

## Forum Review

# Role of Nox Family NADPH Oxidases in Host Defense

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### ABSTRACT

The phagocytic NADPH oxidase is recognized as a critical component of innate immunity, responsible for generation of microbicidal reactive oxygen species (ROS). This enzyme is one representative of the Nox family of oxidases (Nox1–Nox5, Duox1, and Duox2) that exhibit diverse expression patterns and appear to serve a variety of functions related to ROS generation. Mounting evidence now suggests that several of these novel oxidases also serve in host defense, particularly those showing high expression along epithelial surfaces exposed to the external environment. Within these sites, Nox enzymes tend to be located on apical cell surfaces and release ROS into extracellular environments, where they can be used by known antimicrobial peroxidases. Moreover, microbial factors were shown in several cases to cause higher ROS production, either by direct oxidase activation or by inducing higher oxidase expression. Several oxidases are also induced by immune cytokines, including interferon- $\gamma$ , interleukin (IL)-4, and IL-13. Although most of the evidence supporting host defense roles for mammalian nonphagocytic oxidases remains circumstantial, recent evidence indicates that *Drosophila* Duox plays a role in host resistance to infection. Finally, oxidative defense against invading pathogens appears to be an ancient protective mechanism, because related oxidases are known to participate in disease resistance in plants. *Antioxid. Redox Signal.* 8, 1549–1561.

PHAGOCYtic BLOOD CELLS produce large amounts of reactive oxygen species (ROS) in response to a variety of infectious and proinflammatory stimuli. Production of toxic ROS by phagocytes normally occurs in a controlled, contained process during the engulfment of microbes; this process represents an essential arm of the innate immune system. The critical nature of this antimicrobial defense system is evident in chronic granulomatous disease (CGD), where inherited defects in this oxidative response are associated with enhanced susceptibility to bacterial and fungal infections and excess granuloma formation (92). Over the last 20 years, lesions in five distinct genes have been identified that can result in defects in microbial killing by phagocytes [See reviews (21, 84, 92)]. Each of these genes encodes a distinct, essential component of the phagocytic NADPH oxidase complex (phox system), responsible for generation of superoxide anion, a precursor of hydrogen peroxide, hypochlorous acid,

and other potent ROS formed within phagosomes. The most commonly affected gene (*CYBB*) associated with the X-linked form of CGD encodes the enzymatic core of the oxidase, gp91<sup>phox</sup> or Nox2. Three other genes (*CYBA*, *NCF1*, and *NCF2*) encode p22<sup>phox</sup>, p47<sup>phox</sup>, and p67<sup>phox</sup> oxidase components affected in autosomal-recessive forms of CGD. The fifth was initially identified biochemically as an oxidase-supportive ras-like protein, Rac1 or Rac2 (1, 59). A dominant-negative mutation in Rac2 (D57N) was later associated with deficient neutrophil oxidase activity and impaired chemotaxis (5). Much of what has been learned about NADPH oxidase function in phagocytes was derived from studies of CGD patients, together with biochemical approaches, which have advanced our understanding of components needed for complete reconstitution of NADPH oxidase activity *in vitro* (21, 76, 84). Studies in mice with targeted disruption of genes encoding oxidase components (gp91<sup>phox</sup>,

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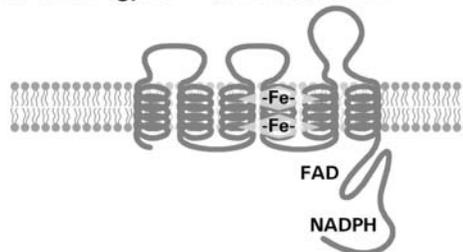
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p47<sup>phox</sup>, or Rac2) confirmed their essential roles in oxidase function, as well as the importance of this enzyme as a primary innate defense system effective against bacterial and fungal infection (50, 74, 88).

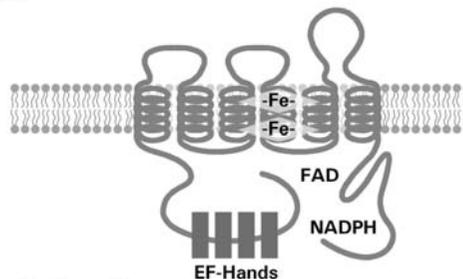
The widely held view that the ROS produced by the phagocyte oxidase act principally as microbicidal agents was challenged recently by the proposal of an alternative mechanism, suggesting that the electrogenic activity of the oxidase, not ROS toxicity, is responsible for microbial killing (3, 85). The model proposes that the rapid production of superoxide anion by the oxidase causes dramatic changes in the phagosomal ionic environment that are required for release and activation of other microbicidal agents (elastase, cathepsin G) that are delivered from insoluble granule stores.

Within the last 10 years, a growing interest has developed in other essential roles served by cellular ROS production, even at much lower levels than are needed in antimicrobial defense (32). Many cells produce and respond to cellular "redox signals," which can act in altering gene-expression patterns, or lead to physiologic and metabolic adjustments to altered oxygen levels, resulting in enhanced cell proliferation, senescence, or apoptosis (32). Redox signals can be transduced through covalent modifications of key regulators that are specific cellular targets of oxidation (87). With the recent growth of information available in genome databases, several novel sources of cellular ROS related to the phagocytic oxidase have been identified that are now designated members of the Nox (or NADPH oxidase) family (Fig. 1). The Nox family in humans encompasses seven unique oxidase genes that encode the core catalytic components of these enzymes: Nox1, Nox2 (a.k.a. gp91<sup>phox</sup>), Nox3, Nox4, Nox5, Duox1, and Duox2 (15, 40, 64). Proposed functions of these novel oxidases based on their expression patterns or the observed effects of their altered or heterologous expression have included roles in regulating cell proliferation, angiogenesis, cell senescence, fertilization, oxygen sensing, biosynthetic processes (thyroid hormone synthesis, extracellular matrix cross-linking), hormone signaling, and regulation of vascular tone (15, 40, 64, 65). In two cases, mutations identified within nonphagocytic oxidase genes have provided clear indications of their physiologic roles: Duox2 is critical in thyroid hormone biosynthesis, because mutations in Duox 2 cause hypothyroidism in humans (73), and Nox3 has a distinct role in the inner ear, because mutations in Nox3 result in deficits in balance and gravity perception in mice (78). Although many tissues express these novel oxidases at levels sufficient to serve as redox-related signaling enzymes, growing evidence suggests that several of these novel nonmyeloid oxidases also function as host defense enzymes. The mounting evidence supporting such a role can be summarized by several general findings: (a) observations regarding tissues and cell types that exhibit abundant expression of these oxidases (*i.e.*, epithelial surfaces exposed to the external environment) (38, 41, 97); (b) observations on the subcellular locations of these oxidases on epithelial surfaces, again suggesting that ROS generated by these enzymes are aimed toward the extracellular environments of these surfaces (31, 54, 91); (c) studies showing that microbial components can act as agonists that stimulate ROS release by these sources (53, 54, 56); (d) observations on proinflammatory signals (cytokines) that can

