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CUSTOM-MADE NEUTROPHILIC GRANULOCYTE DERIVED EXTRACELLULAR VESICLES

Ph.D. Thesis

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List of Abbreviations

?: not communicated

acLDL: acetylated low-density lipoprotein

ADP: adenosine diphosphate

aEV: activated or antibacterial EV

ANCA: anti-neutrophil cytoplasmic antibody

APC: antigen-presenting cell

apoEV: apoptotic EV

ATP: adenosine triphosphate

BAL: bronchoalveolar lavage

BMEC: brain microvascular endothelial cell

C5a: complement component 5a

CD: cluster of differentiation

CINC: cytokine-induced chemoattractants

CLP: cecal ligation and puncture

Contr.: control

COVID-19: coronavirus disease 2019

CR: complement receptor

CXCL: CXC motif chemokine ligand

CXCR: CXC motif chemokine receptor

DC: differential centrifugation

DLS: dynamic light scattering

DNA: deoxyribonucleic acid

EB: erythrosine B

EC: endothelial cell

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

EM: electron microscopy

EV: extracellular vesicle

F: filtration

FasL: Fas ligand (CD95L or CD178)

FC: flow cytometry

FcγR: Fc- (fragment crystallisable) gamma receptor

FLS: fibroblast-like synoviocyte

fMLP: N-formylmethionyl-leucyl-phenylalanine

G-CSF: granulocyte colony-stimulating factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

GPIbα: platelet glycoprotein Ib alpha chain (CD42b)

GRO: growth-related gene product

GTP: guanosine triphosphate

HGF: hepatocyte growth factor

HLA: human leukocyte antigen

HMDM: human monocyte-derived macrophage

HUVEC: human umbilical vein endothelial cell

i.p.: intraperitoneal

ICAM-1: intercellular adhesion molecule 1 (CD54)

IEC: intestinal epithelial cell

IFN: interferon

IL: interleukin

IL-1Ra: IL-1 receptor antagonist

LatrB: latrunculin B

LL37: the only human cathelicidin that is cleaved from an 18 kDa cationic antimicrobial protein (CAP18)

L-NAME: N(γ)-nitro-L-arginine methyl ester

LPS: lipopolysaccharide

Mac-1: macrophage-1 antigen (CD11b/CD18, integrin $\alpha_M\beta_2$ or CR3)

MCP: monocyte chemoattractant protein

M-CSF: macrophage colony-stimulating factor

MFI: mean fluorescent intensity

MHC: major histocompatibility complex

MIP: macrophage infiltrating protein

miRNA or **miR:** microRNA

MoDC: monocyte derived dendritic cell

MPO: myeloperoxidase

MSU: monosodium urate

N.S.: not stimulated

n/a: not applicable

NADPH: nicotinamide adenine dinucleotide phosphate

NET: neutrophil extracellular trap

NK cell: natural killer cell

Nonops.: non opsonised

NOX: NADPH oxidase

ns: not significant

NTA: nanoparticle tracking analysis

Ops.: opsonised

P1EC: primary porcine endothelial cell

PAF: platelet-activating factor

Phox: phagocyte oxidase

PI: propidium iodide

PICD: phagocytosis induced cell death

PKH: fluorescent dyes (named after their discoverer Paul Karl Horan)

PLT: platelet

PMA: phorbol 12-myristate 13-acetate

PMN: polymorphonuclear cells (neutrophilic, eosinophilic and basophilic granulocytes)

PRR: pattern recognition receptor

PS: phosphatidylserine

PSGL-1: P-Selectin glycoprotein ligand-1(CD162)

RLU: relative luminescence unit

RNA: ribonucleic acid

ROS: reactive oxygen species

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SEM: standard error of the mean

sEV: spontaneous EV

TEM: transmission electron microscopy

TF: tissue factor (CD142)

TGF: transforming growth factor

Th cells: T-helper cells

THP-1: human monocytic cell line derived from an acute monocytic leukaemia patient

TNF-α: tumour necrosis factor alpha

TP: thromboplastin (a mixture of phospholipids and TF)

TRPS: tuneable resistive pulse sensing

TXA2: thromboxane A2

UV: ultraviolet

VCAM-1: vascular cell adhesion protein 1 (CD106)

VEGF: vascular endothelial growth factor

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1. Introduction

1.1 Extracellular vesicles

Extracellular vesicles (EV) are phospholipid bilayer delimited particles released spontaneously, upon different stimuli and during apoptosis by both pro- and eukaryotic cells [1]. When eukaryotic EVs are released from multivesicular bodies, they tend to be smaller in size (diameter: 30-100 nm) and are often called exosomes, while a medium-sized (100-1000 nm) population referred to as microparticles, microvesicles or ectosomes can be released by blebbing of the plasma membrane. Large EVs (1-5 μm) are typically generated during apoptosis [2] (Fig. 1.). However, there is overlapping in the size distribution and release mechanism of these populations and there is no consensus on specific markers of the abovementioned subtypes. Therefore, researchers are advised to distinguish between EV populations by physical characteristics (e.g. size), biochemical composition or descriptions of conditions or cells of origin [3].

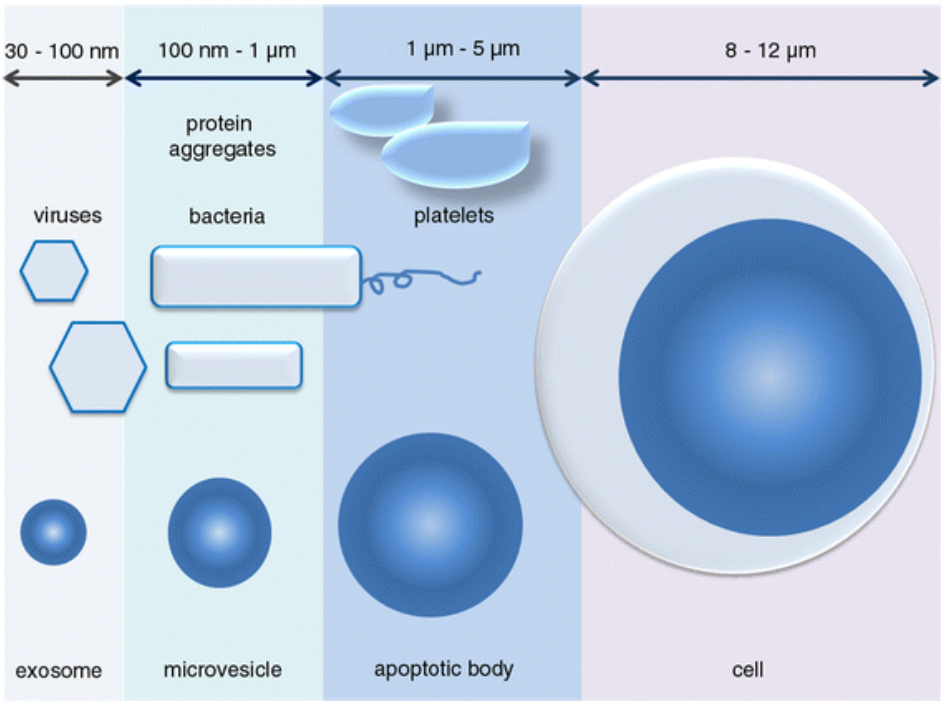

Figure 1. *Dimensions of different EV subtypes* [2].

EVs were first described in 1946 as precipitable factors in platelet-free plasma [4]. In 1967 they were separated by ultracentrifugation from fresh plasma and described as “platelet dust” [5]. Originally, it was thought that EVs were a cellular maintenance or dumping mechanism [1]. Since then, EVs have emerged as versatile packets of biologically active cargo: they can contain lipids, nucleic acids (DNA, RNA, miRNA), carbohydrates and proteins [6]. EVs also expose extracellular facing proteins and lipids derived from the parent cell. Thus, on one hand, EVs are considered as novel means of intercellular communication as they are able to exert influence on diverse biological functions either by transferring their content to other cells or by stimulating receptors of target cells. On the other hand, EVs can affect different extracellular proteins and microorganisms directly, usually by utilizing their specific surface structure and reflecting the functions of the parent cells.

1.1.1 Extracellular vesicles in intercellular communication

Release of EVs, similar to direct cell-to-cell contact and humoral signal transduction, is an important means of intercellular communication. A very large amount of published data reports physiologic processes influenced by EVs. For example, EVs in the cerebrospinal fluid can transport nutrients and growth factors [1], while EVs in the uterine fluid can contribute to sperm capacitation and fertilization [7]. EVs play an important role in embryonic development [8] and successful pregnancy [9]. Urinary EVs can traffic Na^+ transporters and aquaporin-2 along the nephron [10], [11]. EVs in the bronchoalveolar fluid play a role in the response to infectious and allergen stimuli [12], [13].

Similarly, there is a vast amount of evidence about EVs involved in pathophysiological processes. They can transfer receptors [14] and RNA [15], [16] to other cells, thereby promoting tumorigenesis. Malignant cells can even enhance the metastatic behaviour of other cells of the same tumour via EVs [17]. EVs support tissue repair in myocardium [18], liver [19], and kidney [20]. SARS-CoV-2 infected patients showed increased levels of circulating tissue factor positive EVs which could contribute to the characteristic hypercoagulable state in COVID-19 [21]. Similar observations have been made in cancer patients [22].

Intercellular signalling between immune cells is complex and essential, and a multitude of different cytokines participates in their crosstalk. However, it is feasible that EVs can complement these humoral pathways, considering the current knowledge about their role in immune functions [23], [24]. Almost every leukocyte is reported to release EVs affecting function of other cells. Monocytes can enhance the secretion of interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) in airway epithelial cells [25], IL-6 and MCP-1 in podocytes [26] or tumour necrosis factor alpha (TNF-α) and IL-6 in monocytes and macrophages via EVs [27]. T-lymphocyte derived EVs can enhance the secretion of pro- and anti-inflammatory cytokines in monocytes [28], the activation of mast cells [29] and inhibit the NO production of endothelial cells [30]. Natural killer (NK) cells are able to constitutively release EVs with changing composition depending on the environmental factors [1], [31], [32]. Antigen-presenting cells (APCs) can release major histocompatibility- (MHC-) peptide complex containing vesicles during antigen presentation, or send antigenic peptides to the T-lymphocytes [24], [33], [34]. EVs from eosinophil granulocytes may influence the pathogenesis of asthma [35].

1.1.2 Direct effects of extracellular vesicles

Beyond their role in signal transduction, EVs can also have an impact on different extracellular proteins and microorganisms directly.

Platelet-derived EVs account for approximately 25% of the total blood EVs [36] and the very first EV-related observations were based on their procoagulant activity [4], [5]. Since then, these effects have been characterised in great detail and are most likely linked to the phosphatidylserine (PS) expression on the EVs which provides a catalytic surface for the assembly of the coagulation factor complexes [37], [38].

The published procoagulant effects of different EV populations can affect both the intrinsic and the extrinsic pathways in a factor XII dependent manner or by tissue factor (TF) expression, respectively [39]. Interestingly, EVs in the saliva of healthy subjects have also been shown to contain TF and induce clotting. Therefore, it has been suggested

that these EVs could contribute to promoting coagulation when licking a bleeding wound [40].

On the other hand, many studies emphasised an anticoagulant effect of EVs by protein C, protein S, TF pathway inhibitor and plasmin function [41]–[45].

The direct effects of EVs can also play a role in the defence mechanisms against pathogens: tracheobronchial epithelial cells produce antiviral EVs [46], and plants can release EVs which inhibit fungal growth [47]. EVs can also support bone mineralization by enhancing formation of orthophosphate and hydroxyapatite [41]. Urinary EVs express angiotensin-converting enzyme [11] and play a role in the urinary immune defence [48].

In summary, EVs are multipurpose, biologically active particles released by all known cell types [6] with innumerable described effects. Nonetheless, this thesis will focus solely on the role of neutrophilic granulocyte derived EVs.

1.2 Neutrophilic granulocytes

Neutrophils account for 50-70% of the white blood cell count of peripheral blood. They are spherical cells with a diameter of 10-12 μm. The nucleus consists of 3-5 well-defined segments (hence they form part of the polymorphonuclear cell family /PMN/ together with eosinophilic and basophilic granulocytes). Their cytoplasm is granulated, but these granules cannot be stained with acidic nor with basic stains. There are four known types: the lysosomal azurophil (primary) granules and the nonlysosomal specific (secondary), gelatinase (tertiary) and secretory granules [49]. These differ not only in their composition (Table 1.) but also in the tendency for exocytosis: secretory granules are released in great quantities even upon minor stimulation, while azurophil granules (which are basically modified lysosomes) are secreted in smaller amounts – their function is bound mainly to the intracellular (phagosomal) antimicrobial activity [50], [51].

Table 1. *Granule composition of neutrophilic granulocytes (based on* [50]*). Abbreviations: MPO: myeloperoxidase; CR: complement receptor; CD: cluster of differentiation.*

Azurophil (primary) granules	**Specific (secondary) granules**	**Gelatinase (tertiary) granules**	**Secretory granules**
MPO	Lactoferrin	Gelatinase	CR1 (CD35)
Neutrophil elastase	Cathelicidin	Leukolysin	CR3 (CD11b-CD18)
Cathepsin G	Lysozyme	Lysozyme	Formyl peptide receptor
Proteinase 3	Collagenase	Natural-resistance-associated macrophage protein 1	CD14
Azurocidin	Leukolysin	Peptidoglycan recognition protein	CD16
Defensins	Cytochrome b558		
Bacterial permeability-increasing protein	Neutrophil gelatinase-associated lipocalin		

1.2.1 Life cycle of neutrophils

Mature (segmented) neutrophils develop from the myeloid cell lineage through myeloblasts, promyelocytes, myelocytes, metamyelocytes and band (stab) cells. In normal peripheral blood, mostly segmented neutrophils can be found with a smaller percentage (0-4%) of band cells. Although disputed recently by many studies, lifespan of peripheral blood neutrophils is generally thought to be quite short: 6-12 hours when unactivated [52].

Many classical and also rather peculiar forms of neutrophil cell death have been described (Fig. 2.). *Apoptosis* can occur if the cell has not been activated and comes to the end of its life cycle, or if the cell is laden with infectious cargo. In latter case the phenomenon is called *phagocytosis induced cell death (PICD)* [53]. Apoptotic bodies are taken up by macrophages in a process called *efferocytosis*. Furthermore, macrophages can also phagocytose viable neutrophils (termed *phagoptosis*) if they expose PS or other “eat-me” signals, and/or lose “don’t-eat-me” signals on their surface [54]. *Autophagy* is an

intracellular degradation and energy recycling system, by which organelles can be sequestered in autophagosomes. Triggered by phagocytosis-dependent or phagocytosis-independent (e.g. PMA) mechanisms, autophagy is able to detect and eliminate intracellular pathogens that escape from endocytic compartments – a phenomenon often termed xenophagy –, and to culminate in neutrophil cell death [55], [56].

All above mentioned examples represent a coordinated death pathway for neutrophils (with or without infectious cargo) while the cell membrane remains intact. Therefore, they have simultaneous anti-inflammatory and pro-resolving properties leading to the resolution of inflammation and sometimes even to tissue fibrosis. As in these cases cells do not get permeabilised, but PS expression is detectable on the outer surface, these cell death forms can be detected by annexin V labelling, but typically cannot be visualised by the membrane-impermeable nucleic acid dye propidium iodide (PI) [53]. One exception is late apoptosis, where, if the removal of the apoptotic cell (efferocytosis) is delayed, plasma membrane integrity is lost and *secondary necrosis* occurs [57].

