



The role of NADPH oxidases in infectious and inflammatory diseases

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ABSTRACT

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are enzymes that generate superoxide or hydrogen peroxide from molecular oxygen utilizing NADPH as an electron donor. There are seven enzymes in the NOX family: NOX1-5 and dual oxidase (DUOX) 1–2. NOX enzymes in humans play important roles in diverse biological functions and vary in expression from tissue to tissue. Importantly, NOX2 is involved in regulating many aspects of innate and adaptive immunity, including regulation of type I interferons, the inflammasome, phagocytosis, antigen processing and presentation, and cell signaling. DUOX1 and DUOX2 play important roles in innate immune defenses at epithelial barriers. This review discusses the role of NOX enzymes in normal physiological processes as well as in disease. NOX enzymes are important in autoimmune diseases like type 1 diabetes and have also been implicated in acute lung injury caused by infection with SARS-CoV-2. Targeting NOX enzymes directly or through scavenging free radicals may be useful therapies for autoimmunity and acute lung injury where oxidative stress contributes to pathology.

1. Introduction

Reactive oxygen species (ROS) play an important role in multiple cellular processes including metabolism, signaling, and immunity. Cellular ROS are commonly generated from superoxide which is derived from two main sources: the mitochondria via oxidative phosphorylation and through NADPH oxidase (NOX) enzymes [1]. Enzymes in the NADPH oxidase family produce superoxide during normal cellular processes, but also produce superoxide as part of a respiratory burst during phagocytosis [2]. Production of superoxide is a critical cellular process that is required for the generation of other ROS such as peroxynitrite, hydrogen peroxide, hypochlorite, and hydroxyl radicals (Fig. 1). Generation of ROS is necessary for a variety of cellular functions, which are impaired in the absence of superoxide [2]. This review will discuss the importance of NOX enzymes and related proteins in immunity to pathogens, autoimmunity, and inflammation.

1.1. Discovery of NOX enzymes

NOX enzymes were first discovered as the missing component in phagocytic cells like neutrophils in patients with chronic granulomatous disease (CGD) [3]. CGD is caused by any mutations that lead to deficiency in NOX2 activity [4]. CGD patients have an increased susceptibility to certain bacterial and fungal infections and often present with

granulomas, not due to an obvious infection, which is where the name of the disorder is derived. Autoimmune diseases like systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are more common in patients with CGD and mouse models of NOX2 deficiency [5,6]. However, the cause of these aberrant immune responses is not completely understood [4,7].

It has long been known that ROS play an important role in diverse biological processes [8] and that ROS such as superoxide and hydrogen peroxide were produced in phagocytic leukocytes during phagocytosis [9–11]. The production of ROS during phagocytosis was proposed to be microbicidal [9], and it was later determined that this activity was dependent on NADH and NADPH oxidation [12,13]. Segal and colleagues determined that this respiratory burst was independent of mitochondrial-derived superoxide using spectroscopic analysis, which revealed a cytochrome b-like molecule that was present in fractionated phagosomes and separate from mitochondrial cytochrome *b* and endoplasmic reticulum (ER)-associated cytochrome P450 [14]. They also found that this cytochrome *b* peak was missing in patients with CGD [3]. The cytochrome *b* proteins of 91 and 22 kDa were biochemically isolated from granulocyte plasma membranes [15]. The genes coding for the 91 and 22 kDa proteins were mapped to the X chromosome and chromosome 16, respectively, and their gene products were subsequently cloned and characterized [16–19]. The 91 kDa protein, also known as gp91^{phox} or NOX2, is encoded by the *CYBB* gene (Fig. 2A). The 22 kDa or light chain of the cytochrome complex, also known as p22^{phox}, is

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Abbreviations

BCR	B Cell Receptor	NLRP1	Nucleotide-binding oligomerization domain, Leucine rich Repeat, and Pyrin domain containing protein 1
CGD	Chronic Granulomatous Disease	NLRP3	Nucleotide-binding oligomerization domain, Leucine rich Repeat, and Pyrin domain containing protein 3
COVID-19	Coronavirus Disease 2019	NOX	NADPH Oxidase
DC	Dendritic Cell	PB1	Phox and Bem1
DPI	Diphenyleneiodonium	Phox	Phagocytic Oxidase
DUOX	Dual Oxidase	PKC	Protein Kinase C
EGF	Epidermal Growth Factor	PMA	Phorbol 12-Myristate 13-Acetate
EGFR	Epidermal Growth Factor Receptor	PRR	Proline-Rich Region
ER	Endoplasmic Reticulum	PTP1B	Protein-Tyrosine Phosphatase 1B
FAD	Flavin Adenine Dinucleotide	PVPO	Poly(<i>N</i> -Vinylpyrrolidone)
fMLP	<i>N</i> -Formyl-Methionine-Leucyl-Phenylalanine	RA	Rheumatoid Arthritis
G-MDSC	Granulocytic Myeloid-Derived Suppressor Cells	ROS	Reactive Oxygen Species
G6PD	Glucose-6-phosphate dehydrogenase	SARS	Severe Acute Respiratory Syndrome
GILT	γ -Interferon-induced Lysosomal Thiol reductase	SLE	Systemic Lupus Erythematosus
IFN	Interferon	SOD	Superoxide Dismutase
IRF3	Interferon Regulatory Factor 3	TCR	T Cell Receptor
ISG	Interferon-Stimulated Gene	TLR	Toll-Like Receptor
MAVS	Mitochondrial Antiviral Signaling	TNF	Tumor Necrosis Factor
MPO	Myeloperoxidase	TPR	Tetratricopeptide Repeat
NADH	Nicotinamide Adenine Dinucleotide	VEGF	Vascular Endothelial Growth Factor
NADPH	Nicotinamide Adenine Dinucleotide Phosphate	VEGFR	Vascular Endothelial Growth Factor Receptor
NET	Neutrophil Extracellular Trap	XOR	Xanthine Oxidoreductase

encoded by the *CYBA* gene. Since this initial discovery, there have been a total of five NOX enzymes and two dual oxidase (DUOX) enzymes discovered (Fig. 2A) with conserved features.

1.2. NOX enzyme complexes generate superoxide anion

The NOX enzyme complexes are so named because they utilize NADPH as an electron donor to generate superoxide from molecular oxygen [12,13]. The five NOX enzymes (NOX1-5) and two DUOX

enzymes (DUOX1-2) each have six conserved transmembrane domains and a conserved C-terminal domain with FAD and NADPH binding sites (Fig. 2). The main catalytic units of NOX1-4 must form a dimer with the Superoxide-Generating NADPH Oxidase Light Chain Subunit (*CYBA*) for catalytic activity [20]. The activation of NOX1-3 also requires the activity of cytosolic factors for activation. DUOX1 and DUOX2 have an additional transmembrane domain called the peroxidase-like domain (Fig. 2A). NOX5, DUOX1, and DUOX2 also have EF hand domains that are involved in calcium signaling (Fig. 2A). After activation, the enzyme

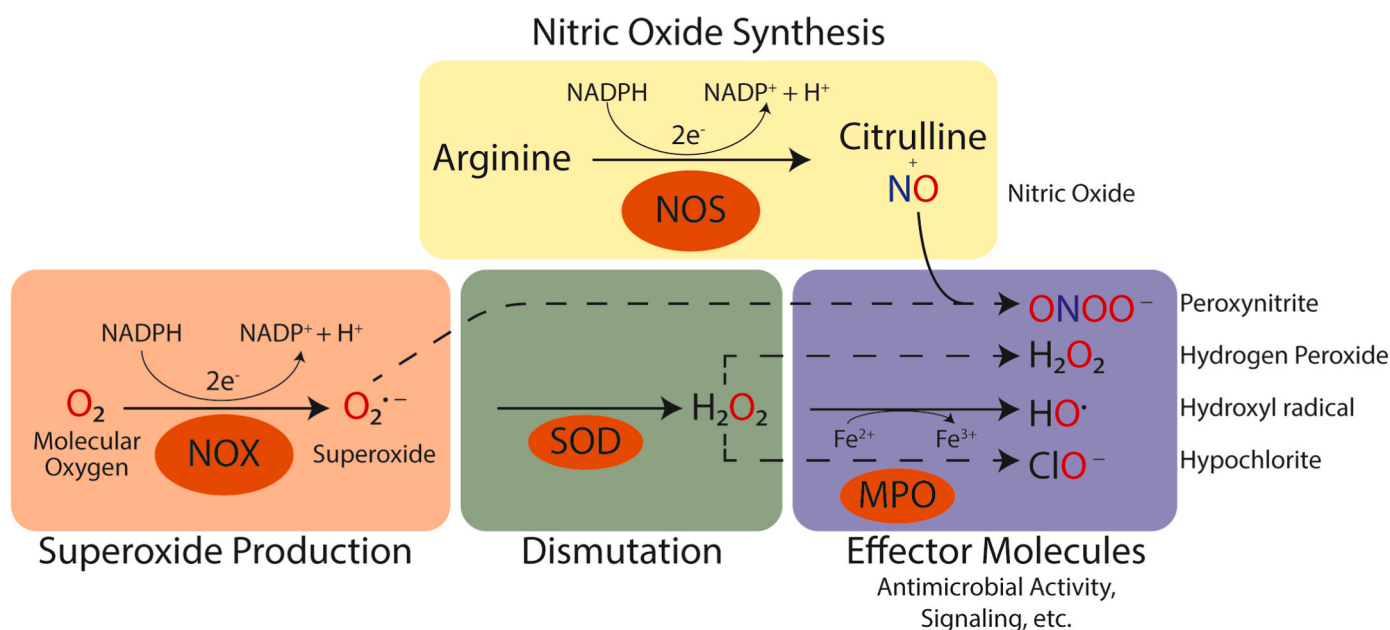
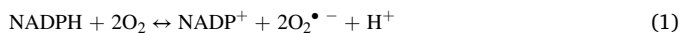


