Review paper

Neutrophil extracellular traps as the main source of eDNA

Zewnątrzkomórkowe pułapki neutrofilowe jako główne źródło eDNA

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Abstract

Neutrophil extracellular traps (NETs) are web-like structures consisting of decondensed DNA together with accompanying proteins, including histones and antimicrobial peptides released from activated neutrophils as part of the first-line defence against pathogens. Despite the protective role of neutrophils, a number of studies indicate that overproduction of NETs followed by accumulation of extracellular DNA (eDNA) and other negatively-charged polyelectrolytes (PE) such as F-actin, contribute to the pathogenesis of some diseases. Neutrophil extracellular traps are also recognised as the structural and functional support of microbial biofilms and should thus be considered as therapeutic targets. Importantly, the chemical nature of PE permits aggregate formation induced by a number of polycations occurring naturally in the human body, including cationic antimicrobial peptides. This review summarises recent reports focused on the clinical significance of NET-derived eDNA and PE and discusses the potential therapeutic strategies to limit the negative consequences of eDNA accumulation.

Streszczenie

Zewnątrzkomórkowe pułapki neutrofilowe (NETs) są strukturami składającymi się ze zdekondensowanej chromatyny wraz z towarzyszącymi jej białkami, w tym histonami oraz peptydami przeciwbakteryjnymi, uwalnianymi z aktywowanych neutrofilów. Uważa się, że stanowią one jedną z pierwszych linii obrony organizmu przed czynnikami infekcyjnymi. Pomimo korzystnego efektu, jaki wywołują w procesie odpowiedzi immunologicznej, niektóre badania wskazują, że nadmierne ich formowanie oraz związane z tym gromadzenie się zewnątrzkomórkowego DNA (eDNA) wraz z innymi polielektrolitami (F-aktyna) odgrywa istotną rolę w patogenezie wielu chorób i stanowi ważny czynnik warunkujący stabilność biofilmu bakteryjnego. Istotne jest również, że natura chemiczna polielektrolitów warunkuje powstawanie agregatów z udziałem polikationów występujących naturalnie w ludzkim organizmie, w tym naturalnych peptydów przeciwbakteryjnych. W pracy podsumowano najnowsze doniesienia dotyczące klinicznego znaczenia zewnątrzkomórkowych pułapek neutrofilowych, a także podstawowe strategie terapeutyczne mogące mieć zastosowanie w terapii schorzeń, których ogniwem patogenetycznym jest to zjawisko.

Introduction

Since its discovery, DNA present in the cell nucleus is considered the most important structural support for a universal biological code for expression of life. Aside from complex and still enigmatic regulation of DNA gene expression in the cell nucleus, a growing amount of evidence indicates that DNA governs some biological functions, often associated with its physicochemical characteristic as a negatively charged biopolymer upon its release to the extracellular space. This characteristic is shared with other polyelectrolyte (PE) biopolymers, including F-actin. PEs are polymers built of charged units. When dissolved in water or polar solvents, PEs carry charges along the macromolecular chain. The total number and density of electric charges determines PE interactions between these charges and their surroundings. Considering the polyelectrolyte nature of DNA and F-actin, the human body contains a significant amount of negatively charged PEs. An important feature of PE biopolymers is the ability of aggregate

formation induced by a variety of polycations such as polyamines, metal ions, and cationic antibacterial peptides (CAPs), which is one of the pathogenic events observed during the course of some diseases, including cystic fibrosis (CF) lung infection, abscess formation, autoimmune disorders, and cancers.

DNA and F-actin accumulation in extracellular compartment

It is accepted that the necrosis process generally induced by infectious and inflammatory factors results in accumulation of negatively charged polyelectrolytes including DNA and F-actin in the extracellular environment. Nevertheless, under physiological settings, excess of biopolymers is eliminated during phagocytosis of cell fragments [1–6]. During the inflammation or infectious exposure, chemotactic migration of neutrophils towards infected/inflamed tissue results in accumulation of PE in the extracellular fluid both as the consequence of formation of neutrophil extracellular traps (NETs) and activity of neutrophil proteases.