The only known cell death form not genetically programmed is *necrosis*. It can be provoked by different destructive impacts like low or high temperature, UV irradiation, radiation, toxic chemical and biological factors with subsequent cellular swelling, disintegration of the cell's nucleus and disturbances in the structure of the cell membrane leading to cell lysis. In some cases, activated neutrophils can develop the same morphological features by a programmed pathway referred to as *necroptosis*. Other circumstances (e.g. activating agents with simultaneous inhibition of reactive oxygen species /ROS/ production) can elicit the cleavage and activation of gasdermin D, thereby forming pores in the plasma membrane and leading to lytic cell death termed *pyroptosis*. In other cases, activating signals cause neutrophils to decondense their chromatin structure, spill their nucleoplasm into the cytoplasm and perforate their membranes. This processed is termed *NETosis* (for detailed description see section 1.2.2) [53], [58].

Necrosis, necroptosis, pyroptosis and NETosis are accompanied with the release of cytoplasmic constituents and pro-inflammatory cytokines in the extracellular space. Hence, these cell death forms are considered being pro-inflammatory. Given the fact that these processes result in disintegration of the plasma membrane integrity, they are detectable by PI staining [53], [57].

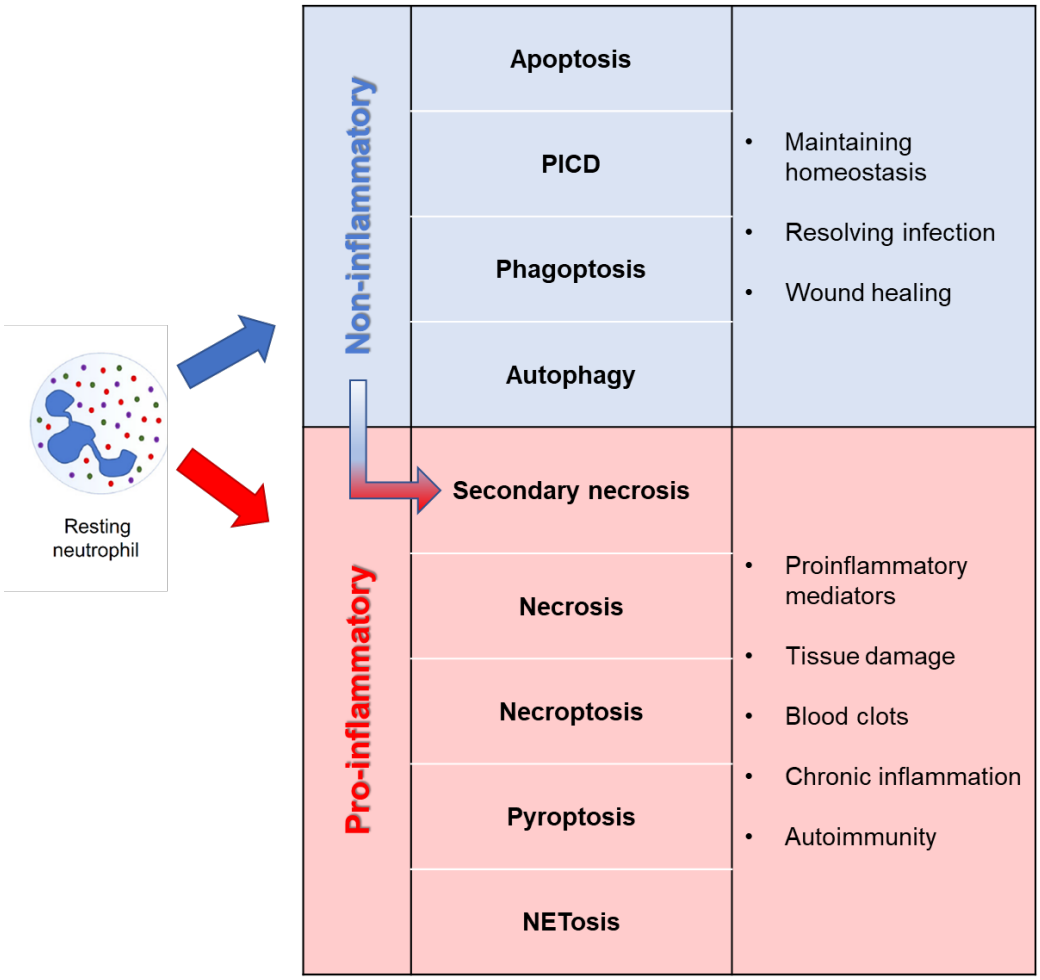

Figure 2. *Main types of neutrophil cell death (based on* [56]*).*

1.2.2 Classic effector functions of neutrophils

Neutrophils are part of the fast-reacting innate immune response, and the principal cells against bacteria and fungi [59]. When they encounter danger signals, they leave the vessels in a complex multi-step process called extravasation (Fig. 3.). After a weaker interaction mediated mostly by selectins (rolling) a stronger bond forms between neutrophils and endothelial cells (adhesion, spreading and crawling) which requires integrins on the surface of the leukocyte [60]. The firm adhesion between the cells during the trans- or paracellular diapedesis ensures the unchanged permeability of the vascular wall in cases where no pro-inflammatory activation is present [61]. The transmigrated neutrophils, guided by diverse chemotactic stimuli (including chemokines, leukotrienes,

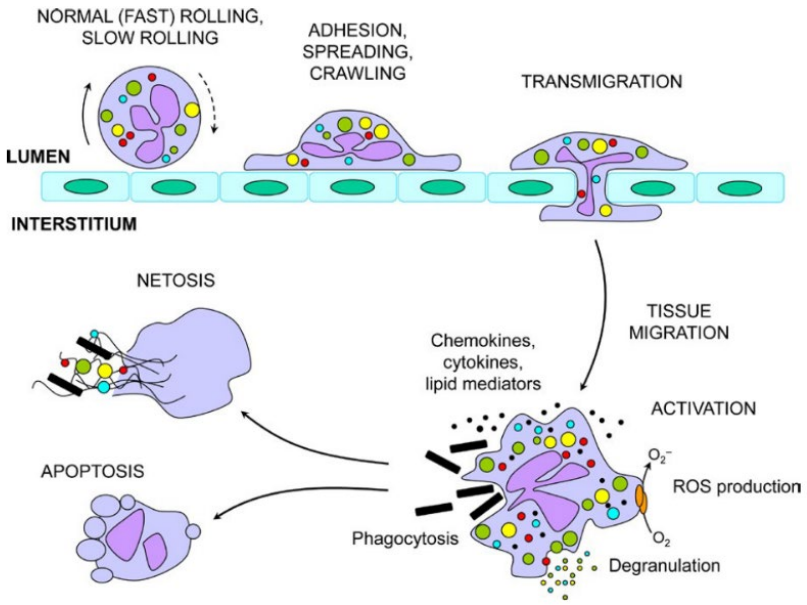

Figure 3. *Stages of neutrophil extravasation and activation* [60].

complement proteins and bacterial fragments), find the infectious foci and encounter the pathogens recognised by opsonin receptors and pattern recognition receptors (PRRs).

If the pathogen can be internalised, phagocytosis occurs, and intracellular killing mechanisms get activated. If, however, the activating agent is immobilised on a surface or too large to be phagocytosed, the very same killing mechanisms are directed towards the extracellular space in a process called “frustrated phagocytosis” [62].

The destruction of the pathogens can be carried out by toxic oxygen metabolites produced by the phagocytic NADPH oxidase (phox) complex (Fig. 4.). It is assembled upon activation in the phagosomal or cell membrane from its membrane bound ($gp91^{phox}$ and $p22^{phox}$) and soluble ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) constituents. One of its regulator proteins (Rac1 or Rac2 – both belong to the family of Rho-GTPases) is associating with $p67^{phox}$ [63]. Its activation is triggered by the phosphorylation of all subunits and the activation of Rac1/2 [64].

When active, the NADPH oxidase generates $O_2^{\bullet -}$ anions in the phagosome or extracellular space from O_2 and electrons transferred from the cytosolic NADPH molecule.

Subsequently, $O_2^{\bullet -}$ anions form H_2O_2 with H^+ ions transported by Hv1 proton channels and V-ATPases. Finally, H_2O_2 is transformed into more toxic HOCl by MPO [65].

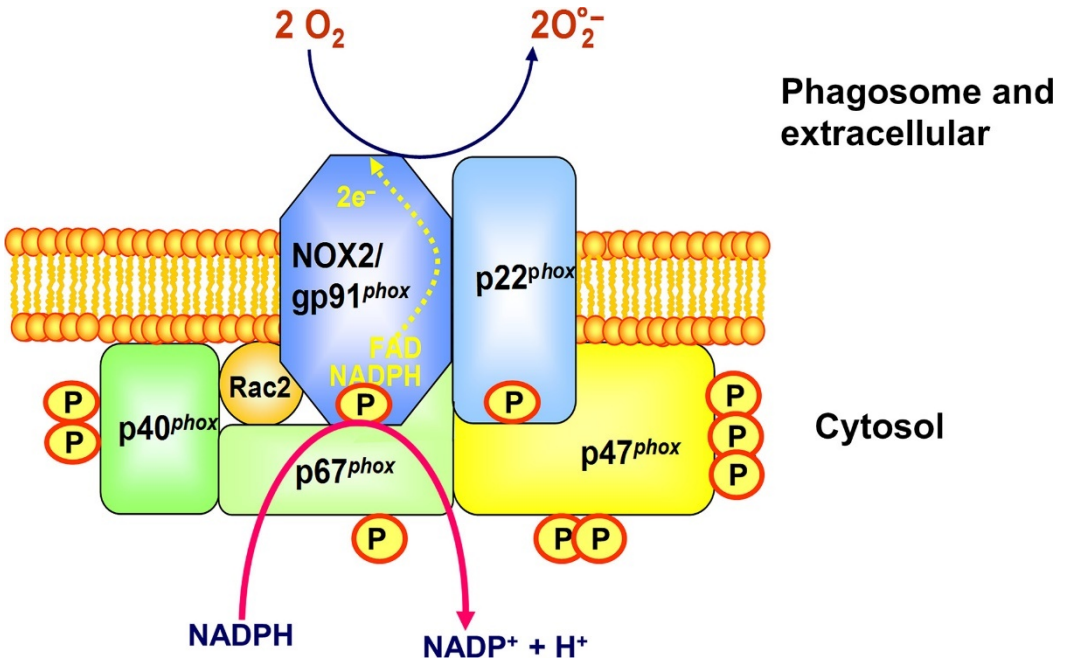

Figure 4. *Structure and phosphorylation sites of the neutrophil NADPH oxidase* [64]. *Abbreviations: NOX: NADPH oxidase.*

Antimicrobial proteins and peptides released from different granule fractions represent another major route of microbe killing in neutrophils [66]. The contribution of these different processes, however, can vary depending on the specific type of microorganism [67].

Historically, mature neutrophils were considered to be terminally differentiated cells lacking the ability to synthetise proteins. In the 1990s, however, it became increasingly clear, that by producing newly manufactured cytokines, neutrophils play a role in the fine regulation of the development and the evolution of inflammatory and immune responses (Table 2.). The first identified and most studied chemokine produced by neutrophils is IL-8, which is not only originating from neutrophils but also targeting them: it enhances cell migration, release of granule enzymes, ROS production and expression of adhesion molecules [68]. The two IL-8 receptors on neutrophils (CXCR1 and CXCR2) have overlapping effects with some functional differences: CXCR2 tends to respond to lower

ligand concentrations than CXCR1, while CXCR1 stimulation results in a wider array of antimicrobial processes [69]. IL-8 is released in larger amounts (i.e. in the order of ng/ml as opposed to pg/ml in case of most of the other secreted products) by neutrophils, and it seems to represent a stereotyped cellular response occurring upon any type of activation [68]. Therefore, by determining the IL-8 secretion of neutrophils one can safely assess the activation state of the cells [70].

Table 2. *Cytokine expression by neutrophils in vitro (based on* [71]*). Abbreviations: IL-1Ra: IL-1 receptor antagonist; GRO: growth-related gene product; MIP: macrophage infiltrating protein; CINC: cytokine-induced chemoattractants; IFN: interferon; G-CSF: granulocyte colony-stimulating factor; FasL: Fas ligand; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; M-CSF: macrophage-CSF; TGF: transforming growth factor.*

Pro-inflammatory cytokines	TNF-α, IL-1α, IL-1β, IL-12
Anti-inflammatory cytokines	IL-1Ra
Chemokines	IL-8, GRO-α, MIP-1α, MIP-1β, CINC
Other cytokines, growth factors	IFN-α, IFN-β, G-CSF, FasL, CD30 ligand, VEGF, HGF
Release under certain conditions	M-CSF, IL-3, GRO-β (CXCL2), IL-18 (IFN-γ inducible factor), TGFα, oncostatin, neurotrophins

1.2.3 Recently discovered functions of neutrophils: Neutrophil extracellular traps

In 1996, neutrophil suicide, distinct from necrosis or apoptosis was described following stimulation with phorbol 12-myristate 13-acetate (PMA) [72]. The observation was repeated by Zychlinsky et al. in 2004 and published as a novel extracellular mechanism able to kill bacteria [73]. Based on these studies neutrophils are capable of decondense their chromatin structure, spill their nucleoplasm into the cytoplasm and perforate their membranes. The process is referred to as neutrophil extracellular trap (NET) formation or NETosis. Some years later, a more rapid, so-called vital form of NETosis was also described, in which case the anuclear cells continue to live, migrate and phagocytose after they have released their chromatin into the interstitial space [74]. In both cases, NETs seem to physically adhere to pathogens, thereby trapping and exposing them to direct

microbicidal effects of the attached proteins (granule proteases, antimicrobial molecules and histones) [58].

1.2.4 Recently discovered functions of neutrophils: Production of extracellular vesicles

As mentioned above, every cell type is able to release EVs – neutrophils are no exceptions either. Neutrophil-derived EVs were first identified in 1991 by Stein and Luzio. Since then, countless direct (e.g. antimicrobial, prothrombotic) and indirect (anti- or pro-inflammatory) effects have been attributed to them, even though these observations are very diverse and sometimes even contradictory. Data from the literature are overviewed in Table 3.

In most publications, the vast majority of neutrophil-derived EVs belongs to the medium-sized population (called microvesicles, microparticles or ectosomes) between 100 and 700 nm diameter. Their appearance on electron micrographs is heterogenous in size, density and structural content both on conventional transmission electron microscopic (TEM) [75] and cryo-TEM [76] images. Neutrophil EVs expose CD66b, CD11b, CD18 and MPO on their surface, and a large part can also be labelled with annexin V due to their PS exposure [77], [78].

The amount of produced EVs is difficult to determine. As EVs are very heterogenous in size, there is no gold standard detection technique for their enumeration. Both single particle detection methods (nanoparticle tracking analysis /NTA/, flow cytometry /FC/, tunable resistive pulse sensing /TRPS/) and bulk measurements (protein and lipid quantification) can lead to inaccurate results, so it is advised to use multiple techniques parallel [3]. It is also informative to compare these quantitative data to a reference population (e.g. spontaneously released EVs with a given cell number and time).

The cargo of the neutrophil-derived EVs arises in large part from the cytoskeleton, granules and mitochondria or belongs to signal proteins [76], [77], [79], [80].

