A. *et al.* Neutrophils Driving Unconventional T Cells Mediate Resistance against Murine Sarcomas and Selected Human Tumors. *Cell* **178**, 346-360 e324, doi:10.1016/j.cell.2019.05.047 (2019).
331 Singhal, S. *et al.* Origin and Role of a Subset of Tumor-Associated Neutrophils with Antigen-Presenting Cell Features in Early-Stage Human Lung Cancer. *Cancer Cell* **30**, 120-135, doi:10.1016/j.ccell.2016.06.001 (2016).
332 Mantovani, A., Marchesi, F., Jaillon, S., Garlanda, C. & Allavena, P. Tumor-associated myeloid cells: diversity and therapeutic targeting. *Cell Mol Immunol* **18**, 566-578, doi:10.1038/s41423-020-00613-4 (2021).
333 De Palma, M., Biziato, D. & Petrova, T. V. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer* **17**, 457-474, doi:10.1038/nrc.2017.51 (2017).
334 Nastasi, C., Mannarino, L. & D'Incalci, M. DNA Damage Response and Immune Defense. *Int J Mol Sci* **21**, doi:10.3390/ijms21207504 (2020).
335 Balkwill, F. R. & Mantovani, A. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol* **22**, 33-40, doi:10.1016/j.semcancer.2011.12.005 (2012).
336 Wculek, S. K., Bridgeman, V. L., Peakman, F. & Malanchi, I. Early Neutrophil Responses to Chemical Carcinogenesis Shape Long-Term Lung Cancer Susceptibility. *iScience* **23**, 101277, doi:10.1016/j.isci.2020.101277 (2020).
337 Canli, O. *et al.* Myeloid Cell-Derived Reactive Oxygen Species Induce Epithelial Mutagenesis. *Cancer Cell* **32**, 869-883 e865, doi:10.1016/j.ccell.2017.11.004 (2017).
338 Albini, A., Bruno, A., Noonan, D. M. & Mortara, L. Contribution to Tumor Angiogenesis From Innate Immune Cells Within the Tumor Microenvironment: Implications for Immunotherapy. *Front Immunol* **9**, 527, doi:10.3389/fimmu.2018.00527 (2018).
339 Belotti, D., Pinessi, D. & Taraboletti, G. Alternative Vascularization Mechanisms in Tumor Resistance to Therapy. *Cancers (Basel)* **13**, doi:10.3390/cancers13081912 (2021).
340 Szczerba, B. M. *et al.* Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature* **566**, 553-557, doi:10.1038/s41586-019-0915-y (2019).

341 Liang, W., Li, Q. & Ferrara, N. Metastatic growth instructed by neutrophil-derived transferrin. *Proc Natl Acad Sci U S A* **115**, 11060-11065, doi:10.1073/pnas.1811717115 (2018).
342 Pham, T. & Rubenfeld, G. D. Fifty Years of Research in ARDS. The Epidemiology of Acute Respiratory Distress Syndrome. A 50th Birthday Review. *Am J Respir Crit Care Med* **195**, 860-870, doi:10.1164/rccm.201609-1773CP (2017).
343 Sigurdsson, M. I., Sigvaldason, K., Gunnarsson, T. S., Moller, A. & Sigurdsson, G. H. Acute respiratory distress syndrome: nationwide changes in incidence, treatment and mortality over 23 years. *Acta Anaesthesiol Scand* **57**, 37-45, doi:10.1111/aas.12001 (2013).
344 Huang, C. *et al.* Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **395**, 497-506, doi:10.1016/S0140-6736(20)30183-5 (2020).
345 Wu, C. *et al.* Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med* **180**, 934-943, doi:10.1001/jamainternmed.2020.0994 (2020).
346 Papazian, L. *et al.* Diagnostic workup for ARDS patients. *Intensive Care Med* **42**, 674-685, doi:10.1007/s00134-016-4324-5 (2016).
347 Bourenne, J., Carvelli, J. & Papazian, L. Evolving definition of acute respiratory distress syndrome. *J Thorac Dis* **11**, S390-S393, doi:10.21037/jtd.2018.12.24 (2019).
348 Zemans, R. L. & Matthay, M. A. What drives neutrophils to the alveoli in ARDS? *Thorax* **72**, 1-3, doi:10.1136/thoraxjnl-2016-209170 (2017).
349 Yang, S. C. *et al.* Luteolin attenuates neutrophilic oxidative stress and inflammatory arthritis by inhibiting Raf1 activity. *Biochem Pharmacol* **154**, 384-396, doi:10.1016/j.bcp.2018.06.003 (2018).
350 Aulakh, G. K. Neutrophils in the lung: "the first responders". *Cell Tissue Res* **371**, 577-588, doi:10.1007/s00441-017-2748-z (2018).
351 Williams, A. E. *et al.* Evidence for chemokine synergy during neutrophil migration in ARDS. *Thorax* **72**, 66-73, doi:10.1136/thoraxjnl-2016-208597 (2017).
352 Cortjens, B. *et al.* Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. *J Pathol* **238**, 401-411, doi:10.1002/path.4660 (2016).
353 Varga, Z. *et al.* Endothelial cell infection and endotheliitis in COVID-19. *Lancet* **395**, 1417-1418, doi:10.1016/S0140-6736(20)30937-5 (2020).
354 Mosleh, W., Chen, K., Pfau, S. E. & Vashist, A. Endotheliitis and Endothelial Dysfunction in Patients with COVID-19: Its Role in Thrombosis and Adverse Outcomes. *J Clin Med* **9**, doi:10.3390/jcm9061862 (2020).
355 Zuo, Y. *et al.* Neutrophil extracellular traps (NETs) as markers of disease severity in COVID-19. *medRxiv*, doi:10.1101/2020.04.09.20059626 (2020).
356 Skendros, P. *et al.* Complement and tissue factor-enriched neutrophil extracellular traps are key drivers in COVID-19 immunothrombosis. *J Clin Invest* **130**, 6151-6157, doi:10.1172/JCI141374 (2020).
357 Arcanjo, A. *et al.* The emerging role of neutrophil extracellular traps in severe acute respiratory syndrome coronavirus 2 (COVID-19). *Sci Rep* **10**, 19630, doi:10.1038/s41598-020-76781-0 (2020).
358 Veras, F. P. *et al.* SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *J Exp Med* **217**, doi:10.1084/jem.20201129 (2020).
359 Watanabe, R. *et al.* Entry from the cell surface of severe acute respiratory syndrome coronavirus with cleaved S protein as revealed by pseudotype virus bearing cleaved S protein. *J Virol* **82**, 11985-11991, doi:10.1128/JVI.01412-08 (2008).

