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FORUM REVIEW ARTICLE

**The dual role of ROS-generating NADPH oxidases in gastrointestinal
inflammation and therapeutic perspectives**

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Abbreviated title : NADPH oxidases in gastrointestinal inflammation

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Significance: Despite their intrinsic cytotoxic properties, mounting evidence indicate that ROS physiologically produced by the NADPH oxidases of epithelial cells (NOX1, DUOX2) and phagocytes (NOX2) are critical for innate immune response and homeostasis of the intestinal mucosa. However, dysregulated ROS production could be a driving factor in inflammatory bowel diseases (IBD).

Recent advances: In addition to NOX2, recent studies have demonstrated that NOX1- and DUOX2-derived ROS can regulate intestinal innate immune defense and homeostasis by impacting many processes, including bacterial virulence, expression of bacteriostatic proteins, epithelial renewal and restitution, and microbiota composition. Moreover, the antibacterial role of DUOX2 is a function conserved in evolution as it has been described in invertebrates, and lower and higher vertebrates. In humans, variants of the *NOX2*, *NOX1* and *DUOX2* genes, which are associated with impaired ROS production, have been identified in very early-onset IBD, but overexpression of NOX/DUOX, especially DUOX2, has also been described in IBD, suggesting that loss-of-function or excessive activity of the ROS-generating enzymes could both contribute to disease progression.

Critical issues: Therapeutic perspectives aiming at targeting NOX/DUOX in IBD should take into account the two sides of NOX/DUOX-derived ROS in intestinal inflammation. Hence, NOX /DUOX inhibitors or ROS inducers should be considered as a function of the disease context.

Future directions: A thorough understanding of the physiological and pathological regulation of NOX/DUOX in the gastrointestinal tract is an absolute pre-requisite for the development of therapeutic strategies that can modulate ROS levels in space and time.

I. Introduction

Inflammation is broadly defined as a beneficial response of the immune system as it allows the organism to defend itself against external aggressions or pathogens and favors restoration of tissue integrity (110). Inflammation is therefore a defense mechanism that is essential for body homeostasis and health. However, when dysregulated or unresolved, the inflammatory response can become chronic and consequently a driving factor for a vast array of diseases, including arthritis, autoimmune diseases and inflammatory bowel disease (IBD) (122).

The gastrointestinal tract represents the largest immune interface that undergoes permanent antigenic pressure from various sources of antigens such as diets, commensal bacteria or pathogens. It thus fulfills several physiological functions (absorption, food metabolism, ion secretion) while coordinating an appropriate immune and inflammatory response to provide defense against potential infection, but also ensure tissue homeostasis (**Figure 1**). Mucosal immune defense relies on specialized epithelial cells that not only constitute a physical barrier against the commensal or pathogenic microbes, but also coordinate the immune response by secreting mucins, cytokines, chemokines, antimicrobial defense molecules like defensins, cathelicidins, RegIII γ , and bacteriostatic proteins like lipocalin 2 (LCN-2 also known as Neutrophil Gelatinase- Associated Lipocalin or NGAL) (114, 120, 129). It also relies on various innate and adaptive immune cells that are scattered throughout the lamina propria (LP) or reside in organized structures such as Peyer's patches (PPs) and mesenteric lymph nodes located below the epithelium (49). Disruption of intestinal homeostasis state results in intestinal disorders such as inflammatory bowel diseases (IBD). IBD represents a group of relapsing chronic disorders, comprising Crohn's disease and ulcerative colitis, all characterized by severe, uncontrolled and recurrent inflammation of the gastrointestinal tract, and arising from a dysregulated immune response to the intestinal microflora. The etiology and pathogenesis of

the disease are multifactorial and may include predisposing genetic background, environmental factors, and gut microbial dysbiosis (35).

Inflammation is closely linked to the generation of reactive oxygen species (ROS) by cells of the microenvironment. ROS are known to be involved in the initiation, progression, and resolution of the inflammatory response (46, 111). In the gastrointestinal tract, epithelial cells and innate immune cells, including resident monocytes/macrophages, dendritic cells or infiltrated neutrophils, are all able to produce ROS in response to inflammatory stimuli through specialized enzyme complexes called NADPH oxidases. This review discusses the dual role of ROS-generating NADPH oxidases in gastrointestinal inflammation and their potential as therapeutic targets in IBD.

II. Properties and endogenous sources of ROS

It is important to know the nature and properties of ROS to better understand their biological impact in the gastrointestinal tract (**Figure 2**). ROS derive from oxygen and are highly reactive towards organic compounds. They are either free radicals or neutral molecules. Free radicals such as superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$) have an unpaired electron in the outer shell, while neutral molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2) have all paired electrons (166). Superoxide anion, the first species generated by oxygen reduction, is a weak oxidant toward biological molecules ($k < 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$). However, $O_2^{\bullet-}$ has indirect toxicity as it is the precursor of other more reactive ROS. $O_2^{\bullet-}$ can undergo spontaneous or enzymatic dismutation by superoxide dismutase to form H_2O_2 , and can react with nitric oxide ($NO^{\bullet}$) to generate peroxynitrite ($ONOO^-$), a highly deleterious molecule. Because of its anionic charge, $O_2^{\bullet-}$ does not diffuse through plasma membranes. However, it has been shown that anionic chlorine channels were capable of facilitating the movement of $O_2^{\bullet-}$ across membranes (121). $O_2^{\bullet-}$ can also cause biological damage by acting as a reductant. H_2O_2 is a relatively stable

molecule and mainly reacts with sulfhydryl groups (SH) in proteins. It can diffuse through biological membranes and this diffusion can be facilitated by aquaporins (16), therefore it can act in an autocrine or paracrine manner. H_2O_2 is also a substrate for catalase, which catalyzes its decomposition into water and oxygen. In addition, H_2O_2 reacts with Cl^- to form hypochlorous acid (HOCl) under the catalytic action of myeloperoxidase, and with $\text{O}_2^{\bullet-}$ in the presence of transition metal ions such as ferrous iron (Fe^{2+}) to form $\text{HO}^\bullet$ in the so-called Fenton reaction. Both HOCl and $\text{HO}^\bullet$ are highly reactive with biomolecules. $\text{HO}^\bullet$ is the most powerful and most toxic oxidant in biology ($k=4 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ for DNA; $k=4 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ for proteins; and $k=7,4 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ for glucose). Finally, the reaction between H_2O_2 and HOCl can generate singlet oxygen ($^1\text{O}_2$), which reacts mostly with lipids in the membranes to induce peroxidation. ROS properties determine their biological impact. At elevated concentrations, their high reactivity with biological molecules can be used as a weapon to kill microorganisms, while at low concentrations, their ability to induce reversible sulfhydryl group oxidation can provide an “on-off” mechanism for protein signaling function (45). Disease states may result from inappropriate and uncontrolled/dysregulated ROS production (73).