Table 3. *Overview of basic characteristics and effects of neutrophil-derived EVs. Bold: data measured on neutrophils. Italic: the candidate's own results. Abbreviations: HMDM: human monocyte-derived macrophage; DC: differential centrifugation; DLS: dynamic light scattering; NTA: nanoparticle tracking analysis; FC: flow cytometry; EM: electron microscopy; HUVEC: human umbilical vein endothelial cell; F: filtration; ?: not communicated; n/a: not applicable; Th cells: T-helper cells; fMLP: N-formylmethionyl-leucyl-phenylalanine; MoDC: monocyte derived dendritic cell; EC: endothelial cell; BMEC: brain microvascular endothelial cell; IEC: intestinal epithelial cell; PLT: platelet; TXA2: thromboxane A2; ECM: extracellular matrix; LatrB: latrunculin B; GM-CSF: granulocyte-macrophage CSF; LPS: lipopolysaccharide; P1EC: primary porcine endothelial cell; PAF: platelet-activating factor; FLS: fibroblast-like synoviocyte; C5a: complement component 5a; CXCL2: chemokine (C-X-C motif) ligand 2 or GRO-β; Ops.: opsonised; Nonops.: non opsonised; EB: erythrosine B; L-NAME: N(γ)-nitro-L-arginine methyl ester; THP-1: human monocytic cell line derived from an acute monocytic leukemia patient; BAL: bronchoalveolar lavage; LL37: the only human cathelicidin that is cleaved from an 18 kDa cationic antimicrobial protein (CAP18); i.p.: intraperitoneal; ANCA: anti-neutrophil cytoplasmic antibody; MSU: monosodium urate; acLDL: acetylated low-density lipoprotein.*

	PMN-EV induction stimulus	Target	Effect	PMN purity	PMN viability	EV isolation method	EV characterization method	EV diameter [nm]	EV storage	References
Unstimulated	Spontaneous release	HMDM	Bacterial killing ↓	? (nuclear morphology analysed with light microscopy)	95% (Trypan Blue)	DC	FC, EM	50-300	-80 °C	[81]
		PMN, HUVEC, plasma	***Anti-inflammatory, PMN ROS production ↓, procoagulant***	***>95%***	***?***	***DC + F***	***FC, DLS, NTA, EM***	***80-1000***	***none***	[82]
	Apoptosis induction	none	No pro-inflammatory effect	?	n/a	no isolation	FC	?	?	[83]
		Monocytes, HMDM	Mostly anti-inflammatory, but IL-10 production of HMDM ↓	>90% (CD15 FC)	n/a	no isolation	FC	?	-70 °C	[84]
		PMN	**ROS production ↓, Leishmania killing ↓**	**>99.9% (Diff-Quik)**	**n/a**	**no isolation**	**FC**	**?**	**n/a**	[85]
		Th cells	Anti-inflammatory	?	n/a	DC + F	FC, NTA	100-400	?	[86]

		HMDM	Anti-inflammatory	?	n/a	no isolation	FC	?	?	[87]
		PMN, HUVEC, plasma	***PMN ROS production delayed, procoagulant***	***>95%***	***?***	***DC + F***	***FC, DLS, NTA, EM***	***80-1000***	***none***	[82]
Bacterial by-products	fMLP	HMDM	Anti-inflammatory	?	?	DC + F	?	?	-80 °C	[88]
		MoDC	Anti-inflammatory, anti-phagocytotic	?	?	DC + F	FC	?	-80 °C	[89]
		HMDM	Anti-inflammatory	?	?	DC + F	none	?	-80 °C	[90]
		Peritoneal macrophages	Anti-inflammatory	?	?	DC	FC, EM	50-500	?	[91]
		NK cells	Anti-inflammatory	95% or 99% (FC)	?	DC	FC	200-1000	?	[92]
		PMN, systemic	**PMN recruitment ↓, PMN-EC interaction ↓**	**?**	**?**	**DC**	**FC**	**?**	**?**	[93]
		HUVEC	Pro-inflammatory, TF expression ↑	?	?	F or DC	FC	?	?	[94]
		HUVEC	Pro-inflammatory	?	?	DC + F	FC	?	?	[95]
		Human coronary endothelial cells	Pro-inflammatory, pro-migratory	?	?	DC + dialysis	FC, TRPS - human, NTA - mouse	280 (human), 165 (mouse)	?	[96]
		BMEC	Vascular permeability ↑	?	?	DC	FC, NTA	100-300	?	[97]
		IEC	Delivers pro-inflammatory miR content, genomic instability, impaired wound healing	Human: ?, Mouse: 85-90%	?	DC	EM	?	?	[98]
		PLT	Arachidonic acid transfer to PLT, causing TXA2 release and subsequent pro-inflammatory EC activation	?	?	ExoQuick-TC kit	?	?	?	[99]
		HUVEC	Non-adherent PMN-derived EVs: anti-inflammatory, vasoprotective. Adherent PMN-derived EVs: pro-inflammatory, vasoreactive	?	?	DC	FC	?	-80 °C	[80]
		HMDM	Pro-inflammatory, bacterial killing ↑	>98%	>98%	DC	FC, NTA, EM	100-200	4 °C <24h	[100]
		HMDM, PMN, systemic	**Pro- and anti-inflammatory, bacterial killing ↑, PMN and macrophage ROS production ↑**	**?**	**?**	**DC**	**FC**	**2000-3000**	**-80 °C**	[101]
		ECM	Neutrophil elastase dependent degradation of ECM	?	>95% (Trypan Blue)	DC	FC, NTA, EM	100	-80 °C or fresh	[102]

		IEC	Disruption of epithelial intercellular adhesion, enhanced transepithelial migration	Human: ?, Mouse: 85-90%	?	DC	FC, EM	100-800	?	[103]
		Vascular permeability	Maintaining the integrity of the microvascular barrier	?	?	no isolation	FC	?	?	[104]
		S. aureus	Binding to opsonised bacteria	>98%	>99% before and after stimulation (Trypan Blue)	DC + F	EM	?	?	[105]
	fMLP or fMLP + LatrB	IEC	Inhibition of epithelial wound healing via MPO delivery	Human: ?, Mouse: 85-90%	?	no isolation	FC, EM	600	?	[106]
	GM-CSF + (?) fMLP	**PMN**	**Pro-inflammatory**	**?**	**?**	**DC + F**	**FC, EM**	**50-120 (purified from 50-500)**	**?**	[107]
	fMLP + LPS	**PMN, HMDM**	**ROS production ↑**	**?**	**?**	**DC**	**FC**	**?**	**?**	[108]
	LPS	P1EC, artery rings	Pro-inflammatory, oxidative stress ↑, TF expression ↑	n/a (splenocytes)	?	DC	TRPS	200-500	?	[109]
		Airway smooth muscle cells	Proliferation	99.5% (Cytospin slide + Protocol Hema 3 staining)	97.75% (ADAM cell counter)	Size-exclusion chromatography	DLS, EM	30-80	-80 °C	[110]
		PLT	Platelet activation and co-aggregation with PMN, delivery of PAF receptor	?	?	DC	none	?	?	[111]
		PLT	Platelet activation	?	?	DC + F	FC	<1000	-80 °C	[112]
Endogenous pro-inflammatory mediators	TNF-α	HDMD, joints, macrophage-FLS co-culture system	Anti-inflammatory	?	?	DC	FC, NTA	70-400	?	[113]
		IEC	Delivers pro-inflammatory miR content, genomic instability, impaired wound healing	Human: ?, Mouse: 85-90%	?	DC	EM	?	?	[98]
		Embryonic kidney cells	Transfer of kinin B1-receptors, calcium influx	?	?	DC	FC, EM	150	-80 °C	[114]
	IFN-γ	IEC	Delivers pro-inflammatory miR content, genomic instability, impaired wound healing	Human: ?, Mouse: 85-90%	?	DC	EM	?	?	[98]

		PMN, HUVEC	**Mainly pro-inflammatory and pro-migratory, but reduced increase in EC permeability upon LPS treatment**	**n/a (stimulation in whole blood)**	**n/a (stimulation in whole blood)**	**? (DC)**	**FC**	**?**	**?**	[115]
	GM-CSF	**PMN, HUVEC**	**Mainly pro-inflammatory and pro-migratory, EC ROS production ↑, but reduced increase in EC permeability upon LPS treatment**	**n/a (stimulation in whole blood)**	**n/a (stimulation in whole blood)**	**? (DC)**	**FC**	**?**	**?**	[115]
	C5a	HMDM	Anti-inflammatory	?	?	DC + F	none	?	-80 °C	[90]
		NK	Anti-inflammatory	95% or 99% (FC)	?	DC	FC	200-1000	?	[92]
		PMN, whole blood	**Pro-inflammatory, ROS production ↑, MPO release ↑**	**?**	**?**	**DC**	**FC**	**300-1000**	**-80 °C**	[116]
	PAF	**PMN, systemic**	**PMN recruitment ↓, PMN-EC interaction ↓**	**?**	**?**	**DC**	**FC**	**?**	**?**	[93]
		PLT	Platelet activation	?	?	DC + F	FC	<1000	-80 °C	[112]
	IL-8	NK	Anti-inflammatory	95% or 99% (FC)	?	DC	FC	200-1000	?	[92]
	CXCL2	Vascular permeability	Maintaining the integrity of the microvascular barrier	?	?	no isolation	FC	?	?	[104]
Pathogens	*M. tuberculosis*	HMDM	Bacterial killing ↓	?, but nuclear morphology analysed with light microscopy	95% (Trypan Blue)	DC	FC, EM	50-300	-80 °C	[81]
	M. tuberculosis	HMDM	Pro-inflammatory, ROS production ↑, autophagy ↑, bacterial killing ↑	>98%	>98%	DC	FC, NTA, EM	100-700	4 °C <24h	[100]
	Ops. *A. fumigatus*	*A. fumigatus*	Antifungal effect	>95%	>98%	DC + F	FC, NTA, EM	?	-80 °C or fresh	[76]
	P. aeruginosa	*P. aeruginosa*	Antibacterial effect	?	?	no isolation	none	?	?	[117]
	Ops. *S. aureus*	Ops. and nonops. *S. aureus, E. coli*	Binding to bacteria, antibacterial effect	>95%	80-85% (EB)	DC + F	FC, DLS, EM	100, 200-800	?	[77]
	Ops. zymosan	*S. aureus, E. coli*	Antibacterial effect	>95%	?	DC + F	FC	?	?	[118]

		PMN, HUVEC, plasma	***Pro-inflammatory, PMN ROS production ↑***	***>95%***	***?***	***DC + F***	***FC, DLS, NTA, EM***	***80-1000***	***none***	[82]
Pharmacological stimuli	PMA	MoDC	Anti-inflammatory, Th2 polarization	?	?	DC	FC, DLS	50-600	-80 °C	[119]
		HMDM	Pro-inflammatory	>98%	>98%	DC	FC, NTA, EM	100-300	4 °C <24h	[100]
		IEC	Inhibition of epithelial wound healing via MPO delivery	Human: ?, Mouse: 85-90%	?	DC	FC, EM	600	?	[106]
		S. aureus	Binding to opsonised bacteria	>98%	>99% before and after stimulation (Trypan Blue)	DC + F	EM	?	?	[105]
		Plasma, NET	Procoagulant (intrinsic), NET-binding	?	?	no isolation	FC, EM	?	?	[120]
		PLT	Platelet activation	?	?	DC + F	FC	<1000	-80 °C	[112]
	PMA + A23187	P1EC, artery rings	Pro-inflammatory, oxidative stress ↑, TF expression ↑	n/a (splenocytes)	?	DC	TRPS	200-500	?	[109]
	A23187	HUVEC	MPO-mediated cytotoxicity	>90% (FC CD66b)	?	DC	FC, EM	<1000	4 °C	[121]
	Ionomycin	*S. aureus*	Binding to opsonised bacteria	>98%	>99% before and after stimulation (Trypan Blue)	DC + F	EM	?	?	[105]
	L-NAME	**PMN**	**Pro-migratory**	**>97% (haemocytometer)**	**>95% (Trypan Blue)**	**DC**	**FC, EM**	**?**	**?**	[122]
Pathophysiological environment	Sepsis	THP-1	Pro-inflammatory, pro-phagocytotic	n/a (peritoneal and BAL EVs)	n/a (peritoneal and BAL EVs)	C	FC	300-1100	?	[123]
		HUVEC, Plasma, ops. *S. aureus*	Pro-inflammatory, procoagulant, binding to ops. bacteria	>95% (FC)	?	DC	FC, NTA	50-800	?	[124]
		Ops. and nonops. *S. aureus, E. coli*	Binding to bacteria	n/a (plasma EVs)	n/a (plasma EVs)	DC + F	FC	?	?	[77]

	Sepsis + LL37	*E. coli*	Antibacterial effect	90% (Giemsa)	?	DC	FC	500-1000	-80 °C	[125]
	Sepsis + thioglycolate i.p.	Peritoneal macrophages, systemic	Pro- and anti-inflammatory, bacterial clearance ↓, mortality ↑	n/a (peritoneal EVs)	n/a (peritoneal EVs)	DC	FC	?	?	[126]
	Cystic fibrosis/Primary ciliary dyskinesia	Airways	Pro-inflammatory	n/a (sputum EVs)	n/a (sputum EVs)	DC	FC	?	4 °C	[127]
	Pancreatitis	Pancreas acinar cells, systemic	Pro-inflammatory, tissue injury ↑	n/a (pancreatic EVs)	n/a (pancreatic EVs)	DC	EM	?	n/a (pancreatic EVs)	[128]
	ANCA vasculitis	none	Procoagulant (extrinsic)	?	?	DC	FC	?	?	[129]
	TNF-α + ANCA	HUVEC	Pro-inflammatory, procoagulant, ROS production ↑	?	?	DC	FC	?	Frozen (no temp. data)	[130]
	Rheumatoid Arthritis + TNF-α	HDMD, joints, macrophage-FLS co-culture system	Anti-inflammatory	?	?	DC	FC, NTA	70-400	?	[113]
	MSU i.p.	Peritoneal macrophages	Anti-inflammatory	n/a (peritoneal EVs)	n/a (peritoneal EVs)	DC	FC, EM	50-500	?	[91]
	Gout	Peritoneal macrophages	Anti-inflammatory	n/a (synovial EVs)	n/a (synovial EVs)	DC	FC, EM	50	?	[91]
	acLDL	Human coronary endothelial cells	Pro-inflammatory, pro-migratory	?	?	DC + dialysis	FC, TRPS - human, NTA - mouse	280 (human), 165 (mouse)	?	[96]
	Hypercapnia	none	Release of EVs carrying IL-1β	?	>80% after EV isolation (Trypan Blue)	no isolation	FC	300-1000	?	[131]
	Hyperglycaemia	none	Release of EVs carrying IL-1β	?	>78% after EV isolation (Trypan Blue)	no isolation	FC	300-1000	?	[132]

1.2.4.1 Effect of neutrophil EVs released without stimulation

Neutrophils are shown to release EVs constitutively without activation (spontaneous EVs or sEVs). Several groups reported either direct anti-inflammatory effects (i.e. decreased antibacterial effect of macrophages) [81] or no occurrence of an investigated, rather pro-inflammatory effect when sEVs were used instead of any activated EV: missing migratory enhancement and endothelial activation [96], [109], equine airway smooth muscle cell proliferation [110], macrophage activation [100], phagocytic ROS production [108], lung ECM destruction [102] or vascular senescence and endothelial ROS production [109].