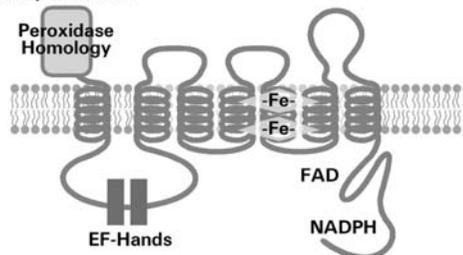
#### Nox1, Nox2 (gp91<sup>phox</sup>), Nox3, Nox4:



#### Nox5:



#### Duox1, Duox2:



**FIG. 1. Structures of Nox family NADPH oxidases.** All oxidases share common structural features identified in the well-characterized catalytic component of the phox system, gp91<sup>phox</sup> (now called Nox2). These include conserved structural motifs involved in binding NADPH, FAD, and two membrane-imbedded heme moieties, which carry electrons from their cytosolic source to some extracytoplasmic compartment. In addition, Nox5 has four EF-hands at its amino terminus that aid direct oxidase activation by calcium. The dual oxidases (Duox1 and Duox2) have an amino-terminal ectodomain with peroxidase homology as well as two calcium-binding EF hands. [Data adapted from Geiszt M and Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* 279: 51715–51718, 2004.]

induce higher expression of several of these ROS generators (38, 61); (e) evidence suggesting functional partnerships between these novel ROS generators and known ROS-dependent antimicrobial systems (*i.e.*, peroxidases) (34, 41); and (f) recent genetic approaches that provide compelling evidence indicating that related oxidases perform antimicrobial defense functions in diverse species, including plants and *Drosophila* (44, 104, 114). Although the importance of NADPH oxidase-dependent bacterial killing by circulating phagocytic cells has been appreciated for decades, the emerging evidence on the novel oxidases suggests that epithelial surfaces rely on similar oxidase-dependent innate microbicidal mechanisms to fight infection, as this interface with the outside represents the true first line of defense against micro-

bial invasion. Direct evidence confirming that the mammalian nonphagocytic oxidases have sufficient oxidative output to be effective antimicrobial systems is still lacking, in part because the cells showing highest Nox or Duox expression are terminally differentiated and are not easily maintained or manipulated in culture. Thus, transgenic Nox or Duox animal models will be crucial in assessing critical host-defense roles of these novel oxidases. This review provides an overview of the functions of the Nox family oxidases, focusing on the recent evidence suggesting that several of these novel oxidases also serve as host defense enzymes.

### *Nox2—the prototypical host defense oxidase of phagocytes*

By their very nature, reactive oxidants are indiscriminate damaging molecules, thus phagocytic cells have developed elaborate mechanisms for controlling their production, both spatially and temporally; several recent reviews provide an excellent overview of these mechanisms (26, 76, 84, 99). In resting cells, the phox system is maintained in a latent, disassociated state: the flavocytochrome b558, composed of gp91<sup>phox</sup> and p22<sup>phox</sup> subunits, is located within intracellular vesicular and granule stores; other regulatory components (p47<sup>phox</sup>, p67<sup>phox</sup>, and p40<sup>phox</sup>) are associated in a cytosolic complex, and Rac1 or Rac2 is maintained as an inactive, GDP-bound complex with RhoGDI (15). When phagocytes encounter and engulf microbes, several receptor types are stimulated (formyl peptide, Fc, and complement receptors) that trigger overlapping signaling pathways, causing the assembly of oxidase components on the membrane and activation of the oxidase (76, 84, 108). This assembly and activation process occurs in a carefully coordinated manner: flavocytochrome-containing vesicles fuse with newly forming phagosomes; the cytosolic regulators undergo phosphorylation-dependent conformational changes and are thereby targeted to specific membranes, where they can interact with the flavocytochrome. Rac1 or Rac2 is independently activated and translocates to assemble with the oxidase effector system. Rac endows the enzyme with guanine nucleotide sensitivity as it interacts with the activator component, p67<sup>phox</sup>, in a GTP-dependent manner. Together these proteins control the flow of electrons through the flavocytochrome. The p47<sup>phox</sup> component acts as a critical phosphorylation-dependent adaptor molecule that bridges interactions between p67<sup>phox</sup> and the flavocytochrome. Both priming and adherence of phagocytes also affect the assembly of Rac and other oxidase components (115). In addition, it appears that other events such as phospholipase A<sub>2</sub> activation are needed after assembly of this complex to initiate oxidase activation (23). The gp91<sup>phox</sup> component of the phagocyte oxidase, now referred to as Nox2, contains all the known electron-carrier (catalytic) functions of the enzyme, as it has binding sites for cytosolic NADPH, FAD, and two membrane-imbedded heme molecules. These are the most-conserved structural features observed in the catalytic centers of all other Nox homologues (Fig. 1). Based on topographic models developed from the phagocytic system, all other Nox oxidases are proposed to use cytosolic sources of electrons (NADPH or NADH), pass them through a flavin intermediate, and then transfer elec-

trons through the membrane to molecular oxygen within some extracytoplasmic compartment (*i.e.*, phagosomes) (21). As the structures and functions of other nonmyeloid oxidases are being described in more detail, it appears that the phox (Nox2-based) enzyme has evolved into one of the most specialized and functionally divergent members of this family, which is presumed to relate to its role as a robust but tightly regulated enzyme in phagocytes. In addition to these post-translational events regulating oxidase activation, inflammatory signals such as interferon (IFN)- $\gamma$  can also act to regulate the expression of most oxidase components (84, 95). Furthermore, microbial factors such as LPS can induce higher expression of Nox2. Thus, both host and pathogen-derived signals can act at the transcriptional and posttranslational levels to enhance the output of ROS by circulating phagocytes.

