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The roles of NADPH oxidase in modulating neutrophil effector responses

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Abstract

Neutrophils are phagocytic innate immune cells essential for killing bacteria via activation of a wide variety of effector responses and generation of large amounts of reactive oxygen species (ROS). Majority of the ROS in neutrophils is generated by activation of the superoxide-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Independent of their anti-microbial function, NADPH oxidase-derived ROS have emerged as key regulators of host immune responses and neutrophilic inflammation. Data from patients with inherited defects in the NADPH oxidase subunit alleles that ablate its enzyme function as well as mouse models demonstrate profound dysregulation of host inflammatory responses, neutrophil hyperactivation and tissue damage in response to microbial ligands or tissue trauma. A large body of literature now demonstrates how oxidants function as essential signaling molecules that are essential for the regulation of neutrophil responses during priming, degranulation, neutrophil extracellular trap formation, and apoptosis, independent of their role in microbial killing. In this review we summarize how NADPH oxidase-derived oxidants modulate neutrophil function in a cell intrinsic manner and regulate host inflammatory responses. In addition, we summarize studies that have elucidated possible roles of oxidants in neutrophilic responses within the oral mucosa and periodontal disease.

Keywords

chronic granulomatous disease; gp91^{phox}; NADPH oxidase; neutrophils; oxidative stress; periodontal disease; reactive oxygen species

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CONFLICT OF INTEREST

None declared.

1 | INTRODUCTION

Reactive oxygen species (ROS) collectively refer to a large group of highly reactive derivatives of oxygen generated as a consequence of metabolic processes or during host stress response. Although associated with oxidative damage, when produced at low regulated levels, oxidants are essential for redox modulation of cellular pathways, immune effector function, cell signaling and anti-microbial responses. The majority of ROS generated in a cell is by the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases or NOX enzyme complexes. NOX enzyme family members NOX1, NOX2, NOX3, NOX4, NOX5 and the dual oxidase (DUOX) enzymes DUOX1, and DUOX2 are membrane associated hetero-oligomeric complexes that are dedicated to generation of ROS by using oxygen as its substrate. NOX enzymes are expressed in a cell specific or tissue specific manner and are rapidly activated in response to external stressors to generate large amounts of ROS. NOX2 or gp91^{phox} is the main catalytic subunit of the leukocyte NADPH oxidase complex. It is highly expressed in phagocytes (neutrophils, monocytes, macrophages, dendritic cells) and at much lower levels in lymphocytes, endothelial cells and colonic epithelial cells.[1,2] NOX2 derived oxidants modulate multiple cellular processes independent of their anti-microbial function. For instance, NADPH oxidase activation is essential for antigen presentation in B cells[3], T cell receptor stimulation[4] and subset polarization.[5,6] In addition, Nox2 has also been detected in colonic epithelial cells where its capacity to produce superoxide (O_2^-) in response to microbial stimulations may facilitate the attachment of commensals such as *Escherichia coli* to the colonic mucus layer.[7] Epithelial cell-derived O_2^- within the gut has been implicated to modulate the composition of the gut microbiome, by altering gene transcription and crosstalk between bacteria. It should be noted that a homolog of Nox2, Noxl is expressed in the epithelial cells in the colon and may functionally overlap with Nox2 in the responses of these cells to the gut microbiome.[8,9] These findings indicate that oxidants play diverse roles in the regulation of host responses. NOX2 has been extensively studied over the last 40–50 years for its role in the activation of the phagocyte oxidative burst (*Phox*), also known as the respiratory burst during phagocytosis; however, recent data from our lab and others now demonstrate a critical role for NOX2-derived ROS in the modulation of neutrophil responses during inflammation. In this review, we will specifically focus on the newly appreciated roles of NOX2 or the leukocyte NADPH oxidase in the regulation of neutrophil effector functions in the context of inflammation and inflammatory disorders.

Neutrophils are the most abundant leukocytes in human blood that are crucial for host innate immune responses during infection and inflammation. In response to soluble or particulate stimuli, neutrophils activate a battery of effector responses including the activation of NADPH oxidase to produce large quantities of ROS. Oxidants generated are crucial for pathogen elimination, and can further activate or modulate other effector responses in neutrophils such as priming, degranulation, apoptosis and formation of neutrophil extracellular traps (NET). Neutrophils are functionally and phenotypically heterogeneous cells that influence the outcome in multiple chronic inflammatory diseases such as arthritis, dermatitis, and periodontitis, thus playing a key role in immune regulation.[10,11] Here we present mechanistic insights derived from published literate on how NADPH oxidase

derived oxidants regulate neutrophil effector responses outside of their role in microbial killing. Excessive ROS generation by neutrophils is often associated with oxidative stress and chronic inflammation, however this view is now being challenged from observations in patients and mouse models where complete loss of NADPH oxidase activity was associated with hyperinflammation. We have summarized recent published data from our laboratory and others that supports the somehow counter-intuitive role of NADPH oxidase in dampening host inflammation and redox regulation of host inflammatory pathways.

