Neutrophil extracellular traps in the fight against biofilm-forming microorganisms: hunters or prey?

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The review presents up-to-date data on the relationships between neutrophil extracellular traps (NETs) and biofilm-forming microorganisms *P. aeruginosa*, *S. aureus*, *Candida* spp. obtained *in vitro* and *in vivo* studies. Up to 80% of human microbial infections are associated with biofilm-forming microorganisms. The formation of highly specialized biofilm communities is one of the main strategies for the survival of bacteria and fungi, significantly increasing their tolerance to aggressive and stressful environmental conditions, chemotherapeutic drugs, and immune system factors, contributing to their persistence and chronicity of the infectious process. The formation of NETs in the process of NETosis is one of the biological mechanisms used by neutrophils in protection against pathogens. Chemoattractants of biofilm origin, as well as those secreted by epithelial and immunocompetent cells, attract and activate migrating neutrophils. However, given that bacteria form fairly large cell clusters and aggregates in biofilms, the process of phagocytosis is sometimes difficult or impossible. Under these conditions, it is logical to assume that the importance of NETs in anti-biofilm immunity increases. However, due to the components of the extracellular biofilm matrix (e.g., Psl exopolysaccharide *P. aeruginosa*), quorum sensing (QS) molecules (e.g., LasR QS system *P. aeruginosa*), enzymes (e.g., LasA protease and LasB elastase *P. aeruginosa*), toxins (e.g., Panton-Valentine leukocidin and AB γ-hemolysin *S. aureus*) and probably other factors yet to be studied, the microorganisms in biofilms are able to influence the signaling systems involved in NETosis, the intensity of the formation of NETs, the sequestration and killing mechanisms in them, sometimes subordinating and using NETs components for their own purposes.

Keywords: *neutrophils; neutrophilic extracellular traps; NETosis; biofilms; biofilm infections; biofilm-forming microorganisms; review.*

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Нейтрофильные внеклеточные ловушки в борьбе с биопленкообразующими микроорганизмами: охотники или добыча?

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В обзоре представлены современные данные о взаимоотношениях нейтрофильных внеклеточных ловушек (НВЛ) и биопленкообразующих микроорганизмов *P. aeruginosa*, *S. aureus*, *Candida* spp., полученные в исследованиях *in vitro* и *in vivo*. До 80% микробных инфекций человека связаны с биопленкообразующими микроорганизмами. Формирование высокоспециализированных сообществ в виде биопленок является одной из основных стратегий выживания бактерий и грибов, значимо повышая их толерантность к действию агрессивных и стрессовых внешних условий, химиотерапевтических препаратов, факторов иммунной системы, способствуя их персистенции и хронизации инфекционного процесса. Образование НВЛ в процессе нетоза является одним из биологических механизмов, используемых нейтрофилами в защите от патогенов. Хемоаттрактанты биопленочного происхождения, а также выделяемые эпителиальными и иммунокомпетентными клетками, привлекают и активируют мигрирующие нейтрофилы. Однако учитывая, что в биопленках бактерии образуют достаточно крупные клеточные кластеры и агрегаты, процесс фагоцитоза порой оказывается затруднен или невозможен. В этих условиях логично предположить, что значимость НВЛ в антибиопленочном иммунитете увеличивается. Однако за счет компонентов внеклеточ-

ного биопленочного матрикса (например, экзополисахарид Psl *P. aeruginosa*), молекул системы quorum sensing (например, quorum sensing-система LasR *P. aeruginosa*), ферментов (например, LasA-протеаза и LasB-эластаза *P. aeruginosa*), токсинов (например, лейкоцидин Пантона–Валентайна и γ-гемолизин АВ *S. aureus*) и, вероятно, других, пока не изученных, факторов микроорганизмы в биопленках способны влиять на сигнальные системы, задействованные в нетозе, на интенсивность формирования НВЛ, механизмы секвестрации и киллинга в них, порой подчиняя и используя компоненты НВЛ для собственных целей.

