PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa

Tirthankar Mohanty,¹ Jonathan Sjögren,¹ Fredrik Kahn,¹ Anas H. A. Abu-Humaidan,¹ Niels Fisker,² Kristian Assing,³ Matthias Mörgelin,¹ Anders A. Bengtsson,⁴ Niels Borregaard,⁵ and Ole E. Sørensen¹

¹Division of Infection Medicine, Department of Clinical Sciences Lund, Lund University, Lund, Sweden; ²Department of Pediatrics and ³Department of Clinical Immunology, Odense University Hospital, Denmark; ⁴Division of Rheumatology, Department of Clinical Sciences Lund, Lund University, Lund, Sweden; and ⁵The Granulocyte Research Laboratory, Department of Hematology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

Key Points

- Saliva induces bactericidal and DNase resistant NETs in the oral cavity via sialyl LewisX-L-selectin signaling.
- Disordered homeostasis in the oral cavity may lead to deficient saliva-mediated NETosis.

Neutrophils are essential for host defense at the oral mucosa and neutrophil defects lead to disordered oral homeostasis. We found that neutrophils from the oral mucosa harvested from morning saliva had released neutrophil extracellular traps (undergone NETosis) in vivo. The NETosis was mediated through intracellular signals elicited by binding of sialyl LewisX present on salivary mucins to L-selectin on neutrophils. This led to rapid loss of nuclear membrane and intracellular release of granule proteins with subsequent neutrophil extracellular trap (NET) release independent of elastase and reduced NAD phosphate-oxidase activation. The saliva-induced NETs were more DNase-resistant and had higher capacity to bind and kill bacteria than NETs induced by bacteria or by phorbol-myristate acetate. Furthermore, saliva/sialyl LewisX mediated signaling enhanced intracellular killing of bacteria by neutrophils. Saliva from patients with aphthous ulcers and Behçet disease prone to oral ulcers failed to induce NETosis, but for different reasons it demonstrated that disordered homeostasis in the oral cavity may result in deficient saliva-mediated NETosis. (Blood. 2015;126(18):2128-2137)

Introduction

Neutrophils are pivotal for host defense.¹,² Neutrophils are continuously recruited to the oral mucosa³ and are important for immune defense and tissue homeostasis in the oral cavity, as illustrated by neutropenia and functional neutrophil deficiencies that are associated with gingivitis, periodontitis, and ulcerations.⁴,⁵ Indeed, oral ulcers are a characteristic feature of the periods of neutrophil nadirs in cyclic neutropenia.⁶ In addition to phagocytosis and release of antimicrobial substances, neutrophils can trap and kill bacteria by extracellular extrusion of nuclear DNA as neutrophil extracellular traps (NETs).⁷ Neutrophils form NETs by 2 distinct mechanisms.⁸ One mechanism is dependent on the reduced NAD phosphate oxidase activation, elastase, and myeloperoxidase (MPO) activity and can be elicited by phorbol-myristate acetate (PMA), Candida albicans, or uric acid crystals,⁷,⁹,¹¹ whereas bacteria and bacterial products can induce NETs by another mechanism involving integrins and Toll-like receptor (TLRs).⁸,¹²,¹⁴ Apart from host defense,¹⁵ NETs have been implicated in diseases like thrombosis, autoimmunity, and gout.¹¹,¹⁶,¹⁷

To study the fate of neutrophils at a mucosal surface, we harvested neutrophils from the oral mucosa in morning saliva and found that oral neutrophils undergo saliva-induced NETosis in vivo mediated by a novel mechanism elicited by sialyl LewisX-L-selectin signaling resulting in bactericidal and DNase-resistant NETs.

Materials and methods

Detailed information about all materials, methods, and patients are found in the supplementary Materials and Methods available on the Blood web site.