2 | COMPOSITION, ASSEMBLY AND ACTIVATION OF THE NADPH OXIDASE COMPLEX

The NADPH oxidase complex is comprised of 2 membrane bound subunits and three cytosolic regulatory subunits, all encoded by distinct genes. The **ph**agocyte **ox**idase or *phox* subunits are referred to by their molecular mass or gene names and assemble together to form an active complex that transfers NADPH-derived electrons to its substrate O_2. The membrane-restricted flavocytochrome$_{b558}$ is a homodimer formed by two integral membrane restricted proteins, the glycosylated gp91^{phox} (NOX2 or CYBB), and the non-glycosylated p22^{phox} (CYBA) subunits. The gp91^{phox} subunit is the main redox center of the complex, and is responsible for the electron transferase activity of the complex. The cytosolic flavin adenine dinucleotide (FAD) domain of gp91^{phox} receives two electrons from NADPH that are sequentially transferred along two internal heme moieties within the membrane-spanning gp91^{phox} core. The p22^{phox} subunit is an obligate binding partner of gp91^{phox} and is essential for the stability of the heterodimer. It also provides docking sites critical for the assembly of the regulatory subunits of the oxidase that translocate from the cytosol.[12] In resting neutrophils, the majority of the flavocytochrome$_{b558}$ stores are localized within the specific (secondary) granules and secretory vesicles. Neutrophil activation or priming causes granule exocytosis delivering the flavocytochrome$_{b558}$ to the plasma and phagosomal membranes.[13]

Activation of the enzyme complex is tightly regulated by a series of protein-protein and lipid binding interactions that mediate the translocation of the cytosolic subunits of NADPH oxidase to the plasma or phagosomal membrane. Concurrent events lead to Rac activation. Rac belongs to the Rho family of GTPases and is an obligate binding partner of the NADPH oxidase enzyme complex. The cytosolic subunits p47^{phox}(Neutrophil Cytosolic Factor 1 [NCF1]), p67^{phox} (NCF2), and p40^{phox} (NCF4), form a tripartite complex in the cytosol of quiescent neutrophils (Figure 1). In resting cells, p47^{phox} is present in an inactive or auto-inhibitory conformation. Upon phagocytosis or receptor ligation, downstream kinases such as protein kinase C (PKC) isoforms, protein kinase A (PKA), phosphoinositide 3-kinase (PI3K) and mitogen activated protein kinases (MAPKs) ERK1/2 and p38 mediate p47^{phox} phosphorylation.[14,15] Phosphorylation induced conformational changes in p47^{phox} exposes its Src-homology (SH) domain that interacts with Proline residues on p22^{phox}.[16] p47^{phox} also contains a PX (*Phox* homology) domains that bind to the transiently generated phosphoinositides within the membrane. This interaction allows for the retention of the subunit on the membrane.[15]

Activation and membrane translocation of p47^{phox} also brings p67^{phox} and p40^{phox} to the membrane. p67^{phox} has an internal activation domain that regulates the transfer of NADPH-derived electrons to the flavin domain of gp91^{phox}.[17] p67^{phox} interacts with GTP-bound Rac, an essential component of the NADPH oxidase holoenzyme. Concurrent to the activation of NADPH oxidase cytosolic subunits, Rac-GDP is converted to its active Rac-GTP form by guanine nucleotide exchange factors (GEFs). Rac-GTP translocates to the membrane and binds to gp91^{phox} and p67^{phox}.[18,22] p47^{phox} and p40^{phox} act as adaptor proteins that positively regulate enzyme assembly on the plasma membrane. p40^{phox} plays a specialized role in regulating NADPH oxidase activation. It binds to the phosphatidylinositol 3-phosphate (PI(3)P) generated in the membrane via its PX domain. Matute et al reported a patient with missense mutations in the PI3P binding domain of p40^{phox} had selective defects in phagosomal ROS generation in neutrophils, while the plasma membrane levels were unaffected.[23,24] Thus, p40^{phox} positively regulates NADPH oxidase function and is essential for high-level O_2^- generation within phagosomes.

Upon complete assembly, the NADPH oxidase transfers NADPH derived-electrons to molecular O_2 generating O_2^-(Figure 1). Large amounts of O_2^- are generated by human neutrophils. For instance, 7–10 nmol min^{-1} 10^{-6} cells of O_2^- are produced on stimulation with phorbol 12-myristate 13-acetate (PMA) as measured by ferric cyto-chrome *C* assays. Other factors contributing to sustained O_2^- production include a quick turnover of the enzyme complex and high affinity for its substrate O_2. The K_m for oxygen (NADPH oxidase substrate) is ~10, equivalent to ~7 mm Hg pO_2. Thus, the NADPH oxidase complex can continue to produce O_2^- in tissues with low oxygen tension such as the gingival cavity.[20,25]

Excessive ROS generation or prolonged activation of the enzyme complex can cause oxidative damage and prolong inflammation. Thus, the activity of NADPH is tightly regulated and triggered only in response to stimuli via complex signaling pathways. Apart from the kinases that mediate subunit phosphorylation and membrane phospholipid generation, activation of voltage gated proton channels, chloride channels and accompanying calcium fluxes also regulate NADPH oxidase activity (reviewed by Nunes et al[18]). The absolute amount of ROS generated is also dependent on the priming status and complexing of activating receptors on neutrophils. Further, neutrophils also contain “deactivation pathways” that lead to disassembly of the NADPH oxidase and terminate O_2^- generation (reviewed by Decoursey et al[26]).

