Abstract. – In response to a range of stimuli, neutrophils produce web-like structures known as neutrophil extracellular traps (NETs). The benefits of NETs in pathogen control are commonly offset by excessive release as part of a pro-inflammatory response, as shown in several disorders. The discovery of potential drugs that regulate NET release has helped to enhance our understanding of the role of NETs in immunological protection, inflammatory diseases, and autoimmune disorders. Emerging evidence has indicated that antimicrobials play an immunomodulatory role by influencing the levels of circulating NETs. Herein, we address NETosis in several disorders and detail the mechanisms of NET-mediated damage in infections. We also aim to evaluate recent evidence on the effects of antimicrobials on NET levels. Relevant keywords were searched in PubMed. Studies were evaluated for their relevance, and a narrative review was written accordingly. Several antibiotics, including beta-lactams and cephalosporins, alter NET formation and degradation in a protective manner, resulting in minimal host organ damage. Additionally, some studies have highlighted the immunomodulatory effects of antivirals and antifungals on NET. Further studies are needed to fully understand the clinical implications of NET-antimicrobial interactions and their underlying mechanisms.

Key Words:
Neutrophil extracellular traps, Infections, Antibiotics, Neutrophils, Immunomodulation, NET interactions.

Introduction

Neutrophils are the first immune cells to be recruited in sites of acute microbial infection, where they perform their functions by identifying, phagocytosing, and eliminating extracellular pathogens. Other neutrophil functions include the production of reactive oxygen species (ROS), chemokine release, and a recently discovered function: the production of neutrophil extracellular traps (NETs). First identified by Brinkmann et al., NETs are extracellular, web-like structures that consist of decondensed neutrophil DNA, histones, and granular proteins.

It has been established that NETs play a vital role in targeting microbes, preventing the dissemination of microbial infection, and colocalizing pathogens with highly toxic antimicrobial substances to enhance their destruction. The role of NETs, however, is not limited to microbial infections, nor is it always protective. Aberrant and massive production of NETs has been implicated in the pathogenesis of several diseases, such as SARS-CoV-2, diabetes, neurologic disorders, chronic obstructive pulmonary disease, cystic fibrosis, and several others. Indeed, the pathological production of NETs is the driving force, and often the mediator, of damage in these conditions.

Antimicrobials play a significant role in host defense against various pathogens. Several antimicrobials demonstrate immunomodulatory effects; however, the mechanisms underlying these effects are not well understood. Given the infamous tendency of NETs to cause extensive damage in other diseases, the existing literature on their possible pathological role in microbial infections and the complex interplay between antibiotics and NETs is limited. In this review, we survey the literature on the role of NETs in bacterial, fungal, and viral infections, as well as the possible interactions between antimicrobials and NETs.
Methods

The MEDLINE/PubMed database was searched for relevant keywords, including “neutrophil extracellular traps”, “NETs”, “antibiotics”, “antifungals”, and “antivirals”. The resulting search was retrieved, and articles were reviewed. We included articles based on their preclinical and clinical relevance to the topic. Then, a narrative review article was written, describing the available literature surrounding the complex interactions between NETs, infections, and antimicrobials.

Mechanism of NET Formation

Once tissue injury ensues, damage-associated molecular patterns and pathogen-associated molecular patterns bind to pattern recognition receptors (PRRs) expressed at the surfaces of host cells, attracting neutrophils and other leukocytes to the site of action. At the site of injury, neutrophils exert their effects by engulfing pathogens, releasing cytokines, producing reactive oxygen species (ROS), and generating NETs. The mechanisms of NET formation are classified according to their dependence on the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme (Nox) for NET release. In Nox-dependent pathways, neutrophil activation occurs via microbial products or phorbol 12-myristate 13-acetate (PMA), a potent chemical used to activate neutrophils in vitro. Additionally, studies have also demonstrated that nanoparticles and molecular structures, such as polyhedral oligomeric silsesquioxanes, are capable of directly inducing NET formation in a dose-dependent manner. Then, neutrophils release calcium from intracellular stores, subsequently activating protein kinase C (PKC) and phosphorylating gp-91phox/Nox2. As a result of these molecular changes, protein arginine deiminase 4 (PAD4) binds to intracellular calcium and enhances ROS production. The newly formed ROS causes cellular damage and, eventually, cell death. Membrane damage causes granular components, such as neutrophil elastases (NE) and myeloperoxidases (MPO), to directly mix with nuclear chromatin and facilitate the process of decondensation. The ‘decorated’ chromatin is then free to be released out of the cell and promotes the extracellular antimicrobial functions of NETs.

