Neutrophils in respiratory syncytial virus disease
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Citation for published version (APA):
Cortjens, B. (2017). Neutrophils in respiratory syncytial virus disease: Untangling the NET

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Chapter 5

Neutrophil Extracellular Traps in Respiratory Disease:
guided anti-microbial traps or toxic webs?

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Paediatric Respiratory Reviews, 2017 January; 21: 54–61
Abstract

Neutrophil recruitment to the airways and lungs is a major hallmark of many respiratory diseases. One of the more recently discovered unique innate immune effector mechanism of neutrophils is the formation of neutrophil extracellular traps (NETs), consisting of an extracellular network of DNA fibers studded with nuclear and granule proteins. Although in the respiratory system NETs contribute to capture and inactivation of bacteria, fungi and viruses, there is a delicate ‘balance’ between aid and damage to the host. Accumulating evidence now suggests that NETs can have direct cytotoxic effects to lung epithelial and endothelial cells and can contribute to airway obstruction. As such, NETs may play an important role in the pathogenesis of respiratory diseases. The purpose of this review is to give an up-to-date overview of the current status of NETs in respiratory diseases. We examine both experimental and clinical data concerning the role of NETs in host defence as well as immunopathology, with special interest to the literature relevant for the paediatric pulmonology community. Finally, we discuss future treatment strategies that may target the formation of NETs in the airways and lungs.

Educational Aims:

The reader will be able:

- To define the process of NETosis and formation of NETs.
- To discuss the potential protective effects of NETs during respiratory diseases.
- To discuss the potential immunopathological effects of NETs during respiratory diseases.
- To discuss potential future treatment options targeting NETs in the airways and lungs.

Future Research Directions:

- Determine the role of NETs in various respiratory diseases in vivo.
- Determine potential age-related differences in NETosis and the role NETs play during pediatric respiratory disease.
- Establish the benefit of anti-NET treatments in (pediatric) respiratory disease.
Introduction

The respiratory system, representing a primary entry site of many distinct microbes, is particularly dependent on strong innate immune surveillance. Neutrophil recruitment to the airways and lungs is a major hallmark of many respiratory diseases, ranging from pulmonary infections to asthma and acute respiratory distress syndrome (ARDS). In the classical paradigm neutrophils display their effector functions in the host defence against bacteria and fungi, however strong recruitment and activation of neutrophils is also seen in viral respiratory infections. Chronic neutrophil-dominant airway infiltrates are seen in paediatric cystic fibrosis (CF) and in severe asthmatic patients, where neutrophils contribute to airway remodelling and mucus hypersecretion.

Neutrophils have three main effector mechanisms for direct anti-microbial activity, although in the last decade new modulatory and effector functions (e.g. immune suppression, tumor suppression) have been discovered. First, neutrophils are known for their phagocytizing capacity, involving the engulfment and killing of opsonized extracellular pathogens with aid of toxic granule proteins and production of reactive oxygen species (ROS). Second, neutrophils can act against extracellular microbes by secreting toxic proteins and enzymes, including myeloperoxidase (MPO), elastase and defensins, from their granules by the process of degranulation. In addition, ROS and a number of pro-inflammatory cytokines are released to the extracellular microenvironment aiding, in the innate immune response.

A third, unique killing mechanism, discovered more recently in addition to phagosome- and granule-mediated killing, involves the formation of neutrophil extracellular traps (NETs). These traps are produced by neutrophils as a last resort suicide mechanism, ensuring effective pathogen killing even after its death. As will be described in more detail below, NETs are large extracellular network-like structures consisting of DNA studded with several granule proteins (e.g. MPO, elastase) and nuclear proteins (e.g. histones). The genomic DNA-strands form the backbone of the NET, organized by the release of modified histones. NETs can expand up to 15 times the size of the originating cell, which tremendously increases the range for effective capture of various large, but also small sized pathogens with subsequent killing or neutralization by the toxic proteins coated on the NETs. NETs were discovered in 2004 by Brinkmann and colleagues who stimulated isolated neutrophils in vitro with potent neutrophil activators (Phorbol 12-myristate 13-acetate (PMA), LPS and CXCL-8/IL-8) to produce NETs and observed bacterial killing by these structures. Currently, the cellular events leading to the formation of NETs are being defined, as well as their precise functions and clinical implications. Given the unique neutrophil biology and their high numbers under both physiologic and pathophysiologic conditions in the lungs, research on NETs will have an impact on the current knowledge regarding the
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Pathophysiology and treatment of many respiratory diseases. In this review we therefore aim to provide an overview of the current literature regarding NETs and their role in respiratory diseases, with special attention to studies relevant for respiratory diseases in children.