360 Brighton, T. A. *et al.* Low-dose aspirin for preventing recurrent venous thromboembolism. *N Engl J Med* **367**, 1979-1987, doi:10.1056/NEJMoa1210384 (2012).
361 Tarantino, E. *et al.* Role of thromboxane-dependent platelet activation in venous thrombosis: Aspirin effects in mouse model. *Pharmacol Res* **107**, 415-425, doi:10.1016/j.phrs.2016.04.001 (2016).
362 Catella-Lawson, F. *et al.* Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *N Engl J Med* **345**, 1809-1817, doi:10.1056/NEJMoa003199 (2001).
363 Vogel, S. *et al.* Platelet-derived HMGB1 is a critical mediator of thrombosis. *J Clin Invest* **125**, 4638-4654, doi:10.1172/JCI81660 (2015).
364 Tilgner, J. *et al.* Aspirin, but Not Tirofiban Displays Protective Effects in Endotoxin Induced Lung Injury. *PLoS One* **11**, e0161218, doi:10.1371/journal.pone.0161218 (2016).
365 Ortiz-Munoz, G. *et al.* Aspirin-triggered 15-epi-lipoxin A4 regulates neutrophil-platelet aggregation and attenuates acute lung injury in mice. *Blood* **124**, 2625-2634, doi:10.1182/blood-2014-03-562876 (2014).
366 Helms, J. *et al.* Thrombomodulin favors leukocyte microvesicle fibrinolytic activity, reduces NETosis and prevents septic shock-induced coagulopathy in rats. *Ann Intensive Care* **7**, 118, doi:10.1186/s13613-017-0340-z (2017).
367 Mutua, V. & Gershwin, L. J. A Review of Neutrophil Extracellular Traps (NETs) in Disease: Potential Anti-NETs Therapeutics. *Clin Rev Allergy Immunol* **61**, 194-211, doi:10.1007/s12016-020-08804-7 (2021).
368 Witalison, E. E., Cui, X., Causey, C. P., Thompson, P. R. & Hofseth, L. J. Molecular targeting of protein arginine deiminases to suppress colitis and prevent colon cancer. *Oncotarget* **6**, 36053-36062, doi:10.18632/oncotarget.5937 (2015).
369 Ricciotti, E. & FitzGerald, G. A. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* **31**, 986-1000, doi:10.1161/ATVBAHA.110.207449 (2011).
370 Shishikura, K. *et al.* Prostaglandin E2 inhibits neutrophil extracellular trap formation through production of cyclic AMP. *Br J Pharmacol* **173**, 319-331, doi:10.1111/bph.13373 (2016).
371 Domingo-Gonzalez, R. *et al.* Inhibition of Neutrophil Extracellular Trap Formation after Stem Cell Transplant by Prostaglandin E2. *Am J Respir Crit Care Med* **193**, 186-197, doi:10.1164/rccm.201501-0161OC (2016).
372 de Bont, C. M., Boelens, W. C. & Pruijn, G. J. M. NETosis, complement, and coagulation: a triangular relationship. *Cell Mol Immunol* **16**, 19-27, doi:10.1038/s41423-018-0024-0 (2019).
373 Hillmen, P. *et al.* The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med* **355**, 1233-1243, doi:10.1056/NEJMoa061648 (2006).
374 Brodsky, R. A. Paroxysmal nocturnal hemoglobinuria. *Blood* **124**, 2804-2811, doi:10.1182/blood-2014-02-522128 (2014).
375 Rena, G., Hardie, D. G. & Pearson, E. R. The mechanisms of action of metformin. *Diabetologia* **60**, 1577-1585, doi:10.1007/s00125-017-4342-z (2017).
376 Gallo, A. *et al.* Metformin prevents glucose-induced protein kinase C-beta2 activation in human umbilical vein endothelial cells through an antioxidant mechanism. *Diabetes* **54**, 1123-1131, doi:10.2337/diabetes.54.4.1123 (2005).
377 Menegazzo, L. *et al.* The antidiabetic drug metformin blunts NETosis in vitro and reduces circulating NETosis biomarkers in vivo. *Acta Diabetol* **55**, 593-601, doi:10.1007/s00592-018-1129-8 (2018).
378 Jimenez-Alcazar, M. *et al.* Host DNases prevent vascular occlusion by neutrophil extracellular traps. *Science* **358**, 1202-1206, doi:10.1126/science.aam8897 (2017).