When PMNs are left unstimulated for several hours or in case of proapoptotic environment (e.g. UV-B/C radiation) apoptotic vesicles (apoEVs) are released. Studies found either direct anti-inflammatory or no pro-inflammatory effects when human [83], [84] or murine [87] macrophages, monocytes [84], other neutrophils [85] and Th cells [86] were exposed to apoEVs. There is only one study reporting a weak, statistically nonsignificant pro-inflammatory effect of apoEVs on LPS stimulated macrophages, where IL-10 production was diminished, although this effect was also accompanied by elevated TGFβ release [84].

1.2.4.2 Effect of neutrophil EVs released upon stimulation with different activators

As presented in Table 3. numerous publications have described diverse effects of EVs originating from activated neutrophils.

fMLP-treated neutrophils tend to secrete anti-inflammatory EVs [88]–[92], [104], albeit they can become pro-inflammatory when also other activators (e.g. GM-CSF or LPS) are present [107], [108]. Besides the anti-inflammatory nature of fMLP-EVs affecting other leukocytes, they seem to activate endothelial cells [93]–[96]. In accordance, coincubation of neutrophils with platelets, ADP and fMLP leads to EV mediated arachidonic acid transfer from neutrophils to platelets, where it fuels the TXA2 synthesis, which in turn leads to endothelial and subsequent neutrophil activation [99].

Sporadic studies investigated the nature of PMN-EVs released upon LPS stimulation, and all of them reported rather pro-inflammatory effects [109], [110].

EVs from PAF [93], IL-8 [92] and CXCL2 [104] activated cells demonstrated anti-inflammatory potential, while complement activated (e.g. C5a) were rated as both anti- [90] and pro-inflammatory [116]. EVs released from neutrophils stimulated with TNF-α, IFN-γ and GM-CSF exhibit a rather pro-inflammatory profile [98], [114], [130] but anti-inflammatory effects have been published as well [113], [115].

Opsonised pathogens deliver multiple strong activating signals to neutrophils via PRRs and opsonin receptors (e.g. Mac-1, FcγR). EVs produced upon stimulation with pathogens are reported to have pro-inflammatory [100], [133] and also anti-inflammatory [81], [92] properties. A work group reported microbicidal effects on *P. aeruginosa* [117] or *A. fumigatus* [76].

PMA, a potent pharmacologic activator of PMN can also induce the production of EVs. As opposed to the powerful overall activating effect of PMA, these EVs seem to be rather anti-inflammatory in nature [100], [119]. On the other hand, when Ca^{2+} ionophores or L-NAME, a NOS inhibitor are used for production, EVs exhibit pro-inflammatory properties [109], [121], [122]. PMA and ionomycin induced EVs also bind to opsonised bacteria via clusters of CR1 [105].

1.2.4.3 Effect of neutrophil EVs released in pathophysiological environments

It is shown in several studies, that neutrophils encountering different types of danger signals in pathological conditions release EVs with different effects.

Sepsis and its animal models are connected to neutrophils in multiple ways – since in most cases the causative agents are bacteria, neutrophils are affected by both the initiation and the effector phase of the disease. Cytokine storms characteristic in sepsis can also both originate from and affect neutrophils.

Due to the complex nature of the disease and the variety of its animal models many neutrophil activation states, and thus, several EV profiles are imaginable.

Kumagai et al. found that injecting mice with the antimicrobial peptide LL-37 after cecal ligation and puncture (CLP) induces EV production with antibacterial potential and reduces bacterial load [125]. Another workgroup reported enhanced phagocytotic activity, pro-inflammatory activation and increased HLA-DR expression of monocytes exposed to EVs released from pulmonal and abdominal sepsis patients [123]. The same group reported an even harmful anti-inflammatory, immunoparalytic effect of peritoneal EVs isolated from CLP mice after injection with thioglycolate, however, elevated systemic IL-6 levels were found [126].

Acute pancreatitis can also be accompanied by severe systemic inflammation, hence there are immunological traits related to sepsis. A recent study showed, that neutrophil-derived EVs associated to NET in an animal model of acute pancreatitis, which contributed to both local and systemic deterioration of inflammation [128].

Neutrophil-derived EVs isolated from the sputum of cystic fibrosis and primary ciliary dyskinesia patients show also pro-inflammatory properties: if administered intratracheally in mice, histopathological analysis shows peribronchial and perivascular infiltrates [127].

High fat diet was shown to increase the neutrophil-derived EV concentration in blood, and the EVs were found to accumulate in atheroprone regions of the vasculature. As acLDL stimulated neutrophils produce EVs with higher amounts of pro-inflammatory miR-155, and EVs were shown to cause endothelial activation, the authors concluded that these EVs contribute to vascular inflammation and atherogenesis [96].

Rheumatological diseases are also shown to be related to neutrophil function and EVs. TNF-α primed neutrophils released EVs when treated with ANCA, and these EVs enhance the ICAM-1 expression of HUVEC [130]. On the other hand, Rhys et al. show that EVs released from neutrophils upon TNF-α treatment are anti-inflammatory in a macrophage-fibroblast-like synoviocyte co-culture system, and this effect is more pronounced if the PMNs are derived from rheumatoid arthritis patients [113]. Intraperitoneal administration of MSU also lead to the production of anti-inflammatory EVs manifesting in decreased IL-1β release and increased TGFβ release of macrophages [91].

Hypercapnia [131] and hyperglycaemia [132] also enhanced EV production of neutrophils. Higher amounts of IL-1β were found in hyperglycaemia induced EVs, which might represent a pro-inflammatory potential [132].

1.2.4.4 Effect of neutrophil EVs on haemostasis

Many pathophysiological conditions, including bleeding and thrombotic disorders are accompanied by elevated levels of EVs [2]. Since EVs expose high amounts of PS on their outer membrane, and some EVs have also been shown to carry TF, many studies describe procoagulant effects of neutrophil-derived EVs.

Some studies report direct procoagulant activity of EVs via the extrinsic pathway by TF expression [129] or via the intrinsic pathway by binding to factor XII [120]. One study showed that TNF-α primed ANCA activated neutrophils released more procoagulant EVs than resting cells [130]. Other studies described an indirect procoagulant activity of EVs by enhancing TF expression of endothelial cells [94] and leading to a secondary generation of procoagulant endothelial microparticles [109].

Other works describe a platelet activating role of neutrophil EVs. One study reports PAF release and consequential platelet activation by EVs released upon endotoxin stimulation of adherent cells [111]. Pluskota et al. show, that conformationally active CD11b/CD18 is enhanced on EVs derived from activated cells, and they are capable of activating platelets with via CD11b/CD18 –glycoprotein Ibα and P-Selectin glycoprotein ligand-1 – P-Selectin interactions [112].

Taken together, basic physical and chemical characteristics of neutrophil-derived EVs are similar in many studies, while the variety of detected effects can be puzzling. The inconsistent data regarding the EVs' effects could arise from the different quality and purity of the PMN isolates, different protocols used for EV preparation, storage conditions of the EV samples and different experimental environment of the investigated target cells. Data are more comprehensible when one considers the different stimulating agents used for EV production, which is the most crucial factor in our opinion. However, there is considerable inconsistency in the results even if we compare the reports using the

same activators. We can hardly find any comparative studies, where two or more types of EVs are parallelly investigated. Without these the effect of specific environmental factors and stimuli on the behaviour of neutrophil EVs cannot be determined, as none or very few basic experimental procedures are matching.

Importantly, to date only eight of the many above presented studies have investigated the effects of neutrophil EVs on neutrophils themselves. As shown in Table 3., these results are also inconsistent and not comparable due to the strongly varying experimental conditions.

1.2.4.5 Characterisation of neutrophil EVs in our laboratory

Earlier studies of our work group demonstrated, that there are at least three different neutrophil-derived EV populations (Table 4.): EVs released from resting cells (sEVs), EVs released from unstimulated dying cells (apoEVs) and EVs released from cells activated with opsonised particles (activated or antibacterial EVs, aEVs) [75], [77], [79], [118], [134]–[136].

The release of sEVs was constitutive and not inhibited by the studied inhibitors or genetic deficiencies of receptors and signalling molecules. apoEVs were released in great amounts when incubating unstimulated neutrophils for a longer period of time (e.g. 24 h). aEV production was dependent on stimulation with opsonised particles. It was also shown, that Mac-1 stimulation via PLCγ2 and Ca^{2+} signalling is necessary and sufficient for aEV release [75], [77], [79], [134].

The protein content of the smaller aEVs and sEVs was high corresponding to their dens appearance on electron micrographs. The larger apoEVs had less protein content and appeared empty on electron microscopic images. The proportion of granule proteins was low in sEVs but high in aEVs and apoEVs. All three populations showed PS expression, but none of them was detectable by PI staining. DNA was only detected in apoEVs, but we found evidence of RNA in all three EV types. None of the investigated EV populations showed signs of ROS production [77], [79], [135], [136].

Table 4. *Summarised characteristics of our three EV populations (results based on* [77], [79]*).*
+ means detected characteristics; - means not detectable characteristics.

	aEV	sEV	apoEV
Size	Peaks at 100 and 500 nm	Peaks at 100 and 500 nm	Peaks at 200 and 800 nm
Protein content	High	High	Low
Granule protein ratio	High	Low	High
PS expression	+	+	+
PI staining	-	-	-
Electron microscopic appearance	Dens, intact	Dens, intact	Empty, intact
RNA concentration (ng/µl)	25.7 ± 4.21	22.83 ± 10.46	66.26 ± 14.79
DNA concentration (ng/µl)	-	-	1.35 ± 0.87
ROS production	-	-	-
Antimicrobial activity	+	-	-

Importantly, only aEVs were capable of forming large aggregates with opsonised and non-opsonised bacteria, which resulted in inhibition of bacterial growth [77]. It was also shown that neutrophil-derived EV numbers in sera of septic patients were significantly increased, and they were more likely to form aggregates with bacteria [77]. Based on these results, a point-of-care microfluidic chip was proposed by another work group, which detects EV-bacteria aggregates characteristic for bacterial infections, not present however in non-infectious inflammation [124].

Our laboratory also showed that the presented characteristics of these neutrophil-derived EV populations depend on the storage techniques. Storage at +20 °C, +4 °C or -20 °C influenced both the morphology and the antibacterial function of EVs, while storage at -80 °C preserved the morphological properties but also lead to partial loss of functional traits. Snap-freezing did not have an influence on these changes, and commonly used cryoprotectants induced EV lysis [136].

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2. Objectives

As presented above, the diverse, often contradictory reports about the effects of neutrophilic granulocyte derived EVs are difficult to interpret. The aim of my Ph.D. work was to use three, in our laboratory's previous publications well defined [75], [77], [79], [118], [134], [136], after isolation freshly applied neutrophil EV populations to evaluate their effects on different physiological processes under comparable conditions.

Initially, I intended to clarify whether these neutrophil-derived EVs are taken up by other neutrophils, monocytes and lymphocytes.

The isolation of neutrophils including their purity and initial viability as well as the used stimuli for EV production and the EV isolation protocols are well established in our research group. On these grounds, we anticipated to obtain a more comprehensive concept of how neutrophils regulate the inflammatory responses of the organism by the release of different EV populations.

In particular, I aimed to investigate the effect of

1. spontaneously released EVs (sEVs)
2. EVs released upon stimulation with opsonised zymosan particles (aEVs) and
3. EVs released upon spontaneous cell death in the course of incubation without activating factors for 24 h (apoEVs)

on

A. other neutrophilic granulocytes
B. endothelial cells and
C. blood plasma.

Finally, it was also important to show, that neutrophils remain viable and do not release significant amounts of NET during our EV production step, so that the detected effects can entirely be attributed to EVs.

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3. Results

Detailed description of the used methods can be found in F. Kolonics et al., JLB, 2020 (for 3.1-3.4) and in Á. M. Lőrincz et al., JEV, 2019 (for 3.5). Venous blood samples were drawn according to the procedures approved by the National Ethical Committee (ETT-TUKEB No. BPR/021/01563-2/2015). HUVEC were harvested from fresh umbilical cords obtained during normal delivery of healthy neonates according to Helsinki Protocol, Semmelweis University Institutional Review Board specifically approved this study (SETUKEB No. 141/2015).

3.1 Interaction of neutrophil EVs with other neutrophils, monocytes and lymphocytes

Neutrophil-derived EVs were shown to be taken up into macrophages [113], but no similar data are available for uptake into neutrophils themselves. The first question to be decided was whether the different populations of neutrophil-derived EVs were all engulfed by leukocytes. The fate of fluorescently labelled neutrophil EVs was followed upon encounter with neutrophils, monocytes or lymphocytes. EVs produced upon stimulation with opsonised zymosan (aEVs) or spontaneously from fresh (sEVs) or apoptotic cells (apoEVs) were labelled with the membrane-localised stain PKH67 and fluorescence was detected by flow cytometry (Fig. 5.).

Figure 5A presents the original data in form of dot plots on the fluorescence distribution at the beginning and at the end of the 45 min incubation time in a representative experiment. Summarised data of the increase of mean fluorescent intensity (ΔMFI) are provided in Figure 5B. As PKH67 is able to form micelles, control measurements were carried out with the stain left in the supernatant of centrifuged EVs. Figure 5B presents the control data as well, which demonstrate whether there was a significant difference between MFI values in the presence or absence of EVs. In 45 min measurable increase of MFI occurred with aEV and apoEV populations in all three cell types. On the other hand, sEVs seem to associate with neutrophils only.

With confocal microscopic imaging I could verify that EVs are engulfed in neutrophils, as opposed to staying only attached on the surface of the cells (Fig. 5C).