Fig. 1. Reactive oxygen species generated from NADPH oxidase-derived superoxide. NADPH oxidase enzymes convert molecular oxygen into superoxide anion ($O_2^{\bullet -}$) using NADPH as an electron donor. Superoxide dismutase enzymes dismutate superoxide into hydrogen peroxide (H_2O_2), which can be converted into hydroxyl radicals (HO^{\bullet}) through the reduction of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) or hypochlorite (ClO^-) by myeloperoxidase. Nitric oxide synthase using electrons from NADPH to oxidize arginine to produce citrulline and nitric oxide (NO). Nitric oxide (NO) reacts with superoxide anion ($O_2^{\bullet -}$) to produce peroxynitrite ($ONOO^-$).

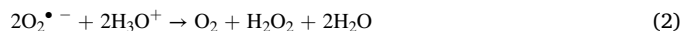
complex utilizes NADPH as an electron donor to convert molecular oxygen to superoxide (Eq. (1)).



Superoxide can also be generated by xanthine oxidase activity of Xanthine Oxidoreductase (XOR) enzymes [21]. XOR is primarily localized to the cytoplasm, but can also be found in the peroxisomes and secreted extracellularly [22,23]. XOR-derived superoxide plays an important role in many physiological processes, which have recently been reviewed in Ref. [21], including commensal microbiome regulation, blood pressure regulation, and immunity. XOR- and NOX-derived superoxide can work cooperatively to maintain superoxide levels. For example, in response to sheer stress, endothelial cells produce superoxide through NOX and XOR pathways and XOR expression and activity is dependent on NOX activity [24]. While this review will focus on NOX-derived superoxide it is important to recognize the contribution of XOR-derived superoxide in physiological processes and disease.

After the generation of superoxide, other ROS can be generated. Peroxynitrite (ONOO⁻) is formed after superoxide reacts with nitric oxide (NO) [25]. Nitric oxide is a product of arginine metabolism by nitric oxide synthase which uses arginine as a nitrogen donor and NADPH as an electron donor to produce citrulline and NO [26,27]. Superoxide can also be converted to hydrogen peroxide by the superoxide dismutase enzymes (SOD), which are critical for maintaining the balance of ROS inside the cells (Fig. 1). There are three superoxide dismutase enzymes, SOD1, SOD2, and SOD3. SOD1 is primarily

cytosolic and utilizes Cu²⁺ and Zn²⁺ ions to dismutate superoxide (Eq. (2)). SOD2 is localized to the mitochondria and utilizes Mn²⁺ to bind to superoxide products of oxidative phosphorylation and converts them to H₂O₂ (Eq. (2)). SOD3 is extracellular and generates H₂O₂ that can diffuse into cells through aquaporins [28,29].

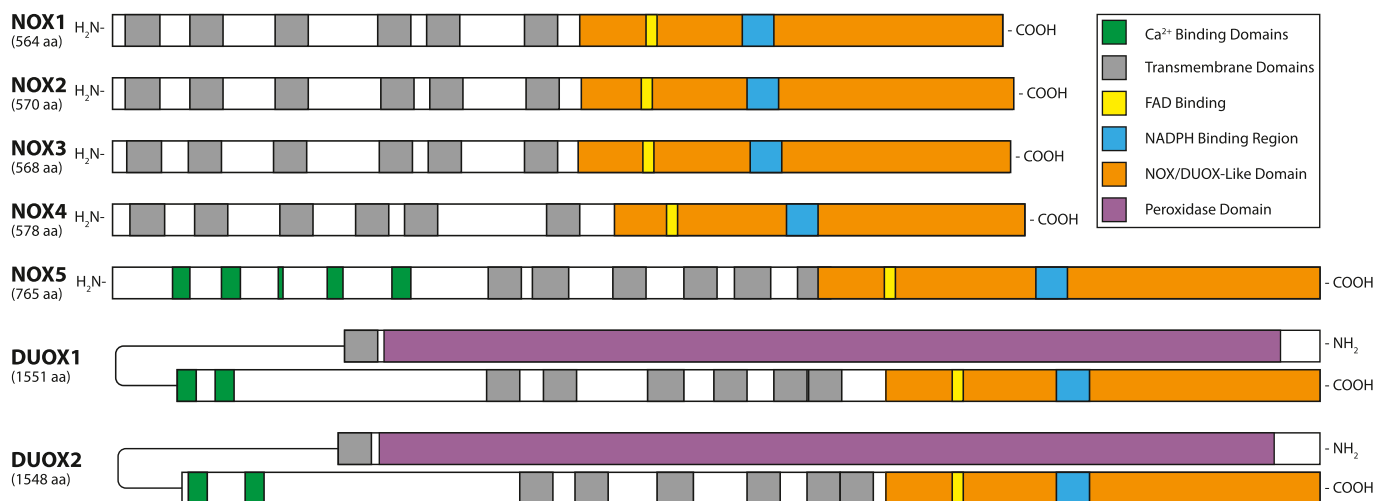


Following the generation of hydrogen peroxide by SOD enzymes, other ROS can be generated (Fig. 1). The enzyme myeloperoxidase (MPO) is responsible for hypochlorite (ClO⁻) formation by utilizing hydrogen peroxide as an oxygen donor and combining it with a chloride ion [30]. A spontaneous Fenton reaction with hydrogen peroxide and ferrous iron (Fe²⁺) results in the production of hydroxyl radicals (HO[•]) [31]. The specific role that each of these ROS play in cellular processes is beyond the scope of this review, but their dependence on superoxide generation highlights the key role of NOX enzymes in a variety of cellular processes.

2. Phagocytic NADPH oxidase 2 complex

The NOX2 complex is the prototypical and best-studied NOX enzyme complex. The NOX2 complex is comprised of two transmembrane proteins encoded by the *CYBB* and *CYBA* genes. The *CYBB* gene, located on the X chromosome, encodes for the cytochrome *b*-245 beta chain subunit also known as gp91^{phox} [18]. The gp91^{phox} heavy chain is initially translated in the ER where mannose side chains are co-translationally

A. Human NADPH Oxidase Enzymes 1-5 and Dual Oxidase 1-2



B. Homology of FAD Binding Site

	FAD Binding
*.....**.....*.....
	xxCPxxSxLEWHPFTLTSAPxE
NOX1	VN C PSI S LLEWHPFTLTSAP E
NOX2	VK C PKV S KLEWHPFTLTSAP E
NOX3	VQ C PAI S SLEWHPFTLTSAP Q
NOX4	LH C PSV S ALENHPPFTL M CP T E
NOX5	LN I PTIARYEWHPFTL I SSAP E Q
DUOX1	IA C LALG T TE Y HPFTLTSAP H E
DUOX2	IA C LALG T TE Y HPFTLTSAP H E

C. Homology of NADPH Binding Site

	NADPH Binding Region
**.....**.....*.....
	VxVLVGAGIGVTPFASILKSxxYKxxxx
NOX1	V A VLVGAGIGVTPFASILKS I W Y K F QCA
NOX2	V V MLVGAGIGVTPFASILKS V W Y K C NN
NOX3	V C VC V AAGIGVTPF A LLKS I W Y K C SEA
NOX4	V S LC V AGGIGVTPFASIL N TLLD --- D
NOX5	H A VL I GAGIG I TPFASIL S IM R HQKR
DUOX1	V S VLVGGGIGVTPFASIL K DLV F K S SVS
DUOX2	V S VLVGGGIGVTPFASIL K DLV F K S SLG

Fig. 2. Protein domains of human NADPH oxidase enzymes 1–2. (A) Conserved domains of human NADPH oxidase enzymes. (B) Amino acid sequences of the conserved FAD binding site in human NADPH oxidase enzymes. (C) Amino acid sequences of the conserved NADPH binding region in human NADPH oxidase enzymes. A “*” indicates residues that are fully conserved, a “.” indicates residues that are strongly conserved, and a “.” indicates residues that are weakly conserved. The consensus sequence is in bold.

added to form a 65 kDa precursor glycoprotein [32–34]. The 65 kDa precursor is further glycosylated in the Golgi network to become the mature 91 kDa glycoprotein [15].