NET induction and immunofluorescence microscopy

Polymorphonuclear leukocytes adhered to coverslips for 15 minutes at room temperature and 15 minutes at 37°C. Neutrophils in RPMI 1640 with 2 mg/mL human serum albumin (HSA) were used as nonstimulated controls. NETs were induced by saliva (undiluted or diluted 1:1 in protein-free saliva), 20 nM PMA, 100 mM glucose oxidase or Staphylococcus aureus (multiplicity of infection [MOI] 30). Cells were fixed, permeabilized, and probed with antibodies against neutrophil elastase¹⁶ or myeloperoxidase (DAKO A0398). Secondary antibodies with Alexa488 and Alexa594 (Lifetech) were added and samples were mounted in Pro-Long Gold anti-fade reagent with 4,6 diamidino-2-phenylindole (DAPI; Lifetech).

NET induction of LAD1 neutrophils

Leukocyte adhesion deficiency type 1 (LAD1) neutrophils were stimulated in solution, cytocentrifuged, and processed for immunohistochemistry similar to neutrophils on coverslips.

Quantification of NETs

Fixed cells were labeled for elastase and stained with DAPI. The elastase positive area in nonstimulated cells with normal polymorphonuclear morphology was

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quantiﬁed using the algorithm developed by Brinkman et al.19 By using an increase in elastase-positive area of 33% as a cutoff for NET formation we always found colocalization of elastase and DNA in cells deﬁned to have undergone NETosis. Image analysis was performed with public domain software (Fiji).

Bacterial culture

*S. aureus* (clinical isolate strain 050701) and *Streptococcus pyogenes* (API) were plated for 6 to 8 hours, and overnight cultures were started from plated bacteria. Bacteria from overnight cultures were harvested and cultured for 3 hours (3 hours day culture), washed, and used. The *S. aureus* (MOI 1:30) from either overnight culture or 3 hours day culture was used for generating NETs. Oral bacteria were harvested from morning saliva from donors and were plated on THY plates and processed similar to *S. aureus* and *S. pyogenes*.

Preparation of salival neutrophils from morning saliva

Saliva from healthy donors (ie, without any known systemic or oral disease) was collected into ice-chilled tubes early in the morning before brushing teeth. Samples were incubated with ﬁxative, diluted, cytocentrifuged, and immunostained.

Collection and preparation of saliva and protein-free saliva

Saliva from healthy donors was collected in the morning after teeth brushing and before additional intake of food. The samples were centrifuged and sterile ﬁltered. Further centrifugation through a 3 kDa ﬁlter (Millipore) yielded protein-free ﬁltrate (protein-free saliva).

Saliva samples from Behçet disease, systemic lupus erythematosus and recurrent aphthous ulcers were centrifuged and supernatant diluted 1:1 in healthy protein-free saliva was used. Saliva samples from normal donors used in parallel were processed similarly.

Deglycosylation of saliva and mucin and collection salival and mucin glycans

Saliva and mucins were incubated with or without PNGase-F to remove N-linked glycans. Retentates with deglycosylated saliva and mucins were resuspended to their original volume in salival buffer. Liberated glycans were lyophilized and resuspended to their original volume in RPMI 1640.

Inhibition experiments

There were 100 µg/mL anti-sialyl Lewis^x^-antibodies (KM93, Millipore) incubated with salival glycans before neutrophil stimulation. For L-selectin blocking, neutrophils were allowed to adhere, and 50 µg/mL L-selectin blocking antibodies (RnDSystems) were incubated with neutrophils for 15 minutes at 37°C before stimulation.

Neutrophils were coincubated with 10 µM MAPK and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) inhibitors U0126 (Tocris Biosciences) and SB202190 (Tocris Biosciences) both during adherence and stimulation with saliva.

Intracellular bacterial killing assay

*S. pyogenes* opsonized with saliva or 20% plasma were resuspended in RPMI 1640 and added to neutrophils (MOI 1:10). Gentamycin was added after 15 minutes during the neutrophil-bacteria coinoculation to eliminate extracellular bacteria. The neutrophils were washed and resuspended in RPMI 1640 with HSA (control), RPMI 1640 with HSA and 50 µg/mL sialyl Lewis^x^ or saliva. DNase (1 U/mL) was added and cells were lysed and plated for culture.