3 | NADPH OXIDASE IN OXIDATIVE KILLING OF MICROBES

Neutrophils are phagocytic cells whose primary function is to defend the host against bacterial and fungal infections. Inherited defects that adversely affect neutrophil numbers or their effector functions are associated with higher susceptibility to recurrent infections indicating that neutrophils are indeed key in the early responses to an infection. To facilitate the recognition of microbial ligands, and their phagocytosis, neutrophils express a large arsenal of receptors. Ligation or activation of Toll like receptors (TLRs), Fc gamma receptors (FcR), G-protein coupled receptors (GPCRs), C-type lectin receptors and integrins on neutrophils potently activates NADPH oxidase, generating large amounts of oxidants.[27,28] Separately, antimicrobial granule proteins (lactoferrin, myeloperoxidase, defensins,

proteases, lysozyme, calprotectin, etc) are rapidly mobilized into phagosomes or into the extracellular environment that aid in microbial killing. Conventionally this has been viewed as a non-oxidative mechanism of killing. However, now it is increasingly apparent that the oxidative (ROS generation) and non-oxidative mechanisms synergize and work in concert to kill bacteria. We refer the readers to excellent reviews by Nauseef et al[29] and Uriarte et al,[30] that discuss the synergy and interdependence of these processes.

Although large amounts of O_2^- are produced during phagocytosis, O_2^- anions have low antibacterial activity and are short lived (microseconds). They either dismutate spontaneously or are enzymatically converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. H_2O_2 is further converted to other forms of ROS with potent anti-microbial activity by the myeloperoxidase-hydrogen peroxide-halide system that converts H_2O_2 to hypochlorous acid on reaction with chloride ions (Cl^-). Myeloperoxidase (MPO) released from neutrophil primary/azurophilic granules into the phagosomes is critical for this process. Neutrophils isolated from patients with MPO deficiency are less effective in killing bacteria, demonstrating a synergy between oxidative and non-oxidative killing pathways.[31] Separately, H_2O_2 can also be oxidized by iron to produce hydroxyl radicals (OH^-) via Fenton chemistry that is highly toxic to bacteria.[27,32] The antimicrobial potential for ROS stems from its ability to cause irreversible damage to bacterial DNA, proteins, and lipids that eventually kills the bacteria. To prolong their survival, several pathogenic bacteria actively produce toxins or effector proteins to prevent the translocation of NADPH oxidase subunits to phagosomes and restrict NADPH oxidase assembly on the phagosome. Pathogens such as *Francisella tularensis, Anaplasma phagocytophilum,* and *Helicobacter pylori* have developed strategies to subvert killing by neutrophils. Virulence mechanisms employed by these pathogens are listed in Table 1.

The critical role of NADPH oxidase in host antimicrobial responses was evident from chronic granulomatous disease (CGD), an immunodeficiency affecting ~1 out of 200 000 individuals in the US. X-linked (*CYBB*) or autosomal recessive mutations in NADPH oxidase genes (*CYBA*, *NCF1*, *NCF2*, and *NCF4*) that ablate enzyme activity cause CGD.[23,24,33–35] CGD patients suffer from life-threatening bacterial and fungal infections resulting in high mortality and morbidity. Over-representation of certain bacterial infections caused by catalase positive microorganisms such as *Staphylococcus aureus, Pseudomonas, Burkholderia cepacia* as well as fungal species such as *Aspergillus* are common. Despite prophylactic use of antibiotics and antifungals, the average life span of CGD patients is short (four decades) and associated with significant morbidity. Thus, the oxidants generated by NADPH oxidase are critical mediators of host anti-microbial defense.

4 | NADPH OXIDASE IN NEUTROPHIL PRIMING AND DEGRANULATION

Neutrophil priming or pre-activation refers to the broad range of phenotypic and molecular changes that poise neutrophils in a state of heightened sensitivity or “readiness” to respond to subsequent secondary stimulation. Circulating neutrophils exist in a relatively quiescent state. Their activation is dynamically regulated and pro-gresses from a quiescent state to an intermediate “primed” state as they transmigrate to the site of infection or injury and encounter low levels of inflammatory cytokines (TNF-α, GM-CSF, IL-1β), chemoattractants

(C5a, fMLF PAF, LTB4) or TLR agonists (LPS, flagellin, lipopeptides). A comprehensive list of neutrophil priming agents can be found in excellent reviews by Miralda et al[36] and El-Benna et al.[37]

Priming augments the microbicidal capacity of neutrophils by enhancing O_2^- production, degranulation responses, inflammatory mediator production, and phagocytic capacity.[36–38] However, priming alone does not cause preassembly of the NADPH oxidase complex. Priming agents can induce partial phosphorylation of NADPH oxidase cytosolic subunits with partial exocytosis of secretory vesicles and secondary granules that contain ~60%−70% flavocytochrome$_{b558}$ to the plasma membrane. GM-CSF and TNF-α induce partial phosphorylation of p47^{phox} at the Ser345 residue via activation of ERK1/2 and p38 MAPKs. Site-directed mutagenesis experiments demonstrated that phosphorylation of Ser345 on p38 is critical for neutrophil priming.[39,40] The TNF-α-induced increase of gp91^{phox} at the plasma membrane is dependent on granule exocytosis.[41] Endotoxin induced priming responses elicited by low doses of lipopolysaccharides (LPS) induce the redistribution of flavocytochrome$_{b558}$ from secondary granules to the plasma membrane[42] and also into the recycling endosomal compartments. Thus changes in the location and/or phosphorylation of NADPH oxidase subunits upon priming leads to the generation of tonic or low basal levels of intracellular ROS critical in regulating the magnitude of neutrophil degranulation responses to secondary stimulation.[43,44]