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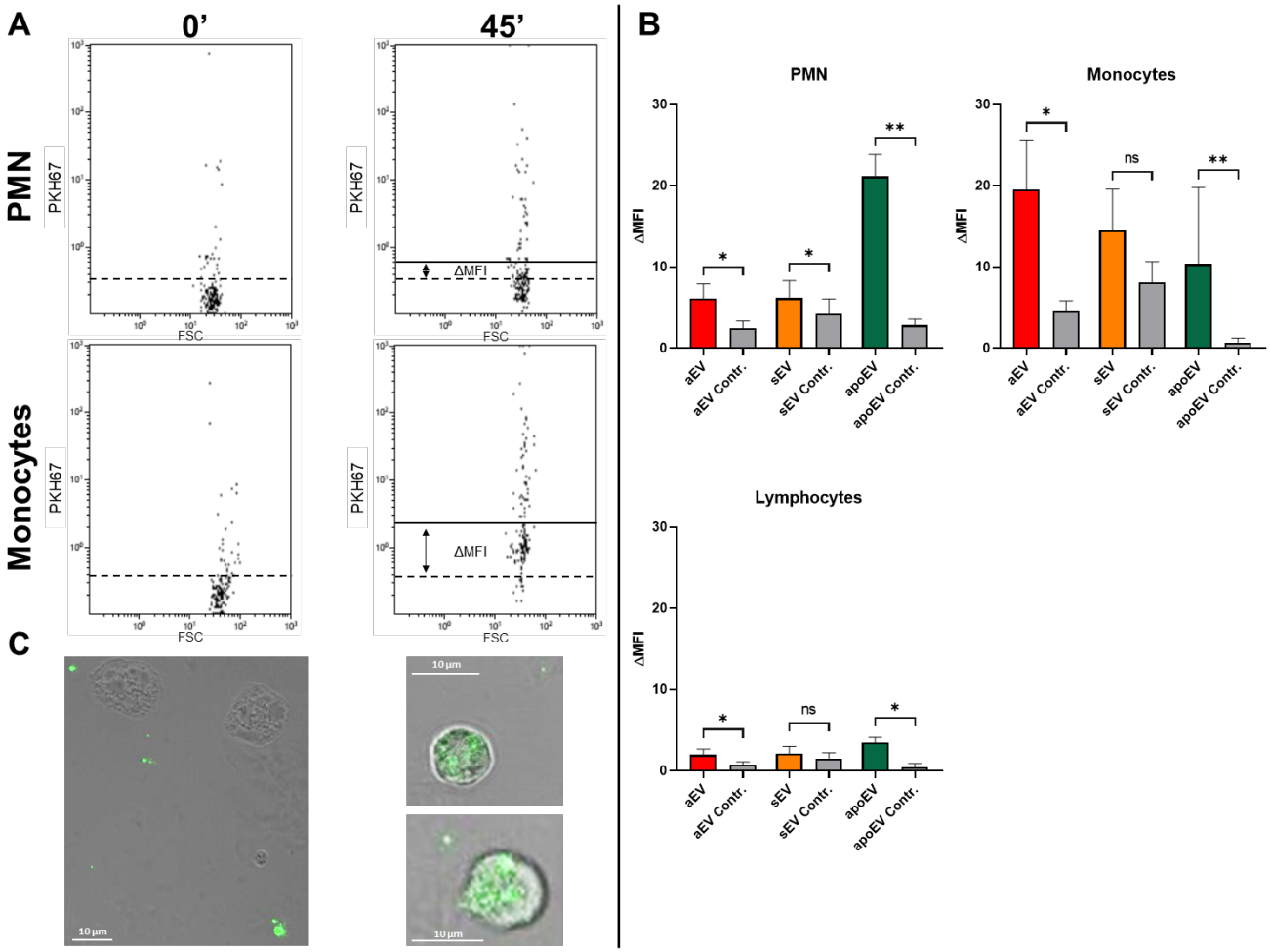

Figure 5. *Interaction of neutrophil EVs with leukocytes.*

A. *Flow cytometric dot plots of PMN and monocytes at 0 min and 45 min of incubation with PKH67 labelled sEVs. Representative data out of 14 similar experiments. Lines indicate the geometric mean of green fluorescence (MFI) at 0 min (dashed) and 45 min (solid).*

B. *Values of ΔMFI after 45 min of incubation with the indicated type of EVs or their controls. Data were compared using paired Student's t-test; n=14 for aEV, n=6 for sEV, n=5 for apoEV (PMN); n=15 for aEV, n=6 for sEV, n=5 for apoEV (monocytes, lymphocytes). Error bars represent mean +standard error of the mean (SEM).*

** represents $p < 0.05$; ** represents $p < 0.01$.*

C. *Representative confocal microscopic images of PMN at 0 min (left) and 45 min (both on the right) of incubation with PKH67 labelled aEVs out of 2 independent experiments.*

Abbreviations: Contr.: control; ns: not significant.

3.2 Effect of neutrophil EVs on other neutrophilic granulocytes

As mentioned above, plenty of studies investigated the effect of neutrophil EVs, but only eight of them discussed effects on neutrophils themselves. As demonstrated in Table 3., six of them described pro-inflammatory effects, while two found rather anti-inflammatory properties (one of these being the effect of apoptotic vesicles). We decided to investigate the effects of our three EV populations on four basic neutrophil functions: chemotactic cell migration, phagocytosis, ROS production and cytokine production.

It is, however, very difficult to investigate the effects of aEVs only. Since they are produced by adding opsonised zymosan to neutrophils, and during the isolation process, they are not separated from the EVs themselves, opsonised zymosan particles remain an inherent part of the aEV fraction. Opsonised zymosan is reported to have an average particle diameter of 3 μm [137] and it is very likely that opsonised zymosan particles stick to EVs, thereby forming larger aggregates, based on our earlier studies [77]. Therefore, we considered any attempt to modify our EV isolation protocol in order to separate zymosan from EVs futile and decided to control our aEV samples by removing the much more vulnerable EV component from the mixture. Since EVs are lipid bilayer bordered structures, they are highly sensitive to hypotonic lysis and mechanical disruption. Hence, in every experiment involving aEVs, we resuspended half of the sample in distilled water and vortexed it for 10 min at maximum intensity. Finally, we centrifuged the sample and resuspended the pellet in the used medium. I will refer to this special control as “lysed aEV”. Given that this control sample consists of a small amount of opsonised zymosan, it is expected to have an effect on neutrophils to some extent. Any additional effect seen in the aEV group compared to this lysed aEV group can safely be attributed to the vesicles.

Considering that sEVs and apoEVs do not contain any exogenous particles, their effect was compared to the absolute negative control designated as “No EV”.

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3.2.1 Effect of neutrophil EVs on the migratory potential

First, we investigated the effect of our EV populations on the migratory potential of neutrophils. We pretreated cells with previously produced sEVs, apoEVs and aEVs for 45 min and put them in a transwell chamber filled with fMLP as chemoattractant.

After one hour of migration, there was no difference in the absolute numbers of transmigrated cells between the different groups (Fig. 6A). Figure 6B-D show the results of the individual experiments as normalised to their adequate controls.

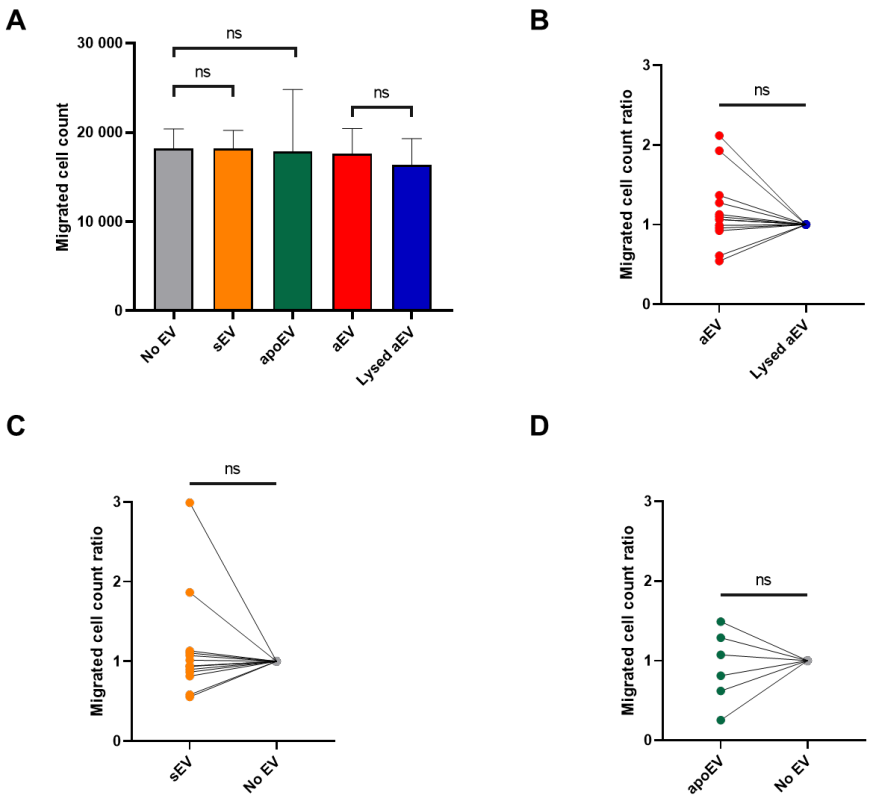

Figure 6. *Effect of EVs on the migratory potential of neutrophils. PMN were pretreated for 45 min with one of the three EV populations or their controls, then placed in a transwell cell culture plate for 1 h. As a chemoattractant, 100 nM fMLP was used. Transmigrated cells were quantified using an acid phosphatase assay.* ***A*** *shows the migrated cell counts in EV- or control-pretreated samples,* ***B-D*** *show the normalised data pairs from each experiment. Data were normalised to their adequate controls ("aEV" to "Lysed aEV", "sEV" and "apoEV" to "No EV"). Raw data were compared using paired Student's t-test; n=13 for aEV & sEV; n=6 for apoEV. Error bars represent mean +SEM.*

3.2.2 Effect of neutrophil EVs on the phagocytotic capacity

Next, we analysed the phagocytotic capacity of neutrophils after 45 min pretreatment with the different EV populations. Pretreated cells were incubated for 20 min with opsonised fluorescent *S. aureus* bacteria (the green fluorescent protein-expressing *USA300* strain was a kind gift of Professor William Nauseef, University of Iowa [138]). Either one concentration of bacteria was used (neutrophil:bacteria ratio 1:30) and samples were taken every 5 min (kinetic measurements), or five different concentrations of bacteria were used (neutrophil:bacteria ratios 1:10, 1:30, 1:100, 1:300 and 1:1000) and the sample was analysed at the end of the incubation period only (maximal capacity measurements). Figure 7. shows that neither the kinetics (panels A, C and E) nor the maximal capacity (panels B, D and F) of neutrophil phagocytosis was affected by pretreatment with the indicated EV populations.

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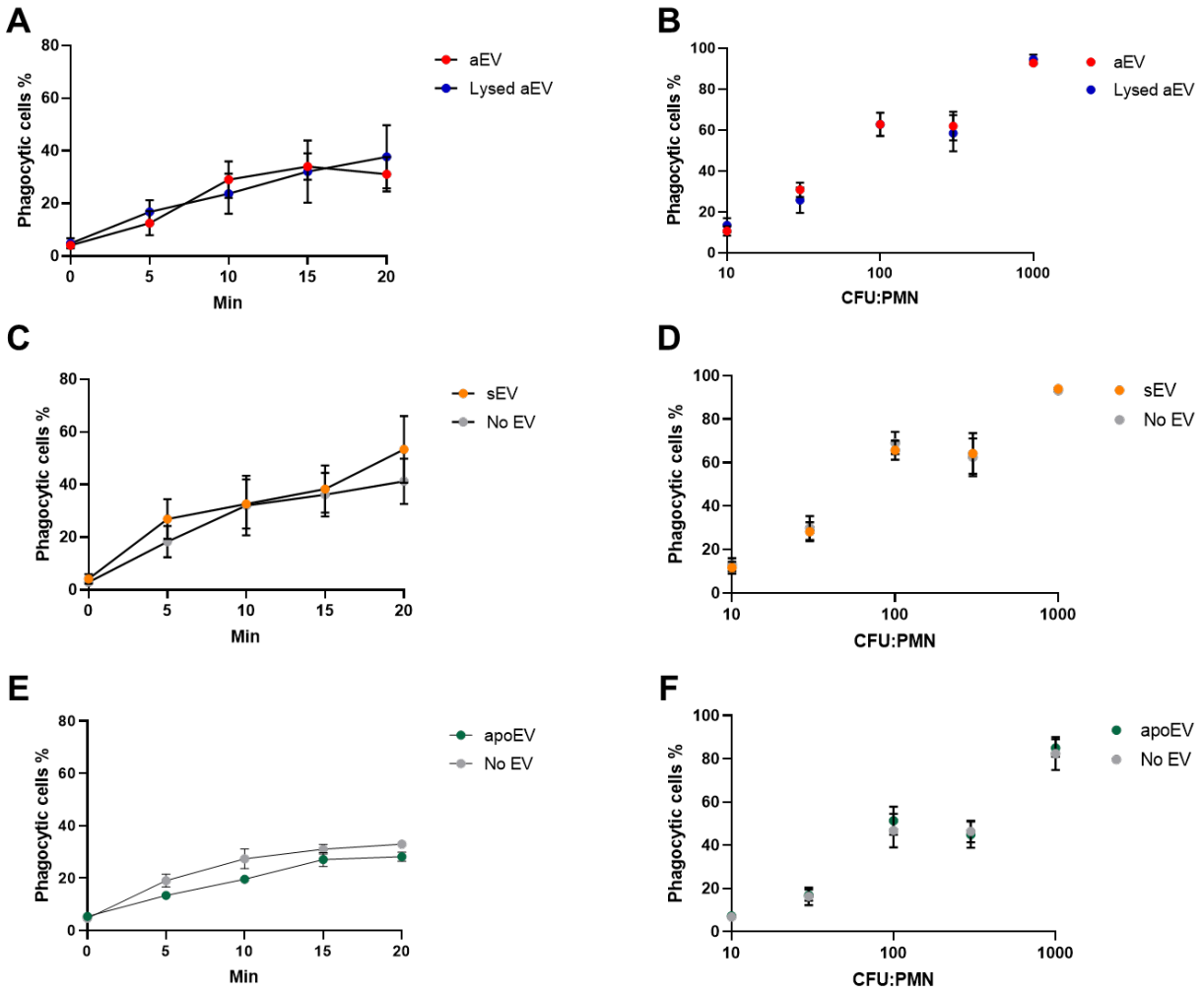

Figure 7. *Effect of EVs on the phagocytosis of neutrophils. PMN were pretreated for 45 min with one of the three EV populations or their controls.* ***A, C, E.*** *Pretreated PMN were incubated with opsonised USA300 bacteria in a 1:30 ratio for 20 min. Samples were taken at 0, 5, 10, 15 and 20 min. Phagocytosis was terminated instantly, then the percentage of phagocytosing PMN was measured in each population.* ***B, D, F.*** *Pretreated PMN were incubated with opsonised USA300 bacteria in 1:10, 1:30, 1:100, 1:300 and 1:1000 ratio for 20 min. Samples were taken only at 20 min. After terminating the phagocytosis, the percentage of phagocytosing PMN was measured in each sample. Fluorescent threshold was determined based on a PMN sample without fluorescent bacteria. Data were compared using paired Student's t-test; n=7 (**B, D**). n=4 (**A, C, E, F**). Error bars represent mean ±SEM.*

3.2.3 Effect of neutrophil EVs on the ROS production

Another classic effector function of neutrophils is the production of superoxide and its derivatives. We pretreated neutrophils with the indicated EV populations and their controls for 45 min, followed by a secondary activation with PMA (or leaving the cells unstimulated) and a 90 min luminescent detection of ROS. Figure 8. shows the raw data (panels A and E) and results of the individual experiments as normalised to their adequate controls (panels B-D and F-H). Panels A-D present the ROS production rate at an early stage (10 min after the secondary activation), while panels E-H present the maximal capacity of the cells (typically occurring between 30 to 40 min after the secondary activation – see representative curves on panel I). While aEVs consistently enhanced both the early and maximal ROS production, sEVs decreased them. Early-stage ROS production was decreased by apoEVs, but the peak of the curve was unaffected. This represents a third kind of EV-related action, i.e. the rightward shift of the curve.

The first five columns of panels A and E show that EV pretreatment alone without secondary activation did not trigger ROS production. aEV and lysed aEV fractions prompted a minor activity – most likely due to the opsonised zymosan residues in these samples, as there was no significant difference between the two.