379 Wang, H. *et al.* Obesity-induced Endothelial Dysfunction is Prevented by Neutrophil Extracellular Trap Inhibition. *Sci Rep* **8**, 4881, doi:10.1038/s41598-018-23256-y (2018).
380 Aly, R. M. Current state of stem cell-based therapies: an overview. *Stem Cell Investig* **7**, 8, doi:10.21037/sci-2020-001 (2020).
381 Chari, S., Nguyen, A. & Saxe, J. Stem Cells in the Clinic. *Cell Stem Cell* **22**, 781-782, doi:10.1016/j.stem.2018.05.017 (2018).
382 Zhang, F. Q. *et al.* Current status and future prospects of stem cell therapy in Alzheimer's disease. *Neural Regen Res* **15**, 242-250, doi:10.4103/1673-5374.265544 (2020).
383 Assmus, B. *et al.* Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* **106**, 3009-3017, doi:10.1161/01.cir.0000043246.74879.cd (2002).
384 Gyongyosi, M. *et al.* Meta-Analysis of Cell-based CaRdiac stUdiEs (ACCRUE) in patients with acute myocardial infarction based on individual patient data. *Circ Res* **116**, 1346-1360, doi:10.1161/CIRCRESAHA.116.304346 (2015).
385 Fisher, S. A., Doree, C., Taggart, D. P., Mathur, A. & Martin-Rendon, E. Cell therapy for heart disease: Trial sequential analyses of two Cochrane reviews. *Clin Pharmacol Ther* **100**, 88-101, doi:10.1002/cpt.344 (2016).
386 Wobma, H. & Satwani, P. Mesenchymal stromal cells: Getting ready for clinical primetime. *Transfus Apher Sci* **60**, 103058, doi:10.1016/j.transci.2021.103058 (2021).
387 Psaltis, P. J. *et al.* Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. *J Cell Physiol* **223**, 530-540, doi:10.1002/jcp.22081 (2010).
388 Dixon, J. A. *et al.* Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. *Circulation* **120**, S220-229, doi:10.1161/CIRCULATIONAHA.108.842302 (2009).
389 Abdalmula, A. *et al.* Immunoselected STRO-3(+) mesenchymal precursor cells reduce inflammation and improve clinical outcomes in a large animal model of monoarthritis. *Stem Cell Res Ther* **8**, 22, doi:10.1186/s13287-016-0460-7 (2017).
390 See, F. *et al.* Therapeutic effects of human STRO-3-selected mesenchymal precursor cells and their soluble factors in experimental myocardial ischemia. *J Cell Mol Med* **15**, 2117-2129, doi:10.1111/j.1582-4934.2010.01241.x (2011).
391 Zhang, W. Y., Ebert, A. D., Narula, J. & Wu, J. C. Imaging cardiac stem cell therapy: translations to human clinical studies. *J Cardiovasc Transl Res* **4**, 514-522, doi:10.1007/s12265-011-9281-3 (2011).
392 Penicka, M. *et al.* One-day kinetics of myocardial engraftment after intracoronary injection of bone marrow mononuclear cells in patients with acute and chronic myocardial infarction. *Heart* **93**, 837-841, doi:10.1136/hrt.2006.091934 (2007).
393 Mansour, S. *et al.* COMPARE-AMI trial: comparison of intracoronary injection of CD133+ bone marrow stem cells to placebo in patients after acute myocardial infarction and left ventricular dysfunction: study rationale and design. *J Cardiovasc Transl Res* **3**, 153-159, doi:10.1007/s12265-009-9145-2 (2010).
394 Losordo, D. W. *et al.* Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. *Circulation* **115**, 3165-3172, doi:10.1161/CIRCULATIONAHA.106.687376 (2007).
395 Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* **410**, 701-705, doi:10.1038/35070587 (2001).