Figure 1: Neutrophil defences against pathogens.

Neutrophils undergo the process of NETosis, which leads to the formation of NETs under influence of various triggers (e.g. cytokines, fungi, viruses, bacteria). These NETs, consisting of extracellular DNA packed with nuclear (e.g. histones) and granule (e.g. MPO, elastase) proteins, can trap and/or neutralize pathogens. Other main effector functions of neutrophils include the classical mechanisms of degranulation with secretion of anti-microbial proteins and phagocytosis of pathogens. This latter process is enhanced through opsonization by the complement system or immunoglobulins. NETs; neutrophil extracellular traps, MPO; myeloperoxidase, PAD4; peptidylarginine deiminase 4.
NETosis

Before discussing NETs in the context of respiratory disease, we here present a brief overview of NETs biology. The cascade of events leading to NET formation is termed NETosis. NETosis can be initiated by various triggers, including direct stimulation by pathogens (bacteria, viruses or fungi) and pro-inflammatory cytokines (e.g. CXCL8/IL-8), and appears dependent on activation of Toll-like receptor (TLR) pathways. NADPH oxidase regulated ROS production is considered to be critical in the process of classical NETosis, as patients with chronic granulomatous disease (CGD), who have impaired NADPH oxidase activity, are unable to produce NETs. However, NADPH oxidase independent NETosis has also been described in response to, for example Staphylococcus aureus and Candida albicans, but these alternative pathways of NETosis are yet largely unexplored.

The production of ROS leads to disintegration of neutrophilic granule membranes with translocation of MPO and elastase to the nucleus. Elastase deposition within the nucleus triggers histone modifications (e.g. citrullination) by Protein Arginine Deiminase 4 (PAD4), which causes chromatin decondensation and nuclear membrane disintegration. Next, the decondensed DNA associates with granule proteins in the cytoplasm before the plasma membrane bursts, spilling these intracellular contents in the form of NETs into the extracellular space. Every step in this process is regulated by different enzymes and signal-transduction cascades. It is important to emphasize that NETosis is a distinct process, not comparable with regulated cell death (apoptosis) or necrosis and that it leads to a different end result: a large maze of DNA fibers networks deposited in the extracellular space which can trap and kill bacteria, fungi and viruses.

Not all neutrophils produce NETs at the same time or rate. PMA, a protein kinase C activator, is among the most potent stimulators of NETosis, which relies on activation of ERK1/2 and p38 MAPK signalling pathways. Yet after high PMA stimulation in vitro only approximately 25% of the neutrophils undergo NETosis. This could reflect an imprinted safe-guard against fulminant NETosis or point towards differential responses to triggers of NETosis in subsets of neutrophils.

The above mentioned process is a form of end stage NET formation, in which the neutrophil dies in the process. Another form of NET formation reported is vital NETosis, in which the neutrophil either uses mitochondrial DNA or its nuclear DNA to form NETs. The NETs produced during vital NETosis are transported in micro-vesicles from the nucleus, through the cytoplasm, to the plasma membrane, without jeopardizing its integrity. Vital NETosis is characterized by fast production of NETs, generally within 30 minutes, compared to hours for ‘normal’ NETosis. The neutrophil survives vital NETosis and remains functional.
as anuclear cytoplasts, still capable of phagocytosis in vitro.\textsuperscript{31} If and how long these anuclear cytoplasts remain functional in vivo is not yet known.

