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NOD1/NOD2 Upregulation in Fusobacterium Nucleatum Induced NETosis

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Abstract
Subgingival plaque contains a variety of pathogenic bacteria that infect the periodontal tissues. Polymorphonuclear neutrophils (PMNs) utilize a number of antimicrobial strategies including phagocytosis and neutrophil extracellular traps (NETs) to defend this microbial challenge. The signaling pathways and specific molecular mechanisms involved in NET formation are still unresolved. Our preliminary data demonstrate that *F. nucleatum* as a model organism for in vitro experiments in activating neutrophils and inducing NETosis via the upregulation of neutrophil's Nucleotide oligomerization domain 1 (NOD1) and NOD2 receptors. However, the pathway by which NOD1 and NOD2 affect NETosis has not been addressed. Our goal was to elucidate the role of NOD1 and NOD2 receptors in the NET formation after stimulation with *F. nucleatum*.

Materials and Methods: We utilized human neutrophils and the HL60 cell line to study NETosis. NETs were induced via PMA and *F. nucleatum*; then fluorescence was measured in 3 hours interval using Sytox Orange assay. Protein arginine deiminase 4 (PAD4), Myeloperoxidase (MPO), and Neutrophil elastase (NE) play a critical role in NETosis, and their loss leads to a deficiency in NET formation. To delineate the mechanism of NOD related NET formation we inhibited both NOD1 and NOD2 receptors in neutrophils using their specific inhibitors followed by stimulation with their specific ligands and PMA. Then we analyzed PAD4 expression using Real-time PCR and a PAD4 enzymatic activity assay. MPO and NE were detected via ELISA to detect their activity in treated neutrophils.

Results: NETosis was successfully induced with *F. nucleatum* in a time depended manner. When neutrophils were treated with NOD1 and NOD2 inhibitors followed by their ligands, we observed a significant down-regulation of PAD4 gene expression with NOD1 inhibition at transcriptional and translational levels tested by real-time PCR and PAD4 activity assay, respectively. Furthermore, supernatant ELISA of treated neutrophils showed a significant increase and decrease of MPO and NE when treated with NOD1/NOD2 ligands and inhibitors, respectively.

Conclusions: Our data illustrate an important pathway linking the NOD1 and NOD2 receptors with NETosis when stimulated with *F. nucleatum*, a key pathogen in periodontal plaque formation. Both NOD1 and NOD2 activity affect MPO and NE production in neutrophils. The downstream events following NOD1 induced NETOsis is related to PAD4 activation.

The consequence of NET formation in periodontitis remains unclear. This will be the first study to elucidate the role of NOD-like receptors in NETosis and downstream signaling network. Activating or inhibiting NET formation in the gingiva may have therapeutic potential.

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NOD1/NOD2 upregulation in *Fusobacterium nucleatum* induced NETosis

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Subgingival plaque contains a variety of pathogenic bacteria that infect the periodontal tissues. Polymorphonuclear neutrophils (PMNs) utilize a number of antimicrobial strategies including phagocytosis and neutrophil extracellular traps (NETs) to defend this microbial challenge. The signaling pathways and specific molecular mechanisms involved in NET formation are still unresolved. Our preliminary data demonstrate that *F. nucleatum* as a model organism for in vitro experiments in activating neutrophils and inducing NETosis via the upregulation of neutrophil's Nucleotide oligomerization domain 1 (NOD1) and NOD2 receptors. However, the pathway by which NOD1 and NOD2 affect NETosis has not been addressed. Our goal was to elucidate the role of NOD1 and NOD2 receptors in the NET formation after stimulation with *F. nucleatum*.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance
ATRA: All-trans-retinoic acid
BIR: Baculovirus inhibitor repeat domain
Cas: Cellular apoptosis susceptibility proteins
cDNA: Complementary DNA
CGD: Granulomatous diseased
Cit H3: Citrullinated histone H3
CRISPR: Clustered regularly interspaced short palindromic repeats
crRNA: CRISPR RNA
DMSO: Di-methylsulfoxide
DNase: Deoxyribonuclease
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GCF: Gingival crevice fluid
GPCR: G-protein-coupled receptors
sgRNA: Single guide ribonucleic acid
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP: Horseradish peroxidase
HS: Horse serum
iEDAP: γ-D-glutamyl-meso-diaminopimelic acid
IL: Interleukin
LLRs: Leucine-rich repeats
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MDP: Muramyl dipeptide
MPO: Myeloperoxidase
NACHT: NAIP, CIITA, HET-E and TP1 domain family
NADPH: Nicotinamide adenine dinucleotide phosphate
NE: Neutrophil elastase
NET: Neutrophil extracellular trap
NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs: Nod-like receptors
NOD: Nucleotide-binding oligomerization domain

PAD4: Peptidylarginine deiminase 4
PAM: Protospacer adjacent motif
PAMPs: Pathogen-associated molecular patterns
PBS: Phosphate buffered saline
PGN: Peptidoglycan
PMA: Phorbol myristate acetate
PMNL: Polymorphonuclear leukocytes
PRRs: Pattern-recognition receptors
PYD: Pyrin domain
qPCR: Quantitative polymerase chain reaction
RIPA: Radioimmunoprecipitation assay buffer
RNase: Ribonuclease
ROS: Reactive oxygen species
RT-PCR: Real time polymerase chain reaction
SD: Standard deviation
SDS: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: Scanning electron microscopy
TAMRA: Tetramethyl rhodamine
TEM: Transmission electron microscope
TLRs: Toll-like receptors
tracrRNA: Trans-activating CRISPR RNA

Chapter 1 : Introduction

Periodontitis: dynamic host-microbial interaction:

Periodontitis is an inflammatory disease that affects the tooth supporting structure, the periodontium. The Periodontium consists of alveolar bone, cementum, periodontal ligaments, and gingiva. Any disturbance to these structures will affect tooth function and eventually may lead to tooth loss (Pihlstrom et al., 2005). In addition, the non-resolving destruction caused by severe periodontitis had a drastic effect on general patient health and is considered to be a risk factor for various systemic diseases (Kim and Amar, 2006). In the United States, 46% of adults suffer from periodontitis, and of that, 8.9% of them were in advanced/severe stage (Eke et al., 2015).

Oral bacteria in the form of microbial biofilms are the initiating and propagating factor in periodontal destruction. It was estimated that dental biofilm contains more than 700-500 distinct bacterial species (Griffin et al., 2011; Moore and Moore, 1994); (Paster et al., 2001; Paster et al., 2006), and several remain uncharacterized (Kroes et al., 1999) (Paster et al., 2006). However, the host immune system plays a crucial role in the disease progression. It was found that tissue damage and destruction was mainly mediated by the host immune system, innate immune cells in particular (Taubman et al., 2005; Van Dyke and Serhan, 2003). Activation of innate immune cells such as Neutrophils will initiate a variety of host protective mechanisms such as opsonization, phagocytosis, activation of

pro-inflammatory signaling cascades, and apoptosis (Janeway and Medzhitov, 2002). The dynamic host-microbial interaction will lead to protective mechanism activation to prevent pathogen invasion. However, over-activation and excessive inflammatory mediator production will also have a destructive effect (Hajishengallis, 2015).

The role of bacteria in periodontal disease: Major periodontal pathogens:

Oral bacteria consists of a mix of aerobic and anaerobic bacterial species (Listgarten et al., 1976). Among these species, subgingival colonies were found to be strongly associated with periodontitis (Haffajee and Socransky, 1994; Holt and Ebersole, 2005). Periodontal bacterial complexes were categorized based on their colonization order and their association with periodontitis. The orange and red bacterial complexes are considered to be periodontally pathogenic (Socransky et al., 1998).

Bacteria including *Porphyromonas gingivalis (P. gingivalis)* and *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* are the major pathogens associated with periodontal lesions (Hajishengallis, 2009; Kononen and Muller, 2014). *Porphyromonas gingivalis (P. gingivalis),* a strictly anaerobic Gram-negative rod from the red complex group, is the keystone pathogen in chronic periodontitis. The pathogenicity of *P. gingivalis* depends mainly on manipulating and modifying the innate immune response (Hajishengallis, 2009; Sochalska and Potempa, 2017). Moreover, the gingipain activity of *P. gingivalis* plays a role in

biofilm-induced cytokine degradation. In fact, our laboratory *in vitro* study of constructed multispecies subgingival plaque showed that around 25% of IL-6, and IL-8 degradation occurred immediately and progressively increased upon exposure to intact biofilm. On the other hand, IL-1β was more susceptible to degradation by the biofilm culture medium components (Guggenheim et al., 2009).

A. actinomycetemcomitans, an anaerobic Gram-negative coccobacillus, is the key periodontal pathogen in aggressive periodontitis (Kononen and Muller, 2014). The production of toxins such as leukotoxin and the cytolethal distending toxins is the major virulence factor of *A. actinomycetemcomitans*. Also, the level of virulence and aggressiveness varies within different serotypes of *A. actinomycetemcomitans*, (Kononen and Muller, 2014).

F. nucleatum, an anaerobic Gram-negative rod from the orange complex group, is one of the most predominant pathogens in gingival inflammation that initiates periodontal disease (Moore and Moore, 1994). Due to its extensive co-aggregation capacity with various bacterial species including *P. gingivalis* and *A. actinomycetemcomitans*, *F. nucleatum* plays an integral role in dental plaque formation and maturation (Figure1) (Guggenheim et al., 2009; Kolenbrander et al., 2006; Zijnge et al., 2010). The *in vitro* strictly anaerobic constructed subgingival biofilm was able to adapt to the aerobic atmospheric conditions, matching the conditions used to propagate mammalian cells (Guggenheim et al., 2009). *F. nucleatum* in particular had a high ability to adapt and reduce an oxygenated

environment, thus supported the growth of various anaerobic microorganisms (Diaz et al., 2000; Guggenheim et al., 2009).

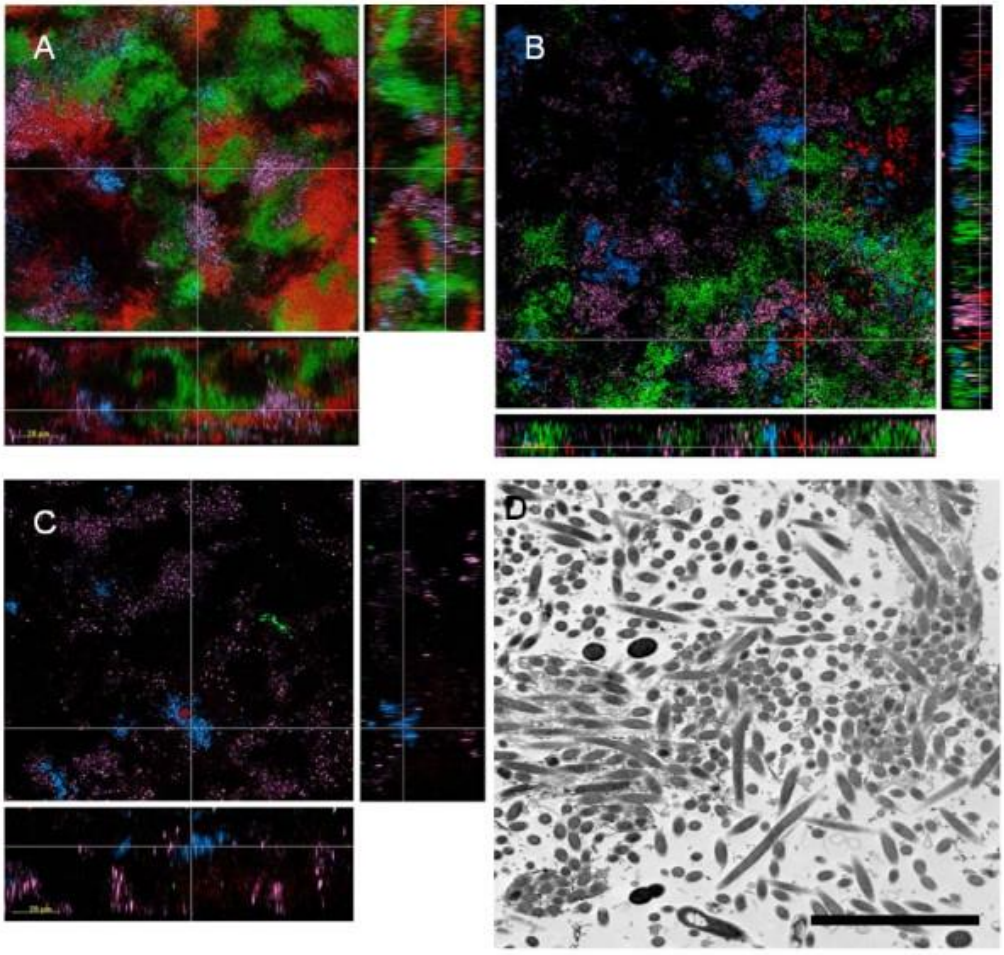


Figure 1: **Multispecies biofilm structure visualized under confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM)**: CLSM images of a 64.5 hours cultured 9-species biofilm stained by multiplex FISH for **(A)** *V. dispar* (purple), *C. rectus* (blue), *F. nucleatum* (red), and *P. intermedia* (green), **(B)** *V. dispar* (purple), *A. naeslundii* (red), *S. intermedius* (green), and *S. oralis* (blue), and **(C)** *V. dispar* (purple), *T. forsythia* (green), *P. gingivalis* (red), and *C. rectus* (blue). Images are 1-*µ*m-thick transverse (large images), sagittal (right) and coronal (bottom) slices at the positions indicated by the fine lines. The length of the bars indicates 20 *µ*m. **(D)** TEM image of a 64.5 hours cultured multispecies biofilm demonstrating the predominance of varius cocci or very short rods (*S. oralis, S. intermedius, V. dispar, P. intermedia*) and of the fusiform *F. nucleatum* cells. Bar = 5 *µ*m (Guggenheim et al., 2009).

The role of the host in periodontal disease: The innate immune response in periodontitis:

Neutrophils:

Neutrophils are the most abundant cells in the innate immune system forming about 50% to 70% of circulating leukocytes (Edwards, 2005). They are easily identified by their multilobulated nucleus, hence also called polymorphonuclear leukocytes (PMNL) (Borregaard and Cowland, 1997). Circulating neutrophils are short-lived cells with a half-life ranges from 8-12 hours (Mayadas et al., 2014). Neutrophils' rapid turnover mandates a basal rate of production to maintain their homeostasis. It has been estimated that bone marrow forms about 1011 neutrophils per day, and more during infection (Edwards, 2005; Furze and Rankin, 2008).

The innate immune system is characterized by a rapid and immediate response (Kenneth, 2011). Being the first line of host defense against most infectious pathogens, neutrophils play a critical role against extracellular bacterial and fungal infections (Borregaard and Cowland, 1997; Mansour and Levitz, 2002; Segal, 2005; Urban et al., 2006). Recent studies have suggested a novel role of neutrophils in both viral and mycobacterial infections (Ramos-Kichik et al., 2009; Saitoh et al., 2012).

Upon activation, neutrophils perform several tasks including being a pathogen killer (Mantovani et al., 2011). Through their phagocytic activity, degranulation, and toxic reactive oxygen species (ROS) production, neutrophils kill their target microbe (Robinson, 2008; Segal, 2005). ROS generation, also known as respiratory burst, cause one of the downsides of prolonged neutrophils activation which is surrounding extracellular tissue damage (Robinson, 2008). In the absence of inflammatory stimuli, neutrophils undergo programmed apoptosis (Branzk and Papayannopoulos, 2013). Also, it was suggested that ROS plays a role in neutrophil apoptosis shortly after the phagocytic process is initiated (Circu and Aw, 2010). Another interesting function that neutrophils perform is the release of neutrophil extracellular traps (NETs), a structure that traps and kills microbes (Mayadas et al., 2014).

Neutrophils role in the periodontium:

Microbes are constantly found in the gingival crevicular fluid and, as a result, a large population of recruited leukocytes also exists, with neutrophils being the most recruited leukocyte type in gingival and periodontal disease (Delima and Van Dyke, 2003; Galicia et al., 2009). Recruited neutrophils were also found in the periodontium of germ-free mice, suggesting its role in maintaining periodontal homeostasis in health (Zenobia et al., 2013). Severe periodontitis was associated with situations such as Severe congenital neutropenia, Leukocyte adhesion deficiency (LAD) I and II, Chediak–Higashi syndrome, Papillon–Lefevre syndrome

(PLS), and the related Haim–Munk syndrome (Nicu and Loos, 2016). Neutropenia patients suffer from a constant reduction in neutrophil-production, and orally manifest severe periodontitis, ulcerations, and Candida infections (Hajishengallis et al., 2016; Nussbaum and Shapira, 2011). Impairment in neutrophil adhesion and migration will lead to an increase in extravascular neutrophils, which is known as neutrophilia. Neutrophilia patients also suffer from severe periodontitis in both their primary and permanent dentition, due to low neutrophils number in the site of infection (Hajishengallis and Hajishengallis, 2014).

On the other hand, excessive activation of neutrophils has a damaging effect on the periodontium, leading to severe bone loss. ROS generation by hyper-responsive neutrophils causes one of the deleterious effects of prolonged neutrophil activation, which is surrounding extracellular tissue damage leading to severe periodontitis (Matthews et al., 2007; Robinson, 2008). Hyper-functional neutrophils were also linked to bone loss in young patients with severe localized periodontitis (Kantarci et al., 2003; Ryder, 2010). Although the link between neutrophils disorders and severe periodontitis is well established, the underlying mechanisms are still to be investigated (Nussbaum and Shapira, 2011).

Neutrophil extracellular traps (NETs):

This phenomenon was first discovered in human neutrophils by (Brinkmann et al., 2004). The term NETosis (NET-associated cell death) was first introduced by (Steinberg and Grinstein, 2007). Subsequent studies showed that other

granulocytes such as mast cells, eosinophils, and macrophages could also release extracellular traps when activated (Goldmann and Medina, 2012). Animals, insects, and plants innate immune system are also capable of extracellular nucleic acids trap formation (Altincicek et al., 2008; Palic et al., 2007; Wen et al., 2009). Robb et al. revealed that extracellular trap formation is a primordial and ancient process that occurs in several invertebrate species, including acoelomates (Robb et al., 2014).

NET is a strategy used by host cells either to prevent or control infections. The antimicrobial function of NET is provided by localizing pathogens and exposing them to antimicrobial components (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012) (Remijsen et al., 2011). To date, a variety of bacteria, fungi, parasites, and even viruses induce NET formation (Branzk and Papayannopoulos, 2013; Remijsen et al., 2011). It has been suggested that NETs could enable killing of microbes and fungi that are too large to be phagocytosed efficiently (Urban et al., 2006). Furthermore, it has been proposed that the balance between apoptosis and NETosis may be based on the strength or the presentation of the stimuli (Mayadas et al., 2014).