Neutrophils contain four different types of granules. The primary (azurophilic) granules contain histotoxic enzymes, including elastase, MPO, and antimicrobial enzymes, cathepsins and defensins. The secondary and tertiary granules contain lactoferrin and matrix metalloprotease 9 (also known as gelatinase B), respec-tively. The secretory vesicles in human neutrophils contain human serum albumin, adhesion molecules and receptors such as CD11b and formyl peptide receptor (FPR1). Degranulation is a hierarchical process that is mediated by tightly regulated pathways.[45] While exocytosis of tertiary granules and secretory vesicles can occur readily, exocytosis of azurophilic granules requires neutrophil priming, MAPK activation and low-level ROS generation.[46,47] Potera et al suggested that NADPH oxidase-derived ROS are essential for preventing excessive degranulation responses. Further, neutrophils isolated from CGD patients that lack NADPH oxidase activity had significantly elevated elastase release from primary granules under both, unstimulated and TNF-α primed conditions.[46] CGD neutrophils also have enhanced basal levels of activation markers like CDiib.[47] These data indicate that NADPH oxidase-derived ROS are necessary to provide a negative feedback loop and prevent excessive degranulation that might lead to tissue damage. Alternatively, hyperactivation of other activation markers may reflect an attempt to compensate for the defect in ROS-mediated microbial killing due to the lack of NADPH oxidase activity.

While priming can facilitate the microbicidal potential of neutrophils, it can sustain or amplify neutrophil responses, thereby exacerbating chronic inflammatory disorders. Primed neutrophils have been observed in blood isolated from patients with systemic insults such as acute lung injury (ALI), trauma and hemorrhagic shock[36] as well as from patients with chronic diseases such as inflammatory bowel disease,[48] arthritis[49] and periodontitis.[50,51]

Thus, understanding the molecular basis of how oxidants govern neutrophil priming responses is essential.

5 | NADPH OXIDASE IN NET FORMATION

NETosis refers to the extrusion of decondensed chromatin that is in-terlaced with neutrophil granular proteins and mitochondrial mate-rial from neutrophils. NETs contain nuclear DNA, are proteolytically active, and form a mesh like structure to trap bacteria in vitro and in vivo. NETs have emerged as an important arm of innate immune anti-microbial responses since their first description by Brinkmann et al.[52] However, the molecular pathways leading to NET formation are diverse and NET formation can occur via a NADPH oxidase dependent or independent manner.

A diverse range of stimuli such as bacterial and fungal pathogens, calcium ionophores, inflammatory cytokines, PMA and immune complexes can all induce NETosis in vitro. In the NADPH oxidase dependent pathway of NET generation, O_2^- is converted to H_2O_2 which is the substrate for MPO. Thus, ROS mediated MPO activation and azurophilic granule mobilization causes the release of neutrophil elastase into the cytosol. Once in the cytosol, neutrophil elastase cleaves F-actin to break down plasma membrane integrity and also translocates to the nucleus to cleave histones promoting chromatin decondensation and subsequent extrusion.[53] Chromatin decondensation is also promoted by the activation of peptidylarginine deiminase 4, an enzyme that citrullinates histones H3.[54] CGD neutrophils fail to form NETs in response to various stimuli.[55,56] The role of ROS in NET formation has also been controversial due to discrepancies in comparing healthy neutrophils treated with ROS scavengers to neutrophils isolated from CGD patients. Separately, the requirement for NADPH oxidase-derived ROS can vary depending on the nature of the activating stimuli. While the NADPH oxidase was dispensable for NET formation in response to *Candida albicans,* CGD neutrophils produced significantly less NETs in response to *Aspergillus fumigatus,* another fungal pathogen associated with life-threaten-ing infections and mortality in CGD patients.

6 | NADPH OXIDASE IN NEUTROPHIL LIFESPAN

Neutrophils are the most abundant immune cell in the blood and are recruited in large numbers to inflammatory sites where their collective histotoxic potential can prolong inflammation. Thus, timely regulation of neutrophil apoptosis is essential for resolution of inflammation and prevention of the release of its cytotoxic cargo into the tissue environment. Neutrophil apoptosis occurs via highly conserved programmed cell death pathways. The extrinsic pathway is triggered via ligation and oligomerization of death receptors (Fas, TRAIL, TNF-α receptors) that lead to the formation of a death inducing signaling complex and subsequent caspase activation. The intrinsic pathway is induced by incompletely characterized factors that precipitate in mitochondrial outer membrane permeabilization and the release of pro-apoptotic proteins (Bcl2 family members) into the cytosol. This leads to activation of executioner caspases.[57–59]

In neutrophils apoptosis is “potentiated” via the activation of the NADPH oxidase and ROS generation. Phagocytosis and consequent NADPH oxidase activation by bacteria trigger a third apoptosis pathway, known as the phagocytosis induced cell death (PICD) pathway.[60–66] Activation of the NADPH oxidase is positively regulated by the numbers of bacteria phagocytosed (multiplicity of infection),[63] as well the complexing of NADPH oxidase activating receptors.[66] Certain pathogens actively subvert NADPH oxidase activity subverting PICD to survive in neutrophils (Table 1). CGD neutrophils lacking NADPH oxidase function are unable to undergo PICD.[67] When injected with heat-killed *S. aureus,* oxidase null mice exhibited defective apoptosis in vivo leading to impaired recognition and subsequent clearance.[68] Delayed neutrophil apoptosis can dysregulate resolution as neutrophils in inflammed tissues can actively sustain inflammation via prolonged production and release of inflammatory mediators.