Previously, it was recognised that destruction of pathogens is achieved mainly through the process of phagocytosis. Recently, a formation of extracellular neutrophil networks generated via release of nuclei content into the extracellular space along with the components of their granularity was demonstrated as a novel approach of neutrophil-mediated microbial killing. The mechanism of NETosis is presented in Figure 1. In neutrophils, bacterial stimuli through the Raf-MEK-ERK signalling pathway induce histone citrullination mediated by peptidylarginine deiminase (PAD), which results in chromatin decondensation, disruption of cellular membrane, and release of DNA with associated proteins, including histones, into the extracellular environment, thus entrapping pathogens [7–11]. The exact mechanism of the NETosis process is still under investigation; nevertheless, there are some data indicating that the formation of neutrophil networks is mainly dependent on production of reactive oxygen species (ROS), which are generated by the NADPH oxidase Nox2, and are required for translocation of neutrophil elastase (NE) from the azurophilic (AZ) granules to the nucleus, resulting in decondensation of chromatin, which is subsequently promoted by myeloperoxidase (MPO) [12]. In this aspect it might be assumed that accumulation of DNA in extracellular space results from a specific physiological mechanism, which prevents sudden elimination of NETs by DNase or by phagocytes at the site of bacteria invasion, or at this setting the intensity of DNA release is much higher than the capacity of DNase to digest DNA and/or the capacity of phagocytosis.

Another outcome of neutrophil accumulation leading to build-up of polyelectrolytes into the extracellular space is proteolytic action of neutrophil proteases (elastase, cathepsin G protease, and proteinase-3) that are released as both free and as a part of the NET component and are very destructive within the surrounding tissue [13, 14]. In effect, DNA and F-actin gathering at infectious sites originate from neutrophils and broken host cells. Necrosis of human cells also causes the release of monomeric actin that can polymerise in the extracellular space by promoting F-actin formation factors such as ionic composition and lack of actin binding proteins that prevent/control F-actin formation in the cytosol. A small part of DNA identified in some purulent fluids results from destruction of pathogen cells [15]. DNA network/aggregates in individual pus samples collected from skin infection before and after treatment are demonstrated in Figure 2. In addition to polyelectrolyte aggregates that accumulate during the process of pus formation at the mucosal surface, especially in the respiratory

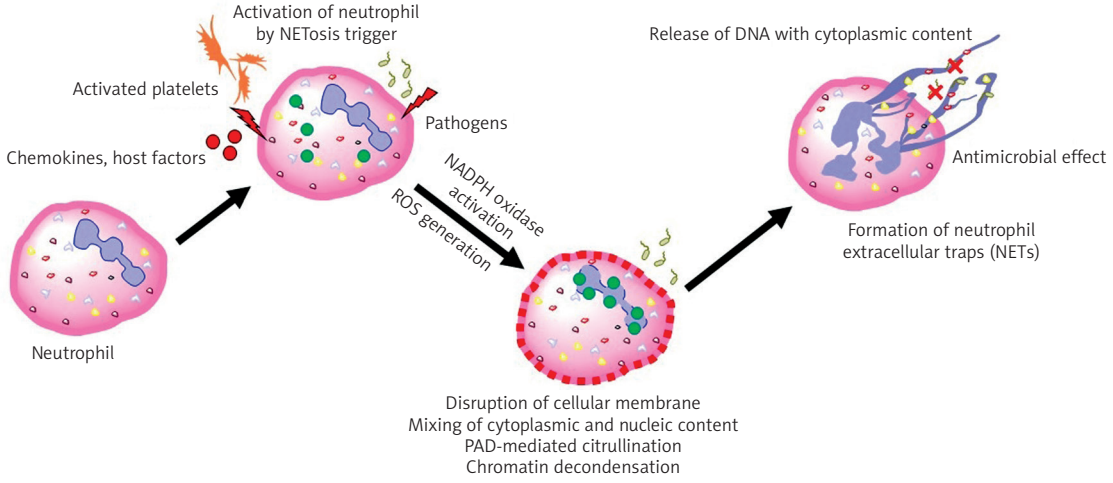

Figure 1. Formation of neutrophil extracellular traps (NETs) might take place in the presence of pathogens, chemokines, and different host factor