Ключевые слова: *нейтрофилы; нейтрофильные внеклеточные ловушки; нетоз; биопленки; биопленочные инфекции; биопленкообразующие микроорганизмы; обзор.*

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Introduction

The discovery by V. Brinkmann and colleagues in 2004 of neutrophil extracellular traps (NETs) as a form of the innate response that binds microorganisms, prevents their proliferation and provides a high local concentration of antimicrobial agents [1, 2] gave rise to the study of the role of NETs along with phagocytosis and degranulation in protection against pathogens and demonstrated their effectiveness against a number of bacterial, fungal, and viral infections [3–9]. However, despite the wide range of countermeasures, the fight against microorganisms is dramatically complicated when they form associations called biofilms, which are involved in the development of persistent infections and destructive inflammatory processes [10, 11].

Main part

Bacteria and fungi are characterized by two different "lifestyles": planktonic and biofilm [12, 13].

In the planktonic existence, single cells or cells in small chains (for example, streptococci) or clusters (for example, staphylococci) "float" without protection against toxic substances, bacteriophages and phagocytes. Therefore, this lifestyle is dangerous for microbes [12].

In the biofilm lifestyle of microorganisms, the formation of cell aggregates surrounded by a self-produced extracellular matrix is one of the survival strategies [14]. Such microbial aggregates may adhere to natural or artificial surfaces (sessile growth, adherent biofilms), for example, to teeth, epidermal cells, venous catheters, artificial joints, or may be located in tissues (non-adherent or suspended biofilms), for example, on mucous membranes, in sputum, or in difficult-to-heal wounds [12]. Thus, a biofilm is a constantly renewed community of microbes on a biogenic or abiogenic substrate, surrounded by an extracellular polymer matrix of exopolysaccharides, extracellular DNA, proteins, RNA and lipids, which protects them from adverse effects, chemotherapeutic drugs and the effects of the host organism, is one of the factors of inter-microbial interaction and communication, promotes horizontal gene transfer, and is a source of nutrients for bacteria during their starvation [13–22].

The use of modern technologies made it possible to establish that the development of biofilms comprises several successive stages:

1) Initial attachment of planktonic bacteria to a surface (substrate);

2) Biofilm maturation;

3) Separation of planktonic forms (dispersion, detachment, scattering of biofilm) with their subsequent migration to new loci [13, 23].

The microorganisms inside biofilms use an intercellular communication system of small signaling molecules, autoinducers, called quorum sensing (QS) [14, 18, 20]. The QS system determines not only the density of population, but also regulates various characteristics, such as bacterial phenotype, virulence factor gene expression, spatial differentiation, and biofilm formation [18, 21]. The release of QS molecules provides fast local communication between cells in the infected area, synchronization of their growth, as well as reactions to changes in temperature and pH of the environment, presence of biocidal compounds, etc. [13, 24]. Up to 80% of human microbial infections, including endocarditis, cystic fibrosis, periodontitis, rhinosinusitis, osteomyelitis, chronic non-healing wounds, meningitis, kidney infections, and post-implantation infections are associated with biofilm-forming microorganisms [13, 20]. It sounds paradoxical, but high doses of antibiotics used to treat biofilm infections promote the formation of antibiotic-resistant bacterial strains. [13].

NETs are DNA fibers, "decorated" with a set of proteins of nuclear, cytosolic, and granular origin, a part of which constitute a relatively constant "proteomic nucleus" regardless of the inducing stimulus, including histones H2A, H2B, H4, lactoferrin, myeloperoxidase

(MPO), neutrophilic elastase (NE), resistin, neutrophilic defensin-2, α-actinin, β-actin, myosin-9, moesin, profilin-1, plastin-2, filamin-A, lipocalin associated with neutrophil gelatinase, α-enolase, glucose-6-phosphate isomerase, transketolase [25–28]. The existence of proteomic variations may be due to the fact that different stimuli trigger different intracellular signaling cascades, which ultimately lead to some changes in the protein composition of NETs. Although another scenario is possible, according to which the adhesion of additional proteins can occur after the release of NETs, depending on their environment [28].

Currently known formation mechanisms are NADPH-oxidase (NOX)-dependent and NOX-independent ones [29, 30]. The best studied is the NOX-dependent mechanism, where the activation stimulus, for example, phorbol 12-myristate 13-acetate (PMA) or bacterial lipopolysaccharide, causes the release of Ca^{2+} reserves into the cytosol, an increase in the activity of protein kinase C and phosphorylation of gp91phox/Nox2 [29, 31]. This process facilitates the assembly of the NADPH oxidase complex, thereby stimulating the generation of reactive oxygen species (ROS) that contribute to the subsequent disintegration of the membranes of the nucleus and granules [9, 29, 32–35]. NE and MPO emerging from the azurophilic granules come into contact with the contents of the nucleus, participating in histone cleavage and chromatin decondensation [9, 29, 34]. The process culminates in the release of decondensed DNA, "decorated" with proteins, into the intercellular space through breaks in the neutrophil cell membrane, which is facilitated by HClO production under the influence of MPO [30] or a pore, the formation of which is assisted by gasdermin D [36], and neutrophil death [9 , 29].