A study by Parker et al. initially suggested that NETs can be induced without the participation of Nox. In vitro administration of calcium ionophores revealed an elevated number of intracellular PAD4-calcium bindings. Ultimately, PAD4 translocates into the nucleus, leading to chromatin decondensation in a Nox-independent manner. Additionally, several studies demonstrated that Nox-independent NET formation can be further provoked via the activation of calcium-activated potassium channels of small conductance and mitochondrial ROS (mROS). mROS was originally thought to have no role in the production of ATP in neutrophils. Recently, however, it has been

Figure 1. A diagram demonstrating the various pathways of NET formation: (a) represents NETosis, which involves apoptosis of the activated neutrophils, (b) shows how NETs can be generated alternatively through mROS-mediated pathways. Therapeutic strategies targeting NETs include PAD4 inhibitors, which inhibit NET production, and enhancers of degradation through DNase. This figure was made using BioRender.
NETs, infections, and antimicrobials: a complex interplay

associated with exacerbated immune responses and the induction of systemic diseases.

**NETs Damage Tissues**

It has been previously demonstrated that NETs induce epithelial and endothelial damage in a dose-dependent manner. Histones, which account for 70% of NET proteins, and MPO seem to play significant roles as mediators of these cytotoxic effects. Saffarzadeh et al. found that inhibiting histones and MPO results in a great reduction of NET-mediated cytotoxicity. Similarly, Xu et al. demonstrated that histone-inhibiting antibodies reduce mortality in mouse models of sepsis. Histones may be a potential therapeutic target in patients in pro-inflammatory states. Other components of NETs, such as human neutrophil peptides 1-3 and LL-37, also result in endothelial and bronchial epithelial cell death by inducing DNA breaks and cytochrome c release. Large complexes also form between NET DNA threads and mucin in small bronchi, causing obstruction and facilitating further microbial growth within the airways. Additionally, MPO and NE degrade glycosaminoglycan heparin sulfate, an essential component of lung tissue, which allows for enhanced neutrophil extravasation and an exacerbated immune response. NE also decreases ciliary speed and damages endothelial actin cytoskeletons, E-cadherin, and VE-cadherin. Collectively, these mechanisms are key players in NET-mediated endothelial and epithelial damage.

NETs also induce thrombosis by enhancing endothelial damage and activating complement systems. Tumor necrosis factor-α, interleukin (IL)-4, and lipopolysaccharide (LPS) stimulate endothelial P-selectin secretion, which aggregates neutrophils and stimulates NET release. Subsequently, NETs possess antibacterial capabilities due to histones, cathespin G, NE, MPO, lactoferrin, antimicrobial peptide LL-37, pentraxin 3, gelatinase, proteinase 3, and peptidoglycan-binding proteins. Accordingly, NETs restrict or destroy bacterial development, including Shigella flexneri, Pseudomonas aeruginosa, Escherichia coli, Shigella sonnei, Salmonella enteritidis, Salmonella typhimurium, Klebsiella pneumoniae, and Staphylococcus. Whole bacteria, as well as cell surface components of gram-positive and gram-negative bacteria, are potent stimuli of NETosis. LPS, a significant component of the outer membrane of gram-negative bacteria, has been observed to stimulate NET formation. It was further demonstrated that neutrophils can distinguish between LPS from various bacterial origins and selectively release NETs. Under serum- and platelet-free conditions, neutrophils generated NETs in response to two of the seven distinct LPS structures examined, with LPS derived from P. aeruginosa (LPS-PA) proving to be particularly powerful. The ability of LPS-palmitic acid to induce NET release may be explained by the fact that P. aeruginosa is well-known for its tactics to evade phagocytosis, such as biofilm production. Likewise, LPS from E. coli (serotype O128:B12; LPS-O128) produced NETosis in similar tissue conditions. Interestingly, however, four additional E. coli LPS serotypes (serotypes O55:B5, O127:B8, O111:B4, and O26:B6) that were also examined did not trigger NET release. A result, LPS-induced NETosis is not only species-specific but also serotype-specific.

**NETs and Bacteria**

NETs were first recognized in viral infections by Narasaraju et al. in a mouse model of influenza pneumonia. Viruses induce NET formation through a variety of mechanisms. Firstly, viruses can engage different PRRs, activating antiviral mechanisms in neutrophils. Virus-infected cells also release many cytokines, such as IL-8 and type-1 interferon, which prepare neutrophils to release antiviral NETs. Additionally, the influenza A virus directly stimulates NET formation; however, the detailed mechanisms of this are not yet well understood.
In COVID-19, the virus binds to angiotensin-converting enzyme 2 and causes excessive production of cytokines (also known as a cytokine storm)\textsuperscript{45}. These cytokines further recruit inflammatory cells by activating PRRs, amplifying the inflammatory response\textsuperscript{45}. Specifically, pyrin domain-containing protein 3 (NLRP3) activates the precursors of the cytokines IL-1β and IL-18. The NLRP3-produced IL-1β then recruits and activates neutrophils, inducing the release of NETs\textsuperscript{46}.