In the organism, $\text{O}_2^{\bullet-}$ can originate from many endogenous sources that can be classified into “non-professional” and “professional” ROS-generating enzymatic systems. Non-professional systems produce $\text{O}_2^{\bullet-}$ as by-products and include mostly enzymatic systems that are usually involved in cellular metabolism such as the mitochondrial respiratory chain, xanthine oxidase, P450 cytochrome, lipoxygenases, cyclooxygenases and NO synthase. Electron leakage from the mitochondrial respiratory chain complex I and III could result in the reduction of molecular oxygen to $\text{O}_2^{\bullet-}$ (87). The mitochondria ability to generate ROS was initially described based on *in vitro* experiments with isolated organelles (147, 160). More recent studies have however shown that spontaneous ROS release by mitochondria in living cells does not occur unless there are changes in the physical state of the membrane, such as fluidity (125). P450 cytochrome and

xanthine oxidase, which are involved in the metabolism of xenobiotics and the catabolism of purine bases, respectively, can also release $O_2^{\circ-}$ when they are functioning (108, 165). Finally, NO synthase, which is responsible for the catabolism of L-arginine with release of NO° , can also produce $O_2^{\circ-}$ when its cofactor tetrahydrobiopterin is oxidized into dihydrobiopterin, resulting in uncoupling of the enzyme (95). In contrast to “non-professional” ROS generating systems, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (or NOX) are multi-subunit enzyme complexes dedicated to the controlled production of $O_2^{\circ-}$ through the single-electron reduction of molecular oxygen, with NADPH as the electron donor. They are therefore considered as “professional” ROS generating enzymes. This family includes seven members, NADPH oxidase 1 to 5 (NOX1-NOX5), and Dual oxidase 1 and 2 (DUOX1, DUOX2), and has been defined on the basis of structural homology with gp91^{PHOX} (now renamed NOX2), the catalytic core of the phagocyte NADPH oxidase identified more than forty years ago (144). Structurally, they all consist of a membrane catalytic core called NOX/DUOX that binds NADPH, flavin adenine dinucleotide (FAD) and two hemes, and through which electrons are transferred to reduce oxygen (94). Except for NOX5, these NOX/DUOX cores are stabilized by another membrane protein, i.e., p22^{PHOX} for NOX1 to 4, and DUOXA1/2 for DUOX1/2. In addition to p22^{PHOX}, NOX1, NOX2, and NOX3 require regulatory cytosolic subunits (detailed below) to be fully functional (12, 94). These different NOX isoforms have tissue/cell selective expression. NOX1 is abundantly expressed in the colon, NOX2 is mainly found in phagocytes, NOX3 is expressed in the inner ear, NOX4 is predominantly found in the kidney, NOX5 is found in lymphoid tissues, and DUOX1/2 are predominantly expressed in the thyroid. However, these NOX isoforms can also be expressed at low levels in other tissues (12, 94).

III. NADPH oxidases found in the gastrointestinal tract and their regulation

The main NADPH oxidases that have been shown to play a physiological/pathophysiological role in the gastrointestinal tract are NOX2, expressed in innate immune cells, and NOX1 and DUOX2, expressed in the epithelium (**Figure 3**).

In the lamina propria, resident monocytes/macrophages, dendritic cells and infiltrated neutrophils all express NOX2. NOX2 structure and regulation have been extensively studied over the past decades (8, 61). A functional phagocytic NADPH oxidase complex consists of the membrane-anchored flavocytochrome b₅₅₈ (the catalytic core of the enzyme composed of gp91^{PHOX} and p22^{PHOX}, phox for phagocyte oxidase), the regulatory cytosolic proteins p47^{PHOX}, p67^{PHOX}, p40^{PHOX}, and the low molecular-weight GTP-binding proteins, Rac 1 or 2. Robust production of O₂^{•-} by NOX2, a phenomenon known as the “respiratory burst”, is the cornerstone of host defense against microbial infections. The vital importance of this enzyme is illustrated by a human genetic disorder called chronic granulomatous disease (CGD), which is a primary immunodeficiency disorder caused by defects in one of the NADPH oxidase subunits and characterized by severe and recurrent life-threatening infections (140). In neutrophils, NOX2 activity is tightly regulated, as its components are segregated in different cellular compartments (membrane and cytosol) ensuring that NOX2 stays inactive in the absence of stimulation. However, upon neutrophil stimulation by agents such as the bacterial chemoattractant peptide formyl-Met-Leu-Phe (fMLF) or opsonized bacteria, p47^{PHOX}, p67^{PHOX} and p40^{PHOX}, which form a trimeric complex in the cytosol, become phosphorylated and, together with Rac1/2, migrate to the membranes where they associate with cytochrome b₅₅₈ to assemble the catalytically active oxidase (13).

NOX1, which is found in the intestinal epithelium, was first cloned from the human epithelial cell line Caco-2 (155). Among normal human tissues, its messenger was found to be mostly expressed in the gastrointestinal tract, and specifically in the colon and the rectum (51, 155). *In*

situ hybridization on mouse colon revealed that NOX1 mRNA was present in epithelial cells that were located in the lower two-thirds of the crypt (50, 112), an area where cells undergo proliferation and differentiation. In contrast, NOXO1 and NOXA1 mRNA are evenly expressed throughout the entire length of the mouse epithelium (9, 112). Nevertheless, expression of NOX1 mRNA in the lower part of the crypt is not a general feature, but rather is dependent on the species. Indeed in the human colon, *in situ* hybridization showed NOX1 mRNA expression along the entire epithelium (156). Few studies have analyzed the expression of NOX1 at the protein level, probably due to the lack of high quality specific NOX1 antibodies. Indeed many NOX1 commercial antibodies have been described to be unable to differentiate between HEK293 cells stably overexpressing NOX1 and control HEK293 cells transfected with empty vectors (38). However, using the antibodies they developed, Fukuyama *et al.* found that NOX1 protein was rather expressed in the upper one-third of the normal human colonic mucosa by immunohistochemistry (48). Therefore, presence of NOX1 mRNA does not always correlate with expression of the NOX1 protein; therefore, it is important to analyze expression both at the mRNA and protein levels. Within epithelial cells, immunohistochemistry on large epithelial cells of the guinea pig colon showed an apical localization of NOX1 (81). NOX1 is closely related to the phagocyte NADPH oxidase in terms of its structure and function (12). It interacts with p22^{PHOX}, and its activation also requires binding to cytosolic regulatory partners, i.e., NOX organizer 1 (NOXO1), the p47^{PHOX} homologue; NOX activator 1 (NOXA1), the p67^{PHOX} homologue; and Rac1 GTPase. As with NOX1, NOXO1 and NOXA1 transcripts are abundantly expressed in the colon (9, 51). In various cell lines transfected with human NOX1, NOXO1 and NOXA1 cDNA, O₂^{•-} is constitutively produced at low levels (51). This has been attributed to permanent association of NOXO1 with p22^{PHOX} and membrane phosphoinositides, due to the lack of auto-inhibitory region (AIR), which is present in p47^{PHOX} but not in NOXO1 (24, 157). In p47^{PHOX}, unless phosphorylation occurs, the AIR domain maintains the protein in an auto-

inhibited conformation, preventing the assembly and activation of NOX2. However, the constitutive activity of NOX1 can be modulated by phosphorylation of NOXA1 and NOXO1. Phosphorylation of NOXA1 by PKA and MAPK was shown to decrease NOX1 activity (85, 88), whereas phosphorylation of NOXO1 by PKC was shown to enhance it (36, 168). Phosphorylation of NOX1 by PKC β I has also been shown to facilitate its assembly and activation (154)

Although DUOX2 is most abundantly expressed in the thyroid (34, 40) where its contribution to the synthesis of thyroid hormones is firmly established as illustrated by DUOX2 mutations in congenital hypothyroidism (138), its expression at the protein level has also been described along the porcine digestive tract (41). This expression was confirmed in human gastrointestinal tissues in the colon, small intestine and duodenum (41). In both porcine and human tissues, immunohistochemistry revealed expression at the apical surface and no expression at the bottom of the crypt (41). In mice, DUOX2 expression is highest in the ileum and is predominantly expressed in differentiated epithelium in the ileum villus tip (58, 151). The maturation factor DUOXA1/2 is required for DUOX1/2 to acquire the correct conformation and localization to the plasma membrane (60). In addition to their function in maturation, DUOXA1/2 form stable functional heterodimers with DUOX1/2 (103, 113), similarly to p22^{PHOX} with gp91^{PHOX}. Recently, it was shown that DUOX2 plays a chaperone-like function with respect to DUOXA2 and this depends on an intramolecular disulfide bond within DUOX2. Suppression of this DUOX2 intramolecular disulfide bond induced DUOXA2 degradation (22). Another feature of DUOX2 is the regulation of its activity by calcium *via* the two EF-Hands motifs located in the cytosolic portion spanning transmembrane domain 1 and 2 and its phosphorylation by PKC under Phorbol Myristate Acetate (PMA) stimulation (4, 136). Unlike NOX2 and NOX1 which produce O₂^{•-}, DUOX2 generate H₂O₂. The underlying mechanisms

might involve the extracellular A-loop of DUOX, which prevents $O_2^{\circ-}$ leakage by facilitating the stabilization and maturation of the DUOX-DUOXA complex (161).

