Changing world of neutrophils

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Abstract

Neutrophilic granulocytes are no longer regarded as cells involved only in the last phase of the immune response with one single – although vitally important – task: engulfing and killing of microorganisms marked by immunoglobulin or complement fragments. In recent years it was shown that neutrophils are actively involved in initiation and organization of the adaptive immune response by releasing various cytokines, interacting with all major types of immune cells, regulating their own lifespan, participating in the anaphylactic reaction and in several classically non-immune functions such as hemostasis, atherogenesis and even insulin resistance. The antibacterial effect is no longer restricted to killing and destruction of microorganisms sequestered in the phagosomal space. Bacteriostasis also occurs at certain locations of the extracellular space, by formation of neutrophil extracellular traps (NETs) that were shown in the last two years to have a significant role in prevention of dissemination of microorganisms. Extracellular vesicles represent a recently discovered form of intercellular communication carried out both by lipids, proteins and nucleic acids. In this review we also summarize the role of neutrophil-derived extracellular vesicles in modifying the function of other cell types as well as their direct antibacterial effect that differs significantly from mechanisms applied either by neutrophils or by the NETs.

Keywords:

Neutrophils; Neutrophil extracellular traps (NETs); Extracellular vesicles; Ectosomes; Microvesicles; Antibacterial effect

Introduction

Neutrophilic granulocytes (also named polymorphonuclear granulocytes [PMN]) play a vital role in innate immune reactions, mainly in defense against pathogenic bacteria and fungi [20]. After their release from the bone marrow they circulate in blood and subsequently they emigrate to peripheral tissues where they find and combat the invaders. PMN are able to engulf particles of almost their own size [62]. Various pattern recognition receptors (PRR) allow only very slow rate of phagocytosis but opsonization of the particles by immunoglobulins or complement fragments augments the rate of phagocytosis by orders of magnitude (Fig. 1.). Isolated PMN are able to engulf up to 50 opsonized bacteria within 30 min (Fig 2.). Phagocytosed microorganisms are sequestered into phagosomes, that are sealed, membrane-surrounded compartments. Assembly and activation of the NADPH oxidase and opening of ion channels in the phagosomal membrane lead to abundant production of superoxide ($O_2^{\cdot -}$), the first component of a cascade of reactive oxygen species (ROS). Simultaneously, the membranes of different granule fractions fuse with the phagosomal membrane and the granule contents are released to the phagosome. Killing and degradation of the microorganism proceed in the small phagosomal space in a concentrated action of the involved ingredients, although the contribution of the individual factors may vary depending on the type of microorganism [89,91]. The molecular details of these processes have been worked out and summarized in recent excellent reviews [11,58,66,75,77,78,80,102,115]. Human pathology indicates the vital necessity of all the above steps as any disturbance of the migration, superoxide production, opsonization or granule production results in serious diseases with repeated, often life-threatening infections [30,46,52,100,112].

The established view of the neutrophils as effector cells coming into play only in the late phase of the immune response and having a single – although vital – task has been largely changed in the last one and a half decades and PMN are shown to play a role in divergent, unexpected functions. Also striking new properties of neutrophils have been discovered. This review highlights some of the new aspects and refers the reader to more specialized papers on these topics. Then we focus on the latest data revealing the contribution of PMN-derived extracellular vesicles both to intercellular communication and to antibacterial defense.

Recently discovered properties and functions of neutrophils

Protein synthesis

Neutrophils were regarded as terminally differentiated cells with very low level, if any, of protein synthesis. This view has been challenged in the 1990s, when it was discovered that stimulated PMN were able to synthetize and release various cytokines [19]. In early studies isolated neutrophils have been activated by lipopolysaccharide (LPS) and release of tumor necrosis factor (TNFα), interleukin 1β (IL-1β), IL-8, transforming growth factor β1 (TGF-β1) or IL-1 receptor antagonist (IL-1ra) was detected [19]. Later PMN were reported to produce a true arsenal of regulatory molecules, such as different chemokines, various pro- and anti-inflammatory cytokines, colony stimulating factors (CSF), and others [66]. The triggers consist of bacterial products as well as CSF, TNFα or interferons (IFN). Human and murine neutrophils differ, however, in the production of several cytokines [66].

Cytokine production expressed as amount per cell was significantly lower in PMN than in monocytes or lymphocytes, but in blood neutrophils largely outnumber any other type of leukocyte. In addition, neutrophils are the first cells that accumulate at an inflammatory site. Thus, cytokine production by neutrophils seemed to have physiological relevance. Indeed, already the first *in vivo* experiments carried out in various animal models of inflammation demonstrated clearly the *de novo* synthesis of several cytokines both at the mRNA and at the protein level [99]. Determination of the gene expression profile of neutrophils from the bone marrow, circulating blood or a skin lesion of mice indicated that transmigration through the vessel wall initiates a new transcription program leading to the synthesis of regulatory and cell-fate determining proteins [11,105,106]. Phagocytosis of opsonized particles via Fc and/or complement receptors also altered the expression of numerous genes, many of them affecting proteins involved in apoptotic pathways [56,57].

