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MINIREVIEW

Antimicrobial actions of dual oxidases and lactoperoxidase

Demba Sarr, Eszter Tóth, Aaron Gingerich, and Balázs Rada*

Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens GA, USA

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The NOX/DUOX family of NADPH oxidases are transmembrane proteins generating reactive oxygen species as their primary enzymatic products. NADPH oxidase (NOX) 1–5 and Dual oxidase (DUOX) 1 and 2 are members of this family. These enzymes have several biological functions including immune defense, hormone biosynthesis, fertilization, cell proliferation and differentiation, extracellular matrix formation and vascular regulation. They are found in a variety of tissues such as the airways, salivary glands, colon, thyroid gland and lymphoid organs. The discovery of NADPH oxidases has drastically transformed our view of the biology of reactive oxygen species and oxidative stress. Roles of several isoforms including DUOX1 and DUOX2 in host innate immune defense have been implicated and are still being uncovered. DUOX enzymes highly expressed in the respiratory and salivary gland epithelium have been proposed as the major sources of hydrogen peroxide supporting mucosal oxidative antimicrobial defenses. In this review, we shortly present data on DUOX discovery, structure and function, and provide a detailed, up-to-date summary of discoveries regarding antibacterial, antiviral, antifungal, and antiparasitic functions of DUOX enzymes. We also present all the literature describing the immune functions of lactoperoxidase, an enzyme working in partnership with DUOX to produce antimicrobial substances.

Keywords: dual oxidase, lactoperoxidase, DUOX, LPO, antimicrobial, NADPH oxidase

Introduction: NADPH oxidases

The family of NADPH oxidases is composed of DUOX1/2 and five additional NOX enzymes: NOX1-5 (Dupuy *et al.*, 1999; De Deken *et al.*, 2000; Bokoch and Knaus, 2003; Lambeth, 2004; Lambeth and Neish, 2014) that generate reactive oxygen species (ROS) as primary products of their enzymatic activities (Lambeth, 2004; Grasberger and Refetoff, 2006; Lambeth and Neish, 2014). A conserved catalytic core responsible for transmembrane electron transfer from the intracellular electron donors to the extracellular compartment to generate superoxide or hydrogen peroxide characterizes this family of enzymes (De Deken *et al.*, 2014). Under normal conditions, most NOX isoforms have very low or no constitutive activity but their expression can be high in disease states. In these disease conditions, the activation of NOX isoforms generates high levels of ROS that can overwhelm the antioxidant system, leading to increased oxidative stress. Oxidative stress is defined as the increase of reduction potential or a large decrease in the reducing capacity of cellular redox couples (Genestra, 2007). In other words, oxidative stress occurs when the production of ROS exceeds the capacity of antioxidant defenses leading to harmful effects on the function and structural integrity of biological tissues. ROS can trigger rapid chain reactions and cause damage to macromolecules such as lipids, proteins, carbohydrates, and nucleic acids [reviewed by (Roy *et al.*, 2017)]. They are believed to be toxic by-products that can cause cellular stress, aging, damage, and cancer (Harman, 1956, 1981; Liu *et al.*, 2004). However, several lines of evidence suggest that ROS generation is an important process of the host innate immune system by promoting killing of invading microorganisms (Rada and Leto, 2008; van der Vliet, 2008; Gattas *et al.*, 2009; Lipinski *et al.*, 2009), and NOX-derived ROS are associated with inflammation [reviewed by (Mittal *et al.*, 2014)]. ROS have been shown to play a role in antimicrobial defenses, and the role of DUOX-derived ROS in innate immune responses against many bacterial, viral, and parasitic infections has been shown. The primary role of the DUOX1 and DUOX2, as well as, the five other NOX proteins (NOX 1–5) is the production of ROS in a wide range of organisms (Geiszt and Leto, 2004; Bedard and Krause, 2007; Bedard *et al.*, 2007; Lambeth and Neish, 2014). While NOX enzymes secrete both, superoxide and hydrogen peroxide as primary products, DUOX proteins generate hydrogen peroxide (H_2O_2) (Bjorkman and Ekholm, 1984; Dupuy *et al.*, 1989). DUOX enzymes have been implicated in a vast array of biological processes including hormone synthesis, fertilization, cell differentiation, cell proliferation, cell death, extracellular matrix formation, vascular regulation, angiogenesis, and host defense mechanisms (Geiszt and Leto, 2004; Lambeth, 2004; Bedard and Krause, 2007; Bedard *et al.*, 2007; Lipinski *et al.*, 2009; Panday *et al.*, 2015; Chen *et al.*, 2017; Kim *et al.*, 2017; Mistry and Brewer, 2017; Prieto-Bermejo and Hernandez-Hernandez, 2017; Ryu *et al.*, 2017). Production of H_2O_2 by

*For correspondence. E-mail: radab@uga.edu; Tel.: +1 706 5423695; Fax: +1 7065425771

NADPH oxidases was thought to be restricted to phagocytes (macrophages and neutrophils) in host defense (Segal, 2005). Later on, however, NADPH oxidase-mediated production of H_2O_2 has been demonstrated in a number of non-phagocytic cells. A recent review has thoroughly analyzed the NOX/DUOX family of NADPH oxidase with lessons learned from knockout mouse models (Sirokmany *et al.*, 2016). Other recent reviews have highlighted the implication of NADPH oxidases in different biological systems including their potential as drug targets and biomarkers in neurodegenerative diseases and their involvement in vascular biology (Jha *et al.*, 2017; Li and Pagano, 2017; Little *et al.*, 2017; Prieto-Bermejo and Hernandez-Hernandez, 2017; Sorce *et al.*, 2017; Wang and Hartnett, 2017).

DUOX discovery

DUOX enzymes are expressed in several tissues and cell types. The generation of ROS by dual oxidases is a crucial step in tyrosine-crosslinking of extracellular matrix, in the fertilization envelope, innate immunity, wound healing, and thyroid hormone biosynthesis (Dupuy *et al.*, 1999; De Deken *et al.*, 2000, 2014; Edens *et al.*, 2001; Wong *et al.*, 2004; Ha *et al.*, 2005; Grasberger *et al.*, 2007; Chavez *et al.*, 2009; Lipinski *et al.*, 2009; Niethammer *et al.*, 2009; Fortunato *et al.*, 2010; Kumar *et al.*, 2010; Song *et al.*, 2010; Hoeven *et al.*, 2011; Moribe *et al.*, 2012; Moribe and Mekada, 2013; van der Hoeven *et al.*, 2015). The *DUOX1* and *DOUX2* genes, previously called *THOX1* and *THOX2*, respectively, were cloned for the first time in human and porcine thyroid gland (Dupuy *et al.*, 1999; De Deken *et al.*, 2000). DUOX1 is the main NADPH oxidase expressed in uroepithelial cells possibly participating in the mechanosensory mechanism of the bladder (Donko *et al.*, 2010). In 2001, Edens *et al.* (2001) reported the cloning of homologous sequences from *Caenorhabditis elegans*, a free-living transparent nematode that lives in soil environment. Both DUOX isoforms are highly expressed in tracheobronchial epithelial cells and in the thyroid gland (Dupuy *et al.*, 1999; De Deken *et al.*, 2000; Geiszt *et al.*, 2003; Harper *et al.*, 2005; Harper *et al.*, 2006). Predominant expression of DUOX1 was found in the respiratory epithelium while DUOX2 is mostly present in the thyroid gland, the salivary and rectal gland epithelia (Geiszt *et al.*, 2003). In airway epithelia, DUOX1 expression is enhanced during epithelial cell differentiation and alveolar maturation (Fischer *et al.*, 2007). DUOX2 is also expressed in the surface epithelia of the lung and the intestine (Geiszt *et al.*, 2003; El Hassani *et al.*, 2005). Expression of DUOX2 has been found elevated in patients with Crohn's disease and in response to *Helicobacter pylori* infections (Szanto *et al.*, 2005; Csillag *et al.*, 2007; Rokutan *et al.*, 2008). Using laser capture microdissection followed by real time quantitative PCR, researchers have found that DUOX2 is predominantly expressed in the tip epithelium of the ileum and colon (Sommer and Backhed, 2015). Here, the expression of DUOX2 in both tissues was induced by a normal microbiota. In the ileum, DUOX2 expression induced by normal microbiota involved NF-B signaling while colonic expression was mediated through MyD88 and p38 MAPK (Sommer and Backhed, 2015).