The *CYBA* gene, located on chromosome 16, encodes for the Superoxide-Generating NADPH Oxidase Light Chain Subunit also known as p22^{phox} [35,36]. The p22^{phox} protein has two transmembrane domains with an extracellular loop and its C-terminal and N-terminal ends on the cytosolic side of the membrane [37]. Mutations in p22^{phox} that affect its function were discovered early on to be one cause of CGD [38]. p22^{phox} has no catalytic activity by itself; however, it is necessary for stabilization of the heterodimer complex with gp91^{phox} [37].

In the absence of p22^{phox}, gp91^{phox} is not able to exit the ER and move to the Golgi network and is degraded by the proteasome [39]. Therefore, individuals deficient for p22^{phox} will also be deficient for gp91^{phox} in phagocytic cells [18,40]. After gp91^{phox} and p22^{phox} are completely translated and gp91^{phox} has been glycosylated into its final 91 kDa form, the heterodimer then moves to the plasma membrane. NOX2 enzyme complexes are also found on the phagosome membranes, secretory vesicles, and peroxidase-negative granules in neutrophils [41]. The C-terminal end of p22^{phox} has a proline-rich region (PRR) that is important for binding of the cytosolic components of the enzyme complex [42].

2.1. NOX2 activation

The membrane-bound NOX2 components, gp91^{phox} and p22^{phox}, require the activity of the activator protein p47^{phox}, the organizer protein p67^{phox}, and the regulatory protein p40^{phox}, which exist in a ternary complex in the cytoplasm [43]. Experiments attempting to create a cell-free NADPH oxidase system demonstrated that membrane components alone were insufficient to reconstitute NADPH oxidase activity and that some unknown cytosolic components were also required [44–47]. The requirement for these cytosolic factors was confirmed in experiments where complementation with cytoplasmic fractions from phagocytic cells from certain patients with autosomal recessive forms of CGD could not restore oxidase activity [48,49].

NOX2 activation and assembly begins with a signaling event after

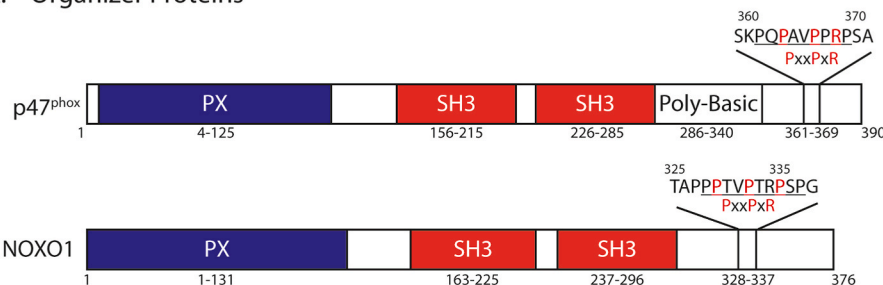
stimulation of formylated peptide receptors, C5a receptor, Fc receptors, or stimulation through a pattern recognition receptor such as toll-like receptor 4 (TLR4) [50–54]. Stimulation with chemicals like concanavalin A or phorbol 12-myristate 13-acetate (PMA) also induces activation of NOX2 [52,55]. What these various signaling pathways have in common is the activation of protein kinase C (PKC) [56]. PKC begins the activation and assembly process by phosphorylating p47^{phox} at serine residues 310 and 328 which causes a conformational change in p47^{phox} that results in its activation and recruitment to the NOX2 complex on the plasma membrane [50]. p47^{phox} recruits the other cytosolic components to the plasma membrane through multiple protein-protein interactions that are detailed in section 2.2.

2.2. Assembly of NOX2 complex

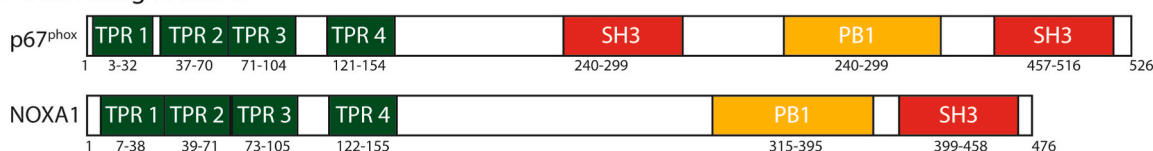
p47^{phox}, the protein encoded by the *NCF1* gene, was first purified from cellular fractions that produced a 47 kDa protein that was necessary to reconstitute a cell-free NADPH oxidase system [57,58]. The *NCF1* gene was cloned and characterized a year later by two independent groups [59,60]. The *NCF1* gene encodes for a 390 amino acid protein (Fig. 3A) that contains a Phox homology (PX) domain at its N-terminus that allows for p47^{phox} to anchor to the plasma membrane through phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂) binding [61–63]. p47^{phox} also has two SH3 domains and a PRR that are required for protein-protein interactions with other members of the NADPH oxidase complex.

p47^{phox} plays an important role in mediating protein-protein interactions required for activation and function of the NOX2 complex. p47^{phox} binds directly to gp91^{phox} and p22^{phox} and also recruits p67^{phox} to the plasma membrane to interact with the NOX2 enzyme complex. In its inactive state, the SH3 domains of p47^{phox} are occluded by intramolecular interactions with the C-terminus of p47^{phox}, an interaction that is undone by activators of oxidase activity [60,64,65]. After activation, p47^{phox} is recruited to the membrane by p22^{phox} via interactions between the SH3 domains of p47^{phox} and the PRR of p22^{phox}. This interaction is dependent on Ile152, Thr153, and Trp193 in p47^{phox} and Pro152, Pro153, Pro156, and Arg158 in p22^{phox} [60,64,66,67]. Indeed,

A. Organizer Proteins



B. Activating Proteins



C. Regulatory Proteins

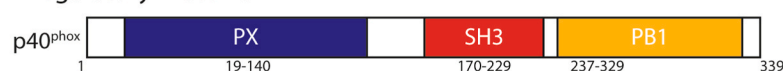


Fig. 3. Protein domains of the NADPH oxidase-associated cytosolic proteins. (A) Protein domains of the organizing proteins p47^{phox} and NOXO1. (B) Protein domains of the activating proteins p67^{phox} and NOXA1. (C) Protein domains of the regulatory protein p40^{phox}.

patients with a Pro156Glu mutation on p22^{phox} are unable to recruit p47^{phox} and p67^{phox} and are deficient in superoxide activity [60,68,69]. p47^{phox} also binds to membrane-bound gp91^{phox} with both of its SH3 domains required for this interaction with gp91^{phox} [70]. Patients with an Asp500Gly mutation in gp91^{phox} are unable to recruit p47^{phox} to the membrane and are deficient in superoxide production [70]. p47^{phox} is also responsible for recruiting p67^{phox} to the NADPH oxidase complex on the membrane through interactions between the PRR of p47^{phox} and the C-terminal SH3 on p67^{phox} [65,68] as well as the interactions between the C-terminal SH3 domain of p47^{phox} with the PRR of p67^{phox} [71]. The binding of p47^{phox} and p67^{phox} is regulated by p40^{phox} [38,72].

The p67^{phox} protein, encoded by the *NCF2* gene, was first purified as part of a cytoplasmic complex capable of complementing an inactive membrane-bound oxidase complex [73,74]. The *NCF2* gene was subsequently cloned [75–77], and it was discovered that several mutations in this gene were also associated with CGD [78,79]. The *NCF2* gene encodes for a 526 amino acid protein that has four tetratricopeptide repeat (TPR) motifs, two SH3 domains, and a Phox and Bem1 (PB1) domain (Fig. 3B). p67^{phox} has two important roles in NOX2 enzyme activation: it recruits the Rac-GTP (RAC1 or RAC2) to the enzyme complex and it is responsible for electron transfer from NADPH to gp91^{phox} [41].

p67^{phox} is recruited to the membrane to interact with the NOX2 complex by p47^{phox}. There are two main interactions between p47^{phox} and p67^{phox}. The first interaction is between the C-terminal SH3 domain of p67^{phox} binding to the PRR of p47^{phox} in a reverse orientation. This interaction is dependent on Asp16 in the C-terminal SH3 domain of p67^{phox} [65,68,80]. The second interaction is between the C-terminal SH3 domain of p47^{phox} which directly binds to p67^{phox} at its PRR that is on the N-terminal side of the SH3 domains [64]. The SH3 domains of p67^{phox} do not bind to the PRR of p22^{phox}, so p67^{phox} must be recruited by p47^{phox} and cannot directly interact with gp91^{phox} and p22^{phox} [81, 82]. The two SH3 domains of p67^{phox} are dispensable for oxidase activity in a cell-free system but are required in whole cells for superoxide production [60,79,80,83,84].

After p67^{phox} is recruited to the membrane-bound components of the NOX2 enzyme complex, it is directly involved in the activation of the NOX enzyme complex. p67^{phox} recruits the GTPase RAC2 through interactions with the TPR motifs on the N-terminal end of p67^{phox} [85,86]. The Rac GTPase assembly with the NOX2 complex is absolutely required for its activity [87]. Ultimately, the activation domain of p67^{phox} interacts with gp91^{phox} and allows for the transfer of electrons from NADPH to the flavin center of gp91^{phox} [88,89].