7 | NADPH OXIDASE IN EFFEROCYTOSIS OF APOPTOTIC NEUTROPHILS

Efferocytosis refers to the ingestion of apoptotic cells by professional phagocytes and other non-phagocytic cells. Prompt removal of apoptotic cells is essential to prevent the loss of cellular integrity and the leakage of cellular contents. Dying cells advertise their presence by the production of several secreted “find me” signals and simultaneously undergo molecular changes that generate “eat me” signals essential for uptake. Up-regulation of phosphatidylserine (PS) in the outer leaflet of the plasma membrane of apoptotic cells provides a key apoptosis associated ligand. PS receptors (TIM4, BAI1, TAM) then regulate the uptake of PS expressing cells and their subsequent clearance.[69,70] Recent studies demonstrated that oxidation of PS at the fatty acyl chains transforms it to a more potent agonist of PS receptors such as CD36.[71,72] The NADPH oxidase is involved in both PS oxidation and externalization that promote the recognition of apoptotic neutrophil and eventual clearance by macrophages.[72,73] NADPH oxidase inhibitors that delayed lyso-PS generation delayed the uptake of apoptotic neutrophils in vitro. In vivo studies using gp91$^{phox-/-}$ mice demonstrated that lower levels of lyso-PS correlated with dysregulated clearance and prolonged inflammation in gp91$^{phox-/-}$ mice during zymosan-induced peritonitis.[73]

We recently demonstrated that NADPH oxidase was also involved in the efferocytic clearance of ingested apoptotic neutrophils by mouse peritoneal exudate macrophages (PEMs). ROS generation positively regulated efferosomal acidification and proteolysis of ingested apoptotic neutrophils. PEMs from gp91$^{phox-/-}$ mice that lack the capacity to generate ROS, exhibited significant delays in the clearance of ingested apoptotic cells. Cross presentation of apoptotic cells associated antigens to CD8 T cells was enhanced leading to CD8 T cell clonal expansion.[74] Ingestion of apoptotic neutrophils is also an important resolution signal and reprograms macrophages from a pro-inflammatory (M1) phenotype to an anti-inflammatory or pro-resolving (M2) and augments the generation of pro-resolving mediators. Impaired efferocytosis in gp91$^{phox-/-}$ mice correlated with skewing of macrophages to the M1 phenotype and reprogramming defects that enhanced inflammation.[75,76] Monocyte derived-macrophages from CGD patients produced significantly lower levels of anti-inflammatory mediators, prostaglandin D2 (PGD2) and TGF-B on efferocytosis.[77] Thus, the NADPH oxidase is essential not only for the proper recognition and digestion of

apoptotic neutrophils, but also plays an essential role in reprogramming efferocytosing macrophages to promote resolution of inflammation. CGD patients are markedly compromised in their ability to produce the anti-in-flammatory mediators PGD2 and TGF-β during the phagocytosis of apoptotic cells.

8 | NADPH OXIDASE MODULATES ACUTE AND CHRONIC INFLAMMATORY RESPONSES IN VIVO

Excessive ROS generation has been associated with oxidative stress in multiple chronic inflammatory disorders such as periodontitis, cardiovascular disease, neurodegenerative disorders and inflammatory diseases.[1] However these associations are based on correlative data linking oxidative stress biomarkers with disease and it is unclear whether the presence of oxidative stress is a cause or consequence of low-grade inflammation associated with these disorders. More di-rect approaches using deletion of NADPH oxidase genes in mouse models that entirely ablates NADPH oxidase derived ROS as well as data from CGD patients all paradoxically showed worse, often profound inflammation in the case of oxidase deficiency (Table 2). For instance, CGD patients are highly susceptible to sterile inflammatory complications, such as granulomatous inflammation in the genitourinary and gastrointestinal tract, and discoid mucocutaneous skin lesions. Further, mice lacking NADPH oxidase activity (due to deletion of *Cybb* [encoding gp91^{phox}]) exhibited hyper-inflammatory responses characterized by elevated levels of pro-inflammatory cytokines and excessive neutrophilic recruitment in multiple models of sterile or microbe-elicited challenge.[74,78‹80] Interestingly, hyperinflammation in these mice was observed even in the absence of active infection. In vivo challenge with heat killed *A. fumigatus* hyphae,[81] LPS,[82] Dectin-1 agonists,[83] zymosan[75,84] and even necrotic cells,[79] all resulted in exuberant neutrophilic inflammation characterized by enhanced levels of pro-inflammatory cytokines, neutrophil and macrophage infiltration and delayed resolution (summarized in Table 2). We recently demonstrated excessive mobilization of neutrophils from the bone marrow reserves in *Cybb*$^{-/-}$ mice in a model of sterile peritoneal injury. Excessive neutrophil mobilization augmented tissue accrual of neutrophils and significantly delayed resolution of inflammation in *Cybb*$^{-/-}$ mice compared to wildtype mice.[79] ROS deficiency also predisposes to autoimmune disorders. Oxidase null mice developed worse disease in models of collagen-induced arthritis,[85,86] mannan-induced psoriasis[85] and developed lupus-like disease with glomerular-nephritis.[87–89] These data indicate that oxidants in fact are essential for suppressing excessive activation of host inflammatory responses triggered by endogenous and microbial stimulation.