Interaction with other immune cells

Neutrophils were regarded as effector cells of the humoral immune response, which eliminate certain pathogens marked by immunoglobulin or complement fragments, without any significant interaction with other immune cells. Discovery of the cytokine release from the neutrophils has profoundly changed this view [66,75].

Neutrophils produce IL-8, a peptide with strong chemotactic effect on neutrophils. A positive feedback via IL-8 may thus contribute to the fast migration of large numbers of neutrophils to inflammatory sites. Other chemokines secreted by PMN are chemoattractants to monocytes [102] or lymphocytes [66] triggering the second wave of cell migration, mostly of mononuclear cells. Furthermore, activated neutrophils produce lipid mediators such as

resolvins and protectins that inhibit neutrophil recruitment [25,58,66] and „find me" and „eat me" signals that initiate the elimination of aged PMN by macrophages [14].

In addition to chemotactic direction of migration, more substantial cross-talk has been demonstrated with all cell types of the immune reactions [66,75]. Some of these interactions are direct, like the effect of B-cell activating factor (BAFF) on survival, maturation and differentiation of B-cells [98], or activation of monocyte-derived dendritic cells (DC) via the integrin Mac1 and DC-SIGN (DC-specific ICAM3-grabbing non-integrin) [113]. In case of NK cells both a direct and an indirect activation by neutrophils has been described, the latter involving a PMN-DC-NK triangle including both contact and humoral effects [24]. Neutrophils are also able to interact with different subsets of T cells, and these interactions seem to be mostly reciprocal [66]. Cross-talk between neutrophils and other immune cells was not only observed *in vitro,* but has been supported in several animal models [66] and in cases of human pathology [75].

Neutrophils have even been proposed as antigen presenting cells. Earlier studies indicated that neutrophils were able to present exogenous antigen to CD8+ T-cells [7] and recent data have shown also antigen-presentation to Th1 and Th17 cells [2]. In confocal microscopic studies neutrophils migrating with fluorescently labeled pathogen were observed in draining lymph vessels [1] and lymph nodes [21]. Moreover, the chemokine receptor CCR7 has been identified as a key player in directing neutrophil migration from the interstitium to the draining lymph nodes [6].

Taken together, experimental evidence supports that neutrophils interact in a complex way with all the players of and exert a regulatory influence on the adaptive immune response.

Lifespan

Neutrophils are regarded as cells with a very short lifespan, spending 8 to 12 hours in the circulation [103]. If they are not involved in inflammatory processes, they undergo spontaneous apoptosis. However, various stimuli, such as cytokines (G-CSF, GM-CSF) and bacterial products were shown to prolong their survival [23]. Most recent *in vivo* measurements estimate an extended half-life up to 5 days for circulating neutrophils [83], although there had been concerns about these data [64].

Interestingly, proteins known to regulate the cell cycle in dividing cells, such as survivin, cyclin-dependent kinases or proliferating cell nuclear antigen (PCNA) were discovered to regulate the survival of neutrophils [119,120]. In contrast to proliferating cells,

in PMN these proteins are localized to the cytosol and act as inhibitors of the apoptotic pathway [120].

Additional functions of granule proteins

Neutrophils possess 4 different types of granules that develop in different stages of maturation in the bone marrow [11]. The contents of these granules are regarded as specific neutrophil proteins involved in killing and degradation of phagocytosed pathogens. However, some of the neutrophil granule proteins have been recently described in other functions or at other locations. As summarized in Table 1, neutrophil granule proteins also affect cell adhesion and migration, apoptosis, or clearance of apoptotic cells. Some of the hydrolases were shown to degrade extracellular (tissue factor pathway inhibitor, TFPI) or intracellular (insulin receptor substrate-1, IRS-1) proteins and influence thereby coagulation and insulin resistance, resp. Selected neutrophil granule proteins are also expressed upon stimulation of endothelial or epithelial cells [11].

Participation of PMN in vital function other than defense against bacteria and fungi

Neutrophils were regarded as cells specialized in elimination of pathogens, mainly bacteria and fungi. In recent years their antimicrobial spectrum has been broadened by demonstration of their involvement in defense against HIV-1 [97], mycobacteria [68] and parasites such as malaria [85] and Leishmania [75,82].

However, the most surprising data support involvement of neutrophils in a vast array of different physiological and pathological processes. PMN were shown to participate in anaphylactic reactions initiated by IgG via murine FcγRIV or human FcγRIIA and mediated by release of platelet activating factor (PAF) from neutrophils [50].

Neutrophils are suggested to participate in atherogenesis as their presence has been detected in atherosclerotic plaques and PMN-depletion resulted in a reduction of the size of lesion [32,75]. Moreover, the neutrophil granule protein cathelicidin LL37 was reported to promote adhesion of monocytes to the vessel wall [31].

Insulin resistance is related to obesity, partly due to alteration of insulin signaling pathway by inflammatory mediators produced in the adipose tissue. Recently, significant accumulation of neutrophils has been demonstrated in adipose tissue and degradation of the signaling molecule IRS-1 was attributed to neutrophil-derived elastase [104].