396 Chen, S. L. *et al.* Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* **94**, 92-95, doi:10.1016/j.amjcard.2004.03.034 (2004).
397 Gnecchi, M., Danieli, P., Malpasso, G. & Ciuffreda, M. C. Paracrine Mechanisms of Mesenchymal Stem Cells in Tissue Repair. *Methods Mol Biol* **1416**, 123-146, doi:10.1007/978-1-4939-3584-0_7 (2016).
398 Gnecchi, M., Zhang, Z., Ni, A. & Dzau, V. J. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* **103**, 1204-1219, doi:10.1161/CIRCRESAHA.108.176826 (2008).
399 Beer, L., Mildner, M., Gyöngyösi, M. & Ankersmit, H. J. Peripheral blood mononuclear cell secretome for tissue repair. *Apoptosis* **21**, 1336-1353, doi:10.1007/s10495-016-1292-8 (2016).
400 Korf-Klingebiel, M. *et al.* Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. *Eur Heart J* **29**, 2851-2858, doi:10.1093/eurheartj/ehn456 (2008).
401 Thum, T., Bauersachs, J., Poole-Wilson, P. A., Volk, H. D. & Anker, S. D. The dying stem cell hypothesis: immune modulation as a novel mechanism for progenitor cell therapy in cardiac muscle. *J Am Coll Cardiol* **46**, 1799-1802, doi:10.1016/j.jacc.2005.07.053 (2005).
402 Gray, M., Miles, K., Salter, D., Gray, D. & Savill, J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* **104**, 14080-14085, doi:10.1073/pnas.0700326104 (2007).
403 Ren, Y. *et al.* Apoptotic cells protect mice against lipopolysaccharide-induced shock. *J Immunol* **180**, 4978-4985, doi:10.4049/jimmunol.180.7.4978 (2008).
404 Gwam, C., Mohammed, N. & Ma, X. Stem cell secretome, regeneration, and clinical translation: a narrative review. *Ann Transl Med* **9**, 70, doi:10.21037/atm-20-5030 (2021).
405 Ankersmit, H. J. *et al.* Irradiated cultured apoptotic peripheral blood mononuclear cells regenerate infarcted myocardium. *Eur J Clin Invest* **39**, 445-456, doi:10.1111/j.1365-2362.2009.02111.x (2009).
406 Lichtenauer, M. *et al.* Intravenous and intramyocardial injection of apoptotic white blood cell suspensions prevents ventricular remodelling by increasing elastin expression in cardiac scar tissue after myocardial infarction. *Basic Res Cardiol* **106**, 645-655, doi:10.1007/s00395-011-0173-0 (2011).
407 Lichtenauer, M. *et al.* Secretome of apoptotic peripheral blood cells (APOSEC) confers cytoprotection to cardiomyocytes and inhibits tissue remodelling after acute myocardial infarction: a preclinical study. *Basic Res Cardiol* **106**, 1283-1297, doi:10.1007/s00395-011-0224-6 (2011).
408 Hoetzenecker, K. *et al.* Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation and vasodilation. *Basic Res Cardiol* **107**, 292, doi:10.1007/s00395-012-0292-2 (2012).
409 Pavo, N. *et al.* Long-acting beneficial effect of percutaneously intramyocardially delivered secretome of apoptotic peripheral blood cells on porcine chronic ischemic left ventricular dysfunction. *Biomaterials* **35**, 3541-3550, doi:10.1016/j.biomaterials.2013.12.071 (2014).
410 Mildner, C. S. *et al.* Secretome of Stressed Peripheral Blood Mononuclear Cells Alters Transcriptome Signature in Heart, Liver, and Spleen after an Experimental Acute Myocardial Infarction: An In Silico Analysis. *Biology (Basel)* **11**, doi:10.3390/biology11010116 (2022).