DUOX structure

Edens *et al.* (2001) suggested a DUOX nomenclature based on the structural features of the protein. The characteristic features of DUOX enzymes have been thoroughly reviewed by Donko *et al.* (2005). *DUOX* genes are located on the human chromosome 15, where they are arranged in a head-to-head configuration, separated by a 16 kb region (Pachucki *et al.*, 2004). *DUOX1* gene shares 83% similarity with *DUOX2* gene. The size of *DUOX1* is 36 kb with 35 exons while *DUOX2* measures 22 kb and contains 34 exons. Structurally, both human DUOX isoforms share a conserved motif, consisting of an N-terminal peroxidase like domain, two calcium-binding sites, six transmembrane domains, and an NADPH oxidase domain. Consistent with this motif, calcium was shown to regulate the production of ROS by both, DUOX1

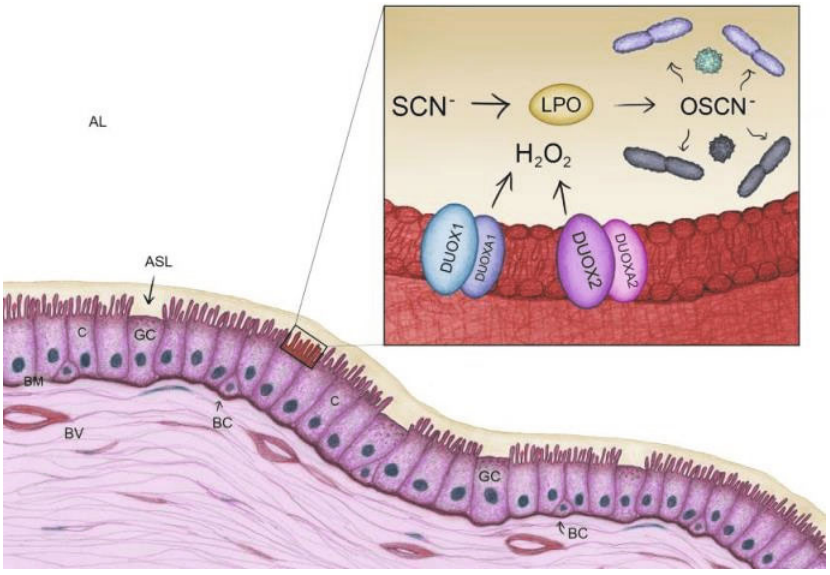

Fig. 1. Model of the DUOX/H_2O_2/LPO/SCN^- antimicrobial system in the respiratory tract. Ciliated (C) airway epithelial cells express DUOX1/DUOXA1 and DUOX2/DUOXA2 complexes in their luminal/apical plasma membrane producing H_2O_2 into the airway surface liquid (ASL). Lactoperoxidase (LPO) uses this H_2O_2 to oxidize its main substrate, thiocyanate (SCN^-) present in large quantities in the ASL into antimicrobial hypothiocyanite ($OSCN^-$). AL, airway lumen; BC, basal cell; BV, blood vessel; GC, Goblet cell.

and DUOX2 (Ameziane-El-Hassani *et al.*, 2005; Rigutto *et al.*, 2009). Rigutto *et al.* (2009) provided evidence that the basal activity of both DUOX enzymes depends on calcium and functional EF-hand motifs. They showed with an optimized functional assay that the two oxidases are differentially regulated by activation of separate intracellular signaling cascades (Rigutto *et al.*, 2009). While DUOX1 is stimulated by forskolin via protein kinase A-mediated phosphorylation on serine 955, in contrast, DUOX2 phosphorylation in induced by phorbol esters via protein kinase C activation associated with high H_2O_2 generation (Rigutto *et al.*, 2009). These results were obtained *in vitro* and await *in vivo* confirmatory studies. Additional works have highlighted the importance of conserved cysteine residues that are localized in the N-terminal domain in enzymatic maturation, independent of structural stabilization (Morand *et al.*, 2004; Meitzler *et al.*, 2013). Intermolecular disulfide bonds support protein interaction between the DUOX enzymes and their maturations factors indicating the complexity of these enzymes (Meitzler *et al.*, 2013). Both DUOX proteins work in partnership and mature with helper proteins called DUOX activators or maturation factors (Fig. 1) (Grasberger and Refetoff, 2006; Morand *et al.*, 2009). DUOX activators (DUOXA1, DUOXA2) dictate maturation, subcellular localization, and the type of ROS produced by establishing stable complexes with DUOX enzymes (Leto *et al.*, 2009; Morand *et al.*, 2009; Hoste *et al.*, 2012; Donko *et al.*, 2014; Ueyama *et al.*, 2015). Intramolecular disulfide bonds in DUOX2 govern its oxidative folding in the endoplasmic reticulum and subsequent covalent interactions with its maturation factor, DUOXA2 (Carre *et al.*, 2015).