Interestingly, a recent study using an *in vivo* method for ROS measurement with the chemiluminescent probe L-012 showed that under steady-state conditions, NOX1-deficient mice have a strongly reduced ROS signal in the gut as compared to wild type mice (27). This suggests that in the intestine, production of ROS *via* NOX2, and probably DUOX2, is negligible as compared to that *via* NOX1 in the absence of inflammatory stimuli or infection (27).

IV. NADPH oxidases-derived ROS in the gastrointestinal tract under physiological conditions: A role in immune defense and homeostasis

The tasks of the intestinal epithelium and immune system are to provide the host with defense mechanisms against pathogenic insults and to maintain gut homeostasis; thus, the expected physiological functions of the NADPH oxidases found in the gastrointestinal tract would be related to these functions. We highlight here the findings that support the contribution of NOX2, NOX1 and DUOX2 to intestinal immune defense and homeostasis.

IV. 1. NOX2 in mucosal immune defense and homeostasis

As an essential component of the neutrophil defensive machinery, NOX2 plays important functions in intestinal innate immune defense. During normal intestinal inflammatory response that may result from epithelial barrier disruption and subsequent translocation of pathogens into the mucosa, neutrophils are recruited at the site of infection under the influence of chemo-attractants where they then engulfed the pathogen to form a phagosome in which massive amount of NOX2-derived ROS and granular enzymes are released. These NOX2-derived ROS act as weapons to kill the microorganisms that have crossed the epithelium, thereby protecting the mucosa (47). In addition to this cytotoxic effect, it has been shown in murine macrophages

and neutrophils, that NOX2-derived ROS can promote phagosome maturation and thus microbial killing by inducing the binding of the autophagic protein LC3 to the phagosome (68). Interestingly, NOX2-derived ROS can also promote the resolution of inflammation in the intestinal mucosa. Therefore, Campbell *et al.* demonstrated that the neutrophil “respiratory burst” can trigger such an increase in oxygen consumption that it rapidly depletes micro-environmental oxygen to level sufficient to stabilize intestinal epithelial cell hypoxia-inducible factor (HIF). Stabilization of HIF elicits the transcription of genes that promote resolution responses important to tissue homeostasis (21). Besides, NOX2-derived ROS have been shown to be important for neutrophil apoptosis and subsequent return to tissue homeostasis (5).

IV. 2. NOX1 in mucosal immune defense and homeostasis

NOX1 regulation by cytokines, PAMPS and Microbiota. - The production of ROS by NOX1 is constitutively low and constant, and is therefore unlikely to exert a direct microbicidal effect. However, several observations point to a role of NOX1 in innate immune defense of the intestine. Thus, a close correlation exists between NOX1 expression level and bacterial burden in the intestine in both human and mice (39, 156). In addition, inflammatory stimuli, including cytokines, Pathogen Associated Molecular Patterns (PAMPS) and bacteria, were all able to modulate the expression and activity of NOX1 subunits in intestinal epithelial cells. Pro-inflammatory cytokines $\text{INF}\gamma$, $\text{IL-1}\beta$, $\text{TNF}\alpha$ and IL-17 (92, 93, 105), LPS from *Helicobacter pylori* (80), flagellin from *S. Enteridis* (81), and pathogenic adherent–invasive *Escherichia coli* LF82 (42) can all increase NOX1 and NOXO1 expression along with ROS production in intestinal epithelial cells. Interestingly, comparison of RNA-seq data from IL-17A -stimulated enteroids (for significantly upregulated genes) versus from the ileum of intestinal epithelial specific- deficient IL17RA mice (for significantly downregulated genes) revealed NOX1 as a gene directly regulated by IL-17 *in vitro* and *in vivo* (90). In addition, the prototype anti-

inflammatory cytokine, IL-10, was shown to significantly inhibit INF γ and TNF α -induced up-regulation of superoxide-producing activity in colonic epithelial cell line by suppressing NOX1 and NOXO1 mRNA and protein (78). *In vivo*, it has been shown that NOX1 expression and ROS generation can be induced by the microbiota in the gut of mice. Depletion of the gut microbiota by antibiotic treatment strongly decreased ROS production and NOX1 expression (107). Moreover, mice kept in a germ-free environment expressed weak level of NOX1 in the colorectum as compared to their counterpart kept in specific pathogen-free environment (58). The observation that NOX1 expression and activity could be regulated by cytokines, PAMPS as well as the microbiota strongly supports a role of NOX1 in mucosal innate immunity and homeostasis.

Mechanisms by which NOX1 contributes to mucosal immune defense and homeostasis ? - In this section we synthesis recent data highlighting the mechanisms through which NOX1 may contribute to mucosal immune defense and homeostasis.

One suggested mechanism is the alteration of bacterial capsule formation and virulence through the disruption of bacterial signaling. Corcionivoschi *et al.* indeed showed that during *Campylobacter jejuni* infection, ROS generated *via* NOX1 (or DUOX2) from epithelial cells inactivate the bacterial tyrosine kinase, Cjtk, resulting in a decrease in the phosphorylation and activity of outer membrane/periplasmic proteins, notably an epimerase involved in polysaccharide biosynthesis and required for capsule formation (28).

Another potential mechanism is the control by NOX1 of the expression of lipocalin 2 (LCN-2, also known as NGAL) (105), a potent bacteriostatic protein known to limit bacterial growth (54). Indeed recently, we demonstrated that upon co-stimulation of colonic epithelial cell line and colon organoids by TNF α and IL-17, NOX1-derived ROS were able to drive the expression of LCN-2 by controlling the expression of I κ B ζ , a non-canonical member of the NF- κ B transcription factor family that acts as a positive regulator of NF- κ B (105). Besides its

bacteriostatic property, LCN-2 was shown to be an important paracrine chemoattractant for neutrophils (142), and through this effect, could also participate to innate immunity.

Induction of chemokine synthesis, which triggers the recruitment of neutrophils, is another means through which NOX1 could contribute to host defense. Notably, it has been shown in large intestinal epithelial cells that activation of NOX1 by *S. Enteridis* flagellin induces IL-8 synthesis through NF- κ B activation (81).

The secretion of mucins into the intestinal lumen by goblet cells also creates the first line of host defense against microbial intruders. It is not clear whether NOX1 is involved in the regulation of mucin secretion. However, it has been shown that ROS generated by a p22^{PHOX} dependent NADPH oxidase are critical to control mucin granule accumulation in colonic goblet cells (128), and that NOX (or DUOX)-derived ROS are upstream of NLRP6 activation, which controls MUC2 secretion in response to TLR ligands in murine colonic explants (17). On the other hand, it has been shown that NOX1-deficient mice have a massive conversion of progenitor cells into goblet cells at the expense of colonocytes associated with increased MUC2 expression, indicating that NOX1 can indirectly impact mucin secretion by controlling progenitor cell fate in the colon (26).