Moreover, the killing of the pathogen is facilitated by increasing the local concentration of antimicrobial components including enzymes, antimicrobial peptides, and histones (von Kockritz-Blickwede et al., 2008). In sepsis, more bacteria were trapped in NETs rather than phagocytized by neutrophils via TLR4-

dependent platelet-neutrophil interaction, suggesting its effectiveness in controlling the spread of infections (Clark et al., 2007).

NET structure and morphology:

NETs are made of de-condensed chromatin bounded to granular proteins. Under high-resolution scanning electron microscopy (SEM) these strand-like structures consist of fragile, smooth stretches with a diameter of 15 to 17 nm. These backbone structures are studded by globular domains that aggregate to form a large 50 nm thread. Proteins forming the NETs are derived mainly from nuclear components, and some cytoplasmic proteins (Brinkmann et al., 2004). In total, about 30 proteins or less were identified to be NET-associated, where most originated from granules and few from the nucleus. All three types of neutrophils granules were found to be involved in the NET process (Brinkmann and Zychlinsky, 2012) The formation of different types of granules in the neutrophils is dependent on their maturation stage. Hence, primary granules form first, followed by secondary and tertiary granules (Kolaczkowska and Kubes, 2013). Primary azurophilic granules include neutrophil elastase, cathepsin G, proteinase 3, neutrophil defensins, and myeloperoxidase. Specific secondary granules include lactoferrin and tertiary granules contain gelatinase (Kolaczkowska and Kubes, 2013). In addition, LL37, a cleaved form of cathelicidin, is an antimicrobial protein formed in neutrophil's peroxidase-negative granules have been identified (Neumann et al., 2014). NETs also contained multiple histones such as H1, H2A,

H2B, H3, and H4. The presence of these histones indicates that nuclear chromatin, not mitochondrial, is the major component in NETs. Azurocidin 1, lysozyme C, bactericidal permeability-increasing protein, and pentraxin three were also recognized to be NET forming proteins (Daigo and Hamakubo, 2012; Jaillon et al., 2007; Lauth et al., 2009). NET integrity is maintained by their DNA content as NET treatment with deoxyribonuclease (DNase) resulted in their degradation. On the other hand, protease treatment left the NETs intact, suggesting that proteins are not essential in NET structure maintenance and they act mainly as antimicrobials (Brinkmann et al., 2004).

NETosis is morphologically different from apoptosis and programmed necrosis. When incubated with *Staphylococcus aureus* toxins, neutrophils undergo cell death with typical necrotic morphology (Figure 2b). In necrosis, neutrophils lose their nuclear structure and start chromatin de-condensation. Also, necrotic cells cytoplasmic organelles, and both their nuclear and cytoplasmic membranes, remain intact (Fuchs et al., 2007). Neutrophils are positively stained by F-actin (a cytoskeleton stain) after undergoing necrosis, but not NETosis (Remijsen et al., 2011). In contrast to apoptosis, NETotic cells do not display “eat-me” signals on their plasma membrane surface before rupture. Apoptotic morphological signs (Figure 2a) such as cellular membrane blebbing, PS expression, nuclear chromatin condensation, and internucleosomal DNA fragmentation is not observed in NETosis (Figure 2c). Positive TUNEL stain is a hallmark of apoptotic cell death,

but not necrotic or NETotic cell death. This indicates the absence of DNA fragmentation in both necrosis and NETosis (Fuchs et al., 2007).

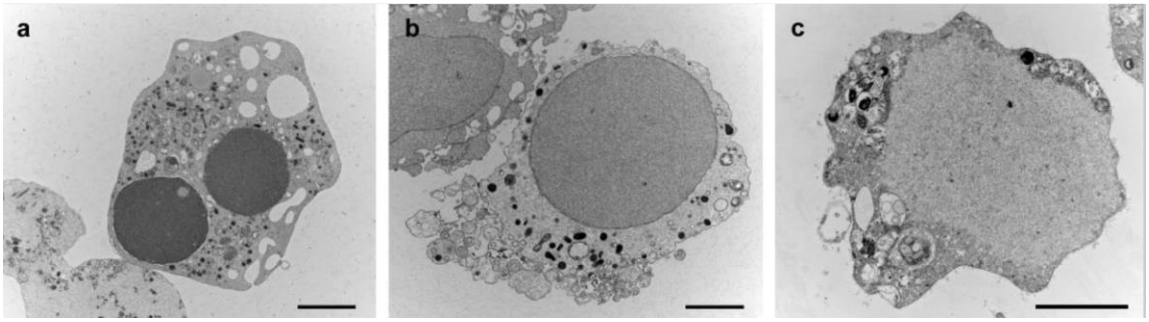


Figure 2: Morphological comparison between NETosis, apoptosis and programmed necrosis: (a) Neutrophil showing characteristic apoptotic morphology, including nuclear condensation and cytoplasmic vacuolization. (b) Neutrophils necrosis with intact nuclear envelope and granules, loss of segregation into eu- and heterochromatin and of the nuclear lobules. (c) Neutrophils undergoing NETosis (Fuchs et al., 2007).

Mechanisms of NETosis:

When stimulated with either phorbol myristate acetate (PMA), Interleukin 8 (IL-8), or lipopolysaccharids (LPS), NET formation is induced (Mayadas et al., 2014; Remijsen et al., 2011), and neutrophils undergo dramatic morphological changes at faster rates when compared to apoptosis (Fuchs et al., 2007). A few minutes after activation, neutrophils start to flatten (Figure 3a, e, and i). An hour later their nuclei lose their characteristic lobules, followed by chromatin de-condensation with an intact nuclear membrane. Concomitantly, a gap forms between the inner and

outer nuclear membrane (Figure 3b, f, and j). 2-hours post-stimulation, vesicles form in the nuclear membranes, that eventually rupture one hour later. At the same time, granular disintegration occurs in the cytoplasm. During these internal membrane disruptions, the plasma membrane of activated neutrophil is still intact. As a result, granular content will mix with leaked chromatin to form a homogenous mass (Figure 3c, g, and k). Finally, cell membrane rupture and the mix are released into the extracellular space forming a NET (Figure 3d, h, and l) (Fuchs et al., 2007). This slow (3 hours) lytic cell death was thought to be the major mechanism of NETosis, but an alternative mechanism that does not involve cell lysis was later proposed. Pilsczek et al. stated that in response to *Staphylococcus aureus*, neutrophils uniquely start a rapid (5-60 min) nuclear NET formation without cell lysis and cell membrane rupture. Post rapid NET formation neutrophils are still able to function as phagocytic cells before they die (Branzk and Papayannopoulos, 2013; Pilsczek et al., 2010).

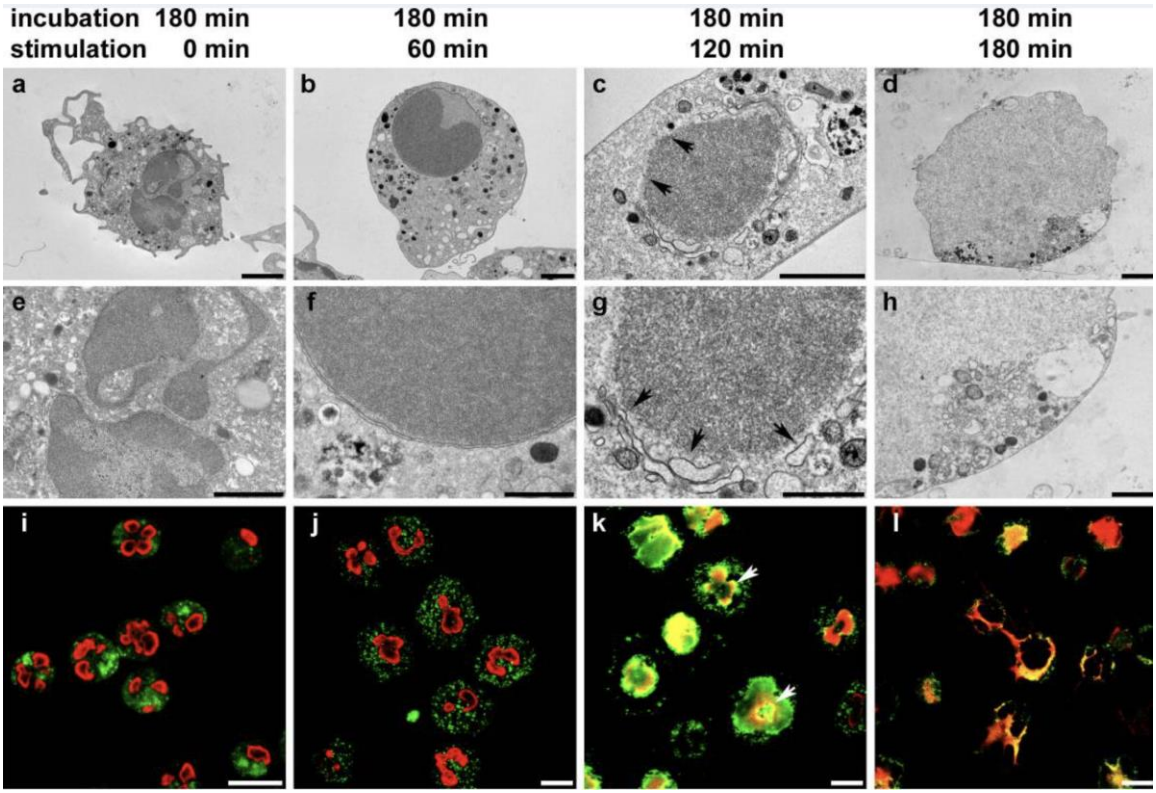


Figure 3: Morphological analysis of neutrophil activation leading to NET formation: Transmission electron microscopy (a–h) and confocal immunomicroscopy (i–l) of neutrophils. Neutrophil were incubated for 180 min without stimulation (a, e, and i) or activated with PMA for 60 min (b, f, and j), 120 min (c, g, and k), or 180 min (d, h, and l) (Fuchs et al., 2007).

It was shown that the production of ROS, followed by protease neutrophil elastase (NE), and myeloperoxidase (MPO) release from granules to the nucleus is required for NET formation. Fuchs et al. reported that ROS production via the NADPH oxidase Nox2 is essential for NET formation. It was found that neutrophils from CGD patients, were incapable of NET formation. It is known that CGD patients suffer from NADPH oxidase enzyme deficiently and hence they lack ROS production. When treated with glucose oxidase (GO) neutrophils regain their ability

of NET production (Fuchs et al., 2007). ROS production in neutrophils inactivates the caspase function directly or indirectly (through NF-kB activation), leading to the blockage of the apoptotic pathway. Moreover, PMA-induced NETosis is not affected when neutrophils are treated with pan-caspase inhibitor zVAD-fmk (Remijsen et al., 2011). On the other hand, ROS can also stimulate apoptosis via caspase activation during complement- or IgG-mediated phagocytosis (Mayadas et al., 2014). When ROS triggers NETosis versus apoptosis remains unclear since it plays a major role in both. Other investigations showed that NADPH oxidase alone is insufficient in triggering NETosis. Increasing intracellular Ca^{2+} levels induces Ca^{2+} -dependent peptidyl arginine deiminase 4 (PAD4) activity, that leads to histone H3 citrullination followed by NET formation (Leshner et al., 2012; Li et al., 2010).

MPO and NE are two of the primary enzymes found abundantly in neutrophils azurophilic granules (Papayannopoulos et al., 2010). It has been shown that neutrophils of patients with complete MPO deficiency fail to form NETs, indicating its importance in NET formation. On the other hand, neutrophils of patients with partial MPO deficiency are capable of NET formation, indicate that low levels of MPO are sufficient to activate NET formation by some cells (Metzler et al., 2011). Treating neutrophils with 4-Aminobenzoic acid hydrazide (ABAH), an MPO inhibitor, abrogates NET formation in most neutrophils, and delays its formation in remaining cells. The incomplete inhibition of MPO by ABAH explains the NET formations in some cells and support the data from MPO-partially deficient

neutrophils (Metzler et al., 2011). Chromatin de-condensation was blocked in neutrophils when treated with NE inhibitors such as GW311616A (NEi) and serum leukocyte protease inhibitor (SLPI), but not with MPO inhibitor (ABAH). Interestingly, chromatin de-condensation is promoted by H4 histone degradation in an NE-depended manner since MPO does not affect histone degradation. Moreover, it has been found that NE translocates first to the nucleus to digest nucleosomal histones, followed by MPO translocation to promote chromatin relaxation. Although, MPO has little effect on chromatin de-condensation, when combined with neutrophil elastase it dramatically enhances the de-condensation process in a dose-depended manner (Papayannopoulos et al., 2010).

Pathogenic role of NETs:

NET formation has been linked to multiple autoimmune, inflammation, coagulation disorders, and cancer metastasis (Branzk and Papayannopoulos, 2013; Brinkmann and Zychlinsky, 2012; Cools-Lartigue et al., 2013; Knight et al., 2012). It has been shown that NET formation was abundantly found in the viscous sputum of cystic fibrosis patients, and placental microdebris in pre-eclampsia patients (Gupta et al., 2007; Marcos et al., 2010). The excessive NET formation was also found to induce deep vein thrombosis by acting a scaffold for platelet aggregation, and coagulation factor activation (von Bruhl et al., 2012). Autoimmune disorders related to NETosis were suggested to be due to excessive production of pro-inflammatory molecules, and autoantigens (Knight et al., 2012).

NETosis and periodontal disease:

Subgingival plaque continuously replenish gingival crevice fluid (GCF) with multiple bacterial species, making it a rich area full of floating dispersed pathogens (Goodson, 2003). Controlling such an environment requires an efficient killing mechanism that can target diverse bacterial species and cover a wider surface area during its attack. Released NETs are found to be capable of performing such a function (Figure 4) (Krautgartner and Vitkov, 2008; Vitkov et al., 2009).

Studies show that NETs and subgingival dispersed bacteria appears in both suppurated and non-suppurated periodontitis, and forms about 78% of GCF. This suggests that subgingival bacteria are the triggers of such mechanisms, and NETosis play a protective role by preventing bacterial adhesion to the crevicular epithelium (Vitkov et al., 2009; Vitkov et al., 2005). Citrullinated histones, one of the prerequisites for the initiation of NETosis were found tremendously increased in most active crevicular neutrophils (Wang et al., 2009).

Heavy bacterial invasion of crevicular epithelium in periodontitis, induces epithelial apoptosis, and subsequently damaged tissue exfoliation to aid in periodontal clearance (Vitkov et al., 2005). In contrast, epithelial tissue in contact with NETs shows no such damage. However, this doesn't preclude its possible destructive effect. It was proposed that bacterial resistance of NETosis via the production of extracellular DNase in the periodontal pocket, is obstructed by GCF outflow. The dilution and repulsion effect of the GCF on the bacterial DNase is an

effective means for suppressing this DNase-mediated resistance to the NET formation (Vitkov et al., 2009). It is also possible that GCF has the same effect on NET proteases and antimicrobial peptides, thus preventing epithelial cell damage.

Studies showed that the prevalence of periodontitis increases with age (Eke et al., 2015; White et al., 2012), and associated with a reduction in NET formation (Hazeldine et al., 2014). However, there was no difference in NET production between aged periodontal and healthy groups (Hazeldine et al., 2014), suggesting age-related reductions in NET formation is independent of its periodontal status (White et al., 2016).

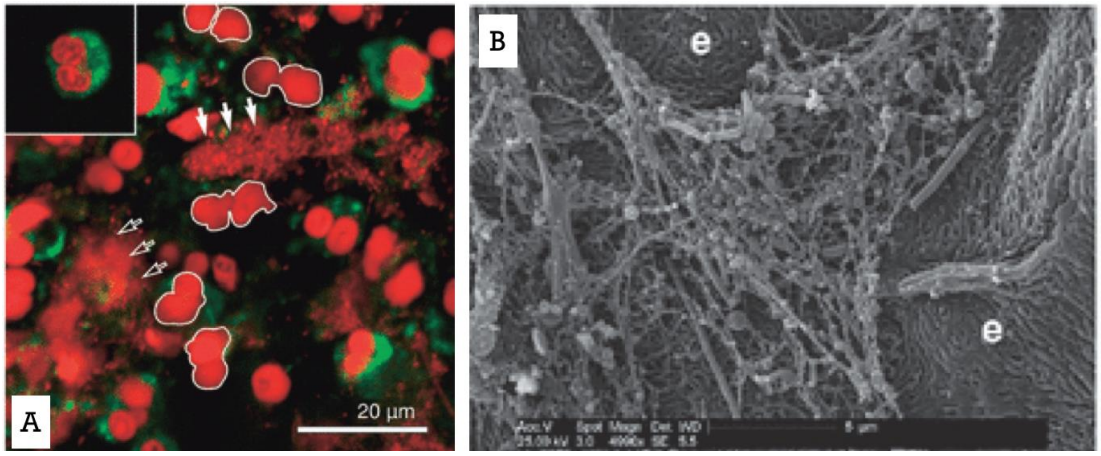


Figure 4: Extracellular neutrophil traps in periodontitis. (a) Confocal laser-scanning microscopy of crevicular exudate samples. Red, propidium iodide; green, human neutrophil elastase. (b) Scanning electron microscopy of pocket epithelium biopsies showing neutrophil extracellular trap entrapped bacteria, lies on the epithelium surface (Vitkov et al., 2009).

The entrapment and restriction function of NETs on pathogens, associated with antimicrobial components release, suggested that NETosis is a protective mechanism that controls infections (Brinkmann et al., 2004; Remijsen et al., 2011). Other studies elucidate the dark side of NETosis, and listed the diseases associated with its formation (Branzk and Papayannopoulos, 2013; Brinkmann and Zychlinsky, 2012; Cools-Lartigue et al., 2013; Gupta et al., 2007; Knight et al., 2012; Marcos et al., 2010; von Bruhl et al., 2012). Moreover, excessive activation of neutrophils has a well-known destructive effect on the periodontium via ROS or excessive release of other cytokines and antimicrobial peptides that relate to the NET formation (Matthews et al., 2007; Robinson, 2008; Ujiie et al., 2007).

Based on these data neutrophil NET formation or deficiency failed to explain its role in the pathophysiology of periodontal disease. NETosis may start as a protective mechanism preventing bacterial penetration and periodontal disease initiation. However, NETosis could be a pathogenic feature if produced excessively and not cleared efficiently.

Many types of oral bacteria are known to induce NETosis (Cooper et al., 2013; Hirschfeld et al., 2015; Palmer et al., 2016), and among the large group of bacterial species colonizing the periodontal flora, the degree of their virulence on the periodontal tissue varies tremendously (Paster et al., 2006). Moreover, the diversity of the bacterial species between individuals and their host immune response (Hajishengallis, 2015; Moore and Moore, 1994; Socransky and Haffajee,

1994) illustrates the difficulty in identifying which derive specific immune functions such as NETosis, over another. Moreover, little is known of the molecular cascade involved in the process of NET formation, concerning oral bacterial stimuli.