**NETs in (paediatric) respiratory disease**

Initially, NETs were discovered in vitro, but they were soon found in vivo in cases of appendicitis and dysentery.\textsuperscript{16} In the following years, NETs were also found in several auto-immune diseases (e.g. SLE, rheumatoid arthritis, psoriasis)\textsuperscript{35-38} and a wide variety of infectious diseases, including bacterial\textsuperscript{16,39-41}, fungal\textsuperscript{42} and viral infections.\textsuperscript{43,44} In this part we will discuss the current literature on NETs in respiratory diseases and their potential role in host defence or immunopathology. Although our main focus is on children, we have also chosen to include studies from the adult literature, as considerable overlap between respiratory diseases independent of age exists. However, it is important to keep in mind that there may be differences in the role and magnitude of response of NETs in children versus adults. For example, Yost et al. found that, in contrast to neutrophils from healthy adults, cord blood neutrophils from term and pre-term neonates fail to produce NETs upon stimulation with LPS (via TLR-4), PMA or live bacteria.\textsuperscript{45} Interestingly, this age-dependent deficiency in NETosis occurred despite having normal NAPDH oxidase levels, and was associated with defective extracellular bacterial killing in vitro. In contrast, others have found evidence of NETosis in neutrophils from neonates in vitro in response to fungal\textsuperscript{46} and LPS/bacterial challenge\textsuperscript{47}, although the formation of functional NETs in neonates may be delayed compared to adults\textsuperscript{47}. In addition, children of only a few months old are able to form NETs in the airways and lungs during viral respiratory infection to a great extent, at least suggesting quick maturation of this specific neutrophil effector mechanism after birth.\textsuperscript{41} Certainly, whether (functional) differences in the formation of NETs between children and adults exist in vivo, and whether this is related to disease outcome in, for example, pneumonia, remains to be elucidated.

**NETs in respiratory host defence**

In line with the non-specific innate immune functions of neutrophils, NETs are known to neutralize a broad range of pathogens, including many causing respiratory disease. NET formation appears an excellent defence mechanism to trap small pathogens, such as virions, and fast moving pathogens, like bacteria, as NET formation effectively increases the surface area of a single neutrophil multiple times.
Anti-bacterial function

NETs have been proven effective in immobilizing and/or killing bacteria in vitro. This also involves many bacteria which are relevant for colonization and infection of the respiratory system, including Pseudomonas aeruginosa, Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Group A streptococci (GAS) and Escherichia coli. In addition, there is evidence for the formation of NETs in the lungs during bacterial pneumonia in vivo. For example, NETs are observed in the alveoli of mice early during pneumococcal pneumonia. Even more importantly, bacterial entrapment by NETs was shown to affect the ability of bacteria to spread from the upper to the lower respiratory tract and to disseminate to the bloodstream. Likewise, Barletta et al. showed that mice with increased NETs production by a deficiency in the anti-inflammatory adenosine A<sub>2B</sub> receptor, have enhanced bacterial clearance from the lungs and increased survival upon challenge with Klebsiella pneumoniae. Importantly, in this study, the enhanced NETosis response of neutrophils was not paralleled by other functions of neutrophils such as phagocytosis or oxidative burst, making a stronger argument for the in vivo beneficial effects of NETs. Together, these studies suggest that NETs contribute to the defence against bacterial respiratory infections. Whether this is true for humans, and in particular, in paediatric disease, is not yet defined.

Several components of the NET are likely responsible for their anti-bacterial effect. For example, cathepsin G has been found to contribute to bacterial killing. In addition, histones, abundantly present on NETs, have anti-bacterial properties via destabilization of the bacterial membrane and contribute to clearance of S. aureus. Inhibition of such granule or nuclear proteins within NETs leads to reduced bacterial killing. Finally, extracellular DNA has anti-bacterial properties through contact mediated chelation of cations, thereby damaging the bacterial membrane. This mechanism was found to be involved in the lysis of P. aeruginosa.