In addition to innate immunity, several studies have revealed the central role of NOX1 in the intestine homeostasis through epithelial renewal, restitution and mucosal healing. Thus, in the DSS-induced colitis model, NOX1-deficient mice have tissue repair deficiency demonstrating the importance of NOX1 for epithelial restitution (79). Furthermore, Annexin A1 (ANXA-1) and some *Lactobacillus* species promote mucosal wound repair by activating NOX1 and ROS production, which then induce intracellular signaling pathways, leading to the proliferation and migration of epithelial cells and tissue repair. Specifically, the cleavage product of ANXA-1 was demonstrated to bind to formyl peptide receptor 1 (FPR1) and to activate NOX1. NOX1 derived-ROS induce oxidative inactivation of the PTEN and PTP-PEST phosphatases, leading

to increased phosphorylation of focal adhesion kinase (FAK) and paxillin, thereby promoting epithelial cell migration and restitution in mechanically-induced wound and DSS-mediated crypt damage models (97). Regarding *Lactobacillus spp*, they were shown upon their ingestion by both *Drosophila melanogaster* and mice to stimulate NOX1-derived ROS production, which in turn promoted cellular proliferation of stem cells in the normal mucosa, indicating a conserved role for NOX1 in epithelial renewal (75). Taking into account the presence of an intact mucus layer in this context, this suggests that direct contact between the bacteria and epithelial cells is not required for this process, which rather involves a secreted intermediate. However, in a mechanical wound model, administration of Lactobacilli also facilitate migration and proliferation of enterocytes adjacent to colonic wounds through the FPR1/NOX1/FAK-ERK axis (3). NOX1 activation by Lactobacilli also promotes cytoprotection against exogenous oxidative insults through activation of the redox-sensitive transcription factor NRF2 that triggers the transcription of antioxidant and detoxifying proteins in both *Drosophila melanogaster* and mice (74). Finally, pro-restitutive microbiota such as *Akkermansia muciniphila*, which were shown to be enriched in the injured microenvironment, exert also their wound repair effect through the FPR1/ NOX1 axis (2). Homeostasis in the intestine also depends on the preservation of microbial balance in the gut. In this regard, NOX1 has been shown to influence this balance as evidenced by the fact that NOX1-deficient mice have an increase in bacterial load in the ileum and an alteration in the gut microbiota composition (107). Moreover, epithelium-restricted p22^{PHOX}-deficient mice (p22^{PHOX} ^{-/-} IEC), which can be considered as equivalent to NOX1^{-/-} IEC mice as other NOXs are not expressed in the epithelium, show a highly augmented abundance of H₂O₂-producing bacteria *Lactobacilli reuteri* and *Lactobacilli murinus* in the cecum and the colon. This change in microbiota composition could represent a compensatory adaptation of the microbiota to confer protection against enteric pathogens (130).

In summary, mechanisms by which NOX1 contributes to mucosal immune defense and homeostasis may include: alteration of bacterial capsule formation, control of bacteriostatic protein expression such as LCN-2, chemokine synthesis, mucin synthesis, epithelium renewal, restitution and healing (**figure 4**).

IV. 3. DUOX2 in mucosal immune defense and homeostasis

Antibacterial role of DUOX2 in invertebrates, lower and higher vertebrates- As detailed below, DUOX2, the other intestinal epithelial NADPH oxidase, has also been demonstrated to be essential in innate immunity and homeostasis of the gastrointestinal tract in several models.

In vitro studies in the Caco-2 human colon cell line demonstrated that binding of the bacterial cell wall component muramyl dipeptides (MDP) to NOD2 induced DUOX2-dependent ROS production which mediate the antibacterial activity of NOD2 against *Listeria monocytogenes*, notably through NFκB activation and interleukin-8 secretion (99).

Most *in vivo* studies elucidating the importance of DUOX in intestinal mucosal immunity derive from studies in invertebrates such as the fly *Drosophila*, the mosquito *Anopheles gambiae*, or the nematode *C.elegans*. In *Drosophila*, ROS produced by DUOX are major effectors of intestinal immunity as ubiquitous- or intestine-restricted deletion of dDUOX (*drosophila* homolog of DUOX), but not dNOX (*drosophila* homolog of NOX), reduces ROS production and increases the mortality rate of flies in response to intestinal bacterial load (64). ROS production by dDUOX upon gut-microbe contact is regulated at two levels, i.e., activation and expression which requires the PLCβ- Ca²⁺ and the p38MAPK-ATF2 axis respectively (62, 63). Interestingly, the p38MAPK-dependent DUOX expression pathway is switched on only under infectious conditions when the gut needs more DUOX to fight infection (63). In addition to the p38MAPK-ATF2 axis, the *drosophila* protein Mesh has also been shown to control the expression of dDUOX to modulate its abundance in response to microbial fluctuation (167). In *Anopheles gambiae* midgut, DUOX provides H₂O₂ to the immunomodulatory peroxidase

(IMPer), which then catalyzes protein cross-linking by forming covalent bonds between tyrosine residues, resulting in reduced permeability to bacteria and *Plasmodium* parasites (91). Finally, in *C. elegans*, generation of ROS by Ce-DUOX1 plays a role in the anti-infectious defense against *Enterococcus faecalis*, as silencing of Ce-Duox1 in the intestine increases susceptibility to this pathogen (23).

In the lower vertebrate zebrafish, the ortholog of the mammalian *DUOX* which is highly expressed in intestinal epithelial cells, was demonstrated to have antibacterial properties against *Salmonella* infection as evidenced by bacterial persistence in *DUOX* knockdown larvae (44).

In higher vertebrates, the use of *DUOXA*^{-/-} mice supplemented with L-thyroxine, which results in alteration of *DUOX* maturation and apical targeting, has allowed to demonstrate a crucial role of gastric epithelial *DUOX2*-derived H₂O₂ in controlling *Helicobacter felis* infection. Therefore, the lack of functional *DUOX2* enzyme leads to increased mucus layer colonization with *Helicobacter*, and exacerbated acute and chronic *Helicobacter*-induced gastritis (57).

Together these observations in invertebrates, and lower and higher vertebrates suggest that the antibacterial role of *DUOX* is a function conserved in evolution.

Mechanisms through which DUOX2 contributes to antibacterial effect and homeostasis.-

Several mechanisms could be involved in the antibacterial effect of *DUOX*-derived H₂O₂. This includes a bacteriostatic effect as low concentrations of H₂O₂ have been shown to be sufficient to suppress *Helicobacter felis* proliferation (57), a repellent effect as *Salmonella typhimurium* invasion of the CHO cell line stably expressing *DUOX2* was shown to be decreased as compared to untransfected cells (19), and the formation of hypothiocyanite (OSCN⁻), a microbicidal agent that derives from the reaction between H₂O₂ and thiocyanate (SCN⁻) under the catalytic action of lactoperoxidase (LPO) (52). Especially in the airways, hypothiocyanite has been shown to eliminate *Staphylococcus aureus* and *Pseudomonas aeruginosa* without being toxic to the host (115). In mice, LPO was detected in the colon, but not in the ileum where

DUOX2 expression is the highest; however, under inflamed conditions such as in glutathione peroxidase-1- and -2-deficient mice, which develop intestinal pathology, significant expression could be detected in the ileum (84). This localization of LPO seems to be compatible with the potential formation of OSCN- in mice. In contrast to mice, LPO expression was not detected in the human gut (135). Interestingly, DUOX2-derived H_2O_2 has been shown to act also as a paracrine chemotactic signal for leukocytes in zebrafish (124), and through this process may contribute to host defense.