Innate immune recognition of oral pathogens: The role of NOD-like receptors (NLR)s:

Innate immune cell responses are non-specific and rely on the recognition of microorganisms through germline-encoded pattern-recognition receptors (PRRs). PRRs recognize the pathogen-associated molecular patterns (PAMPs), a constant microbial structure essential for their survival (Janeway and Medzhitov, 2002). The first identified PRRs were Toll-like receptors (TLRs). TLRs are one of the well-studied PRRs, due to the cardinal role in identifying a variety of PAMPs. Then other PRRs such as C-type lectin receptors, Nod-like receptors (NLRs), and RIG-I-like receptors were identified (Benakanakere and Kinane, 2012).

Nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family is one of the PRRs that sense intracellular microorganisms and trigger a series of signaling cascades to activate pro-inflammatory and antimicrobial genes culminating in the clearance of microorganisms. There are 23 and 34 NLR genes in humans and mice respectively (Franchi et al., 2009). NLRs structure composed of three main protein domains: an amino (N)-terminal domain consisting of caspase recruitment domain (CARD) or pyrin domain (PYD), or baculovirus inhibitor repeat (BIR) domain; a centrally located nucleotide-binding

oligomerization domain (NOD) or (NACHT) that is critical for self-recognition and activation; and a (C)-terminal leucine-rich repeats (LRRs) that is involve in PAMPs recognition (Correa et al., 2012; Geddes et al., 2009; Strober et al., 2006). Despite its critical function in the innate immune response, its role in the neutrophil immune response is not fully characterized.

NOD1/ NOD2 receptors:

NOD1 and NOD2 were the first receptors of the NLR family to be discovered (Figure 5). NOD1 and NOD 2 receptors are successfully upregulated using γ-D-glutamyl-meso-diaminopimelic acid (iEDAP) and muramyl dipeptide (MDP) respectively. iEDAP is found mainly in Gram-negative bacterial peptidoglycan (PGN), while MDP is found in nearly all bacterial PGN (Chamaillard et al., 2003b; Girardin et al., 2003). NOD1/NOD2 stimulation results in the activation of nuclear factor-kB(NF-kB) and mitogen-activated protein kinases (MAPKs), which lead to the production of numerous cytokines and chemokine in response to ligand stimulation (Correa et al., 2012; Geddes et al., 2009; Strober et al., 2006). NF-kB inhibition using (E)-3- [4- methylphenylsulfonyl]-2-propenenitrile (BAY 11-7082) and 6- (phenylsulfinyl) tetrazolo[1,5-b] pyridazine (Ro 106-9920), as well as Acetylsalicylic acid (ASA) results in marked reduction in NETosis in neutrophils (Lapponi et al., 2013). In addition, NOD2 was found to be capable of initiating reactive oxygen and nitrogen species production via induction of nitric oxide synthase and NADPH oxidases (Lipinski et al., 2009; Sorbara and Philpott, 2011). Furthermore, NOD1 and NOD2 receptors were found to be involved in the

recognition of periodontal pathogens, indicating its role in periodontitis (Okugawa et al., 2010). These findings illustrate the importance of NOD1 and NOD2 in periodontitis and their possible role in NETosis.

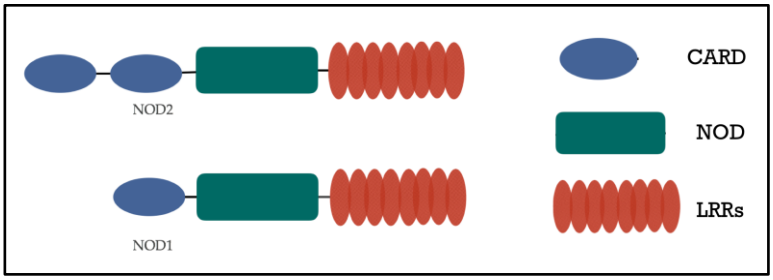


Figure 5: NOD1 and NOD 2 protein domains architecture. N-terminal caspase activation and recruitment domain (CARD) mediate the protein–protein interactions. Centrally located nucleotide-binding domain (NOD) mediate self-recognition and activation. C-terminal leucine-rich repeats (LRRs) mediate ligand recognition.

Summery and aims:

Neutrophils are the most abundant cell type found in the inflamed gingiva of periodontitis patients. It plays a crucial role in eliciting an innate immune response and eliminating microorganisms by phagocytosis and intracellular killing (Galicia et al., 2009; Moutsopoulos et al., 2014). Neutrophil's defense mechanisms involve the formation of NETs which is considered crucial in entrapping and limiting the spread of microorganisms at the site of infection (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012; Remijsen et al., 2011; Vitkov et al., 2009). Periodontal disease is known to be initiated and sustained by common periodontal pathogenic bacteria, including *F. nucleatum*, a gram-negative anaerobe that plays a role in oral biofilm maturation (Kolenbrander et al., 1989; Moore and Moore, 1994). Although many types of bacteria are known to induce NETosis, our preliminary data demonstrate that *F. nucleatum* is optimal for *in vitro* experiments in activating neutrophils and inducing NETosis via the upregulation of neutrophil's NOD1 and NOD2 receptors. NOD-like receptor's role in NETosis still remains unclear. The molecular mechanism of MPO and PAD4 in NETosis is not fully understood. PAD4, MPO, and NE play a critical role in NETosis, and their loss leads to a deficiency in NET formation (Hemmers et al., 2011; Papayannopoulos et al., 2010). However, how they are activated are still under investigation, and the role of NOD-like receptors in their activation has never been investigated. We **hypothesize** that NETosis is mediated by NOD-like receptors via PAD4 or MPO/NE upregulation.

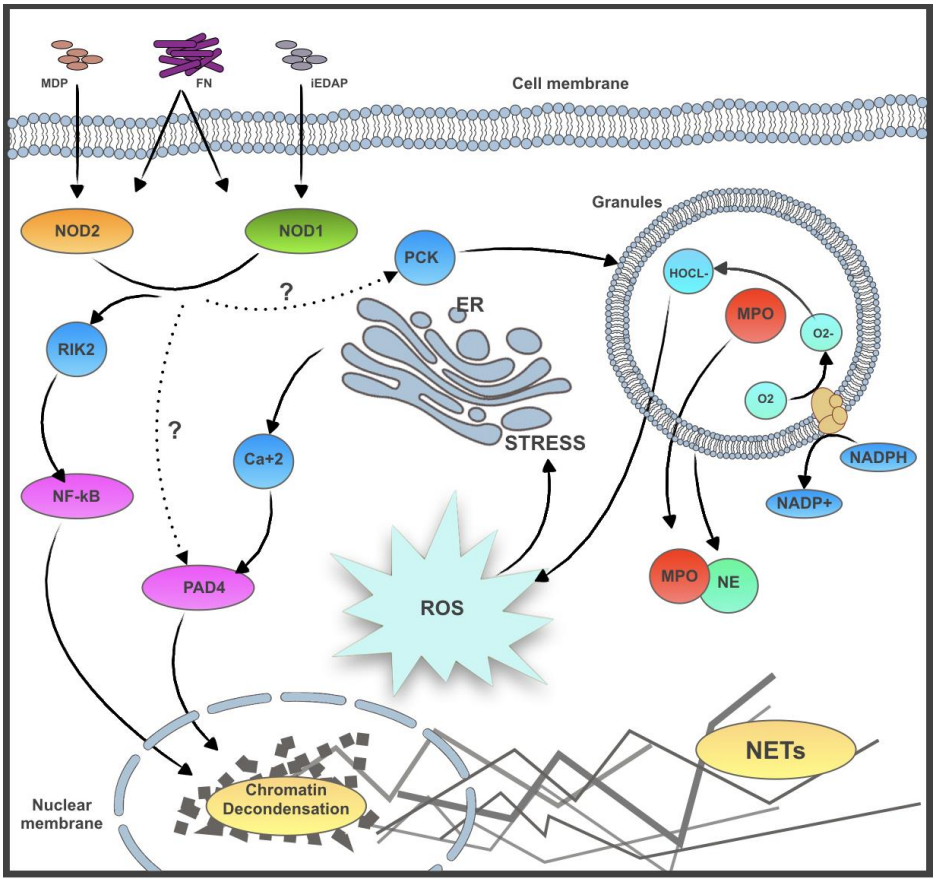


Figure 6: Model summarizing the hypothesized role of NOD1 and NOD2 receptors in the upregulation of PAD 4 and MPO leading to NET formation.

Chapter 2 : Material and Methods

Neutrophils NET production *in vitro:*

Isolation of neutrophils:

Human blood was collected from healthy donors in 10.0 mL, 18 mg K2 EDTA coated Vacutainers (BD Biosciences 366643). To facilitate the study of neutrophils response with minimal effect on its activity, Neutrophils were isolated using EasySep™ Human Neutrophil Enrichment Kit (STEMCELL Technologies, Inc), an immunomagnetic negative selection cell separation method. During isolation venous blood was diluted in RPMI 1640 Gibco™ GlutaMAX™, 25 mM HEPES, supplemented with 2% fetal bovine serum (FBS; GE Healthcare Life Sciences SH30071.03HI). Cells were counted using a hemocytometer and viability (typically >98%) was checked by mixing with trypan blue dye (Sigma, T8154). Neutrophils were re-suspended at the desired concentrations and used immediately.

Stimuli used to induce NETs:

F. nucleatum was used as periodontal pathogen models for in vitro stimulation of peripheral neutrophils NETs production. PMA (Sigma P8139) is an established stimulus for NETosis were also used as control (Table 1).

Cultured bacteria were centrifuged at 700 g for 10 min at room temperature. The supernatant was discarded, and bacterial pellet was washed with PBS then re-suspended in RPMI media. PMA was also diluted in RPMI media.

Table 1 List of stimuli used to induce NETosis.

Bacteria /Other stimulus	Strain /Supplier #	MOI** / Concentration
Fusobacterium nucleatum *	FDC 364	1:10, 1:100
Phorbol 12-myristate 13-acetate (PMA)	Sigma P8139	100 nM

* bacterial stains were purchased from American Type Culture Collection (Manassas, VA, USA).
** Multiplicity of infection.

Bacterial growth conditions:

F. nucleatum was grown in Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions (85% N_2, 10% CO_2 and 10% H_2; Coy Laboratory) at 37 °C.

Functional assay (Fluorometric quantification of NET release):

Cells were suspended in a concentration of 1 × 106 cells/ml in RPMI. Cells were seeded in 96-well microplate (BD FalconTM 353219) and allowed to rest for 20 min in a humidified incubator (37°C, 5% CO2). SYTOX® Orange (Thermo Fisher Scientific, S34861), a cell impermeable nucleic acid stain was added at a concentration of 0.2 µM / ml. Then cells were stimulated with *F. nucleatum* and PMA and incubated for 1-4 hours. Fluorescence was quantified at excitation/emission wavelengths of 540 /570 nm using Infinite M200 microplate reader (Tecan, Mannerdorf, Switzerland) at the time point of 1h, 2h, 3h.

Scanning Electron microscope (SEM):

Cells were seeded onto poly-L-lysine coated 12mm glass coverslips (Corning™ 354085) and incubated for 30 min at 37°C to allow for cell attachment. Then stimulated *F. nucleatum* and visualized by SEM at different time points to study the interaction between the bacteria and the NETs produced. After stimulation, cells were fixed in 4% formaldehyde for 20 minutes, washed once with PBS for 20 minutes and dehydrated by incubating through a graded solution of ethanol diluted in distilled water (20%, 30%, 40%, 50%, 60%, 70%, 90%, 100%, 100%) for 5 minutes each. Samples were then transferred to critical point dryer. First samples were immersed in 100% ethanol within the critical point drier chamber. Then the chamber is slowly filled with liquid CO_2 to replace ethanol. When the chamber is 100% CO_2 heat will be applied at 31°C and 1072 psi to evaporate all the liquid CO_2. The dried sample was then mounted on to Denton Desk II sputter coater for gold coating for 90 seconds. Samples were then analyzed using a JOEL JSM-T330A scanning electron microscope at the Department of Biochemistry, School of Dental Medicine, University of Pennsylvania.

Transmission electron microscopy (TEM):

Cells were fixed with 2.5% glutaraldehyde for 1h at 4°C, and then post-fixed using 1% osmium tetroxide for 1h at 4°C. Cells were dehydrated by incubating in ethanol concentration of (50%, 70%, 85%, 95%, 100%, 100%) twice for 5 minutes each, followed by propylene oxide (PO) incubation for 3 min twice. Cells were then

embedded in Epoxy embedding medium (Sigma 45359) and left to polymerization. After polymerization specimens were cut in sections, range from 500 nm - 2 microns and contrasted with lead citrate. Images were recorded using Hitachi H-7650 transmission electron microscope in a voltage range from 40 kV - 120 kV at Department of Anatomy and Cell Biology, School of Dental Medicine, University of Pennsylvania.

Immunocytochemistry:

Cells were seeded onto poly-L-lysine coated coverslips and incubated for 30 min at 37°C to be attached. Cells were challenged with *F. nucleatum* and PMA. Then cells were fixed for 10 minutes with 4% formaldehyde, permeabilized in 0.5% Triton X-100 for 2 min. Non-specific blocking was done with 10% Horse serum (HS) in PBS for 1 hour at room temperature. Then cells were incubated with primary anti-NE antibody (1:100, Abcam ab21595) and anti-Histone H3 antibody (1:400 Abcam ab5103) overnight at 4C°; washed three times with PBS; followed by Alexa Fluor® 594 or 647 conjugated secondary antibody (Thermo Fisher Scientific) incubation for 1 hour at room temperature. Finally, ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific P36935) was used to stain the nuclei and mount the slide. Images were captured by a fluorescence microscope (Leica DMi8) with the same exposure time for both the control and the experimental group.

Cell Death Quantification (ELISA):

To quantify the death rate after stimulation, first cells were challenged with *F. nucleatum* in the same conditions mentioned previously. After 12 hours of stimulation, apoptosis was measured using Cell-death enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics). Manufacturer instructions were followed.

Neutrophils Gene expression analysis:

RNA isolation:

Cells were then pelleted by centrifugation for 5 minutes at 300 g, the supernatant removed, and the cell pellet re-suspended in 1 ml TRIzol reagent per sample (Invitrogen, 15596026). The mix is transferred in 1.5 ml Eppendorf tubes and placed on a shaker and incubated for 10 min at room temperature. 200 µl of Chloroform (C2432 Sigma) were added for each sample, vortexed for 15 sec and incubated for 3 min at room temperature. This was centrifuged at 12000 g for 15 minutes at 4 °C to separate the protein, DNA and RNA layers. Around 500 µl of aqueous phase (RNA) were transfer to a new tube. One volume of Isopropyl alcohol (Sigma I-9516) were added, mixed and incubate for 10 minutes at room temperature. Then centrifuge at 12000 g for 15 minutes at 4 °C to remove the supernatant. Cell pellet was washed by re-suspension with 1 ml of 75% alcohol by vortexing. The RNA was again pelleted by centrifugation at 7500 g for 5 minutes at 4 °C, the supernatant was carefully removed, and the pellet allowed to air dry

for 10 minutes. The RNA was subsequently re-suspended in 30 µl RNase free water (preheated to 60 °C), Incubated for 2 minutes, then collected by centrifuge.RNA concentration and purity were assessed using a Nanodrop spectrophotometer (Thermo Scientific). For mRNA arrays samples were purified using QIAGEN RNeasy Mini Cleanup Kit (Thermo Scientific 74104). cDNA was synthesized from isolated RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) for gene expression analysis.

Screen gene expression using qPCR array:

At a concentration of 1x106 cells/ ml neutrophils were challenged with *F. nucleatum* (MOI 1:10) for 8 hours. Then qPCR array was used to determine if there is specific gene expression that drives *F. nucleatum* induced NETosis in neutrophils.

Quantitative TaqMan PCR-Array was custom designed to include innate immune, apoptosis and GPCR signaling pathways based on previously published microarray data by (Kinane et al., 2006). After cDNA conversion, the real-time PCR was carried out as per the manufacturer's instructions (Applied Biosystems, CA). The fold increase was calculated as compared to control sample according to ΔΔCT method (Livak and Schmittgen, 2001).

Fold increase data was used to derive heatmap with two-way hierarchical clustering using MeV v4.1 software (rows=genes, columns=samples). The color

scale indicates relative expression: yellow, above mean; blue, below mean; and black, below background.

Real-time PCR:

Real-time PCR (RT-PCR) of NOD1, NOD2 expression from *F. nucleatum, P. gingivalis, A. actinomycetemcomitans* challenged cells with and without PMA was carried out using TaqMan® Fast Advanced Master Mix (Applied Biosystems) following manufacturer instructions. The plate was loaded on to ABI 7500 Real-Time PCR machine. The negative control will be unconverted RNA and reference gene expression GAPDH to normalize the data, using relative quantification normalization method (ΔΔCt) (Livak and Schmittgen, 2001).

HL60 cells as a model for neutrophils:

The acute human promyelocytic leukemia HL60 (ATCC® CCL-240™) cell line has been used to characterize and study the expression of different antigens and functions of myeloid cells. Morphologically, undifferentiated HL- 60 cells resemble promyelocytes, but it can be differentiated in vitro to resemble mature neutrophils using All-trans-retinoic acid (ATRA), and di-methylsulfoxide (DMSO) (Bohnsack and Chang, 1994; Collins, 1987). Therefore, HL60 was used as a model for neutrophil NET formation.

HL60 cell line culture and growth condition:

HL-60 were cultured in RPMI 1640 Gibco™ GlutaMAX™, 25 mM HEPES, supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences SH30071.03HI), 1% penicillin-streptomycin Gibco™ (Life Technologies), 0.05 μg/ml Amphotericin B (Life Technologies), 50 μM β-Mercaptoethanol. Cells were incubated in a humidified incubator (37°C, 5% CO_2) and passaged every 2-3 days.

HL60 cells differentiation:

Cells were seeded at a concentration of $2x10^5$ cells/ml in culture media. 2 days post incubation cells were stimulated to differentiate into neutrophil-like cells by the addition of 1.25% DMSO (Sigma D26650) and μM ATRA (Sigma R2625). Incubated for 3 days. Then cells were washed with PBS and re-suspending at $1x10^6$ cells/ml for use in subsequent experiments.

HL60- Cas9 stable cell line:

Cells were a kind gift from Ali Zamani at University of Pennsylvania School of Medicine. Cells were cultured under the same conditions as HL60. To induce Cas9 expression 1ug /ml of doxycycline (Sigma D9891) were added to cell media for 48 hours.

NOD1 and NOD2 HL60 knock-out: CRISPR/Cas9 system

To investigate the role of NOD-like receptors in NET formation we used IDT Alt-R® CRISPR-Cas9 system to generate NOD1 and NOD2 knock-out HL-60 cell lines and induce NETosis via *F. nucleatum*. Cell-death ELISA kit (Roche Diagnostics), immunofluorescent staining then was performed to visualize and quantify the effect of NOD receptors on the NET formation.