Interestingly, bacteria have also adapted to cope with NETs. Paired clinical P. aeruginosa isolates collected during early and late CF-disease, with an intermediate period of ten years, show marked strain-adaptation with a substantial reduction in NETs-mediated killing. This decrease in the ability of neutralization by NETs does not seem to depend on transformation of the strain to a mucoid type, which are inherently more resistant to NETs. Another example of sophisticated bacterial adaptation is endonuclease (DNase 1) production by S. pneumoniae, which allows these bacteria to degrade the extracellular DNA strings, thereby escaping from entrapment by NETs. This trait, a feature of many streptococcal strains, has been shown to result in increased local spread in the airways and dissemination into the bloodstream in mice. Finally, but most interestingly, S. aureus has adapted in a different way. They produce leukotoxins, which stimulate NET production in vitro. These NETs are
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capable of capturing S. aureus, but are unable to kill the bacteria\textsuperscript{57,58}. Next, the captured S. aureus exploits the NET by converting the DNA backbone to deoxyadenosine, which in turn can induce caspase-3 dependent apoptosis of macrophages. In this way the bacteria prevent macrophages from entering infective areas (e.g. abscesses).\textsuperscript{59} Both enzymes necessary for this conversion (nuclease and adenosine synthase) are secreted by S. aureus.\textsuperscript{59} This demonstrates an unique ability of S. aureus to adapt to the host and develop advanced evasive strategies to survive.

Anti-fungal function

In contrast to the knowledge of antibacterial functions of NETs, much less is known about its function during fungal infections. What is known is that, in vitro, NETs are produced upon stimulation of neutrophils with Candida albicans and Aspergillus fumigatus.\textsuperscript{60,61} In addition, Urban et al. have reported the formation of NETs in the lungs of mice after intranasal challenge with Candida albicans.\textsuperscript{42} Interestingly, calprotectin, one of the granule proteins on the NETs released during NETosis, appears essential in the anti-fungal activity of NETs.\textsuperscript{42,60} Further in vivo evidence that NETs may contribute to the defence against fungi comes from experiments using NAPDH-oxidase deficient mice, which serve as a model for CGD. These mice lack the ability to form NETs in the lungs in response to A. fumigatus pneumonia and show progressive respiratory disease.\textsuperscript{62}

Anti-viral function

While bacteria and fungi can be caught in NETs by size-sequestration, small virions could theoretically float through the mazes of the NET. Yet, viral capture by NETs has been shown to reduce the infectivity of target host cells in vitro, suggesting that NETs can contribute to limiting the spread of viral diseases.\textsuperscript{18,43} Viral binding to NETs is likely being influenced by the positive electrical charge of histones, one of the major proteins (70\%) present on NETs\textsuperscript{63}, causing attraction of negative loaded virions. Respiratory syncytial virus (RSV), a typical respiratory pathogen in infants and young children, induces TLR-4 dependent NETosis\textsuperscript{19}, and entrapment of RSV inside NETs results in less viral attachment and infection in a cultured lung epithelial cell line.\textsuperscript{43}

In addition to simple capture of virions, NETs have also been shown to possess direct anti-viral activity. Virus particles recovered from NETs after entrapment show reduced infectivity.\textsuperscript{18} Such direct anti-viral functions of NETs are probably mediated by exposure to the various granule proteins incorporated in the NET network, such as MPO and defensins. MPO has a direct anti-viral effect against viruses like HIV-1 and cytomegalovirus.\textsuperscript{64,65} Defensins have multiple modes of action which target respiratory viruses like influenza A and RSV.\textsuperscript{66} By exposing these proteins, the NET can function as scavengers against viruses. Interestingly, in
a study by Narayana et al. it is argued, although the evidence presented is limited, that NETs produced in response to influenza virus are less effective in bacterial killing in vitro, suggesting that the structure and protein content of NETs is pathogen specific. If indeed this is the case, this would be relevant for secondary bacterial infections during viral respiratory disease.