Thyroid functions of DUOX

The main function of the DUOX2 isoform has been identified in the thyroid follicle, which is the functional structure responsible for the thyroid hormone biosynthesis, storage, and secretion (Dupuy *et al.*, 1999; De Deken *et al.*, 2000; Ohye and Sugawara, 2010). Follicular thyroid cells are polarized and specialized in the production of thyroxine and triiodothyronine. The first step in thyroid hormone biosynthesis corresponds to the iodination of tyrosyl residues of thyroglobulin, a thyroid-specific protein, synthesized and secreted into the follicular lumen. The iodination reaction is believed to occur at the apical membrane of the cells and the colloid interface [reviewed in (Carvalho and Dupuy, 2013)]. The process involves a number of chemical reactions such as iodide oxidation, tyrosyl radical oxidation, thyroglobulin iodination also named iodine organification, and finally coupling of iodotyrosines to form the thyroid hormone [reviewed by (Carvalho and Dupuy, 2013)]. During thyroid hormone biosynthesis, the enzymatic reactions that involve the oxidation of substrate depends on the presence of H_2O_2 and an enzyme that catalyzes the process named thyroperoxidase. In the thyrocyte, H_2O_2 necessary for hormone biosynthesis is generated at the apical surface through a controlled reaction catalyzed by DUOX2 (Bjorkman and Ekholm, 1984; Virion *et al.*, 1984). Human patients with impaired DUOX2-mediated H_2O_2 production and DUOX2-deficient mice are hypothyroid (Johnson *et al.*, 2007; Grasberger *et al.*, 2012; Carvalho and Dupuy, 2013; Weber *et al.*, 2013; Donko *et al.*, 2014). Several excellent reviews focused on the thyroid consequences of DUOX2 deficiency (Grasberger, 2010; De Deken *et al.*, 2014; Muzza and Fugazzola, 2017; Targovnik *et al.*, 2017). In the present review, we exclusively focus on roles of DUOX1 and DUOX2 in host defense.

Antimicrobial effects of DUOX and LPO

In a landmark study, Geiszt and Leto (2003) demonstrated the presence of DUOX in respiratory and gastrointestinal tract, where expression patterns are consistent with presence of LPO and a host defense function. This work put DUOX proteins as the physiological H_2O_2 sources into the model of the LPO-based antimicrobial system (Fig. 1) (Geiszt *et al.*, 2003). DUOX proteins generate H_2O_2 on the extracellular side of the apical membrane of the airway epithelium (Fig. 1) (Geiszt and Leto, 2004; Schwarzer *et al.*, 2004; Forteza *et al.*, 2005; Shao and Nadel, 2005). H_2O_2 is then metabolized by lactoperoxidase (LPO) (Salathe *et al.*, 1997; El-Chemaly *et al.*, 2003) to oxidize thiocyanate (SCN^-) into the potent microbicidal molecule hypothiocyanite ($OSCN^-$) (Fig. 1) (Reiter, 1978; Thomas and Aune, 1978a, 1978b; Carlsson *et al.*, 1984; Ihalin *et al.*, 1998; Conner *et al.*, 2007; Moskwa *et al.*, 2007; Rada and Leto, 2008).

Additionally, complex interactions between NOX/DUOX enzymes and Toll-like receptors have been shown (TLRs) (Asehnoune *et al.*, 2004; Kawahara *et al.*, 2004; Park *et al.*, 2004, 2006). For example, IL-8 expression and reactive oxygen species generation is linked with interactions between DUOX2 and TLR5 (Joo *et al.*, 2012) and between DUOX1 and TLRs (Boots *et al.*, 2009). Using real-time PCR in primary respiratory tract epithelial cultures after treatment with multiple cytokines, *DUOX1* mRNA expression was increased several-folds with Th2-cytokines (IL-4 and IL-13), whereas *DUOX2* expression was highly induced following treatment with the Th1 cytokine IFNγ suggesting regulation of *DUOX* expression by immunomodulatory cytokines (Harper *et al.*, 2005). Recent works have demonstrated that autophagy promotes an increase in intracellular superoxide levels by regulating DUOX1 during Th2 inflammation of the airway epithelium (Dickinson *et al.*, 2018). This study demonstrated the relationship between autophagy and ROS in airway epithelial cells and implicated DUOX1 in disease pathogenesis (Dickinson *et al.*, 2018).

Thus, several studies have revealed that the human and mammalian airway mucosa contains an oxidative defense mechanism (Gerson *et al.*, 2000; Conner *et al.*, 2002, 2007; Geiszt *et al.*, 2003; Wijkstrom-Frei *et al.*, 2003; Forteza *et al.*, 2005). The three-component system consists of H_2O_2-producing enzyme DUOX1 and DUOX2, the substrate SCN^- and secreted LPO (Geiszt *et al.*, 2003). The LPO-catalyzed reaction between H_2O_2 and SCN^- leads to the formation of $OSCN^-$ (Klebanoff and Luebke, 1965; Klebanoff *et al.*, 1966; Bjorck *et al.*, 1975), which has demonstrated antimicrobial effects (Fig. 1). Table 1 shows a summary of studies implicating DUOX/ H_2O_2 and LPO in antimicrobial functions.

Table 1. Studies on the microbicidal or microbistatic action of the DUOX/H_2O_2/LPO system
Published reports are organized according to microbial class in alphabetical order of names of the microorganisms. LPO, lactoperoxidase; MPO, myeloperoxidase; NE, nasal epithelial cells; TBE, tracheobronchial epithelial cells.

Antibacterial action		
Bacterial species	Experimental system	References
Actinobacillus actinomycetemcomitans	Cell-free system: different LPO and MPO substrates	Ihalin *et al.* (1998, 2003)
Aeromonas hydrophila	Cell-free system	Benoy *et al.* (2000)
Bacillus cereus	Cell-free system: different LPO and MPO substrates	Tenovuo *et al.* (1985)
Borrelia burgdorferi	*Ixodes scapularis* ticks	Yang *et al.* (2014)
Burkholderia cepacia	Human TBE cells	Wijkstrom-Frei *et al.* (2003), Rada *et al.* (2008)
Capnocytophaga ochracea	Cell-free system	Courtois *et al.* (1992)
Citrobacter freundi		Benoy *et al.* (2000)
Eikenella corrodens		Courtois *et al.* (1992)
Enterococcus faecalis	*C. elegans*	Chavez *et al.* (2009), Hoeven *et al.* (2011)
Erwinia carotovora	*Drosophila* gut	Ha *et al.* (2005, 2009b)
Escherichia coli	Cell-free system, Guinea pig milk and saliva	Bjorck *et al.* (1975), Thomas and Aune (1978b), Stephens *et al.* (1979), Marshall and Reiter (1980), Grieve *et al.* (1992), Benoy *et al.* (2000), Bosch *et al.* (2000)
Eubacterium yurii	Cell-free system	Courtois *et al.* (1992)
Fusobacterium nucleatum		
Haemophilus influenzae		Wijkstrom-Frei *et al.* (2003)
Klebsiella pneumoniae		Benoy *et al.* (2000)
Listeria monocytogenes	HEK293, Caco-2 cells: DUOX2 transfection, DUOX2 siRNA	Lipinski *et al.* (2009)
Pasteurella haemolytica	Infection in sheep airways	Gerson *et al.* (2000)
Peptostreptococcus micros	Cell-free system	Courtois *et al.* (1992)
Prevotella intermedia		
Proteus mirablis		Benoy *et al.* (2000)
Pseudomonas aeruginosa	Cell-free system, human and rat TBE cells, human airway secretions, ALX-109, *C. elegans*	Benoy *et al.* (2000), Bosch *et al.* (2000), Wijkstrom-Frei *et al.* (2003), Conner *et al.* (2007), Moskwa *et al.* (2007), Rada *et al.* (2008), Gattas *et al.* (2009), Hoeven *et al.* (2011), Moreau-Marquis *et al.* (2015)
Pseudomonas fluorescens	Cell-free medium, raw milk	Bjorck *et al.* (1975)
Salmonella enterica typhi	Cell-free system	Benoy *et al.* (2000)
Salmonella enteritidis		Benoy *et al.* (2000), Touch *et al.* (2004)
Salmonella schottmuelleri		Benoy *et al.* (2000)
Serratia marcescens		
Shigella dysenteriae		
Shigella sonnei		
Staphylococcus aureus	Cell-free system, Rat TBE cells	Benoy *et al.* (2000), Bosch *et al.* (2000), Moskwa *et al.* (2007)
Staphylococcus citreus	Cell-free system	Benoy *et al.* (2000)
Streptococcus agalactiae		Mickelson (1979), Mickelson and Anderson (1984)
Streptococcus gordonii		Ashby *et al.* (2009)
Streptococcus lactis		Marshall and Reiter (1980)
Streptococcus mutans		Tenovuo and Knuuttila (1977a, 1977b), Soukka *et al.* (1991), Thomas *et al.* (1994), Ashby *et al.* (2009), Welk *et al.* (2009)
Streptococcus sanguinis		Welk *et al.* (2009)
Streptococcus sobrinus		Thomas *et al.* (1994)
Vibrio cholerae		Benoy *et al.* (2000)
Antiviral action		
Viral species	Experimental system	References
Adenovirus	Cell-free system, Human and porcine TBE cells	Mikola *et al.* (1995), Fischer *et al.* (2011)
Echovirus 11	Cell-free system	Mikola *et al.* (1995)
HIV		Pourtois *et al.* (1990)
HSV1		Mikola *et al.* (1995)
H1N1 Influenza A virus	Cell-free system, DUOX2 KO mice, Human NE cells	Kim *et al.* (2013), Cegolon *et al.* (2014), Strengert *et al.* (2014), Kim *et al.* (2015)
H1N2 Influenza A virus	Rat TBE cells	Gingerich *et al.* (2016)
RSV	Cell-free system, Human and porcine TBE cells	Fischer *et al.* (2011), Derscheid *et al.* (2014), El-Fakharany *et al.* (2017)