Periodontal diseases collectively refer to a broad range of inflammatory conditions that affect the supporting structures of the teeth (gingiva, alveolar bone, and periodontal ligament). Neutrophil dysfunction, hyperactivation, and hyper-recruitment have all been associated with worse periodontal disease, pointing to a key role of neutrophils in gingival homeostasis.[11] Interestingly, contrary to the protective role of NADPH oxidase in limiting host inflammation, some studies ascribe excessive ROS generation by neutrophils in response to periodontal bacteria as a significant contributing factor to the pathophysiology of periodontitis.[50,90,91] Whether excessive ROS generation by neutrophils in this case is a

cause or consequence of active inflammation associated with periodontitis is unclear. Proinflammatory cytokines such as TNF-α are often elevated in the gingival crevicular fluid and serum of patients with localized aggressive or chronic forms of periodontitis.[92–94] TNF-α induces phosphorylation and membrane translocation of cytosolic regulatory subunits of the oxidase, resulting in the preassembly of active NADPH oxidase enzyme complex on endosomal membranes. Thus, neutrophils isolated from peritonitis patients with elevated circulating levels of cytokines are likely to have been "primed" in vivo, which accounts for an augmented oxidative burst on secondary in vitro challenge with other agonists as compared to neutrophils isolated from healthy individuals.[18,43]

Others report that inhibiting ROS by the use of ROS inhibitors such as N-acetylcysteine (NAC) ameliorates ROS mediated cytotoxicity, and inflammatory pathways relevant to gingival inflammation in vitro.[95] However, it should be noted that ROS inhibitors, such as NAC, can independently and non-specifically inhibit inflammatory pathways, complicating interpretation.[96] Thus, systematic studies using gene-targeted approaches to specifically delineate the role of NADPH oxidase-derived ROS in periodontal diseases as well as other chronic inflammatory disorders are needed to determine how ROS modulates host inflammatory response.

9 | CONCLUSIONS

Our understanding of the function of the NADPH oxidase has greatly evolved from it being simply an antimicrobial effector to a master regulator of host inflammatory pathways. Besides its role in the regulation of neutrophil effector responses (Figure 2), NADPH oxidase activation is also essential for antigen presenta-tion, autophagy, chemotaxis, and redox signaling in other immune cells. The global role played by the NADPH oxidase in the modulation of host inflammatory responses is also supported by genome wide association studies that link single nucleotide polymorphisms and hypomorphic NADPH oxidase subunit alleles with various chronic inflammatory and autoimmune disorders such as arthritis,[97] lupus,[98–100] and inflammatory bowel disease.[101,102] We along with others demonstrated that oxidants were in fact essential for regulating both the duration and magnitude of host immune responses in multiple murine models. Thus although excessive ROS production might indeed cause oxidative stress, low or tonic levels of ROS are essential to regulate host inflammatory pathways and prevent chronic inflammation. These functions of ROS are independent of its anti-microbial role and we are only beginning to un-derstand the complexities through the use of total or conditional oxidase null mice.

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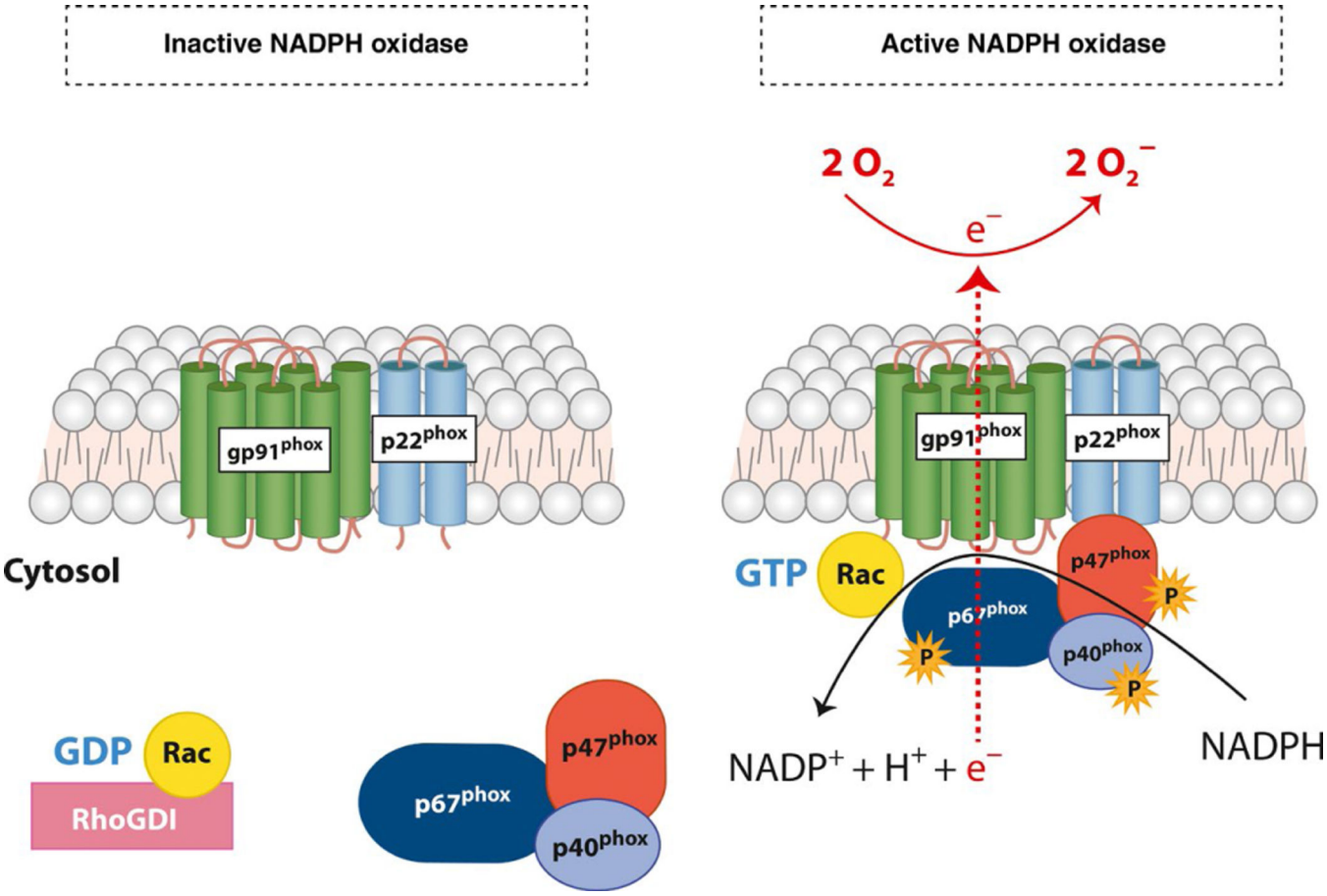