Clustered regularly interspaced short palindromic repeats (CRISPR) are a family of short repetitions of DNA sequences present in many bacteria. CRISPR/Cas system is part of prokaryotes adaptive immune system to protect against viral attacks. It consists of two main components, a single guide RNA (sgRNA) that contain a short protospacer adjacent motif (PAM) to recognizes one strand of the target sequence, and a Cas endonuclease enzyme that induces DNA double-strand breakage. Together, a targeted cleavage in the DNA strand occurs, and nonhomologous end joining (NHEJ) or homologous recombination (HR) repair will facilitate gene editing (Ran et al., 2013; Terns and Terns, 2014). The CRISPR/Cas system has been modified into programmable endonucleases that

facilitate target gene editing. Due to its wide application, high efficiency and simplicity in design, the CRISPR/Cas system is a promising tool for genome engineering in basic sciences, medicine and biotechnology (Liang et al., 2015; Ran et al., 2013).

Guide sequence identification and selection

Using the IDT Alt-R Predesigned CRISPR-Cas9 crRNAs search tool, the optimal sequence for NOD1 and NOD2 gene targeting were selected (Table 2). This tool scans the DNA sequence for CRISPR crRNAs that are 20 nucleotides long followed by an NGG PAM sequence required for Cas9 recognition. The tool also predicts possible off-target matches. Candidate gRNAs are ranked by the accuracy of on-target and off-target activity. After the selection of the optimal crRNAs sequence, (crRNA:tracrRNA) complex were prepared in 10 □M working concentration.

Table 2Targeting sites chosen for NOD1 and NOD2 crRNAs sequence:

GENE	TARGET SEQUENCE (PAM IS UNDERLINED)
NOD1	5’ GTGGCCCTCTTCACCTTCGA **TGG** 3’ 5’ CACCGGCATCCTCAATGAGC **AGG** 3’ 5’ GACGTACCTGGCTCCGACAT **CGG** 3’
NOD2	5’ CAATCCATTCGCTTTCACCG **TGG** 3’

Transfection (electroporation):

48 hours before transfection cells were activated by adding with 1 μg/ ml doxycycline. Then cells were centrifuged at 300 g for 5 min at room temperature, washed with PBS and re-suspending at 1x106 cells/ml. Transfection by electroporation was done using Amaxa® Cell Line Nucleofector® Kit V (Lonza Picturepark). The cell pellet was suspended in a 100 μl of solution V, and mixed with (crRNA:tracrRNA complex) in a final concentration of 2 μM. Then mixed solution was transferred to the cuvette and electroporated and transfected using the recommended program. 500 μl of media with doxycycline (1 μg /ml) was added, and the whole volume was transferred to 12-well plate. Cell sorting was performed 24-hours post transfection.

Colonies selection:

Semisolid media (ClonaCell™-TCS Medium) were prepared by adding 15 ml of liquid media. Sorted cells were diluted to a concentration of 1000 cells/ml. 100 diluted ul cells were mixed very well with ten mL of prepared Semisolid media. Syringe with a 16-gauge needle was used to mix cells to avoid cells loss and inaccurate volume dispense. Then left to rest for 15 min to allow the bubble to rise. The mixer then transfers into ten com culture plate Dispense the mixture in 10 cm

plate. Colonies were collected using 10 μl pipet tip, and transfer to 96-well contain 200 μl of media. Both fast and slow growing colonies were collected for screening.

Knockout cells were single sorted to rule out any mixed colonies. Briefly, cells were washed with PBS then were suspended in 500 μl of 5% FBS Sorting Buffer. Sorting buffer was prepared by adding 1 mM EDTA (Invitrogen 15575), 25 mM HEPES (Thermo Fisher 15630), 5 % FBS (GE Healthcare Life Sciences SH30071.03HI). The cell suspension was filtered and then sorted in 96-well plate using BD Influx cell sorter (BD Biosciences) at the Flow Cytometry and Cell Sorting Resource Laboratory, School of Medicine, University of Pennsylvania.

Confirmation of gene knock-out:

To validate NOD 1 and NOD2 gene knock-out, Real-time PCR (RT PCR) and western blot was performed and compared to control cells.

Immunoblots:

Cell extract was prepared on ice by incubating in 1X RIPA buffer (Cell Signaling #9806) containing Protease Inhibitor Cocktail (Sigma P8340), Phosphatase Inhibitor Cocktail (Sigma P0044). The mix was homogenized using a 30-gauge needle. Protein was collected by centrifuging 14,000g for 20 min at 4°C. Bradford protein assay used to determine protein concentration (Thermo Fisher 23200). 50 µg of total protein was denatured using 1X Laemmli sample buffer (Alfa AesarJ61337). Samples were loaded into NuPAGE™ 4-12% Bis-Tris Protein Gels,

1.5 mm, 15-well (Invitrogen). MagicMark™ XP Western Protein Standard (Thermo Fisher LC5603) and Rainbow™ Molecular Weight Marker (GE Healthcare Life Sciences RPN800E) were run on each gel. The separated proteins were transferred to Polyvinylidene difluoride membrane (PVDF) (Invitrogen LC2002) and blocked in PBS with 0.1% Tween 20 (PBST) and 5% non-fat milk for 1 hour at RT. Then membranes were incubated in NOD1 primary antibody (1:1000, Cell Signaling #3545) and NOD2 primary antibody (1:500, Santa Cruz SC-56168) in blocking buffer overnight at 4°C. Then membranes were washed with PBST and incubated with compatible secondary antibodies at 1:2000 dilution in blocking buffer: anti-rabbit IgG, HRP-linked Antibody (1:2000, Cell Signaling #7074) or anti-mouse IgG, HRP-linked Antibody (1:2000, Cell Signaling #7076). Protein signal was developed using ECL plus™ Western blotting detection reagent (Amersham Biosciences) visualized using Odyssey® Fc Imaging Systems and software (LI-COR, Lincoln, NE).

NOD1 and NOD2 NET related signaling pathway:

PAD4 gene expression:

To better understand the role of NOD1 and NOD2 on the neutrophil NET formation. Isolated neutrophils were seeded in 6-well plate at a concentration of $1x10^6$ cells/ ml and left to rest for 20 min before treatment. Cells were pretreated with 15 μg/ml of NOD 1 inhibitor (ML130, ab142177) and NOD2 inhibitor (GSK717, MilliporeSigma) for 2 hours; followed by stimulation with PMA 100nM and their specific ligand for 4 hours. 1μg/ml C12-iEDAP (InvivoGen) and 10μg/ml MDP (InvivoGen) was used for NOD1 and NOD2 stimulation, respectively. DMSO in control was used as vehicle control. After 4 hours, cell pellets were collected, and total RNA was extracted. For converting RNA to cDNA, we used TaqMan® Fast Advanced Master Mix protocol. Using RT-PCR, we investigated the expression of NOD1 and NOD2 in neutrophils and compared mRNA expression of IL8 and PAD4 between different conditions. Furthermore, we will analyze cell lysate for PAD4 enzyme activity. NE and MPO ELISA of the supernatant were performed to detect any changes in their activities in treated neutrophils.

PAD4 enzyme activity assay:

Isolated neutrophils were treated under the conditions mentioned previously. Cell pellets were collected and lysed. PAD4 enzymatic activity was monitored in 4 hours interval using a fluorescence-quenching sensing strategy. PAD4 substrates TAMRA-(Gly-Arg-Gly-Ala)3 were kindly provided by Prof. David S.

Lawrence (University of North Carolina). PAD 4 substrate fluorescence release is only observed in the presence of PAD4 and Ca^{2+} Since deiminase activity of PAD4 is Ca^{2+}-dependent (Wang et al., 2013).

The sample was prepared by adding 5.2 mg/ml of protein lysate, 5 µM TAMRA-$(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_3$, 5 mM DTT (Sigma 3483-12-3), 4% protease inhibitor cocktail (Sigma P8340), 110 µM of Evans blue as quencher. Assay was initiated by adding 10 mM $CaCl_2$ (Wang et al., 2013). Then samples were plated in 96-well microplate (BD FalconTM 353219). Fluorescence was quantified at excitation/emission wavelengths of 550 /590 nm using Infinite M200 microplate reader (Tecan, Mannerdorf, Switzerland) with a temperature setting of 30 °C.

MPO and NE ELISA:

The culture supernatant of treated cells was collected by snap freezing with liquid nitrogen. One hundred microliters of culture supernatant were used in each well without dilution. MPO and NE were measured in triplicate using ELISA according to the manufacturer's standard protocol (Duoset R&D Systems, DY3174, and DY9167-05). Data acquired using a 96-well plate reader Infinite M200 (Tecan, Mannerdorf, Switzerland).

Statistics:

Statistical analysis was done using GraphPad Prism 6.0 (San Diego, CA). Data were analyzed with one-way ANOVA followed by Tukey's multiple comparison tests. Statistical differences were considered significant at the $p < 0.05$ level.

Chapter 3 : Results

Quantification of *F. nucleatum induced* NETs:

The ability of *F. nucleatum* to produce NETs was assessed using a nucleic acid stain SYTOX® orange. Prior to NET release, the cell membrane is compromised and will eventually rupture (Fuchs et al., 2007). By taking advantage of the compromised membrane integrity, NETs can be quantified using impermeable DNA dyes, such as SYTOX® orange.

PMA is a well-known inducer of NETs (Gupta et al., 2005).Thus, it was used with *F. nucleatum* in this assay. Cells were induced with *F. nucleatum* and PMA for 4 hours. The fluorometric quantification revealed that *F. nucleatum* (MOI 1:10) were capable of inducing NETs successfully when compared to PMA as positive control. NET induction was time depended in a positive manner (Figure 7; $p < 0.0001$), a finding confirmed by NET visualization using TEM and SEM (Next section).

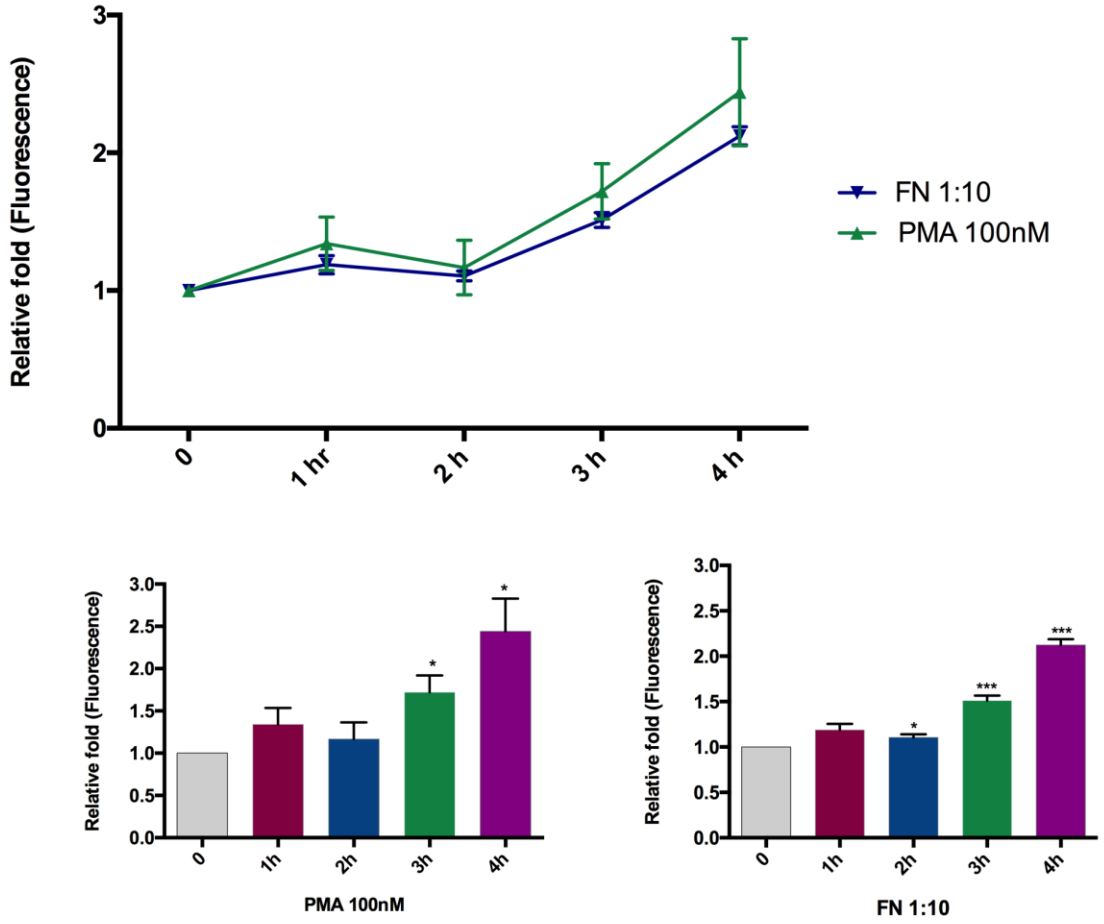


Figure 7: NET release in response to *F. nucleatum* and PMA challenged neutrophils. Freshly isolated neutrophils were challenged with *F. nucleatum* (MOI1:10) or PMA (100nM). NET quantification revealed significant increase in NETs when stimulated with *F. nucleatum*, similar to PMA. Statistical test: One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (*= $p < 0.05$). Values are mean ± SD.

***F. nucleatum* and neutrophils interaction:**

Analysis of the interaction under scanning and transmission electron microscope:

To allow detailed analysis of the interaction between the bacteria and neutrophils, SEM and TEM were performed. Neutrophils were seeded onto poly L lysine coated coverslips and stimulated with *F. nucleatum* at different time point with different concentration. When challenged with *F. nucleatum* (MOI 1:10), morphological changes occur, and neutrophils start to flatten (Figure 8a and b) and eventually releases NETs. Generally, the NETs presented as thin strand-like structures connecting between neutrophils with the bacteria entrapped within these strands (Figure 8c). After 8 hours of stimuli, NETosis can be observed at its maximum (Figure 9). When challenged with a higher concentration of *F. nucleatum* (MOI 1:100) we found more cell death compared to NET release (Figure 10a b and c). Bacterial phagocytosis was also detected via SEM and TEM (Figure 11). Suggesting, that both NETosis and phagocytosis could co-occur in a different rate.

Cell Death ELISA of F. nucleatum challenged neutrophils

To confirm our observation under SEM cell death ELISA was done to detect cytoplasmic histone-associated DNA fragments (H1, H2A, H2B, H3, and H4) (Figure 10d). Data showed that *F. nucleatum* significantly induced histones release and cell death when compared to control.

Detection of citrullinated histone H3 via Immunohistochemistry

Citrullination of histone H3 (Cit H3) by PAD4 plays an essential role in chromatin de-condensation, and subsequently the release of NETs. Immunohistochemistry of Cit H3 was used to detect and visualize NET formation in neutrophils. Staining of citrullinated histone H3 in *F. nucleatum* treated neutrophils indicates that *F. nucleatum* successfully induced chromatin de-condensation via PAD4 activation (Figure 12).

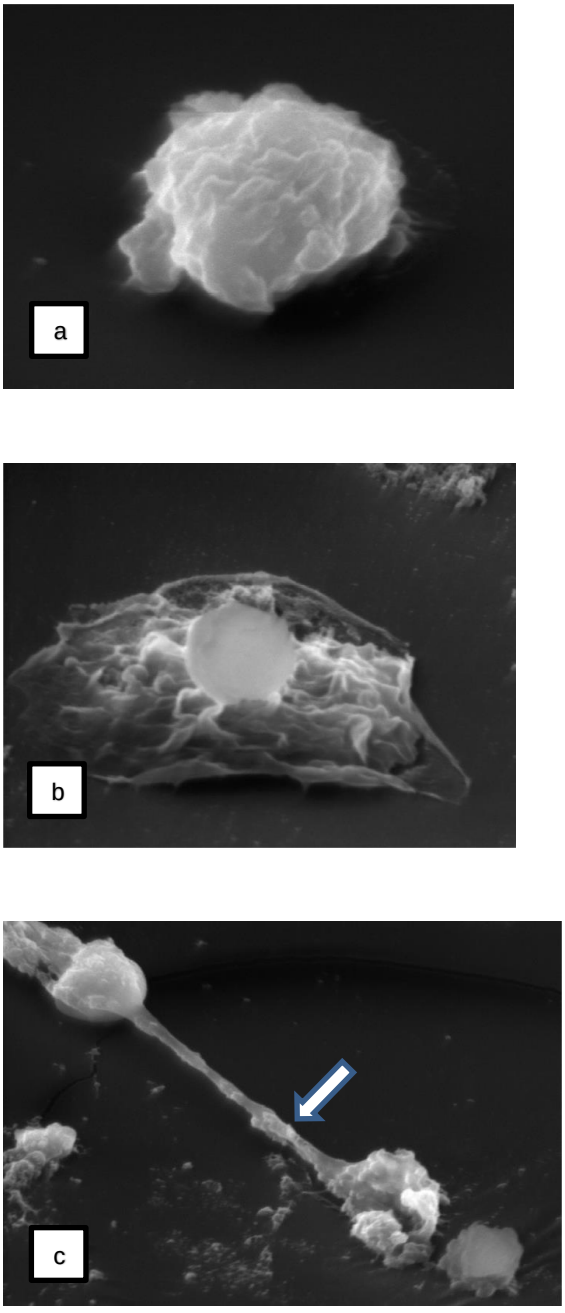


Figure 8: Scanning electron micrographs of neutrophils stimulated with MOI 1:10 of *F.nucleatum.* Typical morphological changes associated with NETosis observed (a & b). NETs are presented as strand-like structure connecting cells (white arrow) (c).

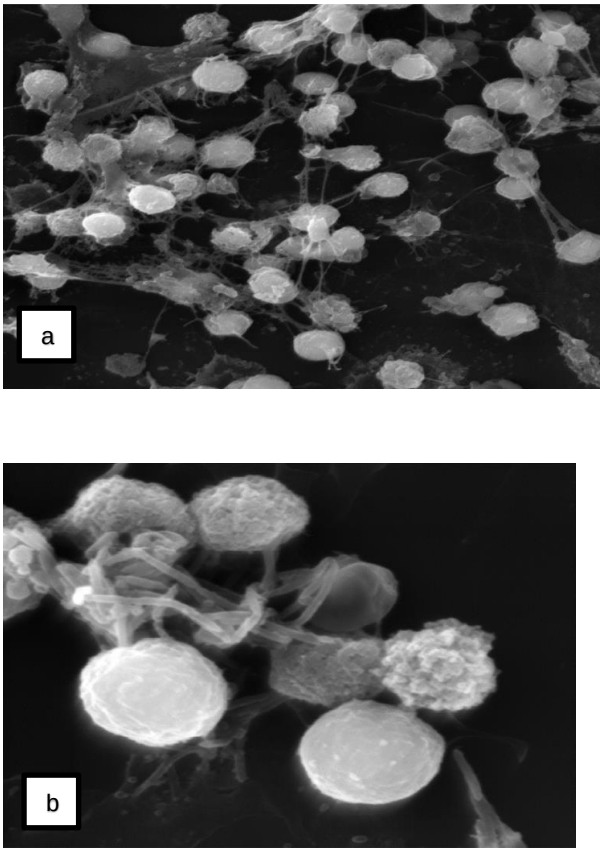


Figure 9: Rapid NET formation upon *F. nucleatum* stimulation. Neutrophils were stimulated with 1:10 MOI of *F. nucleatum* for 8h, fixed and observed under Scanning Electron microscope (a). Magnified image of *F. nucleatum* trapped by neutrophils (b).