The third NADPH oxidase-associated factor is p40^{phox}, which is encoded by the *NCF4* gene. p40^{phox} was first identified by Wientjes et al. (1993) and was shown to have an SH3 domain and an N-terminal domain with sequence similarity to the N-terminal domain of p47^{phox} [81]. Like p67^{phox}, p40^{phox} also has a PB1 domain (Fig. 3C), which mediates its association with p67^{phox} in the inactive cytoplasmic ternary complex [81,90,91]. The p40^{phox} PB1 domain heterodimerizes with the PB1 domain of p67^{phox}, an interaction that can be blocked with an antibody that binds the PB1 domain of p40^{phox} [92–95]. The SH3 domain on p40^{phox} is not required for binding to p67^{phox} and when p67^{phox} is absent in patients with CGD, p40^{phox} and Rac1 are not translocated from the cytosol to the membrane [68,91,96]. The PX domain from p40^{phox} binds to phosphatidylinositol 3-phosphate found on phagosomal membranes [97–102].

The exact role p40^{phox} plays in the activation of the NOX2 enzyme complex is not entirely clear. p40^{phox} is phosphorylated upon activation of NADPH oxidase by fMLP or PMA at amino acids Thr154 and Ser315 [103,104]. After activation, p40^{phox} translocates to the membrane and disassociates from p67^{phox} and p47^{phox} [105]. p40^{phox} has been shown to be a positive regulator of NOX2 activity [106,107]. However, it has also been proposed that p40^{phox} negatively regulates NOX2 activity through its SH3 domain [108]. There is evidence that the SH3 domain of p40^{phox} binds to the C-terminal PRR of p47^{phox} at the same site as p67^{phox}, thus preventing p67^{phox} binding through competition [71].

3. Other NADPH oxidase family large transmembrane catalytic subunits

3.1. NADPH Oxidase 1 (NOX1)

This homologue of gp91^{phox} was first cloned and characterized in 1999 by Suh et al. who demonstrated that it was highly expressed in the colon, but not in leukocytes [109,110]. Activation of NOX1, like that of NOX2, involves homologues of p47^{phox} and p67^{phox} known as NOX organizer 1 (NOXO1) and NOX activator 1 (NOXA1) [111,112]. NOXO1 has homologous SH3 and PX domains to those found in p47^{phox} as well as the conserved PRR (Fig. 3A). NOXA1 also has protein domains homologous to those found in p67^{phox} such as TPR, SH3, and PB1 domains (Fig. 3B). After an activating stimulus like PMA is administered to cells, NOXO1 is phosphorylated at Ser154 which is required for assembly with NOXA1 and subsequent interactions with p22^{phox} [113]. Activation of the NOX1 complex also requires a Rac1 GTPase which is associated with NOXA1 [114–116]. Like NOX2, NOX1 must form a heterodimer with p22^{phox} for activation and superoxide production [117].

Unlike NOX2, NOX1 is not expressed in immune cells, but still plays a role in immunity. NOX1 is primarily expressed in colon epithelial cells and is important for host defense, barrier function, and homeostasis of commensal bacteria [20]. Crosstalk between the commensal bacteria in the colon and NOX1 is important for epithelial homeostasis. Stimulation of formyl peptide receptors on epithelial cells by bacteria stimulates NOX1-dependent ROS production which promotes barrier maintenance through epithelial growth and repair [118,119]. Conversely, production of hydrogen peroxide from NOX1-derived superoxide helps to prevent overgrowth of commensal bacteria [120]. Interestingly, there are catalase-producing commensals like *Escherichia coli* as well as pathogenic bacteria like *Citrobacter rodentium* that can utilize NOX1-derived hydrogen peroxide to support cellular respiration in an otherwise anaerobic environment [121,122].

NOX1 has also been implicated in colon cancer due to its role in regulating cell proliferation and angiogenesis in the colonic epithelium [110,123,124]. Expression of *NOX1* is regulated by the transcription factors GATA-6, HNF-1 α , and CDX2. Expression of these transcription factors is higher in the distal colon than the proximal colon and correlates with NOX1 expression [125]. NOX1 is overexpressed in many epithelial and colon-related cancers as a direct result of k-Ras mutations that result in increased MEK/ERK signaling and activation of GATA-6 [126,127]. NOX1 overexpression in fibroblasts can promote tumorigenesis and angiogenesis through upregulation of VEGF and the VEGF receptors, VEGFR1 and VEGFR2 [124,127]. A novel inhibitor of NOX1, GKT771 has shown efficacy as a complementary treatment to anti-PD1 checkpoint inhibitor therapy in pre-clinical trials in mouse models of colon cancer [128].

3.2. NADPH Oxidase 3 (NOX3)

NADPH Oxidase 3 was identified as a protein with homology to NOX2 located on chromosome 6 [129]. NOX3 is expressed in fetal tissues, but has limited expression in adult tissues and is limited to the colon, testis, and inner ear [129,130]. Stimulation of cells with the PKC activator, PMA, leads to activation of NOX3 via p47^{phox} and p67^{phox} [131]. However, NOX3 also has activity in the absence of PKC stimulation through NOXO1 activity [132,133]. The PMA-independent activation of NOX3 is constitutive due to the interaction of NOX3 with p22^{phox} [132]. Unlike NOX1 and NOX2, the constitutive activity of NOX3 does not require an activating or organizing protein [132]. However, when the activating or organizing proteins are present and activated, NOX3 activity is enhanced [132].

NOX3 is not known to play a role in immune cells or host defense. However, NOX3 activity is involved in the vestibular system in the inner ear [134]. Defects in NOX3 can result in a head-tilt in mice due to otoconia morphogenesis defects [130]. NOX3-derived superoxide has

also been implicated in noise-induced and cisplatin-induced hearing loss [135]. NOX3 expression was shown to increase with cisplatin treatment, age, and noise insults in mice, which correlated to hearing loss [136]. It has been proposed that therapies targeting NOX3 in the inner ear could be used to prevent NOX3-induced hearing loss [135]. Proposed therapies include NOX3-specific siRNA delivery and small molecule inhibitors [137–139]. This would be beneficial as a preventative measure for patients undergoing cisplatin treatment for solid tumors.

NOX3 can also be activated in hepatocytes in response to insulin, which leads to the production of VEGF and the initiation of angiogenesis [140]. Hepatocytes stimulated with palmitate also produce ROS through NOX3, which leads to increased gluconeogenesis and reduced glycogen content [141]. It is thought that this may contribute to insulin resistance in obesity [141,142]. The mechanism has been revealed to be due to increased TNF production that stimulates hepatocytes through the JNK and p38^{MAPK} pathways [129,143,144].

3.3. NADPH Oxidase 4 (NOX4)

NADPH Oxidase 4 was first characterized as a NOX enzyme that is expressed in the kidney with homology to NOX2 [145,146]. NOX4 is also unique compared to the previously discovered NOX enzymes in that it does not require association or activity from cytosolic factors for activation and organization like NOX1, NOX2, and NOX3 [145, 147–151]. NOX4 has been associated with constitutive production of hydrogen peroxide rather than superoxide production [148,152]. It has been shown that when the extracellular loop between transmembrane domains five and six (E-loop) of NOX4 is deleted that NOX4 does in fact generate superoxide, which suggests that the E-loop may have dismutase activity that converts superoxide to hydrogen peroxide before it can be detected by current methods [143,148].

NOX4 was first discovered in the kidney, but is also highly expressed in pulmonary vasculature and endothelial cells and plays an important role in respiratory diseases such as pulmonary fibrosis, asthma, chronic obstructive pulmonary disease, pulmonary vascular diseases, and acute respiratory distress syndrome [153]. NOX4 has also been shown to be expressed in Jurkat T cells. Infection of Jurkat T cells with *Entamoeba histolytica* was shown to induce cell death which was abrogated with siRNA knockdown of NOX4 [154]. However, this has not been shown in primary T cells. NOX4 expression is regulated by several different stimuli including oxygen levels [155–158]. NOX4 expression is also stimulated by angiotensin II, glucose levels, hypoxia, or hyperoxia [159–166]. This change in expression is driven by important transcription factors such as STAT1/STAT3, NRF2, HIF-1 α , NF κ B, Oct-1, SP3, SP1, c-JUN, and E2F [129,167].

3.4. NADPH Oxidase 5 (NOX5)

NADPH Oxidase 5 has an EF-Hand domain (calcium-binding) and is highly expressed in the adult testis, spleen, ovary, placenta, and pancreas and the fetal brain, heart, kidney, liver, lung, skeletal muscle, spleen, and thymus [129]. NOX5 is expressed at lower levels in the adult brain, heart, kidney, liver, lung, prostate, and small intestine [167]. NOX5 is responsible for ROS generation in human sperm [168]. Interestingly, NOX5 is not expressed universally in all mammalian species and is absent in rodents, which makes animal models for studying NOX5 difficult [167]. Unlike its homologues NOX1–4, NOX5 does not require an activating and organizing protein like p47^{phox} or p67^{phox} for activation and can be activated by calcium flux alone [117,169]. Knockout of p22^{phox} or the introduction of mutations in p22^{phox} that abrogate NOX1, NOX2, NOX3, or NOX4 activity does not affect NOX5 activity [170]. Activity of NOX5 is dependent on oligomerization of multiple NOX5 proteins, which bind to each other through the dehydrogenase domain [171]. Binding of phosphatidylinositol (4,5)-bisphosphate directs NOX5 to localize at the plasma membrane through interaction with the N-terminal polybasic region [172].