**NETs and Fungi**

NETs also play a major role in host defense against fungal infections. *Candida albicans*, *Aspergillus fumagatus*, *Histoplasma capsulatum*, *Phialophora verrucosa*, *Paracoccidioides brasiliensis*, and *Scedosporium apiospermum* are some of the fungal species that have been described to induce NET formation\textsuperscript{47}. The mechanisms of NET formation in fungal infections largely depend on the infectious organism. Several components of fungi, such as glucuronoxylomannogalactan, dextrin-2, aspartic protease, mannan, β-glucans, and farnesol, can directly activate neutrophils and induce a cascade of NET formation in human neutrophils\textsuperscript{47}. Interestingly, *C. albicans* biofilms inhibit NET release and reduce fungal killing *in vitro* and animal models *via* reduction of ROS production\textsuperscript{48}. These mechanisms can provide a therapeutic target, as future drugs may be developed to enhance NET formation and reduce immune escape in fungal infections\textsuperscript{47}.

**Antibiotics Affect NET Release and Degradation**

One possible mechanism of antibiotics’ immunomodulatory role is their ability to influence the degree of NET formation in the host. Indeed, different classes of antibiotics, such as β-lactams and macrolides, have demonstrated this effect on NETs (Figure 2). Although studies are limited in this regard, this section highlights the available literature surrounding the effects of antibiotics on NET formation.

**β-lactams and NETs**

β-lactams are the most widely used antibiotics in the world\textsuperscript{49}. Several studies in the literature have described the role of β-lactams in NET modulation in acute and chronic settings. Bysterzycka et al\textsuperscript{50} assessed the effect of amoxicillin on NET release in neutrophils from healthy human donors. The

![Figure 2. Antibiotics are able to decrease or increase NET levels *via* modulation of various steps of the NET pathways. This figure was made using BioRender.](image-url)
authors found that amoxicillin significantly increased NET release in neutrophils stimulated by PMA. Incubation with amoxicillin also resulted in increased phagocytosis of adjacent E. coli, demonstrating the enhancing effects of amoxicillin on neutrophils. Contrarily, studies have found that amoxicillin downregulates NET production in chronic inflammatory states. For example, Moonen et al. demonstrated that periodontal therapy, amoxicillin and metronidazole, significantly reduced NET degradation in chronic periodontitis patients at 3 months, 6 months, and 9 months post-treatment.

Xie et al. also studied the immunomodulatory effects of meropenem and ceftazidime/tazobactam on NET formation, ROS generation, and NADPH activity. Meropenem slightly reduced NETosis, while ceftazidime/tazobactam significantly increased NETosis in PMA-activated human neutrophils but not in resting neutrophils. Both biotics reduced ROS production and NADPH in activated and resting neutrophils. Overall, the study demonstrated that meropenem and ceftazidime/tazobactam modulate ROS-derived NET formation via the protein kinase C (PKC)-protein kinase B (Akt)-mammalian TOR (mTOR) pathway. Contrarily, imipenem, a carbapenem similar to meropenem, has been shown to promote NET formation through a ROS-dependent mechanism in activated mouse neutrophils. The varying results may be due to the differences in the studied species; however, this theory has not been validated yet.

The effects of cefotaxime, a third-generation cephalosporin similar to ceftazidime, on NETs in human neutrophils have also been studied. Unlike ceftazidime, cefotaxime shows no effect on NET release, as discovered by Manda-Handzlik et al. Duan et al., on the other hand, demonstrated that cefotaxime inhibits NET release in activated mouse neutrophils through a ROS-dependent mechanism. The mechanisms underlying the varying effects of ceftazidime and cefotaxime on NETs remain unclear. The differences may be attributed to the combination of ceftazidime with tazobactam, an inhibitor of β-lactamase, studied by Xie et al., instead of the standalone third-generation cephalosporin studied by Manda-Handzlik et al. and Duan et al. In mouse models of sepsis, de-escalation therapy, defined as a switch from broad-spectrum imipenem to narrow-spectrum ceftriaxone, promotes NET formation in early sepsis and inhibits NET formation in the late stages of sepsis. Escalation therapy (the opposite of de-escalation therapy) results in contrasting effects compared to its counterpart.