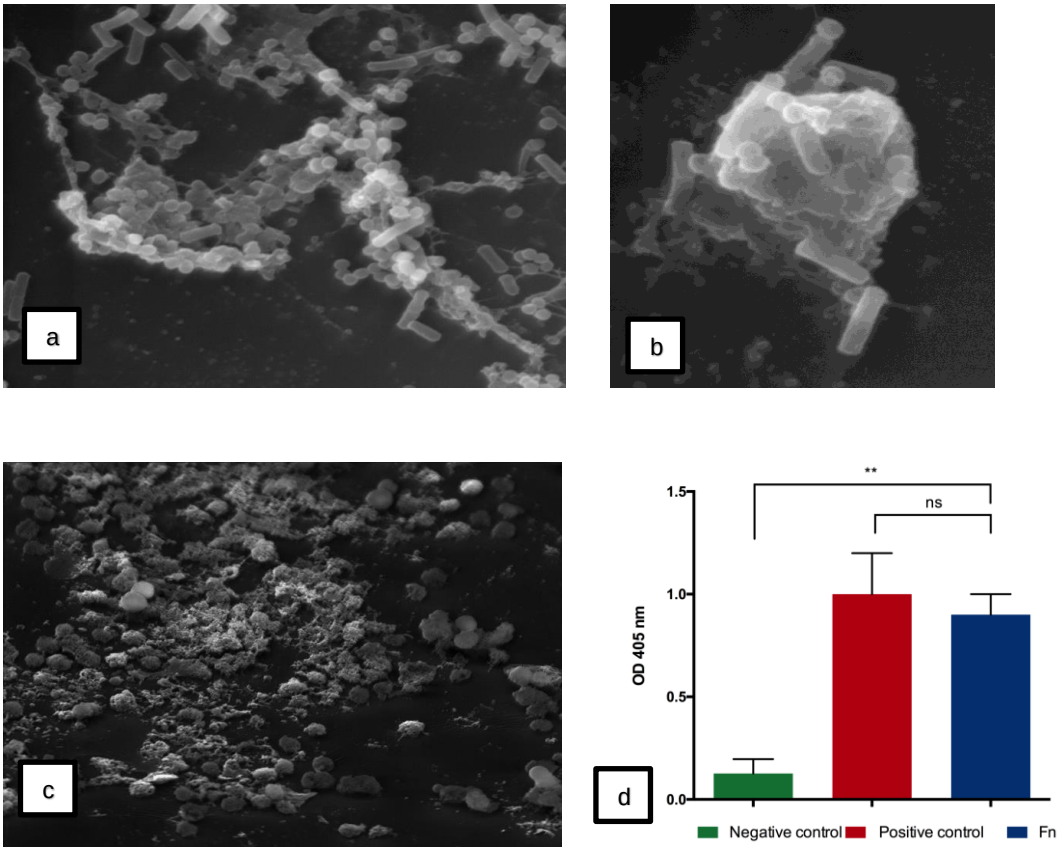


Figure 10: *F. nucleatum* induce neutrophil cell death. Neutrophils were stimulated with 1:100 MOI of *F. nucleatum* for 12h, fixed and observed under SEM (a). Magnified image of *F. nucleatum* invading a neutrophil (b). After 16 hours of stimulation, neutrophils undergo apoptosis and *F. nucleatum* induced cell death (c). Cell-death ELISA showed high cell death rate compared to negative control when challenged with *F. nucleatum* concurring SEM observation. Statistical test: One-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$; *ns* = no significant difference). Results are mean ± SD.

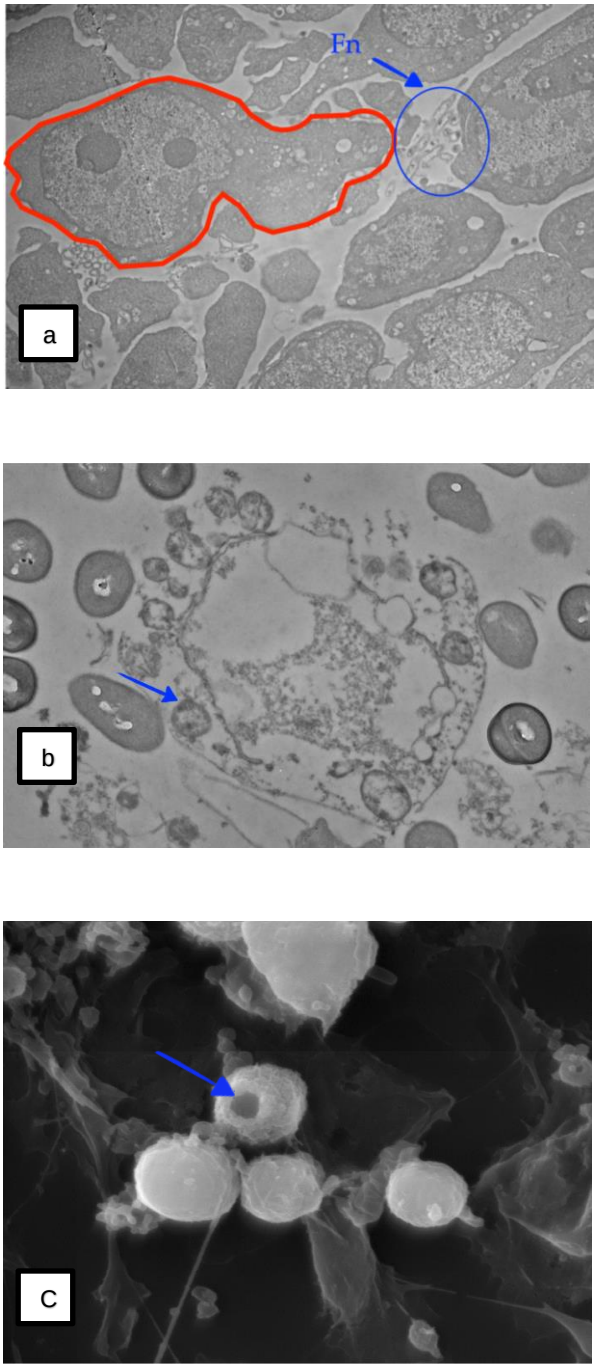


Figure 11: Transmission Electron Microscopic image of *F. nucleatum* challenged neutrophils. TEM showing extension of neutrophils membrane (in red) and granules (a) and *F. nucleatum* inside and outside of a neutrophil (blue) (b). Scanning Electron Microscope shows *F. nucleatum* phagocytosis by a neutrophil (blue arrow) (c).

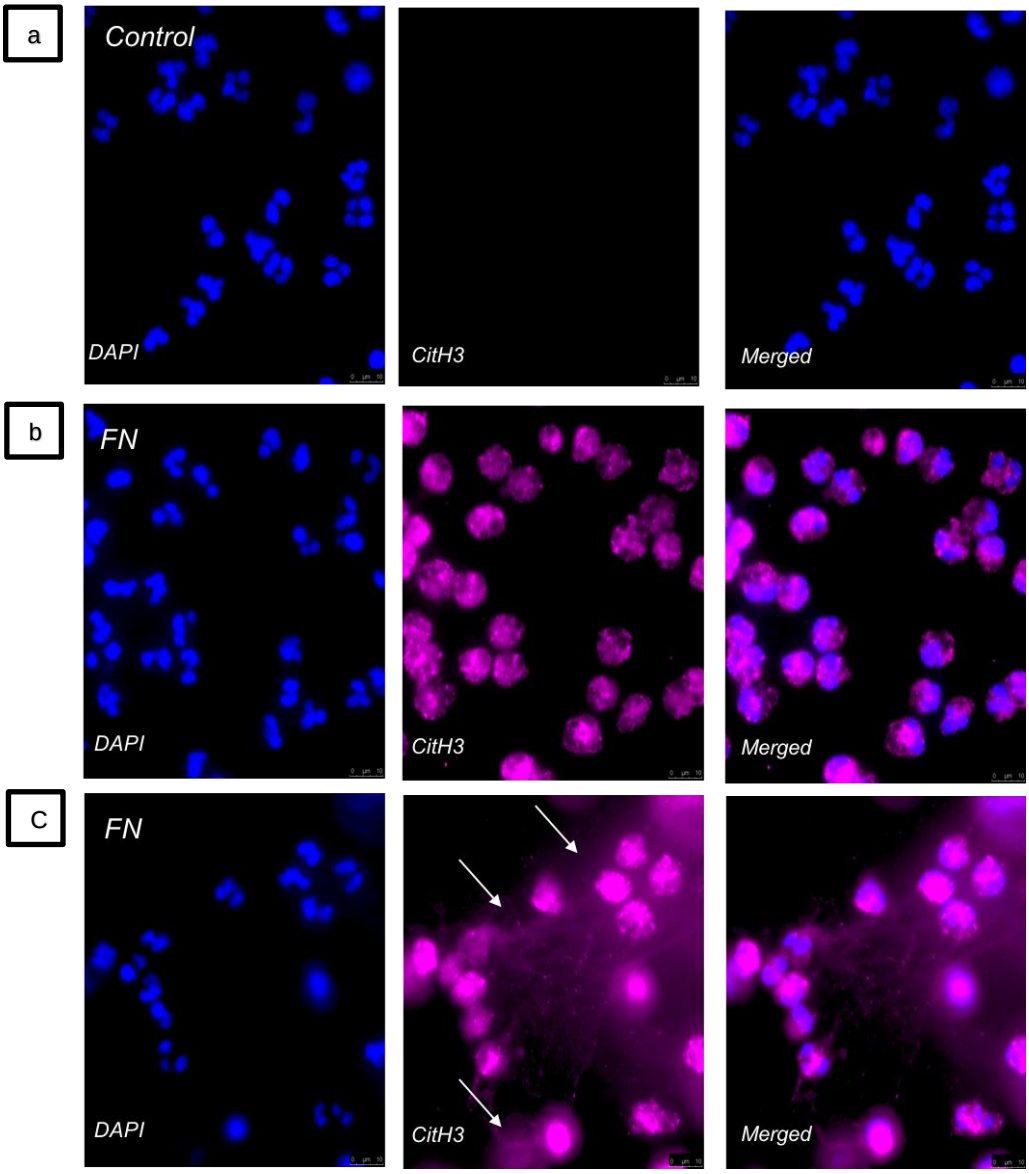
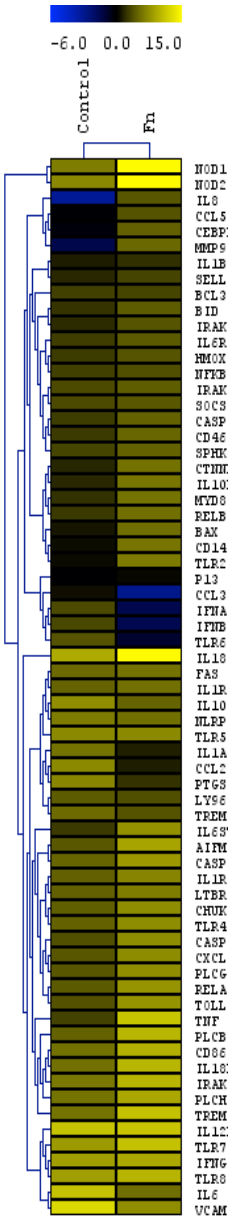


Figure 12: *F. nucleatum* induces PAD activation and the release of de-condensed chromatin in neutrophils. Neutrophils were seeded onto poly L lysine coated coverslips and incubated for 30 min at 37°C to allow for cell attachment. Cells were challenged for 4 hours with *F. nucleatum* (MOI 1:10) or left unstimulated in media and then processed for immunofluorescence. DNA was stained with DAPI (blue), and de-condensed chromatin was stained with anti-Histone H3 (Cit-H3) (pink). *F. nucleatum* challenged neutrophils demonstrated the presence of NETs (white arrow) judged from the presence of citrullinated histone H3. De-condensed chromatin was not observed without stimulation (a). Notice the staining of citrullinated histone H3 (b) followed by chromatin elongation and NET release (c).

Upregulation of NOD1 and NOD2 receptors in *F. nucleatum* challenged neutrophils:

Neutrophils were challenged with *F. nucleatum* at an MOI 1:10 for 8 hours and qPCR was performed on a focused panel of innate immune, apoptosis and GPCR related genes (Figure 13). *F. nucleatum* strongly upregulates NOD1 and NOD2 expression when compared to other genes pool. More specifically, NOD1 and NOD2 expression were 44.8 and 31.8 folds compared to control, respectively. Thus, Indicating the link between NOD1 and NOD2 pathways and *F. nucleatum* induce NETosis.

We measured NOD1 and NOD2 mRNA expression while challenging the cells with *P. gingivalis, A. actinomycetemcomitans,* and *F. nucleatum* to confirm the link between NOD-like receptors and *F. nucleatum* induced NETosis. RT-qPCR data showed that *F. nucleatum* profoundly upregulates NOD-like receptors in comparison with other bacterial strains with and without PMA (Figure 14).



Gene	Control	F. nucleatum (MOI 1:10)
NOD1	6.4	51.2
NOD2	7.1	38.9
IL18	9.3	16.8
IL12B	11.0	11.2
TNF	3.2	11.1
TREM2	5.8	10.9
PLCB2	4.8	10.1
IL18R1	5.7	10.1
IRAK4	6.2	10.0
TLR8	8.5	9.9
CD86	5.7	9.7
IFNG	8.6	9.2
PLCH2	5.8	9.1
AIFM1	4.3	8.9
CASP8	5.0	8.2
RELA	4.4	7.8
TOLLIP	4.0	7.8
IL6ST	2.7	7.7
VCAM1	12.6	7.5
PLCG2	4.2	7.4
CASP3	3.9	7.3
CHUK	4.7	7.3
TLR4	4.8	7.3
CXCL10	4.2	7.3
TLR5	7.2	7.1
IL1RAP	4.8	6.8
LTBR	4.7	6.5
TLR2	0.7	6.4
IL10RA	1.8	6.0
CD14	0.8	5.9
FAS	5.2	5.9
MYD88	2.3	5.8
CTNNB1	1.8	5.7
IL1R1	4.9	5.7
NLRP3	6.4	5.6
RELB	2.7	5.6
IL6	10.9	5.5
BAX	1.1	5.5
MMP9	-1.3	5.2
CD46	2.7	5.1
IL10	7.4	5.0
SPHK1	3.3	4.9
CASP1	2.9	4.8
IRAK2	3.5	4.5
BID	2.4	4.5
IL6R	2.9	4.4
SOCS3	3.6	4.3
IL8	-3.0	4.2
IRAK1	2.0	4.1
HMOX1	2.8	4.1
TREM1	5.4	4.0
CCL5	-0.1	4.0
NFKB1	3.0	3.8
LY96	4.5	3.8
BCL3	3.0	3.3
SELL	1.9	3.2
PTGS2	7.0	2.4
IL1B	1.4	2.2
IL1A	5.8	1.5
CCL20	7.2	1.4
P13	-0.1	0.6
TLR6	4.1	-0.8
IFNA1	3.6	-1.4
IFNB1	3.5	-1.5
CCL3	0.9	-2.9
CEBPB	-0.3	-4.7
TLR7	8.3	-10.9

Figure 13: qPCR-Array of innate immune, apoptosis and GPCR related genes. Neutrophils were challenged with *F. nucleatum* at MOI:10 for 8 hours. Negative control was unchallenged cells in media. (a) represents a heatmap of the genes expression. The color scale indicates fold change in expression, yellow for up-regulated genes, and blue for down-regulated genes. (b) a Layout of the genes shown in heat map and fold changes relative to the control. Numbers are mean change in *F. nucleatum* challenged cells compared to control in two pairs.

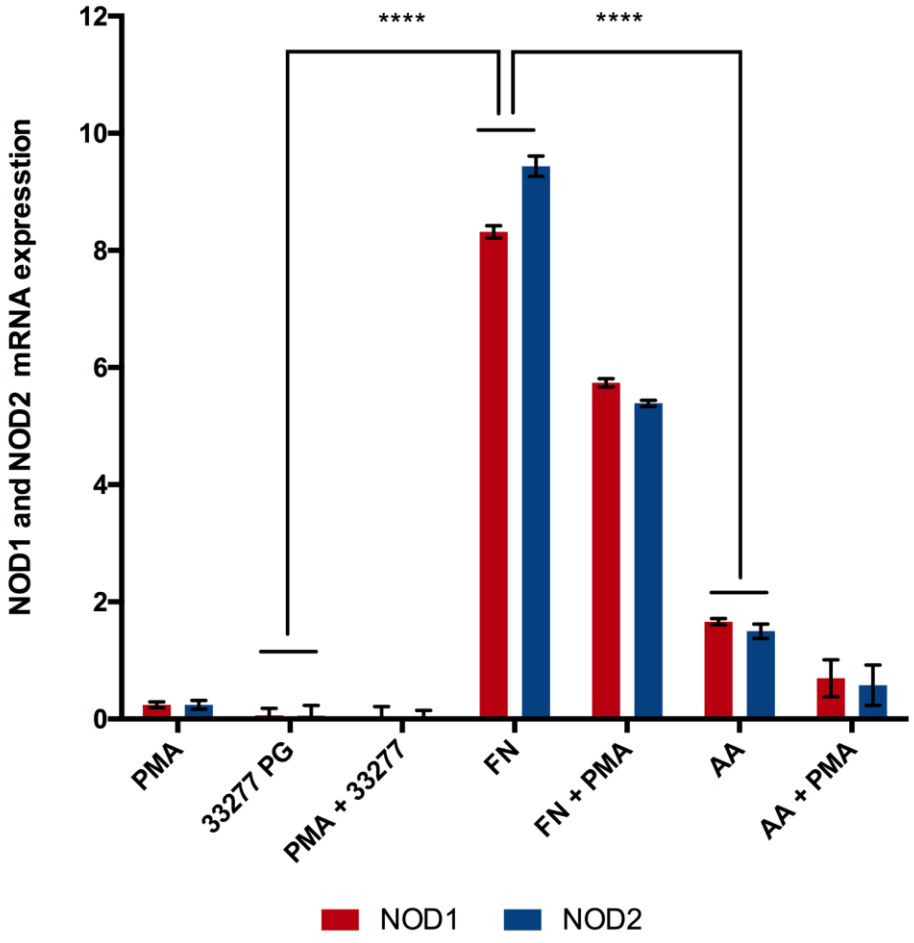


Figure 14: RT-qPCR of NOD1 and NOD2 expression in neutrophils after challenging them with *33277 P. gingivalis (PG), A. actinomycetemcomitans (A.A),* and *F. nucleatum (FN*) with and without PMA. *F. nucleatum* significantly upregulate NOD1 and NOD2 compared to other groups. Statistical test: One-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$). Results are mean ± SD.