Finally, despite the convincing in vitro data, it remains to be investigated whether NETs indeed are important in anti-viral immunity during the various viral diseases of the respiratory system. For example, Hemmers et al. observed comparable viral replication and lung pathology during influenza infection in PAD4<sup>-/-</sup> mice, which have defective NET formation, as compared to wild type mice. This study does not support a major beneficial role in host defence for NETs in vivo, but clearly needs to be extended to other respiratory virus infections.

**NETs in respiratory immunopathology**

Despite the potential beneficial anti-pathogenic function of NETs in respiratory host defence as described above, protection may easily shift towards harm when widespread, out-of-balance, recruitment and activation of neutrophils occurs. This ‘double edged sword’ paradigm concerning the effector functions of neutrophils has been proposed for many diseases, and appears also true for NETosis. Below, we describe two main mechanisms by which NETs may cause adverse effects during respiratory disease (Fig. 2).

**Lung injury**

NETs are studded with extremely toxic proteins that are capable of killing microbes, but may also induce tissue injury. As such, collateral injury to the airways and lungs by NETs may play a role in respiratory diseases, in particular those with prominent neutrophil recruitment, such as ARDS. Indeed, Saffarzadeh et al. showed that the formation of NETs induces cell death of lung epithelial and endothelial cell lines in vitro, suggesting direct toxic effects by NETs to the alveolar-capillary barrier. The injury by NETs observed in this study was mediated by histones and MPO, but not elastase, as shown with the use of blocking antibodies.

In vivo, NETs are observed in the lungs in several settings of acute lung injury. For example, in a mouse model for influenza virus-induced ARDS, NETs are found in the alveoli entangled with the epithelium and within capillary blood vessels. Similarly, extensive formation of NETs occur within the lung microvasculature in animal models of ventilator-induced lung injury and transfusion-related acute lung injury. Intriguingly, this intravascular production of NETs by neutrophils in the lungs may be stimulated by activated
Figure 2: Potential immunopathological roles of NETs during respiratory disease.

NETs produced by neutrophils lead to direct lung epithelial and endothelial cell damage, mediated by toxic proteins (e.g. histones, MPO). Intra-luminal NETs deposition combined with neutrophil induced mucus production lead to airway obstruction. NETs; neutrophil extracellular traps, MPO; myeloperoxidase.

platelets\textsuperscript{30,74,75} and, in turn, histones on NETs may activate platelets\textsuperscript{76}, linking coagulation and lung microthrombi development to NETosis. Importantly, in several of the described experimental models a beneficial effect in terms of decreased lung permeability, improved gas-exchange or even mortality was found when exploiting strategies that target NETs, such as histone-blocking antibodies or inhaled DNase therapy.\textsuperscript{71-73} These studies suggest that NETs, at least under certain conditions, are capable of inducing direct lung injury by attacking both sides of the alveolar-capillary interface.

Airway obstruction

Besides direct tissue damage, NET formation can also lead to more indirect complications. In the respiratory system, this is exemplified by evidence for their role in the development of airway obstruction. In a number of chronic respiratory conditions, including CF, asthma and COPD, airway obstruction by thick DNA- and protein-rich mucus plugs is a pathological hallmark. Since the discovery of NETs, many groups have re-analysed the extracellular DNA content in these mucus plugs of adult patients with these diseases and found that it is mostly...
from neutrophil origin deposited in NETs, as measured by DNA in complex with elastase and MPO.\textsuperscript{77-80} Importantly, it was found that the level of extracellular DNA/NETs inversely correlates with lung function.\textsuperscript{80} For example, the amount of NETs correlates with the degree of airflow limitation, as measured by FEV\textsubscript{1}, in COPD patients.\textsuperscript{81} In addition, studies in patients with CF have linked high serum levels of migration-inhibitory factor (MIF), which serves as a potent stimulant of NETosis in these patients, to poor lung function.\textsuperscript{48} These studies are in line with previous observations that high extracellular DNA content enhances the viscoelasticity of mucus.\textsuperscript{82} As such, the extensive formation of NETs during chronic respiratory diseases can contribute to airway obstruction.