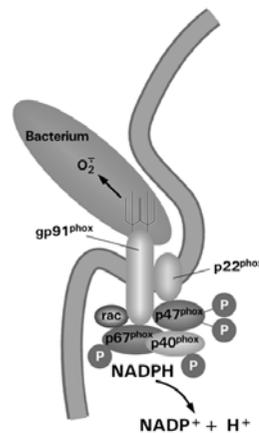
**Nox1.** Nox1 was the first nonphagocytic Nox family oxidase identified and is the closest homologue of Nox2. Early observations on the effects of heterologously expressed Nox1 suggested that this oxidase functions in promoting mitogenesis (97), because NIH 3T3 fibroblasts expressing Nox1 exhibited increased growth, developed a transformed phenotype, and spontaneously formed tumors in nude mice. Hence, this enzyme was initially named *Mox*, for mitogenic oxidase. Later work suggested that the ROS derived from Nox1 could also function in promoting angiogenesis (7). However, these proposed functions were reconsidered recently, because a transforming Ras mutant was also detected in the cell lines used in these studies (64). By far, the highest expression of Nox1 is detected in the colon, where the transcript and protein are detected in the epithelial layer (38, 54, 57). Nox1 mRNA was detected by *in situ* hybridization at highest levels within the lower two thirds of colon crypts, a site where the cells are proliferating and undergoing rapid differentiation (38). However, given the short life span of these cells, it was suggested that Nox1 accumulates and functions within terminally differentiated epithelial cells. Later work showed that the Nox1 protein is detected at highest levels on the luminal surfaces of epithelial cells within the upper portions of crypts, where the epithelial cells reach their most differentiated state (36, 54). Studies in which Nox1 expression was suppressed in colon epithelial tumor lines showed no effect on the proliferation rates of these cells; furthermore, inducers of differentiation, such as IFN- $\gamma$  and 1, 25-dihydroxy vitamin D<sub>3</sub>, halt cell proliferation while causing remarkably enhanced expression of Nox1 in CaCo<sub>2</sub> and HT-29 cells (38). Thus, high Nox1 expression in the colon epithelium did not appear to correlate directly with cell proliferation, and Nox1 was proposed to serve some specialized function in this tissue, such as host defense (38). Consistent with these observations, two groups noted that Nox1 expression is highest in well-differentiated colon tumors and lower in poorly differentiated tumors (36, 38). Fukuyama *et al.* dissociated high Nox1 expression in well-differentiated tumors from Ki-67 expression, which occurs in rapidly proliferating tumors (36). A third group claimed that no correlation existed between Nox1 expression and cell proliferation in various colon tumors (101). Thus, Nox1 expression does not appear to have a direct role in cell proliferation in normal or in cancerous colon cells. A broad survey of a variety of other

tumor specimens did not detect remarkable Nox1 expression in any other tumor tissues (38); the study screened more than 1,200 tumors in human multitissue tumor arrays (NIH-NCI TARP-1 and TARP-2). However, in light of evidence linking high Nox1 expression to pro-inflammatory pathways (*i.e.*, NF- $\kappa$ B activation) (36), the questions of whether Nox1 signaling or its generation of toxic ROS have specific roles in inflammation and carcinogenesis deserve further study.

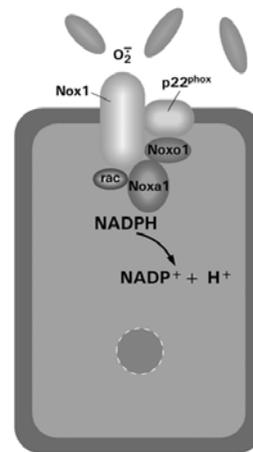
The close structural similarities between Nox1 and Nox2, along with the dramatic induction of Nox1 by IFN- $\gamma$  (38, 61), which is reminiscent of responses of several phox component genes (Nox2, p47<sup>phox</sup>, and p67<sup>phox</sup>), suggested that Nox1 could also serve as a host-defense oxidase. Close examination of the Nox1 promoter elements responsible for IFN- $\gamma$  responsiveness suggests that it has evolved independent of the phox genes, presumably to enable different tissue-specific expression patterns (61). Recently, several groups have noted other remarkable functional similarities between the Nox1- and Nox2-based enzymes. Nox1 was shown to cross-function in place of Nox2 in several models of X-linked CGD (38), indicating that Nox1 activation involves regulatory cofactors, such as p47<sup>phox</sup> and p67<sup>phox</sup>, for full activity. Several laboratories showed that colon epithelial cells express homologues of the phox regulators that are needed to support Nox1 activity (8, 19, 39, 102). The similarities between the multicomponent Nox2 and Nox1 oxidases became further evident, based on observations suggesting p22<sup>phox</sup> and Rac1 involvement in Nox1: p22<sup>phox</sup> appears to associate with and stabilize Nox1, while binding to the p47<sup>phox</sup>-like counterpart, Noxo1 (Nox organizer 1) (4, 19, 102, 106). Rac1 binds to the p67<sup>phox</sup>-like component, Noxa1 (Nox activator 1), in a GTP-dependent manner (102), and may thereby regulate Nox1 activity in a manner analogous to that demonstrated with Nox2 (15). Furthermore, the activity of reconstituted Nox1 systems is affected by mutants of Rac1 or its binding partner, Noxa1, or by Rac1-targeted RNA interference (106). Thus, the colon (Nox1-based) oxidase system functions very much like the phox system, being a regulated, multicomponent enzyme dependent on some of the same or related supportive cofactors for full activation (Fig. 2). Interestingly, human Nox1 demonstrates both constitutive and agonist-dependent (*i.e.*, PMA-stimulated) activities in several reconstituted systems and may not be subject to the same tight controlling mechanisms that regulate Nox2 activity (39, 102). In contrast, the murine Nox1 system exhibits high constitutive activity that is not further enhanced by PMA stimulation (8). Furthermore, it appears that the Nox1 and Nox2 systems have diverged in humans, compared with the murine systems, based on differences in the efficiencies in which the heterologously expressed Nox1 and Nox2 components can cross-function (8, 39, 102).