FIGURE 1.

NADPH oxidase structure and assembly. The membrane-restricted heterodimer of NADPH oxidase is comprised of gp91*phox* and p22*phox* subunits. This heterodimer is known as flavocytochrome$_{b558}$. In an inactive state, the cytosolic subunits p67*phox*, p47*phox*, and p40*phox* remain in the cytosol in a self-inhibitory confirmation. On activation, cellular kinases induce the phosphorylation of cytosolic subunits, releasing the inhibitory confirmation. p67*phox*, p47*phox*, and p40*phox* translocate to the membrane along with Rac-GTP and bind to the flavocytochrome$_{b558}$ forming an active enzyme complex. NADPH-derived electrons are transferred to the substrate molecular oxygen (O_2) generating superoxide (O_2^-) on the other side of the membrane.

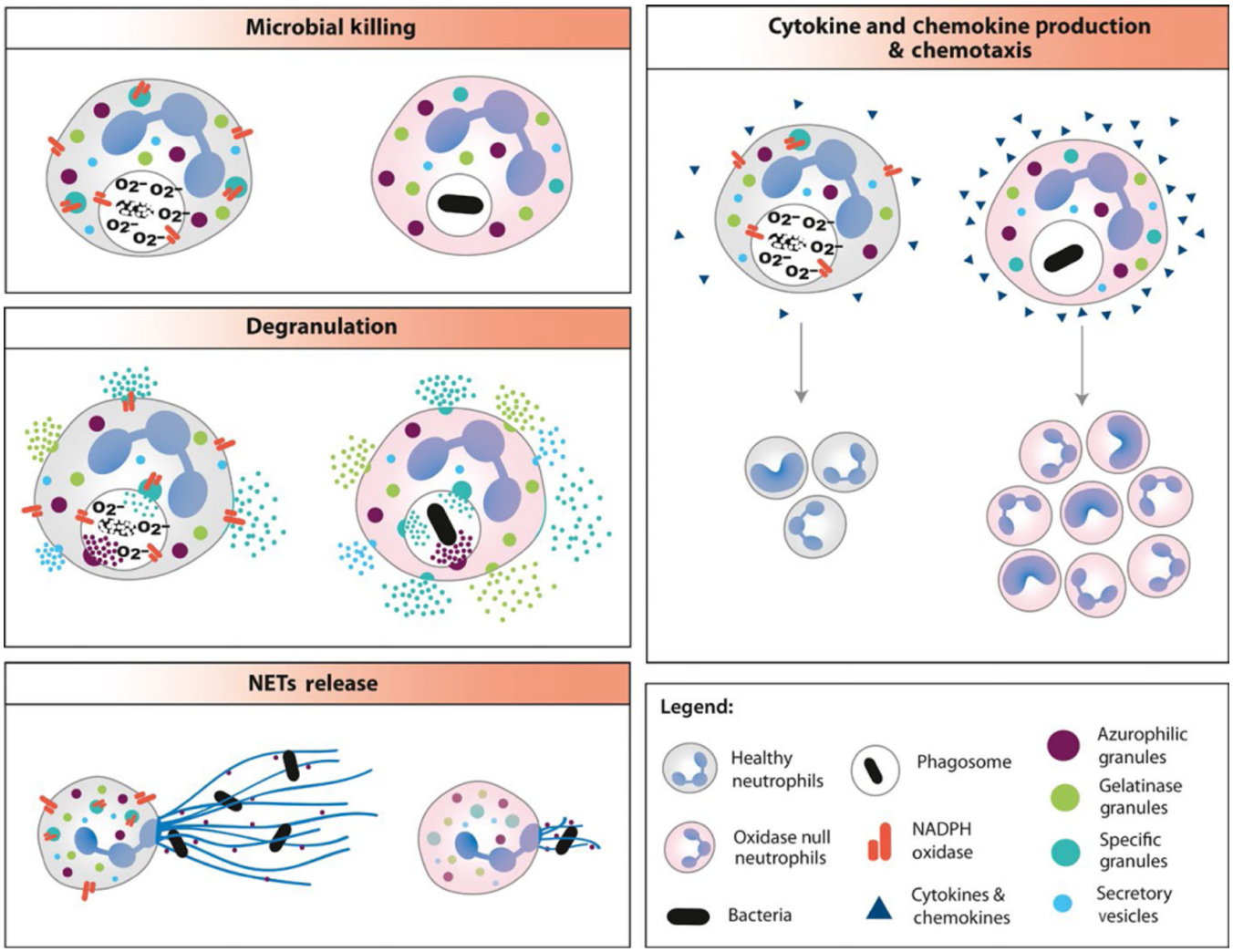

FIGURE 2.