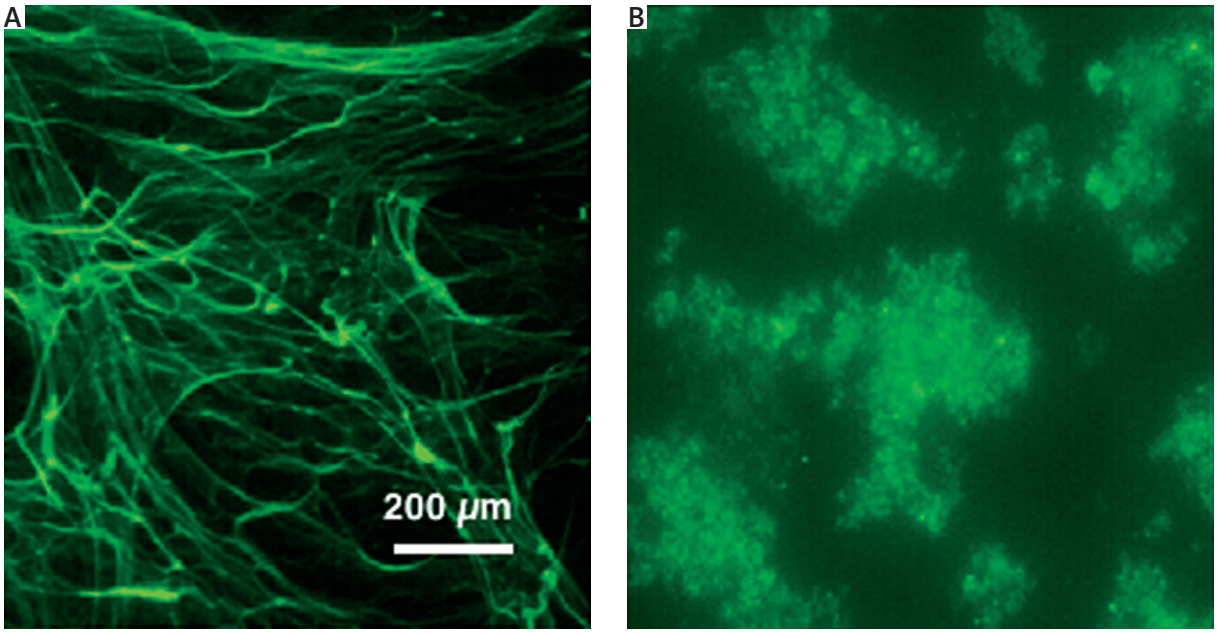

Figure 2. Fluorescence images of DNA network/aggregates (yellow/YOYO-1) in individual pus samples collected from skin infection before (panel **A**) and after (panel **B**) DNase I (3 μg/ml) treatment (30 min at 37°C)

tract of cystic fibrosis subjects, accumulation of extracellular DNA was observed during sterile inflammation within some tissues.

Recently published data demonstrated that formation of NETs followed by accumulation of negatively-charged biopolymers might be a pathogenic event in the development of some diseases. The effects of eDNA presence in extracellular compartments is presented in Figure 3. A number of reports specify the pathogenic role of NETs in the development of au-