Some of the earliest indications suggesting roles for Nox1 in host defense were reported by Rokutan and colleagues, who showed that LPS from pathogenic *Helicobacter pylori* strains causes enhanced ROS release by guinea pig gastric pit cells (56). This was attributed to direct induction of Nox1 by LPS, which acts through activated toll-like receptors (TLRs) 4. LPS also causes coordinate induction of the Nox1-supportive regulator, Noxo1 (53), consistent with the similar tissue-specific expression patterns of Nox1 and Noxo1 (39). Furthermore, TLR4 activation by LPS appears to trigger other

### The Phagocyte Oxidase



### The Colon Oxidase



**FIG. 2. Comparison of closely related multicomponent oxidase systems of phagocytic and colon epithelial cells.** Both oxidases involve common p22<sup>phox</sup> and Rac GTPase components. Regulatory cofactors homologous to the p47<sup>phox</sup> and p67<sup>phox</sup> components, Noxo1 and Noxa1, also appear to serve similar functions in regulating the activity of the colon oxidase: Noxo1 serves as an adaptor protein that bridges interactions between Nox1 and membrane-spanning flavocytochrome subunits (Nox1 and p22<sup>phox</sup>), whereas rac1 shows GTP-dependent binding to Noxa1, which promotes oxidase activation. The phagocytic system is targeted to newly formed phagosomes, whereas the colon system accumulates on the surface of epithelial cells, because superoxide is detected outside of cells. See text for details. [Data adapted from Geiszt M and Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem* 279: 51715–51718, 2004.]

posttranscriptional events that may enhance Nox1-mediated ROS release, including Rac1 activation (53). In contrast to gastric pit cells, colon epithelial cells, which lack TLR4, produce ROS when stimulated through TLR5 activation (54). In the human colon cancer line, T-84, recombinant flagellin from *Salmonella enteritidis*, which binds TLR5, induces higher Nox1 expression and increased ROS release in the presence of Noxo1 and Noxa1; these stimulated cells also show ROS-dependent release of IL-8 (54). Thus, gastric pit and colon epithelial cells recognize different pathogen factors, which can act through distinct innate immune pathways to trigger both transcriptional and posttranslational events leading to enhanced responses involving Nox1. Szanto *et al.* (101) also suggested a host-defense role for Nox1 in the colon when they detected a gradient of higher Nox1 mRNA from the ascending to descending colon segments, correlating Nox1 expression with an increased microbial burden in through tissue. They also detected high Nox1 mRNA levels in interstitial lymphoid cells of the colon, particularly within inflammatory lesions of ulcerative colitis and Crohn disease patients (101). The induction of Nox1 by IFN- $\gamma$  has interesting implications regarding the pathogenesis of inflammatory bowel disease (IBD), because studies in animal models of IBD highlight the importance of imbalances in production of Th1 cytokines, such as IFN- $\gamma$  (16). Furthermore, enhanced oxidant-induced injury to colon epithelial cells has been

noted in IBD (71). Additional work is needed to clarify possible relations between enhanced IFN- $\gamma$  release and Nox1-mediated ROS production in the pathogenesis of IBD.

**Nox3.** Nox 3 is closely related to both Nox1 and Nox2 and also functions as a multicomponent oxidase. Human Nox3 is a more versatile oxidase than Nox1 or Nox2, because it has significant constitutive activity in the absence of any activator or organizer component; however, its activity is enhanced by either one or both regulators of Nox1 or Nox2 (9, 20, 105, 106). Despite these functional similarities with Nox1 and Nox2, there is no evidence suggesting Nox3 functions in host defense. Its critical function was identified in the head-tilt (*het*) mutant mouse strain, which has deficits in balance and gravity perception (78). Lesions in the Nox3 gene of these mice result in defective otoconia morphogenesis in the inner ear. A similar phenotype in the head-slant (*hslt*) mutant mouse strain was attributed recently to lesions in the Nox1 gene (13, 58); thus, Nox1 appears to be an essential regulator of Nox3 in the mouse inner ear.

**Nox4.** Nox4 is structurally and functionally divergent from Nox1 and Nox2. Nox4 exhibits high constitutive activity when expressed alone and is not dependent on the same cytosolic regulators that support Nox1 or Nox2 activity (37, 70, 94). Nox4 forms a stable heterodimeric complex with p22<sup>phox</sup> (4, 70), although its activity is not affected by mutations of the p22<sup>phox</sup> cytoplasmic domain that disrupt its interaction with Nox organizers (Nox1 or p47<sup>phox</sup>) (55). Its high constitutive activity when transfected alone into NIH-3T3 cells is sufficient to inhibit cell growth and cause a senescence phenotype (37, 94). In transfected HEK 293 cells, the enzyme is detected primarily in intracellular sites, consistent with detection of extracellular hydrogen peroxide, not superoxide (70).

The most abundant Nox4 expression is detected in the kidney, as it was originally designated a renal-specific oxidase called Renox (37). This oxidase is also widely detected at lower levels in other tissues, particularly in vascular cells (2, 47, 60, 81). Highest Nox4 expression was initially detected in cortical regions of the murine kidney, specifically in proximal tubules (37). The expression of Nox4 in proximal tubules, together with its high constitutive activity, led to the proposal that Nox4 could serve as a renal oxygen sensor (37), because erythropoietin expression also occurs in proximal tubules (67) or close to proximal tubules (62). ROS have been implicated in oxygen sensing through negative-feedback regulation of erythropoietin expression, independent of hypoxia-inducible factor (49). Interestingly, superoxide dismutase 3 (SOD-3) is also produced in proximal tubules, and disruption of this gene results in an altered erythroid response to hypoxia in mice (98), suggesting that both superoxide production and its conversion to hydrogen peroxide could have roles in regulating erythropoiesis. Nox4 expression in the human kidney is distinctly different from that in the murine kidney, because Nox4 was detected immunohistochemically within human renal distal tubules (94); these authors also suggested that Nox4 could serve in renal oxygen sensing. Since these early speculations, several reports have provided other links between Nox4 and oxygen sensing.

Nox4 expression itself is enhanced in response to hypoxia (98). Although prolyl hydroxylases have been proposed to act as universal oxygen sensors that regulate hypoxia-inducible factors 1 $\alpha$  (17), recently Nox4-derived ROS have been linked to HIF-2 $\alpha$  transcriptional activity in von Hippel-Lindau-deficient renal tumors (69). Furthermore, it appears that Nox4 can transduce signals leading to alterations in expression of oxidative stress-responsive genes (60). Further studies are needed to clarify the relationship of Nox4 expression to renal oxygen sensing.