Neutrophils were also shown to be involved in thrombus formation. They appear at the site of vessel injury instantaneously [27,116] and activated neutrophils express tissue factor [70]. PMN-depletion resulted in serious reduction of thrombus formation [27,116].

Last but not least, neutrophils have been implicated in modulation of tumor growth via several potential mechanisms [66].

Many of the above results have been obtained or supported by detecting the changes following depletion of neutrophils in various animal models. Some remarkable examples are summarized in Table 2.

Extracellular killing by neutrophils

Discovery of the neutrophil extracellular traps (NETs) changed our view on bacterial killing and revealed that neutrophils were also capable to eliminate microorganisms in the extracellular space [15]. NETs represent filamentous structures composed of DNA, histones, granule proteins (mainly elastase and myeloperoxidase) and a few cytosolic proteins. They are formed by a unique mechanism of cell death, called NETosis, when intracellular membranes become disintegrated and granule proteins get access to the nucleus. Eventually, the plasma membrane ruptures and the protein-nucleic acid complex is released [16]. Formation of NETs requires superoxide and other ROS formed thereof. Neutrophils from patients with defective NADPH oxidase (chronic granulomatous disease, CGD) are unable to form NETs, but the process can be rescued by addition of H_2O_2 [37].

NETs were shown to kill various bacteria and *Candida albicans* [15,110] but most recently they were reported to be also involved in defense against human immunodeficiency virus [97]. The mechanism of microbe elimination is not fully understood but probably relies on multiple parallel pathways. Trapping of microorganisms prevents their dissemination and this phase may be based on surface charge interactions. Antibacterial proteins concentrated in NETs, such as histones, defensins and pentraxins, may have a direct toxic effect whereas granule enzymes, such as lysozyme or elastase, may destroy the microbial cell wall or virulence factors. The antimicrobial effect of NETs is critically dependent on the intact DNA structure, as it is inhibited by DNase treatment [15].

In vitro microbe killing effect of NETs was elegantly supported and extended by visualization of live neutrophils by confocal intravital microscopy. Upon bacterial challenge of the skin, extravascular NET formation was observed. In contrast to the *in vitro* data, in live tissues NET formation occurred within minutes and did not involve the death of neutrophils.

Instead, viable but anuclear granulocytes continued to crawl in a chemical gradient and to phagocytose [124]. In a septic model, rapid platelet-dependent NET formation was observed in liver sinusoids that increased trapping of bacteria by four-fold [71]. NETs were also shown to be formed and to be effective in certain viral infections [49]. In all these experiments, destruction of the DNA scaffold of NETs by iv. administered DNase resulted in an increase of bacteremia or number of virus-infected cells [49,71,124]. Thus, NET formation clearly prevents dissemination of microorganisms.

In line with the previous observations, surface expression of nucleases is a critical component of pathogenicity for some bacteria [17] and dissemination of different strains correlated with the type of expressed DNase [117]. Furthermore, in a case of severe Aspergillosis in a CGD patient, negative correlation was found between the ability of neutrophils to form NETs and the severity of infection [10].

In addition to their role in killing of different microorganisms, in recent years NETs were reported to be involved in several other physiological and pathological processes. They were shown to activate dendritic cells via TLR9 receptors [38,61], linking thereby innate and adaptive immune processes. Activation of platelets via TLR2 and TLR4 receptors [101] and degradation of coagulation inhibitory proteins by proteases on the surface of NETs both promote blood clotting which also impairs bacterial dissemination [51,69]. On the other hand, formation of NETs was suggested to contribute to the viscosity of bronchial fluid in cystic fibrosis patients [67], to formation of autoantibodies in systemic lupus erythematosus [44] and to the pathogenesis of autoimmune vasculitis [54]. Most recently, the presence of NETs was demonstrated in atherosclerotic plaques in murine carotid arteries and in human tissues removed by endarterectomy [72] substantiating the involvement of neutrophils in atherogenesis. Disruption of NETs in liver sinusoids by iv. DNase diminished liver damage in septic conditions [71].

Extracellular vesicles: new form of intercellular communication

The first mentioning about extracellular vesicles (EV) was in 1967 when „platelet dust" was described [121]. In the following years there were only sporadic publications, but the field has seen a real boom in the last decade. Extracellular vesicles are seen today as a common way of intercellular communication. Every investigated cell type is able to produce some form of extracellular vesicles, both spontaneously and upon stimulation. EVs are

present in all the body fluids, blood, urine, cerebrospinal fluid and milk having been the most intensively studies [18,107].

The size of EVs varies from approx. 30 nm to more than 1 μm. The smaller vesicles, having a diameter below 100 nm, are mostly referred to as exosomes, they arise from multivesicular bodies. The larger vesicles are named – depending on the authors - microvesicles, ectosomes or microparticles, and they are formed mostly by budding or shedding from the plasma membrane [22,43,107]. However, it seems that the size of EVs presents more a continuous spectrum than discrete populations.