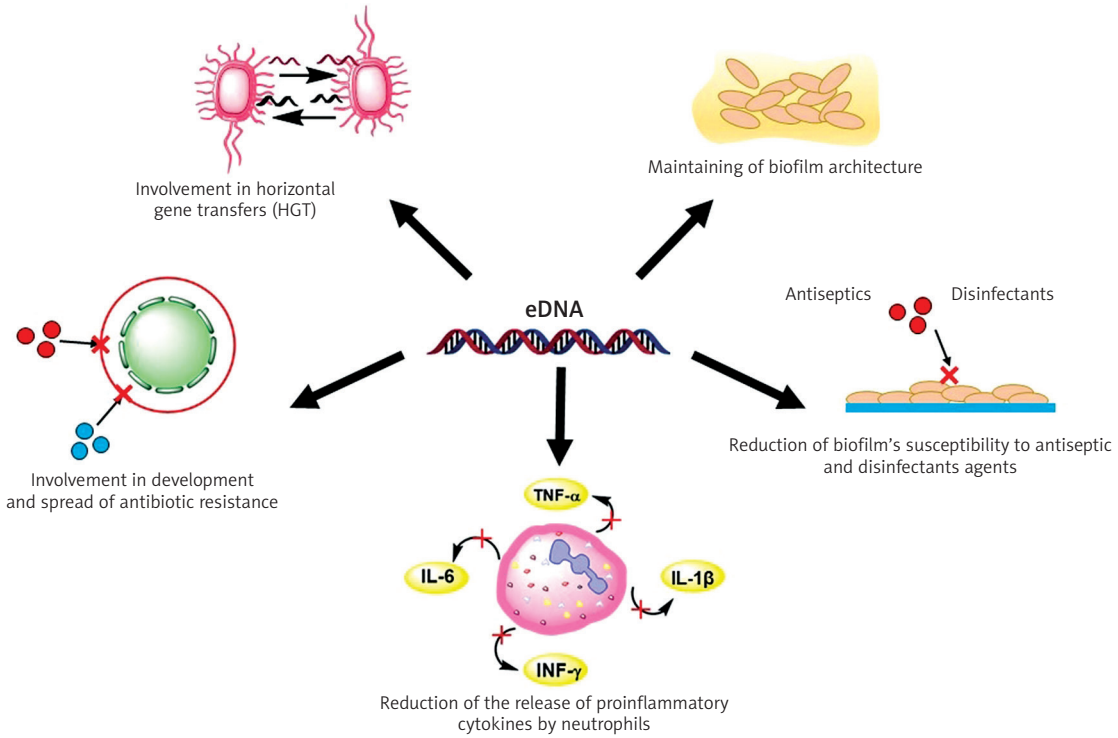

Figure 3. Some consequences of eDNA presence in extracellular matrix

toimmune diseases, which includes NET-mediated stimulation of the modified autoantigens externalisation, induction of type I IFN synthesis, activation of the complement system pathways, and inflammasome machinery. These reports, followed by the data revealing the association of NETs with vasculopathy, endothelial cell dysfunction, formation of atherosclerotic plaque burden, and thrombosis, confirm that the explanation of NET formation might be helpful in the design of modern therapeutic strategies [9]. Fuchs *et al.* demonstrated that the formation and prothrombotic effect of NETs might be limited by commonly used anticoagulant heparin. It was shown that heparin is able to disassemble NETs and remove platelet aggregates from neutrophil networks with an effectiveness comparable to DNase, which is determined by the high affinity of this compound to histones and their removal from the chromatin fibres and destabilisation of NETs [16, 17]. Smith *et al.* demonstrated that NET formation accompanied by impaired neutrophil extracellular trap clearance plays an important role in pathogenesis of systemic lupus erythematosus (SLE) and other autoimmune diseases because NETs/extracellular DNA excess was reported in skin of patients with SLE [18]. Moreover, it was presented that limitation of NET formation using PAD inhibitors considerably affect lupus and atherosclerosis phenotype in murine models [18, 19]. An imbalance between NETs-derived eDNA production and nuclease efficiency is also observed in patients with dry eye disease, which results in ocular surface inflammation [20]. Additionally, increased NETosis also impairs wound healing in diabetes and treatment with plasma DNase 1 because the agent cleaving NETs was demonstrated as the approach to considerably improve re-epithelialisation in diabetic mice [21].

The accumulation of PE in the extracellular environment leads also to the pathological alternations in the lungs of CF patients. Both F-actin and DNA are strong polyelectrolytes, with sufficiently high linear surface charge density to form a condensed layer of counterions [22]. It should be assumed that the physicochemical characteristics of DNA/F-actin aggregates formed at the surface of mucosal membranes differ from that formed within tissues because assembly of negatively charged PEs is governed by local ion composition and the presence of a bundling factor (polycations), such as most cationic antibacterial peptides. Additionally the NET composition might vary based on mechanisms activating NETosis [13, 18, 23]. The composition of DNA-protein complexes that accumulate in airway sputum of CF patients is consistent with NETosis and joins a similar proteomic signature, indicating that the majority of the DNA in sputum is NET derived. Macrophage migration-inhibitory factor (MIF), by promoting mitogen-activated protein kinase, is the leading factor that controls NETosis of polymorphonuclear leukocytes in response to the presence of *P. aeruginosa* in CF airways [24]. Importantly, the accumulation of polyelectrolytes in the lungs of patients with CF contributes to the changes in viscoelastic properties of airway surface fluid and causes rheological abnormalities of CF sputum, as confirmed by the research indicating that enhanced DNA and/or F-actin content directly correlate with increased magnitudes of both elastic and viscous moduli of airway secretions [2, 25, 26]. In effect, dysfunction of ciliary transport and bronchial obstruction represent common manifestations of CF [3, 27].