Bacterial viability assay for NET-bound bacteria

*S. aureus* (clinical isolate strain 0507101) were added (MOI 1:30) to NETs pregenerated with saliva or PMA. NETs were incubated with bacteria and live/dead staining was performed using the BacLight viability kit (Life Technologies) and then mounted in ProLong Gold Antifade Reagent with DAPI (Life Technologies).

NET degradation assay

Neutrophils in ibiTreat chamber slides (ibidi) were exposed to saliva, *S. aureus* and PMA to induce NETs, stained with SYTOX orange (Life Technologies), and photographed. Next, bacteria-free medium from an overnight culture of *S. pyogenes* (AP-1) or fresh saliva was added. Percentage of NET integrity was calculated from ratio of area of staining after addition of saliva or AP-1 supernatant compared with area of staining prior to addition of NET degrading substances.

Ethics statement

Neutrophils and saliva were collected from healthy donors and patients giving informed consent in accordance with the Declaration of Helsinki. The study was approved by the ethics committees of Lund University (2013/728) and the Capital Region of Denmark (H-1-2011-165).

Results

NETs are present in the oral cavity and induced by saliva

Numerous neutrophils present in morning saliva have undergone NETosis as evidenced by strands of extracellular DNA associated with elastase and MPO, a hallmark of NETosis (Figure 1A). The majority of neutrophils isolated from peripheral blood undergo NETosis within 1 hour incubation with saliva demonstrating that the mere exposure of neutrophils to saliva is sufﬁcient to induce NETosis (Figure 1B). See supplemental Appendix A for detailed statistical analysis of all data presented in the ﬁgures.

Saliva contains numerous microorganisms, and microorganisms are potent inducers of NETosis by a mechanism dependent on β2-integrins.8,12,14 Therefore, we examined whether NETosis induced by saliva is dependent on neutrophil β2-integrins. Neutrophils from a patient with LAD1 lacking β2-integrins formed NETs in response to saliva and PMA stimulation, but failed to form NETs in response to bacteria, demonstrating that saliva-induced NETosis is β2-integrin independent (Figure 1B; supplementary Figure 1).

Incubation of neutrophils with protein-free saliva failed to induce NETosis (Figure 1C) demonstrating that a speciﬁc component present in saliva, not merely the hypotonicity of saliva, induces NETosis.

Salivial mucins are responsible saliva-induced NETosis through sialyl Lewis^x^-selectin-mediated signaling

Raising the NaCl concentration in saliva to 140 mM abolished saliva-induced NETosis (Figure 2A). Because the structure of mucins depends on the ionic strength of the medium,20 we tested whether salival mucins induced NETosis. Salival mucins induced NETs when dissolved in buffer with similar ionic concentration as saliva (salival buffer), but not in medium with 140 mM NaCl (Figure 2A).

To investigate the role of the glycans moieties in mucins, N-linked glycans from saliva and isolated salival mucins were removed by peptide-N-glycosidase F (PNGase F) treatment. This reduced the capacity to induce NETosis, both from saliva and from puriﬁed mucins (Figure 2B). The effect of de-glycosylation was most prominent on puriﬁed mucins. A lectin blot to detect N-linked glycans (supplementary Figure 2) demonstrated only partial removal of N-linked glycans by PNGase F-treatment of saliva, probably because PNGase F works best on denatured proteins.21 Puriﬁed glycans released by PNGase F treatment of saliva and mucins induced NETosis, even in isotonic medium (Figure 2B), clearly indicating that glycans present on mucins are the NET inducing component of saliva, but that these...
glycans only induce NETosis when mucins maintain the structure imposed by the ionic conditions in saliva.

Salival mucins contain the sialic acid-containing tetrasaccharide sialyl LewisX, which is a ligand for L-selectin. Removal of sialic acid by sialidase treatment inhibited the NET-inducing activity of saliva (Figure 2C) demonstrating that the NET-inducing activity is due to a sialic acid-containing saccharide. Preincubation of salivary glycans with sialyl LewisX...
antibodies before adding to neutrophils abrogated the NET-inducing activity (Figure 2D). Purified sialyl Lewis\(^{x}\) tetrasaccharide induced NETosis, whereas glucose tetrasaccharide did not (Figure 2E), thus proving that sialyl Lewis\(^{x}\) present in salival mucins is the moiety present in saliva that induces NETosis. Preincubation of neutrophils with blocking anti L-selectin antibodies prior to incubation with saliva inhibited saliva-induced NETosis (Figure 2F), demonstrating that NETosis is induced by sialyl Lewis\(^{x}\) binding to L-selectin on neutrophils.