Summary of the F. nucleatum -neutrophils interaction:

- *F. nucleatum* (MOI 1:10) successfully induced NETosis.
- *F. nucleatum* highly upregulated NOD1 and NOD2 during NET formation
- NOD1 and NOD2 expression is strongly upregulated by *F. nucleatum when* compared to *P. gingivalis and A. actinomycetemcomitans.*

NOD1 and NOD2 knock-out HL-60 cell lines: validation of the role of NOD1 and NOD2 receptors in NETosis.

Knocking out NOD1 and NOD2 using CRISPR-Cas9 system

HL-60 Cas9 stable cell line was treated using 1 μg/ml of doxycycline for 48 hours to activate Cas9 expression. Western blot was done to confirm Cas9 protein induction when compared to control (Figure 15 a). Then cells were transfected with (crRNA: tracrRNA) complex via electroporation. 24 hours post-transfection cells were cultured in semisolid media. In a week, small colonies started to grow (Figure 14 b). At day 14, small and large colonies were selected and plated at 96 well plate for four days. RNA was extracted using RNAqueous™-Micro Kit (Thermo Fisher AM1931). RT-PCR and western blot were performed and confirmed gene knockout (Figure 16). Furthermore, selected colonies were single sorted at 96- well plate to exclude the possibility of mixed population.

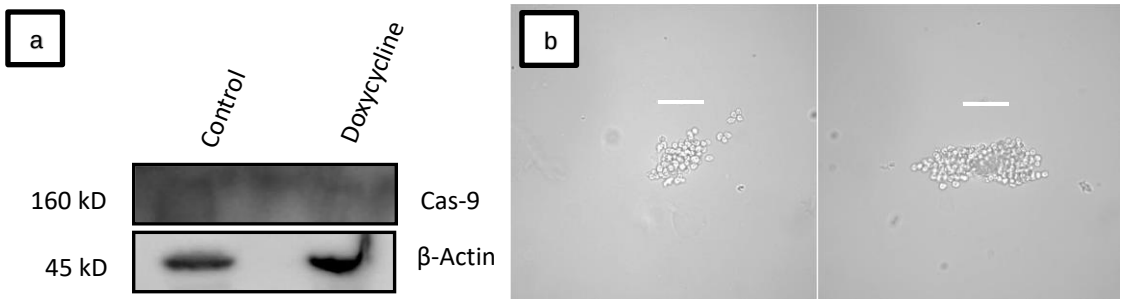


Figure 15: (a) Immunoblot from HL-60 Cas9 stable cell line treated with 1 μg of doxycycline for 48 hours. Cas9 protein (160 kDa) were detected only in doxycycline treated cells. Levels of housekeeping protein β-Actin (45 kDa) were similar between conditions **(b) Image represent a single cell colony formation cultured in a semisolid media.** Cell were seeded in concentration of 1000 cells/ml. Colonies were selected two weeks post transfection and seeded in 96-well plate for colonies expansion and genes knockout validation. Bar represents 100 μm.

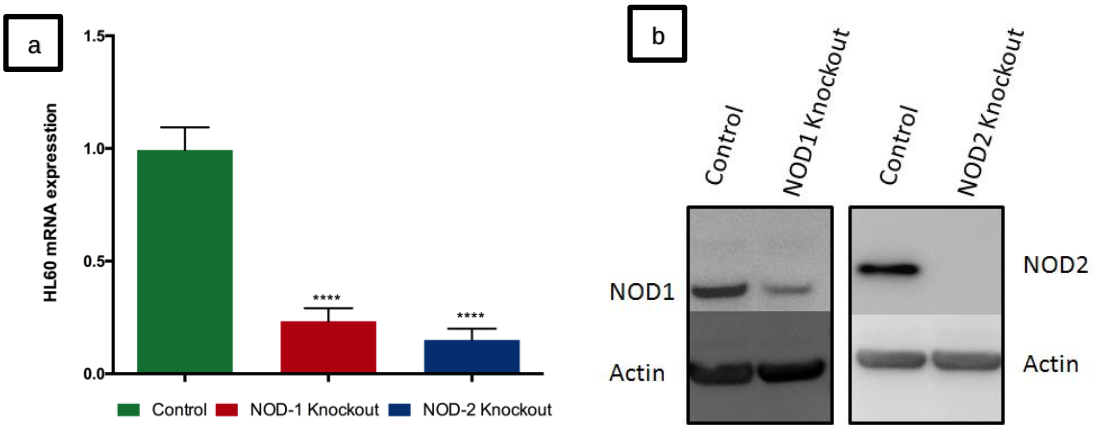


Figure 16: HL-60 Knockout confirmation: RT-PCR of knockout cells showed a significant reduction in NOD1 and NOD2 expression compared to control cells (a). The total protein was subjected to immunoblot to confirm the absence of NOD1 and NOD2 protein expression compared to control. Levels of housekeeping protein β-Actin were similar between samples(b). Statistical comparisons are shown by horizontal bars with asterisks above them (*p < 0.05) determined by one-way ANOVA and Tukey multiple comparison test. Data are expressed as mean ± SD.

Reduction of F. nucleatum induced NET formation in NOD1 and NOD2 knockout HL60

Cell death ELISA of *F. nucleatum* challenged cells showed a significant reduction of histone-associated DNA fragments (H1, H2A, H2B, H3, and H4) in the cytoplasm of NOD1 Knockout cells. On the other hand, NOD2 Knockout cells showed no significant changes (Figure 17).

Utilizing the fact that NETs mainly consist of nuclear DNA (Brinkmann et al., 2004), the fluorescent DNA stain, SYTOX® orange, was used for fluorometric quantification of NET release. When compared to control cells NOD1 and NOD2 knockout HL60 cell line significantly released less NETs when challenged with *F.* nucleatum (MOI 1:10) for 12 hours (Figure 18). Furthermore, neutrophil elastase immunostaining of *F. nucleatum* challenged cells were performed to compare NET formation between NOD1 Knockout HL60 and control (Figure 19).

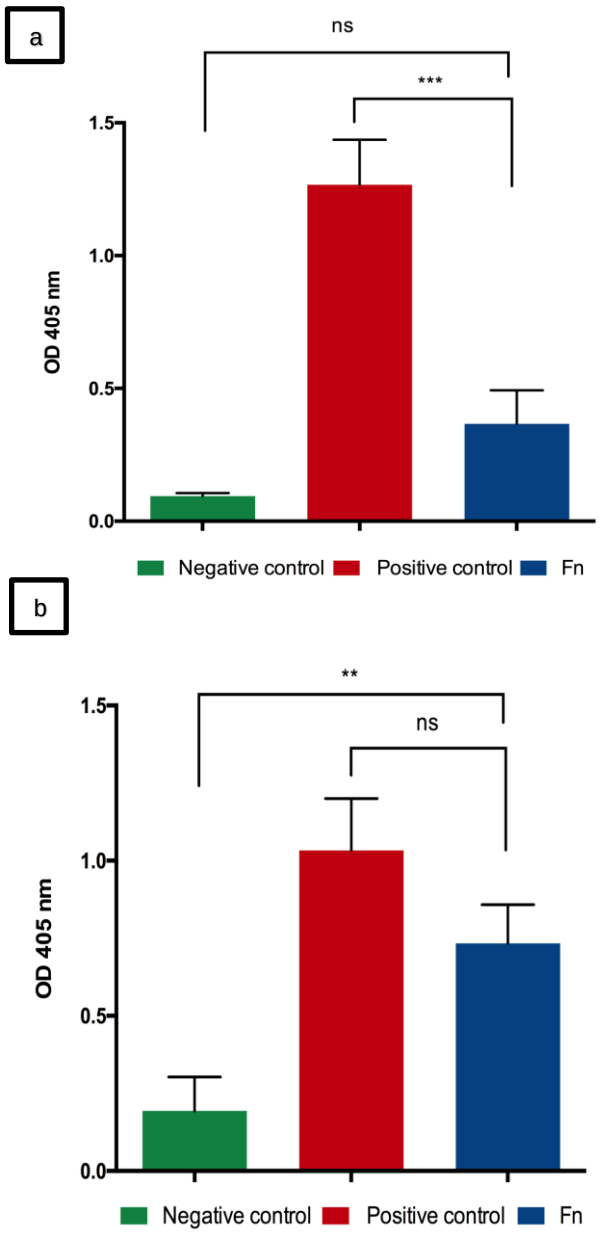


Figure 17: Cell death ELISA of NOD1 and NOD2 knockout HL60 cells: NOD1 Knockout HL60 cells showed a significant reduction in cytoplasmic histone-associated DNA fragments (H1, H2A, H2B, H3 and H4) when challenged *F. nucleatum* (MOI1:10) (a), while NOD2 Knockout HL60 cells revealed insignificant changes in cell death rate. Statistical test: one-way ANOVA and Tukey multiple comparison test (*$p < 0.05$; *ns* = no significant difference). Values represent the means ± SD.

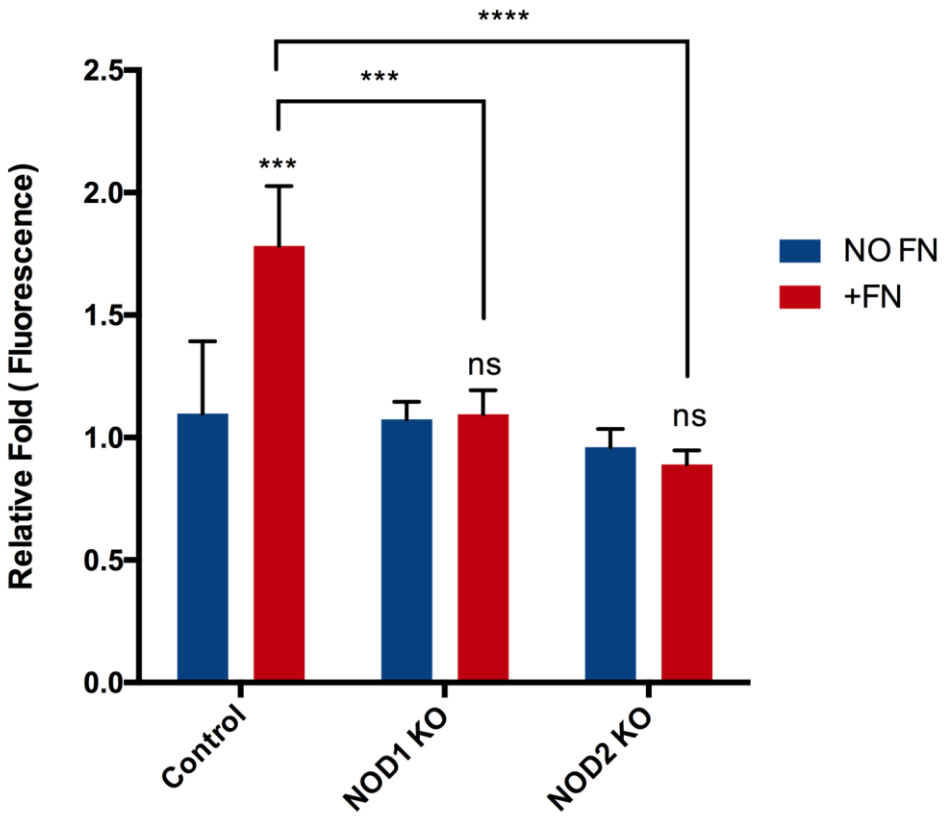


Figure 18: Quantification of NET release in NOD1 and NOD2 Knockout HL-60 cell line. Cells were seeded in concentration of (1 x10^6 cell / ml) with and without *F. nucleatum (FN)* treatment at a MOI 1:10. After 12 hours, NET release were quantified using SYTOX® orange DNA stain. We observed a significant increase in the release of NET from control HL-60 when exposed to *F. nucleatum,* while both NOD! and NOD2 Knockout HL60 didn't produced significant NETs when compared to untreated group and control treated group. Statistical test: one-way ANOVA and Tukey multiple comparison test (*$p < 0.05$; *ns* = no significant difference). Values are the means ± SD.

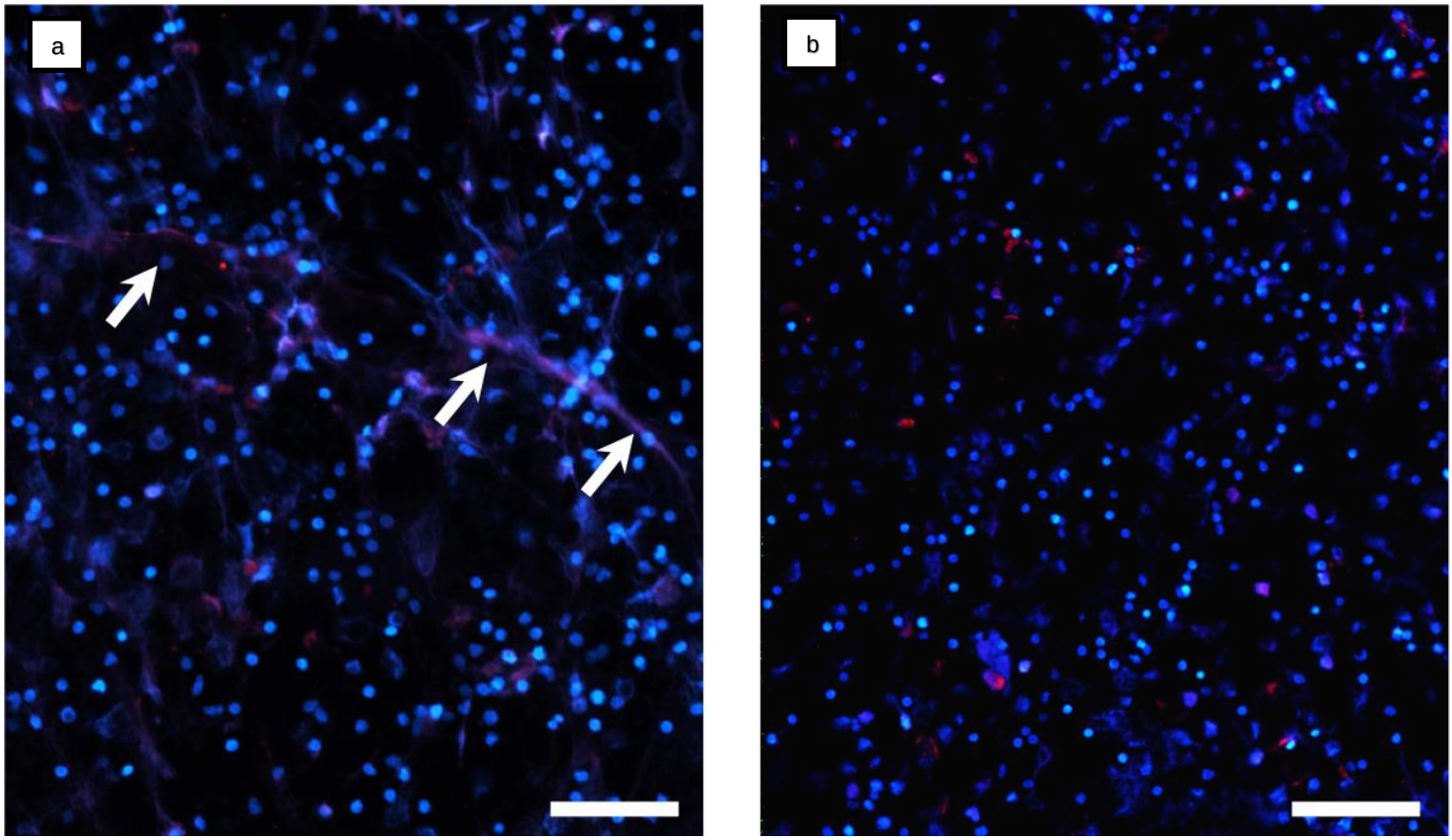


Figure 19: Neutrophil elastase staining on wild type and NOD-1 knockout HL60 cells. NET formation was observed after 12 hour of *F. nucleatum* stimulation. *F. nucleatum* successfully induced NET formation in HL60 wild type cells (a), whereas NOD-1 knockout HL60 cells formed significantly less NETs(b). X20 magnification.

Summary of the role of NOD1 and NOD2 in HL60 NET formation:

- By using CRISPR-Cas9 system we successfully knocked out NOD1 and NOD2 in HL60 cell line
- Knocking out NOD1 and NOD2 significantly reduced NETosis in *F. nucleatum* treated HL60 cells.

NOD1 and NOD2 NET related pathway:

To test the role of NOD-like receptors in neutrophils NET formation, we investigated the pharmacological inhibition of NOD1/NOD2 before challenging with their specific ligands and PMA.

Cells were pretreated with and without ML130 (NOD1-inhibitor) and GSK717 (NOD2-inhibitor) for 2 hours, followed by 4 hours stimulation with their specific ligand: C12-iEDAP (NOD1-ligand), MDP (NOD2-ligand), and PMA.

Activation of NOD1, but not NOD2 upregulate PAD4 expression*:*

We analyzed NOD1, NOD2 and IL-8 mRNA expression using Real-time PCR. Data showed that although C12-iEDAP insignificantly upregulated NOD1 expression, the IL8 expression, which is downstream of this activation (Jeon et al., 2012), was upregulated. On the other hand, pretreated cells with ML130 prior to C12-iEDAP stimulation had a significant downregulation effect on NOD1 and IL8 expression (Figure 20). Furthermore, treating cells with MDP significantly upregulates NOD2 expression, and GSK717 strongly inhibits NOD2 upregulation. IL8 expression with NOD2-inhibitor and/or ligand was also significantly regulated (Figure 21). As expected, PMA had no significant effect on either NOD1 or NOD2 expression. Interestingly, despite the insignificant upregulation of PAD4 with C12-iEDAP, ML130 had a substantial downregulation effect on PAD4 expression.

PAD4 expression of MDP and GSK717 treated cells showed no significant results (Figure 22).