In addition, DUOX2-derived ROS could contribute to MUC3 and MUC5AC secretion in EGF-stimulated Caco-2 colon epithelial cell line by stabilizing tyrosine phosphorylation of the EGF receptor (31).

Finally, an intricate reciprocal relationship exists between the gut microbiota and DUOX2 to maintain homeostasis. In mice, the expression of DUOX2 is strongly induced by normal microbiota in the ileum and the colon through engagement of the NFkBp50/p65 TRIF pathway and the MyD88 and p38MAPK pathways respectively (151). More specifically, the segmented filamentous bacterium, a mucosa-adherent commensal, is a strong inducer of DUOX2 expression and activity in the ileum, and DUOX2 in turn restricts translocation of the bacteria to dampen the microbiota-mucosa induced immune response so to maintain homeostasis (58). In summary, mechanisms through which DUOX2 contributes to mucosal antibacterial effect may comprise: a bacteriostatic and repellent effect of DUOX2-derived H_2O_2 , and the formation of hypothiocyanite which was shown to be microbicide. Furthermore, the reciprocal modulation between DUOX2 and the gut microbiota seems to be prominent in the control of homeostasis.

V. NADPH oxidases-derived ROS in the gastrointestinal tract during pathological conditions: Loss-of-function versus excessive activity of gastrointestinal-associated NADPH oxidases

Taking into account the physiological importance of NOX2, NOX1 and DUOX2 in innate immunity and homeostasis of the intestinal mucosa, one could predict that inappropriate and dysregulated ROS production by these NADPH oxidases, e.g., loss-of-function or excessive activity, would actively contribute to pathological states in the gastrointestinal tract such as IBD. Loss-of-function would impair physiological processes while excessive activity would lead to oxidative stress within the gastrointestinal tract. In this section we discussed experimental data supporting that loss-of-functions as well as excessive NOX/DUOX activity could both contribute to IBD.

V.1 Loss-of-function of gastrointestinal associated NADPH oxidases during pathological conditions

CGD in IBD- Actually, up to 40% of patients with CGD, a disorder caused by defects in one of the NOX2 subunits and characterized by severe infections, have clinical and pathological features resembling Crohn's disease (CD) (106, 164). In addition, common polymorphisms in *NCF4*, which encodes p40^{PHOX}, has been identified as a risk factor for adult-onset IBD, more specifically ileal CD (137, 139). In contrast to adult-onset diseases that may involve host genetic susceptibility and environmental triggers, very early-onset IBD (VEO-IBD) is a subgroup of rare but severe IBD, with disease onset before 6 years of age, and driven by rare genetic variants with high penetrance. Therefore, key players involved in fine-tune regulation of immunity and homeostasis of the intestine are potential affected candidates. Interestingly, variants of several genes encoding NOX2 subunits, including *CYBB* (gp91^{PHOX}), *NCF1* (p47^{PHOX}), *NCF2* (p67^{PHOX}), *NCF4* (p40^{PHOX}) and *RAC1* (Rac1) and *RAC2* (Rac2) have been

identified in VEO-IBD by targeted exome sequencing and tagged SNP genotyping (37). These variants were predicted to result either in impaired ROS production, reduced protein assembly or reduced gene expression, but not a complete loss of function as in CGD patients; thus, they do not trigger evident immunodeficiency (37, 119).

NOX1 in VEO-IBD- Targeted exome sequencing in a cohort of 59 patients with VEO-IBD have identified one patient with the *NOX1* missense mutation p.P330S, and two patients with the missense mutation p.D360N (66). The p.D360N *NOX1* variant is actually a common polymorphism, which is also associated with adult-onset ulcerative colitis (UC) in Ashkenazi Jews, but not in the European decent population (143). p.P330S and p.D360N *NOX1* variants are located in front of the FAD domain 1 (FAD1) and in the FAD domain 2 (FAD2) respectively, and showed a 50%-60% decreased in ROS generation compared to WT *NOX1* (66). In Cos cells, this decrease in ROS generation resulted in enhanced invasion by the enteric pathogen *C. Jejuni* (66). Subsequent work identified 6 other *NOX1* variants by targeted exome sequencing of a cohort of 1878 VEO-IBD patients and whole genome sequencing of an index patient (143). Among them, the p.N122H and p.T497A mutations completely abrogated ROS production in the colonic epithelial cell line, HCT116, reconstituted with WT or *NOX1* variants. In contrast, *NOX1* p.Q293R, p.I67M, p.R287Q and p.Y470H mutations lead to a less dramatic decrease in ROS generation in reconstituted HCT116 (143). However in this study, population statistics also suggest that loss-of-function *NOX1* variants are rare but likely tolerated indicating that *NOX1* mutations have rather a role in susceptibility than in causing highly penetrant disorders that follow Mendelian modes of inheritance. A case of VEO-IBD with rare *NOX1* missense variant, p.R241C, associated with the presence of a common *CYBA* missense variant, p.Y72H, for which the patient was hemizygous and homozygous, respectively, has been recently identified by whole exome sequencing (100). The p.R241C variant is located in the third extracellular loop of *NOX1* and is adjacent to an N-glycosylation site (236-239), whereas

the p.Y72H variant is located in the N-terminal cytosolic tail of p22^{PHOX}. Caco2- cells transfected with either single *NOX1* p.R241C or *CYBA* p.Y72H or with both variants had impaired TNF α -induced ROS production as compared to WT variants. The two variants were shown to synergistically prevent NOX1-mediated antibacterial activity against *Listeria monocytogenes* in Caco-2 cells and to affect NOD2-mediated immune response, notably NF κ B activation and IL-8 secretion (100). *NOX1* genes variants associated with VEO-IBD are shown in **Figure 5**.

DUOX2 in VEO-IBD- Regarding DUOX2, five rare variants have been identified to date. Exome sequencing revealed a *DUOX2* missense mutation, p.P303R, a heterozygous mutation shared by 15 members of the same Ashkenazi Jewish family with high incidence of adult onset CD. This variant leads to a decrease in H₂O₂ production in DUOX2-reconstituted HEK293 cells compared to WT, and could be linked to a defect in trafficking of protein to the cell surface (98). Two other *DUOX2* variants have been identified by exome sequencing in a cohort of 59 patients with VEO-IBD, p.R1211C and p.R1492C, both patients were heterozygous (66). The p.R1211C mutation is located in the polybasic region of the third intracellular loop, and the p.R1492C mutation is located in the NADPH binding domain within the binding pocket. While the functional consequence of the p.R1211C mutation is unclear, the p.R1492C mutation was predicted to weaken binding of NADPH to DUOX2. When co-expressed with DUOX2 in the epithelial H661 cell line, which is devoid of NOX1-5 activity, both DUOX2 variants produced significantly less H₂O₂ than WT DUOX2, although protein expression and cellular localization were not altered. Decreased in H₂O₂ levels caused increased susceptibility to epithelial cell invasion by *C.jejuni*. The thyroid functions of the two patients were unaffected (66). Finally, biallelic inherited mutations of *DUOX2* have been identified in a patient with VEO-IBD (127). Whole exome sequencing identified the missense variant, p.P609S, within the first transmembrane domain, and a second missense variant, p.R286H, in the peroxidase homology

domain of DUOX2. H₂O₂ production in response to thapsigargin was found to be significantly decreased in H661-DUOXA2 cells expressing DUOX2 p.P609S or p.R286H mutants. Here again, thyroid function in the patient was normal (127). Interestingly, Grasberger *et al.* showed that patients with congenital hypothyroidism have increased risk for IBD (59). *DUOX2* genes variants associated with VEO-IBD are shown in **Figure 5**.