Detection of EVs is based mostly on flow cytometric, light scattering or electric resistance measurements. All approaches present potential problems [43]. In flow cytometry the distinction of the smaller vesicles from the noise can be a hard task. Furthermore, immune complexes were shown to overlap with smaller vesicles [42], thus the vesicular structure should always be verified. Most convenient – and reliable – is the detection of vesicles above 300 nm stained with specific fluorescent ligand. Recently, the availability of convenient apparatus based on detection of light scattering or changes of the electric field resulted in the wide-spread usage of vesicle number as the unique parameter for characterization of a given preparation. However, the danger of vesicle fusion, presence of immune complexes or aggregated proteins or contamination with viruses requires careful analysis of the preparation. In our view, the safe approach is still the parallel usage of multiple techniques including electron microscopy for characterization and visualization of the EV preparations.

The composition of EVs is varied. They contain differently enriched collection of both membrane and cytosolic constituents. The discovery of their nucleic acid – mostly mRNA and miRNA – content raised wide perspectives for their application in diagnostics and therapy [111]. In our own experience, the same cells are able to produce EVs of different composition and different functional properties depending on the type of stimulus applied [109]. Differences between spontaneously formed and induced EVs has also been indicated in another study [47]

The physiological or pathological functions related to EVs present a long and colourful list. One of the earliest generally recognized functions was increase of blood coagulation initiated by tissue factor on EVs derived of endothelial cells and platelets. Various tumor cells also produce large numbers of EVs with tissue factor and this could be part of the increase of coagulation generally observed in patients with tumor of different origin [22]. Secretion of leaderless proteins (e.g. IL-1β) occurs in EVs, too [4,88].

Transfer of plasma membrane receptors and proteins has also been demonstrated to occur via EVs. One of the earliest well-documented observations was that exosomes derived from dendritic cells were able to successfully present antigen to T-lymphocytes [90]. In other studies, macrophage-derived exosomes were reported to transfer pathogen-associated molecular patterns of opportunistic intracellular pathogens to uninfected cells [9]. Perhaps the most striking result was the demonstration that oncogenic EGFR of glioblastoma cells was transferred to healthy cells, where it became functional and induced proliferation [3]. Transfer of chemokine receptors [65], FasL [55] and tissue factor [29] via EVs was also reported. Transfer of miRNA has been demonstrated in many cell types [114].

Detailed knowledge is still missing on cell biological processes leading to formation and release of EVs as well as on their fate. However, their existence and role in communication between different cell types can not be neglected.

Role of PMN-derived vesicles in communication

The first report on PMN-derived EVs (named in that paper microparticles) indicated their ability to increase secretion of the cytokine IL-6 from endothelial cells [73]. In a later work it has been demonstrated, that leukocyte-derived EVs were present in blood samples of healthy volunteers and the endogenous EVs were also able to upregulate IL-6 production and tissue factor expression in cultured endothelial cells [74]. In both studies an increase of EV number and effect was achieved by the chemotactic agent fMLP. Anti-neutrophil cytoplasmic antibodies (ANCA) also stimulate the release of microparticles from neutrophils, and these EVs activate endothelial cells to express intercellular adhesion molecule-1 and to secrete IL-6 and IL-8 [47].

Most of the data on PMN-derived EVs (called in these studies ectosomes) come from the laboratory of Dr. Schifferli [26,34-36,39,40,45,93-96]. They characterized the size and surface properties of EVs released upon fMLP-stimulation and came to the conclusion that both the *in vitro* and the *in vivo* generated EVs are right-side out [39,45]. Further, they demonstrated that PMN-derived EVs increased the secretion of the anti-inflammatory cytokine TGF-β1 from monocytes, whereas the stimulated release of IL-8, IL-10 and TNFα was decreased [40]. Secretion of TGF-β1 was induced also in monocyte-derived dendritic cells, where incubation with PMN-EVs interfered with the LPS-induced differentiation process indicated by a decrease of release of inflammatory cytokines, decrease of phagocytosis and T-cell stimulation [34]. The inhibitory effects were initiated by membrane components of PMN-EVs which led to the activation of the Mer tyrosine kinase pathway in

monocytes, resulting eventually in inhibition of transcription factor NF-κB and down-regulation of several pro-inflammatory genes [35]. The rapid release of stored TGF-β1 was independent of the MerTK pathway whereas phosphatidylserine (PS) exposed on the surface of PMN-EVs was necessary but not sufficient for induction of TGF-β1 release [36].

Thus, EVs released from PMN upon chemotactic stimuli communicate a signal to monocytes and monocytic dendritic cells, reducing the inflammatory and favouring the anti-inflammatory response of these cells. However, the specificity of PMN-derived EVs in this process remains to be determined, as EVs produced by stored erythrocytes [94] and platelets [93] have also been reported to down-regulate macrophages and dendritic cells.

In line with the inhibitory effect of PMN-derived EVs upon macrophages, ectosomes released from *Mycobacterium tuberculosis* infected neutrophils decreased the activation of macrophages and prolonged the survival of *M. tuberculosis* [33]. In a recent study, PMN-derived microparticles were shown to stimulate efferocytosis and production of pro-resolving lipid mediators in macrophages [25].