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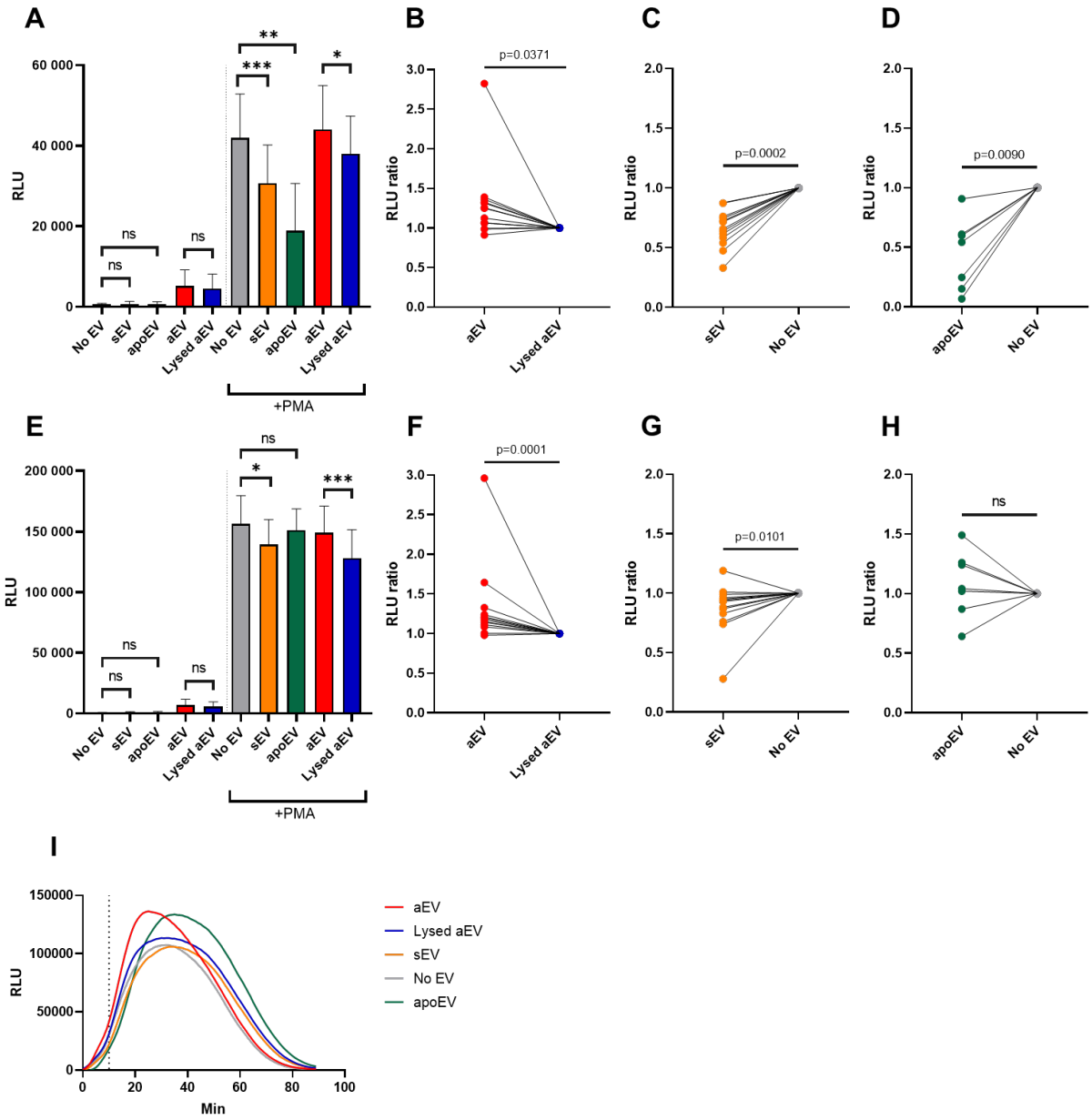

Figure 8. *Effect of EVs on the ROS production of neutrophils. PMN were pretreated for 45 min with the indicated EV or control, then left unstimulated or activated with 100 nM PMA. ROS production was determined at 10 min after activation (**A-D**) and at the peak intensity of the curve, typically at 30 to 40 min (**E-H**). **A** and **E** show the summarised ROS production of the EV-pretreated PMN, **B-D** and **F-H** show the normalised data pairs from each experiment. **I** shows representative curves.*

Data were normalised to their adequate controls ("aEV" to "Lysed aEV", "sEV" and "apoEV" to "No EV"). Raw data were compared using paired Student's t-test; n=13 for aEV & sEV; n=7 for apoEV. Error bars represent mean +SEM.

** represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$.*

Abbreviations: RLU: relative luminescence unit.

3.2.4 Effect of neutrophil EVs on the cytokine production

It is well known that upon stimulation with microbial agents or their derivatives, human neutrophils release cytokines which are able to recruit other neutrophils and other immune cells [139]. Since IL-8 (CXCL8) is the most abundantly secreted cytokine in neutrophils [68], we decided to quantify its secretion with a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit.

We incubated neutrophils with the indicated EV populations and their controls for 3 h and determined the IL-8 concentration in the supernatant after centrifugation of the cells (Fig. 9.). Given the fact that the secretion of IL-8 is reported to be very low in unstimulated human neutrophils [70] we added the lysed aEV sample (i.e. a small amount of opsonised zymosan) to every sEV and apoEV sample in order to make any potential secretion lowering effect detectable.

Panel A shows the raw data while panels B-D demonstrate the results of the individual experiments as normalised to their adequate controls. aEVs enhanced the IL-8 secretion in every single experiment, and a similarly consistent lowering effect was seen with sEVs. On the contrary, apoEVs showed no significant effect on the IL-8 secretion.

Importantly, we failed to detect any measurable amount of IL-8 in the aEV and sEV isolates themselves (apoEV isolates were not assessed).

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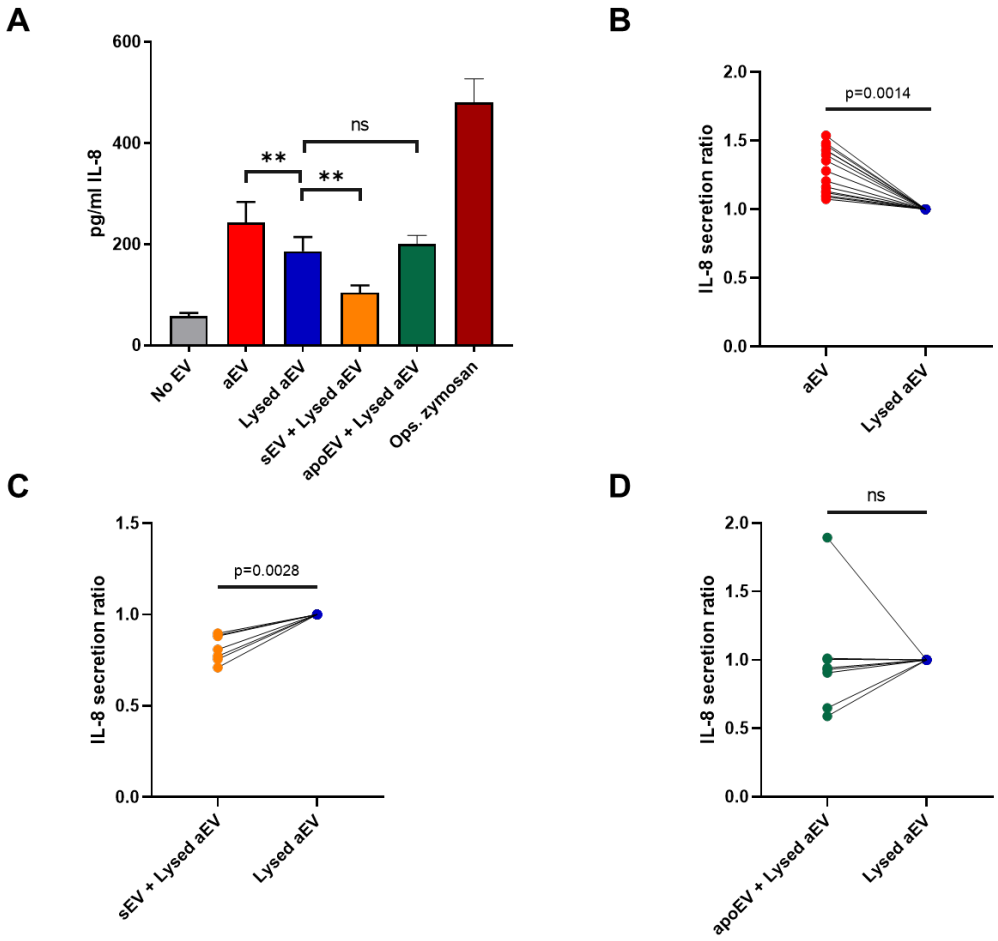

Figure 9. *Effect of EVs on the IL-8 production of neutrophils. PMN were treated for 3 h with one of the three EV populations or their controls. IL-8 amount of the supernatant was quantified with ELISA.* ***A*** *shows the summarised changes in IL-8 production of the EV-treated cells.* ***B-D*** *show the normalised data pairs from each experiment. Data were normalised to their adequate controls ("aEV" to "Lysed aEV", "sEV" and "apoEV" to "No EV"). Raw data were compared using paired Student's t-test; n=15 for aEV; n=7 for sEV; n=8 for apoEV. Error bars represent mean +SEM.*

** represents $p < 0.05$; ** represents $p < 0.01$.*

3.3 Effect of neutrophil EVs on endothelial cells

Endothelial cells represent another cell type that can be affected by neutrophils in their natural environment. We incubated confluent HUVEC layers with the indicated EV populations and their controls for 24 h. IL-8 concentration of the supernatant was determined at 24 h with sandwich ELISA, while E-Selectin expression at 6 h and vascular cell adhesion protein 1 (VCAM-1) expression at 24 h were measured after fixation with cellular ELISA.

Panels A-C of Figure 10. show the raw data while panels D-L demonstrate the results of the individual experiments as normalised to their adequate controls.

HUVECs exposed to aEVs showed signs of pro-inflammatory activation regarding IL-8 release and adhesion molecule expression (Fig. 10. A-F). In contrast, we did not obtain any consistent effects with sEVs and apoEVs (Fig. 10. A-C and G-L).

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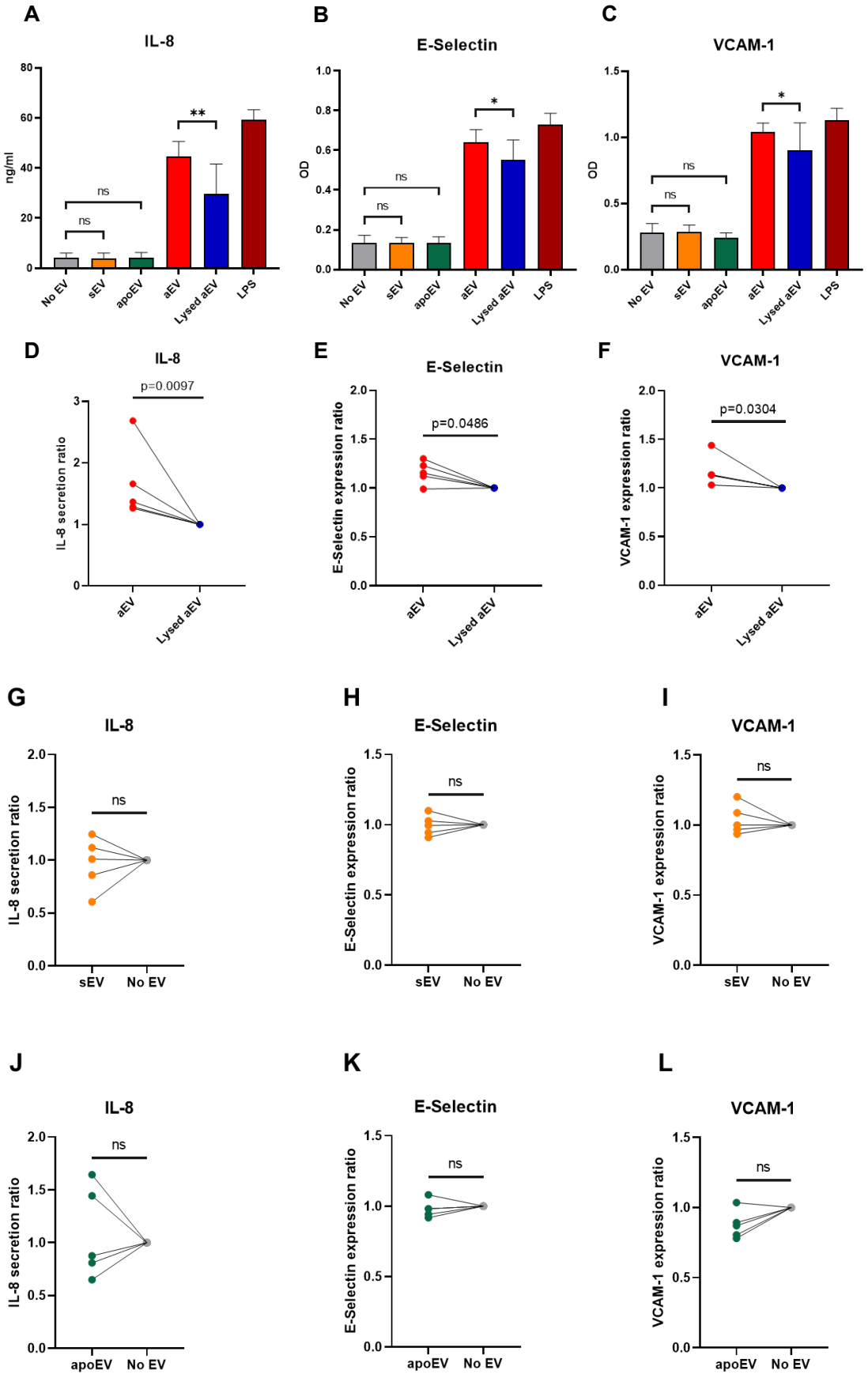

Figure 10. *Effect of EVs on endothelial cells. HUVEC were pretreated for 6 h (E-Selectin) or 24 h (VCAM-1 & IL-8) with one of the three EV populations or their controls. IL-8 amount of the supernatant was quantified with ELISA (**A, D, G, J**). E-Selectin and VCAM-1 expression was determined by cellular ELISA (**B, C, E, F, H, I, K, L**). **A-C** show the summarised changes in IL-8 secretion, E-Selectin and VCAM-1 expression of the EV-treated cells. **D-L** show the normalised data pairs for EV or control treated cells from each experiment (**D-F** for aEV, **G-I** for sEV, **J-L** for apoEV). Data were normalised to their adequate controls ("aEV" to "Lysed aEV", "sEV" and "apoEV" to "No EV"). Raw data were compared using paired Student's t-test; n=5. Error bars represent mean +SEM.*
** represents $p < 0.05$; ** represents $p < 0.01$.*

3.4 Effect of neutrophil EVs on coagulation

As mentioned in the introduction, neutrophil-derived EVs can promote coagulation by enhancing the intrinsic or extrinsic pathway, as well as by activating thrombocytes.

We incubated EVs with recalcified citrated pooled human plasma and measured the change of absorbance (turbidity) to elucidate the effect of our EV populations on plasma clotting. In case of coagulation, the turbidity of the sample showed a sudden increase [140]–[142].

First, we analysed the impact of EVs without the addition of thromboplastin (TP). Under these circumstances, coagulation did not necessarily occur, so we examined the number of coagulated samples. Turbidity changes were monitored for 2 h, spontaneous coagulation occurred typically 15-30 min after recalcification. Figure 11. panels A-C show the absolute numbers of coagulated wells while panels D-F present the percentage of these compared to the overall number of wells used. Frequency of coagulation was almost the same in the aEV and lysed aEV treated samples, while sEVs and apoEVs significantly promoted the occurrence of coagulation.