Overall, β-lactams have mostly been found to mediate protective immunomodulatory effects.

**Macrolides and NETs**

The addition of macrolides to the standard treatment regimen of pneumonia secondary to macrolide-resistant organisms has significantly reduced the mortality of pneumonia patients, demonstrating a possible immunomodulatory role of macrolides. Several studies in the literature have elucidated the effects of macrolides on NET formation. For example, Konstantinidis et al. demonstrated that clarithromycin and azithromycin increased NET formation in healthy human neutrophils in vitro. The authors found that clarithromycin induced the formation of LL-37-bearing NETs in Helicobacter pylori-positive gastritis patients via the autophagy-dependent pathway. Furthermore, clarithromycin-induced NETs reduce the growth of Acinetobacter baumannii and the production of biofilm through the expression of LL-37. It is worth noting that PMA-induced NETs do not demonstrate any effect on the generation of A. baumannii biofilm. LL-37, the active form of human cathelicidin, plays a crucial role in the defense against bacterial and fungal infections. LL-37 may also be involved in wound healing by enhancing vascularization. Arampatzoglou et al. studied the effects of clarithromycin and LL-37-bearing NETs on antibacterial activity and wound healing in type 2 diabetes mellitus (T2DM) patients. NETs of T2DM patients lacked antibacterial activity; however, this effect was reversed by clarithromycin via the production of LL-37-expressing NETs in well-controlled T2DM neutrophils in vitro. Furthermore, the authors demonstrated that clarithromycin-induced NETs enhance wound healing in T2DM by activating and promoting the differentiation of skin fibroblasts. Overall, these results provide further insight into the immunomodulatory effects of clarithromycin and the important role of LL-37 in antimicrobial activities and promoting wound healing.

Interestingly, erythromycin and azithromycin seem to have opposing effects on NETs compared to clarithromycin. In mouse models of emphysema, erythromycin decreases NET levels and ameliorates emphysema by downregulating Th1 and Th17 cells and suppressing myeloid dendritic cells, demonstrating a possible therapeutic indication for erythromycin in emphysema patients. Similarly, azithromycin prevents ROS production and cell degranulation and significantly reduces...
NET formation in healthy human neutrophils\textsuperscript{60}. Hence, macrolides may be able to influence the release of NETs according to the host’s needs.

Other antibiotics, such as enrofloxacin and gentamicin, also influence the process of NET formation. These findings have been summarized in Table I. Studies exploring the effects of antibiotics on NETs in human subjects remain limited. Nonetheless, future preclinical research may yield promising results in employing the complex interplay of antibiotics and NETs as a therapeutic mechanism.

**Antivirals, Antifungals, and NETs**

There are few studies assessing the effects of antivirals and antifungals on NET formation and degradation. Ashar et al\textsuperscript{61} found that treatment of influenza-treated mice with a combination of oseltamivir and a CXC chemokine receptor 2 antagonist improved survival and reduced NET release. Notably, the combination of both drugs had greater effects than either drug alone. Similarly, the combination attenuated lung pathology in piglets with sublethal swine influenza\textsuperscript{61}.

Echinocandin treatment of *C. albicans* biofilms has also been shown\textsuperscript{62} to promote NET release by up to 8-fold compared to non-treated neutrophils. This finding may explain the synergistic anti-*C. albicans* effects seen between phagocytes and echinocandins\textsuperscript{63}. Further studies are needed to explore the immunomodulatory roles of antivirals and antifungals and the mechanisms underlying these roles.

**Clinical Implications**

Overall, preclinical studies have demonstrated that antibiotics influence NET levels in a protective manner, regardless of increases or decreases in such levels. De-escalation therapy results in decreased lung, liver, and intestine damage in mouse models\textsuperscript{53} of sepsis by modulating NET release in the early and late stages of sepsis. Zhu et al\textsuperscript{64} found that dexamethasone reduced neutrophil infiltration, NETs, and bacterial burden in mouse models of *P. aeruginosa keratitis*. Additionally, TobraDex (a combination of tobramycin and dexamethasone) reduced these parameters to a greater extent than dexamethasone\textsuperscript{64}. Accordingly, TobraDex-treated mice exhibited slight corneal damage, while dexamethasone-treated mice suffered severe corneal damage\textsuperscript{64}. These data indicate that the antibiotic-mediated effects on NETs influence the severity of organ damage in bacterial infections.