Neutrophil-derived EVs were also shown to have prothrombotic effects. An increased number of platelet- and PMN-derived microparticles were found in the blood plasma of patients suffering from meningococcal sepsis. These microparticles expressed tissue factor and supported thrombin generation [79]. In another study, stimulation of neutrophils with bacterial endotoxin resulted in shedding of PAF containing microparticles which activated platelets [118]. More recently, the active form of the β_2 integrin Mac-1was detected on the surface of EVs released from activated neutrophils, and the active integrin played a central role in binding to and activation of resting platelets [84].

PMN-derived EV with antibacterial effect

We observed that in isolated PMN different stimulating agents induced the formation of different number of EVs with different composition and different functional properties [109]. Particles (bacteria or zymosan) opsonized with pooled serum initiated the release of microvesicles in the highest quantity whereas chemotactic agents (fMLP, CXCL12), cytokines (TNFα) or lipopolysaccharide (LPS) did not significantly increase the generation as compared to the spontaneous production. Other reports also demonstrated the release of EVs from neutrophils phagocytosing different pathogens [33,41].

Importantly, PMN-derived EVs initiated by opsonized particles were able to impair the growth of bacteria whereas EVs produced spontaneously or upon other stimuli did not interfere with bacterial growth (Fig.3). In induction of the formation of EVs with antibacterial

effect, opsonization of the particles with full serum was critical: heat-inactivation of complement factors resulted in generation of a large number of EV without antibacterial effect (Fig. 3).

The size of PMN-derived EVs ranged from 200 to 800 nm and did not depend on the type of initiating agent. In contrast, the composition of PMN-derived EVs with antibacterial effect differed significantly from that of ineffective EVs. Antibacterial EVs (aEV) were enriched in PMN granule proteins and β_2 integrins. All types of PMN-derived EVs contained metabolic enzymes, many cytoskeletal proteins, some plasma membrane receptors and the membrane components of NADPH oxidase, however the cytosolic oxidase components were mostly missing. Apparently, formation of aEV is associated to specific sorting of cell constitutents into the released EV.

The effect of phagocytosis-induced PMN-derived aEVs proved to be bacteriostatic rather than bacteriocidal and the mechanism of action differed in many respects from the effect of intact PMN (Table 3). PMN-derived aEVs did not engulf bacteria, although their size was comparable or even larger than that of bacteria. PMN-derived aEVs did not produce superoxide and their effect was not influenced by the inhibitor of NADPH oxidase, diphenyl-iodonium (DPI) (Fig. 4A). This observation is substantiated by the lack of the cytosolic components of the NADPH oxidase in aEVs. The most striking difference between the effect of PMN and antibacterial EVs was the total independence of opsonization in the latter case (Fig. 4B). Whether bacteria were opsonized in full serum or in complement-deficient serum or not opsonized at all, their growth was impaired to the same extent by antibacterial PMN-derived vesicles. As PMN have a very low killing activity for non-opsonized bacteria (Fig. 1.), under such conditions, PMN-derived antibacterial EVs proved to be significantly more effective than PMN themselves.

Finally, the spectrum of attacked bacteria is probably also different. Antibacterial EVs were effective against *Staphylococcus aureus* and *Escherichia coli*, but not against *Proteus. mirabilis*, thus they showed some selectivity, which was however not paralleled with Gram staining.

The mechanism of action of PMN-derived antibacterial EVs is strongly associated with their ability to form large aggregates with bacteria (Fig. 5B.). In this process both the number and size of aggregates seem to be important. Summarizing all the experimental conditions investigated, a fairly good negative correlation was obtained between the proportion of large (larger than 1.5 μm) aggregates and bacterial growth. Formation of large aggregates with bacteria seems to depend both on surface charge and density of β_2 integrin on

PMN-derived EVs and requires continuous rearrangement of the actin cytoskeleton (Fig. 5C and D).

The impairment of bacterial growth by PMN-derived aEVs occurred in the extracellular space and in this respect it is similar to the effect of NETs. However, there are numerous differences between NETs and antibacterial EVs (Table 3). Formation and hence effect of NETs depends on superoxide and ROS formed thereof whereas both production and effect of antibacterial EVs is independent of ROS. Generation of NETs requires firm attachment of PMNs to a surface whereas antibacterial EVs were released equally well from suspended or adherent neutrophils. NETs also critically depend on intact DNA structure and can be disrupted by DNase treatment, whereas neither generation nor the antibacterial effect of PMN-derived EVs was influenced by DNase treatment. The kinetics of *in vitro* formation and the initiating agents are also widely different. Finally, the antibacterial effect of EVs depends on the intact vesicular structure, intact cytoskeletal organization (Fig. 5) and glucose supply whereas all these are not required for the antibacterial effect of NETs. However, involvement of granule proteins may be a common property: effect of NETs was shown to depend on granule proteases, and EVs are also enriched in these proteins, suggesting their functional role.