Chromatin decondensation is also assisted by peptidylarginine deiminase 4 (PAD4), an enzyme that converts positively charged histone arginine into neutrally charged citrulline, thereby changing the total charge of molecules and promoting the dissociation of histones and DNA [37]. However, the role of PAD4 in the NOX-dependent NET formation remains a matter of controversy [29]: the data of some researchers evidence that histone citrullination is not a necessary event in the NADPH-oxidase-dependent NET formation [29, 38, 39], while the results obtained by other scientists prove the opposite [29, 40, 41]. In a recent paper published in 2020, H. R. Thiam and et al. found that a chain of sequential cellular events takes place during NETosis and remains the same in different species (e.g., in humans and mice), nuclear localization and citrullinating activity of PAD4 being necessary conditions for decondensation and release of DNA [37].

Calcium ionophores (ionomycin, A23187), some cytokines, phospholipid mediators of inflammation, and uric acid crystals can trigger the NOX-independent mechanism of NET formation [29, 30, 42–47]. On the one hand, the influx and increase in the level of Ca^{2+} in the neutrophil cytosol stimulates calcium-activated potassium channels and the production of mitochondrial ROS [29, 30, 39]; on the other hand, it activates PAD4 and translocates it into the nucleus, which is followed by histone citrullination and chromatin decondensation [29].

NETs and biofilms of Pseudomonas aeruginosa

Chronic biofilm respiratory tract infection caused by Pseudomonas aeruginosa in patients with cystic fibrosis (CF) is the best studied and described biofilm infection in medicine [12]. In CF, there are mutations in the gene encoding the synthesis of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), the protein involved in the transport of bicarbonate and chlorine ions through the membrane [47]. *CFTR* is expressed in many organs, including epithelial cells of the respiratory tract, pancreas, and innate immune cells, in particular, neutrophils [47, 48]. Mutations in the *CFTR* gene lead to the disruption of the normal transport of ions and fluid through the epithelium of the respiratory tract, to the formation of a thick layer of viscous mucus, impaired mucociliary clearance, development of inflammation and chronic bacterial infections leading to impaired lung function and respiratory failure [47]. *P. aeruginosa* infects the respiratory tract of CF patients at an early age and becomes a persistent pathogen in subsequent years due to its ability to form biofilms [47, 49].

Neutrophilic granulocytes play a key role in the elimination of *P. aeruginosa*, including from the respiratory tract. The most effective methods of control are classical phagocytosis and subsequent intracellular killing, while *P. aeruginosa* is resistant to oxygen-dependent mechanisms and is sensitive to the action of oxygen-independent factors such as NE, lysozyme, cathelicidins, and defensins [49]. However, in CF, neutrophils migrating in large amount into the lungs cannot effectively kill bacteria, they exhibit a dysfunctional phenotype, causing damage to lung tissue and impairment of its function [47, 49, 50].

Initially, the secondary necrosis was believed to be the main form of neutrophil death in the respiratory tract in CF, i.e. necrosis after apoptosis due to untimely removal of apoptotic cells [49]. In works dated 2005 [51] and 2009 [52], a team of American researchers found that the complex of DNA and actin protein released during the neutrophil necrosis significantly enhances the biofilm formation of *P. aeruginosa* PAO1 strain *in vitro*, which probably occurs in the respiratory tract of CF patients. Similar results were obtained in the study of another research team from the United States in 2011. They studied the relationship between the biofilm-forming activity of *P. aeruginosa* strain 6294 and neutrophils in the development of keratitis caused by wearing contact lenses [53]. The authors found that the scaffold of F-actin and DNA secreted from necrotic

neutrophils is successfully used by bacteria for the adhesion and further biofilm formation on the surface of a contact lens [53].

However, a number of studies have shown that neutrophils also massively undergo NETosis with NET release, a large amount of which is found in the respiratory tract and sputum of CF patients. [27, 28, 47, 49].