We have also detected strong Nox4 mRNA signals by *in situ* hybridization in medullary regions of the human kidney, particularly within collecting ducts and the renal pelvic epithelium (M. Geiszt and T. L. Leto, unpublished observation). This suggests human Nox4 may serve other functions, such as in host defense, by providing a constitutive source of hydrogen peroxide in urine; this could explain significant hydrogen peroxide levels detected in urine (100  $\mu$ M range) (107). Other plausible functions for the renal oxidase could include roles in oxidation or detoxification of urine wastes or regulation of renal pH or salt resorption, based on the proton and electrogenic activities of all Nox oxidases (21). Gorin *et al.* (43) suggested that a Nox4-based oxidase is responsive to angiotensin II stimulation in mesangial cells. Nox4 and NF- $\kappa$ B activation have been linked to LPS-mediated TLR4 activation, which results in ROS production in heterologously transfected cell models; this study showed that Nox4 can interact directly with the TLR4 (79). Although the physiological settings where such interactions occur were not explored, these experiments suggest the interesting possibility that Nox4 can respond directly to microbial agonists. Other extrarenal functions for Nox4 have been proposed, unrelated to host defense. Nox4 is present in murine osteoclasts, where, together with the phagocytic homologue, it may provide an oxidative basis for bone resorption (113). Nox4 is also detected in vascular smooth muscle and endothelial cells (2, 47, 60, 81). Agonists such as angiotensin II can alter Nox4 expression, although the exact contribution of Nox4 to vascular ROS production has not been explored extensively (65). Nox4 involvement in TGF- $\beta$  signaling was recently described in pulmonary artery smooth muscle cells, in human umbilical vein endothelial cells, and in cardiac fibroblasts (22, 48, 96). Other studies suggest that Nox4 mediates ROS-dependent insulin signaling in 3T3-L1 adipocytes and growth factor signaling in fibroblasts (68, 80); both of these oxidant-generating receptor pathways were proposed to act through inhibition of protein tyrosine phosphatases (103).

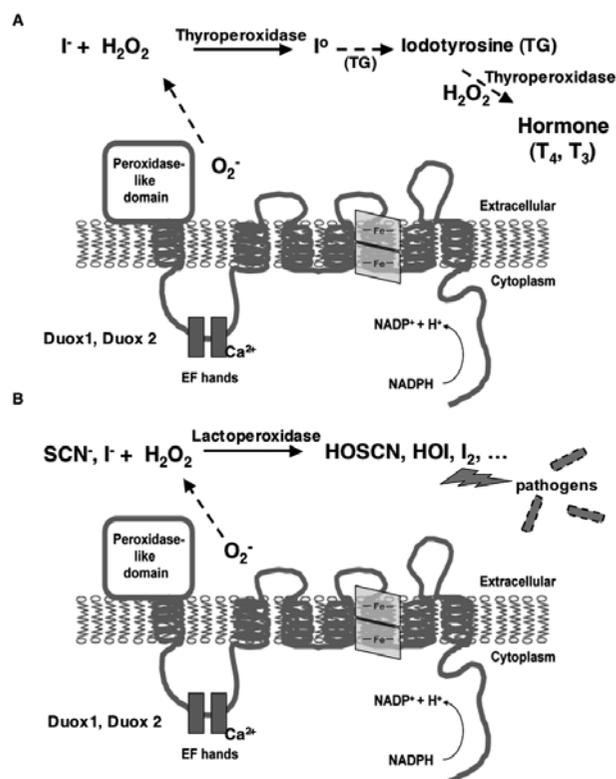
**Nox5.** Like Nox3, Nox5 has a very specialized expression pattern and does not appear to have any role in host defense (10). High Nox5 expression is limited to pachytene spermatocytes of testis and B and T lymphocyte-rich areas of spleen and lymph nodes. Its expression is highest in B cell-rich regions around germinal centers, but not in any circulating lymphocytes (10). The enzyme is directly activated by calcium binding to EF-hand structures that lie N-terminal to the oxidase portion (11). This oxidase is presumed to serve some calcium-dependent signaling function in lymphoid cells. In testis, the expression is highest in pachytene spermatocytes and may

serve some specialized function in spermatogenesis or even in mature sperm, such as in sperm–oocyte fusion.

**Duox1 and Duox2.** Unlike other novel Nox family members, the dual oxidases (Duox1 and 2) were not initially recognized in human genome databases; rather, these oxidases were discovered through efforts aimed at identifying enzymatic sources of hydrogen peroxide needed for thyroid hormone synthesis (25, 29). Earlier biochemical studies on thyroid cells indicated that hydrogen peroxide release was calcium dependent and exhibited other properties of NADPH oxidase activity (28, 66, 75). Hydrogen peroxide is used for the organification of iodide during thyroid hormone synthesis (Fig. 3A). An essential role for Duox2 in thyroid hormone synthesis was confirmed in rare forms of hypothyroidism, where biallelic Duox2 lesions cause severe hypothyroidism, and even single allelic lesions result in mild hypothyroidism (73). Although Duox1 expression is also high in thyroid glands, its distinct role apart from that of Duox2 has not been established. The Duox nomenclature was

proposed based on the dual oxidase structure of these enzymes (30), because these enzymes have amino-terminal peroxidase homology domains in addition to their C-terminal NADPH oxidase (Nox-like) domains (Fig. 1). The dual oxidases appear to have ancient origins, because closely related homologues have been identified in several invertebrate species, including nematodes, fruit flies, and sea urchins (30, 44, 112). Between the two oxidase domains, an additional membrane-spanning segment places the peroxidase-like domain in an extracytoplasmic compartment; furthermore, two calcium-binding EF hands within the first cytoplasmic loop likely mediate direct calcium-dependent activation of these oxidases. Despite the extensive homology of the Duox peroxidase-like domains with other peroxidases, these domains are atypical in that they lack some of the most critical residues conserved in all other well-characterized heme peroxidases (30). Two reports claim the isolated domains from human, *Caenorhabditis elegans*, and *Drosophila* Duox isozymes exhibit peroxidase activity, although no one has shown to date that these proteins indeed contain heme (30, 44).