411 Hoetzenecker, K. *et al.* Mononuclear cell secretome protects from experimental autoimmune myocarditis. *Eur Heart J* **36**, 676-685, doi:10.1093/eurheartj/ehs459 (2015).
412 Altmann, P. *et al.* Secretomes of apoptotic mononuclear cells ameliorate neurological damage in rats with focal ischemia. *F1000Res* **3**, 131, doi:10.12688/f1000research.4219.2 (2014).
413 Haider, T. *et al.* The secretome of apoptotic human peripheral blood mononuclear cells attenuates secondary damage following spinal cord injury in rats. *Exp Neurol* **267**, 230-242, doi:10.1016/j.expneurol.2015.03.013 (2015).
414 Mildner, M. *et al.* Secretome of peripheral blood mononuclear cells enhances wound healing. *PLoS One* **8**, e60103, doi:10.1371/journal.pone.0060103 (2013).
415 Hacker, S. *et al.* Paracrine Factors from Irradiated Peripheral Blood Mononuclear Cells Improve Skin Regeneration and Angiogenesis in a Porcine Burn Model. *Sci Rep* **6**, 25168, doi:10.1038/srep25168 (2016).
416 Hacker, S. *et al.* The secretome of stressed peripheral blood mononuclear cells increases tissue survival in a rodent epigastric flap model. *Bioeng Transl Med* **6**, e10186, doi:10.1002/btm2.10186 (2021).
417 Kasiri, M. M. *et al.* Dying blood mononuclear cell secretome exerts antimicrobial activity. *Eur J Clin Invest* **46**, 853-863, doi:10.1111/eci.12667 (2016).
418 Wagner, T. *et al.* Different pro-angiogenic potential of gamma-irradiated PBMC-derived secretome and its subfractions. *Sci Rep* **8**, 18016, doi:10.1038/s41598-018-36928-6 (2018).
419 Beer, L. *et al.* High dose ionizing radiation regulates micro RNA and gene expression changes in human peripheral blood mononuclear cells. *BMC Genomics* **15**, 814, doi:10.1186/1471-2164-15-814 (2014).
420 Beer, L. *et al.* Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Sci Rep* **5**, 16662, doi:10.1038/srep16662 (2015).
421 Simader, E. *et al.* Tissue-regenerative potential of the secretome of gamma-irradiated peripheral blood mononuclear cells is mediated via TNFRSF1B-induced necroptosis. *Cell Death Dis* **10**, 729, doi:10.1038/s41419-019-1974-6 (2019).
422 Laggner, M. *et al.* Therapeutic potential of lipids obtained from gamma-irradiated PBMCs in dendritic cell-mediated skin inflammation. *EBioMedicine* **55**, 102774, doi:10.1016/j.ebiom.2020.102774 (2020).
423 Laggner, M. *et al.* The secretome of irradiated peripheral blood mononuclear cells attenuates activation of mast cells and basophils. *EBioMedicine* **81**, 104093, doi:10.1016/j.ebiom.2022.104093 (2022).
424 Simader, E. *et al.* Safety and tolerability of topically administered autologous, apoptotic PBMC secretome (APOSEC) in dermal wounds: a randomized Phase 1 trial (MARSYAS I). *Sci Rep* **7**, 6216, doi:10.1038/s41598-017-06223-x (2017).
425 Nemeth, T., Sperandio, M. & Mocsai, A. Neutrophils as emerging therapeutic targets. *Nat Rev Drug Discov* **19**, 253-275, doi:10.1038/s41573-019-0054-z (2020).
426 Morales-Primo, A. U., Becker, I. & Zamora-Chimal, J. Neutrophil extracellular trap-associated molecules: a review on their immunophysiological and inflammatory roles. *Int Rev Immunol* **41**, 253-274, doi:10.1080/08830185.2021.1921174 (2022).
427 Farrera, C. & Fadeel, B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol* **191**, 2647-2656, doi:10.4049/jimmunol.1300436 (2013).
428 Deng, Q. *et al.* Citrullinated Histone H3 as a Therapeutic Target for Endotoxic Shock in Mice. *Front Immunol* **10**, 2957, doi:10.3389/fimmu.2019.02957 (2019).

429 Spinosa, M. *et al.* Resolvin D1 decreases abdominal aortic aneurysm formation by inhibiting NETosis in a mouse model. *J Vasc Surg* **68**, 93S-103S, doi:10.1016/j.jvs.2018.05.253 (2018).
430 Cherpokova, D. *et al.* Resolvin D4 attenuates the severity of pathological thrombosis in mice. *Blood* **134**, 1458-1468, doi:10.1182/blood.2018886317 (2019).
431 Chiang, N. *et al.* Resolvin T-series reduce neutrophil extracellular traps. *Blood* **139**, 1222-1233, doi:10.1182/blood.2021013422 (2022).
432 El Kebir, D., Gjorstrup, P. & Filep, J. G. Resolvin E1 promotes phagocytosis-induced neutrophil apoptosis and accelerates resolution of pulmonary inflammation. *Proc Natl Acad Sci U S A* **109**, 14983-14988, doi:10.1073/pnas.1206641109 (2012).
433 Neubert, E. *et al.* Serum and Serum Albumin Inhibit in vitro Formation of Neutrophil Extracellular Traps (NETs). *Front Immunol* **10**, 12, doi:10.3389/fimmu.2019.00012 (2019).
434 Angeletti, A. *et al.* Neutrophil Extracellular Traps-DNase Balance and Autoimmunity. *Cells* **10**, doi:10.3390/cells10102667 (2021).
435 Laukova, L., Konecna, B., Janovicova, L., Vlkova, B. & Celec, P. Deoxyribonucleases and Their Applications in Biomedicine. *Biomolecules* **10**, doi:10.3390/biom10071036 (2020).
436 Ribeiro, D. *et al.* Calcium Pathways in Human Neutrophils-The Extended Effects of Thapsigargin and ML-9. *Cells* **7**, doi:10.3390/cells7110204 (2018).
437 Brechard, S., Melchior, C., Plancon, S., Schenten, V. & Tschirhart, E. J. Store-operated Ca2+ channels formed by TRPC1, TRPC6 and Orai1 and non-store-operated channels formed by TRPC3 are involved in the regulation of NADPH oxidase in HL-60 granulocytes. *Cell Calcium* **44**, 492-506, doi:10.1016/j.ceca.2008.03.002 (2008).
438 Salmon, M. D. & Ahluwalia, J. Discrimination between receptor- and store-operated Ca(2+) influx in human neutrophils. *Cell Immunol* **265**, 1-5, doi:10.1016/j.cellimm.2010.07.009 (2010).
439 Tintinger, G. R., Theron, A. J., Potjo, M. & Anderson, R. Reactive oxidants regulate membrane repolarization and store-operated uptake of calcium by formyl peptide-activated human neutrophils. *Free Radic Biol Med* **42**, 1851-1857, doi:10.1016/j.freeradbiomed.2007.03.012 (2007).
440 Lytton, J., Westlin, M. & Hanley, M. R. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J Biol Chem* **266**, 17067-17071 (1991).
441 Dan Dunn, J., Alvarez, L. A., Zhang, X. & Soldati, T. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. *Redox Biol* **6**, 472-485, doi:10.1016/j.redox.2015.09.005 (2015).
442 Douda, D. N., Khan, M. A., Grasemann, H. & Palaniyar, N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc Natl Acad Sci U S A* **112**, 2817-2822, doi:10.1073/pnas.1414055112 (2015).
443 Douda, D. N., Yip, L., Khan, M. A., Grasemann, H. & Palaniyar, N. Akt is essential to induce NADPH-dependent NETosis and to switch the neutrophil death to apoptosis. *Blood* **123**, 597-600, doi:10.1182/blood-2013-09-526707 (2014).
444 Pruchniak, M. P. & Demkow, U. Potent NETosis inducers do not show synergistic effects in vitro. *Cent Eur J Immunol* **44**, 51-58, doi:10.5114/ceji.2019.84017 (2019).
445 Rommel, C. *et al.* Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* **286**, 1738-1741, doi:10.1126/science.286.5445.1738 (1999).
446 Zimmermann, S. & Moelling, K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**, 1741-1744, doi:10.1126/science.286.5445.1741 (1999).