One of the powerful inducers of the NET formation is the mobility of *P. aeruginosa* caused by flagellin flagella; therefore, the prevalence of mobile planktonic non-mucoid forms of bacteria in the early stages of the disease causes active NETosis [27, 28, 49, 54]. Another trigger of the NET formation is pyocyanin, exotoxin of *P. aeruginosa* [55]. Its induction through QS signaling correlates with the growth stage of the *P. aeruginosa* biofilm. Although pyocyanin has a wide range of toxic effects, the assumed basis of its toxicity is its penetration through the cell membrane and oxidation of NADPH (i.e., pyocyanin is a non-enzymatic NADPH oxidase) with the formation of superoxide anion and other ROS inside the cell, which results in the development of oxidative stress in target cells [56-58]. However, in 2013, there was established a new mechanism of pyocyanin action, namely the induction of NET formation through the oxidative stress (via ROS, JNK and PI3K enzymes, and autophagy), which is caused not by the direct NADPH oxidation, but by the activation of NADPH oxidase of the neutrophil [55].

While in the respiratory tract, *P. aeruginosa* induces the production of the macrophage migration inhibitory factor by neutrophils – a pro-inflammatory cytokine with autocrine and paracrine action, which, in turn, causes the activation of neutrophilic mitogen-activated protein kinase and the subsequent formation of ROS inside the neutrophil, also potentiating NETosis and inhibiting apoptosis [27].

P. aeruginosa has several QS systems, and the Las system occupies the highest position in the hierarchy, regulating the lower Rhl and Pqs systems [59, 60]. The inducers of NETosis are such important virulence factors of *P. aeruginosa* , QS system controlled LasR, as the enzymes LasA protease and LasB elastase, as well as exotoxins secreted by the type III secretion system (T3SS) [60].

It is important to note that different genetic variants of *P. aeruginosa* induce different mechanisms involved in the NET formation: some of them trigger NOX-dependent pathways, others – ROS-independent ones; the action of the former leads to histone citrullation during NETosis, while the latter do not require PAD4 and citrullination for the release of NETs [60]. Besides that, it was found that the composition of NETs induced by different variants of *P. aeruginosa* always contains a protein that enhances the bactericidal effect of neutrophils, it is specifically targeting gram-negative bacteria such as *P. aeruginosa*, but not always NE and MPO [60].

Thus, a number of direct and *P. aeruginosa*-mediated factors contribute to the NETosis and release of a large amount of NETs since the early stages of the disease. However, sequestration of *P. aeruginosa* by traps at a sublytic concentration of antimicrobial NET-associated proteins does not lead to the complete destruction of bacteria, but, on the contrary, it promotes their microcolonization, aggregation, and finally biofilm formation [28]. During the formation of the *P. aeruginosa* biofilm, the Psl exopolysaccharides of the extracellular matrix interact with the extracellular DNA not only of the bacteria themselves, but also of the NETs, using them as "scaffolding", i.e. a framework for further biofilm formation and mucosa colonization [17, 18, 27, 28, 61]. The retention of *P. aeruginosa* in the scaffolding of the traps and the effect of some NET components on them, for example, the LL-37 protein, promotes bacterial mutagenesis [28, 62]. As the disease progresses, the presence of *P. aeruginosa* in the respiratory tract is accompanied by genetic changes and conversion of a number of characteristics, including the transformation into immobile mucoid forms that constitute biofilms. At the same time, both the loss of flagella and the production of alginate-containing mucus preserve, but decrease the ability of *P. aeruginosa* to induce NETosis [54, 61], and the mucus formation also suppresses the capture and killing of bacteria by NETs, that is reduces the effectiveness of protection [27, 28, 49].

It is of interest that 63% of CF patients with chronic Pseudomonas aeruginosa infection have mutant forms of *P. aeruginosa* with inactivated LasR QS system [60, 63]; however, the emergence of such strains is associated with deterioration of lung function in children and adults with CF [60, 64]. This paradox suggests that *P. aeruginosa* compensates for the loss of virulence factors by other pathogenic mechanisms, in particular, by eluding neutrophil-mediated bactericidal functions through reducing the intensity of NET formation [60].