L-selectin is shed when neutrophils adhere to endothelial cells,\(^{24}\) but only partially.\(^{25}\) Western blot of medium from neutrophils adherent to coverslips demonstrated shedding of L-selectin before stimulation with saliva (Figure 2G). Thus, despite the adherence-dependent partial shedding of L-selectin from neutrophils, saliva-induced NETosis was still L-selectin dependent in our experimental setup.

**NADPH-oxidase and elastase are not required for saliva-induced NETosis that involves the MEK/ERK pathway**

To investigate whether saliva-induced NETosis depends on reduced NAD phosphate (NADPH)-oxidase activity, experiments were performed with neutrophils from 2 patients with chronic granulomatous disease (CGD). CGD neutrophils are incapable of mounting a respiratory burst and did not form NETs in response to PMA, a potent activator of the NADPH oxidase in normal neutrophils. When \(\text{H}_2\text{O}_2\) was provided by exogenous glucose oxidase, the CGD neutrophils formed NETs as expected (Figure 3A). CGD neutrophils formed NETs readily in response to saliva (Figure 3A), demonstrating that this L-selectin induced pathway is independent of the NADPH oxidase.

PMA and \(C. \text{ albicans}\) induce NETosis by an elastase-dependent mechanism.\(^{10}\) Consequently, we investigated whether saliva could induce NETosis in neutrophils from a patient with Papillon-Lefèvre syndrome (PLS). PLS neutrophils lack elastase and are incapable of forming NETs, both in response to PMA or glucose oxidase.\(^{18}\) In contrast, saliva induced robust NETosis in PLS neutrophils (Figure 3B).

**1-selectin-mediated adhesion and degranulation is dependent on p38 MAPK.**\(^{26}\) Preincubation of neutrophils with SB202190, an inhibitor of the MAPK pathway, and U0126, an inhibitor of the MEK/ERK pathway demonstrated that U0126, but not SB202190, inhibited saliva-induced NET formation (Figure 3C). Ten \(\mu\text{M}\) of U0126, used in these experiments, did not inhibit NETosis induced by PMA or by bacteria (supplementary Figure 3). This demonstrates that the intracellular signaling pathways in saliva-induced NETs are different from those in NETs induced by bacteria or PMA.

Due to the differences between saliva-induced NETs and other types of NETs, we examined the composition of proteins in the saliva-induced NETs in the oral cavity. NETs found in the oral cavity and NETs induced by saliva had more calgranulin and hCAP-18 bound than NETs induced by bacteria or PMA (Figure 4D).

**Saliva induces rapid loss of nuclear membrane and release of granule content intracellularly prior to NET-release**

To investigate the nuclear events in saliva-induced NET formation, immunofluorescence microscopy was performed with focus on the nuclear membrane protein SUN2. Perinuclear SUN2 staining was found in nonstimulated polymorphonuclear leukocytes, whereas the perinuclear staining was replaced by disordered nuclear staining of SUN2 and nuclear swelling as early as 15 minutes after saliva stimulation, indicating disruption of the nuclear membrane (Figure 4A). Transmission electron microscopy confirmed the loss of nuclear membrane 15 minutes after saliva stimulation (Figure 4B), whereas the
plasma membrane remained intact judged by scanning electron microscopy (Figure 4C). Confocal immuno
fluorescence microscopy demonstrated colocalization of the cytosolic protein S100A8 and DNA 15 minutes after saliva stimulation (Figure 4D), demonstrating that the loss of nuclear membrane is accompanied by access of cytosolic proteins to the DNA. To investigate the dynamics of neutrophil granules in saliva-induced NETosis, electron microscopy and confocal microscopy were performed to identify markers of azurophil granule matrix proteins (elastase and CD63, respectively) and specific granule matrix proteins and membrane proteins (hCAP-18 and CD18, respectively). Although the granules seemed intact 15 minutes after onset of saliva stimulation there was no colocalization of elastase and CD63 or of hCAP-18 and CD18 (Figure 4E-F), demonstrating intracellular release of granule matrix proteins prior to extracellular release of DNA.