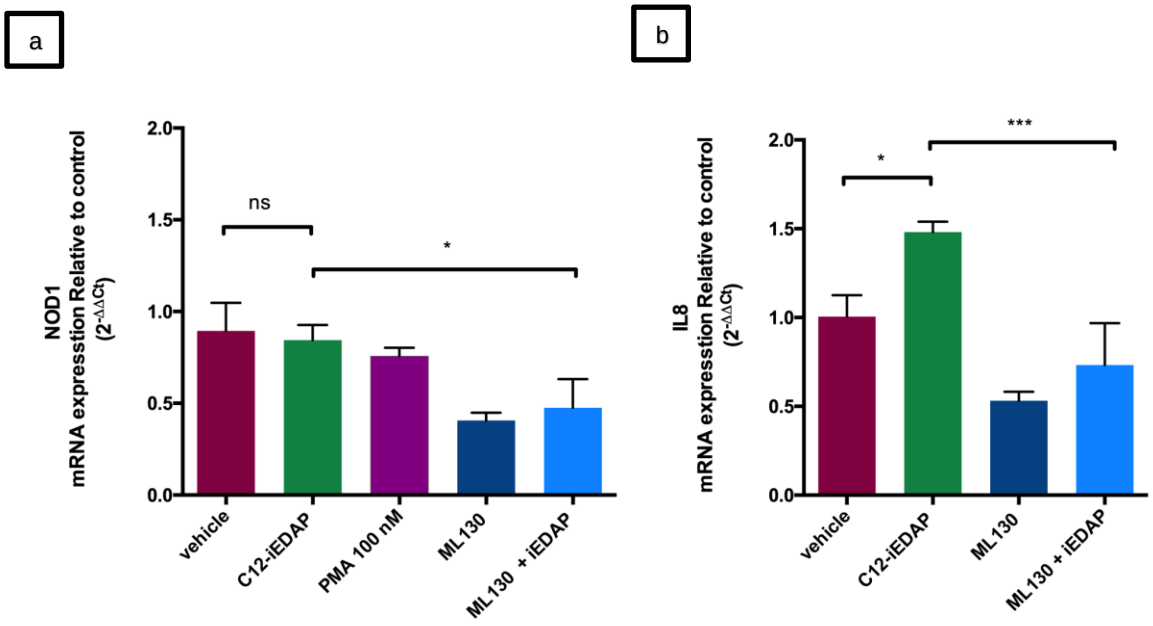


Figure 20: NOD1 and IL-8 gene expression in neutrophils following NOD1 inhibitor/ligand treatment. Cells were subjected to (15 μg/ml) ML130 (NOD1-inhibitor) treatment for 2 hours, followed by stimulation with (1 μg/ml) C12-iEDAP (NOD1-ligand) and PMA (100nM) for 4 hours. Total RNA was isolated for real-time PCR. The NOD1 receptor mRNA expression was unchanged upon NOD1-ligand challenge but was successfully downregulated when treated with its inhibitor prior to ligand stimulation. PMA had no significant effect on NOD1 expression (a). Despite the insignificant increase in NOD1 mRNA expression when treated with C12-iEDAP, its activity observed by IL8 expression was increased and decreased significantly when treated with NOD1 ligand and inhibitor, respectively (b). Results are mean ± S.D. Statistical comparisons are determined using one-way ANOVA followed by Tukey multiple comparison test. (*$p < 0.05$; *ns* = no significant difference).

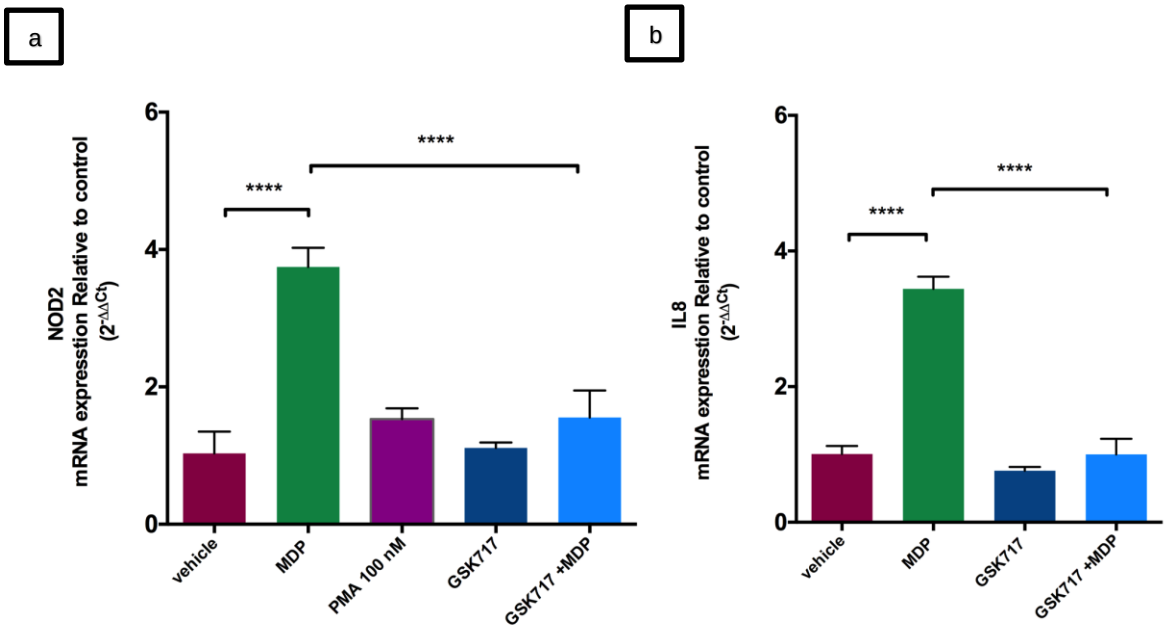


Figure 21: NOD2 and IL-8 gene expression in neutrophils following NOD2 inhibitor/ligand treatment. Cells were subjected to GSK717 (15μg/ml) a specific NOD2-inhibitor treatment for 2 hours, followed by stimulation with (10μg/ml) MDP (NOD2-ligand) and PMA (100nM) for 4 hours. MDP significantly downregulated NOD2 receptor mRNA expression. This rise in expression was successfully inhibited when treated with GSK717 before its stimulation. PMA had no significant effect on NOD2 expression (a). IL8 expression was significantly upregulated and downregulated when treated with NOD2 ligand and inhibitor, respectively (b). Results are mean ± S.D. Statistical comparisons are determined using one-way ANOVA followed by Tukey multiple comparison test (*$p < 0.05$).

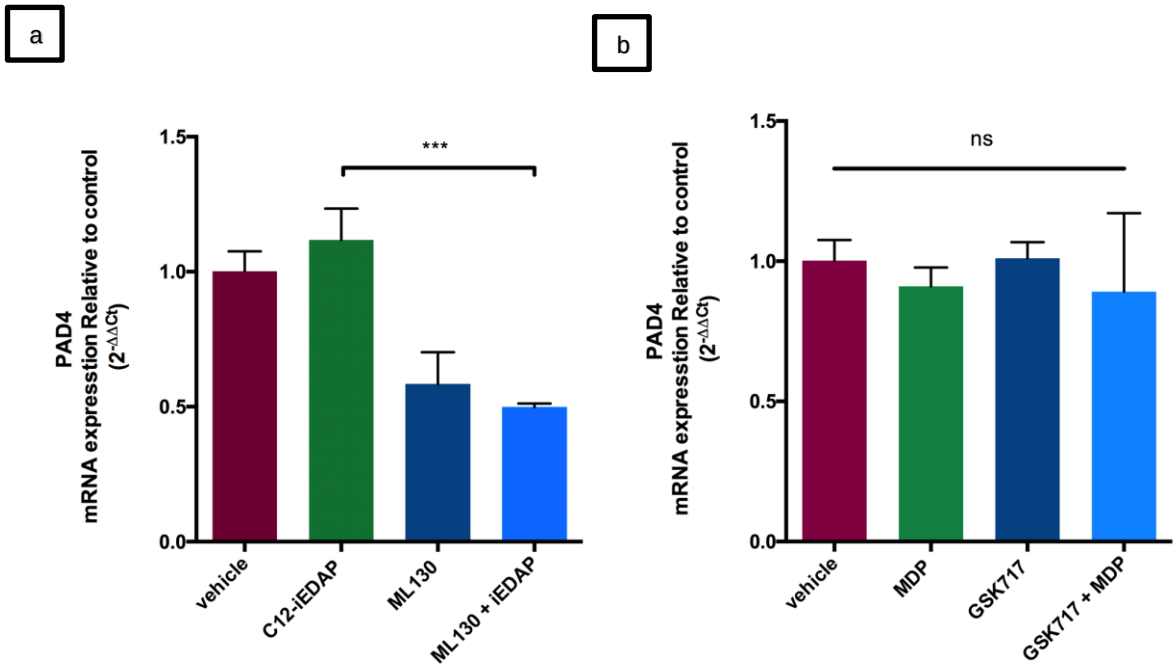


Figure 22: PAD4 mRNA expression in neutrophils following NOD1 and NOD2 inhibitor/ligand treatment. Neutrophils were pretreated with or without (15 µg/ml) ML130 and GSK717 for 2 hours before challenging with (1µg/ml) of C12-iEDAP and (10µg/ml) MDP for 4 hours, respectively. Changes in PAD4 expression of NOD1-ligand treated cells were insignificant but was successfully downregulated upon ML130 pretreatment (a). NOD2 ligand/inhibitor treatments had no significant effect on PAD4 mRNA expression (b). Statistical test: one-way ANOVA and Tukey multiple comparison test (*$p < 0.05$; *ns* = no significant difference).

Tracing PAD4 enzyme activity as result of NOD1 and NOD2 stimulation.

We investigated PAD4 enzymatic activity (post-translational modification) with and without inhibiting NOD-like receptors using Fluorescence Quenching, a fluorescence-based PAD4 activity sensing strategy. In this experiment, we mixed cell lysate from each sample with Evans blue, a fluorescence quenching mediator, and TAMRA-(Gly-Arg-Gly-Ala)$_3$, a PAD4 specific substrate (Figure 23 a). Substrate fluorescence release was monitored in 4 hours interval. We observed a significant increase of PAD4 enzymatic activity with C12-iEDAP treated cells. Moreover, ML130 significantly decreased the enzymatic activity of NOD1 ligand treated cells. MDP had no significant effect on PAD4 activity coinciding with RT-PCR data (Figure 23b). Thus, illustrating the significant role of NOD1 receptor in PAD4 expression at both transcriptional and translational levels.

NOD1 and NOD2 modulate MPO and NE activity

MPO and NE enzymes activity in the culture supernatant were detected using ELISA. We observed a significant increase and decrease of MPO and NE activity when treated with NOD1/NOD2 ligands and inhibitors, respectively. PMA was used as positive control (Figure 24). This confirmed that NOD1 and NOD2 up-regulation is linked to NET formation via MPO and NE activity.

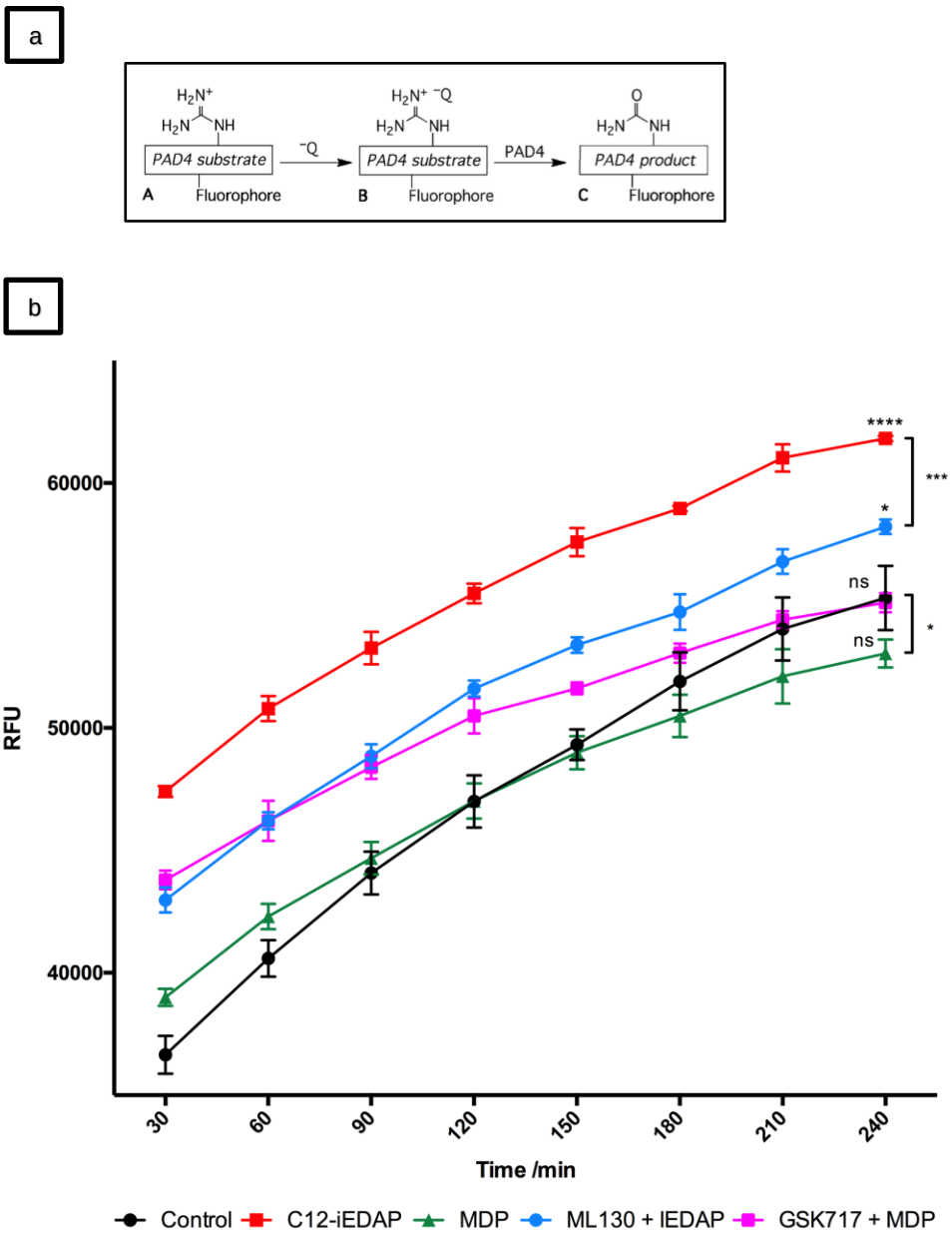


Figure 23: Neutrophils PAD4 activity assay:

(a) When interacting with negatively charged quencher dye, TAMRA tagged PAD4 substrate A forms a non-fluorescent noncovalent complex B. In the presence of citrullinated PAD4, the PAD4 substrate become neutral C and loses its affinity for the dye, and a fluorescence response is observed (Wang et al., 2013)

(b) Fluorescence change of PAD4 substrate (TAMRA-Gly-Arg-Gly-Ala_3) and Evans blue quencher pair in neutrophils lysate upon stimulation with NOD1 and NOD2 ligands, in the presence or absence of inhibitors pretreatment.

Fluorescence release was monitored in 4 hours interval. Increased PAD4 activity were observed only in (1µg/ml) C12-iEDAP treated cells and were significantly reduced in (15µg/ml) ML130 pretreated group. 10µg/ml of MDP had no significant effect on PAD4 enzyme activity, concurring with RT-PCR data. Statistical test: one-way ANOVA and Tukey multiple comparison test (*$p < 0.05$; *ns* = no significant difference).

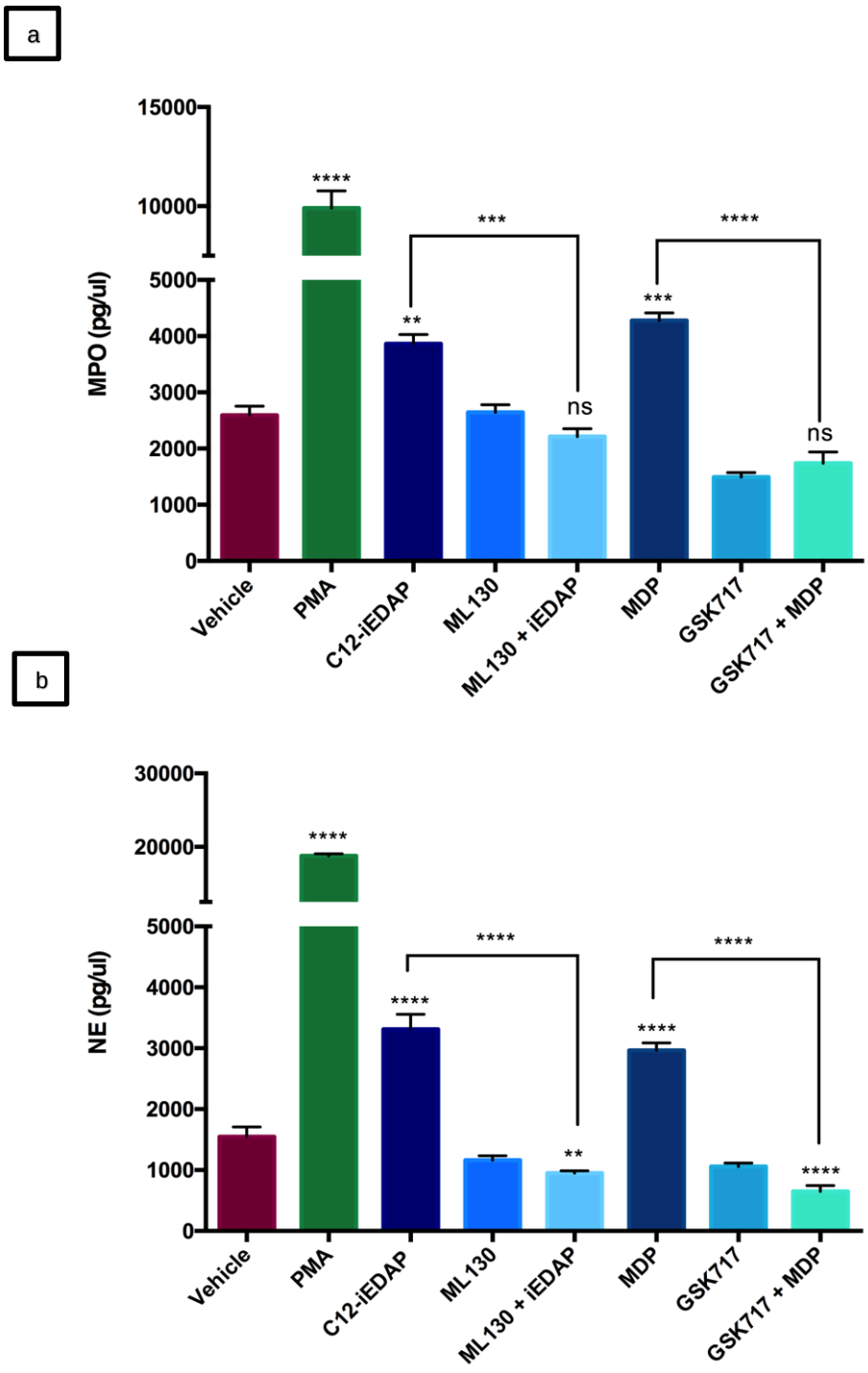


Figure 24: MPO and NE Enzyme-linked immunosorbent assay of supernatant from NOD1/NOD2 ligands and inhibiter treated cells. The negative control was supernatant from non-stimulated cells with DMSO (vehicle). The positive control was supernatant from PMA-stimulated cells. MPO (a) and NE (b) release were

significantly induced by (1µg/ml) C12-iEDAP and (10 µg/ml) MDP ligands. The enzymes induction by NOD1 and NOD2 ligands was significantly downregulated by pharmacological inhibition with (5µg/ml) ML130 and GSK717, respectively. Values represent the mean ± SD. Data were analyzed using on-way ANOVA followed by Tukey multiple comparison test. Statistical comparisons are to the vehicle (*$p < 0.05$; *ns* = no significant difference).