Animal models of NOX-DUOX2 deficiency do not recapitulate human IBD susceptibility- Altogether the above studies suggest that NOX2, NOX1 and DUOX2 loss-of-function is a susceptibility factor for IBD development in humans. However, although NOX2- and DUOXA-deficient mice recapitulate human CGD and congenital hypothyroidism, respectively (56, 131), these mice and NOX1-deficient mice do not develop spontaneous intestinal inflammation. Even under conditions where intestinal inflammation is induced, such as in dextran sulfate sodium (DSS) and trinitrobenzenesulfonic acid (TNBS) models, the consequences of the lack of NADPH oxidases are still unclear, as it can be a worsening factor in some models, but be protective in others (7). Therefore, the role of the NADPH oxidases in IBD might be context-specific and a second hit related to genetics, environment or microbiota may be required to observe significant clinical consequences. In contrast, loss of function mutations of IL-10 in patients with VEO-IBD (86) can be recapitulated in IL-10-deficient mice who develop spontaneous general enterocolitis under conventional breeding (83, 89).

V.2 Excessive activity of gastrointestinal associated NADPH oxidases during pathological conditions

Oxidative stress and IBD- It is clear that a close association exists between oxidative stress and IBD as evidenced by data derived from experimental animal models and clinical studies (15). Experimental IBD is associated with increased biomarkers of oxidative stress such as lipid peroxidation in colon tissue (30, 118), and excessive ROS production has been observed in the

inflamed mucosa of ulcerative colitis (UC) and Crohn's disease (CD) patients in correlation with the degree of inflammation (82, 109, 148). Furthermore, several antioxidant compounds were protective in chemically-induced colitis, supporting a causative role for ROS in the pathogenesis of IBD (72, 126, 145). The source of ROS in IBD has been mainly attributed to activated macrophages and neutrophils, which are massively recruited into the inflamed gut and produce robust amount of ROS through NOX2 (35, 47). The implication of ROS generated by NOX1 and DUOX2 in epithelial cells remains to be determined. We discuss below data from experimental model of NOX/DUOX deficiency and from human IBD/colitis supporting the view that excessive ROS produced by NOX2, NOX1 and DUOX2 could contribute actively to IBD.

Data deriving from experimental model of NOX/DUOX deficiency - It has been shown that NOX2/gp91^{PHOX}-deficient mice are protected against DSS-induced colitis. They exhibit lower body weight loss, less rectal bleeding and fewer histopathological changes, suggesting that gp91^{PHOX} contributes to the development of experimental inflammatory bowel disease (10). In addition, depletion of neutrophils, which abundantly express NOX2, has been shown to ameliorate DSS-induced colitis in rats (123). As NOX2 activation leads to high amount of ROS, it may contribute to pathological inflammation of the intestine through oxidative damage to macromolecules such as lipids, DNA, and proteins, leading to epithelial cell injury and increase in mucosal permeability (109).

There are also some evidence indicating that excessive ROS production by NOX1 is a driving factor in experimental IBD. NOX1 expression was found to be upregulated in different experimental models of intestinal inflammation such as the IL-10-deficient mice model (78) and the chronic DSS-induced colitis (but not acute DSS model) (133). In TNBS-induced colitis, we found that the NOX1 protein was increased (105), although NOX1 mRNA was unaffected and even rather decreased in the colon (170) (and personal data). Furthermore, NOX1

deficiency was also found to attenuate the severity of disease progression in some experimental models of IBD. Thus, mice deficient in the antioxidant enzymes glutathione peroxidase 1 and 2 have severe ileocolitis which was alleviated by NOX1 deficiency (43). In addition, in TNBS-induced colitis, NOX1 deficiency significantly attenuated macroscopic and histological damage scores (105, 170). The mechanisms through which excessive ROS production by NOX1 could induce pathological conditions in the intestine might be linked to uncontrolled synthesis of chemokine and LCN-2 by epithelial cells, but also to dysregulated proliferation of epithelial cells. Indeed, these functions have been shown to be controlled by NOX1 (75, 81, 105) and their dysregulation could lead to abnormal recruitment of inflammatory cells, dysbiosis and cancer processes. Interestingly, NOX1 has been shown to control the proliferation of colon cancer cells (33, 141). Finally, DUOX2 deficiency, which results in a defect in DUOX2 activity, has also been shown to be protective in a mice model of ileocolitis induced by knockdown of glutathione peroxidase 1 and 2 (25).

In summary the protective effect of NOX2, NOX1 and DUOX2 deficiency in some models of experimental IBD suggest that NOX/DUOX-derived ROS are a driving factor in disease progression.

Data from human IBD/colitis- In human, several studies have described the overexpression of NOX/DUOX, during IBD. The increase in NOX1 expression in patients with IBD is less well documented than DUOX2. However, in CD and UC patients, high levels of NOX1 mRNA have been observed in lymphocytes within the gut lesions (156). Moreover, we found an increase in NOX1 protein expression in colon biopsies from inflamed and non-inflamed areas in CDs patients as compared to healthy controls (105). Increased NOX1 expression has also been described in patients with irritable bowel syndrome (1). Interestingly, increased NOX1 expression seems to be also a feature of patients with colon cancer (48, 76, 77, 96, 163), in

which IBD is known to be a risk factor (159). In fact, more than 20% of IBD patients develop colorectal cancer after 30 years of disease progression.

In addition, it is well established that DUOX2 expression is increased in the colon of patients with IBD. Thus, CD patients have increased DUOX2 mRNA expression in non-inflamed colonic mucosal cells as compared to controls (29). Also, Lipinski *et al.* demonstrated a significant increase in DUOX2 mRNA and protein expressions in human colonic biopsies of both CD and UC patients compared to healthy controls (99). While DUOX2 expression was restricted to the apical surface in normal intestinal epithelium, an increase in DUOX2 protein expression was observed in the crypt epithelium of inflamed human colon biopsies (25, 105), raising the possibility that DUOX2 could contribute to crypt epithelial stem cell damage in the context of inflamed colon. Abnormal increase in DUOX2 mRNA expression in ileum of UC and CD patients was also detected in association with Proteobacteria expansion (65), indicating a dynamic interaction between DUOX2 and the microbiota. Besides, 5-aminosalicylic acid (5-ASA), a drug used to treat IBD, was able to upregulate DUOX2 and DUOXA2 during active colitis, suggesting that increased expression of the DUOX2/DUOXA system could reflect a compensatory response to support innate defense, although it could become detrimental by increasing DNA damage (104). Finally, a recent work showed that predicted damaging mutation in *SLC26A3*, a gene encoding for a chloride anion exchanger, in Iceland UC cohorts was potentially associated with enhanced expression of DUOX2 and DUOXA2 in the rectum (162).

Together, these observations support a direct role of NOX1 and especially of DUOX2 in the pathological processes leading to IBD.

VI. Therapeutic perspectives

Current therapeutic management of IBD relies on anti-inflammatory agents (sulfasalazine and corticosteroids), immunosuppressive drugs (azathioprine and cyclosporine), and biological therapy with anti-TNF α as prevailing treatments in moderate to severe IBD. Up to one third of patients do not respond to anti-TNF α treatment, and a similar proportion of patients may experience loss of response after a few years of treatment (14). Therefore, other therapeutic options for IBD, in particular targeting the NOX/DUOX-derived ROS pathway, could be relevant, especially that a recent study showed that DUOXA2 and DUOX2 were among the top ten genes overexpressed in loss-of-responders as compared to anti-TNF α responders (102). However, the therapeutic alternatives must also take into account the dual role of NOX/DUOX-derived ROS in intestinal inflammation. Hence, NOX/DUOX inhibitors or ROS inducers could be recommended based on the cause of the disease, i.e., excessive activity or loss of function of NOX/ DUOX.