Recently, our group has extended these findings of airway obstruction by NETs in adults to acute viral respiratory disease in children.\textsuperscript{43} Severe airway obstruction by DNA-rich mucus is also a prominent feature of acute bronchiolitis by RSV infection in infants. In bronchoalveolar lavage fluid of these patients NETs are detected.\textsuperscript{43} Although RSV particles can be trapped inside NETs, limiting their spread in vitro, we also demonstrated that histopathological examination of calves with severe bovine RSV infection shows widespread airway obstruction by luminal plugs composed of mucins, cellular debris and NETs.\textsuperscript{43} Some of these plugs contain viral antigens, suggesting viral trapping in vivo, but interestingly, the majority of the plugs do not show co-localization with virus. This suggests exaggerated ‘out of balance’ formation of NETs occurs in response to RSV infection, which may lead to occlusion of the airways.

**Future perspective on treatment of NETs**

From the above, it is apparent that NETs are involved in numerous important respiratory diseases. Unfortunately, the current literature specifically addressing NETs in paediatric respiratory disease is very limited. Given the aforementioned potential age-dependent differences in the NETosis response of neutrophils,\textsuperscript{45-47} this clearly frustrates our current view on future pharmacological treatments in children. In addition, a major question remains whether the formation of NETs should be stimulated in the defence against pathogens or should be a target of inhibitory intervention to reduce immunopathology. Finally, there are still gaps in our knowledge regarding the exact mechanisms that induce NETosis, which limits the current number of molecular targets for therapy. Nevertheless, the research field of NETs is moving very quickly and an increasing number of studies are focusing on NETosis modulating strategies. Below, we provide a perspective on potential future therapies addressing the formation of NETs in (paediatric) respiratory diseases.
First, we should further elucidate the exact functions of NETs during respiratory diseases in vivo: under what conditions are they effective guided anti-microbial traps, and when do they become toxic webs? Although many studies show capture or even neutralization of different pathogens by NETs in vitro, the in vivo evidence that NETosis is a major beneficial mechanism in respiratory host defence is more limited. In contrast, there are a number of studies, including in humans, clearly linking NETs to collateral lung injury and airway obstruction. Potentially, the early controlled formation of NETs during infectious respiratory disease to combat the invading pathogens may be followed by an overwhelming, exaggerated, response in NETosis, tipping the balance towards their (in)direct adverse effects. We have hypothesized this may occur in infants with severe RSV bronchiolitis. Likewise, experimental studies suggest that NETs contribute to lung injury during acute lethal influenza pneumonia in mice, whereas in more mild influenza disease (although by a different H1N1 substrain) NETs are not beneficial nor detrimental, as was shown using PAD4 deficient mice.

In addition to these acute respiratory diseases, there is evidence that NETs lose their initial benefit during chronic respiratory disease in time. This can be illustrated by the reduced antibacterial function of NETs against adapted mucoid P. aeruginosa strains that colonize the lungs of CF patients. At this point, the non-functional NETs may add to disease severity. Clearly, future therapeutic strategies that influence the formation of NETs in the airways and lungs therefore should explicitly pay attention to the complex dynamics of (infectious) respiratory diseases.

Second, after we have deciphered the specific role of NETs in a given respiratory disease, the question remains how the formation of NETs needs to be influenced. Both the cellular pathways leading to NETosis and the (granule) protein content of NETs are logical targets but both are incorporated in other main neutrophil effector functions, such as phagocytosis and degranulation. This is illustrated by the fact that CGD-patients, who lack the NADPH-oxidase essential for NETosis, present with severe compromised innate immunity and defective NET formation, whereas MPO-deficient humans, who also have reduced NET formation, are generally not severely immunocompromised. Some of the seemingly conflicting data from animal and human studies may arise from this problem. As such, targeting the pathways or specific proteins of NETs may have unwanted side-effects by influencing the general functioning of neutrophils.