Thus, these data demonstrate a complex dynamic relationship between NETs and *P. aeruginosa* biofilm infection in the respiratory tract of CF patients, it also highlights the potential drawbacks of the NET-based model of protection in the context of this infection [28]. The NETs formed under the action of bacterial triggers capture of *P. aeruginosa*, but the inability to destroy sequestered bacteria, promotes biofilm formation of the latter, on the one hand, and, triggers the process of their pathoadaptation, on the other hand, [27, 28], leading to the formation of even higher resistance to NET-mediated bactericidal activity.

In 2019, the results of the study of the effect of NETs on the biofilm formation of *P. aeruginosa* PAO1 strain in a model of bacterial keratitis in mice were published [65]. The use of multiphoton microscopy, 3D reconstruction in combination with electron microscopy and Gram staining of eye biopsies revealed that already 24 h after the infection, *P. aeruginosa* formed a thick

exopolysaccharide Psl-containing biofilm on the surface of the cornea, and neutrophils migrating through the peripheral limbal vascular network were gathering under this bacterial layer, forming a visible "shield". At the same time, a "dead zone" was found between the neutrophil layer (below) and the bacterial layer (above), filled with DNA in combination with proteins-histones, NE and MPO, i.e. NETs. An important fact is that animals of 3 groups, PAD4, neutrophil elastase and cathepsin C knockout, characterized by impaired NET-forming ability, did not have a "dead zone" in combination with the loss of structured *P. aeruginosa* biofilm detected in wild-type mice. It allowed the authors to suggest that the presence of NETs was responsible for the formation of biofilms by highly replicative planktonic bacteria. At the same time, in all the 3 groups of knockout animals, *P. aeruginosa* was found in the brain 7 days after the inoculation on the cornea, probably passing through the optic nerve canal. Based on this fact, the authors concluded that neutrophils formed a NET-barrier to keep bacteria outside in the form of biofilm and prevent their spread to the brain, "sacrificing" the eye, because planktonic bacteria are much more mobile than biofilm bacteria. Thus, the NET formation is probably an evolutionarily useful mechanism for protecting the brain against infections via the ocular route [65]. At the same time, the authors paid special attention to the fact that the neutrophils under investigation were cells that naturally migrated into tissues, rather than were isolated from the blood, therefore, the system, which they studied *in vivo*, reflected the true complex of relationships between neutrophils and *P. aeruginosa*. When *P. aeruginosa* infects the cornea, the T3SS bacterial system releases the ExoS toxin in the direction of neutrophil accumulation. On the one hand, it stops them under the layer of bacteria, allowing the biofilm to mature; on the other hand, it insites them to form NETs, which, in turn, promote the transition of *P. aeruginosa* from planktonic to biofilm form. The formation of an impenetrable biofilm helps bacteria build resistance to antibiotics, but also inhibits their invasion and spread to the brain. The intravenous administration of bispecific antibodies to animals – to T3SS and exopolysaccharide Psl – promotes the transition from the NET-mediated program of bacterial destruction to the phagocytosis and the intracellular protease-mediated mechanism of their killing, preventing the formation of biofilms. The administration of antibodies combined with local antibiotic treatment demonstrates an effective elimination of the infection and reduction of eye inflammation in mice with formed biofilm [65].

In 2020, a research team from Denmark and the United States published interesting results of the study of the relationship between neutrophils and *P. aeruginosa* biofilms *in vivo* [66]. Using the transmission electron microscopy, the authors revealed a tight contact between neutrophils and *P. aeruginosa* biofilm formed on a silicone implant 24 and 48 h after its installation in the abdominal cavity of mice. Using special dyes together with the Click-iT® technology for *in vivo* labeling of extracellular DNA (eDNA) and confocal laser scanning microscopy, the researchers showed that eDNA strands of neutrophilic origin were localized around the *P. aeruginosa* biofilm, but not inside it, that is they were not a part of it. The investigation of lung tissue sections from CF patients by fluorescence *in situ* hybridization using specific dyes also revealed that fibrils of neutrophil eDNA are located outside, surrounding *P. aeruginosa* biofilms. The immunohistochemical methods for investigation of such components of NETs as histones H3, citrullinated histones H3 (citH3) and NE, both in material from mice and in sections of human lung tissue, showed that citH3 (the main NET marker) are absent inside *P. aeruginosa* biofilms; H3 are localized outside, i.e. around, on the periphery of the biofilm, but not together with it, while NE colocalizes with bacteria in the biofilm. Thus, the authors proposed a hypothesis that *P. aeruginosa* bacterial biofilms *in vivo* do not use the host's eDNA as a scaffold, it is the neutrophil extracellular DNA that acts as a kind of membrane around the biofilm (secondary matrix), limiting the dissemination of bacteria (which is partly consistent with the results of the study by A. Thanabalasuriar et al. [65]) and protecting it against the phagocytosis. The main source of eDNA is the necrotic lysis of neutrophils, while the NETosis makes a very modest contribution to this process. In conclusion, the authors noted that this study was the first one investigating the direct distribution of the host's eDNA and bacteria in chronic bacterial infections *in vivo*. Unlike *in vitro* induction, the stimulation of neutrophils with bacterial biofilms during chronic infections *in vivo* does not seem to induce an active NET formation; however, the necrotic neutrophils do release eDNA, H3 histones, and antibacterial enzymes such as NE [66].