NOX5 can be activated by two different mechanisms: intracellular calcium flux and protein kinase C activation. The C-terminus of NOX5 contains a calmodulin-binding site that increases the sensitivity of NOX5 to calcium-mediated activation [173]. The binding of calcium to the EF-hand domains induces a conformational change in NOX5 which leads to its activation when intracellular calcium levels are high [174]. However, it has been noted that the calcium concentration needed for activation of NOX5 is extremely high and not likely physiological [175] and low levels of calcium-binding to NOX5 can work synergistically with PKC stimulation [176]. It has also been shown that in the presence of ROS that NOX5 is oxidized at cysteine and methionine residues in the Ca²⁺ binding domain thus inactivating NOX5 through a negative feedback mechanism [177,178]. NOX5 can also be activated by PKC- α stimulation [175] after phosphorylation of Thr512 and Ser516 on NOX5 α [16,179].

3.5. Dual Oxidase 1/2 (DUOX1/2)

Two additional proteins with homology to NOX enzymes were discovered in the thyroid. These enzymes were called dual oxidase enzymes 1 and 2 (DUOX1 and DUOX2). Like NOX1–5, these enzymes have six transmembrane domains with a C-terminal domain containing an FAD and NADPH binding site. These enzymes can also convert molecular oxygen to hydrogen peroxide. However, DUOX1 and DUOX2 are more closely related to NOX5 due to the presence of calcium-regulated EF hand domains. DUOX-mediated hydrogen peroxide synthesis is induced transiently after calcium stimulation of epithelial cells [180]. Unlike NOX5, DUOX1 and DUOX2 have an additional transmembrane domain called the peroxidase-homology domain on its N-terminus. DUOX1 and DUOX2 require maturation factor proteins DUOXA1 and DUOXA2, respectively, in order to transition out of the ER to the Golgi [181].

The DUOX enzymes have roles in immune and non-immune physiological processes. DUOX1 and DUOX2 are both expressed in the thyroid gland and are involved in thyroid hormone synthesis. DUOX-derived hydrogen peroxide is utilized by thyroid peroxidase enzymes for the oxidation of iodide [182]. Nonsense and missense mutations in *DUOX2* have been shown to result in hypothyroidism [183,184]. No mutations in the *DUOX1* gene have been linked to hypothyroidism so it is unclear whether *DUOX1* is required for thyroid hormone biosynthesis or whether it acts as a redundant mechanism for defective *DUOX2* [185]. DUOX1 has been detected in bladder epithelial cells where it is thought to function in the sensing of bladder stretch [186]. DUOX enzymes have also been shown to be important for collagen crosslinking in the extracellular matrix in *C. elegans* [187].

DUOX1 is involved in immune cells like macrophages, T cells, and B cells. DUOX1 is expressed in alveolar macrophages where it is important for modulating phagocytic activity and cytokine secretion [188]. T cell receptor (TCR) signaling in CD4⁺ T cells induces expression of DUOX1 which promotes a positive feedback loop for TCR signaling. After TCR signaling, DUOX1-derived hydrogen peroxide inactivates SHP2, which promotes the phosphorylation of ZAP-70 and its subsequent association with LCK and the CD3 ζ chain. Knockdown of DUOX1 in CD4⁺ T cells results in reduced phosphorylation of ZAP-70, activation of ERK1/2, and release of store-dependent calcium [189]. DUOX1 may also play a role in B cell receptor (BCR) signaling. DUOX1 expression is induced by BCR signaling in the presence of IL-4. One study showed that DUOX1-derived hydrogen peroxide negatively regulates B cell proliferation [190]. However, a second study, which used a DUOX1- and DUOX2-deficient mouse, showed that the DUOX enzymes were dispensable for BCR signaling [191]. Further work is necessary to fully understand the role of DUOX1 and DUOX2 in B cells.

More recently it has been appreciated that DUOX enzymes also play important roles in epithelial cells in the airway and gut. DUOX1 is expressed in epithelial cells in the trachea and bronchi and is associated with EGFR signaling after stimulation of TLRs to promote epithelial

homeostasis and repair in response to microbial ligands [192–194]. DUOX2 is also expressed in the airway epithelium and is important for host antiviral (see section 4.3) and antibacterial immunity [195–197].

DUOX2 is also expressed in the tip of epithelial cells in the ileum and colon [198]. Expression of DUOX2 is stimulated by the microbiota through TLRs mediated by MyD88 and TRIF signaling pathways [198]. The role of DUOX in antibacterial host defense has been shown in several animal models including *Drosophila*, *C. elegans*, zebrafish, and mice, which require DUOX enzymes for protection from bacterial insults [199–202]. In mice, DUOX-deficient mice were able to be colonized by *H. felis*, whereas control mice with intact DUOX were not [202].

4. NOX enzymes in immunity

4.1. Phagocytosis and pathogen clearance

NOX2-derived ROS play an important role in pathogen killing in neutrophils and macrophages (Fig. 4). Neutrophils and macrophages phagocytose bacteria and fungi which are then killed in the phagosome [203]. After activation, a respiratory burst occurs where NOX2 is activated and generates superoxide. The generation of superoxide inside the phagosomal lumen creates a change in electrical charge across the phagosomal membrane which can inhibit the further generation of superoxide by NOX2 [204]. This change in electrical charge is counteracted by Hv1 voltage-gated channels which allow for the simultaneous flow of protons into the phagosomal membrane [205]. In the absence of Hv1, NOX2 activity and superoxide production in the phagosome is severely limited [206].

The exact role of superoxide production in the phagosome is somewhat controversial. The dogma in the field is that NOX2-derived superoxide and its downstream products hydrogen peroxide and hypochlorite generated by myeloperoxidase (MPO) directly kill phagocytosed pathogens. However, recent evidence has suggested that proteases delivered to phagosomes by granules are primarily responsible for the microbicidal activity of phagosomes [207]. Indeed, mice deficient for cathepsin G or elastase were more susceptible to *Staphylococcus aureus* and *Candida albicans* infections respectively, despite intact NOX2 activity [207]. Further evidence to support this is the absence of patients identified with deficiencies in MPO that suffer from chronic bacterial infections like patients with CGD [208]. However, mice with MPO deficiencies do have increased susceptibility to infections by certain bacteria or fungi suggesting that MPO is important in some contexts [209].

The controversy surrounding the exact role of NOX2-derived

superoxide and the subsequent activity of MPO in the phagosome is concerned with the pH of the phagosome. After the respiratory burst, the pH of the phagosome increases and becomes alkaline with a pH of approximately 9 [210,211]. This increase in pH is regulated by Hv1 voltage-gated channels and in their absence, the pH rises as high as 11 [210]. This alkaline pH is incompatible with hypochlorite generation by MPO which is optimal at a slightly acidic pH [212,213]. At an alkaline pH, MPO has SOD and catalase activity, which could convert superoxide into hydrogen peroxide and hydrogen peroxide into water [210,214,215]. This would suggest that the role of MPO in the phagosome is to dissipate the ROS generated by NOX2. While the high pH of the phagosome is incompatible with the halogenating activity of MPO, it is compatible with the maximal activity of proteases like elastase, cathepsin G, and proteinase 3 that are present in the phagocytic granules [210]. An increase in the pH and an influx of K^+ are required for the activation of these microbicidal proteases and their release from the negatively charged proteoglycan matrix in the granules [207].

Levine and Segal have proposed that MPO has SOD and catalase activity at a pH of 9 in the phagosome, but in cases where a pathogen cannot be fully engulfed, and the pH is that of the extracellular environment, MPO generates hypochlorite, which assists in killing extracellular pathogens [208]. However, the recently developed rhodamine-based probe, R19-S, which has specificity for hypochlorite, has revealed hypochlorite present in phagosomes of isolated neutrophils infected with *Staphylococcus aureus* [216]. Further evidence for hypochlorite induction in the neutrophil phagosome comes from a recent study that demonstrated the induction of a chlorine-responsive transcription factor, RclR, in *Escherichia coli* after ingestion by neutrophils. The transcription factor was not induced when NOX2 or MPO was inhibited, suggesting that this was indeed due to hypochlorite production in the phagosome [217].