Saliva NETs are more DNase resistant than NETs induced by PMA or bacteria

The finding of NETs in the oral cavity was surprising, given the well-known DNase activity present in saliva.27,28 Accordingly, we investigated the DNase resistance of saliva-induced NETs compared with NETs induced by PMA or bacteria. Although saliva-induced NETs were resistant to the DNase activity of saliva, NETs induced by PMA or by bacteria were readily degraded (Figure 5A). To investigate the susceptibility of bacterial DNases, NETs were incubated with supernatant from S. pyogenes with DNase activity. A concentration of bacterial supernatant that caused complete degradation of NETs induced by PMA or by bacteria caused only minimal degradation of saliva-induced NETs (Figure 5A). Although NETs induced by sialyl LewisX in RPMI 1640 medium instead of saliva buffer were readily degraded by both saliva and AP-1 supernatant (Figure 5B), NETs induced by sialyl LewisX in saliva buffer were DNase resistant. Immunofluorescence microscopy demonstrated increased binding of actin, a known DNase inhibitor, to the DNA of saliva-induced NETs and NETs induced by sialyl LewisX in saliva buffer compared with NETs induced by sialyl LewisX in RPMI 1640, indicating that the hypotonicity of saliva promoted binding of actin (supplementary Figure 5). Only a minor difference in DNase resistance and actin binding was seen in NETs induced by PMA in saliva buffer compared with in RPMI 1640 (Figure 5B; supplementary Figure 5), demonstrating that the ionic

![Figure 4. Intracellular event during saliva-induced NETosis. Neutrophils were left nontreated or were stimulated with saliva for 15 minutes and examined by immunofluorescence microscopy and electron microscopy. All fluorescence micrographs were acquired with a Zeiss LSM700 Axiomager M2 equipped with a 4-stack laser system (405, 488, 555, 635 nm wavelength) using Zen (Zeiss) software, with a 63× oil immersion objective. Electron micrographs were acquired using a JEOL JEM 1230 transmission electron microscope and a JEOL JSM-350 scanning electron microscope with a secondary detector. (A) Immunofluorescence microscopy shows that staining for SUN2 found in the nuclear membrane is dramatically changed after 15 minutes stimulation by saliva. Scale bars represent 10 μm. (B) Transmission electron microscopy demonstrating that the nuclear membrane is lost after stimulation by saliva. Arrow heads indicate nuclear membrane. Scale bars, 50 nm. Larger images found in supplementary Figure 5. (C) Scanning electron microscopy of neutrophils demonstrating no visible changes in the plasma membrane after 15 minutes of saliva stimulation. Scale bars, 100 μm. Larger images found in supplementary Figure 4. (D) Confocal immunofluorescence microscopy for the cytosolic protein S100A8. Scale bars, 10 μm. (E) Confocal immunofluorescence microscopy and immunoelectron microscopy the azurophilic granule protein elastase (green or 5 nm gold particles) and CD63 (red or 10 nm gold particles) present in the azurophil granule membrane. Yellow color depicts colocalization. Many ribosomes of approximately the same size as the 10 nm gold particles are found around the granules in the electron micrograph. Arrow heads indicate elastase in the granule matrix and CD63 in the granule membrane. Scale bars, 10 μm for fluorescence micrographs and 100 nm for electron micrographs. (F) Confocal immunofluorescence microscopy and immunoelectron microscopy demonstrates the specific granule protein hCAP-18 (green or 5 nm gold particles) and CD18 (red or 10 nm gold particles) present in the membrane of specific granules. Yellow color depicts colocalization. Many ribosomes of approximately the same size as the 10 nm gold particles are found around the granules in the electron micrograph. Arrow heads indicate hCAP-18 in the granule matrix and CD18 in the granule membrane. Scale bars, 10 μm for fluorescence micrographs and 100 nm for electron micrographs.](https://www.bloodjournal.org/content/126/18/2132)
composition of saliva, combined with the distinct mechanism of saliva/sialyl LewisX-induced NETosis, is responsible for the increased actin binding to DNA and DNase resistance.