In addition to these studies, the latest reports demonstrate that NETs, by clogging the pancreatic duct, might also contribute to the development of pancreatitis [28] and might also be circulating in blood; however, it could not be employed as a biomarker to assess the progress of antineutrophil cytoplasmic antibody-associated vasculitis (AAV), as previously suspected [29]. Recently, interest in the metabolism of DNA in the epidermis has been raised by the finding that extracellular DNA, in combination with cathelicidin LL-37, is able to activate dendritic cells, which could contribute to the aetiology of psoriasis. In both infectious and non-infectious inflammatory skin diseases, epidermal eosinophils are able to release extracellular traps [17], and DNA might accumulate in the extracellular matrix. DNA can also migrate from an extracellular space to keratinocyte endosomes. In effect, the activation of the innate immune system via TLR9 might occur [30]. The activities of eDNA and LL-37 can be modified when presented in aggregate form. Either mutual inhibition or an increase of activity, depending on the aggregation state, might be observed. Upregulation of LL-37 production and release from keratinocytes in response to injury is temporary, peaking at 48 h and progressively decreasing thereafter. Considering this, it is suggested that interaction between simultaneously released cathelicidin and self DNA might be beneficial for the host. Despite the fact that LL-37 in complex with DNA is characterised by the lack of killing ability against microbials, eDNA-LL-37 aggregates sequester cathelicidin, thus creating a backup of extracellular LL-37, which could be effectively used when secretion of this peptide is diminished. Moreover, the prolonged release of this peptide during infection or injury might be ensured by DNase [30]. There is also some evidence that F-actin and DNA inhibit the antimicrobial activity of extracellular histones released during NET formation, which protects the organism from their high cytotoxicity, tissue injury, and acute inflammatory response observed after release of high amounts of histones to the extracellular environment [31].

Polyamine-induced DNA aggregation

To date, the exact mechanism of NET-mediated autoimmune responses is still not precisely stated;

nevertheless, the latest research focused on the investigation of biological activity of polyamines sheds new light on the importance of these factors in the development of NET-associated pathologies. Polyamines (putrescine, spermidine, and spermine) are linear polycations present in the cells of all living organisms and are involved in a great number of biological functions. However, despite extensive investigation, the ability of polyamines to modulate DNA functions and conformation is not well understood. Polyamines intrinsically form hierarchical assemblies of complex structures in the presence of phosphates. Three compounds, referred to as nuclear aggregates of polyamines (NAPs), were isolated from nuclear extracts of many different cells. NAPs play a crucial role in DNA compaction and functioning [32, 33]. Since cells normally do not secrete spermine, its presence in extracellular space is mostly seen as an indicator of increasing cell death usually associated with cancer development. It is suggested that the presence of NAPs in the neutrophil networks stabilise and protect autoantigens from degradation, resulting in development of excessive immune response. It is also proposed that free polyamines interact with PAD4 and induce the citrullination process during NET formations, which increase their level even in environments with low calcium levels [34].

Condensation of DNA by cationic peptides

A number of reports indicate that the high concentrations of eDNA in the airway fluids of patients with CF are mainly NETs-derived, which confirms that the formation of neutrophil networks in CF subjects plays a major role in the development of lung pathologies. Nevertheless, despite well-established knowledge about the presence of eDNA in amounts higher than 10 mg/ml in CF lugs, the role of this biopolymer in the development of disease was either not considered or attributed to their effects on airway fluid or sputum viscosity [2, 35].