4.2. Macrophage polarization

NOX-derived ROS are important in driving macrophage polarization to a proinflammatory M1 macrophage phenotype and in their absence, anti-inflammatory M2 macrophage differentiation will prevail. In $p47^{phox}$ -deficient mice, a model for CGD, there is more skewing towards an M2 macrophage phenotype [218]. In the absence of NOX2, macrophages have attenuated STAT1 signaling and increased STAT3 signaling which promotes the expression of anti-inflammatory markers such as Arginase-1 [219]. Studies of Type 1 diabetes by our group (see section 5.2) have shown that NOD mice carrying the *Ncf1*^{m1J} mutation, which

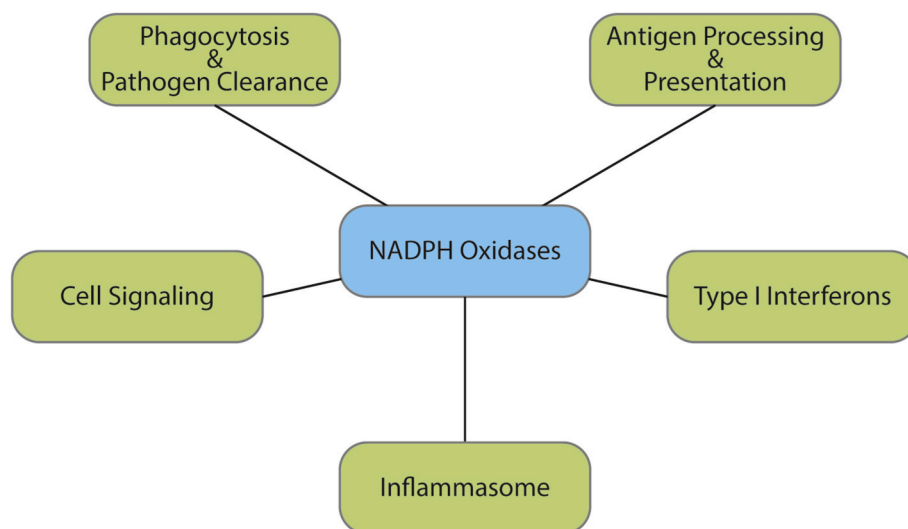


Fig. 4. NADPH oxidase-derived ROS regulate immunity. NOX-derived ROS regulate various aspects of immunity like phagocytosis, pathogen clearance, antigen processing, antigen presentation, type I interferon regulation, inflammasome regulation, and cell signaling.

results in a lack of p47^{phox} activity, exhibit a skewed M2 macrophage phenotype that is partly responsible for delaying spontaneous T1D development [220].

In contrast, NOX4-and DUOX1-derived hydrogen peroxide promotes M2 macrophage polarization. Inhibition of NOX4 in murine bone marrow-derived macrophages results in M1 polarization due to reduced STAT6 activation and increased NFκB activity [221]. In certain disease contexts, NOX4 may be a potential therapeutic target to influence macrophage polarization. In pulmonary fibrosis after asbestos exposure, NOX4 expression in macrophages promotes profibrotic polarization of alveolar macrophages which are resistant to apoptosis [222,223]. In non-small cell lung cancer, expression of NOX4 in the tumor promotes recruitment and polarization of M2 macrophages, which is associated with tumor growth [224]. DUOX1 has also been shown to be expressed in macrophages [225,226]. DUOX1^{-/-} macrophages tend to skew towards a proinflammatory M1 phenotype characterized by IFN-γ, CXCL9, CCL3, and CCL5 secretion. DUOX1^{-/-} macrophages also have enhanced antitumor activity and promote the recruitment of IFN-γ⁺ tumor-infiltrating CD8⁺ T cells [188].

4.3. Antigen processing and presentation

NOX2-derived superoxide is important for pathogen killing in neutrophils and macrophages, but it also regulates antigen processing and presentation in dendritic cells (DCs) (Fig. 4). DCs differ from other phagocytic cells in that their primary function is to process antigens and present them to T cells rather than just destroying pathogens. NOX2 activation through PKC-δ promotes pinocytosis and antigen uptake in DCs through the SSH1-Cofilin pathway [227,228]. In addition to promoting antigen uptake, NOX2 plays a key role in antigen processing within the phagosome by modulating the pH and activity of proteolytic enzymes [229].

Proteolysis in the phagosome is necessary for generating antigens of the correct size for MHC loading. However, too much proteolysis will result in the complete destruction of peptides and poor antigen presentation [229]. Preventing the complete destruction of peptides for antigen presentation requires alkalization of the phagosome, which is driven by NOX2 [230]. Indeed, NOX2-deficient DCs have more acidic phagosomes and increased antigen degradation [230]. Alkalinization of the phagosome is important for optimal activity of proteolytic enzymes which affects the types of antigens that can be presented to T cells [229].

DCs generally have less NOX2 activity in their phagosomes than neutrophils and macrophages, which helps to promote optimal proteolysis [231]. High levels of NOX2 activity result in inhibition of cysteine cathepsins and poor phagosomal proteolysis whereas a lack of NOX2 activity results in high levels of proteolysis and destruction of antigens [232]. High levels of NOX2 activity also result in decreased reduction of disulfide bonds by γ-interferon-inducible lysosomal thiol reductase (GILT), which is necessary for unfolding and linearizing peptides for antigen presentation [229,231]. GILT is a redox-sensitive reductase that is required for disulfide bond reduction and efficient processing of several model antigens [233]. GILT is also required for maintaining optimal proteolysis by cysteine cathepsins [234].

NOX2 activity is also important in promoting cross-presentation of antigens by CD8⁺ DCs [230]. Experimental inhibition of NOX2 by treatment with diphenyleneiodonium (DPI) results in the inhibition of phagosomal alkalization and cross-presentation of model tumor antigens [235]. This phenotype is recapitulated in DCs from patients with CGD [235]. NOX2 is recruited to the endosomes through activity of the SNARE protein VAMP8 [236]. In addition to antigen preservation, NOX2 activity has also been shown to cause lipid peroxidation of endosomal membranes which promotes antigen release from the endosome to the cytosol for cross-presentation [237]. Cross-presentation has also been shown to require activity of Rac2 and not Rac1 for NOX2 activation [238].

4.4. Type I interferon regulation

NOX-generated ROS are also important in regulating type I interferons (IFNs) (Fig. 4). Patients with CGD as well as mice with nonfunctional NCF1 have an elevated type I IFN signature and are more prone to autoimmune manifestations [6]. In mice that are deficient for NCF1, STAT1-dependent gene transcription is increased, which may contribute to development of autoimmune SLE and RA [5,6]. In *Listeria monocytogenes* infection, a lack of NOX2-derived superoxide results in an exaggerated response to type I IFN signaling with increased expression of ISGs. In the case of *Listeria*, this results in the inability to control bacterial spread and mount an effective adaptive immune response [239]. However, this is dependent on the genetic background of mice since non-obese diabetic (NOD) mice have decreased type I IFN signaling, synthesis of ISGs, and a delay in autoimmune diabetes in the absence of NOX2-derived superoxide [240,241].

In viral infections, too much ROS can dampen type I IFN signaling enough to hinder the antiviral response. NOX-derived ROS are required for efficient viral sensing through the mitochondrial antiviral signaling protein (MAVS), and in their absence, MAVS expression is decreased and activation of IRF3 and ISGs is decreased [242]. In the absence of SOD2, ROS levels are elevated and the response to RNA viruses is deficient due to decreased type I IFN production [243]. ROS generation after IFN stimulation is negatively regulated by some ISGs like IFIT2 which can interact with p67^{phox} to downregulate superoxide production [244].

DUOX1 and DUOX2 are required for an efficient antiviral response in airway epithelial cells after influenza A (IAV) infection [193,244]. IAV infection results in the upregulation of DUOX1 and DUOX2 in lung epithelial cells [246] and DUOX2-derived ROS are required for inducing the production of type I and III IFNs during IAV infection [247,248]. It has recently been demonstrated that DUOX1-derived hydrogen peroxide is important for innate immunity during IAV infection by inducing the expression of inflammatory cytokines, recruiting additional immune cells, and generating hypothiocyanite in conjunction with the lactoperoxidase enzyme [245]. DUOX2 expression in the lungs is driven by IFN-β and TNF which induces STAT2 and IRF9-dependent signaling pathways [249]. Expression of MDA5 and RIG-I, which is necessary for detecting IAV replication, is also dependent on DUOX2-derived ROS [250,251]. Inhibition of DUOX2 results in increased IAV replication *in vivo* and *in vitro* [248,250,251].

4.5. The inflammasome

NOX-derived ROS also play a role in regulating the inflammasome (Fig. 4). It has been demonstrated that NOX-derived ROS are necessary for activation of the NLRP3 inflammasome in response to extracellular ATP, silica, and asbestos [252]. Other studies have demonstrated the importance of NOX2-derived ROS for activation of the NLRP1 inflammasome [253,254] and NOX4-derived ROS for activation of the NLRP3 inflammasome [255–257]. The requirement for NOX4 in macrophages for inflammasome activation is specific to the NLRP3 inflammasome; NOX4 is not required for NLRC4, NLRP1, or AIM2 inflammasome activation [258]. Evidence shows that not only can ROS induce inflammasome activation, but that ROS generation is amplified by NLRP3 inflammasome activation as well [259–261]. However, there is also evidence that without NOX2-derived superoxide there is chronically elevated inflammasome activation, highlighting the complexity of interactions between NOX-derived ROS and the inflammasome [262]. Further complicating the relationship, it has been shown that caspase-1 may negatively regulate NOX2 [263].