Table 1. Continued

Antifungal action		
Fungal species	Experimental system	References
Alternaria sp.	Cell-free system	Benoy *et al.* (2000)
Aspergillus flaws		
Aspergillus niger		Popper and Knorr (1997), Benoy *et al.* (2000)
Byssochlamys fulva		Popper and Knorr (1997)
Candida albicans		Lenander-Lumikari (1992), Majerus and Courtois (1992), Bosch *et al.* (2000), Welk *et al.* (2009), Ahariz and Courtois (2010), Kho *et al.* (2012)
Corynespora cassiicola		Benoy *et al.* (2000)
Claviceps sp.		
Corticium salmonicolor		
Mucor rouxii		Popper and Knorr (1997)
Pencillium chrysogeum		Benoy *et al.* (2000)
Phytopthora meadii		
Rhodutorula rubra		Popper and Knorr (1997), Ha *et al.* (2009a, 2009b)
Saccharomyces cerevisiae	Cell-free system, *Drosophila* gut	
Trichoderma sp.	Cell-free system	Benoy *et al.* (2000)
Antiparasitic action		
Parasitic species	Experimental system	References
Plasmodium falciparum	Cell-free system	Malhotra *et al.* (1988)
Plasmodium berghei	*Anopheles gambiae* midgut	Kumar *et al.* (2010)
Toxoplasma gondii	Cell-free system: LPO/I^-, *tachyzoite*	Tanaka *et al.* (2006)

LPO and thiocyanate

LPO is a member of the mammalian heme peroxidase family whose members are best known for their antimicrobial activities (Bafort *et al.*, 2014). LPO is a major antimicrobial protein found in milk, saliva, tears and airway secretions (Allen and Morrison, 1966; Goldman and Smith, 1973; Conner *et al.*, 2002; Wijkstrom-Frei *et al.*, 2003). Other members of this family are myeloperoxidase expressed in neutrophils and macrophages, and eosinophil peroxidase found in eosinophils (Davies *et al.*, 2008; Nauseef, 2018). LPO is a glycoprotein that consists of a single polypeptide chain (around 80 kDa) containing calcium and iron, and is found in the secretions of exocrine glands (Bafort *et al.*, 2014). LPO in the airways is synthesized and secreted by submucosal glands and by the airway epithelium and comprises one percent of the total protein content in the airway surface liquid (Salathe *et al.*, 1997). LPO has been detected previously in human and sheep airways but no data indicated its presence in the murine respiratory tract. We performed immunohistochemistry and detected LPO expression in submucosal tissues, as expected, in murine trachea, as well (Fig. 2). LPO uses hydrogen peroxide to catalyze the oxidation of its preferred halide substrates including I^-, Br^-, F^-, or the pseudohalide SCN^- (Bafort *et al.*, 2014). Based on the second-order rate constants of the reaction between LPO Compound I and its (pseudo) halide substrate, SCN^- is the preferred substrate of LPO followed by I^-, Br^-, and Cl^- (Bafort *et al.*, 2014).

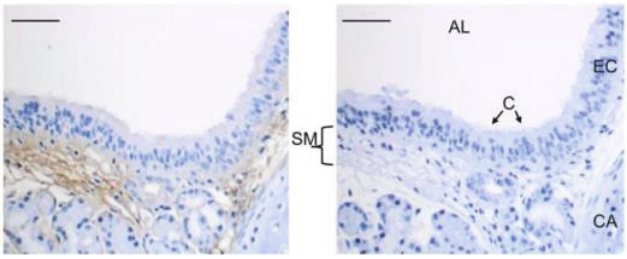

Fig. 2. Submucosal tissue localization of lactoperoxidase in mouse trachea. Trachea of 6–8 week-old C57BL/6J mice were fixed in 4% paraformaldehyde, embedded in paraffin and subjected to immunohistochemistry to detect lactoperoxidase (LPO). Tracheal sections were blocked with Dako Protein Block (serum-free, code #X0909) before probing with primary mouse LPO antibody (Novus Biological, cat#: NBP1-87010, 1/500, rabbit) followed by horseradish peroxidase-labelled secondary anti-rabbit antibody (GBI Labs, Polymer HRP anti-rabbit IgG, cat#: D13-18). Brown staining indicative of LPO localization was developed with 3,3′-diaminobenzidine substrate (left panel). In the right panel the LPO antibody was omitted. Bar indicates 20 μm. AL, airway lumen; C, cilia; CA, cartilage; EC, epithelial cells; SM, submucosa. One representative result, n = 5.