Second, we were curious whether EVs could influence the plasma clotting when it is activated by TP and coagulation occurs in every well. Therefore, we measured the average clotting time in recalcified citrated pooled human plasma after the addition of TP and EV samples. Figure 11. panels G-I show that only apoEVs had a significant accelerative effect

in this experimental setting. The presence of sEVs also elicited a minor decrease of the clotting time, albeit this effect was not statistically significant.

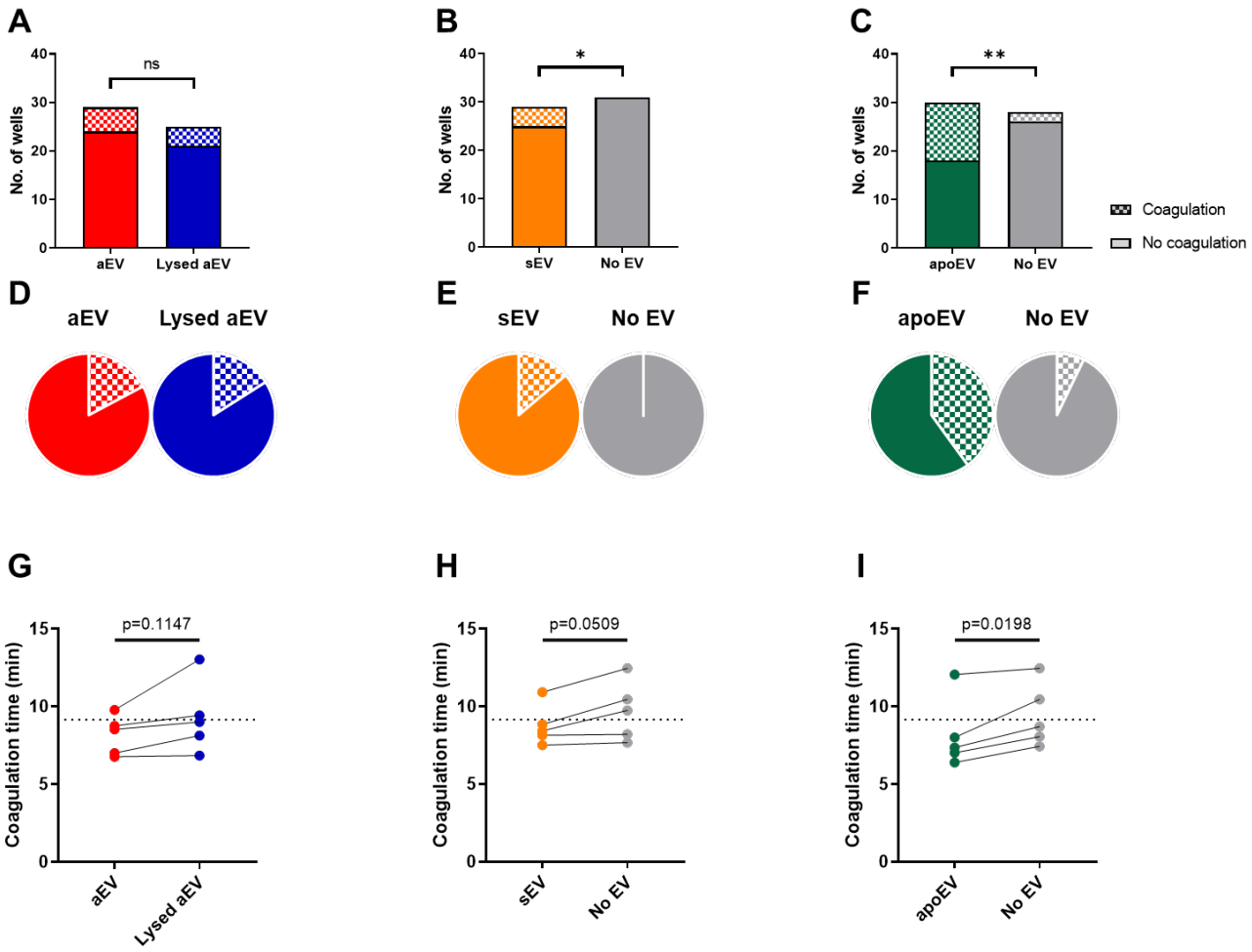

Figure 11. *Effect of EVs on coagulation. One of the three EV populations or their controls were mixed with pooled citrated human plasma in the absence (**A-F**) or presence **(G-I)** of thromboplastin followed by recalcification with Ca-HEPES. **A-C** show the absolute numbers of coagulated and not coagulated wells in each sample. **D-F** represent the percentage of coagulated wells based on the same data. **G-I** show the time needed for 50% of the coagulation process in the thromboplastin treated samples (raw data pairs). The dotted lines on **G-I** show the average coagulation time of the "No EV" samples. Data were compared using Fisher's exact test (**A-F**) and paired Student's t-test (**G-I**). n=29 wells from 7 donors for aEV & sEV; n=30 wells from 6 donors for apoEV (**A-F**). n=5 from 5 donors (**G-I**).*

** represents p < 0.05; ** represents p < 0.01.*

3.5 Assessment of the contribution of NET formation and other cell death forms to the described effects

As described in the introduction, there are multiple neutrophil cell death forms, which can exhibit both pro-inflammatory and anti-inflammatory properties. It is also possible, that NET formation affects the investigated cell and plasma functions. We wanted to elucidate the possible extent of their contribution to the effects described above.

Apart from early apoptosis, all of these cell death forms are accompanied by increased permeability of cell membranes and often also by the release of DNA into the extracellular space [53]. Therefore, we stained our cell isolates with the membrane-impermeable nucleic acid dye PI and measured the intensity of fluorescence for 210 min in not stimulated, opsonised zymosan and PMA stimulated samples (Fig. 12A). As PMA was shown to induce NETosis, it served as positive control in this measurement [58]. Detectable PI staining is only possible in case of DNA release into the extracellular space or when the permeability of the cell membrane is increased.

The dotted line at 20 min represents the end of the incubation period of neutrophils with or without the used stimuli before the isolation of our EV populations. At this point, virtually no PI positivity can be detected. Relevant amount of PI positive cell death forms (late apoptosis, necrosis, necroptosis, pyroptosis or NETosis) occur earliest during the second hour of incubation.

Panels B-D show representative images of PI-stained neutrophils after 20 min (B-D) and 3 h (E-H) when not stimulated (B and E), stimulated with opsonised zymosan (C and F) or with PMA (D and G). Panel H shows PMA activated neutrophils in the presence of DNase after 3 h. Only PMA treated cells after 3 h of incubation show signs of NETosis, i.e. fibrillar PI positive structures (Figure 12G).

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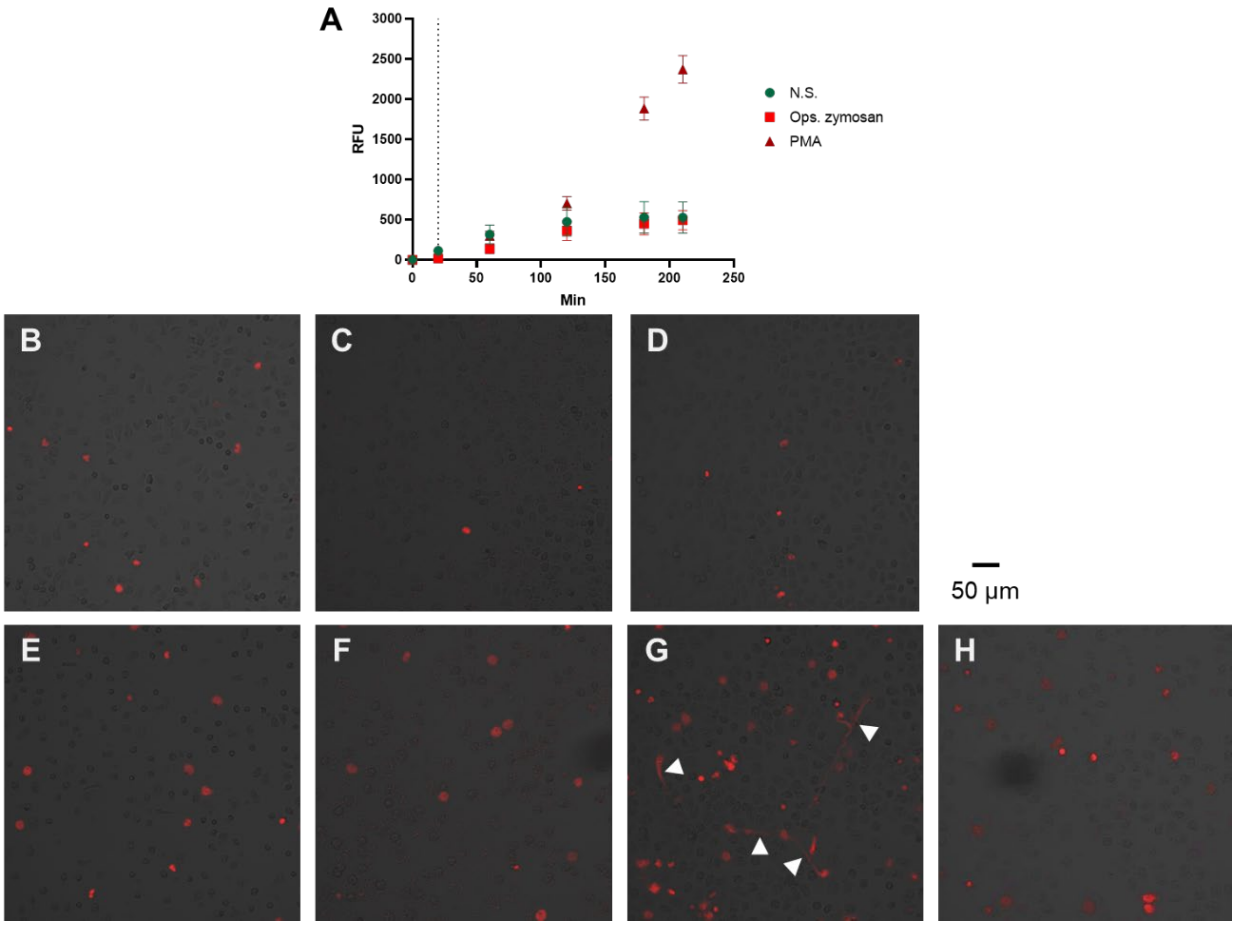

Figure 12. *Assessment of viability and NETosis during neutrophil incubation.* ***A.*** *Propidium iodide fluorescence of opsonised zymosan or PMA stimulated neutrophils compared to non-stimulated cells followed by fluorometry. The dotted line at 20 min represents the end of the incubation period of neutrophils with or without the used stimuli before the isolation of our EV populations. n=3. Error bars represent mean ±SEM.* ***B-D.*** *Propidium iodide fluorescence of neutrophils after 20 min without stimulation (**B**), with opsonised zymosan (**C**) or PMA (**D**) stimulation.* ***E-H****. Propidium iodide fluorescence of neutrophils after 180 min without stimulation (**E**), with opsonised zymosan (**F**) or PMA (**G**) stimulation.* ***H****. Propidium iodide fluorescence after 180 min with PMA stimulation in the presence of DNase. White arrowheads show nucleic acid fibres. Representative pictures out of 3 independent experiments. Abbreviations: N.S.: not stimulated.*

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4. Discussion

Extracellular vesicles emerged as versatile tools in intercellular communication and other non-cellular remote effects mainly in the last two decades. It was shown early that neutrophilic granulocytes also release EVs affecting their environment. Since then, diverse, sometimes even contradictory effects have been described (Table 3.). This extensive inconsistency could arise from differences in (1) the quality, purity, and initial viability of the PMN isolate, (2) the stimulus used for EV production, (3) EV isolation procedures and thus EV populations used, (4) the storage of EV samples and (5) the experimental environment of the investigated target cells. Unfortunately, many of these crucial data are missing in a large part of the studies listed in Table 3.

Our laboratory has a long-time established protocol for the isolation of neutrophils and their EVs. Based on the consistent use of these circumstances, our work group showed earlier that there are at least three different EV populations released by neutrophils corresponding to the current state of the cell: activated EVs (derived from serum-opsonised particle activated neutrophils), spontaneously released EVs and apoptotic EVs. Only aEVs showed antibacterial properties [77]. Many important morphological and functional properties of these populations were characterised by our laboratory (Table 4.).

Our laboratory also showed that fundamental characteristics of these neutrophil-derived EV populations depend on the storage techniques. Thus, we have always applied EVs freshly after isolation.

Based on this knowledge, I carried out a comparative study with the three mentioned EV populations in order to obtain comprehensive data on their functional similarities and differences. I anticipated that at least some of the contradictions presented in Table 3. can be explained by different activation states of the cells of origin and others can be eliminated by the rigorous use of the same elaborated, reproducible protocols for neutrophil preparation, EV isolation and EV treatment.

First, I investigated the uptake of fluorescently labelled neutrophil EVs by other neutrophils, monocytes and lymphocytes. While aEVs and apoEVs were taken up by all three cell types, sEVs seemed to interact only with neutrophils. Based on the presented

results, the uptake of sEVs by monocytes and lymphocytes cannot be excluded entirely, but the lack of statistically significant fluorescence increase suggests a much lower probability of interaction compared to the other seven experimental settings.

An increase of fluorescence in the gated cell population could also arise from the association of EVs with the surface of the cells. With confocal microscopic imaging I could verify that EVs are found in the cells, not only attached to the surface.

The exact characterisation of the uptake mechanism was out of the scope of our project primarily aiming to study the divergent functional consequences of EV treatment. However, many possible uptake mechanisms have been described: different forms of endocytosis (clathrin-mediated, lipid raft-mediated, caveolin-mediated), macropinocytosis, phagocytosis and membrane fusion [143]–[148]. Based on current evidence it has been proposed that not one individual but rather a combination of uptake mechanisms occur [149]. Furthermore, based on the presented results, we cannot definitely conclude that the uptake of EVs is necessary for the seen effects – a surface receptor mediated effect followed by a concomitant uptake would also be an imaginable scenario.

The presented changes of neutrophil and endothelial cell function upon EV treatment suggest a clear pro-inflammatory profile of aEVs: enhancement of ROS production and IL-8 secretion in neutrophils, as well as upregulation of endothelial activation markers. Opsonised zymosan is one of the strongest activating agents of neutrophils, therefore it seems reasonable that the EVs released from these cells also possess immunologically activating properties.

On the other hand, sEVs represent a rather anti-inflammatory nature: they decrease ROS production and IL-8 secretion in neutrophils and do not activate HUVEC. Interestingly, while also exhibiting rather anti-inflammatory properties, apoEVs show some differences when compared to sEVs: they do not inhibit maximal ROS and IL-8 production but decrease the early ROS release thereby right-shifting the ROS curve.

We get a fundamentally different picture when evaluating the effects on coagulation. Here, apoEVs show the clearest, strong procoagulant effect in both settings. sEVs also

exhibit somewhat weaker but still significant procoagulant properties. At the same time, aEVs, which are the only to have antibacterial and pro-inflammatory activity, show absolutely no effect on coagulation.