There have been multiple studies that have linked NOX2-derived ROS and the inflammasome in disease. In chronic kidney disease, oxidative stress can lead to kidney damage due to activation of NOX2 and the NLRP3 inflammasome [264]. In nonalcoholic fatty liver disease in mice, lactate-producing bacteria in the gut can activate NOX2 which results in NLRP3 inflammasome activation and exacerbates disease

[265]. Glucose-6-phosphate dehydrogenase (G6PD)-deficiency results in altered NADPH production. In human peripheral blood mononuclear cells with G6PD-deficiency, there is decreased superoxide production and defective inflammasome activation, which can be ameliorated by exogenous addition of hydrogen peroxide [266].

4.6. Cell signaling

Superoxide and hydrogen peroxide are pleiotropic signaling molecules that can affect a variety of cellular processes ranging from stress adaptation, the antioxidant response, the hypoxic response, and the inflammatory response (Fig. 4). A thorough examination of the role of ROS in cell signaling is beyond the scope of this review and has already been reviewed previously [1,267]. NOX-derived hydrogen peroxide can modulate signaling pathways by triggering redox switches through the oxidation of cysteine and methionine residues [268,269]. Redox switches can be used to promote signaling through a pathway by inactivating protein tyrosine phosphatases through the oxidation of conserved cysteine residues, thus maintaining levels of phosphorylated proteins [270–273]. Redox switches can also direct the degradation of proteins by the proteasome. For example, oxidation of Met145 in calmodulin by peroxynitrite results in its degradation by the proteasome and down-regulation of calcium signaling [268]. A large portion of cellular ROS is derived from superoxide produced by NOX enzymes. However, there are other sources of cellular ROS, such as mitochondrial-derived superoxide, which makes determining the specific contributions of NOX enzymes on signaling pathways more difficult.

The specific role of NOX enzymes in signaling pathways is not always simple to determine when there are multiple NOX enzymes involved such as in the well-characterized epidermal growth factor receptor (EGFR) pathway. Several NOX enzymes have been demonstrated to be involved in the regulation of EGFR signaling. After EGF stimulation, epithelial cells begin to produce ROS which is driven by NOX1 downstream of PI3K signaling [274]. EGF stimulation also activates the ERK pathway which acts to negatively regulate NOX1 activity through the phosphorylation of Ser282 in NOXA1 by ERK [275,276]. EGFR signaling transduction is also modulated by the oxidation of Cys797 in EGFR by hydrogen peroxide derived from NOX2 in A431 cells [277]. NOX4, located in the ER, is also involved in regulating EGFR trafficking through oxidation of PTP1B, which deactivates EGFR by dephosphorylation [278]. In the absence of NOX4, EGFR signaling is decreased due to increased PTP1B activity on EGFR after receptor endocytosis [277]. DUOX1 in the airway is also associated with EGFR signaling after stimulation of TLRs [192–194]. The role of different NOX enzymes in EGFR signaling highlights the key role that NOX enzymes play in cell signaling and the complex nature of their respective roles in these pathways.

5. NOX enzymes in inflammation and autoimmunity

5.1. Rheumatoid arthritis

Studies of NOX2-deficient mice have been used to determine the role of NOX2-derived ROS in autoimmune diseases. However, whether NOX2-derived ROS contribute to or protect from autoimmunity varies depending on the disease and the genetic background of the mice. B10.Q mice homozygous for a mutation in the *Ncf1* gene (*Ncf1^{m1J}* mutant), which results in aberrant splicing and a lack of NCF1 and NOX2 activity, have increased presentation of an autoantigen involved in collagen-induced arthritis. This is thought to be due to upregulation of GILT which facilitates disulfide bond-containing antigen processing [279]. It is worth noting that B10.Q mice are usually resistant to collagen-induced arthritis and have hyporesponsiveness to IL-12 due to a mutation in *Tyk2* [280].

5.2. Type 1 diabetes

Previous work by our group has explored the role of NOX2-derived ROS in the context of Type 1 diabetes (T1D) using a mouse model with the *Ncf1^{m1J}* mutation on the NOD mouse background (NOD.*Ncf1^{m1J}*) [281]. NOD.*Ncf1^{m1J}* mice are protected from spontaneous, adoptively transferred, and virus-accelerated diabetes [220]. An investigation into the mechanism of protection from T1D in these mice has revealed that NOD.*Ncf1^{m1J}* mice have altered macrophage phenotypes. Macrophages from NOD.*Ncf1^{m1J}* mice are skewed more towards an anti-inflammatory M2 phenotype compared to macrophages from NOD mice with intact NOX [281,282]. Macrophages from NOD.*Ncf1^{m1J}* mice also have dysregulated signaling through TLRs and express significantly less proinflammatory cytokines such as TNF and IFN- β after stimulation with TLR ligands [281,282]. In contrast to the B10.Q mice, NOD mice are more prone to Th1 T cell responses and inflammation [283]. These findings suggest that the role of NOX2 in autoimmunity is also heavily dependent on the genetic background of the host.

The diverse biological functions that are regulated or modified by NOX-derived ROS make antioxidant-based therapies attractive for treating diseases associated with oxidative stress. Previous work by our group has investigated the use of a metalloporphyrin-based superoxide dismutase mimetic (SOD mimetic), which acts as a catalytic antioxidant, for the treatment of T1D. We have shown that spontaneous and adoptively transferred diabetes can be delayed in mice pretreated with the SOD mimetic [281]. We have also shown that treatment of macrophages with the SOD mimetic results in decreased TNF, IL-1 β , and ROS production after treatment with inflammatory stimuli due to decreased DNA binding by redox-sensitive transcription factors like NF κ B and SP1 [284].

Our group has also investigated the use of antioxidant-containing biomaterials to treat T1D. We have shown that microcapsules composed of poly(N-vinylpyrrolidone) (PVPON) and the antioxidant tannic acid can be used to deliver antigens *in vivo* to mice to promote antigen-specific tolerance [285]. The goal of this therapy would be to induce tolerance to autoantigens associated with T1D by dampening ROS, which results in antigen hyporesponsiveness [285]. We have also used PVPON and tannic acid-containing biomaterials to encapsulate islets for transplantation into diabetic recipients [286]. Encapsulation with the PVPON and tannic acid-containing biomaterial delays islet allograft and autoimmune-mediated rejection after transplantation into diabetic recipients [286].

6. NOX enzymes in SARS-CoV-2 infection and acute lung injury

NOX-derived ROS play important roles in viral infections and modulate aspects of the innate and adaptive immune responses to infection. DNA and RNA viruses can activate endosomal NOX2 via activation of PKC downstream of sensing by TLR7 or TLR9, which results in the production of hydrogen peroxide. The generation of endosomal hydrogen peroxide results in a suppressed antiviral response and a decrease in antibody production [287]. Studies in mouse models deficient in NOX2 have demonstrated that a lack of NOX2 results in skewing towards a Th1 response and increased production of IgG2c and IFN- γ [288]. Similarly, IgG2 levels were increased in human sera from CGD patients, which suggests a skewing towards Th1 responses [288]. Thus, viruses that can activate NOX2 will be able to dampen the antiviral response, favoring viral replication.

Recent evidence from the COVID-19 pandemic suggests that oxidative stress may be driving acute lung injury in patients with severe SARS-CoV-2 infection (Fig. 5) [289]. NOX2 activation is higher in COVID-19 patients compared to controls and higher in severe COVID-19 cases compared to non-severe cases [290]. Oxidative stress during SARS-CoV-2 infection may be due to activation of the NLRP3 inflammasome in infected cells [291]. It has been hypothesized that increased risk for oxidative stress and severe COVID-19 may be due to suppressed