Thiocyanate (SCN^-) is a pseudohalide thiolate that is universally found in extracellular fluids including saliva, plasma, airway surface liquid (ASL), milk, tears, and gastric juices of mammals (Chandler and Day, 2012). The concentration at which SCN^- is present is variable and can range from 0.01–3 mM, but in the airway surface liquid the SCN^- concentration is estimated to be around 0.4 mM (Wijkstrom-Frei *et al.*, 2003; Chandler and Day, 2012). SCN^- enters the body through dietary uptake or can also be synthesized from cyanide by sulfurtransferase enzymes (Chandler and Day, 2012). Thiocyanate is eliminated in the kidneys and has a half-life of approximately 3 days (Chandler and Day, 2012). SCN^- is transported into the airways after it is first incorporated from the basal side through the Na^+/I^- symporter into the airway epithelium. It is then transported into the airway lumen through anion transporters including the Cystic fibrosis transmembrane conductance regulator (CFTR) and pendrin/SLC26A4 (Suzuki *et al.*, 2016). Both, LPO and SCN^- have been shown to be present in the airway surface liquid

in large quantities (Conner *et al.*, 2002, 2007). Once transported into the ASL, SCN^- is ready to be oxidized into the antimicrobial compound hypothiocyanite by LPO, provided H_2O_2 is present (Moskwa *et al.*, 2007).

Due to SCN^- being transported by the CFTR and its role in antimicrobial defense, it was believed that cystic fibrosis patients have trouble clearing bacteria due to lack of SCN^--mediated microbial killing (Moskwa *et al.*, 2007). However, it was determined that levels of SCN^- were not statistically different between CF and non-CF patients meaning that there could be a backup transport mechanism functioning in CF (Lorentzen *et al.*, 2011). Additionally, it was shown that higher levels of SCN^- in the airways correlated with better lung function in CF patients leading to the hypothesis that SCN^- has a beneficial role (Lorentzen *et al.*, 2011). Since animal cells do not synthesize it, increased levels of SCN^- come from the dietary uptake of cruciferous vegetables such as broccoli and cabbage (Lorentzen *et al.*, 2011). During bacterial challenge in the respiratory tract neutrophils produce hypochlorous acid (HOCl) which is more toxic to host cells than $OSCN^-$ (Lorentzen *et al.*, 2011). SCN^- helps alleviate the overabundance of HOCl because it outcompetes Cl- for Myeloperoxidase (MPO) and H_2O_2, and it also reacts with HOCl directly which leads to the production of more $OSCN^-$ (Lorentzen *et al.*, 2011).

Antibacterial effects of the DUOX/ H_2O_2/LPO system

Antimicrobial effects of the DUOX/ H_2O_2/LPO system have been best demonstrated in bacteria. Table 1 lists all publications reporting microbicidal or microbistatic effects of the DUOX/ H_2O_2/LPO system in a concise manner. Several bacterial species are killed in an *in vitro*, "cell-free" experimental system composed of LPO, H_2O_2, and SCN^-. The "cell-free" characterization refers to the absence of the cellular, host source of H_2O_2. H_2O_2 is either added in a "bolus" manner or provided as the by-product of the enzymatic reaction between glucose and glucose oxidase (Rada *et al.*, 2008). Generating H_2O_2 in this "cell-free" system by an enzymatic reaction better models the slow but steady nature of DUOX-mediated, enzymatic H_2O_2 release in BECs than a bolus-like addition of H_2O_2 (Rada *et al.*, 2008; Rada and Leto, 2010). The "cell-free" model is relatively simple allowing a detailed and easy characterization of LPO-mediated killing of any microorganism including bacteria.

Numerous studies compared LPO substrate preference for microbial killing. The sensitivity of *Actinobacillus actinomycetemcomitans*, a bacterium important in early onset and progressive periodontitis, to LPO-mediated killing is moderate when SCN^- is the substrate, but is enhanced significantly when I^- is provided as alternative substrate in the cell-free system (Ihalin *et al.*, 1998, 2003). *In vitro* growth of *Bacillus cereus*, an oral periodontopathic bacterium, is also inhibited by LPO in an $OSCN^-$ concentration-dependent manner (Tenovuo *et al.*, 1985). Unlike in the case of *A. actinomycetemcomitans*, the antimicrobial efficiency of LPO against *B. cereus* was greater with SCN^- as a substrate than with I^- (LPO did not oxidize Cl^-) (Tenovuo *et al.*, 1985). MPO also killed *B. cereus* that was more efficient with I^- as a substrate compared to Cl^- and SCN^-, respectively (Tenovuo *et al.*, 1985) (Table 1). The LPO-based system is also efficient against a variety of anaerobic bacteria *in vitro* (Courtois *et al.*, 1992). *Salmonella enteritidis* present in different foods and fruit juices was shown to be killed by LPO purified from skim milk and supplemented with SCN^- as substrate (Touch *et al.*, 2004).

LPO is bactericidal or bacteriostatic against a wide variety of *Streptococcus* species. The *in vitro* growth and metabolism of oral streptococci, *Streptococcus mutans* and *S. sobrinus*, are much more effectively inhibited by the H_2O_2/LPO/SCN^- system than H_2O_2 alone (Thomas *et al.*, 1994). An only bacteriostatic effect of the LPO-based *in vitro* system against *Streptococcus lactis* was described and compared to its bactericidal effect against *Escherichia coli* (Marshall and Reiter, 1980). Studies investigating the bactericidal effect of the H_2O_2/LPO/SCN^- system on *Streptococcus agalactiae* indicated LPO/H_2O_2-catalyzed incorporation of SCN^- into bacterial proteins and removal of reactive protein sulfhydryls from a functional role in membrane transport and glucolysis as the likely causes of the antibacterial effect of LPO (Mickelson, 1979; Mickelson and Anderson, 1984). The study by Ashby *et al.* (2009) described that the antimicrobial action of LPO changes the species distribution of *Streptococcus* co-cultures, a finding that can have *in vivo* relevance in the oral cavity.

The LPO-based system is also an efficient *in vitro* killer of *E. coli*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Citrobacter freundi*, *Klebsiella pneumonia*, *Proteus mirablis*, *Salmonella enteritidis*, *Salmonella schottmuelleri*, *Serratia marcescens*, *Shigella dysenteriae*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus citreus*, and *Vibrio cholerae* (Bjorck *et al.*, 1975; Tenovuo and Knuuttila, 1977a, 1977b; Mickelson, 1979; Stephens *et al.*, 1979; Benoy *et al.*, 2000; Bosch *et al.*, 2000) (Table 1). Addition of LPO and SCN^- to milk significantly reduces the burden of psychotrophic bacteria and increases the milk's storage time (Tenovuo and Knuuttila, 1977a). Interestingly, not only bacterial cells, but their secreted toxins can also be attacked by the LPO/H_2O_2/SCN^- system. We have previously shown that the redox-active *P. aeruginosa* exotoxin pyocyanin is degraded and detoxified by LPO using DUOX1-derived H_2O_2 on airway epithelial cells (Rada *et al.*, 2008). LPO-mediated pyocyanin inactivation is LPO and H_2O_2 concentration-dependent (Rada *et al.*, 2008). These results indicate a complex redox interplay between the host epithelium and *P. aeruginosa* in the airways (Rada *et al.*, 2008; Rada and Leto, 2009). All these reports support that the H_2O_2/LPO/SCN^- system is efficient against a wide variety of bacterial species *in vitro* and likely serves as a general, non-specific innate immune mechanism *in vivo* (Fig. 1).