However, theoretical and experimental studies of purified systems show that the highly preferential condensation of polyvalent counterions like CAPs onto the surfaces of linear polyelectrolytes causes formation of F-actin and DNA bundles [36, 37] and the penetration of these bundles into the charged networks of mucin that constitute normal airway and other extracellular fluids. This finding shifts the focus away from the effects of the long cytoskeletal or nuclear polymers on extracellular fluid viscosity as the primary cause for their possible pathological effects to their direct ability to prevent the antimicrobial defence mounted by the infected epithelium and recruited neutrophils. However, polyelectrolyte bundles present in CF sputum trap a large spectrum of molecules including, in addition to different antibacterial peptides, proinflammatory cytokines and proteolytic enzymes. Overall, it should be underlined that cationic antibacterial peptides represent the most important known bundling agent that neutralises repulsive forces between individual filaments in a complex network of sputa. As already mentioned, the bundling power of cationic antibacterial peptides come with the cost of loss of their antibacterial potency because electrostatic interactions govern the ability of antibacterial peptide to recognise the negative surface of bacteria, fungi, or viruses. Clinical conditions in which antimicrobial agents lose activity, aside from chronic lung infection in CF, include potentially all infections associated with pus formation, such as diabetic foot infections or secondary infections after burn injury. In these conditions, antibacterial peptides such as beta defensins and LL-37 are released by the host epithelial cells and haematopoietic cells within the infected tissue in higher amounts compared to their constitutive production, but they fail to kill bacteria or to arrest the progression of the infection [38, 39]. It is accepted that simultaneously release of cationic antibacterial molecules and liberation of negatively charged DNA during NETosis process is an established mechanism leading to elimination of pathogens originally triggering this phenomenon. From a physiologic point of view, such an apparatus might be seen as a common example of negative feedback, which will switch off if the release of antibacterial peptide and DNA leads to pathogen eradication. However, if pathogen invasion leads to chronic persisting colonisation (indicating lack of bacteria killing), development of chronic inflammation, such as that observed in a CF lung, is taking place. We still do not have a full understanding of interplay between release of polymeric DNA, cationic antibacterial peptides, and bacteria responding to those factors. Generally, interactions of CAPs and other polycations with anionic filaments are well described by theories of polyelectrolytes [40–42]. These effects govern the binding of antimicrobials to strong anionic polyelectrolytes such as F-actin and DNA, as well as the resultant 'like-charge attraction', which causes such polyelectrolytes to form bundles and thereby sequester and inactivate antimicrobials. Polyelectrolyte theories and experiment show that there is a subtle balance between sequestration or concentration of CAPs by weaker anionic polymers, such as mucins that normally cover epithelia and concentrate CAPs near the outer cell surface without compromising their antibacterial function, and inhibition of antibacterial activity by stronger and more rigid polyelectrolytes like F-actin or DNA. Previous studies of DNA, F-actin [43], alginate [44], and other anionic filaments present at sites of infection confirm the generality of these biophysical arguments and suggest strategies for design of cationic amphiphiles with reduced binding to linear polyelectrolytes without loss of targeting to bacterial surfaces [45]. Even strong inhibition of cationic antibacterial peptide activity by DNA, F-actin,

or other natural negatively charged biopolymers was observed *in vitro* [1–6]; their interaction with NETs is much more complex within *in vivo* settings. Some reports suggest that DNA release from neutrophils represents an innate immune response to trap, maintain, and buffer the release of cationic defence molecules in exact time-space where the pathogens are trapped. On the other hand, if such mechanisms fail, bacteria trapped in DNA/F-actin networks undergo extensive changes of gene expression leading to the development of bacterial biofilm. Interestingly, the formation of bundles induced by F-actin, DNA or bacteriophages is diminished for synthetic analogues of CAPs, such as ceragenins, which maintain their high antibacterial activity despite the presence of PE at the infection site [46, 47]. Importantly, elevated eDNA and NET levels and their accumulation in biological fluids is a factor activating innate immune responses, which considerably affect the development of such diseases as asthma and chronic obstructive pulmonary disease [48], periodontitis [49], antineutrophil cytoplasmic antibody-associated vasculitis (AAV) [29], or cancer-associated thrombosis [50]. Quantitative analysis of the physical chemistry of polyelectrolytes has been useful for designing possible countermeasures to release endogenous antimicrobial peptides trapped in the polyelectrolyte bundles present in purulent fluids [5, 45, 51–56]. Better penetration through this complex network might be achieved using a nanotechnology approach. The average 3D mesh spacing of CF sputum is 140 ±50 nm (range: 60–300 nm). It might be assumed that nanoparticles up to 200 nm in diameter that do not adhere to CF sputum will diffuse rapidly through this critical barrier by accessing pores that are filled with a low-viscosity fluid [57].