NETs and S. aureus biofilms

Staphylococcus aureus (*S. aureus*) is a known human pathogen that can cause a wide range of diseases, from skin and subcutaneous fat infections to life-threatening invasive nosocomial infections. In case of chronicity of staphylococcal infections, the formation of biofilms is observed both on the implanted structures (heart valves, catheters, implanted joints) and on human tissues [67–71].

In 2018, a research team from the United States found that *in vitro* biofilms of methicillin-resistant *S. aureus*, the USA300 strain, compared with bacteria in the planktonic state, sharply reduce the viability of neutrophils and contribute to their death by NETosis due to their secretory proteins [72]. The production of such staphylococcus virulence factors as Panton-Valentine leukocidin and AB γ-hemolysin (leukocidin hemolysin) plays the main role in the induction of

NETosis by biofilm bacteria. At the same time, the resulting NETs do not affect the biomass of the biofilm and the survival of bacteria in it, i.e. they are ineffective in killing the biofilm bacteria. The results obtained *in vitro* were confirmed by the authors in a model of chronic burn wound infection in pigs, demonstrating that *S. aureus* leukocidins induce the NETosis and contribute to the persistence of bacteria in chronic infections *in vivo*. As noted by the authors, one of the possible reasons for the resistance of *S. aureus* to the bactericidal activity of NETs may be the production of thermonuclease (Nuc) that cleaves the DNA of the traps [72].

In 2019, a research team from the Netherlands published data on the relationship of biofilms of different *S. aureus* strains and their enzyme thermonuclease 1 (Nuc1) with neutrophils *in vitro* [73]. One of the most important components of the extracellular biofilm matrix of *S. aureus* is eDNA, the formation of which is facilitated by the autolysis of bacterial cells, mimicking the apoptosis of eukaryotic cells [74], and which is believed to play a critical role in the stabilization of the biofilm structure [75]. However, as the authors established, at the early stages of *S. aureus* biofilm formation (after 1, 2, 4 h) in IMDM (Iscove's modified Dulbecco's medium), i.e. a medium for the cultivation of mammalian cells, staphylococci already produce Nuc1, an enzyme that destroys DNA, which should logically cause destabilization and remodeling of the biofilm. However, in the experiments performed, an increase in the amount of Nuc1 was parallel to the stable formation of a biofilm. This is consistent with the data of other studies showing that *S. aureus* is not sensitive to DNase I at the early stages of biofilm formation [76, 77]. The authors concluded that the formation of *S. aureus* biofilm in IMDM, unlike tryptic soy broth, i.e. a classical medium for bacteria cultivation, does not depend on eDNA [73].

It was also revealed that *S. aureus* biofilm-induced NETosis is ROS-independent [73]. Moreover, after 90-minute co-incubation of freshly isolated human neutrophils and 3-hour ones, i.e. from early-stage staphylococcal biofilms, the authors observed minimal amounts of NETs in response to wild-type *S. aureus*, while the *nuc*-mutant strain that does not produce thermonuclease 1 induced massive NET formation. On the one hand, the data obtained confirm that early-stage *S. aureus* biofilms are inducers of NETosis; on the other hand, given the revealed ability of biofilm bacteria to produce thermonuclease that destroys the DNA of NETs since the very first hours, like planktonic forms of *S. aureus* do, they prove the ability of staphylococci to actively evade the antimicrobial effect of neutrophils. Further studies are required to clarify the regulating mechanisms of the balance between induction of NETosis and degradation of NETs by *S. aureus* biofilms, which may be associated with the production of not only Nuc, but also other immunomodulatory factors of staphylococci [73].