Summary of NOD1 and NOD2 pathways linked to NETosis:

- NOD1 induced chromatin decondensation via activation PAD4 at a transcriptional and translational level.
- NOD1 and NOD2 affect both MPO and NE by increasing their activity when stimulated.

Chapter 4 : Discussion and Future Directions

Discussion:

Neutrophil extracellular trap (NET) formation is one element in many recently discovered innate immune functions that has garnered much appreciation in the research community due to its link to various types of pathogenic interactions and systemic diseases. NETs consist of webs of DNA which physically trap microorganisms and prevent their spread. Neutrophil anti-bacterial granules are released attached to the DNA structure and function in degrading entrapped microorganisms (Brinkmann et al., 2004). Among these anti-bacterial granules, Neutrophils elastase (NE), and myeloperoxidase (MPO) released from granules to the nucleus were found to be required for NET formation (Papayannopoulos et al., 2010).

Using NE immunostaining of PMA treated neutrophils we observed mixed stages of NET formation. In the early stage, we can see NE translocation to the nucleus, which was reported to precede MPO translocation followed by chromatin de-condensation (Figure 25, blue arrow). In the later stage, the neutrophils nuclei lose its characteristic lobules and chromatin de-condensation with the plasma membrane of activated neutrophil is still intact (Figure 25, yellow arrow). Finally, the cell membrane rupture and the NETs are released into the extracellular space as part of the NET structure (Figure 25, white arrow).

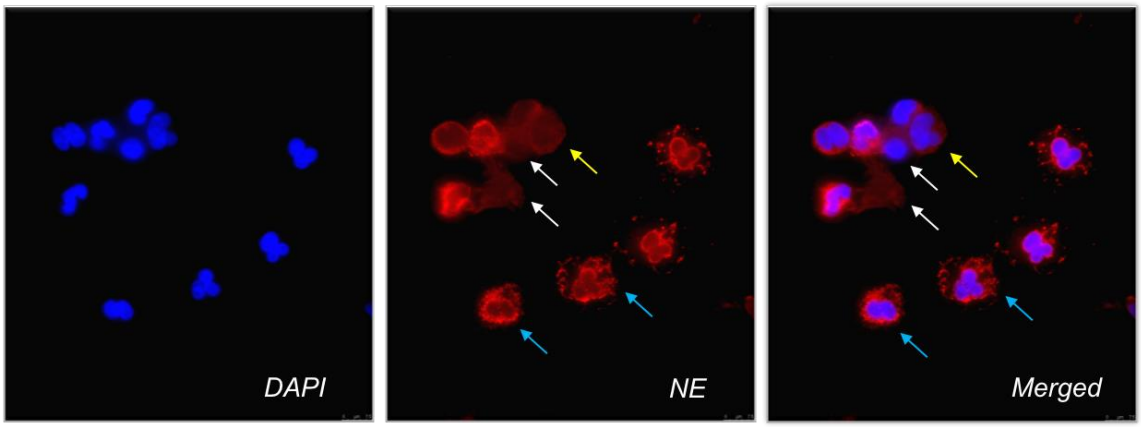


Figure 25: Stages in NET formation in PMA treated neutrophils. Cells ($1x10^6$ cells/ml) were seeded in poly L lysin coated coverslip , 30 min later they were treated with PMA (100nM) for 4 hours. Immunostaining using NE antibody (red) and DAPI (blue) was done to visualize NET formation and localize NE during neutrophil stimulation. Different stages of cell activation were detected. Starting from early stage of NE translocation to the nucleus (*blue* arrow) , followed by loss of characteristic lobules and chromatin decondensation (*yellow* arrow), and ending by NET release (*white* arrow).

Periodontitis is one of the most prevalent infectious diseases in humans (Pihlstrom et al., 2005). *F. nucleatum* has a definite role in periodontitis, due to its remarkable adhesive and adaptation properties (Diaz et al., 2002). Also, it has an essential role in supporting the growth of various bacterial species (Diaz et al., 2002). *F. nucleatum* considered one of the most abundant species in the oral cavity of healthy and diseased individuals (Guggenheim et al., 2009; Signat et al., 2011). Being a bridge bacterium in the interaction between early and late oral colonizing bacteria, it is a key pathogen in the development of dental plaque. Thus, it is linked to various forms of periodontal diseases starting from mild gingivitis to advanced

periodontitis (Guggenheim et al., 2009; Kistler et al., 2013; Zijnge et al., 2010) (Kolenbrander et al., 2006).

Numerous bacterial species have been reported to induce and be trapped by NETs, including periodontal pathogens (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012; Remijsen et al., 2011; Vitkov et al., 2009). *F. nucleatum* (MOI 1:10), was also associated with NETs when analyzed under SEM (Figure 8-9). NETs were observed as a web-like structure that spans between two neutrophils to entrap the bacteria (Figure 8c). In this study *F. nucleatum* was employed to stimulate NET production; as previous work done in our laboratory has demonstrated that *P. gingivalis* induces less NETs. That might be explained by the ability of *P. gingivalis* to manipulate the host innate immune system, thus affecting the ability of neutrophil to kill and produce NETs (Guggenheim et al., 2009; Hajishengallis, 2009; Sochalska and Potempa, 2017). We observed that using a higher concentration of *F. nucleatum* (MOI1:100) will not only lead to a faster and more robust neutrophils activation but also a higher cell death rate than NETosis (Figure 10). Moreover, an MOI of 1:10 is more clinically relevant, hence we decided to use *F. nucleatum* at MOI of 1:10 in all our investigations.

F. nucleatum is one of the largest microbes in the oral cavity. The length of this spindle-shaped rod bacteria ranges from 5 to 10 μm (Bolstad et al., 1996). It is possible that the large size of *F. nucleatum* is what drives neutrophils to NETosis instead of phagocytosis (Urban et al., 2006). It has been suggested that

neutrophils when highly primed by cytokines and exposed to opsonized microbes they will undergo apoptosis, whereas a weaker stimulus will lead to NETosis (Mayadas et al., 2014). *F. nucleatum* is considered to be less virulent when compared to the other red complex pathogen, thus might preferably induce neutrophils NETosis.

While the list of microbes and molecules capable of stimulating NET release is increasing, their induced response is not identical. Bacterial-host interaction elects different immune responses via a varied group of receptors and cytokines (Janeway and Medzhitov, 2002). By using qPCR arrays, custom designed to detect innate immune, apoptosis and GPCR signaling pathways, we sought to investigate which pathway is mostly related to *F. nucleatum* induced NETosis. Interestingly, our data showed that *F. nucleatum* upregulates NOD1 (44.8-folds) and NOD2 (31.8-fold) when compared to control after 8 hours of stimulation that resulted in NET formation (Figure 13). Furthermore, to determine if the upregulation of NOD1 and NOD1 receptors can occur with other types of bacterial stimulation, we challenged neutrophils with *F. nucleatum*, *P. gingivalis*, and *A. actinomycetemcomitans* with and without PMA. We found that between all groups, *F. nucleatum* caused the most significant upregulation with 8.3-folds and 9.4 folds rise in NOD1 and NOD2, respectively (Figure 15). NOD1 and NOD2 receptor are the first NLRs reported as direct intracellular pattern-recognition receptors (PRRs) (Chamaillard et al., 2003a). It has been shown that NLRs are necessary sensors of specific PAMPs. However, the mechanism by which NLRs detect the PAMPs

remains poorly understood, and it is still unclear if they directly bind to PAMPs, or it is only PAMPs that bind to adaptor proteins (Fritz et al., 2006). Given that NOD1 and NOD2 were significantly increased when challenged with *F. nucleatum*, we further analyzed the role of NOD1 and NOD2 receptors in NETosis.

HL60 cell line is a well-known model for neutrophils studies (Bohnsack and Chang, 1994; Collins, 1987). Using the CRISPR-Cas9 gene editing system we developed a NOD1 and NOD2 knockout HL-60 cell line, as a model for our experiments. Although HL60 did not provide a robust model for neutrophil activity and NET formation, it added to our understanding of the role of NOD receptors in a NET release. When challenging NOD1 and NOD2 knockout HL60 with *F. nucleatum* (MOI 1:10), the NET release was significantly reduced when compared to control cells (Figure 18). Quantification of histone-associated DNA fragments (H1, H2A, H2B, H3, and H4) in the cytoplasm of NOD1 Knockout cells was significantly reduced, but NOD2 Knockout cells showed no significant changes compared to control. This indicates that NOD1 but not NOD2 is associated with histones release (Figure 17). In addition, staining with NE revealed that while *F. nucleatum* successfully induced NET formation in HL60 wild-type cells, NOD-1 knockout HL60 cells formed significantly fewer NETs. Thus, confirming that NOD1 and NOD2 receptors play a role in the NET formation, with NOD1 being more associated with histone release. Knowing the importance of NOD1 and NOD2 receptors in NET formation, we further investigate the downstream activation of

these receptors and its link to other essential NET related proteins such as PAD4, MPO, and NE.

Peptidyl-arginine deiminase enzymes (PAD4), catalyze protein transformation into peptidyl-citrulline in a Ca^{2+} dependent manner (Leshner et al., 2012). PAD4 citrullination of histones is essential for chromatin de-condensation as a crucial step for NETs formation (Li et al., 2010). Thus, we investigated the link between the PAD4 enzyme and NOD-like receptors activation in neutrophils. The detection of citrullinated histones indicates the activation of the PAD4 enzyme. *F. nucleatum* (MOI 1:10), successfully induced PAD4 activation and citrullination of histones detected by immunostaining of Cit H3 in neutrophils (Figure 12).

Treating neutrophils with the MDP ligand strongly upregulated NOD2 receptor transcription (Figure 21a), while C12-iEDAP ligand was insignificant in NOD1 upregulation (Figure 20a). Both NOD1 and NOD2 ligands significantly upregulated IL8 expression (Figure 20b, 21b), indicating that despite the insignificant upregulation of NOD1, its receptor activity was increased. Moreover, each bacterial species has a different combination of surface antigens that result in variations in stimulation of host cells. The fact that *F. nucleatum* highly upregulates NOD1 in neutrophils (Figure 15) illustrate that there might be a peptide other than that present in C12-iEDAP that is more specific to the PGN of *F. nucleatum* resulting in NOD1 activation.

NOD1 specific inhibitor (ML130) significantly downregulate PAD4 activity while NOD2 inhibitor (GSK717) had no significant results, confirming that only NOD1 is related to histones released via PAD4 activation. These data coincide with our finding in HL60 knockout NOD1 and NOD2 histones quantification (Figure 17).

The citrullination of proteins by PAD enzymes are regulated at a transcriptional, translational and activation levels (Rodriguez et al., 2009). To determine whether the upregulation or downregulation of PAD4 expression when stimulated with NOD1 and NOD2 inhibitors/ ligands has a significant effect on its enzymatic activity, we used a fluorescence-based assay that allows for the monitoring of PAD4 activity (Wang et al., 2013). We found that PAD4 activity significantly increased with NOD1 ligand stimulation. Moreover, this rise in PAD4 activity was reduced considerably when pretreated with NOD1 inhibitor. On the other hand, NOD2 stimulation and inhibition had no significant effect on PAD4 enzymatic activity (Figure 23b). Thus, confirming that NOD1 but not NOD2 regulate PAD4 at both transcriptional and translational levels.

Myeloperoxidase (MPO) is one of the most abundant proteins in neutrophils (Schultz and Kaminker, 1962), and neutrophils completely deficient of MPO failed to make NETs (Metzler et al., 2011). Neutrophil elastase (NE) formation occurs during the promyelocytic differentiation, then its stored in the azurophilic granules for the life of the neutrophils until its activation (Doring, 1994). NE plays a critical rule in inducing, and its inhibition will impede chromatin de-condensation and NET

release (Papayannopoulos et al., 2010). Based on our knowledge, there are no reports on the role of NOD1 and NOD2 receptors on NE and MPO activity in human neutrophils. It was reported that NOD1 and NOD2 receptors are essential for neutrophil recruitment but do not impair the immune response in $NOD1^{-/-}$ and $NOD2^{-/-}$ mice. Moreover, they stimulated mice neutrophils for 2 hours with Litomosoides sigmodontis antigen (LsAg) or LPS and found that NE and MPO activity in NOD1-/- and NOD2-/- mice were comparable to the wild-type and they are not functionally impaired (Ajendra et al., 2016).

However, in our study, we found that stimulation of NOD1 and NOD2 for 4 hours with their specific ligands resulted in increased activity of both NE and MPO enzymes. Furthermore, inhibition of NOD1 and NOD2 receptors led to a significant reduction in NE and MPO release (Figure 24). It is possible that the insignificant results obtained from $NOD1^{-/-}$ and $NOD2^{-/-}$ mice were a result of inadequate stimulation time (only 2 hours) and that murine neutrophils activity slightly differs than human neutrophils. Also, MPO levels in mice neutrophils is only 10–20% that of human neutrophils (Kalupov et al., 2009; Noguchi et al., 2000; Rausch and Moore, 1975).

Conclusion and future directions:

This is the first study to elucidate the role of NOD-like receptors in NETosis and its downstream targets (Figure 26). To our knowledge, the importance of NOD-like receptor in chromatin de-condensation and PAD4 activation, and their effect on MPO and NE activity have not been addressed previously. I believe that our study will set the ground for a novel approach to one of the most controversial functions of neutrophils. By understanding the processes that govern NET pathway, we can further understand their role in infection and disease.

Furthermore, studying the pathway of NET formation that is related to both oral and systemic health, helps in understanding and targeting the pathways leading to NET related diseases. In addition to NET's role in restricting the spread of infection, there is a growing body of evidence that links NETosis and other various systemic disorders elucidating the importance of an efficient, non-invasive therapeutic modality. A drug therapy that controls the initiation of the NET pathway or the clearance of its byproducts could be the future in treating NET related cancer metastasis, autoimmune, chronic inflammatory and cardiovascular diseases. The periodontal pocket provides an ideal niche to test such therapeutics given its accessibility, its relationship to inflamed tissue and the offending microbal biofilm and the body of local delivery experience in this field.

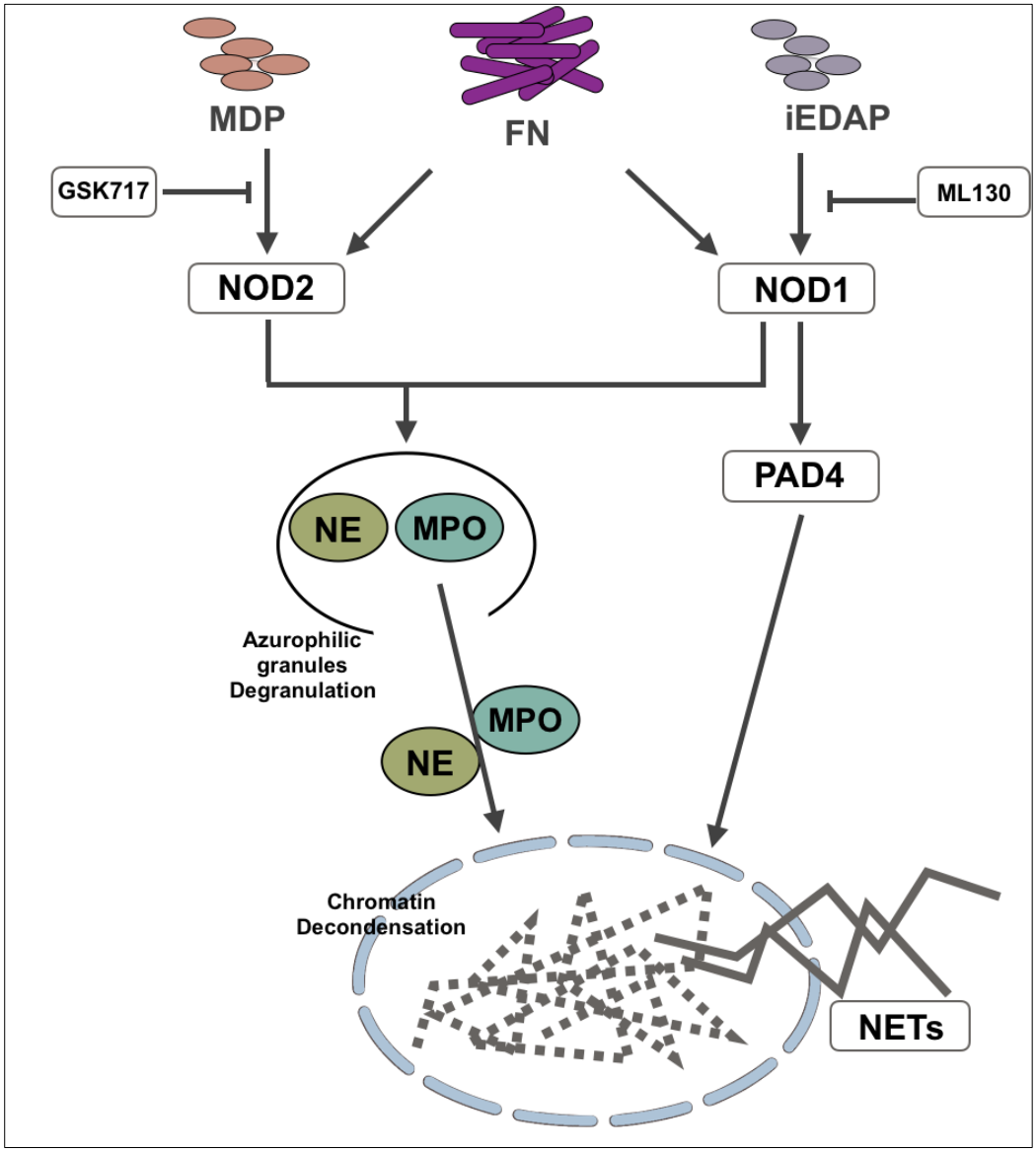


Figure 26: Schematic diagram summarizing NET related NOD1 and NOD2 signaling pathway.

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