DNA and F-actin in bacterial biofilm development

To date, extracellular DNA and F-actin have been shown to function as signalling initiators and structural support in preserving architecture of microbial biofilm [58–60]. From a medical perspective these polyelectrolytes have been proposed as therapeutic targets to prevent biofilm growth and to treat infections [58, 61].

In addition, a cytoskeletal fraction of human neutrophils have a strong stimulating effect on biofilm production, as did purified F-actin. Depleting actin from whole neutrophil lysates diminished this effect [58]. In agreement with these reports, a study performed by our research team demonstrated that the presence of polyelectrolytes at the infection site considerably affect the biofilm formation, although this effect differs among different microorganism strains [62]. Therefore, the employment of PE-depolymerising agents such as gelsolin and thymosin beta 4 or DNase 1 used for dissolution of biofilm-stimulating F-actin and DNA, respectively, might constitute an interesting approach in the eradication of microbial communities [62–64]. The statement that the presence of eDNA is crucial for the biofilm formation and structural integrity is additionally established by the reports demonstrating that the treatment with DNase 1 reduces the attachment of microbial cells to the surface, inhibits the biofilm development, and reduces the mass of mature biofilm [65], which is determined by the impairment of cell-to-cell adhesion and joining elements in microbial aggregates [66, 67]. Importantly, the co-administration of antimicrobial agents with factors that have the ability to disassemble the biofilm polyelectrolyte network, including DNase 1, considerably improves their antibacterial efficiency [5, 68]. As well as the reports indicating the vital role of eDNA in the biofilm structure, it was demonstrated that eDNA acts as an agent affecting the neutrophil-mediated response and triggering the phagocytosis. Bauer *et al.* demonstrated that the supplementation of biofilm material with DNase 1 results in decrease of pro-inflammatory response, which was confirmed by significantly reduced neutrophil activation markers. Extracellular DNA seems to be also engaged in interaction with toll-like receptors (TLR) recognising the pathogen-derived ligands. TLR9, as an intracellular receptor, is required for a response to the unmethylated CpG motifs of bacterial DNA [69]. Upon phagocytosis and bacterial digestion, the bacterial DNA is liberated, and it engages TLR9. TLR9-dependent activation can be triggered not only by phagocytosis of whole bacteria cells, but also by eDNA [70].

Nevertheless, despite the reports indicating the high usefulness of DNase 1 in the improvement of biophysical properties of airway fluids and in eradication of microbial biofilms, it was found that with increasing growing time biofilm seems less susceptible to DNase 1 treatment. It has been hypothesised that this phenomenon is determined by the presence of some proteolytic exoenzymes with the ability to deactivate DNase, because this effect is particularly evident in mature biofilm older than ~80 h [66].

It is also generally accepted that development of microbial biofilm highly contributes to the spreading of an antibiotic resistance. Recently published data demonstrating the role of eDNA in this process have shed new light on the mechanism of this phenomenon. To date, there is evidence confirming that eDNA is a pool for horizontal gene transfer (HTG) for the purpose of genetic recombination of bacterial cells, such as transformation, conjugation, or transduction. Considering the fact that antibiotic resistance may be acquired due to a mutation or an acquisition of resistant genes [66], the presence and high accessibility of eDNA in the biofilm environment provides a readily available gene tool for genetic modifications resulting in the development of antibiotic resistance [66]. Additionally, Wilton *et al.* demonstrated an addi-

tional mechanism of eDNA-mediated development of aminoglycoside resistance of *Pseudomonas aeruginosa*, which is conditioned by acidification of the local biofilm environment, strongly promoting the antibiotic resistant phenotype acquired by modification of lipid A and the synthesis of spermidine on the bacterial outer membrane [71].

Conclusions

The formation of neutrophil networks, despite their protective and beneficial effect in entrapping and eradication of microbial pathogens, should be considered as important factors determining the pathogenesis of a number of diseases, particularly due to the release of negatively-charged biopolymers such as eDNA and F-actin. Considering the above-listed reports, it can be established that further studies aimed at investigating the molecular mechanism of NETosis are justified and needed, particularly for the purpose of novel NET-directed agents, which might be successfully used in the modern therapy of diseases caused by excessive amounts of NETs and accumulation of polyelectrolytes in the extracellular compartments.

Conflict of interest

The authors declare no conflict of interest.

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