Airway epithelial cells express DUOX1 and DUOX2 proteins in their apical plasma membrane that are the major H_2O_2 sources for the H_2O_2/LPO/SCN^- antimicrobial system (Geiszt *et al.*, 2003; Rada, 2017). Air-liquid cultures of differentiated tracheobronbchial epithelial cells provide an excellent, cell-based, *in vitro* model to study the DUOX/ H_2O_2/LPO/SCN^- antimicrobial system (Conner *et al.*, 2002; Rada *et al.*, 2008). We and others showed that $OSCN^-$ kills several respiratory pathogens including *Burkholderia cepacia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* on the surface of air-liquid cultures of differentiated primary tracheobronchial epithelial cell cultures

or in human tracheal secretions (Wijkstrom-Frei *et al.*, 2003; Conner *et al.*, 2007; Moskwa *et al.*, 2007; Rada and Leto, 2008, 2010; Gattas *et al.*, 2009). These measurements show that DUOX-generated H_2O_2 is sufficient to fuel this antimicrobial system in airway cells. We have previously shown that *P. aeruginosa* stimulates DUOX1 activity and H_2O_2 production in airway epithelial cell lines (Rada and Leto, 2010). ALX-109 (an investigational drug consisting of lactoferrin and $OSCN^-$) reduced *in vitro P. aeruginosa* PAO1 biofilm formation (Moreau-Marquis *et al.*, 2015). This drug also increased the ability of tobramycin and aztreonam to inhibit biofilm formation and to reduce established PAO1 biofilms on cystic fibrosis airway epithelial cells (Moreau-Marquis *et al.*, 2015). During *Listeria monocytogenes* infection, DUOX2 interacts and co-localizes with Nucleotide binding oligomerization domain 2 (NOD2) at the plasma membrane and mediates antimicrobial response to prevent cytoinvasion (Lipinski *et al.*, 2009). This study not only demonstrated that DUOX2-derived hydrogen peroxide resulted in direct bactericidal activity, but it also amplified NF-κB signaling in HEK293 cells cultured with the pathogenic bacterium *Listeria monocytogenes* (Lipinski *et al.*, 2009). Another study found that DUOX1 activation is a general response to diverse bacterial triggers (including *Salmonella typhymurium* and *Escherchia coli*) that activate TLRs (Boots *et al.*, 2009).

In vivo studies using non-mammalian animal models also established relevant roles for DUOX proteins in immune defenses. *C. elegans* expresses DUOX1 that generates ROS to trigger protective SKN1 activity via p38 MAPK signaling (Hoeven *et al.*, 2011) suggesting that *C. elegans* DUOX (CreDUOX) is required for pathogen-induced ROS production as a protective immune mechanisms (Hoeven *et al.*, 2011). Using reverse-phase high-performance chromatography and a rhodamine-based sensor for the detection of DUOX-dependent ROS, uracil-induced DUOX activation of *Drosophila* gut innate immunity was shown to be an important mechanism for *E. carotovora* clearance in flies' gut (Chen *et al.*, 2011). Following this interesting discovery, the whole transcriptome profiles of *Drosophila* midgut cells following uracil stimulation revealed the identification of protein kinase genes upregulated by the treatment (Lee *et al.*, 2015a, 2015b) and a clear implication of the Hedgehog (Hh) signaling pathway, initially involved in early embryogenesis, and required for the full activation of DUOX (Lee *et al.*, 2015a, 2015b). An interesting study has shown that intestinal DUOX and an associated peroxidase generates a dityrosine network in *Ixodes scapularis* ticks that protects the insects against infection by *Borrelia burgdorferi*, the causative agent of Lyme's disease (Yang *et al.*, 2014).

In spite of all the previously introduced reports, literature addressing the *in vivo* antimicrobial role of the LPO-based system in mammalian hosts is largely missing. *In vivo* inhibition of airway LPO in sheep leads to significant decrease in bacterial clearance suggesting that LPO plays an important immune role during *Pasteurella haemolytica* infection (Gerson *et al.*, 2000). In summary, the LPO-based oxidative mechanism is likely a major pillar of the first line of antibacterial immune defense in several organs including the airways, oral cavity, eyes and mammary glands.

Antiviral effects of the DUOX/H_2O_2/LPO system

The virucidal effects of H_2O_2/LPO/SCN^- system have been demonstrated in HIV, Herpes Simplex Virus type 1 (HSV1), Respiratory Syncytial Virus (RSV), adenoviruses, echovirus, and influenza A virus (IAV) (Table 1). Treatment of HIV viruses with LPO-generated $OSCN^-$ decreased subsequent viral proliferation in lymphocytes as followed by ELISA specific to the p24 viral capsid protein (Pourtois *et al.*, 1990). H_2O_2 was produced by the glucose/glucose oxidase system (Pourtois *et al.*, 1990). HSV1 is an enveloped DNA virus that is ubiquitous, neurotropic, and the most common pathogenic cause of sporadic acute encephalitis in humans (Whitley, 2006; Steiner, 2011). The virus has the ability to establish and maintain a lifelong infection in neurons with frequent asymptomatic reactivation episode (Tsalenchuck *et al.*, 2014). It is well known that infectious HSV is shed to whole saliva from time to time even during asymptomatic infection (Wittek *et al.*, 1984). The saliva contains several components that have the ability to neutralize the virus *in vitro* (Mikola *et al.*, 1995). The inhibitory effect of $OSCN^-$ against HSV1 was demonstrated but no significant inhibition of the virus was noticed when any of the components (thiocyanate or ROS) were omitted, neither did ROS alone have any inhibitory effect. The complete peroxidase system without any virus did not stress fibroblasts as observed by light microscopy suggesting that $OSCN^-$ does not have any cytotoxic effect on the cells and is indeed the antiviral agent (Mikola *et al.*, 1995). Recently, a comparative analysis of the antiviral activity of camel, bovine, and human LPO against HSV1 revealed that all three proteins were able to inhibit HSV1 in the Vero cell model but the highest anti-HSV1 activity was exhibited by bovine LPO that inhibited HSV1 particles at a concentration of 0.5 μg/μl with the relative activity of 100% (El-Fakharany *et al.*, 2017).

In vivo DUOX2 silencing in mouse nasal mucosa aggravated influenza virus A infection (Kim *et al.*, 2015). Using rat airway epithelial cells, we demonstrated that both DUOX isoforms are expressed by epithelial cells and influenza A viruses are inactivated by epithelial cells via DUOX/LPO/SCN^--dependent mechanism suggesting that the DUOX system is potent at inactivating H1N2 influenza viruses prior to infection of the epithelium (Gingerich *et al.*, 2016). *DUOX1* and *DUOX2* mRNA levels are also significantly increased in nasal epithelium following influenza A infection (Kim *et al.*, 2013). Intracellular ROS generation at 1 h after IAV infection was significantly attenuated in normal human epithelial cells transfected with *DUOX2* shRNA. Influenza virus titers from *DUOX2*-transfected and IAV-infected cells were significantly higher than cells transfected with control shRNA and NP expression by western blot showed a more considerable increase in *DUOX2* knockdown than in control cells. These data show that *DUOX* enzymes represent a dominant source of ROS in response to IAV infection (Kim *et al.*, 2015).