**Antibacterial activity of saliva-induced NETs**

Due to the increased binding of antibacterial proteins from cytosol and specific granules (calgranulin and hCAP-18, respectively) to saliva-induced NETs, we compared the capacity of saliva-induced NETs with NETs induced by PMA and bacteria to bind and kill NET-bound *S. aureus*. Saliva-induced NETs bound a larger number of bacteria than NETs induced by PMA (Figure 5C). Live/dead staining of NET-bound bacteria revealed a significantly higher percentage of dead bacteria in saliva-induced NETs compared with both PMA or bacteria-induced NETs (Figures 5C). NETs induced by sialyl LewisX bound and killed bacteria to a similar extent as saliva-induced NETs and significantly more than PMA-induced NETs (Figure 5D). Consequently, the capacity of saliva-induced NETs to bind and kill bacteria is not due to the binding of saliva components to the NETs, but to the distinct NETosis induced by saliva, resulting in binding of more neutrophil-derived antimicrobial proteins compared with the other types of NETs. The antibacterial effects of saliva induced NETs on oral bacteria was investigated next. Similar to the experiments with *S. aureus*, live/dead staining of NET-bound...
bacteria demonstrated that saliva-induced NETs bound significantly more bacteria cultivated from saliva than PMA-induced NETs, and most of the oral bacteria bound to saliva-induced NETs were dead (Figure 5E).

*Streptococcus pyogenes* survives inside the neutrophil phagosome, probably by inhibiting fusion of azurophil granules with the phagosome. To study the fate of intracellular bacteria in neutrophils undergoing saliva-induced NETosis, neutrophils were stimulated to phagocytose *S. pyogenes* (API). Nonphagocytosed extracellular bacteria were killed with gentamycin, and neutrophils were stimulated by saliva or sialyl LewisX. This caused a 50% to 70% reduction in the number of live intracellular bacteria (Figure 5F). Experiments with oral bacteria cultured from saliva demonstrated similar decrease in intracellular survival of bacteria in neutrophils when stimulated by saliva (Figure 5G). Neutrophils with phagocytosed bacteria underwent NETosis rapidly within 20 minutes of stimulation by saliva, but killing of intracellular bacteria after stimulation by saliva could be observed prior to NET release (supplementary Figure 5).

### Ability of saliva-induced NETs to activate the complement system

PMA-induced NETs are known to activate the complement system. We found that PMA-induced NETs avidly bound complement component C3 both from normal human serum and from heat-inactivated serum (supplementary Figure 5D). However, little C3 was found bound to saliva-induced NETs and NETs from the oral cavity, demonstrating that presence of saliva-induced NETs at the oral mucosal surface does not result in complement activation (supplementary Figure 5E).

### Saliva-induced NETosis is deficient in patients with Behçet disease and recurrent aphthous ulcerations

Behçet disease is a condition of unknown etiology, but it is characterized by ulcerations of mucous membranes with oral ulcers as a defining feature. Although saliva from 5 patients with Behçet disease failed to induce NETosis (Figure 6A), saliva from 5 patients with systemic lupus erythematosus (SLE) also receiving anti-inflammatory medication induced similar NETs similar to saliva from healthy donors (Figure 6A). Most patients with Behçet disease received colchicine (supplementary Table 1 for medication taken by patients with Behçet disease and SLE). However, saliva from a colchicine-treated patient who did not have Behçet disease, induced NETosis and colchicine did not inhibit saliva-induced NETosis (supplementary Figure 6). Western blot demonstrated a similar presence of sialyl LewisX in saliva from patients with Behçet disease and healthy controls (Figure 6B). Buffer changed saliva from patients with Behçet disease-induced NETosis (Figure 6C), whereas mucins diluted in protein-free Behçet saliva failed to induce NETosis, demonstrating that substance(s) in the protein-free fraction of Behçet saliva inhibit(s) the saliva-induced NETosis (Figure 6D) through interactions with mucins.