NETs and Candida spp. biofilms

Candida albicans (*C. albicans*) is a widespread nosocomial fungal pathogen. *C. albicans* lives in the form of biofilms on vascular and urinary catheters, dentures and other medical devices, as well as on mucous membranes, which contributes to its resistance to antifungal agents and protective factors of the macroorganism, significantly reducing the effectiveness of candidiasis treatment [7, 78-80]. Considering that, on the one hand, the release of NETs is the main method of controlling the hyphalic, but not yeast, forms of *C. albicans*, which cannot be phagocytosed due to their size [81], and, on the other hand, *C. albicans* in biofilms is in an aggregated state, it can be assumed that trap formation may be an ideal method for combating the biofilm forms of *C. albicans* [79].

However, in 2016, a research team from the United States that studied the relationship between neutrophils and *C. albicans* biofilms of the SC5314 hyphal strain *in vitro* and *in vivo* (using a model of biofilm infection of the vascular catheter in rats) found that the biofilm forms of *C. albicans*, unlike the planktonic ones, inhibit the release of NETs [78]. Thus, after a 4-hour co-incubation of neutrophils and *C. albicans* biofilms, the formation of NETs was not observed, despite the active migration and adhesion of neutrophils to the fungal hyphae; at the same time, the planktonic forms caused a 20-fold increase in free DNA in a complex with citrullinated histones, becoming entangled in network-like fibrillar structures. The *C. albicans* biofilms disrupted even the PMA-induced trap formation. The authors were the first to determine that the key mechanism for suppressing the release of NETs by *C. albicans* biofilms is associated with inhibition of NADPH oxidase and ROS generation of neutrophils by such components of the extracellular biofilm matrix as α-mannan polysaccharides, but not by soluble molecules. Besides that, the biofilm matrix probably masks the epitopes of the *C. albicans* cell wall, which are recognized by the neutrophil receptors and are necessary for triggering the formation of NETs. Thus, the biofilm lifestyle allows *C. albicans* to avoid NET-mediated killing, contributing to the survival and resistance of biofilms to the neutrophil attack [78]. In addition, the inhibition of NETs may have broader consequences *in vivo*, taking into account their role in preventing the dissemination of microbes, "exposing" epitopes for fungi recognition, and recruiting additional inflammatory cells [78, 82, 83].

In their next study dated 2017, the same research team investigated the reaction of neutrophils to 4 clinical isolates (strains) of *C. albicans* selected for their differences in the biofilm-forming ability, architecture of formed biofilms and degree of filamentation: SC5314 (as in the previous study [78]), 3153, 98-210 and 98-17 [79]. Strain 3153 displayed a biofilm architecture similar to control strain SC5314 by forming a dense biofilm with an outer layer almost entirely composed of hyphal

cells. A noticeably lower degree of hyphae formation was observed in biofilms formed by strains 98-210 and especially 98-17, which contained mainly yeast forms on the biofilm surface. The thickness of the biofilms correlated with the ability to form hyphae: the strains showing the highest degree of filamentation, SC5314 and 3153, formed the thickest biofilms. It was found that, after 4-hour incubation with neutrophils, *C. albicans* biofilms from any strain do not induce the trap formation, inhibiting NETosis, while the planktonic forms of all 4 studied isolates induce the formation of NETs. The data obtained allowed the authors to suggest that the hyphal architecture of biofilms is not critical for inhibiting the release of NETs and even biofilms consisting mainly of yeast morphotypes retain the ability to disrupt neutrophil functions, which confirms the greater significance of other biofilm-specific components, such as extracellular matrix, in the inhibition of trap formation [79]. While the previous study revealed the inhibition of ROS production in neutrophils by biofilm of the SC5314 strain [78], the current study showed that this suppression is strain-dependent [79]. Thus, the biofilm formed by strain 98-210, unlike other isolates, caused the formation of ROS in neutrophils. At the same time, the retention of the NET-suppressing activity of the biofilm of this *C. albicans* strain indicates a possible divergence in the biofilm-induced pathways of inhibition of the ROS and NET formation. Further investigation of these complex inhibitory pathways requires additional studies [79].