Respiratory Syncytial Virus (RSV) is a major cause of acute lower respiratory infection in infants and young children. RSV is a leading cause of bronchiolitis worldwide (Welliver *et al.*, 2010; Collins and Melero, 2011). During RSV infection, airway epithelial host defenses are important components for the inactivation and complete removal of the virus from the respiratory tract. The DUOX-mediated generation

of $OSCN^-$ exhibits conflicting anti-RSV activity (Mikola *et al.*, 1995; Fischer *et al.*, 2011) but iodide supplementation has been shown to protect newborn lambs (McGovern *et al.*, 2016). Because the DUOX/LPO/halide system can generate hypoiodous acid (OI^-) when the iodide concentration is elevated in the airway, a lamb model of RSV was used to test if potassium iodide could enhance the LPO/halide system *in vivo* (McGovern *et al.*, 2016). Newborn lambs were treated with intragastric gavage of potassium iodide that led to a 10-fold increase in iodide concentration in the airway surface liquid (McGovern *et al.*, 2016). Additionally, expiratory effort, gross lung lesions, and pulmonary expression of an RSV antigen were reduced in the potassium iodide-treated lambs compared to untreated control lambs. The authors concluded that high dose of potassium iodide supplementation can be used *in vivo* to lessen the severity of RSV infections by manipulating the LPO-based system (Derscheid *et al.*, 2014). However, LPO-dependent production of $OSCN^-$ did not inactivate adenovirus or RSV but substitution of the LPO substrate SCN^- with iodide (I^-) resulted in a marked reduction of both adenovirus transduction and RSV titer suggesting that the LPO/I^-/H_2O_2 system can contribute to airway antiviral defense (Fischer *et al.*, 2011). However, a treatment *in vivo* with an excess of iodide can induce the blockade of thyroid hormone synthesis (hypothyroidism).

Sendai virus (SeV), also named murine parainfluenza virus type 1 belongs to the genus *Respirovirus* of the *Paramyxoviridae* family. This family is a group of enveloped non-segmented negative strand RNA viruses and include major human and animal pathogens like Newcastle disease virus, parainfluenza viruses, respiratory syncytial viruses, and the bioterrorism threat Nipah Virus. SeV causes a typical respiratory infection and has often been used as a model for respiratory virus infection (Simmons *et al.*, 2002). Using RT-PCR, Fink *et al.* (2008) have shown that SeV infection of the A549 alveolar epithelial cell line induced *DUOX2* mRNA expression. Following this first report, a detailed characterization of *DUOX2* mRNA and DUOX2 protein expression upon SeV infection was performed in different cell line models of alveolar epithelial cells and non-transformed primary normal human bronchial epithelial cells (NHBEs) (Fink *et al.*, 2013). The authors of this study showed first that SeV-induced DUOX2/DUOAX2 expression results from autocrine/paracrine mechanism, second, IFN-β and TNF-α synergize to induce DUOX2 and DUOAX2 expression, third, DUOX2 is essential for airway epithelial cells to mount an antiviral defense, and RSV interferes with the expression of DUOX2 (Fink *et al.*, 2013). Although no direct evidence was provided in this study for the virucidal action of DUOX against SeV, this study showed that airway epithelial cells respond with DUOX up-regulation to viral challenge.

Overall, although the LPO/H_2O_2/SCN^- system has been studied much less for its antiviral activities, accumulating observations indicate its potential to fight viral pathogens, as well. For additional information of the role of NOX/DUOX enzymes expressed in epithelial cells along the respiratory tract in the host defense against respiratory viruses, a review by Grandvaux has revisited our current knowledge (Grandvaux *et al.*, 2015).

Antifungal effects of the DUOX/ H_2O_2/LPO system

Few reports also indicate that the LPO-based system represents an efficient antifungal mechanism. Benoy *et al.* tested the antifungal effect of LPO purified from goat milk in presence of physiological levels of SCN^- (around 400 μM) against several fungal species, and found most of them to be sensitive to LPO-mediated killing: *Aspergillus niger*, *Pencillium chrysogeum*, *Aspergillus flaws*, *Alternaria* sp., *Trichoderma* sp., *Corynespora cassiicola*, *Phytopthora meadii*, *Claviceps* sp., and *Corticium salmonicolor* (Benoy *et al.*, 2000). Mean inhibitory concentrations of LPO antifungal activities were in the range of 62–490 μg/ml LPO (Benoy *et al.*, 2000). Only two species tested, *Candida albicans* and *Pythium* sp., turned out to be resistant to LPO-mediated damage (Benoy *et al.*, 2000). Other studies, however, reported the opposite, killing of *C. albicans* by LPO (Lenander-Lumikari, 1992; Bosch *et al.*, 2000; Ahariz and Courtois, 2010; Kho *et al.*, 2012). The higher sensitivity of *C. albicans* to I^- than to SCN^- as LPO substrates could partially explain these contradictory results (Majerus and Courtois, 1992; Ahariz and Courtois, 2010). Yeast and filamentous forms of *Rhodutorula rubra*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Aspergillus niger*, and *Byssochlamys fulva* were also exposed to the H_2O_2/LPO/SCN^- system in buffer and apple juice and found to be damaged (Popper and Knorr, 1997). Not only bacterial but fungal toxins can also be inactivated by LPO. Different types of aflatoxin (B1, B2A, G1) were efficiently degraded *in vitro* by the LPO/H_2O_2 system, although the enzyme substrate was not specified (Doyle and Marth, 1978). Oxidation of alpha-amanitin, a potent hepatotoxin produced by the *Amanita phalloides* fungus, has also been observed *in vitro* by the LPO/H_2O_2/I^- system (Morris *et al.*, 1979; Zheleva *et al.*, 2000).

Antiparasitic effects of the DUOX/H_2O_2/LPO system

Sporadic observations reported that LPO also has parasiticidal activities. While tachyzoite stage *Toxoplasma gondii*, an obligatory intracellular parasite, is resistant to H_2O_2 *in vitro*, addition of LPO and I^- resulted in enhanced sensitivity of the protozoan (Tanaka *et al.*, 2006). LPO-treated *T. gondii* tachyzoites did not penetrate mouse fibroblasts and could also be killed intracellularly in mouse macrophages (Tanaka *et al.*, 2006). These results suggest that the LPO-based system could inhibit the development of Toxoplasmosis after eating raw meat (Tanaka *et al.*, 2006).