447 Yu, H. P. *et al.* Mechanism of the salutary effects of flutamide on intestinal myeloperoxidase activity following trauma-hemorrhage: up-regulation of estrogen receptor-{beta}-dependent HO-1. *J Leukoc Biol* **79**, 277-284, doi:10.1189/jlb.0705363 (2006).
448 Terry, C. M., Clikeman, J. A., Hoidal, J. R. & Callahan, K. S. Effect of tumor necrosis factor-alpha and interleukin-1 alpha on heme oxygenase-1 expression in human endothelial cells. *Am J Physiol* **274**, H883-891, doi:10.1152/ajpheart.1998.274.3.H883 (1998).
449 Drechsler, Y. *et al.* Heme oxygenase-1 mediates the anti-inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes. *J Immunol* **177**, 2592-2600, doi:10.4049/jimmunol.177.4.2592 (2006).
450 Bauer, I. *et al.* Expression pattern and regulation of heme oxygenase-1/heat shock protein 32 in human liver cells. *Shock* **20**, 116-122, doi:10.1097/01.shk.0000075568.93053.fa (2003).
451 Li, X., Schwacha, M. G., Chaudry, I. H. & Choudhry, M. A. Heme oxygenase-1 protects against neutrophil-mediated intestinal damage by down-regulation of neutrophil p47phox and p67phox activity and O2- production in a two-hit model of alcohol intoxication and burn injury. *J Immunol* **180**, 6933-6940, doi:10.4049/jimmunol.180.10.6933 (2008).
452 Sheppard, F. R. *et al.* Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol* **78**, 1025-1042, doi:10.1189/jlb.0804442 (2005).
453 Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P. & Malik, A. B. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal* **20**, 1126-1167, doi:10.1089/ars.2012.5149 (2014).
454 He, Y. *et al.* HO1 knockdown upregulates the expression of VCAM1 to induce neutrophil recruitment during renal ischemiareperfusion injury. *Int J Mol Med* **48**, doi:10.3892/ijmm.2021.5018 (2021).
455 Cummins, E. P., Keogh, C. E., Crean, D. & Taylor, C. T. The role of HIF in immunity and inflammation. *Mol Aspects Med* **47-48**, 24-34, doi:10.1016/j.mam.2015.12.004 (2016).
456 McGettrick, A. F. & O'Neill, L. A. J. The Role of HIF in Immunity and Inflammation. *Cell Metab* **32**, 524-536, doi:10.1016/j.cmet.2020.08.002 (2020).
457 Zhou, J., Kohl, R., Herr, B., Frank, R. & Brune, B. Calpain mediates a von Hippel-Lindau protein-independent destruction of hypoxia-inducible factor-1alpha. *Mol Biol Cell* **17**, 1549-1558, doi:10.1091/mbc.e05-08-0770 (2006).
458 Mottet, D. *et al.* Role of ERK and calcium in the hypoxia-induced activation of HIF-1. *J Cell Physiol* **194**, 30-44, doi:10.1002/jcp.10176 (2003).
459 Liu, Q., Moller, U., Flugel, D. & Kietzmann, T. Induction of plasminogen activator inhibitor I gene expression by intracellular calcium via hypoxia-inducible factor-1. *Blood* **104**, 3993-4001, doi:10.1182/blood-2004-03-1017 (2004).
460 Berchner-Pfannschmidt, U. *et al.* Chelation of cellular calcium modulates hypoxia-inducible gene expression through activation of hypoxia-inducible factor-1alpha. *J Biol Chem* **279**, 44976-44986, doi:10.1074/jbc.M313995200 (2004).
461 Cramer, T. *et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* **112**, 645-657, doi:10.1016/s0092-8674(03)00154-5 (2003).
462 Walmsley, S. R. *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J Exp Med* **201**, 105-115, doi:10.1084/jem.20040624 (2005).