In 2018, the same group of scientists found that pre-treatment of the *C. albicans* biofilms with drugs from the echinocandin group (anidulafungin, caspofungin, micafungin) promotes the formation of NETs [80], which is likely to be a manifestation of the synergistic action of neutrophils and drugs of this class in the fight against candidiasis [80, 84]. Echinocandins disrupt the integrity of the cell wall of fungal pathogens, causing unmasking of β-glucan, a proinflammatory polysaccharide, which can serve as a trigger for the neutrophil trap formation [80, 85].

In 2019, Polish scientists published the results on the study of the effect of 3 autoregulatory QS molecules of *C. albicans* (farnesol, farnesilic acid, and tyrosol) on the neutrophil trap formation [24]. It is of interest that, on the one hand, farnesol, produced by fungi in response to an increase in cell density, prevents biofilm formation and blocks the transition from blastospores to hyphae. [86]. On the other hand, the blastospores, as particles of smaller sizes than hyphae, induce NETosis in a lesser degree [81]. Perhaps, fungi use farnesol in this way, inhibiting their filamentation and progression of the infection as a way to avoid the attention of neutrophis and survive in the neutrophil-infiltrated environment [24]. However, the Polish scientists were the first to establish that farnesol, but not farnesilic acid or tyrosol, activates the ROS-dependent pathway of neutrophil NETosis and enhances their chemotaxis through CD11b/CD18 and TLR2 receptors. Thus, neutrophils still "hear" the QS language of fungi and this contributes to the defense of the organism against *C. albicans* [24].

Besides *C. albicans*, the relationship between the biofilm forms of *C. glabrata* (one of the most common pathogens of non-*albicans* candidiasis) and NETs was investigated [7]. *C. glabrata* forms only relatively small (1–4 μm) yeast forms, in contrast to *C. albicans* that forms larger (4–7 μm) yeast morphotypes, as well as filamentous forms (pseudohyphae and hyphae). After a 4-hour co-cultivation of neutrophils with 24-hour biofilms of *C. glabrata* from ovoid yeast cells, scanning electron microscopy allowed visualizing of mesh structures emanating from granulocytes, which indicated the formation of NETs, however, the intensity and temporal dynamics of the NET formation were significantly lower than those in response to the planktonic forms of *C. glabrata*. Based on these data, the authors concluded that the delayed and impaired NET release is a potential mechanism for the evasion of *C. glabrata* biofilms from the innate immunity [7].

The use of diphenyleneiodonium, a pharmacological inhibitor of NADPH oxidase [32], did not affect the NETosis induced by the biofilm forms of *C. glabrata*, suggesting the involvement of an alternative ROS-independent pathway to the NET release. At the same time, the authors found that both biofilm and planktonic forms of *C. glabrata* induce the release of NETs through a phagocytosis-dependent pathway that differs from the PMA induction mechanism. This process involves the phagocytosis of yeast cells followed by the extrusion of DNA with citrullinated histones and death of the neutrophil. While the hyphal forms of *C. albicans* are a more potent trigger for NET release than yeast ones [81, 87], the induction of the NETosis by the yeast morphotypes of *C. glabrata* indicates differences in the neutrophilic response and emphasizes the importance of individual study of each host-to-pathogen interaction. [7]. Differences in the NET formation in response to *C. albicans* and *C. glabrata* biofilms may be based on differences in the structures of biofilm architecture and/or extracellular matrix. Thus, despite the fact that *C. glabrata* biofilms, unlike *C. albicans* ones, "allow" NETs to be released, although in a lesser degree than the planktonic forms, the inhibiting NET-modifying activity and impairment of neutrophil functions are a common feature of biofilms of different types of *Candida* that serves to avoid the neutrophilic attack [7].

Conclusion

The influence of biofilm microorganisms on the function of neutrophils, in particular on the NET formation, is ambiguous; sometimes, it goes in different directions and depends on a number of factors, including both the characteristics of the pathogen itself and the conditions of experimental studies *in vitro* and *in vivo*.

However, there is no doubt that microbial biofilms, being the target of neutrophils, try not only to "disarm" the enemy, but also to use its weapon to achieve their own goals. Further detailed study of the relationship between microbes in biofilms and NETs will help both expand our understanding of the persistence mechanisms of pathogens of the biofilm-forming infections and, perhaps, develop new approaches to their treatment.

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