NADPH oxidase derived ROS differentially modulate neutrophil effector responses. NADPH oxidase deficient (oxidase null) neutrophils are deficient in killing of catalase positive microorganisms and *Aspergillus* species. NET production is also compromised in response to select stimuli. ROS deficiency enhances degranulation, cytokine and chemokine generation that might lead to persistent inflammation by recruitment of other immune cells and/or their differential polarization. NADPH, nicotinamide adenine dinucleotide phosphate; NET, neutrophil extracellular traps; ROS, reactive oxygen species

TABLE 1

Bacterial suppression of neutrophil respiratory burst

Pathogen	Mechanism(s)
Anaplasma phagocytophilum	On internalization, the bacterium resides within a protective vacuole that excludes flavocytochrome$_{b558}$[103,104]; down-regulates CYBB (gp91^{phox}) gene expression.[105]
Coxiella burnetii	Membrane translocation of p47^{phox} and p67^{phox} is inhibited, in part by the production of a diffusible acid phosphatase.[106,107]
Francisella	*Francisella tularensis* (live vaccine strain) globally inhibits activation of respiratory burst by preventing flavocytochrome$_{b558}$ as well as cytosolic p47^{phox} and p67^{phox} acquisition on phagosomes.[108] *Francisella novicida* U112 produces an acid phosphatase that prevents phosphorylation and subsequent nuclear translocation of p47^{phox}.[109]
Helicobacter pylori	Although bacteria are internalized rapidly, O_2^- anions are produced extracellularly, but not inside phagosomes. p47^{phox}, p67^{phox} and gp91^{phox} are excluded from the phagosome by unopsonized *H. pylori* strains.[110]
Neisseria gonorrhoeae	Strains lacking Opacity (Opa)-Associated Protein on cell surface prevent the prevent p47^{phox} recruitment to phagosomes.[111]
Yersinia pestis	Produces YopE, a Type 3 secretion system (T3SS) effector protein that has GTPase like activity and inactivates Rac in neutrophils suppressing oxidative bust.[112,113]
Pseudomonas aeruginosa	Produces T3SS effectors ExoS and ExoT that inhibit PI3K signaling essential for activation of NADPH oxidase.[114]
Group A *Streptococcus*	Produces pore-forming toxin Streptolysin that inhibits NADPH oxidase activity via incompletely characterized mechanisms.[115]
Bordetella pertussis	Produces adenylate cyclase toxin-hemolysin (CyaA) that breaks down ATP to cyclic-AMP to activate Src homology region 2 domain-containing phosphatase-1 (SHP-1). SHP-1 inactivates MAPKs (ERK and p38). This reduces p47^{phox} phosphorylation and activation.[116]
Bacillus anthracis	Produces lethal toxin (LTx) and edema toxin (EDx) that interfere with upstream MAPK signaling events preventing optimal activation of NADPH oxidase subunit and subsequent assembly of active enzyme complex. EDx can also upregulate conversion of ATP to cAMP and inhibit ROS via mechanism similar to CyaA.[117]

TABLE 2

Inhibitory role of oxidants during host inflammation

Agonist/model	Inflammatory response
LPS intra-tracheal instillation	1. Neutrophilic alveolitis; enhanced Nf-κB activation associate with aggravated lung injury in p47$^{phox-/-}$ mice.[82,118] 2. Decreased IL-10 production through attenuation of Akt-GSK3-β pathways in p47$^{phox-/-}$ mice.[82]
Zymosan intra-tracheal	Neutrophilic inflammation and augmented inflammatory cytokine production in p47$^{phox-/-}$ mice.[119]
Aspergillus fumigatus	1. Invasive Aspergillosis, neutrophilic inflammation and granulomas in gp91$^{phox-/-}$ mice.[120] 2. Defective autophagy resulting in augmented IL-1β production that worsens inflammation in p47$^{phox-/-}$ mice.[121]
Tobacco smoke exposure	Exacerbated inflammation and lung emphysema in oxidase null (gp91$^{phox-/-}$ and p47$^{phox-/-}$) mice.[122]
Gram negative sepsis	Enhanced PMN alveolar transmigration and acute lung injury in oxidase null mice (gp91$^{phox-/-}$ and p47$^{phox-/-}$).[123]
Systemic inflammatory response syndrome (SIRS)	Early mortality due to hyper-inflammation and advanced lung pathology in oxidase null mice (gp91$^{phox-/-}$).[84]
Necrotic cells/DAMPs	Leukocytosis, neutrophilia and elevated tissue accrual of neutrophils that prolongs inflammation gp91$^{phox-/-}$ mice.[79,80]
Branched β-glucans	Skin granulomas.[81,83]
Collagen induced arthritis	Enhanced T cell autoreactivity, and development of severe arthritis.[85,124]
Influenza model	Increased inflammatory cell infiltration, Th1 driving cytokines in gp91$^{phox-/-}$ mice.[125]