During malaria infection of the mosquito vector *Anopheles gambiae*, DUOX forms a dityrosine network with Immunomodulatory Peroxidase (IMPer) and decreases mosquito gut permeability to immune elicitors (Kumar *et al.*, 2010). This network protects the microbiota by preventing activation of epithelial immunity (Kumar *et al.*, 2010). The authors of this study showed that this mechanism provides a permissive environment for the malaria parasite development in the mosquito as it prevents activation of antimalarial response mediated nitric oxide synthase (NOS) (Kumar *et al.*, 2010). Interestingly, when *DUOX* is silenced, mosquitos mount strong pathogen-specific responses to bacteria and malaria parasites. The authors found that the Oxidation Resistance 1 gene (*OXR1*) decreases the survival of female mosquitos to an oral challenge with ROS. *OXR1* silencing also decreases

the number of *Plasmodium berghei* ookinetes that survive and become oocyts suggesting that malaria parasites in mosquitoes are less susceptible to ROS damage (Kumar *et al.*, 2010). However, susceptibility of *Plasmodium falciparum*, the deadliest human malaria parasite, to a peroxidase-mediated oxygen-dependent microbicidal system has been demonstrated (Malhotra *et al.*, 1988). The *in vitro* study showed that the killing of malaria parasites by H_2O_2, LPO, and SCN^- was not due to red blood cell damage as indicated by the examination of controls after exposure to the different components. Detailed *in vitro* and *in vivo* studies of the role of the DUOX system in malaria are warranted despite the opposite effects of ROS on malaria parasite inside the mosquito gut vector and the host red blood cells. Thus, targets of LPO are not only bacteria and viruses but also include fungal and protozoan pathogens making this innate immune mechanism one of the most general ones in terms of microbial targets.

Concluding remarks

Although data collected in this review prove that the DUOX/H_2O_2/LPO/SCN^- system is a potent, broad spectrum, antimicrobial mechanism, most of these studies were conducted in lower organisms or in *in vitro* cell culture systems. Surprisingly, very few studies addressed the antimicrobial role of this mechanism in mammalian organisms. Such investigations are essential to prove its physiological relevance in the most developed life forms including humans. Table 2 enlists main questions to be addressed on the field in the future.

While negative effects of DUOX1 lung function have already been described (Habibovic *et al.*, 2016), studies are still awaited to show its beneficial role in respiratory or other infections. DUOX1 is expressed in the mouse respiratory tract and cultured murine tracheal epithelial cells suggesting that mice could serve as a great model the study the *in vivo* mechanistic role of DUOX1 in airway infections (Chang *et al.*, 2013; Habibovic *et al.*, 2016). DUOX1-deficient mice are available and provide and ideal tool to address this question (Donko *et al.*, 2010; Rada *et al.*, 2014b; Habibovic *et al.*, 2016) (Table 2). Down-regulation of DUOX1 gene expression in murine airways using instilled siRNA cocktails presents another possible approach (Habibovic *et al.*, 2016). Developing DUOX1-deficient models of larger animals would also provide a feasible option (rat, ferret, pig).

It is important to separate the potential *in vivo* antimicrobial roles of DUOX1 and DUOX2 from each other. Mice that are deficient in both, DUOX1 and DUOX2 (or their corresponding Duox activators) do not represent optimal experimental tools since roles of DUOX1 and DUOX2 cannot be distinguished from each other in these models. Although similar in structure, DUOX1 and DUOX2 are two independent genes with fairly different regulatory mechanisms discovered so far. The main *in vivo* role of DUOX2 is the production of thyroid hormones since DUOX2 deficiency leads to hypothyroidism in both, mice and humans. Interestingly, no study ever reported any problems of DUOX2-deficient mice or human patients with infections. Although not impossible but the lack of these reports makes it unlikely that DUOX2 has a significant *in vivo* immune role. Infection studies performed on mice with global DUOX2 deficiency should be viewed critically since a direct immune role of DUOX2 is very hard to separate from an indirect role via dysfunction in thyroid hormone production. Even if attempts have been made to reintroduce thyroid hormones regularly to DUOX2-deficient mice from early on to circumvent this problem, it is very difficult to ensure that thyroid hormone-fed mice are normothyroid and not hypo- or hyperthyroid. An ideal experimental tool to study the extrathyroid role of DUOX2 in infections would be mice with conditional DUOX2 deficiency only in target cells. There is an urgent need to develop such a mouse model (Table 2).

Similarly, to the case of DUOX1, studies focusing on the *in vivo* antimicrobial role of LPO in mammalian experimental models are also lacking. Although the use of the LPO inhibitor dapsone delivered the most such *in vivo* results until now, more specific experimental tools are required to address LPO. There are no LPO-deficient murine or other mammalian models available to investigate this highly relevant question. In fact, LPO expression has not even been investigated in murine airways so far. Our results presented in Fig. 2 show that LPO is highly expressed in the mouse trachea and, similarly to humans and other mammals studied, it shows a submucosal tissue distribution. Thus, mouse airways express both DUOX and LPO and provide therefore a useful animal model to address the *in vivo* role of this antimicrobial system in infectious diseases.

The field also suffers from lack of antibodies that reliably and specifically recognize only one DUOX isoform. Availability of such research tools would accelerate studies focusing on distinguishing the roles of DUOX1 and DUOX2 in *in vivo* models.

The vast amount of literature cited here shows that the DUOX/H_2O_2/LPO/SCN^- antimicrobial system provides a fast-reacting, powerful, innate immune mechanism for host epithelia to fight several pathogens in a non-specific fashion. A detailed understanding of its mechanism of action is, however, still unclear. How can this oxidative mechanism generating mildly reactive ROS efficiently attack a wide variety of microorganisms? The primary molecular targets of $OSCN^-$ in microbes are likely proteins with SH moiety (Bafort *et al.*, 2014). Altering the function of several such proteins could interfere with general (glycolysis, respiration, nutrient transport) or microbe-specific mechanisms resulting in damage and killing of the targeted microorganisms (Bafort *et al.*, 2014). The exact molecular mechanism of the antimicrobial action of $OSCN^-$ remains, however, to be investigated in the

Table 2. Outstanding questions

- Do DUOX1 and LPO promote antimicrobial functions in murine airways?
- Does DUOX2 promote antimicrobial functions in the airways of normothyroid mice?
- Are DUOX1 or LPO genetic deficiencies present in the human population and associated with increased risks for certain infections?
- What is the molecular mechanism of the antimicrobial action of OSCN-?
- Can the DUOX/H_2O_2/LPO/SCN- antimicrobial system be therapeutically enhanced to boost immune defenses against a wide variety of pathogens?

future.

On the practical side, the large amounts of published results collected here provide a strong basis and enough enthusiasm for the potential therapeutic targeting of the LPO-based system to fight infections. Since delivering a detailed overview about this topic is beyond the scope of this review, we only mention a few key points to be considered. Increasing the concentration of any one or more components of the LPO-based system would theoretically lead to a larger $OSCN^-$ yield and stronger microbicidal activity. Administration of SCN^- or LPO (or both) is more likely a safer approach as enhancing DUOX1 activity in the airways could lead to unwanted allergic side effects (Chang *et al.*, 2013; Rada *et al.*, 2014a; Habibovic *et al.*, 2016). While oral administration is more convenient and could provide more stable airway levels of SCN^- and LPO over time, it has to pass the stomach, get absorbed in the blood and accumulated in the airway surface liquid. On the other hand, nebulized administration delivers SCN^- or LPO directly to its target location, it would, however, have to be repeated regularly as their concentrations are expected to drop rapidly.

While several challenges still lie ahead to have a complete understanding and characterization of the DUOX/LPO-based antimicrobial defense system, it provides a unique, potent, broad spectrum, microbicidal mechanism of interest to both basic and applied research.

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