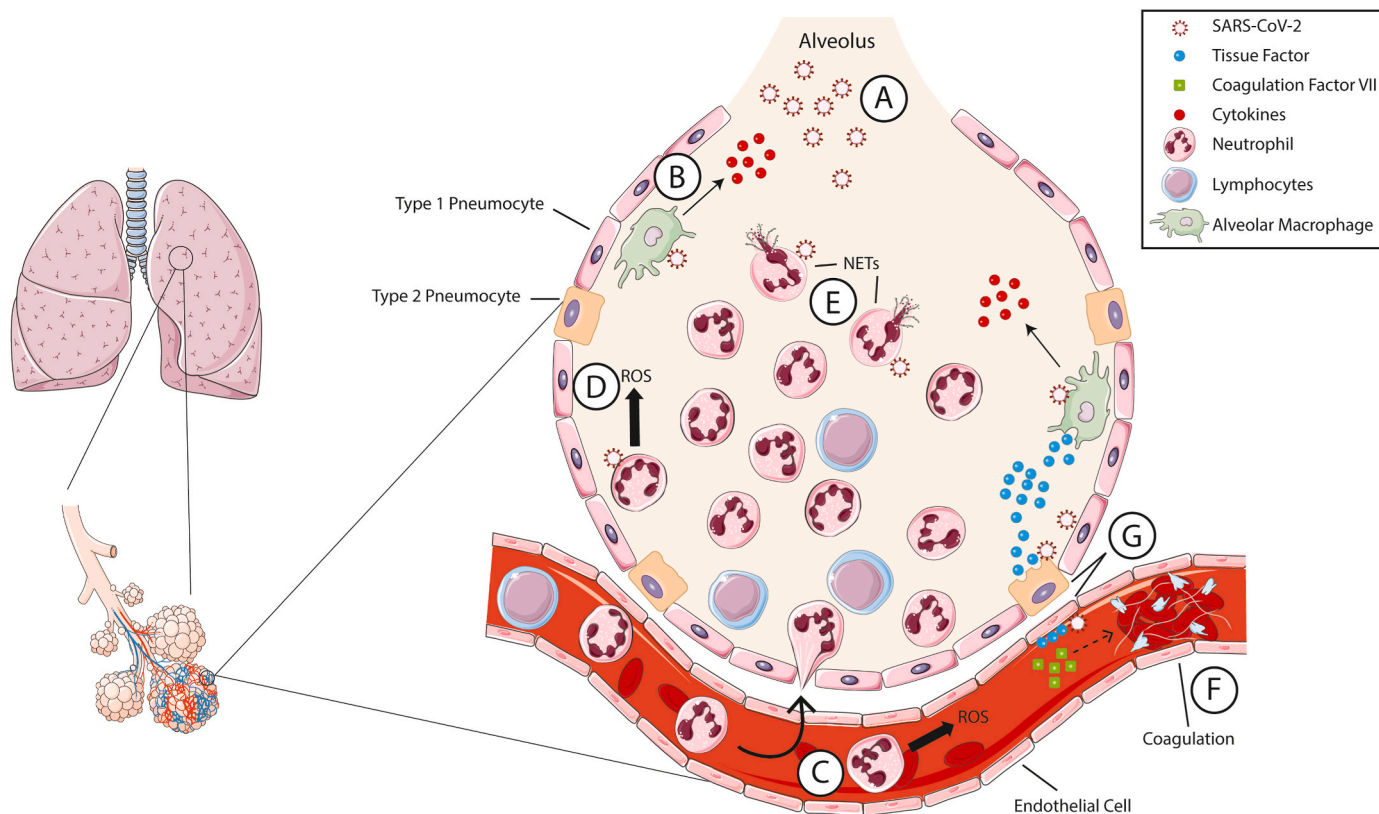


Fig. 5. Acute lung injury during SARS-CoV-2 infection. (A) SARS-CoV-2 inhaled in the lung is first detected by (B) alveolar macrophages which produce proinflammatory cytokines and chemokines to recruit additional immune cells. (C) Neutrophils and lymphocytes are recruited to the lungs. (D) Severe COVID-19 cases are associated with a high neutrophil to lymphocyte ratio. NOX2 is activated in neutrophils which produce ROS in the alveoli driving lung damage. (E) SARS-CoV-2 can also activate NETosis and the release of neutrophil extracellular traps (NETs). (F) Platelet-fibrin thrombi are formed in the lungs causing further tissue damage. (G) Infected endothelial cells and type II pneumocytes in the lungs produce tissue factor which acts on coagulation factor VII to initiate clotting. Some images were modified from Servier Medical Art under a Creative Commons License.

antioxidant responses through the NRF2 pathway, glutathione deficiency, or low levels of SOD3 expression in alveolar type II cells [291–293]. A recent study demonstrated an influx of NOX1 and NOX2 positive granulocytic-myeloid-derived suppressor cells (G-MDSCs) in the lungs of patients with severe COVID-19 complications. The study demonstrated that Arginase-1 positive G-MDSCs depleted L-arginine levels which resulted in impaired T cell and endothelial dysfunction [294]. However, the study did not conclusively demonstrate the role of NOX enzymes in these cells and whether NOX-derived ROS played a role in disease severity.

During SARS-CoV-2 infection, activated neutrophils have been shown to be one of the main sources of ROS production in the lung tissue and a driver of lung tissue damage (Fig. 5A–D) [295,296]. Several studies have demonstrated that increased neutrophil to lymphocyte ratios correlate with more severe disease outcomes [297,298]. Post-mortem analysis of lung tissue of patients with severe COVID-19 showed evidence of neutrophil extracellular traps (NETs) which likely are contributing to lung tissue damage (Fig. 5E) [296]. *In vitro* experiments have demonstrated that SARS-CoV-2 can activate NETs in human neutrophils and that this correlates to increased production of ROS and IL-8 [299]. NETosis can also be induced through Fc α RI engagement by IgA-virus immune complexes. Immune complexes made up of SARS-CoV-2 spike protein pseudotyped lentivirus purified IgA from COVID-19 convalescent patients were able to induce NETosis *in vitro*. NETosis was not seen when using purified serum IgA from COVID-19 naïve patients or when neutrophils were pretreated with the NOX inhibitor DPI [300].

Acute lung injury during COVID-19 also correlates with elevated levels of D-dimer and fibrinogen suggesting that thrombosis may be

contributing to increased mortality in severe cases [297,298]. Indeed, severe COVID-19 cases and COVID-19 deaths have been linked to thrombotic complications like pulmonary embolism [301]. Analysis of post-mortem lung tissue has shown that COVID-19-related deaths appear to be correlated with increased platelet-fibrin thrombi and microangiopathy in the lung (Fig. 5F) [302,303]. NETs from activated neutrophils are likely directly contributing to thrombosis, but there is also evidence to suggest that endothelial cells may be involved [299].

Severe COVID-19 cases have been associated with endothelial cell activation which is present not only in the lungs but also in other vital organs like the heart, kidneys, and intestines [304]. Endothelial cells express the ACE2 receptor which is required for infection by SARS-CoV-2. One hypothesis is that infected endothelial cells produce tissue factor after activation of NOX2, which promotes clotting through interaction with coagulation factor VII (Fig. 5G) [305]. Escher and colleagues reported that treatment of a critically ill COVID-19 patient with anticoagulation therapy resulted in a positive outcome and hypothesize that endothelial cell activation may also be driving coagulation [306]. Studies of SARS-CoV that was responsible for the 2003 SARS epidemic have shown that oxidized phospholipids were found in the lungs of infected patients, which is associated with acute lung injury through promotion of tissue factor expression and initiation of clotting [307,308].

Therapies targeting ROS or NOX enzyme activation may be beneficial in acute lung injury. Given the role of NOX2-derived ROS as a driver of acute lung injury during COVID-19, therapies that target NOX2 enzymes or ROS may be beneficial in severe COVID-19 cases. Pasini and colleagues have extensively reviewed the subject and propose that studies should be performed to assess the use of ROS scavengers and

NRF2 activators as potential COVID-19 therapeutics to be used alone or in conjunction with existing treatments [291]. It has also been proposed that supplementation of vitamin D may also have a positive effect on COVID-19 outcomes through its immunomodulatory effects including inducing downregulation of NOX2 [309]. However, vitamin D has also been shown to upregulate ACE2 which may facilitate viral replication [310]. Therefore, these proposed COVID-19 therapies need testing before their efficacy can be determined.

Targeting NOX enzymes in acute lung injury not caused by COVID-19 may also be beneficial. In acute lung injury caused by renal ischemia-reperfusion, treatment with dexmedetomidine reduces NOX4 activation in alveolar macrophages which correlates with decreased NLRP3 inflammasome activation [311]. Another recent study demonstrated that treatment with the NOX1/NOX4 inhibitor GKT137831 results in less severe acute lung injury after ischemia-reperfusion in mice by dampening the amount of proinflammatory cytokines produced in the lungs [312].

7. Perspectives & conclusions

7.1. Inhibitors of NOX enzymes

Given the role of NOX-derived ROS in various pathologies, specific inhibitors of NOX enzymes have been of interest. Numerous NOX inhibitors have been developed that are pan-NOX inhibitors or are specific to one NOX enzyme (reviewed in Ref. [313]). Inhibitors specific to NOX2 have been developed and shown efficacy in mice [313]. The peptide inhibitor (RKKRRQRRRCSTRIRRL), called NOX2ds-tat, has been shown to prevent p47^{phox} binding to the B-loop of NOX2 [314]. This inhibitor has been shown in a mouse model of ischemic retinopathy to block VEGF overexpression [315]. This inhibitor has also been successfully used to reduce vascular superoxide levels in response to angiotensin II [316]. These proof-of-concept studies demonstrate that NOX inhibitors may have therapeutic benefit for human diseases including COVID-19, type I diabetes, and cardiovascular disease.

7.2. Conclusions

NOX enzymes are an important group of enzymes that produce superoxide which is necessary for the generation of other ROS like hydrogen peroxide, peroxynitrite, and hydroxyl radicals. NOX-derived ROS function in diverse pathways in immunity including immunity to pathogens, generation of an adaptive immune response, cell signaling, and autoimmunity. Understanding the complex biology of NOX enzymes and their regulation will be important for treating a wide variety of diseases. NOX2 is the best-studied NOX enzyme, but more studies of the other NOX enzymes are being conducted to discover their role in health and disease. Future studies investigating the role of antioxidants or inhibitors of NOX enzymes could lead to the development of beneficial therapies for a wide variety of autoimmune and infectious diseases.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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