We also tested saliva from 2 otherwise healthy persons with recurrent oral aphthous ulcers (aphthae), a disease with recurrent formation of noncontagious painful mouth ulcers of unknown etiology. NETs formed readily in response to saliva collected from aphthae-free periods, but saliva from periods with aphthae had reduced ability to form NETs corresponding to the severity of the aphthous ulcers. In one case, failure of saliva to induce NETosis preceded the occurrence of oral ulcers (Figure 7A; supplementary Figure 7A). In the persons with aphthous stomatitis the failure of saliva to induce NETs was paralleled by loss of immunoreactivity for sialyl LewisX (Figure 7B; supplementary Figure 7). Buffer change did not restore the capability of the aphthous saliva to induce NETosis and mucins diluted in protein-free aphthous saliva readily induced NETs (Figure 7C), demonstrating that the protein-free aphthous saliva did not contain substances that inhibited mucin-mediated NETosis. Instead, the deficiency of aphthous stomatitis saliva to induce NETosis was paralleled by the loss of sialyl LewisX.
Discussion

NETs can be induced either through NADPH oxidase activation and elastase activity or by bacteria/bacterial products through a combination of signals from Toll-like receptors and integrins, either TLR4 and CD11a/CD18 or TLR2 and CR3 (CD11b/CD18). We found NETs in the oral cavity with a profile of associated proteins different from these NETs. These distinct oral NETs were induced by a novel mechanism for NET formation elicited by sialyl LewisX-mediated binding of L-selectin to endothelial cells, and this promotes rolling of neutrophils and activation of β2-integrins. However, the binding of L-selectin to sialyl LewisX is of low affinity. When neutrophils were stimulated on coverslips, the lowest concentration of sialyl LewisX capable of inducing significant NETosis was 5 μg/mL corresponding to 6 μM. This indicates that the concentration of sialyl LewisX necessary to induce NETs is significantly higher than that to which neutrophil are exposed during rolling on activated endothelium.

In contrast to NADPH oxidase-dependent NETosis induced by PMA, saliva-induced NET formation occurs more rapidly and is independent of NADPH oxidase and elastase as evidenced by experiments with CGD and PLS neutrophils. In contrast to bacteria-induced NETosis, the saliva-induced NETosis is independent of integrins. NETosis induced by bacteria and saliva occurs rapidly, but with marked differences in the intracellular nuclear events. In bacteria-induced NETs, extracellular DNA is released already after 10 minutes. The bacteria-induced NETs are formed by nuclear budding with dissociation between the inner and outer nuclear membrane with vesicular NET release from the nucleus. Accordingly, intact nuclear membrane can be observed together with extracellular DNA at early time points during NET release. It is only at later stages that the nuclear membrane is disrupted. This mechanism of NETosis is termed vital NETosis and results in nuclei-free cells capable of chemotaxis. In contrast, saliva-induced NET formation involves a complete disruption of the nuclear membrane with nuclear swelling at an early stage prior to any release of extracellular DNA with no evidence of nuclear budding or vesicular DNA release.

Elastase is released from azurophil granules during PMA-induced NETosis neutrophil by a mechanism supposedly similar to the release of cathepsins from lysosomes during autophagy. However, no egress of matrix proteins from specific granules has been described. Granule proteins were intracellularly present prior to DNA release during saliva-mediated NETosis, but neither elastase nor hCAP-18 was found in granules. Normally, neutrophil granules release their content by fusion with the phagosome or the plasma membrane, but this did not seem to occur during saliva-induced NETosis because we did not observe translocation of granule membrane proteins to other organelles. To our knowledge, such intracellular release of neutrophil granule content has not been previously described. We found almost total loss of immunologic reactivity of granule membrane proteins, indicating that the intracellular release of granule contents involves degradation of granule membrane proteins in both azurophil and specific granules. We observed increased killing of intracellular bacteria in saliva-stimulated neutrophils prior to NET release, but after intracellular release of granule proteins. It is, thus, tempting to hypothesize that the intracellular release of antimicrobial granule proteins contributed to the increased intracellular bacterial killing.