Our understanding of the regulation of the NADPH oxidase portions of Duox has been hampered by difficulties in producing the active oxidases in whole transfected cells (24, 72). It is not known whether these oxidases require special cofactors for activation, maturation, or transport to the plasma membrane. Full processing and plasma membrane transport of Duox may require the establishment of a polarized epithelium, because such cells show the highest Duox accumulation. Duox does not appear to be supported by Rac (33) or the cytosolic phox regulators (24), consistent with the fact that autosomal recessive CGD patients do not have any problems with thyroid metabolism. Duox was shown to co-immunoprecipitate with p22<sup>phox</sup>, although its coexpression does not contribute to Duox activity in transfected cells (6, 109). As shown with the Nox2 (gp91<sup>phox</sup>) flavocytochrome, it appears that glycosylation of Duox is a requisite for transport of Duox to the plasma membrane. Human Duox2 possesses a retention signal in the first cytoplasmic loop, which prevents its complete processing and transport to the plasma membrane when heterologously expressed in nonthyroid cells (72). Recently, an active oxidase was detected in broken cell membrane preparations of Duox2-transfected cells that produce an incompletely glycosylated form of Duox2 (6). These preparations showed both calcium- and NADPH-dependent production of superoxide and hydrogen peroxide, whereas membranes from mature thyrocytes containing the fully glycosylated, mature Duox forms produce only hydrogen peroxide. These findings suggest that the C-terminal, Nox-like domains of Duox2 produce superoxide as a primary product, and that the mature peroxidase-like domain may have the activity that converts this to hydrogen peroxide. Duox peroxidase homology domains do not have any striking structural similarities with known superoxide dismutases, although the calcium-binding sequences in other peroxidases are conserved in Duox, which may bind other metals needed for superoxide dismutase activity. The domain is well positioned for such a role, because superoxide is generated within the same molecule, and it could readily accept a second electron from the oxidase portion to form hydrogen peroxide. This hypothesis may explain why all Duox-expressing cells produce hydrogen peroxide, but no detectable superoxide (*i.e.*, thyro-



**FIG. 3. Functional partnerships of H<sub>2</sub>O<sub>2</sub>-generating Duox isozymes with tissue-specific peroxidases.** (A) Thyroid gland hormone synthesis requires both hydrogen peroxide generation and the activity of thyroperoxidase to oxidize iodide ions to form iodine and iodinate tyrosines on thyroglobulin (Tg). (B) The analogous partnership of mucosal surface Duox with known antimicrobial peroxidases found in mucosal secretions (*i.e.*, lactoperoxidase or myeloperoxidase). Lactoperoxidase can form a variety of antimicrobial oxidants based on a substrate specificity similar to that of thyroperoxidase. The pseudo-halide thiocyanate (SCN<sup>-</sup>) is the most relevant substrate in terms of its abundance in saliva and other mucosal secretions, and approaches millimolar concentrations.

cytes, mucosal epithelial cells, sea urchin embryos (28, 41, 46). Furthermore, this would explain the existence of other dedicated peroxidases (*i.e.*, thyroperoxidase, lactoperoxidase, ovoperoxidase) in those tissues or cells exhibiting high Duox expression and activity (see later). Recently, Duox2 was shown to bind directly to thyroperoxidase (109), which would efficiently supply the hydrogen peroxide needed for thyroperoxidase-catalyzed reactions within the thyroid follicle lumen.

Studies on the expression of Duox in invertebrates such as *C. elegans* and the sea urchin have provided insight into other extrathyroid functions of these ancient oxidases. Here these enzymes appear to participate in oxidative cross-linking of extracellular matrix proteins. RNA interference experiments revealed that a Duox isozyme in *C. elegans* is involved in cuticle biosynthesis, because defective cuticle formation is observed in developing worms treated with Duox-specific siRNA (30, 52). A similar blistered-cuticle phenotype is observed in worms with mutations in collagen biosynthesis (51). Edens *et al.* (30) observed a lower content of di- and tri-tyrosine in the cuticles of siRNA-treated worms and suggested that Duox1 expressed in hypodermal cells is responsible for cross-linking of collagen chains during cuticle biosynthesis. In the case of the sea urchins species, *Strongylocentrotus purpuratus* and *Lytechinus variegatus*, Duox isozymes were identified that are thought to be the source of calcium-triggered hydrogen peroxide release that accompanies fertilization of eggs (112). The calcium signal also causes exocytosis of ovoperoxidase, which uses the hydrogen peroxide to catalyze oxidative cross-linking of dityrosine in proteins within the fertilization envelope (46). Thus, barrier formation appears to be a fundamental role for the Duox isozymes, which has preceded its specialized function in thyroid hormone synthesis.

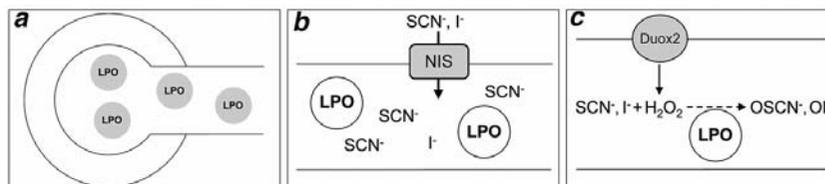
Although Duox expression in mammalian lung and other tissues was recognized early (18, 30), the functional significance of extra-thyroid Duox expression was not appreciated until a histologic survey revealed that Duox transcripts are particularly abundant in epithelial cells along mucosal surfaces and in exocrine glands (41). Using *in situ* hybridization, we detected high Duox expression confined to the epithelial cell layers of salivary gland ducts, major airways (bronchial and tracheal surfaces), and the rectal mucosal boundary. Based on these observations, we suggested that the principal function of the Duox isozymes in these nonthyroid tissues was a role in host defense (41) related to a functional partnership with lactoperoxidase (LPO) (Fig. 3B). It had been known for decades that various secretions (milk, saliva, mucus,

tears) contain the antimicrobial peroxidase, LPO (86), although the sources of hydrogen peroxide supporting LPO activity in these secretions were not clear. LPO is effective against a range of gram-positive and gram-negative bacterial species (14, 77) as well as other fungal (12) and viral pathogens (82). It was suggested that some microbial species themselves produce enough peroxide to support LPO activity in saliva, or that ROS produced by the NADPH oxidase of leukocytes could be involved in some sites. However, fresh saliva collected from healthy, sterile salivary glands contains considerable amounts of hydrogen peroxide that would not be derived from such sources (83). The detection of Duox2 expression specifically within terminal salivary gland duct epithelia has particular significance in the proposal that Duox serves as a hydrogen peroxide source for salivary LPO (41) (Fig. 4). LPO synthesis was detected in early stages of saliva formation within salivary gland acinar cells. Its principal substrate in saliva is thought to be the pseudo-halide thiocyanate, because its levels in saliva approach millimolar concentrations (83). Thiocyanate is oxidized by  $H_2O_2$  to form hypothiocyanite, a broadly effective microbicidal or microbistatic oxidant. Thiocyanate in saliva is likely supplied by the sodium iodide symporter that has been localized within salivary gland intercalated ducts (41). The Duox-LPO antimicrobial system is completely assembled only in late stages of saliva formation, because Duox2 expression is localized within terminal duct epithelial cells and would thereby provide the most labile component of the system  $H_2O_2$  just before delivery into the oral cavity.