463 Klopf, J., Brostjan, C., Eilenberg, W. & Neumayer, C. Neutrophil Extracellular Traps and Their Implications in Cardiovascular and Inflammatory Disease. *Int J Mol Sci* **22**, doi:10.3390/ijms22020559 (2021).
464 Jones, J. E., Causey, C. P., Knuckley, B., Slack-Noyes, J. L. & Thompson, P. R. Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. *Curr Opin Drug Discov Devel* **12**, 616-627 (2009).
465 Ham, A. *et al.* Peptidyl arginine deiminase-4 activation exacerbates kidney ischemia-reperfusion injury. *Am J Physiol Renal Physiol* **307**, F1052-1062, doi:10.1152/ajprenal.00243.2014 (2014).
466 Liu, X. *et al.* PAD4 takes charge during neutrophil activation: Impact of PAD4 mediated NET formation on immune-mediated disease. *J Thromb Haemost* **19**, 1607-1617, doi:10.1111/jth.15313 (2021).
467 Yost, C. C. *et al.* Neonatal NET-inhibitory factor and related peptides inhibit neutrophil extracellular trap formation. *J Clin Invest* **126**, 3783-3798, doi:10.1172/JCI83873 (2016).
468 Janciauskiene, S. M. *et al.* The discovery of alpha1-antitrypsin and its role in health and disease. *Respir Med* **105**, 1129-1139, doi:10.1016/j.rmed.2011.02.002 (2011).
469 van Furth, R., Kramps, J. A. & Diesselhof-den Dulk, M. M. Synthesis of alpha 1-anti-trypsin by human monocytes. *Clin Exp Immunol* **51**, 551-557 (1983).

APPENDIX

Curriculum Vitae

Katharina KLAS, BSc, MSc

18.03.1996, Darmstadt Germany
Adress: Agnesstraße 27, 3400 Klosterneuburg, Austria

phone: +43 680 322 99 36
e-mail: klaskatharina@gmx.at

PROFESSIONAL EXPERIENCE

09.2022 – to date	Sanofi Aventis GmbH Medical Scientific Liaison, Immunology (Dermatology)
11.2019 – 08.2022	Aposcience AG, Vienna Conducting PhD project with company's product (Aposec)
09.2018 – 09.2019	Internship (Master Thesis Project: Distinct distribution of RTN1A in Mouse Skin and lymphoid organs) Laboratory of Prof. Dr. Erwin Tschachler Department of Dermatology, Medical University of Vienna
07.2018 – 08.2018	Internship at Christian Doppler Laboratory for Molecular Stress Research in Peritoneal Dialysis (CDL-MSRPD), Ap.Prof. Priv.Doz. DI Dr. Klaus Kratochwill, Medical University of Vienna
06.2016 – 02.2017	Internship (Bachelor Thesis Project: Involvement of basement membrane laminins in proliferation and migration of mammary epithelial cancer cells) Laboratory of Assoc. Prof. Pekka Katajisto Institute of Biotechnology, University of Helsinki
2015	PR activities for The Asia Pacific Early Mobilization Network 1st Annual Conference on Early Mobilization & Rehabilitation in the ICU 2015, Tokyo Japan
2013	Organizational collaboration at the conference 1st European Conference on Waning & Rehabilitation in Critically ill Patients, International Early Mobilization Network, Vienna

EDUCATION

11.2019 – to date	Medical University of Vienna, PhD Candidate PhD Programme Vascular Biology; *Pharmacological inhibition of NETosis by Aposec (in cooperation with Aposcience AG, Vienna)*
2017-2019	University of Veterinary Medicine, Vienna Master's Programme Comparative Biomedicine Focus: Infection Biomedicine and Tumour Signalling Pathways

2014-2017	IMC FH Krems, University of Applied Science Bachelor's Programme Medical and Pharmaceutical Biotechnology
2009-2014	Hertha Firnberg Schulen for Economics and Tourism, Vienna Educational focus: Buisness Responsibility Management

ADDITIONAL QUALIFICATION

2021	Certificate of the Federation of European Laboratory Science (FELASA) for experimental biomedical studies in animals

CONGRESSES AND MEETINGS

02.2022	48th ADF Annual Meeting, Arbeitsgemeinschaft Dermatologische Forschung Poster presentation
06.2021	ÖGDV Science Days Poster presentation