Investigating the antimicrobial function of NETs by colony counts is challenging because it is impossible to exclude bacteria killed by phagocytosis and bacteria not bound to NETs from the assays. Accordingly, conflicting data have been presented regarding the antimicrobial function of NETs. We cultured bacteria under conditions to obtain ≥99% live bacteria that were then incubated with NETs and subsequently stained with dyes reflecting bacterial viability. This allowed evaluation of both number and viability of bacteria bound to individual NETs, making it possible to exclude bacteria not bound to NETs and bacteria killed by phagocytosis. This revealed that saliva-induced NETs bound a larger number of bacteria than PMA-induced NETs and that significantly more of the NET-bound bacteria were killed by saliva-induced NETs compared with NETs induced by PMA or by bacteria. Increased binding of antimicrobial proteins from cytosol and specific granules paralleled the increased bactericidal activity of saliva-induced NETs compared with other types of NETs. Importantly, NETs induced by isolated sialyl LewisX had similar capacity to bind and kill bacteria as saliva-induced NETs. Therefore, it is the distinct NETs induced by sialyl LewisX, whether isolated or as part of salival mucins and not components present in saliva.
per se that is responsible for the capacity of saliva-induced NETs to bind and kill bacteria.

Neutropenia and functional neutrophil deficiencies are associated with disordered homeostasis in the oral cavity manifested, for example, by oral ulcerations. We demonstrated deficient saliva-induced NET formation in 2 conditions of unknown etiology, but with normal neutrophil count and with oral ulcers as a defining feature, recurrent aphthous stomatitis, and Behçet disease. Indeed, saliva from patients with both conditions failed to induce NETs, but for different reasons. In recurrent aphthous stomatitis, the mucins lost the ability to induce NETs due to loss of sialyl LewisX. Possibly bacterial sialidases produced by the oral flora contribute to the loss of sialyl LewisX in salivary mucins in recurrent aphthous stomatitis. In contrast, sialyl LewisX was present on salivary mucins in Behçet disease. In Behçet disease, components in the protein-free fraction of saliva (saliva fluid) inhibited the ability of the mucins to induce NETs. Because sialyl LewisX salivary mediated neutrophil killing of bacteria from normal oral flora, it is interesting to note that the oral flora is altered in both recurrent aphthous stomatitis and Behçet disease.

Evaluation of just a few parameters: binding of complement components, DNase resistance, and the capacity to bind and kill bacteria, revealed that different mechanisms of NETosis lead to functionally distinct NETs. Although NETs have been implicated in the development of diseases such as nephritis and thrombosis, our study suggests that NETs with antimicrobial activity represent a normal fate of neutrophils in the oral cavity, important for a healthy oral mucosal surface. Future studies will determine whether mucins at other mucosal surfaces interact with neutrophils and thereby influence neutrophil fate by inducing NETosis.

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Author contributions

Contribution: T.M., J.S., M.M., and O.S. designed experiments; T.M., J.S., A.H., and M.M. performed experiments; F.K. analyzed and collected clinical material; N.F., K.A., A.B., and N.B. analyzed and provided clinical samples; T.M., N.B., and O.S. wrote the paper.

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Correspondence: Ole E. Sørensen, Division of Infection Medicine, Nuclear Biology Laboratory, Biomedical Center, B14, Department of Clinical Sciences, Lund University, Tornavägen 10, SE-221 84 Lund, Sweden; e-mail: ole.e.sorensen@med.lu.se.

References


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Correspondence: Ole E. Sørensen, Division of Infection Medicine, Nuclear Biology Laboratory, Biomedical Center, B14, Department of Clinical Sciences, Lund University, Tornavägen 10, SE-221 84 Lund, Sweden; e-mail: ole.e.sorensen@med.lu.se.

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A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa

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