Yet, an accumulating body of evidence shows that NETosis can indeed be successfully influenced by pharmacological strategies, which either stimulate or prevent the formation of NETs. For example, several groups demonstrated increased NETs formation in the presence of statins, despite the fact that statins inhibit oxidative burst in neutrophils. Clinical trials are currently ongoing to investigate this neutrophil-modulatory effect of statins in septic pneumonia patients. In addition, a recent study that focused on the impaired neutrophil...
function during bacterial pneumonia after bone marrow transplantation, showed a beneficial effect of restoring the ability to form NETs upon treatment with cyclooxygenase inhibitors in vitro and in vivo.\textsuperscript{86} Interestingly, this appears in contrast to earlier reports of aspirin-induced inhibition of NETosis\textsuperscript{72,87}, although these studies are not directly comparable because they used different anti-inflammatory agents as well as different respiratory disease models.

At the other end of the spectrum, several strategies that prevent the formation of NETs by inhibiting NETosis have also been exploited. These include inhibition of complement factor C5\textsuperscript{88}, blocking integrin and chemokine activation of neutrophils\textsuperscript{71}, and promisingly, selective inhibition of PAD4\textsuperscript{89}. Besides prevention of NETosis, more direct targeting of the end product, the NET, may also be successful in attenuating adverse effects of NETs. For example, Caudrillier et al. showed that mice treated with histone blocking antibodies have reduced lung edema and mortality in a model for transfusion-related acute lung injury\textsuperscript{72}. In addition, numerous groups have studied whether degrading NETs by attacking the extracellular DNA backbone with exogenous DNase is beneficial in the context of various respiratory diseases. Indeed, both systemic and local (by inhalation) administration of DNase in mice is beneficial in septic lung injury and transfusion-related acute lung injury (TRALI)-models.\textsuperscript{71-73,90} Treatment with recombinant DNase (dornase alfa) is an often used clinically in patients with CF. Interestingly, although DNase reduces atelectasis in children with RSV bronchiolitis\textsuperscript{91}, so far, this therapy hasn’t shown further clinical benefit in randomized controlled trials among patients with mild bronchiolitis.\textsuperscript{92} It is possible that DNase will be more effective in children with severe RSV disease in need of mechanical ventilation.\textsuperscript{93} These children experience more severe airway obstruction and extensive neutrophilic inflammation by a virus and ventilator ‘double hit’, and so may be better candidates for anti-NET therapies.

Although DNase treatment clearly may reduce adverse effects of NETs, it is important to realize degradation of NETs may also have unwanted consequences in the lungs. In vitro, digestion of NETs by nuclease, such as DNase or MNase (micrococcal nuclease), does not reduce their cytotoxicity.\textsuperscript{70} This suggests that the granule and nuclear proteins secreted during NETosis are harmful, independent of whether they are kept contained inside NETs or are being ‘freed’ in the extracellular space by degradation of the DNA backbone. As the extracellular release of several of these proteins, including histones\textsuperscript{94}, elastase and MPO\textsuperscript{95}, is clearly associated with adverse outcomes, DNase treatment may thereby contribute to tissue damage at more distant sites. Similarly, pathogens caught in NETs might again be released after the process of lysis of the NETs, providing renewed dissemination. In support of this, increased infection rates after DNase treatment has been described in idiopathic bronchiectasis patients.\textsuperscript{86}
Conclusion

In this review we aimed to give an up-to-date overview of the current status of NETs in respiratory diseases. Although there is a body of evidence implicating NETs in various important diseases relevant for the pulmonology community, unfortunately only a limited number of studies were found that specifically address the role of NETs in children. NETs can function as (initial) beneficiary guided anti-microbial traps, but they can also contribute to immunopathology by acting as toxic webs, obstructing and damaging the host’s airways and lungs. Indeed, accumulating evidence shows that this complex and dynamic balance between protective and adverse effects often tips towards the latter, making NETs a potential viable new target in the search for novel therapies for many respiratory diseases. The challenge remains to determine if NETs play equivalent roles in adults and children and at which point anti-NET therapy is both possible and beneficial.

Acknowledgements

We would like to thank R.J. Geerdink for his valuable help drafting the illustrations.

Statement of contribution

BC and RB designed and conducted the search. BC, JW and RB interpreted the articles. All authors were involved in writing the paper and had final approval of the submitted and published versions.
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