Clinical studies assessing the interactions between antibiotics and NETs remain limited. An international, multicohort study by Keir et al\textsuperscript{65} assessed NET levels as markers of response to antibiotics in bronchiectasis and asthmatic patients. The authors showed that bronchiectasis patients with the highest

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**Table I. A summary of the various effects of antibiotics on NETs.**

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Antibiotic</th>
<th>Sample type</th>
<th>Effect on NET activities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactams</td>
<td>Penicillins</td>
<td>Amoxicillin</td>
<td>Healthy humans, chronic periodontitis patients</td>
<td>↑ NET release, ↑ degradation</td>
<td>50, 51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ ROS</td>
<td>52</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Ceftazidime</td>
<td>Healthy humans</td>
<td></td>
<td>↑ NET formation</td>
<td>53, 54</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>Healthy humans, mice</td>
<td></td>
<td>↑ NET formation</td>
<td>53, 54</td>
</tr>
<tr>
<td>Carbapenems</td>
<td>Meropenem</td>
<td>Healthy humans</td>
<td></td>
<td>↑ NET formation</td>
<td>56, 58</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>Mice</td>
<td></td>
<td>↓ ROS</td>
<td>52</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Clarithromycin</td>
<td>Healthy humans,</td>
<td>↑ NET formation, ↓ ROS and NET formation</td>
<td>↑ NET formation</td>
<td>58, 59</td>
</tr>
<tr>
<td></td>
<td>Azithromycin</td>
<td>Healthy humans</td>
<td></td>
<td>↑ NET formation</td>
<td>58, 60, 66</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Erythromycin</td>
<td>Mice</td>
<td></td>
<td>↓ ROS and NET formation</td>
<td>59</td>
</tr>
<tr>
<td>Nitroimidazoles</td>
<td>Metronidazole</td>
<td>Chronic periodontitis patients</td>
<td>↑ NET formation</td>
<td>↑ NET formation</td>
<td>67</td>
</tr>
<tr>
<td>Phosphonic acids</td>
<td>Fosfomycin</td>
<td>Mice</td>
<td></td>
<td>↑ NET formation</td>
<td>68</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Gentamicin</td>
<td>Healthy humans</td>
<td></td>
<td>↑ NET formation</td>
<td>54</td>
</tr>
<tr>
<td>Lincomycins</td>
<td>Clindamycin</td>
<td>Healthy humans</td>
<td></td>
<td>↑ NET formation</td>
<td>50</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Methylprednisolone</td>
<td>Canines</td>
<td></td>
<td>↑ NET formation</td>
<td>69</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>Healthy humans</td>
<td></td>
<td>↑ NET release</td>
<td>60</td>
</tr>
</tbody>
</table>
NET levels had more exacerbations, a shorter time to severe exacerbations, and increased mortality. Low-dose azithromycin resulted in decreased NET sputum concentrations over 12 months in both bronchiectasis and asthma patients. Understanding the mechanisms behind antimicrobial-NET interactions may allow physicians to provide tailored therapy targeting various immune functions of the host. Future clinical studies are needed to fully assess the value of NETs as markers of prognosis and response to antibiotic treatment.

Conclusions

Modulating NETs is quickly becoming a therapeutic focus in the treatment of a variety of disease processes. Antibiotics may demonstrate a protective role in reducing organ damage via NET modulation. Clinically, NETs may provide the basis for future prognostic and therapeutic markers in patients with acute and chronic infections. Additionally, antibiotics have been noted to improve wound healing by influencing NET levels in diabetic patients.

Overall, the complex interaction between antibiotics and NETs seems to provide support tailored to the needs of the host, increasing microbial killing, improving wound healing, and reducing organ damage. Targeting NETs in a therapeutic manner may open the door for a multitude of opportunities in the treatment of viral, fungal, and bacterial infections. More research must be undertaken to examine the mode of action of these effects and their effectiveness in clinical, real-life situations.

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Authors’ Contributions


ORCID ID

Tarek Ziad Arabi: 0000-0002-6292-7683
Nader Ashraf Fawzy: 0000-0002-4096-6139
Saleha Abdul Rab: 0000-0003-4179-927X
Ghazi Alabdul Razzak: 0009-0008-7160-6524
Belal Nedal Sabbah: 0000-0001-9131-7430
Khaled Alkattan: 0000-0002-1372-9883
Imad Tleyjeh: 0000-0002-5551-0353
Ahmed Yaqinuddin: 0000-0001-7536-910X.

Conflicts of Interest

The authors declare that they have no conflict of interest to declare.

Data Availability

Data sharing is not applicable to this manuscript, as no data were generated in this study.

Informed Consent

Not applicable.

Ethics Approval

Not applicable.

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