LANGUAGE SKILLS

German	native language (Level C2)
English	fluent in writing and speaking (Level C1-C2)
French	capable of regular conversations and writing (Level B2)
Spanish	basic language skills (A1)

IT SKILLS

MS Office, Adobe Illustrator, GIMP, RStudio/Programming language "R"

SOCIAL COMMITMENT & *HOBBIES*

Make-A-Wish Foundation Austria
Cooking for people in need at "Kochen für die Gruft", Caritas
Riding, Biking, Photography

PUBLICATIONS

The Effect of Paracrine Factors Released by Irradiated Peripheral Blood Mononuclear Cells on Neutrophil Extracellular Trap Formation
Klas K, Ondracek AS, Hofbauer TM, Mangold A, Pfisterer K, Laggner M, Copic C, Direder M, Bormann D, Ankersmit HJ, Mildner M. Antioxidants (Basel) 2022 Aug 11;11(8):1559; doi: 10.3390/antiox11081559

Paracrine Factors of Stressed Peripheral Blood Mononuclear Cells Activate Proangiogenic and Anti-Proteolytic Processes in Whole Blood Cells and Protect the Endothelial Barrier
Copic C, Direder M, Schossleitner K, Laggner M, **Klas K**, Bormann D, Ankersmit HJ, Mildner M. Pharmaceutics 2022, 14(8), 1600; https://doi.org/10.3390/pharmaceutics14081600 - 30 Jul 2022

The secretome of irradiated peripheral blood mononuclear cells attenuates activation of mast cells and basophils.
Laggner M, Acosta GS, Kitzmüller C, Copic D, Gruber F, Altenburger LM, Vorstandlechner V, Gugerell A, Direder M, **Klas K**, Bormann D, Peterbauer A, Shibuya A, Bohle B, Ankersmit HJ, Mildner M. EBioMedicine. 2022 Jul;81:104093. doi: 10.1016/j.ebiom.2022.104093. Epub 2022 Jun 4. PMID: 35671621

Schwann cells contribute to keloid formation.
Direder M, Weiss T, Copic D, Vorstandlechner V, Laggner M, Pfisterer K, Mildner CS, **Klas K**, Bormann D, Haslik W, Radtke C, Farlik M, Shaw L, Golabi B, Tschachler E, Hoetzenecker K, Ankersmit HJ, Mildner M. Matrix Biol. 2022 Apr;108:55-76. doi: 10.1016/j.matbio.2022.03.001. Epub 2022 Mar 10. PMID: 35278628

Severity of thermal burn injury is associated with systemic neutrophil activation.
Laggner M, Lingitz MT, Copic D, Direder M, **Klas K**, Bormann D, Gugerell A, Moser B, Radtke C, Hacker S, Mildner M, Ankersmit HJ, Haider T. Sci Rep. 2022 Jan 31;12(1):1654. doi: 10.1038/s41598-022-05768-w. PMID: 35102298

Secretome of Stressed Peripheral Blood Mononuclear Cells Alters Transcriptome Signature in Heart, Liver, and Spleen after an Experimental Acute Myocardial Infarction: An In Silico Analysis.
Mildner CS, Copic D, Zimmermann M, Lichtenauer M, Direder M, **Klas K**, Bormann D, Gugerell A, Moser B, Hoetzenecker K, Beer L, Gyöngyösi M, Ankersmit HJ, Laggner M. Biology (Basel). 2022 Jan 13;11(1):116. doi: 10.3390/biology11010116. PMID: 35053121

Transcriptional Differences in Lipid-Metabolizing Enzymes in Murine Sebocytes Derived from Sebaceous Glands of the Skin and Preputial Glands.
Klas K, Copic D, Direder M, Laggner M, Prucksamas PS, Gruber F, Ankersmit HJ, Mildner M. Int J Mol Sci. 2021 Oct 27;22(21):11631. doi: 10.3390/ijms222111631. PMID: 34769061

The serine proteases dipeptidyl-peptidase 4 and urokinase are key molecules in human and mouse scar formation.
Vorstandlechner V, Laggner M, Copic D, **Klas K**, Direder M, Chen Y, Golabi B, Haslik W, Radtke C, Tschachler E, Hötzenecker K, Ankersmit HJ, Mildner M. Nat Commun. 2021 Oct 29;12(1):6242. doi: 10.1038/s41467-021-26495-2. PMID: 34716325

Experimental Models for the Study of Hereditary Cornification Defects.
Copic D, Laggner M, Kalinina P, **Klas K**, Tschachler E, Mildner M
Biomedicines. 2021 Feb 26;9(3):238. doi: 10.3390/biomedicines9030238. PMID: 33652877

Distinct Distribution of RTN1A in Immune Cells in Mouse Skin and Lymphoid Organs.
Cichon MA, **Klas K**, Buchberger M, Hammer M, Seré K, Zenke M, Tschachler E, Elbe-Bürger A.
Front Cell Dev Biol. 2021 Jan 15;8:608876. doi: 10.3389/fcell.2020.608876. eCollection 2020. PMID33542915