In vivo relevance of PMN-derived EVs

Blood serum contains EVs derived of many – if not all – cell types. The serum of healthy individuals contains also a detectable amount of PMN-derived EVs. Investigated under *ex vivo* conditions, these vesicles did not form aggregates with bacteria and did not interfere with their growth.

In bacteremic states, an increase in the number of PMN-derived vesicles has been reported. A six fold enrichment of PMN-derived EVs was reported in the blood serum of patients infected with *S. aureus* [109] and over 100-fold increase was detected in patients with meningococcal sepsis [79]. In 3 patients with acute peritonitis, the peritoneal fluid contained over tenfold higher number of PMN-derived microparticles than the control fluid from uninfected patients [87]. An increase of PMN-derived EVs was also reported in different inflammatory diseases (Table 4). Thus, bacterial stimulation results in an enhancement of EV-production also in circulating PMN, similarly to our observation made on isolated PMN.

Importantly, in our experiments, the PMN-derived EVs isolated from the serum of bacteremic patients infected with *S. aureus*, formed *ex vivo* large aggregates with added bacteria, similar to the observation made on antibacterial EVs initiated from isolated PMN.

Whether similar aggregates are formed between PMN-derived EV and bacteria *in vivo*, is presently not known.

The biological significance of the antibacterial effect of PMN-derived EVs detected under *in vitro* condition can not be assessed at present. The observation that antibacterial EVs are fully effective against non-opsonized bacteria, may represent an important factor in the early phase of innate immune reactions. However, on the basis of theoretical calculations, the effect of EVs against opsonized bacteria may also not be negligible. When presented as cell-equivalent, the antibacterial capacity of EVs against opsonized bacteria was smaller than that of PMN and was saturated by lower bacterial load. However, the protein content of EVs is rather low and if we relate the impairment of bacterial growth to protein content of PMN or EV, then we obtain a tenfold higher relative antibacterial capacity for EVs than for PMN. It is thus possible, that PMNs activated by opsonized bacteria, package their antibacterial arsenal into released vesicles that could capture the pathogen and impair its dissemination. Both NETs and blood clotting were shown earlier to prevent pathogen dissemination very effectively [69,71].

PMN-derived EVs with antibacterial capacity may thus represent another PMN-related extracellular mechanism to combat infectious agents.

Conclusion

After several decades, when the attention of immunologists was focused first on various subpopulations of lymphocytes as central players in adaptive immune processes, then later on exciting receptors of innate immunity, in the last period neutrophils have been rediscovered. Several striking properties have been described and participation in a broad spectrum of unexpected functions has been revealed. Discovery of NETs that provide an extracellular mechanism in the fight against microorganisms was accepted first with certain skepticism. It took almost a decade till the *in vivo* significance of NET formation starts to be clarified. It will take certainly years before the true biological role of extracellular vesicles can be assessed. However, it is evident already now that the neutrophils are multifaceted cells that are involved in many more functions than phagocytosis and elimination of engulfed microorganisms.

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Table 1. *Non-classical functions of neutrophil granule proteins*

Protein	Location in the PMN	Other location/ effected cell	Function	Reference
Azurocidin	Azurophilic granules	Endothel cells	Monocyte adhesion	[63]
Cathelicidin LL37	Specific granules	Epithel cells, skin, lung	Apoptosis	[5]
Cathelicidin LL37	Specific granules	Neutrophils	Activation	[76]
Cathelicidin LL37	Specific granules	Monocyte	Chemoattractant	[122]
Cathepsin G Azurocidin Defensin	Azurophil granules	Monocyte	Chemoattractant	[123]
Elastase	Azurophil granules	Lung cells	IRS-1 hydrolysis	[48]
Elastase	Azurophil granules	Hepatocytes	IRS-1 hydrolysis, insulin resistance	[104]
Elastase Cathepsin G	Azurophil granules	Thrombus formation	Hydrolysis of tissue factor pathway inhibitor	[69]
Pentraxin 3	Specific granules	Endothel cells	Binding to P-selectin inhibition of neutrophil transmigration	[28]
Lactoferrin	Specific granules	Endothel cells	Inhibition of migration of neutrophils and eosinophils	[12,13]
Proteinase 3 (PR3)	Azurophil granules	Macrophages	Inhibition of uptake of aged neutrophils into macrophages	[53]
Arginase 1	Gelatinase granules	T-cell	Inhibition of T-cell activation	[58,92,108]

Table 2. *Involvement of PMN in non-classical functions as revealed by PMN-depletion in animal models*

Effect	Reference
Protection against experimental cerebral malaria	[85]
Inhibition of anaphylaxis	[50]
Reduced plaque size in hypercholesterinaemia-induced atherosclerosis	[32]
Protection against deep venous thrombosis	[116]
Decrease of the growth rate of selected tumours	[81]
Inhibition of delayed type hypersensistivity	[59,60]

Table 3. *Comparison of the in vitro antibacterial properties of intact PMN, neutrophil extracellular traps (NETs) and PMN-derived antibacterial EVs (aEVs)*