Another site of high Duox2 expression, which was correlated with LPO expression, is the GI tract. We detected particularly high Duox2 levels in the rectal mucosa by *in situ* hybridization and Northern blotting, although it was also detected at lower levels in the human cecum and colon (41). LPO was also detected in the rectum by Northern blotting. Furthermore, isolated rat rectal glands release  $H_2O_2$  that is enhanced by the calcium ionophore, ionomycin, and inhibited by the flavoprotein inhibitor DPI (41). A recent immunochemical survey of the GI tract confirmed that Duox2 is expressed throughout the porcine digestive tract and also in the human colon, where it localizes to the apical brush border of enterocytes (31). The protein also accumulates in differentiating  $CaCO_2$  colon epithelial cells maintained in confluent long-term cultures.

Renewed interest has developed recently in the LPO/ $H_2O_2$ /thiocyanate system as a major component of the innate defenses of the airways, because LPO was detected at levels as high as 1% of the total protein in airway secretions, and inhi-

**FIG. 4. Proposed assembly of the Duox-lactoperoxidase antimicrobial system during saliva formation.** Lactoperoxidase (LPO) synthesis occurs deep within the serous acini of salivary glands (A). The sodium iodide symporter (NIS), which also transports thiocyanate ( $SCN^-$ ), provides LPO substrates within intercalated ducts (B).

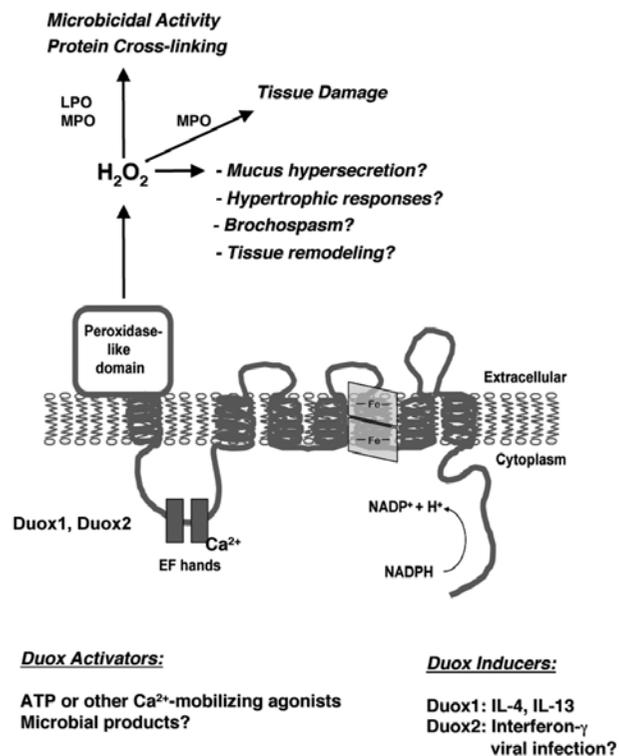


Duox2 produces the most labile component ( $H_2O_2$ ) within major terminal ducts (C), thereby completing this antimicrobial system in the final stages of saliva formation. (Data adapted from Geiszt M, Witta J, Baffi J, Lekstrom K, and Leto TL. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J* 17: 1502–1504, 2003.)

bition of its activity leads to impaired clearance of bacteria from sheep airways (42). We detected LPO expression in serous acini of tracheal and bronchial submucosal glands by *in situ* hybridization (41), whereas others independently detected LPO protein immunochemically at the same site (110). In contrast, Duox1 was detected by *in situ* hybridization as the major oxidase within the surface epithelium of bronchial and tracheal sections (41). Thus, it appears that the sites of expression of the Duox–LPO system are again segregated in these tissues, and the system is complete only on airway surface layers, where Duox1 provides the most labile and limiting substrate. To confirm the importance of Duox1 as a source of hydrogen peroxide for LPO in the airway, ROS production was examined in cultured primary human bronchial epithelial cells. These cells produced extracellular hydrogen peroxide (not superoxide) in response to calcium signals stimulated by ionomycin, which is inhibited by the oxidase inhibitor DPI or by Duox1-targeted antisense oligonucleotides (41).

Since the initial description of functional Duox1 expression in major airways, several groups have confirmed and extended these findings. Duox was detected immunochemically on the apical surface of ciliated epithelial cells [(34, 91), T. Ueyama and T.L. Leto, unpublished data], consistent with its proposed function as a source of  $H_2O_2$  released into the airway lumen. Schwartzer *et al.* (91) suggested that Duox1 is a major source of  $H^+$  production and secretion into the airway based on pharmacologic approaches, although they did not examine ROS production in parallel. Forteza *et al.* (34) showed that thapsigargin or ATP, which also increase intracellular calcium levels, causes coincidental release of  $H_2O_2$  from the apical surface. Thus, any agonist that mobilizes calcium within the apical cytoplasm of airway epithelium could potentially cause Duox-mediated  $H_2O_2$  release. The same group suggested that these cells also concentrate thiocyanate on the apical epithelial surface (35). Recently, Shao and Nadel (93) claimed that ROS produced by Duox1 in airway epithelial cells are involved in mucus hypersecretion through a neutrophil elastase/TNF- $\alpha$ -converting enzyme/EGF-receptor pathway (93); however, their model may in some way involve the ROS produced by the phagocytic oxidase, because their observations implicate p47<sup>phox</sup> and p67<sup>phox</sup>, factors known not to regulate Duox activity (24). It is likely that chronic inflammatory diseases associated with mucus hypersecretion would involve both the Duox in epithelial cells and the phox system in the recruited leukocytes that infiltrate inflamed airways. Under these conditions, the leukocytes would also secrete MPO, a more potent antimicrobial enzyme than LPO, which could also inflict damage to host tissues.