VI.1. Anti-oxidants and NOX/DUOX inhibitors as therapeutic strategies

The use of NOX/DUOX-specific inhibitors for the treatment of IBD would provide a better strategy than antioxidant compounds as it would allow to only target the dysregulated ROS source without impacting the other sources. Indeed, if many antioxidant compounds are protective against IBD in experimental models, there are not many examples of their efficiency in human IBD (18, 55, 116). The lack of clinical efficacy of antioxidants could be due to: their lack of specificity, a higher reactivity rate constant of ROS for biological molecules than for antioxidant compounds, oral administration of antioxidants may not allow reaching optimal concentrations at the site where oxidative stress occurs, and finally, some antioxidants may become pro-oxidants, as is the case with α -tocopherol (Vit.E) that can form the toxic α -tocopheryl radical. A number of studies have indicated that pan-inhibitors of NADPH

oxidases were efficient in preclinical studies in mice. Apocynin (4-hydroxy-3-methoxyacetophenone), an NADPH oxidase inhibitor with antioxidant properties originally extracted from the roots of the Himalayan herb *Picrorhiza kurroa* (67, 149, 153), has been shown to dampen disease severity in a mouse model of TNF α -induced colitis (118) and DSS-induced colitis (71). Interestingly, in the DSS-induced colitis model, apocynin seemed to be even more efficient than sulfasalazine to protect against tissue damage (71). The very low toxicity of apocynin *in vivo* makes this natural compound an interesting option in the treatment of IBD (153). Inhibition of NOX activity by intraperitoneal administration of diphenyleneiodonium (DPI) (2 mg/kg) and VAS2780 (10 mg/kg) also resulted in improvement of DSS-induced colitis with reduced disease activity and decreased cytokine levels (171). Both DPI and VAS2780 inhibit all NOXs (6). Although they were shown to have toxicity *in vivo*, the concentrations used in the above study seemed to be well tolerated (171). A more specific approach would be to selectively target NOX2, NOX1 or DUOX2 in a disease context linked to their excessive activity; however, the ideal inhibitor would be one that would suppress exaggerated activation while preserving the physiological functions of NOX/DUOX; therefore, it is crucial to understand the tight regulation of the activation of the NADPH oxidase isoforms in response to inflammatory stimuli. Regarding NOX2, we have identified a TAT-peptide sequence containing the Ser345 (amino acids 334–347) of p47^{PHOX} that prevents NOX2 hyperactivation mediated by the prolyl isomerase Pin1 (20). This peptide was able to limit TNF α and GM-CSF-induced NOX2 hyperactivation in human neutrophils, while preserving the physiological ability of NOX2 to respond to bacterial N-formyl peptide (32). Thus, the TAT-Ser345 peptide could represent an interesting tool for the treatment of IBD, especially during relapsing periods. ML171 (2-acetylphenothiazine) has been described as a specific NOX1 inhibitor at nanomolar range by high-throughput screening in the colon cancer cell line HT29 using luminol-enhanced chemiluminescence in the presence of horseradish peroxidase

(53), with only low inhibitory activity on other ROS-producing enzymes and NOX isoforms. ML171 has been shown to inhibit ROS production by colonic crypts of *ex vivo* biopsies, as measured by NBT reduction and L-012-enhanced chemiluminescence (143). However, other studies have shown that ML171 is inactive on all NOX isoforms and rather interfered with ROS assays using peroxidases or could potentially act as a scavenger (146, 158). To the best of our knowledge, the effects of ML171 have not been studied in any experimental model of IBD. Several other selective inhibitors of NOX1 have been described, including the NoxA1ds peptide derived from the cytosolic NOXA1 subunit (134), the NF02 peptide derived from the active site of bacterial dehydrogenase (117), the InhNOX1 peptide derived from the proline rich region of the cytosolic NOXO1 subunit (132), NOS31 a natural compound secreted from *Streptomyces* *sp.* (169), and GKT771 a drug developed by Genkyotex (152). None of these inhibitors have been studied in experimental animal models of IBD. Nevertheless, NF02 and NOS31 were shown to inhibit cell migration and invasion of colorectal cancer cells, while GKT771 reduced tumor growth in preclinical models of colorectal cancer and melanoma in immunocompetent mice (117, 152, 169). Concerning DUOX2, its potential inhibition for therapeutic purposes has not been extensively studied, probably due to its central contribution to thyroid hormone synthesis. However, by screening thirty five compounds derived from DPI, Lu *et al.* have identified analogues with a nitro group substitution that have higher selectivity for DUOX2. Based on these findings, they synthesized an isomer with optimized potency against DUOX2 and NOX5 compared to other NOX isoforms (101). This compound could be used to study the potential protective effect of DUOX2 inhibition in rodent models of IBD, especially as rodents do not express NOX5.

VI.2. ROS inducers as therapeutic strategies

Taking in account the current evidence that loss-of-function of NOX/DUOX might be a causal factor in VEO-IBD, the use of a different therapeutic strategy such as ROS inducers could be

considered, with the objective of reversing the phenotype caused by the mutation. This implies that ROS should be delivered at the right place, level and time given their intrinsic cytotoxic nature. Oxidative burst-inducing agents such as phytol have been shown to prevent disease progression in experimental model of arthritis associated with inactivating *NCF1* (p47^{PHOX}) gene polymorphism, with comparable efficiency as standard treatment (anti-TNF α and methotrexate), demonstrating the feasibility of the concept that could potentially be transposed to IBD (69, 70). Probiotics could be used as an alternative source of ROS. Probiotics such as *Lactobacilli* sp have been associated with beneficial effects including maintenance of intestinal homeostasis, limitation of pathogens expansion in IBD and have been used in preclinical trials (11). These beneficial effects could be related to their ability to produce H₂O₂ (130) as demonstrated by a recent work showing that *Lactobacillus johnsonii* mutant strains, with reduced H₂O₂ production, do not promote recovery and restitution in DSS-induced colitis in mice, contrary to wild type *L. johnsonii* strains (150). However, ROS inducer strategies should take into account the possible side effects linked to oxidative stress.

In summary NOX/DUOX inhibitors or ROS inducers could be considered according to the disease context; The ideal inhibitor should limit ROS production by NOX/DUOX without abrogating their physiological functions, whereas the ideal inducer should generate ROS without inducing an oxidative stress.

VII. Conclusions

Despite their intrinsic cytotoxic nature, it is now recognized that ROS produced by epithelial (NOX1, DUOX2) and phagocyte NADPH oxidases (NOX2) play a central role in innate immune defense and homeostasis of the intestine. However, dysregulation of ROS production due to NADPH oxidase loss-of-function or exaggerated activity might contribute to the

development of IBD (**Figure 6**). Indeed, physiological functions are no longer ensured or oxidative stress can settled, respectively. In this context, therapeutic strategies should take into account the two sides of NOX/DUOX-derived ROS, with inhibitory or enhancing-based strategies according to the disease context. This implies a thorough understanding of their regulation in physiological and pathological situations.