Property	PMN	NET	aEV
ROS requirement	Yes	Yes	No
DNase sensitivity	No	Yes	No
Opsonization required	Yes	No	No
Vesicular structure required	Yes	No	Yes
Surface attachment required	No	Yes	No
Engulfment required	Yes	No	No
Intact cytoskeleton required	Yes	No	Yes
Glucose supply required	No	No	Yes
Time of formation	instantaneous	several hours	20 min
Granule proteins required	Yes	Yes	Yes
Effect	Bacteriocidal	Bacteriostatic/ Bacteriocidal	Bacteriostatic

Table 4. *Increased production of PMN-derived EVs in pathological conditions*

State/disease	Investigated fluid	Extent of elevation	Ref
Cystic fibrosis	Sputum	Approx. 2-fold	[86]
Chronic vasculitis	Blood serum	Approx. 2.fold	[26]
Acute vasculitis	Blood serum	Approx. 5-fold	[26]
ANCA-associated vasculitis	Blood serum	50-100 fold	[47]
Rheumatoid arthritis	Synovial fluid	?	[8]

Figure Legends

Fig. 1. Difference in the phagocytosis of opsonized and non-opsonized bacteria by PMN. Uptake of green fluorescent *S. aureus* was followed in time in a flow cytometer.

Fig. 2. Phagocytosis of opsonized bacteria by PMN. Phagocytosis of green fluorescent *S. aureus* by PMN labeled with red-fluorescent antibody against the surface marker CD11b. Phagocytosis was followed in a laser scanning confocal microscope. Pictures were taken of the same visual field at the indicated time-points.

Fig. 3. Differences in number and functional properties of PMN-derived EV induced by different agents. EV formation was induced by S. aureus opsonized in full serum or complement-depleted serum, or tumor necrosis factor (TNFα) or occurred spontaneously (marked "none"). The number of produced EV was determined by flow cytometry (empty columns); bacterial growth was followed by a semi-automated technique (shaded columns).

Fig. 4. Comparison of the effect of (A) inhibition of NADPH oxidase and (B) opsonization on the antibacterial effect of PMN or PMN-derived antibacterial EV. NADPH oxidase was inhibited by 5 μM diphenylene-iodonium (DPI). *S. aureus* was opsonized with full serum.

Fig. 5. Antibacterial EVs form large aggregates with bacteria. The relation of green fluorescent *S. aureus* with different PMN-derived EVs (stained red) was followed for 30 min in a confocal laser scanning microscope. A: spontaneously formed EVs; B. EVs induced by opsonized particles; C. EVs induced by opsonized particles treated with 10 μM cytochalasin B (CB); D. EVs induced by opsonized particles treated with 10 μM latrunculin A. Note that inhibition of continuous rearrangement of the actin cytoskeleton by CB or latrunculin prevents the formation of large aggregates between bacteria and antibacterial EVs.

Figure 1

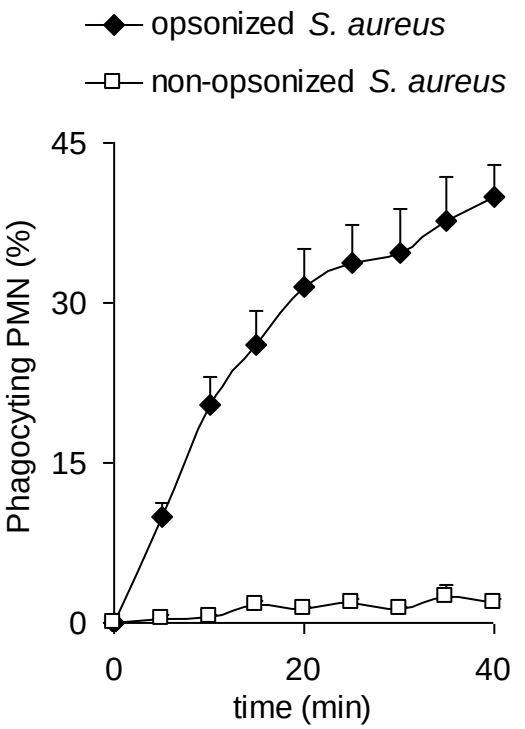

opsonized S. aureus
non-opsonized S. aureus
Phagocyting PMN (%)
45
30
15
0
0
20
40
time (min)