Recent observations on the effects of various cytokines on cultured airway epithelial cells suggest exciting new roles for both Duox isozymes in airway responses to several proinflammatory signals (45) (Fig. 5). About 20% of the Duox transcripts detected in these cells represent Duox2 (45). Duox1 transcript levels are induced by the Th2 cytokines, IL-4 and IL-13, whereas Duox2 transcript levels are dramatically induced by the Th1 cytokine, IFN- $\gamma$  (45) (T. L. Leto and K. Lekstrom, unpublished observations). The induction of these oxidases was correlated with markedly enhanced  $H_2O_2$  release, which is sensitive to the oxidase inhibitors. Further-



**FIG. 5. Proposed physiologic and pathologic roles of Duox in large airways.** Activators of Duox enzymes are thought to act through elevating intracellular concentrations of calcium ions, which can bind directly to these oxidases. Duox expression is increased by a variety of pro-inflammatory cytokine signals occurring during viral infection or allergic asthmatic responses. The proposed downstream consequences of Duox activation can be rapid (*i.e.*, microbial killing, bronchospasm) or long term (tissue remodeling and hypertrophic responses). MPO, myeloperoxidase; LPO, lactoperoxidase.

more, Duox2 production was significantly enhanced by exposure to rhinovirus or the viral mimic, polyinosine:polycytidylic acid, suggesting that Duox2 may be part of an IFN- $\gamma$ -mediated viral-clearance mechanism (45). The IL-13 effects are interesting because this cytokine is considered relevant to the pathogenesis of allergic asthma (111); its effects on epithelial cells suggest a potential role of Duox1 as source of higher ROS production that could promote a variety of downstream pathophysiologic consequences of allergic responses (Fig. 5).

Other chronic inflammatory disease processes in the lung could also involve alterations in Duox function. We suggested several mechanisms by which the cystic fibrosis (CF) phenotype could impair the effectiveness of a Duox/LPO-based innate defense system (41), thereby rendering CF patients more susceptible to infection by some of the same bacterial pathogens that frequently infect CGD patients, who clearly have oxidase-dependent microbial killing defects: (a) the altered ion-transport properties of CF epithelial cells may result in lower transport of thiocyanate; (b) the thicker mucous secretions in CF airways could impair the delivery of LPO or its substrates; or (c) the hypoxic environments of mucopurulent masses in CF lungs could impair ROS production. Alter-

natively, the chronic inflammatory state arising from long-term bacterial infections seen in CF patients may result in higher sustained levels of Duox expression in these patients.

### *An evolutionary perspective on the conserved host defense function of Nox family NADPH oxidases*

*Drosophila melanogaster* is one model system in which a host-defense role for a Duox isozyme has been demonstrated recently (44). *Drosophila* Duox (dDuox) is detected within the lower intestinal tract of flies, and flies in which dDuox is suppressed by RNA interference succumb to infection from microbe-contaminated food. This susceptibility is overcome by transgenic reintroduction of *Drosophila* or human Duox isozymes. Interestingly, dDuox expression is induced to higher levels by infection. The infection-induced expression of dDuox is not dependent on NF- $\kappa$ B activation, nor do the ROS derived from induced dDuox affect other NF- $\kappa$ B regulated pathways. Together these observations indicate that dDuox has a major role in resistance to natural microbial infection.

It appears that oxidative responses to pathogenic attack are indeed very ancient innate defense mechanisms that have existed even before the divergence of the plant and animal kingdoms. It has been known for years that oxidative bursts in plants, triggered by pathogen-derived elicitors, can set into motion several disease-resistance responses downstream of ROS production (63). The oxidants produced not only are microbicidal, but also promote oxidative cross-linking of cell-wall components (barrier formation), trigger hypersensitive cell-death responses, and relay diffusible systemic signals to neighboring cells that can induce other cellular protective genes; all of these processes serve to isolate and limit the infection. Like macrophages, plant cells also produce nitric oxide (27), which appears to act synergistically with ROS to mediate some plant-defense responses including cell death. Several oxidases related to the Nox family have been recognized in plant species, which have been referred to as respiratory burst oxidase homologues (*rboh* genes) (104, 114). These oxidases are most closely related to Nox5, because they also contain two N-terminal, calcium-binding EF-hands that may enable these oxidases to respond directly to elevated calcium levels elicited by pathogen exposure. Among the RBOHs recognized in *Arabidopsis* and *Nicotiana* species, several were shown by gene-silencing and mutagenesis approaches to be the enzymes involved in ROS release and disease-resistance responses triggered by the exposure to pathogens. It is remarkable that both plant and animal species not only generate the same toxic microbicidal agents, but also use very similar signaling pathways leading up to and after the generation of these molecules.

## CONCLUSIONS

Although microbicidal peroxidases have been appreciated as important host-defense components in mucosal secretions for decades (12, 14, 77, 82, 83, 86), it is remarkable that the sources of H<sub>2</sub>O<sub>2</sub> serving these enzymes have remained unclear until recently. Further study is needed to confirm that

Duox and other Nox enzymes serve as adequate sources of H<sub>2</sub>O<sub>2</sub> capable of supporting the antimicrobial activity of LPO and other peroxidases. Although it is difficult to estimate the available H<sub>2</sub>O<sub>2</sub> provided by these novel oxidases in some sites, such as the airway surface layer, these peroxidases are known to be effective at very low H<sub>2</sub>O<sub>2</sub> concentrations (*i.e.*, 20–50  $\mu$ M). Aside from this proposed role in generating microbicidal oxidants, other roles for ROS production related to host-defense functions deserve further consideration. ROS production has long been thought to have a direct role in NF- $\kappa$ B activation and associated downstream effects related to host defense (90). Studies have shown that low-level ROS production resulting from ectopic overexpression of ROS generators can cause NK- $\kappa$ B activation; however, the physiologic context of such manipulations should always be considered. Other signaling functions of ROS generated by epithelial cells on mucosal surfaces may include neutrophil priming, whereby epithelial cells would function as first responders to microbial exposure that would then signal an augmented host-defense response by recruited leukocytes (100). Barrier formation through oxidative cross-linking of extracellular matrix proteins (30), as well as ROS-mediated mucus hypersecretion (93), represents other mechanisms for defending epithelial surfaces from invading pathogens. Finally, another interesting possibility is that the ROS generated on mucosal surfaces may prevent the expression of pathogen virulence genes associated with microbial biofilm overgrowth; recent work showed that quorum sensing and expression of virulence genes by *Staphylococcus aureus* involves an oxidant-sensitive peptide pheromone that can be inactivated by the phagocytic oxidase (89). Animal models in which the expression of these Nox family oxidases are genetically altered should provide valuable opportunities for exploring these suggested roles in host defense.

## ABBREVIATIONS

Nox, NADPH oxidase; Duox, dual oxidase; phox, phagocyte oxidase; ROS, reactive oxygen species; Nox1, Nox organizer 1; Noxa1, Nox activator 1; IFN, interferon; LPS, lipopolysaccharide; TLR, Toll-like receptor; IBD, inflammatory bowel disease; IL, interleukin; MPO, myeloperoxidase; LPO, lactoperoxidase.

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