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List of abbreviations

AIR: auto-inhibitory region

ANXA-1: annexin A1

ATF2: activating transcription factor 2

CD: crohn's disease

CGD: chronic granulomatous disease

DPI: diphenyleneiodonium

DSS: dextran sulfate sodium

DUOX: dual oxidase

DUOXA1/2: dual oxidase maturation factor 2

EGF: Epidermal Growth Factor

ERK: extracellular-signal-regulated kinase (

FAK: Focal adhesion kinase

fMLF: -Formylmethionyl-leucyl-phenylalanine

FPR1: Formyl peptide receptor 1

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HEK293: Human embryonic kidney 293

HIF: Hypoxia-inducible factor

IBD: Inflammatory bowel disease

IL: interleukin

INF γ : interferon gamma

LC3 : microtubule-associated protein 1A/1B-light chain 3

LCN-2: lipocalin 2

LP: lamina propria

LPO: Lactoperoxidase

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinases

MDP: muramyl dipeptide

MPO: Myeloperoxidase

MUC: mucin

NBT: Nitro blue tetrazolium

NCF: neutrophil cytosolic factor

NGAL: neutrophil gelatinase-associated lipocalin

NOD2: nucleotide-binding oligomerization domain 2

NOX : NADPH oxidase

NOXA1 : NADPH Oxidase activator 1

NOXO1 : NADPH Oxidase organizer 1

NRF2 : Nuclear factor erythroid-2-related factor 2

PAMPs : Pathogen Associated Molecular Patterns

PHOX : phagocyte oxidase

PKA : protein kinase A

PKC : protein kinase C

PLC β : Phospholipase C beta

PMA : phorbol 12-myristate 13-acetate

PPs : Peyer's patches

PTEN : Phosphatase and tensin homolog

RegIII γ : Regenerating islet-derived protein 3 gamma

ROS: reactive oxygen species

TNBS: trinitrobenzenesulfonic acid

TNF: Tumor necrosis factor

TLR: toll-like receptor

TRIF: TIR-domain-containing adapter-inducing interferon- β

UC: ulcerative colitis

VEO-IBD: very early onset inflammatory bowel disease

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Figures legends

Figure 1: Colonic epithelial barrier function and role in immune homeostasis.

Several processes contribute to intestinal immune homeostasis. The epithelium layer constitutes a physical barrier against commensal and pathogenic microbes. Goblets cells secrete mucins that cover the epithelium surface and prevent direct contact between epithelial cells and microorganisms. Enterocytes secrete antimicrobial proteins such as RegIII γ and bacteriostatic proteins such as lipocalin-2. The lamina propria contains innate immune cells such as resident macrophages and dendritic cells, and infiltrated neutrophils, but also adaptive immune cells (not shown). Epithelial cells, macrophages, dendritic cells, and infiltrated neutrophils are all able to produce ROS (H_2O_2 or $\text{O}_2^{\circ-}$) in response to inflammatory stimuli through specialized enzyme complexes called NADPH oxidases : NOX1 and DUOX2 in epithelial cells, and NOX2 in immune cells. Goblet cells also likely express NOX1. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

Figure 2: Lewis structure of reactive oxygen species.

Superoxide anion ($\text{O}_2^{\circ-}$), the precursor of other ROS, is produced from many endogenous sources, the major one being NADPH oxidases (NOX/DUOX). $\text{O}_2^{\circ-}$ undergoes spontaneous or superoxide dismutase (SOD)-catalyzed dismutation to form hydrogen peroxide (H_2O_2), and can react with nitric oxide (NO°) to generate peroxynitrite (OONO°), a highly deleterious molecule. H_2O_2 and $\text{O}_2^{\circ-}$ can react in the presence of transition metal ions ($\text{Fe}^{2+}/\text{Cu}^{2+}$) to generate hydroxyl radical (HO°). In addition, H_2O_2 reacts with Cl^- to form hypochlorous acid (HOCl) under the catalytic action of myeloperoxidase (MPO). HOCl and HO° are highly reactive with biomolecules. Finally, the reaction between H_2O_2 and HOCl can generate singlet oxygen ($^1\text{O}_2$),

which is also very unstable. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars

Figure 3: NADPH oxidases found in the gastrointestinal tract.

Resident macrophages, dendritic cells and infiltrated neutrophils express NOX2. Intestinal epithelial cells express NOX1 and DUOX2. A functional NOX2 complex consists of two membrane subunits, gp91^{PHOX} and p22^{PHOX} (phox for phagocyte oxidase), which form the catalytic core of the enzyme, and three regulatory cytosolic subunits p47^{PHOX}, p67^{PHOX}, p40^{PHOX}, and the low molecular-weight GTP-binding proteins, Rac 1 or 2. In neutrophils, NOX2 subunits are segregated in different cellular compartments, ensuring that it stays inactive in the absence of stimulation. Upon neutrophil stimulation, p47^{PHOX}, p67^{PHOX} and p40^{PHOX}, together with Rac1/2, migrate to the membranes where they associate with gp91^{PHOX} and p22^{PHOX} to assemble the catalytically active oxidase. NOX1 binds p22^{PHOX}, and requires the cytosolic regulatory partners NOX organizer 1 (NOXO1), NOX activator 1 (NOXA1), and Rac1 GTPase to be functional. DUOX2 form a stable functional heterodimers with DUOXA2. Its activity is regulated by calcium *via* two EF-Hands motifs. Unlike NOX2 and NOX1 that produce O₂^{•-}, DUOX2 generates H₂O₂. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars

Figure 4: Physiological functions of NOX1 in the gastrointestinal tract.

NOX1-derived ROS have been involved in: **1)** alteration of *C. jejuni* capsule formation and virulence through the disruption of bacterial signaling; **2)** epithelial renewal and restitution in response to the cleavage product of Annexin A1 through the FPR1/ PTEN, PTP-PEST/ FAK, paxillin pathway, but also in response to Lactobacilli through the FPR1/ NOX1/FAK-ERK axis; **3)** cytoprotection against exogenous oxidative insults through activation of the redox sensitive

transcription factor NRF2, which triggers the expression of antioxidant and cytoprotective genes in response to Lactobacilli; 4) control of lipocalin-2 expression in epithelial cells co-stimulated by TNF α + IL-17 through I κ B ζ , a non-canonical member of the NF- κ B transcription factor family that acts as a positive regulator of NF- κ B. Lipocalin-2 is a strong bacteriostatic protein. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars

Figure 5: NOX1 and DUOX2 variants identified in patients with IBD.

A) *Location of the variants in NOX1.* All the variants were associated with very early onset IBD (VEO-IBD), except for the D360N *NOX1* variant that is a common polymorphism associated with adult-onset ulcerative colitis (UC) in Ashkenazi Jews. A rare *NOX1* missense mutation, p.R241C, associated with the presence of a common *CYBA* missense variant, p.Y72H, has been recently identified in a patient with VEO-IBD; **B)** *Location of the variants in DUOX2.* All the variants were associated with very early onset IBD (VEO-IBD), except for the P303R variant, a heterozygous mutation shared by 15 members of the same Ashkenazi Jewish family with high incidence of adult onset Crohn's disease (CD). To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars

Figure 6: The dual role of ROS-generating NOX1/DUOX2 in gastrointestinal inflammation.

Under physiological conditions when their production is controlled, NOX1/DUOX2-derived ROS contribute to intestinal innate immunity and homeostasis by regulating host intracellular signaling pathways, leading to epithelial restitution, expression of cytoprotective genes, and expression of bacteriostatic proteins such as lipocalin 2-(LCN-2). ROS can also alter bacterial signaling, thereby impairing capsule formation and bacterial virulence. However, insufficient

or excessive ROS production by NOX1/DUOX2 could both lead to pathological inflammatory conditions through different mechanisms that are: defective immune response and oxidative stress/tissue damage, respectively, associated with dysregulation of intracellular signaling. Notably, uncontrolled generation of LCN-2, which has bacteriostatic and chemotactic properties, can mount a dysbiosis and increase neutrophil recruitment, thereby amplifying the inflammatory response. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars

Figure 1 (Dang *et al.*)

Figure 2 (Dang *et al.*)

Figure 3 (Dang *et al.*)

Figure 4 (Dang *et al.*)

Figure 5 (Dang *et al.*)

Figure 6 (Dang *et al.*)