Figure 2

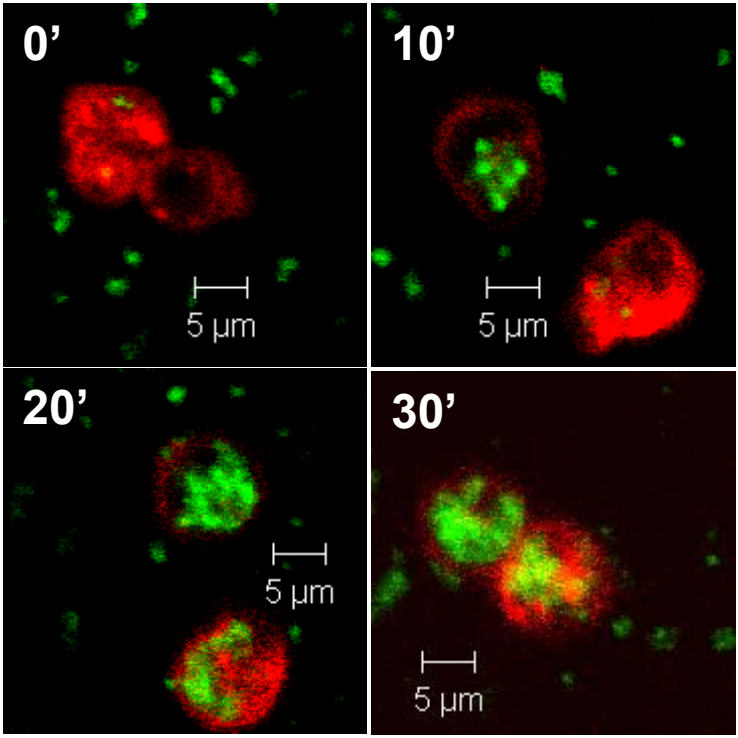


Figure 3

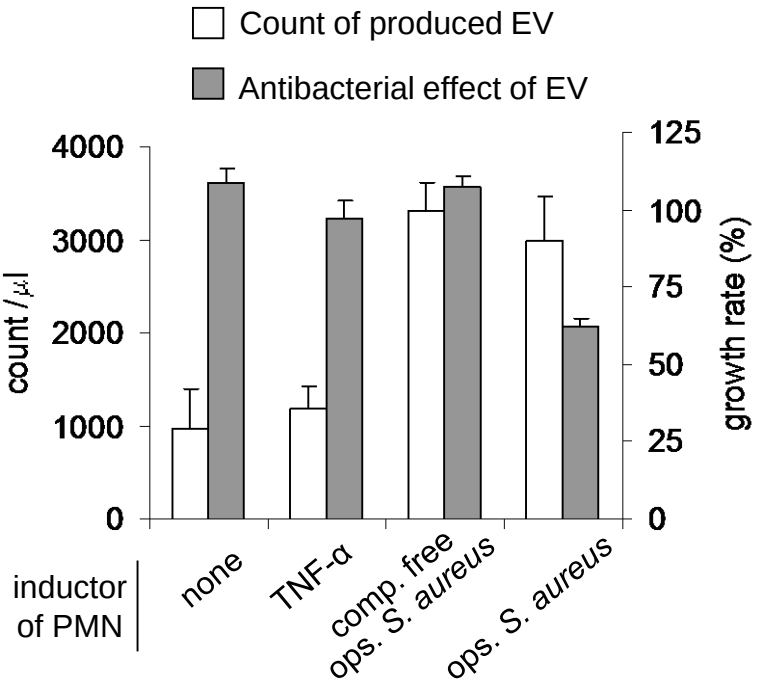
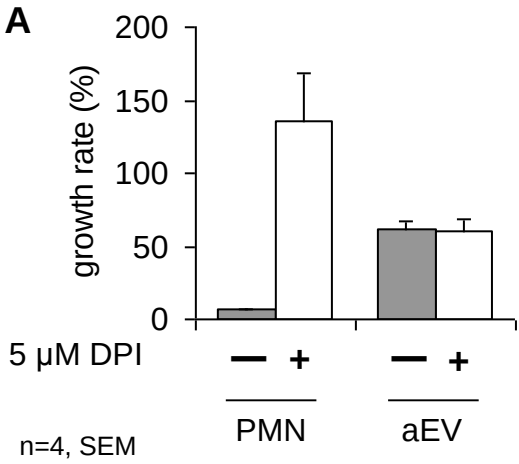

Count of produced EV
Antibacterial effect of EV
count /µl
4000
3000
2000
1000
0
125
100
75
50
25
0
growth rate (%)
inductor of PMN
none
TNF-α
comp. free ops. S. aureus
ops. S. aureus

Figure 4


A
200
150
100
50
0
growth rate (%)
5 µM DPI
—
+
—
+
PMN
aEV
n=4, SEM

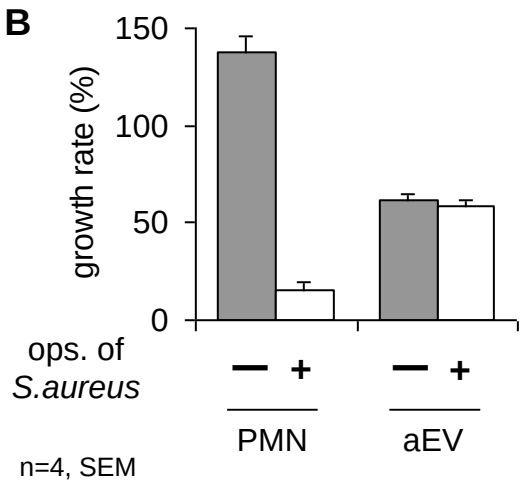

B
150
100
50
0
growth rate (%)
ops. of
S.aureus
—
+
—
+
PMN
aEV
n=4, SEM

Figure 5