Importantly, none of the three EV populations showed any influence on phagocytosis or chemotactic migration of neutrophils, further supporting the selectivity of EV-related actions.

The results regarding the behaviour of neutrophil-derived EVs presented in this thesis are summarised in Table 5.

Table 5. *Summarised characteristics of our three EV populations based on data presented in this thesis. - means not detectable characteristics; arrows represent the observed statistically significant changes upon pretreatment with different EV populations compared to their adequate controls. Pro-inflammatory traits are marked with red, anti-inflammatory effects are marked with green.*

	aEV	sEV	apoEV
Uptake by PMN	+	+	+
Uptake by monocytes	+	-	+
Uptake by lymphocytes	+	-	+
Migration of PMN	-	-	-
Phagocytosis of PMN	-	-	-
Maximal ROS production of PMN	↑	↓	-
Early ROS production of PMN	↑	↓	↓
IL-8 production of PMN	↑	↓	-
IL-8 secretion of HUVEC	↑	-	-
E-Selectin expression of HUVEC	↑	-	-
VCAM-1 expression of HUVEC	↑	-	-
Coagulation (without TP)	-	↑	↑
Coagulation time (with TP)	-	-	↓

The presented effects of neutrophil EVs are most likely caused by multiple mechanisms. The heterogenous time scale alone underlines the variety of possible signal transduction effects. Immediate effects seen in plasma clotting assays were most likely caused by

different surface composition of EVs. Short-term (10-30 min) effects in ROS production could be induced by posttranslational modification. Finally, changes in cytokine secretion of neutrophils and HUVEC as well as surface expression of adhesion molecules on HUVEC occurred after several hours signifying alteration in gene expression.

In experiments where cells (neutrophils or endothelial cells) were treated with the indicated EV populations, we cannot disregard the possibility of EV release during the pretreatment and/or the measurement. In this case, besides the EVs prepared in advance and added to the cells, a distinct EV population released by the treated cells should also be anticipated. This could mask and/or distort the effects of the added EVs. However, we think that this is a universal phenomenon when treating cells with any agents: the treated cells act as a system in which many signalling events can take place, ranging from direct cell to cell contact and autocrine effects to paracrine mediators and EV release. Thus, many forms of EV and cytokine impact can be envisaged in our neutrophil suspensions and endothelial cell cultures in the different experimental setups. However, every treated sample had parallel controls, and the EV production (or any other signalling events) could take place in these as well. Besides that, we typically used EV numbers for the treatment of the cells exceeding the expected amount of EVs produced during the particular measurement: EVs were derived from approximately a 10-fold higher number of neutrophils compared to the treated cell numbers.

Considering that sEVs appear to be produced constitutively (as mentioned in the introduction, their release is not inhibited by any inhibitors or genetic deficiencies of receptors and signalling molecules), and neutrophils go relatively fast in spontaneous apoptosis when not activated, it would be a plausible hypothesis, that sEVs represent a smaller population of apoEVs continuously arising during the neutrophil and EV isolation procedure. This is also credible if one takes the heterogeneity of neutrophil cell age in peripheral blood into consideration. In earlier studies [79] we observed that apoptotic cells released up to 100 times more vesicles than resting cells. If we disregard the debate of recent years about the neutrophil lifetime and take the classical view of 12 hours, approximately 3% of the prevailing cells should die during a 20 min incubation period for sEV production. Thus, we can hypothesise that under our current experimental conditions apoptotic EVs would be roughly in the range of the detected amount of sEVs.

However, we saw in the presented data explicit differences between sEV and apoEV function, which suggests that they are functionally distinct populations. The exact contribution of possible continuous, early apoptosis to the release of sEVs is yet to be determined.

Based on our results and data from Table 3., resting and apoptotic neutrophils tend to release EVs with anti-inflammatory properties. This could be a rational way, as nonactivated and “peacefully” dying immune cells should deliver “calming” signals to other cells. The possibility of this theory is emphasised by the role of pathological neutrophil death in the pathomechanism of autoimmune diseases [150].

In the case of any activating signals, neutrophils seem to release EVs with stepwise more pro-inflammatory potential and even different target cells. Analysing the data from Table 3. it is evident, that fMLP or TNF-α alone does not cause a strong pro-inflammatory EV production: these EVs seem to activate endothelial cells only, which can lead to enhanced extravasation of further immune cells. At the same time, other leukocytes are rather inhibited.

However, in case of further available activating signals (e.g. LPS, GM-CSF) or when activated complement system fragments are present (e.g. C5a, C3bi), neutrophil-derived EVs are clearly pro-inflammatory. Lastly, when neutrophils encounter opsonised pathogens, they release EVs with strong pro-inflammatory and antimicrobial effects (Fig. 13.).

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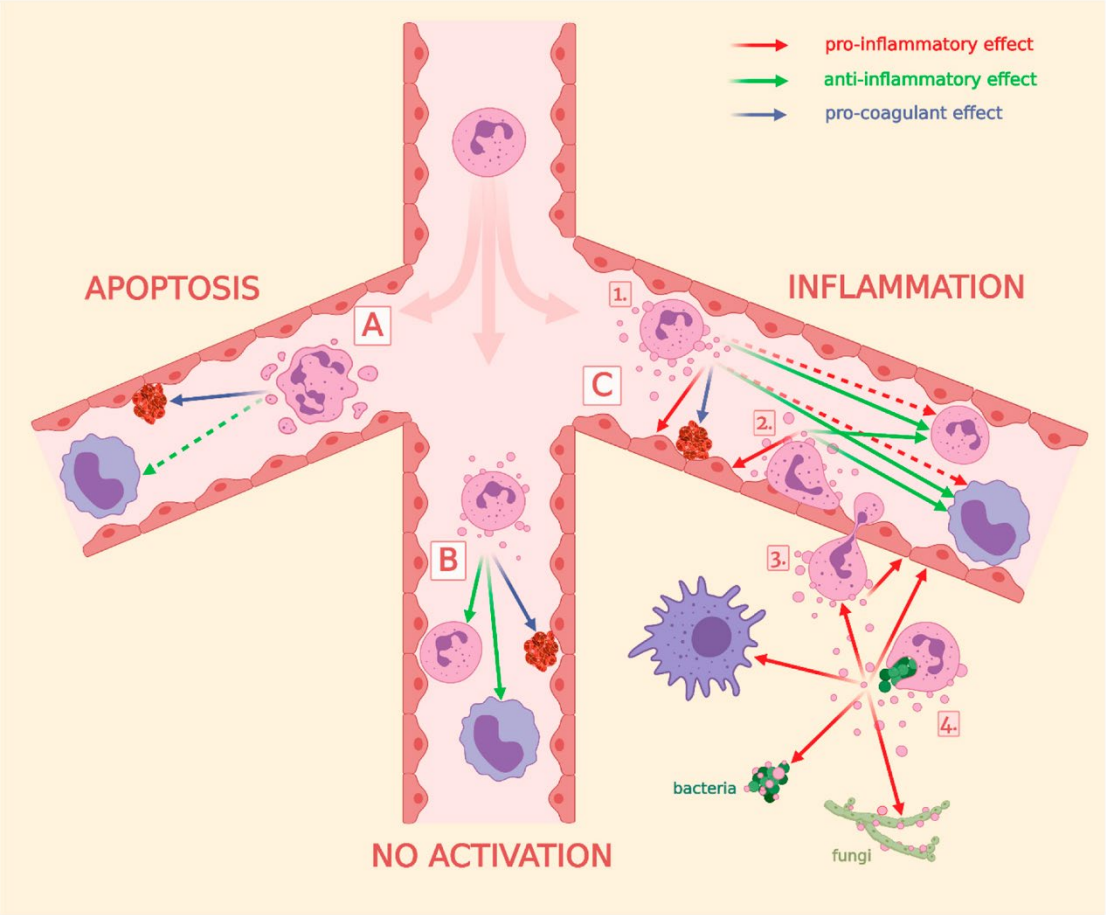

Figure 13. *Selective effects of neutrophil-derived EVs mirror a continuous spectrum of activation states of the cells of origin.* ***A.*** *Effects of apoEVs.* ***B.*** *Effects of sEVs.* ***C.*** *Effects of activated EVs from circulating* ***(1)****, endothelium-attached* ***(2)****, extravasating* ***(3)*** *and phagocytosing* ***(4)*** *neutrophils* [151].

The principle described above resembles the multistep activation pattern of adaptive immune cells: neutrophils too seem to wait after the first stimulus for further reinforcing signals to get fully activated. Thus, we hypothesise that neutrophil EVs represent a signalling model similar to cytokines: there is a continuous spectrum ranging from anti- to pro-inflammatory and even antibacterial EVs with distinctive target cell profiles, and the properties of the currently released, custom-made EVs reflect the activation state of the cell [151].

Such behaviour could account for the puzzling diversity of effects attributed to neutrophil EVs. As not only the protocols and quality of the PMN and EV isolates or the storage

conditions of EVs can differ but also the stimulus used for EV production, a wide spectrum of neutrophil activation states can be envisaged behind the presented results.

It is of utmost importance to standardise these conditions and to specify them precisely in future publications in order to refine our understanding of the functional diversity of neutrophil-derived EVs.

Few previous studies listed in Table 3. have also raised the possibility of divergent neutrophil-derived EV related effects on the immune system [80], [101].

The novelty of this work resides in demonstrating pro-inflammatory and anti-inflammatory signalling via EVs from the very same cell population depending on the environmental conditions during EV biogenesis.

Our future plan is to expand the thorough functional characterisation: by using the same comparative study design on multiple types of activated EVs, we could gain more insight in the exact relationship of different cell activators (e.g. fMLP, LPS, PMA, TNF-α), their receptors, signalling pathways and cargo sorting mechanisms with the functional profile of the released EV populations.

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5. Conclusions

I investigated the functional differences of previously described neutrophilic granulocyte derived EV populations. Based on the results described above, I make the following conclusions:

1. Neutrophil-derived aEVs (derived from serum-opsonised particle activated neutrophils) and apoEVs are taken up by neutrophils, monocytes and lymphocytes. However, sEVs are only taken up by neutrophils themselves. Based on confocal microscopic images, EVs are internalised in neutrophils.
2. Neutrophil-derived EVs do not affect the chemotactic migration of neutrophils to fMLP.
3. Neutrophil-derived EVs do not influence the kinetics nor the maximal capacity of neutrophil phagocytosis.
4. The maximal and early-phase ROS production capacity of neutrophils upon PMA stimulation is enhanced by aEVs and decreased by sEVs. On the other hand, apoEVs only inhibit the early phase of ROS production, leading to a delayed release with the same maximal capacity.
5. IL-8 release of neutrophils is enhanced by aEVs, reduced by sEVs and not affected by apoEVs.
6. Endothelial activation based on IL-8 secretion, E-Selectin and VCAM-1 expression is triggered by aEVs but not by sEVs or apoEVs.
7. Spontaneous coagulation of blood plasma is triggered by apoEVs and sEVs, but not by aEVs. Blood plasma clotting induced by TP is accelerated by apoEVs but not by sEVs or aEVs.
8. Pro-inflammatory, PI positive cell death types (e.g. pyroptosis, necroptosis or necrosis) are not detectable in our neutrophil population within the time frame of PMN preparation and EV production steps, i.e. they are not responsible for the described pro-inflammatory effects.

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6. Summary

Extracellular vesicles are released by every known cell type and represent a novel way of intercellular communication. Neutrophilic granulocytes are the principal phagocytes of the innate immune system and play a crucial role in immunity by engulfing, killing and degrading various microorganisms. A plethora of different, often opposing effects of neutrophil EVs have been described in the last 20 years, even though many differences in the way of elicitation, collection, handling, and storage of the investigated vesicles impede the interpretation of these data.

In our laboratory, three different neutrophil-derived EV populations have been characterised in detail both morphologically and functionally in previous publications: activated EVs (derived from serum-opsonised particle activated neutrophils), spontaneously released EVs and apoptotic EVs.

In my Ph.D. work I aimed to further elucidate the functional diversity of these three EV populations by examining their effects on neutrophil and endothelial function as well as on coagulation under strictly controlled, comparable conditions.

Neutrophil-derived EVs were taken up by the investigated leukocytes. Migration and phagocytosis of neutrophils were unaffected by EVs. Pro-inflammatory processes were promoted by aEVs resulting in enhanced ROS production and cytokine release from neutrophils as well as activation of endothelial cells. In contrast, sEVs exerted anti-inflammatory effects by reducing ROS production and cytokine release from neutrophils. Weak procoagulant effects were also elicited by sEVs.

When cells were treated with apoEVs, maximal ROS production and IL-8 release were unaffected, although a delayed ROS production was observed. Coagulation was strongly promoted by apoEVs.

Based on these data, we propose that neutrophil-derived EVs are custom-made and can have divergent, selective, and sometimes even antagonistic effects depending on the environmental conditions prevailing at the time of the EV production.

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8. Bibliography of the candidate's publications

Publications related to the present thesis:

1. Á. M. Lőrincz, B. Bartos, D. Szombath, V. Szeifert, C. I. Timár, L. Turiák, L. Drahos, Á. Kittel, D. S. Veres, F. Kolonics, A. Mócsai, and E. Ligeti. (2020) Role of Mac-1 integrin in generation of extracellular vesicles with antibacterial capacity from neutrophilic granulocytes. *J. Extracell. vesicles*, vol. 9, no. 1 p. 1698889. doi: 10.1080/20013078.2019.1698889.
   Impact factor (2020): 25.84
   Rank (2020): D1 (17/289 – Cell Biology)

2. F. Kolonics, E. Kajdácsi, V. J. Farkas, D. S. Veres, D. Khamari, Á. Kittel, M. L. Merchant, K. R. McLeish, Á. M. Lőrincz, and E. Ligeti. (Sep. 2020) Neutrophils produce proinflammatory or anti-inflammatory extracellular vesicles depending on the environmental conditions. *J. Leukoc. Biol.*, doi: 10.1002/JLB.3A0320-210R.
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   Rank (2020): Q1 (39/197 – Immunology and Allergy)

3. F. Kolonics, V. Szeifert, C. I. Timár, E. Ligeti, and Á. M. Lőrincz. (Dec. 2020) The Functional Heterogeneity of Neutrophil-Derived Extracellular Vesicles Reflects the Status of the Parent Cell. *Cells*, vol. 9, no. 12doi: 10.3390/cells9122718.
   Impact factor (2020): 6.60
   Rank (2020): Q1 (55/254 – Biochemistry, Genetics and Molecular Biology)

Publications not related to the present thesis:

4. G. Veres, P. Hegedűs, E. Barnucz, R. Zöller, T. Radovits, S. Korkmaz, F. Kolonics, A. Weymann, M. Karck, and G. Szabó. (2013) Addition of Vardenafil into Storage Solution Protects the Endothelium in a Hypoxia-Reoxygenation Model. *Eur. J. Vasc. Endovasc. Surg.*, vol. 46, no. 2, pp. 242–248 [Online].
   Impact factor: 3.07
   Rank: D1 (24/426 – Surgery)

5. C. I. Timár, F. Kolonics, V. Berzsenyi, E. Tamáska, A. Párkányi, M. L. Merchant, D. W. Wilkey, Z. Iványi, K. R. McLeish, E. Ligeti. (2020) Disordered peptides impair neutrophil bacterial clearance and enhance tissue damage in septic patients. *bioRxiv,* [Online preprint], doi: https://doi.org/10.1101/2020.07.31.227017.

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